2nd European Symposium on Enzymes in Grain Processing ESEPG-2

TECHNICAL RESEARCH CENTRE OF FINLAND ESPOO 2000

VTT SYMPOSIUM 207

Keywords: enzymes, grains, cereals, baking, brewing, hydrolases, oxidases, esterases, proteinases, inhibitors, food processing

2nd European Symposium on Enzymes in Grain Processing

ESEGP-2

Helsinki, Finland,
8 - 10 December, 1999

Edited by

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VTT Biotechnology

Organised by

VTT Biotechnology

TNO Nutrition and Food Research Institute

TECHNICAL RESEARCH CENTRE OF FINLAND
ESPOO 2000

ISBN 951–38–5706–9
ISSN 0357–9387

ISBN 951–38–5707–7 (URL: http://www.inf.vtt.fi/pdf/)
ISSN 1455–0873 (URL: http://www.inf.vtt.fi/pdf/)

JULKAISIJA – UTGIVARE – PUBLISHER

Valtion teknillinen tutkimuskeskus (VTT), Vuorimiehentie 5, PL 2000, 02044 VTT
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Cover drawing: Journal of Molecular Biology (1998)275, p. 311, Figure 1.

Text preparing Arja Grahn and Tarja Haapalainen

Otamedia Oy, Espoo 2000

Preface

Cereal grains comprise a range of prominent crops for production of numerous products for human consumption. Grains are the core of human nutrition, and new products are being developed both in traditional product ranges in the bakery, rice and brewing industries and in the manufacture of new ingredients, such as snack foods, pasta and breakfast cereals. In addition, grains are a basal animal feed ingredient. Enzymes are the "power machine" behind all biological reactions, acting as catalysts to speed-up biological reactions. In grain processing, endogenous and added enzymes have always played a decisive role in determining final product quality. For example, the germination of cereal grains, the conversion of malt into wort and beer, and the baking of bread are all mediated through enzymatic reactions. Enzymes can also be used to improve food quality to offer consumers palatable, safe and wholesome foods, as well as to improve the efficiency of food processing.

In the last decade, enzyme research has evolved into improved control of cereal processing. The enzymes used mainly act on starch (amylases), glutein (proteases) and cell-wall components (cellulases, hemicellulases). Recently, other enzymes such as lipases and oxidases have been studied for applications in grain processing. New and better enzymes are increasingly becoming commercially available. The use of molecular biological methods has made it possible to change the genetic regulation of enzyme production. The formation of a specific enzyme may be accelerated or inhibited, which enables the production of tailored enzyme mixtures, and improved efficiency of production processes. This increases the economic viability of enzyme usage, and makes enzymes competitive alternatives to the use of chemicals. Gene technology can also be used to create new, more efficient enzymes by protein engineering, and to change enzyme activity levels in food raw materials. The wider application of enzymes in food processing is anticipated to become one of the key food industry trends over the next 10 years.

The 1st European Symposium on Enzymes in Grain Processing (ESEGP-1) was held in 1996 in Noordwijkerhout, The Netherlands. The meeting was a great success, and we were encouraged to organise the 2nd European Symposium on Enzymes in Grain Processing (ESEGP-2) three years later on December 8 - 10, 1999 in Helsinki, Finland. The objective of ESEGP-2 was to provide a forum where researchers and technologists in the areas of enzyme technology and grain processing, both from academia and industry, could share recent results and discuss the future direction of enzymatic processing of grains. The symposium was jointly organised by VTT Biotechnology and Food Research and TNO Nutrition and Food Research Institute under the auspices of the American Association of Cereal Chemists (AACC).

Over 160 international experts on biotechnology and cereal technology participated in ESEGP-2. The participants were equally from academia and industry, including all major industrial enzyme producers as well as brewing, milling and baking industries. The non-European delegates came from the USA, Japan and Australia. The participants learned about cutting-edge developments in enzyme research and its application in grain processing, from baking better bread to the latest use of gene technology. The papers illustrated the scope of active research and the high scientific level, especially in applied enzyme research, of this field in Europe. One of the driving forces is clearly the activity

of enzyme manufacturers, most of which have research and development facilities in Europe. In addition, grains are one of the most important agricultural products in Europe. However, endogenous cereal enzymes and enzyme inhibitors, which are both very important for processability of grains, have been studied less intensively in Europe. These topics are clearly subject to increasing interest. The amount of research on genetically modified grains has decreased in Europe during the past years. This is clearly due to negative consumer attitudes to genetically modified plants. However, grains could be used in the future as efficient seed factories for production of several valuable products including enzymes, antibodies and vaccines.

This proceedings book includes most of the papers presented either orally or as a poster in ESEGP-2. The book contains general articles giving excellent overviews as well as more detailed scientific reports on the latest research. In the opening paper the carbohydrate composition and structure of grains is reviewed, introducing the reader in the target polymers for enzymes. Subsequently, the latest analytical methods for the structural analysis of polysaccharides and enzyme activities are presented. Endogenous enzyme inhibitors and enzymes are then discussed followed by studies on action mechanisms of microbial enzymes and potential enzyme applications. In addition to hydrolytic enzymes, endo- and exogenous oxidoreductases are presently gaining more interest, and these enzymes are discussed in several papers. Most applications described deal with baking, but uses of enzymes in brewing, pasta and noodle production, and in solubilisation of dietary fibre are also discussed.

We want to acknowledge Ms Päivi Vahala, the secretariat of the symposium, who undertook most of the administrative work for this symposium and assisted in the preparation of the proceedings. A special acknowledgement is dedicated to the European Union, 5th Framework Programme, Quality of Life and Management of Living Resources (QLK1-CT1999-30054) for financial support. In addition, Novo Nordisk, Danisco and Genencor International are thanked for sponsoring the symposium. We hope that the contents of this book will be helpful for food scientists, nutritionists, plant breeders and biotechnologists, as well as business and marketing experts working with grains or enzymes.

On the behalf of the ESEGP-2 Organizing Committee

Maija Tenkanen

Contents

Posters

GRAIN POLYSACCHARIDES, VERSATILE SUBSTRATES FOR ENZYMES

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1 INTRODUCTION

Wheat, rice, maize, barley, sorghum, millet, oats, rye and triticale are the most grown cereal/grain crops in the world. In Sweden the total harvest of cereals was about 5.6 million metric tons during 1998, represented by wheat (40%), barley (30%), oats (21%), triticale (6%) and rye (3%). The wheat is used for baking of bread, in other types of food and also in feed. The rye adds variety to the bread market and is an appreciated cereal both in crisp and soft breads. The barley, oats and triticale are mainly used as feed and only small quantities of barley is used for food and malt production and some oats are used for production of rolled flakes. In the future there is a wish to use naked barley and oats with high β-glucan content in food products. Research also is devoted to the development of high amylose or high amylopectin barley.

Grain polysaccharides mainly include cell wall polysaccharides and starch and will be the focus of this introductory lecture. Since wheat, barley, oats and rye are the traditional cereals grown in Sweden most of the examples in this presentation concern these crops. The content of different constituents in triticale is often somewhere in-between that of its parents, wheat and rye.

2 CONTENT OF STARCH AND DIETARY FIBRE IN SWEDISH CEREAL GRAINS

In our laboratory starch is analysed with enzymatic methods (Åman *et al*., 1994) and dietary fibre, including non-starch polysaccharides, amylase-resistant starch and Klason lignin, with a gas chromatographic-colorimetric-gravimetric method (Theander *et al*., 1995). Mixed linkage (1,3), (1,4)- β-D-glucan, in the following referred to as β-glucan, is analysed selectively with an enzymatic method (Åman and Graham, 1987). β-Glucan, xylans, cellulose and lignin are major components of cereal cell walls. An estimate of the cellulose content can be obtained by subtracting the β-glucan content from the content of glucose residues in the dietary fibre analyses since unprocessed cereals contain very little resistant starch and other glucose containing polymers. The content of xylans can be estimated by adding the values of xylose, arabinose and uronic acid residues from the dietary fibre analyses.

The starch content is highest in wheat (68%) and lowest in oats (46%) (Table 1). Barley and oats are covered cereals and contain about 10 and 25% of fibre-rich husks, respectively, while wheat, triticale and rye are naked. Therefor barley and especially

oats also contain significantly more of dietary fibre than the other cereals. Of the naked cereals rye has the highest content of dietary fibre.

Table 1. Average content of starch, dietary fibre (DF) and main dietary fibre components in Swedish cereal grains (% of dry matter).

Component	Wheat	Rye	Barley	Oats
Starch	68	65	62	46
Total DF	12	15	20	32
β-Glucan	0.8	1.5	3.4	3.2
Xylans	6.0	7.6	7.0	8.0
Cellulose	2.5	3.3	5.3	9.1
Klason lignin	0.8	1.5	2.2	8.4
Σ	80	80	82	78

The β-glucan content is low in wheat, intermediate in rye, and high in barley and oats (Table 1). The reason for this is that the starchy endosperm cell walls of wheat and rye contain about 75% of this polysaccharide wheras the walls of barley and oats only contain about 25%. (Fincher and Stone, 1986). The other main constituent of cereal endosperm cell walls is arabinoxylan. Xylans are also present in the outer parts of the kernels and the content varies from 6% in wheat to 8% in oats. Cellulose as well as the non-carbohydrate part of the dietary fibre complex (Klason lignin), are mainly present in the outer part of the kernels and the contents are highest in the covered cereals, especially oats.

There is a wide variation in the content of starch, dietary fibre and dietary fibre components in the different cereals, respectively. This variation may depend both on cultivar and growing conditions. In a recent investigation we have studied the effects of cultivar, nitrogen fertilisation rate and environment on the yield and grain quality of barley (Oscarsson *et al*., 1998). In this study ten barley cultivars, including covered and naked types, varying in their content of starch, amylose, protein and β-glucan, were grown at different nitrogen fertilisation rates (45, 90 and 135 kg N/ha) and three locations for two different years. Large differences in yield and composition was noted for the different barleys. The yield varied from 3200 to 6700 kg/ha, the protein content from 8–15%, the starch content from 51 to 67% and the β-glucan content from 3.5–5.9%. In general the high amylose and waxy barleys gave low yields and had low starch and high β-glucan contents. The effects of nitrogen fertilisation rate were generally more pronounced than the effects of year and location. The yield and protein content increased with increasing nitrogen fertilisation rate while the starch content decreased. The β-glucan content increased with increased nitrogen fertilisation rate during one of the years while it remained fairly constant during the other year.

3 STARCH COMPOSITION AND STRUCTURE

In cereal grains native starch occurs as small semi-crystalline granules in the amyloplasts of the starchy endosperm. The size, shape and morphology of these granules are dependant on the botanical origin. Wheat, triticale, rye and barley starch all exhibit a bimodal size distribution of large disc-shaped A-granules and small spherical B-granules. In wheat, rye and barley (normal and waxy starch) the size of small granules range from 2 to 10 μm in diameter and the large from 10 to 30 μm for wheat and barley and from 10 to 40 μm for rye (Fredriksson *et al*., 1998). High amylose barley has a population of granules with sizes between the normal small and large granules of barley and smaller large granules than in normal barley. Oat starch granules are polyhedral and small (3–10 μm) and are present in aggregates (about 60 μm), called composite starch.

Table 2. Content of lipid free (FAM), lipid complexed (LAM, percent of total amylose within parenthesis) and total amylose determined by iodine staining (% of starch) and also total amylose determined by gel permeation chromatography (GPC) after debranching (% of glucose units in starch).

Sample	FAM	LAM	Total amylose	Total amylose by GPC
Wheat, Holme	20.4	4.6 (18%)	25.0	28.4
Barley, Golf	21.9	5.5 (20%)	27.4	29.3
Barley, Waxy	3.2	2.4 (43%)	5.6	7.1
Barley, High amylose	29.6	7.5 (20%)	37.1	39.0
Rye, Motto	22.7	3.3 (13%)	26.0	28.6
Oats, Sang	17.2	5.9 (26%)	23.1	29.7

The starch granules contain almost exclusively α-D-glucopyranose residues but also minor amounts of water, lipids, protein (including enzymes) and phosphate groups (low in cereal starches). These minor constituents are of great importance for the starch properties. About 25% of the glucose residues in the granules are present in a 4-linked essentially linear polymer called amylose. Small amounts of the residues have, however, been found to carry short branches at position O-6. The average molecular weight of amylose has been reported to be 10^{5-6} dalton. Total and lipid free amylose (FAM) can be analysed with iodine staining and lipid complexed amylose (LAM) calculated by difference (Morrison, 1995). Starch of oats had a lower content of FAM and a higher content of LAM than the other cereals with normal starch (Table II). LAM constituted only 13% of the amylose in rye starch but 26% in oat starch and as much as 43% in waxy barley starch which has a low amylose content. Total amylose contents were somewhat higher when determined by gel permeation chromatography after debranching compared to when determined by iodine staining.

Highly branched amylopectin of normal starch accounts for about 75% of the glucose residues in the granules. It is a very large molecule (average molecular weight 10^{6-8} dalton) and contains unit chains of 4-linked residues which are connected by 4,6-linked

branching points (5–6%). The unit chains have a polymodal distribution which is in line with the widely accepted cluster model for amylopectin. The clusters are packed together giving rise to alternating crystalline and narrow amorphous lamellae perpendicular to the helix axis (Fig 1; Morrison, 1995). The crystalline regions (for cereals generally A-pattern) are composed of double helices of the linear unit chains, while the narrow amorphous lamellae consist of the branched parts. The combined size of these two layers is 9 nm. At higher level of organisation stacks of these structures form crystalline zones separated by amorphous zones between 120 and 400 nm thick. The structure of the amylopectin in the amorphous zones is not known but Morrison (1995) suggested that the amylose is present in these zones. However, both the location of amylose in the granules and the presence of lipid amylose complexes in intact granules have been questioned (Buleon *et al*., 1998). In addition some starches contain polymers which differ from normal amylose and amylopectin and have been referred to as intermediate material (Manners, 1985).

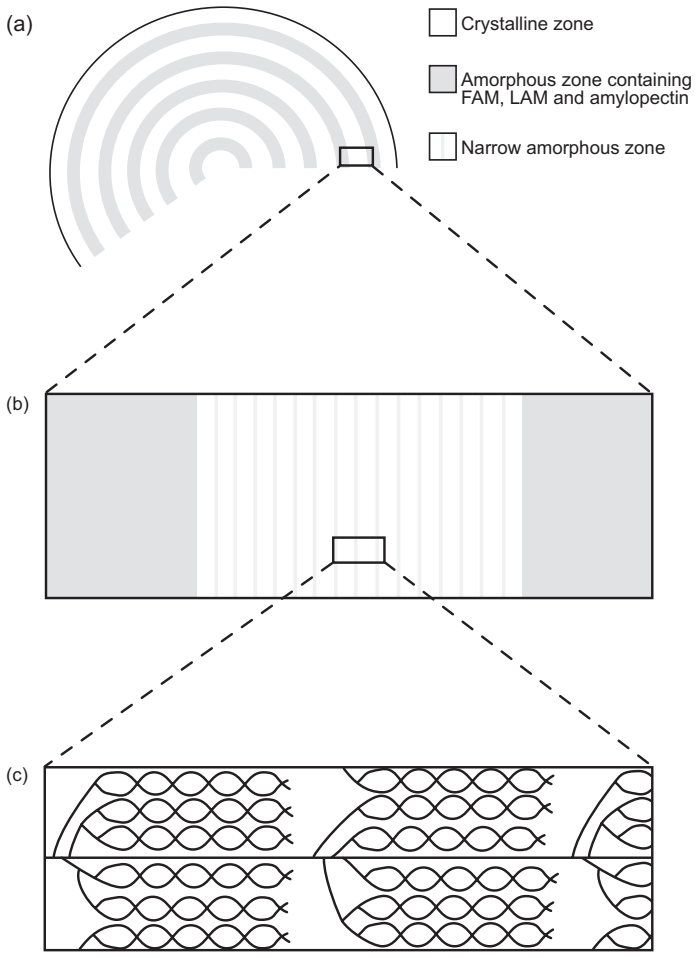

Figure 1. Starch granule (a) with concentric shells of crystalline amylopectin and amorphous zones containing FAM, LAM and amylopectin. The branch points of amylopectin in crystalline shells are arranged in narrow amorphous zones (b) between approximately 16 repeats of microcrystalline bundles (c).

In many starches surface pores and interior channels have been observed (Fannon *et al*., 1992). The channels (around 0.1 μm in diameter) may connect the internal cavity at hilum to external environment (Huber and BeMiller, 2000). For disc-shaped large starch granules of cereals pores are seen at the equatorial groove. Channels will increase the surface area available for reagent infiltration, especially into the less organised area around hileum.

4 CELL WALL POLYSACCHARIDES

The cell walls of plants consist mainly of polysaccharides, structural protein and lignin. These biopolymers are organised, together with smaller amounts of other components such as acetyl groups and phenolic acid residues, in complex three-dimensional structures that are neither uniform nor completely described in different cereal grains or cereal grain fractions (Fincher and Stone, 1986; Carpita and Gibeaut, 1993). A cell wall polysaccharide needs generally to be extracted and purified before the primary structure can be elucidated. It is important to remember that the structure might be changed during the isolation procedure. For example ester groups will be removed during alkaline treatment and arabinofuranosidic residues during mild acid treatment. Other problems may be depolymerization during treatment with shear forces or due to endogenous enzymes which have not been deactivated. It is also possible that only a small fraction is isolated and that this fraction may not be representative of the polysaccharide structure present in the original material.

Once the polysaccharide has been purified it can be subjected to structural analysis by many different methods (Åman and Westerlund, 1996). In order to determine the primary structure in detail several characteristics need to be elucidated such as monosaccharide composition as well as absolute configuration, glycosyl linkage, ring form, anomeric configuration and sequence of each sugar residue. Also position of attachment and structure of any non-carbohydrate component needs to be elucidated. Structure heterogeneity such as variation in degree and type of substitution, distribution of substituents and variation in molecular weight is often a challenge during the isolation and structural elucidation of cell wall polysaccharides.

Cellulose is a homopolysaccharide composed of 4-linked β-D-glucopyranosyl residues (Fincher and Stone, 1986). The individual chains form a helix (two glucose residues per turn) and two hydrogen bonds between adjacent sugar residues stiffen the chain into an extended, ribbon-like and relatively inflexible conformation. Extended chains aggregate to form crystalline microfibrils of up to 25 nm in diameter with about three dozen chains. Although each chain only may be several thousand units long, they begin and end at different places and make very long microfibrils whose ends are rarely detected. The close parallel packing of the molecules permits extensive intermolecular van der Waals interactions and hydrogen bondings. In the primary cell wall cellulose microfibrils is embedded in an essentially random orientation. Extensive extraction of wheat and barley endosperm cell walls dissolves the matrix phase and exposes cellulose microfibrils. In the secondary walls in the outer parts of the grain a high content of microfibrils arranged in parallel sheets can be found. The crystalline arrangement of the

chains result in distinct X-ray diffraction patterns and low solubility. However, in native cellulose both highly ordered crystalline and non-crystalline regions can be found.

β-Glucan in cereal endosperm is built up of about 30% 3-linked and 70% 4-linked β-D-glucopyranosyl residues (Wood *et al*., 1994). About 90% of the polysaccharide is composed of 4-linked residues that occur in groups of two or three separated by single 3-linked residues. The resultant polysaccharide is therefore essentially composed of 3-linked cellotriosyl- and cellotetraosyl units. The molar ratio of cellotriosyl- to cellotetraosyl units is lower for oats (2.1–2.4) than for barley (2.8–3.3), rye (3.0–3.2) and wheat (3.0–3.8) (Wood and Beer, 1998). Isolated fractions of the polysaccharide also contain longer sequences of 4-linked residues (5–15 residues) that may be of importance for the attachment of the polysaccharide in the cell wall matrix and for the degradation of the wall by enzymes. It is believed that the major structural building blocks in β-glucan are randomly distributed but the incorporation of the longer sequences in the structure remain to be elucidated. β-Glucan from different cereals or fractions of cereals vary greatly in extractability, molecular weight and viscosity.

Arabinoxylans are the other major group of polysaccharides in endosperm cell walls of cereals (Fincher and Stone, 1986; Åman and Westerlund, 1996; Vinkx and Delcour, 1996). These heteropolysaccharides consist predominantly of arabinose and xylose residues and are therefore often referred to as pentosans. Arabinoxylan contains a main chain of 4-linked β-D-xylopyranosyl residues, which is substituted at the 2-, 3- or 2- and 3-positions of the xylose residues with essentially terminal α-L-arabinofuranosyl residues. Arabinoxylan may also carry phenolic acid substituents. The substitution pattern of different arabinoxylans vary widely. Acidic xylans are generally present in lignified cell walls, such as the outer parts of the cereal grain. They are extracted with alkali and consist of a backbone of 4-linked β-D-xylopyranosyl residues which is sparsely substituted with short side chains of mainly terminal α-L-arabinofuranosyl, α-D-glucopyranosuronic acid and 4-O-Me-α-D-glucopyranouronic acid residues. Up to 90% of the xylose residues in the backbone may be unbranched and a significant proportion may be acetylated. With hot alkali it is possible to extract a highly branched heteroxylan, especially from maize bran (Chanliaud *et al*., 1995). This xylan has also a backbone of 4-linked β-D-xylopyranosyl residues. About 80% of these xylose residues are branched with monomeric side chains of arabinose, glucuronic acid or xylose residues but also with side chains of arabinose, xylose and sometimes galactose residues. This heteroxylan has a high content of phenolic substituents.

Our studies on water-extractable arabinoxylan in rye grain have shown that two different polysaccharide structures could be isolated (Åman and Westerlund, 1996). One structure, arabinoxylan I, was characterized by a main chain of xylose residues, of which about 40% were substituted at the 3-position by terminal arabinose residues. Using periodate oxidation it was demonstrated that the branched xylose residues were mainly distributed as isolated units or as small blocks of two residues rather than randomly as reported earlier (Åman and Bengtsson, 1991). Structural heterogeneity in terms of distribution of monosubstitued residues was also investigated by separation of intact and slightly xylanase-hydrolysed arabinoxylan I on a Biogel P-6 column and further analysis of collected fractions (Nilsson et. al, 2000). A heterogeneity in the Ara/Xyl ratio was found in the non-hydrolysed arabinoxylan. This heterogeneity was,

however, possible to explain by an uneven distribution of the low amounts of disubstituted xylose residues present in the isolated xylan. In the case of the hydrolysed xylan the change in Ara/Xyl ratio over the column was more pronounced and could not be explained by the distribution of the disubstituted xylose residues alone. It was concluded that the arabinoxylan must consist of either two different polysaccharides or different regions in the same polymer with densely and more sparsely monosubstituted regions. The structure seems to be heterogeneous with respect to substitution density and also with respect to the ratio between mono- and disubstitution.

The other polymeric structure, arabinoxylan II, was isolated after treatment with xylanase. This structure had also a main chain of 4-linked xylose residues, of which about 70% were mainly substituted at both the 2- and 3- positions with terminal arabinose residues. From these studies it can be concluded that mono- and disubstituted residues can be present in different polymers or in different regions of the same polymer. Similar structures have later been isolated from rye without xylanase treatment (Vinkx and Delcour, 1996). In this study smaller amounts of xylose residues substituted at the 2-position by terminal arabinose residues were also detected.

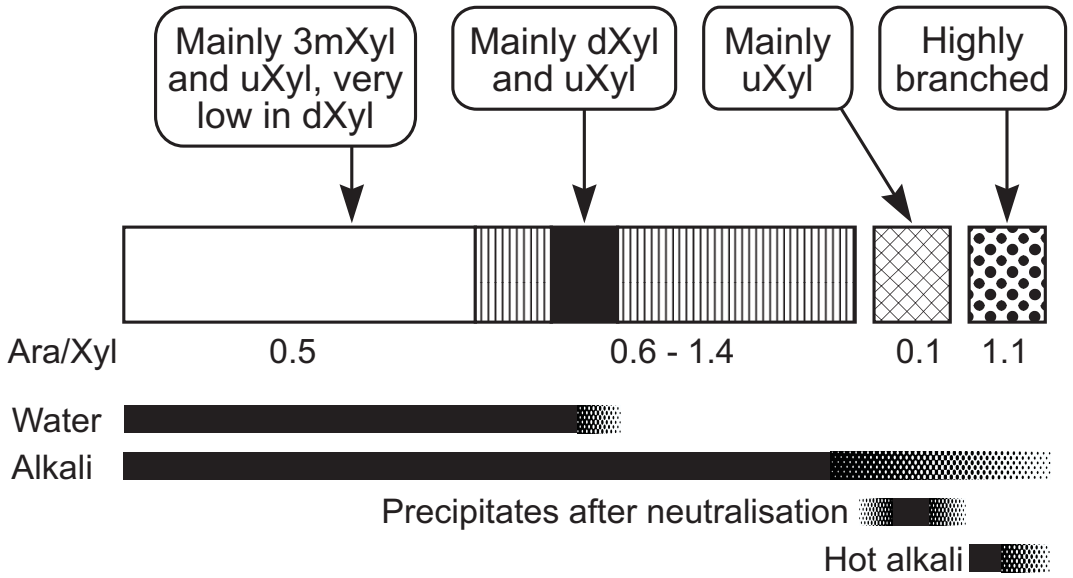

Figure 2. Extractability of rye arabinoxylan classes by different extraction media.

The major portion of (70–80%) of rye arabinoxylan is not extractable with water (Nilsson *et al*., 1996). Sequential extraction of arabinoxylan of rye milling fractions (bran, intermediate fraction and sieved flour) revealed different extractability of different classes of arabinoxylan (Fig 2). Some of the arabinoxylan structures, i.e. low and high Ara/Xyl ratio arabinoxylan, were almost exclusively detected with stronger extractants than water and the major part of the highly branched heteroxylan needed hot alkali to be extracted.

Cell walls of plants contain polymer bound hydroxycinnamic acid derivatives such as ferulic and *p*-coumaric acids (Ishii, 1997). By studying pearled fractions of barley it was

shown that ferulic acid residues were present in all studied fractions of the kernel but peaked in the fractions with the highest content of aleurone layers (1.2% of cell walls) (Nordkvist *et al*., 1984). On the other hand *p*-coumaric acid residues were found in highest concentration in the outermost husk-rich fraction (0.6% of cell walls) and decreased to very low amounts in the starchy endosperm rich fractions. Maize bran is rich in pericarp tissue and contains a heteroxylan as the dominating constituent. Other important constituents are cellulose, phenolic acid residues and structural protein (Saulnier and Thibault, 1999). By using controlled mild acid hydrolysis it was possible to release oligosaccharides with the ester-linked phenolic residues still attached. Three main feruloylated saccharides were identified, which all had the same basic unit with an arabinofuranosyl residue esterified on position O-5 by ferulic acid. From this unit the other two saccharides were built by adding one xylopyranosyl residue on position 2 of the arabinose residue, and futher one galactopyranosyl residue on position 4 of the xylose residue. These saccharides are probably side chains of the heteroxylan which have been released by the cleavage of the acid-labile glycosidic bonds of the arabinofuranosyl residues attached to the main xylan backbone. Both ferulic acid (2.9%) and dehydrodimers of ferulic acid (2.5%) were released from maize bran by alkaline treatment. The 5-5´, 8-O-4´, 8-5´dimers dominated but the 8-8´dimer was also present. Recently the same group have also isolated and structurally elucidated two new 5-5´-diferuloyl oligosaccharides which were also released by mild acid hydrolysis of rye bran (Saulnier *et al*., 1999). In one of the oligosaccharides both phenolic acids of the dimer were esterified to arabinose and in the other oligosaccharide one of the phenolic acid was esterified to arabinose and the other to arabinose which was further substituted with a 2-linked xylose residue. The structure of these saccharides indicate that the heteroxylans in maize bran are covalently cross-linked through dehydrodiferulates.

Cereal endosperm contains a water-extractable arabinogalactan-peptide with a relatively low peak molecular weight (in wheat 5-10 x 10^4 Dalton) (Fincher and Stone, 1974; Loosveld *et al*., 1998). Early studies on wheat have shown that the glycopeptide contains 92% carbohydrate and 8% of a hydroxyproline-rich peptide. The arabinose to galactose ratio is about 0.7. A 4-hydroxyproline-galactoside linkage connects the polysaccharide to the peptide (Strahm *et al*., 1981). The content of this arabinogalactan in wheat flour is 0.2–0.3% (Andersson *et al*., 1994; Loosveld *et al*., 1998). Recent studies on the arabinogalactan structure indicate chains of 6-linked β-D-galactopyranosyl residues which are highly branched (only 10% of the 6-linked galactose residues are unbranched) at the 3-position with terminal α-L-arabinofuranosyl (the only terminal residue detected) or 6-linked β-D-galactopyranosyl residues (Loosveld *et al*., 1998). These data support the early structure proposed by Strahm *et al*. (1981), with branched galactan chains and arabinose present as single residues.

5 CONCLUSIONS

Today most basic structures of grain polysaccharides are known. In many cases, however, structural heterogeneity make a definite determination difficult. In plant structures, such as the starch granule and the cell wall, polysaccharides, together with other components, are organised in complex three-dimensional structures that are neither uniform nor completely described. In the future, significant efforts must be

dedicated to elucidating the structure and properties of these complexes since it is primarily those and not the individual components which will determine the properties in grain products. The different structures present in grain polysaccharides will require many enzymes for their biosynthesis and degradation.

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ENZYMATIC AND MASS SPECTROMETRIC STUDIES OF COMPLEX XYLANS FROM VARIOUS SOURCES

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1 INTRODUCTION

Glucuronoarabinoxylans (GAX) present in the cell walls of Gramineae consist of a β-D-(1,4)-linked xylopyranoside backbone and can be substituted with α-L-arabinofuranose on O2 and/or O3, α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative on O2, and acetyl on (some of) the arabinose or xylose residues (Wilkie, 1979; Brett *et al.*, 1990, Ishii, 1991). Part of the arabinosyl units of the GAX may have a ferulic acid or p-coumaric acid esterified to its O5 (Mueller-Harvey *et al*, 1986). In spite of these general characteristics, the source from which (glucurono)arabinoxylans are extracted strongly determines the specific features with regard to molecular weight, the amount, position and distribution of arabinosyl units over the xylan backbone, and the presence of acidic substituents. A compilation of proposed models for glucuronoarabinoxylans of several origins is shown in Figure 1. However, the presence of acetyl groups is frequently overlooked, mainly due to the commonly used extraction methods including alkali, resulting in the removal of ester groups.

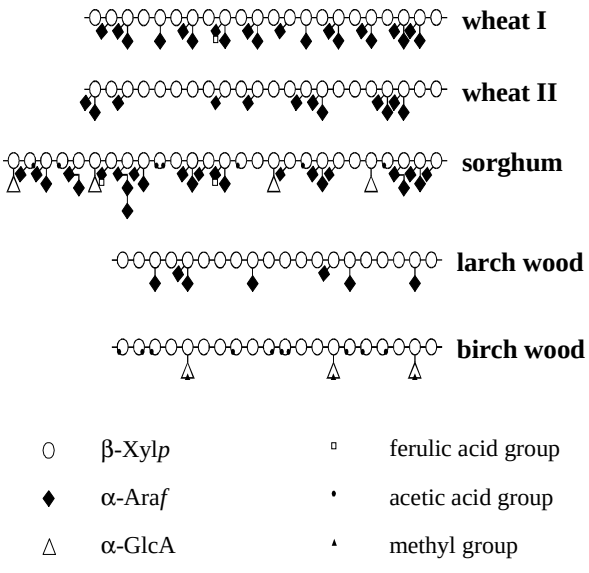

Figure 1. Structural models for cereal (glucurono)arabinoxylans.

Chemically, (glucurono)arabinoxylans are characterised by their sugar (linkage) composition, amount and type of esters present and by differences in charge and molecular weight distribution. The distribution of the (different) substituents over the

backbone can be successfully determined using pure and well defined enzymes (Voragen *et al.*, 1993; Kormelink, 1992) and now-a-days, a wide diversity of enzymes are present to specifically degrade (complex) xylans including various types of endo-xylanases, arabinofuranosidases able to release arabinose from various polysaccharides and specific arabinoxylan arabinofuranohydrolases (AXHs) (Kormelink, 1992; Beldman *et al.*, 1997, Van Laere *et al.*, 1997, Van Laere *et al.*, 1999).

Enzyme digests are usually examined using high-performance ion-exchange chromatography (HPAEC) and unknown components are isolated and characterised by NMR (Gruppen *et al.*, 1992, Kormelink *et al.*, 1993a). Although an extended and useful database has been build up, this procedure for the identification of new (unknown) fragments is laborious and requires mg-quantities of the individual components.

Mid nineties, we started to investigate whether we could use mass spectrometric techniques coming available as a rapid method to examine the nature of compounds present in a complex mixture. At that time, we used HPAEC coupled on-line with mass spectrometry using a thermospray interface (Niessen *et al.*, 1996). Information about the mass of the oligomers obtained, together with the sugar composition of the polysaccharide under investigation and the specificity of the enzyme used enabled us to predict rather precisely the structure of unknown compounds (Schols *et al.*, 1994). Off-line HPAEC electrospray MS after derivatisation of complex enzyme digests enables us to obtain more structural knowledge about the oligosaccharides in enzyme digests (Brüll *et al.*, 1998; Brüll, 1999).

Another development of the last years includes the introduction of Matrix-assisted laser desorption/ionisation Time of flight Mass Spectrometry (Bahr *et al.*, 1994; Kaufmann, 1995) giving good mass accuracy, being easy to operate and requiring hardly any expertise in mass spectrometry. MALDI-TOF MS has also shown to be very suitable to study the presence of ‘unstable’ substituents (e.g. esters) in oligosaccharides providing information which might be lost using other methods of analysis (Daas *et al.*, 1998).

In this paper, we will demonstrate the use of off-line coupling of HPAEC with Maldi Tof MS (matrix assisted laser desorption/ionisation – time of flight – mass spectrometry) with on-line desalting and automated sampling handling in the characterisation of a digest obtained after hydrothermal treatment of a soft wood. In addition, the limitations of the use of enzymes for the characterisation of a complex GAX from maize cell walls are demonstrated.

2 MATERIAL AND METHODS

All materials and methods used were as described in the indicated references.

2.1 OFF-LINE HPAEC MALDI TOF MS COUPLING

In order to obtain molecular mass information of unknown oligosaccharides, an automated system to measure these masses directly from fractions as collected from an

analytical HPAEC run was developed (Kabel *et al.*, 2000). Typical HPAEC conditions include gradients using 100 mM sodium hydroxide in combination with sodium acetate gradients up to 0.5 or 1 M, while such high salt concentrations are known to hinder co-crystallisation of your analyte molecules with the matrix molecules (e.g. 2,5-dihydroxybenzoic acid) for MALDI-TOF MS analysis. To overcome this problem, the HPAEC eluent was desalted on-line using an anion self-regenerating suppressor (ASRS) in series with a cation self-regenerating suppressor (CSRS). In this way, acetate ions were exchanged by hydroxide ions and sodium ions by hydronium ions as obtained by electrolysis of water. The resulting eluent (separated oligosaccharides in water) was fractionated and the collected fractions were analysed by MALDI-TOF MS (Van Alebeek *et al*, 2000a) using a robot-system to mix 1 µl of each fraction with 1 µl of matrix solution. A schematic overview of the experimental set-up is presented in Figure 2. Using conductivity measurements, it was demonstrated that the combination of suppressors used were able to remove ions almost completely when the concentration of NaAc in 100 mM NaOH does not exceed 300 mM at a flow rate of 1 ml/min.

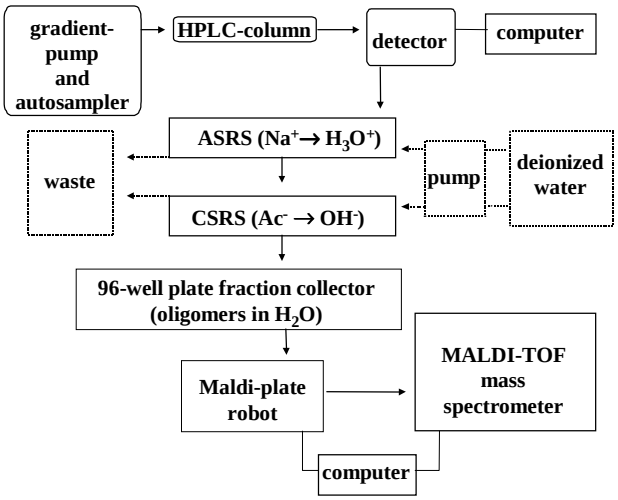

Figure 2. Schematic overview of the experimental set-up for automated coupling of HPAEC to MALDI-TOF MS.

When higher concentrations of salts have to be removed, a lower flow rate is recommended, while also more suppressors in series could be used. The off-line coupling of HPAEC to MALDI-TOF MS was used to analyse a complex mixture of glucuronoxylan oligomers as obtained after a hydrothermal treatment (17 min.; 175 °C) of Eucalyptus wood (Kabel *et al*, 2000). It has been shown that such a treatment mainly solubilizes xylans leaving the abundantly present cellulose rather unchanged. The HPAEC elution pattern of the hydrolysate is rather complex (not shown). Mass spectrometric analysis of the collected fractions (120 µl) gave quite unambiguous results showing that most fractions only contained one single compound as illustrated for some fractions in Figure 3. Mass spectra of a pentamer and a nonamer of pentoses (i.e. xylose since no arabinose is present in the hydrolysate) are easily identified while other /spectra/fractions represent xylo-oligomers containing one 4-O-methyl glucuronic acid moiety. In addition to the strong signal of the single sodium adduct of the acidic

oligomers (mass +23), a weaker signal of oligomers including two sodium ions was also detected. An important advantage of this off-line coupling compared to on-line LC-MS is, that the fractions (although representing only quite small amounts of material due to the analytical column used) remain available for further characterisation using e.g. other mass spectrometric techniques (Iontrap MS^n or post source decay MALDI-TOF MS; Van Alebeek *et al.*, 2000).

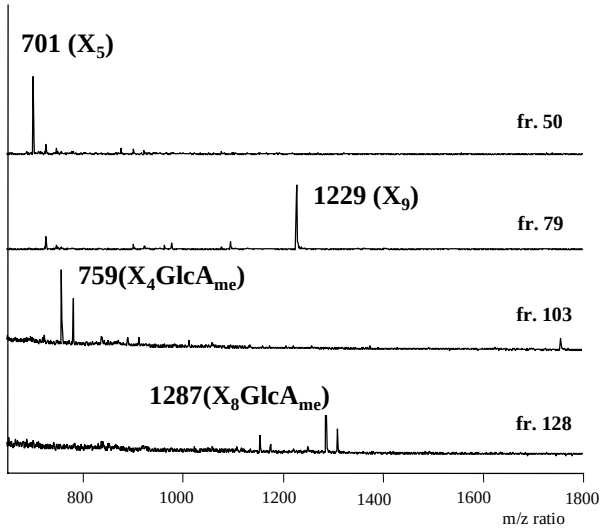

Figure 3. MALDI-TOF mass spectra of four fractions separated by HPAEC of a xylo-oligosaccharide mixture obtained after hydrothermal treatment of Eucalyptus wood. X = xylose, $GlcA_{me}$ = 4-O-methyl glucuronic acid.

2.2 ACETYLATED XYLAN OLIGOSACCHARIDES

Although peak identification in a rather complex elution pattern as obtained from a mixture of oligomers originating from a glucuronoxylan is quite successful, a substantial part of the information concerning the precise structure of the glucuronoxylan oligomers is lost by the elution conditions (pH12) used for the HPAEC separation. As can be seen in Figure 4 representing the 500–750 Dalton range of the MALDI-TOF mass spectrum of the crude hydrolysate, many acetylated oligomers are present as well. Using the specific masses of the various building blocks (e.g. pentose, hexoses, (4-*O*-methyl)hexuronic acid, and acetyl), most of the signals could be identified provisionally. The tentative identification was confirmed by using arabinoxylan acetylesterase (AXAE; Kormelink *et al.*, 1993b) able to specifically remove acetyl groups from acetylated xylans and using an endo-xylanase (type III, Kormelink *et al.*, 1993c) able to hydrolyse glycosidic linkages between xylose residues in a segment of the backbone which is not substituted too much. All enzyme digests were monitored by Maldi Tof MS and peaks were quantified using a signal for hexose-

trimer present in the digest as an 'internal standard' since this compound was not affected during any treatment. Figure 5A and 5B show the relative amounts of acetylated xylotrioses and -tetraoses before and after enzyme treatment. It can be seen that AXAE is able to remove a significant part of the acetyl groups present, but not all as indicated by the endproducts X_3Ac_1 and X_4Ac_1. As expected, the endo-xylanase is not able to act on the acetylated oligomers at all.

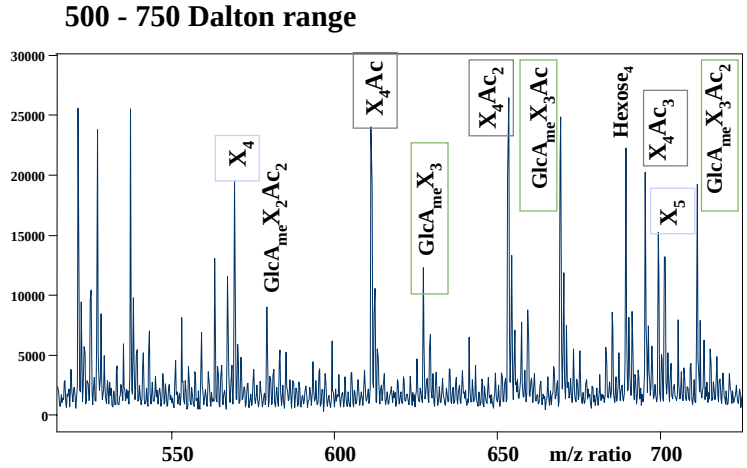

Figure 4. Part of the MALDI-TOF mass spectrum of a eucalyptus wood hydrolysate obtained after hydrothermal treatment. $GlcA_{me}X_3Ac$ represents a tetramer consisting of three xylose residues and one 4-O-methyl-glucuronic acid moiety carrying one acetyl group.

When the xylo-oligosaccharides are treated with alkali before enzyme treatment to remove all acetyl groups, endo-xyl III is able to hydrolyse the higher oligomers to smaller fragments indeed (Fig. 5C and 5D). End products of endo-xyl III were monomer, dimer, trimer and tetramer, although Maldi-Tof MS could not monitor masses below 400 Dalton. When looking to acidic xylose-oligomers, it can be seen that endo-xyl III is able to catalyse hydrolysis of the glycosidic linkages between xyloses although the presence of glucuronic acid substituents result in slightly larger end products.

In conclusion it can be stated that Maldi Tof MS is a rapid and adequate tool to monitor complex mixtures of oligosaccharides and is also quite useful to monitor enzymatic degradation reactions. However, so far, no absolute structure elucidation of the various oligomers using mass spectrometry has been carried out, although it is expected that Iontrap MS^n or post source decay MALDI-TOF MS (Van Alebeek *et al.*, 2000b) will be applicable here as well.

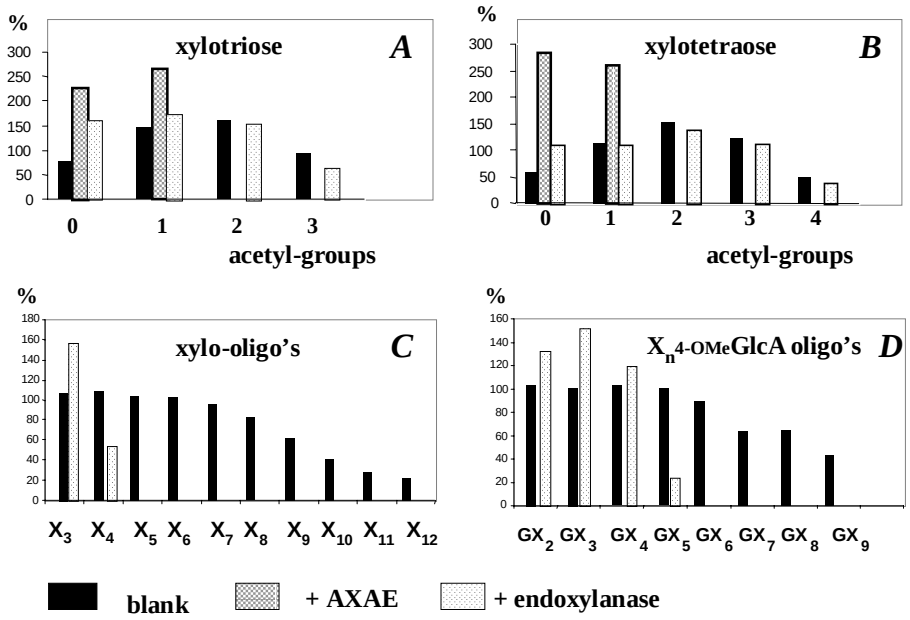

Figure 5. Enzymatic degradation of xylo-oligosaccharides before (A+B) and after (C+D) saponification, studied using Maldi-TOF-MS. AXAE = arabinoxylan acetyl-esterase; X = xylose; G = 4-O-methyl-glucuronic acid.

2.3 GLUCURONO-ARABINOXYLANS FROM MAIZE KERNELS

Within our research towards complex xylans from various cereals and their effect on processing, we also investigated the GAX from maize kernels starting with the cell wall material obtained after removal of lipids, starch and proteins (Huisman *et al.*, 2000). Besides 7% protein and 8% of starch, this CWM-fraction contained about 60% of non-starch polysaccharides of which arabinose, xylose and glucuronic acid accounted for 62% of all sugars (molar ratio ara:xyl:glcA = 0.77:1:0.3). Substitution of the polysaccharides with acetic acid (4.9% w/w) and ferulic acid (1.6% w/w) was found. Extraction with bariumhydroxide, specific for the extraction of arabinoxylans (Gruppen *et al.*, 1991; Bergmans *et al.*, 1996), yielded a rather pure GAX (38% arabinose, 48% xylose and 8% glucuronic acid), although the recovery was rather low (38% of all arabinose, xylose and glucuronic acid present).

Elucidation of the chemical fine structure of the GAX using the enzymatic approach as described by Verbruggen *et al.* (1998) was not successful since (combinations of) various pure and well characterised enzymes (including various xylanases and arabinoxylan hydrolases) were not able to degrade the GAX significantly. However, digestion of the maize GAX with a commercial enzyme preparation from *Humicola insolens* (Ultraflo, Novo Nordisk A/S) resulted in the release of 80% of all arabinose and about 100% of all xylose originally present as monomeric sugar residues. Obviously, the maize GAX was almost completely degraded to monomers by the large variety of enzymes present in Ultraflo, but consequently, all information about the structure of the substrate was lost. Therefore, maize GAX was incubated with a number of enzyme fractions obtained from Ultraflo by Düsterhöft *et al.* (1997). One specific

fraction resulted in a large variety of oligomeric degradation products (Figure 6). In addition to peaks for the monomeric sugars arabinose, xylose and glucuronic acid residues, also the dimer and trimer of xylose can be recognised in the elution pattern. However, the pattern is quite complex (peaks are not always well resolved) and many peaks originate from unknown compounds.

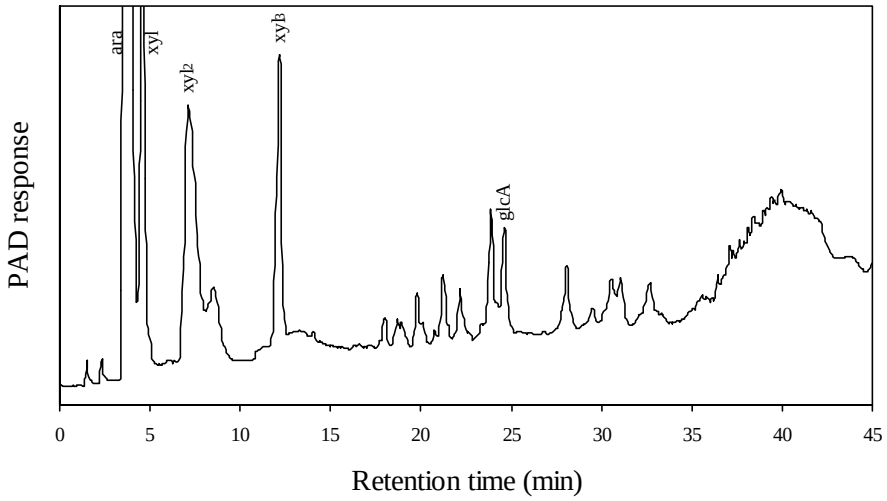

Figure 6. HPAEC elution profile of the digest of maize BE1 after incubation (20 h) with a specific fraction from Ultraflo, rich in arabinoxylan degrading activity.

The MALDI-TOF mass spectrum of the same digest (not shown) illustrated the presence of various series of analogous oligomers. This is demonstrated in Figure 7, showing the 950–1150 Dalton mass range of the mass spectrum. It is clear that Maldi Tof MS is a smart way to demonstrate whether the glucuronic acid moiety is 4-*O*-methyl etherified or not.

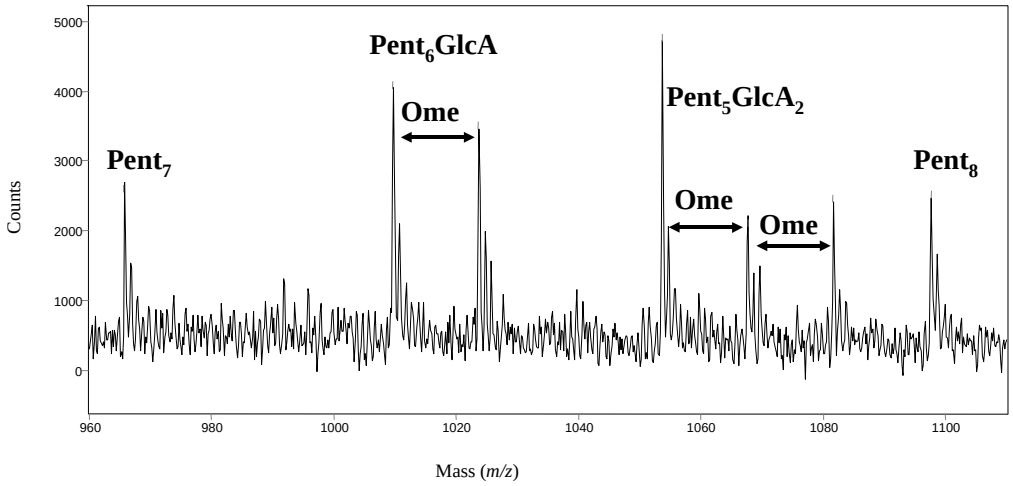

Figure 7. Maldi Tof mass spectrum (950–1150 Dalton mass range) of an arabinoxylan digest from the bariumhydroxide extractable fraction of maize kernels.

The presence of (arabino)xylo-pentamer containing two glucuronic acids is demonstrated as well, while none, only one or both glucuronic acids could be carrying a 4-*O*-methylgroup. Such information could not easily be obtained from HPAEC analysis since it is not known how the retention time of a component is affected by the presence of an additional methoxyl group, and the elution pattern of the enzyme digest is very complex. From the size of these glucuronic acid-containing oligomers (dp 7 to 10), it could be deduced that the glucuronic acids can be very close to each other within the xylan polymer, but are not distributed blockwise. Combination of the data obtained with MALDI-TOF MS with the sugar (linkage) composition of the bariumhydroxide extract, its resistance to endo-xylanase I (Huisman *et al.*, 2000) and knowledge about the composition of the oligomeric side chains from literature (Whistler and Corbett, 1955; Saulnier *et al.*, 1995; Verbruggen *et al.*, 1998) enables the conclusion that glucurono-arabinoxylans from maize kernels are more complex than any other glucurono-arabinoxylan isolated from other sources so far.

In conclusion, it can be stated that the developments in mass spectrometry are giving carbohydrate chemists new and powerful tools in the structural elucidation of complex oligosaccharides. This is especially true when these new MS techniques are used in combination with powerful separation techniques like HPAEC and when pure and well-characterised enzymes are available for the type of carbohydrates under investigation.

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NEW DEVELOPMENTS IN THE MEASUREMENT OF α–AMYLASE, *ENDO*-PROTEASE, β–GLUCANASE AND β–XYLANASE

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1 INTRODUCTION

Over the past 8 years, we have been actively involved in the development of simple and reliable assay procedures, for the measurement of enzymes of interest to the cereals and related industries. In some instances, different procedures have been developed for the measurement of the same enzyme activity (e.g. α–amylase) in a range of different materials (e.g. malt, cereal grains and fungal preparations). The reasons for different procedures may depend on several factors, such as the need for sensitivity, ease of use, robustness of the substrate mixture, or the possibility for automation. In this presentation, we will present information on our most up-to-date procedures for the measurement of α–amylase, *endo*-protease, β–glucanase and β–xylanase, with special reference to the use of particular assay formats in particular applications.

2 α–AMYLASE

A range of substrates are available for the measurement of α–amylase, including:

1 Amylase HR Reagent (Ceralpha Method)[1]; containing end-blocked p-nitrophenyl maltoheptaoside in the presence of excess thermostable α–glucosidase.
2 Amylazyme[2] and Phadebas[R] Tablets; containing dyed and crosslinked starch.
3 Red Starch; a red-dyed, soluble starch substrate.
4 Beta–limit dextrin; for use in starch/iodine assay procedures (e.g. Farrand, International and SKB methods and FIA based procedures).
5 Soluble starch for use in reducing-sugar procedures.

Of the assay procedures available, the only one involving a defined substrate is the Ceralpha method[1] using Amylase HR Reagent. This reagent mixture contains the defined, modified oligosaccharide, "end-blocked p-nitrophenyl maltoheptaoside". The assay is very versatile and can be used to assay cereal, fungal and bacterial α–amylases. The principle of the Ceralpha assay procedure is shown in Figure 1. The replacement of the amyloglucosidase/yeast maltase enzyme mixture by thermostable α–glucosidase allows reagent to be used at temperatures up to 60^{o}C and in the pH range 5.2 to 7.5 (formerly, it could only be used up to 40^{o}C, and in the pH range 5.0 to 6.0). This assay procedure is extremely versatile and has been accepted as a standard by the International Association of Cereal Science and Technology (ICC). It has been successfully evaluated by the U.K. milling industry, and is currently replacing the

Farrand method, which has been in place since 1964. An interlaboratory evaluation by the American Association of Cereal Chemists (AACC) is currently being organised.

The Ceralpha method has been related to other procedures for the assay of α–amylase and equations relating these methods are given in Table 1.

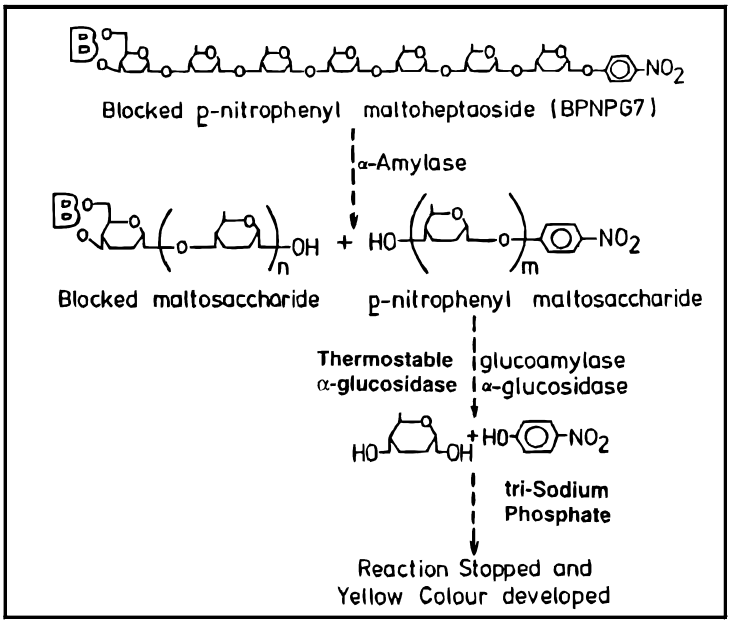


Figure 1. Ceralpha method for the measurement of α–amylase using either amyloglucosidase/yeast maltase mixture (Ceralpha Reagent) or thermostable α–glucosidase (Amylase HR Reagent).

Table 1. Correlation of the Ceralpha method to β–limit dextrin/iodine methods for the measurement of α–amylase.

Method	Source of Enzyme	Regression Equation
International Method (DU)	Malt	DU = Ceralpha (CU) x 0.30
SKB	Malt	SKB = Ceralpha (CU) x 0.57
(AACC)	Fungal	SKB = Ceralpha (CU) x 0.59
	Bacterial	SKB = Ceralpha (CU) x 1.80
Farrand	Wheat	FU = Ceralpha (CU) x 86–1.9
	Fungal	FU = Ceralpha (CU) x 69

Alternative procedures for the measurement of α–amylase involve the use of dyed, crosslinked amylose in tablet form (Amylazyme tablets[2]) or Procion Red dyed soluble starch. The major advantage of the Amylazyme substrate is that it is more robust (harder to contaminate), is more sensitive, and can be used in situations where Amylase HR can't be used. Assay formats based on this substrate are particularly useful for measuring trace levels of α–amylase in materials such as heat-treated flours and food

products containing starch as a thickening agent, and for the measurement of α–amylase in biological washing powders. The Amylazyme method has been adopted by AACC (Method 22–05) for the measurement of α–amylase in cereal flours and malts, following an extensive interlaboratory evaluation. Red Starch is a soluble dyed starch material for the measurement of α–amylase. This can be used in test-tube formats for α–amylase measurement, but it is also useful for detecting α–amylase in agarose and acrylamide gels.

One of the few automated methods for the measurement of α–amylase involves the use of the Scalar flow-injection analysis equipment. The substrate and assay procedure is a modification of the International (American Society of Brewing Chemists; ASBC) method that is based on the reaction of iodine with β–limit dextrin. Until recently, a β–limit dextrin material was available from Rank-Hovis. This material was prepared by treatment of starch with soybean β–amylase. The substrate as supplied was a mixture of β–limit dextrin and maltose in approximately equal proportions. Since Rank Hovis decided not to continue production of this material, Megazyme was approached as a possible alternative manufacturer. The material now produced and supplied by Megazyme, behaves in the assay procedure in a very similar way to the Rank-Hovis product, however, essentially all of the maltose has been removed by ultrafiltration. Consequently, the concentration of the Megazyme β–limit dextrin used in α–amylase assays (1 %) is half the concentration recommended for the Rank-Hovis material. With the Scalar flow-injection analysis procedure for the measurement of α–amylase, the values obtained are essentially identical with the values obtained with the ASBC method (using a β–limit dextrin produced from a special starch provided by ASBC).

3 β–XYLANASE

Several substrates and assay procedures are available for the measurement of β–xylanase (*endo*-1,4-β–xylanase) [2] in enzyme materials and food and feed products, and some of these are summarised in Table 2.

Table 2. Substrates for the assay of β–xylanase.

Substrate	Nature	Assay Procedure
Wheat arabinoxylan	soluble	Reducing-sugar
Wheat arabinoxylan	soluble	Viscometric
Xylazyme AX Tablets	gel particles	Chromogenic substrate
Azo-wheat arabinoxylan	soluble	Chromogenic substrate
Azo-xylan (oat spelts)	soluble	Chromogenic substrate
Azo-xylan (birchwood)	soluble	Chromogenic substrate

In creating standard curves for the various dyed substrates, the β–xylanase used is first standardised in International Units. The Nelson-Somogyi reducing-sugar method is used with wheat arabinoxylan as substrate, and one Unit of activity is defined as the amount

of enzyme required to release one micromole of reducing-sugar equivalents (as xylose) per minute, under the defined assay conditions of temperature and pH. The Nelson-Somogyi reducing-sugar method is one of the few reducing-sugar methods, which gives a stoichoimetric colour response with homologous oligosaccharides of increasing degrees of polymerisation.

Although reducing-sugar methods are useful for standardising the activity of relatively pure *endo*-β–xylanase, they cannot be used for samples containing high levels of reducing sugar, or for preparations with high levels of other enzymes active on the substrate (e.g. β–xylosidase and α–L-arabinofuranosidase). In such cases, viscometric methods, or methods employing dyed xylan substrates must be used. Viscometric assays are highly specific for *endo*-enzymes, particularly if a high viscosity substrate is used. For β–xylanase, the substrate of choice is wheat arabinoxylan. Such assays can be sensitive, specific and accurate, however, unless an automated viscometer is available, the assays are tedious and time consuming. Results are most meaningful if several measurements are taken over an incubation period, and inverse reciprocal viscosity values are determined and plotted against incubation time.

Several chromogenic substrates are available for the specific measurement of *endo*-β–xylanase. Of these, Azo-wheat arabinoxylan (soluble) and Xylazyme AX (gel particles in tablet form) are the most useful and versatile substrates. A comparison of the sensitivity of various soluble chromogenic xylan substrates is given in Figure 2. Of these, Azo-wheat arabinoxylan is the most sensitive. Assays employing Xylazyme AX tablets are approximately 5-times more sensitive than those using Azo-wheat arabinoxylan, consequently, where sensitivity is the major concern, the Xylazyme AX tablets are the substrate of choice.

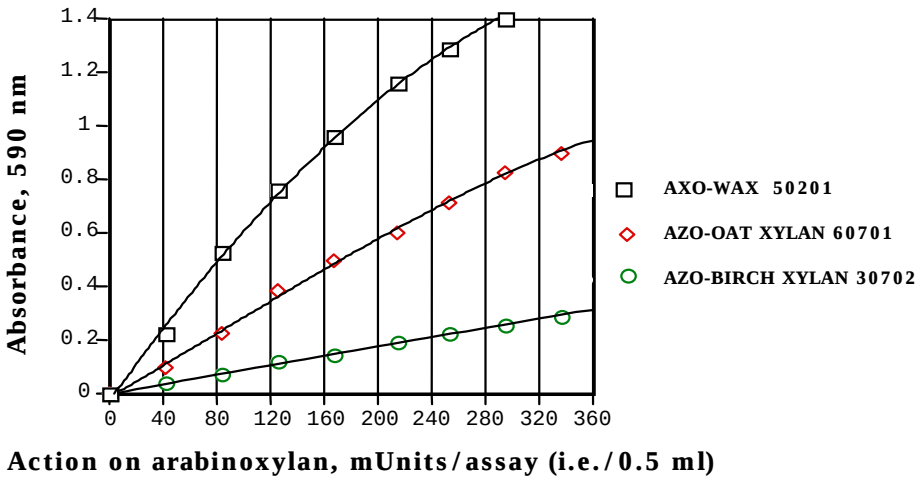


Figure 2. Standard curves relating enzyme activity of pure A. niger xylanase to colour release from Azo-wheat arabinoxylan, Azo-xylan (oat) and Azo-xylan (birchwood).

The measurement of xylanase in industrial enzyme preparations is relatively straight-forward. However, problems arise when measuring trace levels of enzymes in animal

feeds or bread improver mixtures. These problems include adsorption to feed components, inactivation during pelletting and inhibition by specific xylanase inhibitors (such as those identified in wheat flour). The best approach to get a true estimate of the level of enzyme in the feed is through recovery experiments. Basically, a known quantity of a particular xylanase preparation is added to a slurry of the feed, and the recovery of activity is determined using a suitably sensitive substrate, such as Xylazyme AX tablets or Azo-wheat arabinoxylan.

4 β–GLUCANASE AND CELLULASE

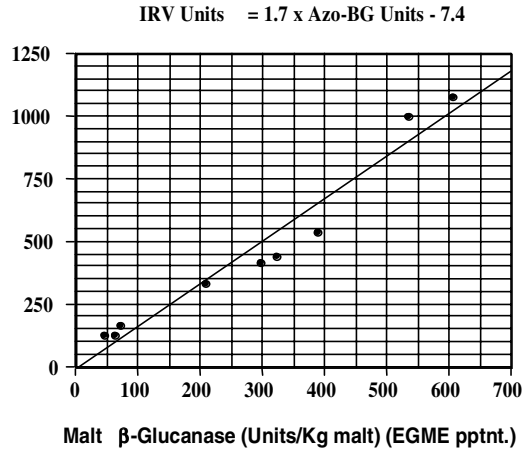
Various substrates are available for the measurement of *endo*-1,4-β–glucanase (cellulase) and *endo*-1,3;1,4-β–glucanase (lichenase, malt β–glucanase) [2] as shown in Table 3. Each of the substrates listed has particular advantages and limitations.

Table 3. Substrates for the assay of cellulase and β–glucanase.

Substrate	Nature	Assay procedure
CELLULASE:		
CM-Cellulose 7M	soluble	Reducing sugar or viscometric
CM-Cellulose 4M	soluble/gel	Reducing sugar
Azo-CM-Cellulose	soluble	Chromogenic substrate
Azo-Barley Glucan	soluble	Chromogenic substrate
Cellazyme C Tabs	gel particles	Chromogenic substrate
Cellazyme T Tabs (tamarind xyloglucan)	gel particles	Chromogenic substrate
Beta–Glucazyme Tabs	gel particles	Chromogenic substrate
1,3;1,4-BETA–GLUCANASE;		
Azo-Barley Glucan	soluble	Chromogenic substrate
Beta–Glucazyme Tabs	gel particles	Chromogenic substrate

Pure barley β–glucan is useful for the assay of purified cellulase and β–glucanase in reducing-sugar assays. It is also used in viscometric assays. The official method of the Institute of Brewing for the assay of malt β–glucanase employs barley β–glucan in a viscometric procedure. The enzyme is mixed with the substrate and viscosity measurements are taken at several times over about 30 min. Inverse reciprocal viscosity (IRV) values are calculated and activities are determined from plots of IRV against incubation time. The assay is relatively accurate and reproducible, but very time consuming; analysis of 10 samples takes a single operator about 2 days. An alternative procedure involves the use of Azo-Barley Glucan[3]. With this substrate and assay procedure, 10 samples can be extracted and analysed in about 1 hour. With the Azo-Barley Glucan method, absorbance values can be converted to International Units of activity through a standard curve, or alternatively can be directly converted to IRV units. A curve relating enzyme activity determined with the Azo-Barley Glucan method

(substrate lot 60602) to IRV units for several malt samples is shown in Figure 3. A range of dyed polysaccharide substrates have been developed for the measurement of cellulase, including Cellazyme C (dyed, crosslinked HE-cellulose), Cellazyme T (dyed, crosslinked xyloglucan) and Beta–Glucazyme (dyed, crosslinked β–glucan). At first glance, one would conclude that the best substrate to assay for enzymes active on barley or oat β–glucan is the Beta–Glucazyme tablets. However, if sensitivity is the major



concern, as is the case for the measurement of trace levels of cellulase in animal feeds, the preferred substrate is Cellazyme T tablets. (see Figure 4).

Figure 3. Curve relating IRV units (IOB method) to malt β–glucanase activity determined with the Azo-Barley Glucan method (substrate lot 60602).

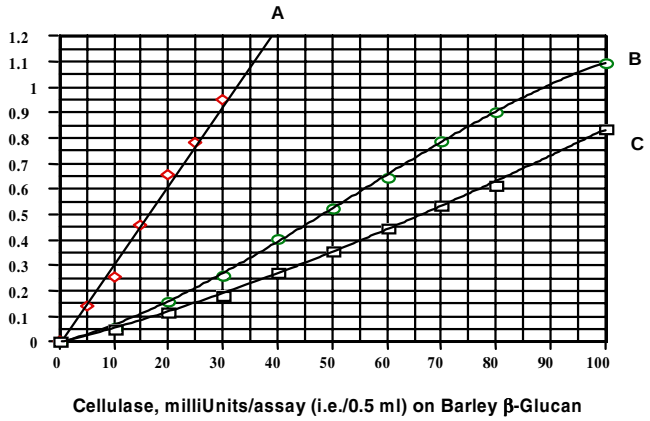


Figure 4. Standard curves relating enzyme activity of pure T. viride cellulase to colour release from Cellazyme C (C), Cellazyme T (A) and Beta–Glucazyme (B) tablets.

When sensitivity is not a constraint, Azo-CMC is the preferred substrate. Firstly, because the level of salt in the sample does not affect the assay, and secondly, the standard curves relating enzyme units (International Units) to absorbance increase were found to be very similar for a range of cellulases. Cellazyme C tablets find widespread

use in the denim industry for standardisation of cellulases used in creating the “stone-washed” effect.

5 PROTEASE

Two commonly used substrates for the assay of *endo*-protease are cow hide azure and Azo-Casein. Cow hide is essentially collagen. We have prepared a dyed, crosslinked collagen product (AZCL-collagen) as an alternative to cow hide azure. The major advantage of AZCL-collagen is that it is less fibrous and can thus be incorporated into tablets (Protazyme OL), which greatly increases the ease of use of this substrate.

However, in comparing a wide range of *endo*-proteases on Protazyme OL tablets (AZCL-collagen) to action on Protazyme AK tablets (AZCL-casein), it was found that the relative rates of hydrolysis of the two substrates were similar. Consequently, since the standard curves with Protazyme AK are more linear than with Protazyme OL, then Protazyme AK is the substrate of choice.

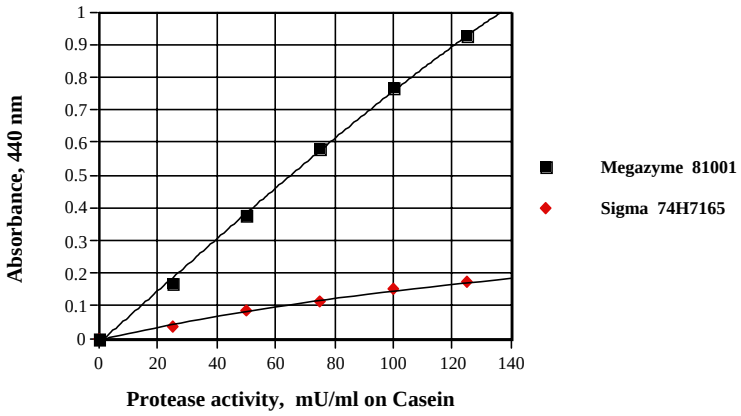


Figure 5. Standard curves for Subtilisin A on Azo-casein (Megazyme, lot 81001 and Sigma, lot 74H 7165). The Subtilisin A was first standardised on casein using tyrosine as the standard.

A disadvantage of the Protazyme tablets is that disintegration of the tablets requires agitation. Thus, a stirrer bath arrangement is required. An alternative substrate is soluble Azo-Casein. Azo-Casein has been commercially available for many years, but most commercial preparations are poorly dyed, and incompletely soluble, resulting in limited sensitivity and poor linearity of the standard curve. We have re-evaluated the dyeing of casein with sulphanilic acid, and have optimised this reaction to give a highly dyed material which is a very effective substrate for *endo*-proteases. In Figure 5, standard curves for Subtilisin A on Azo-Casein from Megazyme (lot 81001) and from Sigma Chemical Co. (lot no. 74H7165), are compared. It is obvious that the Megazyme product is superior in terms of sensitivity and linearity of the standard curve. The sensitivity of *endo*-protease assays using Azo-Casein (Megazyme lot no. 81001), is about one third of that using Protazyme AK tablets. Azo-Casein is a good, non-selective substrate for the assay of *endo*-protease activity. Regression equations for a number of *endo*-proteases on Azo-Casein are given in Table 4.

Table 4. Regression equations for several proteases on Azo-Casein (lot 81001).

Papain (from *Papaya* latex): Protease (milli-Units/mL) = 270 x Absorbance (440 nm) + 7;	R = 0.99
Bromelain (from pineapple stem): Protease (milli-Units/mL) = 460 x Absorbance (440 nm) - 13;	R = 0.99
Ficin (from figs): Protease (milli-Units/mL) = 190 x Absorbance (440 nm) + 3;	R = 0.99
Subtilisin A (from *Bacillus licheniformis*): Protease (milli-Units/mL) = 130 x Absorbance (440 nm) + 4;	R = 0.99
Proteinase K (from *Tritirachium album*): Protease (milli-Units/mL) =140 x Absorbance (440 nm) - 4;	R = 0.99
Fungal protease (*A. niger*; from Sigma Chemical Co.): Protease (milli-Units/mL) = 146 x Absorbance (440 nm) - 4;	R = 0.99

The linear range for each of the proteases was essentially 0.1 to 1.0 absorbance units.

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ENDOGENOUS INHIBITORS OF THE ENDOPROTEINASES AND OTHER ENZYMES OF BARLEY

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1 INTRODUCTION

Barley has long been known to contain compounds that inhibit the activities of enzymes of other species, especially those of insects, and most of these exogenous inhibitors are thought to play roles in protecting the plant or grain from attack. This paper, however, focuses on the endogenous inhibitors; compounds that are present in barley (or its product, malt) that inhibit the activities of barley (or malt) enzymes. Endogenous inhibitors of the barley malt carbohydrate-degrading enzymes α-amylase and limit dextrinase have been studied for many years, and some of the characteristics of these inhibitors will be discussed briefly. However, the majority of this presentation will discuss our recent studies on the compounds that occur in barley and malt and that inhibit the activities of the proteinases that form in barley during the malting process. For the purposes of this paper, the terms proteinase, protease and endoproteinase have been used interchangeably, and refer to enzymes that hydrolyze proteins but which are not primarily exopeptidases.

2 INHIBITORS OF CARBOHYDRATE-DEGRADING ENZYMES

Barley malt contains three major starch-degrading enzymes, α-amylase, β-amylase and limit dextrinase (LD). The activities of two of these, • -amylase and LD, are controlled by the presence of proteinaceous inhibitors that can reduce their activities.

2.1 ALPHA-AMYLASE INHIBITOR

Almost 20 years ago, Mundy *et al*. (1) and Weselake *et al*. (2) simultaneously reported that a protein from barley inhibited the activity of the α-amylase-2 class barley amylases. The α-amylases degrade solubilized starch randomly at (1→4)-α-glycosidic bonds, yielding linear and branched dextrins that can be further degraded by other enzymes. The amylase inhibitor protein was identical to a previously studied specific inhibitor of the bacterial protease subtilisin and had therefore been given the name Barley Amylase Subtilisin Inhibitor (BASI). It appears to be the only one of several barley amylase inhibitors that can inhibit the activity of (germinating) barley.

There appear to be at least six alleles at this locus enzymes. Some of the characteristics of this inhibitor are listed in Table 1. They will not be covered here in detail, since they have been reviewed recently (3). The BASI proteins are very polymorphic and widely distributed among both cultivated and wild barleys (4).

Table 1. Some characteristics of the endogenous barley α-amylase inhibitor (BASI or ISA-1).

Inhibits only the high-pI α-amylase-2 group amylases of barley
MW 19,865, contains 181 amino acid residues, pI is around 7.2
Binds tightly to α-amylase to form the 'α-amylase-3' isoenzyme class
Is synthesized in the endosperm of developing grain; in the aleurone during germination
Coded by a single gene locus, *Isa-1*, located on the barley chromosome 2

2.2 LIMIT DEXTRINASE INHIBITOR

During malting and mashing, the enzyme LD hydrolyzes the α-(1,6)-glycosidic linkages of branched dextrins that form from starch amylopectins. Unless this happens, the β-amylases cannot further degrade the dextrins into sugars that yeasts can utilize during brewing. LD thus plays an important role in brewing, because it allows the complete degradation of branched-chain carbohydrates. Higher levels of the enzyme should, therefore, provide mashes that have increased fermentable sugar levels. Since wort and beer both still contain significant levels of branched dextrins, it seems likely that increasing the activity of this enzyme would lead to an increase in the efficiency of brewing. In 1993, it was reported (5) that two small (~15 kD) proteins in barley inhibited the activity of a purified limit dextrinase preparation. These inhibitors are probably at least partially responsible for the fact that about 60–70% of the limit dextrinase present in mashes was inactive (6, 7). The inhibitors can be removed from the enzyme and its activity recovered by incubating the inhibitor-enzyme complex with either proteases or reducing agents (7).

Table 2. Some characteristics of the endogenous barley limit dextrinase (LD) inhibitor.

Binds to LD in grain or during extraction, forming a 'bound' enzyme class
In malt, about 20% of the LD is present as the 'free' form, 70% in the 'bound' form, but this varies among cultivars
The 'bound' enzyme can be changed to the 'free' form by reducing agents or proteinases
The 'inhibitor' is a mixture of isoforms with MW of 12,686 & 12,928, pI of 6.7 & 7.2 They are coded by a single gene, but are post-translationally modified differently
The inhibitor level drops during malting, but enough remains at the end of malting to restrict the LD activity, leading to worts that have decreased fermentability

2.3 XYLANASE INHIBITOR

While high β-glucan levels are usually responsible for slow mash filtration during brewing, in some cases worts that contained only small amounts of β-glucan have not filtered well. Such problems have often been rationalized by assuming that high levels of wort arabinoxylans caused the problems. Debyser *et al*. (8) reported recently that while barley arabinoxylans were degraded during the brewing of beers from barley malts, this did not happen when beers were prepared from a mixture of barley malt and wheat meal. This indicated that the wheat probably contained an inhibitor that impeded the activity of the barley xylanases. These authors have shown (8, proceedings, this meeting) that such inhibitors do exist in wheat and report that they also exist in barley. Nothing has yet been published about the barley xylanase inhibitors, but it seems likely that they will be similar to those that occur in wheat.

3 PROTEINASE INHIBITORS

3.1 IDENTIFYING INHIBITORS

Almost 35 years ago, Enari *et al*. (9) showed that when unmalted barley was added to malted barley mashes, the nitrogen contents of the resulting worts were significantly lowered. When 50% barley was added, the endoproteinase activity of the mash was lowered by 30% and the brewery fermentation rate was significantly retarded. These results indicated that ungerminated barley contained compounds that inhibited their (malt) endogenous endoproteinases, and it was apparent that at least some of the inhibitors were relatively large. Mikola and Enari (10) later reported that these inhibitors disappeared during the early stages of germination, so that they were totally missing from malt. They also showed that there was not sufficient inhibitor in barley to totally control the proteolytic activities during malting. They therefore proposed that these inhibitors had little or no effect on nitrogen solubilization during malting and brewing. When our studies were started, then, it was unclear whether or not the endogenous barley inhibitors really affected the brewing process.

The ‘soluble nitrogen’ (amino acids, peptides and soluble proteins) levels of worts play important roles in determining beer quality, since they affect many of its aspects, from ‘mouth feel’ (viscosity) to how well the yeast grow during fermentation (yeast food) to beer flavor. If we can determine exactly how proteins are degraded during malting and mashing and how these degradation processes are controlled, then we should be able to scientifically design barleys that will form worts that have nearly perfect soluble protein levels. Several researchers have independently shown that the endoproteinases are the rate-limiting enzymes for the degradation of barley proteins, so it is these enzymes that will need to be controlled to affect the levels of wort soluble protein, and not the exopeptidases (carboxypeptidases and aminopeptidases). For several years, we have been working to define the protein degradation system that operates in barley during malting and mashing and, in order to do this, we obviously must understand what the endogenous endoproteinase inhibitors are, how they function, and how they affect protein solubilization.

3.2 DEMONSTRATING INHIBITORS IN BARLEY AND MALT

We nearly always use malt proteinase preparations to test the activities of the barley and malt inhibitors, since unmalted barley contains only very low levels of endoproteolytic activity. When we first prepared barley extracts and analyzed their abilities to inhibit the activities of crude malt proteinase preparations, no inhibition was detected. It seemed possible that there was inhibition, but that it was being masked by the slight enzymatic activities of the barley extracts being tested, so we boiled them to destroy these proteolytic activities. When this was done, the barley preparations inhibited the malt endoproteinases quite strongly. The reason for this will be discussed later. After we showed that boiled barley preparations contained inhibitors, we tested whether they also occurred in malt extracts. Heated (100°C) malt extracts also inhibited the malt proteinases. Serial dilutions of boiled barley and malt extracts were tested to determine how much each preparation needed to be diluted in order to cause a 50% inhibition of a standard malt proteinase extract. This test showed that, contrary to previous reports, malt not only contained inhibitors, but it contained about 2.5 times as much inhibitory capacity as barley.

3.3 THE SEPARATION OF BARLEY AND MALT INHIBITORS BY ION EXCHANGE CHROMATOGRAPHY

To compare the component inhibitors in the barley and malt extracts, they were subjected to separation by carboxymethyl cellulose (CMC) chromatography and the abilities of the separated fractions to inhibit the activities of a crude malt protease extract were measured. As shown in Figure 1, both inhibitor extracts were separated into multiple fractions. The barley preparation yielded 4 areas of inhibition, while that of malt showed only 3 (11). In accord with our previous studies, the total malt inhibition (the areas of the Fig. 1 graphs in which the activity fell below the control level) was greater than that of the barley sample.

3.4 THE PURIFICATION AND IDENTIFICATION OF TWO ENDOPROTEINASE INHIBITORS

We have used open-column and HPLC chromatographic methods to purify two of the proteinase inhibitors from barley (12–14). Both were small proteins whose amino acid sequences showed homology with the so-called ‘lipid transfer’ proteins that have been called LTP1 (lipid transfer protein 1) and LTP2. While these proteins do share structural similarities with other LTP proteins, neither has yet been demonstrated to transfer lipids. LTP1 has also been called PAPI (**p**robable **a**mylase **p**rotease **i**nhibitor), but had not previously been shown to inhibit any protease enzyme activities. LTP1 probably plays an important role in brewing, since it is apparently the main beer protein that promotes good foam characteristics (15, 16), as well as likely playing a role in regulating the wort soluble protein levels and characteristics. The LTP1 was purified from the inhibition area 3 of Fig. 1, while LTP2 was purified from area 1. This is apparently the first time that LTP2 has been purified from barley or malt. LTP2 was the only inhibitor present in area 1 of the Fig. 1 CMC separation, but fraction 3 contained additional inhibitory species besides LTP1. The inhibition area 2 probably also contains more than one inhibitor, and nothing is known about the inhibitors that comprise the inhibition area 4. The LTP1 and LTP2

proteins appear to have little in common except their names and their abilities to inhibit the malt proteinases, since the LTP2 (MW 7,112) is considerably smaller than LTP1 (MW 9,696) and their amino acid sequences show little homology (17).

3.5 THE INHIBITORS AFFECT MAINLY THE MALT CYSTEINE PROTEINASES

The malt endoproteinases can be separated into about 40 activities using a 2-dimensional (2-D) system that separates them by isoelectric focusing (IEF), followed by polyacrylamide gel electrophoresis (PAGE) (18). By developing the resulting 2-D gels in the presence of various class-specific protease inhibitors, we have shown that proteinases of all four classes (cysteine-, serine-, aspartic- and metallo-) are present in green malt and we have identified the classes of each of the individual activities (18). When the 2-D separated enzyme gels were developed in the presence of either crude inhibitor mixtures or purified LTP1 or LTP2, it was mainly the cysteine proteinases whose activities were inhibited (11), although some of the serine activities were also partially inactivated in the presence of the mixed inhibitors or LTP1 (13). It has also been shown that 0.9 ug of purified LTP1 totally inactivated the ability of a purified 31 kD green malt cysteine endoproteinase to hydrolyze a substrate protein molecule, reduced and alkylated β-purothionin. In the absence of inhibitor, the enzyme quickly reduced the purothionin to small peptides.

3.6 THE INHIBITORS ARE PROBABLY COMPLEXED WITH PROTEINASES IN EXTRACTS (INCLUDING MASHES)

One obvious rationale for why endogenous inhibitors occur in barley and malt is that they may act to inhibit the activities of the proteinases until they are moved to the location where they need to function. If so, this implies that the inhibitors (I) and enzymes (E) are probably localized separately in the grains. However, as soon as the grain or malt is ground and extracted, the inhibitors and enzymes are free to form complexes (E-I). This means that until we figure out how to interrupt these E-I complexes without destroying the enzyme activities, there will always be more enzyme activity in the grain than our measurements indicate. We have tested for the presence of the E-I complexes by using BioGel P-30 size exclusion columns to examine the molecular sizes of the inhibitors and of the E-I complexes. When unheated malt inhibitor extracts were subjected to separation on P-30 columns and the inhibitory activities of the separated fractions were analyzed, both as collected and after being boiled, the inhibitors eluted from the column in the void volume, implying that they were present as part of a large complex. When the inhibitor extract was subjected to boiling prior to being separated on the P-30 column, however, the inhibitory activity eluted as if it were a relatively small molecule. From these and similar experiments, it is very likely that the inhibitors and proteinases interact as soon as their spatial separation inside the grain is destroyed by solubilization, either in these inhibitor preparations or during mashing.

3.7 ATTEMPTS TO DISSOCIATE THE E-I COMPLEX

For studying the proteinase-inhibitor complexes and quantifying the inhibition that occurs in extracts, it would be very useful to be able to dissociate the E-I complexes into their component parts using methods that are gentle enough to preserve the enzymatic activities of the component proteinases. We have attempted to do this by treating malt extracts with various compounds that seemed likely to disrupt the protein complexes; for example detergents, 6 M urea and 5 M sodium chloride. None of these had the desired effect. We had found previously that high cysteine levels strongly increased the proteolytic activities of malt extracts, and it seemed possible that this activity augmentation was due to the cysteine displacing the inhibitor from the enzymes. In addition, cysteine does disrupt the α-amylase-inhibitor complex. Incubation with either 10 and 20 mM cysteine, however, had no detectable effect on the E-I complex. Neither did heating an extract at 70°C, even though this destroyed the proteolytic activity. Heating the extracts to 100°C was the only way we could dissociate the complexes. The released enzyme molecules were, of course, inactive.

3.8 ADDING ENDOGENOUS ENDOPROTEINASE INHIBITORS TO MASHES LOWERS WORT SOLUBLE PROTEIN LEVELS

In the USA, brewers are now convinced that our malting barleys produce malts that have too much proteolytic activity and that thus produce worts containing too much soluble protein. In order to develope barleys whose malts yield worts with lowered soluble protein levels, one could either select for lines that have either lowered endoproteinase levels or increased proteinase inhibitor levels. Because there are many (over 40) different endoproteinases in malt, it will likely be easier to increase the inhibitor levels than to delete sufficient proteinase activities to give the lower soluble protein levels. To determine whether the inhibitors can lower the soluble protein levels of worts, we mashed samples of the malting barleys Morex (6-rowed) and Harrington (2-rowed) in the standard way and also in the presence of an unfractionated Morex malt inhibitor extract. The results (Table 3) show that in the presence of the inhibitor, the soluble protein levels were reduced by about 20%,while none of the other measured characteristics were affected.

Table 3. The effect of adding malt endogenous proteinase inhibitors to mashes.

		Inhibitor added	
Characteristic	Units	No	Yes
Soluble protein,	%	5.41	4.54
Alpha-amylase,	20°DU	78.7	79.0
Diastatic power,	°ASBC	150	155
Beta-glucanase,	ppm	320	388

Since only about 20% of the final wort soluble protein is released during mashing, this means that, in this particular experiment, almost all of the mash endoproteolytic activity was inhibited. Thus, it seems likely that if higher-inhibitor barleys are developed, they will likely yield lower-soluble-protein worts. In addition, if this is done by increasing the LTP1 levels, the beer produced from these barleys should have improved foam characteristics. Alternatively, if brewers are willing to return to using the infusion mashing process (removing a portion of the mash, boiling it and returning it to the mash tun) this process would both: a) destroy the proteolytic activity of the boiled fraction and b) release the inhibitors that were previously bound to the destroyed proteinases. Both of these effects should serve to lower the soluble protein levels of the resultant wort.

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TAXI, A NEW CLASS OF ENZYME INHIBITORS[1]

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1 INTRODUCTION

Wheat arabinoxylans consist of a linear backbone of 1,4-linked β-D-xylopyranose units. L-Arabinofuranosyl residues are attached to the main chain by 1,3- and/or 1,2-α-glycosidic linkages. Phenolic acids, such as ferulic acid and *p*-coumaric acid, can be ester-linked to *O*-5 of the arabinofuranose units.

Arabinoxylan can be degraded by several enzymes. The 1,4-β-D-xylanhydrolases (EC 3.2.1.8), further referred to as endoxylanases, generate unsubstituted and substituted xylo-oligosaccharides. These oligomers are further degraded by β-D-xylosidases (EC 3.2.1.37), releasing β-D-xylose from the non-reducing end. The α-L-arabinose substituents from the main chain are liberated by α-L-arabinofuranosidases (EC 3.2.1.55). Finally, esterases (EC 3.1.1.6) release ferulic and p-coumaric acids esterified to *O*-5 of arabinofuranosyl residues.

Recently, Debyser *et al.*[1,2] obtained evidence for the presence of endoxylanase inhibitors in wheat. While in regular (wheatless) barley malt Pilsner beer brewing the arabinoxylan was degraded by the barley malt xylanolytic system[3], this was significantly less so in the production of Belgian white beers prepared from a mixture of barley malt and wheat wholemeal[1,2].

To demonstrate that cereals contain besides α-amylase[4-7] and protease[8-9] inhibiting proteins also (a) protein inhibitor(s) of endoxylanases we isolated and characterized the *Triticum aestivum* xylanase-inhibitor (TAXI).

In industrial practice microbial endoxylanases are used in breadmaking recipes since they have a positive effect on bread volume[10], presumably because they convert the deleterious water-unextractable arabinoxylans into their beneficial high molecular weight water-extractable counterparts[11]. Therefore, we investigated whether the endoxylanase inhibitor identified here is also active during the breadmaking process.

[1] Based on the paper: W. Debyser, W.J. Peumans, E.J.M. Van Damme, and J.A. Delcour. TAXI, a New Class of Enzyme Inhibitors Affecting Bread Volume, J. Cereal Sci. (1999) 39-43.

2 MATERIALS AND METHODS

Speciality chemicals and microbial enzymes. Azurine-crosslinked wheat arabinoxylan tablets (AZCL-AX), endoxylanase M4 from *Aspergillus niger* and endoxylanase M6 from a rumen microorganism culture filtrate were from Megazyme (Bray, Ireland). The endoxylanases from *Bacillus subtilis* and *Aspergillus aculeatus* were from Puratos (Groot-Bijgaarden, Belgium). Buffers were A: 0.025 M sodium acetate, pH 5.5; B: 0.025 M sodium phosphate pH 6.0; C: 0.250 M sodium acetate pH 5.0; D: 0.025 M sodium acetate, pH 5.0.

Wheat germination. Wheat (*Triticum aestivum* L. cv. Soissons) was germinated under sterile conditions based on procedures by Lai *et al.*[12] and Van Campenhout *et al.* [13]. After surface-sterilization with 0.2% (w/v) silver nitrate for 20 min and rinsing with sterile 0.5 M NaCl and deionised water, the wheat grains were immersed for 6, 4 and 2 h in a solution containing 800 ppm penicillin (Continental Pharma, Brussels, Belgium), 800 ppm streptomycin sulphate (Federa, Brussels, Belgium) and 296 ppm nystatin (Alpha Pharma, Zwevegem, Belgium). The three steeping phases were interrupted by 16 h drying periods. Grains were germinated for 7 days at 20°C under sterile conditions and lyophilized. The microbial contamination was checked by incubating kernels on plate count agar (Oxoid, Hampshire, England). No microbial growth occurred during germination.

Partial purification of endogenous wheat endoxylanase. Whole meal from sterile germinated wheat (400 g) was suspended in 1,600 mL of 0.1 M sodium phosphate buffer (pH 7.0), stirred for 30 min and centrifuged (10,000 *g*, 30 min, 4°C). The supernatant was dialysed against deionised water (48 h, 4°C) and fractionated by anion exchange chromatography on a Source 15Q column (26 x 100 mm, Pharmacia, Uppsala, Sweden). The column was equilibrated with 20 mM Tris[hydroxymethyl]-amino-methane-HCl buffer, (pH 8.0). The fractions with endo-xylanase activity (measured according to an earlier procedure[3]) eluted between 0.3 M and 0.4 M NaCl.

Measurement of endoxylanase activity and inhibition thereof. The endoxylanase activity was measured according to a previously described procedure[3] (buffer A; 30°C). For inhibition measurements, the endoxylanase activity of 0.5 mL endoxylanase solution and 0.5 mL buffer A was compared with that of 0.5 mL endoxylanase solution and 0.5 mL of TAXI or TAXI containing extracts in buffer A. The endoxylanase and TAXI were mixed and the substrate added after 30 min of preincubation. Under the conditions of the assay, one unit (U) corresponds to an increase in extinction at 590 nm as a result of the release of soluble azurine crosslinked arabinoxylan fragments of 1.0.

Purification of endoxylanase inhibitor from wheat flour. Soissons flour (10.0 kg) was suspended in 50.0 L 0.1% (w/v) ascorbic acid in H_2O, extracted overnight at 7°C and centrifuged (7°C, 10,000 g, 30 min). To the supernatant 2.0 g/l $CaCl_2$ was added and the pH was raised to 9.0 with 2.0 M NaOH. The extract was left overnight (7°C) and centrifuged (7°C, 10,000 g, 30 min). The pH was adjusted to 5.0 with 2.0 M HCl. The extract was cation exchanged (SP Sepharose Fast Flow, 90 × 90 mm, Pharmacia). The column was equilibrated with buffer B (500 mL) and a protein fraction was eluted with 800 mL 0.5 M NaCl. This eluate was diluted 5 times, the pH adjusted to 5.0 as above

and cations were exchanged (SP Sepharose Fast Flow, 26 × 100 mm, Pharmacia). The column was equilibrated with buffer B (200 mL) and after a linear salt gradient from 0 to 0.5 M NaCl (800 mL), fractions of 10 mL were, after desalting (PD 10 column, Pharmacia), assayed for endoxylanase inhibition. Eluate (250 µL) was preincubated for 30 min (20°C) with 250 µL of appropriately diluted *A. niger* endoxylanase, the substrate was added and the mixture incubated (60 min, 50°C). The remainder of the procedure was as described above with addition of 5.0 mL 2% (w/v) Trizma base instead of 10.0 mL 1% (w/v). The fractions with inhibitor activity were dialyzed against deionised water (7°C, overnight) and lyophilised. The lyophilised material in buffer C (6.0 mL) was separated on Sephacryl S100 (26 × 670 mm, Pharmacia) with the same buffer. Fractions (2.5 mL) were assayed for inhibitor activity. The active fractions were dialyzed as above and lyophilised (124.5 mg). Fractions of the lyophilised material (5 mg) in buffer D (2 mL) were cation exchanged subsequently (Mono S HR 5/5, Pharmacia) with the same buffer. Fractions eluted in a salt gradient (0 to 0.5 M NaCl) were assayed for endoxylanase inhibition as above. In this way, we obtained 3.08 mg of the inhibitor migrating as a single protein band on SDS-PAGE under non-reducing conditions.

SDS-PAGE and isoelectro focusing. Proteins were analysed by SDS-PAGE on 20% polyacrylamide gels under non-reducing and reducing conditions using the PhastSystem unit (Pharmacia)[14]. The isoelectric point was determined with the same instrument using polyacrylamide gels containing ampholytes (pH 3–9). All gels were silver stained (Pharmacia, Development Technique file 210).

N-terminal amino acid sequencing of proteins. An Applied Biosystems model 477 A gas-phase sequencer, connected on line with an 120 A PTH analyser (Perkin Elmer, Belgium) was used.

Breadmaking. Wheat loaves were prepared in triplicate from 10 g of flour using a straight dough procedure[15] without or with *A. niger* endoxylanase with 8.6% yeast, 1.5 salt, 6% sugar and 5.6 mL water or 4.6 mL water and 1.0 mL enzyme solution. After a fermentation for 60 min at 30°C and a final proof for 25 min, doughs were baked for 13 min at 232°C. Loaf volumes of the triplicates were determined[16].

3 RESULTS

Isolation and characterizaton of TAXI. Using a combination of selective extraction and classical protein purification techniques, a pure preparation of TAXI was obtained. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) of the unreduced protein showed a single polypeptide of ca. 40 kDa, whereas the reduced protein yielded besides the 40 kDa polypeptide also a ca. 30 kDa polypeptide and a ca. 10 kDa peptide (Figure 1A). N-terminal amino acid sequencing of the 40 kDa and 30 kDa polypeptide yielded the same sequence KGLPVLAPVTKXTA, indicating that the 30 kDa polypeptide is derived from the 40 kDa protein. The 10 kDa peptide had the N-terminal amino acid sequence XAPVAKMVLPVAMKEXV. Since the N-termini of TAXI have no sequence similarity with any other known protein, it can be considered as a novel type of protein. TAXI eluted from the gel filtration column with an apparent M_r of ca. 40 kDa and

migrated upon isoelectro focusing as a single band with a pI of ca. 8.8. (Figure 1B). TAXI, already described in a patent application (1997)[17] appears to be similar to the endoxylanase inhibitor purified by Sörensen and Poulsen (1999)[18] but different from the endoxylanase inhibitor isolated by McLauchlan *et al*. (1999)[19]. The latter author found an endoxylanase inhibitor with a molecular weight of 29,000 and with a N-terminal amino acid sequence different from that of TAXI.

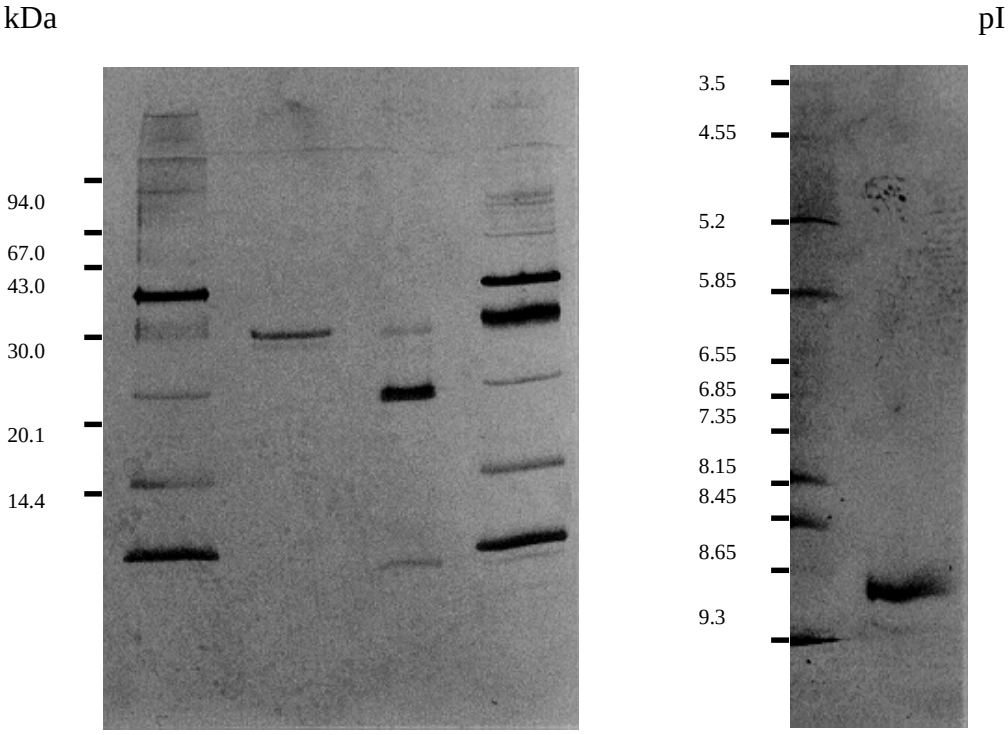

Figure 1. (A) SDS-Page electrophoresis of TAXI. Unreduced and reduced TAXI were loaded in lane 2 and 3 respectively. Low M_r markers (Pharmacia) were loaded in line 1 and 4. (B) Isoelectro focusing of TAXI. TAXI was loaded in lane 1. Markers (Pharmacia) were loaded in lane 2.

<u>*Endoxylanase inhibition by TAXI.*</u> TAXI inhibited several endoxylanases (1.0 U). As shown in Figure 2, both wheat and some microbial endoxylanases were inhibited for up to 80%. However, the endoxylanase from *A. aculeatus* and the endoxylanase M6 were not inhibited in our assay.

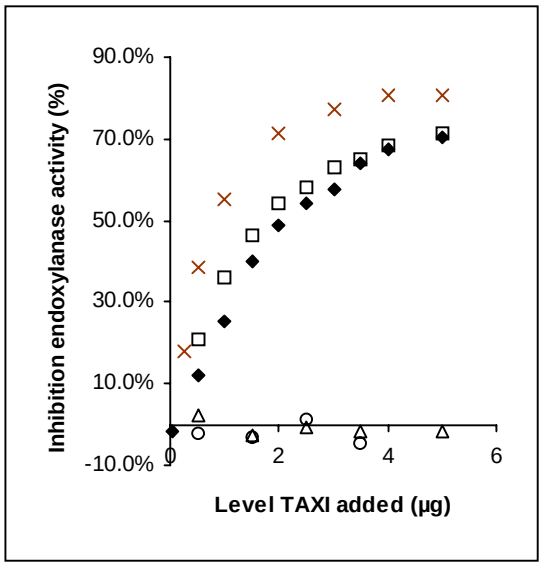

Figure 2. Inhibition of different endoxylanases as a function of the concentration of TAXI. A fixed amount of endoxylanase (1.0 U) was preincubated in the presence of increasing amount of TAXI. Inhibition of the endoxylanase activity is expressed as % reduction of the control. The endoxylanases used were: (☐) *A. niger endoxylanase,* (✕) *Bacillus subtilis endoxylanase,* (◆) *wheat endoxylanase,* (Δ) *A. aculeatus endoxylanase,* (O) *endoxylanase M6.*

Breadmaking. The maximum increase for bread volume by the *A. niger* endoxylanase was ca. 20% (Figure 3). This level of endoxylanase added together with 0.25 mg of purified TAXI did not increase the bread volume. Upon addition of 0.25 mg of purified TAXI the bread volume was reduced by 8%. This observation confirms that the endogeneous wheat flour endoxylanases[20,21] are important for the increase of the bread volume[22] and demonstrates that they are inhibited by TAXI (Figure 2). Accordingly, breeding TAXI-deficient wheat varieties or varieties with low expression levels of this inhibitor may be important for improving breadmaking performance.

A **B**

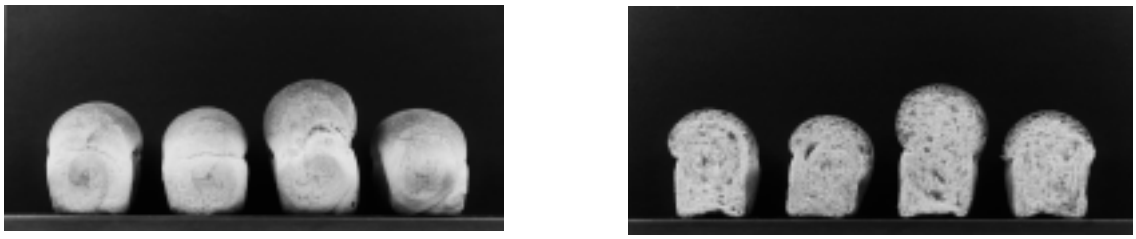

Figure 3. Effect of endoxylanase and TAXI on bread volume. (A) Photograph of bread loaves from 10 g of flour and (B) photograph of cut loaves from the same breads. The volume is listed as the mean ± SD (n=3). From left to right: control loaf (50.2 ±1.5 cc), loaf with 0.25 mg purified TAXI (46.3 ±1.0 cc), loaf with 120 U A. niger endoxylanase (60.0 ±1.7 cc) and loaf with 120 U A. niger endoxylanase and 0.25 mg purified TAXI (50.8 ±1.2 cc).

The identification of TAXI may also explain why only low levels of xylanolytic activity can be measured in bread flour[23] and conflicting results have been reported concerning the relationship between varietal differences in arabinoxylan contents and bread volume[10,24].

4 DISCUSSION

Our work demonstrates for the first time that wheat possesses besides protease and α-amylase inhibitors also endoxylanase inhibitors. Moreover, using a similar approach, we could also demonstrate that rye and barley contain endoxylanase inhibiting activity. If analogy with the role of protease and α-amylase inhibitors holds, endoxylanase inhibitors may very well be involved in plant defence mechanisms. Therefore, their discovery does not only have profound implications in cereal chemistry and technology but is also of great importance for a further unravelling of plant defence systems. Besides the obvious role in wheat breadmaking, efficient hydrolysis of xylans and/or arabinoxylans is highly desirable in many other technologies such as malting and brewing[25,26], rye breadmaking, the production of biscuits, and paper and pulp technologies[27]. Furthermore, the new insights are of significant importance in feed technology because xylan degrading enzymes are used in industrial practice to increase the efficiency of rye and wheat based feed. In conclusion, the discovery of TAXI[17] opens an entirely new area in research since it demonstrates the existence of a group of proteins which are equally relevant for the improvement of plant disease resistance, as well as for nutraceutical or pharmaceutical applications (such as maintaining the structure of dietary fiber material).

5 ACKNOWLEDGEMENTS

W. D., W. J. P. and E. J. M. V. thank the Fonds voor Wetenschappelijk Onderzoek Vlaanderen for research positions as Research Associate, Postdoctoral Fellow and Research Director respectively.

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XYLANASE INHIBITORS FROM CEREALS: IMPLICATIONS FOR BAKING, BREWING AND PLANT TECHNOLOGY

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1 INTRODUCTION

Plants contain a variety of complex polysaccharides, including the cell wall structural polymers cellulose and arabinoxylan and the seed/tuber storage compound starch. They have also evolved with a wide range of enzymes, the glycosyl hydrolases, which catalyse their breakdown. Such enzymes facilitate the mobilisation of reserves from storage organs and seeds during germination, and also direct the restructuring of the plant cell wall during growth. Strict control over such hydrolytic activity in a tissue-specific and/or temporal manner is vital and in germinating seeds the interaction of the plant hormones abscisic acid and the gibberellins is one mechanism in this regulation [1]. Plants have also evolved groups of inhibitor proteins that may represent another level of regulation for glycosyl hydrolases and the level of these inhibitors may themselves be regulated via abscisic acid and the gibberellins. The inhibitors may also have a defense role as these same enzymes, secreted from pathogenic microorganisms, facilitate the penetration and subsequent colonisation of the plant cell wall. Proteinaceous inhibitors have so far been identified for polygalacturonase [2, 3], α-amylase [4], pectin methylesterase [5], invertase [6] and limit dextrinase [7]. The evidence for a xylanase inhibitor had, until recently, only been circumstantial [8, 9] and only in one species, wheat. This paper will describe the independent discovery and characterisation of two different xylanase inhibitors in wheat [10, 11]. It will also discuss the implications for the food and agriculture industry, with particular reference to baking, brewing and plant biotechnology, of the presence of these inhibitors in cereal flour.

2 XYLANASE INHIBITORS IN WHEAT FLOUR : THE EVIDENCE

Xylanases, or more specifically, endo-β-1,4-xylanases hydrolyse the β-1,4-xylan linkages in the arabinoxylan component (hemicellulose) of plant cell walls. They have been grouped into two classes by amino-acid sequence similarities (http://afmb.cnrs-mrs.fr/~pedro/CAZY), family 10 (also called F) and family 11 (also called G) [12]. In plants these enzymes are produced when the need arises to degrade or remodel the cell wall, eg, in germinating barley seed they are involved in the degradation of the endosperm cell wall allowing the rapid entry of other hydrolytic enzymes such as α -amylase to begin the mobilisation of energy reserves. They are also produced as one

component of the battery of hydrolytic enzymes used by plant bacteria and fungal pathogens to invade and colonise plant tissue. They are also produced by saprophytic bacteria and fungi who breakdown dead plant material as their main carbon source.

Microbial xylanases, in particular filamentous fungal xylanases from food-grade organisms such as *Aspergillus* species, have been used for many years as dough improvers in bread making. It is generally agreed that the exogenous xylanases affect the baking process by contributing to the partial solubilisation of the water insoluble arabinoxylan component of the dough, altering its gelling characteristics, leading to increased loaf volume with even crumb structure. It is a feature of these fungal xylanase preparations that their efficiency can vary depending on their source and the variety and grade of flour used. No convincing explanation had been proposed to explain this phenomenon, until 1989, when in a then confidential project, a group led by Professor Jan Maat at the Unilever Laboratorium in Vlaardingen began to find evidence for a proteinaceous moiety in wheat flour which could inhibit fungal xylanases.

a) They found that two xylanases from a commercial enzyme preparation were differentially inhibited by a heat sensitive, water soluble fraction of wheat flour.
b) They could visualise the enzyme/inhibitor complexes using titration curves.
c) They partially purified one inhibitor.

The first evidence to appear in the public domain was in 1997 when the inhibition of endoxylanolytic activity of malt extracts used in the brewing process was detected in the presence of wheat flour extracts suggesting the presence of one or more endoxylanase inhibitors in wheat which could be inactivated by heat treatment [8]. A year later aqueous extracts of wheat flour were found to inhibit pentosanase digestion of wheat arabinoxylan [9].

3 THE PURIFICATION AND CHARACTERISATION OF XYLANASE INHIBITORS FROM WHEAT FLOUR

Following on from the work started at the Unilever's Bakery Products Unit at Vlaardingen, we have purified and characterised a xylanase inhibitor from wheat flour (*Triticum aestivum*, var Soisson) [10]. This inhibitor, which represents the first isolation from any organism, is a glycosylated, monomeric, basic protein with a pI of 8.7–8.9, and while its apparent mass on SDS-PAGE is 29 kDA, its molecular mass by electrospray mass spectroscopy is 30,675 Da. It competitively inhibits family-11 endo-β-1,4-xylanases from both *Aspergillus niger* and *Trichoderma viride*. The K_i value with the former xylanase is 0.35 μM, using soluble wheat arabinoxylan as substrate. The inhibitor has an N-terminal amino-acid sequence of AGGKTGQVTVFWGRN and although in a BLAST search it has a high level of homology with a rice class III chitinase over this short stretch of sequence, it shows insignificant homology over 40 residues of internal sequence and the pure protein shows no measurable chitinase activity.

Following publication of our inhibitor work, data on a second xylanase inhibitor from wheat has been published [11]. Apart from sharing a very basic pI of 8.8, it is

fundamentally different from our inhibitor. It is larger at 40kDa and is a heterodimer, comprising two subunits of 30kDa and 10kDa. It shares no amino sequence homology with our inhibitor, the N-terminal amino-acid sequence of the native 40 kDa protein and the 30 kDa sub-unit is KGLPVLAPVTKXTA, while that of the 10 kDa sub-unit is XAPVAKMVLPVAMLEXV. It is too early to speculate as to the specificities of the two inhibitors as they have been tested against only relatively few xylanases.

4 XYLANASE INHIBITORS FROM OTHER CEREALS

We have also purified 31 kDa and 21 kDa proteins from rye and barley, respectively, with xylanase inhibitor activity. The first ten amino-acid residues of the N-terminus of the rye inhibitor are identical to the wheat inhibitor ie AGGKTGQVTV although it has a slightly larger apparent molecular weight on SDS-PAGE (Figure 1). Sequencing of the first fifteen amino acid residues of the N-terminus and 10 residues of internal sequence of the barley inhibitor gave ADPPPVHDTDGHELR and AYTTCLQSTE respectively. A BLAST search revealed that it is identical to the barley α-amylase/subtilisin inhibitor (BASI) and its ability to inhibit xylanases is thus a novel activity of this multi-functional inhibitor.

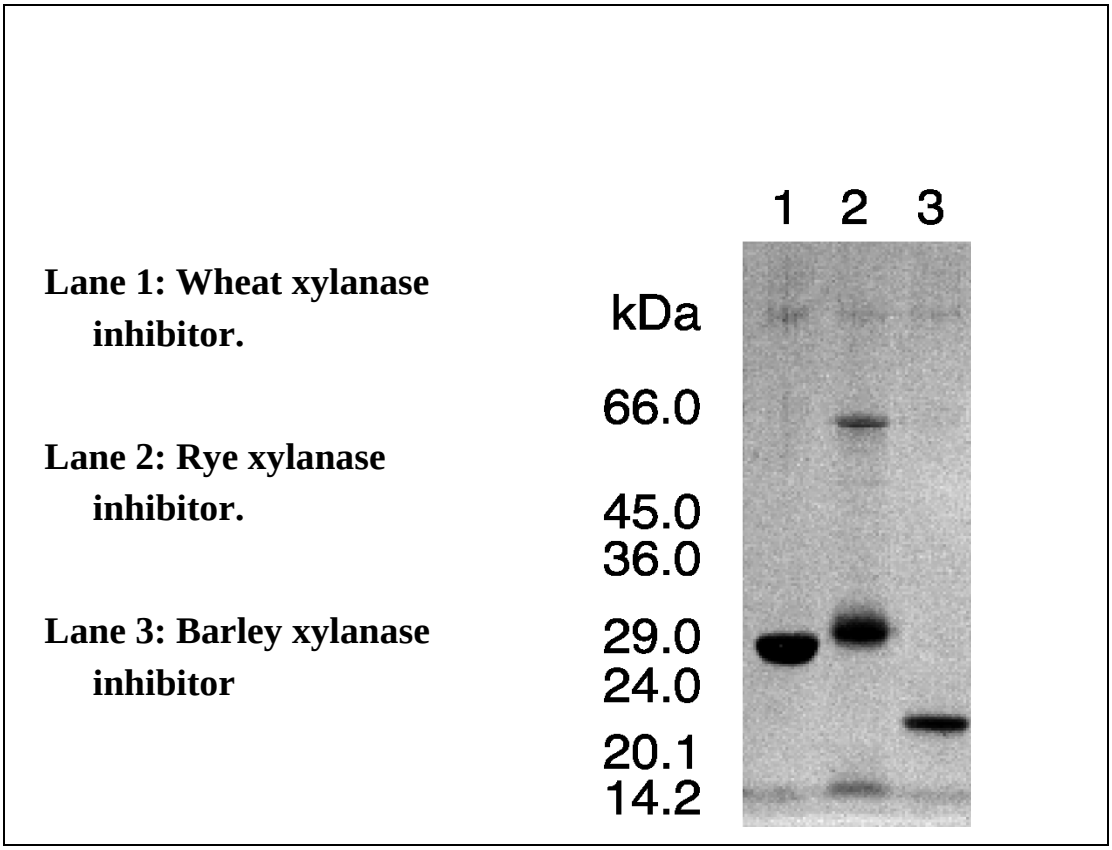

Figure 1. Comparison of the rye and barley xylanase inhibitors with the wheat inhibitor on SDS-PAGE.

5 CURRENT AND FUTURE STUDIES

We are currently working towards an understanding of inhibitor specificity and the binding mechanism of the xylanase / inhibitor complex. We hope to visualise complex formation using titration curves, and use chemical modification to identify important amino acid residues involved in the binding of the xylanase and the inhibitor. This will provide information to guide a programme of site-directed mutagenesis and X-ray crystallography to fully describe the three-dimensional structure and elucidate the mechanism of inhibition. Cloning of the gene that codes for the wheat inhibitor is currently underway. We have designed a series of degenerate oligo-nucleotide primers which are being used to amplify partial clones of the gene from a variety of wheat tissues using PCR. These partial clones will then be used to probe the relevant cDNA libraries to obtain a full-length clone which will then be expressed in the *Pichia pastoris* yeast expression system.

6 XYLANASE INHIBITORS : INPLICATIONS FOR BAKING

The quality of bread is determined by the following criteria: volume, colour, crumb elasticity and flavour. It is common practice to add extra ingredients in bread-making, other than flour, yeast and water, in order to improve the quality. Various bread-improver components such as emulsifiers, oxidising agents and enzymes from plant (barley or wheat malt) or microbial (fungal) origin have been shown to positively influence the bread volume especially when lower quality flours with a low gluten content are being used. Fungal xylanases are commonly used to increase dough machinability, crumb consistency and loaf volume. Optimal baking performance is sometimes also seen when they act in synergy with α-amylases [12]. The commercial enzyme preparations can be either pure recombinant enzymes or a combination of several enzyme activities which vary considerably in composition and ratio of these activities, depending on the source. For a long time it has been known that certain xylanases are more efficient than others as baking improvers, but there was no convincing evidence for why this might be. The isolation and characterisation of two xylanase inhibitors from wheat flour at last provides a logical basis from which to explain this phenomenon. A greater understanding of the specificity of the inhibitors should indicate which types of xylanases are likely to be the most effective bread improvers. This will depend however on an exhaustive comparison of both inhibitors being carried out with a range of family 10 and 11 endoxylanases from bacteria and fungal sources. The overall inhibitory effect of a particular flour will depend not only on the absolute amount, but also on the ratio of each inhibitor present, and to what extent these factors vary between wheat varieties and between different harvests of the same variety.

7 XYLANASE INHIBITORS: IMPLICATIONS FOR BREWING

The endoxylanolytic degradation of arabinoxylans in barley malt is a key part of the brewing process as it lowers wort viscosity and reduces haze formation. Unmalted wheat is used however in the production of Belgian white beers and xylanase inhibitors

from this source have been implicated in a comparative reduction of the endoxylanytic activity seen in this process [8]. As we have also detected xylanase inhibitor activity in barley flour it remains to be seen if this plays a negative role in the efficiency of the barley malting process.

8 XYLANASE INHIBITORS: IMPLICATIONS FOR THE ANIMAL FEED INDUSTRY

There is considerable evidence that the arabinoxylans of cereals such as rye and wheat are responsible for anti-nutritive properties in the diet of monogastric species [13]. In solution, the highly branched arabinoxylans are capable of producing viscous solutions which can result in a reduction in (a) the diffusion rate of enzymes in the intestinal contents, (b) the diffusion of digested nutrients to the gut wall and (c) the rate of intestinal transport [14]. The overall effect of this is a reduction in the nutrient/energy content of the cereal based diet leading to impaired growth of the poultry [15]. As many experiments have shown, supplementation of wheat/rye/barley based poultry diets with xylanases can significantly increase their nutritional value [16]. It is likely however that the endogenous xylanase inhibitors we have detected in rye, barley and wheat flour will significantly reduce their effectiveness and have a negative effect on nutrient availability.

9 XYLANASE INHIBITORS: IMPLICATIONS FOR PLANT BIOTECHNOLOGY

There is evidence that proteinaceous inhibitors of glycosyl hydrolases have evolved in plants as a defence against predation and infection by pathogenic microorganisms. α-Amylase inhibitors are able to interfere with the normal function of the digestive tract of animals and /or insects [17]. All dicotyledonous plants so far examined contain a cell wall associated protein that binds and inhibits fungal endo-α-1,4-D polygalacturonases [18]. There is no evidence as yet that either of the xylanase inhibitors so far described will inhibit the xylanases secreted by pathogenic microorganisms, although it is not unreasonable to suggest that one or both of these inhibitors do play role in plant defence. The implications for plant biotechnology are that these proteins may be manipulated to be expressed in tissues where they not normally found and to be over-expressed in response to infection. It should also be possible to transfer these proteins into crop species where they are not normally found. It also may be possible to engineer xylanase inhibitors to show broad/altered specificity and/or increase their potency. This has already been done in tomato roots with the cysteine proteinase inhibitor, oryzacystatin-1 to increase resistance to the plant parasitic nematode *Globodera pallida* [19].

10 SUMMARY

Xylanases are one of a variety of polysaccharide-degrading enzymes utilised by plants, bacteria and fungi, the activity of which may be regulated to a greater or lesser extent by

inhibitor proteins found in plants. These inhibitor proteins may have evolved in plants to control endogenous processes and help to defend against attack from pathogenic microorganisms. The evidence suggests that these endogenous inhibitors in general have a negative effect on processes in the food and drink industry which exploit polysaccharide-degrading enzymes. Strategies to overcome these problems may include the breeding of cereals with low levels of inhibitor or the engineering of inhibition resistant enzymes. The success of any strategy will depend however on a greater understanding of the specificity of glycosyl-hydrolase inhibitors and the structural determinants which govern the formation of the enzyme/inhibitor complex.

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CELL WALL DEGRADING ENZYMES IN BARLEY

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1 INTRODUCTION

Barley and other members of the Poaceae are characterized by the presence of (1,3;1,4)-β-glucans and arabinoxylans as major components of the matrix phase of their cell walls (Bacic *et al.*, 1988). The compositions of walls from various barley tissues are shown in Table 1. Although cell wall polysaccharides usually account for a relatively small proportion of tissue weight, they can have a larger than expected effect on barley utilization, particularly in the areas of grain technology and nutrition. This effect is generally caused by the capacity of (1,3;1,4)-β-glucans and arabinoxylans to form aqueous solutions of high viscosity. For example, in malting and brewing the (1,3;1,4)-β-glucans and arabinoxylans can adversely affect malt extract values, filtration steps in the brewery, and the quality and stability of the final beer (Bamforth and Barclay, 1993). Similarly, these polysaccharides reduce the digestibility of barley-based stockfeeds in monogastric animals such as poultry and pigs (Fincher and Stone, 1986). On the other hand, they are important components of 'dietary fibre' in human diets and may have a beneficial effect on human digestion and health (Bhatty, 1993).

In this short review, enzymes which depolymerize cell wall polysaccharides in barley are described. Although cellulose is a major component of walls in vegetative tissues (Table 1), little is known about the enzyme systems that are involved in their hydrolysis. Attention will therefore be focussed on the enzymes that hydrolyse arabinoxylans and (1,3;1,4)-β-glucans. These enzymes have been isolated mainly from germinated barley grain, where cell walls are extensively degraded to ensure the free diffusion of other hydrolytic enzymes and substrate breakdown products during mobilization of the starchy endosperm (Fincher, 1989). Some mention will also be made of enzymes that partially degrade (1,3;1,4)-β-glucans during wall loosening in elongating coleoptiles (Sakurai and Masuda, 1978).

Table 1. Composition of cell walls from barley.

Tissue	Major polysaccharides	References
Aleurone of mature grain	71% arabinoxylan 26% (1,3;1,4)-β-glucan 2% cellulose 2% glucomannan	Bacic *et al.* (1981a,b)
Starchy endosperm of mature grain	75% (1,3;1,4)-β-glucan 20% arabinoxylan 2% cellulose 2% glucomannan	Fincher (1975); Ballance and Manner (1978)
4-day-old coleoptiles	55% cellulose 19% arabinoxylan 19% (1,3;1,4)-β-glucan	Sakurai and Masuda (1978)
Young leaves	63% cellulose 16% (1,3;1,4)-β-glucan 11% arabinoxylan 5% pectin	N. Sakurai (personal communication)

2 HYDROLYSIS OF ARABINOXYLANS

Arabinoxylans from barley aleurone or starchy endosperm cell walls consist of a (1,4)-β-xylan backbone to which are appended single α-L-arabinofuranosyl residues, mainly through C(O)3 of the xylopyranosyl residues but also through C(O)2 (Vietor *et al.*, 1993; Figure 1). Other substituents may also be present, but are quantitatively minor (Fincher, 1975; Bacic and Stone, 1981b). For the complete depolymerization of cell wall arabinoxylans in germinated grain, a series of enzymes would be required (Figure 2). So far these enzymes have not been well-characterized, but current information on their specificity and other properties is summarised below.

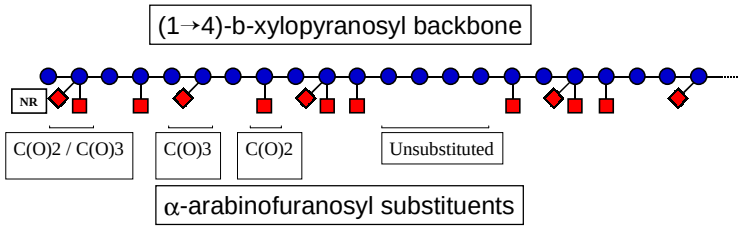

Figure 1. Diagrammatic representation of the structure of cereal arabinoxylans (adapted from Vietor et al., 1993), showing α-L-arabinofuranosyl residues as squares and β-D-xylopyranosyl residues as circles. Some α-L-arabinofuranosyl residues are linked to backbone xylosyl residues through C(O)2, others through C(O)3, and some xylosyl residues are doubly substituted with α-L-arabinofuranosyl residues. Unsubstituted regions of the xylan backbone also exist.

2.1 1,4)-β-XYLAN ENDOHYDROLASES

Three (1,4)-β-xylan xylanohydrolases (EC 3.2.1.8) have been purified from extracts of barley grain 5 days after the initiation of germination (Slade *et al.*, 1989) and a xylanase from isolated aleurone layers has also been described (Benjavongkulchai and Spencer, 1989). Two cDNAs encoding barley (1,4)-β-xylanases have been cloned (Banik *et al.*, 1996). The enzymes are endohydrolases that will rapidly reduce the molecular size of cell wall arabinoxylans (Figure 2). Isoenzyme X-I has a calculated molecular weight of 44,600 and an isoelectric point of 6.1 (Banik *et al.*, 1996). Because the enzymes are difficult to purify and are inherently unstable in purified form (Slade *et al.*, 1989), they have not been characterized in detail. However, expression of (1,4)-β-xylan endohydrolase genes appears to be restricted to the aleurone layer of germinated barley, where the phytohormone gibberellic acid induces both transcription of the gene and secretion of active enzyme (Banik *et al.*, 1997).

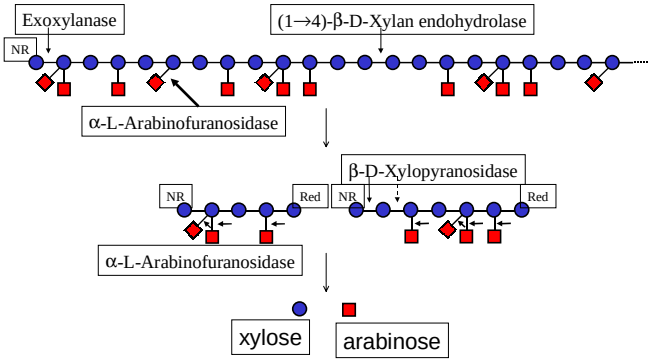

Figure 2. Enzymes that are potentially involved in the complete depolymerization of barley arabinoxylan to monomeric xylose and arabinose units. α-L-Arabinofuranosyl residues are shown as squares and β-D-xylopyranosyl residues as circles. The reducing ends (Red) and nonreducing ends (NR) of the oligosaccharide and polysaccharide chains are indicated.

2.2 α-L-ARABINOFURANOSIDASES

α-L-Arabinofuranosidases are required to remove α-L-arabinofuranosyl substituents from the (1,4)-β-xylan backbone (Figure 2). These enzymes have been detected in grain extracts (Preece and MacDougall, 1958) and are secreted from isolated barley aleurone layers after gibberellic acid treatment (Dashek and Chrispeels, 1977; Banik *et al.*, 1997). However, the enzymes are generally assayed with the synthetic substrate 4-nitrophenyl α-L-arabinofuranoside (4-NPA) and recent work (R.C. Lee and G.B. Fincher, unpublished) indicates that two α-L-arabinofuranosidases purified from extracts of germinated barley grain and assayed against 4-NPA actually have little or no activity against polymeric arabinoxylans. However, another enzyme, designated arabinoxylan arabinofuranosidase, has been detected in the extracts and is capable of removing arabinosyl residues from the polymeric substrate (R.C. Lee and G.B. Fincher, unpublished); this enzyme is under further investigation. These observations raise a

number of questions regarding the removal of α-L-arabinofuranosyl residues during arabinoxylan hydrolysis. For example, are different enzymes required for the hydrolysis of C(O)3-, C(O)2- or doubly-substituted arabinofuranosyl residues, and to what extent do local substitution patterns on the xylan backbone affect the ability of enzymes to hydrolyse α-L-arabinofuranosyl linkages? These questions will be answered through the purification and characterization of individual enzymes and, by analogy with microbial enzymes involved in the hydrolysis of plant heteroxylans, a relatively large number of enzymes might be required (Van Laere *et al.*, 1999).

2.3 XYLOSIDASES AND (1,4)-β-XYLAN EXOHYDROLASES

Enzymes variously designated as β-xylosidases or xylan exohydrolases have been detected in extracts of germinated barley grain (Preece and MacDougall, 1958) and in aleurone layer secretions (Dashek and Chrispeels, 1977; Banik *et al.*, 1997). These could be involved in the hydrolytic removal of β-xylosyl residues from polymeric arabinoxylans or from oligomeric products of endoxylanase action (Figure 2), but again there are no reports of their properties, action patterns, or substrate specificities. We have recently purified a β-xylosidase from extracts of germinated barley grain, using 4-nitrophenyl β-D-xyloside (4-NPX) as a substrate in enzyme assays, and note that the enzyme also has some activity against 4-NPA (R.C. Lee and G.B. Fincher, unpublished). The details of substrate specificity, action pattern and enzymic properties of the barley β-xylosidase remain to be determined.

3 HYDROLYSIS OF (1,3;1,4)-β-GLUCANS

Cell wall (1,3;1,4)-β-glucans from barley are unbranched polysaccharides that contain 1000 or more β-glucosyl residues (Woodward *et al.*, 1983a). About 70% of the β-glucosyl residues are (1,4)-linked and about 30% are (1,3)-linked (Parrish *et al.*, 1960). Within the polysaccharide, single (1,3)-β-glucosyl residues separate blocks of two or three adjacent (1,4)-β-glucosyl residues (Figure 3). Adjacent (1,3)-β-glucosyl residues are not observed and up to 10% by weight of the water-soluble (1,3;1,4)-β-glucan from starchy endosperm cell walls of barley consists of blocks containing 4–14 contiguous (1,4)-β-glucosyl residues; these might be considered as internal 'cellulosic' regions (Woodward *et al.*, 1982b).

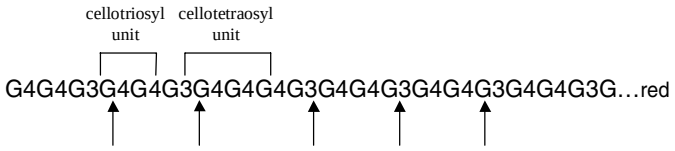

Figure 3. Diagrammatic representation of the structure of cell wall (1,3;1,4)-β-glucans from barley, showing glucosyl residues as 'G', (1,3)- and (1,4)-β-linkages as '3' and '4', respectively, and the reducing end of the polysaccharide as 'red'. Arrows indicate

the point of hydrolysis by (1,3;1,4)-β-glucan endohydrolases of the EC 3.2.1.73 group of enzymes.

During the complete depolymerization of (1,3;1,4)-β-glucans to glucose in germinated barley grain, several types of enzyme would be expected to participate (Figure 4). The substrate specificities, action patterns and other properties of these enzymes are described below.

3.1 Enzymes that Release Large Fragments

There have been several reports of a (1,3;1,4)-β-glucan endohydrolase in germinated barley grain that releases polymeric (1,3;1,4)-β-glucans from cell walls, without hydrolyzing the released material to low molecular mass oligosaccharides (Bamforth, 1982). This enzyme (designated Endo-X in Figure 4) has been named 'β-glucan solubilase' (Bamforth and Martin, 1981), but has not been purified or rigorously characterized. Indeed, it was suggested by Yin and MacGregor (1989) that the enzyme originated from microorganisms that contaminate the surface of germinated grain. However, it is noteworthy that an enzyme with an apparently similar action pattern has been detected in elongating coleoptiles from maize (Labrador and Nevins, 1989) and it is conceivable that an enzyme that specifically hydrolyses (1,3;1,4)-β-glucans only where there are extended blocks of adjacent (1,4)-β-glucosyl residues would generate relatively high molecular mass products. An endo-cellulase with a requirement for 8–10 adjacent (1,3;1,4)-β-glucosyl residues could exhibit such an action pattern, but again the enzyme needs to be purified and characterized in detail.

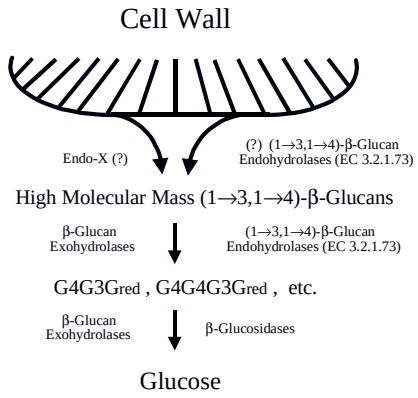

Figure 4. Enzymes that are potentially involved in the release and complete hydrolysis of (1,3;1,4)-β-glucans from barley endosperm cell walls.

3.2 (1,3;1,4)-β-Glucan Endohydrolases

More precise information is available for barley (1,3;1,4)-β-glucan 4-glucanohydrolases of the EC 3.2.1.73 group. These enzymes specifically hydrolyse (1,4)-β-glucosyl linkages which are on the reducing terminal side of (1,3)-β-glucosyl residues (Figure 4), and release oligosaccharides that contain a variable number of (1,4)-β-glucosyl residues

at their non-reducing ends and a single (1,3)-β-glucosyl residue at their reducing ends (Figure 4). Other properties are shown in Table 2.

Table 2. Properties of barley (1,3;1,4)-β-glucan endohydrolases.

Property	Isoenzyme EI	Isoenzyme EII
Apparent molecular mass	30,000	32,000
Amino acids	306	306
Isoelectric point	8.5	10.6
Carbohydrate	0	4% by weight
Expression sites	Scutellum, young vegetative tissue, aleurone	Aleurone

Two (1,3;1,4)-β-glucan endohydrolases from germinated barley grain have been designated isoenzymes EI and EII (Woodward and Fincher, 1982). The three-dimensional (3D) structure of isoenzyme EII has been defined by X-ray crystallography (Varghese *et al.*, 1994). The enzyme adopts an $(\alpha/\beta)_8$ barrel fold and is characterized by a deep substrate-binding cleft that extends across its surface. The open cleft is long enough to accommodate up to eight glucosyl residues of the substrate, it allows the enzyme to bind its substrate at any position along the polysaccharide backbone, and the shape of the cleft is therefore consistent with the enzyme's endo-action pattern. Precise details of enzyme-substrate interactions will now be investigated by diffusing non-hydrolysable S-glycosides (Moreau and Driguez, 1995) into (1,3;1,4)-β-glucan endohydrolase crystals.

3.3 β-GLUCOSIDASES

The (1,3;1,4)-β-oligoglucosides released from (1,3;1,4)-β-glucans by endohydrolase action can be further hydrolysed to glucose (Figure 4). The glucose that is chemically bound in cell wall polysaccharides would thereby become available as an energy source for the developing seedling. One candidate enzyme for the conversion of (1,3;1,4)-β-oligoglucosides to glucose is β-glucosidase, which has been detected in extracts of mature barley grain (Simos *et al.*, 1994; Leah *et al.*, 1995; Hrmova *et al.*, 1996). Properties of two isoforms, designated isoenzymes βI and βII, are compared in Table 3. The enzymes are classified as β-glucosidases because they hydrolyse 4-nitrophenyl β-glucoside (4-NPG), but their preferred substrates appear to be cellodextrins, for which hydrolytic rate increases with the length of the cellodextrin (Leah *et al.*, 1995; Hrmova *et al.*, 1996). They remove single glucose units from the non-reducing ends of substrates, and their action pattern is more typical of a (1,4)-β-glucan exohydrolase than a β-glucosidase. While their native substrates have not been identified unequivocally, the enzymes are able to hydrolyse the (1,3;1,4)-β-oligoglucosides released from (1,3;1,4)-β-glucans by endohydrolases (Figure 4; Hrmova *et al.*, 1996). The (1,3;1,4)-β-oligoglucosides have molecular shapes that are very similar to cellodextrins, which are the preferred substrates of the enzyme, because the (1,3;1,4)-β-oligoglucosides have only one (1,3)-β-glucosyl residue, at its non-reducing terminus (Figure 4).

Table 3. Properties of barley β-glucosidases.

Property	**Isoenzyme BI**	**Isoenzyme BII**
Apparent molecular mass	62,000	62,000
Amino acids	471	471
Isoelectric point	8.9	9.0
Substrate specificity		
4NPG	Active	Active
laminarin	Not active	Not active
(1→3,1→4)-β-glucans	Not active	Not active
(1→4)-β-oligosaccharides	Active	Active
Expression sites	Developing endosperm	Developing endosperm

The primary structure of barley β-glucosidase isoenzyme βII (Leah *et al.*, 1995) has been used to build a molecular model of the enzyme (Hrmova *et al.*, 1998), using as a template the 3D structure of a cyanogenic β-glucosidose from white clover (Barrett *et al*., 1995). A deep funneled-shaped pocket that is lined with hydrophobic amino acid residues is clearly the substrate-binding region of the enzyme. The catalytic amino acids are located near the bottom of the pocket, in a position where they would remove a single glucose unit from the non-reducing terminus of the substrate. The 'dead-end' tunnel or pocket geometry of the substrate-binding region is consistent with the exo-action pattern of the enzyme (Hrmova *et al*., 1998).

Several possible function for the barley β-glucosidases have been reviewed by Leah *et al.*, (1995). The enzyme is synthesized during the late stages of grain development and accumulates in the mature grain; no further synthesis of the enzyme is apparent after germination (Leah *et al.*, 1995). While it must be acknowledged that the true substrates and hence the function of β-glucosidases in germinated barley grain remain undefined at this stage, the weight of current evidence suggests that the enzymes could play a key role in the hydrolysis of (1,3;1,4)-β-oligoglucosides during the complete conversion of cell wall (1,3;1,4)-β-glucans to glucose (Figure 4).

3.4 β-GLUCAN EXOHYDROLASES

The final group of enzymes that might participate in the depolymerization of cell wall (1,3;1,4)-β-glucans is represented by the β-glucan exohydrolases. Two barley β-glucan exohydrolases, designated isoenzymes ExoI and ExoII, hydrolyse the non-reducing terminal glycosidic linkage to release glucose from a broad range of β-oligoglucosides, polymeric β-glucans and aryl β-glycosides (Kotake *et al.*, 1997; Hrmova and Fincher, 1998). The enzymes are true polysaccharide exohydrolases because they rapidly release glucose from polymeric substrates, including cereal (1,3;1,4)-β-glucans. Furthermore, the enzymes can rapidly hydrolyse the (1,3;1,4)-β-oligoglucosides released from (1,3;1,4)-β-glucans by endo-hydrolases (Hrmova and Fincher, 1998). Their properties are summarized in Table 4.

Table 4. Properties of barley β-glucan exohydrolases.

Property	**Isoenzyme ExoI**	**Isoenzyme ExoII**
Apparent molecular mass	69,000	71,000
Amino acids	605	602
Isoelectric point	7.8	8.0
Carbohydrate	4.7% by weight at 3 *N*-glycosylation sites	not known
Substrate specificity		
a) 4NPG	Active	Active
b) (1→3)-, (1→3,1→4)-, (1→3,1→6)-β-glucans	Active	Active
c) (1→2)-, (1→3)-, (1→4)- and (1→6)-β-oligosaccharides	Active	Active
Expression sites	Mainly in scutellum and coleoptiles, also in young leaves and roots	Mainly in scutellum and coleoptiles, also in young roots and leaves

The solution of the 3D structure of barley β-glucan exohydrolase isoenzyme ExoI has allowed the broad substrate specificity of the enzyme to be reconciled in structural terms (Varghese *et al.*, 1999). The enzyme has 605 amino acid residues in all, but two distinct domains that are joined by a 16 amino acid linker can be detected. The active site is located in a relative shallow pocket on the surface of the enzyme, near the interface of the two domains. The pocket is only deep enough to accommodate two β-glucosyl residues. The remainder of the oligosaccharide or polysaccharide substrate projects out of the pocket and away from the surface of the enzyme (Varghese *et al.*, 1999). Substrate binding therefore occurs over a relatively short region at the non-reducing terminus and is relatively independent of the overall shape of the substrate, and hence of the type of glycosidic linkage. The absence of a strict shape requirement would explain the broad substrate specificity of the barley β-glucan exohydrolases.

The broad specificity of the enzymes raises some problems in defining their function. Because the enzymes are also widespread in dicotyledons (Cline and Albersheim, 1981; Crombie *et al.*, 1998), it can be argued that they are less likely to be involved specifically in the hydrolysis of (1,3;1,4)-β-glucans, which are found only in the graminaceous monocotyledons (Bacic *et al.*, 1988). Although some gene expression occurs in the scutellum of germinated grain, mRNAs encoding barley β-glucan exohydrolases are most abundant in the elongating celeoptile (A.J. Harvey and G.B. Fincher, unpublished data). This suggests that the β-glucan exohydrolases function in cell elongation in growing coleoptiles, where the (1,3;1,4)-β-glucan content of cell walls decreases dramatically during growth (Sakurai and Masuda, 1978; Kotake *et al.*, 1997) and where partial hydrolysis or 'loosening' of wall polysaccharides is believed to be a prerequisite for turgour pressure-driven cell expansion (Fry, 1995; Cosgrove, 1999). Unequivocal evidence to support such a function for the barley β-glucan exohydrolases remains to be presented.

4 ACKNOWLEDGEMENTS

This work was supported by grants from the Grains Research and Development Corporation and the Australian Research Council. We thank Professor Peter Colman for his ongoing support of the X-ray crystallography component of these studies.

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CEREAL ESTERASES

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1 INTRODUCTION

Cereals, as members of the grass family, are probably the world's most cultivated plant variety, and over 70% of the world's cultivated acreage being devoted to their production. A number of industries (milling, baking, brewing, etc) are dependent upon a good supply of grain and of this grain undergoing no deterioration during storage. Deterioration of the grain can be caused by a number of factors, from changes in the cell-wall composition due to oxidation, germination or microbial infection. Esterases (and in this review we include lipase-type esterases) have been identified in both stored and developing grain suggesting a wide range of functions, ranging from a putative role in plant pathogen defence to a role in plant cell wall structure affecting both the properties and the processability of the grain components. Esterases are also used as genetic markers in cereals.

2 TYPES OF ESTERASES PRESENT IN CEREAL GRAINS

2.1 PHENOLIC ACID ESTERASES

These esterases hydrolyse the water-soluble or lipid-soluble phenolic esters, or covalently-linked phenolic esters which occur in the plant cell wall. Examples of the soluble compounds are hydroxycinnamoyl esters of glucose, choline, quinic, malonic acid and tartaric acid, galloyl esters of epicatechin and the *p*-coumaroyl esters of anthocyanins [5]. Lipid phenolic acid esters include hydroxycinnamates linked to sterols, terpene alcohols or triterpines, and are usually membrane associated [21]. A preparation of these hydroxycinnamate-sterol esters from rice bran (γ-oryzanol) is commercially available. The most abundant covalent-linked phenolic ester in the plant cell wall is ferulic acid, which is esterified to pectins and heteroxylans [22]. The esterases which have been identified which hydrolyse the hydroxycinnamate ester bonds are known as cinnamoyl esterases (ferulic acid esterase, feruloyl esterase, chlorogenicase), and those purified and characterised to date are mainly microbial. A cinnamoyl esterase was detected in barley with activity against hydroxycinnamic acids [20]. Cinnamoyl esterase activity was detected in wheat (var. Soisson and Durum), rye and rice and is also present in a range of barley varieties of both good malt and feed

quality. The level of cinnamoyl esterase did not relate to the malting properties of the barley variety (Sancho *et al.*, unpublished results). Cinnamoyl esterase activity has also been detected in malted barley, after kilning [3], which suggests that an esterase component in barley is thermostable and thus may have a possible role in opening up the cell wall structure for sugar-releasing enzymes (amylases, xylanases, etc) to work. However, cinnamoyl esterase activity in crude extracts from barley appear to be unstable during refrigerated storage. On the other hand, a crude barley preparation could release free ferulic acid from both soluble and insoluble cell wall materials [9]. A cell wall bound cinnamoyl esterase may also be present in barley. Bamforth [2] argues that the solubilisation of β-glucan during the malting of barley is due to several enzymes, including at least three with different esterase activities (a feruloyl esterase, a general esterase and a carboxylpeptidase). Tannins, which consist of polymerised flavanols containing a galloyl ester-linkage, are present in cereals. Although there are tannases capable of de-esterifying these compounds identified in microorganisms and plants [11], however, there are no reports of cereal tannases.

2.2 ACETIC ACID ESTERASES

These esterases release acetic acid from either artificial compounds and/or from acetylated plant cell wall material. One problem about using an artificial substrate is to determine the physiological role for an enzyme, as not all enzymes which have been identified as having activity against compounds such as naphthol acetate or *p*-nitrophenyl acetate, can act on acetylated plant-derived material, and *vice versa* [4]. Acetylation of a plant polysaccharide alters the physical and chemical properties of the polymer, such as increasing its solubility and changing its interactions with other polysaccharides. The Pir7b protein from rice was found to be active on the synthetic substrate naphthol AS-acetate but not the more common substrate for detecting arylesterases, naphthol acetate, showing the necessity for the anilid moiety in the substrate for enzymic activity [28]. An ‘acetic acid’ esterase with activity against diacetin has been detected in barley malt [10]. An insoluble, inactive form was present in insoluble grain material after the active, soluble form had been extracted.

2.3 PECTIN METHYL ESTERASES

The extent and distribution of methoxy esters in polygalacturonic acid is important in determining the properties of the pectin, such as cell wall density [17] and gelation properties [18]. Pectin methyl esterases (PME) are responsible for the demethylation of pectin prior to the degradation of pectin by polygalacturonase and pectin lyase, as well as involvement in cell wall extension [13] and pollen germination [1]. PME has been detected in maize pollen, with some homology to other plant and fungal PME genes [27].

Acetylcholinesterase;- This esterase has been found in many plant tissues and exhibits properties similar to animal acetylcholinesterases (AchE). Acetylcholine in plants has been shown to be involved in the regulation of enzyme activity and metabolic pathways, and it was proposed that the primary mechanism of action is via the regulation of

membrane permeability to protons and to potassium, sodium and calcium ions [25]. AchE activity was demonstrated at the interface between the stele and cortex of the mesocotyl of maize seedlings. After stimulus, activity was distributed asymmetrically in the coleoptile, the coleoptile node, and the mesocotyl of the stimulated seedlings [12]. The hydrolytic capability of the esterase was greater on the lower side of the horizontally placed seedlings. Therefore, there is a possible role of AchE in the gravity response of the maize seedlings.

2.4 LIPASE-TYPE ESTERASES

Lipases and acylhydrolases (esterases) have been observed in wheat, oat, barley and rice, and studied in some detail because of the problems they cause in rancidity of oils and the loss of baking performance in flours. Many are membrane bound. Although no clear differentiation seems possible between lipases and esterases, esterases exhibit higher affinity on short-chain fatty-acid esters or water soluble molecules. Lipase activity normally increases during germination for the mobilisation of lipid reserves. Wheat germ lipase catalyses the hydrolysis of simple esters, triglycerides, and water-soluble long-chain fatty acids of sorbitan. A commercial wheat germ lipase preparation was fractionated and separated into 3 fractions containing an esterase with activity only on pNPacetate, a lipase with activity on tributyrate, and a non-specific esterase active on both pNPA and tributyrate [6]. The acetylesterase obeyed Mechaelis-Menten kinetics, while the non-specific esterase and the lipase yielded non-linear Lineweaver-Burk plots. The non-specific esterase and the lipase had similar K_m values on tributyrate, however, the components had different heat stabilities. Oat flour contains relatively higher lipase activity compared to other cereals and can also perform acyl transfer into phosphoglycerides [15]. Lipase activity is mainly located in the bran region of the grain (80%) and significant activity also present in the germ.

3 ROLE OF ESTERASES IN CEREALS.

There are a number of reports concerning plant esterases in the literature. ‘Esterase’ zymograms are used as markers for breeding and selectivity, especially for oil producing varieties of seeds. The physiological significance of most of these isozymes has yet to be defined, but some research has shown changes in esterase zymogram profiles with either development or with response to infection. Some examples are given below.

Wheat ‘esterase’ activity is controlled by the Est12, Est1, Est2 and Est5 esterases in seedlings or by the Est1, Est2, Est4, Est5 and Est11 esterases in roots [23]. There is a correlation between the regulatory induced activation of certain physiological and biochemical processes and the activity of leaf and root esterases in the germinating seedlings. The barley seedling esterase complex EST1, 2 and 4 is located at the distal end of chromosome 3- near the MEP1 (malt endopeptidase) site which is related to malting properties and encoded on the CepB locus on chromosome 3 [7]. Esterase activity in polished rice bran is cultivar-dependent [26]. Storage temperature and relative humidity can effect the activities of several enzymes involved in lipid

hydrolysis. The accumulation of free fatty acids in rice seed and the associated rancidity is a problem during storage of the grain. Rice cultivars that were high in esterase activity had higher rates of lipid degradation. Isozyme patterns of maize esterase analysed at different stages of *in vitro* culture of immature embryos show significant changes- a possible association with tissue differentiation [16]? Resistance to drying of wheat seedlings (drying to 12% water content making it cellulase resistant) is to a large extent overcome by the addition of hemicellulase and xylanase to a digestion mixture, and completely overcome if an acetylesterase is included [18]. Changes involving hemicellulose and xylans are therefore implicated in the effects of drying or maturation, including acetylation processes especially in grains that are also mature.

The destruction of barley endosperm tissue by the grain weevil *Calandra granaria* L. is accompanied by the thinning or disappearance of the respective tissue specific esterase isoenzymes Rm21 and Rm28. Hordein fractions remain intact, however [24]. Higher esterase activity was detected in fingermillet seedlings of an infection susceptible genotype compared to that of a resistant genotype [14]. An increase in esterase activity has also been observed in barley upon infection [19]. Isozyme 12 (a general esterase) was found in all developmental stages. Upon infection, isozyme 11 was suppressed in the susceptible genotype, but isozymes 3 (carboxylesterase) and 4 (arylesterase) appeared in the resistant genotype. The esterases hydrolyse the ester-links of different metabolites during development. Carboxylesterases and peroxidases have been localised near infected pea-roots, possibly by catalysing the formation and deposition of suberin, thus forming a barrier that blocks pathogen attack, and so the enzymes may be responsible for motivating a similar response in cereals. In response to infection by a non-host *Pseudomonas syringae* resulted in the transient accumulation of Pir7b esterase transcripts in rice [8]. The Pir7b protein exhibits activity against naphthol AS-acetate but not naphthol acetate, showing the necessity for the anilid moiety [28]. The enzyme showed sequence identity with hydroxynitrile lyases, whose primary function is the release of hydrogen cyanide from cyanogenic glycosides. As rice does not contain these cyanogenic glycosides, a similar function of Pir7b does not seem likely. The protein accumulated in infected leaves, and belongs to a family of esterases widely occurring in plants.

4 FUTURE PROSPECTS FOR CEREAL ESTERASES

A great deal is still required to identify the number of esterases present in cereals, the differences between the role of esterase activity in grain and in the developing plant, and the role of esterases in plant defence. Only within the last year have we seen reports of esterases acting on plant phenolic esters [20, 9], and these only report the detection of activity in crude extracts. The impact of cereal esterases during cereal processing has still to be examined further. The control of lipase activity is important both for the stored grain, but also for the control of lipid degradation during baking and oil production. Phenolic esterase activity was still detectable in malted barley after kilning [3], and so a putative role of a phenolic esterase during brewing can be suggested, perhaps acting as a solubilase on the β-glucan [2].

5 ACKNOWLEDGEMENTS

We would like to thank the Biotechnology and Biological Sciences Research Council, the European Commission (grant number CT097- to AIS), and the British Council through the British-Spanish Acciones Integradas Programme for funding this work.

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POTENTIALITIES OF ENDOGENOUS PEROXIDASES AND FERULOYL ESTERASES IN WHEAT FOR MODIFICATIONS OF ARABINOXYLANS

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1 INTRODUCTION

Arabinoxylans are the major non-starch polysaccharides of wheat. In spite of their low content in wheat flour, water-extractable arabinoxylans (0.3 to 0.7% w/w) have significant effects on dough rheology and bread quality, and are known as anti-nutritive factors in poultry feedings, due to their “water binding capacity” and viscosifying properties.

Arabinoxylans are present in all the cell walls of all tissues in wheat kernel. In starchy endosperm, cell walls contain about 3% of non starch polysaccharides, including water extractable (WEAX) and water unextractable arabinoxylans (WUAX). Arabinoxylans are composed by a backbone of b (1,4) linked D-xylose which may be substituted with arabinose residues, either monosubstituted in position O-3 or disubstituted in positions O-2 and O-3. Some arabinose residues coud be esterified by ferulic acid in position O-5. According to the literature, high variations could be found in the structural features of arabinoxylans, such as the ratio arabinose / xylose (A/X) or substitution pattern of arabinose. More often, it is admitted that arabinoxylans have a rigid rod-like conformation and that the content in arabinose could have an effect on arabinoxylan conformation.

However, recent results obtained in our lab showed that arabinoxylans in solution behaved as random coil and that the substitution degree had no effect on the rigidity (Dervilly *et al.*, 1999). On the other hand, we have observed that WEAX contained small amount of ferulic acid dimers, indicating cross-linking of water-soluble arabinoxylans. Formation of ferulic dimers is well kwnown in the gelation of arabinoxylans with peroxidase, but the presence of such dimers in water-extractable arabinoxylans is new. Their presence could increase the molecular mass and the hydrodynamic volume of arabinoxylans and thus could have an effect on their physico-chemical properties, such as viscosity. However, the mode of control of their levels in arabinoxylans is not known. Two types of enzymes can potentially modify the proportion of dimers : one one hand feruloyl esterase which can release ferulic acid and possibly cut bridges, and on the other hand, peroxidases which can cross-linked ferulic acid and thus create bridges. Contents in ferulic acid and dimers in arabinoxylans could result of the balance between endogenous peroxidase and feruloyl esterase activities. Furthermore, these enzymes could contribute to variation of arabinoxylans physico-chemical behaviour. Therefore, the aim of this work was to investigate the level of feruloyl esterases and peroxidases in wheat flour in relation with the dimers content of WEAX, in order to determine if such endogenous enzymic activities might have an influence on the physico-chemical potentialities of WEAX.

2 MATERIAL AND METHODS

2.1 IN VITRO ASSAY

Peroxidase from horseradish (Sigma Chemicals, St Louis, MO, U.S.A.) exhibited 1100 U/mg, one unit catalysing the oxidation of 1 µmol per min at 25°C using 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) as substrate.

5-O-(transferuloyl) -L-arabinofuranose was previously extracted from maize bran (Saulnier *et al*., 1995a). It was incubated with peroxidase (0.8 mg/mL with 1.1 U/mL) for 30 min at 25°C with various concentrations of hydrogen peroxide (0.5-H_2O_2-2.5 ppm). Reaction mixture was then deesterified and phenolics were analysed as described in Chemical analysis.

2.2 WHEAT SAMPLES

20 french varieties of flour were harvested in 1998. They could be classified as soft or hard varieties.

2.3 DETERMINATION OF ENZYMIC ACTIVITIES

Each step of extraction was carried out at 4°C. Enzymic activities were assayed in triplicate and were expressed in nkat / g flour, one nkat being defined as the amount of enzyme that catalyzes the release of 1 nmol of product per s in the given conditions.

2.3.1 Feruloyl esterases

2 g flour were suspended in 10 mL cold 3-(N-morpholino) propane sulfonic (MOPS) buffer pH 6 containing 10 mM Na_2EDTA, 100 mM NaCl, 1 mM dithioerythritol, 0.02% NaN_3 (Sancho *et al*., 1998). The slurry was homogenised with a metal rod (Kinematica, Suisse, 2 x 1min) and centrifuged 15 min at 5300 g.

5 mL of supernatant were dialysed against deionized water for at least 4 h, and then over night against MOPS buffer 10 mM pH 6. The dialysed extract was freeze-dried and solubilized in 0.5 mL cold deionized water.

5-*O*-(trans-feruloyl)-L-Ara*f* (FA) was extracted from maize bran (Saulnier *et al*., 1995a) and further used as substrate for the quantitation of feruloyl-esterases in the extract. A solution at about 20 nmol L^{-1} in MOPS buffer 100 mmol L^{-1} pH 6 was incubated at 30°C with the extract. The enzymic activity was determined spectrophotometrically at 286 nm (free ferulic acid) and 323 nm (esterified ferulic acid) (Ralet *et al*., 1994). The following molar absorption coefficients determined at pH 6 in MOPS buffer (ε_{286} = 14176 L mol^{-1} cm^{-1}, ε_{323} = 10350 L mol^{-1} cm^{-1} for free ferulic acid, and ε'_{286} = 12465 L mol^{-1} cm^{-1}, ε'_{323} = 19345 L mol^{-1} cm^{-1} for esterified ferulic acid) were used in the calculation.

2.3.2 Peroxidases

0.5 g flour were suspended in 5 mL acetate buffer 100 mM pH 4.7. The slurry was homogenized with a metal rod (Kinematica, Suisse, 1min) and centrifuged 15 min at 5300 g. Peroxidase activity was determined in the supernatant by the use of 2-methoxyphenol and hydrogen peroxide as substrates. 2-methoxyphenol solution was prepared extemporanously (150 µL in 30 mL acetate buffer 100 mM, pH 4.7). H_2O_2 and $CaCl_2$ were solubilized in acetate buffer 100 mM pH 4.7. Reaction medium for the determination of peroxidase activity contained 2.7 mL 2-methoxyphenol, 0.3 mL H_2O_2 / $CaCl_2$, and 50 µL extract. The mixture was incubated at 26°C and its increase in absorbance was followed at 470 nm. The activity was calculated from the linear part of the curve (ε_{470} = 6700 L mol^{-1} cm^{-1} at pH 4.7).

2.4 CHEMICAL ANALYSIS

Monosaccharides of WEAX were analysed by gas liquid chromatography after acidic hydrolysis (sulfuric acid 2 N, 100°C, 2h) and derivation in alditol acetates (Englyst and Cummings, 1988). Analyses were carried out in duplicate and arabinoxylan content was calculated from the sum of arabinose and xylose.

Phenolics were measured in WEAX after saponification in NaOH 2N, 30 min at 35°C in the dark. After adding O-coumaric acid as internal standard, pH was adjusted to 2 by adding 2N HCl. Phenolic acids were extracted with 3 mL ether. The ether phase was transferred and evaporated in a water bath at 40°C. 250 µL methanol / water (50/50 v/v) were added and samples (20 µL) were injected on a HPLC system as previously described (Saulnier *et al*. 1999).

2.5 PHYSICO-CHEMICAL DETERMINATIONS

1 g flour was suspended in 4 mL deionized water and stirred 15 min at room temperature. The slurry was centrifuged (10 min at 10.000 g) and the supernatant was heated 10 min at 100°C in a boiling water bath. After heating, the supernatant was centrifuged again and the pH was adjusted to 2 by adding 1 $mol.L^{-1}$ glycine – HCl buffer. Extract was filtered on 0.45 µm membrane and injected on a high performance size exclusion chromatography (HPSEC) system constituted of two Shodex OH-pack SB HQ 804 and 805 columns eluted at 0.6 mL / min with 50 mM NaNO3, containing 0.02% NaN_3. On-line intrinsic viscosity determination was performed at room temperature using a viscosimeter (T-50, Viscotek) and a differential refractometer (ERC 7517 A). [η] was calculated using Trisec software (Viscotek).

Relative viscosity of flour water extract was measured using capillary viscosimeter as previously described (Saulnier *et al*., 1995b).

3 RESULTS AND DISCUSSION

3.1 ACTION OF PEROXIDASE *IN VITRO*

As the gelation of arabinoxylans with peroxidase has already been described (Figueroa-Espinoza and Rouau, 1998), the products of this reaction were studied in more details in *in vitro* assays. FA was incubated with horse radish peroxidase in the presence of hydrogen peroxide and phenolics were quantified in the reaction medium (Fig. 1). The reaction was very fast and no evolution occured after 30 min. Ferulic acid disappeared as the concentration of hydrogen peroxide increased. In the same time, concentration of dimers increased, demonstrating that peroxidase catalysed the dimerisation of ferulic acid. However, the reaction was quite complex since the formation of dimers reached a maximum and decreased after, suggesting that complex condensation products were formed. Nevertheless, analysis of the dimers showed that 8–5'C represented 95% of the dimers formed, demonstrating that the enzymic reaction allowed the synthesis of almost only one dimer.

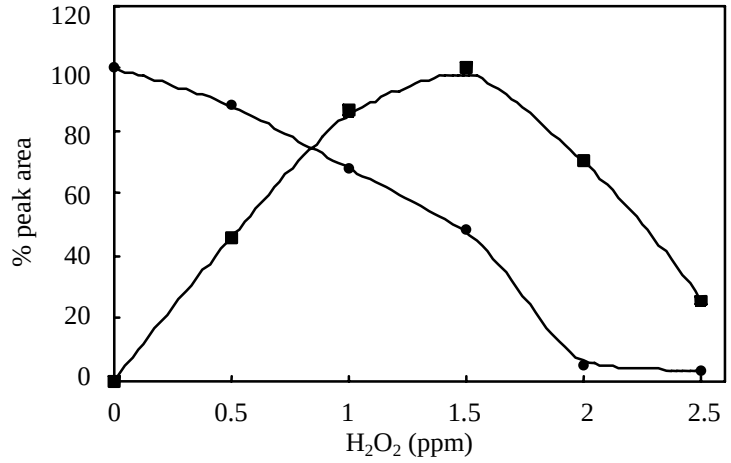

Figure 1. Measurements of ferulic acid (●) *and dimers* (■) *after incubation of feruloyl-arabinose with peroxidase and increasing amount of hydrogen peroxide.*

3.2 CHEMICAL ANALYSIS OF EXTRACTS FROM FLOURS

Aqueous extracts of the 20 french varieties chosen for this study, varied in their arabinoxylan contents from 3.75 to 7.66 mg / g flour (Table).

Variety	WEAX (mg / g)	FA / WEAX (mg / g)	Total dimers / WEAX (mg / g)	5-5' (% total)	8-O-4' (% total)	8-5'C (% total)	Peroxidase (nkat/g flour)	FeE (nkat/g flour)	Relative viscosity	[η] (mL/g)
Albatros	3,75	2,80	0,18	14,58	35,41	50,01	1860.8 ± 309	11.8 ± 1.3	1,37	644
Soissons	3,79	3,26	0,18	16,51	35,33	48,16	2046.7 ± 182	11.2 ± 0.7	1,55	565
Joss	4,07	2,06	0,11	17,96	39,00	43,04	1621.0 ± 98	12.2 ± 0.7	1,39	593
Festival	4,48	2,35	0,15	16,00	29,87	54,12	1729.3 ± 252	10.2 ± 0.3	-	688
Arminda	4,57	2,41	0,19	14,25	29,88	55,87	1471.3 ± 202	13.9 ± 0.1	1,54	642
Capitole	4,69	3,16	0,26	13,78	30,44	55,78	1646.0 ± 78	11.9 ± 0.3	1,58	548
Choisel	4,71	3,75	0,24	15,79	30,77	53,44	1733.0 ± 166	15.2 ± 0.3	1,64	482
Ami	5,09	2,29	0,17	13,11	28,78	58,11	2788.0 ± 203	13.0 ± 0.0	1,73	593
Thésée	5,13	3,66	0,26	13,26	30,05	56,69	1991.5± 110	10.5 ± 1.3	1,94	498
Scipion	5,17	2,70	0,19	13,53	33,50	52,96	1419.8 ± 134	12.8 ± 0.4	1,60	612
Sideral	5,72	2,10	0,15	12,96	31,11	55,93	1720.9 ± 62	10.4 ± 0.0	2,47	582
Feuvert	5,75	3,29	0,21	13,28	33,20	53,52	1281.3 ± 111	13.3 ± 0.7	1,87	519
Rialto	5,85	3,77	0,11	11,40	32,70	55,90	1765.6 ± 162	12.7 ± 0.1	-	504
Apollo	6,07	2,10	0,10	13,23	24,26	62,52	3220.0 ± 132	11.9 ± 0.3	1,81	694
Moulin	6,20	3,44	0,16	12,58	28,47	58,95	2871.8 ± 241	13.2 ± 0.8	1,99	579
Radja	6,44	2,28	0,11	13,93	25,69	60,37	1604.0 ± 199	10.0 ± 0.1	2,07	643
Prinqual	6,79	3,05	0,22	14,37	32,94	52,69	1734.0 ±109	10.1 ± 0.5	2,08	538
Magister	7,30	1,76	0,07	15,85	26,30	57,85	3842.0 ± 426	11.0 ± 0.5	2,45	672
Tarasque	7,58	2,68	0,19	13,21	30,26	56,53	1764.2 ± 165	14.5 ± 0.4	2,40	640
Aubaine	7,66	2,03	0,10	16,72	33,36	49,91	1540.9 ± 301	8.8 ± 0.5	2,69	630
Mean	5,54	2,75	0,17	14,31	31,07	54,62	1982,6	11,9	1,90	593
Std deviation	1,20	0,63	0,05	1,65	3,53	4,47	666,9	1,7	0,40	64
Coeff. variation (%)	21,7	22,9	29,4	11,5	11,4	8,2	33,6	14,3	20,9	10,8

WEAX =Water Extractable Arabinoxylans; FA = Ferulic acid; FeE = Feruloyl esterases; [η] = Intrinsic viscosity

WEAX contents varied largely since the coefficient of variation between the 20 varieties was 21.7%. Ferulic acid contents in WEAX were in the range of 1.7 to 3.8 mg/g, which corresponded to previously reported values (Izydorczyk and Biliaderis, 1995). WEAX contained more or less dimers, which were detected in all extracts. If we express the dimer content as a percentage of ferulic acid, it could vary from 2.8% to 8.2%. Three different dimers were identified in WEAX (Fig. 2) : the 5-5' represented on average 14.3% of the total, the 8-O-4', 31.1% of the total, and the 8-5'C, 54.6% of the total. Thus, the 8-5'C was the major dimer, and as it was supposed to be formed by peroxidase action, peroxidases were measured in flours in order to investigate whether or not dimer level could be controlled by endogenous peroxidase activity. On the other hand, feruloyl-esterases were also measured because they could induce a disappearance of ferulic acid, which could be considered as substrate for peroxidases.

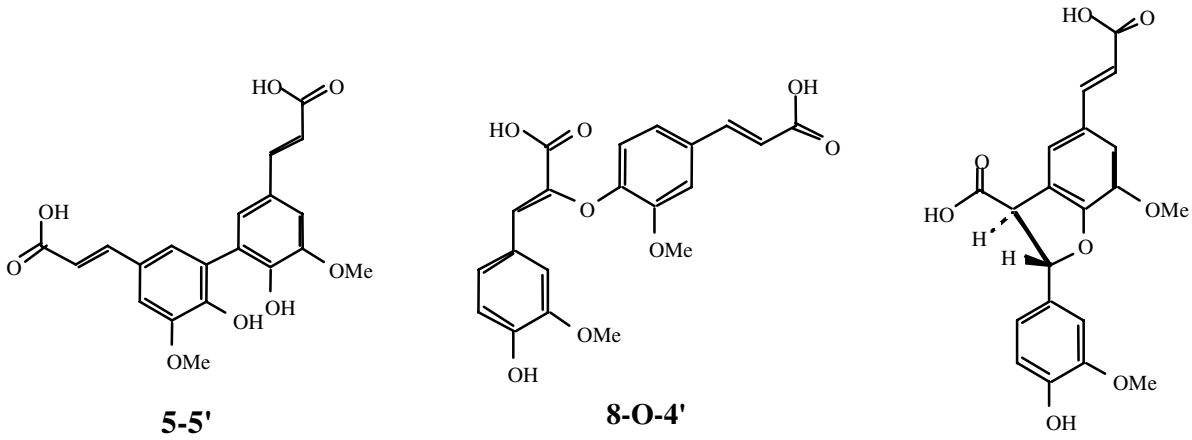

Figure 2. Chemical structure and proportions of dehydrodimers found in wheat flour.

3.3 ENZYMIC ACTIVITIES IN FLOURS

Feruloyl-esterases were found to be very low in flours (mean = 11.9 nkat/g flour, Table) when they were measured towards FA. The coefficient of variation between the 20 varieties was also low (14.3%), showing that feruloyl esterases did not vary so much between the varieties. On the other hand, the use of 2-methoxyphenol as substrate showed very large peroxidase activity (mean = 1983 nkat/g flour) which could vary a lot between varieties (coefficient of variation = 33.6%). Thus, in the flour, or in the grain before flour extraction, peroxidases could efficiently cross-link arabinoxylans by the way of 8-5'C dimer.

3.4 PHYSICO-CHEMICAL PROPERTIES

The average intrinsic viscosity (Table) was 593 mL/g, and the values are in the range of those previously reported (Izydorczyk and Biliaderis, 1995). Some variations were

observed (coefficient of variation = 10.8%) but were rather low compared to variation observed for relative viscosity (coefficient of variation = 20.9%). Intrinsic viscosity reflects the hydrodynamic volume of a macromolecule. It could be affected by conformational modifications, which could be induced by chemical changes such as dimer content in WEAX. On the opposite, relative viscosity is controlled by both the concentration and the hydrodynamic volume of macromolecules. That would explain that the coefficient of variation obtained for intrinsic viscosity was in the same range as that obtained for WEAX content.

3.5 PRINCIPAL COMPONENT ANALYSIS

Figure 3 represented the principal component analysis of the main parametres that were analysed. The two axes of this representation allowed to explain 65% of the variation observed within the samples. According to the first axis, ferulic acid and total dimers varied in a coherent way. Moreover, relative viscosity and WEAX concentration were rather strongly correlated. On the opposite, peroxidase and feruloyl esterase activities were not correlated with any other parameters. Particularly, the variation in content of 8-5' dimer was neither related to intrinsic viscosity of WEAX, nor to peroxidase activity in flour.

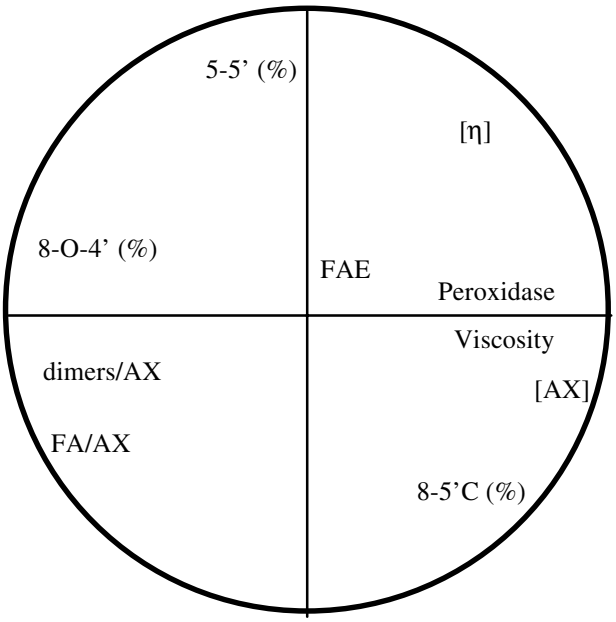

Figure 3. Principal component analysis of the different parameters measured on wheat flour.

4 DISCUSSION

Numerous parameters have been measured on flours from 20 french varieties of wheat. Water extracts exhibited large variations in their relative viscosity, peroxidase activity and WEAX content and some variations in intrinsic viscosity and dimer contents of WEAX. Although they all concerned arabinoxylans and related enzymes, no correlation

was displayed between these parameters, except relative viscosity and WEAX content. However, it has been shown that peroxidase/peroxide-induced changes in physico-chemical properties of arabinoxylans resulted from oxidative cross-linking of ferulic acid moieties (Ng *et al*., 1997, Figueroa-Espinoza and Rouau, 1998).

Much of the difficulty in understanding the effects of peroxidase activity in plants is due to the presence of a large number of isoperoxidases, resulting in a very variably catalytic action towards a wide range of substrates. For instance, early studies on horseradish peroxidases demonstrated the existence of a complex system of isoenzymes (Shannon, 1968 in Robinson, 1991). Thus, it could be of interest to follow peroxidase activity towards ferulic acid instead of 2-methoxyphenol.

An important variability in dehydrodiferulic acid composition was already shown within durum wheat grains (Lempereur *et al*., 1998). In this paper, the 8-O-4' dehydrodimer was predominant whereas it had an intermediate content in our study. On the one hand, we have demonstrated that the major product of *in vitro* enzymic oxidation of ferulic acid was the 8-5'C dehydrodimer, confirming the results previously obtained with methyl ferulate (Wallace and Fry, 1995). On the other hand, we have shown that the 8-5'C was the major dimer in flours, suggesting that it was produced by peroxidase action. Nevertheless, dimer contents of WEAX did not seem to be controlled by peroxidase levels. It is likely that most of the peroxidase activity measured in flours came from germ contamination. Therefore, dimers in WEAX were probably not formed during the water extraction but within the wall, e.g. controlled by wall peroxidase.

The presence of dimers in WEAX have also been found in barley and rye WEAX (unpublished results) and probably influenced the physico-chemical behaviour of these macromolecules. Due to the high level of activity observed for endogenous peroxidase in flours, it is likely that these enzymes could affect the functional properties of WEAX during technological process (breadmaking, brewing…) providing that H_20_2 is available in those food systems.

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SYNERGISTIC INTERACTIONS BETWEEN POLYSACCHARIDE DEGRADING ENZYMES FROM *ASPERGILLUS*

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ABSTRACT

For the biodegradation of plant cell wall polysaccharides, microorganisms require a wide spectrum of enzymes. *Aspergillus niger* and *Aspergillus tubingensis* are fungi, which are commonly used in industry for the production of cell wall degrading enzymes, such as cellulases, xylanases and pectinases.

To study synergy in wheat arabinoxylan degradation, we have used two enzymes hydrolysing the β-1,4-glycosidic linkages of the main chain (endoxylanase and β-xylosidase) and six accessory enzymes (α-L-arabinofuranosidase, arabinoxylan arabinofurano-hydrolase, α-glucuronidase, α-galactosidase, β-galactosidase and feruloyl esterase) from *Aspergillus*. These enzymes were used, alone and in combinations, in incubations using water insoluble pentosan (WIP) isolated from wheat flour. Synergy was observed between most enzymes tested, although not always to the same extent. Degradation of the xylan backbone by endoxylanase and β-xylosidase was influenced most strongly by the action of α-L-arabinofuranosidase and arabinoxylan arabinofuranohydrolase. All accessory enzymes involved in the degradation of WIP positively influenced each other.

Similarly we investigated the degradation of sugar beet pectin (SBP). Degradation of the rhamnogalacturonan backbone stimulated the action of most accessory enzymes, while synergy between the accessory enzymes was also observed. Mixed incubations revealed that an enzyme (feruloyl esterase A), which was previously reported to have no activity on pectin, is in fact able to liberate 58% of the ferulic acid residues in the presence of other enzymes.

1 INTRODUCTION

Xylan is the most abundant hemicellulose structure found in nature. It is located in the cell walls of plants and consists of a β-1,4-linked D-xylose backbone, which can be esterified with acetyl residues. Wheat arabinoxylan is a very complex structure containing a number of different side chains [1, 2]. Attached to the xylan backbone, L-

arabinose is present as monomeric or short oligomeric substituents [3]. Terminal arabinose residues can be esterified with feruloyl or *p*-coumaroyl residues or substituted with galactose residues [4]. The xylan backbone can also be substituted with (4-O-methyl-)glucuronic acid [4].

Another complex plant cell wall polysaccharide is pectin, which consists of two major regions. Pectic 'smooth' regions consist of a galacturonic acid backbone, which can be esterified with acetyl or methyl residues. In the 'hairy' regions the galacturonic acid backbone is interrupted by rhamnose residues, to which long side chains can be attached [5]. These side chains consist of galactose (galactan), arabinose (arabinan) or both (arabinogalactan) and can be esterified with feruloyl residues [6, 7].

The fungus *Aspergillus* produces a wide spectrum of enzymes for the degradation of xylan and pectin. These can be divided into enzymes acting on the main chain and accessory enzymes acting on the side chains of these polysaccharides. Some of these enzymes are specific for either xylan or pectin, whereas others have been shown to act on both structures or also on other polysaccharides. Previous studies have demonstrated synergy between main chain cleaving and accessory enzymes [8, 9, 10], but a detailed study of synergy between accessory enzymes has not been performed. We studied synergy in the degradation of water insoluble pentosan from wheat (WIP) and the hairy regions of sugar beet pectin (SBP) using several main chain cleaving and accessory enzymes (Table 1).

Table 1. Enzymes used in this study.

Enzyme	Symbol	Source	Incubated with
endoxylanase A	XlnA	*A. tubingensis*	WIP
β-xylosidase	XlnD	*A. niger*	WIP
arabinoxylan arabinofuranohydrolase	AxhA	*A. tubingensis*	WIP
α-glucuronidase	AguA	*A. tubingensis*	WIP
α-galactosidase B	AglB	*A. niger*	WIP
arabinofuranosidase B	AbfB	*A. niger*	WIP / SBP
β-galactosidase	LacA	*A. niger*	WIP / SBP
feruloyl esterase A	FaeA	*A. niger*	WIP / SBP
endoarabinase	AbnA	*A. niger*	SBP
endogalactanase	GalA	*A. niger*	SBP
rhamnogalacturonan acetyl esterase	RgaeA	*A. niger*	SBP
rhamnogalacturonan hydrolase A	RhgA	*A. aculeatus*	SBP

2 MATERIALS AND METHODS

Sugar and ferulic acid analysis of water insoluble pentosan from wheat arabinoxylan and sugar beet pectin. Water insoluble pentosan (WIP) from wheat arabinoxylan was isolated as described previously [11]. The sugar analysis involved acid hydrolysis of WIP with 1M sulphuric acid after which the released monomers were determined by HPLC using a Carbopac PA1 column (Dionex Corp., Sunnyvale, Ca) and a pulsed electrochemical detector. The ferulic acid content was determined as described previously [12].

Enzyme incubations. The amount of enzyme necessary to obtain the maximum amount of released monomeric compound after 24 h of incubation when acting alone was determined by incubating WIP or SBP with the individual enzymes, analysing the amount of monomeric compound released in time.

Pre-treatments were carried out for 3 days at 30°C using XlnA or RgaeA and RhgA for WIP and SBP, respectively. The incubated substrate preparations were boiled for 10 min to inactivate the enzymes used for the pre-treatments.

Enzyme incubations were all performed in duplicate at 30°C for 24 h, using 500 µl of a 0.5% WIP suspension or a 1% SBP suspension in 50 or 10 mM sodium acetate buffer (pH 4.5), respectively. Incubating the mixtures for 5 min in a boiling water bath inactivated the enzymes. Undissolved material was removed by centrifugation (14.000 × g, 10 min), after which the supernatant was transferred to a new tube and stored at -20°C.

Xylose, arabinose and galactose concentrations were determined using a Carbopac MA-1 column (Dionex Corp., Sunnyvale, Ca.) and a pulsed amperometric detector (PAD). and isocratic elution with 0.48 M NaOH. Ferulic acid concentrations in the incubations were analyzed by HPLC as described previously [8]. 4-O-methyl-glucuronic acid concentrations were also determined by HPLC using a Carbopac PA-100 column (Dionex Corp., Sunnyvale, Ca) and a PAD. Separation was achieved by using a linear gradient, starting with 100 mM NaOH, 5 mM sodium acetate and ending with 100 mM NaOH, 400 mM sodium acetate at a flow of 1 ml/min over a 20 min period.

3 RESULTS AND DISCUSSION

3.1 COMPOSITION OF WATER INSOLUBLE PENTOSAN (WIP) FROM WHEAT AND SUGAR BEET PECTIN (SBP)

For both substrates the neutral sugar composition and the amount of ferulic acid present was determined as described in Materials and Methods. WIP contained mainly xylose and arabinose, but significant amounts of galactose and 4-O-methyl glucuronic acid were also present (Table 2). In pectin, the main neutral sugar was galactose. Apart from arabinose and rhamnose, small amounts of fucose, glucose and xylose were also detected. Both substrates contained less than 1% ferulic acid.

Table 2. Sugar and ferulic acid content of WIP and SBP (% dry-weight).

	xylose	arabinose	galactose	rhamnose	glucose	4-O-methyl-glucuronic acid	ferulic acid
WIP	32.1	20.7	2.1	-	11.2	6.0	0.49
SBP	<0.1	5.0	9.6	2.4	<0.1	-	0.63

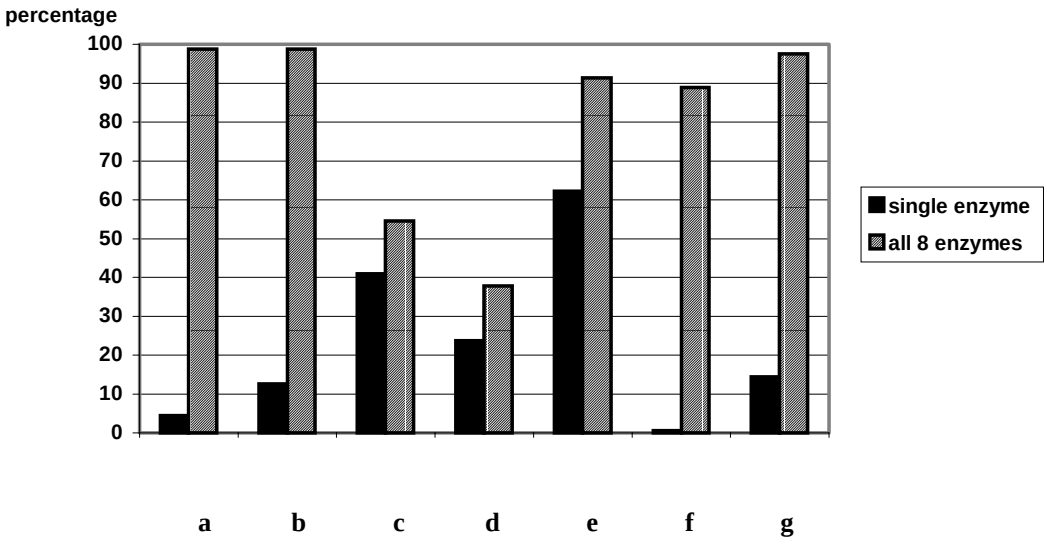
3.2 SYNERGY IN THE DEGRADATION OF WATER INSOLUBLE PENTOSAN (WIP) FROM WHEAT

XlnA and XlnD were only able to release 4.4% and 12.6%, respectively, of the xylose residues from untreated WIP when acting alone. When used together, they were able to release 52.4% of the xylose residues from this substrate (data not shown), whereas 98.7% of the xylose residues were released using all eight enzymes. This indicates that the action of XlnA and XlnD is inhibited by the presence of substituents on the xylan backbone.

AbfB and AxhA are able to release 40.9% and 23.7%, respectively, of the arabinose residues from untreated WIP, whereas the two enzymes together released 62.2%. The same effect was observed on pre-treated WIP, indicating that the enzymes release different arabinose residues from WIP. Using pre-treated WIP, the presence of XlnD reduced the release of arabinose by AbfB (data not shown) suggesting that AbfB prefers longer arabinoxylo-oligosaccharides as substrate. Previous studies [13] suggested that AbfB is not able to remove arabinose from non-terminal residues. The data described here demonstrated that AbfB is able to release 41% of the arabinose residues from intact WIP, indicating that AbfB is in fact capable of removing arabinose from non-terminal xylose residues.

AguA was able to release only 0.6% of the O-methyl-glucuronic acid residues from untreated WIP and 6.7% from pre-treated WIP. However, when AguA and XlnA were used simultaneously 86.7% of these residues were released. This indicates that the action of XlnA is severely affected by the presence of AguA and that the activity of XlnA is necessary to create oligosaccharides, which can be attacked by AguA. The level of synergy observed for AguA and XlnA is higher than that which was observed in a previous study [9], which can be explained by the longer incubation time in this study. A similar effect was observed for the release of ferulic acid. Using pre-treated WIP and all enzymes except XlnA, only 50.4% of the ferulic acid residues were released. When eight enzymes were applied simultaneously, 97.5% of the ferulic acid residues was released.

A



B

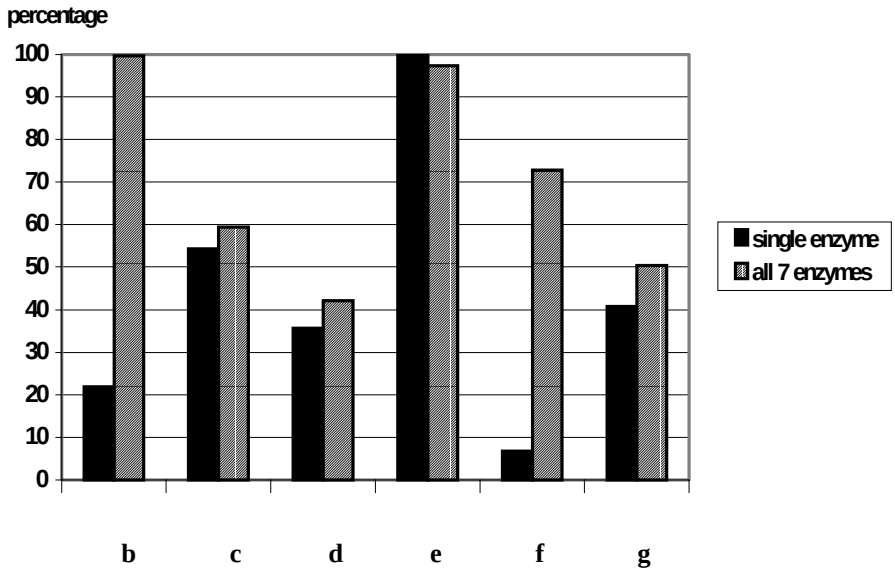


Fig. 1. Synergy in the degradation of untreated WIP (A) and pre-treated WIP (B). Indicated are: a, xylose (XlnA); b, xylose (XlnD); c, arabinose (AbfB); d, arabinose (AxhA); e, arabinose (AbfB+AxhA); f, 4-O-methyl glucuronic acid (AguA); g, ferulic acid (FaeA). All 8 enzymes: XlnA, XlnD, AbfB, AxhA, AguA, AglB, LacA, and FaeA. All 7 enzymes: all enzymes except for XlnA.

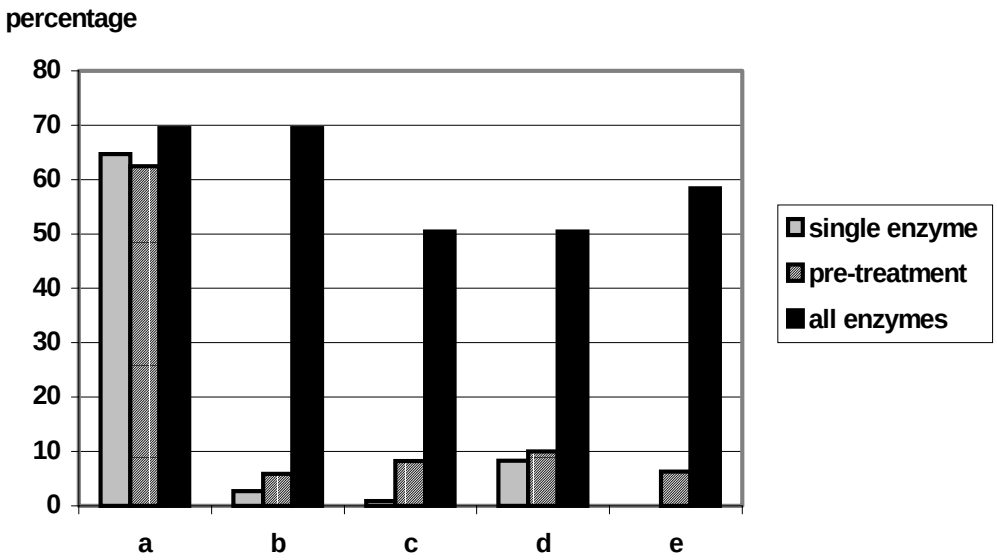
3.3 SYNERGY IN THE DEGRADATION OF THE HAIRY REGIONS OF SUGAR BEET PECTIN (SBP).

AbfB was the only enzyme with a significant activity on untreated SBP when acting alone. The other enzymes had low or no activity on this substrate when acting alone, but their activity was increased in the presence of AbfB (data not shown). The pre-treatment

of pectin with RgaeA and RhgA results in cleavage of the rhamnogalacturonan backbone. This positively affected the activity of all other accessory enzymes, indicating that these enzymes require a more 'open' structure of the substrate for optimal activity.

Galactose release by LacA and GalA is strongly increased by the presence of the other accessory enzymes. This is in agreement with the proposed structure for sugar beet pectin hairy regions, in which short galactan chains connect the longer arabinan chains to the rhamnogalacturonan backbone [14, 15]. By removing the arabinose residues using AbfB and AbnA, the galactan chains become more accessible to LacA and GalA. This effect is even stronger if the rhamnogalacturonan backbone is also cleaved.

Although FaeA was not able to release ferulic acid from intact sugar beet pectin, incubations using all five accessory enzymes and pre-treated sugar beet pectin resulted in the release of 58% of the ferulic acid residues. This contradicts previous papers in which FaeA was reported not to play a role in sugar beet pectin degradation [16, 17]. However, these studies were performed on sugar beet pulp in which the rhamnogalacturonan backbone was not cleaved. The data in this study demonstrates the importance of backbone degradation for the release of ferulic acid by FaeA. Addition of LacA had a strong effect on ferulic acid release from pre-treated SBP by FaeA. This is in agreement with a previous study in which the ability of FaeA to release ferulic acid from sugar beet pectin derived galacto-oligosaccharides, but not from sugar beet pectin derived arabino-oligosaccharides was reported [18].



All enzymes = RgaeA/RhgA pre-treatment, incubation with AbfB, AbnA, LacA, GalA, and FaeA.

Fig. 2. Synergy in the degradation of SBP. Indicated are: a, arabinose (AbfB); b, arabinose (AbnA); c, galactose (LacA); d, galactose (GalA); e, ferulic acid (FaeA).

This study demonstrates a high degree of synergy between heteropolysaccharide degrading enzymes of *Aspergillus*. For the efficient utilisation of these substrates by this fungus it will therefore need to produce these enzymes simultaneously. This has been demonstrated at the molecular level for enzymes involved in xylan degradation. Expression analysis of the encoding genes demonstrated that they are all under the control of the xylanolytic transcriptional activator XlnR [19, 20], except for *abf*B. This is in good correlation with the results of this study, where all accessory enzymes involved in xylan degradation, except AbfB, depend on the presence of other xylanolytic enzymes for an efficient release of the monomeric compounds.

Regulation of genes encoding pectinolytic enzymes has not yet been studied in detail. No indications have been reported for a general transcriptional activator regulating the expression of genes encoding main chain cleaving and accessory enzymes. However, it is remarkable that arabinose release from SBP by AbfB is also not significantly effected by the presence of other enzymes.

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THE EFFECT OF CELL WALL DEGRADING ENZYMES ON THE MICROSTRUCTURE OF RYE CELL WALLS

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ABSTRACT

The effect of cell wall hydrolyzing enzymes, endogenic and exogenic on the cell walls of rye kernel, and rye bread was studied by using fluorescence microscopy and image analysis. Endo-xylanase was able to degrade a small portion of the kernel cell walls. In addition to xylanase, arabinosidase was needed to hydrolyze the inner endosperm cell walls suggesting that the inner endosperm cell walls are more branched than the outer ones. Xylanase treatment of rye dough caused the endosperm cell walls to fragment, and when the dough was treated with 100 nkat xylanase/g flour, small cell wall fragments were observed in the breads. The effect of ferulic acid esterase on the microstructure of rye grains and breads was also studied. Relative to the native grains, weak germination induced minor local changes in the microstructure of cell walls and proteins in the kernels. Kernels of germinated and native grains were milled, and doughs were prepared from the flours. Microstructural examination of dough sections showed germination to cause two types of structural changes in the cell walls: 1) swelling and 2) fading of the blue fluorescence of cell walls. More extensive germination of rye grains at 15°C for 4 days resulted in the production of α-amylase, β-glucanase, endo-β-xylanase and α-arabinosidase. In all the grains the fluorescing cell wall area, measured by computer-assisted image analysis was less than 5% in germinated grains, whereas it was over 30% for non-germinated grains.

1 INTRODUCTION

The main components of rye primary cell walls are arabinoxylans; the β-glucan content is much lower. Ferulic acid is esterified to the arabinoxylans (Harris and Hartley, 1980; Ahluwalia and Fry, 1986). Ferulic acid-arabinoxylan complexes are concentrated in the aleurone layer. No ferulic acid is associated with β-glucan. Structurally, cereal arabinoxylans form a very heterogeneous group, in which the ratio of arabinose to xylose, the pattern of arabinose substitution, the feruloyl group content and the degree of polymerization can vary significantly (Izydorczyk and Biliaderis, 1993).

Soluble rye arabinoxylans are composed of arabinoxylan I characterized by a main chain of xylose residues, of which about 40% were substituted at the 3-position by terminal arabinose residues and arabinoxylan II in which the 70% were substituted at both the 2- and 3-positions with terminal arabinose residues (Åman and Bengtson, 1991). It has been shown that the arabinofuranosyl substituents on the xylan backbone inhibit the action of endo-xylanases on glycosidic linkages in the vicinity of the site of substitution (Kormelink, 1992).

The objective of this paper was to study microstructure of rye grain cell walls by microscopy and image analysis with the aid of pure enzyme. The effect of germination on the cell walls and enzyme activities of cell wall hydrolyzing will also been shown.

2 STAINING OF CELL WALLS

The most commonly used staining systems for cereal cell walls in fluorescence microscopy (Fulcher *et al*., 1989) are presented in Table 1. Some components exhibit autofluorescence. In cereal cell walls, the main sources of autofluorescence are polyphenolic compounds, such as ferulic acid and lignin.

Table 1. The most commonly used stains for cell walls in fluorescence microscopy.

Component	Stain	Colour
Mixed-linked β-glucans	Calcofluor	White/blue
	Congo red	Red

3 EFFECT OF PURE ENZYMES ON MICROSTRUCTURE OF RYE CELL WALLS

Microscopy of thin sections of cereal grains incubated with purified enzymes can be used as a means to localize specific components. Endo-xylanase was able to degrade a small portion of the kernel cell walls. In addition to xylanase, arabinosidase was needed to hydrolyze the inner endosperm cell walls suggesting that the inner endosperm cell walls are more branched than the outer ones. Ferulic acid esterase change only the structural state of aleurone cell walls.

4 EFFECT OF ENDOGENOUS ENZYMES ON ACTIVITIES OF ENZYMES HYDROLYZING CELL WALLS AND ON RYE CELL WALLS

Table 2 shows enzyme activities of rye flours with different falling numbers.

Table 2. Effect of falling number on enzyme activities of rye flours.

Falling number	β-glucanase	Xylanase
262	19	1
244	19	2
172	28	1
62	28	5

Sprouting can cause also changes in the microstructure of rye cell walls. Examination of rye grains with falling number of 62, revealed that cell walls in the subaleurone layer, and in the cells of ventral endosperm, were not stainable by Calcofluor (Fabritius *et al*., 1996). An objective method to quantify visible cell walls in thin sections of rye dough was used for studying differences in the microstructural state of cell walls in case of low and high falling number (Parkkonen *et al*., 1997). The total area of blue fluorescence was 7.3±0.6 and 12.5±0.6, respectively, for doughs prepared from low and high falling-number flour.

Microstructural examination of cell walls showed that endogenous enzymes cause following changes in the doughs: swelling of cell walls and/or fading of cell walls (Autio *et al*., 1998). Addition of microbial xylanase to rye dough caused fragmentation of cell walls in rye bread (Autio *et al*., 1996). Structural changes in other components was also observed: starch granules were highly swollen, when xylanase was added to the dough suggesting that starch granules had more water available due to decreased water-binding of cell walls.

5 CONCLUSIONS

The chemical structure of cell walls is different in different parts of the grain. The aleurone and subaluerone layer is rich in ferulic acid and less branched arabinoxylans. Endogenous or added xylanases may cause following changes in the microstructural level: Fading and disappearance of the blue fluorescence, swelling of cell walls, fragmentation of cell walls and changes in water distribution between cell walls, proteins and starch granules.

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INTERACTIONS BETWEEN LIPOXYGENASE AND OTHER OXIDOREDUCTASES IN BAKING

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1 INTRODUCTION

Redox reactions are of primary importance in the determination of the rheological properties of dough (Grosch, 1986). Most of these reactions if not all are catalyzed or at least influenced by redox enzymes (Nicolas and Drapron, 1983; Van Dam and Hille, 1992). In wheat flour, peptides and proteins containing thiol groups, unsaturated lipids such as polyunsaturated fatty acids and carotenoids, as well as phenolics are the most sensitive to oxidation during dough mixing. Moreover, different ingredients such as ascorbic acid, cysteine, potassium bromate and soya or horse bean flour when added to wheat flour, largely participate to the redox reactions. The purpose of this presentation is first to recall redox reactions during mixing and then to discuss the influence of lipoxygenase and some other oxidoreducing enzymes on these reactions which could explain their effects in breadmaking.

2 REDOX PHENOMENA DURING DOUGH MIXING

Most of the oxygen absorbed by lipid oxidation during dough mixing is used for the oxidation of linoleic and linolenic acids either in the free or the monoglyceride forms (Graveland, 1970a; Tait and Galliard, 1988; Ameille *et al.*, 2000) whereas other fatty acids are not affected (Drapron and Beaux, 1969; Mann and Morrison, 1974; Castello *et al.*, 1998).

It is well known that lipid binding is influenced by the dough-mixing conditions (Daniels *et al.*, 1966; Frazier *et al.*, 1981). Under work-free conditions, lipid binding increases with flour moisture content. Above 30% moisture and after mixing, there is an additional 15% of bound lipids (Daniels, 1975). However when oxygen tension is increased in the mixing chamber, the free lipid level increases (Daniels *et al.*, 1970). Moreover, the same authors showed that the release of bound lipids is increased by higher work input levels whereas lipid binding increased under nitrogen.

The bleaching of carotenoid pigments of wheat flour is a well known effect of enzymatic oxidation during dough mixing (Tsen and Hlynka, 1962; Nicolas, 1978).

Dough mixing affects the sulfhydryl (SH) and disulfide groups of gluten proteins. SH oxidation is associated with aerobic mixing (Sokol *et al.*, 1960) since Bloksma (1963) was unable to detect any significant change in the SH content of dough in the absence of oxygen. Moreover, for a longer period of mixing under nitrogen, Mecham and Knapp (1966) noticed an increasing amount of SH groups. Similarly, it was shown by Jackson

and Hoseney (1986a) that ferulic acid, the predominant phenolic acid in wheat flour pentosans, decreased in concentration when wheat flours were formed into a dough and overmixed.

Several flour additives, used to improve baking quality, have oxidoreducing properties and among them, ascorbic acid (AA) is used worlwide (Grosch and Wieser, 1999). The AA improving action is due to its effect on the sulfhydryl-disulfide exchange reactions. During the dough mixing, AA is rapidly oxidized to dehydroascorbic acid (DHA) (Elkabassany *et al.*, 1980; Nicolas *et al.*, 1980). The latter compound, which is the actual improver, promotes the oxidation of thiol groups to disulfide links in dough.

We shall now examine how these redox reactions are influenced by lipoxygenase and some other oxidoreducing enzymes and their consequences on the dough properties.

3 LIPOXYGENASE (EC 1.13.11.12 : LOX)

Many reviews have been devoted to the effects of lipoxygenase (LOX) in breadmaking (Frazier, 1979; Nicolas, 1979; Faubion and Hoseney, 1981; Nicolas and Drapron, 1983; Grosch, 1986; Nicolas and Potus, 1994).

3.1 EFFECT ON POLYUNSATURATED FATTY ACIDS

In the presence of molecular oxygen, LOX catalyzes the oxidation of polyunsaturated fatty acids (PUFA) containing the cis-cis 1,4 pentadiene system into their corresponding hydroperoxides. It is clear that with regard to this specificity, LOX is involved in the loss of linoleic and linolenic acids in the dough during mixing. This loss, undetectable under anaerobic conditions, increases with mixing under pure oxygen or with added purified LOX (Graveland, 1968). According to Graveland (1970b), in an aqueous extract of flour, hydroperoxy radicals formed from linoleic acid lead to 9- and 13-hydroperoxides (L_1), part of these being reduced to hydroxy acids (L_2). In a flour-water suspension, there is formation of trihydroxy acids (L_4) whereas in a dough, hydroxy-epoxy acids (L_3) are also present and L_1 are undetectable. Kinetic studies during mixing show that L_4 is the hydrolysis product of L_3. According to Graveland (1971), in all cases the peroxy radical is the precursor. L_3 and L_4 are only obtained in the presence of the water-insoluble fraction of flour which redirects LOX-catalyzed reaction. Later, Mann and Morrison (1975), Markwalder *et al.* (1975) and Tait and Galliard (1986) confirmed some of these results, particularly the formation of L_4 in dough.

In addition to free PUFA, wheat LOX is able to catalyse the oxidation of PUFA in monoglycerides (Graveland, 1970a; Morrison and Panpaprai, 1975). Moreover, the presence of fully active soya flour promotes the oxidation of PUFA engaged in triglycerides (Morrison and Panpaprai, 1975). It is well known that soya flour contained enzymes able to act on esterified PUFA (Whitaker, 1991).

3.2 EFFECT ON SULFHYDRYL GROUPS

To explain the loss of SH groups during mixing, it has been suggested that wheat LOX is able to catalyse the oxidation of gluten proteins through co-oxidation of accessible groups of the proteins (Tsen and Hlynka, 1963; Bloksma, 1963). Later, Graveland *et al.* (1978) found a decrease in the SH group content equivalent to the increase in hydroxy acids during mixing. Similarly, Shiiba *et al.* (1991) found a decrease in the SH content of flours supplemented by pure isoenzymes isolated from wheat germ.

3.3 LIPID BINDING AND DOUGH RHEOLOGY

The release effect of bound lipids during mixing has been attributed to the oxidized lipid intermediates formed during the PUFA oxidation catalyzed by LOX (Daniels *et al.*, 1970). According to these authors, these intermediates are able to act on the hydrophobic sites of lipid-binding proteins resulting in the oxidation of SH groups and leading to the release of bound lipids. Later on, Frazier *et al.* (1973) provided further evidence for the influence of LOX. They found that the addition of purified soya LOX resulted in a marked increase in the release of bound lipids during mixing in air. This phenomenon did not occur when mixing was under nitrogen or with heat denaturated soya LOX.

The rheological effects attributed to LOX correspond to an increase of the dough-mixing tolerance (Frazier *et al.*, 1973; Hoseney *et al.*, 1980; Kieffer and Grosch, 1980), and of the dough relaxation time (Frazier *et al.*, 1973, 1977; Hoseney *et al.*, 1980). These improvements result in an enhancement of the bread volume (Frazier, 1979; Shiiba *et al.*, 1991). Recently, Cumbee *et al.* (1997) confirmed the findings of Kieffer and Grosch (1980) who showed that the isoenzyme LOX_2 (optimum pH 6.5), and not the isoenzyme LOX_1 (optimum pH 9), was mainly responsible for the improving effect of soya flour. All these effects have been attributed to the co-oxidation of the flour protein SH groups by LOX. However, Hoseney *et al.* (1980) proposed another mechanism in which the free radicals created by LOX are responsible for the increase in mixing tolerance of dough. According to these authors, these free radicals compete for the activated double-bond compounds (indigenous in flour or created by fast-acting oxidants) which are responsible for the rapid decrease in dough stability. Grosch (1986) casts some doubt on this mechanism since the compounds resulting from the interactions of the activated double-bond compounds with thiyl protein radicals or with lipid free radicals have neither been identified.

3.4 BLEACHING OF CAROTENOID PIGMENTS AND VITAMIN LOSSES

The co-oxidation bleaching activity on carotenoid pigments of LOX is well known. As early as 1934, Haas and Bohn patented the use of enzyme-active soya four as a dough-bleaching agent. However, the carotene-bleaching activity is highly variable among LOX (Weber *et al.*, 1973). Thus wheat LOX isoenzymes and LOX_1 from soya bean have a much lower co-oxidizing activity than the LOX_2 from soya bean (Grosch *et al.*, 1977) and LOX from horse bean (Nicolas *et al.*, 1982).

According to Nicolas (1978), during mixing, the amount of oxidized pigments increases with increasing work input, rate of work input and dough hydration. In addition to carotenoid pigments, tocols were partly destroyed by coupled oxidation during mixing whereas water-soluble vitamins (B_1 and B_6) although sensitive to oxidation were not affected (Drapron *et al.*, 1974). This difference in behaviour could be the result of the relative hydrophobic character of carotenoid and tocols.

3.5 BREAD AROMA

In French breadmaking, horse bean flour added to wheat flour used together with high speed mixing results in an impairment of bread aroma. Drapron *et al.* (1974) showed that this phenomenon was linked to the production of hexanal due the presence of horse bean LOX. Hexanal, the major product of the 13-hydroperoxide scission is probably formed during the baking of dough. With soya bean flour, a similar phenomenon was observed (Hoover, 1979) and mainly attributed to the LOX_2 isoenzyme (Addo *et al.*, 1993).

4 OTHER OXIDOREDUCING ENZYMES

In contrast to LOX, few papers have been devoted to the effects of peroxidase, catalase, polyphenoloxidase, ascorbic acid oxidase, dehydroascorbate reductase, protein disulfide isomerase, glucose and sulfhydryl oxidases.

4.1 PEROXIDASE (POD) AND CATALASE (CAT)

Through their hematin compounds, these enzymes are able to oxidize non enzymatically unsaturated lipids with intermediary free radicals and therefore to promote reactions similar to those obtained with LOX (Eriksson *et al.*, 1971).

Their presence in wheat has long been recognized (Honold and Stahmann, 1968) and several POD isoenzymes have been purified from bread wheat germ (Zmrhal and Machackova, 1978; Billaud *et al.*, 1999). The distribution of POD and CAT activities in different milling fractions was studied by Honold and Stahmann (1968) whereas Kruger and Laberge (1974) and Kruger (1977) examined the activity changes during grain development and maturation.

Some studies have been devoted to their carotenoid bleaching activities during dough mixing (Hawthorn and Todd, 1955; Nicolas, 1978; Gelinas *et al.*, 1998). Based on studies in model solutions, it has been proposed that POD can promote in the dough, the oxidative gelation of pentosans (Geismann and Neukom, 1973, Izydorczyk *et al.*, 1990; Figueroa-Espinoza and Rouau, 1998) and / or the polymerization of proteins (Stahmann, 1977; Matheis and Whitaker, 1984). These effects could explain the improvement of the baking performance of wheat flour by the addition of POD from different origins (Kieffer *et al.*, 1981; Van Oort *et al.*, 1997). Nevertheless, the mechanism remains

unclear because of the questionnable formation of hydrogen peroxide during dough mixing. However, recently, Liao *et al.* (1998) claimed that during fermentation, yeast produced hydrogen peroxide which had an effect on the dough rheology.

4.2 POLYPHENOLOXIDASE (PPO)

Compared to lipoxygenase, POD and CAT, the PPO activity of wheat flour is weak (Honold and Stahmann, 1968; Lamkin *et al.*, 1981; Baik *et al.*, 1994; Park *et al.*, 1997). The activity of wheat is concentrated in the external parts of the grain (Marsh and Galliard, 1986; McCallum and Walker, 1990; Hatcher and Kruger, 1993, 1997).

PPO activity is mainly associated with problems of darkening in food products (Nicolas *et al.*, 1994; Nicolas and Potus, 1994). Only few studies have been devoted to the rheological effects of these enzymes in breadmaking. Using the Brabender extensograph, Kuninori *et al.* (1976) observed a strengthening effect of the dough when supplemented by an aqueous extract of mushroom (rich in PPO). Recently, the uses of laccase and of microbial POD have been patented for improving dough and baked products (Si, 1994a and b).

Two mechanisms can be postulated. The first involved oxidation of ferulic acid esterified to pentosan, leading to the gelation of this fraction (Geismann and Neukom, 1973; Neukom and Markwalder, 1978). The second assumed the polymerisation of proteins by oxidation of the tyrosyl residues or by conjugation between tyrosyl and cysteinyl residues (Matheis and Whitaker, 1984). Using model systems, Nishiyama *et al.* (1979) observed an addition reaction between SH groups and phenolic compounds oxidized by mushroom tyrosinase. A third mechanism has been proposed by Hoseney and Faubion (1981) who postulated a cross-linking between a protein thiyl radical and the ferulic acid esterified to the arabinoxylan fraction of pentosan.

4.3 ACID ASCORBIC OXIDASE AND DEHYDROASCORBATE REDUCTASE

The improving effect of ascorbic acid (AA) is mediated through its oxidation in dehydroascorbic acid (DHA). The latter compound is then able to oxidise two SH groups to form a disulfide bridge (Grosch and Wieser, 1999). Although an AA oxidase has been characterized in wheat flour by numerous authors (Kuninori and Matsumoto, 1963; Grant and Sood, 1980; Pfeilsticker and Roeung, 1980 and 1982; Every *et al.*, 1995 and 1996), the possibility of AA oxidation to DHA by other enzymes (Cherdkiatgumchai and Grant, 1986) or by non enzymatic reactions (Grant, 1974; Every *et al.*, 1995) cannot be ruled out. Moreover, Pfeilsticker and Roeung (1980) claimed that AA oxidase was highly specific for the L-AA, whereas recently, Every *et al.* (1995) indicated that the enzyme had a low specificity for all the stereoisomers of AA. The latter result is in agreement with the results of Elkabassany *et al.* (1980) who found that L-AA and D-AA were oxidized at the same rate in dough.

The reduction of DHA to AA with the concomitant formation of disulfide bonds from thiol compounds is undoubtedly catalyzed by an enzyme, at least when the SH

compound is the reduced form of glutathione (GSH) (Grosch, 1986; Sarwin *et al.*, 1993). Since its first description in wheat by Kuninori and Matsumoto (1964), only few data on the GSH-DHA oxidoreductase (EC.1.8.5.1) are available. Carter and Pace (1965) indicated that the enzyme is mainly concentrated in the germ. Kahnt *et al.* (1975) found that the enzyme was also largely present in flour and bran fractions of different wheat varieties. Boeck and Grosch (1976) have purified DHA reductase from wheat flour to homogeneity. According to these authors and Walther and Grosch (1987), the enzyme was specific for GSH as hydrogen donor and was inactive on cysteine and L-cysteinyl-glycine. Among the stereoisomers of DHA, L-*threo*-DHA was reduced faster than the D and L-*erythro*-DHA which in turn were reduced faster than the D-*threo*-DHA. Recently using a purified fraction of the enzyme, Kaïd *et al.* (1997) obtained a pH optimum close to 7.5, and proposed a sequential mechanism where the enzyme fixed first DHA and then GSH. They also indicated that cysteine and other thiols caused a large activation of the enzymatic formation of ascorbic acid suggesting coupled oxidation of these thiols in agreement with Kieffer *et al.* (1990).

4.4 PROTEIN DISULFIDE ISOMERASE

Numerous authors postulated that SH-disulfide exchange reactions were important to the dough rheological properties (Grosch, 1986). The existence of such reactions were first demonstrated by McDermott and Pace (1961) using thiolated gelatin and later by other workers using radioactive tracers (Redman and Ewart, 1967; Kuninori and Sullivan, 1968; Lee and Lai, 1968).

The involvement of an enzyme catalyzing the exchange is not certain since according to Kuninori and Sullivan (1968), the incorporation of $G^{35}SH$ in dough proteins was not affected by a heat treatment of the flour. However, Grynberg *et al.* (1977, 1978) reported the presence in the germ and endosperm of wheat, of an enzyme which was able to reactivate ribonuclease which had randomly cross-linked disulfide bridges. This enzyme exhibited an optimal activity at pH 7.8 and 35°C and could use GSH as cofactor.

4.5 GLUCOSE OXIDASE AND SULFHYDRYL OXIDASE

Both glucose oxidase and sulfhydryl oxidase use molecular oxygen and produce hydrogen peroxide. Glucose oxidase acts on β-D-glucose which is oxidized in δ-gluconolactone whereas sulfhydryl oxidase acts on thiol groups (GSH is often the best substrate) to form disulfide bridges (Swaisgood, 1980). Contrary to the preceeding enzymatic systems, these two enzymes have never been characterized in wheat flour. Although their uses as ingredients in breadmaking in conjunction with hemicellulases have been patented (Haarisilta *et al.*, 1991; Maat and Roza, 1990; Fok *et al.*, 1994), very few publications are available on their effects in breadmaking (Martinez-Anaya and Jimenez, 1998; Wikstrom and Eliasson, 1998; Vemulapalli *et al.*, 1998). In the above patents, it was claimed that glucose and sulfhydryl oxidases in addition with CAT and hemicellulases improved both the handling properties of dough and the volume and texture of bread. However, since the enzymatic extracts have not been fully

characterized, the activity (or the activities when a synergy is proposed) responsible for the improvement cannot be clearly identified. Nevertheless, it is probable that the hydrogen peroxide produced during catalysis could explain the observed effect by activating the POD system. In addition, a direct effect of sulfhydryl oxidase on the SH groups of dough to form disulfide bridges cannot be ruled out. However, Kaufman and Fennema (1987) indicated that sulfhydryl oxidase was unable to act as a strengthening agent for wheat flour dough. Recently, Poulsen and Bak Hostrup (1998) claimed that hexose oxidase (EC 1.1.3.5) was more efficient than glucose oxidase (EC 1.1.3.4) for improving dough and bread characteristics. This enzyme isolated from red seaweed (*Chondrus crispus*) has a broader specificity since it produces hydrogen peroxide not only from glucose but also from galactose and maltose.

5 INTERRELATIONSHIPS BETWEEN LIPOXYGENASE AND SOME OTHER OXIDOREDUCING ENZYMES

The great number of oxidoreduction reactions occuring during mixing as well as the effects of redox enzymes have been discussed. In many cases if not all, the improving effects of these enzymes in breadmaking are related to their catalytic properties which either enhance crosslinking reactions between gluten proteins and / or pentosans or decrease the amount of small thiol compounds (GSH, cysteine,...) which participate to thiol-disulfide exchange reactions leading to a weakening of gluten.

A tentative overall scheme for the enzyme-catalyzed redox reactions and their interrelationships can be drawn (Fig. 1). LOX, situated at the very center, catalyzes the PUFA oxidation to yield hydroperoxides with intermediate free radicals. These highly reaction products are able to co-oxidize several substances including SH groups as well as lipophilic pigments and vitamins.

Hematin containing substances, including POD and CAT, catalyze also the oxidation of unsaturated lipids but with less specificity and at a much lower rate than LOX. Since these two enzymes destroy hydrogen peroxide, a strong inhibitor of LOX (Mitsuda *et al.*, 1967), they enhance the LOX effect. Moreover, POD, in the presence of hydrogen peroxide is able to catalyze the crosslinking of pentosans and / or proteins.

This is also true for PPO since it oxidizes phenolic compounds and promotes their polymerization. Moreover, through their antioxidant properties, polyphenols are also potent inhibitors of LOX (Richard-Forget *et al.*, 1995). However, this enzyme competes with LOX for oxygen consumption. The enzymatic and non-enzymatic oxidations of ascorbic acid also participate in this competition for the available oxygen (Nicolas *et al.*, 1980). Furthermore, ascorbic acid is an inhibitor of LOX (Walsh *et al.*, 1970; Nicolas, 1978). The DHA formed is reduced back to ascorbic acid by a DHA reductase in the presence of GSH which is oxidized, resulting in disulfide bridge formation (Boeck and Grosch, 1976; Kaïd *et al.*, 1997).

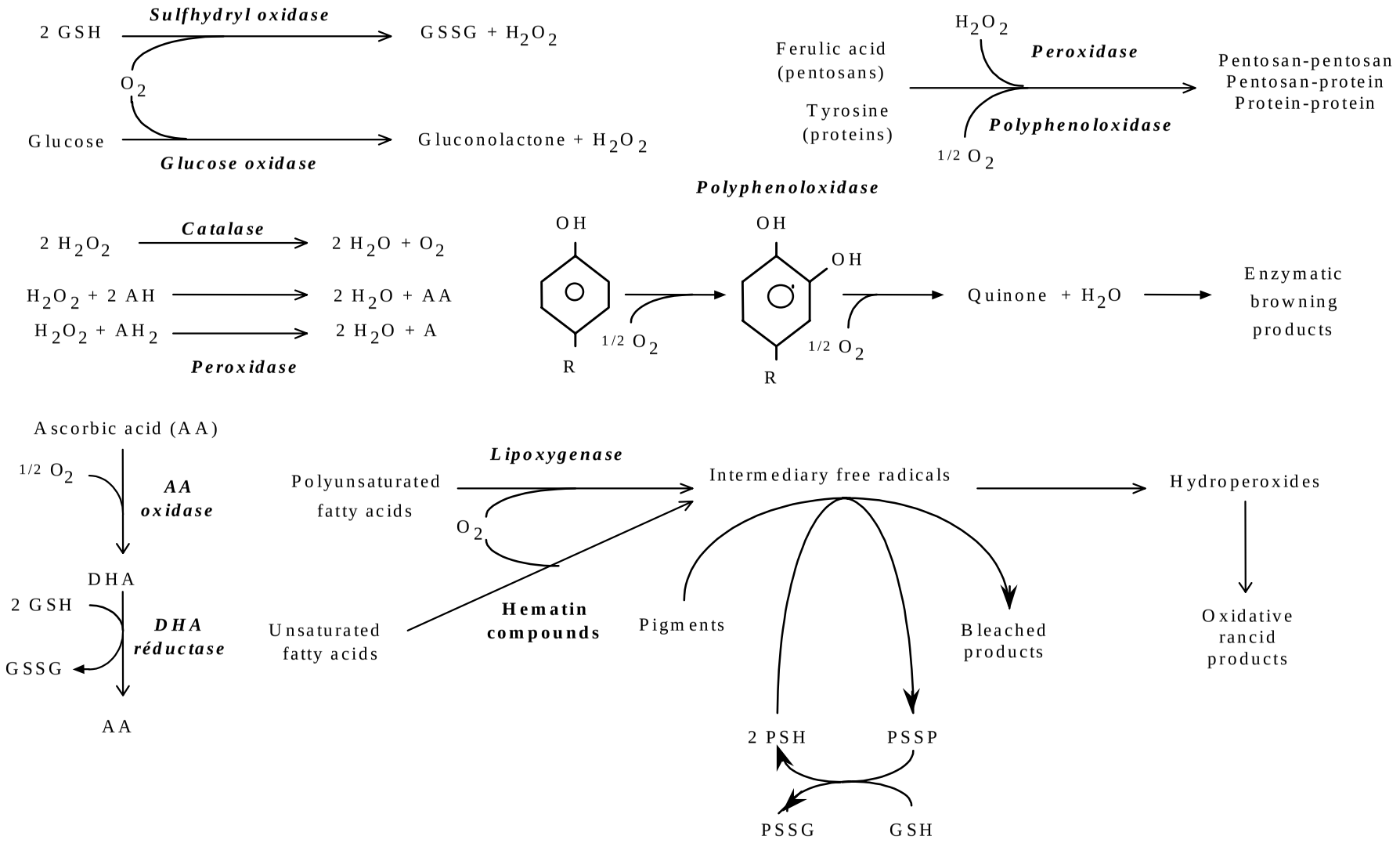

Figure 1. General scheme of the different oxidoreducing systems involved in breadmaking.

Glucose and sulfhydryl oxidases, when added to the dough, compete also for the available oxygen. In addition, they form hydrogen peroxide and therefore activate the POD-catalyzed crosslinking reactions.

Lastly, protein disulfide isomerase also influences the SH-disulfide group interchange which plays a prominent part in the dough rheological properties (Grosch, 1986).

Concerning the uses of redox enzymes in breadmaking, two facts must be recalled. First, special care must be taken when the *in vitro* results obtained for the enzymatic reactions in model systems, are applied to the *in vivo* phenomena observed during dough mixing. The dough conditions are entirely different since the water, and sometimes even the substrates, are present in limited amounts. The particular hydrophobic environment, due to the presence of gluten, might also modify the reaction mechanism. Such a phenomenon was observed for LOX by Graveland (1970a). Also concerning LOX, Pourplanche *et al.* (1992 and 1994) have shown that addition of polyols and sugars to aqueous media resulted in a modification of both the enzyme activity and stereospecificity. Similarly for other enzymes, Rothfus and Kennel (1970) noticed that wheat β-amylase gave an insoluble complex with glutenin and this non-reversible adsorption due to hydrophobic bonding did not alter the kinetic properties of the enzyme but the optimum of activity was shifted from pH 5.5 for the free enzyme to pH 4.5 for the bond one. Wang and Grant (1969) found also that the proteolytic activity of an aqueous wheat flour suspension was higher than that of an aqueous flour extract with a pH optimum shift from 4.4 to 3.8. Recently, Delcros *et al.* (1997) found that LOX and CAT activities were partly lost during mixing whereas POD activity remains unaffected. Moreover, the amounts of LOX and CAT losses were highly dependant on the mixing conditions and on the addition of exogenous oxidoreductases (Rakotozafy *et al.*, 1998).

Secondly, in the absence of added chemical oxidants, oxygen is the only oxidant present for redox enzymes and yeast. Therefore during mixing, a competition among the different redox systems does exist for the available oxygen which unavoidably becomes rapidly limitant. In wheat dough without additives, LOX probably plays a center role for the use of oxygen (together with yeast). Conversely, adding exogenous oxidoreducing enzymes using oxygen, results in a modified balance of the importance of the different redox systems. One must keep in mind that the improving effects on the rheological properties of dough as well as on the bread characteristics will be clearly understood only when the relative importance of the different redox systems will be determined and their targets in dough precisely identified.

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OXIDATION BY GALACTOSE OXIDASE OF ENZYMATICALLY MODIFIED ARABINOGALACTAN-PEPTIDE FOR IMPROVEMENT OF WHEAT FLOUR DOUGH AND BREAD

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1 INTRODUCTION

In the baking industry, many conditioners have been used in order to strengthen the doughs obtained from weak flours, with the aim to provide improved rheological and handling properties and superior volume and texture of the finished baked products. The role of such conditioners is to oxidize the dough, resulting in an increased transformation of sulfhydryl groups of the gluten into disulphide bonds whereby forming a more stable protein matrix. The chemical non-specific oxidizing agents are often objected to by consumers or are not permitted by regulatory bodies. Enzymic oxidants represent an interesting alternative to the conventional flour and dough additives. Glucose oxidase is the most frequently used for bread production. However, it has some limitations because it depends on the presence of glucose in the dough. In fact, the availability of glucose fast decreases after the beginning of the breadmaking process since it is consumed by yeasts. Therefore, the oxidative effect is strong only during mixing.

The aim of this study was to find another enzymatic system which could use a substrate naturally occurring in flours while providing a controlled and lasting oxidation along the breadmaking process [1, 2].

Galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9.), an enzyme secreted by certain filamentous fungi, is capable of oxidizing D-galactose and different substrates containing terminal galactose. The reaction is accompanied by a reduction of molecular oxygen and release of hydrogen peroxide. This is a very stable enzyme with respect to pH, heat and protease treatment. However, the natural content of galactose or oxidizable substrate for galactose oxidase in cereal flours is very low (typically 0.001 to 0.01 wt%). In this work, the possibility to use arabinogalactan-peptide (We-AGP, 0.1 to 0.3% flour), *via* a preliminary enzymatic modification, as a source of oxidizable substrate for galactose oxidase, was investigated.

2 MATERIALS AND METHODS

2.1 ENZYMES

Grindamyl S100 and several commercial or experimental enzyme preparations from *Aspergillus niger* were obtained from Danisco Ingredients (Brabrand, Denmark), an α-L-arabinofuranosidase from Megazyme (Wicklow, Ireland) and a β-galactosidase from Sigma (St Louis, USA). Galactose oxidase was purified from a crude commercial preparation from *Dactylium dendroides* (Sigma) by desalting, ion exchange and hydrophobic interaction chromatographies. The purified enzyme presented a single band after SDS-PAGE gel silver staining with an approximate molecular weight of 69 kDa. An (1-6)-β-D-galactanase was purified from p70 by desalting, ion exchange and two steps of hydrophobic interaction chromatographies. The purified enzyme was homogenous in SDS-PAGE and presented neither arabinofuranosidase nor β-galactosidase activities.

2.2 ABTS ASSAY FOR GALACTOSE OXIDASE

An ABTS reagent (6 mg in 10 mL Na-phosphate buffer, pH 6.4) was mixed with 50 U of galactose oxidase and 20 U of peroxidase, up to a volume of 25 mL. 800 µL of the reagent was mixed with 50 µL of substrate for 30 min at room temperature then read at 420 nm. Enzyme-treated We-AGP (by p70, α-L-arabinofuranosidase, β-galactosidase, (1-6)-β-D-galactanase), galactobiose (6-O-β-D-galactopyranosyl-D-galactose) or galactose were used as substrates.

2.3 ISOLATION, DEPOLYMERISATION AND OXIDATION OF WE-AGP

We-AGP was isolated from Thesee flour by water extraction, heating and ethanol precipitation between 60% and 80% (v/v). Enzyme hydrolyses of We-AGP were performed at room temperature at pH 5.0 and stopped by boiling. Reactions on We-AGP were followed concomitantly with oxidation by galactose oxidase as measured spectrophotometrically (420 nm) using ABTS reagent.

2.4 CHROMATOGRAPHY

Enzyme-treated We-AGP was subjected to gel permeation chromatography on a Superdex 75 HR 10/30column (Pharmacia) and HPLC on a Polyspher CHCA column (Merck) in which polysaccharides, oligosaccharides and monosaccharides were separated at 85°C. The detection was using a refractometric detector.

2.5 DETERMINATION OF THIOL GROUPS

50% Isopropanol/80mM Tris HCl pH 8.5 (1.5 mL) was added to flour or freeze-dried dough (150 mg). DNTB (50 µL) was then added and the reaction took place in the dark

for 10 min under continuous shaking. After centrifugation (14,000 rpm, 5 min) the absorbance was read at 412 nm.

2.6 BAKING TEST

Flour (40 g), dry baker yeast (300 mg), 4.4% NaCl solution (20 mL) and enzyme solution or water (4 mL) were mixed in a mixograph for 5 min and allowed to rest for 5 min before dividing into 3 round pieces of 15 g each. First fermentation was carried out for 30 min at 25°C in 85% humidity in a fermentation chamber. The dough pieces were then shaped, moulded and placed in pans before second fermentation (2.5 h, 25°C). Stickiness was evaluated subjectively on doughs 40 min after the beginning of the breadmaking process. Two breads were baked for 7 min at 250°C while the third dough piece served for biochemical analyses.

3 RESULTS AND DISCUSSIONS

3.1 SCREENING FOR ENZYME CAPABLE OF PRODUCING SUBSTRATE FOR GALACTOSE OXIDASE FROM WE-AGP

The composition of the We-AGP preparation was as follows : protein 37.2%, arabinose 17.6%, galactose 22.8%, xylose 1.9%. Purified We-AGP was not substrate for galactose oxidase as measured by the ABTS assay. Several enzymes preparations from *Aspergillus niger* were incubated with We-AGP. P70, a crude ferment containing several enzyme activities, yielded oxidizable substrate for galactose oxidase. No synergistic effect was obtained by combination of p70 with other tested non-starch polysaccharide degrading enzymes. The optimum pH for the production of substrate by p70 was relatively broad, between pH 4 and pH 6.

3.2 DEPOLYMERIZATION OF WE-AGP

The depolymerization of We-AGP by p70 was followed by SE-HPLC. A progressive loss in high molecular weight compounds was observed on behalf of low molecular weight material. After 18 h of incubation, almost all polymeric material has been degraded.

Galactobiose, galactose and arabinose were produced during the hydrolysis course (Fig. 1a). Arabinose was first released at a high rate (83% of total We-AGP arabinose) whereas galactose was released continuously (24% of total We-AGP galactose). Galactobiose was produced at the beginning of incubation then it was hydrolyzed into galactose. Finally, only 4% of initial We-AGP material was resistant to degradation by p70.

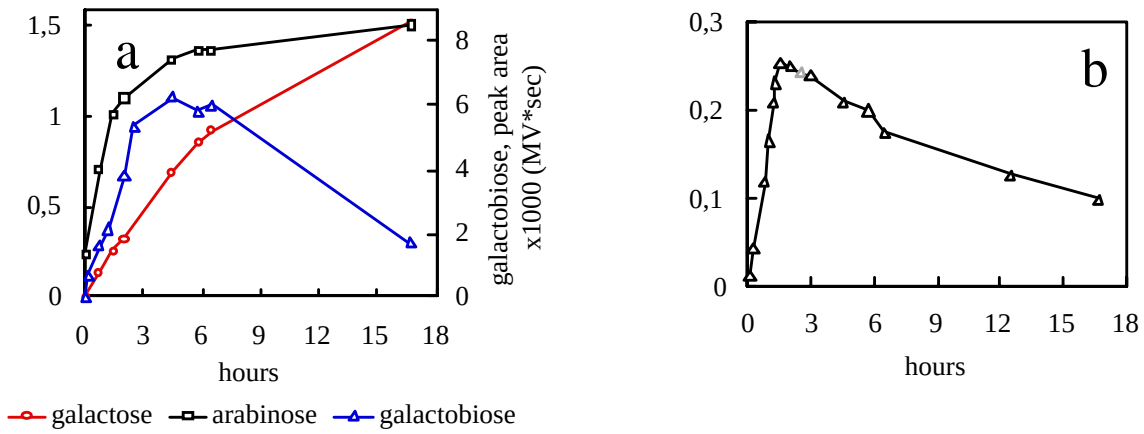

Fig. 1. The release of arabinose, galactobiose and galactose from We-AGP during incubation with p70 (a) and the corresponding production of substrate for galactose oxidase (b).

3.3 Oxidation by galactose oxidase of depolymerization products from We-AGP

The optimal yield in galactose oxidase-ABTS positive substrate was obtained at the beginning of the incubation course of We-AGP with p70, when most of the arabinose was released but a relatively low level of galactose with high amount of galactobiose (Fig. 1b). Further incubation with p70 decreased the yield in oxidizable substrate. On a same galactose equivalent basis, arabinofuranosidase-treated We-AGP was a substrate for galactose oxidase at least 3 times as good as galactose, the latter being itself slightly better than galactobiose. On a molar basis, galactobiose was clearly more favorable than galactose.

3.4 Effect of selected enzymes on oxidation

P70 was fractionated by different chromatography steps which allowed to identified major enzymatic components, namely α-L-arabinofuranosidase, (1-6)-β-D-galactanase and β-galactosidase. Among these three activities, oxidation was observed only when We-AGP was treated by arabinofuranosidase whereas galactanase and galactosidase did not improve the yield in oxidizable substrate (Fig. 2). No synergistic effects were observed with combinations of these three enzymes.

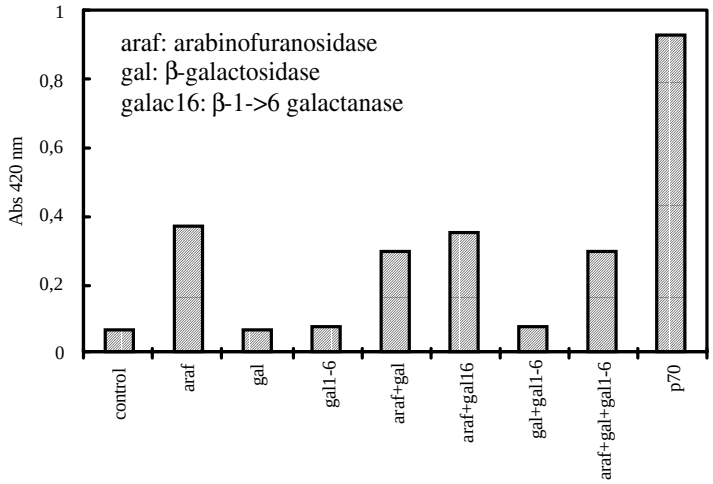

Fig. 2. Incubation of We-AGP with different enzymes foundin p70 followed by oxidation by galactose oxidase.

However, the oxidation of We-AGP was approximately 3 times higher when p70 was used instead of arabinofuranosidase (at a same level of arabinofuranosidase activity), indicating that additional activities to α-L-arabinofuranosidase, but different from (1-6)-β-D-galactanase and β-galactosidase, can improve the transformation of We-AGP into an oxidizable substrate for galactose oxidase.

3.5 EFFECT OF THE GALACTOSE OXIDASE SYSTEM ON THIOL GROUPS

Pure galactose oxidase, alone or in combination with p70, was applied to a model dough system. This experiment showed a significant decrease in thiol groups which was proportionate to the amount of galactose oxidase added (up to 50% decrease of free thiol groups for high levels). This result indicates that substrate is available for galactose oxidase in dough even in absence of added We-AGP degrading enzymes (explained by endogenous enzymes ? other galactose-containing substrate ?). However, when p70 was added, the loss in thiol content was much increased (a total disappearence of free thiol groups was observed for high enzyme levels).

3.6 BAKING TESTS

Small scale baking tests were carried out where especially bread loaf volume and dough stickiness were determined (Table I). The addition of galactose oxidase alone had no effect on dough quality and specific bread volume. P70 alone increased loaf volume but rendered the dough extremely sticky. Combining galactose oxidase and p70 kept the volume increase while correcting the dough stickiness. Similar results were obtained on a large panel of flours. When galactose oxidase was compared to glucose oxidase in the presence of an excess of a bread improving hemicellulase preparation, similar increases in specific loaf volume were obtained, but the dough with galactose oxidase was judged less sticky.

Table I. Effect on specific loaf volumes and dough stickiness of different enzyme combinations applied to the breadmaking of Thesee flour.

enzymes	specific volume increase (%)	stickiness	enzymes	specific volume increase (%)	stickiness
galactose oxidase 150 U	0		Grindamyl S100 500ppm	19	++++
p70 250 μL	16	+++			
p70 250 μL + galactose oxidase 150 U	18				
p70 250 μL + galactose oxidase 225 U	16		Grindamyl S100 500ppm galactose oxidase 150 U	20	+
p70 250 μL + galactose oxidase 113 U	17	++			
p70 375 μL + galactose oxidase 225 U	18				
p70 125 μL + galactose oxidase 75 U	14	+			
p70 375 μL + galactose oxidase 338 U	21	+	Grindamyl S100 500ppm glucose oxidase 100ppm	22	++

4 CONCLUSIONS

The possibility to use a new oxidizing system based on galactose oxidase, for dough and bread improvement, alternative to chemicals and glucose oxidase, was demonstrated in this study. The originality of the system lies in the possibility to control the oxidation at two stages : (i) the rate of production of available substrate by the level of We-AGP depolymerizing enzymes, (ii) the rate of product oxidation by the level of galactose oxidase. Intact flour We-AGP has not been reported in literature to exhibit marked functional properties in breadmaking, unlike arabinoxylans for example. On the other hand, de-arabinosylated We-AGP has proved here to be an excellent substrate for galactose oxidase. However, some questions raised in this study remain to be elucidated, for example : what enzymes are responsible in p70 for the increased yield in oxidizable substrate produced from We-AGP compared to only arabinosidase ? what substrate is used in the flour by galactose oxidase in the absence of added enzymes able of modifying We-AGP ?

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ON THE MECHANISM OF ACTION OF PEROXIDASE IN WHEAT DOUGH

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1 INTRODUCTION

In bread, addition of peroxidase in combination with xylanase has been shown to have a beneficial effect on dough handling properties and on the quality of the final baked product (Van Oort, 1996). It has been suggested (van Oort, 1996) that peroxidases cross-link the arabinoxylans that are liberated by xylanase, via ferulic acid moieties to other arabinoxylans. *In vitro* studies have shown that peroxidase-catalyzed coupling via ferulic acids is feasible (Geissman and Neukom, 1973), as well as the formation of tyrosine dimers (Gross and Sizer, 1959). It has also been suggested that peroxidases can cross-link arabinoxylans to proteins (Neukom, 1976, Matheis and Whitaker, 1984, 1987), but attempt to achieve this *in vitro* have failed (Figueroa and Rouau, 1998).

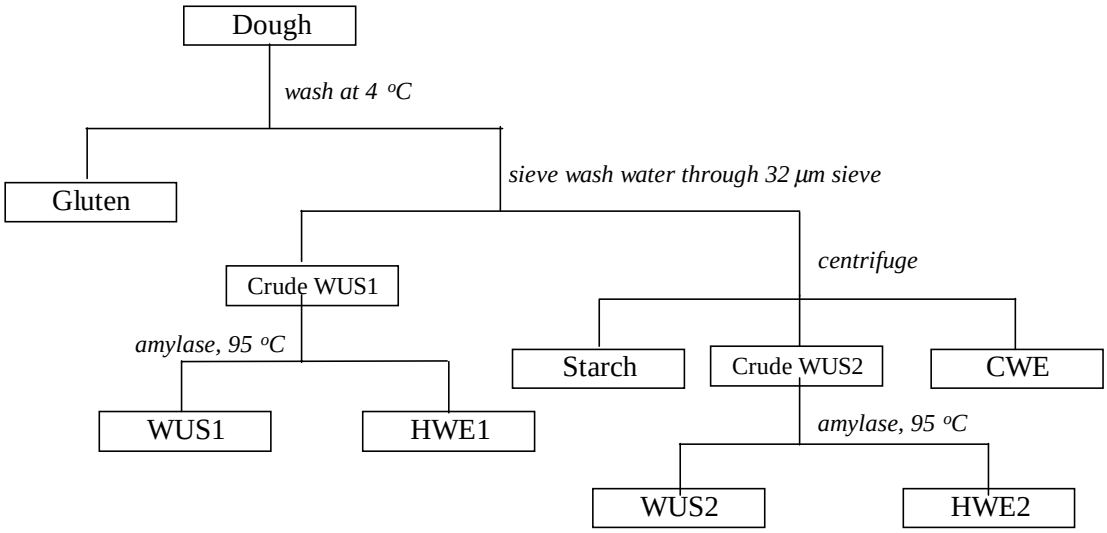
Recently, we have shown that peroxidase does not affect the properties of gluten (Hilhorst *et al*., 1999a), but that peroxidase exerts its positive action most likely by influencing the properties of other fractions in dough. Here we describe which dough fractions are most affected by peroxidase.

2 MATERIALS AND METHODS

Dough's were prepared with 2000 g Kolibri flour as is (Meneba Meel B.V. Rotterdam) 1180 g water, 40 g NaCl, 80 g yeast ("Koningsgist" from Gist-brocades, Delft, NL), 0.04 g ascorbic acid, 0.1 g α-amylase (Biobake 5000, 175 FFA/mg from Quest International BV. Naarden NL) and 0.6 g xylanase ex *Trichoderma* (Biobake CX160, 351 U/mg from Quest International). XYL/POX dough was supplemented with 4.4 mg soy peroxidase (Biobake soy, 35 U/mg from Quest International). Dough's were mixed in a Kemper SP 15 spiral mixer (Neuenkircher Eisengiesserei und Machinenfabrik, Rietberg, Germany), at 300 slow turns and 800 fast turns and frozen. After thawing of the dough's overnight at 4 ^{o}C, they were fractionated into seven fractions according to Scheme 1 as described by Gruppen *et al*. (1989) with minor modifications (Hilhorst *et al*., 1999b). After concentration, CWE, HWE1 and HWE2 were dialyzed extensively against distilled water prior to freeze drying and subsequent grinding.

Analyses for starch, protein, sugar composition and ferulic acid were performed as described in Hilhorst *et al*. (1999a and 1999b).

To investigate the nature of cross-links present, HWE2 and WUS2 underwent three treatments: i) control (5 mg /ml was incubated overnight at room temperature with 0.1 M sodium acetate buffer pH 6.5), ii) alkali (10 mg/ml was incubated overnight at room temperature in 0.5 N KOH) or iii) protease (5 mg/ml was incubated overnight at room temperature with trypsin (Sigma) (0.005 mg /mg) in 0.1 M sodium acetate, pH 6.5). After neutralization with an equal volume acetic acid (KOH incubation), or inactivation in a boiling water bath for 5 min (trypsin incubation), the insoluble material was separated by centrifugation. In the supernatants, the protein and ferulic acid content and the sugar composition were determined. The molecular weight distribution was investigated by High Performance Size Exclusion Chromatography (HPSEC) as described in Hilhorst *et al*. (1999b).



Scheme 1. Fractionation of the dough's.

3 RESULTS

Fractionation of XYL and XYL/POX dough yielded the insoluble fractions Gluten, Starch, WUS1 (large cell wall fragments) and WUS2 (small cell wall fragments), and the soluble fractions CWE (cold water extract), HWE1 (hot water extract from WUS1) and HWE2 (hot water extract from WUS2). The yields and chemical compositions of all fractions were determined. Addition of peroxidase led to an increase in weight, non-starch polysaccharides, protein and ferulic acid in the insoluble fractions.

Comparison of the composition of the individual fractions revealed the largest differences in the HWE2 and WUS2 fractions, whereas WUS1, HWE1 and gluten were hardly affected. Both in HWE2 and WUS2, the proportion of arabinose and xylose increased. In WUS2 the relative contribution of protein had decreased, but the absolute amount had increased. Peroxidase treatment had lowered the ara/xy ratio from 0.83 in XYL WUS2 to 0.63 of XYL/POX WUS2. Apparently, arabinoxylans with a low degree of substitution were added to WUS2 by peroxidase treatment. Such arabinoxylans with a low degree of substitution are present in CWE and HWE2.

Table 1. The composition of HWE2 and WUS2 from XYL and XYL/POX dough.

	HWE2		WUS2	
	XYL dough	XYL/POX dough	XYL dough	XYL/POX dough
Yield (g)	3.2	1.5	1.8	3.0
Protein (%)	12	20	42	35
AXG* (%)	6.7	9.7	6.5	21
Phenolic acid (%)	0.18	0.18	0.09	0.10

* AXG: TOTAL OF ARABINOSE, XYLOSE AND GALACTOSE.

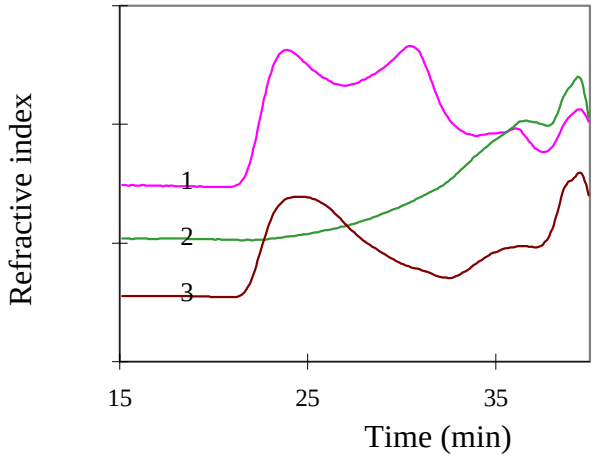


Fig. 1. HPSEC elution pattern with refractive index detection of HWE2 from flour (1), XYL (2) and XYL/POX dough (3).

When the size distribution in HWE2 was analyzed by HPSEC, HWE2 from XYL dough was found to contain mainly low molecular weight arabinoxylans (MW < 50 kDA), whereas HWE2 from XYL-POX dough contained high MW arabinoxylans (> 1000 kDa). Arabinoxylans of the same size were present in the equivalent fraction isolated from wheat flour (Fig.1, trace 1).

To test the hypothesis that peroxidase added arabinoxylans with a low degree of substitution to WUS2 and that these are cross-linked via the ferulic acids, the fractions were incubated with alkali. To release protein that could have been cross-linked to arabinoxylans, the fractions were also incubated with protease. Chemical analysis of the solubilized material showed that alkali released more arabinose and xylose (ara/xy ratio 0.63), and somewhat more protein from XYL/POX WUS2 (Fig. 3B) than from XYL WUS2 (Fig. 3A). The ara/xy ratio for material solubilized from XYL WUS2 was 0.94. This supports the idea that material from HWE2 was added to WUS2.

Analysis of the size distribution (Fig. 4) revealed that material the material released from XYL/POX WUS2 had a high molecular weight (>1000 kDa, RT 22 min). The molecular weight of the material released from XYL/WUS2 was approximately 40 kDa.

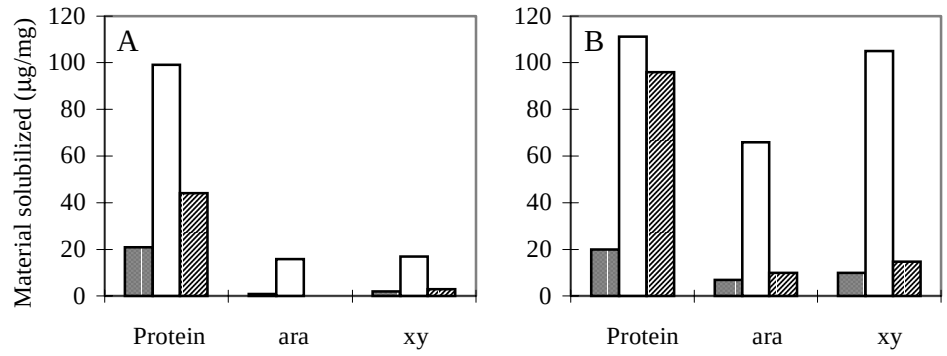


Fig. 2. Amount of protein, arabinose (ara) and xylose (xy) solubilized from WUS2 from XYL dough (A) or from XYL/POX dough (B) after treatment with buffer (grey bar), alkali (white bar) or protease (shaded bar).

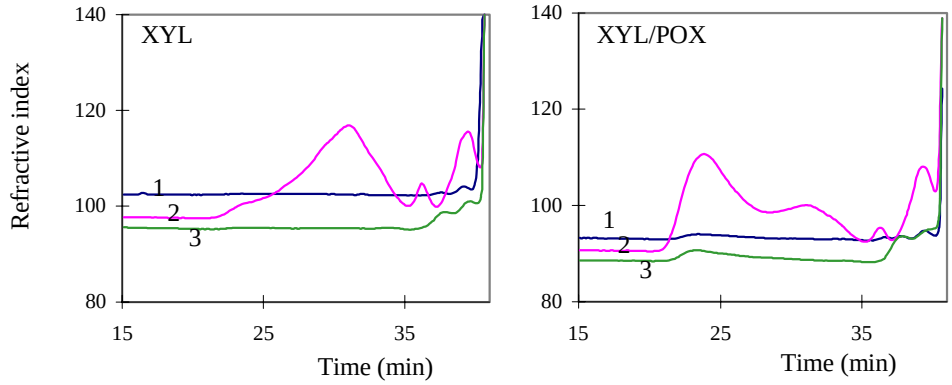


Fig. 3. Hpsec elution patterns with refractive index detection of wus2 from xyl and xyl/pox dough after incubation with sodium acetate buffer (1), alkali (2) or protease (3).

On the basis of these results we conclude that peroxidase acts by cross-linking arabinoxylans via ferulic acids. The cross-linked material becomes insoluble and is isolated as WUS2. Previously we have shown that peroxidase hardly affects gluten properties (Hilhorst, 1999a). The present results confirm this, and show that peroxidase exerts its action in dough by influencing the properties of other fractions, notably WUS2 and HWE2.

4 ACKNOWLEDGEMENTS

This research was supported financially by the Ministry of Economic Affairs, under contract no BIO95030.

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RISK ASSESSMENT STUDIES IN THE CULTIVATION OF TRANSGENIC BARLEY

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ABSTRACT

Molecular breeding is becoming a useful tool in the improvement of plants and plant-based raw materials. Risk assessment studies are needed for the safe commercial use of transgenic plants. Risk assessment for the cultivation of transgenic barley was performed in 1996 and repeated in a larger scale in 1997 in order to provide information on pollen-mediated dispersal of transgenes via cross-fertilization. A transgenic barley line carrying a marker gene coding for neomycin phospho-transferase II (*nptII*) was used as a pollen donor. For maximum resolution a cytoplasmically male-sterile barley line was utilized as recipient and the flow of *nptII* transgene was monitored at distances of 1, 2, 3, 6, 12, 25, 50 and 100 meters from the donor plots of 225 m^2 and 2000 m^2.

In Finnish conditions, the cross-fertilization frequency was found to be low. In male-sterile recipient barley the seed-set was low. In normal male-fertile barley the cross-fertilization frequency varied from 0% to 7%, depending on weather conditions on the heading day. Furthermore, in male-sterile barley the cross-fertilization frequency diminished rapidly with distance and only a few seeds were found at distances of 50 and 100 m. The molecular biological analysis of male-sterile heads revealed that all the seeds at a distance of 1 m were transgenic, but only 3% of the distant seeds (50 m) were transgenic. Due to competing self-produced and non-transgenic background pollen the corresponding figure will be even lower in normal recipient barley.

1 INTRODUCTION

Since 1980, plant genetic engineering has developed and given rise to practical applications. For successful transformation, both cell and tissue culture are needed. Thus gene transfer techniques and tissue culture systems have developed in parallel. The first transgenic barley plants were produced by particle bombardment (Jähne *et al.*, 1994, Ritala *et al.*, 1994, Wan and Lemaux 1994, Hagio *et al.*, 1995). Later on other techniques have also resulted in transgenic barley plants (Funatsuki *et al.*, 1995, Salmenkallio-Marttila *et al.*, 1995, Tingay *et al.*, 1997, Matthews *et al.*, 1997). The use of shoot meristematic cultures derived from germinated dry seeds appears very promising for providing target material for gene transfers (Zhang *et al.*, 1999). However, the most reproducible method has been the bombardment of *Golden Promise* embryos (Wan and Lemaux 1994). The majority of gene transfers aiming at commercial

applications have been carried out using this method (e.g. Jensen *et al.*, 1996, Nuutila *et al.*, 1999).

In general, the risk assessment of transgenic plants should cover the whole area from research and production of transgenic plants to cultivation, processing and use as nutrition. General ecological factors not specifically connected with a particular transgene deserve attention in the first stage. In the risk assessment the potential gene flow is crucial (Eucarpia 1989). Much knowledge is already available in the literature on gene flow in traditional breeding. For example, isolation distances applied successfully in breeding of cultivated species have been composed on the basis of these early studies (e.g. Bateman 1947). However, in order to estimate transgene flow in local cultivation conditions, further studies using modern methods with high resolution are needed. Essential factors that need to be studied in the cultivation of transgenic barley are gene flow between barley fields, survival of the seeds in the field after cultivation, occurrence of established natural populations, and exchange of genetic material with weeds or wild species through hybridization. In this study we concentrated on pollen-mediated dispersal of transgenes via cross-fertilization. The risks of seed dispersal through animal feeding and harvest were not estimated.

2 MATERIALS AND METHODS

2.1 PRODUCTION OF TRANSGENIC BARLEY PLANTS

Microspore culture-derived protoplasts of barley (*Hordeum vulgare* L. cv. *Kymppi*) were transformed by electroporation with the construct pHTT303 (provided by T. H. Teeri, University of Helsinki) carrying the gene coding for neomycin phospho-transferase II (*nptII*). The regenerated plants and their progeny were analyzed by NPTII activity assays and Southern blot hybridization. These confirmed the genomic integration of the transferred gene and that the new trait was inherited by the progeny (Salmenkallio-Marttila *et al.*, 1995).

2.2 EXPERIMENTAL SET-UP FOR RISK ASSESSMENT IN THE CULTIVATION OF TRANSGENIC BARLEY

The experimental set-ups of the field trials of the summers of 1996 and 1997 are presented in Figure 1. The transgenic line carrying the gene coding for neomycin phosphotransferase (*nptII*) was used as a source of homozygous donor pollen (Salmenkallio-Marttila *et al.*, 1995). In addition to the small scale donor plots (225 m^2), a donor area of 2000 m^2 was used during the summer of 1997. A cytoplasmically male-sterile barley line (*Hordeum vulgare* L. cv. *Agneta*, provided by G. Persson, Svalöf-Weibull) was sown as recipient. During the flowering, heads reaching the opening stage were marked daily with colored tapes and in the autumn the heads were collected separately. The resulting seeds were counted individually in the laboratory and the presence of transgene was analyzed by PCR (details in Tammisola *et al.*, manuscript). Detailed weather measurements were continuously recorded at the plots during the experiments.

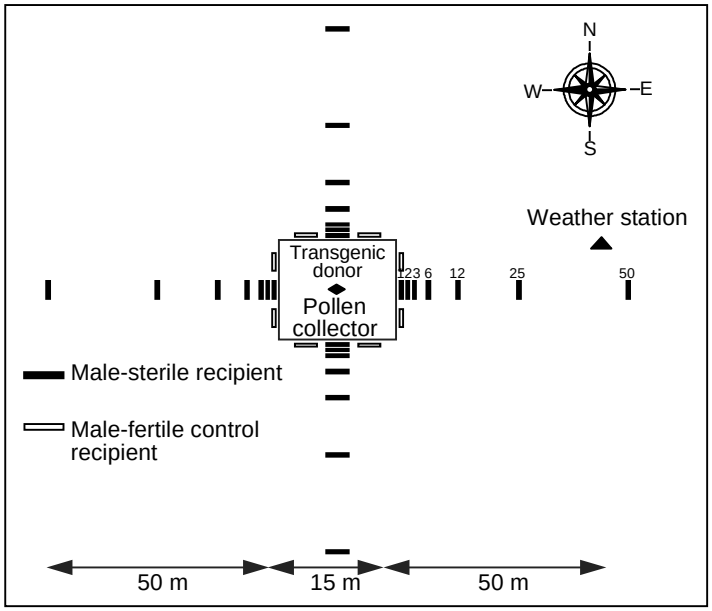

Figure 1. Risk assessment in the cultivation of transgenic barley. Experimental set-up of the field trials in Boreal Plant Breeding, Jokioinen, Finland, in 1996 and 1997.

3 RESULTS

In general, the flow of transgenes is possible through pollen or seed dispersal. The spread of transgenes through pollen flight involves crossing with cultivated barley or with wild relatives. Barley is considered to be a self-pollinating species and in Finland there are no wild relatives with which transgenic barley could cross. Nevertheless, there is a slight possibility of cross-pollination between adjacent barley fields. Our aim was to evaluate the distribution of viable pollen and the consequent potential for transgene flow through cross-fertilization in cultivation scale. The parameters for seed dispersal were not estimated within this study.

The preliminary results from the experimental scale trials of 1996 showed that pollen dispersal was possible up to 50 meters into the dominant wind direction. The applicability of the preliminary model was studied on the basis of the results of scaling up to a cultivation trial in 1997 (Figure 2). The seed-set in the male-sterile recipient diminished dramatically with distance. In the large scale experiment only a few seeds were found at distances of 50 and 100 m. A certain proportion of these seeds originated from contaminating non-transgenic background pollen. The PCR analysis of male-sterile heads revealed that all the seeds at a distance of 1 m were transgenic, but that only 3% of the distant seeds (50 m) were transgenic (Figure 3). The few seeds obtained at 100 m did not germinate and therefore the PCR analysis could not be performed. The non-transgenic pollen is mainly due to leakage of the male-sterility, i.e. occasional formation of pollen in some male-sterile heads. Furthermore the male-fertile plots produced some non-transgenic pollen. In theory, it is also possible that some pollen might have originated from a more distant barley field.

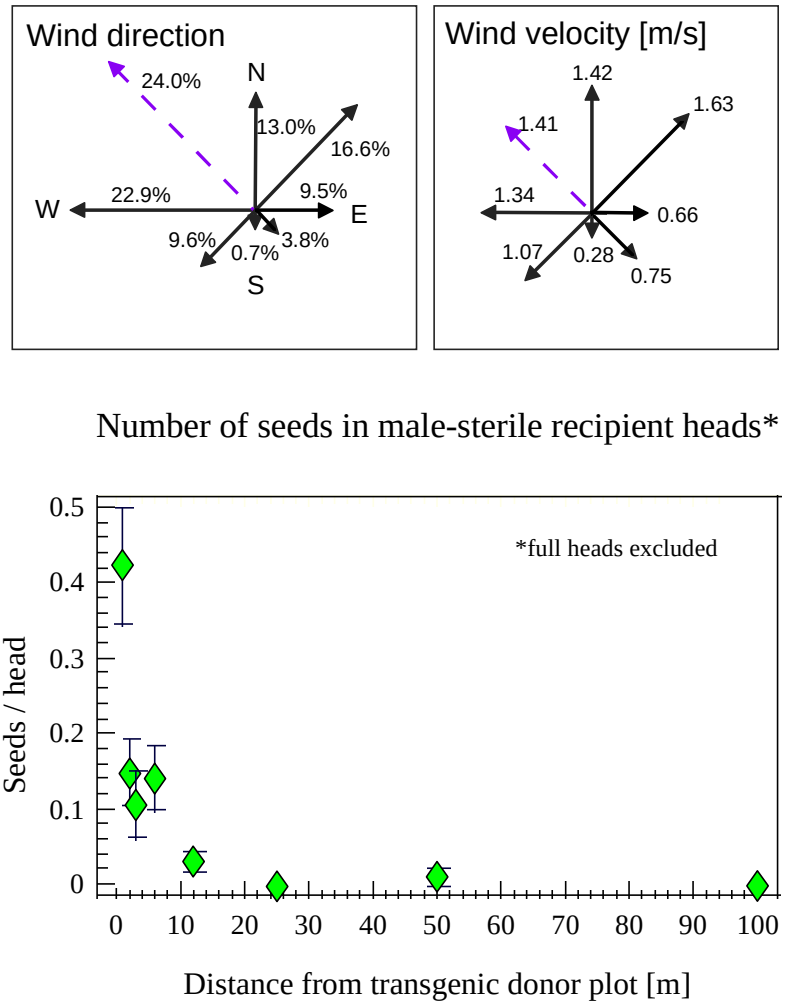

Figure 2. Dispersal of transgenic pollen in the dominant wind direction in the cultivation scale experiment (2000 m^2) in 1997 at Boreal Plant Breeding, Jokioinen, Finland. Pollen dispersal was evaluated as number of seeds in the male-sterile recipient barley line at distances of 1 to 100 m from the homozygous transgenic donor plot.

The cross-fertilization frequency proved to be low in Finnish conditions. On average one seed per male-sterile head was obtained at a distance of 1 m in 1996 and less than half a seed in 1997. The PCR analysis of these seeds revealed that all the seeds obtained were transgenic. The corresponding cross-fertilization frequency in male-fertile plots at the same distance of 1 m varied from 0 to 7% with an approoximate mean of 3 to 4% (Figure 4).

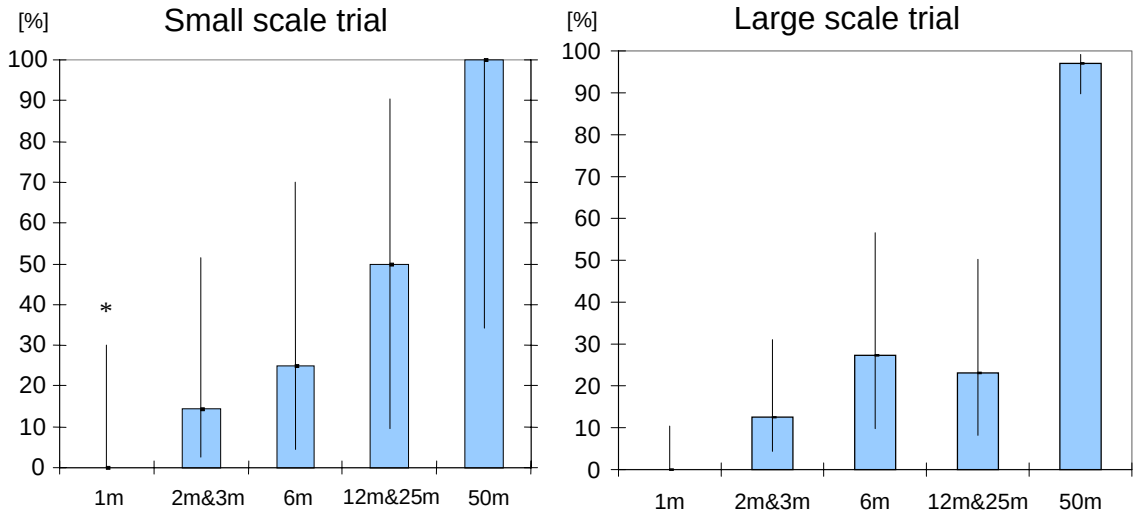

Figure 3. The relative number of non-transgenic seeds in male-sterile plots increased with distance (∗ with 95% confidence interval). The flowers of male-sterile heads were mainly pollinated by non-transgenic background pollen. Such background pollen is to be expected from pollen leakage, i.e. occasional formation of pollen reported to occur in some male-sterile heads.

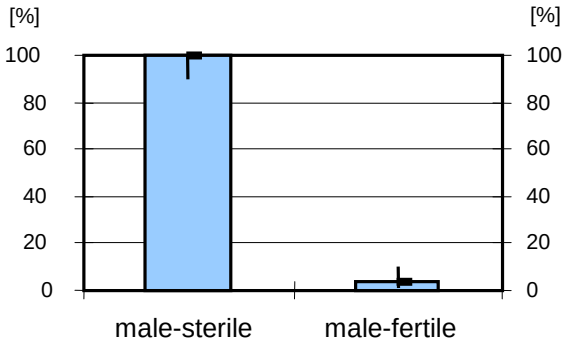

Figure 4. The cross-fertilization frequencies of male-sterile and male-fertile recipient plots (with 95% confidence interval) at a distance of 1 m from the donor area in the cultivation scale experiment in 1997.

4 DISCUSSION

Gene flow has often been studied on the basis of pollen capture records (e.g. Raynor *et al.*, 1972). We also carried out some preliminary experiments with pollen collectors in 1994. The flight of barley pollen was studied in different directions and at different distances from a barley field. In practice the lack of reliable genetic or molecular analyses hindered confirmation of the true origin of the individual pollen grains captured. Thus, the origin of the pollen grains remained obscure. It was also impossible to reliably discriminate pollen of several open-pollinating grass species from barley pollen. Furthermore, no information on the viability of the collected pollen regarding

fertlization was obtained. Thus, the true gene flow should preferably be measured by analyzing the actual seeds produced.

According to the results of this study, transgene flow was low. However, it occasionally bridged a distance of 50 m. The same distance for gene flow was earlier reported with wild barley on the basis of isoenzyme studies (Wagner and Allard, 1991). In our study the cross-fertilization was lower in 1997 than in 1996. One explanation might be the lower overall wind speed in 1997. Furthermore, during the flowering period some very rainy days occurred in 1997, and the wind direction distribution was totally different between the two summers.

The PCR analysis of the seeds collected from male-sterile plots confirmed the existence of contaminating non-transgenic background pollen. This was due to the occasional leakage of male-sterility, causing formation of pollen in some male-sterile heads. Thus, the theoretical maximum resolution could not be obtained. Nevertheless, the 100% cross-fertilization in male-sterile heads at a distance of 1 m from the donor area compared to the cross-fertilization frequency of approximately 4% in male-fertile heads confirms the suitability of male-sterile lines for enhancing resolution in risk assessment studies. The results obtained are definitely more accurate than those obtained from pollen collectors.

5 CONCLUSIONS

It is evident that gene transfer techniques can be of valuable assistance in barley breeding programs. In this context, the risk assessment of cultivation of transgenic plants is essential. Although the risks and benifits depend mainly on the actual trait under consideration, knowledge of transgene flow parameters must be collected so that reliable risk assessment models can be designed, with the aim of safe cultivation of transgenic plants. On the basis of our study, cross-fertilization in male-sterile recipient barley is possible with very low frequency up to 50 meters from the donor area. However, the frequency dramatically decreases with distance and due to self-pollination the possibility of cross-fertilization remains very low in normal cultivated barley. In the future suitably designed risk management procedures implemented in farming will make it possible to transfer the benefits of genetic engineering into the production of plant-based raw materials.

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ENZYMES FOR GRAIN PROCESSING – THE GLOBAL MARKET

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1 THE OVERALL MARKET

The global sales of industrial enzymes have increased throughout the past fifty years and the early dominance of N. America and Europe is now shifting. These two regions have approximately one third of the market each, and considerable trade is now developing throughout the rest of the world. Currently the global sales of enzymes are estimated to be of the order of $US 2 billion.

The previously accepted division of the global market for industrial enzymes into the five sectors, **Detergent, Textile, Starch, Dairy and Other** has been found unsatisfactory for a proper understanding of the distribution of the use of enzymes into many different sectors of industry. Since 1980 I have adopted a more rigorous division into at least 16 sectors. The application of enzymes for grain processing, including the modification of derivatives, is now to be found in seven sectors.

The modification of starches to varying degrees through to glucose, and isomerisation to fructose is the major sector and has held some 15% of the total sales of industrial enzymes for many. It is currently providing enzyme sales of $US 310 millions annually (1998).

In addition to the direct modification of starches, there are enzyme applications in Baking, Textiles, Animal feed, Alcohol production, Protein modification and Flavour production. Today, these markets now have enzyme sales almost equal to the previously largest sector, Detergents, and can be expected to overtake by 2005.

The overall market for enzymes into grain processing was $US 74 millions in 1990, rising to $US 226 millions in 1995, and to $US 540 millions in 1998. By 1998 these applications represented some 26% of all enzymes sales.

1.1 THE ENZYMES

There are at least fifteen different enzyme types involved in these markets, ranging from commodities traded in bulk, through well established intermediate-scale enzymes, to highly specialised catalysts, and some new developments for very particular molecular changes. These are outlined in Table 1.

Table 1. Enzymes involved in processing grain products.

Sector	Commodity	Intermediate	Speciality
Starch	Alpha amylases Glucoamylases Isomerases	Pullulanases Cellulases Xylanases Phospholipases	Maltogenic Maltotriose prodn. Transglucosidases Cyclodextrinases Transferases
Animal Feed	Beat glucanases	Xylanases Cellulases Proteases	Phytase (alpha galactosidase)
Baking	Alpha amylases Glucoamylases	Hemicellulases Proteases	Glucose oxidase (Lipoxygenase and Ferulic acid esterase)
Alcohol	Alpha amylase Glucoamylase	Pullulanase Proteases	Alpha acetolactate decarboxylase
Flavour	Starch syrups		Proteases Lipases Glucosidases Glutaminase
Protein modification	none	Proteases	Proteases Transglutaminase
Textile	Alpha amylases Cellulases	none	none

2 THE CURRENT MARKET (1998)

The enzyme market shares for my seven sectors are given in Table 2. **The Primary Conversion of Cereal Starch** to a range of dextrins and simple sugars and their syrups is the dominant feature at present. However, there are several important intermediate-scale enzymes that contribute significantly to economic efficiency in the processing. In addition, the recent few years have seen the development of commercially viable examples of several exciting speciality enzymes that are enabling the production of highly specific molecules or molecular structures of great value. These include the production of various oligosaccharides with different but useful properties, and the cyclodextrins whose properties and benefits are still being discovered.

Within the **Animal Feed Sector**, there is dominant application of the larger volume and lower cost enzymes. The exceptions are phytase, used to improve the conversion and utilisation of phosphorus, and the probable importance of alpha galactosidase to successful feeding of various vegetable materials to pets.

The Baking Industry has long used fungal alpha amylase to regulate the performance of flour in bread making. Recent demands for extended shelf-life and quality of certain industrial breads has brought the use of glucoamylase, hemicellulase (xylanases) and

some proteases. Proteases are also used to create the right characteristics in biscuit (cookie) doughs. Replacement of chemical additives remains high on the consumer's agenda. Glucose oxidase, and some other enzymes are seen to help in replacing the use of potassium bromate in bread making. New enzymes with high speciality potential are lipoxygenases and ferulic acid esterases.

Table 2. The current sector market (1998)

Sector	**% grain processing enzymes market**	**Sales value $US millions**
Primary Starch Conversion	57.5	310
Animal feed	26	140
Baking	7	40
Textile	6	30
Alcohol	2	12
Flavour	1	6
Protein Modification	0.3	2
		540

The Textile Sector reveals a serious decline in commercial activity, especially in the Far East. This has reduced the demand for cellulases for denim fading, and there is an overall move away from the use of starch sizes for weaving.

Alcohol Production has been largely for the beverage industries in recent years. These activities convert starches to sugars and also use proteases to ensure good nutrition for their yeast, and some find that the removal of acetoin by using aceto-lactate decarboxylase improves the flavour over longer storage times. There is now a strong signal of a recovery in the production of alcohol for chemical use via fermentation. This is increasing the demand for the commodity starch conversion enzymes.

Natural Flavour Creation is very fast growing sector where all manner of enzymic modifications are being tried in the search for acceptable, old styles and exciting new flavours. The main involvement for the grain sector is through the provision of sugars and sugar syrups as primary components. The use of speciality enzymes is confined to seeking very target specific molecular changes and is applied to all material types, including grain derivatives. A major sector is the production of savoury flavours from e.g. wheat gluten, without the use of mineral acids. These are the 'so-called' eHVPs.

This last point leads to **Protein Modification**. Here enzymes are used to change the physical properties of proteins to improve functionality or to so alter the molecular size range of hydrolysed proteins that they have little, or preferably no allergenic potential. The production of specialised diet ingredients for medical use has also tripped over into sports and 'recovery' beverages.

3 FORECASTING THE MARKET

The following comments are my assessment of the future of the enzymes sales into the grain processing market in two stages. From 1998 through to 2005, and then on again to 2010. The key numbers are presented in Table 3.

Table 3. Forecast enzymes sales to grain Processing to 2010.

Sector	1998 $USm	2005 $USm	% change	2010 $USm	% change	Overall % change
Starch	310	325	**5**	340	**5**	**10**
Animal feed	140	220	**50**	310	**40**	**122**
Baking	40	68	**70**	81	**20**	**103**
Textile	30	20	**(-50)**	20	**0**	**(-50)**
Alcohol	12	18	**50**	23	**30**	**92**
Flavour	6	12	**100**	14	**20**	**200**
Protein	2	3	**50**	4	**25**	**100**
	540	664		796		

4 COMMENTS TO THE SECTOR FORECASTS

Primary Starch Conversion will grow a very modest 10% overall. This is largely due to the expected almost static market for the commodity enzymes. It is difficult to forecast a continued rapid rise in the global demand for sweetener syrups. There is likely to be some rearrangement of the geographic locations of the processing plants with some growth in the less developed areas of the world. The application of the speciality enzymes will experience a rapid growth, probably doubling over 5 years.

However, the amount of primary syrup that will be diverted to these products will not be large enough to make a big demand on the syrup supply. In fact, the newer products may supplant some traditional markets for simple sugars. The added value of these speciality enzymes, and the products made with them, will be an attractive segment of this sector during the forecast period.

The Animal Feed Sector is a continuous cause for difficulty when it comes to forecasting enzyme sales. In the past few years there has been a greater growth than had been forecast 5 years ago. This leaves me with the belief that there should be a modest capture of some of the large potential. My estimate is that whilst there likely to be a static position with regard to ruminant rearing in Europe, there may be an increase in N. America. The pig industries show remarkable volatility. Over the forecast period there should be some increased enzyme sales for weaner foods. The greatest probable growth is for the feeding of poultry on a global scale. The Middle East and Asia represent the focus of the earliest development of the market.

Commodity enzymes will be the main gains with slow and steady growth, largely because the industry has very tight cost controls and cannot readily accept significant on-costs.

The speciality market is going to remain largely limited to phytase. This market will continue to hold 30–45% of the total enzymes sales to this sector through the forecast period. Growth will come from an increase in the countries that 'require' improvements to the environmental position and see phytase as a contribution to reducing phosphate contamination. Work with other enzymes will include the greater use of proteases where vegetable protein conversion must be improved to reduce excessive nitrogen contamination of the land. The pet food sector has considerable potential to take up the application of enzymes. This is particularly likely where the requirement to sterilise offals and other slaughter wastes immediately after removal is increased. This stops the autolytic conversion which contributes significantly to the flavour of the final product. Enzymes will have to be added to create this characteristic in sterilised materials.

Overall the Animal feed Sector is forecast to more than double its requirement for enzymes by 2010.

The Baking Sector consumes large amounts of fungal alpha amylase over a period of years, although the amount in any year depends on the harvest quality of bread wheat. It looks as if there will be a minimal requirement for this enzyme in 1998/99. In some European countries and in N. America the production of industrial breads, particularly sliced and wrapped, requires long shelf-life with retained eating quality. This is increasing the demand for hemicellulases and glucoamylases. Glucose oxidase is also increasingly used for the same purpose. However, there are technical problems in getting good value from this enzyme in the baking process. Interest continues to try to substitute for synthetic emulsifiers, and some lipases are gaining acceptance. For biscuit (cookie) production, there is a steady use of proteases to modify gluten so that it relaxes and loses elasticity.

There are challenging developments in the provision of new lipoxygenases that may well find good applications in bleaching flours for both bread and cakes.

New researches have demonstrated that modification of the phenolic compounds that are the precursors to lignin can have large effects on bread dough handling, and also on shelf-life properties. So far the most studied enzymes in this area are ferulic acid esterases. Overall my estimate is that the sales of enzymes to the baking sector will double by 2010.

The Textiles Sector remains lack-lustre and the sales of enzymes for starch desizing are set to fall as less starch is used. The denim washing/fading industry has taken a huge set-back since early 1998. There could be a modest recovery if enzyme prices were to be further reduced, but this is unattractive for the production side. By 2010 it is expected that enzyme sales will have stabilised at a very modest level.

The Alcohol Production Sector is going to increase its consumption of the commodity enzymes for the conversion of starch to sugars, mainly to meet a growing demand for alcohol for use as a chemical feedstock, but derived from sustainable resources. The beverage alcohol sector will not increase its use of commodity enzymes to any great extent. There will be some uptake of speciality enzymes.

Due to the chemical alcohol demand, the sector will grow by about 90% in the forecast period.

The Natural Flavours Sector is growing very fast. The requirement for enzymes to modify materials deriving from grain is a small part of the growth. Both regular and speciality sugars and syrups are used as basic components in many flavours, and it is via these starch sugars that the main use of enzymes will impact on grain products use for flavours. There is a large body of R & D within the flavour industry that is looking at many aspects of the use of enzymes to create/release 'natural flavours'. These include the possible use of grain derivatives other than starch. This is a tricky sector within which to isolate the specific uses of grain products. The overall sales of enzymes to Flavour production are going to grow substantially, with speciality enzymes providing the profitable areas. Multiplication by up to four times the current sales is likely by 2010.

The Protein Modification Sector relates to the processing of isolated grain-derived proteins. Wheat gluten and rice proteins are being increasingly modified for flavours and as the basis for nutraceutical products and medically specified formulations. Maize gluten, as a product of starch extraction, remains a resistant material to modify. There are prospects for improvements using a selection of proteases. The sale of speciality enzymes to this sector is expected to double by 2010. However, the starting point of $US 2m in 1998 is very modest, and this growth does not have a significant impact on sales of enzymes for the forecast period. The modification of grain-derived proteins will account for no more than a third of these minor sales.

5 THE GM IMPACT!

There are big clouds blowing over my crystal ball when I consider this subject.

In the long term it is likely that there will be a global uptake of the use of GM crops.

At this time, it is almost impossible to anticipate when this will start to be significant. We are at the initial stages of working through the concepts and possible consequences, from regulation through to the need for consumer acceptance and confidence.
It can be proposed that the greater the yields and the lower the cost of production of grain crops, the greater is the potential for conversion of part of the crop to derivatives. The lower the base materials price, the greater significance will the processor attach to such other costs as enzyme prices. Significant uptake of GM crop production therefore predicts a downward pressure on enzyme prices. This would be greatest in regard to bulky commodity enzymes. The economic benefit of parallel reduced raw materials cost for the fermentation of the enzymes is extremely marginal.

To stay in the game as a supplier, the commodity enzyme producer will need to find major yield improvements and reductions in downstream processing costs. The commodity enzyme manufacturers have already taken the greatest part of the benefit available by adopting fermentation of GM microbes to get big gains in fermentation productivity. They will need to find another big economic gain if they are to provide enzymes that fit the cost/profit expectations of the processors of starch and protein from GM grain crops.

It may be that the larger starch processors will adopt 'in-house' production of their own enzymes to a greater extent than at present. There are often very positive economic benefits in doing this. The enzymes will still be produced, but will not represent part of the trading market. This would substantially re-order the relative sizes of the enzyme user sectors and a new forecast would look very different than the one presented here.

Currently, most commodity enzymes are very low priced (cheap). If the average G7 inflation over the past 30 years had been applied to the starch conversion enzymes they would be at least ten times more costly than they now are. Most of this stability has come from the improvements made by the enzyme manufacturers, and in recent years this has been from the use of GM microbes as sources.

If there were a major shift back from the use of GM methods, both in crops and also as microbial enzyme sources, there would have to be a one hundred percent increase in fermentation capacity to meet the demand. There would also have to be a substantial rise in enzyme prices.

Consolidation within the members of the industrial enzymes production industry has been particularly active for the past 5 years, and there is no reason to believe that it has finished.

From a strategists point of view this collection of thoughts raises some questions regarding the future of the industrial enzymes industry. How will it respond? Who will respond? Which countries will take the greatest share of the opportunities? Will 'popular feeling' increase the demand for use of enzymes, because they are natural, and they perform natural chemistry.

I hope I shall have some of the answers by the time of ESEGP-3. TG 11/99 ©etc.

ENDOGENOUS AND EXOGENOUS ENZYMES IN MALTING AND BREWING

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ABSTRACT

The conversion of barley into beer is perhaps mankind's oldest and most complex piece of applied enzymology. Although for some companies brewing seems to be more art than science, the major brewing companies (and many of the smaller ones, too) have fine-tuned their operations based on the advances in understanding of the enzymology that underpins the process. And yet the absolute significance of all of the enzymes purported to be involved in the conversion of barley to beer is not rigorously established: c.f. just how important is lipoxygenase? Equally, there are almost certainly some enzymes whose role has been understated, e.g. esterases and peroxidases.

1 THREE KEY ENZYME REACTORS

Table 1 presents an overview of the malting and brewing processes, emphasizing the enzymic processes underway at various stages.
There are three primary 'enzyme reactor' stages in the conversion of barley to beer:

- the barley kernel
- the mash tun
- the yeast cell

Only in one of these, the mash tun, are we considering a 'typical' enzyme reactor, one in which there is an opportunity to address the actions of individual enzymes. Even then, we must recognize that the system is complex and remarkably ill-defined, with a mix of soluble and insoluble substrates, varying levels of inhibitors and activators, interactions of physical (e.g. gelatinization of starch) and chemical as well as enzymic effects. As in so much of brewing, mashing is a compromise, with a set of conditions scheduled to maximize the desirable outcomes from the process stage, whilst minimizing the deleterious impacts.

In the malting and fermentation stages, control is exerted by regulating the behavior of the organisms concerned, *viz.* barley and yeast. Naturally an understanding of the enzymes involved in these organisms is important if control is to be planned systematically. For example the thermolability of malt β-glucanase and the requirement of yeast alcohol dehydrogenase for zinc have a direct impact on approaches to malt kilning (and subsequent mashing) and use of yeast food in fermenter respectively.

Table 1. An overview of the malting and brewing processes, emphasizing the enzymic processes underway at various stages.

Process Stage	Treatments	Events	Endogenous Enzymology	Exogenous Enzymology
Raw barley	**Storage – perhaps to break dormancy**	**Hormonal changes – ill-defined**	**Few enzymes in raw barley: Main ones carboxypeptidase and bound, inactive β-amylase**	
Steeping	**Water added, interspersed by air rests, to raise water content of embryo and endosperm; up to 48h at 14-18 °C**	**Synthesis of hormones by embryo, hydration of 'substrate' (starchy endosperm)**	**No apparent increases reported**	
Germination	**Controlled sprouting ('modification') of grain – typically 4-5 days at 16-20 °C**	**Synthesis of enzymes by aleurone and migration into starchy endosperm; sequential degradation of cell walls, some protein, small starch granules and pitting of large granules**	**Solubilization of β-glucan by solubilases (?) and endo-β-glucanase; degradation of arabinoxylans by arabinofuranosidase and endo-xylanase; partial hydrolysis of proteins by endo-peptidases and carboxypeptidase; development and limited action of α-amylase; splitting of β-amylase from protein Z; synthesis of bound and free limit dextrinase, and activation of latter**	**Microbial flora may contribute enzymes; opportunities for use of selected starter cultures?**
Kilning	**Heating of grain through increasing temperature regime (50-220 °C) for desired properties: enzyme survival, removal of moisture for stabilization, removal of 'raw' flavors, development of 'malty' flavors and color**	**Enzyme survival greater with low temperature start to kilning and lower final 'curing' temperature. Increased heating of malts of increased modification (i.e. higher sugar and amino acid levels) gives increasingly complex flavors and colors via Maillard reactions**	**Some continued action of all enzymes at lower onset temperatures; but then solely an enzyme inactivation issue. Lability of enzymes endo-β-glucanase, limit dextrinase, lipoxygenase> endo-peptidase> β-amylase, lipase> solubilase > α-amylase, peroxidase**	
Malt storage	**3-4 weeks ambient storage, otherwise wort separation problems later**	**Unknown, but may relate to development of cross-links between proteins through oxidation in mashed of unstored malt**	**Lipoxygenase may catalyze this reaction (c.f. enhancement of bread-making by analogous reaction in wheat protein); lipoxygenase decays during malt storage**	
Mashing	**Extraction of milled malt at temperatures between 40 and 75 °C**	**Enzymolysis continued; gelatinization of starch at >62 °C**	**Continued β-glucanolysis favored at low temperatures – also possibly further proteolysis; starch degradation greatly facilitated by gelatinization; balance of enzymes acting faster at higher temperatures with increased destruction of more sensitive ones**	**Use of heat-stable β-glucanase from Bacillus or fungi comprises main use of exogenous enzymes in high malt mashes; use of glucoamylase to promote fermentability (Light beers)**

Use of adjuncts	**Solid adjuncts used in brewhouse, taking advantage of malt enzymes (liquid sugars are products of acid and enzyme action in sugar factory and added at boiling stage)**	**Cereals with higher starch gelatinization temperatures than for barley need pre-cooking before combining with main mash**	**Ditto – also a degree of dilution of malt enzymes, especially with high adjunct use**	**Use of highly heat-resistant α-amylase to promote gelatinization in cooker. Use of amylase, protease, β-glucanase mixtures in main mash**
Boiling	**1-2h at 100 °C, before cooling**	**To sterilize, extract hops, concentrate, and kill all residual enzymes**	**No enzymology**	
Fermentation	**Wort pitched with yeast and fermented for 3-14 days at 6 - 25 °C**	**Fermentation of glucose, maltose, sucrose, maltotriose to alcohol; enzymic production of various flavorsome compounds (alcohols, esters, fatty acids, sulfur-containing compounds etc) Synthesis and removal of diacetyl as an offshoot of amino acid production**	**Embden-Meyerhof-Parnas pathway and offshoots**	**Addition of acetolactate decarboxylase to convert acetolactate precursor to acetoin, thereby circumventing 'natural' route which is non-enzymic breakdown of acetolactate to diacetyl (butterscotch), which is slowly reduced by yeast enzymes to less flavor-active acetoin**
Cold conditioning and filtration	**-1 °C for ≥ 3 days; then filtration**	**Precipitation, sedimentation and removal of solids**	**Slow action by any enzymes 'leaked' from yeast, e.g. proteinases: detrimental to foam**	**Filtration can be limited by viscous polysaccharides, ergo advantage of using β-glucanase in brewhouse (or fermenter). Some use papain as a haze-preventative – but risk of removing foam polypeptides**
Package	**Market- driven**	**Progressive deterioration by chemical reactions, including oxidation**	**Unpasteurized, 'sterile-filtered' beer, may retain some of these enzymes**	**Use of glucose oxidase/catalase as an oxygen scavenger has been suggested**

2 IS THERE A QUICKER WAY?

Chemical engineers coming fresh into the industry are astonished to encounter malting and fermentation processes each lasting a week or more. They are convinced that there must be a better way. Why malt barley? Surely the production of a feedstock for yeast ought to be achievable in a single stage extractor, with raw barley digested by a regulated mix of enzymes of exogenous origin? Indeed, why use barley at all, for surely any source of fermentable carbohydrate could be used, with the characteristics of different beers (notably color and flavor) introduced by additions post-fermentation? In this 'brave new world', acceleration of the fermentation process would be achieved through use of immobilized yeast bioreactors, operating at high temperatures and high substrate concentrations.

It is undeniable that potable alcoholic beverages could be produced in this way, and indeed this could be a way forward for the bulk production of 'cheap and cheerful' potable beverages. They would, however, bear little resemblance in quality to the successful, mainstream beers of today. This is primarily on account of a less than

thorough appreciation of all of the manifestations of flavor: palate (including texture), after-palate and nose.

There is, however, the key aspect of tradition. Many Brewers emphasize their "caring producer" approach, stressing the use of premium raw materials (malt, hops, yeast, water) handled by processes that are *slow* and geared to excellence. Whatismore, it really doesn't make sense to 'take short cuts', bearing in mind that the cost of malt pales virtually into insignificance when compared to other production costs (e.g. packaging and packaging materials), the commitment to marketing and sales, the extent of taxation, quite apart from the invariable on-cost of trying to deal with inferior grist materials. The risk of being exposed for not using premium raw materials is far greater than marginal savings to be made by downgrading the quality of the malt supply, or going as far as to using unmalted barley or other grist materials. Particularly in markets demanding ingredient labeling, the vector is towards 'clean labeling', which is perceived as an opportunity for stressing the presence of positive materials, but also an absence of negatives. For many Brewers the need to use the word 'enzyme' on such a list would be deemed undesirable. For some, it is only in circumstances where perhaps good quality malt is unavailable where compromise in this area can be tolerated.

3 THE CURRENT STATE OF ENZYMOLOGY IN MALTING AND BREWING

Others at this symposium have addressed in detail current knowledge regarding the cell wall-, protein and starch-degrading enzymes in barley. I seek here to highlight where I perceive the gaps in our understanding to be.

3.1 CELL WALLS

There is now a clear understanding of the endo-β-glucanases. However there is much less appreciation of the substrate for the enzymes. Do these enzymes attack the glucan *in situ* in the walls? Is all of the glucan in the wall freely soluble, with the enzyme attacking the glucan once it has leached from the wall as a result of moisture distribution through the grain in steeping? Or is it the case, as some suggest, that there is an initial solubilization of glucan by other enzymes? *In vitro* studies have highlighted that a proportion of the β-glucan is freely soluble in warm water, that increasing proportions are extracted as the temperature is increased, but that use of solvents such as hydrazine and dilute caustic, or enzymes such as carboxypeptidase and ferulic acid esterase can also enhance extractability (1). In short, there is a burning need for better understanding of the architecture of these walls.

3.2 PROTEINS

Whereas the Brewer's interests are best served by a comprehensive degradation of glucans, such is not the case for proteins. There needs to be a controlled generation of amino acids to support a yeast metabolism geared to efficient production of alcohol and the targeted balance of flavor volatiles, and there also needs to be a removal below

critical mass of those proteins which cross-react with polyphenols to cause beer haze. Conversely a beer depends on the presence of hydrophobic polypeptides as the backbone for its foam. Current theories range from a role for a specific protein (Lipid Transfer Protein) in promoting beer foam, after its denaturation in the boiling stage, through to the generation of polypeptides by proteolysis, probably from hordein, during the germination phase of malting, with the resultant ill-defined mix of hydrophobic polypeptides sharing the responsibility for stabilizing the head on beer. Assuming the latter to be significant, then this highlights a previously unsuspected benefit of malting, one which as yet has not been recreated *in vitro* via controlled proteolysis, as would be needed in any barley brewing scenario. However endogenous proteolytic systems have not been analyzed from a perspective of the substrate-product-enzyme interaction. Excessive proteolysis is known to be detrimental to foam quality of beer, focusing recent attention on the significance of proteinase A from yeast. It is now understood that release of this enzyme from stressed yeast is damaging to foaming polypeptides, and it is suspected that this is one of the reasons for decreased foam stability in high gravity brewed beers, with yeast becoming stressed at high wort strengths producing increased level of protease (2).

3.3 STARCH

In most ways, starch degradation is the best characterized of the enzymic events in brewing. Amongst the few substantial areas left for exploration are a better understanding of the differing degradability of starches from different cultivars and of large and small granules. As for cell walls and proteins, then, it is more a focus on substrate and product rather than the enzymes *per se*.

3.4 LIPID

Moreso than the other barley substrates, the fate of lipid in brewing is as much to do with physicochemical effects (e.g. insolubilization) as with enzymic degradation. There is scope for addressing the significance of starch lipids for processing and product quality. Also the impact of lipids in a soluble or insoluble form on the behavior of yeast in fermentation attracts contrary views. Furthermore it is still not understood whether endogenous lipid, in either a free micellar form or in a bound state, can carry through to modify the appearance of beer foam. Certainly the measured stability of foam can recover in hours from the damaging effect of freshly introduced lipid, perhaps as a result of the presence of lipid binding proteins. However it is the possible role of unsaturated fatty acids as precursors of stale flavor in beer which attracts most attention. There is a school of thought that lipoxygenase catalyzes the formation of hydroperoxides during malting and mashing, these products going forward into wort and beer to constitute the precursors of stale flavors. This theory is contested, but is unproven one way or another. Lipoxygenase has been implicated in another key event in the brewing process, namely performance enhancement of stored malt (3). This is an attractive theory, the confirmation (or otherwise) of which should form part of a broader assault on the overall significance of oxygen and oxidation upstream in the brewing process. Thus malt is replete with relatively heat-resistant peroxidases, but the

significance of these activities is not entirely appreciated, save that they are limited for the key substrate peroxide.

3.5 POLYPHENOLS

Certainly some of the polyphenols from the outer layers of the grain are a prime substrate for peroxidases. The major unknowns here, however, are the significance of the polyphenols in determining beer quality. Of course, they form a part of beer haze, yet they may variously have anti- or pro-oxidant activity, and have been claimed to promote mouthfeel and astringency. One of the ways in which they might be antioxidant is through a role as inhibitors of lipoxygenase – but what of other enzymes? Might they also impact on the activity of hydrolases, too?

4 OPPORTUNITIES FOR EXOGENOUS ENZYMES IN BREWING

4.1 WITHIN THE PRESENT PARADIGM

Many Brewers already recognize the value of supplementing the endogenous enzyme complement by the addition of exogenous enzymes, even when using grists of 100% malt. This presents the opportunity to 'even out' unavoidable variations in the consistency of batches of malt, which are invariably heterogeneous to a greater or lesser extent. Furthermore added enzymes can simplify brewhouse operations, e.g. use of glucoamylase in the production of super-attenuated beers, or heat-resistant endo-β-glucanases to avoid a need for low temperature mashing programs and to facilitate very high temperature mashing regimes which might be applied for the production of low alcohol beers. Acetolactate decarboxylase can be used in fermenter to accelerate the maturation process, or to facilitate a 'right first time' approach to fermentation control.

4.2 WITH PARADIGM SHIFTS

Commercial enzymes would be essential in the event of a wholesale re-think of the brewing process (bearing in mind the concerns expressed earlier in this paper). Thus Ahvenainen encouraged us to think of barley fractionation prior to processing of separate streams for different purposes (4). Such an approach would eliminate lengthy maltings processing, have major environmental benefits and reduce concerns for outlets for spent grains. (Regarding the last of these, and within current regimes, enzymes might find application as part of alternative processing treatments leading to secondary fermentation feedstocks.) Genetic modification of barley and yeast is entirely plausible, yet will only succeed for food systems if there is an incontrovertibly explicit direct (e.g. cost, health) or indirect ('green message') benefit for the consumer. Many of the projected goals would simply benefit the processor (viz maltster or brewer) – and, indeed, address issues that can with a greater or lesser degree of discomfort be achieved in alternative ways.

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ENZYME ASSISTED SOLUBILIZATION OF β-GLUCANS FROM RYE BRAN

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1 INTRODUCTION

β-glucans consist of linear chains of D-glucosyl residues joined by β(1→ 4) and β(1→ 3) glycosidic linkages. Rye β-glucans are mainly composed of blocks of 3 or 4 (1→ 4)-linked β-glucosyl units linked by single β(1→ 3)-linkages[1] and the reported[2] molecular weight of rye β-glucan chains (obtained by alkaline extraction at 60°C) is 1.13 x 10^6. This molecular weight suggests an average degree of polymerization of ~ 7000. The presence of the β(1→ 3) bonds renders β-glucans relatively more soluble than cellulose and β-glucan polysaccharides are therefore analyzed as soluble dietary fibres (SDF). In the cell walls of cereal grains, β-glucans are envisaged to be interwoven into the three dimensional wall structure dominated by cellulose microfibrils and arabinoxylans and partially interlocked with polymerizing lignin. There is strong evidence that consumption of soluble dietary fibre polysaccharides, notably cereal β-glucans, can decrease plasma cholesterol levels and thus contribute to the prevention of hypercholesterolaemia and coronary heart disease[3]. In this respect much effort has been focused on oat β-glucans[3,4] while β-glucans from rye have received comparatively limited attention. Rye kernels (*Secale cereale* L.) are known to contain relatively high amounts of arabinoxylans (6–11%)[5] but rye also contains between 1.5–2.5% by weight of mixed-linked β-glucans.[5,6] This is less than oat, but 2–2.5 times more than wheat. The purpose of this work was to investigate the possibilities for releasing SDF, notably β-glucans, from rye bran by controlled enzymatic treatment. After initial suitability screening of different enzyme preparations we studied the efficacy of the hemicellulose preparation Gamanase™ in releasing SDF and β-glucans from rye bran in response to pH and temperature conditions during enzymatic reaction.

2 MATERIALS AND METHODS

Bran: Rye bran was obtained from Havnemøllerne A/S (Fredericia, Denmark) and ground to particle sizes ≤ 0.5 mm. Ground bran samples (50 g) in phosphate buffer (500 mL 0.08 M, pH 6.0) were sequentially treated with α-amylase (5 mL Termamyl 120L, Novo Nordisk; 30 min., 100°C, pH 6.0) and protease (5 mL Alcalase 2,4L FG, Novo Nordisk; 30 min., 60°C, pH 7.5), filtered (50 µm nylon membrane) and washed with doubly distilled water (2 x 100 mL, 60°C) and acetone (2 x 100 mL). The insol-uble rye bran fraction remaining was freeze-dried and stored frozen (–20°C) until use.

Enzymes: Gamanase™ was donated by Novo Nordisk (Bagsværd, Denmark).

Dietary fibre and β-glucans: Soluble dietary fibre (SDF) yields were determined by the enzymatic-gravimetric procedure (method 993.19[7]) using the "Bioquant® Dietary Fibre" kit from Merck (Darmstadt, Germany). β-glucans were quantified with the reagent kit from Megazyme® (Bray, Ireland) for mixed-linkage β-glucans.

Enzyme treatments: Enzyme assisted release of SDF and β-glucans with Gamanase™ at 5% enzyme/substrate ratio (by weight) and 1 hour reaction time was explored versus non-enzyme treated controls in response to pH (4–7) and temperature (24–66°C) in a CCC response surface design. All treatments were halted by heat (75°C, 10 min.).

Statistics: Modde 3.0 Software (Umetri AB, Umeå, Sweden) was used as an aid for experimental designs and data analyses by partial least squares regression. Yield differences were also tested by one-way analyses of variances using Minitab Statistical Software (Addison-Wesley, Reading, MA).

3 RESULTS AND DISCUSSION

In preliminary screening experiments with different plant cell wall degrading enzymes Gamanase™ treatment tended to be superior to other enzyme treatments in releasing SDF from insoluble rye bran, but the enhancement effects of enzyme additions on SDF yields were limited compared to a control without added enzymes (data not shown).

Modification of insoluble rye bran fibres with Gamanase™ resulted in SDF yields of 37–47 g per kg dry, insoluble rye bran fibres (Table 1). The yields varied only little with different pH-temperature combinations during treatment and were similar to the SDF levels obtained in corresponding non-enzyme treated control samples (Table 1). Partial linear regression analysis of the results did not reveal any main effects of the test parameters. However, the proportion of β-glucans in the soluble dietary fibre fractions differed significantly in response to type of treatment and varied from 13–20% by weight of the SDF fraction analyzed (Table 1). Treatment with Gamanase™ generally caused higher yields of β-glucans as compared to treatments without enzyme addition indicating an enrichment effect of Gamanase™ treatment on β-glucan levels in the SDF released. The highest yield of 20% β-glucans in the SDF fraction was obtained with Gamanase™ reacting at pH 4.5 at 60°C (treatment no. 3, Table 1). Gamanase™ is a hemicellulase preparation produced by an *Aspergillus* strain with a stated optimum activity between pH 4–5 and 60–80°C.[8] The Gamanase™ preparation employed is recommended for viscosity reduction in e.g. coffee bean processing,[8] but type and amount of possible endoglucanase activities ("β-glucanase activities") are not stated by the enzyme manufacturer. Gamanase™ was previously found to be effective in catalyzing solubilization of dietary fibre polysaccharides from insoluble wheat fibres with optimum enzyme reaction conditions at pH 6.0, 30°C for 40 min., but the net SDF yield from the wheat fibres in comparison to a non-enzyme treated control was not reported.[9] The available knowledge[5,6] on composition of soluble rye bran fibres suggests that β-glucans constitute ~15–23% by weight of the SDF fraction (1.5–2.5%)

Table 1. Soluble dietary fibre (SDF) yields and relative β-glucan contents in SDF from purified rye bran solubilized by Gamanase™ (hemicellulase) treatment[a] with different pH – temperature combinations.

			SDF (g kg^{-1})[b]		β-glucans / SDF (% w/w)[c]	
Treatment	pH	Temp. (°C)	Control[d]	Gamanase	Control[d]	Gamanase
1	5.5	66	42.3	42.8	16.0^{ab}	19.4^{b,*}
2	6.5	30	39.9	39.9	16.8^{ab}	16.6^{e}
3	4.5	60	40.3	38.9	16.6^{ab}	20.5^{a,*}
4	5.5	24	36.6	37.9	18.6^{a}	18.7^{c}
5	6.5	60	47.6	47.0	15.3^{b}	16.6^{e}
6	4.0	45	39.9	42.5	16.4^{ab}	17.5^{d}
7	5.5	45	43.1	47.0	16.7^{ab}	17.6^{d}
8	7.0	45	44.1	44.9	13.8^{b}	16.2^{d,*}
9	4.5	30	37.1	38.6	14.5^{b}	13.1^{f}
10	5.5	45	44.4	44.6	13.7^{b}	14.1^{f}
11	5.5	45	43.2	44.2	16.9^{ab}	16.9^{de}

[a] Gamanase was dosed at 5% w/w enzyme:substrate ratio; 2.00 g purified wheat bran in 50.0 mL 0.1 M phosphate buffer (at given pH), reaction time 1 hour. [b] Mean values in g per kg insoluble rye bran dry matter (n=2). [c] Values in the same column with the same superscript letters a-f are not significantly different ($p \leq 0.05$). Within the two last rows (β-glucans / SDF) a star indicates a significantly higher mean value ($p \leq 0.05$). [d] Control without enzyme addition.

β-glucans vs. 6–11% heteroxylans in rye grains) which is why the obtained β-glucan ratios of 19–20.5% of SDF may in fact be near the theoretical maximum. Our approach is based on the hypothesis that controlled enzymatic hydrolysis of non-β-glucan polysaccharides loosens the cell wall structure and in turn promotes release and solubilization of high molecular weight β-glucans. Despite the fact that accessibility of depolymerizing carbohydrases to rye cell wall pentosans may be restricted[10] the data obtained demonstrate the plausibility of this hypothesis. More detailed knowledge on the composition and architecture of rye bran materials as well as on the enzymatic action on these complex, insoluble materials are warranted, however, to allow for more targetted hydrolysis for upgrading of rye dietary fibres.

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EFFECTS OF ENZYMES IN PASTA AND NOODLE PRODUCTION

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1 INTRODUCTION

Pasta and noodles are staple foods in many countries and growth in consumption throughout the world has put a demand on improving product quality and increasing the product range.

Noodles differ from pasta in the use of ingredients and processes. Whereas most of pasta products are made from durum semolina and extruded through a metal die, Asian noodles are strips slit from sheeted wheat flour dough. Both product groups have similar quality requirements such as cooking tolerance, biting characteristics or colour stability and quality mostly depends on the raw materials. When pasta is made from non-durum wheat flour, or noodles from poorer quality flour, the end product may easier be overcooked resulting in soft, sticky and mushy texture of the final product.

2 SUMMARY

The application of fungal lipase in the pasta and noodle production offers a new possibility to improve the product quality. This article reviews some of the improving effects of lipase on quality characteristics of pasta or noodles, such as biting quality, stickiness, cooking tolerance or color stability. Furthermore lipase reduces the oil uptake of instant noodles during frying. Addition of lipase improves the quality of noodles and pasta, especially when poorer quality flour is used or pasta produced from non-durum wheat flour.

3 RESULTS

A common problem for noodles is the speckiness, i.e. small dark spots that mainly depend on the ash content and flour quality. Fungal lipase (Novozym 27007) shows positive effect on reducing the speckiness of noodle dough sheets after production and after storage. The enzyme does not only reduce the speckiness of fresh and stored dough sheets, but also stabilises and reduces the speckiness increase over time as shown in Figure 1.

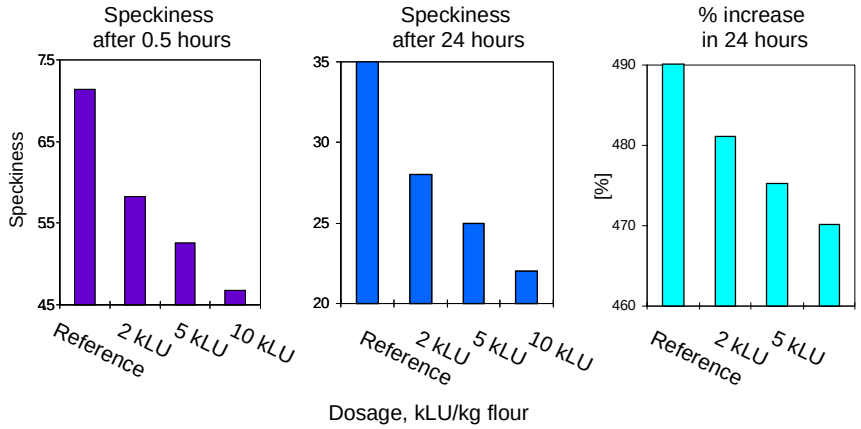


Figure 1. Effect of lipase on speckiness in white salted noodles (dough sheets). Australian Eradu wheat, measured by Branscan.

4 WHITE SALTED NOODLES

Whiteness or brightness is a very important quality parameter for white salted noodles and darkening of raw noodles or noodle dough sheets during production can be a serious problem.

Figures 2 and 3 show colour measurements of raw noodle dough sheets for white salted noodles. The noodles in Figures 2 and 3 are produced from different flours using slightly different production procedures and also different colour measuring equipments. Treatment with lipase increases the white colour and brightness of raw noodles or noodle dough sheets. Further, lipase reduces the darkening rate during storage of raw noodles. After 0.5 and 24 hours raw noodles treated with lipase resulted in significantly less darkness than the control sample. In cooked white salted noodles, brightness is a very important parameter too. As Picture 1 shows very clearly, whiteness/brightness of cooked white salted noodles is increased by treatment with lipase.

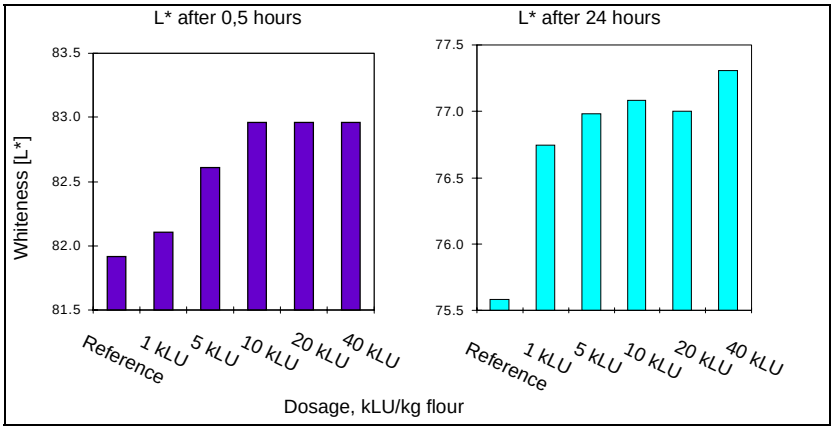


Figure 2. Effect of Lipase on whiteness L in raw white salted noodles, European flour, measured by LabScan XE, HunterLab, CIE L*a*b*.*

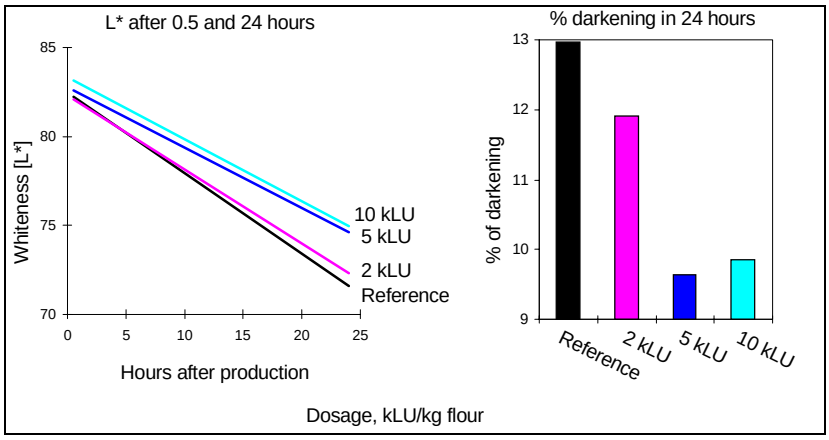
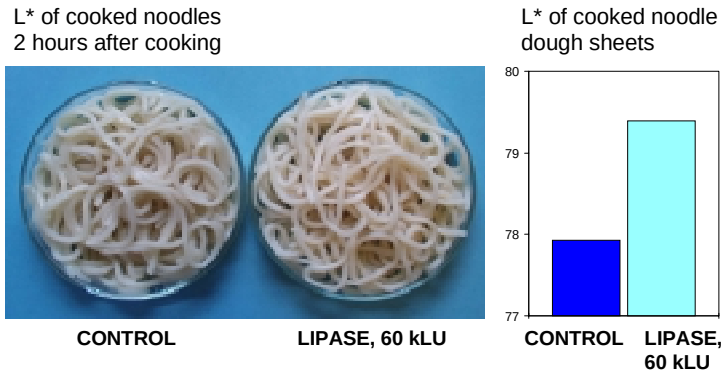


Figure 3. Whiteness of white salted noodles (raw dough sheets), Australia Eradu wheat, measured by Minolta CR310.



Picture 1. Effect of lipase on colour of cooked white salted noodles and cooked noodle dough sheets. Cooking time: 3 minutes, Flour: Meneba type 550, L measured by LabScan XE.*

The graph in Picture 1 shows the L* values of cooked noodle dough sheets. To measure cooked dough sheets instead of cut noodles is more accurate. However due to the increased surface, noodles are even more accessible for oxidation than the noodle dough sheets. Therefore, the colour effect of the lipase may be even more than what is measured in the cooked noodle dough sheets.

Firmness of cooked noodles is another important characteristic being related to biting quality. Firmness can be measured by cutting force of a single noodle strand or by the relative depth of penetration at a cutting force of 0.1 N. The relative depth of penetration at 0.1 N also indicates the firmness of the outer layer of a noodle or pasta strand related to the starch swelling and to the properties during cooking and overcooking tolerance. The firmer the noodles, the higher is the cutting force, but the lower is the relative depth of penetration.

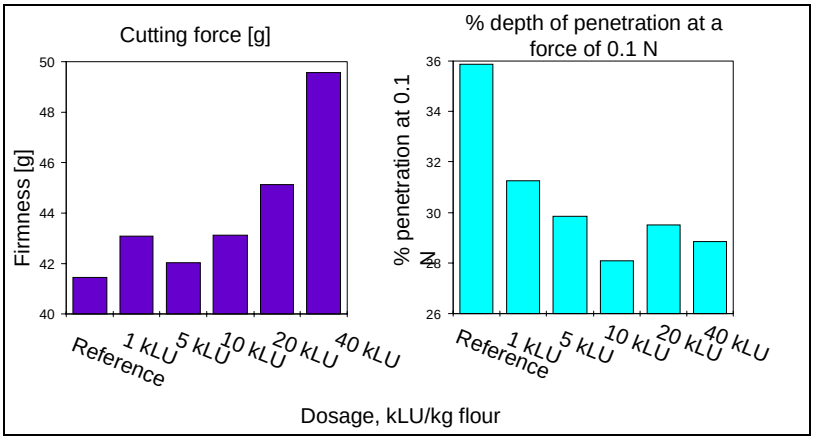


Figure 4. Firmness of cooked white salted noodles measured by Texture Analyser TA.XT2. European flour. Increased cutting force indicates increased firmness, whereas reduced % depth of penetration at 0.1N shows increased firmness of the outer layer of the noodles.

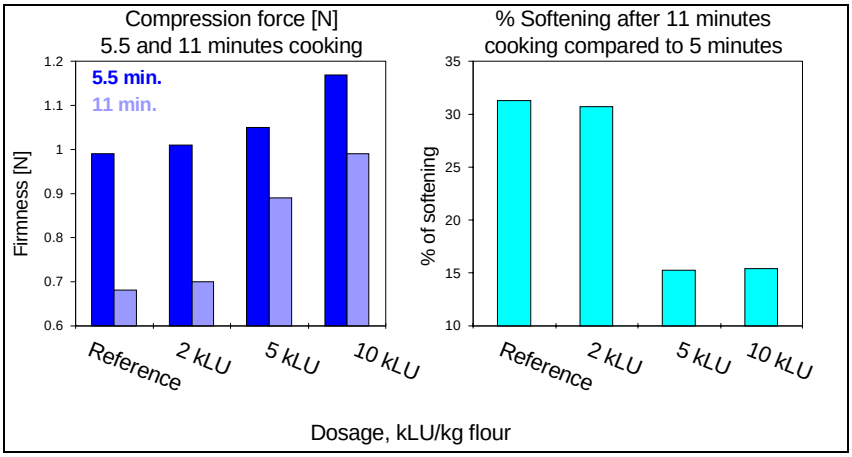


Figure 5. shows the cutting force needed for normal cooked and with 11 minutes over-cooked noodles, whereas the softening degree indicates the overcooking tolerance of the noodles.

Figure 4 shows that the firmness of cooked white salted noodles is increased by treatment of the dough with increasing dosage of lipase. Figure 5 shows, that increasing lipase dosage improves the over-cooking tolerance of noodles. The softening degree of over-cooked reference noodles compared to normal cooked noodles was more than 25%. Noodles treated with lipase have a higher over-cooking tolerance, which results in a lower softening degree during overcooking (around 15% in the used flour and procedure).

Stickiness of cooked white salted noodles was tested by sensory evaluation with triangle tests to compare control noodles to noodles treated with 10 KLU, and 40 KLU respectively.

No difference could be detected between the control and the lower dosage of lipase. But between the control or low dosage of lipase and the higher dosage of lipase, 90 respectively 100% of those that could detect a difference, actually found the control noodles respectively the noodles treated with low dosage of lipase were stickier than with higher dosage of lipase.

5 INSTANT NOODLES

Instant noodles are steamed and oil fried. During production, and already before steaming of the noodles, the colour of the noodle sheet can change a lot within a short time of 30 min only as shown in Figure 6.

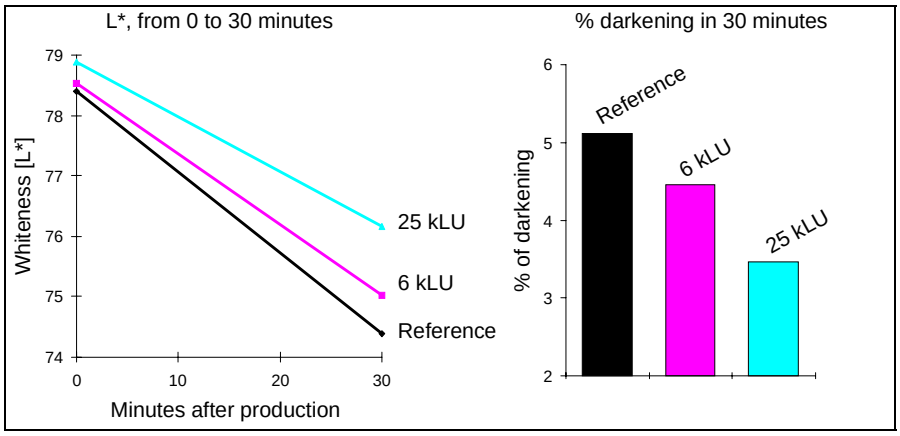


Figure 6. Darkening of instant noodle dough within 30 minutes, flour: Australian Prime Hard, measured by Minolta.

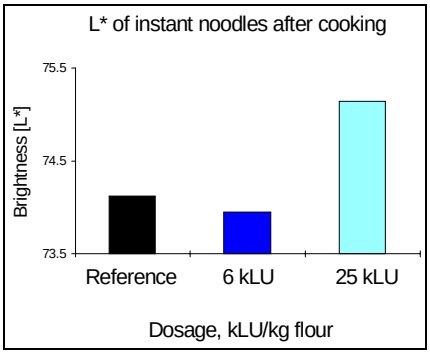


Figure 7. Brightness of cooked instant noodles, flour: Australian Standard White, measured by Minolta CR310.

Within 30 min the control darkened from a L* value of 78 to 74, clearly visible by human eye. L* value of the noodle sheets treated with lipase was significantly higher than the control. Especially it is interesting and important, that by the treatment with lipase, the darkening of the noodle sheets within the 30 minutes – which means during production time frame – is significantly reduced.

In cooked instant noodles, a bright surface again is an important quality parameter. Good quality noodles with smooth surface are brighter than poor quality noodles, which have a mushy and uneven surface. Figure 7 shows the brightness increase in cooked instant noodles when the dough was treated with lipase.

The optimum dosage of lipase concerning its effect on firmness is dependent on the flour type. Lipase was tested in instant noodles using different flour types, representing common wheat flour grades used in the production of instant noodles. Figure 8 shows the effect of lipase on increasing the firmness of instant noodles produced from both Australian Prime Hard and Australian Standard White flour. While Australian Prime Hard is a high protein flour, Australian Standard White is a low protein flour.

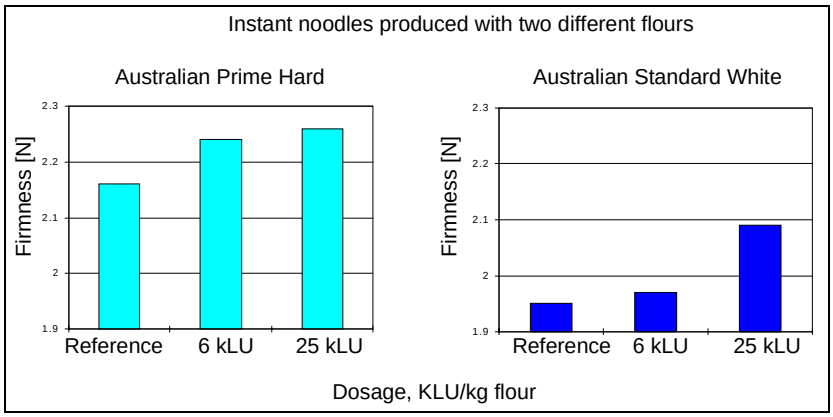


Figure 8. Firmness of instant noodles, measured by maximal cutting force (Newton) of a single noodle strand.

Another very important quality and economical parameter of instant noodles is the oil uptake of noodles during the frying process. Because lipase is able to increase the firmness as well as the rigidity of the outer layer of the noodles due to the increasing formation of amylose-lipids complexes (see below), it can reduce the oil uptake of the instant noodles during frying. Depending on the flour quality, protein content, production formulation and process, the reduction of oil uptake may be in the range of 0.5–3%.

In an alkaline Kan Sui solution (10 g/l Kan Sui concentrate (carbonate) + 58 g/l NaCl + 3–5 g/l mono-sodium glutamate), which is used for production of instant noodles, lipase proofed to be stable during the incubation time of 1 to 4 hours at a pH of 9 and a temperature of 25°C. Therefore lipase is stable during the production process of instant noodles.

6 PASTA PRODUCED FROM BREAD WHEAT FLOUR

When pasta are produced from bread wheat flour, the quality in terms of texture, colour or cooking behaviour differs significantly from the pasta made from durum semolina. The effects of lipase on firmness and stickiness were tested in pasta produced from

bread wheat flour. Firmness was measured as the relative depth of penetration at a cutting force of 0.1N. As shown in Figure 9, lipase increases the firmness of pasta resulting in a lower relative penetration. The sample treated with 60 kLU per kg flour had a firmness close to pasta made from durum semolina.

Furthermore lipase reduces the stickiness of pasta made from bread wheat flour. The spaghetti sample was cooked 1 min longer than the "al-dente" point. Stickiness was measured by Texture Analyser by the integrated adhesive force of the sample towards a withdrawing probe.

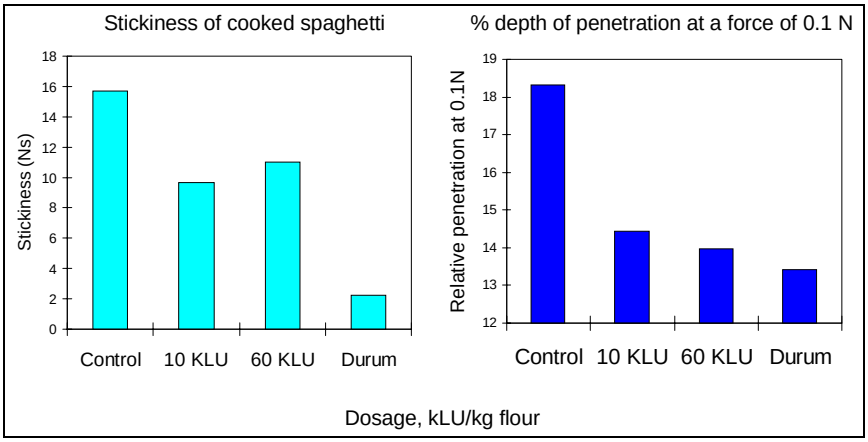


Figure 9. Stickiness and firmness of spaghetti produced from bread wheat flour (European Flour, type 550), measured by Zwick.

7 THE MECHANISM OF LIPASE

The mechanism of the beneficial effect of lipase in bread wheat pasta or noodles is not yet fully elucidated. It may be due to a modification of the protein fraction and of the starch fraction.

Differential Scanning Calorimetry (DSC) measurements of uncooked and cooked pasta produced from bread wheat flour show that complexation of hydrolysis products of lipase with starch takes place. Addition of lipase to pasta did not much change the gelatinisation enthalpy as shown in Table 1. Looking at the melting enthalpy of the amylose-lipid complexes, we see that this melting enthalpy increases by approximately 62% when lipase was added to the dough.

DSC measurements of cooked pasta treated with lipase showed higher amylose-lipid melting enthalpies compared to the control. The results show an increase of around 75% more melting enthalpy in the cooked pasta treated by lipase, indicating that hydrolysis products of lipase do form complexes with amylose. These complexes inhibit the swelling of starch granules and the leakage of amylose during cooking, resulting in a firmer texture and smoother surface. Further, the complex-building capability of the lipase hydrolysis products with amylose reduces leaching of amylose, resulting in less stickiness of the product.

Table 1. DSC measurements of uncooked and cooked pasta produced from bread wheat flour. Dosage of lipase: 60 KLU

Pasta		**Control**	**Lipase**
Uncooked	**Gelatinisation enthalpy**	4.49 J/g db	4.57 J/g db
	Amylose-lipid melting enthalpy	1.41 J/g db	2.29 J/g db
Cooked	**Amylose-lipid melting enthalpy**	0.68 J/g db	1.41 J/g db

It is well known that the formation of amylose-lipid complexes changes the starch fraction of pasta or noodles. The complexes inhibit the swelling of starch granules during cooking. Monoglycerides are currently used for both pasta and noodles for the same purpose.

Another mechanism to explain the effects of lipase can be due to a strengthening of the gluten fraction. Lipase partially degrades the lipids, which aggregate with gluten, resulting in an increase of gluten strength and increase of the elastic property of the gluten in a flour/water dough (Figure 10). This property is part of the reason why lipase can increase the firmness and cooking tolerance of pasta and noodles.

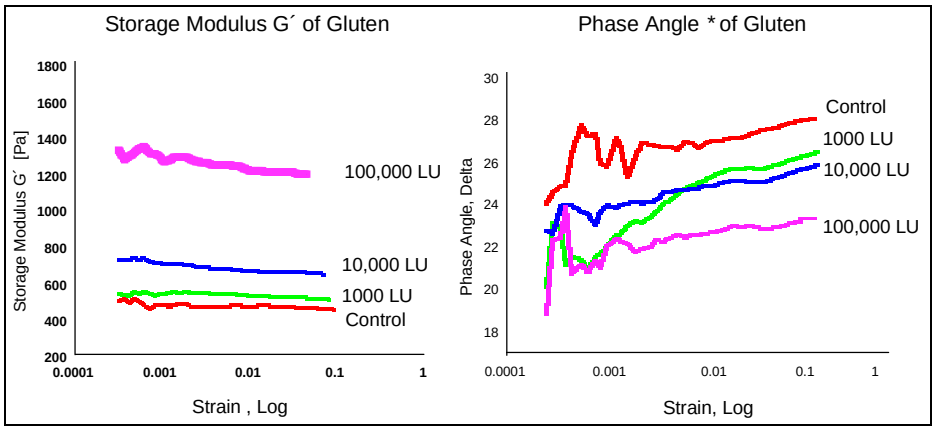


Figure 10. Effect of lipase on rheological properties of wheat gluten, measured by Bohlin Rheometer.

A combined effect on both gluten strengthening and increase of amylose-lipid complexes make this enzyme to be a very good aid to improve the quality of noodles and pasta.

8 ACKNOWLEDGMENT

Silvia Strachan, Fritz Glanzmann, Niki Neyerlin, Antonia Hueber, Novo Nordisk Ferment Ltd., Switzerland; Dr. Ken Quail, BRI Australia Ltd.; Dr. Nasir Azudin, Agrifood Technology, Australia; Dr. Béatrice Conde-Petit, Christoph Zweifel, ETH Zürich, Switzerland; Dr. Andrew Ross, Luise Christiansen, Novo Nordisk, Denmark.

REFERENCES

This article is based on an oral presentation at AACC Annual Meeting October 1999, in Seattle and on an oral presentation at 2nd European Symposium on Enzymes in Grain Processing, ESEGP-2, December 1999, Helsinki, Finland.

AMANO SPECIALITY ENZYMES FOR GRAIN APPLICATIONS

Nigel Robinson

Amano is the World No 1 speciality enzyme producer based in Nagoya, Japan. Amano is not engaged in the business of producing commodity enzymes. We specialise in producing small volumes of very specialised enzymes, suitable for very specific enzymatic applications.

Some of Amano's speciality enzymes, with particular interest to grains processors, are discussed below. Please note that all these enzymes are currently available in commercial quantities.

1. Maltotriose producing enzyme – **AMT1.2L™**
2. Oligosaccharide producing enzyme – **Transglucosidase L "Amano"™**
3. Cyclodextrin producing enzyme – **CGT "Amano"™**
4. Savoury flavour creation enzyme – **UMAMIZYME™.**

These four enzymes represent responses to industrial challenges. The first three are speciality starch processing enzymes, the fourth is for by-product modification. This may be used to add value to a low value by-product!

1 MALTOTRIOSE PRODUCING ENZYME AMT1.2L™ WHY MALTOTRIOSE?

Maltotriose sugars have the following properties: -

i. Can be used to reduce sweetness.

ii. Retains moisture (Humectant)

iii. Resists crystallisation

iv. Allows new syrups stable in acidic and high temperature conditions to be created.

Some successful applications

Baking and Confectionery – Sweets, desserts, pastry, gum, cake etc.

Food Processing with specific requirements – Jams, fish paste etc.

Beverages and innovative beverages – Fruit juice, sports drinks, canned coffee etc

Key Facts about Amano's Maltotriose Producing Enzyme

Amano Name --AMT1.2L™

Optimum Conditions; pH: 6.5, Temperature: 50°C

Description of action

Hydrolyses α-1,4-glucosidic linkages in starch with endo-type reactions producing Maltotriose as the main reaction product.

Organism

Microbacterium imperiale

Yields of more than 60% G3 Maltotriose syrups are practically achievable when used in conjunction with Pullulanase. The yield of Maltotriose is increased from approx. 50% to over 60% by using Pullulanase. This is achieved at the expense of higher oligosaccharides. There is very little change in glucose or maltose components.

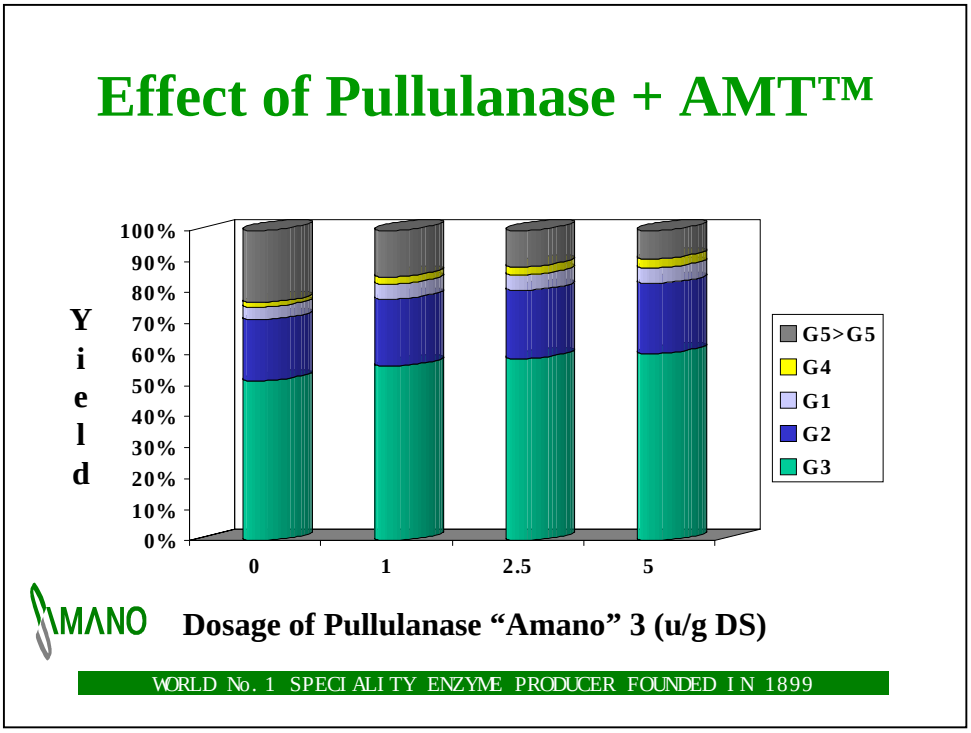


Figure 1. Showing influence of Pullulanase on the yield of Maltotriose.

2 OLIGOSACCHARIDE PRODUCING ENZYME TRANSGLUCOSIDASE L "AMANO"™ WHY BRANCHED OLIGOSACCHARIDES?

i. Pre-biotics provides nutrition for pro-biotics. Improvement of Gastrointestinal Microflora.

ii. Inhibitory effect on dental caries.

Some possible applications

Bakery humectant?

Branched oligosaccharides exhibit low digestibility in humans. They stimulate beneficial microflora – Functional foods.

Mildly sweet and contributes to preservation at high concentration.
Key Facts about Amano's Branched Oligosaccharide Producing Enzyme
Amano name – Transglucosidase L "Amano"™

Organism *Aspergillus niger*
Optimum Conditions; pH: 5.0, Temperature: 65°C

Description of action

Hydrolyses α-1,4-glucosidic linkages to produce α-1,6-glucosidic linkage by a transglucosylation reaction

3 CYCLODEXTRIN PRODUCING ENZYME. WHY CYCLODEXTRINS?

Flavour and fragrance carrier

Supply currently low – international demand is increasing

Cyclodextrins are high value specialities

There appears to be short supply of Cyclodextrins. But a steadily increasing demand?

Key Facts about Amano's Branched Oligosaccharide Producing Enzyme
CGT "Amano"™

Amano name -- CGT "Amano"™

Organism	*Bacillus macerans*
Optimum Conditions;	pH: 6.0, Temperature: 60–65°C
Description of action	Catalyses conversion of starch to mainly cyclohexaamylose (α-CD)

Very fast reaction. in order of 1.5% w/w enzyme to starch dry solids. Reaction takes only minutes.

4 SAVOURY FLAVOUR PRODUCING ENZYME. WHY ENZYMATIC SAVOURY FLAVOURS?

Savoury flavours produced by acid hydrolysis have a long history and have a long history of incorporation into many savoury flavoured products. Enzymaticaly created savoury flavours have the following advantages over ones produced by acid hydrolysis:-

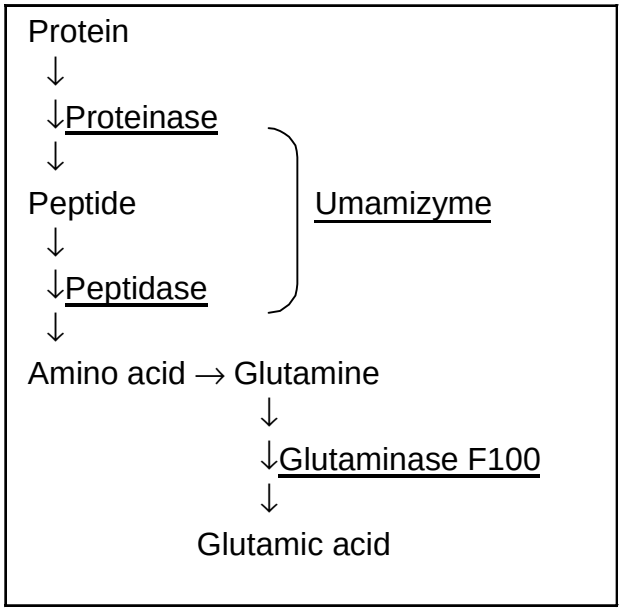
They are 100% Natural!

They meet the demand for propanediol free flavours!

They add value to by-product material e.g. Wheat Gluten.

They can be made with little or no bitterness.

Hydrolysing process



"Umamizyme" has a great deal of proteinase and peptidase.

Figure 2. Showing the two enzyme system of Umamizyme – Protein Hydrolysing steps to produce Savoury flavour.

Table. 1. Umamizyme dose vs. analysis.

Umamizyme in Action

Enzyme Added %	Soy Protein %FAA	Soy Protein Bitterness	Wheat Gluten %FAA	Wheat Gluten Bitterness
1	36	Little	**36**	Little
2	57	Very little	**44**	Very little
4	70	None	**54**	None

Protein solutions were incubated at 45°C for 16 Hr at pH 7.0

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WORLD No.1 SPECIALITY ENZYME PRODUCER FOUNDED IN 1899

Table 2. Umamizyme – Typical Analytical results.

Typical product (Wheat Gluten Substrate)

Analytical data

Parameter	Result
Total Nitrogen	30.0 mg/ml
Protein	196.7 mg/ml
Free Amino Acids	103.6 mg/ml
Glutamic Acid	27.6 mg/ml
Sodium Chloride	11.5%
Amino acids & protein	52.7%
Glutamine / Amino Acids	26.7%

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Table.3. Typical A.A. Profile from Umamizyme action on Wheat Gluten.

Amino Acid	Mg/ml	Amino Acid	Mg/ml
His	2.29	Ala	3.16
Arg	4.86	Pro	9.35
Asn	2.87	Met	3.20
Gln	3.32	Val	5.80
Ser	5.51	Lys	1.97
Asp	0.00	Phe	7.89
Gly	1.22	Ile	5.11
Glu	27.64	Leu	10.99
Thr	3.16	Tyr	4.77

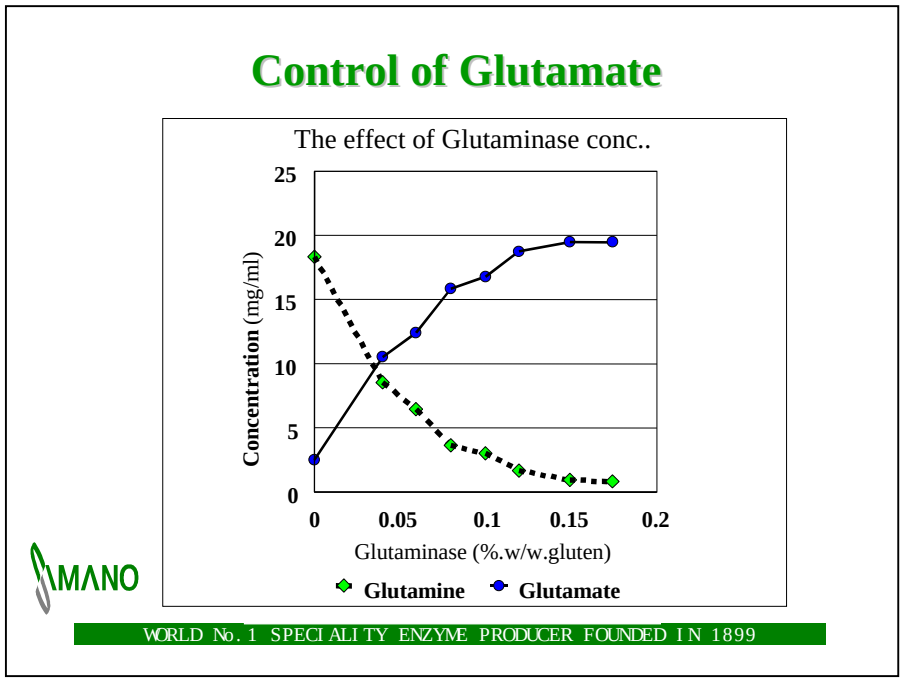

Figure 3. Graph demonstrates the control of Glutamate by Glutaminase.

In acid Hydrolysates, Glutamine appears as glutamate. By using Amano Glutaminase™,this allows the controlled conversion of Glutamine to Glutamate. This makes a very adaptable system where glutamate needs to be excluded or controlled.

Key facts about amano's savoury flavour producing enzyme umamizyme™

Amano name - Umamizyme™
Organism *Aspergillus oryzae*
Optimum Conditions; pH: 7.0, Temperature: 50°C
Description of action Proteinase / Aminopeptidase
Add Glutaminase F "Amano" 100™ to increase Glutamate level.
Safe organism, neutral pH range
One enzyme system producing negligible bitterness
Can be used in conjunction with Amano Glutaminase F100 to create non-declarable glutamate.

5 SUMMARY

We have briefly shown some ideas of how Amano Speciality Enzymes can be used to add value to grain products. The potential for new speciality enzymes is far from exhausted. Amano is always prepared to listen and assess the commercial viability of new and novel enzymes, which address specific industrial needs. In the area of Grains Processing we have proposed the following possibilities: -

AMT1.2L™ syrups	The manufacture of Maltotriose
Transglucosidase L "Amano"™ Oligosaccharides	The manufacture of
CGT "Amano"™ Cyclodextrins	The manufacture of α –
Umamizyme™ flavour	The creation of a savoury base
Glutaminase™ glutamate	The creation of "natural"

There are several innovative, speciality, high value grains products, which can be created using very concentrated, unique, Speciality Enzymes. If you have some interesting ideas and want to develop a novel concept, please talk to us at Amano.

I would like to give thanks to the following contributors and researchers who made this paper possible: -

T. Sakai, K. Takami, S. Mori, K. Hirano, K. Andou, Y.Oyhabu

Also thanks to Tony Godfrey of E.T. Consulting

STARCH DEGRADATION PATTERN IN BREAD AND THE CORRELATION WITH TEXTURAL CHANGES OVER TIME

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1 INTRODUCTION

Several theories have been put forward regarding the factors involved in the firming of bread crumb, or staling. Despite much research on the subject, the mechanism of bread staling remains under debate. Zobel (1996, ref. 1) proposed a comprehensive staling model based only on known starch molecular conformations and properties. In zobel's model gluten is relatively inert to changes over time and appears to have only minor effects on staling. Martin and Hoseney (1991, ref. 2) had previously proposed the divergent hypothesis that bread firming may be a result of hydrogen bond interaction between gelatinised starch and gluten. Certain alpha-amylases inhibit staling of bread. Martin and hoseney proposed that the dextrins formed during hydrolysis interfere with the starch / gluten interaction and thereby retard the staling process. Gerrard *et al.* (1997, ref. 3) later presented evidence that dextrins are not the direct cause of antistaling, but merely act as a marker. The aim of the present study was to determine if the rate of staling is comparable in starch and wheat breads and how various amylases influence texture and specific molecular events in the starch.

2 MATERIALS AND METHODS

All starch breads were formulated to produce bread like crumb structures in the absence of added gluten. These were prepared according to the method described in ref.4, or modified with respect to the presence or absence of pre-gelatinised starch and water content (Table 1). Wheat bread was prepared according to a lean straight dough procedure using a European breadmaking flour. Texture was measured according to a modified version of AACC method 74-09 using a TA.XT2 texture analyser. Firmness was defined as force (g) at 25% compression. For DSC analyses 300mg samples and 500 µl of water were sealed into Hart DSC capsules and scanned at a constant rate of 90°C/hour from 5–95°C. Retrograted amylopectin was defined as the enthalpy of the transition peak area in the temperature range 50–70°C.

Aggregation was measured as turbidity at 700 nm using a spectrophotometer. GPC was performed using a Waters 510 HPLC pump, Waters 717 auto sampler, a Waters 410 differential Refractometer and a Viscotek T60 dual detector. 3 PLGel 20µm MIXED-A7.5x300, GPC columns in series were used. Ion Moderated Partition Chromatography (IMP HPLC) was performed using 2 BIO-RAD Aminex HPX-42A columns connected in series and a Waters 410 RI detector using Milli-Q water as eluent.

3 RESULTS AND DISCUSSION

3.1 STALING RATE IN WHEAT BREAD COMPARED TO STALING RATE IN STARCH MODEL SYSTEMS

The rate of staling was investigated in several model starch bread systems. Several attempts were necessary and the flow of the development work to create a starch bread featuring the same changes in texture as a wheat bread is illustrated in Table 1. Texture changes from day 0 to day 7 are illustrated by Δg/day.

Table 1. Development of model system to feature changes as in a wheat bread. Water contents are expressed as w/w% of dry matter in dough. Texture changes (Δg/day) measured from day 0 to day 7.

	Method	CONTROL Δ g / DAY	NOVAMYL Δg / DAY	CONTROL DSC: degree of retrogradation	NOVAMYL DSC: degree of retrogradation
a	Starch gel	21	5	~ 0%	~ 0%
b	Starch bread 49% + Pregel (Ref4)	86	38	100%	0%
c	Starch Bread 49% - Pregel	83	32	100%	0%
d	Starch bread 42% + Pregel	157	50	100%	19%
e	Starch bread 42% - Pregel (optimized)	162	63	100%	24%
f	Wheat bread 36%	177	68	100%	23%

Starch gels (Table 1: a) did not resemble wheat bread (Table 1: f) regarding staling properties. By modifying the starch bread model system from the original (Table 1: b) it was possible to obtain a staling rate in the starch bread, which was comparable to the staling rate of wheat bread (Table 1: e and f). In addition the effect of an antistaling enzyme (Novamyl) on retrogradation was also comparable in wheat bread and in the optimized starch bread. These results indicate that the modified starch bread model system can be used for investigations on the effects of various amylases on textural changes in breads and changes in starch at the molecular level.

3.2 HOW DO VARIOUS AMYLASES INFLUENCE TEXTURE, AND CAN THESE CHANGES BE ASSOCIATED WITH SPECIFIC MOLECULAR EVENTS?

Degradation of starch can be performed by a host of different amylases, which reflects the complexity of this polymer. In the present study the amylases presented in Table 2 have been investigated.

Table 2. Characteristics of amylases tested in model system.

Enzyme	Nomenclature	pH optimum	Temperature Optimum	Specificity of amylase
BAN	EC 3.2.1.1 1,4-α-D-glucanohydrolase	5,5	70–75 °C	Endo-amylase
Nagase β-amylase	EC 3.2.1.2 1,4-α-D-Glucanmaltohydrolase	6	60 °C	Exo-amylase
AMG	EC 3.2.1.3 1,4-α-D-Glucanglucohydrolase	5-5,5	75 °C	Exo-amylase
Novamyl	EC 3.2.1.133 Glucan 1,4-α-maltohydrolase	5	65 °C	Maltogenic amylase

How do these amylases influence initial texture changes? The softest crumb at Day 0 is obtained with amyloglucosidase (AMG) but on days 1 and 2 Novamyl treated bread is softest. Beta-amylase shows some effect, but not to the same extent as Novamyl. The thermostable endo-acting amylase BAN was dosed at a quite low level in this experiment to illustrate an interesting phenomenon of thermostable endo-amylases. Texture changes measured during the first 2 days after baking are shown in Figure 1.

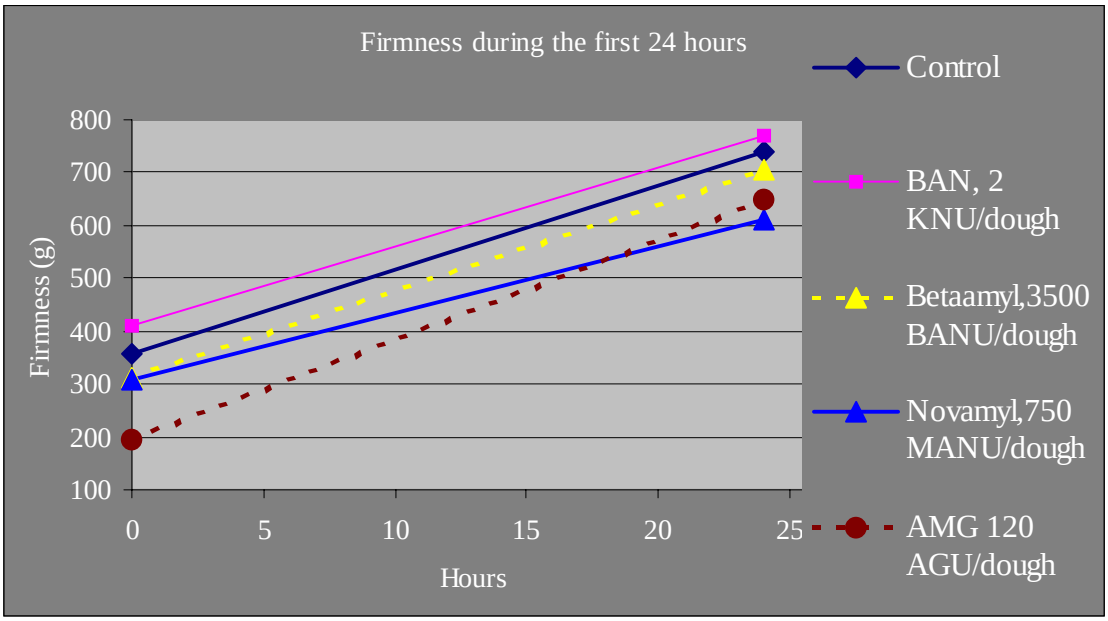


Figure 1. Texture changes of starch bread model system during the first 2 days of storage.

BAN at this very low dosage actually show marginally *increased* firmness of bread crumb during the first 1 ½ days of storage.

Relating this phenomenon to the molecular level, it is well known that solutions of amylose and amylopectin form ordered structures during storage. Already during cooling the amylose fraction starts forming double helices, which will transform into crystals during storage.

When investigating the aggregation of synthetic amylose degraded by BAN, Novamyl or beta-amylase from soybean clear differences in aggregation rate (changes of turbidity at 700 nm) are seen. Such aggregation curves are not as sensitive to dosage as could be expected ~ they actually give a very good picture of differences between amylases. The BAN samples aggregated at the highest rate, followed by Novamyl, whereas the sample treated with beta-amylase is only marginally different from the control. When the rate of aggregation is compared with data from reference 5 showing that the fastest aggregation rate is obtained for dextrins with DP 90 to 110 it is evident that the thermostable endo-amylase hydrolyses the amylose fraction, resulting in dextrins with DP in this range where rapid aggregation is obtained.

Also Novamyl appears to hydrolyse amylose by endo action, but not to the same extent as BAN. These findings are supported by changes in MW distribution measured by GPC after hydrolysis of amylose by BAN, Novamyl and beta-amylase respectively (ref. 6).

The peakshift for BAN and Novamyl is evident, whereas beta-amylase does not change the peak MW of the amylose.

The degradation of amylose by endo acting amylases results in a faster aggregation and a higher rate of formation of ordered structures during cooling of breadcrumb. Data from the literature and our data both point in the same direction. This leads us to the following hypothesis:

Degradation of amylose and the molecular changes caused by this are responsible for the initial firming of bread crumb.

Texture changes during a 7 days storage period are shown in Figure 2.

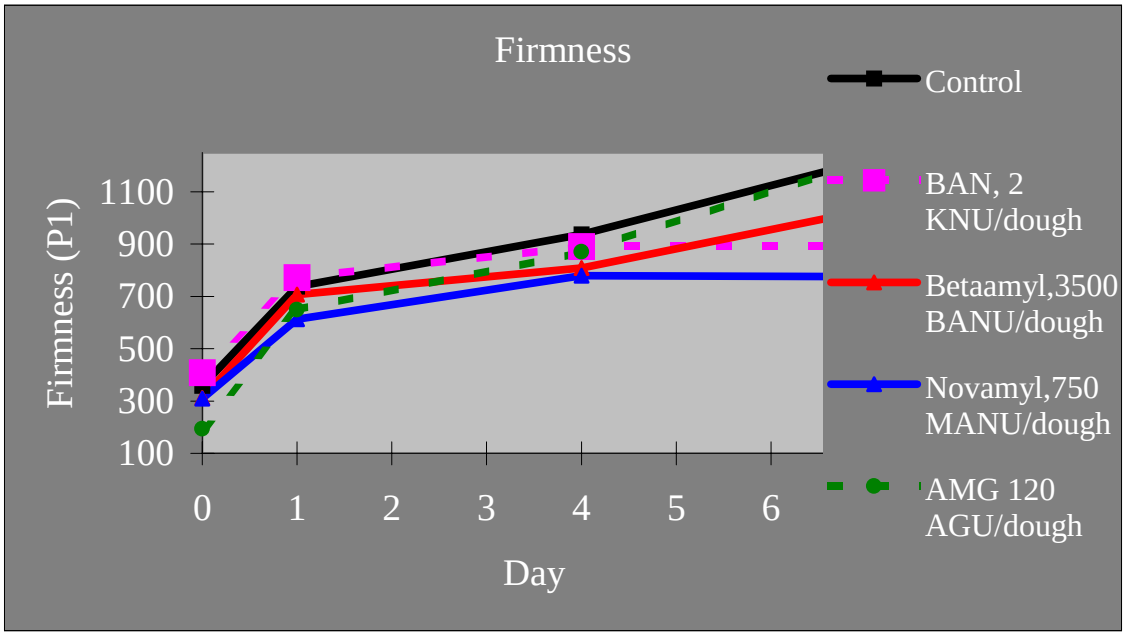
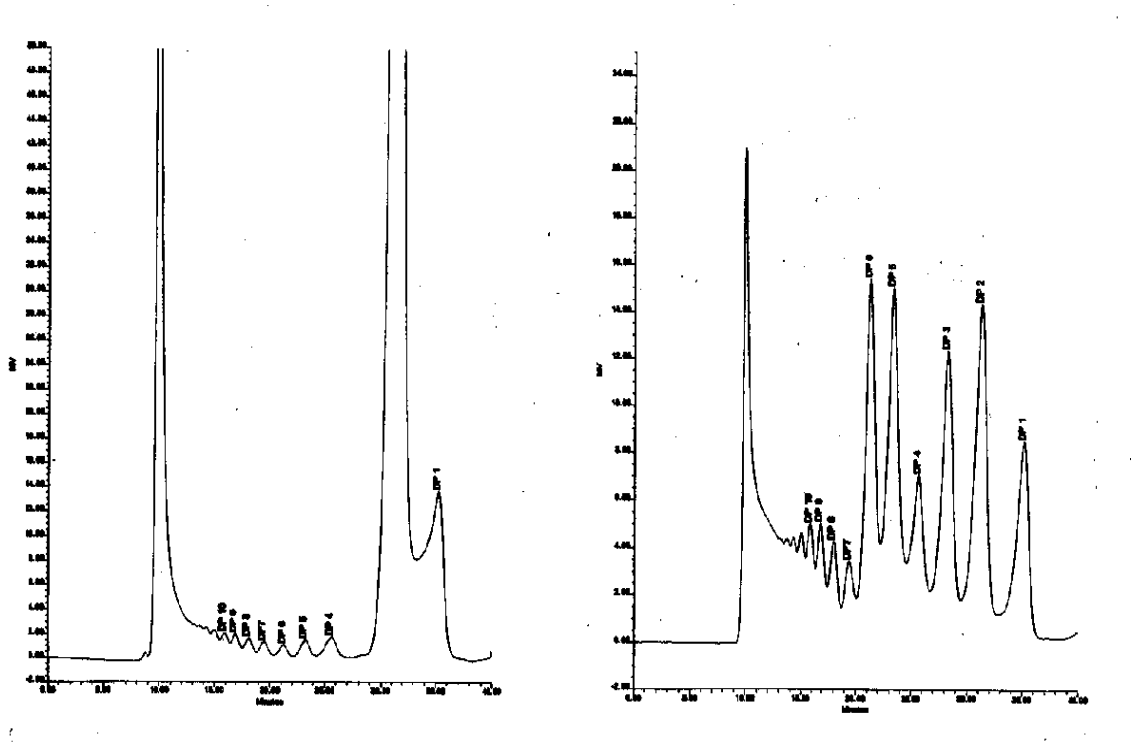


Figure 2. Texture changes of starch bread model system during 7 days storage.

The texture changes caused by the glucose yielding AMG are hardly different from the control, whereas the maltose yielding soy beta amylase does have some antistaling effect. It is evident that the low dosage of the endoamylase BAN and the maltogenic amylase Novamyl show the best antistaling effect. When the selection criteria is the ability to reduce firmness over time, BAN and Novamyl both seem to qualify as antistaling enzymes. However it is commonly known that thermostable endo amylases such as BAN have to be dosed very carefully, as it is very easy to get too high a dosage, which will result in a gummy/sticky crumb without elasticity. Conversely Novamyl is extremely difficult to overdose. In addition to the reduced firmness, Novamyl also retains the elasticity of breadcrumb over time.

Can these differences in texture properties be explained by looking at the molecular level?

When a suspension of amylopectin is hydrolysed by the endo amylase BAN or the maltogenic amylase Novamyl for an extensive period such as 24 hours, and the hydrolysate is analysed by IMP HPLC, the chromatograms shown in Figure 3 are obtained.



Novamyl BAN

Figure 3. IMP HPLC chromatograms of amylopectin hydrolyzed by Novamyl and BAN, respectively

What is noteworthy is that while the endo-amylase BAN hydrolyses or rather liquefies the amylopectin by a total degradation, the maltogenic amylase Novamyl primarily forms DP 1-7, naturally most DP 2, and leaves a limit dextrin ~ an amylopectin with shorter branches but with the backbone of the structure intact. This illustrates that despite Novamyl showing both exo and some endo action, this amylase does not hydrolyse amylopectin as aggressively as the thermostable endo amylases. Novamyl does not liquefy starch.

The retrogradation of samples was determined by DSC and plotted against firming rate of different enzymes to investigate the association between firming rate and retrogradation of amylopectin. Each data point on these graphs represent a different dosage of enzyme.

A good correlation was obtained between firming rate and retrograded amylopectin in the samples relative to the control for BAN and Novamyl, whereas the beta amylase did not show as good a correlation.

These data point in the direction that the action of an exo acting enzyme does not hydrolyse or modify the amylopectin enough to prevent retrogradation of starch, although it does not appear to influence firming rate greatly. The endo acting BAN and the maltogenic amylase influence starch retrogradation, and these changes correlate with firming rate.

A good correlation was obtained between amount of dextrins DP 1-7 in the samples in % of total starch hydrolyzed and firming rate.

The dextrin pattern is however very different in bread samples treated with endo- or maltogenic amylase or exo-amylase. DP 1-7 is determined in the BAN and Novamyl samples whereas only DP 1+2 can be determined in the beta-amylase and AMG samples.

The fact that the dextrin pattern in the enzyme breads is so different, but that the firming rate still shows good correlation to the % of total starch hydrolysed, may suggest that a modification of the starch is responsible for the antistaling effect of certain amylases, and not the presence of specific dextrins.

The presented results have lead us to the conclusion:

The rate of staling in a starch bread model system and in a wheat bread are comparable.

Gluten protein is not needed to explain staling.

Aggregation of amylose is the principle factor causing initial firmness.

Two factors strongly correlate with firming rate: Retrogradation of amylopectin and mount of dextrins below DP7 in crumb, from degraded starch

The ideal antistaling enzyme modifies amylopectin outer chains leaving the backbone of the structure intact.

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UNDERSTANDING AND PREDICTING ENDOXYLANASE FUNCTIONALITY IN BREADMAKING

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1 INTRODUCTION

Endoxylanases are arabinoxylan (AX) degrading enzymes. Despite their widespread use in the breadmaking industry, their mode of action has only recently been elucidated to some extent. Theoretically, endoxylanase functionality in dough is the result of five simultaneous actions on the AX population. Endoxylanases can degrade the water extractable AX (WE-AX) to lower molecular weight. They can cut the water unextractable AX (WU-AX) without solubilising them. They can decrease the WU-AX content through solubilisation. By doing so, they can increase the solubilised AX (S-AX) content. Finally, they can degrade such S-AX. The study of the impact of each of these actions through fractionation reconstitution breadmaking experiments with selective in vitro modification of AX fractions (Courtin *et al*., 1999) together with previous literature data (Kulp and Bechtel, 1963; Maat *et al*., 1992; Gruppen *et al*., 1993; Qi Si *et al*., 1993; Rouau *et al*., 1994; Vanhamel *et al*., 1993; Krishnaray and Hoseney, 1994; Courtin and Delcour, 1998) led to the idea that beneficial functionality of some endoxylanases in breadmaking is based both on lowering the concentration of WU-AX and increasing the level of high molecular weight S-AX in a dough. We hypothesised that endoxylanases with a selectivity towards WU-AX would perform well in breadmaking and that, hence, endoxylanases with a bias towards WE-AX and S-AX would be less effective in improving bread characteristics.

To corroborate this hypothesis, we wanted to find a way of measuring endoxylanase selectivity towards WE-AX and WU-AX, and, secondly, to test it through the use, in breadmaking, of two endoxylanases with different selectivity. The overall objective was to increase understanding of AX and endoxylanase functionality in breadmaking.

2 MATERIALS

All reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. Standard P-82 pullullans were purchased from Showa Denko K.K. (Tokyo, Japan).

Bacillus *subtilis* endoxylanase (enzyme A), free from amylase, glucanase and protease activities, was obtained from Puratos NV (Groot-Bijgaarden, Belgium). Shearzyme (from Aspergillus *aculeatus*, enzyme B) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Enzymes from various other suppliers were tested as well. Endoxylanase activity (nKat/ml) was determined through incubation of the enzyme with excess wheat

WE-AX at 30°C in a sodium acetate buffer (pH 6.0). Reducing sugar formation was then determined by gas liquid chromatography as described by Courtin *et al*. (2000).

Additive free Standaard B and Nachtegaal flours were obtained from Ganda Molens (Antwerpen, Belgium) and Meneba Meel (Rotterdam, The Netherlands) respectively.

WE-AX was isolated from laboratory milled Estica flour as described by Loosveld *et al*. (1998) and was approximately 90% pure. Squeegee starch was prepared as a source of native WU-AX as described by Courtin *et al*. (1999). Besides starch (ca. 80%), WU-AX was the largest fraction (ca. 6.2%).

3 METHODS

3.1 ENZYME SELECTIVITY ASSAY

To obtain preliminary standardisation of endoxylanase activity, the azurine crosslinked AX procedure described in Megazyme Data Sheet 8/94 (Megazyme, Bray, Ireland) was used.Both the WE-AX and the squeegee starch and also a combination thereof were incubated (15 min, 30°C, sodium acetate buffer, 25 mM, pH 6) with an appropriate level of endoxylanase. Solubilisation of WU-AX was measured through total sugar analysis of the supernatants. Degradation of S-AX and degradation of S-AX were assessed through high performance size exclusion chromatography (HP-SEC) and reducing xylose analysis as described by Courtin *et al*. (2000). A selectivity index was defined as follows:

An index value approaching zero implies increased selectivity for WE-AX. If its value approaches infinity, this implies selectivity for WU-AX.

$$I_{Specificity} = \frac{\text{Xylose solubilized from WU - AX}}{\text{Reducing Xylose formed from WU - AX and WE - AX}}$$

3.2 BREADMAKING PROCEDURE

Breadmaking was performed by experienced bakers at both Vamix NV (Gent, Belgium) and Meneba Meel BV (Rotterdam, the Netherlands). A lean recipe (800 g flour, 2.0% yeast, 2.5% sugar, 2.0% salt and 15 ppm ascorbic acid) was used for both flours (Standaard B and Nachtegaal). For Standaard B, dough was mixed for 15 min in an Arthofex mixer and fermentation was 80 min in total. For Nachtegaal, dough was mixed for 9 min in a spiral mixer and fermentation was 160 min. Endoxylanases A and B were added at increasing concentrations (0 to 2.1 nKat/g flour for enzyme A and 0 to 6.0 nKat/g flour for enzyme B). Baking absorption was manually determined.

General dough characteristics during the process (stickiness, elasticity, manually determined baking absorption) and general bread characteristics (loaf volume, crumb structure, initial crumb hardness and staling) were assessed.

3.3 ARABINOXYLAN ANALYSIS

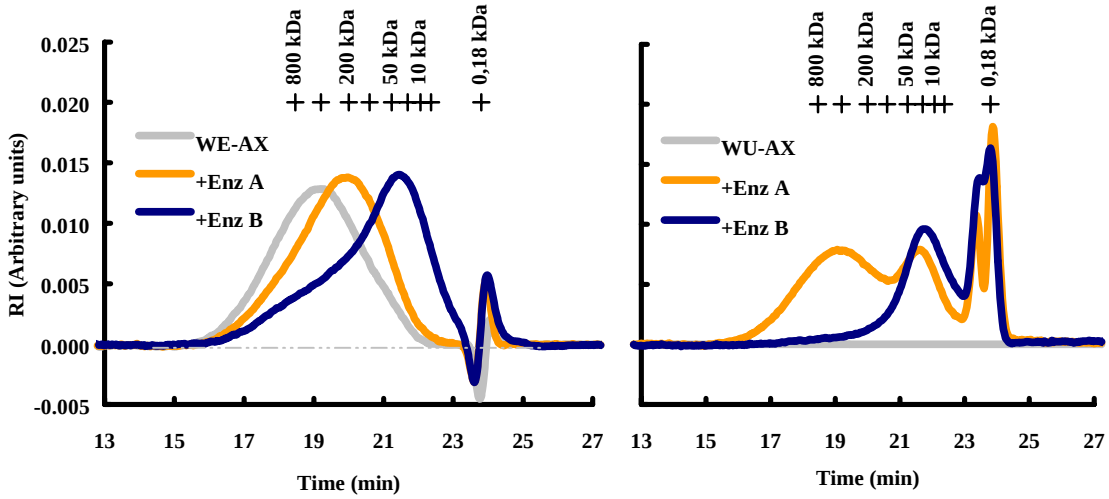
For a study of the AX population after mixing, fermentation and baking, samples withdrawn at the different stages of breadmaking were frozen in liquid nitrogen and freeze dried. After grinding and sieving (250 µm), samples were extracted at 4°C with demineralised water (4°C) for 15 min under continuous shaking. Suspensions were centrifuged (10,000g, 4°C, 15 min) and lyophilised. The dried material was treated with boiling water to inactivate endoxylanases. After a second centrifugation step, the supernatants were analysed for total sugar content and reducing sugar content with gas liquid chromatography and were analysed by HP-SEC (Courtin *et al*., 2000).

4 RESULTS AND DISCUSSION

4.1 ENZYME SELECTIVITY

When screening 10 commercial and experimental endoxylanases with the above described selectivity assay a wide selectivity range was observed. In what follows, the results for the two endoxylanases that were subsequently selected for the breadmaking trials (enzymes A and B) are discussed. Calculated as outlined above, enzyme A and enzyme B had selectivity indices of ca. 150 and ca. 5 respectively.

Figure 1a shows the size exclusion profiles of the degradation of the WE-AX substrate by the two enzymes. The WE-AX substrate was degraded from 400 kDa to 200 kDa by enzyme A and to 20 kDa by enzyme B. When the squeegee starch substrate was incubated with the same enzyme concentrations as in the previous test, enzyme A solubilised WU-AX to a significant extent with the formation of high molecular weight S-AX while enzyme B solubilised much less and much smaller AX fragments. HP-SEC profiles of the S-AX are shown in Figure 1b.



(a) (b)

Figure 1. HP-SEC profiles of (a) WE-AX and (b) squeegee starch incubated with enzymes A and B.

4.2 BREADMAKING

When the enzyme dosages in the recipe were up to the highest levels, baking absorption had to be decreased considerably in order to compensate for dough stickiness (Table 1).

Table 1. Baking absorptions for Standaard B and Nachtegaal flour upon addition of increasing dosages (nKat/g flour) of enzymes A and B.

Enzyme A			Enzyme B		
Dosage	**Standaard B**	**Nachtegaal**	**Dosage**	**Standaard B**	**Nachtegaal**
0	58.7	58.0	0	58.7	58.0
0.08	58.5	58.0	0.12	58.5	58.0
0.17	58.1	--	0.27	57.7	57.0
0.33	57.7	57.5	0.62	57.7	57.5
0.67	55.7	58.0	1.43	57.2	57.0
1.33	53.7	55.0	3.28	56.7	56.5
2.67	52.7	54.0	7.53	55.2	56.0

At lower enzyme dosages, the decrease in baking absorption was minimal to non-existent. Overall, enzyme A gave a fair dough after mixing which improved during fermentation. Enzyme B, on the other hand, gave a good dough after mixing, but its handling properties deteriorated during fermentation.

WU-AX solubilisation at the corresponding steps during the process is represented in Figure 2. As can be seen in Figure 2a, solubilisation sharply increased with dosage of enzyme A. A second observation is that for enzyme A almost all solubilisation was already accomplished after mixing. This is very different for enzyme B. From Figure 2b it is clear that solubilisation after mixing was rather low. It more than doubled after fermentation, but didn't reach the solubilisation accomplished by enzyme A.

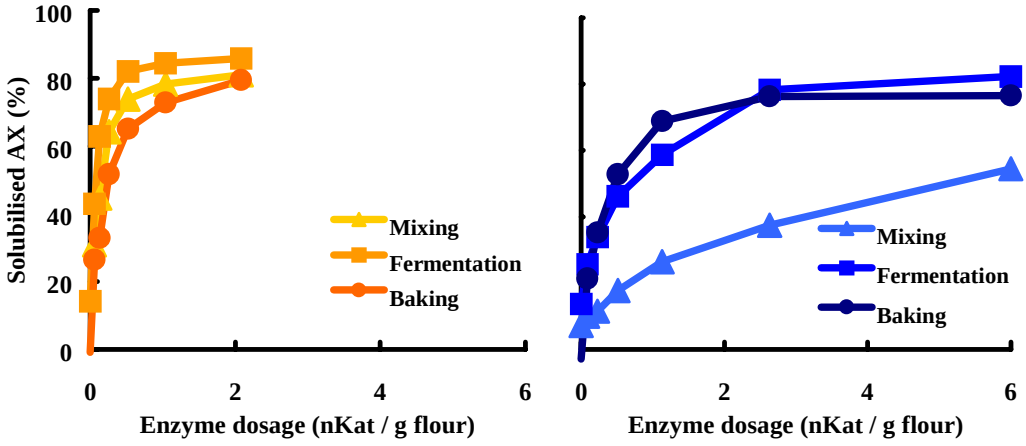
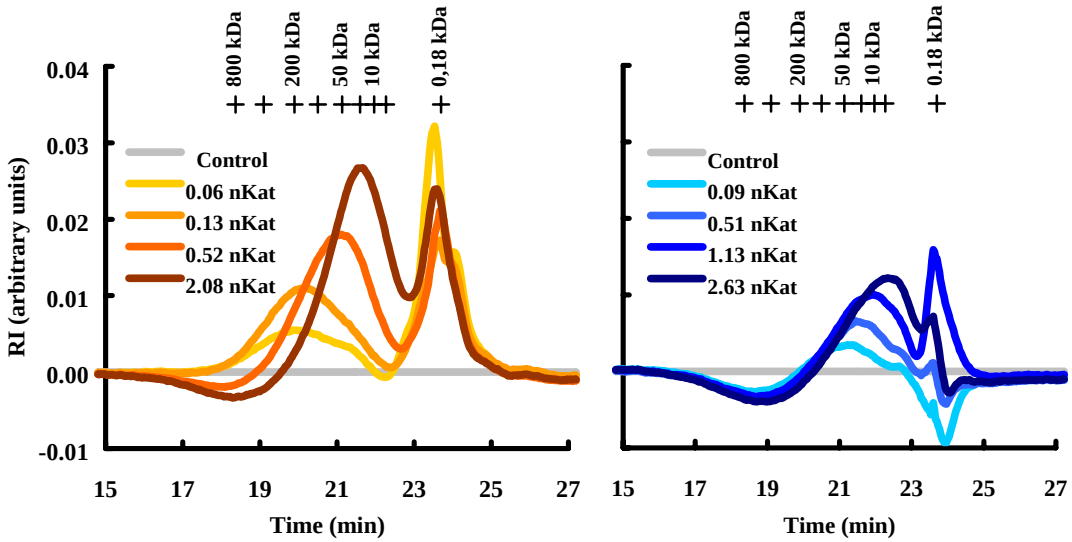


Figure 2. Solubilisation of WU-AX (%) in dough after mixing, fermentation and baking with increasing dosages of (a) enzyme A and (b) enzyme B.

To obtain a clear view on the molecular weight profiles of the WE-AX and S-AX in the dough/bread, size exclusion chromatography profiles of the reference dough/bread extracts were substracted from the profiles made of the endoxylanase added dough/bread extracts. This results in profiles that represent the net solubilisation and degradation of AX in comparison with the reference state. Logically, the reference is then represented as a flat line on the graph.

Figure 3a represents the profiles for Standaard B flour after mixing and with enzyme A added. At low dosages, rather high molecular weight AX were brought in solution after mixing. With the highest dosage, an apparent molecular weight of 20 kDa was obtained. For most dosages, a significant proportion of the AX had a molecular weight exceeding 50 kDa. In contrast, for enzyme B (Figure 3b), most of the AX solubilised were of molecular weight below 50 kDa. At higher dosages, the molecular weight even droped to 1-3 kDa. The area under the curves further indicates that much more AX was solubilised with enzyme A than with enzyme B, as could already be seen in Figure 2 and was predicted from the selectivity index.



(a) (b)

Figure 3. HP-SEC profiles of solubilised WU-AX and degraded WE-AX in dough after mixing with increasing dosages of (a) enzyme A and (b) enzyme B.

The profiles of the AX population after fermentation are similar to those after mixing. Profiles both for enzyme A as B have shifted somewhat towards lower molecular weights. For enzyme B the rise in solubilisation compared to the mixing step is also evident. For baking, profiles are again similar (results not shown).

The dosage-loaf volume response curves are shown in Figure 4. For Standaard B flour (Figure 4a), loaf volume increased with enzyme A up to an optimal concentration and then drops somewhat, probably as an effect of both WU-AX solubilisation and degradation. Enzyme B, on the other hand, first resulted in a small but significant loaf volume decrease, at a dosage resulting in little AX solubilisation but significant

degradation of WE-AX. At higher dosages, as more WU-AX is solubilised, enzyme B resulted in a volume increase of about 5%. For Nachtegaal flour (Figure 4b), the enzyme range used resulted for both enzymes in a plateau level that was reached pretty fast, and was higher for enzyme A than for enzyme B.

Both enzymes improved the crumb structure of the bread produced from both flours. The effect was somewhat more clear with enzyme A. Initial crumb hardness was significantly depressed for both enzymes at increasing concentrations, and for enzyme A somewhat more than for enzyme B. There are probably several factors that can account for this, such as loaf volume and/or gas cell redistribution, water redistribution. Enzyme A did not affect staling rate. Enzyme B showed a small drop in staling rate after 2 days.

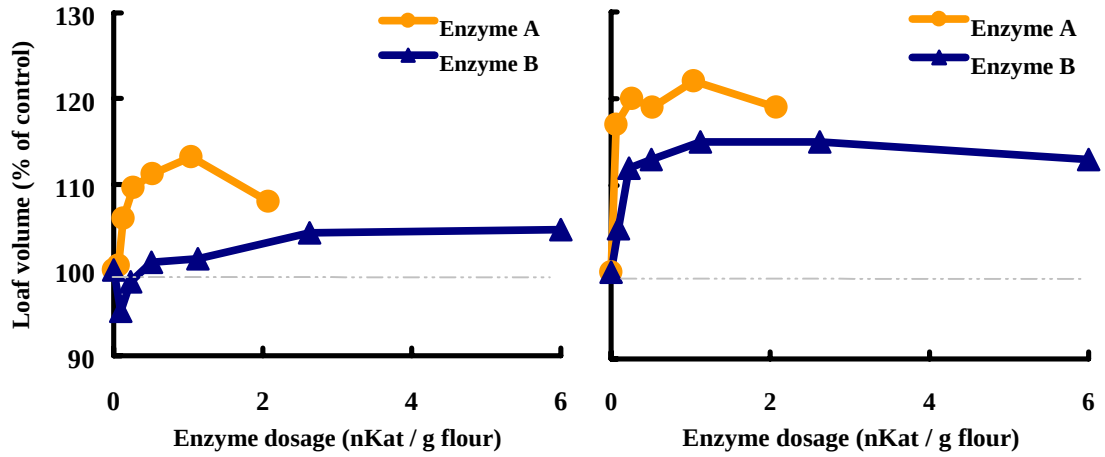


Figure 4. Dosage – loaf volume response curves for breads made from (a) Standaard B flour in a short process and (b) Nachtegaal flour in a long process, with increasing dosage of endoxylanases A and B.

5 DISCUSSION

The results presented above seem to corroborate the hypothesis that both the ability to remove WU-AX and to preserve high molecular weight WE-AX/S-AX are important aspects for endoxylanase functionality in breadmaking.

It is not clear at present why removal of WU-AX is beneficial for breadmaking and why an increased WE-AX/S-AX content has a positive effect. As the one material is water extractable and the other one water unextactable, it is safe to assume that both effects are based on different mechanisms. Indeed, WU-AX, partially or totally embedded in their cell wall structure, are able to hold water. WE-AX, on the contrary, can, by their very nature, not retain water. They can impart viscosity to an aqueous and dough system and have the (theoretical) ability to interact with other flour components more than do their water unextractable counterparts.

The difference in speed at which WU-AX are brought in solution by enzymes A and B during mixing and the corresponding loaf volume responses may indicate that not only the removal of WU-AX is critical, but also the timing at which this process takes place. Part of the larger impact on loaf volume of enzyme A than of enzyme B might be due to the fact that enzyme A is able to solubilise WU-AX largely during mixing, allowing other components (especially gluten) to be better developped or to be better hydrated during the mixing step.

This would also explain why enzyme B has a negative effect on loaf volume at the lowest dosages and a positive effect at the higher ones. Indeed, at the lower dosages, almost no water unextractable material is solubilised, but WE-AX is degraded (as indicated by the negative peak on Figure 3b). At higher dosages, an increased solubilisation takes place during mixing, probably counteracting the negative effect of degradation of the WE-AX to lower molecular weight. This would further imply that, if water content can be controlled and if the endoxylanases are not limited in their breakdown of the WU-AX, both enzymes A and B will have the same effect on loaf volume provided the enzyme dosage is sufficient.

It is clear that the uptake of water by the dough system and the release of water by the degradation of the WU-AX must be balanced in order to keep good dough handling properties without excessive stickiness.

Whether WE-AX functionality can be ascribed to increased gas cell stabilisation, secondary network formation, increased water holding capacity of the flour or another mechanism is not clear from these data.

Furthermore, the molecular mechanism responsible for the difference in activity patterns between enzymes A and B was not investigated. Results obtained by Gruppen *et al*. (1993) would indicate that differences lie in the ability of the enzymes to cut near branch points, which is related to the molecular structure of the enzymes.

6 CONCLUSIONS

We identified two endoxylanases with widely differing properties towards WE-AX degradation and WU-AX solubilisation. In breadmaking, use of these enzymes resulted in different solubilisation of WU-AX as a function of time and different patterns of S-AX and WE-AX degradation. These differences in AX solubilisation and degradation seemed to be related to bread and breadmaking characteristics.

More specifically, the ability to remove WU-AX from dough and the balance between removal of WU-AX and formation of S-AX with high molecular weight are key parameters in endoxylanase functionality. Also the rate at which these processes take place is probably also important.

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TECHNOLOGICAL AND BIOCHEMICAL EFFECTS OF EXOGENOUS LIPASES IN BREADMAKING

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1 INTRODUTION

Despite the fact that lipids represent a minor fraction of wheat flour, they have several functions in breadmaking. Lipids affect the rheological properties and the color of the dough (Frazier *et al*., 1977). These effects are mainly attributed to the free polyunsaturated fatty acids (PUFA) which behave as intermediates in coupled oxidation reactions. During mixing, free PUFA are oxidised by endogenous lipoxygenases and form hydroperoxides and free radicals (Graveland, 1973). These molecules can themselves oxidise the protein thiols and the carotenoid pigments (Hoseney *et al*., 1980; Nicolas *et al*., 1983).

MacRitchie and Gras (1973) observed by reconstitution experiments, that wheat lipids strongly affect the bread volume. These properties vary according to the amount of lipids added to the dough and their polarity. Gan *et al*. (1995) suggested that the changes in bread volumes observed in reconstitution experiments result from a competition between lipids and proteins located at the air-water interfaces. This competition may, either, stabilised or destabilised, gas bubbles.

Wheat endogenous lipase is often described as a key enzyme on the quality of wheat flour during storage. This enzyme improves the quality of the flour after a short time period, the maturating period, but degrades it after a prolonged storage (Castello *et al*., 1998b). Endogenous lipase hydrolyses the TAG into DAG, MAG and FFA. The release of FFA during storage allows to increase the intensity of the oxidation reactions during mixing by supplying more substrates to the endogenous lipoxygenases (Tait and Galliard, 1988). After a prolonged storage, the amount of FFA becomes too high and degrades the quality of the gluten network.

Recently, some autors suggested to use an exogenous 1,3 specific lipase as a bread improver. According to these autors, exogenous lipases improve the rheological properties of the dough, increase the bread volume and delay the bread staling (Olesen *et al*., 1994; Si and Hasen, 1994; Poulsen and Soe, 1996).

The purposes of this work were to verify the technological effects of exogenous lipases in a classical french bread recipe and process, to analyse the biochemical changes

induced by these enzymes in dough and to evaluate the biochemical origins of their technological effects.

2 MATERIALS AND METHODS

The flour used in this work was an untreated, improver-free, straight-grade flour commercially milled by les Moulins Soufflet (Nogent/Seine). The enzyme was a 1,3 specific lipase with an activity of 59000 LU / kg, according to the procedure described by Greenough *et al*. (1996).

2.1 BAKING TESTS (FRENCH BAGUETTES)

Baking tests were performed according to the BIPEA method. 2000 g of wheat flour, 2.5% of dry yeast, 2.2% of salt, 20 ppm AA and 64% of water were mixed in an Artofex type mixer for 4 min at low speed and 21 min at high speed. Doughs were proofed for 20 min at 27°C, weighed out in 350 g pieces, rested for 20 min at 25°C and molded into « french baguettes ». Pieces of dough were then proofed for 120 min, before baking at 250°C for 25 min.

The dough handling properties (elasticity and extensibility) were evaluated, all along the breadmaking process, according to the BIPEA method. The bread volume were evaluated, after cooling, by the rape seed displacement method.

2.2 LIPID ANALYSIS

After mixing or proofing, dough samples were collected, deep frozen into liquid nitrogen and kept in solid carbon dioxide for a maximum of 24 h. Lipids were extracted, fractionated and quantified according to the procedure described by Castello *et al*. (1998a).

3 TECHNOLOGICAL EFFECT OF EXOGENOUS 1,3 SPECIFIC LIPASES

Figure 1 shows that exogenous lipases increase the dough elasticity and decrease the extensibility. If a slight increase of the elasticity allows to improve the dough handling properties, an excess of elasticity degrades these properties.

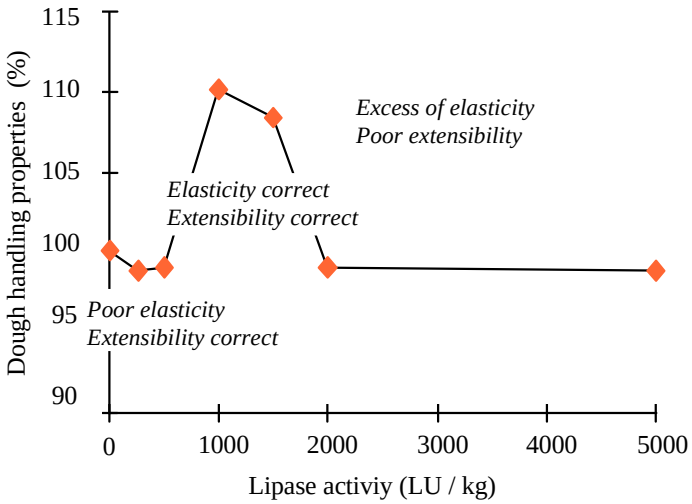

Figure 1. Evolution of the dough handling properties in presence of exogenous lipase compare to a control dough.

Figure 2 shows that exogenous lipases also modify the bread volumes. We observe an optimal concentration which allows to increase the bread volume. This optimal concentration may vary according to the wheat flour properties and the breadmaking process.

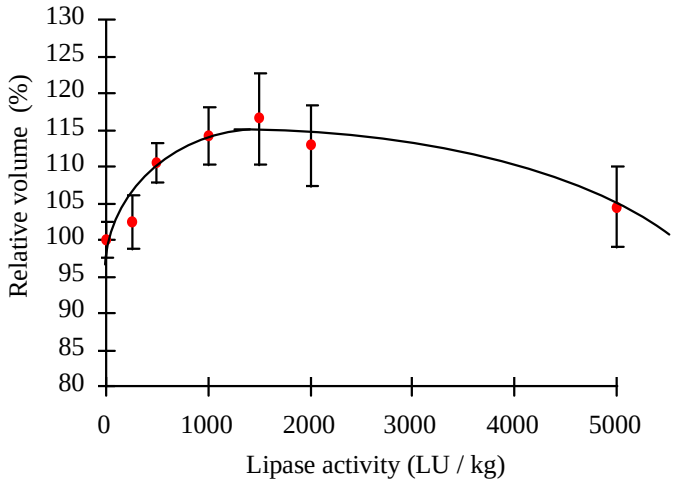

Figure 2. Evolution of the bread volume in presence of exogenous compare to a control dough.

4 BIOCHEMICAL EFFECTS OF EXOGENOUS LIPASES

The biochemical changes induced by an exogenous lipase during dough mixing were analysed as decribed by Castello *et al*. (1998a, 1999).

The results show that exogenous lipases (1,3 specific) hydrolyse TAG and DAG 1-3 fractions into DAG 1-2, MAG and FFA (Figure 3). This reaction indicates that exogenous lipases increase the amount of molecules with emulsifying properties. Among these molecules, the polyunsaturated fractions can be oxidised by endogenous lipoxygenases. This reaction leads to an increase of the lipid oxidation level. The oxidised lipids can themselves co oxidised other molecules such as protein thiols and carotenoid pigments. This means that exogenous lipases increase the level of co oxidation reactions involving lipids.

This pathway is similar to the transformations catalysed by endogenous lipases during the flour maturation period (Castello, 1998b). Exogenous lipases, as well as wheat endogenous lipases, increase the intensity of the oxidation reactions by supplying more substrates to the endogenous lipoxygenases.

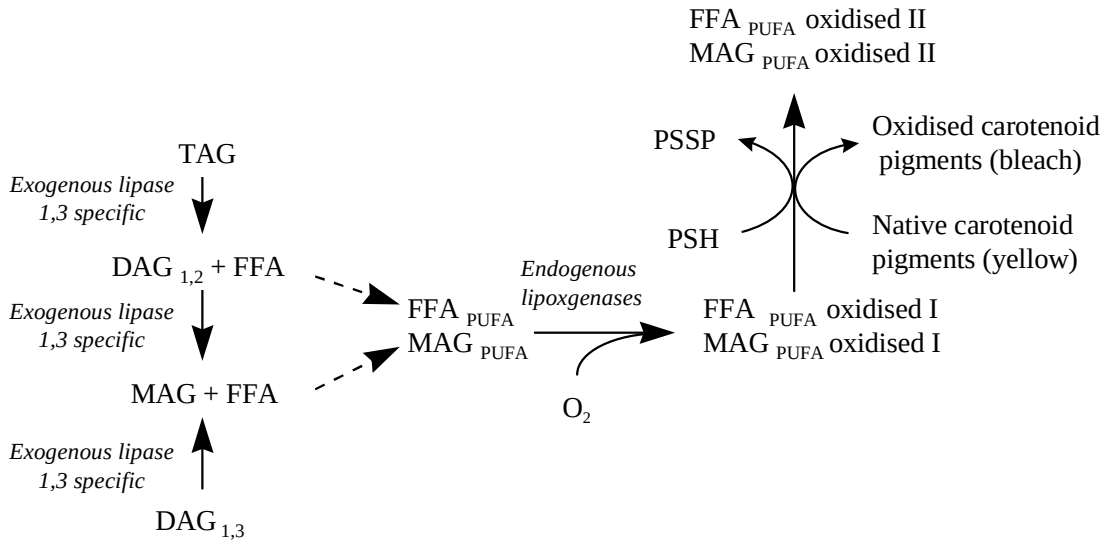

Figure 3. Biochemical changes induced by exogenous lipases during wheat dough mixing.

5 KINETICS OF THE LIPID HYDROLYSIS AND OXIDATION DURING THE BREADMAKING PROCESS

The level of lipids hydrolysis and oxidation were evaluated at the end of mixing and proofing as described by Castello *et al.* (1998a).

Figure 4A shows that the level of lipid hydrolysis at the end of mixing increases linearly with the amount of exogenous lipases added to the dough. During proofing, the lipid hydrolysis, still increases, but it reaches a maximum at about 80%. This kinetic

demonstrates that exogenous lipase is active during the whole breadmaking process, and even during proofing, since at least one half of the hydrolysis occurs during this period.

Figure 4B shows that the evolution of the lipid oxidation is closed to the evolution of lipid hydrolysis. For the lipid oxidation, the reaction occurs mainly during mixing.

These results indicate that the biochemical changes induced by exogenous lipases follow a kinetic which varies according to the enzyme concentration. We think that differences in these kinetics can explain the variations of the technological effects of lipases.

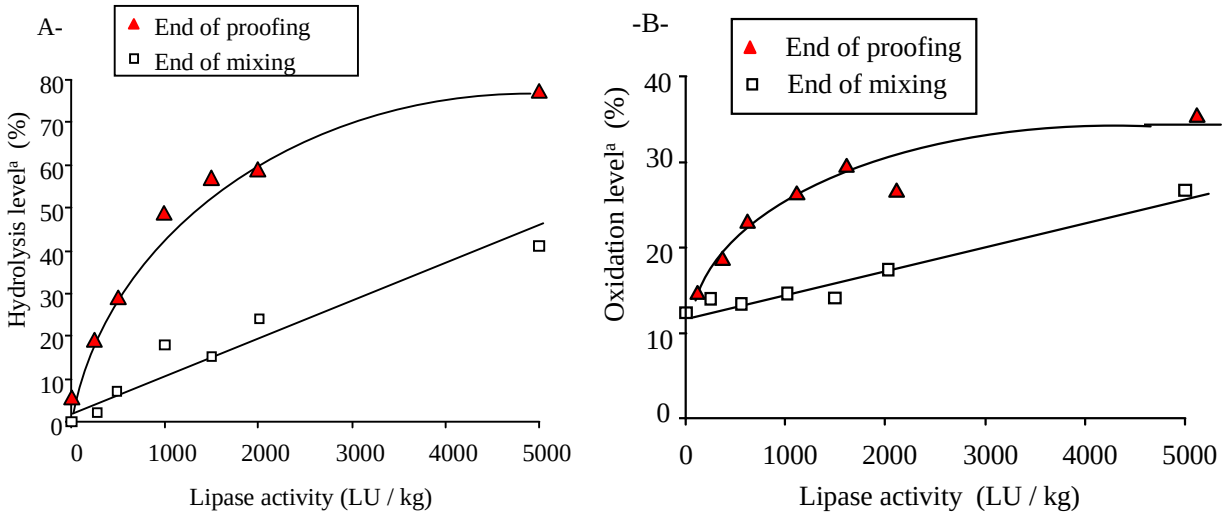

Figure 4. Evolutions of lipid hydrolysis and oxidation levels during the breadmaking process. Calculated as described by Castello et al. (1999).

6 BIOCHEMICAL ORIGINS OF THE TECHNOLOGICAL EFFECTS OF LIPASES

The biochemical changes induced by exogenous lipases can be used to explain their technological effects in breadmaking.

We first suppose that exogenous lipases modify the dough handling properties and the bread volumes, by increasing the coupled oxidation reactions during mixing (Figure 5A). If we compare the dough elasticity (Figure 1) and the levels of lipid oxidation at the end of mixing (Figure 4B), we observe that both of them linearly increase with the amount of lipases added to the dough. We also observed this relationship with a special mixer designed to measure at the same time the dough consistency and the oxygen consumption (Castello *et al.*, 1998c).

We also think that exogenous lipases affect the bread volumes by increasing the oxidation reactions occuring during proofing (Figure 5B). Thus, we observed a linear correlation (data not shown) between bread volumes and the levels of lipid oxidation during proofing.

We then believe that exogenous lipases modify the bread volumes by converting TAG into molecules with higher emulsifying properties. MAG, DAG 1,2 and FFA could, by competing with molecules located at the air water interfaces, stabilise or destabilise the gas as suggested by Gan *et al.* (1995).

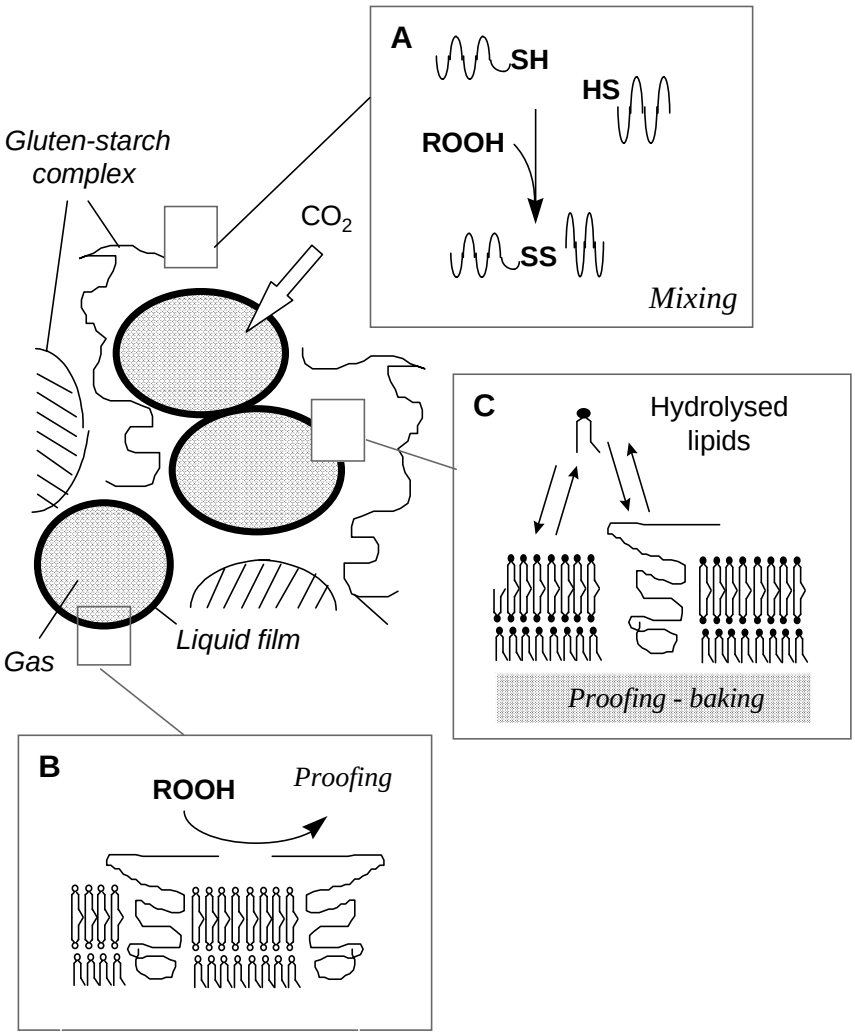

Figure 5. Theoretical models explaining the technological effects of exogenous lipases in breadmaking.

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ENZYME EFFECTS ON BREAD QUALITY AND MICROSTRUCTURE IN HIGH-FIBRE BAKING

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ABSTRACT

We compared the effects of a fungal α-amylase and the combination of α-amylase, lipase and xylanase on high-fibre bread quality and microstructure. The addition of enzymes had a positive effect on the volume, crumb structure and keeping quality of breads supplemented with wheat bran. Microstructural characteristics of the wheat breads were examined by bright field and epifluorescence microscopy. There were notable differences between the samples in the formation of gluten-starch matrix. The observed differences in the swelling of starch during the staling of white wheat bread and the high-fibre breads were consistent with the slower staling rate of the high-fibre breads.

1 INTRODUCTION

Staling of bread is an issue of great importance for the baking industry, as freshness is an important quality parameter of most breads. Staling comprises sensory and physical changes that occur during storage and make the bread less attractive to the consumer. By defining the mechanism of staling it might be possible to improve the keeping qualities of bread. Starch retrogradation and redistribution of water are the most commonly listed causes for bread staling. Although starch has been demonstrated as having a primary role in bread crumb firming, bread also contains other flour constituents such as proteins, pentosans and lipids. Especially in baking with whole meal flours the effects of water-soluble bran components (e.g. pentosans), bran proteins, endogenous enzymes and fibres have been reported to affect the volume and texture of the bread (Laurikainen *et al.* 1998).

In this work the effects of staling on microstructural characteristics of white wheat bread and bread supplemented with 20% wheat bran were examined by bright field and epifluorescence microscopy.

2 MATERIALS AND METHODS

2.1 BAKING

A commercial white wheat flour was used for baking. High-fibre wheat bread was baked with 20% of wheat bran substituted for flour. The optimal water addition was determined in the farinograph. The volume of breads was determined by rape seed displacement. The enzymes used are listed in Table 1.

Table 1. Commercial baking enzymes used.

Enzyme	Substrate
α-Amylase (with β-glucanase side activity): Grindamyl max-life 25, Danisco Ingredients	Starch: amylose and amylopectin (β-glucan)
Xylanase: Pentopan mono BG, Novo Nordisk A/S	Arabinoxylan
Lipase: Lipopan, Novo Nordisk A/S	Triglycerides

Crumb softness and firming rate are important characteristics of bread quality. Crumb firmness was measured at days 0, 1 and 3 to assess the potential shelf life of the breads. The bread crumb firmness was determined as maximum compression force (40% compression, AACC 1983, method 74–09) using the Texture Profile Analysis (TPA) test.

2.2 MICROSCOPY

For confocal laser scanning microscopy pieces of dough were smeared on the objective glass, air dried for 5 to 10 minutes and stained with 0.1% (w/v) Congo red. Images were acquired with Bio-Rad RP/ARG-3 (Bio-Rad Laboratories, Hemel Hempstead, UK) using a 548 nm HeNe laser.

Changes in the appearance and distribution of protein and starch in the breads were studied in samples fixed at the baking day and after storage at room temperature for three days. Pieces of bread crumb from the middle of the loaf were fixed in glutaraldehyde, dehydrated, embedded in plastic and sectioned. For the fluorescence microscopic examination the bread sections were stained with Acid fuchsin and Calcofluor white, and for starch structure studies the sections were stained with Light green and Lugol's solution (Parkkonen *et al.* 1994).

3 RESULTS AND DISCUSSION

3.1 EFFECTS OF BRAN SUPPLEMENTATION ON BREAD QUALITY

Increased consumption of whole grain products and dietary fibre is recommended by health experts on the basis of their positive health effects. Wheat bran is an excellent source of dietary fibre. However, addition of wheat bran in baking results in bread with inferior quality: low volume, poor crumb structure and a bitter flavour. Addition of bran dilutes gluten and starch concentrations in the dough and increases the concentration of insoluble and soluble cell wall material. The bran particles also seem to be mechanically disrupting the structure of the gluten network. This is clearly seen in comparing the confocal images of the wheat dough and the dough supplemented with bran (Fig. 1).

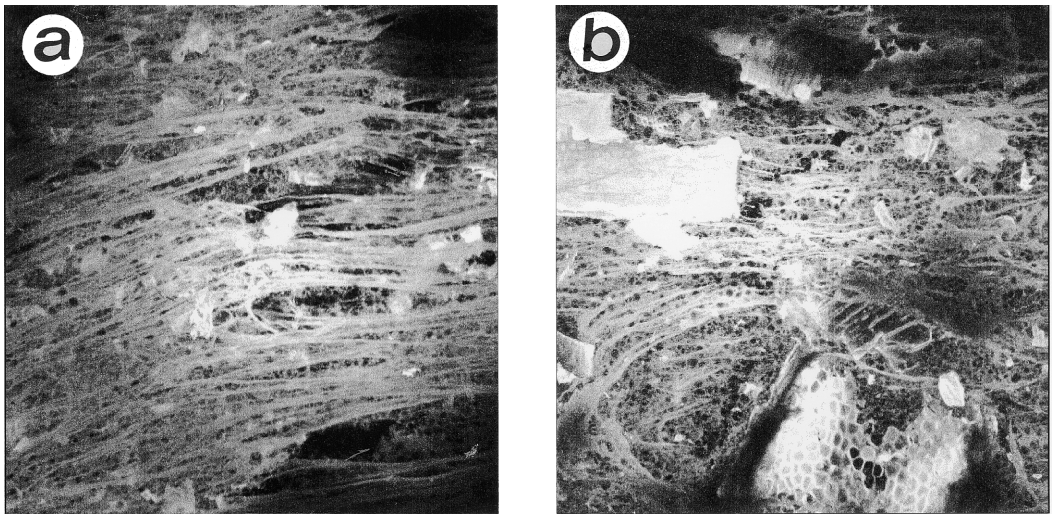

Figure 1. Confocal laser scanning images of the wheat dough (a) and the dough supplemented with wheat bran (b).

Wheat bran supplementation (20%) of wheat flour decreased the specific volume of the breads (Fig. 2). Commercial baking enzymes had a positive effect on the volume and structure of bread supplemented with bran. The fresh bread baked with 20% wheat bran was firmer than the white wheat bread (Fig. 3). However, the firming rate of the high-fibre wheat bread was slower and at day 3 the firmness of the breads was about equal. Commercial baking enzymes had a positive effect on the sofness and firming rate of bread supplemented with wheat bran. The combination of α-amylase, xylanase and lipase was more effective in reducing the staling rate than α-amylase used alone.

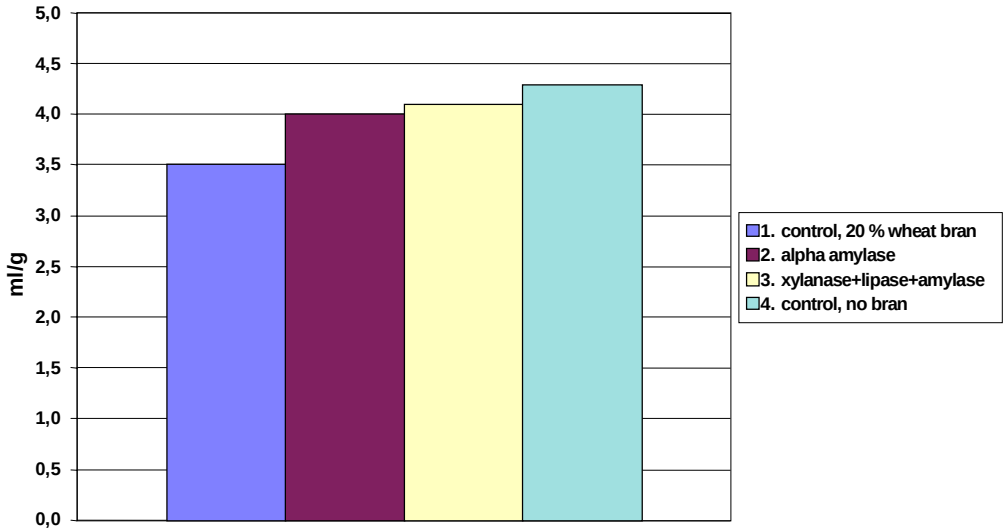

Figure 2. Specific volumes (ml/g) of the test breads.

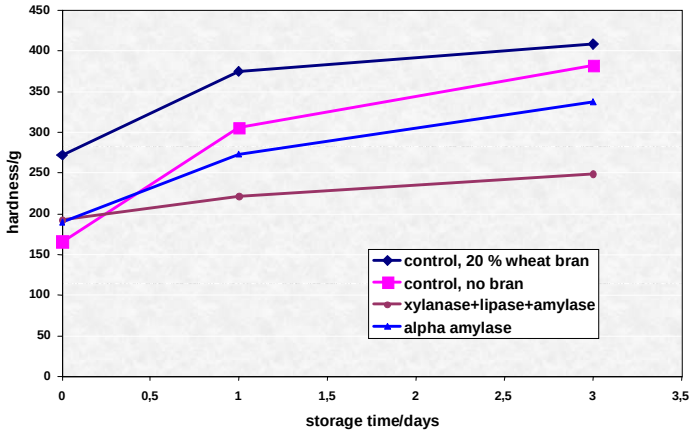

Figure 3. Crumb softness and firming rate. Crumb firmness was measured at days 0, 1 and 3 to assess the potential shelf life of the breads.

3.2 MICROSTRUCTURAL CHANGES DURING STALING

There were notable differences between the samples in the formation of gluten-starch matrix. The structure of the white wheat bread was very homogenous consisting of a continuous gluten matrix with gelatinized starch and occasional small cell wall fragments embedded in it. In the structure of the bread supplemented with wheat bran large bran particles consisting mainly of the pericarp and aleurone layer were a dominating feature. The added enzymes had affected water distribution between the cell wall, starch and gluten phases of the breads. Xylanase treatment has been shown to increase the swelling of starch granules and release of amylose in rye breads (Autio *et al.* 1996). In the 3 d samples starch granules were more swollen than in the samples fixed at baking day and phase separation had taken place. The protein phase had separated from the gelatinized starch and concentrated staining more intensely. The observed differences in the swelling of starch in the staling white wheat bread and the high-fibre breads were consistent with the slower staling rate of the high-fibre breads.

4 CONCLUSIONS

- Wheat bran had a positive effect on the freshkeeping quality of wheat bread.
- Commercial baking enzymes had a positive effect on the volume, structure and freshkeeping quality of high-fibre wheat bread.
- The observed differences in the swelling of starch in the staling white wheat bread and high-fibre wheat bread were consistent with the slower staling rate of the high-fibre bread.

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EXAMINATION OF COMMERCIAL ENZYMES FOR EXTENDING THE SHELF LIFE OF BREAD IN THE UNITED KINGDOM

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Campden & Chorleywood Food Research Association

1 INTRODUCTION

The studies of bread and cake staling, most applicable to the enzyme-based products being developed to-day, were reviewed by Zoebel and Kulp, 1996. The earliest work showed that there are two gel phases in bread crumb, the continuous gluten network and the discontinuous starch phase of gelled granules (Katz, 1934, Sandstedt *et al.* 1954). These phases are initially in intimate contact over the whole surface area of the swollen starch granules. As bread ages, the phases begin to separate and some of the starch granules may be removed from the cell walls and the bread becomes crumbly as well as firm.

It was shown that a baked gluten gel, of a similar moisture content to bread, does not change in firmness with age, but that a mass of gelled starch granules, at an equivalent moisture content, firms in a similar manner to bread (Katz, 1934). More recent studies on gluten by Mita (1990) confirmed that there were no changes in the rheological characteristics of stored gluten gels, which correlated with the firming of breadcrumb. However, the bread crumb firming processes were found to be strongly correlated with the formation of crystallinity in the starch polymers, both in bread and in gelled starch granules. Rigorous studies on the mechanisms of staling in bread and cakes were carried out at CCFRA (Cornford *et al.* 1964, Axford *et al.* 1968, Guy, 1981, Russell, 1982 and 1983).

1.1 THE ROLE OF ENZYMES

Enzymes have a long history of being used as anti-firming agents. Claims for anti-firming effects have been made for all the alpha amylases, including bacterial, fungal, maltogenic and even for cereal types, but not for cereal beta amylase. Other claims are being made for hemi-cellulases and lipases, but not for proteinases. There are conflicting reports about several of these enzymes, as to whether they have true anti-firming effects. It is possible that some of them simply soften the bread by hydrolysis of the starch or protein gel phase during baking, or by increasing the loaf volume, after which it firms at the normal rate to a lower limiting firmness value after 6–8 days storage.

2 CURRENT STUDIES AT CCFRA

Three groups of enzymes, alpha amylases, lipases and xylanases have been claimed to have anti-staling properties. They were examined for their ability to extend the shelf-life of bread in baking trials with breads made from both white and wholemeal flours, using both Chorleywood Bread Process and Spiral mixing technologies. Each enzyme was followed though the processes to study its effect on the processing quality of dough. Samples of bread were examined after baking and cooling for 3 hr for specific volume, moisture content, crumb texture score, crumb firmness and resilience. Further tests of crumb firmness and resilience were carried out at daily intervals during storage trials of up to 7 days at 20 ^{o}C.

Enzymes, which showed good anti-firming effects, were studied in more detail using Sensory Profile Analysis, chemical analysis of the bread and Differential Scanning Calorimetry to examine starch retrogradation.

3 EXPERIMENTAL STUDIES

3.1 GENERAL BAKING PROCEDURES

3.1.1 Recipe

Ingredients	% of flour mass	Batch mass, g
Flour	100	7000
Water	57.8	4046
Yeast	2.5	175
Salt	1.8	126
Ascorbic acid	0.1	0.7
Fat	2.0	140
Fungal enzyme supplement to 80 Farrand units	Added	
Enzymes at different levels of addition based on the manufacturers' recommendation	Added	

3.1.2 Dough making and baking

The doughs were mixed for 3–4 min in a Tweedy mixer to deliver 39 kJ/kg of energy into the dough. The dough was divided (900 g), shaped, proved for 6 min, moulded in four pieces and proved to a fixed height 11cm, for about 45–50 min. The doughs were baked for 30 min at 244 ^{o}C, and cooled for 3h before packing into polythene bags.

3.1.3 Test procedure for assessment of the dough making and baking

1. Dough rheology: unyeasted doughs were mixed and then tested using a frequency sweep in the range 0.1–10 Hz at a strain of 0.0504 (linear region). The test was performed at 25 °C using parallel plate geometry with a gap of 1.1 mm.
2. Dough description by bakers: this assessment is based on the variations in dough from being too stiff, passing through the perfect region of rheology, to being too soft and sticky for use in industrial plant.
3. Moisture of the bread: oven drying methods
4. Firmness and resilience of crumb: the Texture Profile Analysis (TPA) method of Bourne (1970) as adapted to AACC method 74–09, was used with two 2 cylindrical cores of breadcrumb cut from the centre of two loaves.
5. Crumb score: this was made by an experienced bakers on the basis of crumb quality expected in CBP bread, a fine crumb, even distribution of cells and smooth oven spring lines without collapse or too much compression near crusts

3.2 AMYLASES

Trials with amylases were carried out with 4 commercial samples, A–D, recommended for anti-staling performance and our standard fungal amylase, normally employed for loaf volume improvement. The enzymes were tested in white and wholemeal bread manufactured by either CBP or Spiral mixer technology.

Dough characteristics: standard fungal amylase reduced the elastic and viscous modulii of the dough by destroying the damaged starch. This softened the dough before baking and with continuing starch hydrolysis in the baking helped to give an improved volume. All the test amylases gave a similar effect.

Bread characteristics: the effects of added amylases were compared to a control containing a standard fungal amylase at a level required for volume optimisation. All the enzymes gave similar effects on loaf volume, crumb moisture and crumb cellular texture.

Changes in crumb characteristics with age: the four samples of amylase tested gave contrasting results, two were ineffective and two had significant effects, in retarding the firming of the crumb.

The graph of the firming of bread over 4 days versus concentration of the most effective enzyme, A (30 to 120 MANU/100 g of flour) shows a large ant-firming effect (Fig. 1). Enzymes A and B gave similar effects on crumb firmness in white and wholemeal bread made by either the CBP or Spiral technologies.

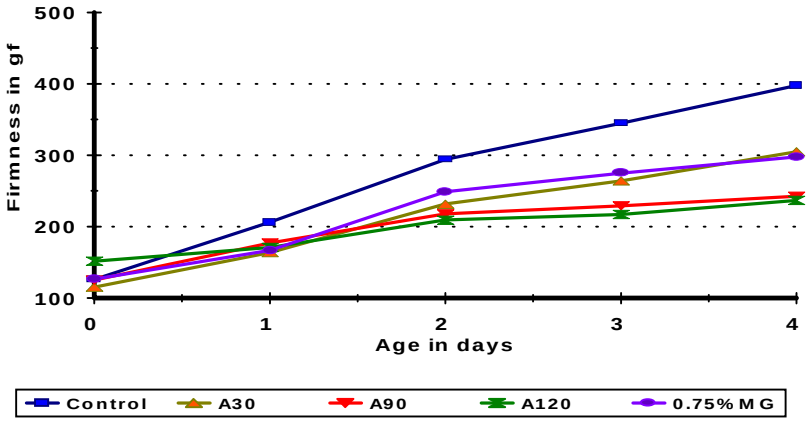

Figure 1. Firmness versus time stored at 20^{o} C for white bread containing amylase A.

The increase in firmness from the initial value at 3 h to that at 96 h is shown in Fig. 2. It falls with increasing enzyme level up to about 110–120 MANU/100 g of flour. There was no improvement in using anymore enzyme, but there were no adverse effects on dough or crumb stickiness when overdosing the enzyme up to 150 MANU/100 g of flour. Enzyme A had a real anti-firming effect because it did not change the initial firmness of the bread. It appears to lower the firming rate and the plateau or limiting firmness value for the bread.

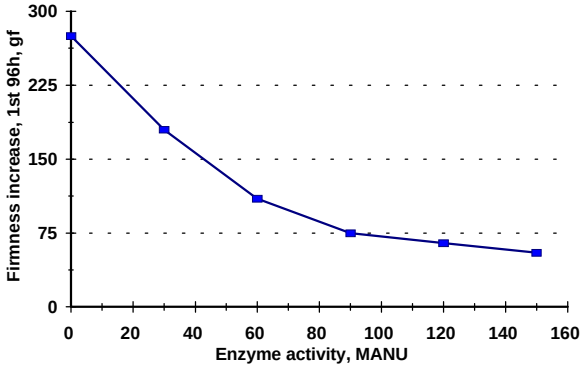

Figure 2. Change in firmness (3 to 96 h) versus enzyme activity for white bread containing amylase A (activity in MANU per 100g of flour).

Studies with DSC to measure the growth of crystallinity in the starch phase of bread showed that amylase A reduced the rate and extent of growth of the crystalline structures. There was a strong correlation (r, 0.95) with the DSC enthalpy measurements and crumb firmness for breads stored at 4 ^{o}C. These results gave support to the major staling hypothesis based on starch crystallisation and also confirmed that amylase A had reduced the plateau value for firmness in standard bread. This meant that the firmness of bread could be kept within an acceptable range for a long period even when all the starch had crystallised.

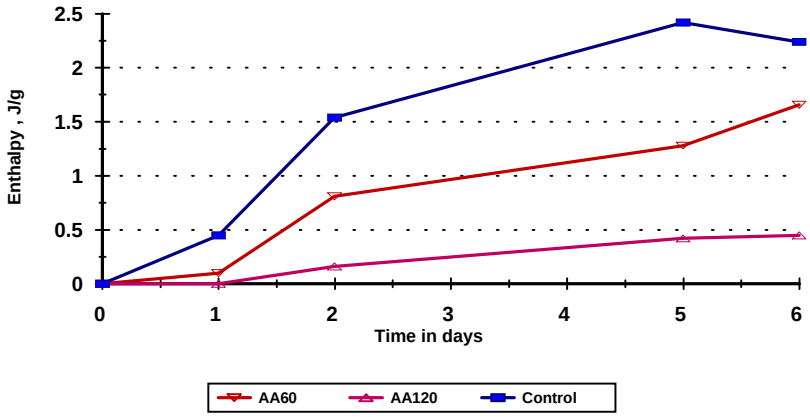

Figure 3. DSC enthalpy versus time for breads containing amylase A (activities 60 and 120 MANU/100 g of flour).

Sensory studies with white bread containing amylase A were carried out over a 96 h period for bread stored at 20^{o}C. They showed that bread, containing amylase A, did not change significantly in most of the sensory attributes for both flavour and texture during the period from 24 to 96 h. Normal control bread staled in terms of the loss of fresh flavour (Strength), the development of stale flavour and harsh aftertaste

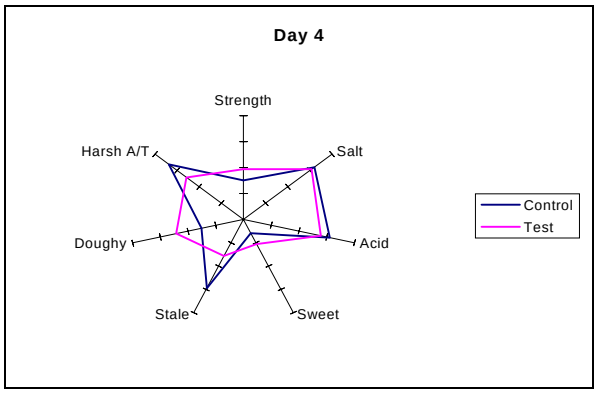

Figure 4. Sensory flavour attributes on day 4 for test bread containing amylase A.

The texture of the control bread changed markedly in terms of the firmness and dryness of the crumb either to the touch (Spring, Dry and Firm) or mouthfeel (Firm 1st bite, Chewy, Dry and Doughy), whereas there was no significant difference between the test bread at 24 and 96 h.

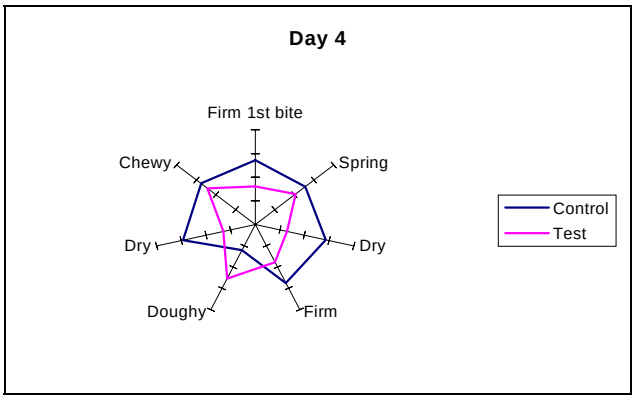

Figure 5. sensory texture attributes on day 4 for test bread containing amylase A.

4 CONCLUSIONS ON THE EFFECTS OF AMYLASE A AND B

These commercial enzymes both contain a maltogenic amylase. In its pure form in amylase A, the maltogenic amylase has a powerful anti-staling performance in all types of UK bread. It reduces both the firming rate and limiting firmness values, which means they can extend the life of bread for periods of weeks rather than days. It has little effect on dough characteristics and present little risk in overdosing during use.
3.3 Hemi-cellulase enzymes

Six samples of enzymes, purified xylanases with no significant amylase or protease activity, were examined. They all gave similar results.

1. Dough characteristics: the xylanase enzymes were active in the dough after mixing and reduced the modulii of elasticity steadily during storage at 25 ^{o}C for 1h. The changes in the dough corresponded to a softening of the dough from the time it was removed from the mixer until it was placed in the oven.

2. Loaf characteristics: it was found that loaf volume increased for two levels of xylanase addition, to a maximum of 110% of the control, and then decreased again. The changes in the moisture content of the bread were not significant at the 5% level but the crumb cellular texture score was improved by the addition of the optimum levels of the enzyme.

3. Changes in crumb characteristics with age: the results from the TPA did not reveal any significant difference between the experimental samples after 1 day ex-oven and the firming rate measured from the graphs appeared to be unaffected by the presence of the xylanase enzymes.

5 CONCLUSIONS ON HEMI-CELLULASES

The action of the xylanases to release water from the pentosans system to soften the gluten matrix was demonstrated for each enzyme tested and the softer dough was found

to give improved oven spring on baking. However, this change did not affect the main mechanism of starch recrystallisation during the firming process.

3.4: Lipases

Two lipases were received which had 1,3 hydrolytic action on triglycerides. They were reported to produce monoglycerides, which would have an anti-firming action. The enzymes were used at one and two times their recommended levels.

1. Dough characteristics: two lipase enzymes were tested in both white and wholemeal bread made with either a spiral mixer or CBP process. After mixing no significant differences were found in the dough quality as judged by the bakers.

2. Loaf characteristics: the only differences, which related to lipase addition, were a small increase in the volume of wholemeal loaves made by the spiral method.

3. Changes in crumb characteristics with age: the results from the TPA showed that the firmness of crumb increased from c. 150 gf at 3 h to 400 gf after 6 days at 20 ^{o}C. There was no significant difference between the samples after 1 day ex-oven and the firming rate measured from the graphs appeared to be unaffected by the presence of the enzymes. Measurements of the levels of monoglyceride in the dough showed that < 0.1% was being produced at the levels of enzyme recommend for use.

6 CONCLUSIONS ON THE LIPASES

The lipases tested had no significant effect on bread staling in terms of crumb firming. This was probably due to the low level of monoglyceride produced by the action of the lipases in the dough.

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IMMUNOLOCALIZATION STUDIES OF BARLEY SERPINS

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1 INTRODUCTION

The serpins constitute a superfamily of proteins (~40 kDa) found in eukaryotes and some viruses. Most serpins are serine proteinase inhibitors, a few are cysteine proteinase inhibitors, and some are non-inhibitory. They are known to be involved in a large range of physiological processes in mammals – from blood coagulation and complement activation to hormone transport and chromatin folding – but the physiological functions of plant serpins are unknown (1).

Serpins are abundant (2–3 mg/g grain) in barley endosperm and are the major antigens in beer (2,3). Active (uncleaved) barley serpins inhibit mammalian serine proteinases of the chymotrypsin family *in vitro* by forming 1:1 irreversible complexes (1). Inactive (cleaved) serpins are stable proteins resistant to boiling, extreme pH, and protease treatment. Barley serpins form heterodimers with β-amylase, and are assumed to influence beer foam stability and haze formation, and are thus of interest for the malting and brewing industry (3).

As a part of a research project continuing to focus on the properties and functions of plant serpins, we have initiated immunomicroscopy studies to localize serpins in barley.

2 MATERIAL AND METHODS

Developing barley (*Hordeum vulgare*) grains about 30 dpa were fixed in 4% paraformaldehyde in PBS, pH 7.4, overnight at 4°C, dehydrated, and embedded in paraffin. Sections of 10 µm were treated with a primary antibody and labelling was detected by the immunogold silver enhancement method (British BioCell International). The antibodies used and their specificities are shown in Table 1.

Table 1. The antibodies and their specificities.

	Monoclonal antibodies		Polyclonal antibodies	
	5C11	8E8	306	360
BSZ4	+	+	+	+
BSZ7	+	-	+	(+)
BSZx	-	++	+	++

3 RESULTS AND CONCLUSIONS

Immunolocalization studies confirm that serpins are present in the starchy endosperm of developing barley grain (Fig. 1). By using antibodies of different specificity we show that barley serpins belonging to distinct subfamilies, BSZ4 and BSZ7, have different but overlapping distributions within the starchy endosperm. Monoclonal antibodies 5C11 and 8E8 gave corresponding labelling to polyclonal R306 and R360, respectively. BSZ4 is mainly located in the central area of the starchy endosperm whereas BSZ7 is strongly expressed in the subaleurone layer. Differences in spatial distribution suggest that serpin subfamilies might differ in their physiological functions.

Previous studies have indicated that barley grain serpins associate with β-amylase *in vitro*. Like serpins now, β-amylase has also been localized in the starchy endosperm of barley grain (4). However, more detailed immunomicroscopy studies are needed to determine whether serpins and β-amylase are co-localized.

BSZx, a third serpin subfamily, has been detected only at the mRNA level (with RT-PCR). We are attempting to locate BSZx using microscopy and highly specific purified antibodies. Progress is also being made towards localizing the serpins in the vegetative tissues.

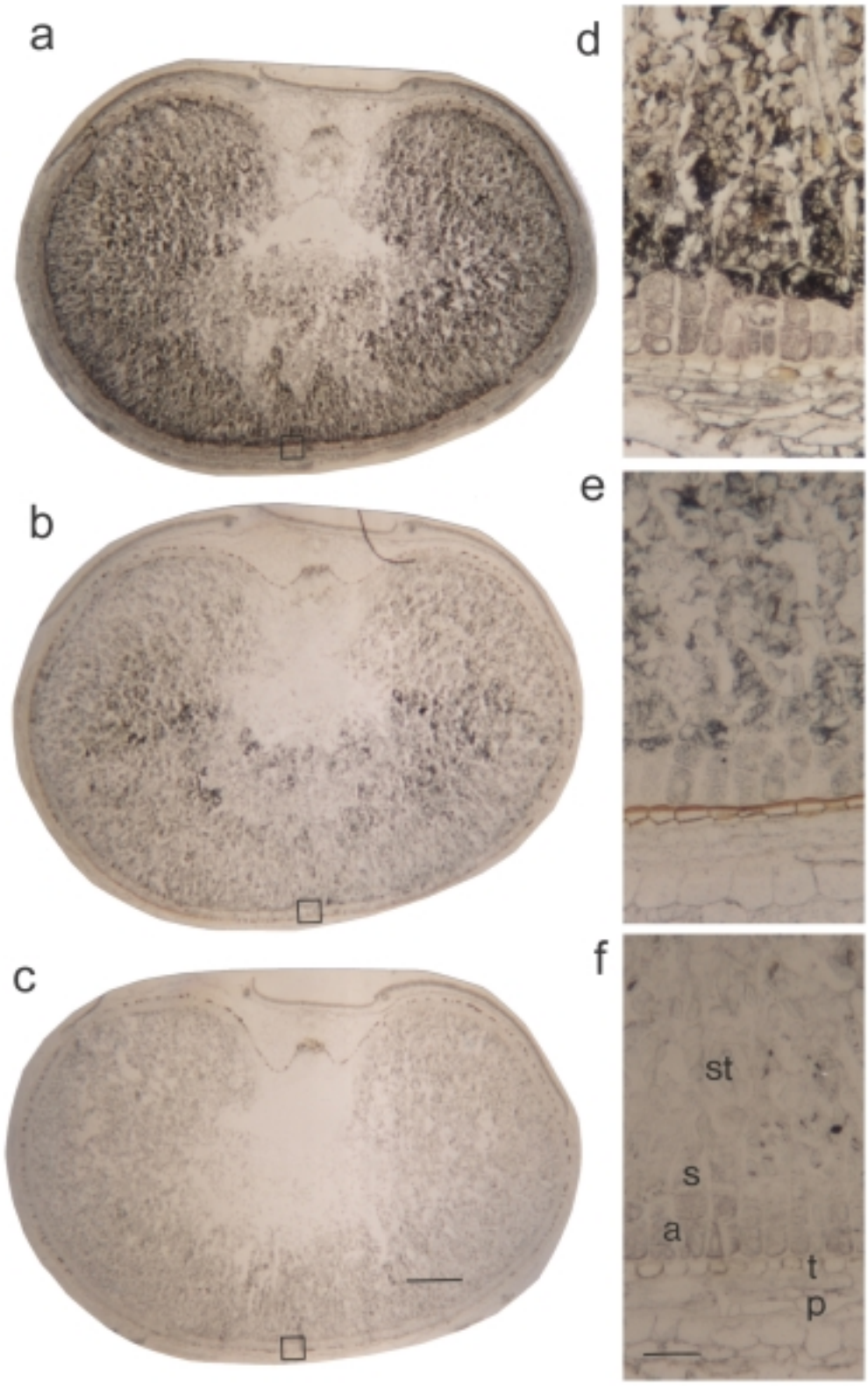

Figure 1. Immunolocalization of serpins in a developing barley grain. Dark silver precipitate shows positive labelling. Serial sections are from the same grain. Sections were treated with ***a, d)*** *polyclonal R306 and* ***b, e)*** *polyclonal R360 antibodies, and* ***c, f)*** *nonimmune control serum. a = aleurone layer, s = subaleurone layer, st = starchy endosperm, t = testa, p = pericarp. Scale bar a-c) 250 µm, d-f) 25 µm.*

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SPECIFIC ANTIBODIES AS A METHOD FOR CHARACTERIZATION OF FIBER SURFACE CHEMISTRY

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1 BACKGROUND

Wood fibers are composed mainly of cellulose, hemicellulose and lignin. Fiber properties such as bonding ability are largely governed by surface chemistry of fibers. FTIR and ESCA have been applied for analysis of wood fibers and papers, but these methods do not give information on the surface distribution of components. Microscopy offers a linkage between chemistry and structure with methods for distinctive and specific staining or labelling of fiber components. Antibodies can be used as specific markers for different carbohydrates. They have been produced against oligosaccharides obtained from xylan and xyloglucan (Takahashi and Sumiya, 1990, Sone and Sato, 1994). Also polysaccharides have been used as antigen (Moore, 1989, Knox *et al*., 1990, Westermark and Vennigerholz, 1995).

2 MATERIALS AND METHODS

Antibodies were produced against different cell wall components using polysaccharides or purified oligosaccharides ($MeGlcAXyl_{2-3}$ or $GalMan_2$) linked to BSA as antigens (Pere *et al.*, 1999). $MeGlcAXyl_{2-3}$ was prepared by β-xylanase and β-xylosidase hydrolysis of xylan (4-O-methylglucuronoxylan from birch, Roth) and $GalMan_2$ by mannanase hydrolysis of galactomannan (from locust beans, Sigma). The oligosaccharides were isolated by anion-exchange chromatography and/or gel chromatography. Polymeric carbohydrates used as antigens were xylan (4-O-methylglucuronoxylan from birch, Roth), glucomannan (from locust beans, Sigma) or pectin (from citrus peels, Fluka).

Antisera were produced in rabbits and tested for their reaction against their own antigen and other wood components by enzyme-linked immunosorbent assay (ELISA). For testing the oligosaccharides were bound to another protein, ovalbumin. The antisera were also checked for their specificity using immunolabelling on fibers of known chemical composition or fibers were treated enzymatically (xylanase or mannanase treatment) to selectively remove components from fiber surface.

Fibers analyzed were: bleached birch kraft pulp (contains xylan, cellulose), bleached pine kraft pulp (contains xylan, glucomannan, cellulose) and spruce thermomechanical pulp (TMP) (contains xylan, glucomannan, pectin, cellulose, lignin).

For microscopic study the fibers were treated with antibodies which were then visualized by a fluorescent labelled secondary antibody. The anti-rabbit IgG was conjugated to FITC (Sigma) or to Fluoro^{TM}Cy^{TM}5 (Amersham Pharmacia Biotech). The labelled fibers were examined either by fluorescence microscopy or by confocal laser microscopy.

3 RESULTS

The antisera were tested for their specificity against several wood derived oligosaccharides linked to ovalbumin as well as against polymeric wood components using the ELISA method (Table 1).

All antisera recognized well their own antigen, but gave some cross-reactivity with other cell wall components. Antisera raised against BSA linked oligosaccharides were stronger than those raised against polysaccharides. Preimmune sera did not give positive reactions with any of the tested components.

Table 1. Specificity of antisera against wood derived components as determined by ELISA.

Target Polysaccharide	Tested compound	Antiserum against		
		$MeGlcAXyl_{2–3}$	$GalMan_2$	Pectin
Xylan	MeGlcA-xylan	+	-	-
	$MeGlcAXyl_{2–3}$	+++	++	±
	Xyl_3	+	-	-
Glucomannan	Glucomannan	-	+	-
	$GalMan_2$	-	+++	-
	Man_2	-	-	-
Pectin	Pectin	-	-	+++
	Polygalacturonic acid	-	-	-
Cellulose	Cellobiose	-	-	-

The specificity of anti-$MeGlcAXyl_{2–3}$ and anti-$GalMan_2$ was also evaluated by labelling bleached birch and pine kraft fibers before and after extensive enzymatic removal of xylan and mannan (Table 2). The amount of label decreased clearly after the removal of corresponding hemicellulose from the fiber surface.

Table 2. Specificity of antisera as analyzed by immunolabelling on bleached birch and pine kraft pulps.

Antiserum	Birch pulp		Pine pulp		
		Xylanase treated[1)]		Xylanase treated[1)]	Mannanase treated[2)]
$MeGlcAXyl_{2–3}$	+++	+	+++	+/-	nd
$GalMan_2$	-	nd	++	nd	+

[1)] 24–28% of xylan removed, 2) 19% mannan removed, nd = not determined

FITC label can be used only for fibers not containig lignin, because lignin and FITC have the adsorption and emission of light at the same regions of light spectrum. Cy5 label has both adsorption and emission at higher wavelength and this label can be used to avoid autofluorescence with lignin containig fibers.

4 SUMMARY

Antibodies were produced against cell wall carbohydrates using purified oligosaccharides linked to BSA and polymeric carbohydrates as antigens. Antibodies recognized well their antigens. Oligosaccharide-BSA produced stronger antiserum than polysaccharides. FITC label is suitable on delignified samples whereas Cy5 label can also be used with lignin containing material. The labelling of fibers was in agreement with the composition of fibers. So this method gives information of the surface distribution of components. Antibodies could also be applied in analysis of different components in cell walls of grains.

5 ACKNOWLEDGEMENTS

Maija Tikka is thanked for the excellent technical assistance. The work was partially financed by National Technology Agency.

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IDENTIFICATION AND MICRO SEQUENCE ANALYSIS OF PROTEINS EXTRACTED FROM BARLEY SEEDS

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1 INTRODUCTION

Barley is a major crop in Denmark and it is used for malting in the brewing industry or as animal feed. Important properties of barley with respect to malting includes the germination index (i.e., the ability to germinate and to do so uniformly), the diastatic power (an indicator of the β-amylase activity) and the protein content. It turns out that these properties vary a lot between various barley cultivars; some are very good for malting (e.g. cv. Alexis) and some are less good (e.g. cvs. Scarlett and Ferment). Proteome analysis is one excellent tool to elucidate the origin of these differences at the protein level.

Proteome analysis encompasses 2D-gel electrophoresis, image analysis and protein identification by either *N*-terminal sequencing or advanced mass spectrometry using sequence information available in public databases (Nørgaard *et al.*, 1998). Using the *N*-terminal sequencing approach the proteins are blotted to a PVDF membrane and visualised by Coomassie staining. Spots of interest are cut out and *N*-terminally sequenced and this information is used for database search. In the approach using mass spectrometry the 2D-gels are either silver stained or Coomassie stained. The protein spots of interest are excised and in-gel digested using a specific protease, e.g. trypsin. The generated peptides are extracted and their masses determined by matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS). The list of peptide masses is then subject to database search, which eventually will identify the protein if included in the database (Shevchenko *et al.*, 1996). If the database search is negative, the remaining peptide mixture is analysed by nano-electrospray ionisation tandem mass spectrometry (ESI-MS/MS). This technique allows the determination of partial sequences of peptides in the mixture, which enables a more efficient database search since also homologous proteins can be found. The overall strategy is summarised in Figure 1.

The aim of the work reported here was to establish the necessary technologies and initiate detailed proteome analysis on barley and malt. Proteins from barley seeds are extracted and separated by 2D-gel electrophoresis and individual proteins are analysed by either electroblotting followed by *N*-terminal sequencing or by using in-gel digestion and mass spectrometry (peptide mapping). The ultimate goal is to identify proteins important for germination and malting.

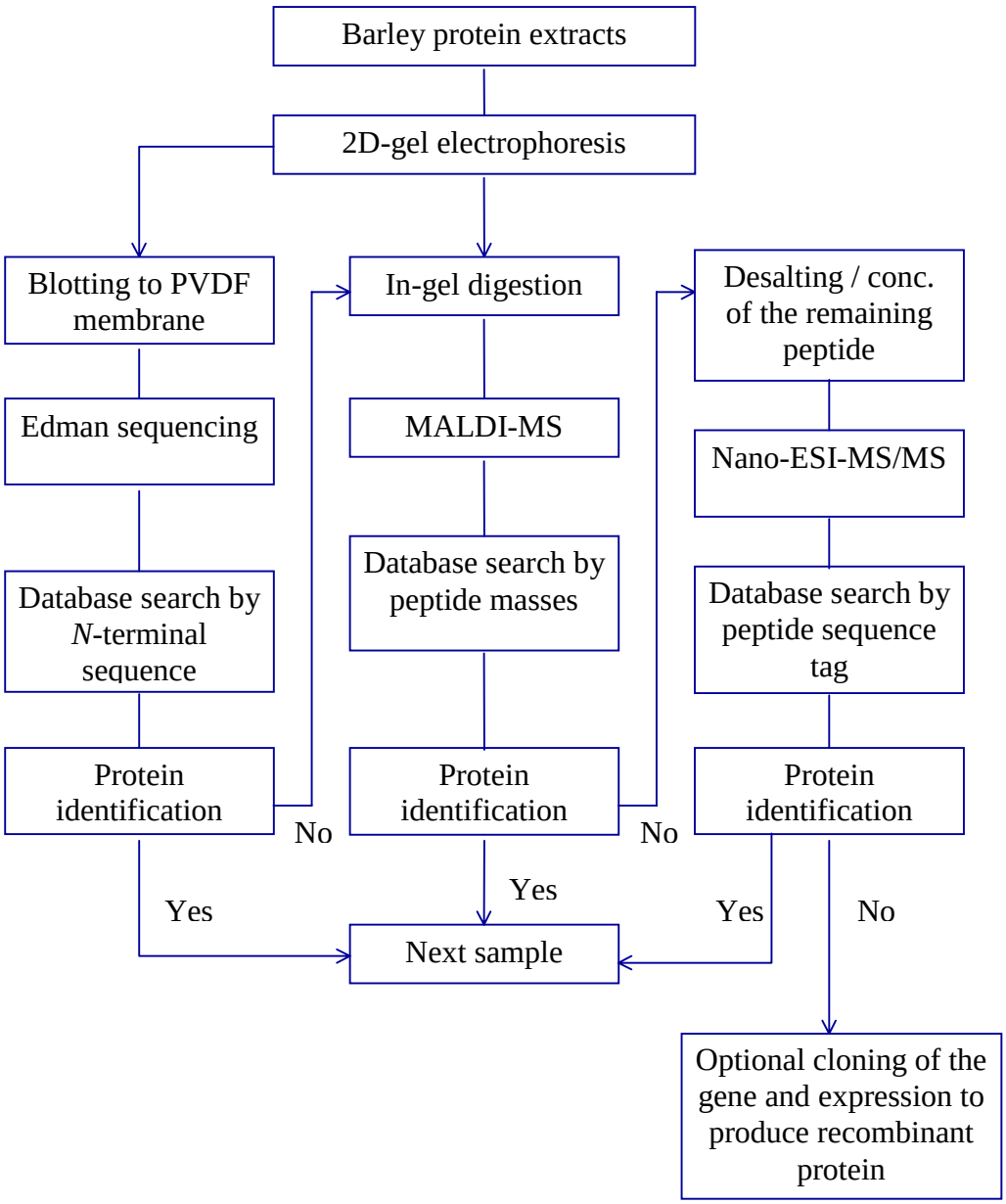

Figure 1. Strategy to be used in this barley proteome project (modified from Shevchenko et al. 1996).

2 MATERIALS AND METHODS

2.1 CHEMICALS

All chemicals used in the work were *pro analysi* grade or better. All aqueous solutions were made from water purified using the Milli-Q system from Millipore. Materials for 2D-gel electrophoresis were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). PVDF membranes were Immobilon™-P Transfer Membranes from Millipore (Bedford, MA).

2.2 PLANT MATERIAL

Barley grains and barley malt were kindly delivered by Danish Malting Group A/S. The barley was field grown in Denmark in 1999.

Protein extraction

Barley grains were frozen at –20°C and portions of 10 g were milled in a cooled mill for 1 min. The proteins were extracted in 5 mM TrisHCl pH 7.5, 1 mM $CaCl_2$ for 30 min with stirring at 4°C. After centrifugation (13000 rpm, Sorvall™ SA-600, 60 min, 4°C) the supernatants were aliquoted in portions of 1 ml and stored at –80°C.

2D-PAGE

2D-gel electrophoresis was performed in accordance with the recommendations from Amersham Pharmacia Biotech (Benkelman *et al.* 1998). The proteins to be focused were acetone precipitated before solubilisation in the rehydration solution (8 M urea, 2% CHAPS [3-(3-cholamido-propyl) dimethylammonio)-1-propanesulphonate] or 2% Triton X-100, 0.5% IPG buffer and a trace of bromophenol blue). The focusing were performed using the IPGphor IEF System. After focusing the strips were equilibrated in SDS equilibration solution (50 mM TrisCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and a trace of bromophenol blue) before running the second dimension on a Multiphor II unit using ready cast gels (ExcelGel SDS XL 12–14) from Amersham Pharmacia Biotech. The LMW standard from Amersham Pharmacia Biotech was used as molecular weight marker. Typically 60 μg and 1 mg protein was loaded on gels for silver staining (Rabilloud 1994) and Coomassie staining, respectively.

Electroblotting and N-terminal sequencing

Proteins separated by 2D-gel electrophoresis were electroblotted to a PVDF membrane in a semi-dry blotting apparatus (NovaBlot Unit) using 10 mM CAPS pH 11.0 as blotting buffer (Matsudaira, 1987). The proteins were transferred at 0.2 mA/cm^2 of gel area over night at room temperature. After electroblotting the PVDF membranes were stained with Coomassie Brilliant Blue R-250 and spots were excised and sequenced using an automated protein sequencer (model 477A, Applied Biosystems).

In-gel digestion and mass spectrometry

In-gel digestion was performed according to the method of Wilm *et al.* (1996). In brief the spots of interest were cut out of the 2D-gel, washed, in-gel reduced with DTT, S-alkylated with iodoacetamide, and digested with sequencing grade modified trypsin (Promega). Peptides were extracted from the gel and micro purified and loaded directly on the target according to Gobom *et al.* (1999). Positive ion reflector mode mass spectra were acquired on a Bruker REFLEX III instrument equipped with delayed extraction technology.

Database search

The *N*-terminal sequences were searched in databases applying the MS-Edman tool, which is a part of ProteinProspector. The programs can be found at the web site: **http://prospector.ucsf.edu/**.

The peptide masses were used for searching in a non-redundant database by the tool ProFound - Peptide Mapping, URL: **http://www.proteometrics.com/**.

3 RESULTS AND DISCUSSION

A typical silver stained 2D-gel is shown in Figure 2. From this gel the pI and the molecular weight (Mw) can be estimated for the separated proteins. About 1200 spots are visualised on a silver stained gel loaded with 60 µg protein and about 350 spots are detected on a PVDF blot from a 2D-gel loaded with 1.6 mg protein (Figure 3). 2D-gel electrophoresis of protein extracts from various barley cultivars show differences in the spot pattern (Figure 4).

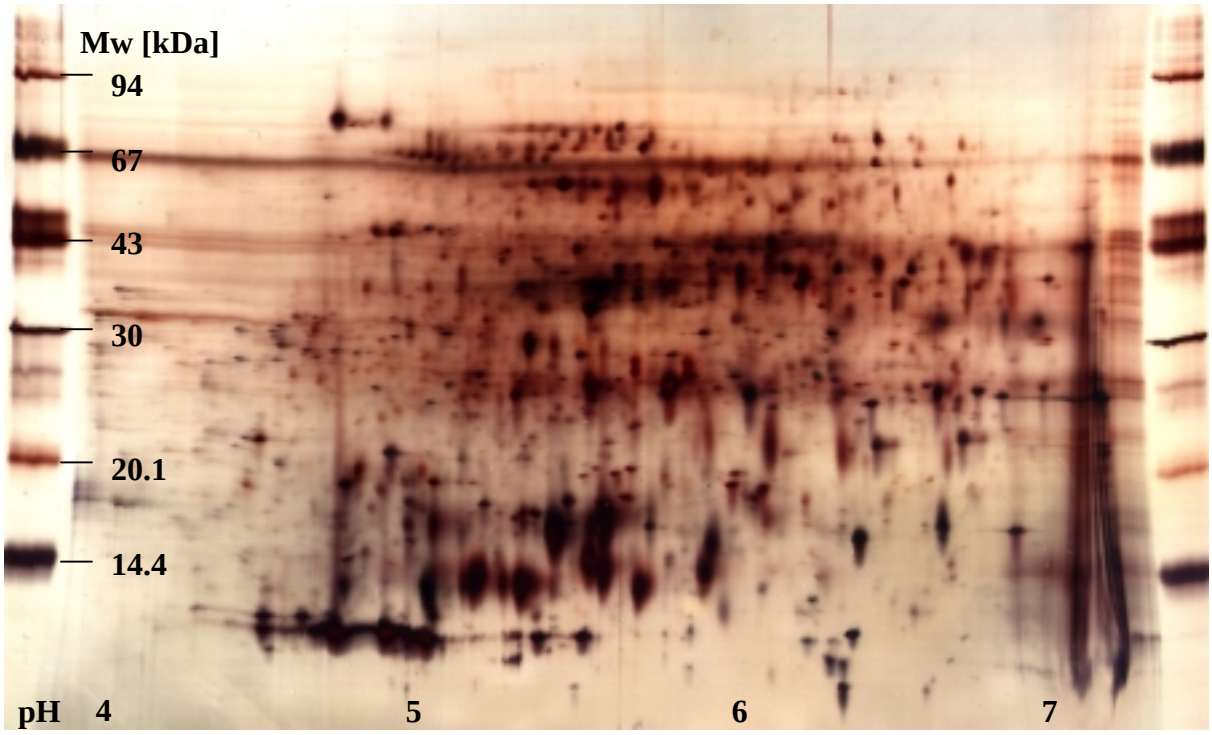

Figure 2. 2D-gel of protein extract from barley Hordeum vulgare seeds cv. Alexis. IEF: pH = 4–7 (the pH scale is approximate). L=18 cm. Protein: 60 µg.

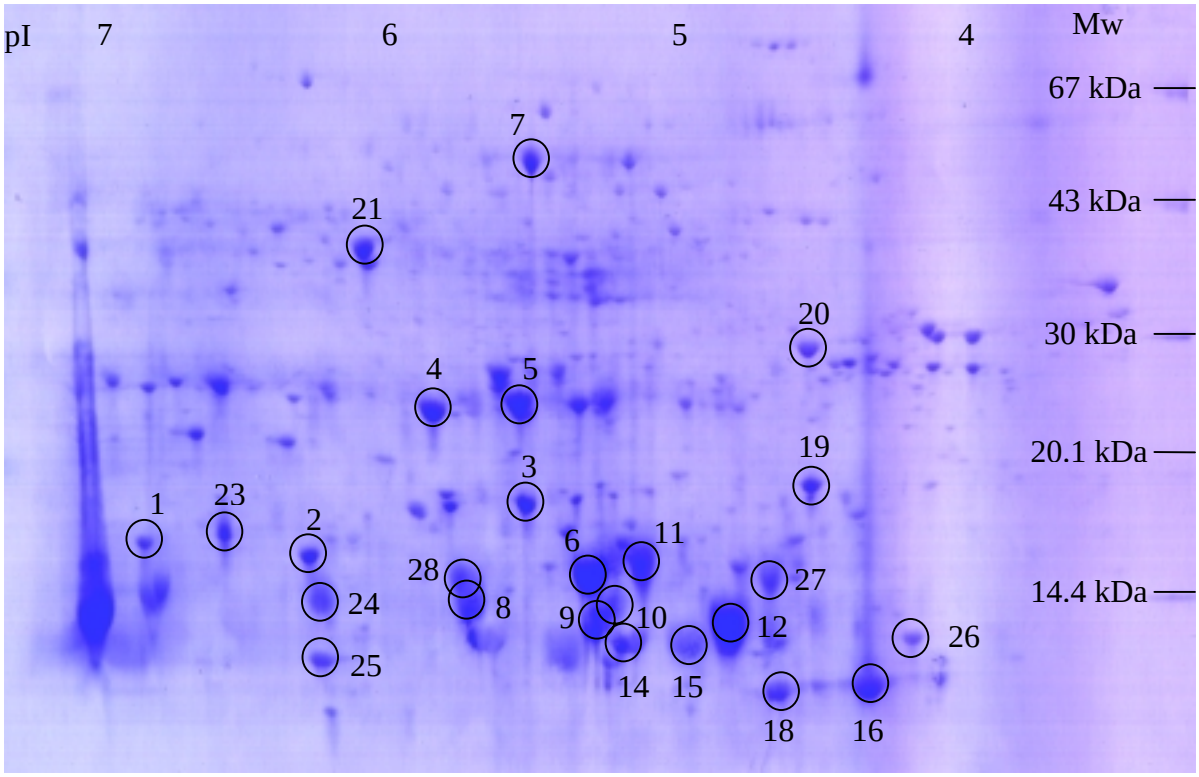

Figure 3. Coomassie stained blot on a PVDF membrane of proteins (1.6 mg) extracted from barley seeds (Hordeum vulgare cv. Alexis) separated on a 2D-gel (IEF pH = 4–7, L = 18 cm, approximate pH scale on figure).

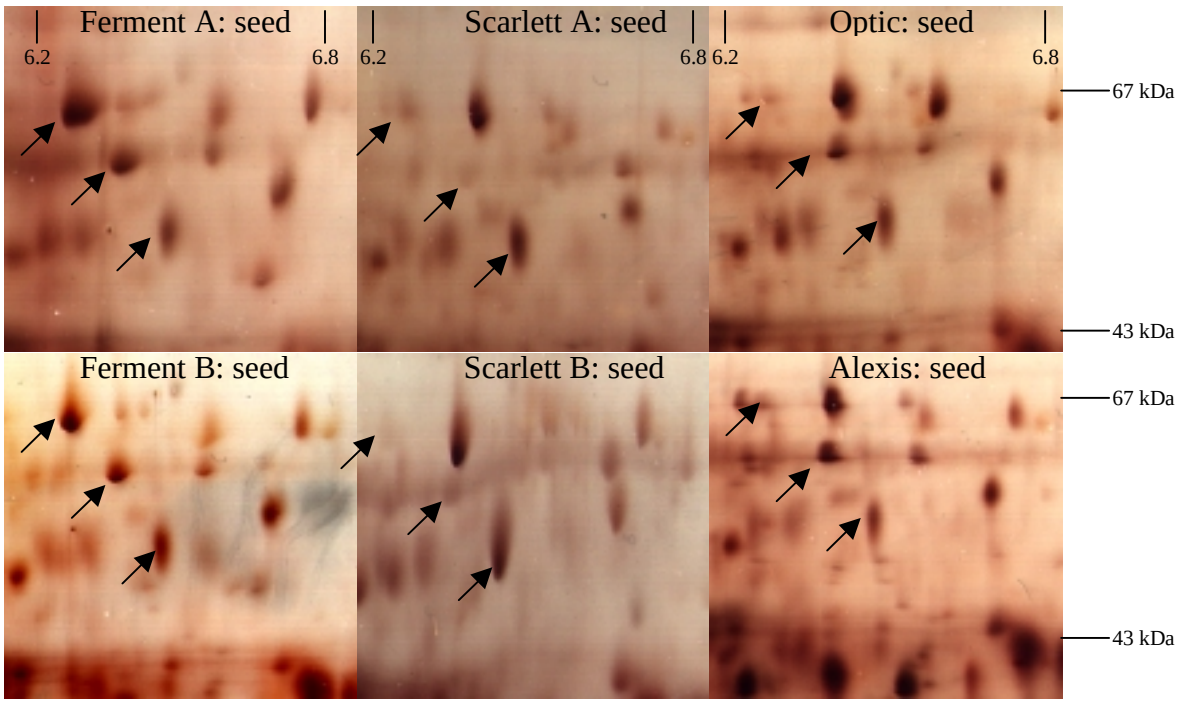

Figure 4. Sections of 2D-gels showing variation in the spot patterns between various barley cultivars. pI=6.2–6.8, Mw = 43–77 kDa.

The *N*-terminal sequences have been determined for 12 protein spots as summarised in Table 1. Furthermore three spots gave very weak partial sequences and six proteins appeared to be *N*-terminally blocked. The data including database entry numbers are given in Table 1. The *N*-terminal sequences led to identification of 11 different proteins. These include two isotypes of chymotrypsin inhibitors and three subunits of the tetrameric α-amylase/trypsin inhibitor (CMa, CMb and CMd). These have been identified in a 2D-gel before (Flengsrud, 1993). The polypeptides have a size of 17–18 kDa and isoelectric points in the range 5.5–5.8. In the case of CMa and CMd two isoforms of the same protein have been identified. For spot #11 it was not possible to determine the isotype of the inhibitor subunit, since the sequences are identical in the *N*-terminal part for all three forms (CMd, CMd2 and CMd3). The calculated isoelectric points for the isotypes vary from 5.23 to 8.00 and spot #11 of pI ≈ 5.5 most likely originates from the isotype CMd.

Table 1. Proteins identified from selected spots shown in Figure 4 by N-terminal sequencing or mass spectrometry of tryptic peptides.

Spot #	N-terminal sequence	Mass spec.	Protein	Database entry	pI	Mw
1	DXGXXFXK	-	n.d.	-	6.9	14.3
2	*N*-terminally blocked	-	n.d.	-	6.5	13.7
3	*N*-terminally blocked	-	n.d.	-	5.8	17.5
4	WG(FA)(EV)(LP)VP	-	n.d.	-	6.1	24.4
5	n.d.	Yes	Triose phosphate isomerase	P34937	5.8	25.2
6	SPGEXXXPGMGYPV SPGEWCWPGMGYPV	Yes	α-amylase inhibitor. HOR V1	P16968	5.7	14.7
7	X(V)XXX(N)(A)	Yes	Enolase	P42895	5.8	53.6
8	TGQYXYAGXGLPXN TGQYCYAGMGLPSN	-	CMa component of tetrameric α-amylase inhibitor	P28041	6.0	11.9
9	XP(GS)E(D)X(N)PG(M)X	-	n.d.	-	5.7	11.9
10	VGXEDAXXPYA VGSEDCTPWTATPITP	Yes	α-amylase/trypsin inhibitor. CMb	P32936	5.6	12.7
11	AAAATDXTPGVA AAAATDCSPGVA	-	CMd component of tetrameric α-amylase inhibitor	P11643	5.5 5.2	14.4 17.3

12	ERDYGEYXRVGKS ERDYGEYCRVGKS	-	trypsin/α -amylase inhibitor. pUP13	g225102	5.3 5.4	12.3 14.7
14	(K)XXGGXX	-	n.d.	-	5.5	11.1
15	SGPXMWXD SGPWMWCD	-	α-amylase inhibitor BDAI-I precursor	P13691	5.4 5.4	11.5 16.4
16	MEGXVPKYPE MEGSVPKYPE	-	Chymotrypsin inhibitor	g167024	4.8 5.3	9.9 9.0
18	XEGSVXKY MEGSVLKYPE	-	Chymotrypsin inhibitor	P16062	5.1 5.2	9.6 8.9
19	*N*-terminally blocked	-	n.d.	-	5.0	19.8
20	*N*-terminally blocked	-	n.d.	-	5.0	31.1
21	IXLVXA(L)GAF	-	n.d.	-	6.4	32.8
23	VKAVAVLTGSEGVKG XKAVAVLTGSEGVXG	-	Superoxide dismutase [Cu-Zn] (fragment)	P34936	6.7	14.4
24	TGQYKYAGMGLPXNX TGQYCYAGMGLPSNP	-	α-amylase inhibitor (CMa)	g18954	6.4 5.9	11.5 15.5
25	*N*-terminally blocked	-	n.d.	-	6.4	7.7
26	*N*-terminally blocked	-	n.d.	-	4.6	12.5
27	(SG)PGEXVSPGXXYF	-	n.d.	-	5.1	14.9
28	AAAATDXXPGVAFE AAAATDCSPGVAFP	-	α-amylase/trypsin inhibitor CMd precursor. CMd2	P11643	6.0 6.1	13.7 18.5

The top line represents the experimental *N*-terminal sequence, pI and Mw. The lower line gives the *N*-terminal sequence from the nun-redundant database (NCBInr.01.08.00) and the theoretical pI and Mw derived from the amino acid sequence.

Spot #5 was identified to contain triose phosphate isomerase by using peptide mapping and mass spectrometry reported previously (Flengsrud, 1993). The yield of the *N*-terminal sequence, however, was too low for reliable identification, although according to the SwissProt database, the *N*-terminus was not blocked.

Spot #7 was identified to contain enolase by using peptide mapping and mass spectrometry. The database search gave seven plant enolases as the highest ranked hits not including the barley enolase. Alignment of the sequences using ClustalW showed that these plant enolases have very high sequence identity (data not shown). It was concluded therefore that spot #7 contained barley enolase. As a final check the databases were searched for the barley enolase without any result, which explains why the barley enolase was not among the hits in the database search.

The spots #6 and #10 were identified by the *N*-terminal sequences and tryptic peptide masses to contain two α-amylase inhibitors, HOR V1 and CMb.

The strategy was to select the largest spots on the gel or on the blot for analysis. The α-amylase inhibitors are indeed among the most abundant water extractable proteins in the kernels, as shown before (Flengsrud, 1993). The inhibitors of the seed are typically specific for insect α-amylases, thus contributing to the protection against pathogen attack.

4 CONCLUSION

Thirteen proteins have been identified. Most interesting was the identification of barley enolase, which is not yet found in the non-redundant (NCBInr.01.08.00) database used by MS-Edman, ProteinProspector. The future work will focus on identification of additional conspicuous protein spots. Special attention will in particular be given to spots unique to specific cultivars or varying in intensity between different cultivars.

5 ACKNOWLEDGEMENTS

The Danish Academy of Technical Sciences (Akademiet for de Tekniske Videnskaber, ATV) is gratefully acknowledged for a Ph.D. fellowship (EF803) to OO.

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ACTIVITY OF ENDOGENOUS α-AMYLASE AND CELL-WALL DEGRADING ENZYMES IN GERMINATING RYE *(SECALE CEREALE L.)*

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ABSTRACT

The activities of endogenous α-amylase, endo-xylanase, β-xylosidase and α-arabino-furanosidase were measured in rye kernels from two varieties (Amilo and Tsulpan3) during 6 days of germination. The activity of the enzymes was low in the non-germinated grain, but increased dramatically during germination. The increase in activity of cell-wall degrading enzymes was strongly correlated to the increase in α-amylase activity. This indicates, that the α-amylase activity in rye grain can be used as a rough indication of the activity of the cell-wall degrading enzymes.

1 INTRODUCTION

Bread making based upon rye is widely practised in Denmark and in other Northern, Eastern and Central European countries. Due to the high content of dietary fibre, lignans and antioxidative phenolics, the consumption of rye is highly recommendable for human nutrition. The quality of rye for baking purposes differs from year to year due to the low sprouting resistance of the rye grain. Rainy summers result in grain samples with high enzymatic activities. This strongly influences the baking quality and causes problems of the bread industry in those years. During rye bread production an enzymatic breakdown of the cell-wall component arabinoxylan (pentosan) is caused by the activity of endogenous enzymes. This breakdown influences the water holding capacity of the arabinoxylan and the quality of the final bread. Intermediate activities of α-amylase and cell-wall-degrading enzymes are required, but too high enzymatic activities are detrimental to the baking performance. The Falling Number of rye is negatively correlated to the logaritm of the α-amylase activity[1] and an exponential increase in α-amylase activity during germination of rye has been found[2]. The change in activity of cell-wall degrading enzymes during germination however, needs to be further investigated. Thus the aim of the present study was to measure the activities of **α-amylase** (1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1), **xylanase** (endo-1,4-β-xylanase, E.C. 3.2.1.8), **xylosidase** (xylan 1,4-β-xylosidase, E.C. 3.2.1.37) and **arabinofuranosidase** (α-L-arabinofuranosid arabinofuranohydrolase, E.C. 3.2.1.55) during germination of rye in order to study, whether the activity of the cell-wall-degrading enzymes is correlated to the increasing α-amylase activity during sprouting.

2 MATERIALS AND METHODS

2.1 RYE SAMPLES

The varieties Amilo (with high sprouting resistance) and Tsulpan3 (with low sprouting resistance) were grown under the same conditions in Denmark and harvested at maturity in 1997. The Falling Number of the grain samples before germination were 357 sec. (Amilo) and 208 sec. (Tsulpan3).

2.2 GERMINATION

5g rye grain were disinfected in 5% Na-hypochlorit solution added 1 drop Tween 20 pr. 20 ml (10 min., room temperature) and neutralised with sterilised water. The kernels were germinated in petri dishes (5 ml sterilised water, 20ºC, no light access) for 1, 2, 3, 4, 5 and 6 days. Two petri dishes per rye variety and day were used for preparation of extracts for the α-amylase assay and 2 petri dishes were used for the pentosanase assay extracts.

2.3 PREPARATION OF EXTRACTS

The grain samples were homogenised and extracted with 0.1 N Na-acetate buffer (pH 5.2 for α-amylase assay, pH 4.5 for pentosanase assays) (3).

2.4 ENZYME ACTIVITY ASSAYS

α-Amylase assay: The activity of alpha-amylase was measured by the Ceralpha method (ICC standard no. 303). Endo-xylanase assay: The activity of endo-xylanase was measured as the break-down of 4-0-methyl-D-glucurono-D-xylan-remazol brilliant blue R. The method was modified after Biely *et al*. (1985) (3, 4). Xylosidase and arabinofuranosidase assays: The activities of xylosidase and arabinofuranosidase were measured by the breakdown of p-nitrophenyl-β-D-xylopyranosid and p-nitrophenyl- α-L-arabinofuranosid respectively. (3).

2.5 CALCULATIONS

All results are calculated as the activity in pkatal (10^{-12} katal) per gram kernel weight before germination.

3 RESULTS AND DISCUSSION

The activities of α-amylase, endo-xylanase, xylosidase and arabinofuranosidase in grain extracts during 6 days of germination are shown in Figures 1, 2, 3 and 4, respectively.

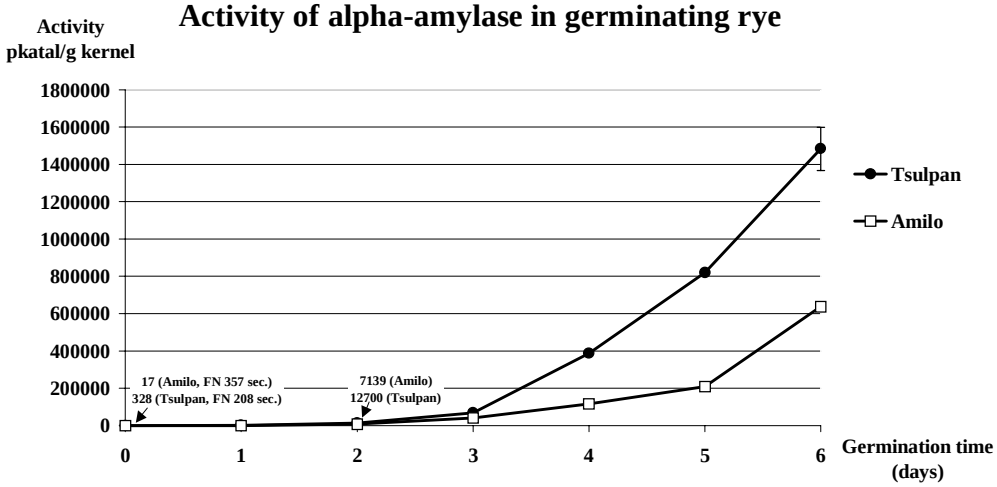

Figure 1: Activity of α-amylase during germination of the rye varieties Amilo and Tsulpan for 6 days (values in pkatal/g kernel weight ± std.dev., n=2).

The variety Amilo had a lower initial activity of α-amylase (17 **p**katal/g) than Tsulpan3 (328 **p**katal/g) which is in accordance with the difference in Falling Number of the two grain samples. As a result of germination the activity of α-amylase increased dramatically and after 6 days of germination Amilo had still the lowest activity (637 **n**katal/g) compared to Tsulpan3 (1483 **n**katal/g).

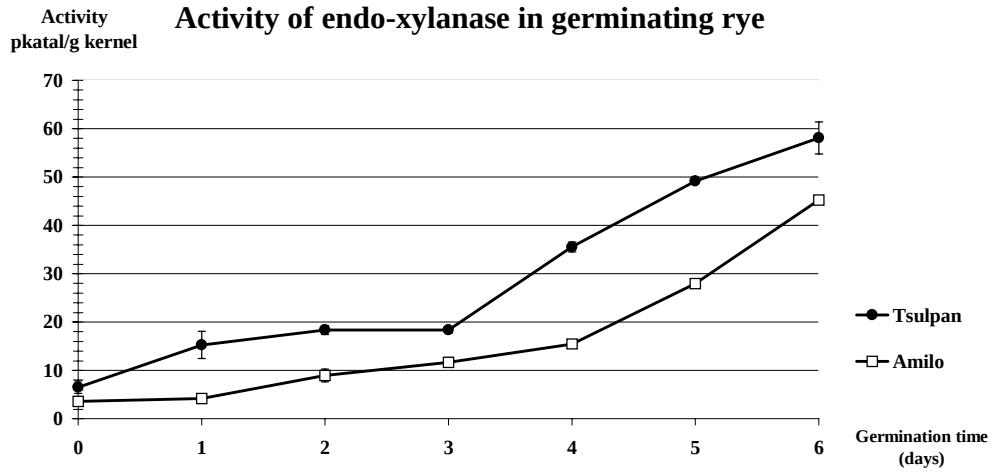

Figure 2. Activity of endo-xylanase during germination of the rye varieties Amilo and Tsulpan for 6 days (values in pkatal/g kernel weight ± std.dev., n=2).

The activity of endo-xylanase was 4 pkatal/g (Amilo) and 7 pkatal/g (Tsulpan3) before germination. During germination for 6 days the activity increased to 45 pkatal/g (Amilo) and 58 pkatal/g (Tsulpan3).

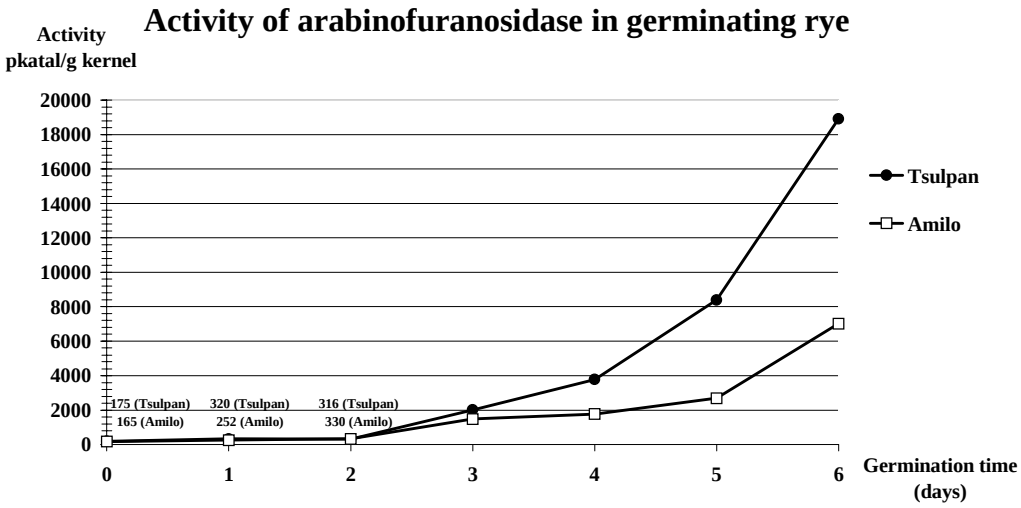

Figure 3. Activity of xylosidase during of the rye varieties Amilo and Tsulpan for 6 days (values in pkatal/g kernel weight ± std.dev, n=2).

The activity of xylosidase before germination was 360 pkatal/g (Amilo) and 481 pkatal/g (Tsulpan3). After 6 days of germination the activity increased to 2539 pkatal/g (Amilo) and 6899 pkatal/g (Tsulpan3).

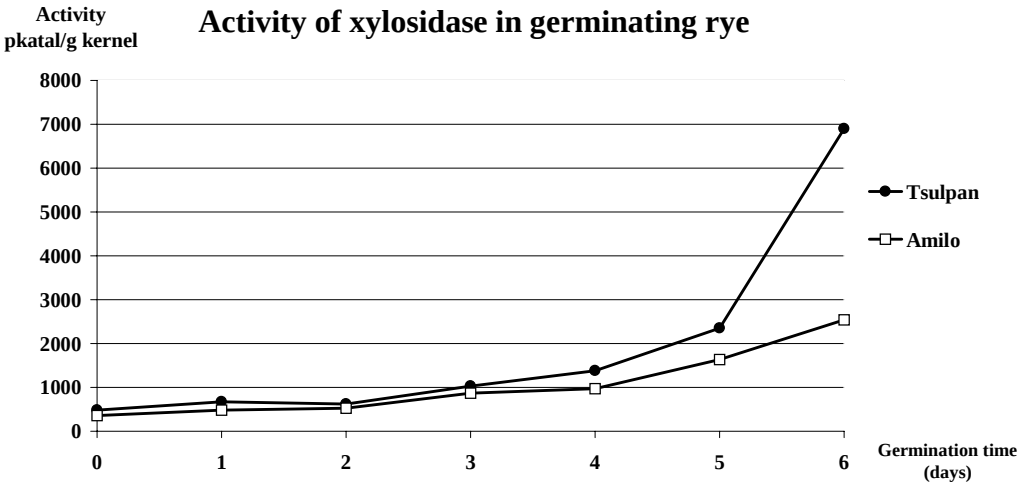

Figure 4. Activity of arabinofuranosidase during germination of the rye varieties Amilo and Tsulpan for 6 days (values in pkatal/g kernel weight ± std.dev, n=2).

The activity of arabinofuranosidase before germination was 165 pkatal/g (Amilo) and 175 pkatal/g (Tsulpan 3). During germination for 6 days the activities increased to 7022 pkatal/g (Amilo) and 18912 pkatal/g (Tsulpan3).

The increase in activities of cell-wall degrading enzymes was closely correlated to the α-amylase activity. The coefficients of correlation between the activities of α-amylase

and the cell-wall degrading enzymes were 0.92 (endo-xylanase), 0.96 (xylosidase) and 0.99 (arabinofuranosidase).

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ISOLATION OF AN ASPARTIC PROTEINASE FROM DORMANT RYE (*SECALE CEREALE L.*) GRAIN

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1 INTRODUCTION

Although the proteolytic activities of rye flours are significantly higher than those of wheat flours and close to those of triticale flours (Madl and Tsen, 1973; Singh and Katragadda, 1980), reported studies about the different proteases in rye are scarce.

Recently, endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase and N-α-benzoyl-arginine-*p*-nitroanilide hydrolysing activities were detected in Humbolt rye whole meal (Brijs *et al*., 1999). After milling, proteolytic enzymes in rye bran extract were concentrated in the fraction precipitating between 35 and 60% ammonium sulphate, which degraded both rye and wheat storage proteins. Pepstatin A, an inhibitor of aspartic proteases, reduced ca. 88% and 75% respectively of the hemoglobin and azocasein hydrolysing activities of the proteases present in this precipitate.

The objective of this study was to isolate and purify the predominant aspartic proteinase from dormant rye grain, and to partially characterise it.

2 MATERIALS AND METHODS

Rye cultivar Humbolt was ground into whole meal with a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). All extraction steps and determination of proteolytic activities were as described before (Brijs *et al*., 1999).

Ammonium sulphate 35–60% rye bran fraction was dissolved (10 mg/mL) in 0.05 M sodium acetate buffer (pH 3.0; 0.5 M sodium chloride) and applied to a pepstatin A-agarose column (10.0 x 1.0 cm), equilibrated with the same buffer. The column was eluted with 0.1 M sodium carbonate buffer (pH 10.0) at 1.0 mL/min. Fractions (1.0 mL) with protease activity were pooled and concentrated.

The purity and M_r of the purified aspartic proteinase were evaluated with native and SDS-PAGE using the PhastSystem (Pharmacia Biotech, Sweden). The proteins were silver stained as described by Pharmacia (Pharmacia LKB Biotechnology, Development Technique File N° 210).

For N-terminal amino acid sequence analysis, the purified enzyme subunits were separated by SDS-PAGE on a 14.5% gradient gel, electroblotted onto polyvinylidene difluoride membrane and subjected to Edman degradation. Sequence analysis was performed on a Applied Biosystems model 477 A gas pulsed liquid-phase sequencer

with on-line phenylthiohydantoin-amino-acid identification (Applied Biosystems 120 A Analyser). Cysteine was not determined.

3 RESULTS AND DISCUSSION

An aspartic proteinase (EC 3.4.23), i.e. RAP (rye aspartic proteinase), was purified from the 35–60% ammonium sulphate rye bran fraction by affinity chromatography on a pepstatin A-agarose column, as shown in Figure 1.

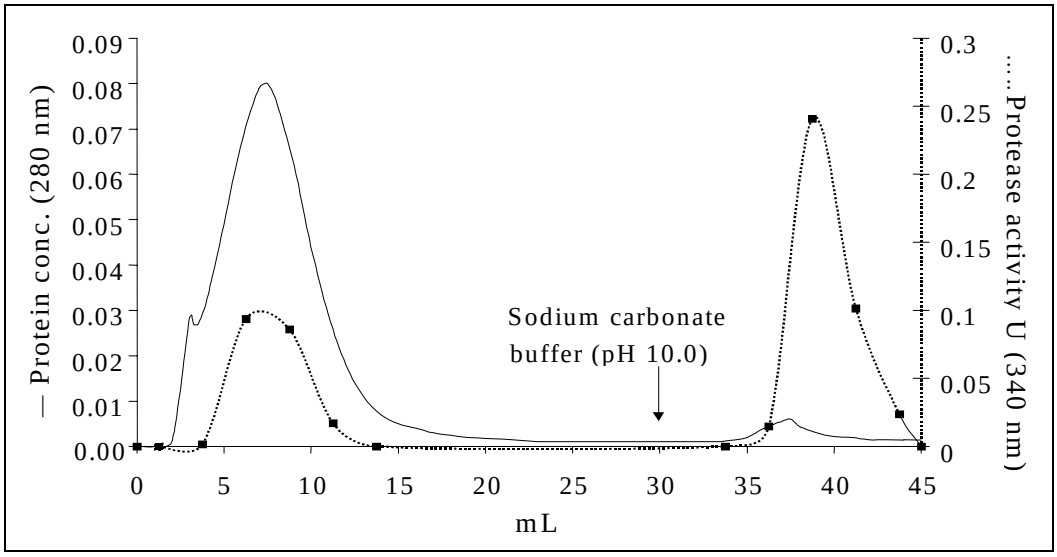


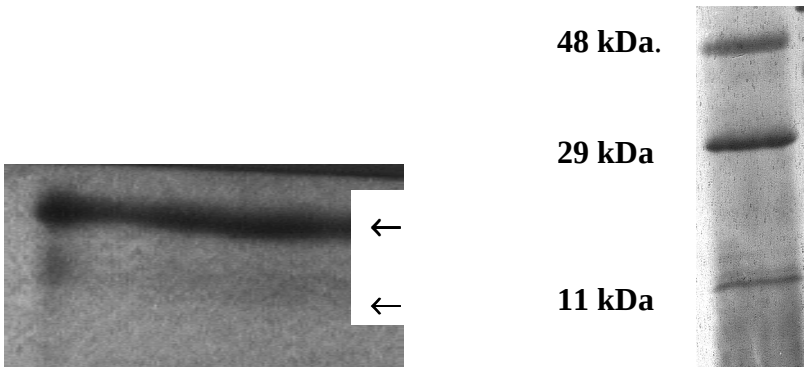
Figure 1. Purification of an aspartic proteinase from the 35–60% ammonium sulphate rye bran fraction on pepstatin A-agarose column (10.0 x 1.0 cm). Flow rate was 1.0 mL/ min, protein (—) was measured at 280 nm and protease activity (...) towards hemoglobin as described before (Brijs et al., 1999). One unit of proteolytic activity (U) corresponds to the liberation of 1 mg of leucine/h at pH 4.0 and 40 °C under the assay conditions.

Native PAGE (Figure 2a) revealed that this partially-purified enzyme preparation contained (two) aspartic proteinase(s) as the activity was totally inhibited by pepstatin A.

SDS-PAGE under non-reducing conditions (Figure 2b) and comparison with literature and sequence data (cfr. infra) indicated a 48-kDa enzyme and 29-kDa and 11-kDa subunits, which very likely stem from a 40-kDa heterodimer. Under reducing conditions (2-mercaptoethanol), the 48-kDa heterodimeric enzyme disappeared and a new protein band of 16-kDa appeared.

The N-terminal amino acid sequence of the different subunits (Figure 3) revealed a high homology of the rye aspartic proteinase (RAP) with *Hordeum vulgare* aspartic proteinase (HvAP; Sarkkinen *et al*., 1992) and with a commercial wheat gluten aspartic proteinase (GLAP; Bleukx *et al*., 1998). In contrast with HvAP, a 32-kDa subunit was not found after reduction with 2-mercaptoethanol. As in the case of HvAP, the 48-kDa

heterodimeric enzyme is probably a precursor-enzyme of the 40-kDa enzyme. The hemoglobin hydrolysing activity was optimal at 50 °C and pH 3.0.



A B

Figure 2. A native page of rye aspartic proteinase (rap) on a 20% polyacrylamide gel; b sds-page of the aspartic proteinase on a 8–25% polyacrylamide gel (in the absence of 2-mercaptoethanol).

HvAP (32 kDa)	NH_2-	S E E E G D I V A L K N Y M N A Q Y F G	
RAP (29 kDa)	**NH_2-**	**S E E E G D I V # L K N Y M N A Q Y F G**	
HvAP (29 kDa)	NH_2-	* * * * * * * * A * * * * * * * * * * *	95%
GLAP (29 kDa)	NH_2-	* * * * * * * * S * * * * * * * * * * *	95%
RAP (16 kDa)	**NH_2-**	**G D P M # N A # E M A V V W N Q Q Y L A**	
HvAP (16 kDa)	NH_2-	A * * * C S * C * * * * * * * * N Q * *	70%
RAP (11 kDa)	**NH_2-**	**E S A V D # A F L G S M P D I E F T I G**	
HvAP (11 kDa)	NH_2-	* * * * * C G * * * * * * * * * * * * *	90%
GLAP (11 kDa)	NH_2-	* * * * * # * S * * * * * * * * * * * *	90%

Figure 3. Comparison of the N-terminal amino acid sequences of the different subunits of the rye aspartic proteinase (RAP) with those of Hordeum vulgare aspartic proteinase (HvAP; Sarkkinen et al., 1992) and a commercial gluten aspartic proteinase (GLAP; Bleukx et al., 1998). Stars (*) in the sequences indicate identity with RAP and the percentages are % identity with the sequence of RAP. The symbol # stands for a non-defined amino acid.

4 CONCLUSIONS

An aspartic proteinases, very similar with HvAP, was isolated from the 35–60% ammonium sulphate bran fraction of rye. Further characterisation of the enzyme and influence on the functionality of some proteins is in progress.

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GERMINATED OATS PROTEINASES THAT HYDROLYZE AVENINS

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1 INTRODUCTION

In the resting seed the storage proteins are generally situated in the endosperm in an insoluble form. During germination, they must be solubilized so that they can be transported to the embryo for use by the growing plantlet. Botanically 'germination' ends when the rootlet emerges from the embryo, but for this report the term is used in a broader sense to include the early phases of plantlet growth.

The proteolytic system of germinated oats is not well known. Sutcliffe and Baset (1973) showed that the oat pH 8.0 casein hydrolyzing activity increased throughout the entire germination period, but that after two days the rate of the increase declined. We have earlier (Mikola and Jones 1999) shown that serine and metalloproteinases are the dominant gelatin-hydrolyzing enzymes of germinated oats at pH 6.2, the pH of the germinating oat endosperm. It was also evident that the addition of 8 mM cysteine (a reducing agent), together with 10 mM calcium raised the azogelatinase activity 2.5-fold and that the cysteine proteinases were the predominant activities under these conditions. According to Shutov and Vaintraub (1987) it is probable that the cysteine proteinases are the major storage-protein-hydrolyzing enzymes in flowering plants.

In oats, the major storage proteins are globulins (salt soluble proteins). Avenins, the oat prolamins, are also present, but in smaller quantities than the globulins (Peterson and Brinegar 1986). The alcohol soluble avenins have molecular weights of 22 kD to 33 kD. The avenins of Finnish oat varieties were compared by Jussila *et al*., (1992), who detected both alpha- and beta-avenins in all of the varieties that they analyzed.

The aim of this study was to characterize the germinated oats proteinases that hydrolyzed avenins. The avenins were hydrolyzed '*in vitro*' by proteinases that were extracted from germinated seeds and the hydrolysis products were analyzed using SDS-PAGE.

2 MATERIALS AND METHODS

Hand-hulled oat seeds (*cv* Veli) were surface sterilized with 1% sodium hypochlorite and were germinated aseptically at 16 °C on 0.5% agar gels. During this process, seed samples were collected every 24 h for eight days and the samples were frozen after the embryos, including the plantlets, were carefully removed. The avenins were extracted from untreated, defatted, whole oat meal that had been previously extracted with

deionized water (1 hr) and centrifuged (10 000 g, 15 min) to remove the albumins. The prolamin fraction was extracted from the albumin-extract precipitate (1 g original weight) with 52% aqueous ethanol (10 ml). The prolamin-extract supernatant was vacuum dried and suspended in 0.675 ml of 52% aqueous ethanol and this fraction was used as a substrate for the endoproteinases. The frozen oat samples were homogenized and enzyme extracts were prepared according to the method of Zhang and Jones (1995). The extraction ratio was 1.5 ml extractant to 1 g fresh weight oats, except for the resting seeds, which were extracted using a ratio of 1 g/ 4 ml buffer.

To study the '*in vitro'* hydrolysis of avenins by oat endoproteinases these proteins were incubated with the enzymes in test tubes at 40 °C, for the times indicated in the figures. The avenin preparation was mixed with buffer and the enzyme extract was added. The buffers used were: pH 3.8 and pH 5.0, Na acetate; pH 6.2, Na citrate. All buffers were 200 mM and cysteine (2 mM) was included in each reaction mixture. To characterize the endoproteinase activity classes, class-specific proteinase inhibitors were used according to the method of Zhang and Jones (1995). The hydrolysis products were analyzed on 12% SDS-PAGE gels under non-reducing conditions. The gels were stained with Coomassie Brilliant Blue R-250.

3 RESULTS

Seed samples were collected every 24 hr for up to eight days of germination. Enzymes from an 8-day germinated sample were used to conduct preliminary experiments to determine the best pH for studying the avenin hydrolyzing proteinases. Since the avenins are all situated in the endosperm and we were studying their hydrolysis, the embryo and plantlet were carefully removed from the seeds and the resulting endosperm material was used for the analyses. The avenin preparation (Fig. 1) contained alpha-avenins that migrated just behind the ion front, to the bottom of the gel. The beta-avenins separated into the beta-1 avenins, which gave two distinct bands that migrated to a position near that of the 31 kD marker protein and the beta-2 avenins, whose major bands moved to a location below that of the 31 kD marker.

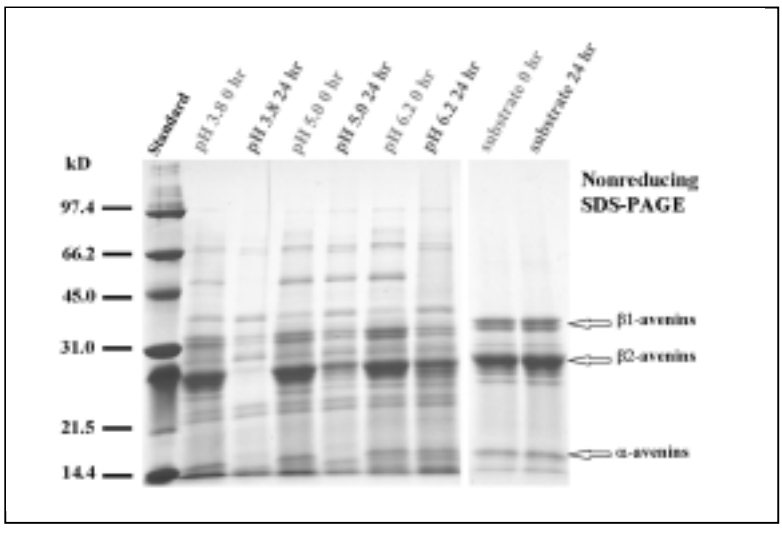

Fig.1 The effect of pH on the hydrolysis of avenin by 8-day germinated seed proteinases.

Endoproteinases that were extracted from 8-day germinated endosperms were incubated with the avenin preparation at pH 6.2, 5.0 and 3.8. The hydrolytic activity was very low at pH 6.2, moderate at pH 5.0, and almost complete hydrolysis occurred at pH 3.8 (Fig. 1). The activities of proteinases that were extracted from seeds germinated for times between 0 to 8 days were then measured at pH 3.8. The alpha-avenin was hydrolyzed by the endoproteinases extracted from 3-day germinated seeds and from those germinated for longer periods (Fig. 2, arrow, lane 3). The beta-2 avenins were partially hydrolyzed by proteinases extracted from the 6-day germinated sample and almost totally degraded by those from 7-day seeds (Fig. 2, arrow, lane 7). Finally the beta-1 avenins were hydrolyzed by activities extracted from 8-day germinated seeds (Fig. 2, arrow, lane 8). The effect of class-specific proteinase inhibitors on the proteinases extracted from 8-day germinated seeds was studied at pH 3.8 (Fig. 3). The addition of E-64, a cysteine proteinase inhibitor, totally inhibited the hydrolysis (Fig. 3). There was no effect when either PMSF (serine proteinase inhibitor), pepstatin A (aspartic proteinase inhibitor), O-phenanthroline or EDTA (metalloproteinase inhibitors) were present.

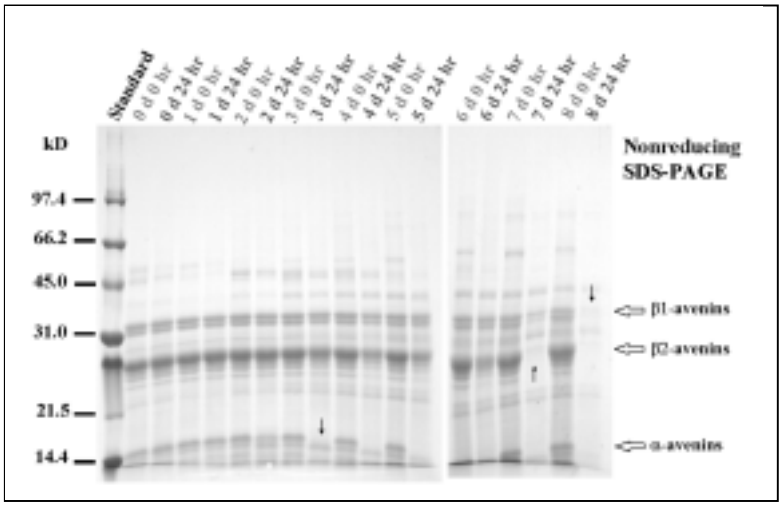

Fig. 2. The development of oat avenin-hydrolysing proteinases during germination, activities measured at pH 3.8.

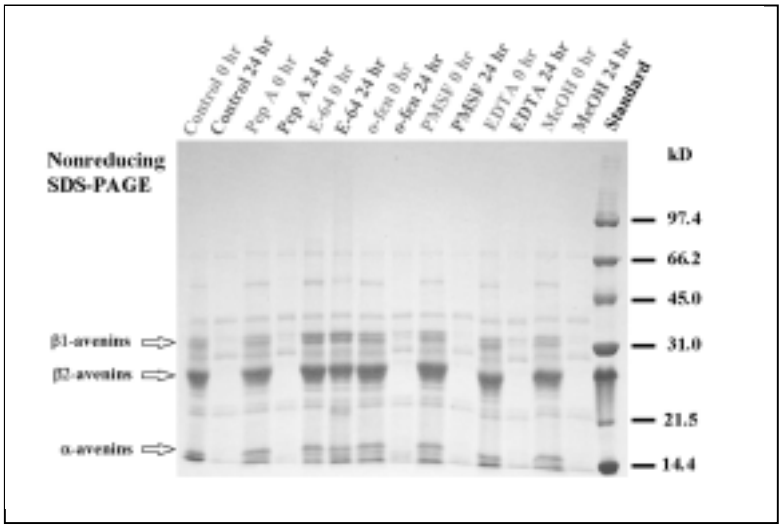

Fig. 3. The effects of class-specific proteinase inhibitors on 8-day germinated seed proteinase activities measured at pH 3.8.

4 DISCUSSION AND CONCLUSIONS

The endoproteinases that were extracted from 8-day germinated oat seeds and which hydrolyzed avenins were active at pH 3.8, giving complete hydrolysis of substrate in 24 h. These proteinases are members of the cysteine proteinase class, as shown by their inhibition by the class-specific inhibitor E-64. The activities that hydrolyzed the beta-avenins only appeared after 8 days of germination. However, the alpha-avenins were hydrolyzed by the proteinases that were extracted from 3-day germinated seeds.

Studies on barley endoproteinases have shown that at least two of the cysteine proteinases can hydrolyze hordeins *in vitro* (Jones & Poulle 1990, Koehler & Ho 1990). We have previously characterized the oat globulin-hydrolyzing proteinases (Mikola and Jones, unpublished results), and these had somewhat different characteristics. Four-day germinated seeds contained proteinases that partially hydrolyzed the globulins and the enzymes of 8-day germinated seeds readily hydrolyzed globulins into peptides that were too small to be detected by 12% SDS PAGE.

Since good protein hydrolysis occurred at pH levels that were much lower than pH 6.2, which is reportedly the pH of the germinated oats endosperm, there is a good chance that pH compartmentalization occurs within the endosperm during germination. The avenin-hydrolyzing proteinases are cysteine- class enzymes. Since the alpha-avenins were hydrolyzed by proteinases that formed in the seed during the early stages of germination, they are probably hydrolyzed by different proteinases than are the beta-avenins, which were hydrolyzed only by enzymes that appeared later in the germination process.

5 ACKNOWLEDGMENTS

We thank the Finnish Ministry of Agriculture and Forestry for financing this study.

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POSSIBLE IMPLICATIONS OF FOUR OXIDOREDUCTASES (POLYPHENOLOXIDASE, CATALASE, LIPOXYGENASE, AND PEROXIDASE) PRESENT IN BREWERY'S BARLEY AND MALT ON ORGANOLEPTIC AND RHEOLOGICAL PROPERTIES OF MASH AND BEER

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1 INTRODUCTION

Organoleptic quality of beer during storage can be dramatically altered as a result of enzymatic oxidation of endogenous polyunsaturated lipids and phenolic compounds originating from brewery's barley. Enzymatic-mediated oxidative reactions can take place during the 3 stages of the process *viz* barley germination, malt kilning and at the beginning of mashing.

In this respect, the action of oxidoreductases can lead to flavour deterioration (development of staling and off-flavours), occurrence of haze, modification of bitterness and astringency, as well as a modification of the colour of the processed product (1, 2). Both **polyphenoloxidase** (**PPO**) and **peroxidase** (**POD**) are able to catalyze the O_2- or H_2O_2- mediated oxidation of endogenous polyphenols in reactive quinonic compounds and give rise to secondary oxidations altering the quality of beer. Moreover, POD are also susceptible to realize oxidative cross-linkings between proteins and / or soluble pentosans (3) and, consequently, may hinder lautering and filterability of beer.

Catalase (**CAT**), by speeding up the dismutation of H_2O_2, a reactive oxygen species, may constitute an *in situ* primary antioxidant system.

Lipoxygenase (**Lox**) catalyzes the oxidation of polyunsaturated free acids. This oxidoreductase is mostly responsible for the production of volatile aldehydes such as *trans* 2-nonenal, originating from 9-hydroperoxide, widely suspected of leading to staling of beer.

2 OBJECTIVES OF THE STUDY

In this work, we have study the evolution of PPO, POD, CAT and Lox during malting of 9 (spring or winter) barley varieties harvested in 1995 and 1996 and the corresponding green and kilned malts.

This study was undertaken to get a better knowledge on the relative importance of these oxidoreductases in the beer deterioration.

3 MATERIAL AND METHODS

3.1 SAMPLE TREATMENTS

Barley (*hordeum vulgare* L.) of spring and winter cultivars was selected and harvested by SECOBRA Recherches and supplied by the French Institute of Brewing and Malting, I.F.B.M. (Nancy, France).

Green malt from barley was prepared in a micromalthouse (2 kg) using standard malting conditions already described in (6). The kilning procedure consisted in 3 successive steps of heating of the germinated barley (6).

Extraction steps of oxidoreductases from kernels are described in (4, 5).

3.2 ASSAYS METHODS

PPO, CAT and Lox activities were routinely assayed at 30 °C by a polarographic method based on consumption (PPO, Lox) or production (CAT) of O_2. Activities were expressed in nkatals or µkatals per mL or g of crude enzymatic extract.

POD activity was routinely assayed at 25 °C by a spectrophotometric method based on the increase in absorbance at 410 nm resulting from the oxidation of ferulic acid in the presence of H_2O_2 and ascorbic acid, acting as a second reductant (for the principle of this chronometric assay, see in (6)). Activity was defined as µkatals per mL or g of crude enzymatic extract.

4 RESULTS

4.1 EVOLUTION OF OXIDOREDUCTASES ACTIVITIES DURING MICROMALTING OF BARLEY

Comparison of PPO, POD, Lox and CAT activity levels present in native barleys, green and kilned malts, presented in Table below, showed that:

Enzymatic activity and variety		Barleys	Green malts	Kilned malts
PPO	Spring	$0.63 \cdot 10^{-3}$–$1.32 \cdot 10^{-3}$	0–$0.42 \cdot 10^{-3}$	0
	Winter	$0.45 \cdot 10^{-3}$–$0.72 \cdot 10^{-3}$	0	0
Lox	Spring	0.14–0.21	0.25–0.35	0.06–0.09
	Winter	0.08–0.12	0.16–0.27	0.02–0.05
POD	Spring	43.3–83.3	80.9–141	57.6–93.0
	Winter	45.4–65.7	55.4–124	61.5–84.7
CAT	Spring	9.34–13.8	156–291	139–183
	Winter	4.35–9.46	139–178	96.03–135

✓ On the whole, **spring (2 rows) barleys** contained significantly more oxidoreductases activities than the **winter (2 or 6 rows)** varieties.

✓ **PPO** activity, very low in barley (~ 1 nkat.g^{-1} dry matter), decreased during malting and totally disappeared at the term of kilning for all barley varieties tested.

✓ Levels of **CAT**, very high in mature barley (~ 9.5 µkat.g^{-1} dry matter), highly increased during germination (× 14–38), in relation with *de novo* synthesis of a second isoform. Kilning process resulted in variable losses (between 8–46%) of activity, according to the thermosensitivity of the isoenzymes. Nevertheless, at the end of malting, kernels contained much more residual activity (× 10–25) than the native grains.

✓ Levels of **Lox** activity determined in barley varieties (~ 140 nkat.g^{-1} dry matter) corresponded quite exclusively to the isoform **Lox 1** which preferentially produces 9-hydroperoxide, a precursor of *trans* 2-nonenal, mainly responsible for staling of beer. The germination step led to an increase (× 1.2–2.3) in global Lox activity along with *de novo* synthesis of the isoform **Lox 2**. The kilning process resulted in an extensive loss (66–88%) of both Lox isoenzymes.

✓ **POD** activity, very high in barley (~ 62 µkat.g^{-1} dry matter) was slightly modified (× 0.8–2) during germination and resulted from the increase in global activity of the different enzymatic fractions contained in green malt. The most heat-resistant POD forms survived the kilning process and globally, malts contained more activity (× 0.9–1.6) than barley kernels.

5 CONCLUSIONS

Results exposed above suggest some possible interventions of these oxidoreductases on organoleptic and rheological properties of mash and beer during the technological process:

✓ Implication of **PPO** on the final colouration of mash and the formation of beer haze seems to be negligible.

✓ Conversely**, POD** could be involved in the oxidative polymerization of phenolic substrates such as endogenous proanthocyanidins, resulting in increased wort colour and turbidity and act defavorably on flavour quality and stability with formation of beer haze.

Moreover, they could be implicated in lautering and filterability problems of mash and beer *via* oxidative cross-linkings between arabinoxylans and / or proteins involving phenolic or thiol residues.

✓ The action of **Lox** (especially **Lox 1**) at the first steps of malting would be predominent for the organoleptic quality (staling) of beer. Moreover, as suggested for POD, Lox could be involved in the creation of oxidative cross-linkings between thiol-rich proteins *via* cooxidative reactions, resulting in macromolecular reticulations, and possibly altering filterability capacity of wort.

✓ Antioxidative capacity of **CAT** during malting and at the beginning of the brewing process could be involved, but its action will depend on endogenous levels of H_2O_2.

6 ACKNOWLEDGMENTS

This work has been financially supported by grants from the French Ministery of Agriculture and Fisheries and Malteurs et Brasseurs de France (Oxidases and Polyphenols: new quality tracers for the brewer's barley).

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OXIDATION OF FERULIC ACID BY PURIFIED WHEAT GERM PEROXIDASE COUPLED WITH DIFFERENT GLUCOSE OXIDASE

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1 INTRODUCTION

Various chemical redox compounds such as ascorbic acid, bromate and iodate were used as bread improvers for a long time. However, depending on the country, some of them such as bromate are either not permitted or could be prohibited in a next future due to the potential hazards (Dupuis, 1997). In order to meet the consumer demand for additives-free products, the baking industry is deeply involved in research for alternatives to these chemical improvers. One candidate for the replacement of oxidizing agents is the glucose oxidase (GOX) (EC 1.1.3.4) which exhibits improving effects in baking (Haarasilta *et al*., 1991; Martinez-Anaya and Jimenez, 1998; Vemulapalli *et al.*, 1998; Wilkström and Eliasson, 1998). GOX catalyzes the conversion of glucose and oxygen into gluconolactone and hydrogen peroxide (Whitaker, 1985). According to Haarasilta and Pullinen (1992), the H_2O_2 formed is able to oxidize the thiol groups in disulfide bonds which explains the dough strengthening. Additionally, according to studies in model systems, it has been proposed that peroxidase (POD) (EC 1.11.1.7) combined with H_2O_2 can promote in dough the oxidative gelation of pentosans (Geissmann and Neukom, 1973; Figueroa-Espinoza and Rouau, 1998) by the cross-linking of their ferulic acid residues. Lastly and although it has never been demonstrated in doughs, the polymerisation of proteins by the H_2O_2-peroxidase system has also been proposed (Matheis and Whitaker, 1984; Bushuk *et al*., 1980).

Recently, the hexose oxidase (HOX) (EC 1.1.3.5), isolated from the red algae *Chondrus crispus*, has been proposed as dough improver (Poulsen and Bak Hostrup, 1998). This enzyme, unlike GOX, can utilize several monosaccharides and oligosaccharides. It catalyzes the conversion of the latters into corresponding lactones with the formation of hydrogen peroxide. Poulsen and Bak Hostrup (1998) compared the effects of HOX and of GOX in dough and bread. They showed that HOX caused increased dough strength and bread volume more efficiently than GOX in the same dosage.

The purpose of this work is to compare the activation of purified wheat germ peroxidase by HOX from *Chondrus crispus* and GOX from *Aspergillus niger* by following ferulic acid oxidation.

2 MATERIALS

Enzymes. Glucose oxidase was extracted from Aspergillus niger. Hexose oxidase, extracted from Chondrus crispus was obtained from Danisco Ingredients (Brabrand, Denmark).

Wheat germ was provided by Les Moulins Soufflet (Nogent sur Seine, France).

3 METHODS

Wheat germ peroxidase purification. *Peroxidase from wheat germ was purified according to Billaud et al. (1999). The major fraction FC1 was the only one used for this study .*

Enzymes assays. *Peroxidase: The peroxidase activity was measured by a spectrophotometric method based on the decrease in absorbance at 310 nm. The reaction mixture contained ferulic acid (100 µM), H_2O_2 (500 µM) and $CaCl_2$ (20 mM) in sodium acetate buffer (0.1 M, pH 5.6). The activity was determined by the initial slope from the linear decrease in absorbance at 310 nm.*

Glucose oxidase (GOX) and hexose oxidase (HOX) : The GOX and HOX activities were determined polarographically at 30 °C, by following the O_2 consumption with a Clark-type oxygen electrode in a 100 mM acetate buffer solution at pH 5.6 saturated by air and containing glucose (50 mM). Activity is expressed in nkat (nmol of oxygen consumed per second in the assay conditions).

4 RESULTS AND DISCUSSION

In order to compare the activation of wheat POD by the H_2O_2 produced by GOX or by HOX, the volumes of each enzyme necessary to obtain the same initial oxygen uptakes were determined for different glucose concentrations (Table 1).

Table 1. GOX and HOX volumes necessary to obtain the same initial oxygen uptake.

[Glucose] used for the assay (mM)	GOX volume (µL)	HOX volume (µL)	Equivalent activity (nkat)
50	25	78	2.7
5	50	55	1.5
2	35	20	0.42

A series of experiments was carried out in order to compare the GOX-POD and the HOX-POD associations. Different amounts of GOX or HOX were associated with a fixed amount of POD (0.042 UA/sec) and ferulic acid oxidation was followed by the decrease in absorbance at 310 nm. Table 2 shows **the time necessary to obtain a**

decrease of 0.4 absorbance units for the GOX-POD or the GOX-POD association for different glucose concentrations in the system.

Table 2. Time necessary to obtain a decrease of 0.4 absorbance units for each association at different glucose concentrations.

Initial [glucose] (mM)	GOX volume (µL)	HOX volume (µL)	Time for GOX-POD (sec)	Time for HOX-POD (sec)
50	25	78	74	55
5	25	78	164	60
2	25	78	286	67
5	50	55	106	71
2	35	20	246	157

The first experiment (line 1 of Table 2) shows that ferulic acid oxidation was slightly faster with the HOX-POD association compared to the GOX-POD.

If the initial glucose concentrations are lowered to 5 mM (line2) or 2 mM (line 3), HOX activates the ferulic acid oxidation by wheat POD more efficiently than GOX does (the time found for GOX-POD is superior than this determined for HOX-POD). The lower the glucose concentration is, the more efficient HOX compared to GOX is. This effect can be attributed to the differences in affinity toward glucose for the two enzymes. We found a Km value of 22 mM for GOX and 1 mM for HOX in our assay conditions. Therefore, when the glucose concentration was decreased from 50 to 2 mM, the production rate of hydrogen peroxide was decreased 9 times for GOX whereas it was only 1.5 times lower for HOX.

Finally, experiments were carried out with the same glucose concentrations (2 and 5 mM) but with adapted amounts of GOX and HOX in order to obtain in both cases, a similar H_2O_2 production rate (Table 1). Table 2 (lines 4 and 5) shows that the activating effects of the two oxidases were much less different than those observed for conditions descibed lines 2 and 3. Nevertheless, the association HOX-POD oxidizes faster ferulic acid than the association GOX-POD does.

A comparison of the oxygen uptake by GOX and HOX shows that if the initial rate is equivalent for both enzymes, the oxygen consumption slows down more rapidly with GOX than with HOX. This phenomenon is not due to an inhibition by hydrogen peroxide since both enzyme kinetics are not affected by H_2O_2 in the concentration range of this study (results not shown). Therefore, it can be assumed that the affinity for oxygen is higher for HOX than for GOX.

5 CONCLUSION

According to Poulsen and Bak Hostrup (1998), HOX is more effective to increase dough stength and bread volume than GOX used in the same dosage. These authors indicate that this is due to the HOX capacity to oxidize several saccharides including glucose and maltose as well as to its higher affinity for glucose. If the efficiency of HOX and (GOX) is linked to the production rate of H_2O_2 which is then used by wheat peroxidase, our results confirm the better efficiency of HOX compared to GOX. The difference between the two enzymes is due to the better affinity of HOX for glucose but probably also for oxygen. This can be of importance in the formulation of enzyme improvers since during mixing, glucose is present in variable and limited amounts in dough. Moreover, other oxidoreductases (e.g. lipoxygenase) and yeast with variable affinities for oxygen are competitors for using this compound.

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VARIATION IN LIPID OXIDATION RATES IN CEREALS – A RESULT OF LIPOXYGENASE ENZYME ACTIVITY OR LIPID AVAILABILITY?

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1 INTRODUCTION

The aqueous processing of cereals can induce serious lipid deterioration due to the increment in the free fatty acids followed by the enzymatic oxidation of the unsaturated fatty acids (Liukkonen *et al*. 1992). Many cereals contain endogenous lipase and lipoxygenase activities, which in the intact grains are suppressed, but when the grain is milled and further processed these enzymes become functional. In cereals, the most abundant oxidizible fatty acids are linoleic acid, C 18:2 (n-6,9), which constitutes typically from one third to over one half of the total fatty acids, and linolenic acid, C 18:3 (n-6,9,12) the amount of which varies from 1 to 10% of total fatty acids.

The lipoxygenase reaction rate of the cereals varies intensely during the different processing stages, while the reasons for these sudden variations are poorly understood. The lipoxygenase activity and the amounts of free acids predict only partially the development of rancidity and it has become clear that changes in the physical organisation of the lipids effects intensely on the development of the rancidity.

The present study evaluates if the variations in the lipoxygenase reaction rate of the flour suspensions are provoked by the changes in the capacity of flours to bind lipids. A reduction in the amount of lipids adsorbed by the flour causes an increase in the amount of lipids exposed to the lipolytic enzymes and thus an increased deterioration rate (Lehtinen and Laakso, 1999). Understanding the relationship between the lipid binding capability and the lipid deterioration would yield an effective tool to predict and control the extent of oxidation occurring during cereal processing.

2 SCHEME OF THE STUDY

In order to mimic the lipase action, flours were suspended with 40 volumes of 0.2 mM Na-phosphate buffer pH 7 containing equal amounts of free micellar oleic and linoleic acids. The former is relatively resistant towards cereal enzymes while linoleic acid is a major substrate for lipoxygenase. The amount of supplemented acids was 15 mg of each acid / g flour.

The supplemented flour suspensions were incubated for 15-min and lyophilised. Total and unbound lipid pools were analysed for fatty acid compositions by analysing either the lyophilised suspension or the n-hexane extract.

Cereals with different lipoxygenase activities, such as wheat, rye, oat, barley, barley malt and oat malt were tested for the lipid binding capability.

2.1 How was the rate of lipoxygenase reaction measured?

The lipoxygenase reaction was followed by 1) measuring oxygen consumption rate of the suspensions containing linoleic acid in excess to lipid binding capacity and 2) by analysing the proportion of supplemented linoleic acid remaining intact in the suspension after 15-min incubation.

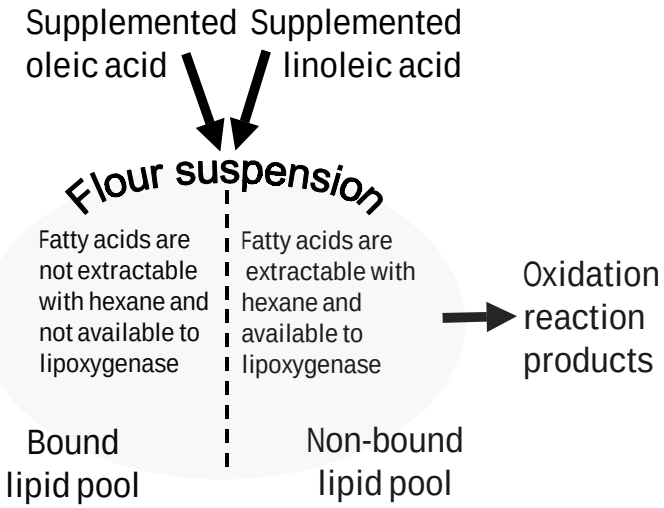

Figure 1. Scheme of the present study.

2.2 How were the unbound and bound lipid pools distinguished?

The hexane extraction was used to collect the unbound lipids from those that were adsorbed to the flour matrices (Figure 1). The total amounts of fatty acids containing both the bound and unbound lipids were evaluated by adding the saponification reagent straight into lyophilised suspensions.

3 RESULTS

3.1 The variation in the lipoxygenase reaction rate of different cereal suspensions

Of the studied flours the barley exhibited highest lipoxygenase reaction rate so that in the barley flour suspension the 15-min mixing was sufficient to reduce the supplemented linoleic acid by 89%. In the wheat and rye flour suspensions the recoveries of supplemented linoleic acid were 46% and 23% respectively. No loss of linoleic acid was observed in the suspensions of oat, oat malt or barley malt.

Unexpectedly, the loss of linoleic acid was not found to strictly correlate with the initial oxygen consumption rates that were measured with the linoleic acid concentration exceeding the lipid binding capacity (Table 1).

Table 1. The total recovery of supplemented linoleic acid and the initial rate of lipoxygenase (LOX) reaction of different cereal suspension.

Flour added to suspension	Total recovery of supplemented linoleic acid	Initial rate of LOX reaction (U)
Barley	11%	1200
Rye	23%	290
Wheat	46%	630
Oat malt	99%	<50
Oat	104%	<50
Barley malt	106%	<50

3.2 THE VARIATION IN THE CAPABILITY OF DIFFERENT CEREALS TO BIND SUPPLEMENTED LIPIDS

The capability to bind supplemented oleic acid was used as a measure of the lipid binding. The total recovery of oleic acid did not change during the mixing, but it's distribution between the n-hexane extractable and non-extractable was different in each flour suspension. Over 70% of the supplemented oleic acid in the barley flour suspension was extractable with n-hexane (Table 2). However, the suspensions of the flours, which exhibited lower rates of lipoxygenase reaction, also released smaller portion, 20–60%, of the added oleic acid in n-hexane extraction.

Table 2. The proportion of supplemented oleic acid recovered in the hexane extract.

Flour added to suspension	Hexane extractable oleic acid
Barley	73%
Rye	62%
Wheat	43%
Oat malt	20%
Oat	17%
Barley malt	21%

4 CONCLUSIONS

An obvious trend that the suspensions of the flours that bind supplemented fatty acids strongly also oxidise linoleic acid slowly was noticed (Figure 2).

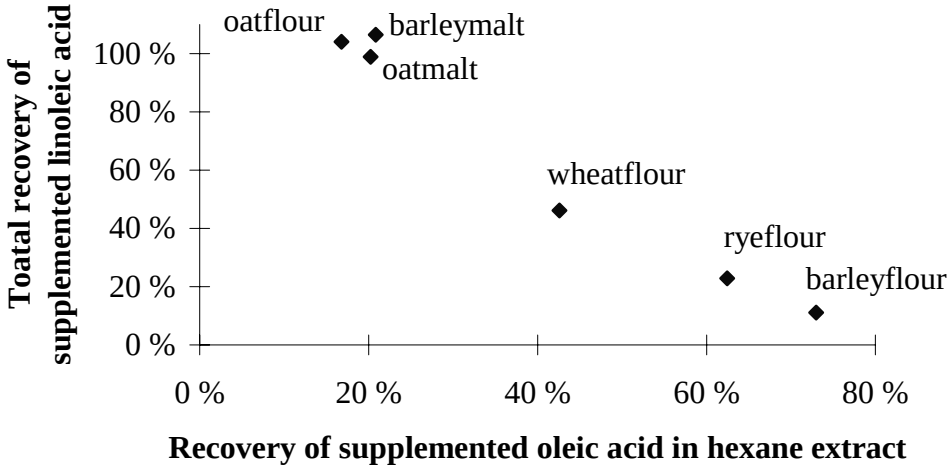

Figure 2. The correlation between the proportion of supplemented linoleic acid remaining intact in the flour suspension and the proportion of supplemented oleic acid recovered in hexane extract.

The wheat flour suspension exhibited a high rate of lipoxygenase reaction when measured using high linoleic acid concentration. However, wheat flour suspension also bound a large amount of supplemented fatty acid. This resulted in a relatively high recovery of the supplemented linoleic acid from the wheat flour suspension. On the other hand, in the rye flour suspension the situation was the opposite. The suspension exhibited slower lipoxygenase reaction rate than wheat flour although the total loss of supplemented linoleic acid was higher after whole incubation period. This was attributed to be due to the lower capability of rye flour to bind lipids, so that the supplemented linoleic acid remained available to lipoxygenase action for the whole incubation period.

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TRANSFORMATION OF BARLEY WITH ANTIFUNGAL PROTEIN GENES

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1 INTRODUCTION

The barley, oat and wheat crops are challenged annually by fungi which can destroy vast acreages of crops. Efforts to clone and express antifungal proteins in barley (*Hordeum vulgare* L.) were begun in response to the recent *Fusarium* outbreak in the upper Midwest of the USA. *Fusarium* infection reduces seed yield and makes the seeds unfit for use in food, feed and beverages. Plants have evolved a variety of mechanisms to cope with the pathogens. One defense against infections is to synthesize pathogenesis-related proteins (PRPs). PRPs are synthesized in response to infections and/or are deposited in fruits and storage organs during normal development. PRPs are classified into five families. Among the PR-5 proteins are the thaumatin-like proteins (TLPs). One group of TLPs, permatins, occurs in barley and other cereal seeds. Further knowledge of their properties could lead to more effective antifungal strategies. Thionins, including the hordothionin storage protein of barley, belong to a class of small, high-cysteine proteins. These have potent antimicrobial properties, which have been known for decades.

2 MATERIALS AND METHODS

Genes for permatin proteins from the starchy endosperm of barley (GenBank AF016238) and oats (GenBank U57787) were cloned and used to transform barley in the sense and antisense orientations. The gene for the barley hordothionin was cloned and used to transform barley (Skadsen *et al*., 1998) using the particle bombardment method. The particle bombardment, culture, selection and regeneration of the bombarded half-embryos followed the procedure of Wan and Lemaux (1994). Excised immature embryos of barley cv. Golden Promise were used as target material. After bombardment, the embryos were cultured on callus induction medium in the presence of a selective agent, bialaphos. After four subcultures, the resistant callus cultures were transferred to a regeneration medium that contained bialaphos. The green plantlets were transferred into Magenta boxes that contained rooting medium supplemented with bialaphos. When the plantlets reached the top of the box they were transferred into soil.

3 RESULTS AND DISCUSSION

From the bombardments with the permatin plasmid three green plants were obtained (regeneration frequency 0.6%). All three plants were PCR positive for the presence of

the *bar*-gene. However, only one of them tested PCR positive for the permatin gene (Fig. 1). The presence of the permatin gene in the transformants was also confirmed with Southern blot hybridization (Fig. 1). The regeneration frequency for the control plasmid (*uidA* and *bar*) was 3.4% (8 green plants). In these experiments, the permatin gene was under the regulation of the constitutive maize uibiquitin promoter. This may be one reason for the low regeneration frequencies, since the physiological function of permatin in barley is not known.

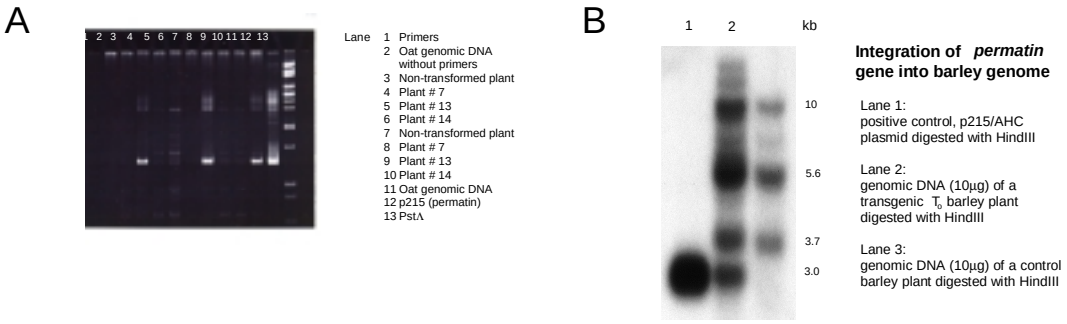

Figure 1. A) PCR analysis of permatin regenerants: Lanes 3–6: DNA extraction was done using the CTAB method (Sigma), lanes 7–14: DNA extraction was done using the DNeasy kit (Qiagen). B) Integration of permatin gene into the barley genome.

Permatin produced by yeast was tested *in vitro* for antifungal activity against *Fusarium*, and, at the same time, another antimicrobial protein isolated from barley, hordothionin, was also included in the experiment and proved to be more active against *Fusarium* than permatin. The gene coding for hordothionin was cloned from barley (Dr. Skadsen) and used in gene transfer experiments. The *bar* gene was used as a selectable marker. From the 240 half-embryos that were bombarded with the thionin construct, 106 green plantlets were regenerated on bialaphos selection. 100 of them were PCR positive for the thionin gene (Fig. 2).

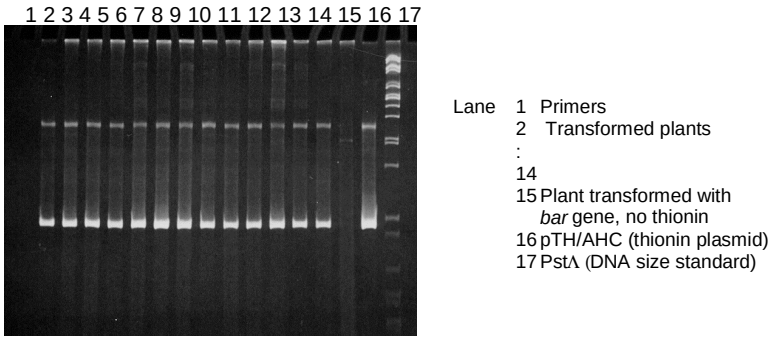

Figure 2. PCR analysis of thionin regenerants: DNA extraction was done using the CTAB method (Sigma).

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PROTEIN ENGINEERING OF THE STABILITY OF *T. REESEI* XYL II

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ABSTRACT

Trichoderma reesei xylanase II has the pH optimum near to five and the half-life at 55°C less than 10 minutes. Several amino acid mutants were constructed to study the activity and stability at elevated temperatures, and pH-dependent activity. The site-directed mutations were made mainly in the environment of the only α-helix present in this enzyme. Although, the single effects shown were quite moderate, a cumulative enhancement in the stability was considerable. The half-life of the best mutants at 65°C was even 50–100-fold compared to the wild-type xylanase. Most of the mutations increased stability at elevated temperatures, but the pH activity curve became narrower. This is probably due to more rigid structure of the mutant enzymes, which helps them to resist unfolding, but which may hinder essential movements in the catalysis.

1 INTRODUCTION

Considerable increase in the thermostability in a group 11/G xylanase has been achieved by addition of intra- and intermolecular disulphide bridges to the B. circulans xylanase /1/. A mutant corresponding to the best one in B. circulans /1/, is marked here as aN. It has a disulphide bond binding the N-terminus of the α-helix to adjacent β-sheet. Other mutants were designed following findings in molecular dynamics simulations, which suggest a site where unfolding is initiated /2/. We constructed mutants to resist denaturation at this site. Their effect is not shown alone but as double mutants with the disulphide bridge, labelled as aNH and aNY. We also made triple mutants, aNHD and aNYD. In this case, a mutation was added to the double mutants. Mutants are coded in a quite non-informative way because they are subjects of a recent patent application.

2 MATERIALS AND METHODS

The correlation between tertiary structure of a xylanase and its properties was studied combining molecular modelling and experimental mutant characterisation. Mutations were visualised and roughly assessed using Swiss-PdbViewer software. Site directed mutagenesis method were from Stratagene and the primers ordered from Europrim. Xylanase activity was assayed as described by Bailey /3/. Enzyme activity for pH-dependence and thermostability was scanned between pH 4–9 and 55–65°C.

3 RESULTS AND DISCUSSION

All the mutants, aN, aNH, aNY, aNHD and aNYD, were active. The aim, to improve the thermostability, was achieved satisfactorily. A cumulative rise in the enzyme stability at 65°C could be seen (Figure 1). The half-life measurements showed some fluctuations from time to time. Over 50-fold stabilisation was achieved by multiple mutations. Constant-time (10 min) incubations at different temperatures, 50–75°C, exhibited a similar trend. From the deactivation curve we estimated the temperature in which half of the initial activity (that of 50°C) was lost. The wild type had lost 50% of its activity during 10 min incubation at 52°C, and corresponding temperatures were 63 and 68°C for the double and triple mutants, respectively (not shown). In the same way as in B. circulans /1/, the disulfide bridge at the N-terminal region of the α-helix provided high stability for the enzyme.

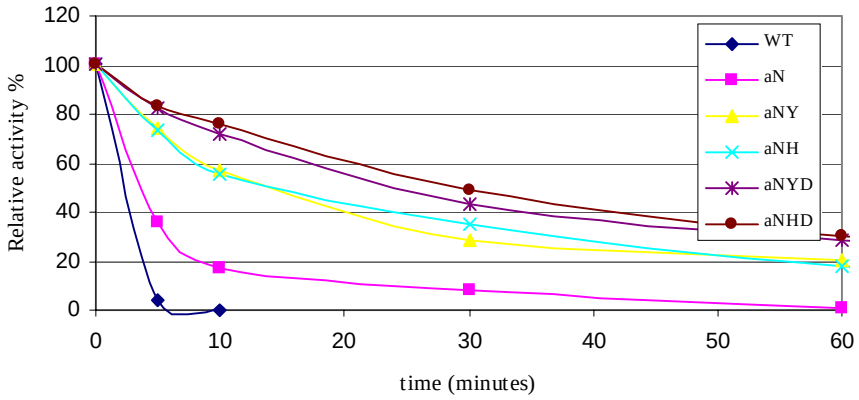

Figure 1. Half-lifes of the wild-type and mutant enzymes at 65°C.

This study shows that the thermostability can be increased considerably by multiple mutations, but the cost can be the narrowing of the optimal pH-range. None of these mutations increased the temperature optimum (55–60°C), which indicates, that stabilising mutations in other regions are needed to improve the enzyme activity at higher temperatures.

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PRODUCTION OF FUNGAL PLANT CELL WALL DEGRADING ENZYMES ON WET-OXIDISED WHEAT STRAW XYLAN

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1 INTRODUCTION

In Denmark, the wet-oxidation (WO) process has been used for fractionating wheat straw [10], where most of the hemicellulose is solubilised mainly as oligomers and polymers and the cellulose retained as a solid. In wheat straw, hemicellulose is an arabino-4-O-methylglucurono xylan, which, in addition to xylose, contains arabinose, 4-O-Me-D-glucuronic acid, and acetic acid substituents [9]. Some arabinosyl groups may be esterified with ferulic or p-coumaric acids. Most micro-organisms used for lactate, ethanol or xylitol production do not produce enzymes for hemicellulose hydrolysis. Acid or enzymatic hydrolysis is needed to convert the sugar polymers to monomers. A problem associated with dilute acid hydrolysis is the poor fermentability of the produced hydrolysates compared with enzymatic hydrolysis.

This study investigated the utilisation of wet-oxidised wheat straw xylan (WO-xylan) for production of enzymes for hydrolysis. Three fungal strains (*Aspergillus oryzae, Aspergillus fumigatus*, and *Trichoderma reesei*) were cultivated on WO-xylan using xylan (Lenzig AG) as a supplementary carbon-source. *A. oryzae* was selected for production of enzymes in larger scale.

2 MATERIALS AND METHODS

2.1 MICROBIAL STRAINS

Aspergillus oryzae VTT D-85248, *Aspergillus fumigatus* VTT D-82195, and *Trichoderma reesei* RUT C-30 were maintained on potato dextrose agar (25°C) under artificial day-light.

2.2 XYLAN SOURCES

Wet-oxidised xylan (WO-xylan): WO was carried out in a loop-reactor constructed at Risø [3]. Wheat straw (60 g/L) was mixed with 6.5 g/L Na_2CO_3 and water before adding 12 bar of oxygen pressure. The suspension was heated to 185°C for 15 minutes [10]. After

pre-treatment, the suspension was filtered to remove the solids from the xylan-rich fraction.

Lenzing xylan (Lz-xylan) was obtained from Lenzig AG (Lenz, Austria) [6].

2.3 CULTIVATION MEDIA

Media containing Mary Mandels minimal medium (MM) were made up either in water with 10 g/L lenzing xylan or directly in the WO wheat straw substrate containing approximately 9 g/L xylan [5]. The pH was adjusted to 4.8–5.0 before sterilisation (121°C, 20 minutes).

2.4 SHAKE FLASK CULTIVATIONS

Four types of media were used for the screening test. *Lz-medium* consisted of MM with 10 g/L lenzing xylan. *Lz-WO-medium* consisted of 25 mL MM with 10 g/L lenzing xylan. After 1 day of cultivation, 25 mL MM in WO wheat straw xylan substrate was added aseptically. *Mixed medium* consisted of a mixture (1:1) of MM with 10 g/L lenzing xylan and MM in WO xylan substrate. *WO-medium* consisted of MM in WO xylan substrate. Shake flasks were inoculated with 2 mL of a 2-day culture and cultivated at 30°C, 200 rpm for a total of 5 days. The biomass was determined by filtration.

2.5 LARGE-SCALE CULTIVATION

The fermentor cultivation was carried out in a BioFlo A fermentor (New Brunswick) with a working volume of 7 L including 500-mL 1 day old inoculum of *A. oryzae* on *Mixed medium* (as described above). The fermentation medium was 2 x MM-medium in WO xylan substrate supplemented with 10 g/L lenzing xylan. The fermentation conditions were: temperature, 30°C; pH 4.0 ± 0.5; DO ≥ 30%, controlled by agitation (400–800 rpm); aeration, 5–10 L/min; foaming control: Struktol. Samples were taken throughout the fermentation and centrifuged (3000 rpm, 15 minutes). The whole broth was separated (5000 rpm, 20 minutes, <10°C). The supernatant was concentrated by ultrafiltration using PCI ES 625 membranes at a pressure of 4 to 5 bars.

2.6 ENZYME ACTIVITY ASSAYS

Endo-1,4-β-D-xylanase activity was determined using 1% w/v solution of birchwood glucurono-xylan (Roth 7500) as substrate [1]. β-Xylosidase activity was assayed using 5 mM p-nitrophenyl-β-D-xylopyranoside [8]. α-Arabinofuranosidase activity was assayed using 2 mM *p*-nitrophenyl-α-L-arabinofuranoside as substrate [7]. Feruloyl esterase activity was measured using 1.8% steam-extracted wheat straw xylan as substrate [12]. Overall cellulolytic activity was assayed by the filter paper assay (FPU) and cellulase (Endo-1,4-β-glucanase) activity against hydroxyethyl cellulose (HEC) [4].

2.7 PROTEIN ASSAY

Soluble protein was measured by the modified Lowry method [11] using bicinchoninic acid after precipitation with 10% w/v trichloracetic acid.

3 RESULTS AND DISCUSSION

The *Aspergillus* strains grew very well on WO-xylan. The *A. oryzae* gave a much higher xylanase activity than *A. fumigatus* (Table I). *T. reesei* grew poorly in the media containing wet-oxidised components. For *A. fumigatus* and *T. reesei* the highest xylanase activity was produced on Lz-xylan alone. Only low cellulase activity could be determined in any of the cultures, though significantly higher activity was found when grown on the mixed medium. Very low activity of feroyl esterase was found, probably because these side groups had been hydrolysed during WO.

Table 1. Production of biomass, protein, endo-xylanase (XYL), β-xylosidase (β-Xyl), α-arabinosidase (α-Ara) and cellulase (HEC) activity by fungi strains on MM with different xylan sources. Shake flask cultures: 5 days at 30°C and 200 rpm.

Strain	Medium	Finalp H	Biomass (g/L)	Protein (g/L)	XYL (nkat/mL)	β-Xyl (nkat/mL)	α-Ara (nkat/mL)	HEC (nkat/mL)
A. oryzae	Lz	2.7	5.3	0.05	227	12.6	6.0	4.4
	Lz-WO	4.6	7.1	0.33	1738	13.6	15.4	8.8
	Mixed	4.6	6.2	0.66	3417	41.7	19.0	24.0
	WO	5.2	7.5	0.95	1861	22.4	16.8	7.8
A. fumigatus	Lz	4.5	3.8	0.09	1018	0.3	0.3	<2
	Lz-WO	8.2	5.3	0.73	224	0.6	0.6	6.6
	Mixed	8.5	4.6	0.92	407	1.7	0.8	22.1
	WO	8.7	5.4	1.97	404	1.0	0.7	3.0
T. reesei	Lz	2.5	4.0	0.02	1018	9.0	5.7	4.7
	Lz-WO	4.8	2.6	0.27	95	0.2	0.5	3.7
	Mixed	5.0	2.6	0.77	14	0.2	0.5	51.9
	WO	5.1	1.4	1.78	14	0.3	0.5	1.8

A. oryzae was selected for larger scale fermentation. The production of xylanase between 55 and 71 hours was very efficient (278 $\mu kat/L \cdot h^{-1}$) and the final level of almost 8000 nkat/mL was excellent compared with previous cultivations of this strain (Figure 1) [2]. Relative lower β-xylosidase and α-arabinosidase activities were found in the larger scale fermentation than in the best shake-flask cultures.

The culture supernatant was concentrated about 4 times by ultrafiltration. A very high xylanase activity was obtained comparable with commercial enzyme preparations (Table 2). A small part (6%) of the total xylanase activity was lost by leaking into the permeate during ultrafiltration, but the yield was still 86%. The other (larger MW) enzymes were retained with a high efficiency. In conclusion, wet-oxidised wheat straw xylan was a suitable carbon-source for production of enzymes for hydrolysis purposes using *A. oryzae* as producing organism.

Table 2. Produced enzyme activities and soluble protein in large-scale cultivation of A. oryzae on WO-xylan supplemented with lenzing xylan and its fractions after ultrafiltration and yields.

Sample	Xylanase (nkat/mL)	β-Xylosidase (nkat/mL)	α-Arabinosidase (nkat/mL)	Protein (g/L)
Supernatant (95 hours)	7825 (100%)	38 (100%)	19 (100%)	1.1 (100%)
UF-concentrate	27515 (86%)	188 (120%)	76 (97%)	4.0 (97%)
UF-permeate	505	0.9	0.7	0.09

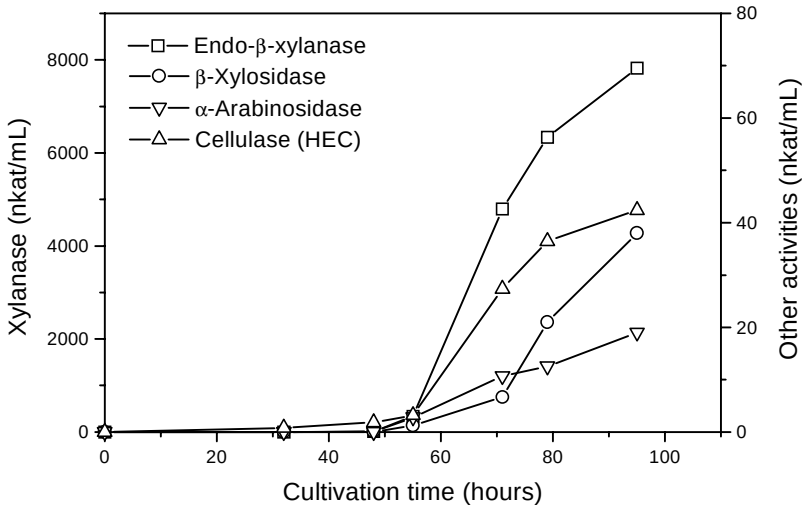

Figure 1. Production of enzymes by A. oryzae on wet-oxidised wheat straw xylan supplemented with 10 g/L lenzing xylan. Conditions: 30°C, pH 3.5–4.5, DO≥30%, 5–10 L air/min.

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PURIFICATION AND CHARACERIZATION OF *ASPERGILLUS NIGER* SULFHYDRYL OXIDASE

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1 INTRODUCTION

Nowadays, there is an increasing demand in the baking industry for alternatives to chemical additives such as potassium bromate due to the potential hazards (Dupuis, 1997). Therefore, in the last decade, an active research is devoted to the use of oxidoreducing enzymes. It is well known that the conversion of gluten thiols to disulfide bonds leads to the wheat flour dough strengthening during mixing. Many oxidoreductases act on the small thiols – e.g. glutathione-dehydroascorbate oxidoreductase (DHA-Red) or sulfhydryl oxidase.

Sulfhydryl oxidase (SOX) oxidizes reduced glutathione (GSH) to its oxidized form (GSSG) by molecular oxygen producing hydrogen peroxide. An addition of exogenous SOX to dough may have two potential benefits. Firstly, the removal of GSH in wheat flour may prevent its participation in SH / SS exchange reactions. These exchanges result in the depolymerization of the gluten proteins and thereby reduce dough elasticity and increase its extensibility (Dong and Hoseney, 1995). Secondly, the H_2O_2 formed is able to activate the wheat peroxidase. The latter catalyzes the oxidative gelation of pentosans by producing diferulate bridges between two ferulic acid residues of arabinoxylan chains (Neukom and Markwalder, 1978).

The aim of this work is to develop a purification procedure of *A. niger* SOX and then to study some of its kinetic properties in order to compare it with the wheat flour DHA-Red. The latter enzyme has been purified according to Kaïd *et al.* (1997).

2 RESULTS

The purification of SOX from *A. niger* has been developed. Optimal conditions have been determined on the solubilized activity from the powder extract followed by double ammonium sulfate precipitations (40%–70% cut). After dialysis, the resulting resolubilized pellet was eluted by ionic exchange chromatography on a DEAE-Sepharose column at pH 5. The major active fraction was 50-fold purified and was devoided of peroxidase activity. More than 99.8% of the contaminating catalase activity was removed by this procedure.

The apparent kinetic constants of SOX have been determined and compared to those obtained for DHA-Red (Table 1).

Table 1. Apparent kinetic constants of SOX and DHA-Red.

		Oxidant	GSH	CSH	γ-glu-cys
Km (mM)	SOX DHA-Red	0.7 (O_2) 0.14 (DHA)	0.5 1.8	2.5 -	10 5.5
Vm (% GSH)	SOX DHA-Red		100 100	1.7 -	30 13

SOX exhibits an optimum activity at pH 5.6 and its apparent Km towards GSH and O_2 were found equal to 0.5 mM and 0.7 mM respectively. The effect of other thiol concentration on the GSH oxidation by SOX has also been analyzed. Cysteine is very slowly oxidized by SOX, whereas a significant activity was found with the dipeptide γ-glu-cys (30% of the Vm obtained with GSH). By comparing SOX and DHA-Red kinetic constants, it can be noticed that both enzymes have GSH as the preferred substrate. However, CSH is not oxidized by DHA-Red. Both SOX and DHA-Red seem to act in the same way on the reactions involving SH / SS exchanges during dough mixing.

The enhancing effect of cysteine obtained on the SOX or DHA-Red activity measured by O_2 uptake or by ascorbic acid formation (results not shown) led to the assumption that three groups of disulfide are produced in GSH / CSH mixtures. A reaction mechanism can be proposed with the transitory formation of thiyl radicals XS° as following :

$$2\,GSH \xrightarrow[\text{SOX (or DHA-Red)}]{} 2\,GS^{\circ} \qquad (1)$$

$$GS^{\circ} + CSH \longrightarrow GSH + CS^{\circ} \qquad (2)$$

$$GS^{\circ} + GS^{\circ} \longrightarrow GSSG \qquad (3)$$

$$GS^{\circ} + CS^{\circ} \longrightarrow GSSC \qquad (4)$$

$$CS^{\circ} + CS^{\circ} \longrightarrow CSSC \qquad (5)$$

The analysis and quantification of the different disulfide groups has been performed by gel filtration and UV detection at 254 nm (Figure 1).

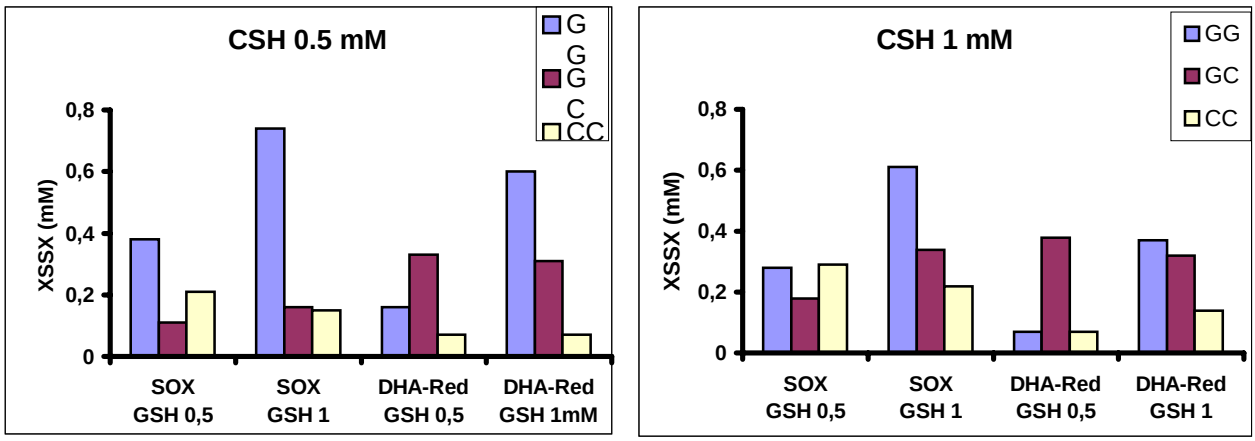

Figure 1. Disulfide formation by SOX or DHA-Red in GSH / CSH mixtures.

From these results, it appears that SOX as well as DHA-Red catalyzes the formation of oxidized glutathione GSSG, cystine CSSC and mixed disulfide GSSC from GSH / CSH mixtures. The relative disulfide proportions depend both on the enzyme and on the initial thiol concentration. Disulfide production increases more with the GSH amount than with CSH. The formation of GSSG and CSSC is greater with SOX than with DHA-Red, and conversely, the latter produces more GSSC. It can be assumed that GSH is the precursor of the thiyl radical (reaction 1) which can react with CSH to generate CS° and GSH (reaction 2). The better efficiency of SOX (compared to DHA-Red) towards CSH can explain the greater amount of CSSC formed by SOX. It is likely that, in the presence of SOX, CSSC is produced by direct CSH oxidation whereas, with DHA-Red, the 3 successive reactions 1, 2 and 5 are necessary .

3 CONCLUSIONS

The developed purification procedure allows to obtain an active SOX fraction devoided of contaminating peroxidase and mostly devoided of the catalase.

The comparison of kinetic constants of SOX and DHA-Red reveals that GSH is the preferred substrate for both enzymes. The broader specificity of SOX and the nature of the oxidizing substrate (O_2 for SOX and DHA for DHA-Red) may be of importance in the incorporation of small thiols in gluten proteins.

The additional production of H_2O_2 by SOX is also expected to activate wheat peroxidase system during dough mixing.

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COMPARISON OF ACTION PATTERN OF TWO XYLANASES FROM *TRICHODERMA REESEI* AND TWO XYLANASES FROM *ASPERGILLUS ORYZAE* ON DIFFERENT XYLANS

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1 INTRODUCTION

Xylans are important components of plant cell walls. The main component in xylans is xylose, which forms the backbone (1,2). Arabinose and 4-O-methylglucuronic acid exist as side groups. In addition several xylans contain acetyl substituents. Some xylans also contain phenolic acid, mainly ferulic acid side groups. The composition and structure of xylans depends on the plant species and may even vary between different parts of the same plant e.g. stem and grain, or even in various parts of the grain e.g. aleuronic layer and endosperm (Table 1).

Table 1. Possible side groups in xylans of different plants.

		MeGlcA	Ara		Ac	Ph
		α-1,2-linked	α-1,3	α-1,2		
Hardwoods		+	-	-	+	-
Softwoods		+	+	-	-	-
Cereals	Stem	+	+	?	+	+
	Grain	?	+	+	+	+

MeGlcA = 4-O-methylglucuronic acid, Ara = arabinofuranose, Ac = acetic acid, Ph = phenolic acid

Xylanases (endo-1,4-β-D-xylanases, EC 3.2.1.8) hydrolyse xylans by cutting the internal linkages in the backbone of the polymer. Most of the xylanases are classified, based on the three dimensional structures, to belong to glycosyl hydrolase families 10 and 11 (3). The structure of xylooligosacchrides formed depends on the starting material and the enzyme used. Family 10 and 11 xylanases have been shown to act differentially on 4-O-methylglucuronoxylan and β-1,3/1,4-xylan (4) but their action on arabinoxylans has not been elucidated. *Trichoderma reesei* and *Aspergillus oryzae* produce both at least two different xylanases (5,6). The action of these enzymes towards various xylans was studied in this work.

2 MATERIALS AND METHODS

Trichoderma reesei xylanases with pI 5.5 and pI 9.0 were purified as described by Tenkanen *et al*. (5) and *Aspergillus oryzae* xylanases with pI 4.9 and pI 6.9 as described

by Bailey *et al*. (6). The N-teminal amino acid sequences from *A. oryzae* xylanases were determined by Edman degradation. 4-O-methylglucuronoxylan from birch was from Roth (Germany) and arabinoxylans from wheat and rye were from Megazymes (Ireland). Birch and pine kraft pulps were obtained from a Finnish pulp mill.

Xylanase activity was assayed by two methods using birchwood xylan as substrate and measuring the amount of reducing ends formed by dinitrosalisylic acids, DNS (7) or using rye xylan as substrate and measuring the reduction on viscosity (8). Both assays were performed at pH 5 and 40°C. The enzymes were dosed to the hydrolysis experiments according to the activity on birch xylan.

The shortest oligosaccharides formed were isolated using anion exchange chromatography (Dowex 1x2 in Cl^- form, Fluka) and gel filtration (Biogel P-2, BioRad) (5). The ^1H and ^{13}C NMR spectra of isolated oligosaccahrides were obtained on a Varian Unity 600 MHz spectrometer. The structures were further analysed by incubation with β-xylosidase from *T. reesei* (9). High-performance anion-exchange chromatography (HPAEC-PAD) was done using Dionex DX 500 chromatograph, CarboPac PA-1 column and Dionex ED 40 Detector (10).

3 RESULTS

The two xylanases from *T. reesei* both belong to the glycosyl hydrolase family 11 (11). The complete amino acid sequence of *A. oryzae* xylanases is not know but the N-terminal amino acid sequence of the pI 4.9 and the pI 6.9 xylanase showed clear homology with family 11 and 10 xylanases, respectively. The four xylanases studied had clearly different specific activities (Table 2). The pI 6.9 xylanase was most "endotype" enzyme as seen from the activity ratio Reducing/Viscosity. Viscosity assay measures only the internal cuts of xylan whereas in reducing end assay monosaccharides and oligosaccharides give high response.

Table 2. Specific activites of T. reesei and A. oryzae xylanases.

Organism	Xylanase	Glycanase family	Specific activity		
			Birch xylan Red. end assay (nkat/mg)	Rye xylan Viscosity assay (vu/mg)	Reducing/ Viscosity
T. reesei	pI 5.5	11	2 600	940	2.8
	pI 9.0	11	6 500	8 050	0.8
A. oryzae	pI 4.9	11	16 200	17 400	0.9
	pI 6.9	10	5 100	12 700	0.4

The structure of oligosaccharides formed by each enzyme was analysed after extensive enzymatic hydrolysis (2000 nkat/g, 48 h, pH 5, 40°C) of less substituted xylans e.g. xylans in hardwood and softwood kraft pulps. The substituted oligosaccharides were isolated and their structures were determined by NMR-spectroscopy and HPAEC-PAD combined with β-xylosidase treatment.

Table 3. Structures of the shortest hetero-oligosaccharides formed from hardwood ($MeGlcAX_n$) and softwood ($AraX_n$) kraft pulps by xylanase treatments.

	T. reesei **pI 5.5**	***T. reesei*** **pI 9.0**	***A. oryzae*** **pI 4.9**	***A. oryzae*** **pI 6.9**
MeGlcA **Xyl – Xyl – Xyl**	-	-	-	+
MeGlcA **Xyl – Xyl – Xyl – Xyl**	+	+	+	-
MeGlcA **Xyl – Xyl – Xyl – Xyl – Xyl**	-	+	+	-
MeGlcA **Xyl – Xyl – Xyl – Xyl – Xyl**	+	**(+)**	**(+)**	-
Ara **Xyl – Xyl – Xyl**	+	+	+	+
Ara **Xyl – Xyl – Xyl – Xyl**	+	**(+)**	+	-
Ara **Xyl – Xyl – Xyl – Xyl**	+	+	**(+)**	-
Ara **Xyl – Xyl – Xyl – Xyl – Xyl**	+	+	+	-

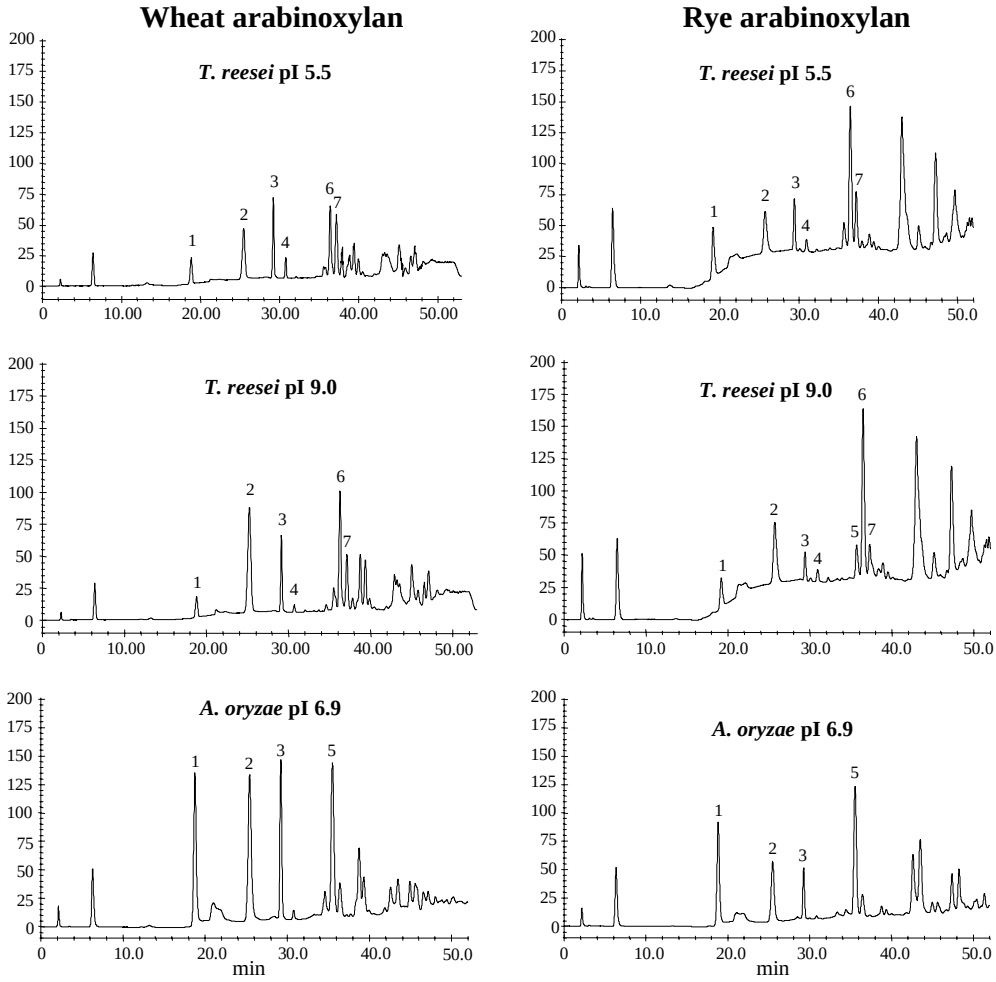
— β-1,4-linkage, | α-1,2-linkage, ⟍ α-1,3-linkage

The difference of *A.oryzae* pI 6.9 xylanase to the other xylanases is further seen in the degradation products (Table 3). It was able to cut the xylosidic linkage closer to the MeGlcA substituted xylose unit than the other three xylanases. It can be also clearly seen that the position of the side group (1,2-linked MeGlcA versus 1,3-linked Ara) affects the action of xylanases.

The action of xylanases on highly substituted wheat (Ara:Xyl = 41:59) and rye (Ara:Xyl = 37:63) arabinoxylans was elucidated by incubating the xylans with lower enzyme dosage (500 nkat/g, 24 h, pH 5, 40°C) after which the hydrolysis products were analysed by HPAEC-PAD (Figure 1). These highly substituted xylans were degraded efficiently by *A.oryzae* pI 6.9 xylanase More xylose and linear xylo-oligomers were formed in the hydrolysis of wheat xylan than that of rye xylan by pI 6.9 xylanase indicating different distribution of arabinose substituents in the xylan.

4 CONCLUSIONS

Xylanases belonging to glycosyl hydrolase family 10 and 11 had clearly different mode of action. Family 10 xylanase (*A. oryzae* pI 6.9) was able to cut the linkage much closer to the side group that family 11 xylanases and it produces substituted xylotrioses. The position of the side group (1,2-linked MeGlcA versus 1,3-linked Ara) affects the action of the xylanases. The differences in the mode of action should be further evaluated in various applications in which xylanases are used for hydrolysis of xylans, for example baking, brewing, feed supplement and bleaching of kraft pulps.



1. Xyl, 2. Xyl_2, 3. Xyl_3, 4. Xyl_4, 5. $AraXyl_3$, 6. $AraXyl_4$, 7. $AraXyl_5$

Figure 1. HPAEC-PAD chromatograms of wheat (left) and rye (right) xylan after hydrolysis by three different xylanases.

5 ACKNOWLEDGEMENTS

Anita Teleman is thanked for the NMR analysis, Helena Simolin for the HPAEC-PAD analysis, Taina Simoinen for the viscosity assays and Riitta Isoniemi for the skilful technical assistance.

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EFFECTS OF DIFFERENT XYLANASES ON THE ARABINOXYLAN SOLUBILISATION FROM RYE FLOUR

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1 INTRODUCTION

The presence of water-extractable arabinoxylans [WEAX] in rye dough increases dough viscosity, bread volume, gas retention, shelf life and improves crumb texture, color and taste. In contrast, water-unextractable arabinoxylans [WUAX] increase crumb firming rate and decrease loaf volume. The solubilisation of AX from WUAX can improve quality in rye bread making, and can be achieved by the use of endo-xylanases. Too much xylanase can result in a dough that is too soft and sticky, due to degradation of WEAX.

Endo-(1,4)-β-D-xylanases (EC 3.2.1.8, (1,4)-β-D-xylan xylanohydrolase) hydrolyse the internal linkages between 4-linked β-D-xylopyranosyl residues in AX, provoking both a decrease in molecular weight and a solubilisation of AX.

The aim of this work was to examine the enzymatic release of high molecular weight AX from rye flour, using three different endo-xylanases: xylanase 1 from *Aspergillus niger*, xylanase 2 from *Talaromyces emersonii*, and xylanase 3 from *Bacillus subtilis*.

2 MATERIALS AND METHODS

A German rye flour type 997 with 93% extraction and 1.06% of ash content, was used for experiments. The enzymatic solubilisation of AX from rye flour was followed by capillary viscometry using an AVS 400 (Schott Geräte, Hofheim/Ts, Germany) viscometer, equipped with an Ostwald capillary tube (water flow time 29.41 s). WEAX was determined according to the semi-automated colorimetric method of Rouau and Surget (1994), using an Evolution II auto-analyser (Alliance Instruments, France). SE-HPLC was performed as described in Figueroa-Espinoza and Rouau (1998).

3 RESULTS AND DISCUSSION

3.1 EFFECT OF XYLANASES 1, 2 AND 3 ON RYE FLOUR AX

The profiles of AX solubilisation from rye flour, were similar for the three xylanases. AX solubilisation increased rapidly until 4 h of reaction, then it raised slowly until it

reached a pseudo-plateau. Around 27% of the WUAX were solubilised after 24 h of reaction with the three xylanases. For a similar extent of AX solubilisation, xylanase 3 presented the highest value of viscosity among these extracts (Figure 1).

Xylanase 2 provoked an extensive depolymerisation of the soluble AX and an extraction of low molecular weight AX, as expressed by the low specific viscosity of extracts [η_{sp}]. Xylanase 3 and 1 solubilised AX of medium and high molecular weight after 1 h of reaction. Then solubilisation was accompanied of a degradation. Results were confirmed by SE-HPLC (not shown).

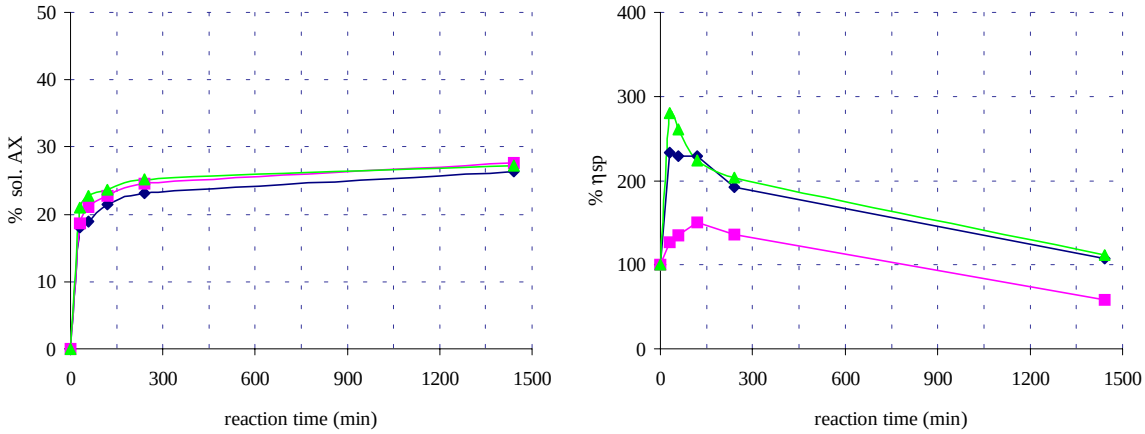

Figure 1. Effect of xylanases (0.5 U) 1 (◆), 2 (■) and 3 (▲) on the solubilisation of AX on rye flour. Solubilisation of AX [% sol. AX] and specific viscosity [% η_{sp}] were determined in reaction mixes.

3.2 Effect of different doses of xylanase 3 on rye flour AX

Five different doses of xylanase 3 were tested. The rate of solubilisation increased with the dose. The maximum values in viscosity were observed with 2.5 units [U] of xylanase 3. Above this dose, important solubilisation of AX was accompanied by a depolymerisation (Figure 2).

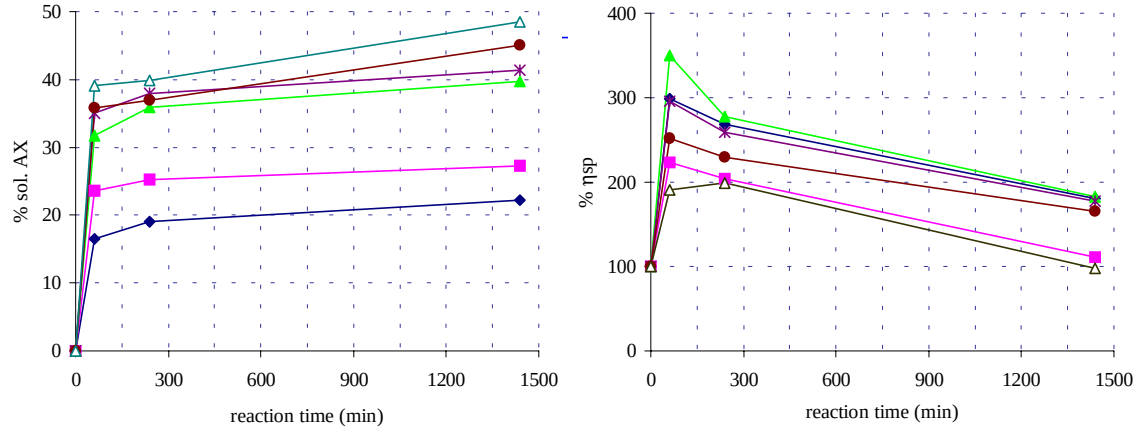

Figure 2. Effect of different doses of xylanase 3 on the solubilisation of AX on rye flour: 0.5 U (■), 2.5 U (▲), 5 U (✳), 10 U (●), 20 U (Δ). Blank (◆).

3.3 EFFECT OF XYLANASE 3 COMBINED WITH XYLANASE 1 ON RYE FLOUR AX

The solubilisation and viscosity profiles were similar with the three different combinations of xylanase 3 and 1 (Figure 3).

The combination of 2.5 U of xylanases 3 and 1 provoked a similar solubilisation as the reference (xylanase 3, dose 2.5 U), but less increase in viscosity and more degradation of AX. From the SE-HPLC profiles (not shown) it was observed that in the presence of xylanase 1 more low molecular weight AX were liberated and more medium size AX were depolymerised.

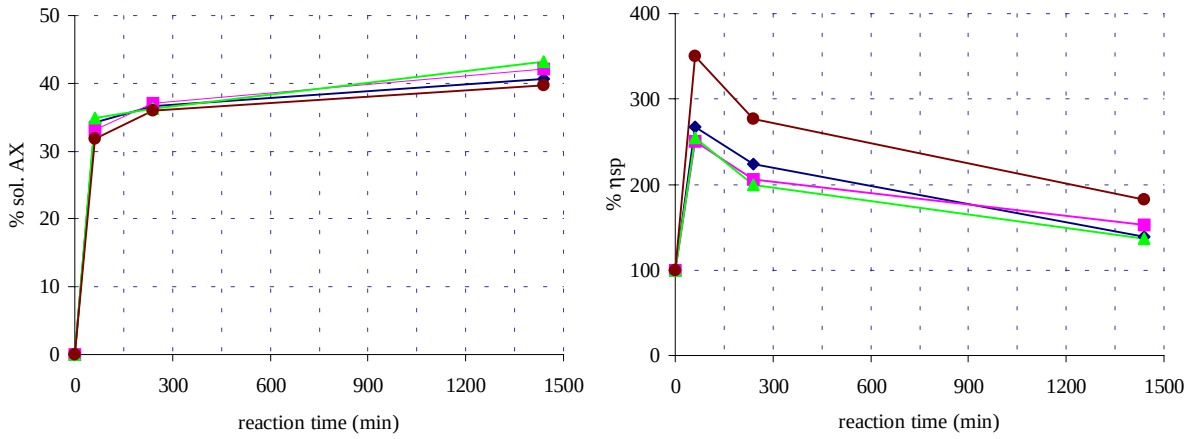

Figure 3. Effect of the combination of xylanases 3 and 1 at different doses on the solubilisation of AX on rye flour. Xyl3 2.5 U (●), *Xyl3 1.25 U + Xyl1 3.75 U* (◆), *Xyl3 2.5 U + Xyl1 2.5 U* (■), *Xyl3 3.75 U + Xyl1 1.25 U* (▲).

4 CONCLUSIONS

Xylanase 3 was the best of the three enzymes used in this study for the extraction of medium and high molecular weight AX.

High doses of xylanase 3 allowed to extract up to 50% of AX after 24 h of reaction, but with a very important depolymerisation.

No synergism was observed between xylanases 3 and 1, either for AX solubilisation or extract viscosity.

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ENDOXYLANASES IN DURUM WHEAT SEMOLINA PROCESSING: SOLUBILIZATION, ACTION OF ENDOGENOUS INHIBITORS AND EFFECTS ON RHEOLOGICAL PROPERTIES

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1 INTRODUCTION

The purpose of this study was to elucidate the effect of different dosages of a number of endoxylanases on spaghetti dough prepared in the farinograph. Endoxylanases of various origin [*Trichoderma viride* (XTV), rumen micro-organisms (XRM), *Bacillus subtilis* (XBS) and *Aspergillus niger* (XAN)] were tested. The changes in waterextractable arabinoxylan (WE-AX) to waterunextractable arabinoxylan (WU-AX) ratio were monitored, as were the gel permeation profiles of the purified AX. At the same time, we wanted to study to what extent the differences in endoxylanase action could be related to the presence of endoxylanase inhibitors (Debyser and Delcour, 1997) in durum wheat.

2 MATERIALS AND METHODS

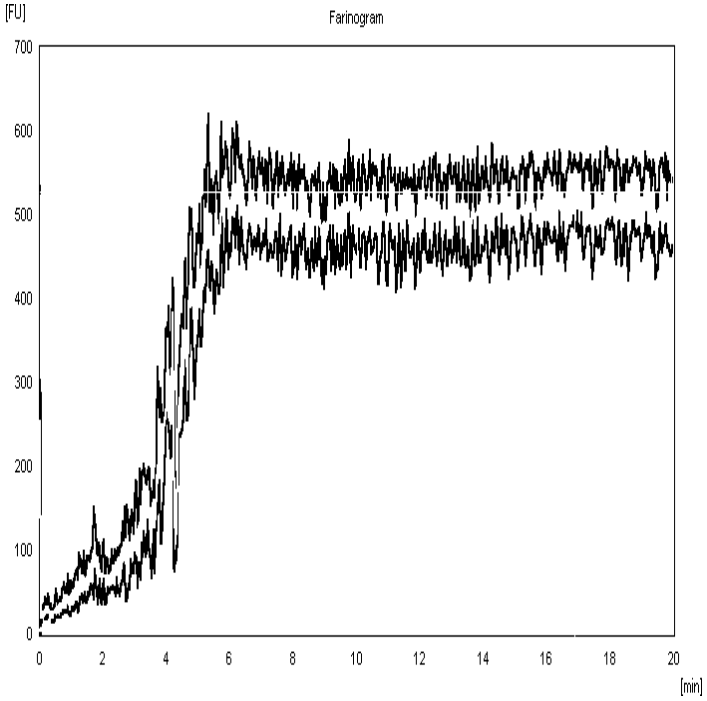
Farinograms were recorded according to the method of Irvine *et al*. (1961). Semolina (50 g, 14.0% moisture basis) was mixed with deionized water (34.9% total water content) in a 50 g stainless steel farinograph bowl connected to a DO-Corder E DCE 330 (Brabender, Duisburg, Germany) operating system. All enzyme units (EU) in this work are expressed as Somogyi Units/ 50 g semolina. The range of activities of the endoxylanases added was between 0 and 75.4 EU. For different reductions in dough water contents, we estimated which enzyme level was required to reach the same farinograph consistency as for the control doughs. Inhibition of the endoxylanases by durum wheat endogenous endoxylanase inhibitors was quantified with the procedure of Debyser *et al*. (1999).

Carbohydrate composition of the doughs and the water extracts thereof was determined by GC. Molecular weights of the WE-AX were determined by HPLC, following purification as described by Loosveld *et al.* (1997).

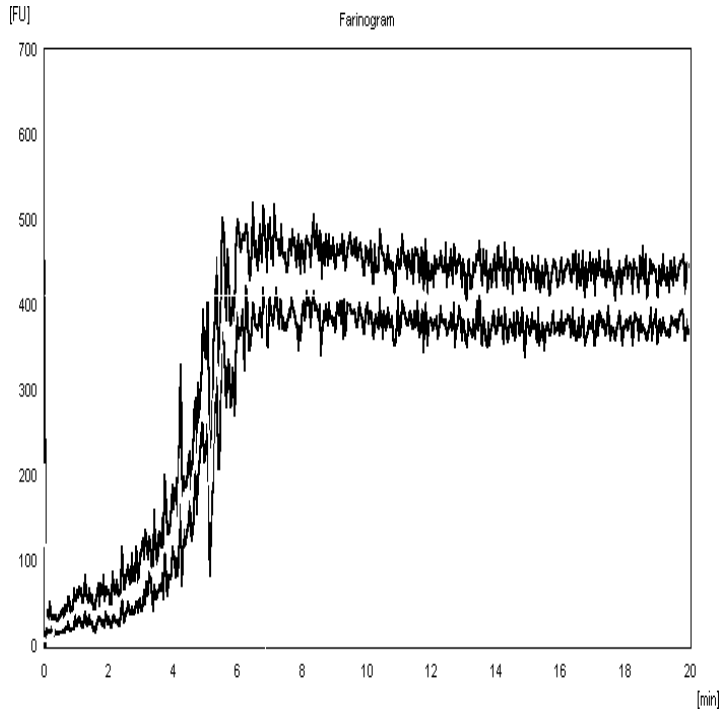
3 RESULTS AND DISCUSSION

Endoxylanases decreased maximal consistency (FU) of semolina doughs significantly [Figure 1 (a), (b) and (c)]. Despite low moisture content of semolina doughs, endoxylanases only needed limited times to be effective since maximal consistency was already reached a few minutes after the addition of enzymes [Figure 1 (a) and (b)].

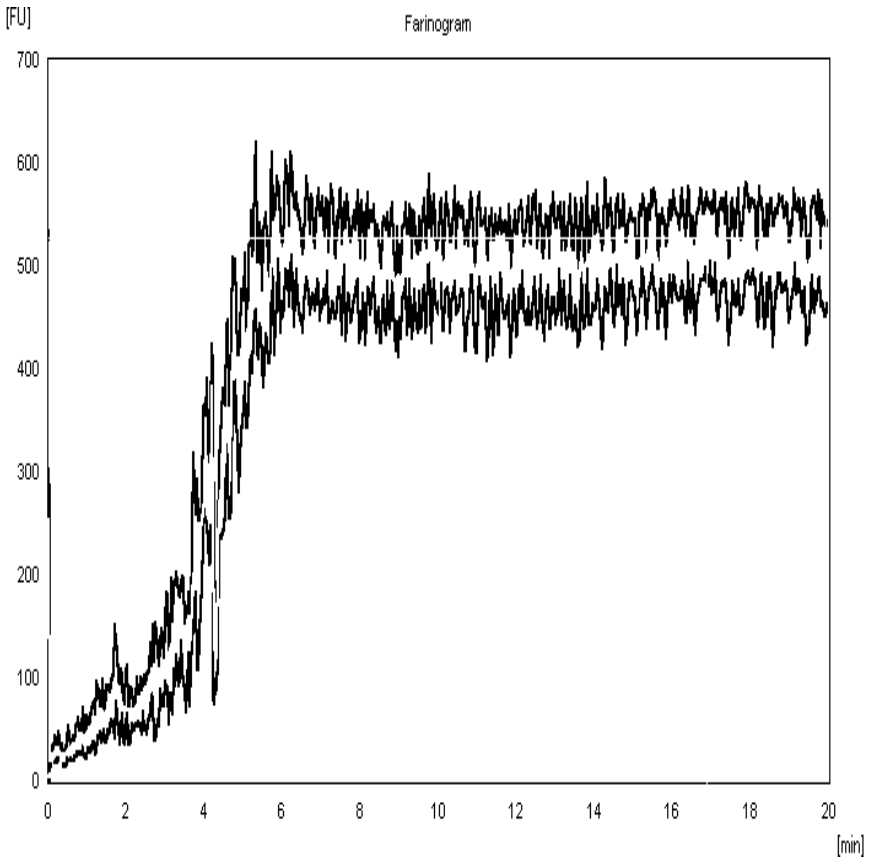
(a)



(b)



(c)



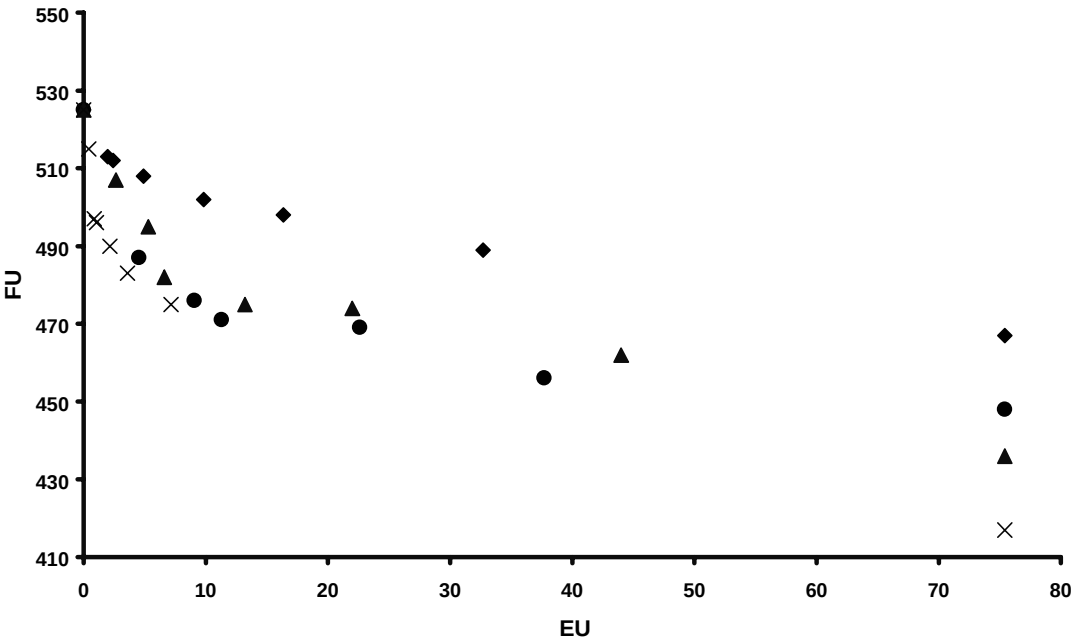


Figure 1. A and b: Farinograms of semolina doughs prepared without added endoxylanases) and with 75.4 EU of XRM endoxylanase). c: Maximal farinograph consistencies (FU) at 34.9% total moisture content of semolina doughs as a function of dosages (EU) of different endoxylanases (XBS ●, XAN ✕, XTV ◆ and XRM ▲).

The decrease of maximal consistency was, for the four enzymes used, dose-dependent [Figure 1(c)].

By reducing the level of added water with 2.0%, 3.0% and 4.5% in the doughs and treating them with a measured dosage of endoxylanase, the maximal consistency of the control dough was restored (Table 1).

Table 1. Endoxylanase dosage (EU) necessary to obtain the same consistency as the control dough (525 FU) by omitting 2%, 3%, and 4.5% of the water added to the semolina doughs.

WATER REDUCTION	XTV	XRM	XBS	XAN
2.0%	**4.0 EU**	**0.5 EU**	**2.0 EU**	**1.0 EU**
3.0%	**7.5 EU**	**2.0 EU**	**3.5 EU**	**2.0 EU**
4.5%	**27.5 EU**	**12.5 EU**	**10.0 EU**	**7.5 EU**

Using endoxylanase during semolina dough preparation results in a solubilisation of AX (Table II) and simultaneously a decrease in the MW of the WE-AX fraction (Figure 2). With higher dosages of endoxylanases, the MW profile of the AX overlaps with that of the AGP peak (Figure 2).

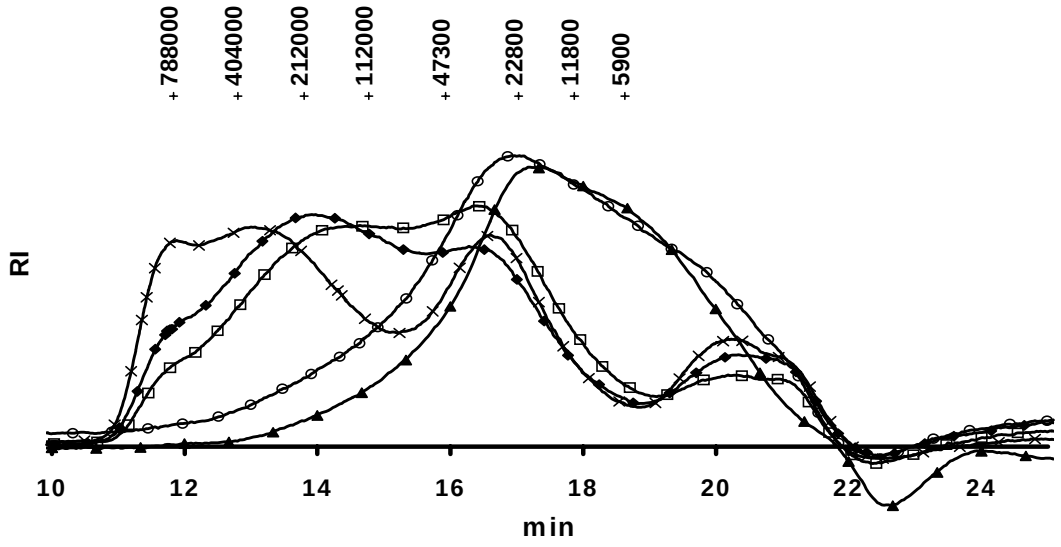


Figure 2. Gel permeation profiles of AX-AGP material, purified from semolina doughs treated with XRM (control ✕, 2.6 EU ◆, 6.6 EU □, 44.0 EU ○, 75.4 EU ▲.)

Both the decreases in maximal consistency [Figure 1 (c)], as well as the levels of water to be omitted (Table 1) were significantly related to the decreases in WE-AX MW (Figure 2) and the percentage of WE-AX solubilised (Table 2) as a result of enzymic action.

Table 2. Non starch polysaccharide composition (%, dry basis) of semolina doughs treated with endoxylanases and subsequently inactivated. AX = [%Xyl + (%Ara - (A/G)%Gal)]*0.88 with A/G = substitution degree of the AGP: 0.68. AG = [%Gal*0.9 + %Gal*(A/G)*0.88]. A/X = [%Ara - (%Gal*(A/G))]/ %Xyl. A/Xs = (% Ara - %Ara control)/ (%Xyl-%Xyl control).*

	AX	**AG**	**A/X**	**A/Xs**
total hydrolysate	2.22	0.37	0.83	
water extract				
control	0.43	0.37	0.52	
Control+XTV				
1.9 EU	0.53	0.34	0.53	0.50
4.9 EU	0.70	0.37	0.53	0.52
32.7 EU	1.16	0.35	0.57	0.58
75.4 EU	1.35	0.37	0.57	0.60
Control+XRM				
2.6 EU	1.12	0.36	0.56	0.57
6.6 EU	1.27	0.38	0.58	0.62
44.0 EU	1.54	0.37	0.58	0.60
75.4 EU	1.58	0.37	0.57	0.59
Control+XBS				
4.6 EU	1.14	0.38	0.54	0.56
11.3 EU	1.44	0.38	0.56	0.58
75.4 EU	1.57	0.36	0.55	0.56
Control+XAN				
0.5 EU	0.75	0.35	0.57	0.58
1.1 EU	1.06	0.37	0.58	0.62
7.2 EU	1.39	0.34	0.60	0.62
75.4 EU	1.62	0.37	0.57	0.59
C.V.	6%	9%	7%	6%

Under the experimental conditions, XRM endoxylanase retained 95% of its activity, while XTV, XBS and XAN retained 3%, 30% and 56% of their activity when in contact with semolina extracts. The above explains part of the observations with the different endoxylanases concerning the decrease in maximal consistency [Figure 1 (c)], degree of solubilization (Table 2), reduction of the apparent MW of the WE-AX (Figure 2) and level of endoxylanase needed to restore the original consistency of the untreated sample when a certain level of the water is omitted (Table 1).

4 CONCLUSIONS

The following conclusions were drawn: (1) Endoxylanases drastically affected the rheological properties of durum semolina pasta doughs prepared in the farinograph. (2) By omitting a certain amount of water (2.0%, 3.0% and 4.5%) and adding a certain level of endoxylanase, the decrease of the maximal consistency was restored. 3) Maximal consistency depended on the level and/ or the MW of the WE-AX. The activity of the endoxylanases was influenced to different extents by durum wheat endogenous endoxylanase inhibitors.

5 ACKNOWLEDGEMENTS

Etn. Joseph Soubry N.V are thanked for supplying pasta samples and the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie (Brussels) for the receipt of a scholarship.

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THE EFFECTS OF WHEAT FLOUR ARABINOGALACTAN-PEPTIDE ON MIXING CHARACTERISTICS OF WHEAT FLOUR DOUGH AND THE RESULTING BREADS

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1 INTRODUCTION

The non-starch polysaccharides of wheat flour consist mainly of arabinoxylans (AXs) and water-extractable arabinogalactan-peptides (WE-AGPs). The research on AXs, although minor constituents of wheat flour, indicates that they play a prominent role in bread-making (Cawley, 1964; Michniewics *et al*., 1992; Biliaderis *et al*., 1995; Courtin and Delcour, 1998; and Courtin *et al*., 1999). To date, no universally accepted functional role in nature has been ascribed to wheat flour water-extractable arabinogalactan-peptide (WE-AGP).

Research on wheat flour WE-AGP has indeed been limited to some structural studies (Fincher and Stone, 1974; Fincher *et al*., 1974; Loosveld *et al*., 1998). The objective of this study was hence to increase insight into the functional role of wheat flour WE-AGP in bread-making. Therefore, the effects of substitution of wheat flour with WE-AGP purified from wheat flour on dough characteristics and the volumes of the resulting breads were investigated.

2 MATERIALS AND METHODS

Flour of the wheat varieties Torfrida and Soissons (harvest 1996) was used in all experiments.

WE-AGP was isolated and purified from wheat flour based on the method described by Loosveld *et al*. (1997).

Farinograms (Farinograph E, Brabender, Duisburg, Germany) of flours substituted with WE-AGP purified from wheat flour (1.0% and 2.0% substitution levels) were recorded at the farinograph absorption (FA) determined for the control flours [dough consistency at the 500 Brabender Unit (BU) line] and at the FA based on a constant dough conistency at the 500 BU line.

Mixograms for the control flours and flours substituted with WE-AGP purified from wheat flour (1.0% and 2.0% substitution levels) were recorded at the FA (500 BU line) with a 10 g mixograph (National Mfg., Lincoln, NE, USA).

Micro-extension tests on dough (500 BU FA, mixing time deduced from mixograph measurements) were performed with a Texture Analyzer TA-XT2 (Stable Micro Systems, United Kingdom) equipped with a Kieffer dough and gluten extensibility rig.

Wheat loaves (10.0 g of flour) were produced using the straight dough bread-making procedure described by Shogren and Finney (1984). Ingredients other than flour, yeast (Bruggeman, Brugge, Belgium) and water were 6.0% sugar (sucrose) (w:w) and 1.5% (w:w) salt.

3 RESULTS AND DISCUSSION

3.1 INFLUENCE OF WHEAT FLOUR WE-AGP ON FA AND DOUGH CONSISTENCY

The baking absorption for wheat flour enriched with WE-AGP purified from wheat flour (1.0 and 2.0% substitution levels) was lower than for the control (no additives) (Table 1). As a logical consequence, a considerable decrease in consistency was observed in doughs substituted with WE-AGP purified from wheat flour when baking absorptions of the control (no additives) were used (Table 1). Wheat flour WE-AGP in doughs possibly acts as plasticizer much as do fat and sugar.

Table 1. Effect of substitution with WE-AGP purified from wheat flour on the FA (constant dough consistency of 500 BU) and on the dough consistency (FA of the control) of Torfrida and Soisssons wheat flour doughs.

Substitution level (%)	**TORFRIDA**		**SOISSONS**	
	FA[a]	BU	FA[a]	BU
0.0	61.5	502	56.1	486
1.0	59.0	425	53.6	408
2.0	56.6	372	51.5	349

[a] FA expressed on 14% moisture content.

3.2 INFLUENCE OF WHEAT FLOUR WE-AGP ON MIXOGRAPH PARAMETERS

Mixograms, recorded on the basis of the 500 BU FA, are shown in Figure 1. Longer mixing times for an optimum dough development were required in the case of substitutution (2.0%) with WE-AGP purified from wheat flour for flour of the wheat variety Torfrida. In contrast, substitution with WE-AGP purified from wheat flour in flour of the wheat variety Soissons did not affect the times to peak. In the case of 2.0% substitution, the profiles had a somewhat increased band width, indicating stronger doughs.

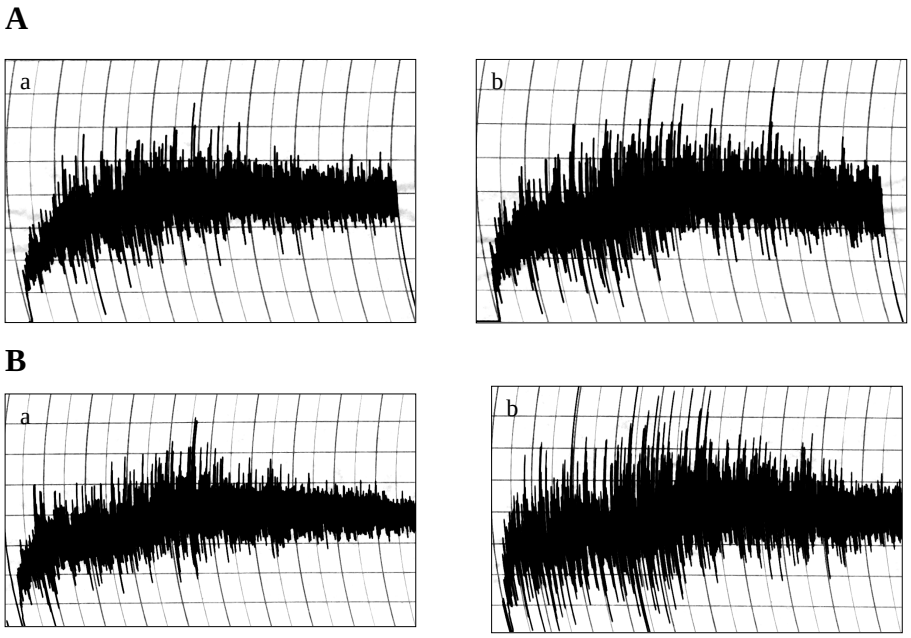

*Figure 1. Mixograms of flour of the wheat variety (**A**) Torfrida (a) control, (b) control with 2.0% WE-AGP purified from wheat flour and (**B**) Soissons (a) control, (b) control with 2.0% WE-AGP purified from wheat flour.*

3.3 INFLUENCE OF WHEAT FLOUR WE-AGP ON EXTENSIGRAPH PARAMETERS

Substitution of flour of the wheat varieties Torfrida and Soissons with 1.0% and 2.0% WE-AGP purified from wheat flour caused a significant increase in maximum resistance to entension (Rmax) and a significant decrease in extensibility (Ex). An increase in Rmax and a decrease in Ex points to an increase in elasticity of a dough at the expense of viscous properties (Hoseney, 1994). No significant differences in areas under the curve were observed (Figure 2).

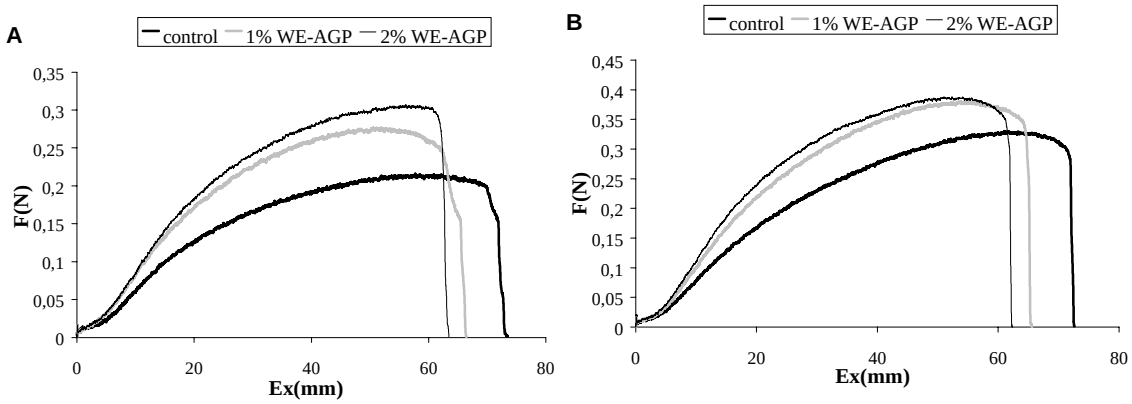

*Figure 2. Effect of substitution with WE-AGP purified from wheat flour on extensigrams of (**A**) Torfrida and (**B**) Soissons doughs.*

3.4 INFLUENCE OF WHEAT FLOUR WE-AGP ON BREAD VOLUME

Substitution with WE-AGP purified from wheat flour caused a slight decrease in bread volume (**Torfrida** : 1.0% substitution : 3.4%; 2.0% substitution : 5.8%; **Soissons** : 1.0% substitution : 4.7%; 2.0% substitution: 7.9%).

4 CONCLUSIONS

Substitution of wheat flour with WE-AGP purified from wheat flour decreased baking absorption. Extended mixing times were required to obtain optimal doughs in the case of WE-AGP substitution. WE-AGP caused a significant increase in maximum resistance to extension and a significant decrease in extensibility of doughs (same consistency, optimal mixing time) pointing to a more elastic dough. In bread-making, WE-AGP caused a slight decrease in bread volume.

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MODIFICATION OF WHEAT GLUTEN WITH OXIDATIVE ENZYMES

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ABSTRACT

Conventional enzymatic treatments in food processing are performed by using hydrolytic enzymes. The utilization of other types of enzymes, such as transglutaminase or oxidative enzymes, is continuously increasing. Transglutaminase (EC 2.3.2.13) catalyzes the reaction between glutamine and e.g lysine residues and creates new peptide linkages into and between the polypeptide chains of proteins. Oxidoreductases, like monophenol monooxygenase (EC 1.14.18.1), catechol oxidase (EC 1.10.3.1) and laccase (EC 1.10.3.2) may also modify proteins. Monophenol monooxygenase, tyrosinase, is known to act on tyrosine. It could be assumed that phenoloxidases may attack also other aromatic amino acids.

VTT Biotechnology and Food Research has worked with laccases for several years and developed applications for different purposes. Applications in pulp and paper, textile, and chemical industry have been successful. Laccase is an ideal enzyme because it does not need any co-factors and it is active towards a variety of structurally different substrates. Laccase starts a radical reaction resulting polymerization. Concerning proteins this may include cross-linking. It has been reported recently that treatment with laccase improves the baking quality of flour (1). The aim here was to study the effects of laccases on the proteins of wheat gluten fraction.

1 MATERIALS AND METHODS

Enzymes: Laccase from the white rot fungus *Trametes hirsuta* was produced and purified at VTT (2). Laccase of *Myceliophthora thermophila* was a commercial preparation by Novo (NS 51003). Laccase activity was estimated using ABTS as substrate (3).

Substrates: L-phenylalanine, L-trypthophan, L-cysteine (Merck) and L-proline (Fluka) were used as model substrates as well as ferulic acid (Sigma) and ferulic acid esterified with wheat straw xylan (VTT/BEL). Gluten preparation was from Sigma.

Treatments: Reactions were performed at room temperature at the optimal pH-values of the enzymes, at pH 5 (0.05M citrate) with *Trametes* laccase and at pH 7 (0.05M phosphate) with *Myceliophthora.*

Oxygen consumption was measured in closed erlenmeyer flasks with 30ml reaction mixture. Substrate concentrations were: amino acids 0.2mM, ferulic acid 0.25mM,

feruloylated xylan 1 mg/ml and gluten 10 mg/ml. Different enzyme doses (140ηkat-400μkat/g substrate) were tested. Reactions were monitored by oxygen electrode (Sensor Link TM Model PCM 800, Orion Research).

Thiol and disulfide groups: Gluten was treated according to the baking conditions. Laccase dose was 140 ηkat/g gluten. Reaction mixture, 3g gluten/ 30ml buffer, was mixed with magnetic stirrer (50 rpm) for 7 min and left without stirring for 1 h. Buffer solutions were discharged and samples of residual insoluble gluten were taken for estimation of dry weight and SH/ SS-groups. Approximately 30mg gluten was taken to analyse SH- or SS-groups by using Ellmans reagent (4).

Molecular mass distribution: Gluten, 2g/ 20 ml buffer, was treated with high laccase dose (12 μkat/ g gluten), mixed vigorously (500rpm) and aerated by pressurized air for 1 h. Buffer solutions were discharged and samples of residual insoluble gluten were freeze-dried. Dry material was dissolved (mg/ml) and fractionated in dimethylsulfoxide containing 0.5% LiCl. Chromatography was performed at 70°C using Mixed B HPLC-column (Polymerlabs, Amersham), flow rate was 0.5ml/min and detection by PDI 2000-dual beam laser (Precision Detectors, Amersham).

Storage modulus G': Gluten was treated according to the baking conditions (see estimation of SH/ SS-groups). The storage modulus of residual insoluble gluten was estimated by using StressTech Constant Stress Rheometer (ReoLogica Instruments AB) (5).

Baking was performed with laccase doses 14 ηkat/ g mixed flour (85% wheat and 15% graham, both from Melia Oy). Enzymes were mixed with water and added to the other dough components. Baking was according to the conventional practice at VTT BEL. Loaf volume of bread was measured by rapeseed displacement. Crumb firmness during storage (20°C; 0; 24 and 72 h) was determined using Texture Analyzer (TA-XT2 Staple Micro Systems) according to AACC 74-09.

2 RESULTS

2.1 OXIDATION OF GLUTEN AND OTHER FLOUR COMPONENTS

Both laccases oxidized gluten with similar efficiency (Fig. 1a). However, *Myceliophthora* laccase was more effective than *Trametes* laccase towards the main amino acids of gluten: phenylalanine, proline, trypthophan, and especially cysteine (Fig. 1b). The more conventional substrates for phenoloxidases, free ferulic acid and ferulic acid esterified with xylan, both potentially present in flour, were oxidized by ten times higher rate (results not shown).

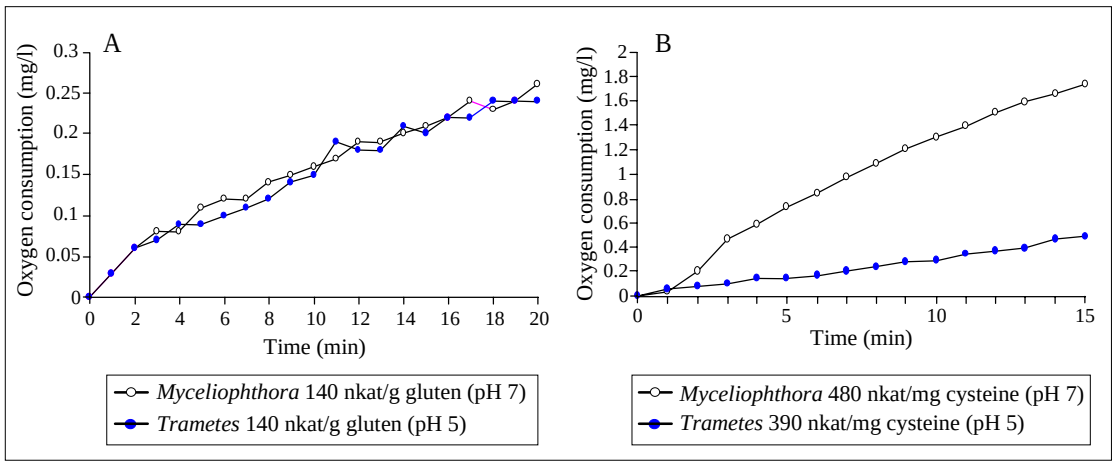

Figure 1. A) Oxygen consumption by laccases in gluten solution, B) Oxygen consumption by laccases in 0.2 M cysteine solution.

2.2 Effect on free thiol and disulfide groups

The amount of free thiol groups decreased and that of disulfide bridges increased slightly by laccase treatments (Fig. 2).

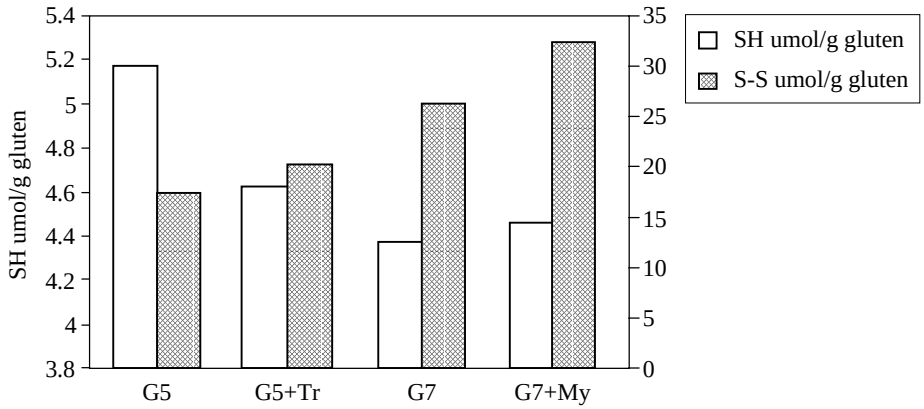

Figure 2. Effect of laccases on the free thiol and disulphide bridges of gluten. G5: gluten at pH 5, G7: gluten at pH 7, Tr: Trametes laccase, My: Myceliophthora laccase.

2.3 Molecular mass distribution

Laccase treatment decreased the molecular mass of gluten (Fig. 3). Modification was most remarkable by *Trametes* laccase.

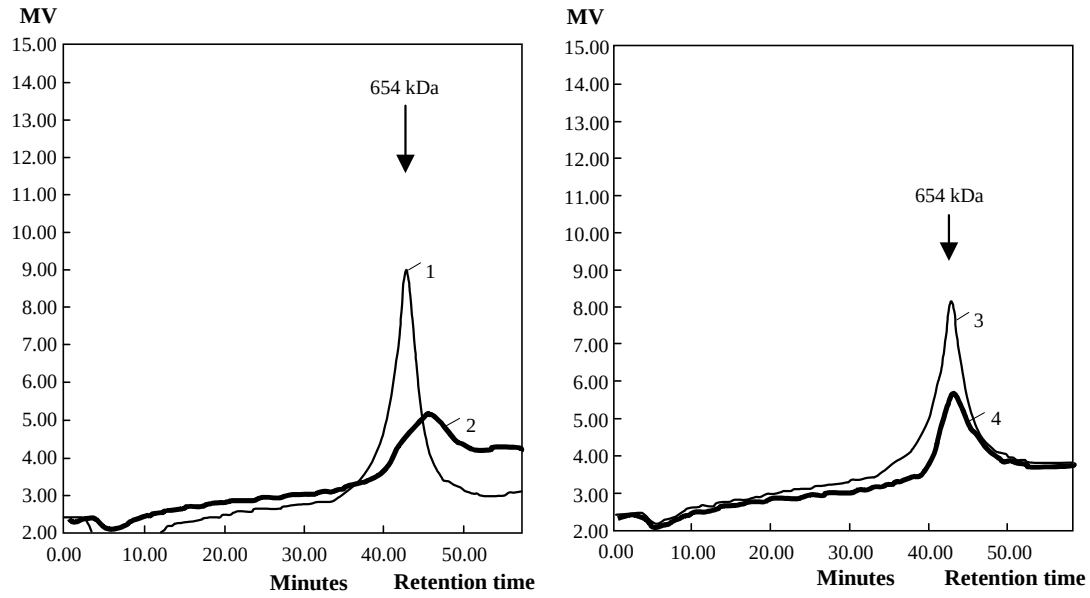

Figure 3. Molecular mass distrubution of gluten.

Samples: 1. Gluten at pH 5, 2. Gluten treated with *Trametes* laccase at pH 5, 3. Gluten at pH 7, 4. Gluten treated with *Myceliophthora* laccase.

2.4 STORAGE MODULUS

Storage modulus G´ was decreased by *Trametes* laccase but slightly increased by *Myceliophthora* laccase (Fig. 4).

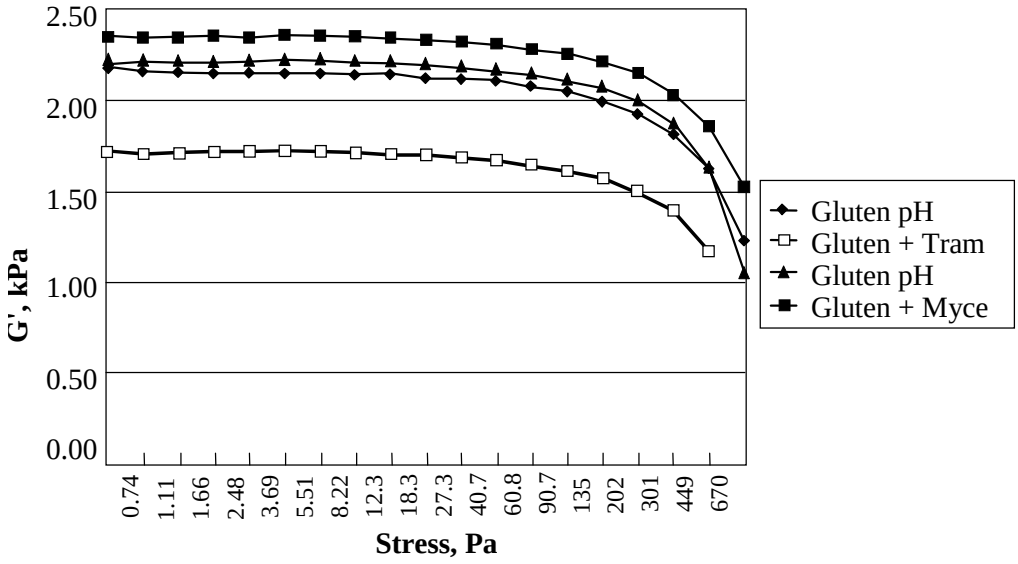

Figure 4. Storage modulus G' of gluten.

2.5 BAKING

Myceliophthora laccase increased the loaf volume and decreased the crumb firmness of bread. *Trametes* laccase did not effect on these parameters (Fig. 5).

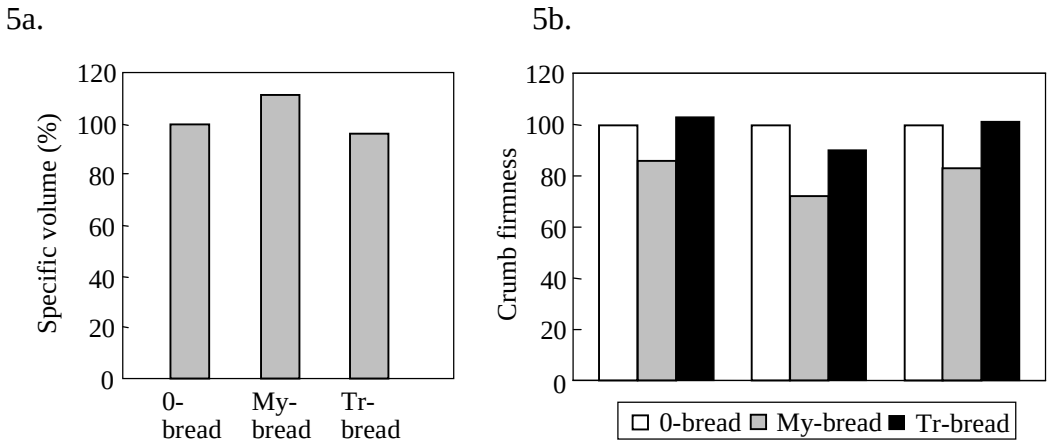

Figure 5. a) Loaf volume of bread (results have been reported as ratio of normal bread and enzyme bread, so that specific volume of normal bread is 100%). My = Myceliophthora, Tr = Trametes; b) Crumb firmness of bread; 0, 1 and 3 days after baking (results have been reported as ratio of normal bread and enzyme bread, so that hardness of normal bread is 100%). My = Myceliophthora, Tr = Trametes.

3 CONCLUSIONS

Laccases modified gluten by oxidizing thiol groups and increasing the amount of disulfide bridges. However, the properties of gluten modified by *Trametes* laccase were different from those modified by *Myceliophthora* laccase. This may be due to the different pH-values used.

Trametes laccase (pH 5) obviously degraded gluten aggregates and thus molecular mass and storage modulus were decreased. With *Myceliophthora* laccase (pH 7) disulfide bridges appeared to be more stabile and gluten aggregates were rearranged. This resulted a slight decrease of molecular mass but an increase of storage modulus.

During baking the pH of flour is close to 7. *Myceliophthora* laccase modified gluten and improved bread quality, while *Trametes* laccase was mainly inactive due to the unoptimal pH-value.

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ENZYMES AS STABILIZERS OF BREAD STRUCTURE

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ABSTRACT

The aim of this project was to study the effects of enzymes (α-amylase, two laccases, lipase and xylanase) on the structure of mixed wheat bread (85 wheat /15 graham flour), especially when baked from prefermented frozen dough. Preparations containing bacterial α-amylase, two laccases, lipase, xylanase, a mixture of xylanase and laccase or a mixture of α-amylase, lipase and xylanase were added to the dough. The dough was divided into two parts for different baking methods: 1) immediate baking after final proofing and 2) freezing for 1 week after prefermentation. Specific volume and firmness of the breads were analysed. The mixtures of enzymes were most effective in improving loaf volume and retaining softness of bread with both baking methods. Freezing of dough affected negatively the ability of lipase and laccase 2 to improve the quality of bread.

1 INTRODUCTION

Frozen dough baking is a rapidly developing and expanding baking technology with several economic advantages. The use of prefermented frozen doughs offers time saving in the final processing of frozen dough to bread. However, the gluten network of prefermented dough is under extension and highly sensitive to damage from ice crystals (Räsänen, 1998). In conventional bread baking several different types of enzymes are used to improve processing and baking quality. Our aim was to improve the structure of mixed wheat bread baked from prefermented frozen dough by using added enzymes.

2 RESULTS AND DISCUSSION

Breads were baked with a mixture of whole meal and wheat flour (15% whole meal wheat flour of the total flour weight) and different enzymes (Table 3). The properties of the two laccases have been reported by Virtanen *et al*. (2000). The dough was divided into two parts for different baking methods. 1) immediate baking after final proofing and 2) freezing for 1 week after prefermentation. A control bread without enzyme addition was baked with both methods. The volumes of breads were determined by the rape seed displacement method. Crumb firmness was measured at days 0, 1 and 3 to assess the potential shelf life of the breads. The bread crumb firmness during storage was determined as maximum compression force (40% compression, AACC 1995, method 74-09) using the Texture Profile Analysis (TPA) test.

Table 1. The effect of laccase and xylanase or their mixtures on the specific volume (ml/g) of bread.

Baking method	Control	Xylanase	Laccase 1	Laccase 2	Xylanase+ laccase 1	Xylanase+ laccase 2
Fresh	4.0	4.6	4.2	4.2	4.4	4.8
Frozen	3.6	4.2	3.6	3.8	4.5	4.0

Enzymes increased the specific volume of fresh baked breads by 5–20% (Table 1). According to a patent by Si (1994) laccase and xylanase increased the specific volume of white pan bread. Laccase 1 did not have any effect on the volume of bread, when baked from prefermented frozen dough. Laccase 2, xylanase and the mixtures of enzymes increased the specific volume of these breads by 5–25% when compared to the control.

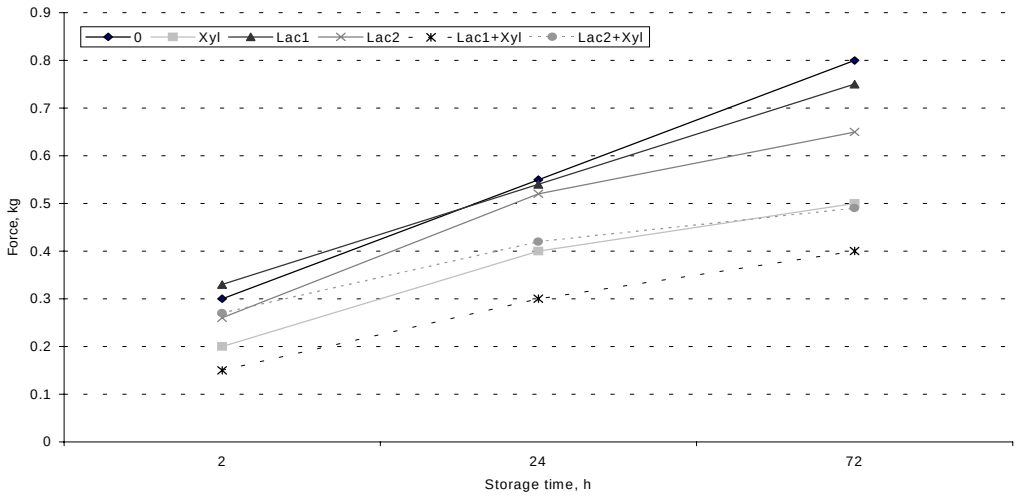

Figure 1. The effect of laccase and xylanase or their mixtures on the firming rate of breads baked from prefermented frozen dough.

Crumb softness and firming rate are important characteristics of bread quality. Xylanase, alone and in combination with both laccases retarded the firming rate of breads significantly (Figure 1). Bread with laccase 1 was harder than bread with laccase 2 during the 3 day storage.

Table 2. The effect of α-amylase, lipase and xylanase or their mixtures on the specific volume (ml/g) of bread.

Baking method	Control	Xylanase	Lipase	α-amylase	Xylanase+lipase+ α-amylase
Fresh	4.3	4.9	4.5	4.6	5.2
Frozen	3.4	3.8	3.4	3.9	4.3

Enzymes increased the specific volume of fresh baked breads by 5–20% (Table 2). Lipase did not have any effect on the volume of bread baked from prefermented frozen

dough. Xylanase, α-amylase and the mixture of enzymes increased the specific volume of these breads by 10–25% when compared to the control bread.

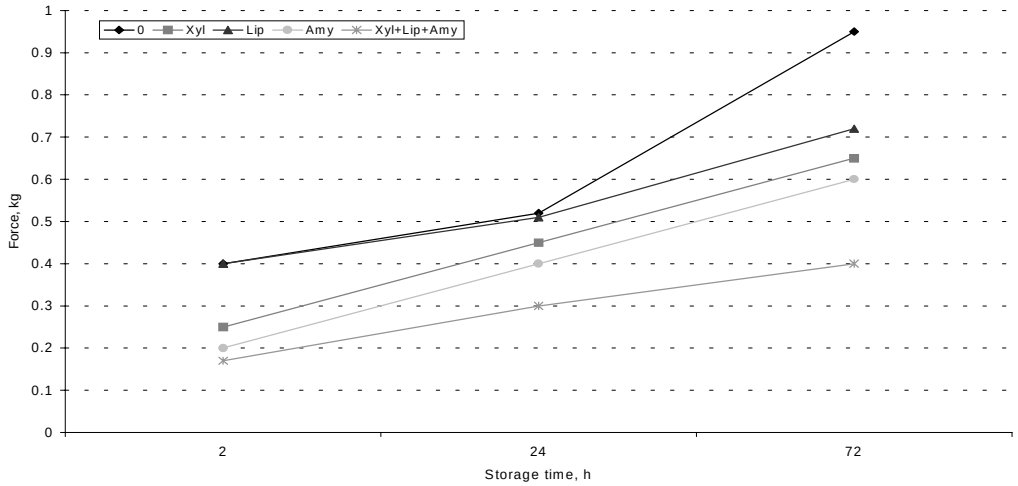

Figure 2. The effect of xylanase lipase and α-amylase or their mixtures on the firming rate of breads baked from prefermented frozen dough.

Xylanase, α-amylase and the mixture of enzymes retarded the firming rate of breads significantly (Figure 2). Bread with lipase was softer than control only after 3 days storage. The results reported in the study of Gil *et al*. (1999) showed also that addition of bacterial α-amylase, specially to blends of enzymes also containing lipase and pentosanase significantly increased the shelf life of white pan bread.

The following conclusions may be drawn from the results

1. Without freezing of dough, all the enzymes improved the specific volume of bread and retarded the firming rate during storage significantly compared to the control bread.
2. Specific volume of bread baked from prefermented frozen dough increased significantly by addition of α-amylase, xylanase, laccase 2 or the mixtures of enzymes when compared to the control bread. Also the firming rates of these breads were slower than the firming rate of the control. When added alone, lipase or laccase 1 did not have any positive effect on the volume or on the staling rate of these breads.

Table 3. The enzymes used and their main effects observed in prefermented frozen dough baking.

Enzyme	Main substrate in bread flour	Main effects observed in **prefermented frozen** dough baking
α-Amylase: TS E-216, Danisco Ingredients Ltd	Starch: amylose and amylopectin	Softer crumb Extended shelf life (20–50%) Larger volume (10%)
Xylanase: Pentopan mono BG, Novo Nordisk A/S	Arabinoxylan	Softer crumb, fine and regular pore structure Larger volume (10–15%) Extended shelf life (20–40%)
Lipase: Novozym 677 BG, Novo Nordisk A/S	Triglycerides	Smaller volume (–5%) Extended shelf life (5%)
Laccase 1: *Myceliopthora thermophilus*, Novo Nordisk A/S	Phenolic compounds	Shorter shelf life (–5%)
Laccase 2: *Trametes hirsuta*, VTT		Larger volume (5–10%) Extended shelf life (15–20%)
Enzyme combinations		
Laccase 1 + xylanase	Phenolic compounds, arabinoxylan	Larger volume (15%) Extended shelf life (20–40%)
Laccase 2 + xylanase	Phenolic compounds, arabinoxylan	Larger volume (15%) Extended shelf life (15–30%)
α-Amylase + Xylanase + Lipase	Arabinoxylan, starch and triglycerides	Softer crumb, fine and regular pore structure. Extended shelf life (25–50%) Larger volume (20%)

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BAKING OF HIGH FIBER WHEAT BREAD

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ABSTRACT

Our aim was to produce good quality wheat bread containing up to 10–12% of dietary fiber by optimizing the baking process. Reduction of bran particle size improved crumb structure and mouthfeel but the volume of the bread was not improved. Prefermentation of the wheat bran with yeast or with yeast and lactic acid bacteria improved loaf volume and crumb structure. The bread had added flavour and good and homogenous pore structure. Elasticity of the crumb was excellent. Commercial baking enzymes also had a positive effect on the volume, structure and flavour of bread supplemented with wheat bran. The combination of α-amylase, xylanase and lipase was the most effective in extending the shelf-life of the bread supplemented with wheat bran.

1 INTRODUCTION

Increased consumption of whole grain products and dietary fiber is recommended by health experts on the basis of their positive health effects. On the basis of scientific evidence the FDA has approved several claims related to fiber intake and decreased risk of heart disease and cancer. Wheat bran is an excellent source of dietary fiber. However, addition of wheat bran in baking results in bread with inferior quality: low volume, poor crumb structure and a bitter flavour. Dough machinability is also poor. Pretreatments of the wheat bran and addition of baking enzymes can be used to improve dough properties and bread quality.

Our aim was to produce good quality wheat bread containing up to 10–12% of dietary fiber by optimizing the baking process. The amount of fiber corresponds to 3 g/serving, the amount of wheat bran fiber cited to reduce the risk of colon cancer in aa approved health claim of the Food and Drug Administration.

2 MATERIALS AND METHODS

Breads were baked with 20% wheat bran (dry matter basis). The volume of breads was determined by rape seed displacement. Breads: 1. Control, 20% wheat bran, 2. Prefermentation of the bran with instant active dry yeast (4 h, 28 °C), 3. Prefermentation of the bran with instant active dry yeast and *Lactobacillus brevis* (16 h, 25 °C), 4. Enzymes I: Xylanase Pentopan mono BG, Novo Nordisk A/S; lipase Novozym 677, Novo Nordisk A/S; and α-amylase Grindamyl max-life, Danisco Ingredients, 5. Enzymes II: α-amylase Grindamyl max-life, 6. Control, no bran.

The bread crumb firmness during storage was determined as maximum compression force (40% compression, AACC 1983, method 74–09) using the Texture Profile Analysis (TPA) test.

3 RESULTS

The breads containing 20% wheat bran on a flour basis had low volume and poor crumb structure. Reduction of bran particle size improved crumb structure and mouthfeel but the volume of the bread was not improved (Data not shown). Prefermentation of the wheat bran with yeast (bread 2) or with yeast and lactic acid bacteria (bread 3) improved loaf volume and crumb structure (Fig. 1). The bread had added flavour and good and homogenous pore structure. Elasticity of the crumb was excellent. Commercial baking enzymes (breads 4 and 5) also had a positive effect on the volume, structure and flavour of bread containing wheat bran.

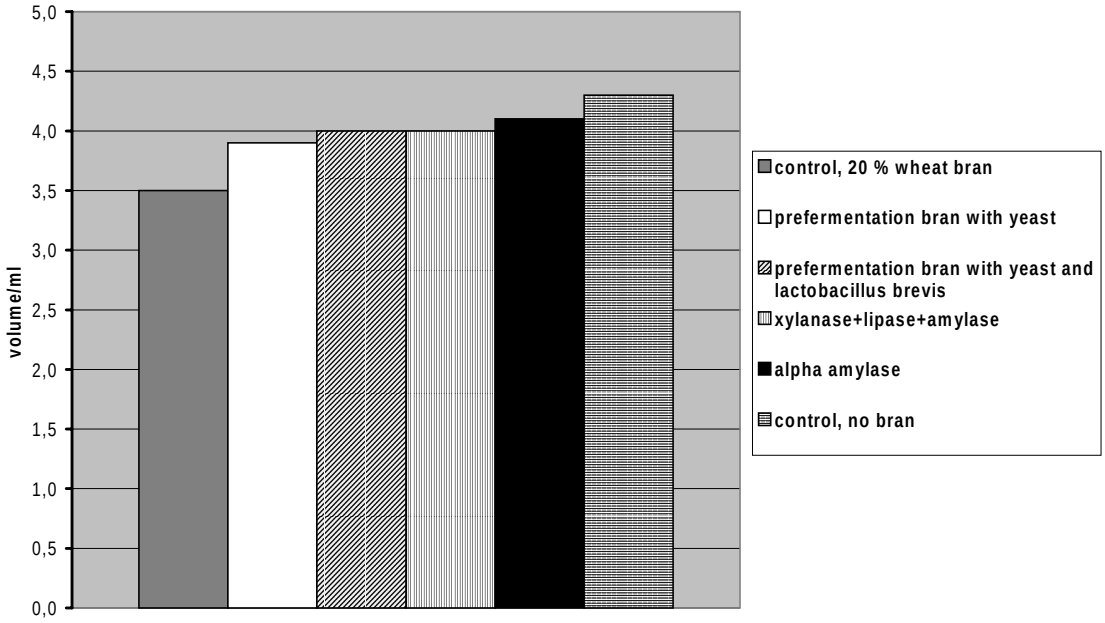

Figure 1. Effects of added wheat bran, prefermentation of bran and commercial baking enzymes on specific volumes of the test breads.

Crumb softness and firming rate are important characteristics of bread quality. Prefermentation of the wheat bran with yeast (bread 2) or with yeast and lactic acid bacteria (bread 3) improved crumb structure and elasticity of the bread. The bread crumb was softer and had a much slower staling rate than the crumb of the control bread (Fig. 2). Commercial baking enzymes (breads 4 and 5) also had a positive effect on the softness and firming rate of bread containing wheat bran. The combination of α-amylase, xylanase and lipase was the most effective in extending the shelf-life of the bread supplemented with wheat bran.

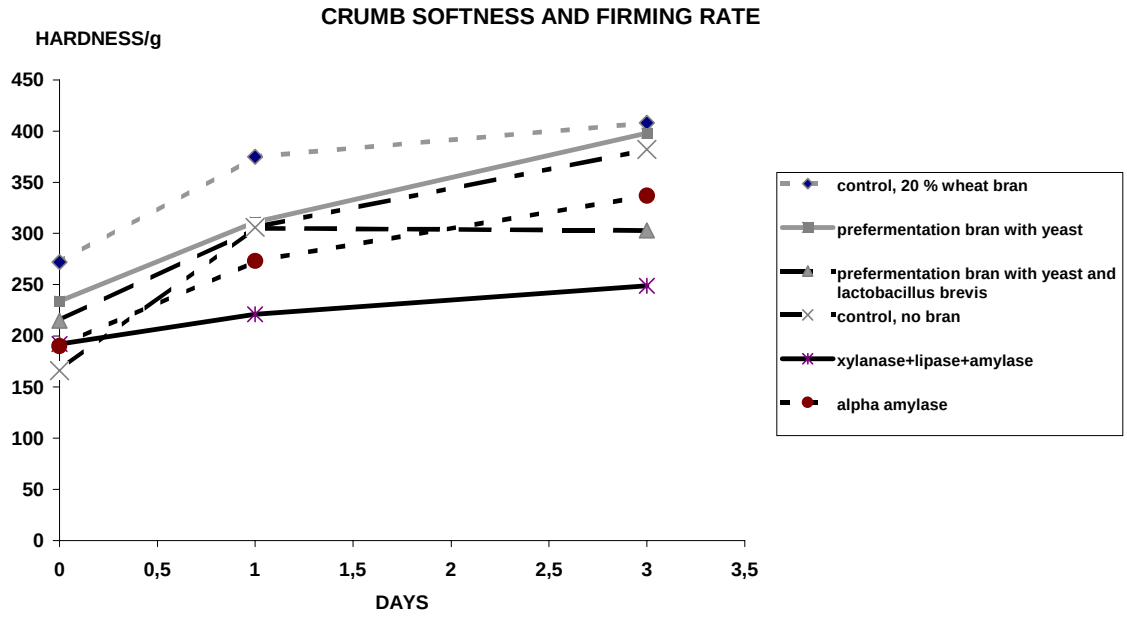

Figure 2. Crumb softness and firming rate. Crumb firmness was measured at days 0, 1 and 3 to assess the potential shelf life of the breads.

4 CONCLUSIONS

By optimizing the baking process it is possible to produce good consumer quality wheat bread containing up to 10–12% of dietary fiber.

- Reduction of bran particle size improved crumb structure and mouthfeel.
- Prefermentation of the wheat bran with yeast or with yeast and lactic acid bacteria improved loaf volume, crumb structure and shelf-life. The bread had added flavour and good and homogenous pore structure. Elasticity of the crumb was excellent.
- Commercial baking enzymes also had a positive effect on the volume, structure and shelf-life of bread containing wheat bran.

PRODUCTION OF LOW-CARBOHYDRATE BEER USING YEAST CO-IMMOBILISED WITH AMYLOGLUCOSIDASE

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1 INTRODUCTION

Dietetic beer is produced for customers who wish to have or must have a low nutrient diet (e.g. diabetics). In normal beer production although all the starch is degraded, some of it is not degraded to sugars and fermented, but only to dextrins which remain unfermentable in the beer. To produce dietetic beer, these dextrins must also be broken down further to sugars and fermented to alcohol. Consequently, a higher concentration of alcohol is automatically produced in the dietetic beer (in many countries the law states that this must be reduced again). With the usual mash processes starch degradation came to a halt at a final attenuation value of 80–85%. At this stage, there is insufficient time and residual enzyme activity present to degrade the remaining dextrins. Therefore, a variety of methods can be applied to achieve the necessary 100% attenuation, such as (a). using malt with high amylase activity (b). performing mashing for long time and very intensively (c). adding malt extract or malt flour at the beginning of the fermentation and (d). addition of enzymes at the start of the fermentation process. Very often a combination of these methods are applied.

In this project the objective was to reduce the carbohydrate content in beer by using yeast co-immobilised with amyloglucosidase in a continuous system.

2 MATERIALS AND METHODS

2.1 CO-CROSSLINKING OF AMYLOGLUCOSIDASE

Mix 15ml of water and 100mg of albumin at room temperature, then add 3ml of amyloglucosidase solution, mix thoroughly and put on ice. Add 60ml of ice cold acetone slowly and 2ml of 25% glutaraldehyde and stir for 2 hours at 25^{o}C. Centrifuge at 1500rpm for 5 min, and discard the supernatant. Wash twice with 50ml of distilled water and centrifuge again (1500rpm for 5 min), then resuspend in water.

2.2 CO-IMMOBILIZATION OF AMYLOGLUCOSIDASE AND YEAST IN ALGINATE BEADS

Add 1.2g (dry weight) yeast to 50ml resuspension of co-crosslinked amyloglucosidase and mix well. Add 0.6g of sodium alginate and stir until the alginate is dissolved. Then fill the mixture into a bead machine and allow drops to fall into a 0.1M $CaCl_2$ solution (Fig. 1 and Fig. 2).

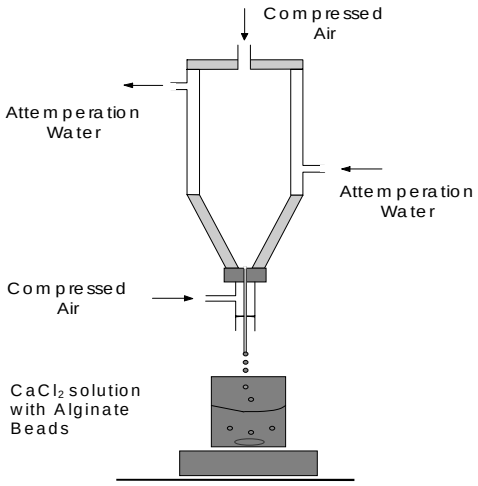

Fig.1: Production of alginate beads

Figure1. Production of alginate beads.

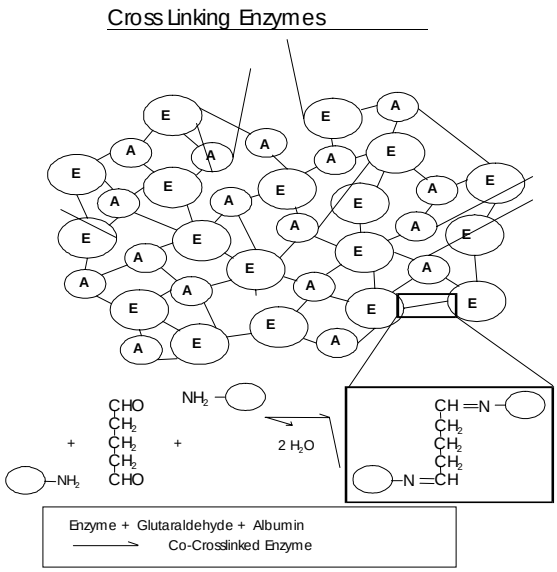

Figure 2. Co-Cross linking of enzyme, glutaraldehyde and Albumin.

2.3 PRODUCTION OF LOW CARBOHYDRATE BEER

The ability of the beads to reduce the carbohydrate content of the beer was studied in a repeated batch system and a continuous system using a multi-stage fluidised bed bioreactor (Fig. 3).

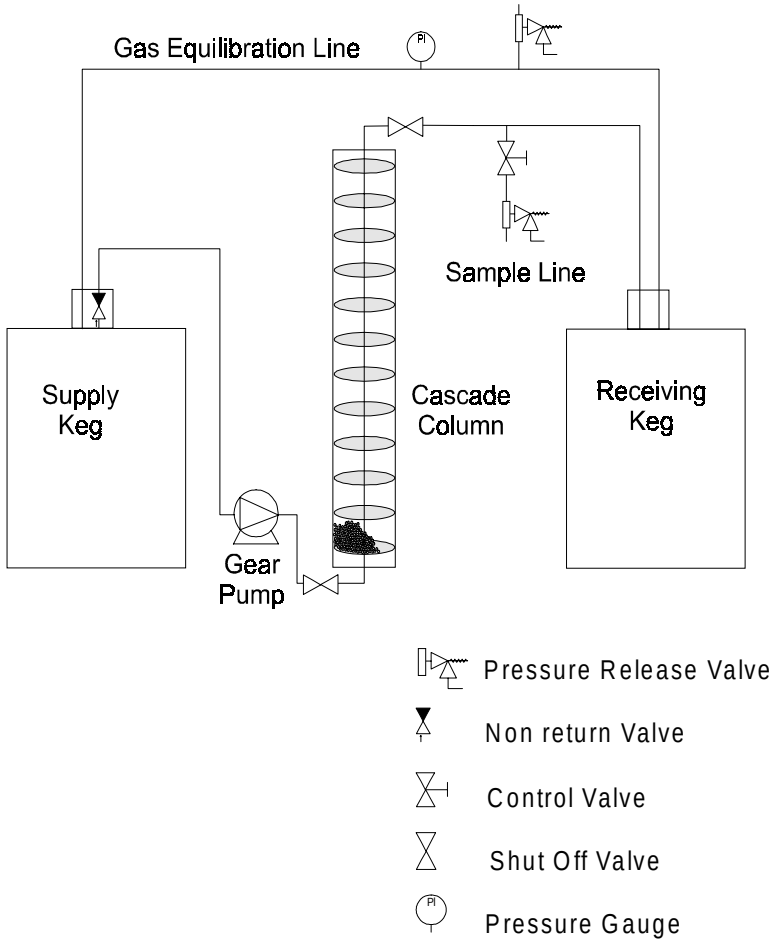

Figure 3. Schematic diagram of the multi-stage fluidised bed bioreactor.

2.4 ANALYSIS

Alcohol and Density:	Analytica EBC 9.2.2
Amyloglucosidase activity:	Spectrophotometer
Colour:	AVM comparometer
Haze:	Hazemeter
Free amino nitrogen:	Analytica EBC 9.9.10
Total Nitrogen:	Analytica EBC 9.9.1

3 RESULTS

The amyloglocosidase activity was evaluated in the beads as well as in the low carbohydrate beer. In the batch system only a 5% loss of the enzyme activity was determined over a period of 400h. No significant loss in activity was observed in the continuous system over a period of 2 months. In the repeated batch system the apparent attenuation increased from 74% to 106% as the alcohol content increased from 4% to 6.9% in a period of 4 h (Fig. 4). In the continuous system a dilution rate of 1,13ml

beer/h x ml (volume of liquid in column) was achieved and the apparent attenuation increased from 74% to 104%, and the alcohol content increased from 4% to 5.7% (Fig. 5).

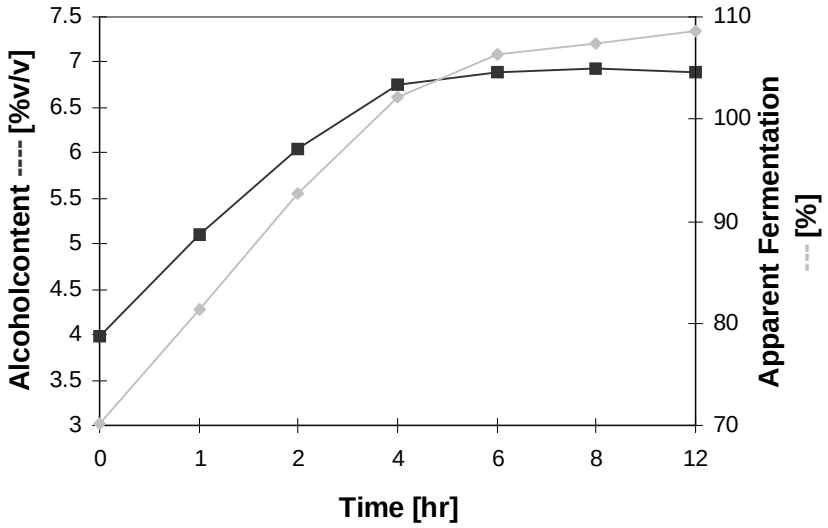

Figure 4. Development of Alcohol content and apparent fermentation in a batch system.

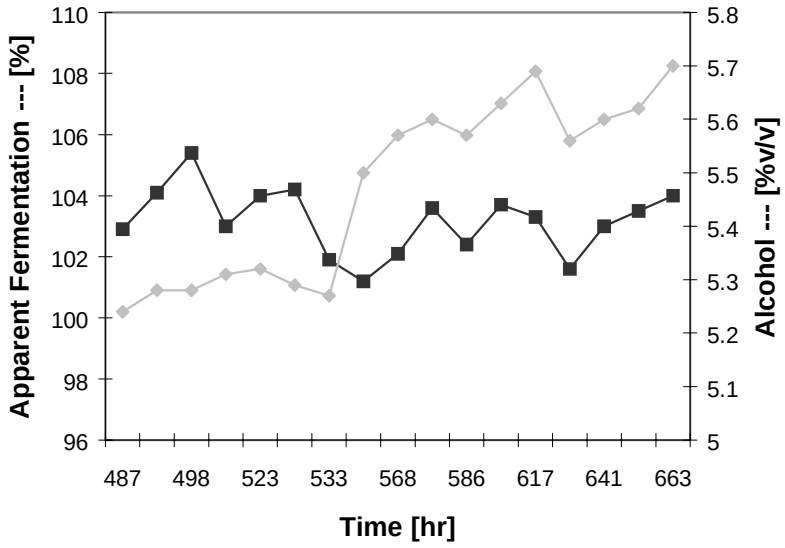

Figure 5. Development of apparent fermentation and alcohol content in a multi-stage fluidised bed bioreactor.

4 CONCLUSION

In conclusion, it was shown that yeast co-immobilised with amyloglucosidase can be efficiently applied to reduce the carbohydrate content of beer as a continuous process using a multistage fluidised bed bioreactor.

THE USE OF RIBOFLAVIN BINDING PROTEIN TO IMPROVE FLAVOUR STABILITY OF BEER

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1 INTRODUCTION

Riboflavin and its derivatives FMN and FAD (Fig. 1) are present as cofactors of a wide variety of enzymes in barley, hop and yeast, the main ingredients of beer. During the brewing process riboflavin is released from the enzymes, but not further degraded.

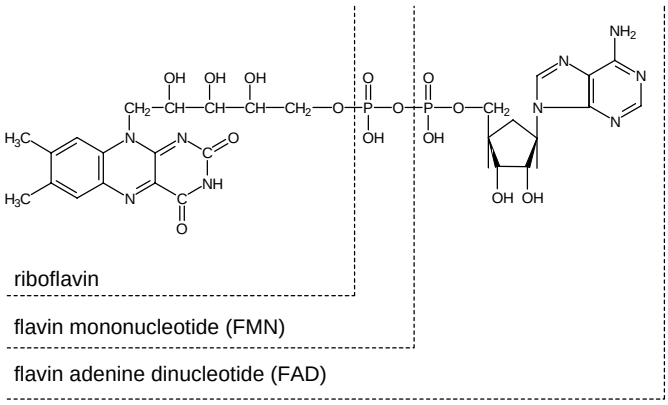

Figure 1. Riboflavin, FMN and FAD.

Riboflavin has for a long time been implicated as a photosensitiser in the formation of sunstruck flavour in beer [1–3]. In addition, it has been postulated that riboflavin and other flavins [4–6] are also involved in the formation of reactive oxygen species (ROS), which initiate a chain reaction in beer, thus leading to the formation of stale flavour in general (Fig. 2). Consequently, the removal of riboflavin from beer would significantly improve the stability of beer flavour towards light.

2 RESULTS

The riboflavin content of beer was determined by fluorometric titration with the apo-riboflavin binding protein (apo-RfBP), purified from chicken egg white [8,9]. This protein binds riboflavin in a 1:1 ratio, with a high affinity at neutral pH [10]. The binding of riboflavin to apo-RfBP almost completely quenches the riboflavin fluorescence (Fig. 3).

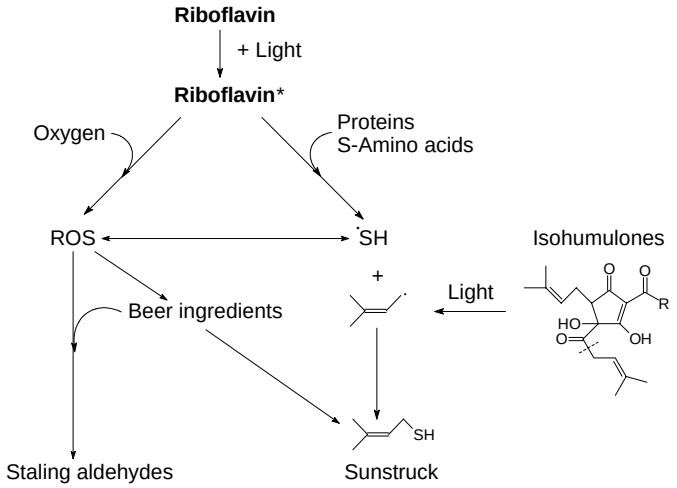

Figure 2. Proposed pathway for the formation of sunstruck and other off-flavours in beer. Modified after [3,6,7].

Since the relationship between the fluorescence intensity and the riboflavin concentration of a solution is linear (data not shown), the riboflavin content of the solution can be calculated from the fluorescence titration curve (Fig. 3). The riboflavin levels of the different types and brands of beer investigated were all in the range of 0.5–1.0 µM. Beer contains only a very small amount of FAD (data not shown) and does not contain FMN [11].

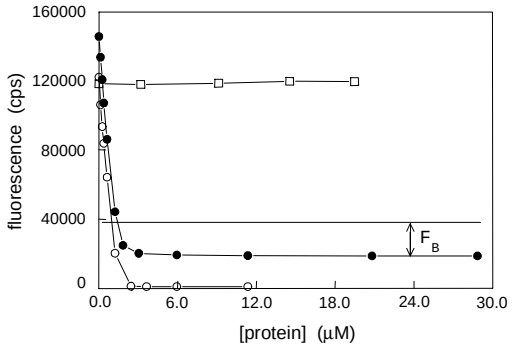

Figure 3. Fluorometric titration of solutions of riboflavin with apo-RfBP (F) *and* (M)*, and BSA* (G)*. Solutions* (F) *and* (G) *contained 1.0 µM riboflavin + 50 mM NaPi, pH 7.0; solution* (M) *additionally contained 300 µl lager beer; all in a final volume of 1 ml. Fluorescence units are cps, counts per second. F_B, fluorescence of the riboflavin in the beer. Excitation wavelength 450 nm, emission wavelength 525 nm. All measurements were performed at room temperature.*

Possible sources of riboflavin in beer are the malt, hop and yeast. Hopped beer was found to have the same riboflavin content as unhopped beer (Table 1). Literature data show that the maximum contribution of yeast to the riboflavin content of beer is approximately 50 nM [12,13]. The high riboflavin concentration of the sweet wort sample (Table 1), as compared to the riboflavin content of the final product, can be

explained by dilution effects during spargeing (washing out of sugars from the filter bed). Obviously, the origin of riboflavin in beer is the malt.

Table 1. Riboflavin content of sweet wort and beer.

sample	[riboflavin] (μM)	s.d. (μM)	*n*
sweet wort	1.41	± 0.13	4
unhopped lager beer	0.81	± 0.02	5
hopped lager beer	0.80	± 0.02	5

The riboflavin content is the average of *n* determinations, from fluorometric apo-RfBP titration of the sample; s.d., standard deviation. Dilution of the samples: sweet wort, 100 μl; hopped and unhopped beer, 300 μl; added to 50 mM NaPi, pH 7.0, to a final volume of 1 ml. The sweet wort did not contain hop; the hopped and unhopped beers (obtained from special pilot brews) were identical except for the presence and absence, respectively, of hop.

Table 2. Effect of addition of apo-RfBP to model beer solutions on sunstruck flavour formation.

[riboflavin] (μM)	[isohumulone] (ppm)	[cystein] (ppm)	[apo-RfBP] (μM)	sunstruck flavour
2.7	-	-	-	-
2.7	20	10	-	++
2.7	20	10	0.3	+
2.7	20	10	1.3	+/-
2.7	20	10	2.7	-
2.7	20	10	13.5	-

All samples contained 5% ethanol + 0.1 M citric acid/Pi, pH 4.0. The samples were illuminated for 4 hours, at 30 °C. (++), marked sunstruck flavour; (-), not detectable.

Besides its use in the determination of riboflavin levels in beer, apo-RfBP might also provide a way to selectively remove riboflavin from beer. The effect of apo-RfBP on the light-induced formation of ROS and sunstruck flavour was studied in model beer solutions (containing riboflavin, ethanol, isohumulones and traces of oxygen, in a pH 4.0 buffer). EPR studies showed that upon illumination, hydroxyl and hydroxyethyl radicals were formed in such model beer solutions. The formation of these radicals could successfully be suppressed by the addition of apo-RfBP to the solutions, prior to illumination. A slight excess of apo-RfBP (1.5 times the amount of riboflavin present) already reduced the amount of radicals formed by two-third (data not shown). Apo-flavodoxin from *Azotobacter vinelandii* was about equally effective as apo-RfBP in

suppressing radical formation. Organoleptic assessment clearly indicated that the addition of apo-RfBP to the model beer solutions reduced sunstruck off-flavour formation (Table 2).

3 CONCLUSIONS

Fluorometric titration with apo-RfBP proved to be a simple, reliable and sensitive method for the determination of the riboflavin content of beer. Riboflavin in beer was found to originate mainly from the malt.

Apo-RfBP effectively inactivates riboflavin as a photosensitiser in model beer solutions. Upon binding, riboflavin looses its ability to generate free radicals as well as sunstruck flavour. Addition of apo-RfBP to beer is likely to block the detrimental activity of riboflavin, which would minimise the light sensitivity and the radical-related lightstruck off-flavour formation in beer.

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THE USE OF MALTED BARLEY AND COMMERCIAL ENZYMES IN UNMALTED SORGHUM BREWING

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1 INTRODUCTION

Traditional problems associated with unmalted sorghum brewing include incomplete saccharification, lautering difficulties, poor fermentability, beer foam and colloidal instability and flavour problems. The effects of the addition of malted barley at increasing percentages (0–100%) to unmalted sorghum grists together with industrial enzymes were evaluated.

2 MATERIALS AND METHODS

The cereal varieties used in this study were a Nigerian sorghum variety Fara Fara and an Irish malted barley variety Cooper. Mashing was carried out in a standard type EBC mash bath using a commercial type program used in the production of sorghum beer (Figure 1). Industrial enzymes Hitempase 2XL, Bioferm L and Bioprotease N-100L were added at recommended dose levels of 0.05% per weight of grist. At mashing off a range of wort parameters were evaluated, including filterability,% extract, pH, colour, viscosity, total soluble nitrogen, free amino nitrogen and high and low molecular weight nitrogen fractions. All methods used were carried out in accordance with standard EBC, IOB and MEBAK analytical brewing methods.

Product name	**Type**	**Opt Temp**	**Function**
Hitempase 2XL	Thermostable α-amylase from *Bacillus lichenformis*	90–95 °C	Used for effective gelatinisation of starch
Bioprotease N-100P	Proteolytic enzyme from *Bacillus subtilis*	50 °C	For the production of Free Amino Nitrogen
Bioferm	Fungal α-amylase from *Aspergillus oryzae*	55–60 °C	For effective liquefaction of starch

	Unmalted Sorghum	**Malted Barley**
Moisture	9.75%	7.02%
Protein (dry wt)	9.83%	9.75%
Extract (dry wt)	76%	79%
Fat	4.0%	1.5%
Gelatinisation temp	69–75 °C	64 °C

3 RESULTS

Pale wort production is a problem when brewing with 100% unmalted sorghum. With the introduction of malt the colour increases linearly. As the % malt was increased there was a linear increase in viscosity and a corresponding decrease in pH.

Measuring the mash filterability at laboratory scale gives a rough indication as to potential lautering times and degree of starch granule/endosperm degradation. From Figure 1 it can be seen that as the % malt is increased, the lautering time decreases. From the graph it can also be seen that at sorghum levels as low as 20% a negative impact on filterability is observed.

The % extract (Figure 2) gives an indication of the degree of modification of both the sorghum and malt with regard to protein and starch breakdown. As the % malt is increased, the % extract content also increases.

The Kolbach index (degree of soluble nitrogen) increased from 10% (100% sorghum) to 40% (20% sorghum) indicating that as the % malted barley was increased, more of the available proteinaceous material was solubilised (Figure 3).

High molecular weight nitrogen has a positive effect on foam stability. From Figure 4 it is seen that as the % malt is increased, there is a linear increase in the high molecular weight nitrogen fractions. Therefore the inclusion of malt could have a positive effect on the foam stability of the resultant beer.

Free amino nitrogen is necessary for adequate fermentation. Levels of 100–140mg/L are reported to be essential for yeast metabolism. As the % malt increases (Figure 5) the free amino nitrogen increases accordingly. Therefore the fermentation performance is enhanced as the % malt is increased.

4 CONCLUSIONS

With the addition of just 20% malted barley, there were increases of

63% in total soluble nitrogen

91% in high molecular weight nitrogen

64% in free amino nitrogen

Therefore the addition of malt together with commercial enzymes could significantly improve the brewhouse performance when brewing with unmalted sorghum. These results suggest an improvement in the potential for brewing high quality beers with unmalted sorghum.

Figure 1

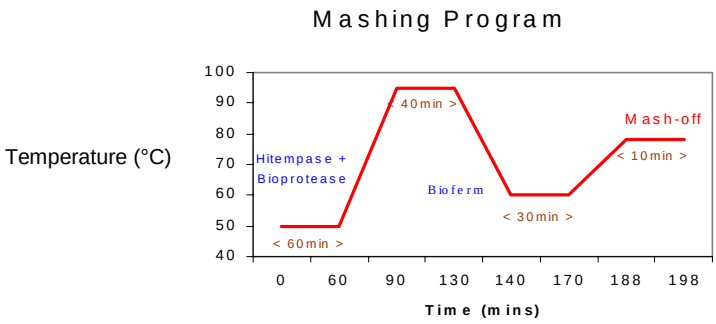


Figure 2

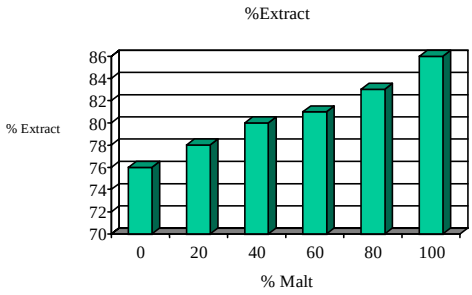


Figure 3

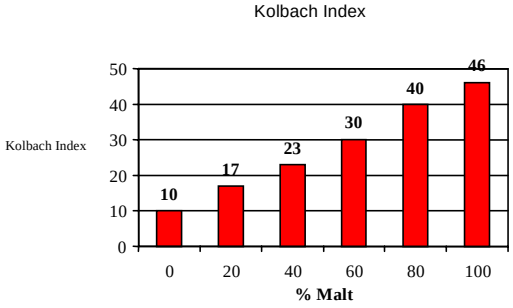


Figure 4

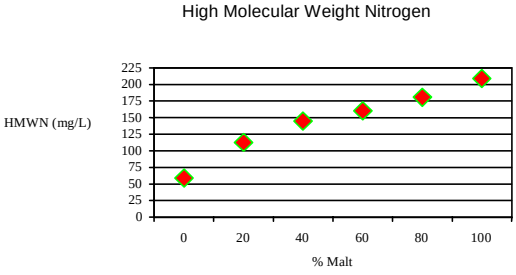
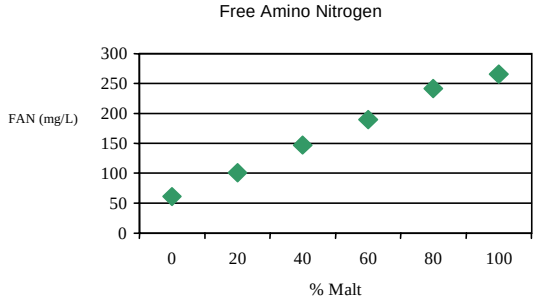


Figure 5



OPTIMISATION OF ENZYME ACTIVITY IN A MIXED BARLEY / MALT BREW USING BIOLOGICAL ACIDIFICATION

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[2] National Food Biotechnology Centre, University College Cork, Ireland

1 INTRODUCTION

Reduction in pH of mash is a beneficial and desirable event in the beer brewing process. A number of positive effects result from pH decrease during mashing. These include improved enzyme activity (especially proteolytic enzymes), which in turn leads to many benefits during processing such as reduced lautering time, β-glucan content and viscosity, also a higher fermentability and foam and taste stability of beer. Standard procedure in Irish and UK breweries is to acidify the mash or the cast wort by the addition of either mineral acids, artificially-produced organic acids, calcium sulphate or calcium chloride.

The objective of this project is to develop an alternative biological acidification procedure through the use of defined starter culture systems in a mash which has a high barley content. Lactic acid strains were isolated from barley using a mash program, which allowed thermophilic malt micro-flora propagation. Their individual performances were assessed in a wort environment, and the strain to achieve the greatest pH reduction was used for a mash biological acidification. The resulting wort was compared to an unacidified mash on the basis of pH, filtration time, viscosity, colour, buffer capacity, extract, β-glucan and zinc contents.

2 MATERIALS AND METHODS:

2.1 PROPAGATION MASH

50g of malt was milled with a Buhler Miag Disc Mill, DLFU (0.2mm), and mashed-in with 300ml of water at 49°C for 24 hours. At the end of this holding period strains were isolated and identified.

2.2 DILUTE WORT MASH

100g of malt grist was mashed-in with 400ml of water at 50°C. It was held for 30 minutes, increased again to 60°C for 15 minutes, 65°C for 20 minutes, 72°C for 30 minutes, 78°C for 5 minutes, and finally filtered. The wort was diluted to 10% w/w extract content.

2.3 ACIDIFIED STOCK SOLUTION PREPARATION

Individually the strains were inoculated into 50ml of dilute wort and incubated at 48°C for 48 hours. Using a Thoma 10 * 10^6 cell ml^{-1} were inoculated into 250ml of dilute wort, and again incubated at 48°C for 48 hours. The strain which attained the lowest pH was chosen.

2.4 BIOACIDIFICATION MASH

60g of barley and 40g of malt grist was mashed-in at 35°C with 400ml of water. No greater than 10% of the acidified stock solution was added to the mash to reduce the pH. The mash was held for 10 minutes, then heated to 45°C for 10 minutes, 50°C for 15 minutes, 55°C for 15 minutes, 60°C for 15 minutes, 65°C for 20 minutes, 72°C for 30 minutes, 78°C for 5 minutes and cooled to 20°C.

A mash of the same grist combination was prepared unacidified. Both resulting worts under went a series of analysis.

Wort analysis

Analysis	Method
pH	Analytica-EBC 8.17
Filtration Time	Analytica-EBC 4.5.1
Colour	Analytica-EBC 4.7.1
Viscosity	Analytica-EBC 8.4
Zinc	Analytica-EBC 8.11
β-glucan	Analytica-EBC 8.13
Extract	FOSS
Buffer Capacity	MEBAK 2.18

3 RESULTS AND DISCUSSION

Overall the *P.acidilactici* strain gave the best performance in wort producing the lowest pH value 4.15 (Fig. 1) and also showing good growth. This strain was chosen for further studies in barley/malt mashes. The addition of the strain lead to a decrease of the mash pH by 0.168 units compared to the unacidified mash (pH of acidified mash 5.46, pH of control mash 5.63). The decrease in pH of the mash had a number of positive effects on the mash and wort such as a decrease in colour (control 9.02 EBC units, acidified mash 8.70 EBC units), β-glucan (control 310.66 mg/l, acidified 300.02 mg/l), viscosity (unacidified 0.2457 MPAs, acidified 0.2404 MPAs), and filtration time (Fig. 2). It also lead to an increase in buffer capacity (unacidified 20.158, acidified 23.449), and zinc content (Fig. 3). Similar results were obtained at the 10hl pilot scale brewery at University College Cork.

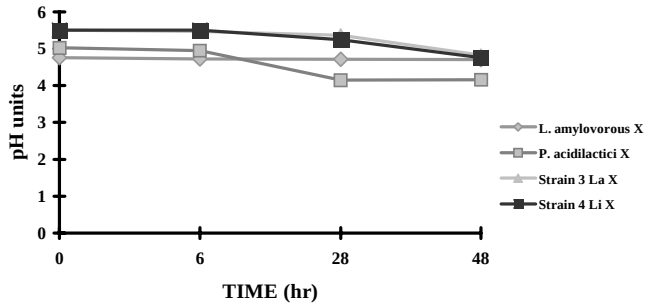

Figure 1. Effect of strains of lactic acid bacteria isolated from barley on wort pH.

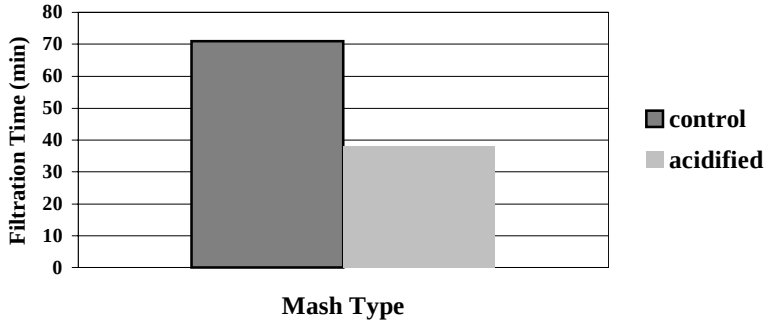

Figure 2. Effect of biological acidification on filtration time of a 60:40 barley malt mash.

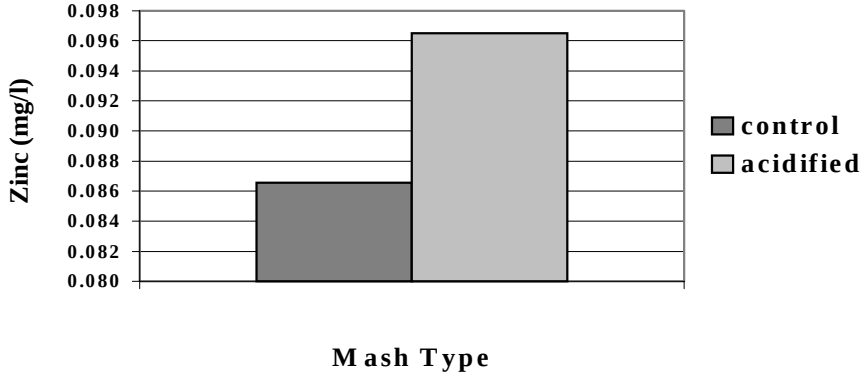

Figure 3. Effect of biological acidification on the zinc content of a 60:40 barley malt mash.

4 CONCLUSION

In conclusion, *P. acidilactici* was successfully used to acidify mash and had positive effects on the processibility of mash and wort.

ACKNOWLEDGEMENTS

This Research has been part-funded by grant aid under the Food Sub-Programme of the Operational Programme for Industrial Development which is administrated by the Department of Agriculture, Food and Forestry and was supported by national and EU funds.

ENZYMATIC EXTRACTION OF ARABINOXYLO-OLIGOSACCHARIDES FROM RYE BRAN IN PILOT SCALE

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ABSTRACT

Arabinoxylan is the most abundant dietary fiber component of rye. It is partly water-soluble, and provides fermentable substrates for colon fermentation. It has been suggested that xylo-oligosaccharides could be bifidogenic. The aim of this study was to prepare water-soluble arabinoxylo-oligosaccharides from rye bran with the aid of xylanases, in order to study their degradation in *in vitro* fermentation.

1 METHOD

Rye bran was extruded with Clextral BC 45. Extruded rye bran (160 kg) and water (1500 l) were mixed (Fig. 1) in a pilot fermentor at 50^{o}C. Xylanase was added (Pentopan Mono BG, Novo, 300 g). The suspension was stirred at 50^{o}C for two hours and finally cooled. The solid material was allowed to settle overnight under cooling. The supernatant (ca 650 l) was first decanted and then centrifuged by Alfa-Laval separator, evaporated by the falling film evaporator (MTK-PE-1,1,6) and lyophilized in Atlas pilot freeze dryer.

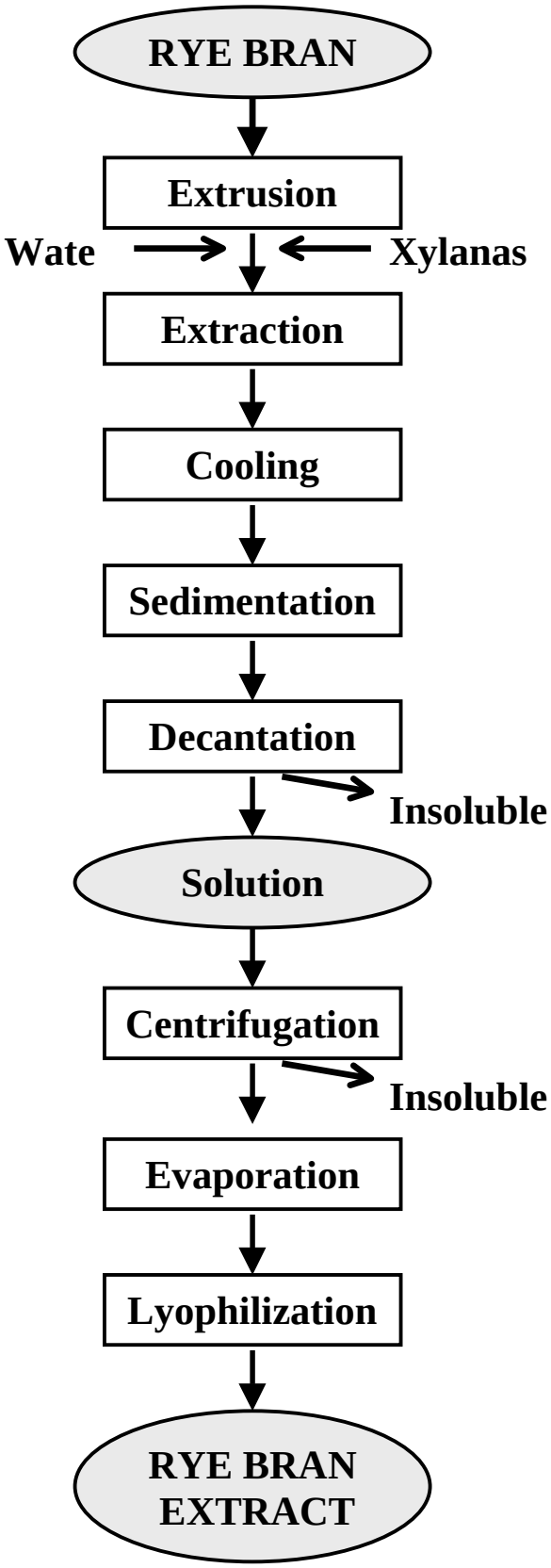

Figure 1. Extraction of arabinoxylo-oligosaccharides in pilot scale.

2 RESULTS

9.4 kg dry rye bran extract was extracted from 160 kg of rye bran. The main components of the starting material and the product – rye bran and rye bran extract – are shown in Table 1. The recovery of pentosan was 9%.

Table 1. The main components of rye bran and rye bran extract. Percentages are on wet basis.

	Rye bran (%)	**Rye bran extract (%)**
Pentosan	18	27
β-Glucan	3.2	4.4
Fructan	7.1	21
Starch	24	12
Protein	16	9.1
Fat	4.3	2.3
Ash	6.0	8.1
Moisture	7.4	6.0

The total carbohydrate content of the extract was 72% on wet basis (Table 2) the main carbohydrates being pentosans of different molar masses (27%), but also the fructan content in the water solubles was rather high (21%). Pentosans were mixtures of poly- and oligosaccharides and only traces of pentose sugars were detected. The poly- and oligosaccharides composed mainly of xylose and arabinose. The specific xylo-oligosaccharides quantitated were xylobiose, xylotriose, xylotetraose and xylopentaose, the sum of which was 8.8%. The content of the undigestible carbohydrates was 73% calculated from the total carbohydrates in the rye bran extract.

Table 2. The carbohydrate composition of the rye bran extract. Percentages are on wet basis.

COMPONENT	%
PENTOSAN; (ARABINOSE+XYLOSE)*0.88	**26.7**
XYLOBIOSE	***(4.9)***
XYLOTRIOSE	***(2.3)***
XYLOTETRAOSE	***(0.9)***
XYLOPENTAOSE	***(0.7)***
β-GLUCAN	**4.4**
FRUCTAN	**21.2**
UNDIGESTIBLE CARBOHYDRATES	52.3
STARCH	**12.0**
ARABINOSE	**0.02**
XYLOSE	**0.5**
SUCROSE	**5.5**
MALTOSE	**1.7**
CARBOHYDRATES, TOTAL	72.0

THE USE OF VARIOUS ENZYME PREPARATIONS FOR LIQUEFACTION AND SACCHARIFICATION OF CEREAL MASHES, VISCOSITY CHANGES AND TOTAL ETHANOL YIELD

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1 INTRODUCTION

Viscosity of media is important technological feature throughout the whole process in distillery. High viscosity influences negatively energy consumption, separation properties of mashes, disturbs fermentation (mash lifting) and distillation. It also lowers starch conversion to saccharides, which means serious losses in total ethanol yield.

The aim of the study was to test in laboratory scale an effect of use different standard enzyme preparations to mash viscosity, and to supplement their effectiveness with special viscosity-reducing enzymes. The changes in ethanol yield were also followed.

2 MATERIALS

Three types of cereals from the 1997 harvest (wheat- variety Astella, triticale- variety Kolor and Ring) were used as a raw-material.

Enzyme mixtures used in this study were product of Novo Nordisk A/S, DK. In the standard combinations recommended levels of liquefaction •-amylases Termamyl 120L and BAN 240L together with saccharification preparation SAN Super 240L were added. Preparations with cellulase activity (Celluclast 1,5L), xylanase activity (Shearzyme L) and pentosanase-arabanase activity (Ultraflo L) were added in selected mashes.

Mashes were inoculated with 0.30 g l^{-1} of active dry yeast *Saccharomyces cerevisiae* SIHA DF 639 (E. Begerow GmbH., D).

3 METHODS

Progress of viscosity in single worth was measured with HAAKE VT 500 viscometer before liquefaction, after each enzyme addition (taking in account recommended temperature and reaction times), after fermentation and in stillages.Constant temperature was 50 °C.

Ethanol concentration was measured by HPLC 4001 (LP Prague, Czech Republic), column packing: Ostion LGKS 0800 in a Ca^{2+} cycle. Detection: refractiometer RIDK 1101, eluent: demineralized water.

Table 1. Preparations of mashes.

code	variety	enzyne preparations
P-2	wheat Astella	BAN, SAN
P-3	wheat Astella	BAN, SAN, Celluclast
P-4	wheat Astella	BAN, SAN, Ultraflo
P-5	wheat Astella	BAN, SAN, Shearzyme
P-6	wheat Astella	Termamyl, BAN, SAN
P-7	wheat Astella	Termamyl, BAN, SAN, Ultraflo
P-8	wheat Astella	BAN, SAN, Celluclast, Shearzyme
P-9	wheat Astella	Termamyl, BAN, SAN, Celluclast, Shearzyme
P-10	wheat Astella	Termamyl, BAN, SAN, Celluclast
P-11	wheat Astella	Termamyl, BAN, SAN, Shearzyme
TK-2	triticale - Kolor	BAN, SAN
TR-2	triticale - Ring	BAN, SAN
TK-3	triticale - Kolor	BAN, SAN, Celluclast
TR-3	triticale - Ring	BAN, SAN, Celluclast
TK-10	triticale - Kolor	Termamyl, BAN, SAN, Celluclast
TR-10	triticale - Ring	Termamyl, BAN, SAN, Celluclast

Table 2. Viscosity of mashes [Pa.S].

	Mashes	Worths			Fermented worths	
Code	Before saccharification	After Termamyl	After BAN	After SAN	After fermentation	stillages
P-2	0,0021	-	0,0089	0,0084	0,0087	0,0037
P-3	0,0020	-	0,0040	0,0040	0,0050	0,0025
P-4	0,0021	-	0,0086	0,0092	0,0090	0,0037
P-5	0,0025	-	0,0062	0,0053	0,0047	0,0027
P-6	0,0024	0,0019	0,0142	0,0140	0,0121	0,0074
P-7	0,0020	0,0190	0,0025	0,0024	0,0056	0,0203
P-8	0,0023	-	0,0026	0,0036	0,0027	0,0027
P-9	0,0017	0,0089	0,0021	0,0035	0,0048	0,0044
P-10	0,0018	0,0205	0,0082	0,0111	0,0061	0,0024
P-11	0,0020	0,0210	0,0130	0,0104	0,0093	0,0358
TK-2	0,0103	-	0,0109	0,0092	0,0022	0,0025
TR-2	0,0032	-	0,0049	0,0073	0,0047	0,0021
TK-3	0,0023	-	0,0031	0,0032	0,0020	0,0018
TR-3	0,0021	-	0,0023	0,0025	0,0019	0,0023
TK-10	0,0020	0,0192	0,0106	0,0110	0,0108	0,0049
TR-10	0,0017	0,0133	0,0071	0,0105	0,0043	0,0020

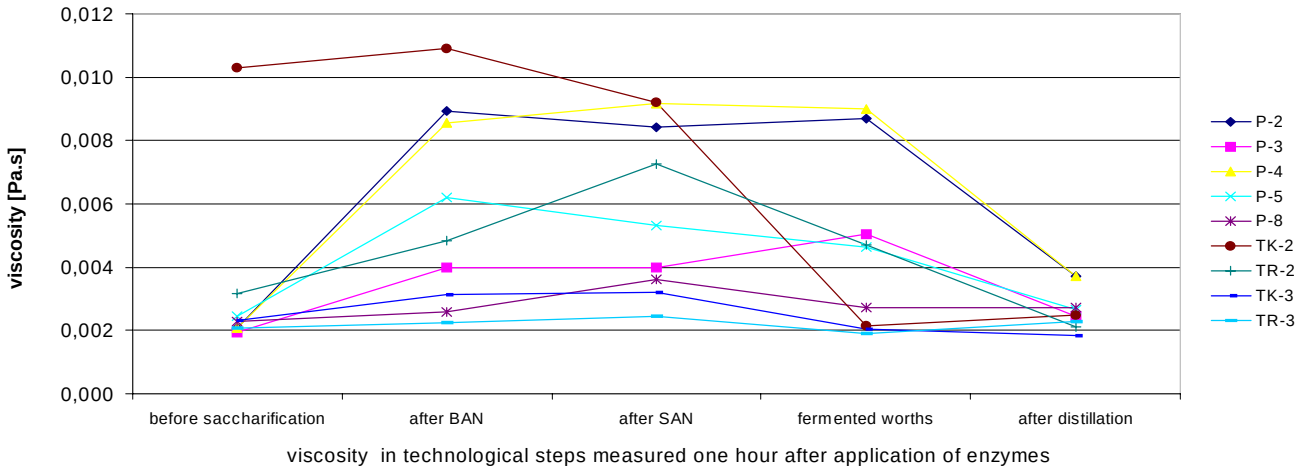

Figure 1. Viscosity of worths treated without Termamyl.

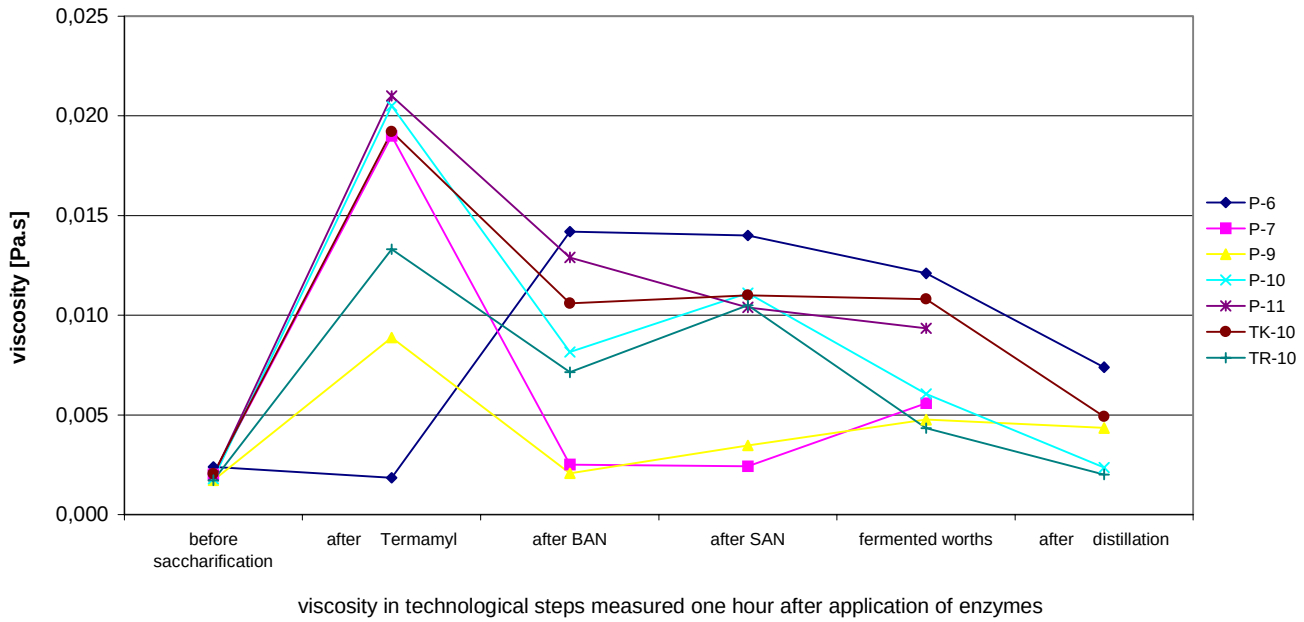

Figure 2. Viscocity of worth treated with termamyl.

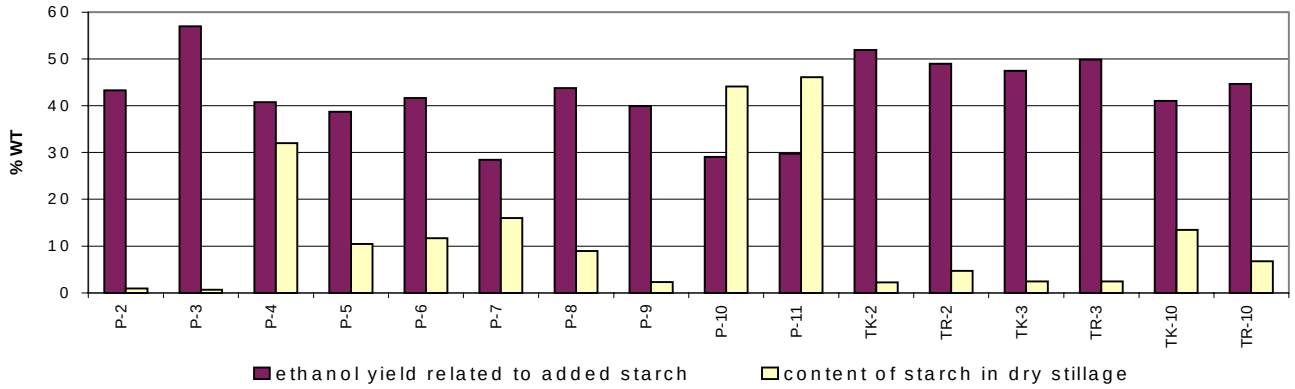

Figure 3. Comparison of ethanol yields and content of unutilized starch.

4 CONCLUSION

The best results were obtained, when mixture of BAN, SAN Super and Shearzyme was added. In this case viscosity of stillage was always the lowest (0,005 Pa.s). Viscosity curves of these mixtures demonstrated the lowest viscosity values in all measured worths during whole process. On the other hand, all combinations with Termamyl showed higher viscosity at the initial stage of hydrolysis (0,025 Pa.s) compared to combinations with BAN (0,01 Pa.s).

5 SUBSTRATE

Triticale as a substrate appeared to reduce viscosity in comparison with wheat at beginning of heating up the mashes, especially by variety triticale Ring, where higher activity of endogenous enzymes seems to be the reason.

6 ETHANOL YIELD

Influence of mixture of BAN, SAN Super and Celluclast resulted in the highest ethanol yields, for wheat and triticale mashes (57,0% and 50,5% respectively), calculated as ethanol yield related to added starch.

7 ACKNOWLEDGEMENT

This work was supported by the grant No. EP 0960986668 from the National Agency for Agricultural Research of Czech Republic nad the Research intention No. J19/98:223300005 from Ministry of Education, Youth and Sport of Czech Republic.

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NOVO Nordisk, DK, Application sheet.

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IMPROVEMENT OF OAT BY GENETIC ENGINEERING

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1 INTRODUCTION

In Finland the annual oat (*Avena sativa* L.) production of about 1.3 million tons ranks second only behind barley. Finland is one of the major oat producers in the world and a considerable part of the harvest is exported. The Finnish share of the worldwide oat trade is approximated to be 20%. Traditionally, the main use of oats has been for animal feed but in recent years reports of the beneficial nutritional and physiological effects of oat products have increased interest in oats as an important food source.

The Finnish oat cultivars are generally well adapted to the growth conditions in Finland (e.g. humid, cool climate). In order to improve the Finnish oat cultivars better to meet the requirements of the food industry, modern biotechnical methods are being used. Our first aim is to develop oat varieties with resistance to BYDV (barley yellow dwarf virus) through gene transfer.

2 MATERIALS AND METHODS

Six Finnish oat cultivars were used. The cultivars Kolbu, Rhiannon, GP-1 and *Avena sterilis* were used as controls (see Table 1). Cell cultures were started from mature embryos of all of these cultivars. From two Finnish cultivars leaf bases were also used. The induction of embryogenic cell cultures from mature embryos of oat was performed according to Somers et al. (1992). The leaf base culture method was a modification of the method of Gless et al. (1998). Gene transfer was accomplished using particle bombardment.

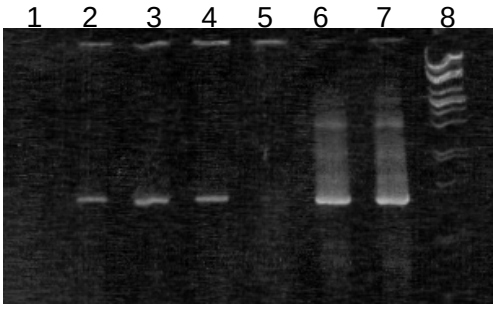
3 RESULTS

Callus was obtained from all the cultivars used. Out of the ten cultivars tested, all regenerated into green plants in the culture system used. The marker gene *nptII* was successfully transferred into four cultivars. Gene transfer experiments with the BYDV resistance gene have been commenced and the first transformants from these experiments have been regenerated and rooted on selection medium and transferred in soil in the greenhouse. The presence of the selection marker *bar* has been confirmed with PCR.

Table 1. Tissue culture responses of different oat cultivars.

Cultivar	Mature embryos		Leaf bases		Gene transfer
	callus	regeneration	callus	regeneration	(*nptII* or *bar*)
Aarre	yes	yes	-	-	-
Aslak	yes	yes	yes	yes	yes
Katri	yes	yes	-	-	yes
Lisbeth	yes	yes	-	-	-
Puhti	yes	yes	-	-	-
Veli	yes	yes	yes	yes	yes
Kolbu	yes	yes	-	-	yes
Rhiannon	yes	yes	-	-	-
GP-1	yes	yes	-	-	yes
Avena sterilis	yes	(yes)	-	-	-

- = not tried



Lane	
1	reagent blank
2,3,4	transgenic oats
5	control oats
6	positive control (BYDV)
7	positive control (pAHC25)
10	molecular size standard

Figure 1. PCR analysis of the presence of bar gene in oats transformed with the BYDV plasmid.

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Published by

Vuorimiehentie 5, P.O.Box 2000, FIN-02044 VTT, Finland
Phone internat. +358 9 4561
Fax +358 9 456 4374

Series title, number and report code of publication

VTT Symposium 207
VTT–SYMP–207

Author(s)
Simoinen, Taina & Tenkanen, Maija (eds.)

Title

2nd European Symposium on Enzymes in Grain Processing ESEPG-2

Abstract

Cereal grains are the core of human nutrition, and new products are being developed both in traditional product ranges in the bakery, rice and brewing industries and in the manufacture of new ingredients, such as snack foods, pasta and breakfast cereals. In addition, grains are a basal animal feed ingredient. In grain processing, endogenous and added enzymes have always played a decisive role in determining final product quality. For example, the germination of cereal grains, the conversion of malt into wort and beer, and the baking of bread are all mediated through enzymatic reactions. Enzymes can also be used to improve food quality to offer consumers palatable, safe and wholesome foods, as well as to improve the efficiency of food processing. The enzymes used nowadays mainly act on starch, glutein and cell-wall components. Recently other enzymes, such as lipases and oxidases, have been studied in connection with grain processing. New and better enzymes are increasingly becoming commercially available. The wider application of enzymes in food processing is anticipated to become one of the key food industry trends over the next 10 years.

This symposium proceedings book includes most of the papers presented in the 2nd European Symposium on Enzymes in Grain Processing (ESEGP-2) on December 8 - 10, 1999 in Helsinki, Finland. The book contains general articles giving excellent overviews as well as more detailed scientific reports on the latest research. It covers present know-how from the carbohydrate composition and structure of grains and endogenous enzyme inhibitors to action mechanisms of endogenous and microbial enzymes, as well as the state of the art of using enzymes in grain processing. We hope that the contents of this book would be helpful for food scientists, nutritionists, plant breeders and biotechnologists as well as business and marketing experts working with grains or enzymes.

Keywords
enzymes, grains, cereals, baking, brewing, hydrolases, oxidases, esterases, proteinases, inhibitors, food processing

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ISBN
951–38–5706–9 (soft back ed.)
951–38–5707–7 (URL: http://www.inf.vtt.fi/pdf/)

Project number
B9SU00011

Date	Language	Pages	Price
December 2000	English	339 p.	G

Name of project
QLK1-CT1999-30054

Commissioned by
Technical Research Centre of Finland (VTT),
European Union (EU)

Series title and ISSN
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1455–0873 (URL: http://www.inf.vtt.fi/pdf/)

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