Food Research International 42 (2009) 963–975

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

The role of corn starch, amaranth flour, pea isolate, and *Psyllium* flour on the rheological properties and the ultrastructure of gluten-free doughs

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ARTICLE INFO

Article history:
Received 9 January 2009
Accepted 22 April 2009

Keywords:
Gluten-free
Dough
Amaranth
Pea isolate
Psyllium
Rheology
CLSM
SEM

ABSTRACT

The removal of gluten from bakery products, in order to produce foods (mainly based on gluten-free cereal flours and starch) for people with celiac disease, impairs dough's capacity to properly develop during leavening and baking. The main aim of this research was to produce and evaluate some experimental gluten-free (GF) doughs containing different levels of corn starch, amaranth flour (to enhance the nutritional benefits), pea isolate (to increase the protein content) and *Psyllium* fiber (as thickening agent and fiber source) in order to study the influence of the different ingredients on the rheological properties and on the ultrastructure of the doughs. *Psyllium* fiber generally enhanced the physical properties of the doughs, due to the film-like structure that it was able to form, and the most complex among the experimental formulations looked promising in terms of final bread technological and nutritional quality even when compared to two different commercial GF mixtures.

1. Introduction

Gluten is the structure-forming complex in wheat, responsible for the viscoelastic properties needed to produce good quality baked products. Interactions of gliadins and glutenins through covalent and non-covalent bonds to form gluten complexes result in viscoelastic dough that has the ability to withstand stresses applied during mixing and to retain gas during fermentation and baking, producing a light baked product (Lindsay & Skerritt, 1999). Unfortunately, this complex can be harmful for people suffering from celiac disease (CD) or from other allergic reactions or intolerances to gluten consumption. CD, in particular, is a chronic malabsorption disorder of the small intestine caused by exposure to gluten in the genetically predisposed individual (Laurin, Wolving, & Falth-Magnusson, 2002): it is characterized by a strong immune response to certain amino acid sequences found in the protein fractions of wheat, barley, and rye (Fasano & Catassi, 2001; Thompson, 2001). The only effective treatment for celiac people is a strict adherence to a gluten-free diet throughout their lifetime.

The replacement of gluten in bakery products is a major technological challenge, as it is the essential structure-building protein. Its removal impairs dough's capacity to properly develop during kneading, leavening and baking. The absence of gluten often results in a liquid batter rather than a dough and can result in breads with a crumbling texture, poor color and other post-baking quality defects (Gallagher, Gormley, & Arendt, 2004). Thus, substances that imitate the viscoelastic properties of gluten, in order to provide structure and retain gas, are always required. Recently, there has been an increasing interest in gluten-free (GF) breads, whose formulations mainly involve the incorporation of starches of different origin, dairy proteins, other non-gluten proteins, gums, hydrocolloids, and their combinations, into a GF flour base (mostly rice and corn flour) that could simulate the viscoelastic properties of gluten and could result in maintaining structure, mouthfeel, acceptability and shelf-life of the finished products. However, currently, many GF breads available on the market are of low technological and nutritional quality, particularly when compared to their wheat counterparts, exhibit a dry crumb and have poor mouthfeel and flavor.

GF products are frequently produced with the addition of various proteins to a starchy base, to increase their nutritional value. The incorporation of dairy proteins has long been established in the baking industry, but legumes can also be a good supplement for cereal-based foods since they increase the protein content

Abbreviations: ANOVA, analysis of variance; BU, brabender units; CD, celiac disease; CLSM, confocal laser scanning microscopy; D, dough; FITC, fluorescein isothiocyanate; FWA, farinographic water absorption; G', storage modulus or elastic modulus; G'', loss modulus or viscous modulus; $|G^*|$, complex shear modulus; GF, gluten-free; HPMC, hydroxy-propyl-methyl-cellulose; LSD, least significant difference; LVE, region of linear viscoelasticity; SEM, scanning electron microscopy; tan δ, loss tangent or damping factor (DF).

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doi:10.1016/j.foodres.2009.04.017

and complement the nutritional value of cereal proteins. Cereals are deficient in lysine, one of the essential amino acids for the human diet, while legumes have a high level of this amino acid; simultaneously, cereal proteins are able to complement legume proteins in the essential amino acid methionine (Iqbal, Khalil, Ateeq, & Khan, 2006). Although the most-used legume protein is the soybean protein, due to its valuable functional properties (Ranhorta, Loewe, & Puyat, 1975; Ribottan et al., 2004), pea proteins can also be successfully used in bakery products, obtaining an enrichment in proteins and improving biological value of these products (Tömsközi, Lásztity, Haraszi, & Baticz, 2001).

Pseudocereals such as buckwheat and amaranth can also be useful for the above purpose. The protein content of amaranth (*Amaranthus* spp.) (11.7–18.4%) is generally higher than that of wheat (Berghofer & Schoenlechner, 2002, chap. 7) and contains acceptable levels of essential aminoacids (particularly lysine, tryptophan, and methionine), which are found in low concentrations in cereals and leguminous grains of common usage; structural characteristics of these proteins influence their functional properties (Avanza, Puppo, & Añón, 2005). The fat content of amaranth is also interesting and it is characterized by a high amount of unsaturated fatty acids, with a very high level of linoleic acid (Berghofer & Schoenlechner, 2002, chap. 7; Lucisano, Mariotti, Pagani, & Caramanico, 2006). Starch comprises the main component of the carbohydrates and significant amounts of calcium, iron, potassium, phosphorous, vitamins, and dietary fiber are present.

Gums and hydrocolloids are essential ingredients in GF bread formulations for improving the texture and the appearance of the final products. Due to their structure forming properties, their addition assures higher dough consistency, improved gas retaining capacity and longer shelf-life. In this respect, it has been proved that the most effective gum and hydrocolloid compounds are hydroxypropyl-methyl cellulose (HPMC), locust bean gum, guar gum, carrageenan, and xanthan gum (Christianson, Gardner, Warner, Boundy, & Inglett, 1974; Gallagher et al., 2004; Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007). The association of HPMC (2 g/100 g rice flour) with *Psyllium* fiber (1 g/100 g rice flour) in the formulation of GF bread gave good results in terms of loaf volume due to the formation of a weak gel network, capable of trapping CO_2 by virtue of the gelling and water-absorbing abilities of *Psyllium* fiber, and of the heat-induced gelation of HPMC (Haque & Morris, 1994). Higher additions (5%, 7.5%, 10%) of *Psyllium* to a GF formulation determined an increased fiber content of the bread (190–450% higher than that of the control bread) and a softer crumb during a 4-day storage period. *Psyllium*, besides being an excellent source of natural soluble fiber, has been widely recognized for its cholesterol-lowering effect and insulin sensitivity improvement capacity (You, Perret, Parker, & Allen, 2003). The enrichment of GF baked products with dietary fiber has proved to be necessary, since celiac patients generally have a low intake of fiber attributed to their GF diet (Thompson, 2000).

The main aim of this research was to produce and evaluate some experimental GF doughs containing different levels of corn starch, amaranth flour (to enhance the nutritional benefits), pea isolate (to increase the protein content) and *Psyllium* flour (as thickening agent and fiber source), in order to: (1) study the influence of the different ingredients on the rheological properties and on the ultrastructure of the dough; and (2) develop healthier breads that contain higher protein and dietary fiber levels. For these purposes, the experimental GF doughs were compared to those obtained from two commercial GF bread mixes. Moreover, rheometry and microscopy approaches (limited up till now in studies of GF dough properties), were both used, with the microscopic images generated by scanning electron microscopy and confocal laser scanning microscopy, to develop a fundamental understanding of the ultrastructure of GF doughs and constituents as a good basis for further improvement.

2. Materials and methods

2.1. Raw materials

2.1.1. Origin

The raw materials used to produce the GF formulations were of commercial origin: the corn starch was supplied by Molino Quaglia SpA (Vighizzolo D'Este, PD, Italy), the *Psyllium* fiber by Giulio Gross Srl (Trezzano sul Naviglio, MI, Italy) and the pea isolate (Pisane F9) was provided by Prodotti Gianni SpA (Milan, Italy). Amaranth (*Amaranthus hypochondriacus*) was furnished as seeds by *Cooperativa Quali* (Tehuacàn, Mexico). Amaranth seeds were ground to flour at DiSTAM, just before use, by a laboratory mill (Labormill 4RB; BONA srl, Monza, MI, Italy), and three milling fractions (coarse, medium, fine) were obtained. On the basis of previous studies, a mixture of 30% fine fraction and 70% medium fraction was used for this research. This blend was characterized by the following particle size distribution: 48% $\geqslant$ 200 μm, 125 μm $\leqslant$ 36% < 200 μm, 16% < 125 μm.

2.1.2. Chemical characterization

The raw materials were characterized for their moisture (AACCN°44–15A; AACC, 1983), protein (AACCN°46–11; AACC, 1983), lipid (ICCN°136; ICC, 1999), fiber (Prosky, Asp, Schweizer, DeVries, & Furda, 1988), total starch and damaged starch ("Total Starch Assay Kit", "Starch Damage Assay Kit"; Megazyme International Ireland Ltd., Bray Business Park, Bray, Co., Wicklow, Ireland) contents. Data obtained from these characterizations are listed in Table 1 and are the average of at least three determinations.

2.1.3. Scanning electron microscopy

The ultrastructure of corn starch, amaranth flour, pea isolate, and *Psyllium* fiber was observed by means of scanning electron microscopy (SEM). Powders were mounted on aluminum stubs, sputter-coated with gold, and their ultrastructures were imaged in a LEO438 VP SEM (LEO Electron Microscopy Ltd., Cambridge, UK) under high vacuum conditions (10^{-4} Pa) at an accelerating voltage of 20 kV.

2.2. Gluten-free formulations

2.2.1. Experimental GF bread formulations

Corn starch, amaranth flour, pea isolate, and *Psyllium* fiber were used to produce six experimental GF formulations (Table 2). Two

Table 1
Composition of the raw materials involved in the experimental GF formulations.

	Moisture (g/100 g)	Protein (g/100 g db)	Lipids (g/100 g db)	Total starch (g/100 g db)	Damaged starch (g/100 g db)	Fiber (g/100 g db)
Corn starch	13.70 ± 0.01	–	0.69 ± 0.02	>95[a]	1.91 ± 0.06	–
Amaranth flour	12.01 ± 0.06	15.78 ± 0.05	8.16 ± 0.06	55.04 ± 0.70	9.31 ± 0.11	8
Pea isolate	5.93 ± 0.04	86.99 ± 0.01	8.55 ± 0.03	0.38 ± 0.01	–	–
Psyllium fiber	9.66 ± 0.01	2.14 ± 0.23	0.70 ± 0.05	–	–	>95[a]

[a] As reported on the product label.

levels were used for amaranth flour (0% or 40%), pea isolate (1% or 6%) and *Psyllium* fiber (0% or 2%), while corn starch levels were varied according to the amounts of the other ingredients. The relative proportions of different dough ingredients were based on previous trials performed with the same raw materials.

2.2.2. Commercial GF bread mixtures

Two commercial GF bread mixtures available on the market (coded C1 and C2) were included in the study, in order to compare their properties with those of the experimental formulations. Their ingredients, as declared on their respective labels, are listed in Table 2. The ultrastructure of these powders was evaluated by SEM, according to the conditions reported in Section 2.1.3.

2.3. Gluten-free dough

2.3.1. GF dough production

A sample of each mixture (300 g) was placed in the Farinograph mixer (OHgG, Duisburg, Germany) and the amount of water required to yield a dough consistency of 200 BU (brabender units) after 15 min mixing (FWA) was added. The choice of the 200 BU value as an optimal consistency for GF dough handling derived from previous studies carried out in our laboratories. At the end of the 15 min mixing, the GF dough was divided into aliquots (with care taken to not disturb or destroy the inherent structure developed during the mixing phase), and the moisture content, the rheological properties, and the ultrastructure of these samples were evaluated.

2.3.2. GF dough moisture

The moisture content of each dough sample was evaluated by determining the loss in weight of an exactly weighed 5 g sample after 1.5 h at 130 °C. The results are the average of at least three replicates.

2.3.3. GF dough rheological properties

The rheological properties of the different GF dough formulations were studied by dynamic oscillatory measurements performed on a Physica MCR100 rheometer (PHYSICA Messtechnic GmbH, Ostfildern, Germany). A parallel plate geometry (25 mm diameter, 2 mm gap) was used, with corrugated plates to prevent dough slippage. The temperature was regulated at 30 °C by a circulating bath and a controlled Peltier system. After loading, the excess dough was trimmed and a thin layer of paraffin oil was applied to the edge of the exposed sample to prevent moisture loss during measurements. The dough was allowed to rest for 30 min in order to equilibrate stresses, before starting the test. The following tests were performed on each sample: (1) *strain sweep test*, in the range of 0.01–100%, at 1 Hz frequency; (2) *frequency sweep test*, in the range of 1–20 Hz, at 0.05% strain; (3) *temperature sweep test*, in the range of 30–90 °C, at 0.05% strain and 1 Hz, using a gradient equal to 4 °C/min. Each test was performed at least three times. Data were analyzed using US200/32 v.2.50 rheometer software (PHYSICA Messtechnic GmbH, Ostfildern, Germany).

2.3.4. GF dough ultrastructure

A confocal laser scanning microscope (CLSM, LSM5 Pascal, Zeiss, Thornwood, NY) was used to observe the ultrastructure of GF doughs. First, to determine the appropriate conditions for the CLSM dough observations, the individual ingredients of the experimental recipes and the commercial mixtures C1 and C2 were checked for their own auto-fluorescence. The absence of endogenous autofluorescence was determined. As regards GF dough, the samples were frozen immediately after their production and kept at −80 °C before and after cryo-section. The cryo-sectioning was used to remove dough surface irregularities, producing a very flat surface, and to scrape off the outer layers of the dough, making the inner part of each sample accessible for observation. After different trials, a 30 μm thick dough section was judged as reasonable for our purposes. Before observations, dough sections on the slide were thawed at room temperature, stained with FITC solution (0.05% w/v in 0.5 mM NaOH, pH 8.0) to reveal protein, and dried at room temperature in a dark environment due to light sensitivity of FITC. In order to simultaneously detect starch and protein distribution (Lee, Ng, Whallon, & Steffe, 2002), non-confocal transmitted polarized light and confocal fluorescence images were collected with the same instrument from a single area of each dough sample: simple polarized light allowed the imaging of starch granules (as distinct from air bubbles or lipids), while fluorescence allowed the imaging of protein matrix. The overlay of the two images collected from the same area gave the final image of the GF dough network. Water (10×, 20×) and oil immersion (⩾40×) objectives were used. For both transmitted and fluorescence images, the 488 line of a dual-line argon ion laser was used. A band pass 505–600 barrier filter was selected for the detection of FITC. At least two replicates were made for each dough sample.

2.4. Statistical analysis

Analysis of variance (ANOVA) and significant correlations were performed on the data adopting the least significant difference (LSD) and Pearson correlation analysis procedure, respectively, to test the statistical significance of the differences between means at a $P < 0.05$ confidence level. Data were processed using *Statgraphics®Plus* v. 5.01 (StatPoint, Inc., Herndon, Virginia, USA).

3. Results and discussion

3.1. GF dough farinographic water absorption, moisture and appearance

In preliminary studies on GF mixtures carried out at our laboratories, a farinographic consistency equal to 200BU ± 20 was evidenced as the adequate condition to properly form a GF dough able to sustain further transformations. Fig. 1 shows the amount of water required by the six experimental formulations and the two commercial GF mixtures to reach the 200 BU consistency after 15 min mixing. Generally, the higher the corn starch and the pea isolate content (see sample 1 vs. 2 and 3), the higher the FWA of the mixture. The requirement for water increased substantially, as expected, when the *Psyllium* fiber was present in the formulation (1 vs. 4), due to the hydrophilic nature of this biopolymer (You et al., 2003). The substitution of 2% *Psyllium* fiber for 2% corn starch caused a *"FWA percentage increase"* {calculated as [(FWA sample "A" – FWA sample "B")∗100/FWA sample "A"]} equal to 34% for sample 1 vs. sample 4, 21% for 2 vs. 5, and 21% for 3 vs. 6, indicating that the increase was even more evident when no amaranth flour or pea isolate were present in the dough recipe. The presence of a higher amount of pea isolate in the formulation (i.e., 2 vs. 3, 5 vs. 6) caused a *FWA percentage increase* as well (6.2% and 5.9%, respectively), but the effect was much smaller than the one originated by *Psyllium* fiber. On the contrary, the substitution of 40% amaranth flour for 40% corn starch resulted in decreases in the FWA (i.e., 1 vs. 3, 4 vs. 6), especially when no *Psyllium* fiber was present in the mixture (1 vs. 3). This phenomenon could be due to the larger particle size (hence lower surface area) of the amaranth flour in comparison to that of corn starch, as SEM images demonstrated (*figures shown later*). The FWA values of C1 and C2 were in the range of those of the experimental GF mixtures.

The moisture contents of the different GF doughs are also shown in Fig. 1. Although the FWA range of values was widespread

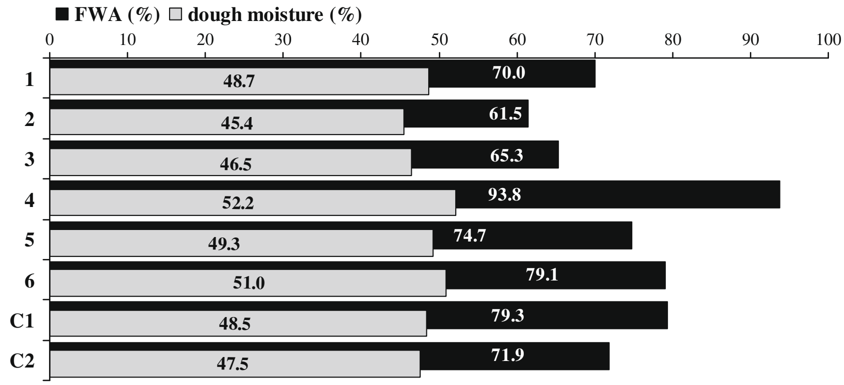


Fig. 1. Farinographic water absorption (FWA, %) of the experimental (1–6) and commercial (C1 and C2) GF mixtures (see Table 2 for details) required to reach a 200BU dough consistency after 15 min mixing. On the same graph, the moisture (%) of the final doughs is reported.

Table 2
Experimental GF formulations and commercial GF mixtures.

	Experimental GF bread formulations			
	Corn starch (g/100 g)	Amaranth flour (g/100 g)	Pea isolate (g/100 g)	*Psyllium* fiber (g/100 g)
1	94	0	6	0
2	59	40	1	0
3	54	40	6	0
4	92	0	6	2
5	57	40	1	2
6	52	40	6	2
Commercial GF bread mixtures				
C1	Ingredients: corn starch, sugar, chicory fibers (inulin), fiber of *Psyllium* seeds, thickener (guar gum), corn maltodextrin			
C2	Ingredients: rice flour, potato starch, sugar, thickener E464 (hydroxypropylmethylcellulose, HPMC) and locust bean gum, salt, emulsifier E471 (mono- and diglycerides of fatty acids)			

(from 61.5% up to 93.8%), the corresponding moisture contents of the doughs ranged narrowly between 45.4% and 52.2%. The ANOVA, however, highlighted statistically significant differences ($P < 0.05$) among all these samples. A high correlation ($r = 0.918$) was found between the FWA of the mixtures and the moisture content of the corresponding dough: as expected, the higher the FWA, the higher the dough moisture. The 2% *Psyllium* fiber in the formulation had an undoubted role in these properties, by virtue of its gelling and water-absorbing abilities.

Although the different GF doughs (D) had the same final farinographic consistency after 15 min mixing (200BU), their appearances (Fig. 2) were very different in relation to the main constituents of the mixtures. The worst product in terms of handling and workability was D1, made mainly of corn starch (94%). The presence of 2% *Psyllium* fiber in place of 2% corn starch (D4) was enough to determine big changes in both structure and workability of the resulting dough, indicating that *Psyllium* fiber, despite the higher amount of water required to form a dough, could act as an improver of the cohesion of starchy matrix. When amaranth was added (D3), the color of the sample also changed.

3.2. GF dough rheological properties

During oscillatory measurements samples are subjected to harmonically varying stress or strain and results are usually sensitive to chemical composition and physical structure. Dynamic tests are used to calculate the frequency-dependent viscoelastic moduli G' (storage modulus or elastic modulus) and G'' (loss modulus or viscous modulus), which are respectively the real and imaginary parts of the complex shear modulus $|G^*|$ ($G' + iG''$). The loss tangent or damping factor (DF) is defined as: $\tan\delta = G''/G'$. It is challenging to measure the properties of a dough system, since with time it changes continuously due to physical and chemical factors (*e.g.,* evolution of the water/components interactions, enzymatic reactions, relaxation of the stresses induced during mixing) (Létang, Piau, & Verdier, 1999). Consequently, between the end of mixing

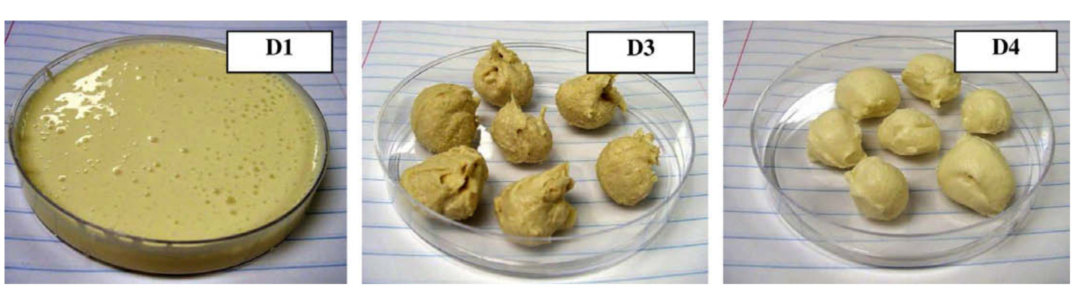


Fig. 2. Appearance of some experimental GF doughs (D), all with the same 200BU final farinographic consistency after 15 min mixing. D4 vs. D1: 2% *Psyllium* fiber in place of 2% corn starch; D3 vs. D1: 40% amaranth flour in place of 40% corn starch.

and the beginning of the rheometrical measurement, each stage has been timed carefully.

3.2.1. Strain sweep test

The evaluation of the region of linear viscoelasticity (LVE) of a sample is a fundamental step. When materials are tested in their linear range, their characteristics do not depend on the magnitude of the stress, the magnitude of the deforming strain, or the rate of application of the strain: if linear, an applied stress will produce a proportional strain response (Steffe, 1996). The strain sweep curves of the experimental and commercial GF doughs are shown in Fig. 3. The limits of the region of linear viscoelasticity were identified by the "*LVE range analysis method*" (US200/32 v. 2.05 Rheometer Software) and reported in Table 3. No relationships between the different water levels in the GF doughs and the values of dynamic moduli were found. Experiments have been conducted on wheat dough systems in order to see the influence of water content (Hibberd, 1970; Navickis, Anderson, Bagley, & Jasberg, 1982) and it was observed that high water contents gave lower dynamic moduli. In our studies on GF doughs, the absolute values of the elastic modulus G' and the loss modulus G'' (Fig. 3A, and B) were actually not dependent on the FWA of the mixtures nor on the moisture content of the corresponding dough.

Looking at the strain sweep curves, generally both G' and G'' decreased after a certain limit. GF dough G' was found to decrease more than G'' and, as a result, tan δ (G''/G') increased with the strain amplitude. The presence of 2% *Psyllium* fiber in place of 2% corn starch (1 vs. 4, 2 vs. 5, 3 vs. 6) seemed to have no effect on the G' LVE of the corresponding dough: the ANOVA did not reveal a statistically significant difference ($P < 0.05$) among the above mentioned doughs (Table 3). The sample based mainly on corn starch (D1) exhibited rheological behavior very different from the others, but its tan δ showed a trend similar to the one of D4 and C1. The two commercial dough systems exhibited different behaviors: C1 was quite similar to the majority of the experimental GF doughs, while C2 had similar elastic and viscous values and, as a result, its tan δ diverged from those of the experimental GF.

The length of the LVE of G' can be used as a measurement of the stability of a sample structure, since structural properties are usually best related to elasticity. A sample that has a long LVE region is indicative of a well-dispersed and stable system. When the *Psyl-*

Table 3
Strain sweep tests[A] of experimental and commercial GF bread doughs.[B]

GF dough	G' LVE (γ, %)	G'' LVE (γ, %)	$\|G^*\|$ LVE (γ, %)
1	0.065 a ± 0.002	0.138 a ± 0.002	0.065 a ± 0.001
2	0.162 b ± 0.021	0.147 a ± 0.016	0.119 b ± 0.007
3	0.346 c ± 0.030	0.229 de ± 0.027	0.249 d ± 0.011
4	0.105 a ± 0.001	0.255 e ± 0.008	0.099 b ± 0.001
5	0.181 b ± 0.016	0.200 cd ± 0.019	0.147 c ± 0.009
6	0.341 c ± 0.036	0.298 f ± 0.028	0.275 d ± 0.025
C1	0.076 a ± 0.011	0.153 ab ± 0.011	0.071 a ± 0.010
C2	0.184 b ± 0.015	0.189 bc ± 0.004	0.124 bc ± 0.006

Values followed by the same letter in the same column are not significantly different, $P < 0.05$.
[A] LVE = region of linear viscoelasticity, G' = storage modulus or elastic modulus, G'' = loss modulus or viscous modulus, $|G^*|$ = complex modulus.
[B] See Table 2 for formulations and ingredients.

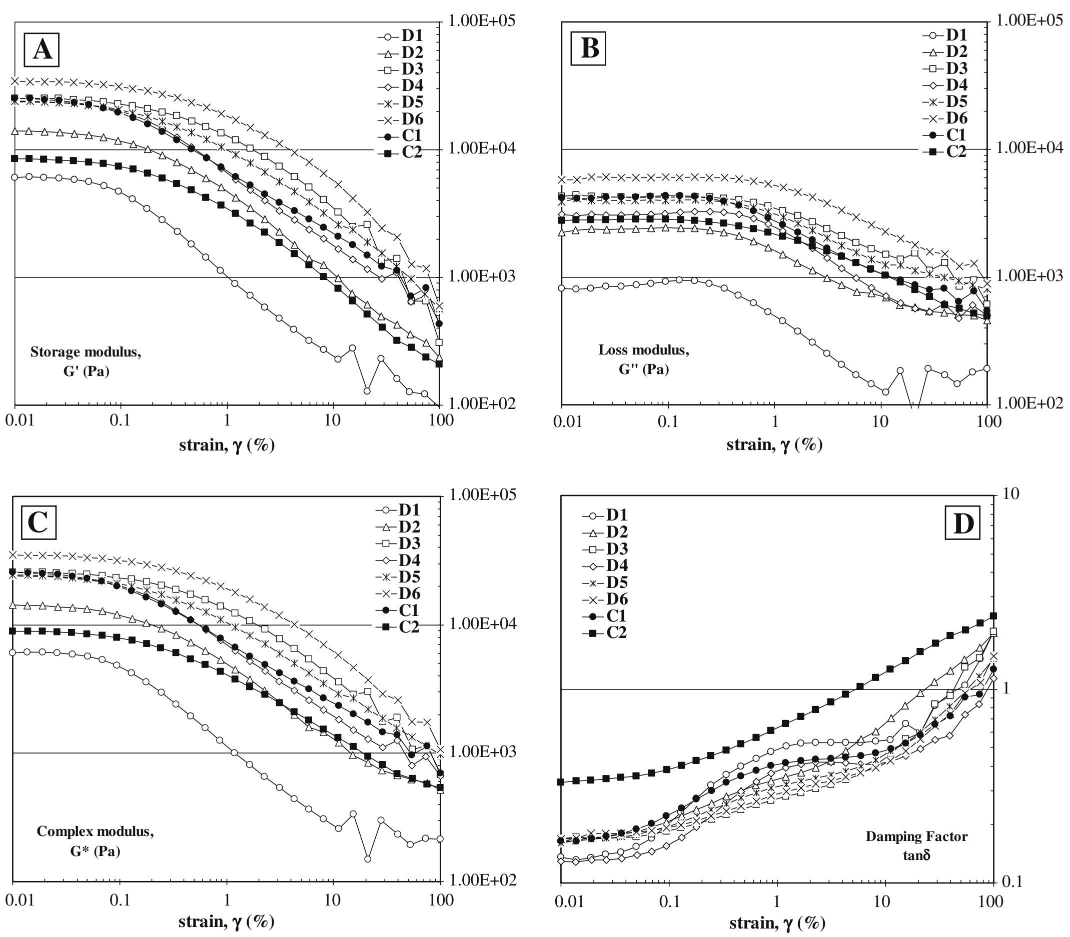


Fig. 3. Strain sweep curves (1 Hz, 30 °C) of the experimental and commercial GF dough samples (*curves are the mean of at least two replicates*).

lium fiber was present, the LVE region was longer (D4 vs. D1, D5 vs. D2, D6 vs. D3), indicating more stable systems. Generally, a drop in G' started to occur above 0.05% strain and became larger above 1% strain, suggesting the progressive breakdown of the GF dough structure beyond these deformation levels. The use of 0.05% strain level for the subsequent frequency and temperature sweep tests was thus proposed.

3.2.2. Frequency sweep test

The frequency sweep shows how the viscous and elastic behavior of the material changes with the rate of application of strain or stress, while the amplitude of the signal is held constant (Steffe, 1996). The frequency sweep curves of the GF doughs are shown in Fig. 4. Although all the dough samples had the same final farinographic consistency (200BU), it is evident how much they differed in terms of fundamental rheological properties. No relationships were found between the different water levels in the GF doughs and the values of their respective dynamic moduli.

For all the formulations in the whole range of frequencies, G' was greater than G'' suggesting a solid elastic-like behavior of the GF doughs. Similar behaviors were reported by Lazaridou et al. (2007). G' was also strictly frequency-dependent and increased with increasing frequency, while for G'' this effect was evident only at higher frequencies (with the exception of C2). Because the difference between G' and G'' was high, tan δ was low. Sample C2, which mixed to a very stable dough (based on the farinograph), gave lower values for both G' and G'' and a lower difference between the two; tan δ, as a consequence, was the highest, thus showing a good balance between the two moduli. On the contrary, high G' and low G'' generally reflect a more rigid and stiff material whose tan δ is small.

In general, the presence of amaranth in the recipe determined higher values of G' and G'' (Fig. 4), whether the *Psyllium* fiber was absent (D1 vs. D3) or present (D4 vs. D6). The incorporation of amaranth into existing food formulations in order to modify their functional properties and their nutritional quality, as well as to create entirely new-products, is a great challenge. D5 and D6, characterized by the most complete recipe and different only in the pea isolate level, had the highest G' values. They also possessed very similar mechanical spectra, indicating that a higher presence of protein derived from pea isolate did not influence the rheological properties of the dough when *Psyllium* fiber was present. However, the effect of pea isolate was marked in the absence of *Psyllium* fiber (D2 vs. D3): the substitution of 5% corn starch in place of 5% pea isolate, in fact, made the dough more solid. In the recipe characterized only by starch and pea isolate (D1), the influence of the addition of *Psyllium* fiber (D4) was very evident at the lowest frequencies studied, while it tended to decrease when frequency increased; that, despite their clearly different appearance (Fig. 2D1 and D4). The commercial sample C2 showed a specific trend: its G' values were the lowest among the others, while its G'' values were similar to those of one group of samples at low frequencies and to those of another group of sample at high frequencies; moreover, C2 G' and G'' values were quite similar to each other, and so C2 tan δ values were the highest in the whole range of frequencies investigated. C2 is characterized by the presence of rice and potato starches, very different in size, shape and functional properties, and by the presence of HPMC and locust bean gum, frequently used for its thickening, stabilizing, gelling and emulsifying properties.

The results of the application (US200/32 v. 2.05 Rheometer Software) of an exponential equation (*Power Law Equation:* $y = a{\cdot}x^{b}$)

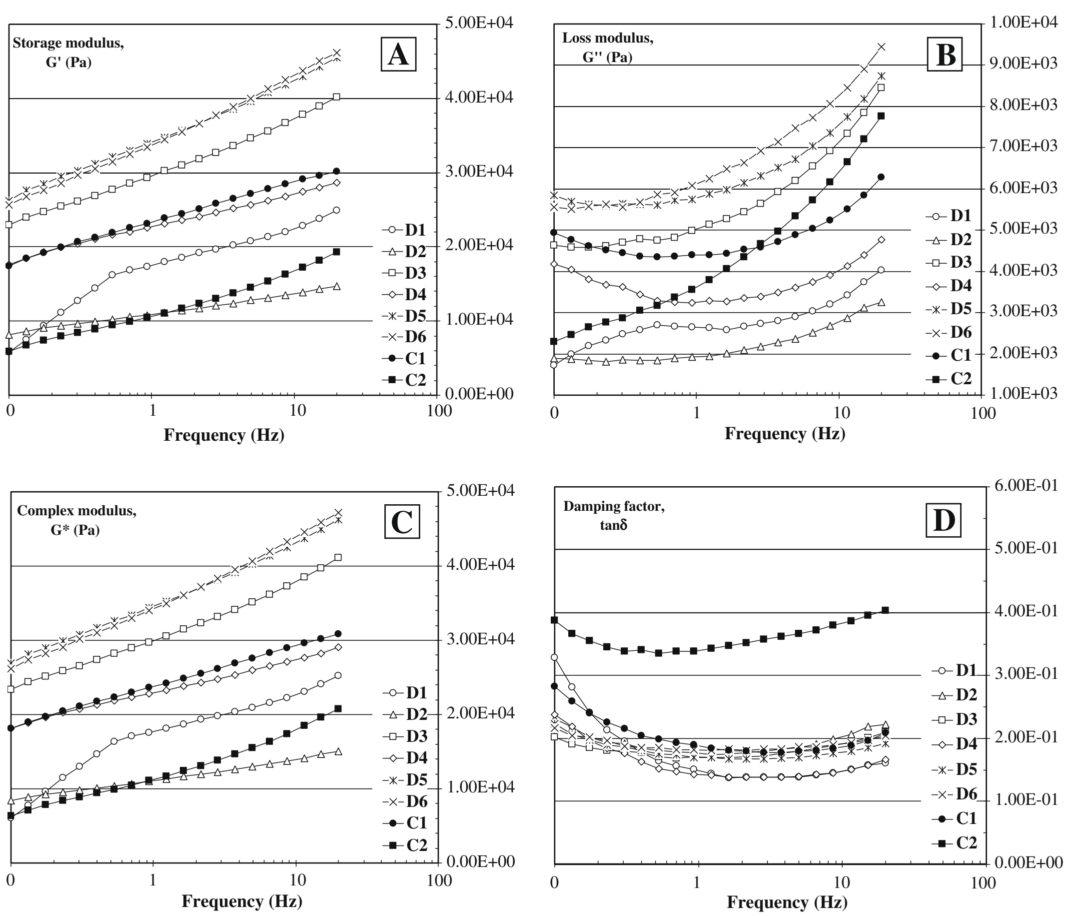
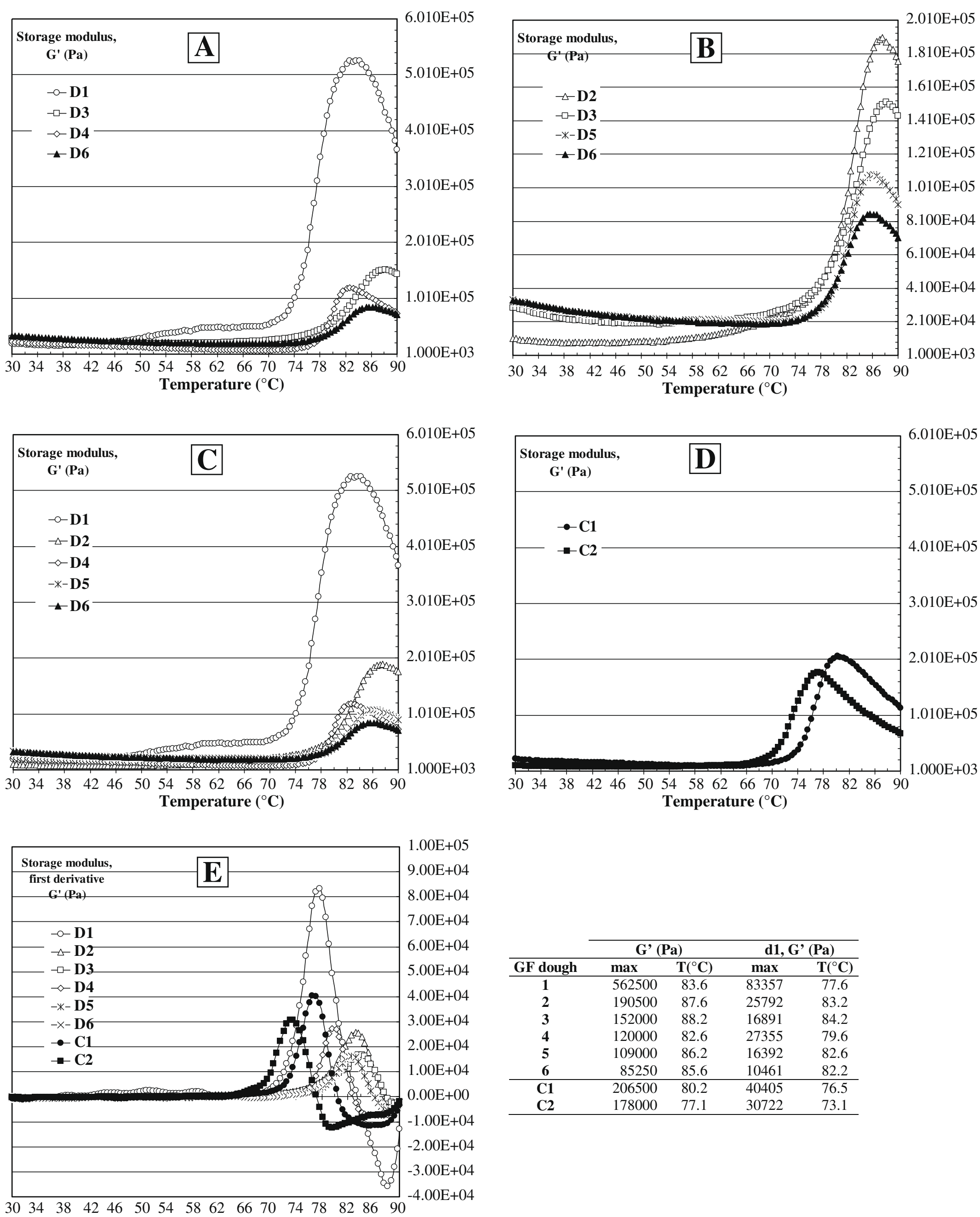


Fig. 4. Frequency sweep curves (0.05%γ, 30 °C) of the experimental and commercial GF dough samples (*curves are the mean of at least two replicates*).

Table 4
Application of the power law equation to the frequency sweep test[A] of experimental and commercial GF bread doughs.[B]

GF dough	G'		G''		$\|G^*\|$	
	a	b	a	b	a	b
1	14,588 b ± 1225	0.220 c ± 0.136	2578 a ± 572	0.108 a ± 0.077	14,861 a ± 1348	0.215 c ± 0.132
2	10,822 ab ± 89	0.104 ab ± 0.004	2087 a ± 122	0.104 bc ± 0.001	11,025 a ± 64	0.104 ab ± 0.003
3	29,469 d ± 845	0.102 ab ± 0.001	5342 de ± 169	0.112 a ± 0.004	29,953 c ± 862	0.103 ab ± 0.001
4	22,416 c ± 947	0.086 a ± 0.000	3647 b ± 265	0.020 a ± 0.006	22,729 b ± 981	0.084 a ± 0.001
5	33,871 e ± 3094	0.100 ab ± 0.006	6162 ef ± 431	0.075 abc ± 0.002	34,435 d ± 3120	0.099 a ± 0.005
6	33,578 e ± 1680	0.110 abc ± 0.000	6413 f ± 364	0.102 bc ± 0.001	34,188 d ± 1720	0.109 abc ± 0.001
C1	23,007 c ± 2204	0.100 ab ± 0.001	4714 cd ± 522	0.043 ab ± 0.003	23,504 b ± 2268	0.097 a ± 0.001
C2	10,478 a ± 1516	0.206 bc ± 0.013	3758 bc ± 720	0.226 d ± 0.008	11,137 a ± 1671	0.209 bc ± 0.011

Values followed by the same letter in the same column are not significantly different, $P < 0.05$.
[A] G'=storage modulus or elastic modulus, G'' = loss modulus or viscous modulus, $|G^*|$ = complex modulus.
[B] See Table 2 for formulations and ingredients.



GF dough	G' (Pa) max	G' (Pa) T(°C)	d1, G' (Pa) max	d1, G' (Pa) T(°C)
1	562500	83.6	83357	77.6
2	190500	87.6	25792	83.2
3	152000	88.2	16891	84.2
4	120000	82.6	27355	79.6
5	109000	86.2	16392	82.6
6	85250	85.6	10461	82.2
C1	206500	80.2	40405	76.5
C2	178000	77.1	30722	73.1

Fig. 5. Temperature sweep curves (0.05%γ, 4 °C/min) of the experimental and commercial GF dough samples; (E) represents the first derivatives (D1) of the curves (*curves are the mean of at least two replicates*).

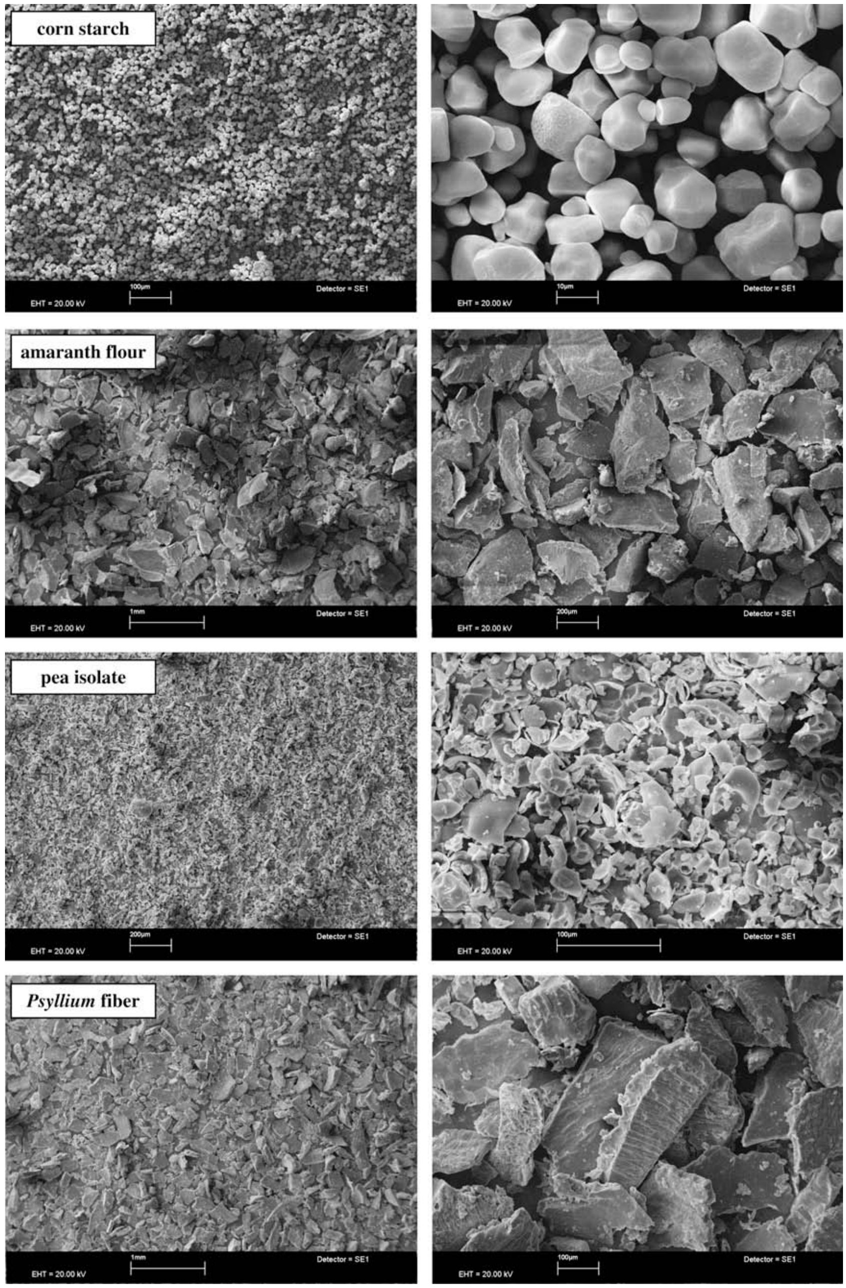


Fig. 6A. SEM images of the raw materials used in the experimental GF mixtures (images are at different magnifications to optimize views of raw material particles).

that fits very well ($r > 0.95$) all the frequency sweep curves are reported in Table 4. The used variables were: frequency (x) and G', G'' or G^* (y). "a" is a consistency index, and the exponent "b" is the slope of the curve in a log–log plot of (y) versus the frequency (x) and it is related to the strength and the nature of the gel. D5 and D6, characterized by the most complex recipes, had the highest G' consistency indices. The absence of *Psyllium* fiber in the mixture caused the collapse of G', especially when the lowest amount (1%) of pea isolate was present (D2 vs. D5). The lowest "*a*" value occurred for C2. With regard to "*b*" values, the higher the value of the exponent, the higher the dependence of the structure on strain: the less sensitive was D4; when *Psyllium* was not present (D4 vs. D2) the "*b*" values increased substantially, suggesting a higher dependence of the structure (mainly based on corn starch) on strain. C2 had a high "*b*" value, which could be associated with its higher resemblance to a viscous liquid with low elasticity, in comparison to the other samples.

The low deformation conditions used for the oscillatory measurements are often inappropriate to practical processing situations, because they are carried out at rates and conditions very different from those experienced by the dough during processing or baking. These low strain levels, which allow measurements but do not disturb or destroy inherent structure, are of great value in studying the influence and actions of ingredients because dynamic mechanical parameters are highly sensitive to changes in polymer type and concentration as well as water content (Lazaridou et al., 2007).

So many combinations of substitute materials could be used in attempts to replicate the fundamental role of gluten in GF products, that explanations for the phenomena taking place in GF doughs during a non-conventional baking process present a real challenge. Usually a bread-baking process is divided into the cold (mixing, proofing) and hot (baking) stages. Besides strain and frequency sweep tests, temperature sweep tests were performed to monitor modifications linked to the baking stage.

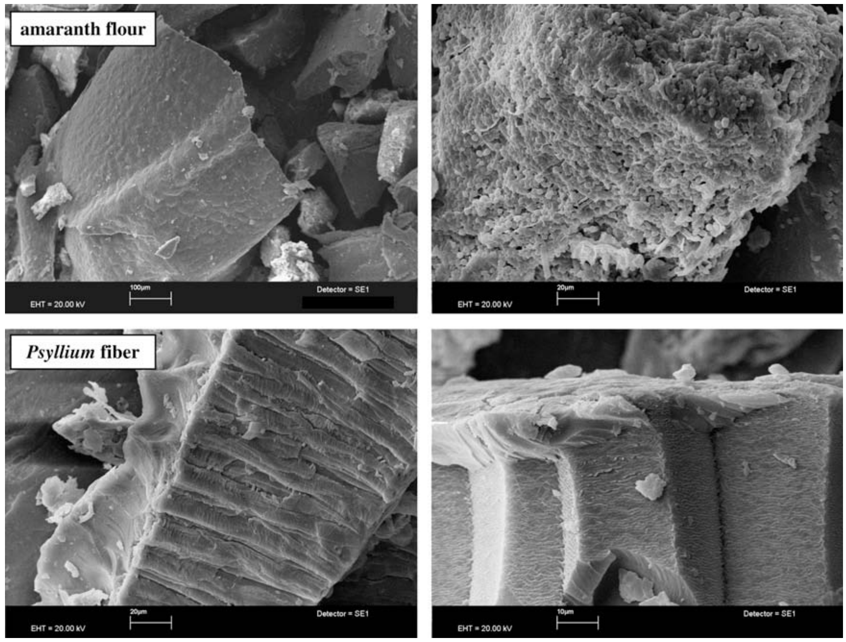


Fig. 6B. More detailed images of amaranth flour and *Psyllium* fiber.

3.2.3. Temperature sweep test

The temperature sweep curves (Fig. 5A–D) resemble the first stages of the amylographic test and they are a reflection of the biochemical and physical reactions that occur during baking. Obviously, starch gelatinisation does not take place under the same conditions in a slurry as it does in a dough, both because the water availability is altered and because pasting properties can be modified by the presence of other ingredients. In these temperature sweep tests, the water availability did not influence the values of G' and G'', but the moduli (G' in particular) were strongly influenced by the different ingredients composing the mixtures. D1 showed the highest increase in both G' and G'' values during the heating phase; in comparison, more complex recipes highlighted lower values of the moduli. In particular, the presence of amaranth (Fig. 5A) slowed the increases in G' and G'' during heating, both with or without *Psyllium* fiber in the dough (D1 vs. D3, D4 vs. D6, respectively). The higher amount of pea isolate (Fig. 5B) decreased the G' peak, too, both with or without *Psyllium* fiber in the dough (D2 vs. D3, D5 vs. D6, respectively); its influence was more evident on G' at temperatures lower than the starch gelatinization temperatures. In all cases studied, the substitution of 2% corn starch in place of 2% *Psyllium* fiber slowed the corn and amaranth starch gelatinization (Fig. 5C) and this may be due to *Psyllium* fiber's high competition for water. The temperature sweep curves of C1 and C2 doughs (Fig. 5D) differed from the others. Starch pasting properties are largely modified by hydrocolloid addition, although the extent of their effect depends upon their origin, chemical structure and concentration (Guarda, Rosell, Benedito, & Galotto, 2004). The hydrocolloid HPMC included in sample C2, for instance, is known to be a bread improver regarding its effect on fresh bread quality and its anti-staling properties (Guarda et al., 2004).

The table included in Fig. 5 highlights the maximum value reached by G' of each sample during the heating phase and the temperature at which it occurred; it also shows the maximum value of the first derivative of each G' curve and, again, the temperature at which it occurred. The first derivative was obtained applying the Savitzky–Golay first derivative algorithm (TableCurve™ 2D, Jandel Scientific Software, UK) to G' temperature sweep curves. As expected, doughs characterized by the higher starch contents (D1, D4, C1, and C2) exhibited an early onset of gelatinisation: the phenomenon was accelerated and reached the highest speed at temperatures between 73.1 and 79.6 °C; for all the other doughs the increase in G' was slowed and the maximum gelatinization speed was reached in the range 82.2–84.2 °C (Fig. 5E).

3.3. GF dough ultrastructure

3.3.1. Scanning electron microscopy (SEM)

Before evaluating the ultrastructure of the GF doughs, SEM observations of each of the components in the experimental formulations and of the commercial GF powders were carried out. Fig. 6A and Fig. 6B shows a selection of SEM images of the raw materials used to produce the experimental GF formulations, while Fig. 7 is related to the commercial GF mixtures. Corn starch is well known (Fig. 6A), however, images of amaranth flour, pea isolate, and *Psyllium* fiber (Fig. 6A and Fig. 6B) are not common in the literature. Microscopic information on the morphology of each ingredient thus obtained was to help in the interpretation of confocal laser scanning microscopy images, especially in the case of GF dough samples, for which limited information on ultrastructure is available in the literature. Fig. 6B, in particular, shows detailed images of amaranth flour and *Psyllium* fiber. At higher magnifications, the complex ultrastructure of these raw materials became more evident. Amaranth flour was characterized by the presence of many wide plates of teguments (width: even $\geqslant$600 μm) together with specific small starch granules (diameter: 0.5–2 μm) or agglomerates of starch granules (width: ~80 μm). *Psyllium* powder was formed of many particles with highly organized networks and elevated specific surfaces, that were presumed to enable high water absorption through capillarity. The more varied composition of the commercial GF mixture C2 (characterized, as reported on the label, by the presence of potato starch, rice starch, hydrocolloids and more), in comparison to that of C1 (characterized by less ingredients), was distinguished on SEM micrographs (Fig. 7).

3.3.2. Confocal laser scanning microscopy (CLSM)

Fig. 8A and Fig. 8B exhibits the overlay of the images obtained by simple polarized light and fluorescence to the same area: in red, the starch granules; in green, the protein matrix. The gluten matrix is the major determinant of the rheological characteristics of a wheat dough, such as elasticity, extensibility, resistance to stretch, mixing tolerance, and gas holding ability. Its replacement represents a big technological challenge, as it is an essential structure-building protein, contributing to the appearance and crumb structure of many baked products (Lazaridou et al., 2007). In contrast to that of in wheat doughs, a homogeneous and continuous matrix cannot be found in GF samples.

At low magnification, D1 (Fig. 8A), characterized by the highest content of starch, seemed to be more compact and continuous than the other samples (black holes are air bubbles incorporated into the dough during the mixing phase). Nevertheless, when higher magnifications were used, the single starch granules in D1 appeared separated, with only some of them covered by pea protein. Given this lack of ultrastructural organization, starch granules are free to swell and gelatinize because no specific network was formed. This loose structure could explain the quickest and the greatest increase in G' during temperature sweep tests on the high starch content dough samples. The weakest dough from a rheological point of view (D1), also had the weakest matrix in terms of compactness and continuity as visualized with CLSM.

When 2% *Psyllium* fiber was added to this formulation in place of 2% corn starch (Fig. 8A, D4), some of the starch granules appeared to be linked, forming groups of objects; thus this hydrocolloid seemed to provide more stability to the dough matrix, as already seen at the macroscopic level in Fig. 2. This effect was even more evident when D2 and D5 were compared (2% *Psyllium* fiber in place of 2% corn starch, in the presence of 40% amaranth). *Psyllium* influence on the properties of a starch-based dough was also confirmed by fundamental rheological experiments; in particular, the smaller increase in G' during heating of dough containing *Psyllium* fiber (in comparison to those in which this hydrocolloid was not present), could be explained not only by the competition for water due to *Psyllium*'s extremely strong gelling and water-absorbing abilities, but also by the creation of a thin network (composed by protein and hydrocolloid) able to limit starch swelling and gelatinisation. Upon baking, this phenomenon could have great importance for the shelf-life of GF bread.

When other ingredients were included in the formulations, the ultrastructure of the resulting doughs changed. Amaranth (Fig. 8A, D2, D3, D5 and D6) seemed to have a double effect in dough: on the one hand, the presence of teguments in high amounts (as already seen on SEM images, Fig. 6A and Fig. 6B) brought about large discontinuity in the matrix; on the other hand, amaranth starch granules were so small (about 0.5–2 μm diameter) and the protein content was so high that amaranth seemed to act as a filler of the dough matrix. When the higher amount of pea isolate (6%) was present, as expected, proportionally more green area was seen, indicating a larger protein matrix. The more complex formulations (containing all the ingredients) (Fig. 8B, D5 and D6) exhibited a quite continuous and homogeneous network in the dough, accounting for the smallest increases in their G' during heating.

The different ultrastructure of the two commercial GF samples (C1 and C2) in comparison to that of the experimental GF doughs, already appreciable by SEM observations (Fig. 7), was confirmed by CLSM images. C1 dough appeared quite homogeneous and regular. Potato and rice starch granules, rice starch granules completely enveloped by protein, and different structures attributable to hydrocolloids, made the C2 dough ultrastructure quite inhomogeneous; nevertheless, and perhaps due to the well-balanced mixture of the properties of the different ingredients, its empirical and fundamental rheological properties came out as one of the best of those investigated.

Looking at the end-use of the experimental GF bread mixtures formulated and investigated, starch-based ones could give rise to quick bread staling, because the lack of an embedding structure allows starch to rapidly swell and gelatinize. The addition of protein and fiber sources (here, pea isolate and amaranth) improved the experimental GF doughs from both a physical (rheological and ultrastructural) and nutritional point of view, and a delay in the staling phenomenon of these samples could be expected. The presence of *Psyllium* fiber generally enhanced the physical properties of the studied experimental GF doughs, due to the film-like structure

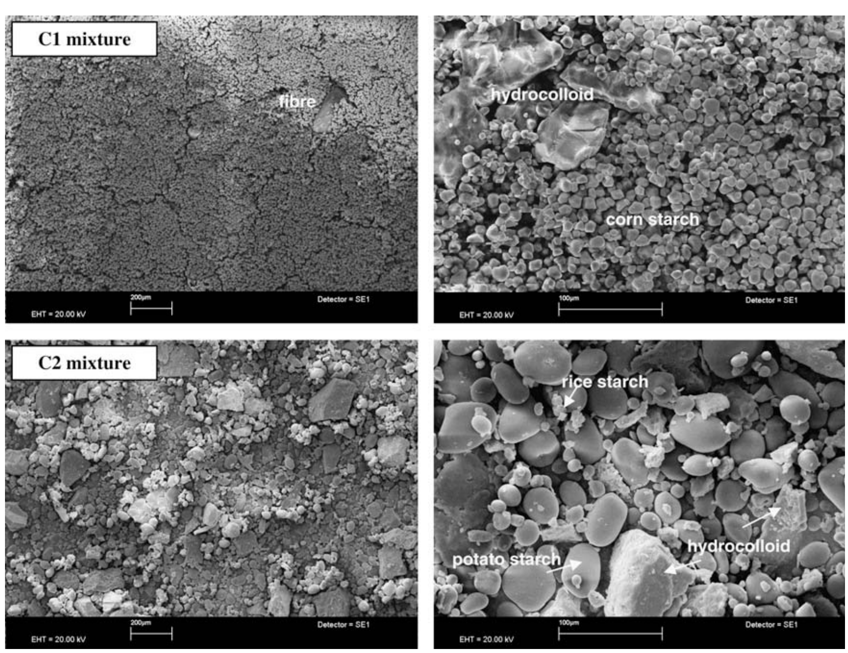


Fig. 7. SEM images of the two commercial GF bread powders.

that it was able to form. Thus the formation of a continuous protein phase and of a film-like structure appears to be critical for the workability of a GF dough. Generally, the more complex experimental formulations (containing corn starch, amaranth flour, pea

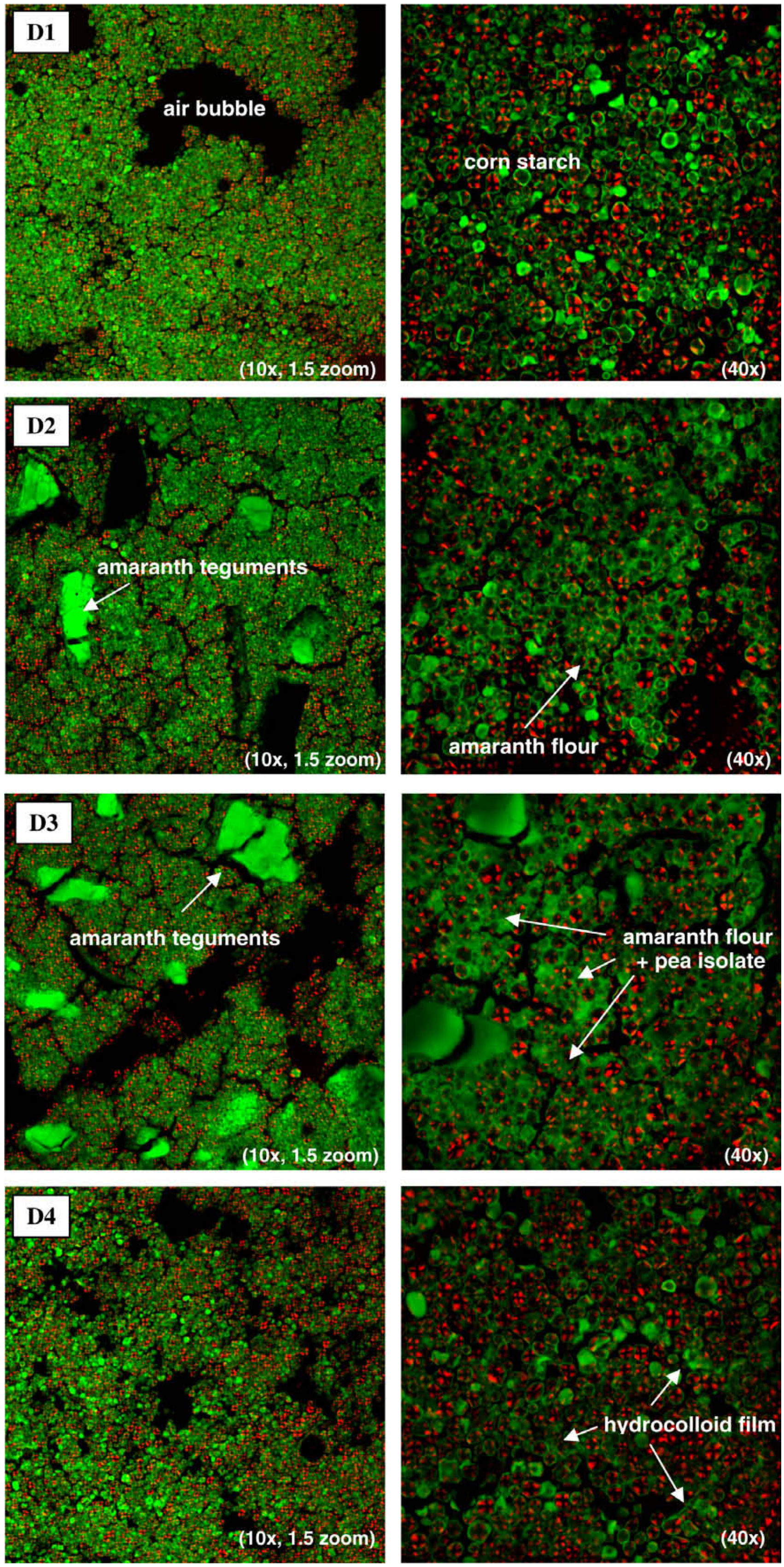


Fig. 8A. CLSM images of the experimental (D1–D6).

isolate and *Psyllium* fiber) investigated in this research looked promising in terms of final bread technological and nutritional quality, even when compared to commercial mixtures already present on the market. The combination of fundamental rheology

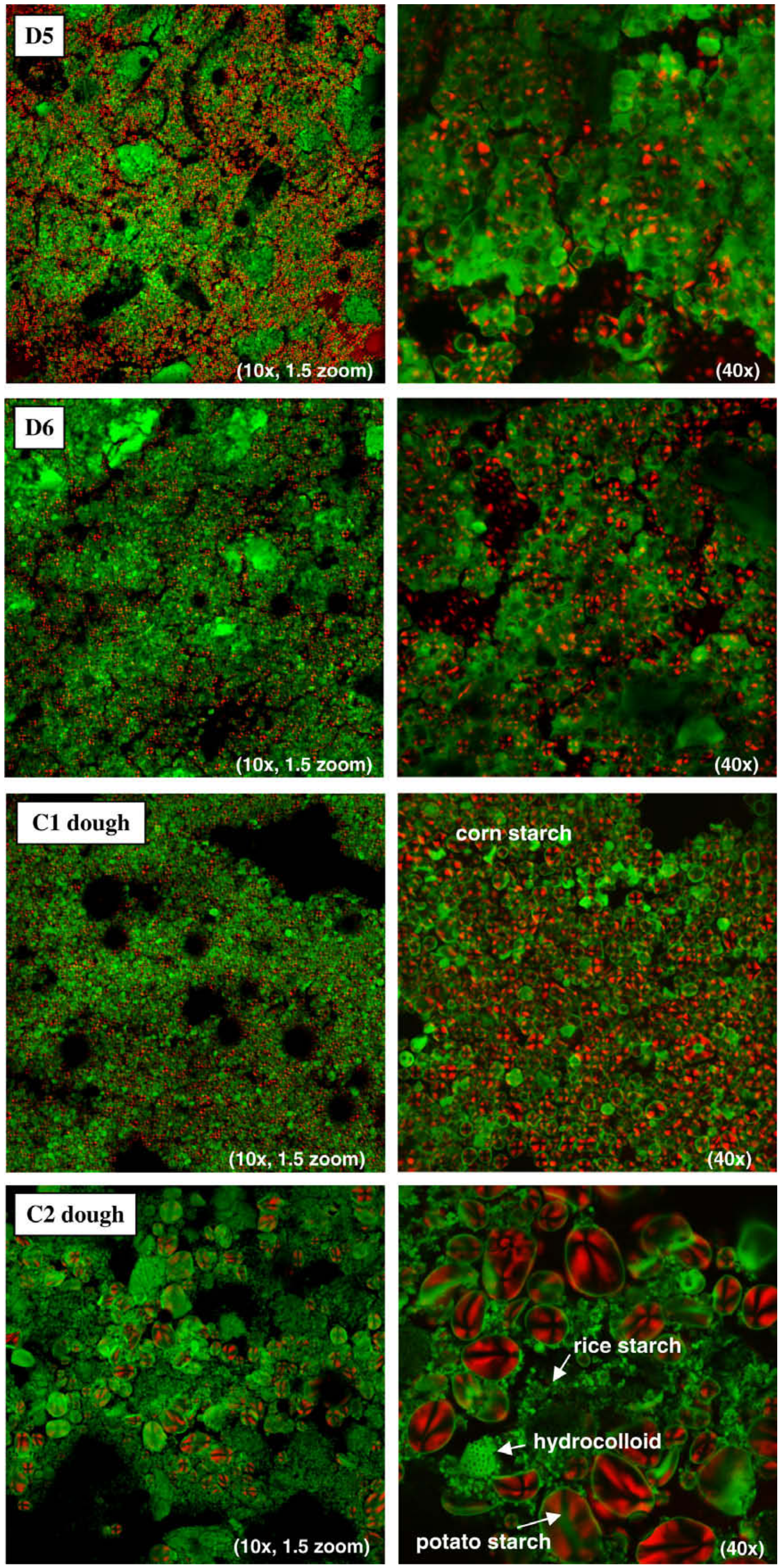


Fig. 8B. Commercial (C1 and C2) gluten-free doughs.

and ultrastructural microscopy proved to be very useful in the understanding of GF dough properties.

Acknowledgments

We thank Dr. Ritu Saini for her precious help with the CLSM. This work was supported in part by funds from the Italian Ministry for University (MIUR, Ministero dell'Istruzione, dell'Università e della Ricerca).

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