Effect of Carboxymethyl Chitin-Glucan on the Activity of Some Hydrolytic Enzymes in Maize Plants

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Activity of some hydrolytic enzymes (acid and basic phosphatase, nonspecific esterase, leucine aminopeptidase, β-glucosidase, chitinase) and peroxidase was investigated in the roots and leaves extracts isolated from maize plants sprayed with carboxymethyl chitin-glucan (CM-CG) solution. Application of CM-CG, prepared from the mycelium of the industrially used filamentous fungus *Aspergillus niger* resulted in the decrease of the activity of acid and alkaline (basic) phosphatase in the roots and leaves extracts. Activity of β-glucosidase significantly decreased in extracts isolated from leaves but was unaffected in roots extracts. On the other hand, activity of nonspecific esterase, and leucine aminopeptidase was significantly stimulated in extracts from leaves while inhibited in roots extracts. Significant increase was also detected in the activity of chitinase isolated from leaves. Activity of peroxidase remained unchanged both in roots and leaves extracts. Effect of applied fungal polysaccharide on the enzymatic activities of some enzymes involved in the plant protection mechanisms may imply possible utilization of such polysaccharides.

Phytopathogenic microorganisms activate a wide range of host genes especially at the infection site leading to significant changes in host physiological processes. The timing of enzyme activation always depends on the degree of host-parasite compatibility showing higher stimulation in compatible interaction [1]. Induction of enzymes or proteins involved in disease resistance response has also been demonstrated by application of biologically derived products from cell walls of different fungi [2—4]. For example, chitosan (β-1,4-linked glucosamine polymer) induced accumulation of phytoalexins in tomato plants [5], fungal glucan activated tobacco glycine-rich protein (GRP) gene which resulted in enhanced resistance to viral infection [6], yeast mannan or glucomannan significantly decreased tobacco mosaic and tobacco necrosis virus infection in cucumber and bean plants [7], *etc.*

Role of hydrolytic enzymes in host—pathogen interactions was studied by several authors [8, 9]. Even though hydrolases do not play key role in the host resistance, they are involved not only in the degradation of host substances but they may also attack the invader itself. On the other hand, proteolytic enzymes are involved in senescence, and increased activity of epidermal exoprotease could be a consequence of the local tissue "senescence" [1, 10].

The purpose of this paper was to evaluate activities of several hydrolases (acid and basic phosphatase, nonspecific esterase, leucine aminopeptidase, β-glucosidase, and chitinase) in leaves and roots of maize plants sprayed with CM-CG solution, prepared from a polysaccharide complex isolated from the mycelium of the industrial strain of *Aspergillus niger*.

EXPERIMENTAL

All reagents used were of analytical purity grade. The substrates used for enzyme activity assays were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA).

Preparation and Characterization of Carboxymethyl Chitin-Glucan

The crude, water-insoluble chitin-glucan complex was isolated from the cell walls of the industrial strain of the filamentous fungus *Aspergillus niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovak Republic). The mycelium (wet weight 100 g) was subjected to a hot alkaline (250 cm^3 of 1 M-NaOH, 100 °C) digestion for 1 h. The alkali-insoluble residue was washed several times with distilled water and freeze-dried. The dry sample contained 2.24 % of nitrogen, which corresponded to a

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content of *ca.* 30 % of chitin. Solubilization of the isolated chitin-glucan complex was achieved by means of carboxymethylation as described by *Machová et al.* [11]. The degree of substitution of the obtained carboxymethylated chitin-glucan complex was 0.43 as determined by means of potentiometric titration with KOH [12]. In order to improve water solubility of the obtained CM-CG, it was further treated by ultrasonication using a 20 kHz sonicator UZD 300 (PERSON-Ultragen, Nitra, Slovak Republic) [13]. After 60 min ultrasonic treatment, a well soluble sample of CM-CG was isolated by gel filtration and its relative molecular mass was determined using high-performance liquid chromatography (HPLC) to be 57000 [11]. This preparation was subsequently used in the plant experiments.

Plant Experiments

Seeds of maize (*Zea mays* L. cv. TO 360) were germinated in darkness at the temperature of 25 °C and relative humidity of 98 %. Three-day-old maize seedlings were transferred to 6000 cm^3 glass jars containing half strength Hoaglands nutrient solution [14] and cultivated in a growth chamber (E 8 Conviron, Canada) at the temperature of 24 °C and 12 h photoperiod (irradiance 70 μmol m^{-2} s^{-1}; 400—700 nm). After two weeks half of the plants was sprayed with 0.05 % Tween solution (control) while the second half with 0.05 % Tween solution containing 0.1—0.5 mg cm^{-3} of carboxymethyl chitin-glucan (treated plants). 24 h after the CM-CG application (16—83 μg $plant^{-1}$), plants were harvested and immediately frozen in liquid nitrogen. The samples were ground in a cold mortar in liquid nitrogen and resulting fine powder was re-homogenized (1:2, g cm^{-3}) in extraction buffer (100 mM-Tris buffer, pH 8.0) with homogenizer (Heidolph DIAX 900, Keilheim, Germany). After filtration, the homogenate was fractionated using centrifugation at 1500 *g* (pellet discarded) and supernatant was re-centrifuged at 12000 *g*. The resulting supernatant was used for determination of the activity of all studied enzymes.

Enzyme Activity Assays

Total protein content in the extracts was determined with the Bradford method [15]. Bovine serum albumin was used for calibration. Enzyme activities were determined spectrophotometrically at appropriate wavelengths. The "Easy Reader" (STL-Laborinstruments, Austria) with 96 well EIA-plate (Costar, USA) was used for the quantification of enzyme activities. Specific enzyme activities were expressed as $A \times \mu g^{-1}$ protein $\times$ min^{-1} (A – observed absorbance at the specified wavelength). Changes in enzyme activities are expressed as a percentage of a value calculated for control experiment.

If not specified differently, the following reagents and conditions have been used for the assays of the enzymes: 100 mm^3 buffer solution, 50 mm^3 sample (blank – H_2O), 50 mm^3 substrate, incubation at 30 °C for 60 min, absorbance read at 405 nm. For acid phosphatase (EC 3.1.3.2), the buffer was 0.1 M-sodium acetate (pH 5.2) and the substrate used was 2 mg of 4-nitrophenyl phosphate in 1 cm^3 redistilled H_2O, the reaction was stopped by adding 50 mm^3 of 0.4 M-sodium phosphate (pH 7.2); for alkaline (basic) phosphatase (EC 3.1.3.1), the buffer was 0.025 M-glycine-NaOH (pH 9.6) and 1 mM-$MgCl_2$ and the substrate used was 2 mg of 4-nitrophenyl phosphate in 1 cm^3 redistilled H_2O; for nonspecific esterase (EC 3.1.1.2), the buffer was 0.1 M-Tris-HCl (pH 7.2) and the substrate used was 2 mg of 4-nitrophenylacetate in 20 % acetone; for β-glucosidase (EC 3.2.1.21), the buffer and substrate was 0.1 M-sodium acetate (pH 5.0) containing 0.5 mg of *p*-nitrophenyl D-glucopyranoside, the incubation was stopped by adding 100 mm^3 of 10 % Na_2CO_3; for chitinase (EC 3.2.1.14), the buffer was 0.1 M-Tris-HCl (pH 7.2) with the dissolved substrate poly(*N*-acetyl-D-glucosamine) in concentration 2—4 mg cm^{-3}, absorbance measurements were carried out at 544 nm; for leucine aminopeptidase (EC 3.4.11.1), the buffer was 0.1 M-Tris-HCl (pH 7.2) and substrate used was 2 mg of 1-leucine-*p*-nitroanilide dissolved in 20 % acetone.

RESULTS AND DISCUSSION

In plants, stress symptoms caused by abiotic and biotic factors are accompanied by the changes in the level of many proteins, that reflect the alterations in gene expression. In our earlier work [1, 16] we have found significant changes in the activity of hydrolytic enzymes during pathogenesis. Due to the fact that some purified cell-wall components isolated from pathogenic fungi are able to induce changes in host plant which usually decrease the infectivity of pathogen, we focused our effort on the study of the effect of exogenously applied CM-CG complex (manufactured from the cell-wall preparation from *Aspergillus niger*) on the activity of plant hydrolytic enzymes. These enzymes are involved in a number of different reactions not only during the pathogenesis but also during other abiotic stresses [16]. In the preliminary experiments using different concentrations of CM-CG in the range mentioned in Experimental, we have found that concentrations higher than 16 μg $plant^{-1}$ did not cause significant changes in enzyme activities different from that caused by the lowest one (*i.e.* 16 μg $plant^{-1}$). For this reason, in all further experiments only that concentration of CM-CG was applied.

From the results obtained in our experiments it is evident that application of CM-CG selectively influences the activity of these enzymes. The activities of

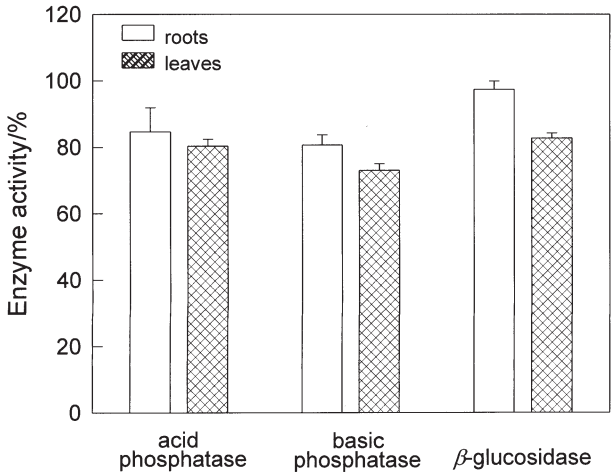

Fig. 1. Enzyme activity in maize plants (roots and leaves) 24 h after spraying the plant with CM-CG (16 μg per plant) expressed as the percentage of enzyme activity in control plants determined spectrophotometrically (acid phosphatase – control Δ_{405} 1.75×10^{-3} – roots, Δ_{405} 2.45×10^{-3} – leaves; basic phosphatase – control Δ_{405} 2.97×10^{-4} – roots, Δ_{405} 3.35×10^{-4} – leaves; β-glucosidase – control Δ_{405} 1.27×10^{-2} – roots, Δ_{405} 2.15×10^{-2} – leaves).

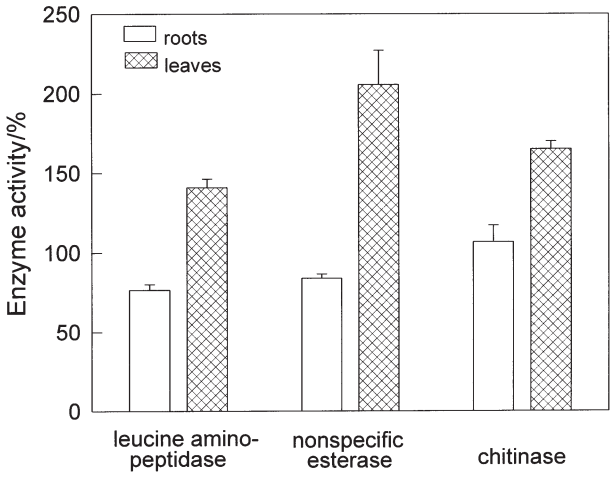

Fig. 2. Enzyme activity in maize plants (roots and leaves) 24 h after spraying the plant with CM-CG (16 μg per plant) expressed as the percentage of enzyme activity in control plants determined spectrophotometrically (leucine aminopeptidase – control Δ_{405} 8.35×10^{-4} – roots, Δ_{405} 1.27×10^{-3} – leaves; nonspecific esterase – control Δ_{405} 8.25×10^{-3} – roots, Δ_{405} 1.25×10^{-2} – leaves; chitinase – control Δ_{544} 1.15×10^{-4} – roots, Δ_{544} 6.35×10^{-4} – leaves).

both types of phosphatases (acid and basic) were significantly decreased after CM-CG treatment (Fig. 1). The extent of inhibition was very similar with acid and basic phosphatases with the tendency of lower inhibition in roots than in leaves. A very similar pattern showed CM-CG treatment on the activity of β-glucosidase isolated from leaves (Fig. 1). Only minor, insignificant inhibition of β-glucosidase was determined in the roots extract. Completely different impact of CM-CG treatment was observed in the activity of leucine aminopeptidase and nonspecific esterase (Fig. 2). Leucine aminopeptidase activity increased 24 h after spraying the leaves with CM-CG up to 50 % above the activity of control plants. At the same time, almost 25 % decrease in the activity of leucine aminopeptidase was measured in roots extract. Even higher increase was found in the activity of nonspecific esterase in extract isolated from leaves. In this case, the activity was two times higher than in the control plants. Surprisingly, activity of nonspecific esterase was significantly decreased in the extract from roots.

Increased activities of chitinase and peroxidase after exogenously applied fungal polysaccharides were demonstrated by *Slováková et al.* [7] in cucumber and bean leaves. Due to the fact that after inoculation of leaves with tobacco mosaic virus or tobacco necrosis virus, polysaccharide-induced elevation of the content of both enzymes has gradually decreased, the authors have suggested that these polysaccharides are not directly associated with virus inactivation but act rather indirectly by inducing defence mechanisms of the host. In contrast to the results of these authors no significant increase of chitinase activity was determined in barley epidermal cells inoculated with powdery mildew [9]. Contrary to these data, we have observed strong increase of chitinase activity especially in the leaves (almost 70 % growth in comparison with the control plants), while only moderate increase was found in the roots (Fig. 2).

Despite the fact that peroxidases are ubiquitous enzymes that are expressed in plants in response to biotic and abiotic stresses [17], application of CM-CG on barley leaves had only negligible effect on the activity of peroxidase (results not shown). Similarly, low effect on peroxidase activity was found after spraying cucumber leaves with another cell-wall polysaccharide, glucomannan [7]. On the other hand, it is necessary to stress that despite the fact that peroxidase activity was only weakly influenced by glucomannan in crude extract from leaves, in the intercellular fluid isolated from the same leaves activity of peroxidase was significantly higher than in control plants.

Based on our results, we can conclude that spray application of CM-CG prepared from the cell walls of *Aspergillus niger* on maize leaves induced different but significant changes in the content of most of the studied enzymes. The impact of CM-CG on the enzymes involved in defence mechanism of plants against biotic stresses (nonspecific esterase, leucine aminopeptidase, β-glucosidase) was very similar to the impact of viral or fungal pathogens [7, 9] on the activity of these enzymes. We have previously demonstrated that application of CM-CG provided protection of bean and

cucumber against viral infection [18]. Both chitin and glucan are the major cell-wall components of fungi, including those acting as plant pathogens. It is therefore possible that subjecting plants to the fungal polysaccharide preparations can mimic the pathogen attack and thus stimulate plant defence responses [19—21]. There is enough experimental evidence that chitin and chitosan-based oligomeric and polymeric materials stimulate plant growth, protect them from viral and fungal pathogens, and improve productivity [22—28]. Our data suggest that influencing the production of plant hydrolases might be one of the components of the complex mechanism of polysaccharide-induced plant protection responses and imply that pretreatment of plants with isolated components of fungal cell-wall polysaccharides can be used as a potential means of plant protection against biotic agents.

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