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Changes in glycosidase activities during galactoglucomannan oligosaccharide inhibition of auxin induced growth

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Abstract

The inhibition of 2,4-D-induced elongation growth by galactoglucomannan oligosaccharides (GGMOs) in pea stem segments (*Pisum sativum* L. cv. Tyrkys) after 18 h of incubation results in changes of extracellular, intracellular and cell wall glycosidase activities (β-D-glucosidase, β-D-mannosidase, β-D-galactosidase, β-D-xylosidase, α-D-galactosidase, and α-L-arabinosidase). GGMOs lowered the glycosidase activities in the extracellular fraction, while in the cell wall fractions their activities were markedly increased. The intracellular enzyme α-D-galactosidase increased while the β-D-galactosidase decreased in activity in response to the GGMO treatment. Extracellular enzymes showed low values of activities in comparison with intracellular and cell wall glycosidases. It is evident that GGMOs can alter auxin induced elongation and glycosidase activities in different compartments of the cell, however, the mode and site of their action remains unclear.

Keywords: *Pisum sativum* L. cv. Tyrkys; Fabaceae; Elongation growth; Glycosidases; Galactoglucomannan oligosaccharides

1. Introduction

The plant cell wall is a complex molecular structural network that undergoes dynamic changes during cell extension or elongation (Masuda, 1990). Cosgrove (1999) suggested that the primary agent of wall extension is expansion along with some enzymes that secondarily modulate its action. However, oligosaccharides derived from plant polysaccharides may also have regulatory functions in cell growth, morphogenesis, and defense reactions (York et al., 1984). These effects have been demonstrated with xyloglucan fragments, oligogalacturonides, cello-oligosaccharides, xylomannooligosaccharides, lipochitooligosaccharides and others (Branca et al., 1988; McDougal and Fry, 1988; Lorences et al., 1990; Priem et al., 1990; Hoson and Masuda, 1991; Dumville and Fry, 2000). In our previous work (Auxtová et al., 1995; Auxtová Šamajová et al., 1996) we reported on the inhibitory effect of galactoglucomannan oligosaccharides on the 2,4-D-induced elongation growth in pea and spruce stem segments. The biochemical basis of this inhibition is unknown. Auxin induced cell wall loosening, which precedes cell elongation, is connected with biochemical modifications of cell wall components. In some reports glycanases (Masuda, 1990; Acebes and Zarra, 1992; Hoson, 1993; Cosgrove, 1999; Kotake et al., 2000) and xyloglucan transglycosylase (XET) (Fry and Matthews, 1992; Potter and Fry, 1994; Nishitani, 1995; Barrachina and Lorences, 1998; Cosgrove, 1999) have been suggested to be involved in this process. In other studies (Murray and Bandurski, 1975; Tanimoto and Igari, 1976; Masuda et al., 1988; Hrmova et al., 1997), glycosidases participating in the auxin induced elongation growth, are reported. Because glycosidases act in an exo-fashion they could be responsible for the modification of side chains of cell wall oligo- or polysaccharides affecting the mechanical properties as well as growth of the cell wall (Fry, 1995).

The aim of the present work was to screen for changes in glycosidase activities in response to tissue treatment with galactoglucomannan oligosaccharides, to inhibit 2,4-D-induced elongation growth. In addition,

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to determine their potential role in the regulation of this process in a model system utilizing pea stem segments (*Pisum sativum* L. cv. Tyrkys).

2. Results and discussion

Previous work had demonstrated an inhibitory effect of galactoglucomannan oligosaccharides (GGMOs, d.p. 4–8, composed of Gal (4.5%), Glc (25.1%) and Man (70.4%)) on the 2,4-D-induced elongation growth of pea stem segments (Auxtová et al., 1995). The highest inhibition of elongation growth was observed at 10^{-10} M concentration of GGMOs and was used in these studies focused on changes in glycosidase activities after 18 h of incubation. Total enzymes activity in the whole cell system (Fig. 1) showed relatively high values for α- and β-galactosidases in control, 2,4-D and 2,4-D + GGMOs treated samples compared to all the other glycosidases. However, α-galactosidase activity increased (f) (a–f, degrees of significancy), while β-galactosidase decreased its activity (a) in the presence of GGMOs. All other enzymes studied showed non-significant differences in their activities in comparison with α- and β-galactosidases. The inhibition of 2,4-D-induced elongation growth by GGMOs coincided with changes in glycosidase activities in extracellular, intracellular-soluble and cell wall fractions.

In the extracellular fraction, auxin substantially increased the activity of the most glycosidases monitored in this study (Fig. 2), although the real values of these activities were small compared to the total activity (Fig. 1). The activities of β-D-glucosidase (b), α-D-galactosidase (d), β-D-galactosidase (a) and α-L-arabinosidase (b) decreased when treated with GGMOs compared with samples treated with auxin, while β-D-mannosidase remained unaffected (N) and a slight increase in β-D-xylosidase activity (f) (Fig. 2). It can be concluded that GGMOs significantly decreased the effect of auxin in this fraction and may participate in the regulation of extracellular glycosidases. Besides, these glycosidases act in the extracellular space and they are not involved in process of cell wall loosening.

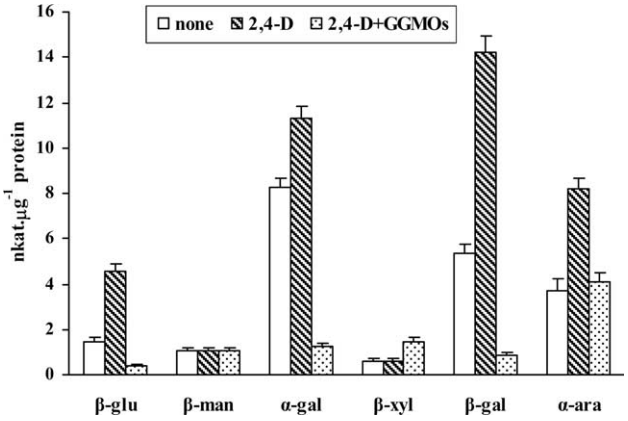

Fig. 2. Changes of glycosidases activity in the extracellular fraction during 2,4-D-induced elongation growth inhibited by GGMOs in pea stem segments (β-glu = β-D-glucosidase, β-man = β-D-mannosidase, α-gal = α-D-galactosidase, β-xyl = β-D-xylosidase, β-gal = β-D-galactosidase, α-ara = α-L-arabinosidase).

The highest levels of glycosidase activities were in the intracellular-soluble fraction (Fig. 3). In the presence of GGMOs there was a significant decreased in β-D-mannosidase (b), β-D-xylosidase (a), β-D-galactosidase (a), and α-L-arabinosidase (f) activities, while β-D-glucosidase activity remained unaffected (N) and α-D-galactosidase significantly increased (d) compared with 2,4-D treated samples. β-D-galactosidase had the highest level of all the enzyme activities and showed the most prominent changes in activity in response to 2,4-D and 2,4-D + GGMOs treatments. There was no correlation between the elongation growth induced by 2,4-D and the

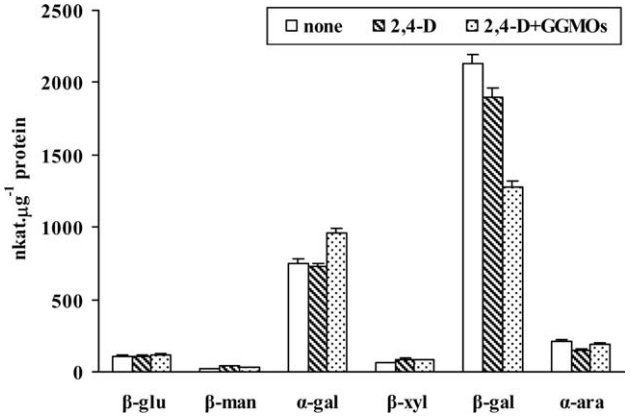

Fig. 1. Sum of all glycosidases activities during 2,4-D-induced elongation growth inhibited by GGMOs in pea stem segments (β-glu = β-D-glucosidase, β-man = β-D-mannosidase, α-gal = α-D-galactosidase, β-xyl = β-D-xylosidase, β-gal = β-D-galactosidase, α-ara = α-L-arabinosidase).

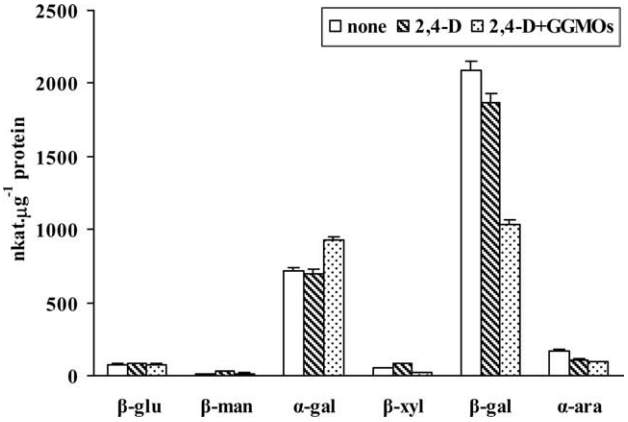

Fig. 3. Changes of glycosidases activity in the intracellular-soluble fraction during 2,4-D-induced elongation growth inhibited by GGMOs in pea stem segments (β-glu = β-D-glucosidase, β-man = β-D-mannosidase, α-gal = α-D-galactosidase, β-xyl = β-D-xylosidase, β-gal = β-D-galactosidase, α-ara = α-L-arabinosidase).

β-D-galactosidase activity, because 2,4-D did not increase β-D-galactosidase activity compared to the control. However, GGMOs significantly lowered the activity of this enzyme compared with the control and 2,4-D treatment. It indicates that oligosaccharides may be involved in the regulation of β-D-galactosidase activity. On the other hand only α-D-galactosidase increased in samples with exogenous supplementation of GGMOs. It is possible that GGMOs can induce synthesis of α-D-galactosidase, which in turn would catalyse the cleavage of α-D-galactosyl residues from GGMOs, as well as from other potential substrates. Removing branch galactosyl residues could inactivate GGMOs in their inhibition effect on elongation growth induced by auxin (Auxtová et al., 1995), and in this way participate in the regulation of elongation growth. This hypothesis is correct only if α-D-galactosyl residues are essential for the inhibition activity of GGMOs. The bioassay on pea stem segments using modified GGMOs supports this hypothesis (Fig. 4). The results show significantly lower inhibition of elongation growth induced by 2,4-D when the α-D-galactose content of GGMOs was reduced by α-D-galactosidase to 2.4 mol% compared with non-modified oligosaccharides (4.5 mol%). Because α-D-galactosyl residues appear to be essential for the inhibition activity of GGMOs, the α-D-galactosidase could be involved in a feeback mechanism regulating the activity of GGMOs in 2,4-D-induced elongation growth of pea stem segments.

Cell wall glycosidases monitored in this study increased in the presence of GGMOs compared with control and 2,4-D treatment (Fig. 5). Glycosidase activities within cell wall fractions (ion-extractable, EDTA-extractable, tightly bound) displayed different patterns of change (Figs. 6 and 7). The ion- and EDTA-extractable fractions are characterized by low values of

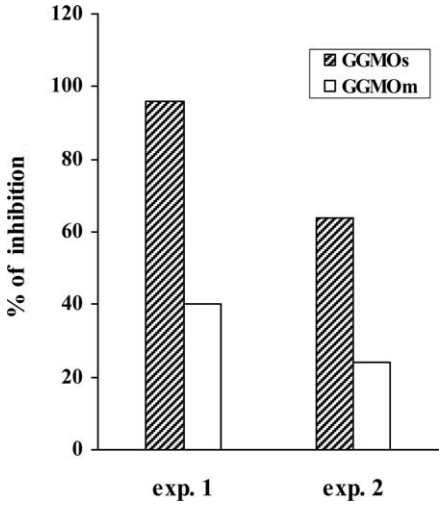

Fig. 4. Effect of native (GGMOs) and modified (GGMOm) oligosaccharides on 2,4-D-induced elongation growth of pea stem segments. Segments were measured after 18 h. Data are presented as the inhibition (%) of 2,4-D-stimulated growth. The growth variations in two independent experiments are caused by the preparation and the natural variation of plant material used. Differences between GGMOs and GGMOm treated samples were highly significant (a).

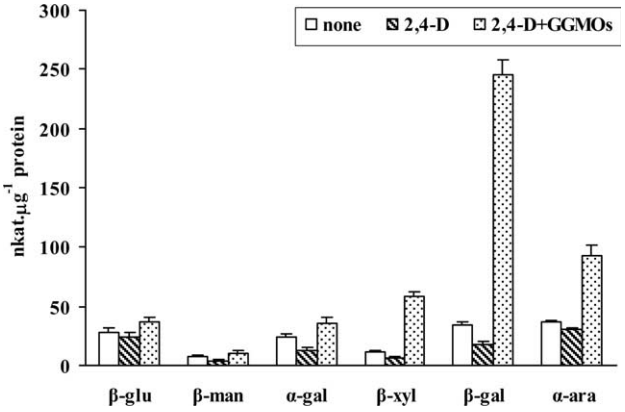

Fig. 5. Changes of total glycosidases activity in the cell wall fraction during 2,4-D-induced elongation growth inhibited by GGMOs in pea stem segments (β-glu = β-D-glucosidase, β-man = β-D-mannosidase, α-gal = α-D-galactosidase, β-xyl = β-D-xylosidase, β-gal = β-D-galactosidase, α-ara = α-L-arabinosidase).

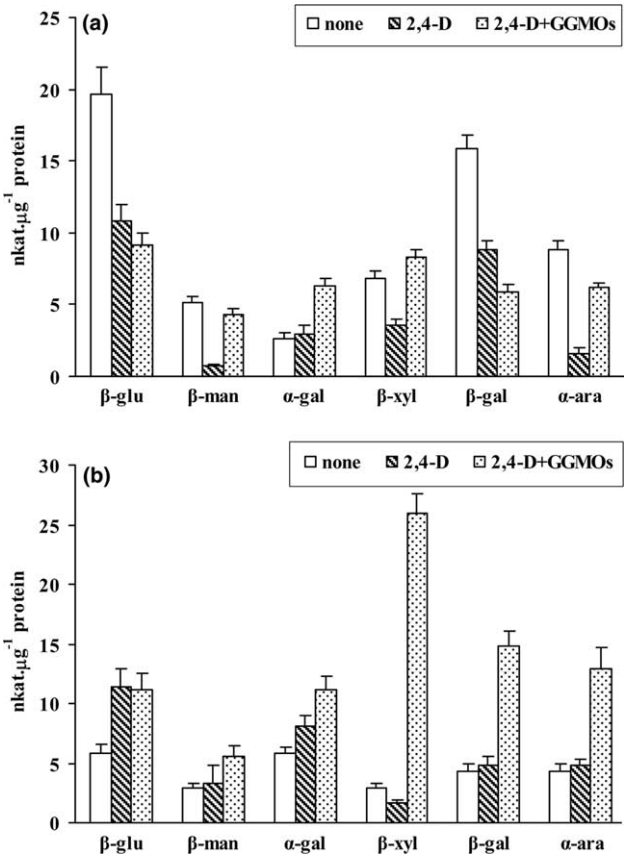

Fig. 6. Changes of ion-extractable (a) and EDTA-extractable (b) glycosidases activity in the cell wall fraction during 2,4-D-induced elongation growth inhibited by GGMOs in pea stem segments (β-glu = β-D-glucosidase, β-man = β-D-mannosidase, α-gal = α-D-galactosidase, β-xyl = β-D-xylosidase, β-gal = β-D-galactosidase, α-ara = α-L-arabinosidase).

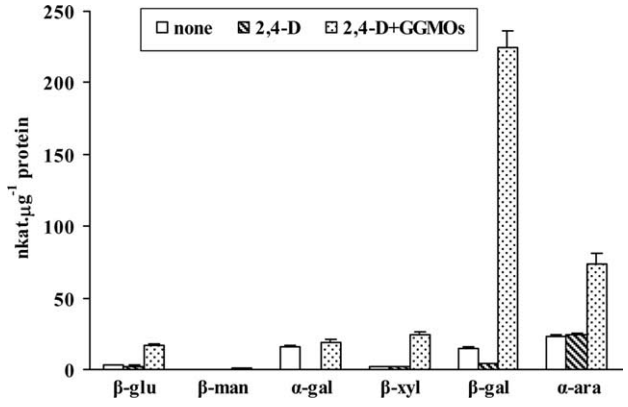

Fig. 7. Changes of tightly bound nonextractable glycosidases activity in the cell wall fraction during 2,4-D-induced elongation growth inhibited by GGMOs in pea stem segments (β-glu = β-D-glucosidase, β-man = β-D-mannosidase, α-gal = α-D-galactosidase, β-xyl = β-D-xylosidase, β-gal = β-D-galactosidase, α-ara = α-L-arabinosidase).

enzyme activities compared to the tightly bound fraction. The most prominent changes seemed to be due to GGMO treatment on the total cell wall glycosidase activities of β-D-galactosidase (a), α-L-arabinosidase (c) and β-D-xylosidase (a).

In the ion-extractable cell wall fraction (Fig. 6(a)) a significant increase of α-D-galactosidase activity (d) was determined while the activity of β-D-glucosidase (d) and β-D-galactosidase (a) decreased. In addition, β-D-mannosidase (b), β-D-xylosidase (b) and α-L-arabinosidase (b) were inhibited in the presence of 2,4-D, while their activity increased in the presence of GGMOs.

In the EDTA-extractable cell wall fraction GGMOs increased the activity of all the enzymes studied (Fig. 6(b)), except for β-D-glucosidase. It indicates that GGMOs have no effect on the activity of this enzyme and are not involved in its regulation. However, β-D-glucosidase (f) is the only enzyme in this fraction that activity increased after auxin treatment. The significant increase of α-L-arabinosidase (d), β-D-galactosidase (b) and in particular of β-D-xylosidase(a) activity in the presence of GGMOs was unexpected concerning the fact that growth-inhibition effect of these oligomers is the highest after 18 h. If the auxin-induced elongation is suppressed by oligomers and glycosidases activity is increasing, subsequently these enzymes probably are not involved in the regulation of elongation growth, but they have other roles.

The tightly bound glycosidases (Fig. 7) showed the highest values of enzymes activity of all cell wall fractions. The presence of 2,4-D did not increase any of these activities. It indicates that auxin itself does not affect glycosidases in the cell wall after 18 h of incubation, while GGMOs resulted in significantly increased of glycosidase activities. The highly significant increase of these enzymes under conditions of the highest inhibition of growth is remarkable. The participation of β-D-glucosidase and α-D-galactosidase (Nevins 1970; Murray and Bandurski, 1975) in elongation growth have been reported. While other observations (Evans, 1974) suggest that the action of these glycosidases is not essential in auxin-induced cell wall loosening. Auxin-induced increase of β-D-galactosidase activity in short-term courses (1–5 h), not only in the induced elongation growth but also in cases when elongation growth was suppressed by mannitol, has been described (Tanimoto and Igari, 1976). However, the subcellular localization of glycosidases tested was not clear because the enzymes activity was estimated in the whole stem segments. The high β-D-galactosidase activity is often connected with fruit ripening, where a significant effect of this enzyme on pectic polysaccharides was described (Priem and Gross, 1992). It is known that glycosidases have low aglycone specificity, and therefore it is difficult to specify their natural substrates and roles in different compartments of the plant cells (Fry, 1995). From the results presented in Fig. 7 it is evident that there is no relationship between auxin-induced elongation growth and the increase of cell wall glycosidase activities after 18 h of incubation.

2.1. Conclusions

In conclusion, the cell wall glycosidases (ion-extractable, EDTA-extractable and tightly bound) showed entirely different activity patterns compared to the extracellular and intracellular fractions. However, the values of ion- and EDTA-extractable cell wall glycosidases activity were small compared to the tigthly bound cell wall fraction. Tigthly bound cell wall glycosidases did not show any substantial changes in their activities in response to auxin-induced elongation growth. The results indicate that 2,4-D has no influence on glycosidases in the elongation growth and that the tigthly bound glycosidases are not involved in cell wall loosening. The increase in the activity of all tigthly bound glycosidases after GGMOs treatment, but predominately of β-D-galactosidase and α-L-arabinosidase, under conditions of the highest inhibition growth is surprising. Therefore we can assume that these glycosidases can cause significant changes or disintegration in the cell wall network resulting in inhibition of cell wall loosening. However, this statement is not in agreement with the generally accepted idea that exo-enzymes are able to make only cosmetic changes (removal of non-reducing termini sugar residues from various wall polysaccharides or glycoproteins) in comparison with endo-enzymes.

Extracellular glycosidases showed the lowest values of activities of all fractions tested. From the results it is evident that GGMOs can be involved in their regulation, but their glycosidase values are negligible.

The most prominent glycosidases of the intracellular-soluble fraction, α- and β-D-galactosidase, showed

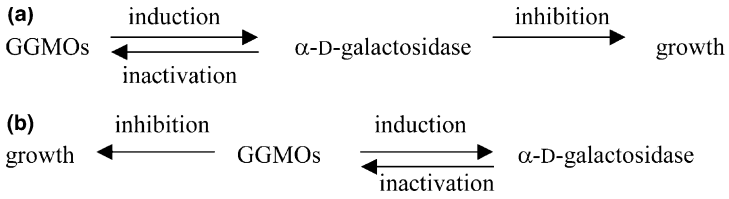

Fig. 8. Proposed mechanism(s) of GGMOs action in the elongation growth in an dependent (a) and/or independent (b) manner.

markedly different activity depending on the exogeneous supplementation of GGMOs. Oligosaccharides induced α-D-galactosidase activity, while the activity of β-D-galactosidase was significantly reduced. Lowering of β-D-galactosidase is in a consonance with the anti-auxin effect of GGMOs. However, the increase of α-D-galactosidase activity after 18 h, when the growth-inhibiting effect of GGMOs was the highest, is surprising. We can assume two possible explanations of this phenomenon. The induction of α-D-galactosidase by GGMOs and the inhibition of elongation growth could be either dependent or independent processes. When the process is dependent GGMOs enhance the α-D-galactosidase activity which is directly responsible for inhibition of cell wall loosening by an unknown mechanism. Besides, α-D-galactosidase can retroactively inactivate GGMOs by feedback spliting off their side chains (Fig. 8(a)). In the independent process oligosaccharides could enhance α-D-galactosidase activity which retroactively inactivates GGMOs by removing the galactosyl side-chains and in this way indirectly regulate the elongation growth. However, the mode of GGMOs action is not through the α-D-galactosidase but through an other unknown way (Fig. 8(b)).

To obtain a more detailed picture of these processes it is necessary to follow the growth dynamics and glycosidases changes during cell wall loosening under auxin and oligosaccharides treatment. Despite of the shortcomings mentioned we can conclude, that the biochemical and physiological changes during the elongation growth under GGMOs treatments confirm the active role of these oligosaccharides in the growth process, although the mechanism of their action is still not understood.

3. Experimental

3.1. Plant material

Pea (*P. sativum* L., cv. Tyrkys) was obtained from Selgen Slovakia.

3.2. Preparation and incubation of segments

Pea stem segments (6 mm) were cut below the apical hook from the third internode of eight-days old seedlings as described previously (Auxtová et al., 1995). The excised segments were incubated (18 h) with GGMOs (10^{-10} M) in 5 mM potassium phosphate buffer pH 6.1 supplemented with 1% sucrose at 24 °C. After 90 min of incubation 2,4-D was added to a final concentration of 0.9 μM. Two control groups of segments were used (1) segments incubated in buffer and (2) segments incubated in buffer supplemented with 2,4-D (Auxtová et al., 1995).

3.3. Pea stem bioassay

Bioassays were performed as described previously (Auxtová et al., 1995) and the effect of GGMOs (10^{-10} M) on the 2,4-D-induced elongation growth of pea stem segments after 18 h was calculated according to McDougal and Fry (1988).

3.4. Preparation of galactoglucomannan oligosaccharides

Oligosaccharides were prepared by partial acid depolymerization of galactoglucomannan from spruce (*Picea abies* L. Karst) secondary cell walls (Capek et al., 2000) with 0.4 M TFA for 70 min at 100 °C. After evaporation of the acid a mixture of mono- and oligosaccharides was separated on a column (200 × 2.5 cm) of Bio-Gel- P2 by water elution. Fractions (5 ml) were collected and analyzed for the carbohydrate content by phenol–sulphuric acid assay (Dubois et al., 1956). The degree of polymerization (d.p.) was identified by comparison with elution volumes of malto-oligosaccharides (Serva, Germany) used as reference standard. The resulting mixture of GGMOs d.p. 4–8 was used for bioassays.

3.5. Preparation of GGMOs with reduced content of α-D-galactosyl residues

Partially degalactosylated oligosaccharides were prepared by treatment of GGMOs with α-D-galactosidase (EC 3.2.1.22) from Green Coffee Beans (Sigma, USA) in 50 mM sodium acetate buffer pH 6.1 at 31 °C during 170 h as described previously (Bilisics and Kubačková, 1988). The enzyme cleaved under these conditions melibiose but not lactose. The reaction was stopped by heating at 100 °C, the mixture was deionized (Ion exchanger V, Merck, Germany) and from the hydrolyzates after evaporation a mixture of partially degalactosylated GGMOs on the column of Bio-Gel-P2 have been recovered.

3.6. Monosaccharide analysis

The constituents of oligosaccharides with d.p. 4–8 were identified by gas chromatography after hydrolysis with 2 M TFA at 120 °C for 90 min, reduction with $NaBH_4$ and conversion to alditolacetates, using myo-inositol as an internal reference (Bilisics et al., 1982). Hewlett Packard chromatograph, model 5711A, equiped with a flame-ionizing detector and a stainless-steel column (200×0.3 cm) packed with 3% SP-2340 over Chromosorb W AV DMCS (80–100 mesh) was employed for gas chromatography at 180–210 °C (4 min), 2 °Cmin^{-1}. The carrier-gas flow (N_2) was 28.5 mlmin^{-1}.

3.7. Preparation of cell fractions

All operations were performed at 0–4 °C. After incubation (18 h) of pea segments (6–18 g) in buffer solution, plant material was filtered off and washed with distilled water under vacuum. The filtrates were combined and used for the preparation of extracellular fraction (Masuda et al., 1985; Masuda et al., 1988). The resulting plant material was homogenized in fivefold (v/w) 10 mM sodium phosphate buffer containing 20 mM 2-mercaptoethanol with an X-Press Cell Disintegrator (LKB-Biotec, Sweden) at −25 °C and 200 MPa pressure. The homogenate was filtered through Miracloth and washed with distilled water. The combined filtrates were centrifuged at 3000 g/20 min and used for the preparation of intracellular-soluble fraction. The crude cell walls were used for isolation of cell wall glycosidases.

3.8. Preparation of intracellular-soluble and extracellular fractions

Preparation of two fractions was performed identically from the above mentioned combined filtrates after removal of possible debris by centrifugation at 20,000 *g*/20 min (4 °C) and a successive precipitation with ammonium sulfate at a 80% saturation of the solutions followed by centrifugation of the precipitates at 20,000 *g*/20 min. The obtained preparations were dissolved in a solution containing 5 mg of ammonium sulfate/1 ml and stored after lyophilization at −30 °C with negligible loss of enzyme activities (up to 10%) (Bahl and Agrawal, 1969).

3.9. Purification of cell wall material

Purified cell walls for the subsequent stepwise extraction of glycosidases were prepared from the crude cell wall fraction by the method of Masuda et al. (1988). Crude cell walls were suspended and stirred at room temperature in 0.005% sodium deoxycholate for 2 h and filtered through Miracloth. The retained material was washed with distilled water. The preparation resuspended in water was stored after lyophilization at −30 °C with negligible loss of enzyme activities (up to 10%).

3.10. Extraction of cell wall glycosidases

Extraction of glycosidases from the purified cell walls (Masuda et al., 1988) was performed stepwise with 0.2 M NaCl in 50 mM Tris pH 8.0 (2 h, 4 °C, Tris extractable fraction (McCleary, 1983; Sekhar and DeMason, 1990) and 3 M LiCl in 10 mM sodium citrate buffer pH 5.5 (48 h, 4 °C, LiCl extractable fraction) (Labrador and Nicolas, 1985; Hatfield and Nevins, 1986; Seara et al., 1988). The fractions were combined and contained ion-extractable ionically bound glycosidases. Further extraction of glycosidases from cell walls was made by 0.5% EDTA in 50 mM sodium phosphate pH 6.8 (32 °C, 48 h, EDTA extractable fraction) (Masuda et al., 1988). After repeated dialysis against 100 mM sodium citrate buffer (pH 5.0) all fractions were concentrated on Amicon PM 10 and used for the subsequent determination of glycosidase activities. Remaining material after these extractions, containing tightly bound enzymes, was designated as the non-extractable fraction.

3.11. Enzyme assay

The activity of soluble glycosidases as well as enzymes tightly-bound to cell wall after adding model substrates were determined at pH optimum photometrically at 410 nm (Masuda et al., 1985). The release of *p*-nitrophenol from model substrates has been determined: *p*-nitrophenyl-α-galactoside for α-galactosidase (pH 5.0), *p*-nitrophenyl-β-D-glucoside for β-D-glucosidase (pH 5.5), *p*-nitrophenyl-β-D-mannoside for β-D-mannosidase (pH 5.3), *p*-nitrophenyl-β-D-galactoside for β-D-galactosidase (pH 5.4), *p*-nitrophenyl-β-D-xyloside for β-D-xylosidase (pH 5.0), and *p*-nitrophenyl-α-L–arabinoside for α-L-arabinosidase (pH 4.9). The assays were performed in 100 mM sodium acetate buffer at 37 °C. Specific activities of the enzymes were expressed as nkat μg^{-1} of protein. All experiments were repeated three times with three replicates.

3.12. Protein assay

Protein in the soluble fraction was assayed according to Bradford (1976) employing bovine serum albumin as reference. In the insoluble fraction the protein content was determined according to Lowry (1951) and by the elementar analysis (% $N \times 6.25$).

3.13. Statistics

All experiments were evaluated by the use of Student's *t*-test and the statistical program ANOVA – Statgraphic 5.0 (a, b, c, d — $P < 0.001$, 0.002, 0.005, 0.01; e,

f – $P < 0.02$, 0.05 are different degrees of significancy, N – non-significant - shown directly in the text).

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