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Glycanase activities associated with cell walls of *Cicer arietinum* L. epicotyls

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Abstract

The effect of the proteins extracted with 1 M LiCl from the *Cicer arietinum* etiolated epicotyl cell walls on the degradation of polysaccharides extracted with alkali was studied. The hemicellulosic polysaccharides were fractionated into three fractions extracted with 4 % KOH, 4 % KOH containing 8 M urea, and 24 % KOH. The protein extract showed exo-glycanase activity against all three hemicellulosic fractions whilst endo-glycanase activity was shown mainly against the hemicellulosic polysaccharides extracted with 4 % KOH.

Additional key words: autolysis, arabinogalactan, hemicellulose, pectin.

Introduction

The autolytic process of plant cell walls has been reported using isolated cell walls derived from gramineae (Huber and Nevins 1979), dicot (Labrador and Nicolás 1985 and Revilla *et al.* 1986) and gymnosperm (Llamazares *et al.* 1987) shoot axes. Previous studies carried out with *Cicer arietinum* L. epicotyls demonstrated that their cell walls autolytically released both polysaccharides, which included an acidic arabinogalactan and xyloglucan, and monosaccharides which were mainly galactose with smaller amounts of arabinose, xylose and glucose (Revilla *et al.* 1986). Furthermore, α- and ß-D-galactosidase, α- and ß-D-glucosidase, α-D-arabinosidase, and ß-D-xylosidase are associated with *Cicer arietinum* cell walls (Dopico *et al.* 1989). ß-D-galactosidase, which releases only monomeric galactose from the cell wall, has been proposed as the main enzyme responsible for autolysis in the cell walls of *Cicer arietinum* (Dopico *et al.* 1990). However, it is not likely that exo-glycanases have much impact on the release of polymeric components (*e.g.*

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arabinogalactan and xyloglucan) from the cell wall. In this paper, we report the effect of both endo- and exo-glycanases extracted from the chickpea cell walls on three cell wall hemicellulosic polysaccharide preparations.

Materials and methods

Biological material: *Cicer arietinum* L. seeds were soaked in running tap water at 25 °C for 24 h, and germinated on moistened filter paper in darkness at 25 °C. After four days, when the epicotyls were in the fast growth phase, they were harvested.

Extraction of cell wall protein: Cell walls were prepared as described by Revilla *et al.* (1986). Freshly harvested epicotyls (100 g of fresh mass) were homogenized in an *Omnimixer* (*Sorvall*, Newtown, USA) with 250 cm^3 of ice cold 50 mM sodium citrate-phosphate buffer, pH 5.5. The homogenate was filtered through a glass fiber filter (*Whatman GF/A*), washed with ice cold buffer, acetone at -20 °C, and again with ice cold buffer. During the washing procedure the cell wall preparation was maintained in suspension by continuous stirring.

Proteins were extracted from the cell wall preparation by washing twice with 100 cm^3 of 50 mM sodium citrate-phosphate buffer, pH 5.5, containing 1 M LiCl at 4 °C for 24 h (Acebes and Zarra 1992). After the extraction, the wall suspension was filtered through *Miracloth* and washed with an equal volume of 50 mM sodium citrate-phosphate buffer, pH 5.5. The protein extracts and washings were combined, dialyzed against the same buffer and concentrated with an ultrafiltration cell (*Amicon PM 10*). All the subsequent operations were carried out at 4 °C. The concentrated extract was centrifuged (10 000 *g*, 20 min) and any precipitate was discarded. Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. About 150 μg protein per g of epicotyl was obtained.

Hemicellulose extraction: 4-d-old chickpea epicotyls were fixed in boiling methanol, rehydrated, treated with pronase and homogenized. After that, the homogenate was treated with hog pancreas α-amylase, and then washed with water, acetone, methanol-chloroform (1:1, v/v), ethylether and air dried (Lorences and Zarra 1986). The dried cell walls were depectinated with 20 mM ammonium oxalate, pH 4.0 (1 h, 70 °C) four times (2.5 v/m). The hemicellulosic polysaccharides were extracted as described by Nishitani and Masuda (1983). Depectinated cell walls were sequentially extracted (2.5 v/m) for 1 d each three times with 4 % KOH under nitrogen atmosphere, 4 % KOH containing 8 M urea, and 24 % KOH under the same conditions. The extracts were neutralized with acetic acid, dialyzed against distilled water and centrifuged (10 000 *g*, 20 min). The three supernatants were lyophilized and referred as to KI, KI-U and KII hemicellulose fractions, respectively.

Enzyme assays: Reaction mixtures containing 3 mg of total sugars of KI, KI-U or KII hemicellulose fractions, respectively, dissolved in 1.5 cm^3 of 50 mM sodium citrate-phosphate buffer, pH 5.5, containing 0.02 % sodium azide, and 1.5 cm^3 of protein extract (130 - 250 μg protein cm^{-3}) were incubated for different periods of time at

30° C. One drop of toluene was added to the reaction mixture to prevent bacterial contamination. A protein extract boiled for 30 min incubated in the same conditions was used as control.

Hemicellulase activity was measured reductometrically using the Nelson-Somogyi method (Somogyi 1952) and as the decrease in viscosity of the incubation medium as measured with an *Ostwald* viscosimeter (*Cannon-Fenske*, Barcelona, Spain).

Molecular mass distribution of the reaction products: After incubation of each fraction with protein extract, ethanol at a final concentration of 80 % was added and the mixture was centrifuged at 10 000 *g* for 30 min. The precipitated polysaccharides were dried, dissolved in 2 cm^3 of 1 M NaOH, applied on a column of *Sepharose CL-4B* (92 × 1.6 cm) and eluted with the same solution. 2 cm^3 fractions were collected and the amount of total sugars of each fraction was measured by the phenol-sulfuric acid method (Dubois *et al.* 1956).

The supernatant, with ethanol-soluble sugars was also studied. Total, reducing and neutral sugars were measured. After that, the remaining volumes of each fraction were dried using a vacuum rotary-evaporator, dissolved in 2.1 cm^3 of 10 mM citrate-phosphate buffer, pH 6.0, and applied on a column of *Bio Gel P-2* (92 × 1.6 cm), eluted with the same solution. 2 cm^3 fractions were collected and the amounts of total sugars determined.

Neutral sugar composition of ethanol-soluble and ethanol-insoluble sugars were determined by gas liquid chromatography as described by Lorences and Zarra (1986).

Results

Three hemicellulosic fractions were obtained from depectinated cell walls of chick-pea. The KI and KII were the main hemicellulosic fractions while the KI-U was present in a lower amount (Table 1). The fraction KI was mainly composed of

Table 1. Neutral sugar composition of the hemicellulosic fractions extracted from cell walls of chickpea epicotyls. Depectinated cell walls were sequentially extracted with 4 % KOH, 4 % KOH containing 8 M urea, and 24 % KOH. The extracts neutralized with acetic acid, dialyzed against distilled water, and water-soluble materials were considered as KI, KI-U and KII hemicellulosic fractions, respectively. The neutral sugar analysis was performed by GLC.

Saccharide [molar %]	Fraction KI	KI-U	KII
Total saccharides	40.1 ± 2.3	13.7 ± 1.4	46.2 ± 1.4
Fucose	4.1 ± 0.2	3.6 ± 0.7	3.0 ± 0.2
Arabinose	33.2 ± 1.3	8.2 ± 0.2	9.6 ± 0.4
Xylose	3.0 ± 0.1	4.7 ± 0.4	22.8 ± 0.6
Mannose	0.8 ± 0.2	5.1 ± 1.1	4.6 ± 0.1
Galactose	37.2 ± 0.6	12.5 ± 0.3	18.1 ± 0.5
Glucose	21.6 ± 1.6	65.9 ± 1.5	41.9 ± 0.6

arabinose, galactose and glucose. The presence of an arabinogalactan in this fraction has been described for dicot axis (Nishitani and Masuda 1981). The fraction KI-U, a minor fraction in the cell wall of chickpea, was enriched in glucose with low amount of arabinose and galactose. The KII fraction was mainly composed of glucose, xylose and galactose with small amounts of arabinose, fucose and mannose.

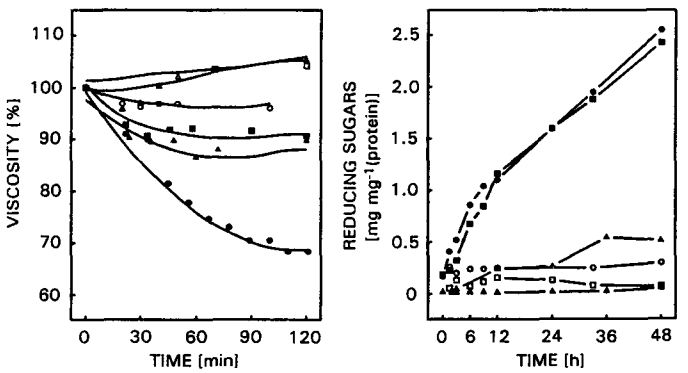

Fig. 1. Effect of the protein extract on the viscosity of the KI (*circles*), KI-U (*triangles*) and KII (*squares*) fractions. *Closed symbols* - active proteins, *open symbols* - inactive boiled protein extract. The effect of active protein extract was significantly different from the controls as checked by Student's *t*-test. $P < 0.001$, < 0.002 and < 0.002 for KI, KIU and KII, respectively.

Fig. 2. Effect of the protein extract on the release of reducing sugars from KI (*circles*), KI-U (*triangles*) and KII (*squares*) fractions. *Closed symbols* - active proteins, *open symbols* - inactive boiled protein extract. $P < 0.01$, < 0.05 and < 0.01 for KI, KIU and KII, respectively.

The three hemicellulosic fractions were incubated with the proteins extracted from the cell wall and the decrease in viscosity was measured (Fig. 1). The protein extract showed activity against the three fractions, the activity against the KI being higher than against KI-U and KII fractions. Furthermore, the glycanase activity of the protein extract against the three fractions was also measured as the increase in the amount of reducing sugars (Fig. 2). The activity against both KI and KII fraction was high, but it was low against the KI-U fraction. The high activity of the protein extract against the KI fraction measured as decrease in viscosity as well as increase in reducing sugars suggests the presence of both exo- and endo-glycanases able to degrade the polysaccharides present in that fraction. However, in the case of the KII fraction the exo-glycanases must be the main activity.

After the incubation of the three different fractions with the protein extract, the molecular mass distribution of the polysaccharides of the KI fraction suffered a dramatic shift towards the low molecular mass region (Fig. 3). The peak which eluted in the void volume region was strongly reduced and a peak in the region between the void volume and 510 kDa appeared after the incubation. This result was in good agreement with the presence in the protein extract of endo-glycanases. The molecular mass distribution of both KI-U and KII fractions was slightly shifted to the low molecular mass region (Fig. 3).

The total saccharides present in the ethanol-soluble reaction products are shown in Fig. 4. The activity was higher against the KI and KII fractions and lower against the KI-U. The ethanol-soluble saccharides released from the three fractions by the protein extracts after an incubation period of 9 h were chromatographed on a Bio Gel P-2 column (Fig. 5). The ethanol-soluble sugars from the KI fraction showed two

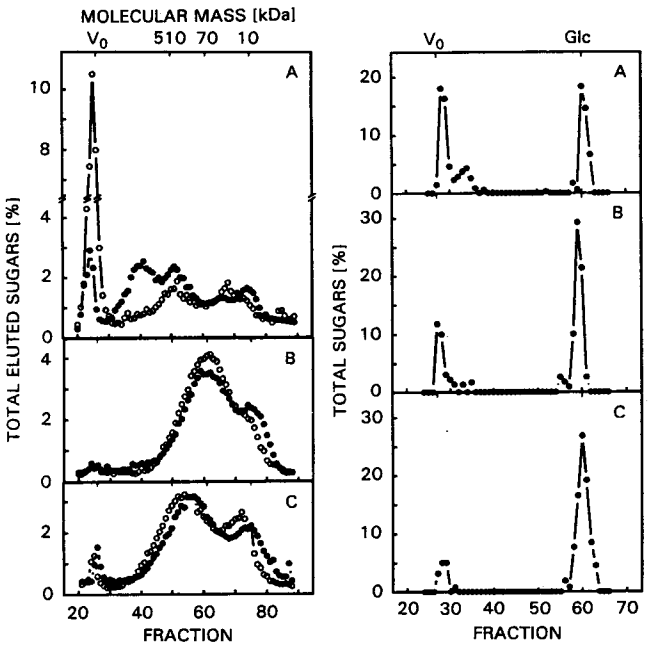

Fig. 3. Effect of the protein extract on the molecular mass distribution of the ethanol-insoluble polysaccharides from the KI (*A*), KI-U (*B*) and KII (*C*) fractions. The different fractions were incubated with the protein extract for 0 (*open circles*) and for 24 h (*closed circles*). Calibration scale using authentic dextrans is shown at the top. V_0 - void volume.

Fig. 5. Bio Gel P-2 chromatography of the ethanol soluble sugars released after incubation of the KI (*A*), KI-U (*B*) and KII (*C*) fraction with the protein extract for 9 h. V_0 - void volume, Glc - glucose.

peaks. The first peak, with a small shoulder, eluting in the void volume, accounted for a 55 % of the total recovered sugars. It was mainly composed by arabinose, its relative amount being higher than 90 %. The second one eluted in the region corresponding to monosaccharides, and it was mainly composed of glucose (more than 90 %) and a small amount of galactose (*ca.* 7 %). The ethanol-soluble sugars released from the fraction KI-U showed also two peaks when they were subjected to Bio Gel P-2 chromatography (Fig. 5). The peak corresponding to the void volume was smaller (29.5 %), as compared with the same peak in the KI fraction. It was mainly composed of arabinose (*ca.* 80 %), with small amounts of xylose and glucose. The second peak corresponding to monosaccharides accounted for a 70 % of the total recovered sugars, galactose and glucose being the main components. The ethanol-

soluble sugars released from the KII fraction showed also two peaks (Fig. 5). The first one, that eluted in the void volume region, was very small (12 %) and was

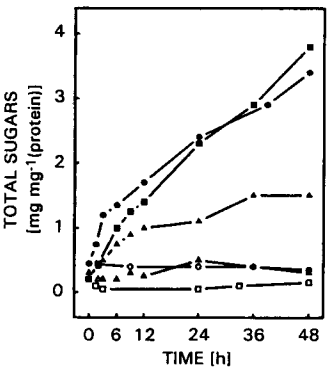

Fig. 4. Effect of the protein extract on the amount of total sugars present in the ethanol-soluble fraction from KI (*circles*), KI-U (*triangles*) and KII (*squares*) fractions. *Closed symbols* - active proteins, *open symbols* - inactive boiled protein extract. $P < 0.01$, < 0.01 and < 0.005 for KI, KIU and KII, respectively.

composed of arabinose (*ca.* 90 %). The second one in the monosaccharides eluting region, composed by galactose (41 %), glucose (38 %) and xylose (14 %). In general, the relative amount of total sugars eluting in the void volume region decreased from KI through KII fractions, while the monosaccharide peak increased. Arabinose was the main component of the void volume peak from the three fractions. Galactose and glucose were the main components of the monosaccharide peaks.

Discussion

The increase in the amount of reducing sugars caused by the protein extract when incubated with the three hemicellulosic fractions (Fig. 2), as well as the release of a monosaccharide fraction (Fig. 5), is in agreement with the presence of different exo-glycanases (*i.e.* galactosidase and glucosidase), as it has been previously shown (Dopico *et al.* 1989). A β-D-galactosidase extracted from the cell walls with 3 M LiCl has been postulated as the main enzyme involved in the autolytic process in the chickpea cell walls. Such enzyme was able to release only monomeric galactose from the different cell wall fractions (Dopico *et al.* 1990). However, it is not likely that exo-glycanases have much impact on the cell wall components and, therefore, may not be the main enzyme responsible for cell wall autolysis (Fry 1988).

Our results clearly show the presence of endo- and exo-glycanases in the proteins extracted from the cell wall with 1 M LiCl as has been postulated in previous work (Revilla *et al.* 1986). The main effect of endo-type glycanases was on the KI fraction as indicated by the important decrease in viscosity (Fig. 1), and the shift in the molecular mass distribution of the total polysaccharides (Fig. 3). The protein extract was able to release from the KI fraction ethanol-soluble oligosaccharides with

a degree of polymerization higher than 10 mainly composed of arabinose (Fig. 5). Since arabinose domains are a constituent of arabinogalactan (Keegstra *et al.* 1973), which is constituent of the KI fraction (Nishitani and Masuda 1981, 1983 and Table 1), it seems possible to suggest that such endo-glycanase activity was able to degrade the arabinogalactan to some extent. Furthermore, a polymeric component enriched in arabinose was released when the chickpea cell walls were incubated with a partially purified protein extracted with 3 M LiCl (Dopico *et al.* 1989). Thus, some glycanases other than ß-D-galactosidase must be acting during chickpea cell wall autolysis. At least some endo-glycanase might play an important role during autolysis facilitating the action of exo-glycanases (*e.g.* glucosidase and galactosidase) in order to release monomeric glucose and galactose (Dopico *et al.* 1990). The presence of an endo-glycanase able to degrade an arabinogalactan is in agreement with the release of arabinogalactan during the autolysis of isolated chickpea cell walls, as it has been previously found (Revilla *et al.* 1986).

A similar glycanase system able to degrade an arabinogalactan from cell walls has been also found in gymnosperm plants (Llamazares *et al.* 1987, Acebes and Zarra 1992). Thus, it seems that the presence in the cell wall of glycanases able to degrade arabinogalactan is a general phenomenon for higher plants.

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