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Chemical modification and fractionation of pea stem polysaccharides

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Pea stem tissue was fractionated by chemical and enzymatic methods and the water soluble fractions analyzed by NMR spectroscopy and high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD). The obtained results indicate that arabinogalactoxyloglucan in similar purity could be isolated by one-step extraction with 2% NaOH or 5% $ZnCl_2$, as well as by two-step treatment with pronase and α-amylase. By quaternization under alkaline conditions with 3-chloro-2-hydroxyprophyltrimethylammonium chloride (CHMAC), a mixture of arabinogalactan and galacturonan modified with trimethylammoniumhydroxypropyl (TMAHP) group (10.8% yield) could be isolated. By extraction with 2% NaOH, a fraction in 19.0% yield could be obtained, contrary to 2.4% yield of polysaccharide when treated with 5% $ZnCl_2$. Treatment with pronase and amylase have given 1.5 or 4.5% yields of soluble frations, while the subsequent residue extracted with ammonium oxalate gave galacturonic acid oligomers (2.1% yield). Direct extraction with $(NH_4)_2(COO)_2$ and without enzymatic pretreatment solubilized only a small part of the material (0.9% yield) consisting of three polysaccharide fractions having the highest molecular weight from all obtained fractions. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Annual plants represent an important source of polysaccharides. The primary cell wall composition of these polysaccharides differs from that of the secondary cell wall. The main difference is in the presence of pectic and hemicellulose polysaccharides, protein content and degree of lignification (York *et al.*, 1985). Pea (*Pisum sativum* L. cv. Tyrkys) stem tissue used for elongation growth studies (Auxtová *et al.*, 1995) also represents a good material for research on fractionation methodologies of the primary cell wall polysaccharides. Related species were studied (Talbott & Ray, 1992), and xyloglucan, arabinan/galactan, and pectic polysaccharides were isolated. There are indications that in the cells of pea plants, arabinogalactans are linked to proteins (Chasan, 1994) and that these components are involved in different biological roles.

It is known that chemical procedures degrade pectin and hemicellulose polymers more dramatically than enzymatic methods (York *et al.*, 1985; Aldington & Fry, 1993). In our previous studies on wood materials (Antal *et al.*, 1984) and annual plants (Šimkovic *et al.*, 1992) we have used quarternary ammonium alkylating agents for modification and fractionation of plant cell walls. Also, treatment with $ZnCl_2$ was applied on wheat straw (Šimkovic *et al.*, 1994), which resulted in hydrolysis of polysaccharide side chains. In the present paper the goal was to isolate polysaccharides by direct chemical modification of pea stems with CHMAC and to compare these fractions with those isolated by known methods. The materials obtained were analyzed by NMR spectroscopy and the composition of fractions compared with HPAEC–PAD analysis using the same gradient method.

EXPERIMENTAL METHODS

Seeds of pea, *Pisum sativum* L. cv. Tyrkys, were grown in trays of moist vermiculite for 9–10 days in the dark at 24°C (Auxtová *et al.*, 1995). The stems cut between roots and apocotyls were dissected into segments and homogenized in an excess of water and lyophilized. The dry material obtained represented 6.5% of fresh mass weight, containing 7.1% of nitrogen and 1.5% of Klason lignin.

The fractionation procedures used are listed in Table 1. According to the first procedure (P1), the freshly cut segments were extracted with 2% NaOH (1:10 w/v ratio) for 2 h at 100°C. The mixture was dialyzed (8–15 kDa MWCO, Serva), separated into the water-soluble part and water-insoluble residue, and then lyophilized.

Table 1. Yields of fractions obtained by fractionation of pea stems

Procedure	Method	Yield (%)	Fraction
P1	Extraction NaOH	19.0[a]	XG[b], AG[c], PGA[d], degraded proteins
		12.7[e]	
P2	Quaternization	10.8[f]	TMAHP-AG, PGA
		13.4[g]	
P3	Pehydrolysis	2.4[h]	XG
	$ZnCl_2$	36.1[i]	
P4	Enzymatic Pronase	1.5[j]	XG, AG
		31.9[k]	
	α-Amylase	4.5[l]	XG
		23.8[m]	
P5	Extraction $(NH_4)_2(COO)_2$	2.1[n]	PGA oligosaccharides
		15.2[o]	
		0.9[p]	XG, PGA, degraded proteins and unknown
		21.0[r]	

[a] water-soluble fraction, N = 4.43%;
[b] xyloglucan;
[c] arabinogalactan;
[d] polygalacturonan;
[e] insoluble residue, N = 0.29%;
[f] water-soluble fraction, N = 7.30%;
[g] insoluble residue, N = 1.10%;
[h] water-soluble fraction, N = 3.12%;
[i] insoluble residue, N = 2.83%;
[j] water-soluble fraction, N = 2.21%;
[k] insoluble residue, N = 3.08%;
[l] water-soluble fraction from residue after α-amylase treatment, N = 0.45%;
[m] insoluble residue, N = 3.78%;
[n] water-soluble fraction, N = 1.37%;
[o] insoluble residue, N = 0.85%;
[p] water-soluble fraction obtained by direct extraction of stems, N = 0.54%;
[r] insoluble residue, N = 2.10%.

When quaternized (P2), the pea stems (5.75 g of dry mass, containing 4.67 mol of water) were mixed with 5.75 g of CHMAC (50% solution, containing 0.16 mol of water) and 5.75 g of solid NaOH and stirred for 24 h at 22°C and subsequently for 4 h at 80°C and worked up as described above.

For the $ZnCl_2$ pretreatment (P3), the freshly cut stems (131.2 g) were homogenized in water (400 cm^3) and lyophilized (8.6 g, N = 7.07%). Part of this sample (2.65 g) was mixed with 5% $ZnCl_2$ solution (265 cm^3; HCl was added to achieve complete $ZnCl_2$ solubilization) and vacuum-evaporated to dryness. The sample was mixed with ethanol (96% solution containing 5% HCl) and allowed to stand overnight and separated to eluant and residue (0.96 g, N = 2.83%) by washing with the acidic ethanol and water. The eluant was dialyzed and lyophilized.

For enzymatic fractionation (P4), the segments (170.4 g) were homogenized in 1000 cm^3 of CH_3COONH_4 (0.05 M)/$CaCl_2$ (0.015 M) buffer (pH 8.0) and treated with pronase (ex *Streptomyces griseus*, Koch-Light, 26 mg) for 150 h (5 mg additions every 24 h) at 37°C. After enzyme denaturation (5 min heating at 100°C), the sample was separated to the water-soluble part (dialysis, lyophilization) and residue (lyophilization), followed by analogical pretreatment of the residue (3.37 g) with α-amylase (ex *B. subtilis*, Koch-Light, 50 mg) in 500 cm^3 of KH_2PO_4 buffer (pH 7.0) for 100 h at 25°C, from which the water-soluble part and the residue (2.51 g) were obtained.

The insoluble residue (3.61 g), after amylase treatment (P4), was extracted (P5) with ammonium oxalate (0.02 M, pH 4) at 70°C for 1 h. The pH was adjusted to 7 with KOH solution and separated to eluant (dialysis, lyophilization) and residue (lyophilization). For direct extraction with ammonium oxalate, stems (126.12 g) were homogenized with water (400 cm^3) in the presence of NaN_3 (5 g). After dialysis and lyphilization (2.33 g, 28.9% yield), the ammonium oxalate extraction was run under the same conditions as above. The insoluble residue was extracted for a second time in the same way and the eluants were combined, dialyzed, and lyphilized.

HPAE chromatography was performed with a

Dionex metal-free BioLC on CarboPac PA-1 (4×250 mm) column and detected using a pulsed amperometric detection equipped with a gold working electrode. The electrochemical detector operated in the pulsed amperometric mode. The column was eluted at 1 cm^3/min using a linear gradient from 0 to 100% of 1 M AcONa in 100 mM NaOH over 40 min.

All the other methods used were described previously (Šimkovic *et al.*, 1992).

RESULTS AND DISCUSSION

The pea stems contain proteins which complicate the fractionation. According to the rule which multiplies percentage of nitrogen by 6.25 (Selvedran & O'Neill, 1987), the starting material contains 44.2% of protein. By pretreatment with 2% NaOH at 100°C (Table 1, P1), the protein could be degraded to small fragments and the insoluble residue free of nitrogen obtained. According to the ^{13}C-NMR spectrum, the water-soluble fraction is a mixture of degraded proteins (182.4, 131.7, 130.3, 116.7, 12.6–43.5 ppm) and arabino- (111.6 and 108.6 ppm), galacto- (105.5 ppm), -xylo- (99.6 ppm), -glucan (103.3 ppm; Ryden & Selvedran, 1990) and some galacturonic acid oligomers (174.5 and 98.3 ppm; Hricovíni *et al.*, 1991). According to other ^{13}C-NMR data (Groman *et al.*, 1994), arabinogalactan could be also considered as a component not chemically linked to xyloglucan. On the basis of gel filtration chromatography it was also suggested that arabinose and galactose are included in a side-chain of pectin and also as components of arabinogalactan not linked to pectin (Talbott and Ray, 1992). As could be seen from HPAEC–PAD profile (Fig. 1a), there is only one polymeric component in the mixture, which is eluted from the column at 35–37 min.

When the material is quaternized under alkaline conditions (P2), then more heterogeneous poly-

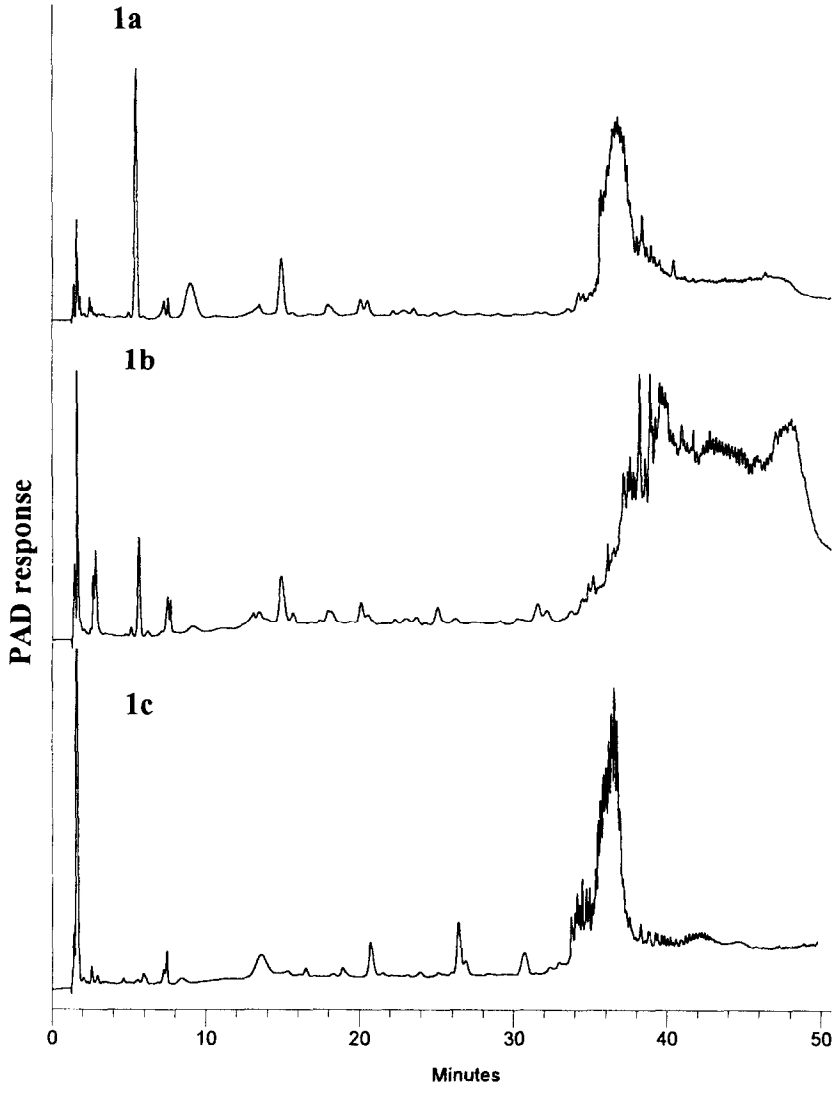

Fig. 1. HPAEC–PAD profiles of water-soluble fractions isolated by: (a) 2% NaOH extraction; (b) quaternization with CHMAC; (c) acidic $ZnCl_2$ hydrolysis. Elution using a linear gradient from 0 to 100% of 1 M AcONa in 0.1 M NaOH.

saccharide fraction is obtained in comparison to P1, as shown on the HPAEC profile (Fig. 1b). Surprisingly, the ^{13}C-NMR spectrum gave only two signals in the anomeric region, at 108.6 (arabinofuranose) and 105.5 ppm (galactopyranose). In the ^{1}H-NMR spectrum, which could be considered as more sensitive to minor components, as well as signals at 5.12, 4.68, and 4.65 ppm, there is also a broad H-1 signal of galacturonan at 5.04 ppm (Ló *et al.*, 1994). The quaternized fraction represented the highest yield from all water-soluble samples (Table 1) which might be affected by the presence of trimethylammoniumhydroxyprophyl groups indicated with high nitrogen content as well as NMR signals (68.6, 67.9, 62.7, 55.3, 54.4, and 53.5 ppm). The assigmnent is supported by the predominant intensity of these signals as well as results obtained by the modification of corn cobs with CHMAC (Šimkovic *et al.*, 1992). The yield of insoluble residue is about the same as for extraction with 2% NaOH (P1).

The $ZnCl_2$ pretreatment (P3) resulted in a lower yield of water-soluble fraction than obtained by P1 and P2 methods (Table 1). According to the HPAEC profile (Fig. 1c), the mixture is slightly more hydrolyzed but still contains a similarly retained polymeric component as that observed for the P1 mixture. The ^{1}H-NMR spectrum indicates the presence of galactose (4.51 ppm), glucose (4.67ppm), xylose and arabinose (4.98 ppm), xylose (5.12 ppm), and fucose (5.26 ppm) H-1 signals. According to the splitting constants (1.25–3.43 Hz), fucose, arabinose, and xylose have α-glycosidic bonds, while glucose and galactose are β-glycosidically bonded, (7.79 Hz). These data are characteristic for xyloglucan structures (Hisamatsu *et al.*, 1992). The nitrogen content of this fraction is lower than analogous fractions obtained by P1 and P2 treatment (Table 1). The yield of water-insoluble residue is the highest of all fractions obtained, and it was not affected by the presnce of salts as concluded from the elemental analysis (C = 41.59%, H = 6.30%, while for the untreated freeze-dryed stems, C = 35.92% and H = 7.07%).

By treatment with pronase (Table 1, P4), a water-soluble eluant, with lower yield than fractions obtained by P1, P2, and P3 procedures, was obtained. According to the ^{13}C-NMR spectrum, this fraction contained a mixture of cleaved peptides and polysaccharides. The individual monosaccharide components could be assigned to arabinose (110.4, 108.6, and 107.7 ppm), galactose (104.5 and 104.3 ppm), glucose (103.9, 103.2, 102.9, and 102.4 ppm) and xylose (101.1 ppm). The ^{1}H-NMR spectrum confirmed the presence of fucose and xylose (5.28 and 5.03 ppm) linked on xyloglucan (Hisamatsu *et al.*, 1992). There were three H-1 signals at 5.23, 5.20, and 5.13 ppm, which did not fit with known NMR data for xyloglucan-linked arabinoses (York *et al.*, 1995). From these data, it is assumed that the fraction is a mixture of arabinogalactan and xyloglucan. The solubilization of polysaccharide by pronase treatment supports the possibility of protein linkage to arabinogalactan (Chasan, 1994). The HPAEC profile (Fig. 2a) is more complicated than fractions obtained by extraction with NaOH or $ZnCl_2$. The residue obtained after pronase treatment contained much more nitrogen than residue after NaOH treatment (Table 1). By subsequent fractionation of the residue after pronase treatment with α-amylase, the water-soluble fraction with higher yield than obtained by pronase or $ZnCl_2$ treatments (Table 1) was obtained. HPAEC profile (Fig. 2b) indicates only one type of polymer present. Because the NMR spectrum of this fraction contained in the anomeric region, the arabino- (109.9 ppm), galacto- (106.0 ppm), xylo- (99.8 and 99.3 ppm) and gluco- (102.9 and 102.1 ppm) signals, it is assumed that the polysaccharide is xyloglucan, containing chemically linked arabinose and galactose. Because the HPAEC profiles of P1, P3, and P4 amylase-solubilized fraction (Figs 1 and 2) contained the polysaccharide component at the same retention time (35–38 min), it is probable that this broad band is related to the xyloglucan component of these mixtures.

The fraction obtained by ammonium oxalate extraction (Table 1, P5) after pretreatment with pronase and amylase (see Experimental) also gave a small yield. According to the ^{13}C-NMR data, these are galacturonic acid oligomers (101.6, 100.9, 100.6, and 100.2 ppm; Hricovíni *et al.*, 1991) probably linked with some arabinofuranose units (109.7 ppm). There is no polymer present in this fraction, as can be seen from the HPAEC profile (Fig. 2c). The direct extraction of tissue with ammonium oxalate resulted in water-soluble fraction with the smallest yield from all fractions obtained (Table 1). According to the ^{1}H-NMR spectrum there were some protein residues present (8.69 ppm) as well as some unusual anomeric signals at 5.86, 5.60, 5.51, and 5.45 ppm, which might be related to some branched sugar units. There were also some ascribed (York *et al.*, 1995; Hisamatsu *et al.*, 1992) anomeric signals typical for xyloglucan saccharide units at 5.37, 5.31 (arabinose), 5.29 (fucose), 4.92 (xylose), and 4.69 ppm (galactose). Some minor polygalacturonic acid signals at 5.11 and 5.04 ppm indicate the presence of pectin. All these signals were so broad that no coupling constants could be determined, indicating the polymeric character of the analyzed mixture. The HPAEC–PAD profile (Fig. 2d) presented the mixture of oligomers and three polysaccharide fractions. The band eluted after 42 min was not present in any of the previous fractions.

The enzyme treatments, although using non-pectolytic enzymes, also resulted in pectin degradation, as conluded from the dramatic difference between the two ammonium oxalate extracts (Figs 2c and 2d). The solubilized fraction after amylase treatment (Fig. 2b) contained xyloglucan, while the subsequent amonium oxalate extract (Fig. 2c) contained PGA oligomers. We

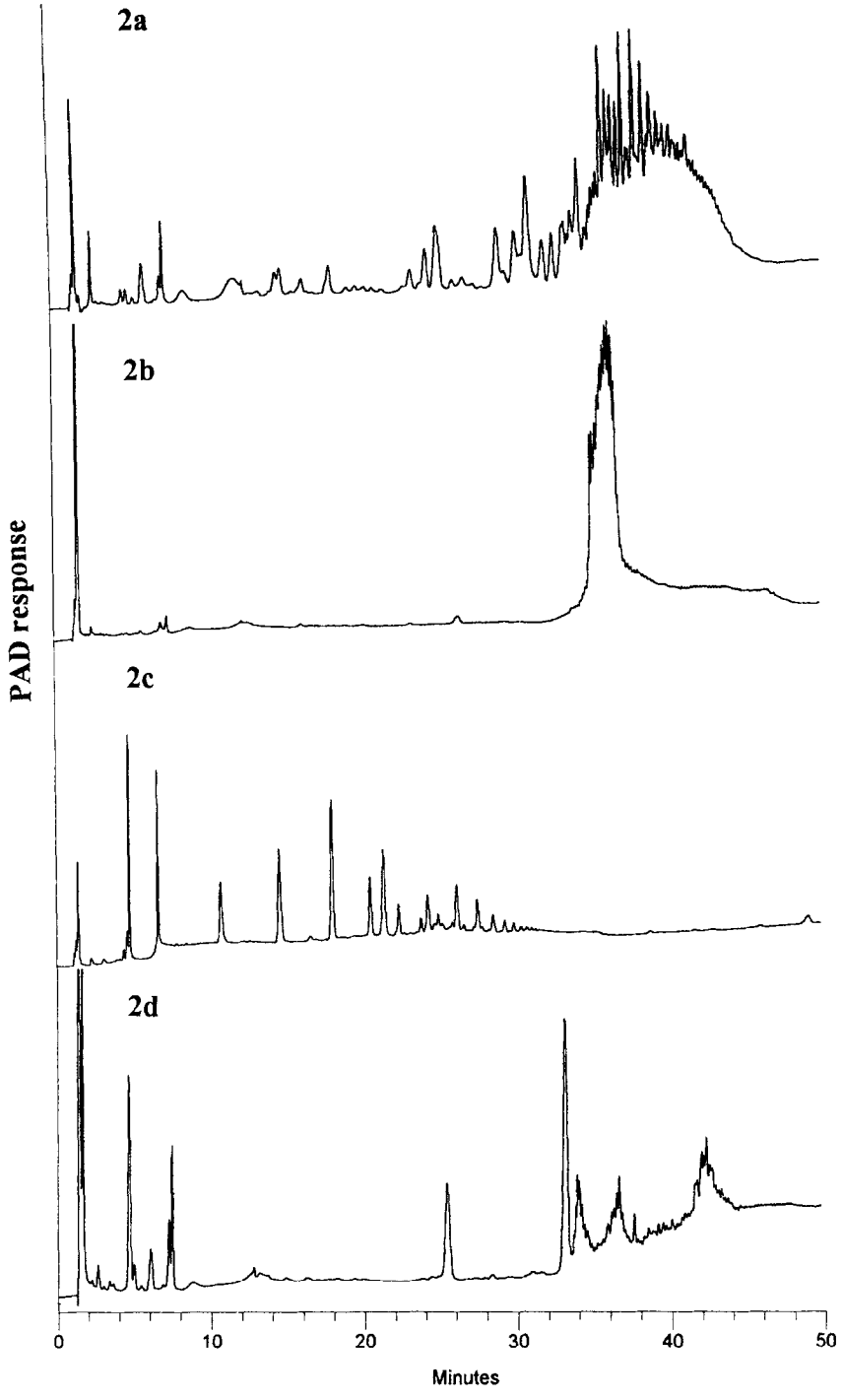

Fig. 2. HPAEC–PAD profiles of water-soluble fractions obtained by (a) pronase treatment and subsequent (b) α-amylase treatment; (c) ammonium oxalate treatment after pronase and amylase pretreatment; (d) direct ammonium oxalate extraction. Same conditions as in Fig. 1.

think that these two fractions should not be combined as was done before (Talbott & Ray, 1992). The yield of ammonium oxalate extract is lower than reported for other sources (O'Neil *et al.*, 1990). The HPAE–PAD chromatogram (Fig. 2c) shows a homological row of oligosaccharides. The two-step enzymatic pretreatment with pronase and amylase (P4), as well as alkaline extraction (P1) and $ZnCl_2$ pretreatment (P3), solubilized xyloglucan fractions. Xyloglucan isolated with pronase might contain longer side chains than that isolated with an α-amylase, which might cleave xylopyranose units linked with α-glycosidic linkage to a glucan main chain. This enzyme could be considered as not specific enough to distinguish between α-glycosidic bonds of xylo- and glucopyranose. The HPAE–PAD profile of the pronase-solubilized fraction (Fig. 2a) is more complicated than the α-amylase-solubilized fraction (Fig. 2b). This might be due to the presence of arabinogalactan-protein

components in the mixture solubilized by pronase treatment. The chemically isolated fractions are obtained in higher yields (Table 1). Most of the data published with HPAE–PAD instruments on similar oligosaccharides (Lerouge *et al.*, 1993; McDougall and Fry, 1991; Spiro *et al.*, 1993; An *et al.*, 1994; Whitecombe *et al.*, 1995) did not contain polysaccharides in the mixture together with oligosaccharides. Chemical extraction with ammonium oxalate after pronase and α-amylase pretreatment gave oligogalacturonides which were free from polysaccharides. All other extraction steps solubilized polysaccharides predominantly.

CONCLUSIONS

Pea stem cell walls could be purified from proteins with 2% NaOH solution more effectively than with pronase. The fractions extracted with NaOH contain arabinogalactan, xyloglucan, and galacturonic acid oligomers. Pronase and α-amylase treatment solubilizes arabinogalactan-protein and xyloglucan in similar yields as $ZnCl_2$ treatment. Also, pectin is degraded by these non-pectolytic enzymes, because of the difference in composition of oxalate extracts obtained with and/or without use of enzymes. Quaternization with CHMAC cleaves TMAHP-arabinogalactan fragments without xyloglucan being present in the soluble fraction.

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