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Characteristics of Pectic Polysaccharides from Rice Shoots

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Pectic polysaccharides from the starch-free cell wall preparation of rice (*Oryza sativa*) shoots have been extracted in sequence with cyclohexane-*trans*-1,2-diaminetetra-acetate (CDTA) and Na_2CO_3. The total amount of polysaccharides extracted with the agents was estimated as approximately 1% of the cell walls. The extracted polysaccharides were fractionated by DEAE-Trisacryl M ion-exchange chromatography yielding five fractions, and the monosaccharide composition and molecular mass were determined for the major fractions. The acidic pectic polymers were constructed from homogalacturonan and rhamnogalacturonan containing the "hairy" region with galactosyl and arabinosyl side-chains. The solubilized pectic polysaccharides after treatment with two pectolytic enzymes accounted for 0.4～0.6% of the starch-free cell walls.

Key words: Cell wall, *Oryza sativa*, Pectic polymer.

INTRODUCTION

Pectin or pectic polysaccharides, which are major constituents of the primary cell walls and middle lamella, are covalently interconnected to the other carbohydrate polymers serving as structural elements of cell walls thus forming a tightly cross-linked matrix network (Carpita and Gibeaut 1993). The structural features of pectic polysaccharides have been extensively studied by the techniques of chemical analysis and enzymic degradation and, therefore, the picture of the polysaccharides appears to be accurate (O'Neill et al. 1990). However, since the investigations of pectic polysaccharides thus far have focused on dicotyledonous plants, the polysaccharides in monocotyledons have yet to be fully understood. The present work describes the characteristics of pectic polysaccharides in the cell walls from rice shoots.

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MATERIALS AND METHODS

1. *Plant material.*

Rice (*Oryza sativa* L. cv. Nipponbare) plants were grown at the Research Institute farm for 3 weeks from seeds under normal cultural conditions and irrigated as necessary, as described by Konno and Tsumuki (1993).

2. *Preparation of cell walls and pectic polysaccharides.*

The shoots were harvested, surface-sterilized in 1% (v/v) NaOCl for 20 min, rinsed in distilled water, dried with paper and weighed. Shoots (ca 500 g fresh weight) were homogenized in 100 mM K-phosphate (pH 7.0) containing 1 M NaCl and 10 mM 2-mercaptoethanol, using a Waring Blender for 5 min at 0°C. The homogenates were filtered through a sintered glass filter under suction at 2~4°C; the filtrate was discarded. The wall residues retained on the filter were washed with ethanol and acetone, and then treated with Actinase E (Kaken Chemical Co., Japan) and α-amylase (pancreas Type I-A, Sigma Chemical Co., USA) as described previously (Konno et al. 1984).

Pectic polysaccharides were solubilized from the cell walls by sequential extraction with 50 mM cyclohexane-*trans*-1,2-diaminetetra-acetate (CDTA, Na-salt) at 20°C for 8 hr and 50 mM Na_2CO_3 at 1°C for 20 hr, as described by Redgwell and Selvendran (1986).

3. *Ion-exchange chromatography.*

Each aliquot (100~150 mg sugar content) of CDTA- and Na_2CO_3-soluble polysaccharides was dialyzed against 20 mM K-phosphate (pH 6.0) and fractionated on a DEAE-Trisacryl M (IBF Biotechnics, Villeneuve-la-Garenne, France) column (2.0×20 cm), previously equilibrated with the same buffer, by stepwise elution with buffer and buffer containing 125, 250 and 500 mM NaCl, and finally with 200 mM NaOH. The eluate was collected as 4-ml fractions and assayed for total sugars.

4. *Gel-permeation chromatography.*

The sample (5~10 mg sugar content) was applied to a column (1.5×90 cm) of Bio-Gel A-1.5m, previously equilibrated with 50 mM Na-acetate (pH 5.2) containing 20 mM EDTA, and eluted with the same buffer. The eluate was collected as 1.8-ml fractions and assayed for total sugars.

5. *Sources of enzymes.*

Endo-pectate lyase (EC 4.2.2.2) and endo-polygalacturonase (EC 3.2.1.15) were purified to electrophoretic homogeneity from cell homogenates of *Erwinia carotovora Er.* (Konno 1988) and from the culture filtrate of *Kluyveromyces fragilis* (IAM 4763) (Konno et al. 1987), respectively, as described.

6. *Enzymic digestion.*

For the endo-pectate lyase treatment, the sample (5~10 mg) was suspended in 10 ml of 50 mM Tris-HCl (pH 8.6) containing 0.9 unit of enzyme and 0.5 mM $CaCl_2$. After incubation at 30°C for 48 hr with continuous stirring in the presence of a drop of toluene and 0.02% (w/v) Na-azide acting as bacteriostatic, the reaction mixture was adjusted to pH 5.0 and stopped by heating at 100°C for 3 min. The reaction mixture for endo-polygalacturonase treatment contained the sample and enzyme (10 units) in 10 ml of 50 mM Na-acetate (pH 5.0). The reaction conditions were the same as described for the endo-pectate lyase. Cell walls (1 g dry weight) were treated with endo-pectate lyase or endo-polygalacturonase as described above. After incubation, the wall residues were separated from digestion products by filtration on glass fiber filters. The products were further filtered with a Molcut L (UFP2-LGC with 10 kDa cut-off; Millipore Corp., USA) to remove the enzymes.

8. *De-esterification of pectic polymer.*

The methoxyl and acetyl groups in acidic polymer were saponified with 10 mM NaOH. After gently stirring for 90 min in an ice-water bath, the solution was adjusted to pH 5.0 with 50 mM glacial acetic acid and dialyzed against distilled water.

9. *Analyses.*

The contents of total sugar and galacturonic acid in each sample were estimated by phenol-H_2SO_4 (Dubois et al. 1956) and *meta*-hydroxydiphenyl (Blumenkrantz and Asboe-Hansen 1973) methods, respectively. Neutral sugars were analyzed by gas-liquid chromatography as their alditol acetate derivatives following trifluoroacetic acid hydrolysis (Albersheim et al. 1967).

RESULTS AND DISCUSSION

The cell wall materials (crude cell walls) obtained after homogenization of shoots were prepared by successive washing with hot ethanol, acetone and ether. Finally, the crude cell walls were treated with protease and α-amylase to remove protein and starch. Ten grams (fresh weight) of shoots yielded 0.6 g (dry weight) of crude cell walls and 0.15 g (dry weight) of starch-free cell wall preparation. The cell walls (100 mg dry weight) were composed of 28.9 mg of xylosyl, 8.64 mg of arabinosyl, 2.45 mg of glucosyl, 1.53 mg of galactosyl, 0.084 mg of rhamnosyl, 0.049 mg of mannosyl and 2.63 mg of galacturonosyl residues. Thus, the noncellulosic neutral glycosyl and galacturonosyl residues accounted for 41.7% and 2.6% of the cell walls, respectively.

The bulk of pectic polysaccharides was extracted in sequence with

Table 1. Monosaccharide composition of pectic fractions of cell walls from rice shoots.

Fraction	Weight % of total sugars						
	Rha	Ara	Xyl	Man	Gal	Glc	GalUA
CDTA-sol.	0.48	7.06	1.40	Tr	5.30	1.25	84.5
C-1	1.38	47.4	2.59	—	39.0	1.93	7.73
C-2	4.91	28.3	2.21	—	25.0	0.42	39.2
C-3	2.55	3.24	0.45	0.05	2.58	0.48	90.7
Na_2CO_3-sol.	—	19.6	24.9	—	5.75	3.29	46.5
N-1	0.051	40.6	36.7	0.60	16.1	0.88	5.10
N-2	1.48	35.8	39.6	Tr	12.3	1.39	9.41
N-3	7.24	15.0	10.9	—	8.17	2.35	56.4
Treated C-2	6.23	44.2	Tr	—	47.4	—	2.01

Data represent the mean of two analyses, each run in duplicate. Tr; trace, Rha; rhamnose, Ara; arabinose, Xyl; xylose, Man; mannose, Gal; galactose, Glc; glucose, GalUA; galacturonic acid.

CDTA and Na_2CO_3 from the starch-free cell wall preparation. The pectic polysaccharides solubilized with CDTA and Na_2CO_3 accounted for $0.60\pm0.02\%$ and $0.42\pm0.06\%$, respectively, of the cell wall.

Table 1 shows the monosaccharide compositions of pectic polysaccharides. The CDTA- and Na_2CO_3-soluble polysaccharides contained 84.5% and 46.5% of galacturonic acid, respectively, while these polysaccharides contained different amounts of neutral sugars, mainly arabinosyl, xylosyl and galactosyl residues together with minor amounts of glucosyl and rhamnosyl residues. The composition of rice pectic polysaccharides thus differs considerably from those of pectic polysaccharides derived from carrot cell cultures (Konno et al. 1986) and several dicotyledonous plants (Darvill et al. 1980).

The extracted pectic polysaccharides were chromatographed by DEAE-Trisacryl M to separate the polysaccharides into neutral and acidic polymers. Each of the CDTA- and Na_2CO_3-soluble polysaccharides could be fractionated into five polymers, as illustrated in Figs. 1 and 2. The yields were 14.1 mg of fraction C-1, 8.9 mg of fraction C-2, 16.1 mg of fraction C-3, 0.9 mg of fraction C-4 and 1.2 mg of fraction C-5 from 116 mg of CDTA-polysaccharides, and 14.7 mg of fraction N-1, 10.0 mg of fraction N-2, 11.5 mg of fraction N-3, 0.9 mg of fraction N-4 and 2.8 mg of fraction N-5 from 95.6 mg of Na_2CO_3-polysaccharides. Thus, the chromatographic recoveries were ca 36% for CDTA-polysaccharides and ca 42% for Na_2CO_3-polysaccharides. As pointed out by Redgwell and Selvendran (1986), the results may be due to the irreversible adsorption of uronic acid-rich polymers on the anion-exchangers. This problem remains in the present experiment.

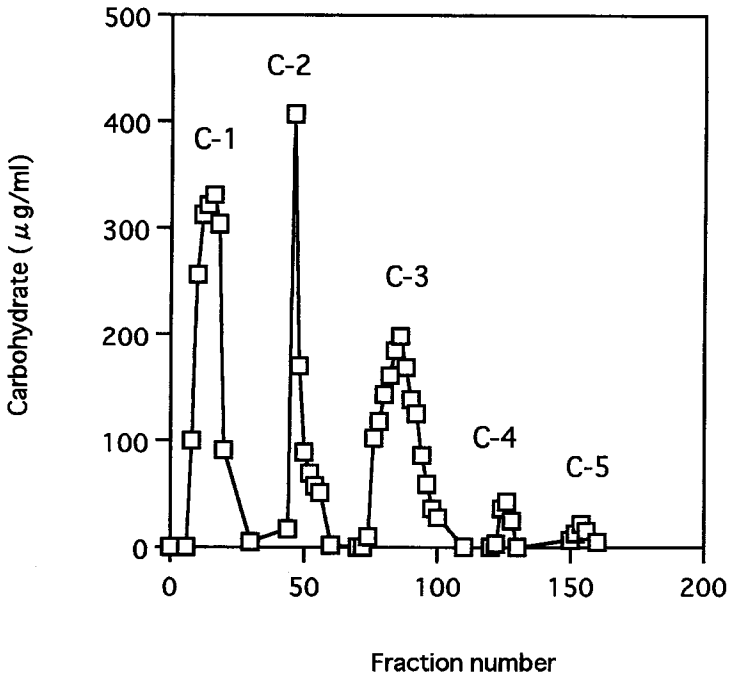

Fig. 1. Ion-exchange chromatography of the CDTA-soluble polysaccharides on DEAE-Trisacryl M. The column was eluted with 20 mM K-phosphate buffer (pH 6.0) (fractions 1-30), buffer containing 125 mM NaCl (fractions 31-60), buffer containing 250 mM NaCl (fractions 61-110) and buffer containing 500 mM NaCl (fractions 111-130), and with 200 mM NaOH (fractions 131-170), respectively.

The main fractions, C-1, C-2, C-3, N-1, N-2 and N-3, were pooled separately and dialyzed against water, and the monosaccharide composition was determined (Table 1). Fractions C-1 and N-1, which represented 34.2% and 36.8% of the yield, did not bind to the DEAE-Trisacryl M column in 20 mM K-phosphate (pH 6.0), and contained neutral polymers not covalently linked to the acidic polymers. Fraction C-1 had a high content of arabinosyl and galactosyl residues, suggesting a sugar composition typical for an arabinogalactan. Fractions N-1 and N-2 comprised a high arabinosyl-to-xylosyl residue ratio (ca 77 wt %), suggesting that the fraction comes from a part of an arabinoxylan located in the hemicellulosic fraction of cell walls. The apparent molecular masses of fractions C-1 and N-1 were 56 kDa and 30 kDa, respectively, as estimated by gel-permeation on a Bio-Gel A-1.5m column by comparison with the elution volume of linear dextrans (T-500, T-70 and T-40; Pharmacia, Uppsala, Sweden).

Fractions C-2 and N-3 were retained on the DEAE-Trisacryl M column and were more enriched in arabinosyl, galactosyl and galacturonosyl resi-

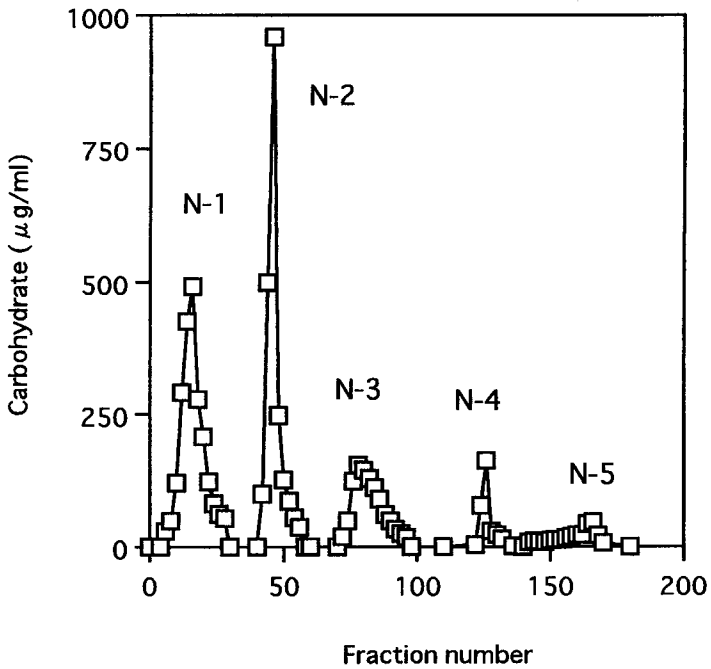

Fig. 2. Ion-exchange chromatography of the Na_2CO_3-soluble polysaccharides on DEAE-Trisacryl M. The column was eluted with 20 mM K-phosphate buffer (pH 6.0) (fractions 1-30), buffer containing 125 mM NaCl (fractions 31-60), buffer containing 250 mM NaCl (fractions 61-110) and buffer containing 500 mM NaCl (fractions 111-140), and with 200 mM NaOH (fractions 141-180), respectively.

dues than the other fractions. It is possible that fractions C-2 and N-3 contain so-called "hairy" or ramified fragments (De Vries et al. 1982) which are comprised mostly of arabinosyl and galactosyl side-chains, such as arabinogalactan and galactan, and short-chains. Fraction C-3 contained a high proportion of galacturonosyl residues (more than 90 wt %), suggesting that the polymer is a homogalacturonan.

2. *Enzymic degradation of isolated cell walls and pectic polymer.*

In general, pectic polysaccharides have been extracted from isolated cell walls with chelating agents such as EDTA, ammonium oxalate and hexametaphosphate. Since the traditional pectic extraction with hot aqueous solutions of these agents has been suggested to cause breakdown of pectic polysaccharides by autohydrolysis and by *trans*-eliminative degradation (Selvendran and O'Neill 1987), the method of Jarvis (Jarvis et al. 1981), in which pectic polysaccharides are solubilized with CDTA and Na_2CO_3 under a mild condition, was used in this experiment. However, these procedures

may be less sensitive. On the other hand, several pectolytic enzymes purified from phytopathogenic bacteria are able to attack isolated cell walls directly and solubilize effectively pectic fragments as the digestion products. Using this method, pectic fractions solubilized from cell walls in cell cultures of sycamore (York et al. 1985), carrot (Konno et al. 1986) and liverwort (Konno et al. 1987), and carrot roots (Konno and Yamasaki 1982) have been studied extensively in detail.

To estimate the amounts of pectic polysaccharides in rice, the cell walls from rice shoots were then submitted to the action of purified endo-pectate lyase from *Erwinia carotovora Er.* and endo-polygalacturonase from *Kluyveromyces fragilis*. After a sufficient reaction time, the carbohydrate released by each enzyme was 4.1～6.0 mg from 1 g dry cell walls, and accounted for 0.4～0.6% of rice cell walls. These values were similar to those obtained by solubilization with CDTA-treatment, indicating that the enzyme solubilizes effectively the pectic fraction of rice cell walls. Unlike the cell walls from dicotyledonous plants, the walls of rice shoots possess a very small amount of pectic polysaccharides, which is consistent with the suggestion that the cell walls from monocotyledonous plants contain only minor amounts of pectic polysaccharides (Jarvis et al. 1988).

To analyze the "hairy" fragments, fraction C-2 with a molecular mass of 82 kDa was submitted to the action of purified endo-polygalacturonase. This enzyme degraded 15.9% of glycosyl linkages in fraction C-2. The prevailing concept is that endo-polygalacturonase hydrolyzes only non-esterified 1, 4-α-D-galacturonosyl linkages in acidic polymers (Burns 1991). It is conceivable that the methylesterified galacturonosyl residues in the polymer would block further degradation by the enzyme. Thus, fraction C-2 was de-esterified by treatment with base prior to the enzyme treatment. Even after an exhaustive reaction, however, the extent of the degradation of fraction C-2 was the same as that of native fraction C-2. It can be deduced, therefore, that the limit of hydrolysis is ascribed to the existence of some enzyme-insusceptible side-chains in the polymer. After the treatment of fraction C-2 with the enzyme, the carbohydrate was resolved into two peaks by the Bio-Gel A-1.5m gel-permeation chromatography (Fig. 3). The products eluted at an inclusion volume of the column were predominantly galacturonosyl residues. Whereas undegraded polymer was composed of 47.4 (w/w) galactosyl, 44.2% arabinosyl and 6.35% rhamnosyl residues, as identified by GLC, and 2.01% galacturonosyl residues. Actually, the content of galacturonosyl residues in the treated fraction C-2 was significantly reduced, but the galactosyl and arabinosyl residues were fairly resistant (Table 1). This strongly suggests that fraction C-2 contains the "hairy" region with

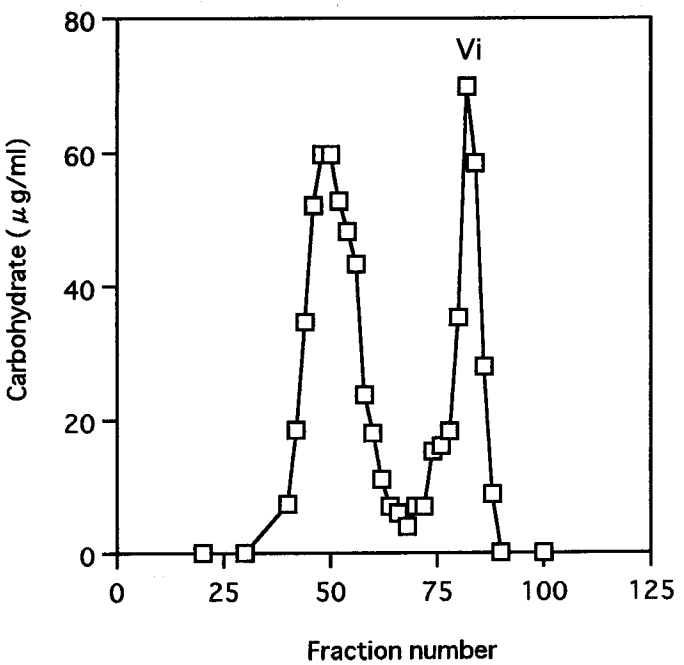

Fig. 3. Gel-permeation chromatography of the enzyme-treated fraction C-2 on Bio-Gel A-1.5m. Experimental conditions as described in MATERIALS AND METHODS. The included volume (Vi) was determined with glucose.

galactosyl and arabinosyl side-chains, as mentioned above.

The degradation of cell walls has been considered to increase dramatically during some events in the plant's life, such as seed germination, proliferation, development and senescence, during cell growth (Taiz 1984). The change depends on the alteration in amount and/or structure of pectic polysaccharides serving as structural elements of cell walls, a process which could involve the action of endogenous or exogenous glycan-hydrolytic enzymes. The characteristics of the hydrolytic enzymes should be helpful in elucidating the metabolic events *in situ* of pectic polysaccharides of cell walls during cell growth.

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イネ幼植物から調製した細胞壁に含まれるペクチン質の性状

今野晴義・積木久明・山崎良樹

イネ幼植物から調製した細胞壁から，50 mM の1, 2-シクロヘキサンジアミン四酢酸と炭酸ナトリウムにて，ペクチン質を化学的に抽出した．抽出される全ペクチン質に含まれる糖含量は，乾燥細胞壁重の約１％に相当した．両者の薬剤で抽出されたペクチン質を DEAE-Trisacryl M カラムクロマトグラフィーによって，５種の多糖画分に分画し，主要３画分の構成糖組成と分子量を検討した．1, 2-シクロヘキサンジアミン四酢酸で抽出され上記カラムに未吸着の中性多糖画分は，アラビノース，ガラクトースから成り，分子量56 kDa であった．また，炭酸ナトリウムで抽出されカラムに未吸着の中性多糖画分は，アラビノース，キシロース，ガラクトースから成り，分子量30 kDa であった．一方，酸性多糖画分は，中性糖側鎖を持ったラムノガラクツロナンとホモガラクツロナンを含んでいた．

Erwinia carotovora Er. からのエンド-ペクチン酸リアーゼと *Kluyveromyces fragilis* からのエンド-ポリガラクツロナーゼの精製標品を，イネ細胞壁に作用させたところ，0.4～0.6％に相当するペクチン質断片が可溶化された．一方，上述したカラムクロマトグラフィーで得られた82 kDa のラムノガラクツロナンにエンド-ポリガラクツロナーゼを作用させると，多量のガラクツロン酸が遊離され，また，未分解画分は90％以上のガラクトースとアラビノースから成っていた．この結果から，この酸性多糖画分の“hairy”領域は，エンド-ポリガラクツロナーゼの作用できないガラクトースとアラビノースのみから成る側鎖であった．

キーワード：イネ細胞壁，酵素分解，ペクチン質