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PECTIC POLYSACCHARIDES OF RICE ENDOSPERM CELL WALLS

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Abstract—Two pectic polysaccharide fractions were purified from rice endosperm cell walls Methylation analysis including carboxyl-reduction and also selective enzymolysis using purified α-L-arabinofuranosidase suggested that both have a main chain consisting of (1 → 4)-linked galacturonic acid and (1 → 2)-linked rhamnose residues which are mostly substituted at the O-4 position Side chains mainly consisting of (1 → 5)-linked arabinose and (1 → 4)-linked galactose residues appear to be attached to the O-4 position of the (1 → 2)-linked rhamnose residues Over 80% of arabinose residues in the side chains are considered to be localized in the outer chains or arabinan regions

INTRODUCTION

Pectic polysaccharides are widely distributed throughout the land plants as components of cell walls and middle lamellae [1, 2] However, the detailed structural features of these pectic polysaccharides are not yet fully understood, especially those from monocot cell walls There is no report of the isolation of polygalacturonan or rhamnogalacturonan-type polysaccharides from the cell walls of grasses and cereals, as far as we know, although some authors have reported the presence of galacturonic acid and its dimer and also galacturonosyl rhamnose in the hydrolysate of these cell walls [3–5]

We have reported the chemical composition of rice endosperm cell walls [6] and some structural features of the hemicellulosic polysaccharides which were isolated [7, 8] In this paper, we describe the isolation and structural analysis of pectic polysaccharides from rice endosperm cell walls

RESULTS

Two pectic polysaccharide fractions (Pec-I and Pec-II) were purified from rice endosperm cell walls by extraction with hot ammonium oxalate solution, fractional precipitation, ion exchange chromatography and gel filtration Each of the purified polysaccharides showed a single symmetrical peak on sedimentation analysis The yields were about 10% of the crude pectin and 1 5% of the cell wall for each fraction

Table 1 shows the sugar composition and $[\alpha]_D$ value of each fraction Most of the uronic acid was identified as galacturonic acid by PC The uronic acid content of Pec-I was about half of that in Pec-II Arabinose, galactose and rhamnose were the major neutral sugar components of both fractions, though Pec-I contained a larger amount of arabinose and xylose as compared to Pec-II Apiose and 2-methylfucose, which have recently been identified as components of the rhamnogalacturonan II-type polysaccharide [9], were not detected by GC/MS analysis of the hydrolysates of rice endosperm pectic polysaccharides

Table 1 Sugar composition and $[\alpha]_D$ values of the purified pectins

	Mole %*	
Component sugar	Pec-I	Pec-II
Rham	10 1	17 7
Ara	35 2	18 5
Xyl	14 3	4 1
Gal	18 3	18 2
Glc	5 0	4 9
Uronic acid†	17 0	36 7
$[\alpha]_D$	6 1° (c0 083, H_2O)	80 3° (c0 122, H_2O)

*Estimated by GC of alditol acetates on column A Peak areas were converted to molar ratios using a molar response factor [29]

†Estimated by the carbazole method [26] in terms of galacturonic acid

To clarify the structure of these acidic polysaccharides, methylation analysis was performed by two different methods In the first method, the purified polysaccharide was first methylated using the procedure of Hakomori [10], then part of the methylated polysaccharide was 'carboxyl-reduced' with $LiAlD_4$ to give Me-Red-polysaccharides [11] In the second method, the polysaccharide was first 'carboxyl-reduced' with water-soluble carbodiimide and $NaBH_4$ [12], then methylated following Hakomori to give Red-Me-polysaccharides Table 2 shows the results of the methylation analysis of Pec-I and also Pec-II The results suggest that the rhamnose residues in both fractions are (1 → 2)-linked, and a large part is substituted at the O-4 position The presence of (1 → 4)-linked galacturonic acid is suggested by the increase of 2,3,6-tri-*O*-methylgalactose in the Red-Me-polysaccharides, and also by the increase of 2,3-di-*O*-methyl-

Table 2 Results of the methylation analysis of purified pectins

Component	Linkage indicated	Molar ratio (%)*					
		Pec-I			Pec-II		
		Original	Red-Me-†	Me-Red-‡	Original	Red-Me-†	Me-Red-‡
3,4-Me_2-Rham	$\rightarrow 2(Rham_p)1\rightarrow$	2 4	2 7	1 6	6 7	5 9	4 0
3-Me-Rham	$\rightarrow 2(Rham_p)1\rightarrow$ 4 ↑	12 6	11 1	11 3	21 1	13 8	19 9
2,3,5-Me_3-Ara	$(Ara_f)1\rightarrow$	1 5 9	13 1	17 1	10 8	6 5	9 1
2,3-Me_2-Ara	$\rightarrow 5(Ara_f)1\rightarrow$	31 6	25 2	34 6	26 3	17 2	26 4
2-Me-Ara	$\rightarrow 5(Ara_f)1\rightarrow$ 3 ↑	4 3	3 4	4 7	1 1	0 9	2 2
2,3-Me_2-Xyl	$\rightarrow 4(Xyl_p)1\rightarrow$	2.8	2 2	2 1	2 0	2 3	1 1
2- and 3-Me-Xyl	$\rightarrow 4(Xyl_p)1\rightarrow$ 3 ↑ and ↑ $\rightarrow 4(Xyl_p)1\rightarrow$ 2 ↑	9 7	5 3	7 7	3 9	1 6	2 6
2,3,4,6-Me_4-Gal	$(Gal_p)1\rightarrow$	6 5	7 4	5 4	13 0	10 2	10 1
2,3,4-Me_3-Gal	$\rightarrow 6(Gal_p)1\rightarrow$	0 8	0 4	0 8	1 1	—	1 0
2,4,6-Me_3-Gal	$\rightarrow 3(Gal_p)1\rightarrow$	1 6	1 6	1 4	3 7	—	3 7
2,3,6-Me_3-Gal	$\rightarrow 4(Gal_p)1\rightarrow$	9 4	22 1	3 8	8 0	36 2	3 9
2,6-Me_2-Gal	$\rightarrow 4(Gal_p)1\rightarrow$ 3 ↑	2 5	2 9	3 1	2 0	2 3	2 3
2,3-Me_2-Gal	$\rightarrow 4(Gal_p)1\rightarrow$ 6 ↑	—	0 6	6 4	—	—	13 9
2,3,4,6-Me_4-Glc	$(Glc_p)1\rightarrow$	—	2 9	—	0 4	3 2	—

*Estimated by GC of alditolacetates on column B Peak areas were converted to molar ratios using a molar response factor [29]
†Carboxyl-reduced with water-soluble carbodiimide and $NaBH_4$[12], then methylated [10]
‡Methylated [10], then carboxyl-reduced with $LiAlD_4$ [11]

galactose in the Me-Red-polysaccharides The identification of 2,3-di-*O*-methyl-1,4,5,6-tetra-*O*-acetylgalactitol labelled with deuterium at the O-6 position in the Me-Red-polysaccharides confirmed that the compound was derived from the (1 → 4)-linked galacturonic acid residues in the original polysaccharide The (1 → 4)-linked galacturonic acid and (1 → 2)-linked rhamnose residues can be considered to form the main chain of these acidic polysaccharides on the basis of comparison with other pectic polysaccharides isolated from several dicots [1, 2]

The efficiency of the carboxyl-reduction of the second method (over 90%, as estimated from the determination of the uronic acid content) was better than the first method, in which the yield of the reduction product, 2,3-di-*O*-methylgalactose, was very low (under 50% of the expected value based on the uronic acid content of original polysaccharide) The low recovery of the reduction product in the first method may be partially explained by the low efficiency of the reduction step itself and the loss of uronic acid residues during Hakomori-methylation by β-elimination

Table 2 also suggests that the arabinose residues in these pectic polysaccharides are (1 → 5)-linked and the galactose residues are (1 → 4)-linked. Similar linkages have been detected in various pectic polysaccharides isolated from dicot cell walls [1, 2], however, the low degree of branching in the rice endosperm pectic polysaccharides, 4–12% of (1 → 5)-linked arabinose and 20% of (1 → 4)-linked galactose residues is a characteristic structural feature

The identification of a small amount of terminal glucose in the Red-Me-polysaccharides indicates the presence of terminal glucuronic acid residues in the original polysaccharides, though part could be derived from contaminating arabinoglucuronoxylan [8] Glucuronic acid was reported to be linked to fucose and galactose residues in the pectic polysaccharides of soybeans [13], lemon peel [14] and lucerne [15]

To obtain more detailed information about the structure of the side chains of the pectic polysaccharides of rice endosperm cell walls, the carboxyl-reduced Pec-I and II were degraded enzymatically using purified α-L-arabinofuranosidase from *Rhodotorula flava* [16] then the degraded polysaccharides were methylated and hydrolysed as usual The results were then compared with the results of methylation analysis of carboxyl-reduced Pec-I and II without enzymatic modification

Table 3 shows the sugar composition of the carboxyl-

Table 3 Sugar composition of carboxyl-reduced pectins before and after α-L-arabinofuranosidase treatment

Component sugar	Mole %			
	Pec-I		Pec-II	
	Before	After	Before	After
Rham	14 6	21 2	19 1	27 0
Ara	37 7	10 9	20 5	4 1
Xyl	8 9	13 9	6 1	4 0
Gal	36 1	50 5	51 6	61 8
Glc	2 6	3 5	2 7	3 1

*Estimated as same as for Table 1

reduced pectic fraction both with and without enzymatic modification Over 80% of the arabinose residues in the carboxyl-reduced pectic polysaccharides were removed by treatment with purified L-arabinofuranosidase, indicating that these residues are located in the outer chains, or as arabinan chains The results of the methylation analysis of the enzymatically modified fractions (Table 4) show that the residual arabinose residues in these fractions are also (1 → 5)-linked, but the reason why they were not attacked by the enzyme is not yet clear There are three possible explanations some other sugar residues, e g galactose, may be attached to these arabinose residues; the anomeric configuration of these arabinose residues is not in the α-L-form, the enzyme could not attack these residues because of some change in the conformation of the polymer after the partial degradation.

DISCUSSION

There is little knowledge regarding the structure of the pectic polysaccharides of monocot cell walls, except for reports on those from the sisal leaf [17] and the onion bulb [18] The results of the structural analysis of Pec-I and II which were purified from rice endosperm cell walls indicate the presence of pectic polysaccharides similar to those of dicot cell walls Pec-I and II appear to be rhamnogalacturonan I-type polysaccharides, based on the classification of Darvill *et al* [1], since they contain no apiose, 2-methylfucose or 2-methylxylose, and also the rhamnose residues are (1 → 2)- and (1 → 2),-, (1 → 4)-linked Ishii [18] detected the presence of (1 → 3)- and (1 → 3)-, (1 → 4)-linked rhamnose in the intermediate MW fraction of the pectic polysaccharide solubilized from onion bulb cell walls by endo-pectin lyase and classified it as a rhamnogalacturonan II-type polysaccharide, although apiose, 2-methylfucose and 2-methylxylose were not detected in this fraction We [6] reported previously that the pectic polysaccharides of rice endosperm cell walls contained high MW (main) and low MW (minor) components Both Pec-I and II belong to the high MW fraction and the possibility of the presence of a rhamnogalacturonan II-type polysaccharide in the latter fraction still remains

Pec-I and II have similar structural features, judging

Table 4 Methylation analysis of the carboxyl-reduced and α-L-arabinofuranosidase treated pectins

Component	Linkage indicated	Molar ratio (%)*	
		Reduced and arabinosidase treated Pec-I	Reduced and arabinosidase treated Pec-II
3,4-Me_2-Rham	→ 2($Rham_p$)1 →	6 5	8 7
3-Me-Rham	→ 2($Rham_p$)1 → 4 ↑	19 3	19 4
2,3,5-Me_3-Ara	(Ara_f)1 →	2 0	0 9
2,3-Me_2-Ara	→ 5(Ara_f)1 →	5 2	11 6
2,3-Me_2-Xyl	→ 4(Xyl_p)1 →	14 2	2 8
2- and 3-Me-Xyl	→ 4(Xyl_p)1 → 3 and ↑ 4(Xyl_p)1 → 2 ↑	4 1	—
2,3,4,6-Me_4-Gal	(Gal_p)1 →	13 5	15 8
2,3,4-Me_3-Gal	→ 6(Gal_p)1 →	0 9	—
2,3,6-Me_3-Gal	→ 4(Gal_p)1 →	27 0	35 0
2,6-Me_2-Gal	→ 4(Gal_p)1 → 3 ↑	2 2	1 4
2,3,4,6-Me_4-Glc	(Glc_p)1 →	4 0	4 3

*Estimated as same as for Table 2

from the results of methylation analysis, although Pec-I has a lower uronic acid content and a higher ratio of branched rhamnose residues Both fractions seem to be contaminated with a small amount of highly branched arabinoxylans, which are a major constituent of the matrix phase of the rice endosperm cell walls [8] Most of (1 → 4)-linked xylose and part of the terminal arabinose residues in the methylated Pec-I and II might originate from the arabinoxylans The amounts of contaminated arabinoxylans are estimated at about 5% (Pec-II) or 10–20% (Pec-I) of these fractions from the result of sugar composition and also methylation analysis

The main chains of the rhamnogalacturonan-type polysaccharides are generally considered to be composed of rhamnose and galactose, though the ratio is variable depending on the origin of the polysaccharide [1, 2] The ratio of rhamnose to galacturonic acid in Pec-I and II isolated from rice endosperm cell walls, 1 2, is similar to that of rhamnogalacturonan I obtained from suspension-cultured sycamore cell walls [19], but is higher than that obtained from lemon peel [14], rape seed hull [20] and tobacco leaf [21] pectins Pec-I and II might also be characterized by the high proportion of the (1 → 2)-, (1 → 4)-linked rhamnose residues, suggesting the highly branched nature of the main chain of them However, it cannot be concluded that these results fully describe the intact polysaccharide in the living cell walls, because it is well known that pectic polysaccharides easily break by β-elimination under fairly mild conditions, and it cannot be rejected that these purified polysaccharides might be linked to other parts of intact polymers in the cell wall, for example, to the low MW fraction [6], which is rich in galacturonic acid Some authors [22, 23] suggested that pectin has two regions which differ according to the content of galacturonic acid

The side chains of Pec-I and II mainly consist of (1 → 5)-linked arabinose and (1 → 4)-linked galactose residues and most of the arabinose residues seem to be localized in the outer chains or arabinan regions These side chains may be attached to the O-4 position of (1 → 2)-linked rhamnose residues in the main chain, as recently identified in rhamnogalacturonan-I of suspension-cultured sycamore cell walls [24] The side chains of rhamnogalacturonan-I of sycamore cell walls are considered to have a very complicated structure [24] and more work, including specific degradation experiments, will be needed to clarify this point further

EXPERIMENTAL

Plant material Rice (*Oriza sativa*, cultivar Norin 29) was harvested in Ibaraki prefecture, Japan

Source of enzyme α-L-Arabinofuranosidase was purified from the culture filtrate of *Rhodotorula flava* by the method of Uesaka *et al* [16] After purification by CC on SP-Sephadex, DEAE-Sephadex and Sephadex G-100, the enzyme preparation showed an activity of 95 3 units/mg protein on araban and did not act on xylan, laminaran, CMC and starch

General methods The total carbohydrate content was determined by the PhOH–H_2SO_4 method [25] The uronic acid content was determined by the carbazole method [26], corrected for neutral sugar For the analysis of the component sugar, the polysaccharides were hydrolysed with 1 N H_2SO_4 at 121° for 2 hr The neutral sugars in the hydrolysate were converted into their corresponding alditol acetates and analysed by GC using a column of 3% ECNSS-M on Gas Chrom Q (column A, 0 3 × 200 cm) [27] or a glass capillary column coated with Silar-10C (column B, 0 28 mm × 30 m or 50 m) [28] Peak areas were converted to the molar ratios using the appropriate response factor [29] GC/MS was carried out with a Hitachi model M-80 mass spectrometer using EI ionization and also CI with isobutane as the reactant gas PC of uronic acids was performed on Toyo No 50 filter paper with two solvent systems, (a) EtOAc–C_5H_5N–HOAc–H_2O (5 5 1 3) and (b) EtOAc–HOAc–H_2O (9 2 2) Sedimentation analyses of purified polysaccharides were performed with a Hitachi model UCA-1A ultracentrifuge using a 0 6% soln of purified polysaccharides in distilled H_2O at 55430 rpm at 20°

Methylation analysis Methylation of the acidic polysaccharides were performed using the method of Hakomori [10] after acetylation The introduction of methyl group did not proceed adequately without prior acetylation A portion of the methylated polysaccharides was reduced with $LiAlD_4$ [11], to give the methylated and carboxyl-reduced (Me-Red-) polysaccharides Another part of the original polysaccharides was dissolved in H_2O and reduced using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate and $NaBH_4$(× 2) [12] The carboxyl-reduced polysaccharides obtained were then methylated by the method of Hakomori, to give carboxyl-reduced and methylated (Red-Me-) polysaccharides The methylated polysaccharides were hydrolysed by heating with 90% HCO_2H at 100° for 2 hr, and then with 1 M TFA at 121° for 1 hr The partially methylated sugars obtained were converted into the corresponding alditol acetates and analysed by GC and GC/MS using column B

Purification of pectic polysaccharides Rice endosperm cell wall was prepared from milled rice flour as previously described [6] Pectic substances were extracted from the cell wall by refluxing with an excess of 0 25% $(CO_2NH_4)_2$ (18 hr × 3), and the resulting soln was dialysed against H_2O and lyophilized (yield 1 62 g from 10 g cell wall) The crude pectin preparation (1 g) was dissolved with H_2O (100 ml), then cetyl pyridinium chloride was added to this soln until the turbidity did not increase The ppt was collected by centrifugation, dissolved in 1 M NaCl and reprecipitated by the addition of EtOH The ppt was dissolved with H_2O, dialysed against 0 05 M K-Pi buffer (pH 6 0) and applied to a column of DEAE-cellulose (5 × 20 cm) which was equilibrated with the same buffer Acidic polysaccharides were eluted stepwise with a buffer containing different concns of NaCl Pectic polysaccharides which were rich in uronic acid were recovered in the 0 2 M and 0 5 M NaCl fractions, respectively These two fractions were then applied to a column of Sephadex G-100 (5 × 82 cm) which was equilibrated with 0 05 M NH_4OAc and eluted with the same soln The void vol fraction was collected and lyophilized to give the purified polysaccharides These fractions were designated as Pec-I (0 2 M NaCl fraction) and Pec-II (0 5 M NaCl fraction) (Yield 99 6 mg for Pec-I and 99 8 mg for Pec-II)

Enzymatic modification of carboxyl-reduced pectin by α-L-arabinofuranosidase Pec-I and II were first reduced with water-soluble carbodiimide and $NaBH_4$ as described above, then the carboxyl-reduced Pec-I or Pec-II (2–3 mg) was dissolved in 50 mM acetate buffer (pH 4 0) and incubated with purified *R flava* α-L-arabinofuranosidase (0 15 ml) overnight at 40° The reaction mixture was dialysed against H_2O, heat inactivated and centrifuged The supernatant soln was used for the sugar analysis and also for the methylation analysis

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