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Two Chains of Rhamnogalacturonan II Are Cross-Linked by Borate-Diol Ester Bonds in Higher Plant Cell Walls[1]

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Polysaccharide moiety of the boron-polysaccharide complex (T. Matoh, K. Ishigaki, K. Ohno, J. Azuma [1993] Plant Cell Physiol 34: 639–642) isolated from radish (*Raphanus sativus*) roots has been shown to be rhamnogalacturonan II by glycosyl-linkage analysis and the presence of diagnostic monosaccharides, including apiose, aceric acid, 2-*O*-methylfucose, and 3-deoxy-D-*manno*-2-octulosonic acid. Removal of boron from the complex reduced the molecular weight by one-half without causing a significant increase in the number of reducing end groups, indicating that boron, as boric acid, links two rhamnogalacturonan II chains together to form the boron-polysaccharide complex.

Boron (B) is an essential microelement for higher plants (reviewed by Loomis and Durst, 1992); however, its primary function is not known. A number of metabolic disorders that are consequences of B deficiency have been discussed in recent reviews (Dugger, 1983; Parr and Loughman, 1983; Loomis and Durst, 1992). Skok and McIlrath (1958) pointed out that the locus of B in the plant cell was little known, and the issue has never been resolved. We have shown that in cultured tobacco cells more than 98% of the B is present in the cell wall (Matoh et al., 1992), and that a BPC can be isolated from radish (*Raphanus sativus*) cell walls (Matoh et al., 1993a). In this paper, the structure of the polysaccharide moiety of the radish BPC is reported and its physiological function is discussed.

MATERIALS AND METHODS

Preparation of the BPC

The BPC was prepared from radish (*Raphanus sativus*) roots as described previously (Matoh et al., 1993a) with some modifications. Briefly, radish roots were grated and squeezed, and the macerated tissue was freeze-dried. The dried tissue (4 g) was treated with Pectinase-SS (0.1%, w/v; Kyowa Chemical Products, Nishi-Nakajima, Osaka, Japan) in 400 mL of 20 mM sodium acetate, pH 4.0, for 48 h at 25°C on a rotary shaker (130 rpm). The suspension was centrifuged (10,000*g* for 15 min), and the supernatant was adjusted to pH 8.0 with 2 M Tris and then applied to a DEAE-Sepharose column (4.8 × 60 cm, Cl^- form, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0. The column was eluted with a 6-L linear gradient of 0 to 0.5 M NaCl in the column buffer, and the fractions containing B were pooled and dialyzed. The BPC was purified by rechromatography on the same DEAE-Sepharose column. Fractions containing B were subjected to gel filtration using a Superdex 75 column (2.6 × 60 cm, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl. B-rich fractions were pooled, dialyzed against water, and lyophilized.

Partial Hydrolysis of the BPC with Acid

The BPC (1 mg) was incubated in 0.1 M HCl (1 mL) for 15 min at 25°C. After neutralization with NaOH, an aliquot (100 μL) was analyzed by size-exclusion chromatography (YMC-pack Diol-120, 300 × 8 mm [YMC, Karasuma-Oike, Kyoto, Japan], Shimadzu 6A HPLC system). The column was equilibrated and eluted at a flow rate of 0.5 mL min^{-1} with 50 mM sodium acetate, pH 5.2, containing 0.2 M NaCl. Saccharides were detected fluorometrically (Shimadzu RF 530 detector) after postcolumn labeling of their reducing termini with 2-cyanoacetamide (Honda et al., 1980) at excitation and emission wavelengths of 331 and 383 nm, respectively.

The BPC (10 mg) was partially hydrolyzed with 0.1 M TFA (5 mL) at 40°C for 24 h (Spellman et al., 1983). The hydrolysate was adjusted to pH 9 with 1 M NH_4OH and applied to a DEAE-Sepharose column (1.3 × 4 cm) equilibrated with 30 mM NH_4HCO_3, and the flowthrough was collected and lyophilized. This fraction is referred to as the neutral fraction of the partial hydrolysate. Further fractionation was carried out on a Bio-Gel P-2 column (1.6 × 90 cm, Bio-Rad) equilibrated with 50 mM sodium acetate, pH 5.2. The two fractions (fractions I and II) were pooled separately, desalted by passage through a column of Dowex 50W-X8 (H^+ form, 1 × 2 cm), and lyophilized.

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Abbreviations: BPC, boron-polysaccharide complex; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; RG-II, rhamnogalacturonan II.

Glycosyl Composition and Methylation Analyses

Alditol acetates and trimethylsilylated methyl glycosides were prepared according to York et al. (1986) and analyzed by GLC and GLC-MS. Methylation analysis was carried out without pre-reduction as described by Harris et al. (1984) except that sodium carbanion was used. Fraction II was worked up according to York et al. (1985). Partially methylated alditol acetates were analyzed by GLC and GLC-MS. Quantification was made based on GLC peak area and response factors that were determined empirically. When authentic standards were not available, the effective carbon-response factors (Sweet et al., 1975) were used. The factor for the derivative of aceric acid residue was assigned to be 0.70 in glycosyl composition analysis (Thomas et al., 1989), and that for the methylated derivative of 5-linked KDO residue was assigned to be 0.87 (Sweet et al., 1975).

GLC was performed on a Shimadzu GC-15A equipped with a 15-m DB-225 capillary column (J & W Scientific). The oven temperature program was as follows: 140 to 220°C at 2°C min^{-1}, and 220°C for 20 min. GLC-MS was performed on a Shimadzu GC-14A QP2000 system (electron-impact ionization mode at 60 eV) equipped with a 15-m DB-225 capillary column. For the analysis of alditol acetates and trimethylsilyl ethers, the temperature program used was the same as that for GLC. For analysis of partially methylated alditol acetates, the following oven temperature program was used: 100 to 220°C at 2°C min^{-1}, and 220°C for 20 min.

Determination of Molecular Weight

The molecular weights of the BPC and its fragments were determined with MALDI-TOF MS (Bruker, Fällanden, Switzerland) TOF-MS, model REFLEX II equipped with a 337 nm laser) using a nominal potential of 20 kV and 2,5-dihydroxybenzoic acid as the matrix.

^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were recorded with a Bruker ARX-500 spectrometer operated at 500 MHz at 70°C. Samples were dissolved in deuterium oxide, and chemical shifts were reported relative to internal acetone (δ 2.235 ppm).

B and Sugar Assay

B content was determined by the 2,4-dinitro-1,8-naphthalenediol method (Matoh et al., 1993b). Total sugars were assayed by the phenol-sulfuric acid method (Dubois et al., 1956) using Glc as a standard. Uronic acids were measured by the *m*-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid as a standard. 2-Keto-3-deoxy sugars were determined by the modified thiobarbituric acid method (York et al., 1985). Reducing end groups were determined as a Glc equivalent by the 2-cyanoacetamide assay (Honda et al., 1982).

RESULTS AND DISCUSSION

Acid hydrolysis (0.1 M HCl for 15 min at 25°C) of the BPC released B and decreased its molecular weight by one-half with only a slight increase (14%) in the proportion of reducing end groups (Table I). On size-exclusion chromatography, the elution time of the BPC increased by the treatment from 17.8 to 19.0 min (Fig. 1) without yielding any detectable amounts of other, smaller fragments. Trace amounts of oligosaccharide were detected in the untreated BPC (22–25 min in Fig. 1a). Those fragments may result from degradation of the BPC during storage and did not increase significantly after the acid treatment (Fig. 1b). The fragment generated by the treatment was homogeneous in terms of size and charge based on its single symmetric elution peak on columns of Diol-120 and DEAE-Sepharose (data not presented). These results indicate that the acid treatment of the BPC generates two identical polysaccharide chains. The B in the BPC was shown by ^{11}B-NMR to be present as a tetravalent 1:2 borate-diol complex (Matoh et al., 1993a), and since the reducing end group did not increase significantly (Table I), the formation of only one component with the simultaneous release of B suggests that B cross-links two identical polysaccharide chains through B-diol ester bonds. Since the B content determined gravimetrically was 0.23% (Matoh et al., 1993a) and its molecular weight is 9900 (Table I), 1 mol of BPC contains 2.1 mol of B.

The glycosyl composition of the BPC was determined by GLC and by colorimetric assays. Taking recovered sugars as 100%, the mol % of the components was as follows: 2-*O*-methylfucose (3%), rhamnose (8%), Fuc (2%), 2-*O*-methylxylose (4%), aceric acid (1%), Ara (7%), apiose (12%), Gal (7%), 2-keto-3-deoxy sugars (5%, KDO equivalent), and uronic acid (51%, galacturonic acid equivalent). The glycosyl-residue composition, except for uronic acid, is very close to that of the RG-II isolated from the sycamore cell wall (Stevenson et al., 1988). Uronic acid percentage for the BPC is rather high compared to that for sycamore RG-II. This may be due to the different hydrolyzing enzyme employed. Glycosyl-linkage analysis of the BPC revealed the presence of at least 11 different linkages, including 3,4-linked fucosyl, and 3- and 2,3,4-linked rhamnosyl residues. Because these component monosaccharides and glycosyl-linkages are diagnostic of RG-II (Thomas et al., 1989), further analysis was carried out using the methods developed for sycamore RG-II (Spellman et al., 1983).

The neutral fraction of the partial TFA hydrolysate consisted of two major peaks (fractions I and II) as revealed by Bio-Gel P-2 column chromatography (Fig. 2). Neither fraction contained uronic acid. Fraction II gave a positive response in the thiobarbituric acid assay, which indicates the

Table I. *Effects of acid hydrolysis on the B-polysaccharide complex of radish root cell walls*

Sample	Boron/Sugar[a]	Molecular Weight[c]	Reducing Termini/ Total Sugar
	wt %[b]		*mol %*
Intact	0.30	9894	7.2
Acid-hydrolyzed	0.01	4927	8.2

[a] Hydrolysate was thoroughly dialyzed against water before determining B. [b] Sugars were determined colorimetrically. [c] Determined by MALDI-TOF MS.

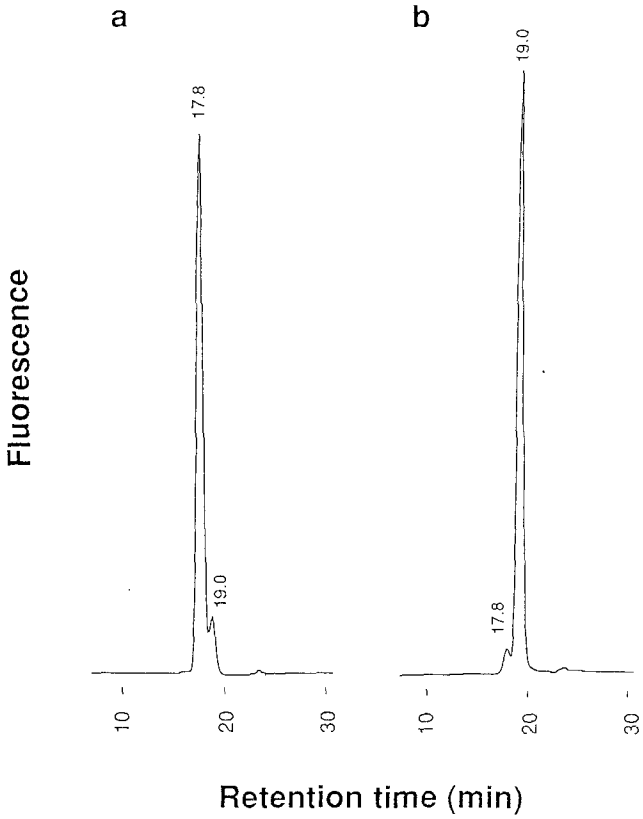

Figure 1. Chromatogram for the radish B-polysaccharide complex before and after acid hydrolysis. One milligram of BPC was incubated in 1 mL of 0.1 M HCl and a 100-μL aliquot was subjected to size-exclusion chromatography. a, Intact BPC (without hydrolysis); b, BPC incubated in 0.1 M HCl at 25°C for 15 min. Saccharides were detected fluorometrically using postcolumn labeling of the reducing termini with 2-cyanoacetamide.

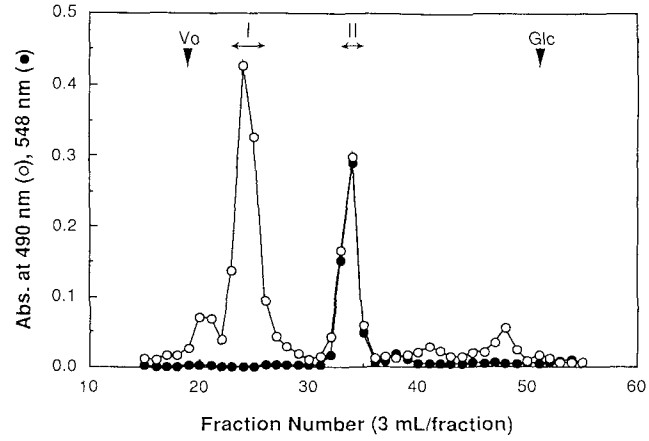

Figure 2. Chromatogram for the neutral fractions of the TFA hydrolysate of the BPC on a Bio-Gel P-2 column (1.6 × 90 cm). Fractions (3 mL) were collected and assayed for total sugars (○) and 2-keto-3-deoxy sugars (●). Tube numbers 23 to 26 and 33 to 35 indicated by the double-headed arrows were pooled separately as fractions I and II, respectively. The single-headed arrows indicate the void volume and the elution volume for Glc, respectively.

presence of 2-keto-3-deoxy sugars in this fraction. The glycosyl composition of fraction I is shown in Table II. The molar ratio of each monosaccharide except for aceric acid was approximately unity. MALDI-TOF MS of fraction I gave a peak of m/z 892.6 (average of two measurements), which may correspond to the $[M\text{-}H_2O]^+$ ion of a hexasaccharide composed of one methylfucosyl, one rhamnosyl, one aceric acid, one arabinosyl, one apiosyl, and one galactosyl residue (m/z 910.8). Thus, the oligosaccharide in fraction I was considered to be a hexasaccharide. Glycosyl linkages of these residues determined by methylation analysis are listed in Table III. Comparing these characteristics with those of the sycamore RG-II (Spellman et al., 1983), the terminal nonreducing rhamnosyl residue was not detected in the hexasaccharide derived from the radish BPC. Consistent with this finding, Puvanesarajah et al. (1991) reported that RG-II isolated from Pectinol AC lacks a terminal nonreducing α-L-rhamnosyl residue, since Pectinol AC may contain α-L-rhamnosidase activity. The Pectinase-SS used here may also contain the same activity.

The 2-keto-3-deoxy sugar in fraction II was identified as KDO by GLC-MS of its trimethylsilylated derivative (data not presented). In addition to KDO, the monosaccharide detected was rhamnose (Table II) and the molar ratio was approximately 1:1. Methylation analysis showed the presence of a terminal nonreducing rhamnosyl and a 5-linked KDO residue (Table III). The apparent inequality of the molar ratio was probably due to a low recovery of the KDO derivative. The ^{1}H-NMR spectrum of fraction II contained a signal in the anomeric region (at δ 5.2 ppm), indicating that the rhamnosyl residue was α-linked (J 2.9 Hz). Therefore, the oligosaccharide in fraction II was identified as α-Rha-(1 → 5)-KDO, identical to the disaccharide derived from sycamore RG-II (York et al., 1985).

The occurrence in radish root BPC of oligosaccharide fragments that are characteristic of sycamore RG-II indicates that the polysaccharide moiety of the BPC is RG-II. Initially, sycamore RG-II had been reported to consist of approximately 60 glycosyl residues based on its elution pattern on gel filtration (Melton et al., 1986), but more recently Stevenson et al. (1988) determined RG-II to have approximately 30 glycosyl residues by end-group analysis.

Table II. *Glycosyl composition of the oligosaccharides released from the radish B-polysaccharide complex by TFA hydrolysis*

Residue	Molar Ratio
Fraction I	
2-*O*-Methylfucose	0.98[a]
Rhamnose	1.00
Aceric acid	0.53[a]
Arabinose	0.85
Apiose	1.09
Galactose	1.04
Fraction II	
Rhamnose	1.00
KDO	0.88[b]

[a] Calculated using the effective carbon-response factor (see text).
[b] Determined colorimetrically.

Table III. *Methylation analysis of the oligosaccharides released from the radish B-polysaccharide complex by TFA hydrolysis*

Residues	Position of O-CH_3 Groups	Deduced Linkage	Mol %[a]
Fraction I			
2-*O*-Methylfucose	2,3,4	Terminal	33
Rhamnose	2,4	3-Linked	16
Arabinose	2,3,4	Terminal (pyranose)	25
Apiose	2,3	3′-Linked	Trace
Galactose	3,6	2,4-Linked	26
Fraction II			
Rhamnose	2,3,4	Terminal	82
KDO	2,4,6,7,8	5-Linked	18

[a] Calculated using the effective carbon-response factor (see text).

If the sycamore RG-II is in fact RG-II dimer cross-linked by B, the size determined by gel filtration and end-group analysis should be that of the dimer and monomer, respectively. This may be the reason for the apparent discrepancy in the size of RG-II. Here we define BPC as a dimeric RG-II cross-linked by B-diol ester bonding.

It has often been observed that B in the cell walls of mature tissues cannot be reutilized by elongating cells, suggesting that the B-binding site fixes B so tightly and that the BPC accumulates as the cell matures. As in the case of rice RG-II (Thomas et al., 1989), radish BPC may occur in intact cell walls as a component of a long chain of pectic polymers. Since the BPC contains 2.1 mol of B, the two pectic chains will be cross-linked at two sites in the RG-II region. The loop formed between the knots, either in the RG-II region or between the neighboring RG-II region along the chain, may bundle other polysaccharide chains. Teasdale and Richards (1990) speculate that B plays a key role in contributing to cell-wall strength during the expansionary phase of cell growth by participating in reversible formation of a carbohydrate gel surrounding the cellulose microfibrils. Such a mode of function of B in cell walls was also predicted by Torssell (1956). Our speculation is particularly pertinent to their proposal. Future efforts will focus on how B-RG-II complexes contribute to the stabilization of cell walls.

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