Pergamon

PII: S0031-9422(96)00539-0

Phytochemistry, Vol. 44, No. 2, pp. 243–248, 1997

0031-9422/97 $17.00 + 0.00

A BORON–RHAMNOGALACTURONAN-II COMPLEX FROM BAMBOO SHOOT CELL WALLS

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(*Received in revised form* 21 *June* 1996)

Key Word Index—bamboo; *Phyllostachys edulis*; Gramineae; boron; cell walls; pectic polysaccharide; rhamnogalacturonan-II.

Abstract—A boron–polysaccharide complex was isolated from a Driselase digest of bamboo (*Phyllostachys edulis*) shoot cell walls by successive DEAE Sepharose FF, Bio-Gel P-10 and Mono Q HR 5/5 chromatography. The complex contained 0.15% boron (w/w). The glycosyl residue and linkage composition analyses of the polysaccharide moiety of the complex identified the polysaccharide as a rhamnogalacturonan-II (RG-II), a structurally complex pectic polysaccharide present in the primary cell walls of all higher plants. ^{11}B NMR spectroscopy showed that boron was present as a tetrahedral borate-diol diester. Removal of boron from the complex decreased the M_r by half without any loss of glycosyl residues, suggesting that boron cross-links two RG-II molecules. The boron–RG-II complex from bamboo (a monocot) shoot cell walls has almost the same structure as that of sugar beet (a dicot) cell walls. The results demonstrate that the structure of boron–RG-II complex is very similar in dicots and monocots. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The first evidence that boron is an essential micronutrient for higher plants was provided by Warington [1]. Boron deficiency produces various anatomical, physiological and biochemical changes, and numerous functions for boron have been proposed [2]. However, the definitive primary functions of boron in plants are still unknown.

Numerous reports have noted changes in cell wall structure as a result of boron deficiency [3–5]. Matoh *et al.* [6] reported that 98% of boron in tobacco cells is located in the cell walls, and Hu and Brown [7] concluded that boron in the cell walls of squash (*Curcurbita pepo* L.) and tobacco (*Nicotiana tabacum*) is associated with pectin. Matoh *et al.* [8] isolated a boron–polysaccharide complex from radish (*Raphinus sativus* L.) root and indicated that the polysaccharide is similar to rhamnogalacturonan-II (RG-II) [9], which constitutes pectin together with homogalacturonan and rhamnogalacturonan-I [10].

Recently, a boron–polysaccharide complex was isolated from dicot sugar beet (*Beta vulgaris* L.) cell walls and the polysaccharide was identified as RG-II [11]. Dicots have larger amounts of pectic polysaccharide than monocots [10], and require 6.5 times more boron than do monocots (average 5.7 ppm) [12]. As far as we know, there has been no report on the structure of the boron complex in monocots. In this contribution, a description of the isolation of a boron–polysaccharide complex from monocot bamboo shoot cell walls, and the characterization of the RG-II polysaccharide by structural analysis is presented.

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RESULTS

Isolation of boron–RG-II complex

Bamboo shoot cell walls were found to contain 5.42 $\mu g\ g^{-1}$ boron. This value is almost one-eighth the amount contained in sugar beet (43.4 $\mu g\ g^{-1}$) [11]. Driselase digests of bamboo shoot cell walls were separated into two fractions by ultrafiltration (10 000 molecular weight cut off). The retentate of the ultrafiltration was applied to a DEAE Sepharose FF column. The boron–polysaccharide complex was adsorbed on the column and eluted with a linear gradient of $HCOONH_4$ buffer (Fig. 1(A)). The fraction containing boron was purified further by gel filtration on Bio Gel P-10 (Fig. 1(B)). The polysaccharides separated into two fractions by this chromatography, of which only boron was detected in the later eluting fraction. This latter fraction was then applied to a Mono Q HR 5/5 column, with the adsorbed boron–polysaccharide complex subsequently eluted with a linear gradient of $HCOONH_4$ buffer (Fig. 1(C)). This fraction was used for the structural characterization of the boron–polysac-

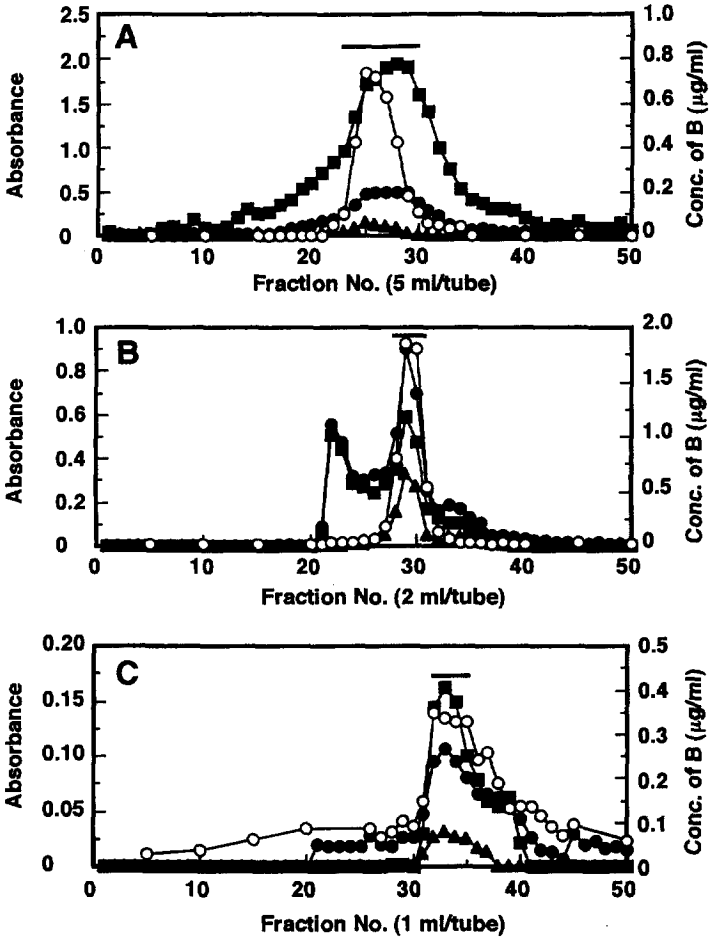

Fig. 1. Purification of the boron–RG-II complex: (A) anion-exchange chromatography on DEAE Sepharose Fast Flow; (B) gel-permeation chromatography on Bio Gel P-10; (C) anion-exchange chromatography on Mono Q HR 5/5. Fractions were assayed for boron (○), KDO (▲), hexose (■) and uronic acid (●) by ICP-MS, thiobarbituric acid assay, anthrone and *meta*hydroxybiphenyl methods, respectively. Boron and polysaccharide containing fractions were pooled, as indicated (bold line), for further purification.

charide complex as follows. The ^{1}H NMR spectrum of the complex gave signals at δ 2.0 and 2.1, indicating the presence of acetyl groups (data not shown). The M_r of the boron–polysaccharide complex was determined by calibration by HPSEC with pullulan narrow molecular weight standards and by MALDI-TOF-MS to be 8200 and 9200, respectively. The M_r of the complex from bamboo is almost the same as that of sugar beet (10 500 by HPSEC, 8900 by MALDI-TOF-MS) [11]. The MALDI-TOF-MS signals corresponding to the molecular ions are 800–1800 amu wide at half peak height. These broad peaks may, in part, result from the lengths of the 1,4-linked α-D-galacturonan backbone which are on average 7–9 [10].

Composition of the boron–RG-II complex

The purified boron–polysaccharide complex contained 0.15% boron (w/w). This value is almost the same as the boron content of the boron–RG-II complex from sugar beet (0.12%, w/w) [11]. The glycosyl residue composition of the boron–polysaccharide complex is given in Table 1, together with the boron–RG-II complex from sugar beet [11] and RG-II from rice (*Oryza sativa*) [10] for reference. The boron complex

Table 1. Glycosyl residue composition (mol. %) of the boron–RG-II complex from bamboo shoot cell walls

Glycosyl residue	Bamboo	Sugar beet*	Rice†
Rha	18.6	11.3	18.2
Fuc	3.2	1.6	4.9
2Me-Fuc	4.6	3.3	5.3
Ara	12.1	10.9	10.0
2Me-Xyl	4.5	4.9	7.3
Apiose	6.7	4.5	9.0
Gal	8.3	12.4	12.3
GlcA	3.2	7.0	6.3
GalA	28.7	37.7	26.7
Aceric acid	–‡	–‡	–‡
Kdo	8.3	5.3	–‡
Dha			–‡

*Ishii *et al.* [11].
†O'Neill *et al.* [10].
‡Present but not quantified.

from bamboo had 31.9% of uronic acid residues. GLC-mass spectrometry analysis of the alditol acetates from the boron–polysaccharide complex obtained after acid hydrolysis, reduction and per-*O*-acetylation showed the presence of 2-*O*-methylfucose (*m/z* 117, 171, 231 and 275) [13], 2-*O*-methylxylose (*m/z* 117, 201 and 261) [13], and apiose (*m/z* 145, 187 and 302) [14], in addition to rhamnose, fucose, arabinose and galactose. KDO (3-deoxy-D-*manno*-2-octulosonic acid) and DHA (3-deoxy-D-*lyxo*-2-heptulosaric acid) were measured together using a modified thiobarbituric acid assay [15] and accounted for 8.3%. Aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) was detected but not quantified. These unusual glycosyl residues are characteristic of the pectic polysaccharide RG-II [10]. Glycosyl linkage analysis of the boron–polysaccharide complex is shown in Table 2, where the complex contained 3-linked and 2,3,4-linked rhamnosyl, 3,4-linked fucosyl and 2-linked glucuronic acid residues, which are characteristic of RG-II. Based on the similarity of the glycosyl linkage composition of the boron–polysaccharide complex to that of RG-II [10, 11], together with the presence of 2-*O*-methylfucose, 2-*O*-methylxylose, apiose, aceric acid and thiobarbituric acid positive sugar residues, we concluded that the polysaccharide moiety of the complex was RG-II.

Table 2. Glycosyl linkage composition (mol. %) of the boron–RG-II complex from bamboo shoot cell walls

Glycosyl linkage*		Bamboo	Sugar beet†	Rice‡
Rha	T-Rha	4.6	4.5	8.6
	2-Rha	2.3	4.3	tr
	3-Rha	5.1	5.3	5.9
	2,3-Rha	1.6	0.8	ND
	2,4-Rha	0.8	0.9	ND
	2,3,4-Rha	5.2	6.5	3.7
Fuc	3,4-Fuc	5.8	3.3	4.9
2Me-Fuc	T-Fuc	6.7	5.6	5.3
Apiose	3′-Api	5.2	5.0	9.0
Ara	T-Araf	6.4	6.3	5.8
	T-Arap	ND	2.5	0.0
2Me-Xyl	T-Xyl	ND	4.1	7.3
Gal	T-Gal	11.0	5.0	6.8
	2,4-Gal	7.6	5.2	5.5
	3,4-Gal	ND	4.3	0.0
	3,6-Gal	ND	0.3	ND
GalA	T-GalA	7.2	10.0	10.2
	4-GalA	5.6	14.3	7.9
	3,4-GalA	10.7	ND	3.1
	2,4-GalA	4.8	ND	2.8
	2,3,4-GalA	7.8	4.7	2.7
GlcA	2-GlcA	3.1	7.1	6.3

ND, not determined; tr, trace.
*T, non-reducing terminal rhamnosyl, etc.
†Ishii *et al.* [11].
‡O'Neill *et al.* [10].

^{11}B *NMR spectroscopy of the boron–RG-II complex*

^{11}B NMR spectroscopy is useful for characterizing the esters formed by the interaction of boric acid and borate with diols [16]. The ^{11}B NMR spectrum of the boron–RG-II complex (Fig. 2) contains a sharp peak at δ −9.45, indicating that the boron–RG-II complex is present as a tetrahedral borate-diol diester [16]. A peak due to free boric acid was not detected (δ 0.00). Boron in the boron–RG-II complex from sugar beet was also present as a tetrahedral borate-diol diester [11].

Acid treatment of the boron–RG-II complex

The M_r of the boron–RG-II complex from bamboo shoot cell walls was decreased to about one-half by the treatment with 0.5 N HCl for 30 min at 30°. The M_r of the boron–RG-II complex by MALDI-TOF-MS before and after the treatment was 9200 and 4100, respectively.

Treatment of boron–RG-II from bamboo shoot cell walls with 0.1 M TFA for 16 hr at 40° cleaved some of the side-chains from boron–RG-II. The TFA treated boron–RG-II was separated by HPSEC. Each peak (peak 1, 19.5 min; peak 2, 22.5 min; peak 3, 27.2 min; peak 4, 32.6 min; peak 5, 35.3 min) was separately collected. By a modified thiobarbituric acid assay of peaks 1, 2 and 3, it was estimated that 29% of KDO or DHA was released by TFA treatment. The negative-ion FAB-mass spectroscopy spectrum of peak 3 gave an intense ion at *m/z* 1139 $[M-H]^-$, whereas the positive-ion FAB-mass spectroscopy had peaks at *m/z* 1141 $[M+H]^+$ and 1163 $[M+Na]^+$. The mass spectroscopy data showed that the M_r of the compound associated with peak 3 was 1140. Glycosyl composition analysis of peak 3 showed that it consisted of Rha, Ara, 2-*O*-Me-Fuc, Gal, aceric acid and apiose residues. The ratio of these residues was Rha/Ara/2-*O*-Me-Fuc/Gal 2:1:1:1. Aceric acid and apiose were not quantified. The ^{1}H NMR spectrum of peak 3 gave signals at δ 2.0 and 2.1, indicating the presence of acetyl groups (data not shown). These results show that the peak 3 com-

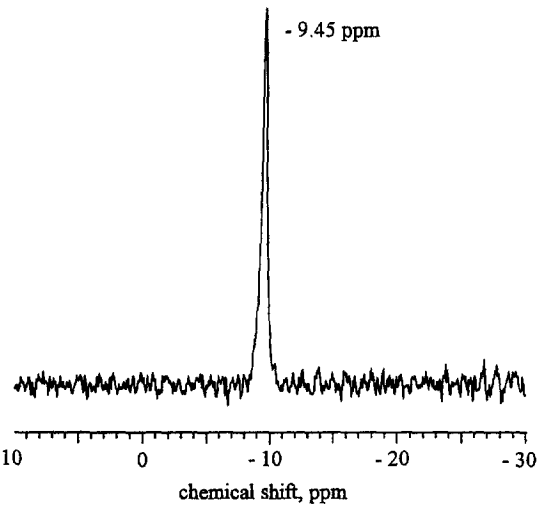

Fig. 2. A ^{11}B NMR spectrum of the boron–RG-II complex from bamboo shoot cell walls (35 497 scans). A sample (2.2 mg) was dissolved in 0.5 ml H_2O to give a solution of pH 4.8. Chemical shifts are referenced to external 0.1 M boric acid at δ 0.00.

pound consists of two acetyl groups, two Rha residues, and one residue each of Ara, 2-*O*-Me-Fuc, Gal, AceA and Api. The structure of the fragment seems to be the same as the side-chain of RG-II of sycamore (*Acer pseudoplatanus*) [17]. GLC analysis of alditol acetate of peak 4 gave only a Rha residue. 5-Linked KDO was also detected by the alditol acetate method described by York *et al.* [15]. Peak 4 was determined to be Rha-(1→5)-KDO. The glycosyl composition analysis of peak 5 by GLC of alditol acetate, showed only an Ara residue.

DISCUSSION

It is well known that monocots have smaller amounts of pectic polysaccharides and require smaller amounts of boron than do dicots [12]. In this study, a boron–polysaccharide complex was isolated from cell walls of bamboo (monocot) shoots. The polysaccharide moiety of the complex was determined to be RG-II by glycosyl composition and linkage analyses (Tables 1 and 2). Treatment of the complex with 0.1 M TFA at 40° for 16 hr gave a heptasaccharide and a disaccharide, which have been identified previously as side-chains of sycamore RG-II [15, 17]. The results strongly indicated that the polysaccharide moiety of the complex is RG-II. The boron–RG-II complex from bamboo contained 0.15% boron (w/w), which was present as a tetrahedral borate-diol diester. HCl treatment decreased the M_r by MALDI-TOF-mass spectroscopy of the complex by one-half (9200 to 4100), without any cleavage of glycosidic bonds. The results suggest that boron cross-links two molecules of RG-II by tetrahedral borate-diol diester bonds. Recently, a similar B–RG-II complex was isolated from sugar beet cell walls [11], which were found to contain 0.12% boron (w/w), which was present as a tetrahedral borate-diol diester [11]. HCl treatment decreased the M_r found by MALDI-TOF-mass spectroscopy of the complex by half (8900 to 4200, unpublished data).

RG-II is one of the components of pectin, and is now known to be present in the primary walls of all higher plants [10]. As far as we know, there have been no reports on the biological or structural functions of RG-II. In this study we have demonstrated that the boron–RG-II complex exists in bamboo shoot cell walls, and that the structure of the boron–RG-II complex is very similar in sugar beet (dicots) and bamboo (monocots). It should be emphasized that boron, an essential micronutrient, binds selectively to RG-II, which is a highly conserved structure in all higher plants. These findings suggest that boron may play an important role in cell wall synthesis and/or structure.

Possible glycosyl residues that could form tetrahedral borate-diol diesters would have two free hydroxyl groups in the *cis* position. Apiose and KDO have *cis*-hydroxyl groups in RG-II. One molecule of RG-II contains two apiosyl residues and one KDO residue. If boron cross-links two molecules of RG-II, it may be possible that boron links two of four apiose residues, two KDO residues or binds between apiose and KDO residues. To identify such structures, it will be necessary to elucidate the linkage positions between boron and glycosyl residues in the boron complex.

During submission of our manuscript, it was reported that boron–RG-II from radish (a dicot) roots has very similar properties to sugar beet boron–RG-II [18], and hence also that from bamboo.

EXPERIMENTAL

Plant material and preparation of cell walls. Fresh, young bamboo (*Phyllostachys edulis* A. and C. Riv.) shoots were purchased in May 1993 from a Nohara Agricultural Farm (Ibaraki Prefecture). The cell walls were isolated as alcohol insoluble solids as described previously [9], and contained 5.42 μg^{-1} g boron.

Isolation of boron–RG-II. Bamboo shoot cell walls were hydrolysed with Driselase [20], and the solubilized material was ultrafiltered (Amicon Hollow fibre HIP 10-20, Amicon) [20]. The retentate (>10 000 mol. wt. cutoff) was applied to a DEAE Sepharose Fast Flow (25 × 430 mm) column equilibrated with 50 mM $HCOONH_4$ buffer (pH 7.0). After the column was washed with 500 ml of the same buffer, the adsorbed material was eluted from the column with a linear gradient of $HCOONH_4$, from 50 to 1000 mM (total volume 500 ml), at a flow rate of 43 ml hr^{-1}. The eluate was fractionated into 5-ml portions. Fractions 23–30 were pooled and lyophilized. The lyophilized material was dissolved in 1 ml 50 mM $HCOONH_4$ buffer (pH 7.0) and applied to a Bio-Gel P-10 column equilibrated with 50 mM $HCOONH_4$ buffer (pH 7.0). The elution was done using the same buffer at a flow rate of 4 ml hr^{-1}. The eluate was fractioned into 2-ml portions, with fractions 28–31 combined and lyophilized. The lyophilized boron–polysaccharide complex was further applied to a Mono Q HR 5/5 (Pharmacia) column equilibrated with 100 mM $HCOONH_4$ buffer (pH 7.0). After the column was washed with 10 ml of the same buffer, the adsorbed material was eluted from the column with a linear gradient of $HCOONH_4$, from 100 to 1000 mM (total volume 60 ml), at a flow rate of 1 ml min^{-1}. The eluate was fractionated into 1-ml portions, with fractions 32–35 pooled and lyophilized. The boron–polysaccharide complex thus obtained was used as purified boron–RG-II.

Acid treatment of RG-II. Purified boron–RG-II (3 mg) was treated with 0.1 M TFA (0.5 ml) for 16 hr at 40°. The mixture was lyophilized, and separated by HPSEC.

Analytical methods. Neutral sugars, uronic acid and KDO/DHA concentrations were determined using the anthrone method [21], *m*-hydroxybiphenyl method [22] and the modified thiobarbituric acid assay [15], respectively.

Neutral glycosyl residue compositions were determined by GLC of their alditol acetate derivatives [23]. Combined neutral and acidic glycosyl residue com-

positions were determined by GLC of their trimethylsilyl methyl ester methyl glycoside derivatives [23].

Glycosyl linkage compositions were determined using a modification [24] of the procedure described by Hakomori [25]. Polysaccharides were per-*O*-methylated with methylsulphinyl methyl potassium and iodomethane, and the resulting products were isolated using Sep-Pak C_{18} cartridges (Waters, Milford, MA) [26]. The methyl esters of uronic acid residues in the per-*O*-methylated samples were reduced with Super-deuteride (1 M lithium triethylborodeuteride in tetrahydrofuran; Aldrich) as described by York *et al.* [27]. The glycosyl linkage compositions were then determined by GLC-MS of the partially methylated, partially acetylated alditol acetate derivatives [27].

Boron determination. The boron concentration in each fraction was determined using an inductively coupled plasma mass spectrometer (ICP-MS) SII SPQ8000A (Seiko Instruments Inc.). The sample solution was introduced to the ICP-MS by flow injection analysis (FIA) using a JASCO PU-980i pump and a Rheodyne 9125 injection valve. The outlet of the injector was connected to the nebulizer of the ICP-MS with PEEK (poly ether ether ketone) tubing. FIA conditions: carrier H_2O; flow rate 1 ml min^{-1}; injection volume 20 μl.

NMR spectroscopy. ^{11}B NMR spectra of the boron complex were recorded at 25° on a JEOL JNM-A600 spectrometer operated at 192.5 MHz without field lock using 5 mm diameter quartz-glass tubes. A sample (2.2 mg) was dissolved in 0.5 ml H_2O to give a solution of pH 4.8. Chemical shifts (δ) are reported relative to external boric acid (0.1 M) at δ 0.00. NMR conditions: number of data points 2048; observation frequency range 28 900 Hz; pulse width 15 μs (90°); pulse repetition time 0.11 s; exponential broadening factor 15 Hz. For the ^1H NMR spectrum, a sample (2.0 mg) was dissolved in D_2O and spectrum recorded at 500 MHz using a JEOL JNM-A500 spectrometer at 500 MHz.

High-performance size-exclusion chromatography (HPSEC). The weight-average molecular weight (M_w) of the boron–RG-II complex was determined by HPSEC using a Shimadzu LC6A liquid chromatograph with a refractive index detector (Shimadzu model RID-6A) and a Superdex peptide HR 10/30 column (Pharmacia), eluted with 200 mM $HCOONH_4$ buffer (pH 7.0) at a flow rate of 0.5 ml min^{-1}. The column was calibrated with pullulan narrow M_w standard (P-5, M_w 5800; P-10, 12 200; P-20, 23 700) (Showa Denko K.K., Tokyo).

FAB-MS. FAB-MS spectra were recorded on a JEOL HX 110 A mass spectrometer (JEOL, Tokyo, Japan) operating in the negative-ion and positive-ion modes, with accelerating voltages of 10 kV. A portion (1 μl) of the ammonium salt formed oligosaccharide in water was mixed with 1 μl glycerol/thioglycerol (1:1 v/v) on the probe tip of the mass spectrometer.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Determination of the M_r of boron–RG-II and its acid hydrolysate by MALDI-TOF-MS was done using a TofSpec-SE (Micromass Ltd, Manchester, U.K.) equipped with a nitrogen laser (337 nm). Matrix solution was prepared by dissolving 20 mg 2,5-dihydroxybenzoic acid in 1 ml acetonitrile/water (1:1). Aqueous sample solutions (*ca* 1 mg/1 ml) were mixed 1:1 with matrix solution and a portion (1.5 μl) deposited on the target.

Acknowledgement—This work was supported by a research grant (Biomedia Projects BMP-96-V-1-3) from the Japanese Ministry of Agriculture, Forestry and Fisheries.

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