ELSEVIER

Carbohydrate Research 284 (1996) 1–9

CARBOHYDRATE RESEARCH

Isolation and characterization of a boron–rhamnogalacturonan-II complex from cell walls of sugar beet pulp

Tadashi Ishii [a,*], Toshiro Matsunaga [b]

[a] *Forestry and Forest Products Research Institute, P.O. Box 16, Tsukuba Norin Kenkyu Danchinai, Ibaraki 305, Japan*

[b] *National Institute of Agro-Environmental Sciences, Kannondai, Tsukuba, Ibaraki 305, Japan*

Received 3 October 1995; accepted in revised form 20 December 1995

Abstract

A boron (B)–polysaccharide complex was isolated from a Driselase digest of sugar beet (*Beta vulgaris* L.) cell walls by ion-exchange and gel-permeation chromatography. The complex contained 0.12% B (w/w). The polysaccharide moiety contained 2-*O*-methylfucose, rhamnose, fucose, 2-*O*-methylxylose, arabinose, apiose, galactose, aceric acid, galacturonic, and glucuronic acids residues, and thiobarbituric acid-assay positive sugars, presumably 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) and 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha). Methylation analysis, together with glycosyl composition analysis, showed that the polysaccharide was a typical rhamnogalacturonan-II (RG-II), a structurally complex pectic polysaccharide present in the primary cell walls of plants. ^{11}B NMR spectroscopy showed that the B was present as a tetrahedral borate–diol diester. Approximately 70% of B was released by treating the B–RG-II complex at pH 4.8 at 40 °C.

Keywords: Boron; Cell walls; Pectic polysaccharide; Rhamnogalacturonan-II; Sugar beet

1. Introduction

Boron (B) is an essential micronutrient for higher plants. Boron deficiency produces various anatomical, physiological, and biochemical changes and numerous functions for B have been proposed [1]. However, the primary functions of B in plants are not known. Boron deficiency symptoms first appear at growing points and are characterized by cell-wall abnormalities [2]. Hu and Brown [3] reported that in squash and tobacco most

* Corresponding author.

SSDI S0008-6215(96)00010-9

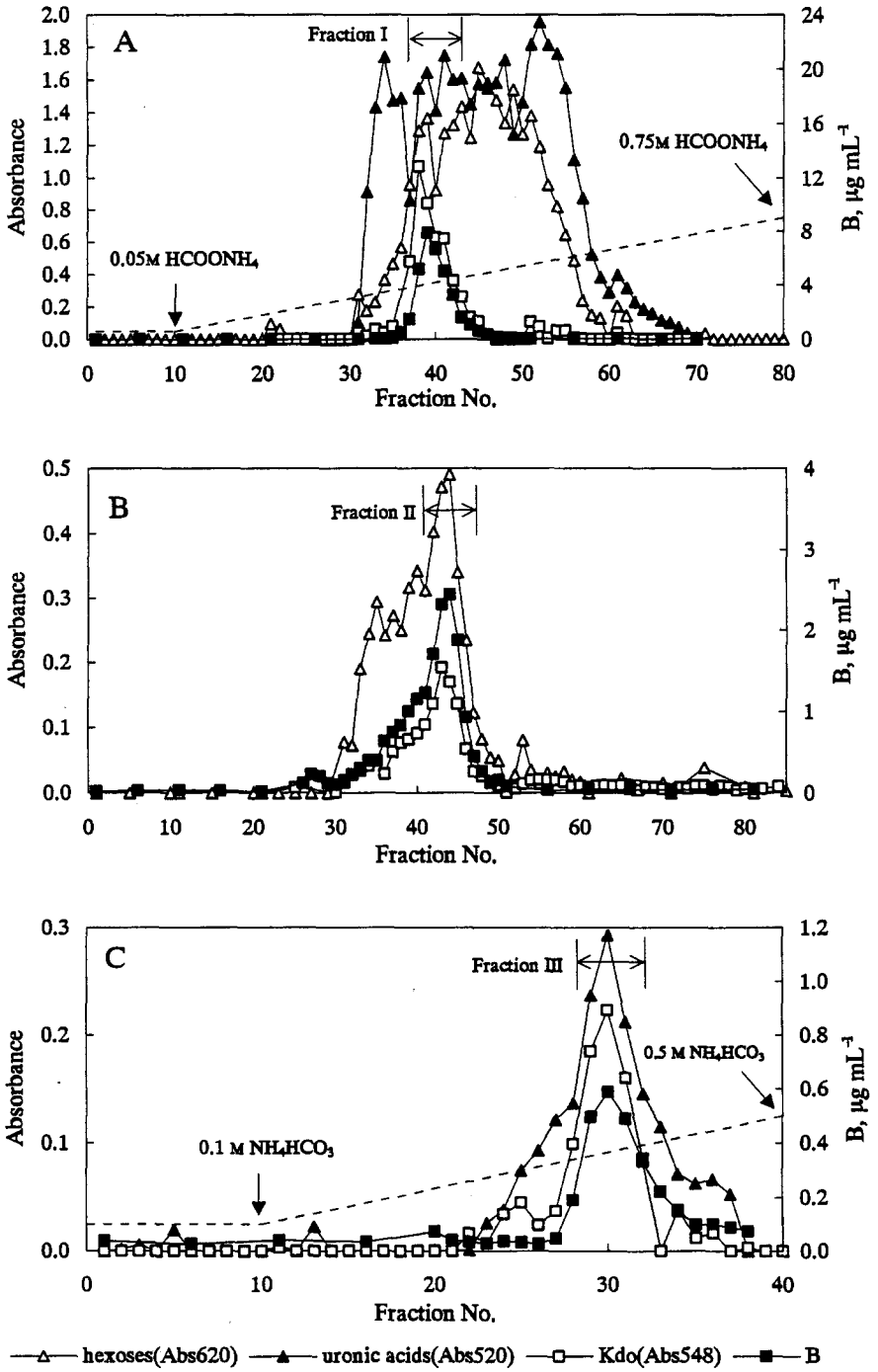
of the cellular B is located in the cell wall and that in the cell walls, B is associated with pectin. Matoh et al. [4] isolated a boron–polysaccharide complex from radish root and reported that ~ 60% of B in the cell wall was associated with this polysaccharide. Very recently Kobayashi et al. [5] indicated that the polysaccharide of the B complex was quite similar to rhamnogalacturonan-II (RG-II), which together with rhamnogalacturonan-I (RG-I) and homogalacturonan make up the pectic complex [6]. We have isolated and purified a B–polysaccharide complex from a Driselase digest of sugar beet cell walls by following the chromatographic separations using assays for B, uronic acid, and thiobarbituric acid-reactive components. The polysaccharide moiety was shown by glycosyl-residue and glycosyl-linkage composition analyses to be RG-II and the form of the B determined by ^{11}B NMR spectroscopy.

2. Materials and methods

Plant material.—Sugar beet pulp was a gift from Hokkaido Prefectural Senkon Agricultural Experimental Station (Senkon, Hokkaido). The pulp was refluxed with MeOH for 16 h and the alcohol-insoluble residue (AIR) was used as the cell wall material. AIR contained 43.4 μg B/g.

Isolation of B–RG-II complex.—Sugar beet cell walls were digested with Driselase [7] and the solubilized material was dialyzed against deionized H_2O with 10,000 mol wt cutoff dialysis membrane (Amicon Hollow fiber HIP10-20) [8]. The retentate was applied to a column (2.5 × 43 cm) of DEAE-Sepharose Fast Flow that had been equilibrated with 50 mM HCO_2NH_4 buffer, pH 7.0. The column was washed with equilibration buffer (500 mL) to remove unbound material. The column was then eluted with a linear gradient from 50 mM to 1 M HCO_2NH_4, pH 7.0 (total volume 1 L). Fractions (5 mL) were collected and assayed for B, thiobarbituric acid-assay positive sugars, hexoses, and uronic acids contents. Fraction I (fractions no. 37–43 in Fig. 1A) was pooled and freeze-dried. Fraction I was loaded onto a column (1.5 × 90 cm) of Bio Gel P-10, equilibrated with 200 mM HCO_2NH_4 buffer, pH 7.0, and eluted with the same buffer. Fractions (2 mL) were collected and assayed for B, thiobarbituric acid-assay positive sugar residues and hexose contents. Fraction II (fractions no. 41–47 in Fig. 1B) was pooled, dialyzed against deionized H_2O with 1000 mol wt cutoff dialysis membranes (Spectropor-7), and freeze-dried. Fraction II was loaded onto a column (1.5 × 25 cm) of Q-Sepharose Fast Flow, equilibrated with 100 mM NH_4HCO_3, pH 7.8, and the column was washed with the equilibration buffer (250 mL). The column was then eluted with a linear gradient from 100 to 500 mM NH_4HCO_3, pH 7.8 (total volume 500 mL). Fractions (5 mL) were collected and assayed for B, thiobarbituric acid-assay positive sugar residues, and uronic acids content. Fraction III (fractions no. 28–32, Fig. 1C) was pooled, desalted as above, and freeze-dried.

Fig. 1. Purification of B–RG-II complex by (A) ion-exchange chromatography on DEAE-Sepharose Fast Flow, (B) gel-permeation chromatography on Bio Gel P-10, and (C) ion-exchange chromatography on Q-Sepharose Fast Flow. Fractions were assayed for B (■), Kdo (□), hexose (△), and uronic acid (▲) by ICP-MS, thiobarbituric acid assay, anthrone, and metahydroxybiphenyl methods, respectively. Boron and polysaccharide-containing fractions were pooled as indicated for further purification.


A
Fraction I
0.05M HCOONH4
0.75M HCOONH4
Absorbance
B, μg mL-1
Fraction No.
B
Fraction II
Absorbance
B, μg mL-1
Fraction No.
C
Fraction III
0.1 M NH4HCO3
0.5 M NH4HCO3
Absorbance
B, μg mL-1
Fraction No.
hexoses(Abs620)
uronic acids(Abs520)
Kdo(Abs548)
B

Acid treatment of B–RG-II complex.—The B complex (2 mg) was treated with 0.5 M HCl (0.5 mL) for 30 min at 25 °C, neutralized with 1 M NaOH (0.25 mL), dialyzed against deionized water, and freeze-dried. The B content and mol wt of the sample were determined and glycosyl-composition and glycosyl-linkage analyses were performed as described below.

Analytical methods.—Neutral sugars, uronic acids, and Kdo concentrations were determined by the following methods, respectively; the anthrone method [9], the *m*-hydroxydiphenyl method [10], and the modified thiobarbituric acid assay [11].

Neutral glycosyl-residue compositions were determined by GLC of their alditol acetate derivatives [8]. Combined neutral and acidic glycosyl-residue compositions were determined by GLC of their trimethylsilyl methyl ester methyl glycoside derivatives [8].

Glycosyl-linkage compositions were determined using a modification of the Hakomori procedure [12]. Polysaccharides were per-*O*-methylated with methylsulfinylmethyl potassium and iodomethane, and the resulting products were isolated using Sep-Pak C_{18} cartridges (Waters, Milford, MA) [13]. The methyl esters of uronic acid residues in the per-*O*-methylated samples were reduced with Super-deuteride (1 M Li-triethylborodeuteride in tetrahydrofuran, Aldrich, Milwaukee, WI) as described by York et al. [14]. The glycosyl-linkage compositions were then determined by GLC–MS of the partially methylated, partially acetylated alditol acetate derivatives [14].

Boron determination.—The B concentrations in each fraction were determined with an inductively coupled plasma-mass spectrometer (ICP-MS) SII SPQ8000A. 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 were carrier H_2O, flow rate 1 mL min^{-1} and injection volume 20 μL.

NMR spectroscopy.—^{11}B NMR spectra of the B complex were recorded at 25 °C on a JEOL JNM-A600 spectrometer operated at 192.6 MHz without field lock using 5-mm diameter quartz-glass tubes. Chemical shifts (δ) are reported relative to external boric acid (0.1 M) at δ 0.0 ppm. NMR conditions were as follows: number of data point 2048, observation frequency range 28,900 Hz, pulse width 15 μs (90°), pulse repetition time 0.11 s, exponential broadening factor 15 Hz. For ^{1}H NMR spectrum, a sample was dissolved in D_2O and the same spectrometer was operated at 600.1 MHz.

High-performance size-exclusion chromatography (HPSEC).—The weight-average mol wt (MW) of the B complex was determined by HPSEC using a Shimadzu LC6A liquid chromatograph with a refractive index detector (Shimadzu model RID-6A) and a YMC Pack Diol 120 column (0.8 × 30 cm) (YMC, Kyoto, Japan), eluted with 200 mM HCO_2NH_4, pH 7.0, at 0.8 mL min^{-1} at 25 °C. The column was calibrated with pullulan narrow mol wt standards (P-5, MW = 5800; P-10, MW = 12,200; P-20, MW = 23,700; Showa Denko K.K., Tokyo).

3. Results

Isolation of a B–RG-II complex.—Driselase digests of sugar beet cell walls were separated into two fractions with a membrane filter of mol wt cutoff 10,000. Most of the

Table 1
Glycosyl-residue composition of B–RG-II complex from sugar beet cell walls

Glycosyl residue [a]	B–RG-II	Sycamore [b]
Rha	11.3	12.4
Fuc	1.6	2.8
2Me–Fuc	3.3	3.5
Ara	10.9	10.8
2Me–Xyl	4.9	4.8
Apiose	4.5	12.2
Gal	12.4	9.0
GlcA	7.0	3.2
GalA	37.7	31.2
Aceric acid	[c]	3.5
Kdo	5.3	3.5
Dha	5.3	3.5

[a] Values expressed as mol%.
[b] Stevenson et al. [15].
[c] Present but not quantified.

B in the enzymatic digest was present in the retentate of the membrane filtration. The retentate material was separated into neutral (unbound) and acidic (bound) fractions by ion-exchange chromatography on DEAE-Sepharose Fast Flow (Fig. 1A). The fraction containing B was further purified on Bio Gel P-10 (Fig. 1B), which proved ineffective in separating any B–polysaccharide complex from higher mol wt material. An additional pass on Q-Sepharose Fast Flow was carried out to achieve a further purification (Fig. 1C). On all three chromatograms (Fig. 1A–C), B and thiobarbituric acid-assay positive material co-eluted. Fractions from Q-Sepharose chromatography were pooled to give Fraction III as shown in Fig. 1C and when this was chromatographed on HPSEC it gave a single symmetrical peak (not shown). The mol wt of the complex was determined to be 10,500 by calibration of HPSEC with pullulan narrow mol wt standards.

Composition of B–RG-II complex.—The purified complex contained 0.12% B (w/w). The glycosyl residue composition of the B complex is given in Table 1, together with RG-II from sycamore for ref. [15]. The B complex had 45% uronic acid residues. GLC–MS analysis of alditol acetates from the B complex obtained after acid hydrolysis, reduction, and per-*O*-acetylation showed the presence of 2-*O*-methylfucose (*m/z* 117, 171, 231, and 275) [16], 2-*O*-methylxylose (*m/z* 117, 201, and 261) [16] and apiose (*m/z* 145, 187, and 302) [17], in addition to rhamnose, fucose, arabinose, and galactose. Kdo and Dha were measured together by a modified thiobarbituric acid assay and accounted for 5.3%. 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 [6]. 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). Glycosyl-linkage analysis of the B complex is shown in Table 2 together with RG-II from sycamore [15]. The B complex contained 3-linked rhamnosyl, 2,3,4-linked rhamnosyl, 3,4-linked fucosyl, and 2-linked glucuronic acid residues, which are characteristic of RG-II. Based on the

Table 2
Glycosyl-linkage composition of B–RG-II complex from sugar beet cell walls

Glycosyl linkage [a]		B–RG-II	Sycamore [b]
Rha	T-Rha [c]	4.5	6.6
	2-Rha	4.3	tr
	3-Rha	5.3	5.7
	2,3-Rha	0.8	nd
	2,4-Rha	0.9	nd
	2,3,4-Rha	6.5	4.5
Fuc	3,4-Fuc	3.3	4.5
2MeFuc	T-Fuc	5.6	4.8
Apiose	3′-Apiose	5.0	10.9
Ara	T-Ara*f*	6.3	6.1
	T-Ara*p*	2.5	nd
2MeXyl	T-Xyl	4.1	4.5
Gal	T-Gal	5.0	4.9
	2,4-Gal	5.2	5.6
	3,4-Gal	4.3	nd
	3,6-Gal	0.3	nd
GalA	T-GalA	10.0	10.3
	4-GalA	14.3	8.8
	3,4-GalA	nd	7.3
	2,4-GalA	nd	4.6
	2,3,6-GalA	4.7	1.5
GlcA	2-GlcA	7.1	6.3

[a] Values expressed as mol%.
[b] Stevenson et al. [15].
[c] T, non-reducing terminal rhamnosyl, etc.; tr, trace; nd, not determined.

similarity of the glycosyl-linkage composition of the B–polysaccharide complex to that of sycamore RG-II [15], together with the presence of 2-*O*-methylfucose, 2-*O*-methylxylose, apiose, aceric acid, and thiobarbituric acid positive-sugar residues, we conclude that the polysaccharide moiety of the B complex was RG-II.

^{11}B NMR spectroscopy of the B–RG-II complex.—^{11}B NMR spectroscopy is useful in characterizing the esters formed by the interaction of boric acid and borate with diols [18]. The ^{11}B NMR spectrum of the B–RG-II complex (Fig. 2) contains a strong signal at δ −9.5, indicating that the B complex is present as a tetrahedral borate–diol diester [18]. A weak signal at δ 0.0 was assigned to boric acid. As free boric acid would be completely separated from the B complex during purification processes, the boric acid detected in the B–RG-II complex was probably liberated from the B complex during dialysis, sample preparation, and NMR measurement. The liberation of B from the B–RG-II complex in H_2O was determined by ^{11}B NMR spectroscopy. A solution of the B complex in H_2O has a pH of 4.8. When the complex was kept at 25 °C for 5 and 12

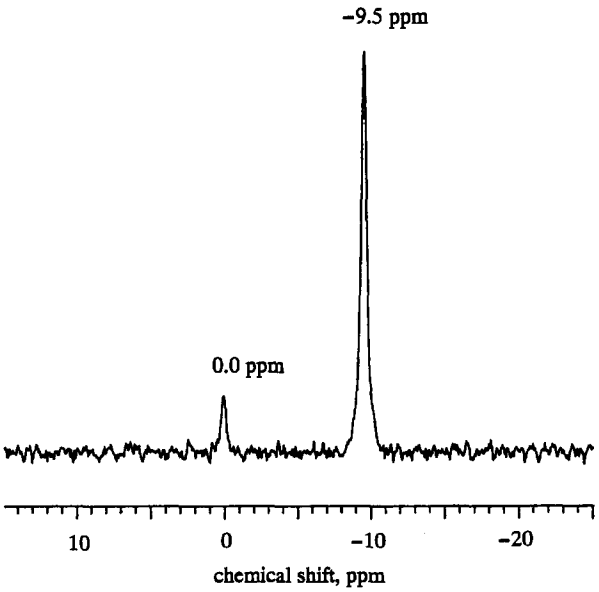


Fig. 2. A ^{11}B MNR spectrum of the B–RG-II complex from sugar beet cell walls (64,540 scans). A sample (2.2 mg) was dissolved in 0.5 mL of H_2O to give a solution of pH 4.8. Chemical shifts are referenced to external 0.1 M boric acid at 0.0 ppm.

days, the percentage B lost from the complex, based on the signal intensities of diester and boric acid, was 9 and 15%, respectively. The B complex degraded faster at 40 than at 25 °C. Treatment of the complex at 40 °C for 5 days caused 64% degradation. After 10 days, 71% of the complex degraded. The pH of the B complex which had been treated at 40 °C for 10 days was adjusted to 7.9, and then ^{11}B NMR spectrum recorded. The signal intensities of boric acid and borate–diol diester did not change. The mol wt of the B complex treated at 40 °C was measured by HPSEC to be about 9500.

Acid treatment of B–RG-II complex.—The B–RG-II complex was treated with 0.5 M HCl solution for 30 min at 25 °C. The HCl-treated complex contained negligible amount of B (less than 0.002%). Dilute acid treatment of the B complex reduced its mol wt to 7000, determined by HPSEC. However, glycosyl-composition and linkage analyses showed that there was no significant structural difference between the B–RG-II complex and the HCl-treated complex (data not shown).

4. Discussion

Matoh et al. [4] isolated a uronic acid-rich B–polysaccharide complex from radish roots and very recently Kobayashi et al. [5] indicated that the polysaccharide moiety of a B–polysaccharide complex from the same source was similar to an RG-II. We have now isolated a B–polysaccharide complex from a sugar beet cell-wall preparation and for the

first time identified the polysaccharide moiety as an RG-II by structural analysis. ^{11}B NMR spectroscopy confirmed that B bound RG-II to form terahedral borate–diol diester in the complex.

RG-II is one of the components of pectin. It was originally isolated from cell walls of suspension-cultured sycamore cells and is now known to be present in the primary walls of all higher plants [6]. The primary structure of RG-II is very similar among dicots, monocots, and gymnosperms [6]. RG-II has a complex structure, its biosynthesis requires at least 20 glycosyl transferases, large amounts of energy, and precise regulation of these reactions. To date, there have been no reports on the biological and structural functions of RG-II. The association of RG-II with B found here may relate to the function of RG-II in plant cell walls.

Loomis and Durst [2] postulated that the apiose–borate esters cross-links were the auxin-sensitive acid growth link in vascular plants on the basis of the results that the apiose–borate esters had $pK'_a < 5$ and that the components equilibrated rapidly and the equilibrium was pH dependent. The borate ester found in the B–RG-II complex appears to be different from the apiose–borate ester since our results show that in the B–RG-II complex there is no equilibrium between boric acid, borate monoester, and borate diester, but rather only the borate diester is present in the complex. We observed that the B–RG-II complex released boric acid gradually at pH 4.8 at 25 or 40 °C and that the borate-diol did not reform when the pH of the solution was adjusted to pH 7.9. Furthermore, there was no signal corresponding to borate monoester at δ −11.5 to −14.5 [18].

We found that the B–RG-II complex released B at pH 4.8 without significant change in mol wt Treatment of B–RG-II with 0.5 M HCl for 30 min at 25 °C released B completely and decreased the mol wt of the polysaccharide by 30% (from 10,500 to 7000). HCl treatment may cleave acid-labile glycosyl linkages involving residues such as Kdo and apiose in RG-II. It is reported that approximately 50% of rhamnose-Kdo disaccharide, 35% of apiose-containing octa- and nona-saccharides are released from sycamore RG-II by treatment with 1 M AcOH for 6 h at 40 °C [11], and with 0.1 M TFA for 16 h at 40 °C [19], respectively. Nevertheless the decrease in apparent mol wt by 3500 is difficult to explain in terms of cleavage of acid labile glycosyl linkages in the polysaccharides.

Our determination of the mol wt of the B complex was based on calibration with pullulan standards without regard for charge, conformation, and intrinsic viscosity differences between the RG-II and the pullulans. Removal of B and cleavage of the acid-labile glycosidic side chains may cause some changes in retention properties of HPSEC. Stevenson et al. [15] determined, by reducing-end measurements, that the mol wt of RG-II from sycamore was ~ 5000. Doco and Brillouet [20] reported that wine RG-II has a mol wt of 5300 and 9800, as determined by the low-angle laser-light-scattering method and HPSEC with pullulan standards, respectively. The mol wt of the B–RG-II complex, determined here by HPSEC to be 10,500, might be an over-estimate.

Further studies are required to determine the mol wt of the complex exactly by mass spectrometry. Hopefully this paper clarifies the nature of the polysaccharide in the B–RG-II complex. What is needed is a determination of the location of the borate binding sites on the polysaccharide.

Acknowledgements

We thank Drs P. Albersheim and M. O'Neill (Complex Carbohydrate Research Center, the University of Georgia, Athens, GA, USA) for critically reading the manuscript and Dr. M. Fujita (Hokkaido Prefectural Senkon Agricultural Experimental Station, Senkon, Hokkaido) for providing the sugar beet roots, and Mrs Y. Takeda for editorial assistance. This work was supported by a research grant (Biomedia Projects, BMP-96-V-1-1) from the Ministry of Agriculture, Forestry, and Fisheries.

References

[1] B.J. Shelp, in U.C. Gupta (Ed.), *Boron and Its Role in Crop Production*, CRC Press, Boca Raton, pp 53–85.
[2] W.D. Loomis and R.W. Durst, *BioFactors*, 3 (1992) 229–239.
[3] H. Hu and P.H. Brown, *Plant Physiol.*, 105 (1994) 681–689.
[4] T. Matoh, K. Ishigaki, K. Ohno, and J. Azuma, *Plant Cell Physiol.*, 34 (1993) 639–642.
[5] M. Kobayashi, T. Matoh, and J. Azuma, *Plant Cell Physiol. Suppl.*, (1995) 139.
[6] M. O'Neill, P. Albersheim, and A. Darvill, in P.M. Dey and J.B. Harborne (Eds), *Methods in Plant Biochemistry*, Vol. 2, Academic Press, London, 1990, pp 415–441.
[7] T. Ishii, *Plant Cell Physiol.*, 35 (1994) 701–704.
[8] T. Ishii, *Mokuzai Gakkaishi*, 41 (1995) 669–676.
[9] Z. Dische, in R. Whistler and M. Wolfrom (Eds.), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, pp 478–512.
[10] N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
[11] W.S. York, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 138 (1985) 109–126.
[12] S. Hakomori, *J. Biochem.*, 55 (1964) 205–208.
[13] T.J. Waeghe, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
[14] W.S. York, A.G. Darvill, M. McNeil, T.T. Stevenson, and P. Albersheim, *Methods. Enzymol.*, 118 (1985) 3–40.
[15] T.T. Stevenson, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 182 (1988) 207–226.
[16] P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lonngren, *Chem. Common. Univ. Stockholm*, (1976) 1–20.
[17] A.G. Darvill, M. McNeil, and P. Albersheim, *Plant Physiol.*, 62 (1978) 418–422.
[18] M. Van Duin, J.A. Peters, A.P.G. Kieboom, and H. Van Bekkum, *Tetrahedron*, 40 (1984) 2901–2911.
[19] A.J. Whitcombe, M.A. O'Neill, W. Steffan, P. Albersheim, and A.G. Darvill, *Carbohydr. Res.*, 271 (1995) 15–29.
[20] T. Doco, J.-M. Brillouet, *Carbohydr. Res.*, 243 (1993) 333–343.