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Note

Rhamnogalacturonan II, a dominant polysaccharide in juices produced by enzymic liquefaction of fruits and vegetables

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Abstract

Rhamnogalacturonan II (RG-II), a small complex pectic polysaccharide, is released from apple (*Malus domestica*), carrot (*Daucus carota*), and tomato (*Solanum lycopersicum*) by treatment with two commercial liquefying enzyme preparations. RG-II was isolated by size-exclusion chromatography from apple, tomato, and carrot juices obtained by enzymic liquefaction. All the RG-IIs contained the diagnostic sugars, apiose, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, aceric acid, Kdo and Dha. Glycosyl-linkage compositions of the neutral and acidic sugars, including aceric acid, were consistent with the hypothetical model described for sycamore RG-II confirming the conservation of RG-II in plants. Thus, when pectinolytic enzyme preparations are used to process fruits and vegetables, RG-II is released as a main soluble polysaccharide fraction while other pectic polysaccharides are heavily degraded. © 1997 Elsevier Science Ltd.

Keywords: Rhamnogalacturonan II; Apple; Carrot; Tomato; Pectin; Pectic polysaccharide; *Malus domestica*; *Solanum lycopersicum*; *Daucus carota*; Enzymic liquefaction; Pectinases

1. Introduction

Cell walls determine, to a large extent, the qualities attributed to fruits and vegetables and their processing characteristics [1,2]. Pectic polysaccharides are abundant in primary cell walls and have been the subject of many studies [3–5]. Three pectic polysaccharides have been isolated from primary cell walls [3], homogalacturonan, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II). Rhamnogalacturonan II was first isolated after treatment of sycamore (*Acer pseudoplatanus*) cell walls by a fungal endopolygalacturonase [6]. The presence of RG-II has been reported in the walls of Douglas fir [7], rice [8], onion [9], kiwi fruit [10], radish [11], *Bupleurum falcatum* roots [12], *Arabidopsis thaliana* [13] and *Panax ginseng* [14] leaves, sugar-beet pulp [15] and in a commercial enzyme preparation Pectinol AC [16]. RG-II has also been isolated from red wine by the authors [17] and characterized as a major component of wine polysaccharides [18].

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RG-II is a small complex polysaccharide containing twelve different glycosyl residues including several rare "diagnostic" monosaccharides such as apiose [6], 2-*O*-methyl-L-Fuc [6], 2-*O*-methyl-D-Xyl [6], aceric acid (3-*C*-carboxy-5-deoxy-L-Xyl) [16], Kdo (3-deoxy-D-*manno*-octulosonic acid) [19] and Dha (3-deoxy-D-*lyxo*-heptulosaric acid) [20]. The backbone of RG-II is built of 1,4-linked α-D-galacturonosyl residues carrying four oligosaccharide side-chains [3,5,21]. Recently, Kobayashi et al. [11] and O'Neill et al. [22] have shown that RG-II is present predominantly as a dimer in plant cell walls. This dimer is cross-linked by borate-diol diesters, located on one apiosyl residue [22], and seems to play an important role in the structure and functions of pectic polysaccharides in primary cell walls [22].

The composition and structure of fruit pectic polysaccharides have been extensively studied [23–30]. De Vries et al. [29] proposed a general model for apple pectin based on alternating linear α-(1 → 4)-galacturonan chains and hairy regions containing most neutral sugars. Schols et al. [30] determined the structure of hairy regions as branched, highly acetylated rhamnogalacturonans, carrying side-chains composed of arabinose, galactose or xylose. Numerous studies have reported the presence and composition of polymers including rhamnogalacturonan rich regions isolated from kiwi [11], apple [25], carrot [24,27], potato [27], pear [27], grape [31], and also from apple juice manufactured by the liquefaction process [30]. Several enzymes able to cleave the rhamnogalacturonan backbone of hairy regions have been reported [32–36]. Enzyme preparations containing high levels of pectinolytic activities (hydrolases, lyases, esterases) are widely used in the fruit-processing industry to increase yields, improve liquefaction, clarification and filterability of juices [37].

As far as we are aware, the presence of RG-II in juices obtained directly by enzymic liquefaction of fruit and vegetables by an industrial process has not been reported. This paper reports that RG-II is present as one of the main polysaccharides in juices obtained by enzymic liquefaction of apples (*Malus domestica*), tomatoes (*Solanum lycopersicum*), and carrots (*Daucus carota*).

2. Results and discussion

Total soluble polysaccharides present in apple, carrot and tomato juices initially and after 2 and 24 h of enzymic liquefaction were isolated by ultrafiltration. Molecular size distributions were determined by

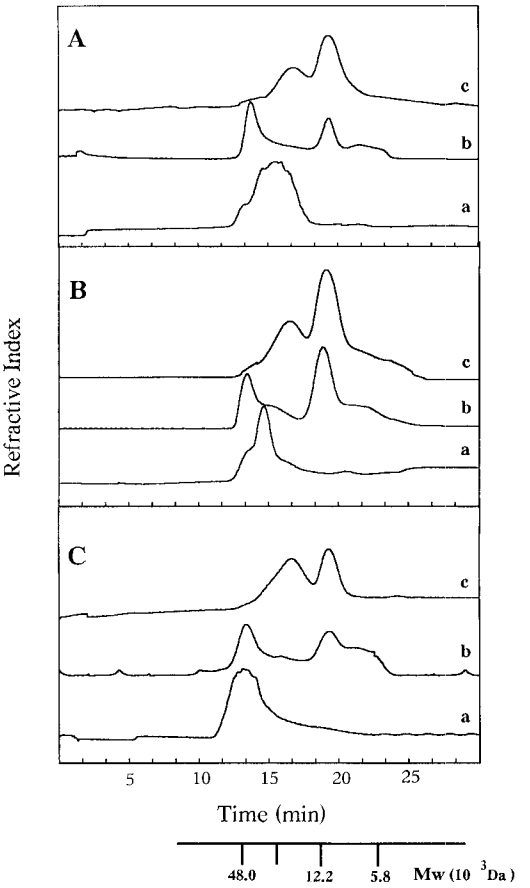

Fig. 1. Superdex-75 high-resolution size-exclusion chromatography of total soluble polysaccharides obtained from apples (A), carrots (B) and tomatoes (C) initially (a), and after 2 (b) and 24 hours (c) of enzymic liquefaction with 0.1% of Rapidase LIQ® and Pectinex® Ultra-SPL.

Table 1
Composition analysis (mole percentage) of RG-II fractions isolated from red wine, apple, carrot and tomato juices obtained by the liquefaction process

	Red wine [a]	Apple	Carrot	Tomato
Rha	16.8	17.4	14.3	11.6
2-*O*-CH_3-Fuc	3.7	4.8	5.0	4.4
Fuc	3.0	5.5	4.7	4.0
Apiose	6.2	5.4	9.1	7.0
Ara	11.2	16.8	14.0	12.4
2-*O*-CH_3-Xyl	2.8	2.9	4.0	3.3
Gal	6.6	6.4	7.8	8.1
GalA	38.2	33.0	28.3	35.5
GlcA	3.3	2.8	2.7	4.4
Aceric acid	1.2	1.5	3.5	2.3
Kdo	2.6	1.1	2.3	4.0
Dha	4.4	2.5	4.6	3.0

[a] According to ref. [18].

high resolution size-exclusion chromatography on a Superdex-75 HR column (Fig. 1). After 24 h of liquefaction with Rapidase LIQ® and Pectinex® Ultra-SPL, two pectic populations eluted at 16.7 and 19.1 min, respectively. Ultrafiltration retentates were fractionated by size-exclusion chromatography on a Sephacryl S-200 HR column. The fractions which eluted at the same time as isolated wine RG-II [18] were collected. Glycosyl-residue compositions (Table 1) of the isolated fractions indicated that they were composed of at least 85% of RG-II with the characteristic composition including the diagnostic monosaccharides, apiose, 2-*O*-methyl-L-Fuc, 2-*O*-methyl-D-Xyl, aceric acid, Kdo and Dha. GalA, Ara, Rha and Gal were found to be the dominant monosaccharides. Molar ratios were close to the composition reported for sycamore and isolated wine RG-II [3,18]. Kdo, Dha and aceric acid contents were lower than the theoretical values expected, but these acidic sugars are known for their acid-lability and are therefore difficult to determine quantitatively [13,20]. The amount of each isolated fraction indicated that RG-II was present approximately at 350, 700 and 170 mg/L, respectively, in apple, carrot and tomato

Table 2
Glycosyl linkage composition (mole percentage) of RG-II fractions isolated from apple, carrot and tomato juices obtained by the liquefaction process

Glycosyl residue	Linkage	Residue [a]	Apple	Carrot	Tomato
Rhamnosyl	Rha*p* [b]-(1 →	B6, C2	3.2	5.8	4.4
	→ 2)-Rha*p*-(1 →	B6	5.2	2.1	1.8
	→ 3)-Rha*p*-(1 →	B2	3.1	3.1	3.9
	→ 2,4)-Rha*p*-(1 →		2.4	2.4	–
	→ 2,3)-Rha*p*-(1 →		–	0.8	0.5
	→ 2,3,4)-Rha*p*-(1 →	A2	4.6	3.9	6.2
2-*O*-Me-Fucosyl	2-*O*-Me-Fuc*p*-(1 →	B4′	2.5	2.4	2.5
Fucosyl	→ 3,4)-Fuc*p*-(1 →	A3	3.4	2.9	3.3
Apiosyl	→ 3′)-Api-(1 →	A1 or B1	3.8	3.9	4.8
	→ 2,3,3′)-Api-(1 →	A1 or B1	2.8	2.2	3.2
Arabinosyl	Ara*f*-(1 →	B7, D2	6.1	8.5	4.9
	Ara*p*-(1 →		1.9	0.7	1.8
	→ 2)-Ara*p*-(1 →	B5	–	2.2	0.9
	→ 2)-Ara*f*-(1 →		–	0.9	1.8
	→ 3)-Ara*f*-(1 →		2.8	0.5	0.6
	→ 4 or 5)-Ara-(1 →		2.3	–	0.9
	→ 2,3)-Ara*p*-(1 →	B5	3.6	2.2	1.1
2-*O*-Me-Xylosyl	2-*O*-Me-Xyl *p*-(1 →	A3′	4.7	2.2	2.4
Galactosyl	Gal*p*-(1 →	A5	4.1	3.7	5.6
	→ 3)-Gal*p*-(1 →		1.2	1.8	2.0
	→ 4)-Gal*p*-(1 →		–	2.2	–
	→ 3,6)-Gal*p*-(1 →		3.2	3.8	1.5
	→ 3,4)-Gal*p*-(1 →		0.4	1.7	1.3
	→ 2,4)-Gal*p*-(1 →	B4	3.0	3.8	4.1
Galacturonosyl	Gal*p*A-(1 → [c]	A2′, A2″, Backbone	9.9	10.0	11.4
	→ 4)-Gal*p*A-(1 →	Backbone	9.7	8.4	8.8
	→ 3,4)-Gal*p*A-(1 →	Backbone	6.6	5.2	5.5
	→ 2,4)-Gal*p*A-(1 →	Backbone	4.5	4.5	5.7
	→ 2,3,4)-Gal*p*A-(1 →	Backbone	2.2	1.8	4.1
Glucuronosyl	Glc*p*A-(1 →	A4	–	1.0	0.9
	→ 2)-Glc*p*A-(1 →	A4	2.3	3.7	3.8
Aceryl	→ 2)-Ace*f*A-(1 → [d]	B3	0.4	0.2	0.3

[a] According to ref. [18].
[b] Rha*p*[b]-(1 → = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol, etc.
[c] 6-Dideuterated ether.
[d] 3′-Dideuterated ether.

juices. These values corresponded, respectively, to ~ 400, 800 and 200 mg of RG-II per kg of fresh apples, carrots and tomatoes.

Juice RG-IIs were permethylated [39] and then the methyl-esterified carboxyl groups were reduced with lithium triethylborodeuteride prior to hydrolysis, reduction and acetylation [20,40]. The identification of all the derivatives and especially the presence of C-6 dideuteriohexitols arising from acidic monosaccharides were confirmed by GC–EI–MS analyses. Glycosyl-linkage compositions of the three juice RG-IIs (Table 2) were all consistent with the theoretical model of RG-II since most methyl ethers obtained correspond to known glycosyl residues of RG-II [3,8,17,18,21,38,41]. Equivalent mole percentages were found for the 2,3,4-linked rhamnosyl, 3,4-linked fucosyl, terminal non-reducing 2-*O*-methyl-xylosyl, and 2-linked glucuronosyl residues, all originating from side-chain A, the 2-*O*-Me-Xyl containing-oligosaccharide [5,18]. This was also the case for the terminal non-reducing 2-*O*-methyl-fucosyl, 2,4-linked galactosyl, 3-linked rhamnosyl and 2-linked arabinosyl residues belonging to side-chain B, the aceric acid-containing oligosaccharide [5,18]. Apiose was present as 3′-linked and 2,3,3′-linked residues.

The terminal non-reducing, 4-linked, 3,4-linked, 2,4-linked galacturonosyl residues correspond to the homogalacturonan backbone given in the model [18,21,38,41]. The presence in all samples of 2,3,4-linked galacturonosyl residues supports the hypothesis that one of the 4-linked Gal*p*A residues in the backbone may be substituted with two oligosaccharide side chains [5,18]. Aceric acid, the specific sugar of RG-II, was present in all fruit-derived RG-IIs and could be identified as being 2-linked as previously described [18,42]. Dha and Kdo were not analysed in this study as they were destroyed under the acidic conditions used to cleave glycosidic linkages [20].

RG-II exists in plants both as a monomer and as a dimer that is cross-linked by borate di-esters [11,18,22]. These two forms, well separated by high resolution size-exclusion chromatography on Superdex-75 HR (respective elution times of ~ 19.0 and 21.0 min) [18,22], have the same glycosyl-residue compositions but differed by their MW (4750 and 9500, respectively), and by the specific presence of boron and of 2,3,3′-linked apiose in dimeric RG-II [18,22]. The Superdex-75 profiles (Fig. 2) show that RG-II was present mainly as a dimer in apple, carrot and tomato juices. Boron was only detected in the dimeric RG-II (dRG-II) peak collected separately from the whole apple RG-II preparation but not in the

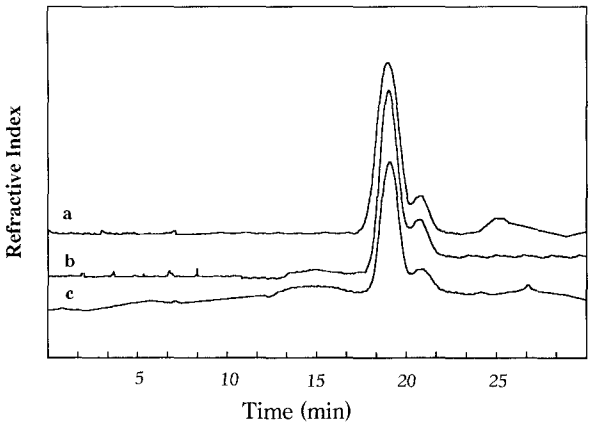

Fig. 2. Superdex-75 high-resolution size-exclusion chromatography of apple (a), carrot (b) and tomato (c) isolated RG-IIs.

corresponding monomeric RG-II (mRG-II) peak (M. O'Neill, personal communication). This result is in accordance with the presence of 2,3,3′-linked apiosyl residue (Table 2) since this residue is assumed to be involved in the formation of tetrahedral 1,2 borate diol diester bonds located on C-2 and C-3 of apiose [18,22].

This study has shown that a low molecular weight pectic polysaccharide can be isolated from juices obtained by enzymic liquefaction of apples, carrots and tomatoes. Glycosyl-residue and glycosyl-linkage analyses confirm that its structure resembles the theoretical model of sycamore RG-II [3,5] which was released by treatment of isolated cell walls with a purified endo-polygalacturonase. This structural similarity confirms the conservation of this polysaccharide among primary plant cell walls [3,5] and indicates that RG-II can be defined as an enzyme-resistant pectic polysaccharide. The use of liquefying enzymes during fruit and vegetable processing induces an enrichment of the derived products in RG-II whereas other pectic polysaccharides like homogalacturonan or rhamnogalacturonan rich regions are heavily degraded. RG-II which represents 1–8% of dicots cell walls, is a major soluble polysaccharide when fruits and vegetables are processed with liquefying industrial enzyme preparations. It is now necessary to assess the technological consequences of its presence in fruit and vegetable-derived products.

3. Experimental

Fruit and vegetable materials.—Apples (*Malus domestica* — Golden delicious), carrots (*Daucus carota*) and tomatoes (*Solanum lycopersicum*) were

bought at the local market. 100 g were peeled, sliced and crushed in waring blender (2 min) and treated at 45 °C with 0.1% of both Rapidase LIQ® (Gist Brocades, The Netherlands) and Pectinex® Ultra-SPL (Novo Ferment, Switzerland) in the presence of 3 mM ascorbic acid. Samples were removed before the addition of enzymes (initial) and after 2, 4 and 24 hours of incubation. After enzyme inactivation at 100 °C, total polysaccharides were recovered from the ultrafiltration retentates on centricon membrane (Amicon, MW Cut Off 30 kDa). RG-II was purified from ultrafiltration retentates by size-exclusion chromatography on a Sephacryl S-200 HR column (1.6 × 95 cm; Pharmacia) equilibrated in 50 mM sodium acetate buffer pH 5.2 containing 50 mM NaCl. The fractions which eluted at the same volume as an isolated red wine RG-II sample (10 mg) [18] were collected.

Analytical methods.—Molecular weight distributions of ultrafiltration retentates (1 mL), initial and after 2 and 24 hours of enzymatic incubation were examined by high resolution size-exclusion chromatography on a Superdex-75 HR column (1.3 × 30 cm; Pharmacia) equilibrated at 0.6 mL/min in 30 mM ammonium formate buffer pH 5.2 [18] and calibrated with narrow pullulan molecular-weight standards (P-5, MW = 5800; P-10, MW = 12,200; P-20, MW = 23,700; P-50, MW = 48,000; Showa Denko Japan). Neutral and acidic glycosyl residues were analysed by GC of their trimethylsilyl methyl-ester methyl glycoside derivatives [18,38].

Glycosyl-linkage determination.—Glycosyl-linkages were determined by GC–MS of the partially methylated alditol acetates [18,38–40]. The derivatization procedure included the reduction of carboxyl groups with lithium triethylborodeuteride (Superdeuteride®, Aldrich, USA).

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References

[1] M. Knee, *Phytochem.*, 12 (1973) 1543–1549.
[2] A.G.J. Voragen, H.A. Schols, H.A.I. Siliha, and W. Pilnik, *ACS Symp. Ser.*, 310 (1986) 230–247.
[3] M. O'Neill, P. Albersheim, and A.G. Darvill, in P.M. Dey (Ed.), *Methods in Plant Biochem.*, Vol. 2, Academic Press, London, 1990, pp 415–441.
[4] N.C. Carpita and D.M. Gibeaut, *The Plant J.*, 3 (1993) 1–30.
[5] P. Albersheim, J. An, G. Freshour, M.S. Fuller, R. Guillen, K.-S. Ham, M.G. Hahn, J. Huang, M. O'Neill, A. Whitcombe, M.V. Williams, W.S. York, and A.G. Darvill, *Biochem. Soc. Trans.*, 22 (1994) 374–378.
[6] A.G. Darvill, M. McNeil and P. Albersheim, *Plant Physiol.*, 62 (1978) 418–422.
[7] J.R. Thomas, M. McNeil, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 83 (1987) 659–671.
[8] J.R. Thomas, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 185 (1989) 261–277.
[9] S. Ishii, *Phytochemistry*, 21 (1982) 778–780.
[10] R.J. Redgwell, L.D. Melton, D.J. Brasch, and J.M. Coddington, *Carbohydr. Res.*, 286 (1992) 287–302.
[11] M. Kobayashi, T. Matoh, and J.-L. Azuma, *Plant Physiol.*, 110 (1996) 1017–1020.
[12] H. Yamada, M. Hirano, and H. Kiyohara, *Carbohydr. Res.*, 219 (1991) 173–192.
[13] E. Zablackis, J. Huang, B. Müller, A.G. Darvill, and P. Albersheim, *Plant Physiol.*, 107 (1995) 1129–1138.
[14] K.-S. Shin, H. Kiyohara, T. Matsumoto, and H. Yamada, in J. Visser (Ed.), *Pectins and Pectinases*, Elsevier, Amsterdam, 1996, in press.
[15] T.Ishii and T. Matsunaga, *Carbohydr. Res.*, 284 (1996) 1–9.
[16] M.W. Spellman, M. McNeil, A.G. Darvill, P. Albersheim, and K. Henrik, *Carbohydr. Res.*, 122 (1983) 115–129.
[17] T. Doco and J.-M. Brillouet, *Carbohydr. Res.*, 243 (1993) 333–343.
[18] P. Pellerin, T. Doco, S. Vidal, P. Williams, J.-M. Brillouet, and M.A. O'Neill, *Carbohydr. Res.*, 290 (1996) 183–197.
[19] W.S. York, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 138 (1985) 109–126.
[20] T.T. Stevenson, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 179 (1988) 269–288.
[21] V. Puvanesarajah, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 218 (1991) 211–222.
[22] M. O'Neill, D. Warrenfeltz, K. Kates, P. Pellerin, T. Doco, A.G. Darvill, and P. Albersheim, *J. Biol. Chem.*, 271 (1996) 22923–22930.
[23] P. Massiot, J.-F. Thibault, and X. Rouau, *J. Sci. Food Agric.*, 49 (1989) 45–57.
[24] P. Massiot, A. Baron, and J.-F. Drilleau, *Carbohydr. Polym.*, 25 (1994) 145–154.
[25] C.M.G.C. Renard, H.A. Schols, A.G.J. Voragen, J.-F. Thibault, and W. Pilnik, *Carbohydr. Polym.*, 15 (1991) 13–32.
[26] C.M.G.C. Renard, M.-J. Crépeau, and J.-F. Thibault, *Carbohydr. Res.*, 275 (1995) 155–165.
[27] H.A. Schols and A.G.J. Voragen, *Carbohydr. Res.*, 256 (1994) 83–95.
[28] H.A. Schols, E. Vierhuis, E.J. Bakx, and A.G.J. Voragen, *Carbohydr. Res.*, 275 (1995) 343–360.

[29] J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.*, 2 (1982) 25–33.
[30] H.A. Schols, M.A. Posthumus, and A.G.J. Voragen, *Carbohydr. Res.*, 206 (1990) 117–129.
[31] L. Saulnier, J.-M. Brillouet, and J.-P. Joseleau, *Carbohydr. Res.*, 182 (1988) 63–78.
[32] H.A. Schols, C.C.J.M. Geraeds, M.J.F. Searle-van Leeuwen, F.J.M. Kormelink, and A.G.J. Voragen, *Carbohydr. Res.*, 259 (1990) 105–115.
[33] M.J.F. Searle-van Leeuwen, L.A.M. van den Broek, H.A. Schols, G. Beldman, and A.G.J. Voragen, *Appl. Microbiol. Biotechnol.*, 38 (1992) 347–349.
[34] T. Sakamoto and T. Sakai, *Carbohydr. Res.*, 206 (1995) 77–91.
[35] P. Azadi, M. McNeil, C. Bergmann, A.G. Darvill, and P. Albersheim, *Glycobiology*, 5 (1995) 783–789.
[36] M. Mutter, I.J. Colquhoun, H.A. Schols, G. Beldman, and A.G.J. Voragen, *Plant Physiol.*, 110 (1996) 73–77.
[37] A.G.J. Voragen and W. Pilnik, ACS Symp. Ser., 389 (1989) 223–247.
[38] W.S. York, A.G. Darvill, M. McNeil, T.T. Stevenson, and P. Albersheim, *Methods Enzymol.*, 118 (1986) 3–40.
[39] S.I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
[40] P. Pellerin, S. Vidal, P. Williams, and J.-M. Brillouet, *Carbohydr. Res.*, 277 (1995) 135–143.
[41] T.T. Stevenson, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 182 (1988) 207–226.
[42] M.W. Spellman, M. McNeil, A.G. Darvill, P. Albersheim, and A. Dell, *Carbohydr. Res.*, 122 (1983) 131–153.