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Note

Modification of di- and tetrasaccharides from shark cartilage keratan sulphate by refined anhydromethanolic hydrochloric acid-treatments and evaluation of their specific desulphation

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Abstract

Highly sulphated keratan di- and tetrasaccharides were prepared from keratan sulphate (KS) of shark cartilage by enzymatic digestion with keratanase II and subsequent chromatography. The tetrasaccharide fraction carrying four sulphate groups was completely desulphated by 100 mM anhydromethanolic hydrochloric acid (MeOH–HCl) treatment at room temperature for 16 h. The conditions for the desulphation reaction by MeOH–HCl treatment were examined using sulphated keratan di- and tetrasaccharides as substrates by means of reversed phase high performance liquid chromatography (HPLC) and/or capillary electrophoresis, followed by the preparation of partially desulphated keratan oligosaccharides. Sulphate substitution patterns of monosulphated keratan disaccharide and trisulphated keratan tetrasaccharide were evaluated by methylation analysis. The results suggested that 6-*O*-sulphate groups of Gal moieties are cleaved faster than those of GlcNAc moieties under the present conditions adopted for the MeOH–HCl treatment of KS-derived oligosaccharides.

Keywords: Keratanase II; Keratan sulphate; Methylation analysis; Oligosaccharide; Desulphation

Keratan sulphate (KS) is a member of glycosaminoglycan family that possesses a linear polysaccharide structure in which the main repeating disaccharide unit comprises Gal and GlcNAc, sulphated at C-6 of Gal and/or C-6 of GlcNAc. KS is classified into two main categories according to the structure of linkage region.[1–5] Scudder and co-workers[6] isolated a series of sulphated oligosaccharides by *endo*-β-galactosidase-digestion of bovine corneal KS peptideglycan.[7] Later, Hounsell and co-workers[8] characterized these KS-derived oligosaccharides by NMR techniques, and showed that the repeating disaccharide unit comprises [D-GlcNAc-Gal]. KS from the cartilage of an elasmobranch fish, shark, is highly sulphated at almost every C-6 position of Gal residue as well as GlcNAc.[9–11] Since keratanase II[12] possesses the substrate specificity different from that of *endo*-β-galactosidase, we prepared KS-derived di- and tetrasaccharides, which are composed of [D-Gal-GlcNAc] units, from shark cartilage by keratanase II-digestion and subsequent chromatography. Selective desulphation of the sulphated oligosaccharides was studied by methanolic hydrochloric acid (MeOH–HCl) treatment.[13] It was found that partial desulphation reactions of KS-derived di- and tetrasaccharides by MeOH–HCl treatment under the optimum conditions removed preferentially the sulphate groups at C-6 of Gal at the initial stage of the reactions.

To evaluate the optimum HCl concentration, keratan tetrasaccharide carrying four sulphate groups, with repeating disaccharide unit [D-Gal(6S)-GlcNAc(6S)],[11] was subjected to desulphation with MeOH–HCl con-

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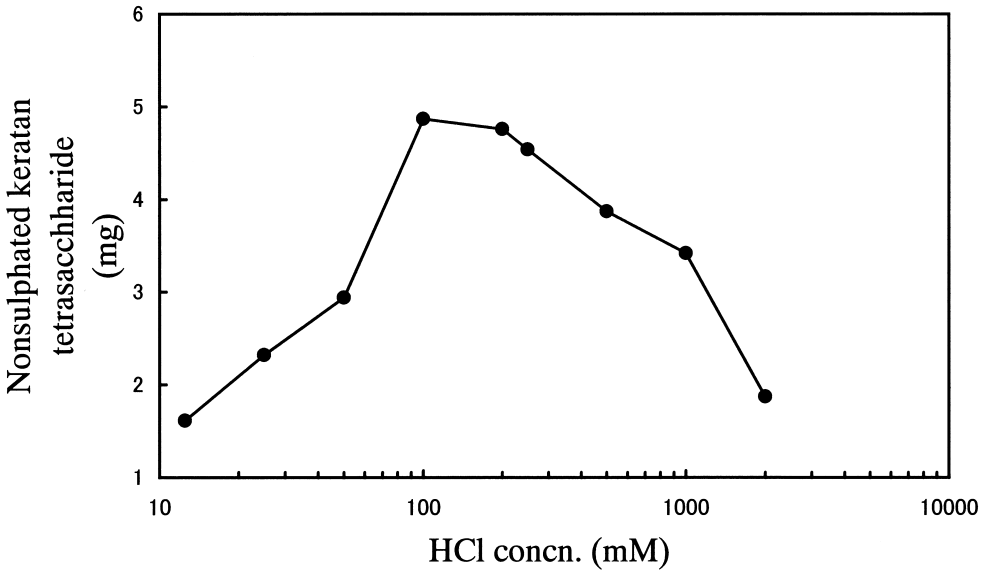

Fig. 1. HCl concentration-dependent changes in desulphation of keratan tetrasaccharide isolated from shark cartilage by MeOH–HCl treatment. The degree of desulphation was represented as the amount of liberated nonsulphated keratan tetrasaccharide by the reaction, which was determined by reversed phase HPLC equipped with a DaisoPak ODS column.

taining various concentrations of HCl. As shown in Fig. 1, the degree of desulphation was evaluated by calculating the content of nonsulphated keratan tetrasaccharide determined by reversed phase high performance liquid chromatography (data not shown). The profile was interestingly bell-shaped. The optimum condition was attained at 100 mM HCl concentration. It is possible that HCl below 100 mM would be insufficient to cleave sulphate ester bonds and that HCl above 100 mM would degrade nonsulphated keratan tetrasaccharide by the cleavage of glycoside linkages. The optimum HCl concentration found (100 mM) for tetrasulfated keratan tetrasaccharide was slightly higher than those reported previously.[6,13,14]

To evaluate the optimum reaction time, keratan tetrasaccharide was treated with 100 mM MeOH–HCl at various reaction times. As shown in Fig. 2, the Capillary electrophoresis (CE) electropherograms afforded five major peaks for keratan tetrasaccharide-derived products at five discontinuous migration times. The five peaks correspond to non- (fastest), mono-, di-, tri-, and tetrasulphated tetrasaccharides (slowest), because the higher negative charge/mass ratio of a substance due to sulphate content the slower migration time of the substance on the CE electropherogram in the positive mode.[15] With increasing reaction time, peak 1 (starting material) decreased, whereas peaks 2–4 increased and then decreased. Peak 5 increased and finally became predominant at 8 h-treatment (Fig. 2f). CE electropherogram of 16 h-reaction products gave only peak 5 (data not shown).

The reaction time-dependent changes in the contents of tri-, di-, and monosulphated tetrasaccharides (peaks 2–4) were bell-shaped. The maximum contents of tri-, di-, and monosulphated tetrasaccharides were attained at 30 min, 1 h, and 4 h, respectively (Fig. 2). In the case of keratan disaccharide, the reaction time-dependent change in the content of monosulphated disaccharide was bell-shaped, and the maximum content was attained at 2 h (data not shown). From these results, the optimum reaction times for the preparation of monosulphated disaccharide, and non- and trisulphated tetrasaccharides were found to be 2 h, 16 h, and 30 min, respectively. Table 1 shows the composition analysis of partially and completely desulphated keratan tetrasaccharide prepared by MeOH–HCl treatments.

To determine the desulphated position(s) of keratan oligosaccharides by the action of MeOH–HCl, monosulphated keratan disaccharide and trisulphated keratan tetrasaccharide were examined by methylation analysis. The PMAAs from the former disaccharide contained only 2,3,4,6-tetra-*O*-methyl-galactitol, and those from the latter tetrasaccharide contained equimolar amounts of 2,3,4,6-tetra-*O*-methyl-galactitol, 2,4,6-tri-*O*-methyl-galactitol, 2,3,4-tri-*O*-methyl-galactitol, and 2,4-di-*O*-methyl-galactitol. From these results, KS-derived disaccharide bearing one sulphate group was confirmed to be authentic Gal-GlcNAc(6S), and KS-derived tetrasaccharides carrying three sulphate groups were confirmed to be equimolar mixture of authentic Gal-GlcNAc(6S)-(1→3)-Gal(6S)-GlcNAc(6S) and authentic Gal(6S)-GlcNAc(6S)-(1→3)-Gal-GlcNAc(6S). In both cases, it was found that one sulphate residue was removed from the starting oligosaccharides. Thus, it was confirmed that methylation analysis could also be useful for the determination of sulphate substitution patterns in oligosaccharides, in addition to glycosyl

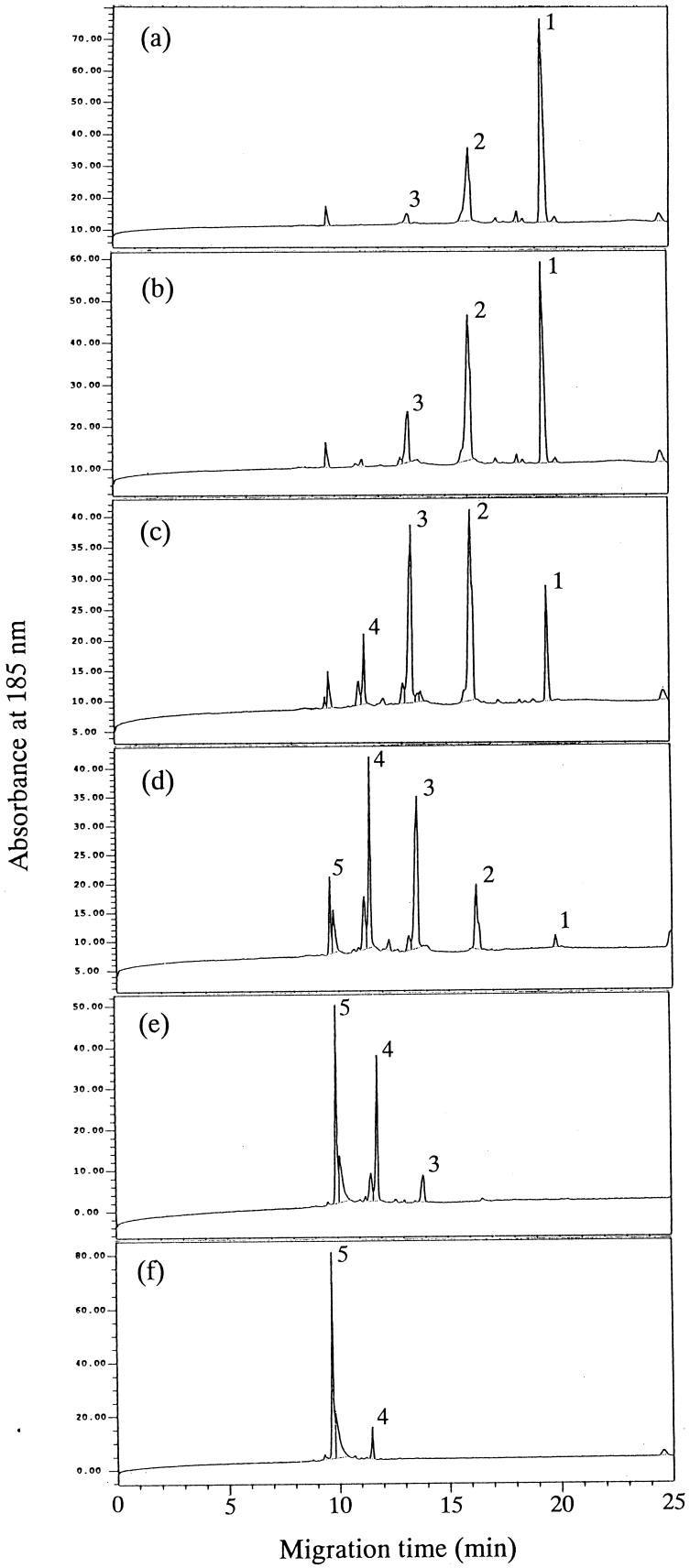

Fig. 2. CE electropherograms of desulphated products of keratan tetrasaccharide from shark cartilage by 100 mM MeOH–HCl treatments at rt for 15 min (a), 30 min (b), 1 h (c), 2 h (d), 4 h (e), and 8 h (f). Peaks indicated by numbers 1–5 represent tetra-, tri-, di-, mono-, and nonsulphated keratan tetrasaccharides, respectively.

Table 1
Chemical composition analysis of partially and completely desulphated keratan tetrasaccharide derivatives prepared by MeOH–HCl treatments

Peak [a]	Component		
	Gal (mmol/g)	GlcNAc (mmol/g)	Sulfate (mmol/g)
1	1.50 (1.89) [b]	1.59 (2.00)	3.13 (3.94)
2	1.68 (1.92)	1.75 (2.00)	2.47 (2.82)
5	2.26 (1.88)	2.40 (2.00)	0.07 (0.06)

[a] Peaks 1, 2 and 5, which are corresponding to those shown in Fig. 2, are tetrasulphated keratan tetrasaccharide (starting material), trisulphated keratan tetrasaccharide, and nonsulphated keratan tetrasaccharide, respectively.

[b] Numbers in parentheses represent relative molar ratios, as taking GlcNAc as 2.00.

linkage position. The authors previously reported the usefulness of this method for the determination of sulphate substitution patterns in the fucose branches of the sea cucumber glycosaminoglycan.[16]

The 6-*O*-sulfate groups of Gal residues in the sulphated oligosaccharides are cleaved faster than those of GlcNAc residues under the present conditions adopted for the MeOH–HCl treatment of KS-derived oligosaccharides possibly due to effect from the axial O-4 of Gal residues. This specific desulphation of C-6 of Gal in KS-derived oligosaccharides would be useful, because there has been no enzyme to remove the sulphate groups at C-6 of Gal. *N*-Acetylglucosaminidase-6-sulphate sulphatase, which removes the sulphate groups at C-6 of GlcNAc in KS, was identified previously.[17–19] In addition, it should be notified that the present desulphation method specific to C-6 of Gal is an alternative procedure to produce KS-derived oligosaccharide derivatives in which only one 6-*O*-sulphate group is exclusively removed from one of the Gal residues, instead of the established procedure to produce corresponding keratan oligosaccharides by keratanase II digestion of bovine corneal KS peptideglycan.[12]

1. Experimental

1.1. Materials

KS from shark cartilage and Keratanase II[12] from Gram-negative becterium were obtained from Seikagaku Corporation (Tokyo, Japan). Anhydromethanolic hydrochloric acid (MeOH–HCl) reagent (2.05 M) was purchased from Tokyokasei Co. (Tokyo, Japan). Other reagents used were of analytical grade.

1.2. Analytical methods

Columns and resins; Analytical (ϕ 6.0 × 150 mm) and preparative DaisoPak ODS columns (ϕ 20 × 500 mm) were purchased from Daiso Co. (Osaka, Japan). SP2330 capillary column (ϕ 0.25 mm × 30 m) was purchased from Supelco Co. (Bellefonte, PA). TSK-gel G-2,500PW$_{XL}$ column (ϕ 7.5 × 300 mm) was purchased from Tosoh Co. (Tokyo, Japan). Cellulofine GCL-90m was obtained from Seikagaku Corporation (Tokyo, Japan). AG 1 × 4 resin was purchased from BioRad (Hercules, CA).

HPLC procedure (1); HPLC was performed with a TSK-gel G-2,500PW$_{XL}$ column equilibrated with 0.2 M NaCl at 35 °C and a flow rate of 0.6 mL/min, with monitored by spectrometry at 210 nm. HPLC procedure (2); HPLC was performed with an analytical DaisoPak ODS column equilibrated with distilled water at 35 °C. The sample was eluted isocratically with distilled water at 1 mL/min, monitored by 210 nm. CE procedure; CE was performed with a fused silica capillary column (ϕ 75 μm × 60 cm) equilibrated with 50 mM sodium tetraborate (pH 9.5) at room temperature (rt) in the positive mode at 12 kV, monitored at 185 nm.

1.3. Preparation of keratan oligosaccharides by keratanase II-digestion of KS

Twelve grams of KS from shark cartilage and 10 U of keratanase II were dissolved in 2 L of 50 mM AcONa (pH 6.5) containing 0.05% NaN_3. This digestion mixture was incubated at 37 °C with stirring for 24 h. After reaction, enzymatic digests were partially purified by EtOH precipitation (75% EtOH concentration; v/v). The supernatant containing the target product was concentrated to dryness, and then dissolved in 10 ml of 0.5 M NaCl. The solution was applied to Cellulofine GCL-90m column (ϕ 3.5 × 110 cm) equilibrated with 0.5 M NaCl. Elution was performed with 0.5 M NaCl. Anthrone-positive fractions[20] were monitored for their molecular weights by HPLC procedure (1). The keratan di- and tetrasaccharide fractions were separately combined, concentrated, desalted, and lyophylized. The yields of keratan di- and tetrasaccharides were 1.1 and 0.5 g, respectively.

1.4. Refinement of desulphation condition (HCl concentration) of keratan tetrasaccharide by MeOH–HCl treatment

Anhydromethanolic hydrochloric acid (MeOH–HCl) reagents containing various concentrations of HCl, 2, 1,

0.5, 0.25, 0.2, 0.1, 0.05, 0.025, and 0.0125 M, were prepared. To 10 mg each of keratan tetrasaccharide was added 2 mL each of the MeOH–HCl reagents. The reaction mixture was incubated at rt with stirring for 16 h. For termination of the reactions, appropriate concentrations of NaOH were added to neutralize the reaction mixtures. A portion (4 μL) of the reaction mixture was analyzed by HPLC procedure (2). The amounts of nonsulfated keratan tetrasaccharide generated were plotted against corresponding HCl concentrations.

1.5. Refinement of desulphation condition (reaction time) of keratan di- and tetrasaccharides by MeOH–HCl treatment

To 5 mg each of keratan di- and tetrasaccharide was added 5 mL of 100 mM MeOH–HCl as described above. The reaction was performed at rt with stirring for 15 and 30 min, and 1, 2, 4, 8, and 16 h. At the certain reaction times, 500 μL-aliquot of each reaction mixture was withdrawn and neutralized with 0.2 M NaOH. After removing MeOH under reduced pressure, 1 mL of distilled water was added to the dryness. A portion (100 nL) of the reaction mixture was analyzed by CE procedure. From the time-dependent changes in the electropherograms, desulphation degrees of keratan di- and tetrasaccharides were evaluated.

1.6. Preparation of nonsulfated keratan tetrasaccharide, mono-desulphated keratan di- and tetrasaccharides by refined MeOH–HCl treatments

Keratan tetrasaccharide (4 g) was suspended in 400 mL of 100 mM MeOH–HCl reagent, and the reaction mixture was incubated at rt with stirring for 16 h. Resultant nonsulfated keratan tetrasaccharide was purified essentially according to HPLC procedure (2) using preparative DaisoPak ODS column, yielding 1.96 g. To 5 g each of keratan di- and tetrasaccharides was added 500 mL of 100 mM MeOH–HCl reagent, and the reaction mixture was incubated at rt with stirring for 4 and 1 h for keratan di- and tetrasaccharide, respectively. The reaction mixture was neutralized by the addition of 0.2 M NaOH. After removing MeOH under reduced pressure, the dryness obtained was dissolved in 250 mL of distilled water, and was applied to a BioRad AG 1×4 column (ϕ 3.0×25 cm) equilibrated with 0.2 M NaCl. Elution was performed with a linear gradient from 0.5 to 1.25 M NaCl for keratan disaccharide-derived products, or with a linear gradient from 0.5 to 2.5 M NaCl for keratan tetrasaccharide-derived products. The fractions containing mono-desulphated keratan di- and tetrasaccharides were combined, desalted, and lyophilized, respectively. The yields of mono-desulphated keratan di- and tetrasaccharides were 1.31 and 1.66 g, respectively. The purity of each product was confirmed by CE procedure.

1.7. Chemical composition analysis of keratan tetrasaccharide derivatives

Neutral sugar was determined by the anthrone method,[20] using authentic D-galactose as the standard. Hexosamine was determined essentially according to the previous method,[21] using authentic D-glucosamine as the standard. Sulfate was determined as previously reported.[22]

1.8. Estimation of sulphate substitution pattern of keratan oligosaccharide derivatives by methylation analysis

To evaluate the desulphated position(s) of keratan oligosaccharides, monosulphated keratan disaccharide and trisulphated keratan tetrasaccharide were subjected to per-*O*-methylation by the modification by Sandford and Conrad[23] of the method of Hakomori,[24] and per-*O*-methylated oligosaccharides were purified as described by Waeghe and co-workers.[25] The methylated oligosaccharides were hydrolyzed with 2 M TFA at 121 °C for 1 h, and reduced with sodium borodeutride, and then acetylated with acetic anhydride. The resulting PMAAs were analyzed by GC–MS with a Shimadzu QP2,000-type mass spectrometer (Tokyo, Japan) equipped with a Supelco SP2330 capillary column in the splitless mode. The column was programmed to run at 50 °C for 2 min and then to 170 °C at 30 °C/min. Column temperature was further raised to 235 °C at 4 °C/min and held for 15 min.

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References

1. Baker, J. P.; Cifonelli, J. A.; Roden, L. *Biochem. J.* **1969**, *115*, 11.
2. Funderburgh, J. L.; Caterson, B.; Conrad, G. W. *J. Biol. Chem.* **1987**, *262*, 11634–11640.
3. Oeben, M.; Keller, R.; Stuhlsatz, H. W.; Greiling, H. *Biochem. J.* **1987**, *248*, 85–93.
4. Bray, B. A.; Lieberman, R.; Meyer, K. *J. Biol. Chem.* **1967**, *242*, 3373–3380.
5. Nieduszynski, I. A.; Huckerby, T. N.; Dickenson, J. M.; Brown, G. M.; Tai, G. H.; Morris, H. G.; Eady, S. *Biochem. J.* **1990**, *271*, 243–245.

6. Scudder, P.; Tang, P. W.; Hounsell, E. F.; Lowson, A. M.; Mehmet, H.; Feizi, T. *Eur. J. Biochem.* **1986**, *157*, 365–373.
7. Roden, L.; Baker, J. R.; Cifonelli, J. A.; Mathews, M. B. *Methods Enzymol.* **1972**, *28*, 110–112.
8. Hounsell, E. F.; Feeney, J.; Scudder, P.; Tang, P. W.; Feizi, T. *Eur. J. Biochem.* **1986**, *157*, 375–384.
9. Furuhashi, T. *J. Biochem. (Tokyo)* **1961**, *50*, 546–547.
10. Seno, N.; Meyer, K.; Anderson, B.; Hoffman, P. *J. Biol. Chem.* **1965**, *240*, 1005–1010.
11. Brown, G. M.; Huckerby, T. N.; Morris, H. G.; Abram, B. L.; Nieduszynski, I. A. *Biochemistry* **1994**, *33*, 4836–4846.
12. Nakazawa, K.; Ito, M.; Yamagata, T.; Suzuki, S. Substrate specificity of keratan sulfate degrading enzymes (*Endo*-β-galactosidase, keratanase and keratanase II) from microorganisms. In *Keratan sulfate. Chemistry, Biology, and Chemical pathology*; Greiling, H.; Scott, J. E., Eds.; The Biochemical Society: London, 1989; pp 99–110.
13. Kantor, T. G.; Schbert, M. S. *J. Am. Chem. Soc.* **1956**, *79*, 152–153.
14. Toma, S.; Di Ferrante, D. T.; Tenni, R.; Di Ferrante, N. *Carbohydr. Res.* **1981**, *88*, 93–105.
15. Lamari, F.; Militsopoulou, M.; Gioldassi, X.; Karamanos, K. *Fresenius' J. Anal. Chem.* **2001**, *371*, 157–167.
16. Kariya, Y.; Watabe, S.; Kyogashima, M.; Ishihara, M.; Ishii, T. *Carbohydr. Res.* **1997**, *297*, 273–279.
17. Kresse, H.; Paschke, E.; Fingura, K. V.; Gilberg, W.; Fuchs, W. *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 6822–6826.
18. Matalon, R.; Wappner, R.; Deanching, M.; Brant, I. K.; Holwitz, A. *Ann. Clin. Lab. Sci.* **1982**, *12*, 234–238.
19. Hopwood, J. J.; Elliott, H. *Biochem. Int.* **1983**, *6*, 141–148.
20. Tsiganos, C. P.; Muir, H. *Anal. Biochem.* **1966**, *17*, 495–501.
21. Hurst, R.; Settine, J. M. *Anal. Biochem.* **1981**, *115*, 88–92.
22. Kariya, Y.; Sakai, T.; Kaneko, T.; Suzuki, K.; Kyogashima, M. *J. Biochem. (Tokyo)* **2002**, *132*, 335–343.
23. Sandford, P. A.; Conrad, H. E. *Biochemistry* **1966**, *5*, 1508–1516.
24. Hakomori, S. *J. Biochem. (Tokyo)* **1964**, *55*, 205–208.
25. Waeghe, T. J.; Darvil, A. G.; Mcneil, M.; Albersheim, P. *Carbohydr. Res.* **1983**, *123*, 281–304.