Biosci. Biotechnol. Biochem., **74** (9), 1895–1900, 2010

Selective Hydrolysis of the 3,6-Anhydrogalacotosidic Linkage in Red Algal Galactan: A Combination of Reductive Acid Hydrolysis and Anhydrous Mercaptolysis

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Received April 21, 2010; Accepted June 1, 2010; Online Publication, September 7, 2010
[doi:10.1271/bbb.100306]

Here we report a simple method for the structural analysis of red algal galactan containing 3,6-anhydrogalactose. Structural heterogeneity in the galactan was demonstrated by this method. For selective hydrolysis of 3,6-anhydrogalactosidic linkages in the galactan, conditions for reductive mild acid hydrolysis were examined by characterizing the resulting oligosaccharide alditols by anhydrous mercaptolysis. Residues other than alditols at the reducing ends, including labile 3,6-anhydrogalactose, were liberated quantitatively as diethyl dithioacetal derivatives, whereas alditols at the reducing ends were not derivatized and were liberated as alditols intact. The liberated sugars were then separated and measured quantitatively by gas-liquid chromatography. Heating of agarose in reductive hydrolysis with 0.3 M trifluoroacetic acid in the presence of an acid-stable reducing agent, 4-methyl morpholine borane, at 80 °C for 90 min and for 90 °C for 45 min was found to be optimum for the selective hydrolysis of 3,6-anhydrogalactosidic bonds, without detectable cleavage of other glycosidic bonds.

Key words: 3,6-anhydrogalactose; algal galactan; heterogeneity; mercaptolysis; reductive hydrolysis

Various biological activities have been found in marine algal polysaccharides.[1–3] However, since algal polysaccharides in nature are generally heterogeneous in size and composition, the structure-activity relationships of these saccharides are not fully established yet.

Red algal galactans such as agar, carrageenans, and porphyran contain 3,6-anhydrogalactose (AG) as a major component monosaccharide.[4] Since AG is easily destroyed by harsh acidic conditions such as acid hydrolysis and methanolysis, which are generally used in compositional and structural analysis of polysaccharides, the details of the structure of these algal galactans remain to be elucidated.

Stevenson and Furneaux[5] reported a double-hydrolysis procedure in which mild acid hydrolysis was employed to cleave only 3,6-anhydrogalactosidic linkages, followed by reduction with sodium borohydride. The resulting oligosaccharide alditols with 3,6-anhydrogalactitol at the reducing ends were fully hydrolyzed for compositional analysis by gas-liquid chromatography (GLC). They also developed a reductive hydrolysis procedure in which mild acid hydrolysis is carried out in the presence of an acid-stable reducing agent, 4-methyl morpholine borane (MMB), to obtain oligosaccharide alditols with 3,6-anhydrogalactitol at the reducing ends.[5] Since then, these methods have been modified by several other groups.[6–8] However, the degree of hydrolysis of the 3,6-anhydrogalactosidic bonds in the galactans was evaluated by determining the resulting AG-ol, because AG, unlike AG-ol, cannot be measured directly by these methods. Therefore, the behavior of the remaining AG residues during the reaction period remains substantially unknown.

We have reported a method for the determination of component monosaccharides of red algal galactans by which all the component sugars including AG were liberated quantitatively as diethyl dithioacetal (diethyl mercaptal) derivatives by anhydrous mercaptolysis[9,10] (Fig. 1A). For structural analysis of red algal galactans, we planned to design a simple method including reductive mild acid hydrolysis and anhydrous mercaptolysis. Using this method, reducing end residues and residues other than reducing ends should be measurable independently as alcohols and diethyl dithioacetals respectively in each monosaccharide, including AG (Fig. 1B). Therefore, not only the resulting AG-ol at the reducing ends but also native AG can be mearured simultaneously. Conditions for the selective hydrolysis of 3,6-anhydrogalatosidic bonds were examined by tracing the amounts of AG and AG-ol, as well as other component sugar derivatives, during the reaction period.

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Abbreviations: AcOH, acetic acid; AG, 3,6-anhydrogalactose; AG-ol, 3,6-anhydrogalactitol; EtSH, ethanethiol; Gal-ol, galactitol; GLC, gas-liquid chromatography; HMDS, 1,1,1,3,3,3-hexamethyldisilazane; Man-ol, mannitol; 2-*O*-Me-AG, 2-*O*-methyl-3,6-anhydrogalactose; 6-*O*-Me-Gal, 6-*O*-methyl-galactose; 6-*O*-Me-Gal-ol, 6-*O*-methyl-galactitol; MMB, 4-methyl morpholine borane; TFA, trifluoroacetic acid; TMCS, trimethylchlorosilane

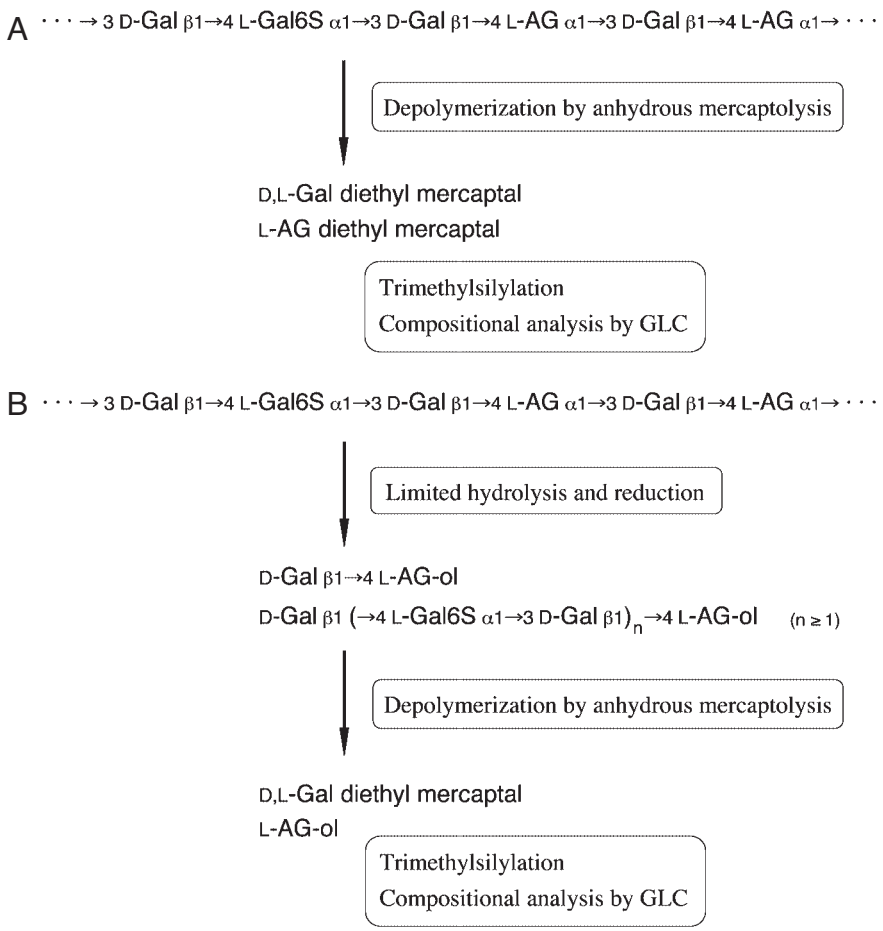

Fig. 1. Scheme for the Structural Analysis of Red Algal Galactan Containing 3,6-Anhydrogalactose by Reductive Acid Hydrolysis and Anhydrous Mercaptolysis.

A, Component monosaccharides of the galactan, including labile 3,6-anhydrogalactose, were liberated quantitatively as diethyl dithioacetal derivatives by anhydrous mercatolysis. B, 3,6-Anhydrogalactosidic linkage in the algal galactan was cleaved by reductive mild acid hydrolysis. The resulting oligosaccharide alditols were subjected to anhydrous mercaptolysis. Reducing end residues and residues other than at reducing ends were liberated as alcohols and diethyl dithioacetals respectively, in the monosaccharides. After trimethylsilylation, these sugar deivatives were separated and determined quantitatively by GLC. When the hydrolysis of 3,6-anhydrogalactosidic linkage but not galactosidic linkage is completed, Gal diethyl dithioacetal and AG-ol are detected, as in B, with the formation of neither Gal-ol nor AG diethyl dithioacetal. Generally, part of D-Gal is 6-*O*-methylated, and L-Gal is either 6-*O*-sulfated or in the 3,6-anhydride form. Gal6S, galactose 6-sulfate.

In addition, a method involving mild acid hydrolysis followed by reduction with sodium borohydride was also evaluated.

Materials and Methods

Reagents. The reagents used in this study were of highest grade, unless stated otherwise. The following reagents were purchased from commercial sources: acetic acid (AcOH), trifluoroacetic acid (TFA), ethanethiol (EtSH, first grade), MeOH, sodium borohydride ($NaBH_4$, for amino acid analysis), 1,1,1,3,3,3-hexamethyldisilazane (HMDS), and trimethylchlorosilane (TMCS) from Wako Pure Chemical Industries (Tokyo); hydrogen chloride-methanolic reagent 10 (for esterification) from Tokyo Chemical Industry (Tokyo); pyridine (silylation grade) from Pierce (Rockford, IL); agarose I (for electrophoresis) from Dojin (Kumamoto); and D-mannitol (D-Man-ol), D-Gal, D-galactitol (D-Gal-ol), 6-*O*-methyl-D-galactose (6-*O*-Me-D-Gal), 3,6-anhydro-D-galacstose (D-AG), and 4-methyl morpholine borane (MMB) from Sigma-Aldrich (St. Louis, MO). 6-*O*-Methyl-D-galactitol (6-*O*-Me-D-Gal-ol) and 3,6-anhhydro-D-galactitol (D-AG-ol) were prepared by reducing 6-*O*-Me-D-Gal and D-AG with $NaBH_4$, respectively. Each of these sugar alchlols was purified by gel filtration on Sephadex G-15 (GE Healthcare, Tokyo) before use.

Mild acid hydrolysis and reduction. The conditions for the hydrolysis of 3,6-anhydrogalactosidic linkages in method I and method II described below were essentially those of Stevenson and Furneaus.[5] Agarose was used as a representative AG-containing galactan, because its structure has been established to be a linear polymer of alternating 3-linked β-D-Gal and 4-linked α-L-Gal in which part of D-Gal is 6-*O*-methylated and all of L-Gal occurs as L-AG. The degrees of hydrolysis of anhydrogalactosidic bonds and other glycosidic bonds in the hydrolyzate were evaluated by quantitative determination of the component sugar diethyl dithioacetals (Gal, 6-*O*-Me-Gal, and AG: internal and non-reducing end residues) and their alcohols (Gal-ol, 6-*O*-Me-Gal-ol, and AG-ol: reducing end residues).

Selective hydrolysis and simultaneous reduction with MMB (method I). To an agarose sample (1 mg) dissolved in distilled water (0.9 ml), MMB (8.1 mg/0.1 ml of distilled water) and 3 M TFA (0.11 ml) were added. The resulting 0.3 M TFA solution containing MMB was heated at 80 or 90 °C for an appropriate period of time, and the hydrolyzate was neutralized with 1 M NH_3. The solution was then separated by gel filtration on Toyopearl HW-40 fine (1.0 × 30 cm × 2, Tosoh, Tokyo) with distilled water as the eluent in order to remove the excess reagents in the solution. Elution of the carbohydrate components was monitored with a refractive index detector (Shimadzu RID-6A, Shimadzu, Kyoto, Japan) and by TLC of the eluted fractions. Fractions containing carbohydrate components were collected and lyophilized. The sample was then redissolved in 1 ml of distilled water. A 100-µl aliquot was taken, mixed in a glass vial with D-Man-ol (10 µg) as internal standard, and lyophilized for GLC analysis.

Selective hydrolysis and subsequent reduction with $NaBH_4$ (method II). An agarose sample (100 µg) was mixed with D-Man-ol (10 µg) as

internal standard in a glass vial, and the sample was heated with 0.5 ml of 0.1 M TFA at 80 °C for an appropriate period of time. The reactant was then lyophilized to remove acid and solvent. The resulting oligosaccharides were reduced to their oligosaccharide alditols by the addition of 0.5 mg-$NaBH_4$/0.1 ml-1 M NH_3. After the solution was left for 1 h at room temperature, excess $NaBH_4$ was destroyed by the addition of 3 M AcOH. The sample solution was then quickly passed through a Dowex 50-x8 (H^+ form, Dow Chemical, Midland, MI) column at 4 °C, and the effluent collected was lyophilized. The boric acid in the solid material was removed by repeated codistillation with MeOH.

Anhydrous mercaptolysis. Anhydrous mercaptolysis of the samples was carried out as described previously.[9,10] Briefly, to the dried sample (100 µg), 1.0 ml of 0.5 M HCl in EtSH:MeOH (2/1, v/v), which had been prepared by mixing 1 volume of 1.5 M HCl/MeOH with 2 volumes of EtSH, was added. The sample was then heated at 60 °C for 6 h, concentrated with a stream of nitrogen, and trimethylsilylated with pyridine:TMCS:HMDS (5/1/1, v/v/v). Sugar composition was determined by GLC on a Shimadzu GC-14A (Shimadzu, Kyoto) equipped with a DB-1 capillary column (0.25-µm thickness, 0.25 mm × 25 m, J&W Scientific, Folsom, CA). Identification of sugar derivatives was done by comparing their retention times on GLC with those of authentic compounds and/or by EI-mass spectral analysis on GC-MS (Shimadzu GC-MS QP-5000, Shimadzu).

Separation and characterization of oligosaccharide alditols. An agarose sample (agarose I, 100 mg) was dissolved in 10 ml of distilled water by heating in a boiling water bath. After cooling to around 40 °C, 1.11 ml of 3 M TFA and 150 mg of MMB (solid) were added to form a 0.3-M TFA solution in the presence of MMB. The solution was heated at 80 °C for 90 min, followed by neutralization with AcOH. The oligosaccharide alditols in the solution were concentrated by lyophilization and redissolved in 2.0 ml of 0.1 M NH_4HCO_3. The sample was fractionated by gel filtration on a Superdex 30 column (1.6 × 87.6 cm) with 0.1 M NH_4HCO_3 as the eluent. An aliquot (10 µl) of the fractions was spotted onto a Silicagel 60 TLC plate (Merck, Darmstadt, Germany), and developed with 1-buthanol:AcOH:water (2:1:1, v/v/v). The carbohydrates on the plate were visualized with diphenylamine reagent.[11,12] The hexose contents of the fractions were determined by the anthrone method.[13]

Results and Discussion

Reductive mild acid hydrolysis of agarose in the presence of MMB (method I) and mild acid hydrolysis of agarose followed by reduction with $NaBH_4$ (method II)

Examples of typical chromatograms obtained by GLC by method I (reductive hydrolysis) are shown in Fig. 2. As described previously,[9] since neither anomeric nor structural isomers occur in diethyl dithioacetal derivatives as in alditols, the components were detected as single peaks (Fig. 2A). Therefore, the chromatograms obtained were very simple as compared to those obtained after methanolysis or acid hydrolysis. As can be seen clearly in Fig. 2A, the AG in agarose was detected as AG diethyl dithioacetal (peak e) along with the dithioacetals of 6-*O*-Me-Gal and Gal (peaks f and g, respectively). Only trace amounts of 2-*O*-Me-AG (peak d) were detected. Figure 2B shows the results of reductive hydrolysis at 80 °C for 15 min. Even at this early stage of hydrolysis, new peaks derived from AG residues at the reducing ends appeared (peaks a and b, 2-*O*-Me-AG-ol and AG-ol respectively) along with concomitant decreases in internal and non-reducing end AG residues (peaks d and e, 2-*O*-Me-AG and AG respectively). The ratio of hydrolyzed 3,6-anhydrogalactosidic linkages to total 3,6-anhydrogalactosidic linkages in the galactan can be calculated as follows:

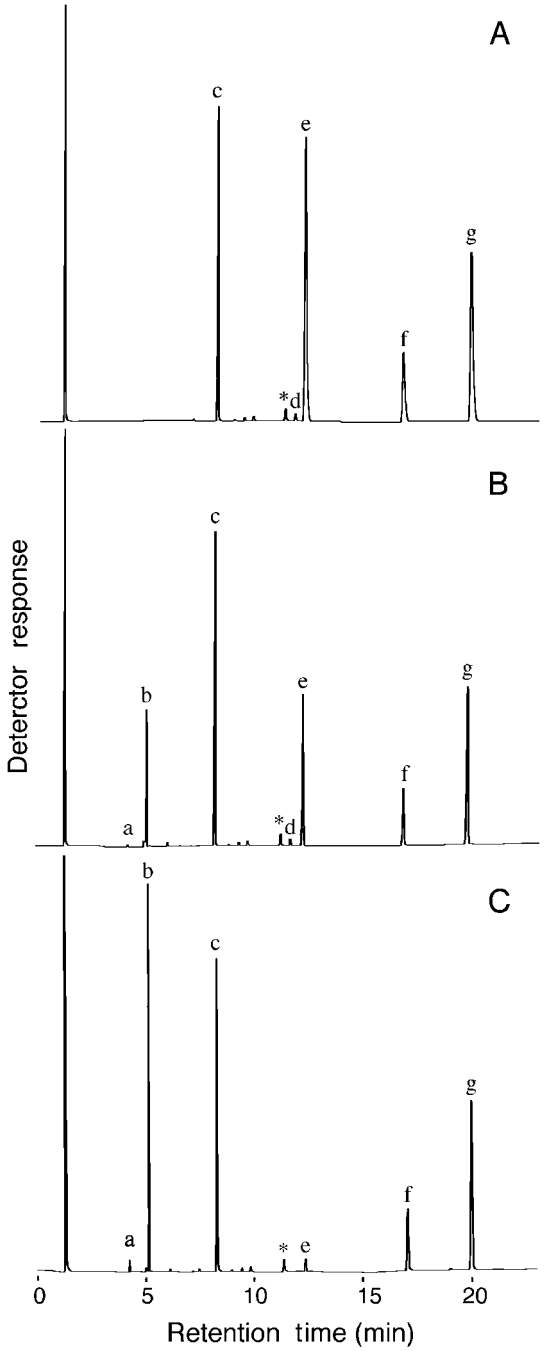

Fig. 2. Compositional Analysis of the Reductive Hydrolyzate of Agarose by GLC.

Agarose was hydrolyzed reductively in 0.3 M TFA in the presence of MMB at 80 °C for 0 min (A), 15 min (B), and 90 min (C). After hydrolysis, the samples were mercaptolyzed anhydrously at 60 °C for 6 h. The monosaccharide derivatives thus obtained were trimethylsilylated and analyzed by GLC. Column, DB-1; column oven temperature, 180 °C → 240 °C at 2 °C/min; injection temperature, 250 °C; detector temperature, 280 °C; carrier gas, He. Peaks a, 2-*O*-Me-AG-ol; b, AG-ol; c, Man-ol (internal standard); d, 2-*O*-Me-AG; e, AG; f, 6-*O*-Me-Gal; g, Gal; *, phthalate (contaminated from the screw cap of the sample vial).

[amount of AG-ol]/[sum of amounts of AG and AG-ol]. At the final stage of the reaction (Fig. 2C), most of AG was detected as AG-ol (peak b), but AG did not disappear completely (peak e). On the other hand, other component sugars, such as Gal and 6-*O*-Me-Gal, were detected dominantly as diethyl dithioacetals (peaks f and g respectively) without the formation of corresoponding alcohols throughout the reaction period.

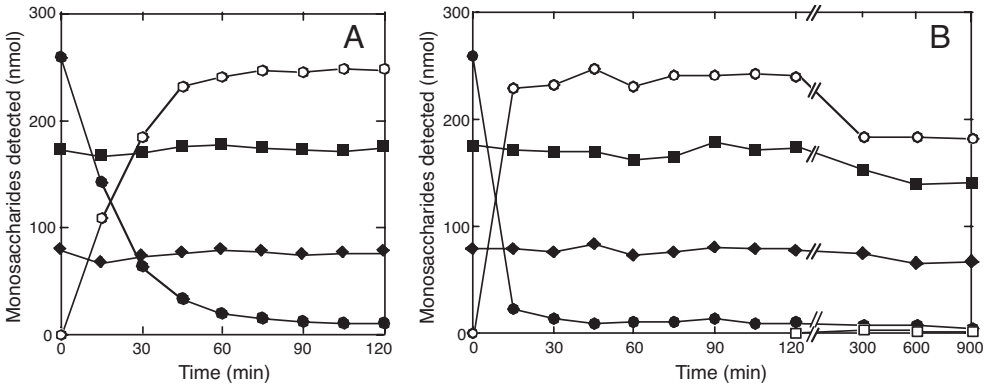

Fig. 3. Time Course of the Reductive Hydrolysis of 3,6-Anhydrogalactosidic Linkages in Agarose.
Agarose was hydrolyzed reductively in 0.1 M TFA in the presence of MMB at 80 °C (A) and at 90 °C (B). After hydrolysis, the samples were mercaptolyzed and analyzed by GLC as in Fig. 2. ○, AG-ol; ●, AG; ■, Gal; □, Gal-ol; ◆, 6-*O*-Me-Gal.

Table 1. Sugar Compositions of Agarose and Oligosaccharide Alditols from Agarose

		Gal	6-*O*-Me-Gal	AG	2-*O*-Me-AG	Gal-ol	6-*O*-Me-Gal-ol	AG-ol	2-*O*-Me-AG-ol
Agarose	nmol	173.3	79.2	259.3	8.0	n.d.	n.d.	n.d.	n.d.
	molar ratio	0.67	0.31	1.00	0.03	—	—	—	—
Oligosaccharide	nmol	171.8	76.3	11.3	n.d.	n.d.	n.d.	252.2	7.7
Alditols	molar ratio	0.68	0.30	0.04	—	—	—	1.00	0.03
Fr. 93 in Fig. 5	molar ratio	0.66	0.34	n.d.	n.d.	n.d.	n.d.	1.00	0.03
Fr. 87 in Fig. 5	molar ratio	1.36	1.12	0.69	n.d.	n.d.	n.d.	1.00	0.63

Oligosaccharide alditols were obtained by the reductive hydrolysis in 0.3 M TFA at 80 °C for 90 min. A sample (100 μg of agarose or oligosaccharide alditols) was mercaptolyzed anhydrously and analyzed as described in the text.

Changes in the GLC-determined amounts of the sugar derivatives during the reaction period at 80 °C are shown in Fig. 3A. As the reaction period increased, the amount of AG decreased, with concomitant increases in the amount of AG-ol. After reductive hydrolysis for 15 min, 45% of the AG detected in the original sample disappeared, whereas almost the same amount of new AG-ol appeared (Fig. 3A, Fig. 2B). A plateau was reached after 90 min, at which 95% of the AG was detected as AG-ol (Fig. 3A, Fig. 2C). In contrast, throughout the reaction period, the amounts of Gal and 6-*O*-Me-Gal remained basically unchanged, and the corresponding alcohols were undetected (Fig. 2). These data strongly suggest that anhydrogalactosidic bonds were selectively hydrolyzed without hydrolyzing other glycosidic bonds, and that the resulting oligosaccharides with AG at the reducing end were reduced simultaneously to stable oligosaccharide alditols with AG-ol at the reducing end. The sugar compositions of agarose and the resultant oligosaccharide alditols obtained after reductive hydrolysis at 80 °C for 90 min are summarized in Table 1. Based on the composition of the oligosaccharide alditols, almost all of them were composed of galactosyl-(3,6-anhydrogalactitol), in which one third of Gal residues were 6-*O*-methylated.

Similar results were obtained when reductive hydrolysis was carried out at 90 °C (Fig. 3B). Most of the 3,6-anhydrogalactosidic linkages were selectively hydrolyzed until 30 min, and the oligosaccharides alditols produced were stable up to a reaction time of 120-min, but an apparent decrease in AG-ol was observed when the reaction time exceeded 300 min. The amounts of Gal and 6-*O*-Me-Gal decreased gradually after 120 min.

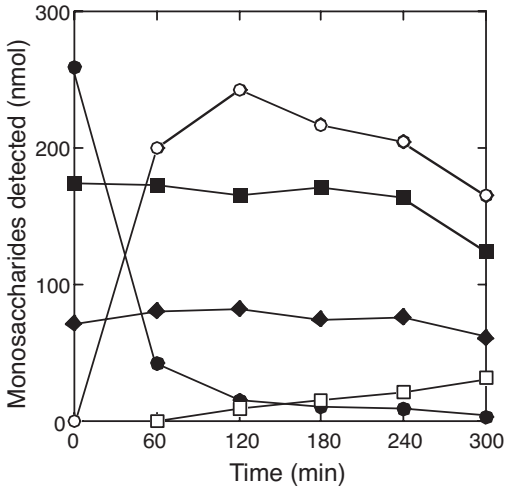

Fig. 4. Time Course for the Hydrolysis of Glycosidic Linkages in Agarose, Followed by Reduction with $NaBH_4$.
The detailed conditions are described in the text. Symbols are the same as in Fig. 3.

Therefore, the optimum conditions for the selective hydrolysis of 3,6-anhydrogalactosidic bonds were concluded to be 90 min at 80 °C and 45 min at 90 °C in 0.3 M TFA in the presence of MMB. However, it is noted that part (4–5%) of the AG was not converted to AG-ol even on heating in 0.3 M TFA at 90 °C for 120 min. It is unclear why all the anhydrogalactosidic bonds were not hydrolyzed, but this might be related to the observation that the sulfated AG residues were more resistant to acidic conditions than the non-sulfated AG residues.[5,14]

Figure 4 shows the results for method II, which involved mild acid hydrolysis in 0.1 M TFA at 80 °C

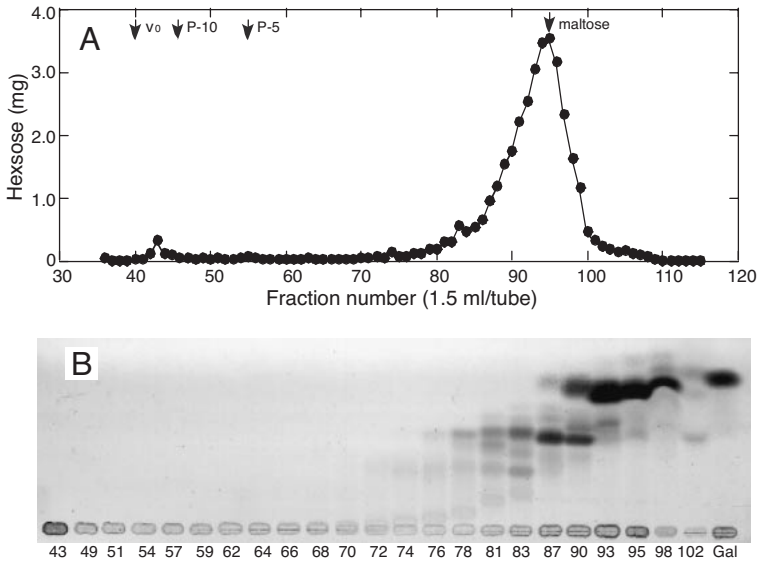

Fig. 5. Gel Filtration of Oligosaccharide Alditols Obtained by Reductive Hydrolysis of Agarose (A) and TLC of the Eluted Fractions (B).
The detailed conditions are described in the text. Arrows indicate the elution positions of pulluran molecular weight makers (v_0, (void volume), P-200 (MW 186 000); P-10 (MW 12 200); P-5 (MW 5 300)) and maltose (MW 342). The Degrees of polymerization of the oligosaccharide alditols were deduced from the elution volumes of these markers.

followed by $NaBH_4$ reduction. The highest amount of AG-ol was obtained at 120-min reaction, and the amount is almost identical to those obtained by method I at 80 °C for 90 min and at 90 °C for 45 min (Fig. 3). These results indicate that the destruction of AG in the acidic condition was negligible until a reaction time of 120 min at 80 °C. However, the amount of AG-ol obtained by method II did not make a plateau: after it reached a maximum, it decreased with prolonged heating in 0.1 M TFA at 80 °C. Moreover, Gal-ol formation was apparent for method II. We expect this formation to be accompanied by the destruction of AG at the reducing ends. However, this method should be useful when oligosaccharides with native AG but not AG-ol at the reducing ends are required.

Kazlowski has reported methods for the preparation and separation of neoagaro- and agaro-oligosaccharides from agarose by β-agarase digestion and by mild acid hydrolysis.[15] Similar results, including the formation of even-numbered oligosaccharides from red algal galactans, have been reported by several other researchers.[16,17] On the other hand, Yu[18] and Yang *et al.*[19] reported recently the formation of odd-numbered oligosaccharides by mild acid hydrolysis of κ-carrageenan and both κ-carrageenan and agarose, respectively. In their studies, residues at the reducing end were determined to be Gal, not AG. These results appear to have resulted from the destruction of AG at the reducing end, as observed for meethod II. The latter researchers,[19] however, reported the exclusive formation of odd-numbered oligosaccharide alditols obtained by reductive hydrolysis in the presence of MMB.

Structural heterogeneity in agarose

The optimum conditions for the selective hydrolysis of 3,6-anhydrogalactosidic bonds obtained in this study, *i.e.*, heating in 0.3 M TFA in the presence of MMB at 80 °C for 90 min, was applied to agarose, and the resulting oligosaccharide alditols were separated by gel filtration on Superdex 30. Figure 5 shows the elution profile of the reductive hydrolyzate of agarose and TLC of the eluted fractions. As expected, most of the hydrolyzate was composed of dimers, but tetramers and other oligomers were also detected (typically, Fr. 78–90, Fig. 5). The occurrence of oligosaccharides other than dimers clearly indicates the presence of structural heterogeneity in agarose, although agarose is believed to be a linear polymer of alternating 3-linked β-D-Gal and 4-linked α-L-Gal in which part of D-Gal is 6-*O*-methylated and all the L-Gal occurs as L-AG. The molar ratios of component sugars of Frs. 93 (dimer) and 87 (tetramer and others) are shown in Table 1. As described above, sulfated AG residues have been reported to be more resistant to acidic conditions than non-sulfated AG ones,[5,14] and the sugar composition in Fr. 87 suggests a D-Gal→L-AG (sulfated)→D-Gal→2-*O*-Me-L-AG sequence for agarose, where half of D-Gal is 6-*O*-methylated. The presence of substantial amounts of 2-*O*-Me-AG-ol and native AG in Fr. 87 (tetramers and others) but not in Fr. 93 (dimers) may be a clue to understanding the detailed structure of agarose.

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