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Quantitative Anhydrous Mercaptolysis of Algal Galactans Followed by HPLC of Component Sugars

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This paper describes a new HPLC method for the sugar analysis of algal galactans containing 3,6-anhydrogalactose, which is readily destroyed during usual acid hydrolysis and methanolysis. By anhydrous mercaptolysis of galactans at 60°C for 6 h with the newly developed solvent system, 0.5 N HCl/[ethanethiol:methanol (2:1, v/v)], component sugars, including 3,6-anhydrogalactose, were liberated quantitatively as their diethyl dithioacetals. The resultant sugar diethyl dithioacetals were found to have strong UV-absorption (absorption maximum of 3,6-anhydrogalactose diethyl dithioacetal in water, 191-192 nm; molar extinction coefficient, 4,400). The sugar diethyl dithioacetals were then resolved simultaneously within 15 min by reversed phase HPLC with 30% acetonitrile as the eluent and detected by UV-absorption at 215 nm. Amounts of sugar diethyl dithioacetals less than 50 pmol can be determined without further derivatization.

Key words: algal galactans, anhydrogalactose, anhydrous mercaptolysis, dithioacetal, HPLC.

3,6-Anhydrogalactose (AG) is a major constituent of such red algal galactans as agar, porphyran, and carrageenan (*1*). Because of such characteristics as their gel-forming ability, increasing amounts of agar and carrageenan have been used in the foods, cosmetics, and chemical industries. The AG residue in galactans is very sensitive to the harsh acidic conditions of acid hydrolysis and methanolysis commonly used to analyze the compositional and structural nature of polysaccharides. Attempts have been made to stabilize AG residues during acid-catalyzed cleavage by mercaptolysis (*2*), methanolysis (*3, 4*), acetolysis (*5*), formolysis (*6*), bromine oxidation during hydrolysis (*7*), or double hydrolysis-reduction (*8*), but these techniques have met with only limited success and none has been used widely. Consequently, quantitative determinations of AG in galactans have been carried out entirely by colorimetric methods (*9-11*), and the detailed structures of AG-containing galactans have not yet been fully elucidated although some are reported to have biological activities (*12-18*).

Mercaptolysis is the only known method by which AG can be isolated from galactans as its diethyl dithioacetal (diethyl mercaptal) (Fig. 1). Araki and Hirase prepared AG diethyl dithioacetal as well as galactose diethyl dithioacetal by the mercaptolysis of agar (*2*). The mercaptolysis was carried out by dissolving agar in concentrated hydrochloric acid with ice-cooling and stirring the mixture with occasional additions of ethanethiol (ethyl mercaptan) for 48 h to give free AG diethyl dithioacetal. The method is time consuming and requires complicated manipulations; nevertheless less than one-fourth of the AG residues can be obtained as AG diethyl dithioacetal (*2*).

We have developed a new chromatographic method for the sugar analysis of AG-containing algal galactans. The galactans are mercaptolyzed anhydrously with a newly developed solvent system, 0.5 N HCl/[ethanethiol:methanol (2:1, v/v)], at 60°C for 6 h. Under these conditions, component sugars including AG are liberated quantitatively to give the corresponding sugar diethyl dithioacetals. The resultant sugar diethyl dithioacetals show strong UV-absorption, although the native monosaccharides have practically no UV-absorption. Because of their UV-absorption, less than 50 pmol of sugar diethyl dithioacetals can be determined by reversed phase HPLC.

EXPERIMENTAL PROCEDURES

Reagents—The reagents used in this study were of special grade unless otherwise specified. The following were purchased from commercial sources: liquefied HCl gas (>99.7%), Turumi Soda (Kanagawa); ethanethiol (EtSH, ethyl mercaptan, first grade), Wako (Osaka); acetonitrile (HPLC grade), Katayama (Osaka); Agarose I, II, and III for electrophoresis, Dojin (Kumamoto); κ-carrageenan, D-galactose (D-Gal), 6-*O*-methyl-D-galactose (D-MeGal), and L-arabinose (L-Ara), Sigma (St. Louis, MO); DE-52 anion-exchange cellulose, Whatman (Maidstone, England). The diethyl dithioacetals of L-AG, D-Gal, D-

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Abbreviations: AG, 3,6-anhydrogalactose; Ara, arabinose; EtOH, ethanol; EtSH, ethanethiol; Gal, galactose; MeGal, 6-*O*-methyl-galactose; MeOH, methanol.

MeGal, and L-Ara were prepared essentially according to Araki and Hirase (*2*), and purified by HPLC under the conditions described below. Dry sheets of Susabinori, *Porphyra yezoensis*, were purchased from a local market.

Preparation of Porphyran—Dry sheets (10 g) of Susabinori were cut into small pieces and homogenized in 1,000 ml of 85% ethanol (EtOH) using a generator shaft-type homogenizer. The homogenate was heated at 75°C with constant stirring for 1 h and filtered to remove 85% EtOH-soluble substances. This extraction was repeated 3 times. The residue was washed with methanol (MeOH) and dried *in vacuo* to obtain 7.5 g of partially decolorized powder. This powder (7.0 g) was extracted with 2,000 ml of distilled water at 95°C with constant stirring for 1.5 h. The extract was centrifuged at 8,000 × *g* for 20 min and the supernatant was concentrated to 600 ml in a rotary evaporator. Sodium acetate and acetic acid were added to the concentrate to make a 0.5 M sodium acetate solution, pH 5.0, and then EtOH was added to a concentration of 38% (v/v). After 1 h, the ethanolic solution was centrifuged to remove precipitates. EtOH was added to the supernatant to a concentration of 60% and the precipitate formed was collected by centrifugation. The precipitate was dissolved in water, dialyzed against distilled water, and lyophilized to obtain 1.4 g of porphyran. The porphyran was further fractionated by chromatography on a DE-52 anion-exchange column eluted with a linear NaCl gradient into two fractions, the first eluted with 0.2-0.4 M NaCl and the second with 0.4-0.7 M NaCl. These fractions were dialyzed against distilled water and lyophilized.

Anhydrous Mercaptolysis—Methanolic and ethanethiolic HCl were prepared by blowing dry HCl gas slowly into MeOH and EtSH, respectively. The solvent systems used for the anhydrous mercaptolysis of algal galactans were: Solvent A, 0.5 N HCl/EtSH; Solvent B, 0.5 N HCl/[EtSH:MeOH (2:1, v/v)], prepared by mixing one volume of 1.5 N HCl/MeOH with two volumes of EtSH; and Solvent C, 1.0 N HCl/[EtSH:MeOH (2:1, v/v)], prepared by mixing one volume of 3.0 N HCl/MeOH with 2 volumes of EtSH. A galactan sample (100 μg) was placed in a glass vial with a Teflon lined screw cap, lyophilized, and dried in a vacuum desiccator over P_2O_5. Then, the sample was heated with 1 ml of one of the solvents at 60 or 80°C for appropriate periods of time. The mercaptolyzate was evaporated to dryness with a stream of nitrogen, and the residue was dissolved in 500 μl of distilled water containing 25 μg of L-Ara diethyl dithioacetal as the internal standard. An aliquot (10 to 50 μl) was directly applied onto the HPLC system described in the following section.

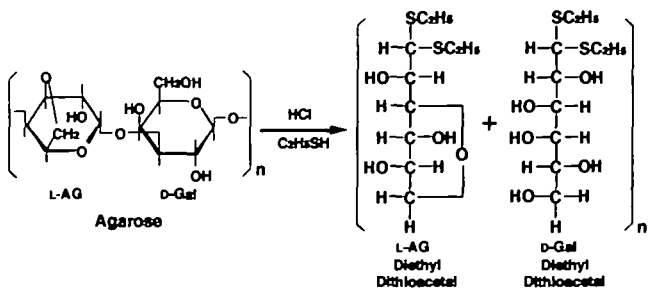

Fig. 1. **Mechanism of mercaptolysis.**

HPLC—Reversed phase HPLC was carried out on a Shimpak HRC-ODS column (0.6 cm i.d. × 15 cm, 5 μm in particle diameter, Shimadzu, Kyoto) using 30% acetonitrile (v/v) as the eluent at a flow rate of 0.5 ml/min at room temperature. Elution of the sugar diethyl dithioacetals was monitored at 215 nm with a UV-detector (Tosoh UV-8010, Tosoh, Tokyo).

Standard Curve—A standard mixture for the determination of sugar diethyl dithioacetals was prepared by mixing known amounts (usually 25 μg each) of diethyl dithioacetals of AG, MeGal, and Gal. The mixture was lyophilized and redissolved in 500 μl of distilled water containing 25 μg of L-Ara diethyl dithioacetal and analyzed by HPLC as described in the preceding section. The peak area ratio *versus* the weight ratio of each sugar to L-Ara was plotted.

Absorption Spectrum—UV-absorption spectra of native monosaccharides and their diethyl dithioacetals in distilled water were recorded on a Hitachi 228A Spectrophotometer (Hitachi, Tokyo).

GLC—After mercaptolysis of the sample as described in the preceding section, the mercaptolyzate was evaporated to dryness with a stream of nitrogen. The sugar diethyl dithioacetals were trimethylsilylated with a mixture of 100 μl of pyridine, hexamethyldisilazane, and trimethylchlorosilane (5:1:1, v/v), and analyzed by GLC on a Shimadzu GC-14A instrument (Shimadzu) equipped with a capillary column (DB-1, 0.25 mm i.d. × 25 m, J&W Scientific, Folsom, CA).

Colorimetric Determination—AG in galactans was determined with the resorcinol reagent as described by Yaphe and Arsenault (*9*) or with the thymol reagent as described by Matsuhiro and Zanlungo (*10*). Simultaneous determination of Gal and AG in galactans with the anthrone reagent was also carried out according to Yaphe (*11*).

RESULTS AND DISCUSSION

Preparation of Ethanethiolic HCl—Anhydrous ethanethiolic HCl was prepared by blowing dry HCl gas slowly into EtSH. The maximum concentration of HCl in EtSH at room temperature was found to be approximately 0.8 N. Probably due to the poor solubility of HCl in EtSH, the HCl concentration of ethanethiolic HCl solutions decreased gradually even when kept in the cold room. However, 0.5 N ethanethiolic HCl could be used for at least one month when kept at 5°C.

UV-Spectra of Sugar Diethyl Dithioacetals—Diethyl dithioacetals of AG, Gal, and MeGal were prepared essentially according to Araki and Hirase (*2*), purified by HPLC, and their UV-spectra in distilled water were recorded (Fig. 2). Although untreated monosaccharides showed almost no UV-absorption, sugar diethyl dithioacetals exhibited strong UV-absorption; their absorption maxima were almost the same, 191-192 nm, with molar extinction coefficients to be 4,400, 4,600, and 4,600, for the diethyl dithioacetals of AG, Gal, and MeGal, respectively.

HPLC—Anhydrous mercaptolysis was carried out in one of the three solvent systems, and the resultant sugar diethyl dithioacetals were analyzed by HPLC. Figure 3 shows an HPLC profile from Agarose II mercaptolyzed with 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] (Solvent B) at 60°C for 6 h. The individual component sugars were detected as single peaks since neither anomeric nor structural isomers

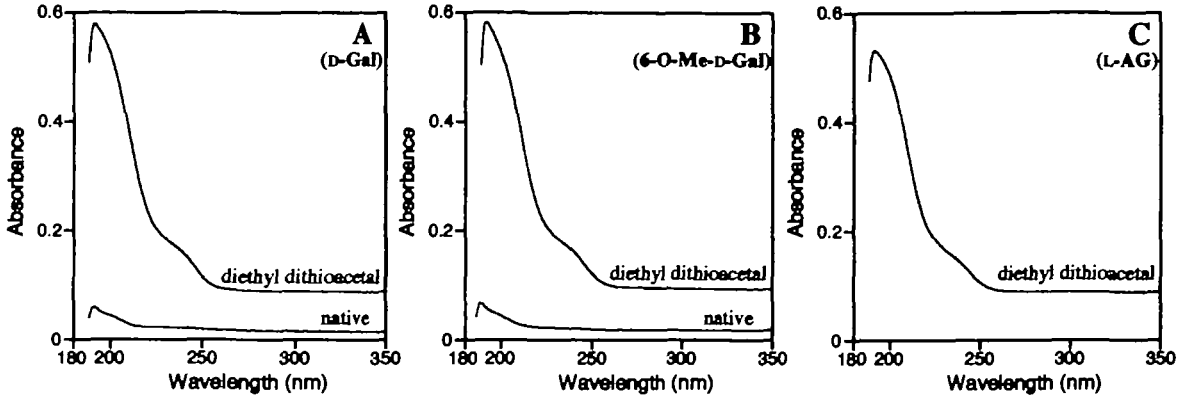

Fig. 2. **UV-spectra of native monosaccharides and their diethyl dithioacetals.** Native monosaccharides and their diethyl dithioacetals were dissolved in distilled water to make 0.1 mM solutions. A, D-Gal; B, D-MeGal; C, L-AG.

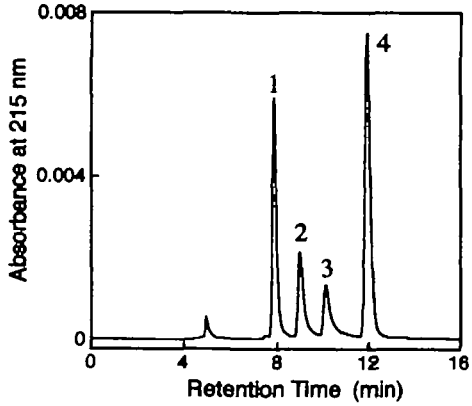

Fig. 3. **HPLC profile of component sugar diethyl dithioacetals released from Agarose II by anhydrous mercaptolysis.** Agarose II (100 μg) was heated with 1.0 ml of 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] at 60°C for 6 h. The mercaptolyzate was evaporated to dryness and redissolved in 0.5 ml of distilled water containing 25 μg of L-Ara diethyl dithioacetal as an internal standard. A 10-μl aliquot was subjected to reversed phase HPLC. Column, Shimpak HRC-ODS (0.6 cm i.d.×15 cm); eluent, 30% acetonitrile; flow rate, 0.5 ml/min; detection, A_{215}. Numbers above the peaks indicate: 1, D-Gal diethyl dithioacetal; 2, L-Ara diethyl dithioacetal (internal standard); 3, D-MeGal diethyl dithioacetal; 4, L-AG diethyl dithioacetal.

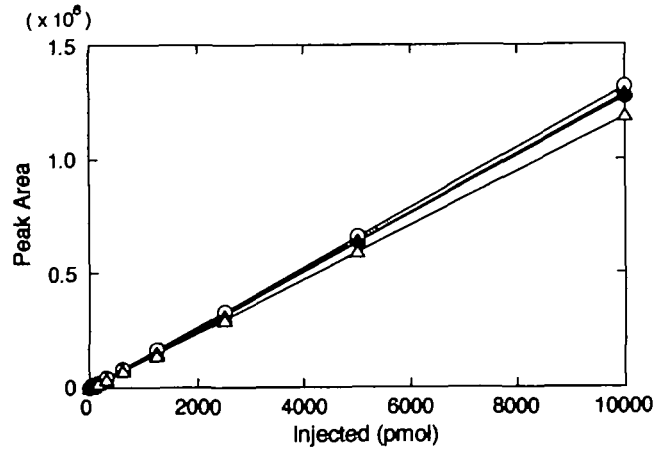

Fig. 4. **Linear correlations between amounts of sugar diethyl dithioacetals injected and detector responses.** Sugar diethyl dithioacetals in the range of 40 to 10,000 pmol were subjected to HPLC and detected at 215 nm with a UV-detector. Symbols are: ○, D-Gal diethyl dithioacetal [(pmol)=131.6×(peak area)+191.1, r=0.99999]; ●, L-AG diethyl dithioacetal [(pmol)=127.4×(peak area)+1083, r=0.99997]; ▲, D-MeGal diethyl dithioacetal [(pmol)=129.2×(peak area)−4143, r=0.99997]; △, L-Ara diethyl dithioacetal [(pmol)=119.1×(peak area)−1950, r=0.99999]. The signal to noise ratio at the 20-pmol injection was greater than 20.

are produced by mercaptolysis (Fig. 1). Although the detection wavelength was set at 215 nm in this study to obtain a stable baseline, it can be varied depending on instrumental conditions and available sample sizes. Under the conditions used, less than 50 pmol of sugar diethyl dithioacetal could be determined without any special arrangement of the instrument. Furthermore, under the conditions used, sugar diethyl dithioacetals show extremely linear responses on HPLC in the range of 40 to 10,000 pmol (R^2>0.9999) (Fig. 4). Therefore, an internal standard is not always necessary for the determination of these sugars.

Optimum Conditions for Anhydrous Mercaptolysis—Optimum conditions for anhydrous mercaptolysis were examined using agarose, the basic structure of which consists of the repeating disaccharide unit (4L-AGα1→3D-Galβ1→)$_n$. The time course for the anhydrous mercaptolysis of Agarose II at 60°C is shown in Fig. 5, A–C. With 0.5 N HCl/EtSH (Solvent A, Fig. 5A), the component sugars of Agarose II seemed to be released almost quantitatively after 12-h mercaptolysis. However, one or two out of 20 samples were observed to carbonize slightly during heating. This carbonization was considered to reflect the poor solubility of galactan in EtSH. Therefore, to increase the solubility of galactan in the solvent, we added MeOH to the solvent system. The incorporation of MeOH into the solvent system was very effective in increasing the solubility of galactan, and no carbonization was observed during the anhydrous mercaptolysis of galactan. Since the solvent system contained MeOH, the formation of sugar methyl glycosides along with sugar diethyl dithioacetals was supposed. Then, part of the mercaptolyzate was trimethylsilylated and analyzed for sugar methyl glycosides by GLC, since they are not detected by absorption at 215 nm. As a result, monosaccharide diethyl dithioacetals were quantitatively formed, without any formation of methyl glycosides, during anhydrous mercaptolysis in the HCl solvent contain-

ing MeOH at a ratio of 2:1 (v/v) of EtSH to MeOH (data not shown). This indicates that mercaptolysis occurs preferentially in the solvent system composed of EtSH and a limited amount of MeOH. The ratio of EtSH:MeOH (2:1, v/v) was thought to be critical for the preferential mercaptolysis since sugar methyl glycosides begin to form when the solvent system contains MeOH beyond EtSH/MeOH (2:1, v/v).

Compared to the results obtained with Solvent A, which contained no MeOH, the maximum releases of component sugars were attained more rapidly with 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] (Solvent B), and also the maximum amounts of released sugar diethyl dithioacetals were slightly higher (Fig. 5, A and B). Furthermore, the sugar diethyl dithioacetals were very stable in Solvent B through 48 h of mercaptolysis. These results suggest that slight destruction or incomplete liberation of sugar diethyl dithioacetals occurs during the anhydrous mercaptolysis with Solvent A due to the lesser solubility of galactan in EtSH. The results obtained by mercaptolysis in 1.0 N HCl/[EtSH:MeOH (2:1, v/v)] (Solvent C, Fig. 5C) were similar to those with Solvent B, although the resultant sugar diethyl dithioacetals decreased gradually during prolonged mercaptolysis. At a higher temperature (80°C, Fig. 5, D-F), similar results were obtained to those at 60°C, but the sugar diethyl dithioacetals formed were less stable. Therefore, the optimum conditions for the anhydrous mercaptolysis of galactans were determined to be: heating at 60°C for 6 h with 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] (Solvent B). The optimum conditions were applicable to the anhydrous mercaptolysis of not only L-AG-containing galactans, but also κ-carrageenan, which is composed mainly of D-Gal and D-AG (Fig. 6).

Recommended Procedure—The following is a recommended procedure for the sugar analysis of AG-containing galactans by the present method: (I) 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] is prepared by mixing one volume of 1.5 N HCl/MeOH with two volumes of EtSH. (II) A sample of galactan (100 μg) is added to a small vial with a Teflon lined screw cap, lyophilized, and dried in a vacuum desiccator over P_2O_5. One milliliter of 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] is added to the vial, which is capped tightly and heated at 60°C for 6 h. (III) The mercaptolyzate is evaporated to dryness with a stream of nitrogen to remove the acid and solvents. The residue is dissolved in 500 μl of distilled water containing 25 μg of L-Ara diethyl dithioacetal as the internal standard. An aliquot (10 to 50 μl) is analyzed by HPLC as described in "EXPERIMENTAL PROCEDURES."

Analysis of Galactans—Using the optimum conditions

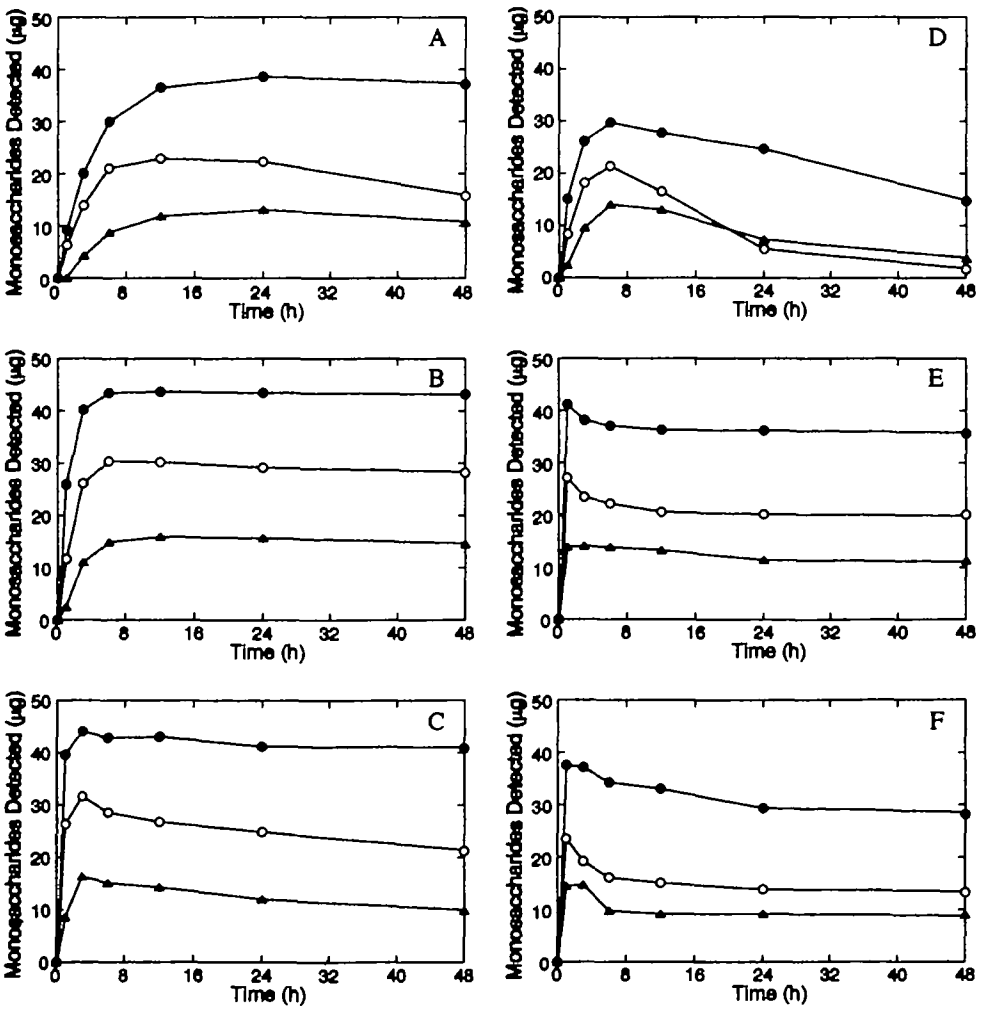

Fig. 5. **Anhydrous mercaptolysis of agarose.** Agarose II (100 μg) was heated with 1.0 ml of 0.5 N HCl/EtSH (A, D), 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] (B, E), or 1.0 N HCl/[EtSH:MeOH (2:1, v/v)] (C, F) at 60°C (A-C) or 80°C (D-F). The mercaptolyzates were analyzed by HPLC as described in the legend to Fig. 3. Symbols are: ○, D-Gal diethyl dithioacetal; ●, L-AG diethyl dithioacetal; ▲, D-MeGal diethyl dithioacetal.

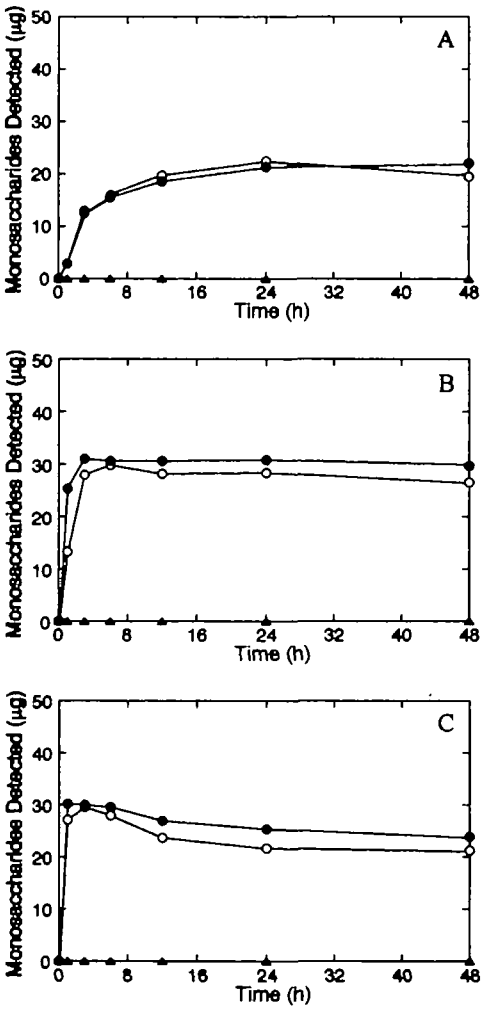

Fig. 6. **Anhydrous mercaptolysis of carrageenan.** κ-Carrageenan (100 μg) was heated with 1.0 ml of 0.5 N HCl/EtSH (A), 0.5 N HCl/[EtSH:MeOH (2:1, v/v)] (B), or 1.0 N HCl/[EtSH:MeOH (2:1, v/v)] (C) at 60°C. The mercaptolyzates were analyzed by HPLC as described in the legend to Fig. 3. Symbols are: ○, D-Gal diethyl dithioacetal; ●, D-AG diethyl dithioacetal; ▲, D-MeGal diethyl dithioacetal.

described above, the sugar compositions of some algal galactans were determined. As shown in Table I, the AG contents of galactans determined by the present method agree well with the levels determined colorimetrically. On the other hand, the Gal contents of galactans as determined by the method are significantly lower than those obtained by the anthrone method. Since the anthrone method can not differentiate between MeGal from Gal in galactans, this contradiction might be due to the occurrence of notable amounts of MeGal in galactans. Porphyran and κ-carrageenan contain sulfate groups (sodium salt); therefore, their total sugar contents were determined to be approximately 60%.

It is well known that red algal galactans are composed of Gal and AG and that part of the Gal is methylated or sulfated. Because of the lack of chromatographic methods for the compositional analysis of galactans, their structures and heterogeneity have not been fully elucidated, although their physicochemical properties are utilized for various purposes. To demonstrate the heterogeneity of porphyran, we fractionated porphyran into two fractions by DEAE-cellulose chromatography and analyzed them by the present method. The analysis revealed remarkable compositional heterogeneity in porphyran (Table I). Similar results were obtained with agarose. According to the manufacturer, Agarose I, II, and III are characterized by their gel-forming abilities: gel strength of Agarose I, >800 g/cm^2; Agarose II, 600-800 g/cm^2; Agarose III, <600 g/cm^2. Although the structure of agarose is believed to be a repeating disaccharide unit, $(4\text{L-AG}\alpha 1\rightarrow 3\text{D-Gal}\beta 1\rightarrow)_n$ (Fig. 1), some differences in sugar composition are observed (Table I). This compositional heterogeneity must be closely related to gel-forming abilities. Stevenson and Furneaux proposed a gas chromatographic method for the compositional analysis of algal galactans in which the 3,6-anhydrogalactosidic bonds in galactans are selectively hydrolyzed by mild acid hydrolysis, the resultant oligosaccharides containing AG at the reducing ends are reduced, and then hydrolyzed completely to the constituent monosaccharides (*8*). However, the method is complicated and has not been used widely. Quemener *et al.* investigated methanolysis conditions for the compositional analysis of algal galactans (*4*). They methanolyzed galactans under the

TABLE I. **Sugar compositions of algal galactans analyzed by the present method and conventional colorimetric methods.**

	Agarose I			Agarose II			Agarose III		
		(%)			(%)			(%)	
	AG	Gal	MeGal	AG	Gal	MeGal	AG	Gal	MeGal
Mercaptolysis[a]	46.2±0.51	34.4±0.36	15.7±0.24	43.8±1.95	32.5±2.34	16.6±0.69	45.8±0.34	35.8±1.64	9.7±0.43
Resorcinol[b]	49.4			47.2			48.4		
Thymol[c]	47.3			45.1			47.1		
Anthrone[d]	47.1	42.1		46.4	40.6		42.9	55.9	
	Porphyran (0.2-0.4 M NaCl fraction)			Porphyran (0.4-0.7 M NaCl fraction)			κ-Carrageenan		
		(%)			(%)			(%)	
	AG	Gal	MeGal	AG	Gal	MeGal	AG	Gal	MeGal
Mercaptolysis[a]	11.9±0.32	45.4±1.17	0.2±0.01	3.1±0.30	50.8±1.09	0.3±0.04	31.1±1.23	32.1±1.16	1.2±0.07
Methanolysis[e]		46.9±3.17	0.5±0.05		51.6±8.30	0.5±0.09		28.7±4.18	0.8±0.07
Resorcinol[b]	15.5			4.3			29.5		
Thymol[c]	15.4			4.5			33.0		
Anthrone[d]	15.7	61.7		4.9	66.3		27.4	39.9	

[a]Analytical values by the present method. Data are expressed as mean±SD (n=3-5). [b]AG in galactans was determined by the resorcinol method (*9*). [c]AG in galactans was determined by the thymol method (*10*). [d]Simultaneous determinations of Gal and AG in galactans were carried out by the anthrone method (*11*). [e]Methanolysis of galactans was carried out in 1.0 N HCl/MeOH at 60°C for 6 h. The monosaccharide methyl glycosides were trimethylsilylated and analyzed by GLC. Data are expressed as mean±SD (n=3).

optimized conditions, 0.125 N HCl/MeOH at 85°C for 1 h, with a yield of only 70% of the constituent AG from agarose. They also described a method for distinguishing agar and carrageenan by methanolysis in 0.001 N HCl/MeOH at 100°C for 1 h to cleave the 3,6-anhydrogalactosidic bonds to yield agarobiose and carrabiose dimethyl acetals which were employed to characterize agar and carrageenan, respectively. However, these methods are circuitous and require large amounts of sample since the released mono- and disaccharides were detected by a refractive index detector following HPLC.

The method we present here is very simple and sensitive; component sugars including AG can be liberated quantitatively as their diethyl dithioacetals by heating galactans at 60°C for 6 h in the newly developed solvent system, 0.5 N HCl/[EtSH:MeOH (2:1, v/v)], and 40 to 10,000 pmol of liberated sugar diethyl dithioacetals can be determined directly by reversed phase HPLC with UV-detection without complicated manipulations. This method can be applied to other aldoses (D-xylose, L-fucose, L-rhamnose, D-glucose, and D-mannose), but not to ketoses, amino sugars, or sialic acids.

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