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Component Sugars of the Polysaccharide of the Red Seaweed *Grateloupia Elliptica*

Susumu HIRASE, Choji ARAKI*1 and Kyoko WATANABE

Department of Chemistry, Faculty of Industrial Arts, Kyoto Technical University, Matsugasaki, Kyoto

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The extraction of *Grateloupia elliptica* with hot water afforded a mucilageous sulfated polysaccharide, which was then purified through precipitation as a complex with cetyl pyridinium chloride. In order to examine the component sugars, the polysaccharide was subjected to complete hydrolysis and methanolysis, the respective products of which were then carefully fractionated both by crystallization and chromatography in order to isolate the individual products. The results obtained, coupled with the data of quantitative estimation, have shown that the bulk of the polysaccharide is composed of D-galactose, L-galactose, 3, 6-anhydro-D-galactose, and sulfate, existing in the approximate molar proportion of 9 : 1 : 2 : 8. In addition to these main components, the presence of small amounts of D-xylose, 2-*O*-methyl-L-galactose, 4-*O*-methyl-D-galactose and 2-*O*-methyl-3, 6-anhydro-L-galactose has also been established. These compounds have been identified on the crystalline basis. The partially-methylated sugars isolated above have seldom or never previously occurred in polysaccharides.

*Grateloupia elliptica**2 is one of the red seaweeds commonly occurring along the coastlines of Japan and Korea. It contains water-soluble, mucilageous polysaccharide, which is utilized as a thickener for wall plasters. However, little has been known as to its chemical structure. The present paper will report the presence of small amounts of rare sugars as well as greater amounts of common sugars in the polysaccharide obtained from *G. elliptica*.

The polysaccharide was extracted from the seaweed with hot water and precipitated with ethanol. Fractionation was first attempted with potassium chloride,¹⁾ which has frequently been used for separating carrageenan and similar polysaccharides into κ- and λ-fractions. However, the polysaccharide under investigation was insensitive to this fractionating reagent. Purification was therefore carried out by precipitating the polysaccharide as a complex with cetyl pyridinium chloride,²⁾ and by subsequently regenerating the polysaccharide therefrom with sodium chloride. The resulting purified polysaccharide, in sodium salt-form, was obtained as a fibrous powder, consisting of 25% sodium sulfate.

In order to examine the component sugars, the polysaccharide was subjected to complete acid hydrolysis, and the products were separated into acidic and neutral products by means of ion-exchange resins. The former products were submitted to experiments to be reported in the future, while the latter products were carefully fractionated, both by crystallization and chromatography on the columns of cellulose powder as well as charcoal-Celite. The predominant product isolated was D-galactose, contaminated with a one-ninth quantity of L-galactose. In addition, small amounts of D-xylose, 2-*O*-methyl-L-galactose, and 4-*O*-methyl-D-galactose were also isolated in crystalline forms.

D-Xylose had been detected on paper chromatograms of the hydrolysates of almost all the red seaweed polysaccharides so far examined. However, its isolation has been reported on only a few occasions.³⁾⁴⁾ 2-*O*-Methyl-L-galactose is a new compound, although its enantiomorph had been reported on several occasions in the synthetic field.⁵⁾⁶⁾ A comparison of the isolated crystalline sugar and its crystalline anilide with the corresponding synthetic D-compounds has established that they are optical antipodes to each other. The isolation of 4-*O*-methyl-D-galactose from a seaweed polysaccharide is again the first reported instance, although its enantiomorph has recently been isolated from the agar prepared from *Gelidium amansii*.³⁾

On the other hand, 3, 6-anhydro-galactose, commonly found in red seaweed polysaccharides, can not be obtained from the hydrolysis products, as any anhydro-sugar would have been decomposed

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[*1] Present address: Shijonawate Women's College, Daitō-shi, Osaka.

[*2] The Japanese name is "Tanbanori" or "Hoguro".

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by the action of acid during hydrolysis.⁷⁾ The presence of the anhydro-D-sugar has been proved by the isolation of its dimethyl acetal from the complete methanolysis products of the polysaccharide. In addition, a trace of 2-*O*-methyl-3, 6-anhydro-L-galactose dimethyl acetal has also been isolated. This compound has been identified by its conversion into crystalline 2-*O*-methyl-3, 6-anhydro-L-galactonic acid⁸⁾ through hydrolysis, followed by oxidation with bromine. 2-*O*-Methyl-3, 6-anhydro-L-galactose has never been obtained from any intact polysaccharide, although it had been obtained from the degradation products of methylated agarose.⁸,⁹⁾

The occurrence of partially-methylated sugars has been exemplified in many polysaccharides, both of land plant and seaweed origin. However, it is interesting to note that, in the polysaccharide under investigation, both 2-*O*-methyl-galactose and its 3, 6-anhydride have the L-configuration, in spite of the D-configuration of the bulk of the galactose and its 3, 6-anhydride components. No reasonable explanation for this is possible at the present time.

Apart from the minor components, a quantitative determination has shown that D-galactose, L-galactose, 3, 6-anhydro-D-galactose and sulfate exist in the approximate molar proportion of 9 : 1 : 2 : 8. This composition seems to suggest that the polysaccharide under investigation is an intermediary one between the extremes of κ- and λ-carrageenan.¹⁰⁾

Experimental

General Procedure. Solutions were evaporated under reduced pressure at about 40°C. The melting points are uncorrected.

Paper chromatography was performed on Toyo Filter Paper No. 51 in the ascending manner. Unless otherwise stated, the irrigant was a 1-butanol - acetic acid - water mixture (4 : 1 : 2 v/v). The spray reagents used were (a) aniline hydrogen phthalate¹¹⁾ to detect reducing sugars and (b) *o*-aminophenol in ethanol acidified with phosphoric acid¹²⁾ to detect 3, 6-anhydrogalactose derivatives in methanolysis products.

Paper electrophoresis was carried out in a 0.2 M sodium borate buffer (pH 10)¹³⁾ on Toyo Filter Paper No. 51 (30 cm long) at 400—600 V and 15—25 mA/15 cm. The sugars were located by spraying the reagent (a) described above.

Polysaccharide. *a) Extraction.* The air-dried alga (20 g), which had been found in Korea, was treated with water (2 *l*) in a boiling-water bath for 2 hr with occasional stirring. The mixture was then cooled to about 60°C and filtered through a bed of Celite under mild suction, and the residue was washed with hot water. The filtrate and washings were combined, evaporated to about 500 m*l*, and poured into three volumes of ethanol with stirring. The precipitates were collected by filtration through fine cloth and lyophilyzed with acetone, the crude polysaccharide being obtained as slightly-colored fibers; yield, 10.8 g.

b) Purification. The crude polysaccharide (10.0 g) was dissolved in water (500 m*l*), a small amount of insoluble material was removed by centrifugation, and a solution of cetyl pyridinium chloride (10 g) in water (100 m*l*) was stirred in. The resulting precipitates of a complex were collected by centrifugation, washed with water, and then dissolved in a 25% sodium chloride solution (400 m*l*). After being re-centrifuged, the solution was poured into three volumes of ethanol. The precipitates were collected by centrifugation and kept in an vacuum oven at 30°C in order to remove the ethanol. They were again dissolved in water (400 m*l*), and the solution was dialysed against running water until free of chloride, and then poured into three volumes of ethanol. The precipitates were collected by centrifugation, lyophilyzed with acetone, and finally dried in an vacuum oven at 40°C. The polysaccharide in sodium-salt form was obtained as a colorless powder; yield, 8.4 g, $[\alpha]_D^{15}$ +45.0° (*c* 0.91, water).

c) Analysis. The sulfate was estimated by hydrolysing the polysaccharide with 2 N hydrochloric acid for 6 hr and then determining the resulting sulfuric acid as the precipitate of barium sulfate in the usual way. The galactose was estimated by the paper chromatographic method of Wilson,¹⁴⁾ and 3, 6-anhydrogalactose, by the colorimetric method with a resorcinol reagent following Yaphe.¹⁵⁾

All the analyses were carried out in duplicate. The following mean values were obtained: sulfate (as SO_3Na), 25.0; galactose (as $C_6H_{10}O_5$), 51.8; 3, 6-anhydrogalactose (as $C_6H_8O_4$), 8.1; N (Kjieldahl), 0.15%. The galactose was assumed to be composed of D- and L-isomers in the ratio of 9 : 1, as will be described below.

Hydrolysis of the Polysaccharide. The polysaccharide (10.9 g on a moisture-free basis) was heated with N sulfuric acid (100 m*l*) in a boiling water bath for 15 hr. The solution was neutralized with barium carbonate, filtered, and then deionized by passing it through columns of Amberlite IR-120 (300 m*l*) and Amberlite IR-45 (300 m*l*) in succession. The resins were thoroughly washed with water. The effluents and washings were combined and evaporated to a syrup (7.0 g). Paper chromatography with the reagent (a) showed the presence of galactose and very small amounts of xylose and mono-*O*-methyl-galactoses.

D- and L-Galactose. The syrup obtained above afforded crude galactose on crystallization from methanol; yield, 4.6 g. Recrystallization from methanol gave pure D-galactose; yield, 3.20 g, mp and mmp 165°C, $[\alpha]_D^{18}$ +80.0° (24 hr, *c* 1.0, water).

The mother liquor of recrystallization was evaporated

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to a syrup; yield, 1.31 g, $[\alpha]_D^{18}$ +67.1° (24 hr, *c* 0.85, water). This value of specific rotation, plus the observation that it showed a single spot of galactose on a paper chromatogram, indicated that the syrup was composed of D-galactose (1.20 g) and its L-isomer (0.11 g).

The filtrate from the crude crystals of galactose described above was evaporated to a syrup (2.3 g), which was then chromatographed on a cellulose powder column (4.5×30 cm) with a 1-butanol - water mixture (6 : 1 v/v) as an eluant. Fractions of 14 m*l* were collected and examined by paper chromatography. The following two fractions were obtained: fraction I, containing xylose and mono-*O*-methyl-galactoses; yield, 0.68 g, $[\alpha]_D^{15}$ −4.2° (*c* 0.68, water); fraction II, containing galactose only; yield, 1.08 g, $[\alpha]_D^{15}$ +10.7° (*c* 0.65, water). The former fraction was investigated as will be described below, while the latter fraction was assumed to be composed of D-galactose (0.62 g) and its L-isomer (0.46 g), the proportion being based on the optical rotation. Thus D-galactose amounted to 5.02 g and L-galactose, to 0.57 g, in total, the ratio being 9 : 1.

D-Xylose. Fraction I, described above, was rechromatographed on a charcoal-Celite (1 : 1) column (2.5×16 cm), which was eluted with water and then 1.5% ethanol in water. Fractions of 50 m*l* were collected and examined by paper chromatography, and the fractions having the same contents were combined and evaporated to dryness. The three fractions shown in Table 1 were obtained.

TABLE 1. RE-CHROMATOGRAPHY OF FRACTION I

Fraction	Yield g	$[\alpha]_D^{16}(H_2O)$ °	Sugars identified on paper chromatograms
Ia	0.40	+9.5	Xylose
Ib	0.08	−12.5	Xylose, mono-*O*-methylgalactoses
Ic	0.16	−41.4	Mono-*O*-methylgalactoses

Fraction Ia in Table 1 gave D-xylose on crystallization from methanol; yield, 0.21 g; mp 138—141°C. Recrystallization from methanol gave pure D-xylose; mp 140—143°C, $[\alpha]_D^{15}$ +60.5°→+18.3° (*c* 0.76, water). The literature values are mp 145°C and $[\alpha]_D$ +93.6°→ +18.8° (water). Admixture with an authentic sample showed no depression of the melting point.

2-*O*-Methyl-L-galactose. By paper electrophoretic examination, fraction Ic in Table 1 was shown to be a mixture of 2-*O*-, 4-*O*- and 6-*O*-methylgalactose (M_G, 0.51, 0.40, and 0.86 respectively), among which the first one was predominant and the last seemed to exist in only a trace quantity. When the fraction was dissolved in a small volume of ethanol, 2-*O*-methyl-L-galactose crystallized in prisms; yield, 0.09 g, mp 142—145°C. It was purified by recrystallization from ethanol; mp 147—149°C, $[\alpha]_D^{14}$ −52.0° (5 min)→ −93.3° (72 hr, *c* 0.75, water). These values are in agreement with those reported for its enantiomorph except for the sign of specific rotation: mp 145—148°C and $[\alpha]_D^{18}$ +52°→ +94° (water);6) mp 147—149°C and $[\alpha]_D$ +53°→ +82.6° (water).5) The R_f values observed were 0.32 in a 1-butanol - acetic acid - water mixture (4 : 1 : 2 v/v), 0.70 in a phenol-water mixture (3 : 1 w/w), and 0.27 in ethyl acetate - pyridine - water mixture (6 : 2 : 1 v/v). These values are in agreement with those observed for an authentic sample of the synthetic D-sugar.6)

Found: C, 43.35; H, 7.11%. Calcd for $C_7H_{14}O_6$: C, 43.29; H, 7.27%.

N-Phenyl-2-O-methyl-L-galactosylamine. A solution of 2-*O*-methyl-L-galactose (0.05 g) and aniline (0.03 g) in ethanol (2 m*l*) was refluxed for 4 hr and then evaporated. The residual anilide crystallized spontaneously. It was twice recrystallized from acetone-hexane; mp 148—151°C, $[\alpha]_D^{14}$ +79.4° (10 min) → +37.8° (44 hr, not constant, *c* 0.63, ethanol). The melting point disagreed with the melting point 165°C reported by McCreath and Smith for its enantiomorph.6) 2-*O*-Methyl-D-galactose and its anilide were, therefore, synthesized according to the method of McCreath and Smith. The resulting anilide melted at 146°C in agreement with the L-compound obtained above. The specific rotation $[\alpha]_D^{14}$ −79.5° (10 min) → −27.4° (44 hr, *c* 0.41, ethanol) was also in agreement with that of the L-compound except that its direction was opposite.

Found: C, 57.75; H, 7.01; N, 5.15%. Calcd for $C_{13}H_{19}O_5N$: C, 57.98; H, 7.11; N, 5.20%.

4-*O*-Methyl-D-galactose. The filtrate from the crystals of 2-*O*-methyl-L-galactose described above was evaporated to a syrup (0.05 g), which was then chromatographed on Toyo Filter Paper No. 514 (40×40 cm, 4 mm thick) with an ethyl acetate - pyridine - water mixture (6 : 2 : 1 v/v). Under these conditions, 2-*O*-, 4-*O*-, and 6-*O*-methyl-galactose have R_f values of 0.27, 0.20 and 0.25 respectively. As a result, two fractions were obtained: one contained 2-*O*-methyl-L-galactose contaminated with a trace of 6-*O*-methylgalactose (yield, 17 mg), and the other contained 4-*O*-methyl-D-galactose, contaminated again with a trace of 6-*O*-methyl-galactose (yield, 10 mg). From the former fraction, 2-*O*-methyl-L-galactose (mp 145—147°C) was obtained, while, from the latter fraction, 4-*O*-methyl-D-galactose was crystallized from a drop of methanol; mp 200—203°C, $[\alpha]_D^{31}$ +66.6° (10 min)→ +86.6° (72 hr, *c* 0.30, water). The reported values are: mp 207°C and $[\alpha]_D^{20}$ +62°→ +92° (water);16) mp 218—221°C and $[\alpha]_D^{24}$ +61°→$[\alpha]_D^{22}$ +83° (water).17) It migrated to the same position as an authentic sample of its enantiomorph,8) both on a paper electrophoretic strip (M_G 0.40) and on paper chromatograms (R_f 0.28 in a 1-butanol - acetic acid - water mixture (4 : 1 : 2 v/v), R_f 0.66 in a phenol-water mixture (3 : 1 w/w), and R_f 0.20 in a ethyl acetate-pyridine-water mixture (6 : 2 : 1 v/v)).

Found: C, 43.45; H, 7.16%. Calcd for $C_7H_{14}O_6$: C, 43.29; H, 7.27%.

3, 6-Anhydro-D-galactose Dimethyl Acetal. The Polysaccharide (1.7 g on a dry basis) was methanolysed with 3% methanolic hydrogen chloride (20 m*l*) for 20 hr, after which the neutral methanolysates were isolated in the usual way as a syrup; yield, 1.07 g, $[\alpha]_D^{28}$ +61.8° (*c* 0.34, water). Paper chromatography with the reagent (b) showed the presence of 3, 6-anhydrogalactose dimethyl acetal (R_f 0.67) and a trace of 2-*O*-methyl-3, 6-anhydrogalactose dimethyl acetal (R_f 0.92). When the syrup was chromatographed on a

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16) E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, **1946**, 506.

17) R. W. Jeanloz, *J. Am. Chem. Soc.*, **76**, 5684 (1954).

cellulose powder column (3.0×40 cm) with a 1-butanol - water mixture (6 : 1 v/v) as an eluant, 3, 6-anhydro-D-galactose dimethyl acetal was obtained as a syrup (yield, 0.15 g; $[\alpha]_D^{30}$ +27.2° (*c* 0.92, water)). Both the R_f value and the specific rotation were in approximate agreement with the reported values.4)

3, 6-Anhydro-D-galactosazone. The dimethyl acetal obtained above was hydrolysed to the reducing sugar, which was then converted into phenylosazone in the usual way;18) mp 219°C, $[\alpha]_D^{15}$ $+72.2° \rightarrow +50.0°$ (*c* 0.36, pyridine-methanol (2 : 3)). These physical constants are in exact agreement with the reported values.4,18) Admixture with an authentic sample showed no depression of the melting point.

2-*O*-Methyl-3, 6-anhydro-L-galactose Dimethyl Acetal. *a) Methanolysis of the Polysaccharide.* Since the presence of a trace of 2-*O*-methyl-galactose dimethyl acetal was indicated in the methanolysis experiments described above, larger amounts of the polysaccharide (26.4 g on a moisture-free basis) were subjected to complete methanolysis in the same way; the neutral methanolysates were obtained as a syrup.

b) Isolation. The syrup obtained above was repeatedly extracted with boiling acetone. After the acetone had been removed by evaporation, the extract was dissolved in water and the resulting solution was percolated in a Soxhlet apparatus with ether until the 2-*O*-methyl-3, 6-anhydro-galactose dimethyl acetal had completely been extracted (about 44 hr) as monitored by paper chromatography. The syrup (0.23 g) obtained from the extract on evaporation was shown by paper chromatography with the reagent (b) to be a mixture of 3, 6-anhydro-galactose dimethyl acetal and its 2-*O*-methyl derivative. The mixture was separated twice on columns of potato starch (3×30 cm), with a 1-butanol - water mixture (6 : 1 v/v) as the eluant. The faster-moving component, obtained as a syrup, was 2-*O*-methyl-3, 6-anhydro-L-galactose dimethyl acetal, contaminated with its corresponding methyl β-glycoside, as shown by paper chromatography with cyclohexanol saturated with water9) as the solvent and with the reagent (b); yield, 0.06 g, $[\alpha]_D^{13}$ ±0° (*c* 0.57, water).

c) 2-O-Methyl-3, 6-anhydro-L-galactonic Acid. The syrup obtained above was hydrolysed to the reducing sugar by heating it in 0.1 N hydrobromic acid (1 m*l*) in a boiling water bath for 1 hr. The solution was then treated with bromine (0.5 m*l*) in the usual way to oxidize the sugar. When the product was isolated in the usual way and then crystallized from ethyl acetate, 2-*O*-methyl-3, 6-anhydro-L-galactonic acid was obtained; mp 135—138°C, $[\alpha]_D^{13}$ −73.2° (*c* 0.41, water). The reported values are mp 142°C and $[\alpha]_D^{12}$ −70.0° (water).8) Admixture with an authentic sample9) showed no depression of the melting point.

Found: C, 43.61; H, 6.20%. Calcd for $C_7H_{12}O_6$: C, 43.75; H, 6.29%.

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18) C. Araki and K. Arai, Collection of Treatises in Commemoration of 45 th Anniversary of Kyoto Technical College, p. 84 (1948).