The Presence of Pyruvate Residues in λ-Carrageenan and a Similar Polysaccharide (Commemoration Issue Dedicated to Professor Sango Kunichika On the Occasion of his Retirement)

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The Presence of Pyruvate Residues in \( \lambda \)-Carrageenan and a Similar Polysaccharide

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It has been found that \( \lambda \)-carrageenan from Gigartina tenella and a polysaccharide from Grateloupia elliptica contain 1.5% and 2.9%, respectively, of pyruvic acid. Evidence has also been provided to indicate that pyruvate residues occur in acetal linkages with \( C_4 \) and \( C_6 \) of D-galactose residues in the macro-molecules.

In an earlier work one of the present writers reported that commercial agar made from the red seaweed Gelidium amansii contained 1.06% of pyruvic acid,\(^1\) which was shown to be linked through acetal linkages with \( C_4 \) and \( C_6 \) of D-galactose residues in the polysaccharide molecule.\(^2\)\(^-\)\(^3\) This was the first reported instance in polysaccharide chemistry for the presence of pyruvate residues. Later, Araki\(^4\) and Young, Duckworth and Yaphe\(^5\) showed that pyruvic acid occurred widely in various agars prepared from a variety of agarophytes, while the polysaccharides from certain species of fungi were also shown to contain pyruvic acid.\(^6\) In the present paper we report the presence of pyruvic acid in \( \lambda \)-carrageenan and a similar seaweed polysaccharide.

The carrageenan used in this work was prepared from Gigartina tenella. It is composed of two different sulfated polysaccharides, \( \kappa \)- and \( \lambda \)-carrageenan. The former polysaccharide contains D-galactose, 3,6-anhydro-D-galactose and sulfate in the molar proportion \( 1 : 0.98 : 1.17 \)\(^7\) and its chemical structure has been reported in our recent papers.\(^8\)\(^-\)\(^9\) The latter polysaccharide differs from the former one in the composition, especially with respect to the anhydro-sugar content, the molar proportion of D-galactose, 3,6-anhydro-D-galactose and sulfate being \( 1 : 0.16 : 0.89 \). The structure of this polysaccharide is being investigated in our laboratory, although that of \( \lambda \)-carrageenan from Chondrus crispus was reported by Dollan and Rees.\(^10\)

In order to examine whether pyruvic acid is present or not, 2,4-dinitrophenylhydrazine was added to the hydrolytic solution of each polysaccharide according to the procedure reported previously by one of the present writers for agar.\(^1\) Pyruvic acid 2,4-dinitrophenylhydrazone was isolated as crystals from the \( \lambda \)-carrageenan, but not from the \( \kappa \)-carrageenan. On quantitative analysis by the method of Duckworth and Yaphe using lactate dehydrogenase,\(^11\) the \( \lambda \)-carrageenan was shown to contain 1.5% of pyruvic acid. This amount was equivalent to one for about every twenty sugar residues. While, \( \kappa \)-carrageenan contained no pyruvic acid.

In an attempt to examine the manner of linkages, the \( \lambda \)-carrageenan was subjected

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to methanolysis, which had been proved effective in the case of agar to produce a broken-
down fragment with a bound pyruvate residue. When the methanolysates were tri-
methylsilylated and analysed by gas liquid chromatography, there were revealed the
peaks with exactly the same retention times as an authentic sample of trimethylsilylated
methyl 4,6-O-(1-carboxymethoxyethylidene)-α,β-D-galactosides. From this result, it is
most likely that pyruvate occurs as 4,6-O-(1-carboxymethyliidene)-D-galactose in the λ-
carrageenan. This pyruvated residue should be connected at either 1,2- or 1,3-positions.
The latter case is preferred, because 1,2-linked galactose has never occurred in any red
seaweed polysaccharide as well as in λ-carrageenan from C. crispus. This manner of
linkages (Fig. 1) is exactly the same as that proved for agar. Carrageenan has long been
investigated by many chemists, but the present work is the first to prove the existence of
pyruvate residues in λ-carrageenan.

Similar investigation has been carried out for the polysaccharide from Grateloupia
elliptica, which resembles λ-carrageenan in composition and properties. Quantitative
analysis showed that this polysaccharide contained 2.92% of pyruvic acid. This content
was equivalent to one for about every eleven sugar residues. The result of methanolysis
experiment indicated that pyruvate residues occurred again in the same acetal linkages
as those described above (Fig. 1).

In the case of agar, recent results of fractionation reported independently by Izumi and
Duckworth and Yaphel have shown that pyruvate residues are enriched in a fraction
with little sulfation. Furthermore, the latter workers also suggested from enzymatic study
that pyruvate residues occurred remote from sulfated regions of the molecule. This
concept of rivalry of pyruvate and sulfate does not seem applicable to λ-carrageenan and
the Grateloupia polysaccharide, both of which are much more highly sulfated (SO₃ about
20%) than agar (SO₃ less than 10%). From their sulfate contents, it is most likely that
these polysaccharides are composed almost entirely of sulfated sugar residues. Then,
pyruvate residues would have to occur between these sulfated residues.

**EXPERIMENTAL**

**Preparation of Polysaccharides.** κ- and λ-Carrageenan: Crude carrageenan
was prepared by extraction of Gigartina tenella with hot water and precipitation with
ethanol. It was fractionated with 0.2 M-potassium chloride solution to give precipitating
κ-carrageenan and non-precipitating λ-carrageenan. Experimental details and analysis
of κ-carrageenan were reported previously. Analysis of λ-carrageenan carried out in the
present work gave the following results: galactose (C₆H₁₀O₅) 48.0%; 3,6-anhydrogalactose
(C₆H₁₀O₅) 6.8%; sulfate (SO₃) 21.1%.
The Grateloupia Polysaccharide: The crude polysaccharide was prepared by extraction of Grateloupia elliptica with hot water and precipitation with ethanol. It was purified through its cetyl pyridinium complex. Experimental details were reported previously. Analytical results were: galactose (C₆H₁₀O₅) 51.8%; 3,6-anhydrogalactose (C₆H₈O₄) 8.1%; sulfate (SO₃) 18.9%.

Pyruvic Acid 2,4-Dinitrophenylhydrazone. According to the procedure reported previously, a polysaccharide sample (2.0 g) was hydrolysed with 0.4N-hydrochloric acid at 100° for 3 hr. 2,4-Dinitrophenylhydrazine (0.5 g.) in warm 4N-hydrochloric acid (50 ml.) was added and the mixture was left at room temperature for 3 hr. It was extracted with ethyl acetate (20 ml., 5 times), and the combined extracts were filtered, washed once with water (10 ml.) and then treated with 5% sodium carbonate solution (10 ml., 5 times). The combined carbonate extracts were evaporated at 40° under reduced pressure to 15 ml. and acidified with concentrated hydrochloric acid. Precipitated pyruvic acid 2,4-dinitrophenylhydrazone was filtered off, washed with cold water, dried and purified by recrystallization from ethyl acetate-ethanol.

The hydrazone obtained from A-carrageenan: yield 48 mg. (0.79% of the polysaccharide on the free acid basis); m.p. 218–219°, not depressed on admixture with an authentic sample.

Anal. Found: C, 40.18; H, 3.11; N, 20.14%. Calcd. for C₉H₃₀₆N₄: C, 40.30; H, 3.01; N, 20.89%.

The hydrazone obtained from the Grateloupia polysaccharide: yield 130 mg. (2.1% of the polysaccharide on the free acid basis); m.p. 218–219.5°, not depressed on admixture with an authentic sample.

Anal. Found: C, 40.25; H, 3.06; N, 20.87% (calculated values are given above).

No corresponding product was obtained from κ-carrageenan.

Quantitative Determination of Pyruvic Acid. The procedure of Duckworth and Yaphe was followed with a slight modification. A polysaccharide sample (3–5 mg.) was hydrolysed at 100° for 4 hr. with 0.04N-oxalic acid (3 ml.). The solution was neutralized with calcium carbonate and filtered, and the filtrate was diluted to 10 ml. with water. To a part (2 ml.) of this solution was added 0.3M-triethanolamine (1 ml.) and 0.075% NADH in 0.1% sodium bicarbonate solution (0.3 ml) and the initial absorbance was read at 340 mµ. Lactate dehydrogenase (0.02 ml) was then added and the absorbance was measured until a constant value was obtained. The pyruvic acid content was obtained by comparing the difference between the initial and final absorbance with a standard curve, which had been prepared for pyruvic acid solutions of different concentrations. The results were as follows. κ-Carrageenan: nil; λ-carrageenan: 1.50%; the Grateloupia polysaccharide: 2.92%.

Methanolysis of Polysaccharides. A polysaccharide sample (100 mg.) suspended in 3% methanolic hydrogen chloride (2 ml.) was heated in a sealed tube at 80° for 20 hr. The hydrogen chloride was removed by neutralization with excess of silver carbonate and filtration. The filtrate was evaporated at 40° under reduced pressure to a syrup, which was then taken in water (10 ml.), and the solution was allowed to pass through Amberlite IR-120 (5 ml.) and Amberlite IR-45 (10 ml) in succession. The deionized solution was evaporated at 40° under reduced pressure to a syrup, which was dried at 40° under reduced pressure until a constant weight was obtained.

(334)
Pyruvate Residues in λ-Carrageenan

Table 1. Retention Times (min.) of Standard Compounds.

<table>
<thead>
<tr>
<th>Trimethylsilyl ethers</th>
<th>Column 1 a)</th>
<th>Column 2 b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl D-xylosides</td>
<td>4.2; 4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3,6-Anhydro-D-galactose dimethyl acetal</td>
<td>5.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Methyl D-galactosides</td>
<td>7.2; 8.2; 9.2</td>
<td>1.1; 1.3</td>
</tr>
<tr>
<td>Methyl 4,6-O-(1-carbomethoxyethylidene)-a,β-D-galactosides</td>
<td>12.0; 13.8</td>
<td>3.6; 4.9</td>
</tr>
</tbody>
</table>

a: 2.5% SE-30-Chromosorb W (2 m.), at 160°.
b: 2.5% ECNSS-M-Chromosorb W (2 m.), at 150°.

Fig. 2. Gas liquid chromatograms of trimethylsilylated (TMS) methanolysates of λ-carrageenan.

(A) 2.5% SE-30 on Chromosorb W (2 m) at 160°;
(B) 2.5% ECNSS-M on Chromosorb W (2 m) at 150°.

Peak a: solvent; b: TMS methyl xylosides; c: TMS 3,6-anhydrogalactose dimethyl acetal; d: TMS methyl galactosides; e: TMS methyl 4,6-O-(1-carbomethoxyethylidene)-galactosides.

Gas Liquid Chromatography of the Methanolysates. A small portion (10 mg.) of the methanolysates obtained above was trimethylsilylated with pyridine (0.5 ml.), hexamethyldisilazane (0.2 ml.) and trimethylchlorosilane (0.1 ml.). To the reaction mixture was added chloroform (2 ml.), and the solution was washed with cold water (1 ml., 3 times), dried with anhydrous magnesium sulfate, and evaporated under reduced pressure to dryness. The product was analysed by gas liquid chromatography with two different columns. Identification of the observed peaks was carried out by comparison with retention times of authentic samples (Table 1). In the methanolysates of both λ-carrageenan (Fig. 2) and the Grateloupia polysaccharide were detected a small amount of methyl 4,6-O-(1-carbomethoxyethylidene)-a,β-galactosides in addition to methyl galactosides and small amounts of 3,6-anhydrogalactose dimethyl acetal and methyl xylosides.

ACKNOWLEDGMENT

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(3) S. Hirase, ibid., 30, 75 (1957).
(8) S. Hirase and K. Watanabe, ibid., 45, 1529 (1972).
(9) S. Hirase and K. Watanabe, ibid., 45, 1839 (1972).