

The Presence of Pyruvate Residues in λ -Carrageenan and a Similar Polysaccharide

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It has been found that λ -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 C₄ and C₆ 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 C₄ and C₆ 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, Araki⁴⁾ and Young, Duckworth and Yaphe⁵⁾ 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 λ -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, κ - and λ -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 respects 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 λ -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 λ -carrageenan, but not from the κ -carrageenan. On quantitative analysis by the method of Duckworth and Yaphe using lactate dehydrogenase,¹¹⁾ the λ -carrageenan was shown to contain 1.5% of pyruvic acid. This amount was equivalent to one for about every twenty sugar residues. While, κ -carrageenan contained no pyruvic acid.

In an attempt to examine the manner of linkages, the λ -carrageenan was subjected

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Pyruvate Residues in λ -Carrageenan

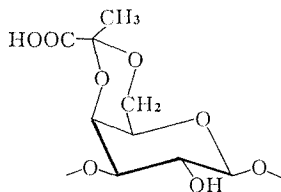


Fig. 1. 1,3-Linked 4,6-*O*-(1-carboxyethylidene)-D-galactose residue.

to methanolysis, which had been proved effective in the case of agar to produce a broken-down fragment with a bound pyruvate residue.^{2,3)} When the methanolysates were trimethylsilylated 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-carbomethoxyethylidene)- α,β -D-galactosides. From this result, it is most likely that pyruvate occurs as 4,6-*O*-(1-carboxyethylidene)-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*.¹⁰⁾ 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.¹²⁾ 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 Izumi¹³⁾ and Duckworth and Yaphe¹⁴⁾ 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.^{5,15)} 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 (SO_3 about 20%) than agar (SO_3 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.¹⁾ Analysis of λ -carrageenan carried out in the present work gave the following results: galactose ($\text{C}_6\text{H}_{10}\text{O}_5$) 48.0%; 3,6-anhydrogalactose ($\text{C}_6\text{H}_8\text{O}_4$) 6.8%; sulfate (SO_3) 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.¹²⁾ Analytical results were; galactose ($C_6H_{10}O_5$) 51.8%; 3,6-anhydrogalactose ($C_6H_8O_4$) 8.1%; sulfate (SO_3) 18.9%.

Pyruvic Acid 2,4-Dinitrophenylhydrazone. According to the procedure reported previously,¹⁾ 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 λ -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 $C_9H_8O_6N_4$: 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 Yaphe¹¹⁾ 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 $m\mu$. 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.

Pyruvate Residues in λ -Carrageenan

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

Trimethylsilyl ethers	Column 1 ^{a)}	Column 2 ^{b)}
Methyl D-xylosides	4.2; 4.5	0.5
3,6-Anhydro-D-galactose dimethyl acetal	5.6	1.1
Methyl D-galactosides	7.2; 8.2; 9.2	1.1; 1.3
Methyl 4,6- <i>O</i> -(1-carbomethoxyethylidene)- α,β -D-galactosides	12.0; 13.8	3.6; 4.9

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

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

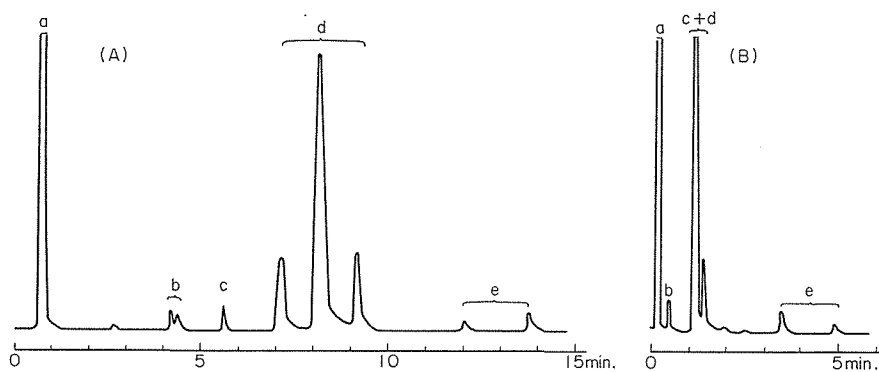


Fig. 2. Gas liquid chromatograms of trimethylsilylated (TMS) methanolysates of λ -carrageenan.

(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)- α,β -galactosides^{2,3)} in addition to methyl galactosides and small amounts of 3,6-anhydrogalactose dimethyl acetal and methyl xylosides.

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