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β-Carrageenan: Isolation and characterization

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β-Carrageenan, essentially devoid of ester sulfate, was isolated from the hot aqueous extracts of alkali-modified *Eucheuma gelatinae*, *Eucheuma speciosa*, and *Endocladia muricatum* by precipitating the more anionic moieties with a quaternary ammonium salt, isolating the fractions that did not precipitate, then treating these with an anion-exchange cellulose. The β-carrageenan was characterized by chemical analysis, optical rotation, and NMR. Gelling was found to be ion-independent, with $T_g = 31–33°C$ and $T_m = 63–70°C$. Specific optical rotations of the isolated β-carrageenan samples were more positive than the κ-, λ-, and ι-carrageenans with which they were compared, while agarose, its stereoisomer, exhibited a negative specific rotation. Electrophoresis gels made from β-carrageenan were used to separate DNA fragments which exhibited faster migration than on an agarose gel of comparable concentration, indicating that β-carrageenan has a less restrictive pore structure.

INTRODUCTION

The authors have obtained β-carrageenan fractions with ion-independent gelling properties from *Eucheuma gelatinae*, *Eucheuma speciosa*, and *Endocladia muricatum* and have characterized them by chemical analysis, optical rotation, infrared and nuclear magnetic resonance spectroscopy, and gelling and melting temperatures. The existence of β-carrageenan subunits in other carrageenans was first reported by Greer and Yaphe (1984) using enzymatic cleavage of carrageenan extracts of *Eucheuma gelatinae*, followed by IR and ^{13}C-NMR spectroscopy on the isolated fragments. Subsequently, Mollion *et al.* (1987), in characterizing the carrageenan from *Rissoella verruculosa*, again demonstrated the existence of β-carrageenan fragments. However, the isolated carrageenans, before enzymatic hydrolysis, contained considerable ester sulfate, the lowest being 15·0%. Their paper states that β-carrageenan 'always co-exists with ω'(-carrageenan). ω-Carrageenan is defined as a 6-sulfated β-carrageenan. Shi *et al.* (1987) demonstrated the existence of β-carrageenan segments in carrageenan extracted from *Eucheuma gelatinae* by using ^{13}C-NMR spectroscopy on the hybrid extracts. Zablackis and Santos (1986) isolated a new type of carrageenan, α-, from *Catenella nipae* which they characterized as a β-carrageenan substituted with ester sulfates in the 4-position.

In its idealized form, β-carrageenan consists of multiple units of carrabiose and contains no sulfate ester moieties. Carrabiose is the repeating structure of the entire family of carrageenans which are obtained from various red macroalgae, or Rhodophytes, and consists of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-D-galactopyranose units. Agars and the purified component agaroses are also obtained from red macroalgae, or seaweeds, but in other genera than those that produce carrageenans. Agars contain the agarobiose backbone, which in its idealized form consists of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose units. Thus, β-carrageenan is the D,D analog of the D,L agarose. Because of the wide utility of the agaroses in life science applications, as well as the existence of so few neutral, ion-independent gelling agents for aqueous systems, isolation and characterization of the properties of β-carrageenan seemed appropriate.

While ester sulfate values of the authors' best preparations were low (<0·85%), like idealized agarose, β-carrageenan completely devoid of ester sulfate may only exist as a concept. Analogous to the terms Duckworth and Yaphe (1971) used to describe agarose,

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perhaps it is fitting to describe β-carrageenan as: that mixture of carrageenan molecules with the lowest charge content, fractionated from a whole complex of molecules called carrageenan, all differing in the extent of masking with charged groups. Unlike the ion-dependent gelling carrageenans, κ- and ι-, the β-carrageenan apparently does not require a 4-sulfate on the galactose subunit for gel formation.

EXPERIMENTAL

Materials

Sun-dried seaweeds were used as raw materials. *Eucheuma gelatinae* was obtained from both the Phillippines and Hunan Island, People's Republic of China. The *Eucheuma speciosa* was obtained through the efforts of the Dr Gerald Kraft family, Melbourne, Australia, and the *Endocladia muricatum* was provided by Dr Maxwell Doty, University of Hawaii.

Methods and results

Isolation and purification

Purified β-carrageenan was prepared by reacting a marine macroalga that contains β-carrageenan and/or a precursor with potassium hydroxide, extracting the carrageenans, treating the extracted carrageenans with an aqueous quaternary ammonium salt to precipitate the more anionic fraction, isolating the partially purified β-carrageenan, then treating an aqueous solution of this with DEAE cellulose for further purification. Alternatively, an aqueous extract was obtained, followed by alkali treatment, then quaternary ammonium salt precipitation of the more anionic fractions.

Although the example of the method given describes the isolation of β-carrageenan from *Eucheuma gelatinae*, similar methods were used to isolate β-carrageenan from *Eucheuma speciosa*, and *Endocladia muricatum*.

Isolation of β-carrageenan from Eucheuma gelatinae. β-carrageenan was isolated from the marine macroalga, *Eucheuma gelatinae*, by covering 20 g of the dried seaweed with 400 ml of 20% (w/v) potassium hydroxide. To complete the conversion of the 6-sulfated galactose segments to 3,6-anhydrogalactose units, the suspension was heated without agitation for 3 h at 85°C, using a water bath. The treated seaweed was rinsed continuously, using cold water, until the pH of the wash was 8–9. Usually 2–3 h was required. The seaweed was then transferred to a beaker containing 500 ml of 30% (w/v) sodium chloride and allowed to ion-exchange overnight to the Na^+ form. Excess salts were removed by washing with cold water. Enough water was added just to cover the weed and the mixture was then heated in a boiling-water bath for 1 h at 95°C. After 1 h, the softened seaweed and water were allowed to cool slightly and the mixture then milled in a Waring blender. The paste was returned to the boiling-water bath and held at 95°C for another hour, maintaining the pH of the paste between 8 and 9. To this, 30 g of Hyflo Super-Cel filter aid (Manville Service Corporation, Lompoc, CA) was added and the mixture pressure filtered through a piece of No. 50 Whatman filter paper. The collected and combined filtrate and hot-water wash were heated to 70°C and, with stirring, added to 2× volume of 99% isopropyl alcohol warmed to 40°C. The coagulum (coag) was recovered by pouring the mixture through finely woven nylon cloth and squeezing to remove excess alcohol. To wash the coag, it was allowed to stand in 1× volume of 80% isopropyl alcohol for 30 min. The coag was recovered as above, squeezed to remove excess alcohol, and dried at 55°C in a forced hot-air oven. Once dried, the sample was weighed to determine yield, which was typically 30–40%. In the aforementioned example, 7·59 g (38% yield) was recovered and then ground through a 20-mesh screen. To isolate the β-carrageenan, the dried product was dissolved in 759 ml distilled water (1%, w/v), using heat. To the heated (40°C) extract was added 114 ml of 5% Hyamine 3500 solution (Rohm & Haas), 15% (v/v), and the mixture was placed in a boiling-water bath for 20 min. The precipitate which formed was separated by centrifugation at 4000 rpm for 10 min. A small portion of the clear supernatant solution was checked for additional precipitables by adding a few drops of Hyamine 3500. If and when more precipitate formed, additional Hyamine was added to the total volume of the supernatant which was centrifuged again as above to remove the precipitate. To the clear supernatant containing the crude β-carrageenan, 10% aqueous sodium chloride was added at 1% of the total volume to convert any anionic polymers to the Na^+ form. The solution was heated to 70°C in a microwave oven, after which it was added, with stirring, to 2× volume of 99% isopropyl alcohol warmed to 40°C. After several hours, a floc formed. The coag was collected by vacuum filtration through No. 50 Whatman filter paper and dried at 50°C. Once dried, the recovered sample weighed 0·287 g (1·44%, based on the weight of the weed used). The dried product was ground through a 20-mesh screen. Ten milliliters of a 1% aqueous solution was prepared from this product, using heat to dissolve the β-carrageenan. Upon cooling to room temperature, a relatively firm, somewhat elastic gel formed.

Using another approach, dry *Eucheuma gelatinae* (20 g) was suspended in 400 ml of distilled water and the pH of the solution was adjusted with 1·0 N NaOH to 7·0–8·0. The solution was heated to 95°C in a microwave oven, transferred to a boiling-water bath, and further heated with occasional stirring for 3 h. The pH of the solution was monitored every 30 min. After 3 h, the seaweed was milled in a Waring blender and the pH

adjusted if necessary. The paste was then diluted to 900 ml with distilled water, 30 g of filter aid added, and the paste pressure filtered. This collected filtrate was coagulated in 2× volume 99% isopropyl alcohol and recovered as above. The coag was dried at 60°C, ground through a 20-mesh screen, then dissolved by dispersing in 600 ml distilled water and heating to a boil. Using an exhaust hood, 5·98 g of sodium borohydride (lot no. 784162, Fisher Scientific, Fair Lawn, NJ) was added along with 1-octanol to reduce foaming. This solution was transferred to a boiling-water bath and heated to 95°C for 20 min. An equal volume of 20% KOH (600 ml) was added and the solution heated to 95°C for 2 h. After 2 h, the pH was adjusted with acetic acid to 8·0–9·0. The solution was then coagulated in 2× volume 99% isopropyl alcohol. The coagulum was washed twice in 80% isopropyl alcohol, recovering it between washes then drying at 60°C. The dried coag was ground and then dissolved in distilled water with heat. To the hot solution, 5% Hyamine 3500 was added at a level equal to 15% of the total volume, then the mixture heated in a boiling-water bath for 2 min. Filter aid (15 g) was added and the solution pressure filtered. The collected filtrate was checked with extra Hyamine as above and remained clear. The filtrate was then coagulated in 2× volume 99% isopropyl alcohol with 10% NaCl added at 1% of the total volume. The coag was recovered and washed in 60% isopropyl alcohol as above. After the coag was hardened in 99% isopropyl alcohol, it was dried at 60°C. The dried coag weighed 0·862 g, a 4·31% yield. Analysis of this material gave similar results as material isolated above.

Purification of β-carrageenan. One gram of a composite sample of β-carrageenan was suspended in 100 ml distilled water and heated with constant stirring to dissolve the β-carrageenan (70°C). This 1% (w/v) β-carrageenan solution was cooled to 40°C. With constant stirring, a solution of FMB 451-5 QUAT (Huntington Laboratories, Huntington, IN) was added at a level (3 ml) of 3× the milliequivalents of sulfate in the crude β-carrageenan. This solution was incubated at 37°C, a temperature higher than that at which β-carrageenan gels. After 2 h of incubation, additional FMB 451-5 QUAT was added to check for more flocculation. To this, with stirring, 3 g Hyflo Super-Cel filter aid was added. This slurry was then poured into a 100 ml pressure filter, containing a piece of No. 50 Whatman filter paper previously coated with 8 g filter aid and preheated with 100 ml boiling distilled water. Pressure was applied (25 psi) and the filtrate collected and maintained at a temperature of 40°C. The filter cake was rinsed with 100 ml hot distilled water and this wash was combined with the primary filtrate. This solution was then coagulated in 400 ml of 99% isopropyl alcohol. To check for residual β-carrageenan in the aid cake, the cake was rinsed with an additional 50 ml hot distilled water and the filtrate poured into 100 ml 99% isopropyl alcohol. The coagulated β-carrageenan was allowed to cool to room temperature (20°C) and recovered by centrifugation at 3500 rpm for 10 min. After the supernatant was removed by decantation, the pellet was transferred to and dispersed in 400 ml of 60% isopropyl alcohol. After 30 min, the coagulum was recovered on a fine woven cloth by vacuum filtration. Excess alcohol was removed by squeezing. The coagulum was washed in 400 ml of 2% aqueous sodium chloride (w/v) for 30 min. The coagulum was filtered through cloth, as above, squeezing out excess liquid and then washing the coagulum in 2× volume distilled water for 30 min, then hardened in 2× volume 99% isopropyl alcohol for 30 min. The coagulum was again filtered, squeezed to remove excess alcohol and then dried overnight (16 h) in an airdraft incubator at 37°C. Once dried, the sample was weighed to determine yield, typically about 50%. In this experiment, 0·512 g (51·2%) was obtained. This was ground through a 40-mesh screen. A 1% solution of this partially purified β-carrageenan was prepared by first dispersing the powder in distilled water, then heating the mixture to 70°C. A 2·5% slurry of DEAE cellulose was also prepared by suspending 1·25 g DEAE cellulose (lot no. 68F-0744, Sigma Chemical Co., St Louis, MO) in 50 ml distilled water and stirring for 10 min. The DEAE cellulose powder was recovered on a piece of No. 54 Whatman filter paper by vacuum filtration. The washed DEAE cellulose powder was added to the β-carrageenan solution and the mixture allowed to stir at about 37°C. After 2 h, the DEAE cellulose was removed by vacuum filtration using a piece of No. 54 Whatman filter paper. The DEAE cellulose was rinsed with 50 ml hot distilled water and the filtrate mixed with the previously filtered β-carrageenan. The β-carrageenan was then coagulated in 200 ml of 99% isopropyl alcohol. The coagulum was collected on a finely woven nylon cloth by vacuum filtration and then transferred to 200 ml of 60% isopropyl alcohol. After 30 min, the coagulum was again recovered on a finely woven cloth and the excess alcohol removed by squeezing. The coagulum was hardened in 200 ml of 99% isopropyl alcohol for 30 min. The coagulum was again recovered and then dried overnight at 37°C. A 60·8% yield (0·304 g) was obtained. At a temperature just above its gelling point, a 1% aqueous solution of the product obtained from this protocol exhibited no perceptible metachromasia when Toluidine Blue was added, indicating essentially no ester sulfate residues. Metachromasia is a color change produced by interaction of anionic polysaccharides, such as carrageenans, with a dye. With carrageenans, the resulting interaction, or 'stacking' of the dye with

the sulfate ester groups causes a shift in light absorption from 633 nm for the uncombined Toluidine Blue to 540 nm for the combined form (Güven & Güvener, 1985).

Upon cooling the 1% solution of purified β-carrageenan formed a relatively firm, resilient, somewhat opaque gel. Because of the limited amount of purified product, a standard gel strength measurement could not be done. With 10 ml of a 1% gel prepared in a 20 ml beaker, a single-break gel strength of 146 g/cm^2 was obtained using a Marine Colloids™ Gel Tester, Model GT-2 (FMC, Rockland, ME). This gel strength is comparable to that obtained for bacteriological grade agar.

Characterization

Chemical analysis

Moisture was determined by the weight loss of the samples in a vacuum oven at 75°C overnight. Ash was determined by heating the samples from the moisture determination at 550°C in a platinum crucible until they became white and then determining the total weight of the residues. Sulfate was determined gravimetrically as barium sulfate following nitric acid digestion and destruction of the excess acid with sulfate-free formaldehyde. Total chloride content was determined by silver nitrate colorimetry test for Cl^-. The cationic content was determined by atomic absorption spectrophotometry. Pyruvate was determined by the lactate dehydrogenase method (Duckworth & Yaphe, 1970) and 3,6-anhydrogalactose was determined using resorcinol.

Optical rotation

Specific rotation values were determined using the sodium D line (589 nm) while maintaining the samples at 60°C. Agarose and carrageenan samples were used as standards. The results obtained for 0·45 μm membrane-filtered, 1·5% (w/v) solution are given in Table 2. These results confirm that the β-carrageenan is indeed a member of the carrageenan and not the agarose family.

Gelling and melting temperature determinations

These determinations were done at 1·5% concentrations using the dynamic method of raising the temperature of the surrounding water bath at the rate of about 0·5°C/min for the melting temperature, and lowering the temperature at about the same rate for the gelling temperature. The results were as follows:

T_g: 31–33°C
T_m: 63–70°C

Infrared and NMR spectroscopy

Using standard procedures, infrared and ^{13}C-nuclear magnetic resonance spectra were obtained. Interpretation of these confirm the carrageenan backbone structure and virtual absence of κ-carrageenan or any other sulfated fragments. The infrared spectra confirmed that the authors' purified fractions contained no ester sulfate groups attributable to κ- or ι-carrageenans. A peak at *c.* 900 cm^{-1} indicated the presence of the unsubstituted galactose moiety. With NMR, the resonance shown at 96·1 ppm is assigned to the anomeric carbon of the 1,4-linked units and is the fingerprint for β-carrageenan. While the spectrum of the isolated, but not purified, β-carrageenan contained this peak as well as a minor amount of the 96·3–96·9 ppm peak assigned to κ-carrageena, the spectra of the purified fractions contained only that of the β-carrageenan.

DNA electrophoresis

The separating ability of a β-carrageenan gel as an electrophoretic medium was examined. To prepare a framing gel, a 1% (w/v) SeaKem® LE agarose (FMC BioProducts, Rockland, ME) solution was prepared by dissolving 0·15 g agarose in 15 ml 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA (pH 8·0)). This was used to cast a gel, 3 mm thick, in a mini submarine gel electrophoresis chamber (model no. 750, Aqueboque Machine Shop, Aqueboque, NY). After the gel had solidified, three of the side lanes were removed by cutting. To fill this space, a 1% (w/v) β-carrageenan solution was prepared by dissolving 0·035 g of the

Table 1. Results of chemical analysis (corrected for moisture)

Analysis	Isolated β-carrageenan	Purified β-carrageenan
Moisture (%)	13·14	15·75
Ash (%)	2·73	1·50
Total sulfate (%)	1·73	0·84
Free sulfate (%)	0·00	0·00
Cl^- (%)	na	0·07
Mg^{2+} (%)	0·002	0·00
Ca^{2+} (%)	na	0·00
K^+ (%)	na	0·02
Na^+ (%)	0·10	0·21
Pyruvate (%)	na	0·01
3,6-Anhydrogalactose (%)	35·98	38·24

na, Not available.

Table 2. Specific rotation values

Sample	[α]
Purified β-carrageenan	+68·6
κ-carrageenan	+53·6
λ-carrageenan	+48·9
ι-carrageenan	+44·9
Agarose from *Pterocladia*	−33·1

Table 3. Comparison of DNA migration in β-carrageenan vs agarose gels

DNA fragment size (kb)	(kD)	Distance migrated (mm)	
		1% agarose	1·06% β-carrageenan
23·1	15000	5·0	8·5
9·4	6200	8·5	13·7
6·6	4300	10·5	15·7
4·3	2800	18·5	19·0
2·3	1500	20·5	24·0

isolated β-carrageenan in 3·5 ml 1× TAE buffer. This solution, while warm, was poured into the agarose framing gel and allowed to cool and solidify. The wells were loaded with 5 μl of a heat-treated sample of *Hind* III/λ DNA digest (334 ng total DNA) containing Bromphenol Blue as a tracking dye. Electrophoresis was performed at 5 V/cm for 2 h. At the end of 2 h, the gel was removed and stained in an ethidium bromide solution (1 μg/ml) for 25 min, followed by destaining in distilled water for 40 min. The gel was examined and photographed under a UV light source (Fotodyne, model 3-4400). DNA migration was considerably faster in the β-carrageenan than in the agarose, particularly for the larger fragments, as shown in Table 3.

Searching for an explanation of the ability of certain gels to separate large DNA molecules, Stellwagen and Stellwagen (1992) have found that when the gels of different types of agarose, polyacrylamide and β-carrageenan were compared using transient electrical birefringence, the agaroses exhibited anomolous orientation effects, whereas the polyacrylamide and β-carrageenan did not. However, both the agaroses and β-carrageenan gave large birefringence signals, indicating that the noncovalent hydrogen bonds joining the polysaccharide fibers allow a high degree of orientation of the gel matrix.

DISCUSSION

We have shown that ion-independent, gelling β-carrageenan can be isolated from *Eucheuma gelatinae*, *Eucheuma speciosa*, and *Endocladia muricatum*, and that the resulting material, like its stereoisomeric biopolymer, agarose, can be used as a gel electrophoresis medium. Just as there are many members of the agarose family — depending on the species, where harvested, stage of growth cycle, isolation, and purification methods — the same undoubtedly holds for β-carrageenan.

It is important to obtain optical rotation values in characterizing the extracts of Rhodophyta. The first isolate of what we thought was β-carrageenan was a mixture of β-carrageenan and agarose. Because of the negative value obtained for the optical rotation, the raw material used was examined and found to be a mixture of the carrageenanophyte *Eucheuma gelatinae* and a similar-appearing agarophyte, *Gracilaria eucheumoides*. If one examines the optical rotation data, the progression of the increase in values from ι → κ → β seems to be dependent on the decreasing ester sulfate content. However, no direct comparisons are possible since the ι- and κ-carrageenans contain 4-sulfate ester groups while the β-carrageenan does not. Also, λ-carrageenans contain little or no 3,6-anhydro-D-galactose, while the others do. As is well known with substituted mono-, di-, and small oligo-saccharides, the nature and degree of substitution can significantly affect optical rotation values.

Although the particular samples of seaweed used yielded only small amounts of β-carrageenan, it is conceivable that significantly increased yields could be obtained from other species, the same species harvested at other locations or at different times in their growth cycles, and/or by alternative isolation procedures. A patent application covering gelling β-carrageenan has been filed.

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