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Purification, identification, and characterization of D-galactose-6-sulfurylase from marine algae (*Betaphycus gelatinus*)

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ABSTRACT

We extracted and purified D-galactose-6-sulfurylase from *Betaphycus gelatinus* by ammonium sulfate precipitation, ion exchange chromatography and hydrophobic interaction chromatography and investigated the desulfation of carrageenan by the purified enzyme. The purity of the enzyme increased 4.9 fold with approximately 3.7% yield of the crude extract. It was able to catalyze the conversion of μ- to κ-carrageenan. The purified enzyme was a monomeric protein with a molecular weight at about 65 kDa. The maximum activity of the enzyme was observed at pH 7.0 and temperature 40 °C. 26% of the total sulfate was removed from the carrageenan when treated with 18 U purified enzyme. The conversion from μ- to κ-carrageenan was confirmed through the IR spectral analysis of both the control and enzyme treated carrageenan. This study proved that there is a congeneric enzyme that has the same mechanism with alkali treatment on carrageenan from a new kind of red algae *Betaphycus gelatinus*, which is an alternative way of alkali treatment to the production of carrageenan. The existence of precursor μ-carrageenan in *Betaphycus gelatinus* was evidently found in this study.

1. Introduction

Carrageenans are hydrophilic (water soluble) and highly sulfated galactans which occur as a matrix material in numerous red algae (Rhodophyta). The common feature of being linear polysaccharide is a repeating structure of alternating 1,3-linked β-D-galactophyranosyl and 1,4-linked α-D-galactophyranosyl units. Three major types of carrageenan have been found in literature as a major group according to the position of sulfated esters and by the occurrence of 3,6-anhydroring in the α-linked residues, namely κ-carrageenan, ι-carrageenan, and λ-carrageenan.[1] Among them κ-carrageenan is mostly used in the food and cosmetic industries as gelling, stabilizing, and thickening agent due to its high water holding capacity, mechanical strength, biocompatibility, and biodegradability.[2,3]

Researches of several decades focused on the desulfation of carrageenan to achieve the higher gel quality such as, gel strength, viscosity, etc. This is based on the fact that the sulfated esters in carrageenans block the formation of double helices between the molecules and reduce the gel strength.[4,5] By removing the sulfate from the precursors to form 3,6-androgalactose ring, namely μ-, ν-, γ-, λ-carrageenan, to the stable formation κ-, ι-, β-, and Θ-carrageenan, respectively, transition can be achieved.[6,7] Traditional alkali treatment have already achieved the process though it is not eco-friendly. The commonly used ways are sinking the seaweed into low concentrations of alkali solutions, for example potassium hydroxide,[8] sodium hydroxide,[9,10] and sodium carbonate[11] at higher temperatures (70–110 °C) for only few hours. μ-Carrageenan has been found in many red seaweed, like *Kappaphycus alvarezii*, *Mastocarpus stellatus* (formerly *Gigartina stellata*), *Chondrus crispus*, and *Eucheuma striatum*. The transition from the precursor μ-carrageenan to κ-carrageenan has been proved by ^{1}H NMR or ^{13}C NMR.[12–16]

As far back as in the early 1970s, Rees found that the extracts from *Porphyra umbilicalis* was able to remove the sulfate from porphran and reduce the viscosity of the gel solution.[17] Later he found out that L-galactose-6-sulfate was able to convert to L-3,6-anhydrogalactose due to catalyzing activity of the enzyme, which has the same mechanism as the alkali treatment with carrageenan to remove the sulfate.[18] Hemmingson et al. demonstrated the conversion using ^{13}C NMR in the molecular level by cultivating the seaweed with labeled $NaH^{13}CO_3$ after 35 years.[19] Lawson et al.[14] found that, the enzyme extracted from *Mastocarpus stellatus* took part in the transition of the double-helix of carrageenan, which belonged to transferase (cyclizing) group. But the elimination which occurred at 6-sulfate or 2,6-disulfate remained unknown. Wong et al.[15] partially purified the enzyme from *Chondns crispus* and

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proved the μ-carrageenan was converted to κ-carrageenan, and the amount of sulfate released was equal to the synthesis of the D-3,6-anhydrogalactose. Enzymes that had similar function and ability to remove the sulfate of agar and improve the quality were also found in *Gracilaria dura* and have also been investigated.[20] Electrophoretic homogeneity enzyme sulfurylase has been recently purified from *Eucheuma striatum* and was specific on μ-carrageenan.[16]

All the enzymes mentioned above were extracted from red algae. In our previous work we proved that, there is a sort of enzyme in the plant itself to catalyze the transition of the precursors to the terminal formation.[16] With the foundation of our work, we continued the research about purification of sulfurylase from another red algae (*Betaphycus gelatinus*) formerly *Eucheuma gelatinae*. The objective of this study was to purify D-galactose-6-sulfulyrase from *Betaphycus gelatinus* to provide a novel and eco-friendly alternative than alkali treatment method to modify the μ-carrageenan and convert it to κ-carrageenan.

2. Materials and methods

2.1. Materials

Betaphycus gelatinus was collected manually on the shore at Hainan Island, China, and identified by Hainan Provincial Fisheries Research Institute. After harvest, *Betaphycus gelatinus* were cleaned and brought to lab under cool condition and then stored at −80 °C (New Brunswick, U410). All the other chemicals used were of HPLC grade and obtained from Sigma (St. Louis, MO, USA).

2.2. Preparation of the carrageenan

The *Betaphycus gelatinus* was washed carefully to remove the sand and shells before use. Then the seaweeds were smashed in a refiner. 2500 mL steamed water was added to 50 g seaweed and the suspension was heated at 80 °C for 2 h. After this, the suspension was centrifuged at 8000 rpm for 5 min. Then the solution was diluted and NaCl was added to the supernatant to 0.05% and stirred for 2 h. Centrifugation at 8000*g* for 10 min was carried out to remove the undissolved carrageenan. The supernatant was dialyzed against tap water for 72 h and then in ultrapure water for 24 h. The dialyzed carrageenan solution was condensed to 5% volume using rotary evaporator and was precipitated in 3 fold volumes of 95% ethanol. Precipitate was then collected using cotton clothes and washed twice with ethanol (95%) and then vacuum dried at 45 °C. The carrageenan was dried to balance weighed and kept in desiccator to preserve.[7,21]

2.3. Extraction and purification of sulfurylase

The *Betaphycus gelatinus* was thawed and washed properly with cold water before use. Then the seaweeds were grounded in liquid nitrogen and defrosted in precooled extracting buffer (50 mM Tris–HCl, pH 9.5, 500 mM KCl and 10 mM β-mercaptoethanol) at a ratio of 1:3 (w/v). The suspension was stirred gently overnight at a temperature of 4 °C and all the subsequent procedures during extraction and purification were maintained at this temperature. Then the suspension was centrifuged at 12,000*g* for 15 min using a refrigerated centrifuge (J-20×PI, Beckman, USA) and the proteins in supernatant were precipitated by the addition of 80% ammonium sulfate at a slow rate. The precipitate was then collected after centrifugation at 24,990*g* for 15 min and redissolved in a buffer containing 50 mM Tris–HCl (pH 7.1) and 10 mM β-mercaptoethanol (buffer A) and then dialyzed in the same buffer for 24 h. The buffer was changed in every 4 h.

Purification was carried out using AKTA Avant 25 (GE Healthcare) and the prepacked columns were purchased from GE Healthcare. The dialyzed crude enzyme was loaded on HiTrap DEAE FF column (16/10, GE Healthcare), which have been previously equilibrated with buffer B solution (50 mM Tris–HCl, pH 8.5, 10 mM β-mercaptoethanol) until effluent A_{280} was negligible. The column was first washed with buffer B to remove unbound proteins and then eluted at a flow rate of 1 mL min^{-1} with an increasing step gradient of NaCl in buffer B. The active fractions were collected at 200 mM NaCl and desalted using centrifugal enrichment tubes (Macrosep Advance, 3k, Pall, USA) and refreshed with 3 fold volume of buffer A.

The active part of the enzyme was brought to 30% saturation and then loaded on Phenyl FF (high sub) (16/10, GE Healthcare) which has been previously equilibrated with buffer C (50 mM Tris–HCl, pH 8.3, 30% $(NH_4)_2SO_4$, and 10 mM β-mercaptoethanol). The gel was washed with buffer B at a flow rate of 1.5 mL min^{-1}. The elution was carried out at a decreasing step gradient from 30% $(NH_4)_2SO_4$ to 0% $(NH_4)_2SO_4$ at the same flow rate. The active fractions were pooled and desalted.

At each and every step of the purification, the active fractions were analyzed by SDS–PAGE (Gel Doc™ XR, Bio-Rad) as described by Azevedo et al.[22], using 10% polyacrylamide gel. Molecular weight markers of 14.4–97.4 kDa (Genei, Bangalore) were also run simultaneously at 120 V. Quantification of proteins was carried out according to the Bradford method using bovine serum albumin as a standard.[23]

2.4. Enzymatic activity assay

The sulfurylase activity in *Betaphycus gelatinus* was detected by measuring the concentration of sulfate released after incubation of the enzyme with hybrid carrageenan. The standard reaction mixture contained 100 μL of each protein fraction in 50 mM Tris–HCl, pH 7.1, and 800 μL of hybrid carrageenans (0.5%, w/v) in ultra-purewater. The reaction was performed at 45 °C for 6 h to 15 h and was ended by adding 50 μL of 6 N HCl. The fraction taken at the beginning of the reaction was used as a reference. One unit of the D-galactose-6-sulfurylase activity is defined as the amount of enzyme causing production of 1 μmol sulfate min^{-1} at optimal conditions of temperature and pH. The High Performance Anion Exchange Chromatography (HPAEC) method was used to test the content of the free sulfate in the reaction solution.[24]

Buffer solutions used to determine the pH dependence of D-galactose-6-sulfurylase activity were prepared as follows: 50 mM acetic acid/sodium acetate (pH 4.0–6.0), 50 mM Tris–HCl (pH 6.5–9.0), and 50 mM glycine–NaOH (pH 9.5–10.0). The incubation temperatures ranging from 10 °C to 70 °C were tested at pH 7.0 to determine the optimum temperature for the enzyme. Several kinds of metal ion and organic solvents were studied to examine their effect on the enzymatic activity. The Michaelis parameters were determined at the optimum conditions (pH 7.0 and 40 °C). Kinetics experiments were conducted using extracted carrageenan as substrate in pure water with concentrations ranging from 0.005% to 2% (w/v).

2.5. Desulfation of carrageenan

For the determination of the desulfation of the carrageenan extracted from *B. gelatinus*, 0.5% (w/v) solution of carrageenan was prepared in ultra-pure water. After incubation of hybrid carrageenans with 0–20 U of purified D-galactose-6-sulfurylase at 40 °C for 15 h, the precipitate collected after centrifugation at 15,000*g* was washed with ethanol (50% v/v) and dehydrated with acetone. The acetone and residual water was removed by vacuum drying at 40 °C. The content of sulfate in carrageenan was detected according to the method described by Verma et al.[25]