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Purification and characterization of D-Gal-6-sulfurylase from *Eucheuma striatum*

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ABSTRACT

D-Gal-6-sulfurylase catalyzing the conversion of μ-carrageenan into κ-carrageenan was extracted from *Eucheuma striatum* and purified by ammonium sulfate precipitation, hydrophobic interaction chromatography and ion exchange chromatography. The purified enzyme was a monomeric protein with a molecular mass of about 65 kDa as shown in SDS–PAGE. The maximum activity of the enzyme was observed at pH 7.0 and temperature 40 °C. K_m value for μ-carrageenan was 4.31 mM, and the corresponding V_{max} was 0.17 mM min^{-1}. The carrageenan treated with 10 U of the purified enzyme exhibited 7.1-fold increase in gel strength with a removal of 30% sulfate groups. ^{1}H NMR spectral analysis of the control and enzyme treated carrageenan confirmed the conversion of μ- into κ-carrageenan and highlighted the specificity of Gal-6-sulfurylase for μ-carrageenan. This Gal-6-sulfurylase provides an eco-friendly and alternative for alkali treatment method to produce high gel strength κ-carrageenan.

1. Introduction

Carrageenans are water-soluble sulfated polysaccharides extracted from numerous red algae (Rhodophyta; Doyle, Giannouli, Rudolph, & Morris, 2010). They share a common galactan backbone of alternating disaccharide-repeating 1,3-linked-β-D-galactopyranose (β-Gal, unit G) and 1,4-linked-α-D-galactopyranose (α-Gal, unit D) forming a linear chain (Jouanneau et al., 2010). The difference in the number and position of sulfated esters (S) and by the occurrence of 3,6-anhydro ring in the α-linked residues (An Gal, unit A) give rise to three major types of carrageenans sorted with assigned Greek letters; κ-carrageenan (kappa, G4S-DA), ι-carrageenan (iota, G4S-DA2S) and λ-carrageenan (lambda, G2S-D2S, 6S; Knutsen, Myslabodski, Larsen, & Usov, 1994; Rees, 1970; Usov, 1998). The type of κ-carrageenan due to its physical, thermal, and chemical properties as well as its ability to interact with other food polymers, such as starch and proteins, is widely used as gelling, stabilizing, and viscosity-binding agents (Usov, 2011) in food, cosmetics and pharmaceutical industries (Bravo, Arimon, Valle-Delgado, Garcia, & Durany, 2008; Huang, Kennedy, Li, Xu, & Xie, 2007).

However, the native or unprocessed κ-carrageenan has very heterogeneous chemical structures, and usually contains the fraction of the biosynthetic precursor named μ-carrageenan (G4S-D6S; Bellion, Brigand, Prome, Welti, & Bociek, 1983; Van de Velde & De Ruiter, 2002). Evidence from X-ray diffraction (Anderson, Campbell, Harding, Rees, & Samuel, 1969) and optical rotation (Lawson & Rees, 1970; Mckinnon, Rees, & Williamson, 1969; Rees, Steele, & Williamson, 1969) indicates that the presence of sulfated esters in μ-carrageenan deteriorates its gel strength by blocking the formation of double helices among the molecules. Alkali treatment could remove the sulfate groups from μ-carrageenan to enhance the gel strength (Rees, 1972; Yaphe & Duckworth, 1972). The widely used industrial step involves soaking the red algae in a strong solution of alkali hydroxide (up to 10% NaOH/KOH) at elevated temperature (70–90 °C) for a few hours (up to 5 h; Al-Alawi, Al-Marhubi, Al-Belushi, & Soussi, 2011; Freile-Pelegrín & Robledo, 2007; Viana, Noseda, Duarte, & Cerezo, 2004). The alkali treated carrageenans generally exhibit higher gel strength than the native extracts, however, this treatment has disadvantages including the decrease of carrageenan yields, and generation of an alkali effluent (Freile-Pelegrín & Robledo, 2007; Freile-Pelegrín, Robledo, & Azamar, 2006). Thus, there is a growing need to develop eco-friendly technologies for recovery of products from bioresources. Recently, an alternative method of subjecting the temperate red seaweed to dark has been used to improve the gel quality of carrageenan, however, this dark treatment reaction taken a long time (more than 10 days; Villanueva, Hilliou, & Sousa-Pinto, 2009).

The enzymatic catalysis for improving the gel strength of carrageenan has been reported. The reported enzymes include D-Gal-2,6-sulfurylase and sulfohydrolase. The former enzyme from *Choudrus crispus* could catalyze the conversion of ν- into ι-carrageenan (Genicot-Joncour et al., 2009), however, this enzyme was specific to ν-carrageenan and was inactive on μ-carrageenan.

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The latter enzyme was reported to catalyze the conversion of μ-carrageenan into κ-carrageenan by removing the sulfate groups (Wong & Craigie, 1978), but so far the purified enzyme was not obtained yet. In this paper, the D-Gal-6-sulfurylase from *Eucheuma striatum* was purified by sequential chromatography procedure to a natural electrophoretic homogeneity for the first time. The purified Gal-6-sulfurylase exhibited significant advantages in purity and enzymatic activity for μ-carrageenan. In addition, Gal-6-sulfurylase treated with carrageenan could obviously improve the gel strength. Therefore, the Gal-6-sulfurylase was purified in this study to provide an eco-friendly and alternative for alkali treatment method to produce high gel strength κ-carrageenan.

2. Experimental

2.1. Materials

E. striatum was provided by Hainan Provincial Fisheries Research Institute and collected in May 2011 on the shore at Hainan Island, China. The specimens were carried in a cool pack to the laboratory under cool conditions, frozen in liquid nitrogen and stored at −80 °C. Unmodified κ-carrageenan was extracted and purified from *E. striatum* according to the method of Freile-Pelegrín et al. (2006). The polysaccharide was composed of about 11.2% μ-carrageenan moieties according to ^{1}H NMR analysis. The present hybrid carrageenans were used to screen and study Gal-6-sulfurylase activities. Ultra pure water used for experiment was prepared in laboratory from a Milli-Q system (Millipore, USA). All other chemicals were analytical grade and obtained from Sigma (St. Louis, MO, USA).

2.2. Extraction and purification of Gal-6-sulfurylase

Frozen *E. striatum* was ground in liquid nitrogen and allowed to thaw in cold extracting buffer (50 mM Tris–HCl, pH 9.5, 500 mM KCl, and 10 mM 2-β-mercaptoethanol) at a proportion of 1:3 (w/v). The suspension was gently stirred overnight at 4 °C, and all of the following fractionation and purification steps were performed at this temperature. The suspension was centrifuged at 12,000 × g for 15 min and proteins in the supernatant were precipitated with 80% ammonium sulfate saturation. Precipitate was collected after centrifugation at 15,000 × g for 15 min and dissolved in buffer A (50 mM Tris–HCl, pH 7.1, and 10 mM 2-β-mercaptoethanol). The solution, containing some polysaccharides as well as the active protein, was dialyzed against same buffer for 24 h with the change of buffer at an interval of 4 h. The dialyzed enzyme solution was brought to 30% $(NH_4)_2SO_4$ saturation and loaded on Phenyl Sepharose 6 Fast Flow column previously equilibrated with buffer B (50 mM Tris–HCl buffer, pH 7.1, 30% $(NH_4)_2SO_4$, and 10 mM 2-β-mercaptoethanol). The gel was washed with buffer B at a flow rate of 1.5 mL min^{-1} until effluent A_{280} was negligible. Elution of the bound proteins was achieved by applying a linearly decreasing gradient from 30% to 0% $(NH_4)_2SO_4$ at a flow rate of 1.5 mL min^{-1}. All of the active fractions were pooled and dialyzed for 36 h in buffer A.

The desalted enzyme solution was further purified with DEAE Sepharose CL-6B column previously equilibrated with buffer A. The column was washed with buffer A to remove the unbound proteins and eluted at a flow rate of 1 mL min^{-1} with an increasing step gradient of NaCl in buffer A. The active fractions were collected and dialyzed about 6 h against buffer A.

At each step of purification, the active fractions were analyzed by SDS–PAGE (Laemmli, 1970) using 12% polyacrylamide gel and molecular weight markers of 14.4–97.4 kDa (Genei, Bangalore) were also run simultaneously at l20 V. Protein quantification was performed according to Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.3. Gal-6-sulfurylase activity assay

Gal-6-sulfurylase activity was determined by measuring the concentration of sulfate released after incubating with hybrid carrageenans. The standard reaction mixture contained 100 μL of each protein fraction in 50 mM Tris–HCl, pH 7.1, and 100 μL of hybrid carrageenans (1.4%, w/v) in the same buffer. The reaction was conducted at 45 °C for 6–15 h and terminated by the addition of 0.2 ml of 6 N HCl. The fraction taken at zero time was used as a reference. The release of sulfate was measured by high-performance anion-exchange chromatography (HPAEC; Genicot-Joncour et al., 2009). One unit of the Gal-6-sulfurylase activity is defined as the amount of enzyme causing production of 1 μmol sulfate per minute at optimal conditions of temperature and pH.

2.4. Enzymological experiments with Gal-6-sulfurylase

Buffer solutions for the determination of pH dependence of D-Gal-6-sulfurylase activity were prepared as follows: 0.1 M acetic acid/0.1 M sodium acetate (pH 4.0–6.0), 0.1 M Tris–HCl (pH 6.0–9.0), and 0.1 M glycine–NaOH (pH 9.0–12.0). The optimum temperature for the purified Gal-6-sulfurylase activity was measured at pH 7.0 over a temperature range of 10–60 °C. Different metal ion and organic solvents were studied to examine their effect on the enzymatic activity. Apparent K_m and V_{max} were determined using a Lineweaver–Burk plot. Kinetic data for the Gal-6-sulfurylase reaction with μ-carrageenan as a substrate was obtained with substrate concentrations ranging from 0.1% to 2.5% (w/v), corresponding to 0.27–6.76 mM μ-carrabiose units. The reactions were performed at the optimum conditions (pH 7.0 and 40 °C).

2.5. Determination of sulfate content and gel strength of carrageenan

For the determination of gel strength 1.4% (w/v) solution of carrageenan was prepared in 50 mM Tris–HCl (pH 7.0) buffer. After incubation of hybrid carrageenans with 0–12 U of purified Gal-6-sulfurylase at 40 °C for 15 h, the content of sulfates and gel strength of carrageenan were determined. The amount of sulfate content in sample was determined by the method of Verma, Swaminathan, and Sud (1977). Gel strength (g/cm^2 at 20 °C) was measured on discs of carrageenan (6 cm diameter, 3 cm height) using Texture Analyzer (Model TAXT2J/25, Stable Micro System, Godalming, Surrey, UK) with a 2 cm^2 probe area and operating at a crosshead speed of 0.5 mm s^{-1}. Gel was aged overnight at 4 °C and equilibrated for 20 min at 20 °C before measurement. Gel strength was measured as the required weight to break the gel.

2.6. ^{1}H NMR spectroscopy

The composition of hybrid carrageenans was determined by ^{1}H NMR spectroscopy. One-dimensional ^{1}H NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer equipped with an indirect 5-mm gradient probehead ^{1}H/^{13}C/^{31}P at an ambient temperature. Prior to analysis, samples were dissolved at concentration of about 10 mg mL^{-1} in D_2O. Chemical shifts (δ) are expressed in ppm in reference to an internal standard (DSS). No suppression of the HOD signal was performed.