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Purification and characterization of κ-carrageenase from the marine bacterium *Pseudoalteromonas porphyrae* for hydrolysis of κ-carrageenan

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ABSTRACT

A bacterial strain LL1 producing κ-carrageenase was isolated from the decayed seaweed collected from Yellow Sea, China and identified as *Pseudoalteromonas porphyrae*. The extracellular κ-carrageenase in the supernatant of cell culture of the marine bacterium *P. porphyrae* LL1 was purified to homogeneity with a 202.6-fold increase in specific κ-carrageenase activity as compared to that in the supernatant by ultrafiltration, gel filtration chromatography, and anion-exchange chromatography. According to the data from sodium dodecyl sulfatepolyacrylamide gel electrophoresis, the molecular mass of the purified enzyme was estimated to be 40.0 kDa. The purified enzyme could actively convert κ-carrageenan into tetrasaccharides, but poorly convert λ-carrageenan. The optimal pH and temperature of the purified enzyme were 8.0 and 55 °C, respectively. The enzyme was significantly stimulated by Mg^{2+} and Ba^{2+}. The enzyme was inhibited by phenylmethylsulfonyl fluoride (PMSF), iodoacetic acid, EDTA, EGTA and 1,10-phenanthroline. The K_m and V_{max} values of the purified enzyme for κ-carrageenan were 4.4 mg/ml and 0.1 mg/min ml, respectively. The amino acid sequence (NPQPHIAKPGQTWILQEKRS) of N-terminus of the purified enzyme was identical to that of N-terminus of the deduced protein encoded by the gene encoding κ-carrageenase cloned from the marine bacterium.

1. Introduction

Carrageenans are gel-forming linear sulfated galactans extracted from certain marine red algae. They consist of D-galactose residues linked by alternating α-1,3 and β-1,4 linkages. Depending on the presence of a 3,6-anhydro bridge in the α-l,4-linked galactose residue and on the position of sulfate substituents, they are referred to as κ-, ι-, or λ-carrageenans. κ-Carrageenan is an alternating galactan of 1,3-linked β-D-galactopyranose 4-*O*-sulfate and 1,4-linked 3,6 anhydro-α-D-galactopyranose and occurs in the cell wall of certain species of red alga, such as *Chondrus* sp., *Gigartina* sp., *Eucheuma* sp. and *Iridaea* sp. [1].

κ-Carrageenase (EC 3.2.1.83) cleaves the internal β(1-4) linkages of carrageenans, yielding oligogalactans of the neocarrabiose or neoagarobiose series and belongs to family 16 of glycoside hydrolases. As the galactan hydrolase displays a strict substrate specificity, it obviously recognizes the pattern of sulfation on the digalactose repeating units. It is a useful tool for the structural analysis of the cell walls and protoplast isolation from red alga. After hydrolysis of κ-carrageenan using κ-carrageenase, sulfated oligosaccharides obtained can show potential anti-tumor activities [2], anticoagulation, anti-inflammation, anti-thrombosis, and viral inactivation [3].

Various marine bacteria that secrete galactan endo-hydrolases, including producers of κ-carrageenases, ι-carrageenases, and λ-carrageenases have been obtained. All of these bacteria belong to the genera *Pseudoalteromonas, Alteromonas, Pseudomonas*, *Vibrio, Zobellia* and *Cytophaga* and were isolated from marine environments [2]. The structural genes of the κ-carrageenase of *Pseudoalteromonas carrageenovora, Zobellia galactanovorans* and *Alteromonas fortis* have been described in detail [4].

In the present study, a marine bacterium producing κ-carrageenase was isolated from the decayed seaweed collected from Yellow Sea, China and identified as *Pseudoalteromonas porphyrae*. Its extracellular κ-carrageenase was purified and characterized and the gene encoding the κ-carrageenase was cloned from this marine bacterium.

2. Materials and methods

2.1. Bacterial strain

P. porphyrae LL1 (the collection number 2E01119 at the Marine Microorganisms Culture Collection of China) was isolated from the decayed seaweed collected from Yellow Sea, China and preserved at the laboratory.

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2.2. Media

2216E medium contained tryptone 1.0%, yeast extract 0.5%, NaCl 1.0%, $FePO_4$ 0.001% [5]. The seed culture medium consisted of carrageenan 0.2%, $NaNO_3$ 0.2%, yeast extract 0.1%, NaCl 1.5%, $MgSO_4{\cdot}7H_2O$ 0.05%, $FePO_4$ 0.001%, $K_2HPO_4{\cdot}12H_2O$ 0.1%, $CaCl_2$ 0.01%, pH 7.5. The κ-carrageenase production medium was composed of carrageenan 0.1%, beef extract 0.2%, NaCl 2.0%, $K_2HPO_4{\cdot}3H_2O$ 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05% and $FePO_4$ 0.001%.

2.3. DNA extraction and 16S rDNA amplification

P. porphyrae LL1 was aerobically grown in the 2216E medium at 25 °C overnight. The genomic DNA was extracted and purified from the culture using the TIANamp Bacteria DNA kit (TIANGEN, China) and used as the template for PCR. 16S rDNA of the bacterium was PCR amplified using the primers: the forward primer: 5′-AGAGTTTGATC(C/A)TGGCTCAG-3′ and the reverse primer: 5′-TACGG(C/T)TACCTTGTTACGACTT-3′. The PCR was performed in a total volume of 50.0 μl PCR mixture containing 5.0 μl of ExTaq buffer, 4.0 μl of 2.5 mM dNTPs, 1.0 μl of 50 μM each primer, 1.0 μl of the genomic DNA (10.0 ng/ml), 37.0 μl of sterile deionized water and 1.0 μl of ExTaq DNA polymerase. The conditions for the PCR amplification were as follows: initial denaturation at 94 °C for 10 min, denaturation at 94 °C for 45 s, annealing temperature at 51 °C for 45 s, extension at 72 °C for 1 min, final extension at 72 °C for 8 min. PCR was run for 30 cycles and the PCR cycler was GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA, USA). The PCR products were sequenced by Shanghai Sangon Company.

2.4. Phylogenetic analysis of the bacterium

The 16S rDNA sequence obtained above was aligned using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences which shared over 98% similarity with currently available sequences were considered to be the same species and multiple alignments were performed by using ClustalX 1.83 and the phylogenetic tree was constructed by MEGA 4.0 [6].

2.5. Production of κ-carrageenase

P. porphyrae LL1 was cultivated for 28 h at 28 °C in 250-mL Erlenmeyer flasks with 75.0 ml of the production medium by shaking at 160 rpm. After 600 ml of the culture was centrifuged at 10000 × *g* and 4 °C for 5 min, the supernatant obtained was concentrated to a volume of 30.0 ml by ultrafiltration with a 5-kDa-cutoff™ membrane with a Labscale TFF System (Millipore) and the concentrate was filtered using sterile 0.2 μm membrane. These concentrated supernatants were stored at 4 °C and was used as the crude κ-carrageenase preparation.

2.6. Determination of κ-carrageenase activity

Because the results have shown that the κ-carrageenan degradation proceeded by a linear increase of the number of cleavage within 1 h, κ-carrageenase activity in the crude κ-carrageenase preparation obtained above and of the purified enzyme described below was determined as follow. 50.0 μl of the enzyme was mixed with 250.0 μl of 0.2% κ-carrageenan solution prepared with 0.02 M sodium phosphate butter (pH 8.0) and the mixture was incubated at 55 °C for 30 min. After that, the reaction in the mixture was immediately stopped by heating it at 100 °C for 10 min. The amount of reducing sugar released from κ-carrageenan was determined using Nelson-Somogyi [7]. One unit of κ-carrageenase activity was defined as the amount of enzyme causing release of reducing sugars equivalent to 1.0 mg reducing sugar from κ-carrageenan in 1 min under the assay conditions. The enzyme that had been heated at 100 °C for 10 min prior to the reaction was used as the control. Protein concentration was measured by the method of Bradford, and bovine serum albumin served as standard [8].

2.7. Purification of κ-carrageenase

The crude κ-carrageenase preparation obtained from the previous step was applied to DEAE Sepharose Fast Flow anion-exchange column (2.5 cm × 30 cm) that had been equilibrated with 20.0 mM sodium phosphate buffer (pH 7.2) and the unbound proteins were then eluted with 20.0 mM sodium phosphate buffer (pH 7.2) using ÄKTA™ prime with Hitrap™ (Amersham, Biosciences, Sweden). At a flow rate of 0.5 ml/min, 5.0 ml fractions were collected. The κ-carrageenase-positive fractions were combined and were concentrated to 10.0 ml by filtration through an AmiconYM3 (MW cut-off 10,000) membrane. The concentrate was applied to Sephadex ™ G-75 column (Pharmacia 2.5 cm × 100 cm) and the column was eluted with 20.0 mM sodium phosphate buffer (pH 8.0) by using ÄKTA™ prime with Hitrap™ (Amersham, Biosciences, Sweden). At a flow rate of 0.5 ml/min, 2.0 ml fractions were collected. It was found that there were five peaks of the elute and only the elute of peak 2 corresponding to elution time from 80 to 130 min had κ-carrageenase activity. The κ-carrageenase-positive fractions were combined and were concentrated to 5.0 ml by filtration through an AmiconYM3 (MW cut-off 10,000) membrane.

2.8. SDS polyacrylamide gel electrophoresis

The purity and molecular mass of the κ-carrageenase in the concentrated fractions showing the activity was analyzed in non-continuous denaturing SDS-PAGE [9] according to the instructions offered by the manufacturer with a Two Dimensional Electrophoresis System (Amersham, Biosciences, Sweden) and the gels was stained by Coomassie Brilliant Blue R-250 [10]. The molecular mass standards for SDS-PAGE comprised phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa).

2.9. Determination of N-terminal amino acid sequence of the purified κ-carrageenase

To determine the N-terminal amino acid sequence, the band of the purified κ-carrageenase on SDS-PAGE as described above was electroblotted to a polyvinlymethylformamide (PVDF) membrane (Bio-Rad) with a CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer system. Electroblotting was conducted at 200 mA for 1 h. The membrane was stained with Ponceau S to visualize the protein. The band was excised and submitted for N-terminal amino acid sequencing with an ABI PROCISE™ 492cLC protein sequencer.

2.10. κ-Carrageenan hydrolysis

The reaction mixture containing 200 μl of 3.2 U/ml of the concentrated enzyme and 100 μl of 0.2% of κ-carrageenan in sodium phosphate buffer (0.02 M, pH 8.0) was incubated at 50 °C for 10 h. After that, κ-carrageenase in the mixture was immediately inactivated by heating at 100 °C for 15 min. The end-products of κ-carrageenan hydrolysis were determined by thin layer chromatography [11]. The reaction mixture in which the κ-carrageenase was inactivated prior to addition by heating at 100 °C for 15 min was used as controls.

2.11. Effects of pH and temperature on κ-carrageenase activity

The optimal temperature for activity of the enzyme was determined at temperatures of 40, 45, 50, 55, 60 and 65 °C. Temperature stability of the purified enzyme was tested by pre-incubating the enzyme at different temperatures ranging from 0 to 65 °C for 1 h, residual activity was measured as described above immediately. Here, the relative κ-carrageenase activity of the pre-incubated sample at 4 °C was regarded as 100%.

The effect of pH on the enzyme activity was determined by incubating the purified enzyme in 20 mM sodium phosphate buffer between pH 4.0 and 10.0. pH stability was tested via 6-h pre-incubation of the purified enzyme in 20.0 mM sodium phosphate buffer at different pH values ranging from 4.0 to 10.0 and at temperature of 4 °C. The remaining activities of κ-carrageenase were measured immediately after this treatment with the standard method as mentioned above.

2.12. Effects of different metal ions and protein inhibitors on κ-carrageenase activity

To examine effects of different metal ions on κ-carrageenase activity, enzyme assay was performed in the reaction mixture as described above with various metal ions at a final concentration of 10.0 mM. The activity assayed in the absence of metal ions was defined as control. The metal ions tested include Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Ba^{2+}, K^{+}, Co^{2+}, Ni^{2+}, and Li^{+}.

The effects of protein inhibitors (1,10-phenanthroline, EGTA, PMSF, EDTA, SDS and iodoacetic acid at a final concentration of 5.0 mM, respectively) on κ-carrageenase activity were measured in the reaction mixture as described above. The purified enzyme was pre-incubated with the respective compound for 1 h at 4 °C, followed by the standard enzyme assay as described above. The relative activity assayed in the absence of the protein inhibitors was regarded as 100%.

2.13. Determination of kinetic parameters

To obtain K_m and V_{max} for the purified κ-carrageenase, 250.0 μl of 0.25, 0.5, 1.0, and 2.0 mg/ml of κ-carrageenan in 20.0 mM sodium phosphate buffer (pH 8.0) was mixed with 50.0 μl of the purified κ-carrageenase, respectively, and the mixture was incubated at 55 °C for 30 min and the reaction was stopped immediately by heating at 100 °C for 10 min. K_m and V_{max} values were obtained from Lineweaver–Burk plot.

2.14. Measurement of substrate specificity

The reaction mixtures containing 50.0 μl of 3.2 U/ml of the concentrated enzyme and 250.0 μl of 0.20% of κ-carrageenan, ι-carrageenan, λ-carrageenan, agar and chitin (all the polysaccharides were purchased from Sigma) in sodium phosphate buffer (0.02 M, pH 8.0) were incubated at 55 °C for 30 min, respectively. After that, κ-carrageenase in the mixtures was immediately inactivated by heating at 100 °C for 15 min. The reducing sugar in the mixtures was determined as described above.