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Purification and characterization of a carboxymethyl cellulase from *Artemia salina*

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ABSTRACT

Brine shrimp (*Artemia salina*) belong to a group of crustaceans that feed on microalgae and require a cellulase enzyme that can be used in ethanol production from marine algae. Protein with potential cellulase activity was purified and the activity analyzed under different conditions. After initial identification of cellulase activity by CMC cellulase, surface sterilization and PCR using 16s rRNA primers was conducted to confirm that the cellulase activity was not produced from contaminating bacteria. The enzyme was purified by ammonium sulfate fractionation, gel filtration, and ion exchange chromatography. After the final purification, a 70-fold increase in specific enzyme activity was observed. SDS–PAGE results revealed that the cellulase enzyme had a molecular mass of 96 kDa. Temperature, pH, and salinity values were found to be optimal at 55 °C, pH 8.0, and 600 mM NaCl, respectively. Specifically, the enzyme showed a fivefold increase in enzyme activity in seawater compared to 600 mM NaCl in phosphate buffer. Further analysis of the purified enzyme by molecular spectrometry showed no match to known cellulases, indicating this enzyme could be a novel halophilic cellulase that can be used for the production of bioethanol from marine macroalgae.

1. Introduction

Biofuels produced from biomass are expected to be an alternative energy source in the future, which in liquid or gaseous form are usually produced from plant matter and residues, and believed to reduce harmful carbon emissions [1].

Algae include diverse species inhabiting freshwater and seawater and ~50% of global biomass is thought to be generated in the marine environment [2]. Compared to most productive land plants, algae are capable of producing high yields of carbohydrates, lipids, and proteins over a short period of time, which can be processed to generate biofuels. Algae are generally divided into microalgae and macroalgae based on their morphology and size. Microalgae are usually single-celled and do not have to spend energy for distribution and transportation of storage molecules; thus, they are very effective at producing storage materials such as starch, glycogen, and cellulose, which can be used for biofuel production [3]. Similar to microalgae, macroalgae grow at a fast rate and yield large amounts of biomass. In addition, macroalgae can be cultured on a large scale, even three-dimensionally, by seeding onto thin, weighed strings suspended over a larger horizontal rope [4].

The production of ethanol from biomass is generally performed in four steps: pretreatment, enzymatic hydrolysis, fermentation, and distillation. Sometimes saccharification and fermentation (SSF) are conducted simultaneously [5]. The pretreatment that produces polysaccharides from biomass is usually conducted by using diluted acid (0.2–2.5%) and temperatures between 130 °C and 210 °C, but this complex and energy-intensive pretreatment is not necessary for macroalgae because they do not contain lignins [3]. The use of extremely low acid conditions for the pretreatment of macroalgae biomass can simplify downstream processing such as neutralization and waste treatment, and reduces equipment costs [6].

Similar to the cellulosic biomass from other plant sources, that from algae can be enzymatically hydrolyzed using cellulase enzymes and converted to simple sugars that can be fermented to ethanol [3]. Cellulose is a major polysaccharide component of plants and algae cell walls. Although cellulose is a common carbohydrate, only a few organisms have the ability to utilize it efficiently. Cellulase, an important inducible enzyme synthesized mostly by microorganisms during their growth on cellulosic materials, is required to release glucose prior to the production of ethanol. Most biotechnologically significant cellulases are derived from bacteria and fungi [7,8]. Due to their origin, most cellulases have limited activity under high-salt conditions such as hydrolysis of polysaccharides derived from marine macroalgae, and the raw macroalgal biomass needs to be desalted before processing [9]. Therefore, a cellulase that is active under a high-salt condition is desirable for bioethanol production from marine macroalgae.

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In this study, *Artemia salina* was used as the source of a new salt-tolerant cellulase. *A. salina* is a primitive invertebrate belonging to a group of crustaceans in the kingdom Animalia [10]. This species can survive under extreme conditions including high salinity due to its special adapting abilities against environmental stresses. *A. salina* feeds on microalgae and uses cellulase for the digestion of the microalgae cell wall components. Additionally, considering the high-salt habitat of this organism, the cellulase was expected to be active under high-salt conditions, which was confirmed by the purified enzyme in the present study.

2. Materials and methods

2.1. A. salina culture

Lyophilized cysts of *A. salina* (Inve Aquaculture, Salt Lake City, UT, USA) coated with iron were washed using 70% ethanol for 2 min in a 1.5 mL tube, and then the ethanol was removed by standing the tube on a magnetic stand. The cysts were incubated in a 20 L plastic tank containing autoclaved seawater for 48 h at 28 °C with fluorescent light and mild aeration. After visible signs of hatching, the volume was reduced using an autoclaved nylon mesh, and the empty cysts were removed using a magnetic stand. Pure *A. salina* was collected on autoclaved nylon mesh and used for homogenization.

2.2. Preliminary test for cellulose activity and bacterial contamination

Hatched *A. salina* was ground in liquid nitrogen and resuspended in 50 mM phosphate buffer (pH 7.0 with 10 mM β-mercaptoethanol, 5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM EDTA). The homogenate was centrifuged at 8000*g* for 5 min and then the supernatant was filtered through a 0.2 μm syringe filter. The crude homogenate, supernatant, and filtrate (20 μL) were placed on a 1.5% agar plate containing 1% carboxymethylcellulose (CMC) and incubated for 30 min at room temperature. Cellulast (Novozymes, Bagsvaerd, Denmark) of 70 endoglucase units (EGU) in 20 μL 50 mM phosphate buffer was used as a positive control. The carboxymethyl cellulolytic activity was confirmed by the Congo Red overlay method [11]. The crude homogenate supernatant and the filtrate were streaked on Luria Broth plates and Marine 2216 agar (Difco, Franklin Lakes, NJ, USA), respectively, and incubated at 37 °C and 25 °C, respectively, overnight to observe any bacterial growth. Total DNA was extracted from the crude homogenate using phenol/chloroform and PCR was conducted using two universal primers for the bacterial 16S rRNA gene (27F: 5′-AGAGTTTGATGGCTCAG-3′, 1492R: 5′-TACGGYTACCTTGTTACGACTT-3′) [12].

2.3. Ammonium sulfate fractionation

All purification steps were performed at 4 °C except where otherwise specified. Hatched *A. salina* was separated from the empty cyst as above, collected on nylon mesh, and washed with sterile distilled water. The collected *A. salina* was ground in liquid nitrogen using a mortar and pestle, and resuspended in 50 mM phosphate buffer (pH 7.0) containing 10 mM β-mercaptoethanol, 5 mM PMSF, 1 mM EDTA, and 10% sucrose in 100 mL buffer to a 10 g sample ratio. The homogenate was centrifuged at 10,000*g* for 20 min. The supernatant was saved and the pellet was resuspended with 100 mL of 50 mM phosphate buffer (pH 7.0) with 10 mM β-mercaptoethanol and 1 mM EDTA, and centrifuged as above. The two supernatants were pooled and subjected to ammonium sulfate fractionation. Solid ammonium sulfate was slowly added to the homogenate in a stepwise manner from 10% to 70% in 10% increments with constant stirring for 70 min. For each ammonium sulfate concentration, the mixture was allowed to stand for 30 min and then centrifuged at 10,000*g* for 15 min. The pellet was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) with 10 mM β-mercaptoethanol and 1 mM EDTA, and used for the cellulose activity test. The supernatant was used for the next fractionation with a 10% increase in ammonium sulfate.

2.4. Enzyme assay

Cellulase activity was measured by the 3,5-dinitrosalicylic acid (DNS) method [13], which determines the amount of reducing sugars liberated by the cellulase from 1% CMC solubilized in 50 mM phosphate buffer (pH 7.0). Purified cellulose (500 μL) was incubated with 500 μL of 1% CMC for 30 min at room temperature and the reaction was stopped by adding 1 mL DNS solution. Treated samples were boiled for 5 min and cooled at room temperature, and then the optical density was measured at 550 nm. The cellulase activity was determined using a calibration curve for glucose (Sigma–Aldrich, Gillingham, Dorset, UK). One unit of activity was defined as the amount of enzyme that released 1 μM of glucose equivalents from substrate per minute. The specific activity was expressed in μmol/min/mg.

2.5. Cellulase purification and amino acid sequence analysis

Fractions from 50% to 70% ammonium sulfate fractionations were pooled and used for enzyme purification. The pooled sample was first filtered through a 0.45 μm syringe filter and concentrated using VIVASPIN 20 with a 10,000 MWCO (Sartorius Stedim Biotech, Aubagne, France) at 1700*g* for 45 min. The concentrated enzyme was purified using gel filtration chromatography and ion exchange chromatography using the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech, Piscataway, NJ, USA). For gel filtration chromatography, preparations were applied to a HiLoad 16/60 Superdex 75 (GE Healthcare Life Sciences, Piscataway, NJ, USA) equilibrated with 50 mM phosphate buffer (pH 7.0 with 10 mM β-mercaptoethanol, 50 mM NaCl, and 5% glycerol) at a flow rate of 1 mL/min and collected by an autofraction collector. Every fraction of 0.3 mL was collected and assayed for cellulase activity. The active fractions were dialyzed overnight with 50 mM Tris–HCl buffer (pH 8.0 with 10 mM β-mercaptoethanol, 50 mM NaCl) and further processed in a RESOURCE Q 6 mL column (GE Healthcare Life Sciences), which was equilibrated with elution buffer (50 mM Tris–HCl, pH 8.0, with 50 mM NaCl). Elution was achieved with a linear gradient of 5–100 mM NaCl in equilibration buffer at a flow rate of 1 mL/min. Every 0.3 mL fraction was collected and assayed for cellulase activity, and the active fractions were collected and concentrated using VIVASPIN 20 with a 10,000 MWCO (Sartorius Stedim Biotech) at 1700*g* for 45 min and used for the analyses described below. The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 12% polyacrylamide gel with a protein marker (GenDepot, Barker, TX, USA).

The amino acid sequence of the purified protein was determined by ultra- performance liquid chromatography (UPLC) and mass spectrophotometry at Yonsei Protomics Research Center, Yonsei University, Seoul, Korea.

2.6. Optimum conditions for enzyme activity

The optimum temperature, pH, and salinity of the purified cellulase were determined by incubating the 50 μL of purified cellulase with 50 μL of 1% CMC in 50 mM phosphate buffer (pH 7.0) under different conditions. The reaction was conducted in a 96 well ELISA plate. To determine the optimum temperature, the