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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

reaction mixtures were incubated for 30 min at different temperatures ranging from 4 °C to 60 °C. For the optimum pH, the purified cellulase was mixed with 1% of CMC in 50 mM phosphate buffer that ranged from pH 3.0 to pH 12.0 and incubated at room temperature. For optimum salinity, the purified cellulase was mixed with 1% of CMC in 50 mM phosphate buffer (pH 7.0) with salinity ranging from 0.1 M to 1.0 M for 30 min at room temperature. The reactions were stopped by adding 100 μL DNS solution, and enzyme activity was measured using the enzyme assay procedures described above. The values represent the percentages of the enzyme activity as compared to the observed maximum activities under optimum conditions.

2.7. Effect of metal ions on cellulase activity

The effects of various metal ions on purified cellulase activity were determined by preincubating the enzyme with individual metal ions of 1 mM ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, KCl, $MgCl_2$, $MnCl_2$) in 50 mM phosphate buffer (pH 7.0) at room temperature for 30 min. The enzyme activity was measured as described above. The activity assayed in the absence of metal ions was recorded as 100%.

3. Results

3.1. Verification of cellulase from A. salina

The cellulase activity from the crude extract of hatched *A. salina*, the supernatant after centrifugation, and the filtrate were tested for the presence of cellulase. As shown in Fig. 1, all samples showed cellulase activity on a 1.5% agar plate containing 1% CMC and were stained with Congo Red. When the three samples were spread on LB and Marine 2216 agar plates, no bacterial colony growth was observed. Furthermore, no PCR products for the 16s rRNA gene was amplified from the samples.

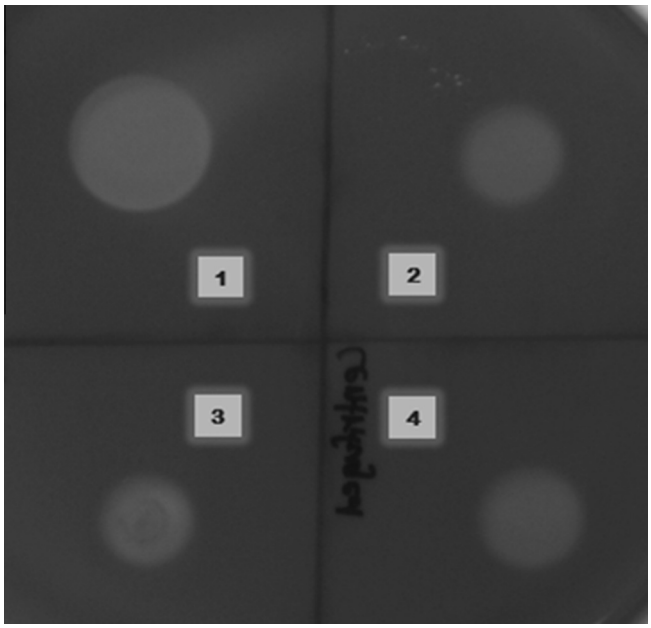

Fig. 1. Pretest for the presence of cellulase activity from *A. salina* extract by the Congo Red overlay method. Samples (20 μL) were tested with 1% CMC for 30 min and stained with Congo Red. (1) Positive control, 70 EGU of cellulast; (2) crude homogenate; (3) filtrate from 0.2 μm syringe filter; and (4) supernatant after 5 min centrifugation at 8000*g*.

3.2. Purification and molecular characterization of cellulase

The crude enzyme obtained from *A. salina* was precipitated using ammonium sulfate up to 80% saturation in 10% increments. When each fraction was tested for cellulase activity, most of the cellulase activity was observed in the protein fractions precipitated with 60–70% ammonium sulfate. The proteins pooled from these two fractions showed cellulase of 1.1 U/mg (Table 1).

These two pooled fractions were used for further purification by gel filtration chromatography and ion exchange chromatography. In the gel filtration chromatography, the protein peak was observed in fractions numbered 11–13 after the initial flowoff (Fig. 2). In the cellulase activity test, these fractions also showed cellulase activity. When the fractions were pooled and cellulase activity was tested, the specific enzyme activity was 11.6 U/mg (Table 1). Notably, these protein contaminating fractions also showed a high concentrations of NaCl, which are shown in the dotted line in Fig. 2. The active fractions 11–13 of the gel filtration chromatography were pooled, concentrated, and further purified using ion exchange chromatography. When the purified protein was subjected to SDS–PAGE analysis, a major protein with an estimated molecular mass of 96 kDa was observed (Fig. 3). The partial amino acid sequence of the purified protein was analyzed using a mass spectrometer after trypsin digestion. Although peptide sequences with a diverse molecular weight were verified from the analysis, none of the peptides showed any sequence similarity to known proteins (Table 2).

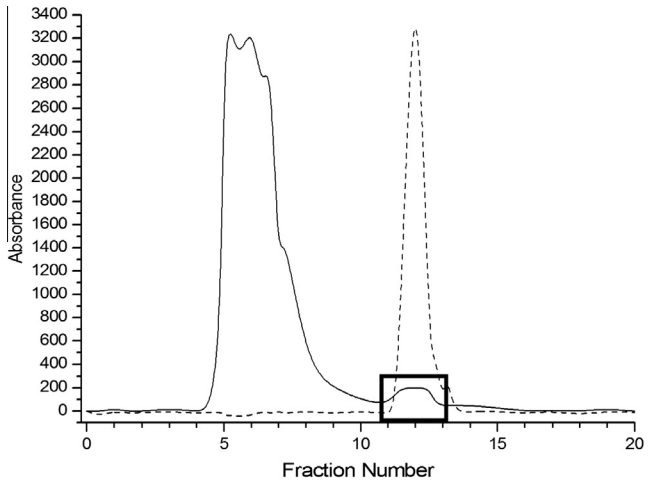

Fig. 2. Enzyme purification by gel filtration chromatography. Fractions (300 μL) were collected and assayed for cellulase activity and the NaCl concentration was determined. Protein concentration is indicated by the black line, and the dash represents the NaCl concentration. The boxed area denotes the fractions showing cellulase activity.

Table 1
Purification steps of the cellulase enzyme isolated from *A. salina*.

Purification steps	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield
Crude enzyme	2680	1420.4	0.5	–	100
Ammonium sulfate precipitation	552	590.6	1.1	2.2	41.6
Superdex 75	3	34.8	11.6	23.2	2.5
RESOURCE Q6	0.5	18.1	35.1	70.2	1.3

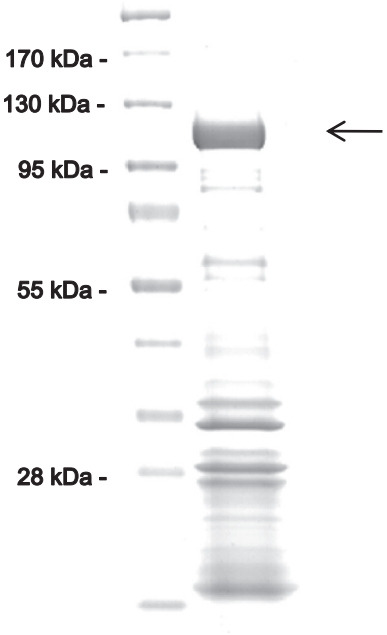

Fig. 3. SDS–PAGE analysis of the purified cellulase. The purified protein after ion exchange chromatography was analyzed on 12% SDS–PAGE (lane 1) with a molecular weight marker (lane M). The arrow points to the band used for mass spectrophotometry.

Table 2
Amino sequence of peptide from trypsin digestion of cellulase enzyme isolated from *A. salina*.

Peptide sequence	Molecular weight (Da)
IKDDR	645.34
SRTLLPR	841.51
RDIELDR	915.48
LSSSFDFSR	1044.5
VTISSTSSTSR	1124.57
RGPSLAHYFR	1202.63
GNTEYKLAVDR	1264.64
MACMLVQDKR	1282.58
MLQQAIQENFR	1376.69
AKSWLAASPCLPK	1427.76
AWIFTNITDQDR	1488.72
SQETIGELLSGGFR	1492.75
LLIMDTMMVQDR	1496.7
ELAILMGSLLDEVTR	1674.89
ENIDALKEVLPSFPR	1726.93
NNINLAIVAMVNYTAIR	1889.02
ISLADWTIASVWAFKSAK	1993.07
ISLADWTIASVWAFKSAK	1993.07
FFKNNFFSNSTESVTSESQR	2355.06

3.3. Effect of temperature on the cellulase activity

The effect of temperature on the purified enzyme was determined after 30 min of incubation at temperatures ranging from 4 °C to 60 °C. The enzyme activity increased as the temperature increased up to 55 °C, and then declined (Fig. 4A). The purified enzyme exhibited the highest activity at 55 °C and the relative activity of the purified enzyme at 4 °C, 10 °C, 20 °C, 25 °C, 30 °C, 40 °C, 50 °C, 55 °C, and 60 °C were 38 ± 6.2, 56 ± 4.0, 62 ± 2.9, 67 ± 4.2, 70 ± 4.1, 77 ± 3.3, 84 ± 3.8, 90 ± 2.8, 100 ± 2.0, and 84 ± 6.3, respectively.

3.4. Effect of pH on the cellulase activity

The effect of pH on the cellulase activity of the purified protein was examined at various pH values ranging from pH 3.0 to pH 12.0.

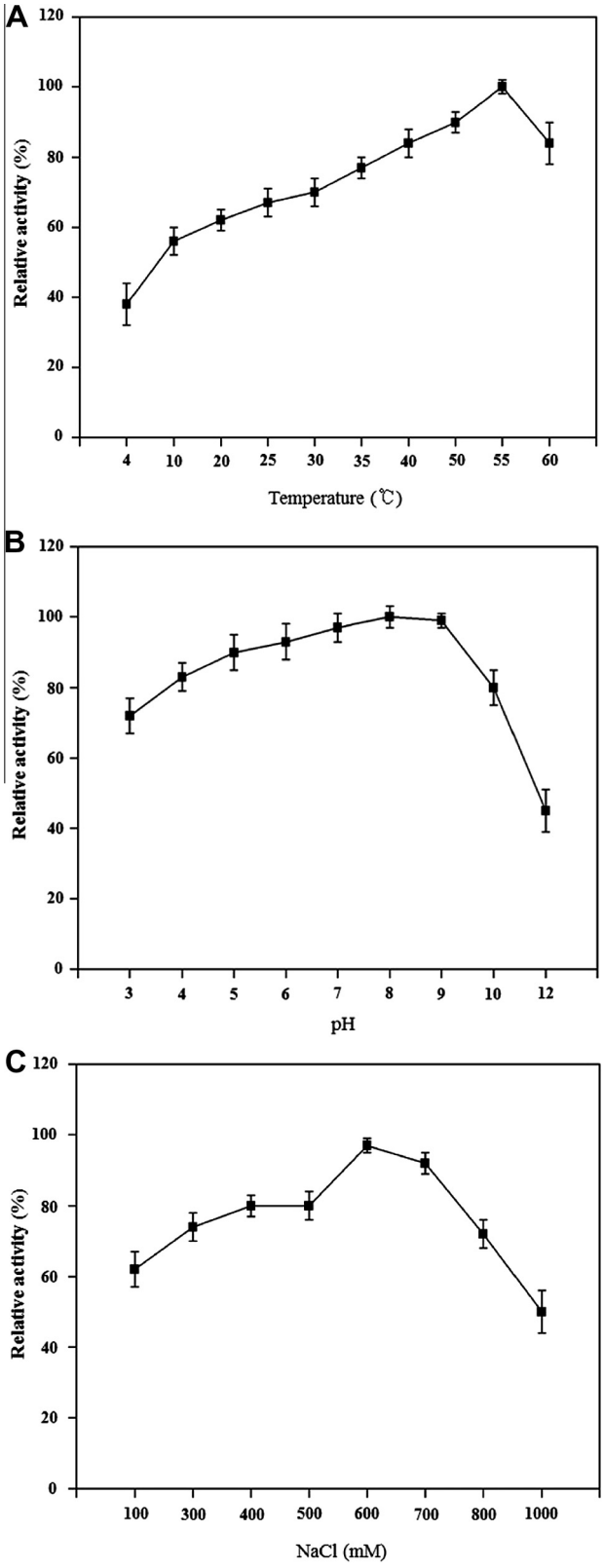

Fig. 4. Effect of temperature (A), pH (B) and salinity (C) on cellulase activity. The activity of purified cellulase was measured at different temperatures, pH and NaCl concentrations using a phosphate buffer (pH 7.0).

The enzyme activity increased as the pH increased to pH 8.0 at which point the activity was the highest and the activity decreased rapidly afterward (Fig. 4B). The relative enzyme activity at pH values of 3, 4, 5, 6, 7, 8, 9, 10, and 12 were 72 ± 4.9, 83 ± 3.8, 90 ± 5.0,

Table 3
Effects of various metal ions on cellulase activity from *A. salina*.

Metal ions (1 mM)	Residual activity (%)
None	100
EDTA	83
$CaCl_2$	124
$CoCl_2$	420
$CuCl_2$	236
$FeSO_4$	88
KCl	94
$MgCl_2$	138
$MnCl_2$	188

93 ± 5.2, 97 ± 4.3, 100 ± 3.1, 99 ± 2.0, 80 ± 5.3, and 45 ± 6.4, respectively. As shown in Fig. 4B, the enzyme showed over 60% activity compared to the highest activity at pH 8.0 at values ranging from pH 3.0 to pH 10.0.

3.5. Effect of salinity on the cellulase activity

The effect of salinity on the cellulase activity of the purified protein was measured at various NaCl concentrations ranging from 0.1 M to 1 M for 30 min at room temperature. The enzyme activity increased in accordance with the increase in NaCl concentration up to 600 mM and sharply declined afterward (Fig. 4C). The relative enzyme activity at the NaCl concentration of 100 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, and 1000 mM was 62 ± 5.6, 74 ± 4.4, 80 ± 3.6, 80 ± 4.5, 100 ± 2.2, 92 ± 3.4, 72 ± 4.5, and 50 ± 6.4, respectively. When the purified enzyme was tested with seawater, the enzyme showed 500% increased activity compared to its 100% activity at 600 mM.

3.6. Effect of metal ions on the cellulase activity

The cellulase activity in the presence of various metal ions of the same concentration (1 mM) was compared in phosphate buffer, and the results are shown in Table 3. The enzyme showed the highest activity in the presence of $CoCl_2$ followed by $CuCl_2$, $MnCl_2$, $MgCl_2$, and $CaCl_2$. In contrast, the presence of KCl, $FeSO_4$, and EDTA showed inhibition of the enzyme activity although the inhibition was less than 20%.

4. Discussion

Microalgae and macroalgae are regarded as new and promising bioenergy sources that can solve the problems such as the scarcity of food and high cost for processing when using the first- and second-generation biofuels [3]. Bioethanol production from algae requires four processing steps: pretreatment, enzymatic hydrolysis, fermentation, and distillation. Of these four steps, we focused on the hydrolysis of cellulosic polymers. Specifically, we looked for a cellulase that can be active under high-salt conditions such as seawater in which macroalgae are cultivated so that a laborious desalting process can be avoided.

Although cellulase activity has been detected from many invertebrates including marine vertebrates, most are believed to be produced from symbiotic microorganisms living in the gut of these animals [6,14]. After initial confirmation of the cellulase activity from the homogenate of *A. salina* (Fig. 1), we tested for possible bacterial contamination. As the initial step, we surface-sterilized the cyst before hatching, and sterile seawater was used for hatching. No colony appeared on the LB and Marine agar plates. For further confirmation, PCR amplification of the bacterial 16S rRNA gene was performed and no PCR product was detected. Although other microorganisms may not have been detected, the data suggested that the e cellulase activity originated from *A. salina*.

Ammonium sulfate fractionation, gel filtration, and ion exchange chromatography were performed to purify the novel cellulase enzyme. Notably, during purification, the protein fractions that contained the cellulase activity showed a high NaCl concentration (Fig. 2). These results are in agreement with the effects of salt concentration on enzyme activity as discussed below. After the last purification, specific activity was 35.1 U/mg, which was a 70-fold increase compared to the crude enzyme before purification (Table 1). The specific activity after final purification was comparable to activity purified from various microorganisms, which ranged from 3.8 U/mg to 71 U/mg [7,8,15].

The SDS–PAGE showed a major protein band with a molecular mass of ~96 kDa and a small protein band with smaller molecular weight, which we ignored considering the relative amount of the major band (Fig. 3). Cellulolytic enzymes include diverse enzymes such as endo-β-1,4-glucanase (EG), exo-β-1,4-cellobiohydrase (CBH), and β-glucosidase that are classified into 78 families in 10 clans [16]. Therefore, the molecular weights of these cellulases are diverse. For example, a thermostable cellulase isolated from blue mussel has a molecular weight of ~20 kDa [17], yet another thermophilic cellulase from *Panenibacillus* sp. strain B39 has a molecular weight of 148 kDa [15]. Further confirmation of the molecular weight of the cellulase purified in this study by amino acid sequencing, or the cloning of the gene encoding this protein, is necessary.

As the molecular weight of cellulases differ, the optimum temperature, pH, and effects of metal ions are also different for each cellulase and usually show a broad range of optimum conditions. When considering cellulases from marine invertebrates, the cellulase isolated from blue mussel showed an optimum pH of 4.6 with over 80% activity in the pH range of 4.0–5.5 [17]. Additionally, the enzyme showed a broad optimum activity in the temperature range of 30–50 °C but its activity dropped rapidly above 50 °C. In another example, the cellulase isolated from abalone showed an optimal temperature and pH of 38 °C and 6.3, respectively [18]. The cellulase purified in this study also showed a broad optimum activity in pH and temperature but with a higher pH (pH 8.0) and temperature (55 °C) at the highest enzyme activity (Fig. 4A and B). Therefore, the broad optimal range of temperature and pH of this cellulase is not unusual.

The effect of NaCl concentration on the enzyme activity is an interesting characteristic. As shown in Fig. 4C, the activity increased up to 600 mM NaCl and decreased afterward. When this enzyme was tested in seawater, the activity increased fivefold, compared to the activity at 600 mM NaCl. The NaCl concentration in seawater is typically 3.5%, equivalent to 598 mM. Therefore, the highest activity at 600 mM NaCl concentration is consistent with the NaCl concentration in seawater. The activity increase in seawater compared to pure NaCl can be explained by the presence of other metal ions in seawater. As shown in Table 2, the presence of metal ions such as $CoCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, and $CaCl_2$ increased the enzyme activity. Compared to the metal concentration used in this experiment (1 mM), the concentrations of metal ions are much lower. For example, the concentration of Co in the North Pacific was 4–50 pM on the surface and 10–20 pM in deep water and that of Cu was 0.5–1.3 nM on the surface and 4.5 nM in deep water [19]. Although the effect of each metal ion at different concentrations was not determined, the higher enzyme activity in seawater could have resulted from the additional effects of NaCl and metal ions present in seawater. The high activity of this enzyme in seawater is an important characteristic because this enzyme can be used for the hydrolysis of biomass from marine cultures without the desalting process.

For the confirmation of the purified protein as a cellulase and future application of the information for cloning this gene for the production of recombinant protein, we analyzed the purified

protein using mass spectrometry. Although over 20 peptide sequences were obtained, none matched any previously reported cellulase sequence. Although further confirmation by cloning the gene is necessary, this enzyme appears to be a novel cellulase with high activity in seawater, which could be applicable in bioethanol production from marine macroalgae without the desalting process of the raw materials.

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