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Article

Partial Purification and Characterization of Halotolerant Alkaline Protease from *Halomonas marisflava* KCCM 10457 Isolated from Salt-fermented Food

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Halotolerant protease produced by *Halomonas marisflava* KCCM 10457 was partially purified through ammonium sulfate precipitation and Sephacryl S-200HR gel permeation chromatography. Optimal pH and temperature of protease were 11.0 and 45°C, respectively. Enzyme activity was inhibited by Cu^{2+}, Hg^{2+}, Fe^{2+}, and Fe^{3+}, and selectively inhibited by *p*-chloromercuribenzoic acid (PCMB), suggesting this enzyme is cysteine protease. The enzyme is halotolerant, because it retained 77% of original activity in presence of 3.33 M NaCl. The protease showed broad substrate specificity to various natural proteins; BSA, casein, egg albumin, gelatin, and hemoglobin.

Key words: *halotolerant, alkaline protease, broad substrate specificity,* Halomonas marisflava

Proteases are the most important kind of enzymes from an industrial viewpoint. Alkaline proteases are particularly important to the detergent industry, leather processing, pharmaceuticals, and production of protein hydrolysates,[1,2] with a large proportion of commercial alkaline proteases mainly derived from *Bacillus* species. Recently, various microbial strains producing alkaline protease were isolated.[3-5] Most investigators have concentrated on obtaining thermostable alkaline protease rather than halotolerant protease due to the versatility of thermostable alkaline protease, although halotolerant proteases have advantages of proteolytic hydrolysis acceleration and consequent decrease in the production time of oriental salt-fermented foods such as soy, anchovy, shrimp, and sand lance sauces. Nevertheless, only a few reports are available on the saline-tolerant protease from microbial sources.[6-8] In addition, the mechanism of protease hydrolysis of soy and fish proteins at high NaCl concentration remains unclear. Therefore, it is meaningful to isolate microorganisms that produce protease with superior NaCl tolerance. In our previous study, we isolated a moderately halophilic bacteria *Halomonas marisflava* KCCM 10457 strain that produced an extracellular salt-tolerant protease and optimized the medium conditions for the enzyme production.[9] Remarkably, the crude salt-tolerant protease retained about 80% of its original activity in the 2.1 M NaCl reaction system.[9] The objective of the present investigation was to evaluate the properties of salt-tolerant protease.

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Materials and Methods

Fermentation and partial purification of enzyme. *H. marisflava* KCCM 10457 was grown aerobically in a suspension culture medium as previously described.[9] After 48 h fermentation, the culture broth was centrifuged at 3,000 × g for 15 min at 4°C. The supernatant was precipitated by ammonium sulfate salting-out (30~80% saturation), and the enzyme precipitate obtained was centrifuged at 12,000 × g for 15 min at 4°C. The resulting pellet was dissolved in a small amount of 50 mM glycine-NaOH buffer (pH 11.0), and the enzyme solution was loaded for gel permeation chromatography using a Sephacryl S-200HR column (2 × 140 cm) equilibrated with the same buffer. The column was developed at 36 m*l*/h. The fractions containing protease were pooled and concentrated using membrane (Amicon Ultra-15, MWCO 10,000). The concentrated enzyme was used for further characterization studies. Protease activity was assayed according to a previously established method.[9] One unit of protease activity was equivalent to the amount of enzyme required to liberate 1 μg of tyrosine per 10 min under the assay conditions.

Reaction properties of partially purified protease. The optimum pH of alkaline protease was determined using 1% casein as a substrate dissolved in different buffers (acetate, pH 5.0; phosphate, pH 6.0~7.0; Tris-HCl, pH 8.0~9.0; glycine-NaOH, pH 10.0~12.0 at 40°C). The optimum temperature of protease activity was determined using a reaction mixture incubated at different temperatures (25 to 60°C). For determination of halo-tolerance, NaCl was added to the enzyme reaction mixture at various concentrations (0 to 3.33 M) for measurement of the protease activity. To determine the inhibitory activity of protease, the enzyme was pre-incubated with 1 mM each PCMB and phenylmethanesulfonyl fluoride (PMSF), and 5

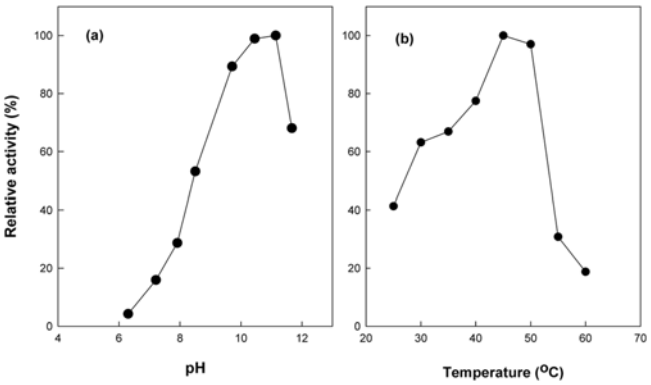

Fig. 1. Effects of pH (panel a) and temperature (panel b) on the activity of *H. marisflava* KCCM 10457 protease.

mM EDTA in 50 mM glycine-NaOH buffer (pH 11.0) at 40°C for 1 h. The effect of various metal ions at 10 mM was studied by pre-incubating with protease enzyme for 1 h prior to measuring the enzyme activities. To determine the proteolytic activity of protease for natural proteins, enzyme activity was measured using different protein substrates (BSA, casein, egg albumin, gelatin, and hemoglobin).

Results and Discussion

Effect of pH and temperature on protease activity. The extracellular protease from *H. marisflava* KCCM 10457 was partially purified at 30~80% $(NH_4)_2SO_4$ saturation through Sephacryl S-200HR gel permeation chromatography, resulting in a yield of 24.3% and a purification fold of 11.0. The partially purified protease was active in the narrow alkaline range with an optimum pH of 11.0 and showed a drastic decrease of activity at below pH 10 (Fig. 1(a)). The proteolytic activity increased progressively with temperature, reaching maximum at 45°C (Fig. 1(b)), then significantly decreased at above 50°C.

Effect of inhibitors and metal ions on protease activity. Effects of specific inhibitors on the protease activity are shown in Table 1. The enzyme was resistant to inhibition by chelating reagent (EDTA). PCMB was the most potent inhibitor, and PMSF inhibited the enzyme activity by 35%. PCMB strongly inhibited the cysteine residue at or near the active site, causing a complete inhibition of the enzyme activity, which suggests that the enzyme is a cysteine protease. The activity was measured by incubating the enzyme with various metal ions at 10 mM. Mg^{2+} and Ni^{2+} ions enhanced the enzyme activity by 60 and 18%, respectively (Table 1). Notably, the enzyme-inactivating cations were either sulfhydryl-reactive metal ions (Hg^{2+}) or redox-active metal ions, such as those of iron and copper; the most remarkable inhibition was observed in the presence of Cu^{2+}, Fe^{2+}, Fe^{3+}, and Hg^{2+} ions.

Aside from the alkaline optimum pH (pH 11.0) and moderately low optimum temperature (45°C), the effect of protease inhibitors on the enzyme activity also differed from those of the reported proteases with different halotolerant or halophilic properties. In general, proteases are classified to four families according to functional amino acid residues arranged in a particular configuration to form the active site; serine protease, cysteine protease, aspartic protease, and metalloprotease. Because the proteases from halophilic bacteria such as *Halobacterium halobium*[10,11] and *H. mediterranei*,[12] and halotolerant bacteria such as *B. pumilus*,[6] *Bacillus* sp.,[7,13] and *B. licheniformis*[14] were strongly inhibited by PMSF, these proteases are classified as serine proteases. However, activity of protease from *H. marisflava* KCCM 10457 is significantly inhibited by PCMB and moderately by PMSF. In the case of cysteine, its presence at a certain critical site is corroborated by the significant inhibitory effect of Hg^{2+} ion such as β-galactosidase from *Bacillus* sp.[15] In this respect, it is tempting to speculate that protease from KCCM 10457 may belong to the cysteine protease family.

Table 1. Effects of various inhibitors and metal ions on the activity of alkaline protease from *H. marisflava* KCCM 10457

Inhibitors/metal ions	Concentration (mM)	Relative activity (%)
Control		100.0
EDTA	5.0	85.1
PCMB	1.0	3.7
PMSF	1.0	64.3
$CaCl_2$	10.0	101.0
$CoCl_2$	10.0	97.0
$CuCl_2$	10.0	0
$FeCl_2$	10.0	26.3
$FeCl_3$	10.0	23.2
$HgCl_2$	10.0	0
$MgCl_2$	10.0	159.6
$MnCl_2$	10.0	89.2
$NiCl_2$	10.0	118.2
$ZnCl_2$	10.0	47.2

Effect of NaCl concentration on protease activity. Figure 2 shows the effect of sodium chloride on the protease activity. The enzyme activity retained 77% of its original level in the presence of 3.33 M NaCl (19% NaCl). Salt-tolerance characteristics of proteases are of great interest in the production of salt-fermented foods. However, only few reports have been published on the salt-tolerant protease except for some from halophiles, archaebacteria, and fungi.[6-8] To the best of our knowledge, there have been no previous reports on salt-tolerant protease from *H. marisflava*. Although studies showed that the activities of some proteases from halophilic bacteria increased with increasing NaCl concentration over the range 2-5 M,[10-12] in our study, activity of protease from *H. marisflava* KCCM 10457 slightly decreased with increasing NaCl concentration, suggesting that the extracellular protease produced from *H. marisflava* KCCM 10457 was halotolerant alkaline protease.

Substrate specificity of protease. To further assess the

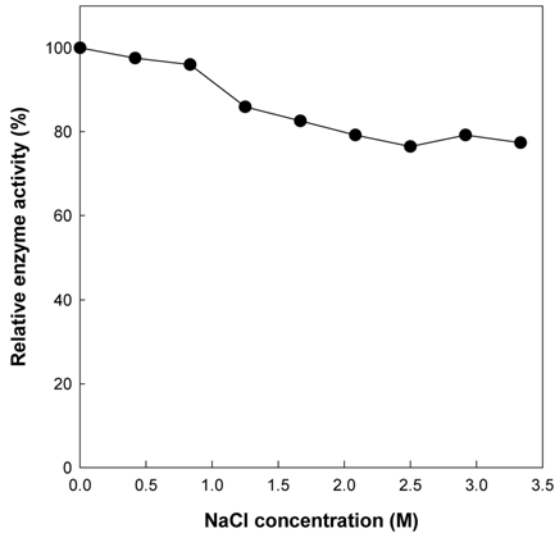

Fig. 2. Effect of sodium chloride on the activity of *H. marisflava* KCCM 10457 protease. The enzyme was assayed in the presence of sodium chloride (0 to 3.3 M).

Table 2. Effects of various protein substrates on alkaline protease activity

Proteins (1.7 mg/m*l*)	Activity (U/m*l*)	Relative activity (%)
Casein	3.38	100
BSA	6.92	205
Egg albumin	8.38	248
Gelatin	13.79	408
Hemoglobin	2.17	64

application of protease from *H. marisflava* KCCM 10457 for other uses, an experiment was carried out using various proteins as substrates (Table 2). Results showed that the protease was able to hydrolyze all tested proteins, although at different extents. Gelatin was found to be the most preferred substrate (13.8 U/m*l*), followed by egg albumin (8.4 U/m*l*), BSA (6.92 U/m*l*), casein (3.38 U/m*l*), and hemoglobin (2.17 U/m*l*). Broad substrate specificity and salt-tolerance are important properties when considering the application of alkaline protease as a detergent ingredient and starter in salt-fermented foods. It should be noted that the enzyme of our preparation exhibited the highest activity towards gelatin; in contrast to the reports that majority of alkaline protease preferred casein as the substrate.[16-18] The broader specificity of the alkaline protease from *H. marisflava* implies that it may be advantageous over a wide variety of stains for use in detergents.

Only few reports have been made on the extracellular protease of *Halomonas* species.[19,20] The present results on the enzyme properties demonstrate that the extracellular protease from *H. marisflava* KCCM 10457 is significantly different from the already known proteases from other halotolerant microorganisms in many aspects (Table 3). Because *H. marisflava* KCCM 10457 can secrete an alkaline salt-tolerant protease, its protease may find potential applications in the detergent industry and for use to improve the manufacture of oriental salt-fermented foods. Further comparative studies on cloning and sequencing of its gene may assist in the identification and production of this salt-tolerant protease.

Acknowledgements

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Table 3. Comparison of bacterial halotolerant protease properties

Strains	Opt. pH	Opt. temp. (°C)	Molecular mass (kDa)	Chemical inhibitor	Salt tolerance (Remaining activity)	Reference
Bacillus subtilis NCIM No.64	9.7	72	28	PMSF	100% at 5 M NaCl	7
Bacillus pumilus JB05	10.5	58	-[a]	PMSF	70% at 2.5 M NaCl	6
Bacillus sp. JB-99	11.0	70	-	PMSF	65% at 5 M NaCl	13
Halomonas sp. ES10	11.0	35	-	EDTA	65% at 1 M NaCl	18
Halomonas marisflava KCCM 10457	11.0	45	-	PCMB	77% at 3.3 M NaCl	this work

[a]Data not available.

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