A Halophilic Serine Proteinase from *Halobacillus* sp. SR5-3 Isolated from Fish Sauce: Purification and Characterization

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A halophilic bacterium was isolated from fish sauce, classified, and named *Halobacillus* sp. SR5-3. A purified 43-kDa proteinase produced by this bacterium showed optimal activity at 50 °C and pH 9–10 in 20% NaCl. The activity of the enzyme was enhanced about 2.5-fold by the addition of 20–35% NaCl, and the enzyme was highly stabilized by NaCl. It was found to be a serine proteinase related to either chymotrypsin or subtilisin. It absolutely preferred Ile at the P2 position of substrates. Thus, the enzyme was found to be a halophilic serine proteinase with unique substrate specificity.

Key words: moderately halophilic bacteria; *Halobacillus* sp.; serine proteinase; fish sauce; fluorescence resonance energy transfer substrate (FRETS)

Extremely and moderately halophilic bacteria dominate in saline environments (0.5% to saturated NaCl). They have developed an efficient metabolism for the utilization of proteins and amino acids.1) Proteinases from extremely halophilic bacteria have been studied and characterized.1–9) On the other hand, few extracellular proteinases from moderately halophilic bacteria have been characterized in detail. Among these that have been characterized are proteinases from *Pseudomonas* sp. A-14,10) *Pseudoalteromonas* sp. CP76,11) and *Salinivibrio costicola* 18AG.12)

Obtained under conditions of high-salt environment, Thai fish sauce (nam-pla) is one of many condiments prepared from fish in concentrated brine. This clear, brown, salty liquor contains peptides, amino acids, calcium, and vitamin B. Fish proteins are digested by the action of microorganisms and fish proteinases.13) An example of the former is a serine proteinase purified from a moderately halophilic bacterium, *Filobacillus* sp. RF2-5.14) Fish sauce contains a variety of moderately halophilic bacteria producing proteinases. These proteinases from moderately halophilic bacteria are applicable in producing fish sauce in a shorter time, and in other food production.

In this study, we screened fish sauces made in Thailand for moderately halophilic bacteria producing proteinases, in order to find unique enzymes that can be applied for purposes such as improvement in fish sauce production. One of the isolates was classified and named *Halobacillus* sp. SR5-3, and its halophilic serine proteinase was purified and characterized.

Materials and Methods

Chemicals. Bacitracin was purchased from Wako Pure Chemical Industries (Osaka, Japan). NHS-activated Sepharose 4 Fast Flow was purchased from Amersham Biosciences (Tokyo, Japan), Hammerstein casein was from Merck (Damstadt, Germany), a bincinchoninic acid (BCA) Protein Assay Kit from Pierce Chemical (Rockford, USA), and Suc-Ala-Ala-Pro-Phe-MCA was from the Peptide Institute (Osaka, Japan). All other reagents were of guaranteed grade.

Screening for proteinase-producing halophilic bacteria and its culture condition. The fish sauce samples were plated on JCM no. 377 medium (10% [w/v] NaCl, 0.5% [w/v] casamino acids, 0.5% [w/v] yeast extract, 0.2% [w/v] KCl, 0.3% [w/v] trisodium citrate, 2% [w/v] MgSO4•7H2O, 0.036% [w/v] FeCl2•4H2O, 0.0036% [w/v] MnCl2•4H2O, 2% [w/v] agar,

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; MAPI, a-microbial alkaline proteinase inhibitor; FRETS, fluorescence resonance energy transfer substrate; Nma, 2-(N-methylamino)benzoyl; D-A2pr, D-2,3-diamino propionic acid; Dnp, 2,4-dinitrophenyl
pH 7.2) containing 1% skim milk, and incubated at 37 °C for 7 d. Optimization of proteinase production was tested by adding a variety of nitrogen sources and carbon sources such as ami ami, casamino acid, yeast extract, soybean powder, lactose, starch, and sucrose. After glutamic acid fermentation, the byproduct, containing 4% of glutamic acid, was referred to ami ami. Samples were obtained periodically and their proteinase activities were assayed using casein as substrate in the presence and in the absence of 10% NaCl. The phenotypic characteristics and 16S rRNA gene sequence were determined as previously described.\textsuperscript{15–17)}

Enzyme purification. The selected isolate, SR5-3, was cultivated in modified JCM no. 377 medium, which contained 1% (w/v) yeast extract, 0.32% (w/v) aspartic acid, 0.32% (w/v) glycine, and 2% (v/v) ami ami as carbon and nitrogen sources, pH 7.2 at 30 °C. Culture broth after 48 h cultivation was centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was used for the subsequent enzyme purification procedures.

Step 1. Ammonium sulfate precipitation. Solid ammonium sulfate was added into 2,700 ml of the supernatant to make an 80% saturated final concentration (561.1 g/l), and kept overnight at 4 °C. The precipitate was collected by centrifugation (8,000 rpm for 20 min at 4 °C). Two hundred ml of 50 mM Tris–HCl buffer, pH 8.0 (buffer A) was added to dissolve the precipitate.

Step 2. Bacitracin-Sepharose column chromatography. A Bacitracin-Sepharose column was prepared by the method of Stepanov and Budenskaya.\textsuperscript{18)} The Bacitracin-Sepharose 4 Fast Flow was packed in the column (2 × 20 cm) with a bed volume of 50 ml. The column was equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 4 M NaCl and 1 mM CaCl\textsubscript{2} (buffer B). The sample diluted with buffer B was loaded onto the column, and then the column was washed with buffer B. The proteinases were eluted by a stepwise method with buffer B containing 15% (v/v) and 20% (v/v) ethanol. The purity of the purified enzyme was analyzed by 12.5% SDS–PAGE after the sample was boiled with 2% SDS and 2-mercaptoethanol for 3 min. It was also analyzed by native PAGE (16% PAGE). Proteinase activity was detected by zymogram, as previously reported.\textsuperscript{11)} In the case of the zymogram using casein as a substrate, the enzyme was mixed with SDS, but not boiled, and then subjected to casein-zymogram. After electrophoresis, the gel was rinsed in 2.5% Triton X-100 in order to remove SDS for 30 min, and then incubated in 25 mM Tris–HCl, pH 7.5. After incubation for 1 h at 37 °C, the gels were stained with amido black.

Analysis of molecular mass and amino-terminal sequence. The analyses were carried out according to the method described in previous papers.\textsuperscript{14,19)}

Assay of proteinase activity and protein concentration. Proteinase activity was assayed at 37 °C for 30 min by the casein Folin-Ciocalteau method described previously\textsuperscript{20)} in the presence of 10% NaCl. One unit of enzyme activity was defined as the enzyme quantity that liberates 1 μg of tyrosine per ml of the reaction mixture per min.\textsuperscript{14)} In the case of the MCA-peptide method,\textsuperscript{22) the peptidase activity was assayed in the presence of the optimal NaCl concentration of 20% NaCl. Fifty μl of diluted enzyme and 440 μl of 25 mM Tris–HCl buffer containing 20% NaCl, pH 7.5, were preincubated at 37 °C for 30 min. After preincubation, the reaction was started by adding 10 μl of 98 μM of MCA-peptide substrate, and incubated at 37 °C for 20 min. The enzyme reaction was terminated by the addition of 2 ml of 15% (v/v) acetic acid. The fluorescence intensity was measured at λex = 360 nm and λem = 460 nm. One relative fluorescence unit of enzyme activity was defined as the enzyme quantity that liberates 1 μ mol of AMC per ml of the reaction mixture per min. Protein concentration was estimated by BCA protein assay\textsuperscript{21)} with crystalline bovine serum albumin (Sigma-Aldrich, St. Louis, USA) as a standard.

Characteristics of the proteinase. Optimum pH and temperature. The effect of pH on the purified enzyme activity was measured at various pH values at 37 °C for 20 min. The effect of temperature was measured at different temperatures at pH 7.5 for 20 min. For these assays, MCA-peptide was used as a substrate.

pH and thermal stability. pH stability at various pHs (pH 6.0–12.0, 37 °C, 3 h) and thermal stability (30–70 °C, pH 7.5, 2 h) were studied.

Effect of NaCl on activity and stability. The activity of the purified enzyme was measured at various NaCl concentrations at pH 7.5 using casein or Suc-Ala-Ala-Pro-Phe-MCA as a substrate. For the effect of NaCl on stability, the purified enzyme was incubated at various concentrations of NaCl at 37 °C at pH 7.5 for 24 h. Then the remaining activity was measured at pH 7.5 using casein or Suc-Ala-Ala-Pro-Phe-MCA as a substrate. For these assays, the NaCl concentration was adjusted to 10% (w/v) for casein as substrate and to 20% for MCA-peptide as substrate.

Effects of proteinase inhibitors on activity. The effects of various proteinase inhibitors on proteinase activity were examined. After preincubuation of the purified enzyme with inhibitors (PMSF, EDTA, EGTA, TLCK, TPCK, E-64, leupeptin, chymostatin, and MAPI) at 37 °C for 30 min, the residual activity was assayed using MCA-peptide substrate.

Analysis of substrate specificity using FRETS-25Xaa-libraries. FRETS combinatorial libraries were used for the analysis of substrate specificity. The details are described in previous reports.\textsuperscript{14,22,23)}
Results and Discussion

Screening for proteinase-producing strains from Thai fish sauce

A total of 50 bacterial strains were isolated from a fish sauce made in Thailand using the spread plate technique on JCM no. 377 medium containing 1% skim milk and 10% NaCl. Of the strains, 10 strains exhibited caseinolytic activity on the plate. The SR5-3 strain showed the highest activity on the plate containing 10% NaCl, and it was selected for further study.

Classification of the SR5-3 strain

The SR5-3 strain was an aerobic Gram-positive rod-shaped bacterium with spherical or ellipsoidal endospores. Colonies of the strain were white-cream pigmented and showed positive reactions to hydrolysis of casein, gelatin, tyrosine, and DNA. Acid was produced from L-arabinose, D-fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, trehalose, and xylose. Indole formation, nitrate reduction, MR-VP, urease, utilization of starch, and arginine were negative. The SR5-3 strain grew at up to 30% NaCl, and its optimal growth was at 10% NaCl.

The 16S rDNA sequence similarity of the SR5-3 strain (DDBJ accession no. AB185150) was 97.1% to *Halobacillus trueperi* DSM 10404T, 97.0% to *Halobacillus karajensis* DSM 14948T, and 96.8% to *Halobacillus litoralis* SL-4T. Phylogenetic analysis revealed that this strain belonged to genus *Halobacillus* (data not shown). It showed differences from the closest strain, *Halobacillus trueperi* DSM 10404T, in pigmentation, acids from mannitol and xylose, and hydrolysis of casein and urease. Based on these results, it is assumed that the SR5-3 strain is a new species of *Halobacillus*.24)

Production of the proteinase

Production of proteinase of the SR5-3 strain was enhanced 100-fold by the addition of aspartic acid, glutamic acid, and glycin into the JCM no. 377 medium. This result is similar to a report on the activity of a halophilic proteinase from *Halobacterium halobium*.4) Proteinase activity in the culture broth of the SR5-3 strain reached its maximum at late exponential growth phase (1.17 U/ml, substrate: casein), but its productivity gradually decreased at stationary phase.

Our preliminary experiments showed that the SR5-3 strain produces two kinds of proteinases. One of them is a non-halophilic proteinase (peak A), and the other is a halophilic proteinase (peak B) (data not shown). More details will be provided later.

Purification of the proteinase

In the elution profile of the bacitracin-affinity column chromatography, peak A showed specific activity of 13.3 U/mg protein for casein substrate and 0.7 U/mg protein for MCA-peptide substrate in the presence of 10% NaCl, whereas the corresponding values for Peak B were 163 U/mg protein for casein substrate and 82 U/mg protein for MCA-peptide as a substrate in the presence of 10% NaCl. We are not aware of any previous reports showing that moderately halophilic bacteria produce two such types of proteinases. We focused this study on the halophilic proteinase (peak B) that might be involved in fish sauce production. The purification of the halophilic proteinase is summarized in Table 1. From a 2.7 L culture, the proteinase was purified 270-fold, with the final yield of 39%. SDS-PAGE analysis of the enzyme revealed a single band of 43 kDa (Fig. 1A). The purified proteinase mixed with SDS but not boiled was subjected to casein-zymogram to detect proteolytic activity. After electrophoresis of the enzyme in SDS–polyacrylamide gels containing copolymerized casein, SDS was removed and proteinase activity was detected (Fig. 1B).

Amino-terminal sequence analysis

The N-terminal amino acid sequence of the enzyme was determined to be


The DDBJ databases revealed that the most similar enzyme is a 36.9 kDa-serine proteinase from *Bacillus* sp. Ak.1, with 87% identity.25)

Characteristics of the proteinase

The halophilic proteinase from the SR5-3 strain showed maximal activity at pH 10.0 at 50 °C (Fig. 2A, C). The enzyme was stable in a broad pH range from pH 5.0 to 8.0 (Fig. 2B), and was also stable up to 50 °C (Fig. 2D, closed triangles). The addition of CaCl$_2$ to the enzyme solution enhanced stability up to 60 °C (Fig. 2D, closed circles).

In order to determine the type of proteinase, various proteinase inhibitors were used: serine proteinase inhibitors, PMSF, TLCK, chymostatin (carboxy termi-
nal = phenylalaninal), leupeptin (carboxy terminal = argininal), and α-MAPI (carboxy terminal = phenylalaninal); a cysteine proteinase inhibitor, E-64; and a metalloproteinase inhibitor, EDTA-2Na. Among these, PMSF, chymostatin, and MAPI were found to inhibit proteinase activity almost completely, while leupeptin and E-64 showed 10–15% inhibition (Table 2). Therefore, the proteinase from the SR5-3 was identified as a serine proteinase. Among the serine proteinases, it was assumed to be a chymotrypsin-type or a subtilisin-type, but not a trypsin-type.

The effects of NaCl on the enzyme activity were analyzed at various concentrations of NaCl (0–30%) using N-succinyl-Ala-Ala-Pro-Phe-MCA or casein as a substrate. Peptidase activity increased about 2.5-fold in the presence of 20–35% NaCl as compared with that of a control (Fig. 3A). In the case of casein as a substrate, proteinase activity was suppressed in the presence of a high content of NaCl (Fig. 3A). One of the reasons the enzyme activity against casein decreased at higher salt concentrations is that casein becomes less hydrophobic.

**Fig. 1.** SDS-Polyacrylamide Gel Electrophoresis of the Purified Enzyme.

12.5% polyacrylamide gel was used for analysis. A, Stained with Coomassie brilliant blue R250: lane 1, purified proteinase after Bacitracin-Sepharose column; lane 2, Marker: bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and soybean trypsin inhibitor (21.5 kDa). B, Zymogram: lane 1, purified proteinase after Bacitracin-Sepharose column.

**Fig. 2.** Effect of pH on Activity (A) and Stability (B), and Effect of Temperature on Activity (C) and Stability (D).

In the presence of 20% NaCl, Suc-Ala-Ala-Pro-Phe-MCA assay was used for characterization of the proteinase. A. The buffers used were: (△) 25 mM KH$_2$PO$_4$/NaOH (pH 6.0–8.0); (●) 25 mM H$_3$BO$_3$/NaOH (pH 8.0–10.0); (●) 25 mM NaHCO$_3$/NaOH (pH 10.0–11.0); and (■) 25 mM Na$_2$HPO$_4$/NaOH (pH 11.0–12.0). Maximal peptidase activity was shown as 100%. Enzyme assay was carried out at 37°C for 20 min. B, The pH of the purified proteinase was adjusted at various pHs by: (△) 25 mM citrate–NaOH (pH 4.0–6.0); (●) 25 mM KH$_2$PO$_4$/NaOH (pH 6.0–8.0); and (■) 25 mM H$_3$BO$_3$/NaOH (pH 8.0–10.0) buffer. The enzyme was incubated at 37°C for 3 h, then residual peptidase activity was measured at pH 7.5 for 20 min. C, Peptidase activity was measured at 30–70°C at pH 7.5 for 20 min. D, After heat treatment at various temperatures (30–70°C) at pH 7.5 for 2 h; (●) with 2 mM CaCl$_2$, and (△) without CaCl$_2$ at pH 7.5, the remaining activity was measured at pH 7.5 for 20 min.
The purified enzyme was incubated at various concentrations of NaCl at 37°C or Suc-Ala-Ala-Pro-Phe-MCA as a substrate at pH 7.5. The relative proteinase/peptidase activity was measured using casein (■) or Suc-Ala-Ala-Pro-Phe-MCA (▲) as a substrate at pH 7.5. The relative proteinase/peptidase activity was defined as the percentage of activity with respect to the maximum proteinase/peptidase activity detected in the assay used.

### Table 2. Effects of Inhibitors on the Activity of Halophilic Proteinase

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Final conc.</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>PMSF</td>
<td>1 mM</td>
<td>99</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>100 μM</td>
<td>93</td>
</tr>
<tr>
<td>α-MAPI</td>
<td>50 μM</td>
<td>87</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 mM</td>
<td>13</td>
</tr>
<tr>
<td>TLCK¹</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>E-64</td>
<td>10 μM</td>
<td>15</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>10 μM</td>
<td>0</td>
</tr>
</tbody>
</table>

¹TLCK, L-1-chloro-3-[4-tosylamido]-7-aminoo-2-hepanone HCl

and the original conformation or folding was lost, making the substrate unavailable as a target for proteolytic activity. The stability of the proteinase increased drastically in the presence of 20–35% of NaCl, but was completely lost under conditions of low concentrations of NaCl (Fig. 3B). Thus, these data indicate that the 43 kDa-proteinase from Halobacillus sp. SR5-3 is a halophilic proteinase, or is salt-activated.

**Substrate specificity of the proteinase**

A novel type of fluorescence energy transfer (FRET) combinatorial library, which has been proven to be a sensitive and reliable substrate for determination of the substrate specificity of proteinases, was used in order to determine the substrate specificity of the purified proteinase from Halobacillus sp. SR5-3. The proteinase was found to prefer Leu, Gln, and Ala at the P₁ position (Fig. 4A), and Ile at the P₂ position of the peptide substrates. As for the preference at the P₁ position, the proteinase preferred Arg->Ala->Phe->Val->Glu in FRETS-25Leu, and Phe->Arg->Val->Ala->Glu in FREFS-25Gln of substrates (Fig. 4B, C).

One of the unique points in the substrate specificity of the proteinase is that, without exception, it preferred Ile at the P₂ position. This suggests to us that the S₂ subsite of the enzyme might be large and hydrophobic in nature. Another point is that the proteinase preferred Met residue at the P₁ position of the substrate as well as Leu, Gln, and Ala. There are few reports on serine proteinases that prefer Met at the P₁ position.

Similar substrate specificity with a slightly different preference at the P₁ (Phe, Met, and Thr) was observed in the halophilic proteinase from Filobacillus sp. RF2-5 isolated by us from Thai fish sauce.

The enzymatic properties of SR5-3 proteinase were compared with a 36.9 kDa-serine proteinase from Bacillus sp. Ak.1, which showed the highest identity in the primary sequence among the proteinases reported so far. Some differences were observed in their properties. For example, the optimal pH and temperature of the SR5-3 proteinase were pH 10.0 and 50°C using MCA-substrate in the presence of 20% NaCl and without Ca²⁺ ion, while the optimal pH and temperature of the proteinase from Ak.1 were pH 7.5 and 75°C in the presence of 5 mM Ca²⁺. The SR5-3 proteinase almost lost its activity after 2 h at 70°C in the presence of 2 mM Ca²⁺ and 20% NaCl, whereas the proteinase from the Ak.1 retained 50% of initial activity after 40 h at 75°C in the presence of 5 mM Ca²⁺. SR5-3 proteinase was less thermostable than Ak.1 proteinase.

Thus we succeeded in purifying and characterizing a unique proteinase from a moderately halophilic Halobacillus sp. SR5-3 isolated from fish sauce made in Thailand. The uniqueness of the enzyme is: (1) its activity was enhanced about 2.5-fold by the addition of 20–35% NaCl, and it was highly stabilized by NaCl, and (2) it preferred Ile at the P₂ positions of substrates.

The proteinase described here, as well as 49 kDa-proteinase from Filobacillus sp. RF2-5, might be involved in the degradation of fish protein during fermentation at high salt concentrations. In the future,
the fermentation period of fish sauce, which is usually about one year, might be reduced by the addition of moderately halophilic proteinases from bacteria such as *Halobacillus* sp. SR5-3 and *Filobacillus* sp. RF2-5, and extremely halophilic proteinases.

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