Purification and Characterization of Serine Proteinase from a Halophilic Bacterium, *Filobacillus* sp. RF2-5

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In order to find a unique proteinase, proteinase-producing bacteria were screened from fish sauce in Thailand. An isolated moderately halophilic bacterium was classified and named *Filobacillus* sp. RF2-5. The molecular weight of the purified enzyme was estimated to be 49 kDa. The enzyme showed the highest activity at 60°C and pH 10–11 under 10% NaCl, and was highly stable in the presence of about 25% NaCl. The activity was strongly inhibited by phenylmethane sulfonyl fluoride (PMSF), chymostatin, and C11-microbial alkaline proteinase inhibitor (MAPI). Proteinase activity was activated about 2-fold and 2.5-fold by the addition of 5% and 15–25% NaCl respectively using Suc–Ala–Ala–Pro–Phe–pNA as a substrate. The N-terminal 15 amino acid sequence of the purified enzyme showed about 67% identity to that of serine proteinase from *Bacillus subtilis* 168 and *Bacillus subtilis* (natto). The proteinase was found to prefer Phe, Met, and Thr at the P₁ position, and Ile at the P₂ position of peptide substrates, respectively. This is the first serine proteinase with a moderately thermophilic, NaCl-stable, and NaCl-activatable, and that has a unique substrate specificity at the P₂ position of substrates from moderately halophilic bacteria, *Filobacillus* sp.

**Key words:** halophilic bacterium; *Filobacillus* sp.; serine proteinase; fluorescence resonance energy transfer substrate (FRETS)

Many proteinases from mesophilic microorganisms have been purified and characterized, but proteinases from moderately halophilic microorganisms that are able to grow optimally between 0.5 and 2.5 M salt¹ have not been extensively studied. Some previous studies on proteinases from moderately halophilic bacteria are as follows: Duong Van Qua et al. (1981)² and C. Sanchez-Porro et al. (2003).³

Fish sauce is fermented from fish in concentrated brine (4.4–5.1 M NaCl). Degradation of fish protein is accomplished by the action of both bacterial proteinases and endogenous fish enzymes. Hence, fish sauce is a good screening source for isolation of halophilic bacteria that show proteinase activity. Such halophilic bacteria and proteinases are used to produce fish sauce in a shorter time, and they can be applied in other food production. There are several previous studies on proteinases from bacteria isolated from fish sauce,⁴,⁵ but few proteinases from moderately halophilic bacteria have been purified and studied in depth so far.⁵ Hence, there are possibilities to find proteinases with unique characteristics from fish sauce.

In this study, in order to find a unique proteinase and to apply it in fish sauce production, we conducted screening for proteinase-producing moderately halophilic microorganisms from fish sauces in Thailand. One of the isolates was named *Filobacillus* sp. RF2-5. It’s serine proteinase was purified and characterized.

**Materials and Methods**

**Materials.** DEAE-Sepharose CL-6B and MonoQ HR 5/5 were purchased from Amersham-Biosciences. Hammarsten casein was purchased from Merck. A BCA (bicinchoninic acid) Protein Assay Kit was purchased from Pierce Chemical Co. Suc–Ala–Ala–Pro–Phe–pNA and Suc–Ala–Ala–Phe–pNA were purchased from the Peptide Institute Inc., Osaka, Japan. All other reagents were of guaranteed grade.

**Screening and taxonomic analysis of strain RF2-5.** Bacterial strain RF2-5 was isolated from a fish sauce in Thailand (Rayong Fish Sauce Industry, Rayong Province) using the spread plate technique on JCM168 medium containing 1% skim milk and 10% NaCl.
morphological, physiological, and biochemical characteristics were determined as previously described. The 16S rDNA sequence was determined as previously reported.

**Media and growth conditions.** The medium used for production of proteinase was modified JCM168 medium (1% yeast extract, 0.1% sodium glutamate, 0.3% Tri-sodium citrate, 2% MgSO$_4$-7H$_2$O, 0.2% KCl, 10% NaCl, 0.036% FeCl$_2$, 0.00036% MnCl$_2$, 1% lactose, and 0.2% starch, pH 7.2). A seed culture of strain RF2-5 was inoculated into a 500 ml glass flask containing 100 ml of the modified JCM 168 medium (1%, v/v). Cultivation was conducted at 30°C for 3 d at 120 strokes/min.

**Purification of proteinase.**

*Step 1. Ammonium sulfate precipitation.* Solid ammonium sulfate was added to 1,600 ml of the cell-free culture broth to make an 80% saturated final concentration. The resulting precipitate was collected by centrifugation. Four hundred ml of 20 mM sodium phosphate buffer, pH 8.0 (buffer A) was added to dissolve the precipitate, and then the solution was dialyzed against the same buffer overnight at 4°C.

*Step 2. DEAE-Sepharose CL-6B column chromatography.* The dialyzed was put on a DEAE-Sepharose CL-6B column (φ35 × 110 mm) previously equilibrated with buffer A. The column was washed with the 5 bed volume of buffer A, followed by the same buffer containing 0.3 M NaCl. The proteinase was eluted with a linear 0.3–0.7 M NaCl gradient in buffer A and the eluate was collected in 5.5 ml fractions. Proteinase fractions were collected, then dialyzed against buffer A overnight at 4°C.

*Step 3. MonoQ column chromatography.* The enzyme solution was then applied to a MonoQ column (φ5 × 50 mm) in an FPLC system equilibrated with buffer A containing 0.4 M NaCl and eluted with a linear 0.4–0.9 M NaCl gradient. The enzyme fractions were collected.

**Analysis of molecular mass and the amino-terminal sequence.** A 12.5% polyacrylamide gel was used to determine the purity of the purified proteinase. Protein bands were stained with Coomassie brilliant blue R-250. The molecular mass of the purified proteinase was determined by comparison of its electrophoretic mobility with that of marker proteins. The amino-terminal sequence of the purified proteinase was identified by the method of Matsudaaira, using an Applied Biosystems Model 476A protein sequencing system.

**Assay of proteinase activity and protein determination.** Proteinase activity was assayed at 37°C for 2 h by the casein Folin–Ciocalteau method described in our previous paper in the presence of 10% NaCl (w/v). To 0.35 ml of 1.14% Hammarsten casein solution containing 25 mM Tris–HCl, pH 7.5, 10% (w/v) NaCl, 50 μl of the enzyme solution was added. After incubation for 2 h, the reaction was stopped by the addition of 0.4 ml of 0.44 M trichloroacetic acid, followed by centrifugation at 15,000 × g for 10 min. The supernatant (0.5 ml) was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of Folin–Ciocalteau reagent. The optical density of the color developed at 37°C for 20 min was measured at 660 nm. One unit of enzyme activity was defined as the enzyme quantity that liberates 1 μg of tyrosine per ml of the reaction mixture per minute.

Protein concentration was estimated by bichinchoninic acid with crystalline bovine serum albumin (Sigma Chemical) as the standard.

**Optimum pH.** Proteinase activity of the purified enzyme was measured at various pH values under standard assay conditions using casein as a substrate at 37°C for 2 h. The buffer used was 25 mM KH$_2$PO$_4$/NaOH (pH 6.0–8.0), 25 mM H$_2$BO$_3$/NaOH (pH 8.0–10.0), 25 mM NaHCO$_3$/NaOH (pH 10.0–11.0), 25 mM Na$_2$HPO$_4$/NaOH (pH 11.0–12.0), or 25 mM NaOH–KCl (pH 12.0–13.0).

**Optimum temperature.** Proteinase activity of the purified enzyme was measured at 20, 30, 40, 50, 60, and 70°C for 2 h at pH 7.5.

**pH stability.** The pH of the purified proteinase was adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0 8.0, 9.0, and 10.0 using 25 mM citrate/NaOH, KH$_2$PO$_4$/NaOH, and H$_2$BO$_3$/NaOH buffer. The enzyme was incubated at 30°C for 2 h, then residual proteinase activity was measured at pH 7.5 for 2 h.

**Thermal stability.** The purified proteinase was incubated at various temperatures (20, 30, 40, 50, 60, and 70°C) for 1 h at pH 7.5 and the remaining activity was assayed at pH 7.5 for 2 h at 37°C.

**Effect of NaCl on proteinase activity.** The proteinase activity of the purified enzyme was measured at various NaCl concentrations (0, 5, 10, 20, and 30% (w/v)) using casein (final conc. = 1.0% (w/v)) or Suc–Ala–Ala–Phe–pNA (final conc. = 100 μM) as a substrate at pH 7.5. In the case of pNA substrate, the assay was carried out by rate assay at 37°C for 30 min at pH 7.5.

**Effect of NaCl on proteinase stability.** The purified enzyme was incubated at various concentrations of NaCl (0, 5, 10, 20, and 30% (w/v)) at 30°C for 24 h at pH 7.5. In the case in which Suc–Ala–Ala–Phe–pNA was the substrate, the purified enzyme was incubated at various concentrations of NaCl at 30°C for 96 h at pH 7.5. Then the NaCl concentration was adjusted to 10% and the remaining activity was determined.

**Analysis of substrate specificity using FRET-25Xaa-libraries.** FRETs (fluorescence resonance energy trans-
fer substrate) combinatorial libraries (Peptide Institute, Osaka, Japan) were used for the analysis of substrate specificity. FRETs-25Xaa contains a highly fluorescent 2-(N-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal d-2,3-diamino propionic acid (d-A2pr) residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the ε-amino function of Lys, as shown below:

\[
P_3 \quad P_2 \quad P_1 \\
d-A2pr(Nma)\text{-Gly}\text{-Zaa}\text{-Yaa}\text{-Xaa}\text{-Ala}\text{-Phe}\text{-Pro}\text{-Lys(Dnp)}\text{-d-Arg}\text{-d-Arg}
\]

Xaa represents the fixed position of the 19 natural amino acids excluding Cys. A mixture of 5 amino acid residues (P, Y, K, I, and D) was incorporated at the Yaa position along with a mixture of 5 amino acid residues (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provided a peptide mixture of 25 combinations of each Xaa series, resulting in a combinatorial library with a total of 475 peptide substrates in 19 separate pools.

First, favored amino acids at the P1 position were determined at 37°C for 5 min in 50 mM H3BO3/NaOH buffer, pH 10. The fluorescence intensities at \( \lambda_{ex} = 360 \text{ nm} \) and \( \lambda_{em} = 465 \text{ nm} \) were measured. Then the P1 position was fixed with the favored amino acid residue and subjected to determination of preference at the P2 and P3 positions by LC–MS analysis.

**Results and Discussion**

**Screening of proteinase producing strain from fish sauce in Thailand**

Initially, 33 strains were isolated from the fish sauce in Thailand using the spread plate technique on JCM medium no. 168 containing 1% skim milk and various concentrations of NaCl less than 20%. Among these, 13 strains corresponding to 39% of the total isolated strains showed caseinolytic halo-forming colonies on the plate. Strain RF2-5 showed the highest proteinase activity on the plate containing 10% NaCl, and was used for further study.

**Classification of strain RF-2-5**

Strain RF2-5 is an aerobic gram-positive rod-shaped bacterium with terminal endospore. The colonies are circular, white, and low convex. The cells are motile. It showed positive reactions to catalase, oxidase, and urease, hydrolysis of casein and gelatin, and growth at 45°C and growth in 0 to 25% (w/v) NaCl. The optimum NaCl concentration for growth of RF2-5 was about 15% (w/v) after 3 d of cultivation at 30°C in the modified JCM168 medium. Nitrate reduction was negative. Acid was not produced from D-glucose, D-galactose, D-xylose, D-mannose, D-fructose, or sucrose.

The 16S rDNA sequence of RF2-5 (DDBJ accession no. AB191344) showed 97.3% similarity to the type Filobacillus milosensis ATCC 700960, 95.7% similarity to Bacillus halodalkaliphilus DSM 5271, and 94.6% similarity to Gracilibacillus dipsosauri ATCC 700347. Based on the 16S rDNA sequence alignment, the strain RF2-5 was located in the lineage of the genus Filobacillus. The strain was classified and named Filobacillus sp. RF2-5.

**Purification of the proteinase**

The production of proteinase by Filobacillus sp. RF2-5 was entirely dependent on the presence of yeast extract but not on the presence of casamino acids in the modified JCM168 medium, and the proteinase activity was detected after late log phase and reached maximum after stationary phase (about 3 days) (data not shown). The purification of the proteinase is summarized in Table 1. The proteinase was purified 64-fold with a specific activity of 83.1 U/mg protein and a final yield of 5%. SDS–PAGE analysis of the purified enzyme revealed a single band with a molecular weight of 49 kDa (Fig. 1).

**Amino-terminal amino acid sequence analysis**

The N-terminal amino acid sequence of the proteinase from Filobacillus sp. RF2-5 was determined to be A–L–D–T–G–V–(X)–W–D–((X; unidentified amino acids)–P–A–L–K–E–(X; unidentified amino acids). A comparison of this sequence with those available in the DDBJ databases revealed about 67% identity to a serine proteinase, bacillopeptidase F from Bacillus subtilis 16814, and about 67% identity to an extracellular 90 kDa serine proteinase from Bacillus subtilis (natto). The molecular weight of the proteinase from Filobacillus sp. RF2-5 was about half of that of bacillopeptidase F, and the N-

<table>
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<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total act. (U)</th>
<th>Total protein (mg)</th>
<th>Specific act. (U/mg protein)</th>
<th>Yield (%)</th>
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<td>100</td>
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<td>MonoQ 5/5 HR</td>
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<td>150</td>
<td>1.8</td>
<td>83.1</td>
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terminal amino acid sequence of the proteinase from *Filobacillus* sp. RF2-5 showed homology to the amino acid sequences from 227 amino acid to 239 amino acid of bacillopeptidase F. Perhaps the proteinase from *Filobacillus* sp. RF2-5 might be processed after expression at the middle of the molecule, corresponding to about 227 amino acid residue of bacillopeptidase F. To confirm this assumption, we intend to obtain a gene encoding the proteinase. The cloning of the protease encoding gene should allow us to clarify the nature of this serine proteinase.

**Characteristics of the proteinase**

The optimum pH and temperature of the purified proteinase was pH 10–11 and 60 °C (Fig. 2A and B). Thus the enzyme was slightly or moderately thermophilic. It was stable over a broad pH range from 5 to 10 at 30 °C for 2 h (Fig. 2C) and up to 50 °C (Fig. 2D) after treatment for 1 h at pH 7.5 (Table 2).

The pH of fish sauce during its fermentation (about 1 year) is in the range between pH 5.2 to 6.3. As described above, the optimum pH of the purified proteinase from *Filobacillus* sp. RF2-5 is pH 10 to 11. It is suggested that the proteinase from *Filobacillus* sp. RF2-5 might be involved in the degradation of fish proteins during long-term fermentation, even under as low a pH as pH 6, because the proteinase still has 40% of the maximum activity at pH 6.

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

A 12.5% polyacrylamide gel was used for analysis. Markers: bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and soybean trypsin inhibitor (21.5 kDa).

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

(A) The buffers used were: ○, 25 mM KH₂PO₄/NaOH (pH 6.0–8.0); □, 25 mM H₃BO₃/NaOH (pH 8.0–10.0); ▲, 25 mM NaHCO₃/NaOH (pH 10.0–11.0); △, 25 mM Na₂HPO₄/NaOH (pH 11.0–12.0); and ◆, 25 mM NaOH–KCl (pH 12.0–13.0). Maximal proteolytic activity is shown as 100%. (B) Proteinase activity was measured at 20–70 °C for 2 h at pH 7.5. (C) The pH of the purified proteinase was adjusted to various pH by: ○, 25 mM citrate–NaOH (pH 3.0–6.0); □, 25 mM KH₂PO₄/NaOH (pH 6.0–8.0); and ▲, 25 mM H₃BO₃/NaOH (pH 8.0–10.0) buffer. The enzyme was incubated at 30 °C for 2 h, then the residual proteinase activity was measured at pH 7.5 for 2 h. (D) After heat treatment at various temperatures (20 to 70 °C) for 1 h at pH 7.5, the remaining activity was measured at pH 7.5 for 2 h.
In order to determine the type of proteinase, the following proteinase inhibitors were used: as serine proteinase inhibitors, PMSF, TLCK, TPCK, chymostatin (carboxy terminal = phenylalanine), SBTI, leupeptin (carboxy terminal = arginine), and C11-MAPI (carboxy terminal = phenylalanine); cysteine proteinase inhibitors, E-64; and metalloproteinase inhibitors, EDTA-2Na. PMSF, chymostatin, and MAPI were found to inhibit the proteinase activity strongly, whereas the other proteinase inhibitors did not show any inhibitory activity (Table 2). These results suggest that the proteinase purified from Filobacillus sp. RF2-5 was a serine type of proteinase. Based on the inhibition profile of the proteinase against aldehyde-type inhibitors (chymostatin, MAPI, and leupeptin), it is possible that the serine proteinase from Filobacillus sp. RF2-5 was chymotrypsin- or subtilisin-type proteinase, not trypsin-type proteinase. This speculation is in good agreement with that of primary specificity, shown in Fig. 4A (phenylalanine residue at the P1 position = 100%, arginine residue at the P1 position = ~50%).

A kinetic study was carried out using N-succinyl-Ala–Ala–Phe–pNA and N-succinyl-Ala–Ala–Pro–Phe–pNA as substrates. The kcat, Km, and catalytic efficiency (kcat/Km) of the enzymes for each substrate were 3.8 s⁻¹, 0.115 mM, 33.0 s⁻¹.mM⁻¹ and 2.58 s⁻¹, 0.226 mM, 11.5 s⁻¹.mM⁻¹ respectively. Assays were done at 37°C for 5 min in 50 mM H3BO3/NaOH buffer (pH 10).

Proteinase activity was measured at NaCl concentrations between 0 and 30% using casein or N-succinyl–Ala–Ala–Phe–pNA as substrate. High activity was detected in the range of 0–5% NaCl using casein, but casein was coagulated in the higher NaCl assay conditions and thus this did not reflect a true characteristics of the proteinase (Fig. 3A). In contrast, high activity was observed in the range of 5–30% of NaCl and maximal activity in the range of 15–25% NaCl using N-succinyl–Ala–Ala–Phe–pNA (Fig. 3A). Interestingly, proteinase activity was activated about 2-fold by the addition of 5% NaCl and about 2.5-fold by the addition of 15–25% NaCl. At higher NaCl concentrations (~30% NaCl), high proteinase activity was still detected.

The stability of the proteinase under various conditions was also examined. The enzyme was almost stable at 25% NaCl using casein as a substrate (Fig. 3B), and more than 80% of activity was measured at 5–20% NaCl using N-succinyl–Ala–Ala–Phe–pNA as substrate (Fig. 3B).

**Substrate specificity using FRET combinatorial libraries**

A novel type of fluorescence energy transfer (FRET)

![Fig. 3. Effect of NaCl on the Activity (A) and Stability (B) of the Purified Proteinase.](image)

(A) The proteinase activity of the purified enzyme was measured at various NaCl concentrations using casein (●) or Suc–Ala–Ala–Phe–pNA (▲) as substrate at pH 7.5. (B) The purified enzyme was incubated at various concentrations of NaCl (0, 5, 10, 20, and 30% (w/v)) at 30°C for 24 h at pH 7.5. In the case in which Suc–Ala–Ala–Phe–pNA was incubated at various concentrations of NaCl at 30°C for 96 h at pH 7.5. Then the remaining activity was measured. The relative proteinase activity was defined as the percentage of activity with respect to the maximum proteinase activity detected in the assay.
combinatorial libraries, which has proven to be a sensitive and reliable substrate for determination of the substrate specificity of serine proteinase, \(^{12}\) were used to determine the purified proteinase from \textit{Filobacillus} sp. RF-2-5. The proteinase was found to prefer Phe, Met, and Thr at the P\(_1\) position (Fig. 4A), and Ile at the P\(_2\) position (Fig. 4B) of the substrates respectively.

According to the results of LC–MS analysis, the cleaved site by the proteinase was deduced between Phe and Ala of FRETS-25F. The product cleaved between Phe and Pro of the substrate was not detected. This suggests that Ile at the P\(_2\) site is important for cleavage by the proteinase. To date, no serine proteinase has exhibited a preference for Ile at the P\(_2\) position.

Moreover, there is only one report on serine proteinase which prefers Met at the P\(_1\) position.\(^ {12}\) Thus the serine proteinase from \textit{Filobacillus} sp. RF-2-5 is unique in terms of its substrate specificity.

Based on the data described here, we compared the properties of the serine proteinase of \textit{Filobacillus} sp. RF-2-5 with other proteinases.

(1) \textit{Filobacillus milosensis} gen. nov., sp. nov. was reported as a new halophilic bacterium in 2001,\(^ {13}\) but it was reported as a proteinase non-producing strain.

(2) As for moderately halophilic bacteria, the following two reports were compared: Duong Van Qua \textit{et al.} reported purification and characterization of a halophilic proteinase (M.W. = 120 kDa) from \textit{Pseudomonas} sp. (A-14).\(^ {2}\) The enzyme is a metalloproteinase. C. Sanchez-Porro \textit{et al.} reported purification and characterization of 38 kDa proteinase, CP1 from the moderately halophilic bacterium \textit{Pseudoalteromonas} sp. CP76.\(^ {3}\) This proteinase is a serine-metallo proteinase, as described by the authors. Thus the serine proteinase from \textit{Filobacillus} sp. RF2-5 is different from these enzymes.

(3) Regarding proteinases from fish sauce bacteria, the following two reports were compared: C. Thongthai \textit{et al.} reported caseinolytic and gelatinolytic activity by the extremely halophilic archaeobacterium \textit{Halobacterium salinarium} from fish sauce (nam pla).\(^ {4}\) But the detailed characteristics of this proteinase (proteinase type, M.W., etc.) have not been clarified and thus it is difficult to compare it with the proteinase from \textit{Filobacillus} sp. RF-2-5. In addition, S. Chaiyanan \textit{et al.} reported three extracellular proteinases with molecular weights of approximately 100 kDa, 42 kDa, and 17 kDa from the moderately halophilic \textit{Halobacillus thailandensis} sp. nov. isolated from fish sauce.\(^ {5}\) Among them, 100 kDa- and 17 kDa-proteinase are classified into serine proteinase, the molecular weight (49 kDa) of serine proteinase from \textit{Filobacillus} sp. RF2-5, however, is different. Thus the serine proteinase from \textit{Filobacillus} sp. RF-2-5 is clearly different from these enzymes.

Accordingly, this is the first report of a serine proteinase that is moderately thermostable, NaCl-stable, and NaCl-activatable, and with a unique substrate specificity at the P\(_2\) position of substrates from moderately halophilic bacteria, \textit{Filobacillus} sp. The proteinase from \textit{Filobacillus} sp. RF-2-5 might be useful for the degradation of fish protein during fermentation at high salt concentrations and might be useful for reduction of the fermentation period.

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References


