



## Purification and Characterization of Cold-Adapted Metalloprotease from Deep Sea Water Lactic Acid Bacteria *Enterococcus Faecalis* TN-9

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### Abstract

This paper investigated a 3-step purification and characterization of a protease from *Enterococcus faecalis* TN-9, a bathypelagic lactic acid bacteria. The purification procedure includes precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , then ion-exchange chromatography with DEAE-Sephadex A-25 and DEAE Cellulofine A-500. Native PAGE analysis indicates a single protease band. The molecular weight is 30 kDa by SDS-PAGE analysis, and 69 kDa by gel chromatography analysis. It proves that the optimal temperature for protease reaction is 30 °C, and the optimal pH is 7.5-8.0. The reaction is stable while pH is 6.0-9.5 and temperature is under 45 °C. The relative activity is 6.1% at 0 °C. The enzyme is totally deactivated with heat treatment at 60 °C or over. The protease is partially inhibited by EDTA-2Na,  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Ag}^{2+}$ ,  $\text{Co}^{2+}$  and Pepstatin A.  $\text{Zn}^{2+}$  shows obvious activation to the protease.  $K_m$  and  $V_{max}$  of purified protease acting on azocasein are 0.098 % and 72 mg/(h·mg) respectively. This protease is one of gelatinase with N-terminal sequence of VGSEVTLKNS, and shows characteristics of a cold-adapted metalloprotease.

**Keywords:** Deep sea water, Lactic acid bacteria, Cold-adapted enzyme, Metalloprotease, Azocasein

### 1. Introduction

The cold-adapted enzymes from cold-adapted organisms or other microbial life in extreme environments have been widely studied in recent years. Reports are often on cold-adapted enzymes from marine microorganism. Shi et al (2005, p. 258-263; 2006, p. 72-75) separated and purified a strain of cold-adapted enzyme from a psychrophilic bacteria *Bacillus cereus* SYP-A2-3 acquired from samples collected from glacier. Nushin Aghajari et al (2003, p. 636-647) and Denner E B et al (2001, p. 44-53) isolated a strain of psychrophilic bacteria from samples of Antarctic and studied on the cold-adapted metalloprotease. Miyamoto K et al (2002, p. 416-421) acquired and purified two types of cold-adapted

metalloprotease (MprI and MprII) from marine microorganism *Alteromonas* sp. strain O-7. However, we have not found any report on the cold-adapted protease isolated from marine lactic acid bacteria.

Study described in this paper isolated 25 strains of lactic acid bacterium from deep sea water at Toyama Bay of Japanese Sea. Among them *Enterococcus faecalis* strain TN-9 (Atsushi Hayashi, 2007, p. 58-64) shows liquefying gelatin, fermented and solidified litmus milk,  $\beta$ -galactosidase activity and other characteristics. Oral administration has been proved safe by sub-acute toxicity study of this bacteria strain in rats (Chie Motonaga, 2007, p. 191-196). Therefore, *E. faecalis* TN-9 is expected promising in the research and development of health foods containing lactic acid bacteria. Based on above-mentioned results, the study in this paper isolated, purified and characterized the protease from lactic acid bacteria *E. faecalis* TN-9.

## 2. Materials and methods

### 2.1 Materials and equipments

Bacteria stain: lactic acid bacteria *E. faecalis* strain TN-9, stored and provided by Nichinichi Pharmaceutical Co., Ltd; Lactobacilli MRS Broth from USA; Hinute SMP provided by Fuji Oil Co. of Japan; Azocasein from Sigma of USA; DEAE-Sephadex A-25 from Pharmacia of Sweden; DEAE-Cellulofine A-500 and Toyopearl HW-55s provided by Chisso of Japan; standard protein provided by Wako of Japan; and other commonly used reagents made in Japan. Key instruments: U-2000 spectrophotometer from Hitachi of Japan and KUBOTA 7780 centrifuger from Kubota of Japan.

### 2.2 Methodology

#### 2.2.1 Culture of strain and collection of culture media

##### (1) Culture medium

Take MRS agar plate as solid culture media. Liquid culture media is a mixture of A, B and C, where A is composed of 1.5 g Hinute SMP, 2.4 g D-Glucose, 0.2 g NaCl and 70 ml distilled water; B is composed of 18.0 g  $K_2HPO_4$  and 100 ml distilled water; C is composed of 0.05 g  $FeSO_4 \cdot 7H_2O$ , 0.58 g  $MgSO_4 \cdot 7H_2O$ , 0.03 g  $MnSO_4 \cdot 5H_2O$ , 0.10 g  $ZnSO_4 \cdot 7H_2O$  and 100 ml distilled water. Begin by formulating A and B, in turn autoclaving at 121 °C for 15 min, then sterilize C by sterilizing filter (DISMIC-25CS/0.45  $\mu m$ , made in Toyo of Japan), finally mix A, B and C at the ratio of 7:2:1 in aseptic environment, ready for culture of strain.

##### (2) Culture of strain and collection of culture media

On the MRS medium, cut marker lines on the -80 °C stored bacteria strain, culture the slides at 30 °C for 48 h. Select and inoculate the independent bacterial colonies into 50 ml the above-mentioned liquid medium. After static culture at 30 °C for 18 h, transfer it into 800 ml the same liquid medium to have the second static culture at 30 °C for 18 h. Centrifuge at 4 °C (12,000 $\times g$ , 10 min) to collect supernatant as crude enzyme solution for isolating and purifying protease.

#### 2.2.2 Determination of protease activity

Protease activity is measured by the TCA-azocasein assay based on the methods proposed by Hagihara B et al (1958, p. 185-194) and Thomas J B et al (1986, p. 139-145). 100  $\mu l$  protease solution with specific concentration is added into 400  $\mu l$  1.25% (w/v) phosphate-buffered saline (100 mM Na-K phosphate buffer with pH of 7.5, called buffer A); react at 30 °C for 10 min (shaking at 170 times per minute) in water bath; adding 1 ml 10% (w/v) trichloroacetic acid (TCA) to stop the reaction; centrifuge (16,000 $\times g$ , 20 min, 10 °C) the resulting product to remove undigested azocasein. The absorbance (A335) of the obtained supernatant is measured at 335 nm with a spectrophotometer (Hitachi, U-2000). Protease activity is calculated from the amount of L-tyrosine, which is achieved from A335. One unit of protease activity is defined as the amount of enzyme for 1  $\mu g$  of L-tyrosine which is released from the substrate per minute under the above conditions.

#### 2.2.3 Determination of protein

Protein is measured by the Lowry process (Lowry O H, 1951, 265-275), with bovine serum albumin (Sigma of USA) as criterion.

#### 2.2.4 Polyacrylamide gel electrophoresis (PAGE)

Referring to the method proposed by Reisfeld R A et al (1962, p. 281-283), Native-PAGE is carried out under the following conditions: 2.5% (w/v) stacking gel, 7.5% (w/v) separation gel (pH 4.0), alanine-acetic acid (pH 4.5) as electrolyte bath, anode/cathode reversely-connected NA-1311 Electrolyte Bath (NIHON EIDO Co. Ltd. Tokyo), and electrophoresis at a constant current of 2.5 mA for 3.5 h per column. Referring to the method proposed by Weber K and Osborn M (1969, p. 4406-4412), SDS-PAGE is carried out under following conditions: 7.5% (w/v) polyacrylamide gel and 0.10% (w/v) SDS-0.10 M phosphate buffer (pH 7.2) as electrolyte bath, electrophoresis at a constant current of 6.0 mA for 3 h per column. Use BSA (79,000), Aldolase (42,000), Carbonic Anhydrase (30,000), Trypsin Inhibitor (20,000) and Lysozyme (14,000, from Wako of Japan) as reference standards for molecular weight. Stain for 1 h with 0.25% (w/v)

Coomassie Brilliant Blue R-250 solved in the mixture of ethanol, acetic acid and deionized water at the ratio of 9:2:9; destain for 1 h with the mixture of ethanol, acetic acid and deionized water at the ratio of 25:8:65, further with the mixture of ethanol, acetic acid and deionized water at the ratio of 10:15:175.

### 2.2.5 Gel chromatography

Gel chromatography is carried out at 20 ml/h by using Toyopearl HW-55s column (25×900 mm) and buffer A containing 0.2 M NaCl as eluent. Reagents and kits for molecular weight measurement: Ferritin (440 kDa), Catalase (240 kDa),  $\gamma$ -globulin (160 kDa), Bovine serum albumin (67 kDa) and Ovalbumin (43 kDa, from Wako of Japan).

### 2.2.6 Isolation and purification of enzyme

Collect supernatant as crude enzyme by centrifuging 3,200 ml culture medium at 12,000×g, 4 °C for 10 min. Slowly add  $(\text{HN}_4)_2\text{SO}_4$  powder of saturation 0-60% (w/v); stir and sediment for 30 min. Centrifuge the mixture and have the sediment resolved in 40 ml 100 mM Tris-HCl buffer (buffer B, pH 7.5). The resulting filtrate from semi-permeable membrane is forced through buffer B-balanced DEAE-Sephadex A-25 column (26×180 mm); uncombined protein is then removed by washing with 450mL buffer B; obtained product is finally eluted with 720 ml buffer B at 50 ml/h (elution gradient is 0→2.5 M NaCl). Determine protease activity and protein amount of each fraction (6.0 ml/tube), collect the fractions with high protease activity, apply buffer A-balanced DEAE-Cellulofine A-500 column (26×180 mm) after dialysis by buffer A for 24 h (change buffer at the 3<sup>rd</sup> hour and the 9<sup>th</sup> hour from the beginning of dialysis), remove other proteins by rinsing with 450mL buffer A, then elute with 720 ml buffer A at 40 ml/h (elution gradient is 0→2.0 M NaCl). Determine protease activity and protein amount of each fraction (3.0 ml/tube); collect the fractions with high protease activity. The purified enzyme is obtained by dialysis with buffer A for 24 h.

### 2.2.7 Determination of subunit molecular weight

Using SDS-PAGE, find subunit molecular weight from  $R_f$ -Log  $M_r$  chart, wherein  $R_f$  represents relative mobility of standard protein with certain molecular weight ( $M_r$ ) under SDS-PAGE condition.

### 2.2.8 Determination of optimal reaction temperature and optimal pH

The optimal reaction temperature and optimal pH are found from relative activities of enzyme at different temperature (0-80 °C) and in different buffers of different pH, buffers such as 100 mM acetate solution (pH 3.2-5.5), 100 mM phosphoric acid solution (pH 4.8-9.0), 100 mM Tris-HCl solution (pH 7.5-9.5), 100 mM carbonic acid solution (pH 9.0-12.0), relative activities of enzyme in turn is measured by Azocasein Method with the highest enzyme activity being defined as 100%.

### 2.2.9 Thermal stability and pH stability

Investigate the thermal stability after holding at 20-80 °C for 10 min and then quick-cooling in ice bath, as well as pH stability after storing respectively in the above buffers (pH 3.2-12.0) and then dialyzing at 4 °C overnight, by measuring the residual activity of enzyme according to the above-mentioned procedure.

### 2.2.10 Determination of kinetic constants

Use buffer A to formulate azocasein substrates with different concentrations of 0.02%, 0.04%, 0.08%, 0.16%, 0.32%, 0.64%, 1.25%, 2.50%, wherein the amount of enzyme is 1.5 g. Based on Lineweaver-Burk plotting method,  $K_m$  and  $V_{max}$  are obtained from the enzyme activity of each substrate.

### 2.2.11 Effects of inhibitor, metal ions and denaturant on enzyme activity

Solve inhibitor, metal ions and denaturant respectively in deionized water to obtain concentrated solutions; add the concentrated solutions in 0.5 ml purified enzyme solution (1.0 unit) to formulate the specimen with final concentrations of 1.0 mM and 0.1 mM, keep them at 25 °C for 30 min. Effects of inhibitor, metal ions and denaturant on enzyme activity are investigated by determination of residual enzyme activities of the obtained specimen according to the above-mentioned procedure with the enzyme-free system as blank control and pure enzyme solution as 100%.

### 2.2.12 Activation experiment on deactivated protease

Add 0.5ml 2.0 mM EDTA-2Na solution in 0.5 ml purified enzyme solution, keep it at 25 °C for 30 min; then add various 2.0 mM metallic salt solutions at the amount of 0.5 ml, keep it at 25 °C for 30 min. Effects of various metallic ions on activation of EDTA-2Na-deactivated protease are investigated by determination of the residual enzyme activities of the specimen according to the above-mentioned procedure with EDTA-2Na-free system and the metallic salt-free system as blank controls respectively.

### 2.2.13 $\text{NH}_2$ -terminal amino-acid sequence analysis

$\text{NH}_2$ -terminal amino-acid sequence analysis is carried out by SHIMADZU PPSQ-10 protein sequence analyzer (Shimadzu of Japan) after electro blotting the purified enzyme based on the method proposed by Matsudaira P (1987, p. 10035-10038).

### 3. Results and discussions

#### 3.1 Purification of protease

After purifying the crude enzyme by ammonium sulfate precipitation (Fraction 2) and two-step anion-exchange chromatography with DEAE-Sephadex A-25 (Figure 1, Fraction 3) and DEAE-Cellulofine A-500 (Figure 2), The specific enzyme activity of the obtained specimen is increased from 25 U/mg to 12,300 U/mg. The purification fold is 492, and the recovery rate reaches 23% (Table 1).

From the purification procedure, we can see that ammonium sulfate precipitation is a simple and effective means for initial fractionation of proteins. 80% protease is recovered and the specific enzyme activity is increased by 120 folds. This is because that ammonium sulfate doesn't affect the enzyme activity; and not only is Hinute SMP-mainly-contained culture medium suitable for *E. faecalis* TN-9 to develop and produce large amount of protease, but also its residual protein is easy to be removed by ammonium sulfate precipitation process, making it easy for further purification.

Native PAGE analysis shows only one protease band, indicating lack of isozyme in purified protease specimen (Figure 3A); SDS-PAGE analysis also shows only one protease band, indicating an electrophoresis grade for the purification of protease. The subunit molecular weight is calculated as 30 kDa. Molecular weight measured by gel chromatography analysis is 69 kDa, indicating that the natural state of protease is a dimer consisting of two subunits. Mäkinen et al (1989, p. 3325-3334) isolated and purified a protease with molecular weight of 31.5 kDa from *Streptococcus faecalis* (OG1-10) and proved it to be a new neutral endoprotease (Gelatinase, EC3.4.24.4).

During purification and while using Tris-HCl buffer (100 mM, pH 7.5), protease is absorbed to DEAE series anion exchange resin adhesive, then eluted with NaCl solution. However, protease can not be absorbed to DEAE series anion exchange resin adhesive nor CM resin adhesive while using phosphoric acid buffer (100 mM, pH 6.0-7.5) instead. In addition, during PAGE analysis, protease does not show any movement in alanine-acetic acid solution (pH 4.5) in anode/cathode normal-connected NA-1311 Electrolyte Bath; on the other hand, protease moves towards cathode when anode and cathode are reversely connected. This is explained that the protease shows electronegative in Tris-HCl buffer with pH higher than 7.0, neutral in phosphoric acid buffer with pH of 7.0, and electropositive in alanine-acetic acid buffer with pH of 4.5. Therefore, the isoelectric point of this protease should be between pH 4.5-pH7.0.

#### 3.2 Study on the properties of protease

##### 3.2.1 Optimal reaction temperature and optimal pH of the enzyme

The optimal reaction temperature and optimal pH of the purified protease are 30 °C (Figure 4A) and 7.5-8.0 (Figure 4C) respectively. The optimal pH of gelatinase isolated from *S. faecalis* (Strain OG1-10) reported by Mäkinen (1989, p. 3325-3334) is 6-8.

##### 3.2.2 Thermal stability and pH stability of the enzyme

The relative activity of this purified protease is 6.1% at 0 °C (Figure 4A), and 65.1% at 15 °C; while that of cold-adapted protease isolated from psychrophile bacteria *Bacillus cereus* SYP-A2-3 from glacier is about 6.0% at 0 °C, and 60% at 25 °C. It indicates that this protease, with larger low temperature enzyme activity, shows characteristics of cold-adapted enzyme (Shi, Jinsong, 2005, p. 258-263; 2006, p. 72-75). The activity of this purified protease maintains 100% after heat treatment at 45 °C for 10 min, drops sharply from 50 °C, and is completely inactivated over 60 °C (Figure 4B). This indicates that this protease is unstable to heat. The residual activity is over 89% with pH in the range of 6.0-9.5, and maintains over 70% in phosphoric acid buffer even with pH decreased to 4.8 (Figure 4D), demonstrating that this protease is more stable in phosphoric acid buffer.

##### 3.2.3 Determination of kinetic constants

Based on Lineweaver-Burk plotting method,  $K_m$  and  $V_{max}$  are respectively found to be 0.098% and 72 mg/(h·mg) from the relation between enzyme activity and concentration of azocasein substrate.

##### 3.2.4 Effects of inhibitor, metal ions and denaturant on enzyme activity

As illustrated in Table 2, the purified protease is partially inhibited by  $\beta$ -mercaptoethanol, Pepstatin A, Chymostatin, DTT and EDTA-2Na, where the inhibiting rate of EDTA-2Na is highest, up to 42%. It is not inhibited by PMSF, TLCK and Leupiptin, inhibited to certain extend by  $Hg^{2+}$ ,  $Cu^{2+}$ ,  $Ag^+$ ,  $Ni^{2+}$  and  $Co^{2+}$ . However,  $Zn^{2+}$ ,  $Fe^{3+}$ ,  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $Fe^{2+}$  and IAA increase enzyme activity (Table 3). In addition, EDTA-2Na-deactivated enzyme (apoenzyme) can be activated by  $Zn^{2+}$ , indicating that  $Zn^{2+}$  is the main metal ion to catalyze chemical reactions begins with the binding of the substrate to the active site on the enzyme. Meanwhile, the protease is inhibited by the inhibitor of aspartic acid Pepstatin A, suggesting that aspartic acid is abundant at the active site on the enzyme and active in catalyzing chemical reactions.

##### 3.2.5 $NH_2$ -terminal amino-acid sequence analysis

10 NH<sub>2</sub>-terminal amino-acid starting with V (Val) sequence (VGSEVTLKNS) are proved. By using FASTA sequence similarity analysis with NCBI database, the analysis are in accordance with that of gelatinase (EC 3.4.24.4) isolated from *S. faecalis* (Strain OG1-10) (Pirkko-Liisa, Mäkinen, 1989, p. 3325-3334) and Coccolysin (EC 3.4.24.30) (Pirkko-Liisa, Mäkinen, 1994, p. 981-985). It suggests that the metalloprotease isolated from *E. faecalis* TN-9 is a gelatinase. In 1994, Mäkinen et al isolated and purified Coccolysin (EC3.4.24.30) from *E. faecalis* (OG1-10) and concluded that Coccolysin is one of Gelatinase based on amino-acid sequence analysis. Although Pirkko-Liisa, Mäkinen et al (1989, p. 3325-3334; 1994, p. 981-985) isolated and determined gelatinase (EC 3.4.24.4) and coccolysin (EC 3.4.24.30) sequentially, the study on the characteristics of cold-adapted metalloprotease isolated from *E. faecalis* hasn't been reported so far.

#### 4. Conclusion

This paper illustrated the purification process and common characteristics of extracellular protease isolated from lactic acid bacteria *E. faecalis* TN-9 from deep sea water. The electrophoresis-grade metalloprotease acquired by 3-step purification process has a specific activity 12,300 U/mg, a recovery rate 23%, a subunit molecular weight 30 kDa and Native molecular weight 69 kDa and an isoelectric point is between pH 4.5 and pH 7.0. These properties indicate that the protease is a metalloprotease with Zn<sup>2+</sup>-dependent active site. The protease has 10 N-terminal sequence (VGSEVTLKNS) the same as Gelatinase (EC3.4.24.4) and Coccolysin, indicating the isolated protease is one of Gelatinase for classification point of view. The purified protease has a relative activity 6.1% at 0 °C, a maximum relative activity at 30 °C and between pH 7.5 and pH 8.0. The activation protease is in the most stable state between pH 6.0 and pH 9.5 and at temperature below 45 °C, drops sharply above 50 °C, is completely inactivated above 60 °C. Meanwhile, phosphoric acid buffer is more beneficial to the storage and activation of this protease. Km and Vmax of purified protease acting on azocasein are 0.098 % and 72 mg/(h·mg) respectively.

The purified metalloprotease exhibits certain features of cold-adapted enzyme, especially in low-temperature catalyst and thermal instability. Therefore it is concluded as a cold-adapted metalloprotease. It is shown by an incomplete literature search that this is the first report on the characteristics of cold-adapted metalloprotease isolated from lactic acid bacterium in deep sea so far. Atsushi Hayashi et al (2007, p. 58-64) believes that *E. faecalis* TN-9 most likely comes from muddy cold seabed or excrement of psychrophilic fish at the bottom of deep sea. Our study further validates the conclusion. Cold-adapted enzymes in many research reports come from psychrophilic bacteria in extreme cold environments such as glacier, marine environment and alike. Most psychrophilic bacteria secrete cold-adapted enzymes. Gerday C et al (2000, p. 103-107) believes that as dominating species of cold ecosystem, cold-adapted microorganisms live widely in polar region, glacier, ice sea, deep sea and other year-around cold environments. Cold-adapted microorganisms can produce some efficient cold-adapted enzymes to maintain normal metabolism and other complex functions.

Application of cold-adapted protease isolated from bacteria has bright future in food industry. It can improve the quality, stability and solubility of food. It could be produced in industrial scale, serving as an inexpensive and abundant enzyme source., It is generally toxic free, capable of processing food at low temperature and is generally free of side effect as the enzymes deactivate rapidly at moderate temperature (Ryoma Miyake, 2007, p. 4849-4856; Wang, Yujing, 2004, p. 8-15; Nakagawa T, 2004, p. 383-387; Gu, Jing, 2002). The purified cold-adapted protease studied in this paper has a maximum activity at 30 °C, and is deactivated at 60 °C within only 10 min, therefore meets the enzyme-processing requirements for food at low temperature. In addition, the safety of the oral administration of *E. faecalis* TN-9 has been proved. Not only can the purified enzyme from culture media be applied to food industry, but also is the culture media itself and bacterium strain applied in food industry directly. In all, the application of lactic acid bacteria *E. faecalis* TN-9 and the cold-adapted metalloprotease from it has promising future in food industry.

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Table 1. Summary of purification of protease

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture supernatant	3.5×10 <sup>5</sup>	14,265	25	1	100
Ammonium sulfate	2.8	95	2,950	118	80
DEAE-Sephadex A-25	0.8	12.8	6,250	250	23
DEAE-Cellulofin A-500	0.8	6.5	12,300	492	23

Table 2. Effects of inhibitor, denaturant and reducing agent on the activity of protease

Compound	Concentration	Relative activity (%)
Inhibitor		
None	0	100
PMSF	10 µg/ml	105
TLCK	10 µg/ml	100
Leupiptin	10 µg/ml	100
PCMB	10 µg/ml	105
ICH <sub>2</sub> COOH	1.0 mM	98
EDTA-2Na	0.1 mM	58
Pepstatin A	10 µg/ml	81
n-Octyl alcohol	1.0 mM	99
Chymostatin	10 µg/ml	91
IAA	10 µg/ml	124
AEBSF	10 µg/ml	100
Phosphoramidon	10 µg/ml	119
Antipain	10 µg/ml	100
BHH	10µg/ml	100
Denaturant and reducing agents		
None	0	100
SDS	1.0 mM	102
Urea	1.0 mM	101
DTT	10 µg/ml	96
β-mercaptoethanol	1.0 mM	68

Table 3. Effects of metal ions on the activity of protease

Metal ion	Relative activity (%)	
	Concentration	
	1.0 mM	0.1 mM
None	100	100
Zn <sup>2+</sup>	116	109
Fe <sup>3+</sup>	112	100
Mn <sup>2+</sup>	107	103
Mg <sup>2+</sup>	106	100
Fe <sup>2+</sup>	104	100
Co <sup>2+</sup>	78	88
Ni <sup>2+</sup>	74	82
Ag <sup>+</sup>	72	82
Cu <sup>2+</sup>	27	51
Hg <sup>2+</sup>	8	10

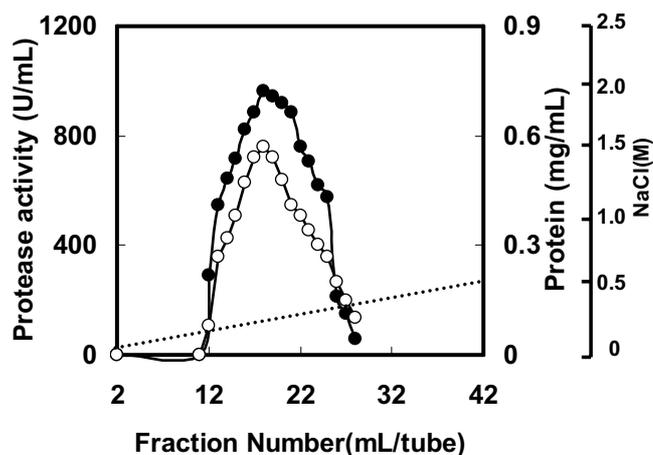


Figure 1. Column chromatography of protease on DEAE-Sephadex A-25

The enzyme solution (Fraction 2) was applied to a column (26×180 mm) of DEAE-Sephadex A-25 equilibrated with buffer A. The column was washed with 450 ml buffer A, then proteins were eluted with a linear gradient of 0 to 2.5 M NaCl solved in 720 ml buffer A. Fractions of 6.0 ml were collected at flow rate of 60 ml/h. Protein concentration and protease activities were assayed.

● represents enzyme activity; ○ represents protein concentration; ··· represents NaCl.

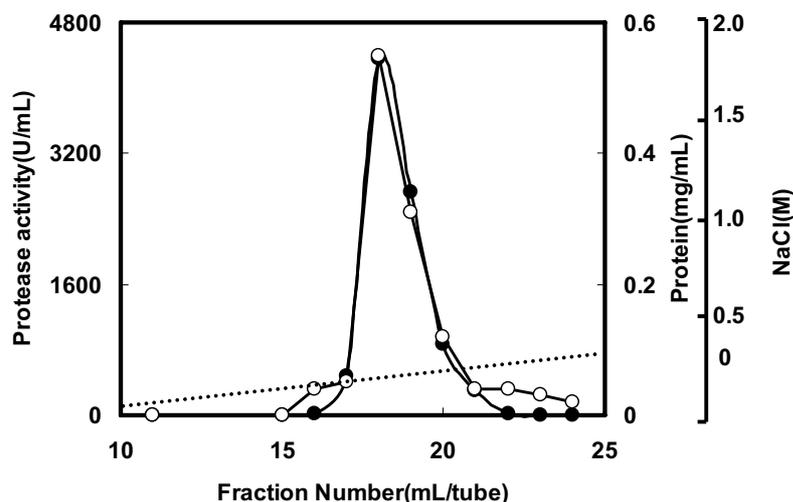


Figure 2. Column chromatography of protease on DEAE-Cellulofine A-500

The enzyme solution (Fraction 3) was applied to a column (26×180 mm) of DEAE-Cellulofine A-500 equilibrated with buffer A. The column was washed with 450 ml buffer A, then proteins were eluted with a linear gradient of 0 to 2.0 M NaCl solved in 720 ml buffer A. Fractions of 3.0 ml were collected at flow rate of 40 ml/h. Protein concentration and protease activities were assayed.

● represents enzyme activity; ○ represents protein concentration; ··· represents NaCl.

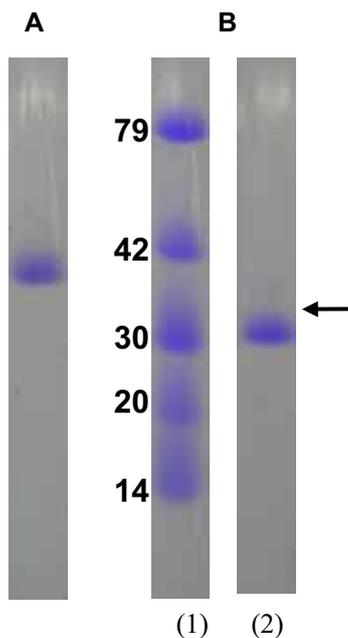


Figure 3. Native PAGE (A) and SDS-PAGE (B) of protease.

A: Native PAGE, the purified enzyme (5.0  $\mu$ g) was run on a 7.5% (w/v) gel with pH 4.0 at 2.5 mA/tube for 3.5 h in a running buffer (pH 4.5) of  $\beta$ -alanine-acetic acid.

B: SDS-PAGE, the purified enzyme (5.0  $\mu$ g) denatured with SDS was run on a 7.5% (w/v) gel containing 0.10% (w/v) SDS at 6.0 mA/tube for 3.0 h in a running buffer (pH 7.2) of 0.10% (w/v) SDS-0.10 M sodium phosphate.

The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent mixture of ethanol, acetic acid and water at the ratio of 9:2:9.

B(1): molecular weight markers: Lysozyme (14,000), trypsin inhibitor (20,000), carbonic anhydrase (30,000), aldolase (42,000) and bovine serum albumin (79,000).

B(2): purified enzyme.

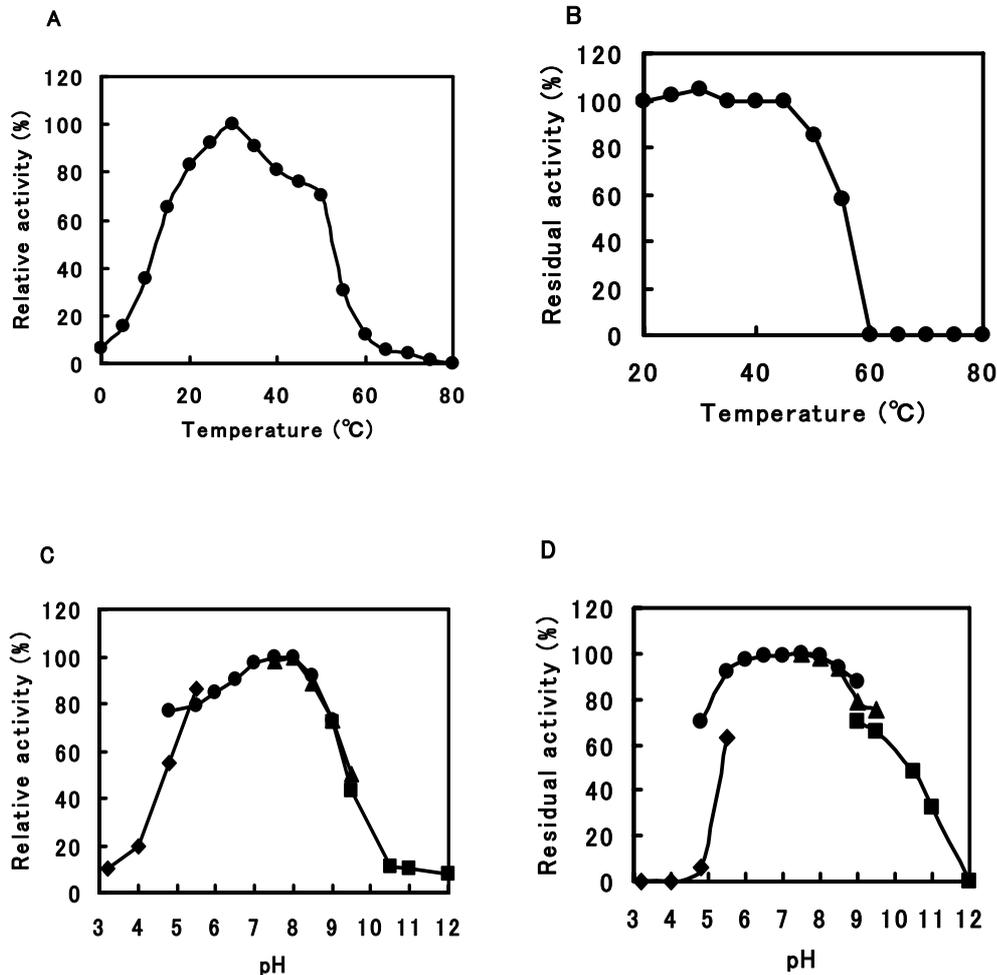


Figure 4. Effects of temperature on activity (A) and stability (B), and effects of pH on activity (C) and stability (D) At the amount of 1.25 % (w/v), Azocasein assay was used for characterization of the protease.

A: Protease activity was measured at 0-80 °C with pH of 7.5 for 10 min.

B: After heat treatment at various temperatures (20-80 °C) with pH of 7.5 for 10 min.

C: The buffer used was: (◆) 100 mM Ac (pH 3.2-5.5); (●) 100 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> (pH 4.8-9.0); (▲) 100 mM Tris/HCl (pH 7.5-9.5) and (■) 100 mM NaCO<sub>3</sub>/NaHCO<sub>3</sub> (9.0-12.0). Maximal activity of protease was defined as 100%. Enzyme assay was carried out at 30 °C for 10 min.

D: pH of the purified protease was adjusted in different buffers: (◆) 100 mM Ac (pH 3.2-5.5); (●) 100 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> (pH 4.8-9.0); (▲) 100 mM Tris/HCl (pH 7.5-9.5) and (■) 100 mM NaCO<sub>3</sub>/NaHCO<sub>3</sub> (pH 9.0-12.0) buffer. The enzyme was incubated at 4°C overnight, then the residual activity of protease was measured at pH 7.5 for 10 min.