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Purification and Characterization of Two Novel Halotolerant Extracellular Proteases from *Bacillus subtilis* Strain FP-133

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Bacillus subtilis **strain FP-133, isolated from a fermented fish paste, synthesized two novel halotolerant extracellular proteases (expro-I and expro-II), showing activity and stability at concentrations of 0–20% (w/v) NaCl. Each protease was purified to homogeneity and characterized. The purified expro-I was a non-alkaline serine protease with an optimum pH of 7.5, although most serine proteases from *Bacillus* strains act at the alkaline side. The molecular mass of expro-I was 29 kDa. The purified expro-II was a metalloprotease with a molecular mass of 34 kDa. It was activated by Fe^{2+}, which has never been reported as a bacterial protease activator. At a concentration of 7.5% (w/v) NaCl, both proteases preferred animal proteins to vegetable proteins as natural substrates. In addition, under saline conditions, expro-I and II showed high catalytic activity toward gelatin and casein respectively.**

Key words: halotolerant protease; *Bacillus subtilis*; neutral serine protease; Fe-containing metalloprotease; endoprotease

The saline fermentation process, involved in the production of various protein-rich foods, is important to prevent putrefaction and development of food poisoning, as well as to produce desired flavor.[1)] That process concerns hydrolysis of protein as the main process. Proteases able to maintain high activities under moderate saline conditions are thus essential. Halophilic proteases are less suitable for this process, because they need at least 12.5% (w/v) NaCl for expression of high activity.[2)] Therefore, halotolerant proteases, which are active at both low and high concentrations of NaCl, are needed.

Several halotolerant proteases have been purified from microorganisms involved in saline food fermentation. Some of them are produced by *Filobacillus* sp. RF2–5 isolated from fish sauce,[3)] *Penicillium chrysogenum* Pg222 from cured ham,[4)] and *Pseudoalteromonas* sp. strain CP7 from soil,[5)] but there have been few reports on halotolerant proteases from the main industrial protease producer *Bacillus subtilis*.[6)]

B. subtilis is a highly favorable bacterium for protease production, because it is non-pathogenic, well explored as a model of gram-positive bacteria, and capable of synthesizing various types of protease.[7–9)] Since *B. subtilis* is classified as a halotolerant bacterium growing well in a range of 0–10% of NaCl, it can be expected to produce several types of halotolerant protease. To obtain an optimum performance of protease under saline conditions, it is necessary that the character of the halotolerant protease is well understood.

In this study, we purified and characterized two halotolerant extracellular proteases (expro-I and expro-II) from *B. subtilis* strain FP-133, isolated previously. The hydrolytic activity of the enzymes for some natural proteins was also measured to determine potential prospects for a wide range of industrial uses.

Materials and Methods

Materials. Casamino acids were purchased from Difco Laboratories (Detroit, MI); yeast extract and Polypepton were from Nihon Seiyaku (Tokyo); casein Hammerstein grade was from ICN Biomedicals (Aurora, OH); *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide was from Sigma-Aldrich (St. Louis, MO); Gly-Leu, was from Peptide Institute (Osaka, Japan); DE52 cellulose and CM52 cellulose were from Whatman (Madison, WI); CM-Toyopearl 650S, Phenyl-Toyopearl 650M, and Toyopearl HW-55 SF were from Tosoh (Tokyo); and other chemicals used were of guaranteed grade.

Strain and culture conditions. Bacillus subtilis strain FP-133 isolated from Terasi, a Southeast Asian traditional fish paste fermented under saline conditions, was used throughout this study. Various carbon and energy sources and nitrogen sources were evaluated to establish the optimum conditions for the production of halotolerant proteases. The basal medium consisted of 5 g NaCl,

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1 g KH_2PO_4, 0.3 g $Na_2HPO_4 \cdot 12H_2O$, and 0.02 g $MgSO_4 \cdot 7H_2O$ in 100 ml H_2O. The pH of the medium was adjusted to 5.5. Carbon and energy sources (1% w/v each at final concentration), including D-arabinose, D-fructose, D-galactose, D-mannose, D-raffinose, D-glucose, lactose, maltose, and glycerol and nitrogen sources (1% w/v) including yeast extract, Polypepton, malt extract, Casamino acids, and casein were added to the basal medium.

Four milliliters of pre-culture was transferred into 400 ml of medium. It was then incubated at 30 °C at 140 rpm on a reciprocal shaker for 36 h. Cell growth was measured by monitoring absorbance at 660 nm (OD_{660}). Protease activity was measured as caseinolytic activity, as described below.

Enzyme assays. Protease (caseinolytic) activity was assayed by a modification of the method of Kunitz.[10] The reaction mixture (800 µl) consisted of 0.5% (w/v) casein Hammerstein grade in 20 mM Tris–HCl buffer (pH 7.3) (buffer A) containing 7.5% (w/v) NaCl. The reaction was started by adding 100 µl of enzyme solution. After incubation for 30 min at 37 °C, the reaction was stopped by adding 450 µl of 10% (w/v) trichloroacetic acid, and then kept on ice for another 10 min, followed by centrifugation at 15,000 × *g* for 10 min. The absorbance of the supernatant was measured against a blank (non-incubated sample) at 280 nm. One unit of activity was defined as the amount of enzyme that made an increase in absorbance of 1 in 1 h under the conditions described. Protein concentrations were measured by the method of Lowry *et al.*[11]

For the kinetic study of protease I (expro-I) produced by strain FP-133, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide was used as a substrate. The reaction mixture, composed of 1 ml of buffer A containing the substrate at various concentrations and 7.5% (w/v) NaCl and 0.15 ml of enzyme (3 µg) solution, was incubated at 45 °C. Absorbance at 410 nm was measured every 5 min to estimate released *p*-nitroaniline.[12] A molar extinction coefficient of 4.0×10^3 for *p*-nitroaniline was used.

The kinetic study of protease-II (expro-II) was carried out using Gly-Leu as a substrate, by a modification of the method of Kessler and Yaron.[13] A reaction mixture composed of 0.8 ml of buffer A containing the substrate at various concentrations, 7.5% (w/v) NaCl, and 0.1 ml of enzyme (2 µg) solution was incubated at 45 °C. The reaction was initiated by adding enzyme solution to the reaction mixture. After 30 min, the reaction was stopped by adding 1 ml each of ninhydrin reagent and 4 M sodium acetate buffer (pH 5.5). The released amino acids were then measured by the ninhydrin method.[12] A molar extinction coefficient of 1.5×10^3 at 570 nm for an equimolar mixture of glycine and L-leucine was used.

Purification of enzymes. All purification steps were performed at 4 °C. The culture, harvested at an early stationary growth phase, was centrifuged at 15,000 × *g* for 10 min to obtain the supernatant as a protease source (fraction 1, 500 ml). Fraction 1 was brought to 85% saturation of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 15,000 × *g* for 10 min and filtration through Whatman filter no. 2 paper (Whatman, Madison, WI) and dissolved in buffer A. The solution was dialyzed overnight against 20 mM Tris–HCl buffer (pH 7.3) containing 10% sucrose (buffer B). The dialyzed solution (fraction 2, 80 ml) was loaded onto a DE52 cellulose column (1.6 × 12.5 cm) previously equilibrated with buffer B. When the column was washed with buffer B, proteases were eluted and active fractions were pooled (fraction 3, 50 ml). The other proteases were not eluted with a linear gradient of 0–0.4 M NaCl in buffer B. Fraction 3 was dialyzed against 20 mM potassium phosphate buffer (pH 6.4) containing 10% (w/v) sucrose (buffer C). The dialyzed solution (fraction 4, 50 ml) was loaded onto a CM52 cellulose column (2.2 × 16.5 cm) equilibrated with buffer C. When the column was washed with buffer C, one peak with protease activity (expro-II) appeared and the active fractions were pooled (fraction 5, 35 ml). Other proteins were eluted with a linear gradient of 0–0.3 M NaCl in buffer C. Another protease (expro-I) was obtained from this chromatography and the active fractions were pooled (fraction 6, 45 ml). Fraction 6 were loaded onto a CM-Toyopearl column (2.2 × 13 cm) equilibrated with buffer C. Proteins were eluted with a linear gradient of 0–0.3 M NaCl in buffer C and the active fractions were pooled (fraction 7, 25 ml). Fraction 7 was loaded onto a Phenyl-Toyopearl column (1.6 × 11.5 cm) equilibrated with buffer C containing 1 M $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient of 1.0–0 M $(NH_4)_2SO_4$. The purity of the enzyme in each fraction was verified by polyacrylamide gel electrophoresis (PAGE). Fractions showing a single band on a polyacrylamide gel were pooled.

Fraction 5, containing expro-II, was loaded onto a Phenyl-Toyopearl column (1.6 × 11.5 cm) equilibrated with buffer C, containing 0.8 M $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient of 0.8–0 M $(NH_4)_2SO_4$, the active fractions were pooled, and the solution was dialyzed overnight against buffer C. The dialyzed solution (fraction 8, 20 ml) was loaded onto a DE52 cellulose column equilibrated with buffer C. When the column was washed with buffer C, expro-II was eluted. Fractions showing a single band on a SDS–polyacrylamide gel were pooled. The other proteases were not eluted with a linear gradient of 0–0.3 M NaCl in buffer C.

Determination of molecular masses. The purified enzyme solution was concentrated to 1.0 ml with a collodion bag (Sartorius, Goettingen, Germany). The concentrated sample and size markers (catalase 210 kDa, γ-globulin 160 kDa, bovine serum albumin 67 kDa, ovalbumin 45 kDa, and myoglobin 17 kDa) were loaded onto a column (2 × 92 cm) of Toyopearl HW-55 SF

equilibrated with buffer C, containing 0.2 M NaCl and eluted with the same solution. The molecular mass of the subunit of the enzymes was determined by SDS–PAGE.[14] An LMW peptide calibration kit for SDS–PAGE (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.) was used for size markers.

Determination of NH_2-terminal amino acid sequence. The purified enzyme was electroblotted by the method of Matsudaira.[15] The NH_2-terminal amino acid sequence was identified with a Shimadzu PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan).

Effects of temperature, pH, and NaCl concentration on the activity and stability of the purified enzymes. The effect of temperature on enzyme activity was determined by the method described above at temperatures from 30 to 80 °C in 5 °C increments. The optimum pH of the enzyme was determined at 37 °C using the following 20 mM buffers: sodium acetate (pH 3.0–5.5), sodium-potassium phosphate (pH 5.0–8.0), Tris–HCl (pH 7.0–9.5), and sodium carbonate-bicarbonate (pH 9.0–11.0). The effect of NaCl concentration on caseinolytic activity was tested using 20 mM sodium-potassium phosphate buffer (pH 6.5) containing 0–20% (w/v) NaCl.

The effect of temperature on the stability of the enzyme was determined by incubating the enzyme (7.5 μg) at 0–75 °C for 10 min. The remaining activity was then measured under optimum conditions. The effect of pH on the stability of the enzyme was determined by dialyzing the enzyme (7.5 μg) against buffers with various pHs at 4 °C overnight, and the remaining activity was measured. For the study of halostability, the enzyme (7.5 μg) was incubated in buffer B, containing 0–20% (w/v) NaCl at 4 °C for 24 h. The remaining activity was assayed.

Substrate specificity. The reaction mixture consisted of 0.4% (w/v) protein in 400 μl of 50 mM sodium phosphate buffer (pH 6.5) containing 7.5% (w/v) NaCl and 150 μl of enzyme (4 μg) solution. After incubation at 37 °C for 1 h, 0.1 ml of the mixture was withdrawn and the increase in the amount of free amino groups was determined by the ninhydrin method.[12] The activity for each substrate was expressed as the amount of amino group (μmol as glycine) per h·μg·enzyme.

The preparation of natural substrates for expro-I and II was initiated with inactivation of possible enzymes contained in the materials by heating at 120 °C for 10 min. The materials were ground in order to extract proteins with 20 mM Tris–HCl buffer (pH 7.3). After filtration of the extracts with Whatman filter no. 2 paper, the protein concentrations of the filtrate were estimated by the method of Lowry *et al.*[11]

Assay of iron. The Fe content in expro-II was measured with a Hitachi polarized Zeeman 180–80 atomic absorption spectrophotometer (Hitachi, Tokyo).

Results

Effects of carbon, energy, and nitrogen sources on protease production

Among the nine carbon and energy sources tested, lactose and D-galactose promoted the production of extracellular proteases in the presence of 1% (w/v) casein as nitrogen source, although the two substrates did not increase the cell growth of *B. subtilis* strain FP-133. On the other hand, the production of the proteases was not increased by D-glucose or D-fructose, despite promotion of cell growth. We observed that 1% (w/v) lactose was optimal for the production of halotolerant extracellular proteases.

Then we tested the effect of nitrogen source on protease production in the medium containing 1% (w/v) lactose as carbon and energy source. Of the five nitrogen sources tested, yeast extract led to the highest activity of protease in the culture. Polypepton promoted protease production moderately. Then we examined the optimum combination of yeast extract and Polypepton to give the highest protease production. The culture medium containing 1% (w/v) each of yeast extract and Polypepton was most favorable for the production of proteases and their recoverability; when *B. subtilis* strain FP-133 was incubated in this medium, proteins including proteases in the culture were precipitated with $(NH_4)_2SO_4$ without difficulty. On the basis of these results, the medium containing 1% (w/v) each of lactose, yeast extract, and Polypepton was established for the production of extracellular proteases. Under these conditions, OD_{660} of 9 in the cell growth and units/OD_{660} of 540 in the production of proteases were obtained. On the other hand, in the original medium containing 1% (w/v) D-glucose as carbon and energy source and 1% (w/v) casein as nitrogen source, OD_{660} of 11 in the cell growth and units/OD_{660} of 21 in the production of proteases were obtained, showing that under the optimum conditions, the protease productivity of strain FP-133 increased 26-fold.

Purification of proteases and their molecular properties

Table 1 is a summary of a typical enzyme purification for expro-I and II from *B. subtilis* strain FP-133. The specific activities of the final preparations of expro-I and II were 270 and 120 units per mg with overall recoveries of 6 and 0.7% respectively. The final enzyme preparations of expro-I and II showed 100- and 44-fold increases respectively in their specific activities. The final preparation of expro-I showed single protein bands on polyacrylamide and SDS–polyacrylamide gels (Fig. 1). That of expro-II also showed a single band on SDS–polyacrylamide gel. Expro-II was not observed by native PAGE using pH 9.5,[16] 8.0,[17] or 4.3[18] gels.

The molecular masses of expro-I and II were 29 and 33 kDa by gel filtration on Toyopearl HW-55S and 29 and 34 kDa by SDS–PAGE respectively. These findings indicate that the two proteases are monomers.

Table 1. Summary of Purification of Expro-I and II

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	7,200	2,700	2.7	100
$(NH_4)_2SO_4$	1,400	150	5.3	20
DE52	1,100	26	42	15
Expro-I				
CM52	680	18	38	9
CM-Toyopearl	440	7.6	58	6
Phenyl-Toyopearl	460	1.7	270	6
Expro-II				
CM52	290	11	27	4
Phenyl-Toyopearl	96	0.7	140	1.3
2nd DE52	49	0.4	120	0.7

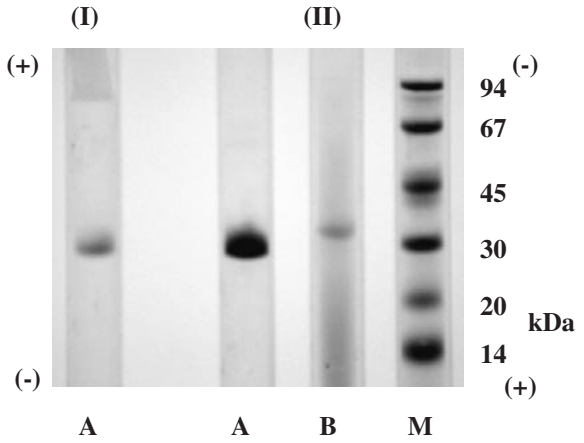

Fig. 1. Native PAGE (I) and SDS–PAGE (II) of Purified Expro-I (A) and Expro-II (B).

I, Native PAGE. The purified enzyme (10 µg) was run on 7.5% (w/v) gels of pH 4.3 in a running buffer (pH 4.5) of β-alanine and acetic acid.[18] II, SDS–PAGE. The purified enzymes (10 µg each) and markers (M) were run on 12.5% (w/v) gels containing 0.1% (w/v) SDS in sodium phosphate buffer (pH 7.2).[14] The gels used for native PAGE and SDS–PAGE were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent of ethanol–acetic acid–H_2O (9:2:9, v/v).

General properties of the purified proteases

The optimum pH, effect of temperature on enzyme activity, pH stability, thermostability, kinetic values, and N-terminal amino acid sequences of the purified expro-I and II are listed in Table 2. Based on the DDBJ database, the N-terminal amino acid sequence of expro-I showed 76% identity to pro-subtilisin from *Bacillus* sp. DJ-4,[19] under accession no. AY627764, whereas the N-terminal amino acid sequence of expro-II did not show any identity to those from other proteins. Because of the optimum pH for activity, the two proteases exhibited the characteristics of neutral protease.

Effect of salt concentration on the activity and stability of the proteases

As Fig. 2 shows, expro-I and II maintained significant activities at a concentration of 20% (w/v) NaCl, although they exhibited the highest activity in the absence of NaCl. In addition, high salt concentrations were still favorable for the storage of the two enzymes. Expro-I maintained 100% activity even when it was stored in 20% (w/v) NaCl for 24 h at 4 °C, whereas expro II kept 76% activity under the same conditions.

Effects of inhibitors, denaturing agents, and metal ions on enzyme activity

Expro-I was strongly inhibited by serine protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), and chymostatin (Table 3). However, other serine protease inhibitors, chymotrypsin-like serine protease inhibitor, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), and leupeptin, did not inhibit the protease. The metalloprotease inhibitor EDTA, and the cysteine protease inhibitors *p*-chloromercuribenzoic acid (PCMB), ICH_2COOH, and $HgCl_2$ did not inhibit expro-I. In addition, expro-I was strongly inhibited by the denaturing and reducing agents SDS, dithiothreitol (DTT), and β-mercaptoethanol, but not by urea (Table 3). On the other hand, expro-II was markedly inhibited by the metalloprotease inhibitor EDTA. Expro-II was inhibited

Table 2. Characteristics of Expro-I and II

Parameter	Expro-I	Expro-II
Protease class	Serine protease	Metalloprotease
Molecular mass	29 kDa (native) 29 kDa (denatured)	33 kDa (native) 34 kDa (denatured)
Optimum pH	7.5	8
Temperature with highest activity	60 °C	45 °C
pH stability (≥ 70% act.)	5.5–10	5.5–9
Thermostability	70 °C	50 °C
Kinetic value	K_m: 0.72 mM V_{max}: 1.2 mM *p*-nitroaniline/min·mg	K_m: 50 mM V_{max}: 270 µM glycine or L-leucine/min·mg
NH_2-terminal amino acid sequence	AESVPYGVSEIKAPAL	ADATGXGGNQXTGQNY

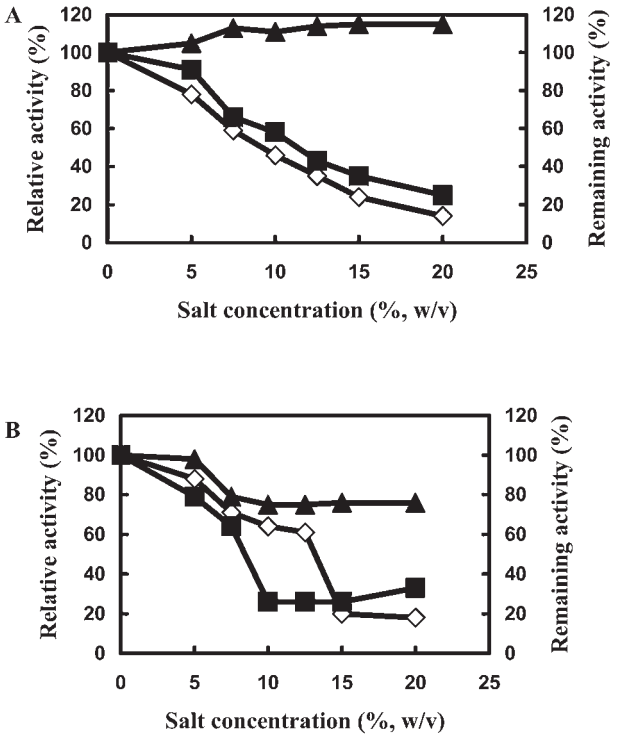

Fig. 2. Effects of Salt Concentration on the Activity and Stability of Expro-I (A) and Expro-II (B).

The activities of expro-I and II were measured at various concentrations of KCl (■) and NaCl (◇) at 37 °C at pH 7.5. The enzymes were incubated at various concentrations of NaCl (▲) at 4 °C overnight, and then the remaining activity was measured in the presence of 7.5% (w/v) NaCl.

by DTT and slightly by SDS. Expro-I and II were not inhibited by the aspartic protease inhibitors pepstatin A, phenacyl bromide,[20] or *n*-octyl alcohol.[20,21]

Among the metal ions tested, expro-I was strongly inhibited by Ni^{2+}, Ca^{2+}, Cu^{2+}, and Zn^{2+} (Table 4). On the other hand, expro-II was not much inhibited by these metal ions. The activity of expro-II increased in the presence of Fe^{2+}. When expro-II was treated with 10 mM EDTA, its activity was completely lost, but was recovered by the addition of Fe^{2+}, Fe^{3+}, and Zn^{2+} (Table 4). Other metal ions examined did not recover the activity of the apoenzyme. Then we analyzed the iron in expro-II and found that the protease contained 1.0 mol of Fe per mol of protein.

Substrate specificity

Expro-I and II hydrolyzed gelatin, casein, ovalbumin, and bovine serum albumin, with the highest activity for gelatin and casein respectively (Table 5). Among the natural substrates tested, mackerel meat, shrimp meat, and tofu proteins were more susceptible to hydrolysis by expro-I and II than the other proteins (Table 5).

Discussion

In this study, we found two extracellular halotolerant proteases, expro-I and II, in the culture of *B. subtilis*

Table 3. Effects of Inhibitors and Denaturing and Reducing Agents on the Activities of Expro-I and II

Compound	Relative activity (%)	
	Expro-I	Expro-II
Inhibitor[a]		
None	100	100
PMSF	0	125
Chymostatin	19	79
TLCK	95	104
Leupeptin	95	82
DFP	0	118
PCMB	104	107
ICH_2COOH	96	86
$HgCl_2$	98	110
EDTA 2Na	71	25
Pepstatin A	94	82
n-Octyl alcohol	94	84
Phenacyl bromide	91	97
Denaturing and reducing agent[b]		
None	100	100
SDS	15	78
Urea	136	100
DTT	9	43
β-mercaptoethanol	1	67

[a]A reaction mixture (500 µl) containing 0.5% (w/v) casein, enzyme (4 µg), 7.5% (w/v) NaCl, and 1 mM of each inhibitor in 50 mM sodium phosphate buffer (pH 6.5) was incubated at 37 °C for 30 min. The remaining activity was then measured, as described in the text.

[b]A reaction mixture (400 µl) containing 0.5% (w/v) casein, enzyme (4 µg), 7.5% (w/v) NaCl, and 2% (w/v) SDS, 4 M urea, 0.1% (w/v) DTT, or 0.1% (v/v) β-mercaptoethanol in 50 mM sodium phosphate buffer (pH 6.5) was incubated at room temperature for 1 h. The remaining activity was then measured.

Table 4. Effects of Metal Ions on the Activities of Expro-I and II[a]

Metal ion	Relative activity (%)	
	Expro-I	Expro-II
Holoenzyme		
None	100	100
Fe^{2+}	110	121
Fe^{3+}	103	83
Mn^{2+}	85	106
Mg^{2+}	69	74
Ni^{2+}	7	60
Ca^{2+}	11	97
Cu^{2+}	0	38
Zn^{2+}	0	81
Apoenzyme[b]		
None	—[c]	0
Fe^{2+}	—	65
Fe^{3+}	—	74
Mn^{2+}	—	0
Zn^{2+}	—	14

[a]The experiments were carried out as described in Table 3, using 1 mM of metal ions.

[b]Apoenzyme was prepared by incubating expro-II (5 µg) with 10 mM EDTA·2Na at 4 °C for 1 h, when enzyme activity was completely lost. After removal of EDTA·2Na by dialysis, the apoenzyme was dialyzed against a buffer containing 1 mM of each metal ion for 4 h at 7 °C, and then the recovered enzyme activity was measured.

[c]The apoenzyme of expro-I was not prepared.

Table 5. Substrate Specificity of Expro-I and II

Substrate	μmol Gly/h·μg·enzyme	
	Expro-I	Expro-II
Casein	44	42
Gelatin	52	36
Bovine serum albumin	25	17
Ovalbumin	18	9.1
Mackerel meat protein	21	19
Shrimp meat protein	11	17
Shrimp shell protein	1.0	7.0
Soybean protein	0.19	6.1
Tofu[a] protein	6.1	11
Okara[b] protein	1.8	4.5
Beef protein	11	5.1

[a]A traditional Japanese food made from soybeans; soybean curd.
[b]Waste produced in the process of tofu manufacture.

strain FP-133, and they were purified to homogeneity. The purified proteases showed activity at concentrations of 0–20% (w/v) NaCl. Some reports state that *B. subtilis* secretes several types of extracellular protease, including the alkaline serine protease subtilisin, neutral protease A, bacillopeptidase F, metalloprotease, neutral protease B, and cell wall-associated extracellular protease.[9,22,23] However, only one halotolerant protease from *Bacillus subtilis* has been reported.[6] It acts optimally on the alkaline side, although this bacterium was apparently equipped with halostress response mechanisms like K^+ uptake and synthesis of compatible solutes.[24] This is the first report describing neutral halotolerant proteases produced by a *B. subtilis* strain, because expro-I and II had an optimal pH between 7.5 and 8, which is favorable for industrial use.

Lactose and D-galactose promoted more protease production by strain FP-133 than D-glucose or glycerol, although lactose and D-galactose did not increase its cell growth. In *Penicillium expansum*[25] and *Yersinia ruckeri*,[26] the synthesis of extracellular proteases is promoted under malnutrition conditions rather than the best condition for cell growth. In these microorganisms, including strain FP-133, protease production appears to be sensitive to catabolite repression by rapidly assimilable sugars such as D-glucose, D-fructose, and glycerol.

Expro-I was strongly inhibited by a broad spectrum of serine protease inhibitors. In addition, the NH_2-terminal amino acid sequence of expro-I showed 76% identity to pro-subtilisin from *Bacillus* sp. DJ-4.[19] Therefore, expro-I was classified as a serine protease. The reducing agents β-mercaptoethanol and DTT inhibited the enzyme. β-Mercaptoethanol has been reported to stabilize cysteine proteases by protecting the oxidation of sulfhydryl groups in proteins, but β-mercaptoethanol would cause the alteration of structure of enzymes by reducing disulphide bonds in the proteins.[27] Expro-I probably lost its activity due to alteration of the protein structure in the presence of β-mercaptoethanol and DTT.

The optimum pH for expro-I was 7.5. This is different from that of most serine proteases from *B. subtilis*, which show an optimum pH of 10.[7,23,28]

Expro-I and II showed high activity for gelatin, casein, albumin, ovalbumin, and bovine serum albumin, indicating that the two enzymes are endoproteases.

Expro-II was strongly inhibited by EDTA, and its identity as a metalloprotease was confirmed by treating holo- and apo-types of this enzyme with metal ions (Table 4). The prominent roles of Fe^{2+} in the enhancement of activity of the holoenzyme and in the recovery of activity of the apoenzyme were observed. In addition, this enzyme contained 1 mol of Fe per mol of protein. This is the first report on a bacterial metalloprotease containing Fe and activated by Fe^{2+}. Several proteases from animals[29] and *Saccharomyces cerevisiae*[30] have been reported to retain Fe^{2+} in their protein molecules. There were no NH_2-terminal amino acid sequences up to 16 residues of other proteins showing identity to that of expro-II.

At any given pH, expro-II was adsorbed on neither anion nor cation exchangers. This feature was in accord with the failure of expro-II protein bands to appear on pH 9.5, 8.0, and 4.3 gels in native PAGE. However, when expro-II was run on the hydrophobic resin Phenyl-Toyopearl, it was adsorbed on the resin and eluted with $(NH_4)_2SO_4$. These findings suggest that expro-II is dominated by small uncharged and hydrophobic amino acid residues, such as L-alanine and glycine, which probably bring the low net electric charges and hydrophobicity to the protein molecule of expro-II. In the near future, we will clone the gene encoding expro-II and determine the complete amino acid sequences of the protein. Another explanation for these characteristic has been suggested by Scopes,[31] who describes slippery proteins that are difficult to bind to any adsorbents due to non-electrostatic interactions and uneven distribution of charges over protein surfaces.

When we used Gly-Leu, a K_m value of 50 mM was obtained, which is higher than that of expro-I for *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, although different substrates of peptide were used (Table 2). These results suggest that expro-II has low affinity for peptides as compared with expro-I.

The halotolerant proteases expro-I and II showed a broad range of NaCl concentrations for the expression of activity and stability (Fig. 2) as compared with halophilic proteases. For example, an extracellular serine protease from the halophilic archaeon *Natrialba magadii* does not show any activity at a concentration less than 5.8% (w/v) NaCl.[32] Some reports suggest that the amino acid composition of a protein determines its halophilism or halotolerance.[33] In general, the halophilism of an enzyme strengthens in the predominant existence of acidic amino acid residues, an increased amount of small hydrophobic amino acid residues such as glycine, L-alanine, and L-valine, and a decreased amount of aliphatic amino acid residues, but no reports describing the characteristics of amino acid composition in halotolerant enzymes have been published. They will

be elucidated by determining the complete amino acid sequences of expro-I and II produced by *B. subtilis* strain FP-133.

At a concentration of 7.5% (w/v) NaCl, expro-I and II preferred to hydrolyze animal proteins rather than vegetable proteins. These results suggest the application of the proteases in fish- or meat-based food fermentation. In addition, gelatin was markedly susceptible to hydrolysis by the proteases. Since gelatin is a denatured form of collagen, the two proteases should be useful for the preparation of hydrolyzates from collagen, which is needed in the pharmaceutical, cosmetic, and food industries.

References

1) Margesin, R., and Schinner, F., Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles*, **5**, 73–83 (2001).
2) Ventosa, A. J., Nieto, J., and Oren, A., Biology of moderately halophilic aerobic bacteria. *Micribiol. Mol. Biol. Rev.*, **62**, 504–554 (1998).
3) Hiraga, K., Nishikata, Y., Namwong, S., Tanasupawat, S., Takada, K., and Oda, K., Purification and characterization of serine protease from a halophilic bacterium *Filobacillus* sp. RF2–5. *Biosci. Biotechnol. Biochem.*, **69**, 38–44 (2005).
4) Benito, M. J., Rodriguez, M., Nunez, F., Asensio, M. A., Bermudez, M. E., and Cordoba, J. J., Purification and characterization of an extracellular protease from *Penicillium chrysogenum* Pg222 active against meat proteins. *Appl. Environ. Microbiol.*, **68**, 3532–3536 (2002).
5) Sanchez-Porro, C., Mellado, E., Bertoldo, C., Antranikian, G., and Ventoza, A., Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76. *Extremophiles*, **7**, 221–228 (2003).
6) Kembhavi, A. A., Kulkarni, P., and Pant, A., Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM no. 64. *Appl. Biochem. Biotechnol.*, **38**, 83–92 (1993).
7) Adinarayana, K., Ellajah, P., and Prasad, D. S., Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS Pharm Sci. Tech.*, **4**, 1–9 (2003).
8) Kawamura, F., and Doy, R. H., Construction of *Bacillus subtilis* double mutant deficient extracellular alkaline and neutral protease. *J. Bacteriol.*, **160**, 442–444 (1984).
9) Valbuzzi, A., Ferrari, E., and Albertini, A. M., A novel member of the subtilisin-like protease family from *Bacillus subtilis*. *Microbiol.*, **145**, 3121–3127 (1999).
10) Kunitz, M., Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen. Physiol.*, **30**, 291–310 (1947).
11) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
12) Prescott, J. M., and Wilkes, H., *Aeromonas* aminopeptidase. In "Methods in Enzymology" Vol. XLV part B, ed. Lorand, L., Academic Press, New York, pp. 530–543 (1976).
13) Kessler, E., and Yaron, A., Extracellular aminopeptidase from *Clostridium histolyticum*. In "Methods in Enzymology" Vol. XLV part B, ed. Lorand, L., Academic Press, New York, pp. 544–545 (1976).
14) Weber, K., and Osborn, M., The reliability of molecular weight determination by dodecyl sulfate–polyacrylamide gel electrophoresis. *J. Biol. Chem.*, **244**, 4406–4412 (1969).
15) Matsudaira, P., Sequence from picomole quantities of protein electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.*, **262**, 10035–10038 (1987).
16) Davis, B. J., Disc electrophoresis. II. Method and application to human serum proteins. *Ann. NY Acad. Sci.*, **121**, 404–427 (1964).
17) Williams, D. E., and Reisfeld, R. A., Disc electrophoresis in polyacrylamide gels: extension to new conditions of pH and buffer. *Ann. NY Acad. Sci.*, **121**, 373–381 (1964).
18) Reisfeld, R. A., Lewis, U. J., and Williams, D. E., Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*, **195**, 281–283 (1962).
19) Choi, N. S., Chang, K. T., Maeng, P. J., and Kim, S. H., Cloning, expression and fibrinogenolytic properties of a subtilisin DJ-4 gene from *Bacillus* sp. DJ-4. *FEMS Microbiol. Lett.*, **236**, 325–331 (2004).
20) Bankus, J. M., and Bond, J. S., Some commercially available proteases. In "Proteolytic Enzymes, a Practical Approach," eds. Beynon, R., and Bond, J. S., Oxford University Press, New York, p. 308 (2001).
21) Tang, J., Competitive inhibition of pepsin by aliphatic alcohols. *J. Biol. Chem.*, **240**, 3810–3815 (1965).
22) Park, C. H., Lee, S. J., Lee, S. G., Lee, W. S., and Byun, S. M., Hetero- and autoprocessing of the extracellular metalloprotease (Mpr) in *Bacillus subtilis*. *J. Bacteriol.*, **186**, 6457–6464 (2004).
23) Wu, X. C., Lee, W., Tran, L., and Wong, S. L., Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.*, **173**, 4952–4958 (1991).
24) Boch, J., Kempf, B., Schmid, R., and Bremer, E., Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the *gbsAB* genes. *J. Bacteriol.*, **178**, 5121–5129 (1996).
25) Dahot, M. U., Cultivation of *Penicillium expansum* on rice husk powder for protease production. *J. Islamic Academic Sci.*, **6**, 29–33 (1993).
26) Secades, P., and Guijarro, J. A., Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions in production. *Appl. Environ. Microbiol.*, **65**, 3969–3975 (1999).
27) Scopes, R. K., "Protein Purification: Principle and Practice," Springer-Verlag, New York, pp. 195–196 (1982).
28) Rao, M. B., Tanksale, A. M., Ghatge, M. S., and Deshpande, W., Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.*, **62**, 597–635 (1998).
29) Gifford, A. H. T., Klippenstein, J. R., and Moore, M. M., Serum stimulates growth and proteinase secretion by *Aspergillus fumigatus*. *Infect. Immun.*, **70**, 19–26 (2002).
30) Hiromura, M., and Sakurai, H., Intracellular metal transport proteins. *RIKEN Rev.*, **35**, 23–25 (2001).
31) Scopes, R. K., "Protein Purification: Principle and

Practice," Springer-Verlag, New York, pp. 67–90 (1982).
32) Gimenez, M. I., Studdert, C. A., Sanchez, J. J., and De Castro, R. E., Extracellular protease of *Natrialba magadii*: purification and biochemical characterization. *Extremophiles*, **4**, 181–188 (2000).
33) Madern, D., Ebel, C., and Zaccai, G., Halophilic adaptation of enzymes. *Extremophiles*, **4**, 91–98 (2000).