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**Purification and characterization of a halotolerant intracellular protease from *Bacillus subtilis* strain FP-133**

[Running title: Halotolerant intracellular protease]

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**Abstract**

A halotolerant strain FP-133, able to grow at concentrations of 0-12.5% (w/v) NaCl, was isolated from a fish paste and identified as *Bacillus subtilis*. *B. subtilis* strain FP-133 produced an intracellular protease which showed catalytic activity under saline conditions. The enzyme was purified to homogeneity 143-fold with a yield of 0.9%. The purified enzyme showed an optimum activity at a concentration of 5% (w/v) NaCl. After storage in 7.5% (w/v) NaCl at 4°C for 24 h, the enzyme kept 100% of its activity. The molecular mass of the protease was determined to be 59 kDa by gel filtration; the protein consisted of four subunits each with a molecular mass of 14 kDa. The enzyme showed aminopeptidase activity. It acted on L-leucyl-*p*-nitroanilide, L-leucyl-*β*-naphthylamide, and oligopeptides containing glycine, L-histidine, or L-leucine. The $K_m$ and $V_{max}$ values for L-leucyl-*p*-nitroanilide were 18 μM and 2.2 mM/h mg, respectively. The enzyme was activated by $Fe^{2+}$, $Fe^{3+}$, and $Ni^{2+}$ in synergism with $Mg^{2+}$.

*Keywords*: Intracellular protease; Halotolerant; *Bacillus subtilis*; Aminopeptidase; Exoprotease

## Introduction

A saline environment produces stress to non-halophilic microorganisms by decreasing water activity and increasing $Na^+$ concentration inside the cells (PADAN and KRULWICH 2000). However, some of these microorganisms can tolerate and grow from 0 to 10% (w/v) of NaCl. These microorganisms are classified as halotolerant microorganisms (KUSHNER 1978).

The extracellular proteases produced by halotolerant microorganisms have been reported to show halotolerance (STUDDERT *et al*. 1997; BENITO *et al.* 2002; SANCHEZ-PORRO *et al.* 2003). However, there is no report about the halotolerance of intracellular proteases from these microorganisms, although obviously the cell membrane is not able to completely prevent the internal increase and adverse effect of $Na^+$ (PADAN and KRULWICH 2000). Halophilic intracellular proteases, with distinguished characteristics in amino acid compositions and inability to work in the absence of salt have been reported (SEITZ *et al.* 1997; WILSON *et al.* 1999).

Thus, this study was aimed to obtain information on the halotolerance of an intracellular protease from a halotolerant microorganism. The isolate, *B. subtilis* strain FP-133 from a fish paste, produced an intracellular protease in addition to extracellular ones. This study deals with the purification and characterization of the halotolerant intracellular protease. Since an intracellular protease plays an essential role in the physiological process of a cell, the result of this study will enrich the understanding of the salt tolerant mechanism of halotolerant bacteria.

## Materials and methods

**Screening of halotolerant protease producing bacteria:** Approximately 1 g of fermented food or soil collected from Hyogo prefecture areas in Japan was suspended in 10 ml of 0.8% (w/v) NaCl solution; one drop of the suspension was spread on a plate of skim milk medium containing 5 g NaCl, 2.5 g skim milk, 1 g D-glucose, 0.5 g yeast extract (Nihon Seiyaku, Tokyo, Japan), 1 g $KH_2PO_4$, 0.3 g $Na_2HPO_4 \cdot 12H_2O$, 0.1 g $NH_4NO_3$, 0.02 g $MgSO_4 \cdot 7H_2O$, and 1.5 g agar in 100 ml deionized water. The plate was incubated at 30°C for 3-5 days. Each bacterial colony surrounded by a clear zone was transferred to a skim milk medium slant. The isolated strains were then cultivated in 10 ml of casein medium, in which skim milk was replaced by 1% (w/v) casein (Wako Pure Chemical Industries, Osaka, Japan) without agar. After 24 h of cultivation, cultures were centrifuged and the supernatant was then used for the measurement of protease activity. Of the isolates, strain FP-133 showed the highest activity of extracellular protease at a concentration of 15% (w/v) NaCl and was used for further studies.

**Identification of strain FP-133:** Morphological and biochemical parameters, such as Gram reaction, flagella type, catalase and oxidase activities, and sugar assimilation tests were determined using conventional methods (KOMAGATA 1985). Quinones were analyzed as described by COLLINS and JONES (1981). The cellular fatty acid composition was determined by the MIDI (Sherlock Microbial Identification System) method (MILLER

1982). The 16S rRNA gene was sequenced by the method reported by EDWARDS *et al.* (1989).

**Effects of NaCl concentrations on the growth of strain FP-133 and production of intracellular and extracellular proteases:** Ten milliliters of casein medium containing 0, 3, 5, 10, 15, or 20% (w/v) of NaCl was inoculated with strain FP-133 and then incubated at 30°C for 24 day. The culture was then transferred to 400 ml of the same medium in a 3 l flask and cultivated at 30°C with shaking at 140 rpm for 24 h. Growth was monitored by measuring the optical density at 660 nm ($OD_{660}$). In addition, cells were harvested by centrifugation at 15,000×*g* for 10 min, followed by washing with 0.8% (w/v) NaCl solution. The supernatant of the culture was collected for the assay of extracellular protease. The washed cells were disrupted with a Kubota 201M ultrasonic oscillator (Kubota Shoji, Tokyo, Japan) three times for 5 min. at 180 W. After removing intact cells and cell debris, the supernatant, designated as cell extracts, was obtained and used for the assay of intracellular protease.

**Culture conditions for the production of intracellular protease:** Ten milliliters of modified casein medium containing 5% (w/v) NaCl was inoculated with strain FP-133 and then incubated at 30°C for 1 day. The culture was transferred to 400 ml of the same medium in a 3-l flask. The cultivation, cell harvest, and cell extracts preparation were carried out essentially as mentioned above.

**Enzyme assays:** Protease activity was assayed by the modified method of KUNITZ (1947). The reaction mixture (800 μl) consisted of 0.5% (w/v) casein (Hammersten grade; ICN Biomedicals, Inc., OH, USA) in 20 mM Tris-HCl buffer pH 7.3 (buffer A) containing 7.5% (w/v) NaCl. The reaction was started by adding 150-300 μl of enzyme solution. After incubation for 45-60 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 at 280 nm. One unit of activity was defined as the amount of enzyme that increased an absorbance of 1 in 1 h under the described conditions. Protein concentrations were measured by the method of LOWRY *et al.* (1951).

Aminopeptidase activity was assayed by the method of BENITO *et al.* (2002). The nitroanilide derivative of L-leucine (Wako Pure Chemical Industries) was used as a substrate. The reaction mixture containing 0.2 ml of purified enzyme solution (50 μg/ml), 0.4 ml of substrate (various concentrations), and 0.4 ml of methanol was incubated at 45°C for 3 h. The reaction was stopped by adding 0.5% (w/v) acetic acid. *p*-Nitroaniline released was measured spectrophotometrically at 390 nm. The molar extinction coefficient of $4.4 \times 10^3$ /M·cm for *p*-nitroaniline was used. The $K_m$ and $V_{max}$ values were determined using the double reciprocal of the Michaelis-Menten equation according to the Hans Lineweaver-Burk formula.

**Purification of intracellular protease:** All enzyme purification steps were carried out at 4°C. Cells harvested at the late exponential phase ($OD_{660}$ of 10-12) were stored at -20°C.

The frozen cells were thawed and suspended in 10 volumes of buffer A. Cell disruption and cell extract preparation were carried out as mentioned above.

The cell extracts were brought to 40% saturation with $(NH_4)_2SO_4$ and centrifuged at 15,000×*g* for 15 min. The precipitate dissolved in buffer A was dialyzed against buffer A containing 7.5 % (v/v) glycerol (buffer B). An extra centrifugation step was needed to remove insoluble proteins from the dialyzate.

The clear dialyzed solution was applied onto a DE52 cellulose (Whatman, Madison, Wis., USA) column (2.7×22 cm) equilibrated previously with buffer B. Proteins were eluted with a linear gradient of 0-0.4 M NaCl in buffer B. Active fractions were pooled and dialyzed overnight against buffer B.

The dialyzed solution was applied onto a DEAE-Cellulofine A-800 (Seikagaku Kogyo, Tokyo) column (2.2×15 cm) equilibrated previously with buffer B. Proteins were eluted with a linear gradient of 0-0.3 M NaCl in buffer B. The purity of the enzyme in each fraction was determined by PAGE. Fractions showing a single protein band on a gel were pooled.

**Determination of molecular mass:** The purified enzyme solution was concentrated to 1.0 ml with a collodion bag (Sartorious, Goettingen, Germany). The concentrated sample and size markers were loaded onto a column (2x92 cm) of Toyopearl HW-55 SF (Tosoh, Tokyo) equilibrated with buffer B containing 0.2 M NaCl and eluted with the same buffer. Size markers used were purchased from Boehringer Manheim (Manheim, Germany). The molecular mass of the subunit of the enzyme was determined by SDS-PAGE on 12.5%

(w/v) polyacrylamide gels (WEBER and OSBORN 1969). The LMW and peptide calibration kit for SDS (Amersham Bioscience, Piscataway, NJ, USA) were used as size markers.

**Determination of $NH_2$-terminal amino acid sequence:** The purified enzyme was electroblotted onto a PVDF sequencing membrane Immobilon membrane (Nihon Millipore Kogyo, Yomezawa, Japan) by the method of MATSUDAIRA (1997). The $NH_2$-terminal amino acid sequence was identified with a Shimadzu PPSQ-10 protein sequencer (Kyoto, Japan).

**Effects of temperatures, pHs, and salt concentrations on the activity and stability of the enzyme:** The effect of temperatures on protease activity was determined as above at temperatures from 30 to 80°C in 5°C increments. The optimum pH of the enzyme was determined at 60°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 effects of NaCl and KCl concentrations on the activity were tested using 20 mM sodium-potassium phosphate buffer (pH 6.5) containing 0-20% (w/v) NaCl.

The effect of temperatures on the enzyme stability was tested by incubating purified enzyme (7.5 μg) at 0-70°C for 15-60 min. The remaining activity was then measured under the optimum conditions. The effect of pH on the enzyme stability were tested by dialyzing purified enzyme (7.5 μg) against buffer with various pHs, overnight at 4°C and measuring the remaining activity. For the study of halostability, the purified enzyme (7.5 μg) was

incubated in buffer A containing 0-20% (w/v) NaCl at 4°C for 24 h. The remaining activity was assayed at a concentration of 7.5% (w/v) NaCl.

## Results

### *Screening and identification of halotolerant protease producing bacteria*

The isolated strain FP-133 was a gram-positive and spore-forming rod of 0.7x2.3-3.1 μm. It was motile with peritrichous flagella and showed oxidase activity, but not catalase activity. Strain FP-133 produced acid from sucrose, D-glucose, D-fructose, D-mannose, and lactose. The predominant quinone (more than 83% of total quinones) in the cell was menaquinone with seven units of isoprenoid (MK-7). The cellular fatty acid composition of the strain was dominated by *iso*-$C_{15}$ (24.2%), *anteiso*-$C_{15}$ (37.0%), and *iso*-$C_{17}$ (13.8%). These properties were traits displayed by the genus of *Bacillus* (CLAUS and BERKELEY 1986). This was confirmed by the analysis of sequenced 16S rRNA gene (1,541 bp, DDBJ, EMBL, and GenBank nucleotide sequence databases with accession no. AB 192294), which was 99.5, 99.5, and 99.4% identical to those of three *Bacillus subtilis* strains (accession nos. E37857, AB110598, and AB06537). The strain FP-133 was thus identified as *Bacillus subtilis*.

We found protease activity in the cells of *B. subtilis* strain FP-133, in addition to the culture fluid.

*Effects of NaCl concentrations on the growth of strain FP-133 and protease production*

Of the NaCl concentrations tested, the strain reached the maximum growth at a concentration of 5% (w/v) NaCl within 24 h of cultivation (Fig. 1). The decrease in growth became significant at concentrations of 15 and 20% (w/v) NaCl. Although the strain grew well in the absence of NaCl, the production of halotolerant intracellular protease was little observed. The enzyme was produced in the presence of NaCl, and maximally at a concentration of 5% (w/v) NaCl.

On the other hand, the high production of halotolerant extracellular protease was observed at a concentration of 0-5% (w/v) NaCl . When the cells were cultivated at a concentration of 20% (w/v) NaCl, the production of extracellular protease was hardly observed. The different production pattern of the two fractions of protease indicates that the halotolerant extracellular protease is different from the intracellular one. We tried to purify and characterize the halotolerant intracellular protease in this study.

Figure 2 shows that at a concentration of 5% (w/v) NaCl strain FP-133 produced the intracellular protease exponentially phase with a specific activity of 49 mU/mg protein. We then harvested cells cultivated for 36 h and used them for the purification and characterization of the enzyme.

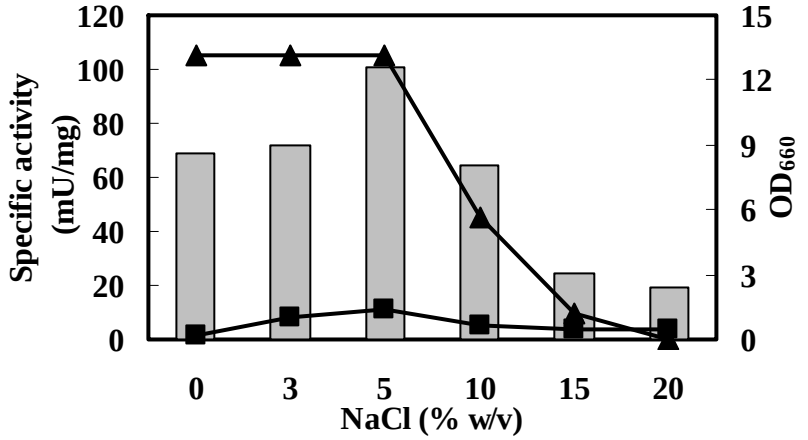


Fig.1

The effects of NaCl concentrations on the growth and halotolerant protease production by strain FP-133. Strain FP-133 was cultivated in the casein medium containing 0-20% (w/v) NaCl. After 24 h of cultivation, the growth was measured at 660 nm (bar). Intracellular (■) and extracellular (▲) protease activities were measured in the presence of 7.5% (w/v) NaCl.

*Purification of halotolerant intracellular protease*

Table 1 shows the summary of a typical enzyme purification. The specific activity of the final preparation of the enzyme from *B. subtilis* strain FP-133 was 3.3 U/mg with overall recovery of 0.87%. The final enzyme preparation showed a 140-fold increase in its specific activity. The final preparation of the enzyme showed a single protein band on polyacrylamide and SDS-polyacrylamide gel (Fig. 3).

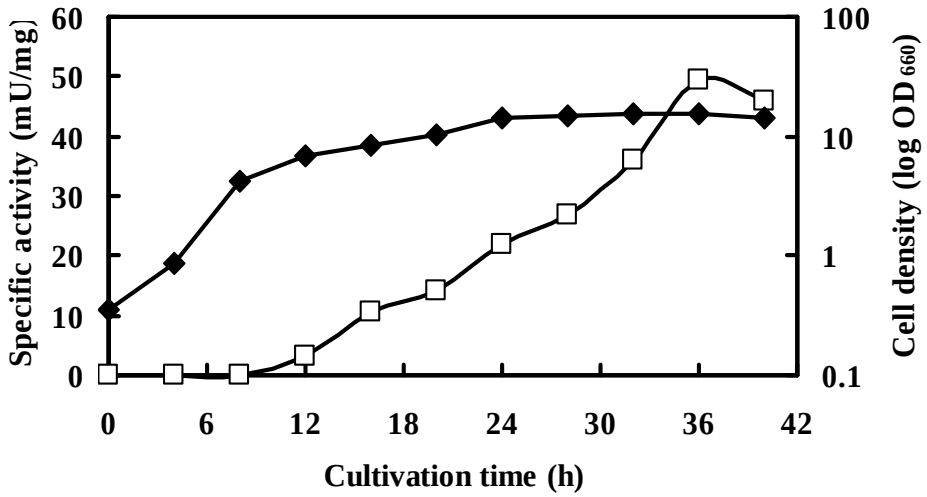


Fig. 2

Growth of strain FP-133 and production of a halotolerant intracellular protease. Strain FP-133 was cultivated in the medium containing 5% (w/v) NaCl. Growth was estimated by measuring optical density at 660 nm (♦) and protease activity (□) was measured in the presence of 7.5% (w/v) NaCl.

Table 1

Summary of purification of halotolerant intracellular protease

| Fraction | Total Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Recovery (%) |
|---|---|---|---|---|
| 1: Cell extracts [a] | 150 | 6,300 | 0.024 | 100 |
| 2: Ammonium sulfate | 36 | 1,000 | 0.036 | 24 |
| 3: DE52 | 1.7 | 2.9 | 0.59 | 1.1 |
| 4: DEAE-Cellulofine A-800 | 1.3 | 0.4 | 3.3 | 0.87 |

[a] Cell extracts were prepared from 25 g of cells (wet weight).

*Molecular properties of the enzyme*

The apparent molecular masses were determined to be 59 kDa by gel filtration and 14 kDa by SDS-PAGE (Fig. 3). These findings indicate that the enzyme was a homotetramer with a subunit molecular mass of 14 kDa.

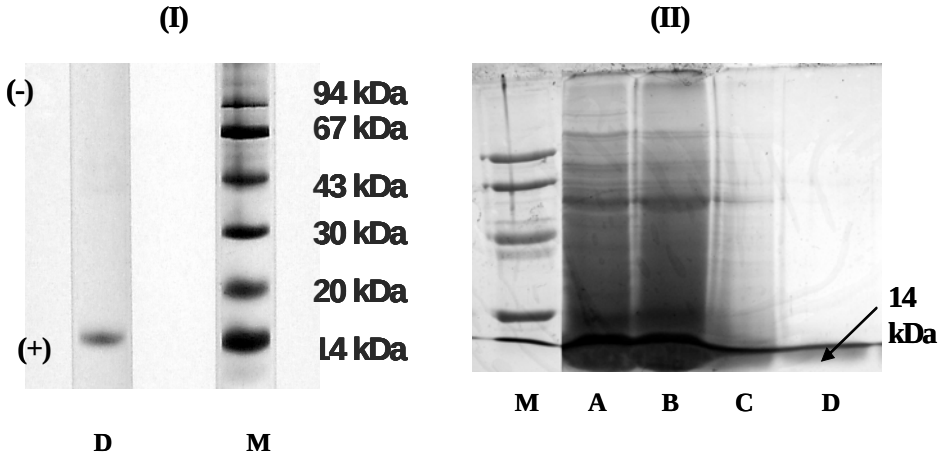


Fig. 3

SDS-PAGE of the purified enzyme on disc (I) and slab gels (II). The enzyme (10 μg) and markers were run on 12.5% (w/v) gels containing 0.1% (w/v) SDS. The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent of ethanol/acetic acid/$H_2O$ (9:2:9). A, cell extract; B, $(NH_4)_2SO_4$ fraction, C, DE 52 fraction; D, DEAE cellulofine fraction; and M, standard molecular markers consisting of α-lactalbumin (14.4 kDa); soybean trypsin inhibitor (20.1 kDa); carbonic anhydrase (30 kDa); ovalbumin (43 kDa); bovine serum albumin (67 kDa); and phosphorylase *b* (94 kDa).

*Effects of temperatures, pHs, and salt concentrations on the activity and stability of the purified enzyme*

The purified protease showed the maximum activity at 60°C and maintained more than 80% activity from 30 to 65°C. At 60°C and a concentration of 7.5% (w/v) NaCl, the enzyme showed activity in a range of pH 5.5 to 7.5. The optimum pH for activity was 6.5. No activity was measurable at pH 5 and 8. The enzyme maintained more than 60% of activity from pH 5.5 to 7.5.

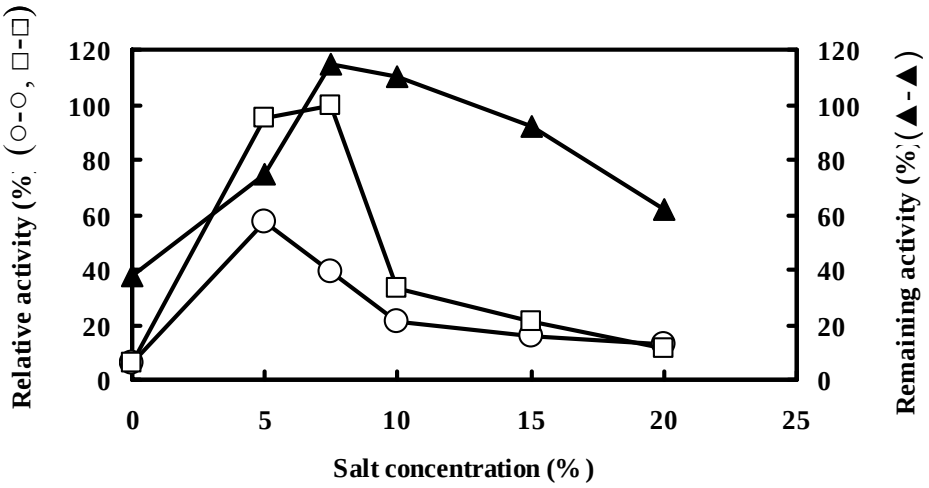


Fig. 4

Effects of salt concentrations on the activity and stability of the enzyme. Protease activity of the purified enzyme was measured at various concentrations of KCl (□) and NaCl (○) at 60°C and pH 6.5. The enzyme was incubated at various concentrations of NaCl and 4°C overnight and the remaining activity for casein was measured at a concentration of 7.5% (w/v) NaCl (▲).

Figure 4 shows that the optimum activity was obtained at a concentration of 5% NaCl. In the absence of NaCl, activity was observed. The enzyme had a higher activity in the presence of KCl than NaCl. In addition, the NaCl concentrations from 5 to 20% (w/v) stabilized the enzyme during storage more than without NaCl.

*Substrate specificity of the enzyme*

Of the synthetic oligopeptides tested, the protease showed the highest activity for L-leucyl-*β*-naphthylamide and significant activity for L-leucyl-*p*-nitroanilide and tripeptides containing L-leucine, glycine, and L-histidine (Table 2A). Table 2B shows that among the five proteins tested, gelatin and bovine serum albumin were hydrolyzed extensively by the enzyme. The enzyme showed a considerable activity for casein, globulin, and myoglobin.

The $K_m$ and $V_{max}$ values for L-leucyl-*p*-nitroanilide were 18 μM and 2.2 mM/h mg, respectively

Table 2

Substrate specificity of the enzyme. The reaction mixture consisted of 0.4% (w/v) peptide or protein in 0.4 ml of 50 mM sodium phosphate buffer (pH 6.5) containing 7.5% (w/v) NaCl and 0.15 ml of purified enzyme (4 μg) solution. After incubation at 37°C for 12 h, the amount of amino acids and peptides released in 0.1 ml of the reaction mixture was measured by the ninhydrin method (REIMEDES and KLOSTERMEYERS 1976). The hydrolysis of peptides was confirmed by thin layer chromatography on a TLC cellulose plastic sheet (Merck, Darmstad, Germany) with a solvent of methanol/6 N $HCl/H_2O$/pyridine (80:4:26:10, by vol.).

| A. Peptide | Relative activity (%) |
|---|---|
| L-leucyl-*β*-naphthylamide | 100 |
| L-leucyl-*p*-nitroanilide | 61 |
| Z-Ala-Leu-$NH_2$ | 9 |
| His-Leu | 72 |
| Gly-L-Phe | 8 |
| Gly-Gly-His | 55 |
| Gly-Gly-Gly-Gly | 43 |
| Gly-Gly-Gly | 46 |
| Gly-Pro | 2 |
| DL-Ala-Gly | 0 |
| L-Leu-L-Tyr | 0 |
| L-Leu-Met-ester HCl | 0 |
| Benzoyl-L-Tyr-*p*-nitroanilide | 1 |
| Gly-Leu | 0 |
| Carbobenzoxy-Gly-Phe | 0 |
| L-Leu-Gly | 19 |
| L-Glu-Cys-Gly | 11 |
| B. Protein | |
| Casein | 24 |
| Gelatin | 100 |
| Bovine serum albumin | 47 |
| Myoglobin | 18 |
| Globulin | 26 |

*Effects of inhibitors and metal ions on enzyme activity*

The protease was strongly inhibited by the cysteine protease inhibitor $HgCl_2$ and the iron ion chelator Tiron. Other cysteine inhibitors such as *p*-chloromercuribenzoic acid (PCMB) and $ICH_2COOH$ also inhibited the enzyme (Table 3).

Table 3

Effect of inhibitors on the enzyme activity. The activity was measured in the reaction mixture (0.5 ml), consisting of 0.5% (w/v) casein, enzyme (4μg), 7.5% (w/v) NaCl, and 50 mM sodium phosphate buffer (pH 6.5) in the presence of inhibitor.

| Inhibitor | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | - | 100 |
| $HgCl_2$ | 1 | 0 |
| PCMB | 1 | 48 |
| $CH_2ICOOH$ | 1 | 58 |
| Tiron | 1 | 17 |
| PMSF | 0.2 | 133 |
| PMSF | 1 | 83 |
| EDTA·2Na | 0.2 | 80 |
| EDTA·2Na | 1 | 53 |

Among the metal ions tested, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ inhibited the enzyme a little, while $Fe^{2+}$, $Fe^{3+}$, and $Ni^{2+}$ enhanced the activity of the protease (Table 4). Furthermore, the activity of the apo-form of the enzyme obtained by treatment with EDTA was recovered by each enhancer ion synergistically with $Mg^{2+}$. The recovered activities were more than those of the native enzyme. When the apoenzyme was treated with each ion in the absence of $Mg^{2+}$, the recovered activity was much lower than in the presence of $Mg^{2+}$.

Table 4

Effects of metal ions on the enzyme activity. The activity was measured as the same as in Table 3. Apoenzyme was prepared by incubating the enzyme with 10 mM EDTA·2Na at 4°C for 1 h, until enzyme activity was completely lost. After removal of EDTA by dialysis, the apoenzyme was dialyzed against buffer containing each metal ion for 4 h at 7°C and then the recovered activity was measured.

| Metal | Concentration (mM) | Relative activity (%) |
|---|---|---|
| A. Holoenzyme | | |
| None | | 100 |
| $Fe^{2+}$ | 1 | 108 |
| $Fe^{3+}$ | 1 | 142 |
| $Zn^{2+}$ | 1 | 60 |
| $Cu^{2+}$ | 1 | 60 |
| $Co^{2+}$ | 1 | 73 |
| $Ba^{2+}$ | 1 | 93 |
| $Mn^{2+}$ | 1 | 53 |
| $Ca^{2+}$ | 1 | 60 |
| $Ni^{2+}$ | 1 | 133 |
| $Mg^{2+}$ | 1 | 80 |
| B. Apoenzyme | | 0 |
| Apoenzyme + $Mg^{2+}$ | 1 | 21 |
| Apoenzyme + $Fe^{3+}$ | 1 | 20 |
| Apoenzyme + $Fe^{3+}$ + $Mg^{2+}$ | 1 | 120 |
| Apoenzyme + $Fe^{2+}$ | 1 | 30 |
| Apoenzyme + $Fe^{2+}$ + $Mg^{2+}$ | 1 | 110 |
| Apoenzyme + $Ni^{2+}$ | 1 | 35 |
| Apoenzyme + $Ni^{2+}$ + $Mg^{2+}$ | 1 | 205 |

*$NH_2$-terminal amino acid sequence*

The *$NH_2$*-terminal amino acid sequence of the enzyme was determined to be L-V-A-P-F-P-G-P-T-P-N-X-L-X-V-Q-V.

## Discussion

*B. subtilis* strains were reported to grow well up to a concentration of 7% (w/v) NaCl (CLAUS and BERKELEY, 1986). *B. subtilis* strain FP-133 isolated in this study was able to grow at concentrations of 0-10% (w/v) NaCl and optimally in 5% (w/v) NaCl. Based on the optimum NaCl concentration for growth, strain FP-133 was classified as a halotolerant bacterium, but not halophilic one that needs at least 12.5% (w/v) of NaCl for growth (KUSHNER 1978)

We purified and characterized a protease from cell extracts of *B. subtilis* strain FP-133. The purified enzyme showed activities at concentrations of 0-20% (w/v) NaCl. Therefore, the protease can be classified as a halotolerant enzyme. This is the first report describing a halotolerant intracellular enzyme, although some halophilic intracellular enzymes including proteases have been reported (SEITZ *et al.* 1997; WILSON *et al.* 1999). Most intracellular proteases reported from *B. subtilis* were serine proteases (VALBUZZI *et al.* 1999; SHEEHAN and SWITZER, 1990; STRONGIN *et al*. 1978) and none of them was reported to show halotolerance. One study on halotolerant extracellular protease produced by *B. subtilis* was reported by KEMBHAVI (KEMBHAVI *et al.* 1993). This enzyme is a serine protease showing its activity on the alkaline side.

At almost any given salt concentration of the cultivation medium, high activity for casein as the substrate was shown by the extracellular instead of the intracellular fraction from *B. subtilis* FP-133 (Fig. 1). This result was in accord with the substrate specificity of intracellular protease indicating that the enzyme was an exoprotease, showing an

aminopeptidase activity. An exoprotease generally requires at least one free terminus close to the cleavage site, so that they have little action on intact protein (BANKUS and BOND, 2001). Contrarily, the extracellular protease of strain FP-133 showed much higher activity for casein than that of intracellular protease, indicating that the extracellular fractions are endoproteases (SETYORINI *et al*. 2006).

Unlike the halophilic proteases from *Natrialba magadii* (GIMENEZ *et al*. 2000) and *Natronococcus occultus* (STUDDERT *et al*. 1997) which need a high concentration of NaCl for the activity and stability, the protease from strain FP-133 could express and maintain its activity in the absence of NaCl (Fig. 4). However, the protease from strain FP-133 showed a higher activity and stability in the presence of salt than in the absence of salt. Similar results were reported for halotolerant extracellular proteases from *Penicillium chrysogenum* Pg222 (BENITO *et al*. 2002) and *Pseudoalteromonas* sp. strain CP76 (SANCHEZ-PORRO 2003).

The intracellular protease from strain FP-133 had higher protease activity in the presence of KCl than NaCl (Fig. 4). These results are possibly explained by the nature of the cytoplasm, which is generally characterized by a high concentration of KCl (OREN 1999). There is a general observation that $K^+$ is preferred for intracellular protein folding, whereas $Na^+$ shows less function or even disturbed this process (PADAN and KRULWICH 2000).

The protease from strain FP-133 distinctly showed an aminopeptidase activity, because it readily hydrolyzed *β*-naphthylamide and *p*-nitroanalide derivatives of amino acids as substrates (PRESCOTT and WILKES 1976). The aminopeptidase property of this enzyme

was emphasized by the inhibitory effects of cysteine protease inhibitors (Table 3), which also inhibit the aminopeptidase from *Debaromyces hensenii* (BOLUMAR *et al*. 2002).

Among the metal ions tested, $Fe^{2+}$, $Fe^{3+}$, and $Ni^{2+}$ promoted the activity of the purified intracellular protease from strain FP-133 (Table 4). The incubation of the apoenzyme with these ions recovered the activity. When the apoenzyme was incubated with each ion in the presence of $Mg^{2+}$, the recovered activity was much higher than when it was done without $Mg^{2+}$. We didn't have any data showing the role of $Mg^{2+}$ on the recovery of the activity. However, since the medium used for the cultivation contained $Mg^{2+}$, this ion may play a significant role in the synthesis of the enzyme. The aminopeptidase I from *E. coli* has been reported to require $Mg^{2+}$ for activity, beside $Mn^{2+}$ (RAO *et al.* 1998). The intracellular amiopeptidase from *Streptomyces rimosus* is activated by $Co^{2+}$, $Zn^{2+}$, and $Ni^{2+}$.

Because the enzyme from strain FP-133 lost activity by treatment with EDTA, the enzyme is assumed to retain possible metals. Although the amount of the purified protease was insufficient to analyze metals, we are going to conduct the experiments on the analysis of cofactors, including metals, in this enzyme in the near future.

In this study, we found a halotolerant intracellular protease in the isolated *B. subtilis* strain FP-133 from a fish paste and characterized the enzyme. The halotolerance of intracellular enzymes including the present protease may play a role in the survival of a halotolerant strain in moderately saline environment.

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