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Purification and characterization of a novel salt-tolerant protease from *Aspergillus* sp. FC-10, a soy sauce koji mold

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A novel salt-tolerant protease produced by *Aspergillus* sp. FC-10 was purified to homogeneity through anion-exchange chromatography, preparative isoelectric-focusing electrophoresis, and gel filtration chromatography, with an overall recovery of 12.7%. This protease demonstrated an optimum pH range of 7.0–9.0 for activity, with a stable pH range of 5.0–9.0. The optimum process temperature at pH 7.0 was 65°C. The enzyme has a molecular mass of 28 kDa and was deduced as a monomer with an isoelectric point of 3.75. Enzyme activity was strongly inhibited by 5 mM of $HgCl_2$ and $FeCl_3$, and significantly inhibited by 5 mM of $CuSO_4$, $FeSO_4$, and $MnCl_2$. The activity of this purified protease was inhibited by Na_2·EDTA; however, leupeptin, pepstatin A, PMSF, and E-64 did not affect the activity. Based on the N-terminal amino acid sequence and amino acid composition, this purified protease should be classified as a member of the deuterolysin family. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 253–258.

Keywords: salt tolerant; metalloprotease; koji; deuterolysin

Introduction

Soy sauce is made from steam-treated soybeans and roasted wheat in both industrial and traditional koji fermentation processes. During such fermentations, molds secrete elevated quantities of proteases, essential for efficient solubilization and hydrolysis of the soybean protein. However, the protease activities decline significantly when harvested koji is mixed with high levels of sodium chloride solution to make a mash [7,13,14,26]. This phenomenon results in an extension of the time required for the enzymatic hydrolysis of soy protein and, consequently, for the overall maturation of the soy sauce. Many studies have investigated methods for elevating koji mold protease activity, so as to promote the utilization efficiency of raw materials, to shorten the time for the maturation process, and to improve the quality of the final soy sauce products [11,12,24]. Nevertheless, there has been no mention of a salt-tolerant protease for use in the manufacture of soy sauce. The development of such a protease from koji mold would effectively accelerate the proteolytic hydrolysis, and result in reduced time for soy sauce production. In our previous study, we isolated an aflatoxin-negative *Aspergillus* sp. FC-10 strain that produced a particular extracellular salt-tolerant protease [26]. It was remarkable that the partially purified salt-tolerant protease retained about 50% of its original proteolytic activity in an 18% NaCl solution [26].

The current study focuses on this particular salt-tolerant protease as part of a program to evaluate *Aspergillus* sp. FC-10 for soy sauce fermentation, and specifically production and purification of this protease. The characteristics, including the effects of pH, temperature, metallic salts, and several group-specific protease inhibitors, and the N-terminal sequence and amino acid composition of this purified protease were determined in this study.

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Materials and methods

Chemicals

Sodium dodecyl sulfate (SDS), Na_2·EDTA and electrophoresis reagents (Sigma Chemical, St. Louis, MO), DEAE-Sepharose CL-6B, Sepharcyl S-200 and standard proteins (Amersham Pharmacia Biotech, Uppsala, Sweden), and protein-assay reagents and ampholytes (Bio-Lyte 3/10; Bio-Rad Laboratories, Hercules, CA, USA) were obtained from their respective suppliers. All other chemicals used were of analytical grade.

Koji mold and preparation of spore inoculum

Aspergillus sp. FC-10 was isolated from soil in the Taipei area of northern Taiwan and was maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) containing 5% (*w/v*) sodium chloride. The spore suspension was prepared by adding 10 ml of sterilized distilled water to a slant containing a fully grown mycelial mat on saline PDA medium and mixing the resulting suspension vigorously. Solid fermentation substrate was prepared by mixing 100 g of defatted soybean flakes with 120 ml of water and autoclaving the suspension at 1.2 kg/cm^2 for 15 min, followed by mixing with 100 g of ground roasted wheat. The substrate was then inoculated with 5 ml of spore suspension and mixed thoroughly to make a koji. The koji was then transferred to a perforated stainless-steel tray and cultured at 30°C and relative humidity of 92% for 2–3 days. When a green-yellow mass appeared as a result of mold growth and sporulation, the koji was transferred to a hot-air oven maintained at 40°C for 6 days. Subsequently, the dried koji was blended as spore inoculum and stored in a tightly sealed plastic bottle at 4°C until required.

Solid-state fermentations and preparation of crude protease extracts

One kilogram of defatted soybean mixed with 1.2 l of distilled water was autoclaved and then combined with 1 kg of ground roasted wheat to prepare the koji medium. The medium was cooled

to room temperature, inoculated with 3 g of spore inoculum, and subsequently dispersed onto several perforated stainless-steel trays. Each tray was loaded with fermenting koji to approximately a 3-cm thickness and incubated first at 30°C for 28 h, and then at 25°C for 24 h. The koji mold culture was turned over slightly at around 22 and 28 h of incubation in order to lower the koji temperature and to provide aeration.

The koji was sampled at intervals and immediately processed to prepare protease extracts by the following method modified from Hirose [5]. One hundred grams of koji was mixed with 2 l of distilled water containing 0.9% sodium chloride. The mixture was left standing at ambient temperature for 4 h with occasional stirring and then centrifuged at 5000×*g* for 20 min. The supernatant was assayed for protease activity and used for further purification.

Protease activity was assayed according to a method modified from Anson [1], using 1.5% Hammarsten-milk casein as the substrate. The effect of the 18% NaCl solution on proteolytic activity was determined by adding NaCl to the reaction mixtures to obtain a final concentration of 18%. One unit of protease activity was defined as the amount of enzyme that liberates 1 μg of tyrosine per minute in a reaction mixture at 30°C.

Protease detection and purification of the salt-tolerant protease

Protease profiles of crude protease extract were preliminarily detected on the gel by active stain after a native-PAGE process (170 V at 4°C, with the separating gel containing 12.5% acrylamide). The developed gel was removed from the fixed plates and immediately placed face to face on a 7.5% acrylamide gel containing 0.1% casein as the substrate, and incubated at 40°C for 1 h. Wet filter papers were used to pad and cover the gels to avoid damage to the reacting gels through drying. After incubation, both gels were stained with Coomassie brilliant blue R 250 for 1 h, followed by washing of the gels with a destaining solution containing acetic acid and methanol.

Purification of the salt-tolerant protease is described as follows. Five liters of crude protease extract was concentrated by diafiltration using a spiral-wound module ultrafiltration column (MWCO: 10 kDa, Advanced Biotechnology Laboratories, Taipei, Taiwan) to a final volume of 1 l. Ten liters of distilled water was added and diafiltered again to a final volume of around 500 ml. The retentate was centrifuged at 5000×*g* for 20 min to remove the insoluble component. The supernatant was lyophilized to produce crude protease powder which was stored at −20°C. Next, 650 mg of crude-protease powder was dissolved in 20 ml of 0.01 M phosphate buffer (pH 7.0) and subjected to a DEAE-Sepharose CL-6B column (2.6 by 40 cm) previously equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was then eluted with 600 ml of the same buffer followed by 800 ml of a linear gradient of 0–0.5 M NaCl in 0.01 M phosphate buffer (pH 7.0). Sequential 10-ml aliquots of eluate were collected with a fraction collector, with each fraction evaluated for proteolytic activity and protein content. Fractions demonstrating proteolytic activity were pooled and concentrated by ultrafiltration (MWCO: 10 kDa), followed by overnight dialysis against distilled water at 4°C, and then purified as follows. One milliliter of Bio-Lyte 3/10 (40%) solution was added to 50 ml of the protease dialysate, and this was applied to the chamber of a Bio-Rad Rotofor preparative isoelectric-focusing (prep. IEF) electrophoresis apparatus which was operated at a constant power of 12 W for 5 h at 4°C. The focusing process was terminated when the voltage had stabilized for approximately 30 min. All 20 fractions were collected for protein and protease activity determinations. The most active fractions were combined and dialyzed against a 0.5 M NaCl solution containing 5% glycerol to remove ampholytes, followed by ultrafiltration (MWCO: 10 kDa). The resultant protease concentrate together with 1 ml of 0.1% blue dextran 2000 were loaded onto a Sephacryl S-200 gel filtration column (Amersham Pharmacia Biotech; 2.6 by 90 cm) previously equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 0.5 M NaCl, and eluted with the same buffer solution at a flow rate of 5 cm h^{-1}. The eluate was collected in 2.5-ml fractions.

Characterization of the purified protease

The molecular mass of this protease was estimated using SDS-PAGE according to the method described by Laemmli on a 12.5% acrylamide gel [8]. The low-molecular-weight calibration kit (containing phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin) available from Pharmacia was employed as the protein standard. The isoelectric point of the purified protease was estimated using a Pharmacia multiphor system with an isoelectric-focusing polyacrylamide gel (5%, 0.2 mm thick) in a pH gradient of 3.5–9.3. The reference proteins used were amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85, 7.35), lentil lectin (pI 8.15, 8.45, 8.65), and trypsinogen (pI 9.30). To measure the effects of temperature and pH on protease activity, assays were carried out at different temperatures (30–70°C) and at different pHs, using acetate (pH 3–6), phosphate (pH 6–9), and glycine–NaOH buffers (pH 9–10). The effects of such group-specific protease inhibitors leupeptin, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64), phenylmethanesulphonyl fluoride (PMSF), pepstatin A, and Na_2·EDTA, and various metallic ions, on the purified protease were studied by incubating the enzyme solution with reagents or metallic ions in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature. Residual activity was measured under NaCl-free conditions, and inhibition or activation was expressed as a percentage of the activity without the effector. Protein levels were determined by measuring absorption at 280 nm or by using Bradford's method [2] with bovine serum albumin as the standard.

Determination of the N-terminal sequence and amino acid composition of the salt-tolerant protease

The active protease band, separated by SDS-PAGE, was transferred onto a PVDF membrane. The N-terminal amino acid sequence of the enzyme was analyzed by automatic Edman degradation using a protein sequencer (model ABI 476A, PE Biosystems, CA). The amino acid composition of the salt-tolerant protease was determined after hydrolysis with 6 N HCl at 150°C for 90 min, and amino acid analysis using a high-performance amino acid analyzer (Model 6300, Beckman Coulter, Fullerton, CA).

Results

Extracellular protease production by Aspergillus sp. FC-10 grown on solid-state substrate

Figure 1 shows the proteolytic activities of the koji extracts assayed in NaCl-free and in 18% NaCl solutions. During the

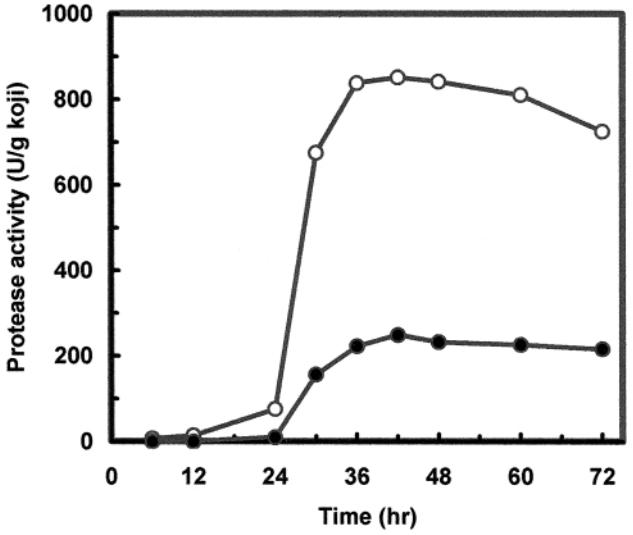

Figure 1 Time course for the production of protease by *Aspergillus* sp. FC-10 grown on solid medium. ○——○, assayed in NaCl-free solution; ●——●, assayed in 18% NaCl solution. Each point represents the mean activity of two samples. Experimental details are in the text.

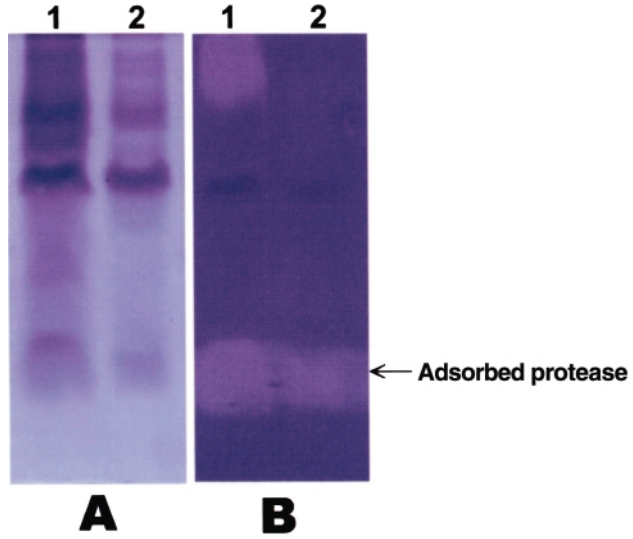

Figure 2 Protease profiles of the protease extract from *Aspergillus* sp. FC-10. Panel A: native-PAGE. Panel B: proteolytic activity profiles of protease showing the corresponding positions on panel A. Lane 1: crude protease extract, lane 2: the adsorbed fraction from DEAE Sepharose CL-6B column chromatography.

first 24 h of incubation, only slight activities were seen in both cases. Thereafter, the proteolytic activities increased rapidly to the maximum levels at 36 h of incubation. The protease activities were about 800 U g^{-1} koji in the NaCl-free solution, and about 250 U g^{-1} koji in the 18% NaCl solution. Subsequently, the proteolytic activity remained high for a further 12 h, and then decreased slightly up to 72 h of incubation. During the first 24 h of incubation, the mold grew very fast with the formation of a white mycelial mat on the solid substrate. Thereafter, a green-yellow mass began to appear, indicating that sporulation had occurred. Based on this information, 48-h-old cultures of *Aspergillus* sp. FC-10 were used for preparation of crude protease extracts.

Protease profile of crude protease extract and purification of the salt-tolerant protease

Native-PAGE and protease activity staining profiles of crude protease extract revealed two protein bands demonstrating proteolytic activities (lane 1 in Figure 2). Purification of the salt-tolerant protease was carried out according to the procedures described above. About 80% of protease activity was recovered after ultrafiltration and lyophilization. The elution pattern for DEAE-Sepharose CL-6B chromatography is shown in Figure 3, with two major protein peaks reflecting the proteolytic activities. The first protease passed through the column freely with the void volume, and the second protease was eluted by 0.01 M phosphate buffer (pH 7.0) containing 0.2 M NaCl. The adsorbed protease (lane 2 in Figure 2) that revealed the salt tolerance was collected for preparative IEF electrophoresis. The resulting fraction with a pH of around 4.2 demonstrated the greatest proteolytic activity. The active fractions were pooled and dialyzed to remove ampholytes, then concentrated by ultrafiltration for gel filtration. After Sephacryl S-200 column chromatography, the extracellular salt-tolerant protease was purified to homogeneity. The purification steps and yields are summarized in Table 1. In this purification process, 62.3 mg of protease was obtained. The yield was 12.7%, with a specific activity of 168 U mg^{-1} and a 13.8-fold purification efficiency. The electrophoresis profiles of the salt-tolerant protease-related fractions are shown in Figure 4. The native-PAGE profiles of the crude, partially purified, and final purified salt-tolerant protease are shown in Figure 4A. Electrophoresis of the purified salt-tolerant protease under denaturing conditions (SDS-PAGE) revealed a single protein band with an estimated subunit molecular mass of 28 kDa (Figure 4B). In addition, the purified protease, both heated and unheated, exhibited the same migration distances in the 12.5% acrylamide gel (containing 0.1% SDS), indicating that the purified protease was a monomer (one polypeptide chain). The pI of this purified protease was pH 3.75 as determined by slab isoelectric-focusing electrophoresis (Figure 4C).

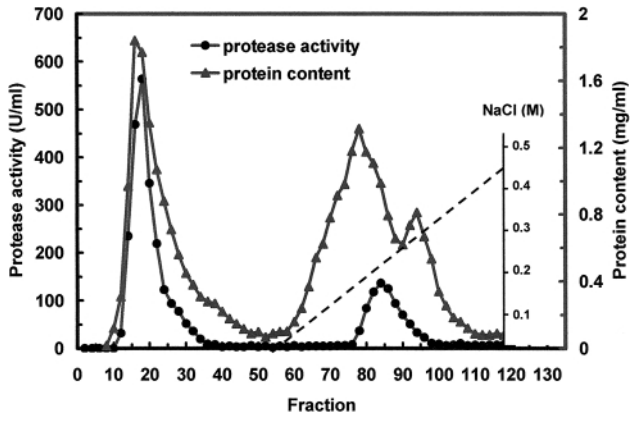

Figure 3 Fractionation of the protease by DEAE-Sepharose CL-6B column chromatography. ●——●, protease activity; ▲——▲, protein content; – – –, NaCl concentration. Experimental details are in the text.

Table 1 Summary of the procedures for purification of the salt-tolerant protease from *Aspergillus* sp. FC-10

Step	Activity (unit)	Protein (mg)	Specific activity ($U\ mg^{-1}$)	Yield (%)	Purification (fold)
Crude extract	82200	6790	12.1	100	–
Ultrafiltration and lyophilization	64300	591	109	78.3	9.0
DEAE-Sepharose CL-6B	15400	177	87.0	18.7	7.2
Preparative IEF	13700	112	123	16.7	10.1
Sephacryl S-200HR	10400	62.3	168	12.7	13.8

Effect of pH and temperature on the activity of the purified salt-tolerant protease

The optimal pH for maximum activity of the purified salt-tolerant protease was in the range of 7.0–9.0, and the optimum temperature was 65°C. The purified protease was stable in a pH range of 5.0–9.0, retaining more than 90% of its original activity after a 3-h incubation under these pH conditions. The protease was stable at 55°C, retaining more than 80% of its activity when treated at 60°C for 3 h, and being completely inactivated when heated at 65°C for 3 h. Some properties of this purified salt-tolerant protease are summarized in Table 2.

Effect of metallic salts and reagents on the activity of the purified salt-tolerant protease

The effects of various metallic salts and group-specific reagents on the activity of this protease are summarized in Table 3. The activity was almost completely inhibited by 5 mM of $FeCl_3$ or $HgCl_2$, partially inhibited by $CuSO_4$, $MnCl_2$, or $FeSO_4$, and significantly inhibited by Na_2·EDTA. Other metallic salts or reagents were judged to have no effect on this enzyme's activity. These results, taken *in toto*, suggest that the purified salt-tolerant protease is a metalloprotease.

N-terminal sequence and amino acid composition of the purified salt-tolerant protease

The N-terminal amino acid sequence was analyzed using an automatic protein sequencer. The sequence of the first 12 N-terminal amino acids was determined to be T-E-V-T-D-X-K-G-D-A-E-E- (X represents an ambiguous residue). The amino acid composition of this enzyme is shown in Table 4. The purified protease demonstrated low level of methionine and high levels of alanine, aspartic acid, and glutamic acid.

Discussion

There have been very few reports on NaCl-tolerant filamentous fungi and their utilization. Sterflinger's [25] study of the NaCl tolerance of rock-inhabiting meristematic fungi revealed that all

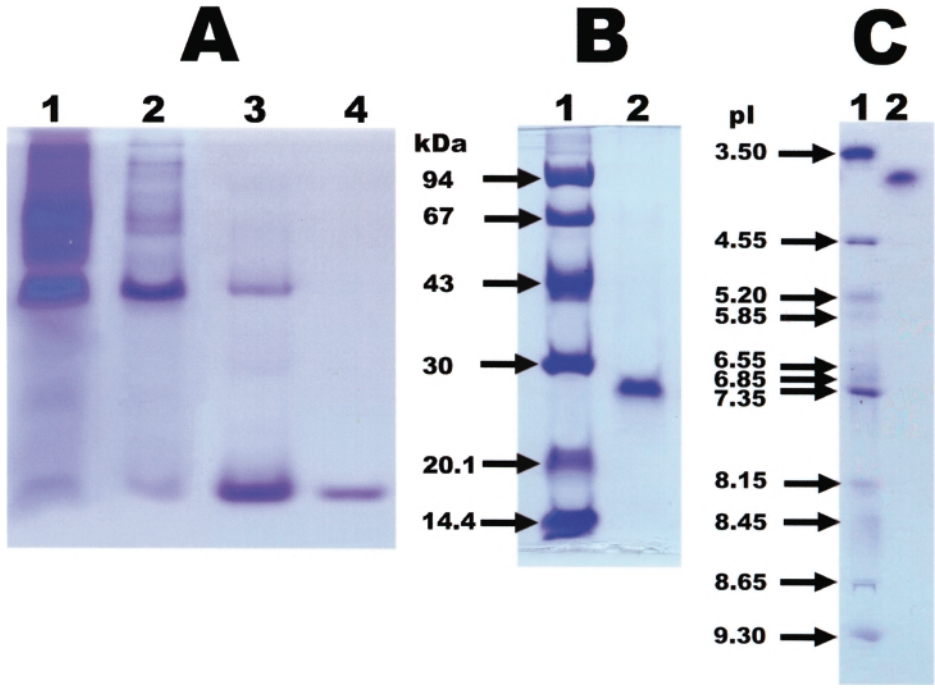

Figure 4 PAGE analysis for the salt-tolerant protease. Panel A: 12.5% native PAGE. Lane 1, crude-protease powder; lane 2, adsorbed protease fractions from DEAE-Sepharose CL-6B chromatography; lane 3, protease fractions from preparative isoelectric-focusing electrophoresis; and lane 4, purified protease by Sephacryl S-200 gel-filtration chromatography. Panel B: 12.5% SDS-PAGE of the purified protease. Lane 1, molecular masses of the standard proteins; lane 2, purified protease. Panel C: slab isoelectric-focusing electrophoresis. Lane 1, pIs of the standard proteins; lane 2, pI of the purified protease under study. Coomassie brilliant R-250 was used for protein staining in all panels.

Table 2 Some properties of the purified salt-tolerant protease from *Aspergillus* sp. FC-10

Property	Value
Molecular mass	28 kDa
Isoelectric point	3.75
Optimum pH	7.0–9.0
pH stability	5.0–9.0 for 3 h at room temperature
Optimum temperature	65°C
Thermal stability	55°C for 3 h
	80% residual activity at 60°C for 3 h
N-terminal sequence	T-E-V-T-D-X-K-G-D-A-E-E-[a]

[a]X represents an ambiguous residue.

isolates grew better without NaCl, and the growth rate decreased with increasing NaCl concentration for all except a halophile. Our previous research found that some molds possess salt-tolerant characteristics and have the potential to produce extracellular salt-tolerant proteases. However, only a few filamentous fungi can survive on agar with high levels of NaCl. We fortunately found that *Aspergillus* sp. FC-10, isolated from Taipei area soil, secretes two proteases that can be easily separated from each other with a DEAE-Sepharose CL-6B column [26]. One of these two proteases (adsorbed by the DEAE-Sepharose CL-6B resins) was salt-tolerant. In this study, we purified this salt-tolerant protease to homogeneity and investigated its characteristics (Table 2). This salt-tolerant protease was a 28-kDa metalloprotease. There are several species of *Aspergillus* known to secrete metalloprotease [3,4,6,7,10,14,16,18–23,27,28], and some species-specific characteristics have been elaborated. Among them, some of their genes have been studied and cloned [4,6,19,22,23,27]. Based on the database from BLAST and FASTA [9,17], the N-terminal amino acid sequence of the present salt-tolerant protease is very similar to that of the neutral protease II of *Aspergillus oryzae* [6,27]. The sequence of the first 12 N-terminal amino acids of the present salt-tolerant protease was T-E-V-T-D-X-K-G-D-A-E-E- (X represents an ambiguous residue), while that of the neutral protease II of *A. oryzae* is T-E-V-T-D-C-K-G-D-A-E-S-; only two amino acid residues differ between these two. However, they differ in molecular mass, optimum pH, and thermal stability (19 kDa, pH 6.5, and stable at 100°C for the neutral protease II of *A. oryzae*) [4,6,27]. Thus, it seems reasonable to consider the present salt-tolerant protease as a novel protease. The neutral protease II of *A. oryzae* is a zinc-containing metalloprotease, and is now designated as a deuterolysin (EC 3.4.24.39) according to the Enzyme Nomenclature Commission [15]. The typical deuterolysin reveals an extraordinary resistance to thermal stressors even at 100°C [3,6,21,28]. In our studies, the purified protease retained around 50% of its original activity in an 18% NaCl solution (data not shown). Recently, some properties of deuterolysin from different fungi were reported by Ichishima [6]. By comparing these characteristics with those derived from our preliminary research, we suggest that this purified salt-tolerant protease be classified as a member of the deuterolysin family along with other members such as MEP20 from *Aspergillus flavus* [18,19] and *Aspergillus fumigatus* [10,18], metalloendopepti-

Table 4 Amino acid composition of the salt-tolerant protease from *Aspergillus* sp. FC-10

Amino acid	Concentration (nmol/50 μl)	Relative content (%)
Aspartic acid	3.75	10.78
Threonine	2.86	8.20
Serine	1.77	5.10
Glutamic acid	4.76	13.68
Proline	1.39	3.98
Glycine	2.16	6.19
Alanine	5.25	15.08
Half cystine	0.56	1.62
Valine	1.05	3.01
Methionine	0.02	0.05
Isoleucine	0.60	1.73
Leucine	2.74	7.86
Tyrosine	2.08	5.96
Phenylalanine	0.56	1.61
Histidine	0.76	2.19
Lysine	1.52	4.36
Arginine	0.61	1.74
NH_3	2.38	6.83

Table 3 Effect of metallic salts and reagents on the activity of the salt-tolerant protease from *Aspergillus* sp. FC-10

Reagent	Residual activity (%)	Reagent	Residual activity (%)
None	100	$MnCl_2$	68
$BaCl_2$	90	$ZnCl_2$	100
$CaCl_2$	93	Urea (8 M)	100
$CuSO_4$	45	2-Mercaptoethanol (10 mM)	100
$FeSO_4$	71	Na_2·EDTA (10 mM)	36
$FeCl_3$	10	Leupeptin (0.1 mM)	98
$HgCl_2$	10	Pepstatin A (1 mM)	100
KCl	100	PMSF[a] (10 mM)	94
$MgCl_2$	96	E-64[b] (1 mM)	100

The protease was incubated with 5 mM of metallic salts or reagents (as indicated) at room temperature for 30 min, and then the residual activity was measured under NaCl-free conditions.
[a]PMSF: phenylmethanesulphonyl fluoride.
[b]E-64: *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine.

dases from *Grifola frondosa* and *Pleurotus ostretus* [16], and penicillolysin from *Penicillium citrinum* [16].

Since *Aspergillus* sp. FC-10 can secrete a superior salt-tolerant protease, it may be a potential strain for use in the manufacture of soy sauce. Further comparative studies dealing with cloning and sequencing may assist in the identification and production of this saline-tolerant protease. In addition, extensive safety evaluations of this strain are still required to assess the suitability of *Aspergillus* sp. FC-10 for commercial applications.

Acknowledgements

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