JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Purification and Characterization of a Novel Extracellular Tripeptidyl Peptidase from *Rhizopus oligosporus*

Jia-Shin Lin,[†,‡,§] Shuo-Kang Lee,[†,‡] Yeh Chen,[‡] Wei-De Lin,[‖,⊥] and Chao-Hung Kao*[,‡]

[‡]Department of Biotechnology, and [§]Department of Food Science and Technology, Hungkuang University, 34 Chung-Chie Road, Taichung 43302, Taiwan

[‖]Department of Medical Research, China Medical University Hospital, 2 Yuh-Der Road, Taichung 40447, Taiwan

[⊥]School of Post-Baccalaureate Chinese Medicine, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan

ABSTRACT: A novel extracellular tripeptidyl peptidase (TPP) was homogenously purified from the culture supernatant of *Rhizopus oligosporus* by sequential fast protein liquid chromatography. The purified enzyme was a 136.5 kDa dimer composed of identical subunits. The effects of inhibitors and metal ions indicated that TPP is a metallo- and serine protease. TPP was activated by divalent cations, such as Co^{2+} and Mn^{2+}, and completely inhibited by Cu^{2+}. Enzyme activity was optimal at pH 7.0 and 45 °C with a specific activity of 281.9 units/mg for the substrate Ala-Ala-Phe-*p*NA. The purified enzyme catalyzed cleavage of various synthetic tripeptides but not when proline occupied the P1 position. Purified TPP cleaved the pentapeptide Ala-Ala-Phe-Tyr-Tyr and tripeptide Ala-Ala-Phe, confirming the TPP activity of the enzyme.

KEYWORDS: Tripeptidyl peptidase, *Rhizopus oligosporus*, metalloprotease, serine protease

INTRODUCTION

Tripeptidyl peptidases (TPPs) cleave peptide bonds to liberate tripeptides sequentially from the free N terminal of poly peptides. TPPs are classified as TPP I (EC 3.4.14.9) and TPP II (EC 3.4.14.10) based on their catalytic mechanisms, molecular weights, and cellular localization.[1] TPP I is an acidic lysosomal protease that exhibits broad substrate specificity; its inhibitory profiles indicate that it may represent an unusual serine protease.[2−4] TPP II is a high-molecular-weight extralysosomal serine protease with a neutral pH optimum that exhibits both exo- and endoproteolytic activities.[5−8] In mammals, TPPs may play important roles in intracellular protein turnover,[1] antigen presentation,[9] peptide hormone and neuropeptide production,[10] bacteria-induced apoptosis,[11] and neurodegenerative disorders.[2]

In addition to the mammalian TPPs, a secreted tripeptidyl aminopeptidase (TAP) from *Streptomyces lividans*[12] and three secreted sedolisins (SedB, SedC, and SedD) from *Aspergillus fumigatus*[13] with TPP activity have been identified. Moreover, a protease classified as a prolyl tripeptidyl peptidase (PTP) specific for peptides with a proline residue in the P1 position has also been characterized.[14−17] TPPs in microorganisms may play substantial roles in provision of nutrients and may also contribute to pathogenesis.[13,15,16,18] TPPs are used in industrial hydrolysis of protein sources[13] and in activating zymogens.[14,17]

Rhizopus oligosporus, a filamentous fungus belonging to the class zygomycetes, is economically and medically important. It has been used for production of industrial enzymes,[19−21] treatment of wastewater,[22] and production of antibiotics that inhibit Gram-positive bacteria.[23] In the food and fermentation industries, *R. oligosporus* has been used in east and southeast Asia to produce soybean tempeh since ancient times, and there is increasing interest in this food worldwide[24] as a low-fat, high-protein food with high antioxidant activity.[25] Secretion of proteases from *R. oligosporus* appears to be a critical factor in the production of high-quality tempeh.[26] Furthermore, *R. oligosporus* has potential as a protease producer because it does not produce toxins.[27] The regulation and production of secretory acid proteases by *R. oligosporus* during fermentation have been extensively studied.[28,29] However, the biochemical properties of this specific protease produced by this fungus have not been reported. Therefore, our objective was to purify and characterize the proteases secreted by *R. oligosporus*.

MATERIALS AND METHODS

Materials. The chromogenic substrates listed in Table 4 were purchased from Bachem AG (Bubendorf, Switzerland). The peptides Ala-Ala-Phe-Tyr-Tyr (AAFYY) and Ala-Ala-Phe (AAF) were synthesized at Mission Biotech Co., Ltd. (Taipei, Taiwan) using a Symphony Multiple Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ). Pepstatin A, phenylmethylsulfonyl fluoride (PMSF), Pefabloc SC, E-64, *N*-ethylmaleimide, iodoacetic acid, *β*-mercaptoethanol, and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO), and Ala-Ala-Phe-chloromethyl ketone was obtained from New England Biolabs (Ipswich, MA). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich.

Strains, Media, and Culture Conditions. *Rhizopus microsporus* var. *oligosporus* BCRC31750 (*R. oligosporus*) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and grown in DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$) under aerobic conditions at 30 °C for 36 h on an orbital shaker at 150 revolutions

Received: May 12, 2011
Revised: September 9, 2011
Accepted: September 10, 2011
Published: September 11, 2011

per minute (rpm). TPP medium (1% glucose, 11.2 mM KH_2PO_4, 7 mM KCl, 2.1 mM $MgSO_4 \cdot 7H_2O$, 0.04 mM $FeCl_2 \cdot 4H_2O$, 0.04 mM $CoCl_2 \cdot 6H_2O$, and 0.07 mM $ZnCl_2$ at pH 7.0) supplemented with different nitrogen sources, i.e., 2% casein, skim milk, beef extract, bovine serum albumin (BSA), Soytone, or NH_4Cl, was used to induce TPP activity.

Purification of Proteases. *R. oligosporus* (10^7 spores) was cultured in 400 mL of TPP medium containing 2% Soytone as the sole nitrogen source at 30 °C for 3 days. Cells were removed by centrifugation (8000*g* at 4 °C for 30 min), and the supernatant was collected for enzyme purification. All purification steps were performed using an AKTA purifier 10 system (GE Healthcare Biosciences, Uppsala, Sweden) at 4 °C.

The culture supernatant was concentrated by ultrafiltration using an Amicon Ultra-15 10K Centrifugal Filter Device (10 kDa cutoff; Millipore, Bedford, MA) and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0). The dialyzed supernatant was loaded onto a HiTrap Q Sepharose Fast Flow column (GE Healthcare Biosciences), previously equilibrated with 50 mM sodium phosphate buffer (equilibration buffer; pH 7.0). TPP was eluted at a flow rate of 0.2 mL/min using the equilibration buffer with a NaCl stepwise gradient of 0−55 mM (10 mL), 55 mM (12 mL), 55−155 mM (10 mL), 155 mM (12 mL), and 155 mM−1.0 M (5 mL). TPP activity in the eluted fractions was measured using Ala-Ala-Phe-*p*-nitroanilide (Ala-Ala-Phe-*p*NA) as the substrate. TPP-containing fractions were pooled and dialyzed against 50 mM sodium phosphate buffer (pH 6.5) in an Amicon Centrifugal Filter Device.

The pooled fractions from the HiTrap Q Sepharose Fast Flow column were further purified on a MonoQ HR 5/5 high-performance column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 6.5). TPP was eluted using a NaCl gradient of 0−120 mM (5 mL), 120−270 mM (15 mL), and 270 mM−1.0 M (3 mL) in 50 mM sodium phosphate buffer (pH 6.5) at a flow rate of 0.15 mL/min. The active fractions were pooled, concentrated, and dialyzed against 50 mM sodium phosphate buffer (pH 6.5). The pooled sample was then subjected to gel filtration through a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 6.5). Elution of TPP was performed using a 50 mM sodium phosphate buffer (pH 6.5) at a flow rate of 0.4 mL/min. Fractions with the highest TPP activity were pooled, concentrated by ultrafiltration, and then stored at −20 °C.

Electrophoresis and Protein Determination. The proteins obtained from each chromatography step were separated using 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to standard protocols.[30] After electrophoresis, the gel was stained with 0.1% silver nitrate. Broad-range protein markers were used to estimate protein size (6.5−200 kDa; Bio-Rad Laboratories, Richmond, CA). Protein concentrations were quantified using a Bio-Rad Protein Assay Kit with BSA as the standard.

Estimation of the Molecular Mass. The molecular mass of the purified enzyme was estimated by both size-exclusion chromatography using a Superdex 200 column and SDS−PAGE. The purified enzyme and a solution of standard molecular-weight markers, such as β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa), were loaded separately onto a Superdex 200 column, using 50 mM sodium phosphate (pH 6.5) as the mobile phase at a flow rate of 0.4 mL/min. Blue Dextran (2000 kDa) was used to determine the void volume. Protein bands on the polyacrylamide gels were analyzed by densitometry, and protein sizes were estimated using Kodak 1D Image Analysis Software (Windows, version 2.0.3; Eastman Kodak, Rochester, NY).

N-Terminal Amino Acid Sequencing. The purified enzyme on the polyacrylamide gel was electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF, Millipore) in 3-(cyclohexylamino)-1-propanesulfonic acid buffer at pH 11.0 and stained with Coomassie Blue R-250. The stained protein band was excised from the membrane and subjected to N-terminal amino acid sequencing. The N-terminal sequence was determined by the Edman degradation method using an ABI Procise 494 protein sequencer (Applied Biosystems, Foster City, CA).

Enzyme Activity Assay. The activity of purified TPP was determined spectrophotometrically as described by Exterkate.[31] The reaction mixture, containing 100 mM sodium phosphate buffer (pH 7.0), 1 mM $CoCl_2$, 1 mM Ala-Ala-Phe-*p*NA as the substrate, and the purified enzyme (20 ng), was incubated at 45 °C for 30 min. The reaction was terminated by the addition of acetic acid to a final concentration of 30%, and the absorbance at 405 nm was measured using a Hitachi U3000 spectrophotometer (Hitachi, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μM *p*NA per minute at 45 °C, with *p*NA as the standard.

Enzyme Characterization. The optimal temperature for TPP was determined at temperatures ranging from 15 to 65 °C for 30 min in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM $CoCl_2$ and 1 mM Ala-Ala-Phe-*p*NA as the substrate. The optimal pH for TPP was determined by measuring its activity at 45 °C for 30 min over a pH range of 4.0−9.0 using the following buffers: 100 mM glycine-HCl (pH 4−6), sodium phosphate (pH 6−8), potassium phosphate (pH 6−8), and Tris-HCl (pH 7−9).

To determine the effects of protease inhibitors and reducing agents on TPP activity, the purified enzyme (20 ng) was incubated in 100 mM sodium phosphate (pH 7.0) with each of the chemical agents at 30 °C for 30 min, followed by measurement of its activity under the standard assay conditions described above. To investigate the effects of metal ions on TPP activity, the purified enzyme was incubated at 30 °C for 30 min in 100 mM sodium phosphate buffer (pH 7.0) with 10 mM ethylenediaminetetraacetic acid (EDTA) or 5 mM dipicolinic acid. After the chelating agent was removed by centrifugal ultrafiltration (Amicon Ultra-15 10K Centrifugal Filter Device), the divalent metal salt solution was added and incubation was continued for an additional 30 min before the enzyme assay. TPP activity was assayed using the standard procedure in the absence of metal ions and chelating agents. To avoid interference by the anions, all metal ions were added as chlorides (Table 3). Substrate specificity was examined using a range of synthetic tripeptidyl-*p*NAs, dipeptidyl-*p*NAs, and monopeptidyl-*p*NAs at a final concentration of 1 mM (Table 4) under the standard assay conditions.

TPP Activity of the Enzyme. The TPP activity was investigated using the AAFYY synthetic pentapeptide. The peptide was dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM $CoCl_2$ to a final concentration of 1 mM, and purified TPP (20 ng) was added and incubated at 45 °C for 30 min. The reaction was terminated by the addition of acetic acid to a final concentration of 30%, and the samples were then analyzed by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). The HPLC/MS/MS system consisted of two PerkinElmer Series 200 LC micro pumps, a Series 200 Autosampler (PerkinElmer Co., Waltham, MA), and an AB Sciex API 2000 triple quadrupole mass spectrometer with a TurboIonSpray probe (Applied Biosystems). The data were processed using AB Sciex software (Analyst, version 1.3.2; Applied Biosystems).

HPLC analysis was conducted using a reversed-phase Polaris C18-A column (2 mm inner diameter × 50 mm; particle size, 3 μm; Varian, Inc., Palo Alto, CA). The mobile phases were (A) 0.1% formic acid and (B) acetonitrile, and the flow rate was 0.1 mL/min. The linear gradient was initiated with 10% B and was increased to 95% B within 10 min. For MS/MS detection, the TurboIonSpray, orifice voltages, temperature, collision energy, and entrance potential were set to 5500 V, 60 V, 200 °C, 16 eV, and −9.5 V, respectively. The collision gas (nitrogen) was maintained at a pressure of 2.3×10^{-5} Torr. The positive-ion mode and multiple-reaction monitoring (MRM) mode were used to detect AAFYY (m/z 634.3−453.2) and AAF (m/z 308.2−166.1), respectively. Total analysis time was 10 min for each sample.

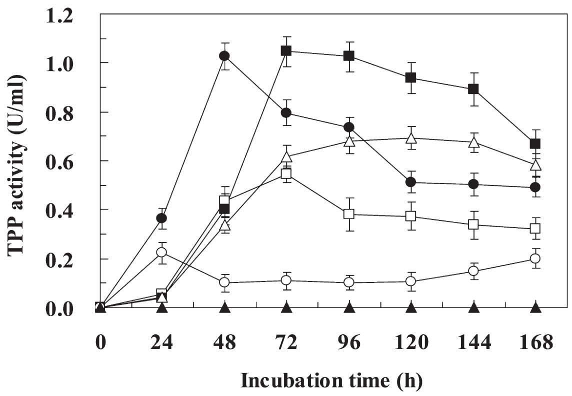

Figure 1. Effects of nitrogen sources on TPP production of *R. oligosporus*. Cells were cultivated in TPP medium containing 2% casein (□), 2% Soytone (■), 2% BSA (△), 2% skim milk (●), 2% beef extract (○), or NH_4Cl (▲) as the sole nitrogen source at 30 °C with shaking at 150 rpm for 7 days. The data are means of three independent experiments, and the bars indicate standard deviations.

RESULTS AND DISCUSSION

Production of *R. oligosporus* TPP. TPP production from *R. oligosporus* reached a maximum of ∼1.0 unit/mL after 48 and 72 h of incubation when the cells were cultivated in medium containing skim milk or Soytone as the sole nitrogen source, respectively (Figure 1). When the cells were cultured in TPP medium containing casein, BSA, or beef extract as the sole nitrogen source, the maximum activity of TPP was ∼0.5, 0.6, and 0.2 unit/mL, respectively. No detectable activity was found when NH_4Cl was used as the sole nitrogen source. TPP enzyme activity was induced by organic nitrogen sources, which may indicate that this enzyme plays a role in the provision of nutrients. Proteolytic enzymes are secreted by a variety of fungi and likely supply nitrogen for cell survival when inorganic nitrogen in the environment is depleted.[28,32] The extracellular carboxyl proteinase of *R. oligosporus* is also suppressed when amino acids are used as a nitrogen source, while the enzyme activity is enhanced when the medium contains protein as the sole nitrogen source.[28]

Purification of *R. oligosporus* TPP. The concentrated supernatant of *R. oligosporus* BCRC31750 exhibiting TPP activity (18.5 units/mg) was subjected to chromatography using a HiTrap Q Sepharose Fast Flow column and eluted with a stepwise NaCl gradient. The chromatographic profile is shown in Figure 2A. The column yielded four peaks, of which that eluting at 155 mM NaCl was active. The specific activity of the collected active fractions was 24.2 units/mg, with a yield of 22.3%. The active fractions were fractionated on a MonoQ HR 5/5 column. All of the proteins present in fractions 25−31 were active (Figure 2B), and the target enzyme, with specific activity of 29.5 units/mg and a yield of 7.1%, was obtained. All active fractions were further subjected to chromatography on a HiLoad 16/60 Superdex 200 prep-grade column, which resulted in elution of five protein peaks, among which only the third peak showed proteolytic activity (Figure 2C). A summary of the purification steps for TPP produced by *R. oligosporus* is shown in Table 1. The enzyme was purified 15.4-fold from the culture supernatant, with a yield of 0.9% and a specific activity of 285.4 units/mg.

Low purification yields were also observed with TPP II purified from rat liver and from *Arabidopsis thaliana*, with 2 and 5% yields,

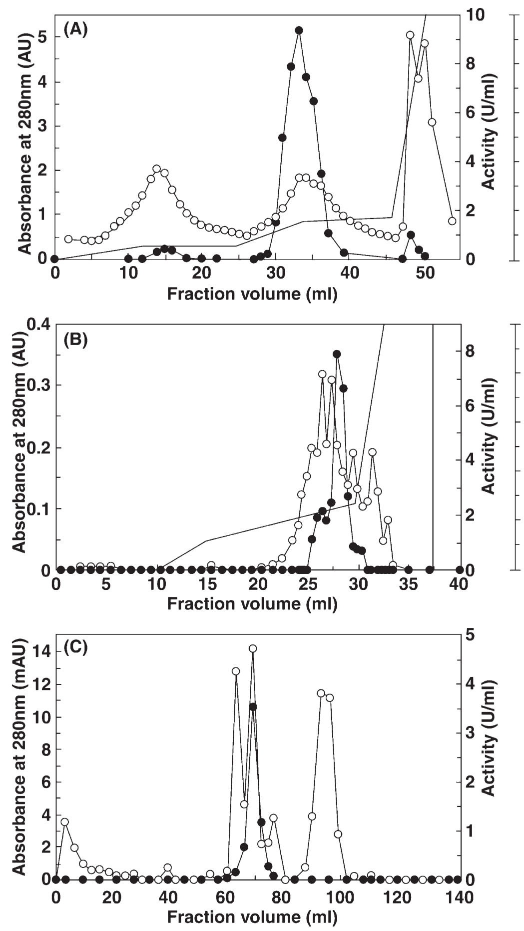

Figure 2. Elution profiles for chromatographic purification of TPP from the culture supernatant of *R. oligosporus*. (A) Anion-exchange chromatography on a Q Sepharose column. (B) Anion-exchange chromatography on a MonoQ column. (C) Gel filtration chromatography on a Superdex 200 column. The experimental conditions are described in the Materials and Methods. (○) Absorbance at 280 nm, (●) proteolytic activity, and (—) NaCl gradient.

respectively.[7,8] The yields of the enzyme purified from *Prevotella nigrescens* and *Streptomyces mobaraensis* were also low, ∼8.3 and 4%, respectively.[15,17] In our previous work, ammonium sulfate precipitation was used to concentrate TPP, which resulted in almost the complete loss of TPP activity. Whether the low yield of the *R. oligosporus* enzyme reflects greater initial heterogeneity or greater instability of the enzyme remains to be determined.

Purity and Molecular Mass. The purity of the enzyme was confirmed by SDS−PAGE after silver staining (Figure 3). The purified protein migrated as a single band on the denaturing gel with an apparent molecular mass of ∼70 kDa (lane 4 of Figure 3). The molecular mass of the native enzyme determined by gel filtration chromatography was ∼136.5 kDa, indicating that purified TPP consisted of two subunits with similar molecular masses.

PTP from *Porphyromonas gingivalis* is a homodimeric cell surface-associated serine protease with two 77 kDa subunits,[33] while the enzyme from *P. nigrescens* is a 56 kDa intracellular protease.[15] TAPs from *S. lividans* and *S. mobaraensis* have molecular masses of 55 and 53 kDa, respectively.[12,17] *A. fumigatus* was reported to secrete the proteases SedB (60−90 kDa), SedC (65 kDa), and SedD (70−100 kDa), which possess TPP

Table 1. Purification of TPP from *R. oligosporus* BCRC31750

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	purification (fold)	yield (%)
supernatant	17.0	314.78	18.5	1	100
Q Sepharose	2.90	70.13	24.2	1.3	22.3
MonoQ	0.76	22.42	29.5	1.6	7.1
Superdex 200	0.01	2.85	285.4	15.4	0.9

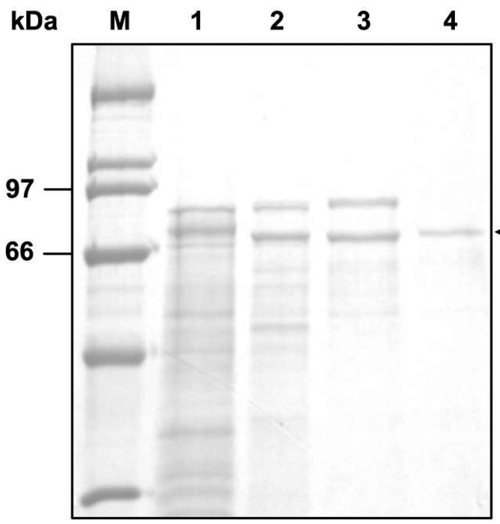

Figure 3. SDS−PAGE analysis of TPP purification from *R. oligosporus*. After electrophoresis, the gel was stained with silver nitrate. Lane M, molecular mass markers; lane 1, supernatant of the *R. oligosporus* culture broth; lane 2, eluted fraction with TPP activity from the Q Sepharose column; lane 3, eluted fraction with TPP activity from the MonoQ column; lane 4, eluted fraction with TPP activity from the Superdex 200 column. The arrow indicates the position of the monomer at 70 kDa.

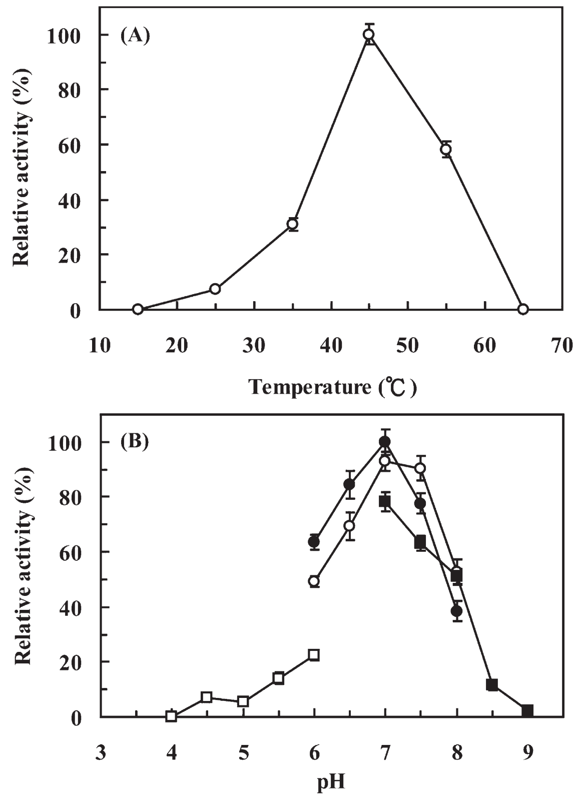

Figure 4. Effects of the (A) temperature and (B) pH on the activity of purified TPP. (A) Temperature dependence was determined in 100 mM sodium phosphate buffer (pH 7.0) at various temperatures for 30 min. (B) The effect of pH on TPP activity was determined at 45 °C for 30 min in a mixture containing purified TPP (20 ng) in 100 mM glycine-HCl buffer at pH 4−6 (□), 100 mM sodium phosphate buffer at pH 6−8 (●), 100 mM potassium phosphate buffer at pH 6−8 (○), and 100 mM Tris-HCl buffer at pH 7−9 (■). The enzyme activity was measured as described in the Materials and Methods. The data are means of three independent experiments, and the highest activity was defined as 100%. The bars indicate standard deviations.

activity.[13] The molecular mass of rat spleen TPP I was estimated to be 47 kDa by SDS−PAGE and 64 kDa by gel filtration chromatography.[3] TPP II from rat liver, *Drosophila melanogaster*, and *A. thaliana* is composed of a single 135−150 kDa subunit that forms an oligomeric complex with a native molecular mass of ≥1000 kDa.[5−8] Unlike the known TPPs from prokaryotic and eukaryotic organisms, *R. oligosporus* TPP represents a novel homodimeric extracellular TPP.

N-Terminal Amino Acid Sequence of TPP. The N-terminal amino acid sequence of purified TPP was identified as "S-K-I-Q-V-K-Y-A-T-T-P-K-M-S-T". Protein homology searches revealed no significant similarity with published TPP amino acid sequences in the National Center for Biotechnology Information (NCBI) GenBank database. The highest similarity was observed to *Polysphondylium pallidum* aminopeptidase N (GenBank EFA77142.1) spanning amino acid residues 297−307, indicating that the purified enzyme possesses aminopeptidase activity. For protein identification, the N-terminal sequence was also searched for within the genome database for *Rhizopus oryzae* RA 99-880 (http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/Blast.html); however, no detectable similarity was observed.

Effects of the Temperature and pH on TPP Activity. The optimum temperature for TPP activity was determined at pH 7.0 using the substrate Ala-Ala-Phe-*p*NA; TPP was found to be active between 35 and 55 °C, with maximum activity at 45 °C (Figure 4A). The effect of pH on TPP activity was studied using Ala-Ala-Phe-*p*NA as the substrate at 45 °C. TPP was found to be active between pH 6.0 and 8.0, with maximum activity at pH 7.0 in sodium phosphate buffer (Figure 4B). The relative activities at pH 6.0 and 8.0 in sodium phosphate buffer were about 63 and 39%, respectively, of that at pH 7.0. Only about 10% of the optimum activity was observed at pH 5.5 and 8.5. These results are similar to those for PTP from *P. gingivalis*[16] and TAP from *S. mobaraensis*,[17] which had an optimum pH of 7.0−7.5. An optimum alkaline pH (7.5−8.5) was reported for TAP from *S. lividans*,[12] while an optimum acidic pH (5.0−6.0) was observed for SedB, SedC, and SedD from *A. fumigatus*.[13] Mammalian TPP II has an optimum pH of 7.5,[6,8] while an acidic environment is preferable for TPP I.[3,34]

Table 2. Effects of Protease Inhibitors and Reducing Agents on TPP Activity

inhibitor	concentration (mM)	relative activity (%)[a]
none		100 ± 2.5
pepstatin A	0.1	97.1 ± 2.3
	1	95.4 ± 2.2
phenylmethylsulfonyl fluoride	0.1	65.9 ± 1.3
	1	39.1 ± 1.1
Ala-Ala-Phe-chloromethylketone	0.1	43.2 ± 0.9
	1	40.9 ± 1.1
Pefabloc SC	1	70.6 ± 1.4
E-64	0.1	97.0 ± 2.0
	1	89.0 ± 1.7
N-ethylmaleimide	1	102.0 ± 2.3
iodoacetic acid	1	103.9 ± 2.6
β-mercaptoethanol	1	94.0 ± 2.2
dithiothreitol	1	95.2 ± 2.0

[a] TPP activity assayed in the absence of inhibitors was defined as 100%.

Table 3. Effects of Chelating Agents and Various Metal Ions on TPP Activity[a]

compound	concentration (mM)	relative activity (%)[b]
none	0	100.0 ± 3.3
EDTA	10	10.3 ± 1.0
EDTA + $MgCl_2$	10 + 1	19.1 ± 0.9
EDTA + $CaCl_2$	10 + 1	66.1 ± 2.5
EDTA + $ZnCl_2$	10 + 1	0
EDTA + $NiCl_2$	10 + 1	52.9 ± 1.9
EDTA + $CuCl_2$	10 + 1	0
EDTA + $MnCl_2$	10 + 1	233.0 ± 4.2
EDTA + $CoCl_2$	10 + 1	269.3 ± 4.2
dipicolinic acid	5	3.1 ± 0.2
dipicolinic acid + $MgCl_2$	5 + 1	26.2 ± 1.1
dipicolinic acid + $CaCl_2$	5 + 1	77.9 ± 3.5
dipicolinic acid + $ZnCl_2$	5 + 1	7.2 ± 0.2
dipicolinic acid + $NiCl_2$	5 + 1	101.0 ± 3.8
dipicolinic acid + $CuCl_2$	5 + 1	0
dipicolinic acid + $MnCl_2$	5 + 1	288.7 ± 6.0
dipicolinic acid + $CoCl_2$	5 + 1	312.3 ± 5.8

[a] The purified enzyme was incubated at 30 °C in 100 mM sodium phosphate buffer (pH 7.0) with 10 mM EDTA or 5 mM dipicolinic acid for 30 min. After the chelating agent was removed by centrifugal ultrafiltration (Amicon Ultra-15 10K Centrifugal Filter Device), 1 mM of the indicated divalent metal salt solution was added and incubation was continued for an additional 30 min before enzyme assay. [b] TPP activity assayed in the absence of metal ions and inhibitors was regarded as 100%.

Effects of Enzyme Inhibitors and Metal Ions on TPP Activity. To determine the nature of the peptidase, the effects of various inhibitors on enzyme activity were examined (Table 2). TPP was inhibited by the serine protease inhibitors PMSF (1 mM) and Pefabloc SC (1 mM), with ~60 and 30% inhibition, respectively. A tripeptidyl chloromethyl ketone analogue, Ala-Ala-Phe-chloromethyl ketone, was also inhibitory. The aspartyl protease inhibitor pepstatin A had no effect on enzyme activity. E-64 (1 mM) inhibited activity slightly, while other cysteine protease inhibitors (*N*-ethylmaleimide and iodoacetic acid) had no effect on enzyme activity. The cysteine protease activator agents dithiothreitol (1 mM) and β-mercaptoethanol (1 mM) were also ineffective.

These inhibition results are similar to those for TPP from *P. nigrescens*. Enzyme activity was inhibited by diisopropylfluorophosphate (DIFP) by 60% at 1 mM and 70% at 10 mM and was reduced to about 40% in the presence of Pefabloc SC (1 mM).[15] PTP from *P. gingivalis* was strongly inhibited by the serine protease inhibitors DIFP and Pefabloc SC, while PMSF had no effect.[16] Low effectiveness of PMSF and differing degrees of inhibition have also been observed for dipeptidyl peptidases II from various organisms and tissues.[35] Variations in inhibition profiles for different serine protease families remain poorly understood. Our results indicate that purified TPP was inhibited, if not strongly, by serine protease inhibitors and may be classified as a serine protease.

The effects of chelating agents and various metal ions on the activity of TPP were examined at pH 7.0 and 45 °C with Ala-Ala-Phe-*p*NA (Table 3). TPP was strongly inhibited by chelating agents, such as 10 mM EDTA (~90%) and 5 mM dipicolinic acid (~97%), suggesting that it is a metalloenzyme. After EDTA was removed by ultrafiltration, the enzyme activity could be strongly reactivated and enhanced to ~233 and 269.3% of the original activity by 1 mM Mn^{2+} or Co^{2+}, respectively. The addition of 1 mM Mg^{2+}, Ni^{2+}, or Ca^{2+} caused slight recovery of TPP activity to about 19.1, 52.9, and 66.1%, respectively, whereas the addition of 1 mM Zn^{2+} or Cu^{2+} inhibited the activity. Similarly, upon treatment of the enzyme with dipicolinic acid, ~288.7, 312.3, and 101% of the original activity could be reactivated by 1 mM Mn^{2+}, Co^{2+}, or Ni^{2+}, respectively. The addition of 1 mM Zn^{2+}, Mg^{2+}, or Ca^{2+} increased TPP activity to about 7.2, 26.2, and 77.9%, respectively, whereas the addition of 1 mM Cu^{2+} inhibited residual enzyme activity. When TPP was not treated with chelating agents, 1 mM Co^{2+} or Mn^{2+} stimulated activity by ~294.5 and 162.8%, respectively. Zn^{2+} reduced TPP activity by up to 50%. Moreover, 0.1 mM $CuCl_2$ inhibited 88.2% of the original activity and completely inhibited activity at 1 mM (data not shown).

On the basis of the above results, the peptidase purified from *R. oligosporus* was identified as a metallo-dependent TPP, which has not previously been reported in eukaryotes. The enzyme activity of TAP from *S. mobaraensis* was found to be enhanced by Ca^{2+} and reduced by up to half in the presence of chelating agents.[17] However, in contrast to TPP, TAP from *S. mobaraensis* is a proline-specific tripeptidyl and tetrapeptidyl peptidase.[14,17] Dipeptidyl peptidase III, belonging to the serine protease family, is also a metalloprotease harboring a conserved HEXXXH motif as a Zn-binding site, which provides a rationale for substrate recognition.[36] The role of metal ions in proteolytic activity of TPP from *R. oligosporus* remains to be determined, including the exact function of metal ions in catalysis and the enzyme proteolytic mechanism.

Proteolytic Activity of TPP. Specificities of purified TPP for various chromogenic substrates are summarized in Table 4. The enzyme most actively cleaved Ala-Ala-Phe-*p*NA, and hydrolysis of Ala-Ala-Ala-*p*NA was also efficient (about 60% of the relative activity toward Ala-Ala-Phe-*p*NA). About 10% relative activity was detected for Phe-Pro-Ala-*p*NA and Pro-Leu-Gly-*p*NA. No activity toward Ala-Phe-Pro-*p*NA was observed in the purified enzyme. Hydrolysis of dipeptidyl-*p*NA, monopeptidyl-*p*NA, and N-terminally blocked tripeptidyl-*p*NA substrates was also not detected. These results indicated that purified TPP had no endoproteolytic activity and catalyzed most tripeptidyl-*p*NA substrates, except when proline occupied the P1 position. Moreover, these data also indicated that purified TPP was free of any contamination with aminopeptidase, dipeptidyl peptidase, and endopeptidase activities.

Table 4. Substrate Specificity of Purified TPP

substrate (1 mM)	specific activity (units/mg)	relative activity (%)[a]
Ala-Ala-Phe-*p*NA	281.9 ± 8.1	100
Ala-Ala-Ala-*p*NA	169.8 ± 6.1	60.2
Ala-Phe-Pro-*p*NA	0[b]	0
Phe-Pro-Ala-*p*NA	31.6 ± 3.8	11.2
Pro-Leu-Gly-*p*NA	28.7 ± 2.2	10.1
N-succinyl-Ala-Ala-Phe-*p*NA	0	0
N-succinyl-Ala-Ala-Ala-*p*NA	0	0
Gly-Pro-*p*NA	0	0
Gly-Arg-*p*NA	0	0
Ala-*p*NA	0	0
Phe-*p*NA	0	0
Pro-*p*NA	0	0

[a] The rate of hydrolysis is expressed as a percentage of the activity compared to that obtained using Ala-Ala-Phe-*p*NA as the substrate. Values are means of three independent experiments, with a maximum sample mean deviation of ±3%. [b] No detectable activity observed under the assay conditions.

Substrate specificity of *R. oligosporus* TPP was similar to that reported for mammalian TPP I and TPP II, which show the highest activity toward Ala-Ala-Phe-*p*NA.[3,8] SedB, SedC, and SedD from *A. fumigatus* also efficiently catalyze Phe-Pro-Ala-*p*NA and Ala-Ala-Phe-*p*NA.[13] The TPPs described above show no catalytic activity toward the P1-Pro tripeptide substrates. In contrast, PTPs isolated from *S. mobaraensis*,[14,17] *P. nigrescens*,[15] and *P. gingivalis*[16] specifically hydrolyze tetra-, tri-, and/or dipeptide substrates with a P1-proline residue.

To further demonstrate the proteolytic activity of TPP, the synthetic peptide AAFYY was used as the substrate and the resulting reaction products were analyzed by HPLC/MS/MS. Using the full-scan mode for MS analysis, the most abundant $[M + H]^+$ ions for AAFYY and AAF standards were *m*/*z* 634.3 and 308.2, respectively; these were selected as the precursor ions for MS/MS analysis. Figure 5 shows the product ion spectra for AAFYY and AAF obtained by infusion in the positive-ion mode. The primary product ions for AAFYY were *m*/*z* 290.2, 345.2, and 453.2 (Figure 5A). The primary product ions for AAF were *m*/*z* 143.1, 166.1, and 237.1 (Figure 5B). The MS/MS transitions *m*/*z* 634.3−453.2 and *m*/*z* 308.2−166.1 were then selected for AAFYY and AAF analysis, respectively, using MS/MS in the MRM mode. To determine AAFYY and AAF using HPLC/MS/MS analysis, the standard peptides (10 μM each) were separated by a Polaris C18-A column and subjected to MS/MS. AAFYY and AAF were eluted at retention times of 6.65 and 5.60 min, respectively, in the MRM chromatograms (panels C and D of Figure 5). When purified TPP was incubated with the peptide

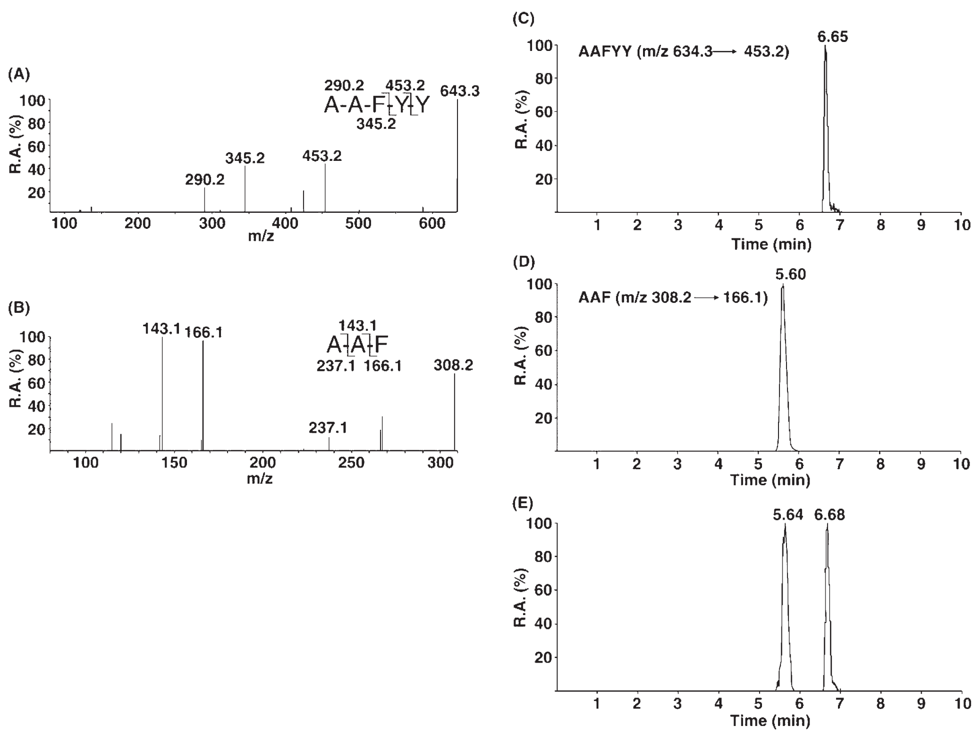

Figure 5. HPLC/MS/MS analysis of the proteolytic activity of TPP. Positive electron spray ionization product ion mass spectra of (A) AAFYY and (B) AAF. The MS/MS transitions of *m*/*z* 634.3−453.2 and *m*/*z* 308.2−166.1 were selected for detection of AAFYY and AAF, respectively. Mass chromatograms for (C) AAFYY and (D) AAF. Standard solutions (10 μM) of AAFYY and AAF in 100 mM sodium phosphate buffer (pH 7.0) were separated using a Polaris C18-A column and detected under MRM mode. (E) Mass chromatogram for AAFYY cleaved by purified TPP. The catalytic conditions are described in the Materials and Methods. RA = relative abundance.

AAFYY, a mass peak consistent with the degradation product AAF was detected, confirming the TPP activity of the enzyme (Figure 5E).

AUTHOR INFORMATION

Corresponding Author

*Telephone: +886-4-2631-8652, ext. 5611. Fax: +886-4-2652-5386. E-mail: chkao@sunrise.hk.edu.tw.

Author Contributions

†These authors contributed equally to this work.

Funding Sources

This work was supported by Grants 98-2313-B-241-001 and 98-2313-B-241-006-MY3 from the National Science Council of Taiwan.

ABBREVIATIONS USED

AAF, Ala-Ala-Phe; AAFYY, Ala-Ala-Phe-Tyr-Tyr; BSA, bovine serum albumin; DIFP, diisopropylfluorophosphate; DPY, dextrin, polypeptone, and yeast extract; EDTA, ethylenediaminetetraacetic acid; HPLC/MS/MS, high-performance liquid chromatography/tandem mass spectrometry; MRM, multiple-reaction monitoring; PMSF, phenylmethylsulfonyl fluoride; *p*NA, *p*-nitroanilide; PTP, prolyl tripeptidyl peptidase; PVDF, polyvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TAP, tripeptidyl aminopeptidase; TPP, tripeptidyl peptidase

REFERENCES

(1) Tomkinson, B. Tripeptidyl peptidases: Enzymes that count. *Trends Biochem. Sci.* **1999**, *24*, 355–359.

(2) Rawlings, N. D.; Barrett, A. J. Tripeptidyl-peptidase I is apparently the CLN2 protein absent in classical late-infantile neuronal ceroid lipofuscinosis. *Biochim. Biophys. Acta* **1999**, *1429*, 496–500.

(3) Vines, D.; Warburton, M. J. Purification and characterisation of a tripeptidyl aminopeptidase I from rat spleen. *Biochim. Biophys. Acta* **1998**, *1384*, 233–242.

(4) Page, A. E.; Fuller, K.; Chambers, T. J.; Warburton, M. J. Purification and characterization of a tripeptidyl peptidase I from human osteoclastomas: Evidence for its role in bone resorption. *Arch. Biochem. Biophys.* **1993**, *306*, 354–359.

(5) Renn, S. C.; Tomkinson, B.; Taghert, P. H. Characterization and cloning of tripeptidyl peptidase II from the fruit fly, *Drosophila melanogaster*. *J. Biol. Chem.* **1998**, *273*, 19173–19182.

(6) Balow, R. M.; Ragnarsson, U.; Zetterqvist, O. Tripeptidyl aminopeptidase in the extralysosomal fraction of rat liver. *J. Biol. Chem.* **1983**, *258*, 11622–11628.

(7) Book, A. J.; Yang, P.; Scalf, M.; Smith, L. M.; Vierstra, R. D. Tripeptidyl peptidase II. An oligomeric protease complex from *Arabidopsis*. *Plant Physiol.* **2005**, *138*, 1046–1057.

(8) Balow, R. M.; Tomkinson, B.; Ragnarsson, U.; Zetterqvist, O. Purification, substrate specificity, and classification of tripeptidyl peptidase II. *J. Biol. Chem.* **1986**, *261*, 2409–2417.

(9) Seifert, U.; Maranon, C.; Shmueli, A.; Desoutter, J. F.; Wesoloski, L.; Janek, K.; Henklein, P.; Diescher, S.; Andrieu, M.; de la Salle, H.; Weinschenk, T.; Schild, H.; Laderach, D.; Galy, A.; Haas, G.; Kloetzel, P. M.; Reiss, Y.; Hosmalin, A. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat. Immunol.* **2003**, *4*, 375–379.

(10) Kida, E.; Golabek, A. A.; Walus, M.; Wujek, P.; Kaczmarski, W.; Wisniewski, K. E. Distribution of tripeptidyl peptidase I in human tissues under normal and pathological conditions. *J. Neuropathol. Exp. Neurol.* **2001**, *60*, 280–292.

(11) Hilbi, H.; Puro, R. J.; Zychlinsky, A. Tripeptidyl peptidase II promotes maturation of caspase-1 in *Shigella flexneri*-induced macrophage apoptosis. *Infect. Immun.* **2000**, *68*, 5502–5508.

(12) Krieger, T. J.; Bartfeld, D.; Jenish, D. L.; Hadary, D. Purification and characterization of a novel tripeptidyl aminopeptidase from *Streptomyces lividans* 66. *FEBS Lett.* **1994**, *352*, 385–388.

(13) Reichard, U.; Lechenne, B.; Asif, A. R.; Streit, F.; Grouzmann, E.; Jousson, O.; Monod, M. Sedolisins, a new class of secreted proteases from *Aspergillus fumigatus* with endoprotease or tripeptidyl-peptidase activity at acidic pHs. *Appl. Environ. Microbiol.* **2006**, *72*, 1739–1748.

(14) Umezawa, Y.; Yokoyama, K.; Kikuchi, Y.; Date, M.; Ito, K.; Yoshimoto, T.; Matsui, H. Novel prolyl tri/tetra-peptidyl aminopeptidase from *Streptomyces mobaraensis*: Substrate specificity and enzyme gene cloning. *J. Biochem.* **2004**, *136*, 293–300.

(15) Fujimura, S.; Ueda, O.; Shibata, Y.; Hirai, K. Isolation and properties of a tripeptidyl peptidase from a periodontal pathogen *Prevotella nigrescens*. *FEMS Microbiol. Lett.* **2003**, *219*, 305–309.

(16) Banbula, A.; Mak, P.; Bugno, M.; Silberring, J.; Dubin, A.; Nelson, D.; Travis, J.; Potempa, J. Prolyl tripeptidyl peptidase from *Porphyromonas gingivalis*. A novel enzyme with possible pathological implications for the development of periodontitis. *J. Biol. Chem.* **1999**, *274*, 9246–9252.

(17) Zotzel, J.; Pasternack, R.; Pelzer, C.; Ziegert, D.; Mainusch, M.; Fuchsbauer, H. L. Activated transglutaminase from *Streptomyces mobaraensis* is processed by a tripeptidyl aminopeptidase in the final step. *Eur. J. Biochem.* **2003**, *270*, 4149–4155.

(18) Oda, H.; Saiki, K.; Tonosaki, M.; Yajima, A.; Konishi, K. Participation of the secreted dipeptidyl and tripeptidyl aminopeptidases in asaccharolytic growth of *Porphyromonas gingivalis*. *J. Periodontal Res.* **2009**, *44*, 362–367.

(19) Takaya, N.; Yamazaki, D.; Horiuchi, H.; Ohta, A.; Takagi, M. Intracellular chitinase gene from *Rhizopus oligosporus*: Molecular cloning and characterization. *Microbiology* **1998**, *144*, 2647–2654.

(20) Casey, A.; Walsh, G. Identification and characterization of a phytase of potential commercial interest. *J. Biotechnol.* **2004**, *110*, 313–322.

(21) Jin, B.; van Leeuwen, H. J.; Patel, B.; Doelle, H. W.; Yu, Q. Production of fungal protein and glucoamylase by *Rhizopus oligosporus* from starch processing wastewater. *Process Biochem.* **1999**, *34*, 59–65.

(22) Ozsoy, H. D.; Kumbur, H.; Saha, B.; van Leeuwen, J. H. Use of *Rhizopus oligosporus* produced from food processing wastewater as a biosorbent for Cu(II) ions removal from the aqueous solutions. *Bioresour. Technol.* **2008**, *99*, 4943–4948.

(23) Yamada, O.; Sakamoto, K.; Tominaga, M.; Nakayama, T.; Koseki, T.; Fujita, A.; Akita, O. Cloning and heterologous expression of the antibiotic peptide (ABP) genes from *Rhizopus oligosporus* NBRC 8631. *Biosci., Biotechnol., Biochem.* **2005**, *69*, 477–482.

(24) Nout, M. J.; Kiers, J. L. Tempe fermentation, innovation and functionality: Update into the third millenium. *J. Appl. Microbiol.* **2005**, *98*, 789–805.

(25) Chang, C. T.; Hsu, C. K.; Chou, S. T.; Chen, Y. C.; Huang, F. S.; Chung, Y. C. Effect of fermentation time on the antioxidant activities of tempeh prepared from fermented soybean using *Rhizopus oligosporus*. *Int. J. Food Sci. Technol.* **2009**, *44*, 799–806.

(26) Wang, H. L.; Hesseltine, C. W. Studies on the extracellular proteolytic enzymes of *Rhizopus oligosporus*. *Can. J. Microbiol.* **1965**, *11*, 727–732.

(27) Gumbira-Sa'id, E.; Doelle, H. W.; Greenfield, P. F.; Mitchell, D. A. Protein enrichment of sago starch by solid-state fermentation with *Rhizopus* spp. *World J. Microbiol. Biotechnol.* **1991**, *7*, 419–427.

(28) Farley, P. C.; Ikasari, L. Regulation of the secretion of *Rhizopus oligosporus* extracellular carboxyl proteinase. *J. Gen. Microbiol.* **1992**, *138*, 2539–2544.

(29) Heskamp, M. L.; Barz, W. Expression of proteases by *Rhizopus* species during Tempeh fermentation of soybeans. *Nahrung* **1998**, *42*, 23–28.

(30) Sambrook, J.; Fritsh, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(31) Exterkate, F. A. Location of peptidases outside and inside the membrane of *Streptococcus cremoris*. *Appl. Environ. Microbiol.* **1984**, *47*, 177–183.

(32) Farley, P. C.; Sullivan, P. A. The *Rhizopus oryzae* secreted aspartic proteinase gene family: An analysis of gene expression. *Microbiology* **1998**, *144*, 2355–2366.

(33) Ito, K.; Nakajima, Y.; Xu, Y.; Yamada, N.; Onohara, Y.; Ito, T.; Matsubara, F.; Kabashima, T.; Nakayama, K.; Yoshimoto, T. Crystal structure and mechanism of tripeptidyl activity of prolyl tripeptidyl aminopeptidase from *Porphyromonas gingivalis*. *J. Mol. Biol.* **2006**, *362*, 228–240.

(34) Watanabe, Y.; Kumagai, Y.; Fujimoto, Y. Acidic tripeptidyl aminopeptidase in rat liver tritosomes: Partial purification and determination of its primary substrate specificity. *Biochem. Int.* **1992**, *27*, 869–877.

(35) Sentandreu, M. A.; Toldrá, F. Partial purification and characterisation of dipeptidyl peptidase II from porcine skeletal muscle. *Meat Sci.* **2001**, *57*, 93–103.

(36) Baral, P. K.; Jajcanin-Jozic, N.; Deller, S.; Macheroux, P.; Abramic, M.; Gruber, K. The first structure of dipeptidyl-peptidase III provides insight into the catalytic mechanism and mode of substrate binding. *J. Biol. Chem.* **2008**, *283*, 22316–22324.