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Novel Fibrinolytic Enzyme in Fermented Shrimp Paste, a Traditional Asian Fermented Seasoning

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A novel fibrinolytic enzyme was purified from fermented shrimp paste, a popular seasoning used in Asian countries. The enzyme is a monomer with an apparent molecular weight of 18 kDa, and it is composed primarily of β-sheet and random coils. The N-terminal amino acid sequence was determined to be DPYEEPGPCENLQVA. It is a neutral protease with an optimal activity from pH 3 to 7. No inhibition was observed with PMSF, Pepstatin A, E64, and 1,10-phenanthroline, but the enzyme was slightly inhibited by EDTA and Cu^{2+}. It was relatively specific to fibrin or fibrinogen as a protein substrate, yet it hydrolyzed none of the plasma proteins in the studies. In vitro, the enzyme was resistant to pepsin and trypsin digestion. It also had an anticoagulant activity measured with activated partial thrombin time and prothrombin time tests. The novel fibrinolytic enzyme derived from traditional Asian foods is useful for thrombolytic therapy. In addition, this enzyme has a significant potential for food fortification and nutraceutical applications, such that its use could effectively prevent cardiovascular diseases.

KEYWORDS: Fibrinolytic enzyme; fermented shrimp paste; anticoagulant activity; cardiovascular diseases; plasmin; nutraceutical; functional foods

INTRODUCTION

Cardiovascular disease is a main contributing cause of death in the world, inflicting a huge physical, emotional, and financial toll on its victims and their families (*1*). The underlying pathophysiological process in myocardial infarction and stroke is the formation of a fibrin clot, which adheres to the unbroken wall of the blood vessels. Accumulation of fibrin in the blood vessels can interfere with blood flow and may severely damage the heart tissue, leading to irregular heartbeat, cardiac arrest, or death. Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by thrombin (*2*). The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase−plasminogen complex (*3*, *4*). The basis of fibrinolytic therapy is the intravenous administration of an exogenous plasminogen activator, which lyses the thrombus and restores the blood flow to the area of ischemia (*5*, *6*). The three fibrinolytic agents that are currently being used for this purpose include urokinase, streptokinase, and genetically engineered tissue plasminogen activator (t-PA). However, these enzymes are expensive, and patients may suffer from undesirable side effects such as gastrointestinal bleeding, allergic reactions, and resistance to repercussion (*7*−*9*). Therefore, the search for safer thrombolytic agents from other sources continues.

Recently, potent fibrinolytic enzymes have been discovered from fermented food products, such as Japanese natto (*10*, *11*), Korean Chungkook-Jang soy sauce (*12*, *13*), skipjack Shiokara (*14*, *15*), and marine creatures (*16*). In particular, oral administration of natto or its enzyme can effectively enhance the release of an endogenous plasminogen activator in both animal models and human subjects (*17*). A novel fibrinolytic metalloprotease from edible mushroom was isolated and characterized (*18*).

Because of the promising biological benefits from consuming food sources of fibrinolytic enzymes, we have extensively explored new sources of fibrinolytic enzymes from Asian fermented food products, to provide potential health benefits for humans as nutraceutical ingredients. A total of 13 Asian fermented food seasonings have been screened for fibrinolytic activity. A unique strong fibrinolytic enzyme was discovered in fermented shrimp paste, which is a popular seasoning in Asian countries.

MATERIALS AND METHODS

Materials. Commercial fermented seasonings were purchased from local Chinese grocery stores (Toronto, ON). A fermented shrimp paste (1-month-fermented product containing 10−15% NaCl) was provided by SL Food Co. Ltd., Hong Kong. Human plasmin (0.35 units/mg), bovine fibrinogen, bovine immunoglobulin IgG, thrombin, bovine hemoglobin, protease inhibitors [phenylmethanesulfonyl fluoride (PMSF), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), pepstatin A, 2,4-dinitrophenol (DNP), *trans*-epoxysuccinyl-L-leucyamino(guanidin)-butane (E64), ethylenediaminetetraacetic acid (ETA), 1,10-phenanthroline, and collagenase inhibitor], porcine pepsin, and porcine trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was from Fisher Scientific (Fair Lawn, NJ), and DL-dithiothreitol (DTT) was from Wako Pure Chemicals Industries Ltd. (Tokyo, Japan). Dialysis cellulose membrane (1 kDa cutoff) was from Spectrum Laboratories Inc. (Rancho Dominguez, CA), and ultrafiltration

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membrane (10 kDa cutoff) was from Millipore (Canada) Ltd. (Nepean, ON). Thrombin (1 unit/μL), the Superdex 75HR column (1.0 × 24 cm), and the Sephasil Protein C4 5 μm ST 4.6/250 column (0.46 × 25 cm) were from Amersham Pharmacia Biotech (Uppsala, Sweden). Buffer salts and other chemicals used were of reagent grade and were obtained from Sigma Chemical Co.

Fibrin Plate Assay. A fibrin plate assay (*19*, *20*) was used to measure fibrinolytic activity of the samples. In the assay, 0.35 unit/mg of human plasmin (Sigma Chemical Co.) was used as a standard fibrinolytic protease. The fibrin plate was prepared by dissolving 0.8% (w/v) of bovine fibrinogen in 0.8% (w/v) of agarose solution at 37 °C. The fibrinogen solution was mixed with 0.2% (v/v) of 1 unit/μL of thrombin protease (Amersham Pharmacia Biotech) and poured into a 5 cm glass Petri dish. The dish was left at room temperature for 2 h to form a fibrin clot layer. A hole (2 mm in diameter) was punched for sample application on the fibrin plate. After incubation of the plates for 17 h at room temperature, the diameter of the clear zone was measured, and fibrinolytic activity was expressed in plasmin units, which was determined from the plasmin standard curve.

Crude Enzyme Preparation. Each of the 13 traditional Asian fermented food samples, including fermented black beans, instant soybean paste, fermented shrimp paste, pickled gouramy fish, tempeh, light soy sauce, sweet bean paste sauce, yellow bean sauce, red fermented bean curd, white fermented bean curd, fermented cow's milk, glutinous rice in wine, and stinky bean curd powder, was homogenized with 100 mM Tris-buffered saline (TBS), at pH 8.0, to extract crude enzymes. Insoluble materials were removed by centrifugation at 12000*g* for 1 h at 4 °C. The supernatant liquid was assayed for fibrinolytic activity by the fibrin plate assay.

Purification of Fibrinolytic Enzymes. After all 13 samples had been screened, a strong fibrinolytic enzyme was found in only fermented shrimp paste. The crude enzyme extract of fermented shrimp paste was prepared as described. To remove most of the impurities, the supernatant liquid was filtered with Whatman no. 1 filter paper and dialyzed with a 1 kDa cutoff cellulose membrane (Spectrum Laboratories Inc.) against deionized water for 2 days at 4 °C. The dialyzed protein sample was freeze-dried. A 10 mg/mL crude fermented shrimp paste enzyme solution was prepared from the freeze-dried sample in 10 mM phosphate-buffered saline (PBS) at pH 7.0. About 5 mg/0.2 mL PBS of crude enzyme was applied to the Superdex 75HR column (1.0 × 24 cm) (Amersham Pharmacia Biotech), which was equivalent in 10 mM PBS, pH 7.0. The column was eluted with the same buffer at a flow rate of 0.5 mL/min using a Bio Logic Workstation pump (Bio-Rad Laboratories, Hercules, CA). The fraction containing fibrinolytic activity was collected and concentrated with a 10 kDa cutoff ultrafiltration membrane (Millipore Canada Ltd). Reversed phase chromatography by using a Sephasil Protein C4 5 μm ST 4.6/250 column (0.46 × 25 cm) (Amersham Pharmacia Biotech) was performed to separate the active enzyme fraction collected from gel filtration. The column was eluted with a 10 mM ammonium acetate (buffer A) and 10 mM ammonium acetate in 75% acetonitrile (buffer B) gradient at pH 7.0. The fraction containing fibrinolytic activity was collected and dialyzed with a 3.5 kDa cutoff cellulose membrane (Spectrum Laboratories, Inc.) against deionized water for 1 day to remove acetonitrile. The dialyzed protein sample was concentrated with a 10 kDa cutoff Centricon centrifugal filter (Millipore Canada Ltd.).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out on a 15% polyacrylamide gel under constant voltage at 150 V for 1 h (*21*). The sample was treated with 5% (v/v) of β-mercaptoethanol at 90 °C for 10 min. Wide-range and low molecular weight standard proteins (Sigma Chemical Co.) were used as molecular markers. The gels were stained with silver staining according to the method of Heukeshoven and Dernick (*22*).

Protein Assay. The Bio-Rad DC assay kit (Bio-Rad Laboratories) was used to determine protein concentration in the experiment. The assay was a modified version of the Lowry method, and BSA (Fisher Scientific) was used as a protein standard.

N-Terminal Amino Acid Sequence Analysis. Amino acid terminal sequence analysis of the isolated enzyme was carried out with a PE Applied Biosystems model 476A/494 protein sequencer (Perkin-Elmer Instruments, Norwalk, CT).

Amino Acid Composition. Amino acid analysis of the enzyme was carried out with a Perkin-Elmer Applied Biosystems model 130A PTC amino acid analyzer.

Circular Dichroic (CD) Analysis. The far-UV spectrum of the enzyme was determined using a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Pure enzyme (0.116 mg/mL) was resolved in 10 mM phosphate buffer at pH 7.0. Buffer and sample were filtered through a 0.22 μm filter and degassed before use. The enzyme spectrum was obtained by scanning the sample three times, from 190 to 250 nm, using a quartz cuvette with a path length of 0.1 cm (Japan Spectroscopic Co., Ltd.). Phosphate buffer was used to establish a baseline, which was subtracted from the sample spectrum. The mean residue weight ellipticities were calculated, using a mean residue weight of 110, as described by Mulkerrin (*23*), and expressed in degrees centimeters squared per decimole. The percentages of the different secondary structures, including α-helix, β-sheet, β-turn, and random coil, were estimated using the Jasco protein secondary structure estimation program, based on the method of Yang et al. (*24*).

Effect of pH on Activity. The pH effect on fibrinolytic activity of the enzyme was assayed from pH 2.0 to 12.0. The enzyme in 10 mM PBS at pH 7.0 was mixed with different buffers in the range of 2−12. The buffers used and their pH ranges were 0.05 N HCl (pH 2), 50 mM sodium acetate buffer (pH 3−5), 50 mM phosphate buffer (pH 6−8), 50 mM carbonate buffer (pH 9 and 10), and 0.05 N NaOH (pH 12). The reaction mixtures were incubated for 1 h at 37 °C, and the enzyme activities were measured by fibrin plate. Maximum activity was expressed as 100%, and others were compared to the maximum activity.

Effect of Temperature on Activity. The effect of temperature on fibrinolytic activity was studied at various temperatures at pH 7.0. The enzyme in 10 mM PBS at pH 7.0 was heated for 10 min at different temperatures starting from 30 to 90 °C (30, 40, 50, 60, 65, 70, 80, and 90 °C). The enzyme was applied to the fibrin plate, and the relative activities were calculated as a percentage of the maximum activity.

Effects of Protease Inhibitors and Metal Ions. Nine protease inhibitors (Sigma Chemical Co.), including PMSF, EDTA, DTT, DNP, E64, TLCK, pepstatin A, 1,10-phenanthroline, and collagenase inhibitor, were used to characterize the fibrinolytic enzyme. Each inhibitor was dissolved in deionized water and mixed with pure enzyme (in 10 mM PBS at pH 7) at 37 °C for 10 min. Enzyme activity at two different inhibitor concentrations, 1 and 2 mM, was measured by using the fibrin plate assay. The effects of various divalent ions, including Mg^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{2+} ions, on fibrinolytic activity were investigated. Metal chlorides (Sigma Chemical Co.) were dissolved in deionized water and mixed with pure enzyme (in 10 mM PBS at pH 7.0) at 37 °C for 10 min. The concentration of the reaction mixture was 1 mM, and the enzyme activity was determined by fibrin plate assay. The relative activity was calculated on the basis of the activity of the pure enzyme without any metal chlorides under the same experimental conditions.

Substrate Specificity. The purified enzymes in 10 mM PBS at pH 7.0 were incubated with 1 mg/mL of bovine fibrinogen (Sigma Chemical Co.) in a ratio of 1:50 (w/w) at 37 °C for various time periods (0, 15, 20, 60, 120, and 180 min). The fibrinolytic degradation pattern was determined by SDS-PAGE using a 10% polyacrylamide gel. Gel was stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 and destained using a 40% (w/v) methanol and 7% acetic acid solution. Pure enzymes in 10 mM PBS at pH 7.0 were incubated with 1 mg/mL of each of the four plasma proteins in a ratio of 1:50 (w/w) at 37 °C for 3 h. The plasma proteins used were BSA (Fisher Scientific), bovine immunoglobulin G (IgG) (Sigma Chemical Co.), thrombin (Th), and hemoglobin (Hb) (Amersham Pharmacia Biotech). SDS-PAGE of the reduced reaction mixtures was done on a 10% polyacrylamide gel, and the proteins were visualized with Coomassie Blue.

Anticoagulant Activity. The anticoagulant activity of the fermented shrimp paste enzyme was measured with active partial thrombin time (APTT) and prothrombin time (PT) tests (Sigma). Fresh human blood was collected in 3.8% sodium citrate. To measure APTT, the serum was centrifuged at 2500*g* for 15 min. In the assay, 100 μL of human serum was incubated with various amounts of the pure enzyme (0, 1.25, 2.5, 5, and 10 μg) at 37 °C for 1 min. One hunder microliters of APTT reagent (Sigma Chemical Co.) was added, and the reaction mixture

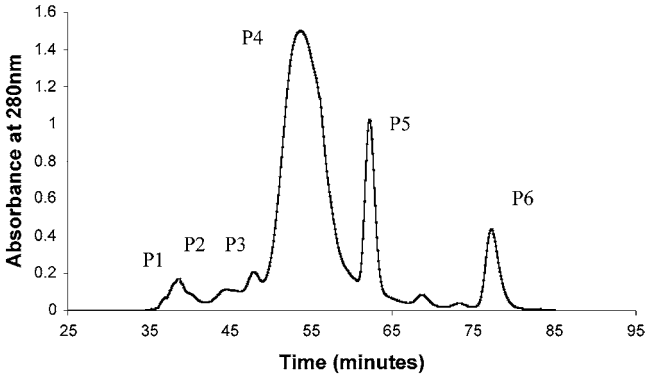

Figure 1. HPLC elution profile of fermented shrimp paste enzyme from the Superdex 75HR column. The active enzyme faction is signified by an asterisk in the profile.

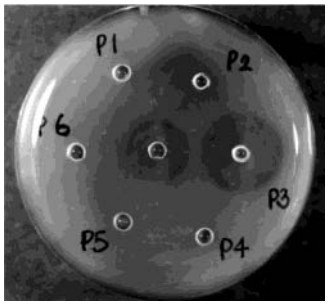

Figure 2. Fibrinolytic activities of the fractions, peaks 1–6, eluted from the Superdex 75HR column. Plasmin was used as a positive control.

was incubated for another 3 min. The reaction was initiated by adding 100 μL of 20 mM $CaCl_2$. To measure PT, the serum was centrifuged at 2500*g* for 15 min. In the assay, 100 μL of human serum was incubated with various amounts of pure enzyme (0, 1.25, 2.5, 5, and 10 μg) at 37 °C for 3 min. The reaction was initiated by adding 200 μL of ThromboMax with calcium reagent (Sigma Chemical Co.). Human serum without the addition of the enzyme was used as a control. This experiment was carried out under approval from the University of Guelph, Human Subjects Committee.

Effects of Digestive Enzymes on Activity. The crude enzymes in HCl (pH 2) and PBS (pH 8) were incubated with 4% (w/w) of pepsin and 0.2% (w/w) of trypsin extracted from porcine stomach mucosa (Sigma Chemical Co.), respectively, at 37 °C for 1 h and overnight, respectively. Enzyme activities were measured by the fibrin plate assay, and the hydrolysis patterns were determined by SDS-PAGE using a 15% polyacrylamide gel. Proteins were stained with silver nitrate.

Statistical Analysis. Data were analyzed by ANOVA (SPSS version 8.0 for Windows; SPSS, Chicago, IL), and means were separated by Duncan's multiple-range test. Significance was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Crude enzyme extracts from fermented shrimp paste were subjected to a Superdex 75HR column on the basis of their differential sedimentation in a gravitational field related to their molecular weight, ranging from 3 to 70 kDa (**Figure 1**). The enzyme was eluted with 10 mM PBS at pH 7.0. As shown in **Figure 1**, six different fractions (P1−P6) were eluted from the gel matrix, and the third peak (P3) contained the majority of enzyme activity (**Figure 2**). This active fraction came off the column right before the contaminated dye in fermented shrimp paste (P4), implying it is a low molecular weight protein. According to SDS-PAGE analysis, the active fraction (P3) collected from gel filtration consisted of at least three different proteins and many minor impurities (data are not shown). This crude enzyme fraction was then purified on the basis of the reversed phase chromatography. A pH 7.0 buffer system was

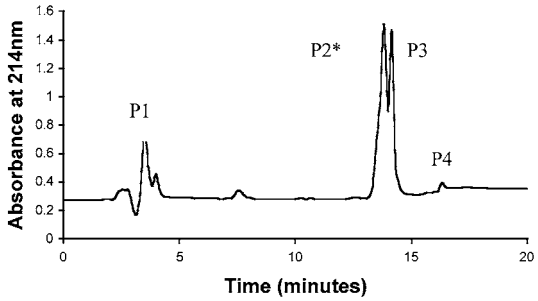

Figure 3. HPLC elution profile of fermented shrimp paste enzyme from the Sephasil Protein C4 5 μm ST 4.6/250 column. The active enzyme faction is signified by an asterisk in the profile.

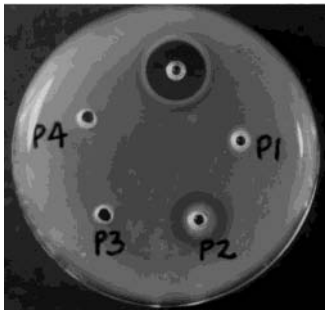

Figure 4. Fibrinolytic activities of the fractions, peaks 1–4, eluted from the Sephasil Protein C4 5 μm ST 4.6/250 column. Plasmin was used as a positive control.

chosen to elute the enzyme from a Sephasil protein C4 column, as preliminary data indicated that separation of enzyme was optimized at neutral pH. As shown in **Figure 3**, four fractions, in increasing order from lowest to highest hydrophobicity, were eluted from the column. According to the fibrin plate assay shown in **Figure 4**, the second peak (P2) was shown to have fibrinolytic activity. This active enzyme fraction (P2) was enlisted under extremely hydrophobic conditions (100% buffer B), indicating that the enzyme has highly hydrophobic characteristics and is stable in such a hydrophobic environment. This is unique because many biologically active components are inactivated with 100% acetonitle. According to SDS-PAGE analysis, the fibrinolytic enzyme from fermented shrimp paste was a monomer with an apparent molecular mass of 18 kDa (**Figure 5**). The purification results are summarized in **Table 1**. The enzyme was purified 12.7-fold with an 18.2% recovery, and it has a specific activity of 2.54 units/mg.

The N-terminal amino acid sequence of the first 15 residues of the enzyme is shown in **Figure 6**. In comparison to other fibrinolytic enzymes that have been discovered from various fermented foods, no homology in the N-terminal sequence could be observed. We could not identify it with any other existing protein sequence from a protein data bank and thus concluded that the enzyme is a novel enzyme. The amino acid composition of the enzyme is summarized in **Table 2**. The enzyme consisted of a high content of proline (12.04 mol %), which has unique imino bonds. The enzyme contains rich charged amino acids such as Asx, Glx, and Lys, which are unexpected from the Sephasil protein C4 column profile (**Figure 3**). It is suggested that the enzyme is expected to have a more or less compact structure because of its high content of proline residues. This may be responsible for its being stable in hydrophobic solvent.

The secondary structure of the enzyme was obtained using CD. The observed spectrum of the enzyme and the proportion of secondary structures are shown in **Figure 7** and **Table 3**. The result indicated that the enzyme had a β-type of protein

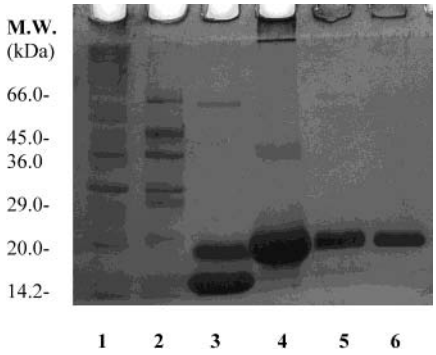

Figure 5. SDS-PAGE of the isolated fibrinolytic enzyme: (lane 1) wide-range molecular weight standard proteins; (lane 2) low molecular weight standard proteins; (lane 3) crude enzyme extract; (lane 4) partially purified enzyme eluted from gel Superdex 75HR column; (lanes 5 and 6) pure fibrinolytic enzyme eluted from C4 5 μm ST 4.6/250 column. The samples were treated with 1% of SDS with β-mercaptoethanol. Electrophoresis was carried out on a 15% polyacrylamide gel at 150 V. Proteins were stained with silver nitrate.

Table 1. Purification of a Fibrinolytic Enzyme from Fermented Shrimp Paste[a]

procedure	total protein (mg)	total activity (units)	specific activity (units/mg)	purification (fold)	yield (%)
crude enzyme	5.0	0.99	0.20	1	100
Superdex 75 HR gel filtration	0.36	0.39	1.08	5.4	39.4
Sephasil protein C4 5 μm ST 4.6/250	0.07	0.18	2.54	12.7	18.2

[a] Data are calculated as the mean (SEM < 5%). The units of activity are calculated on the basis of the plasmin standard (3.5 units/mg).

with 56% of β-sheet. The CD estimation of 30.7% of random coil, 11.2% of β-bend, and only 1.8% of α-helix agreed with that predicted by amino acid composition analysis, as a high percentage of proline residues in the enzyme favored less compact and random secondary structures.

Table 2. Amino Acid Composition of the Enzyme

amino acid	mol (%)	amino acid	mol (%)
Asx	13.47	Pro	12.04
Glx	10.77	Tyr	2.93
Ser	6.71	Val	5.21
Gly	10.62	Met	0.82
His	1.58	Ile	2.52
Arg	3.24	Leu	4.01
Thr	9.75	Phe	1.69
Ala	6.90	Lys	7.74

Table 3. Secondary Structure of the Enzyme

secondary structure	%[a]	secondary structure	%[a]
α-helix	1.8	β-turn	11.2
β-sheet	56.3	random coil	30.7

[a] Calculated as percentages from the CD analysis, using the Jasco protein secondary structure estimation program based on the method of Yang et al. (*24*).

The fibrinolytic activity of the enzyme was examined in various buffers, ranging from pH 2 to 12. The relative activity of the enzyme was plotted against the pH range as shown in **Figure 8**. The enzyme was active in a wide range of pH. The fibrinolytic activity of the enzyme was maximized between pH 3.0 and 7.0, indicating that it was a neutral enzyme. The result was consistent with the amino acid composition analysis, which suggested that there were approximately equal amounts of acidic and basic amino acids in the enzyme. The enzyme remained active at extremely high and low pH values, but the relative activities were decreased to 65 and 56%, respectively. In accordance with the pH stability of the enzyme, it will be best to deliver the enzyme in a neutral medium because the enzyme activity will be decreased under acidic condition. If the enzyme is to be prescribed as pills or capsules, for example, an enteric coating should be used to protect the enzyme from stomach acids.

	1			5				10				15		
Fermented shrimp paste (a novel enzyme)	D	P	Y	E	E	P	G	P	C	E	N	L	Q	V	A
Natto (nattokinase)	A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	
Chungkook-Jang (CK)	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	
Bacillus subtilis BK-17	A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A
Tofuyo (SMCE)	A	Q	T	V	P	Y	G	I	P	Q	I	K	A	D	
Skipjack "Shiokara" (katsuwokinase)	I	V	G	G	Y	E	Q	Z	A	H	S	Q	P	H	Q
Armillariella mellea	X	X	Y	N	G	X	T	X	S	R	Q	T	T	L	V

Figure 6. Comparison of N-terminal amino acid sequence of the enzyme with other fibrinolytic enzymes. The first 14–15 amino acid residues at the N terminus of the enzymes are shown (*13*, *14*, *18*, *26*, *27*).

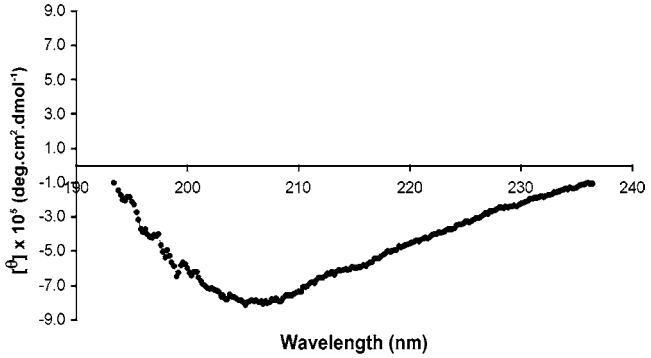

Figure 7. Far-UV spectrum of the enzyme. The spectrum was obtained by scanning the sample three times, from 190 to 250 nm. A 10 mM phosphate buffer was used as the blank.

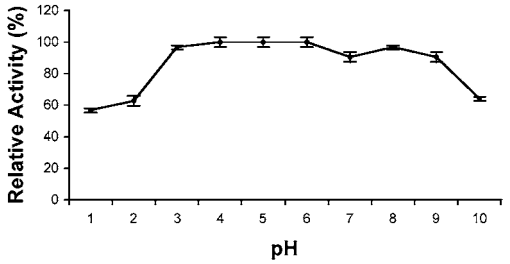

Figure 8. Effect of pH on enzyme activity at 37 °C.

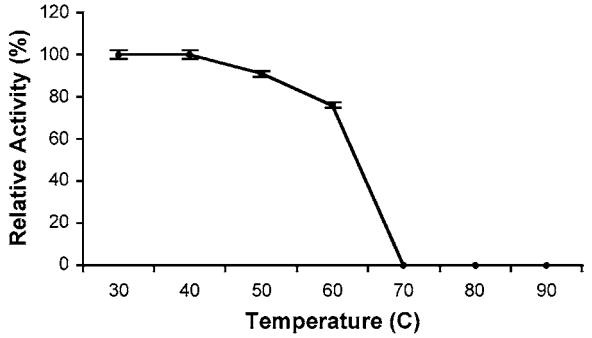

Figure 9. Effect of temperature on enzyme activity at pH 7.0.

The thermal activity of the fiblinolytic enzyme was examined after incubation at different temperatures for 10 min at pH 7. The relative enzyme activity was plotted against the temperature range as shown in **Figure 9**. The enzyme activity was stable between 30 and 40 °C. The enzyme became less active when the temperature rose above 40 °C, and it was completely denatured at temperatures >60 °C. On the basis of the temperature effect on the fibrinolytic activity of the enzyme, the cooking temperature of fermented shrimp paste should be properly controlled. To use fermented shrimp paste as a functional food in preventing heart attack, a low cooking temperature is recommended since the enzyme will be denatured at temperatures >60 °C. Fermented shrimp paste, for instance, can be used as a dipping sauce or as an ingredient of salad dressing. Like Japanese nattokinase, the enzyme can be extracted from fermented shrimp paste and consumed as a nutraceutical in the form of potions or capsules.

The effects of protease inhibitors on fibrinolytic activity of the enzyme are summarized in **Table 4**. In general, there are four classes of proteases, namely, serine proteases, aspartic proteases, cysteine proteases, and metalloproteases. The isolated enzyme was not inhibited by PMSF, TLCK, or pepstatin A, indicating that it was not a serine protease or an aspartic protease. The enzyme was not a cysteine protease either because

Table 4. Effects of Protease Inhibitors and Metal Ions on Fibrinolytic Activity[a]

	relative activity (%)	
reagent	1 mM	2 mM
PMSF[b]	96.7	88.9
EDTA	94.7	77.8
DTT[c]	99.3	77.8
DNP[d]	100	100
E64[e]	100	100
TLCK[f]	98.7	100
pepstatin A	96.7	88.9
1,10-phenanthroline	100	100
collagenase inhibitor	100	100
Mg		93.8
Cu		75.0
Zn		93.8
Hg		93.8
Fe		96.9

[a] Data are calculated as the mean (SEM < 5%) of two sets of experiment. [b] Phenylmethanesulfonyl fluoride. [c] Dithiothreitol. [d] 2,4-Dinitrophenol. [e] *trans*-Epoxysuccinyl-L-leucylamido(quantidino)butane. [f] Trypsin inhibitor.

it was not affected by E64 and DNP. In addition, the enzyme appeared not to be a metalloprotease because it was not inhibited by metalloprotease inhibitors, such as 1,10-phenanthroline and collagenase inhibitor. Its activity, however, was slightly inhibited by EDTA, which forms strong complexes with divalent and higher oxidation state cations such as ferric ion, zinc ion, copper ion, and calcium ion. Therefore, the isolated enzyme was determined to be a novel enzyme, which could not be classified into one of the four classes of protease enzymes, but it shared some similarities with metalloproteses. An inhibition of enzyme activity by DTT predicted that there might be a disulfide linkage in the activie site of the enzyme or the enzyme was denatured by DTT. The effects of cations on enzyme activity were investigated. As shown in **Table 4**, the enzyme was insensitive to calcium ion, mercuric ion, magnesium ion, and ferric ion. Yet significant inhibition was observed when the enzyme was incubated with copper ion. In general, the cations perform a specific role in the modulation of enzyme activity, whereas anions play a more general role in the modulation of the activity of enzymes (*25*). It was hypothesized that copper ion intrudes on the enzyme-catalyzed system by binding to carboxyl groups of the enzyme. In other words, the carboxyl group could be an essential component of the active site for the enzyme function.

The degradation pattern of bovine fibrinogen by purified enzyme is shown in **Figure 10**. The results indicated that the alpha subunit of the multimeric fibrinogen was resistant to the enzyme digestion because its protein band at 64 kDa was intact after 3 h of incubation. The beta and gamma subunits, however, were more susceptible to the proteolytic degradation because they were dissociated into three polypeptides with lower molecular weights. Whether these three polypeptide chains belonged to the beta or gamma subunit has yet to be determined.

As the fibrinolytic enzyme will be delivered into the circulatory system in order to obtain its beneficial effects, it is important that the enzyme does not break down any blood proteins that are not involved in the blood-clotting cascade. The enzyme was incubated with each of the four typical plasma proteins, including BSA, immunoglobulin G, thrombin, and hemoglobin, and the degradation patterns of these proteins by the enzyme are shown in **Figure 11**. The results showed that none of these plasma proteins was broken down by the enzyme after incubation at 37 °C for 1 h. Thus, the fermented shrimp paste enzyme was very specific to fibrin or fibrinogen as protein

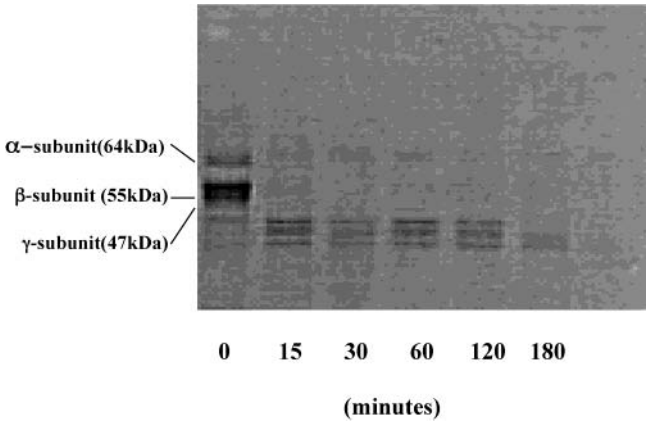

Figure 10. Degradation pattern of bovine fibrinogen by the enzyme. Electrophoresis was carried out on a 10% polyacrylamide gel, and proteins were stained with Coomassie Blue.

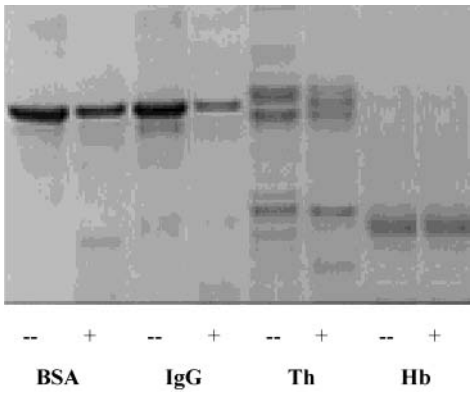

Figure 11. Degradation pattern of bovine plasma proteins, including bovine serum albumin (BSA), immunoglobulin G (IgG), thrombin (Th), and hemoglobulin (Hb), by the enzyme. Electrophoresis was carried out on a 10% polyacrylamide gel, and proteins were stained with Coomassie Blue. The substrate proteins incubated with shrimp paste enzyme at 37 °C for 3 h are specified by "+" signs, and those without enzyme are specified by "–" signs.

substrate, cleaving beta or gamma subunits without affecting the alpha subunits of fibrinogen. The enzyme might not interfere with the normal composition of the plasma proteins while it exerts its fibrinolytic activity in the circulatory system. To further confirm the substrate specificity of the enzyme, the active site of the enzyme could be examined through the coupling between the enzyme and synthetic protein substrates, such as synthetic thrombin substrate and synthetic plasmin substrate. On the basis of the nature of the active site of the fibrinolytic enzyme, the catalytic mechanism, including the binding mechanism and the release mechanism, of the enzyme could be elucidated as well.

The anticoagulant activity of fermented shrimp paste enzyme is expressed as APTT and PT, which are common clinical tests for measuring defects in the intrinsic and extrinsic pathways of the blood-clotting system, respectively, in **Figure 12**. The APTT of human serum was substantially delayed for more than a minute in the presence of 10 μg of isolated enzyme. However, the PT was not prolonged evidently even at the highest enzyme concentration. These results indicated that the enzymes could act as an anticoagulant as well as a fibrinolytic agent. It might inhibit the blood-clotting cascade by enhancing the anticoagulant activity of naturally occurring blood factors. With its high proteolytic activity, the enzyme might also break down any one of the blood-clotting factors involved in the intrinsic pathway

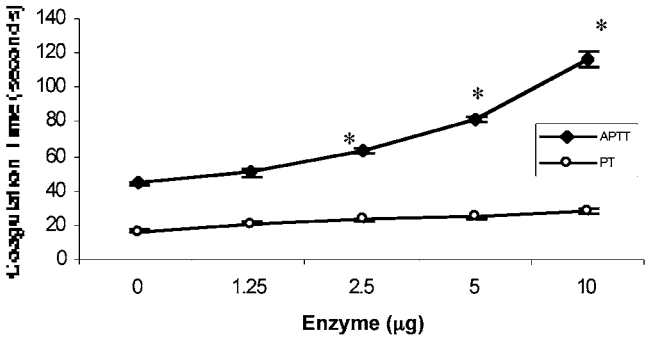

Figure 12. Anticoagulant activity of the enzyme. The blood-clotting time was measured by active partial thrombin time (APTT) and prothrombin time (PT). A significant delay in coagulation time ($p < 0.05$) is signified by an asterisk in the graph.

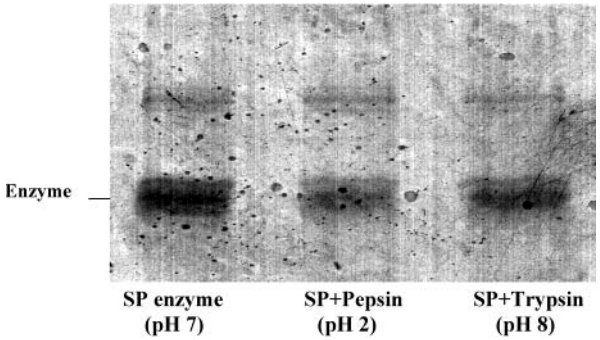

Figure 13. Effects of pepsin and trypsin on the enzyme. Electrophoresis was carried out on a 15% polyacrylamide gel, and proteins were stained with silver nitrate. SP refers to the enzyme in the figure.

of the cascade, leading to abolition of both thrombin generation and platelet aggregation.

The crude enzyme was incubated under two different physiological conditions in vitro, an acidic pH with pepsin in the stomach and a mild alkaline pH with trypsin in the small intestine models. According to the reduced SDS-PAGE in **Figure 13**, the enzyme band at 18 kDa was intact in both experimental conditions. Also, the enzyme remained active (fibrin plate assay data are not shown). Thus, the enzyme was resistant to pepsin and trypsin digestion.

Fermented shrimp paste is traditionally made by using fresh silver shrimp and salt (10−15% w/w). The mixture is crushed and kept for 2 days at room temperature. The partially fermented shrimp paste is then blended into a smooth paste and kept under the sun for another 2−30 days. The finished product is stored in a plastic or wooden bucket, or it is packed into glass bottles for retail sale. The fermentation process is bacteria from air, utensils, or ingredients that are present in nature. However, no commercial cultures are used. To determine whether this fibrinolytic enzyme is a natural component of shrimp or a derivative from the fermentation process of shrimp, shrimp muscle and intestine were both tested for fibrinolytic activity by using the fibrin plate method (data not shown). Neither of them showed fibrinolytic activity, and hence, it was concluded that the enzyme was produced during the natural fermentation stage. The source of this enzyme has yet to be determined. We have isolated four bacterial strains from this product. The identification and isolation of bacteria that produce this novel enzyme are in progress in our laboratory. Kim et al. isolated a *Bacillus* sp. strain that produces a strong fibrinolytic enzyme from Chunkook-Jang, a traditional Korean fermented soybean source (*13*). *Bacillus subtilis* for the production of domestic "natto" in Taiwan is another source of a potent fibrinolytic

enzyme (*26*). A potent fibrinolytic enzyme (nattokinase; NK) was previously isolated from a traditional fermented food in Japan, "nato", by Sumi et al. (*10*). This enzyme is an extracellular serine protease produced from *Bacillus natto*. Sumi et al. further demonstrated that oral administration of natto or NK capsules enhances fibrinolysis in canine plasma in an experimental thrombosic model (*17*). Recently, Jeong et al. also isolated a fibrinolytic enzyme from *B. subtilis* BK-17 strain (*27*). Thus, the shrimp paste might contain *Bacillus* spp. but produces a novel fibrinolytic enzyme. To confirm the physiological functions of the enzyme, the next step will be to examine the intestinal absorption of the enzyme in vitro by using human intestinal epithelial cells and to measure the fibrinolytic activity of the enzyme in the blood and the organs using animal model systems.

The novel fibrinolytic enzyme derived from a traditional Asian food is useful for thrombolytic therapy like other potent fibrinolytic enzymes, such as nattokinase and earthworm enzyme. It will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, because large quantities of enzyme can be conveniently and efficiently produced. In addition, this enzyme has a significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases.

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