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Purification and Characteristics of Fibrinolytic Enzyme from *Chongkukjang*

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Abstract

***Bacillus* sp. strain K-1, which produces a strong fibrinolytic enzyme, was isolated from *chongkukjang*, a traditional Korean fermented soybean paste. The fibrinolytic enzyme was purified from *chongkukjang* base by using ammonium sulfate fractionation and chromatographic techniques. Purified enzyme, CK K-1 was demonstrated to be homogeneous by SDS-PAGE and isoelectric focusing electrophoresis, and has molecular mass of a 12.4 kDa and a pI of 8.0. The optimal reaction pH value and temperature were 8.0 and 40°C, respectively. Phenyl-methyl-sulfonyl-fluoride (PMSF; serine protease inhibitor), ethylene-diamine-tetra-acetic acid (EDTA; metallo protease inhibitor), copper ion, ferric ion and lead ion inhibited the enzyme activity. These results indicated that the fibrinolytic enzyme is a metallo-serine protease and different from *nattokinase* and *chongkukjangkinase*.**

Key words: fibrinolytic enzyme, *chongkukjang*, soybean, chromatography, zymography

INTRODUCTION

Fibrin is the primary protein component of a blood clot, which is formed from fibrinogen by thrombin (1). When fibrin accumulates in the blood vessels as a result of an imbalance of fibrin clot formation and fibrinolysis, blood circulation is impaired and leads to myocardial infarction and other cardiovascular diseases. Typical thrombolytic agents used therapeutically include urokinase (2), streptokinase (3) and a tissue-type plasminogen activator (4). However, they have disadvantages such as high cost, systemic hemorrhage, short half-life, and intravenous administration except for urokinase.

Due to the limitation of present thrombolytic agents, a new method for treating thrombosis has been widely sought after. Food-grade microorganisms in fermented foods produce fibrinolytic enzymes, so these foods can be consumed daily as nutraceutical foods to prevent thrombosis and other related diseases.

Groundbreaking studies on fibrinolytic enzymes by Sumi et al. (5,6), Kim and Choi (7), and Kim et al. (8) have received much attention. These groups have purified and characterized proteolytic enzymes with strong fibrinolytic activity from traditional fermented foods, such as *natto* and *shiokara* (5,6), *doen-jang* (7), and *jeot-gal* (8). *Doen-jang* and *natto* are fermented soybean products popular in Korea and Japan, respectively, and have been consumed for several hundred years. *Jeot-gal* and *shiokara* are fermented fish products used as seasoning in Korea and Japan, respectively. 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 (9). Furthermore, a novel fibrinolytic metalloprotease from edible mushroom was also isolated and characterized (10).

However, in previous *chongkukjangkinase* studies, the fibrinolytic enzymes were purified from the supernatant of the microorganism culture broth but not *chongkukjang* itself, which is a traditional Korean fermented soybean paste.

In this paper, we first report the purification and partial characterization of the fibrinolytic enzyme from *chongkukjang* base fermented with *Bacillus* sp. strain K-1 isolated from traditional *chongkukjang*.

MATERIALS AND METHODS

Chongkukjang preparation

Chongkukjang was manufactured by using *Bacillus* sp. strain K-1, which produces a strong fibrinolytic enzyme, isolated from *chongkukjangs* collected from various regions in Korea. Species K-1 was selected based upon growth rates, intensity of ammonianic odor and amount of slime and identified as *Bacillus circulans* K-1 by

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using mini API. After soaking and cooking of selected whole soybeans, the cooked soybeans were cooled to 60°C and fermented by incubation at 50°C for 48 hr with 3% *Bacillus* sp. strain K-1. *Chongkukjang* base was dried by lyophilization and grounded to fine powder. The powdered *chongkukjang* was added to 3 volumes of hexane. The mixtures of *chongkukjang* and hexane were allowed to stand at 4°C for 1 day. Defatted *chongkukjang* was lyophilized and used for the following experiments.

Crude enzyme preparation

Defatted *chongkukjang* powder was suspended in 25 volumes of 20 mM Tris buffer (pH 8.0) at 4°C for 2 days and then filtered through cheese-cloth. After centrifugation (10,000 *g*, 20 min, 4°C), the supernatant was saturated to 70 % with ammonium sulfate and the precipitates were dialyzed against 20 mM Tris/HCl (pH 8.0) buffer.

Purification of fibrinolytic enzyme

The crude enzyme solution was applied to a DE52 column (Whatman, Maidstone, UK, 6×30 cm) which was preeqilibriated with 20 mM Tris buffer (pH 8.0) and eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer. The active fractions were pooled, dialyzed, and applied to Toyo-pearl butyl column (TosoHaas, Stuttgart, Germany, 4×16 cm) equilibriated with 20 mM Tris buffer (pH 8.0) containing 4 M ammonium sulfate and eluted with a linear gradient of 4 M to 0 M ammonium sulfate at flow rate of 1.0 mL/min. For further purification, the concentrated active fractions were applied to a Sephadex G-100 column (Whatman, Maidstone, UK, 2×90 cm) with 20 mM Tris buffer (pH 8.0) containing 0.1 M NaCl.

Protein determination

The protein concentration was estimated according to Bradford (11) with bovine serum albumin as the standard. During column chromatography, concentrations of protein in the fraction were monitored by measuring their absorbance at 280 nm.

Fibrinolytic assay

Fibrinolytic activity was determined by the fibrin tube method, a modification of the fibrin plate method of Astrup (12). The fibrinogen solution of 500 μL [0.6% (w/v) fibrinogen in sodium borate buffer (50 mM sodium borate:0.2 M boric acid=1:4), pH 7.8] was mixed with 25 μL of thrombin solution (200 NIH unit/mL) in a test tube. After the tubes were allowed to stand for 30 min at room temperature to form fibrin clots, 30 μL of sample solution was dropped into each tube and incubated at 37°C for 5 hr. An equal volume of plasmin solution (1 NIH unit/mL) was incubated in a test tube as a calibrator. The fibrinolytic activity was estimated by measuring the weight of the dissolved solution and extrapolated to plasmin activity.

SDS-PAGE and fibrin zymography

SDS-PAGE was performed on 12% gels following the method of Laemmli (13). Fibrin zymography assay was determined by the method of Kim and Choi (14). Briefly, resolving gel solution (12%) containing 0.12% (w/v) fibrinogen was prepared and then, quickly thrombin (1 unit/mL) solution and N,N,N′,N′-tetramethylethylenediamine were added to the gel solution to a final concentrations of 0.1 μunit/mL and 0.028% (v/v), respectively. After washing the gel with 50 mM Tris buffer (pH 7.4) containing 2.5% Triton X, the gel was incubated in 30 mM Tris buffer (pH 7.4) containing 200 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 at 37°C for 16 hr and stained with Coomassie blue R-250 solution. Broad pI calibration kits (protein pI 3.50~9.30) were used as markers in the pI value determinations.

Effect of pH on stability of enzyme

The optimal pH for the proteolytic activity was determined by varying the pH of the reaction mixture between 4 and 11. The enzyme (1 μg) was dissolved in ether citrate buffer (50 mM, pH 3~6), Tris-HCl buffer (50 mM, pH 7~9) or phosphate buffer (50 mM, pH 10~11), and incubated at 4°C for 48 hr. The enzyme activity was measured using the fibrin tube assay. The aximum activity was expressed as 100 %, and the others were as a percentage of the maximum activity.

Effect of temperature on stability of enzyme

The effect of temperature on fibrinolytic activity was studied at various temperatures at pH 8.0. The enzyme in 10 mM PBS at pH 8.0 was heated for 10 min at different temperatures from 30 to 80°C (30, 40, 50, 60, 70, 80°C). The enzyme activity was measured using the fibrin tube assay and the relative activities were calculated as a percentage of the maximum activity.

Effects of protease inhibitors and metal ions

Phenyl-methyl-sulfonyl-fluoride (PMSF, serine protease inhibitor) and ethylene-diamine-tetra-acetic acid (EDTA, metallo protease inhibitor) were used to characterize the fibrinolytic enzyme purified from *chongkukjang*. Each inhibitor was dissolved in deionized water and mixed with pure enzyme (1 mM in 10 mM PBS at pH 8) at 37°C for 10 min. Enzyme activity was measured by using the fibrin tube assay. The effects of various divalent ions, including Ca^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Ba^{+} and Na^{+}, K^{+}, on fibrinolytic activity were also investigated. Metal chlorides (Sigma, Missouri, USA) were dissolved in deion-

ized water and mixed with pure enzyme (in 10 mM PBS at pH 8) at 37°C for 10 min. The concentration of the reaction mixture was 5 mM, and the enzyme activity was determined using the fibrin tube 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.

RESULTS

The protein (CK K-1) with fibrinolytic activity from *chongkukjang* base was purified to electrophoretic homogeneity by the combination of various chromatographic steps: ammonium sulfate fractionation, DE-52 cellulose anion exchange chromatography and Toyo-pearl gel filtration. Specific activity and fold purification during purification are summarized in Table 1. About 22-fold purification for the fibrinolytic enzyme was obtained after Toyo-pearl chromatography, resulting in a considerable loss of total enzyme activity. After ammonium sulfate fractionation, 94% of the fibrinolytic enzyme was recovered.

There was much loss of the fibrinolytic enzyme after DE-52 cellulose anion exchange chromatography (Yield 13%) compared to the ammonium sulfate precipitation (Yield 94.3%). The final enzyme yield after Toyo-pearl filtration was 7.5%. The specific activity of purified fibrinolytic enzyme was 0.352 units/mg protein when determined with plasmin as the standard (Table 1).

The elution profile from the DE-52 cellulose column is shown in Fig. 1. Fibrinolytic enzymes were eluted when 0.63~0.66 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) was applied. These fractions were pooled and then applied onto the Toyo-pearl butyl column. Large quantities of protein without fibrinolytic activity were removed and one single peak with fibrinolytic activity was obtained (data not shown).

Employing the above procedures, it was possible to

Table 1. Purification table of the fibrinolytic enzyme from *chongkukjang* base

	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Yield (%)	Purification fold
Water extract	29156	477	0.016	100	1
Ammonium sulfate	9205	450	0.05	94.3	3.1
DE-52 cellulose	196	62	0.317	13	19.8
Toyo-pearl	102	35.9	0.352	7.5	22

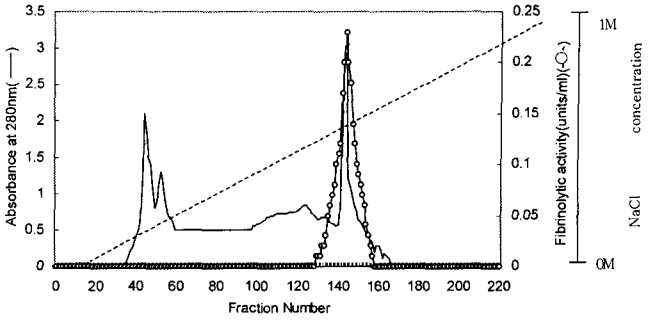

Fig. 1. DE52-cellulose ion exchange chromatography.

obtain pure fibrinolytic enzyme, CK K-1 that appeared homogeneous on fibrin zymography as shown in Fig. 2. Buffer extracts and 70% ammonium sulfate-saturated precipitates had shown the strongest fibrinolytic activity at 25.7 kDa on the fibrin zymogram with 12% SDS-PAGE (Fig. 2; Lane 1, 2). However, most of the 25.7 kDa enzyme was lost after DE-52 column chromatography and 12.4 kDa enzyme with potent fibrinolytic activity was obtained. The pH stability of the purified enzyme was examined in various buffers, ranging from pH 4.0 to 11.0. The relative activity of the enzyme was plotted against the pH range as shown in Fig. 3. The fibrinolytic activity of the CK K-1 was maximum between pH 7.0 and 11.0 at room temperature for 48 hr, indicating that it was alkaline enzyme. The enzyme remained active at extremely high pH values, and the relative activities at pH 11.0 were sustained to 85% of that of pH 8.0.

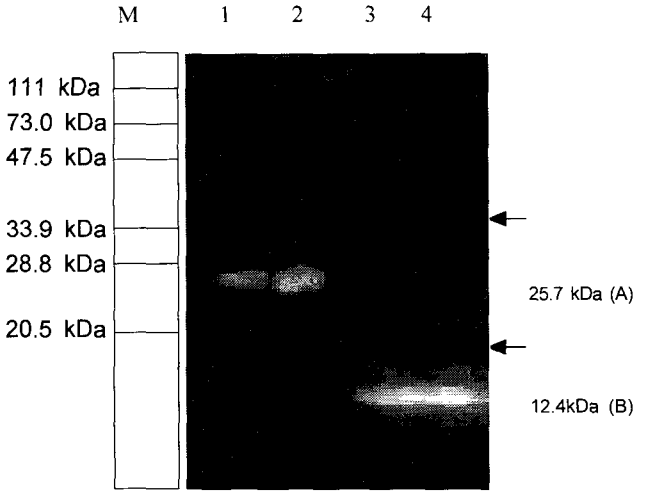

Fig. 2. Zymograph assay of the purified enzyme.
Molecular weight of the fibrinolytic enzyme was calculated by comparison with six molecular markers (M). Lane 1: water extract of *chongkukjang* base (400 μg), lane 2: ammonium sulfate precipitate (400 μg), lane 3: active fractions from DE52-cellulose column chromatography (200 μg), lane 4: active fractions from Toyo-pearl column chromatography (100 μg).

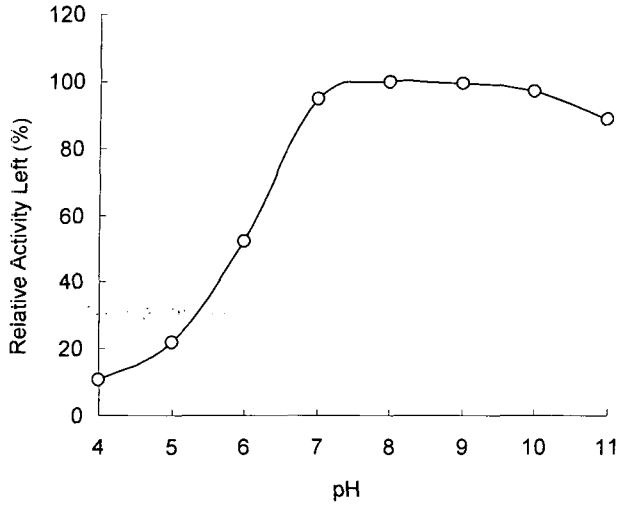

Fig. 3. Effects of pH on stability of CK K-1.
The effects of pH on the enzyme stability were measured over a range of pH 4.0 to 11.0 by using different buffer systems. After incubation of the enzyme over this pH range for 48 hr at 4°C, the residual enzyme activity was measured.

The thermal stability of the CK K-1 was examined after incubation at different temperatures for 10 min at pH 8.0. The remaining relative enzyme activity was plotted against the temperature range as shown in Fig. 4. The enzyme was stable up to 30°C and 40°C. The enzyme became denatured when the temperature increased above 40°C, and it was completely denatured at temperatures above 60°C. It was also observed that above 70% of the enzyme's activity remained after incubation at 40°C for 1 hr (data not shown).

The effects of protease inhibitors on fibrinolytic activity of the CK K-1 are summarized in Table 2. In general, there are four classes of proteases; namely, serine proteases, aspartic proteases, cysteine proteases, and metalloproteases. The CK K-1 was completely inhibited by PMSF, which is a well-known inhibitor of serine protease.

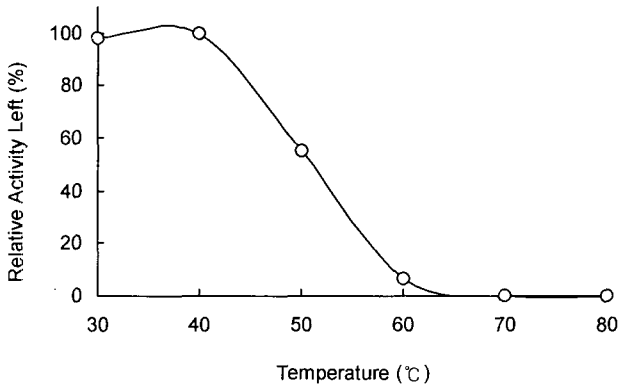

Fig. 4. Effects of temperature on stability of the purified enzyme. The residual enzyme activity was assayed after incubation at the indicated temperature for 10 min at pH 8.

Table 2. Effects of metal ions and inhibitors on the fibrinolytic activity (%) of the purified enzyme (after Toyo-pearl column step)

Metal ions (5 mM) /Inhibitors (1 mM)	Relative activity (%)
None	100
Na^+	100
K^+	100
Ca^{2+}	100
Co^{2+}	96
Mg^{2+}	89
Zn^{2+}	71
Ba^{2+}	57
Cu^{2+}	35
Fe^{2+}	32
Pb^{2+}	0
PMSF	0
Mercaptoethanol	44
SDS	50
EDTA	53

Furthermore, EDTA, SDS, and mercapto-ethanol also inhibited the fibrinolytic activity of CK K-1. EDTA forms strong complexes with divalent and higher oxidation state cations such as ferric ion, zinc ion, copper ion, and calcium ion. The effects of cations on enzyme activity are shown in Table 2. The enzyme activity was not sensitive to Ca^{2+}, Co^{2+}, Mg^{2+}, Na^+, and K^+, while significantly inhibited by Cu^{2+}, Fe^{2+}, and Ba^+. CK K-1 activity was completely destroyed by Pb^{2+}.

DISCUSSION

In recent years, fibrinolytic enzymes have been discovered in several traditional fermented foods, such as *natto*, *shiokara* and *chongkukjang*. *Chongkukjang* and *natto* are fermented soybean products, which are popular in Korea and Japan, respectively, and have been eaten for several hundred years. We isolated *Bacillus* sp. strain K-1 from traditional *chongkukjang* (15) and identified the strain based on Bergey's manual of systematic bacteriology (16).

We have detected a protein with a strong fibrinolytic activity from *chonkukjang* made by *Bacillus* sp. strain K-1, which was different from other fibrinolytic enzymes previously reported. For the isolation and purification of the enzyme with the greatest fibrinolytic activity, the bacteria was cultured in a broth. In this experiment, hexane-defatted *chongkukjang* base powder was subjected to the extraction of fibrinolytic enzyme with 20 mM Tris buffer (pH 8.0), which was useful for the isolation of the fibrinolytic enzyme.

We found that the molecular weight of a protein with fibrinolytic activity changed during the isolation process. It seems that a 25.7 kDa protein has fibrinolytic activity

in crude extract and ammonium sulfate precipitate, while a 12.4 kDa protein appeared to have activity in fractions from DE-52 and Toyo-pearl butyl column chromatography. These results might suggest that the fibrinolytic enzyme remains intact in *chonkukjang*, and its buffer extract or dialyzed ammonium sulfate precipitates is protected against other proteases by unknown mechanism. One possible explanation might be that the 25.7 kDa enzmyme was partially hydrolyzed to 12.4 kDa protein with its active site intact. The final molecular weight of the isolated CK K-1 from *chongkukjang* was calculated to be 12.4 kDa by both SDS-PAGE and fibrin autograph, which was smaller than that of *nattokinase* (27.7 kDa) (5), *chongkukjang* kinase (28.2 kDa) (17), *kimchi* (38.3 kDa) (18), *joet-gal* (41 kDa) (8) or *shiokara* enzyme (35 kDa) (6). These findings might indicate that the molecular size of fibrinolytic enzymes excreted by microorganisms varies depending upon the species used for fermentation.

The pH stability study showed that CK K-1 is very stable at alkaline pH. Therefore, it will be the best to deliver this enzyme in an alkaline 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.

On the basis of the temperature effect on the fibrinolytic activity of the enzyme, the cooking temperature of fermented soybean paste, *chongkukjang*, should be properly controlled. To use *chongkukjang* as a functional food in preventing cardiovascular diseases, a low cooking temperature is recommended, since the enzyme will be denatured at temperature above 60°C. Like Japanese *natto* (5), it is recommended that *chongkukjang* be eaten raw, without heat treatment.

The results of the inhibition test showed that PMSF, EDTA, SDS, or mercapto-ethanol inhibited the CK K-1. In addition, the enzyme activity was strongly inhibited by the presence of Pb^{2+}, Cu^{2+}, Fe^{2+} or Ba^{+}. PMSF is a well-known inhibitor of serine protease. In general, the cations perform a specific role in the modulation of enzyme activity, whereas anions play a general role in the modulation of the activity of enzymes (19). It was hypothesized that ferric ion, barium ion, copper ion and lead 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. These results indicate that the CK K-1 is a serine protease containing a metal binding site.

Sumi et al. (9) have reported that fibrinolytic activity, the amounts of tissue type plasminogen activator (t-PA) and fibrin degradation product in plasma increased approximately 2-fold after oral administration of *nattokinase*. A similar result was reported with administration of urokinase. Furthermore, in our previous study, increased fibrinolytic activity in plasma was detected in rats fed with *chongkukjang* base powder. The CK K-1 isolated from Korean traditional *chongkukjang* has also been recognized as being safe for human. In view of these reports, we are continuing to study this enzyme's properties in an attempt to develop the enzyme for use as a thrombolytic agent.

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