Purification and Characterization of the Fibrinolytic Enzyme Produced by *Bacillus subtilis* KCK-7 from Chungkookjang

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Abstract A fibrinolytic enzyme has been found in several bacteria isolated from fermented food. This study was carried out to investigate the purification and characteristics of the fibrinolytic enzyme produced by *Bacillus subtilis* KCK-7 originated from Chungkookjang. The fibrinolytic enzyme was purified to homogeneity from the culture supernatant using ammonium sulfate fractionation and chromatographies on DEAE-cellulose and on Sephadex G-100. The final specific activity of the purified enzyme increased 11.0-fold, and the protein amount in the purified enzyme was about 16% of that in the culture supernatant. The molecular weight of the purified enzyme was estimated to be about 45,000 by SDS-PAGE. The optimum pH and temperature for the enzyme activity were pH 7.0 and 60°C, respectively. The enzyme activity was relatively stable up to 60°C over the pH range of 7.0– 10.0. The fibrinolytic enzyme activity increased by Ca^{2+} and Cu^{2+}, whereas it was inhibited by Hg^{2+} and Ba^{2+}. In addition, it was severely inhibited by PMSF and DFT. It is suggested that the purified enzyme was a serine protease for the fibrinolysis. The purified enzyme could completely hydrolyze fibrin *in vitro* within 8 h. Hence, it is suggested that the purified enzyme can be put into practice as an effective thrombolytic agent.

Key words: Fibrinolytic enzyme, purification, *Bacillus subtilis*, Chungkookjang

Microorganisms generally produce diverse proteases which differ in their specificities, and numerous investigations have been performed to find production and industrial applications of microbial proteases and lipases [1, 9, 12, 16, 17]. For fibrinolysis, it has been reported that there are some proteases of microbial origin showing fibrinolytic specificity. Studies on the streptokinase from *Streptococcus haemolyticus* [22, 26] and the staphylokinase from *Staphylococcus aureus* [2, 20] have been extensively carried out.

Blood clots are formed by the conversion of fibrinogen into fibrin through the proteolytic action of thrombin (EC3.4.21.5) and the subsequent formation of insoluble fibrin clots. The fibrin clots are dissolved by the hydrolytic action of plasmin (EC3.4.21.7), which is activated from plasminogen by a tissue a plasminogen activator [23]. It is generally called fibrinolysis when fibrin is hydrolyzed. Hemostasis is an elaborate and complicated function, where the fibrin formation and fibrinolysis are well balanced in the biological system. However, when the fibrin hydrolysis is not complete due to a balance disorder, thrombosis, such as a myocardial infarction, can occur [30]. Intravenous administration of urokinase and streptokinase, which are capable of degrading fibrin, has been widely used for this thrombosis therapy. Unfortunately, these enzymes have a low specificity for fibrin and they are also expensive. The tissue plasminogen activator (tPA) has been developed for the treatment of thrombosis because of its high efficacy and stronger affinity for fibrin [27]. Also, the nattokinase, which is produced from *Bacillus* sp. NAT in the traditional Japanese fermented food of natto, has been known as a fibrinolytic protease. In fact, the oral administration of this nattokinase has been found to be effective in enhancing the fibrinolytic activity in plasma and the production of tPA [29]. An enzyme from *Aspergillus terricola*, terrilytin, was also proven *in vitro* to possess a potent fibrinolytic activity and therefore prevent the formation of fibrin in blood [8].

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In addition, similar fibrinolytic enzymes have been obtained from *Fusarium pallidoroseum* [4].

When this type of fibrinolytic enzyme is produced from food-grade microorganisms in food, it is likely to be quite effective in preventing thrombosis and other related diseases. In addition to natto in Japanese food, soybean paste and Chungkookjang have a long history of being taken as fermented soybean products in Korea. As a matter of fact, Chungkookjang has been eaten for several hundred years. For the last few decades, microorganisms demonstrating fibrinolytic activity have been isolated from fermented foods [7, 10, 29, 32]. Therefore, it is very likely that microorganisms exist that excrete fibrinolytic enzymes in Korean fermented foods. Recently, investigations have been conducted on the isolation and medium optimization of fibrinolytic bacteria from traditional Chungkookjang in order to develop a fibrinolytic enzyme for use as a thrombolytic agent [10, 19]. As a result, bacteria which were identified as *Bacillus subtilis* KCK-7 were isolated, producing fibrinolytic enzyme. In this study, the purification and characteristics of the fibrinolytic enzyme from *Bacillus subtilis* KCK-7 are reported.

MATERIALS AND METHODS

Microorganism and Cultivation

Bacillus subtilis KCK-7 showing fibrinolytic activity was isolated in the laboratory from different versions of traditional Chungkookjang that were purchased from local markets and households in Korea [10]. Bacteria were cultivated in 500-ml Erlenmeyer flasks containing 200 ml of culture broth, adjusted at pH 7.0 with 1 N of NaOH, for 48 h at 37°C on a rotary shaker at 150 rpm. The composition of culture broth consisted of 5% soluble starch, 0.5% cellobiose, 0.3% beef extract, 0.5% peptone, 0.02% Na_2HPO_4, and 2% raw soybean meal.

Enzyme Assay

Fibrinolytic enzyme activity was assayed by the modified method of Fayek and El-Sayed [5]. This enzyme reaction was carried out by adding 0.5 ml of enzyme solution into 3 ml of 0.6% fibrin solution in 0.1 M Mcllavain buffer (pH 7.0) at 50°C for 10 min. The reaction was stopped by adding 3 ml of 0.4 M trichloroacetic acid (TCA), and the solution was allowed to stand for 30 min. The resulting solution was then filtered through Whatman No. 2 filter paper. Successively, 5 ml of Na_2CO_3 solution and 1 ml of 1 N Folin reagent were added to 1 ml of filtrate. After the reaction mixture had been allowed to stand for 30 min at room temperature, the absorbance of the above solution was measured at 280 nm, which was then converted to an equivalent amount of tyrosine using a standard curve. One unit (U) of fibrinolytic activity was defined as the amount

of enzyme releasing 1 μmol of tyrosine equivalent per min.

Enzyme Purification

Bacterial cells were harvested from 500-ml culture broth by centrifugation of 8,000 ×g for 15 min. The remaining culture supernatant was collected as crude enzyme solution, and ammonium sulfate was added to lead an enzyme precipitation. The resultant precipitate of crude enzyme was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The crude enzyme solution was dialyzed against the same buffer for 12 h at 4°C, and then concentrated using polyethyleneglycol (PEG, M.W. 20,000) with a dialysis bag. The concentrated enzyme solution was applied onto DEAE-cellulose ion-exchange column (3 cm×25 cm), preequilibrated with the same buffer, and eluted with a linear 0- 0.5 M NaCl gradient at a flow rate of 1 ml/min at 4°C. Active fractions were pooled and concentrated by using PEG with a dialysis bag. For further purification, gel filtration with a Sephadex G-100 column (1.5 cm×60 cm) was successively performed in the same buffer at 4°C. The active fractions were collected and precipitated with acetone, followed by lyophilization. The concentration of protein was determined according to the Lowry method with bovine serum albumin as a standard protein [21].

Molecular Weight Determination

The molecular weight of the purified enzyme was estimated by SDS-PAGE assay. Phosphorylase (M.W. 97,000), BSA (M.W. 66,000), oval albumin (M.W. 45,000), carbonic anhydrase (M.W. 31,000), and trypsin inhibitor (21,000) were used as standard protein markers. SDS-PAGE was conducted according to the procedure of Laemmli [15] using a 12% polyacrylamide gel. Electrophoresis was performed at 30 mA constant current and the gel was stained with Coomassie brilliant blue R250.

Patterns for Fibrin Degradation

Reaction of the purified enzyme with 0.6% fibrin solution was carried out for 2, 4, 6, and 8 h, respectively. The reaction solutions were then filtered through Whatman No. 2 filter paper and the filtrate was analyzed by SDS-PAGE to identify the patterns of fibrin degradation.

Effect of pH and Temperature on the Enzyme Activity

The fibrinolytic activity of the enzyme was assayed at various temperatures of 30- 80°C at pH 7 in 0.1 M Mcllavain buffer. The enzyme activity was also determined at an optimum temperature over a pH range of 4- 10 in 0.1 M Mcllavain buffer, pH 4- 7; 50 mM Tris-HCl buffer, pH 8- 10.

Effect of pH and Temperature on the Enzyme Stability

The enzyme solution was allowed to stand for 60 min at a pH range of 4- 10 at 25°C and concurrently incubated
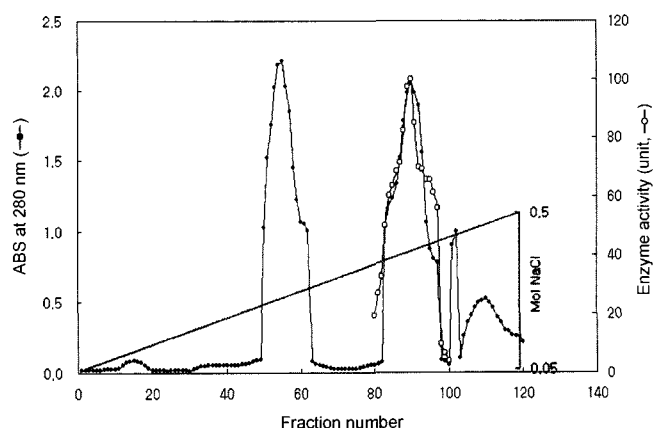
Fig. 1. Chromatography of fibrinolytic enzyme from *Bacillus subtilis* KCK-7 on DEAE-cellulose column.

The protein was applied to the column (3×25 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8). The protein was eluted with a 0-0.5 M NaCl linear gradient at the flow rate of 60 ml/h at 4°C. The fraction volume was 6.5 ml.

Fig. 2. Gel filtration chromatography of fibrinolytic enzyme from *Bacillus subtilis* KCK-7 on Sephadex G-100.

The protein was eluted through the column (1.5×60 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) at the flow rate of 0.7 ml/min at 4°C. The fraction volume was 6.5 ml.

for 60 min at a temperature range of 30–80°C in water bath at pH 7.0. An appropriate volume of the enzyme solution was taken at each pH level and temperature after the incubation, and the remaining fibrinolytic activity was assayed in 0.1 M of McIlavain buffer (pH 7.0) at 40°C.

Effect of Metal Ions and Inhibitors on the Enzyme Activity

The effects of metal ions were investigated using $NiCl_2$, $AlCl_3$, $CoCl_2$, $CaCl_2$, KCl, $FeCl_3$, $MgCl_2$, $CuSO_4$, and $HgSO_4$. The effects of protease inhibitors were also studied using EDTA, *p*-chloromercuribenzoate, iodoacetic acid, phenylmethyl sulfonylfluoride (PMSF) and diisopropylfluorophosphate (DFP). The purified enzyme was preincubated in the absence and the presence of bivalent cations such as Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, and Hg^{2+} and other inhibitors with a final concentration of 1 mM and 5 mM in 0.1 M McIlavain buffer (pH 7.0) for 60 min at 40°C. An appropriate volume of the incubated solution was taken and the fibrinolytic activity was measured at the optimum pH and temperature. The remaining activity was expressed as a percentage of the activity originally measured without any effectors.
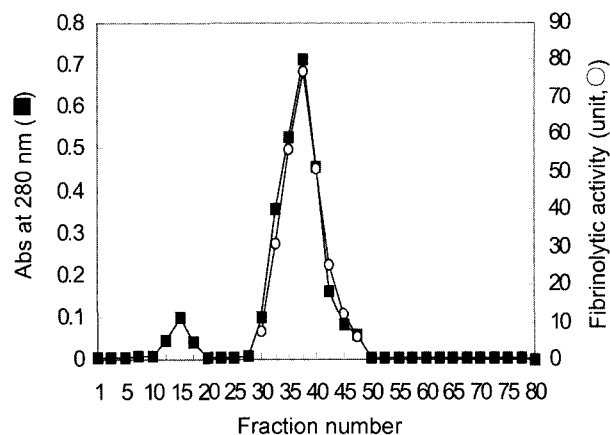
RESULTS AND DISCUSSION

Purification of Fibrinolytic Enzyme from *Bacillus subtilis* KCK-7

The fibrinolytic enzyme was purified from the culture supernatant of *Bacillus subtilis* KCK-7, cultivated for 48 h at 37°C. Figure 1 shows the elution patterns obtained from ion-exchange chromatography on DEAE-cellulose. As shown in Fig. 1, four protein peaks were obtained and they were eluted from the column with a linear gradient of NaCl. Most fibrinolytic activity was largely detected in the relatively large protein peak (fraction numbers 83– 100) that was eluted at 0.35 M of NaCl. The active fractions of protein peaks by ion-exchange chromatography were collected, and continuously applied onto the Sephadex G-100 gel column, which was equilibrated with 50 mM Tris-HCl buffer. Filtration profiles of the protein eluents through Sephadex G-100 column are shown in Fig. 2. One large protein peak with fibrinolytic activity could be separated in fractions 30 to 50. Results of these purification procedures are summarized in Table 1. The final specific activity of purified enzyme was 117.6 units/mg protein, which indicated that the enzyme was purified 11.4-fold with an approximately 20% yield.

Table 1. Summary of purification procedures for the fibrinolytic enzyme from *Bacillus subtilis* KCK-7.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture broth	8,800.8	852.0	10.33	100.0	1.0
$(NH_4)_2SO_4$	5,730.1	169.0	33.91	65.1	3.3
DEAE-Cellulose	2,209.8	25.3	87.34	25.1	8.5
Sephadex G-100	1,728.1	14.7	117.56	19.6	11.4

The fibrinolytic enzyme assay was carried out with 0.6% of fibrin in 0.1 M McIlavain buffer (pH 7.0) at 50°C for 10 min. The enzyme unit (U) was defined as the amount of enzyme producing 1 μmol of tyrosine per min.
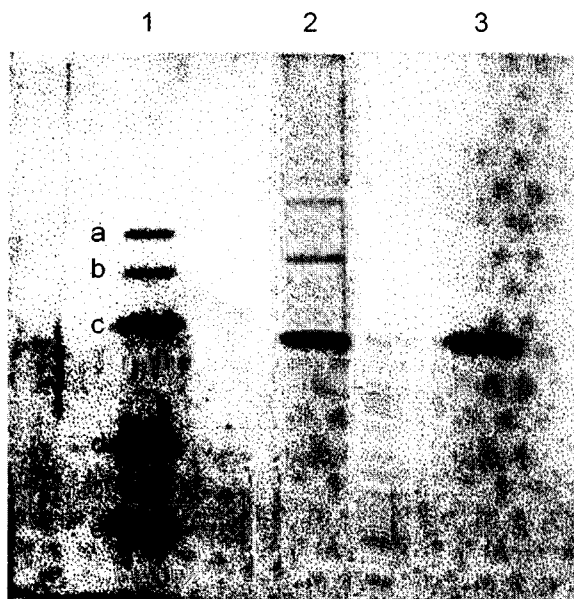
Fig. 3. SDS-PAGE of fibrinolytic enzyme from *Bacillus subtilis* KCK-7 isolated from Chungkookjang.

Lane 1: standard proteins; a:phosphorylase B (97,000), b:bovine serum albumin (66,000), c:oval albumin (45,000), d:carbonic anhydrase (31,000), e:trypsin inhibitor (21,000); Lane 2:after ion-exchange chromatography; Lane 3: after gel-filtration chromatography.

Fig. 4. The molecular weight determination of the fibrinolytic enzyme from *Bacillus subtilis* KCK-7 by SDS-PAGE.

a: Phosphorylase B (97,000); b: bovine serum albumin (66,000); c: oval albumin (45,000); d: carbonic anhydrase (31,000); e: trypsin inhibitor (21,000); s: sample.

The electrophoretic patterns of the protein eluents from the active protein peaks for each chromatography purification were investigated on the SDS-PAGE gel, as shown in Fig. 3. The active protein peaks from the ion-exchange column exhibited a few protein bands on the SDS-PAGE gel. However, the electrophoretic pattern of the active protein peak from the gel-filtration chromatography showed only one protein band, as shown in Fig. 3. This result indicated that the proteins of different molecular weights without any fibrinolytic activity in the active peaks of ion-exchange chromatography could be removed by gel filtration.

The molecular weight of the fibrinolytic enzyme finally purified by gel filtration was estimated to be approximately 45,000 by SDS-PAGE (Fig. 4). This value is higher than that of the enzyme from bacteria which were isolated from natto in Japan [7], but lower than that (M.W. 51,000) of the fibrinolytic enzyme from serpent [3]. However, it is in agreement with the molecular weight (M.W. 41,000) of fibrinolytic enzyme from *Bacillus* sp. that was isolated from fermented fish [13] and from Shrimp Jeot Gal [11], and that (M.W. 44,000) of fibrinolytic protease from *Bacillus* sp. isolated in soybean paste [18]. Noh *et al.* [25] isolated the different bacterial strains from Kimchi secreting extracellular fibrinolytic enzyme, and reported that *Micrococcus luteus* had fibrinolytic activity by enzymes of 45 kDa and 50 kDa M.W., but *Bacillus amyloliquefaciens* secreted only one protease of 29 kDa containing fibrinolytic activity.
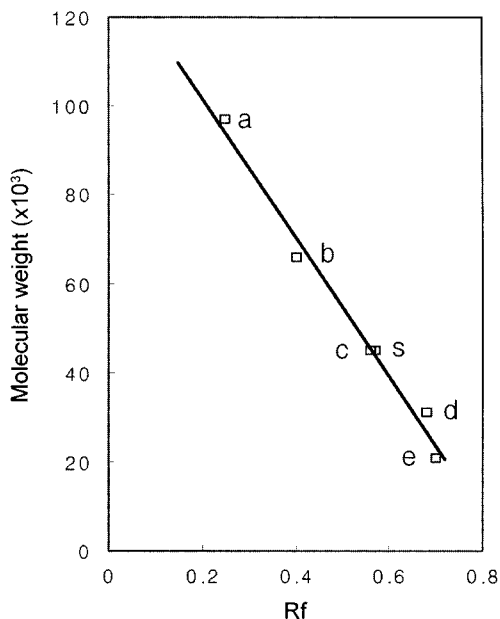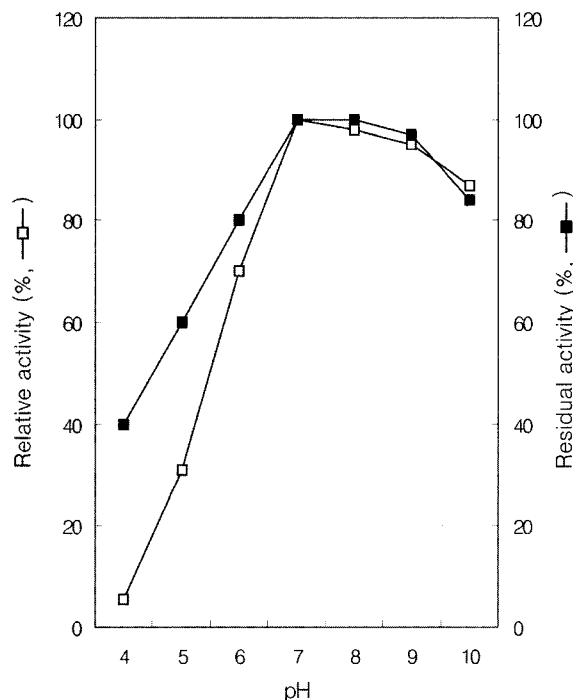

Fig. 5. Activity and stability of the fibrinolytic enzyme from *Bacillus subtilis* KCK-7 at various pHs.

The enzyme activity (□) was assayed in the pH range of 4- 10, respectively in 0.1 M McIlavain buffer for pH 4- 7 and in 50 mM Tris-HCl buffer for pH 8- 10 at 4°C. The enzyme stability (■) was measured by incubating it for 1 h at different pHs at 25°C. After incubation, the enzyme assay was performed in 0.1 M McIlavain buffer (pH 7.0) at 50°C.

Effect of pH and Temperature on the Activity and Stability of the Fibrinolytic Enzyme

The optimum pH for fibrinolytic activity was examined over a pH range from 4 to 10. Figure 5 shows that the enzyme was most active at pH 7.0. The enzyme activity was relatively higher in the alkaline region than in the acidic region. The pH stability of the enzyme was also investigated in a range of pH 4- 10 by measuring the residual enzyme activity. As shown in Fig. 5, the pH stability was also considerably higher in the neutral and alkaline regions (pH 7- 10) than in the acidic region (pH 4-6). Consequently, the fibrinolytic enzyme from *Bacillus subtilis* KCK-7 was considered to show the properties of neutral and alkaline protease. Kim *et al.* [14] reported that the stability of fibrinolytic enzyme from *Bacillus* sp. strain CK11-4 decreased largely above pH 11.0, although the enzyme was very stable in a range of pH 7- 10 at 30°C for 20 h.

The effect of temperature on the activity and stability of the fibrinolytic enzyme was also studied in a range of 30- 80°C at pH 7.0. Figure 6 shows that the optimum temperature for the fibrinolytic activity was approximately 60°C. This optimum temperature belongs to a range for other fibrinolytic enzymes such as 65°C as reported by Yoo *et al.* [31]. Kim *et al.* [14] suggested that the fibrinolytic enzyme from Chungkookjang, one of Korea's traditional fermented foods, is thermostable and exhibited a maximum activity at
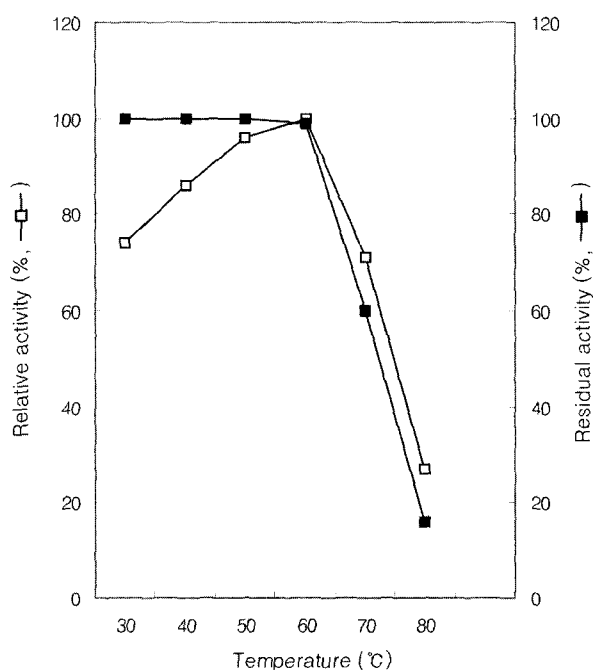
Table 2. Effect of metal ions on the activity of the fibrinolytic enzyme from *Bacillus subtilis* KCK-7.

Metal	Relative activity (%)	
	1 mM	5 mM
None	100	100
$NiCl_2 \cdot 6H_2O$	100.4	103.7
$AlCl_3 \cdot 6H_2O$	80.5	71.7
$CoCl_2 \cdot 6H_2O$	97.2	95.8
$CaCl_2$	105.7	115.5
KCl	97.3	94.6
$FeCl_3$	102.5	107.1
$MgCl_2$	98.4	96.1
$BaCl_2 \cdot 2H_2O$	80.1	69.2
$CuSO_4$	111.3	137.5
$HgSO_4$	69.7	58.9

The enzyme was preincubated with various metal ions in 0.1 M McIlavain buffer for 60 min at 30°C. After incubation, the mixture was subjected to the fibrinolytic enzyme assay. The results were expressed as percent (%) relative activity to that of none.

70°C. The residual activities of 55% and 14% were shown at 70°C and 80°C, respectively. The purified enzyme was stable up to 60°C, and its stability decreased at higher temperatures. Thermostable alkaline protease from *Bacillus licheniformis* was reported to be very stable up to 60°C, but about 70% of activity was lost at 90°C [6].

Effect of Metal Ions and Inhibitors on the Enzyme Activity

The effects of metal ions on fibrinolytic activity are presented in Table 2. Under the conditions applied, Ca^{2+} and Cu^{2+} at 5 mM increased the fibrinolytic activity by 15-40% when compared to the control in the absence of metal ion. Al^{3+} and Ba^{2+} showed a somewhat inhibitory effect on the enzyme activity, and Hg^{2+} particularly exhibited a

Fig. 6. Effect of temperature on the activity and stability of the fibrinolytic enzyme from *Bacillus subtilis* KCK-7.
The enzyme activity (□) was assayed at temperatures of 30- 80°C in 0.1 M McIlavain buffer. The enzyme stability (■) was measured after keeping it for 1 h at the different temperatures and pH 7, and the enzyme assay was performed in 0.1 M McIlavain buffer (pH 7.0) at 50°C.

Table 3. Effect of various inhibitors on the activity of the fibrinolytic enzyme from *Bacillus subtilis* KCK-7.

Inhibitors	Relative activity (%)	
	1 mM	5 mM
None	100	100
EDTA[a]	73.5	63.0
EGTA[a]	82.6	67.2
o-Phenanthroline	73.1	60.4
KCN	98.1	75.7
PMSF[a]	11.5	3.7
DFP[a]	10.9	4.3

The enzyme was preincubated with various inhibitors in 0.1 M McIlavain Buffer (pH 7.0) for 60 min at 30°C. After incubation, the mixture was subjected to the enzyme assay. The results were expressed as percent (%) relative activity to that of none.
[a]EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol tetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride; DFP, diisopropyl fluorophosphates.

marked inhibition. Ni^{2+}, Fe^{2+}, Mg^{2+}, and K^+ at 1 mM had no inhibitory effect on the enzyme activity. The effects of other protease inhibitors were also examined in 0.1 M McIlavain buffer at pH 7.0. As shown in Table 3, the enzyme was highly inactivated by protease inhibitors such as phenylmethyl sulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) at 1 mM, which were specific for the inhibition of serine proteases.

Thus, it is suggested that the fibrinolytic enzyme from *Bacillus subtilis* KCK-7 was a serine protease, essentially requiring a hydroxyl group for its catalytic activity. This was very similar to other reports for the fibrinolytic enzymes, like nattokinase [7], CK enzyme [14], and shiokara enzyme [24].

However, EDTA and o-phenanthronine showed a slightly inhibitory effect on the enzyme activity. Lee *et al.* [18] recently reported that the activity of fibrinolytic enzyme showed a severe inhibition effect of EDTA but the activity was restored to a large extent in the presence of cobalt ion. Kim *et al.* [13] also reported that fibrinolytic enzyme from *Bacillus* sp. KA38 originated from fermented fish was suggested to be a metalloenzyme. In addition, the other

Fig. 7. Degradation patterns of fibrin by the purified fibrinolytic enzyme from *Bacillus subtilis* KCK-7 isolated from Chungkookjang with time.

The enzyme was incubated with 0.6% fibrin solution in 0.1 M McIlavain buffer (pH 7.0) at 40°C for 8 h. The reaction mixture was taken out at every 2 h, and analyzed by SDS-PAGE. Lane 1: after 2 h reaction; lane 2: 4 h reaction; lane 3: after 6 h reaction; lane 4: after 8 h reaction.

fibrinolytic enzymes produced by *Fusarium pallidoroseum* [4], *Bacillus subtilis* [5], and *Pleurotus sajor-caju* [28] were also reported to be metalloproteases.

Degradation of Fibrin by the Purified Fibrinolytic Enzyme

Reaction of the purified enzyme with 0.6% fibrin solution was carried out for 2, 4, 6, and 8 h, respectively, at 40°C. After the filtration process of the reaction mixtures, the hydrolytic pattern of reaction products in the filtrate was analyzed on the SDS-PAGE gel with time. As shown in Fig. 7, *in vitro* degradation of fibrin appeared to be relatively slow in the initial phase of the reaction. A considerable amount of untreated fibrin was seen to remain even 4 h after reaction. Finally, the residual fibrin almost disappeared due to the hydrolysis at 8 h after the reaction. The hydrolysis of fibrin by the enzyme produced several low molecular weight polypeptides, which were initially detected in the positions lower than the untreated fibrin band on the gel and ultimately degraded into the smallest limit polypeptide to be accumulated with reaction time. *In vitro* fibrinolysis by the purified enzyme from *Bacillus subtilis* KCK-7 was completed stepwise with the fibrin during 8 h of reaction. It could then be concluded that the purified enzyme was very specific for the fibrin hydrolysis through this experiment. Hence, this study suggests that the purified enzyme can be practically applied as an effective thrombolytic agent.
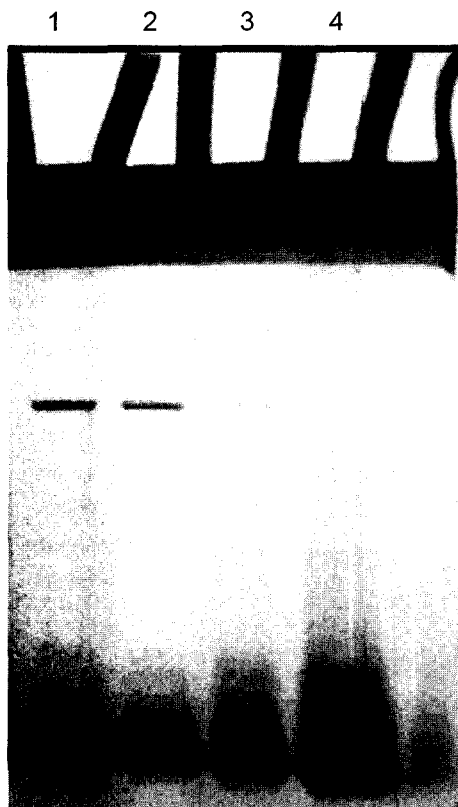
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