Purification and Characterization of a Fibrinolytic Enzyme from *Bacillus* sp. KDO-13 Isolated from Soybean Paste

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Abstract A microorganism producing fibrinolytic enzyme was isolated from Korean traditional soybean paste and identified as *Bacillus* sp. KDO-13. The fibrinolytic enzyme was purified to homogeneity by ammonium sulfate fractionation, ion-exchange chromatography on DEAE-celluose, and gel chromatography on Sephadex G-100 of the culture supernatant of *Bacillus* sp. KDO-13. The molecular weight of the purified enzyme was estimated to be 44,000 by SDS-PAGE. The optimum pH and temperature for the enzyme activity were pH 8.0 and 50°C, respectively. The enzyme activity was relatively stable at pH 7.0–9.0 and temperature below 50°C. The activity of the enzyme was inhibited by Al^{3+} and Hg^{2+}, but activated by Co^{2+} and Ni^{2+}. In addition, the enzyme activity was potently inhibited by EDTA and *o*-phenanthroline. The purified enzyme could completely hydrolyze a fibrin substrate within 6 h *in vitro*, and had a low K_m value for fibrin hydrolysis. It was concluded that the purified enzyme was a metalloprotease with relatively high specificity for fibrinolysis, and thus, could be applied as an effective thrombolytic agent.

Key words: Fibrinolytic enzyme, fibrin, *Bacillus* sp., soybean paste, metalloprotease

Blood clots are formed through the conversion of fibrinogen into fibrin by the proteolytic action of thrombin (EC3.4.21.5), and subsequently, insoluble fibrin clots are formed. The fibrin clots are dissolved by the hydrolytic action of plasmin (EC3.4.21.7), which is activated from plasminogen by a tissue plasminogen activator [19]. Generally, the hydrolysis of fibrin is called fibrinolysis. The fibrin clot formation and the fibrinolysis are well balanced in the biological system. However, when the fibrin is not hydrolyzed due to any disorder, thrombosis such as myocardial infarction can occur [26]. 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 are also expensive. The tissue plasminogen activator (tPA) has been developed for the treatment of thrombosis because of its good efficacy and stronger affinity for fibrin [22].

It has also been reported that there are some proteases of microbial origin showing fibrinolytic specificity. Among these fibrinolytic proteases, streptokinase from *Streptococcus haemolyticus* [17, 21] and staphylokinase from *Staphylococcus aureus* [1, 15] have been most extensively studied. The practical usage of these fibrinolytic proteases for thrombolytic therapy has been assessed through oral administration tests. The nattokinase, which is produced from *Bacillus* sp. NAT in the traditional Japanese fermented food of natto, has also 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 [24]. An enzyme from *Aspergillus terricola*, terrilytin, was also proved *in vitro* to possess a potent fibrinolytic activity and therefore prevent the formation of fibrin in blood [7]. In addition, similar fibrinolytic enzymes have been obtained from *Fusarium* sp.[4].

When this type of fibrinolytic enzyme is produced from food-grade microorganisms in food, it is likely to be

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quite effective in preventing thrombosis and other related diseases. Similarly to the natto in Japanese food, a traditional soybean paste has been used as a fermented soybean product in Korean food. In fact, this traditional soybean paste has been eaten for several hundred years. Since this traditional soybean paste is a fermented food, it has been well known to contain many microorganisms including bacteria, which produce various kinds of proteases with increased activity [11]. Therefore, it is very likely, as in the case of natto, that there exists a microorganism that excretes fibrinolytic enzyme in the Korean traditional soybean paste (Doenjang). Recently, an investigation was conducted on the isolation of fibrinolytic bacteria from traditional soybean paste in order to develop the fibrinolytic enzyme for use as a thrombolytic agent [13, 14]. As a result, a bacterium was isolated, producing fibrinolytic enzyme, which was identified as *Bacillus* sp. KDO-13. In this study, the purification and characteristics of the fibrinolytic enzyme from *Bacillus* sp. KDO-13 are reported.

MATERIALS AND METHODS

Microorganism and Cultivation

Bacillus sp. KDO-13, having a strong fibrinolytic activity, was isolated in the laboratory from traditional soybean pastes that were purchased from local markets and households in Korea [14]. The bacterium was cultivated in 500-ml Erlenmeyer flasks containing 200 ml of culture broth at pH 7.0, adjusted with 1 N of NaOH, and at 37°C for 48 h 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% $CaCl_2$, and 1% α-soybean powder.

Enzyme Assay

Fibrinolytic enzyme activity was determined according to the method of Fayek and El-Sayed [5] and Kim [10] with some modifications. This enzyme reaction was carried out by adding 0.5 ml of enzyme solution to 3 ml of 0.6% fibrin solution in 0.1 M McIlavain buffer (pH 7.0) at 40°C for 10 min. The reaction was stopped by the addition of 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 with Whatman filter paper No. 2. Subsequently, 1 ml of filtrate was mixed with 5 ml of Na_2CO_3 solution and 1 ml of 1 N Folin reagent, and the mixture was stood for 30 min in the room temperature. The absorbance of the above mixture was measured at 280 nm with a conventional spectrophotometer, which was equivalent to the amount of tyrosine converted based on 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 the 500-ml culture broth by centrifugation at 8,000 ×g for 15 min. The remaining culture supernatant was collected as a crude enzyme solution, and ammonium sulfate was added to precipitate the enzyme. The resulting 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 by using polyethyleneglycol (PEG, M.W. 20,000) with a dialysis bag. The concentrated enzyme solution was applied onto a DEAE-cellulose ion-exchange column (3 cm×25 cm) preequilibrated with the same buffer, and eluted with a NaCl gradient of 0–0.5 M with 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 the standard protein [16].

Molecular Weight Determination

The molecular weight of the enzyme was determined by SDS-PAGE with standard marker proteins such as phosphorylase (M.W. 97,000), bovine serum albumin (M.W. 66,000), oval albumin (M.W. 45,000), carbonic anhydrase (M.W. 31,000), and trypsin inhibitor (M.W. 21,000). SDS-PAGE was carried out according to the method of Laemmli [12] using 12% polyacrylamide gel and the gel was stained with Coomassie Brilliant Blue R 250.

Fibrin Hydrolysis Pattern

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 with Whatman filter paper No. 2 and the filtrate was analyzed by SDS-PAGE to identify the pattern of fibrin hydrolysis.

Effects of pH and Temperature on the Enzyme Activity

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

Effects of pH and Temperature on the Enzyme Stability

The enzyme solution was left at 25°C for 1 h at the pH range of 4–10, and concurrently incubated for 1 h at pH 7.0 and a temperature range of 30–80°C in a water bath. 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 Mcllavain buffer (pH 7.0) at 40°C.

Effects of Metal Ions and Inhibitors

The effects of metal ions were investigated by 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 by 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 Mcllavain 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 original activity measured without any effectors.

Measurement of Kinetic Constants

The kinetic constants, K_m and V_{max}, for the fibrinolysis by the purified enzyme were determined by Lineweaver-Burk plot with a substrate of fibrin at various concentrations of 2–10 mg/ml in 0.1 M of Mcllavain buffer (pH 7.0).

RESULTS AND DISCUSSION

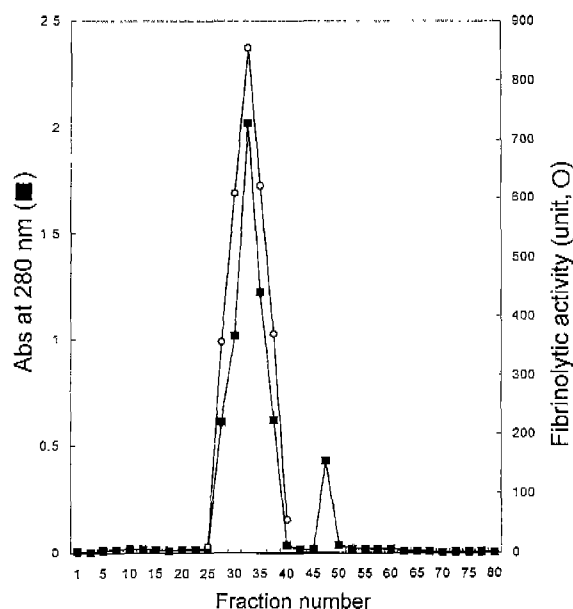### Purification of Fibrinolytic Enzyme from *Bacillus* sp. KDO-13

Bacillus sp. KDO-13 is an aerobic, Gram-positive, spore-forming, and catalase-producing bacterium. Optimum pH and temperature for growth were 7 and 37°C, respectively
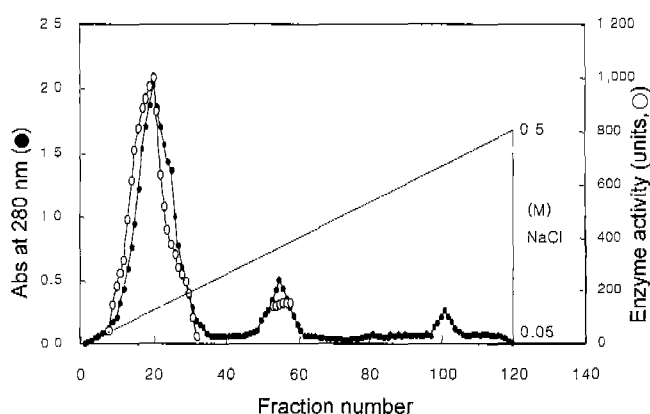
[14]. The fibrinolytic enzyme was purified from the culture supernatant of *Bacillus* sp. KDO-13, cultivated for 48 h at the optimum temperature. After the precipitation and dialysis of the enzyme protein, the chromatographies were performed. The fractionation of fibrinolytic enzyme by ion-exchange chromatography on DEAE-cellulose was performed first, as shown in Fig. 1. Two active protein peaks were eluted from the column with an increasing linear concentration of NaCl. Most of the fibrinolytic activity was mainly detected in the relatively large protein peak that was eluted first with 0.2 M of NaCl gradient, and the minor active protein peak was eluted with 0.3 M of NaCl gradient. Active fractions of both peaks from the ion chromatography were collected, and subsequently applied to the Sephadex G-100 gel column which was equilibrated with 50 mM of Tris-HCl buffer. The filtration profile of the protein eluents through Sephadex G-100 column are shown in Fig. 2. Only one large protein peak with fibrinolytic activity could be separated. These purification procedures described above are summarized in Table 1. After the final purification step, the purified enzyme had a specific activity of 931.3 units/mg protein, indicating that the enzyme was purified 9.1-fold with approximately 16% of its yield.

The electrophoretic patterns of the protein eluents from the active protein peaks for each chromatographic purification were investigated on the SDS-PAGE gel (Fig. 3). The active protein peaks from the ion-exchange column exhibited a few protein bands on the SDS-PAGE gel.

Fig. 1. Ion-exchange column chromatography of fibrinolytic enzyme from *Bacillus* sp. KDO-13 on DEAE-celluose.
The protein sample was applied to the column (3×25 cm) equilibrated with 50 mM of Tris-HCl buffer (pH 8.0). The protein was eluted with 0–0.5 M of the NaCl linear gradient at the flow rate of 1 ml/min at 4°C. The fraction volume was 6 ml.

Fig. 2. Gel filtration column chromatography of fibrinolytic enzyme from *Bacillus* sp. KDO-13 on Sephadex G-100.
The protein was eluted through the column (1.5×60 cm) equilibrated with 50 mM of Tris-HCl buffer (pH 8.0) at the flow rate of 0.75 ml/min at 4°C. The fraction volume was 4 ml.

Table 1. Purification steps for the fibrinolytic enzyme from *Bacillus* sp. KDO-13.
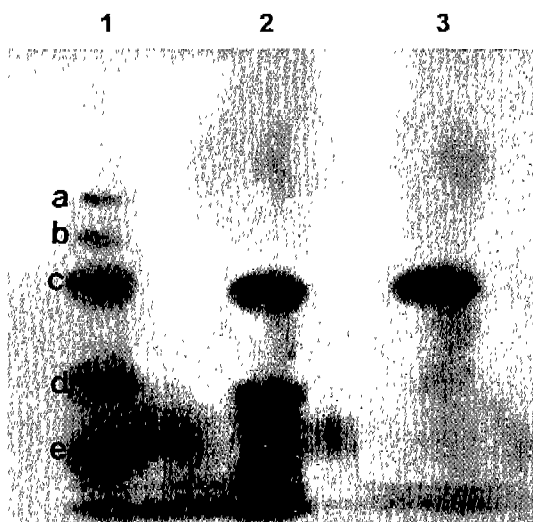
Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture broth	90,188	878.0	102.72	100.0	1.0
(NH$_4$)$_2$SO$_4$	54,744	178.0	307.55	60.7	3.0
DEAE-Cellulose	22,817	30.5	748.10	25.3	7.3
Sephadex G-100	14,249	15.3	931.31	15.8	9.1

The enzyme assay for the fibrinolysis was carried out with 0.6% of fibrin in 0.1 M Mcllavain buffer (pH 7.0) at 40°C for 10 min. The reaction was stopped by 0.4 M trichloroacetic acid and stood for 30 min. Then, it was filtered and mixed with 1 N folin reagent. The absorbance of the reaction mixture was measured at 280 nm. The enzyme unit (U) was defined as the amount of enzyme producing 1 μmol of tyrosine per min. The protein concentration was determined according to the Lowry method.

However, the electrophoretic pattern of the active protein peak from the gel filtration chromatography showed only one protein band (lane 3, Fig. 3). This result indicated that the proteins of different molecular weight did not contain fibrinolytic activity, and thus, the active peaks could be removed through the gel filtration procedure. In addition, it was assumed that there existed two types of active enzymes having a different charge but the same molecular weight, since two active enzyme peaks were separated from the ion-exchange chromatography and identified as one active enzyme band on the SDS-PAGE and gel filtration steps. Further investigation, such as the determination of pI, is in progress. For the following experiment in the present report, the active enzyme that was purified by gel filtration from the main large peak of the ion-exchange chromatography was employed.

Molecular Weight

The molecular weight of the fibrinolytic enzyme finally purified through gel filtration was estimated to be about

44,000 by SDS-PAGE, as shown in Fig. 3. This value is higher than that of the fibrinolytic enzyme which was isolated from natto in Japan [6], and it is also lower than that (M.W. 51,000) of serpent fibrinolytic enzyme [3]. However, it is in good agreement with the molecular weight (M.W. 41,000) of the fibrinolytic enzyme from a *Bacillus* sp. that was isolated from fermented fish [8] as well as that (M.W. 45,000) of alkaline protease from *B. thermoruber* [18].

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

The pH optimum for fibrinolytic activity was determined over the pH range of 4 to 10. As shown in Fig. 4, the enzyme had the optimum pH of 8.0, and was relatively higher in activity in the neutral and alkaline region than in
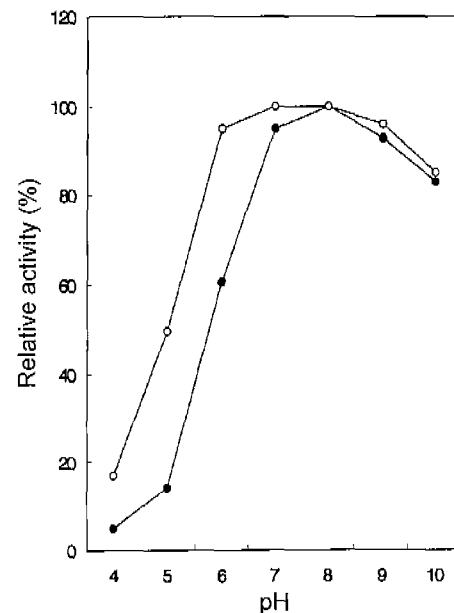

Fig. 3. SDS-PAGE of fibrinolytic enzyme from *Bacillus* sp. KDO-13.

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. Effect of pH on the activity and stability of the fibrinolytic enzyme from *Bacillus* sp. KDO-13.

The enzyme activity (●) was assayed in the pH range of 4–10, respectively in 0.1 M Mcllavain buffer for pH 4–7 and in 50 mM Tris-HCl buffer for pH 8–10 at 40°C. The enzyme stability (○) was measured by incubating it for 1 h at the various pH values and 25°C. After the incubation, the enzyme assay was performed in 0.1 M Mcllavain buffer (pH 7.0) at 40°C.
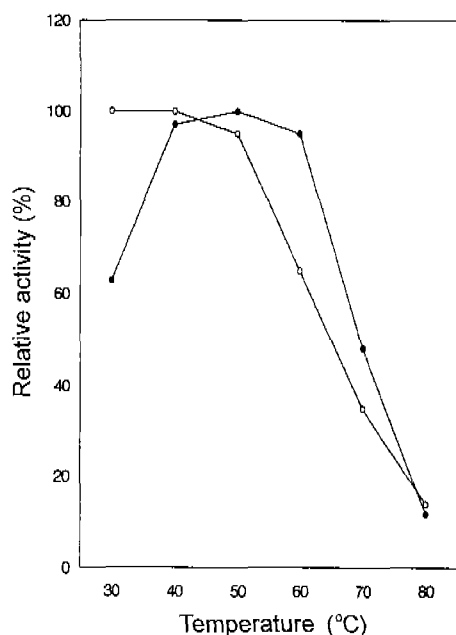
Fig. 5. Effect of temperature on the activity and stability of the fibrinolytic enzyme from *Bacillus* sp. KDO-13.

The enzyme activity (●) was assayed at various temperatures of 30–80°C and pH 7 in 0.1 M Mcllavain buffer. The enzyme stability (○) was measured by keeping it for 1 h at the various temperatures and pH 7, and the enzyme assay was performed in 0.1 M Mcllavain buffer (pH 7.0) at 40°C.

the acidic region. The pH stability of the enzyme was also investigated in the range of pH 4–10 by measuring the remaining enzyme activity. As shown in Fig. 4, the pH stability was also considerably higher in the neutral and alkaline region (pH 7–10) than in the acidic region (pH 4–6). Thus, the fibrinolytic enzyme from *Bacillus* sp. KDO-13 was shown to be a neutral and alkaline protease. According to the report made by Kim *et al.* [9], the stability of fibrinolytic enzyme from *Bacillus* sp. strain CK11-4 decreased considerably above pH 11.0, although the enzyme was very stable in the 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 examined in the range of 30–80°C at pH 7.0. As shown in Fig. 5, the optimum temperature for the enzyme activity was approximately 50°C. This optimum temperature was in agreement with a range for other fibrinolytic enzymes, such as 40°C, reported by Kim *et al.* [8] and 65°C by Yoo *et al.* [27]. It was reported that the fibrinolytic enzyme from Chungkookjang, one of the Korean traditional fermented foods, was thermostable and exhibited a maximum activity at 70°C [9]. However, the purified enzyme was stable only at 50°C and its stability sharply decreased at higher temperatures (Fig. 5). The residual activities of 65, 35, and 14% were shown at 60, 70, and 80°C, respectively. Thermal inactivation was observed more rapidly at pH 5.5 than at pH 7.0 (data not shown).

Table 2. Effect of metal ions on the activity of the fibrinolytic enzyme from *Bacillus* sp. KDO-13.

Metals	Relative activity (%)	
	1 mM	5 mM
None	100.0	100.0
$NiCl_2 \cdot 6H_2O$	109.0	121.0
$AlCl_3 \cdot 6H_2O$	81.4	69.1
$CoCl_2 \cdot 6H_2O$	113.0	141.0
$CaCl_2$	99.1	102.8
KCl	95.1	94.8
$FeCl_3$	87.8	84.7
$MgCl_2$	102.1	99.3
$BaCl_2 \cdot 2H_2O$	92.4	88.0
$CuSO_4$	106.4	120.1
$HgSO_4$	6.1	2.7

The enzyme was preincubated with various metal ions in 0.1 M of Mcllavain buffer for 60 min at 40°C. After incubation, the mixture was subjected to the fibrinolytic enzyme assay. The results were expressed as percent (%) relative activity to that of none.

Effects of Metal Ions and Protease Inhibitors on the Enzyme Activity

The effects of metal ions on the fibrinolytic activity are represented in Table 2. Under the conditions employed, Ni^{2+}, Cu^{2+}, and Co^{2+} at 5 mM increased the enzyme activity by 20–40% when compared to the control without metal ion. On the contrary, Al^{3+}, Ba^{2+}, and Fe^{3+} showed some inhibitory effect, while Hg^{2+} particularly exhibited a marked inhibition. However, Ca^{2+}, Mg^{2+}, and K^+ had no inhibitory effect on the enzyme activity. The effects of other protease inhibitors were also examined in 0.1 M Mcllavain

Table 3. Effect of various inhibitors on the activity of the fibrinolytic enzyme from *Bacillus* sp. KDO-13.

Inhibitors	Relative activity (%)	
	1 mM	5 mM
None	100.0	100.0
EDTA[a]	30.3	17.7
EGTA[a]	38.1	29.2
o-Phenanthroline	6.3	1.6
Oxalic acid	88.2	71.1
$KMnO_4$	84.0	76.3
SDS	98.4	43.5
L-Cysteine	97.1	86.8
KCN	97.6	71.4
PMSF[a]	98.1	86.9
DFP[a]	96.8	91.4

The enzyme was preincubated with various inhibitors in 0.1 M Mcllavain buffer (pH 7.0) for 60 min at 40°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, phenylmethylsulfonylfluoride; DFP, diisopropyl fluorophosphates.

Table 4. Effect of EDTA and CoCl₂ on the activity of the fibrinolytic enzyme from *Bacillus* sp. KDO-13.

CoCl₂ (mM)	EDTA (mM)	Relative activity (%)
0	0	100.0
0	1	30.3
0	3	21.4
0	5	17.7
1	0	103.7
1	1	57.8
1	3	46.7
1	5	30.4
5	0	141.4
5	1	89.5
5	3	67.1
5	5	40.8

The enzyme was preincubated with various concentrations of CoCl₂ and EDTA in 0.1 M McIlavain buffer (pH 7.0) for 60 min at 40°C. After incubation, the mixture was subjected to the enzyme assay. The results were expressed as percent (%) relative activity to that in the absence of CoCl₂ and EDTA.

buffer at pH 7.0. The results are shown in Table 3. The enzyme activity was slightly inhibited 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 like nattokinase, CK (fibrinolytic enzyme from *Bacillus* sp. CK11-4), and shiokara enzyme [6, 9, 20].

EDTA and *o*-phenanthroline showed a very potent inhibitory effect on the enzyme activity. In particular, the inhibitory effect of *o*-phenanthroline was shown to be the strongest among the inhibitors tested. In general, EDTA and *o*-phenanthroline has been known to remove the essential metal ion from the enzyme molecule to bring about the inactivation of the enzyme. The inhibition effect of EDTA was further investigated in the absence and the presence of cobalt ion with various concentrations (Table 4). The relative activity of the fibrinolytic enzyme greatly decreased with increasing EDTA concentrations upto 5 mM. The decreased enzyme activity due to EDTA was reversed in the presence of excess amount of cobalt ion. The degree of the activity recovery was increased by increasing the concentration level of cobalt ion. These results indicated that the enzyme activity was largely dependent on the combined effect of cobalt metal ion and the metal chelating agents such as EDTA and *o*-phenanthroline. Thus, it was strongly suggested that the fibrinolytic enzyme from *Bacillus* sp. KDO-13 was a metalloprotease essentially requiring metal ions for its catalytic activity. This feature was very similar to other reports for the fibrinolytic enzymes. Fayek and El-Sayed [5] reported that the activity of the fibrinolytic enzyme from *Bacillus subtilis* increased in the presence of copper ion. According to Kim *et al.* [8], fibrinolytic enzyme isolated from fermented fish was a metalloenzyme, because
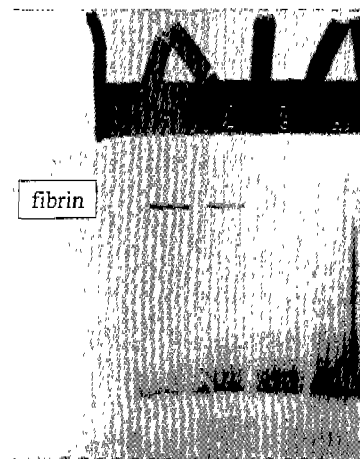

Fig. 6. Hydrolysis pattern of fibrin by the purified fibrinolytic enzyme from *Bacillus* sp. KDO-13 with time.

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

it was highly inhibited by EDTA and two metalloprotease inhibitors such as 2,2'-bipyridine and *o*-phenanthroline. In addition, the fibrinolytic enzymes from *Fusarium pallidoroseum* and *Pleurotus sajor-caju* were also reported to be metalloproteases [4, 23].

Hydrolysis 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. After filtration of the reaction mixtures, the hydrolytic pattern of reaction products in each of the filtrates was analyzed on the same SDS-PAGE gel to study the reaction progress (Fig. 6). *In vitro* degradation of fibrin appeared to be relatively slow in the initial phase of the reaction, and a considerable amount of untreated fibrin was seen even after a 4 h reaction. Finally, most of the fibrin disappeared due to the hydrolysis after 6 h of the reaction. The hydrolysis of fibrin by the enzyme produced several low molecular weight polypeptides, which were detected in the positions lower than the untreated fibrin band on the gel, and ultimately degraded into the smallest polypeptide, which was accumulated during the reaction. *In vitro* fibrinolysis by the purified enzyme from *Bacillus* sp. KDO-13 was stepwise completing with the fibrin within the 8-h reaction. It was concluded that the purified enzyme was very specific for the fibrin hydrolysis.

The fibrin-specificity of the purified enzyme was kinetically compared to other reported enzymes. In order to evaluate the kinetic constants for the fibrinolytic reaction by the purified enzyme, the initial velocities of the enzyme reactions were determined at various concentrations of the fibrin substrate. As shown in Fig. 7, the kinetic constants, K_m and V_{max}, for the fibrinolysis were determined using a
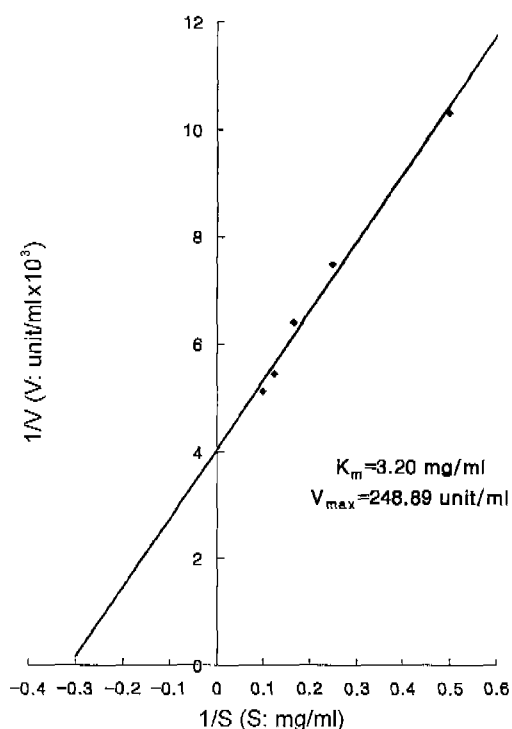
Fig. 7. Lineweaver-Burk plot for the hydrolysis of fibrin by the fibrinolytic enzyme of *Bacillus* sp. KDO-13.

The enzyme activity was assayed with various concentrations (2–10 mg/ml) of fibrin in 0.1 M McIlavain buffer (pH 7.0).

Lineweaver-Burk plot. The reaction properly followed the Michaelis-Menten kinetics. The apparent K_m and V_{max} for the fibrinolysis were calculated to be 3.2 mg/ml and 249 U/ml, respectively. The K_m value, which means an apparent binding affinity for a substrate, was either higher than that of the alkaline protease (1.3 mg/ml) reported by Bae and Park [2] or lower than that of the alkaline protease (8.2 mg/ml) reported by Takami *et al.* [25]. However, it was in good agreement with the K_m value for the fibrinolytic enzyme from *Bacillus subtilis* K-54 [27]. The present results showed that the purified enzyme showed a relatively high affinity for fibrin hydrolysis. Hence, it is suggested that the purified enzyme can be applied as an effective thrombolytic agent. The efficacy of the purified enzyme is under further investigation.

Acknowledgment

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