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Purification and Biochemical Characteristics of Fibrinolytic Enzyme from *Streptomyces corcohrussi* JK-20

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A fibrinolytic enzyme of *Streptomyces corcohrussi* from soil sediment was purified by chromatography using DEAE-Sephadex A-50 and Sephadex G-50. The analysis of SDS-polyacrylamide gel suggested that the purified enzyme is a homogeneous protein and the molecular mass is approximately 34 kDa. The purified enzyme showed activity of 0.8 U/ml in a plasminogen-rich fibrin plate, while its activity in a plasminogen-free fibrin plate was only 0.36 U/ml. These results suggested that the purified enzyme acts as a plasminogen activator. The fibrinolytic activity of the enzyme under the supplementation of protease inhibitors, ε-ACA, t-AMCHA and mercuric chloride in the enzyme reaction was less than 24%, indicating that it could be modulated by the plasmin and/or fibrinogen inhibitors involved in the fibrinogen-to-fibrin converting process. As time passed, Zn^{2+}, a heavy metal ion, inhibited the activity to 34.1%. The optimum temperature of the purified enzyme was approximately 50°C and over 92% of the enzyme activity was maintained between pH 5.0 and 8.0. Therefore, our results provide a potential fibrinolytic enzyme as a noble thrombolytic agent from *S. corcohrussi*.

Key words : Fibrinolytic enzyme, plasmin inhibitor, plasminogen activator, *Streptomyces corcohrussi*

Introduction

The typical fibrinolytic enzymes such as tissue plasminogen activator (*t*-PA) and urokinase (*u*-PA, EC 3.4.21.31) have been used as thrombolytic agents [3]. The fibrinolytic enzymes produced by the microbes including streptokinase [17] and staphylokinase [1] have been extensively investigated, however, they are not in a common use because of the side effects such as gastrointestinal bleeding, allergic reactions and resistance to repercussion [3,4,25]. The fibrinolytic enzymes from *Bacillus* spp. have also been interested in the thrombolytic agent because of a high efficiency in the fibrinolytic reactions such as plasmin activation. A variety of extracellular and intracellular proteases including Subtlisin Carsberg [22], nattokinase [19], amylosachariticus [26], *aprE* [20], Subtilin J [8] and BK II [9] have been produced by the *Bacillus* spp.

However, the enzymes such as tissue plasminogen activators, urokinase, streptokinase and staphylokinase were not widely used in the medical treatment for the blood-clotting disease because of the high cost and unstable enzymatic properties in the temperature [3,4]. The fibrinolytic enzymes from the *Streptomyces* genus including *S. megasporus* were previously reported [5,7], however, the effect of protease inhibitors on a fibrinolytic enzyme of the genus has not been reported. Thus, this study aimed to develop a fibrinolytic enzyme from *S. corcohrussi*, which can be modulated by the protease inhibitors for a therapeutic potential. In this paper, we report the purification of a fibrinolytic enzyme from *S. corcohrussi* and its biochemical characteristics including the effect of protease inhibitors on the fibrinolytic enzyme.

Materials and Methods

Isolation of a *Streptomyces* strain and culture condition

The soil sediments isolated in Busan, Korea was suspended in a 10% (w/v) sterile sodium chloride solution. To isolate *Streptomyces* spp. producing a fibrinolytic enzyme, 50 μl of the diluted sample was smeared on the plate containing an ISP medium (pH 7.5) supplemented with 1% glucose, 0.1% yeast extract, 0.1% polypeptone, 0.1% beef extract and

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1.5% agar. The colonies from the plate were aerobically cultured at 28°C for 4 days and the resulting culture was centrifuged for 8,000 rpm at 10 min. The harvested cells were analyzed for the morphological, physiological and biochemical characteristics including the production of a fibrinolytic enzyme [2].

Enzyme purification and SDS-polyacrylamide gel electrophoresis

The cultured cells were removed by centrifuging at 10,000 rpm for 15 min and ammonium sulfate was slowly added to the supernatant up to the 75% saturation. The precipitate was harvested by centrifuging at 10,000 rpm for 15 min and dialyzed with 5 l of 20 mM Tris-HCl (pH 7.5) five times. All purification steps were performed at 4°C and protein concentration was measured according to the method of Bradford [6]. The enzyme was purified by chromatography method using the DEAE-Sephadex A-50 and Sephadex G-50 gel filtration columns. In DEAE-Sephadex A-50 column, the enzyme was eluted by a linear gradient from 0.1 to 0.5 M sodium chloride and 70% (w/v) ammonium sulfate precipitation. In Sephadex G-50 column, the enzyme was purified by gel filtration using the Tris-HCl buffer as an elution buffer. The enzyme solution obtained in the chromatography method was then lyophilized. SDS-polyacrylamide gel electrophoresis (PAGE) of the lyophilized protein was performed according to the method of Laemmli [15]. The 10-15% gradient polyacrylamide and 4% stacking gels were used in performing SDS-PAGE.

Estimation of fibrinolytic activity

Fibrinolytic activity was determined by fibrin plate methods [2] using plasminogen-free and plasminogen-rich plates. The plasminogen - free fibrin plate was supplemented with fibrinogen solution [2.5 ml of 1.2% (w/v) human fibrinogen (Sigma, USA) in 0.1 M sodium phosphate buffer, pH 7.4], 10 U of thrombin solution (Sigma, USA) and 1% agarose. Fibrin plates were heated at 80°C for 30 min. A plasminogen-rich fibrin plate was supplemented with 1.5% fibrinogen, 5 U of plasminogen and 1% agarose.

A hole (5 mm in diameter) was punched for sample application on the fibrin plate and the sterilized paper disc was placed on the plate. To observe fibrinolytic activity, 100 μl of the purified protein solution was carefully dropped to the disc and incubated at 37°C for 18 hr. The activity of fibrinolytic enzyme was determined by measuring the halo zone on the fibrin plate and plotting to the standard curve.

Effect of the metal ions and protease inhibitors

The effect of metal ions including Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Hg^{2+} on the fibrinolytic activity was observed. The effect of the protease inhibitors ε-aminocaproic acid (ε-ACA), t-4-aminomethyl-cyclohexane carboxyl acid (t-AMCHA), mercuric chloride, tosyllysine chloromethylketone (TLCK), *p*-chloromercuribenzonic acid (PCMB), ρ-tosyl-L-arginine methylester hydrochloride (TAME) and diisopropyl fluorophosphate (DIFP) on the activity was also analyzed. Fibrinolytic activity of the enzyme was measured spectrophotometrically by supplementing with the chromogenic substrate in the enzyme reaction. The reaction mixture (1 ml) contained 20 μl of enzyme solution, 5×10^{-4} M chromogenic substrate and 0.1 M sodium phosphate buffer (pH 7.4). After incubation for 5 min at 37°C, the amount of liberated *p*-nitroaniline was determined from the spectrophotometric absorption at 405 nm. One unit of amidolytic activity was expressed as μmol of the substrate hydrolyzed per min per mg of the protein at 25°C.

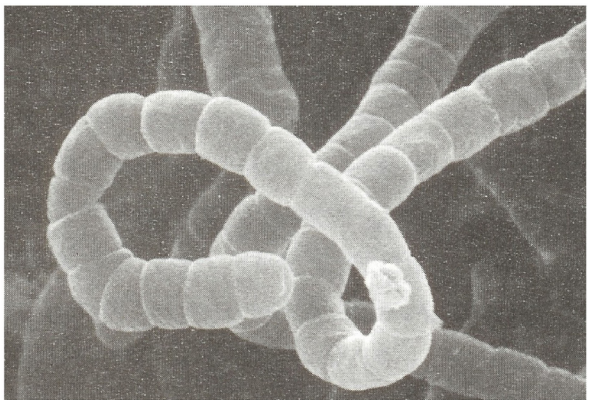
Effects of temperature and pH on the enzyme activity

The optimum temperature for the enzyme activity was determined by keeping the purified enzyme in 10 mM phosphate buffer (pH 7.4) for 30 min at various temperatures (20, 30, 40, 50, 60, 70 and 80°C). The optimal pH of the enzyme was determined between pH 3.0-11.0, using the following buffer systems: 0.05 M citrate buffer (pH 4.0), 0.05 M sodium phosphate (pH 5.0-7.0), 0.05 M Tris-HCl (pH 8.0-9.0) and 0.05 M glycine-NaOH (pH 10.0), respectively. Fibrin plates of different pH values were prepared using different buffers. All experiments were carried out at least three times.

Results

Isolation of microbial strains showing fibrinolytic activity

Twelve microbial strains showing fibrinolytic activity were isolated in the soil sediment. Out of the strains, an isolate was identified and its physiological characteristics were investigated. Scanning electron micrograph revealed that the isolated microbe possessed an aerial mycelium and cylindrical spores showing the smooth surface (Fig. 1). The biochemical and physiological characteristics were shown in



100 nm

Fig. 1. Scanning electron micrograph of fibrinolytic enzyme-generating *Streptomyces corcohrussi* JK-20.

Table 1. The strain was denoted as *Streptomyces corcohrussi* JK-20. A fibrinolytic enzyme was purified from the strain and the biochemical characteristics were also investigated.

Purification of a fibrinolytic enzyme and SDS-PAGE

The fibrinolytic enzyme was purified by the steps listed in Table 2. After the ammonium sulfate precipitation, ethanol precipitation, DEAE-Sephadex A-50 chromatography method and Sephadex G-50 gel filtration method were performed to purify the enzyme. As a result, the analysis of SDS-polyacrylamide gel showed only single polypeptide (Fig. 2). The apparent molecular mass of the purified fibrinolytic enzyme was estimated to be approximately 34 kDa. The specific activity of the enzyme was 809-folds higher than that of culture supernatant.

The proteolytic activity of the enzyme was observed by fibrin plate assay for 5 hr, respectively (Fig. 3). The fibrinolytic activity of purified enzyme in plasminogen-rich fibrin plate was more than 0.8 U/ml, however, the activity in plasminogen-free fibrin plate was less than 0.36 U/ml (Fig. 3C). These results indicate that the enzyme acts as a plasminogen activator.

Table 1. Physiological characteristics of the purified fibinolytic enzyme from *S. corcohrussi* JK-20

Physiological characteristics	Assimilation
Coagulation of milk	+
Peptonization of milk	−
Hydrolysis of starch	+
Melanoid pigment	−
Liquefaction of gelatin	+
Reduction of nitrate	−
Hydrolysis of starch	+
Assimiliation of	
D-Glucose	+
L-Arabinose	±
D-Xylose	+
D-Mannitol	±
D-Fructose	±
L-Rhamnose	+
Sucrose	±
Raffinose	+
D-Galactose	+
Cellobiose	+
Cellulose	−
Inulin	±
Melibiose	+
Optimum growth temperature	24°C
Optimum growth pH	6.0

+: assimilation, −: no assimilation

Effect of the protease inhibitors and the metal ions

Influence of protease inhibitors on the fibrinolytic activity was estimated (Table 3). Relative activity of the purified enzyme on the addition of ε-ACA, t-AMCHA, mercuric chloride and TLCK was about 17.9, 21.2, 23.6 and 31.4%, respectively. In addition, PCMB, TAME and DFP inhibited the activity to 45, 48 and 57%, respectively. These findings suggest that the fibrinolytic activity of the enzyme could be significantly reduced by the plasmin inhibitor such as ε-ACA and/or presence of the multiple binding sites on the fibrinogen molecule to convert fibrin.

In addition, the effect of metal ions on the fibrinolytic activity was observed (Table 3). The addition of Zn^{2+} to the reaction decreased the enzyme activity by 66%, whereas the addition of Co^{2+} to the reaction increased the activity by 31%.

Table 2. Purification processes of the fibrinolytic enzyme from *S. corcohrussi* JK-20

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Folds
Culture supernatant	22,163	35,375	1.6	100	1
Ethanol precipitation	756.2	25,648	34	73	21.3
DEAE-Sephadex A-50	8.6	4,723	549	13	343
Sephadex G-50	0.4	518	1,295	1.4	809

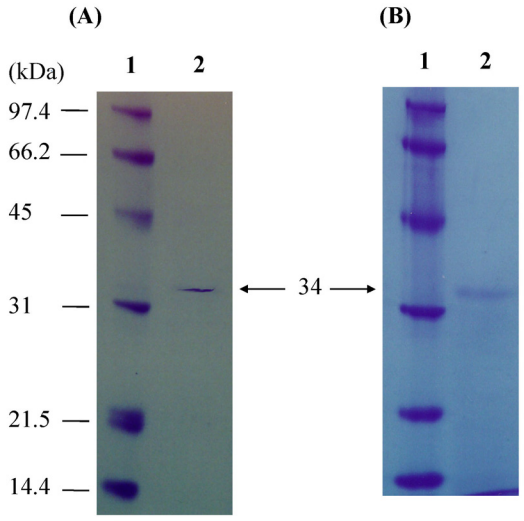


Fig. 2. Polyacrylamide gel electrophoresis of the fibrinolytic enzyme purified by DEAE-Sephadex A-50 chromatography and Sephadex G-50 gel filtration. A: enzyme from DEAE-Sephadex A-50 chromatography, B: enzyme from Sephadex G-50 gel filtration (lane 1, molecular mass markers; lane 2, fibrinolytic enzyme purified from *S. corcohrussi* JK-20). The enzyme showed only single polypeptide on SDS-PAGE gels, which was in the same position. The arrow indicates the fibrinolytic enzyme purified from the isolated *Streptomyces* strain.

Effects of temperature and pH on fibrinolytic activity

The estimation of fibrinolytic activity measured at temperature from 20 to 80°C showed that the activity was the highest at 50°C and inactivated by 40% at temperature over 70°C (Fig. 4A). It was also maintained over 74% for 30 min at temperature between 40 and 50°C, indicating that the enzyme was significantly stable at the temperature. The enzyme remained active at pH values ranging from 5.0 to 10.0 (Fig. 4B). Especially, the fibrinolytic activity of the enzyme was optimal between pH 6.0 and 8.0. However, it was significantly decreased at pH below 5.0. These results indicate that the enzyme purified from *S. corcohrussi* is active in a broad pH range.

Table 3. Effect of metal ions and protease inhibitors on the activity of the purified fibrinolytic enzyme

Metal ions or inhibitors	Relative activity (%)
None	100
ε-ACA	15±2.9
t-AMCHA	19±2.2
Mercuric chloride	21±2.6
TLCK	28±3.4
PCMB	51±3.9
TAME	48±4.2
DFP	40±3.3
EDTA	72±3.7
Ca^{2+}	110±4.2
Co^{2+}	136±4.1
Cu^{2+}	81±3.5
Mg^{2+}	92±3.8
Mn^{2+}	83±2.6
Zn^{2+}	32±2.1
Hg^{2+}	65±2.8

The concentration of protein inhibitors and metal ions added to the reaction mixture was 1 mM.
The results are expressed as the relative percentage (%) of the relative activity. All experiment was performed in triplicate.

Discussion

Many studies have reported the production of fibrinolytic enzymes [10-14,16,18,21,23,24,27] but the generation of fibrinolytic enzymes from the *Streptomyces* genus has not been

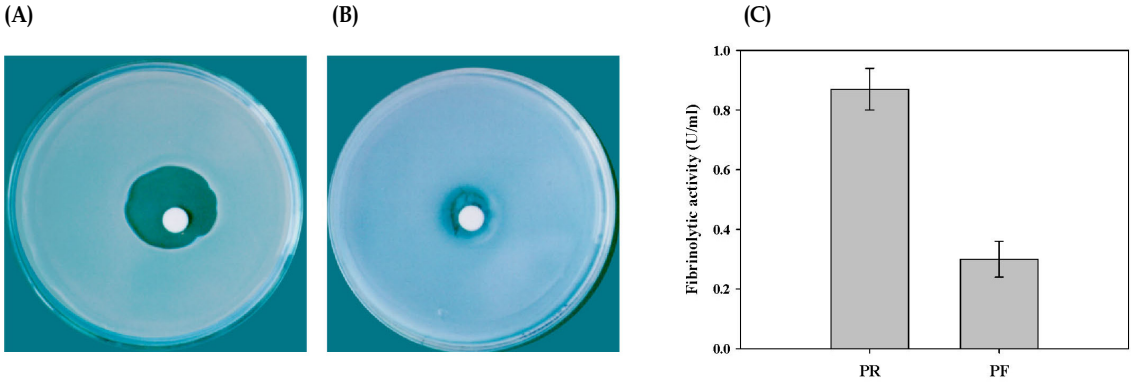


Fig. 3. Fibrinolytic activity in plasminogen-rich fibrin plate (A), plasminogen-free fibrin plate (B) and estimation of the fibrinolytic activity (C). The results in (C) represent the mean±SD from the duplicate of three independent experiments. (SD: standard deviation). PR, plasminogen-rich fibrin plate: PF, plasminogen-free fibrin plate.

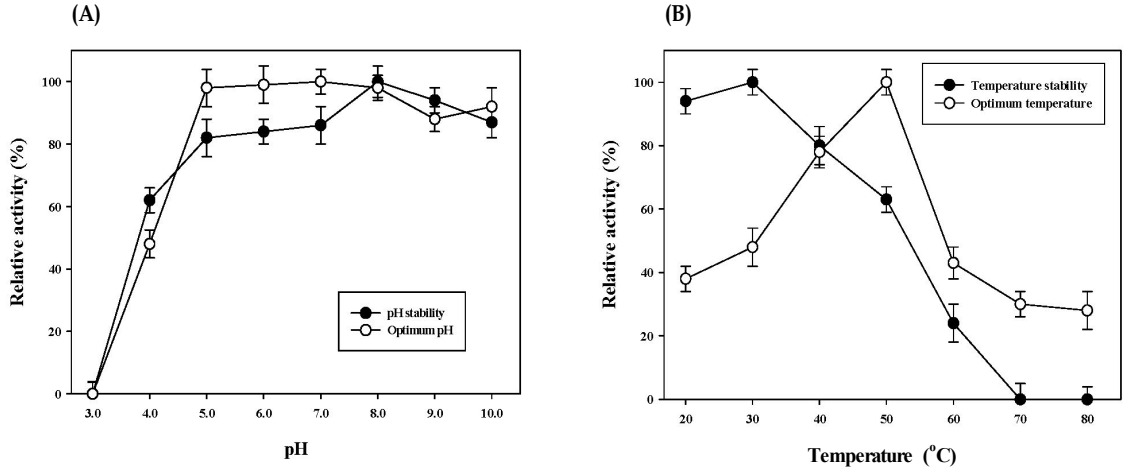


Fig. 4. Optimum temperature (A) and pH (B) of the fibrinolytic enzyme purified from *S. corchorussi* JK-20. The results represent the mean±SD from the duplicate of three independent experiments. (SD: standard deviation).

widely investigated [5,7]. For a therapeutic potential, we have newly identified *S. corcohrussi* capable of producing a fibrinolytic enzyme and investigated its biochemical characteristics.

Scanning electron micrograph of the isolate showed an aerial grey mycelium and the spore chain consisting of about 10-40 spores (Fig. 1). The optimum growth temperature and pH of *S. corcohrussi* JK-20 were 24°C and 6.0, respectively. The isolate hydrolyzed starch and coagulated milk (Table 1). An extracellular fibrinolytic enzyme of *S. corcohrussi* JK-20 was purified by the chromatography method using DEAE-Sephadex G-50 and Sephadex G-50 (Table 2). The apparent molecular weight of the enzyme obtained by SDS-PAGE was estimated to be about 34 kDa (Fig. 2). The fibrinolytic activity in plasminogen-rich and plasminogen-free fibrin plates was over 0.8 U/ml and below 0.36 U/ml, respectively (Fig. 3C). The result indicates that the fibrinolytic enzyme from the newly isolated *S. corcohrussi* JK-20 acts as a plasminogen activator.

In addition, effect of metal ions and protease inhibitors on the enzyme activity was estimated (Table 3). The addition of Zn^{2} and $Hg^{2+}$to the enzyme reaction decreased fibrinolytic activity by 34% and 32%, respectively. Notably, the addition of Co^{2+} augmented the activity by 32%. In a while, the addition of the protein inhibitors, t-AMCHA, mercuric chloride and TLCK to the enzyme reaction decreased the activity by 79, 76 and 69%, respectively. Especially, the addition of the plasmin inhibitor, ε-ACA in the reaction mixture inhibited the activity over 82%. These results indicate that the fibrinolytic process occurring by the enzyme could down-regulated by the plasmin inhibitor and/or presence of the multiple binding domains on the fibrinogen molecule to convert fibrin. The enzyme purified from *S. corcohrussi* showed high stability at 50°C and its optimal pH ranged in 5.0 and 8.0 (Fig. 4). The enzymatic features were distinguished from the known fibrinolytic enzyme from *S. megasporus* strain SD5 resistant to temperature ranging from 37 to 60°C and the range between pH 6.0 and 9.0 [7].

In conclusion, the fibrinolytic enzyme of *S. corcohrussi* JK-20 was isolated in the soil sediment and investigated for the biochemical characteristics. It acted as a plasminogen activator, which could degrade fibrin. Therefore, these results provide a fibrinolytic enzyme, as a noble thrombolytic agent from *S. corcohrussi* JK-20.

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초록 : *Streptomyces corcohrussi* JK-20 유래 혈전용해효소의 순수분리 및 이의 생화학적 특성 규명

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토양에서 생육하는 *Streptomyces corcohrussi*의 혈전용해효소가 DEAE-Sephadex A-50 그리고 Sephadex G-50 젤 여과를 이용한 크로마토그라피 방법에 의해 순수분리 되었다. SDS-PAGE 분석결과, 분리된 효소는 단일 단백질이고, 그 분자량은 약 34 kDa 이라는 것을 알 수 있었다. 순수분리된 효소의 혈전용해활성은 plasminogen-rich fibrin plate 에서 0.8 U/ml 이었으나, plasminogen-free fibrin plate 에서의 그 효소활성은 0.36 U/ml 이하이었다. 이러한 결과로, 순수분리된 효소가 plasminogen activator 로 작용한다는 것을 알 수 있었다. 단백질 저해제인 ε-ACA, t-AMCHA 와 mercuric chloride 의 존재시에 그 혈전용해활성은 24% 이하이었는데, 이러한 결과는 이들 plasmin 저해제 그리고(혹은) fibrinogen을 fibrin으로 전환시키는 과정과 관련된 fibrinogen 저해제에 의해 이 효소가 조절될 수 있음을 나타낼 수 있다. 한편으로, 중금속 이온인 Zn^{2+}은 그 활성을 58% 감소시켰다. 순수 분리된 효소의 최적 온도는 약 50°C 이었고, 그 효소활성의 92% 이상은 pH 5.0과 8.0 사이에서 유지되었다. 그러므로, 이러한 결과들은 하나의 강력한 혈전용해효소를 제공해서, *S. corcohrussi* 유래 새로운 혈전용해제의 개발에 기여하도록 한다.