Purification and Biochemical Characteristics of a 45 kDa Fibrinolytic Enzyme from a Halophile

Do Hyoung Kim, Jeong Uck Park[1,2], Min Jeong Seo[1], Min Jeong Kim[2], Hye Hyeon Lee[2], Yung Hyun Choi[3], Woo Hong Joo[4] and Yong Kee Jeong[1,2]*

Center for Food and Drug Analysis, Busan, Korea
[1]*Department of Medical Bioscience, Dong-A University, Busan, Korea*
[2]*Department of Biotechnology, Dong-A University, Busan, Korea*
[3]*Department of Biochemistry, College of Oriental Medicine, Dong-Eui University, Busan, Korea*
[4]*Department of Biology, Changwon National University, Changwon, Korea*

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A fibrinolytic enzyme producing *Bacillus* sp. J-19 was isolated from the popular Korean seasoning, pickled anchovy. The fibrinolytic enzyme was purified to homogeneity by chromatographic methods including ethanol precipitation and gel-filtration using Sephadex G-50. Compared to the crude enzyme extract, the specific activity of the enzyme increased 1021-fold with a recovery of 23%. The purified enzyme was estimated to be approximately 45 kDa by SDS-PAGE. Especially, the amidolytic activity in the presence of the synthetic substrate for serine protease (H-D-Ile-Pro-Arg-pNA, S-2288) represented approximately 17 U/mg. In addition, more than the 60% activity of the 45 kDa fibrinolytic activity was maintained in the presence of up to 30% (w/v) sodium chloride. These findings could provide a unique fibrinolytic enzyme, leading to a potential thrombolytic agent.

Key words : Amidolytic activity, *Bacillus* sp., fibrinolytic enzyme, halophile, serine protease

Introduction

Recently, cardiovascular diseases such as acute myocardial infarction have posed significant causes of death on the glove. The World Health Organization (WHO) reported that heart diseases have been involved in approximately 29% of the total mortality rate in the world [20]. The myocardial infarction and stroke causes the formation of a fibrin clot adhering to the unbroken wall of blood vessels, resulting in the cardiovascular diseases. The main protein component of the blood clot, fibrin is normally formed from fibrinogen by the action of thrombin (EC 3.4.21.5). The accumulation of fibrin in the blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular diseases [4,31]. Insoluble fibrin fiber is normally hydrolyzed by plasmin generated from plasminogen activator.

However, the fibrinolytic therapy may reflect the disadvantages by the intravenous administration of an exogenous plasminogen activator and expensiveness [7,19]. Those problems can be considerably overcome by food intake possessing the fibrinolytic enzymes [3,8,24,28] such as Japanese natto [27], Korean Chungkook-Jang soy sauce [14], edible honey mushroom [15], tofuyo [25], Chinese douchi and sufu [23,32] and fermented shrimp paste [33]. In this paper, a fibrinolytic enzyme from a halopile, *Bacillus* sp. J-19, which was screened from a Korean traditional seasoning, pickled anchovy, was purified and biochemically characterized for the development of a unique thrombolytic agent.

***Corresponding author**
Tel : +82-51-200-7557, Fax : +82-51-206-0848
E-mail : ykj9912@dau.ac.kr

Materials and Methods

Isolation of a bacterial strain and culture condition

The pickled anchovy obtained from a fish market in Korea was suspended in a 10% sterile sodium chloride solution at a dilution rate of 1:1. 100 μl of the diluted sample was spread on using a Luria-Bertani (LB) medium consisting of 1% bacto-tryptone (Sigma, USA), 2% sodium chloride and 0.5% bacto-yeast extract (Sigma, USA) and was aerobically cultured at 25°C overnight. The cell morphology was determined by a scanning electron microscopy and motility was assessed by direct microscopic observation during growth. The identification of the isolate was performed by using the API 20NE kit (Biomérieux, France).

Menaquinone extraction

The isolated cells from the pickled anchovy were aerobically cultured in the LB broth and harvested at 10,000 rpm for 10 min. The harvested cells were washed with a 50 mM potassium phosphate buffer. Quinones were extracted by adding 20 ml of chloroform and methanol at a ratio of 2:1 (v/v) and mixing for 10 min. The extracted quinone solution was filtered using a filter paper (110 mm in diameter, No. 2, Whatman, England) and concentrated using a vacuum rotary evaporator (APU-980, USA). 2 ml of hexane and distilled water was added to the concentrated extract and mixed well, leading to the quinone in a hexane layer. The quinone solution was concentrated by repeating these processes three times and subjected to quantitative thin layer chromatography (TLC, Merck Kiesel-gel 60 F254, 0.5 mm thickness) using petroleum benzene/diethylether (9:1, v/v) as a developing solution. The quinone band was detected using a spectrophotometric method at 270 nm and was assessed by HPLC. The length of a HPLC column and eluting solution was 4.6×250 mm and methanol-isopropyl ether (4:1, v/v), respectively. The flow rate was 0.8 ml/min at room temperature.

Crude enzyme preparation

The cultured cells were removed by centrifuging at 12,000 rpm for 15 min and ammonium sulfate was slowly added to the supernatant up to the 80% saturation. The mixture of supernatant and ammonium sulfate was allowed to stand at 4°C for 12 hr to harvest the extracellular proteins. The precipitate was subjected to centrifugation at 12,000 rpm for 15 min and dialyzed using 5 l of 20 mM Tris-HCl (pH 7.5) six times.

Enzyme purification and electrophoresis

All purification steps were performed at 4°C. Protein concentration was measured according to the method of Bradford [5]. The crude enzymes were saturated up to 75% by using ethanol and followed by centrifugation at 14,000 rpm for 30 min under 4°C. The precipitate was then dialyzed against 5 l of 20 mM Tris-HCl (pH 7.5) at 4°C for 12 hr three times. The enzyme solution was collected by the filtration through a Sephadex G-50 column using the Tris-HCl buffer, lyophilized and subjected to HPLC. SDS-PAGE was done according to the method of Laemmli [17] by using a 10-15% gradient polyacrylamide gel and 4% stacking gel at 4°C.

Biochemical analysis of the purified fibrinolytic enzyme

Fibrinolytic activity was determined by both plasminogen-free fibrin plate method and plasminogen-rich fibrin plate method [2]. Plasminogen - free fibrin plate was made up of the fibronogen solution [2.5 ml of 1.2% human fibrinogen (Sigma, USA) in 0.1 M sodium phosphate buffer, pH 7.4], 10 U of thrombin solution (Sigma, USA) and 1% agarose. Plasminogen-rich fibrin plate was made up 2 ml of 1.5% fibrinogen and 5 U of plasminogen. The sterilized paper disc (5 mm in diameter) was placed on the fibrin plate. To observe the fibrinolytic activity of the enzymes, 100 µl of the purified protein solution was carefully dropped to the disc and incubated at 37°C for 18 hr. The activity of a fibrinolytic enzyme was determined by measuring the dimension of the clear zone on the fibrin plate and plotting to the standard curve made by varying the quantity of plasmin.

Amidolytic activity was measured spectrophotometrically by using the chromogenic substrates. 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 *p*-nitroaniline that was liberated was determined from the spectrophotometric absorption at 405 nm. One unit of amidolytic activity was expressed as nM of substrate hydrolyzed per minute by the enzyme.

Effect of the protease inhibitors

The effect of the protease inhibitors were studied by using diisopropyl fluorophosphate (DFP), tosyllysine chloromethyl-ketone (TLCK), phenylmethan sulphonyl fluoride (PMSF), Mercuric chloride, ε-aminocaproic acid (ε-ACA), t-4-aminomethyl-cyclohexan carboxyl acid (t-AMCHA) and p-tosyl-L-arginine methylester hydrochloride (TAME). The concentration of all the protease inhibitors in the reaction was 10 mM.

Results

Isolation of *Bacillus* sp. J-19 from the pickled anchovy

Five microbial strains conveying the fibrinolytic activity were screened from the pickled anchovy. Among them, a strain possessing a substantial fibrinolytic activity and the protein degrading capability on the LB plate supplemented

with 1% skim milk was used for identification and physical characterization. This strain was a motile, spore-forming and aerobic rod. The scanning electron micrograph and biochemical assay using the API 20NE identification kit showed that the isolated strain belonged to the *Bacillus* genus (Fig. 1). The biochemical difference between the isolated microbe and reference strain was shown in Table 1. In addition, the presence of menaquinone-7 (MK-7), which has been known as Vitamin K2 occurring in Natto and metabolized by the microbe, was clearly observed on HPLC (Fig. 2). This finding indicates that this strain could conserve a feature of the *Bacillus* genus. Thus, the isolated microbe was denoted as *Bacillus* sp. J-19 and used for a unique fibrinolytic enzyme throughout this work.

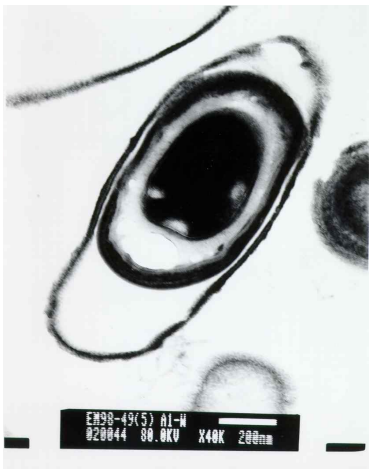

Fig. 1. SEM micrograph of *Bacillus* sp. J-19 isolated from the pickled anchovy.

Table 1. The biochemical characteristics of *Bacillus* sp. J-19 isolated from the pickled anchovy and *B. subtilis* ATCC 6633

Characteristics	*B. subtilis* ATCC 6633	*Bacillus* sp. J-19
Morphology	Rod	Rod
Spore-forming	+	+
Gram stain	+	+
Assimiliation of		
D-Glucose	+	+
D-Sucrose	+	+
D-Mannitol	+	−
L-Arabinose	+	−
D-Lactose	−	+
D-Sorbitol	+	−
Rhamnose	−	−
Citrate	+	−
Inositol	+	−
5-Ketogluconate	−	−

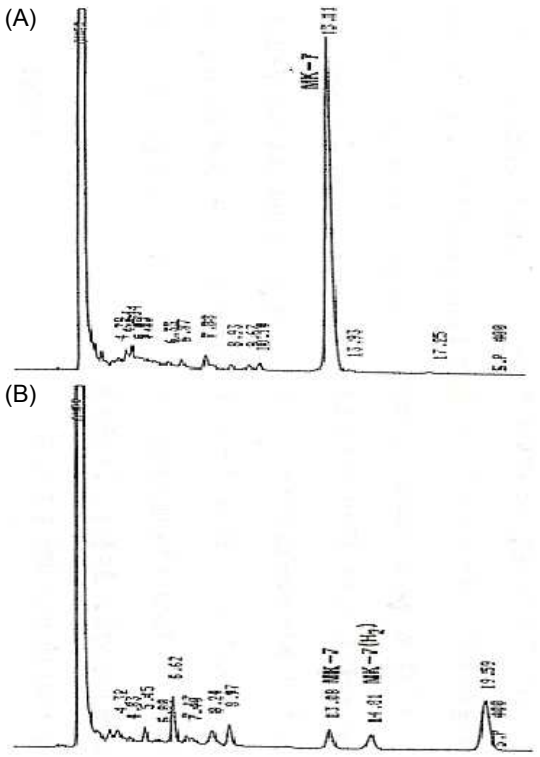

Fig. 2. A HPLC chromatogram of the menaquinone from the isolate, *Bacillus* sp. J-19 (A) and menaquinone standard (B).

Purification of a fibrinolytic enzyme and estimation of amidolytic activity

The fibrinolytic enzyme was purified to electrophoretic homogeneity by the steps listed in Table 2. After the ammonium sulfate precipitation, the subsequent ethanol precipitation and Sephadex G-50 gel filtration were used to purify the enzyme to homogeneity. A Sephadex G-50 column yielded only single polypeptide showing a high fibrinolytic activity on a SDS-PAGE gel as well as a native gel. The apparent molecular mass of the purified fibrinolytic enzyme was estimated to be approximately 45 kDa on the gels (Fig. 3), which was distinguished from that of other fibrinolytic enzymes reported [10,13,14,21,23,27,35]. The 45 kDa fibrinolytic enzyme formed a clear zone on the plasminogen-rich fibrin plate (Fig. 4). This result may reflect that it was able to degrade fibrin clots by forming plasmin from plasminogen (ie. plasminogen activator type). In addition, the amidolytic activities for the synthetic substrates, serine protease, plasmin, thrombin and urokinase were approximately 16.9, 32.2, 2.2 and 18.6 U/mg, respectively (Table 3).

Effect of the protease inhibitors

The influence of the protease inhibitors on the activity

Table 2. Purification steps of the fibrinolytic enzyme from *Bacillus* sp. J-19

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Culture supernatant	3,335	7,905	2.4	100	1
Ammonium sulfate precipitation	115.3	6,588	57.1	83.3	23.8
Ethanol precipitation	6.8	3,944	580	49.9	241.7
Sephadex G-50	0.7	1,824	2451.6	23	1021.2

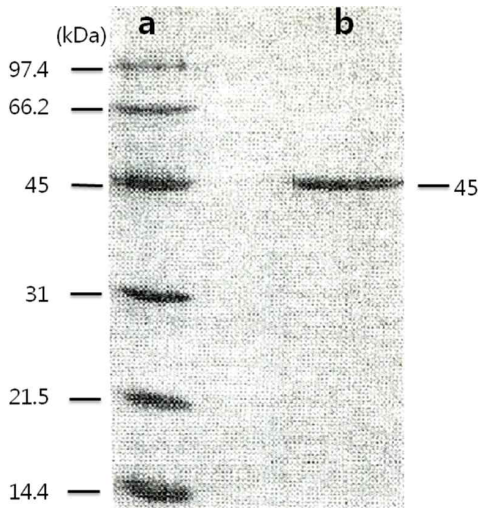

Fig. 3. Polyacrylamide gel electrophoresis of the fibrinolytic enzyme generated from *Bacillus* sp. J-19. Lane a, protein molecular mass markers; lane b, the fibrinolytic enzyme purified by the ethanol precipitation and Sephadex G-50 gel filtration. The enzyme showed only single polypeptide on the SDS-PAGE and native gels, which was in the same position. The arrow indicates the purified fibrinolytic enzyme from the *Bacillus* strain.

was observed, as shown in Table 4. The activity of the purified fibrinolytic enzyme was substantially decreased by the several inhibitors such as TLCK. In comparison with the enzyme supplementing with no inhibitor, the relative activity

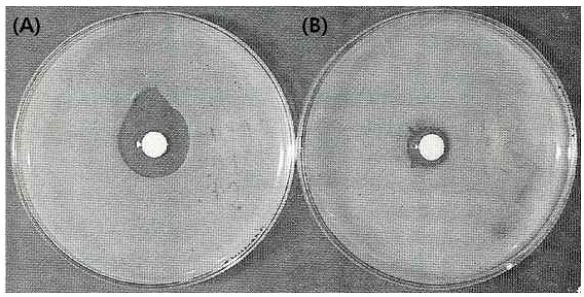

Fig. 4. Comparison of the fibrinolytic activity on the plasminogen-rich fibrin plate (A) and plasminogen-free fibrin plate (B).

of the enzyme by TLCK, t-AMCHA, ε-ACA and TAME were less than 17.1%, indicating that the fibrinolytic enzyme could conserve more than one binding domain for fibrinogen. However, that of the fibrinolytic enzyme by the other inhibitors including DFP, PCMB and mercuric chloride were more than 88%, representing that there may be no specific domain site for these inhibitors on fibrinogen.

Effect of sodium chloride on the fibrinolytic enzyme

To observe an effect of sodium chloride on the activity, the 45 kDa fibrinolytic enzyme was assessed at the various concentrations of sodium chloride. As shown in Table 5, its fibrinolytic activity at the 5, 10, 15, 20 and 25% concentration of sodium chloride showed 83, 81, 78, 75 and 73%, respectively,

Table 3. Amidolytic activity of the 45 kDa fibrinolytic enzyme, nattokinase, subtilisin BNP', subtilisin Carlsberg and CK for the synthetic substrates

Substrate	Enzyme activity[a]				
	45 kDa enzyme (nmol/min/mg protein)	Nattokinase (nmol/min/mg protein)	Subtilisin BNP, (μM/min/mg protein)	Subtilisin Carlsberg (μmol/min/mg protein)	CK (μmol/min/mg protein)
H-D-Val-Leu-Lys-pNA[b]	32.2 (100)	68.5 (100)	119.7 (100)	462.5 (100)	424.3 (100)
H-D-Ile-Pro-Arg-pNA[c]	16.9 (52.5)	ND	ND	ND	ND
H-D-Phe-Pip-Arg-pNA[d]	2.2 (6.8)	14.0 (20.4)	6.8 (5.7)	50.3 (10.9)	21.7 (5.1)
H-D-Val-Leu-Arg-pNA[e]	18.6 (57.8)	13.5 (19.7)	3.4 (2.6)	25.9 (5.6)	16.9 (4.0)

The values in parentheses are percentages calculated on the basis of enzyme activity to H-D-Val-Leu-Lys-pNA. Each value is the mean of three determinations. ND indicates "not determined".

[a]Enzyme activity was expressed as nmol or μmol of substrate hydrolyzed per minute by 1 mg of the protein.

[b]Synthetic substrate for plasmin. [c]Synthetic substrate for serine protease. [d]Synthetic substrate for thrombin.

[e]Synthetic substrate for kallikrein.

Table 4. Effect of the protease inhibitors on the activity of the 45 kDa fibrinolytic enzyme

Inhibitor	Relative activity (%)[a]
None	100
DFP	91±2
PCMB	93±2
Mercuric chloride	90±2
TLCK4	8±0.4
t-AMCHA	5±0.2
ε-ACA	15±2.1
TAME	2±0.2

[a]Protease activity relative to the 45 kDa fibrinolytic enzyme which was taken as 100%. The values shown are the averages of at least three independent experiments. The concentration of all the inhibitors was 10 mM.

Table 5. Effect of the sodium chloride concentration on the activity of the 45 kDa fibrinolytic enzyme

Sodium chloride concentration (%)	45 kDa fibrinolytic activity (%)[a]
0	100
5	81±3.3
10	80±2.6
15	78±2.9
20	75±3.1
25	73±2.9
30	63±2.8

[a]Fibrinolytic activity relative to the 45 kDa fibrinolytic enzyme which was taken as 100%. The values shown are the averages of at least three independent experiments.

in comparison with the activity of the enzyme without supplementing with sodium chloride. In addition, the activity of the 45 kDa enzyme at a 30% concentration of the salt still remained more than 60%. This finding reflects that the purified enzyme from *Bacillus* sp. J-19 could maintain its activity at a high concentration of salt and be a halophile.

Discussion

The typhical 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]. However, they are of human origin and cost-effective. To overcome these problems, the fibrinolytic enzymes produced by microbes including streptokinase produced by *Streptococcus haemolyticus* [18] and staphylokinase produced by *Staphylococcus aureus* [1] have been extensively investigated, however, they are not in common use because of side effects such as gastrointestinal bleeding, allergic reactions and resistance to repercussion [3,4,29]. The fibrinolytic enzymes from *Bacillus* sp. have been interested in the thrombolytic agent in that it showed high efficiency in the fibrinolytic reactions such as plasmin activation. A variety of extracellular and intracellular proteases including nattokinase [9,21], amylosachariticus [30,35], subtilin J [11] and subtilisin E [10,22] were produced by the *Bacillus* sp.

In this report, we purified and biochemically characterized a unique fibrinolytic enzyme from *Bacillus* sp. J-19 in the pickled anchovy, which has been a traditional seasoning in Korea. An extracellular fibrinolytic enzyme produced by *Bacillus* sp. J-19, which was able to survive in the presence of up to 30% sodium chloride (Table 5) was purified to electrophoresis homogeneity such as Sephadex G-50 gel filteration. The apparent molecular mass in size was estimated to be about 45 kDa by SDS-PAGE (Fig. 3). This finding was differentiated from other fibrinolytic enzymes from that of subtilisin KA38 (41 kDa) [16], subtilisin E (55.8 kDa) [34], subtilisin NAT (27.7 kDa) [9], subtilisin CK (28.2 kDa) [14], subtilisin DFE (28.0 kDa) [23], subtilisin IMR-NK1 (31.5 kDa) [6], subtilisin KK (38 kDa) [26] and Bacillokinase (31 kDa) [12].

As shown in Table 3, the amidolytic activity of the purified fibrinolytic enzyme on several chromogenic substrates was compared with those of Nattokinase [8], Subtilisin BNP′ [26], Subtilisin Carlsberg [26] and CK [14], respectively. The purified fibrinolytic enzyme from the halophile exhibited a significant specificity to the synthetic substrates for plasmin (H-D-Val-Leu-Lys-pNA, S-2251), thrombin (H-D-Phe-Pip-Arg-pNA, S-2238) and kallikrein (H-D-Val-Leu-Arg-pNA, S-2266). Of note, the amidolytic activity of the fibrinolytic enzyme to the synthetic substrate for serine protease (H-D-Ile-Pro-Arg-pNA, S-2288) was 17 U/mg. In addition, the influence of the protease inhibitors on the activity was estimated (Table 4). The fibrinolytic activity from the halophile was decreased more than 81% under the presence of the 10 mM TLCK, t-AMCHA, ε-ACA and TAME, respectively. These findings demonstrate that the enzyme may conserve more than one binding domain for fibrinogen on the fibrinolytic enzyme. However, other inhibitors including DFP, PCMB and mercuric chloride did not have a significant effect on the protease activity. In addition, the 45 kDa fibrinolytic enzyme was distinguished from other known enzymes in that it activity was maintained in the presence of 30% (w/v) sodium chloride (Table 5).

In conclusion, the 45 kDa fibrinolytic enzyme from *Baclus* sp. J-19 isolated from the pickled anchovy was purified and assessed for its biochemical characteristics. Of note, it was distinguished from other known fibrinolytic enzymes with regard to the molecular mass in size and a tolerance to the high concentration of salt. The purified fibrinolytic enzyme was a plasminogen activator type degrading fibrin clots by plasmin activated from plasminogen (Fig. 4). Therefore, these findings could provide the unique fibrinolytic enzyme from the pickled anchovy and contribute to the development of a potent thrombolytic agent.

Acknowledgement

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초록 : 호염성균 유래 45 kDa 혈전용해효소의 순수분리와 생화학적 특성

김도형 · 박정욱[1,2] · 서민정[1] · 김민정[2] · 이혜현[2] · 최영현[3] · 주우홍[4] · 정영기[1,2]*
(식품의약품안정청, [1]동아대학교 의약생명공학과, [2]동아대학교 생물공학과, [3]동의대학교 한의과대학 생화학 교실, [4]창원대학교 생물학과)

혈전용해효소를 생산하는 *Bacillus* sp. J-19가 보편적인 한국의 조미제인 멸치젓갈에서 분리되었다. 그 혈전용해효소는 에탄올 침전, Sephadex G-50을 이용한 젤 여과법 등을 포함하는 일련의 크로마토그래피 방법으로 순수분리 되었다. 조효소 추출액과 비교해서, 그 효소의 비활성은 1021배 증가하였고, 수율은 23%이었다. 순수분리한 효소의 분자량은 SDS-PAGE 상 약 45 kDa이었다. 특히, 합성기질인 serine protease (H-D-Ile-Pro-Arg-pNA, S-2288)에 대한 아미드활성은 약 17 U/mg이었다. 또한, 그 45 kDa 혈전용해효소의 60% 이상의 활성이 30% (w/v) sodium chloride 의 존재 하에서도 유지되었다. 이러한 발견들은 특이한 혈전용해효소를 제공해서, 실용적인 혈전용해제 개발을 유도할 수 있다.