Journal of Life Science 2007 Vol. 17. No. 5. 606~612 ©JLS / ISSN 1225-9918

Biochemical Characterization of a Protease with Fibrinolytic Activity from Maggots of *Protaetia brevitarsis*

Jeong Hyun Chang, Ji Young Jo, Yeong Jin Kim[1], Sun Yi Lee[1], Hyo Jin Cho[1], Sun Nyoung You[1], Kwang Youn Kim[1], Byoung Keun Park[2], Soon Cheol Ahn[1] and Heun Young Kwon*

Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan 609-757, Korea
[1]*Department of Microbiology and Immunology, Pusan National University School of Medicine, Busan 602-739, Korea*
[2]*Jangsaeng Doraji Co., Ltd., 1218-9 Jungja-ri, Geumgok-myeon, Jinju-City, Gyeongnam, 660-833, KOREA*

Received March 28, 2007 / Accepted April 30, 2007

Fibrin clots remained in blood vessels can be one of the serious factor caused cardiovascular disease, such as ischemia, infarction and necrosis The development of an antithrombotic and thrombolysis solvent is necessary to prevent and treat these diseases. In this study, the fibirinolytic protease was prepared from the maggots of *Protaetia brevitarsis* using ammonium sulfate fractionation and desalting column. The optimum pH and temperature for the enzyme activity were pH 9.0 and 50℃, respectively. The enzyme activity was relatively stable at pH 7.0-9.0 and temperature below 60℃. The activity of the enzyme was strongly inhibited by phenylmethanesulfonyl fluoride. And the activity of the enzyme was inhibited by Ca^{2+} and Zn^{2+}, but it was not by Mg^{2+} and Fe^{2+} ions. In these experimental results, we have speculated that the enzyme derived from maggots of *Protaetia brevitarsis* is a serine protease with a strong fibrinolytic activity.

Key words – Fibrinolytic enzyme, protease, maggots of *Protaetia brevitarsis*, phenylmethanesulfonyl fluoride

Introduction

Blood clots are formed through the conversion of fibrinogen into fibrin by the proteolytic action of thrombin. Thus, fibrin is a major structural component of blood clots. The normal vascular endothelium maintains blood fluidity by inhibiting blood coagulation and platelet aggregation and promoting fibrinolysis [9]. The fibrinolytic system, which dissolves fibrin formed from fibrinogen by thrombin and which also maintains blood flow at vascular injury sites, is an important component of the normal hemostatic response. The major protease of the fibrinolytic enzymes is plasmin. The fibrin clots are dissolved by the hydrolytic action of plasmin, which is activated from plasminogen by a tissue plasminogen activator [10]. Accordingly, fibrinolytic enzymes have therapeutic potential for the treatment of thrombosis in man. Lately, there has been great interest in the search for new thrombolytic agents from various origins with particular reference to sources.

*Corresponding author
Tel : +82-51-510-0561, Fax : +82-51-510-0569
E-mail : hykwon@cup.ac.kr

The typical fibrinolytic enzymes used therapeutically include urokinase [20], streptokinase and a tissue-type plasminogen activator (tPA) [13]. They are plasmin activators that convert plasminogen to plasmin which degrades fibrin. These enzymes have been used for the past 30 years and reported as useful therapeutics for cardiovascular diseases related to thrombus[18]. Except streptokinase, these enzymes are of human origin and generally safe. Many side effects such as systemic hemorrhage occur because these enzymes have been no affinity to thrombi [4]. However these enzymes and are very expensive [11] and their half lives are very short [17].

Recently researchers have attempted to develop a better fibrinolytic enzymes from natural sources including snake venoms [5] and insects [6]. Sumi *et al.* isolated *Bacillus natto* from a tranditional soybean fermented food in Japan, and purified a fibrinolytic enzyme nattokinase from *B. natto* [11]. And they reported that nattokinase increased the fibrinolytic activity in plasma and the production of tPA [16]. This identification of nattokinase has triggered a strong interest in other Asian traditional medicine. Thus, In this study, we characterized a protease with fibrinolytic activity from the *Protaetia brevitarsis* extracts.

Materials and Methods

Materials

The maggots of *P.brevitarsis* were purchased at local market in Yeong-Do, Busan. Thrombin, sheep fibrinogen, PMSF (phenylmethyl sulfonylfluoride), EDTA (ethylenedamine tetraacetate), DDT (dichloro-diphenyl-trichloroethane), EGTA (ethyleneglycol tetraacetate), and SDS (sodium dodecyl sulfate) were products of Sigma (St. Louis, USA). Most of the other reagents and chemicals were commercial sources and were of the best grade available.

Fractionation of a fibrinolytic protease

All of the steps were carried out at 4℃, unless stated otherwise. One hundred gram of the maggot was homogenized in 40 ml of distilled water (D.W), and extracted at 4℃ for 24 hr. For removalof any insoluble materials, the homogenate was centrifuged at 13,000 x g for 5 min (4℃). The supernant was collected, filtered using a filter paper, and one more filtered with a membrane filter of 0.45 μm (Sartorius, Goettingen, Germany). The crude proteinswere then salted out with ammonium sulfate. The precipitate was suspended in 1ml of a 1/15 M phosphate buffer (pH 7.4) and filtered using a desalting column. And it was used for enzyme reaction.

Preparation of fibrin plate

The fibrinolytic activity of enzyme from the maggots of *P. brevitarsis* was determined by the fibrin plate method of Astrup and Mulertz with slight modification [1]. The fibrin plate, 3 mm thick fibrin gel and 9 cm in diameter, was prepared with 10 ml of 0.6% fibrinogen (Sigma, Louis, MO, USA) in a fibrin plate buffer (0.15 M NaCl and 50 mM potassium phosphate buffer [pH 7.0]). The fibrinogen solution was filtered through a filter paper (0.05 mm, Wattman, Maidstone, England). Next, 10 ml of the mixture was distributed into each sterile petri-dish (100 mm, SPL Life Sciences, Gyeonggi-do, Korea), and 0.1 ml of thrombin (10 NIH units/ml) solution (Sigma) was slowly added and solidified for 1 h at room temperature [15]. The crude enzyme solution of maggots of *P. brevitarsis*was spotted directly onto the fibrin plate, and then incubated at 37°C for 18 hr. The clear zone formed by the crude enzyme was expressed as a fibrinolytic activity.

Preparation of skim milk agar plate

The proteolytic plate was prepared using Luria-Bertain (LB)agar plate containing 0.5% skim milk (w/v). A hole (2 mm in diameter) was punched for sample application on the proteolytic plate. To measure the protease activity, the supernatant (50 ul) of lysate was spotted on the skim milk plate and then incubation at 37°C for 24 hr. The clear zone was measured and expressed as its protease activity.

Protease enzyme activity : casein hydrolysis

The casein method was applied to assay caseinolytic activity as follows [3] : 250 μl of 0.6% hamster casein as a substrate was mixed in 100 μl of the 1/15 M sodium phosphate buffer (pH 7.0) and 20 μl of the crude enzyme solution, and stored for 30 min at 37°C. The reaction was stopped by using 250 μl of TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid) for 10 min at room temperature. The reactants were centrifuged at 15,000 x g for 20 min. The mixture of 0.1 ml supernatant with 1 ml of 0.55 M Na_2CO_3 and 0.1 ml of Folin-Ciocalteu was storedat room temperature for 10 min. The mixture was measured at 660 nm using a spectrophotometer (Hitachi High-Technologies co. Japan).

Effect of pH and temperature on the enzyme activity

Effects of pH and temperature on the proteolytic, caseinolytic and fibrinolytic activities of the enzyme were evaluated. The optimal pH for the proteolytic activityof the enzyme was determined with varying the pH of the reaction mixture between 3 to 11. *P. brevitarsis* fibrolytic enzyme was dissolved in either 0.5 M citrate buffer (pH 3-5), Tris-HCl buffer 0.1M (pH 5-9), 0.1 M carbonic acid buffer (pH 9-11), and incubated at 37℃ for 2 hr. And temperature dependency of the proteolytic activty was determined at different temperatures (20, 30, 40, 50, 60, and 70℃). The enzyme was incubated at each temperature for 1 hr and then its proteolytic activity was determined using hamster casein and fibrinas substrates.

Effect of pH and temperature on the enzyme stability

Effects of pH and temperature on the stability of the proteolytic enzyme (caseinolytic and fibrinolytic) were evaluated. The pH stability was determined in different buffers with pH ranges from 3 to 11; 0.5 M citrate buffer for pH 3-5, 0.1 M Tris-HCl buffer for pH 5-9, and 0.1 M carbonic acid buffer for pH 9-11. The 0.6% casein was dissolved in 4.0 ml of each buffer and incubated for 2 hr at

37℃. And temperature dependency of the enzyme stability was determined under standard condition at different temperatures (20, 30, 40, 50, 60, 70 and 100℃). The enzyme was incubated at each temperature for 1 hr and then its proteolytic activity was determined using hamster casein and fibrin as substrates. Then the solutions were neutralized and measured the degradation of fibrin and casein by using the procedures of Robbins et al. [14].

Effect of metal ions and various inhibitors on the enzyme acivity

The effects of metal ions were investigated by using $MgSO_4$, $FeSO_4$, $CuSO_4$, $CaCl_2$ and $ZnSO_4$. The effects of protease inhibitors were also checked by using phenylmethyl sulfonylfluoride (PMSF), EDTA, EGTA, DDT. In order to determine the sensitivity of the fibrinolytic protease towards various inhibitors, and metal ions, the crude enzyme was preincubated in the absence and the presence of 5 and 10 mM of bivalent cations such as Mg^{2+}, Fe^{2+}, Cu^{2+}, $Ca^{2+}$and Zn^{2+} and other inhibitors with a final concentration of 5 and 10 mM. An appropriate volume of the incubated solution was taken and the fibrinolytic activity was measured at the optimum pH(9.0) and temperature (50℃). The remaining activity was expressed as a percentage of the original activity measured without any effectors.

Results and Discussion

Proteolytic activity assay on the fibrin and skim milk plate

Biological active proteases are commonly found in the venome from western diamondback Rattle snake [19]. In this investigation, crude extract of maggot of *P. brevitarsis* was assayed by fibrin or skim milk plate analysis. The maggot of *P. brevitarsis*, as shown in Fig. 1. represented the strong enzyme activity on the fibrin and skim milk plate.

Effects of pH and temperature on the caseinolytic activity and stability of the enzyme

Effects of pH and temperature on the caseinolytic activity of the enzyme were evaluated. As shown in Fig. 2A, optimum pH of the enzyme was 7.0 to 9.0 than in the

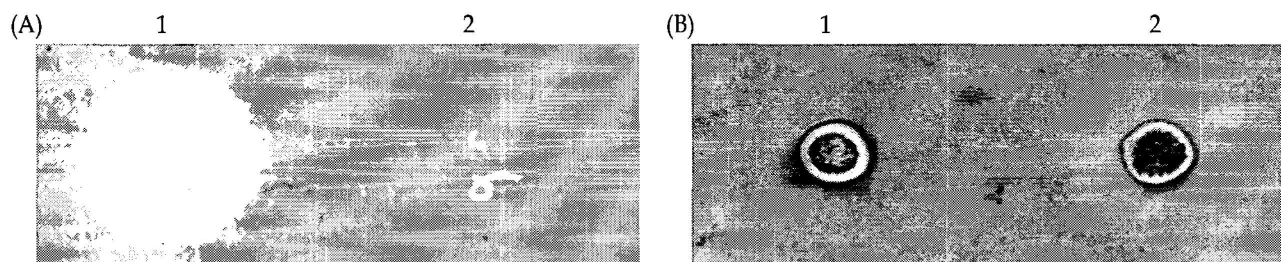

Fig. 1. Proteolytic activity assay on the fibrin plate or skim milk agar plate with the homogenized maggot of *Protaetia brevitarsis*. (A) Fibrinolytic activity (10ul) on the fibrin plate. (B) Proteolytic activity (50 ul) on the skim milk agar plate. Lane 1: control, Lane 2: crude enzyme of maggot of *Protaetia brevitarsis*.

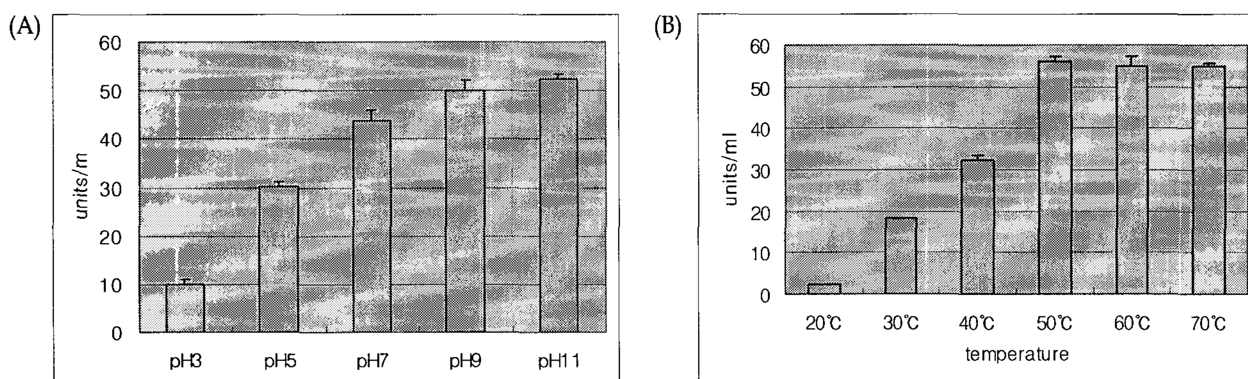

Fig. 2. Effect of pH (A) and temperature (B) on the activity of the caseinolytic enzyme from *Protaetia brevitarsis*. The enzyme activity was assayed in the pH range of 3-11 by using 0.5 M citrate buffer for pH 3-5, 0.1 M Tris-HCl buffer for pH 5-7 and 0.1M carbonic acid buffer for pH 9-11 at 37℃. The enzyme activity was assayed at different temperatures of 20-70℃ and pH 7 in 0.1 M Tris-HCl buffer.

acidic region (pH 3.0-5.0). The pH stability of the enzyme was also investigated in the range of pH 3.0-11.0 by measuring the remaining enzyme activity. As shown in Fig. 3A, the pH stability was also considerably higher in the neutral and alkaline region (pH 7.0-11.0) than in the acidic region (pH 3.0-5.0). Thus, the caseinolytic enzyme from *P. brevitarsis*, was shown to be a neutral and alkaline protease. The effect of temperature on the activity and stability of the caseinolytic enzyme were also examined in the range of 20-70°C at pH 9.0. As shown in Fig. 2B, the optimum temperature for the caseinolytic activity was approximately 50°C. The optimum temperature was in agreement with one of other caseinolytic enzymes reported by Kim [7].

Effects of pH and temperature on the fibrinolytic activity and stability of the enzyme

Effects of pH and temperature on the fibrinolytic activity of the enzyme were evaluated. The optimum pH for activity of the fibrinolytic enzyme from *P. brevitarsis* is shown in Fig. 4A. The relative fibrinolytic activity of the enzyme was well maintained in the range of pH 9.0 to pH 11.0. The pH stability of the enzyme was also investigated in the range of pH 3.0-11.0 by measuring the remaining enzyme activity. As shown in Fig. 5A, the pH stability was also considerably higher in the neutral and alkaline region (pH 7.0-11.0) than in the acidic region (pH 3.0-5.0). Thus, the fibrinolytic enzyme from *P. brevitarsis*, was shown to be a neutral and alkaline protease. According to the report of Kim [8], the stability of fibrinolytic enzyme from *Bacillus* sp. strain CK11-4 decreased considerably above pH 7-10. The effect of temperature on the fibrinolyic activity and stability of the enzyme was also examined in the range of 20-100°C at pH 9.0. As shown in Fig. 4B, the optimum temperature for the fibrinolytic activity of the enzyme was

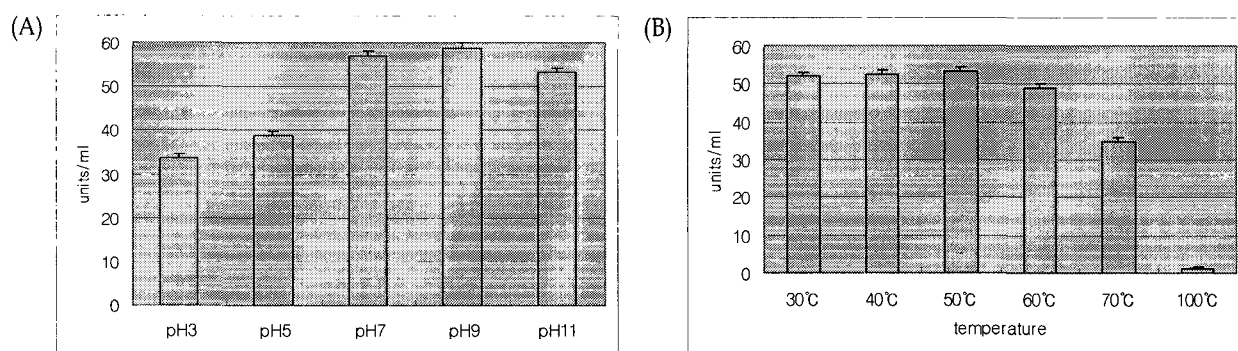

Fig. 3. Effect of pH (A) and temperature (B) on the stability of the caseinolytic enzyme from *Protaetia brevitarsis*. The enzyme stability was assayed in the pH range of 3-11, by using 0.5 M citrate buffer for pH 3-5, 0.1 M Tris-HCl buffer for pH 5-7 and 0.1M carbonic acid buffer for pH 9-11 at 37℃. The enzyme stability was measured by incubating it for 30 mins at the various pH values and 37℃. After the incubation, the enzyme assay was performed in 0.1M Tris-HCl buffer (pH 7.0). The enzyme activity was measured by keeping it for 2 hr at the different temperatures, and the enzyme assay was performed in 0.1 M Tris-HCl buffer (pH 7). One unit of activity was defined as nmol substrate hydrolyzed per min per ml enzyme.

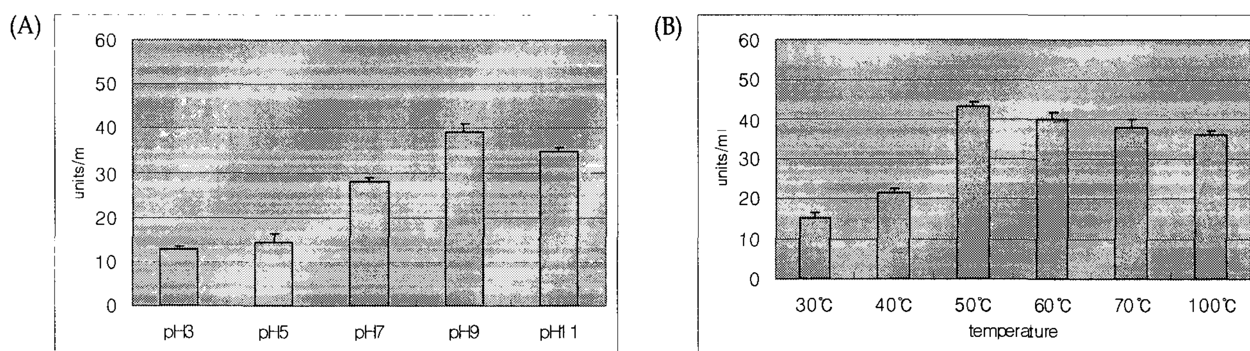

Fig. 4. Effect of pH (A) and temperature (B) on the activity of the fibrinolytic enzyme from *Protaetia brevitarsis*. The enzyme activity was assayed in the pH range of 3-11 by using 0.5 M citrate buffer for pH 3-5, 0.1 M Tris-HCl buffer for pH 5-7 and 0.1 M carbonic acid buffer for pH 9-11 at 37℃. The enzyme activity was assayed at different temperatures of 20-70℃ and pH 7 in 0.1 M Tris-HCl buffer. One unit of activity was defined as nmol substrate hydrolyzed per min per ml enzyme.

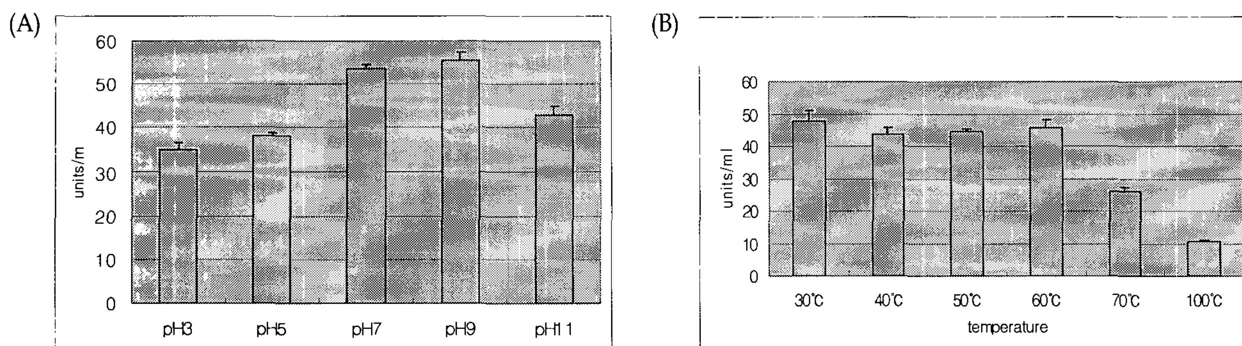

Fig. 5. Effect of pH (A) and temperature (B) on the stability of the fibrinolytic enzyme from *Protaetia brevitarsis*. The enzyme stability was assayed in the pH range of 3-11, by using 0.5 M citrate buffer for pH 3-5, 0.1 M Tris-HCl buffer for pH 5-7 and 0.1M carbonic acid buffer for pH 9-11 at 37℃. The fibrinolytic enzyme stability was measured by incubating it for 30 mins at the different pH values and 37℃. After the incubation, the enzyme assay was performed in 0.1M Tris-HCl buffer (pH 7.0). The enzyme activity was measured by keeping it for 2 h at the various temperatures, and the enzyme assay was performed in 0.1 M Tris-HCl buffer (pH 7).

approximately 60°C. The optimum temperature was in agreement with fibrinolytic enzymes from *Bacillus subtilis* reported by Yoo *et al.* [21].

Effects of protease inhibitors and metal ions on the enzyme activity

Historically, serine proteases were first recognized among the digestive system enzymes and found to be widely distributed in nature. The specific serine proteases which catalyze the hydrolysis of polypeptides for the biological purpose of digestion, blood clotting, clot lysis, sensing pain, and chemically opening insect cocoons are known to have common structural features [2]. The effects of various protease inhibitors on the fibrinolytic activity from maggot of *P. brevitarsis* are represented in Table 1.

Table 1. Effect of various inhibitors on the fibrinolytic enzyme from maggot of *Protaetia brevitarsis*

Inhibitors	Concentration (mM)	Relative activity (%)
None		100
PMSF	5	16.0
	10	8.6
EGTA	5	119.7
	10	93.9
EDTA	5	98.4
	10	92.0
DDT	5	58.9
	10	39.3
SDS	5	96.5
	10	93.6

Abbreviations: PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenedamine tetraacetate; EGTA, ethyleneglycol tetraacetate; DDT, dichloro-diphenyl-trichloroethane.

The enzyme activity was significantly inhibited by protease inhibitors such as PMSF and DDT at 5 or 10 mM, which were specific for the inhibition of serine proteases like nattokinase. However, it was not inhibited by EDTA, EGTA, and SDS. The effects of metal ions on the fibrinolytic activity are represented in Table 2. Under the conditions employed, Mg^{2+} and Fe^{2+} at 5 or 10 mM have no inhibitory effect on the enzyme activity when compared to the control without metal ion. On the contrary, Cu^{2+} showed slightly inhibitory effect, while Ca^{2+}, and Zn^{2+} exhibited a significant inhibition.

Therefore, these results suggest that the fibrinolytic enzymes from *P. brevitarsis* is a member of the serine protease family. In conclusion, we characterized a new serine fibrinolytic protease from the maggot of *P. brevitarsis*. The present results showed that the purified enzyme showed a relatively high activity for fibrin hydrolysis. Hence, it is

Table 2. Effects of metal ions on the fibrinolytic enzyme activity from maggot of *Protaetia brevitarsis*

Metal ion Inhibitors	Concentration (mM)	Relative activity
None		100
Mg^{2+}	5	100.7
	10	97.7
Fe^{2+}	5	99.8
	10	97.2
Cu^{2+}	5	79.0
	10	75.4
Ca^{2+}	5	55.6
	10	11.2
Zn^{2+}	5	55.4
	10	55.1

suggested that the purified enzyme can be applied as an effective thrombolytic agent.

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초록 : *Protaetia brevitarsis의 maggot로부터* fibrinolytic activity을 가진 protease의 생화학적 특성 연구

장정현 · 조지영 · 김영진[1] · 이선이[1] · 조효진[1] · 유선녕[1] · 김광연[1] · 박병근[2] · 안순철[1] · 권헌영*
(부산가톨릭대학교 보건과학대학 임상병리학과, [1]부산대학교 의과대학 미생물학 및 면역학 교실, [2]주식회사 장생도라지)

혈전(fibrin clot)은 심혈관계 질환을 일으키는 주요 인자로서 전신의 미세동맥이나 모세혈관 내에서 형성되어 주의 조직이나 장기에 혈류의 공급방해가 생겨 허혈, 경색, 나아가 괴사까지도 발생시킨다. 혈전이 원인이 되어 발생한 심혈관계 질환을 예방 혹은 치료할 목적으로 기존으로 사용되고 있는 항혈전제(antithrombolytic drug)나 혈전용해제(thrombolytic drug)의 개발을 위해 많은 연구들이 진행되고 있다. 본 연구에서는 치료목적으로 사용할 혈전용해제를 분리하고자 *Protaetia brevitarsis*의 maggot로부터 ammonium sulfate 분획과 desalting column을 이용하여 fibrinolytic protease를 분리하고 생화학적 특성을 조사하였다. 활성의 최적 pH는 9.0였고 최적온도는 50℃였다. pH 7.0-9.0 사이와 온도 60℃이하에서는 비교적 활성이 안정성을 나타냈다. 효소활성이 phenylmethanesulfonyl fluoride에 의해 강하게 저해되고 있는 것으로 보아 serine protease로 추정되며 금속이온에 의한 영향을 조사해 본 결과 Ca^{2+}과 Zn^{2+}에 의해서는 저해되지만 Mg^{2+}와 Fe^{2+}에 의해서는 저해를 받지 않았다.