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Isolation and characterization of a fibrinogenolytic enzyme from *Macrovipera mauritanica* snake venom

朝 鮮 大 學 校 大 學 院

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뱀독으로부터 피브리노겐을 분해하는 단백질
분해효소의 분리 및 특성 분석

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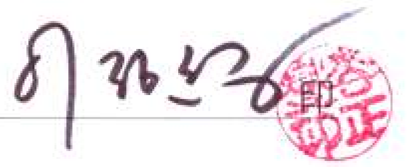
委　員 朝鮮大學校 敎授  印

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TABLE OF CONTENTS

TABLE OF CONTENTS ········· i

LIST OF TABLES ········· iii

LIST OF FIGURES ········· iv

ABSTRACT ········· 1

I. INTRODUCTION ········· 3

II. MATERIALS AND METHODS ········· 7

II-1. Materials ········· 7

II-2. Purification of MMF from *Macrovipera mauritanica* snake venom ········· 7

II-3. Fibrinogen clotting time (FCT) assay ········· 8

II-4. Fibrinogenolytic assay ········· 8

II-5. Fibrinolytic assay ········· 9

II-6. Design of a fluorogenic substrate for MMF and proteolytic activity assay ········· 9

II-7. Vascular permeability assay ········· 10

II-8. Type IV collagen digestion by MMF ········· 10

II-9. Effect of MMF on plasminogen and prothrombin cleavage ········· 11

II-10. Effect of inhibitors and divalent ions on MMF activity ········· 11

II-11. Effects of temperature and pH on MMF activity ········· 12

II-12. SDS-PAGE analysis ··· 12
II-13. N-terminal sequencing ··· 13

III. RESULTS AND DISCUSSION ··· 14

III-1. Purification of MMF ··· 14
III-2. Determination of N-terminal amino acid sequence ··· 18
III-3. Characterization of enzymatic property of MMF ··· 18
III-3-1. Proteolytic activity of MMF ··· 18
III-3-2. Effect of divalent ions and protease inhibitors on MMF enzyme activity ··· 29
III-3-3. Effects of pH and temperature on the MMF activity ··· 29
III-3-4. Vascular permeability induced by MMF ··· 34

IV. 적 요 ··· 40

V. References ··· 42

LIST OF TABLES

Table. 1. Purification summary of MMF ··· 17

Table. 2. Comparison of the N-terminal amino acid sequence of MMF with those of other related proteases ··· 19

Table. 3. Kinetic values of MMF ··· 28

Table. 4. Effect of various inhibitors on the enzyme activity of MMF ··· 31

Table. 5. Effect of various divalent ions on the enzyme activity of MMF ··· 33

LIST OF FIGURES

Fig. 1. Hemostatic pathway of blood coagulation in part ···· 6
Fig. 2. Purification steps of a fibrinogenolytic enzyme MMF ···· 15
Fig. 3. SDS-PAGE analysis of the purified MMF ···· 16
Fig. 4. Time-dependent degradation of fibrinogen by MMF ···· 21
Fig. 5. Degradation of cross-linked fibrin by MMF ···· 22
Fig. 6. Effect on fibrinogen clotting time by MMF ···· 23
Fig. 7. Cleavage of prothrombin and plasminogen by MMF ···· 24
Fig. 8. Analysis of cleavage pattern of fibrinogen by MMF ···· 26
Fig. 9. Fluorogenic assay with MMF ···· 27
Fig. 10. Effect of various inhibitors on fibrinogenolytic activity of MMF ···· 30
Fig. 11. Effect of various divalent ions on fibrinogenolytic activity of MMF ···· 32
Fig. 12. Effect of pH on the fluorogenic activity of MMF ···· 35
Fig. 13. Effect of temperature on the fluorogenic activity of MMF ···· 36
Fig. 14. Vascular permeability induced by MMF ···· 38
Fig. 15. Cleavage of type IV collagen by MMF ···· 39

ABSTRACT

Isolation and characterization of a fibrinogenolytic enzyme from *Macrovipera mauritanica* snake venom

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Snake venom contains various enzymes and peptides showing anticoagulant and fibrinogenolytic activities. In this study, a fibrinogenolytic enzyme designated to as MMF was purified and characterized from crude snake venom of *Macrovipera mauritanica*. For the purification, Superdex 75, Source Q, and Mono Q column chromatographic steps were employed in order. MMF was purified to homogeneity as judged by its migration profile in SDS-polyacrylamide gel stained with coomassie blue. The purified enzyme showed a molecular mass of about 27 kDa. MMF was composed of single polypeptide and its amino-terminal 10 amino acid sequence was found to be NH_2-QRFAPRYIEL-COOH, identical to that of a fibrinolytic metalloproteinase

from the venom of *Vipera lebetina*, except for the first amino acid glutamine. MMF could prolong 2.2 times the fibrinogen clotting time (FCT), compared to that of non-treated control. Among substrate proteins tested, fibrinogen was the most susceptible one for MMF. The α- and the β-chains of fibrinogen were completely digested by MMF until 20 min and 8 h at 37℃, respectively. As shown in other fibrinogenolytic enzymes from snake venoms, the γ-chain of fibrinogen seemed to be resistant to the enzyme cleavage. The cleavage pattern on cross-linked fibrin by MMF was similar to the case of fibrinogen. MMF could cleave the peptide bond between Lys^{413} and Leu^{414} in the α-chain of fibrinogen. Based on the cleavage site of MMF, a fluorogenic peptide substrate was designed and synthesized as follows: Abz-His-Thr-Glu-Lys-Leu-Val-Thr-Ser-Lys-Dnp-NH_2. Using the synthetic fluorogenic substrate, enzyme kinetics was determined as follows: K_M = 1.5 x 10^{-2} M/L, V_{max} = 2.33 x 10^{-7} M/min, and turnover rate = 0.31. The optimal pH and temperature for the enzyme activity of MMF were about pH 5.5 and 30℃, respectively. The enzyme activity of MMF could be inhibited by metalloproteinase inhibitors (1,10-PT, DTT, and EDTA), but not by serine protease inhibitors (TLCK and TPCK), suggesting that MMF may be a metalloproteinase. In addition, MMF increased vascular permeability as observed by Miles assay. MMF could partially cleave type IV collagen that is an extracellular matrix components *in vitro*. Taken together, the results obtained by this study suggest that MMF is a fibrinogenolytic metalloproteinase and also can induce vascular permeability through digesting type IV collagen.

I. INTRODUCTION

Envenomation by venomous snakes in humans induced many potential effects, but just a few broad categories are of major clinical significance. They are flaccid paralysis, systemic myolysis, coagulopathy and hemorrhage, renal damage and failure, cardiotoxicity, and local tissue injury at the bite site. Each of these may cause a number of secondary effects, each with potential morbidity and mortality (White, 2005). Among the specie's of snake, venoms of Viperidae, Elapidae and Crotalidae snake contain fibrin(ogen)olytic enzymes that affect hemostasis, directly acting on vessel walls, platelet function, fibrinogen and other factors of the hemostatic system (Braud, Bon & Wisner, 2000), shown in Fig. 1. The hemostatic effects by snake venom have been studied since 1700s. Fontana (1787) observed that blood remained to fluid state without blood clotting in animals dying from viper envenomation. This effect was most likely due to the combined action of fibrinogenolytic and defibrinogenating enzymes found in the venom. Later, Mitchell and Reichert (1886) studied and described the effect of Crotalidae venom on a number of animal organs including blood. They made the observation that in animals dying soon after envenomation the blood coagulable, but blood from animals whose death was delayed was incoagulable. Interestingly, Elapidae venoms were observed to possess fibrinogenolytic activity but not fibrinolytic activity (Swenson and Markland, 2005).

Macrovipera mauritanica is a venomous viper species found in northwestern Africa. The three species of Macrovipera genus currently recognized as subspecies of *M. lebetina*. A number of proteins purified from

M. lebetina venom have been reported (Aaspollu *et al*., 2005; Gasmi *et al*., 1997; Gasmi *et al*., 2001; Saidi *et al*., 1999; Samel *et al*., 2002; Siigur *et al*., 1996; Siigur *et al*., 1998; Trummal *et al*., 2000). The snake venom contains serine proteases (α-fibrinogenase, β-fibrinogenase, and factor V activator) and metalloproteinases (fibrinolytic enzyme lebetase and factor X activator) that affect coagulation and fibrinolysis.

The fibrin(ogen)olytic metalloproteinases are classified as α- and β-fibrin(ogen)ase according to their proteolytic preference toward the Aα- or Bβ-chain of fibrin(ogen) (Leonardi *et al*., 2007). However, identification as either an α- or β-fibrin(ogen)ase is not absolute since there is significant degradation of the alternate chain with increasing time of incubation. These direct acting endoproteinases do not require any other factors for activity. Furthermore, they do neither release fibrinopeptides A or B and nor induce fibrin clot formation (Markland, 1998). In contrast to the fibrin(ogen)olytic metalloproteinases, fibrin(ogen)olytic serine proteases cleave the Bβ-chain preferentially with lower activity directed toward the Aα-chain, although there are a number of exceptions. The γ-chain does not appear to be a specific target as there are virtually no reports of fibrin(ogen)oytic snake venom enzyme whose specificity is uniquely directed to this chain (Swenson and Markland, 2005).

Based on their domain structures, snake venom metalloproteinases (SVMPs) has been classified into four classes as follows: (1) Class P-I includes proteases with molecular masses of 20-30 kDa having only a metalloproteinase domain; (2) Class P-II comprises proteins with molecular masses of 30-60 kDa that are composed of metalloproteinase and disintegrin domains; (3) Class P-III includes proteases composed of metalloproteinase

domain, disintegrin-like domain and cysteine-rich domain; (4) Class P-IV proteases show a characteristics that the P-III domain structure and lectin-like domains are connected by disulfide bonds. According to the presence or absence of certain domains on the classes, the proteases give an effect against hemorrhagic, fibrinolytic, apoptotic, or coagulation system (Fox and Serrano, 2005).

The vascular system has the critical function of supplying tissues with nutrients and clearing waste products. Thus, vascular permeability is essential for the health of normal tissues and is also an important characteristic of many disease states in which it is greatly increased. Permeability is also an extremely complicated process, that is affected by many different variables (Nagy *et al.*, 2008). The effect of vascular permeability may be related with proteolytic degradation of the basement membrane proteins by the certain enzymes of venom. Basement membrane (BM) is a complex extracellular matrix structure comprising several major components, such as laminin, type IV collagen, and fibronectin. BM is a supramolecular lattice which, among other functions, provides mechanical stability to the capillary vessel structure (Escalante *et al.*, 2006).

In this study, a fibrinogenolytic enzyme named MMF was purified and characterized from *Macrovipera mauritanica* snake venom. The purified enzyme was characterized in terms of its fibrinogenolytic and fibrinolytic activities. In addition, the proteolytic activity of MMF using a synthetic substrate designed on the basis of its cleavage site was examined. The increase of vascular permeability by MMF was also demonstrated through the Miles assay.

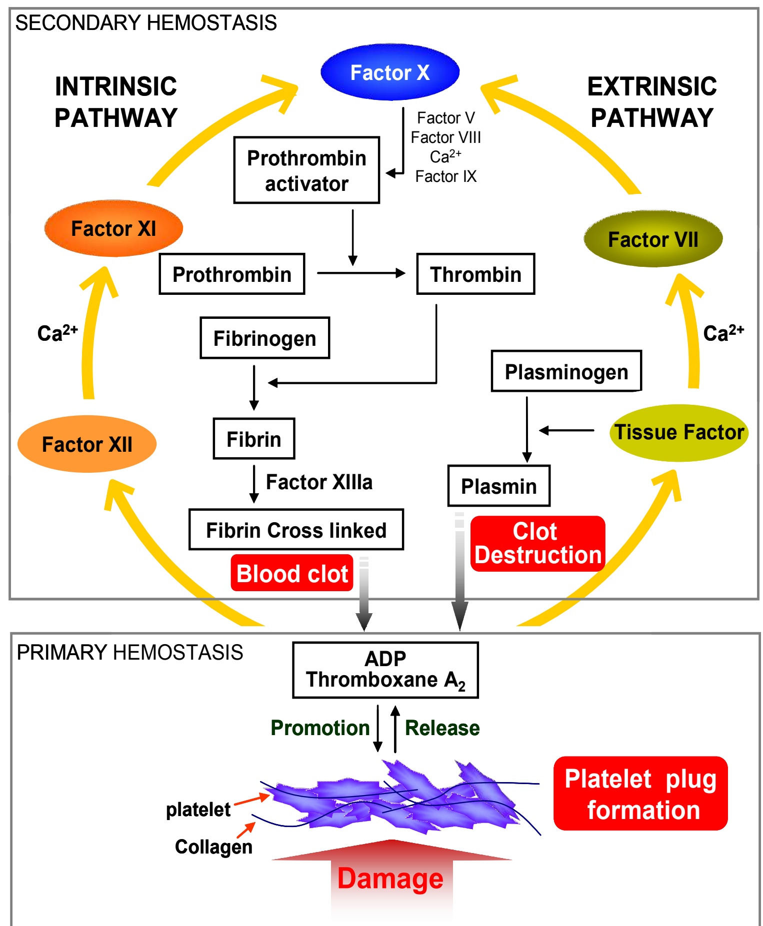


Fig. 1. Hemostatic pathway of blood coagulation. Coagulation is initiated by vascular injury, leading to reflex vasoconstriction near the injured vessel. The injured vascular endothelium makes platelet adhesion, interacting with vWF, collagen, fibronectin and vitronectin released by the subendothelium. The platelet adhesion releases the contents of their granules such as ADP and thromboxane A_2, which promote the platelet aggregation. Following cascade is formation of the fibrin clot resulted from activation of fibrinogen by thrombin, which is activated from prothrombinase complex. Finally, fibrin cross-linked from fibrin by factor XIIIa forms more tightly blood clot with platelet plug. After clot formation, bleeding is stopped, and fibrin network is degraded by the fibrinolytic system, which mainly acted by plasmin (Braud *et al.*, 2000, Norris, 2003).

II. MATERIALS AND METHODS

II-1. Materials

The lyophilized snake venom of *Macrovipera mauritanica* was purchased from Latoxan (Valence, France). All chromatographic columns were obtained from Amersham Biosciences (Uppsala, Sweden). KC1 coagulometer was from Sigma (St. Louis, MO, USA). Plasminogen was purchased from Roche Applied Science (Mannheim, Germany). Human prothrombin was obtained from CalBiochem (Darmstadt, Germany). Protein molecular weight markers were obtained from Fermentas (Darmstadt, Germany). Human fibrinogen, α-thrombin, factor XIIIa, type IV collagen, EGTA, EDTA, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline (1,10-PT), N, N-methylene-bis- acrylamide, TEMED, Trizma base and other chemicals used were obtained from Sigma (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Bio-Rad (Rich-mond, Calif). The synthetic fluorogenic peptide substrates used in this study were synthesized from GenScript Co. (New Jersey, USA).

II-2. Purification of MMF from *Macrovipera mauritanica* snake venom

The lyophilized snake venom (121.8 mg) was dissolved in 1.5 ml of 20 mM sodium acetate buffer (pH 5.5) containing 100 mM NaCl. The insoluble materials were removed by centrifugation at 9,000 x*g* for 5 min,

and the resulting supernatant (total 40.6 mg protein) was fractionated three times on a Superdex 75 10/300 GL column equilibrated with 20 mM sodium acetate (pH 5.5) containing 100 mM NaCl at a flow rate of 0.5 ml per min. Fractions from the column were assayed using the fibrinogen clotting time (FCT) assay described in next section. The active fractions were pooled and applied on to a Source^{TM} 15Q 4.6/100 PE column equilibrated with 20 mM sodium acetate (pH 5.5), in which the bound proteins were eluted with a linear gradient of 0 to 0.4 M NaCl at flow rate of 1.0 ml per min, and then further fractionated on a Mono Q^{TM} HR 5/5 column equilibrated with the same buffer. The bound proteins were eluted with linear gradient of 0 to 0.2 M NaCl at flow rate of 0.5 ml per min. The fractions obtained from this last chromatographic step were analyzed on a 12% SDS-PAGE under reducing condition and stored at -20℃ until used.

Protein concentrations were determined with Bradford reagent (Sigma) according to the manufacturer's instructions, or using Nanodrop 1000 spectrophotometer (Thermo, SCIENTIFIC).

II-3. Fibrinogen clotting time (FCT) assay

Fifty microliters of thrombin (2.5 U/ml) solution pre-incubated at 37℃ for 5 min was mixed with 100 μl of fibrinogen (0.5%) solution with different concentrations of MMF with or without 1 mM EDTA. The clotting time was monitored using a KC1 coagulometer (Sigma, St. Louis, MO, USA).

II-4. Fibrinogenolytic assay

The fibrinogenolytic activity was determined by incubating MMF (5.5 μg/ml) with fibrinogen (3.47 mg/ml) in 20 mM Tris-HCl (pH 8.0) buffer containing 0.2 M NaCl at 37℃. The final volume was adjusted to 140 μl to achieve a molar ratio of MMF versus fibrinogen of approximately 1:50. Fifteen microliters aliquots were taken from reaction mixture at interval times (0 to 24 h) and separated by 12% SDS-PAGE to examine the cleavage pattern.

II-5. Fibrinolytic assay

Cleavage assay of cross-linked fibrin by MMF was performed as described previously (Chang *et al*., 2005). Thirty microliters of reaction mixture containing 0.7 mg/ml of fibrinogen, 0.13 U/ml of thrombin, 0.13 U/ml of factor XIIIa, and 1 mM $CaCl_2$ in 20 mM Tris-HCl (pH 8.0) and 0.2 M NaCl was incubated at room temperature for 1 h. For the detection of MMF-cleaved cross-linked fibrin, 2 μg of MMF was added to the reaction sample and incubated at room temperature for 30 min. The reaction was terminated by the addition of a 5 μl of 6X SDS-PAGE sample buffer followed by heating at 100℃ for 2 min and then electrophoresed on 8% SDS-polyacrylamide gel.

II-6. Design of a fluorogenic substrate for MMF and proteolytic activity assay

The design of the fluorogenic peptide {(2-amino benzene)-His-Thr-Glu-Lys-Leu-Val-Thr-Ser-(dinitro-phenol)-amide} was based on the cleavage site of MMF obtained through the cleavage analysis of human fibrinogen (Lys^{413}- Leu^{414}) and synthesized by GenScript (USA). This peptide was designed so that the fluorescence derived from the C-terminal 2-amino benzene group (λ_{ex}=320 nm, λ_{em}=435 nm) was strongly quenched by the N-terminal dinitro-phenol (Dnp) group. Hydrolysis of a peptide bond between the two groups generates a fluorescence that can easily determine the enzyme activity. Typically, the reaction mixture (100 μl) was composed of 20 μg/ml of MMF and various concentrations of the synthetic substrate (dissolved in 30% dimethylformamide) in the reaction buffer (20 mM sodium acetate, pH 5.5 and 0.1 M NaCl). The reaction mixture was pre-incubated separately, mixed, and the fluorescence was monitored for 15 min at 37℃ using a 96 well plate reader (Molecular Devices).

II-7. Vascular permeability assay

Vascular permeability was evaluated by a modification of the Miles assay (Yamazaki *et al*., 2007). After etherigation with diethyl ether, a guinea pig (250-300 g) was shaved on its back and given an intracardiac injection of Evan’s Blue dye (65 mg/kg) in PBS buffer. MMF (10 μg) was diluted into PBS buffer and injected intradermally into the shaved area on the back of

the animal. After 10 min, animal was sacrificed and back skin was excised. For quantification of dye leakage, the spot excised from skin was incubated in formamide at 60℃ for 48 h and the amount of extracted dye was determined by using the absorbance at 620 nm.

II-8. Type IV collagen digestion by MMF

Type IV collagen (0.16 mg/ml) was incubated with MMF (0.04 mg/ml) in 200 μl of 20 mM sodium acetate and 0.1 M NaCl (pH 5.5) at 37℃. At various intervals times (1 to 6 h), 30 μl aliquot each were withdrawn, mixed with 6X SDS-PAGE sample buffer, and then analyzed on SDS-PAGE.

II-9. Effect of MMF on plasminogen and prothrombin cleavage

MMF (3 μg) was incubated with plasminogen or prothrombin (40 μg in 60 μl of 20 mM sodium acetate, pH 5.5, 0.1 M NaCl) at 37℃. Fifteen μl aliquots were taken at the time interval of 2, 4, and 24 h to analyze on a 10% SDS-PAGE.

II-10. Effect of inhibitors and divalent ions on MMF activity

MMF (3 μg) was pre-incubated with 1 mM of various inhibitors (DFP, PMSF, TLCK, TPCK, EDTA, 1,10-PT, bestatin, aprotinin, DTT, and EGTA) or

various divalent ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) in 30 μl of 20 mM sodium acetate buffer containing 0.1 M NaCl (pH 5.5) at 37℃ for 10 min. To analysis of the effects on fibrinogenolytic activity, MMF (0.5 μg) was incubated with fibrinogen (19.2 μg) for 20 min at 37℃ and then submitted to an 12% SDS-PAGE under reducing condition. To examine their effects on MMF proteolytic activity, MMF (1 μg) was used together with the fluorogenic peptide assay as described above.

II-11. Effect of temperature and pH

To investigate the effect of temperature on the enzyme activity of MMF, MMF was incubated at 25 to 70℃ for 30 min in a 20 mM sodium acetate buffer (pH 5.5) containing 0.1 M NaCl and their samples were assayed with fluorogenic substrate as described in section II-6. The effect of pHs on the enzyme activity of MMF was also assayed with fluorogenic substrate in buffers having different pHs. The buffers used in these assays were 50 mM sodium acetate (pH 4-5.5), 50 mM potassium phosphate (pH 6-7.5), 50 mM Tris-HCl (pH 8-8.5), and 50 mM glycine-NaOH (pH 9-10.5) buffers, system as described previously (Chang *et al*., 2005).

II-12. SDS-PAGE analysis

SDS-PAGE was performed according to the method of Laemmli (1970). Samples to be analyzed were mixed with an equal volume of 2X SDS-PAGE sample buffer. The samples were heated at 100℃ for 1 min and

loaded onto 8%, 10%, or 12% gel. After electrophoresis, protein bands were visualized by staining with Coomassie blue. Molecular weight markers used consisted of β-galactosidase (*E. coli*, 116 kDa), bovine serum albumin (bovine plasma, 66 kDa), ovalbumin (chicken egg white, 45 kDa), lactate dehydrogenase (porcine muscle, 35 kDa), restriction endonuclease Bsp981 (*E. coli*, 25 kDa), β-lactoglobulin (bovine milk, 18.4 kDa), lysozyme (chicken egg white, 14.4 kDa).

II-13. N-terminal sequencing

Protein samples were subjected to electrophoresis on 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11.0) containing 10% methanol. The membrane was stained with Coomassie blue, followed by destaining. Target bands were excised from the membrane and N-terminal sequencing was performed by Korea Basic Science Institute (KBSI, Seoul, Korea).

III. RESULTS AND DISCUSSION

III-1. Purification of MMF

MMF was purified from *Macrovipera mauritanica* venom by three-step chromatographic procedures (Fig. 2). Chromatography of *Macrovipera mauritanica* snake venom on a Superdex 75 column resulted in the separation of about five peaks (Fig. 2A). The proteins present in the fractions of third peak (fraction numbers 12 to 14) showed anticoagulant effect on fibrinogen clotting (data not shown). Further chromatography of the pooled fractions was performed on a Source Q, in which one main peak appeared when the bound proteins were eluted by NaCl gradient from 0 to 0.4 M (Fig. 2B). The fractions of main peak finally applied to a Mono Q column to obtain pure enzymes (Fig. 2C). Through the three chromatographies, approximately 2.8 mg of MMF could be purified from 121.8 mg crude venom. As shown in Table 1, the specific activity of MMF was 1,000 U/mg and the purification yield was 2.3%. MMF appeared to be homogeneous on SDS-PAGE with an estimated molecular mass of about 27 kDa (Fig. 3). This size is similar to a hemorrhagic metalloproteinase BlaH1 isolated from *Bothrops lanceolatus* venom (28 kDa) (Stroka *et al*., 2005) and a non-hemorrhagic metalloproteinase VIF isolated from *Vipera lebetina* venom (26 kDa) (Gasmi *et al*., 1997; Gasmi *et al*., 2000).

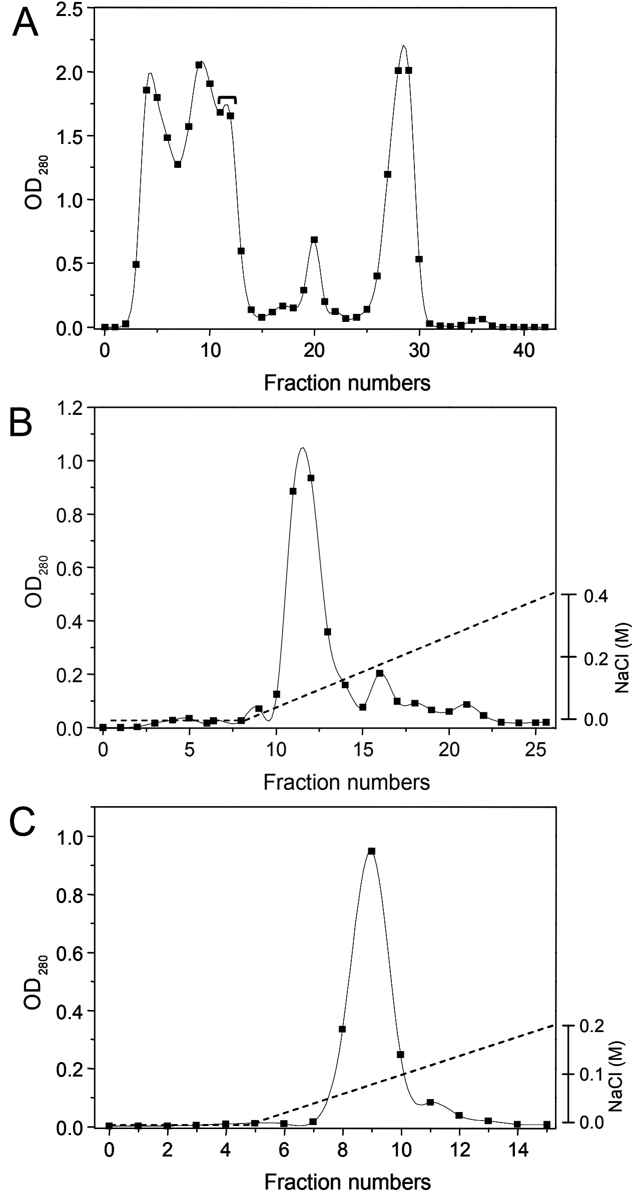


Fig. 2. Purification steps of a fibrinogenolytic enzyme MMF. (A) First step: gel filtration on Superdex 75 column. The solid bar indicates the pooled the fractions. (B) Second step: anion exchange chromatography on Source Q column. (C) Final step: anion exchange chromatography on Mono Q column.

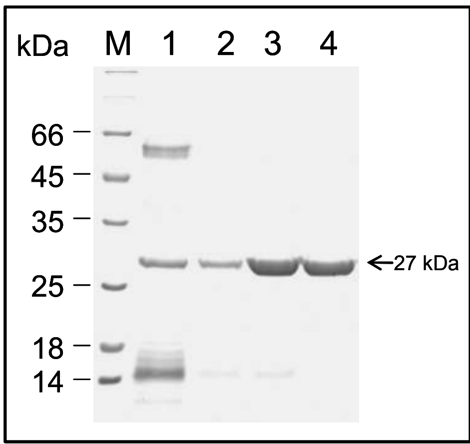


Fig. 3. SDS-PAGE analysis of the purified MMF. Proteins obtained from each purification step were electrophoresed on 12% polyacrylamide gel. Lanes M, Protein size makers; 1, Crude snake venom; 2, Superdex 75; 3, Source Q; 4, Mono Q.

Table 1. Purification summary of MMF.

Steps	Total Protein (mg)	Total Activity (U)[a]	Specific Activity (U/mg)	Yield (%)[b]
Crude venom	121.8	19,500	160	100
Superdex 75	16.8	12,800	762	13.8
Source Q	4.2	3,360	800	3.5
Mono Q	2.8	2,800	1,000	2.3

[a]One unit (U) was defined as the amount of the enzyme digesting 4 μg of fluorogenic peptide substrate in 10 min.
[b]Total activity in the crude snake venom was assigned the value of 100%.

III-2. Determination of N-terminal amino acid sequence

Macrovipera mauritanica snake belongs in the *Macrovipera* family with *Vipera lebetina*. Snake venom of *Vipera lebetina* contains factor X activator, factor V activator, and serine protease, except for the metalloproteinase (Siigur *et al*., 2001).

The N-terminal amino acid sequence of MMF was determined to be NH_2-QRFAPRYIEL-COOH, showing a significant similarity to those of some fibrinogenolytic enzymes as described in Table 2. The average sequence homologies between MMF and the four proteases (BAP1, Lebetase-II, Lebetase-4, and VIF) were 90% (Table 2). Both VIF and Lebetase isolated from *Vipera lebetina* snake venom have been characterized as a non-hemorrhagic and a fibrin(ogen)lytic metalloproteinase, respectively (Gasmi *et al*., 1997; Gasmi *et al*., 2001; Gasmi *et al*., 2000; Saidi *et al*., 1999; Samel *et al*., 2002; Siigur *et al*., 1998; Trummal *et al*., 2000). In addition, BaP1 isolated from *Bothrops asper* is a hemorrhagic metalloproteinase (Escalante *et al*., 2000; Escalante *et al*., 2004; Fernandes *et al*., 2006; Gutierrez *et al*., 1995; Loria *et al*., 2003; Rucavado *et al*., 1995; Watanabe *et al*., 2003).

III-3. Characterization of enzymatic property of MMF

III-3-1. Proteolytic activity of MMF

Most snake venom metalloproteinases (SVMPs) possess proteolytic

Table 2. Comparison of the N-terminal amino acid sequence of MMF with those of other related proteases.

Snake venom (Enzyme name)	N-terminal sequence	Identity (%)
Macrovipera mauntanica (MMF)	Q R F A P R Y I E L	100
Bothrops asper (BAP1)	Q R F S P R Y I E L	90
Vipera lebitina (Lebetase-II)	Q R F E P R Y I E L	90
Vipera lebitina (Lebetase-4)	Q R F D P R Y I E L	90
Vipera lebitina (VIF)	E R F A P R Y I E L	90

activity towards fibrinogen and/or fibrin. Similarly, MMF could digest the α- and the β-chains of human fibrinogen, which was completely digested within 20 min and 8 h, respectively (Fig. 4). The fibrinolytic activity of MMF was also determined using cross-linked fibrin as described previously (Chang *et al*., 2005). As shown in Fig. 5, MMF could cleave cross-linked fibrin to make fibrin degrading products, but not digest γ-γ chains effectively.

Many metalloproteinases isolated from various venomous snakes, such as ammodytase (Leonardi *et al*., 2007), patagonfibrase (Peichoto *et al*., 2007), BmooMP-I (Bernardes *et al*., 2008), VIF (Gasmi *et al*., 2000), and Lebetase (Siigur *et al*., 1998) also preferentially hydrolyze the α-chain followed by digesting the β-chains of fibrinogen and fibrin. However, BmooMP-I somewhat differs with other proteases, due to it also can digest the γ-chain of fibrinogen. Some metalloproteinases such as Leuc-a (Bello *et al*., 2006) and halysase (You *et al*., 2006) can only cleave the α-chain of fibrinogen.

Thrombin-induced clotting time could be inhibited by MMF in dose-dependent manner, that was prolonged 2.2 times compared to that of non-treated control (Fig. 6). In addition, the anticoagulant effect of MMF could be inhibited by the addition of EDTA (Fig. 6). These results suggest that MMF can delay thrombin-induced clotting time.

In this study, it was also examined the effect of MMF on plasminogen and prothrombin activations. When MMF was incubated with prothrombin or plasminogen in the presence of chromogenic substrate for thrombin or plasmin, there were no amidolytic activities (data not shown), although the protease could digest plasminogen and prothrombin (Fig. 7). These results

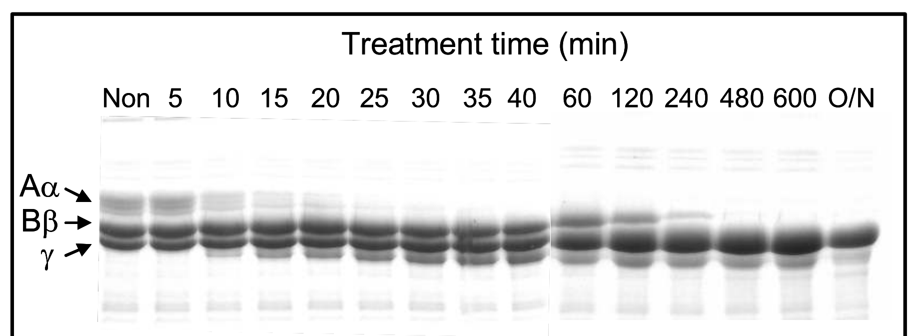


Fig. 4. Time-dependent degradation of fibrinogen by MMF. MMF was added to fibrinogen solution at a molar ratio of 1:50 and then incubated at 37℃ for the indicated time intervals. The samples were electrophoresed on 12% polyacrylamide gel. Non means fibrinogen only and O/N indicates overnight incubation at 37℃.

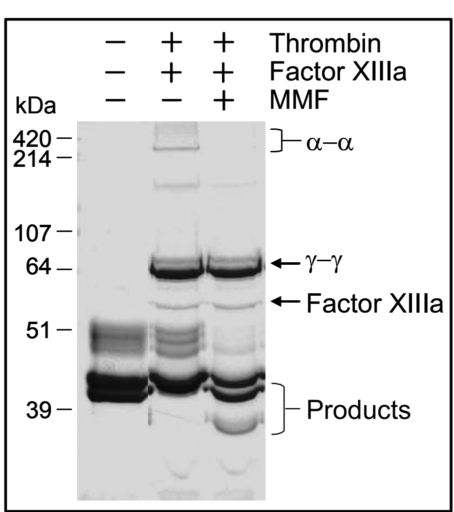


Fig. 5. Degradation of cross-linked fibrin by MMF. Polymerization of fibrin was initiated by the addition of fibrinogen, thrombin, and factor XIIIa and $CaCl_2$ followed by incubation at room temperature for 1 h. The cross-linked fibrin was reacted with MMF at room temperature for 30 min followed by SDS-PAGE using 8% gel.

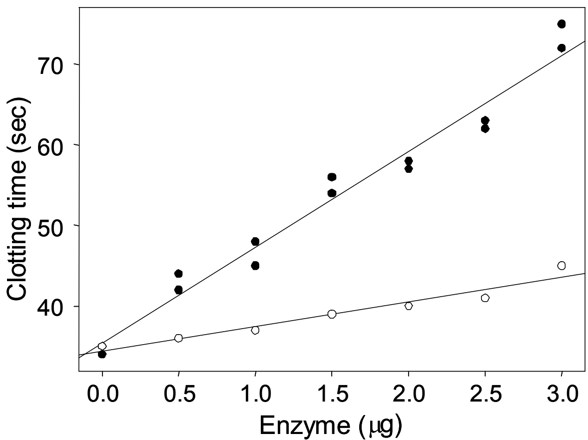


Fig. 6. Effect on fibrinogen clotting time by MMF. Fibrinogen was pre-incubated with different concentrations of MMF at 37℃ for 5 min and fibrinogen clotting time (FCT) was determined by adding thrombin into the mixture. The fibrinogen clotting time delayed by MMF was compared with non-treated sample. Symbols ○, + EDTA; ●, - EDTA.

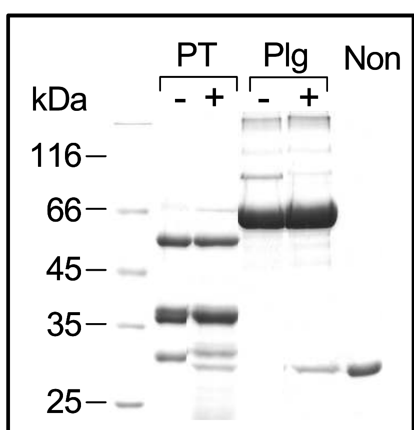


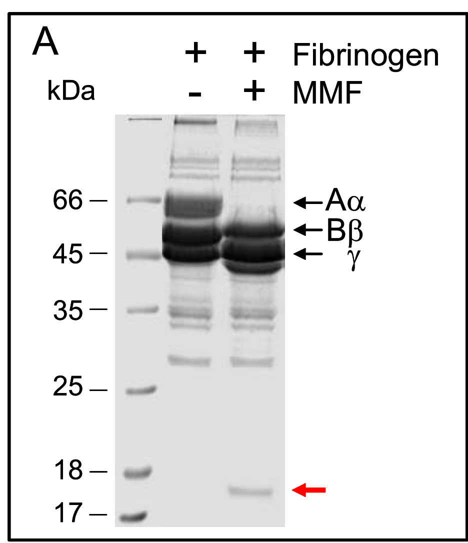
Fig. 7. Cleavage of prothrombin and plasminogen by MMF. Prothrombin and plasminogen were incubated with or without MMF for 24 h at 37℃. After incubation, the reaction mixtures were analyzed by 10% SDS-PAGE under reducing condition. PT, Prothrombin; Plg, Plasminogen; Non, MMF only.

suggest that MMF cannot have roles as plasminogen and prothrombin activators. In contrast with MMF, metalloproteinases basparin A (Loria *et al*., 2003) and bothrojaractivase (Berger, Pinto & Guimaraes, 2008) from *Bothrops asper* and *Bothrops jararaca*venom, respectively, can activate prothrombin.

To analysis the cleavage pattern of human fibrinogen by MMF, fibrinogen was incubated with 1 μg of MMF for 30 min and the resulting peptides were seperated by SDS-PAGE (Fig. 8A). Among the fragments, appoximately 17.5 kDa band was used for analysis of N-terminal amino acid sequence to reveal the cleavage site for MMF. The N-terminal sequencing of the fragment showed that MMF specifically cleaved the peptide bond between Lys^{413} and Leu^{414} contained in the α-chain of fibrinogen (Fig. 8B).

Similar with MMF, fibrolase (Swenson and Markland, 2005) and lebetase (Trummal *et al*., 2000) cleave the α-chain of fibrinogen preferentially at same site. However, ammodytase (Leonardi *et al*., 2007) cleaves at Glu^{593}-Phe^{540} and Glu^{441}-Leu^{442} of the fibrinogen.

Unfortunately, MMF could not cleave effectively azocasein when it was used as a substrate. As a result, azocasein assay could not use for MMF activity assay. With that reason, MMF-specific fluorogenic substrate was designed and synthesized on the basis of its cleavage site (Lys^{413}-Leu^{414}) found in the α-chain of fibrinogen as follows: Abz-His-Thr-Glu-Lys-Leu-Val-Thr-Ser-Lys-Dnp-NH_2. As shown in Fig. 9, the synthetic substrate was effectively used by MMF as a substrate in dose-dependent manner. Using this fluorogenic substrate, the enzymatic kinetics could be measured as shown in Table 3.



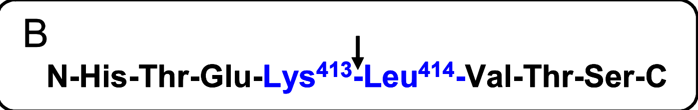


Fig. 8. Analysis of cleavage pattern of fibrinogen by MMF. (A) MMF (1 μg) was incubated with 140 μg of fibrinogen at 37℃ for 20 min and then separated on 12% SDS-polyacrylamide gel. An arrow on the bottom of gel indicates a band used for N-terminal sequencing to reveal the cleavage site for MMF. (B) Amino acid sequence of fibrinogen in part and the cleavage site of MMF (An arrow indicates the cleavage site).

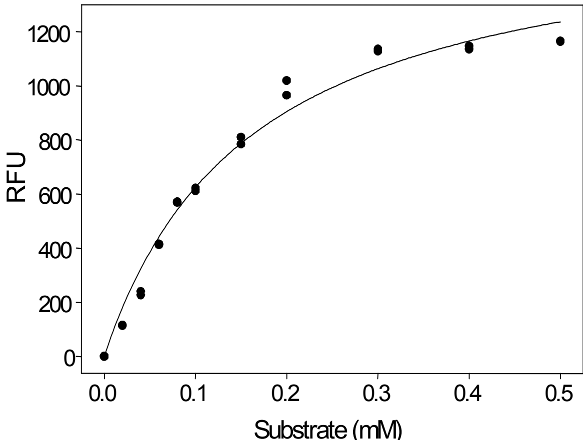


Fig. 9. Fluorogenic assay with MMF. MMF (2 μg) and various concentrations (0 to 0.5 mM) of the fluorogenic peptide substrate were separately pre-incubated for 10 min at 37℃ and then mixed together. Fluorescence was monitored at λ_{ex}=320 nm and λ_{em}=435 nm for further 15 min.

Table 3. Kinetic values of MMF

V_{max} (M/min)	K_M(M/L)	Turnover rate
2.33×10^{-7}	1.5×10^{-2}	0.31

III-3-2. Effect of divalent ions and protease inhibitors on MMF enzyme activity

The enzyme activity of MMF was inhibited by metalloproteinase inhibitors including EDTA, 1,10-phenanthroline (1,10-PT), and EGTA. However, the inhibitors of serine protease such as TLCK and TPCK had no effect on the activity. These results suggest that MMF may be a metalloproteinase. A reducing reagent DTT also completely inhibited the enzyme activity of MMF, suggesting that the reduction of disulfide bonds in MMF diminished the activity (Fig. 10 and Table 4). Some divalent ions such as Ca^{2+} and Mg^{2+}, in particular Zn^{2+} has been regarded as an important factor in snake venom proteins. Therefore, effects of the divalent cations on the enzyme activity of MMF were examined (Fig. 11 and Table 5). The enzyme activity of MMF was inhibited by Cu^{2+}, Ni^{2+}, and Zn^{2+}, but other divalent ions had no effect.

It has been reported that ussurenase from *Agkistrodon blomhoffii ussuresis* venom (Sun, Liu and Greenaway, 2006) and Lebetase from *Vipera lebetina* venom (Siigur *et al*., 1998) are not affected by Mg^{2+} and Ca^{2+} on their enzyme activities, while Cu^{2+} inhibit them. However, BmooMP-I from *Bothrops moojeni* venom (Bernardes *et al*., 2008), patagonfibrase from *Philodryas patagoniensis* venom (Peichoto *et al*., 2007) and halysase from *G. hays* (You *et al*., 2006) require Ca^{2+} for their activities.

III-3-3. Effects of pH and temperature on MMF activity

The general proteolytic activity of MMF was analyzed by fluorogenic peptide assay, as previously described. As shown in Fig. 12, the optimal pH for the enzyme activity of MMF was around 5.5. The stable range of pH for

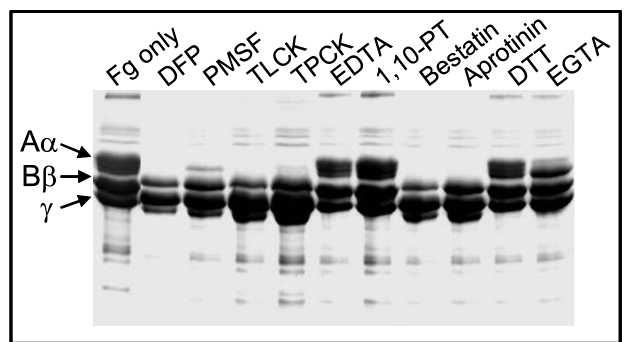


Fig. 10. Effect of various inhibitors on fibrinogenolytic activity of MMF. MMF was pre-incubated with or without the inhibitors at 37℃ for 10 min. The reaction samples were electrophoresed on 12% SDS-polyacrylamide gel. Fg, Fibrinogen.

Table 4. Effect of various inhibitors on the enzyme activity of MMF

Additive	Activity (%)
Control	100
DFP	72.7
PMSF	27.3
TLCK	103.5
TPCK	99.7
EDTA	0
1,10-PT	1.2
Bestatin	101.8
Aprotinin	97.9
DTT	0
EGTA	3.1

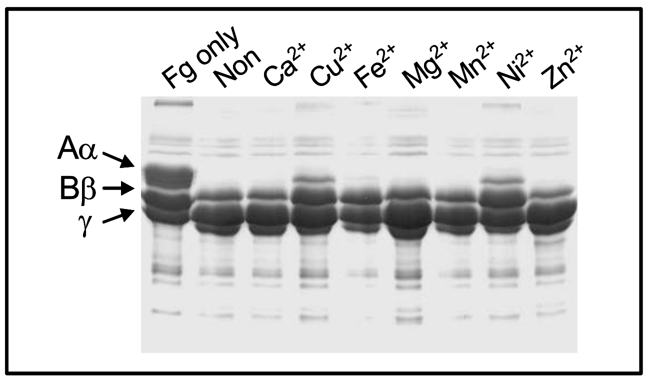


Fig. 11. Effect of various divalent ions on fibrinogenolytic activity of MMF. MMF was pre-incubated with or without divalent ions at 37℃ for 10 min. The reaction samples were electrophoresed on 12% SDS-polyacrylamide gel. Fg, Fibrinogen; Non, non-treated.

Table 5. Effect of various divalent ions on the enzyme activity of MMF

Additive	Activity (%)
Control	100
Ca^{2+}	101.8
Cu^{2+}	17.6
Fe^{2+}	88.6
Mg^{2+}	98.2
Mn^{2+}	104.1
Ni^{2+}	43.2
Zn^{2+}	91.8

the enzyme activity was a little acidic condition from 5.5 to 6.5. This differs from other SVMPs that have stable pH at usually natural or a little alkalic conditions (Bernardes *et al*., 2008; Siigur *et al*., 1998; Sun *et al*., 2006).

MMF exhibited its maximal activity in the temperature ranging from 25 to 40℃ (Fig. 13). The enzyme activity of MMF dropped sharply under alkaline conditions or higher temperature, with a completely loss of activity at pH 10.5 or above 60℃.

III-3-4. Vascular permeability induced by MMF

Vascular permeability characterized by the movement of fluids and molecules from blood stream to the extravascular space is integral for various biological processes. Generally, vascular leakage is enhanced by serveral effectors such as inflammatory mediators, vascular endothelial growth factor (VEGF), or vascular basemembrane (Yamazaki et al., 2007). One of these, vascular basemembrane comprised several components including laminin, type IV collagen, and fibronectin provides stability of the vascular structure (Braud *et al.*, 2000, Norris, 2003). Miles assay has been often used for examining vascular permeability. Briefly, a dye such as Evan's blue that binds noncovalently to albumin is injected intravenously and its accumulation is measured at some later time at a skin test site. Permeability is defined as the amount of albumin-dye complex after Evan's blue injection (Nagy *et al.*, 2008).

The Miles assay showed a leakage spot of Evan's blue at skin 10 minutes later by 10 μg of MMF injection (Fig. 14A) and the leakage amount of dye could be determined about 8 μg (Fig. 14B).

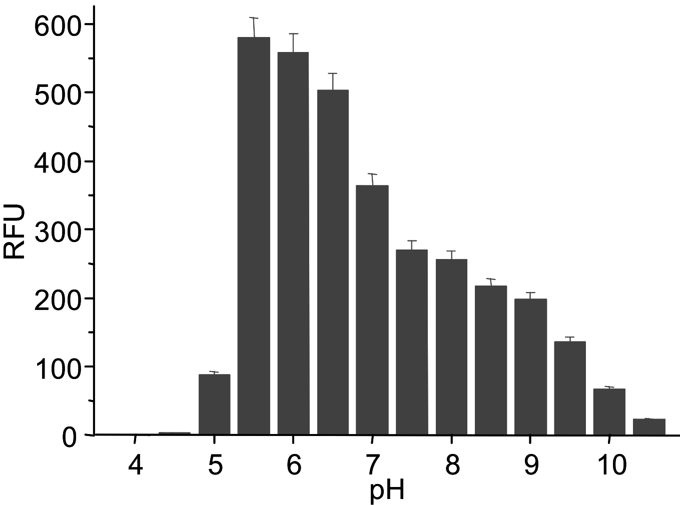


Fig. 13. Effect of pH on the fluorogenic activity of MMF. Fluorogenic activity was assayed with fluorogenic peptide substrate (80 nM) as described previously at various pHs using the buffer systems as described in Materials and Methods.

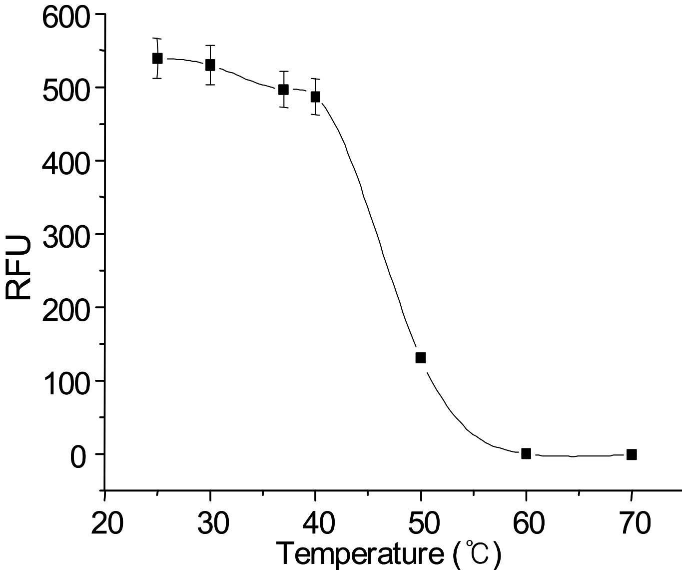
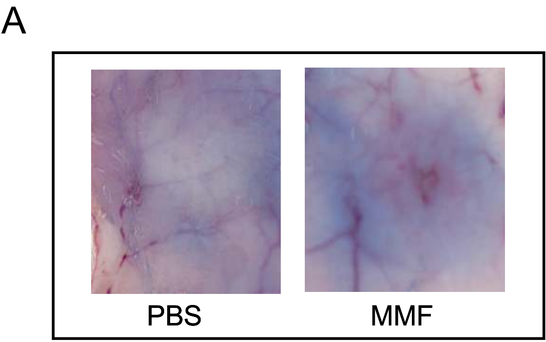


Fig. 14. Effect of temperature on the fluorogenic activity of MMF. MMF was incubated at various temperatures ranging from 25 to 70℃ for 30 min in 20 mM sodium acetate buffer (pH 5.5). The fluorogenic activity was assayed using fluorogenic peptide substrate as descried previously.

A



B

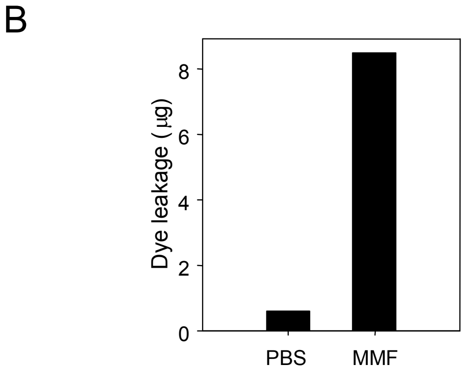


Fig. 15. Vascular permeability induced by MMF. MMF was injected intradermally into the back skin of guinea pig. After 10 min, the guinea pig was scarified and the skin was carefully removed to observe a vascular permeability. For quantification of dye leakage, the spot was excised and incubated with formamide and the amount of extracted dye was determined by measuring the absorbance at 620 nm.

Furthermore, MMF could digest type IV collagen, which is a major component of the basemembrane, within 5 h (Fig. 15). These results suggest that increase of vascular permeability induced by MMF may be related with its cleavage of type IV collagen.

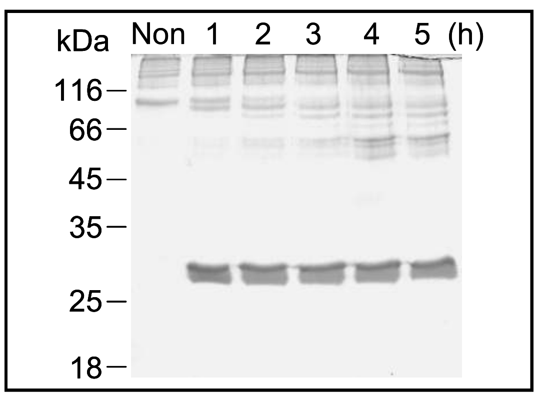


Fig. 16. Cleavage of type IV collagen by MMF. Type IV collagen was incubated with MMF at 37℃ for various time periods (1, 2, 3, 4, and 5 h) and analyzed by SDS-PAGE on 12% gel under reducing condition. Non, collagen only.

IV. 적 요

밤독으로부터 피브리노겐을 분해하는 단백질

분해효소의 분리 및 특성 분석

이 은 희

지도교수 : 이 정 섭

조선대학교 대학원

생명공학과

뱀독은 항 응고 작용과 피브리노겐을 분해하는 활성을 지닌 여러가지 펩타이드 및 효소를 함유하고 있다. 본 연구에서는 *Macrovipera mauritanica* 뱀독으로부터 피브리노겐을 분해하는 단백질 분해효소를 정제하고 그 특성을 분석하였다. 이 분해효소의 정제에는 Superdex 75, Source Q 및 Mono Q 칼럼 등 세 단계의 크로마토그래피를 사용하였으며 정제한 효소를 MMF라 명명하였다. MMF는 단일 폴리펩타이드로 구성되었으며 약 27 kDa의 분자량을 가지고 있었다. MMF의 N-말단 아미노산 서열은 "NH_2-QRFAPRYIEL-COOH"였다. 여러가지 단백질을 기질로 사용한 결과, MMF는 피브리노겐을 가장 효과적으로 절단하는 단백질 분해효소였다. MMF는 피브리노겐의 알파와 베타사슬을 각각 20분과 8시간내에 모두 절단하는 특성을 지니고 있었다. 그러나 피브리노겐의 감마사슬은 MMF에 의해 절단되지 않았는데 이는 대부분의 뱀독 유래 피브리노겐 분해 단백질효소가 지니는 공통의 특성이다. 트롬빈과 혈액응고인자인 FXIIIa를 이용하여 교차연결시킨 피브린 또한 MMF에 의해 절단되었는데, 이 경우에서도 γ-γ 사슬은 MMF에 의해 절단되지 않았다. MMF가 절단된 약 17.5 kDa 크기의 피브리노겐 펩타이드의 N-말단 아미노산 서열을 분석한 결과,

MMF는 Lys^{413}과 Leu^{414}를 연결하는 펩타이드 결합을 가수분해함을 확인하였다. MMF의 이 절단자리를 포함하는 아미노산 서열을 이용하여 Abz-His-Thr-Glu-Lys-Leu-Val-Thr-Ser-Lys-Dnp-NH_2 서열을 지닌 형광성 펩타이드 기질을 합성하였다. 이 펩타이드 기질을 이용하여 MMF의 효소활성을 분석한 결과, K_M값은 1.5×10^{-2} M/L였으며 분자활성은 0.31이었다. MMF의 효소 활성을 위한 최적의 pH는 약 5.5였으며, 최적 온도는 30℃였다. MMF의 효소 활성은 1.10-PT, DTT 및 EDTA에 의해 억제 되지만 TLCK, bestatin 및 aprotinin에 의해서는 억제되지 않았다. 이러한 결과는 MMF는 금속-함유 단백질 분해효소이며 MMF내의 이황화결합이 효소활성에 중요한 역할을 하고 있음을 제시하는 것이다. 기니피그를 이용한 혈관투과성 유도 특성을 분석한 결과, 10 μg의 MMF는 10분 후 혈관 투과성을 증가시켰으며, 유출된 색소양은 약 8 μg이었다. MMF는 또한 세포간질을 구성하는 콜라겐 IV를 분해하는 효소활성을 지니고 있었다. 이와 같은 결과는 MMF가 콜라겐 IV를 분해하여 혈관투과성을 증가시킬 가능성을 시사하는 것이다. 본 연구에서 얻은 이상의 결과들은 뱀독 유래의 MMF는 피브리노겐을 선택적으로 분해하는 금속-함유 단백질 분해효소임을 보여주는 것이다.

V. References

Aaspollu, A., Siigur, J. and Siigur, E. (2005) cDNA cloning of a novel P-I lebetase isoform Le-4. *Toxicon* **46,** 591-594.

Bello, C.A., Hermogenes, A.L., Magalhaes, A., Veiga, S.S., Gremski, L.H., Richardson, M. and Sanchez, E.F. (2006) Isolation and biochemical characterization of a fibrinolytic proteinase from *Bothrops leucurus* (white-tailed jararaca) snake venom. *Biochimie* **88,** 189-200.

Berger, M., Pinto, A.F. and Guimaraes, J.A. (2008) Purification and functional characterization of bothrojaractivase, aprothrombin-activating metalloproteinase isolated from *Bothrops jararaca* snake venom. *Toxicon* **51,** 488-501.

Bernardes, C.P., Santos-Filho, N.A., Costa, T.R., Gomes, M.S., Torres, F.S., Costa, J.*, et al.* (2008) Isolation and structural characterization of a new fibrin(ogen)olytic metalloproteinase from *Bothrops moojeni* snake venom. *Toxicon* **51,** 574-584.

Braud, S., Bon, C. and Wisner, A. (2000) Snake venom proteins acting on hemostasis. *Biochimie* **82,** 851-859.

Chang, A.K., Kim, H.Y., Park, J.E., Acharya, P., Park, I.S., Yoon, S.M.*, et al.* (2005) *Vibrio vulnificus* secretes a broad-specificity metalloprotease capable of interfering with blood homeostasis through prothrombin activation and fibrinolysis. *J Bacteriol* **187,** 6909-6916.

Escalante, T., Franceschi, A., Rucavado, A. and Gutierrez, J.M. (2000) Effectiveness of batimastat, a synthetic inhibitor of matrix metalloproteinases, in neutralizing local tissue damage induced by BaP1, a hemorrhagic metalloproteinase from the venom of the snake

bothrops asper. *Biochem Pharmacol* **60,** 269-274.

Escalante, T., Rucavado, A., Kamiguti, A.S., Theakston, R.D. and Gutierrez, J.M. (2004) Bothrops asper metalloproteinase BaP1 is inhibited by alpha(2)-macroglobulin and mouse serum and does not induce systemic hemorrhage or coagulopathy. *Toxicon* **43,** 213-217.

Escalante, T., Shannon, J., Moura-da-Silva, A.M., Gutierrez, J.M. and Fox, J.W. (2006) Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. *Arch Biochem Biophys* **455,** 144-153.

Fernandes, C.M., Zamuner, S.R., Zuliani, J.P., Rucavado, A., Gutierrez, J.M. and Teixeira Cde, F. (2006) Inflammatory effects of BaP1 a metalloproteinase isolated from *Bothrops asper* snake venom: leukocyte recruitment and release of cytokines. *Toxicon* **47,** 549-559.

Fox, J.W. and Serrano, S.M. (2005) Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon* **45,** 969-985.

Gasmi, A., Chabchoub, A., Guermazi, S., Karoui, H., Elayeb, M. and Dellagi, K. (1997) Further characterization and thrombolytic activity in a rat model of a fibrinogenase from *Vipera lebetina* venom. *Thromb Res* **86,** 233-242.

Gasmi, A., Srairi, N., Guermazi, S., Dekhil, H., Karoui, H. and El Ayeb, M. (2001) Amino acid structure and characterization of a heterodimeric disintegrin from *Vipera lebetina* venom. *Biochim Biophys Acta* **1547,** 51-56.

Gasmi, A., Srairi, N., Karoui, H. and El Ayeb, M. (2000) Amino acid

sequence of VIF: identification in the C-terminal domain of residues common to non-hemorrhagic metalloproteinases from snake venoms. *Biochim Biophys Acta* **1481,** 209-212.

Gutierrez, J.M., Romero, M., Diaz, C., Borkow, G. and Ovadia, M. (1995) Isolation and characterization of a metalloproteinase with weak hemorrhagic activity from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **33,** 19-29.

Leonardi, A., Fox, J.W., Trampus-Bakija, A. and Krizaj, I. (2007) Ammodytase, a metalloprotease from *Vipera ammodytes ammodytes* venom, possesses strong fibrinolytic activity. *Toxicon* **49,** 833-842.

Loria, G.D., Rucavado, A., Kamiguti, A.S., Theakston, R.D., Fox, J.W., Alape, A. and Gutierrez, J.M. (2003) Characterization of 'basparin A,' a prothrombin-activating metalloproteinase, from the venom of the snake *Bothrops asper* that inhibits platelet aggregation and induces defibrination and thrombosis. *Arch Biochem Biophys* **418,** 13-24.

Markland, F.S., Jr. (1998) Snake venom fibrinogenolytic and fibrinolytic enzymes: an updatedinventory. Registry of Exogenous Hemostatic Factors of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost* **79,** 668-674.

Nagy, J.A., Benjamin, L., Zeng, H., Dvorak, A.M. and Dvorak, H.F. (2008) Vascular permeability, vascular hyperpermeability and angiogenesis. *Angiogenesis* **11,** 109-119.

Norris, L.A. (2003) Blood coagulation. *Best Pract Res Clin Obstet Gynaecol* **17,** 369-383.

Peichoto, M.E., Teibler, P., Mackessy, S.P., Leiva, L., Acosta, O., Goncalves,

L.R., *et al.* (2007) Purification and characterization of patagonfibrase, a metalloproteinase showing alpha-fibrinogenolytic and hemorrhagic activities, from *Philodryas patagoniensis* snake venom. *Biochim Biophys Acta* **1770,** 810-819.

Rodrigues, V.M., Soares, A.M., Guerra-Sa, R., Rodrigues, V., Fontes, M.R. and Giglio, J.R. (2000) Structural and functional characterization of neuwiedase, a nonhemorrhagic fibrin(ogen)olytic metalloprotease from *Bothrops neuwiedi* snake venom. *Arch Biochem Biophys* **381,** 213-224.

Rucavado, A., Lomonte, B., Ovadia, M. and Gutierrez, J.M. (1995) Local tissue damage induced by BaP1, a metalloproteinase isolated from *Bothrops asper* (Terciopelo) snake venom. *Exp Mol Pathol* **63,** 186-199.

Saidi, N., Samel, M., Siigur, J. and Jensen, P.E. (1999) Lebetase, an alpha(beta)-fibrin(ogen)olytic metalloproteinase of *Vipera lebetina* snake venom, is inhibited by human alpha-macroglobulins. *Biochim Biophys Acta* **1434,** 94-102.

Samel, M., Subbi, J., Siigur, J. and Siigur, E. (2002) Biochemical characterization of fibrinogenolytic serine proteinases from *Vipera lebetina* snake venom. *Toxicon* **40,** 51-54.

Siigur, E., Aaspollu, A., Tu, A.T. and Siigur, J. (1996) cDNA cloning and deduced amino acid sequence of fibrinolytic enzyme (lebetase) from *Vipera lebetina* snake venom. *Biochem Biophys Res Commun* **224,** 229-236.

Siigur, J., Aaspollu, A., Tonismagi, K., Trummal, K., Samel, M., Vija, H., *et al.* (2001) Proteases from *Vipera lebetina* venom affecting coagulation and fibrinolysis. *Haemostasis* **31,** 123-132.

Siigur, J., Samel, M., Tonismagi, K., Subbi, J., Siigur, E. and Tu, A.T. (1998) Biochemical characterization of lebetase, a direct-acting fibrinolytic enzyme from *Vipera lebetina* snake venom. *Thromb Res* **90,** 39-49.

Stroka, A., Donato, J.L., Bon, C., Hyslop, S. and de Araujo, A.L. (2005) Purification and characterization of a hemorrhagic metalloproteinase from *Bothrops lanceolatus* (Fer-de-lance) snake venom. *Toxicon* **45,** 411-420.

Sun, M.Z., Liu, S. and Greenaway, F.T. (2006) Characterization of a fibrinolytic enzyme (ussurenase) from *Agkistrodon blomhoffii ussurensis* snake venom: insights into the effects of Ca^{2+} on function and structure. *Biochim Biophys Acta* **1764,** 1340-1348.

Swenson, S. and Markland, F.S., Jr. (2005) Snake venom fibrin(ogen)olytic enzymes. *Toxicon* **45,** 1021-1039.

Trummal, K., Vija, H., Subbi, J.and Siigur, J. (2000) MALDI-TOF mass spectrometry analysis of substrate specificity of lebetase, a direct-acting fibrinolytic metalloproteinase from *Vipera lebetina* snake venom. *Biochim Biophys Acta* **1476,** 331-336.

Watanabe, L., Shannon, J.D., Valente, R.H., Rucavado, A., Alape-Giron, A., Kamiguti, A.S., *et al.* (2003) Amino acid sequence and crystal structure of BaP1, a metalloproteinase from *Bothrops asper* snake venom that exerts multiple tissue-damaging activities. *Protein Sci* **12,** 2273-2281.

White, J. (2005) Snake venoms and coagulopathy. *Toxicon* **45,** 951-967.

Yamazaki, Y., Nakano, Y., Imamura, T. and Morita, T. (2007) Augmentation of vascular permeability of VEGF is enhanced by KDR-binding proteins. *Biochem Biophys Res Commun* **355,**693-699.

You, W.K., Jang, Y.J., Chung, K.H., Jeon, O.H. and Kim, D.S. (2006) Functional roles of the two distinct domains of halysase, a snake venom metalloprotease, to inhibit human platelet aggregation. *Biochem Biophys Res Commun* **339,** 964-970.

감사의 글

지금와서 생각해보면, 학부 시절에 어떤 꿈을 가지고 들어왔는지 잊고 지낼 정도로 정신없이 여기까지 왔다는 생각이 듭니다. 하지만 저에게 학부때부터 4년이란 시간은 나 자신을 끊임없이 돌아보게 하였던 짧지만은 않은 시간들이었습니다. 오지 않을 것 처럼 멀게만 느껴지던 졸업을 하게 되니, 항상 옆에서 지켜봐주던 많은 지인들이 떠오릅니다.

제가 학부 시절때 이정섭 교수님만 보고 이 실험실을 들어오고 싶은 마음에 교수님을 찾아뵈었을때 교수님이 '너가 나를 찾아올지는 몰랐구나' 라는 말씀이 지금도 생생하게 기억이 납니다. 근데 벌써 이렇게 졸업을 한다니, 감회가 새롭습니다. 부모님이외에 저에게 따뜻한 조언들과 가끔은 따끔한 가르침들이 저에게 얼마나 많은 도움이 되었는지 모르겠습니다. 너무 감사드립니다. 이정섭 교수님..^^ 저에게 틈틈이 좋은 말씀 주시는 전홍성 교수님, 박윤경 교수님, 모든 생명공학과 교수님들께 감사하다는 말씀드리고 싶습니다.

실험실 생활이 홀로 하는 것이 었다면, 많은 외로움과 힘겨움이 있었을텐데, 지금 실험실 멤버들이 있어 많은 힘이 되었습니다. 항상 많은 짐을 어깨에 매고 있으면서도 후배를 챙기고 미소를 잃지 않은 정은언니, 제가 막막해서 주저앉아 있으면 옆에와서 항상 챙겨줘서 너무 고마워요.!! 그리고 저의 석사 동기들..어디 학회나 연수를 갈 때 항상 세트로 갔던 종우오빠ㅋㅋ, 학부때부터 계속 같이 생활하면서 위안이 될 때가 많았어요. 이제는 오빠도 조금은 외도(?)하는 모습을 보고 싶네요^^, 그리고 또 한명의 동기, 으리씨^^, 참 말이 필요없는 동기..답답하고 짜증날 때 둘이 커피마시면서 얘기하면서 풀고, 재미난일 있을 때 같이 왁자지껄 웃어주고, 힘든 일 있을때 묵묵히 서로 지켜봐주었던 동기이자 친구인 으리야. 고맙고, 항상 힘내라!!. 지금 현재 우리 실험실의 막둥이 재영오빠..한숨쟁이..ㅋㅋ. 우리 실험실에서 어울리지(?) 않는 귀여움과 넉살을 부리시는 오빠 덕에 웃을때가 많아서 좋았어요. 지금은 다른데로 떠나고 없지만, 실험을 많이 가르쳐줬던 Alan박사님, 남희언니, 그리고 잠시 잠깐이었지만 기억에 남는 진선 선생님 모두들 감사했습니다. 실험실 멤버이외에도 많은 과 선

배들과 동기들 모두가 어울러 있어서 재미난 일들이 있었던것 같습니다.

현재 졸업에 이르기 까지 힘든일도 많았고, 고심한적도 많이 있었는데 그때마다 옆에 있는 친구들이 얼마나 힘이 되는지 알게되었던 것 같습니다. 대학교 1학년 때부터 저랑 죽마고우였던 성희씨~^^ 항상 내 얘기 한없이 들어주고 얘기해주고 이제는 옆에 너같은 친구가 있다는 존재감이 문득 외로울때 힘이 나게하더라. 고마운거 알지?? 보내기 싫지만 결혼해서 행복하게 살아야 된다!!. 같은 길을 걸어가고 있어 많은 동질감이 형성되고 위안이 되었던 우리 바게지니!!너랑 같이 졸업해서 좋다. 우리같이 성공해보자! 같은 광주에 없지만 내 마음속에 늘 그 자리에 있는 연경, 형래, 경원이, 그리고 가끔씩 연락해서 힘내라고 응원해주는 고등학교 동창들 보라, 에리, 수미 모두 고맙다.

마지막으로, 이들을 생각하면 마음이 따뜻해지고 편안해지는, 그리고 항상 내편을 들어주는 가족이 있어 저는 늘 행복한 사람이라고 생각합니다. 처음에 이쪽과를 선택했을때 전망이 없다고 몇 년동안 반대하셨던 그러나 지금은 우리집에도 자랑거리 하나있어야 된다고 누구보다도 저를 지지해주시는 우리 아빠. 저 막둥이로 놓으셔서 한갑이 벌써지나셨는데도 불구하고 저한테만은 부족함없이 할려고 지원해주는 거 말은 안하지만 항상 감사하게 생각합니다. 무슨일이 생겨도 항상 무조건적으로 내편을 들어주고 이해해주는 우리 엄마, 너무 사랑합니다.!! 매일 집에 갈때마다 전화하면 한없이 한풀이 들어주고, 힘내라고 응원해주었던 우리 큰언니, 그리고 묵묵히 지켜보면서 막내 동생이라고 아껴주는 우리 작은언니, 오빠. 다들 항상 고마워~^^.

저는 지금 작은 언덕하나 넘었을 뿐이라고 생각합니다. 앞으로도 항상 당당한 모습과 큰 포부를 가지는 여성으로 거듭나기 위해 노력하겠습니다.

저작물 이용 허락서

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논문제목	한글 : 뱀독으로부터 피브리노겐을 분해하는 단백 질 분해효소의 분리 및 특성 분석				
	영어 : **Isolation and characterization of a fibrinogenolytic enzyme from *Macrovipera mauritanica* snake venom**				

가. 본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(   ) 반대(   )

2008년 12월 일

저작자 : 이 은 희 (서명 또는 인)

조선대학교 총장 귀하