J. Toxicol. Pub. Health
Vol. 17, Supplement, pp. 97-104, 2001

Biochemical Characterization of Serine Proteases with Fibrinolytic Activity from *Tenodera sinensis* (Praying Mantis)

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ABSTRACT *: Three types of proteases (MEF-1, MEF-2 and MEF-3) were purified from the egg cases of Tenodera sinensis using ammonium sulfate fractionation, gel filtration on Bio-Gel P-60 and affinity chromatography on DEAE Affi-Gel blue gel. The proteases were assessed homogeneous by SDS-polyacrylamide gel electrophoresis and have molecular weight of 31,500, 32,900 and 35,600 Da, respectively. The N-terminal regions of the primary structure were compared and they were found to be different each other. MEFs readily digested the Aα - and Bβ-chains of fibrinogen and more slowly the γ-chain. The action of the enzymes resulted in extensive hydrolysis of fibrinogen and fibrin, releasing a variety of fibrinopeptides. MEF-1 was inactivated by Cu^{2+} and Zn^{2+} and inhibited by PMSF and chymostatin. MEF-2 was inhibited by PMSF, TLCK, soybean trypsin inhibitor. MEF-3 was only inhibited by PMSF and chymostatin. Antiplasmin was not sensitive to MEF-1 but antithrombin III inhibited the enzymatic activity of MEF-1. MEF-2 specifically bound to antiplasmin Among the chromogenic protease substrates, the most sensitive one to the hydrolysis of MEFs was benzoyl-Phe-Val-Arg-p-nitroanilide with maximal activity at pH 7.0 and 30°C. MEF-1 preferentially cleaved the oxidized B-chain of insulin between Leu15 and Tyr16. In contrast, MEF-2 specifically cleaved the peptide bond between Arg23 and Gly24. D-dimer concentrations increased on incubation of cross-linked fibrin with MEF-1, indicating the enzyme has a strong fibrinolytic activity.*

Key Words *: Serine protease, Fibrinolytic activity, Purification, Properties, Tenodera sinensis*

I. INTRODUCTION

Many antithrombotic, anticoagulant and fibrinolytic agents have been searched from natural sources including mammals and invertebrates. Insects are the most abundant species in the world. Mantids are a family of insects in the order Orhtoptera, or crickets, grasshoppers, roaches, and mantids. Mantids egg cases have been used for the purpose of strengthening the kidney function in the traditional medicines. We found a very potent fibirinolytic activity from the egg cases of mantids. Peptide bond cleavage is one of the most frequent and important enzymatic modifications of proteins. Recent studies of proteolytic enzymes have focused on their regulatory roles in a variety of physiological processes. Among the most thoroughly studied regulatory proteases are those associated with coagulation, fibrinolysis and the complement system (Neurath, 1989).

Fibrinolysis dissolves existing thrombi through the action of plasmin on fibrin (Walker *et al.*, 1985). Over the last ten years, thrombolytic therapies have played major roles in the early treatment of myocardial infarction. More effective thrombolytic agents have been identified and characterized from vampire bat (Cartwright, 1974; Gardell *et al.*, 1989), snake venoms (Bajwa *et al.*, 1982; Siigur and Siigur, 1991; Zhang *et al.*, 1995), microorganisms (Fujita *et al.*, 1993; Kim *et al.*, 1996) and earthworm (Nakajima *et al.*, 1993; Mihara *et al.*, 1993) marking a new era in the early treatment of heart attack. Recently, Wang *et al.* demonstrated the presence of fibrinolytic activity in the egg cases of the praying mantis Tenodera sinensis (Wang *et al.*, 1989). Any specific component has yet to be attributed to its biochemical properties.

In the present study, we have identified, purified and characterized the fibrinolytic protease, mantis-egg

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Abbreviations: E64: L-carboxy-trans-2,3-epoxypropyl-leucylamido(4-guanidino)butane, EDTA: ethylene diamine tetraacetic acid, EGTA: ethylene glycol tetraacetic acid, PITC: phenylisothiocyanate, PMSF: phenylmethanesulfonyl fluroide, RP-HPLC: reversed phase-high performance liquid chromatography, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TFA: trifluroacetic acid, TLCK: tosyl-lysine chloromethylketone, TPCK: tosylamido-2-phenylethyl chloromethyl ketone.

fibrolase (MEF) from the egg cases of *Tenodera sinensis*, including proteolytic specificity and fibrin(ogen)olytic activity.

II. MATERIALS AND METHODS

1. Chemicals

The mantis egg cases were purchased at a local market in Beijing, China. Bio-Gel P-60 (medium) and DEAE Affi-Gel blue gel was purchased from Bio-Rad (Hercules, CA). Human thrombin, bovine fibrinogen, PMSF, soybean trypsin inhibitor, aprotinin, benzamidine and the chromogenic protease substrates, benzoyl-Pro-Phe-Arg-*p*-nitroanilide, benzoyl-Phe-Val-Arg-*p*-nitroanilide, benzoyl-Gly-Pro-*p*-nitroanilide, tosyl-Gly-Pro-Arg-*p*-nitroanilide, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, boc-Ala-Ala-Pro-Ala-*p*-nitroanilide, tosyl-Gly-Pro-Lys-*p*-nitroanilide and boc-Gly-Gly-Leu-*p*-nitroanilide were from Sigma Chemicals (St. Louis, MO). Chymostatin, elastatinal, E64, TPCK, TLCK, human fibrinogen (plasminogen free) and antiplasmin were from Calbiochem (La Jolla, CA). Antithrombin III was from Chromogenix AB (M–lndal, Sweden).

2. Purification of the Proteases

MEFs were isolated by a combination of ammonium sulfate fractionation, gel filtration, and affinity chromatography. The egg case powder (240 g) was suspended in 2.5 *l* of 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and any insoluble material was removed by centrifugation at 8,000 × g for 30 min at 4°C. Ammonium sulfate was added to the supernatant up to 60%, centrifuged and the supernatant was decanted away from the pellet. Ammonium sulfate was again added up to 90%, centrifuged and the supernatant decanted away. The resulting precipitate was suspended in 15 m*l* of 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl. The resulting solution was applied on a Bio-Gel P-60 column (83×3 cm) equilibrated with 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.5) and the column was eluted with the same buffer at a flow rate 11 m*l*/h. Fractions showing optimal fibrinolytic activity were pooled, dialyzed overnight against 50 mM Tris-HCl containing 10 mM NaCl, pH 7.4 at 4°C. The sample was concentrated using a Diaflo UM 10 from Amicon Co (Beverly, CA) and then loaded onto a DEAE Affi-Gel blue gel chromatography column (7.8×2.5 cm) equilibrated with the same buffer. The non-interacting solutes were washed from the column with equilibration buffer and the bound fraction was eluted using a linear sodium chloride gradient from 10 to 200 mM in Tris-HCl (pH 7.4) at a flow rate of 11.3 m*l*/h. Fractions showing maximal fibrinolytic activity were pooled and concentrated.

3. Fibrinolytic Assay and Protein Determination

Fibrinolytic activity was assessed by applying a 10 m*l* sample to a fibrin plate generated by thrombin-mediated polymerization. Samples were allowed to incubate for 17 hours at 37°C and the activity was quantified by measuring the area of lysis on the plate and converting to plasmin unit (Astrup and Mullertz, 1952). Protein concentration was determined using the method of Bradford (Bradford, 1976).

4. Determination of Molecular Weight and Isoelectric Point of MEFs

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970) using a 4% stacking and 10% resolving polyacrylamide gel. Isoelectric focusing was performed using a Bio-Rad's Model 111 Mini IEF Cell (Hercules, CA) according to manufacturer's procedure. Gels were visualized with either Coomassie Brilliant Blue R250 or silver staining.

5. Determination of N-terminl Amino Acid Sequence and Amino Acid Composition Analysis of MEF

The *N*-terminal amino acid sequence of purified MEFs was determined using an Applied Biosystems Procise 491 amino acid sequencer at the Korea Basic Science Center in Seoul. Compositional analysis was carried out by first derivatization of the amino acids with phenylisothiocyanate (PITC) followed by RP-HPLC (Williams *et al.*, 1998).

6. Proteolytic Activity Assay

Proteolytic activity was measured using azocasein

as substrate according to previously described methods (Beynon and Kay, 1978). The reaction mixture, composed of 1 ml of azocasein (2 mg/ml in 0.2 M sodium borate buffer, pH 7.8) and 1 μg of MEF, was incubated at 37°C. After 1 hr, 0.25 ml of the mixture was transferred to a 1.5 ml tube containing 1 ml of 5% (w/v) trichloroacetic acid and mixed. The tubes were then centrifuged at 11,000 × g for 5 min and the absorbance of the supernatant was measured at 340 nm.

7. Effects of Divalent Metal Cations and Inhibitors

MEFs (1 μg) was preincubated with : 5 mM of Ca^{2+}, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, 10 mM benzamidine, 5 mM EDTA, 5 mM EGTA, 5 mM cystein, 0.02, 0.2 and 2 mM PMSF, 10 mM β-mercaptoethanol, 0.01 and 0.05 mM iodoacetate, 0.03 and 0.3 mM E64, 50 μM soybean trypsin inhibitor, 50 μM aprotinin, 0.1 mM TPCK, 0.1 mM TLCK, 0.001, 0.05 and 0.1 mM elastatinal, and 0.001, 0.05 or 0.1 mM chymostatin, 1 μM antiplasmin, and 0.1 μM antithrombin III at 37°C for 2 hours. The residual enzyme activity was determined using the azocasein assay.

8. Characterization of Proteolytic Specificity

Oxidized insulin B chain (0.5 mg/ml) was incubated with MEF (3 μg) at 37°C in 50 mM Tris-HCl buffer, pH 7.4. At various time intervals, aliquots were withdrawn from the digestion mixture, heated at 100°C for 5 min and then analyzed by RP-HPLC. It was performed using C_8 HPLC column from Applied Biosystems (San Jose, CA) using a linear gradient from 5% to 75% acetonitrile in water, containing 0.05% trifluoroacetic acid at a flow rate 1 ml/min for 40 min. The absorbance of the effluent was detected at 210 nm. Each peak was collected, hydrolyzed in 6 M HCl for 24 hr at 110 °C and analyzed for amino acids.

9. Amidolytic Activities on Chromogenic Substrates

Amidolytic activities were measured spectrophotometrically using the chromogenic protease substrates, benzoyl-Pro-Phe-Arg-*p*-nitroanilide, benzoyl-Phe-Val-Arg-*p*-nitroanilide, benzoyl-Gly-Pro-*p*-nitroanilide, tosyl-Gly-Pro-Arg-*p*-nitroanilide, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, boc-Ala-Ala-Pro-Ala-*p*-nitroanilide, tosyl-Gly-Pro-Lys-*p*-nitroanilide and boc-Gly-Gly-Leu-*p*-nitroanilide. Activities were tested by mixing MEF [1 μg/200 μl of 50 mM Tris-HCl (pH 7.4)] with 300 μl of a 0.5 mM substrates. After continuous measurement for 5 min at 37°C with a temperature regulated spectrophotometer, the amount of *p*-nitroaniline released was determined by measuring the change in absorbance at 405 nm ($\varepsilon = 9.65 \times 10^3\ M^{-1} \cdot cm^{-1}$, Tris-HCl, pH = 7.4).

10. Analysis of Degradation Products of Fibrinogen

Fibrinogenolytic activity was tested by incubating 200 μl of human fibrinogen (1 mg/ml) in 40 mM Tris-HCl, pH 7.4 containing 0.1 M NaCl with 1 μg of MEF at 37°C. At various time intervals (0.5, 1, 3, 5, 10, 20 and 30 min) aliquots were taken from the reaction mixture and mixed with an equal volume of sample buffer containing β-mercaptoethanol, boiled and then electrophoresed (Laemmli, 1970). Alternatively, 6 ml of fibrinogen (10 mg/ml in 50 mM Tris buffer, pH 7.4) was incubated at 37°C with 1 ml of either MEF (6 μg/ml), plasmin (0.1 U/ml) or thrombin (25 U/ml). At various time intervals, aliquots of each incubation mixture of MEF and plasmin were boiled for 5 min and then filtered using a 0.45 μm filter. The mixture containing thrombin was centrifuged at 250 × g for 30 min and the supernatant was filtered. Degradation products of fibrinogen/fibrin produced by MEF, plasmin or thrombin were compared using RP-HPLC. Chromatography was performed using a linear gradient from 5% to 75% acetonitrile in water, containing 0.05% trifluoroacetic acid at a flow rate 1 ml/min for 40 min.

11. D-dimer Assay

Partially cross-linked fibrin was prepared from fresh recalcified human plasma. In brief, 1 ml of fresh platelet poor plasma was mixed with 2 ml of 0.15 M Tris-HCl buffer pH 7.8 containing 5 mM $CaCl_2$. After addition of 10 μl of 2.5 M $CaCl_2$, the whole mixture was incubated for 4 hr at 37°C. The fibrin clot was

then squeezed and washed exhaustively with the same buffer. The cross-linked fibrin was resuspended in 1 ml of 0.15 M Tris-HCl buffer, pH 7.8 and incubated with 1 μg of MEF. At various time intervals (1, 3, 5 and 7 hr), aliquots (5 μl) were withdrawn from the digestion mixture and assayed using a TintElize D-dimer kit of Biopool Co (Ume, Sweden) according to the manufacturer's instructions.

III. RESULTS

1. Purification

The isolation of MEFs was achieved by a combination of three purification steps. The crude extract containing 110 mg of protein showed a specific activity of 0.02 U/mg. Ammonium sulfate fractionation between 60 to 90% gave maximal fibrinolytic activity. Gel-filtration chromatography of the 60~90% ammonium sulfate fraction on Bio-Gel P-60 could yielded a single fraction with the specific activity increased 30 times. The final step in purification was accomplished using DEAE Affi-Gel blue chromatography with a linear sodium chloride gradient from 0.01 M to 0.2 M. Two fractions, flow-through and bound fractions, were observed. Both fractions could hydrolyze fibrinogen, fibrin and azocasein. The bound fraction had a specific fibrinolytic and azocaseinolytic activity of 102.4 U/mg and 56.1 U/mg, respectively. The total protein of MEF-1 in the unbound fraction was about 0.23 mg having a total fibrinolytic activity of 8.49 U and a specific activity of 336 U/mg using azocasein as substrate. The bound fraction also showed another proteolytic activity. This was different from MEF-1 and indicated that it contained another type of proteases. In order to remove dye and other contaminants, MONO-Q anion-exchange chromatography was used. Only a fraction C of them showed a fibrinolytic activity and it was entirely homogeneous on SDS-PAGE. MEF-3 was purified to 1,635 folds compared to the crude extract based on fibrinolytic activity.

2. Biochemical Properties of the Fibrinolytic Enzyme

MEF-1 was found to be homogeneous by SDS-PAGE and the apparent molecular mass of the protease was estimated to be 31,500 Da (Fig. 1). MEF-1 had a molecular weight of 32,800 Da as determined by gel filtration on TSK-3000 SW (data not shown) indicating the enzyme is monomeric. MEF-1 has an isoelectric point is 6.1 as determined by isoelectric focusing. The optimum pH for proteolytic activity was found to be 7.0 and the enzyme was stable between the pH range of 4-11. At pH values below 4.0 enzyme activity was greatly reduced. The optimum temperature was 30°C and the enzyme activity was stable between 10°C to 30°C.

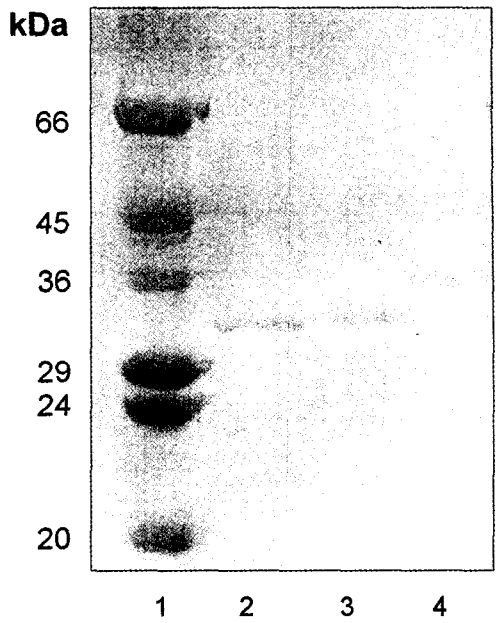

Fig. 1. SDS-PAGE analysis of mantis egg proteases under denaturing conditions. *Lane 1*, mixture of marker proteins, bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da) and α-lactalbumin (14,200 Da); *lane 2* MEF-2, *lane 3*, MEF-3.

MEF-3 gave a single band migrating with an apparent molecular weight 35,600 Da (Fig. 2). In contrast, molecular weight of MEF-2 was determined as 31,500 Da (Hahn *et al.*, 1999) and 33,000 Da (Hahn *et al.*, submitted), respectively. It was identified as monomer through HPLC gel filtration chromatography using TSK-3000 SW HPLC column. Its isoelectric point was determined as 6.0. Glycosylation was not detected by Thymol-H_2SO_4 method. The optimal temperature and pH for the activity were found to be at 30°C and pH 7.0 It was stable in the range of 10~50°C and pH 5.0-10.0. V_{max} and K_m value were calcu-

lated as 0.0163 mM/sec and 0.0544 mM regarding to benzoyl-Phe-Val-Arg-*p*-nitroanilide.

3. Determination of *N*-terminal Sequence

The *N*-terminal amino acid sequence of MEF-1 was determined to be Ala-Asp-Val-Val-Gln-Gly-Asp-Ala-Pro-Ser and compared with other sequences in the nucleic acid and protein databases found in GenBank, Swiss-Prot, PDB and PIR using the BLAST program (NCBI, Bethesda, MD). Only a serine protease from *Penicillium citrium* showed 70% homology to this *N*-terminal peptide (Yamamoto *et al.*, 1993).

The *N*-terminal amino acid sequence of MEF-3 was Ala-Thr-Gln-Asp-Asp-Ala-Pro-Pro-Gly-Leu-Ala-Arg-Arg. When compared with sequence of other poteases, MEF-3 shows 84% homology with the protease from *Tritirachium album*. The *N*-terminal sequence of MEF-3 was different from other serine proteases related to blood coagulation and fibrinolytic system, such as trypsin, plasmin and thrombin (Fig. 2)

4. Effect of Inhibitors

The effect of various compounds on the activity of the purified fibrinolytic protease is shown in Table 2. Activity was strongly inhibited by PMSF and chymostatin but not by elastinal, aprotinin, TLCK, TPCK and benzamidine and soybean trypsin inhibitor. EDTA, EGTA, cysteine, β-mercaptoethanol, iodoacetate and E64 were ineffective. Antiplasmin moderately inhibited the enzymatic activity, while antithrombin III strongly inhibited its activity even at a very low concentration (0.1 μM). The proteolytic activity of MEF-1 was also inactivated by Cu^{2+} and Zn^{2+}. PMSF almost completely inhibited the activity of MEF-3 at a concentration of 200 μM. In contrast, other protease inhibitors such as TLCK, soybean trypsin inhibitor, aprotinin and benzamidine did not affect the proteolytic activity of MEF-3. Chymostatin also inhibited the proteolytic activity of MEF-3 at a concentration of 10 μM. MEF-1 and MEF-3 are similar each other in light of the effects of the inhibitors on the azocaseinolytic activity. As opposed to MEF-1 and -3, MEF-2 was inhibited in the presence of a low concentration of TLCK, soybean trypsin inhibitor and aprotinin (unpulished data). These results strongly suggest that MEF-3 is a chymotrypsin-like protease rather than a plasmin-like protease.

5. Fibrin(ogen)olytic Activity

The activity of MEF-1 and MEF-3 on fibrinogen and fibrin clots was determined by SDS-PAGE and RP-HPLC. MEF-1 readily cleaved the Aα- and Bβ- chains of fibrinogen and the γ-chain more slowly. Incubation of fibrinogen and fibrin clots with MEF-1 resulted in several fibrinopeptides, indicating extensive hydrolysis of the molecule and resembled the nonspecific action of the serine protease, plasmin. Thrombin, a specific serine protease, released only two major peptide products corresponding to fibrinopeptides A and B.

6. Determination of D-dimer Concentration

D-dimer represents a cross-linked fibrin degradation product that originated from the breakdown of the fibrin clot network during the bodys repair mech-

MEF-1	A-N-V-V-Q-G-D-P-S
MEF-2	I-V-G-G-E-E-A-V-A-G-D-F-P-X-I-V
MEF-3	A-T-Q-D-D-A-P-P-G-L-A-R-R
Penicillium citrium	A-N-V-V-G-S-N-V-P-S-W-G-L-A-R-I
Tritirachium album	A-T-Q-E-D-A-P-W-G-L-A-R
Thrombin	I-V-E-G-S-D-A-E-I-G-M-S-P-W-Q
Trypsin	I-V-G-G-Y-T-C-G-A-N-T-V-P-Y-Q
Chymotrypsin	I-V-G-D-E-E-A-V-P-G-S-W-P-W-Q
Plasmin	V-V-G-G-C-V-A-T-P-H-S-W-P-W-Q
Subtilisin BPN'	A-Q-S-V-P-Y-G-V-S-Q-I-K-A-P

Fig. 2. Homology of *N*-terminal amino acid sequence of mantids egg proteases and other serine proteases. Subtilisin BPN' (extracelluar protease from *Bacillus amyloliquefaciens*).

anisms. The value of D-dimer increased in proportion to the length of incubation time of 1, 3, 5 and 7 hr, representing 0.12, 1.14, 2.22 and 8.15 μg, respectively.

IV. DISCUSSION

The fibrinolytic activity of crude extracts from mantis egg case has been reported previously (Wang *et al.*, 1989). Here we describe the purification and the characterization of a fibrinolytic serine protease (MEF-1). MEF-1 and 3 have been purified in two chromatographic steps. The final recovery was about 385% and its purification factor was increased 1,850 fold. The high recovery of purification suggest the presence of possible inhibitors in the crude extract. MEF-1 and MEF-2 were shown to be 31.5 and 33 kDa monomeric endopeptidases. They were sensitive to serine protease inhibitors, especially to PMSF and chymostatin. However, other serine protease inhibitors including elastinal, aprotinin, TLCK, TPCK, benzamidine and soybean trypsin inhibitor had no marked effect on proteolytic activity. Cysteine protease inhibitors such as iodoacetate or E64 did not affect MEF-1 activity. Metalloprotease inhibitors, EDTA and EGTA also did not inhibit their proteolytic activities Antithrombin III is a naturally occurring inhibitor of Factor Xa and thrombin (Factor IIa), especially in the presence of heparin. The fact that MEF-1 was inhibited by antithrombin III indicates it may some common properties of blood coagulation factors. Its substrate specificity is much related to trypsin or thrombin in light of amidolytic activity. These results suggest that MEF-1 is a serine protease and its active site might be different from those of trypsin, chymotrypsin or subtilisin. A specific activity on fibrin by MEF-1 was 36.9 U/mg being higher than plasmin (9.89 U/mg). The presence of low molecular weight fibrinopeptide products from incubating fibrin(ogen) with either MEF-1 (0.4 U) or plasmin (0.4 U) indicates that MEF-1 has greater fibrinolytic activity than plasmin. The analysis of the cleavage site showed a preference between Leu^{15}-Tyr^{16}. This specificity was also observed among the serine proteases isolated from microorganisms (Yamamoto *et al.*, 1993; Johansen *et al.*, 1968; Ichishima *et al.*, 1986). But these microbial enzymes cannot cleave C-terminal to hydrophilic amino acids of the insulin B chain. Interestingly, MEF-1 also cleaves between Tyr^{26}-Thr^{27}, which is an uncommon site for a protease. The serine protease from *Penicillium citrinum* has a similar proteolytic specificity and *N*-terminal sequence to MEF-1. However, the serine protease from *Penicillium citrinum* has a much higher isoelectric point (9.4). Mammalian proteases including pepsin, trypsin and elastease also showed different cleavage sites from MEF-1 (Ryle *et al.*, 1968; Wang and Carpenter, 1967; narayanon and Anwar, 1969). The above results suggest that the protease from *Tenodera sinensis* has a different specificity from other serine proteases from animals and microorgansims. Fibrinolytic activity of MEF-1 was demonstrated using SDS-PAGE and RP-HPLC. HPLC analysis showed that MEF-1 cut fibrin(ogen) in a different fashion than plasmin and thrombin. Thrombin only cleaves α and β, but not the γ chain of fibrinogen (Hahn *et al.*, 1996). Plasmin is a serine protease with relatively broader specificity than thrombin. It hydrolyzes not only Arg-X but also Lys-X, and can dissolve insoluble fibrin polymers (Francis *et al.*, 1980). MEF-1 has an even broader specificity than plasmin on insulin and chromogenic protease substrates. These results suggest that MEF-1 has a strong activity on fibrinogen.

D-dimer is a protein which is released into the circulation during fibrin clot breakdown and is indicative of a blood clot being formed and broken down (Kornberg *et al.*, 1992). Digesting fibrin with MEF-1, the value of D-dimer increases continuously suggesting that MEF-1 may be a potent naturally occurring candidate for fibrinolytic therapy.

What are the physiological roles of MEFs in mantis eggs? They may work on the regulatory function of insects. They may be the proteins acting on the development of young mantis. They are similar to one of the proteases in the egg of silk worm specializing the selective degradation of yolk proteins (Maki and Yamashita, 1997). They may also associate with egg activation and then disappear (Ruder *et al.*, 1990). It should be investigated whether MEFs induces the lysis of thrombi *in vivo* and its physiological role in mantis eggs.

ACKNOWLEDGEMENT

This work was supported by Brain Korea 21 Project.

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