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Purification and Characterization of a Novel Serine Protease with Fibrinolytic Activity from *Tenodera sinensis* (Chinese Mantis) Egg Cases

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Mantis egg fibrolase (MEF-3) was purified from the egg cases of *Tenodera sinensis* using ammonium sulfate fractionation, gel filtration on Bio-Gel P-60, DEAE Affi-Gel blue gel affinity chromatography, and MONO-Q anion-exchange chromatography. This protease had a molecular weight of 35,600 Da as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions and its isoelectric point was 6.0. The *N*-terminal amino acids sequence was Ala-Thr-Gln-Asp-Asp-Ala-Pro-Pro-Gly-Leu-Ala-Arg-Arg. This sequence was 80% homologous to the serine protease from *Tritirachium album*. MEF-3 readily digested the α- and β-chains of fibrinogen and more slowly the γ-chains. It showed strong proteolytic and fibrinolytic activities. Phenylmethanesulfonyl fluoride and chymostatin inhibited its proteolytic activity, while EDTA, EGTA, cysteine, β-mercaptoethanol, elastinal, tosyl-lysine chloromethylketone, and tosyl-amido-2-phenylethyl chloromethyl ketone did not affect its proteolytic activity. Among the chromogenic protease substrates, the most sensitive one to the hydrolysis of MEF-3 was benzoyl-Phe-Val-Arg-*p*-nitroanilide. Based on these experimental results, we speculated that MEF-3 is a serine protease with a strong fibrin(ogen)olytic activity.

Keywords: Fibrinolytic activity, Properties, Purification, Serine protease, *Tenodera sinensis*.

Introduction

When a blood vessel is damaged, the hemorrhage reaction would occur to stop blood loss. After the hemorrhage reaction stops and blood vessel tissue is regrown, the cross-linked fibrin polymer to be formed is dissolved by endogeneous plasmin. However, if any defect in the balance of the coagulation and fibrinolysis systems occurs, many cardiovascular related diseases such as hypertension, myocardial infarction, and strokes are induced. Much effort have been invested for the treatment of such diseases, including the removal of thrombi. The plasminogen activators such as streptokinase, urokinase, t-PA, and rt-PA (recombinant tissue-type plasminogen activator) have been used for the past 30 years and reported as useful therapeutics for cardiovascular diseases related to thrombus (Marder and Sherry, 1988; Verstraete *et al.*, 1995). However, many side effects such as systemic hemorrhage occur because these thrombolytic agents have no affinity to fibrin (Haber *et al.*, 1989) and their half-lives are very short (Tanaka *et al.*, 1996).

Recently, we have attempted to develop a better thrombolytic agent from natural sources including snake venoms (Hahn *et al.*, 1995; 1996) and insects (Hahn *et al.*, 1999). During the purification of a fibrinolytic protease from *Tenodera sinensis*, we found that two different types of proteases also existed in the fraction eluted from the dye-binding affinity chromatography (Hahn *et al.*, 1999). In the present report, we purified and characterized a novel serine protease (MEF-3) from the egg cases of *Tenodera sinensis*.

Materials and Methods

Materials The mantis egg cases (Sangpioxiao) were purchased at a local market in Beijing, China. Bio-Gel P-60 (medium), DEAE Affi-Gel Blue gel, thrombin, bovine fibrinogen, PMSF, soybean

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trypsin inhibitor, aprotinin, benzamidine, and chromogenic substrates (benzoyl-Pro-Phe-Arg-*p*-nitroanilide, benzoyl-Phe-Val-Arg-*p*-nitroanilide, tosyl-Gly-Pro-Lys-*p*-nitroanilide, suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, boc-Ala-Ala-Pro-Ala-*p*-nitroanilide, boc-Gly-Gly-Leu-*p*-nitroanilide) were products of Sigma (St Louis, USA). Chymostatin, elastinal, phenylmethyl sulfonyl fluoride (PMSF), tosyl-amido-2-phenylethyl chloromethyl ketone (TPCK) and tosyl-lysine chloromethylketone (TLCK), were purchased from Calbiochem (La Jolla, USA). Most of the other reagents and chemicals were commercial sources and were of the best grade available.

Purification of a fibrinolytic protease, MEF-3 MEF-3 was isolated by a combination of ammonium sulfate fractionation, gel filtration, affinity chromatography, and high-performance liquid chromatography. The egg case powder (240 g) was suspended in 2.5 L of 50 mM Tris·HCl (pH 7.4) containing 100 mM NaCl, and any insoluble material was removed by centrifugation at 8000 × g for 30 min at 4°C. Ammonium sulfate was added to the supernatant up to 60%, centrifuged, and the pellet was decanted from the supernatant. Ammonium sulfate was again added up to 90%, centrifuged, and the supernatant decanted. The resulting precipitate was suspended in 15 ml of 50 mM Tris·HCl (pH 7.4) containing 100 mM NaCl. The resulting solution was applied onto a Bio-Gel P-60 column (83 × 3 cm) equilibrated with 50 mM Tris·HCl buffer containing 100 mM NaCl (pH 7.4) and the column was eluted with the same buffer at a flow rate of 11 ml/h. Fractions showing a fibrinolytic activity were pooled and 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, USA) 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 ml/h. Fractions showing maximal fibrinolytic activity were pooled and concentrated as previously described (Hahn *et al.*, 1999a). The fraction from the above steps was filtered using 0.45 μm filter (Millipore), was injected to a MONO-Q HR5/5 anion-exchange column (Pharmacia) equilibrated with 50 mM Tris HCl-0.01 M NaCl (pH 7.4), and then eluted with a linear gradient from 0.01 to 1.0 M NaCl in the same buffer at a flow rate of 1 ml/min.

Proteolytic activity Proteolytic activity was measured using azocasein as substrate as described previously (Beynon and Kay, 1978).

Fibrinolytic assay and protein determination Fibrinolytic activity was assessed by applying a 10 μl sample to a fibrin plate generated by thrombin-mediated polymerization. Samples were incubated for 17 h at 37°C and 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 (1976) using bovine serum albumin as a standard.

Molecular weight determination SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using 10% polyacrylamide gel and 4% stacking gel. The protein standards used were β-lactoalbumin (14,200), soybean trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000).

pI value determination Iso-electrofocusing was performed with the Bio-Rad Model 111 Mini Isoelectric focusing cell (Richmond, USA) according to the manufacturer's procedure.

***N*-terminal amino acid sequence determination** After SDS-PAGE, the band indicating MEF-3 was transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting (Matsudaira, 1995) and then used for the analysis of the *N*-terminal sequence. It was determined using an Applied Biosystems Procise 491 amino acid sequencer at the Korea Basic Science Center in Seoul. Compositional analysis was carried out, first by derivatization of the amino acids with phenylisothiocyanate (PITC) followed by reversed-phase high-performance liquid chromatography (Willis and Tu, 1988).

Fibrinogenolytic activity Fibrinogenolytic activity was examined using 1 mg/ml bovine fibrinogen solution in 50 mM Tris·HCl (pH 7.4) (Willis and Tu, 1988).

Effect of protease inhibitors on proteolytic activity MEF-3 (1 μg) was preincubated with the inhibitors (5 mM EDTA, 5 mM EGTA, 5 mM cystein, 10 mM 2-mercaptoethanol, 2 mM PMSF, 100 μM elastinal, 100 μM TPCK, 100 μM TLCK, 50 μM soybean trypsin inhibitor, 50 μM aprotinin, and 100 μM benzamidine, respectively) at 37°C for 1 h. The residual activity was determined using azocasein as a substrate.

Effects of pH and temperature The optimal pH for the proteolytic activity was determined by varying the pH of the reaction mixture between 3 to 11. MEF (1 μg) was dissolved in either citrate buffer (50 mM, pHs 3–6), Tris·HCl buffer (50 mM, pHs 7–9) or CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer (50 mM, pHs 10–11), and incubated at room temperature for 2 h. Temperature dependency of the purified enzyme was determined under standard conditions at different temperatures (10, 20, 30, 40, 50, 60, 70, and 80°C). The enzyme (1 μg) was incubated at each temperature for 15 min and then its proteolytic activity was determined using azocasein as substrate.

Amidolytic activity Amidolytic activity was 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-3 [1 μg/200 μl of 50 mM Tris·HCl (pH 7.4)] with 300 μl of a 0.5 mM of substrates. After a 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 ($\varepsilon = 9.65 \times 10^3 M^{-1} \cdot cm^{-1}$). Kinetic constants ($V_{max}$ and K_m) were calculated using the Hans plot.

Results

Purification of MEF-3 The protease of MEF-3 was purified and its typical profile is shown in Fig. 1. The fraction with fibrinolytic activity was pooled and applied onto the DEAE Affi Gel-blue affinity column. The unbound fraction represented MEF-1 (Fig. 1B) and 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. Four fractions were collected (Fig. 1C). Only a fraction C of them showed fibrinolytic activity and it was entirely homogeneous on SDS-PAGE. The purification result of MEF-3 is summarized in Table 1. MEF-3 was purified 1635-fold compared to the crude extract based on fibrinolytic activity.

Biochemical properties MEF-3 gave a single band migrating with an apparent molecular weight of 35,600 Da (Fig. 2). In contrast, molecular weights of MEF-1 and -2 were determined as 31,500 Da (Hahn *et al.*, 1999) and 33,000 Da (unpublished data), respectively. It was identified as a monomer through HPLC gel filtration chromatography using a TSK-3000 SW HPLC column. Its isoelectric point was determined to be 6.0. Glycosylation was not detected by the Thymol-H_2SO_4 method. The optimal temperature and pH for the activity were found to be at 30°C and pH 7.0, respectively. It was stable in the range of 10–50°C and pHs 5.0–10.0. V_{max} and K_m values were calculated as 0.0163 mM/sec and 0.0544 mM, respectively, with regards to benzoyl-Phe-Val-Arg-*p*-nitroanilide.

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

Effect of protease inhibitors on proteolytic activity 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, as shown in Table 2. Chymostatin also inhibited the proteolytic activity of MEF-3 at a concentration of 10 μM. MEF-1 and MEF-3 are similar to 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

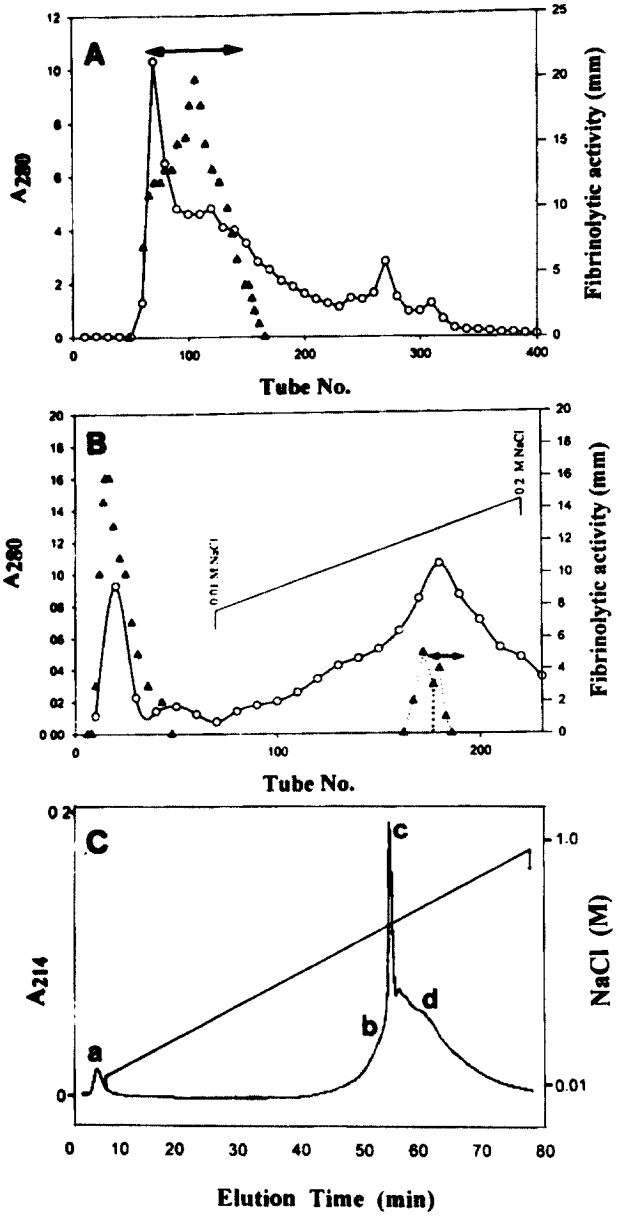

Fig. 1. Fractionation of egg case extract. Purification of MEF-3 was accomplished by using (**A**) Bio-Gel P-60, (**B**) DEAE Affi-Gel blue gel, (**C**) Mono-Q anion-exchange chromatography. **A.** The elution was performed with 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl at a flow rate of 11 ml/h. The elution profiles were monitored by reading the absorbance at 280 nm (−○−). Fibrinolytic activity, based on fibrin plate assay, is shown as a dotted line (···▲···) and the active fractions were pooled (<—>). **B.** About 2 mg of material pooled from the previous step was applied to the column. Elution was performed with a linear salt gradient of 0.005 M to 0.2 M at a flow rate of 11.3 ml/h. The elution profiles were monitored by spectrophotometry at A_{280} (−○−). Fibrinolytic activity was measured (···▲···). **C.** The fraction from the above steps was injected to MONO-Q anion-exchange column equilibrated with 50 mM Tris-HCl-0.01 M NaCl (pH 7.4) and then eluted with a linear gradient from 0.01 to 1.0 M NaCl in the same buffer at a flow rate of 1 ml/min.

Table 1. Purification of serine protease MEF-3 from *Tenodera sinensis* egg cases.

Purification step	Protein (mg)	Azocaseinolytic activity (U/mg)[1]	Fibrinolytic activity (U/mg)	Fibrinolytic activity/ Azocaseinolytic activity	Total activity (unit)[2]	Yield (%)[3]	Purification fold
Crude extract	110.00		0.02	2.2	100	100	
Ammonium sulfate fractionation (60–90)	89.70	17.20	0.30	0.017	26.9	1223	15
Bio-Gel P-60	2.36	148.00	10.00	0.067	23.6	1072	500
MONO-Q HPLC	0.054	153.10	32.70	0.214	1.8	80	1635

1) One unit of azocaseinolytic activity is defined as the amount of enzyme which causes a net increase of 1.0 of absorbance at 340 nm in 1 h.
2) One unit is defined as the amount of enzyme that will hydrolyze 1.0 μmol of tosyl-Gly-Pro-Lys-*p*NA per minute at 25°C.
3) Yield was calculated based on the total fibrinolytic activity.

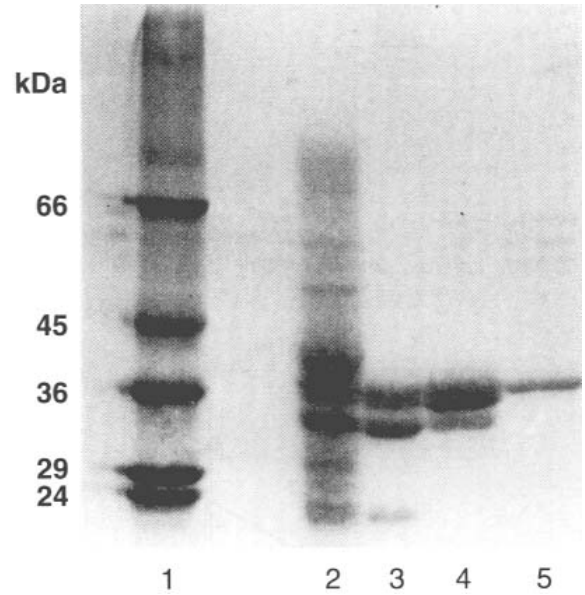

Fig. 2. SDS-PAGE analysis of MEF-3 under denaturing conditions. Lane 1, mixture of marker proteins, bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da) and α-lactalbumin (14,200 Da); lane 2, ammonium sulfate precipitate (60–90%); lane 3, fraction from Bio-Gel P-60; lane 4, fraction from DEAE Affi-Gel blue (MEF); lane 5, fraction from MONO-Q step.

MEF-3	A-T-Q-D-D-A-P-F-G-L-A-R-R
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
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. 3. Homology of *N*-terminal amino acid sequence of MEF-3 and other serine proteases. Subtilisin BPN' (extracelluar protease from *Bacillus amyloliquefaciens*).

Table 2. Effect of some inhibitors on azocaseinolytic activity of MEF-3[1].

Inhibitor	Concentration (mM)	Relative activity (%)
None		100
EDTA	5	103
EGT	5	103
Cysteine	5	98
2-Mercaptoethanol	10	110
PMSF	2	2.6
Chymostatin	0.1	5.2
Elastatinal	0.1	107
TPCK	0.1	110
TLCK	0.1	111
Soybean trypsin inhibitor	0.05	107
Aprotinin	0.05	109
Bezamidine	10	111

[1] MEF-3 (1 μg) was incubated in 10 mM Tris·HCl (pH 7.4) at 37°C for 1 h with several protease inhibitors. Azocaseinolytic activity was determined as described in Materials and Methods. Values represent the mean of triplicate experiments.

aprotinin (Hahn *et al.*, unpublished). These results strongly suggest that MEF-3 is a chymotrypsin-like protease rather than a plasmin-like protease.

Amidolytic activity The amidolytic activity of MEF-3 was investigated with several chromogenic substrates and compared with that of other proteases. MEF-3 showed the highest activity towards benzoyl-Phe-Val-Arg-*p*-nitroanilide, a substrate for thrombin and trypsin. Its specific activity was 82.2 U/mg (Table 3).

Fibrinogenolytic activity The fibrinolytic activity of MEF-3 was demonstrated using SDS-PAGE (Fig. 4). MEF-3 readily digested α-, β-, and γ- chains of fibrinogen within several minutes. On the other hand, fibrinogen incubated for 24 h without MEF-3 was not digested at all. It should be investigated whether proteases in mantis egg cases induce the lysis of thrombi *in vivo*.

Table 3. Amidolytic activity on chromogenic substrates of MEF-3.

Chromogenic substrates	MEF-3 (mmol/min/mg)	Original proteases
Benzoyl-Phe-Val-Arg-*p*-nitroanilide	82.2	Trypsin/Thrombin
Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide	26.2	Cathepsin G
Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide	24.2	Factor Xa
Tosyl-Gly-Pro-Lys-*p*-nitroanilide	17.2	Plasmin
Benzoyl-Pro-Phe-Arg-*p*-nitroanilide	2.6	Kallikrein
Tosyl-Gly-Pro-Arg-*p*-nitroanilide	12.9	Thrombin
Boc-Ala-Ala-Pro-Ala-*p*-nitroanilide	1.8	Elastase
Boc-Gly-Gly-Leu-*p*-nitroanilide	0.9	Subtilisin A

Thrombin (0.1 U) and plasmin (0.01 U) were used in the test, and had values for tosyl-Gly-Pro-Arg-*p*-nitroanilide and tosyl-Gly-Pro-Lys-*p*-nitroanilide of 0.015 and 0.024 (A_{405}/min), respectively.

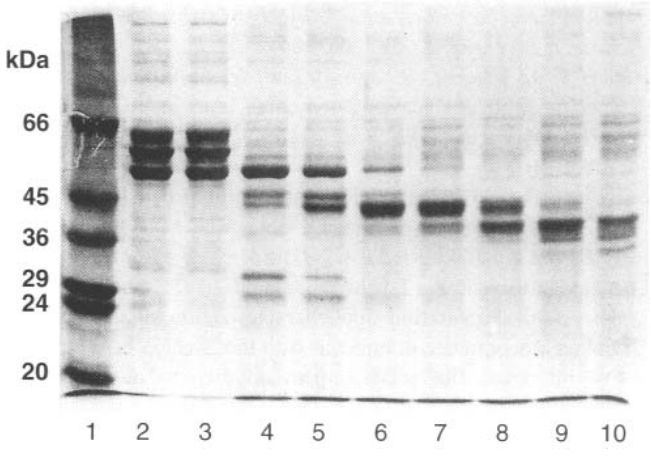

Fig. 4. SDS-PAGE analysis of reduced bovine fibrinogen after digestion with MEF-3. Lane 1, mixture of marker proteins, bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200); lanes 2 and 3, fibrinogen control without MEF-3 after 0 min and 30 min incubation; lanes 4–10, the incubation products of fibrinogen and MEF-3 after 0.5, 1, 3, 5, 10, 20, and 30 min incubation, respectively.

Discussion

The fibrinolytic serine proteases (MEF-1) were purified from the crude extracts of mantis egg cases previously (Hahn *et al.*, 1999). We purified another serine protease, MEF-3, from the egg cases of *Tenodera sinensis*. The final recovery was about 80% and its purification factor was increased 1635-fold. The high recovery of purification suggests the presence of possible inhibitors in the crude extract. Its biochemical properties were similar to those of MEF-1 but its *N*-terminal sequence is different (Salvesen and Nagase, 1990). As chymostatin, an inhibitor of a chymotrypsin-like serine protease and most cysteine proteases, inhibited MEF-3 by 95% at the concentration of 0.1 mM, it may be related to trypsin and chymotrypsin. However, two inhibitors, TPCK and TLCK, which are active site-directed proteinase modifying agents of trypsin and chymotrypsin, did not affect its activity. The amidolytic activity of MEF-3 was similar to that of trypsin. These contradictory results suggest that MEF-3 is related to the enzyme of the digestive system, but it requires a further investigation for clarification.

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 (Park *et al.*, 1998). For example, subtilisin from *Bacillus subtilis* and related species are well-known proteases and they are used for commercial purposes (Kame *et al.*, 1973). Proteolytic enzymes in *Actinomycetes* play major roles in physiology, including diverse functions such as germination and sporulation under lignolytic conditions (Dosoretz *et al.*, 1990). What is the physiological role of MEF-3 in mantis eggs? It may work on the regulatory function of insects. It may be the protein acting on the development of the young mantis. It is similar to one of the proteases present in the egg of silk worm specializing in the selective degradation of yolk proteins (Maki and Yamashita, 1997). It may also be associated with egg activation and then disappear (Ruder *et al.*, 1990). As a similar reported finding, *Tenebrio molitor* cathepsin L-like protease playing a role in metamorphosis was only detected at the larvae, pupae, and adult stages, but not at the embryo stage (Jang *et al.*, 1998). Antibodies produced against MEFs should give a strong suggestion about the development of the mantis in its life cycle.

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