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Purification and Biochemical Characterization of a Serine Protease with Fibrinolytic Activity from Maggots of *Mimela splendems*

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Maggot fibrolase (MsMg-1) was purified from the maggots of *Mimela splendems* using ammonium sulfate fractionation, DEAE Affi-gel affinity chromatography. This protease had a molecular weight of 85 kDa as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions. It showed strong proteolytic and fibrinolytic activities. The purified enzyme was strongly inhibited by phenylmethanesulfonyl fluoride, Mn^{2+}, and Zn^{2+} but it was not by EDTA, EGT, Mg^{2+}, Ca^{2+}, and Li^{2+} ions. In these experimental results, we have speculated that MsMg-1 is a serine protease with a strong fibrinolytic activity.

Key Words: Fibrinolytic activity, Maggot of *Mimela splendems*, Serine protease

INTRODUCTION

The normal vascular endothelium maintains blood fluidity by inhibiting blood coagulation and platelet aggregation and promoting fibrinolysis (Lskutoff et al., 1977). When a blood vessel is damaged, a complex hemostatic response occurs in order to stop blood loss. After the hemorrhage reaction stop, the cross-linked fibrin polymer to be formed is dissolved by endogenous plasmin (Harber et al., 1989; Patthy et al., 1990). If any defect in the balance of the coagulation and fibrinolysis systems occurs many cardiovascular related diseases such as hypertension, myocardial infarction, cholesterolemia, and strokes are induced. Much effort have been invested for the treatment of such diseases, including the removal of thrombi. For the removal of thrombi, due to activation of plasminogen. Plasminogen activation is initiated by release on the plasminogen activators such as urokinase (Colman et al., 2001), tPA (Colman et al., 2001), streptokinase (Colman et al 2001), rt-PA (recombinant tissue-type plasminogen activator) and which catalyzes the proteolytic conversion of the inactive zymogen to the active broad-spectrum proteinase, plasmin. These activators have been used for the past 30 years and reported as useful therapeutics for cardiovascular diseases related to thrombus (Verstrate et al., 1995). However, many side effects such as systemic hemorrhage occur because these activators have been no affinity to thrombi (Haber et al., 1989) and their half lives are very short (Tanaka et al., 1996). Recently researchers have attempted to develop a better thrombolytic agents (Marder et al., 1988) from natural sources including snake venoms (Hahn et al., 1995) and insects (Hahn et al., 1999). In this study, we purified a serine protease like protease with fibrinolytic activity from the whole maggot body. And now we study the characterization of the purified enzyme.

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MATERIAL AND METHODS

1. Materials

The living maggots of *Mimela splendems* were purchased at local farm in Po-Hwang. DEAE Affi-gel, thrombin, sheep fibrinogen, chromogenic substrates (Tosyl-Gly-Pro-Lys-*p*-nitroanilide), PMSF, EDTA, EGT were products of Sigma (St Louis, USA). Most of the other reagents and chemicals were commercial sources and were of the best grade available.

2. Purification of a fibrinolytic protease (MsMg-1)

All of the steps were carried out at 4℃, unless stated otherwise. 40 g of the living maggots were homogenized

in 100 ml of 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and suspended in the same buffer, and homogenated for 24 h. For remove of any insoluble material, the homogenate was centrifuged at 10,000 x g for 1 h. Solid ammonium sulfate was added to the supernatant until 50% saturation was achieved. The pellet was discarded after centrifugation at 10,000 x g, for 1 h and the supernatant was pooled. Additional solid ammonium sulfate was added to the supernatant until 70% saturation was achieved. The resulting precipitate was collected by centrifugation at 10,000 x g for 1 h and dissolved in a minimal volume of the 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl buffer. The solution was twice dialyzed for 16 h against 100 volumes of the same buffer. After centrifugation at 10,000 x g for 1 h, the 50~70% ammonium sulfate fraction was applied to a DEAE Affi-gel (2×40 cm), which had been equilibrated with the column buffer and then washed with the same buffer until the absorbance of effluent at 280 nm fell to near zero. The proteins were eluted with a linear gradient from 250 mM to 400 mM NaCl in the same buffer at a flow rate of 1 ml/min. Fractions (fraction No 43 to 47) showing maximal fibrinolytic activity were pooled (not shown) and stored at -20℃.

3. Preparation of the normal fibrin plate and standard fibrin plate

In each petri dish (dia. 5 cm) 3.0 ml of 0.6% (wt/vol) fibrinogen solution in a 50 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl was mixed with 10 μl of 0.5% (wt/vol) brillient cresyl blue. The petri dishes are placed on a horizontal plastic plate and added 40 μl of thrombin solution (100 units/ml). The solution in the petri dish was allowed to stand for 1 h at room temperature in order to form a fibrin clot layer. For preparation of standard fibrin plate, the plate was incubated for 50 min at 75℃.

4. Enzyme assay on fibrin plate and protein determination

Enzyme activity was determined by the fibrin plate methods as previously described (Tge et al., 1952). Twenty microliters (30 μg of protein)) of the sample solution were dropped into holes previously made on a fibrin plate using a micro pipete and incubated at 37℃ for 16 h. An equal volume (3 μg) of plasmin solution (1 NIH unit/ml) was incubated into a hole on the plate as a standard protease. The enzyme activity was estimated by measuring the area of digested fibrin on the plate. Protein concentration was determined using the method of Bradford (1976) using bovine serum albumin as a standard.

5. Proteolytic activity

The protease activity was determined with azocasein as the substrate in a modified method of Ling (2001). A 100 μl enzyme solution and a substrate (0.5% azocasein in water and 250 mM Tris-HCl pH 8.0) were preincubated at 37℃ for 5 min prior to mixing and incubation at 37℃ for 30 min. The reaction was halted by the addition of 150 μl of 10% trichloroacetic acid and the reaction mixture was incubated for 5 min at room temperature, and centrifuged at 9,000 x g for 5 min. Then 120 μl of the supernatant was transferred to a microtiter plate that contained 150 μl of 1 N NaOH. The resulting color development was measured at A_{405} on the reader. One unit (Beyon et al., 1978) of protease activity

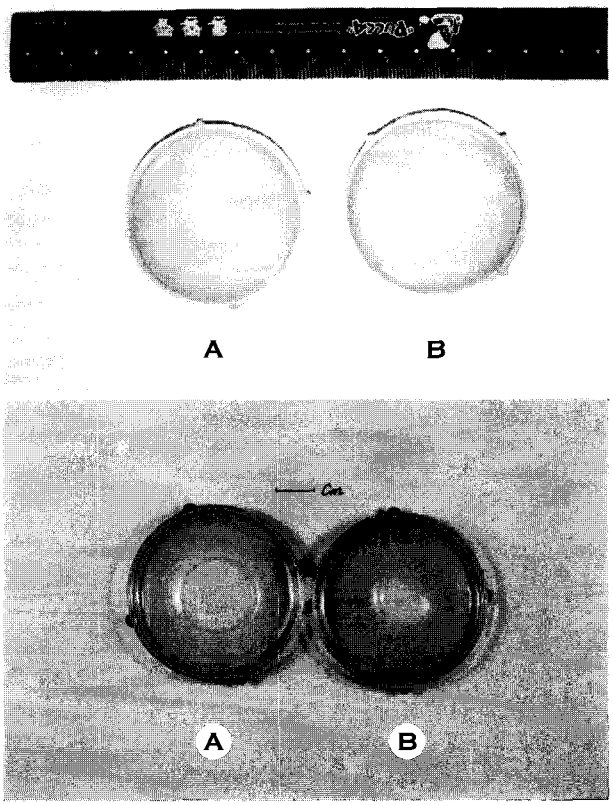

Fig. 1. Fibrinolytic activity assay on the fibrin plate (0.6%) with the homogenized of maggot *Mimela splendems*. Upper **A**, 20 μl (30 μg protein) of sample were loaded on the hole; **B,** 20 μl (3 μg) of plasmin were loaded on the hole. Low fibrin plate is result on the standard plate. **A,** 20 μl (30 μg protein) of sample were loaded on the hole; **B,** 20 μl (3 μg) of plasmin were loaded on the hole.

was defined as the amount of enzyme per ml that is required to cause an increase in absorbance of 0.001 at 405 nm.

6. Molecular weight determination

SDS-PAGE was performed according to the method of Laemmli (1970) by using 5% stacking acrylamide gel and 10% running gel. Proteins were stained with Coomassie brilliant blue R-250. Samples for SDS-PAGE were prepared by heating at 100℃ for 5 min in a medium (pH 6.8) containing 1.6% SDS, 4.2% 2-mercaptoethanol, 0.17 M Tris-0.047 M HCl, 11.2% glycerol, and 0.04% bromphenol blue to denature proteins. The samples were electrophoresed with

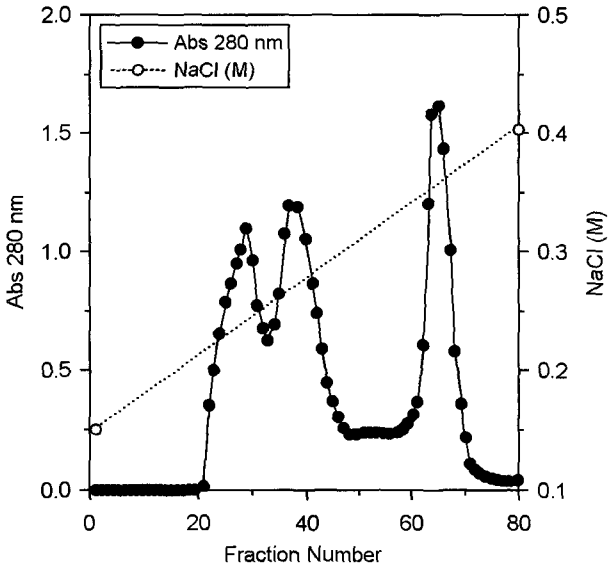

Fig. 2. Fractionation maggot mimela splendems extract. Purification of MsMg-1 was accomplished by DEAE Affi-Gel. The elution profiles were monitored by assay the Bradford method (-●-). About 242 mg of protein pooled from the ammonium sulfate fraction was applied to the column (2.8×40 cm). Elution was performed with a linear salt gradient of 250 mM to 400 mM at a flow rate of 8.0 ml/h.

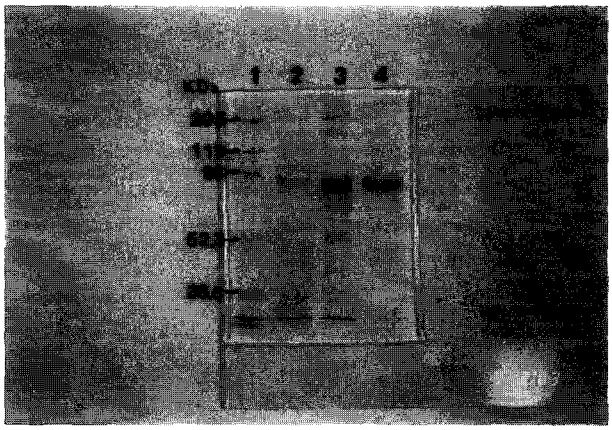

Fig. 3. SDS-PAGE of serine protease from maggot *Mimela splendems*. Electrophoresis was carried out on a 10% polyacrylamide containing 0.1% SDS. The gel was stained with Coomassie Brilliant blue R-250. A molecular weight standard was shown in lane 1: (1) myosin (205,000 Da), (2) β-galactosidase (119,000 Da), (3) phosphorylase a (98,000 Da), (4) glutamate dehydrogenase (52.300 Da), (5) glyceraldehyde-3-phosphate dehydrogenase (36.800 Da). Lane 2: crude extract; Lane 3: ammonium sulfate precipitated fraction 50~70%, Lane 4: purified enzyme.

Table 1. Purification of serine protease MsMg-1 from maggot

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	340	2,775	291.4	0.105	100	1
Amm. Sulfate fractionation (50~70)	17	242	208.4	0.86	71.5	8.2
DEAE-Affi gel	10	0.42	47.4	112.9	16.3	1,075

Table 2. Effect of some inhibitors on azocaseinyltic activity of MsMg-1

Inhibitors	Concentration (mM)	Relatively activity
None		100
PMSF	2	1.85
EDTA	5	98.5
EGT	5	97.8

Abbreviations: DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenedamine tetraacetate; EGT, ethylene glycol tetraacetate

Table 3. Effects of metal ins on the activity of the purified fibrinolytic protease

Metal ion (1.0 mM)	Relative activity (%)
None	100
Mg^{2+}	109
Mn^{2+}	32
Zn^{2+}	21
Ca^{2+}	107
Li^{2+}	112

molecular weight markers at 30 mA for 2 h. Gels were immediately stained with 0.1% Coomassie Brilliant blue R-250 and destained with a mixture of 30% (v/v) methanol and 10% (v/v) acetic acid mixture. The standard proteins were myosin (205 kDa), β-galactosidase (119 kDa), phosphorylase a (98 kDa), glutamate dehydrogenase (52.3 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36.8 kDa).

7. Effect of protease inhibitors on proteolytic activity

In order to determine the sensitivity of the purified fibrinolytic protease towards various inhibitors such as PMSF, EDTA, EGT, metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Li^{2+}), 2 μg of MsMg-1 was preincubated with the inhibitors (5 mM PMSF, 5 mM EDTA, 5 nM EGT) at 37℃ for 1 h. The residual activity was determined using azocasein as a substrate (Cho et al., 1999). Effects of metal ions on the enzyme were investigated by adding the divalent metal ions to the reaction mixture after dialyzing the purified enzyme solution. The final concentration of each metal ion was 1 mM.

8. Amidolytic activity

Amidolytic activity was measured spectrophotometrically using the chromogenic protease substrate, Tosyl-Gly-Pro-Lys-*p*-nitroanilide. 20 μl of plasminogen (0.2 unit/ml) was added in the 20 μl of elution sample and incubated at 37℃ for 5 min. 70 μl of substrate were added in the sample mixture and incubated at 37℃ for 10 min, and for the stop reaction, 300 μl of 20% acetic acid were added. The amount of *p*-nitroaniline released was determined by measuring the change in absorbance at 405 nm. Kinetic constants were calculated using the standard calibration curve.

RESULTS AND DISCUSSION

Biological active proteases are commonly found in the venom from western diamondback Rattlesnake (Willis et al., 1988). In this investigation, crude extract of maggot was assayed by fibrin plate analysis. The maggot of *Mimela splendems*, as shown in Fig. 1. showed the highest enzyme activity on the fibrin plate. The activity was compared with that of the urokinase. Standard plate (heated plate) showed higher fibrinolytic activity than that of normal plate. The protease of maggot of *Mimela splendems* was purified and its typical profile is shown in Fig. 2. The enzyme active fractions pooled were concentrated by 50~70% ammonium sulfate saturation and dialyzed in 50 mM Tris-HCl buffer (pH 7.4). After centrifugation at 10,000 x g for 30 min, the supernatant was pooled and applied onto the DEAE Affi-Gel affinity column (2.8×40 cm). Active fraction was pooled and stored at -20℃ until use. It was entirely homogeneous on SDS-PAGE. The purification result of MsMg-1 is summarized in Table 1. MsMg-1 was purified 1,075-fold compared to the homogenate based on fibrinolytic activity with a recovery of 16.3%. SDS-PAGE revealed a single band of 85 kDa (Fig. 3). In contrast, molecular weight of MEF-3 was determined 35,600 Da (Cho et al., 1999). It was quite different to the molecular weight compared with that of MsMg-1. 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 (Cho et al., 1999). The fibrinolytic protease activity of he purified enzyme was inhibited by PMSF (Table 2). However, it was not inhibited by EDTA, and EGT. In preliminary experiments, enzyme was inhibited by Mn^{2+} and Zn^{2+} (Table 3). But it was not inhibited by Mg^{2+}, Ca^{2+}, and Li^{2+}.

Therefore, these results suggest that the fibrinolytic enzyme is a member of the serine protease family. In conclusion, we isolate and purified a new serine fibrinolytic protease from the maggot of *Mimela splendems*. However, the identification of the partial amino acid sequence was not carried out. Therefore, we are presently investigating the information for the characterization and the gene of the maggot fibrinolytic protease.

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