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Purification and Characterization of a Serine Protease (CPM-2) with Fibrinolytic Activity from the Dung Beetles

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Catharsius protease-2 (CPM-2) was isolated from the body of dung beetles, *Catharsius molossus*, using a three step purification process (ammonium sulfate fractionation, gel filtration on Bio-Gel P-60, and affinity chromatography on DEAE Affi-Gel blue). The purified CPM-2, having a molecular weight of 24 kDa, was assessed homogeneously by SDS-polyacrylamide gel electrophoresis. The *N*-terminal amino acid sequence of CPM-2 was composed of X Val Gln Asp Phe Val Glu Glu Ile Leu. CPM-2 was inactivated by Cu^{2+} and Zn^{2+} and strongly inhibited by typical serine proteinase inhibitors such as TLCK, soybean trypsin inhibitor, aprotinin, benzamidine, and α_1-antitrypsin. However, EDTA, EGTA, cysteine, β-mercaptoethanol, E64, and elastatinal had little effect on enzyme activity. In addition, antiplasmin and antithrombin III were not sensitive to CPM-2. Based on the results of a fibrinolytic activity test, CPM-2 readily cleaved Aα- and Bβ-chains of fibrinogen and fibrin, and γ-chain of fibrinogen more slowly. The nonspecific action of the enzyme resulted in extensive hydrolysis, releasing a variety of fibrinopeptides of fibrinogen and fibrin. Polyclonal antibodies of CPM-2 were reactive to the native form of antigen. The ELISA was applied to detect quantities, in nanograms, of the antigen in CPM-2 protein.

Key words: Catharsius protease (CPM-2), Fibrinolytic activity, *Catharsius molossus*

INTRODUCTION

The dung beetle (*Catharsius molossus*), as its name suggests, eats dung from the ground, and is found throughout the world. It has been used in East Asia as a crude form of drugs in treating constipation, tumors and anal fistula, and is presently reared as a pharmaceutical insect in China. According to previous research, a serine protease (CPM-1) from *Catharsius molossus* was purified and characterized, suggesting it was a new candidate for fibrinolytic therapy (Ahn *et al.*, 2003).

The incidence of thrombotic disorders including cerebral strokes, myocardial infarctions, and venous thromboembolism are on the rise throughout the world. During the past decade, treatments for thrombotic and vascular disorders have been developed to improve therapeutic efficacy and safety (Ouriel *et al.*, 2002). As agents for vascular occlusive diseases, streptococcal streptokinase, urokinase, and human tissue type plasminogen activator (t-PA) are widely used (Collen and Lijnen, 1991). However, high doses of these thrombolytic drugs commonly cause rapid thrombolysis leading to the systemic generation of plasmin, degradation of clotting factors, and potentially, to bleeding complications. More effective thrombolytic agents have been identified and characterized such as, nattokinase in vegetable cheese natto from microorganisms (Fujita *et al.*, 1993), lumbrokinase from earthworms (Nakajima *et al.*, 1993; Mihara *et al.*, 1993) and insects (mantis) (Hahn *et al.*, 1999, 2001) making them potent naturally occurring candidates for fibrinolytic therapy.

In oriental regions, a number of traditional treatment regimens have been developed for thrombotic diseases and many of these crude drugs have been evaluated through the purification and characterization of their active substances (Ahn *et al.*, 2002). Up to now, there has been a dearth of research on the digestive enzymes of *Catharsius molossus* and other related insects. This study

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was designed to characterize a new fibrinolytic enzyme of the digestive system from the whole body of the beetle, evaluating its proteolytic specificity and fibrin (ogen) olytic activity.

MATERIALS AND METHODS

Chemicals

Catharsius molossus was purchased at a local market in Beijing, China. Bio-Gel P-60 (medium) and DEAE Affi-Gel blue were obtained from Bio-Rad (Hercules, CA). Human thrombin, human fibrinogen, anti-mouse IgG alkaline phosphatase, soybean trypsin inhibitor, aprotinin, benzamidine, antithrombin III, EDTA (ethylene diamine tetraacetic acid), EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*, tetraacetic acid] 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 purchased from Sigma Chemicals (St. Louis, MO). Chymostatin, elastatinal, E64 [L-*carboxy-trans*-2, 3-epoxypropyl-leucylamido (4-guanidino) butane], TPCK (tosyl-2-phenylethyalaine chloromethyl ketone), TLCK (tosyl-lysine chloromethyl ketone), human fibrinogen (plasminogen free) and antiplasmin were purchased from Calbiochem (La Jolla, CA).

Purification of the fibrinolytic protease, CPM-2

CPM-2 was isolated by a combination of ammonium sulfate fractionation, gel filtration, and affinity chromatography. *Cartharsius molossus* (100 g) was suspended in 500 mL of 40 mM Tris-HCl (pH 7.4) 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 up to 60% of the supernatant, after which it was centrifuged and decanted away. Ammonium sulfate was then added to up to 90% of the supernatant, and it was once again centrifuged and removed. The precipitate was suspended in 15 mL of 40 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The resulting solution was applied to a Bio-Gel P-60 column (88×2.6 cm) equilibrated with a 40 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.4) at 4°C, and the column was eluted with the same buffer at a flow rate of 20 mL/h. Fractions showing optimal fibrinolytic activity were pooled and dialyzed overnight against 50 mM Tris-HCl (pH 7.4) containing 10 mM NaCl at 4°C. The sample was concentrated using a DIAFLO UM 10 from Amicon (Beverly, CA) and then loaded onto a DEAE Affi-Gel Blue gel chromatography column (17×2.6 cm) equilibrated with the same buffer. The non-interacting solutes were washed from the column with the equilibration buffer. The bound fractions were eluted using a linear sodium chloride gradient from 10 to 200 mM in Tris-HCl (pH 7.4) at a flow rate of 20 mL/h. Fractions showing maximal fibrinolytic activity were pooled and concentrated as previously described.

Fibrinolytic assay and protein determination

Fibrinolytic activity was assessed by applying a 10 μL sample to a fibrin plate generated by thrombin-mediated polymerization. The activity was quantified by measuring the lysis area on the plate and converted to plasmin units (Astrup and Mullertz, 1952). Protein concentration was determined using the Bradford method (Bradford, 1976).

Determination of molecular weight and isoelectric point of CPM-2

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed following the Laemmli method (Laemmli, 1970), using a 4% stacking and 12% resolving polyacrylamide gel. The gels were visualized with either Coomassie Brilliant Blue R250 or silver staining.

Determination of *N*-terminal amino acid sequence of CPM-2

The *N*-terminal amino acid sequence of the purified CPM-2 was determined using an Applied Biosystems Precise 491 amino acid sequencer at the Korea Basic Science Center in Seoul (Williams *et al.*, 1998).

Proteolytic activity assay

Proteolytic activity was measured using azocasein as a substrate following previously described methods (Hahn *et al.*, 1999, 2001; Beynon and Kay, 1978).

Effects of divalent metal cations and inhibitors

CPM-2 (1 μg) was preincubated with 5 mM of Ca^{2+}, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, 1, and 10 mM benzamidine, 5 mM EDTA, 5 mM EGTA, 5 mM cysteine, 10 mM β-mercaptoethanol, 0.05 mM iodoacetate, 0.01 mM E64, 0.05 mM soybean trypsin inhibitor, 0.05 mM aprotinin, 0.1 mM TPCK, 0.1 mM TLCK, 0.1 mM elastatinal, 0.1 mM chymostatin, 16 nM antiplasmin, 16 nM antithrombin III, and 16 nM antitrypsin at 37°C for 2 h. Residual enzyme activity was determined using the azocasein assay (Hahn *et al.*, 1999, 2001).

Effects of pH and temperature

The optimal pH for proteolytic activity was determined by varying the pH of the reaction mixture between 3 to 11. CPM-2 (1 μg) was dissolved in either a citrate buffer (50 mM, pH 3-6), Tris-HCl buffer (50 mM, pH 7-9) or phosphate buffer (50 mM, pH 10-11), and incubated at

37°C for 2 h. Temperature dependency of the purified enzyme was determined under standard conditions at different temperatures. The enzyme (1 μg) was incubated at each temperature for 15 min, after which its proteolytic activity was determined using the azocasein assay as described above.

Amidolytic activities on chromogenic substrates

Amidolytic activities were measured spectrophotometrically using the chromogenic protease substrates as previously reported (Hahn *et al.*, 1999, 2001). Activities were tested by mixing CPM-2 [1 μg/200 μL of 50 mM Tris-HCl (pH 7.4)] with 300 μL of 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 ($\varepsilon = 9.65\times10^3$ $M^{-1}\cdot cm^{-1}$, Tris-HCl, pH=7.4).

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 either CPM-2 (1 μg/mL), plasmin (0.1 U/mL), or thrombin (25 U/mL) at 37°C. At various time intervals (0, 1, 3, 5, 10, 30, and 60 min), aliquots were taken from the reaction mixture and added to an equal volume of the sample buffer containing β-mercaptoethanol and were then boiled and electrophoresed in a 10% polyacrylamide gel in reducing conditions (Laemmli, 1970).

Production of anti-CPM-2 antibody

Briefly, polyclonal antibodies were raised by injecting them into Balb/c mice and then used as the primary antibodies for ELISA. The purified CPM-2 was emulsified with an equal volume of Freund's complete adjuvant and the mixture was injected intraperitoneally to two Balb/c mice. A series of 3 booster injections were given at 3 week intervals in a similar way to that of the first injection, except that Freund's incomplete adjuvant was used. Blood samples were taken from the tail vein one week after each immunization, and the antibody titers of the plasma were determined by ELISA as described below. Each animal was bled from the tail vein and the sera obtained were stored at -80°C.

Determination of CPM-2 in polyclonal antibody

The titer of the primary antibody was determined by reading the absorbance after a two-fold serial dilution of the antibody. The highest absorbance of the sample was 3.3 and the lowest value was 1.1 and the typical background reading was 0.11. Since the convection point was observed to be in the range of 100 to 500-fold dilutions, the primary antibody was fixed at 400-fold dilutions. Quantitative analysis indicated that the minimal concentration of CPM-2 in the solution was 10 ng/mL and the maximum concentration was 500 ng/mL.

RESULTS

Purification of CPM-2

CPM-2 was isolated using a three step purification process. The crude extract, containing 83 mg of protein, showed a specific activity of 2.0 U/mg. Ammonium sulfate fractionation between 60 and 90% produced maximum fibrinolytic activity. Gel-filtration chromatography of the 60-90% ammonium sulfate fraction on Bio-Gel P-60 yielded three fractions with specific activity increased approximately ten times (Fig. 1A). The final step of the purification

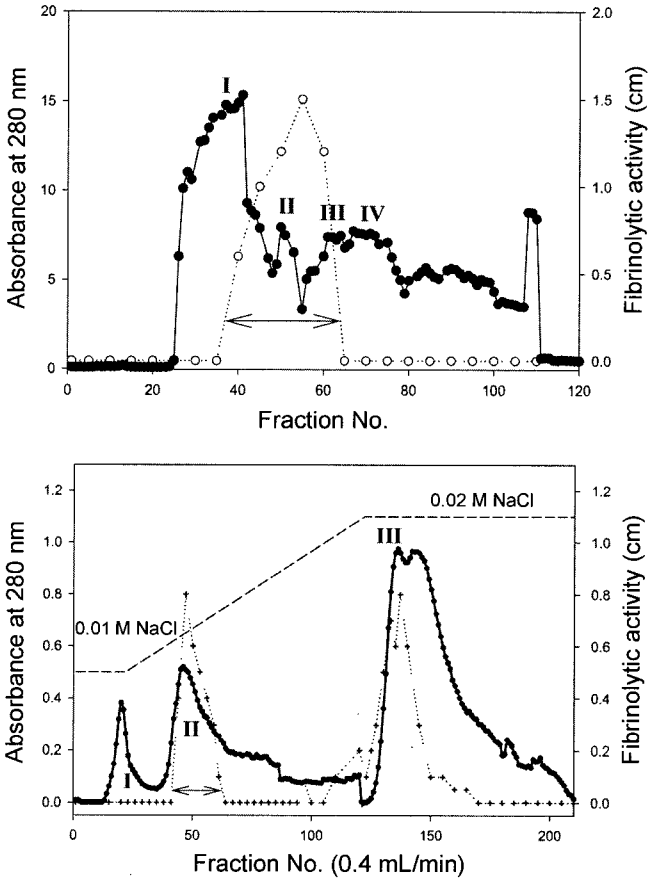

Fig. 1. Purification of *C. molossus* serine protease (CPM-2) by gel permeation chromatography and affinity chromatography (A). Chromatogram of the protein elution of Bio-Gel P-60. The elution was performed with 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl at a flow rate of 20 mL/h. The elution profiles were monitored by reading the absorbance at 280 nm (-●-). Fibrinolytic activity measured (..○..) and the active fractions were pooled (↔). (B) Elution profiles from DEAE Affi-Gel bule gel monitored by spectrophotometry at A_{280} (-●-). Fibrinolytic activity was measured (-+-) and the active fractions were pooled (↔).

Table I. Purification of a serine protease from *Catharsius molossus*

Purification step	Protein (mg)	Azocaseinolytic activity (U/mg)[a]	Fibrinolytic activity (U/mg)[b]	Total activity (units)	Yield (%)[c]	Purification fold
Crude extract	83.00	13.6	2.0	166	100	1
Ammonium sulfate Fractionation (60-90%)	43.23	19.4	4.3	185.9	112	2
Bio-Gel P-60	11.17	42.0	47.1	526.1	317	24
DEAE Affi-Gel blue	0.064	40.8	79.3	5.8	3	40

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

process was accomplished by using DEAE Affi-Gel Blue gel chromatography with a linear sodium chloride gradient from 0.01 M to 0.2 M. Three bound fractions were observed (Fig. 1B). The three fractions (I~III) hydrolyzed fibrinogen, fibrin, and azocasein. The total amount of protein from CPM-2 in the third bound fraction (III) was approximately 0.064 mg with a total fibrinolytic activity of 5.8 U and a specific activity of 40.8 U/mg, using azocasein as a substrate. These results are shown in Table I. CPM-2 was found to be homogeneous by SDS-PAGE with a molecular weight of 24 kDa (Fig. 2). The same result was obtained by gel filtration chromatography on TSK-3000 SW (data not shown), indicating a monomeric structure protease.

Effects of the fibrinolytic enzyme on pH and temperature

The optimum pH for proteolytic activity was 9.8. The enzyme was stable between pH ranges of 4-11, at pH values below 4.0 enzyme activity was greatly reduced. The optimum temperature was 40°C, while enzyme activity was stable between 10°C to 50°C (data not shown).

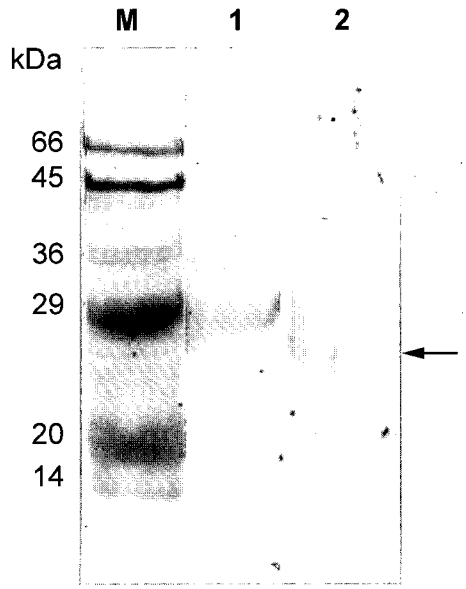

Fig. 2. SDS-PAGE analysis of CPM-2 under denaturing conditions, using 4% stacking and 12% resolving polyacrylamide gels; M: molecular weight markers; lane 1, CPM-1; lane 2, CPM-2.

Effect of inhibitors and divalent metal ions

The effect of various compounds on the purified fibrinolytic protease activity is shown in Table II. The activity was strongly inhibited by chymostatin, TLCK, soybean trypsin inhibitor, aprotinin, benzamidine, and α_1-antitrypsin (Table II). Antiplasmin and antithrombin III were not sensitive to

Table II. Effect of some inhibitors on azocaseinolytic activity of CPM-2[a]

Inhibitor	Concentration (mM)	Relative activity (%)
None		100.0 ± 1.4
EDTA	5	98.3 ± 3.0
EGTA	5	99.3 ± 2.0
Cysteine	5	101.6 ± 0.8
β-Mercaptoethanol	10	100.1 ± 3.8
Iodoacetate	0.05	80.5 ± 2.4
E64	0.01	100.3 ± 1.2
PMSF	2	92.7 ± 1.5
Chymostatin	0.1	63.2 ± 1.4
Elastatinal	0.1	103.4 ± 3.8
TPCK	0.1	88.3 ± 4.6
TLCK	0.1	67.8 ± 1.9
Soybean trypsin inhibitor	0.05	69.0 ± 0.9
Aprotinin	0.05	68.9 ± 1.2
Benzamidine	10	68.3 ± 0.7
	1	79.1 ± 1.6
α_2-Antiplasmin	16[b]	101.6 ± 3.5[c]
Antithrombin III	16[b]	96.0 ± 3.3[c]
α_1-Antitrypsin	16[b]	70.1 ± 0.7[c]

[a]CPM-2 (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 the materials and methods section. Values represent the mean of triplicate experiments.
[b]Means nM.
[c]Represents the mean of triplicate experiments.

Table III. Effect of the divalent cations on azocaseinolytic activity of CPM-2[a]

Divalent	Cation concentration (mM)	Relative activity (%)
None		100 ± 1.7
$CaCl_2$	5	163 ± 3.8
$MgCl_2$	5	167 ± 1.3
$BaCl_2$	5	165 ± 1.9
$CuCl_2$	5	28 ± 0.7
$ZnCl_2$	5	26 ± 1.3
$MnCl_2$	5	144 ± 6.7

[a]CPM-2 (1 μg) was preincubated in 10 mM Tris-HCl (pH 7.4) with different divalent cations at 37°C for 2 h. Azocaseinolytic activity was determined as described in the materials and methods section. Values represent the mean of triplicate experiments.

CPM-2, while antitrypsin slightly inhibited its activity even at very low concentrations (16 nM). Cu^{2+} and Zn^{2+} also inactivated the proteolytic activity of CPM-2 by 72% and 74%, respectively. As with other insect trypsins, CPM-2 was unaffected by metallic cofactors such as calcium ions, magnesium ions, barium ions, and manganese ions (Table III).

Fibrin (ogen) olytic activity

The activity of CPM-2 on fibrinogen and fibrin clots was determined by SDS-PAGE (Fig. 3) and RP-HPLC (data not shown). CPM-2 readily cleaved Aα- and Bβ-chains of fibrinogen (Fig. 3) and fibrin (data not shown), and γ-chains of fibrinogen more slowly.

Determination of *N*-terminal amino acid sequence and analysis of amino acid composition of CPM-2

The *N*-terminal amino acid sequence of CPM-2 was determined to be X Val Gln Asp Phe Val Glu Glu Ile Leu and compared with other sequences in the protein databases using the BLAST program (NCBI, Bethesda, MD). The *N*-terminal amino acid sequence of the enzyme did not match any other insect serine protease.

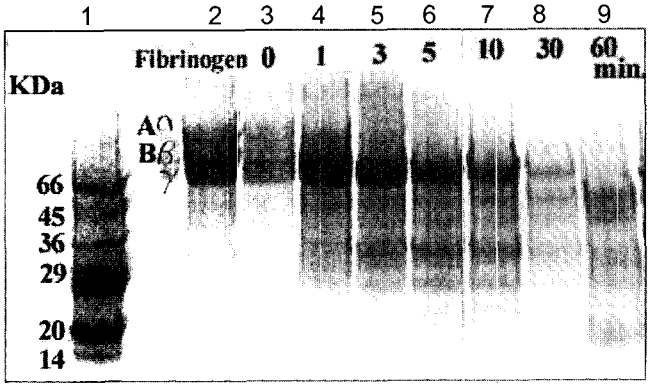

Fig. 3. SDS-PAGE analysis of reduced bovine fibrinogen after digestion with CPM-2 in a 10% gel in reducing conditions. *Lane 1*, molecular weight markers, *lane* 2, fibrinogen control without CPM-2 after 0 min incubation; *lane 3-9*, fibrinogen and CPM-2 after 0, 1, 3, 5, 10, 30, and 60 min incubation, respectively.

Amidolytic activity on chromogenic substrates

Amidolytic activity of CPM-2 was evaluated against several chromogenic protease substrates, which are specific to serine protease. CPM-2 displayed activity toward benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, tosyl-Gly-Pro-Arg-*p*-nitroanilide, tosyl-Gly-Pro-Lys-*p*-nitroanilide, and benzoyl-Phe-Val-Arg-*p*-nitroanilide (each substrate for Factor Xa, thrombin, plasmin, and trypsin or thrombin, respectively, Table IV). The specific activity of the substrate for thrombin was 15.47 U/mg.

Western blot analysis of CPM-2 antigen

To detect the CPM-2 antigen, Coomassie Brilliant Blue staining of the membrane and antigen detection after probing with polyclonal antibodies were used, and the locations of the two bands were found to be precisely consistent, which indicated that polyclonal antibodies were raised to the CPM-2 antigen (data not shown).

Determination of CPM-2 in polyclonal antibody

The titer of the primary antibody was determined by reading the absorbance after a two-fold serial dilution of the antibody. The highest absorbance of the sample was 3.3 and the lowest value was 1.1 and the typical background reading was 0.11. Since the convection point was observed to be in the range of 100 to 500-fold dilutions B (serum 500:1), C (serum 250:1), and D (serum 100:1)], the

Table IV. Amidolytic activity on synthetic protease substrates in CPM-2

Synthetic protease substrate	mmole/min/mg	Proteases[a]
Benzoyl-Phe-Val-Arg-*p*-nitroanilide	1.67±0.34	Trypsin and thrombin
Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide	0.67±0.45	Cathepsin G
Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide	3.10±1.99	Factor Xa
Tosyl-Gly-Pro-Lys-*p*-nitroanilide	14.44±3.76	Plasmin[b]
N-Benzoyl-Pro-Phe-Arg-*p*-nitroanilide	2.08±0.87	Kallikrein
Tosyl-Gly-Pro-Arg-*p*-nitroanilide	15.47±2.69	Thrombin
Boc-Ala-Ala-Pro-Ala-*p*-nitroanilide	N.D[c]	Elastase
Boc-Gly-Gly-Leu-*p*-nitroanilide	N.D	Subtilisin A
Benzoyl-Gly-Pro-*p*-nitroanilide	0.04±0.02	Prolyl endopeptidase

[a]The substrates are effective against the proteases listed.
[b]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, respectively.
[c]N.D: Any increase of absorbance was not detected.

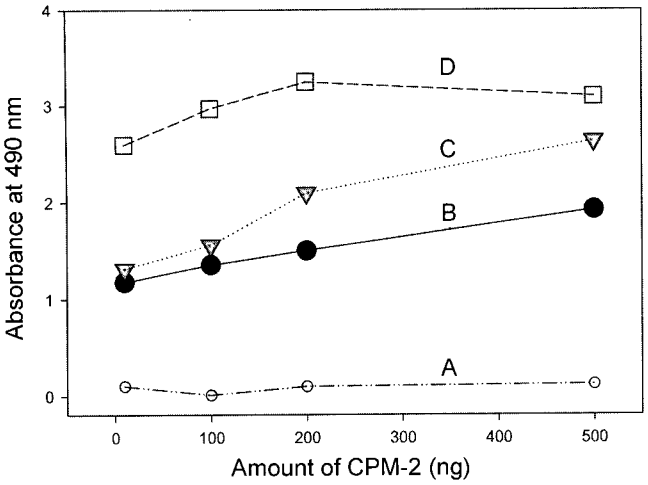

Fig. 4. Measurement of polyclonal antibody of CPM-2: direct ELISA for a detection of CPM-2. Polyclonal antibody (serum) in coating buffer was serially diluted so that A (BSA), B (serum 500:1), C (serum 250: 1), and D (serum 100:1) were immunoassayed with coating CPM-2 (100-500 ng/mL).

conjugate (anti-mouse IgG alkaline phosphatase) was fixed at 1000-fold dilutions. Quantitative analysis indicated that the minimal concentration of CPM-2 in the solution was 10 ng/mL and the maximum concentration was 500 ng/mL (Fig. 4). In other words, the binding capacity of CPM-2 to the plate ranged from 100 ng/mL to 500 ng/mL when considering a nonspecific binding of CPM-2 to the plate.

DISCUSSION

A fibrinolytic serine protease (CPM-2) was purified in a three step process from dung beetles (Table I). The final recovery was about 3% of the original and its purification increased by a factor of 40-fold. CPM-2 has a molecular weight of approximately 24 kDa. The optimal activity of CPM-2 at a basic pH suggests localization of the enzyme in the guts. In comparing it with other serine proteases, the *N*-terminal peptide of CPM-2 did not match any other sequences in the protein databases using the BLAST program (NCBI, Bethesda. MD).

CPM-2 was sensitive to typical serine protease inhibitors, especially to TLCK, soybean trypsin inhibitor, aprotinin, and benzamidine. However, cysteine protease inhibitors (E64), other serine protease inhibitors (elastatinal and PMSF), and metalloprotease inhibitors (EDTA and EGTA) exerted little effect under the same conditions. Also, the inhibition of CPM-2 by α_1-antitrypsin suggests that this property is common in trypsin-like serine proteases belonging to digestive enzymes (Ahn *et al.*, 2003).

The specific activity of CPM-2 on fibrin (79.3 U/mg) was higher than that of plasmin (9.9 U/mg). The fibrinolytic activity of CPM-2 was demonstrated using SDS-PAGE. The lysis of thrombin only cleaves Aα and Bβ, but the γ chain of fibrinogen more slowly. (Hahn *et al.*, 1996). CPM-2 is a typical trypsin-like serine protease. Trypsin preferably attacks the Arg site instead of plasmin, which preferably attacks Lys (Francis *et al.*, 1980). Suggesting CPM-2 is a naturally occurring candidate for fibrinolytic therapy. Further studies on the physiological functions of CPM-2 as well as *in vivo* lysis of thrombi are required.

REFERENCES

Ahn, M. Y., Hahn, B. S., Ryu, K. S., and Cho, S. I., Effects of insect crude drugs on blood coagulation and fibrinolysis system. *Natural Product Sciences*, 8, 66-70 (2002).

Ahn, M. Y., Hahn, B. S., Ryu, K. S., Kim, J. W., Kim, I., and Kim, Y. S., Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, *Catharsius molossus*. *Thrombosis Res.*, 112, 339-347 (2003).

Astrup, T. and Müllertz, S., The fibrin plate method for estimating of fibrinolytic activity. *Arch. Biochem. Biophys.*, 40, 346-351 (1952).

Beynon, R. J. and Kay, J., The inactivation of native enzymes by a neutral proteinase from rat intestinal muscle. *Biochem. J.*, 173, 291-298 (1978).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese bovine insulin. *Biochemistry*, 6, 215-224 (1967).

Collen, D. and Lijnen, H. R., Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* ,78, 3114-3124 (1991)

Dametto, M., David, A. P., Azzolini, S. S., Campos, I. T. N., Tanaka, A. M., Gomes, A., Andreotti, R., and Tanaka, A. S., Purification and characterization of a trypsin-like enzyme with fibrinolytic activity present in the abdomen of horn fly, *Haematobia irritans* (Diptera: Muscidae). *J. Protein Chem.*, 19, 515-521 (2000).

Francis, C. W., Marder, V. J., and Barlow, G. H., Plasmic degradation of cross-linked fibrin: Characterization of new macromolecular soluble complexes and model of their structure. *J. Clin. Invest.*, 66, 1033-1043 (1980).

Fujita, M., NoMura, K., Hong, K., Ito, Y., Asada, A., and Nishimura, S., Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. *Biochem. Biophys. Res. Commun.*, 197, 1340-1347 (1993).

Hahn, B. S., Cho, S. Y., Ahn, M. Y., and Kim, Y. S., Purification and characterization of a plasmin-like protease from *Tenodera sinensis* (Chinese mantis). *Insect Biochem. Mol. Biol.*, 31, 573-581 (2001).

Hahn, B. S., Cho, S. Y., Wu, S. J., Chang, I. M., Baek, K. H., Kim, Y. C., and Kim, Y. S., Purification and characterization of a serine protease with fibrinolytic activity from *Tenodera*

sinensis (Chinese mantis). *Biochim. Biophys. Acta*, 1430, 376-386 (1999).

Hahn, B. S., Yang, K. Y., Park, E. M., Chang, I. M., and Kim, Y. S., Purification and molecular cloning of a thrombin-like enzyme from *Agkistrodon caliginosus* (The Korean viper). *J. Biochem.* (Tokyo), 119, 835-843 (1996).

Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685 (1970).

Nakajima, N., Mihara, H., and Sumi, H., Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus*. *Biosci. Biotech. Biochem.*, 57, 1726-1730 (1993).

Ouriel, K., Current status of thrombolysis for peripheral arterial occlusive disease. *Ann.Vas. Surg* ., 16, 797-804 (2002).

Williams, K., Neice, R. L., Aterton, D., Fowler, A. V., Kutny, K., and Smith, A. J., The size, operation and technical capabilities of protein and nucleic acid core facilities. *FASEB J.*, 2, 3124-3130 (1998).