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A novel fibrin(ogen)olytic trypsin-like protease from Chinese oak silkworm (*Antheraea pernyi*): Purification and characterization

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ABSTRACT

A novel fibrin(ogen)olytic protease from *Antheraea pernyi* (important economically insect), named cocoonase, was isolated by a combination of ion-exchange chromatography and gel filtration. Furthermore, the characterization of cocoonase was investigated using fibrin(ogen)olytic, thrombolysis, and hemorrhagic assays. The NH_2-terminal sequence (**IVGGY SVTID KAPYQ**) was established by Edman degradation. Based on the N-terminal sequencing, cocoonase cDNA has been cloned by means of RT-PCR and 5′RACE. It is composed of 261 amino acid residues and possesses the structural features of trypsin-like serine protease. The purified cocoonase showed specific esterase activity on N-β-benzoyl-L-arginine ethyl (BAEE), and the kinetic constants, Km and Vmax were 2.577×10^{-3} mol/L and 4.09×10^{-3} μmol/L/s, respectively. Cocoonase showed strong activities on both fibrin and fibrinogen, preferentially hydrolyzed Aα and Bβ chains followed by γ-chains of fibrinogen. Cocoonase exhibited a thrombolysis activity both in vitro (blood-clot lysis activity assay) and in vivo (carrageenan-induced thrombosis model). These findings indicate that *A. pernyi* cocoonase ia a novel fibrin(ogen)olytic enzyme and may have a potential clinical application as an antithrombotic agent.

1. Introduction

Thrombosis is a leading cause of morbidity and mortality throughout the world. Anticoagulants, antiplatelet drugs and fibrinolytic drugs are acknowledged approaches to treat thrombotic diseases. There have been major advances in the development of new anticoagulants and antiplatelet drugs, but the area of new fibrinolytic drugs has lagged [1]. Existing fibrinolytic drugs are plasminogen activators (PAs) or their variants which can convert from plasminogen to plasmin. Although they are effective in dissolving the thrombi, all these agents have potential for hemorrhagic complications [2]. Direct fibrinolytic agents, such as plasmin [3] and alfimeprase [4], have been developed in attempt to increase thrombolytic efficacy and reduce their hemorrhagic potential. However, effective use of direct fibrinolytic drugs has required local catheter of agents into the thrombus to bypass circulating plasma inhibitors. Moreover, phase III studies with alfimeprase were suspended, because primary endpoints were not met [5]. Therefore, the research for safer and more effective fibrinolytic enzymes from various sources needs to be continued.

Over the last decade, many fibrinolytic enzymes have been identified and characterized from a variety of sources, such as snake venom [6,7], microorganisms [8,9], and insects [10,11]. In a previous publication, Meiser identified a salivary trypsin-like protease of the *Panstrongylus megistis* possessed fibrinolytic activity [12], indicating that some insect salivary proteins are potential thrombolytic agents. During the latter stage of eclosion of some Lepidoptera insects, cocoonases are secreted by their maxillary glands to facilitate the escape of moth from the cocoon [13]. The Chinese oak silkworm *Antheraea pernyi* (Saturniidae, Lepidoptera) is one of the most well-known silk-producing species. In order to investigate whether cocoonase has fibrinolytic activity, we characterized the sequence of cDNA encoding a cocoonase of *A. pernyi* and further efforts have been made to evaluate the enzymatic character, hemorrhagic activity and fibrinolytic activity.

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2. Materials and methods

2.1. Sample preparation

All these studies were performed with cocoonase isolated from maxillary gland fluid of Chinese oak silkmoths (*A. pernyi*) collected from the coast of Dandong prefecture in China and stored at −80 °C before use. The maxillary gland fluid was secreted during the latter stages of eclosion and was collected after the pupae were incubated at 22 °C for several days. All procedures used during the experiments were approved by the Animal Care Committee at the Dalian University of Technology.

2.2. Purification of cocoonase from the maxillary gland fluid of A. pernyi

The proteinase (cocoonase) was isolated from the maxillary gland fluid of *A. pernyi* by a combination of ion-exchange chromatography on SP FF and gel filtration on Sephadex G-50. Briefly, 10 ml maxillary gland fluid was centrifuged at 12,000*g* at 4 °C for 15 min. The supernatant was applied to a SP FF column pre-equilibrated with 20 mM acetate–acetate buffer solution, pH 6.0. The column was eluted with 20 mM acetate–acetate buffer solution, pH 6.0, followed by a linear NaCl gradient elution (0–1.0 M), with an elution flow rate of 1.0 ml/min (Fig. 1A). The active fraction (Sp-6) was concentrated, desalted, loaded onto a Sephadex G-50 column (1.6 × 96 cm), and eluted by 50 mM Tris–HCl buffer, pH 8.0, with an elution flow rate of 1.0 ml/min (Fig. 1B). A single peak (Se-2) with enzymatic activity was obtained from this step. The absorbance at 280 nm was monitored during protein elution. All solutions were collected and stored at −20 °C until they were used. The activities of all the solutions were assessed utilizing the esterase assay described below.

2.3. N-terminal amino acid sequencing

The proteinase purified from maxillary gland fluid as described above were analyzed for N-terminal sequence by the automated Edman degradation method (Proteomics International Pty Ltd., Australia). N-terminal sequence homology was matched against BLAST database search.

2.4. cDNA cloning and sequence analysis

Total RNA was extracted using Trizol (Invitrogen) from the maxillary gland of *A. pernyi* according to the manufacturer's protocol. For cloning of 3′ flanking region of cocoonase gene, the degenerate primers, N–F primer: 5′ ATH (A/C/G) GTN (A/C/G/T) GGN (A/C/G/T) ATH (A/C/T) TAY (C/T)W (A/T)SN(A/C/G/T) 3′, was synthesized according to the N-terminal amino acid sequence of the cocoonase. After first strand cDNA was synthesized from total RNA using the supplied 3′RACE adaptor, the cDNA was then subjected to PCR using inner primer of the 3′RACE which are complimentary to the anchored adapter. A 680 kb band was obtained and cloned into pMD18-T for sequencing. For cloning of 5′ flanking region of cocoonase gene, two nested specific primers, C-R1: 5′ CAC CGT CGA TTG CTA TAT C 3′, C-R2: 5′ TAT ATT ATC AAC AAG ACA ATG 3′ were synthesized according to the above sequencing results. A PCR product was got by using those nested primers after total RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) and a RNA adapter was ligated to the RNA population using T4 RNA ligase according to RLM-RACE kit manual, which was cloned into pMD18-T for sequencing.

2.5. Sequence alignment and phylogenetic analysis

The amino acid sequences from different species used for homologous alignment and phylogenetic analysis were downloaded from GenBank database. Multiple sequence alignments were carried out using Clustal X software version 2.0 [14]. A phylogenetic tree was constructed by MEGA version 5 using Neighbor-joining method based on a matrix of pair wise distances estimated under the Jones–Thornton–Taylor (JTT) model with bootstrap test of 1000 replications [15].

2.6. Esterase activity

Esterase activity and apparent kinetic parameters, Km and Vmax, were estimated according to the method of Blanco and Guisán [16] with a little modification. Briefly, 200 μL of purified enzyme was added to 2.8 ml 0.5 mM BAEE in 50 mM Tris–HCl buffer of pH 8.0, and the increase in absorbance was measured at 253 nm every 2 s for 5 min. One unit of arginine-esterase activity was calculated as the amount of enzyme that hydrolyses 1 μM BAEE/min under the conditions described. The initial reaction velocity (expressed in ΔA/min) was plotted as a function of the injected BAEE concentration and the apparent Km and Vmax were estimated by fitting the experimental points with the Lineweaver–Burk plot.

2.7. Fibrinogenolytic and fibrinolytic activities

Fibrinogenolytic activity was determined using a modified fibrinogenolytic assay [17]. Briefly, an aliquot of 100 μL of bovine

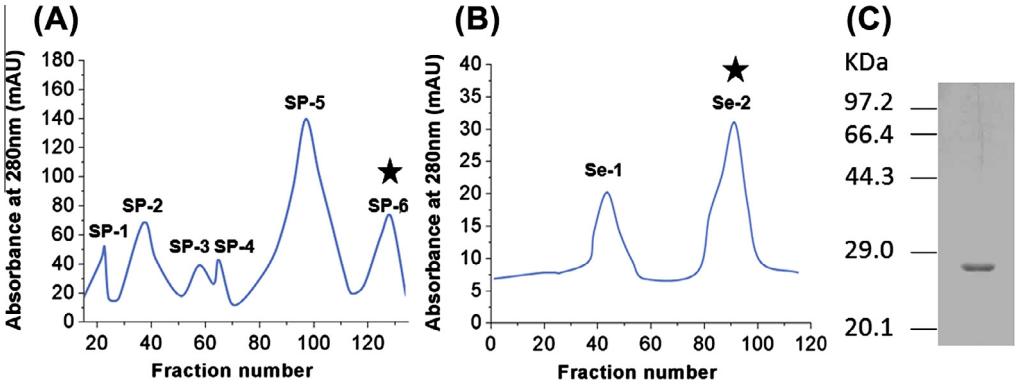

Fig. 1. Purification of cocoonase from the maxillary gland fluid of *A. pernyi*. (A) The supernatant of centrifuged maxillary gland fluid from *A. pernyi* was introduced into a cation-exchange chromatography on a SP FF column. The interesting peak was indicated by a star (SP-6). (B) The active fraction from "SP-6" was then subjected to Sephadex G-50 gel filtration column (1.6 × 96 cm), eluted by 50 mM Tris–HCl pH 8.0 with a flow rate of 1.0 ml/min. Also, the starred peak (Se-2) was interested. (C) The purified proteinase with molecular weight of approximately 28 kDa was then resolved by 12% SDS–PAGE, visualized by Coomassie blue staining. Lane: *A. pernyi* cocoonase.

fibrinogen (0.2% W/V, essentially plasminogen freein 50 mM Tris–HCl buffer, pH 8.0) was incubated with 1.5 μg of cocoonase for different time intervals (15, 30, 60, 90, 120, 150 and 180 min) or with different concentrations of cocoonase (0, 0.5, 1, 1.5, and 2.5 μg) for 30 min. Reactions were carried out at 37 °C and terminated by adding denaturing solution (10 M urea, 4% SDS, and 4% β-mercaptoethanol) and heating at 100 °C for 15 min before subjected to 10% SDS–PAGE gels. Fibrinolytic activity was assayed by a modified fibrin agarose plate assay [18]. A mixture consisting of 4.5 ml of 1% agarose (prepared in 50 mM Tris–HCl, 100 mM NaCl, pH 7.5), 0.5 ml of 0.2% (w/v) bovine fibrinogen (prepared in 100 mM Tris–HCl, pH 8.0), and 200 μL of 100 U/ml human thrombin was poured into a petri dish, and the clot was allowed to stand for 1 h at room temperature. Then, the sample solutions ware carefully placed onto the plate and incubated for 6 h at 37 °C. The diameters of the lytic circles were measured. In the fibrin plate assay, a clear transparent region was observed in which fibrin was hydrolyzed and its diameter was used as an indicator of the fibrinolytic activity. The fibrinolytic activities of samples were calculated as the lytic circle compared to standard urokinase unit.

2.8. Thrombus dissolving model

Carrageenan-induced mouse tail thrombosis model was used to evaluate thrombus dissolving in vivo as described by Kondo et al. [19,20] with slight modifications. 0.3% (w/v) carrageenan (dissolved in Phosphate Buffered Saline) was injected into the claw of the mice. Cocoonase was injected into mice intracutaneously at doses of 50, 100, 150, 200 μg after 24 h of carrageenan injection. The frequency of infarction and the length of the infarcted region at the tail tip were recorded at 24 and 48 h after cocoonase injection. The control group was inoculated with PBS.

2.9. Blood-clot lysis activity assay

An in vitro thrombolysis effect of cocoonase was estimated by mice blood-clot lysis activity assay. Briefly, fresh peripheral blood was drawn from adult mice without any anticoagulants. After natural blood coagulation, serum was completely removed after clot formation. The blood clots were weighed and transferred to 1.5 mL eppendorf tubes. The blood clots were then incubated with purified cocoonase (20 or 30 μg) at 37 °C for 3 h. PBS alone and urokinase (20 U/tube) were tested as negative control and positive control. Lysed fluid was completely absorbed from each tube with filter paper and the blood clots were then re-weighed. Weight differences before and after incubation were calculated and the dissolution rates in treated and untreated samples were recorded.

2.10. Hemorrhagic activity

Hemorrhagic activity was evaluated using a modified skin test procedure described by Kondo et al. [20]. Briefly, different doses of cocoonase (0.5, 1.25, 2.5 mg/kg body weight) were intradermally injected into the dorsal depilated skin of mice (20–22 g). Mice were injected with 0.1 ml of physiological saline solution as controls. Twenty-four hours after cocoonase injection, the skins were removed and diameters of the hemorrhagic spots were measured on the inside surfaces. The minimum hemorrhagic dose (MHD) was defined as the amount of protease producing a hemorrhagic spot with a diameter of 10 mm.

Table 1
Purification of cocoonase from *Antheraea pernyi*.

Purification step	Total protein (mg)	Esterase activity[a]		Yield (%)	Purification fold
		Specific activity (unit/mg)	Total activity (unit)		
Crude extract	25.6	122.4	3133	100	1
SP FF	0.78	947.2	739	23.6	7.7
Sephadex G-50	0.21	1340	281	9	10.9

[a] Esterase activity was measured by using the esterase activity assay as described above. An enzyme unit was calculated as the amount of enzyme that hydrolyses 1 μM BAEE/min under the conditions as described above.

3. Results

3.1. Purification of cocoonase from the maxillary gland fluid of A. pernyi

Purification of this new proteinase was carried out by the combination of various column chromatographic steps and esterase activity was used as an index for purification. As indicated in Fig. 1A, six protein absorption peaks (280 nm) appeared after flowing past the column of SPFF. The sixth peak (SP-6) contained esterase activity was further purified using gel filtration on a Sephadex G-50 column, which was eluted by 50 mM Tris–HCl buffer, pH 8.0 (Fig. 1B). The fraction of the second eluted peak (Se-2) had maximal esterase activity, and showed a single protein band in SDS–PAGE with molecular weight of approximately 28 kDa (Fig. 1C). The purified protease was designated as *A. pernyi* cocoonase because of its functional homolog with *Bombyx mori* cocoonase [21,22]. The purified esterase (or esterolytic) enzyme activity yields were summarized in Table 1.

3.2. Identification and characterization of cocoonase

The N-terminal amino acid sequence of cocoonase was investigated by automated Edman degradation, and the result is **IVGGY SVTID KAPYQ**. Based on the amino acid sequence of N-terminus, degenerate primers were designed to amplify 3′-RACE fragment encoding cocoonase. Three recombinant clones of 3′-RACE fragment were sequenced and two clones carried identical insert with deduced protein sequences that matched trypsin-like protein sequences in the GenBank. Full-length cDNA sequences of this protein were successfully amplified and sequenced from mRNA using RT-PCR and 5′RACE cloning protocols. The fragment was cloned into the pMD 18-T vector and sequenced. The entire cDNA of cocoonase that contained a 783 bp ORF was assembled (GenBank accession HM011050), and the amino acid sequence was then deduced from the cDNA sequence, which encoding a polypeptide of 261 amino acid residues and containing 8 cysteine residues paired in 4 disulphide bridges. A 20-residue signal peptide (MYKYY FLLLAC AIFWK DGSC) was predicted by using SignalP program [23].

The result of multiple sequence alignment between *A. Pernyi* cocoonase and 7 homologous trypsin-like precursors proteins from other species indicated that the amino acid sequence of *A. pernyi* cocoonase containing the conserved residues of typical trypsin proteinases (Fig. 2A). These residues included a highly conserved trypsin-like N-terminal sequence (IVGG), and three conserved residues of active siteHis77, Asp210, and Ser216, which form the catalytic triad in serine proteinase [24]. Six cysteine residues, predicted to exist in disulfide bridge configurations among trypsins and chymotrypsins, exhibit the same conserved pattern of cysteine residues found in other serine proteinases. The residues Gly233, and Gly243, which define the substrate-binding pocket, are

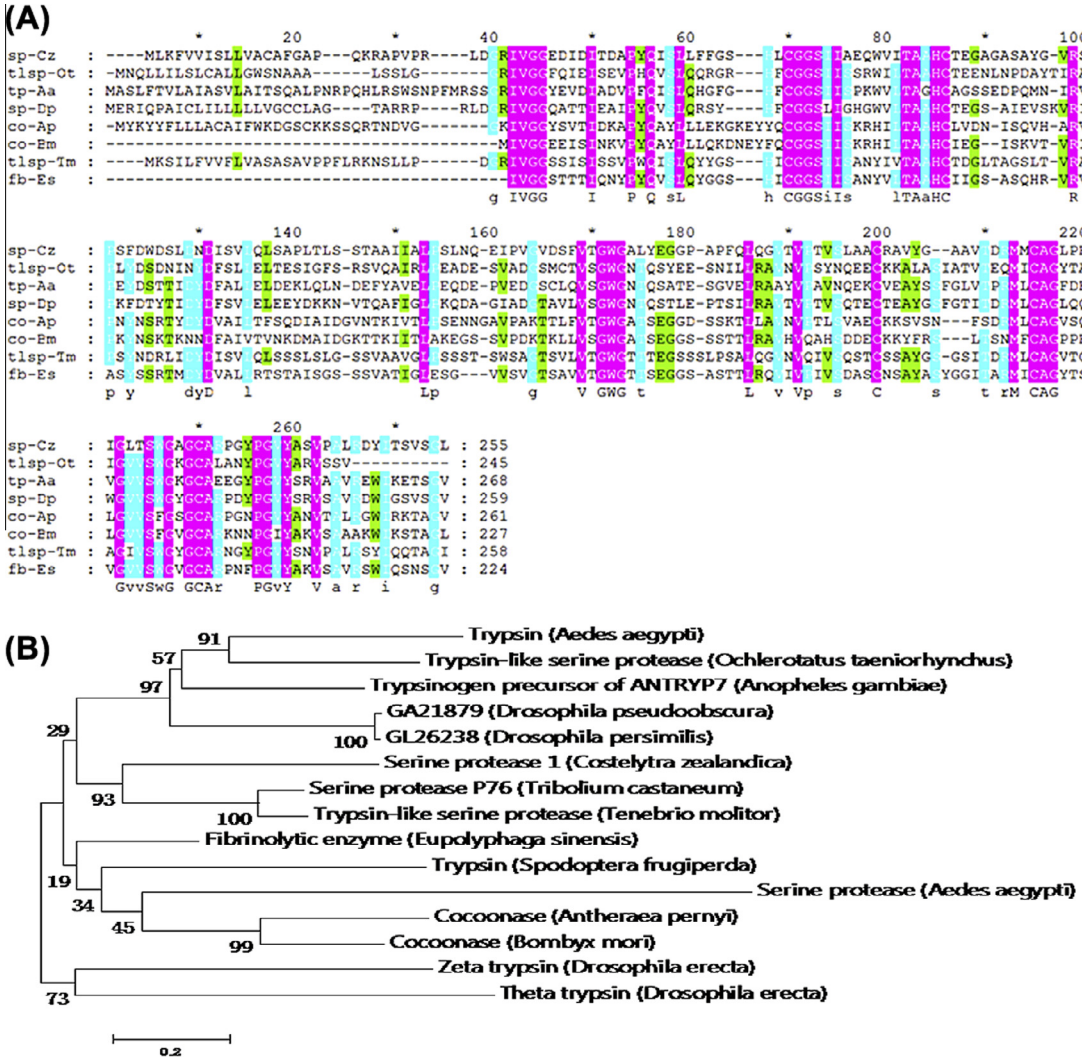

Fig. 2. Phylogenetic tree analysis of cocoonase from *A. Pernyi*. (A) Alignment of the amino acid sequence of cocoonase from *A. Pernyi*with other 7 homologous trypsin-like enzymes from different species. sp-Cz: serine protease from *C. zealandica*, tlsp-Ot: trypsin-like serine protease from *O. taeniorhynchus*, tp-Aa: trypsin from *A. aegypti*, sp-Dp: serine protease from *D.persimilis*, co-Ap: cocoonase from *A. pernyi*, co-Bm: cocoonase from *B.mori*, tlsp-Tm: trypsin-like serine protease from *T.molitor*, fb-Es: fibrinolytic enzyme from *E. sinensis*. (B) The phylogenetic tree was constructed based on their amino acid sequences by the neighbor-joining and pairwise-deletion methods of MEGA computer program. The bold numbers on the branches indicate the proportions of the bootstrap replicates.

conserved in trypsin-like proteinases and Asp 81 was predicted to be located at the bottom of the binding pocket.

Mutual evolutionary relationships of trypsin-like proteinases from different species were analyzed by phylogenetic tree constructed based on their amino acid sequences by the neighbor-joining method. And the phylogenetic tree indicated that *A. pernyi* cocoonase is more related to *B. mori* (Fig. 2B).

3.3. Esterase activity

Cocoonase showed a specific activity of 1.34×10^3 U/mg of enzyme on the trypsin substrate N-benzoyl-L-arginine ethyl ester. One unit of arginine-esterase activity was calculated as the amount of enzyme that hydrolyses 1 μM of BAEE/min under the conditions as mentioned above. The kinetic parameters Km and Vmax of cocoonase on the substrate BAEE were 2.577×10^{-3} mol/L and 4.09×10^{-3} μmol/L/s respectively .

3.4. Fibrinogenolytic and fibrinolytic activities

In order to determine whether cocoonase was potentially capable of interfering with the blood coagulation system, the fibrinogenolytic and fibrinolytic activity of cocoonase was examined. The fibrinogenolytic activity of cocoonase was assayed with bovine fibrinogen (plasminogen free) as substrate and the hydrolysis of fibrinogen was analyzed using SDS–PAGE analysis (Fig. 3A and B). Cocoonase cleaved fibrinogen into multiple distinct fragments. The time course study also revealed that cocoonase hydrolyzed the Aα-chain, Bβ-chain of bovine fibrinogen much faster than the γ-chain, and the γ-chain could be completely cleaved with increasing time (Fig. 3A). The dose-dependent digestion pattern showed that cocoonase degraded preferentially the Aα-chain and Bβ-chain of fibrinogen, while the γ-chain was also substantially cleaved when the concentration of cocoonase was 25 μg/ml or higher (Fig. 3B).

In addition, the fibrinolytic activity of cocoonase was assayed using fibrin agarose plate assay. It showed that the addition of cocoonase led to the formation of a clear hollow (Fig. 3C). As compared with standard urokinase, the fibrinolytic activity of cocoonase was calculated as 487.2 UK/mg.

3.5. In vivo and in vitro thrombolysis activities

In order to investigated whether cocoonase has thrombolysis effect in vivo, a carrageenan-induced mouse tail thrombosis model

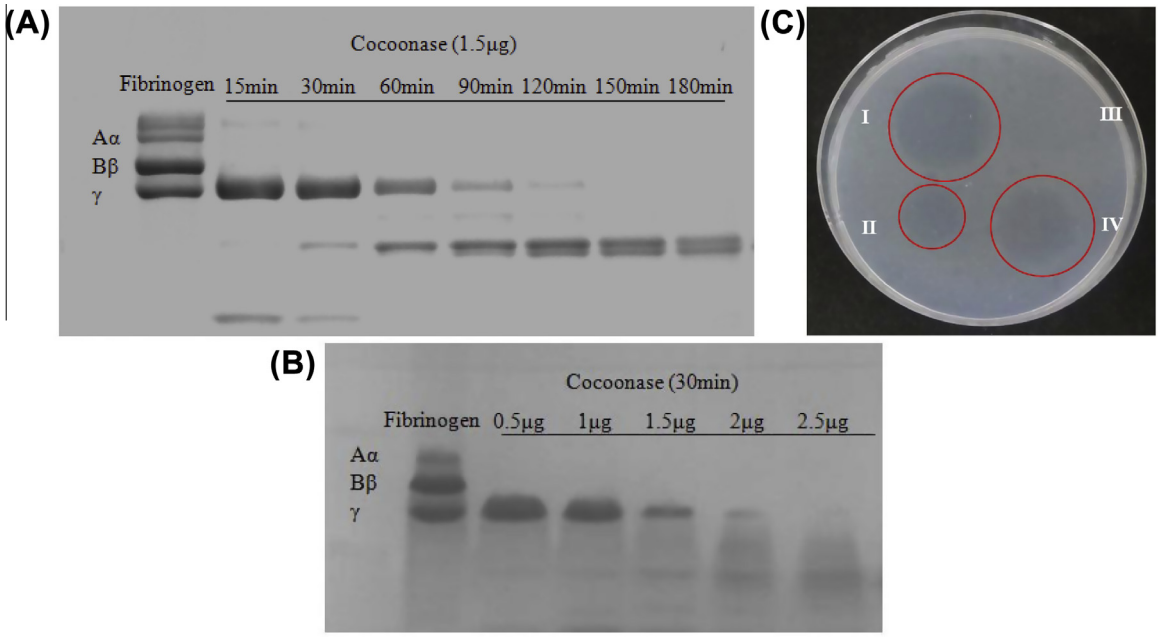

Fig. 3. Analyses of fibrinolytic and fibrinogenolytic activities of cocoonase. (A) An aliquot of 100 μl of bovine fibrinogen was incubated with 1.5 μg of cocoonase for different time intervals (15, 30, 60, 90, 120, 150 and 180 min). Reactions were carried out at 37 °C and then subjected to 10% SDS–PAGE gels, visualized by Coomassie blue. (B) Bovine fibrinogen was incubated with different concentrations of cocoonase (0, 0.5, 1, 1.5, and 2.5 μg) at 37 °C for 30 min, analyzed by SDS–PAGE (10% gel). (C) Detection of the enzymatic activity of cocoonase on fibrin plate. The samples were dropped onto the fibrin plate and incubated at 37 °C for 6 h. (I) cocoonase (0.5 mg/ml); (II) cocoonase (0.25 mg/ml); (III) Tris–HCl buffer (Negative control); (IV) 5U urokinase (Positive control).

Table 2
Effect of cocoonase on mice tail thrombosis in vivo ($n = 3$).

Groups	Dose (μg/mice)	Length of thrombus/length of tail (%) 24 h post-injection	Length of thrombus/length of tail (%) 48 h post-injection
PBS	–	31.69 ± 2.06	30.57 ± 4.30
Urokinase	100 U	28.72 ± 2.24	6.41 ± 5.31**
Cocoonase	50	29.70 ± 3.10	25.45 ± 4.28
Cocoonase	100	29.95 ± 3.76	20.21 ± 5.60
Cocoonase	150	29.46 ± 3.95	13.98 ± 4.05*
Cocoonase	200	28.58 ± 1.61	7.52 ± 1.70**

* $P < 0.05$.
** $P < 0.01$, compared with PBS group, Mean ± S.E. ($n = 3$) are presented.

Table 3
The thrombolytic effect of cocoonase in vitro ($n = 3$).

Groups	Dose	Dissolution rate (%)
PBS	–	5.4 ± 0.8
Urokinase	20 U	74.1 ± 2.4**
Cocoonase	20 μg	91.4 ± 3.1**
Cocoonase	30 μg	100 ± 0**

** $P < 0.01$, compared with PBS group, Mean ± S.E. ($n = 3$) are presented.

was established and used for the tests. An average length of 30.57 ± 4.30% tail length thrombus was formed in saline control group. The thrombus length was reduced to 7.52 ± 1.70%, 13.98 ± 4.05%, 20.21 ± 5.60%, and 25.45 ± 4.28% tail length respectively by cocoonase administration of 200, 150, 100, 50 μg/mice at 48 h post-injection, similar to the effect produced by urokinase in mouse tail thrombosis model (Table 2).

The in vitro thrombolysis effect of cocoonase was estimated by mice blood clots lysis activity assay. As illustrated in Table 3, Fig. 4A and Fig. 4B, cocoonase significantly decreased the thrombus weight in dose-dependent manner, and 30 μg of cocoonase could completely scavenge the thrombus after 3 h treatment. These results were consistent with the fibrinolytic effect of cocoonase on fibrin in vitro.

3.6. Hemolysis safety assay

When intradermally injected through dorsal skin of mice, cocoonase did not induce any myotoxicity, lethality and hemorrhage up to a dose of 1.25 mg/kg body weight and displayed hemorrhagic activity with an MHD of 2.5 mg/kg bodyweight (Fig. 4C). Control mice injected with saline alone showed no hemorrhagic activity (Fig. 4D).

4. Discussion

Thrombosis remains a leading cause of morbidity and mortality throughout the world despite the development of antithrombotic drugs for treatment of arterial and venous thrombosis [1]. A greatest unmet clinical need exists for developing improved antithrombotic drugs because current existing thrombolytics may produce moderate to severe side effects such as bleeding [2]. Therefore, development of effective, yet safe thrombolytics needs to be continued.

Fibrinolytic enzymes have been isolated from various organisms, especially the snake venom, but there are few reports on the enzymes from insects. In this study, a novel direct-acting fibrin(ogen)olytic proteinase (cocoonase) was purified and characterized from the maxillary gland fluid of *A. pernyi* for the first time. Native cocoonase is composed of 261 amino acid residues and containing 8 cysteine residues paired in 4 disulphide bridges. A 20-residue signal peptide is predicted using SignalP program. Fellow by the signal peptide, a N-terminal segment (at positions 21–33) of 13 natural amino acid residues ending in a basic lysine residue, suggesting that tryptic cleavage (Arg-Ile or Lys-Ile bond) might

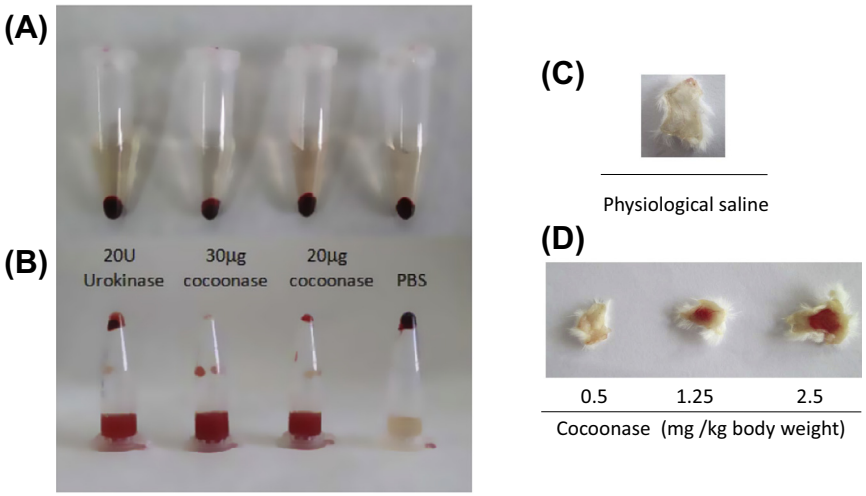

Fig. 4. Blood-clot lysis activity assay and hemorrhagic activity assay. (A) and (B) The blood clots were incubated with purified cocoonase (20 or 30 μg) at 37 °C for 3 h. PBS alone and 20 U urokinase were tested as negative control and positive control. (C) Hemorrhagic activity induced by 100 μl physiological saline. (D) Hemorrhagic activity induced by purified cocoonase at doses of 0.5, 1.25, 2.5 mg/kg body weight respectively.

be involved in activation. The sequence "IVGG" at positions 34–37 is highly conserved in many trypsin and chymotrypsin like proteinases and marks the N-terminal of the active proteinases. BLAST research revealed that it possessed the conserved catalytic triad (His77, Asp210, and Ser216) in serine proteinases. Scan of the PROSITE database reinforced that the deduced cocoonase sequence belongs to the trypsin family of serine proteases which contains a conserved histidine active site sequence pattern [LIVM]-[ST]-A-[STAG]-H-C and a conserved serine active site sequence pattern [DNSTAGC]-[GSTAPIMVQH]-x(2)-[DE]-SG-[GS]-[SAPHV]-[LIVMFYWH]-[LIVMFYSTANQH]. Spatial model revealed that the cocoonase protein folded into a two β-barrel structure bearing thirteen β-strands and two α-helices, and shared remarkable topological similarity with the typical structure of trypsin-like serine proteases.

Cocoonase showed strong activities on both fibrinogen and fibrin (Fig. 3), indicated that cocoonase is a direct-acting fibrino(geno)lytic enzyme like eupolytin1 isolated from mid-guts of the medicinal insect of *E. Sinensi* [10]. No fibrin clot was observed when pre-incubated with fibrinogen, indicating that cocoonase acted directly on fibrinogen in a different manner than thrombin. Fibrinogenolytic activity of cocoonase was inhibited by serine protease inhibitors such as PMSF and DTT reinforcing cocoonase is a trypisin-like serine protease. Different from some known fibrino(gen)olytic enzymes which only hydrolyze Aα- or/and Bβ-chains of fibrinogen, cocoonase is capable of digesting all three chains, Aα-, Bβ-, and γ-chains of fibrinogen and showed no activity on fibrinogen-clotting. Based on its fibrino(gen)olytic activity, we hypothesized that cocoonase had antithrombotic functions both in vitro and in vivo. As illustrated in Table 1, the average length of thrombus in cocoonase injected mice was significantly reduced as compared with control group. The experiments of in vitro blood clot lysis indicating that 30 μg of cocoonase could dissolve the whole blood clot (Table 3) which shown similar effects as urokinase. The mechanism of anti-thrombus can be explained that cocoonase directly degrades fibrinogen and fibrin, and leads to the decrease of blood viscosity, thus prevents the formation of thrombus or dissolves the existed thrombus. The action of cocoonase is independent of plasminogen content in tissues and cocoonase is not inhibited by PAI-1 (type 1 plasminogen activator inhibitor). Since cocoonase could hydrolyze fibrinogen as well as fibrin, it not only hydrolyzes preformed fibrin but also inhibits fibrin generation.

To evaluate the risk of hemorrhage, we performed an in vivo assay by intradermal injection of cocoonase into the dorsal skin of mice. Cocoonase did not induce any myotoxicity, lethality and hemorrhage up to a doses of 1.25 mg/kg body weight indicating that it was devoid of hemorrhagic activity. Because it has a significant specificity for fibrin and naturally expressed in the maxillary gland fluid of *A. pernyi* [13], cocoonase may decrease hemorrhagic risk.

Further investigation is needed to identify the precise mechanisms of degradation of fibrin and fibrinogen, and evaluate the possibility to be used as safe "direct-acting" antithrombotic drug.

In this study, we purified and characterized a novel non-hemorrhagic serine proteinase, cocoonase, from the Chinese oak silkworm *A. pernyi*. Our data indicate that cocoonase may dissolve thrombus by directly degrading fibrinogen and fibrin. Because cocoonase is highly expressed by moths in virtually pure form [13], our findings indicate that *A. pernyi* may have a potential clinical application as a antithrombotic agent.

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