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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, phylogenetic analysis and 3D modeling

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 fibrinogen (0.2% W/V, essentially plasminogen freein 50 mM

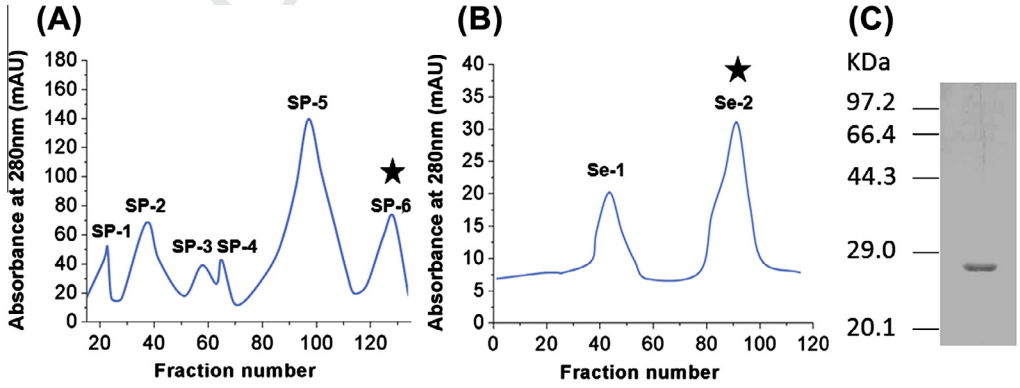

Fig. 1. Purification of cocoonase from the maxillary gland fluid of *A. pernyi*. (A) The supernatant of centrifuged maxillary gland fluid from *A. pernyi* was introduced into a cation-exchange chromatography on a SP FF column. The interesting peak was indicated by a star (SP-6). (B) The active fraction 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.

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