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Activation of prothrombin by two subtilisin-like serine proteases from *Acremonium* sp.

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Abstract

Two novel subtilisin-like serine proteases (AS-E1 and -E2) that activate prothrombin have been identified in a culture of the fungus *Acremonium* sp. The enzymes were purified through repeated hydrophobic interaction chromatography. The N-terminal sequences of AS-E1 (34.4 kDa) and AS-E2 (32 kDa) showed high similarity to the internal sequences of two distinct subtilisin-like hypothetical proteins from *Chaetomium globosum*. Both enzymes proteolytically activated prothrombin to meizothrombin(desF1)-like molecules, while the activation cleavage seemed to occur at a site (Tyr^{316}-Ile^{317}) that is four residues proximal to the canonical Xa cleavage site (Arg^{320}-Ile^{321}). Both enzymes inhibited plasma clotting, possibly due to extensive degradation of fibrinogen and production of meizothrombin(desF1)-like molecule.

Keywords: Subtilase; Subtilisin-like serine protease; Prothrombin; Meizothrombin(desF1); Blood coagulation; Hemostasis

The blood coagulation is an important defense system, protecting the body against blood loss from injured vessels. Prothrombin is a vitamin K-dependent zymogen that is converted to thrombin during the penultimate step of the blood coagulation cascade. Under physiological conditions, prothrombin is activated to thrombin on cell surfaces by the prothrombinase complex consisting of Xa, Va, and phospholipid membranes [1]. Although Xa alone is capable of the activation, the rate is $<10^{-5}$ times as compared to the activation by the prothrombinase [2]. Thrombin promotes plugging of damaged vessels by activating platelets and converting fibrinogen to a fibrin clot [3,4]. In addition to the hemostatic role, thrombin is also involved in the inflammation processes [5]. Proteases from foreign sources are thought to be virulence factors in inflammatory events occurring at infected sites. An example is several snake venom enzymes that activate prothrombin [6]. With regard to microbial proteases that activate prothrombin, only a few enzymes have been studied in detail. These include metalloproteinases from *Staphylococcus aureus* [7] and *Bacillus megaterium* (bacillolysin MA) [8], and cysteine proteinases from *Porphyromonas gingivalis* (gingipains) [9].

In our attempts of a screen of microorganisms that modulate coagulation and fibrinolytic systems, we have found that a fungus produced potent enzymes that activate prothrombin. In the present study, we describe the identification, purification, and properties of two novel enzymes belonging to a family of the serine protease subtilase.

Experimental procedures

Microorganism and purification of AS-E1 and -E2. Strain F11177 was originally isolated from a soil sample and identified as *Aremonium* sp. based on morphological studies and 28S rDNA D1–D2 domain sequence (Supplementary Methods). The enzymes were produced as described in Supplementary Methods. Culture supernatant (451 ml) was brought to

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75% saturation of $(NH_4)_2SO_4$ for 30 min and centrifuged to obtain pellet, which was then suspended with 9 ml of TBS (20 mM Tris–HCl and 150 mM NaCl, pH 7.4). The resulting supernatant was diluted with four volumes of TBS, brought to 40% saturation with $(NH_4)_2SO_4$ and subjected to chromatography on a Butyl-Toyopearl column (20 ml) equilibrated with TBS/$(NH_4)_2SO_4$ (40% saturation). The column was washed sequentially with 63 ml each of TBS containing 40%, 30%, and 20% saturations of $(NH_4)_2SO_4$. Activity was eluted with 63 ml of TBS/$(NH_4)_2SO_4$ (10% saturation). Active fraction was brought to 40% saturation of $(NH_4)_2SO_4$ and subjected to HPLC on a Protein-pack G-butyl column (Waters; 10×100 mm) equilibrated with TBS/$(NH_4)_2SO_4$ (40% saturation). The column was developed for 30 min with TBS/$(NH_4)_2SO_4$ (40% saturation), 60 min with a gradient of $(NH_4)_2SO_4$ (40–0%) in TBS, and 70 min with TBS at a flow rate of 0.5 ml/min, affording two purified proteins with an activity to promote prothrombin activation.

Enzyme assay. For determination of amidolytic activity, appropriate amount of enzyme was incubated with 0.1 mM Spectrozyme TH (H-D-hexahydrotyrosol-Ala-Arg-*p*-nitroanilide; American Diagnostica, Greenwich, CT, USA) in 50 μl of TBS/T/Ca (TBS containing 0.1% Tween 80 and 2 mM $CaCl_2$) at 37 °C. The hydrolysis of the chromogenic substrate was monitored at 405 nm. For determination of prothrombin activation, enzyme was incubated with 0.1 mM Spectrozyme TH in the presence or absence of 20 nM human prothrombin (Haematologic Technologies, Essex Junction, VT, USA) in 50 μl of TBS/T/Ca at 37 °C, and the change in absorbance at 405 nm was measured. The differences between values obtained in the presence and absence of prothrombin were plotted against t^2 to obtain initial rate of generation of thrombin activity, which was normalized using human α-thrombin (Sigma, St. Louis, MO, USA) as standard and expressed as α-thrombin equivalent.

Zymography. For casein zymography, AS-E1 (3 ng) or AS-E2 (15 ng) was resolved on nonreduced SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel containing 1 mg/ml casein. After electrophoresis, gels were washed for 30 min with 2.5% (wt/vol) Triton X-100 twice and then incubated in TBS/T/Ca for 16 h at 37 °C. After staining with Coomassie brilliant blue R-250, proteolytic activity was observed as a clear area on a blue background. For fibrinogen zymography, prothrombin (2 μM) was treated with 3 nM of either AS-E1 or -E2 at 37 °C for 30 min, and aliquot of the mixture was subjected to nonreduced SDS–PAGE on a 12.5% gel. The gel was washed as described above and finally washed thrice with water, followed by overlaying the gel to a fibrinogen indicator gel [2 mg/ml human fibrinogen and 0.8% agarose in 75 mM Tris–HCl and 22 mM NaCl, pH 7.8] [10]. After incubation at 37 °C for 18 h, the indicator gel was processed for image scanning.

Characterization of prothrombin cleavage. Prothrombin (2 μM) was incubated with either AS-E1 or -E2 at 37 °C for 30 min in TBS/T/Ca and then subjected to reduced or nonreduced SDS–PAGE on 12.5% gels. When using Xa (25 pM), the reaction mixture was further supplemented with Va (100 pM) and 50 μM phosphatidylcholine–phosphatidylserine (3:1, mol/mol). Where indicated, 3 μM dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA; Haematologic Technologies) was included in the reaction mixture to inhibit thrombin-mediated cleavages.

Measurement of clot formation. Citrated human plasma (200 μl) was mixed with 60 μl of AS-E1 or -E2 (0.07–4 μM) in PBS. Plasma clot formation was initiated by adding 40 μl of 133 mM $CaCl_2$, and thrombelastogram was recorded at 37 °C on a ROTEM Gamma (Pentapharm, Munich, Germany). For measurement of fibrinogen clotting, 120 μl of 10 mg/ml human fibrinogen and 120 μl of varying concentrations of AS-E1 or -E2 were mixed in TBS/T/Ca at 37 °C for 30 min. Clot formation was initiated by adding 60 μl of 10 nM α-thrombin. Turbidometric assay for fibrinogen clotting was assayed in microplates using similarly prepared reaction mixtures. After addition of α-thrombin, the change in absorbance at 630 nm was monitored.

Results and discussion

From a screen of microorganisms that modulate coagulation and fibrinolytic systems, we identified a soil isolate F11177 as a producer of a potent activity that promoted prothrombin activation. The strain was taxonomically classified based on morphological studies and nucleotide sequence of the D1–D2 domain of 28S rDNA (see Supplementary Methods). The D1–D2 domain sequence was highly similar (98% identity) to the sequence of the ascomycetous fungi *Chaetomium* species, including *C. globosum*. The morphological studies, however, suggested that strain F11177 did not have characteristic ascospores but had features of the genus *Acremonium* sp. Therefore, the strain was identified as *Acremonium* sp. F11177, an anamorph of *Chaetomium* sp. When incubated with prothrombin, the culture supernatant had significant activity to hydrolyze Spectrozyme TH (a chromogenic substrate for thrombin), while slight activity was seen in the absence of prothrombin (Fig. 1A). The activity to promote prothrombin activation could be obtained by subtracting values obtained with the incubation in the absence of prothrombin from values obtained in its presence, followed by plotting the differences versus t^2 (Fig. 1A, *inset*).

Purification of AS-E1 and -E2

From 451 ml of culture supernatant, 120 and 570 μg of AS-E1 and E2 were purified through salting out and repeated hydrophobic interaction chromatographies (Fig. 2B), where AS is named after "*Acremonium* subtilase". Both proteins were nearly homogeneous on both reduced and nonreduced SDS–PAGE (Fig. 1C), with apparent molecular masses of 34.4 kDa (AS-E1) and 32 kDa (AS-E2), as judged from reduced gels. The N-terminal sequences of both proteins partly resemble to each other (Fig. 1D and E), and following database search suggested that the two proteins belonged to a family of subtilisin-like serine proteases, called subtilases (Supplementary Fig. 1). Each enzyme appeared to form a dimer (as well as oligomers) as judged from size-exclusion chromatography (Fig. 1F). The zymography on casein gel suggested that the major active enzyme species was a dimer for AS-E1, while additional active smear bands corresponding to multimers were seen in AS-E2 (Fig. 1G).

Identification of AS-E3 from cDNA cloning

An initial database search with AS-E1 N-terminal sequence identified several related sequences. From the consensus of these sequences, we designed several combinations of degenerate oligonucleotide primers to clone cDNAs by reverse transcription-polymerase chain reaction on RNA from strain F11177. One combination resulted in the production of a cDNA segment, from which a full-length clone was obtained (see Supplementary Methods). Amino acid sequence deduced from the cDNA matched the N-terminal sequence of neither AS-E1 nor -E2. Therefore, the cDNA was designated AS-E3. From recent database search, we found that AS-E3 was highly similar to a hypothetical protein from *C. globosum*, Q2H5N4 (TrEM-