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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

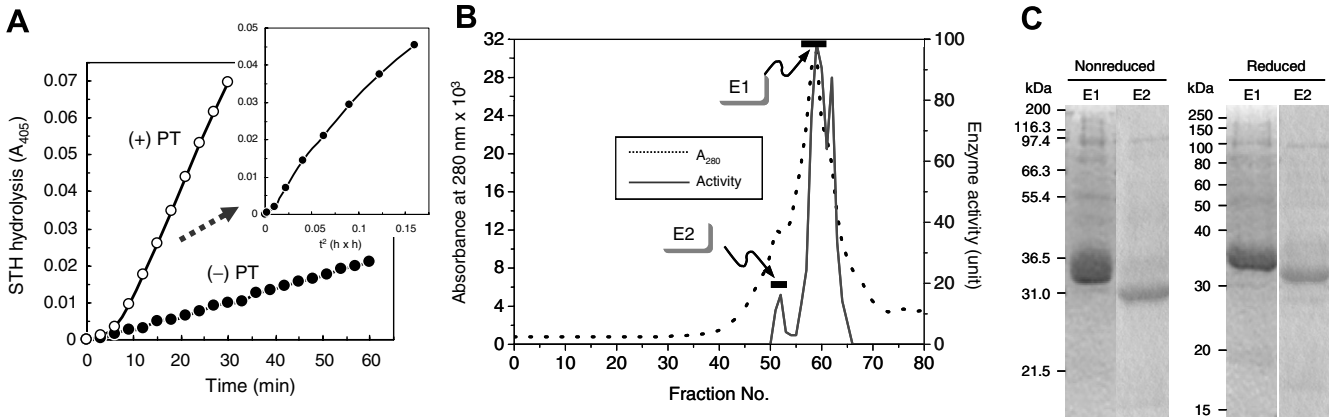
From a screen of microorganisms that modulate coagulation and fibrinolytic systems, we identified a soil isolate F11177 as a producer of a potent activity that promoted prothrombin activation. The strain was taxonomically classified based on morphological studies and nucleotide sequence of the D1–D2 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-



D

Cycle	1	2	3	4	5	6	7	8	9	10
Amino acid	D	N	V	P	W	G	L	A	R	I
Recovery (pmol)	12.4	9.5	12.3	8.2	7.9	6.1	8.0	11.5	5.3	11.1
	11	12	13	14	15	16	17	18	19	20
	S	H	R	T	T	G	A	T	S	Y
	3.7	3.8	4.3	3.3	4.3	5.2	10.0	3.0	2.9	4.3
	21	22	23	24	25	26	27	28	29	30
	V	Y	D	D	S	A	G	E	G	T
	6.8	5.1	5.2	6.5	2.9	8.3	3.5	1.6	3.2	2.4
	31	32	33	34	35	36	37	38	39	40
	C	S	Y	I	I	D	T	G	I	Y
	-	2.0	2.5	2.9	2.9	3.1	1.6	3.1	2.2	1.9

E

Cycle	1	2	3	4	5	6	7	8	9	10
Amino acid	A	Y	V	S	Q	S	G	A	P	W
Recovery (pmol)	13.2	22.6	23.4	10.2	19.3	8.9	9.9	13.4	10.7	8.0
	11	12	13	14	15	16	17	18		
	G	L	G	R	I	S	H	K		
	9.6	11.8	10.4	6.9	14.2	3.5	3.3	5.3		

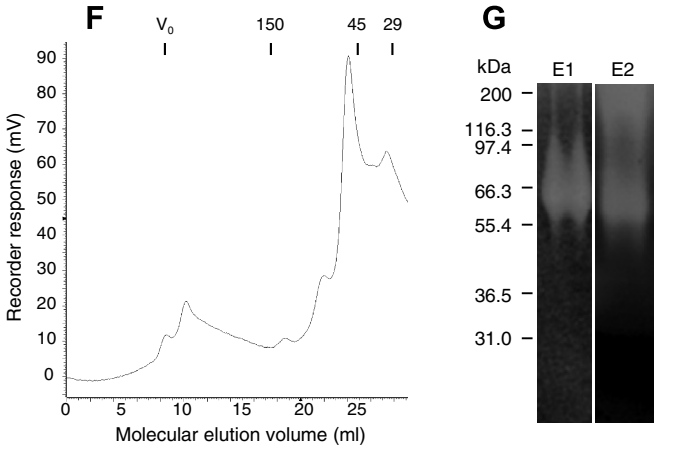


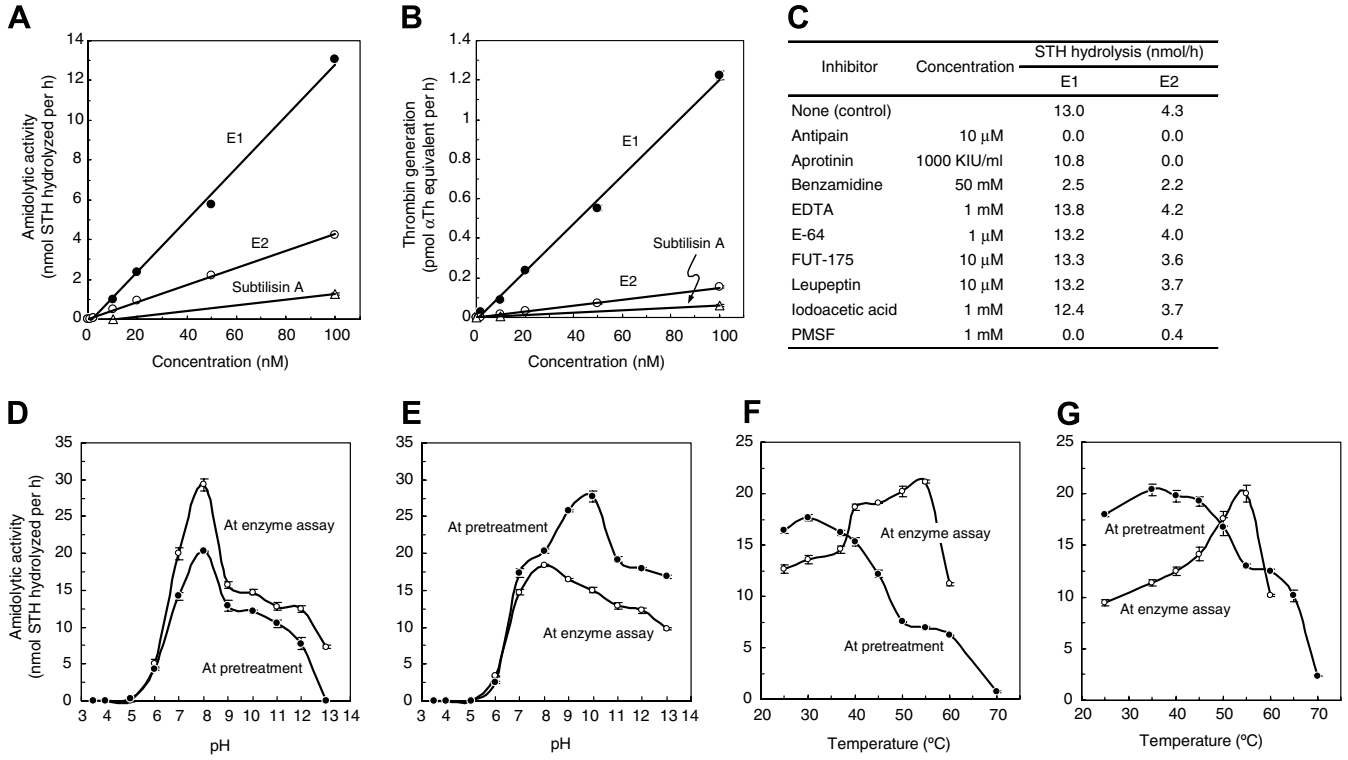
Fig. 1. Purification of proteases that activate prothrombin. (A) A representative result of a screen for prothrombin-activating activity. Culture supernatant (0.01 μl) from *Acremonium* sp. F11177 was incubated with Spectrozyme TH (STH) in the presence or absence of prothrombin (PT). The difference between the two values ["(+) PT" subtracted by "(−) PT"] was also plotted against t^2 (*inset*) to obtain initial rate of thrombin activity generation. (B) Final enzyme purification was achieved by chromatography on a Protein-pack G-butyl column. Bars represent fractions pooled for AS-E1 and -E2. Bar represents fractions pooled. One unit is defined as the activity that hydrolyzes 1 nmol of STH per hour under the standard assay conditions. (C) Purified AS-E1 (7.5 μg) and -E2 (3 μg) were treated with 2 mM PMSF (to prevent enzyme autoproteolysis), boiled for 5 min in SDS-sample buffer and subjected to SDS–PAGE under nonreducing and reducing conditions. (D,E) N-terminal amino acid sequences of AS-E1 and -E2. (F) Size-exclusion chromatography of AS-E2. (G) Casein-gel zymography after resolving on nonreduced SDS–PAGE. The samples were treated neither with PMSF nor boiling before electrophoresis.

BL) (Supplementary Fig. 1). Similarly, 38/40 and 15/18 of amino acids in N-terminal sequences of AS-E1 and -E2, respectively, were found to be identical to internal sequences of two other distinct hypothetical proteins from *C. globosum* [Q2GUF9 (TrEMBL) and Q2GYU7 (TrEMBL), respectively]. Thus, we concluded that AS-E1, -E2, and -E3 were variants of corresponding hypothetical proteins from *C. globosum*, while functional properties of these have remained unknown in spite of their sequence similarity to subtilisin.

Characterization of enzyme activity

Substrate specificity of AS-E1 and -E2 is shown in Supplementary Table 1. Among the chromogenic substrates tested, Spectrozyme TH was the preferred substrate for both enzymes (2.92 and 0.86 nmol/h/pmol enzyme under standard conditions). Succinyl-Ala-Ala-Ala-*p*-nitroaniline was hydrolyzed by AS-E2 (but not by E1) at a significantly slower late (0.06 nmol/h/pmol). As shown in Fig. 2A, the activities of AS-E1 and -E2 to hydrolyze Spectrozyme TH were roughly 10 and 3 times higher than the activity of subtilisin A (0.26 nmol/h/pmol). With regard to prothrombin activation, AS-E1 was 7 times more potent than AS-E2 (Fig. 2B). To date no subtilase had been recognized to activate prothrombin, while the present data suggest that some types of subtilases may activate prothrombin. Indeed, subtilisin A was positive in activating prothrombin, whereas its activity was 1/20 and 1/3 of AS-E1 and -E2, respectively (Fig. 2B).

As shown in Fig. 2C, both enzymes were strongly inhibited by antipain and phenylmethanesulfonyl fluoride (PMSF). Benzamidine (50 mM) inhibited AS-E1 by 80%, while its inhibition of AS-E2 was 49%. On the other hand, AS-E2 was completely inhibited by aprotinin at 1000 KIU/ml, whereas its inhibition of AS-E1 was only partial (17%).



Inhibitor	Concentration	STH hydrolysis (nmol/h)	
		E1	E2
None (control)		13.0	4.3
Antipain	10 μM	0.0	0.0
Aprotinin	1000 KIU/ml	10.8	0.0
Benzamidine	50 mM	2.5	2.2
EDTA	1 mM	13.8	4.2
E-64	1 μM	13.2	4.0
FUT-175	10 μM	13.3	3.6
Leupeptin	10 μM	13.2	3.7
Iodoacetic acid	1 mM	12.4	3.7
PMSF	1 mM	0.0	0.4

Fig. 2. Enzymatic properties of AS-E1 and -E2. Amidolytic activity toward Spectrozyme TH (A) and prothrombin activation promoting activity (B) were determined at the indicated concentrations of each enzyme. (C) The sensitivity of AS-E1 and -E2 to various inhibitors was examined by incubating each enzyme at 100 nM in the presence of the indicated substances under standard conditions using Spectrozyme TH as substrate. (D–G) The enzymatic activities of AS-E1 and -E2 were characterized using Spectrozyme TH as a substrate. (D,E) AS-E1 (100 nM) and AS-E2 (460 nM) were assayed for activity either at the indicated pH (○) or at pH 7.4 after preincubation at the indicated pH for 20 min at 25 °C (●). Buffers used were 200 mM sodium acetate (pH 3.5–5.6), 200 mM sodium phosphate (pH 5.8–8.0), and 100 mM glycine–NaOH (pH 9–13). (F,G) AS-E1 (100 nM) and AS-E2 (460 nM) were assayed for activity either at the indicated temperature (○) or at 25 °C after preincubation at the indicated temperature for 20 min at 25 °C in TBS/T/Ca (●). The concentration of AS-E1 and -E2 at pretreatment was 125 and 575 nM, respectively. Each value represents the mean ± SD from triplicate determinations.

EDTA, E-64, iodoacetic acid, leupeptin, and FUT-175 (6-amidino-2-naphthyl 4-guanidinobenzoate) showed only slight or no effects.

AS-E1 had optima at pH 8 and 55 °C (Fig. 2D and F). AS-E1 kept >50% of its activity after treatment at 25 °C for 20 min at pH 7–11, while the enzyme completely lost its activity by treatment at pH < 5 and 13 (Fig. 2D). When AS-E1 was treated at temperature above 40 °C for 20 min, the enzyme activity was reduced considerably, while the enzyme was stable at 25–40 °C (Fig. 2F). AS-E2 was similar to AS-E1 with respect to pH and temperature optima, whereas AS-E2 showed higher stability to high temperature and pH as compared with AS-E1 (Fig. 2E and G).

The above data suggest that both enzymes are alkaline serine proteases. These results are consistent with the observation that the sequences of AS-E1 and -E2 show significant similarity to sublilases, a broad family of subtilisin-like serine proteases [11].

Prothrombin cleavage

Both enzymes caused specific cleavages of prothrombin at relatively low concentrations (enzyme–substrate ratios of ~1:1000). When analyzed by nonreduced SDS–PAGE, the pattern of cleavage resembled that by factor Xa, an enzyme responsible to physiological activation of prothrombin (Fig. 3A). In spite of close similarities in apparent cleavage patterns, the major active molecular species generated by AS-E1 and -E2 were meizothrombin(desF1)-like molecules (molecular mass around 56 kDa) as judged from fibrinogen zymography (Fig. 3B). This result indicated that AS-E1 and -E2 cleaved prothrombin at sites distinct from those cleaved by Xa. Indeed, the analyses of the cleaved products on nonreduced SDS–PAGE demonstrated unique cleavages (Fig. 3C–G). Factor Xa cleaves prothrombin at Arg^{320}-Ile^{321} to afford meizothrombin as an intermediary thrombin species, which is subsequently cleaved at Arg^{271}-Thr^{272} to produce α-thrombin (Fig. 3C and I). The resulting thrombin cleaves Arg^{155}-Ser^{156} bond to yield the "byproducts" F1 and F2. AS-E1 seemed to cleave Arg^{155}-Ser^{156} first to afford fragments 1 and 3 (Fig. 3D, F, and I). This notion was supported by the observation that the addition of DAPA, a specific inhibitor of thrombin, did not affect the cleavage pattern (Fig. 3D, right panel). AS-E1 caused additional cleavages at Thr^{45}-Ala^{46}, Tyr^{316}-Ile^{317}, and an unidentified site in the protease domain, yielding the additional frag-

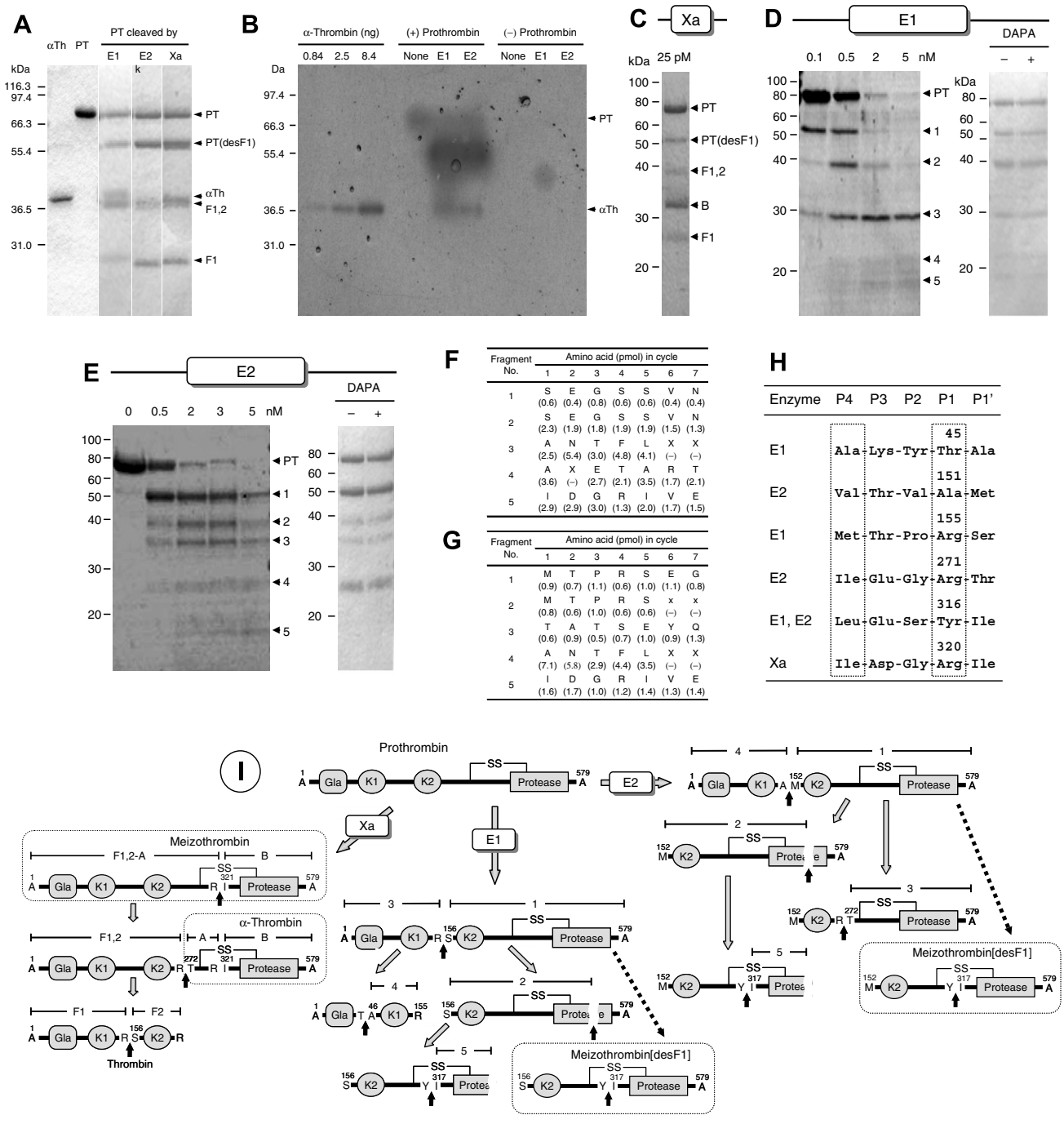


Fig. 3. Characterization of prothrombin cleavage by AS-E1 and -E2. (A) Prothrombin (2 μM) was incubated at 37 °C for 30 min with 2 nM AS-E1, 3 nM AS-E2 or 0.025 nM Xa with 0.1 nM Va and 50 μM phospholipids (prothrombinase complex), and aliquots were subjected to nonreduced SDS–PAGE. The positions of prothrombin (*PT*), α-thrombin (*αTh*), as well as prothrombin fragments generated by the Xa cleavage [prothrombin(desF1), F1,2 and F1] are shown. (B) Samples prepared as described in (A) were subjected to fibrinogen zymography. The amount of prothrombin degradation products applied to electrophoresis was 1.44 μg. As controls, no enzyme reaction mixture and reaction mixtures without prothrombin were also resolved. (C–E) Prothrombin that was incubated with enzymes as described in (A) was resolved on reduced SDS–PAGE. Where indicated, DAPA was included during the reaction. The positions of thrombin B-chain (given as B) and other fragments are shown. Prothrombin fragments generated by AS-E1 and -E2 are numbered. (F,G) N-terminal amino acid sequences of the numbered fragments are shown. (H) Amino acids near the sites cleaved by AS-E1 and -E2 are shown. (I) Possible structures of prothrombin fragments generated by cleavages with AS-E1 and -E2 as well as with Xa are shown schematically. *Solid line* represents a polypeptide chain. Domain structure is represented as a circle or a box: *Gla*, Gla domain; *K*, kringle domain; *protease*, serine protease domain. *Solid arrow* denotes cleavage site. *Dashed box* shows active enzyme species, while meizothrombin(desF1) is hypothetical.

ments 2, 4, and 5 (Fig. 3D). The cleavage site at Tyr^{316}-Ile^{317} is four residues proximal to the canonical Xa cleavage site (Arg^{320}-Ile^{321}). Although no fragment corresponding to thrombin B-chain was detected, we propose that the active molecular species generated by AS-E1-mediated prothrombin cleavage be a meizothrom-

bin(desF1)-like molecule with Ile^{317} as an N-terminus of B-chain (Fig. 3I) from the following notions. First, an activity migrates to a position similar to that of meizothrombin(desF1) on fibrinogen zymography (Fig. 3B). Second, a fragment with Ile^{317} as an N-terminus (fragment 5) can be found even after extensive degradations of prothrombin with high concentrations of AS-E1 (Fig. 3D), but any fragments starting with Ile^{321} are not found.

AS-E2 produced similar results as compared with those produced by AS-E1, while some positions of cleavage were different. The first cleavage seemed to occur at Ala^{151}-Met^{152} (instead of Arg^{155}-Ser^{156} by AS-E1), affording fragments 1 and 4. In addition, a unique cleavage at Arg^{271}-Thr^{272} to yield fragment 3 was observed (Fig. 3E, G, and I). These cleavages seemed to be due to AS-E2 activity itself because DAPA did not affect the proteolysis pattern (Fig. 3E, right panel). The AS-E2-mediated prothrombin activation products may also be the meizothrombin(desF1)-like molecule from similarity to the results obtained with AS-E1 (Fig. 3I).

Fig. 3H summarizes the positions of prothrombin cleavage by AS-E1 and -E2. Subtilisin, a canonical subtilase, recognizes hydrophobic and aromatic amino acids both at P1 and P4 [12]. On the other hand, kexins, which comprise the family E member of the subtilase superfamily, recognize Arg at P1. Kexins are characterized as that these have Asp^{166} at the bottom of the S1 pocket (instead of Gly^{166} in subtilisin) [11]. Thus, amino acid at position 166 in subtilases (the numbering is based on Ref. [11]) is important in determining specificity at P1. With respect to AS-E1 and -E2, amino acid at that position is predicted to be Tyr^{166} (residue 331 with symbol # in Supplementary Fig. 1). The two enzymes apparently recognized hydrophobic amino acids at P4, whereas their P1 recognition seemed to be less stringent, and they accepted even polar or basic residues at P1 (Fig. 3H).

Effects on plasma clotting

We asked whether AS-E1 and -E2 promote blood coagulation because the two enzymes directly activate prothrombin. We first tested their effects on clotting of human plasma using thrombelastography. AS-E1 and -E2 did not enhance but inhibit coagulation of plasma (Fig. 4A): no clot was formed at higher concentrations (>460 nM). When fibrinogen alone was used to monitor

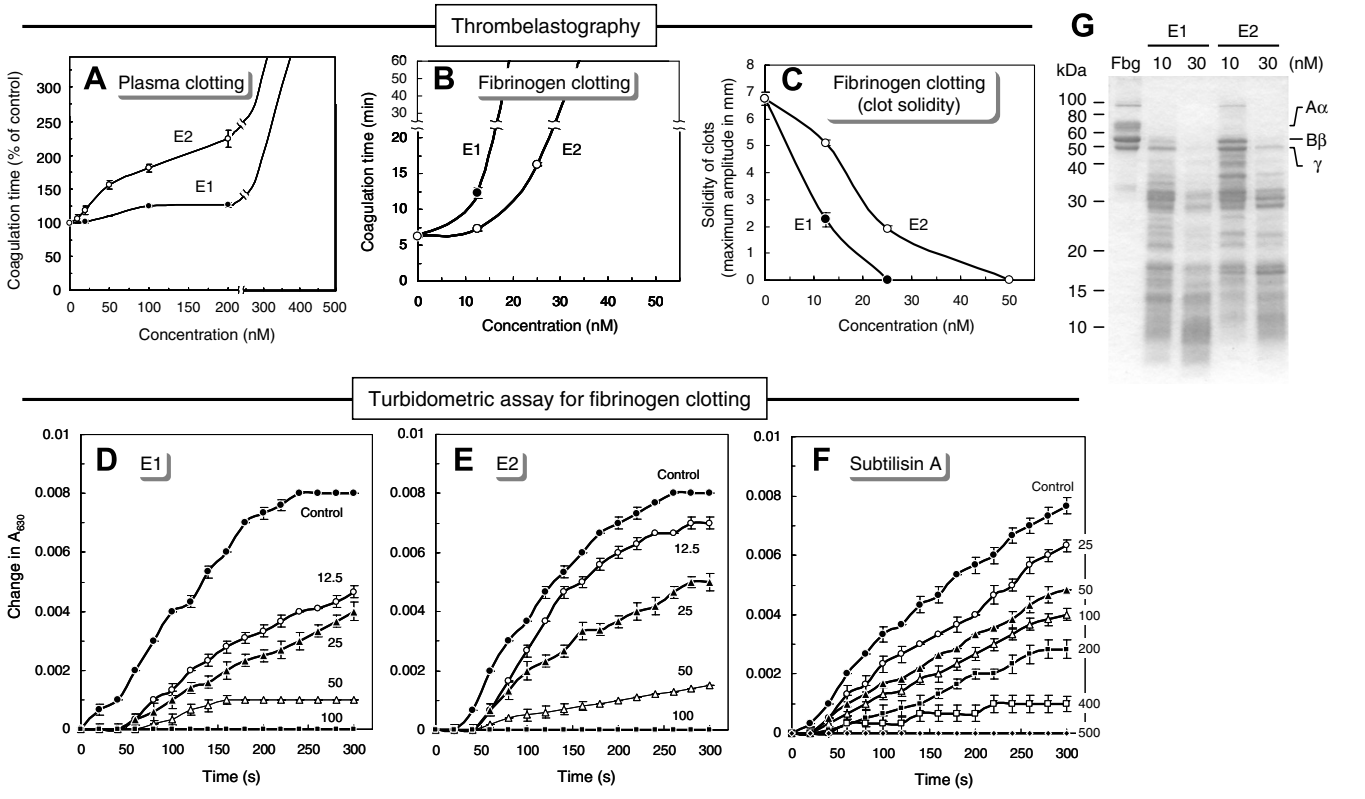


Fig. 4. Effects of AS-E1 and -E2 on the clotting of plasma and fibrinogen. (A) AS-E1 and -E2 at the indicated concentration was added to human plasma, and subjected to thrombelastography. Parameter shown is coagulation time (time required for the initiation of clot formation). Control coagulation time was 10.5 ± 2.5 min. (B,C) Thrombelastography was performed with fibrinogen after treatment with either AS-E1 or -E2 at the indicated concentrations. Coagulation time (B) and clot solidity (maximum amplitude) (C) are shown. Each value represents the average of duplicate determinations. (D–F) Fibrinogen clotting was measured by turbidometry after treatment with AS-E1 (D), AS-E2 (E) or subtilisin A (F) at the indicated concentrations (in nM). Each value represents the mean ± SD from triplicate determinations. (G) Patterns of fibrinogen degradation were examined after treatment with the indicated concentrations of AS-E1 or -E2 for 30 min at 60 °C, followed by reduced SDS–PAGE. The amount of degradation products applied was 10 μg, while the amount of fibrinogen standard (*Fbg*) was 2.5 μg. The positions of Aα, Bβ, and γ chains of fibrinogen are shown.

thrombin-induced coagulation, both enzymes also were inhibitory with respect to coagulation time and solidity of clots formed (Fig. 4B and C). The inhibition of coagulation was further confirmed by a turbidometric assay for fibrinogen clotting (Fig. 4D and E). Subtilisin A also inhibited fibrinogen clotting, although it was several times less potent than AS-E1 and -E2 (Fig. 4F). These results suggested that the inhibition of plasma clotting was in part due to degradation of fibrinogen. Indeed, fibrinogen was highly sensitive to degradation by AS-E1 and -E2, affording similar degradation products (Fig. 4G). Among the three subunits of fibrinogen, Aα chain appeared to be the most sensitive and was completely degraded by AS-E1 and -E2 at 10 nM (enzyme–substrate ratio at ~1:1400 by mol). Because the pattern of the fibrinogen cleavages was so complicated, we could not assign critical cleavage(s) responsible for the impairment of clotting.

The inhibition of plasma clotting by AS-E1 and -E2 can be explained as follows. Prothrombin and fibrinogen are similarly sensitive to cleavage by these enzymes (enzyme–substrate ratio at ~1:1000 is sufficient to specific cleavage), while the major product of prothrombin activation was meizothrombin(desF1)-like molecule. Meizothrombin(desF1) is far less potent than α-thrombin in causing fibrinogen clotting, but these are equally potent in activation of protein C (an anticoagulant zymogen) [13,14]. Thus, meizothrombin(desF1) may behave as an anticoagulant, rather than procoagulant. This notion is demonstrated with prothrombin Dhahran (R271H) and a recombinant mutant prothrombin R157A/R268A. These mutants are activated by Xa to generate a stable form of meizothrombin(desF1) or meizothrombin. Patients with prothrombin Dhahran display impaired blood coagulation [15], and prothrombin R157A/R268A inhibits experimental thrombosis [16]. Therefore, in addition to degradation of fibrinogen, the formation of meizothrombin(desF1)-like molecule may be the mechanism of the plasma clotting inhibition by AS-E1 and -E2.

Although the anticoagulant properties of these enzymes remain hypothetical under physiological conditions, these may play a role in part in tissue invasion of the microorganism in lesions affected by *Acremonium* and *Chaetomium* species, which cause onychomycosis (nail infection), peritonitis, corneal ulcers, and eumycotic mycetoma, as well as opportunistic infections in immunocompromised patients [17,18].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.133.

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