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Activation of prothrombin by ASP, a serine protease released from *Aeromonas sobria*

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Abstract The effect of a serine protease (ASP) secreted from *Aeromonas sobria* on plasma coagulation was investigated. Proteolytically active ASP promoted human plasma coagulation in a dose-dependent manner. Consistent with the preference for a factor Xa-specific oligo-peptide substrate, ASP produced enzymatic activity from human prothrombin but not from factors IX and X. ASP cleaved prothrombin to produce enzymatically active 37 kDa-fragment displaying the same molecular mass as α-thrombin. ASP is the first bacterial serine protease that produces α-thrombin, through which ASP may contribute to the induction of thrombotic tendency in disseminated intravascular coagulation complicated with sepsis caused by *A. sobria* infections.
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1. Introduction

Aeromonas species are facultative anaerobic, Gram-negative rods [1]. *Aeromonas* infection commonly presents through the digestive tract, with associated gastroenteritis and often develops to sepsis [1,2]. Shock and disseminated intravascular coagulation (DIC) are common and deadly consequence in the sepsis patients, with a high mortality rate. As a putative virulence factor we have purified a 65 kDa-serine protease (ASP) from the culture supernatant of *Aeromonas sobria* [3], which is more virulent than other *Aeromonas* species. ASP induces vascular leakage and blood pressure lowering through activation of the kallikrein/kinin system [3]; suggesting an involvement of ASP in the septic shock. Whether ASP is associated with the induction of DIC has yet to be elucidated.

Thrombotic tendency is a prominent clinical feature of DIC that occurs in as many as 40% of sepsis patients and often leads to multiple organ failure [4]. The coagulation system is initiated by complex formation of tissue factor and factor VII/VIIa [5], and mediated by a cascade reaction of the proteolytic activation of factors IX, X and prothrombin, leading to the ultimate product α-thrombin, which converts fibrinogen to a fibrin clot and causes platelet aggregation. It is generally accepted that the DIC associated with sepsis is triggered by the expression of tissue factor on monocytes and endothelial cells activated by endotoxins, and/or cytokines released from various cells, including leukocytes, stimulated by the infection [6]. However, bacterial proteases, released into the circulation during sepsis, possibly activate either of the zymogens of the coagulation system, and ultimately yield α-thrombin. To study the potential virulence activity of ASP through interaction with plasma proteins, we investigated the effect of ASP on activating the coagulation system.

2. Materials and methods

2.1. Materials

Human prothrombin, factors IX and X, were purchased from Enzyme Research Laboratories. Methylsulphonyl-D-cyclohexylglycyl-glycyl-L-arginine-4-methylcoumaryl-7-amide (MS-D-CHG-Gly-Arg-MCA) was purchased from Pentapharm Ltd. (Basel, Switzerland). Other MCA substrates were obtained from the Peptide Institute (Minoh, Japan). Human α-thrombin was purchased from Calbiochem (San Diego, CA, USA). Thromborel® S and STA APTT reagent were purchased from Dade Behring (Eschborn, Germany) and from Diagnostica Stago (Asnières, France), respectively. Horseradish peroxidase conjugated-streptavidin and biotinylated Phe-Pro-Arg-chloromethylketone were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Bachem (King of Prussia, PA, USA), respectively. Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Normal human plasma was prepared by centrifugation of a mixture of 9 volumes of freshly drawn blood from healthy volunteers and 1 volume of 3.8% (w/v) sodium citrate.

2.2. Purification of ASP

ASP was purified from the culture supernatant of a clinically isolated *A. sobria* strain, according to the method described previously [3]. Enzyme preparations demonstrated to be homogenous, revealing a single 65 kDa band on an SDS–polyacrylamide gel under both reduced and non-reduced conditions [3].

2.3. Clotting assay

Plasma coagulation time was measured with KC4Δ (Trinity Biotech, Bray, Ireland) according to the method detailed previously [7].

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2.4. Measurement of enzymatic activity

Ten microliter of an MCA substrate (10 mM) was added to 600 μl of ASP solution (10 nM in 50 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl) and incubated at 37 °C, measuring 7-amino-4-methyl coumarin (AMC) (fluorescence at 440 nm with excitation at 380 nm) with fluorescence spectrophotometer (Model 650-40, Hitachi) monitored with a recorder. For the activated coagulation factor assay, 10 mM Boc-Asp(OBzl)-Pro-Arg-MCA (for thrombin) [8], 10 mM Z-Pyr-Gly-Arg-MCA (for factor Xa) [8] or 5 mM MS-D-CHG-Gly-Arg-MCA (for factor IXa) [9] were used. In the case of the activated factor IX assay, the buffer was supplemented with ethylene glycol (30% final concentration) to enhance the catalytic activity of factor IXa against the substrate [9,10].

Table 1
Cleavage of various oligo-peptide substrates by ASP

Substrate	AMC release (nM/min)
Boc-Ile-Glu-Gly-Arg-MCA	82
Boc-Leu-Ser-Thr-Arg-MCA	64
Pyr-Arg-Thr-Lys-Arg-MCA	45
Boc-Glu-Lys-Lys-MCA	45
Boc-Leu-Lys-Arg-MCA	7.0
Boc-Phe-Ser-Arg-MCA	2.2
Boc-Asp(OBzl)-Pro-Arg-MCA	2.0
Boc-Glu(OBzl)-Ala-Arg-MCA	1.8
Boc-Leu-Thr-Arg-MCA	1.5
Boc-Val-Leu-Lys-MCA	1.3
Boc-Gly-Arg-Arg-MCA	1.0
Z-Pyr-Gly-Arg-MCA	0
Pro-Phe-Arg-MCA	0
Z-Phe-Arg-MCA	0
Boc-Gln-Gly-Arg-MCA	0
Boc-Leu-Gly-Arg-MCA	0
Pyr-Gly-Arg-MCA	0
Arg-MCA	0
Suc-Ala-Ala-Pro-Phe-MCA	1.8
Suc(OMe)-Ala-Ala-Pro-Val-MCA	1.2
Gly-Pro-MCA	0.4
Ac-Asp-Glu-Val-Asp-MCA	0.4

Boc, *t*-buthyloxycarbonyl; Pyr, L-pyroglutamyl; Asp(OBzl), [-(2*S*)-2-amino-3-(benzylocarbonyl)propionyl]; Glu(OBzl), [-(2*S*)-2-amino-3-(benzylocarbonyl)butanoyl]; Z, benzyloxycarbonyl; Suc, succinyl; Suc(OMe), *N*-methoxysuccinyl; Ac, acetyl.

2.5. Blotting of enzymatically active thrombin

Enzymatically active thrombin was detected using biotinylated Phe-Pro-Arg-chloromethylketone [11].

2.6. Statistics

Statistical analysis was performed using an unpaired Student's *t*-test. Values were expressed as means ± S.D. ($n = 4$).

3. Results

3.1. Cleavage of oligo-peptide substrates by ASP

ASP cleaved some substrates with Arg at the P1 site, especially those that contained a hydrophobic amino acid at the P4 site, whilst little or no cleavage was observed for other substrates with Arg at the P1 site (Table 1). In accordance with its high structural similarity to furin [12], ASP also cleaved substrates with paired basic amino acid residues at the carboxy-terminus, but had negligible activity for substrates with an amino acid residue other than Arg at the P1 site.

3.2. Effect of ASP on human plasma coagulation

Human plasma was incubated with ASP and examined APTT for the intrinsic coagulation pathway. ASP shortened APTT in a dose-dependent manner at concentrations as low as 30 nM (Fig. 1A). Since ASP treated with DFP, a serine protease-specific inhibitor, did not affect APTT, the proteolytic activity of the enzyme must be linked to its coagulation promoting effects. ASP exerted a similar effect on the extrinsic coagulation pathway, as seen by PT shortening in a dose- and protease activity-dependent manner (Fig. 1B). ASP itself did not convert human fibrinogen to a fibrin clot (data not shown). These results suggest that enzymatically active ASP can promote plasma coagulation in both of the coagulation pathways.

3.3. Effect of ASP on coagulation factor zymogens

The effect of ASP on both coagulation pathways (Fig. 1A and B) indicates that ASP activates any of factors IX, X, or prothrombin, common factors to both pathways. ASP gener-

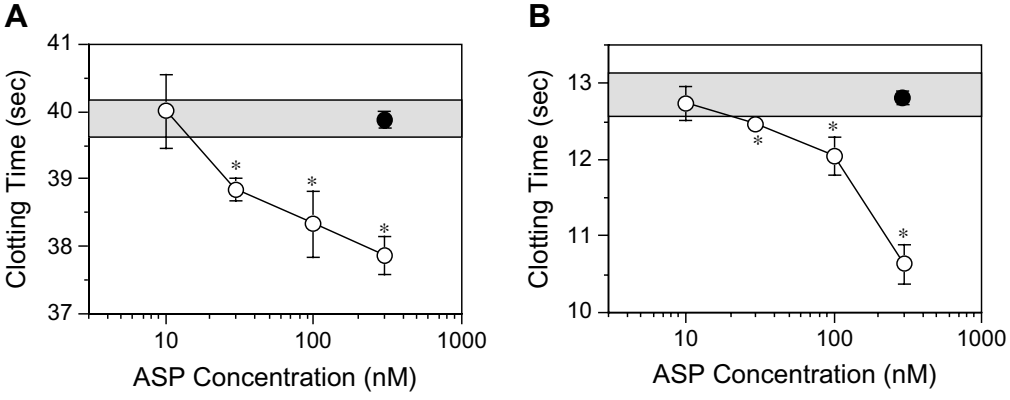

Fig. 1. Promotion of plasma coagulation by ASP. (A) Activated partial thromboplastin time (APTT) of human plasma treated with ASP. Forty-five microliter of plasma and 5 μl of protease were incubated in a plastic cell at 37 °C for 1 min, followed by the addition of 50 μl STA APTT. The mixture was incubated for 3 min, and after the addition of 50 μl of 25 mM $CaCl_2$, the clotting time was measured. (B) Prothrombin time (PT) of human plasma incubated with ASP. Forty-five microliter of plasma and 5 μl of ASP were incubated in a plastic cell at 37 °C for 3 min. Fifty microliter of Thromborel® S was added and the clotting time was measured. ASP concentrations in plasma are shown. (○) non-treated ASP; (●) DFP (1 mM)-inactivated ASP. Dashed lines are the borders of controls which were assayed using 10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl (TBS) instead of ASP. $^{*}P < 0.01$ versus controls.

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