Review

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Aeromonas sobria serine protease (ASP): a subtilisin family endopeptidase with multiple virulence activities

DOI 10.1515/hsz-2016-0344
Received December 29, 2016; accepted April 7, 2017; previously published online April 21, 2017

Abstract: *Aeromonas sobria* serine protease (ASP) is secreted from *Aeromonas sobria*, a pathogen causing gastroenteritis and sepsis. ASP resembles *Saccharomyces cerevisiae* Kex2, a member of the subtilisin family, and preferentially cleaves peptide bonds at the C-terminal side of paired basic amino acid residues; also accepting unpaired arginine at the P_1 site. Unlike Kex2, however, ASP lacks an intramolecular chaperone N-terminal propeptide, instead utilizes the external chaperone ORF2 for proper folding, therefore, ASP and its homologues constitute a new subfamily in the subtilisin family. Through activation of the kallikrein/kinin system, ASP induces vascular leakage, and presumably causes edema and septic shock. ASP accelerates plasma clotting by α-thrombin generation from prothrombin, whereas it impairs plasma clottability by fibrinogen degradation, together bringing about blood coagulation disorder that occurs in disseminated intravascular coagulation, a major complication of sepsis. From complement C5 ASP liberates C5a that induces neutrophil recruitment and superoxide release, and mast cell degranulation, which are associated with pus formation, tissue injury and diarrhea, respectively. Nicked two-chain ASP also secreted from *A. sobria* is more resistant to inactivation by α_2-macroglobulin than single-chain ASP, thereby raising virulence activities. Thus, ASP is a potent virulence factor and may participate in the pathogenesis of *A. sobria* infection.

Keywords: C5a; chaperone; kinin; shock; thrombin; vascular leakage.

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Introduction

Aeromonas species is a genus of facultative anaerobic, waterborne Gram-negative rods (Jones and Wilcox, 1995), and most commonly implicated as a cause of gastroenteritis (Janda and Brenden, 1987; Farraye et al., 1989; Deutsch and Wedzina, 1997; Schiavano et al., 1998). *Aeromonas* infections are caused via wounds or the digestive tract, and often develop into systemic infections, such as peritonitis, meningitis, pneumonia and sepsis (Janda and Abbott, 1998). The majority of patients inflicted with *Aeromonas* sepsis are infants under 2 years of age and immunocompromised adults with multiple underlying medical complications that include mostly malignancy, hepatobiliary diseases, or diabetes. Mortality rates for the patients with sepsis generally range from 25% to 50% (Janda et al., 1994; Duthie et al., 1995). Patients with severe myonecrosis caused by this bacterium also develop sepsis, and more than 90% of the patients die (Janda and Abbott, 1996; Lin et al., 1996). Septic shock is a major cause of death of these patients, but the mechanisms underlying this fatal complication have not been fully elucidated.

Three *Aeromonas* species, *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* were mainly recovered from clinical samples (Janda et al., 1984). *Aeromonas hydrophila* (50%) and *A. caviae* (72%) were most commonly recovered from the gastrointestinal tract and wounds, whereas *A. sobria* was more often associated with the gastrointestinal tract and bacteremia (68%). *Aeromonas sobria* along with *A. hydrophila* accounted for 88% of all blood isolates (Janda et al., 1984) and *A. sobria* is more pathogenic than *A. hydrophila* (Daily et al., 1981).

Aeromonads produce a number of putative virulence factors, including hemolysins, enterotoxins, and proteinases (Janda, 1991). A serine protease, referred to as *A. sobria* serine protease (ASP), was previously cloned from *A. sobria*, and recombinant protein was subsequently purified from the culture supernatants of bacteria transformed with an expression plasmid (Okamoto et al., 2000; Kobayashi et al., 2009a). Using the recombinant

protein, we investigated the role of ASP in the pathogenesis of diseases caused by *A. sobria*, and obtained evidence strongly suggesting that ASP may be a virulence factor of this pathogen. In this review, we will describe the physicochemical properties, maturation process and virulence activities of ASP.

Activity and substrate specificity

ASP shows its highest activity at pH 7.5 and loses its activity after heating at 60°C for 10 min (Yokoyama et al., 2002). ASP is inactivated by serine protease inhibitors, such as {4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)} and diisopropyl fluorophosphate (DFP), but not by metalloprotease inhibitors such as EDTA, EGTA, 1,10-phenanthroline and phosphoramidon, or the cysteine protease inhibitors such as [E-64, *N*-ethylmaleimide (NEM)] and [*p*-chloromercuribenzoate (PCMB)] (Yokoyama et al., 2002). From the response to these inhibitors, ASP is determined as a serine protease, but it is resistant to soybean trypsin inhibitor (SBTI). Kex2 is a serine protease isolated from *Saccharomyces cerevisiae* (Siezen and Leunissen, 1997). Because of a similarity in the substrate specificity and the structure, ASP and Kex2 (Kobayashi et al., 2009a) are assigned into the kexin subfamily of the subtilisin family (Siezen and Leunissen, 1997). Like kex2, ASP preferentially cleaves peptide bonds at the C-terminal side of paired basic amino acid residues, especially Lys, rather than Arg, at the P_1 site (Kobayashi et al., 2006), but accepts both amino acid residues (Kobayashi et al., 2006; Nitta et al., 2007; Murakami et al., 2012a). On the other hand, Kex2 has a strict specificity for Arg at the P_1 site (Rockwell et al., 2002). When arginine residue is present at the P_1 site, ASP does not always require a basic amino acid residue at the P_2 site, as exemplified by its cleavage for t-butyloxycarbonyl(Boc)-Ile-Glu-Gly-Arg-4-methylcoumaryl-7-amide (MCA) and Boc-Leu-Ser-Thr-Arg-MCA (Nitta et al., 2007; Murakami et al., 2012a). In these substrates, amino acid residue is either Gly or Thr at the P_2 site and Ile or Leu at the P_4 site, which is consistent with the preference of subtilisin and kex2 for hydrophobic side chains at the S_4 subsite (Grøn et al., 1992; Rheinnecker et al., 1993; Rockwell and Fuller, 1998), the binding site for the P_4 residue in the protease. ASP exerts no cleavage for Boc-Gln-Gly-Arg-MCA, Boc-Leu-Gly-Arg-MCA or Pyroglutamyl-Gly-Arg-MCA, and poor cleavage for Boc-Leu-Thr-Arg-MCA (Nitta et al., 2007), indicating the importance of hydrophobic residue at the P_4 site in substrate selection by this endopeptidase. As described below, this substrate preference of ASP is closely linked to its virulence that may be associated with the pathophysiology of diseases caused by *A. sobria* infection.

Structure and maturation process

Structural chemistry

As shown in Figure 1, the precursor of ASP is a 624-amino acid protein containing a 24-amino acid signal peptide (Okamoto et al., 2000), a subtilisin-like catalytic domain (431 residues) with two disulfide bridges (Cys_4-Cys_{24} and Cys_{301}-Cys_{326}), and a P-domain (169 residues) at the C-terminus (Kobayashi et al., 2009a). In contrast, the precursor proteins of subtilisin and kex2 contain a long propeptide at the N-terminus but subtilisin lacks the P-domain.

The catalytic triad of ASP is Asp_{78}, His_{115} and Ser_{336} and the consensus substrate-binding sequence is located at Asn_{333}-Gly-Thr-Ser_{336} (Okamoto et al., 2000). The binding site for substrate P_1 residue (S_1 subsite) is constituted with Gly_{179}, Gly_{217}, Glu_{242} and Asp_{247} for ASP, whereas Gly_{274}, Asp_{277}, Gly_{313}, Asp_{320}, Asp_{325} and Glu_{350} for Kex2 (Holyoak

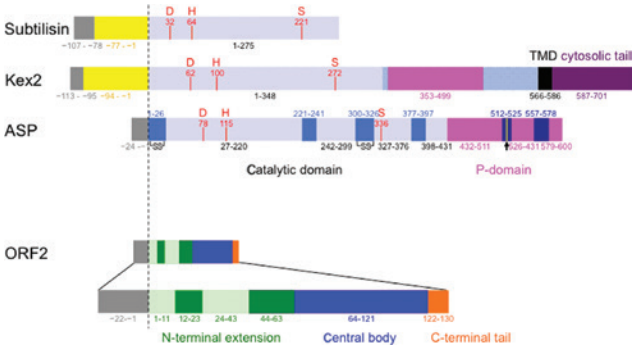

Figure 1: The schematic structures of subtilisin, kex2, ASP and ORF2 precursor proteins.
Subtilisin has only the catalytic domain (faint blue), whereas Kex2 includes the catalytic domain (faint blue), P-domain (magenta), the transmembrane domain (TMD) (black) and cytosolic tail (purple). The signal peptide (gray) and the propeptide (intramolecular chaperone) (yellow) are included in their precursors. ASP precursor is composed of the signal peptide (gray), the catalytic domain (faint purple) with four protrudent subdomains (light blue) and P-domain (magenta) with two occluding subdomains (dark blue). SS and arrow indicate the positions of disulfide bonds and the nick of two-chained ASP, respectively. Residues of catalytic triad are shown in red. The precursor of ORF2, magnified below, was constituted by the signal peptide (gray), the N-terminal extension (green) with flexible subdomains (light green), central body (blue) and the C-terminal tail (orange). Numbers of amino acid residues begin from the N-terminal residues (dashed line) of respective mature proteins.

et al., 2003). ASP resembles Kex2 in the substrate specificity and the structure, but contains additional four protrudent subdomains, Gly_3-Pro_{26}, Asn_{221}-Phe_{241}, Gly_{300}-Cys_{326} and Gln_{377}-Glu_{397}, in the catalytic domain, and two occluding subdomains, Gly_{512}-Thr_{525} and Gly_{557}-Asn_{578}, in the P-domain (Figure 1). Together with the difference in Ca^{2+} binding sites (Kobayashi et al., 2009a), these subdomains influence ASP substrate preference, causing a substrate specificity difference between ASP and Kex2.

Maturation process

As noted above, the precursor proteins of subtilisin and kex2 contain the 77- and 94-amino acid N-terminal propeptides, respectively (Figure 1, shown in yellow). These propeptides are essential for the proper folding of the tertiary structure of the catalytic domain and referred to as 'intramolecular chaperones' (Zhu et al., 1989). Such a propeptide is missing from ASP, instead ORF2 (open reading frame 2), a protein encoded by a gene located just downstream of the *asp* gene, substitutes for the lacking propeptide. In the absence of concomitant ORF2 production, ASP is inactive and degraded inside the cell (Okamoto et al., 2000), indicating the necessity of ORF2 as an external chaperone for constructing the stable (and active) form of ASP.

The ORF2 gene (*orf2*) is composed of 456 base pairs and encodes a protein of 152 amino acid residues (Okamoto et al., 2000) (Figure 1). The mature form of ORF2 consists of an N-terminal extension (63 residues), a central body (58 residues), and a C-terminal tail (9 residues) (Figure 1). The latter two are structurally similar between ORF2 and the subtilisin N-terminal propeptide, but the N-terminal extension is absent in the intramolecular chaperone of subtilisin. This N-terminal extension is also absent in other subtilisin-like proteases (Kobayashi et al., 2015). Since deletions in the N-terminal or C-terminal regions of ORF2 significantly lessen the ability of ORF2 to bind ASP and form active ASP, both the N-terminal extension and the C-terminal tail participate in a proper folding of ASP (Kobayashi et al., 2015). The N-terminal extension possesses two flexible subdomains (Figure 1), which, together with the C-terminal tail, another flexible portion, appears to be important for the proper folding of ASP. Indeed, the structure of ASP within the ASP-ORF2 complex was almost identical to that of active ASP, except the active site is blocked by the C-terminal tail of ORF2.

ORF2 is synthesized in the cytoplasm as a precursor with a 22-residue signal peptide (Figure 1) that is requisite for the translocation across the inner membrane. After the removal of the signal peptide the mature form enters the periplasmic space (Nomura et al., 2002). The P-domain is not bound to ORF2 in the ASP-ORF2 complex, however, the maturation of ASP was disturbed when more than six amino acid residues were deleted from the C-terminus of the protease (Kobayashi et al., 2009b). This suggests that ORF2 requires a three-dimensional structure involving His595 of ASP, the sixth residue from the C-terminus, to interact with premature ASP for proper folding. The ASP maturation process that occurs in the presence of ORF2 is as follows: (1) by transcription of the *asp* and *orf2* genes, the proteins are synthesized in the cytosol, (2) after the translocation to the periplasmic space through the inner membrane together with the removal of respective signal peptides, these proteins form a complex and ASP folding occurs with ASP active site blocked by the C-terminal tail of ORF2 in order to protect ASP from digestion by other proteases, (3) through the outer membrane, the complex moves to the extracellular space, where active ASP dissociates from ORF2 presumably due to the alkaline pH milieu and degrades ORF2 (Kobayashi et al., 2015).

Because of a similarity to kex2 in the substrate specificity and the structure, ASP is at present classified into the kexin subfamily of the subtilisin family (Okamoto et al., 2000; Kobayashi et al., 2009a). However, like other proteases in the kexin subfamily, kex2 has an N-terminal propeptide as an intramolecular chaperone (Brenner and Fuller, 1992). Operons consisting of genes homologous to *asp* and *orf2* are found in five genera: *Aeromonas*, *Vibrio*, *Shewanella*, *Chromobacterium*, and *Pseudoalteromonas* (Kobayashi et al., 2015). ASP homologues from these genera lack intramolecular chaperones but possess substitutive external chaperones. Therefore, ASP and its homologues are different from other kexin subfamily proteases in the maturation mechanism, thus constituting a new subfamily of proteases that utilize external chaperones for a proper folding and self-protection during the translocation to the extracellular space.

Virulence activities

Edema and shock induction

The number of cases of *Aeromonas* bacteremia in England and Wales from 1990 to 2004 was between 47 and 116 annually, and the overall incidence was about 1.5 per million population (Janda and Abbott, 2010). *Aeromonas* septicemia is mainly associated with immunocompromised patients, but it can occur in apparently

healthy adults, usually arising from severe wounds infected by aeromonads. In such cases of septicemia, the mortality rate is over 90% (Janda et al., 1996; Lin et al., 1996). Leech therapy has been reported to cause *Aeromonas* septicemia (Haycox et al., 1995). Aeromonads recovered from such diseases have been identified mostly as *A. veronii* bv. *sobria* (‘*A. sobria*’). Septic shock is one of common symptoms of *Aeromonas* septicemia but its underlying mechanism has not been clarified. It is possible that ASP secreted from *A. sobria* acts on plasma proteins during septicemia and plays a critical role in the onset of septic shock.

The activation of plasma kallikrein/kinin system is initiated by the activation of coagulation factor XII (also known as Hageman factor) on a negatively charged surface, which leads to the conversion of plasma prekallikrein to kallikrein that liberates bradykinin (BK) from high molecular weight kininogen (HK) (Cochrane and Griffin, 1982) (Figure 2). The plasma levels of the components of the kallikrein/kinin system are low in sepsis patients (Smith-Erichsen et al., 1982; Kalter et al., 1985; Hesselvik et al., 1989; Pixley et al., 1995), strongly suggesting the activation and subsequent consumption of the components. BK (RPPGFSPFR), the final product of activation of the system, binds to BK-B_2 receptor on vascular endothelial cells (Regoli and Barabé, 1980) and induces vascular leakage (VL). VL at sites of infection or inflammation causes ‘edema’, local swelling. Systemic VL leads to the lowering of blood pressure due to a decrease in circulating blood volume (Figure 2). Indeed, the activation of the plasma kallikrein/kinin system in an animal model caused lethal hypotension (Pixley et al., 1992; Pixley et al., 1993). Hence, activation of the kallikrein/kinin system and/or direct kinin release from kininogens seems to contribute to the pathogenesis of septic shock.

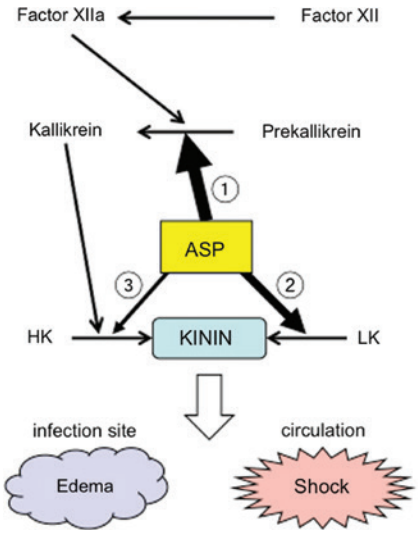

Figure 2: Acting points of ASP in the kallikrein/kinin system. Numbers indicate the order of contribution to kinin generation. Factor XIIa, active factor XII; HK, high moleculer weight kininogen; LK, low moleculer weight kininogen. Kinin release by ASP causes edema at the infection site of *A. sobria* and shock in circulation.

VL induction

We analyzed the role of ASP in the activation of the kallikrein/kinin system (Imamura et al., 2006). Interestingly, ASP, but not DFP-inactivated ASP, induced VL in a dose-dependent manner, starting at an enzyme concentration of 30 nM, after injecting into the guinea pig skin. The VL increased by 1.8-fold at 10 min after ASP injection and disappeared at 30 min. Approximately 90% of the maximal VL detected at 10 min was inhibited by HOE140, a BK B_2 receptor-specific antagonist (Wirth et al., 1991; Rhaleb et al., 1992), and partially by diphenhydramine, a histamine H_1 receptor antagonist, indicating a major contribution of BK to the ASP-induced VL. These results suggested that ASP induced VL through activation of kallikrein/kinin system in the guinea pig, being terminated by α_2-macroglobulin (α_2-MG), a sole ASP inhibitor in plasma (Murakami et al., 2012b). As BK B_2 receptor-dependent VL activity was also generated from human plasma after incubation with ASP (Imamura et al., 2006), ASP can exert the activity in human diseases caused by *A. sobria* infection.

Prekallikrein activation

To examine the role of ASP in the activation of the prekallikrein, we first examined whether ASP activates factor XII or prekallikrein. SBTI, a potent inhibitor for plasma kallikrein (Wuepper and Cochrane, 1972; Imamura et al., 1984), reduced more than 60% of the ASP-induced VL, while not affecting the enzymatic activity of ASP (Yokoyama et al., 2002). An approximately 40% of VL activity production from human plasma by ASP was also inhibited by SBTI, suggesting the activation of human factor XII and/or prekallikrein by ASP. We therefore examined whether ASP activates these factors, and revealed that ASP generated kallikrein activity from human prekallikrein, but no enzymatic activity was detected when human factor XII was incubated with ASP (Imamura et al., 2006). These findings suggest a mechanism that ASP activates only prekallikrein and resulting kallikrein releases BK from HK. As SBTI inhibited ASP-induced VL better than

diphenhydramine, and it also inhibited more than 70% of BK B_2-receptor-dependent VL, BK liberation by kallikrein, which was converted from prekallikrein by ASP, is a major contributor to VL. In fact, the strong substrate preference of ASP for basic amino acid at the P_1 position (Kobayashi et al., 2006) is identical to the cleavage site requisite for prekallikrein conversion to kallikrein (-Ser-Thr-Arg-↓-Ile-) (Chung et al., 1986). No serine protease of the bacteria origin has been reported as a prekallikrein activator; thus, ASP is so far the only bacterial serine proteinase having this activity.

Kinin release from kininogens

Plasma contains two types of kininogens; low molecular weight kininogen (LK) and HK and they may be targets of ASP for direct kinin release (Figure 2). We found that incubation of both types of human kininogens with ASP generated VL activity; it yielded 2- to 3-fold more VL activity from LK than HK (Imamura et al., 2006) when these kininogens were used at their normal plasma concentrations (Müller-Esterl, 1986), indicating the direct kinin release by ASP from kininogens. The cleavage between Arg_{371} and Ser_{372} (Takagaki et al., 1985) for kinin release from HK is supported by the ASP preference for basic amino acid at the P_1 position (Kobayashi et al., 2006). Besides ASP, staphopain A (ScpA) from *Staphylococcus aureus* (Imamura et al., 2005), streptopain (SpeB) from *Streptococcus pyogenes* (Herwald et al., 1996), 56K-protease from *Serratia marcescens* (Maruo et al., 1993), and subtilisin from *Bacillus subtilis* (Maruo et al., 1993) were reported to release kinin directly from human kininogens. Thus, the ability to directly release kinin from kininogens is not unique to ASP and not limited to serine-type proteases from bacteria.

Blood pressure lowering

As ASP can generate kinin, this protease may be a cause of low blood pressure (BP) that occurs in septic shock patients with *A. sobria* septicemia. We tested this possibility by injecting ASP into guinea pigs. Intra-arterial injection of ASP (1.5 nmole/100 µl PBS/kg body weight) induced a BP drop (by 20.3 ± 2.7 mm Hg), which did not occur by enzymatically inactive ASP (by 3.0 ± 3.3 mm Hg) (Imamura et al., 2006). Notably, ASP-induced BP drop was almost completely inhibited by HOE140 (by 4.1 ± 2.5 mm Hg). Thus, we have demonstrated that ASP in the bloodstream can lower BP by releasing kinin.

Kinin generated in the blood stream can bind directly to BK B_2 receptors on endothelial cells and cause BP drop via vasodilation through the production of nitric oxide and prostacyclin (Leeb-Lundberg et al., 2005). We have shown that kinin is a major cause of BP drop by ASP, in contrast to a marginal contribution by the histamine pathway that requires histamine release from mast cells. Thus, ASP secreted from *A. sobria* can generate kinin in the circulation and induce systemic VL and vasodilatation, presumably contributing to the onset of septic shock. The role of ASP in the induction of edema and shock via the kallikrein/kinin system is summarized in Figure 2. The biggest contribution of ASP to kinin release is by the conversion of prekallikrein to kallikrein, followed by direct kinin release from LK and HK in this order. Whether kinin is release at *A. sobria* infection site or the circulation likely determines whether ASP causes edema or septic shock.

Blood coagulation disorder

Thrombotic tendency is a prominent clinical feature of disseminated intravascular coagulation (DIC) that occurs in as many as 40% of sepsis patients and often leads to multiple organ failure (Levi and ten Cate, 1999). The coagulation system is initiated by the complex formation between tissue factor (TF) and factor VII/VIIa (Nemerson, 1988), followed by the proteolytic activation of factors IX, X and prothrombin in this order. The final product α-thrombin causes platelet aggregation and converts fibrinogen to a fibrin clot. It is generally accepted that DIC associated with sepsis is triggered by the expression of TF on monocytes and endothelial cells after stimulation by endotoxins and/or cytokines secreted from various types of cells, including leukocytes (Marshall, 1997). In addition, bacterial proteases, released into the circulation during sepsis, may activate coagulation factors IX, X and/or prothrombin and subsequently generate α-thrombin, accelerating thrombus formation that takes place in DIC. On the other hand, the same proteases may degrade coagulation factors, causing bleeding tendency, a symptom of DIC at a late phase. To determine whether ASP plays a role in blood coagulation disorder, we examined the effects of ASP on human plasma clotting and activation of coagulation factors.

Prothrombin activation

The activated partial thromboplastin time (APTT) is an indicator to evaluate the intrinsic coagulation pathway.

ASP shortened human plasma APTT in a dose- and protease activity-dependent manner at a concentration as low as 30 nM (Nitta et al., 2007). ASP also shortened prothrombin time (PT), an indicator of the extrinsic coagulation pathway. The shortening of both APTT and PT by ASP suggests that this protease promotes plasma coagulation and can participate in the onset and exacerbation of DIC. As ASP itself did not convert human fibrinogen to a fibrin clot, it must have shortened APTT and PT by activating either of factors IX, X, or prothrombin, common factors in both pathways. ASP, but not DFP-inactivated ASP, rapidly generated thrombin activity from prothrombin but no enzymatic activity from factors IX and X (Nitta et al., 2007). Thus, it is clear that ASP directly activated prothrombin by proteolytic action.

In accordance with its ability to rapidly generate thrombin activity from prothrombin, ASP cleaved prothrombin within 1 min and produced fragments of 55, 37, 29 and 24 kDa within 15 min (Nitta et al., 2007). The 37 kDa-product was identified as α-thrombin by two N-terminal amino acid sequences (TATSEYQTFFNPR and IVEGSDAEIGMSPWQVMLFR) which are identical to those of α-thrombin light and heavy chains (Degen et al., 1983), respectively, and by the labeling with biotinyl-ε-aminocaproyl-D-Phe-Pro-Arg-chloromethylketone that specifically binds to the enzymatically active forms of thrombin. These data clearly indicate that ASP promotes coagulation through α-thrombin generation. In comparison to the indirect generation mediated by factor IXa and/or Xa, the direct generation of α-thrombin from prothrombin lessens the inactivation opportunities by plasma protease inhibitors and do not require phospholipids, important cofactors in blood coagulation. Furthermore, platelet aggregation was also induced by ASP-treated prothrombin, which, together with the promotion of coagulation, may lead to thrombus formation, the major cause of death in DIC (Levi and ten Cate, 1999).

Meizothrombin is an intermediate form of thrombin and produced from prothrombin via the cleavage between Arg_{320} and Ile_{321}. It has an enzymatic activity for oligo-peptide substrates without any reduction in its molecular weight (Degen et al., 1983; Rosing et al., 1986). The finding that the 72 kDa-molecule whose molecular weight was almost identical to that of prothrombin was labeled with biotinyl-ε-aminocaproyl-D-Phe-Pro-Arg-chloromethylketone after incubation of prothrombin with ASP (Nitta et al., 2007) suggested that this molecule was meizothrombin. The shift from 72 kDa to 55 kDa appears to occur via cleavage at the site between fragment 1 and fragment 2 (Figure 3), where there is no disulfide bond connection between the two fragments; thus, detaching a molecule containing fragment 1. At this step, the involvement of meizothrombin autolysis (Doyle and Mann, 1990) cannot be excluded. ASP then cuts off fragment 2 from the 55 kDa-product by cleaving between Arg_{271} and Thr_{272}, yielding α-thrombin (Figure 3). β- and γ-thrombin can be generated from α-thrombin through autolysis or limited proteolysis by trypsin and similar enzymes and are enzymatically still active for synthetic oligo-peptide substrates (Chang, 1986). The 29 and 24 kDa-fragments generated by

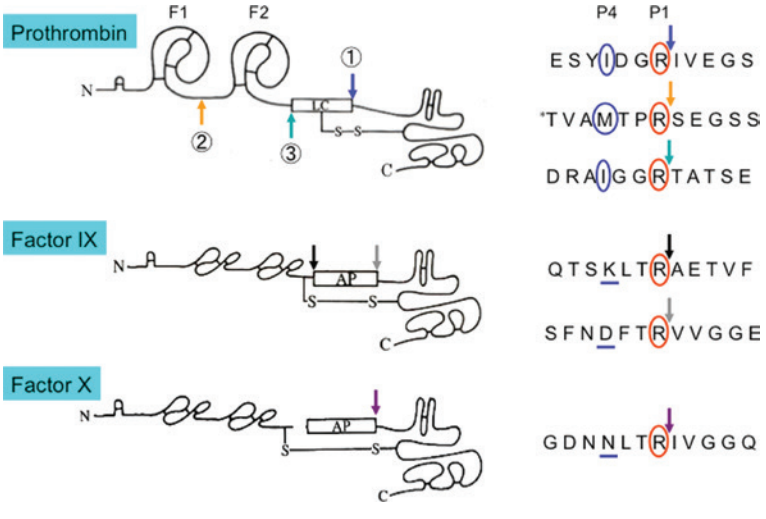

Figure 3: Amino acid sequences around cleavage sites for the activation of three coagulation factors.
Arrows in prothrombin indicate cleavage sites by ASP and numbers show the cleaving order. Arrows in factors IX and X indicate cleavage sites for activation. Arginine residues (R) at the P_1 sites of cleavage for activation are encircled in red. Residues at the P_4 sites are encircles in blue for prothrombin and underlined in blue for factors IX and X. *a putative second cleavage site of prothrombin by ASP. F1, fragment 1; F2, fragment 2; LC, light chain; AP, activation peptide; -S-S-, disulfide bond.

ASP from prothrombin were not labeled with biotinyl-ε-aminocaproyl-D-Phe-Pro-Arg-chloromethylketone (Nitta et al., 2007), which excluded creation of β- and γ-thrombin by the protease.

The ASP-induced cleavage of peptide bonds at the C-terminal side of arginine residues, exemplified by the activation of human prekallikrein (-Arg_{371}-↓-Ile_{372}-) (Imamura et al., 2006), is consistent with the cleavage sites requisite for α-thrombin generation from human prothrombin (-Arg_{271}-↓-Thr_{272}- and -Arg_{320}-↓-Ile_{321}-) (Degen et al., 1983). However, it is puzzling that ASP did not activate factors IX and X, because these factors are also activated by the cleavage at the C-terminal side of arginine residues (Kurachi and Davie, 1982; Leytus et al., 1984). Like subtilisin and kex2 (Grøn et al., 1992; Rheinnecker et al., 1993; Rockwell and Fuller, 1998), ASP prefers hydrophobic amino acid residues at the P_4 site (Nitta et al., 2007; Murakami et al., 2012a). Prothrombin has two target sites for ASP with Ile at the P_4 site, whereas those in factor IX and factor X are Lys or Asp and Asn, respectively (Kurachi and Davie, 1982; Degen et al., 1983; Leytus et al., 1984) (Figure 3). The difference in the P_4 residues, requisite for the activation of these zymogens, explains why ASP activates prothrombin but not factors IX and X.

Cysteine proteases (gingipains-R) secreted from the major periodontal disease pathogen *Porphyromonas gingivalis* (Imamura et al., 2001) and metalloproteases from *Staphylococcus aureus* (Wegrzynowicz et al., 1981) or *Vibrio vulnificus* (Chang et al., 2005) have been shown to activate human prothrombin. Metalloproteases from *Serratia marcescens* and *Pseudomonas aeruginosa* are capable of releasing thrombin activity from bovine prothrombin (Kaminishi et al., 1994). No serine protease of bacterial origin has been reported as a prothrombin activator; thus, ASP is currently the only bacterial serine protease that can activate this coagulation factor.

Fibrinogen degradation

Fibrinogen, a plasma protein, is converted by thrombin to a fibrin clot (Mann and Lundblad, 1987) that plugs bleeding sites, thereby protecting the host from blood loss. Therefore, degradation of either fibrinogen or fibrin results in increased hemorrhagic tendency, which is a prominent symptom of the late phase DIC and a common and potentially deadly complication of sepsis patients (Levi, 2001). The ability of *Aeromonas* species to bind fibrinogen (Ascencio et al., 1990) suggests that ASP may degrade fibrinogen, thereby impair plasma clottability. As expected, ASP at concentrations starting from 3 nM prolonged fibrinogen thrombin time (TT, an indicator of fibrinogen clottability) at the normal plasma concentration of 3 mg/ml (Halkier, 1991) (Imamura et al., 2008). ASP also prolonged human plasma TT at concentrations starting from 10 nM (Imamura et al., 2008), indicating the ability of ASP to reduce clottability of fibrinogen in plasma. The involvement of plasmin-mediated fibrinogen degradation in this ASP effect is highly unlikely because ASP does not directly activate plasminogen. From these results we concluded that ASP impaired plasma clotting through fibrinogen cleavage. The experiments using the culture supernatants of either *asp* gene-disrupted or –transgenic *A. sobria* strains revealed that ASP accounted for the majority of serine protease activity and fibrinogen clottability-impairing activity of the wild type *A. sobria* (Imamura et al., 2008).

When incubated with ASP, Aα-chain of fibrinogen was completely degraded within 2 min, followed by Bβ-chain degradation. γ-chain was considerably resistant to ASP. Judging from the relation between fibrinogen concentration and TT (Imamura et al., 1995), more than 80% of fibrinogen lost clottability after a 3-min incubation with 30 nM ASP (Imamura et al., 2008). The rapid cleavage of Aα-chain by ASP, which is also seen by the physiological fibrinogenolytic protease plasmin (Pizzo et al., 1972), indicates that this chain is a preferred substrate and that the cleavage of Aα-chain by ASP was responsible for the loss of plasma clottability caused by *A. sobria*. As decribed above, ASP is a subtilisin-like protease and preferentially cleaves peptide bonds at the C-terminal side of the paired basic amino acid residues (Kobayashi et al., 2006). The fact that such paired residues are less frequent in the γ-chain (two pairs) than in the Aα- or the Bβ-chains (seven pairs) (Halkier, 1991) may be associated with the γ-chain being relatively resistant to ASP, but it does not provide an explanation as to why ASP degrades Aα-chain faster than Bβ-chain. Aα-chain has a flexibly attached and mobile structure in the two third of the C-terminal portion (αC domain), whereas Bβ-chain form a comparatively rigid structure (Kollman et al., 2009); thus αC domain is presumably more accessible to ASP than Bβ-chain. In fact, we found that αC domain was highly sensitive to the proteolysis by staphopains, cysteine proteases from *Staphylococcus aureus*, but other two chains were resistant to the proteases (Ohbayashi et al., 2011; Imamura, 2014). Since αC domains are important for lateral association of fibrinogen protofibrils in the fibrin clot formation (Collet, et al., 2005), degradation of the domains impairs fibrinogen clottability. The difference in the three-dimensional structure between Aα- and Bβ-chains may explain the preference of ASP to cleave Aα-chain.

A subtilisin-like serine protease from group B *Streptococcus* degrades human fibrinogen and a mutant strain deficient of this protease was 10 times less virulent in a neonatal rat sepsis model of group B *Streptococcus* infection (Harris et al., 2003). These data support our notion that, with the fibrinogen-cleaving activity, ASP is an important virulence factor of *A. sobria*. With regard to fibrinogenolytic activity, ASP is less potent than gingipain K isolated from *Porphyromonas gingivalis* (Imamura et al., 1995), but the activity of ASP to cleave fibrinogen Aα-chain is comparable to that of the *S. aureus* cysteine protease SspB (Ohbayashi et al., 2011).

Induction of inflammation, tissue injury and impairment of the host defense system

Anaphylatoxin C5a is a 74-amino acid fragment released from the α-chain of the complement 5th component (C5). C5a is a potent inducer of neutrophil chemotaxis (Marder et al., 1985), superoxide release and phagocytosis (Goldstein and Weissmann, 1974; Sacks et al., 1978; Mollnes et al., 2002). C5a also stimulates mast cells to release histamine that causes VL (Kikuchi and Kaplan, 2002). These C5a activities mimic responses observed at an early stage of inflammation, such as neutrophil accumulation and edema formation. Thus, C5a is an important mediator of inflammation.

During the activation of the complement system, C5a is generated by C5-convertase that cleaves C5 at the C-terminal side of the Arg_{74} residue (DiScipio et al., 1983). This cleavage site matches the substrate preference of ASP (Imamura et al., 2006, Nitta et al., 2007). ASP-induced dermal VL was partially inhibited by an anti-histamine drug (Imamura et al., 2006), indicating the involvement of histamine released from mast cells in the skin. Taken together, these findings suggest that C5a may be generated by ASP at the infection site of *A. sobria*.

C5a activity generation from C5

Neutrophil chemotactic activity (NCA) is a representative biological activity of C5a (Fernandez et al., 1978). Interestingly, we found that the incubation of human C5 at the normal plasma concentration of 350 nM (Halkier, 1991) with ASP resulted in the generation of NCA in a dose dependent manner at an ASP concentration range starting at 3 nM and the activity of ASP reached a plateau at 30 nM (Nitta et al., 2008). The activity of NCA generated by 30 nM ASP was comparable to that of 10 nM recombinant C5a. The generation of NCA was first detected at 7 min and peaked at 60 min, and completely inhibited by the C5a receptor antagonist N-methyl-Phe-Lys-Pro-D-cyclohexylalanine-D-cyclohexylalanine-D-Arg (Konteatis, et al., 1994). Pronounced neutrophil accumulation was induced by intradermal injection of ASP-treated C5 into the guinea pig skin, indicating the ability of ASP-cleaved C5 to attract neutrophils *in vivo* (Nitta et al., 2008). ASP-treated C5 also induced neutrophil superoxide release via C5a receptor. Finally, VL-inducing activity of ASP-treated C5 was completely blocked by a histamine H_1 receptor antagonist diphenhydramine (Nitta et al., 2008). Thus, ASP-cleaved C5 does not directly cause VL but it can induce VL by triggering mast cell degranulation and subsequent histamine release. No significant VL activity was found from human C3 by ASP, indicating that ASP is incapable of releasing C3a, another anaphylatoxin.

C5a liberation from C5

ASP was capable of cleaving C5 within 2 min at several sites, and in 7 min released a fragment with a molecular mass of 10.5 kDa that was recognized by an antibody against C5a (Nitta et al., 2008). The amount of this fragment increased in an incubation time-dependent manner. Since ASP could also liberate C5a from C5 in human plasma, the ASP action on C5 can occur *in vivo*.

The Arg_{74} residue at the C-terminus of C5a is indispensable in the binding to its receptor (Fernandez and Hugli, 1978), hence ASP must cleave C5 at the C-terminal side of Arg_{74} as the P_1 site. C3a is also released by cleavage at the C-terminal side of Arg_{77} (de Bruijn and Fey, 1985), however, ASP cannot generate VL activity from human C3. Unlike C5, C3 has an -Arg_{64}-Arg_{65}- sequence (Halkier, 1991). It is possible that ASP cleaves preferentially the peptide bond at the C-terminal side of the paired basic amino acid residues before cutting at the C-terminal site of Arg_{77} in C3. The truncated form of C3a lacking 12-amino acid peptide from its C-terminus seems to be unable to trigger mast cell degranulation.

It has been reported that a 30-kDa serine proteinase isolated from the house dust mite *Dermatophagoides farinae* (Maruo et al., 1997) and cysteine proteinases purified from the culture supernatants of *Porphyromonas gingivalis* (Wingrove et al., 1992; DiScipio et al., 1996) generate C5a from human C5. In these reports, NCA generated from C5 by the proteases was suggested to be due to C5a, but the molecule responsible for the activity was not identified. Thus, ASP is the only proven C5a-releasing microbial protease that generates C5a.

Pathological consequences of C5a release

The harmful effects of ASP-induced C5a release in the *A. sobria* infection sites are shown in Figure 4. Oral intake of histamine-rich foods is known to induce gastrointestinal symptoms in humans, such as diarrhea and flatulence (Wöhrl et al., 2004). Therefore, histamine released from mast cells in response to ASP-generated C5a in the mucosa of the digestive tract may be responsible for diarrhea caused by *A. sobria* infection (Deutsch and Wedzina, 1997; Janda and Abbott, 1998). Histamine also brings about edema in aeromonad-infected wounds and lungs (Janda and Abbott, 1998), and may lead to the onset of acute respiratory distress syndrome (Janda and Duffey, 1988). C5a can cause neutrophil accumulation at sites of infection, which results in pus formation seen in cellulitis and furuncles. Furthermore, C5a provokes neutrophils to release superoxide and enhances neutrophil elastase release in the presence of lipopolysaccharides from Gram-negative bacteria (Fittschen et al., 1988), such as *A. sobria*. Superoxide and elastase injure tissues. The ability of C5a to induce the expression of TF, the initiator of the blood coagulation, in monocytes (Muhlfelder et al., 1979) and endothelial cells (Ikeda et al., 1997), may suggest a link of C5a generation by ASP to thrombotic tendency that leads to the onset of DIC.

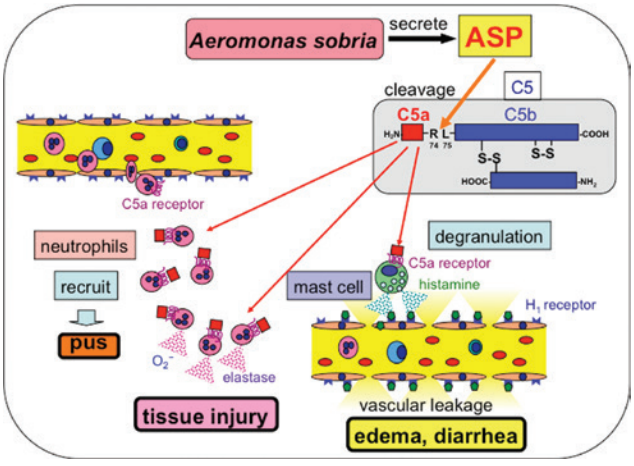

Figure 4: Potential role of C5a generated by ASP at *Aeromonas sobria* infection sites.
ASP secreted from *A. sobria* releases C5a from C5 by cleaving between Arg_{74} and Leu_{75}. C5a binds to C5a receptor on neutrophils and recruits them to the lesion from the circulation, leading to pus formation seen in cellulitis and furuncles caused by this bacterium. Neutrophils stimulated by C5a release superoxide and elastase that cause tissue injury. C5a elicits mast cell degranulation by binding to C5a receptor and released histamine induces H_1-receptor-dependent vascular leakage that is associated with edema in the infection sites and diarrhea in the mucosa of the infected digestive tract. C5a and histamine are shown by red rectangle and green pentagon, respectively.

The complement system is a key component of the humoral defense against invasion by bacteria. After C5a release, C5b, the remaining part of C5, leads to the formation of the membrane attack complex that causes lysis of bacteria, and the accumulation of C5a-recruited phagocytes, which appear to be detrimental to *A. sobria*. However, by incubation of C5 with ASP a molecule smaller than C5b was produced before releasing C5a (Nitta et al., 2008), indicating that C5 is first cleaved inside C5b by ASP. The truncated form of C5b is probably incapable of acting as an opsonin and leading to the formation of the membrane attack complex. Moreover, C5a level is elevated in sepsis (Nakae et al., 1994) and the exposure of neutrophils to C5a at the concentrations detectable in the plasma of sepsis patients can cause neutrophil dysfunction and paralysis of signaling pathways via phosphorylation of p42/p44 mitogen-activated protein kinases (desensitization) (Huber-Lang et al., 2002). Such poor responses of neutrophils may be further enhanced by decreased number of neutrophil C5a receptor, as shown in a cecal ligation and puncture-induced rat sepsis model (Guo et al., 2003). Therefore, C5 cleavage and subsequent C5a generation by ASP may impair bacteria elimination by the complement system and neutrophils, facilitating a long survival of *A. sobria* and further growth and spread. This ASP effect can expedite the onset and exacerbation of sepsis, particularly in immunocompromised patients.

Virulence of nicked ASP

In addition to single-chain ASP (sASP) we discussed above, *A. sobria* also secretes two-chained ASP (nASP) that has a nick between Gln_{519} and Leu_{520} in the P-domain (Figure 1) with the cut-off C-terminal peptide tail non-covalently still bound to the protease (Kobayashi et al., 2009a for overall structure of sASP and nASP, see Murakami et al., 2012a). The nick is created by a protease of the bacterium other than ASP, because an *A. sobria* mutant in which ASP was inactivated by the replacement of His_{115} (one of the catalytic triad residues) with Ala also secretes nASP. *Aeromonas sobria* culture supernatants contain nASP in a quantity comparable with that of sASP. nASP possesses almost the same catalytic activity as sASP for oligopeptide substrates but is less potent in fibrinogen degradation and prothrombin activation (Murakami et al., 2012a). As the C-terminal region of nASP is embedded in the protease body (Kobayashi et al., 2009a), a conformational change by the nick is probably slight. However, the flexibility of

the C-terminal fragment presumably increases and interferes with the access of large molecule substrates to the catalytic domain of ASP, resulting in a decreased proteolytic activity for large molecules, such as protherombin and fibrinogen. Surprisingly, however, nASP exerts stronger proteolytic activity than sASP for these substrates when they are in human plasma. We found that this opposite result was caused because α_2-MG, a sole ASP inhibitor in plasma (Murakami et al., 2012b), inhibited nASP more than twofold slower than sASP (Murakami et al., 2012a). Proteases have been shown to be immediately trapped by α_2-MG when they cleave the bait region of the inhibitor (Harpel, 1973). Therefore, the retarded formation of the α_2-MG complex with nASP (Murakami et al., 2012a) suggests slower cleavage of the bait region by nASP, which allows nASP to maintain its proteolytic activity in plasma longer than the activity of sASP. To mitigate α_2-MG control for ASP, *A. sobria* might nick ASP at the tail region by its own protease, prolonging ASP proteolytic activity at infection sites. Thus, nASP practically increases the virulence of *A. sobria*, thereby exacerbating diseases caused by this pathogen. nASP may be an example of evolutionary changes that occur in bacterial proteases for a longer survival in the host.

Conclusions

ASP causes VL by activation of the kallikrein/kinin system and neutrophil recruitment by C5a generation. These are associated with edema and pus formation seen in the infection sites of *A. sobria*, such as cellulitis and gastroenteritis. Superoxide and elastase released from C5a-activated neutrophils cause tissue injury. Mast cells in the intestinal mucosa are also stimulated by C5a and release histamine that induces gastrointestinal symptoms, such as diarrhea. In the circulation, ASP can activate the kallikrein/kinin system, leading to shock by lowering BP (Figure 5). ASP perturbs the blood coagulation system by thrombin generation and fibrinogen degradation together with TF induction of phagocytes and endothelial cells by C5a, which may contribute to the onset of DIC (Figure 5). In contrast to the activation of acute inflammatory responses, ASP-generated C5a can desensitize neutrophils. Furthermore, the cleavage of C5b by ASP may weaken the host-defense against bacteria by disturbing the generation of opsonin C5b and the formation of the membrane attack complex, thus facilitating a longer survival of *A. sobria* and exacerbation of sepsis (Figure 5). These ASP virulence activities are elevated by the creation of a nick in ASP. Thus, ASP can function as a virulence factor with multiple actions; accordingly this protease is potentially a therapeutic target. Antibodies against ASP and inhibitors of this enzyme together with BK B_2 receptor antagonists may be applied to the treatments for *A. sobria* infections. Other *Aeromonas* spp. also secrete serine proteinases similar to ASP (Husslein et al., 1991; Coleman and Whitby, 1993; Esteve and Birbeck, 2004). Therefore, the benefit of ASP-targeted therapies is further extended, and antibodies and inhibitors for these proteolytic enzymes

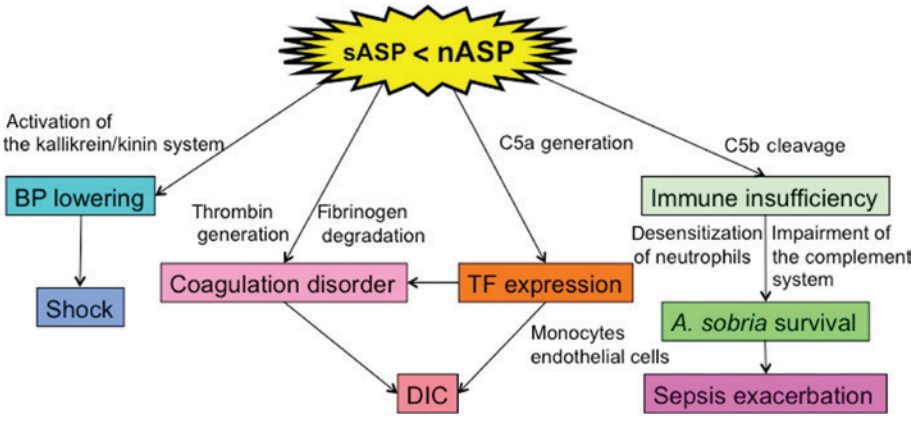

Figure 5: Potential pathogenesis of ASP in sepsis.
ASP-elicited blood pressure (BP) lowering through activation of the kallikrein/kinin system and coagulation disorder caused by thrombin generation and fibrinogen degradation can be associated with the onset of septic shock and disseminated intravascular coagulation (DIC), respectively. Induction of tissue factor (TF) expression on monocytes and endothelial cells by ASP-generated C5a also contributes to coagulation disorder and the onset of DIC. C5 cleavage and subsequent C5a generation may lead to immune insufficiency by impairment of the complement system and desensitization of neutrophils, facilitating a longer survival of *A. sobria* and exacerbating sepsis. These virulence activities of the single-chained ASP (sASP) can be elevated by the nicked ASP (nASP).

may become effective drugs for the diseases caused by infection of aeromonads.

Acknowledgments: We thank Dr. T. Yoshimura, Department of Pathology and Experimental Medicine, Okayama University for his critical reading and editing of this manuscript. We also thank Dr. H. Kobayashi, Laboratory of Molecular Microbiological Science, Faculty of Pharmaceutical Sciences, Hiroshima International University for his invaluable comments on ASP properties.

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