Cleavage specificity of the serine protease of *Aeromonas sobria*, a member of the kexin family of subtilases

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**Abstract**

Subtilisin-like proteases have been grouped into six families based on a sequence of the catalytic domain. One of the six is the kexin family, of which furin is a representative protease. All members of the kexin family, except one, are from eukaryotes. The one prokaryotic protease is a serine protease of *Aeromonas sobria* (ASP). Here, we examined the substrate specificity of ASP based on the cleavage of short peptides. The results showed that ASP preferentially cleaves the peptide bond following two basic residues, one of which is Lys, but not the bond following a single basic residue. This indicates that the tertiary structure around the catalytic domain of ASP resembles, but is not identical to that of furin. Prekallikrein was cleaved into four fragments by ASP, indicating that the protein must be cleaved at specific sequences.

**Introduction**

Subtilisin-like proteases, subtilases, are widely found in Archaeabacteria, eubacteria, and eukaryotes. These enzymes have been classified into six families based on the structure of their catalytic domains: subtilisin, thermitase, proteinase K, lantibiotic peptidase, pyrolysin, and kexin (pro-protein convertases). The first five families each contain at least two bacterial proteases, however, the kexin family contains only one bacterial protease, a serine protease of *Aeromonas sobria* (ASP) (Siezen & Leunissen, 1997). The eukaryotic proteases of the kexin family are involved in important biological reactions such as hormone processing (Dupuy et al., 1994). Therefore, elucidating the origin of these eukaryotic proteases is important to our understanding of the development of mammals. Phylogenetic analysis suggests that eukaryotic proteases of the kexin family have differentiated from ASP. Actually, the amino acid sequence around the active site of furin resembles that of ASP, with an identity in this region of 30% (see Fig. 5). However, the genetic distance between ASP and other eukaryotic proteases of this family is considerable (Siezen & Leunissen, 1997). In order to clarify the relationship between these eukaryotic proteases of the kexin family and ASP, further examinations on the activity of ASP are necessary.

We previously demonstrated that ASP enhances vascular permeability in rat skin (Yokoyama et al., 2002). The enhancement of vascular permeability induces the leakage of tissue fluid containing nutrients, and such fluid is favorable for bacteria to survive in the locus of infection. Analysis of the mode of action of ASP in rat skin using inhibitors suggested that ASP stimulates the bradykinin-releasing pathway by activating the plasma kallikrein. To support this hypothesis, it is necessary to clarify the cleavage specificity of ASP, which we report in this study.

**Materials and methods**

**Proteases**

ASP was purified from a culture supernatant of *Aeromonas sobria* 288 by successive column chromatographies as described previously (Okamoto et al., 2000). The purified preparation of ASP gave a single band on SDS-
polyacrylamide gel electrophoresis at a position of 65,000 Da. The size is almost identical to the calculated value (64,313.7) from the amino acid sequence (Okamoto et al., 2000). Subtilisin A (Subtilisin Carlsberg) and trypsin (from bovine pancreas) were purchased from Sigma (St Louis, MO, USA). These preparations were dissolved in 10 mM Tris-HCl (pH 7.5) at a concentration of 200 μg of protein mL\(^{-1}\).

**Cleavage of peptide substrates**

Fluorogenic peptide substrates, peptidyl-4-methylcoumaranyl-7-amides (MCA), were used to examine the cleavage motif of ASP. The peptides used were as follows: Boc–Glu–Lys–Lys–MCA (Boc; t-Butyloxycarbonyl), Pyr–Arg–Thr–Lys–Arg–MCA (Pyr; t-Pyroglutamyl), Boc–Gly–Lys–Arg–MCA, Boc–Arg–Val–Arg–Arg–MCA, Suc–Ala–Ala–Pro–Phe–MCA (Suc; Succinyl), Boc–Gly–Arg–Arg–MCA, Z–His–Glu–Lys–MCA (Z; Benzoyloxy carbonyl), and Boc–Phe–Ser–Arg–MCA. These peptides were purchased from Peptide Institute Inc. (Osaka, Japan). They were dissolved in dimethylformamide at a concentration of 10 mM and these solutions were diluted immediately before use with 11 mM sodium phosphate buffer (pH 7.5) to a concentration of 1 mM.

The cleavage reaction was initiated by adding 50 μL of the protease solution to 700 μL of the solution containing peptidyl–MCA at a concentration of 1.1 mM. After incubation at 37 °C for 30 min, 750 μL of acetic acid (130 mM) was added to stop the reaction. The fluorescence of the solution at λ\(_{ex}\) = 340 nm and λ\(_{em}\) = 440 nm was measured with a fluorescence spectrophotometer (Model F-4500, Hitachi Co., Tokyo, Japan).

**Peptide library method**

FRETS-25Xaa libraries (Peptide Institute Inc.) were used as substrates for the peptide library method. FRETS stands for fluorescence resonance energy transfer substrate. As shown below, FRETS-25Xaa contains a highly fluorescent 2-((N-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal d-2,3-diamino propionic acid (d-A2pr) residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the ε-amino function of Lys.

**Structure of FRETS-25Xaa**

\[ \text{d-A2pr(Nma)} – \text{Gly–Zaa–Yaa–Xaa–Ala–Phe–Pro–Lys(Dnp)–d–Arg–d–Arg} \]

Zaa: Phe, Ala, Val, Glu, Arg, Yaa: Pro, Tyr, Lys, Ile, Asp

Xaa represents the fixed position where each of the 19 natural amino acids excluding Cys is incorporated. In this experiment, we used five kinds of FRETS. Xaa amino acid residues of these five FRETS peptides were Lys, Arg, Trp, Gly, and Glu.

One of five amino acid residues (Pro, Tyr, Lys, Ile, and Asp) was incorporated at the Yaa position along with one of five amino acid residues (Phe, Ala, Val, Glu, and Arg) at the Zaa position for each fixed Xaa. This provides a peptide mixture of 25 combinations of each Xaa series. When an enzyme of interest cleaves any peptide bond between d-A2pr(Nma) and Lys(Dnp) in the substrate, the fluorescence at λ\(_{ex}\) = 340 nm and λ\(_{em}\) = 440 nm increases in proportion to the release of the Nma fluorophore from the internal Dnp quencher.

Stock solutions of ASP and trypsin were diluted with 10 mM sodium phosphate buffer (pH 7.5). FRETS dissolved in DMSO at 1 mM was added to these protease solutions to give a concentration of 100 μM. The concentrations of ASP and trypsin in the mixture were 430 and 12 ng mL\(^{-1}\), respectively. After incubation at 37 °C for 30 min, an equal volume of acetic acid (130 mM) was added to the solution. The fluorescence intensity at λ\(_{ex}\) = 340 nm and λ\(_{em}\) = 440 nm for each sample was measured immediately.

**LC-MS analysis**

FRETS-25K hydrolyzed with ASP was analyzed by liquid chromatography-mass spectrometry (LC-MS). An ODS[C18] column (YMC Co. Ltd., Kyoto, Japan) was used for LC. Eluent A was H\(_2\)O containing 0.05% TFA and eluent B was CH\(_3\)CN containing 0.05% TFA. The gradient was 10–40% of eluent B in A, over 50 min. One hundred microliters of sample was injected. The effluent was monitored for fluorescence intensity at λ\(_{ex}\) = 340 nm and λ\(_{em}\) = 440 nm. The three main peaks were further analyzed by MS. From the value obtained by the analysis with MS, the structure of the cleaved product in each peak was deduced.

**Cleavage of prekallikrein by ASP**

In all, 5 μg of human prekallikrein (EMD Bioscience Inc., La Jolla, CA) was incubated with ASP in 15 μL of 10 mM sodium phosphate buffer (pH 7.5) at 37 °C for 30 min. The amount of ASP in these samples ranged from 1.25 to 10 μg. After incubation, the reaction was analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

**Results**

**Amidolytic activity of ASP**

In order to examine the NH\(_2\)-terminal part of the accessible bond cleaved by ASP, fluorogenic peptide substrates listed in Fig. 1 were incubated with ASP at a concentration of 1.0 μg 750 μL\(^{-1}\) at 37 °C for 30 min. After the incubation, the amount of 7-amino-4-methyl-coumarin released upon...
hydrolysis of the MCA peptide was measured. As shown in Fig. 1a, the substrate presenting the highest level of fluorescence after treatment with ASP was Boc–Glu–Lys–Lys–MCA. The fluorescent intensity of Pyr–Arg–Thr–Lys–Arg–MCA was low compared with that of Boc–Glu–Lys–Lys–MCA, however, it was higher than that of the other peptides used. Two peptides, Boc–Gly–Lys–Arg–MCA and Boc–Arg–Val–Arg–Arg–MCA were cleaved to a small extent during the incubation with ASP. The remaining substrates, Suc–Ala–Ala–Pro–Phe–MCA, Boc–Gly–Arg–Arg–MCA, Z–His–Glu–Lys–MCA, and Boc–Phe–Ser–Arg–MCA, were not cleaved as a result of incubation with ASP.

To clarify the specificity of cleavage by ASP, the substrates were cleaved with trypsin (10 ng 750 μL⁻¹) and subtilisin (50 ng 750 μL⁻¹). The substrates, which showed low or negligible reactivity in the incubation with ASP, exhibited fairly strong fluorescence after incubation with trypsin or subtilisin (Fig. 1b and c). This means that there was no problem with the quality of these peptides.

These results indicate that ASP prefers the peptide bond following two basic amino acid residues including lysine, but does not recognize the bond following a single basic residue.

Analysis of substrate specificity using FRETS 25Xaa libraries

We incubated five kinds of FRETS with ASP at a concentration of 430 ng mL⁻¹. The Xaa amino acid residues of these five FRETS are Lys, Arg, Gly, Glu, and Trp. The cleavage of these FRETS peptides was done in 10 mM sodium phosphate buffer (pH 7.5) at 37 °C for 30 min. The FRETS presenting the highest level of fluorescence contained Lys at position Xaa (FRETS-25K) (Fig. 2a). The FRETS presenting the second highest level of intensity was FRETS-25R containing an Arg residue at position Xaa. To confirm the accuracy of the results obtained, we cleaved each of these FRETS with trypsin at a concentration of 12 ng mL⁻¹. The FRETS exhibiting the strongest fluorescence after incubation with trypsin was FRETS-25R, while the fluorescent intensity of FRETS-25K after incubation with trypsin was considerably lower (Fig. 2b). This indicates that these FRETSs are suitable as substrates in the assays, and that ASP prefers Lys at P₁, i.e. the amino acid immediately preceding the site of cleavage.

Subsequently, we analyzed the products of FRETS-25K hydrolysis by ASP using LC-MS to identify the N-terminal segments generated by the treatment. A 100 μL volume of the FRETS-25K solution treated with 430 ng mL⁻¹ ASP in

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Fig. 1. Cleavage of fluorogenic peptide substrates by serine protease of Aeromonas sobria, trypsin, and subtilisin. The substrates listed in the figures were treated with serine protease of A. sobria, (ASP) (a), trypsin (b), and subtilisin (c) and the fluorescence intensity generated was measured as described in the text. The amount of ASP, trypsin, and subtilisin in each sample (750 μL) was 1 μg, 10 ng, and 50 ng, respectively.

Fig. 2. Cleavage of FRETS-25Xaa libraries by serine protease of Aeromonas sobria and trypsin. Each FRETS-25Xaa was treated with serine protease of A. sobria (ASP) (a) and trypsin (b) as described in the text. The concentration of ASP and trypsin in the reaction mixture was 430 and 12 ng mL⁻¹, respectively. After incubation, the fluorescence intensity of each sample at λex = 340 nm and λem = 440 nm was measured.
the above experiment was fractionated by LC. The elution profile of the LC is shown in line A of Fig. 3. To confirm that these peaks were generated directly from FRETS by enzymatic cleavage, we cleaved FRETS-25K with half the amount of ASP (215 ng mL\(^{-1}\) of ASP) and separated the reaction mixture by LC under the same conditions (line B of Fig. 3). The elution profile of line B was almost identical to that of line A, though the height of the peaks of line B was less than that of the corresponding peaks of line A. This suggested that most peaks of line A were directly derived from FRETS by cleavage with ASP and therefore could be analyzed to determine the site of cleavage by ASP.

We selected three peaks (peaks 1–3 in Fig. 3) and determined the mass values of the components of these peaks by MS. Then we predicted the sequences of the components from the mass value. The results are shown in Table 1.

The amino acid residue at the carboxy terminal end of each of the three segments is lysine. The second amino acid residue from the carboxy terminal end of these three segments is also lysine. Therefore, it can be concluded that lysine is the most suitable amino acid residue at P\(_1\) and P\(_2\) for cleavage by ASP.

### Fragmentation of prekallikrein by ASP

The result of incubation of human prekallikrein with ASP is shown in Fig. 4. As the prekallikrein (86 kDa) is a glycoprotein, it does not appear as a sharp band. Judging from the size, the band indicated as 65 kDa might be ASP. Other bands on the gel, which are indicated as 43, 36, 33 and, 28 kDa, are assumed to be fragments of the prekallikrein generated by cleavage with ASP. Appearance of these fragments after treatment with ASP shows that the prekallikrein is cleaved at a limited number of sites by ASP.

### Discussion

In these experiments, we examined the cleavage specificity of ASP using short peptide substrates and showed that ASP prefers a Lys residue at both positions P\(_1\) and P\(_2\) relative to the cleavage site. It is said that results obtained using short peptide substrates reflect the sequence-based cleavage specificity of the corresponding protease, because these short peptides lack any significant and stably folded structure (Kato et al., 1980). Therefore, we believe that the conclusions reached in this work are relevant to the substrate specificity of ASP.

As described, mammalian proteases of the kexin family function as pro-protein convertases. These proteases are named SPCs (subtilisin-like pro-protein convertases)

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**Table 1.** Analysis of cleaved products of FRETS-25K by mass spectrometry

<table>
<thead>
<tr>
<th>Peak number*</th>
<th>Relative area (^{1})</th>
<th>Mass value (^{1})</th>
<th>Predicted sequence (^{6})</th>
<th>Theoretical molecular weight (^{1})</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.000</td>
<td>622.2</td>
<td>D–A2pr(\text{Nma})–G–A–K–K</td>
<td>622.4</td>
</tr>
<tr>
<td>3</td>
<td>0.722</td>
<td>698.4</td>
<td>D–A2pr(\text{Nma})–G–F–K–K</td>
<td>698.4</td>
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<tr>
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<td>0.451</td>
<td>650.4</td>
<td>D–A2pr(\text{Nma})–G–V–K–K</td>
<td>650.4</td>
</tr>
</tbody>
</table>

*Peaks on the elution profile shown in Fig. 3.

\(^{1}\)Relative ratio of each peak to the area of peak 1.

\(^{2}\)Value measured by mass spectrometry.

\(^{6}\)Predicted from mass value.

\(^{1}\)Calculated from the predicted sequence.
Fig. 4. Fragmentation of prekallikrein by ASP. Human prekallikrein (5 μg) was incubated with ASP in 15 μl of 10 mM sodium phosphate buffer (pH 7.5) at 37 °C for 30 min. The amount of ASP in each sample was 1.25 μg (lane 2), 2.5 μg (lane3), 5.0 μg (lane 4), and 10.0 μg (lane 5). ASP was not added to the sample for lane 1. After incubation, sodium dodecyl sulphate (SDS)-sample buffer containing SDS and 2-mercaptoethanol was added to each solution. After heating at 100 °C for 5 min, these samples were separated by SDS-polyacrylamide gel electrophoresis. The gel was stained with Comassie brilliant blue. Sizes of bands in the gel are shown by numbers at the side. (Bergeron et al., 2000). Furin is the best characterized protease among SPCs, and strongly cleaves the peptide bond following two basic residues (Rockwell et al., 2002). Our study showed that ASP prefers the peptide bond following two basic residues including Lys. Both proteases, ASP and furin, resemble each other in the respect of preference of the bond following two basic residues.

Recent analysis of the structure of substrate recognized by furin showed that an Arg at P4 increases the cleavage of substrate (Rockwell et al., 2002). Our study using Boc–Gly–Arg–Arg–MCA and Boc–Arg–Val–Arg–Arg–MCA indicates that an Arg residue at P4 enhances the cleavage of the substrate by ASP as well as by furin; the former was not cleaved at all, but the latter was cleaved, though the extent of the cleavage was slight (Fig. 1). Similarly, it is worthy to note that the sensitivity of Pyr–Arg–Thr–Lys–Arg–MCA to ASP is higher than that of Boc–Gly–Arg–Arg–MCA, though both substrates contain Lys–Arg immediately upstream of MCA. The influence of the amino acid at P4 on the cleavage is therefore observed for both ASP and furin, and this suggests there is a relationship between ASP and furin.

However, the substrate structure recognized by ASP is not identical to that recognized by furin. That is, furin exhibits extremely stringent specificity for Arg at position P1 (Rockwell et al., 2002; Henrich et al., 2003). In contrast, the ability of ASP to recognize Arg at P1 is low. The most preferable amino acid at P1 for ASP is Lys (Table 1 and Fig. 1).

With furin, the P1–Arg side chain extends through the kinked Ser253–Asp258 ‘entrance frame’ into the S1 pocket, sandwiched between the strictly conserved segments Ser253–Gly255 and Ser293–Asn295 (Henrich et al., 2003, 2005). The terminal guanidyl group is perfectly packed into a flat groove lined by the carboxylate groups of Asp258 and Asp306 and the carbonyl groups of Ala292 and Pro256.
would degrade the prekallikrein into pieces. The fact that ASP strongly prefers the peptide bond following two basic residues including Lys supports the idea that the cleavage of prekallikrein by ASP is restricted. According to the NCBI data base, rat prekallikrein (number; P14272) has six dibasic residues (2KR, 3RK, 1KK), and these sites are candidates for cleavage by ASP. However, it remains unknown whether all these sites are cleaved by ASP. We incubated human prekallikrein in vitro with ASP and found that only four fragments are generated by the incubation (Fig. 4). Four amino acid sequences of human prekallikrein are registered in NCBI (P03952, NP002248, AAF9940, and NP000883), and these are identical except for one amino acid residue (Ser at position 143 of NP000883). There are five dibasic residues (4KR, 1RR) at positions 73, 139, 285, 550, and 604 in the sequences of these prekallikreins. The dibasic amino acid residue at position 604 is RR. Though the amino acid sequence of human prekallikrein used in this experiment has not been published, it is likely that these dibasic amino acid residues are conserved. As described, kallikrein is glycosylated. Therefore, the mobility of the fragments generated by the cleavage with protease on SDS-gel do not correspond to the molecular size calculated from the amino acid sequence. Therefore, we cannot infer the site of prekallikrein cleavage by ASP from the mobility of the fragment generated on the SDS-gel. However, judging from the number of fragments, i.e. 4, it was likely that ASP cleaved some of these dibasic residues preferentially. Subsequently, the remaining dibasic residues in these fragments might be cleaved by ASP with low efficiency. The faint bands observed between 33 and 28 kDa in Fig. 4 may reflect such cleavage with low efficiency. The results suggest that ASP cleaves the prekallikrein at specific sites and a fragment generated by the digestion might be the activated kallikrein.

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