Substrate Specificity at the P1’ Site of *Escherichia coli* OmpT under Denaturing Conditions

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Though OmpT has been reported to mainly cleave the peptide bond between consecutive basic amino acids, we identified more precise substrate specificity by using a series of modified substrates, termed PRX fusion proteins, consisting of 184 residues. The cleavage site of the substrate PRR was Arg140-Arg141 and the modified substrates PRX substituted all 19 natural amino acids at the P1’ site instead of Arg141. OmpT under denaturing conditions (in the presence of 4 M urea) cleaved not only between two consecutive basic amino acids but also at the carboxyl side of Arg140 except for the Arg140-Asp141, -Glu142, and -Pro141 pairs. In addition to Arg140 at the P1 site, similar results were obtained when Lys140 was substituted into the P1 site. In the absence of urea, an aspartic acid residue at the P1’ site was unfavorable for OmpT cleavage of synthetic decapetides, the enzyme showed a preference for a dibasic site.

Key words: *Escherichia coli*; outer membrane protein OmpT endopeptidase (EC 3.4.21.87); substrate specificity; denaturing conditions

*Escherichia coli* OmpT is an outer membrane-bound endopeptidase consisting of 297 amino acid residues. It belongs to the serine peptidase family S18 and the deduced amino acid sequence has high similarity to those of the *Yersinia pestis* plasminogen activator, *Salmonella typhimurium* E protein, *E. coli* OmpP, and *Shigella flexneri* SopA proteins. The physiological function of OmpT has been suggested to be involved in *E. coli* pathogenicity and the inactivation of antimicrobial peptides.

The hydrolysis of T7 RNA polymerase, human interferon gamma, and cyclin A by this enzyme has been reported. In some cases, undesired degradation of recombinant proteins expressed by *E. coli* also occurs. OmpT has the ability to process recombinant fusion proteins such as cholesterol esterase/lipase, cholera toxin B subunit, and *Staphylococcus aureus* V8 protease derivative. The narrow substrate specificity contributes to OmpT being a useful processing enzyme for recombinant fusion proteins.

Although OmpT primarily cleaves the peptide bond between consecutive basic residues, not all dibasic sites were cleaved. Moreover, OmpT is known to cleave monobasic amino acid sites such as Arg-Met, Arg-Ala, and Arg-Val.

Recently Dekker *et al.* have investigated the substrate specificity for the P2, P1, P1’, and P2’ positions of OmpT using synthetic peptides called SPOT peptide libraries. Here, we identified the more precise substrate specificity by using a series of modified substrates, termed PRX fusion proteins, consisting of 184 residues, but not synthetic peptides, under denaturing conditions. As regards the specificity for the P1’ position, our finding corresponded with their results in the point of the less exclusive preference although there were some differences in the cleavage efficiencies. OmpT is associated in inclusion bodies and active in the presence of high concentrations of urea. Therefore, as soon as inclusion bodies containing a fusion protein are dissolved in denaturation solution with urea, OmpT can cleave the fusion protein to release the target peptide without external addition of any protease. Thus, OmpT has a suitable property for processing fusion proteins when they are in inclusion bodies. In this paper, long and free peptides were used as substrates under denaturing conditions. Hence, our results could be applied to site-specific cleavage of protein and peptide when we consider the application of OmpT as a processing protease.

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Abbreviations: β-gal11754H, a truncated *E. coli* β-galactosidase derivative; GLP-1, glucagon-like peptide-1 (7–36) amide; GLP-1 [G], the C-terminally glycine extended precursor peptide of human GLP-1 (7–36)


**Materials and methods**

**Materials.** Plasmid pG117S4HompPRR (Fig. 1A) was used to construct the mutated fusion protein variants and as a template for PCR amplification. It is a pBR322-derived high-copy plasmid that encodes the PRR fusion protein the expression of which is under the control of the *E. coli lac* promoter. This fusion protein is designed to produce the recombinant C-terminally glycine extended precursor peptide of human GLP-1 (7-36) (GLP-1 [G]). *E. coli* JM109 was used as the host for plasmid construction. *E. coli* W3110 M25, which is an OmpT protease-deficient mutant derived from *E. coli* W3110, was used for expression of the fusion proteins. DNA restriction enzymes were purchased from New England Biolabs, Beverly, MA, USA. Dynorphin A 1-13 was purchased from the Peptide Institute, Osaka, Japan. Benzamidine Sepharose 6B was obtained from Amersham Pharmacia Biotech, Uppsala, Sweden. Both YMC Protein RP and YMC A-302 HPLC columns were obtained from YMC, Kyoto, Japan. Synthetic peptides RR (Glu-Leu-Arg-Leu-Tyr-Arg-Arg-His-His-Gly) and RD (Glu-Leu-Arg-Leu-Tyr-Asp-Asp-Asp-Asp-Asp-Asp-His-His-Gly) were synthesized with a Model 433A peptide synthesizer (PE Biosystems, Foster City, CA, USA). Synthetic peptide KR (Glu-Leu-Arg-Leu-Tyr-Lys-Arg-His-His-Gly) and RN (Glu-Leu-Arg-Leu-Tyr-Arg-Asn-His-His-Gly) were purchased from Sawady Technology, Tokyo, Japan. Protein concentrations were measured by the BCA assay (Pierce, Rockford, IL, USA).

**Construction of plasmids.** The fusion protein (PRR, 184 residues) consists of a truncated *E. coli β*-galactosidase (β-gal117S4H) as a protective peptide fused to the precursor of human glucagon-like peptide-1 (GLP-1 [G]) via a linker peptide. It has an OmpT cleavage site at Arg<sup>153</sup>Arg<sup>154</sup> defined as the P1 and P1' sites in the linker peptide, respectively. The codon corresponding to the P1' site on the plasmid pG117S4HompPRR (Fig. 1A) was acted on by site-directed mutagenesis to generate 19 different fusion protein genes of type PRX (where X denotes a substituted amino acid-residue at the P1' site), as substrates for OmpT as shown in Fig. 1B. The expression plasmids pG117S4HompPRRX were constructed as follows. The plasmid pG117S4HompPRRX was used as a template for PCR amplification. The upper primer 5'-ACCCC AGGCT TTACA CTTTA-3' is located in the *E. coli lac* promoter-operator region. The lower primer 5'-TTTAC GCCGT AGATG NNGCG ATACA GGG-3' is complementary to the fusion protein linker sequence containing the changes at the codon of the P1' site (where NNN is complementary to the desired codon for the amino acid to be tested in the P1' site). The PCR product was digested with *Pvu*I and *Bam*HI and purified from the agarose gel. It was inserted into the same site of pG117S4HompPRRX to create the series of mutations at the P1' site. For the construction of the fusion protein genes that have Lys instead of Arg at the P1 site, the expression plasmids pG117S4HompPKA, pG117S4HompPKS, pG117S4HompPKK, pG117S4HompPKR, pG117S4HompPKD and pG117S4HompPKE were constructed. PCR amplification was done by using the upper primer 5'-ACCCC AGGCT TTACA CTTTA-3' and the lower primer 5'-CCGG ATCCG TATAC GGC-3' (NNN designates AGC for PKA, AGA for PKK, TTT for PKK, ACC for PKR, GTC for PRD, or TTC for PKE) complementary to the fusion protein linker. PCR products were digested with *Pvu*I and *Bam*HI. The DNA fragment was inserted into the same site of pG117S4HompPRRX to generate these fusion protein genes. The nucleotide sequences of each mutated fusion protein gene were confirmed using an A. L. F. Sequencer (Amersham Pharmacia Biotech.).

**Preparation of fusion proteins.** OmpT protease-deficient *E. coli* W3110 M25 cells harboring each fusion protein expression plasmid were grown in 400 mL of Luria Bertani medium containing 10 μg/mL of tetracycline at 37°C overnight. The cells were harvested by centrifugation and disrupted by sonication. The expressed fusion proteins were found as insoluble inclusion bodies. This insoluble fraction was harvested by centrifugation and then washed with 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 1% Triton X-100. The remaining insoluble fraction was further washed with deionized water and suspended in deionized water to an OD<sub>600</sub> for the suspension equal to 100, which was used as substrate. It was stored at -20°C until use.

**Preparation of OmpT.** OmpT protease was expressed in *E. coli* W3110 transformed with the plasmid pOmpTTcE that was constructed by replacing the coding region of the fusion protein in pG117S4HompPRRX with the ompT gene cloned by Sugimura. The OmpT protease expressing *E. coli* cells was grown in a 3-L fermentor with 2 L of the medium of Mori et al. at 37°C for 12 hours. The cells were harvested by centrifugation. After washing with 50 mM Tris-HCl (pH 7.5) twice, the cells were homogenized using a Manton-Gaulin homogenizer. The membrane extract was prepared from the whole membrane fraction by the method of Sugimura and Nishihara, and the extract (120 mL) was put on a benzamidine Sepharose 6B column (8 mL) equilibrated with buffer A (50 mM Tris-HCl [pH 7.5] containing 0.1% Triton-X100). The column was washed with 10 column volumes of buffer A, and the adsorbed proteins were eluted with buffer A containing 0.3 M NaCl. The active fractions were analyzed by
SDS-PAGE. Only the fraction showing a single band was used as a purified OmpT protease.

**OmpT protease activity assay.** For measurement of OmpT protease activity, the substrate dynorphin A was used according to the method of Sugimura and Nishihara. A 20-μL reaction mixture was analyzed by reverse phase HPLC using a YMC Protein RP column. Elution was done with a linear gradient of 10 to 15% acetonitrile in a 0.1% trifluoroacetic acid solution at a flow rate of 1 mL/min, the effluent being monitored by absorbance at 214 nm. One unit of OmpT protease activity was defined as that cleaving 1 μmol of dynorphin A per min at 25°C.

**Cleavage of fusion proteins.** A 10-μL portion of the substrate inclusion bodies suspension (OD_{600} = 100) of each fusion protein was solubilized in 20 μL of 10 mM urea. Sodium phosphate (1 mM, pH 7.0) and EDTA (50 mM) were added to give final concentrations of 50 mM and 2 mM, respectively. The reaction was started by the addition of 5 μL of 4 units/mL OmpT in a total volume of 50 μL. The reaction mixtures were incubated for 30 min at 25°C and analyzed by SDS-PAGE. The band intensities on the gel were measured by scanning with a computing densitometer Model 300A (Molecular Dynamics, Sunnyvale, CA, USA) after Coomassie Brilliant Blue R250 staining.

**Identification of the cleavage sites.** The OmpT reaction product from each fusion protein was purified from the reaction mixture described above. An equal volume of 12% acetic acid and 4 M urea was added to the reaction mixture. After centrifugation, the supernatant was put on a YMC Protein RP column. Elution was done with a linear gradient of 21.5 to 32% acetonitrile in a 0.1% trifluoroacetic acid solution at a flow rate of 1 mL/min. The peptide fragment containing GLP-I [G] was pooled and amino acid sequence was analyzed with a Model 477A protein sequencer (PE Biosystems, Foster City, CA, USA).

**Calculation of kinetic constants.** Kinetic data were obtained by incubating various concentrations of the synthetic decapeptides, RR, KR, and RN with 0.11 μM, 0.23 μM, and 90 μM of constant OmpT concentrations, respectively. Peptide concentrations were chosen where possible to surround the K_m. The reactions were done as described in "OmpT protease activity assay," except for substituting these peptides instead of dynorphin A as the substrate. A reaction mixture (20 μL) was analyzed by reverse phase HPLC with a YMC A-302 column. Elution was done with a linear gradient of 10 to 40% acetonitrile in a 0.1% trifluoroacetic acid solution at a flow rate of 1 mL/min, the effluent being monitored by absorbance at 214 nm. The amount of the cleaved peptide Glu-Leu-Arg-Leu-Tyr-Arg (from RR and RN), or Glu-Leu-Arg-Leu-Tyr-Lys (from KR) was estimated from the peak area of the chromatogram. Data were fitted to the Michaelis-Menten rate equation.

**Results and discussion**

**Effects of the P1′ site on OmpT cleavage**

OmpT was originally described as a protease cleaving the bonds between two consecutive basic amino acids (Arg-Arg, Arg-Lys, Lys-Lys, and Lys-Arg); since then monobasic sites such as Arg-Met, Arg-Ala, and Arg-Val have been reported as targets. In order to investigate the effects on OmpT cleavage of the P1′ site, other amino acids were used to replace the residue at the P1′ position of the cleavage site in the fusion proteins PRX (Fig. 1B). All of the PRX fusion proteins were prepared in the OmpT protease-deficient *E. coli* W3110 M25 strain. OmpT cleavage of the twenty fusion proteins was done in the presence of 4 M urea to solubilize the fusion proteins. It has been observed that OmpT proteolysis is active even under extreme denaturing conditions (8 M urea). As shown in Fig. 2, all of the fusion proteins were cleaved except for PRD (lane D) and PRE (lane E). These results indicate that acidic amino acids are disallowed at the P1′ site for cleavage. The cleavage efficiency of the fusion protein was affected by the amino acid species at the P1′ site. The cleavage of PRR and PRK was more efficient than that of the other fusion proteins. The results of N-terminal amino acid sequences of the 4.9-kDa cleavage products showed that all of the cleaved fusion proteins, except for PRP, were cleaved at Arg140-X141, but PRN, PRY, PRG, PRH, and PRW were also moderately cleaved at Arg177-Leu178 as well (Table 1). PRP was slightly cleaved at Arg177-Leu178 but cleavage at Arg140-Pro41 was not detected. Pro at the P1′ site is unfavorable for cleavage probably for structural reasons. These results indicate that OmpT cleaves at the same site in these fusion proteins where the P1′ site carries any natural amino acid except for Asp, Glu, or Pro.

Recently Dekker et al. have investigated the substrate specificity for the P2, P1′, and P2′ positions of OmpT using synthetic peptides called SPOT peptide libraries. As regards the specificity for the P1′ position, our finding corresponded with their results in the point of the less exclusive preference. Furthermore, it was confirmed that basic amino acids were preferred and acidic amino acids were not allowed at the P1′ position. However, there were some differences in the cleavage efficiencies. In the SPOT peptide analysis, Arg-Cys, Arg-Tyr, and Arg-Trp were reported as poor substrates (0–5% cleavage). In contrast, we observed higher cleavages for these sequences (36%, 22%, and 6.2%, respectively). In the case of Arg-Pro sequence, 10% cleavage was detect-
ed in the SPOT peptide analysis. However, we could not detect cleavage for Arg-Pro in our fusion protein. These might be due to the difference of the substrates (the number of amino acid residues, the peptide sequences, immobilized or free peptides) or the reaction conditions (pH, incubation time, with or without urea). Detailed analysis will be needed to clarify the differences.

In the fusion protein PRR, there is another potential OmpT cleavage site at Arg14-Lys15. The cleavage at this site could not be detected even under denaturing conditions. This might be due to the existence of acidic residues (Asp and Glu) at P2' and P4' position of Arg14-Lys15 sequence. Dekker et al. showed that peptides containing an acidic residue at P2 or P2' were not substrates for OmpT and suggested that long-range electrostatic interactions are important for the formation of the enzyme-substrate complex.10 In this context, the use of long and free peptides as substrates is significant. When we consider the application of OmpT as a processing protease under denaturing conditions, our results could be applied to site-specific cleavage of proteins and peptides.

Effects of a Lys residue at the P1 site on cleavage

Though Arg at the P1 site seemed essential for cleavage, the Arg140 at P1 was nevertheless replaced by Lys. Arg141 at the P1' site was also replaced by the six amino acids shown in Fig. 3A. After the PKXs were incubated with or without OmpT, the reaction mixtures were analyzed by SDS-PAGE (Fig. 3B). PKR, PKK, PKA, and PKS were cleaved, but PKD and PKE were not cleaved by OmpT. These results approximately corresponded with those of the fusion protein PRXs (Arg at the P1 site). Moreover, the
Substrate Specificity of OmpT under Denaturing Conditions

Fig. 2. OmpT Cleavage of Fusion Protein PRX.
The inclusion body suspension consisting of fusion protein PRX was incubated under denaturing conditions (in the presence of 4 M urea) at 25°C for 30 min and then analyzed by SDS-PAGE (16%). Relative cleavage efficiency was calculated from the intensity of the 4.9-kDa cleavage product including GLP-1 [G] as described in "Materials and methods". Mr, Molecular mass marker; O, purified OmpT; -, without OmpT; +, with OmpT (0.4 unit/ml); ND, not determined. A, PRA; V, FRV; L, PRL; I, PRI; P, PRP; F, PRF; W, PRW; M, PRM; G, PRG; S, PRS; T, PRT; C, PRC; Y, PRY; N, PRN; Q, PRQ; D, PRD; E, PRE; K, PRK; R, PRR; H, PRH.

band intensities on the SDS-PAGE of the 4.9-kDa cleavage product were measured with a densitometer. As in the case of Arg at the P1 site, OmpT efficiently cleaved at dibasic sites (lane R and K). By N-terminal amino acid sequence analysis of each 4.9-kDa cleavage product, fusion protein PKS, PKA, PKR, and PKK were cleaved at Lys\textsuperscript{140}-Ser\textsuperscript{141}, Lys\textsuperscript{140}-Ala\textsuperscript{141}, Lys\textsuperscript{140}-Arg\textsuperscript{141}, and Lys\textsuperscript{140}-Lys\textsuperscript{141}, respectively (Table 2). These results show that OmpT cleaves at the same site after replacement with Arg, Lys, Ser, or Ala at the P1' site even when Lys is present at the P1 site. A basic amino acid at the P1 site is likely to be essential for cleavage because the fusion protein, which has Ala\textsuperscript{140} (P1)-Arg\textsuperscript{141} (P1'), was not cleaved (data not shown).

Kinetics measurements with synthetic peptides
Analysis with the fusion proteins as substrates indicated that OmpT cleaved most bonds of the type Arg-X except where X was Asp, Glu, or Pro. The enzyme also cleaved at Lys-Lys, Lys-Arg, Lys-Ser, and Lys-Ala. In addition, dibasic sites were cleaved more efficiently than monobasic sites in the presence of 4 M urea. In order to confirm these observations in the absence of urea and to measure OmpT specificity for the P1 and P1' sites, kinetic constants were measured.
using the synthetic decapeptides, RR, KR, RN, and RD in the absence of urea. The sequences of these decapeptides are indicated in Table 3. The differences among decapeptides RR, RN, and RD are that the P1' site is Arg, Asn, or Asp, respectively. The $K_m$, $k_{cat}$, and catalytic efficiencies ($k_{cat}/K_m$) of each substrate are shown in Table 3. The $K_m$ on RR was lower than that on RN, and the $k_{cat}$ on RR was higher than that on RN (1600-fold). RD was not detectably hydrolyzed. Therefore, OmpT prefers the basic amino acid Arg over the neutral amino acid Asn and refuses the acidic amino acid Asp at the P1' site. It seems that OmpT recognizes the charge of the amino acid rather than the structure of the P1' residue. Furthermore, these results suggest that OmpT can cleave at monobasic sites except for Arg-Asp, Arg-Glu, and Arg-Pro even under native conditions. These results are comparable with those for fusion protein cleavage under denaturing conditions. In a comparison be-
tween RR and KR, the catalytic efficiency of KR decreased to half of that of RR. This suggests that Arg is preferable to Lys at the P1 site for the cleavage by OmpT.

In these experiments, we studied the P1' site specificity of OmpT using long and free peptide substrates. We are continuing to study the influence of PRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

1. Wig. PRKPRKKPKSPKAPKD*PKE* RKsADE ELRLYKIRHHGSG

2. PKD, (Fig. 8) PKRPKKPKSPKAPKD*PKE* RKsADE ELRLYKIRHHGSG

3. Cleavage PKRPKKPKSPKAPKD*PKE* RKsADE ELRLYIRHHGSG

4. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

5. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

6. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

7. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

8. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

9. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

10. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

11. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

12. PKD, PRKPRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**

Table 1. Cleavage Site of Fusion Proteins PRX by OmpT

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<tr>
<th>Fusion protein</th>
<th>PRK</th>
<th>PRKPRRPRIPRSPRCPRFPRVPRAPRYPRNPRMpRQPRH*PRTPRWPRPPRLPRG'PRD--PRE**</th>
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<td>PRK</td>
<td>K</td>
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<tr>
<td>PRK</td>
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<tr>
<td>PRE**</td>
<td>E</td>
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* PRH and PRG were equally cleaved at two sites.
** The cleavage products of PRD and PRE were not detected on SDS-PAGE (Fig. 2) and HPLC.

Table 2. Cleavage Site of Fusion Proteins PKR, PKK, PKS, PKA, PKD, and PKE by OmpT

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>PKR</th>
<th>PKK</th>
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<th>PKA</th>
<th>PKD*</th>
<th>PKE*</th>
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<td>P1' residue</td>
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<tr>
<td>Cleavage sites</td>
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<td>ELRLYK1AHHGSG</td>
<td>ELRLYK1DHHGS</td>
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* The cleavage products of PKD and PKE were not detected on SDS-PAGE (Fig. 3B) and HPLC.

Table 3. Kinetic Constants of OmpT for Cleavage of Decapeptides without Urea

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sequence</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}$ (s⁻¹)</th>
<th>$k_{\text{cat}}/K_m$ (s⁻¹ µM⁻¹)</th>
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<td>30</td>
<td>62</td>
<td>2.1</td>
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</table>

The adjacent amino acid residues on the cleavage by OmpT.

References

12) Yam, C. H., Siu, W. Y., Kaganovich, D., Ruderman, J. V., and Poon, R. Y. C., Cleavage of cyclin A at the adjacent amino acid residues on the cleavage by OmpT.


