Identification of Catalytic Residues of Pepstatin-insensitive Carboxyl Proteinases from Prokaryotes by Site-directed Mutagenesis*

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Pepstatin-insensitive carboxyl proteinases from *Pseudomonas* sp. (PCP) and *Xanthomonas* sp. (XCP) have no conserved catalytic residue sequences, -Asp-Thr-Gly- (Asp is the catalytic residue) for aspartic proteinases. To identify the catalytic residues of PCP and XCP, we selected presumed catalytic residues based on their high sequence similarity, assuming that such significant sites as catalytic residues will be generally conserved. Several Ala mutants of Asp or Glu residues were constructed and analyzed. The D170A, E222A, and D328A mutants for PCP and XD79A, XD169A, and XD348A mutants for XCP were not converted to mature protein after activation, and no catalytic activity could be detected in these mutants. The specificity constants toward chromogenic substrate of the other PCP and XCP mutants, except for the D84A mutant of PCP, were similar to that of wild-type PCP or XCP. Coupled with the result of chemical modification (Ito, M., Narutaki, S., Uchida, K., and Oda, K. (1999) J. Biochem. (Tokyo) 125, 210–216), a pair of Asp residues (170 and 328) for PCP and a pair of Asp residues (169 and 348) for XCP were elucidated to be their catalytic residues, respectively. The Glu222 residue in PCP or Asp79 residue in XCP was excluded from the candidates as catalytic residues, since the corresponding mutant retained its original activity.

Acid proteinases, having their proteolytic activities in acidic pH regions, have recently been called aspartic proteinases, since a pair of carboxyl groups of aspartic acid residues has been shown to be involved in their catalytic function (1). These enzymes are inactivated by pepstatin (2), acetyl pepstatin (3), diazoacetyl-dl-norleucine methyl ester, and 1,2-epoxy-3-(p-nitrophenoxy)propane (4).

In 1972, Murao et al. (6, 7) isolated three new types of proteinases, designated acid proteinase A, B, and C, from *Scytalidium lignicolum* ATCC 24568. Proteinases A and C were insensitive to all of the inhibitors as mentioned above (8, 9), while proteinase B was inhibited by 1,2-epoxy-3-(p-nitrophenoxy)propane but insensitive to pepstatin and diazoacetyl-dl-norleucine methyl ester (8, 10). These enzymes had unique substrate specificities (10–14). The complete amino acid sequence of proteinase B was quite different from that of other aspartic proteinases (15). Acid proteinases having similar properties to those of *Scytalidium*-type proteinases, called pepstatin-insensitive carboxyl proteinases, subsequently have been found widely distributed among fungi (16–21), bacteria (22, 23), and even thermophilic bacteria (24–27).

Recently, CLN2, encoding pepstatin-insensitive lysosomal peptidase, has been discovered in classical late infantile neuronal ceroid lipofuscinosis patients' brains (28). The amino acid sequence of CLN2 gave a significant match with those of PCP and XCP. The transcript of CLN2 mRNA was widely distributed in human tissues. This paper was the first example showing indirectly that a pepstatin-insensitive carboxyl proteinase exists in mammal tissues.

Of pepstatin-insensitive carboxyl proteinases, *Pseudomonas* sp. 101 carboxyl proteinase (PCP) and *Xanthomonas* sp. T-22 carboxyl proteinase (XCP) were the first and second isolated enzymes from prokaryotes (22, 23). These enzymes were not inactivated by acetyl pepstatin, diazoacetyl-dl-norleucine methyl ester, and 1,2-epoxy-3-(p-nitrophenoxy)propane but were inhibited by tyrostatin (N-isovaleryl-tyrosyl-leusyl-tyrosinal) (29). We have cloned and sequenced the PCP and XCP genes (30, 31) and developed efficient expression systems for both enzymes as zymogens in *Escherichia coli* cells. The zymogens were converted to mature enzymes through autoproteolytic activations under acidic pH conditions. The COOH-terminal pro-region of XCP (192 amino acid residues) was not essential for the forming of active mature XCP (31). The primary structures of PCP and XCP showed no homology to pepstatin-sensitive carboxyl proteinases (aspartic proteinase) reported so far, whereas approximately 52% identity (65% similarity) existed between PCP and XCP (Fig. 1). Furthermore, the conserved catalytic residues (-Asp-Thr-Gly-) for aspartic proteinases did not exist in both enzymes.

As reported previously, it was verified that a pair of carboxyl groups was associated with the catalytic functions of PCP and XCP by the zinc(II)-pyridine-2-azo-3-p-dimethylaniline method and kinetics analysis (33, 35). Recently, we have shown that Asp146 and Glu222 residues of PCP were involved in the catalytic function, probably as substrate binding sites by differential labeling using N,N-dicyclohexylcarbodiimide and tyrostatin (36). Catalytic residues of PCP and XCP have not yet been identified.

To identify the catalytic residues of PCP and XCP, we took a molecular biological approach based on their high sequence similarities. This paper describes the construction and expres-
sion of plasmids for mutant PCPs and XCPs and the identification of catalytic residues based on their autocatalytic mature-
ations and enzyme activities.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases and modifying enzymes were purchased from Nippon Gene (Toyama, Japan) or Toyobo (Osaka, Japan). AmpliTaq DNA polymerase Stoffel fragment and Dye terminator cycle sequencing kit were obtained from Perkin-Elmer (Chiba, Japan).

**Materials**

**E. coli** JM109 (741 ‘McrA’), recA1, endA1, gyrA96, thi-1, hsdR17(rk^K^, m^K^), supE44, relA1, 0lacZD15, lacY1 (supE4, hsdS^B^, thi, 0lac-proAB), [P’ traD36 proAB lacI^Q^ZAM15)], and TG1 (supE4, hsdS^B^, thi, 0lac-proAB), [P’ tralacD36 proAB lacI^Q^ZAM15]) were used as hosts. Plasmids, pUC19, pKK223-3, and pUK223-3 were used for expression and sequencing. Bacteria were grown in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride, pH 7.0), 2× YT broth (1.6% tryptone, 2.4% yeast extract, 0.5% glycerol, 1.25% K_2HPO_4, and 0.38% KH_2PO_4, pH 7.0).

**DNA Manipulation**

The general procedures for DNA manipulation were based on those described in Sambrook et al. (37). Protocol for nucleotide sequencing was recommended by the respective manufacturers.

**Proteinase Activity**

Proteinase activity was determined by Anson’s method with a modification using casein as a substrate (6). One unit of enzyme was defined as the liberation of 1 μg of tyrosine per ml of reaction mixture per min.

**Polymerase Chain Reaction**

The amplification was performed in 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3.75 mM MgCl_2, 0.16 mM dNTPs, 10 ng of MV18-2, pUKCP2212, or pUKCP4192 (30, 31), 20 pmol of each primer, and 5 units of Taq polymerase in a total volume of 100 μl. The amplification conditions consisted of 25 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 90 s, and extension at 72 °C for 90 s. The primers and vectors for mutation are shown in Table 1 and Fig. 2, respectively.

**Construction of PCP Expression Plasmid, pUKCP2212**

Construction of a superior PCP expression plasmid, pUKCP2212, was carried out as follows. pUKCP2212 (30) was digested with EcoRI and HindIII, and the resultant 0.9-kilobase pair fragment was inserted into the EcoRI and HindIII site of pUK223-3 (31). pKCP221 was digested with EcoRI, and the resultant 1.6-kilobase pair fragment was cloned into the EcoRI site of plasmid constructed above. The resultant plasmid was designated pUKCP221. MV18-2 (30) was amplified with a sense primer 10-M1 and an antisense primer 704R. The PCR product was digested with PstI, and the resultant fragment was inserted into the Smal–PstI site of pKK223-3. pCPP221 was digested with PstI, and the resultant fragment was not inserted into the Smal–PstI site of plasmid constructed above. The resultant plasmid was designated pKCP221. MV18-2 (30) was amplified with a sense primer 10-M1 and an antisense primer 704R. The PCR product was digested with PstI, and the resultant fragment was inserted into the Smal–PstI site of pUKCP221. The resultant plasmid was designated pUKCP221.

**Construction of PCP Mutant Plasmids**

p84A and p225A—A 910-bp fragment of pUKCP2212 was amplified with a sense primer 10-M1 and an antisense primer D84ARV. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with NdeI and XbaI, and the fragment was cloned into pUKCP2212 from which the fragment containing the PCP gene was removed. The resultant plasmid was designated pUK84A. A 960-bp fragment of pUKCP2212 was amplified with a sense primer, SD84A, and an antisense primer, 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with NdeI and XbaI, and the fragment was ligated into the NheI site of pD84ARV. The mutant plasmid was designated pD84A. pD225A was constructed by the same strategy described above using a sense primer SD225A and an antisense primer D225ARV. The mutant plasmid was designated pD225A.

### Table 1

**PCR primers for the construction of PCP and XCP mutants**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-M1</td>
<td>5’-AGGAGCTCATATGGAATGTCGAC-3’</td>
</tr>
<tr>
<td>2501RX</td>
<td>5’-TTCTAGAAGCTTTGACCCACAGGC-3’</td>
</tr>
<tr>
<td>SD84A</td>
<td>5’-CGCTAGCCATCGCTTGGTGTCG-3’</td>
</tr>
<tr>
<td>D84ARV</td>
<td>5’-CGCTAGCCATCGCTTGGTGTCG-3’</td>
</tr>
<tr>
<td>SD124A</td>
<td>5’-AGGAGCTCATATGGAATGTCGAC-3’</td>
</tr>
<tr>
<td>D124ARV</td>
<td>5’-AGGAGCTCATATGGAATGTCGAC-3’</td>
</tr>
<tr>
<td>SD170A</td>
<td>5’-CTGAGCCCGGTATGAAATGACT-3’</td>
</tr>
<tr>
<td>D170ARV</td>
<td>5’-CTGAGCCCGGTATGAAATGACT-3’</td>
</tr>
<tr>
<td>SD225A</td>
<td>5’-TGGAGCTCATATGGAATGTCGAC-3’</td>
</tr>
<tr>
<td>SD225ARV</td>
<td>5’-TGGAGCTCATATGGAATGTCGAC-3’</td>
</tr>
<tr>
<td>SD265A</td>
<td>5’-CGCTAGCCATCGCTTGGTGTCG-3’</td>
</tr>
<tr>
<td>SD265ARV</td>
<td>5’-CGCTAGCCATCGCTTGGTGTCG-3’</td>
</tr>
<tr>
<td>SD328A</td>
<td>5’-GAGGCTACGCTGGTATATGCGGACAGCCG-3’</td>
</tr>
<tr>
<td>SD328ARV</td>
<td>5’-GAGGCTACGCTGGTATATGCGGACAGCCG-3’</td>
</tr>
<tr>
<td>SE217A</td>
<td>5’-GAGGCTACGCTGGTATATGCGGACAGCCG-3’</td>
</tr>
<tr>
<td>SE217ARV</td>
<td>5’-GAGGCTACGCTGGTATATGCGGACAGCCG-3’</td>
</tr>
<tr>
<td>SE222A</td>
<td>5’-GAGGCTACGCTGGTATATGCGGACAGCCG-3’</td>
</tr>
<tr>
<td>SE222ARV</td>
<td>5’-GAGGCTACGCTGGTATATGCGGACAGCCG-3’</td>
</tr>
<tr>
<td>E1M7</td>
<td>5’-GGAAATCCAGGACCATCCATGAGATT-3’ EcoRI</td>
</tr>
<tr>
<td>C4RM4</td>
<td>5’-TICAGAGTTCTCCAGGCACTGCGGAGC-3’ Stop</td>
</tr>
<tr>
<td>XSD79A</td>
<td>5’-GGCTACGCGCATGCGGATCTGAT-3’</td>
</tr>
<tr>
<td>XD79ARV</td>
<td>5’-GGCTACGCGCATGCGGATCTGAT-3’</td>
</tr>
<tr>
<td>XOVS169</td>
<td>5’-GGCTCCAGGGCGCTTGGTCTCAG-3’</td>
</tr>
<tr>
<td>XOVS169ARV</td>
<td>5’-GGCTCCAGGGCGCTTGGTCTCAG-3’</td>
</tr>
<tr>
<td>YSS230A</td>
<td>5’-GGGCGCCACGTGGTAAGAGCAGGGCC-3’</td>
</tr>
<tr>
<td>YSS230ARV</td>
<td>5’-GGGCGCCACGTGGTAAGAGCAGGGCC-3’</td>
</tr>
<tr>
<td>YSS235A</td>
<td>5’-GGACTTCACGCTGGTAAGAGCAGGGCC-3’</td>
</tr>
<tr>
<td>YSS235ARV</td>
<td>5’-GGACTTCACGCTGGTAAGAGCAGGGCC-3’</td>
</tr>
<tr>
<td>704R</td>
<td>5’-AGATGCCATTTTCGGGGCTGAC-3’</td>
</tr>
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</table>

The amplification was performed in 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 3.75 mM MgCl_2, 0.16 mM dNTPs, 10 ng of MV18-2, pUKCP2212, or pUKCP4192 (30, 31), 20 pmol of each primer, and 5 units of Taq polymerase in a total volume of 100 μl. The amplification conditions consisted of 25 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 90 s, and extension at 72 °C for 90 s. The primers and vectors for mutation are shown in Table 1 and Fig. 2, respectively.
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Expression of Recombinant PCPs and XCPs in E. coli Cells

Wild-type and mutant plasmids were transformed into E. coli JM109 cells. Recombinant PCPs and XCPs were expressed by the method of Oda et al. (30, 31) with a slight modification. Western hybridization of expressed proteins was carried out according to the method of Towbin et al. (38), by using rabbit anti-PCP or anti-XCP antibodies and alkaline phosphatase-conjugated rabbit IgG antibodies.

Purification of Recombinant PCPs

E. coli JM109 harboring recombinant plasmid was cultured at 30 °C in 2× YT medium containing 0.1 mg/ml ampicillin and 1% glucose without optical density at 600 nm reached 2, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the cultivation was continued for 3 h. The cells collected by centrifugation from a 1-liter of cultured medium were suspended in 20 mM phosphate buffer, pH 6.5 (buffer A). The suspension was sonicated and centrifuged at 8000 × g for 20 min. The supernatant was fractionated with ammonium sulfate at 80% saturation. After standing at 4 °C overnight, the precipitate was collected by centrifugation (20,000 × g, 20 min) and dialyzed against 50 mM acetate buffer, pH 4.8 (buffer B). The dialysate was centrifuged, and the supernatant was loaded onto a column of DEAE-Sepharose CL-6B (1.5 × 15 cm) equilibrated previously with

Construction of XCP Mutant Plasmids

pD124A—A 1030-base fragment of pUCKCP2212 was amplified with a sense primer 10-M1 and an antisense primer D124ARV. The amplified fragment was inserted into the Smal site of pUC19. The lac promoter and the PCP gene were inserted in opposite directions. The resultant plasmid was designated pD124ARV. A 580-base fragment of pUCKCP2212 was amplified with an antisense primer SD124A and an antisense primer 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with Aor51HI and XbaI, and the fragment was ligated into the NdeI—XbaI site of pUCKCP2212, from which the fragment containing the PCP gene was removed. The mutant plasmid was designated pD124A.

pD700A—A 1170-base fragment of pUCKCP2212 was amplified with a sense primer 10-M1 and an antisense primer D700ARV. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was designated pD700ARV. A 690-base fragment of pUCKCP2212 was amplified with a sense primer SD700A and an antisense primer 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with NdeI and Ddel, and the fragment was digested with Ddel and XbaI. Both resultant fragments were ligated into the NdeI—XbaI site of pUCKCP2212 from which the fragment containing the PCP gene was removed. The mutant plasmid was designated pD700A.

pD265A—A 1450-base fragment of pUCKCP2212 was amplified with a sense primer SD265A and an antisense primer 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was designated pS265ARV. A 1450-base fragment of pUCKCP2212 was amplified with a sense primer SD265A and an antisense primer 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with Ddel and XbaI, and the fragment was cloned into the PvuII—XbaI site of pUCKCP2212 from which the fragment containing the PCP gene was removed. The mutant plasmid was designated pD265A.

pD328A—A 1640-base fragment of pUCKCP2212 was amplified with a sense primer 10-M1 and an antisense primer D328ARV. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with NdeI and SpI, and the resultant fragment was cloned into the NdeI—SpI site of pUCKCP2212 from which the fragment containing the PCP gene was removed. The mutant plasmid was designated pD328ARV. A 1640-base fragment of pUCKCP2212 was amplified with a sense primer SD328A and an antisense primer 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The lac promoter was amplified with a sense primer SD328A and an antisense primer 2501RX, and the fragment was ligated into the Smal site of pUC19. The lac promoter and the PCP gene were inserted in opposite directions. The resultant plasmid was digested with NdeI and SpI, and the fragment was ligated into the SpI site of pD328ARV. The mutant plasmid was designated pD328A.

pE217A and pE222A—A 560-base fragment of pUCKCP2212 was amplified with a sense primer SE217A and an antisense primer 2501RX. The amplified fragment was inserted into the Smal site of pUC19. The lac promoter and the PCP gene were inserted in the same directions. The resultant plasmid was designated pE217A. A 1300-base fragment of pUCKCP2212 was amplified with a sense primer 10-M1, and an anti- sense primer E217ARV. The amplified fragment was inserted into the Smal site of pUC19. The resultant plasmid was digested with NdeI and SpI, and the fragment was ligated into the NdeI—SpI site of pUCKCP2212 from which the fragment containing the PCP gene was removed. The mutant plasmid was designated pE217A. pE222A was constructed by the same strategy described above using a sense primer SE222A and an anti- sense primer E222ARV. The mutant plasmid was designated pE222A.

pD710A—A 1230-base fragment of pUCKXCPAC192 was amplified with a sense primer E1M7 and an antisense primer XOV-348ARV. The amplified fragment was inserted into the Smal site of pUC19. The lac promoter and the PCP gene were inserted in opposite directions. The resultant plasmid was digested with EcoRI and HindIII, and the fragment was cloned into the EcoRI—HindIII site of pD710ARV from which the fragment of the XCP gene was removed. The mutant plasmid was designated pD710A.
buffer. Recombinant PCP was eluted with a linear gradient of sodium chloride from 0 to 0.5 M. The concentrated sample was loaded onto a column of Sephadex G-75 (2.5 × 90 cm) equilibrated with buffer B containing 10 mM CaCl$_2$ and 0.02% NaN$_3$. Active fractions were pooled and stored at −20°C until use. In the cases of some mutants, a part of the purification procedures was changed. For D84A, after treatment with ammonium sulfate, the enzyme solution was loaded onto a CM-Sepharose CL-6B column (1.5 × 90 cm) equilibrated with buffer B. Recombinant PCP was eluted with a linear gradient of sodium chloride from 0 to 0.5 M. For D124A, after treatment with ammonium sulfate, the enzyme solution was loaded onto a Sephadex G-75 (2.5 × 90 cm) equilibrated with buffer B and active fractions were eluted with a linear gradient of sodium chloride from 0 to 0.5 M.

**Purification of Recombinant XCPs**

Expression in *E. coli* JM109 was carried out by the method described above except for the activation. After sonication, the supernatant was diluted 6-fold with 60 mM sodium acetate buffer, pH 4.7, containing 12 mM CaCl$_2$ and then incubated at 37 °C for 5 h. The enzyme solution was fractionated with ammonium sulfate at 80% saturation. After standing at 4 °C overnight, the precipitate was collected by centrifugation (20,000 g, 20 min) and dialyzed against buffer C overnight. The dialysate was loaded onto a DEAE-Sepharose CL-6B column (1.5 × 15 cm) equilibrated with buffer C, and active fractions were eluted with a linear gradient of sodium chloride from 0 to 0.5 M.

**Kinetic Analysis**

Kinetic analysis was performed at 37 °C in 0.1 M sodium formate buffer, pH 3.5 using Lys-Pro-Ala-Leu-Phe-Nph-Arg-Leu as a chromogenic peptide substrate. The cleavage of the substrate between Phe and Nph was monitored spectrophotometrically by following the decrease in absorbance at 300 nm. Initial rate was measured with six concentrations of substrate, and kinetic constants $V_{\text{max}}$ and $K_{\text{cat}}$ were calculated from Lineweaver-Burk plots. $k_{\text{cat}}$ was derived from $V_{\text{max}} = k_{\text{cat}}[E]_0$, where [E]$_0$ is the enzyme concentration.

**CD Spectra**

The CD spectra were measured using a Jasco model J-720 spectropolarimeter at 25 °C.

**Electrophoresis**

SDS-polyacrylamide gel electrophoresis was done according to the method of Laemmli (39). The enzyme preparation was electrophoresed at room temperature. The gel was stained with Coomassie Brilliant Blue R-250 in order to detect protein bands.

**RESULTS**

In order to identify the catalytic residues of PCP and XCP, we selected eight presumed catalytic residues based on their high sequence similarity between both enzymes (Fig. 1). Several Ala mutants of Asp or Glu residues were constructed. They were D84A, D124A, D170A, D225A, D265A, and D328A of PCP and XD79A, XD169A, XE230A, XE235A, and XD348A of XCP. The construction of each recombinant plasmid was confirmed by the existence of newly introduced restriction sites.

**Expression of Recombinant Wild-type PCP and XCP**—In our previous study, *E. coli* JM109 cells carrying pKCP221 produced about 1 mg of recombinant PCP in 1 liter of cultured medium (30). In order to carry out a mutagenesis study, a superior expression system for recombinant enzymes was essential. A plasmid, pUKCP221, was derived from pUK223–3, which was constructed for efficient expression of recombinant XCP (31). These plasmids are shown in Fig. 2. pUKCP221 contained tandem Shine-Dalgarno (SD) sequences and the replication origin from pUC19. Cultivation conditions for recombinant PCP production were optimized. When *E. coli* JM109 harboring pUKCP2212 was cultured in 2 × YT medium at 30 °C, about 20 mg of PCP was produced in 1 liter of cultured medium. On the other hand, *E. coli* JM 109 harboring pUKXCPA192 produced about 15 mg of XCP in 1 liter of cultured medium under the conditions described above.

**Expression of Mutant PCPs in *E. coli***—According to the previous report (30), recombinant wild-type PCP in *E. coli* cells was produced as a precursor protein (62 kDa). The molecule was processed and secreted into the periplasmic space as a 49-kDa inactive protein. The protein was autokatalytically converted to 40-kDa mature PCP under acidic conditions. *E. coli* cells carrying wild-type and mutant plasmids, pUKCP2212, pD84A, pD124A, pD170A, pD225A, pD265A, pD328A, pE217A, and pE222A produced immunoreactive proteins (wild-type, D84A, D124A, D170A, D225A, D265A, D328A, E217A, and E222A, respectively) against the anti-PCP antibody. As shown in Fig. 3, D84A, D124A, D225A, D265A, and E217A produced 62-kDa precursor protein and 43-kDa inactive partially processed PCP. After dialysis against 50 mM acetate buffer, pH 4.8, precursor proteins of D124A, D225A, D265A, and E217A were converted to 40-kDa mature protein. D124A showed slightly lower proteinase activity than that of wild-type PCP. D225A, D265A, and E217A showed almost the same proteinase activities as wild-type PCP. D84A was converted to 40-kDa mature protein after dialysis with 50 mM acetate buffer, pH 4.8, and incubation at 25 °C for 36 h, but the proteinase activity was...
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Expression plasmid for PCP and XCP precursors. Expression vector pUK223-3 was constructed from plasmid pKK223-3 and pUC19, in which the tac promoter, multicloning site, the transcriptional terminator signal, rrrBT2, were derived from pKK223-3, and the replication origin (ori) of the plasmid from pUC19. The potential Shine-Dalgarno (SD) sequence of both enzymes and the tac promoter are indicated by boxes. The dotted underline shows the introduced EcoRI site. The restriction sites that were lost during the construction are indicated in parentheses.

0.2% of that of wild-type PCP (duplicate runs). On the other hand, D170A, D328A, and E222A were not processed autocatalytically in E. coli cells. These mutants resulted in complete losses of their enzyme activities.

Enzymatic Properties of Mutant PCPs—Wild type, D84A, D124A, E217A, D225A, and D265A were purified from cell-free extracts of E. coli JM109 cells as described under “Experimental Procedures.” As shown in Fig. 4, the purified enzymes except for D84A showed a single protein band at around 40 kDa corresponding to the authentic enzyme on SDS-polyacrylamide gel electrophoresis. The D84A was very unstable and partially purified 4.8-fold with 19.2% recovery. To confirm their structures, the CD spectra were taken for authentic PCP and recombinant PCPs (wild type, D84A, D124A, E217A, D225A, and D265A) as shown in Fig. 5. The spectral patterns of these proteins except for D84A and D124A were identical. The variation in the CD spectrum of D84A was thought to be caused by the impurity of the enzyme or structural change due to the replacement of a carboxyl group. Table II shows kinetic parameters toward chromogenic substrate of wild-type and mutant PCPs. The $K_m$, $k_{cat}$, and catalytic efficiency ($k_{cat}/K_m$) values of authentic PCP were reported to be 6.3 $\mu$m, 51.4 $s^{-1}$, and 8.2 $\mu$m$^{-1}s^{-1}$, respectively (34). These values of wild-type PCP were the same as those reported so far. The measured $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values of the purified mutant PCPs were nearly the same value as that of wild-type PCP except for D84A and D225A. Each value of D84A was changed significantly (Table II). The $K_m$ and $k_{cat}$ values of D84A were 149 $\mu$m and 0.055 $s^{-1}$, respectively. The $k_{cat}/K_m$ value of D84A was nearly 4 orders of magnitude lower than that of wild-type PCP. In the case of D225A, the $K_m$ value increased, while the $k_{cat}$ value remained nearly the same as that of wild-type PCP. The $k_{cat}/K_m$ value of D225A was 4 times lower than that of wild-type PCP.

Expression of Mutant XCPs in E. coli Cells—When the COOH-terminal Pro-region deletion mutant (Δ192) was expressed in E. coli cells, a 64-kDa precursor protein composed of NH$_2$-terminal Pro-region and mature XCP was detected in the cell-free extracts, and it was converted into active XCP after incubation at pH 4.8 and 37 °C (31). E. coli JM109 cells carrying wild-type and mutant plasmids, pUKXCPΔC192, pXD79A, pXD169A, pXE230A, pXE235A, and pXD348A, produced immmunoreactive proteins (wild type, XD79A, XD169A, XE230A, XE235A, and XD348A) against the anti-XCP antibody. As shown in Fig. 3, XE230A and XE235A produced a 64-kDa precursor protein. After activation, these mutant XCPs were autocatalytically converted to 42-kDa mature protein, which has proteinase activity. On the other hand, XD79A, XD169A, and XD348A produced a 64-kDa precursor protein, but these mutant XCPs were not processed to active enzymes under acidic conditions.

Enzymatic Properties of Mutant XCPs—Wild type, XE230A, and XE235A were purified from cell-free extracts of E. coli JM109 cells as described under “Experimental Procedures.” As shown in Fig. 4, the purified mutant XCPs were confirmed to show a single protein band at around 42 kDa corresponding to the authentic enzyme on SDS-polyacrylamide gel electrophoresis. As shown in Fig. 6, the CD spectral pattern of XE230A was different from that of the wild-type XCP. Table III shows kinetics parameters toward chromogenic substrate of wild-type and mutant XCPs. The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values of the authentic XCP were reported to be 3.6 $\mu$m, 52.2 $s^{-1}$, and 14.5 $\mu$m$^{-1}s^{-1}$, respectively (34). These values of A192 were nearly the same as those of wild-type XCP. The $k_{cat}/K_m$ values of XE230A and XE235A toward chromogenic substrate were 6 times lower than that of wild-type XCP. These results indicate that the replacement of Glu230 and Glu235 residues in XCP caused structural changes in their three-dimensional structure, and the activities of the mutant XCPs decreased.

DISCUSSION

In order to identify the catalytic residues of pepstatin-insensitive carboxyl proteinases from Pseudomonas sp. (PCP) and Xanthomonas sp. (XCP), the high sequence similarities between PCP and XCP were used as the strategy for identification. Oda et al. (33) have demonstrated that PCP has a pair of active carboxyl residues participating in the catalytic function, as do aspartic proteinases such as pig pepsin. We searched for conserved Asp or Glu residues in their primary structures. As shown in Fig. 1, eight amino acid residues were chosen as candidates for their catalytic residues. Mutant PCPs (D84A, D124A, D170A, E217A, E222A, D225A, D265A, and D328A) and mutant XCPs (XD79A, XD169A, XE230A, XE235A, and XD348A) were constructed by site-directed mutagenesis.

Recombinant PCP expressed in E. coli cells was produced as a 62-kDa precursor protein, which was converted to 43-kDa inactive protein by E. coli cells. The 43-kDa protein was autocatalytically converted to 40-kDa active PCP under acidic conditions. In the case of XCP, COOH-terminal Pro-region deleted XCP (△192) was autocatalytically converted directly to a 42-kDa mature protein under acidic conditions.

Aspartic proteinases from mammals are synthesized as inactive precursors and subsequently activated to become active proteinases (40, 41). In pepsin, intramolecular pepsinogen activation was accomplished in the active site (center), and the active site mutant did not have any activity (42).

Upon the destruction of the catalytic residue(s) by site-directed mutagenesis, mutant PCPs and XCPs cannot be processed to mature protein under acidic conditions, and their proteinase activities will be completely lost.

The D170A, E222A, D328A, XD79A, XD169A, and XD348A mutants for XCP, respectively. As shown in Fig. 7, sequence comparison around Asp$^{84}$, Asp$^{170}$, and Asp$^{228}$ residues in PCP revealed significant similarities among PCP,
XCP, and the CLN2 protein. These sequences were also conserved in a thermostable and pepstatin-insensitive carboxyl proteinase, Kumamolysin from thermophilic bacteria (data not shown). Based on these results described above, we thought that these three amino acid residues were important for the structure and/or function of the pepstatin-insensitive carboxyl proteinase family.

D84A in PCP was processed to mature protein after prolonged incubation under acidic conditions and revealed proteinase activity (Fig. 3). The $k_{cat}/K_m$ value of partially purified D84A toward chromogenic substrate was 4 orders of magnitude lower than that of wild-type PCP (Table II). According to the Western blotting analysis, a significant difference in expression levels between D84A and wild-type PCP was not observed (Fig. 3). These results suggested that molecular activity of D84A was lower than that of wild-type PCP. In general, intramolecular enzyme reaction proceeds faster than intermolecular reaction. Hence, we concluded that weak proteinase activity of D84A sufficed for the autocatalytic maturation. According to the CD spectrum of the partially purified D84A, it was suggested that the three-dimensional structure of the enzyme was changed (Fig. 5). This variation may cause the instability of D84A. XD79A in XCP (corresponding to D84A in PCP) did not show any autocatalytic processing and proteinase activity. Consequently, we concluded that Asp84 in PCP was one of the amino acid residues involved in the catalytic function, probably as a substrate binding site.

D170A and D328A in PCP did not show any autocatalytic processing and proteinase activities. XD169A and XD348A, corresponding to Asp 170 and Asp 328 residues in PCP, respectively, also did not show any autocatalytic maturation and proteinase activity as described above in PCP mutants. Therefore, we concluded that a pair of amino acid residues, Asp170 and Asp328, found in PCP and a pair of amino acid residues, Asp169 and Asp348, found in XCP are involved in their catalytic functions as catalytic residues, respectively.

D124A in PCP showed proteinase activity 60% of that of wild-type PCP (Fig. 3). The $k_{cat}/K_m$ value toward the chromogenic substrate of the purified D124A was almost the same value as that of wild-type PCP (Table II). According to the CD spectrum, it was suggested that mutation of Asp124 in PCP causes some structural changes in the mutant (Fig. 5). This mutant may be unstable during the maturation. These results excluded the possibility of Asp124 in PCP (Asp123 in XCP) being a catalytic residue or substrate binding site.

E222A in PCP (corresponding to XE235A in XCP) did not show any autocatalytic maturation and proteinase activity. On the other hand, XE235A in XCP showed almost the same proteinase activity as wild-type XCP, and the $k_{cat}/K_m$ value was 16% of that of the control (Table III). Furthermore, as reported previously, we demonstrated that Glu 222 in PCP was elucidated to be involved in its catalytic function as a substrate binding site, using the differential labeling method (36). Based on these results, we concluded that Glu222 in PCP and Glu235 in XCP were involved in their catalytic functions as catalytic residues, respectively.

E217A in PCP (corresponding to XE230A in XCP) showed proteinase activity, and the $k_{cat}/K_m$ value was about 70% of that of the control (Table II). XE230A in XCP showed autocatalytic maturations and proteinase activities (Fig. 3), although the $k_{cat}/K_m$ value was about 17% of that of the control (Table III). The locations of the Glu217 and Glu222 in the primary...
structure in the PCP molecule are very close to each other (Fig. 1). These results suggest that the Glu 217 residue in PCP (corresponding to the Glu 230 residue in XCP) may be involved in substrate binding, but not so significant.

D225A in PCP was converted to mature protein and showed proteinase activities (Fig. 3). The CD spectrum of this mutant was similar to that of wild-type PCP (Fig. 5). These results suggested that the Asp225 residue in PCP was not involved in catalytic function or substrate binding.

Two catalytic aspartic residues in pepsin, Asp₃ and Asp₂₁₅, are located in two independent domains (43). Distance between the residues was important for structural stability as an active form. The number of amino acid residues between the Asp¹⁷₀ and Asp³₂₈ residues in PCP (corresponding to the Asp¹⁶₉ and...
Asp residues in XCP, respectively) was 159 (180), approximately similar to the distance (184 residues) between the two Asp residues in pepsin. As shown in Fig. 7, a pair of catalytic aspartic acid residues in PCP was conserved in CLN2. Distance between the residues in CLN2 was close to that of PCP. Based on the data described above, it was strongly suggested that PCP, XCP, and CLN2 are also two-domain proteins.

Taking into consideration the present results, we concluded that a pair of Asp residues (Asp\(^{170}\) and Asp\(^{322}\)) in PCP and a pair of Asp residues (Asp\(^{169}\) and Asp\(^{348}\)) in XCP are powerful candidates for their catalytic residues, respectively. Furthermore, we predicted that a pair of Asp residues (Asp\(^{165}\) and Asp\(^{328}\)) in CLN2 is also a candidate for its catalytic residues.

According to the exploration of subsite binding specificities of PCP and XCP using chromogenic substrates, their S\(_{\text{b}}\) subsites were elucidated to be apparently different from each other (33–35). The Asp and Glu residues in PCP, corresponding to the Asp\(^{79}\) and Glu\(^{235}\) residues in XCP, were also strongly suggested to be involved in their catalytic functions, probably as substrate binding sites.

Three-dimensional structure analysis of the complex of PCP and the competitive inhibitor tyrostatin is under investigation. We hope to obtain more high level information to support our results.

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Identification of Catalytic Residues of Pepstatin-insensitive Carboxyl Proteinases from Prokaryotes by Site-directed Mutagenesis
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