Engineering of Porcine Pepsin

ALTERATION OF S1 SUBSTRATE SPECIFICITY OF PEPсин TO THOSE OF FUNGAL ASPARTIC PROTEINASES BY SITE-DIRECTED MUTAGENESIS

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The S1 substrate specificity of porcine pepsin has been altered to resemble that of fungal aspartic proteinase with preference for a basic amino acid residue in P1 by site-directed mutagenesis. On the basis of primary and tertiary structures of aspartic proteinases, the active site flap mutants of porcine pepsin were constructed, which involved the replacement of Thr-77 by Asp (T77D), the insertion of Ser between Gly-78 and Ser-79 (G78(S)S79), and the double mutation (T77D/G78(S)S79). The specificity of the mutants were determined using p-nitrophenylalanine-based substrates containing a Phe or Lys residue at the P1 position. The double mutant cleaved the Lys-Phe(4-NO2) bonds, while wild-type enzyme digested other bonds. In addition, the pH dependence of hydrolysis of Lys-containing substrates by the double mutant indicates that the interactions between Asp-77 of the mutant and P1 Lys contribute to the transesterification process. The double mutant was also able to activate bovine trypsinogen to trypsin by the selective cleavage of the Lys6–Ile7 bond of trypsinogen. Results of this study suggest that the structure of the active site flap contributes to the S1 substrate specificity for basic amino acid residues in aspartic proteinases.

Aspartic endopeptidases (EC 3.4.23._) comprise a group of enzymes whose proteolytic activities are dependent on two aspartyl residues, Asp-32 and Asp-215, in pepsin numbering (1). Mammalian and fungal enzymes have been extensively characterized, and their three-dimensional structure has been determined at high resolution (2–7). The enzymes of this family are similar in their tertiary structures, the active site directed mutagenesis. On the basis of primary and tertiary structures of aspartic proteinases, the active site-flap mutants of porcine pepsin were constructed, with preference for a basic amino acid residue in P1 by site-directed mutagenesis. According to these reports, although the hydrogen bonding pattern between the main chain of inhibitor and enzyme is well conserved in all inhibitor-enzyme complexes, the differences in the size of subsites, of which residues make van der Waals contacts with the side chain of inhibitor, may control the substrate specificities in aspartic proteinases. Several attempts to examine the structural determinants of substrate specificities of aspartic proteinases by site-directed mutagenesis have been made recently (17–23). These provided direct evidence that the subtle differences in the structures of substrate binding sites of aspartic proteinases were sufficient to alter their substrate specificities.

Aspartic proteinases generally show specificity for the cleavage of the bond between hydrophobic residues occupying the S1–S2 subsites. However, fungal enzymes also have preferences for a Lys residue in the P1 position, which leads to activation of trypsinogen by cleavage of the Lys6–Ile7 bond (24–27). The S1 subsite is formed by several hydrophobic residues in the neighborhood of the catalytic Asp-32 and by the residues on the active site flap. The sequence alignments of mammalian and fungal enzymes reveal that Asp-77 and Ser-79 on the flap are conserved in all family members able to activate trypsinogen, but Asp-77 is replaced by Ser or Thr and Ser-79 is deleted in those unable to do so (Table I). Asp-77 is shown to be the binding site to P1 Lys in a substrate by crystallographic study of penicillopepsin (EC 3.4.23.20) (11) and site-directed mutagenesis studies of aspergillopepsin I (EC 3.4.23.18) (20, 21) and rhizopuspepsin (EC 3.4.23.21) (22).

Pepsin (EC 3.4.23.1) is a typical aspartic proteinase produced in the gastric mucosa of vertebrates as a zymogen form. This enzyme has been extensively characterized, and its three-dimensional structure has been determined at high resolution (2, 3, 9). Porcine pepsin, in particular, has been studied as a model to analyze the structure-function relationship of the aspartic proteinases.

The present studies investigated whether the active site flap controls the S1 subsite specificity of aspartic proteinases, and for this we generated several mutants of porcine pepsin by site-directed mutagenesis and analyzed their enzymatic properties. The substitution of Thr-77 by Asp and the insertion of Ser between Gly-78 and Ser-79, corresponding to the fungal enzymes, conferred upon porcine pepsin the ability to hydrolyze the substrate containing a Lys residue at the P1 position. These experiments provide positive evidence of the importance of the active site flap of aspartic proteinases in the S1 subsite specificity.

EXPERIMENTAL PROCEDURES

Materials—Porcine pepsin, bovine trypsinogen, and bovine hemoglobin were from Sigma. Pro-Thr-Glu-Phe(4-NO2)-Arg-Leu was ob-
Comparison of the amino acid sequence of the active site flap in the aspartic proteinase family (pepsin numbering)

TABLE I

The amino acid sequences of porcine pepsin, human pepsin, human cathepsin D, human renin, mucorpepsin, aspergillopepsin I, penicillopepsin, rhizopuspepsin, and endotheiopain are aligned. The mutated positions are indicated in boldface characters.

| Residue number | Porcine pepsin | Human pepsin | Human cathepsin D | Human renin | Mucorpepsin | Aspergillopepsin I | Penicillopepsin | Rhizopuspepsin | Endotheiopain | Candidapepsin |
|----------------|---------------|--------------|------------------|-------------|-------------|------------------|----------------|---------------|---------------|-------------|
| 70             | L             | V             | F                | T           | L           | W                | W              | W             | F             | Y           |
| 71             | S             | S             | D                | S           | G           | T                | E              | D             | I             | G           |
| 72             | S             | T             | I                | G           | T           | Y                | M              | M             | G             | Y           |
| 73             | T             | Y             | Y                | Y           | G           | D                | G              | Y             | G             | D           |
| 74             | S             | G             | S                | G           | G           | D                | S              | S             | S             | G           |
| 75             | M             | T             | T                | G           | F           | G                | A              | A             | S             | G           |
| 76             | T             | G             | M                | Y           | M           | L                | A              | A             | S             | G           |
| 77             | G             | G             | G                | T           | G           | S                | S              | S             | S             | S           |

Retained from Calbiochem. Pro-Thr-Glu-Lys-Phe(4-NO₂)-Arg-Leu and Ac-Ala-Ala-Lys-Phe(4-NO₂)-Ala-Ala-NH₂ were custom-synthesized by TANA Laboratories (Houston, TX). Aspergillopepsin I was purified by the method described previously (28).

Synthesis of Porcine Pepsinogen cDNA and Construction of Expression Plasmid—Total RNA from porcine gastric mucosa was extracted and purified by the modified acid guanidinium thiocyanate/phenol/chloroform RNA extraction method (29). Porcine pepsinogen cDNA was synthesized by reverse transcriptase-polymerase chain reaction (PCR) (30) using the oligonucleotides 5'-CGCACATATGCTGACGAGTC-GCGCTGTG-3' and 5'-GGAGGAATTCAGCGACGGCCGAGGACCC-3', based on cDNA sequence of porcine pepsinogen reported by Lin et al. (31). Sense primer was designed to delete the signal sequence and introduce a translation initiation codon (Met) for direct expression. Plasmids, pUC119 and pET12a, were purchased from Takara Shuzo.

Site-directed Mutagenesis—Site-directed mutagenesis was performed by the method of Kunkel et al. (32). The following mutagenic primers were used: T77D, 5'-CACCTATGGCGACGGATCCAGCATGA-3'; T77G/8/S79, 5'-TCACATGGTACCGTTCAAGCTAGTCCGCTGCTG-3' and 5'-GGAGGAATTCAGCGACGGCCGAGGACCC-3', based on cDNA sequence of porcine pepsinogen reported by Lin et al. (31). Sense primer was designed to delete the signal sequence and introduce a translation initiation codon (Met) for direct expression. The NdeI/SalI fragment was inserted into pET12a, which was designated as pETPP, to express porcine pepsinogen under the control of a T7 promoter.

Overexpression and Purification of Recombinant Porcine Pepsin—The structure of porcine pepsinogen cDNA was reported earlier by Lin et al. (31). Therefore, porcine pepsinogen cDNA was cloned by reverse transcriptase-PCR, using total RNA from porcine gastric mucosa as a template. PCR primers were designed for direct expression in the E. coli pET system. The amplified cDNA was dideoxy-sequenced to verify whether the enzyme sequences were determined by the method of Ichishima (36) in 50 mM sodium citrate, pH 2.0, containing 1.25% acid-denatured hemoglobin at 30 °C. Trypsinogen activation was determined as described previously (20).

Kinetic Characterizations of Mutant Porcine Pepsins—Three chromogenic peptide substrates, Pro-Thr-Glu-Lys-Phe(4-NO₂)-Arg-Leu (peptide A), Pro-Thr-Glu-Lys-Phe(4-NO₂)-Arg-Leu (peptide B), and Ac-Ala-Ala-Lys-Phe(4-NO₂)-Ala-Ala-NH₂ (peptide C), were used as substrates for kinetic analyses. Assays with peptides A and B were performed as described by Dunn et al. (37) and with peptide C by the method of Hofmann and Hodges (38). The kinetic constants, K_m and V_max, were determined from plots of initial rates versus substrate concentrations with ranges of 0.010–1.000 mM for all substrates. Values for V_max were derived from V_max = k_cat [E], where [E] is the enzyme concentration.

Product Analysis—Purified enzymes (0.35 μg) were incubated in total volume of 100 μl of 20 mM sodium acetate, pH 3.5, containing 10 mM of each peptide substrate (peptides A, B, and C) for 8 h at 37 °C (enzyme/substrate, 1/1,000 mol/mol). Cleavage products were separated by reverse-phase high performance liquid chromatography on a TSK ODS-120T column (4.0 x 250 mm, Tosoh, Tokyo, Japan) and confirmed by amino acid sequence analysis.

pH Dependence Studies—The circular dichroism (CD) spectra were measured with a Jasco J-700 spectropolarimeter at room temperature in 1 mM potassium phosphate buffer, pH 7.0.

RESULTS

Overexpression and Purification of Recombinant Porcine Pepsin—The structure of porcine pepsinogen cDNA was reported earlier by Lin et al. (31). Therefore, porcine pepsinogen cDNA was cloned by reverse transcriptase-PCR, using total RNA from porcine gastric mucosa as a template. PCR primers were designed for direct expression in the E. coli pET system. The amplified cDNA was dideoxy-sequenced to verify whether it was synthesized correctly. The sequence was identical with that reported by Lin et al. (31) except for the substitution of Tyr-242 by Asp. Porcine pepsin has been sequenced in several laboratories by protein and cDNA sequencing (39, 40), and these studies indicated that there are two variants at position 242 of amino acid sequence (Asp or Tyr).
Folded by solubilization with 8 m urea and subsequent dialysis under alkaline pH as described under "Experimental Procedures." Porcine pepsin was purified from the refolded pepsinogen preparation by acidification and subsequent chromatography on a RESOURCE Q column and gave a single band on SDS-PAGE (Fig. 1, lane 2). The NH2-terminal sequence of recombinant pepsin was found to be predominantly Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-... which is known as the NH2-terminal sequencing. These results were reported in the activation of native porcine pepsinogen by Kageyama and Takahashi (42). The secondary structures of native and recombinant porcine pepsin were analyzed by CD spectrometry. The CD spectral data showed that the spectrum of the recombinant enzyme was essentially superimposable on that of the native enzyme (data not shown). The native and recombinant enzymes were almost equal in specific activity for hydrolysis of acid-denatured hemoglobin (82 and 83 millikatals/kg, respectively).

**Molecular Properties of Mutant Porcine Pepsins**—The mutant porcine pepsins, T77D, G78(S)S79, and T77D/G78(S)S79, were purified by the same method as wild-type pepsin, and the purities of the enzymes were judged by SDS-PAGE (Fig. 1). The NH2-terminal sequences of the mutants were the same as that of wild-type enzyme. The secondary structures of recombinant wild-type and mutant pepsins were analyzed by CD spectrometry to determine whether localized or global changes of structures were induced by the mutations. The CD spectra data showed that the spectra of the mutants were essentially superimposable on that of the wild-type enzyme (data not shown). These results suggest that no major conformational alterations occurred in the mutant enzymes.

**Enzymatic Properties of Mutant Porcine Pepsins for Protein Substrates**—Proteolytic activities for acid-denatured hemoglobin and trypsinogen-activating activities due to limited proteolysis of the Lys6–Ile7 bond of trypsinogen were measured with wild-type and mutant pepsins. The specific activities of mutant enzymes, T77D, G78(S)S79, and T77D/G78(S)S79, for hemoglobin hydrolysis were determined to be 53, 8, and 50 millikatals/kg, respectively. Each mutant enzyme effectively hydrolyzed hemoglobin although the activity of G78(S)S79 mutant was 10 times lower than that of wild-type enzyme. The specific activities of wild-type, T77D, and T77D/G78(S)S79 enzymes for trypsinogen activation were 0.04, 0.01, and 18.5 microkatals/ml, respectively, and no detectable activity was found for G78(S)S79 enzyme, which indicates that the activity of trypsinogen activation was significantly increased only by the double mutant. However, it was still 300-fold less than the activity of aspergillopepsin I. SDS-PAGE analysis showed that trypsinogen was converted to trypsin by T77D/G78(S)S79 mutant alone (Fig. 2) and the cleavage site was found to be Lys6–Ile7 by NH2-terminal sequencing. These results indicated that the double mutation in the active site flap altered substrate specificity of porcine pepsin to those of fungal aspartic proteinases, aspergillopepsin, penicillopepsin, and rhizopospapsin.
To obtain information on recognition of a Lys residue at the P1 position by the T77D/G78(S)S79 double mutant, the kinetic parameters for this double mutant were determined at various pH values using three peptide substrates containing a Phe or Lys in P1 (Fig. 3). In all substrates, the $K_m$ values were pH independent, and above pH 4 the values of $k_{cat}$ and $k_{cat}/K_m$ were controlled by the dissociation of a carboxyl group with a $pK_a$ of about 5. Below pH 4, however, the $k_{cat}$ and $k_{cat}/K_m$ values for the P1 Lys substrates (peptides B and C) decreased as pH declined, while there was no significant change in $k_{cat}$ and $k_{cat}/K_m$ values for the P1 Phe substrate (peptide A).

The pH-dependent changes of substrate preference are shown in Fig. 4. The P1 Lys/Phe preference increased as pH rose, which indicates that the dissociation of a carboxyl group as Asp-77 may affect the preference of a Lys residue at the P1 position.

## DISCUSSION

The double mutation in porcine pepsin, the substitution of Thr-77 by Asp, and the insertion of Ser between Gly-78 and Ser-79 (T77D/G78(S)S79), successfully altered the $S_1$ subsite specificity of pepsin to those of fungal aspartic proteinases. Some fungal aspartic proteinases, aspergillopepsin I, penicillopepsin, rhizopuspepsin, and others, are able to activate trypsinogen by cleavage of its Lys6–Ile7 bond, showing an affinity values for the P1 Lys substrates (peptides B and C) decreased as pH declined, while there was no significant change in $k_{cat}$ and $k_{cat}/K_m$ values for the P1 Phe substrate (peptide A).

The pH-dependent changes of substrate preference are shown in Fig. 4. The P1 Lys/Phe preference increased as pH rose, which indicates that the dissociation of a carboxyl group as Asp-77 may affect the preference of a Lys residue at the P1 position.

### TABLE II

| Substrate | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$) | Ratio$^a$ |
|-----------|------------|----------------------|-------------------------------|---------|
| Pro-Thr-Glu-Phe-Phe(NO$_2$)-Arg-Leu | 0.04 | 61 | 1500 | 1.0 |
| Wild type | 0.20 | 20 | 100 | 1.0 |
| T77D/G78(S)S79 | 0.17 | 2.0 | 12 | 1.0 |
| T77D/G78(S)S79 | 0.06 | 23 | 380 | 1.0 |
| Aspergillopepsin I | 0.01 | 18 | 1800 | 1.0 |
| Pro-Thr-Glu-Lys-Phe-Phe(NO$_2$)-Arg-Leu | 0.18 | 0.10 | 0.56 | 0.0056 |
| Wild type | ND$^b$ | ND$^b$ | ND$^b$ | ND$^b$ |
| T77D/G78(S)S79 | 0.15 | 1.1 | 7.3 | 0.019 |
| Aspergillopepsin I | 0.02 | 13 | 650 | 0.36 |
| Ac-Ala-Ala-Lys-Phe-Phe(NO$_2$)-Ala-Ala-NH$_2$ | 0.01 | 18 | 1800 | 1.0 |
| Wild type | — | ND | ND | ND |
| T77D/G78(S)S79 | 0.15 | 1.1 | 7.3 | 0.019 |
| Aspergillopepsin I | 0.10 | 8.0 | 80 | 0.044 |

$^a$ Ratio is $k_{cat}/K_m$, value relative to that with Pro-Thr-Glu-Phe-Phe(NO$_2$)-Arg-Leu as substrate in each enzyme.

$^b$ Cleavage occurred between Glu–Lys bond.

$^c$ No detectable cleavage was observed.

$^d$ Cleavage occurred between Phe(NO$_2$)–Ala bond.
for the anionic lysine side chain in S₁ (26). It is known that Asp-77 (pepsin numbering) of these enzymes is the binding site to the Lys residue in P₁ by means of site-directed mutagenesis of aspergillopepsin I (20, 21) and rhizopuspepsin (22), as pointed out for the case of inhibitor binding to penicillopepsin (11). In porcine pepsin, the hydrophobic side chain can be accommodated in the S₁ subsite, while basic residue cannot. As expected, the double mutant pepsin was able to accept the lysine residue in P₁, which led to trypsinogen activation, although it still showed a preference for the hydrophobic residue in P₁. However, the single mutants, T77D and G78(S)S79, were not able to hydrolyze the substrates containing a Lys residue in P₁ and also exhibited lower catalytic efficiency (kₗ/kₘ) for the P₁ Phe substrate than those of wild-type and double mutant enzymes. The double mutant exhibited a similar preference for peptide C as did aspergillopepsin I, while for peptide B the catalytic efficiency of double mutated pepsin was about 20-fold lower than that of aspergillopepsin I; this was mainly due to the smaller Kₘ values of aspergillopepsin I for peptides A and B than those of the double mutated pepsin. The differences in the substrate affinity may be a consequence of alterations in the structures of other substrate binding sites (S₄, S₃, S₂, S₂', and S₁') than the S₁ and S₁' subsites.

Fig. 5 shows the structures of S₁ subsites of human pepsin and penicillopepsin complexed with pepstatin and statine-based inhibitor, Iva-Val-Val-LySta-OEt (Iva, isovaleryl; LySta, 4S,3S-4-amino-3-hydroxy-6-methylheptanoic acid), respectively. The hydroxy group of the transition state analogue, statine (4S,3S-4-amino-3-hydroxyl-6-methylheptanoic acid) or LySta, is hydrogen-bonding to the oxygen atoms of two catalytic aspartyl side chains, and the positions of the analogue and catalytic apparatus are essentially superimposable in both complexes despite the differences in the flap structures. The flap structure of human pepsin is included in the typical “class 2” β-hairpin loop, while that of penicillopepsin is an anomalous structure, which has been included in the “class 3” β-hairpin loop according to Milner and Poet (44). Schematic models of the flap structure of human pepsin and penicillopepsin show that Ser-79 of penicillopepsin is inserted while human pepsin is not (Fig. 6). However, the hydrogen bonds between the main chain of the inhibitor and the enzyme are well conserved in both complexes, which may contribute to the proper orientation of
Alteration of $S_1$ Substrate Specificity of Porcine Pepsin

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The effects of the dissociation of the $\beta$-carboxyl group of Asp-77 in double mutated pepsin on the recognition of Lys residue at the $P_1$ position were examined by pH-dependent hydrolysis of two types of peptide substrates containing Phe or Lys at $P_1$. The major differences in pH activity profiles were the $k_{cat}/K_m$ values below pH 4. These values for Phe substrates (peptides B and C) decreased as the pH dropped, while there was no significant change for Phe Phe substrate (peptide A). The $K_m$ values were pH independent in all cases. These results are different from those of the studies that the existence of Lys or Arg residues at the $P_4$, $P_5$, $P_6$, $P'_4$, and $P'_5$ positions in the substrates influences the $K_m$ values with little effect on $k_{cat}$ in porcine pepsin (45, 46); the ionic interactions between the basic residues in the substrate and the side-chain carboxylates in the $S_1$, $S_2$, $S_3$, $S'_1$, $S'_2$, and $S'_3$ substrates contribute to substrate affinity. In double mutated pepsin, it is likely that the dissociation of Asp-77 side chain contributes to the stabilization of the transition state when substrates contain Lys residue at the $P_1$ position rather than substrate affinity. The electrostatic interaction between Asp-77 side chain and $P_1$ Lys of the substrates could play an important role in the recognition of Lys residue at the $P_1$ position. In aspgilopepsin I, however, the substitution of Asp-77 by Asn hardly affected the $P_1$ Lys/Phe preference compared with wild-type enzyme, whereas the substitution of Asp-77 by other amino acids (Glu, Ser, and Thr) and the deletion of Ser-79 reduced the $P_1$ Lys/Phe preference with the decrease in the $k_{cat}$ values for the $P_1$ Lys substrates. This indicates that the recognition of basic $P_1$ side chain is not dependent on the specific electrostatic interactions between $P_1$ side chain and Asp-77 side chain of the enzyme (21). From a site-directed mutagenesis study of rhizopuspesein, Lowther et al. (22) concluded that the presence of Asp at position 77 has the potential to establish an extensive hydrogen-bonding network between the enzyme and the substrate containing Lys residue at the $P_1$ position. In the double mutated pepsin, although the dissociation of the $\beta$-carboxyl group of Asp-77 would be dispensable to accommodate the Lys residue in the $S_1$ pocket, it enhances the stabilization of the transition state complex when substrates contain a Lys residue at the $P_1$ position. This may be due to the formation of the electrostatic interaction or the changes of the hydrogen-bonding pattern between Lys residue in $P_1$ and Asp-77 of the mutant pepsin as the pH is higher.

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FIG. 6. Schematic models of flap structure of porcine pepsin (A) and penicillopepsin (B). Dashed lines show hydrogen bonds. The shading indicates a unit which is structurally inserted as compared with porcine pepsin.
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