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Subsite Preferences of Pepstatin-Insensitive Carboxyl Proteinases from Bacteria[1]

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***Pseudomonas* sp. 101 carboxyl proteinase (PCP) and *Xanthomonas* sp. T-22 carboxyl proteinase (XCP), the first and second unique carboxyl proteinases from prokaryotes to be isolated and characterized, are not inhibited by the classical carboxyl proteinase inhibitor pepstatin. In this study, we elucidated their subsite preferences by using a series of synthetic chromogenic substrates, Lys-Pro-Ile(P_3)-Glu(P_2)-Phe*Nph-Arg(P_2')-Leu(P_3') (Nph is *p*-nitrophenylalanine, Phe*Nph is the cleavage site) with systematic substitutions at the P_3, P_2, P_2', and P_3' positions. Among 45 substrates tested, the best substrate for PCP had a Leu replacement at the P_2 position ($k_{cat}=27.2\ s^{-1}$, $K_m=4.22\ \mu M$, $k_{cat}/K_m=6.43\ \mu M^{-1}\cdot s^{-1}$), and that for XCP had an Ala replacement at the P_3 position ($k_{cat}=79.4\ s^{-1}$, $K_m=6.05\ \mu M$, $k_{cat}/K_m=13.1\ \mu M^{-1}\cdot s^{-1}$). PCP and XCP preferred such charged amino acid residues as Glu, Asp, Arg, or Lys at the P_2' position. This suggested that the S_2' subsites of PCP and XCP are occupied by hydrophilic residues, similar to that of pepstatin-insensitive carboxyl proteinase from *Bacillus coagulans* J-4 [Shibata *et al.* (1998) *J. Biochem.* 124, 642-647]. In contrast, the S_2' subsite of pepstatin-sensitive carboxyl proteinases (aspartic proteinases) is hydrophobic in nature. Thus, the hydophilic nature of the S_2' subsite appears to be a distinguishing feature of pepstatin-insensitive carboxyl proteinases.**

Key words: bacterial proteinase, carboxyl proteinase, kinetic property, pepstatin-insensitive, subsite preferences.

Carboxyl proteinases, formerly called acid proteinases, are distributed in a wide range of organisms, including animals, plants, and microbes. These enzymes are classified into two groups on the basis of the pepstatin sensitivity (*1-4*).

One group is the pepstatin-sensitive carboxyl proteinases, inhibited by pepstatin (*5*), S-PI (acetyl pepstatin) (*6*), and the active-site directed affinity labeling reagents 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) (*7*) and diazoacetyl-DL-norleucine methyl ester (DAN) (*8*). In porcine pepsin, a pair of aspartyl residues, Asp^{32} and Asp^{215}, have been revealed to be essential for catalytic function (*9*). Therefore, pepstatin-sensitive carboxyl proteinases are called aspartic proteinases (*10*). Extensive sequence similarity has been observed among the enzymes of this group (*11*), and their tertiary structures, as far as they have been examined, are also very similar (*12-15*). These enzymes have evolved from the same ancestral gene.

The other group is the pepstatin-insensitive carboxyl proteinases. In 1972 Murao and Oda *et al.* isolated four distinct pepstatin-insensitive carboxyl proteinases, A-1, A-2, B, and C, from *Scytalidium lignicolum* ATCC 24568 (*16-19*). None of them were inhibited by pepstatin, S-PI, DAN, or EPNP, with the exception of carboxyl proteinase B, which is inhibited by EPNP. Moreover, these enzymes showed unique substrate specificities (*20-25*). The amino acid sequence of carboxyl proteinase B is quite different from those of aspartic proteinases (*26*). These pepstatin-insensitive carboxyl proteinases have been found in a wide variety of microorganisms, such as *Aspergillus, Pseudomonas, Xanthomonas*, and even thermophilic *Bacillus* (*27-37*). We are now focusing on pepstatin-insensitive carboxyl proteinases from prokaryotes.

Pseudomonas sp. 101 carboxyl proteinase (PCP) (*32*) and *Xanthomonas* sp. T-22 carboxyl proteinase (XCP) (*33*) are the first and second unique carboxyl proteinases to be purified from prokaryotes, regardless of the pepstatin sensitivity. PCP and XCP are not inhibited by pepstatin, S-PI, DAN, or EPNP; but they are inhibited by a competitive inhibitor, tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal) (*38*) [$K_i=2.6$ nM for PCP (*39*) and 2.1 nM for XCP (*40*)]. Recently we have cloned, sequenced, and expressed both PCP and XCP genes (*41, 42*). PCP is produced as a precursor comprising an amino-terminal prepro region (215 a.a.) and the mature region (372 a.a.).

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Abbreviations: S-PI, acetyl pepstatin; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; DAN, diazoacetyl-DL-norleucine methylester; PCP, *Pseudomonas* sp. 101 carboxyl proteinase; XCP, *Xanthomonas* sp. T-22 carboxyl proteinase; J-4, *Bacillus coagulans* J-4 carboxyl proteinase; Nph, *p*-nitrophenylalanine; a.a., amino acid residues.

XCP is produced as a precursor comprising an amino-terminal prepro region (237 a.a.), the mature region (398 a.a.), and a carboxyl-terminal pro region (198 a.a.). The primary structures of PCP (*43*) and XCP show no similarity to those of any pepstatin-sensitive carboxyl proteinases (aspartic proteinases). However, they exhibit high sequence identity (51.5%) to each other. Moreover, the consensus catalytic site sequence, Asp*-Thr-Gly (Asp*: catalytic residue), of pepstatin-sensitive carboxyl proteinases is also absent in both PCP and XCP. On the other hand, it was confirmed by the zinc(II)-PAD (pyridine-2-azo-*p*-dimethylaniline) method (*44*) and kinetic analysis (*39*) that PCP and XCP have a pair of catalytic carboxyl residues.

In this study, as a part of understanding the structure-function relationships of PCP and XCP, the substrate specificities of PCP and XCP were investigated by using a series of synthetic chromogenic substrates, which have the general structure Lys-Pro-Ile(P_3)-Glu(P_2)-Phe*Nph-Arg(P_2')-Leu(P_3') (cleavage site; Phe*Nph, Nph; *p*-nitro-L-phenylalanine). The amino acid residues of the P_3, P_2, P_2', and P_3' positions were systematically substituted in order to explore several subsites of the enzymes. The data obtained in this study, together with data on the alcohol-resistant pepstatin-insensitive carboxyl proteinase from *Bacillus coagulans* J-4 (*45*), strongly suggest that the pepstatin-insensitive carboxyl proteinases from prokaryotes are characterized by the hydrophilic nature of the S_2' subsite.

MATERIALS AND METHODS

Enzymes—The wild-type recombinant PCP and XCP were prepared as described previously (*41, 42*). Both gave single bands on SDS polyacrylamide gel electrophoresis. The protein concentrations were determined by amino acid analysis.

Peptide Substrates—The peptide substrates were synthesized and characterized as described previously (*46, 47*). They have the general structure Lys-Pro-Ile(P_3)-Glu(P_2)-Phe-Nph-Arg(P_2')-Leu(P_3') (P_3, P_2, P_2', P_3': various amino acids, Nph is *p*-nitrophenylalanine). All were pure by HPLC criteria and were readily soluble in water. Aqueous stock solutions of approximately 5 mM were prepared. The substrate concentrations were determined by amino acid analysis. In all cases, the position cleaved by PCP and XCP was between the Phe and Nph residues.

Determination of Kinetic Parameters—The cleavage of a substrate between the Phe and Nph residues was monitored at 37°C and pH 3.5 in 0.1 M sodium formate buffer. The average decrease in absorbance over the range 284-324 nm was monitored by using a Hewlett Packard 8452 Diode Array Spectrophotometer. For each kinetic assay, six tubes containing a mixture of the buffer and the enzyme were preincubated for 3 min at 37°C. Then substrates of at least six different concentrations were added and the absorbance changes were monitored using a multi-cell transporter. The initial linear rate of hydrolysis was measured to give the initial velocity (v). The K_m and V_{max} values were calculated by Lineweaver-Burk plotting, with at least six initial substrate concentrations. The k_{cat} values were obtained with the equation: $V_{max} = k_{cat}[E]$, where [E] is the enzyme concentration. The estimated precision of K_m and k_{cat} values is within 20%.

RESULTS AND DISCUSSION

Aspartic proteinases (pepstatin-sensitive carboxyl proteinases) are involved in human diseases: *e.g.*, renin in hypertension and human immunodeficiency virus proteinase in AIDS. Kinetic and X-ray crystal structure analyses of aspartic proteinases have revealed that they have active sites consisting of at least seven subsites (*48*) and a distinct preference for cleaving between two hydrophobic residues (*49*). The subsites are located on both sides of the active site, four on the one side and three on the other. The preferred residue at each subsite forming such an extended binding cleft can be elucidated by using systematic series of synthetic substrates.

We have focused on the structure-function relationships of pepstatin-insensitive carboxyl proteinases from prokaryotes. These studies will play an important role in understanding the evolution of carboxyl proteinases. As reported previously, we have analyzed the substrate specificities of PCP and XCP by using the first series of substrates (parent substrate: Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu). In spite of their high sequence similarity, PCP and XCP showed quite different substrate specificities: PCP had a strict preference for substrates, while XCP showed a broad specificity.

In this study, the binding specificity of the S_3, S_2, S_2', and S_3' subsites of PCP and XCP was investigated by assaying their kinetic parameters with a new series of substrates (parent substrate: Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu). This new series was utilized because it yielded more favorable kinetic than the first series. Thus, it was possible to obtain significantly more information on the preferences of PCP and XCP. The data are shown in Tables I-IV, respectively. In addition, we compared the specificity constants of PCP, XCP, and J-4 for substrates with the same P_2 residue but either Ile or Ala at the P_3 position (Table V).

(1) The Effect of the P_3 Position—In aspartic proteinases, the interactions at the S_3 subsite with inhibitor or substrate were reported to be significant (*49-51*). The effect of the P_3 replacement on hydrolysis of the new series is summarized in Table I. In the case of PCP, the peptide having Ala at the P_3 position was the best substrate. The specificity constant, k_{cat}/K_m, was 3.30 $\mu M^{-1} \cdot s^{-1}$. The P_3 Lys derivative was particularly unacceptable to many aspartic proteinases (data not shown). However, PCP cleaved effectively the peptides with such basic amino acid derivatives as Arg or Lys at P_3. In contrast, the k_{cat}/K_m values for the derivatives with a negatively charged amino acid residue, Glu or Asp, decreased to about 13-21% of that for the parent substrate. PCP could not cleave the substrates having Phe and Tyr at the P_3 position. Thus, the S_3 subsite of PCP may be occupied by hydrophobic residues and negatively charged amino acid residues.

In the case of XCP, the substrate having Ala at the P_3 position showed the highest specificity constant, k_{cat}/K_m = 13.1 $\mu M^{-1} \cdot s^{-1}$, among 45 substrates tested in this series. The k_{cat}/K_m values decreased in the following order: Ala, Arg, Ile, Ser, Gln. Like PCP, XCP preferred substrates with a positively charged amino acid residue at the P_3 position to those with a negatively charged residue. In the case of Phe and Tyr derivatives, which PCP could not

hydrolyze, the k_{cat}/K_m values of XCP were 57 and 39% of that for the parent substrate, respectively. Thus, the S_3 subsite of XCP may be occupied by hydrophobic and negatively charged amino acid residues, similar to PCP.

Dunn *et al.* reported that most aspartic proteinases prefer a bulky and hydrophobic amino acid residue at the P_3 position (*46*). However, in the case of *Endothia parasitica*, the S_3 subsite has been shown to be occupied by negatively charged and hydrophobic amino acid residues by X-ray crystal structure analysis and substrate specificity. The S_3 subsite of microbial enzymes may be much more versatile than that of mammal aspartic proteinases. Indeed, the S_3 subsite of PCP and XCP was shown to be similar in nature to that of *Endothia parasitica* proteinase.

(2) The Effect of the P_2 Position—The most critical subsite preference of aspartic proteinases was observed in the S_2 subsite (*49, 50*). From experiments with the first series of substrates, we reported that the hydrophobic interaction of the S_2 subsite of PCP was particularly important, as in the case of cathepsin D and E (*40*). In this study, we investigated the effect of the P_2 replacement on hydrolysis of the second series (Table II). PCP cleaved the peptide containing the P_2 Leu derivative with the highest specificity constant ($k_{cat}/K_m = 6.43\ \mu M^{-1}\cdot s^{-1}$) among all substrates tested. The k_{cat}/K_m value was about twice of that for the parent substrate. As reported previously, PCP preferred the P_2 Leu derivative in the earlier series of substrates. The k_{cat}/K_m value was $8.16\ \mu M^{-1}\cdot s^{-1}$. These data are in close agreement with each other. The substrate with P_2 Lys was much less preferred. PCP cleaved the first series of substrates less effectively than the second series because the P_2 position of the first series of substrates was fixed as a Lys residue. This feature was also observed for cathepsin D and E (*52, 53*).

XCP hydrolyzed more effectively substrates having a small Ala or Ser residue at the P_2 position. The k_{cat}/K_m values were $12.1\ \mu M^{-1}\cdot s^{-1}$ and $10.9\ \mu M^{-1}\cdot s^{-1}$, respectively. It was reported that the P_2 Pro substrate was disfavored by cathepsin E (*52*), porcine pepsin (*54*), and other aspartic proteinases (data not shown). XCP hydrolyzed effectively the substrates having Pro residue at the P_2 position, similar to J-4. XCP preferred smaller amino acid residues at this position than PCP. A similar tendency has been observed for J-4 (*45*). Thus, XCP showed properties between PCP and J-4 in the P_2 preference.

In the case of porcine pepsin, the k_{cat}/K_m values for such bulky and hydrophobic amino acid residues as Nle, Leu, Ile were similar (*54*). PCP and XCP preferentially hydrolyzed

TABLE I. **Kinetic parameters for hydrolysis of peptides with P_3 substitutions by PCP and XCP.**

Substrate P_4 P_3 P_2 P_1 P_1' P_2' P_3'	PCP k_{cat} (s^{-1})	PCP K_m (μM)	PCP k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	XCP k_{cat} (s^{-1})	XCP K_m (μM)	XCP k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
Lys Pro **Ala** Glu Phe Nph Arg Leu	57.6±1.64	17.4±0.45	3.30±0.09	79.4±1.13	6.05±0.35	13.1±0.79
Ile*	32.4±1.64	11.1±1.64	2.92±0.46	102±1.76	10.6±0.75	9.58±0.70
Arg	53.1±1.12	22.4±0.87	2.37±0.10	105±2.32	9.32±0.69	11.2±0.86
Ser	20.4±0.30	8.75±0.44	2.33±0.12	36.7±0.33	3.90±0.14	9.42±0.35
Lys	56.3±0.83	27.5±1.39	2.04±0.11	96.4±2.72	18.3±1.13	5.27±0.36
Asn	47.1±1.95	24.7±2.10	1.91±0.18	38.3±0.98	11.5±0.96	3.33±0.29
Gln	24.5±0.24	16.2±0.50	1.52±0.05	73.9±0.83	13.0±0.72	5.67±0.32
Val	14.8±0.39	11.5±1.23	1.29±0.14	28.1±0.95	9.95±0.95	2.83±0.29
Glu	3.82±0.14	6.12±0.77	0.62±0.08	62.4±2.35	12.0±1.14	5.18±0.53
Thr	7.92±0.27	13.2±1.51	0.60±0.07	37.0±0.73	7.10±0.55	5.21±0.41
Asp	9.68±0.48	24.2±3.61	0.40±0.06	18.1±0.37	13.9±0.58	1.31±0.06
Leu	3.10±0.13	8.84±1.13	0.35±0.05	15.3±0.26	11.4±0.54	1.35±0.07
Phe	P.C.			57.5±2.74	10.5±1.36	5.47±0.75
Tyr	P.C.			8.45±0.21	2.24±0.18	3.78±0.31

* indicates the parent substrate. P.C., poorly cleaved under the standard assay conditions (0.1 M sodium formate buffer, pH 3.5).

TABLE II. **Kinetic parameters for hydrolysis of peptides with P_2 substitutions by PCP and XCP.**

Substrate P_4 P_3 P_2 P_1 P_1' P_2' P_3'	PCP k_{cat} (s^{-1})	PCP K_m (μM)	PCP k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	XCP k_{cat} (s^{-1})	XCP K_m (μM)	XCP k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
Lys Pro Ile **Leu** Phe Nph Arg Leu	27.2±0.42	4.22±0.47	6.43±0.73	16.8±0.51	2.14±0.37	7.87±1.37
Glu*	32.4±1.64	11.1±1.64	2.92±0.46	102±1.76	10.6±0.75	9.58±0.70
Thr	22.9±0.81	10.6±1.00	2.17±0.22	30.5±0.82	4.51±0.43	6.75±0.67
Ala	27.7±0.73	13.1±0.90	2.12±0.16	60.5±0.68	5.00±0.28	12.1±0.68
Nle	14.8±0.21	7.13±0.44	2.07±0.13	39.8±1.16	66.2±3.73	0.60±0.04
Val	15.7±0.80	7.82±0.96	2.00±0.27	26.9±0.56	4.45±0.38	6.03±0.53
Asp	20.9±0.99	10.6±1.39	1.97±0.27	18.6±0.14	2.60±0.10	7.14±0.27
Ser	14.8±0.51	9.99±0.78	1.48±0.13	56.8±1.34	5.23±0.44	10.9±0.95
Arg	12.5±0.70	10.0±1.54	1.25±0.20	43.3±1.22	13.5±1.23	3.20±0.31
Pro	29.3±0.88	27.5±1.76	1.07±0.08	57.9±1.16	7.82±0.56	7.40±0.55
Ile	18.3±0.46	17.0±0.84	1.07±0.06	19.4±0.81	7.16±1.10	2.71±0.43
Gln	10.4±0.18	20.7±1.08	0.50±0.03	48.8±0.77	12.6±0.58	3.87±0.19
Asn	8.79±0.64	18.9±2.49	0.46±0.07	35.7±0.61	5.31±0.44	6.72±0.57
Lys	9.75±0.22	56.4±2.52	0.17±0.01	16.6±0.53	17.3±1.37	0.96±0.08
His	2.93±0.05	22.9±0.97	0.13±0.01	23.1±0.58	10.1±0.94	2.30±0.22

* indicates the parent substrate.

the substrate with hydrophobic side chain Leu at the P_2 position. But the k_{cat}/K_m for β-branched Ile was 17 and 34% of that of the P_2 Leu derivatives, respectively. Furthermore, they showed poor cleavage of the P_2 Nle derivative. Thus, PCP and XCP showed strict preferences for bulky and hydrophobic amino acid residues at the P_2 position.

(3) The Effect of the P_2' Position—The S_2' subsite of aspartic proteinases reported so far has been shown to be hydrophobic by X-ray crystal structure analysis. We examined the specificity of the S_2' subsite of PCP and XCP (Table III). In the case of PCP, the k_{cat}/K_m value of the P_2' derivatives in the earlier series of substrates did not vary greatly. In this study, differences were found in the k_{cat}/K_m value in the new series of substrates. A Glu residue at the P_2' position was found to yield the most favorable substrate in this series. PCP preferred the substrates having a charged amino acid residue, Asp, Arg, or Lys, at the P_2' position, which suggested us that the S_2' subsite of PCP mainly consisted of hydrophilic residues. In addition, PCP showed a low K_m value for the P_2' Leu derivative. We also observed a low K_m value for the P_2' Leu derivative in the first series. These data suggested the hydrophobic nature of the interactions between the S_2' subsite of PCP and the P_2' position of substrates. But their k_{cat} values were also low. Therefore, PCP did not show high activity.

The most effective substrate for XCP was the parent substrate with Arg at the P_2' position. XCP also hydrolyzed effectively the substrate having a negatively charged Glu residue at the P_2' position, similar to PCP (Table III). The P_2' Asp derivative was less favorable than the P_2' Glu derivative. This characteristic has been observed for J-4. XCP does not prefer such hydrophobic amino acid residues as Leu, Ile, or Val at the P_2' position.

Thus, these results indicated that the hydrophilic interactions between the S_2' subsites of both enzymes and the P_2' position of substrates are most important. This feature may be considered as a common characteristic of bacterial pepstatin-insensitive carboxyl proteinases (PCP, XCP, and J-4) reported so far.

(4) The Effect of the P_3' Position—The effect of the P_3' replacement is summarized in Table IV. PCP preferred the substrates having Ser and Asp at the P_3' position. The k_{cat}/K_m values increased approximately 1.5 times over that for the parent substrate. PCP did not prefer the P_3' Arg derivative. The k_{cat}/K_m values for bulky hydrophobic Ile and Leu (parent substrate) were almost the same, whereas their k_{cat} and K_m values were different. That is, PCP showed different preferences for P_3' Ile and Leu.

XCP preferred the substrates with Asp or Leu at this position. The k_{cat}/K_m values are approximately 10 $\mu M^{-1} \cdot s^{-1}$. The k_{cat} value of the P_3' Ile derivative was 27% of that for the parent substrate. This k_{cat}/K_m value of the P_3' Ile derivative was thus lower than that of the parent substrate. In the same way, XCP did not prefer the Ala derivative. The difference of side chain of Leu and Val did not greatly affect the preference of the S_3' subsite of XCP. XCP hydrolyzed the P_3' Asp derivative more effectively than that with a positively charged amino acid residue (Arg). Thus, XCP favored small hydrophilic residues or bulky hydrophobic residues at the P_3' position.

(5) Comparison of the Two Series of Substrates—Finally, we compared the specificity constants of PCP, XCP, and J-4 for the substrates with the same P_2 residue but having either a bulky P_3 Ile residue (second series) or a

TABLE III. **Kinetic parameters for hydrolysis of peptides with P_3' substitutions by PCP and XCP.**

Substrate		PCP			XCP		
P_5 P_4 P_3 P_2 P_1 P_1' P_2'	P_3'	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Lys Pro Ile Glu Phe Nph Glu	**Leu**	33.3±1.13	9.01±1.15	3.70±0.49	189±6.19	21.7±1.55	8.71±0.68
	Asp	33.0±1.56	9.46±1.17	3.49±0.46	87.9±2.76	21.3±1.59	4.13±0.33
	Arg*	32.4±1.64	11.1±1.64	2.92±0.46	102±1.76	10.6±0.75	9.58±0.70
	Lys	54.1±0.08	18.9±0.24	2.87±0.37	43.7±1.76	15.8±1.31	2.77±0.26
	Ala	19.0±0.52	8.27±0.87	2.30±0.25	99.1±3.55	17.4±1.47	5.68±0.52
	Leu	8.38±0.31	5.67±0.81	1.48±0.22	31.0±0.60	28.3±1.39	1.10±0.06
	Ser	11.8±0.52	14.0±1.35	0.85±0.09	40.4±1.41	16.4±1.85	2.46±0.29
	Asn	12.9±0.42	30.9±2.22	0.42±0.03	32.7±1.98	18.3±2.34	1.79±0.25
	Ile	5.24±0.10	15.3±1.03	0.34±0.02	16.1±0.59	9.77±0.89	1.65±0.16
	Val	5.37±0.31	28.0±2.61	0.19±0.02	8.57±0.33	13.8±1.21	0.62±0.06

* indicates the parent substrate.

TABLE IV. **Kinetic parameters for hydrolysis of peptides with P_3' substitutions by PCP and XCP.**

Substrate		PCP			XCP		
P_5 P_4 P_3 P_2 P_1 P_1' P_2'	P_3'	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Lys Pro Ile Glu Phe Nph Arg	**Ser**	44.4±2.19	10.1±1.08	4.41±0.52	71.3±2.08	9.82±0.93	7.26±0.72
	Asp	40.7±0.97	9.62±0.70	4.23±0.33	101±2.27	9.95±0.74	10.1±0.79
	Ile	10.9±0.24	3.70±0.33	2.92±0.27	27.3±0.69	8.24±0.67	3.31±0.28
	Leu*	32.4±1.64	11.1±1.64	2.92±0.46	102±1.76	10.6±0.75	9.58±0.70
	Ala	15.5±0.22	6.97±0.35	2.23±0.12	24.7±0.59	7.63±0.57	3.24±0.26
	Val	11.6±0.41	5.47±0.77	2.11±0.31	30.9±0.60	5.32±0.34	5.80±0.39
	Tyr	9.87±0.24	6.27±0.81	1.57±0.21	27.6±0.85	9.44±0.85	2.92±0.28
	Phe	10.7±0.48	7.40±1.26	1.44±0.25	26.7±0.79	7.46±0.75	3.58±0.37
	Arg	3.64±0.08	5.42±0.60	0.67±0.08	15.5±0.23	6.30±0.31	2.47±0.13

* indicates the parent substrate.

TABLE V. **Specificity constants of PCP, XCP, and J-4 for hydrolysis of P_2 derivatives of two series of substrates.**

Amino acid P_2	PCP		XCP		J-4[a]	
	P_3 Ile	P_3 Ala	P_3 Ile	P_3 Ala	P_3 Ile	P_3 Ala
	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Lys	0.17	0.15	0.96	4.35	0.004	0.012
Ala	2.12	2.48	12.1	2.81	0.628	0.769
Asp	1.97	0.52	7.14	5.63	0.226	0.227
Leu	6.43	8.16	7.87	14.5	0.035	0.003
Arg	1.25	0.38	3.20	2.14	0.013	0.010
Ser	1.48	0.80	10.9	13.3	0.161	0.137

[a]Shibata *et al.* (*45*).

small Ala residue (first series) (Table V). The best substrate for PCP had the Leu substitution at the P_2 position in both series. Thus, PCP showed almost the same preference for the P_3 position (Ile or Ala). In general PCP preferred the series having bulky Ile at the P_3 position over the series of substrate with small Ala. These results suggested that the S_2 and S_3 pockets of PCP are large enough to accommodate such bulky amino acid residue as Leu or Ile.

XCP hydrolyzed the substrate with Leu at the P_2 position more effectively than that with Ala in the first series substrates. In the new series, XCP preferred the P_2 Ala or Ser derivatives. Thus, XCP showed different preferences for the P_2 residue in the two series. This suggested that a bulky Ile residue at the P_3 position may be difficult to fit in the available space of the S_3 pocket of XCP. Thus we can say that the S_2 pocket of XCP is large enough to accommodate Leu, but the S_3 pocket of XCP might be smaller than that of PCP. These data also suggest a linkage between the S_3 and S_2 subsites. The total space available can be occupied by only certain combination of residues in P_3 and P_2.

J-4 preferred the Ala derivatives in both series of substrates. This preference was so strict that J-4 did not cleave other derivatives effectively. This suggested that the S_2 pocket of J-4 is much narrower than those of PCP or XCP.

By employing two series of chromogenic substrates, the substrate specificities of three bacterial enzymes, PCP, XCP, and J-4 were elucidated in detail. On the basis of the K_m and k_{cat}/K_m values for the two series, the new series was found to consist of generally better substrates for PCP and J-4 than the earlier series. This may be because the P_2 Lys residue is rejected electrostatically by PCP and J-4. XCP cleaved the substrates with Lys at the P_2 position more effectively than PCP. As described above, PCP and XCP showed unique substrate specificities at the P_2 and P_2' positions, respectively, compared to aspartic proteinases reported so far. It was suggested that the interaction of the S_2 subsite of PCP with the P_2 position of the substrates was hydrophobic, as in the case of cathepsin D and E. On the other hand, the hydrophobic interaction of the S_2 subsite of XCP with the P_2 position of the substrates was less much important than for PCP and many aspartic proteinases. In addition, the P_2 Pro derivative of the new series was acceptable to XCP, similar to J-4. This is one of the unique features of XCP and J-4.

Thus PCP and XCP differed considerably in substrate specificity. The S_2' subsite of PCP and XCP may consist of hydrophilic residues, similar to that of J-4. This characteristic is very different from those of aspartic proteinases reported so far and appears to be a common feature of pepstatin-insensitive carboxyl proteinases from prokaryotes.

Further information on the subsite structures of PCP and XCP will be obtained by X-ray crystal structure analyses in the near future. Analysis of the three-dimensional structure of PCP is in progress.

It was recently reported that normal brain has a pepstatin-insensitive lysosomal carboxyl proteinase related to a fatal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis (LINCL) (*55*). Sequence comparisons showed significant sequence similarities between this lysosomal enzyme, PCP, and XCP. This is the first demonstration of a pepstatin-insensitive carboxyl proteinase in mammals.

We hope this study will contribute to the understanding of pepstatin-insensitive lysosomal enzyme related to lysosomal storage disease.

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