J. Biochem. 120, 845-850 (1996)

Substrate Specificities of Pepstatin-Insensitive Carboxyl Proteinases from Gram-Negative Bacteria[1]

Masaaki Ito,* Ben M. Dunn,† and Kohei Oda*,[2]

*Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606; and †Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL 32610, USA

Received for publication, June 20, 1996

Pseudomonas carboxyl proteinase (PCP), isolated from *Pseudomonas* sp. 101, and *Xanthomonas* carboxyl proteinase (XCP), isolated from *Xanthomonas* sp. T-22, are the first and second examples of unique carboxyl proteinases [EC 3.4.23.33] which are insensitive to aspartic proteinase inhibitors, such as pepstatin, diazoacetyl-DL-norleucine methylester, and 1,2-epoxy-3(*p*-nitrophenoxy)propane. The substrate specificities of PCP and XCP were studied using a series of synthetic chromogenic peptide substrates with the general structure, P5-P4-P3-P2-Phe-Nph-P2′-P3′ (P5, P4, P3, P2, P2′, P3′: a variety of amino acids, Nph is *p*-nitro-L-phenylalanine, and the Phe-Nph bond is cleaved). PCP and XCP were shown to hydrolyze a synthetic substrate, Lys-Pro-Ala-Leu-Phe-Nph-Arg-Leu, most effectively among 28 substrates. The kinetic parameters of this peptide for PCP were $K_m = 6.3\ \mu M$, $k_{cat} = 51.4\ s^{-1}$, and $k_{cat}/K_m = 8.16\ \mu M^{-1} \cdot s^{-1}$. The kinetic parameters for XCP were $K_m = 3.6\ \mu M$, $k_{cat} = 52.2\ s^{-1}$, and $k_{cat}/K_m = 14.5\ \mu M^{-1} \cdot s^{-1}$. PCP showed a stricter substrate specificity than XCP. That is, the specificity constant (k_{cat}/K_m) of each substrate for PCP was in general $< 0.5\ \mu M^{-1} \cdot s^{-1}$, but was drastically improved by the replacement of Lys by Leu at the P2 position. On the other hand, XCP showed a less stringent substrate specificity, with most of the peptides exhibiting reasonable k_{cat}/K_m values ($> 1.0\ \mu M^{-1} \cdot s^{-1}$). Thus it was found that the substrate specificities of PCP and XCP differ considerably, in spite of the high similarity in their primary structures. In addition, tyrostatin was found to be a competitive inhibitor for XCP, with a K_i value of 2.1 nM, as well as for PCP ($K_i = 2.6$ nM).

Key words: carboxyl proteinase, Gram-negative bacteria, kinetic property, pepstatin, substrate specificity.

Carboxyl proteinases, formerly called acid proteinases, are classified into two groups on the basis of their sensitivity to inhibitors: pepstatin (isovaleryl-pepstatin)-sensitive and -insensitive carboxyl proteinases (*1-3*).

Pepstatin-sensitive carboxyl proteinases, represented by porcine pepsin, are blocked by such inhibitors as pepstatin (*4*), S-PI (acetyl pepstatin) (*5*), diazoacetyl-DL-norleucine methylester (DAN) (*6*), and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (EPNP) (*7*). In porcine pepsin, a pair of aspartyl residues, Asp^{32} and Asp^{215}, have been revealed to be essential for its catalytic function (*8*). Therefore, pepstatin-sensitive carboxyl proteinases are called aspartic proteinases (*9*). Extensive sequence similarity has been observed among the enzymes belonging to this group (*10*).

In 1972, Murao *et al.* reported that several pepstatin-insensitive carboxyl proteinases are produced by *Scytalidium lignicolum* (*11-14*). None of them are inhibited by pepstatin, S-PI, DAN, or EPNP, with the exception of carboxyl proteinase B, which is inhibited by EPNP. Furthermore, these enzymes have unique substrate specificities (*15-20*). The primary structure of carboxyl proteinase B shows no similarity with those of other carboxyl proteinases reported so far (*21*). The existence of enzymes having characteristics similar to those of *S. lignicolum* has been reported in fungi (*22-26*), bacteria (*27, 28*), and thermophilic bacteria (*29-32*).

Among these enzymes, *Pseudomonas* carboxyl proteinase (PCP), a pepstatin-insensitive carboxyl proteinase from *Pseudomonas* sp. 101 (*27*), and *Xanthomonas* carboxyl proteinase (XCP), a pepstatin-insensitive carboxyl proteinase from *Xanthomonas* sp. T-22 (*28*), are the first and second carboxyl proteinases to be isolated from prokaryotes and characterized, regardless of their pepstatin sensitivity. PCP and XCP are not inhibited by pepstatin, S-PI, DAN, or EPNP, but are inhibited by tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal; K_i, 2.6 nM for PCP) (*33*).

Recently, Oda *et al.* cloned, sequenced, and expressed the PCP (*34*) and XCP (*35*) genes. In the case of PCP, the enzyme is produced as a precursor comprising an NH_2-terminal prepro region (215 residues) and the mature PCP

[1] This work was supported in part by a Grant-in-Aid for Scientific Research (C), 06660105, and a Grant-in-Aid for International Scientific Research (Joint Research), 08044202, from the Ministry of Education, Science, Sports and Culture of Japan (K.O.).

[2] To whom correspondence should be addressed. Tel.: +81-75-724-7763, Fax: +81-75-724-7710, E-mail: bika@ipc.kit.ac.jp

Abbreviations: DAN, diazoacetyl-DL-norleucine methylester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; PCP, *Pseudomonas* carboxyl proteinase; XCP, *Xanthomonas* carboxyl proteinase.

(372 residues). On the other hand, XCP is produced as a precursor comprising an NH_2-terminal prepro region (237 residues), the mature XCP (398 residues), and a COOH-terminal pro region (198 residues). This is the first case of a carboxyl proteinase that has an additional COOH-terminal pro region in its precursor. Furthermore, the amino acid sequence of PCP was determined by chemical methods (*36*). From the results, it was clarified that the amino acid sequences of PCP and XCP show no similarity to those of any other carboxyl proteinases; however, they exhibit high sequence similarity (51.5% identity) to each other. Moreover, the consensus catalytic site sequence, -Asp*-Thr-Gly- (Asp*: catalytic residue), of pepstatin-sensitive carboxyl proteinases is also absent. On the other hand, it was confirmed that PCP has a pair of catalytic carboxyl residues by the zinc(II)-PAD (pyridine-2-azo-*p*-dimethylaniline) method (*37*) and kinetic analyses (*38*).

In the present study, we investigated the substrate specificities of PCP and XCP using chromogenic peptide substrates to clarify the catalytic properties of pepstatin-insensitive carboxyl proteinases. It was found that PCP and XCP have different substrate specificities, in spite of the high similarity in their primary structures.

MATERIALS AND METHODS

Enzymes and Inhibitor—PCP was prepared as described previously (*27*). It gave a single protein band on SDS-polyacrylamide gel electrophoresis. The protein concentration was determined from the absorbance at 280 nm ($E^{1\%}_{1cm}=16.6$).

XCP was prepared as described previously with further purification by DEAE Sephadex A-50 column chromatography (*28*). It also gave a single protein band on SDS-polyacrylamide gel electrophoresis. The protein concentration was determined from the absorbance at 280 nm ($E^{1\%}_{1cm}=11.7$).

Tyrostatin, a specific inhibitor of PCP and XCP, was prepared as described previously (*33*). The inhibitor was found to be homogeneous on HPLC analysis. Its concentration was precisely determined by amino acid analysis.

Peptide Substrates—The peptide substrates used in this study were synthesized and characterized as described previously (*39, 40*). They have the general structure, P5-P4-P3-P2-Phe-Nph-P2′-P3′ (P5, P4, P3, P2, P2′, P3′: a variety of amino acids, and Nph is *p*-nitro-L-phenylalanine). All were pure as to HPLC criteria and readily soluble in water. Aqueous stock solutions of approximately 5 mM were prepared. The concentrations were precisely determined by amino acid analysis. In all cases, the position in these substrates cleaved by carboxyl proteinases was between the Phe and Nph residues (*38*).

Determination of Kinetic Parameters for Chromogenic Substrates—Stock substrate solutions were diluted with buffer for rate determinations. 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, as the decrease in absorbance at 300 nm. The initial linear rate of this change was measured to give the initial velocity (v). The K_m and V_{max} values were estimated 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.

RESULTS AND DISCUSSION

Substrate Specificity for Synthetic Chromogenic Substrates—Pepstatin-sensitive carboxyl proteinases have an extended active-site cleft (*41*), which can accommodate at least seven amino acids of a substrate in the S4-S3′ subsites (nomenclature of Schechter and Berger) (*42*), so that cleavage can occur between the two hydrophobic residues occupying the P1-P1′ positions (*39, 43*). On the other hand, the subsite structure of pepstatin-insensitive carboxyl proteinases has not been well clarified.

In the previous paper, Oda *et al.* reported the substrate specificity of PCP, as determined using chromogenic peptide substrates with the general structure, P5-P4-P3-P2-P1-Nph-Arg-Leu (P5, P4, P3, P2, P1: a variety of amino acids, and P1-Nph is the scissile peptide bond) (*38*). PCP cleaved between two hydrophobic amino acid residues preferably, especially Phe residues, like pepsin and cathepsin D. The cleavage at the P1-Nph positions of substrates by PCP was influenced by replacement with different amino acid residues at the P5, P4, P3, P2, and also P1 positions.

In order to further examine the substrate specificities and kinetic properties of pepstatin-insensitive carboxyl proteinases (PCP and XCP), we have designed and synthesized 28 different chromogenic substrates with the general structure, P5-P4-P3-P2-Phe-Nph-P2′-P3′. A series of experiments was designed to provide an insight into the subsite interaction at each position. The varied amino acids include Ala, a small hydrophobic amino acid; Asp, a small residue that can be negatively charged; Ser, a small polar residue that can donate a hydrogen bond; Leu, a larger hydrophobic residue; and Arg, a positively charged amino acid. The kinetic constants of one substrate, Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu (peptide No. 1 in Table I), as a control parent peptide, will be discussed.

(1) The effect of the P5 position: The effect of the P5 position in a substrate for PCP has been determined (Table I) (*38*). A substrate having Lys at the P5 position is the most favorable for hydrolysis by PCP. On the replacement of Lys by other amino acid residues, the kinetic parameters for PCP were not drastically changed.

In the case of XCP, the replacement of Lys at the P5 position by Asp decreased the k_{cat}/K_m value to only 9% of the control value. On the other hand, the replacement of Lys by Arg did not change this kinetic constant. Thus, the S5 subsite of XCP can accept positively charged residues, but not negatively charged side chains. XCP may have one or more acidic amino acid(s) in its S5 subsite.

The effects of replacement at the P5 position for pepsin (*44*), human cathepsin D (*45*), human cathepsin E (*46*), and rhizopuspepsin (*47*) have been determined using the same set of substrates. Cathepsin E showed high activity toward a substrate having Lys at the P5 position, but showed poor kinetic parameters for all other substituted substrates. The substrate specificity of cathepsin E as to the P5 position was similar to that of PCP.

(2) The effect of the P4 position: The effect of the P4 position in a substrate for PCP has been reported (Table I) (*38*). A substrate having Pro at the P4 position was the most favorable. The replacement of Pro at the P4 position by Leu decreased the k_{cat} value to about 26% of the control value. The replacement of Pro by Asp, Arg, or Ser de-

TABLE I. **Kinetic parameters for the hydrolysis of chromogenic peptide substrates by carboxyl proteinases.** All reactions were carried out at 37°C in 0.1 M sodium formate buffer, pH 3.5 (PCP and XCP) or pH 4.5 (human cathepsin E). Kinetic parameters were determined as described under "MATERIALS AND METHODS."

No	Substrate								PCP			XCP			Human cathepsin E[a]		
	P5	P4	P3	P2	P1	P1′	P2′	P3′	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
1	Lys-	Pro-	Ala-	Lys-	Phe-	Nph*-	Arg-	Leu	95	14.5	0.15[b]	11.3	49.1	4.35	150	11	0.07
2	Ala-								100	6.2	0.06[b]	7.3	13.7	1.88	25	0.5	0.02
3	Asp-								98	5.9	0.06[b]	14.0	5.4	0.39	46	0.4	0.01
4	Leu-								43	4.0	0.09[b]	3.9	5.5	1.41	29	0.6	0.02
5	Arg-								125	5.2	0.04[b]	13.4	34.5	2.57	39	0.5	0.01
6	Ser-								55	5.5	0.10[b]	6.8	9.8	1.44	50	0.3	0.01
7		-Ala-							120	11.4	0.10[b]	8.1	18.3	2.26	160	2.0	0.01
8		-Asp-							122	1.0	0.01[b]	9.5	15.1	1.59	250	3.0	0.01
9		-Leu-							30	3.8	0.13[b]	2.9	21.9	7.55	58	0.5	0.009
10		-Arg-							77	0.9	0.01[b]	4.0	7.3	1.83	116	0.5	0.004
11		-Ser-							123	2.2	0.02[b]	5.6	3.5	0.63	250	2.0	0.008
12			-Asp-						98.6	25.4	0.26	7.7	77.8	10.1	48	4.4	0.09
13			-Leu-						37.1	15.3	0.41	14.1	69.4	4.92	38	6.0	0.15
14			-Arg-						77.7	24.3	0.31	11.7	46.9	4.01	22	3.0	0.14
15			-Ser-						65.0	25.0	0.38	4.6	58.4	12.7	73	5.0	0.07
16				-Ala-					15.4	38.2	2.48	2.6	7.3	2.81	59	41	0.7
17				-Asp-					13	6.8	0.52[b]	5.4	30.4	5.63	100	10	0.1
18				-Leu-					6.3	51.4	8.16	3.6	52.2	14.5	9	15	1.6
19				-Arg-					79.7	30.1	0.38	7.0	15.0	2.14	50	7	0.14
20				-Ser-					23.2	18.6	0.80	3.9	51.7	13.3	48	33	0.7
21							-Ala-		78.3	10.2	0.13	8.5	26.9	3.16	120	4.0	0.03
22							-Asp-		110	15.8	0.14	21.3	57.8	2.71	PC**	PC	PC
23							-Leu-		12.6	1.5	0.12	5.1	2.1	0.41	40	3.0	0.08
24							-Ser-		89.2	9.5	0.11	27.1	36.7	1.35	60	2.0	0.03
25								-Ala-	120	36.1	0.30	14.2	68.4	4.82	50	2.0	0.04
26								-Asp-	91.4	27.6	0.30	20.9	81.4	3.89	52	1.0	0.02
27								-Arg-	142	39.9	0.28	9.7	50.0	5.15	62	2.0	0.03
28								-Ser-	141	37.9	0.27	17.5	68.2	3.90	45	1.0	0.02

*Nph, *p*-nitrophenylalanine. **PC, poorly cleaved under the standard assay conditions. [a]Rao-Naik *et al.* (*46*). [b]Oda *et al.* (*38*).

creased the k_{cat}/K_m value to only 7–13% of the control one. The replacement of Pro at the P4 position by Ala or Leu did not change this specificity constant. PCP preferred hydrophobic amino residues at the P4 position. Accordingly, it was suggested that the S4 subsite of PCP was occupied by hydrophobic residues that exhibited hydrophobic interactions with the P4 position of the substrate.

In the case of XCP, Leu at the P4 position was found to yield the most favorable substrate. The k_{cat}/K_m value decreased on substitution at the P4 position in the following order, Leu > Pro > Ala > Arg > Asp > Ser. Substrates having a hydrophobic residue at the P4 position were easily cleaved by XCP. It was suggested that the S4 subsite of XCP was also occupied by hydrophobic residues, similar to that of PCP.

In human cathepsin E, it has been reported that hydrophobic interactions are particularly important at the P4 position based on the results of three-dimensional modeling and kinetic analysis (*46*).

(3) The effect of the P3 position: In the case of PCP, the replacement of Ala at the P3 position by Leu or Ser increased the k_{cat}/K_m value to approximately 3 times the control value.

For XCP the replacement of Ala at the P3 position by Asp or Ser resulted in favorable changes in the kinetic constants: the K_m values decreased to about one half and the k_{cat}/K_m values increased to approximately 2 or 3 times those for the control peptide, respectively. But, the replacement of Ala at the P3 position by Leu or Arg in a substrate did not change the kinetic parameters. It was suggested that PCP and XCP have different characteristics as to the P3 position of a substrate.

In the case of human cathepsin E, the replacement of Ala at the P3 position by Leu or Arg increased the k_{cat}/K_m value to 2 times the control value (*46*). Thus, PCP and XCP have slightly different characteristics from cathepsin E as to interaction with the P3 position of a substrate.

(4) The effect of the P2 position: The kinetic parameters for PCP were improved by the replacement of Lys at the P2 position by Ala, Asp, Leu, or Ser. The replacement of Lys by Leu caused a drastic change: the K_m value decreased to only 7%, the k_{cat} value increased by approximately 4 times, and the k_{cat}/K_m value increased to about 54 times. The k_{cat}/K_m value (8.16 $\mu M^{-1}\cdot s^{-1}$) was the highest among those of the 28 different substrates tested. The replacement of Lys at the P2 position by Ala increased the k_{cat}/K_m value to 17 times of that for the control substrate. Substrates having large hydrophobic residues at the P2 position were easily cleaved by PCP. A substrate having Lys or Arg at the P2 position was not a good substrate. It was suggested that hydrophobic interaction of the S2 subsite of PCP with the P2 position of a substrate was particularly important.

For XCP, the replacement of Lys at the P2 position by Leu also resulted in favorable changes in the kinetic parameters: the K_m values decreased to approximately 1/3, and the k_{cat}/K_m values increased to about 3 times the

control value. The k_{cat}/K_m value (14.5 $\mu M^{-1} \cdot s^{-1}$) was the highest among those of the 28 different substrates tested. The replacement of Lys at the P2 position by Ser also increased the k_{cat}/K_m value, similar to the replacement by Leu. The replacement of Lys at the P2 position by Ala, Asp, or Arg did not cause significant changes in the specificity constants. In general, these k_{cat}/K_m values for XCP were higher than those for PCP.

The specificities of cathepsin D (*48*) and cathepsin E (*46*) for the P2 position have been determined using the same set of substrates. As shown in Table II, the substrate specificity of PCP for the P2 position was similar to that of cathepsins D and E, with a low tolerance for positively charged residues (Lys and Arg). The S2 subsites of cathepsins D and E have been shown to be largely hydrophobic in nature. These findings may suggest that the S2 subsite of PCP is similar to those of cathepsins D and E, rather than that of XCP.

(5) The effect of the P2′ position: In the case of PCP, the kinetic parameters did not significantly change on the replacement of Arg at the P2′ position by Ala, Asp, or Ser. The replacement of Arg by Leu having a branched hydrophobic side chain decreased the K_m value to about 13% of that of the control peptide. However, the k_{cat} value of the substrate was only 9-16%, compared with the values of other substituents. Consequently, the specificity constant of the substrate is equivalent to those of other substrates.

Turning to XCP, the K_m value of a substrate having Leu at the P2′ position was lower than those of other P2′ substituted substrates. The k_{cat} value of the substrate was only 4-8%, compared with those of other substituted substrates. Similar characteristics were observed for PCP.

Thus, in the cases of both PCP and XCP, hydrophobic interaction between the S2′ subsite and the P2′ position of a substrate was observed. The S2′ subsite of most aspartic proteinases has been shown to be hydrophobic in nature by X-ray crystal structure analysis. Cathepsin E poorly cleaved a substrate having Asp at the P2′ position under the standard assay conditions (*46*). This unique characteristic of cathepsin E was not observed for either PCP or XCP.

(6) The effect of the P3′ position: Finally, the nature of the amino acid residue at the P3′ position was examined (Table I). The kinetic parameters for PCP and XCP did not change on the replacement of Leu at the P3′ position by Ala, Asp, Arg, or Ser. A similar trend has been observed for human cathepsin E (*46*), which has at least 7 subsites between S4 and S3′.

In this study, we examined the substrate specificities of PCP and XCP using 28 chromogenic synthetic peptide substrates. As shown in Table I, the substrate specificity of PCP was stricter than that of XCP. That is, the specificity constant (k_{cat}/K_m) of each substrate for PCP was in general $<0.5\ \mu M^{-1} \cdot s^{-1}$, but was drastically improved by the replacement of Lys by Leu at the P2 position. Similar characteristics were observed for cathepsin E (Table I) (*46*). On the other hand, XCP showed a less stringent substrate specificity, with most of the peptides exhibiting reasonable k_{cat}/K_m values ($>1.0\ \mu M^{-1} \cdot s^{-1}$). These characteristics are similar to those of rhizopuspepsin (*47*).

As described above, the rates of hydrolysis by PCP and XCP were influenced by the replacements at the P5, P4, P3, P2, and P2′ positions in a substrate. These results suggest that PCP and XCP have at least 7 subsites (S5-S2′). PCP exhibits a high sequence similarity with XCP (51.5% identity) (*35*). However, PCP showed a different substrate specificity, compared with that of XCP. The structures of the S2, S3, and S5 subsites of PCP may be different from those of XCP. Further information on the subsite structures of PCP and XCP will be obtained by three-dimensional structure analysis. Thus, we can conclude that the substrate specificities of PCP and XCP are clearly different from each other, in spite of the high similarity in their primary structures.

Inhibition Constant by Tyrostatin of XCP—In 1989, Oda *et al.* found a peptidic inhibitor for PCP and XCP, named tyrostatin, from *Kitasatosporia* sp. 55 (*33*). The chemical structure of tyrostatin is *N*-isovaleryl-tyrosyl-leucyl-tyrosinal. The COOH-terminal tyrosinal residue of tyrostatin is essential for the inhibition. Tyrostatin specifically inhibits PCP and XCP, *i.e.* it does not inhibit other pepstatin-insensitive carboxyl proteinases. Tyrostatin is a competitive inhibitor of PCP. The K_i value is 2.6 nM at pH 3.5 and 37°C with Arg-Pro-Ala-Lys-Phe-Nph-Arg-Leu as a substrate (*38*).

In this study, we determined the inhibition constant of tyrostatin for XCP. Among the substrates described above,

TABLE II. **Specificity constants for the hydrolysis by carboxyl proteinases of a series of chromogenic substrates, Lys-Pro-Ala-P2-Phe-Nph*-Arg-Leu, with variations at the P2 residue.**

No.	P2 residue	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)			
		PCP	XCP	Human cathepsin D[a]	Human cathepsin E[b]
1	Lys	0.15[c]	4.35	NC**	0.07
16	Ala	2.48	2.81	0.15	0.7
17	Asp	0.52[c]	5.63	0.30	0.1
18	Leu	8.16	14.5	1.00	1.6
19	Arg	0.38	2.14	NC	0.14
20	Ser	0.80	13.3	0.07	0.7

*Nph, *p*-nitrophenylalanine. **NC, not cleaved under the standard assay conditions. [a]Scarborough *et al.* (*48*). [b]Rao-Naik *et al.* (*46*). [c]Oda *et al.* (*38*).

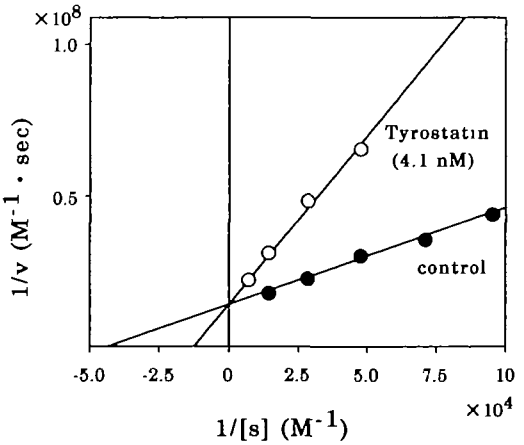

Fig. 1. **Inhibition of XCP by tyrostatin.** XCP (2.5 nM) was incubated with tyrostatin (4.1 nM) with four different concentrations (21, 35, 70, and 140 μM) of the substrate in 0.1 M sodium formate buffer (pH 3.5) in the presence of 0.5% methanol at 37°C. The substrate used was Lys-Pro-Ala-Lys-Phe-Nph-Ser-Leu (peptide No. 24). The initial rates were determined by analysis of the time-courses of the reactions. Tyrostatin was found to be a competitive inhibitor of the enzyme, with a K_i value of 2.1 nM.

Lys-Pro-Ala-Lys-Phe-Nph-Ser-Leu (peptide No. 24) was selected because of its poor K_m value ($K_m = 27.1\ \mu M$). This facilitates observation of competitive inhibition. As shown in Fig. 1, tyrostatin was also a competitive inhibitor for XCP, with a K_i value of 2.1 nM at pH 3.5 and 37°C.

On the basis of these results, we designed and synthesized several tyrostatin derivatives to modify the catalytic residue(s) of PCP and XCP (*3*). Among the resultant compounds, *N*-benzyloxycarbonyl-tyrosine-2,3-epoxypropylester inactivated XCP, but not PCP. *N*-Benzyloxycarbonyl-phenylalanine-2,3-epoxypropylester inactivated PCP and XCP. In addition, PCP and XCP were inactivated by 1,2-epoxyethylbenzene, similar to pepsin (*7*). We are now trying to identify the catalytic residue(s) of PCP and XCP by chemical modification, using such compounds. We are also trying to identify the catalytic residues of PCP and XCP by site-directed mutagenesis based on the high sequence similarity between PCP and XCP. We hope to identify the catalytic residues of PCP and XCP by means of these two different methods.

We are grateful to Professor S. Hara and Dr. K. Hayashi (Department of Chemistry and Materials Technology, Kyoto Institute of Technology) for the amino acid analysis. We also thank E. Takahashi and T. Watanabe for purification of the PCP and XCP.

REFERENCES

1. Murao, S. and Oda, K. (1985) Pepstatin-insensitive acid proteinases in *Aspartic Proteinases and Their Inhibitors* (Kostka, V., ed.) pp. 379-399, Walter de Gruyter, Berlin
2. Oda, K. and Murao, S. (1991) Pepstatin-insensitive carboxyl proteinases in *Structure and Function of the Aspartic Proteinases. Genetics, Structures, and Mechanisms* (Dunn, B.M., ed.) pp. 185-201, Plenum Press, New York
3. Oda, K., Takahashi, S., Shin, T., and Murao, S. (1995) Pepstatin-insensitive carboxyl proteinases in *Aspartic Proteinases: Structure, Function, Biology, and Biomedical Implications* (Takahashi, K., ed.) pp. 529-542, Plenum Press, New York
4. Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M., and Takeuchi, T. (1970) Pepstatin, a new pepsin inhibitor produced by actinomycetes. *J. Antibiot.* **23**, 259-262
5. Murao, S. and Satoi, S. (1970) New pepsin inhibitors (S-PI) from *Streptomyces* EF-44-201. *Agric. Biol. Chem.* **34**, 1265-1267
6. Rajagopalan, T.G., Stein, W.H., and Moore, S. (1966) The inactivation of pepsin by diazoacetyl-norleucine methyl ester. *J. Biol. Chem.* **241**, 4295-4297
7. Tang, J. (1971) Specific and irreversible inactivation of pepsin by substrate-like epoxides. *J. Biol. Chem.* **246**, 4510-4517
8. Tang, J., Sepulveda, P., Marciniszyn, J., Jr., Chen, K.C.S., Huang, W.-Y., Tao, N., Liu, D., and Lanier, J.P. (1973) Amino-acid sequence of porcine pepsin. *Proc. Natl. Acad. Sci. USA* **70**, 3437-3439
9. Kay, J. (1985) Aspartic proteinases and their inhibitors in *Aspartic Proteinases and Their Inhibitors* (Kostka, V., ed.) pp. 1-17, Walter de Gruyter, Berlin
10. Delaney, R., Wong, R.N.S., Meng, G., Wu, N., and Tang, J. (1987) Amino acid sequence of rhizopuspepsin isozyme pI 5. *J. Biol. Chem.* **262**, 1461-1467
11. Murao, S., Oda, K., and Matsushita, Y. (1972) New acid proteases from *Scytalidium lignicolum* M-133. *Agric. Biol. Chem.* **36**, 1647-1650
12. Murao, S., Oda, K., and Matsushita, Y. (1973) Isolation and identification of a microorganism which produces non *Streptomyces* pepsin inhibitor and *N*-diazoacetyl-DL-norleucine methylester sensitive acid proteases. *Agric. Biol. Chem.* **37**, 1417-1421
13. Oda, K. and Murao, S. (1974) Purification and some enzymatic properties of acid protease A and B of *Scytalidium lignicolum* ATCC 24568. *Agric. Biol. Chem.* **38**, 2435-2444
14. Oda, K., Torishima, H., and Murao, S. (1986) Purification and characterization of acid proteinase C of *Scytalidium lignicolum* ATCC 24568. *Agric. Biol. Chem.* **50**, 651-658
15. Oda, K., Murao, S., Oka, T., and Morihara, K. (1975) Some physicochemical properties and substrate specificity of acid protease B of *Scytalidium lignicolum* ATCC 24568. *Agric. Biol. Chem.* **39**, 477-484
16. Oda, K., Murao, S., Oka, T., and Morihara, K. (1976) Some physicochemical properties and substrate specificities of acid proteases A-1 and A-2 of *Scytalidium lignicolumn* ATCC 24568. *Agric. Biol. Chem.* **40**, 859-866
17. Morihara, K., Tsuzuki, H., Murao, S., and Oda, K. (1979) Pepstatin-insensitive acid proteases from *Scytalidium lignicolum*. Kinetic study with synthetic peptides. *J. Biochem.* **85**, 661-668
18. Morihara, K. (1981) Comparative specificity of microbial acid proteinases in *Proteinases and Their Inhibitors: Structure, Function, and Applied Aspects* (Turk, V. and Vitale, L.J., eds.) pp. 213-222, Mladinska Knjiga-Pergamon Press, Ljubljana, Oxford
19. Oda, K. and Murao, S. (1976) Action of *Scytalidium lignicolum* acid proteases on insulin B-chain. *Agric. Biol. Chem.* **40**, 1221-1225
20. Majima, E., Oda, K., Murao, S., and Ichishima, E. (1988) Comparative study on the specificities of several fungal aspartic and acidic proteinases towards the tetradecapeptide of a renin substrate. *Agric. Biol. Chem.* **52**, 787-793
21. Maita, T., Nagata, S., Matsuda, G., Maruta, S., Oda, K., Murao, S., and Tsuru, D. (1984) Complete amino acid sequence of *Scytalidium lignicolum* acid protease B. *J. Biochem.* **95**, 465-475
22. Chang, W.J., Horiuchi, S., Takahashi, K., Yamasaki, M., and Yamada, Y. (1976) Effects of acid protease-specific inhibitors on the acid proteases from *Aspergillus niger* var. *macrosporus*. *J. Biochem.* **80**, 975-981
23. Oda, K., Terashita, T., Kono, M., and Murao, S. (1981) Occurrence of *Streptomyces* pepsin inhibitor-insensitive carboxyl proteinase in Basidiomycetes. *Agric. Biol. Chem.* **45**, 2339-2340
24. Terashita, T., Oda, K., Kono, M., and Murao, S. (1981) *Streptomyces* pepsin inhibitor-insensitive carboxyl proteinase from *Lentinus edodes*. *Agric. Biol. Chem.* **45**, 1937-1943
25. Terashita, T., Oda, K., Kono, M., and Murao, S. (1984) *Streptomyces* pepsin inhibitor-insensitive carboxyl proteinase from *Ganoderma lucidum*. *Agric. Biol. Chem.* **48**, 1029-1035
26. Kobayashi, H., Kusakabe, I., and Murakami, K. (1985) Purification and characterization of a pepstatin-insensitive carboxyl proteinase from *Polyporus tulipiferae (Irpex lacteus)*. *Agric. Biol. Chem.* **49**, 2393-2397
27. Oda, K., Sugutani, M., Fukuhara, K., and Murao, S. (1987) Purification and properties of a pepstatin-insensitive carboxyl proteinase from a Gram-negative bacterium. *Biochim. Biophys. Acta* **923**, 463-469
28. Oda, K., Nakazima, T., Terashita, T., Suzuki, K., and Murao, S. (1987) Purification and properties of an S-PI (pepstatin Ac)-insensitive carboxyl proteinase from a *Xanthomonas* sp. bacterium. *Agric. Biol. Chem.* **51**, 3073-3080
29. Murao, S., Ohkuni, K., Nagao, M., Oda, K., and Shin, T. (1988) A novel thermostable, S-PI (pepstatin Ac)-insensitive acid proteinase from thermophilic *Bacillus* novosp. strain MN-32. *Agric. Biol. Chem.* **52**, 1629-1631
30. Murao, S., Ohkuni, K., Nagao, M., Hirayama, K., Fukuhara, K., Oda, K., Oyama, H., and Shin, T. (1993) Purification and characterization of kumamolysin, a novel thermostable pepstatin-insensitive carboxyl proteinase from *Bacillus* novosp. MN-32. *J. Biol. Chem.* **268**, 349-355
31. Toogood, H.S., Prescott, M., and Daniel, R.M. (1995) A pepstatin-insensitive aspartic proteinase from a thermophilic *Bacillus* sp. *Biochem. J.* **307**, 783-789
32. Prescott, M., Peek, K., and Daniel, R.M. (1995) Characterisation of a thermostable pepstatin-insensitive acid proteinase from a *Bacillus* sp. *Int. J. Biochem. Cell Biol.* **27**, 729-739
33. Oda, K., Fukuda, Y., Murao, S., Uchida, K., and Kainosho, M.

(1989) A novel proteinase inhibitor, tyrostatin, inhibiting some pepstatin-insensitive carboxyl proteinases. *Agric. Biol. Chem.* **53**, 405-415
34. Oda, K., Takahashi, T., Tokuda, Y., Shibano, Y., and Takahashi, S. (1994) Cloning, nucleotide sequence, and expression of an isovaleryl pepstatin-insensitive carboxyl proteinase gene from *Pseudomonas* sp. 101. *J. Biol. Chem.* **269**, 26518-26524
35. Oda, K., Ito, M., Uchida, K., Shibano, Y., Fukuhara, K., and Takahashi, S. (1996) Cloning and expression of an isovaleryl pepstatin-insensitive carboxyl proteinase gene from *Xanthomonas* sp. T-22. *J. Biochem.* **120**, 564-572
36. Hayashi, K., Izu, H., Oda, K., Fukuhara, K., Matsuo, M., Takano, R., and Hara, S. (1995) The primary structure of pepstatin-insensitive carboxyl proteinase produced by *Pseudomonas* sp. No. 101. *J. Biochem.* **118**, 738-744
37. Nakatani, H., Hiromi, K., Satoi, S., Oda, K., Murao, S., and Ichishima, E. (1975) Studies on the interaction between *Streptomyces* pepsin inhibitor and several acid proteinases by means of a zinc(II)-dye complex as a probe. *Biochim. Biophys. Acta* **391**, 415-421
38. Oda, K., Nakatani, H., and Dunn, B.M. (1992) Substrate specificity and kinetic properties of pepstatin-insensitive carboxyl proteinase from *Pseudomonas* sp. No. 101. *Biochim. Biophys. Acta* **1120**, 208-214
39. Dunn, B.M., Jimenez, M., Parten, B.F., Valler, M.J., Rolph, C.E., and Kay, J. (1986) A systematic series of synthetic chromophoric substrates for aspartic proteinases. *Biochem. J.* **237**, 899-906
40. Dunn, B.M., Scarborough, P.E., Davenport, R., and Swietniki, W. (1994) Analysis of proteinase specificity by studies of peptide substrates in *Methods in Molecular Biology* (Dunn, B.M. and Pennington, M.W., eds.) Vol. 36, pp. 225-243, Humana Press, Totowa, NJ
41. Tang, J., James, M.N., Hsu, I.N., Jenkins, J.A., and Blundell, T.L. (1978) Structural evidence for gene duplication in the evolution of the acid proteases. *Nature* **271**, 618-621
42. Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157-162
43. Dunn, B.M., Valler, M.J., Rolph, C.E., Foundling, S.I., Jimenez, M., and Kay, J. (1987) The pH dependence of the hydrolysis of chromogenic substrates of the type, Lys-Pro-Xaa-Yaa-Phe-(NO_2)-Phe-Arg-Leu, by selected aspartic proteinases: Evidence for specific interactions in subsites S_3 and S_2. *Biochim. Biophys. Acta* **913**, 122-130
44. Rao, C., Scarborough, P.E., Lowther, W.T., Kay, J., Batley, B., Rapundalo, S., Klutchko, S., Taylor, M.D., and Dunn, B.M. (1991) Structure-function database for active site binding to the aspartic proteinases in *Structure and Function of the Aspartic Proteinases. Genetics, Structures, and Mechanisms* (Dunn, B.M., ed.) pp. 143-147, Plenum Press, New York
45. Scarborough, P.E., Richo, G.R., Kay, J., Conner, G.E., and Dunn, B.M. (1991) Comparison of kinetic properties of native and recombinant human cathepsin D in *Structure and Function of the Aspartic Proteinases. Genetics, Structures, and Mechanisms* (Dunn, B.M., ed.) pp. 343-347, Plenum Press, New York
46. Rao-Naik, C., Guruprasad, K., Batley, B., Rapundalo, S., Hill, J., Blundell, T., Kay, J., and Dunn, B.M. (1995) Exploring the binding preferences/specificity in the active site of human cathepsin E. *Proteins* **22**, 168-181
47. Lowther, W.T., Chen, Z., Lin, X.L., Tang, J., and Dunn, B.M. (1991) Substrate specificity study of recombinant *Rhizopus chinensis* aspartic proteinase in *Structure and Function of the Aspartic Proteinases. Genetics, Structures, and Mechanisms* (Dunn, B.M., ed.) pp. 275-279, Plenum Press, New York
48. Scarborough, P.E., Guruprasad, K., Topham, C., Richo, G.R., Conner, G.E., Blundell, T.L., and Dunn, B.M. (1993) Exploration of subsite binding specificity of human cathepsin D through kinetics and rule-based molecular modeling. *Protein Sci.* **2**, 264-276