J. Biochem. 124, 642-647 (1998)

Substrate Specificity of Pepstatin-Insensitive Carboxyl Proteinase from *Bacillus coagulans* J-4[1]

Masahiro Shibata,*,[2] Ben M. Dunn,† and Kohei Oda*,[3]

Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Sakyo-ku, Kyoto, 606-8585; and †Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, FL 32610-0245, USA

Received for publication, May 7, 1998

***Bacillus coagulans* J-4 carboxyl proteinase, designated as J-4, is characterized as alcohol-resistant and insensitive to aspartic proteinase inhibitors such as pepstatin, diazoacetyl-DL-norleucinemetylester, and 1,2-epoxy-3-(*p*-nitrophenoxy)propane. Here, its substrate specificity was elucidated by using two series of chromogenic substrates, Lys-Pro-Ala-Lys-Phe*Nph (*p*-nitrophenylalanine:* is cleavage site)-Arg-Leu (XVI) and Lys-Pro-Ile-Glu-Phe*Nph-Arg-Leu (RS6), in which the amino acid residues at positions P_5-P_2, P_2', and P_3' were systematically substituted. Kinetic parameters were determined for both sets of peptides. J-4 was shown to hydrolyze Lys-Pro-Ala-Ala-Phe-Nph-Arg-Leu most effectively among the XVI series. The kinetic parameters of this peptide were $K_m = 20.0 \pm 3.24$ μM, $k_{cat} = 15.4 \pm 0.71$ s^{-1}, and $k_{cat}/K_m = 0.769 \pm 0.128$ $\mu M^{-1} \cdot s^{-1}$. Among the RS6 series, Lys-Pro-Ile-Pro-Phe-Nph-Arg-Leu was hydrolyzed most effectively. The kinetic parameters of this peptide were $K_m = 13.7 \pm 1.30$ μM, $k_{cat} = 9.65 \pm 0.38$ s^{-1}, and $k_{cat}/K_m = 0.704 \pm 0.072$ $\mu M^{-1} \cdot s^{-1}$. These systematic analyses revealed that J-4 had a unique preference for the P_2 position: J-4 preferentially hydrolyzed peptides having an Ala or Pro residue in the P_2 position. Other carboxyl proteinases preferred peptides having hydrophobic and bulky amino acid residue such as Leu in the P_2 position. Thus, J-4 was found to differ considerably in substrate specificity from the other carboxyl proteinases reported so far.**

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

Carboxyl proteinases, formerly called acid proteinases, are classified into two groups: pepstatin (isovaleryl-pepstatin)-sensitive and pepstatin-insensitive (*1-3*).

Pepstatin-sensitive carboxyl proteinases are inhibited by pepstatin (*4*), S-PI (acetylpepstatin) (*5*), diazoacetyl-DL-norleucinemetylester (DAN) (*6*), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) (*7*). In porcine pepsin, a pair of aspartic acid residues, Asp^{32} and Asp^{215}, was shown to be essential for catalytic function (*8*). Likewise, most other carboxyl proteinases have been shown to contain two active aspartic acid residues at the catalytic site. Thus, pepstatin-sensitive carboxyl proteinases are called aspartic proteinases (*9*). Extensive sequence similarity has been observed among the enzymes of this family (*10*). Amino acid sequences around the two catalytic aspartyl residues are well conserved as -Asp*-Thr-Gly-(Asp*: catalytic residues). These enzymes are also similar to each other in their tertiary structure (*11-14*). And they have been shown to utilize a common catalytic mechanism for the hydrolysis of substrates.

On the other hand, in 1972, Murao *et al.* isolated carboxyl proteinases A, B, and C from *Scytalidium lignicolum* ATCC 24568 (*15-18*). These enzymes are not inhibited by pepstatin, S-PI, or DAN and only carboxyl proteinase B is inhibited by EPNP. In addition to this inhibition profile, these enzymes have unique substrate specificities (*19-24*). Carboxyl proteinase B also differed considerably in its amino acid sequence from previously reported aspartic proteinases (pepstatin-sensitive carboxyl proteinases) (*25*). In subsequent studies, enzymes having similar properties to *Scytalidium* carboxyl proteinases have been isolated from fungi (*26-30*), bacteria (*31, 32*), and even thermophilic bacteria (*33-36*).

Among these pepstatin-insensitive carboxyl proteinases, *Pseudomonas* sp. No. 101 carboxyl proteinase (PCP) (*31*), and *Xanthomonas* sp. T-22 carboxyl proteinase (XCP) (*32*) are the first and second examples of unique carboxyl proteinases from prokaryotes, regardless of their pepstatin-sensitivity. These enzymes are not inhibited by the inhibitors mentioned above, but are inhibited by tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal) (*37*). PCP and XCP have no sequence similarity to any other carboxyl

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

[2] Present address: Department of Cell Biology and Anatomy 1, Osaka University Medical School, Suita, Osaka, 565-0871.

[3] To whom correspondence should be addressed. Tel: +81-75-724-7763, Fax: +81-75-724-7760, 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.

© 1998 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

proteinases (*38-40*); and they lack the consensus catalytic site sequence, -Asp*-Thr-Gly-(Asp*: catalytic residue). On the other hand, it was verified that two catalytic carboxyl groups were associated to catalysis of these enzymes by the zinc(II)-PAD (pyridine-2-azo-*p*-dimethylaniline) method (*41*) and kinetic analyses (*42*).

Ozaki *et al.* (*43*) isolated *Bacillus coagulans* J-4, which produced a pepstatin-insensitive carboxyl proteinase. This is the third example of a pepstatin-insensitive carboxyl proteinase from a prokaryote (mesophile). The molecular weight and optimum pH were found to be 45,000 and pH 3.0, respectively. Some of the unique features of this enzyme are as follows: (i) the enzyme activity is not inhibited by tyrostatin, which differs from PCP and XCP; (ii) the enzyme is stable in 30% ethanol at room temperature for more than one month, and retains 80% of original activity in 15% ethanol; (iii) the partial amino acid sequence of J-4 has similarity to that of kumamolysin, which was isolated from a thermophilic bacterium, *Bacillus* novosp. MN-32 (*34*).

In this paper, we describe the substrate specificity of the alcohol-resistant and pepstatin-insensitive carboxyl proteinase from *Bacillus coagulans* J-4. The substrates used were two series of chromogenic substrates. J-4 was found to differ considerably in substrate specificity from other carboxyl proteinases (*44*), especially in the P_2 position.

MATERIALS AND METHODS

Enzyme—J-4 was purified from a crude preparation (kindly supplied by Daiwa Kasei, Osaka) by column chromatographies on Sephadex G-100 and DEAE-Sephadex A-50. It was homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentration was determined by amino acid analysis.

Peptide Substrates—The peptide substrates used in this study were synthesized and characterized as described previously (*45, 46*). They have the general structure P_5-P_4-P_3-P_2-Phe-Nph-P_2'-P_3' (where P_5, P_4, P_3, P_2, P_2', and P_3' are various amino acids, Nph is *p*-nitrophenylalanine). All were pure on HPLC and readily soluble in water. Stock peptide solutions were made in filtered distilled water. The concentrations were quantified by amino acid analysis.

Determination of Kinetic Parameters—The cleavage of each substrate between Phe and Nph residues was monitored at 37°C and pH 3.5 in 0.1 M sodium formate buffer by averaging the decrease in absorbance measured over the range of 284-324 nm a Hewlett Packard diode array spectrophotometer. For kinetic assays, six tubes containing a mixture of the buffer and the enzyme were prewarmed for 3 min at 37°C. After the preincubation, five different concentrations of substrate were added, and the absorbance was monitored using a multicell transporter. The initial rate of this change was measured to give the initial velocity (v). The K_m and V_{max} values were derived from direct fitting by using a nonlinear regression analysis program, with at least five values of initial substrate concentration. k_{cat} values were obtained from the equation: $V_{max} = k_{cat}$ [E], where [E] is the enzyme concentration. The estimated error of the derived K_m and k_{cat} values is within 20%. Under the same conditions, the cleavage of the peptides was also examined by HPLC and amino acid analysis to confirm that each substrate had been cleaved at the Phe-Nph bond.

RESULTS AND DISCUSSION

Despite the overall structural similarities among pepstatin-sensitive carboxyl proteinases (aspartic proteinases), each proteinase must have subtle distinctions that enable it to fulfill its own function(s) in its own environment. Pepstatin-sensitive carboxyl proteinases have an extended active-site cleft, which can accommodate at least seven amino acids residues of a substrate in the S_4-S_3' subsites (*46*), so that cleavage can occur between two hydrophobic residues occupying the P_1*P_1' positions (*47*).

On the other hand, pepstatin-insensitive carboxyl proteinases have not been extensively studied. Oda and Ito *et al.* reported substrate specificities of pepstatin-insensitive carboxyl proteinases from *Pseudomonas* sp. (PCP) and *Xanthomonas* sp. (XCP) using chromogenic substrates (*42, 44*). They found that substrate specificities of these enzymes differ considerably, in spite of the high similarity in their primary structures.

To examine the substrate specificity of J-4, we used two series of chromogenic substrates: Lys-Pro-Ala-Lys-Phe*-Nph-Arg-Leu (XVI series) and Lys-Pro-Ile-Glu-Phe*-Nph-Arg-Leu (RS6), in which the amino acid residues at positions P_5-P_2, P_2', and P_3' were systematically substituted. The amino acids introduced in these positions include: Ala, a small hydrophobic residue; Leu, a larger hydrophobic residue; Arg, a positively charged residue; Asp, a small residue that can be negatively charged; and Ser, a small polar residue that can form a hydrogen bond. These series present a variety of potential interactions that might be favorable or unfavorable for interaction at any of the subsites. The kinetic parameters for hydrolysis of XVI and RS6 substrate series by J-4 are summarized in Tables I-V.

(1) The Effect of the P_5 Position—Table I summarizes the effect of the P_5 position on hydrolysis of the XVI substrate series by J-4. J-4 preferentially cleaved the peptides with a positively charged Lys or Arg residue in the P_5 position, which suggests that the S_5 subsite of J-4 might be negatively charged. Ito *et al.* reported that both PCP and XCP favored peptides having Lys in the P_5 position (*44*). In these points, J-4 is similar to PCP and XCP. It differs, however, in that PCP and XCP cleaved all peptides but J-4 could not cleave the Ser, Asp, and Ala derivatives, and J-4 showed poor kinetic constants for the other three substrates compared to those of PCP and XCP.

(2) The Effect of the P_4 Position—The k_{cat} value with the Leu substituent was 1.5-2.7 times higher than those of the other substituents, and the K_m value was lowest of all. The specificity constant for the Leu-containing peptide was over 25 times higher than those of the other substituents. The S_4 subsite of J-4 may be occupied by hydrophobic residues that exhibit hydrophobic interactions with the P_4 position of the substrate. PCP and XCP also preferentially hydrolyze peptides having hydrophobic amino acid residues in the P_4 position. From the results of three-dimensional modeling and kinetic analysis, it has been reported that hydrophobic interactions at the P_4 position are important in cathepsin E (*49*). In its preference for the P_4 position, therefore, J-4 seems to be identical to other carboxyl proteinases. These hydrophobic interactions seem to be important for both pepstatin-sensitive and pepstatin-insensitive carboxyl proteinases.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

TABLE I. **Kinetic parameters for hydrolysis of XVI substrates by J-4.**

P_6	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 ($\mu M^{-1} \cdot s^{-1}$)
Lys	Pro	Ala	Lys	Phe	Nph	Arg	Leu	0.21±0.01	16.8±2.87	0.012±0.002
Arg								0.12±0.01	14.3±1.27	0.008±0.001
Leu								0.18±0.01	50.8±9.93	0.004±0.001
Ser								N.C.		
Asp								N.C.		
Ala								N.C.		
	Leu							4.24±0.70	11.3±0.68	0.375±0.066
	Ala							2.85±0.12	57.6±5.70	0.050±0.005
	Arg							1.12±0.06	28.7±4.26	0.039±0.006
	Asp							1.32±0.08	64.5±9.00	0.020±0.003
	Ser							1.55±0.14	107±16.4	0.015±0.003
		Asp						0.43±0.02	17.1±1.71	0.025±0.003
		Arg						0.63±0.04	30.7±4.46	0.021±0.003
		Ser						0.23±0.01	21.6±1.85	0.011±0.001
		Leu						N.C.		
			Ala					15.38±0.71	20.0±3.24	0.769±0.128
			Asp					3.09±0.19	13.6±2.40	0.227±0.042
			Ser					2.86±0.11	20.9±2.30	0.137±0.016
			Arg					0.36±0.02	36.6±6.16	0.010±0.002
			Leu					0.61±0.06	217±45.3	0.003±0.001
						Ala		N.C.		
						Asp		N.C.		
						Leu		N.C.		
						Ser		N.C.		
							Arg	0.49±0.02	26.3±3.47	0.019±0.003
							Ser	0.56±0.06	33.7±5.85	0.017±0.003
							Ala	0.33±0.03	63.8±12.7	0.005±0.001
							Asp	0.35±0.05	78.4±20.2	0.004±0.001

Nph is *p*-nitro-L-phenylalanine. N.C., not cleaved under standard condition (0.1 M sodium formate buffer, pH 3.5).

TABLE II. **Kinetic parameters for hydrolysis of P_3-substituted RS6 substrates by J-4.**

P_6	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 ($\mu M^{-1} \cdot s^{-1}$)
Lys	Pro	Arg	Glu	Phe	Nph	Arg	Leu	4.22±0.16	14.2±1.52	0.297±0.033
		Ile*						5.21±0.20	21.2±2.24	0.246±0.027
		Thr						2.82±0.08	15.1±1.35	0.187±0.018
		Glu						1.97±0.07	10.6±1.20	0.186±0.022
		Gln						2.96±0.08	21.1±1.53	0.140±0.011
		Lys						3.97±0.06	33.4±1.40	0.119±0.005
		Asn						1.17±0.04	12.1±1.49	0.097±0.012
		Ser						1.25±0.05	13.2±1.66	0.095±0.013
		Leu						0.89±0.03	30.4±3.14	0.029±0.003
		Asp						0.42±0.03	21.9±0.17	0.019±0.001
		Phe						N.C.		

Nph is *p*-nitro-L-phenylalanine. * indicates the parent substrate. N.C., not cleaved under standard condition (0.1 M sodium formate buffer, pH 3.5).

TABLE III. **Kinetic parameters for hydrolysis of P_2-substituted RS6 substrates by J-4.**

P_6	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 ($\mu M^{-1} \cdot s^{-1}$)
Lys	Pro	Ile	Pro	Phe	Nph	Arg	Leu	9.65±0.38	13.7±1.30	0.704±0.072
			Ala					15.7±0.70	25.0±2.63	0.628±0.071
			Asn					2.63±0.04	5.43±0.48	0.487±0.046
			Glu*					5.21±0.20	21.2±2.24	0.246±0.027
			Asp					3.64±0.23	16.1±2.76	0.226±0.042
			Ser					4.08±0.44	25.3±4.96	0.161±0.036
			Nle					1.75±0.08	18.8±2.38	0.093±0.013
			Thr					0.56±0.03	33.3±2.62	0.017±0.002
			Arg					0.22±0.01	16.6±2.49	0.013±0.002
			His					0.21±0.01	27.0±3.13	0.008±0.001
			Val					0.12±0.01	23.4±3.58	0.005±0.001
			Lys					0.15±0.01	41.6±6.58	0.004±0.001
			Ile					0.15±0.01	44.4±4.46	0.003±0.000

Nph is *p*-nitro-L-phenylalanine. * indicates the parent substrate.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

(3) The Effect of the P_3 Position—The effect of the P_3 position was examined in hydrolysis of XVI and its derivatives and the RS6 series (Tables I and II). The best P_3 replacement was Asp in the XVI series and Arg in the RS6 series. Comparison of the specificity constant for Lys-Pro-Arg-Lys-Phe-Nph-Arg-Leu (0.021) with that for Lys-Pro-Arg-Glu-Phe-Nph-Arg-Leu (0.297) reveals that the RS-6 series is hydrolyzed roughly 15 times more efficiently.

Among the RS6 substrates, the specificity constant with the Leu substituent was about 10% of that with an Ile substituent. In addition, the Phe substituent yielded a peptide that was not hydrolyzed. These results suggest that J-4 has very strict steric selection at the S_3 subsite. The observation that J-4 cleaves peptide with Arg at P_3 stands in sharp contrast to the properties of porcine pepsin, which does not favor Arg substitution (*51*).

(4) The Effect of the P_2 Position—Previously, Ito *et al.* reported that the hydrophobic interaction of the S_2 subsite of PCP with the P_2 position of the XVI series substrates was particularly important (*44*), and that the S_2 subsite of PCP may be similar to those of cathepsin D (*50*) and cathepsin E (*49*), rather than that of XCP.

The preference of J-4 for this position revealed some unique features. Tables I and III summarize the kinetic parameters for hydrolysis of XVI and RS6 substrates series by J-4. The specificity constant for hydrolysis of the XVI peptide having an Ala residue in P_2 position was 0.769 $\mu M^{-1} \cdot s^{-1}$. Those of RS6 peptides with Pro and Ala in position P_2 were 0.704 and 0.628 $\mu M^{-1} \cdot s^{-1}$, respectively. These values were highest of all substrates tested in this study. The order of specificity constants presented in Tables I and III showed that J-4 preferentially hydrolyzed the peptides having a Pro, Ala, or Asn residue in the P_2 position. The side chains of these residues are comparably small. Moreover, the specificity constants of the peptides having a negatively charged amino acid residue (Asp in the XVI substrate, Glu or Asp in the RS6 substrate) in the P_2 position were higher than those with a positively charged residue (Lys and Arg for both substrate series). The Leu substituent in XVI, and Ile and Val substituents in RS6 gave poor substrates for hydrolysis by J-4. These results suggest that the S_2 subsite of J-4 is small and occupied by positively charged amino acid residues.

J-4 hydrolyzed the peptide having a Pro residue in the P_2 position. This peptide was not hydrolyzed by cathepsin E (*49*), porcine pepsin (*51*), and other pepstatin-sensitive carboxyl proteinases (data not shown). We also summarize the specificity constants for hydrolysis of XVI peptides with P_2 substituents by several carboxyl proteinases (*48–51*) (Fig. 1). Only J-4 preferentially hydrolyzed the peptide having an Ala residue in the P_2 position rather than a Leu residue. These findings make J-4 unique among the carboxyl proteinases reported to date.

(5) The Effect of the P_2' Position—J-4 did not hydrolyze any peptide in which the Arg in XVI P_2' position was replaced by another amino acid residue (Table I). In the RS6 substrate series, differences appeared in the specificity constants for hydrolysis (Table IV). J-4 preferentially hydrolyzed peptides having Arg, Glu, or Lys residues in the P_2' position. This suggests that the S_2' subsite of J-4 is occupied by a hydrophilic amino acid residue, or it is largely exposed to the solvent. However, the replacement of Glu by Asp caused a decrease in the specificity constant. The reason could be that the side chain of Asp is smaller than Glu. That is, the S_2' subsite of J-4 may be slightly larger than those of other carboxyl proteinases.

In the case of PCP and XCP, the specificity constants of the XVI substrate with Arg at P_2' were almost equivalent to

TABLE IV. **Kinetic parameters for hydrolysis of P_2'-substituted RS6 substrates by J-4.**

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 ($\mu M^{-1} \cdot s^{-1}$)
Lys	Pro	Ile	Glu	Phe	Nph	Arg*	5.21±0.20	21.2±2.24	0.246±0.027
						Glu	5.46±0.19	33.5±3.37	0.163±0.017
						Lys	2.87±0.07	19.5±2.13	0.147±0.016
						Ala	1.38±0.04	36.7±3.04	0.038±0.003
						Leu	1.51±0.08	53.8±7.55	0.028±0.004
						Asp	1.95±0.17	96.4±13.8	0.020±0.003
						Asn	1.31±0.12	82.1±15.6	0.016±0.003
						Val	0.67±0.03	48.4±5.24	0.014±0.002
						Ser	0.69±0.06	74.3±11.0	0.009±0.001
						Ile	N.C.		

Nph is *p*-nitro-L-phenylalanine. * indicates the parent substrate. N.C., not cleaved under standard condition (0.1 M sodium formate buffer, pH 3.5).

TABLE V. **Kinetic parameters for hydrolysis of P_3'-substituted RS6 substrates by J-4.**

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 ($\mu M^{-1} \cdot s^{-1}$)
Lys	Pro	Ile	Glu	Phe	Nph	Arg	Leu*	5.21±0.20	21.2±2.24	0.246±0.027
							Asp	8.74±0.46	78.3±8.37	0.112±0.013
							Ser	8.22±0.50	79.4±9.70	0.104±0.014
							Phe	4.61±0.37	49.9±9.35	0.092±0.019
							Ala	3.13±0.09	42.2±2.92	0.074±0.006
							Arg	2.14±0.08	29.5±2.46	0.073±0.007
							Ile	2.56±0.06	37.8±2.75	0.068±0.005
							Val	4.05±0.25	61.8±8.60	0.065±0.010
							Tyr	3.29±0.17	51.9±5.43	0.063±0.007

Nph is *p*-nitro-L-phenylalanine. * indicates the parent substrate.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

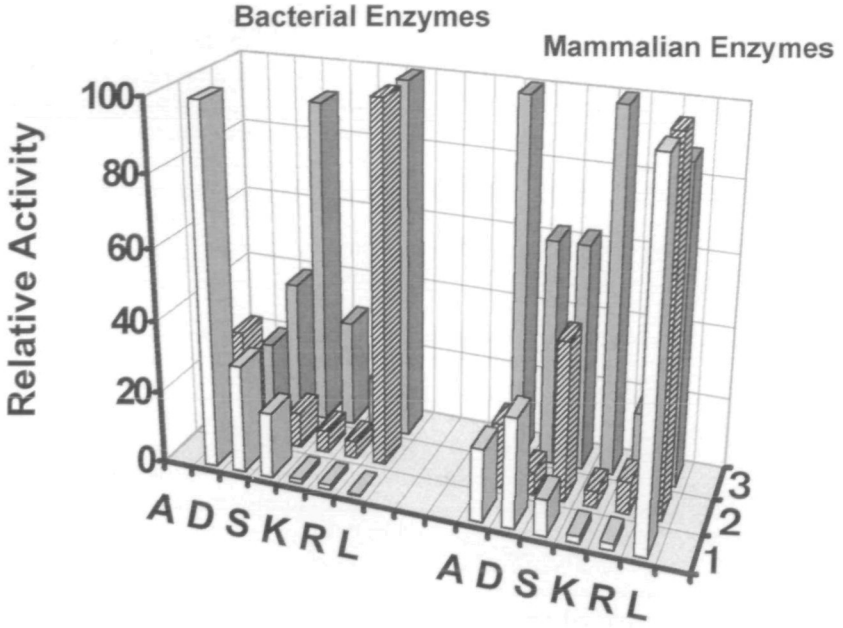

Fig. 1. **Comparison of specific constants for hydrolysis of XVI series peptides with P_2 substitutions by several carboxyl proteinases.** Specific constants of each enzyme for P_2-substituted XVI (Lys-Pro-Ala-P_2-Phe-Nph-Arg-Leu) are shown as relative activity. In each case, the highest activity was taken as 100%. Bacterial enzymes: row 1, J-4 (the highest value was observed for the Ala derivative, $k_{cat}/K_m = 0.77$ $\mu M^{-1} \cdot s^{-1}$); row 2, PCP (the highest value was observed for the Leu derivative, $k_{cat}/K_m = 8.16$ $\mu M^{-1} \cdot s^{-1}$); row 3, XCP (the highest value was observed for the Leu derivative, $k_{cat}/K_m = 14.5$ $\mu M^{-1} \cdot s^{-1}$). Mammalian enzymes: row 1, cathepsin D (the highest value was observed for the Leu derivative, $k_{cat}/K_m = 1.00$ $\mu M^{-1} \cdot s^{-1}$); row 2, cathepsin E (the highest value was observed for the Leu derivative, $k_{cat}/K_m = 1.60$ $\mu M^{-1} \cdot s^{-1}$); row 3, pepsin (the highest value was observed for the Ala and Lys derivatives, $k_{cat}/K_m = 0.80$ $\mu M^{-1} \cdot s^{-1}$).

the values derived with other substituents. However, the K_m values of the peptide with a Leu substituent were much lower than those of other substrates (data not shown). These results indicate that the hydrophobic interactions between the S_2' subsite and the P_2' position of substrates are important for the enzymes PCP and XCP. The S_2' subsite of most aspartic proteinases has been shown to be hydrophobic in nature by X-ray crystal structure analysis. The results in Table IV indicate that the S_2' subsite of J-4 is very different from that of other carboxyl proteinases.

(6) The Effect of the P_3' Position—The effect of the P_3' position was also examined. The kinetic parameters are summarized in Tables I and V. The specificity constant for hydrolysis of the peptide having a Leu residue in the P_3' position by J-4 was highest of all substituents in this position. However, no significant difference was observed between substituents in this position. A similar tendency was observed for human cathepsin E, which has at least 7 subsites from S_4 to S_3' (*49*).

In this study, we examined the substrate specificity of J-4 by using two series of chromogenic peptide substrates. J-4 preferentially hydrolyzed the RS6 substrates rather than the XVI substrates. The reason for this seems to be a difference in affinity between S_2 subsite of J-4 and P_2 position of the substrates. In its specificity for position P_2 of the substrate, J-4 was notably different from other carboxyl proteinases (Table III and Fig. 1).

We are now cloning the gene of J-4 to obtain more detailed information on the structure-function relationships of J-4. Hitherto, detailed studies on the specificity of pepstatin-insensitive carboxyl proteinases have been lacking. Kinetic analyses and information on the tertiary structures of J-4 would be useful to elucidate the catalytic mechanisms of this family. Comparison of this pepstatin-insensitive carboxyl proteinases family with the classical pepstatin-sensitive carboxyl proteinases family may provide further understanding of the evolution of carboxyl proteinases.

We wish to thank Mr. S. Ito for purification of J-4 carboxyl proteinase.

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 Biochemical 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 inhibitor (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. Bott, R., Subramanian, E., and Davies, D.R. (1982) Three-dimensional structure of the complex of the *Rhizopus chinensis* carboxyl proteinase and pepstatin at 2.5-Å resolution. *Biochemistry* **21**, 6956-6962
12. James, M.N.G. and Sielecki, A. (1983) Structure and refinement of penicillopepsin at 1.8 Å resolution. *J. Mol. Biol.* **163**, 299-361
13. Andreeva, N.S., Zdanov, A.S., Gustchina, A.E., and Fedorov, A.A. (1984) Structure of ethanol-inhibited porcin pepsin at 2-Å

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

resolution and binding of the methyl ester of phenylalanyl-diiodotyrosine to the enzyme. *J. Biol. Chem.* **259**, 11353-11365
14. Pearl, L. and Blundell, T. (1984) The active site of aspartic proteinases. *FEBS Lett.* **174**, 96-101
15. Murao, S., Oda, K., and Matsushita, Y. (1972) New acid proteases from *Scytalidium lignicolum* M-133. *Agric. Biol. Chem.* **36**, 1647-1650
16. Murao, S., Oda, K., and Matsushita, Y. (1973) Isolation and identification of a microorganism which produces non *Streptmyces* pepsin inhibitor and *N*-diazoacetyl-DL-norleucine methylester sensitive acid proteases. *Agric. Biol. Chem.* **37**, 1417-1421
17. 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
18. 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
19. 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
20. Oda, K., Murao, S., Oka, T., and Morihara, K. (1976) Some physicochemical properties and substrate specificities of acid protease A-1 and A-2 of *Scytalidium lignicolum* ATCC 24568. *Agric. Biol. Chem.* **40**, 859-866
21. 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
22. 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
23. Oda, K. and Murao, S. (1976) Action of *Scytalidium lignicolum* acid proteases on insulin B-chain. *Agric. Biol. Chem.* **40**, 1221-1225
24. 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
25. 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
26. 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
27. 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
28. 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
29. 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
30. Kobayashi, H., Kusakabe, I., and Murakami, K. (1985) Purification and characterization of pepstatin-insensitive carboxyl proteinase from *Polyporus tulipiferae* (*Irpex lacteus*). *Agric. Biol. Chem.* **49**, 2393- 2397
31. Oda, K., Sugitani, 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
32. 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
33. Murao, S., Ohkuni, K., Nagano, 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
34. 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
35. 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
36. 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
37. 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
38. 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
39. 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
40. 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
41. 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 zinc(II)-dye complex as a probe. *Biochim. Biophys. Acta* **391**, 415-421
42. 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
43. A. Ozaki *et al.* (1993) Japan Patent, 5-211869
44. Ito, M., Dunn, B.M., and Oda, K. (1996) Substrate specificity of pepstatin-insensitive carboxyl proteinases from Gram-negative bacteria. *J. Biochem.* **120**, 845-850
45. 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
46. 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, Human Press, Totowa, NJ
47. Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157-162
48. Kay, J. and Dunn, B.M. (1992) Substrate specificity and inhibitors of aspartic proteinases. *Scand. J. Clin. Lab. Invest.* **52**, 23-30
49. 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
50. 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
51. Rao, C. and Dunn, B.M. (1995) Evidence for electrostatic interactions in the S_2 subsite of porcine pepsin in *Aspartic Proteinases: Structure, Function, Biology, and Biochemical Implications* (Takahashi, K., ed.) pp. 91-94, Plenum Press, New York

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012