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Subsite Preferences of Pepstatin-Insensitive Carboxyl Proteinases from Prokaryotes: Kumamolysin, a Thermostable Pepstatin-Insensitive Carboxyl Proteinase[1]

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Kumamolysin, a carboxyl proteinase from *Bacillus* novosp. MN-32, is characterized by its thermostability and insensitivity to aspartic proteinase inhibitors such as pepstatin, diazoacetyl-DL-norleucine methylester, and 1,2-epoxy-3-(*p*-nitro-phenoxy)propane. Here, its substrate specificity was elucidated using two series of synthetic chromogenic substrates: P_5-P_4-P_3-P_2-Phe*Nph (*p*-nitrophenylalanine: *cleavage site)-P_2'-P_3', in which the amino acid residues at the P_5-P_2, P_2' and P_3' positions were systematically substituted. Among 74 substrates, kumamolysin was shown to hydrolyze Lys-Pro-Ile-Pro-Phe-Nph-Arg-Leu most effectively. The kinetic parameters of this peptide were K_m = 41 ± 5 μM, k_{cat} = 176 ± 10 s^{-1}, and k_{cat}/K_m = 4.3 ± 0.6 $\mu M^{-1} \cdot s^{-1}$. These systematic analyses revealed the following features: (i) Kumamolysin had a unique preference for the P_2 position. Kumamolysin preferentially hydrolyzed peptides having an Ala or Pro residue at the P_2 position; this was also observed for the pepstatin-insensitive carboxyl proteinase from *Bacillus coagulans* J-4 [J-4; Shibata *et al.* (1998) *J. Biochem.* 124, 642–647]. Other carboxyl proteinases, including *Pseudomonas* sp. 101 pepstatin-insensitive carboxyl proteinase (PCP) and *Xanthomonas* sp. T-22 pepstatin-insensitive carboxyl proteinase (XCP), preferred peptides having hydrophobic and bulky amino acid residue such as Leu at the P_2 position. (ii) Kumamolysin preferred such charged amino acid residues as Glu or Arg at the P_3' position, suggesting that the S_3' subsite of kumamolysin is occupied by hydrophilic residues, similar to that of PCP, XCP, and J-4. In general, the S_3' subsite of pepstatin-sensitive carboxyl proteinases (aspartic proteinases) is hydrophobic in nature. Thus, the hydrophilic nature of the S_3' subsite was confirmed to be a distinguishing feature of pepstatin-insensitive carboxyl proteinases from prokaryotes.

Key words: carboxyl proteinase, pepstatin-insensitive, subsite preferences, substrate specificity, thermostable enzyme.

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

Pepstatin-sensitive carboxyl proteinases are inhibited by pepstatin (*5*), S-PI (acetyl pepstatin) (*6*), diazoacetyl-DL-norleucine methylester (DAN) (*7*), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) (*8*). In porcine pepsin, a pair of aspartic acid residues (Asp32 and Asp215) was shown to be essential for the catalytic function (*9*). 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 (*10*). Extensive sequence similarity has been observed among the enzymes of this family (*11*). 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 structures (*12–15*). Furthermore, 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 (*16–19*) [Scytalidopepsin A, EC 3.4.23.31; Scytalidopepsin B, EC 3.4.23.32]. These enzymes are not inhibited by pepstatin, S-PI, or DAN. Only carboxyl proteinase B is inhibited by EPNP. In addition to this inhibition profile, these enzymes have unique substrate speci-

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Abbreviations: a.a., amino acid; DAN, diazoacetyl-DL-norleucine methylester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; J-4, *Bacillus coagulans* J-4 carboxyl proteinase; kumamolysin, *Bacillus* novosp. MN-32 carboxyl proteinase; Nph, *p*-nitrophenylalanine; S-PI, acetyl pepstatin; PCP, *Pseudomonas* sp. 101 carboxyl proteinase; XCP, *Xanthomonas* sp. T-22 carboxyl proteinase.

ficities (*20–25*). Carboxyl proteinase B also differs considerably in its amino acid sequence from previously reported aspartic proteinases (*26*). In subsequent studies, enzymes having similar properties to *Scytalidium* carboxyl proteinases have been isolated from fungi, bacteria, and even thermophilic bacteria (*27–36*).

Among these pepstatin-insensitive carboxyl proteinases, the carboxyl proteinases from *Pseudomonas* sp. 101 (PCP) (*32*) [Pseudomonapepsin; EC 3.4.23.37], *Xanthomonas* sp. T-22 (XCP) (*33*) [Xanthomonapepsin; EC 3.4.23.33], *Bacillus coagulans* J-4 (J-4) (*34*), and *Bacillus* novosp. MN-32 (kumamolysin) (*35*) were isolated from prokaryotes. PCP and XCP are the first and second examples of unique carboxyl proteinases from prokaryotes, regardless of their pepstatin-sensitivity. J-4 is characterized by its alcohol resistance. Kumamolysin is the first thermostable, pepstatin-insensitive carboxyl proteinase from bacteria. It shows its maximum proteolytic activity at 70°C and pH 3.0. The last two enzymes are produced by the same genus, *Bacillus*. None of these enzymes is inhibited by the reagents mentioned above (pepstatin, S-PI, DAN, or EPNP). Among them, PCP and XCP alone are inhibited by tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal) (*37*) [K_i = 2.6 nM for PCP (*38*), and 2.1 nM for XCP (*39*)].

PCP (*40*), XCP (*41*), J-4 (unpublished data), and kumamolysin (unpublished data) exhibit no sequence similarity to any other carboxyl proteinases, except for a pepstatin-insensitive lysosomal carboxyl proteinase, CLN2, found in the human brain (*42*). However, they exhibit high sequence identity with each other. The identities of these enzymes to PCP are: XCP = 52%, J-4 = 35%, and kumamolysin = 36%, respectively. These data suggest that they have the same origin, but different from that of the pepsin family. Moreover, they lack the consensus catalytic site sequence, -Asp*-Thr-Gly- (Asp*: catalytic residue), of aspartic proteinases. On the other hand, it was confirmed by pH-dependent kinetic analysis that PCP and XCP have a pair of catalytic carboxyl residues essential for their catalytic functions (*38*). Quite recently the catalytic residues were identified as Asp170 and Asp328 in PCP, and Asp169 and Asp348 in XCP by means of chemical modification (*43*) and site-directed mutagenesis (*44*).

In 1997, it was reported that the normal brain has a pepstatin-insensitive lysosomal carboxyl proteinase, CLN2, related to a fatal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis (LINCL) (*42*). Sequence comparisons revealed significant sequence similarities among CLN2 and PCP, XCP, J-4, and kumamolysin. The identities of these enzymes to CLN2 are: PCP = 31%, XCP = 29%, J-4 = 34%, and kumamolysin = 35%, respectively. This is the first demonstration of a pepstatin-insensitive carboxyl proteinase in mammals.

In this study, to facilitate understanding of the structure-function relationships of bacterial carboxyl proteinases with related sequences, the substrate specificity of kumamolysin was investigated using two series of synthetic chromogenic substrates, compared to the results of similar studies on other members of this family (*34*, *39*, *45*). It was found that kumamolysin had an unusual preference for the P_2 position of substrates, and that kumamolysin was characterized by the hydrophilic nature of the S_2' subsite. We hope this study will contribute in establishing the subsite preference of the pepstatin-insensitive lysosomal enzyme, CLN2, related to lysosomal storage disease.

MATERIALS AND METHODS

Enzymes—The gene for wild-type recombinant kumamolysin (Oda *et al.*, unpublished) was expressed in *E. coli* cells, and purified by three step column chromatography on DEAE-Sepharose F.F., gel filtration Sephadex G-75 and Mono Q. The purified enzyme was found to be homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentration was determined from the molar extinction coefficient (ε = 51,600).

Peptide Substrates—The peptide substrates used in this study were synthesized and characterized as described previously (*46*, *47*). The first series has the general structure, Lys(P_5)-Pro(P_4)-Ala(P_3)-Lys(P_2)-Phe*Nph-Arg(P_2')-Leu(P_3') (Nph is *p*-nitrophenylalanine, Phe*Nph is a cleavage site), with systematic substitutions at the P_5, P_4, P_3, P_2, P_2', and P_3' positions. The second series has the general structure, Lys-Pro-Ile(P_3)-Glu(P_2)-Phe*Nph-Arg(P_2')-Leu(P_3'), with systematic substitutions at the P_3, P_2, P_2', and P_3' positions. All were pure as to HPLC criteria and readily soluble in water. The substrate concentrations were determined by amino acid analysis.

Determination of Kinetic Parameters—The cleavage of each substrate between Phe and Nph was monitored at 60°C and pH 3.5 in 0.1 M sodium formate buffer. The average decrease in absorbance over the range of 284–324 nm was monitored using a Hewlett Packard 8452 Diode Array Spectrophotometer. For each kinetic assay, six tubes containing a mixture of the buffer and the enzyme were prewarmed for 3 min at 60°C. Following this pre-incubation, the substrates were added and the absorbance changes were monitored using a multi-cell transporter. The linear rate of the absorbance change 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 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 to confirm that each substrate had been cleaved at the Phe-Nph bond by HPLC and amino acid analysis.

RESULTS

In this study, the binding specificity of kumamolysin was investigated by assaying its kinetic parameters with both series of substrates. The data are summarized in Tables I–V, compared with those of J-4 (*34*). Kumamolysin and J-4 were produced by the same genus, *Bacillus*, the former with a thermophilic bacterium, and the latter with a mesophilic bacterium, respectively. In addition, high sequence identity (69%) was observed between them.

(1) The Effect of the P_5 Position—Table I summarizes the effect of P_5 replacement on the hydrolysis of the first series substrates (parent substrate: Lys-Pro-Ala-Lys-Phe*Nph-Arg-Leu). Kumamolysin preferentially cleaved the peptides with an Ala or Ser residue at the P_5 position, which suggested that the S_5 subsite of kumamolysin might be small. In contrast, the Ala or Ser derivatives were not cleaved by J-4 (*34*).

(2) The Effect of the P_4 Position—Kumamolysin cleaved the substrate having Leu at the P_4 position most effectively. The specificity constant (k_{cat}/K_m) was 0.474 ± 0.054 $\mu M^{-1} \cdot s^{-1}$. This was 3.8–31.6 times higher than those of the other substituents. The second best substrate was the Ala derivative. In the case of J-4 (*34*), the best and second substrates are also the peptides with Leu and Ala substitutions at the P_4 position, respectively.

(3) The Effect of the P_3 Position—The effect of the P_3 position was examined by hydrolysis of the first and second

TABLE I. **Kinetic parameters for hydrolysis of the first substrates series by Kumamolysin and J-4.**

Substrate P5 P4 P3 P2 P1 P1' P2' P3'	Kumamolysin k_{cat} (s^{-1})	Kumamolysin K_m (μM)	Kumamolysin k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	J-4 k_{cat} (s^{-1})	J-4 K_m (μM)	J-4 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Lys Pro Ala Lys Phe Nph Arg Leu	0 41±0 03	56 0±7 3	0 007±0 001	0 21±0.01	16.8±2.9	0 012±0 002
Ala	0 54±0 03	38 2±5 1	0 014±0 002	N C		
Ser	0 27±0 02	21 1±4 4	0 013±0 003	N C		
Leu	0 26±0 01	26 9±3 2	0 010±0 001	0 18±0 01	50 8±9 9	0 004±0 001
Arg	0 25±0.03	103 ±19	0 002±0 001	0.12±0 01	14 3±1 3	0 008±0.001
Asp	N C			N C		
Leu	20 3±1 0	42 8±4 5	0 474±0 054	4 24±0 70	11 3±0 7	0 375±0.066
Ala	6 50±0 58	51 7±9 2	0 126±0 025	2 85±0 12	57 6±5 7	0 050±0 005
Arg	1 33±0 08	12 8±2 2	0 104±0 019	1 12±0 06	28.7±4 3	0.039±0 006
Asp	1 46±0 11	34 9±6 4	0 042±0 008	1 32±0 08	64 5±9 0	0 020±0 003
Ser	2 35±0.25	162 ±26	0 015±0 003	1 55±0 14	107 ±16	0 015±0 003
Asp	0 64±0 04	35 1±5 6	0 018±0 003	0 43±0 02	17 1±1 7	0 025±0 003
Ser	0 59±0 06	57 7±10 6	0 010±0 002	0 23±0 01	21.6±1 9	0 011±0 001
Arg	1 13±0 10	142 ±22	0 008±0 001	0 63±0 04	30 7±4 5	0 021±0.003
Leu	N C			N C		
Ala	98.3±7 1	63 6±8 9	1 55 ±0 24	15 4±0 7	20 0±3 2	0 769±0.128
Ser	19 9±1.8	64 5±11 3	0 308±0 061	2 86±0 11	20 9±2 3	0 137±0 016
Asp	18 1±0 6	133 ±7	0 136±0 008	3 09±0 19	13 6±2 4	0 227±0 042
Arg	0 98±0 05	15 4±3 0	0 064±0 013	0 36±0 02	36.6±6 2	0 010±0.002
Leu	N C			0 61±0 06	217 ±45	0 003±0 001
Ala	N C			N C		
Asp	N C			N C		
Leu	N.C			N C		
Ser	N C			N C		
Arg	1 04±0 03	12 2±1 0	0 085±0 007	0 49±0 02	26 3±3 5	0 019±0 003
Ser	0.45±0 04	52 7±9 0	0 009±0 002	0.56±0 06	33 7±5 9	0 017±0 003
Ala	0 22±0 02	56 7±8 6	0 004±0 001	0 33±0 03	63 8±12 7	0.005±0 001
Asp	0 19±0 02	67 4±10 4	0 003±0.000	0 35±0 05	78.4±20.2	0 004±0.000

Nph, *p*-nitro-L-phenylalanine. N.C., not cleaved under the standard conditions (0.1 M sodium formate buffer, pH 3.5). Data for J-4 (see Ref. *34*).

TABLE II. **Kinetic parameters for hydrolysis of P_3-substituted second series substrates by Kumamolysin and J-4.**

Substrate P5 P4 P3 P2 P1 P1' P2' P3'	Kumamolysin k_{cat} (s^{-1})	Kumamolysin K_m (μM)	Kumamolysin k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	J-4 k_{cat} (s^{-1})	J-4 K_m (μM)	J-4 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Lys Pro Lys Glu Phe Nph Arg Leu	13 3±0 8	24 6±3 3	0 541±0 080	3 97±0 06	33 4±1 4	0 119±0 005
Gln	16 1±1 6	37 6±6 9	0 428±0 089	2 96±0 08	21 1±1 5	0 140±0 011
Ile*	25 4±2 1	60 3±8 2	0 421±0 067	5 21±0 20	21 2±2 2	0 246±0 027
Thr	16 7±2 0	46 9±9 4	0 356±0 083	2 82±0 08	15 1±1 4	0 187±0 018
Glu	13 1±0 8	40 4±5 1	0 324±0 046	1 97±0 07	10 6±1 2	0 186±0 022
Ala**	13 0±0 7	40 6±4 6	0 320±0 040	2 57	23 9	0 107
Arg	32 3±2.7	119 ±13	0 272±0 038	4 22±0 16	14 2±1 5	0 297±0 033
Val**	18 6±1 6	105 ±13	0.178±0 026	7.06	37 8	0 187
Asn	4 62±0 29	37 7±4 8	0 123±0 017	1 17±0.04	12 1±1 5	0 097±0 012
Ser	9 95±0 51	100 ±7	0 100±0 009	1 25±0 05	13 2±1 7	0 095±0 013
Phe	3 76±0.30	38 9±6 3	0 100±0 017	N.C		
Leu	4 44±0 35	57 5±8 1	0 077±0 012	0 89±0 03	30 4±3 1	0 029±0 003
Asp	3 12±0 37	65 4±13 0	0.048±0 011	0 42±0 03	21 9±0 2	0 019±0 001
Tyr	N C			N C		

Nph, *p*-nitro-L-phenylalanine. N.C., not cleaved under the standard conditions (0.1 M sodium formate buffer, pH 3.5). * indicates the parent substrate. Data for J-4 (see Ref. *34*). ** indicates data for J-4 obtained with a Beckman DU7400 Spectrophotometer.

series of substrates (Tables I and II). The best P_3 derivative was the Asp one in the first series and the Lys one in the second series. Their specificity constants, k_{cat}/K_m values, were 0.018 ± 0.003 and 0.541 ± 0.080 $\mu M^{-1} \cdot s^{-1}$, respectively. That is, the second series substrate was hydrolyzed roughly 30 times more effectively. The replacement of Lys by Gln, Ile, Thr, or Glu decreased the specificity constant, k_{cat}/K_m value, in that order. These results suggested that kumamolysin exhibits much more versatile selection at the S_3 subsite. J-4 (*34*) showed the same tendency.

(4) The Effect of the P_2 Position—The preference of kumamolysin for the P_2 position revealed some unique features, similar to those shown by J-4 (*34*). Tables I and III summarize the kinetic parameters for the hydrolysis of the first and second substrate series by kumamolysin and J-4. The best P_2 replacement was the Ala one in the first series and the Pro one in the second series. Their specificity constants, k_{cat}/K_m values, are 1.55 ± 0.24 and 4.30 ± 0.61 $\mu M^{-1} \cdot s^{-1}$, respectively. These values are the highest for all substrates tested in this study. J-4 also effectively cleaved the Pro or Ala derivative in the second series of substrates.

(5) The Effect of the P_2' Position—We examined the specificity of the S_2' subsite of kumamolysin (Tables I and IV). In the case of the first series of substrates, kumamolysin could not cleave the substrates in which Arg at the P_2' position was replaced by other amino acid residues. This result was same as for J-4 (*34*). On the other hand, in the case of the second series of substrates, differences were found in the k_{cat}/K_m values. A Glu residue at the P_2' position was found to yield the most suitable substrate in this series. The par-

TABLE III. **Kinetic parameters for hydrolysis of P_2-substituted second series substrates by Kumamolysin and J-4.**

Substrate	Kumamolysin			J-4		
P5 P4 P3 P2 P1 P1' P2' P3'	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Lys Pro Ile Pro Phe Nph Arg Leu	176 ±10	40 9±5 3	4 30 ±0 61	9 65±0 38	13 7±1 3	0 704±0 072
Ala	48 5±2 8	42 6±4 9	1 14 ±0 15	15 7±0 7	25 0±2 6	0 628±0 071
Ser	52 7±4 6	64 2±11 7	0 821±0 166	4 08±0 44	25 3±5 0	0 161±0 036
Asp	28 6±1 4	40 5±4 2	0 707±0 081	3 64±0 23	16 1±2 8	0 226±0 042
Nle	20 2±1 0	46 8±5 4	0 432±0 055	1 75±0 08	18 8±2 4	0 093±0 013
Glu *	25 4±2 1	60 3±8 2	0 422±0 067	5 21±0 20	21 2±2 2	0 246±0 027
Gln**	4 79±0 29	37 7±4 5	0 127±0 017	1 67	18 1	0 092
Asn	9 14±0 95	73 0±13 2	0 125±0 026	2 63±0 04	5 43±0 48	0 487±0 046
Thr	3 48±0 22	44 7±4 9	0 078±0.010	0 56±0 03	33 3±2 6	0 017±0 002
His	3 00±0 35	83 1±14 4	0 036±0 008	0 21±0 01	27 0±3 1	0 008±0 001
Ile	0 30±0 01	12 8±1.0	0 024±0 002	0 15±0 01	44 4±4 5	0 003±0 000
Leu**	0 84±0 09	41.0±8 3	0 021±0 005	0 31	8 87	0 035
Val	0 73±0 06	82.5±12.0	0 009±0 001	0 12±0 01	23 4±3 6	0 005±0 001
Arg	0 46±0 04	76 7±9 3	0 006±0 001	0 22±0 01	16 6±2 5	0 013±0 002
Lys	0 32±0.02	67 1±5 7	0 005±0.000	0 15±0 01	41 6±6 6	0.004±0.001

Nph, *p*-nitro-L-phenylalanine. * indicates the parent substrate. Data for J-4 (see Ref. *34*). ** indicates data for J-4 obtained with a Beckman DU7400 Spectrophotometer.

TABLE IV. **Kinetic parameters for hydrolysis of P_2'-substituted second series substrates by Kumamolysin and J-4.**

Substrate	Kumamolysin			J-4		
P5 P4 P3 P2 P1 P1' P2' P3'	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Lys Pro Ile Glu Phe Nph Glu Leu	22.7±2.6	43.8±9.0	0 518±0 123	5 46±0.19	33 5±3 4	0 163±0 017
Arg*	25 4±2 1	60.3±8 2	0.422±0.067	5.21±0.20	21.2±2.2	0.246±0 027
Leu	23 9±1 3	109 ±9	0.220±0 022	1 51±0.08	53 8±7 6	0 028±0 004
Ala	7 70±0 32	37 1±4 2	0 207±0.025	1.38±0 04	36 7±3 0	0 038±0 003
Lys	10 9±1.3	128 ±24	0.085±0 019	2 87±0.07	19 5±2 1	0 147±0 016
Ile	1.82±0.12	28.5±3.4	0 064±0.009	N.C		
Val	5 04±0.63	88 3±18 1	0.057±0 014	0 67±0 03	48 4±5 2	0 014±0 002
Ser	2 60±0 23	47 3±8 2	0 055±0 011	0 69±0 06	74 3±11 0	0 009±0 001
Asp	8 78±0 95	225 ±39	0 039±0 008	1 95±0 17	96 4±13 8	0 020±0 003
Asn	3 48±0 55	430 ±87	0 008±0 002	1 31±0 12	82 1±15 6	0 016±0 003

Nph, *p*-nitro-L-phenylalanine. N.C., not cleaved under the standard conditions (0.1 M sodium formate buffer, pH 3.5). * indicates the parent substrate. Data for J-4 (see Ref. *34*).

TABLE V. **Kinetic parameters for hydrolysis of P_3'-substituted second series substrates by Kumamolysin and J-4.**

Substrat P5 P4 P3 P2 P1 P1' P2' P3'	Kumamolysin k_{cat} (s^{-1})	Kumamolysin K_m (μM)	Kumamolysin k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	J-4 k_{cat} (s^{-1})	J-4 K_m (μM)	J-4 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Lys Pro Ile Glu Phe Nph Arg Tyr	15 9±0 8	24.8±2 6	0 641±0 074	3.29±0 17	51.9±5 4	0.063±0 007
Phe	31 9±2.4	63 4±7 4	0 504±0.070	4 61±0 37	49.9±9 4	0.092±0.019
Leu*	25 4±2 1	60 3±8 2	0 422±0 067	5 21±0 20	21 2±2 2	0 246±0 027
Nle	34 2±3 2	119 ±16	0 289±0 047	N C		
Val	13 1±0 7	58.9±5 2	0.222±0 026	4 05±0 25	61 8±8.6	0.065±0.010
Asp	21 7±1 8	109 ±13	0 198±0 029	8 74±0 46	78 3±8 4	0.112±0.013
Ile	12 5±0 8	69 7±8.8	0 179±0.025	2 56±0 06	37 8±2 8	0 068±0 005
Ala	12 4±1 5	99 5±18 2	0.125±0 027	3 13±0 09	42 2±2 9	0 074±0 006
Arg	15 8±0 8	154 ±12	0 102±0 009	2 14±0 08	29 5±2 5	0 073±0 007
Ser	11 5±1 4	129 ±20	0 089±0 018	8 22±0 50	79 4±9 7	0.104±0 014

Nph, *p*-nitro-L-phenylalanine. * indicates the parent substrate. Data for J-4 (see Ref. *34*).

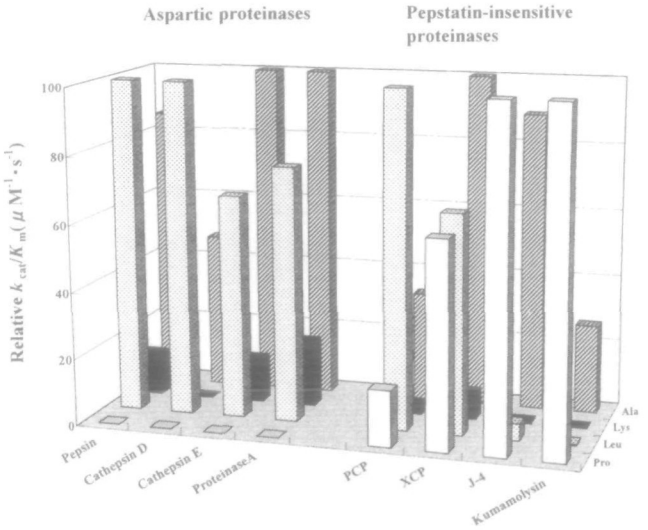

Fig. 1. **Comparison of the specific constants for hydrolysis of peptides with P_2 substitutions of K-P-I-P_2-F-Nph-R-L by several carboxyl proteinases.** The specific constants for each enzyme for a P_2-substituted second substrate (K-P-I-P_2-F-Nph-R-L) are shown as the relative activity. In each case, the highest activity for Pro, Leu, Lys, and Ala derivatives was taken as 100%. Aspartic proteinases: Porcine pepsin (the highest value was observed for the Leu derivative, k_{cat}/K_m = 1.40 $\mu M^{-1}\cdot s^{-1}$); human cathepsin D (the highest value was observed for the Leu derivative, k_{cat}/K_m = 1.61 $\mu M^{-1}\cdot s^{-1}$); human cathepsin E (the highest value was observed for the Ala derivative, k_{cat}/K_m = 3.0 $\mu M^{-1}\cdot s^{-1}$); *Saccharomyces cerevisiae* proteinase A (the highest value was observed for the Ala derivative, k_{cat}/K_m = 0.316 $\mu M^{-1}\cdot s^{-1}$). Pepstatin-insensitive carboxyl proteinases: PCP (the highest value was observed for the Leu derivative, k_{cat}/K_m = 6.43 $\mu M^{-1}\cdot s^{-1}$); XCP (the highest value was observed for the Ala derivative, k_{cat}/K_m = 12.1 $\mu M^{-1}\cdot s^{-1}$); J-4 (the highest value was observed for the Pro derivative, k_{cat}/K_m = 0.704 $\mu M^{-1}\cdot s^{-1}$); Kumamolysin (the highest value was observed for the Pro derivative, k_{cat}/K_m = 4.30 $\mu M^{-1}\cdot s^{-1}$).

ent substrate (Arg at the P_2' position) was the second most favorable substrate. The specificity constants of the Leu derivative and other ones were half or less. J-4 showed the same tendency.

(6) The Effect of the P_3' Position—The effects of the P_3' substitution are summarized in Tables I and V. Kumamolysin hydrolyzed most effectively the substrate having Tyr at the P_3' position in the second series. The specificity constant, k_{cat}/K_m value, was approximately 1.5 times higher than that for the parent substrate (Leu derivative). The specificity constant decreased in the following order of P_3' substituents, Tyr, Phe, Leu, Nle, and Val. J-4 (*34*) favored the substrate with Leu at this position, but, on the whole, substitution at this position did not result in significant differences.

DISCUSSION

Aspartic proteinases (pepstatin-sensitive carboxyl proteinases) are involved in human diseases: *e.g.*, renin in hypertension and human immunodeficiency virus proteinase in AIDS. Aspartic proteinases have an extended active-site cleft (*48*), which can accommodate at least seven amino acids of a substrate in the S_4-S_3' subsites [nomenclature of Schechter and Berger (*49*)], so that cleavage can occur between two hydrophobic residues occupying the S_1-S_1' sites. 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 (PCP, XCP, J-4, and kumamolysin) from prokaryotes. As a part of these studies, we have analyzed the substrate specificities of PCP and XCP using two series of substrates [parent substrate of first series: Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu (*39*), and parent substrate of second series: Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu (*45*)], with systematic substitutions at the P_6-P_2, P_2', and P_3' positions. In spite of their high sequence identity (52%), PCP and XCP showed quite different substrate specificities: PCP had a strict preference for substrates, while XCP showed a broad specificity (*39*). The best substrate for PCP had a Leu replacement at the P_2 position, and that for XCP an Ala replacement at the P_3 position among the second series substrates (*45*). 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 J-4 (*34*). In contrast, the S_2' subsite of aspartic proteinases is hydrophobic in nature. We have

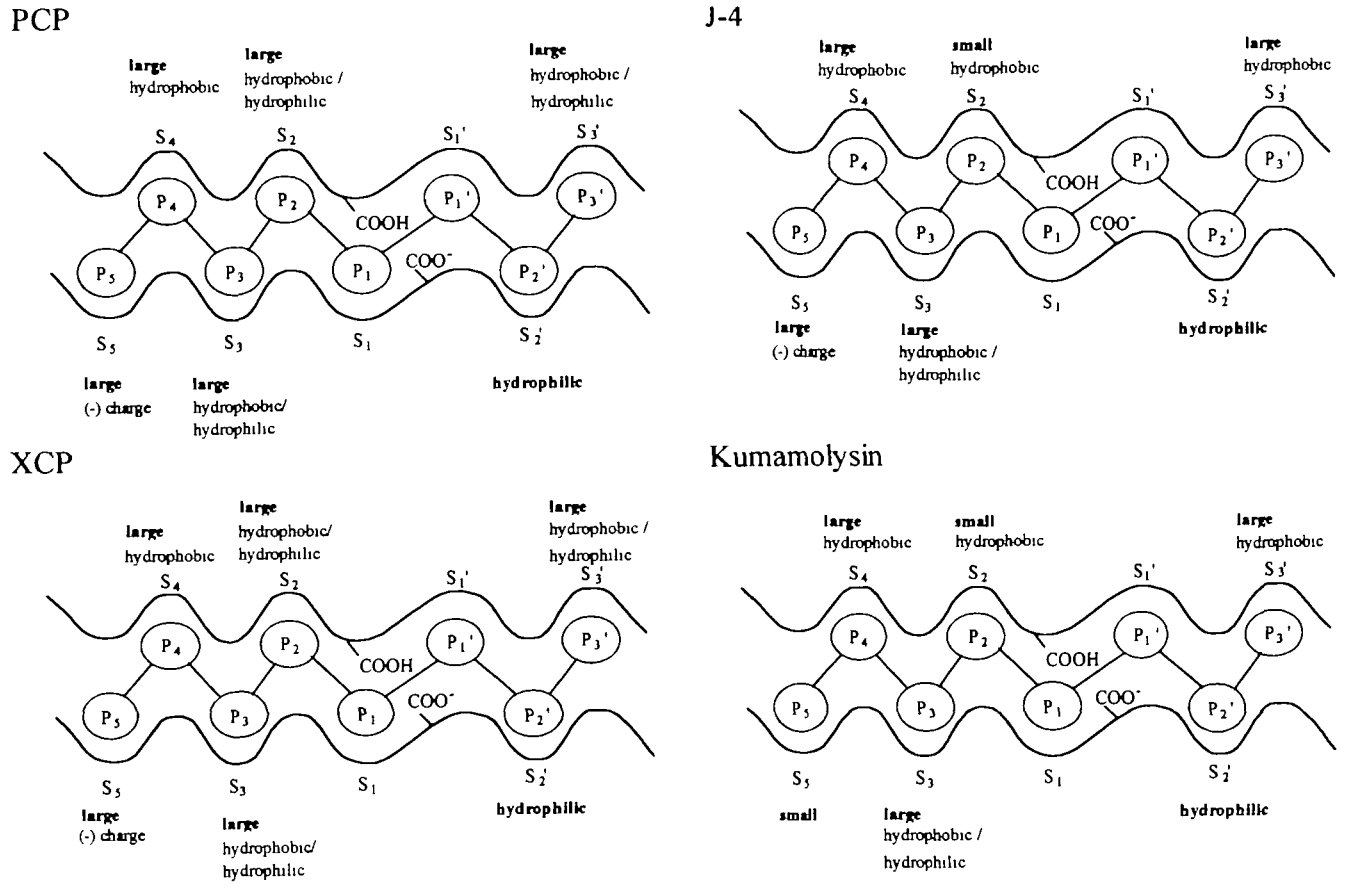

Fig. 2. **Presumed subsite structures of pepstatin-insensitive carboxyl proteinases from bacteria.** See the following references: PCP (*39*, *45*), XCP (*39*, *45*), and J-4 (*34*), respectively.

also analyzed the substrate specificity of *Bacillus coagulans* J-4 pepstatin-insensitive carboxyl proteinase, designated as J-4 (*34*). J-4 is characterized as being alcohol-resistant. J-4 preferentially hydrolyzed peptides having an Ala or Pro residue at the P_2 position. Other carboxyl proteinases, including PCP and XCP, preferred peptides having a hydrophobic and bulky amino acid residue such as Leu at the P_2 position. Thus, J-4 was found to differ considerably in substrate specificity from the carboxyl proteinases reported so far.

As part on our study on the structure-function relationships of bacterial carboxyl proteinases with related sequences, the substrate specificity of kumamolysin was determined and compared to the results of similar studies on other members of this family.

(1) The S_5 Subsite—Kumamolysin preferentially cleaved the peptides with an Ala or Ser residue at the P_5 position, which suggested that the S_5 subsite of kumamolysin might be small. PCP, XCP (*39*, *45*), and J-4 (*34*) favored peptides having Lys at the P_5 position. Cathepsin D exhibited the same nature (*50*). In this point, kumamolysin was different from PCP, XCP, J-4 (*34*), and cathepsin D. Kumamolysin showed poor kinetic constants compared to those of PCP and XCP, as observed for J-4.

(2) The S_4 Subsite—Based on the results of three-dimensional modeling and kinetic analyses, it has been reported that hydrophobic interactions at the P_4 position are important in cathepsin E (*51*). The S_4 subsite of pepstatin-insensitive carboxyl proteinases (PCP, XCP, and J-4) was reported to be hydrophobic. Kumamolysin showed the same nature as these enzymes. In its preference for the P_4 position, kumamolysin seems to be identical to other carboxyl proteinases. These hydrophobic interactions seem to be important for both pepstatin-sensitive and pepstatin-insensitive carboxyl proteinases.

(3) The S_3 Subsite—In previous reports, it was noted that interactions at the S_3 subsite of aspartic proteinases with inhibitors or substrates might be important (*52*, *53*). Kumamolysin cleaved most effectively the Lys derivative in the second series of substrates. The specificity constant was $0.541 \pm 0.080\ \mu M^{-1} \cdot s^{-1}$. Many aspartic proteinases can not accept a Lys derivative as to the P_3 position (data not shown). As for aromatic amino acid derivatives, PCP (*45*) and J-4 (*34*) could not cleave the substrate having Phe or Tyr at the P_3 position. In contrast, XCP (*45*) could cleave both substrates. In the case of kumamolysin, the substrate having Tyr at the P_3 position could not be cleaved, but that having Phe at the P_3 position was cleaved. Kumamolysin could distinguish the difference in the -OH groups of Phe and Tyr residues. These results suggested that these four proteinases show different selectivities at the P_3 position.

(4) The S_2 Subsite—The most critical subsite preference of aspartic proteinases was observed for the interaction in the S_2 subsite (*52*, *53*). The best P_2 replacement was an Ala

residue in the first series (k_{cat}/K_m = 1.55 ± 0.24). The best and second best substrates in the second series were the Pro and Ala derivatives, respectively. Their specificity constants, k_{cat}/K_m values, are 4.30 ± 0.61, and 1.14 ± 0.15 $\mu M^{-1}\cdot s^{-1}$, respectively. J-4 showed a similar tendency, but the specific constant was about one-sixth of that of kumamolysin. In the case of the parent substrate (Glu derivative), the value was about one-tenth of that of the Pro derivative. These results suggested that kumamolysin exhibits considerably high selectivity at the S_2 subsite. In contrast, the Lys derivative in this series showed the lowest specificity constant (k_{cat}/K_m = 0.005 ± 0.000 $\mu M^{-1}\cdot s^{-1}$). This is one explanation for why the first series of substrates, having a Lys residue at the P_2 position, showed such lower susceptibilities than those of the second series substrates. This feature has also been observed for PCP (*39*, *45*) and J-4 (*34*). The specificity constants, k_{cat}/K_m, of the Ala derivatives in the first and second series substrates were very similar, 1.55 ± 0.24 $\mu M^{-1}\cdot s^{-1}$ and 1.14 ± 0.15 $\mu M^{-1}\cdot s^{-1}$, respectively. Based on these data, we expected that the Pro derivative in the first series must be cleaved much faster than Ala derivatives, but the specificity constant was 1.34 $\mu M^{-1}\cdot s^{-1}$ (data not shown). These data indicated that the P_3 position of these substrates (P_3 Ala in the first series, and P_3 Ile in the second series) is also involved in their susceptibility.

Thus, kumamolysin preferred a small amino acid residue at the P_2 position. PCP (*39*, *45*) and XCP (*39*, *45*) have a preference for a Leu residue at this position. In contrast, J-4 (*34*) and kumamolysin have a preference for the Pro and Ala derivatives, not the Leu derivative as to this position. The specificity constant, k_{cat}/K_m value, of the Leu derivative for kumamolysin was about 0.5% that of the Pro derivative. As described in the introduction, PCP (*38*) and XCP (*39*) are inhibited competitively by tyrostatin (*N*-isovaleryl-tyrosyl-**leucyl**-tyrosinal) (*37*), but J-4 and kumamolysin are not inhibited by tyrostatin. These differences in the susceptibility against tyrostatin are well correlated with the substrate specificities reported here.

(5) The S_2' Subsite—The S_2' subsite of aspartic proteinases reported so far has been shown to be hydrophobic in nature by X-ray crystal structure analysis. As shown in Table IV, kumamolysin preferred substrates having a charged amino acid residue, Glu or Arg, at the P_2' position, which suggested that the S_2' subsite of kumamolysin mainly consists of hydrophilic residues. PCP (*45*), XCP (*45*), and J-4 (*34*) show the same tendency as kumamolysin. Thus, these results indicated that the hydrophilic interactions between the S_2' subsites of such pepstatin-insensitive carboxyl proteinases as PCP, XCP, J-4, and kumamolysin, and the P_2' position of substrates are most important. The S_2' subsites of pepstatin-insensitive carboxyl proteinases are very different from those of aspartic proteinases reported so far. It was strongly suggested that this feature is a common characteristic of bacterial pepstatin-insensitive carboxyl proteinases.

(6) The S_3' Subsite—Kumamolysin cleaved effectively a peptide with a large hydrophobic residue at the P_3' position, especially an aromatic amino acid. To the best of our knowledge, kumamolysin is the first enzyme that prefers such an aromatic amino acid residue as Tyr or Phe at the P_3' position. Moreover, the specificity constants, k_{cat}/K_m values, for large hydrophobic residues, Leu and Nle were 66 and 45% that for the Tyr derivative, respectively. Thus, kumamolysin favored bulky and hydrophobic residues, suggesting that the S_3' subsite of kumamolysin may be large on the whole. J-4 (*34*) favored the substrate with Leu at this position, but, on the whole, derivatives as to this position did not lead to significant differences. PCP (*45*) preferred substrates having Ser or Asp residue, and secondly Leu at this position. XCP (*45*) preferred substrates having Asp or Leu at this position. Thus, PCP and XCP preferred small hydrophilic residues or large hydrophobic residues at the P_3' position, and did not prefer substrates having an aromatic amino acid. We can say that these four bacterial carboxyl proteinases have different specificities as to the P_3' position.

Based on these results, the following features of kumamolysin and other members of this family were elucidated:

1) Kumamolysin preferentially hydrolyzed the second series of substrates rather than the first one. The reason for this seems to be a difference in affinity between the S_2 subsite of kumamolysin and the P_2 position of the substrates.

2) Kumamolysin preferred peptides having an Ala or Pro residue at the P_2 position, similar to J-4 (*34*). Aspartic proteinases reported so far (*e.g.*, pepsin, cathepsin D, cathepsin E, and yeast proteinase A) can not cleave the P_2 Pro derivative at all, but kumamolysin, J-4 (*34*), XCP (*45*), and PCP (*45*) could cleave this peptide, as shown in Fig. 1. Significantly, the P_2 Pro derivative was the best substrate among the P_2 derivatives. In the specificity for position P_2 of a substrate, kumamolysin and J-4 (*34*) were notably different from other carboxyl proteinases including PCP (*45*) and XCP (*45*). The unique preference as to the P_2 position is one of the reasons why kumamolysin and J-4 are insensitive to tyrostatin (*37*), which has a Leu residue at the P_2 position.

3) Kumamolysin preferred such charged amino acid residues as Glu or Arg at the P_2' position, like PCP (*45*), XCP (*45*), and J-4 (*34*) did. Thus, the hydrophilic nature of the S_2' subsite was confirmed to be a distinguishing feature of pepstatin-insensitive carboxyl proteinases from prokaryotes.

4) The presumed subsite structure of kumamolysin is illustrated in Fig. 2, compared with those of other members of this family.

Accordingly, we can conclude that the substrate specificities of PCP (*39*, *45*), XCP (*39*, *45*), J-4 (34), and kumamolysin are clearly different from each other. *Bacillus* novosp. MN-32 (*35*) grows at high temperature (60–70°C) and must have adapted to such a severe environment. Therefore, kumamolysin might have such unique substrate specificity in spite of its high identity to other pepstatin-insensitive carboxyl proteinases.

The results of these kinetic analyses and the information on the structure–function relationship of kumamolysin would be useful for elucidating the catalytic mechanisms of this family. Moreover, comparison of the pepstatin-insensitive carboxyl proteinase family with the aspartic proteinase (pepstatin-sensitive carboxyl proteinase) family may facilitate further understanding of the evolution of carboxyl proteinases, and also contribute to the understanding of the pepstatin-insensitive lysosomal enzyme, CLN2, related to lysosomal storage disease.

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