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Thermolysin: Kinetic Study with Oligopeptides

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Thermolysin is a well-known protease which exhibits its specificity against hydrophobic amino acid residues such as L-leucine, L-phenylalanine, *etc.* whose amino groups donate the susceptible peptide bonds (amino-endopeptidase). The present study was undertaken to investigate the effects of neighboring residues surrounding the sensitive amino acid residues at the amino-side in peptide substrates. For the purpose, a kinetic study was made using various synthetic oligopeptides such as Z-A-$(Gly)_n$-Leu-Ala or Z-Gly-Leu-$(Gly)_n$-B (A or B = various D- or L-amino acid residues; $n = 0$, 1 and/or 2; the arrow shows the bond split) as substrates. Other kinetic or inhibition studies were also made. These studies indicated that the specificity is affected by at least three amino acid residues on the N-terminal side and by two amino acid residues on C-terminal side from the sensitive amino acid residue (at amino-side) in peptide substrates. The effect of each of the five neighboring amino acid residues for appearance of the specifiicity was similar with that of the corresponding one which had been observed in a neutral protease of *Bacillus subtilis*, but that was not completely the same.

Thermolysin is a thermo-stable protease produced by *Bacillus thermoproteolyticus*. It was discovered by Endo [1], and its enzymatic specificity was first studied by Matsubara *et al.* [2], using beef cytochrome *c* as substrate, who assumed that the enzyme is specific for peptide bonds containing the amino group of hydrophobic amino acid residues such as L-leucine, L-isoleucine, L-phenylalanine, *etc.* This assumption was supported by a further study by the same authors using insulin [3], tobacco mosaic virus protein [3], and spinach ferredoxin [4], and by an independent study by us [5] using oxidized insulin B chain as substrate. This suggested that the enzyme is useful for amino acid sequence determination of peptides or proteins as well as trypsin, chymotrypsin, *etc.* Indeed, the significance of the enzyme has later been realized in sequence determinations of *Pseudomonas fluorescens* azurin [6], maleyl peptide T9 (around residue 102) in chymotrypsin [7], and cyanogen bromide fragment F_{III} in carboxypeptidase A [8].

Specificity against synthetic peptides also was studied by Ohta and Ogura [9], Matsubara [10], and Morihara *et al.* [5,11]. A systematic study by us [12] using Z-Gly-X-NH_2 (X = various amino acid residues of D- or L-configuration; the arrow shows the bond split) as substrates indicated that the highest activity was obtained when X was L-leucine, or L-phenylalanine, well reflecting the specificity against large moleular peptides or proteins. These characteristics of specificity have since been found to be general [12,13] for those proteases from microorganisms, called neutral proteases, which are optimally active at neutral pH and are inhibited by EDTA but unaffected by DFP. These enzymes may also be called as amino-endopeptidases, as proposed by Millet and Acher [14].

The specificities of various neutral proteases from bacterial origin, including thermolysin, were shown [15] to be affected by at least the next five residues adjacent to the sensitive amino acid residue in peptide substrates. A further study of *B. subtilis* neutral protease [16,17] with various synthetic peptides somewhat clarified the contribution to specificity of each of these five amino acid residues in peptide substrates. The present kinetic study of thermolysin with synthetic peptides was undertaken to compare the influences of neighboring residues on the specificity against the amino acid residue donating the amino group to be hydrolyzed with those found with the *B. subtilis* enzyme.

Unusual Abbreviations. DFP, diisopropyl phosphofluoridate; Z, benzyloxycarbonyl.

EXPERIMENTAL PROCEDURE

Materials

Thermolysin (1 × crystallized) was kindly supplied by Dr. S. Endo of Daiwa Kasai Co. (Osaka), which was used in this study without further purification.

Various synthetic peptides such as Z-Gly-X-NH_2 (X = L-serine, L-alanine, L-valine, L-isoleucine, L-leucine, D-leucine, L-phenylalanine, L-tyrosine, and L-tryptophan), Z-Ala-Leu, Z-Pro-Leu-NH_2, Z-Gly-Pro-Leu-Gly, and Z-Gly-Pro-Leu-Gly-Pro were obtained from the Peptide Center at the Institute for

Protein Research of Osaka University. Peptides such as Z-Gly-Gly-NH_2, Z-Tyr-Gly-NH_2, and Z-Tyr-Ser-NH_2, were obtained from the Cyclo Chemical Corporation, California. The other peptides used were synthesized according to the method described in the previous papers [16,27]; they are Z-Gly-Leu-B (B = amide, glycine, D-alanine, L-alanine, L-leucine, and L-phenylalanine), Z-Gly-Leu-Gly-B (B = glycine, D-alanine, L-alanine, and L-phenylalanine), Z-A-Leu-Ala (A = D-alanine, L-alanine, and L-phenylalanine), Z-A-Gly-Leu-Ala (A = glycine, D-alanine, L-alanine, and L-phenylalanine), Z-A-Gly-Gly-Leu-Ala (A = D-alanine, L-alanine, and L-phenylalanine), Z-Phe-X-Ala (X = glycine, L-serine, L-alanine, L-valine, L-isoleucine, D-leucine, L-leucine, L-phenylalanine, and L-tyrosine), Phe-Leu-Ala, Phe-Gly-Leu-Ala, Phe-Gly-Gly-Leu-Ala, and Z-Ala-D-Leu-Ala.

Except when specified, the constituent amino acids were all of the L-configuration. Abbreviated designations of amino acid derivatives, peptides, or the derivatives obey the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature.

Methods

The hydrolysis of various synthetic peptides was determined as follows: A reaction mixture (1 or 5 ml) containing 0.1 M Tris-buffer of pH 7.0 (except where specified), 2.5 mM $CaCl_2$, an appropriate concentration of peptide and a suitable amount of enzyme was incubated at 40°. At various intervals, 0.1 ml (or 0.5 ml) of the reaction mixture was withdrawn and put into a test tube which contained 1 ml (or 2 ml) of a mixture of 0.5 M citrate buffer (pH 5) and 0.01 M EDTA solution to stop further hydrolysis. The extent of hydrolysis was measured by the ninhydrin method of Yemm and Cocking [18]. The color yields by the ninhydrin method of the compounds, based on L-leucine as 100%, were described in the previous papers [16,17]. The sites of action of the enzyme were determined by paper chromatography of the hydrolyzates, or by the usual dinitrophenyl method.

In all cases, satisfactory Michaelis-Menten kinetics were observed, and plots of 1/[S] *vs.* $1/v$ (Lineweaver-Burk plots) permitted the fitting of definite straight lines. For each determination of K_m and V_{max} derived from such plots, initial rates were measured from five (or more) values of the initial substrate concentration [S]. Depending upon the rate of enzymatic cleavage, the enzyme concentration was suitably adjusted; it was assumed that the molecular weight was 37500 [19]. This enzyme concentration was used to calculate k_{cat} from V_{max} values. The inhibition constant (K_i) was determined from plots of $1/v$ *vs.* [I] for two substrate concentrations. The value on the abscissa that corresponds to the point where these two lines intersect is equal to $-K_i$.

RESULTS

pH-Activity Profile

The optimum pH of thermolysin was determined using Z-Gly-$\underset{\uparrow}{}$Leu-Ala as substrate. The result is shown in Fig. 1, which indicates that the enzyme was most active at or near to pH 7. Similar results were obtained when Z-Gly-$\underset{\uparrow}{}$Leu-NH_2, Z-Ala-$\underset{\uparrow}{}$Leu-Ala and Z-Gly-Pro-$\underset{\uparrow}{}$Leu-Gly-Pro were used as substrates. The optimum pH was between 7 and 9, when casein was used as substrate [1]. The effects of pH on the kinetic parameters with Z-Gly-Leu-Ala were determined further (one example of a Lineweaver-Burk plot, measured at pH 7, is shown in Fig. 2). The K_m values at various pH values are shown in the inset of Fig. 1, they indicate that the change of proteolytic coefficients in the pH range from 5 to 9 mainly concern catalysis (k_{cat}) and only little the binding (K_m).

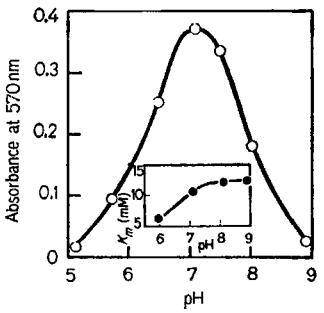

Fig. 1. ***Effect of pH for hydrolysis of Z-Gly-Leu-Ala by thermolysin.*** **The reaction mixture (1 ml) contained 0.1 M acetate buffer (pH 5—6), or 0.1 M Tris-buffer (pH 6.5—9) at various pH values, 2.5 mM $CaCl_2$, 10 mM Z-Gly-Leu-Ala, and an appropriate amount of enzyme (0.4 μg/ml). The reactions were performed at 40°, and aliquots (0.1 ml) were removed for ninhydrin analysis after 10 min reaction times. The inset shows K_m values determined as a function of pH**

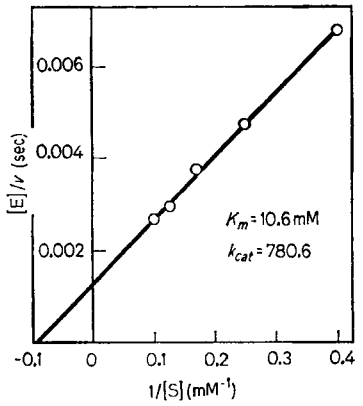

Fig. 2. ***Lineweaver-Burk plot on Z-Gly-Leu-Ala.*** **The reaction mixture (1 ml) contained 0.1 M Tris-buffer of pH 7.0, 2.5 mM $CaCl_2$, and 0.4 μg enzyme. The concentration of Z-Gly-Leu-Ala varied from 2.5 to 10 mM. The reactions were performed at 40°, and aliquots (0.1 ml) were removed at 3-min intervals for ninhydrin analysis. Initial velocities were taken at 10—20% hydrolysis**

Table 1. *Kinetics of Z-Gly-Leu-(Gly)$_n$-B and Z-A-(Gly)$_n$-Leu-Ala hydrolysis*
Groups I, II, III, IV and V shows the peptide series Z-Gly-Leu-B, Z-Gly-Leu-Gly-B, Z-A-Leu-Ala, Z-A-Gly-Leu-Ala and Z-A-Gly-Gly-Leu-Ala (A or B = various amino acid residues), respectively. The positions of amino acid residues (P) in each peptide are numbered as P_1, P_2, *etc.*, respectively, for those toward the NH_2-end from the cleavage site which is shown by the arrow, and as P_1', P_2', *etc.*, respectively, for those toward the COOH-end. The kinetic study was carried out according to the method described in Experimental Procedure, and one example is shown in Fig. 2. The initial substrate concentrations used were in the ranges of approx. 25 mM

Group	Peptides P_4---P_3---P_2---P_1-↓-P_1'---P_2'---P_3'	K_m	k_{cat}	k_{cat}/K_m
		mM	sec^{-1}	$sec^{-1} \times mM^{-1}$
I	Z---Gly---Leu--NH_2	20.6	105.0	5.1
	Z---Gly---Leu---Gly	10.8	65.4	6.1
	Z---Gly---Leu---Ala	10.6	780.6	78.1
	Z---Gly---Leu--D-Ala	16.6	5.2	0.3
	Z---Gly---Leu---Leu	2.6	374.0	143.9
	Z---Gly---Leu---Phe	2.4	120.0	50.0
II	Z---Gly---Leu--Gly--Gly	11.8	62.4	5.3
	Z---Gly---Leu--Gly--Ala	13.6	418.9	30.8
	Z---Gly---Leu--Gly--D-Ala	13.4	25.0	1.9
	Z---Gly---Leu--Gly--Phe	11.0	131.6	12.0
III	Z---Ala---Leu--Ala	4.9	996.9	203.4
	Z--D-Ala--Leu--Ala		Negligibly small	
	Z---Phe---Leu--Ala	0.58	398.3	678.0
	H---Phe---Leu--Ala		Negligibly small	
IV	Z---Gly--Gly---Leu--Ala	12.2	362.0	29.7
	Z---Ala--Gly---Leu--Ala	8.6	5208.0	605.6
	Z--D-Ala-Gly---Leu--Ala	16.0	625.0	39.0
	Z---Phe--Gly---Leu--Ala	0.91	446.4	490.5
	H---Phe--Gly---Leu--Ala	4.6	53.0[a]	11.5
V	Z---Ala--Gly--Gly---Leu--Ala	7.5	50.0	6.6
	Z--D-Ala-Gly--Gly---Leu--Ala	7.1	127.5	18.0
	Z---Phe--Gly--Gly---Leu--Ala	3.0	21.9	7.3
	H---Phe--Gly--Gly---Leu--Ala	4.8	150.6[b]	50.2

[a] A rough comparison of the color yields of the released peptides in the digest, using a densitometer, revealed that Phe-Gly-Leu-Ala : Phe-Gly : Leu-Ala is about 0.85:1.35:1.0. Therefore, the calculation was made by the factor of Leu-Ala × [1.0 + (1.35 — 0.85)].

[b] A rough comparison of the color yields of the released peptides in the digest, using a densitometer revealed that Phe-Gly-Gly-Leu-Ala : Phe-Gly-Gly : Leu-Ala is about 0.9:1.5:1.0. Therefore, the calculation was made by the factor of Leu-Ala × [1.0 + (1.5 — 0.9)].

KINETIC STUDY

Z-A-(Gly)$_n$-Leu-Ala and Z-Gly-Leu-(Gly)$_n$-B

To investigate the effects of the amino acid residues adjacent to the sensitive L-leucine residue in peptide substrates, a kinetic study was undertaken using various synthetic peptides such as Z-A-(Gly)$_n$-Leu-Ala and Z-Gly-Leu-(Gly)$_n$-B (A or B = various amino acid residues of D- or L-configuration; n = 0, 1 and/or 2). These peptides were all split at the peptide bond containing the amino group of L-leucine, as expected [12]. The results are summarized in Table 1, where the positions of amino acid residues (P) in each peptide are numbered as P_1, P_2, *etc.*, respectively, for those toward the NH_2-end from the cleavage site, and as P_1', P_2', *etc.*, respectively, for those toward the COOH-end.

The table indicates that the effect of the kind of A or B decreases with its distance from the catalytic point and that k_{cat}/K_m is very dependent on the kind of amino acid residue A or B in these peptides as follows: L-leucine, L-alanine, L-phenylalanine > glycine, amide > D-alanine at P_2'; L-alanine > L-phenylalanine > glycine > D-alanine at P_3'; L-phenylalanine > L-alanine > glycine ⋙ D-alanine at P_1; L-phenylalanine, L-alanine > D-alanine, glycine at P_2; D-alanine > L-phenylalanine, L-alanine at P_3. From the kinetic study, it may be deduced that the amino acid residue at P_1 relates largely to binding (K_m) but little to catalysis (k_{cat}), while those at the other positions, P_2, P_3, P_2' and P_3', relate to binding and/or catalysis depending upon the kind of amino acid residue present. Interestingly, D-alanine is more effective than L-alanine at P_3, mainly relating to catalysis.

The presence of an α-amino group at P_1 or P_2 markedly inhibits the hydrolysis. The small susceptibility of Phe-Gly-↑-Leu-Ala as compared with the corresponding Z-peptide is related to both the decrease of k_{cat} value and the increase of K_m value.

Table 2. *Kinetics of Z-Gly-X-NH_2, Z-Tyr-X-NH_2 and Z-Phe-X-Ala*

Groups I, II and III show the peptide groups of Z-Gly-X-NH_2, Z-Tyr-X-NH_2 and Z-Phe-X-Ala (X = various amino acid residues), respectively. Because of the low solubility of some of these peptides, their kinetic parameters were determined at much lower concentrations than their K_m values as seen in the table. The reaction mixture contained 1.7% ethanol and the other conditions were described in Fig. 2

Group	Peptides P_2---P_1-↓-P_1'---P_2'	Substrate concentrations (approx.)	K_m	k_{cat}	k_{cat}/K_m
		mM	mM	sec^{-1}	$sec^{-1} \times mM^{-1}$
I	Z---Gly---Gly--NH_2	36		Negligibly small	
	Z---Gly---Ser--NH_2	36	600	1.6	0.003
	Z---Gly---Ala--NH_2	20	600	44.5	0.074
	Z---Gly---Val--NH_2	6	71.5	22.6	0.32
	Z---Gly---Ile---NH_2	7.5	33.3	59.3	1.8
	Z---Gly---Leu--NH_2	8.0	33.3	89.0	2.7
	Z---Gly--D-Leu-NH_2	8.0		Negligibly small	
	Z---Gly---Phe--NH_2	6	14.7	38.5	2.6
	Z---Gly---Tyr--NH_2	18	14.7	1.6	0.11
	Z---Gly---Trp--NH_2	10		Negligibly small	
II	Z---Tyr---Gly--NH_2	7.5	20.8	0.125	0.006
	Z---Tyr---Ser--NH_2	2.5	2.3	0.36	0.16
III	Z---Phe---Gly--Ala	2.5	7.4	7.8	1.05
	Z---Phe---Ser--Ala	2.5	1.6	3.0	1.9
	Z---Phe---Ala--Ala	2.5	2.6	21.9	8.4
	Z---Phe---Val--Ala	2.5	0.78	169.0	216.7
	Z---Phe---Ile---Ala	1.5	0.82	148.0	205.5
	Z---Phe---Leu--Ala	2.5	0.72	416.0	577.8
	Z---Phe--D-Leu-Ala	2.5		Negligibly small	
	Z---Phe---Phe--Ala	0.8	0.45	162.0	360.0
	Z---Phe---Tyr--Ala	2.5	0.72	7.1	9.9

In contrast, the presence of an α-amino group at P_3 little affects the hydrolysis, and rather promotes it in comparison with the corresponding Z-peptides, mainly relating to catalysis. As seen in the correlation among the three peptides Z-Gly$\underset{\uparrow}{-}$Leu-NH_2, Z-Gly$\underset{\uparrow}{-}$Leu-Ala and Z-Phe$\underset{\uparrow}{-}$Leu-Ala, the hydrolysis of a peptide bond containing the amino group of L-leucine (corresponding to P_1') is doubly increased by double replacement with suitable amino acid residues at both P_1 and P_2'.

Z-Gly-X-NH_2, Z-Tyr-X-NH_2 and Z-Phe-X-Ala

To clarify the specificity against P_1' in more detail, the following experiment was undertaken using Z-Gly-X-NH_2, Z-Tyr-X-NH_2 and Z-Phe-X-Ala (X = various amino acid residues) as substrates. Because of the low solubility of some of the peptides, their kinetic parameters were determined at much lower concentrations than their K_m values (see Table 2). The table indicates that the proteolytic coefficient (k_{cat}/K_m) is markedly affected by the amino acid residue (X) at P_1', as follows; L-leucine, L-isoleucine, L-phenylalanine, L-valine > L-alanine, L-tyrosine > L-serine, glycine ≫ D-alanine for both Z-Gly-X-NH_2 (group I) and Z-Phe-X-Ala (group III). The difference, *i.e.* specificity, relates with both binding (K_m) and catalysis (k_{cat}).

The table further indicates that Z-Tyr-X-NH_2 (group II) is much more sensitive than Z-Gly-X-NH_2 when X is glycine or L-serine. Comparison of the peptides Z-Tyr-X-NH_2 and Z-Phe-X-Ala (X = glycine or L-serine) indicates that they have similar K_m values but that their k_{cat} values differ by a factor of 8—60. When Z-Gly-X-NH_2 and Z-Phe-X-Ala are compared, where X is the same residue, increased hydrolysis is observed, related to both the decrease of the K_m value and the increase of k_{cat}. This would mean that substitution of L-tyrosine or L-phenylalanine in place of glycine at P_1 reduces the K_m value, and substitution of L-alanine in place of amide at P_2' causes an increase of k_{cat}, independent of the nature of the amino acid residue at P_1'.

Z-Gly-Pro-Leu-Gly and Z-Gly-Pro-Leu-Gly-Pro

The previous paper [12] indicated that the hydrolysis of Z-Pro-Leu-NH_2 was negligible. A comparative study was made using the three peptides Z-Pro-Leu-NH_2, Z-Gly-Pro-Leu-Gly and Z-Gly-Pro-Leu-Gly-Pro, and the results are summarized in Table 3, where the result of Z-Gly-Leu-NH_2 is presented for comparison. It was ascertained that cleavage of the former three peptides occurred at the Pro-Leu bond. As mentioned above (Table 1), glycine and amide at P_2' made almost the same contribution to hydrolysis.

Table 3. *Effects of neighboring residues on the hydrolysis of a peptide bond Pro-Leu*
The reaction mixture (1 ml) containing 0.1 M Tris-buffer (pH 7), 1 mM substrate, 2.5 mM $CaCl_2$, 10% dimethylformamide, and 0.01 mg enzyme was kept at room temperature. After 7 and 20 h of incubation, the percentage of hydrolysis was determined

Peptides	Hydrolysis after	
P_3---P_2---P_1-↓-P_1'---P_2'---P_3'	7 h	20 h
	%	%
Z---Gly--Leu--NH_2	54	100
Z---Pro--Leu--NH_2	2	3
Z---Gly--Pro--Leu--Gly	17	41
Z---Gly--Pro--Leu--Gly---Pro	36	72

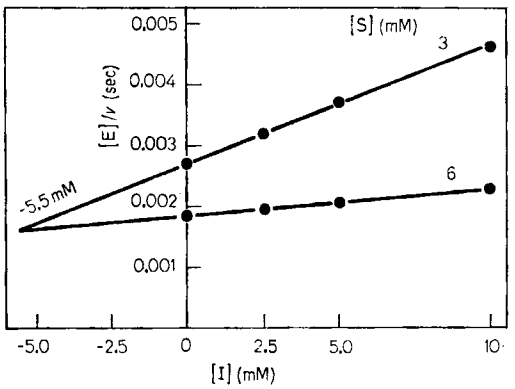

Fig. 3. *Dixon-plot of Z-Ala-Leu.* As substrate, Z-Ala-Leu-Ala was used, the concentrations of which were adjusted at 3 and 6 mM as seen in the figure. The inhibitor concentration (Z-Ala-Leu) was as seen in the figure, and the enzyme concentration was 0.2 μg/ml reaction mixture. The other conditions and the method of determination were as described in Fig. 2

Thus, the differences in the activities of these peptides must be ascribed to the elongation of the peptide chain towards the NH_2- or COOH-end. The kinetic parameters determined under the conditions described in Table 1 indicated that the K_m and k_{cat} (sec^{-1}) of Z-Gly-Pro-Leu-Gly were 12.8 mM and 11.2, respectively, and those of Z-Gly-Pro-Leu-Gly-Pro were 13.3 mM and 41.6, respectively.

INHIBITION STUDY

As shown in the previous paper [12], a Z-dipeptide such as Z-Gly-Leu, Z-Ala-Leu, *etc.* is not susceptible to the enzyme unless the α-carboxyl group of the peptide is blocked with an amide or amino acid residue. Therefore an inhibition study was undertaken using Z-Ala-Leu, as seen in Fig. 3. The peptide shows competitive inhibition, with a K_i value similar to the K_m value of corresponding Z-tripeptide such as Z-Ala-Leu-Ala (refer to Table 1). Similar inhibition studies were also made, using Z-Ala-D-Leu-Ala, Z-D-Ala-Leu-Ala and Z-Ala as

Table 4. *Inhibition constants, K_i of various peptides*
All experiments were performed with Z-Ala-Leu-Ala (3 and 6 mM) as substrate. The inhibitor concentrations are described in the table, and the other conditions and methods are described in Fig. 3

Inhibitor	Concentration of I	Type of inhibition	K_i
	mM		mM
Z---Ala---Leu	0, 2.5, 5, 10	Competitive	5.5
Z---Ala--D-Leu--Ala	0, 20, 40, 80	Competitive	32
Z--D-Ala--Leu---Ala	0, 20, 40, 80	Competitive	60
Z---Ala	0, 10, 20, 30	Inhibition[a]	30

[a] Not clear whether competitive or not.

inhibitors, for the hydrolysis of Z-Ala-Leu-Ala. The results, summarized in Table 4, would indicate that both amino acid residues at P_1 and P_1' are essentially significant in the binding of peptide substrates, blocking of the amino group of P_1 being requisite for binding though the carboxyl group of P_1' is not necessarily related to it.

As seen in Table 1, the hydrolysis of an α-amino free tripeptide such as Phe-Leu-Ala was negligible. The inhibition study, however, was difficult because of the high ninhydrin color. An experiment was therefore undertaken with a reaction mixture containing 10 mM inhibitor (Phe-Leu-Ala) and 2 mM substrate (Z-Phe-Leu-Ala), other conditions being exactly the same as those described in Table 4. No inhibition occurred.

DISCUSSION

The present study somewhat clarified the effects of several neighboring amino acid residues surrounding the susceptible peptide bond on the appearance of the specificity in thermolysin (Table 1). The specificity against P_1' was also studied using either Z-Gly-X-NH_2 or Z-Phe-X-Ala as substrate (Table 2). These results are summarized in Table 5, where results with a neutral protease from *B. subtilis* are presented for comparison. They indicate that the enzymes are very similar, not only in their specificity against P_1' but also with regard to the effects of amino acid residues neighboring the site of cleavage in peptide substrates on their specificity. Therefore, the following discussion will be limited to the differences between the enzymes.

Some difference is observed in the side chain specificity at P_1': The specificity of thermolysin against aromatic amino acid residues such as L-phenylalanine, L-tyrosine, *etc.* is higher than that of the *B. subtilis* enzyme while the specificity of the former enzyme against aliphatic amino acid residues such as L-leucine, L-alanine, *etc.* seems to be lower than that of the latter enzyme. The former case is well reflected in the hydrolysis of oxidized insulin B chain; Phe(25)-

Table 5. *The effect of substrate structure on proteolytic coefficient of thermolysin*
The numbers are the ratios of the proteolytic coefficients (k_{cat}/K_m) for the pairs of substrates given, which were described in Table 1 and 2: For example, the numbers in L/D (Ala) at P_2 were calculated from the proteolytic coefficients (k_{cat}/K_m) of Z-Ala-Gly-Leu-Ala and Z-D-Ala-Gly-Leu-Ala, those of Ala/Gly at P_2 were made from the coefficients of Z-Ala-Gly-Leu-Ala and Z-Gly-Gly-Leu-Ala, those of masked by Z/α-NH_2 at P_2 were made from the coefficients of Z-Phe-Gly-Leu-Ala and Phe-Gly-Leu-Ala, and those of masked by Gly/α-COOH at P_1' were made from the coefficients of Z-Gly-Leu-Gly and Z-Gly-Leu. As to P_1', the numbers were calculated from the result of Z-Phe-X-Ala in Table 2. For comparison, the results of *B. subtilis* neutral protease [16, 17] are presented in the table, where T and S show thermolysin and *B. subtilis* neutral protease, respectively

Replacement	Position of amino acid residue in peptide substrate											
	P_3		P_2		P_1		P_1'		P_2'		P_3'	
	T	S	T	S	T	S	T	S	T	S	T	S
L/D Ala	0.5	2.5	16	16	$>10^4$ [a]	$>10^4$			260	4104	16	26
Leu							$>10^4$ [a]	$>10^4$				
Ala/Gly			20	14	3	3	8	42	13	38	6	6
Leu/Gly							550	1040	24	35		
Phe/Gly			17	101	7	32	343	174	8	41	2	3
Tyr/Gly							9	<1				
Masked by Z/α-NH_2	0.1	1.1	43	38	$>10^3$ [a]	$>10^3$						
Masked by Gly/α-COOH							$>10^3$ [a]	$>10^3$	0.9	2.2		1.2

[a] Unpublished data.

Tyr(26) was split by thermolysin but this was not observed with the *B. subtilis* enzyme, as shown in the previous paper [5]. Nevertheless, the effect of L-phenylalanine at each of the neighboring positions P_1, P_2, and P_2' in peptide substrates on the specificity of thermolysin seems to be lower than that in the *B. subtilis* enzyme.

A striking difference between these enzymes is observed as regards the effect of the kind of amino acid residue at P_3. At P_3 in thermolysin, D-alanine promotes the hydrolysis more markedly than does L-alanine, and the presence of a free α-amino group at this position does not interfere with the hydrolysis but rather promotes it compared with blocking of the group by Z. This was not observed with the *B. subtilis* enzyme. The proteolytic coefficients of thermolysin against various peptide substrates such as Z-Gly-Leu-NH_2, Z-Phe-Leu-Ala, *etc.* are not so different from those of the *B. subtilis* enzyme but the K_m values of the former enzyme, are in general, smaller than those of the latter [16, 17]; for example, the K_m values of Z-Phe-Leu-Ala are 0.7, and 3.7 mM, for each enzyme, respectively.

The present study using oligopeptides indicated that the specificity against P_1' is enhanced or inhibited by the five amino acid residues surrounding the sensitive residue at P_1', depending upon the kind of amino acid residues occupying the five positions. Nevertheless, the points of cleavage in large molecular peptides or proteins well reflect [2—8] the specificity against the amino acid residue at P_1', as deduced from the study using Z-Gly-X-NH_2 or Z-Phe-X-Ala as substrates (Table 2). This may indicate that the effects of neighboring residues on the specificity against P_1' are cancelled in the hydrolysis of large molecular peptides or proteins.

The above conflict could be interpreted as follows: A peptide sequence of ---Gly-Phe-Ser-Ala-Gly--- situated at an internal position of a long peptide chain, must at first be split by thermolysin at the peptide bond of Gly-Phe because of the specificity against the amino acid residue at P_1'. The released peptide fragment Phe-Ser-Ala-Gly--- is not split at the peptide bond of Phe-Ser because of not only the small specificity against the residue at P_1' but also the inhibitory effect of α-amino group at P_1. That is, the peptide bond Phe-Ser in the above peptide sequence could scarcely be split even though suitable amino acid residues surrounded the L-serine residue. While the hydrolysis of Z-Phe-Ser-Ala is promoted about 1000-fold compared with that of Z-Gly-Ser-NH_2, being almost the same degree with that of Z-Gly-Leu-NH_2 (refer Table 2); and the hydrolysis of either the bond of Z-Phe or Ser-Ala in the peptide (Z-Phe-Ser-Ala) does not occur at all. When glycine residue in the above peptide sequence (—Gly-Phe-Ser-Ala-Gly—) is replaced with L-proline, the hydrolysis of Pro-Phe is disturbed, but the promoting effect of L-phenylalanine at P_1 for hydrolysis of the peptide bond of Phe-Ser is at the same time diminished. So, the hydrolysis might also scarcely be observed.

The amino acid composition of thermolysin [19] is remarkably different from that [20] of a neutral

protease from *B. subtilis*. The former enzyme is thermostable [1] but the latter one is not [21]. Recently, thermolysin has been shown [22] to be a zinc enzyme, as was also found for *B. subtilis* enzyme [21,23]. This and the present study would indicate that the structure of active site is similar in both enzymes, though the whole structure is remarkably different.

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