ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 165, 72-79 (1974)

Comparative Study of Various Serine Alkaline Proteinases from Microorganisms

Esterase Activity Against *N*-Acylated Peptide Ester Substrates

KAZUYUKI MORIHARA, TATSUSHI OKA, AND HIROSHIGE TSUZUKI

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, 553 Japan

Received March 13, 1974

Hydrolyses of *N*-acylated peptide ester substrates by various serine alkaline proteinases from bacterial and mold origin were compared using Ac- or Z-$(Ala)_m$-X-OMe (m = 0–2 or 0–3; X = phenylalanine, alanine, and lysine) as esterase substrates. The results indicated that the esterase activities of these enzymes were markedly promoted by elongating the peptide chain from P_1 to P_2 or P_3 with alanine, irrespective of the kind of the amino acid residue at the P_1-position (amino acid residues in peptide substrates are numbered according to the system of Schechter and Berger (1)). The effect of the kind of amino acid residue at the P_2-position was further determined using Z-X-Lys-OMe (X = glycine, alanine, leucine, or phenylalanine) as esterase substrates. Alanine was the most efficient residue as X with subtilisins and *Streptomyces fradiae* Ib enzyme, while leucine or phenylalanine were most efficient with the enzymes from *Streptomyces fradiae* II, *Aspergillus sojae*, and *Aspergillus melleus*. All the serine alkaline proteinases tested in this study were sensitive to Z-Ala-Gly-PheCH_2Cl, the dependence of inhibition on the inhibitor concentration differed among the enzymes.

In a previous paper (2), we showed that the amidase activity of subtilisin BPN′, a serine alkaline proteinase from *Bacillus amyloliquefaciens*, is markedly increased by elongating the peptide chain on the N-terminal side from the point of cleavage in *N*-acylated peptide amide substrates. We observed a similar situation with the other subtilisins (3) and with a serine alkaline proteinase (Ib fraction) from *Streptomyces fradiae* (4). With other serine alkaline proteinases from *Streptomyces* and *Aspergillus* genus, however, it was difficult to determine the effect of elongation of the peptide chain on N-terminal side, because these enzymes are inert to peptide amide substrates (4).

More recently, Gertler *et al.* (5–7) have shown that Ac-$(Ala)_3$-OMe is an efficient esterase substrate for subtilisin BPN′ and serine alkaline proteinases from *Streptomyces griseus* (Pronase) and *Aspergillus sojae*, as seen with pancreatic elastase (8). The result suggested that the three amino acid residues (P_1–P_3)[1] on the N-terminal side from the splitting point in peptide substrates are important for enzymatic action of serine alkaline proteinases from microorganisms, independent of the species of origin, a conclusion which could not be arrived at from our study using *N*-acylated peptide amide substrates.

Our present study was undertaken to confirm the above conclusion. For this purpose we used Ac- or Z-$(Ala)_m$-X-OMe (m = 0–2 or 0–3; X = phenylalanine, alanine, and lysine) as esterase substrates for various serine alkaline proteinases from bacterial and mold origin, including those which were inert on *N*-acylated peptide amide substrates. Accordingly, by varying

[1] The amino acid residues in a peptide substrate are designated by the numbering of Schechter and Berger (1); those on the N-terminal side of the splitting point are called P_1, P_2, etc., and those on the C-terminal side P_1', P_2', etc.

the number m in the substrate we could determine the effect of the length of the peptide chain on the N-terminal side of the splitting point on the hydrolysis; while by changing the nature of X we could investigate the effect of the kind of the amino acid residue at the P_1 position in promoting the hydrolysis. Further study, to clarify the specificities of these enzymes at the P_2 position, was done using Z-X-Lys-OMe (X = glycine, alanine, leucine, and phenylalanine) as esterase substrates.

MATERIALS AND METHODS

Enzymes. Subtilisins Carlsberg (crystals) and BPN′ (crystals) were supplied by Novo Industri A/S, Copenhagen, and Nagase & Co., Osaka, respectively. Two serine alkaline proteinases from *Streptomyces fradiae* ATCC 3535, known as fractions Ib and II (crystals), were prepared by the method described in previous papers (9, 10). Highly purified serine alkaline proteinase from *Aspergillus sojae* was kindly donated by Dr. K. Hayashi of Kikkoman Institute, Chiba. The semialkaline proteinase of *Aspergillus melleus* (twice recrystallized) was obtained from Seikagaku Kogyo, Tokyo, and was further purified by column chromatography on DEAE-Sephadex and Sephadex G-75 in the usual way. All these enzymes were sensitive to iPr_2P-F[2] and were most active at alkaline pH range, belonging to the serine alkaline proteinase group (10). The homogeneity of these enzymes was checked by discelectrophoresis at pH 7.5.

Substrates and inhibitors. The ester substrates Ac-$(Ala)_m$-OMe (m = 3 and 4), Z-$(Ala)_m$-Lys-OMe (m = 0–2), and Z-X-Lys-OMe (X = glycine, D- and L-alanine, L-leucine, and L-phenylalanine) were synthesized according to the method described previously (11, 12). The synthesis of Z-Ala-Phe-OMe was also described in the literature (13). The other ester substrates were synthesized as described below.[3] The chloromethyl ketone derivatives, Z-PheCH_2Cl, Z-Ala-PheCH_2Cl, and Z-Ala-Gly-PheCH_2Cl, were synthesized according to the method described in a previous paper (14).

Ac-Ala-OMe. To the pyridine solution (30 ml) of Ala-OMe-HCl (46.7 mM), acetic anhydride (63.5 mM) was added dropwise at room temperature. After 1-hr agitation, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in ethylacetate (200 ml), which was then neutralized by powdered $NaHCO_3$ in presence of water saturated with NaCl, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. An oily syrup was obtained as the product. Yield, 77%.

Ac-Ala-Ala-OMe. Ala-Ala-OMe-HCl (4.98 mM), prepared according to the method described previously (18), was dissolved in pyridine (10 ml), which was then treated with acetic anhydride (10.6 mM) as mentioned above. The product was crystallized from ethylacetate–petroleum ether. Yield, 91.4%; mp 148.5–149.5°C.

Ac-Phe-OMe. Phe-OMe-HCl (10.2 mM) was treated with acetic anhydride (21.2 mM) as mentioned above, with pyridine (20 ml) as the solvent. The product was crystallized from ethylacetate–petroleum ether. Yield, 93.6%; mp 89–90°C.

Ac-Ala-Phe-OMe. Ala-Phe-OMe-HCl, prepared by catalytic hydrogenolysis of the corresponding Z-derivative (10.2 mM), was treated with acetic anhydride (21.2 mM), with pyridine (20 ml) as the solvent. The product was crystallized from ether–petroleum ether (1:1, by volume). Yield, 86.5%; mp 136–137°C.

Z-Ala-Ala-Phe-OMe. Z-Ala-ONP (14.5 mM) and Ala-Phe-OMe, prepared by neutralization of the hydrochloride (14.4 mM) in cold, were coupled, with CH_2Cl_2 (70 ml) and chloroform (70 ml) as the solvents. The product was crystallized from ether. Yield, 86.2%; mp 193.5–194°C.

Ac-Ala-Ala-Phe-OMe. Ala-Ala-Phe-OMe-HCl, prepared by catalytic hydrogenolysis of the corresponding Z-derivative (6.11 mM), was treated with acetic anhydride (10.6 mM), with pyridine (10 ml) as the solvent. The product was crystallized from ethanol–petroleum ether. Yield, 94.7%; mp 208–210°C.

Determination of enzymatic activity. Esterase activity was determined using a Radiometer type TTT1 pH-stat equipped with a syringe buret, a type SBR2c recorder, and a thermostatically controlled reaction vessel (30°C). Reactions were carried out in 0.1 M KCl at pH 7.5, with 0.05 N NaOH as titrant. In all cases, the data were in accordance with Michaelis–Menten kinetics over the range of substrate concentration (~10 mM) employed; five to eight runs were performed for each determination of K_m and k_{cat} (V per molar equivalent of enzyme); the enzyme concentration was chosen to give reliable data for the initial rate of hydrolysis. In the calculation of k_{cat}, the molecular weights of subtilisin BPN′ (or Carlsberg), *St. fradiae*

[2] Abbreviations: iPr_2P-F, diisopropyl phosphofluoridate; Z-PheCH_2Cl, benzyloxycarbonyl L-phenylalanine chloromethyl ketone; Z-Ala-PheCH_2Cl, benzyloxycarbonyl L-alanyl-L-phenylalanine chloromethyl ketone; Z-Ala-Gly-PheCH_2Cl, benzyloxycarbonyl L-alanyl-glycyl-L-phenylalanine chloromethyl ketone. Abbreviated designations of amino acid derivatives, peptides, or the derivatives obey the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature. Except where specified, the constituent amino acids were all of the L-configuration.

[3] The data on elemental analysis were presented for editorial review.

Ib and II, *Asp. sojae*, and *Asp. melleus* enzymes were taken as 27,600 (15), 20,000 (9), 17,700 (9), 25,500 (16), and 30,300 (17), respectively.

Inactivation study. Inactivation by various chloromethyl ketone derivatives was carried out as follows: The reaction mixture (5 ml) containing 0.05 M Tris buffer (pH 7.0), 10^{-3} or 10^{-4} M inhibitor, 5×10^{-6} M enzyme, and 10% dioxane was kept at 40°C. At various intervals, the remaining activity was determined using Ac-Tyr-OEt as substrate. The esterase activity was followed in a pH-stat, at pH 8.0 and 30°C. Since the inactivation reactions were all pseudo-first order the k (sec^{-1}) values of the reagents were calculated for the respective enzymes, as described in the previous paper (14).

RESULTS

Hydrolysis of Ac- and Z-(Ala)$_m$-X-OMe

Data on the hydrolysis of Ac-(Ala)$_m$-OMe (m = 1–4) by various serine alkaline proteinases from microbial origin are shown in Table I, the results with pancreatic elastase being presented for comparison. The esterase activities of all the microbial enzymes tested were markedly increased by elongating the peptide chain with alanine, e.g., with the increase of the number of m in the ester substrates. Similar promotion of hydrolysis is observed with pancreatic elastase; this has previously been found by Thompson and Blout (19), Gertler and Hofmann (8), and by us (20).

The increase of esterase activity on elongation of the peptide chain from P_1 to P_2 (e.g., Ac-Ala-OMe and Ac-(Ala)$_2$-OMe) is very marked (40- to 200-fold increase) with the microbial enzymes, mainly due to increased k_{cat} values, except with the *St. fradiae* Ib enzyme. The increase of activity by elongation of the peptide chain from P_2 to P_3 (Ac-(Ala)$_2$-OMe and Ac-(Ala)$_3$-OMe) is less marked (5- to 10-fold increase), being mainly related to a decrease in K_m values. With the *St. fradiae* II enzyme and

TABLE I

HYDROLYSIS OF Ac-(Ala)$_m$-OMe BY SERINE ALKALINE PROTEINASES FROM MICROORGANISMS[a]

Substrate	Serine alkaline proteinase from											
	Subtilisin BPN′			Subtilisin Carlsberg			*St. fradiae* Ib			*St. fradiae* II		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)
↓												
Ac-Ala-OMe	36.3	9.1	0.25	60	210	3.5	83.3	83.3	1.0			0
Ac-(Ala)$_2$-OMe	5.8	305	52.5	13	2300	177	2.3	340	148	91	60	0.66
Ac-(Ala)$_3$-OMe	0.45	255	566	0.83	1046	1260	0.42	267	636	12.5	60	4.8
Ac-(Ala)$_4$-OMe	0.18	175	995	0.28	885	3053	0.4	280	700	1.8	72	40
	Asp. sojae			*Asp. melleus*			Panc. elastase[b]			Panc. elastase[c]		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)
↓												
Ac-Ala-OMe	96.8	28.9	0.3	194	28.3	0.15	76	8.1	0.11	170	8.2	0.049
Ac-(Ala)$_2$-OMe	44.5	1150	25.7	68.5	2000	29.3	9.2	4.4	0.48	22	49	2.2
Ac-(Ala)$_3$-OMe	32.1	3720	116	6.7	1540	230	0.28	17.3	62	0.4	120	300
Ac-(Ala)$_4$-OMe	7.0	2090	300	4.7	1970	419	0.056	17.3	310	0.067	120	1800

[a] The esterase activity was determined in the presence of 0.1 M KCl at pH 7.5 and 30°C; [S] = ~10 mM.

[b] Data from Morihara and Oka (20). The kinetic parameters of Ac-(Ala)$_4$-OMe were determined under the conditions described in Methods using crystalline pancreatic elastase obtained from Mann Research Laboratory, New York.

[c] Data from Thompson and Blout (19).

pancreatic elastase, further increase of hydrolysis was observed on elongating the peptide chain from P_3 to P_4 (Ac-$(Ala)_3$-OMe and Ac-$(Ala)_4$-OMe), mainly related to the K_m values; whereas this was not necessarily observed with the other microbial enzymes.

Data for the hydrolyses of Ac-$(Ala)_m$-Phe-OMe (m = 0–2) by the microbial enzymes are shown in Table II, the results with α-chymotrypsin being added for comparison. Here, also, the esterase activities of the enzymes were markedly increased by elongating the peptide chain with alanine from P_1 to P_2 or P_3 (Ac-Phe-OMe and Ac-Ala-Phe-OMe or Ac-$(Ala)_2$-Phe-OMe); and 80- to 500-fold increase in hydrolysis on elongation from P_1 to P_3, related mainly to binding (K_m) with the bacterial and *Streptomyces* enzymes, and to catalysis (k_{cat}) with the mold enzymes. The increase in hydrolysis on elongation of the peptide chain is less with α-chymotrypsin than with the microbial enzymes.

Data on the hydrolyses of Z-$(Ala)_m$-Lys-OMe (m = 0–2) are shown in Table III, together with results with trypsin. Elongation of the peptide chain from P_1 to P_2 or P_3 (Z-Lys-OMe and Z-Ala-Lys-OMe or Z-$(Ala)_2$-Lys-OMe) with alanine caused a 300- to 1000-fold increase in hydrolysis by the microbial enzymes, relating both to K_m and k_{cat} values, but had little effect on trypsin hydrolysis.

Hydrolysis of Z-X-Lys-OMe

The esterase activities of the microbial enzymes against Z-X-Lys-OMe (X = various amino acid residues) are shown in Table IV, the results with trypsin being added for comparison. The effect on hydrolysis of the kind of amino acid residue X at P_2-position differs considerably depending upon the species of the enzyme producer, in the following decreasing orders: alanine > leucine > phenylalanine, glycine with subtilisins BPN′ and Carlsberg; leucine ≥ alanine > phenylalanine, glycine with *St. fradiae* Ib enzyme; leucine > alanine, phenylalanine glycine with *St. fradiae* II enzyme; and leucine, phenylalanine > alanine > glycine with *Asper-*

TABLE II

HYDROLYSIS OF Ac-$(Ala)_m$-Phe-OMe BY SERINE ALKALINE PROTEINASES FROM MICROORGANISMS[a]

Substrate	Serine alkaline proteinase from											
	Subtilisin BPN′			Subtilisin Carlsberg			*St. fradiae* Ib			*St. fradiae* II		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)
↓												
Ac-Phe-OMe	28	100	3.7	11.4	540	47.4	23	51	2.2	20	120	6
Ac-Ala-Phe-OMe	1.1	254	224	0.87	1643	1888	2.2	303	138	3.3	231	70
Ac-$(Ala)_2$-Phe-OMe	0.32	297	941	0.31	1179	3800	0.37	247	667	0.32	261	816

Substrate	*Asp. sojae*			*Asp. melleus*			α-chymotrypsin[b]		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)
↓									
Ac-Phe-OMe	23.0	78.3	3.4	22.4	53.7	2.4	0.57	31.4	55
Ac-Ala-Phe-OMe	13.5	799	59.2	10.7	1080	101	0.05	16.4	330
Ac-$(Ala)_2$-Phe-OMe	6.7	2620	391	2.1	2170	1033	0.03	60	2000

[a] The methods are described in Table I.

[b] Data from Morihara and Oka (20).

TABLE III

HYDROLYSIS OF Z-$(Ala)_m$-Lys-OMe BY SERINE ALKALINE PROTEINASES FROM MICROORGANISMS[a]

Substrate	Serine alkaline proteinase from											
	Subtilisin BPN'			Subtilisin Carlsberg			*St. fradiae* Ib			*St. fradiae* II		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)
↓												
Z-Lys-OMe	20	47	2.3	12	102	8.5	7.6	18.5	2.4			0
Z-Ala-Lys-OMe	1.4	414	296	0.8	1000	1250	0.55	303	551	0.5	75	150
Z-$(Ala)_2$-Lys-OMe	0.16	454	2790	0.16	1100	6875	0.1	290	2900	0.18	75	417

Substrate	*Asp. sojae*			*Asp. melleus*			Trypsin[b]		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1} \cdot sec^{-1}$)
↓									
Z-Lys-OMe	31.1	175	5.6	17.8	148	8.3	0.23	101	440
Z-Ala-Lys-OMe	13.3	1730	130	6	1485	248	0.039	68	1750
Z-$(Ala)_2$-Lys-OMe	1.45	2800	1940	0.7	1840	2630	0.079	106	1350

[a] The methods are described in Table I.

[b] Data from Morihara and Oka (20).

gillus enzymes. However, stringent stereospecificity at this position is seen with all the microbial enzymes.

With the most efficient residue, alanine, at P_2, hydrolysis by subtilisins and *St. fradiae* Ib enzyme is 7- to 9-fold that with glycine at the position; whereas the most efficient leucine and phenylalanine residues promote hydrolysis by *St. fradiae* II and *Aspergillus* enzymes to over 100 times the value with glycine at P_2. These promotions mainly relate to changes in K_m values. The effect of the kind of amino acid residue X at the position is much less with trypsin than with the microbial enzymes; whereas considerable stereospecificity at the position is seen with the former enzyme.

Effect of Peptide Chloromethyl Ketone Derivatives

In previous papers (3, 4, 14), we showed that peptide chloromethyl ketone derivatives such as Z-Ala-Gly-PheCH_2Cl markedly inactivate various serine alkaline proteinases from microbial origin, but not the *St. fradiae* II and *Asp. melleus* enzymes, which are used in this study. Nevertheless, as with other serine alkaline proteinases, the esterase activities of both the latter enzymes are markedly increased on elongating the peptide chain on the N-terminal side from the splitting point in peptide ester substrates, as mentioned above. We therefore reinvestigated the sensitivity of the two enzymes to peptide chloromethyl ketone derivatives.

We found this time that, although the *St. fradiae* II and *Asp. melleus* enzymes were only slightly inhibited by Z-Ala-Gly-PheCH_2Cl at a concentration of 10^{-4} M, other conditions being as described in Methods; both enzymes were sensitive when the inhibitor concentration was 10^{-3} M, the former enzyme then being inactivated almost completely within 60 min and the latter enzyme within 20 min. The inactivation reactions were pseudo-first-order, and the k values (sec^{-1}) of Z-Ala-Gly-PheCH_2Cl ([I] = 10^{-3} M) were calculated as 8.5×10^{-4} with the *St. fradiae* II enzyme and 2.12×10^{-3} with the *Asp. melleus* enzyme.

Inactivation of either enzymes by Z-PheCH$_2$Cl was negligible, even with 10^{-3} M of the inhibitor. An experiment with Z-Ala-PheCH$_2$Cl, could not be done, however, because of the low solubility in the reaction mixture containing 10% of dioxane.

Inactivation of *Asp. sojae* enzyme by Z-PheCH$_2$Cl, Z-Ala-PheCH$_2$Cl, and Z-Ala-Gly-PheCH$_2$Cl was determined at a concentration of 10^{-4} M, other conditions being as described in Methods; the k values (sec^{-1}) of the reagents were 1, 7, and 90 $\times$ 10^{-5}, respectively. Inactivation increased with an increase in length of the peptide chain of the inhibitor, as seen with various serine alkaline proteinases in previous studies (3, 4, 14).

DISCUSSION

The present study shows that the esterase activities of all the serine alkaline proteinases from bacterial and mold origin tested are markedly increased by elongating the peptide chain of peptide ester substrates from P_1 to P_2 or P_3 with alanine, irrespective of the kind of amino acid residue at the P_1 position. A similar promotion of hydrolysis by elongation of the substrate peptide chain is seen with pancreatic elastase, though such promotion of hydrolysis by α-chymotrypsin is small in comparison, and with trypsin it is negligible.

An X-ray study of subtilisin BPN' inactivated with peptide chloromethyl ketone derivatives indicates (21) that the CO of Ser-125 and the NH and CO of Gly-127 in the backbone chain of the enzyme molecule are implicated in hydrogen bonding in an antiparallel β-pleated arrangement with the tripeptide chloromethyl ketones. A similar situation may obtain with the other serine alkaline proteinases from microbial origin. Therefore, the increased susceptibility of the *N*-acylated tripeptide ester substrates of these enzymes might be

TABLE IV

HYDROLYSIS OF Z-X-Lys-OMe BY SERINE ALKALINE PROTEINASES FROM MICROORGANISMS[a]

Substrate	Serine alkaline proteinase from											
	Subtilisin BPN'			Subtilisin Carlsberg			*St. fradiae* Ib			*St. fradiae* II		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})
↓												
Z-Gly-Lys-OMe	10	414	41	6.6	1046	159	4.2	267	64	5.5	21.1	3.8
Z-Ala-Lys-OMe	1.4	414	296	0.8	1000	1250	0.55	303	551	0.5	75	150
Z-D-Ala-Lys-OMe	33	8.8	0.3	25	11.5	0.5	9.5	4.5	0.5			0.0
Z-Leu-Lys-OMe	1.2	230	190	0.8	658	823	0.28	200	714	0.04	30	750
Z-Phe-Lys-OMe	1.0	45.5	46	1.1	177	161	0.5	54	108	0.4	40	100

Substrate	*Asp. sojae*			*Asp. melleus*			Trypsin[b]		
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1}. sec^{-1})
↓									
Z-Gly-Lys-OMe	66	1287	20	40	1010	25	0.11	84	760
Z-Ala-Lys-OMe	13.3	1730	130	7.4	1845	248	0.039	68	1750
Z-D-Ala-Lys-OMe	50	22.5	0.5	40	32	0.8	0.14	2.7	19
Z-Leu-Lys-OMe	0.7	2142	3060	0.38	1683	4430	0.016	22.4	1400
Z-Phe-Lys-OMe	0.5	530	1060	0.5	3160	6320	0.054	46	850

[a] The methods are described in Table I.

[b] Data from the literature (12).

due to their ability to form three hydrogen bonds, as has been assumed with subtilisin BPN'.

The degree of promotion of sensitivity to microbial enzymes by elongation of the peptide chain in peptide ester substrates seems to be higher when the P_1 position is occupied by alanine (Ac-$(Ala)_m$-Ala-OMe) or lysine (Z-$(Ala)_m$-Lys-OMe) than when it is occupied by phenylalanine (Ac-$(Ala)_m$-Phe-OMe). An exception to this is the hydrolysis of Z-$(Ala)_m$-Lys-OMe by the *Aspergillus* enzymes, a situation which may be ascribed to the comparatively high esterase activity of the mold enzymes against Z-Lys-OMe.

It can be said, therefore, that the effect of elongation on hydrolysis by microbial enzymes is more marked the less specific the amino acid residue occupying the P_1 position in a peptide ester substrate. This leads to the suggestion that the effect of elongation is greater or lesser depending on whether the side-chain of the residue at P_1 is fixed loosely or tightly into the corresponding specific site (hydrophobic pocket, if present) in the enzyme molecule. If this is in fact so, it would explain why serine alkaline proteinases from microbial origin show broad specificity against large molecular peptide or protein substrates. A similar consideration has been made (20) on the difference in promotion of hydrolysis on elongation of the peptide chain in peptide ester substrates among the four serine proteinases, trypsin, α-chymotrypsin, pancreatic elastase, and subtilisin BPN'.

There are considerable differences among the microbial enzymes in the effect of the kind of the amino acid residue at the P_2 position, as seen in the hydrolysis of Z-X-Lys-OMe. With subtilisin BPN', it has been shown (2) that the most efficient amino acid residue at the P_2 position is alanine when Z-X-Leu-NH_2 is used as amidase substrates. An identical result is obtained in the present study using Z-X-Lys-OMe as esterase substrates. This may indicate that leucine and lysine at P_1 position in the respective peptide series occupy the same hydrophobic pocket in the enzyme molecule. The X-ray study with subtilisin BPN' (21) indicates that there is not enough space to accomodate a residue larger than alanine at the subsite (S_2) corresponding to the P_2 position of the peptide substrate. The space of subsite S_2 in the *St. fradiae* Ib enzyme may be similar to that of subtilisin BPN'.

With the enzymes from *St. fradiae* II, *Asp. sojae* and *Asp. melleus*, the most efficient amino acid residue at the P_2 position is leucine or phenylalanine, as seen with α-chymotrypsin (22, 23). An X-ray study of γ-chymotrypsin inactivated by chloromethyl ketone derivatives indicates (24) that a bulky, nonpolar residue at P_2 (e.g., leucine or valine) should interact with Ile-99 in subsite S_2 by van der Waals contact. A similar situation may exist with the *Streptomyces* and *Aspergillus* enzymes, a point which will be resolved by future X-ray study.

In our previous paper (4), we showed that peptide chloromethyl ketone derivatives such as Z-Ala-Gly-PheCH_2Cl are not always potent inhibitors against serine alkaline proteinases from microbial origin; serine proteinases from *St. fradiae* II and *Asp. melleus* were inactivated only slightly by tripeptide chloromethyl ketone. The present study, however, shows that both these enzymes become considerably sensitive to Z-Ala-Gly-PheCH_2Cl when the inhibitor concentration is raised to 10^{-3} M in the reaction mixture. It seems, therefore, that peptide chloromethyl ketone derivatives are efficient inhibitors against most serine alkaline proteinases from microbial origin, just as their corresponding peptide esters or amides are readily susceptible to hydrolysis by the enzymes.

ACKNOWLEDGMENTS

We are greatly indebted to Novo Industri A/S, Copenhagen, and to Dr. K. Hayashi of Kikkoman Institute, Chiba, for their supply of subtilisin Carlsberg (crystals) and highly purified preparation of *Asp. sojae* alkaline proteinase, respectively.

REFERENCES

1. SCHECHTER, I., AND BERGER, A. (1967) *Biochem. Biophys. Res. Commun.* **27,** 157.
2. MORIHARA, K., OKA, T., AND TSUZUKI, H. (1970) *Arch. Biochem. Biophys.* **138,** 515.
3. MORIHARA, K., TSUZUKI, H., AND OKA, T. (1971) *Biochem. Biophys. Res. Commun.* **42,** 1000.

4. MORIHARA, K., OKA, T., AND TSUZUKI, H. (1971) *Arch. Biochem. Biophys.* **146,** 297.
5. GERTLER, A., AND HAYASHI, K. (1971) *Biochim. Biophys. Acta* **235,** 378.
6. GERTLER, A., AND TROP, M. (1971) *Eur. J. Biochem.* **19,** 90.
7. GERTLER, A. (1971) *Eur. J. Biochem.* **23,** 36.
8. GERTLER, A., AND HOFMANN, T. (1970) *Can. J. Biochem.* **48,** 384.
9. MORIHARA, K., OKA, T., AND TSUZUKI, H. (1967) *Biochim. Biophys. Acta* **139,** 382.
10. MORIHARA, K., AND TSUZUKI, H. (1969) *Arch. Biochem. Biophys.* **129,** 620.
11. THOMPSON, R. C., AND BLOUT, E. R. (1973) *Biochemistry* **12,** 51.
12. MORIHARA, K., AND OKA, T. (1973) *Arch. Biochem. Biophys.* **156,** 764.
13. SCHRÖDER, E. (1964) *Ann. Chem.* **679,** 207.
14. MORIHARA, K., AND OKA, T. (1970) *Arch. Biochem. Biophys.* **138,** 526.
15. MATSUBARA, H., KASPER, C. B., BROWN, D. M., AND SMITH, E. L. (1965) *J. Biol. Chem.* **240,** 1125.
16. HAYASHI, K., FUKUSHIMA, D., AND MOGI, K. (1967) *Agr. Biol. Chem. (Tokyo)* **31,** 642, 1171, and 1237.
17. ITO, M., AND SUGIURA, M. (1968) *Yakugaku Zasshi* **88,** 1576, 1583, and 1591.
18. MORIHARA, K., OKA, T., AND TSUZUKI, H. (1969) *Arch. Biochem. Biophys.* **135,** 311.
19. THOMPSON, R. C., AND BLOUT, E. R. (1970) *Proc. Nat. Acad. Sci. USA* **67,** 1734.
20. MORIHARA, K., AND OKA, T. (1973) *Fed. Eur. Biochem. Soc. Lett.* **33,** 54.
21. ROBERTUS, J. D., ALDEN, R. A., BIRKTOFT, J. J., KRAUT, J., POWERS, J. C., AND WILCOX, P. E. (1972) *Biochemistry* **11,** 2439.
22. YAMASHITA, T., AND IZUMIYA, N. (1959) *J. Biochem. (Tokyo)* **46,** 991.
23. YAMASHITA, T. (1960) *J. Biochem. (Tokyo)* **48,** 846.
24. SEGAL, D. M., POWERS, J. C., COHEN, G. H., DAVIES, D. R., AND WILCOX, P. E. (1971) *Biochemistry* **10,** 3728.