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ON THE SPECIFICITY OF *PSEUDOMONAS AERUGINOSA* ALKALINE PROTEINASE WITH SYNTHETIC PEPTIDES

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SUMMARY

The specificity of *Pseudomonas aeruginosa* alkaline proteinase was studied using various synthetic peptides as substrates. Since it was found that the smallest peptide which is sensitive to the enzyme is a Z-tripeptide, in which an internal peptide bond is split, the primary specificity against the amino acid residue at either side of the splitting point was determined using various Z-tripeptides as substrates. The results indicated that the enzyme shows specificity against L-alanine at the imino side and L-phenylalanine at the carbonyl side of the splitting point, though its specificity is not as stringent as that seen with usual metal neutral proteinases.

An experiment using L-alanine oligomers (from tetramer to hexamer) as substrates showed that the proteolytic activity increases drastically with increase in chain length, suggesting that the hydrolysis is markedly affected by amino acid residues more distant from the catalytic point in a peptide substrate ("secondary interaction"). The effect of secondary interaction was studied using various Z-tetra- or Z-pentapeptide substrates, which indicated that the specificity against peptides larger than Z-tripeptides was determined by secondary interaction, rather than by primary specificity, the presence of a hydrophobic or bulky residue being required at positions distant, rather than adjacent to the splitting point.

INTRODUCTION

Pseudomonas aeruginosa produces two proteinases which are separable by column chromatography on DEAE-cellulose[1]. Both the enzymes have been crystallized and their physicochemical and enzymatic properties clarified[2–6]. One of the enzymes is most active in a neutral pH range, and as it is active on elastin it can be

* Abbreviations used are: $iP_2{}_2P$-F, diisopropyl phosphofluoridate; *p*Cl-HgBzO$^-$, *p*-chloromercuribenzoic acid; Tos-PheCH_2Cl, tosyl-L-phenylalanine chloromethyl ketone; Tos-LysCH_2Cl, tosyl-L-lysine chloromethyl ketone. Ac-, acetyl; -OMe, methoxy-; Bz, benzoyl-; -OEt, ethoxy-; Z-, benzyloxycarbonyl-; -OBz, benzyloxy-; -OBu^t, *tert.*-butoxy- and -ONp, *p*-nitrophenoxy-. Abbreviated designations of amino acid derivatives, peptides or their derivatives conform to the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature (BBA 263 (1972) 205–212). Except when specified, the constituent amino acids were all of the L-configuration.

called an "elastase" (ref. 6). Its molecular weight and isoelectric point are 39 500, and pH 5.9, respectively. The other enzyme is most active at slightly alkaline pH values, and might therefore be called an "alkaline proteinase" (ref. 2). This enzyme does not hydrolyze elastin[7], being clearly different from the "elastase". Its molecular weight and isoelectric point are 48 400 and pH 4.0, respectively.

Both the enzymes are inactivated by various metal chelators but not by iP_2P-F*, *p*Cl-HgBzO$^-$, Tos-PheCH_2Cl, Tos-LysCH_2Cl, soybean trypsin inhibitor and potato inhibitor[2,4,6]. The elastase is a typical metal chelator-sensitive neutral proteinase[8,9] which shows specificity against bulky or hydrophobic amino acid residues such as L-leucine, L-phenylalanine, *etc.* at the imino side of the splitting point. The enzyme might therefore be termed a "neutral proteinase" instead of elastase. The metal content of the elastase has not been determined, but like the other neutral proteinases from bacterial origin, such as that from *Bacillus subtilis*[10], thermolysin[11], *etc.*, the enzyme may be a Zn^{2+} enzyme.

The alkaline proteinase does not contain a significant amount of any metal ion except Ca^{2+} (1–2 gatoms of Ca^{2+} per mole of enzyme)[4]. Its specificity determined using oxidized insulin B chain as substrate, is remarkably different[8] from that of the elastase produced by the same species, *i.e.* its specificity does not resemble that of usual neutral proteinases. Since no regularity in specificity could be deduced from the points of cleavage in the B chain, the present study on the specificity was further undertaken.

Recently, it has become clear that the specificity of some proteinases is determined not only by the amino acid residues(s) nearest to the splitting point, but also by amino acid residues more distant from the catalytic point in a peptide substrate. The former specificity may be called "primary specificity", and the latter effect "secondary interaction", as has been proposed for pepsin by Fruton[12]. With respect to the *P. aeruginosa* alkaline proteinase, not only the primary specificity but also the effect of secondary interaction on its hydrolysis were studied using various synthetic peptides as substrates.

MATERIALS AND METHODS

Enzyme

The crystalline proteinase of *P. aeruginosa* IFO 3080 (twice recrystallized) was prepared according to the method described previously[2]. The preparation was found to be homogeneous in disc electrophoresis at pH 8.

Substrates

Ac-Leu-OMe, Ac-Phe-OMe, Ac-Tyr-OMe, Bz-Arg-OEt, Z-Leu-NH_2, Z-Tyr-NH_2, Bz-Arg-NH_2, Z-Gly–Leu, Z-Gly–Phe, Z-Phe–Leu, Z-Tyr–Leu, Leu–Gly–Gly, Gly–Pro, Z-Gly–Pro–Leu–Gly–Pro, and Z-Gly-X-NH_2 (X = L-alanine, L-valine, L-leucine, D-leucine and L-tyrosine) were supplied from the Peptide Center at the Institute for Protein Research at Osaka University. Ac-Lys-OMe, Z-Ala–Gly, Z-Ala–Ala, Z-Ala–Leu, Z-Leu–Leu, Z-Gly–Gly-NH_2, Z-Ala–Leu-NH_2, Z-Tyr–Gly-NH_2, Z-Tyr–Ser-NH_2, $Z\text{-}Gly_3$, $Z\text{-}Gly_4$, $Z\text{-}Gly_5$, Ala_3, Ala_4, Ala_5 and Ala_6 were obtained from the Cyclo Chemical Corporation, Los Angeles. Z-Gly–Pro–Gly–Gly–Pro–Ala was supplied by Mann Research Laboratory, New York.

Z-Gly–Leu-X (X = glycine, D- and L-alanine, L-leucine, and L-phenylalanine), Z-X-Leu–Ala (X = D- and L-alanine, and L-phenylalanine), Z-Phe-X-Ala (X = glycine, L-alanine, L-serine, L-valine, D-leucine, L-isoleucine, L-phenylalanine, and L-throsine), Z-X-Gly–Leu–Ala (X = glycine, D- and L-alanine, and L-phenylalanine), Z-X-Phe–Leu–Ala (X = glycine, and L-alanine), Z-Gly–Leu–Gly-X (X = glycine, and L-alanine), Z-Gly–Leu–Gly–Gly-X (X = D- and L-alanine), Z–Ala–Gly–Gly–Leu–Ala, Z–Phe–Leu–Ala–Ala, and Z-Gly–Gly–Leu-NH_2 were synthesized according to the method described previously[7,8,13–15]. Ac-Ala-OMe was synthesized in this laboratory by the usual method. Ala–Leu-NH_2, Gly–Leu–Ala, and Phe–Gly–Leu–Ala were prepared by catalytic hydrogenolysis (in the presence of palladium black) of the corresponding Z-peptides in the usual manner. The other peptides were synthesized as described below.

Hydrolysis of synthetic substrates

Esterase activity was determined with the aid of a Radiometer type TTT1 pH-stat equipped with a syringe burette, a type SBR2c recorder, and a thermostatically controlled reaction vessel (30 °C). The reaction was carried out in 0.1 M KCl at pH 7.5, in which 0.05 M NaOH was used as titrant.

Either amidase or peptidase activity was determined as follows: a reaction mixture (1 ml) containing 0.05 M Tris buffer of pH 7.0, an appropriate concentration of peptide, and a suitable amount of enzyme was incubated at 40 °C. At 3-min intervals, 0.1 ml of the reaction mixture was withdrawn and put into a test tube containing 1 ml of a mixture of 0.5 M citrate buffer (pH 5) and 0.01 M EDTA solution to prevent further hydrolysis. The extent of hydrolysis was measured by the ninhydrin method of Yemm and Cocking[16]. The sites of enzyme action upon substrates were determined by paper chromatography of the hydrolyzates in comparison with authentic compounds, by the usual 2,4-dinitrophenyl method, or by paper-electrophoresis in a current of 1200 V at pH 2 (0.6 M formic acid and 2 M acetic acid, 1:1, by vol.) for 50 min.

The color yields by the ninhydrin method of Gly–Gly, Gly–Ala, Gly–Leu, Gly–Pro, Ala–Ala, Ser–Ala, Val–Ala, Ile–Ala, Leu–Gly, Leu–Ala, Leu–Leu, Leu–Phe, Phe–Ala, Tyr–Ala, and Gly–Gly–Ala, based on L-leucine as 100%, have been shown in previous papers[13–15]. The ninhydrin color yields of Ala_3 and Gly–Leu–Ala, based on L-leucine as 100%, were found to be 84 and 67%, respectively. The rate of hydrolysis of Z-Gly–Pro–Gly–Gly–Pro–Ala was determined using Gly–Pro as the standard, as authentic Gly–Pro–Ala was not available.

Kinetic study

In all cases, satisfactory Michaelis–Menten kinetics were observed, and plots of $1/v$ *vs* $1/S$ (Lineweaver–Burk plots) permitted the fitting of definite straight lines. For each determination of K_m and V derived from such plots, initial rates were measured from 5 (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 48 000 (ref. 3). This enzyme concentration was used to calculate k_{cat} from V 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 $-K_i$.

Synthesis of peptides

Z-Ala-OBu^t. Z-Ala (112 mM) was mixed with isobutene (125 ml) in the cold in the presence of conc. H_2SO_4 (2 ml), with dichloromethane (400 ml) as the solvent. The reaction was carried out in the usual manner. After neutralization with 5% $NaHCO_3$, the reaction mixture was concentrated *in vacuo*. The residue was dissolved in ethylacetate, which was washed with 5% $NaHCO_3$ and distilled water. The solution was concentrated *in vacuo* to give a syrup. Yield, 93.6%. Analysis, calcd for $C_{15}H_{21}O_4N$ (279.4): C, 64.50; H, 7.58; N, 5.01. Found: C, 64.72; H, 7.73; N, 4.84.

Z-Ala-Ala-OBu^t. Z-Ala-ONp (148 mM) and Ala-OBu^t, prepared by catalytic hydrogenolysis (palladium black) of the corresponding Z-derivative (165 mM) and by neutralization with cold triethylamine, were coupled in the usual manner, with dichloromethane as the solvent. The product was crystallized from ether–petroleum ether. Yield, 81.6%; m.p. 69–70.5 °C. Analysis, calcd for $C_{18}H_{26}O_5N_2$ (350.4): C, 61.70; H, 7.48; N, 7.99. Found: C, 61.46; H, 7.25; N, 8.25.

Z-Gly-Ala-Ala-OBu^t. Z-Gly-ONp (5.80 mM) and Ala-Ala-OBu^t, prepared as above from the corresponding Z-peptide (6.00 mM), were coupled, with dichloromethane (20 ml) as the solvent. The product was crystallized from ether–petroleum ether. Yield, 57.5%; m.p. 90–92 °C. Analysis, calcd for $C_{20}H_{29}O_6N_3$ (407.5): C, 58.96; H, 7.17; N, 10.31. Found: C, 59.20; H, 7.35; N, 10.25.

Z-Gly-Ala-Ala. The above peptide (2.01 mM) was treated with 1 M HCl in acetic acid (12 ml). After concentration *in vacuo*, the product was crystallized from ethyl acetate. Yield, 83.9%; m.p. 170–171 °C. Analysis, calcd for $C_{16}H_{21}O_6N_3 \cdot 0.25$ H_2O (355.9): C, 54.00; H, 6.09; N, 11.81. Found: C, 54.09; H, 6.00; N, 11.73.

Z-Val-Ala-Ala-OBu^t. A mixed acid carboxylic anhydride was prepared in the usual manner (below −5 °C) from Z-Val (12.1 mM) and ethyl chloroformate (12.1 mM) in the presence of triethylamine (12.5 mM), with dichloromethane (30 ml) as the solvent. To the cooled solution, a mixed solution of dichloromethane (10 ml) and chloroform (10 ml) containing Ala-Ala-OBu^t (11.1 mM) prepared as above was added. The product was crystallized from ethylacetate–ethanol–petroleum ether. Yield, 73.6%; m.p. 150–152 °C. Analysis, calcd for $C_{23}H_{35}O_6N_3$ (449.6): C, 61.45; H, 7.85; N, 9.35. Found: C, 60.91; H, 7.77; N, 9.48.

Z-Val-Ala-Ala. The above peptide (2.01 mM) was treated with 1 M HCl in acetic acid (12 ml). The product was crystallized from ether. Yield, 81.2%; m.p. 196–198 °C. Analysis, calcd for $C_{19}H_{27}O_6N_3$ (393.4): C, 58.00; H, 6.92; N, 10.68. Found, C, 57.44; H, 7.01; N, 10.71.

Z-Ala-Ala-OBz. A mixed carboxylic acid anhydride was prepared in the usual manner from Z-Ala (10.0 mM) and ethyl chloroformate (11.0 mM) in the presence of triethylamine (11.4 mM), with dichloromethane (25 ml) as the solvent. To the cooled solution, a dichloromethane (15 ml) solution of Ala-OBz, prepared by neutralization of Ala-OBz-TosH (10.0 mM) with triethylamine, was added. The product was crystallized from ethyl acetate–ether–petroleum ether. Yield, 74.8%; m.p. 137–139 °C. Analysis, calcd for $C_{21}H_{24}O_5N_2$ (384.4): C, 65.61; H, 6.29; N, 7.29. Found: C, 65.38; H, 6.38; N, 7.10.

Z-Ala-Ala-Ala. A mixed carboxylic acid anhydride was prepared from Z-Ala

(6.04 mM) and ethyl chloroformate (6.20 mM), with dichloromethane (15 ml) as the solvent. To the cooled solution, a mixed solution of dichloromethane (15 ml), chloroform (10 ml) and dimethylformamide (10 ml) of Ala–Ala, prepared by catalytic hydrogenolysis of Z-Ala–Ala-OBz and by neutralization with triethylamine, was added. The product was crystallized from ether. Yield, 12.6%; m.p. 216–217 °C. Analysis, calcd for $C_{17}H_{23}O_6N_3$ (365.4): C, 55.88; H, 6.35; N, 11.50. Found: C, 55.74; H, 6.65; N, 11.27.

Z-Leu–Ala–Ala-OMe. The method was described in a previous paper[14].

Z-Leu–Ala–Ala. The above ester (2.78 mM) was saponified in the usual manner. The product was crystallized from ethanol–ether. Yield, 82.4%; m.p. 185–186.5 °C. Analysis, calcd for $C_{20}H_{29}O_6N_3$ (407.5): C, 58.96; H, 7.17; N, 10.31. Found: C, 58.83; H, 7.29; N, 9.91.

Z-Gly-Gly-OEt. Z-Gly-ONp (5.15 mM) and Gly-OEt, prepared by neutralization of the hydrochloride (5.14 mM) with cold triethylamine, were coupled in the usual manner, with dichloromethane (30 ml) as the solvent. The product was crystallized from ether. Yield, 68%; m.p. 81–82 °C. Analysis, calcd for $C_{14}H_{18}O_5N_2$ (294.3): C, 57.14; H, 6.16; N, 9.52. Found: C, 57.01; H, 6.15; N, 9.62.

Z-Gly-Gly-NHNH$_2$. A methanol solution (25 ml) containing Z-Gly–Gly-OEt (6.22 mM) and hydrazine-hydrate (52.4 mM) was stirred for about 5 h at room temperature. The product was crystallized from ethanol. Yield, 99.2%; m.p. 167–169 °C. Analysis, calcd for $C_{12}H_{16}O_4N_4$ (280.3): C, 51.42; H, 5.76; N, 19.99. Found: C, 51.19; H, 5.57; N, 20.17.

Z-Gly–Gly–Gly–Leu. Z-Gly–Gly-N_3 was prepared from the hydrazide (5.66 mM) in the usual manner, with $NaNO_2$ (5.65 mM) in 1.5 ml of distilled water, in the presence of 7 ml methyl cyanide and 8.5 ml of 2 M HCl below 5 °C. To the reaction mixture, a methyl cyanide solution (8 ml) of Gly–Leu (5.4 mM), prepared by catalytic hydrogenolysis of the corresponding Z-peptide, was added in the cold (below 5 °C), and the pH was maintained at about 8 with triethylamine. The reaction mixture was kept for one night in the cold. After concentration *in vacuo*, the residue was washed with 0.5 M HCl, water, and ethyl acetate. Yield, 81%; m.p. 197–199 °C. Analysis, calcd for $C_{20}H_{28}O_7N_4$ (436.5): C, 55.04; H, 6.47; N, 12.83. Found: C, 55.21; H, 6.46; N, 13.00.

Z-Gly–Leu-OBz. Z-Gly-ONp (9.50 mM) and Leu-OBz, prepared by neutralization of Leu-OBz-TosH (9.53 mM) with triethylamine, were coupled in the usual manner, with dichloromethane (80 ml) as the solvent. The product was purified by column chromatography on silica gel (100–200 mesh) using chloroform as the effluent. A syrup was obtained. Yield, 75.8%. Analysis, calcd for $C_{23}H_{28}O_5N_2 \cdot 0.5\ H_2O$ (421.5): C, 65.54; H, 6.93; N, 6.65. Found: C, 65.68; H, 6.82; N, 6.62.

Z-Gly–Gly–Leu. Z-Gly-ONp (7.08 mM) and Gly–Leu, prepared by catalytic hydrogenolysis of Z-Gly–Leu-OBz (7.13 mM), were coupled in the usual manner, with dimethylformamide (35 ml) as the solvent. The product was crystallized from methanol–ether–petroleum ether. Yield, 40.3%; m.p. 153–154 °C. Analysis, calcd for $C_{18}H_{25}O_6N_3$ (379.4): C, 56.98; H, 6.64; N, 11.08. Found: C, 57.14; H, 6.83; N, 11.00.

Z-Ala–Gly–Gly–Leu. Z-Ala-ONp (2.78 mM) and Gly–Gly–Leu, prepared by catalytic hydrogenolysis of the corresponding Z-peptide (2.76 mM), were coupled in the usual manner, with dimethylformamide (15 ml) as the solvent. The product was purified by column chromatography on silica gel using a chloroform solution con-

taining various concentrations of methanol as the effluent. The product was crystallized from methanol–ether. Yield, 41.8%; m.p. 129–131 °C. Analysis, calcd for $C_{21}H_{30}O_7N_4 \cdot 0.5\ H_2O$ (495.5): C, 54.89; H, 6.80; N, 12.19. Found: C, 54.51; H, 6.94; N, 12.38.

Z-Ala–Phe–Gly–Ala. Z-Ala-ONp (9.05 mM) and Phe–Gly–Ala, prepared by catalytic hydrogenolysis of the corresponding Z-peptide (8.89 mM), were coupled in the usual manner, with dimethylformamide (20 ml) and dichloromethane (40 ml) as the solvent. The product was cryatallized from methanol–ether. Yield, 45.7%; m.p. 189–190 °C. Analysis, calcd for $C_{25}H_{30}O_7N_4 \cdot 0.5\ H_2O$ (507.5): C, 59.16; H, 6.16; N, 11.04. Found: C, 59.07; H, 6.12; N, 10.83.

Z-Ala–Leu–Gly–Gly. Z-Ala-ONp (4.04 mM) and Leu–Gly–Gly (4.12 mM) were coupled in the usual manner in the presence of triethylamine (4.22 mM), with dimethylformamide (30 ml) and distilled water (3 ml) as the solvent. The product was crystallized from methanol–ether. Yield, 49.2%; m.p. 154–155.5 °C. Analysis, calcd for $C_{21}H_{30}O_7N_4$ (450.5): C, 55.99; H, 6.71; N, 12.66. Found: C, 55.95; H, 6.64; N, 12.28.

RESULTS

Some enzymatic characteristics

Effect of Co^{2+}. The effects of various metal ions ($2 \cdot 10^{-3}$ M of $CaCl_2$, $ZnCl_2$, $CoCl_2$, $NiCl_2$, and $MnCl_2$) on the enzymatic activity were determined using Z-Ala–Phe–Gly–Ala as substrate. The results indicated that Co^{2+} alone promoted the rate of hydrolysis, among the metal ions tested. The effect of the concentration of Co^{2+} on hydrolysis was examined, as shown in Table I. It indicates that a considerable concentration of Co^{2+} ($> 5 \cdot 10^{-3}$ M) is required for maximum promotion of hydrolysis, though the requisite concentration differs considerably depending upon whether preincubation

TABLE I

EFFECT OF CONCENTRATION OF Co^{2+} ON THE ENZYMATIC ACTIVITY OF *P. aeruginosa* ALKALINE PROTEINASE

The reaction mixture (1 ml) contained 10 mM peptide (Z-Ala–Phe–Gly–Ala), a suitable amount of enzyme (0.005–0.02 mg), 0.05 M Tris buffer (pH 7.0) and various concentrations of $CoCl_2$ as shown in the table. Both preincubated and non-preincubated enzyme preparations were used; preincubated enzyme was treated with $CoCl_2$ solution ($1 \cdot 10^{-2}$ M) for one night at about 4 °C. Initial velocities were determined at 40 °C.

$CoCl_2$ (M)	*Relative activity*	
	Without pre-incubation	*With pre-incubation*
0	1	1
$1 \cdot 10^{-5}$		1.2
$1 \cdot 10^{-4}$		1.6
$1 \cdot 10^{-3}$		4.7
$2 \cdot 10^{-3}$	2.2	6.3
$5 \cdot 10^{-3}$		8.8
$1 \cdot 10^{-2}$	4.3	8.4
$2 \cdot 10^{-2}$	5.2	9.5
$5 \cdot 10^{-2}$	7.1	

with Co^{2+} has taken place or not. At the optimum concentration, the rate of hydrolysis is increased several-fold.

The following experiment was therefore carried out in the reaction mixture containing $1 \cdot 10^{-2}$ M of $CoCl_2$, using the proteinase solution preincubated with Co^{2+} ($1 \cdot 10^{-2}$ M) at about 4 °C overnight.

Effect of pH. The previous paper[2] indicated that with casein as substrate the proteinase is most active at pH 8–10. It is well known that the optimum pH for protein substrates is not always the same as that for synthetic substrates. The effect of pH on the kinetic parameters of Z-Phe–Ala–Ala and Z-Ala–Phe–Gly–Ala was determined. The K_m, and k_{cat}/K_m values at various pH values are summarized in Fig. 1. The proteolytic coefficient (k_{cat}/K_m) is rather higher in the neutral pH range, than at alkaline pH, though both the K_m and k_{cat} values increase with increase in the pH value. Similar behavior was also observed with the other peptides, so the following kinetic study was made at pH 7.0.

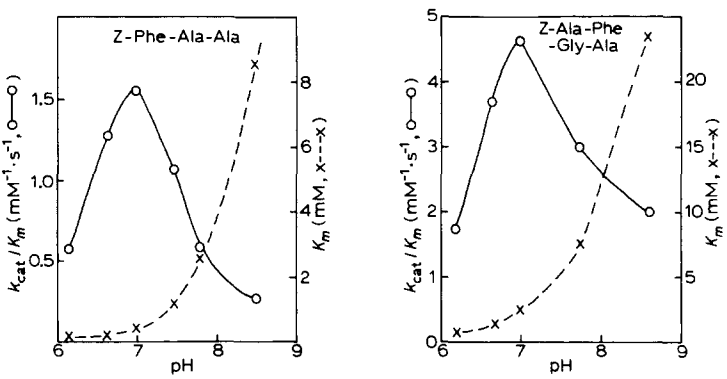

Fig. 1. Effect of pH on the kinetic parameters of *P. aeruginosa* alkaline proteinase. The reaction mixture contained $1 \cdot 10^{-2}$ M $CoCl_2$ and 0.1 M Tris buffer of various pH values. The other methods are described in Materials and Methods.

A preliminary experiment on hydrolysis of synthetic substrates. A preliminary experiment was made using various *N*-acylated amino acid derivatives, Z-dipeptides, Z-dipeptide amides, and Z-tripeptides as substrates. The results, which are summarized in Table II, indicate that the enzyme is inert against small molecular substrates such as *N*-acylated amino acid esters or amides, and Z-dipeptides, while it is moderately active on Z-dipeptide amides and considerably so on Z-tripeptides. The Z-dipeptide amides and Z-tripeptides are all susceptible at their internal peptide bond. Z-free dipeptide amides and tripeptides are insensitive. It was thus found that the smallest peptide sensitive to the enzyme is a Z-tripeptide (including Z-dipeptide amide), in which an internal peptide bond is susceptible. The following experiment was then carried out using various types of Z-tripeptides or more large peptide substrates.

Primary specificity

The primary specificity of the proteinase against the amino acid residues at the imino or carbonyl side of the splitting point in peptide substrates, was determined

TABLE II

HYDROLYSIS OF VARIOUS SYNTHETIC SUBSTRATES BY THE *P. aeruginosa* ALKALINE PROTEINASE

The reaction mixture contained 2.5 mM substrate, 1 μM enzyme, and $1 \cdot 10^{-2}$ M $CoCl_2$. For determination of esterase activity, the reaction was carried out in 0.1 M KCl at pH 7.5 and 30 °C. For either amidase or peptidase activity, the reaction mixture contained 0.05 M Tris buffer (pH 7.0), which was kept at 37 °C. After 20 h reaction, the percentage of hydrolysis was determined; −, ±, + and ++ show <5, 5–10, 10–20 and >20% of hydrolysis, respectively. For Z-Leu-NH_2 and Z-Ala-Leu-NH_2 owing to the small solubility of the substrate, the reaction mixture contained 15% dimethylformamide and 4 μM enzyme, the other conditions being the same as above.

(A) N-Acylated amino acid derivatives
Ac-Ala-OMe −, Ac-Leu-OMe −, Ac-Phe-OMe −, Ac-Tyr-OMe −, Ac-Lys-OMe −,
Bz-Arg-OEt −, Z-Leu-NH_2 −, Z-Tyr-NH_2 −, Bz-Arg-NH_2 −.
(B) Z-Dipeptides
Z-Gly–Leu −, Z-Gly–Phe −, Z-Ala–Gly −, Z-Ala–Ala −, Z-Ala–Leu −, Z-Leu–Leu −,
Z-Phe–Leu −, Z-Tyr–Leu −.
(C) Z-Dipeptide amides
Z-Gly–Gly-NH_2 −, Z-Gly–Ala-NH_2 ±, Z-Gly–Val-NH_2 ±, Z-Gly–Leu-NH_2 ±,
Z-Gly-D-Leu-NH_2 −, Z-Gly–Tyr-NH_2 ±, Z-Ala–Leu-NH_2 +, Ala–Leu-NH_2 −,
Z-Tyr–Gly-NH_2 +, Z-Tyr–Ser-NH_2 +.
(D) Z-Tripeptides
Z-Gly–Gly–Gly −, Z-Gly–Leu–Gly ±, Z-Gly–Leu–Ala ++, Z-Gly–Leu-D-Ala −,
Z-Ala–Ala–Ala ++, Ala–Ala–Ala −, Z-Ala–Leu–Ala ++, Z-D-Ala–Leu–Ala −,
Z-Phe–Leu–Ala ++, Z-Phe-D-Leu–Ala −, Z-Phe–Gly–Ala ++.

using Z-Phe–X-Ala or Z-X–Ala–Ala and Z-X–Leu–Ala (X = various amino acid residues) as substrates. Since these peptides were susceptible at the peptide bonds as shown by the arrows, it was possible to examine the specificity against the amino acid residues at either side of the splitting point.

Z-Phe-X-Ala. The results are summarized in Table III, which indicates that the susceptibility of Z-Phe–X-Ala is X dependent; L-alanine > L-phenylalanine > L-leucine, L-tyrosine, L-serine, glycine ≫ D-leucine. Stringent stereospecificity is observed. The kinetic study for hydrolysis of these peptides would indicate that the effect of X relates to both binding and catalysis. A kinetic study on Z-Phe–Ile–Ala and Z-Phe–Val–Ala was difficult because of the small ninhydrin values of Ile–Ala and Val–Ala. However, the rates of hydrolysis at 2 and 20 h under the conditions of Table II were almost equal to those of Z-Phe–Leu–Ala. Therefore, it may be considered that the proteolytic coefficients of these peptides are comparable with that of Z-Phe–Leu–Ala. Z-Phe-D-Leu–Ala showed a competitive inhibition for hydrolysis of Z-Ala–Phe–Gly–Ala; the K_i value calculated from the Dixon plots is considerably higher than the K_m value of the corresponding L-peptide.

Z-X-Ala–Ala and Z-X-Leu–Ala. The results are summarized in Table IV. The susceptibility of Z-X–Ala–Ala or Z-X–Leu–Ala is dependent upon the kind of X as

TABLE III

KINETIC PARAMETERS OF HYDROLYSIS OF Z-Phe-X-Ala BY *P. aeruginosa* ALKALINE PROTEINASE

The reaction mixture contained 0.05 M Tris buffer (pH 7.0), $1 \cdot 10^{-2}$ M $CoCl_2$ and a suitable amount of enzyme, which was kept at 40 °C. The substrate concentrations used are as seen in the table. Inhibition study was made using Z-Ala-Phe-Gly-Ala ([*S*] = 2 and 5 mM) as substrate, where [*I*] was 0, 1, 2, 3 and 5 mM. The other methods are described in Materials and Methods. The arrows show the bond split.

Peptides	[*S*] (*mM*)	K_m (*mM*)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Z-Phe–Gly–Ala	0.3–1.5	1.8	0.09	0.05
Z-Phe–Ser–Ala	0.5–2.0	1.8	0.12	0.07
Z-Phe–Ala–Ala	0.2–1.0	0.4	0.62	1.55
Z-Phe–Leu–Ala	0.1–1.0	0.4	0.04	0.1
Z-Phe-D-Leu–Ala			(K_i = 3.5 mM)	
Z-Phe–Phe–Ala	0.05–0.6	0.2	0.06	0.3
Z-Phe–Tyr–Ala	0.05–0.6	0.2	0.01	0.05

TABLE IV

KINETIC PARAMETERS OF HYDROLYSIS OF Z-X-Ala–Ala OR Z-X-Leu–Ala BY *P. aeruginosa* ALKALINE PROTEINASE

The methods are described in Table III.

Peptides	[*S*] (*mM*)	K_m (*mM*)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Z-Gly–Ala–Ala	1.0–7.5	7.7	0.62	0.08
Z-Ala–Ala–Ala	0.5–3.0	2.7	0.92	0.34
Z-Val–Ala–Ala	1.0–4.5	2.5	0.4	0.16
Z-Leu–Ala–Ala	0.5–4.0	1.8	0.63	0.35
Z-Phe–Ala–Ala	0.2–1.0	0.4	0.62	1.55
Z-Ala–Leu–Ala	0.3–2.0	1.5	0.16	0.11
Z-D-Ala–Leu–Ala			(K_i = 4.2 mM)	

follows; L-phenylalanine > L-leucine, L-alanine > L-valine > glycine ≫ D-alanine. Stringent stereospecificity is observed. The kinetic parameters of these peptides would indicate that the effect of X relates to the K_m value rather than to k_{cat}. Z-D-Ala–Leu–Ala showed a competitive inhibition for hydrolysis of Z-Ala–Phe–Gly–Ala, whose K_i value is somewhat higher than the K_m value of the corresponding L-peptide.

Effect of secondary interaction on hydrolysis

Effect of molecular size. To investigate the effect of molecular size of peptide substrates on the enzymatic activity, an experiment was performed using L-alanine oligomers (from trimer to hexamer) as substrates. The results are summarized in Table V, which indicates that the proteolytic coefficient (k_{cat}/K_m) increases drasti-

TABLE V

KINETIC PARAMETERS OF HYDROLYSIS OF VARIOUS L-ALANINE OLIGOMERS BY *P. aeruginosa* ALKALINE PROTEINASE

The methods are described in Table III. The dashed arrow shows the bond which is less susceptible.

Peptides	*[S] (mM)*	K_m *(mM)*	k_{cat} (s^{-1})	k_{cat}/K_m $(mM^{-1} \cdot s^{-1})$
Ala–Ala–Ala		Negligible hydrolysis		
Ala–Ala–Ala–Ala	0.5–6.0	8.7	1.6	0.18
Ala–Ala–Ala–Ala–Ala	0.5–2.5	1.2	2.5	2.1
Ala–Ala–Ala–Ala–Ala–Ala	0.1–1.0	0.5	3.9	7.8

cally with increase in chain length, relating mainly to binding (K_m) rather than to catalysis (k_{cat}). Ala_4 was split at the internal peptide bond releasing Ala_2. The hydrolysis of Ala_5 resulted in the release of Ala_2 and Ala_3 and it is indeterminable whether the cleavage was at the second peptide bond from the N-terminus of the peptide or at the third one.

The main product of hydrolysis of Ala_6 was Ala_3, which had a proteolytic activity considerably higher than that of Ala_5. This would indicate that the hydrolysis is influenced by the residues of at least the six positions (P_1–P_3 and P_1'–P_3') in a peptide substrate. The amino acid residues in a peptide substrate are numbered P_1, P_2, *etc.* to the N-terminus, and P_1', P_2', *etc.* to the C-terminus from the catalytic point, using the numbering of Schechter and Berger[17]. The effect of the amino acid residues at P_2, P_2', P_3 and P_3', respectively, on the hydrolysis were found to be as follows.

Effect of P_2'. The effect was determined using Z-Gly–Leu-X as substrates, as shown in Table VI. The susceptibility to the enzyme is markedly dependent on X; L-leucine > L-phenylalanine, L-alanine ≫ glycine, NH_2 ≫ D-alanine, H. Stringent stereospecificity is observed. A kinetic study was not made on Z-Gly–Leu-NH_2, Z-Gly–Leu–Gly and Z-Gly–Leu-D-Ala because of their extremely small hydrolysis (Table II). The peptides all showed a competitive inhibition, whose K_i values are

TABLE VI

KINETIC PARAMETERS OF HYDROLYSIS OF Z-Gly–Leu-X BY *P. aeruginosa* ALKALINE PROTEINASE

Inhibition study was made using Z-Ala–Phe–Gly–Ala ([S] = 2 and 5 mM) as substrate, where [I] was 0, 5, 10, 15 and 20 mM. The other methods are described in Table III.

Peptides	*[S] (mM)*	K_m *(mM)*	k_{cat} (s^{-1})	k_{cat}/K_m $(mM^{-1} \cdot s^{-1})$
Z-Gly–Leu-NH_2		(K_i = 10 mM)		
Z-Gly–Leu–Gly		(K_i = 12 mM)		
Z-Gly–Leu–Ala	1.0–5.0	2.9	0.12	0.04
Z-Gly–Leu-D-Ala		(K_i = 18 mM)		
Z-Gly–Leu–Leu	0.5–2.0	0.9	0.10	0.11
Z-Gly–Leu–Phe	0.2–1.0	0.6	0.04	0.07

considerably higher than the K_m value of Z-Gly–Leu–Ala. This and the other kinetic study would indicate that the amino acid residue at P_2' relates to both binding (K_m) and catalysis (k_{cat}) and that to be susceptible to the enzyme it should be hydrophobic or bulky.

Effect of P_2. The effect was determined using Z-X-Gly–Leu–Ala, Z-X-Phe–Leu–Ala and Z-X-Phe–Gly–Ala as substrates, as seen in Table VII. Elongation of the peptide chain at P_2 with a hydrophobic or bulky amino acid residue results in a remarkable increase in hydrolysis (approx. 80-fold) in each peptide series (A, B and C), relating mainly to catalysis (k_{cat}). Stringent stereospecificity is observed at the position. Z-Gly–Phe–Leu–Ala was susceptible at the position shown by the arrow, which might indicate that a hydrophobic residue must be present at P_2 rather than at P_1 (a Z-residue is regarded as a hydrophobic residue).

TABLE VII

KINETIC PARAMETERS OF HYDROLYSIS OF Z-X-Gly–Leu–Ala, Z-X-Phe–Leu–Ala, AND Z-X-Phe–Gly–Ala BY *P. aeruginosa* ALKALINE PROTEINASE

The methods are described in Table III.

Group	*Peptides*	*[S] (mM)*	K_m *(mM)*	k_{cat} (s^{-1})	k_{cat}/K_m $(mM^{-1}\cdot s^{-1})$
A	Z-Gly–Leu–Ala	1.0–5.0	2.9	0.12	0.04
	Z-Gly–Gly–Leu–Ala	2.0–6.0	5.4	0.97	0.18
	Z-Ala–Gly–Leu–Ala	2.0–6.0	4.8	15.5	3.2
	Z-D-Ala–Gly–Leu–Ala	2.0–15	20.0	0.11	0.005
	Z-Phe–Gly–Leu–Ala	1.0–5.0	2.3	4.6	2.0
B	Z-Phe–Leu–Ala	0.1–1.0	0.4	0.04	0.1
	Z-Gly–Phe–Leu–Ala*	0.2–1.5	0.9	0.56	0.62
	Z-Ala–Phe–Leu–Ala*	0.3–1.5	0.6	5.3	8.8
C	Z-Phe–Gly–Ala	0.3–1.5	1.8	0.09	0.05
	Z-Ala–Phe–Gly–Ala	1.0–5.0	2.5	11.4	4.6

* For longer incubation, the other peptide bonds were split as shown by the dashed arrows; Z-Gly–Phe–Leu–Ala, Z-Ala–Phe–Leu–Ala.

Effect of P_3'. The effect was determined using Z-Gly–Leu–Gly–Gly-X and Z-Ala–Gly–Gly–Leu-X as substrates. The results are summarized in Table VIII. The proteolytic coefficient of Z-Gly–Leu–Gly–Gly–Ala is more than 100 times that of Z-Gly–Leu–Gly–Gly, indicating the significance of a hydrophobic residue at P_3' in the peptide, in which the increase of hydrolysis relates mainly to catalysis. On the

TABLE VIII

KINETIC PARAMETERS OF HYDROLYSIS OF Z-Gly–Leu–Gly–Gly-X AND Z-Ala–Gly–Gly–Leu-X BY *P. aeruginosa* ALKALINE PROTEINASE

The methods are described in Table III.

Group	*Peptides*	*[S] (mM)*	K_m *(mM)*	k_{cat} (s^{-1})	k_{cat}/K_m $(mM^{-1} \cdot s^{-1})$
A	Z-Gly–Leu–Gly–Gly	1.0–15	11.0	<0.05	<0.005
	Z-Gly–Leu–Gly–Gly–Ala	1.0– 5.0	4.3	27.8	6.5
	Z-Gly–Leu–Gly–Gly-D-Ala			Negligibly small	
B	Z-Ala–Gly–Gly–Leu	2.0–15	5.4	6.8	1.26
	Z-Ala–Gly–Gly–Leu–Ala	1.0–10	9.0	50.3	5.6

other hand, the proteolytic coefficient of Z-Ala–Gly–Gly–Leu–Ala is only several times higher than that of Z-Ala–Gly–Gly–Leu, the increase being mainly related to catalysis. These results suggest that the contribution of P_3' to the hydrolysis varies depending upon the kind of amino acid residues at P_2 and/or P_2' in the substrate.

Effect of P_3. The significance of P_3 in the hydrolysis is not so clear as that of the positions mentioned above, though an influence is suggested by the following results; (1) Z-free tetrapeptides were much less susceptible to the enzyme than the corresponding Z-peptides were: the K_m and k_{cat} (s^{-1}) of Phe–Gly–Leu–Ala were 17.3 mM and 0.65, respectively, the proteolytic coefficient being considerably smaller than that of the corresponding Z-peptide shown in Table VII. This might indicate a significance of P_3 in the hydrolysis, when the Z-residue is regarded as an amino acid residue. (2) Z-Phe–Leu–Ala–Ala was the most efficient substrate (K_m = 0.5 mM; k_{cat} (s^{-1}) = 21.2) among all the synthetic peptides tested in this study. The remarkable susceptibility of the C-terminal peptide bond, wherein P_2' is absent, may be due to the participation of P_3 for hydrolysis.

Effect of secondary interaction on the appearance of primary specificity

Both $Z\text{-}Gly_4$ and $Z\text{-}Gly_5$ were insensitive to the *P. aeruginosa* alkaline proteinase, indicating that the presence of a side-chain at some position in the peptide is essential for susceptibility. Some susceptibility was conferred when any one of the four glycine residues in $Z\text{-}Gly_4$ was replaced by a hydrophobic or bulky residue such as L-alanine or L-leucine, as demonstrated by the hydrolysis of Z-Gly–Leu–Gly–Gly, Z-Gly–Gly–Leu-NH_2 and Z-Gly–Gly–Gly–Leu. The innermost peptide bond was the one mainly split, though the rates of hydrolysis of these peptides were too small for the kinetic parameters to be determined.

When any two amino acid residues in $Z\text{-}Gly_4$ were replaced by hydrophobic residues, susceptibility to the enzyme increased considerably, the most internal

TABLE IX

KINETIC PARAMETERS OF HYDROLYSIS OF THE OTHER PEPTIDES BY *P. aeruginosa* ALKALINE PROTEINASE

The methods are described in Table III.

Peptides	*[S] (mM)*	K_m *(mM)*	k_{cat} (s^{-1})	k_{cat}/K_m $(mM^{-1} \cdot s^{-1})$
Z-Gly–Gly–Leu–Ala	2.0–6.0	5.4	0.97	0.18
Z-Ala–Leu–Gly–Gly*	1.0–5.0	2.3	1.1	0.48
Z-Gly–Leu–Gly–Ala	1.0–5.0	2.4	0.36	0.15
Z-Ala–Gly–Gly–Leu	2.0–15	5.4	6.8	1.26
Z-Gly–Pro–Gly–Gly–Pro–Ala	1.0–9.0	5.3	0.6	0.11

* For longer incubation, the other peptide bond was split as follows; Z-Ala–Leu–Gly–Gly.

peptide bond being mainly split. Among the four peptides, Z-Ala–Gly–Gly–Leu, in which glycine residues occupy both sides of the splitting point, was extremely susceptible, as seen in Table IX. The kinetic study indicates that the difference is mainly related to catalysis and little to binding. This indicates that the insensitive Gly–Gly bond becomes susceptible to the enzyme when both P_2 and P_2' positions are occupied by hydrophobic residues in a Z-tetrapeptide.

Z-Gly–Pro–Gly–Gly–Pro–Ala, which is known[18] as a suitable substrate for clostridial collagenase, was considerably susceptible to the alkaline proteinase at the same peptide bond that the clostridial collagenase attacks. The kinetic parameters are as given in Table IX. The smaller proteolytic coefficient as compared with that of Z-Ala–Gly–Gly–Leu–Ala might be ascribed to the L-proline residues at P_2 and P_2', and also to the absence of a hydrophobic residue at P_3. Z-Gly–Pro–Leu–Gly–Pro, which is also known[19] as a substrate for clostridial collagenase, was slightly susceptible to the enzyme but at a different peptide bond from that hydrolyzed by the clostridial enzyme.

DISCUSSION

The present study indicates that the smallest peptide being effectively sensitive to the *P. aeruginosa* alkaline proteinase is a Z-tripeptide, in which an internal peptide bond is susceptible. The susceptibility is negligible when the carboxyl-terminal amino acid residue is small, such as amide or glycine. Z-free tripeptide is also insensitive to the enzyme. It would seem therefore that susceptibility requires a peptide backbone whose free α-amino and α-carboxyl groups are blocked with hydrophobic or bulky residues. These characteristics are exactly the same as those observed with metal-neutral proteinases[20] obtained from *B. subtilis*, *B. thermoproteolyticus* (thermolysin), *P. aeruginosa*, *etc.* Further, the *P. aeruginosa* alkaline proteinase was found in this study to be most active against synthetic peptides at neutral pH values. The enzyme might, therefore, be regarded as a kind of metal-neutral proteinase.

Nevertheless, a considerable difference is observed between the *P. aeruginosa* proteinase and usual metal-neutral proteinases in their specificity against the amino acid residue whose imino group donates the susceptible peptide bond; the former enzyme is specific against L-alanine, while the latter ones against L-leucine, L-isoleucine, and L-phenylalanine. The specificity of the *P. aeruginosa* proteinase is much less stringent than that of the metal-neutral proteinases; the proteolytic coefficient for Z-Phe–Ala–Ala is about 30-fold that for Z-Phe–Gly–Ala with the former enzyme, while the coefficient for Z-Phe–Leu–Ala is about 1000-fold that for Z-Phe–Gly–Ala with *B. subtilis* neutral proteinase[14].

On the other hand, the effect of the kind of amino acid residue whose carbonyl group donates the susceptible peptide bond, is not great for either the *P. aeruginosa* alkaline proteinase or metal-neutral proteinases[13,21]; in both Z-X–Ala–Ala and Z-X–Leu–Ala, the peptide with L-phenylalanine as X is hydrolyzed 20–30 times as rapidly as that having glycine as X by either enzyme. That is to say, usual metal-neutral proteinases show much more stringent specificity against the amino acid residue at the imino side of the splitting point than that against that at the carbonyl side, while the *P. aeruginosa* proteinase specificity is not so stringent and is similar for the residue on either side of the splitting point.

A more striking difference is observed between the *P. aeruginosa* alkaline and usual metal-neutral proteinases in their proteolytic coefficients of these Z-tripeptides. The k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$) of Z-Phe–Ala–Ala, a most efficient substrate for the enzyme, is 1.55 by the *P. aeruginosa* alkaline proteinase, while those of Z-Phe–Leu–Ala by *B. subtilis* neutral proteinase and thermolysin are 943.4 (ref. 13) and 577.8 (ref. 21), respectively. The remarkable difference mainly relates to their k_{cat} values. Nevertheless, the proteinase activities of these enzymes against various protein substrates are not so different from each other. This might be resolved by considering that a fairly large molecular size is required as peptide substrates for development of maximum hydrolysis of the *P. aeruginosa* alkaline proteinase.

The study using L-alanine oligomers (from tetramer to hexamer) as substrates, indicates that the proteolytic activity increases drastically with the increase in chain-length. The main product from Ala_6 was Ala_3, indicating that the hydrolysis is influenced by all the positions, P_1–P_3 and P_1'–P_3', in the peptide. A study of the effect of the kind of amino acid residues at positions distant from the catalytic point (*i.e.* secondary interaction), *e.g.* at P_2–P_3 and P_2'–P_3', showed that a hydrophobic or bulky amino acid residue has a significant influence but a glycine or D-amino acid residue has not. This would indicate that the effect of amino acid residues at these positions is ascribed to their side chains and not to their main chains. It has been shown that elongation of the peptide chain with glycine residues at one or both sides from the catalytic point results in a remarkable increase in hydrolysis by pepsin[22] or substilisins[15,23]. This is considerably different from the situation with *P. aeruginosa* alkaline proteinase.

It is noticeable that the primary specificity of the proteinase, determined using Z-tripeptides as substrates, is not always reflected in the hydrolysis of Z-tetra- or

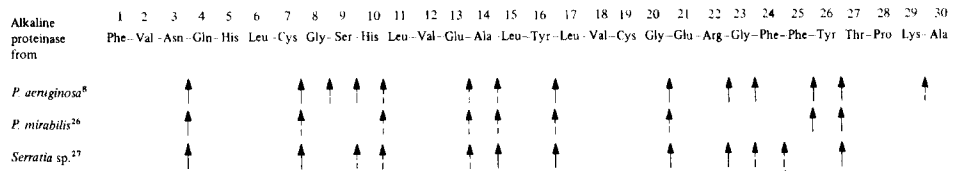

Fig. 2. Points of cleavage in oxidized insulin B chain by various proteinases. The arrows indicate principal sites of action; the dashed arrows indicate bonds less readily attacked.

Z-pentapeptides. For example, Z-Ala–Gly–Gly–Leu was much more susceptible to the enzyme than the other Z-tetrapeptides, which contain two hydrophobic residues at either side of the splitting point (P_1 or P_1' and P_2 or P_2'). This would indicate that the susceptibility of a peptide bond in peptide substrates is dependent upon the kind of amino acid residues at P_2 and P_2', rather than on those at P_1 and P_1'. A similar case has been observed with papain[24] or streptococcal proteinase[25]; the chief requirement for hydrolysis was the presence of a bulky side chain at P_2.

The above consideration on specificity may further lead to an assumption that rather small amino acid residues border the splitting point when it acts on peptides of large molecular size. This can be seen in the hydrolysis of oxidized insulin B chain as shown in Fig. 2 (the points of cleavage were identical in the presence or absence of Co^{2+}, unpublished data). The peptide bonds containing the carbonyl and/or imino groups of glycine are all susceptible to the enzyme; *viz.*, Cys–Gly (7–8), Gly–Ser (8–9), Gly–Glu (20–21), Arg–Gly (22–23) and Gly–Phe (23–24), are five of the eleven main points of cleavage. The other four susceptible peptide bonds, Asn–Gln (3–4), Ser–His (9–10), Glu–Ala (13–14) and Tyr–Thr (26–27), contain rather small amino acid residues at either side of the splitting point.

It is of interest to note that an alkaline proteinase from *Proteus mirabilis*[26] or *Serratia sp.*[27] shows very similar specificity against oxidized insulin B chain, as seen in Fig. 2. These enzymes are also sensitive to metal chelators but not to the other inhibitors such as *p*Cl-HgBzO$^-$, iP2$_2$*P*-F, *etc.* The specificity of the *Serratia* enzyme was determined by using various synthetic peptides[27], and the results indicate that the following peptides are sensitive to the enzyme;

Bz-Gly–Leu-NH_2

Lys–Phe–Ile–Gly–Leu–Met-NH_2

Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg

The *P. aeruginosa* proteinase hydrolyzes Z-Gly–Leu-NH_2 slightly at the same peptide bond, as shown in this paper. Although the last two of the above peptides have not been tested with the *P. aeruginosa* enzyme, its specificity against peptides agrees well with the view on specificity of the *P. aeruginosa* proteinase mentioned above. The specificity of the *Proteus* enzyme has not been determined with synthetic peptides as substrates.

Collagenase-like proteinases have been found to be produced by *P. aeruginosa*[28] and *Serratia marcescens*[29]; these hydrolyze Z-Gly–Pro–Gly–Gly–Pro–Ala at the same

peptide bond as clostridial collagenase does. The enzymes, however, hydrolyze native collagen only slightly. These enzymes are most active against usual protein substrates within an alkaline pH range, and are sensitive to metal chelators but not to *p*Cl-HgBzO$^-$ or $iP2_2P$-F. The present study indicates that the *P. aeruginosa* alkaline proteinase used here is also active on the peptide. Therefore, it might be said that the *P. aeruginosa* proteinase presented here is representative of a type which is distributed in Gram-negative bacteria.

In conclusion, it may be said that the specificity of the *P. aeruginosa* alkaline proteinase is determined by secondary interaction rather than by primary specificity when it acts on large molecular peptides. Secondary interaction is seen to govern the specificity of most proteinases to a greater or lesser extent, and its influence on *P. aeruginosa* proteinase is the most marked that has yet been encountered.

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