Studies on the Specificity of *Bacillus subtilis* Neutral Protease with Synthetic Substrates*

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ABSTRACT: The substrate specificity of a neutral protease obtained from *Bacillus subtilis* strain AM was studied using simple dipeptide substrates. The effects of variations in the amino acids contributing the carboxyl and amino groups of the bond cleaved and the presence of the free carboxyl or amino group on the apparent K_m, V_{max}, and pseudo-first-order rate constants for the enzyme-catalyzed hydrolysis of these substrates were determined. It was found that the enzyme requires a dipeptide backbone with an alanine, valine, isoleucine, norleucine, leucine, or phenylalanine as the amino acid whose amino group formed the peptide bond. The rate of hydrolysis varied with the amino acid; the best rates were observed with leucine. The presence of the free α-carboxyl or α-amino group greatly diminished the enzymatic hydrolysis.

An enhancement in the rate of hydrolysis was obtained by the presence of an alanyl, seryl, threonyl, or histidyl group as the amino acid whose carboxyl group formed the bond. This latter effect was secondary, obtained only if the former structural requirements were fulfilled.

A number of proteolytic enzymes, some of which have alkaline pH optima, are produced by *Bacillus subtilis* and inhibited by diisopropylfluorophosphate (Guntelberg and Ottesen, 1954; Ottesen and Schellman, 1957; Matsubara and Nishimura, 1958). Recently McConn *et al.* (1964) and Tsuru *et al.* (1965) reported the isolation of the lesser known neutral protease and described some of its chemical and physical properties. This enzyme, unlike the alkaline protease, is inhibited by chelating agents such as EDTA and reactivated by zinc, cobalt, and manganese. It is therefore of interest to compare this bacterial enzyme with other metal-containing proteolytic enzymes with the aim of obtaining further information concerning the mechanism of action of the metal-containing proteases.

The elucidation of the nature and molecular environment of the bond catalytically hydrolyzed by *B. subtilis* neutral protease is necessary before the nature of the enzyme catalysis can be investigated. The use of simple dipeptide substrates in which the individual amino acids and the absence and presence of free carboxyl and amino groups are varied is very useful to obtain such information. Hypotheses derived from these simple systems can then be tested by identifying the points of cleavage in larger peptides resulting from enzyme-catalyzed hydrolyses. The specificity requirements for *B. subtilis* strain AM neutral protease catalysis have been under study and some attempts at its general definition are presented here.[1]

Experimental Section

Materials

Z-Gly-Trp-NH_2, Z-Trp-Leu-NH_2, Z-Phe-Gly, Z-Ala-Ser-OMe, and Pro-Phe-NH_2 were purchased from New England Nuclear Corp. Bz-Gly-Leu-NH_2, Z-Tyr-Gly-NH_2, and Z-Gly-Phe were purchased from Mann Research Laboratories, Inc., and Z-Pro-Leu-Gly-NH_2 was obtained from the Sigma Chemical Co. All the other peptides were purchased from the Cyclo Chemical Corp. The substrates were used without any further treatment.

The purity of most of these dipeptides was checked by thin layer chromatography and all those which proved to be substrates were reanalyzed for carbon, hydrogen, and nitrogen yielding good agreement between the theoretical and found values.

These di- and tripeptide substrates were prepared directly in the appropriate buffers because of the low solubilities. *N-trans*-Cinnamoylimidazole (Nutritional Biochemicals Corp.) stock solutions were prepared in spectroquality acetonitrile (Matheson Coleman and Bell) which had been purified by the procedure described by Lewis and Smyth (1939).

Stock solutions of *p*-nitrophenyl acetate (Sigma Chemical Co.) and Z-Gly-ONp (Cyclo Chemical Co.) were also prepared in acetonitrile. Hippuryl-DL-phenyllactic acid and *N*-benzoyl-L-arginine ethyl ester·HCl were purchased from Nutritional Biochemicals Corp. Stock solutions of *N*-acetyl-L-tyrosine ethyl ester (Mann Research Laboratories) were prepared in purified acetonitrile. Only reagent grade salts and deionized water (conductivity 5×10^{-7} mho) was used throughout these studies. Crystalline subtilisin was purchased from Nutritional Biochemicals Corp.

The neutral protease used in these studies was ob-

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[1] Abbreviations used: Z, benzyloxycarbonyl; ONp, *p*-nitrophenoxy; Nle, norleucyl; Sar, sarcosyl; Nva, norvalyl; FDNB, fluorodinitrobenzene; Abu, L-α-aminobutyryl.

tained from *B. subtilis* strain AM fermentation beers. The enzyme preparation was a DEAE-treated ammonium sulfate fractionated preparation which had been purified by column chromatography on hydroxylapatite. The enzyme was homogeneous by the criterion of ultracentrifugation, disc gel electrophoresis, and gel exclusion chromatography (methods to be published). Nevertheless, the enzyme preparations were treated with 10^{-3} M diisopropylfluorophosphate to inactivate any traces of subtilisin that might be present. Oxidized ribonuclease (bovine pancreas) and insulin β chain (carboxymethylated and reduced) were purchased from Mann Research Laboratories.

Methods

Protease Assay. The protease activity of enzyme preparations was determined by the casein digestion method of Kunitz (1947) as modified and described by McConn *et al.* (1964). The neutral protease-catalyzed hydrolysis of Z-Thr-Leu-NH_2 under first-order conditions was the basis for a more accurate measure of the enzyme activity.

Ninhydrin Determinations. The enzyme-catalyzed hydrolysis of di- and tripeptide substrates was followed by determining the production of ninhydrin-reactive groups. The reactions solutions contained about 3×10^{-3} M substrate and 0.1–1.0 mg/ml of enzyme in the appropriate buffer. At intervals 0.1-ml aliquots were withdrawn and diluted to 1.0 ml with water, and ninhydrin determinations were made according to the method described by Greenstein and Winitz (1961) and first reported by Rosen (1957).

Thin Layer Chromatography. The reaction products of these hydrolyses were identified by the use of one-dimensional chromatography on type K301R2 silica gel coated sheets of polyester (Eastman Kodak Co.). The solvent system consisted of 1-butanol–acetone–acetic acid–5% ammonia–water (4.5:1.5:1:2) as described by Randerath (1963). This also provided a method of showing the purity of the substrates. The bond between the amino acids was thus shown to be the one cleaved.

Kinetic Studies. The neutral protease-catalyzed hydrolysis of many of the substrates was studied by means of a Radiometer pH-Stat (type SBR-2C) fitted with an autoburet (type A Bulb) and a 0.25-ml syringe. The reactions were run in a jacketed reaction cell connected to a water bath at 25.0 ± 0.1° with a steady stream of nitrogen layered over the solutions to minimize CO_2 absorption. The substrates were prepared in 0.1 M KCl.

Esterase Activity. The hydrolysis of *p*-nitrophenyl acetate and Z-Gly-ONp was followed spectrophotometrically at 340 and 400 mμ using a Cary 14 PM recording spectrophotometer. The hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester was observed at 253 mμ (Schwert and Takenaka, 1955), *N*-acetyl-L-tyrosine ethyl ester at 237 mμ, hippuryl-DL-β-phenyllactic acid at 270 mμ (Bender *et al.*, 1965), and *N-trans*-cinnamoylimidazole at 335 mμ (Bender *et al.*, 1962).

FDNB End-Group Analysis. A preliminary attempt was made to obtain information concerning the specificity of the bonds cleaved by neutral protease in larger peptides and to see how this correlated with the specificity requirements obtained from the simple dipeptides studied. The amino groups liberated by the enzyme-catalyzed hydrolysis of oxidized ribonuclease and the insulin β chain were reacted with FDNB and the N-terminal amino acids identified after hydrolysis by two-dimensional paper chromatography. The procedure of Redfield and Anfinsen (1956) was used for the FDNB end-group analysis.

Results

The initial screening experiments to determine the amino acids required for specificity with respect to simple substrates utilized the detection of ninhydrin-reactive products liberated on hydrolysis. The reactions were carried out at 25 and 37° using substrate concentrations of the order of 10^{-3} M and enzyme concentrations up to 1 mg/ml of reaction solution. Aliquots of the reaction mixture were withdrawn at intervals for total incubations up to 70 hr and ninhydrin determinations were made. Table I shows the results obtained with a number of synthetic substrates. The absence of an observed reaction means that no increase in the absorbancy at 570 mμ was observed over the time course of the experiment. Thin layer chromatography was used to identify the products and thereby demonstrated that only the dipeptide bond was being cleaved. A requirement for alanine, valine, leucine, norleucine, isoleucine, and phenylalanine as the amino acid contributing the amino group of the peptide bond hydrolyzed is seen from the table. Reversing the position of the amino acids such as in Z-Ala-Gly-NH_2, Z-Leu-Gly-NH_2, Z-Ile-Gly-NH_2, and Z-Phe-Gly-NH_2 resulted in the loss of the enzyme-catalyzed hydrolysis. The absence of a reaction with Z-Gly-Phe and Gly-Phe-NH_2 suggested that the enzyme is an endopeptidase, not readily catalyzing the hydrolysis of peptide bonds adjacent to the free amino or carboxyl end of the chain. Likewise, Gly-Leu-NH_2 and Z-Gly-Leu, although cleaved by the enzyme, were hydrolyzed at a very much slower rate than the Z-Gly-Leu-NH_2.

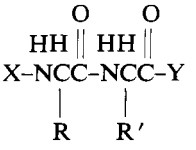
In order to quantitatively measure the effects observed, initial rate data were obtained using the pH-Stat and ninhydrin determinations for the determination of K_m values and catalytic rate constants. The problem of substrate insolubility and large values for K_m made it difficult to obtain rate data over the ideal substrate range for good Lineweaver–Burk (1934) plots. On the other hand, at lower substrate concentrations good pseudo-first-order kinetics were obtained and provided a good tool for comparing the rates of enzyme-catalyzed hydrolyses of the various substrates of interest. When the substrate concentration falls below the K_m, the rate expression for an enzyme-catalyzed reaction becomes $V = k_{cat}(E)(S)/K_m$. Under these conditions it is easy to obtain pseudo-first-order constants ($k = (k_{cat}(E)/K_m)$. These constants of course do not distinguish between variation in k_{cat} or K_m

TABLE I: The Effect of Substituent Variation on the Neutral Protease-Catalyzed Hydrolysis of Dipeptides.[a]

Substrate	Reaction Obsvd	Substrate	Reaction Obsvd	Substrate	Reaction Obsvd
Z-Gly-Gly-NH_2	−	Z-Leu-Trp-NH_2	−	Z-Gly-Nle-NH_2	+
Z-Gly-Ala-NH_2	+	Ac-Trp-NH_2	−	Z-Abu-Leu-NH_2	+
Z-Ala-Ala-NH_2	+	Z-Gly-Leu-NH_2	+	Z-Pro-Leu-Gly-NH_2	+
Z-Ala-Gly-NH_2	−	Z-Ala-Leu-NH_2	+	Z-Gly-Phe-NH_2	+
Z-Sar-Ala-NH_2	−	Z-β-Ala-Leu-NH_2	−	Z-Gly-Phe	−
Z-β-Ala-β-Ala-NH_2	−	Z-Leu-Gly-NH_2	−	Z-β-Ala-Phe-NH_2	−
Z-β-Ala-Ala-NH_2	−	Z-Sar-Leu-NH_2	−	Z-Phe-Gly-NH_2	−
Z-Gly-Ser-NH_2	−	Gly-Leu-NH_2	+	Z-Phe-Gly	−
Z-Ala-Ser-OMe	−	Z-Gly-Leu	+	Pro-Phe-NH_2	−
Z-Ser-Gly-NH_2	−	Z-Trp-Leu-NH_2	+	Z-Tyr-Gly-NH_2	−
Z-Gly-Val-NH_2	+	Z-Pro-Leu-NH_2	+	Z-Tyr-Leu-NH_2	+
Z-Nva-Gly-NH_2	−	Bz-Leu-NH_2	−	Z-Tyr-Gly-OMe	−
Z-Gly-Pro-NH_2	−	Ac-Leu-NH_2	−	Ac-Tyr-NH_2	−
Z-Gly-Met-NH_2	−	Z-Gly-Gly-Leu-NH_2	+	Z-Tyr-Tyr-NH_2	−
Z-Met-Gly-NH_2	−	Z-Gly-Ile-NH_2	+	Bz-Gly-Tyr-NH_2	−
Z-Leu-Met-NH_2	−	Z-Ile-Gly-NH_2	−	Z-Sar-Tyr-NH_2	−
Z-Gly-Trp-NH_2	−				

[a] All reactions were carried out at pH 7.0–7.5, Tris buffer (ionic strength, 0.2), 25°, and monitored by ninhydrin determinations.

but afford a good means of comparison. The product of this pseudo-first-order rate constant and the K_m yields the value k_{cat}(E) or the V_{max}, and this value can be compared to the V_{max} obtained from the Lineweaver–Burk plot. This affords a means of checking the accuracy of the K_m values. The rate constants are reported here as k/(E) which is equal to k_{cat}/K_m when E is expressed in molar units. Using these methods the effect of variation in R and R′ on the enzyme-catalyzed hydrolysis was examined. All of the kinetic



data using the pH-Stat were obtained at pH 8.0, 0.1 M KCl, and 25.0°. Table II shows the effect of variation of R′ on the k/(E) and K_m values. No reaction was observed using Z-Gly-Gly-NH_2 as a substrate, *i.e.*, when R′ is a hydrogen. An increase in the size of the chain yielded a greater value of k/(E). The isobutyl constituent gave the best rate enhancement. The effect of varying R (maintaining the isobutyl R′) on the rate constants are given in Table III. An eightfold increase in the value of k/(E) is observed in going from Z-Gly-Leu-NH_2 to Z-Thr-Leu-NH_2. This effect is also seen when we compare Z-Gly-Ala-NH_2 (k/(E) = 0.43 × 10^{-4} sec^{-1} $(mg/ml)^{-1}$) and Z-Ala-Ala-NH_2 (k/(E) = 1.68 × 10^{-4} sec^{-1} $(mg/ml)^{-1}$). It is important to point out that this effect is observed only when R′ is the appropriate group. The k/(E) for Z-His-Leu-NH_2 is 341.5 × 10^{-4} sec^{-1} $(mg/ml)^{-1}$ while Z-His-Gly-NH_2 is not hydrolyzed.

Even though a factor of 10^3 is observed in the values of k/(E) in going from Z-Gly-Ala-NH_2 to Z-Thr-Leu-NH_2 the values of K_m for these compounds are 1.04 and 1.22 × 10^{-2} M, respectively, and the total variation in K_m for all the substrates studied falls within a factor

TABLE II: The Effect of Variations of R′ on the Neutral Protease-Catalyzed Hydrolysis of Dipeptides.[a]

Substrate	k/(E) × 10^4 sec^{-1} $(mg/ml)^{-1}$[b]	K_m × 10^2 M
Z-Gly-Gly-NH_2	No reaction	
Z-Gly-Ala-NH_2	0.43	1.04
Z-Gly-Val-NH_2	6.95	2.16
Z-Gly-Nle-NH_2	11.70	3.60
Z-Gly-Leu-NH_2	54.57	2.94
Z-Gly-Phe-NH_2	3.47	0.32
Z-Gly-Tyr-NH_2	≦0.12[c]	−

[a] Reactions were monitored by pH-Stat at pH 8.0, 0.1 M KCl, 25.0 ± 0.1° (S_0 ~10^{-3} M). [b] k is the pseudo-first-order rate constant; k/(E) is equal to k_{cat}/K_m when E is expressed in molar concentration. E is given here in milligrams per milliliter. [c] Obtained from initial rate approximation.

TABLE III: The Effect of Variations of R on the Neutral Protease-Catalyzed Hydrolysis of Dipeptides.[a]

Substrate	$k/(E) \times 10^4$ sec^{-1} $(mg/ml)^{-1}$	$K_m \times 10^2$ M
Z-Gly-Leu-NH_2	54.47	2.94
Bz-Gly-Leu-NH_2	33.41	2.13
Z-Ala-Leu-NH_2	147.7	1.01
Z-Ser-Leu-NH_2	249.2	0.47
Z-Thr-Leu-NH_2	406.5	1.22
Z-Met-Leu-NH_2	27.18	—
Z-His-Leu-NH_2	341.5	0.38
Z-Tyr-Leu-NH_2	381.47	—

[a] Reactions monitored by the pH-Stat at pH 8.0, 0.1 M KCl, 25.0 ± 0.1° (S_0, $\sim 10^{-3}$ M).

TABLE IV: The Effect of Free Carboxyl or α-Amino Group on the Neutral Protease-Catalyzed Hydrolysis.[a]

Substrate	$k/(E) \times 10^4$ sec^{-1} $(mg/ml)^{-1}$	k(Z-Gly-Leu-NH_2)/ k(X-Gly-Leu-Y
Z-Gly-Leu-NH_2	54.57	1
Gly-Leu-NH_2	$\leqq 0.51$[b]	107
Z-Gly-Leu	$\leqq 1.38$[b]	39
Z-Gly-Leu-OMe	$\leqq 0.10$[b]	545
Z-Ser-Leu-NH_2	249.29	1
Z-Ser-Leu-OMe	$\leqq 0.15$[b]	1661

[a] Reactions were monitored by the pH-Stat, 0.1 M KCl, pH 8.0, 25.0 ± 0.1°. [b] Pseudo-first-order rate constants obtained from initial rate approximations and represent maximum values.

of 10. This indicates that the side-chain variation primarily had an effect on the catalytic rate constant.

The effect of a free carboxyl or free α-amino group is shown in Table IV. A decrease of over a 100-fold in the rate constant is observed when the α-amino group is free while the free carboxyl decreases the rate constant by about 40. Since these reactions are exceedingly slow even with high concentrations of enzyme (2 mg/ml), the rate constants were approximated from the initial rate of hydrolysis and the substrate concentration. This is not simply a charge effect since the methyl ester was considerably worse than the free acid. This is vividly demonstrated by comparing Z-Ser-Leu-NH_2 and Z-Ser-Leu-OMe ($(k(NH_2))/k(OMe) = 1661$). These are maximal rates calculated by assuming pK's of 8.0 and 9.6 for the α-amino group of leucine amide or leucine methyl ester and free acid, respectively.

The tripeptides Z-Pro-Leu-Gly-NH_2 and Z-Gly-Gly-Leu-NH_2 were hydrolyzed by the neutral protease. The Z-Pro-Leu-Gly-NH_2 yielded a $k/(E)$ of 7.4×10^{-4} sec^{-1} $(mg/ml)^{-1}$ at pH 8.0 and a K_m of 9.36×10^{-3} M. A $k/(E)$ of 17.7×10^{-4} sec^{-1} $(mg/ml)^{-1}$ and a K_m of 1.15×10^{-3} M were obtained for the neutral protease-catalyzed hydrolysis of Z-Gly-Gly-Leu-NH_2 (pH-Stat, pH 8.0, 0.1 M KCl). Little to no hydrolysis was obtained for Z-Gly-Tyr-NH_2 and Bz-Gly-Tyr-NH_2, respectively, by the enzyme. The Z-Gly-Tyr-NH_2 yielded a value of $k/(E)$ of 0.12×10^{-4} sec^{-1} (mg/ml^{-1}). No evidence of reaction was observed with Z-α-Glu-Tyr, a pepsin substrate. The effect of subtilisin on a few of these substrates had been examined. While subtilisin was over 20-fold better than the neutral protease with respect to Z-Gly-Tyr-NH_2, the neutral protease was over 70-fold better with Z-Ala-Leu-NH_2 and 20-fold better with Bz-Gly-Leu-NH_2.

Preliminary experiments using oxidized ribonuclease A and the reduced β chain of insulin (carboxymethylated) were performed to identify the amino-terminal groups liberated upon hydrolysis by the neutral protease. The peptides were incubated with the enzyme for various lengths of time and the dinitrophenyl derivatives of the products of hydrolysis were prepared. The DNP-amino acids were identified by two-dimensional paper chromatography after acid hydrolysis. Leucine and phenylalanine appear as the N-terminal amino acid liberated on incubation with enzyme early in the hydrolysis of both the insulin β chain and the oxidized ribonuclease. These are only preliminary results and work is currently in progress to identify the exact points of cleavage of the insulin β chain by neutral protease.

Esterase Activity. The neutral protease appeared not to catalyze the hydrolysis of *p*-nitrophenyl acetate, *N*-CBZ-glycine *p*-nitrophenyl ester, *N*-benzoyl-L-arginine ethyl ester, *N*-acetyltyrosine ethyl ester, and *N*-*trans*-cinnamoylimidazole. Hippuryl-DL-phenyllactic acid, a good substrate for carboxypeptidase, also proved not to be a substrate for the protease.

Enzyme Assay. The neutral protease-catalyzed hydrolysis of simple dipeptide substrates was also used as the basis of an enzyme assay. Figure 1 shows the effect of enzyme concentration on the initial rate of hydrolysis of Bz-Gly-Leu-NH_2 at pH 8.0 as monitored by the pH-Stat. Under the conditions of the experiment ($S_0 = 1.549 \times 10^{-2}$ M, 0.1 M KCl, 25.0 ± 0.1°) the ratio of V_{init}:(E) = 3.13 (units/sec) $(mg/ml)^{-1}$ was obtained. An initial velocity of a unit per second represents 2.5×10^{-8} equiv of base/sec or about 2×10^{-5} M/sec under experimental conditions. Since the K_m for Bz-Gly-Leu-NH_2 is 2.13×10^{-2} M at pH 8.0, it is not possible to carry out reactions under substrate concentrations that are high above this value. It is, therefore, not possible to obtain zero-order or near zero-order conditions which are ideal for rate assays.

On the other hand, it is possible to use low substrate

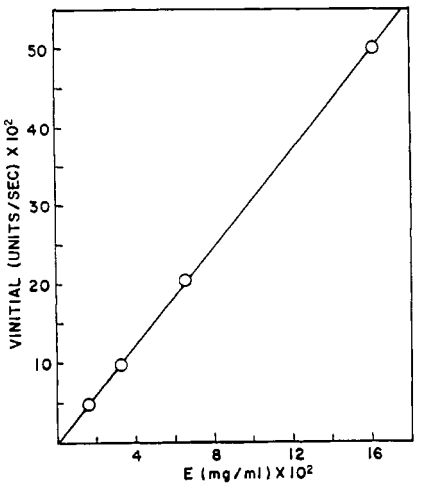


FIGURE 1: The effect of enzyme concentration on the initial rate of hydrolysis of hippuryl-L-leucine amide. Reactions were carried out at pH 8.0, 0.1 M KCl, 25.0 ± 0.1° using a pH-Stat. One unit is equivalent to the uptake of 2.5×10^{-8} equiv of base ($S_0 = 1.55 \times 10^{-2}$ M).

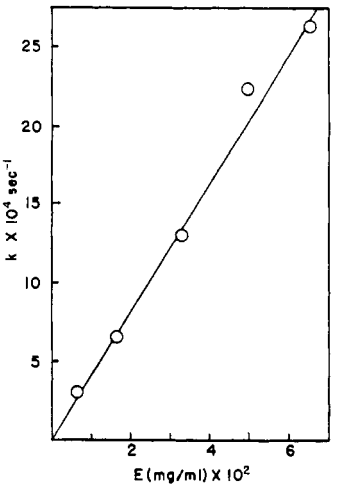


FIGURE 2: The effect of enzyme concentration on the pseudo-first-order rate constants for the hydrolysis of *N*-benzyloxycarbonyl-L-threonyl-L-leucine amide. Reactions were carried out at pH 8.0, 0.1 M KCl, 25.0 ± 0.1° using a pH-Stat ($S_0 = 1.55 \times 10^{-3}$ M).

concentrations and obtain pseudo-first-order conditions. The pseudo-first-order rate constants obtained from these reactions as mentioned earlier are equal to $k_{cat}(E)/K_m$. Since k_{cat} and K_m are constants at any given pH, the rate constants are direct functions of the enzyme concentration. Also, since variations in the substrate concentrate (provided they remain low) and the method of monitoring the reaction do not effect the rate constant obtained, enzyme-catalyzed hydrolysis under pseudo-first-order conditions would serve as an excellent kinetic assay. The substrate Z-Thr-Leu-NH_2 which has a high K_m and a high value of $k/(E)$ was chosen for this assay. Figure 2 shows the relationship between the pseudo-first-order-rate constants obtained and the enzyme concentration. Substrate concentrations of 1.64×10^{-3} M were used.

Discussion

The results presented here strongly indicate that the neutral protease is an endopeptidase. The presence of a free carboxyl, free amino, or an ester adjacent to the bond cleaved markedly diminished or even prevented catalytic hydrolysis. A difference of 1600-fold in the $k/(E)$ values was obtained in going from Z-Ser-Leu-NH_2 to the methyl ester. Similarly over a 500-fold difference was observed in comparing the Z-Gly-Leu-NH_2 and its methyl ester.

The need for a dipeptide backbone is strongly indicated from the results shown in Tables I and IV. The enzyme was unable to cleave Ac-Leu-NH_2 and Bz-Leu-NH_2.

The lack of observable enzyme-catalyzed hydrolysis of Z-β-Ala-Leu-NH_2, Z-β-Ala-β-Ala-NH_2, Z-Ala-β-Ala-NH_2, and Z-β-Ala-Phe-NH_2 points out the need of an α-amino acid peptide chain. Likewise, the inhibitory effect of the sarcosyl replacement for glycine in Z-Gly-Ala-NH_2 and Z-Gly-Leu-NH_2 indicates the need for the unhindered amide bond adjacent to the bond cleaved.

The specificity requirements of a dipeptide backbone with the amino and carboxyl group blocked do not give a structure which can serve as a substrate. No hydrolysis of Z-Gly-Gly-NH_2 by neutral protease was observed but the introduction of a methyl, isopropyl, *n*-butyl, isobutyl, or phenyl group resulted in progressively better enzymatic hydrolysis. The position of the side chain is critical since Z-Ala-Gly-NH_2, Z-Val-Gly-NH_2, Z-Phe-Gly-NH_2, Z-Ile-Gly-NH_2, and Z-Leu-Gly-NH_2 were not cleaved by the enzyme. Therefore, in order to serve as a substrate the dipeptide must have a group other than H in the R′ position. The seryl derivative as opposed to alanyl had no activity, and likewise only slight hydrolysis of the tyrosyl derivative as compared to the phenylalanyl derivative was observed. The need for a strictly hydrophobic side chain is, therefore, indicated with leucine being the best amino acid. If these absolute structural requirements are fulfilled, further enhancement in hydrolysis rates is obtained by the presence of a side chain on the amino acid whose carboxyl group is involved in the bond.

Unlike the requirements for substituent R′, introduction of a substituent containing a hydroxyl or an imidazole at R results in an increased rate of catalysis. The over-all effects of these substituents resulted in differences of $k/(E)$ of about a factor of 1000, whereas the apparent K_m values varied a maximum of 10 with no correlation between high values of $k/(E)$ and low values of K_m. It might be suggested that the backbone of the peptide chain primarily accounts for the over-all

binding to the enzyme. The substituents have a more pronounced effect on the observed catalytic rate constants. The preliminary results of the N-terminal amino acid released upon neutral protease hydrolysis of oxidized ribonuclease and insulin β chain agree with the dipeptide results.

The results of the substrate specificity studies of neutral protease reported by McConn *et al.* (1964) bear out these observations. The lack of enzyme-catalyzed hydrolysis of Ala-Leu, Leu-Leu, Z-Gly-Phe, Phe-Phe, Val-Val, Gly-Gly, glutathione, and tetraglycine agree with the proposed specificity requirements of the enzyme. The reported hydrolysis of Z-Tyr-Gly-NH_2 was not observed, however, with the enzyme used in these studies. Since the starting crude enzymes used in the two purifications were not obtained from the same source and different methods of purification were used, slight differences in the neutral protease might exist.

It is of interest to compare this bacterial zinc protease with the better known mammalian carboxypeptidases. The most striking contrast is the absolute requirement for a free carboxyl group by the carboxypeptidases and the greatly diminished neutral protease-catalyzed hydrolysis of substrates containing it.

On the other hand, there are some interesting similarities. The nature of the amino acid contributing the amino group of the peptide bond cleaved by the carboxypeptidases is of importance in the specificity. This has been shown to be true also for neutral protease. Whereas the aromatic side chains in this position are better than aliphatic ones for carboxypeptidase A, neutral protease favors an aliphatic substituent but also works well with a phenyl group. The Z-Gly-Leu-NH_2 was better than Z-Gly-Phe-NH_2 by a factor of 15. Though the carboxypeptidases do not catalyze the hydrolysis of these dipeptides, Neurath and DeMaria (1950) found that the Z-Gly-Phe was considerably more susceptible to carboxypeptidase cleavage than the Z-Gly-Leu.

Hanson and Smith (1948) reported the requirement of an α peptide for carboxypeptidase and this is reported here for the *B. subtilis* enzyme. Similar observations pointing to the need of a hydrogen atom on the peptide bond adjacent to the one cleaved are seen from the results reported here with Z-Sar-Leu-NH_2 and the observation with carboxypeptidase reported by Snoke and Neurath (1949). They observed a more than 2000-fold decrease in rate of hydrolysis of Bz-Sar-Phe as compared to Bz-Gly-Phe.

Recently Matsubara (1966) reported some studies on the specificity of thermolysin, another bacterial protease. The enzyme requires the absence of free amino and carboxyl groups from the immediate vicinity of the susceptible bond, and a bulky hydrophobic side chain on the amino acid whose amino group is involved in the peptide bond. Both Z-Gly-Phe-NH_2 and Z-Gly-Leu-hydroxide were good substrates whereas Z-Gly-Gly-NH_2 was not readily cleaved. The thermolysin appears to have a substrate specificity very similar to that of the neutral protease.

In light of these specificity requirements, the lack of observed esterase activity with the esters studied is understandable. An ester analogous to one of the dipeptide substrates, such as the leucic amide ester of benzyloxycarbonylglycine should be investigated. These studies are in progress.

Acknowledgment

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