Studies on the *Bacillus subtilis* Neutral-Protease- and *Bacillus thermoproteolyticus* Thermolysin-Catalyzed Hydrolysis of Dipeptide Substrates*

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ABSTRACT: A series of furylacryloyl dipeptide substrates for the *Bacillus subtilis* neutral protease and *Bacillus thermoproteolyticus* thermolysin have been synthesized and their enzyme-catalyzed hydrolyses were studied. The hydrolysis of these peptides which include furylacryloyl-Gly-Leu-NH_2, furylacryloyl-Gly-Phe-NH_2, furylacryloyl-Gly-Ile-NH_2, furylacryloyl-Leu-Leu-NH_2, furylacryloyl-Thr-Leu-NH_2, furylacryloyl-Leu-Ile-NH_2, and furylacryloyl-Phe-Phe-NH_2 was readily monitored spectrophotometrically at 345 mμ. Although both enzymes have very similar specificities, requiring a hydrophobic substituent on the amino acid donating the amino group of the peptide bond cleaved, quantitative differences were observed in the values of k_{cat}/K_m particularly when leucine was replaced by phenylalanine in the amino-donating position. Both enzymes exhibited identical bell-shaped pH profiles with pH optima near neutrality, for the enzyme-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 and furylacryloyl-Gly-Phe-NH_2. The values of k_{cat}/K_m increased in D_2O over H_2O for furylacryloyl-Gly-Leu-NH_2 with both enzymes. The decreases in the K_I for Z-His-Gly-NH_2 and in the apparent K_m values for other substrates suggest that an important effect of D_2O was to increase the enzyme–substrate binding and thus lower the K_m for the neutral protease dipeptide substrates.

The *Bacillus subtilis* neutral protease and the *Bacillus thermoproteolyticus* thermolysin have very similar substrate specificities toward both dipeptides and proteins despite the marked difference in the thermal stability of the enzymes. Both appear to be metalloendopeptidases with a specificity toward peptide bonds in which the amino group was contributed by amino acids having hydrophobic side chains such as leucine and phenylalanine (Matsubara *et al.*, 1965, Matsubara, 1966; Feder, 1967; Morihara, 1967). Identical bonds were cleaved in the β chain of insulin by both enzymes and these were similar to those cleaved in dipeptide substrates (Matsubara *et al.*, 1966; Feder and Lewis, 1967; Morihara *et al.*, 1968). Neither enzyme exhibits esterolytic activity toward a number of esters. Studies on the mechanism of action of these enzymes therefore have involved the use of di- or tripeptides, the hydrolysis of which does not lend itself to spectrophotometric monitoring techniques. The preparation of specific dipeptide substrates which undergo spectral perturbation when the dipeptide bond was hydrolyzed would be most useful.

Bernhard and coworkers (Bernhard *et al.*, 1965; Charner and Bernhard, 1967) described the synthesis and use of β-arylacryloyl derivatives for spectrophotometric identification of chymotrypsin and subtilisin acyl-enzyme intermediates. McClure and Neurath (1966) reported the use of *N-trans*-3-(2-furylacryloyl)-L-phenylalanine and *O-trans*-3-(2-furylacryloyl)-DL-β-phenyl lactate as chromophoric amide and ester substrates for carboxypeptidase A.

A number of furylacryloyl dipeptide substrates for the neutral proteases have been prepared which permit the dipeptide hydrolysis to be monitored spectrophotometrically (Feder, 1968). The study of the neutral-protease- and thermolysin-catalyzed hydrolysis of these substrates has yielded kinetic information concerning the comparative specificity of these two enzymes and some mechanistic implications.

Experimental Section

Materials

Crystalline thermolysin (*B. thermoproteolyticus* Rokko) was obtained from Daiwa Kasei Co., Ltd., Osaka, and used without further purification. The neutral protease in these studies was obtained from *B. subtilis* (NRRL B3411) fermentation beers by alcohol fractionation. Further purification was obtained by chromatography on hydroxylapatite (Keay, 1969) followed by Sephadex G-75 gel filtration. The enzyme was treated with 10^{-3} M DFP to inactivate any traces of subtilisin that might be present.

Z-His-Gly-NH_2,[1] Z-Thr-Leu-NH_2, Z-Gly, Leu-NH_2, Z-Gly-Leu-NH_2, and furylacryloyl-Ala-Phe-NH_2 were purchased from Cyclo Chemical Corp.

Bz-Gly-Leu-NH_2 was purchased from Mann Research Laboratories.

Deuterium oxide (isotope purity 99.8% minimum) and deuterium chloride (38% DCl in D_2O) were purchased from Mallinckrodt Chemical Co. Only Reagent grade salts and deionized water (conductivity below 5×10^{-7} mho) were used throughout these studies.

Methods

Protease determinations using casein substrate were carried out as described by Anson (1938). Protein concentra-

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[1] Abbreviations used are: Z, benzyloxycarbonyl; Bz, benzyl.

tion was determined by the biuret method (Gornall *et al.*, 1949). Standard curves were made using crystalline bovine serum albumin as the protein standard.

The enzyme-catalyzed hydrolysis of the furylacryloyl dipeptides was monitored spectrophotometrically at 345 mμ on a Cary 14 PM recording spectrophotometer equipped with a thermostated cell compartment at 25.0 ± 0.1° as described for furylacryloyl-Gly-Leu-NH_2 (Feder, 1968).

The enzyme-catalyzed hydrolysis of Z-Thr-Leu-NH_2, Z-Gly-Leu-NH_2, and Z-Gly-Phe-NH_2 was monitored spectrophotometrically at 225 mμ. A decrease in absorbance was observed as the dipeptide bond was hydrolyzed. The hydrolysis of Bz-Gly-Leu-NH_2 was monitored spectrophotometrically at 283 mμ. It was necessary to use a screen as a blank for these reactions because of the high absorbance of the substrate. A decrease in absorbance was observed upon the hydrolysis of the glycyl–leucine bond of Bz-Gly-Leu-NH_2 with an $\Delta\epsilon_{283\ m\mu}$ value of 31.8.

Zinc determinations were made by atomic absorption using a Beckman atomic absorption accessory, with an acetylene–air laminar flow burner, attached to a Beckman DU spectrophotometer and 10-in. recorder. Measurements were carried out at 213.9 mμ with 4.8 psi of acetylene, 20 psi of supporting air, and 8 mA through the zinc hollow cathode lamp. Standard curves for zinc were made over the range of 25–200 ppb of zinc.

The enzyme concentration for both the *B. subtilis* neutral protease and the thermolysin was based upon the activity of the purified crystalline enzyme toward casein or furylacryloyl-Gly-Leu-NH_2. The crystalline thermolysin had an activity toward casein of 13.2×10^6 units/g in our assay. This material contained 1.0 g-atom of zinc/38,000 molecule weight of protein. Latt *et al.* (1969) reported a similar zinc content for thermolysin. Ohta and coworkers (1966) reported a molecular weight of 37,500 for this enzyme. Thus there appeared to be one zinc atom per molecule of the enzyme. A value of $9.46 \times 10^3\ M^{-1}\ sec^{-1}$ was obtained for k_{cat}/K_m for the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 in pH 7.2 Tris buffer (μ = 0.1) basing the molar enzyme concentration on either the zinc concentration or protein concentration.

The neutral protease, however, was quite unstable. Purified enzyme lost activity when stored at −70°. The presence of autolysis products from the neutral protease made estimation of molarity difficult when based on the protein value. A linear relationship between the zinc concentration and the casein protease units has been reported by Yasunobu and coworkers (McConn *et al.*, 1964, 1967). A linear relationship also has been observed between the zinc concentration of the enzyme at various stages of purification and the first-order rate constants ($k = k_{cat}(E)/K_m$) for the neutral-protease-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2. Assuming one atom of zinc per molecule of enzyme (McConn *et al.*, 1964), k_{cat}/K_m was calculated using the zinc concentration as the enzyme molarity. Consistent values were obtained between various fractions and the purest fresh preparations corrected to 100% active enzyme. This yielded an activity of 25×10^6 units/g of protease toward casein substrate and a value of $5.06 \times 10^3\ M^{-1}\ sec^{-1}$ for k_{cat}/K_m for the neutral-protease-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 in pH 7.2 Tris buffer (μ = 0.1). Thus the enzyme molarity was estimated routinely from the first-order rate constants for the enzyme-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 ($k = k_{cat}(E)/K_m$) divided by the representative values of k_{cat}/K_m.

A Beckman Expandomatic pH meter was used to determine the pH before and after reaction. The pD was calculated from the pH meter reading according to Glasoe and Long (1960) (pD = pH meter reading + 0.40).

Synthesis of Furylacryloyl Derivatives of Dipeptides

3-(2-Furyl)acryloyl Chloride. Practical 2-furanacrylic acid (Eastman) was recrystallized from dimethylformamide–water to give yellow needles, mp 141–142.5°. To 20 g (0.145 mole) of recrystallized 2-furanacrylic acid was added 30 ml of reagent thionyl chloride (Fisher, d = 1.655, *ca.* 0.42 mole). The flask was fitted with a drying tube of $CaSO_4$ and the mixture was allowed to stand at room temperature for 2 hr at which time solution was complete. Excess thionyl chloride was stripped off at reflux temperature and reduced pressure. The brown-black residue was distilled at oil pump pressure (5.0 mm) to give 21.0 g (93%) of pale yellow liquid which crystallized in the receiver. The 3-(2-furyl)acryloyl chloride (mp 32–34°) was stable in excess of 6 months when stored at −20° and was used without further purification.

3-(2-Furyl)acryloylglycol-L-leucinamide. To 3.6 g of glycyl-L-leucinamide hydrochloride [mp 219–220°, $[\alpha]_D^{20}$ −20.3° (*c* 5, H_2O)] (0.016 mole) suspended in 40 ml of redistilled pyridine was added 2.95 g of crystalline 3-(2-furyl)acryloyl chloride (0.019 mole). The yellow complex slowly dissolved as did the glycyl-L-leucinamide hydrochloride. After 2 hr at room temperature, solution was complete and the reaction mixture was evaporated to dryness under reduced pressure. Water (20 ml) was added and the mixture was again evaporated to dryness. The oil was dissolved in 25 ml of ethyl alcohol, diluted to 100 ml with hot distilled water, treated with Norit, and filtered. Slow crystallization gave 3.9 g (80%) of furylacryloyl-Gly-Leu-NH_2 (mp 175–176°). *Anal.* Calcd for $C_{15}H_{21}N_3O_4$: C, 58.61; H, 6.86; N, 13.67. Found: C, 58.45; H, 6.81; N, 13.48.

3-(2-Furyl)acryloyl-L-phenylalanyl-L-phenylalaninamide. To 500 mg of L-phenylalanyl-L-phenylalaninamide hydrochloride (1.44 mmoles, Cyclo Chemical Corp.), suspended in 4.0 ml of redistilled pyridine, was added 290 mg of 3-(2-furyl)-acryloyl chloride (1.86 mmoles). The mixture was stirred at room temperature for 2 hr. Solution was almost complete and then precipitate began to form. The mixture was stirred for an additional hour and then heated on a steam bath for 5 min. The suspension was cooled and diluted with 50 ml of distilled water. Solid was recovered by filtration and recrystallized from ethyl alcohol–water. Filtration gave 536 mg (86.5%) of fine needles of furylacryloyl-Phe-Phe-NH_2 (mp 259–260.5°). *Anal.* Calcd for $C_{25}H_{25}N_3O_4$: C, 69.59; 5; 84.H, N, 9.74. Found: C, 69.52; H, 5.86; N, 9.66.

3-(2-Furyl)acryloyl-L-leucyl-L-isoleucinamide. To 200 mg of L-leucyl-L-isoleucinamide hydrochloride (0.715 mmole) suspended in 5.0 ml of anhydrous pyridine was added 146 mg of 3-(2-furyl)acryloyl chloride (0.94 mmole). The mixture was stirred at room temperature for 2 hr and then heated on a steam bath for 15 min. After addition of 15 ml of water followed by 15-min stirring at room temperature, the mixture was diluted to 50 ml with water. The product was collected by filtration and recrystallized from ethyl alcohol–water to give 181 mg (70%) of furylacryloyl-Leu-Ile-NH_2 (mp

TABLE 1: Comparison of Neutral-Protease- and Thermolysin-Catalyzed Hydrolysis of Some Dipeptide Substrates.[a]

Substrate		Neutral Protease		Thermolysin	
	$(S_0) \times 10^4$ M	$k_{cat}/K_m \times 10^{-3}$ M^{-1} sec^{-1}	$(E_0) \times 10^7$ M	$k_{cat}/K_m \times 10^{-3}$ M^{-1} sec^{-1}	$(E_0) \times 10^7$ M
Furylacryloyl-Gly-Leu-NH_2	9.60	5.06	20.77	9.46	16.51
Z-Gly-Leu-NH_2	15.37	0.98	15.63	1.48	18.25
Bz-Gly-Leu-NH_2	3.41	0.77	24.85	1.15	16.86
Furylacryloyl-Thr-Leu-NH_2	7.22	39.76	12.63	20.03	4.42
Z-Thr-Leu-NH_2	13.25	4.87	8.07	4.03	9.44
Furylacryloyl-Leu-Leu-NH_2[b]	0.59	29.50	6.64	16.20	4.42
Furylacryloyl-Gly-Ile-NH_2	8.87	7.00	12.63	5.33	4.42
Furylacryloyl-Leu-Ile-NH_2[b]	0.55	22.98	6.64	5.54	8.70
Furylacryloyl-Gly-Phe-NH_2	11.16	0.23	67.08	2.62	13.13
Z-Gly-Phe-NH_2	13.84	0.07	12.73	1.55	18.25
Furylacryloyl-Ala-Phe-NH_2	2.50	0.68	56.17	16.27	32.49
Furylacryloyl-Phe-Phe-NH_2[b]	0.40	32.97	20.10	368.2	1.05

[a] All reactions carried out in pH 7.2 Tris buffer (μ = 0.1). [b] Buffers contained 5% dioxane; expanded scale of the spectrophotometer used when monitoring these reactions. Experimental details described in section on Methods.

278–279°). *Anal.* Calcd for $C_{19}H_{29}N_3O_4$: C, 62.79; H, 8.04; N, 11.56. Found: C, 62.97; H, 8.05; N, 11.51.

3-(2-Furyl)acryloylglycyl-L-phenylalaninamide. PROCEDURE AS FOR FURYLACRYLOYL-Leu-Ile-NH_2. From 665 mg of glycyl-L-phenylalaninamide hydrochloride (2.59 mmoles, New England Nuclear) was obtained 815 mg (92%) of recrystallized furylacryloyl-Gly-Phe-NH_2 (mp 211.5–213°). *Anal.* Calcd for $C_{18}H_{18}N_3O_4$: C, 63.51; H, 5.33; N, 12.35. Found: C, 63.47; H, 5.67; N, 12.16.

3-(2-Furyl)acryloyl-L-leucyl-L-leucinamide. PROCEDURE AS FOR FURYLACRYLOYL-Leu-Ile-NH_2. From 300 mg of L-leucyl-L-leucinamide hydrochloride·H_2O (1.01 mmoles, Cyclo Chemical Corp.) was obtained 296 mg (81%) of recrystallized furylacryloyl-Leu-Leu-NH_2 (mp 223–224.5°). *Anal.* Calcd for $C_{19}H_{29}N_3O_4$: C, 62.79; H, 8.04; N, 11.56. Found: C, 62.63; H, 8.06; N, 11.50.

3-(2-Furyl)acryloylglycyl-L-isoleucinamide Monohydrate. PROCEDURE AS FOR FURYLACRYLOYL-Leu-Ile-NH_2. From 178 mg of glycyl-L-isoleucinamide hydrochloride (0.80 mmole) was obtained 222 mg (85%) of recrystallized furylacryloyl-Gly-Ile-NH_2·H_2O (mp 228–229°). *Anal.* Calcd for $C_{15}H_{23}N_3O_5$: C, 55.37; H, 7.12; N, 12.92. Found: C, 55.53; H, 7.00; N, 12.93.

3-(2-Furyl)acryloyl-L-threonyl-L-leucinamide. To 35 mg of palladium black (Engelhard lot 1190), suspended in 6 ml of 1.0 N HCl, was added 480 mg of Z-Thr-Leu-NH_2 (1.31 mmoles, Cyclo Chemicals, lot M 3425) in 30 ml of absolute methyl alcohol. The system was pressurized to 30 psi with H_2 and allowed to reduce overnight with shaking. The mixture was filtered and evaporated to dryness to give crude Thr-Leu-NH_2·HCl. The crude product was dissolved into 15 ml of redistilled pyridine. To the solution was added 215 mg of pure furylacryloyl chloride. The mixture was stirred at room temperature for 7 hr. After addition of 3.0 ml of distilled water, the solution was evaporated to dryness. Repeated attempts to crystallize the product were unsuccessful. Evaporation to dryness did give a glassy solid which was leached with 20 ml of water to remove salts. The residue was dissolved into absolute ethanol, transferred to a tared flask, and evaporated under oil pump vacuum to give 235 mg of crude furylacryloyl-Thr-Leu-NH_2. This was used without further purification.

Furylacryloyl-Gly-OEt. To a suspension of 13.9 g (0.1 mole) of Gly-OEt-HCl in 200 ml of toluene containing 50 ml of redistilled pyridine was added the 15.6 g of 3-(2-furyl)-acryloyl chloride dissolved in toluene. The mixture was stirred at room temperature for 2 hr and then warmed on a steam bath. Much of the initial precipitate dissolved. Solvent was removed under reduced pressure. The solid was triturated with a solution of 15 ml of concentrated HCl in 150 ml of distilled water. After filtration, the solid was washed with H_2O and then dissolved in absolute ethanol. The solution was evaporated to dryness and then recrystallized from 100 ml of absolute ethanol to give 17.9 g of product (mp 127–127.5°).

Furylacryloyl-Gly. To 3.7 g (0.0166 mole) of furylacryloyl-Gly-OEt dissolved in 20 ml of absolute ethanol was added 20 ml of distilled water and 1.0 g of KOH. The solution was boiled in an open vessel until volume was reduced to *ca.* 30 ml. After cooling to 0°, concentrated HCl was added dropwise to a pH of 1. A precipitate formed immediately on acidification. Product was washed twice with 30-ml aliquots of distilled H_2O to give 3.0 g (mp 207–210°). Repeated recrystallization from 50:50 ethanol–water gave a product (mp 213–215°). *Anal.* Calcd for $C_9H_9NO_4$: C, 55.38; H, 4.64; N, 7.18. Found: C, 55,77; H, 4.77. Analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

Results

A comparison of the neutral-protease- and thermolysin-catalyzed hydrolysis of a series of dipeptide substrates is

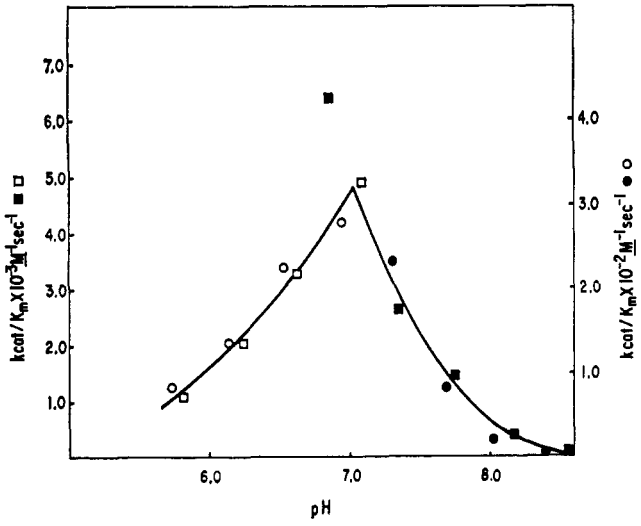

FIGURE 1: Comparison of pH dependencies of neutral-protease- and thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Phe-NH_2. (○) Neutral protease ($(E_0) = 6.71–10.37 \times 10^{-6}$ M), (□) thermolysin ($(E_0) = 1.31 \times 10^{-6}$ M), (●, ■) Tris buffer ($\mu = 0.1$), (○, □) cacodylate buffer ($\mu = 0.1$), and $(S_0) = 1.116 \times 10^{-3}$ M. Experimental details are given in section on Methods.

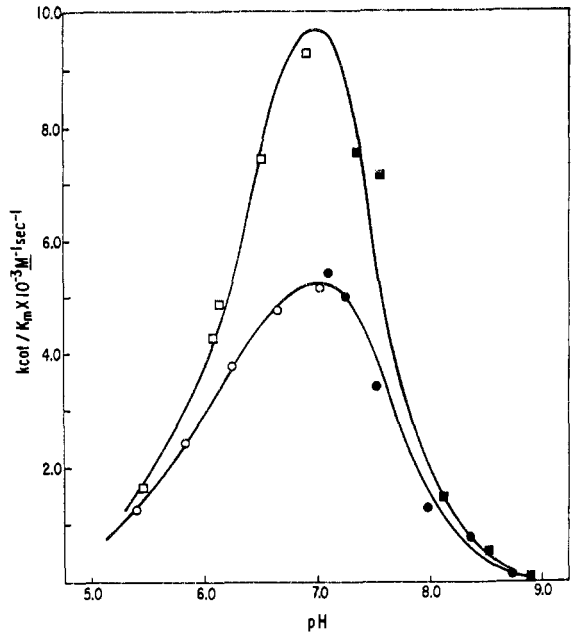

FIGURE 2: Comparison of pH dependencies of neutral-protease- and thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2. (○) Neutral protease ($(E_0) = 2.20 \times 10^{-6}$ M), (□) thermolysin ($(E_0) = 2.39 \times 10^{-6}$ M), (●, ■) Tris buffer ($\mu = 0.1$), (○, □) cacodylate buffer ($\mu = 0.1$), and $(S_0) = 1.02 \times 10^{-3}$ M.

shown in Table I. Limited solubility and high K_m values of the furylacryloyl dipeptide substrates precluded determination of dependable K_m values so that all of these reactions were carried out at low substrate concentration under pseudo-first-order conditions. The furylacryloyl-blocked dipeptides showed considerable increase in k_{cat}/K_m over the corresponding carbobenzoxyl and benzoyl derivatives. Thus, k_{cat}/K_m values of 5.06×10^3 and 9.46×10^3 sec^{-1} M^{-1} were obtained for the neutral-protease- and thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 at pH 7.2 Tris buffer ($\mu = 0.1$), respectively, while Z-Gly-Leu-NH_2 yielded values of 9.77×10^2 and 1.484×10^3 sec^{-1} M^{-1} and Bz-Gly-Leu-NH_2 yielded values of 7.68×10^2 and 1.15×10^3 M^{-1} sec^{-1}, respectively. The most pronounced specificity difference between the enzymes at the dipeptide level was observed when phenylalanine was the amino acid donating the amino group of the bond cleaved. Secondary effects due to the substituents on the amino acid donating the carboxyl group of the peptide bond cleaved were qualitatively similar for both enzymes. Values of 7.9 and 2.11 were obtained for the ratios of k_{cat}/K_m for furylacryloyl-Thr-Leu-NH_2/furylacryloyl-Gly-Leu-NH_2 as catalyzed by neutral protease and thermolysin, respectively. Considerable effects were obtained by a benzyl substituent on the carboxyl-donating amino acid. The ratio of k_{cat}/K_m values for furylacryloyl-Phe-Phe-NH_2/furylacryloyl-Gly-Phe-NH_2 was 143 for neutral protease and 140 for thermolysin. Furylacryloyl-Gly-Ile-NH_2 was about equivalent to furylacryloyl-Gly-Leu-NH_2 for the neutral protease but only about half as effective as the latter for thermolysin. Thus it appears that although the major specificity requirements for dipeptide substrates is quite similar for both enzymes the quantitative effects are different.

A comparison of the pH dependence of the neutral-protease- and thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Phe-NH_2 is shown in Figure 1. Identical pH profiles were obtained in spite of a considerable difference in the relative magnitude of k_{cat}/K_m for both enzymes. Similar bell-shaped pH profiles also were obtained for the neutral-protease- and thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 as shown in Figure 2. Both enzymes were inhibited by phosphate and to a lesser extent by cacodylate as compared with acetate buffers. The pD dependence

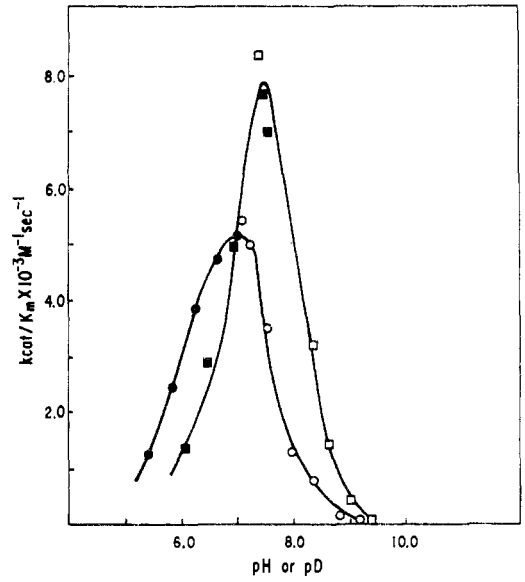

FIGURE 3: The pH and pD dependence of the neutral-protease-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2. (○) H_2O, (□) D_2O, (○, □) Tris buffer ($\mu = 0.1$), (●, ■) cacodylate buffer ($\mu = 0.1$). $(E_0) = 2.20–2.39 \times 10^{-6}$ M $(S_0) = 0.98$ -- 1.02×10^{-3} M. Additional details in text.

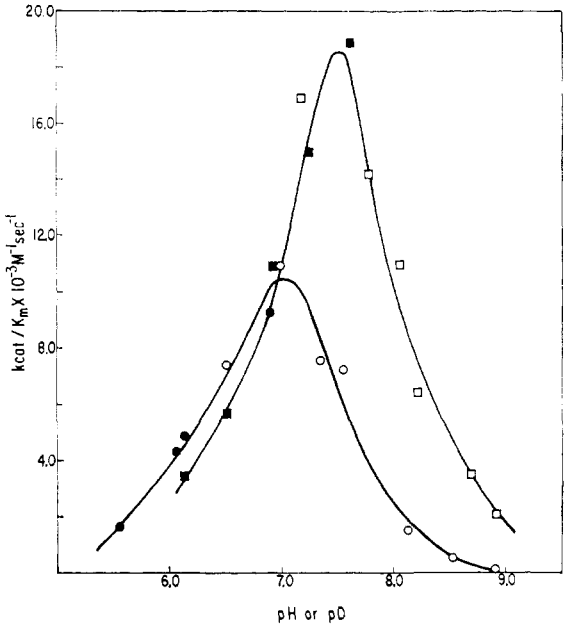

FIGURE 4: The pH and pD dependence of the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2. (○) H_2O, (□) D_2O, (○, □) Tris buffer (μ = 0.1), (●, ■) cacodylate buffer (μ = 0.1). (E_0) = 0.86–2.39 × 10^{-6} M and (S_0) = 0.96–1.02 × 10^{-3} M. Additional details in text.

of the neutral-protease-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 is shown in Figure 3. An upward shift of about 0.5 pH unit was observed with an increase in the value of k_{cat}/K_m in D_2O over H_2O. A similar D_2O effect was obtained with thermolysin (Figure 4).

As indicated above it was not possible to obtain dependable values of K_m and k_{cat} for either the neutral-protease- or thermolysin-catalyzed hydrolyses of furylacryloylglycyl-L-leucinamide or any of the other furylacryloyl dipeptides due to their limited solubilities and high K_m values. Consequently, the direct effect of D_2O on k_{cat} or K_m could not be determined. Increases in k_{cat}/K_m in D_2O could occur as a result of decreased values of K_m, increased k_{cat} values, or even decreased values of k_{cat} but greater decreased values of K_m. However, a few indirect approaches were used to study D_2O effects on the binding of substrates to neutral protease. Z-His-Gly-NH_2 is an inhibitor of neutral protease. Under pseudo-first-order conditions ((S) ≪ K_m) one cannot distinguish competitive or noncompetitive inhibition but both yield the rate expression

$$\frac{dp}{dt} = \frac{k_{cat}(E)(S)}{K_m(1 + (I)/K_I)}$$

and the first-order rate constant obtained is

$$k = \frac{k_{cat}(E)}{K_m(1 + (I)/K_I)}$$

The ratio of the first-order rate constants, π, in the presence and absence of inhibitor yield $1 + (I)/K_I$ from which the value of K_I is readily determined.

TABLE II: Effect of Z-His-Gly-NH_2 on Neutral-Protease-Catalyzed Hydrolysis of Furylacryloyl-Gly-Leu-NH_2.[a]

(I) × 10^2 M		$k \times 10^3$ sec^{-1}	$\pi = k_0/K_I$	$K_I \times 10^2$	pH or pD
0		8.27	1.22	6.33	7.01
	H_2O				
1.393		6.78			7.01
0		10.41			7.49
	D_2O				
1.402		6.85	1.52	2.70	7.49

[a] (S_0) = 9.46 × 10^{-4} M, Tris buffer (μ = 0.1). Experimental details in text.

$$\pi = \frac{k_{uninhib}}{K_{inhib}} = 1 + (I)/K_I$$

The effect of Z-His-Gly-NH_2 upon the first-order rate constants for neutral-protease-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 was determined in H_2O and D_2O and K_I values were obtained. These results are shown in Table II. A K_I of 6.33 × 10^{-2} M was obtained in H_2O for Z-His-Gly-NH_2 at pH 7.01 while values of 2.70 × 10^{-2} and 3.30 × 10^{-2} M were obtained in D_2O at pD values of 7.40 and 8.0, respectively. Thus, it appeared that this dipeptide inhibitor was better bound to the enzyme in D_2O than in H_2O.

It is also possible to measure the apparent K_m of a competitive substrate to furylacryloyl-Gly-Leu-NH_2 in the presence of H_2O and D_2O. The rate expression for the hydrolysis of one substrate in the presence of a competing substrate is given by

$$\frac{dp_1}{dt} = \frac{k_{cat_1}(E_0)(S_1)}{(S_1) + K_{m_1}(1 + (S_2)K_{m_2})}$$

Only the $(S)/K_m$ of the competing substrate appears in the rate expression. Under pseudo-first-order rate conditions the first-order rate constants, k, obtained is given by

$$k_1 = \frac{k_{cat_1}(E_0)}{K_{m_1}(1 + (S_2)/K_{m_2})}$$

The ratio of k in the presence and absence of the competing substrate yields, π, which is equivalent to $1 + (S_2)/K_{m_2}$.

If the rate constants are obtained from initial rate data at different concentrations of S_1 but constant S_2 one can readily evaluate this data for K_{m_2}.

Likewise, if the reaction with S_1 is much faster than with S_2 so that it is over before much of S_2 has reacted one might assume a constant concentration of S_2 for the determination of K_{m_2}. A few competitive substrates were thus examined with furylacryloyl-Gly-Leu-NH_2. An apparent K_m of 8 × 10^{-2} was obtained for the neutral-protease-catalyzed hydrolysis of Z-Thr-Leu-NH_2 at pH 7.0 Tris buffer (μ = 0.1) in H_2O. In D_2O an apparent K_m of 2.15 × 10^{-2} M was obtained.

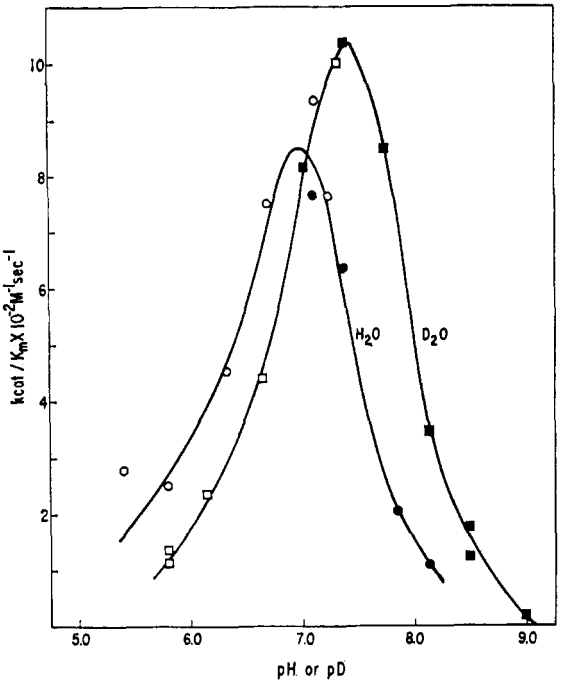

FIGURE 5: The pH and pD dependence of the neutral-protease-catalyzed hydrolysis of Bz-Gly-Leu-NH_2. (●, ■) Tris buffer (μ = 0.1); (○, □) cacodylate buffer (μ = 0.1). $(E_0) = 7.77 \times 10^{-6}$ M and $(S_0) = 1.69 \times 10^{-2}$ M. Additional details in text.

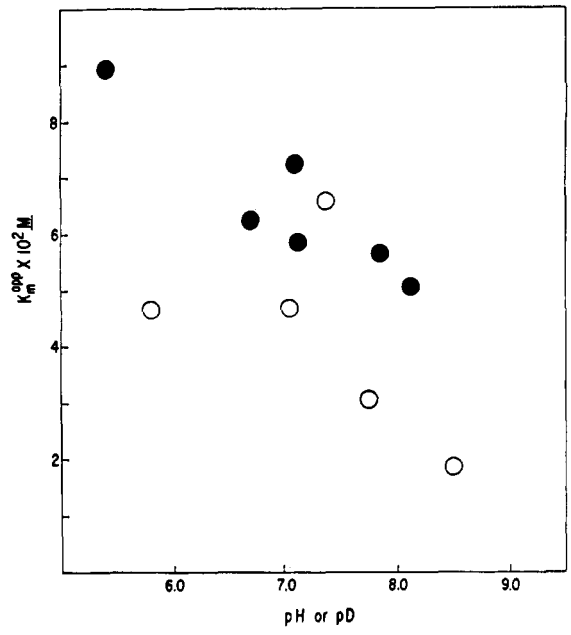

FIGURE 6: The pH and pD dependence of the $K_{m,app}$ for the neutral-protease-catalyzed hydrolysis of Bz-Gly-Leu-NH_2. (●) H_2O and (○) D_2O. $(E_0) = 7.77 \times 10^{-6}$ M and $(S_0) = 1.69 \times 10^{-2}$ M. Additional details in text.

The concentration of the competing substrate Z-Thr-Leu-NH_2 was 5.39×10^{-3} M while the furylacryloyl-Gly-Leu-NH_2 concentration was varied between 3.20×10^{-4} and 16.0×10^{-4} M. It had been observed that larger polypeptides apparently were better bound to the neutral protease than the simple dipeptide substrates based upon effects as competitive substrates to furylacryloyl-Gly-Leu-NH_2. Bovine serum albumin was examined as a competitive substrate with the neutral protease. Understandably it would be most difficult to write a true K_m for the bovine serum albumin molecule since there are a number of sites per molecule for reaction and the peptide products are also substrates. However, one can approximate an overall apparent K_m for bovine serum albumin by using initial conditions and considering most initial reactions about equivalent. As a competitive substrate to furylacryloyl-Gly-Leu-NH_2 in pH 7.15 Tris buffer (μ = 0.1) the bovine serum albumin yielded an apparent K_m of 1.30×10^{-4} M. This is at a concentration of bovine serum albumin of 1.480×10^{-4} M, assuming a molecular weight of 66,500 (Schachman, 1963). In D_2O at a pD of 7.58 an average value of 0.80×10^{-4} M for K_m apparent was obtained.

Because of the accumulation of considerable product in following complete reactions with some of the dipeptide substrates, the inhibition constants were determined for leucinamide, carbobenzoxylglycine, and furylacryloylglycine. These were determined from the first-order rate constants for the neutral-protease-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 in the presence and absence of inhibitor as described above. A K_I value of 0.19 M was obtained for leucinamide at pH 7.20 Tris buffer with an inhibitor concentration of 0.119 M. The Z-Gly and furylacryloyl-Gly yielded K_I values of 0.161 and 0.10 M at inhibitor concentrations of 5.25×10^{-2} and 5.16×10^{-3} M, respectively, under the same conditions. No effect of product inhibition would therefore be expected using substrate concentrations of furylacryloyl-Gly-Leu-NH_2 of 9.60×10^{-4} M. No effect on k_{cat}/K_m was obtained when different concentrations of substrate were used with a constant enzyme concentration as would be expected if any significant product inhibition were observed.

The K_m for the neutral-protease-catalyzed hydrolysis of Bz-Gly-Leu-NH_2 was lower than that for furylacryloyl-Gly-Leu-NH_2. Although it was still not possible to obtain substrate concentrations above the K_m, the $(S)/K_m$ values were much better than those for furylacryloyl-Gly-Leu-NH_2 and the reactions were not pseudo first order. A FORTRAN IV program was written to obtain complete Lineweaver–Burk plots from single reactions run to near completion. Least-squares values were obtained from the data for calculation of the V_{max}, K_m, and V_{max}/K_m. Standard errors were calculated by the computer and only the reactions which yielded reproducible data repeatedly, were used. A small but significant D_2O effect on k_{cat}/K_m was observed as shown in Figure 5.

Figure 6 shows the pH and pD dependence of the $K_{m,app}$ values. An increase in K_m was observed as the pH increased. However, the values in D_2O were significantly lower than those in H_2O over the pH range studied.

Discussion

The use of *N*-furylacryloyl-blocked dipeptides has permitted simple spectrophotometric monitoring for the hydrolysis of dipeptide substrate as catalyzed by neutral proteases. The similarity in specificities between the *B. subtilis* neutral protease and the *B. thermoproteolyticus* thermolysin allow the same substrates to be used for simple spectrophotometric assay of both of these enzymes. At the dipeptide level quantita-

tive differences in k_{cat}/K_m for the neutral-protease- and thermolysin-catalyzed hydrolysis of various substrates suggest slight specificity differences between these enzymes. The major differences seem to center about the effect of the phenylalanine or leucine in the amino-donating position of the dipeptide bond. Similar differences have been reported by Morihara and coworkers (1967, 1968). Secondary effects associated with the side chain of the amino acid donating the carboxyl of the peptide bond were quite similar for both enzymes as demonstrated by the increases in k_{cat}/K_m obtained for Z-Thr-Leu-NH_2, furylacryloyl-Ala-Phe-NH_2, furylacryloyl-Thr-Leu-NH_2, furylacryloyl-Phe-Phe-NH_2, furylacryloyl-Leu-Leu-NH_2, and furylacryloyl-Leu-Ile-NH_2. Generally it appeared that the benzoyl- or carbobenzoxyl-blocked dipeptides had smaller K_m values (measureable) than the corresponding furylacryloyl dipeptides.

It is interesting to compare Bz-Gly-Leu-NH_2 and furylacryloyl-Gly-Leu-NH_2. At pH 7.2 the $K_{m,app}$ for neutral-protease-catalyzed hydrolysis of Bz-Gly-Leu-NH_2 was 6.5×10^{-2} M. Although not as dependable, the K_m for furylacryloyl-Gly-Leu-NH_2 appeared to be in excess of 0.1 M. If we then calculate the k_{cat} values for both of these substrates the effect of a furylacryloyl substitution for a benzoyl group resulted in a tenfold increase in k_{cat} while the $K_{m,app}$ decreased in half. The $K_{m,app}$ values appear to be considerably smaller for serum albumin than the dipeptide substrate. Recently, Morihara and coworkers (1968, 1969) have reported studies with larger peptides and suggested that the neutral protease and thermolysin have an active site accommodating up to six amino acids of the substrate. Although essentially the same specificity was observed from the neutral-protease- and thermolysin-catalyzed hydrolysis of large polypeptides as with the simple dipeptide substrates, the differences could show up kinetically both in the catalytic and binding parameters.

It is interesting to note that Morihara and coworkers (1968) have reported different pH dependencies for neutral protease and thermolysin with casein substrate. The neutral protease yielded a sharp optimum at pH 7.0, similar to what has been reported here with dipeptides while the thermolysin displayed a broad pH optimum between pH 7 and 8.5. No such difference has been observed between the neutral protease and thermolysin with both furylacryloyl-Gly-Leu-NH and furylacryloyl-Gly-Phe-NH_2. Because of the possibility of a large binding site, differences between the neutral protease and thermolysin which are not seen with small peptides could appear with larger polypeptide or protein substrates. When one compared the activity of these two enzymes toward casein as compared with a simple dipeptide this difference was quite evident. The k_{cat}/K_m values for the neutral-protease- and thermolysin-catalyzed hydrolyses of furylacryloyl-Gly-Leu-NH_2 at pH 7.20 Tris buffer ($\mu = 0.1$) were 5.06×10^3 and 9.46×10^3 sec^{-1} M^{-1}, respectively. Expressing $k/(E)$ ($k = k_{cat}(E_0)/K_m$) for the neutral-protease- and thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 using units of protease activity toward casein for the enzyme concentration, values of 5.4×10^{-6} and 18.82×10^{-6} sec^{-1} $(units/ml)^{-1}$, respectively, were obtained.

The pH dependence of the $K_{m,app}$ for the neutral-protease-catalyzed hydrolysis of Bz-Gly-Leu-NH_2 yielded decreasing values as the pH increased. A bell-shaped pH dependence of k_{cat} was obtained with a dependence upon two ionizable groups with pK values close to neutrality. Although it was not possible to determine the K_m for furylacryloyl-Gly-Leu-NH_2 and furylacryloyl-Gly-Phe-NH_2, if a similar pH dependence would exist then the pH profiles of k_{cat} would also be bell shaped, reflecting a dependence on two ionizable groups both near neutrality. Whether one or both of these might be an imidazole is certainly speculative at this point.

The D_2O effects observed upon both enzymes seem to be in part at least an effect on the K_m of the substrate. The K_I of Z-His-Gly-NH_2 was significantly decreased in D_2O over H_2O. The $K_{m,app}$ values for the competitive substrates Z-Thr-Leu-NH_2 and bovine serum albumin were also significantly lowered in D_2O. Finally the pH and pD dependencies of the $K_{m,app}$ values for the neutral-protease-catalyzed hydrolysis of hippurylleucinamide was decreased by about 60% in D_2O as compared with H_2O. Bender and Hamilton (1962) reported a value for $K_{m,app}$ $(H_2O)/K_{m,app}$ (D_2O) of 2.2–6.0 for the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester. They suggested a more tightly bound substrate in D_2O than in H_2O particularly with a substrate such as trimethyl acetate which has large hydrocarbon-like parts. Likewise, the K_m values for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan ethyl ester were about twice as large in H_2O as in D_2O as reported by Bender and coworkers (1964). There thus appears to be some observations with another proteolytic enzyme in which the $K_{m,app}$ values decreased in D_2O. The neutral protease substrates have hydrophobic side chains and similar D_2O effects on $K_{m,app}$ or K_I values were observed. Further efforts on the effect of D_2O on the binding of simple molecules might yield information which will help define the nature of the enzyme–substrate complex. The shift of the pH profile of about 0.5 pH unit to higher pH in D_2O is consistent with the effect of D_2O on the pK's of acids (Hogfield and Bigeleisen, 1960). It does not, however, identify the ionizable groups involved.

In conclusion, the use of a furylacryloylamino-blocking substituent has permitted the kinetic study of enzyme-catalyzed hydrolysis of dipeptides by simple spectrophotometric techniques. This should prove to be a useful tool for the mechanism of proteolytic enzymes with peptidic substrates, as opposed to the esterolytic catalysis of these enzymes.

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Chicken Pepsinogens and Pepsins. Their Isolation and Properties*

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ABSTRACT: Pepsinogens A, D, and C have been isolated from the gastric mucosae of chickens using ion-exchange and molecular sieve chromatography and were found to have molecular weights of approximately 42,000. Pepsinogens A and D are closely related in their amino acid compositions, electrophoretic mobilities, and stabilities. Pepsinogen C is significantly different in these properties. Unlike swine pepsin, chicken pepsins A and D are stable at neutral pH and have molecular weights similar to their precursors. They contain over twenty of the approximately thirty basic amino acid residues originally present in the respective precursors. Pepsin C has a molecular weight of about 38,500, indicating that a larger peptide fragment is removed from pepsinogen C during the conversion process.

Multiple forms of pepsinogen,[1] which yield enzymes active at acid pH, have been found in the gastric mucosae of several species. In the most extensive studies, three or four pepsinogens have been extracted from the gastric mucosae of the swine (Ryle, 1960, 1965), chicken (Levchuk and Orekhovich, 1963), human (Seijffers *et al.*, 1963), and dogfish (Merrett *et al.*, 1969). The purification and properties of three chicken pepsinogens and the pepsins derived from them are the subject of this communication. Their immunological interrelationships are reported in the accompanying paper (Donta and Van Vunakis, 1970a).

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[1] The nomenclature adopted by various authors for the pepsinogens has not been uniform. Because of the similarities of isolation techniques and elution patterns from ion-exchange columns, we have used the system adopted by Ryle (1965) for the swine pepsinogens, as recommended by the Commission on Enzymes of the International Union of Biochemistry.

Materials and Methods

Unless otherwise stated, materials were similar to those used in the study of the dogfish pepsinogens by Merrett *et al.* (1969). All chromatographic and preparative procedures were carried out at 0–5°. DE-11 and DE-52 ion-exchange cellulose (Whatman) and Sephadex G-100 (Pharmacia) were used for the isolation of the pepsinogens. Sodium phosphate buffer, pH 6.9, was used in most of the purification procedures since it was found to give the best separation of the proteins. However, in one scheme, the protein isolations were carried out in Tris buffer, pH 7.5.

Proteolytic and potential proteolytic activities were determined using hemoglobin (Anson, 1938) or milk (Herriott, 1938) as substrates. Peptidase activity was determined by measuring the extent of hydrolysis of the synthetic substrate Cbz-L-Glu-L-Tyr (CGT) using the ninhydrin assay (Moore and Stein, 1954). Details of these methods, as well as the modifications used, are outlined in Merrett *et al.* (1969).

Proteins were concentrated with an Amicon Corp. Diaflo ultrafiltrator, using a UM-1 membrane (10,000 mol wt cutoff). Analytical acrylamide gel electrophoresis was performed by the method of Ornstein and Davis (1964), except that no sample gel was used. Acrylamide gel electrophoresis in sodium