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Synthesis and Hydrolysis by Pepsin and Trypsin of a Cyclic Hexapeptide Containing Lysine and Phenylalanine

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1. A cyclic hexapeptide, *cyclo*(-Gly_2-Phe_2-Gly-Lys-), and the corresponding open-chain hexapeptides, Gly_2-Phe_2-Gly-Lys and Phe-Gly-Lys-Gly_2-Phe, have been synthesized and their susceptibilities to the hydrolytic action of pepsin and trypsin were determined.

2. The cyclic peptide was hydrolyzed slowly by trypsin to a hexapeptide Gly_2-Phe_2-Gly-Lys, the value of the Michaelis constant for this reaction being $K_m = 0.010$ M, whereas Phe-Gly-Lys-Gly_2-Phe was hydrolyzed rapidly to yield Phe-Gly-Lys and Gly_2-Phe; $K_m = 0.00022$ M.

3. The cyclic peptide was not cleaved by pepsin at all, but Gly_2-Phe_2-Gly-Lys was hydrolyzed rapidly at a Phe-Phe bond; $K_m = 0.0091$ M.

4. The cyclic peptide inhibits the hydrolysis of Gly_2-Phe_2-Gly-Lys by pepsin in a linear noncompetitive manner, the value of the inhibition constant being $K_i = 0.004$ M.

From the studies using synthetic substrates it has been demonstrated that pepsin hydrolyzes preferentially the substrates with aromatic amino acids on one or both sides of the susceptible peptide bond [1]. Although several linear peptides have been synthesized for the substrate of pepsin, a cyclic peptide substrate has never yet been known. Kailova *et al.* have reported that *cyclo*(-Gly-Phe-Leu-)$_2$ was not cleaved by pepsin and no inhibitory effect of this compound toward the enzyme was observed [2].

On the other hand, a few cyclic peptide substrates for trypsin have been synthesized. In previous communications from this laboratory it was shown that *cyclo*(-Gly_5-Lys-) [3] and *cyclo*(-Gly_2-Lys-)$_2$ [4] were completely hydrolyzed by trypsin at the lysine carbonyl linkage, whereas dipeptide anhydrides which contain L-lysine were not hydrolyzed by the enzyme [5]. Kenner *et al.* have reported that *cyclo*(-Gly-Lys-Gly-Lys-Gly-) was not cleaved by trypsin nor did the peptide inhibit the tryptic cleavage of the corresponding linear pentapeptide [6]. These results suggest that a certain minimum ring size may be necessary for the hydrolysis of cyclic peptides by the enzyme.

Abbreviations. Abbreviations follow the IUPAC-IUB Commission on Biochemical Nomenclature Symbols for Amino-Acid Derivatives and Peptides Recommendations (1971). Amino acid symbols except Gly denote the L-configuration.

Enzymes (CBN Recommendations 1972). Pepsin (EC 3.4.23.1); trypsin (EC 3.4.21.4); chymotrypsin (EC 3.4.21.1).

In a previous paper from this laboratory, several oligopeptides of a type A-X-Y-B (X and Y, Tyr or Phe) were synthesized and the effect of the elongation of peptide chains at positions of A and B was examined [7]. Among the synthesized substrates, Gly_2-Phe_2-Gly and Gly_2-Phe-Tyr-Gly are most susceptible to the hydrolysis by pepsin. In this connection we attempted to examine a mode of action of pepsin on cyclic peptides. It is expected that a cyclic peptide composed of glycine and phenylalanine residues exclusively is sparingly soluble in an acidic buffer [8], and the presence of a lysine residue in such a peptide seems to render adequate solubility in the buffer. Therefore, in the present study *cyclo*(-Gly_2-Phe_2-Gly-Lys-) (compound XV, see Table 1) was synthesized and the kinetic behavior of this peptide toward pepsin and trypsin was examined. In addition, the corresponding linear peptides, Gly_2-Phe_2-Gly-Lys (XVII) and Phe-Gly-Lys-Gly_2-Phe (XIX) were prepared and subjected to the action of the enzymes. Furthermore, the experiments investigating inhibition by *cyclo*(-Gly_2-Phe_2-Gly-Lys-) towards pepsin action were carried out using the synthetic and protein substrates.

The cyclic benzyloxycarbonyl-substituted cyclic hexapeptide (XIV) was obtained by two different routes shown in Fig. 1 and 2.

The cyclization reaction of the linear hexapeptide derivative (VIII) was achieved by means of the azide method in pyridine [9] in an excellent yield. As shown

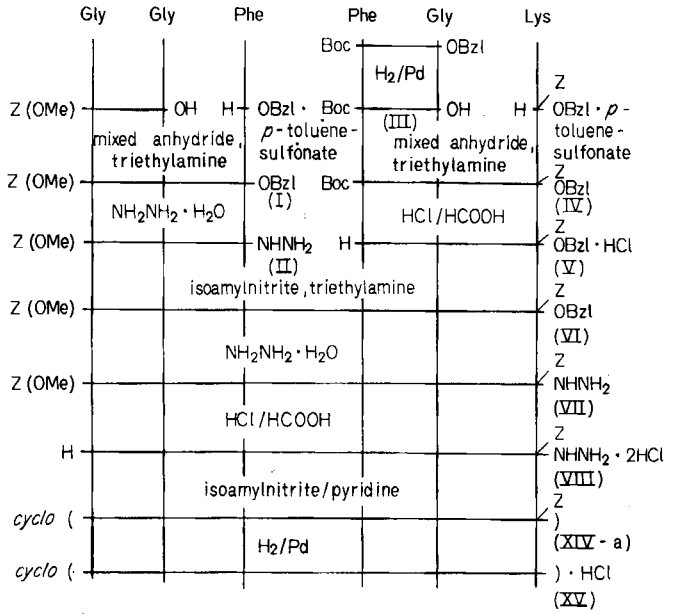

Fig. 1. *Synthesis of the cyclic hexapeptide (XV)*

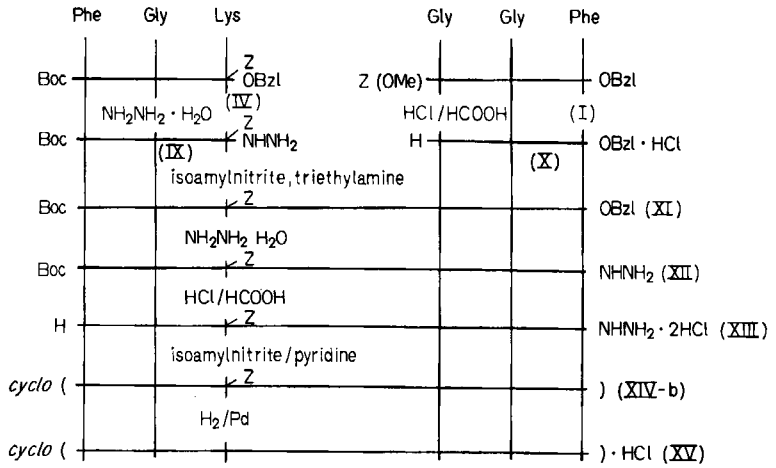

Fig. 2. *Synthesis of the cyclic hexapeptide (XV)*

in Fig. 3, two linear hexapeptides (XVII and XIX) were prepared from the intermediates of the synthesis of XIV.

MATERIALS AND METHODS

SYNTHETIC PROCEDURES

All melting points are uncorrected. Thin-layer chromatography was carried out on Merck Silica gel G with the following solvent systems: R_F^1, chloroform–methanol (5:1 v/v); R_F^2, *n*-butanol–acetic acid–water (4:1:5 v/v, upper phase). Spots of materials possessing free amino groups on a thin-layer plate were detected by spraying ninhydrin, and those of the amino-group-blocked materials by spraying 40% hydrobromic acid and then ninhydrin. To determine the molecular weight of synthetic peptide (XIV), dimethylformamide was used as a solvent for Hitachi Osmometer, type 115.

Mixed Anhydride Coupling

Z(OMe)-Gly$_2$-Phe-OBzl (I). To a chilled solution of Z(OMe)-Gly$_2$-OH [10] (5.93 g, 20 mmol) and triethylamine (2.8 ml, 20 mmol) in tetrahydrofuran

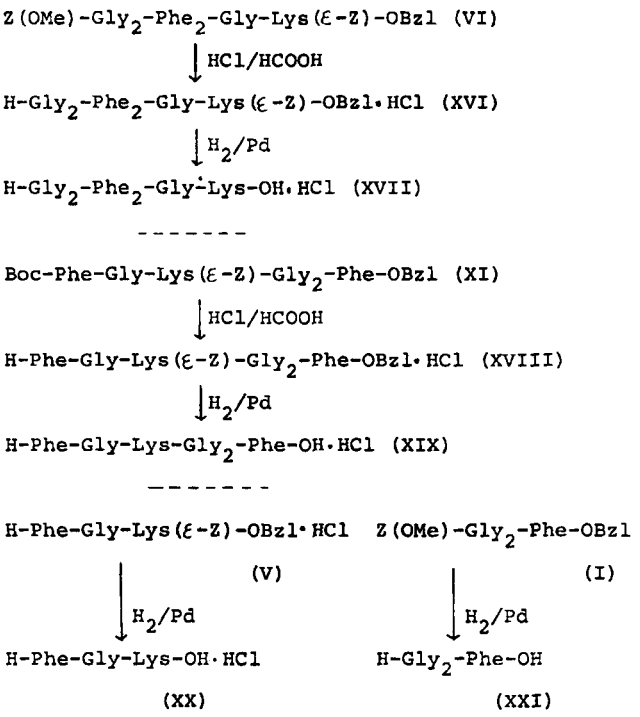

Fig. 3. *Syntheses of the linear tripeptides and hexapeptides*

(40 ml), isobutyl chloroformate (2.62 ml, 20 mmol) was added. After 10 min a chilled mixture of H-Phe-OBzl · *p*-toluenesulfonate [11] (8.55 g, 20 mmol) and triethylamine (2.8 ml, 20 mmol) in chloroform (40 ml) was added to the solution. The reaction mixture was stirred for 1 h at 0 °C, allowed to stand overnight at room temperature, and evaporated *in vacuo*. The residual oil was dissolved in ethyl acetate, then washed successively with 10% citric acid, 4% $NaHCO_3$ and water, and dried over Na_2SO_4. The filtrate was evaporated, and the residual oil was solidified by the addition of ether and petroleum ether. It was recrystallized from methanol–ether–petroleum ether; yield, 7.85 g (74%).

Boc-Phe-Gly-Lys(ε-Z)-OBzl (IV). III (5.80 g, 18 mmol) was coupled with H-Lys(ε-Z)-OBzl · *p*-toluenesulfonate [4] (9.75 g, 18 mmol). The product was recrystallized from ethyl acetate–methanol–ether; yield, 8.79 g (72%).

Preparation of Acyl Peptide Hydrazides

Z(OMe)-Gly_2-Phe-$NHNH_2$ (II). A solution of I (1.07 g, 2 mmol) and hydrazine hydrate (0.97 ml) in dimethylformamide (10 ml) was allowed to stand at room temperature for 2 days. The solution was evaporated, and water was added to the residue. The resulting crystals were collected; yield, 896 mg (98%).

Z(OMe)-Gly_2-Phe_2-Gly-Lys(ε-Z)-$NHNH_2$ (VII). VI (1.20 g, 1.2 mmol) in dimethylformamide (12 ml) was converted to the hydrazide and the product was recrystallized from dimethylformamide–methanol; yield, 1.06 g (95%).

Boc-Phe-Gly-Lys(ε-Z)-$NHNH_2$ (IX). IV (1.32 g, 2 mmol) in methanol (20 ml) was converted to the hydrazide; yield, 1.17 g (98%).

Boc-Phe-Gly-Lys(ε-Z)-Gly_2-Phe-$NHNH_2$ (XII). XI (655 mg, 0.7 mmol) was converted to the hydrazide and the product was recrystallized from dimethylformamide–ethanol; yield, 540 mg (90%).

Azide Coupling

Z(OMe)-Gly_2-Phe_2-Gly-Lys(ε-Z)-OBzl (VI). To a solution of II (1.37 g, 3 mmol) in dimethylformamide (15 ml) containing 3.6 N HCl in dioxane (1.73 ml) at −30 °C was added isoamylnitrite (0.45 ml, 3.2 mmol). After 15 min, disappearance of the hydrazide was ascertained by the detection method for hydrazide [12]. To the reaction mixture was added triethylamine (0.87 ml, 6.2 mmol). After 5 min at 0 °C, a solution of V (2.01 g, 3.2 mmol) in dimethylformamide (10 ml) containing triethylamine (0.45 ml, 3.2 mmol) was added. The reaction mixture was stirred for 3 days at 0 °C, and evaporated *in vacuo*. Ethyl acetate was added to the residue, and the solution was washed with 10% citric acid, 4% $NaHCO_3$ and water. It was dried over Na_2SO_4, and evaporated. The residual oil was crystallized by the addition of ethanol and ether. It was recrystallized from dimethylformamide–ethanol–ether; yield, 2.40 g (80%).

Boc-Phe-Gly-Lys(ε-Z)-Gly_2-Phe-OBzl (XI). IX (1.08 g, 1.8 mmol) was coupled with X (0.81 g, 2 mmol) and the product was recrystallized from dimethylformamide–ethyl acetate–ether; yield, 1.53 g (91%).

Cyclization Reaction

cyclo*(-Gly_2-Phe_2-Gly-Lys(ε-Z)-) (XIV) (a) from VIII.* To a chilled solution of VIII (333 mg, 0.4 mmol) in dimethylformamide (2 ml) containing 3.6 N HCl in dioxane (0.22 ml) was added isoamylnitrite (0.056 ml). After 20 min the solution was dropped into pyridine (133 ml) under stirring at 0 °C and the stirring was continued for 2 days. The solution was evaporated *in vacuo* and the residual oil was solidified by the addition of methanol. The solid was collected by filtration, washed with 2% HCl and water, and dried over P_2O_5 at room temperature and reduced pressure. The product was recrystallized from dimethylformamide–methanol; yield, 230 mg (79%).

cyclo(-*Gly*$_2$-*Phe*$_2$-*Gly-Lys*(ε-*Z*)-) *(XIV) (b) from XIII.* XIII (416 mg, 0.5 mmol) was treated in the same manner as described above; yield, 235 mg (65%).

Deblocking with Acidolysis

H-Phe-Gly-Lys(ε-*Z*)-*OBzl* · *HCl (V).* A solution of IV (1.31 g, 2 mmol) in 0.11 N HCl in 99% formic acid (20 ml) was allowed to stand for 20 min at room temperature, and evaporated. The resulting oil was solidified by the addition of ether; yield, 1.27 g (99%).

H-Gly$_2$-*Phe-OBzl* · *HCl (X), H-Phe-Gly-Lys*(ε-*Z*)-*Gly*$_2$-*Phe-NHNH*$_2$ · *2 HCl (XIII), and H-Phe-Gly-Lys*-(ε-*Z*)-*Gly*$_2$-*Phe-OBzl* · *HCl (XVIII).* I (1.60 g, 3 mmol), XII (536 mg, 0.62 mmol), and XI (0.62 g, 0.66 mmol) were treated by the same manner as described for V. Yields; X, 1.17 g (96%); XIII, 499 mg (96%); XVIII, 0.53 g (92%).

H-Gly$_2$-*Phe*$_2$-*Gly-Lys*(ε-*Z*)-*NHNH*$_2$ · *2HCl (VIII), and H-Gly*$_2$-*Phe*$_2$-*Gly-Lys*(ε-*Z*)-*OBzl* · *HCl (XVI).* VII (1.05 g, 1.14 mmol) and VI (1.00 g, 1 mmol) were treated similarly in the presence of anisole (0.3–0.5 ml for 1 mmol). Yields; VIII, 0.92 g (97%); XVI, 0.86 (99%).

Deblocking with Hydrogenolysis

Boc-Phe-Gly-OH (III). A solution of Boc-Phe-Gly-OBzl [7] (8.25 g, 20 mmol) in methanol (250 ml) was hydrogenated in the presence of palladium black. After 4 h, the filtrate from the catalyst was evaporated *in vacuo*. The crystals were collected with the aid of a mixture of ether and petroleum ether, and recrystallized from methanol–ether–petroleum ether; yield, 6.39 g (99%).

cyclo(-*Gly*$_2$-*Phe*$_2$-*Gly-Lys-*) · *HCl (XV).* A suspension of XIV (291 mg, 0.4 mmol) in acetic acid (16 ml) was hydrogenated. The filtrate was evaporated to dryness. The residue was dissolved in N HCl (0.44 ml) and the solution was again evaporated. The residual solid was recrystallized from methanol–ether; yield of air-dried material, 238 mg (88%).

H-Gly$_2$-*Phe*$_2$-*Gly-Lys-OH* · *HCl (XVII), H-Phe-Gly-Lys-Gly*$_2$-*Phe-OH* · *HCl (XIX), H-Phe-Gly-Lys-OH* · *HCl (XX), and H-Gly*$_2$-*Phe-OH (XXI).* XVI (150 mg, 0.17 mmol), XVIII (275 mg, 0.31 mmol), V (126 mg, 0.2 mmol), and I (674 mg, 1.26 mmol) were hydrogenated as described for XV. In the case of V 50% methanol (7 ml) was used as a solvent. Solvent systems for recrystallization of XVII, XIX, XX, and XXI were water–isopropyl alcohol, water–ethanol–ether, water–ethanol–ether, and water–ethanol respectively. Yields of air-dried materials; XVII, 102 mg (87%); XIX, 190 mg (86%); XX, 68 mg (80%); XXI, 309 mg (88%).

ENZYMATIC ASSAY

Enzymes

Pepsin employed was a twice-crystallized sample from Worthington Biochemical Corporation and trypsin was a salt-free crystalline sample from Nutritional Biochemical Corporation. Both enzymes were used without further purification. Casein according to Hammersten was purchased.

Assay for Pepsin Action

All reactions were run at 37 °C, and 0.2 M sodium citrate buffers were used to control the pH in the range of 1.5–4.0. The enzyme weighed out was dissolved in 0.005 M citrate buffer at pH 4.18 before use. The enzyme solution (0.2 ml) was added to a 2-ml assay flask containing the pertinent substrate dissolved in the buffer (1.0 ml). The mixture was diluted to the specified volume with water, the final concentration of the buffer being 0.1 M. At selected time intervals an aliquot was withdrawn and added into an empty flask, immediately immersed in a bath (100 °C) for 60 s, and subjected to assays by amino acid analyzer and paper chromatography as described below.

For measurement of kinetic constants, 0.2 M phosphate buffer (pH 2.3) or 0.2 M sodium citrate buffer (pH 2.3) was used as mentioned previously [13].

Assay for Trypsin Action

The procedure of tryptic reaction was similar to the one described in the literature [14]. The enzyme solution in 0.001 N HCl was added to a 2-ml assay flask containing the pertinent substrate dissolved in a buffer at a given concentration. The solution was diluted to 2 ml with water, the final concentration of Tris buffer and borate buffer being 0.1 M and 0.05 M respectively. Incubation was effected at 30 °C, and the reaction was stopped by the addition of N HCl (0.1 ml per 0.5 ml of reaction mixture). The rates of hydrolysis of the substrate were followed by paper chromatography and an amino acid analyzer.

Assay for Inhibition of Pepsin Action

To the solution containing substrate and inhibitor (*cyclo*(-Gly$_2$-Phe$_2$-Gly-Lys-)) dissolved in 0.2 M phosphate buffer (pH 2.3) or dilute HCl (pH 1.8) was added the enzyme solution. Gly$_2$-Phe$_2$-Gly-Lys and casein were used as the substrate. The same procedure as described in the section of Assay for Pepsin Action was carried out for Gly$_2$-Phe$_2$-Gly-Lys. Casein was dissolved in dilute HCl (pH 1.8) to the concentration

of 0.6% and the hydrolysis was followed spectrophotometrically by essentially the same procedure as described in the literature [15].

Calculation of Constants

Proteolytic coefficients (C) were estimated from $C = K/E$, where $K = \log[100/(100 - \%\text{hydrolysis})] \cdot \text{min}^{-1}$, and E is the protein concentration in mg of protein nitrogen per ml of test solution. Maximum proteolytic coefficients (C_{max}) were estimated by means of $C_{max} = k_3/2.3\ K_m$ where K_m is a Michaelis constant and k_3 is a rate constant [16]. For non-competitive inhibition, K_i is the inhibition constant defined in the following equation:

$$E + I \underset{k_{-i}}{\overset{k_{+i}}{\rightleftharpoons}} EI \qquad K_i = k_{-i}/k_{+i}.$$

Analysis of Reaction Products by Amino Acid Analyzer and Paper Chromatography

In previous papers the authors described a procedure using an amino acid analyzer for quantitative determination of each component in an incubation mixture of a substrate with pepsin [7,13]. In the present study satisfactory separation and determination of at least one component in an incubation mixture were obtained with the following conditions: the apparatus used was a Hitachi amino acid analyzer model KLA-3B; the column, 0.6 × 10 cm with spherical resin at a flow rate of 60 ml/h and the jacket temperature of 55 °C; buffers: (condition a) after 10 min with a standard 0.2 M sodium citrate at pH 4.25, the system was changed to 1.4 M sodium citrate buffer at pH 5.9; (condition b) after 10 min with 1.4 M sodium citrate buffer at pH 5.9, the system was changed to 0.2 M trisodium citrate containing 1 M NaCl and 0.02 M NaOH. The rate of hydrolysis was determined under the condition (a) for the linear hexapeptide and under the condition (b) for the cyclic hexapeptide.

The quantitative evaluation of Gly_2-Phe, Phe-Gly-Lys, Gly_2-Phe_2-Gly-Lys or Phe-Gly-Lys-Gly_2-Phe produced from the corresponding substrate was made on the basis of the color values known for these compounds with the amino acid analyzer. Elution volume (ml) and color yield (%) of the prepared compounds are shown below.

Gly_2-Phe_2-Gly-Lys; 86 ml in condition (a), 29 ml and 145.5% in condition (b). Phe-Gly-Lys-Gly_2-Phe; 98 ml in condition (a), 30 ml and 103.7% in condition (b). Gly_2-Phe; 25 ml and 98.7% in condition (a). Phe-Gly-Lys; 63 ml and 176.1% in condition (a). Percentage of the color yield at 570 nm was determined with L-leucine = 100% under the conditions indicated above. *cyclo*(-Gly_2-Phe_2-Gly-Lys-) was not eluted under the conditions mentioned.

Paper chromatography was carried out on Toyo Roshi No. 52 paper with the following solvent systems: solvent A, *n*-butanol–acetic acid–water (4:1:2 v/v) and solvent B, *n*-butanol–acetic acid–pyridine–water (15:3:10:12 v/v). The procedure was the same as described previously [13].

RESULTS

Synthesis

Table 1 gives the analytical data for the products and the intermediate peptides.

The homogeneities of free peptides prepared above were established by paper electrophoresis, paper chromatography and amino acid analysis. The electrophoretic mobilities, the R_F values in paper chromatography and the results of amino acid analysis of the free peptides are shown in Table 2.

Paper Chromatography of Reaction Mixtures

The course of the enzymatic reaction was followed by means of paper chromatography of each reaction mixture over a period of time.

Action of Pepsin. Gly_2-Phe_2-Gly-Lys (5 mM) was incubated with pepsin (0.1 mg per ml) at pH 2.3. Soon after 30 min the spots of Gly_2-Phe and Phe-Gly-Lys could be detected on a paper chromatogram. The starting substrate Gly_2-Phe_2-Gly-Lys disappeared after 24 h. *cyclo*(-Gly_2-Phe_2-Gly-Lys-) (0.01 M) was incubated with large amount of pepsin (4 mg per ml) for up to 48 h at pH 2.3. The cyclic hexapeptide was not cleaved at all even after 48 h. Solvent system A was used.

Action of Trypsin. Phe-Gly-Lys-Gly_2-Phe (10 mM) and *cyclo*(-Gly_2-Phe_2-Gly-Lys-) (4 mM) were digested with trypsin (0.1 mg and 2 mg per ml) at pH 8.5 and 8.0 respectively. As expected, the enzyme cleaved these two substrates at the Lys-Gly bond, thus giving rise to the peptides Phe-Gly-Lys and Gly_2-Phe, or the open chain Gly_2-Phe_2-Gly-Lys, soon after 0.5–1 h digestion. Both of the starting substrates, Phe-Gly-Lys-Gly_2-Phe and *cyclo*(-Gly_2-Phe_2-Gly-Lys-), disappeared after 5 h incubation. Solvent system A was used for Phe-Gly-Lys-Gly_2-Phe, and solvent B for *cyclo*(-Gly_2-Phe_2-Gly-Lys).

pH–Activity Curves

The pH dependence of hydrolysis catalyzed by pepsin and trypsin was examined for all synthetic

Table 1. *Analytical data of synthetic peptides*
In the last two columns the upper set of figures represent theoretical values and those in the lower set represent the experimental values. Theoretical values given for VIII, XV, XVII, XVIII, XIX, and XX correspond to hemihydrate, trihydrate, dihydrate, hemihydrate, trihydrate, and monohydrate respectively. DMF, dimethylformamide

Compound	R_F^1	R_F^2	melting point	$[\alpha]_D^{22}$	Analysis			Molecular weight
					C	H	N	
			°C					
I	0.82		115–116	− 3.3 (*c* 2, DMF)	65.28 65.22	5.86 5.77	7.88 7.75	
II	0.41		163–164.5	− 1.2 (*c* 2, DMF)	57.76 57.73	5.95 5.90	15.31 15.08	
III	0.30		165	− 5.1 (*c* 2, DMF)	59.61 59.51	6.88 6.82	8.69 8.66	
IV	0.73		127	−16.9 (*c* 2, methanol)	65.86 65.86	6.87 6.87	8.30 8.24	
V	0.62		84– 95	+ 9.1 (*c* 1.5, methanol)	61.09 61.07	6.57 6.50	8.91 8.84	
VI	0.65		156–159	−23.5 (*c* 2, DMF)	64.85 64.61	6.15 6.08	9.80 9.66	
VII	0.48		191–194	−28.7 (*c* 2, DMF)	61.09 60.92	6.22 6.21	13.64 13.47	
VIII	0.04		200 (decomp.)	−49.7 (*c* 1.7, DMF)	54.22 54.26	6.23 6.20	14.98 14.65	
IX	0.51		178–179	−14.3 (*c* 2, DMF)	60.18 60.21	7.07 7.16	14.04 13.87	
X	0.45		158–159	− 2.5 (*c* 2, methanol)	59.18 59.18	5.96 6.12	10.35 10.35	
XI	0.55		155–156	− 8.8 (*c* 2, DMF)	64.15 64.16	6.57 6.66	10.48 10.44	
XII	0.52		214	−11.0 (*c* 2, DMF)	60.05 59.82	6.68 6.69	14.66 14.57	
XIII		0.55	212–220 (decomp.)	+14.6 (*c* 2, DMF)	54.80 55.41	6.17 6.47	15.14 15.15	
XIV from VIII		0.76	279–287 (decomp.)	−61.8 (*c* 1, DMF)	62.71 62.63	6.23 6.25	13.47 13.30	728 739
from XIII		0.76	280–287 (decomp.)	−61.2 (*c* 1, DMF)				
XV		0.33	246–248 (decomp.)	−52.2 (*c* 1, methanol)	52.66 52.38	6.78 6.54	14.33 14.04	
XVI		0.67	192–194 (decomp.)	−46.2 (*c* 2, DMF)	61.95 61.80	6.24 6.08	11.24 11.05	
XVII		0.32	208–213 (decomp.)	−11.9 (*c* 1, water)	52.66 52.20	6.78 6.63	14.33 13.96	
XVIII		0.70	185–189 (decomp.)	+13.3 (*c* 2, DMF)	61.31 61.35	6.29 6.26	11.12 10.92	
XIX		0.32	202–206 (decomp.)	+19.2 (*c* 1, water)	51.31 51.23	6.89 6.77	13.96 13.81	
XX		0.24	209–214 (decomp.)	+43.4 (*c* 1, water)	50.43 50.32	7.22 6.96	13.84 13.69	
XXI			222–223 228–230 [17]	+35.8 (*c* 1, water) +35.6 [17]	–	–	–	

Table 2. *Electrophoretic mobilities compared to lysine, R_F values and the results of the amino acid analyses of reference compounds*
Toyo Roshi No. 52 paper was used for electrophoresis and chromatography. The solvent system for estimating R_{Lys} was formic acid–acetic acid–methanol–water (1:3:6:10 v/v) pH 1.8. Solvent B, *n*-butanol–acetic acid–pyridine–water (15:3:10:12 v/v); solvent A, *n*-butanol–acetic acid–water (4:1:2 v/v)

Compound	R_{Lys}	R_F		Amino acid ratio		
		solvent B	solvent A	Gly	Phe	Lys
Gly_2-Phe_2-Gly-Lys	0.51	0.48	0.37	3.10	2.00	0.97
Phe-Gly-Lys-Gly_2-Phe	0.51	0.52	0.30	3.09	1.85	1.00
cyclo(-Gly_2-Phe_2-Gly-Lys-)	0.53	0.79	0.70	2.98	1.97	1.00
Gly_2-Phe	0.59	0.40	0.43			
Phe-Gly-Lys	0.85	0.28	0.20			

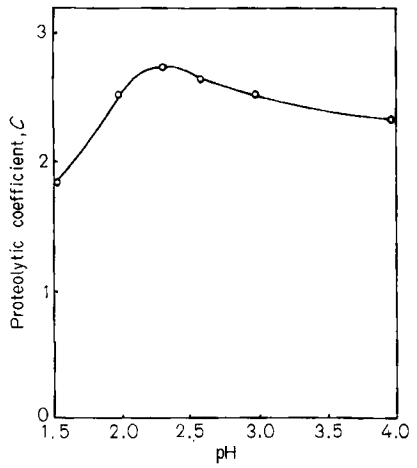

Fig. 4. *The pH dependence of the hydrolysis of Gly_2-Phe_2-Gly-Lys by pepsin at 37 °C.* Substrate concentration is 0.005 M. Sodium citrate (0.2 M) is the buffer at pH 1.5–4.0

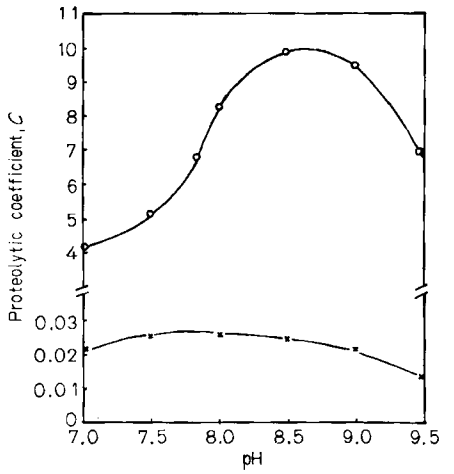

Fig. 5. *The pH dependence of the hydrolysis of linear and cyclic hexapeptides by trypsin at 30 °C.* Substrate and enzyme concentrations are: 10 mM for Phe-Gly-Lys-Gly_2-Phe and 0.1 mg per ml for trypsin; 4 mM for *cyclo*(-Gly_2-Phe_2-Gly-Lys) and 2.0 mg per ml for trypsin. Tris (0.1 M) is the buffer at pH 7.8–9.5 and phosphate (0.1 M) at pH 7.0–8.0 for Phe-Gly-Lys-Gly_2-Phe (○). Borate (0.05 M) is the buffer at pH 7.0–9.5 for *cyclo*(-Gly_2-Phe_2-Gly-Lys-) (×)

peptides except the cyclic hexapeptide which was not cleaved by pepsin at any pH, the results being shown in Fig. 4 for peptic action and Fig. 5 for tryptic action. The substrate of pepsin Gly_2-Phe_2-Gly-Lys has a pH optimum at 2.3. The optimum pH of trypsin found for Phe-Gly-Lys-Gly_2-Phe is close to 8.5, and for *cyclo*(-Gly_2-Phe_2-Gly-Lys-) it appears to be a broad range of 7.5–8.5. Several papers from this laboratory have reported that the optimum pH value is 8.5 for the peptide substrates, for example, Gly_2-Lys-Gln_n (n = 1, 2, 3 and 4), with trypsin [14], and 2.3 for Gly_2-Phe_2-Gly with pepsin [7]. For the purpose of comparing the relative susceptibilities of the cyclic and linear hexapeptide to trypsin the reaction was performed at pH 8.5 and 30 °C.

Kinetic Studies

Measurements of initial rate of hydrolysis at different concentrations of the substrates and the use of a Lineweaver-Burk plot give the values of K_m, k_3 and C_{max} for the hydrolysis of the substrates by the enzymes [16, 18]. Under the conditions given in the Materials and Methods section it was found that the hydrolysis of the peptides tested obeyed first-order kinetics within the extent of experimental error in all cases.

Pepsin Action. The substrate Gly_2-Phe_2-Gly-Lys was tested at 0.003–0.01 M concentrations in 0.1 M phosphate buffer (pH 2.3) and 0.1 M sodium citrate buffer (pH 2.3). The kinetic constants calculated here and those obtained previously in the hydrolysis of Gly_2-Phe-Gly [7] are given in Table 3. The hydrolytic rates of Gly_2-Phe_2-Gly-Lys in two different kinds of buffers are approximately the same (Table 3).

Table 3. *Kinetic constants of peptides hydrolysed by pepsin*
The data for Gly_2-Phe_2-Gly at pH 2.3 and 37 °C are from Yonezawa *et al.* [7]. pH 2.3 (0.1 M phosphate buffer); temperature, 37 °C

Substrate	K_m	k_3	C_{max}
	M	min^{-1}	$min^{-1} \times M^{-1}$
Gly_2-Phe_2-Gly-Lys	0.0091	0.089	4.0
	0.013[a]	0.13[a]	4.3[a]
cyclo(-Gly_2-Phe_2-Gly-Lys-)	0.0044[b]	0	0
Gly_2-Phe_2-Gly	0.0027	0.012	1.9

[a] pH 2.3 (1 M sodium citrate buffer); temperature 37 °C.
[b] This is the K_i value for the inhibition of pepsin-catalyzed hydrolysis of Gly_2-Phe_2-Gly-Lys by the cyclic hexapeptide.

Table 4. *Kinetic constants of peptides hydrolysed by trypsin*
pH 8.5 (0.1 M Tris-HCl buffer); temperature, 30 °C. *cyclo*-(-Gly_2-Phe_2-Gly-Lys-) was incubated pH 8.5 (0.05 M borate buffer containing 10% dimethylformamide); temperature, 30 °C. The data for Gly_2-Lys-Gly_3 at pH 8.5 and 30 °C are from Yamamoto and Izumiya [20]. The data for *cyclo*(-Gly_2-Lys-Gly_2-Lys-) at pH 8.5 and 30 °C are from Nishikawa (unpublished observation)

Substrate	K_m	k_3	C_{max}
	M	min^{-1}	$min^{-1} \times M^{-1}$
Phe-Gly-Lys-Gly_2-Phe	0.00022	0.16	310
cyclo(-Gly_2-Phe_2-Gly-Lys-)	0.010	0.00072	0.031
Gly_2-Lys-Gly_3	0.0035	0.29	37
cyclo(-Gly_2-Lys-Gly_2-Lys-)	0.012	0.00082	0.028

Trypsin Action. Because of the poor solubility of the cyclic hexapeptide, it was tested at 0.002 – 0.005 M initial substrate concentrations in a borate buffer (pH 8.5) containing 10% dimethylformamide. The reduction of initial activity by the addition of dimethylformamide was not more than 5%. Table 4 gives the kinetic constants of the substrates determined here and those obtained previously in the hydrolysis of Gly_2-Lys-Gly_3 [19] and *cyclo*(-Gly_2-Lys-)$_2$.

Inhibition of Pepsin by the Cyclic Hexapeptide. The inhibitory effect of *cyclo*(-Gly_2-Phe_2-Gly-Lys-) on the hydrolysis of Gly_2-Phe_2-Gly-Lys by pepsin was examined. The inhibition reaction was tested at three substrate concentrations [S], and the cyclic hexapeptide was tested at four levels of concentration [I] including the absence of the cyclic peptide. The values of initial velocity (v_i) obtained were used for $1/v_i$ *versus* 1/[S] plots according to the method of Lineweaver and Burk [18]. The cyclic hexapeptide behaves as a non-competitive inhibitor of pepsin in this assay (Fig. 6), and a value of K_i has been calculated to be 0.0044 M.

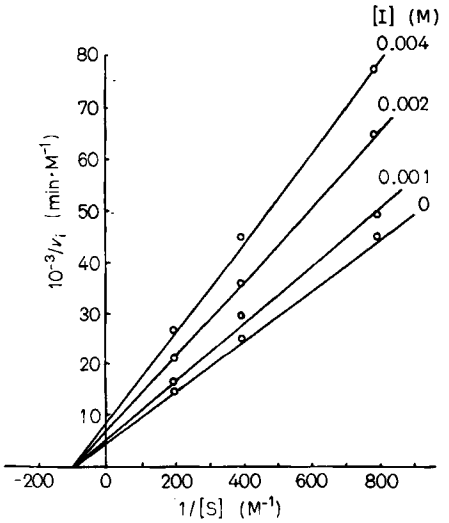

Fig. 6. *Inhibition of peptic hydrolysis of Gly_2-Phe_2-Gly-Lys by* cyclo*(-Gly_2-Phe_2-Gly-Lys-) at pH 2.3 (0.2 M phosphate) and 37 °C.* [S], initial substrate concentration; [I], concentration of the cyclic hexapeptide; v_i, initial velocity. The enzyme concentration is 2.28 μg N/ml

The inhibitory action of the cyclic hexapeptide was further confirmed with regard to the protease activity of pepsin using casein as a substrate. The rate of hydrolysis is described to 88% and 83% at 0.002 M and 0.004 M with respect to the inhibitor.

DISCUSSION

Thus far the studies of the hydrolysis of cyclic substrates have been done merely qualitatively, and none of the kinetic constants have been reported. The primary purpose of the present study involves a quantitative comparison of the susceptibility of the cyclic substrate with that of the linear substrate. From the analogy to cleavage manners of linear hexapeptides, we expected that *cyclo*(-Gly_2-Phe_2-Gly-Lys-) might be cleaved by pepsin at a Phe-Phe peptide bond and also by trypsin at a Lys-Gly bond. However, the cyclic hexapeptide was not hydrolyzed at all even when a high concentration of pepsin was used. On the other hand, the cyclic hexapeptide was hydrolyzed by trypsin. The optimum pH of the cyclic substrate is lower and broader than that of the linear substrate.

Inouye *et al.* have reported that an increase in the concentration of citrate buffer (pH 4.0) causes a marked decrease in the initial rate of peptic cleavage of Z-His-Phe(NO_2)-Phe-OMe [20]. In the case of the hydrolysis of Gly_2-Phe_2-Gly-Lys, considerable differ-

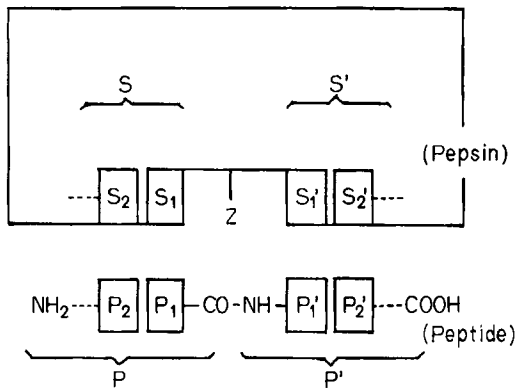

Fig. 7. *Schematic representation of the active center of pepsin combined with a peptide substrate.* Z represents the catalytic site containing two carboxyl groups. P and P' correspond to X and Y respectively in the reaction scheme

ence in C_{max} was not observed between two kinds of buffers, 0.1 M citrate and 0.1 M phosphate at pH 2.3 (Table 3). Yonezawa *et al.* have shown that the elongation from Gly to Gly_2 in regard to the B residue of A-Tyr_2-B did not alter the level of susceptibility [7]. In accord with this fact, Table 3 indicates that C_{max} values for Gly_2-Phe_2-Gly and Gly_2-Phe_2-Gly-Lys are approximately on the same level, though K_m and k_3 values for these substrates are markedly different. In contrast to these data, it was reported that Z-Phe-Leu-Ala_2 is 50 times more susceptible than Z-Phe-Leu-Ala [21].

For the comparison of the susceptibility of the substrate to tryptic action, the rates of hydrolysis of the cyclic and linear hexapeptides were determined at pH 8.5. Table 4 indicates that *cyclo*(-Gly_2-Phe_2-Gly-Lys-) is hydrolyzed 10^4 times more slowly than Phe-Gly-Lys-Gly_2-Phe, and this decrease in C_{max} is mainly due to the decrease in k_3 rather than the increase in K_m. It would be of interest to note that two cyclic hexapeptides cited in Table 4 resemble each other closely in the magnitude of values of K_m, k_3 and C_{max}. Although k_3 for Phe-Gly-Lys-Gly_2-Phe is nearly the same as for Gly_2-Lys-Gly_3, the value of K_m in the former substrate is lower by a factor of about 16, showing the stronger binding of the former substrate to trypsin. This effect may be derived from the replacement of the terminal glycine residues with more hydrophobic phenylalanine residues. At this point it is pertinent to note that the rate of hydrolysis of the amide or ester derivatives of some aminoacyl-L-lysine peptides by trypsin was markedly influenced by the nature of the N-terminal amino acid residue of the substrate [22]. Yamamoto has also observed similar results in the case of Gly_2-Lys-X-Gly_2 (X various amino acid residues) (unpublished observation).

Although the cyclic hexapeptide, *cyclo*(-Gly_2-Phe_2-Gly-Lys-), is resistant to hydrolysis by pepsin, it inhibits the cleavage of the linear hexapeptide, Gly_2-Phe_2-Gly-Lys. It is evident from Fig. 6 that the character of the inhibition is not competitive but clearly linearly non-competitive [23]. This observation suggests that the cyclic hexapeptide may bind both to free enzyme (E) and to the intermediate (EY), usually called amino-enzyme [24,25]. The rate equation relating to this situation is obtained according to King *et al.* [26], and leads to the kinetic behavior shown in the reaction scheme below. As will be seen from Table 3, the K_i value for the cyclic hexapeptide

$$\begin{array}{ccccccc} EI & & & & EY \cdot I & & \\ \Updownarrow & & & & \Updownarrow & & \\ E & \rightleftharpoons & E \cdot XY & \rightleftharpoons & EY & \rightleftharpoons & E. \\ & & & & + X & + Y & \end{array}$$

is smaller than the K_m value for Gly_2-Phe_2-Gly-Lys, suggesting that the former binds to pepsin more firmly than does the latter.

For the mechanism of peptic hydrolysis, Knowles proposed a hypothesis that the split of the peptide bond of a substrate is brought about by two carboxyl groups located in the catalytic site of pepsin, one an un-ionized and the other an ionized carboxyl group [27,28]. Although the binding site for side-chains of the substrate is not explicit at present, it would be reasonable to assume the presence of two subsites (S and S') occupied by the side-chains of the substrate. A schematic figure according to the concept of subsite developed by Schechter *et al.* [29] is shown in Fig. 7. It is well recognized that the nature of the amino acid in the P_1 position is decisive in determining the catalytic efficiency in peptic hydrolysis of the P_1-P_1' bond [7,30]. According to the reaction scheme, binding of the inhibitor, *cyclo*(-Gly_2-Phe_2-Gly-Lys-), takes place at the S position on pepsin, since the scheme requires that the inhibitor binds both to the free enzyme and to the amino-enzyme. In order to grasp the mode of inhibition caused by the cyclic hexapeptide, further investigations may be required.

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