The Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-3,5-dibromotyrosine at pH 2

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Abstract: The pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine has been investigated at pH 2.0 and 25.0° and found to proceed at a rate intermediate between that of the rapidly hydrolyzed substrate, N-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine, and that of the unsubstituted dipeptide, N-acetyl-L-phenylalanyl-L-tyrosine. Under these conditions the Michaelis constant for the hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine is 9.3×10^{-5} *M*, and the catalytic rate constant has a value of 1.97×10^{-2} sec^{-1}. One of the products of the reaction, N-acetyl-L-phenylalanine, acts as a competitive inhibitor with a K_I of 1.4×10^{-4} *M*. Some suggestions that warrant further investigation can be made concerning the factors affecting the rapidity of the hydrolysis of dipeptides by pepsin.

Early in the 1950's it was determined that the pH optimum for the hydrolysis of several dipeptide substrates of pepsin is 2.[2] Since then kinetic studies on N-acetyl-L-phenylalanyl-L-tyrosine have shown that, at 35°, K_M is approximately 2×10^{-3} *M* and that k_{cat} is between 2 and 15×10^{-2} sec^{-1}.[3-5] Recently, the kinetics of the pepsin-catalyzed hydrolysis of a halogen-containing dipeptide, N-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine, was reported.[6] This is the fastest known substrate for pepsin, and the constants reported ($K_M = 7.5 \times 10^{-5}$ *M*, $k_{cat} = 2 \times 10^{-1}$ sec^{-1} at 35°) show that a major part of the rate effect relative to N-acetyl-L-phenylalanyl-L-tyrosine is attributable to the significantly stronger binding of the larger halogen-containing substrate. With this in mind we have attempted to determine the cause of this much stronger binding by studying the kinetics of the hydrolysis of analogous substrates substituted in the 3 and 5 positions of the tyrosine moiety with bromo, nitro, and other groups. This paper contains the results obtained to date for the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine.

Experimental Section

Syntheses. N-Acetyl-L-phenylalanyl-L-3,5-dibromotyrosine was prepared by the following route. N-Carbobenzyloxy-L-phenylalanyl-L-tyrosine (Cyclo Chemical Corp., Lot-M-1738) was hydrogenated to give L-phenylalanyl-L-tyrosine.[7] This was acetylated and saponified[8] to make N-acetyl-L-phenylalanyl-L-tyrosine. A portion of this material (3.1 g, 0.00863 mole) in 30 ml of glacial acetic acid was placed in a reaction flask, and 4.35 g of bromine (0.0272 mole) in 10 ml of glacial acetic acid was dropped into the ice-cooled flask over a 15-min period. The mixture was stirred for 21 hr at room temperature. It was then concentrated on a rotary evaporator for 30 min at 30°, diluted to 250 ml, and allowed to stand for 15 min. The precipitate which formed was filtered and immediately recrystallized from ethanol–water to give 2.95 g of long needles, mp 214–218° dec. Recrystallization of this crude product gave 2.32 g (0.00439 mole), 52.4% yield, of crystals that melted at 218–219.5° dec. Further recrystallization did not change the melting point for N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine, $[\alpha]^{26}D$ +32.3° (*c* 2, pyridine).

Anal. Calcd for $C_{20}H_{20}Br_2N_2O_5$: C, 45.48; H, 3.82; Br, 30.25. Found: C, 45.54; H, 3.97; Br, 30.17.

Materials. Ninhydrin, Aldrich Lot N-800, was recrystallized from H_2O (decolorizing carbon), mp 251–253° dec.

Methyl Cellosolve, Fisher Lot 741581, was distilled over molecular sieves and stored in a tightly stoppered dark glass bottle. A peroxide test with NaI solution was faint or negative before the methyl cellosolve was used in the kinetic procedure.

Various lots of Baker Analyzed reagent or Baker and Adamson absolute methanol were used as received, as was Worthington pepsin, Lot PM 707. Distilled water was deionized on an ion-exchange column. $SnCl_2 \cdot 2H_2O$, Baker Analyzed reagent, was used.

The citrate buffer of pH 5.14 was prepared by dissolving 21 g of citric acid monohydrate in 200 ml of 1 *N* NaOH and diluting to 1 l. in a volumetric flask; 1 *N* NaOH was added until the pH was 5.14.

The phosphate buffer of pH 2, 5% MeOH, was prepared by dissolving 0.65 g of KH_2PO_4 and 6.95 g of H_3PO_4 (85%) in 2 l. of H_2O and adding 105 ml of MeOH, pH 2.004 at 25°.

All pH measurements were taken on a Beckman research pH meter. Optical density measurements were taken on a Beckman Model DU spectrophotometer.

Enzyme solutions were prepared fresh daily by dissolving 50 mg of pepsin in 2.5 ml of H_2O and adding 2.5 ml of pH 2 buffer. Solutions were centrifuged for 30 min at 10,000 rpm at 4°. At 25° solutions were clear for at least 48 hr.

Ninhydrin solutions were also prepared fresh daily by mixing a solution of 0.6 g of ninhydrin in 15 ml of Methyl Cellosolve with a solution of 0.024 g of $SnCl_2 \cdot 2H_2O$ in 15 ml of citrate buffer of pH 5.14.

Kinetic Measurements. The method used to follow the kinetics was a modification of the Moore and Stein method,[9] utilizing the fact that the only ninhydrin-positive material in the system is the L-3,5-dibromotyrosine produced as the reaction proceeds. The general method was as follows: 10.0 ml of a pH 2.00, H_3PO_4–KH_2PO_4 buffer (5 vol % methanol, ionic strength 0.02) was pipetted into each of two test tubes and equilibrated at 25.0 ± 0.25°. To one was added the appropriate amount of 0.005 *M* substrate (and inhibitor in the inhibited runs) solution in methanol. To the other was added an equal amount of methanol (and inhibitor solution in the inhibited runs). The reaction was initiated by adding 25 μl of enzyme solution to each test tube ($[E]_0 = 7.0 \times 10^{-7}$ *M*). At designated times 500-μl aliquots were delivered into 1 ml of ninhydrin solution in a 3-ml volumetric flask. The neck of the flask was rinsed with 100-μl of 50% ethanol, and the stopper was taped tightly in place. The flasks were then heated at 100.0 ± 0.3° for 15 min, immediately placed in an ice bath for 3 min, and then diluted to the mark with 50% ethanol. The optical density was then read at 570 mμ. Optical densities were related to a standard

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Table I. Studies on Dipeptide Substrates of Pepsin at pH 2.0

	Temp, °C	10^5K_M, M	10^2k_{cat}, sec^{-1}	10^4K_I,[a] M	pK_a $-CO_2H$	pK_a $-OH$	pK, NH_2
N-Acetyl-L-phenylalanyl-L-tyrosine[b]	37	240	14		4.48[c]	11.47[d]	
N-Acetyl-L-phenylalanyl-L-tyrosine[e]	35	195	4.66		3.40[e]		
N-Acetyl-L-phenylalanyl-L-3,5-dibromotyrosine	25	9.3 ± 2.9	1.97 ± 0.31	1.4	4.20[c]	7.48[c]	
N-Acetyl-L-phenylalanyl-L-3,5-diiodotyrosine[f]	35	7.5	20	Not observed	4.20[c]	7.55[c]	
Tyrosine[g]					2.20	10.07	9.11
Dibromotyrosine[g]					2.17	6.45	7.60
Diiodotyrosine[g]					2.12	6.48	7.82
N-Acetyl-L-diiodotyrosine[g]						6.95	

[a] For N-acetyl-L-phenylalanine. [b] Reference 4. [c] Apparent pK_a values, taken in 50% MeOH for CO_2H, 25% MeOH for OH at room temperature. [d] Spectrophotometrically determined both at 2930 and 2745 A in 3% MeOH solutions, this laboratory. [e] Reference 3. [f] Reference 6. [g] J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 4, aqueous values.

curve generated independently with 3,5-dibromotyrosine under the same conditions as above.

pK_a Determinations. The apparent pK_a' values for the carboxyl and phenolic hydroxyl groups of N-acetyl-L-phenylalanyl-L-tyrosine, N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine, and N-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine were determined either titrimetrically or spectrophotometrically. For example, in the case of N-acetyl-L-phenylalanyl-L-tyrosine, the pK_a' of the phenolic hydroxyl group was determined from measurements on a Cary 14 recording spectrophotometer at two different wavelengths, 2930 and 2745 A, at pH 6, 9, 11, 12, and 14. Measurements were taken in 50% MeOH for the carboxyl group and 25% MeOH for the hydroxyl group.

Kinetic Scheme

A kinetic scheme which fits the observations made on the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine is shown below. E represents pepsin, S is the dipeptide, P_1 is N-acetylphenylalanine, and P_2 is L-3,5-dibromotyrosine.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

$$E + P_1 \underset{k_{-I}}{\overset{k_I}{\rightleftharpoons}} EP_1$$

$$K_M = (k_{-1} + k_{cat})/k_1$$

$$K_I = k_{-I}/k_I$$

As mentioned in the Results and Discussion section, we have measured the competitive inhibition which one of the products, P_1, exerts on the hydrolysis of S. The K_I value which we have observed for N-acetyl-L-phenylalanine is 1.4×10^{-4} M which is very close to the K_M found for the substrate, 9.3×10^{-5} M. It has been shown[10] that for this type of kinetic scheme, under conditions were $K_I \approx K_M$, apparent first-order kinetics should be observed in a run at any given substrate concentration although the value of the first-order constant found will vary with the initial substrate concentration when K_M and $[S]_0$ are approximately equal. In conformity with this expectation, individual runs in the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine obeyed first-order kinetics. Reaction velocities were calculated with a high-speed computer using a program described elsewhere.[11,12]

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(12) F. W. Carson and E. T. Kaiser, *J. Am. Chem. Soc.*, **88**, 1212 (1966).

Results and Discussion

The kinetic constants[13] and other data obtained in this study and in related studies are summarized in Table I. A Lineweaver–Burk plot[14] of the rate observations made for the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine is shown in Figure 1. The value of K_I for competitive inhibition by N-acetyl-L-phenylalanine, one of the products, was determined from a Dixon plot.[15]

A comparison of the values of K_M for the three dipeptide substrates listed in Table I shows that placing two halogen substituents in the tyrosine portion of N-acetyl-L-phenylalanyl-L-tyrosine markedly decreased K_m. If the Michaelis constants are taken as indicators of the strength of binding of substrate, then it would appear that the diiodo-substituted dipeptide binds best to the enzyme, the dibromo compound binds slightly less well, and the unsubstituted dipeptide binds considerably less strongly.

The situation with respect to the k_{cat} values found is less clear because of the spread in the literature data for N-acetyl-L-phenylalanyl-L-tyrosine.[3,4] However, if Silver's recently reported value for k_{cat} is accepted,[3] it would seem that the k_{cat} for the dibromo dipeptide is close to that for the unsubstituted compound (taking into consideration the difference in the temperatures at which measurements were made). The value of k_{cat} for the diiodo dipeptide on the other hand is considerably greater.

Sufficient information is not yet available to distinguish among the factors which may be involved in the binding of dipeptides and in the catalysis of their hydrolyses. We have thought of a number of possible reasons why the K_m values are substantially smaller for the halogenated dipeptides than for the unsubstituted compound, N-acetylphenylalanyl-L-tyrosine. One explanation might be that the larger size of the substituted tyrosine nucleus in the halogen-substituted dipeptides leads to a more perfect fit of the substrate with the enzyme. Another possibility is that the polarizable electrons of the halogen substituents interact with some

(13) A recent paper by J. Tang, *J. Biol. Chem.*, **240**, 3810 (1965), reported an examination of the inhibition of pepsin by aliphatic alcohols at pH 3.5. A "15% inhibition" by methanol was observed at a concentration of 0.5%. The importance of this effect at pH 2 is unknown. In general, however, V_{max} should be unaffected by this inhibitor which is competitive.
(14) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
(15) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

electrophilic center at the binding site of the enzyme. An alternative to these explanations is that the observation of the lower Michaelis constants for the halogen-substituted dipeptides may depend in some way on the electron-withdrawing inductive effect of the halogen atoms on the aromatic ring of the tyrosine nucleus. In this regard the powerful influence of the bromo and iodo substituents is evidenced by the pK_a' OH values measured for the substituted phenolic groups as shown in Table I. The pK_a' OH values for the hydroxyl groups of the tyrosine residues in N-acetylphenylalanyl-L-3,5-dibromotyrosine and N-acetylphenylalanyl-L-3,5-diiodotyrosine are much lower than that in the unsubstituted dipeptide.

Another point which requires explanation is that in a recent report of work on the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-diiodotyrosine, inhibition by the product, N-acetyl-L-phenylalanine, was not found[6] despite the observation of competitive inhibition by the same product in studies on the hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine[3] (see Table I). In our investigation of the hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine, we found inhibition by N-acetyl-L-phenylalanine although the value of K_I which we measured cannot be compared directly to that observed in the hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine since Silver, *et al.*,[3] did not report a value for K_I.

In summary, our results indicate that the hydrolytic behavior of the dibromo dipeptide lies in between that observed for the diiodo dipeptide and that of the substituted dipeptide. In order to further elaborate on the causes of trends in both K_m and k_{cat}, we are systematically varying the polarizability and size of substituents. For these reasons the kinetics of the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dinitrotyrosine and related dipeptides are now under study.

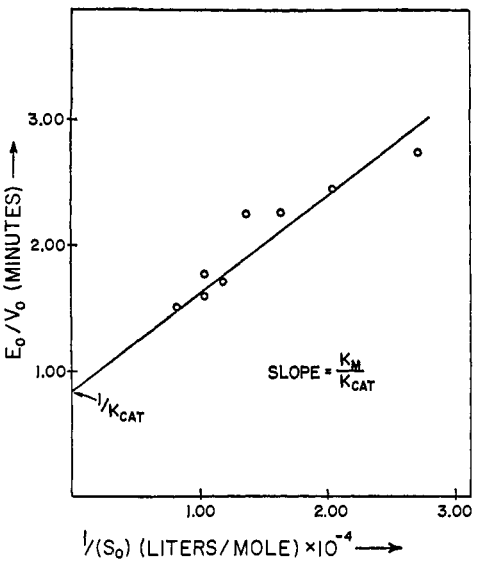

Figure 1. Lineweaver–Burk plot for the hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine by 7×10^{-7} *M* pepsin at pH 2.0.

Communications to the Editor

A Total Synthesis of a Natural Prostaglandin

Sir:

Prostaglandins, a family of C_{20} prostanoic acids of the same carbon skeleton[1] first isolated by von Euler[2] and Goldblatt[3] as crude extracts from sheep seminal vesicular glands or human seminal plasma, are now known to be physiologically important lipids[1,4-6] widely distributed in nature.[1,7] In an elegant series of chemical, spectral, and crystallographic studies Bergstrom and co-workers first isolated PGE_1 (prostaglandin E_1), (15*R*)-9-oxo-11α,15-dihydroxyprost-13-*trans*-enoic acid,[1c] in pure form[8] and established its structure (I).[1a,b,9,10] We now report the synthesis (see Scheme I) of the racemic ethyl ester of (15*R*)-9-oxo-11α,15-dihydroxyprostanoic acid (dihydro-PGE_1, XIVa), a biologically potent[11,12] naturally occurring[13] metabolite of PGE_1.

Formylation (sodium hydride–ethyl formate) of 3-ethoxy-2-cyclopentenone (II)[14] afforded nearly quantitative yields of the sodium salt of the 5-hydroxymethylene derivative III. Heterogeneous reaction of III in chloroform with ethyl bromoacetate and triphenylphosphine gave IV[15] in 70% yield, mp 64–65°, λ_{max}^{EtOH} 251 (ϵ 14,500) and 260 mμ (ϵ 14,300), prepared also, after neutralization of III (although less conveniently), with carbethoxymethylenetriphenylphosphorane.

Acid hydrolysis of IV yielded the enol Va[15] [mp 155–160°, λ_{max}^{EtOH} 275.5 mμ (ϵ 17,900)] which, after treatment

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(15) Analysis within journal requirements and compatible nmr spectra.