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The Kinetics of the Pepsin-Catalyzed Hydrolysis of N-Carbobenzoxy-L-Glutamyl-L-Tyrosine by Determination of the Spectrophotometer

by

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合成基質 N-Carbobenzoxy-L-glutamyl-L-tyrosine의 Pepsin 加水分解反應의 分光光度法에 依한 速度論的 硏究

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ABSTRACT

The kinetics of the pepsin-catalyzed hydrolysis of N-carbobenzoxy-L-glutamyl-L-tyrosine at pH 3.5 and 37C° were determined by a spectrophotometric technique. The pepsin used was further purified on a Sephadex G-75 column. The kinetics data were $Km=1.7\times10^{-3}M$, $-\triangle F^{\circ}=3.99$Kcal/mole, and $k_3=2.1\times10^{-2}$ sec^{-1}. An analysis of the above data and other investigators' data obtained from some dipeptides led to the following conclusions.

(1) Phenylalanyl residues in a synthetic peptide are bound to pepsin more strongly than glutamyl or tyrosyl residues, supporting the theory that a part of the binding region of the active center is hydrophobic.

(2) Dipeptides are bound to pepsin principally through their side chains and the binding involves both side-chain residues.

(3) The nature of amino acids in dipeptides R_2-R_1 affect the k_3 values.

INTRODUCTION

The kinetics of the pepsin-catalyzed hydrolysis of the synthetic substrate has been studied and reported by Casey[1], Baker[2,3], Green[4], Bovey,[5] Herriott[6], Zeffren[7], etc.

In this communication, the porcine pepsin(EC 3. 4. 4. 1) is purified by means of a column of Sephadex G-75. When the N-Carbobenzoxy-L-glutamyl-L-tyrosine is hydrolyzed with that pepsin so purified, the rate of the hydrolysis is measured by the method of E. Zeffren[7] modified by the researcher, using a spectrophotometer. The kinetics data obtained by the aforementioned researchers were all checked, comparing them with the procured by me through experiments. The same research resulted in an investigation of the action of different amino acid residues on both sides of the sensitive peptide bond in the synthetic substrate on enzyme-substrate complex forming and hydrolysis of the substrate. The kinetics data on hand are Michaelis' constant Km and molecular activity coefficient k_3 and standard free energy change when forming enzyme-substrate complex, all made available for such an investigation. In order to compare the binding energy of the enzyme-substrate complex between themselves, it is assumed that K_m is approximately equal to K_s[8] The value of $-\triangle F^\circ$(Table 1) is calculated based on K_m, but it is interim.

EXPERIMENTAL

Materials

Ninhydrin and methylcellosolve are obtained from E. Merck. They are used for further purification by the E. Zeffren[7] method. Crystalline porcine pepsin is obtained from Worthington Biochemical Corp., the synthetic substrate Z-Glu-Tyr from Institute for Protein Research, Osaka University, and Bovine Hemoglobin from Difico Laboratory.

The citrate buffer solution of pH 5. 1, the phosphate buffer solution of pH 1. 8, the enzyme solution and the ninhydrin solution are prepared by the E. Zeffren method. The citrate buffer solution of pH 3. 5 and the acetate buffer solution of pH 5. 1 are prepared by the J. Tang[9,10] method.

Purification of Enzyme

The purification method is based on the J. Tang[9,10] method. The porcine pepsin used is that crystallized three times. When this purified pepsin is incubated together with Z-Glu-Tyr at pH 3. 5 and 37C°, the rate of free tyrosine production increased as time elapsed. The total ninhydrin positive material in the incubation mixture solution is greater than that calculated at the rate of production of free tyrosine.

These facts suggest that commercially prepared pepsin is a mixture of autodigestion of the enzyme. The hydrolysis of Z-Glu-Tyr might be effected by a contaminated protein in the enzyme solution. This

difficulty could be overcome by further purification of the enzyme through a column of Sephadex G-75(2×150cm), sodium acetate buffer pH 5.1 used for eluating. When this purified pepsin was incubated with Z-Glu-Tyr, the rate of free tyrosine production appeared to be the first order.

Determination of Pepsin Activity

The activity of pepsin was measured with Bovine hemoglobin by the Rajagopalan[11] procedure. The optical density was measured by using the spectrophotometer (Unicam SP 500) at 280mμ. As a result of the said measurement, the specific activity of pepsin was found to be 2505± 125 units/mg(mean of five determination), when assayed with denatured hemoglobin (1.77%) as substrate at pH 1.8 and 30 C°. One pepsin unit was defined as amount of the enzyme that produces in this assay an increase in the optical density of 0.01 above the blank. The increase in optical density was proportional to the enzyme concentration, within the range of pepsin concentration used in this assay with hemoglobin as the substrate. In calculating the molar pepsin concentration, a molecular weight of 35,000 was assumed(1mg=0.0286μmole).

Kinetics of the Hydrolysis of Z-Glu-Tyr by Pepsin

In this system, the rate of reaction was measured by the Moore[12] method modified, because of the production of the free L-tyrosine where the ninhydrin-positive material exists only in the incubation mixture. A series of tubes ocntaining 0.2 ml of stock purified pepsin solution(e_o=2.8 ×10^{-6}M) and 1.0 ml of stock substrate solution was incubated at 37C° and pH 3.5 for 0∼8hrs. After incubation, to the 0.1 ml incubated sample of each tube was added 0.5 ml of the ninhydrin solution, and the mixture was heated at 100C° for 15 minutes, then cooled and diluted.

The optical density was measured by using 1-cm cells in a Unicam SP 600 spectrophotometer at 570 mμ. For a comparative experiment, the optical density was measured in relation to the incubation solution devoid of substrate or enzyme.

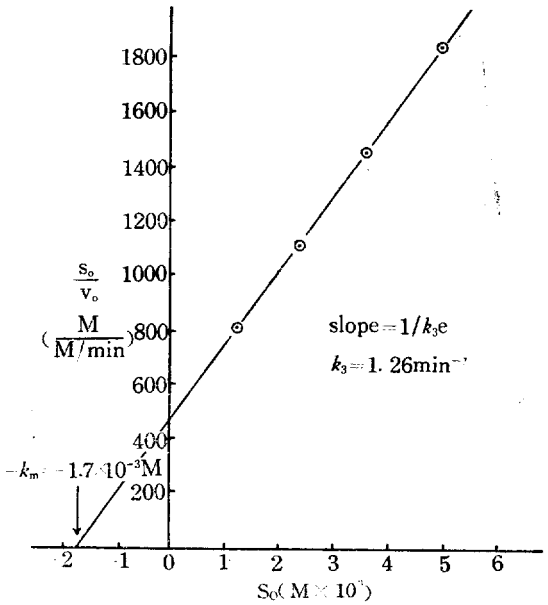

Fig. 1. Determination of Michaelis constant, K_m, and molecular activity coefficient, k_3, for the hydrolysis of Z-Glu-Tyr by pepsin at pH 3.5, 37C°. The concentration of pepsin was 2.8×10^{-6}M. The incubation was carried out for 8 hours.

TABLE 1. Reaction constants for pepsin with synthetic substrates.

Substrate	pH	Temp. (C°)	$S_o \times 10^4$ (M)	$e \times 10^6$ (M)	$Km \times 10^3$ (M)	$-\triangle F^{\circ a)}$ (Kcal/mole)	$k_3 \times 10^2$ (sec^{-1})
Ac-Tyr-Tyr[b)]	2	37			6.1	3.1	1.5
Ac-Phe-Tyr[c)]	2	35	1.16~13.6	13.4~15.8	1.95	3.8	4.66
Z-Glu-Tyr[d)]	3.5	37	12~49	2.8	1.7	3.99	2.1
Z-Phe-Tyr[c)]	2	35	0.73~3.29	12.0~13.8	0.214	5.2	1.24

※ S_o, initial substrate concentration; k_3, molecular activity coefficient; e, enzyme concentration.

a) The standard free energy of association are calculated using the expression $-\triangle F^\circ = 2.303\ RT \log K$, where $R = 1.986$ cal. deg^{-1}. $mole^{-1}$, and K, the association constant=the reciprocal of K_m.

b) Reference 3.

c) Reference 14.

d) The data are reliable to 5%.

After correction for ninhydrin-reacting material present at the start, the amount of hydrolysis was calculated according to a standard absorbance curve of tyrosine. No correction was made for production of additional ninhydrin color owing to the products of selfdigestion of pepsin nor for reduction in ninhydrin color to loss of enzyme activity during incubation. Such effects were small and tended to offset each other. Results were plotted as S_o/V_o vs. S_o by Lineweaver-Burk [13] method.

RESULTS

A Comparison of Commercially prepared Pepsin and Sephadex G-75 Purified Pepsin Action on the Rate of Hydrolysis of Z-Glu-Tyr.

The pepsin was purified by the J. Tang[9, 10] method. The effect of pepsin on the rate of hydrolysis of Z-Glu-Tyr was compared with the pepsin prepared commercially, and that purified by a column of Sephadex G-75, the results coinciding with the J. Tang.[9,10]

K_m and k_3 for the Peptic Hydrolysis of Z-Glu-Tyr

The values of these kinetic constants for the hydrolysis of the Z-Glu-Tyr by purified pepsin were obtained from plots of S_o/V_o vs. S_o(Fig. 1. based on Equa. 1).

$$S_o/V_o = \frac{S_o}{V_{max}} + \frac{K_m}{V_{max}} \quad \cdots\cdots\cdots\cdots(1)$$

Substrate concentrations both higher and lower than K_m were used. The Km was obtained from the intercept on the abscissa and k_3 from the slope of the experimental line. The results are as indicated in Table 1. Also in Table 1 is the standard free energy of binding of pepsin with substrate, calculated on the assumption that Km is equal to K_s. This assumption entails

the view that k_3 is much amaller than k_2 in the expression K_m $(k_2+k_3)/k_1$ hence $K_m=K$[15], And then, in Table 1, K_m, k_3, and $-\triangle F°$, these gained by using the other synthetic substrate are indicated with Z-Glu-Tyr substrate.

DISCUSSION

The primary purpose of this study was to assess what influence the presence and the relative position of phenylalanine, tyrosine and glutamic acid in a dipeptide have on the susceptibility of the CONH bond to peptic hydrolysis. A study of the kinetic and thermodynamic data given in Table 1 indicates that the binding energy of the synthetic substrate to pepsin and the rate of pepsin-catalyzed hydrolysis of synthetic substrate are both affected by the kind of the residue of amino acid possessing both sides of the sensitive peptide bond. It has been noticed that the binding energy of the dipeptide in Table 1 increased in the following order:

Ac-Tyr-Tyr-<Ac-Phe-Tyr≃Z-Glu-Tyr <Z-Phe-Tyr.

Thus, when a tyrosyl residue is replaced by a phenylalanyl residue in either position of the dipeptide or acetyl group is replaced by a carbobenzoxy group, there is an increase of the binding energy. For example, it may be noticed that the binding energy of Z-Phe-Tyr is greater than that of Z-Glu-Tyr by about 30% (1.21 Kcal). These increases of the binding energy upon replacing a hydroxyl group with a hydrogen atom or a glutamyl residue with a phenylalanyl residue support the theory that a region of the active center is hydrophobic.[9,10]

The binding energy of Ac-Phe-Tyr compared with that of Z-Glu-Tyr, it will be revealed that the binding energy of the latter increases more than that of the former when the former CH_3- group is replaced by the HO-CO-CH_2-CH_2CH- chain. But the effect is not great, and the difference is small, if any. It seems that these effects depend upon the different conditions of experiment. On the other hand, it may be found that the Z-Phe-Tyr of the value of $\triangle F°$ increases remarkably, since it is Z-Phe-Tyr in which the glutamyl residue of Z-Glu-Tyr is replaced by the phenylalanyl residue. These observations prove the fact that the phenyl group of Z-Phe-Tyr may be more effective than the HO-CO-CH_2-CH_2-CH- chain of Z-Glu-Tyr in forming the hydrophobic bond between an active site of enzyme and a substrate binding site(active site). The binding data provide further evidence for the view[5,15] that aromatic dipeptides are bound to the enzyme through their side chains and that binding involves both side-chain residues.

With reference to the influence of phenylalanine, tyrosine and glutamic acid on the catalytic hydrolysis of the dipeptide, the value of k_3 in Table 1 indicates that the position and the nature of the amino acid in the compound are both important. In the Z-Glu-Tyr and the Z-Phe-Tyr substrate, when a glutamyl residue as N-terminal group of the dipeptide was occ-

upied in place of a phenylalanyl residue, the k_3 value of the Z-Glu-Tyr was greater than that of the other.

Here again some effects on k_3 may be noticed according to different kinds of the residue of amino acid. Observing these, some effects on them different in their mutual binding position of amino acid residue(for example, Ac-Tyr-Phe and Ac-Phe-Tyr) seem worth considering to inquire into it. The k_3 values provide some information about the nature of the rate-limiting step in the catalytic hydrolysis of the dipeptide R_2-R_1.

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