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Competitive Inhibition of Pepsin by Carboxylic Acids

by

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脂肪酸에 依한 Pepsin의 競走的 억제

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ABSTRACT

In order to obtain the more effective evidence, supporting the hypothesis which have been previously described by former report that[1] pepsin (EC 3. 4. 4. 1) forms a hydrophobic bond with the nonpolar side chain of its substrate, the inhibitory effect of carboxylic acids(from formic acid to iso-butyric acid) on the activity of pepsin to the synthetic dipeptide, N-Carbobenzoxy-L-glutamyl-L-tyrosine, was discussed. The kinetic study showed that the inhibition by carboxylic acids was competitive. The K_i decreased with increasing size of the inhibitor molecule. The $-\Delta F^\circ$ increased linearly with increasing number of carbon atoms in the hydrocarbon chain of the inhibitor.

It was confirmed that the hydrophobic bond between more than one side chain of amino acid residues(phenylalanine) in the binding region of the active center of pepsin and the side chain of amino acid residues in the substrate was formed as the first step of its enzymic mechanism. The inhibitory effect of carboxylic acids was due to the competition of the hydrocarbon group of the carboxylic acids with the side chain of the substrate for the hydrophobic binding site(the side chain of phenylalanine) of the pepsin.

INTRODUCTION

In various preteolytic enzymes, a theory [1,2,3] that a part of the binding region of their active center is hydrophobic is presented. In the pepsin (EC 3.4.4.1), it is known that substrates are bound to pepsin by means of a hydrophobic bond. In order to explain the mechanism of pepsin catalyzed hydrolysis, in the first place, the substrate specifity of this enzyme must be known, and in the second place, how the substrate is bound with enzyme is to be known. The data presented in the previous communication confirm the view that pepsin exhibits preference for aromatic amino acid residues on both sides of the susceptible peptide bond. Judging from the fact that phenylalanyl residues are bound to pepsin more strongly than tyrosyl residues, binding of substrates with pepsin is supposed to be hydrophobic through their side chains.

So, in this communication, the carboxylic acids affecting the pepsin-catalyzed hydrolysis were observed by examining the probability of hydrophobic bond more precisely. If the hypothesis is correct, the hydrocarbon group of carboxylic acids and substrate will react competitively upon the hydrophobic site expected in the active center of pepsin, That is, the carboxylic acids will take a role as competitive inhibitor against pepsin-catalyzed hydrolysis. For the reason that the carboxylic acids were selected, the competitive inhibition of aliphatic alcohols against pepsin was caused by the hydrocarbon groups of alcohols. Therefore, through the Tangs' report[3] in which pepsin-substrate forms the hydrophobic bond in the initial reaction of enzyme, the reaction effects on pepsin of the compound (carboxylic acids, open chain hydrocarbons, and cyclic hydrocarbons) with a hydrocarbon group was observed to see if the hydrocarbon group assumes effect as a competitive inhibition. In the second place, the carboxylic group is supposed not to affect the hydrophobic inter-reaction between enzyme and substrate, and so it can be proved by Cann[4] that the formation of the enzyme-substrate complex between serum albumin and pepsin is inhibited by a long chain fatty acid. In the third place, there is a theory[5] that the α-carboxylate group adjacent to the sensitive peptide bond of substrate inhibits the pepsin reaction.

Therefore, an observation was made in order to see what effect is caused by carboxylate in the same concentration existing in the buffer solution. The N-Carbobenzoxyl-L-glutamyl-L-tyrosine was used as a synthetic substrate in this experiment. And the carboxylic acid ranging from formic acid to butyric acid in the various concentrations was used as inhibitor. The binding of the pepsin with substrate was proved to be hydrophobic through an observation of the kinetics of the pepsin-catalyzed hydrolysis.

EXPERIMENTAL

Materials

Tyrsine was obtained from E. Merck, and crystallized twice. The α-chymotrypsin(twice crystallized) was obtained from Worthington CDT. The Z-Glu-Tyr substrate and N-benzoyl-L-arginine amide and N-acetyl-L-tyrosine amide were obtained from Institute for Protein Research, Osaka University(Japan). The carboxylic acids highest in purity were used.

Determination of Enzyme Activity

The activity of pepsin was measured in the same method as in the former report. The activity of trypsin was measured with N-benzoyl-L-arginine amide as substrate by the Laskowski[6] method. The activity of α-chymotrypsin was measured with N-acetyl-L-tyrosine amide as substrate by the Laskowski[6] method, too.

Purification of Commercially Prepared Pepsin

In this experiment, the purifing method of pepsin was based on the J. Tang[2,3] method.

Determination of the Rate of Enzyme Action

The rate of enzyme action was measured by the same method previously. In order to observe the effect of inhibition, the solution of reactant was in cubated, adding a variety of the concentration of car boxylic acid. And then, the ionic strength was maintained constantly by using 0. 1M KCl. The various concentrations of carboxylic acids were added to the standard solution of tyrosine, and then, the standard curve was plotted by the method aforementioned.

RESULTS

Effect of Carboxylic Acids on Pepsin Activity

The inhibitive effect of the carboxylic acids ranging from formic acid to butyric acid upon the pepsin activity was also observed through the use of Z-Glu-Tyr substrate. As a result of this, the degree of inhibition could be observed to be roughly in propotion to the incresae of molecular weight of the carboxylic acids as in Table 1. But in the case of formic acid, the mechanism of the inhibition was of non-peculiarity. For the purpose of observing and comparing the inhibitive effects of the carboxylic acids upon the proteolytic enzyme with one another, the effect of α-chymotrypsin and trypsin was also observed.

As a result, the α-chymotrypsin had something of such an inhibitive effect as pepsin, and the inhibitive effect of trypsin did not appear to the extent of 1.4% concentration of carboxylic acid. After these were incubated with carboxylic acids, the reactivation of pepsin was observed to become reactive through the removal of carboxylic acids by dialysis.

Kinetics

The mechanism of hydrolysis of pepsin substrate is not yet clear, but the Hofstee plot presents straight lines as in Fig. 1-6 in this report. Thus the hydrolysis of Z-

Glu-Tyr substrate was also observed to obey to the Michaels-Menten kinetics, and the mechanism of hydrolysis was suggestive like the following Equa. (1)as observed formerly.

$$\text{Pepsin}+\text{Z-Glu-Tyr} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\text{Michaelis-Menten complex}}{\overset{\text{Pepsin}}{\text{Z-Glu-Tyr}}}$$

$$\rightleftharpoons \overset{\text{pepsin}}{\text{Z-Glu. COOH}} \quad \text{Tyr. COOH}$$

intermediate in which pepsin molecular is connected by a covalent linkage

$$\xrightarrow{k_3} \text{Pepsin}+\text{Z-Glu. COOH}+\text{Tyr. COOH} \quad \cdots\cdots(1)$$

$$S_o/V_o=\frac{S_o}{V_{max}}+\frac{K_m}{V_{max}} \quad \cdots\cdots(2)$$

$$S_o/V_i=\frac{S_o}{V_{max}}+\frac{K_m}{V_{max}}\left(1+\frac{i}{K_i}\right) \quad \cdots(3)$$

$$V_o/V_i=1+\frac{K_m}{K_i}\left(\frac{i}{K_m+S_o}\right) \quad \cdots\cdots(4)$$

When the acetic acid was used as inhibitor, Fig. 1 and Fig. 2 got plotted S_o/V_i against S_o by Equa. 2 and Equa. 3 according to this, and it was obvious that the two kinds of acid were competitive inhibitors within the range of lower concentration. It has been observed that n-butyric acid is greater than acetic acid in the competitive inhibitor. But, it did not appear to be the competitive inhibition at the concentration above 90 mM of acetic acid and 71 mM of n-butyric acid.

The reason is that, as observed, the existance of carboxylic acid in higher concentration menaces to the maintenance of the optimal pH 3.5 and gives an inhibitive effect like the active structure upon the active center of enzyme. The Fig. 3-6 plotted V_o/V_i against i by Equa. 4 the inhibitive effect on the substrate of the different concentrations of each acid. Through this, K_i of each comptitive inhibitor was determinded, of which the result is shown in the Table 1. In Table 1-2, it has been observed that as carbon atom in the molecular carboxylic acid increases in number, the K_i decreases like the aliphatic alcohol. This is a reciprocal of K_i, in other words, which means the increase

TABLE 1. Inhibition constant Ki, of carboxylic acids

Carboxylic acids	$S_o\times10^3$ (M)	$i\times10^3$ (M)	$K_i\times10^3$ (M)	Average $K_i\times10^3$ (M)
Acetic	1.2	48 ; 90	96	87.3
	2.3	〃	87	
	3.5	〃	79	
Propionic	1.2	35 ; 71	64	
	2.3	〃	72	64.3
	3.5	〃	57	
n-Butyric	1.2	36 ; 70	48	
	2.3	〃	51	46.3
	3.5	〃	40	
iso-Butyric	1.2	35 ; 69	50	
	2.3	〃	38	44.0
	3.5	〃	44	

※ Abbreviations used:Z-Glu-Tyr, N-carbobenzoxy-L-glu-tamyl-L-tyrosine; So, initial substrate concentration;Vo, initial velocity of the reaction without inhibitor;V_i, initial velocity of the reaction with inhibitor;e, enzyme concentration;i, inhibitor concentration;K, Michaelis constant without inhibitor;V_{max}, maximum volocity without inhibitor; K_i, inhibitor constant.

of affinity to the enzyme of inhibitor. So the increase in number of carbon atoms in acid molecular seems to increase the inhibitive effect.

TABLE 2. Values for $-\triangle F^{\circ}{}_{i}^{a)}$, $-\triangle F^{\circ}{}_{HC}^{b)}$, $-\triangle F^{\circ}{}_{phe}^{c)}$

Carboxylic acid	Average $K_i \times 10^3$ (M)	$-\triangle F^{\circ}{}_{i}$ Kcal/mole	$-F^{\circ}{}_{HC}$ Kcal/mole	$-\triangle F^{\circ}{}_{phe}$ Kcal/mole
Acetic	87.3	1.5	—	—
Propionic	64.3	1.8	0.3	0.21
n-Butyric	46.3	1.9	0.4	—
iso-Butyric	44.0	1.9	0.4	—

a) $\triangle F_i$: Standard free energy of formation of pepsin-inhibitor complex, calculated from expression $-\triangle F^{\circ}{}_{i}=2,303RT \log K$, where K=the reciprocal of average values of K_i

b) $\triangle F^{\circ}{}_{HC}$: Standard free energy of formation of complex between pepsin and the hydrocarbon chain of carboxylic acid The data was abtained by subtracting $\triangle F^{\circ}{}_{i}$ of acetic acid from all the values of $\triangle F^{\circ}{}_{i}$.

c) $\triangle F^{\circ}{}_{phe}$: Standard free energy of formation of hydrophobic bonds of maximum strength between a side chain of phenylalanine and the side chains of two amino acids with the same structure as the hydrocarbon chains of two carboxylic acids.

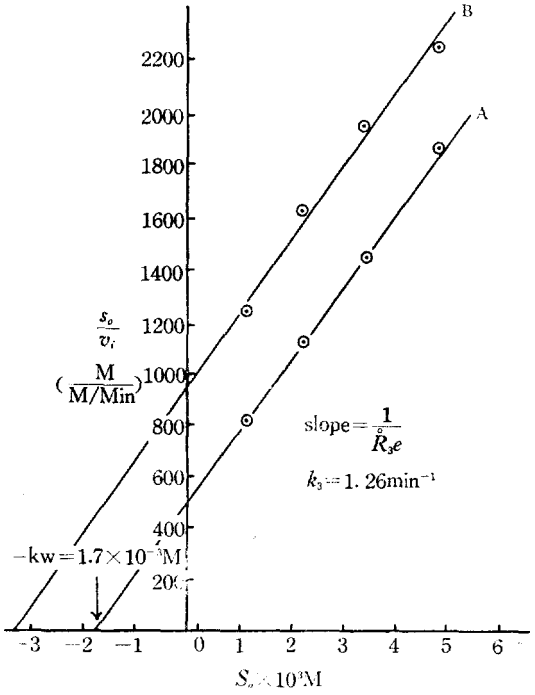

Fig. 1. Repsin-catalyzed of Z-Glu-Tyr inhibited by acetic acid. Hofstee plot where the inhibitor concentrations are: A, no inhibitor, and B, 90mM.

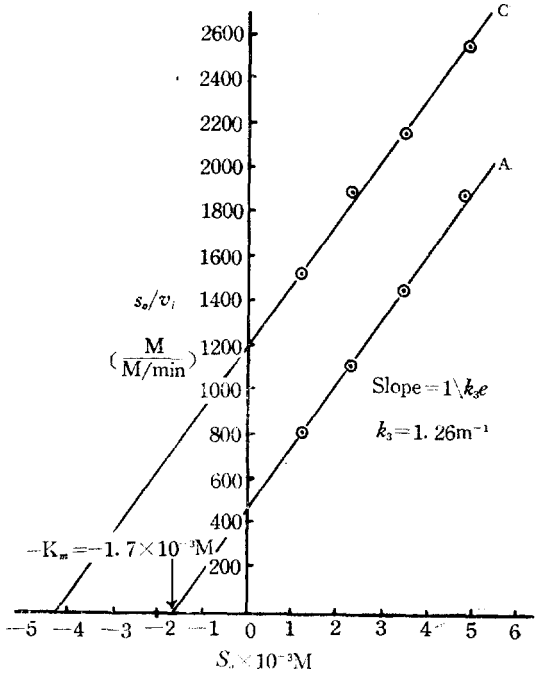

Fig. 2. Pepsin-catalyzed hydrolysis of Z-Glu-Tyr inhibited by n-butyric acid. Hofstee plot where the inhibitor concentrations are:A, no inhibitor, and C, 70 mM.

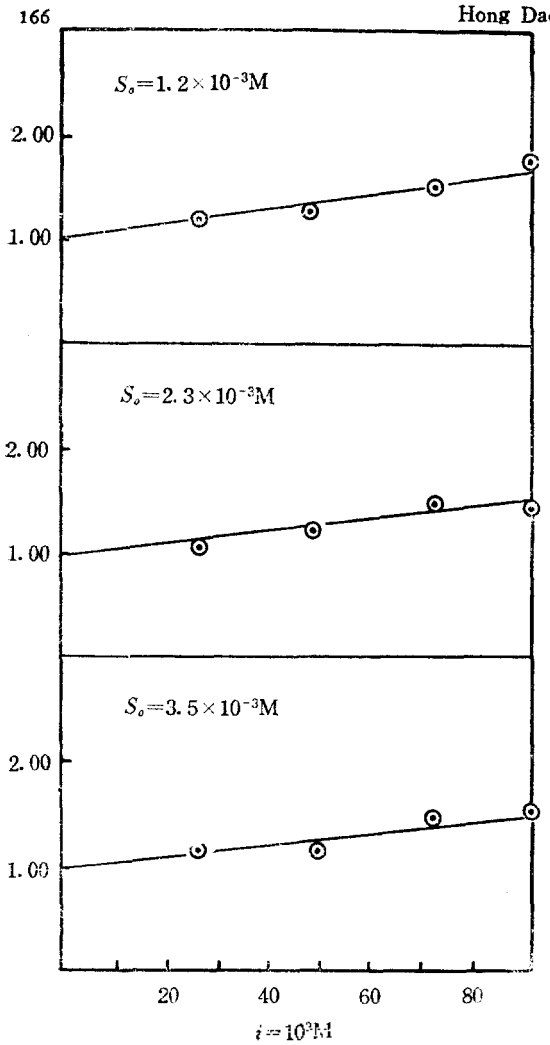

Fig. 3. Acetic acid (Plot of V/V vs. i)

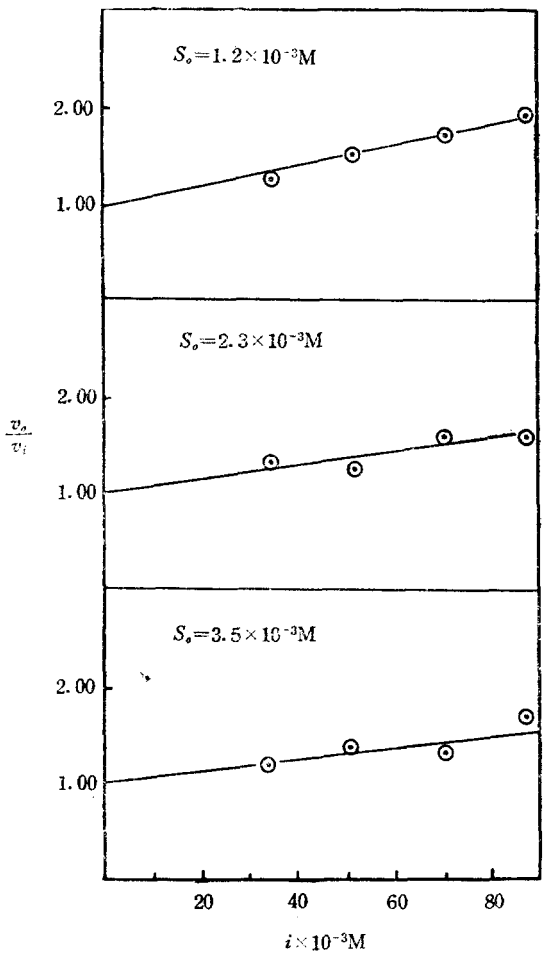

Fig. 4. Propionic acid (Plot of V/V vs. i)

DISCUSSION

When the Fig. 3-6 were observed in regard to the inhibition of carboxylic acid, it was found that the form of inhibition of pepsin-catalyzed hydrolysis of Z-Glu-Tyr substrate with acids were not a partial competitive inhibition. As a result of the above facts, when the enzyme and the substrate were about to form a enzyme-substrate complex at the initial reaction, the pepsin clearly suffered competitive inhibition by carboxylic acids. It means plainly that the binding site of the active center of the enzyme is inhibited competitively by acids and substrate which are mutual. There is no possibility that the active center of enzyme is inhibited competitively with the caboxyl group of carboxylic acid and water molecular both mutual.

It was reported by Inouye[5] that the α-carboxylate group adjacent to the sensitive peptide bond in the substrate appeared to be the inhibition effect, but the carb-

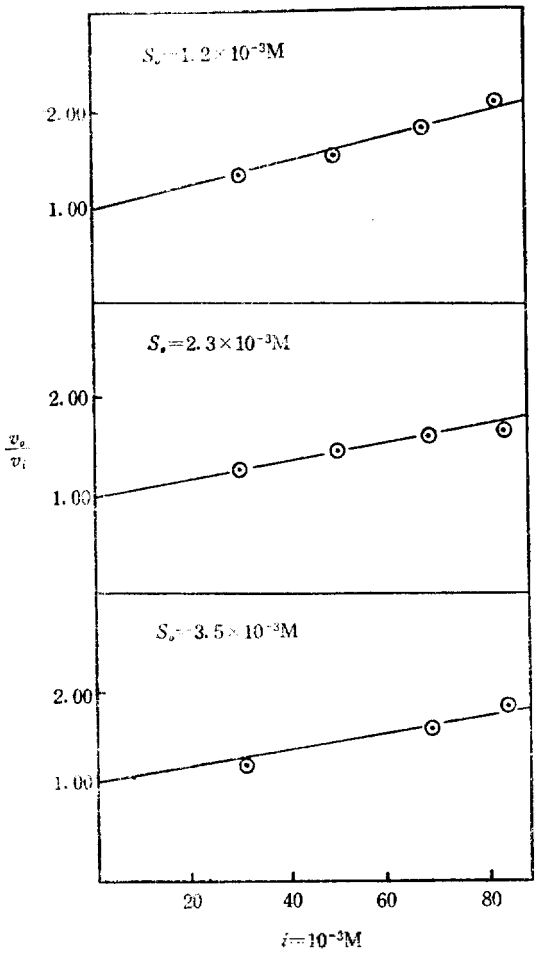

Fig. 5. n-Butyric acid(Plot of V/V vs. i)

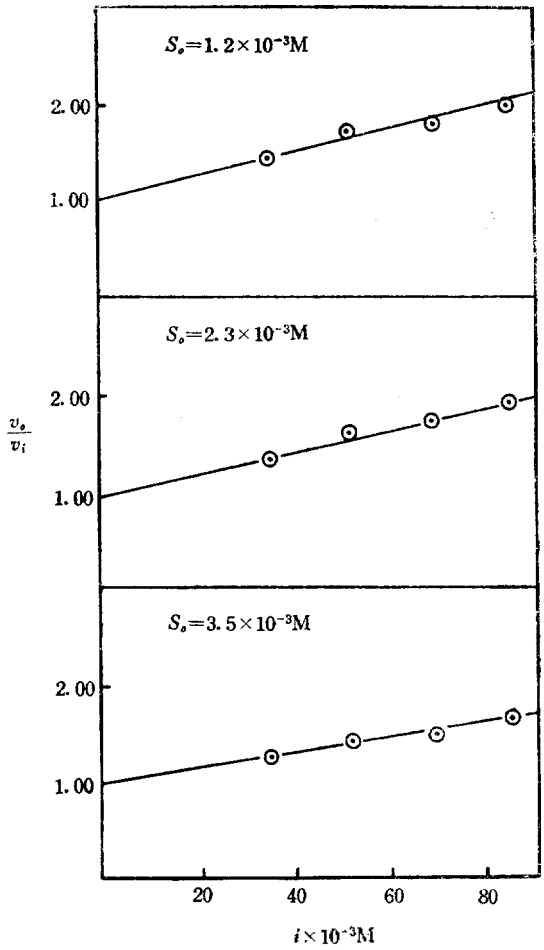

Fig. 6. iso-Butyric acid(Plot of V/V vs. i)

oxylate ion in the buffer solution did not appear effective. The reason is that the rate of the hydrolysis of the substrate by pepsin is equal to the rate of the reaction of the buffer solution made from the acid and its Na-salt in which, great as the difference in concentration is, the part of hydrocarbon group is not great in difference. And the same result was also obtained within the range of the concentration of carboxylate ion used in the reaction solution of this experiment. It is presumed that the effect of competitive inhibition by acid probably occurred through the hydrocarbon group in acid molecular as revealed in this experiment. This may be quite proved by the fact that the degree of the inhibitor is in proportion to the increase in number of the carbon atoms in the acid molecular(cf. Table 1).

Thus, the part of hydrocarbon group in acid molecular and side-chain in substrate reacts competitively on the binding site of the active center of enzyme. That the degree of the competitive inhibition depends upon the size of the part of hy-

drocarbon group, means any binding site of the active center of enzyme to be hydrophobic, this fact concluding the same consequence as in J. Tangs[2,3] and the former[1] report.

In other words, inhibition by carboxylic acid depends on the comptitive reaction the hydrocarbon group of carboxylic acid molecular and the substrate of the hydrophobic site of enzyme.

It seems to be true that in the Tangs' report the hydrophobic bond forms between the pepsin and the side-chain of residue of amino acid in the substrate in the initial enzyme action or between that and one more hydrophobic side-chain of the binding site of the active center of enzyme.

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