BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 3472—3474 (1970)

Competitive Inhibition of Pepsin by Carboxylic Acids

Hong Dae SHIN

Department of Chemistry, College of Liberal Arts and Sciences, Pusan National University, Pusan, Korea

(Received July 25, 1969)

In order to obtain evidence for Tang's hypothesis that pepsin (EC 3.4.4.1) forms a hydrophobic bond with the nonpolar side chain or its substrates, the inhibitory effect of carboxylic acids (from formic acid to isobutyric acid) on the activity of pepsin to the synthetic dipeptide, *N*-carbobenzoxy-L-glutamyl-L-tyrosine, was studied. Kinetic study showed that the inhibition by carboxylic acids was of competitive type. The inhibitor constant K_i decreased with an increase in the size of the inhibitor molecule. The free energy change of formation of complex between pepsin and hydrocarbon chain of carboxylic acid, $-\Delta F_{HC}$, increased linearly with the increase in the number of carbon atoms in the hydrocarbon chain of the inhibitor. It was suggested that the hydrophobic interaction between the side chain of amino acid residues in the binding region of the active center of pepsin and the hydrocarbon side chain of the inhibitor was the cause of the inhibition.

A theory[1,2] that a part of the binding region of their active center is hydrophobic was presented for various proteolytic enzymes. In pepsin (EC 3.4.4.1), it is known that substrates are bound to pepsin through hydrophobic bond.[2] The data given by Tang[1] indicated 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 in nature. In this communication, the effect of carboxylic acids with aliphatic side chain on pepsin-catalyzed hydrolysis is described in order to examine the hydrophobic character of binding. If the hypothesis is correct, the hydrocarbon group of carboxylic acids and the substrate will bind competitively to the hydrophobic region of the active center of pepsin. Thus the carboxylic acids will act as competitive inhibitor against pepsin-catalyzed hydrolysis. In fact, the competitive inhibition of aliphatic alcohols against pepsin was observed by Tang.[2] Moreover, it was reported by Cann[3] that the formation of the enzyme-substrate complex between serum alubumin and pepsin is inhibited by a long chain fatty acid.

The inhibition of carboxylic acids ranging from formic acid to butyric acid on the pepsin-catalyzed hydrolysis of *N*-carbobenzoxyl-L-glutamyl-L-tyrosine (Z-Glu-Tyr) was studied to see the effect of hydrocarbon side chain of the carboxylic acids.

Experimental

Materials. Ninhydrin and methylcellosolve were obtained from E. Merck, and were purified by the E. Zeffren method.[4] Crystalline porcine pepsin and α-chymotrypsin (twice crystallized) were obtained from Worthington Biochemical Corp. *N*-Carbobenzoxyl-L-glutamyl-L-tyrosine substrate, *N*-benzoyl-L-arginine amide and *N*-acetyl-L-tyrosine amide were obtained from the Institute for Protein Research, Osaka University (Japan), and boving hemoglobin from Difco Laboratory. Carboxylic acids of highest purity were used.

Purification of Enzyme. Purification was carried out following Tang's method.[1,2]

Enzyme Activity. The activity of pepsin was measured with bovine hemoglobin by the Rajagopalan method.[5] The optical density was measured with a spectrophotometer (Unicam SP 500) at 280 mμ. From the results, the specific activity of pepsin was found to be 2505 ± 125 units/mg (mean of five determinations), when assayed with denatured hemoglobin (1.77%) as substrate at pH 1.8 and 30°C. One pepsin unit was defined as the amount of enzyme that produces (in the assay) an increase in the optical density of 0.01 above the blank. The increase in optical density was proportional to enzyme concentration, within the range of pepsin concentration used in the assay with hemoglobin as the substrate. In calculating the molar pepsin concentration, a molecular weight of 35000 was assumed (1 mg=0.0286 μmole). The activity of trypsin was measured with *N*-benzoyl-L-arginine amide as substrate by the Laskowski method[6]. The activity of α-chymotrypsin was also measured with *N*-

1) J. Tang, *Nature*, **199**, 1094 (1964).
2) J. Tang, *J. Biol. Chem.*, **240**, 3810 (1965).
3) J. Cann, *J. Biol. Chem.*, **237**, 707 (1962).
4) E. Zeffren and E. T. Kaiser, *J. Amer. Chem. Soc.*, **88**, 3129 (1966).
5) T. G. Rajagopalan, S. Moore and W. H. Stein, *J. Biol. Chem.*, **241**, 4940 (1966).
6) M. Raskowski, S. P. Coliwick and N. O. Kaplan (Ed.), "Methods in Enzymology," Vol. 11. Academic Press, Inc., New York (1955). pp. 22, 26.

Table 1. Inhibition constant, K_i, of carboxylic acids

Carboxylic acids	$S_0 \times 10^3$ (M)	$i \times 10^3$ (M)	$K_i \times 10^3$ (M)	Average $K_i \times 10^3$ (M)
Acetic acid	1.2	48 ; 90	96	87.3
	2.3	48 ; 90	87	
	3.5	48 ; 90	79	
Propionic acid	1.2	35 ; 71	64	64.3
	2.3	35 ; 71	72	
	3.5	35 ; 71	57	
n-Butyric acid	1.2	36 ; 70	48	46.3
	2.3	36 ; 70	51	
	3.5	36 ; 70	40	
Isobutyric acid	1.2	35 ; 69	50	44.0
	2.3	35 ; 69	38	
	3.5	35 ; 69	44	

acetyl-L-tyrosine amide as substrate by the Laskowski method.[6]

Kinetic Measurements. The rate of enzyme action was measured by modified Moore's method.[7] In order to observe the effect of inhibition, the solution of reactant was incubated at various concentrations of the carboxylic acids added. Incubation mixtures consisted of 1.0 m*l* of inhibitor solution at pH 3.5, 1.0 m*l*, of stock substrate solution*, 0.2 m*l*, of stock enzyme solution**, and 0.1 m*l* of HCl in order to adjust the pH of final mixture to 3.5. Ionic strength of incubation mixture was adjusted to 0.1 with 0.1 M KCl. (*, **, Each stock solution was prepared with 0.2 M sodium citrate buffer, pH 3.5).

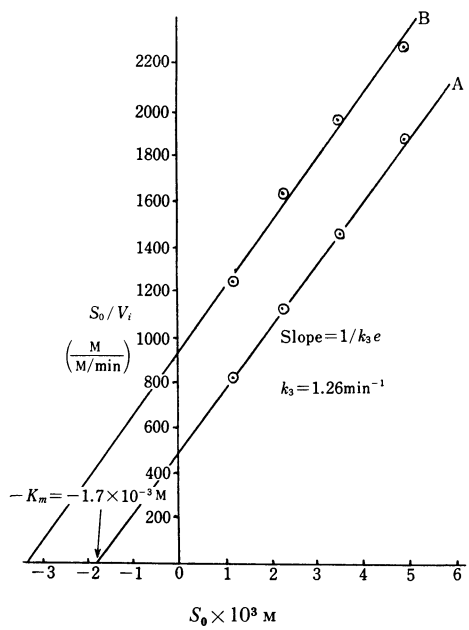

Fig. 1. Hofstee plots for the pepsin-catalyzed hydrolysis of Z-Glu-Tyr with or without the inhibition by acetic acid. The inhibitor concentrations are; A, no inhibitor; and B, 90 mM acetic acid.

7) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

Results

Effect of Carboxylic Acids on Pepsin Action. The inhibitory effect of the carboxylic acids from formic acid to butyric acid upon the pepsin activity was observed with the use of Z-Glu-Tyr as substrate. The result shows that the degree of inhibition is roughly proportional to the increase in the molecular weights of carboxylic acids as shown in Table 1. However, in the case of formic acid, inhibition was non-competitive. For the purpose of comparing the inhibitory effects of carboxylic acids upon different proteolytic enzymes, the same study was carried out with α-chymotrypsin and trypsin. With α-chymotrypsin was observed such

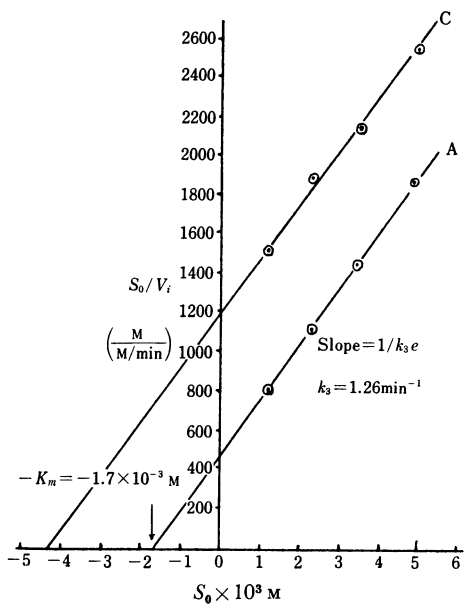

Fig. 2. Hefstee plots for the pepsin-catalyzed hydrolysis of Z-Glu-Tyr with or without the inhibition by *n*-butyric acid. The inhibitor concentrations are; A, no inhibitor, and C, 70 mM *n*-butyric acid.

an inhibitory effect by carboxylic acids as the in case of pepsin. The inhibitory effect on trypsin did not appear up to 1.4% concentration of carboxylic acids. Reversibility of inhibition was confirmed by the removal of carboxylic acids by dialysis.

Kinetics. The Hofstee plot for the hydrolysis of Z-Glu-Tyr showed straight lines as shown in Figs. 1 and 2 in accordance with the Michaelis-Menten equation (1).

$$\text{Pepsin} + \text{Z-Glu-Tyr} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \overset{\text{Pepsin}}{\text{Z-Glu-Tyr}} \xrightarrow{k_3} \text{Pepsin} + \text{Z-Glu}\cdot\text{COOH} + \text{Tyr}\cdot\text{COOH} \quad (1)$$

$$S_0/V_0 = S_0/V_{\max} + K_m/V_{\max} \quad (2)$$

$$S_0/V_i = S_0/V_{\max} + K_m/V_{\max}(1+i/K_i) \quad (3)$$

$$V_0/V_i = 1 + K_m/K_i[i/(K_m+S_0)] \quad (4)$$

where

S_0=initial substrate concentration
V_0=initial velocity of the reaction without inhibitor
V_i=initial velocity of the reaction with inhibitor
$V_{\max}$=maximum velocity without inhibitor
K_m=Michaelis constant without inhibitor
K_i=inhibitor constant
i=inhibitor concentration

The results with acetic acid and *n*-butyric acid as inhibitor, are shown in Figs. 1 and 2 as the plot S_0/V_i *vs.* S_0 according to Eqs. (2) and (3). It is obvious that the two kinds of acid were competitive inhibitors within the concentration range studied. It has been observed that *n*-butyric acid is a stronger competitive inhibitor than acetic acid. The competitive inhibition did not appear up to 90 mM concentration of acetic acid and 71 mM concentration of *n*-butyric acid. In Fig. 3 plots V_0/V_i *vs.* i by Eq. (4) shows the inhibitory effect at different concentrations of each acid. From the plots, K_i of each competitive inhibitor was determined, and the result is shown in Table 1. We see in Tables 1 and 2, that K_i decreases as the number of carbon atoms in the carboxylic acid molecule increases, as was observed with a series of aliphatic alcohols.[2]

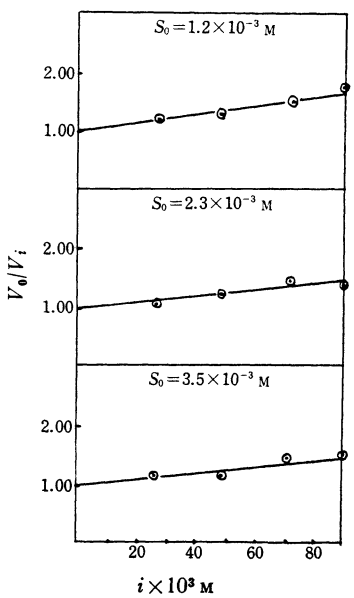

Fig. 3. V_0/V_i *vs.* i plots for the hydrolysis of Z-Glu-Tyr by pepsin inhibited by acetic acid at pH 3.5 (0.2 M sodium citrate buffer); temperature, 37°C.

TABLE 2. VALUES FOR $-\Delta F_i^{0a)}$, $-\Delta F_{HC}^{0b)}$

Carboxylic acids	average $K_i\times10^3$ (M)	$-\Delta F_i^0$ kcal/mol	$-\Delta F_{HC}^0$ kcal/mol
Acetic acid	87.3	1.5	
Propionic acid	64.3	1.8	0.3
n-Butyric acid	46.3	1.9	0.4
Isobutyric acid	44.0	1.9	0.4

a) ΔF_i^0: Standard free energy change of formation of pepsin-inhibitor complex, calculated from the equation, $-\Delta F_i^0=2.303\boldsymbol{R}T\log 1/K_i$.
b) ΔF_{HC}^0: Standard free energy change of formation of complex between pepsin and the hydrocarbon chain of carboxylic acid. The date was obtained by subtracting ΔF_i^0 of acetic acid from all the values of ΔF_i^0.

Discussion

The results of the present study show that the pepsin clearly underwent competitive inhibition by carboxylic acids. Since the concentration of carboxylate ion does not affect the rate of pepsin-catalyzed reaction,[8] it is not likely that the observed competitive inhibition is caused by the carboxyl group of carboxylic acids. Thus, it is probable that the effect of competitive inhibition by acid is caused by the hydrocarbon group in the acid molecule. This is supported by the fact that the degree of the inhibition increases with the increase in the number of the carbon atoms in the acid molecule (*cf.* Table 1). Thus the binding site of the active center of enzyme may be considered to be hydrophobic, in line with Tang's result.[1,2] In other words, inhibition by carboxylic acid depends on the competition between the hydrocarbon group of carboxylic acid molecule and the substrate on the hydrophobic binding site of enzyme. It is likely that hydrophobic bond is formed between the side chain of amino acid residue in the substrate molecule and the hydrophobic region of the binding site of the active center of enzyme.

8) K. Inoyue, I. M. Voynick, G. R. Delpierre and J. S. Fruton, *Biochemistry*, **5**, 2473 (1966).