MECHANISM OF INHIBITION OF PEPSIN BY PEPSTATIN

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(Received for publication February 9, 1972)

Pepstatin, a specific inhibitor of acid proteases, binds tightly to pepsin. Although the binding is not of the covalent nature, the inhibition roughly follows the stoicheometrical mode. Pepstatin can be used to titrate pepsin. Formation of an equimolar pepsin-pepstatin complex can be shown by gel filtration. Diacetylpepstatin, which has weaker activity than pepstatin competitively inhibits pepsin with a dissociation constant of 7.3×10^{-6} M. Data of pepstatin binding of chemically modified pepsins suggested that pepstatin binds with the active site surrounded by two aspartic acid moieties.

Pepstatin, isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid, was discovered in streptomyces culture filtrates and has been shown to be a specific inhibitor of acid proteases[1,2,3]. It shows a strong protective effect against pylorus-ligated rat stomach ulcer, and an effect against human gastric ulcer has been observed in clinical studies. A specific inhibitor of acid proteases is a biochemical tool useful for analysis of the role of these enzymes in stomach and duodenal ulcers. In this connection, the mode of inhibition of pepsin is thought to be interesting. In this paper, we report the strong binding of pepstatin with pepsin in the equimolar ratio and our studies suggesting the site in pepsin binding with this inhibitor.

Materials and Methods

Enzyme, inhibitors and others

Pepsin (twice crystallized) was purchased from Sigma Chemical Co., U.S.A. (lot 28B-1900) and used without further purification. Pepstatin was prepared by fermentation of *Streptomyces argenteolus* var. *toyokaensis* as previously described[1]. Diacetylpepstatin was prepared from pepstatin as previously described[1]. Radioactive pepstatin (specific activity was 2.2×10^5 dpm/μg) was kindly prepared by Dr. T. KOMAI, National Institute of Health, Tokyo, by exposing pepstatin to tritium gas. N-Acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) was purchased from Sigma Chemical Co., U.S.A., Sephadex G-50 (medium) from Pharmacia, Sweden, and *p*-bromophenacyl bromide from Tokyo Kasei Kogyo Co., Japan. All other reagents employed were analytical grade.

Determination of the initial velocity of peptic hydrolysis

The ANSON method slightly modified as previously described[2] was used for the assay of peptic activity, using hemoglobin as the substrate. For enzyme titration and kinetic study APDT was used as the substrate, because it was the most rapidly hydrolyzed of the peptide substrates commercially available. A 2 mM APDT solution in 4 mM NaOH was diluted before used to the desired concentration with 0.01 N HCl. To each test tube 3.2

ml of substrate solution containing desired amount of pepstatin or diacetylpepstatin was added and preincubated for 3 minutes at 37°C. The reaction was started by addition of 0.2 ml of 0.001 N HCl containing 60 μg of pepsin except as otherwise noted. After the incubation at 37°C peptic hydrolysis was stopped by addition of 0.1 ml of 0.7 N NaOH and the released diiodotyrosine was determined by ninhydrin method slightly modified by JACKSON *et al*[4]. The substrate concentration used was 10^{-4} M for enzyme titration experiment but in case of the kinetic study it was varied in a range of $5\sim16\times10^{-5}$ M.

Binding of ^{3}H-pepstatin to pepsin

The mixture of pepstatin and pepsin was passed through a column of Sephadex G-50 (2×55 cm) equilibrated with eluting buffer at a cold room. Three kinds of eluting buffers were employed. They were 0.05 M KCl-HCl buffer, pH 2.0, 0.05 M acetate buffer, pH 5.5, and 0.05 M Tris-HCl buffer, pH 7.2. The eluate was fractionated every 3 g by a fraction collector. Protein content of each fraction was determined by absorption at 280 mμ and pepsin activity of each fraction was measured by hydrolysis of hemoglobin. The radioactivity of ^{3}H-pepstatin in 0.1 ml of each fraction was determined in a Beckman Liquid Scintillation System using 6 ml of BRAY's scintillation solution[5]. The inhibitory activity of pepstatin was determined by the method previously described[2].

Preparation of chemically modified pepsins

Two aspartic acid residues in pepsin active site were modified by esterification with *p*-bromophenacyl bromide or/and α-diazo-*p*-bromoacetophenone as described by ERLANGER *et al.*[6] Modification was confirmed by the determination of hemoglobin hydrolyzing activity.

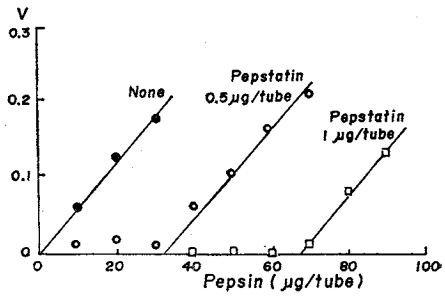
Results and Discussion

The inhibitory activity of pepstatin is dependent on the concentration of pepsin as described in a previous paper[2]. When pepsin was used at a concentration of 2, 0.5, 100, 18 μg/ml for hydrolysis of casein, hemoglobin, N-acetyl-L-phenylalanyl-L-tyrosine or N-acetyl-L-phenylalanyl-L-diiodotyrosine, respectively, pepstatin concentration exhibiting 50 % inhibition was 1.5×10^{-8} M, 4.5×10^{-9} M, 1.1×10^{-6} M or 2.3×10^{-7} M, respectively. This proportionality of pepstatin concentration to pepsin concentration suggests that pepstatin would bind to pepsin tightly. The evidence supporting this suggestion will be described in this paper.

As shown in Fig. 1, the rates of peptic hydrolysis of APDT at different pepsin concentrations with a constant amount of pepstatin were determined and plotted against the amount of pepsin. Two straight lines in the presence of pepstatin have identical slope with a straight line determined in the absence of pepstatin. This plot suggests irreversible binding, but pepstatin is a reversible inhibitor because dialysis for 2 days of pepsin-pepstatin complex separated by Sephadex gel filtration releases free pepstatin out of the dialysis bag, and pepstatin is recovered from the complex by extraction with butanol or by standing in alkaline condition inactivating pepsin. It is thought that pepstatin binds to pepsin so

Fig. 1. Plots of initial velocity against pepsin concentration at different concentrations of pepstatin.

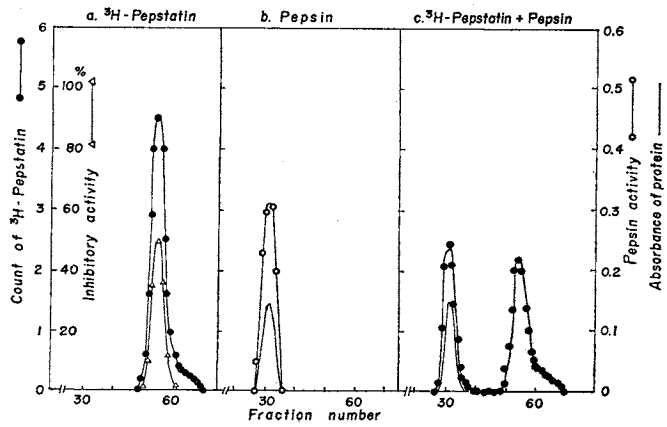
Initial velocity was determined using 10×10^{-5} M APDT as substrate as described in the text. Pepsin concentration was varied from 90 μg per 3.4 ml of incubation mixture. These incubation mixtures contained none, 0.5 μg and 1.0 μg of pepstatin.



tightly that the dissociation of enzyme-inhibitor complex is very slow. The amounts of pepsin indicated at the intersection with the horizontal line in the presence of pepstatin in Fig. 1 must be equivalent to the amount of inhibitor added. As a method of titrating pepsin normality has never been established, pepstatin is very useful for this purpose. In this experiment 1 μg of pepstatin was equivalent to 65 μg of pepsin. Considering that the molecular weights of pepstatin and pepsin are 686 and 35,000, respectively, it is concluded that one molecule of pepstatin combines with one molecule of pepsin and the active pepsin molarity used in this experiment was calculated to be 77% of that calculated from the weight.

Fig. 2. Binding of ^{3}H-pepstatin to pepsin shown by Sephadex G-50 gel-filtration.

Column of Sephadex G-50 (2×55 cm) was previously equilibrated and eluted with 0.05 M KCl-HCl buffer, pH 2.0. 40 μg of ^{3}H-pepstatin (a), 1.8 mg of pepsin (b) and the mixture of 40 μg of ^{3}H-pepstatin and 1.8 mg of pepsin (c) were each chromatographed on Sephadex G-50. Pepsin and pepstatin activity and radioactivity of pepstatin were determined. Pepsin was estimated as absorption at 280 mμ.



It was also proven by another experiment with Sephadex G-50 gel filtration that pepstatin is a tight binding inhibitor. Pepstatin labeled with tritium was used and the experimental condition in detail is described in the legend to Fig. 2. The mixture of pepsin and ^{3}H-pepstatin were incubated at 37°C for 10 minutes and passed through Sephadex G-50 column. As shown in Fig. 2 c) ^{3}H-pepstatin appeared in two peaks. The second peak corresponded to free pepstatin (Fig. 2 a) (and the first peak corresponded to that of pepsin (Fig. 2 b)). It means that ^{3}H-pepstatin binds to pepsin tightly. Pepsin activity in this pepsin-pepstatin complex was 1.3% of free pepsin activity in hemoglobin hydrolizing activity. The same result was obtained at pH 2.0 and pH 5.5. When 0.05 M Tris-HCl buffer of pH 7.2 in which pepsin was denatured was used as eluting buffer, pepstatin did not bind to pepsin. Pepstatin did not bind to pepsinogen, so it was concluded that pepstatin binds tightly to active form of pepsin.

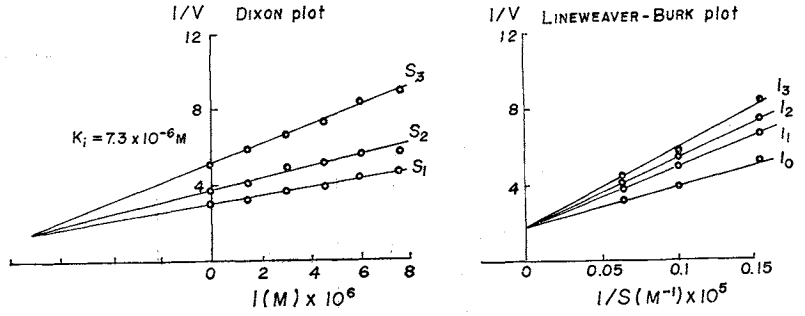
Since inhibition of pepsin by pepstatin appears to be of the pseudo-irreversible type, kinetic analysis has many limitations. Noncompetitive inhibition in LINEWEAVER-BURK plot and upwards curvature in DIXON plot reported in our preliminary paper[1] can be explained as mutual depletion system[7]. From the results described in this paper our previous conjecture of multiple binding of pepstatin to pepsin should be corrected. Diacetylpepstatin is less active than pepstatin and can be used for kinetic studies. Diacetylpepstatin was a competitive inhititor as shown in LINEWEAVER-BURK plot and DIXON plot (Fig. 3) using APDT as the substrate. Its dissociation constant was calculated to be 7.3×10^{-6} M. If pepstatin is a competitive inhibitor like diacetylpepstatin,

Fig. 3. Competitive inhibition of pepsin by diacetylpepstatin.

Substrate was N-acetyl-L-phenylalanyl-L-diiodotyrosine and the pepsin concentration was 17.7 μg/ml.

(a) LINEWEAVER-BURK plot The concentrations of pepstatin were : I_0, no inhibitor ; I_1, 3.05×10^{-6} M ; I_2, 4.58×10^{-6} M ; and I_3, 6.11×10^{-6} M.

(b) DIXON plot The concentrations of substrate were : S_1, 16×10^{-5} M ; S_2, 10×10^{-5} M; and S_3, 6.45×10^{-5} M.



velocity of peptic hydrolysis in the presence of pepstatin is given by equation (1)[8].

$$V=\frac{kS}{2}\left[\sqrt{\left(\frac{K_t}{K_m}+\frac{I_t-E_t}{K_m+S}\right)^2+\frac{4E_t}{K_m+S}\cdot\frac{K_t}{K_m}}-\left(\frac{K_t}{K_m}+\frac{I_t-E_t}{K_m+S}\right)\right] \quad \text{eq. (1)}$$

When equation (1) is applied to our experimental data, dissociation constant of pepsin-pepstatin complex is roughly estimated to be less than 3×10^{-9} M. Further investigation on the inhibitory mechanism of pepstatin is now in progress using a more suitable substrate for the kinetic study.

The structure of pepstatin is quite different from that of any synthetic substrates of pepsin but pepstatin is thought to be a very strong competitive inhibitor from the data obtained on diacetylpepstatin. In another approach to study the inhibitory mechanism, pepsin was chemically modified in the active site and the binding of ^{3}H-pepstatin to modified pepsins was examined by gel filtration method as described before. Two different β-carboxyls of aspartic acid residues at or near the active site of pepsin can be modified by *p*-bromophenacyl bromide and α-diazo-*p*-bromoacetophenone[6]. It has been thought that the aspartic acid substituted by the former does not play an essential role in catalysis but is

Fig. 4. Binding of ^{3}H-pepstatin to modified pepsin shown by Sephadex G-50 gel-filtration.

0.05 M Acetate buffer, pH 5.5 was used as eluting buffer. Chromatography was carried out on the mixture of 60 μg ^{3}H-pepstatin and 1.8 mg of pepsin or modified pepsin as follows: pepsin with no modification (a), pepsin treated with *p*-bromophenacyl bromide (b), pepsin treated with α-diazo-*p*-bromoacetophenone (c) and pepsin treated with *p*-bromophenacyl bromide and α-diazo-*p*-bromoacetophenone (d). Absorbance of protein at 280 mμ (-----) and radioactivity of ^{3}H-pepstatin (——) were determined.

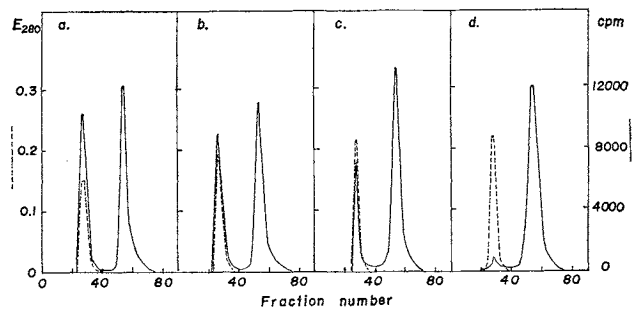


Table 1. Effect of chemical modification of pepin

Modification	Relative enzyme activity	Binding of pepstatin
No modification (Pepsin)	100	100
p-Bromophenacyl bromide	25	81
α-Diazo-*p*-bromoacetophenone	0.74	64
p-Bromophenacyl bromide and α-diazo-*p*-bromoacetophenone	0.41	10

involved in substrate binding and the aspartic acid substituted by the latter is involved in catalytically active site because this modification causes almost complete inactivation of pepsin as seen in the other modifications with various diazocarbonyl compounds[9~14]. Both these aspartic acid moieties can be esterified by reaction with α-diazo-*p*-bromoaceptophenone of pepsin already treated with *p*-bromophenacyl bromide. The affinities of these modified pepsins to ^{3}H-pepstatin were tested by Sephadex G-50 gel filtration as shown in Fig. 4. Results are summarized in Table 1, comparing the radioactivity distributed in protein fractions. Enzyme activity of modified pepsins was shown by relative activity to pepsin. Pepsin monoesterified by either reagent still binds with pepstatin, though the binding is lowered to about 60 % of that of active pepsin. However, pepsin modified by both reagents showed reduced binding activity for pepstatin, about 10 % of native pepsin. This result suggests that the two substituted aspartic acid residues exist near one another in the three dimensional structure of pepsin and the binding site for pepstatin is near the two aspartic acid moieties. A concept of hydrophobic binding site was proposed by TANG[15] to explain the specificity of pepsin for protein substrates. In digestion of proteins by pepsin, peptide linkages which are not expected to be hydrolyzed from action of pepsin on synthetic peptides are rapidly cleaved. The structure of pepstatin is also different from that of any synthetic substrate. Hydrophobicity can be thought to play an important role in binding of pepstatin to pepsin, because pepstatin and pepsin are not bound by a covalent bond.

Acknowledgement

We wish to express our sincere thanks to Dr. TOMOYOSHI KOMAI, National Institute of Health, Tokyo, for preparing radioactive pepstatin.

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