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BACITRACIN AND GRAMICIDIN S AS INHIBITORS OF CARBOXYLIC PROTEINASE

V. M. Stepanov, M. V. Gonchar, and G. N. Rudenskaya

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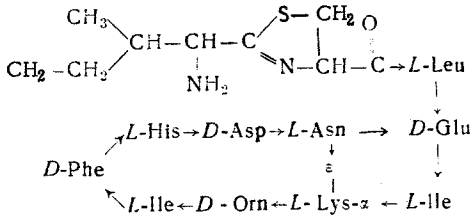
The antibiotic bacitracin — a mixture of cyclic polypeptides of similar structure — is produced by *Bac. licheniformis*. Below we give the structure of the main representative of the family of bacitracins — bacitracin A [1]:



Makinen [2] investigated the action of bacitracin on a number of proteolytic enzymes. It was found that it inhibits the papain-catalyzed hydrolysis of N_α-benzoyl-D,L-arginine 2-naphthylamide and the subtilisin-, papain-, and leucine-aminopeptidase-catalyzed hydrolysis of L-leucine 2-naphthylamide. The K_I values for the inhibition of papain, subtilisin, and leucine aminopeptidases by bacitracin are 5.0, 4.5, and 2.0 mM, respectively. On the other hand, bacitracin shows no capacity for inhibiting aminopeptidase B and trypsin, and α-chymotrypsin is inhibited feebly. In view of the fact that bacitracin has proved to be a suitable ligand for the affinity chromatography of carboxylic proteinases, we have studied its capacity for inhibiting a typical enzyme of this class — porcine pepsin.

As the pepesin substrate we selected a protein substrate — hemoglobin. The proteolytic activity was determined by a modification of Anson's method [3]. It was found that the dependence of the reciprocal rate of cleavage ($1/\Delta E_{280}$) on the reciprocal concentration of hemoglobin ($1/[S_0]$) obeys a linear law at least in the range of concentrations of substrate of from 1 to 20 mg/ml. A graphical calculation of the apparent value of K_M for the hydrolysis of hemoglobin by pepsin (Fig. 1, line a) gives a value of 5.7 mg/ml or 0.35 mM (calculated to one hemoglobin subunit).

Treatment of the kinetic results on the cleavage of hemoglobin by pepsin in the presence of bacitracin at various concentrations of the substrate and inhibitor in the Lineweaver–Burk coordinates (see Fig. 1, lines a, b, and c) shows that the dependence of $1/\Delta E_{280}$ on $1/[S_0]$ has the form of a family of straight lines intersecting on the axis of ordinates. Consequently bacitracin inhibits porcine pepsin as a fully competitive, or close to fully competitive, inhibitor. Analysis of the kinetic results on inhibition by the simplified Dixon method (Fig. 2) in the coordinates $1/\Delta E_{280}$ versus [I] gives for K_I a value of 3.1 mg/ml or 2.3 mM. Thus, bacitracin inhibits pepsin with approximately the same efficiency as papain, subtilisin,

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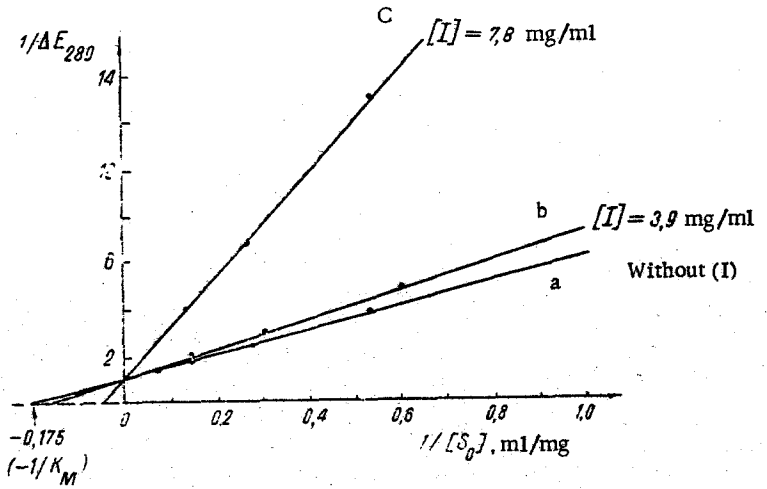
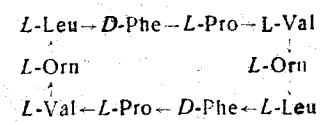


Fig. 1. Determination of $K_{M(app)}$ for the hydrolysis of hemoglobin by pepsin (a) and for the type of inhibition of pepsin by bacitracin (a, b, c). The abscissa of the point of intersection of the straight line a with the axis of abscissas is equal to the reciprocal of K_M with a negative sign. The point of intersection of the bundle of straight lines a, b, and c on the axis of ordinates shows a competitive type of inhibition of pepsin by bacitracin.

and leucine aminopeptidase. This is probably connected with the broad specificity of the enzymes under consideration and the greater extension of the corresponding substrate-binding zones.

Analysis of the influence of another antibiotic cyclopeptide — gramicidin S —



on the hydrolysis of hemoglobin by horse pepsin showed that this peptide is also a pepsin inhibitor with $K_I \sim 2$ mM.

The capacity of bacitracin and gramicidin for inhibiting pepsin is apparently due to the presence of hydrophobic amino acids characteristic for the specificity of pepsins — phenylalanine, isoleucine, and leucine — in these antibiotics.

The values of K_I for the inhibition of pepsin by gramicidin S and bacitracin are close to K_M of the best peptide substrates of pepsin. Thus, for example, the values of K_M for the hydrolysis of the peptides N-Ac-L-Tyr-L-Phe-OH, N-Ac-L-Phe-L-Tyr-OH, and N-Ac-L-Tyr-L-Tyr-OH are 2.0, 2.2, and 6.1 mM, respectively [4].

V. M. Stapanov et al. [5-7] have used gramicidin S as a ligand for the affinity chromatography of a series of carboxylic proteinases — porcine and equine pepsins, a mixture of bovine pepsin and rennin, aspergillopepsin A produced by *Asp. awamori*, and a carboxylic proteinase from an insectivorous plant. The results that we obtained directly confirm the hypothesis put forward previously [7] according to which gramicidin S may act as a pepsin inhibitor. On the other hand, the value of K_I permits a more definite idea of the nature of the forces participating in the interaction with the affinity solvent. We may note that the amount of ligand in the affinity sorbents based on gramicidin S that were investigated was usually 0.3-2 μmole/ml. When pepsin was chromatographed on such solvents at pH ∿4.5, practically complete binding of the enzyme was usually observed, which could not have been expected if the process depended only on specific interactions of the ligand with the enzyme. We assume that in addition to specific interactions of the ligand and the enzyme a large contribution to the binding of pepsin by affinity sorbents based on the antibiotics under consideration is given by nonspecific ionic interactions of pepsin, which is negatively charged under

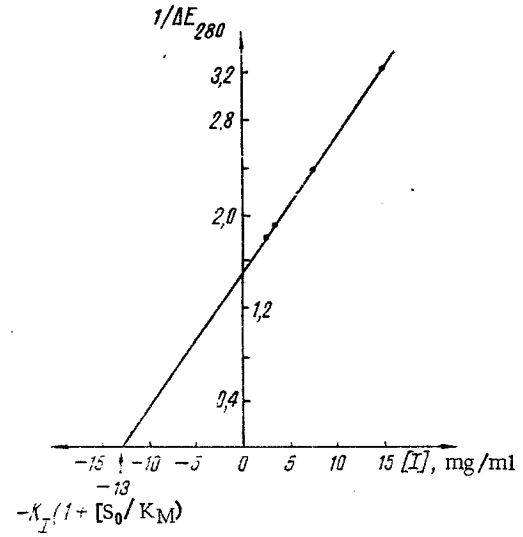


Fig. 2. Determination of K_I for the inhibition of pepsin by bacitracin by Dixon's method. The concentration of antibiotic has been plotted along the axis of abscissas. The abscissa of the point of intersection of the straight line with the axis of abscissas is equal to $-K_I\ (1 + [S_0]/K_M)$; in this series of experiments, $[S_0]$ was 17.9 mg/ml. K_M = 5.7 mg/ml (see Fig. 1).

the conditions of chromatography, with the positively charged isourea derivatives formed on the addition of gramicidin to Sepharose activated by cyanogen bromide.

M. M. Chernaya et al. [8] have shown that nonspecific ionic interactions play a large role in the binding of pepsin with sorbents containing peptide ligands. There is indirect evidence that gramicidin S can also inhibit other proteinases. Thus, for example, T. I. Vaganova et al. [9] have found that (gramicidin S)—Sepharose gives good results in the isolation of bacterial metalloproteinases. As can be seen from literature information and the results of the present work, bacitracin is an inhibitor of a fairly wide range of proteinases. It is possible that a whole series of polypeptide antibiotics containing fairly diverse sets of amino acids in the molecule is capable of inhibiting various classes of proteinases possessing a broad specificity and having fairly extended substrate-binding sections.

EXPERIMENTAL

The work was carried out with porcine pepsin purified by chromatography on amino-Silochrome [10] and having a specific activity of 50 units/mg and equine pepsin obtained from gastric juice with the aid of ion-exchange and affinity chromatography [6] and having a specific activity of 56 units/mg, "Serva" bacitracin (GFR) and an industrial preparation of gramicidin S purified by recrystallization from ethanol.

DETERMINATION OF THE CONSTANTS OF INHIBITION BY BACITRACIN AND GRAMICIDIN S. Inhibition of Porcine Pepsin by Bacitracin. A solution of bacitracin in 0.06 M HCl (25 mg/ml) was prepared. An aliquot (50-60 µl) of the antibiotic solution was added to 1 ml of a solution of hemoglobin (1-20 mg/ml) in 0.06 M HCl, the solution was thermostated at 37°C, and then an aliquot (50-70 µl) of the enzyme solution (0.3 mg/ml) was added. After 10 min, 5 ml of 5% trichloroacetic acid was added to the samples, the precipitate was eliminated by filtration through a "Filtrak"-90 paper filter (GDR), and the optical density of the filtrate at 280 nm was measured. In parallel with the main samples, control samples were set up to which no enzyme was added. The activity of the pepsin was expressed in units of increase in the optical density of the filtrate at 280 nm (ΔE_{280}). The experiment was performed at various concentrations of substrate and inhibitor. Treatment of the experimental results of the inhibitor of porcine pepsin by bacitracin is given in Figs. 1 and 2. K_I = 3.1 mg/ml (or 2.3 mM).

Inhibition of Equine Pepsin by Gramicidin S. To 1 ml of a solution of hemoglobin in 0.06 M HCl (20 mg/ml) was added 50 µl of an ethanolic solution of gramicidin S (50 mg/ml), the solution was thermostated at 37°C, and then 70 µl of a solution of equine pepsin (0.2 mg/ml was added. In parallel, the activity of the pepsin was determined at the same concentration of substrate and ethanol but in the absence of the antibiotic. The value of K_I was calculated analytically on the basis of a model of competitive inhibition by means of the equation

$$K_I = \frac{[I]}{(v_0/v - 1)([S_0]/K_M - 1)}.$$

where $[S_o]$ = 17.9 mg/ml is the concentration of hemoglobin; [I] = 2.23 mg/ml is the concentration of gramicidin S; and $K_{M(app)}$ = 5.7 mg/ml is the Michaelis constant for the hydrolysis of hemoglobin by equine pepsin (it was determined in the same way as for porcine pepsin, see Fig. 1, line a); and a is the ratio of the activities of pepsin in the absence and in the presence of gramicidin S, which under the given conditions is 1.21. Calculation gave K_I = 2.6 mg/ml (or 2.0 mM).

SUMMARY

1. It has been shown that polypeptide antibiotics — bacitracin and gramicidin S — inhibit carboxylic proteinases — porcine and equine pepsins. Bacitracin inhibits pepsin with K_I = 2.3 mM, and gramicidin S with $K_I \sim 2$ mM.

2. The results obtained permit a theoretical foundation for the use of bacitracin and gramicidin S and also of other polypeptide antibiotics as ligands for the biospecific chromatography of proteinases.

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STUDY AND ISOLATION OF VANADYLPORPHYRINS FROM THE PETROLEUM OILS OF THE BUZACHI PENINSULA

R. Nasirov and A. Kushalieva

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The porphyrin fragments of petroleum oils discovered by A. Treibs in 1934 [1] have long attracted the attention of research workers. All porphyrin pigments are related compounds of the porphins. A fairly large number of porphyrins differing from one another by their side-chain substituents is known: the majority of them have been obtained synthetically, and a considerable number have been isolated from biological sources [2].

Several types of porphyrins have been detected in petroleum oils and ancient sediments [3, 4]. Porphyrins may be present in petroleum oils both in the free form and in the form of complexes with heavy-metal ions as a vanadium complex [5], an iron complex [6], and a nickel complex [7, 8]. The complexes of other metals with porphyrin have not yet been identified with certainty.

There is no information in the literature on the isolation and structure of the porphyrin fragments of the petroleum oils of Kazakhstan. We have limited ourselves to determining the vanadylporphyrins in the petroleum oils of the Buzachi peninsula and to isolating them.

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