IDENTIFICATION OF THE CARBOXYL GROUP OF PEPSIN REACTING WITH DIAZOACETAMIDE DERIVATIVES

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Three types of specific inhibitors of pepsin were described: α-bromoketones (Erlanger, Vratsanos, Wassermann and Cooper, 1966), α-diazoketones (Hamilton, Spona and Crow, 1967; Delpierre and Fruton, 1966), diazoacetamide derivatives (Rajagopalan, Stein and Moore, 1966; Stepanov,Lobareva and Mal'tzev,1967). It was shown that p-bromophenacylbromide (Erlanger,Vratsanos,Wassermann and Cooper,1966) and N-diazoacetyl-N'-2,4-dinitrophenyl-ethylenediamine (Stepanov,Vaganova 1967) interacted with β-carboxyl groups of aspartic acid residues in hog pepsin. To identify the aspartic acid residue participating in the reaction with the inhibitor of given type it is necessary to determine the neighbouring amino acids.

We found that N-diazoacetyl-N'-2,4-dinitrophenyl-ethylenediamine (DDE) reacted in pepsin molecule with β-carboxyl group of aspartic acid residue in the sequence Val-Asp situated in the C-terminal part of pepsin polypeptide chain.

Materials and Methods.

Hog pepsin purified by chromatography on DEAE cellulose was used. Proteolytic digestion was performed with "Orysine" - commercial preparation of proteinases of Aspergillus oryzae.

Pepsin was treated with DDE as follows. To the solution of

1 g of pepsin in 1 l of 0.1 M acetate buffer, pH 5.0, cooled to 14°, 10 ml of 0.1 M cupric acetate was added. After 10 min. 150 mg of freshly prepared DDE in 15 ml of wet acetone was added at once to the pepsin solution under vigorous agitation. After standing at 14° for 20 min, proteolytic activity of the mixture was measured (Anson,1938). It was found that the residual activity was about 0.7 per cent of initial value.

Excess of the reagent and its decomposition products were removed on Sephadex G-25 column (80x7.5 cm) pre-equilibrated with water. The first portion of the eluate containing the protein and small quantity of highly aggregated yellow substance - probably product of decomposition of the inhibitor - was collected and transferred on DEAE cellulose column. Chromatography conditions and elution pattern are shown on the Fig.1. It should be mentioned that the yellow products of inhibitor decomposition were held strongly by DEAE cellulose and could not be eluted under usual conditions.

Results and Discussion.

Fractins 1 and 2 (Fig.1) appear obviously as a result of cleavage of inhibited pepsin. In some experiments the third yellow peak corresponding to the inhibited pepsin appears immediately after Fraction 2. Fraction 2 contains the inhibitor residue whereas Fraction 1 does not. Determination of basic amino acids on Amino Acid Analyser revealed the presence of one lysine and two arginine residues in the Fraction 2 which is devoid of histidine. The only histidine residue of pepsin was found in Fraction 1. From the distribution of basic amino acid residues it might be concluded that the Fraction 2 corresponds to the C-terminal region of pepsin molecule where

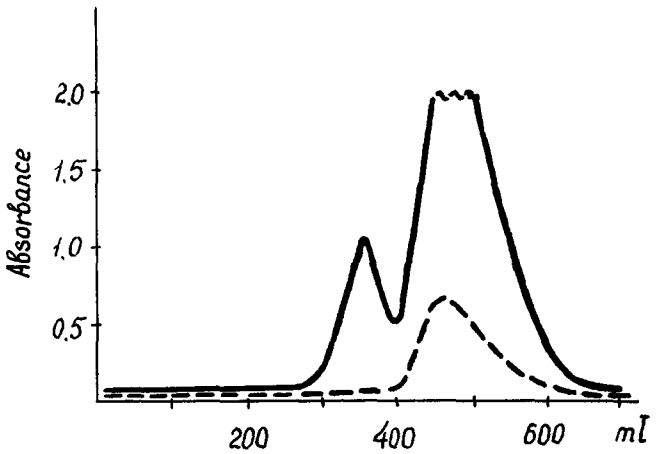

Fig.I. Chromatography of DDE-treated pepsin on DEAE cellulose. 300 mg of pepsin treated with DDE was chromatographed on DEAE cellulose column (3.5 x 25 cm). Elution performed with 0.I M acetate buffer,pH 5.0 with NaCl concentration gradient from 0 to I.0 M. The rate of elution I5 ml per hr. 5 ml fractions were collected. All procedures were performed at 5°.

Solid line - absorbance at 280 mμ , broken line - absorbance at 360 mμ .

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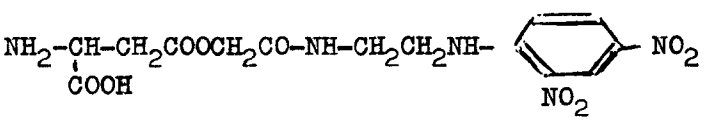
all arginine and lysine residues are accumulated. (Stepanov et al.,1967).

The close investigation of the cleavage of inhibited pepsin does not come into the scope of this paper but it should be indicated that the picture obtained resembled strongly that observed in the course of chromatography of alkali denatured pepsin (Ginodman, Greil and Orekhovich, 1961). We suppose therefore that the DDE-treated hog pepsin becomes more liable to the limited proteolysis by traces of active pepsin escaped from the inhibition.

Fraction 2 was collected, desalted on Sephadex G-25 and lyophilised. 75 mg of this fraction in 15 ml of 0.1 M acetate buffer, pH 5.0, was digested 21 hr. at 37° with 10 mg of "Orysine". Hydrolysate was repeatedly extracted with n-butanol The yellow upper layer was evaporated and subjected to fractionation by paper electrophoresis at pH 2.2 and 700 v during 2.5 hr. Three yellow bands were separated, their distances from the origin being 0 cm (fraction A), 2.3 cm. (fraction B) and 5.5 cm (fraction C).

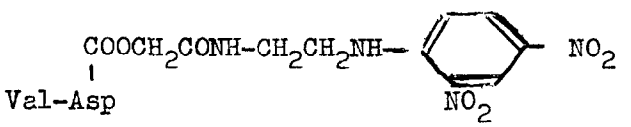
Fraction A gives negative ninhydrin reaction and corresponds presumably to the product of cleavage of enzyme-inhibitor bond.

Fraction B gives brown colour with ninhydrin. After acid hydrolysis of this fraction (5.7 N HCl, 105°, 24 hr.) only aspartic acid was found. Quantitative determination revealed that aspartic acid was bound with inhibitor residue in 1:1 ratio. Incubation of this substance at pH 9 and 37° lead to cleavage of inhibitor-aspartic acid bond. The following structure should be ascribed to this substance:



Fraction C was purified by gel-filtration on water-equilibrated Sephadex G-10 column (23 x 1 cm) . The first portion of the eluate containing yellow substance was collected, evaporated and chromatographed on polyamide thin-layer plate (Polyamide Woelm) in 30 per cent ethanol. The substance containing dinitrophenyl residue (substance C) moved in this solvent slowly whereas the contaminating free peptides migrated with the solvent front. 0.3 μ mol of the

substance C (quantity determined on the basis of UV absorption at 360 mμ) was hydrolysed with 5.7 N HCl, 24 hr. at 105°. In this hydrolysate 0.24 μmol of aspartic acid and 0.21 μmol of valine was found. After dinitrophenylation of 0.3 μmol of the substance C (pH 9, 40°, 2 hr.) and hydrolysis (5.7 N HCl, 24 hr., 105°) 0.19 mol of dinitrophenyl-valine was found. N-terminal position of valine being thus established the following structure should be ascribed for C:



Thus, DDE attacs in hog pepsin the unique carboxyl group of aspartic acid residue participating in the sequence Val-Asp.

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