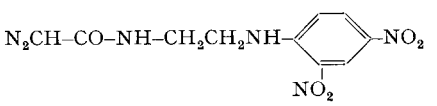
BBA 61156

Coloured inhibitors of pepsin

Various diazoacetyl derivatives were suggested recently as reagents for the chemical modification of enzymes. Diazoacetylglycine amide has been shown to react with carboxyl groups of ribonuclease (EC 2.7.7.16)[1]. RAJAGOPALAN, STEIN AND MOORE[2] succeeded in specificaly inhibiting hog pepsin (EC 3.4.4.1) with diazoacetyl-DL-norleucine methyl ester at pH 5.0 in the presence of cupric ions. Although the mechanism of pepsin inhibition is not yet established, a great deal of indirect evidence points to the carboxyl groups of aspartic or glutamic acid as the most probable sites of inhibitor attachment to the enzyme molecule. It should be mentioned that other inhibitors suggested for pepsin, *i.e.* diphenyldiazomethane[3], *p*-bromophenacylbromide[4,5], diazoketones[6,7] are also supposed to interact specifically with the pepsin carboxyl group.

Obviously, the use of coloured reagents for the inhibition of pepsin would greatly facilitate the elucidation of the mechanism of their action on the enzyme and also the location of their attachment sites to the polypeptide chain. It is the purpose of this paper to describe the synthesis of a coloured inhibitor and its reaction with pepsin.

N-Diazoacetyl-*N'*-2,4-dinitrophenylethylenediamine (DDE) was prepared as follows.



To 4.10 g of phthaloylglycine in 50 ml of dry methylene chloride, 2.02 g of triethylamine was added. To this solution cooled to $-5°$, 2.72 g of isobutylchloroformate were added slowly, the mixture was kept 15 min at $-5°$ then treated with 4.52 g of mono-dinitrophenylethylenediamine[8] in methylene chloride. *N*-Phthaloylglycyl-*N'*-2,4-dinitrophenylethylenediamine appeared immediately as a yellow precipitate. After 1 h the product was collected and treated with 100 ml of hot ethanol. The yield was 5.6 g (69%). The substance is practically insoluble in water and in common organic solvents with the exception of boiling formic acid. The compound had a m.p. of 304–305°. (Found: C, 52.06; H, 3.48; N, 17.11. $C_{18}H_{15}O_7N_5$ requires C, 52.31; H 3.65; N, 16.94.)

1.6 g of *N*-phthaloylglycyl-*N'*-2,4-dinitrophenylethylenediamine in 20 ml of ethanol and 2 ml of water was refluxed for 1 h with 2 ml of hydrazine hydrate. The solvents and hydrazine hydrate were removed on flash evaporator, the residue was placed in 40 ml of hot water and acidified to pH 2 with HCl. Precipitated phthaloylhydrazide was filtered off, the filtrate was evaporated and a yellowish red residue was recrystallised from boiling ethanol. The yield of chlorohydrate of *N*-glycyl-*N'*-2,4-dinitrophenylethylenediamine 1 g. Sometimes the preparation contains an admixture of hydrazine chlorohydrate which can be removed by crystallisation from a small volume of water. When subjected to paper electrophoresis at pH 2.2 the substance moves toward the cathode as one yellow spot, giving a positive ninhydrin

Abbreviation: DDE, *N*-diazoacetyl-*N'*-2,4-dinitrophenylethylenediamine.

reaction. The compound had a m.p. of 193–195°. (Found: C, 37.33; H, 4.48; N, 21.95. $C_{10}H_{14}N_5O_5Cl$ requires C, 37.56; H, 4.41; N, 21.90%.)

100 mg of *N*-glycyl-*N'*-2,4-dinitrophenylethylenediamine·HCl and 300 mg of sodium nitrite were dissolved in 5 ml of water. The pH of this solution was lowered to 4.0–4.2 by careful addition of glacial acetic acid. The mixture became turbid and separation of the yellow precipitate began. When necessary more acetic acid was added to prevent a rise in pH. After 30 min at 18°, the precipitate was collected and washed with a small volume of cold water. The yield of *N*-diazoacetyl-*N'*-2,4-dinitrophenylethylenediamine was 50 mg. The infrared spectrum of this compound shows a band at 2114 cm^{-1} characteristic for aliphatic diazo compounds.

Inhibition of pepsin with DDE was performed as follows. 50 mg of hog pepsin purified by chromatography on DEAE-cellulose[9] were dissolved in 50 ml of 0.04 M acetate buffer (pH 5.0). To this solution 0.5 ml of 0.1 M copper acetate was added, the mixture was incubated for 10 min at 15°, and then a solution of 10 mg of freshly prepared DDE in 0.2 ml of wet acetone was added quickly with vigorous agitation. After 15 min at 15° proteolytic activity was determined in an aliquot of the reaction mixture using haemoglobin as substrate[10]. As a control, the pepsin solution was subjected to the same treatment in the absence of DDE. Proteolytic activity of pepsin treated with DDE does not exceed 1% of the initial value, whereas in the control the activity was not changed.

The reaction mixture was centrifuged for 1 h at 40 000 rev./min to remove decomposition products of excess DDE. The clear yellow solution was applied to a Sephadex G-25 column (4 cm × 40 cm) pre-equilibrated with water. The column was eluted with water (5 ml/min). Fractions containing protein were combined and lyophilised. The yield of inhibited pepsin was 31 mg. A solution of this protein in 48 ml of water had an absorbance of 0.780 at 280 mμ and 0.310 at 360 mμ. The ultraviolet spectrum of pepsin inhibited with DDE is shown in Fig. 1. Since the absorbance at 360 mμ being caused by the dinitrophenylamino group of inhibitor, one can calculate the content of inhibitor in the sample of protein. Taking into account the fact that the ε_M of the dinitrophenylamino group is 15 000, one can determine that the sample contains 0.99 μmole of inhibitor. This solution was evaporated and hydrolysed with 5.7 M HCl (24 h at 105°). Determinations made with an Amino Acid Analyser revealed the presence of 0.93 μmole of lysine and 2.10 μmoles

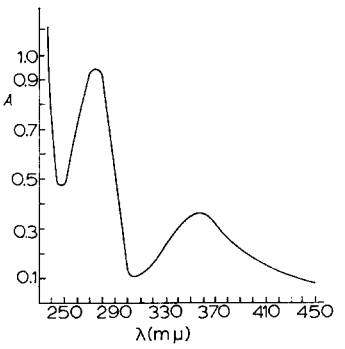


Fig. 1. Ultraviolet absorption spectrum of DDE-inhibited pepsin. The spectrum was measured in water at a concentration 0.6 mg/ml in 1-cm cells.

of arginine in the hydrolysate, which corresponds to 1 μmole of pepsin[11]. Thus DDE-inhibited pepsin contains one inhibitor residue per protein molecule.

It is obvious that the manner of DDE action on pepsin strongly resembles that of diazoacetyl-DL-norleucine methyl ester which also combines with enzyme in a 1:1 ratio[2]. We found that DDE did not interact with hog pepsin previously inhibited with diazoacetyl-DL-norvaline methyl ester — a close homologue of diazoacetyl-DL-norleucine methyl ester.

DDE can also be used for inhibition or chemical modification of other enzymes. Thus treatment of calf rennin (EC 3.4.4.3) with DDE under the conditions described in this paper for the inhibition of pepsin leads to complete loss of its rennin activity.

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Received January 2nd, 1968

Biochim. Biophys. Acta, 151 (1968) 719–721

BBA 61160

Heterogeneity of pig heart creatine kinase

In addition to the well-established pattern of isoenzymes for soluble creatine kinases (ATP:creatine phosphotransferase, EC 2.7.3.2), recent data support the existence of further heterogeneity at the subcellular level. JACOBS, HELDT AND KLINGENBERG[1] produced the first evidence for the existence of creatine kinase in highly purified mitochondria from skeletal muscle, as well as from heart and brain of the rat. They demonstrated electrophoretically that the enzyme of mitochondrial origin differs from the well known isoenzymes of the soluble fraction. Using guinea-pig brain, SWANSON[2] found differences in kinetic behaviour, both in rates and in pH optima, between crude extracts of the soluble enzyme and that found associated with a mitochondrial suspension. However, he was unable to solubilize this latter enzyme. Further, OTTAWAY[3] has produced evidence for a creatine kinase associated with the myofibrillar fraction of ox heart muscle.

These results prompt us to report the first purification and crystallization, from