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CHROMATOGRAPHY OF ACID PROTEINASES AND CHYMOTRYPSIN ON A SORBENT CONTAINING 2,4-DINITROPHENYL RESIDUES

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SUMMARY

A sorbent obtained by treatment of cyanogen bromide-activated Sepharose 4B with mono-N-DNP*-hexamethylenediamine has been shown to be effective in the affinity chromatography of pepsin, pepsinogen and acid proteinase from *Aspergillus awamori*. It is considered that 2,4-dinitrophenyl residues of the sorbent interact specifically with the hydrophobic zone of the enzyme, which may belong to the substrate binding site. The chromatography of chymotrypsin on the same sorbent supports this assumption.

INTRODUCTION

Affinity chromatography of pepsin on sorbents that contain covalently bound substrates or their analogues has been described earlier[1,2]. We found that a specific sorbent of another type can be used to this purpose. A study of the modification of pepsin with various mono-DNP*-diamines, *e.g.*, mono-N-DNP-hexamethylenediamine in the presence of water-soluble carbodiimide, led to the assumption that these DNP-amines are capable of being selectively bound to the hydrophobic zone localized somewhere on the enzyme surface[3,4]. If this assumption is correct, the interaction of DNP moieties with pepsin can be used in affinity chromatography. In order to check this hypothesis, we have synthesized a sorbent bearing covalently bound DNP groups by treatment of cyanogen bromide-activated Sepharose 4B (ref. 5) with mono-N-DNP-hexamethylenediamine. The sorbent was found to be useful for the chromatography of swine pepsin, pepsinogen and pepsin-like acid proteinase from *Aspergillus awamori* and chymotrypsin.

MATERIALS AND METHODS

Enzymes

Two preparations of swine pepsin were used: (1) pepsin purified by two-fold chromatography on DEAE-cellulose[6] with a specific activity of 56 units/mg and

* DNP = 2,4-dinitrophenyl.

(2) a commercial preparation of crude pepsin with a specific activity of 11 units/gm. Acid proteinase from *Aspergillus awamori* was partially purified on ECTEOLA-cellulose and had a specific activity of 35 units/mg (ref. 7). Swine pepsinogen was purified as described previously[8] and had a potential specific activity of 49 units/mg.

α-Chymotrypsin (recrystallized three times) purchased from Serva (Heidelberg, G.F.R.) contained 60–70% of active enzyme. Crystalline bovine trypsin was obtained from the Leningrad slaughterhouse (U.S.S.R.).

The α-chymotrypsin activity was assayed against N-3-carboxypropionyl-L-phenylalanine *p*-nitroanilide[9] and acetyl-L-tyrosine ethyl ester as substrates.

The proteolytic activity of acid proteinases was measured against haemoglobin[10]. One unit of activity was assumed to be equal to the amount of enzyme which, under the conditions employed, gives a ΔE_{280} of 1.0 in the trichloroacetic acid supernatant.

Ligand

1-Chloro-2,4-dinitrobenzene (20.3 g) in ethanol (160 ml) was added slowly to a solution of hexamethylenediamine (26.5 g) in ethanol (140 ml). The mixture was stirred for 20 min at 20° and refluxed for 1 h. The yellow precipitate of the bis-DNP derivative was collected on a heated funnel and washed with ethanol (25 ml). The filtrate was acidified with concentrated hydrochloric acid to pH 1–2 and evaporated to dryness. The residue was dissolved in hot 6 *N* hydrochloric acid (250 ml), the precipitate, after cooling, was collected and washed on the filter with 20 ml of 6 *N* hydrochloric acid, then dissolved in hot water (125 ml) and precipitated by the addition of an equal volume of 12 *N* hydrochloric acid. Yield, 22 g; m.p., 160–161°. Found: C, 45.05; H, 6.08; N, 17.80%. Calculated for $C_{12}H_{18}N_2O_4 \cdot HCl$: C, 45.25; H, 6.01; N, 17.58%.

The substance moves as a single yellow ninhydrin-positive spot when subjected to paper electrophoresis (pH 2.2 and 5.6) or paper chromatography in *n*-butanol–acetic acid–water (4:1:1); R_F, 0.65; $\lambda_{max.}$, 267 nm, 362 nm (E_M^{362} = 15,000 in water).

Sorbents

To 7.5 ml of Sepharose 4B (Pharmacia, Uppsala, Sweden), 0.75 g of cyanogen bromide in 22.5 ml of cold water were added at pH 11 (maintained by adding 5 *N* sodium hydroxide solution). The mixture was stirred for 10 min at 20° and pH 10.8–11.2. The activated Sepharose 4B was filtered off, rinsed with cold 0.1 *M* sodium hydrogen carbonate (pH 8) and added to a solution of 160 mg of mono-N-DNP-hexamethylenediamine hydrochloride in 0.1 *M* sodium hydrogen carbonate–dimethylformamide (1:1), pH 10. After stirring for 16 h at 4°, the sorbent was washed successively with 0.1 *M* sodium hydrogen carbonate–dimethylformamide, dimethylformamide–water, water, 0.1 *M* acetate buffer (pH 5.6), 1 *M* sodium chloride solution in the same buffer and a 20% solution of isopropanol in the latter mixture. The extensive washing was continued until the absorbance of the effluent at 360 nm became negligible.

The ligand concentration of the orange-yellow sorbent was estimated as follows. One millilitre of modified Sepharose was dried to constant weight under vacuum. The dry substance was dissolved by heating it at 100° with 15 ml of 66% trifluoroacetic acid. The solution obtained after appropriate dilution with 20% trifluoroacetic acid had a UV spectrum with a maximum at 360 nm, characteristic of

DNP-amines, which can be used to calculate the content of DNP-amine ($E_M^{360} = 15{,}000$). This procedure seems to be more reliable and convenient than hydrolysis of the sorbent with 5.7 *N* hydrochloric acid at 105° for 24 h, which results in a dark solution showing a featureless UV spectrum.

The acid proteinases were chromatographed on columns equilibrated with 0.1 *M* acetate buffer (pH 5.6), which has been used for the preparation of all eluents.

RESULTS AND DISCUSSION

The treatment of cyanogen bromide-activated Sepharose 4B with mono-N-DNP-hexamethylenediamine yields a sorbent with a sufficiently high content of DNP ligand corresponding to a "concentration" of approximately 9 m*M* of the latter in the Sepharose matrix. The relatively long polymethylene chain minimizes steric hindrance by serving as an "arm" and might eventually contribute to hydrophobic enzyme–sorbent interaction.

The study of the modification of pepsin with mono-N-DNP-hexamethylenediamine and water-soluble carbodiimide has shown that at a 2.5 m*M* concentration of DNP-amine the enzyme binding site became saturated. In agreement with these results, it has been shown that DNP-Sepharose with a comparable "concentration" of the attached ligand binds pepsin well, its capacity being close to 0.2 μmoles of the enzyme per 1 ml of the sorbent. Hence DNP-Sepharose has the typical effectivity limits of affinity sorbents.

The strength of pepsin binding by DNP-Sepharose is demonstrated by the fact that the enzyme cannot be eluted with 1 *M* sodium chloride solution or 20% isopropanol. Effective elution was achieved with a 20% solution of isopropanol in 1 *M* sodium chloride solution. In this sense, DNP-Sepharose is comparable with the sorbent containing the covalently attached tripeptide substrate analogue ε-aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester[2]. It must be emphasized that the capacity of the sorbent as well as the conditions of enzyme elution depend strongly on the ligand content and may change considerably if the column has been in use for a long period.

Evidently, the important role in the interaction of DNP-Sepharose with pepsin is due to hydrophobic contacts that are known to govern the substrate specificity of this enzyme. It seems probable that the DNP groups of the sorbent interact with the hydrophobic zones of the enzyme, which participate in the formation of the Michaelis complex. However, it cannot be excluded that these hydrophobic zones do not belong to the substrate binding site and the interaction observed is rather similar to "hydrophobic chromatography".

The ability of DNP-Sepharose to interact with the hydrophobic sites of enzymes seems to be confirmed by the fact that chymotrypsin, but not trypsin, can be chromatographed on this sorbent. The chromatography results in the separation of small amounts of inactive proteins corresponding to 10–20% of the enzyme applied to the column (Fig. 1). The active enzyme can be eluted with 0.1 N acetic acid or a 20% solution of isopropanol in 1 *M* sodium chloride solution. It is noteworthy that the Sepharose 4B with an approximately 9 m*M* concentration of covalently attached ε-aminocaproyl-D-phenylalanine methyl ester cannot bind chymotrypsin.

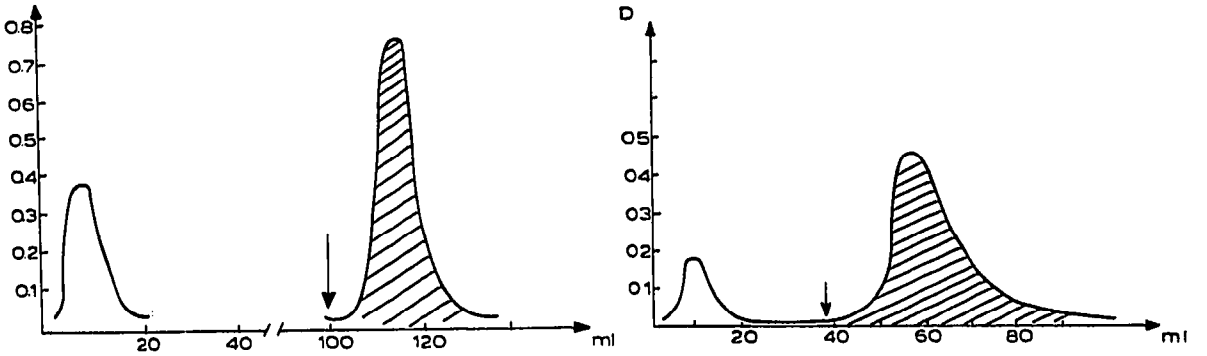

Fig. 1. Chromatography of chymotrypsin on DNP-Sepharose. A 10-mg amount of chymotrypsin in 5 ml of 0.05 *M* Tris–HCl buffer (pH 8) was applied to 5 ml DNP-Sepharose in a column with a content of DNP-ligand of 5.8 μmoles/ml. The sorbent was washed with the same buffer and eluted with 0.1 *M* acetic acid. For details, see the legend to Fig. 2.

Fig. 2. Chromatography of pepsin on DNP-Sepharose. A 10-mg amount of pepsin in 3 ml of acetate buffer (pH 5.6) was applied to a DNP-Sepharose column (volume 7.6 ml). The column was washed with 1 *M* NaCl, 10% isopropanol in 1 *M* NaCl and eluted with 20% isopropanol in 1 *M* NaCl, as shown by the arrow. Solid line: eluate absorbance at 280 nm. The peak possessing proteolytic activity is hatched.

Although it would be premature to postulate the mechanism of the sorption process, it is obvious that DNP-Sepharose is a very efficient sorbent for the chromatography of pepsin and its analogues. Even the preparation of pepsin obtained by chromatography on DEAE-cellulose, which has been used extensively for sequence studies, could be liberated from impurities, presumably the products of pepsin autolysis (Fig. 2), and its specific activity increased to 61 units/mg. This preparation

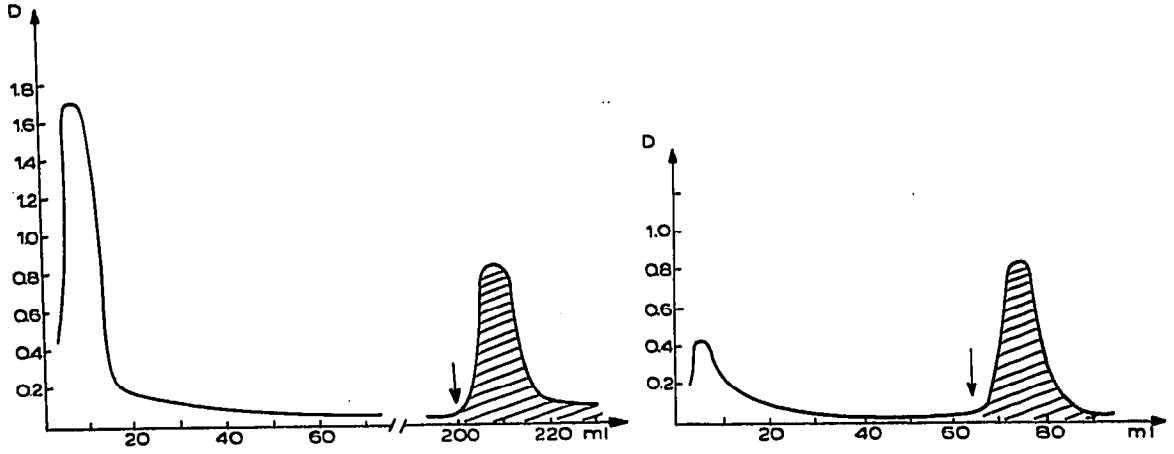

Fig. 3. Chromatography of crude pepsin preparation on DNP-Sepharose. A 45-mg amount of pepsin in 10 ml of acetate buffer (pH 5.6) was applied to the column containing 3 ml of the sorbent. Elution was performed with 20% isopropanol in 1 *M* NaCl. For further details, see the legend to Fig. 2.

Fig. 4. Chromatography of *Aspergillus awamori* acid proteinase on DNP-Sepharose. A 10-mg amount of the proteinase in 3 ml of pH 5.6 buffer was applied to 3 ml of DNP-Sepharose in a column. Elution was carried out with 1 *M* NaCl. For further details, see the legend to Fig. 2.

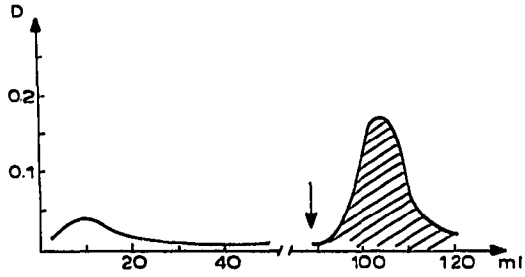

Fig. 5. Chromatography of pepsinogen on DNP-Sepharose. A 3-mg amount of pepsinogen in 2.5 ml of pH 5.6 buffer was applied to 2.5 ml of DNP-Sepharose in a column. Elution was carried out with 1 *M* NaCl. For further details, see the legend to Fig. 2.

shows one band when subjected to disc electrophoresis at pH 5.3. Chromatography of commercial crude pepsin (Fig. 3) permitted the removal of a substantial amount of inert proteins and low-molecular-weight substances, which resulted in a 4.5-fold increase in the specific activity. The yield calculated on the activity basis was 89%, with a specific activity of 51 units/mg.

Pepsin-like acid proteinase from *Aspergillus awamori* also binds fairly well to DNP-Sepharose and can be easily purified from non-proteolytic proteins (Fig. 4). The elution of this enzyme proceeds more readily than that of pepsin, *i.e.*, merely by application of 1 *M* sodium chloride solution. The specific activity increases to 44–46 units/mg, which corresponds to essentially pure enzyme.

Pepsinogen can also be chromatographed on DNP-Sepharose (Fig. 5). This procedure permits one to remove from pepsinogen purified on DEAE-cellulose up to 7% of inert proteins. The zymogen interaction with the sorbent is substantially weaker than that of pepsin and it can be eluted with 1 *M* sodium chloride solution. It should be remembered that the binding of pepsinogen to the sorbent containing covalently attached tripeptide is also weaker than that of pepsin[11]. Presumably, the interaction of pepsinogen with DNP-Sepharose should be attributed to a partially formed substrate binding site on the surface of the zymogen.

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