Chromatography of Lipophilic Proteins on Adsorbents Containing Mixed Hydrophobic and Ionic Groups

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(Received 23 November 1971)

Proteins with binding sites for molecules with hydrocarbon moieties may be purified by affinity chromatography (Cuatrecasas & Anfinsen, 1971) on adsorbents containing hydrocarbon groups. Assuming that the adsorbent support material is inert, two types of adsorbent may conceivably be used for this purpose. The first type contains hydrocarbon groups only. In an aqueous solvent phase this adsorbent may be expected to have some of the properties of an oil/water interface, including the ability to denature many proteins. The second type contains mixed hydrocarbon and ionic groups. By suitable choice of these ionic groups and of pH, selective electrostatic repulsion may be introduced between adsorbent and protein, in opposition to the hydrophobic bonding between the protein's binding site and the adsorbent's hydrocarbon group. This type of adsorbent is expected to be capable of finer discrimination between lipophilic proteins and to have less-severe denaturing effects than the first type. This communication reports initial studies on the use of adsorbents of the second type.

Agarose was selected as the adsorbent support material. The methods of Cuatrecasas (1970) were used in the following modification procedures. A 50 ml portion of settled Sepharose 6B (lot no. 5073; Pharmacia, London W.13, U.K.) in water was activated with CNBr and then coupled with 1,10-diaminodecane in 0.1 M-sodium tetraborate buffer, pH 9.5, at 5°C. The diaminodecane was used at saturation concentration in order to restrict coupling to one amino group. The resulting AD-agarose* was washed free of uncoupled reagents. When this material is used at pH values below 9.5 the free amino groups are expected to be positively charged. Half of the AD-agarose, in an equal volume of water, was treated with a large excess of succinic anhydride (0.2 g/ml of settled gel), 5 M-NaOH being added dropwise to maintain the pH at 6, the temperature being 5°C. Complete reaction of the free amino groups was achieved in about 4 h. The resulting CPAD-

agarose, after thorough washing, was used at pH values of 5 or above, when the free carboxyl groups were expected to be negatively charged.

AD-agarose and CPAD-agarose are able to interact with a protein by hydrophobic interactions involving the C₁₀ chains and by electrostatic interactions involving the terminal ionic groups. In the following discussion it is assumed that (1) these interactions are relatively independent of each other, (2) no other interactions occur to a significant degree, (3) the hydrophobic interactions are pH-independent, (4) the electrostatic interactions are determined only by pH, and (5) there are no binding sites on the protein with specific affinity for the adsorbent ionic groups. Thus, when chromatography is carried out at the isoelectric pH of the protein, adsorption will be due to hydrophobic bonding alone. By changing the pH so as to introduce a net charge on the protein of the same sign as the charge on the adsorbent ionic groups, the net adsorptive force will be diminished as a result of electrostatic repulsion. It is thus possible, in principle, to vary the strength of adsorption continuously from maximum hydrophobic adsorption at the isoelectric point to zero adsorption at the pH where hydrophobic bonding is exactly annulled by electrostatic repulsion.

The validity of this selection mechanism was tested by chromatography of bovine serum albumin on a column of CPAD-agarose. Bovine serum albumin is able to bind a number of detergents, including long-chain alkyl sulphates and alkyl sulphonates (Reynolds *et al.*, 1967); part, at least, of the binding energy is likely to be hydrophobic. In chromatography experiments unbound protein, if any, emerged shortly after sample application to the column. Thereafter protein adsorbed by mainly electrostatic forces was eluted by adding 0.5 M-NaCl to raise the ionic strength of the buffer. Finally, protein adsorbed by mainly hydrophobic forces was eluted by including sodium dodecyl sulphate (1.5%, v/v) or butan-1-ol (10%, v/v) in the buffer. The experiment was repeated at a series of pH values. The isoelectric point of bovine serum albumin was taken as 4.7–4.9 (Young, 1963). A typical set of results is shown in Fig. 1. Except for the small

* Abbreviations: AD-agarose, aminodecyl-agarose; CPAD-agarose, *N*-(3-carboxypropionyl)aminodecyl-agarose.

Fig. 1. Chromatography of bovine serum albumin on a column of CPAD-agarose at various pH values

Small columns (4 cm $\times$ 0.8 cm) of CPAD-agarose were equilibrated with buffer. A 0.1 ml portion of a 10% (w/v) solution of bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) was introduced at A. From A to B the column was developed with buffer only. Between B and C the buffer contained 0.5M-NaCl. From C to the end of the run the buffer contained 1.5% (w/v) sodium dodecyl sulphate. Chromatography was carried out at room temperature (22°C). Buffer compositions were as follows: at pH 5.0, 50mM-sodium citrate; at pH 6.5 and 8.0, 50mM-sodium phosphate; at pH 9.5, 50mM-sodium glycinate. In each case the pH was readjusted after the addition of salt or detergent. E_{280} in the column effluent was monitored continuously by using a Gilford 2000 recording spectrophotometer with flow-cell attachment. The flow rate was maintained at 0.5 ml/min by means of a peristaltic pump. Recorder traces in the presence of sodium dodecyl sulphate have been corrected for background absorbance. Recovery of protein was approx. 100% in all cases.

amount near the isoelectric point, no bovine serum albumin is adsorbed by electrostatic attractive forces in the pH range 5–9.5. At pH 5 and 6.5 nearly all the bovine serum albumin is bound hydrophobically; presumably at pH 6.5 the hydrophobic interaction is still strong enough to offset completely the small charge repulsion. However, as the pH is increased to 8.0 and then to 9.5, the fraction of protein that is not adsorbed increases at the expense of the hydrophobically bound protein, a consequence of the increasing charge repulsion. The double peak of hydrophobically bound protein observed in all of these experiments may reflect heterogeneity in the binding sites on bovine serum albumin; it is known that heterogeneous sites bind detergents (Reynolds

et al., 1967). Alternatively or additionally, the hydrocarbon adsorbent groups may be heterogeneous on account of different environments, i.e. it may be incorrect to assume that the adsorbent matrix (of CNBr-treated agarose) is completely inert.

In spite of these drawbacks, the behaviour of bovine serum albumin on CPAD-agarose is in excellent agreement with the charge-repulsion proposal outlined above. This encourages the belief that adsorbents such as AD-agarose and SAD-agarose may find a use in the selective elution of lipophilic proteins adsorbed from complex protein mixtures. The technique is likely to be especially useful when it is required to separate two particular classes of lipophilic proteins from such mixtures. First, proteins with relatively weak affinity for the C_{10} chains on the adsorbent, but that have isoelectric points outside the 'normal' range of about 5–8, are best adsorbed at the isoelectric pH on an adsorbent that repels most 'normal' proteins at this pH. Secondly, proteins with 'normal' isoelectric points may be successfully adsorbed if the hydrophobic affinity for the C_{10} chains is strong enough; adsorption of this class of proteins is, in theory, best performed near the pH limit at which hydrophobic adsorption is balanced by charge repulsion. Proteins that enjoy the joint advantages of strong hydrophobic adsorption and 'abnormal' isoelectric point may be adsorbed by either procedure, provided they are not denatured by the strong hydrophobic forces likely to operate at the isoelectric pH. Proteins with low hydrophobic affinity for the adsorbent combined with a 'normal' isoelectric point are unlikely to be substantially purified on these adsorbents. In all cases the adsorbed protein may subsequently be eluted by the appropriate pH change, or by means of a detergent. Improved resolution of bound proteins may be obtained by eluting with pH or detergent gradients. In the chromatography of crude biological extracts, a preliminary extraction of lipid material is probably essential.

Hydrophobic-affinity chromatography has been used as a step in the purification of aspartate transcarbamoylase (EC 2.1.3.2) from wheat germ. This allosteric enzyme (Yon, 1971) has a high affinity for the C_{10} chain of CPAD-agarose. A crude preparation of the enzyme from defatted wheat germ was chromatographed on a column of CPAD-agarose (sample volume/column volume ratio 1:5) equilibrated and developed with 50mM-sodium phosphate buffer, pH 8.0, at 5°C. A large protein peak emerged in the void volume (80% of total protein). A second protein peak was released when the buffer included 0.2M-NaCl. Neither of these peaks contained enzyme activity. Addition of 0.2% (w/v) sodium deoxycholate to the buffer, and omission of NaCl, released a third peak containing 5% of the total protein and 40% of the enzyme activity. Thus

an eightfold purification was achieved in this step. Enzyme activity could also be eluted by making the buffer 2% (v/v) in acetone or by raising the pH to 10.

Much of this work was carried out during the tenure of a U.K. Commonwealth Scholarship, for which I am grateful.

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