Enzyme Purification by Hydrophobic Chromatography: an Alternative Approach Illustrated in the Purification of Aspartate Transcarbamoylase from Wheat Germ

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Two adsorbents containing similar numbers of hydrocarbon (C₁₀) chains but different numbers of carboxyl groups were made by chemical modification of Sepharose. The use of these adsorbents to purify proteins, under conditions where hydrophobic adsorption is partly resisted by electrostatic repulsion, is illustrated in the purification of aspartate transcarbamoylase (EC 2.1.3.2) from wheat germ.

The surfaces of a considerable number of solute proteins contain exposed hydrophobic 'pockets' such as binding sites or subsites or the crevices at subunit junctions, with different proteins showing great variation in the number, size and accessibility of these 'greasy spots'. Hydrophobic interaction between these proteins and suitable adsorbents offers an attractive method of protein chromatography. Hydrophobic adsorbents are readily made by coupling 1-aminoalkanes or their derivatives to Sepharose by the cyanogen bromide method of Axen *et al.* (1967). Using these adsorbents a number of workers have begun to apply 'hydrophobic chromatography' (Shalteil & Er-el, 1973) to the purification of enzymes and other proteins (Yon, 1972a; Er-el *et al.*, 1972; Hofstee, 1973; Shalteil & Er-el, 1973; Peters *et al.*, 1973; Jakubowski & Pawelkiewicz, 1973).

The adjustment of column hydrophobicity, so as to adsorb but not denature the proteins of interest, has been approached in two ways. Er-el *et al.* (1972), Hofstee (1973) and Shalteil & Er-el (1973) used a series of alkyl-Sepharoses, or ω -aminoalkyl-Sepharoses of various short alkyl chain lengths, from which the appropriate one was found by trial. I have explored an alternative approach, which uses columns with strongly hydrophobic (C₁₀) alkyl chains bearing negatively charged groups (Yon, 1972a). By selecting a pH value at which the protein of interest is also negatively charged, it is possible to control the binding force by the introduction of electrostatic repulsion. Fine control of the charge on the protein, and hence of the repelling force, is achieved by varying the pH. Preliminary results in the application of this method in purifying wheat-germ aspartate transcarbamoylase have been described (Yon, 1972a). In the present paper an improved version of the method, using two adsorbents, has been incorporated in a four-step scheme for purifying the enzyme to apparent homogeneity. To my knowledge, this is the first successful application of the method.

Experimental

The preparation of ω -aminodecyl-Sepharose and CPAD-Sepharose* (I), by the methods of Cuatrecasas (1970), has been described (Yon, 1972a). PMAD-Sepharose (II) was prepared identically to CPAD-Sepharose except for the substitution of pyromellitic dianhydride (1,2,4,5-benzenetetracarboxylic dianhydride; Ralph N. Emanuel, Wembley, Middx., U.K.) for succinic anhydride. In the preparation of both adsorbents, treatment of ω -aminodecyl-Sepharose with the appropriate anhydride was repeated, if necessary, until all the amino groups had reacted, as judged by the trinitrobenzenesulphonic acid test (Cuatrecasas, 1970). A high (saturating) concentration of pyromellitic dianhydride was used to minimize the incidence of the diamide product.

The assay of aspartate transcarbamoylase has been described (Yon, 1972b). Protein in column effluents was monitored as E₂₈₀; otherwise protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis was performed at pH 9.5 in a Shandon apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.) according to the manufacturer's instructions. Protein bands were

(I) Sepharose-N-[CH₂]₁₀-NH-CO-[CH₂]₂-CO₂⁻

* Abbreviations: CPAD-Sepharose, *N*-(3-carboxypropionyl)aminodecyl-Sepharose; PMAD-Sepharose, *N*-pyromellitylamino-decyl-Sepharose.

stained with Coomassie Brilliant Blue. The gels were stained and de-stained as described by Weber & Osborn (1969).

Results

Purification of wheat-germ aspartate transcarbamoylase. Stages 1 and 2: Extraction with heating and acetone fractionation. These stages were essentially the same as a method described previously (Yon, 1972b) except that the defatted wheat germ was extracted into buffer containing 50mM-sodium phosphate, 10mM-thioglycolic acid and 60 μ M-UMP, adjusted to pH7.5 with 5M-NaOH.

All the following operations were done at 4°C and (except for the final dialysis) in the presence of the allosteric inhibitor, UMP (60 μ M). In the following description, 'standard buffer' means 0.1M-Tris containing 60 μ M-UMP, adjusted to pH8.5 with acetic acid.

Stage 3: hydrophobic chromatography. Two similar columns (6cm $\times$ 2.5cm), of PMAD- and CPAD-Sepharose respectively, were equilibrated with standard Tris buffer. The acetone fraction

(110ml) was adjusted with 1M-NaOH to pH8.5 and pumped on to the PMAD-Sepharose column, followed by standard buffer until the effluent was free of protein. Enzyme activity emerged in the unbound, break-through, peak of protein, but a considerable amount of protein was bound and could be eluted by 0.2M-NaCl in standard buffer, followed by 0.1M-trisodium phosphate, pH12, containing 0.2% (v/v) butan-1-ol (Fig. 1a). Pooled active fractions were then pumped on to the CPAD-Sepharose column, followed by a standard buffer wash. Again the major fraction of the proteins emerged in the break-through peak, but the enzyme was strongly bound. Some protein, free of enzyme activity, was eluted by including 0.2M-NaCl in the standard buffer wash. After further washing with standard buffer (free of NaCl) the enzyme was eluted by changing to 10mM-glycine-NaOH buffer, pH10, containing 0.2% butan-1-ol and 60 μ M-UMP. Fractions (2ml) were collected into tubes in each of which 2ml of 0.1M-Tris-acetate (containing 60 μ M-UMP) had been previously placed, to lower the pH and dilute the butanol immediately on elution of the enzyme. Considerable protein remained adsorbed on the column and could be eluted with 0.1M-

Fig. 1. Hydrophobic chromatography of the acetone fraction on (a) PMAD-Sepharose followed by (b) CPAD-Sepharose. The enzyme-containing sample was introduced at (1) in each case. The sample in (a) was 110ml of the acetone fraction, and in (b) the pooled active fractions from (a). The loaded columns were developed with the following buffers as indicated: (2), standard Tris buffer; (3), 0.2M-NaCl in standard Tris buffer; (4), 0.01M-glycine-NaOH buffer, pH10, containing 0.2% butan-1-ol; (5) 0.1M-trisodium phosphate, pH12, containing 0.2% butan-1-ol. All buffers contained 60 μ M-UMP. The flow rate was 4ml/min. Other details are given in the text. Enzyme activity is expressed relative to the most active fraction in each case. —, Protein (E_{280}); ●, relative activity.

trisodium phosphate, pH12, containing 0.2% butan-1-ol (Fig. 1b). The enzyme could also be eluted by 0.2% butan-1-ol at pH8.5 (in standard buffer) or, without butanol, by 10mM-glycine-NaOH, pH10; in either case, however, the enzyme was eluted as a broad flat peak. The combined use of butanol and pH10 results in the elution of the enzyme in a smaller volume. The low yield at this step is probably the result of denaturation.

In routine use this step is shortened by pumping the acetone fraction on to the two columns connected in series, with the PMAD-Sepharose column leading, then washing with standard buffer until the effluent from the CPAD-Sepharose column is free of protein. The PMAD-Sepharose column is set aside and further development of the CMAD-Sepharose column is as described above.

Stage 4: hydroxyapatite chromatography. A column of hydroxyapatite (5cm×1.2cm) was equilibrated with standard buffer. The pooled active fractions from the previous stage were adjusted to pH8.5 and pumped on to this column, followed by 500ml of standard buffer. The enzyme was then eluted by changing the buffer to 25mM-sodium phosphate, pH8.5, containing 60μM-UMP. The pooled active fractions (80ml) were concentrated to about 2ml in a dialysis bag suspended in 50% (w/v) Carbowax 20M in 50mM-sodium phosphate buffer, pH7.5, and then dialysed against two changes (2 litres each) of the same buffer. Slight turbidity was removed by filtration through a small cellulose filter.

The results of a typical purification are given in Table 1.

Purity of the enzyme. In the best results achieved, polyacrylamide-gel electrophoresis (50μg of protein per gel) showed a single intensely staining band of protein on an otherwise clear gel. This band had a mobility of 0.26 relative to Bromophenol Blue and coincided with a single band of enzyme activity in a duplicate gel. Some results were less satisfactory, with up to four faint additional protein bands.

Effect of PMAD-Sepharose. When the enzyme

was purified by a scheme omitting the PMAD-Sepharose column, but otherwise identical with the above, polyacrylamide-gel electrophoresis of the product showed 15–20 bands, of which two stained with intensities similar to the enzyme band. Presumably these are lipophilic proteins binding strongly to both adsorbents, unlike the enzyme, which binds only to CPAD-Sepharose. Omission of PMAD-Sepharose allows these proteins to come through to the final product.

Discussion

It has been suggested that the adsorption of charged lipophilic proteins on similarly charged columns carrying hydrocarbon groups is determined by the resultant effect of opposing forces, namely a short-range entropic force leading to hydrophobic bond formation and a longer-range electrostatic repulsion force (Yon, 1972a). It could be asked whether the enzyme and adsorbents used in this study behave according to this model. That wheat-germ aspartate transcarbamoylase is negatively charged at pH8.5 has been confirmed electrophoretically. Whether the electrical properties of the two adsorbents are predominantly determined by structures (I) and (II) is more difficult to prove. To evaluate the possible effects of positive charges (Hofstee, 1973) in these adsorbents, the chromatographic mobilities of several small molecules bearing hydrophobic and/or ionic moieties were studied. The results suggested that (a) PMAD-Sepharose is more negatively charged than CPAD-Sepharose and (b) any positive charges that may occur are effectively swamped by the negative charges (R. J. Yon, unpublished work). Possibly the sources of positive charge found by Hofstee (1973) have pK_a values lower, or not much higher, than 8.5, in which case many of them would be uncharged at this pH, unlike the carboxyl groups, which would be fully charged.

Since the two adsorbents were made from the same batch of ω -aminodecyl-Sepharose, they carry the same density of C_{10} groups. The difference in their

Table 1. *Purification of aspartate transcarbamoylase from wheat germ*

One unit of enzyme activity catalyses the formation of 1μmol of carbamoylaspartate/min under the standard assay conditions (Yon, 1972b).

Stage	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor	
					Overall	Over previous stage
Heat-treated supernatant	26200	143	0.0055	100	1	1
Acetone fraction	1810	114	0.063	80	11.5	11.5
Pooled fractions after hydrophobic chromatography	26.5	35.6	1.34	24.8	244	21.2
Concentrated hydroxyapatite fractions	1.76	11.0	6.25	7.7	1140	4.7

affinity for the enzyme is most readily explained by their difference in negative charge, which repels the enzyme strongly enough to prevent hydrophobic-bond formation in PMAD-Sepharose, but not in CPAD-Sepharose. Raising the pH can prevent hydrophobic bonding in CPAD-Sepharose by increasing the negative charge on the enzyme. The hydrophobic nature of the binding to CPAD-Sepharose is indicated by the ease of elution with organic solvents or detergents in low concentrations, and by the failure of attempts to elute the enzyme at pH 8.5 with 100 column volumes of 0.2M-NaCl in standard buffer, or with the same volume of buffer containing either one of the substrates or the inhibitor UMP, at concentrations at least 20 times the K_m or K_i values (Yon, 1972b). The aromatic ring of PMAD-Sepharose appeared to have no effect on the enzyme.

The purification of aspartate transcarbamoylase demonstrates that columns with similar hydrocarbon moieties but different numbers of charged groups, combined with suitable choice of pH, can be used to discriminate between lipophilic proteins by a

different mechanism from the alkyl-Sepharoses used by other workers. Both approaches to selective hydrophobic chromatography will probably find other applications in studies of lipophilic proteins.

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