Biospecific-Elution Chromatography with 'Impihlytes' as Stationary Phases

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Six out of seven enzymes tested (four of them nicotinamide nucleotide-dependent dehydrogenases) showed differences in chromatographic behaviour in the presence and absence of their biospecific ligands, when chromatographed on immobilized amphipathic ampholytes ('impihlytes') as stationary phases. Some enzymes were adsorbed more tightly, others less tightly, in the presence of ligands. These results have implications for enzyme purification in general, and for some types of affinity chromatography in particular.

With reference to the column chromatography of active proteins such as enzymes, the term 'biospecific elution' will be used to denote a change in elution behaviour (reflecting tighter or weaker adsorption) that occurs when a biospecific ligand of the protein is included in the mobile phase, no such change being observed in the presence of related, but non-biospecific, compounds. We are concerned here with biospecific elution of proteins that are adsorbed by non-biospecific forces (i.e. not involving functional binding sites on the protein predominantly). One type of biospecific-elution effect, biospecific desorption, has been quite frequently reported in relation to ion-exchange chromatography [see Pogell & Sarngadharan (1971), von der Haar (1974) and references therein].

Interest in my laboratory is focused on the use, in protein chromatography, of stationary phases that have a mixed population of hydrophobic, positively and negatively charged groups. To emphasize the multifunctional nature of these adsorbents, we have described them in recent papers as immobilized amphipathic ampholytes, abbreviated to 'impihlytes' (Yon *et al.*, 1976*a,b*). The main impihlytes being studied are *N*-(3-carboxypropionyl)aminoalkyl-Sepharoses, having the putative structures shown below:

The octyl and decyl homologues (CPAO- and CPAD-Sepharose respectively) are most frequently used. Although originally recommended as spacers for affinity chromatography (Cuatrecasas, 1970) they have useful properties in their own right (Yon,

1972*a*, 1974; Yon & Simmonds, 1975; Simmonds & Yon, 1976; Yon *et al.*, 1976*a,b*). In the present paper their use for biospecific-elution chromatography is described.

Experimental

Rabbit muscle lactate dehydrogenase and pig heart malate dehydrogenase were from Sigma, Kingston-upon-Thames, Surrey, U.K. Yeast alcohol dehydrogenase was from Boehringer, London W.5, U.K. Calf intestinal alkaline phosphatase and all buffer components and inorganic salts were from BDH, Poole, Dorset, U.K. Other materials have been listed (Yon & Simmonds, 1975). CPAD- and CPAO-Sepharose were prepared, by using diaminodecane and diamino-octane respectively, as described by Yon & Simmonds (1975). Both gels contained 15–18 μmol each of carboxyl and cationic (mainly isourea) groups/ml of settled gel, as determined by acid-base titration.

Wheat-germ aspartate transcarbamoylase was partially purified (Yon, 1972*b*). Crude preparations of wheat-germ ornithine transcarbamoylase and malate dehydrogenase were obtained from the supernatant (30 min at 6000*g*_{av}) of an aqueous extract of wheat germ (300 g in 2 litres of water, adjusted to pH 7.0). Ornithine transcarbamoylase was precipitated between 1.6*M*- and 3.3*M*-(NH_4)₂SO₄, redissolved in 250 ml of 5*mM*-Tris/acetate, pH 7.5, dialysed extensively against this buffer and chromatographed on a column (250 ml volume, 4 cm diam.) of DEAE-cellulose equilibrated with the same buffer. The column was developed with a gradient (0–0.32*M*) of sodium acetate, and the active fractions (220 ml) were pooled and used for the experiments below. Malate dehydrogenase was precipitated from the aqueous extract between 2.3*M*- and 4.3*M*-

(NH₄)₂SO₄ and redissolved in 150ml of 50mM-sodium phosphate, pH7.5, containing 2M-(NH₄)₂SO₄. This was adsorbed on a column (30ml volume, 2.5cm. diam.) of CPAO-Sepharose equilibrated with the same buffer-salt solution. The column was developed with a decreasing (2M-0) gradient of the salt. The active fractions (170ml) were pooled, dialysed into 10mM-sodium phosphate, pH7, and used for the experiments below. All enzyme preparations, including the commercial enzymes, were dialysed into the chromatography equilibration buffer before use.

Malate dehydrogenase, alcohol dehydrogenase and alkaline phosphatase were assayed as described by Bergmeyer *et al.* (1974) and lactate dehydrogenase was assayed as described by Bergmeyer & Bernt (1974). Aspartate transcarbamoylase was assayed as described by Yon (1972b). Ornithine transcarbamoylase was assayed as described by Marshall & Cohen (1972), except that the buffer was 0.1M-Tris/acetate, pH8.5, and citrulline was determined as described by Prescott & Jones (1969).

Results

Chromatography experiments with the wheat-germ enzymes were designed to detect small biospecific effects. In each case, chromatography in the presence of one or a combination of ligands was compared with a control experiment, performed simultaneously, in which sample composition, batch of adsorbent, column geometry, flow rate, temperature, pH, ionic strength and the compositions of buffers and gradients were identical except that the ligand was replaced by a similar but non-biospecific compound. Under these conditions, biospecific-elution effects were shown by each of the wheat-germ enzymes.

When wheat-germ aspartate transcarbamoylase was chromatographed on CPAD-Sepharose (Fig. 1a), a substantial change in the elution profile occurred in the combined presence of one substrate, L-aspartate, and UMP, a potent end-product inhibitor (Yon, 1972b). In the presence of the ligands there is less enzyme in the void-volume peak, and a higher pH is required to elute the adsorbed enzyme, indicating that the enzyme-ligand complex is adsorbed more tightly than the free enzyme. By successive passages through a column of CPAD-Sepharose, in the presence and absence of ligands, a substantial purification of the enzyme may be achieved, since in the second passage the enzyme is specifically displaced away from impurities that co-chromatographed with it in the first passage. This method of purification has been successfully applied (J. E. Grayson & R. J. Yon, unpublished work).

A second case in which ligand binding led to tighter adsorption was provided by the chromato-

Fig. 1. *Biospecific elution of three wheat-germ enzymes* All experiments were done on small columns (1.5 ml volume, 0.8 cm diam.) at a flow rate of 0.1 ml/min and at 22°C. Ligands, or ligand substitutes, were present in the enzyme sample, in the equilibrating and washing buffers and in the gradients. (a) Chromatography of aspartate transcarbamoylase on CPAD-Sepharose in the presence of (○) 60 μM-UMP and 5 mM-L-aspartate or (●) 60 μM-phosphate and 5 mM-D-aspartate. The equilibrating buffer was 50 mM-Tris/50 mM-glycine adjusted to pH8.5 with acetic acid, and the pH gradient (----) was formed by adjusting the same buffer with NaOH. (b) Chromatography of ornithine transcarbamoylase on CPAO-Sepharose in the presence of (○) 5 mM-L-ornithine and 5.6 mM-phosphate, pH7.0, or (●) 5 mM-L-ornithine and 5.0 mM-β-glycerophosphate, pH7.0, both of these solutions being adequately self-buffered. The sample was 15 ml of the partially purified enzyme, and the column was developed with a 0-0.1 M-sodium acetate gradient (----). (c) Chromatography of malate dehydrogenase on CPAD-Sepharose in the presence of (○) 0.1 mM-NADH or (●) 0.1 mM-pyrophosphate. The sample was 2.5 ml of the partially purified enzyme, the equilibrating and starting buffer was 10 mM-sodium phosphate, pH7.3, and the column was developed with a 0.01-0.10 M-sodium phosphate gradient, pH7.3 (----).

Fig. 2. Biospecific desorption of pig heart malate dehydrogenase from CPAD-Sepharose by 0.1 mM-NADH

Changes in the column eluent were introduced as shown by arrows: S, enzyme sample in buffer; B, buffer wash; PP, 0.1 mM-sodium pyrophosphate in buffer; N, 0.1 mM-NADH in buffer. Column size and other conditions were as for Fig. 1, except that the flow rate was 0.2 ml/min and various concentrations of sodium phosphate buffer, pH 7.0, were used. Enzyme loading, buffer concentration and total recovery for each experiment were as follows: (a) 200 μg of enzyme, 5 mM-phosphate, 7%; (b) 100 μg of enzyme, 10 mM-phosphate, 82%; (c) 300 μg of enzyme, 15 mM-phosphate, 100%; (d) 100 μg of enzyme, 50 mM-phosphate, 100%.

graphy of wheat-germ ornithine transcarbamoylase on CPAO-Sepharose (Fig. 1b). The effect occurred only in the combined presence of a substrate, L-ornithine, and a product, phosphate, thereby suggesting the existence of a dead-end ternary complex (enzyme-phosphate-ornithine). However, the biospecific displacement of the eluted enzyme peak was quite small and could not be used as the basis of a substantial purification step.

The effect of NADH on the elution profile of wheat-germ malate dehydrogenase (Fig. 1c), with CPAD-Sepharose as adsorbent, provided an example of ligand binding leading to weaker adsorption and partial desorption. This was the largest biospecific change among the three wheat-germ enzymes studied. Indeed, in this case, but not with aspartate transcarbamoylase or ornithine transcarbamoylase, the change produced by the ligand was large enough

Fig. 3. Biospecific desorption of rabbit muscle lactate dehydrogenase from CPAD-Sepharose by 0.1 mM-NADH. Enzyme loading, buffer concentration and total recovery for each experiment were as follows: (a) 88 μ g of enzyme, 10 mM-phosphate, 70%; (b) 200 μ g of enzyme, 5 mM-phosphate, 53%. All other conditions were as for Fig. 2.

for frontal displacement of a substantial part of the adsorbed enzyme to be possible (i.e. displacement of the enzyme by an abrupt change from the presence to the absence, or vice versa, of the ligand in the eluent).

Large biospecific effects were also observed in the chromatography of pig heart malate dehydrogenase and rabbit muscle lactate dehydrogenase on CPAD-Sepharose, with NADH as bioligand. Pig heart malate dehydrogenase was used to investigate some of the factors affecting optimal frontal elution of the enzyme by NADH (Fig. 2). The ionic strength of the buffer and the loading of the column were two of these factors. At low sodium phosphate buffer concentration (5 mM) the column could be loaded to quite a high content (200 μ g of enzyme/ml column volume) without any sign of enzyme leakage in the effluent. However, under these conditions attempts to achieve a sharp, frontal 'pulse' at 0.1 mM-NADH were ineffectual (Fig. 2a). At considerably higher buffer concentrations and/or loading, substantial amounts of enzyme 'leaked' (Fig. 2c) or appeared in the void volume (Fig. 2d), so that a subsequent ligand pulse displaced only a fraction of the recovered enzyme. These experiments suggested that optimal conditions for frontal elution were obtained when, for a given buffer concentration, the column

was loaded just short of the point at which 'leaking' occurred. These conditions are approached at 10 mM-phosphate buffer concentration with a loading of 100 μ g of enzyme/ml column volume (Fig. 2b). Frontal elution of rabbit muscle lactate dehydrogenase was similarly optimized (Fig. 3).

Weakened adsorption in the presence of NAD⁺ or NADH was also observed for yeast alcohol dehydrogenase (CPAD-Sepharose as adsorbent), but was not optimized for frontal elution. No biospecific effects could be detected in the chromatography of calf intestinal alkaline phosphatase by the technique of Fig. 1, by using CPAD-Sepharose as adsorbent, phosphate as bioligand and an NaCl gradient in 0.05 M-Tris/acetate, pH 7.5, for development.

Discussion

A biospecific-elution effect in imphilyte chromatography was first observed, in the course of purification trials, with wheat-germ aspartate transcarbamoylase. In a preliminary search for further examples, the seven enzymes reported above were studied, of which six gave clear evidence of biospecific-elution effects. The single 'failure' was intestinal alkaline phosphatase, and it is by no means certain that phosphate is the best choice of bioligand for this enzyme. Thus although these seven enzymes are an admittedly small sample, and contain a group (the dehydrogenases) that are structurally and functionally similar, nevertheless it is tentatively suggested that imphilyte chromatography may offer a high probability of finding a usable biospecific effect for many more enzymes. The use of this technique, which it is proposed to call 'biospecific imphilyte chromatography', appears especially promising in purifying nicotinamide-nucleotide-dependent dehydrogenases. On the other hand, an imphilyte has a high capacity for non-specific adsorption, so that the separation of a single protein from a complex mixture poses a number of experimental problems, most of which are poorly understood at the present time.

It has been recognized for several years that some of the adsorbents used in affinity chromatography, particularly those with hydrocarbon spacers coupled by the CNBr procedure, are capable of gross non-specific adsorption (Barry & O'Carra, 1973; O'Carra *et al.*, 1974). Some of these, notably the immobilized-NAD adsorbents designed for NAD-linked dehydrogenases, are also imphilytes, having a mixture of hydrophobic, positive and negative groups. They are therefore capable of showing biospecific desorption effects (such as those in Figs. 2 and 3), which might be interpreted as 'competitive' desorption from a true affinity adsorbent, even though the adsorption could be non-biospecific. Hence,

unless there is independent evidence of biospecific adsorption, a purification on one of these columns could just as likely be by imphilyte chromatography as by affinity chromatography. These comments will not, of course, apply to more recently developed matrix systems in which hydrophobic and ionic groups have largely been eliminated. They do, however, apply to the increasingly popular 'Blue Sepharose', in which the dye Cibacron Blue is coupled to a Sepharose matrix (Easterday & Easterday, 1974), and which has been used as an 'affinity' adsorbent for several proteins.

References

- Barry, S. & O'Carra, P. (1973) *Biochem. J.* **135**, 595–607
- Bergmeyer, H. U. & Bernt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) pp. 574–579, Academic Press, New York and London
- Bergmeyer, H. U., Gawehn, K. & Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 425–522, Academic Press, New York and London
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
- Easterday, R. L. & Easterday, I. M. (1974) in *Immobilized Biochemicals and Affinity Chromatography* (Dunlap, R. B., ed.), pp. 123–133, Plenum Press, New York
- Marshall, M. & Cohen, P. P. (1972) *J. Biol. Chem.* **247**, 1641–1653
- O'Carra, P., Barry, S. & Griffin, T. (1974) *Methods Enzymol.* **34**, 108–126
- Pogell, B. M. & Sarngadharan, M. G. (1971) *Methods Enzymol.* **22**, 379–385
- Prescott, L. M. & Jones, M. E. (1969) *Anal. Biochem.* **32**, 408–419
- Simmonds, R. J. & Yon, R. J. (1976) *Biochem. J.* **157**, 153–159
- von der Haar, F. (1974) *Methods Enzymol.* **34**, 163–171
- Yon, R. J. (1972a) *Biochem. J.* **126**, 765–767
- Yon, R. J. (1972b) *Biochem. J.* **128**, 311–320
- Yon, R. J. (1974) *Biochem. J.* **137**, 127–130
- Yon, R. J. & Simmonds, R. J. (1975) *Biochem. J.* **151**, 281–290
- Yon, R. J., Simmonds, R. J. & Grayson, J. E. (1976a) *Advances in the Chromatographic Fractionation of Macromolecules: Chem. Soc. Symp.*, Ellis Horwood Ltd., Chichester, in the press
- Yon, R. J., Simmonds, R. J. & Grayson, J. E. (1976b) *Abstr. Int. Congr. Biochem. 10th, Hamburg*, p. 197, Bronners Druckerei Breidenstein KG, Frankfurt am Main