

Purification of Glycolytic Enzymes by using Affinity-Elution Chromatography

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1. A systematic procedure for the purification of enzymes by affinity-elution chromatography is described. Enzymes are adsorbed on a cation-exchanger, and eluted with ligands specific for the enzyme concerned. 2. All of the glycolytic and some related enzymes present in rabbit muscle can be purified by the affinity-elution technique. The pH range for adsorption and elution of each enzyme was found, and the effects of minor variations of conditions are described. 3. A description of experimental conditions suitable for affinity elution of each enzyme is given, together with special features relevant to each individual enzyme. 4. Theoretical considerations of affinity elution chromatography are discussed, including its limitations, advantages and disadvantages compared with affinity-adsorption chromatography. Possible developments are suggested to cover enzymes which because of their adsorption characteristics are not at present amenable to affinity-elution procedures.

The use of the term affinity chromatography in the purification of enzymes generally refers to the adsorption of enzymes on insoluble matrices containing covalently attached ligands relevant to the enzymes in question. The specificity of the procedure occurs at this adsorption stage, and subsequent elution from the matrix has generally been non-selective, by using solutions such as strong salts to disrupt the ligand-enzyme association. Although undoubtedly an ideal method in theory, the practice of affinity chromatography does have problems, in particular the difficulty and expense of developing a suitable adsorbent in the first place. The actual, as opposed to theoretical, capacity of the adsorbent is often small; in addition, non-specific adsorption effects can be a substantial complication (O'Carra, 1974).

An alternative affinity procedure, known variously as 'biospecific elution', 'substrate elution' or 'affinity elution', has been used occasionally, though not often in a systematic manner. This procedure makes use of a substrate's or other ligand's ability to change the binding characteristics of an enzyme to a general protein adsorbent such as CM-, phospho- or DEAE-cellulose. The selective affinity occurs at the elution stage in the chromatography. Some examples that may be cited are the purification of fructose biphosphatase (EC 3.1.3.11) (Black *et al.*, 1972; Nimmo & Tipton, 1975), fructose biphosphate aldolase (EC 4.1.2.13) (Pogell, 1962; Penhoet & Rutter, 1975), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Chilla *et al.*, 1973), myokinase (EC 2.7.4.3) (Heil *et al.*, 1974; Feldhaus *et al.*, 1975), phosphoglucose isomerase (EC 5.3.1.9) (Tilley *et al.*, 1974), phosphoglycerate mutase (EC 2.7.5.3) (Sasaki *et al.*, 1975) and

aminoacyl-tRNA synthetases (EC 6.1.1.-) (von der Haar, 1973).

Compared with affinity-adsorption chromatography, affinity-elution chromatography has both advantages and disadvantages. The main advantage is that the process of development of a suitable adsorbent is eliminated; conventional ion-exchange materials are used. Moreover, the capacity of modern ion-exchange materials is such that gram quantities of enzymes can be adsorbed on quite modest-sized columns. Disadvantages include the necessity of being able to adsorb the enzyme on an ion-exchanger having the same charge as the substrate/ligand to be used for elution. Non-selectivity at the adsorption stage also means that it is less suitable for purifying an enzyme which makes up only a small fraction of the total protein present: this is the situation for which affinity-adsorption procedures are most suited.

The glycolytic enzymes in rabbit muscle constitute the major portion of the sarcoplasmic proteins (Czok & Bücher, 1960; Scopes, 1968), individual enzymes representing between 0.5 and 20% of the water-soluble proteins in muscle. This is the situation where in affinity chromatography selectivity at the adsorption stage is less important than capacity, for which affinity-elution techniques are most suited. The present paper describes the use of affinity elution for the purification of each of the glycolytic enzymes from rabbit muscle. These enzymes are mostly isoelectric in the pH range 6–8, and conditions can be found for each of them to adsorb on cation-exchangers; all the enzymes have negatively charged substrates which can be used for elution.

Although most experimenters are only concerned

with one particular enzyme, the procedures described allow for many separate enzymes to be isolated from the one original extract. Descriptions of multiple enzyme purifications from two different sources are given in the following paper (Scopes, 1977).

Materials

Rabbit muscle from freshly killed animals was extracted with 3 vol. of 30mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, by homogenization in a 3-litre-capacity stainless-steel Waring Blendor. The homogenate was then centrifuged at 4800g for 45 min, and the liquid decanted from the insoluble residue. Extracts not used immediately were adjusted to pH7.0 with 1M-Tris and stored frozen at -25°C . Special biochemicals included all glycolytic intermediates and related compounds, ATP, ADP, AMP, NAD^+ , NADH and NADP^+ . They were obtained from both Sigma Chemical Co., St. Louis, MO, U.S.A., and Boehringer und Soehne, Mannheim, Germany. Coupling enzymes for the assay procedures, if not obtained by the purification procedures described in the present paper, were made as described previously (Scopes, 1973). CM-cellulose was the microgranular form (CM-52) from Whatman. Phosphocellulose was the fine-grade material supplied by Sigma. Zwitterionic buffers (Mes,* Mops, Tricine) were obtained from Sigma; it was found necessary to recrystallize the Mops from 80% (v/v) ethanol, as it contained an impurity which inactivated certain enzymes, notably pyruvate kinase. Chromatographic columns used in these procedures were the Wright range of poly(methyl methacrylate) columns (Wright Scientific Co., Kenley, Surrey, U.K.), the most useful sizes being the adjustable types of dimensions $2\text{cm}^2 \times 15\text{cm}$, $4\text{cm}^2 \times 15\text{cm}$, $8\text{cm}^2 \times 15\text{cm}$ and $16\text{cm}^2 \times 30\text{cm}$. In addition, fixed-dimension columns of $8\text{cm}^2 \times 30\text{cm}$ and $16\text{cm}^2 \times 90\text{cm}$ were used for desalting.

Methods and Results

Enzyme assays

All enzymes were measured in systems designed to oxidize or produce NAD(P)H. All buffers included 3mM-magnesium acetate, 50mM-KCl, 0.2mM-EDTA and 0.2mg of bovine serum albumin/ml. Except for the phosphorylase assay, buffers were used at 30mM concentration, being imidazole at pH7.0, triethanolamine, pH7.5, or Tris, pH8.0, each adjusted with HCl. NADH, if used, was at a concentration of 0.1mM. Other components were as follows.

Phosphorylase (EC 2.4.1.1). 20mM-Potassium phosphate buffer pH7.2, 1mg of glycogen/ml,

* Abbreviations: Mes, 2-(*N*-morpholino)ethanesulphonic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

0.2mM- NADP^+ , 0.5mM-AMP, 2 units of phosphoglucumutase/ml, 0.5 unit of glucose 6-phosphate dehydrogenase/ml.

Phosphoglucumutase (EC 2.7.5.1). Tris buffer, 1.0mM-glucose 1-phosphate, 0.01mM-glucose 1,6-bisphosphate, 0.2mM- NADP^+ , 0.5 unit of glucose 6-phosphate dehydrogenase/ml.

Phosphoglucose isomerase (EC 5.3.1.9). Tris buffer, 1.0mM-fructose 6-phosphate, 0.2mM- NADP^+ , 0.5 unit of glucose 6-phosphate dehydrogenase/ml.

Phosphofructokinase (EC 2.7.1.11). Tris buffer, 1.0mM-fructose 6-phosphate, 1.0mM-ATP, 0.2mM-phosphoenolpyruvate, NADH, 2 units each of pyruvate kinase and lactate dehydrogenase/ml.

Aldolase (EC 4.1.2.13). Triethanolamine buffer, 0.5mM-fructose 1,6-bisphosphate, NADH, 2 units of triose phosphate isomerase/ml, 0.5 unit of glycerol phosphate dehydrogenase/ml.

Triose phosphate isomerase (EC 5.3.1.1). Triethanolamine buffer, 0.5mM-glyceraldehyde 3-phosphate, NADH, 0.5 unit of glycerol phosphate dehydrogenase/ml.

Glycerol phosphate dehydrogenase (EC 1.1.1.8). Triethanolamine buffer, 1.0mM-fructose 1,6-bisphosphate, preincubated with 0.2 unit of aldolase/ml and 1 unit of triose phosphate isomerase/ml, NADH.

Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12). Imidazole buffer, 5mM-3-phosphoglycerate, 1mM-ATP, NADH, 2 units of phosphoglycerate kinase/ml.

Phosphoglycerate kinase (EC 2.7.2.3). Triethanolamine buffer, 4mM-ATP, 10mM-3-phosphoglycerate, NADH, 10 units of glyceraldehyde phosphate dehydrogenase/ml.

Phosphoglycerate mutase (EC 2.7.5.3). Triethanolamine buffer, 2mM-3-phosphoglycerate, 0.1mM-2,3-bisphosphoglycerate, 0.5mM-ADP, NADH, 2 units each of enolase, pyruvate kinase and lactate dehydrogenase/ml.

Enolase (EC 4.2.1.11). Imidazole buffer, 0.4mM-2-phosphoglycerate, 0.5mM-ADP, NADH, 2 units each of pyruvate kinase and lactate dehydrogenase/ml.

Pyruvate kinase (EC 2.7.1.40). Imidazole buffer, 1mM-ADP, 0.5mM-phosphoenolpyruvate, NADH, 2 units of lactate dehydrogenase/ml.

Lactate dehydrogenase (EC 1.1.1.27). Imidazole buffer, 2mM-pyruvate, NADH.

AMP kinase (*myokinase*, EC 2.7.4.3). Triethanolamine buffer, 1mM-ATP, 2mM-AMP, 0.2mM-phosphoenolpyruvate, NADH, 5 units each of pyruvate kinase and lactate dehydrogenase/ml.

Creatine kinase (EC 2.7.3.2). Tris buffer, 20mM-creatine, 1mM-ATP, 0.2mM-phosphoenolpyruvate, NADH, 5 units each of pyruvate kinase and lactate dehydrogenase/ml.

Protein was measured by u.v. absorption at 205nm and 280nm (Scopes, 1974), and specific activities are

expressed as units (μmol of substrate transformed/min) per mg of protein at 25°C .

Buffers for chromatography on CM- and phosphocellulose were chosen to be non-interacting with the matrix, i.e. negatively charged, and to have the maximum buffering power for a given ionic strength. Buffers to satisfy these conditions must be uncharged in the acid form and so have a single negative charge in the basic form. There are few buffers which satisfy these criteria in the pH range above 5; cacodylate, barbitone and borate are about the only simple buffers, and do not cover the range properly. However, zwitterionic buffers are now available to cover the whole range, and have been used exclusively in this study. Those mainly used have been Mes (pK6.2), Mops (pK7.2) and Tricine (pK8.1). For lower pH values, picolinic acid (pK5.4) has been used; it has the disadvantage of a strong u.v. absorption. Buffer solutions were made by titrating 10mM of base (Tris for the low-pH buffers, KOH for pH above 7.0) with 1M solutions of the relevant buffer to the pH required. Buffer solutions also included 0.2mM-EDTA as a routine protection against heavy-metal contamination. Thus the ionic strength of the standard buffers was always 0.01 (mol/litre); it was necessary to add KCl to increase the ionic strength in certain cases. The standard buffers used were: Tris/Mes, pH6.0; Tris/Mes, pH6.5; KOH/Mops, pH7.2; KOH/Tricine, pH8.0. Although the converse procedure of elution from anion-exchangers such as DEAE-cellulose or DEAE-Sephadex has not often been attempted (mainly for want of enzymes with positively charged ligands), buffers for such a procedure would consist of 10mM acid (e.g. acetic acid, HCl) titrated with bases such as diethanolamine (pK9.0), Tris (pK8.2), triethanolamine (pK7.5), imidazole (pK7.0) or histidine (pK6.0).

Preliminary fractionation of tissue extracts

Although the method can be used with a tissue extract directly applied to the column (provided that the extract has been clarified by high-speed centrifugation and dialysed or desalted appropriately), a preliminary fractionation is desirable. $(\text{NH}_4)_2\text{SO}_4$ precipitation separates most of the non-protein material, and divides groups of enzymes into separate fractions. As a routine rabbit muscle extracts were fractionated either into five parts similar to those of Czok & Bücher (1960), or four parts (Scopes, 1969), depending on what enzymes were required. Fractionation was carried out at pH6.0, at $15\text{--}20^\circ\text{C}$, with 20–30min allowed for equilibration before centrifugation. Fraction A was precipitated between zero and 45% saturation of $(\text{NH}_4)_2\text{SO}_4$, fraction B at 45–55% satn., fraction C at 55–65% satn., and fraction D at 65–75% satn. Fraction E was precipitated at 75–80% satn., with adjustment to pH8.0 with 5M- NH_3 ; precipitation of fraction E was allowed to

continue at 4°C overnight. In the four-step fractionation, fractions A and E were as above, fraction BC was precipitated at 45–60% satn., and fraction CD at 60–75% satn. Each fraction was taken up in a small volume of 50mM-triethanolamine buffer, pH7.5 (stored at $+4^\circ\text{C}$ as a slurry if not worked up immediately), then desalted by passage through a gel-filtration column pre-equilibrated with 0.2mM-EDTA (acid form) adjusted to pH8.0 with Tris. Desalting columns were packed with Sephadex G-25 (coarse grade), and were of dimensions $8\text{cm}^2 \times 30\text{cm}$ or $16\text{cm}^2 \times 90\text{cm}$. The latter was capable of desalting up to 300ml of sample at a flow rate of 500ml/h.

Operating procedures for affinity elution column

As the methods involve stepwise changes in adsorption characteristics of the enzyme, it was not always necessary, and often undesirable, to have long columns. The best system was to have a wide column enabling a fast flow rate, with protein adsorbed on as much as the top one-third of the matrix material. Up to 2g of adsorbed protein could usually be accommodated on a column of about 100cm^3 of adsorbent. A column height 1–3 times its diameter was the normal system used. Flow rates were of the order of 20ml/h per cm^2 .

The overall procedure used for affinity elution of an enzyme depends very much on the purpose of the operation. To prepare multiple enzymes from one column will obviously require more steps than a procedure aiming for only one particular enzyme. The first step is to find conditions in which the enzymes are adsorbed on the column; the present paper illustrates the use of CM-cellulose, and in a few cases phosphocellulose, as adsorbent. A Mes buffer at pH6.5 is a good starting point, since few enzymes are unstable on the column at this pH, and many will be adsorbed quantitatively. Table 1 lists the adsorption behaviour of various rabbit muscle enzymes on CM-cellulose columns in these conditions. The muscle enzymes from another species would not necessarily behave in the same fashion, as the pI values are likely to differ.

After desalting of the appropriate sample, buffer was added to make the same ionic strength and pH as that to be used on the column. A concentrated stock buffer solution was used for this purpose, and final adjustment of pH was made by using either 1M-Tris or a 1M solution of the relevant buffer acid. The solution was diluted with buffer if necessary to decrease the protein concentration to below 15mg/ml, and then run on to the pre-equilibrated column.

Once proteins had been adsorbed on the column, the next stage was to wash with a buffer which did not quite elute the desired enzyme. This was done by stepwise pH increases of 0.5–0.8 unit at a time, by using the standard buffers described above, with no change in ionic strength. If the enzyme was eluted

Table 1. *Adsorption characteristics of rabbit muscle glycolytic enzymes on a column of CM-cellulose at pH 6.5 (Tris/Mes buffer), 4°C, I = 0.01*

Enzyme	Adsorbed at pH 6.5	Eluted at pH (without substrate)
Phosphorylase	Partially	6.5–7.0
Phosphoglucumutase	No	6.0–6.5
Phosphoglucose isomerase	Yes (partially denatures)	7.0–7.5
Phosphofructokinase	Yes	>8
Aldolase	Yes	>8
Triose phosphate isomerase	No	5.8–6.0
Glyceraldehyde phosphate dehydrogenase	Yes	>8
Glycerol phosphate dehydrogenase	Partially	6.5–7.0
Phosphoglycerate kinase	Yes	8.0–8.5
Phosphoglycerate mutase	Partially (de-phospho-enzyme only)	6.5–7.0
Enolase	Yes	7.0–7.5
Pyruvate kinase	Yes	7.5–8.0
Lactate dehydrogenase	Yes	>8.0
Creatine kinase	No	6.3–6.6
AMP kinase	Yes	6.8–7.2

with a particular buffer, this indicated that with the previous lower-pH buffer addition of negatively charged substrate would probably have eluted the enzyme specifically. For example, pyruvate kinase adsorbs at pH 6.5; washing with pH 7.2 buffer does not elute this enzyme. Going up to pH 8.0, however, causes elution of pyruvate kinase, together with other proteins. At pH 7.2, inclusion of 0.5 mM substrate (phosphoenolpyruvate) causes specific elution of pyruvate kinase. Although this addition of substrate causes only a small increase in ionic strength, it has in some cases still been sufficient to cause some non-specific elution of proteins. To avoid this occurrence, a 'dummy-substrate wash' was carried out before applying the substrate. This wash consisted of the buffer plus a compound which has roughly equivalent ionic properties to the real substrate; EDTA has generally been used for this purpose. 'Non-specific' proteins were then washed out before application of the substrate.

Thus a scheme for isolation of pyruvate kinase is to adsorb fraction BC (after heat-treatment) at pH 6.5 on CM-cellulose, wash with Mops buffer, pH 7.2, containing an additional 0.5 mM-EDTA, and then, when no more protein is eluted, change to Mops buffer containing 0.5 mM-phosphoenolpyruvate; pyruvate kinase is eluted specifically. It is important to be aware of the specific binding properties of the enzymes when planning an elution procedure. In the example of pyruvate kinase, most evidence suggests that K^+ ions are involved in the binding of phosphoenolpyruvate (Kayne, 1973), and so these should preferably be present in the buffer; the Mops buffer should be the K^+ form. Kinetic studies suggest a random-order type of reaction for muscle pyruvate kinase (Ainsworth & MacFarlane, 1973), so ADP^{3-} should also be able to elute the enzyme. However, ADP did not elute the enzyme under these conditions. Similarly with lactate dehydrogenase, no amount of pyruvate will specifically elute this enzyme, but NADH at pH 8.0, at a concentration of only 0.1–0.2 mM, very effectively removes the enzyme from the column. Lactate dehydrogenase has an ordered mechanism of reaction, with nucleotide binding first (Everse & Kaplan, 1973).

Fig. 1 illustrates the adsorption and elution pH ranges for rabbit muscle enzymes on columns of CM-cellulose. Most enzymes would be denatured if adsorbed at pH values below 5.5; the lowest pH value usable for each enzyme has been determined in only a few cases. Total adsorption occurs in the shaded ranges indicated, but at the tapered higher-pH end the totality of adsorption will depend on factors such as length of column and volume of sample applied. If a partition coefficient is defined as the proportion of enzyme adsorbed on the matrix in a given volume of the column, then its value is unity at the lower pH values, decreasing to zero at high pH. If the value is intermediate, then the enzyme will be held back on the column, but continuous washing with buffer will eventually elute all the enzyme. Addition of substrate or other ligand has the effect of lowering the partition coefficient of the enzyme concerned. The areas enclosed by dotted lines in Fig. 1 indicate pH ranges where without substrate the partition coefficient is high, but with substrate it is decreased substantially so that specific elution occurs. The ideal choice of pH value depends on column dimensions and amount of enzyme present. If the pH chosen is too low, then the partition coefficient may not be decreased sufficiently on addition of substrate, resulting in slow elution of the enzyme. This is undesirable, since the enzyme becomes highly diluted, has more chance of being contaminated with another protein which is also slowly coming off the column non-specifically, and a large volume of sometimes expensive ligand solution is required. On the other hand, if the pH is too high, the partition coefficient may already be decreased

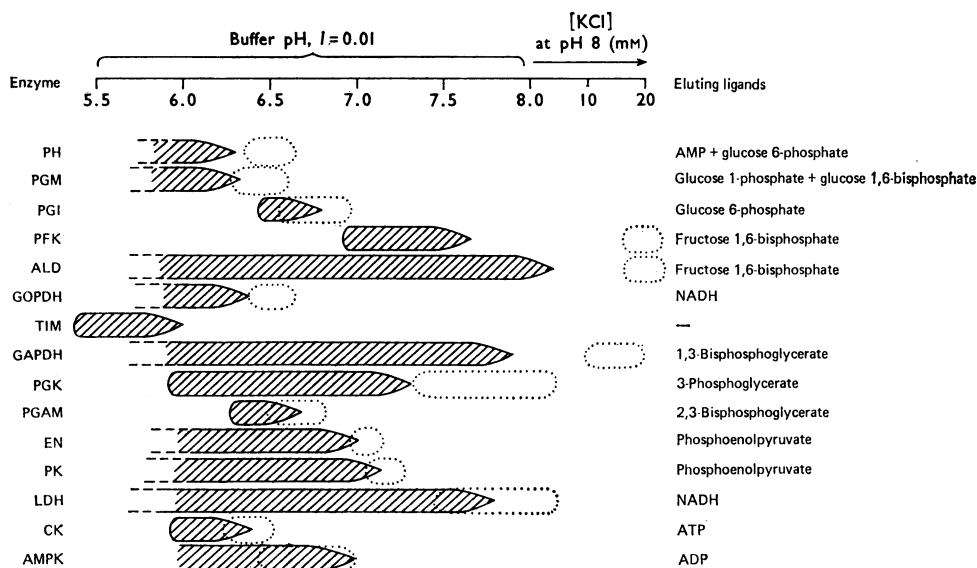


Fig. 1. Adsorption and affinity elution of rabbit muscle glycolytic enzymes on CM-cellulose at $I = 0.01$, 4°C . The hatched pH ranges indicate suitable conditions for adsorption of the enzyme on a column of CM-cellulose. In examples where the lower end of the range is closed, the enzyme is unstable at pH values below this, and so although adsorbed, may be denatured. In the other examples with open ends of ranges, the enzyme may be stable at lower pH values, but relevant experiments have not been carried out. In general, few enzymes can be recovered if adsorption is at or below pH 5.5. The pH areas enclosed by dotted lines indicate the approximate range at which the enzyme can be eluted by using between 0.2 and 0.5 mM of the ligand indicated on the right-hand side of the Figure. Abbreviations for enzymes: PH, phosphorylase; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; ALD, aldolase; GOPDH, glycerol phosphate dehydrogenase; TIM, triose phosphate isomerase; GAPDH, glyceraldehyde phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; EN, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase; CK, creatine kinase; AMPK, AMP kinase.

sufficiently to cause some elution of the enzyme in the 'dummy-substrate' wash before substrate is introduced. These situations are illustrated diagrammatically in Fig. 2, as applying to enolase; the pH for this enzyme is quite critical, and intermediate values from the standard buffers are used.

Thus the ideal conditions are dependent on a variety of factors, including the buffer composition and the amount of enzyme involved relative to the dimensions of the column. Generally speaking there is a range of about 0.5 pH unit over which affinity elution can be carried out. When changing the pH, a total of only 2-3 times the column volume of the new buffer need be washed through before adding the substrate, to lessen the chance of 'leakage' of the desired enzyme before affinity elution commences. In Table 2 are listed conditions suitable for an affinity elution column to purify a particular glycolytic enzyme from rabbit muscle, assuming that no other enzyme is required. In some cases a heat pretreatment to remove unwanted proteins is desirable; this has been indicated, along with the best $(\text{NH}_4)_2\text{SO}_4$ fraction to start with. Adsorption on the column is carried out in the cold, but it is not always necessary to elute the enzymes at low temperature; when the

pH is around 7, negligible denaturation occurs at room temperature. The charge introduced per 100000 daltons is indicated in Table 2. This is calculated on the basis of the number of negative charges on the ligand species presumed to bind to the enzyme, and on the subunit molecular weight of the enzyme concerned (Scopes & Penny, 1970). The best specific activities of the eluted preparations are also indicated. In most cases these are in the range 75-100% of the highest specific activities reported for these enzymes.

Behaviour of individual enzymes

Phosphorylase. This enzyme, which is almost entirely in the *b* form in these extracts, partially adsorbs on CM-cellulose or phosphocellulose at pH 6.5, and completely at pH 6.0. The pale-yellow band of adsorbed phosphorylase can be seen clearly on a CM-cellulose column. Washing the column with pH 6.5 buffer causes the band to move down very slowly, and become more diffuse. Inclusion of 0.2 mM activator (AMP) and 0.2 mM inhibitor (glucose 6-phosphate) together in the buffer speeds up the movement of the band considerably, though not sufficiently to cause it to be eluted with the ligand front; it is eluted a little later, depending on the dimensions of

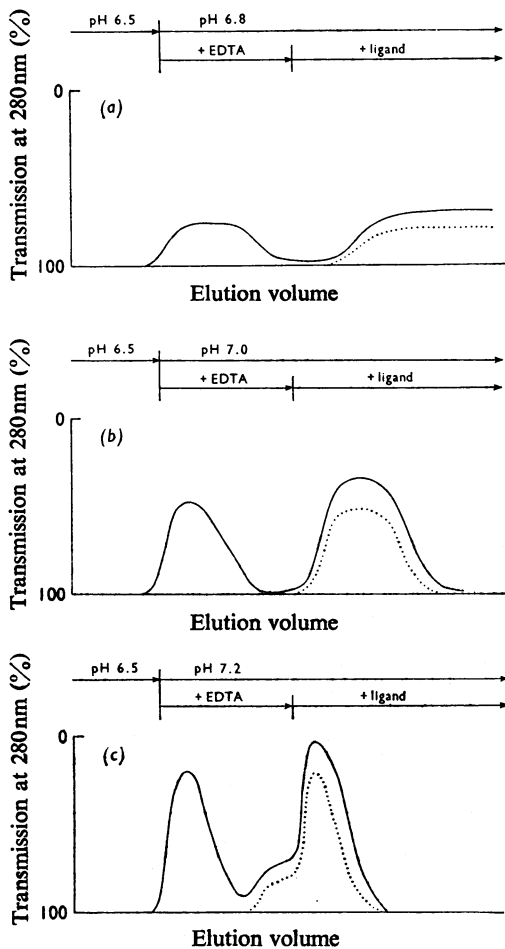


Fig. 2. Diagrammatic representation of the effect of buffer pH on affinity elution of an enzyme

The situation approximates to the behaviour of enolase; a small amount of contaminant protein is removed by increasing the pH, then application of ligand (phosphoenolpyruvate or 2-phosphoglycerate for enolase) elutes the enzyme. (a) Use of an elution pH that is too low, (b) correct pH and (c) use of a pH that is too high.

the column. Thus the principle of the method works adequately for phosphorylase. Although this may not be preferable to the standard procedure for purifying phosphorylase (Fischer & Krebs, 1958), it could be useful for removing contaminating glycogen-metabolizing enzymes from phosphorylase, or vice versa. Inclusion of glycogen in the buffer does not affect the elution, and use of the other substrates (P_i, glucose

1-phosphate) does not help, as the enzyme has rather low affinities for these.

Phosphoglucumutase. This enzyme has a rather low pI, but will adsorb on either phosphocellulose or CM-cellulose at pH 6.0. As extracted, the enzyme exists in a variety of forms, including phosphorylated and dephosphorylated enzyme. The dephosphorylated enzyme only binds glucose 1,6-bisphosphate, whereas the phospho-enzyme only binds a monophosphorylated glucose (Ray *et al.*, 1973). Thus, to elute all the enzyme specifically, a mixture of glucose 1-phosphate and glucose 1,6-bisphosphate should be used. Conversion of these into glucose 6-phosphate by the enzyme's action may cause specific elution of some phosphoglucose isomerase, if present. The isomerase may be removed before the fraction (BC) is applied to the column by a heat-denaturation step at slightly acid pH (5.5), in which conditions the mutase is stable. Enzyme purified by this scheme has had a specific activity of up to 750 units/mg.

Phosphoglucose isomerase. Phosphoglucose isomerase is rather unstable at slightly acid pH values; consequently, although it adsorbs on CM-cellulose at pH 6.5, it tends to be denatured on the column at this pH. Phosphocellulose adsorbs the enzyme more strongly and most of the enzyme will adsorb on a phosphocellulose column at pH 7.0. Elution with either glucose or fructose 6-phosphate is successful, at a concentration of 0.5 mM substrate. Only one negative charge is added for every 30000 daltons at this pH, which is one of the smallest charge-density changes that has successfully eluted an enzyme.

Phosphofructokinase. This enzyme may be described as a 'sticky' enzyme; although it needs an ionic strength of about 0.1 mol/litre at pH 8 to elute it from DEAE-cellulose, it can also be adsorbed on CM-cellulose at pH 7, and requires an ionic strength nearly as high to elute it. However, impure preparations which still have membranous material associated with the enzyme (for instance, the propan-2-ol-precipitated fraction of Ling *et al.*, 1965) do not adsorb on CM-cellulose; only preparations previously eluted from DEAE-cellulose are suitable for affinity elution from CM-cellulose. A great variety of substrates and inhibitors could be used to elute the enzyme specifically; for instance, fructose 1,6-bisphosphate, which binds very tightly to the enzyme, causes elution in conditions close to those needed for aldolase (below) or for fructose 1,6-bisphosphatase. To avoid multiple enzyme elution, a scheme utilizing the strong binding of citrate at a pH where the enzyme exhibits allosteric behaviour has been used. The enzyme was adsorbed on CM-cellulose at pH 6.9, and the column washed with pH 7.2 buffer containing 75 mM-KCl (100 mM-KCl elutes the enzyme at this pH). The column wash was then changed to buffer plus 70 mM-KCl plus 1 mM-sodium citrate, and phosphofructokinase was eluted. To maintain

Table 2. Some buffer systems and ligands for affinity elution of rabbit muscle glycolytic enzymes

The best $(\text{NH}_4)_2\text{SO}_4$ fraction to use for a particular enzyme is indicated. In the asterisked (*) examples, a heat pretreatment for 5 min at pH 5.5, 50°C before desalting removed proteins (mainly creatine kinase) which might be adsorbed and denatured on the column, and so interfere with the running of the column. The pre-elution wash buffer always included extra EDTA at the concentration indicated in the next column for ligands, to represent 'the dummy substrate'. CBN recommended abbreviations have been used: Glc, glucose; Fru, fructose; Grn, fructose; Grn, dihydroxyacetone; Gra, glyceraldehyde; Gri, glycinate; Prv, enolpyruvate.

Enzyme	$(\text{NH}_4)_2\text{SO}_4$ fraction	Adsorbent	Buffer, pH for absorption	Buffer, pH for pre-elution wash and elution	Ligands in buffer to cause elution, concn. (mM)	Charge introduced per 100000 daltons	Approximate specific activity (at 25°C) of eluted enzyme
Phosphorylase	A	CM-cellulose	Mes, 6.0	Mes, 6.5	AMP, 0.2+Glc-6-P, 0.2	4	18
Phosphoglucosmutase	BC*	Phosphocellulose	Mes, 6.0	Mes, 6.5	Glc-1-P, 0.2+Glc-1,6-P ₂ , 0.05	3 or 6	750
Phosphoglucose isomerase	BC	Phosphocellulose	Mops, 7.2	Mops, 7.2	Glc-6-P, 0.5 or Fru-6-P, 0.5	3.5	750
Phosphofructokinase	—	CM-cellulose	Mops, 6.9	Mops, 7.2+75 mM-KCl	Citrate, 1.0	3.5	140
Aldolase	BC, B	CM-cellulose	Mops, 7.2	Tricine, 8.0+20 mM-KCl	Fru-1,6-P ₂ , 0.2	10	18
Triose phosphate isomerase	CD*, D*, E*	CM-cellulose	Picolinate, 5.5	Mes, 5.8	Grn-P, 0.5	7.5	4000
Glycerol phosphate dehydrogenase	BC*	CM-cellulose	Mes, 6.0	Mes, 6.5	NADH, 0.2	5	220
Glyceraldehyde phosphate dehydrogenase	E	CM-cellulose	Mops, 7.2	Tricine, 8.0	NAD ⁺ , 0.2+Gra-3-P, 0.2+P _i , 1.0	14	150
Phosphoglycerate kinase	CD, D	CM-cellulose	Mes, 6.5	Tricine, 8.0	Gri-3-P, 0.5	6	600
Phosphoglycerate mutase	BC	Phosphocellulose	Mes, 6.5	Mes, 7.2	Gri-2,3-P ₂ , 0.2	16	1000
Enolase	CD, C	CM-cellulose	Mes, 6.5†	Mops, 7.0†	Gri-2-P or Prv-P, 0.5	7.5	50
Pyruvate kinase	BC	CM-cellulose	Mes, 6.5	Mops, 7.2	Prv-P, 0.5	5	300
Lactate dehydrogenase	BC	CM-cellulose	Mops, 7.2	Tricine, 8.0	NADH, 0.2	5	450
Myokinase	CD	CM-cellulose	Mes, 6.0	Mes, 6.5	ADP, 0.5	14+	800
Creatine kinase	CD, D	CM-cellulose	Mes, 6.0	Mes, 6.2	ATP, 0.5	7	100

† Magnesium acetate (1.0 mM) included in buffers to stabilize enzyme.

enzyme activity during the procedures, all buffers contained 1 mM-dithiothreitol.

Aldolase. The review on purification methods for mammalian muscle aldolases (Penhoet & Rutter, 1975) described the use of affinity elution from phosphocellulose. Except for the use of non-interacting buffers and a much lower substrate concentration, the present method is very similar. With so much enzyme to be eluted, the protein (subunit) concentration in the eluent is as high as the substrate concentration, i.e. 0.2 mM or 8 mg/ml. It is preferable to remove the substrate from the enzyme as soon as possible after elution to avoid inactivation; this is even more important if aldolase B, the liver enzyme, is isolated by the same procedure (Chappel *et al.*, 1976). Aldolase is also a 'sticky' enzyme, and some salt must be added to the pH 8 buffer to loosen its adsorption before adding the substrate.

Triose phosphate isomerase. This enzyme has several electrophoretic forms (Krietsch *et al.*, 1970) all of which have rather low pI values. However, the enzyme is completely adsorbed on CM-cellulose at pH 5.5. To elute the enzyme, glyceraldehyde 3-phosphate or dihydroxyacetone phosphate could be used as substrate (they are instantly interconverted on contact with the enzyme on the column), or more economically fructose 1,6-bisphosphate that has been pretreated with a little aldolase plus triose phosphate isomerase; 0.5 mM-fructose 1,6-bisphosphate at equilibrium forms about 0.5 mM-dihydroxyacetone phosphate in these conditions. However, at pH 5.5–6.0 very little of the substrate is in the correct ionic form, so the method barely works for rabbit enzyme. Trout muscle isomerase, which has a much higher pI (R. K. Scopes, unpublished work), can be adsorbed on CM-cellulose, then eluted with substrate at pH 7.2. Purification of the chicken muscle enzyme by affinity elution is described in the following paper (Scopes, 1977).

Glycerol phosphate dehydrogenase. This enzyme can be adsorbed at pH 6.0, and eluted with NADH at pH 6.5. It is presumed that, like lactate dehydrogenase, the enzyme has an ordered mechanism of reaction, and will only bind NADH or NAD⁺ to the free enzyme.

Glyceraldehyde phosphate dehydrogenase. Purification of glyceraldehyde phosphate dehydrogenase from rabbit muscle by (NH₄)₂SO₄ fractionation and crystallization is one of the simplest classical enzyme-purification procedures. Nevertheless, the affinity-elution procedure works well, and is adaptable to purifying the enzyme from other species. The sequence of reaction first makes compulsory the presence of NAD⁺, then glyceraldehyde phosphate must be bound before phosphate. All three substrates together (but no two in combination) cause elution of the enzyme, at a protein concentration limited by that of the substrates present. Alternatively, NADH alone

can be used for elution, but the enzyme is unstable in these conditions (Amelunxen & Grisolia, 1962). 1,3-Bisphosphoglycerate can also be used (see below), but the indicated procedure is preferable.

Phosphoglycerate kinase. The rabbit muscle enzyme is most conveniently eluted with 3-phosphoglycerate, but any of the other three substrates/products can be used. 1,3-Bisphosphoglycerate should theoretically be the most efficient, since it has the most negative charges and the highest affinity for the enzyme. But preparation of this substrate, salt-free, is not convenient. Moreover, it tends to elute glyceraldehyde phosphate dehydrogenase at the same time. Although the pI for rabbit phosphoglycerate kinase is reported to be 7.0, it is retained on CM-cellulose in Tricine buffer, pH 8.0, then eluted effectively with 3-phosphoglycerate.

Phosphoglycerate mutase. Evidence suggests that both mutases in the glycolytic sequence have similar reaction mechanisms (Ray *et al.*, 1973; Britton *et al.*, 1972). Thus phosphoglycerate mutase requires 2,3-bisphosphoglycerate to form the phospho-enzyme as a reaction intermediate. Nevertheless, it appears that most of the enzyme exists as the dephosphorylated form, at least by the time that the initial fractionation procedure has been carried out. The enzyme will mostly adsorb on phosphocellulose at pH 6.5, though only partly or not at all on CM-cellulose. The adsorbed de-phospho-enzyme can be eluted very specifically and effectively by using 0.2 mM-2,3-bisphosphoglycerate. An interesting observation is that, when samples containing haemoglobin were applied to the column, addition of the 2,3-bisphosphoglycerate caused the pink haemoglobin band to move slowly down the column, reflecting the fact that 2,3-bisphosphoglycerate binds to haemoglobin also, as an allosteric effector.

Enolase. Enolase requires Mg²⁺ ions to maintain its integrity, so all buffers used for the preparation of enolase have included 1 mM-magnesium acetate. The enzyme is eluted from CM-cellulose by substrates in Mops buffer, pH 7.0; however, phosphoenolpyruvate is also a substrate for the adjacent enzyme pyruvate kinase, which is eluted at the same pH. Some phosphoglycerate kinase may also be eluted, perhaps by conversion of the enolase substrate through into 3-phosphoglycerate by traces of mutase remaining on the column. In any case, these three can easily be separated subsequently by gel filtration.

Pyruvate kinase. As mentioned above, pyruvate kinase can be eluted at pH 7.2 with phosphoenolpyruvate. Enolase will also be eluted under these conditions, but enolase contamination can be removed before application of the fraction (BC) to the column by heat-denaturation treatment. Pyruvate kinase is particularly stable to heat at neutral pH, and this fact is made use of in the classical purification procedure (Tietz & Ochoa, 1958); 10 min at 60°C,

pH7, will remove all the enolase. ADP^{3-} (0.5mM) does not release the pyruvate kinase from the column.

Lactate dehydrogenase. The muscle isoenzyme M_4 adsorbs on CM-cellulose quantitatively at pH7.2 or lower. It can be eluted with NADH at pH7.2, but more effectively at pH8.0. This procedure is very easy to carry out, and can be scaled to gram quantities at a time if desired. It cannot of course be applied to the heart-type isoenzyme, which will not adsorb on a cation-exchanger.

AMP kinase. AMP kinase (adenylate kinase, myokinase) can be eluted from CM-cellulose at pH6.5 with ADP, preferably 0.5mM-ADP containing 0.5mM-magnesium acetate, which then contains roughly equal amounts of ADP^{3-} and MgADP^- , both substrates of the enzyme. Preliminary fractionation to concentrate the relatively small amount of this enzyme is desirable, and an acid treatment at pH2 is a useful step. The transition-state analogue P^1P^5 -di(adenosine-5') pentaphosphate has been used very successfully for affinity elution of AMP kinase (Feldhaus *et al.*, 1975).

Creatine kinase. Muscle contains a large amount of creatine kinase, an enzyme which is unstable in slightly acid conditions (Scopes, 1965). It can be purified with the aid of conventional DEAE-cellulose chromatography, although contamination with triose phosphate isomerase and phosphoglucomutase is difficult to avoid. The enzyme will adsorb on phosphor or CM-cellulose below pH6.5, but at pH lower than 6.0, even in the cold, it denatures quite quickly on the column. Nevertheless, it is possible to obtain a very pure preparation of the enzyme by adsorbing fraction CD on an ice-cold column of CM-cellulose at pH6.0, then eluting the enzyme at pH6.2–6.5 with ATP (no Mg^{2+} ions) as ligand: the HATP^{3-} ions bind strongly to creatine kinase, acting as an inhibitor of the normal enzymic reaction (Nihei *et al.*, 1961).

Discussion

The procedures for purifying enzymes by affinity elution represent an alternative approach to affinity-chromatographic techniques. Affinity chromatography is generally taken to imply the use of an adsorbent which has an affinity for the enzyme being isolated, and until recently less attention was paid to the role of specific elution from such an adsorbent. Specific elution from any adsorbent is possible provided that binding of the ligand alters the interaction between the enzyme and the adsorbent, so that the partition coefficient between adsorbent and liquid phase is decreased, ideally from 1 to 0. Although the affinity-elution technique has been used by many workers (see the introduction), particularly from phosphocellulose, there has often been an element of non-specific elution in these procedures because of increased ionic strength due to introduction of

sometimes quite unnecessarily high concentrations of substrate. The present work has attempted to eliminate these effects, first by finding conditions such that only small substrate concentrations are necessary (0.1–1.0mM), and secondly by utilizing 'dummy-substrate' washes, which result in no change of ionic strength when the substrate (or other ligand) is subsequently introduced. CM-cellulose has been used wherever possible; phosphocellulose has been avoided, not because it is not suitable, but because it is a more variable product, it titrates in the pH range used, and has less quantifiable specific effects. In particular it has some specific attraction for the active sites of phosphate ester-metabolizing enzymes, which can be exploited to advantage. Both phosphoglucose isomerase and phosphoglycerate mutase behaved better on this adsorbent, and it is likely that a new set of conditions can be found for each enzyme with phosphocellulose as adsorbent. Several different buffers with pH values between 5.5 and 8.0 have been used, followed by increasing ionic strength at pH8.0, if this was necessary. An alternative procedure is to use fewer buffers, but at a variety of ionic strengths according to the enzyme being eluted; this procedure is illustrated in the following paper (Scopes, 1977).

Most of the rabbit glycolytic enzymes have isoelectric points in the range 6–8; consequently it was possible to find conditions for each where they would adsorb on a cation-exchanger, without the pH being low enough to cause denaturation. This also mostly applies to the same enzymes from other mammalian sources, but those of lower vertebrates are more variable; for example some fish have considerably more acidic enzymes, yet some are more basic (Scopes, 1968). Invertebrate, plant and bacterial enzymes tend to be more acidic, and so the methods described here may not often be suitable for enzymes from such sources, unless there is a positively charged ligand that can be used after adsorption on an anion-exchanger. A preliminary estimate of the basicity of the enzyme of a species, compared with that of the rabbit, can be made by using simple gel-electrophoretic methods, or more precisely by using isoelectric focusing.

The effectiveness of the procedure depends on the ability of the ligand to weaken the enzyme's binding to the adsorbent. The amount of ligand required for this will depend on several things, such as its dissociation constant (and this may be affected by the enzyme being bound to the matrix), the number of charges on the effective ligand form, the size of the active subunit (the last two factors being classed together as 'charges introduced per 100000 daltons'; Table 2) and possible conformation changes after ligand binding. Single-substrate enzymes will also react to equilibrate substrate with product(s). This would be a problem with hydrolases if the enzyme did not have substantial affinities for the products; competitive inhibitors

would be better ligands in this case. Even if the substrate binds very tightly to the enzyme, the amount used must be at least equivalent to the amount of enzyme active site. For instance, if about 1 g of aldolase (typically from 300g of muscle) is being eluted from a column, the concentration should be at least 4 mg/ml to elute it in a reasonable total volume; 4 mg/ml is 0.1 mM-aldolase active site, and so at least 250 ml of 0.1 mM substrate (fructose 1,6-bisphosphate) is required. The use of a higher concentration is more effective in this case; the enzyme can be eluted at over 10 mg/ml, and can then be crystallized by adding $(\text{NH}_4)_2\text{SO}_4$ without a concentration step. It is preferable to have enzymes eluted at not less than 0.5 mg/ml to maintain stability.

It is assumed that the main effect that a substrate or other ligand has in eluting an enzyme is to neutralize the charge which is responsible for the enzyme binding to the column. It is also quite possible that a simple conformational change may alter the surface charge distribution and so cause elution (von der Haar, 1974). Enolase, which requires Mg^{2+} ions to maintain its dimeric integrity (Brewer & DeSa, 1972), can be specifically eluted by Mg^{2+} ions from a CM-cellulose column (though in poor yield, as activity is lost by adsorbing the enzyme in the absence of Mg^{2+}). Yet Mg^{2+} ions on binding to enolase ought to increase the positive charge and so make the enzyme adsorb more strongly. Clearly a substantial conformational change is more important in this case.

The behaviour of most of the enzymes in these experiments and the comparative elution behaviours of the enzymes from different species (Scopes, 1977, and unpublished work) suggest that in most cases the mechanism of affinity elution is a straightforward charge effect, a lowering of the effective pI by binding negatively charged ligand. For instance, creatine kinase can be eluted with HATP^{3-} from CM-cellulose, but a high concentration (20 mM) of uncharged creatine has no effect, although both compounds are known to bind to the free enzyme. Also, among the most effective procedures are those in which the substrates have 4 or more negative charges, e.g. fructose 1,6-bisphosphate for aldolase, or 2,3-bisphosphoglycerate for phosphoglycerate mutase.

As with conventional affinity-chromatography techniques, the reaction mechanism, whether it be ordered or random binding of substrates to a multi-substrate enzyme, is an important factor, and if the mechanism is unknown it may be deduced from experiments based on these techniques. Pyruvate kinase is not eluted by ADP^{3-} in conditions where phosphoenolpyruvate $^{3-}$ will elute the enzyme; this suggests an ordered mechanism of reaction, in contrast with the conclusions of Ainsworth & MacFarlane (1973) based on kinetic studies. But there are other possible explanations of this observation;

for instance it could be that the dissociation constant for ADP^{3-} from a binary complex with the enzyme is much greater than its reported K_m (10^{-5} M). Alternatively a conformational change caused by binding one of the substrates may be a more important factor controlling the elution characteristics.

If this method is unsuccessful simply because suitable conditions for adsorbing the enzyme on the appropriate ion-exchanger cannot be found, then it is theoretically possible that suitably charged ligand analogues might be developed. For example an acidic enzyme with a negatively charged or neutral substrate may be adsorbed on DEAE-cellulose, then eluted by a substrate analogue containing positively charged substituents which give the analogue an overall positive charge. Since most enzymes have negatively charged ligands, the converse problem is unlikely to occur often, and would probably be easier to solve.

The following paper (Scopes, 1977) describes multiple enzyme purifications from muscle extracts by using the affinity-elution techniques described here.

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