Multiple Enzyme Purifications from Muscle Extracts by using Affinity-Elution-Chromatographic Procedures

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1. Starting with $(\text{NH}_4)_2\text{SO}_4$ fractions of muscle extracts, procedures for purifying four to six separate enzymes from each fraction by using affinity-elution-chromatographic techniques are described. 2. Schemes for purifying 12 separate enzymes from rabbit muscle, and eight from chicken muscle extracts, are included. In nearly all cases the overall procedure involves three steps: the initial $(\text{NH}_4)_2\text{SO}_4$ fractionation, the ion-exchange chromatography with affinity elution of the enzyme, and gel filtration. The specific activities of the enzymes so purified are comparable with the highest values in the literature. 3. The five schemes described include illustrations of affinity elution of the separate enzymes at different pH values, at different ionic strengths and in combination with conventional gradient elution. They also include stepwise adsorption on columns at different pH values. 4. Separation of two electrophoretically differing forms of phosphoglycerate kinase was achieved by gradient affinity elution from CM-cellulose. The lower-pI form was eluted by a lower concentration of substrate than the higher-pI form.

Methods for purifying individual glycolytic and related enzymes from rabbit muscle by using affinity-elution techniques were described in the preceding paper (Scopes, 1977). Although most investigators are interested in only one or perhaps two different enzymes, it may be desirable to purify others to use, for example, in assaying the enzymes or the substrates being investigated. In this laboratory all glycolytic enzymes are required in large quantities both for assaying purposes and for metabolic reconstitution studies (Scopes, 1973). Use of the affinity-elution procedure has been widely adopted by us (Scopes & Fifis, 1975; Stewart & Scopes, 1975; Chappel *et al.*, 1976; Scopes, 1977), and the present paper describes procedures for purifying several different enzymes simultaneously from extracts of rabbit muscle.

In addition, methods for purifying some of the corresponding chicken muscle enzymes by affinity elution are described.

Materials and Methods

Materials and basic methods were as described in the preceding paper (Scopes, 1977). In addition, chicken muscle from freshly killed hens was obtained and extracted exactly as the rabbit muscle. $(\text{NH}_4)_2\text{SO}_4$ fractions obtained as described were dissolved in a small volume of Tris/HCl buffer, pH 8.0, and the pH was adjusted to 8.0 with 1M-Tris before desalting. The fractions were often stored as a slurry at 4°C before desalting, since it was not possible to work up more than one fraction at a time.

In all cases procedures relate to material from 1 litre

of extract (approx. 350 g of muscle), and the columns used were always 16 cm² cross-section and close to 5 cm tall unless otherwise indicated. The buffers used in chromatography were 10 mM of base (Tris, KOH, NaOH) adjusted to the required pH with α -picolinic acid, Mes,* Mops or Tricine as appropriate. The buffers also contained 0.2 mM-EDTA.

Results

Five schemes are presented here (three for rabbit enzymes, two for chicken) which cover 12 different enzymes. Phosphorylase (EC 2.4.1.1), phosphofructokinase (EC 2.7.1.11), glycerol phosphate dehydrogenase (EC 1.1.1.8) and glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) are not included; alternative techniques are more convenient for these enzymes (Scopes, 1973). In particular, phosphofructokinase is mostly destroyed by $(\text{NH}_4)_2\text{SO}_4$ fractionation at pH 6.0; phosphorylase and glyceraldehyde phosphate dehydrogenase form the major portions of fractions A and E respectively, and each can be crystallized directly after suitable treatments.

Scheme (a): purification of pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), aldolase (EC 4.1.2.13) and phosphoglucomutase (EC 2.7.5.1) from the 45–60% -satd. $(\text{NH}_4)_2\text{SO}_4$ fraction

Fraction BC contains between 70 and 80% of each of the aldolase, lactate dehydrogenase and pyruvate

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

kinase present in the extract, these enzymes being the major protein components of the fraction. Proteins in smaller amounts include creatine kinase (EC 2.7.3.2), phosphoglucumutase, phosphoglycerate mutase (EC 2.7.5.3), phosphoglucose isomerase (EC 5.3.1.9) and enolase (EC 4.2.1.11); some of these are more concentrated in other fractions. The procedure described here gives four of the main components, together with some others isolated in lesser overall yield.

Fraction BC from 1 litre of extract was first de-salted, then adjusted to pH 6.5 with Tris/Mes buffer, and diluted to a protein concentration of between 10 and 15 mg/ml (approx. 400 ml). A CM-cellulose column (of dimensions 16 cm² × 10 cm) in Tris/Mes buffer was used first, and the sample run on under gravity (300–400 ml/h). The sample was washed in with buffer, and KCl was added to the buffer to a concentration of 20 mM. Approx. 300 ml of this was pumped in (300 ml/h), and a protein peak, which included most of the phosphoglucose isomerase in the fraction, was obtained. This isomerase could be further purified by affinity elution from phospho-

Fig. 1. Diagram of a sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis slab containing samples relevant to Schemes (a) and (b)

Sample 1: fraction BC complete; 2: fraction BC not adsorbed on CM-cellulose at pH 6.5; 3: phosphoenolpyruvate-eluted pyruvate kinase, Scheme (a); 4: pyruvate kinase after gel filtration (Fig. 2); 5: lactate dehydrogenase eluted by NADH, Schemes (a) and (b); 6: aldolase eluted by fructose 1,6-bisphosphate, Schemes (a) and (b); 7: phosphoglucumutase eluted by glucose phosphates, Scheme (a); 8: phosphoglucose isomerase eluted by glucose 6-phosphate, Scheme (b); 9: phosphoglycerate mutase eluted by 2,3-bisphosphoglycerate, Scheme (b). Abbreviations: PGM, phosphoglucumutase; PK, pyruvate kinase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; EN, enolase; CK, creatine kinase; ALD, aldolase; LDH, lactate dehydrogenase; PGAM, phosphoglycerate mutase.

Fig. 2. Separation of impurities from pyruvate kinase by gel filtration

The fraction applied to the Sephadex G-150 column (8 cm² × 90 cm) was the phosphoenolpyruvate-eluted fraction in Fig. 3, after concentration by ultrafiltration to 25 ml. ····, Pyruvate kinase. —, Percentage transmission at 280 nm.

cellulose as described in the preceding paper (Scopes, 1977).

After the column had been equilibrated with Tris/Mes/KCl buffer, 0.5 mM-phosphoenolpyruvate was added to the buffer [a 'dummy-substrate' wash (Scopes, 1977) was not necessary at this higher ionic strength], which specifically eluted pyruvate kinase, somewhat contaminated with enolase, phosphoglycerate mutase and phosphoglycerate kinase. These contaminants are clearly identifiable by their subunit sizes on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). They were present because during the affinity-elution step the enolase and mutase combined to convert the added phosphoenolpyruvate through 2-phosphoglycerate into 3-phosphoglycerate, so causing affinity elution of all four enzymes; however, pyruvate kinase was the major component, and being a much larger molecule than the other enzymes was subsequently completely separated from the others by gel filtration on Sephadex G-150 (Fig. 2).

The column now contained only lactate dehydrogenase and aldolase in significant amounts. The buffer was changed to KOH/Mops, pH 7.2, containing 10 mM-KCl, and after 200 ml of this had been passed into the column 0.2 mM-NADH was added to it (150 ml), specifically eluting lactate dehydrogenase. The column was next washed with KOH/Mops buffer, pH 7.2, containing 20 mM-KCl, followed by the same buffer containing 0.2 mM-fructose 1,6-bisphosphate (200 ml), which eluted the aldolase. The purities of these preparations can be judged from Fig. 1. The complete elution diagram from this column is shown in Fig. 3. All elution diagrams in this

Fig. 3. Elution diagram for rabbit muscle fraction BC adsorbed to a CM-cellulose column at pH 6.5 The enzymes obtained by affinity elution were pyruvate kinase (----), lactate dehydrogenase (.....) and aldolase (-.-.-). —, Percentage transmission at 280nm in 3mm-path-length cell. For details of procedure, see the text.

Fig. 4. Affinity elution of phosphoglucumutase from a CM-cellulose column at pH 6.0 The fraction applied was that protein not adsorbed at pH 6.5 (see Fig. 3). ····, Phosphoglucumutase. —, Percentage transmission at 280nm. For details of procedure, see the text.

paper are typical profiles selected from several separate experiments.

The final step in this scheme was to take the protein that had not adsorbed on the CM-cellulose column, lower its pH to 6.0 with 1 M-Mes, and run it on to another CM-cellulose column (16 cm² × 5 cm), equilibrated at pH 6.0. The enzymes adsorbed included phosphoglucumutase, creatine kinase and phosphoglycerate mutase. The latter was denatured and could not be recovered from the column. After a short wash of the column with Tris/Mes buffer, pH 6.0, containing an additional 0.5 mM-EDTA, the wash was changed to Tris/Mes, pH 6.0, containing both 0.5 mM-glucose 1-phosphate and 0.1 mM-glucose 1,6-bisphosphate (100 ml). Both phospho and dephospho forms of phosphoglucumutase were eluted from the column, the latter being phosphorylated in the process. This preparation was essentially pure; it has been crystallized from (NH₄)₂SO₄ in the form of large needles in the presence of various bivalent metal ions.

Creatine kinase could be obtained from this column by the same procedure as described below in Scheme (c); however, less than one-third of the creatine kinase from the original extract is present in fraction BC, and so the overall yield is not high. A diagram of the elution pattern from the second column is shown in Fig. 4. The activities of the enzymes and the specific activities of the purified preparations are summarized in Table 1.

Scheme (b): purification of phosphoglucose isomerase, phosphoglycerate mutase, lactate dehydrogenase, aldolase and phosphoglucumutase from the 45–60% -satd.-(NH₄)₂SO₄ fraction

This scheme is an alternative to Scheme (a) as far as lactate dehydrogenase, aldolase and phosphoglucumutase are concerned; it has been developed for isolation of the other two enzymes. Fraction BC was desalted, then taken to pH 7.0 with Tris/Mops buffer. Two columns in tandem were used, the first being CM-cellulose, the second phosphocellulose. The sample was diluted to about 400 ml, and passed

through the two columns, which were then washed with a little buffer. Of the five enzymes noted, only phosphoglucumutase was not adsorbed on either column, and could subsequently be purified on CM-cellulose at pH 6.0 as described in Scheme (a). The CM-cellulose column was worked up for lactate dehydrogenase and aldolase exactly as described in Scheme (a). The phosphocellulose column retained the phosphoglucose isomerase and phosphoglycerate mutase. The isomerase was eluted in the same pH 7.0 buffer, by using 0.5 mM-glucose 6-phosphate. The mutase was eluted, still at pH 7.0, by using 0.2 mM-2,3-bisphosphoglycerate. Pyruvate kinase was also adsorbed on the phosphocellulose column, but elution of this enzyme was not attempted. The diagram of elution from the phosphocellulose column is illustrated in Fig. 5, and the activities of the enzymes eluted from it and from the CM-cellulose columns are listed in Table 2. The purities of the enzymes as obtained by affinity elution are illustrated in Fig. 4; as with the other examples, the impurities could mostly be removed by gel filtration.

Scheme (c): purification of enolase, phosphoglycerate kinase (EC 2.7.2.3), triose phosphate isomerase (EC 5.3.1.1), creatine kinase and myokinase (EC 2.7.4.3) from the 60–75% -satd.-(NH₄)₂SO₄ fraction

Fraction CD contains more than 50% of the enolase, creatine kinase, myokinase and phosphoglycerate kinase from the extract. Triose phosphate isomerase is only partially precipitated at 75% -satd. (NH₄)₂SO₄, and there are approximately equal amounts of this enzyme in each of fractions CD, E and the supernatant from E.

After desalting or dialysis, fraction CD was made to pH 6.6 with Tris/Mes buffer; the buffer included 1.0 mM-magnesium acetate to stabilize enolase, and 10 mM-2-mercaptoethanol as a general protective measure for all of these enzymes. The sample, in a volume of approx. 300 ml, was applied to a CM-cellulose column in pH 6.6 buffer. Nearly all of the creatine kinase, and all of the triose phosphate

Table 1. *Summary of purifications in Scheme (a)*

The 'highest literature specific activities' are extrapolated to assay conditions used in the present experiments (Scopes, 1977). References: ^a, Bondar & Pon (1969); ^b, Pesce *et al.* (1967); ^c, Penhoet & Rutter (1975); ^d, Hashimoto & Handler (1966).

	Pyruvate kinase	Lactate dehydrogenase	Aldolase	Phospho- glucumutase
Activity in 1 litre of original extract (k-units)	235	480	42.5	135
Activity in fraction BC (from 1 litre) (k-units)	162	356	32.5	69
Activity not adsorbed at pH 6.5 (k-units)	0	7	0	69
Activity affinity-eluted (k-units)	122	228	27.5	56
Specific activity affinity-eluted (units/mg)	260	490	16.5	650
Specific activity after gel filtration (units/mg)	340	600	17.0	750
Highest literature specific activity (units/mg)	340 ^a	610 ^b	18.0 ^c	600 ^d
Final yield of enzyme from 1 litre (mg)	320	380	1350	68

Fig. 5. Elution diagram for the phosphocellulose column at pH 7.0 in Scheme (b)

The fraction applied was BC after it had been passed through a CM-cellulose column at pH 7.0. ····, Phosphoglucose isomerase; ----, phosphoglycerate mutase. —, Percentage transmission at 280 nm. For details of procedure, see the text.

Table 2. Summary of purifications in Scheme (b)

The 'highest literature specific activities' are extrapolated to assay conditions used in the present experiments (Scopes, 1977). References: ^a, Dyson & Noltmann (1968); ^b, Grisolia & Cleland (1968); ^c, Pesce *et al.* (1967); ^d, Penhoet & Rutter (1975); ^e, Hashimoto & Handler (1966).

	Phospho- glucose isomerase	Phospho- glycerate mutase	Lactate dehydro- genase	Aldolase	Phospho- gluco- mutase
Activity in 1 litre of original extract (k-units)	142	440	435	45	145
Activity in fraction BC (from 1 litre) (k-units)	88	260	320	33	72
Activity not adsorbed on CM-cellulose (k-units)	88	260	10	0	72
Activity affinity-eluted (k-units)	62	245	210	27	66
Specific activity affinity-eluted (units/mg)	540	840	500	17.0	620
Specific activity after gel filtration (units/mg)	740	1000	*	*	710
Highest literature specific activity (units/mg)	760 ^a	1050 ^b	610 ^c	18.0 ^d	600 ^e
Final yield of enzyme from 1 litre (mg)	74	205	420	1590	79

* Enzymes were crystallized without gel-filtration step.

isomerase, passed through the column. (At pH 6.5 or lower, substantial amounts of creatine kinase were retarded on the column.) The sample was washed in with 200 ml of buffer containing an additional 0.5 mM-EDTA, and the myokinase was eluted by replacing the additional EDTA with 0.5 mM-ADP. The protein eluted contained, not only all the myokinase, but also the small amount of creatine kinase that had been held back on the column, which in fact accounted for most

of the protein. Separation of the two kinases was readily achieved by a subsequent gel filtration on Sephadex G-100 or G-75.

The column pH was raised slightly, to pH 6.8 (Tris/Mes buffer, including 0.5 mM-EDTA as dummy substrate), by using 200 ml of buffer. The EDTA was next replaced by phosphoenolpyruvate, causing elution of enolase, somewhat contaminated with pyruvate kinase, for the same

Fig. 6. Elution diagram for rabbit muscle fraction CD adsorbed on a CM-cellulose column at pH 6.6. The enzymes obtained by affinity elution were myokinase (.....), enolase (-.-.-) and phosphoglycerate kinase (-----). —, Percentage transmission at 280 nm. For details of procedure, see the text.

Fig. 7. Affinity elution of creatine kinase from CM-cellulose after adsorption at pH 6.0. The fraction applied was that protein not adsorbed on CM-cellulose at pH 6.6 (see Fig. 6)., Creatine kinase. —, Percentage transmission at 280 nm. For details of procedure, see the text.

reason as the converse contamination was obtained in Scheme (a). After all the enolase had been obtained, the column was washed with pH 8.0 buffer (NaOH/

Tricine containing 0.5 mM-EDTA as dummy substrate). As the pH rose, protein was eluted, including some haem pigments. After about 250 ml of buffer

had been washed into the column, the EDTA was replaced by 0.5mM-3-phosphoglycerate. Phosphoglycerate kinase was eluted from the column by this procedure, and it contained only a little contaminant, which was completely removed by gel filtration. The elution diagram for this column is illustrated in Fig. 6.

The protein that had not adsorbed on the column at pH 6.6 was adjusted to pH 6.0 with 1M-Mes, cooled to 0°C, then run on to another ice-cold CM-cellulose column of the same dimensions, equilibrated with Tris/Mes buffer, pH 6.0. Creatine kinase was totally

adsorbed, but triose phosphate isomerase was not. The column was washed with 200ml of cold Tris/Mes buffer, pH 6.2, containing 0.5mM-EDTA and no magnesium acetate, and the creatine kinase was eluted by replacing the EDTA with 0.5mM-ATP (Fig. 7).

Finally, triose phosphate isomerase could be adsorbed by further lowering the pH of the fraction which did not adsorb at pH 6.0 to 5.5 with picolinic acid, and applying it to a third CM-cellulose column equilibrated with Tris/picolinate, pH 5.5. The sample was washed in with Tris/Mes buffer, pH 6.0. As the

Fig. 8. Elution of triose phosphate isomerase from CM-cellulose after adsorption at pH 5.5. The fraction applied was that protein not adsorbed on CM-cellulose at pH 6.0 (see Fig. 7). ····, Triose phosphate isomerase. —, Percentage transmission at 280nm. For details of procedure, see the text.

Table 3. Summary of purifications in Scheme (c)

The 'highest literature specific activities' are extrapolated to assay conditions used in the present experiments (Scopes, 1977). References: ^a, Kress *et al.* (1966); ^b, Wold (1971) (assumes $A_{280}^{1\text{mg/ml}} = 0.90$); ^c, Scopes (1969); ^d, Mahowald *et al.* (1962); ^e, Krietsch *et al.* (1970).

	Myokinase	Enolase	Phospho- glycerate kinase	Creatine kinase	Triose phosphate isomerase
Activity in 1 litre of original extract (k-units)	78	80	225	116	3250
Activity in fraction CD (from 1 litre) (k-units)	45	47	144	64	990
Activity not adsorbed at pH 6.5 (k-units)	0	0	0	48	990
Activity affinity-eluted (k-units)	32	27	117	38.5	700*
Specific activity affinity-eluted (units/mg)	210	36	550	95	4600*
Specific activity after gel filtration (units/mg)	1410	66†	720	98	6000
Highest literature specific activity (units/mg)	1500 ^a	90 ^b	720 ^c	100 ^d	6000 ^e
Final yield of enzyme from 1 litre (mg)	20	355	145	345	105

* Not 'affinity' procedure, see the text.

† With $A_{280}^{1\text{mg/ml}} = 0.63$; specific activity would be 92 with $A_{280}^{1\text{mg/ml}} = 0.90$ (Wold, 1971).

pH slowly rose, the isomerase was eluted, in a fairly pure state (Fig. 8). As indicated in the preceding paper (Scopes, 1977), affinity elution of rabbit

Fig. 9. Diagram of a dodecyl sulphate/polyacrylamide-gel electrophoresis slab containing samples relevant to Scheme (c)

Sample 1: fraction CD complete; 2: fraction CD not adsorbed on CM-cellulose at pH 6.6; 3: ADP-eluted myokinase (+creatine kinase); 4: myokinase after gel filtration; 5: phosphoenolpyruvate-eluted enolase; 6: enolase after gel filtration; 7: phosphoglycerate kinase eluted with 3-phosphoglycerate; 8: creatine kinase eluted with ATP; 9: triose phosphate isomerase eluted by raising pH. Abbreviations: see legend to Fig. 1; and TIM, triose phosphate isomerase; MK, myokinase.

muscle triose phosphate isomerase is not successful, since at the suitable pH (approx. 5.8) the enzyme has low affinity for its substrates.

A summary of the procedure is given in Table 3. The purity of the enzymes obtained in this Scheme can be judged by comparison of the specific activities with the highest recorded values in the literature (Table 3), and from the diagram of the sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram (Fig. 9).

The impurities present were in most cases enzymes which bind the same ligand as the one being used in the elution procedure. Thus myokinase was contaminated with creatine kinase, and enolase with pyruvate kinase. Impurities in each case were removed by gel filtration, resulting in virtually pure enzyme, as indicated in Fig. 9 and by the specific activities in Table 3.

Scheme (d): purification of myokinase, triose phosphate isomerase, enolase and two forms of phosphoglycerate kinase from the 60–70% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of chicken muscle

This procedure for chicken muscle is included to illustrate how the methods can be adapted to purify the enzymes from other species. The same main enzymes are present in chicken muscle fraction CD as in that from rabbit muscle, and except for creatine kinase, which is too acidic in chicken muscle to adsorb on a cation-exchanger in a stable pH range, all have been isolated by very similar steps. Comparison of the

Fig. 10. Elution diagram for chicken muscle fraction CD adsorbed on CM-cellulose at pH 6.5

The enzymes obtained by affinity elution were myokinase (·····), triose phosphate isomerase (·-·-·-), enolase (-·-·-), and phosphoglycerate kinase (----). —, Percentage transmission at 280 nm. For details of procedure, see the text.

Table 4. Summary of purifications in Scheme (d)

The 'highest literature specific activities' are extrapolated to assay conditions used in the present experiments (Scopes, 1977). References: ^a, Kress *et al.* (1966) (for rabbit enzyme); ^b, Putman *et al.* (1972); ^c, Wold (1971) (for rabbit enzyme); ^d, Scopes (1969) (for rabbit enzyme). I and II refer to the lower- and higher-pI forms of phosphoglycerate kinase respectively.

	Myokinase	Triose phosphate isomerase	Enolase	Phosphoglycerate kinase
Activity in 1 litre of original extract (k-units)	110	2500	155	200
Activity in fraction CD (from 1 litre) (k-units)	93	850	85	145
Activity not adsorbed at pH 6.5 (k-units)	0	10	0	0
Activity affinity-eluted (k-units)	70	575	81	115
Specific activity affinity-eluted (units/mg)	950	4600	89	I 500 II 120
Specific activity after gel filtration (units/mg)	1450	6000	*	I 700 II 650
Highest literature specific activity (units/mg)	1500 ^a	5800 ^b	90 ^c	720 ^d
Final yield of enzyme from 1 litre (mg)	43	86	910	I 83 II 59

* Crystallized without gel-filtration step.

Fig. 11. Diagram of a dodecyl sulphate/polyacrylamide-gel-electrophoresis slab containing samples relevant to Scheme (d)

Sample 1: fraction CD complete; 2: ADP-eluted myokinase; 3: myokinase after gel filtration; 4: dihydroxyacetone phosphate-eluted triose phosphate isomerase; 5: triose phosphate isomerase after gel filtration; 6: phosphoenolpyruvate-eluted enolase; 7: enolase after gel filtration; 8: 3-phosphoglycerate-eluted phosphoglycerate kinase I; 9: 3-phosphoglycerate-eluted phosphoglycerate kinase II. Abbreviations: see legends to Figs. 1 and 9.

electrophoretic mobilities of the rabbit and chicken enzymes (Scopes, 1968) suggests that enolase, being more basic in chicken, should adsorb more firmly, whereas triose phosphate isomerase should adsorb less readily. In practice, the isomerase adsorbs more strongly, whereas the enolase behaves about the same, as do myokinase and phosphoglycerate kinase. These

four enzymes are adsorbed on and eluted from a single CM-cellulose column.

Application of the sample is the same as for the rabbit muscle fraction (Scheme c), namely in a Tris/Mes buffer containing 1 mM-magnesium acetate and 10 mM-mercaptoethanol, but the pH used was 6.5. The first elution of myokinase by ADP was also carried out under the same conditions. The myokinase was not contaminated with creatine kinase, as none of the latter enzyme had adsorbed on the column. Triose phosphate isomerase had adsorbed on the column, and could be eluted after the myokinase by raising the pH to 6.9 (Tris/Mes), and then adding substrate mixture generated enzymically from fructose 1,6-bisphosphate (Scopes, 1977) at pH 6.9. After this, enolase was eluted with 0.5 mM-phosphoenolpyruvate, also at pH 6.9, and the pH was then raised to 8.0 with NaOH/Tricine. Phosphoglycerate kinase in chicken muscle appears to consist of two components (Scopes, 1968), and these can be separated either by successive elutions with different concentrations of substrate (0.5 mM-3-phosphoglycerate for the less basic of the two, 1 mM-3-phosphoglycerate for the other), or preferably with a linear substrate gradient. After washing with 250 ml of NaOH/Tricine buffer, pH 8.0, containing additional 0.5 mM-EDTA, 150 ml of this buffer was set up, 150 ml of NaOH/Tricine buffer containing 1.5 mM-3-phosphoglycerate being mixed in to form a linear gradient; the two phosphoglycerate kinase components were eluted separately, with a substantial amount of another protein contaminating the second form. A summary of this procedure is given in Table 4, and the elution diagram in Fig. 10. Purities of the enzymes obtained are illustrated diagrammatically by the sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram in Fig. 11.

Fig. 12. Elution diagram for chicken muscle fraction BC adsorbed on DEAE-cellulose at pH 7.2

The enzymes obtained by gradient elution were phosphoglucumutase (·····) and phosphoglycerate mutase (----). ·-·-·, KCl concentration —, Percentage transmission at 280 nm. For details of procedure, see the text.

Table 5. Summary of purifications in Scheme (e)

The 'highest literature specific activities' are extrapolated to assay conditions used in the present experiments (Scopes, 1977). References: ^a, Hashimoto & Handler (1966) (for rabbit and fish enzyme); ^b, Torralba & Grisolia (1966); ^c, Pesce *et al.* (1967); ^d, Penhoet & Rutter (1975) (for rabbit enzyme).

	Phospho- glucumutase	Phospho- glycerate mutase	Lactate dehydrogenase	Aldolase
Activity in 1 litre of original extract (k-units)	195	550	540	31.0
Activity in fraction BC (from 1 litre) (k-units)	110	345	355	20.5
Activity not adsorbed on DEAE-cellulose (k-units)	1	0	330	20.1
Activity affinity-eluted (k-units)	84*	230*	186	17.7
Specific activity affinity-eluted (units/mg)	500*	1000*	560	18.3
Specific activity after gel filtration (units/mg)	660	—†	—†	—†
Highest literature specific activity (units/mg)	600 ^a	1000 ^b	660 ^c	18.0 ^d
Final yield of enzyme from 1 litre (mg)	65	230	335	970

* Not 'affinity' procedure.

† Crystallized without gel-filtration step.

Scheme (e): purification of the two glycolytic mutases from chicken muscle, together with lactate dehydrogenase and aldolase

This procedure has been included, although part of it does not involve affinity elution. Fraction BC from chicken muscle, after heat-treatment to remove creatine kinase, contains only two major components acidic enough to adsorb on DEAE-cellulose at pH 7.2. These are phosphoglucumutase and phosphoglycerate mutase. A simple ionic-strength gradient elutes the two enzymes separately, each sufficiently pure to be able to be crystallized. Proteins not adsorbed on the DEAE-cellulose can then be adsorbed on CM-cellulose for affinity elution of lactate dehydrogenase and aldolase.

Fraction BC was taken to pH 5.5 with 1 M-acetic

acid, then heated at 45°C for 10 min, cooled to 20°C, the pH adjusted to 7.5 with 1 M-Tris, and the denatured protein removed by centrifugation for 10 min at 10000g. It was then desalted on a Sephadex G-25 column, and run on to a DEAE-cellulose column in Tris/Mops buffer, pH 7.2. The column was washed with 200 ml of Tris/Mops buffer, pH 7.2, then a linear gradient in KCl was applied by mixing in 400 ml of 0.15 M-KCl in Tris/Mops buffer to 400 ml of the buffer alone. First phosphoglucumutase was eluted, and then phosphoglycerate mutase. The elution diagram for this column is shown in Fig. 12, and the activity details in Table 5.

The protein that did not adsorb on the DEAE-cellulose column was run straight on to a CM-cellulose column, equilibrated in the same Tris/Mops

Fig. 13. Elution diagram for chicken muscle fraction BC adsorbed on CM-cellulose at pH 7.2

The fraction applied was that protein not adsorbed to DEAE-cellulose (see Fig. 12). Enzymes obtained by affinity elution were lactate dehydrogenase (····) and aldolase (----). —, Percentage transmission at 280 nm. For details of procedure, see the text.

Fig. 14. Diagram of a dodecyl sulphate/polyacrylamide-gel-electrophoresis slab containing samples relevant to Scheme (e)

Sample 1: fraction BC complete; 2: fraction BC not adsorbed on DEAE-cellulose; 3: phosphoglucumutase obtained by gradient elution; 4: phosphoglycerate mutase obtained by gradient elution; 5: NADH-eluted lactate dehydrogenase; 6: fructose 1,6-bisphosphate-eluted aldolase. Abbreviations: see legend to Fig. 1.

buffer; the two columns were in fact coupled together in tandem during sample application. This column was washed with Tris/Mops buffer containing 5 mM-KCl, and NADH was then added to the buffer to elute lactate dehydrogenase, giving enzyme of specific activity 560 units ($\mu\text{mol}/\text{min}$) per mg of protein. Then further KCl was added before elution of aldolase. It was found necessary to use 30 mM-KCl for the pre-elution wash for chicken muscle aldolase, as it adsorbs more strongly on the column than does the rabbit enzyme. As in the rabbit muscle example, the enzyme was specifically eluted with 0.2 mM-fructose 1,6-bisphosphate, giving an enzyme which was essentially pure (specific activity 17). The elution diagram for these enzymes is shown in Fig. 13. The procedure is summarized in Table 5, and the electrophoretogram is shown in Fig. 14.

Discussion

The procedures outlined in this paper demonstrate that affinity-elution chromatography can be used for relatively large-scale purification of several different enzymes from one extract. For multiple purifications there are several possible stages where separation can be achieved. Ideally, all enzymes would be adsorbed on one column, then each eluted successively by stepwise changes of pH and ionic strength, and inclusion of ligands. In practice this is rarely satisfactory; the

pH needed to adsorb the lowest pI protein (on a cation-exchanger) is likely to be so low as to denature other proteins. Even if the denatured proteins are not among the enzymes being purified, this would be undesirable, as the coagulated protein clogs the columns. Also, a protein present in relatively small amounts still needs a column large enough to adsorb it together with other proteins present in larger quantity, leading to high dilutions and possible loss of activity of the small component. Because of these factors two or more columns have been used in most cases, a system of stepwise adsorption as well as stepwise elution. By using the zwitterionic buffers as described it was possible to lower the pH after passage through one column without changing the ionic strength, so that the adsorption characteristics of each enzyme were unaltered. Each column was separately worked up with stepwise affinity elution, for each of the major enzymes present.

Some of the schemes described took only two or three trials before arriving at a satisfactory procedure; others took considerably longer. Certain enzymes have very narrow ranges of conditions where affinity elution can be carried out. Differences of only 0.2 pH unit or changed column dimensions can result in enzymes either being eluted too soon or not appearing when expected. However, the conditions for other enzymes, particularly those eluted at pH values above 7, were less critical. This probably reflects a greater degree of change of adsorptive properties on binding of ligand, but also relevant is the fact that pH is less critical because proteins have few titratable groups in the pH range 7–8.

In cases where more than one form of enzyme was present owing to genetic or epigenetic factors, it has been possible to separate the enzymes by a substrate gradient. Separation of the five lactate dehydrogenase isoenzymes has been reported from an affinity adsorption column, by using an NADH gradient (Brodelius & Mosbach, 1973). In the present example two forms of phosphoglycerate kinase from chicken muscle (one of which is probably an epigenetic modification of the other, as peptide 'maps' are indistinguishable; T. Fifis, unpublished work) were nearly completely separated by a 3-phosphoglycerate gradient. In another example (Stewart & Scopes, 1975), the genetically distinct A and B isoenzymes of phosphoglycerate kinase (VandeBerg *et al.*, 1975) were completely separated by using a substrate gradient.

Although few of the affinity-eluted enzymes could be described as 'pure', contaminants were usually not more than 20% of the protein, so they could be removed either by direct crystallization of the enzyme, or in most cases by gel filtration. It is fortuitous that contaminating proteins for the most part had molecular sizes quite different from the major enzyme present. After gel filtration, the specific activities of the

enzymes were close to (and in one case in excess of) the highest reported specific activities (extrapolated to the assay conditions used in the present experiments). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis confirmed the homogeneity of the preparations. The electrophoretic technique was also valuable in indicating the likely identity of impurities eluted with the enzyme from the column, from known subunit sizes of the major protein components (Scopes & Penny, 1970). Certain results have been anomalous, in particular the properties of enolases isolated by this technique. The subunit sizes for the chicken and rabbit enzymes were estimated to be 50000 ± 2000 and 47000 ± 2000 daltons respectively. The latter is somewhat larger than reported, but the former is similar to the size of the fish enzymes (Wold, 1971), being further evidence for a larger polypeptide chain in non-mammalian enolases. The extinction coefficients at 280 nm of both enolases, based on protein determination by u.v. methods (Scopes, 1974) were each 0.63 (for 1 mg/ml), compared with values around 0.90 in the literature.

The principles of affinity-elution chromatography as applied to rabbit muscle glycolytic enzymes are described in the preceding paper (Scopes, 1977). The successful adaptation of the methods to chicken muscle enzymes as described here demonstrates the general usefulness of the procedure. Since adsorption on ion-exchange materials is dependent on net charge, some knowledge of the relative isoelectric points of the species' enzymes is useful; this can be obtained by gel-electrophoresis techniques (Scopes, 1968). It has been possible to purify phosphoglycerate kinase from a wide variety of species by using this technique, and several other enzymes have also been purified from kangaroo muscle extracts (R. K. Scopes & T. Fifis, unpublished work).

For multiple enzyme purification, as a 'half-way house' between conventional ion-exchange chromatography and affinity-adsorption chromatography, affinity-elution chromatography combines beneficial factors of each, with many of the disadvantages removed.

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