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PURIFICATION AND PARTIAL CHARACTERIZATION OF ALCOHOL DEHYDROGENASE, FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE AND THE CYTOPLASMIC FORM OF MALATE DEHYDROGENASE FROM *DROSOPHILA MELANOGASTER*

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Abstract—A purification scheme for the cytoplasmic form of malate dehydrogenase (s-MDH) of *Drosophila melanogaster* is presented which is superior to any previously reported method. In addition, this scheme can also be used to obtain alcohol dehydrogenase (ADH) and FDP aldolase. Gel filtration experiments reveal an oligomeric molecular weight of 69,000 for s-MDH, and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate indicates subunit molcular weights of 32,100 for s-MDH, 24,600 for ADH and 34,000 for FDP aldolase. The amino acid composition of *Drosophila melanogaster* s-MDH and FDP aldolase are reported.

Key Word Index: *Drosophila melanogaster*, malate dehydrogenase, alcohol dehydrogenase, FDP aldolase

INTRODUCTION

Naturally occurring enzyme variants offer an excellent opportunity to test evolutionary hypotheses that relate the biochemical properties of particular enzymes to the fitness of the organism as a whole (Clarke, 1975; Koehn, 1978). They can also serve as probes of structure function relationships in these enzymes. *Drosophila melanogaster* alcohol dehydrogenase (ADH, EC 1.1.1.1) is a good example of such a system (Fletcher *et al.*, 1978; Retzios and Thatcher, 1979; Thatcher and Retzios, 1980; Chambers *et al.*, 1981, 1983).

We are also interested in comparing the structures and properties of cytoplasmic (soluble) malate dehydrogenase (s-MDH, EC 1.1.1.37) obtained from a variety of *D. melanogaster* lines. The development of efficient purification protocols is most important to facilitate such a study because the mass culture of *D. melanogaster* larvae and the purification of enzymes are both labour-intensive activities. Thus, it is advantageous to be able to isolate several proteins from a single batch of starting material. Here, we report an improved technique for the combined purification of s-MDH and ADH based on the purification of Sofer and Ursprung (1968), and McReynolds and Kitto (1970). This new scheme yields products which are consistently pure after three column chromatographic stages. An initially unidentified by-product of the purification scheme is shown to be FDP aldolase (fructose-1,6-bisphosphate aldolase EC 4.1.2.13). This enzyme is obtained in large quantities but in a largely inactive form.

MATERIALS AND METHODS

Drosophila melanogaster

The lines used are derived from single wild-caught flies (McDonald Ranch, Napa County, California) made isogenic for the whole second chromosome (containing the structural loci for ADH and s-MDH). Larvae were raised by mass culture on standard cornmeal–molasses–agar medium, harvested at third instar by washing in tap water and flotation in 12% (w/v) sucrose and stored at −70°C until required.

Supplies

Whatman CM-32 carboxymethylcellulose was purchased from Reeve Angel, hydroxylapatite (Biogel HTP) from Bio-Rad and DEAE–Sephadex (A50) and Sephadex G-150 (40–120 μ) from Pharmacia Fine Chemicals who also supplied the molecular weight standards: aldolase, ovalbumin, α-chymotrypsinogen and ribonuclease. Bovine serum albumin was obtained from Armour Pharmaceutical Co. Eastman Organic Chemicals supplied materials for polyacrylamide gel electrophoresis. Substrates and cofactors for enzyme assays and activity stains and materials for starch gel electrophoresis were obtained from Sigma Chemical Co. Hydrochloric acid and mercaptoethanesulphonic acid were provided by Pierce Chemical Co. All other chemicals used were reagent grade.

Affinity chromatography media

5′-AMP-Sepharose was obtained from Pharmacia Fine Chemicals and NAD^+-Agarose types 1, 3 and 4 from P. L. Biochemicals, Inc. Blue Dextran Sepharose (0.64 μmol/ml packed bed) prepared by the method of Ryan and Vestling (1974) was a gift from Dr M. E. Knuth. Blue Sephadex G-150 (0.87 μmol dye/ml packed bed) was prepared from Sephadex G-150 and Cibacron Blue F3GA (Polysciences, Inc.) by the method of Easterday and Easterday (1974). Dye substitution in these media was determined according to Chambers (1977).

Enzyme assays

All assays were carried out at room temperature, which was 23°C with a range of ±2°C, using a Gilford Model 250 recording spectrophotometer. ADH activity was measured by the method of McDonald *et al.* (1977) with propan-2-ol

and NAD^+ as substrate. MDH activity was measured by the rate of oxaloacetate reduction according to McReynolds and Kitto (1970). For both assays we define one unit (U) of enzyme activity as the change in absorbance per minute at 340 nm equivalent to the conversion of one micromole of NAD^+ to NADH.

Protein assay

We used the Coomassie blue G-250 binding assay (micro method) of Sedmak and Grossberg (1977). Bovine serum albumin was used as a standard.

Polyacrylamide gel electrophoresis

The systems described by Laemmli (1970) and Davies (1964) were employed to check the purity of isolated enzymes. Duplicate 7.5% polyacrylamide "native" gels (Davis, 1964) without a stacking layer were used for each sample. One gel from each set was stained for protein by immersion for 1 hr in 0.2% Coomassie blue R in 5% methanol and 7.5% acetic acid; the gel was then diffusion-destained for 24 hr in the same solvent. The second gel was stained for enzyme activity following the methods of Ayala *et al.* (1972). The denaturing system of Laemmli (1970) was employed without modification, utilizing 10% gels. Bovine serum albumin, ovalbumin, α-chymotrypsinogen, and ribonuclease were used as molecular weight standards for SDS gels.

Starch gel electrophoresis

The systems described by Ayala *et al.* (1972) were employed.

Concentration of protein solution

This was achieved by nitrogen pressure filtration of dilute protein solutions in an Amicon Pressure cell Model 202 fitted with a PM10 membrane at a pressure of 35–42 kg cm^{-1}.

Amino acid analysis

Proteins were reduced and S-carboxymethylated (Crestfield *et al.*, 1963) prior to hydrolysis in 6 M HCl *in vacuo* at 110°C for 24, 48 or 96 hr. Analyses were performed on a Durrum Model D-500 amino acid analyzer. A sample of S-carboxymethyl-s-MDH was also hydrolysed with 3 M mercaptoethanesulphonic acid according to Thatcher (1977).

Gel filtration

Determination of the oligomeric molecular weights of s-MDH and ADH were carried out according to Andrews (1965). A G-150 Sephadex column (50 × 2.5 cm i.d.) equilibrated with 50 mM potassium phosphate buffer pH 7.5 containing 1 mM 2-mercaptoethanol and 1 mM EDTA was employed. A flow rate of 20 ml/hr was maintained. The column was calibrated with aldolase (158,000), ovalbumin (45,000), α-chymotrypsinogen (25,800) and ribonuclease A (13,700). Standard proteins were applied at a concentration of 10 mg/ml in equilibrium buffer (1 ml total volume). Purified s-MDH (0.5 mg) was applied separately in 1 ml equilibration buffer.

RESULTS

Affinity chromatography

We attempted, first, to purify MDH and ADH by general ligand affinity chromatography. Analytical scale trials of affinity chromatography media were very disappointing. Neither 5′-AMP-Sepharose nor any type of NAD^+-agarose tested appeared to bind any of the enzymes of interest to us. The technical difficulties associated with the use of these media to purify *Drosophila* alcohol dehydrogenase have recently been described by Lee *et al.* (1979) and Leigh-Brown and Lee (1979). Both Blue Dextran Sepharose and Blue Sephadex G-150 proved capable of binding ADH and both MDHs under similar conditions (50 mM potassium phosphate or Tris–HCl buffer pH 7.5 containing 1 mM EDTA and 1 mM 2-mercaptoethanol, protein concentration of dialysed 75% ammonium sulphate fraction 10 mg/ml). ADH activity could be recovered from Blue Sephadex G-150 in good yield (up to 80% of activity applied to the column) and often in homogeneous condition with NAD^+ washings as low as 0.1 mM concentration. Elution was not facilitated by the presence of up to 3 mM hydroxylamine. No effective scheme was found for either form of malate dehydrogenase.

Our regime for ADH was not suited to large-scale preparations, because a bed volume of 0.67 ml per mg of protein applied was required to ensure that all ADH was bound to the column. The method is, however, applicable to analytical scale preparations. Caution is advised when using preparations obtained in this way since it has been shown that interconversion of the molecular forms of *D. melanogaster* ADH occurs in the presence of NAD^+ (Jacobson *et al.*, 1972). However, this probably only occurs when the cofactor preparation employed contains small amounts of acetone (Schwartz *et al.*, 1979; Winberg *et al.*, 1983).

Purification scheme

The limited success of affinity chromatography made it necessary to revert to a more classical procedure of enzyme purification. This, although not nearly as elegant, results in the successful purification of s-MDH and ADH from a single batch of starting material.

Our purification scheme is shown in outline in Fig. 1.

Stage 1. Three hundred grammes of frozen *D. melanogaster* larvae are suspended in 600 ml 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM 2-mercaptoethanol and 1 mM EDTA and allowed to thaw. The larvae are then disrupted in a Waring blender for 30 sec at full speed. The extract is centrifuged at 16,000 ***g*** for 30 min at 4°C to remove insoluble debris. The same centrifugation conditions are used at all subsequent stages. Re-extraction of the precipitate yields only a 5% increase in total enzyme activity at an approx. 5-fold lower specific activity and is, therefore, not used.

Stage 2. The supernatant is filtered through glass wool to remove the surface fatty layer. After the addition of 14 ml of a 2% (w/v) protamine sulphate solution in extraction buffer per 100 ml of filtrate the solution is stirred gently for 30 min and centrifuged.

Stage 3. Solid ammonium sulphate is added to the supernatant from stage 2 to 45% saturation. The mixture is stirred for 30 min and centrifuged. Although only a small increase in specific activity is achieved, we find this step to be necessary in order to remove phenoloxidase activity or activators thereof (see Dickinson and Sullivan, 1975).

Stage 4. The supernatant from stage 3 is raised to 75% saturation by the addition of solid ammonium sulphate. The solution is stirred for 30 min and centrifuged.

Stage 5. The precipitate from stage 4 is dissolved in 10 mM potassium phosphate buffer pH 6.5 containing 1 mM 2 mercaptoethanol and 1 mM EDTA to a final volume of approx. 100 ml. The solution is dialysed twice against 2 l of the same buffer (2 hr per dialysis). The small amount of resultant precipitate is removed by centrifugation.

Stage 6. The supernatant is applied to a hydroxylapatite column equilibrated with 10 mM potassium phosphate buffer, pH 6.5, containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The column is developed with a combined pH and concentration gradient. See Fig. 2 for elution profile.

When a longer (40–45 × 5 cm i.d.) column is employed or when a more diluted sample is applied (170 ml total volume), both ADH and s-MDH are retained on the column and are observed to elute on either side of the first emerging protein peak. Here, with a shorter (30 cm bed length) column, 50–60% of the ADH actvity emerges at breakthrough. Since combining the two ADH pools does not affect the yield or purity of the enzyme obtained, such short

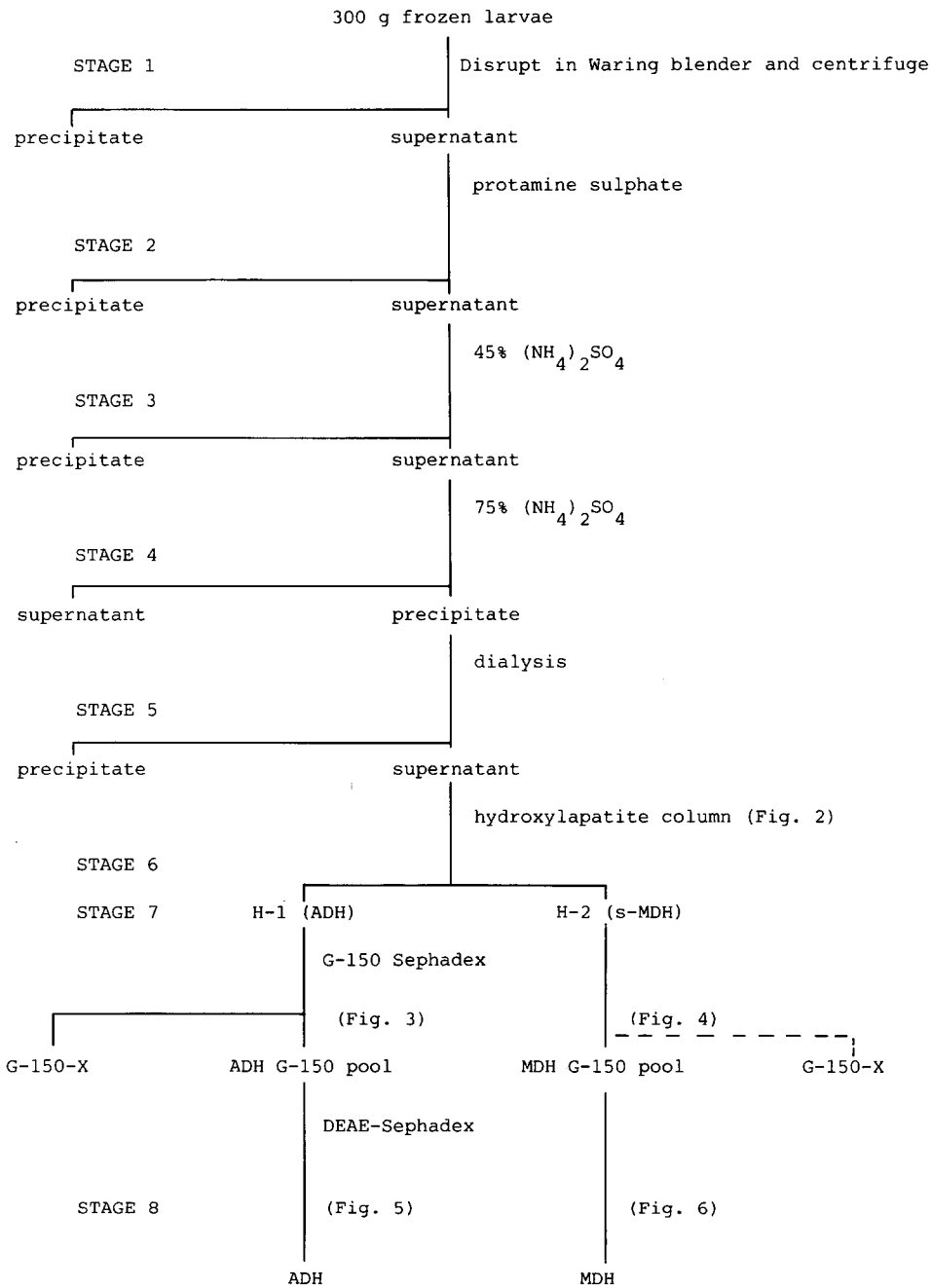

Fig. 1. Purification scheme for the isolation of s-MDH, ADH and FDP aldolase from frozen *D. melanogaster* larvae. Details of procedures are given in the text. All stages are carried out at 0–4°C. All buffers contain 1 mM EDTA and 1 mM 2-mercaptoethanol.

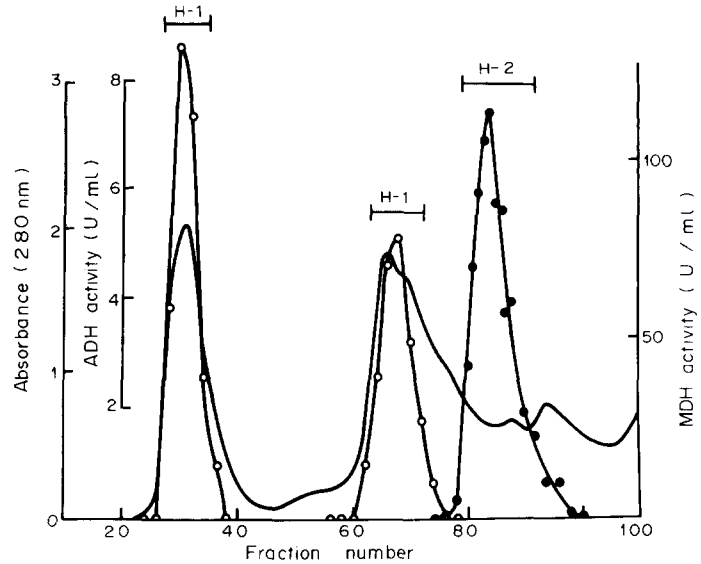

Fig. 2. Elution profile of the hydroxylapatite column (30 × 5 cm i.d.). The 75% ammonium sulphate pellet was first dialysed against 10 mM potassium phosphate buffer, pH 6.5, containing 1 mM 2-mercaptoethanol and 1 mM EDTA (final sample volume = 130 ml). The column was developed at 70 ml/hr with a linear gradient consisting of 2 l of equilibration buffer and 2 l of 250 mM potassium phosphate, pH 7.5, containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The fraction size was 20 ml. Fractions were monitored for total protein by the absorbance at 280 nm (line without cirlces) and for ADH (open circles) and MDH (black circles) enzyme activities. Fractions were pooled as indicated by the bars.

hydroxylapatite columns have been used for reasons of speed and economy.

Fractions containing ADH and s-MDH activity were pooled separately (pools H-1 and H-2, respectively, in Fig. 1). The s-MDH pool contains no m-MDH activity, as judged by protein and activity staining of pool H-2 samples run on polyacrylamide gels. The m-MDH is retained on the column and is not eluted by the gradient system employed. (Final condition: 250 mM potassium phosphate pH 7.5 containing 1 mM 2-mercaptoethanol and 1 mM EDTA.) A satisfactory scheme for elution of m-MDH from hydroxylapatite remains to be investigated in detail.

Stage 7. Pools H-1 and H-2 are concentrated by precipitation at 70 and 80% saturation of ammonium sulphate respectively and subsequent centrifugation. A buoyant foam of denatured protein is observed in both pools and is composed mostly of FDP aldolase (see later). The resultant pellets are dissolved in 50 mM Tris–HCl buffer, pH 8.3, containing 1 mM 2-mercaptoethanol and 1 mM EDTA, giving a final volume of approx. 10 ml. These pools are then subjected to gel filtration of Sephadex G-150 (see elution profiles in Fig. 3 for ADH and in Fig. 4 for s-MDH). Fractions containing enzyme activity are pooled and concentrated by ultrafiltration. The elution profile for ADH pool H-1 (Fig. 3) shows a protein peak G-150-X emerging after the void volume but before ADH. The size of this peak varies between preparations and depends on how much of the denatured foam is resuspended with the pellet proper. Examination of this material by polyacrylamide gel electrophoresis shows that it contains only a single protein. Smaller amounts of similar material are also obtained after

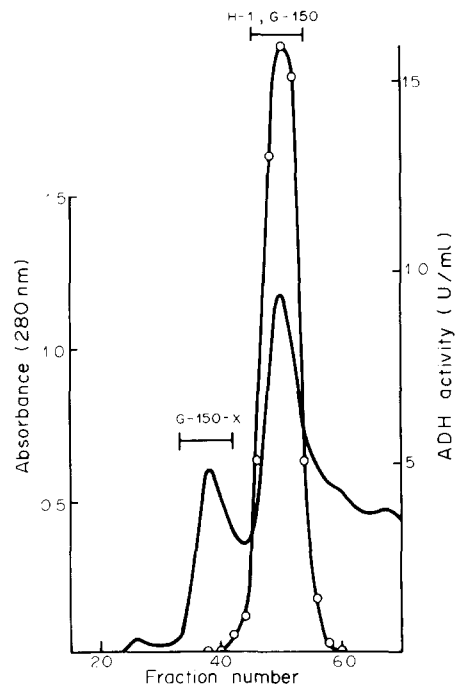

Fig. 3. Elution profiles of a G-150 Sephadex column (50 × 5 cm i.d.). The sample was hydroxylapatite pool H-1 (ADH) after concentration (volume = 15 ml). The column was eluted with 50 mM Tris–HCl pH 8.3 containing 1 mM 2-mercaptoethanol and 1 mM EDTA at 40 ml/hr; 12 ml fractions were collected. Fractions were monitored for total protein by the absorbance at 280 nm (line without circles) and for ADH activity (open circles). Fractions were pooled as indicated by the bars.

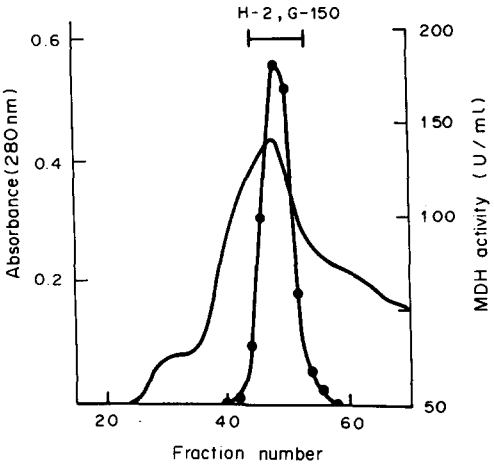

Fig. 4. Elution profiles of a G-150 Sephadex column. The sample was hydroxylapatite pool H-2 (s-MDH) after concentration (volume = 10 ml). Conditions as in Fig. 2. Absorbance at 280 nm (line without circles); MDH activity (black circles). Fractions were pooled as indicated by the bar.

gel filtration of s-MDH pool H-2, giving a combined total of between 40 and 70 mg.

Stage 8. Concentrated enzyme pools H-1, G-150 (ADH) and H-2, G-150 (s-MDH) are put through a final purification stage consisting of ion-exchange chromatography on DEAE–Sephadex (see elution profiles in Fig. 5 for ADH and in Fig. 6 for s-MDH).

Enzyme assays and protein determinations are carried out at each stage. A purification table for a particular preparation is given in Table 1. Figures 2–6

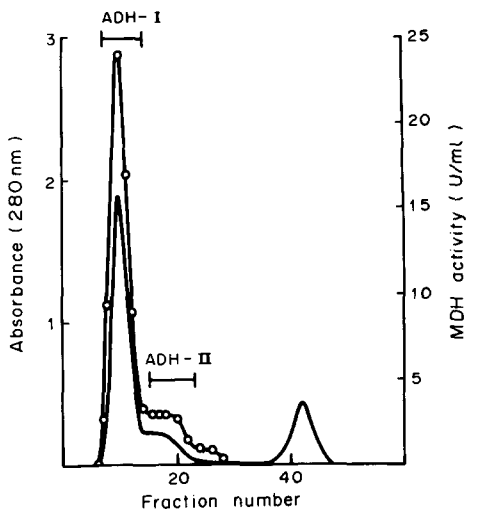

Fig. 5. Elution profiles of a DEAE–Sephadex column (12 × 2.5 cm i.d.). The sample was the ADH pool after gel filtration (Fig. 2) and concentration (volume = 9.5 ml). The column was washed with equilibration buffer (50 mM Tris–HCl pH 8.3 containing 1 mM 2-mercaptoethanol and 1 mM EDTA) to 15 fractions; then a linear gradient (total volume 360 ml) of equilibration buffer and 250 mM potassium chloride in the same buffer was applied. The flow rate throughout was 30 ml/hr; 6-ml fractions were collected. Fractions were monitored for total protein by absorbance at 280 nm (line without cirlces) and for ADH activity (open circles). Fractions were pooled as indicated by the bar.

Table 1. Purification table for a *D. melanogaster* strain

Stage	Totals						Specific activity ($U \times 10^{-3}$)		Yield (%)		Purity (fold)	
	Volume (ml)	Enzyme activity (U/ml)		Protein (mg/ml)	Activity ($U \times 10^{-3}$)		Protein (mg)					
		ADH	MDH		ADH	MDH		ADH	MDH	ADH	MDH	ADH	MDH
1. Crude extract	770	2.34	77	14.3	1.81	59.3	11,011	0.16	5.39	100	100	1	1
2. Protamine sulfate supernatant	870	2.17	62.8	7.75	1.89	54.7	6743	0.28	8.10	104	92	1.71	1.50
3. 45% $(NH_4)_2SO_4$ supernatant	970	2.0	59.5	5.38	1.95	57.7	5219	0.37	11.1	108	97	2.28	1.94
4. 75% $(NH_4)_2SO_4$ pellet	105	16.2	503	37.7	1.71	52.8	3960	0.43	13.3	94	89	2.63	2.48
5. After dialysis	130	15.1	392	25.0	1.97	50.9	3250	0.61	15.7	109	86	3.69	2.91
6a. Hydroxylapatite pool H-1	410	4.34	—	1.05	1.78	—	431	4.13	—	98	—	25.2	—
6b. Hydroxylapatite pool H-2	290	—	67.5	0.53	—	19.6	154	—	127	—	33	—	23.6
7a. ADH G-150 pool	147	8.36	—	0.91	1.23	—	134	9.16	—	68	—	56	—
7b. s-MDH G-150 pool	140	—	95.7	0.51	—	13.4	71.4	—	188	—	23	—	34.8
8a. ADH DEAE pool	55	12.2	—	0.62	0.672	—	34.1	19.8	—	37	—	120	—
8b. s-MDH DEAE pool	50	—	217	0.24	—	10.9	11.8	—	920	—	18	—	171

Procedures are given in the text and in Fig. 1. The strain used (M092) carries the electrophoretically slow form of ADH.

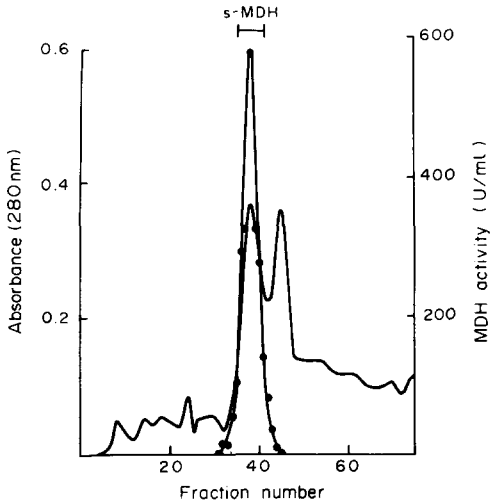

Fig. 6. Elution profiles of a DEAE–Sephadex column. The sample was the s-MDH pool after gel filtration (Fig. 3) and concentration (volume = 10 ml). Conditions as in Fig. 5. Absorbance at 280 nm is indicated by the line without circles; MDH activity is shown with black circles. Fractions were pooled as indicated by the bar.

show the elution profiles for this particular preparation. The yields of s-MDH and ADH are 18 and 37% respectively.

Purity and properties of products

Polyacrylamide gel electrophoresis reveals in the case of s-MDH an identical two-banded pattern for protein and activity. Pool ADH-I contains unmodified ADH dimers (form ADH-5) and appears as a single band on gels stained for protein or ADH activity. This enzyme form can be rapidly interconverted *in vitro* to the faster migrating derivatives ADH-3 and ADH-1 by incubation with NAD^+ and acetone (Chambers and Fletcher, unpublished; Winberg *et al.*, 1983). The modified forms ADH-3 and ADH-1 contain, respectively, one and two subunit equivalents of an abortive ternary complex (Schwartz *et al.*, 1979). Pool ADH-II shows the familiar 2- or 3-banded pattern. FDP aldolase (pool G-150-X) exhibits a single broad band with a mobility less than that of ADH-F. Homogeneity of the samples was confirmed by use of SDS-containing polyacrylamide gels. All three proteins give single bands. The estimated subunit molecular weights are: 24,600 for ADH, 32,100 for s-MDH, and 34,000 for FDP aldolase (Fig. 7).

Determination of oligomeric molecular weights by gel filtration

The oligomeric molecular weight determined by gel filtration on the calibrated G-150 Sephadex column (Fig. 8) was 69,000 for s-MDH. Repeated experiences with gel filtration on G-150 Sephadex of partially purified material (75% ammonium sulphate pellet) which contains m-MDH, s-MDH and ADH activities reveal a single symmetrical peak of MDH activity and a single ADH peak of equal width. We conclude from this that the mitochondrial and cytoplasmic forms of *D. melanogaster* MDH have very similar if not identical molecular weights and that s-MDH is dimeric. The ADH and MDH peaks are separated by less than one percent of the total column volume. In addition, the ammonium sulphate precipitated pools from the hydroxylapatite column (Fig. 2) have similar elution profiles on G-150 Sephadex (see Figs 3 and 4). We calculate an apparent molecular weight for the ADH dimer of 67,000 (Fig. 8). The expected value is 49,200 from our SDS polyacrylamide gel electrophoresis experiments and 54,800 calculated from the ADH protein sequence of Thatcher (1980).

This anomalous gel filtration property of ADH may be an artifact occurring in concentrated protein mixtures rich in enzyme. This behaviour is not seen with dilute pupal extracts prepared in 50 mM Tris–HCl pH 8.3 containing 2 M urea (see Batterham and Chambers, 1981, for details). When such material is run on G-150 Sephadex in pH 8.3 buffer but lacking urea, a value of 52,000 is obtained for the molecular weight of the ADH oligomer (Chambers and Batterham, unpublished).

It should be noted that co-elution of both malate dehydrogenase and alcohol dehydrogenase from G-150 Sephadex can be helpful. Figure 9 shows a scheme which we have used to obtain the three enzymes from a single batch of larvae.

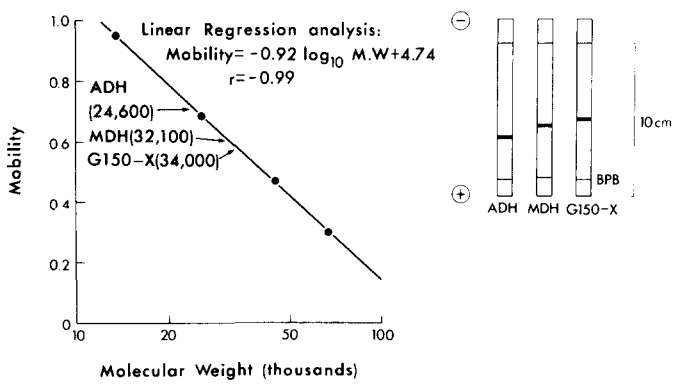

Fig. 7. Calibration curve for SDS–gel electrophoresis. Diagrams of sample gels are shown to scale on right (loading 25 μg protein). BPB—bromophenol blue marker of dye front.

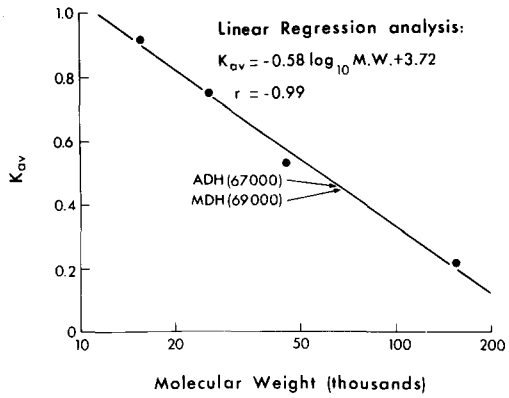

Fig. 8. Calibration curve for molecular weight determination of ADH and MDH oligomers. Details are given in the text.

Amino acid analysis

The amino acid compositions of our ADH samples agree well with that of Thatcher (1977). The amino acid compositions of s-MDH and FDP aldolase are given in Table 2.

Evidence that protein G-150-X is FDP aldolase

The protein material eluted before ADH from the G-150 Sephadex column was not immediately recognized as FDP aldolase and was provisionally named G-150-X. This protein is consistently obtained in large amounts by our purification method. However, Fletcher (1980) soon recognized that the amino acid composition of this product shown in Table 2 closely resembled that of *D. melanogaster* FDP aldolase (Brenner-Holzach, 1979).

Table 3. The average residue deviation (ARD) between the amino acid composition of *Drosophila* FDP aldolases from different sources

Comparison		ARD Value
Between two* G-150-X preparations		0.73
Several G-150-X preparations vs	1.	1.56
standard aldolase‡	2.	2.17
	3.†	1.72
Mean composition G-150-X (preps 1–3) vs standard aldolase		1.21
s-MDH§ vs standard aldolase		31.30
ADH‖ vs standard aldolase		80.50

*Samples 1 and 2 (Chambers, unpublished). †Data from Table 2. ‡Brenner-Holzach (1979). §Table 2 data adjusted to 354 residues. ‖Data (Chambers, unpublished) adjusted to 354 residues.

Amino acid compositions were adjusted to a total of 354 residues, i.e. the total number of residues in *Drosophila* aldolase according to Brenner-Holzach (1979) minus cysteine, methionine and tryptophan (total 10 residues) because reliable values were not available for these amino acids in all data sets. ARD values were calculated as described in the text.

Table 3 shows a comparison of several preparations of our G-150-X material with the published composition for FDP aldolase. For each comparison we calculated the average residue deviation (ARD), i.e. the squared differences between residue values averaged over all residue types, asp, thr, ser, etc. Comparisons between our G-150-X preparations and FDP aldolase give low ARD values (range 0.5–2.5). To demonstrate that the similarity was not artifactual, we compared the compositions of two unrelated proteins from the same organism (s-MDH and ADH) to the published FDP aldolase composition, making allowance for differences in subunit sizes as explained (Table 3, legend). High ARD values were obtained in both comparisons.

The analysis clearly supports the hypothesis of Fletcher (1980). The subunit molecular weight of G-150-X, 34,000, together with its elution position on

Table 2. The amino acid composition of s-MDH and G-150-X (FDP aldolase) of *D. melanogaster*

	s-MDH		Protein X	
Residue	Ratio (relative to Asx)	Residues/ molecule‖	Ratio (relative to Asx)	Residues/ molecule¶
Cys*	0.10	3.0	0.16	4.9
Asx	1.00	29.3	1.00	30.6
Thr†	0.52	15.2	0.66	20.3
Ser†	0.95	27.7	0.58	17.9
Glx	1.02	29.8	1.20	36.8
Pro	0.50	14.5	0.51	15.6
Gly	0.92	26.9	0.85	26.1
Ala	1.13	32.9	1.20	36.8
Val‡	1.04	30.5	0.80	24.5
Met	0.24	6.9	0.06	1.8
Ile‡	0.48	14.1	0.54	16.5
Leu	0.78	22.6	0.97	29.8
Tyr	0.21	6.1	0.32	9.8
Phe	0.33	9.7	0.24	7.4
His	0.15	4.3	0.12	3.7
Lys	0.70	20.5	0.71	21.8
Arg	0.32	9.3	0.49	15.0
Trp§	+	1	—	—
Total		304.3		319.3

Data are calculated from compositions obtained after analysis of one-fifth aliquots of three 0.25 mg samples of S-carboxymethylated proteins hydrolyzed for 24, 48, or 96 hr.

*Determined as S-carboxymethyl cysteine. †Corrected for destruction during hydrolysis. ‡Corrected for slow release during hydrolysis. §From sample hydrolysed in 3M mercaptoethanesulfonic acid. Tryptophan was noted to be present but could not be quantified. One residue per molecule is included in the composition on the basis of evidence from peptide maps (Chambers, unpublished). ‖A subunit molecular weight of 32,100 is used. ¶A subunit molecular weight of 34,000 is used.

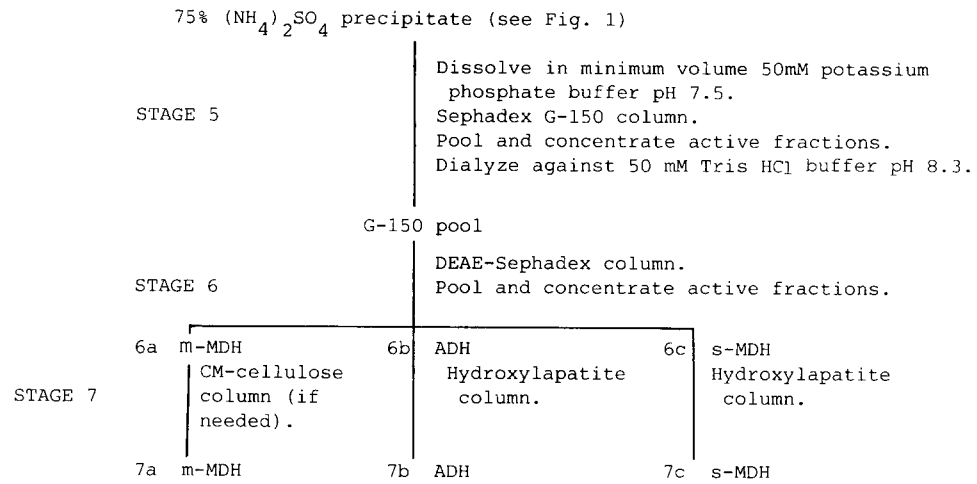

Fig. 9. Purification scheme for the isolation of s-MDH, m-MDH and ADH from *D. melanogaster* larvae. All stages carried out at 0–4°C. All buffers contained 1 mM EDTA and 1 mM 2-mercaptoethanol.

the Sephadex column, suggests that it is a tetramer, which is the usual size and subunit structure for aldolases (see Brenner-Holzach, 1979).

Subsequent investigations (Chambers, unpublished) have shown that FDP aldolase activity monitored by the assay system of Fernandez-Sousa *et al.* (1979), co-elutes with ADH retained on the hydroxylapatite column, and coincides with the G-150-X elution position on the Sephadex column. Native polyacrylamide gels of G-150-X pools stained for FDP aldolase show identical patterns of activity stain and protein. However, when FDP aldolase activity is assayed throughout the preparation, it can be seen that activity is lost rapidly as the purification progresses. Only 1% of total starting FDP aldolase activity remains at the G-150-X stage. The high protein yield of this material suggests that the enzyme is being inactivated during the preparation. Polyacrylamide gel electrophoresis investigations support this view. The enzyme is progressively converted into a faster migrating low activity form presumably by deamidation or some other non-specific inactivation mechanism.

DISCUSSION

We report here an improved scheme for the purification of cytoplasmic MDH from *D. melanogaster*, which also produces purified ADH and FDP aldolase. The yield of s-MDH is 18% of total malate dehydrogenase activity (representing about 36% of the total s-MDH activity). The enzyme is consistently obtained pure and is stable for days at 4°C.

The method we have developed is superior to the procedures described for the isolation of *D. virilis* s-MDH by McReynolds and Kitto (1970) and by Narise (1979). The yield and degree of purification achieved are similar to those of Hay and Armstrong (1976) who do not, unfortunately, report an electrophoretic examination of their preparations for contaminating proteins. Although the affinity chromatography procedure of Lee *et al.* (1979) seems slightly more effective than our scheme (see below), the conventional procedure has the advantages that it does not require specially prepared resins or specific adducts for elution and that it can be used for large-scale preparations. For analytical scale preparations the procedure of Lee *et al.* (1979) seems particularly good. They obtain a similar yield to us (23% starting activity) but achieve a higher degree of purification (376-fold) of a product that appears homogeneous of SDS polyacrylamide gels.

It is possible then that our final pool contains some inactivated enzyme. Indeed, McReynolds and Kitto (1970) and Narise (1979) report instability in *D. virilis* s-MDH preparations. We have not investigated the stabilizing effect of adding 0.1% (w/v) bovine serum albumin to s-MDH reported by Hay and Armstrong (1976) because our s-MDH preparations were destined for protein chemical experiments.

The scheme in Fig. 1 is at least as effective for the purification of ADH as the affinity procedure of Leigh-Brown and Lee (1979) and has advantages of simplicity and scale. Our scheme also produces a high yield of FDP aldolase protein but since the enzyme is inactivated it cannot be considered preferable to that of Brenner-Holzach and Leuthardt (1972). This particular version of our preparation does not give m-MDH, but changes can be implemented to make this possible.

Our procedure is a modification of a previously used one (see Fig. 9). The older scheme gives an inferior yield of s-MDH (5–8% of starting activity), although it has been successfully applied to isolate cytoplasmic MDH from honey bee larvae (Snyder *et al.*, 1979). The scheme illustrated has the advantage that m-MDH can be purified from the DEAE–Sephadex pool using a CM cellulose column as described by McReynolds and Kitto (1970). The results of Hay and Armstrong (1976) suggest that in our present (Fig. 1) procedure either m-MDH is precipitated during the pH 6.5 dialysis step (stage 5) or that it remains bound to the hydroxylapatite column, from which it could be removed by a prolonged wash with the final elution buffer (250 mM potassium phosphate pH 7.5 with 1 mM 2-mercaptoethanol and 1 mM EDTA).

The older procedure shown in Fig. 9 has two additional useful features. First, it is a simple large-scale procedure for obtaining ADH. Second, ion exchange chromatography on DEAE–Sephadex allows rapid separation of s-MDH and m-MDH activities for analytical work. The method is probably superior in yield and scale to preparative electrophoretic methods that have been used (see Alahiotis, 1979a,b). Alternatively, a similar separation could be achieved by means of hydroxylapatite at pH 7.5 after Hay and Armstrong (1976).

We have isolated s-MDH from several lines of *D. melanogaster* in quantities large enough to begin chemical characterization of the enzyme. Our experiments show that the enzyme consists of identical subunits of molecular weight 32,100. This, together with an oligomeric molecular weight of 69,000 (compare to 68,000 reported for the mitochondrial form of the enzyme from *D. virilis* by McReynolds and Kitto, 1970), suggests that the enzyme is a dimer. This is further supported by the fact that individuals heterozygous for electrophoretic variants at the s-MDH locus show a triple-banded pattern in a wide variety of *Drosophila* species (more than 30 different species have been studied in our laboratory): see also Hay and Armstrong (1976) and O'Brien (1973). The structure of cytoplasmic MDH is, therefore, similar in *Drosophila* to that observed in other organisms (see Banaszak and Bradshaw, 1975, for a review).

We have used our method to purify ADH from several lines of *D. melanogaster* carrying the electrophoretically slow (ADH-S) and fast (ADH-F) forms of the enzyme. The structures of these enzymes have been compared by peptide mapping (Ayala, 1982; but see Fletcher, 1980 for details). Other studies (Fletcher *et al.*, 1978 and Chambers *et al.*, 1981) have served to demonstrate that the same *Adh* alleles are segregating in natural populations on three continents (Europe, U.S.A. and Australia). They have also identified the amino acid sequence difference between ADH-F and ADH-S (Lys 192 for Thr in ADH-S) and revealed a different substitution in a heat-resistant form of ADH (ADH-FCh.D.) isolated in Australia (Ser 214 for Pro). Many of these data have recently been confirmed by DNA sequencing of *Adh* genes from natural populations of *D. melanogaster* (Kreitman, 1983).

FDP aldolase is consistently obtained in large quantities but in a form which is not suitable for enzymological experiments. However, this material is perfectly satisfactory for peptide mapping and sequencing. The small subunit size of this abundant protein together with the large body of information already available concerning its primary, tertiary and quaternary structure (Brenner-Holzach and Smit, 1982; Brenner-Holzach and Zumsteg, 1982), make it a good candidate for planned comparative studies between populations and between species of *Drosophila*.

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