Purification and Enzyme Stability of Alcohol Dehydrogenase from *Drosophila simulans*, *Drosophila virilis* and *Drosophila melanogaster adh^s*

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Three alcohol dehydrogenases from *Drosophila simulans*, *Drosophila virilis* and *Drosophila melanogaster adh^s* (which possesses an alloenzyme with slow electrophoretic mobility) were purified essentially to homogeneity. The purification procedure involves a new step of affinity chromatography, which efficiently lowers the amount of contaminants in the final preparation, producing a very stable enzyme. The purification procedure developed consists of a salmine sulphate precipitation, two CM-Sepharose CL-6B column-chromatography steps, an affinity-chromatography step and a Sephacryl gel filtration. A minimum of 30-fold purification is obtained and the yield is not less than 34%. The isoelectric points and molar absorption coefficients were determined.

Previous purifications of alcohol dehydrogenase (EC 1.1.1.1) of *Drosophila melanogaster* (Sofer & Ursprung, 1968; Schwartz *et al.*, 1975; Thatcher, 1977) produced preparations that were highly unstable, presumably because of the presence of proteinase contaminants in the final preparation. The half-life of the enzyme was reported to be 4 h at 4°C and 200 h at 0°C. The main aim of the present purification procedure was to obtain a very stable enzyme preparation free from proteinases and peptidases to avoid inactivation and degradation of the enzyme. An affinity-chromatography step was devised to lower the amount of contaminants in the final enzyme preparation.

Experimental

Materials

An isogenic strain of *Drosophila melanogaster* with *adh^s* (slow) allele constructed by Dr. B. Clarke (Department of Genetics, University of Nottingham, Nottingham, U.K.) was used in this experiment. A strain from *Drosophila simulans* homozygous for the *adh* gene was obtained as follows. An original wild population of *D. simulans* caught in El Prat de Llobregat (near Barcelona, Spain) had been kept in the laboratory for 8 years. A single female from this population was crossed with a male, and a female descendant was back-crossed with the original parent. Six successive inbreeding generations were raised with the progeny of that cross. The same crosses were performed with flies from a *Drosophila virilis* population sent to us by Dr. S.

Ohba from Tokyo (Japan). The *adh* gene appears to be monomorphic in all the populations studied of *D. simulans* and *D. virilis*.

The flies from these strains were grown continuously in large Perspex [poly(methyl methacrylate)] cages (60 cm × 60 cm × 75 cm) fitted with muslin tops. The usual corn-meal/agar medium provided in trays was used to feed the flies. Adults were harvested with a modified vacuum cleaner. The flies were stored frozen at –15°C until required.

Acrylamide and *NN'*-methylenebisacrylamide were from Eastman Kodak Chemical Co. (Rochester, NY, U.S.A.). Bio-Gel HTP hydroxyapatite was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). CM-Sepharose CL-6B, Blue Sepharose and Sephacryl were from Pharmacia Fine Chemicals (Uppsala, Sweden). LKB Produkter (Bromma, Sweden) provided Ampholines, pH 3.5–10, 9.0–11, 4.0–6.0 and 5.0–8.0. Bovine serum albumin, ovalbumin, horse myoglobin, horse cytochrome *c* and NAD⁺ were from Serva Feinbiochemica (Heidelberg, Germany). Carbonic anhydrase from bovine erythrocytes was from Sigma (London) Chemical Co. (Poole, Dorset, U.K.) and horse liver alcohol dehydrogenase from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). All other laboratory reagents and biochemicals were from Merck (Darmstadt, Germany).

Spectrophotometric measurements

All spectrophotometric measurements were made on a Beckman DB-G recording spectrophotometer.

Alcohol dehydrogenase activity was quantified by

measuring the rate of increase in absorbance at 340nm. One unit of activity reduces $1\mu\text{mol}$ of NAD^+ /min at 25°C . In the standard assay 0.15ml of propan-2-ol [6.5% (v/v) in buffer] and 0.15ml of NAD^+ (10mg/ml in buffer) were added to 0.75ml of 10mM-sodium phosphate buffer, pH8.0. The reaction was initiated by the addition of 0.15ml of enzyme solution and the initial velocity was recorded.

U.v. spectra of the purified enzyme were recorded after calibration of the spectrophotometer with a holmium filter. The protein concentration was determined by amino acid analysis.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in a thin-layer system with a modified discontinuous system of King & Laemmli (1971). The concentrations of acrylamide and NN' -methylenebisacrylamide in the upper gel were 3 and 0.08% respectively. Sodium dodecyl sulphate was at 0.1% concentration. The lower gel contained 12.5% acrylamide, 0.33% NN' -methylenebisacrylamide and 0.1% sodium dodecyl sulphate. The buffer was the same as that used by King & Laemmli (1971). Gels were first fixed in

methanol/acetic acid/water (5:1:5, by vol.) before staining in 0.1% (w/v) Coomassie Blue. The destaining solution was acetic acid/methanol/water (7:5:88, by vol.). The fixation, staining and destaining of the gel were performed at 37°C .

Electrofocusing

Analytical thin-layer gel electrofocusing in polyacrylamide gels was performed as described by Karlsson *et al.* (1973), the only difference being that the gel used was 0.8 mm thick.

Determination of protein concentration

Protein concentration in all the purification steps was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. In the final enzyme preparations it was also determined by amino acid analysis. The samples were hydrolysed for 24h and analysed on a Beckman 119C automatic analyser.

Results

Purification rationale

The purification procedures for the three enzymes from different *Drosophila* species differ slightly

Fig. 1. Thin-layer gel electrofocusing in polyacrylamide gel of alcohol dehydrogenase of *D. melanogaster*, *D. simulans* and *D. virilis*

The gels were prepared and electrophoresed as described by Karlsson *et al.* (1973). (a), (b), (g) and (h) are samples from *D. melanogaster*; (c), (d), (i) and (j) are samples from *D. simulans*; (e), (f), (k) and (l) are samples from *D. virilis*. From (a) to (f) the gel was incubated for alcohol dehydrogenase activity and from (g) to (l) the gel was fixed and stained as described by Billcliffe & Arbuthnott (1974) for the detection of protein bands. (a), (c), (e), (g), (i) and (k) are crude homogenates; (b), (d), (f), (h), (j) and (l) are samples from the final preparation.

mainly because of differences in the isoelectric points of the three enzymes; these indicate the pH values to be used in the ion-exchange chromatography.

Fig. 1 illustrates the pI values in the crude and pure preparations of the three species. The regression line of pH value versus distance (cm) is $4.55 = y - 0.49x$ and the correlation coefficient $r = 0.99$. The isoelectric points of alcohol dehydrogenase are 8.28, 8.01 and 7.63 for *D. simulans* enzyme, 7.56, 7.03 and 6.67 for *D. melanogaster* enzyme and 8.01, 7.56 and 7.43 for *D. virilis* enzyme. The first value corresponds to the most intense band and the one that appears in the pure preparations.

Purification of alcohol dehydrogenase from Drosophila

The purification of the alcohol dehydrogenase from *D. simulans* is reported in detail, and the

modifications required for the enzymes from the other two species are given below.

Frozen flies (100g wet wt.) were blended with 230ml of 2mM-sodium phosphate buffer, pH 7.0, containing 1.1% (v/v) propan-2-ol and 0.2% (v/v) 2-mercaptoethanol. The blending was performed in three 1 min periods with 1 min intervals. The slurry was centrifuged at 23 000g for 30 min at 4°C, and the supernatant was filtered through glass-wool to eliminate fatty deposits. Next 14 ml of 2% (w/v) salmine sulphate was added per 100ml of supernatant. The precipitate was removed by centrifugation at 23 000g for 30 min. The supernatant was fractionated with $(\text{NH}_4)_2\text{SO}_4$. The solution was first adjusted to 35% $(\text{NH}_4)_2\text{SO}_4$ saturation. After centrifugation at 23 000g for 30 min the precipitate was discarded. The supernatant was then adjusted to 70% $(\text{NH}_4)_2\text{SO}_4$ saturation and the solution centrifuged at 23 000g for 30 min. The precipitate was

Table 1. *Purification data on the isolation of alcohol dehydrogenase from D. melanogaster adh^S, D. simulans and D. virilis*

The purification procedure was performed as described in the text. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. In the different steps the data in the first row are for the preparation of *D. simulans*, the second for *D. melanogaster* and the third for *D. virilis*

	Volume (ml)	Alcohol dehydrogenase activity (units/ml)	Total alcohol dehydrogenase activity (units)	Yield (%)	Total protein (mg)	Specific alcohol dehydrogenase activity (units/mg)	Purification factor
Dry wt. (% of wet. wt.)	23.7 25.3 27.4						
Alcohol dehydrogenase activity (units/g dry wt.)	23.4 33.7 26.5						
After salmine sulphate	240.0 291.0 248.0	2.3 3.0 2.9	554.0 874.0 725.6	— — —	1752.0 2647.0 2329.0	0.3 0.3 0.3	— — —
After first Sepharose	58.0 73.0 110.0	6.4 6.9 3.7	369.0 506.0 406.6	66.6 58.0 56.0	152.0 175.0 219.0	2.4 2.9 1.8	7.8 8.8 6.0
After Blue Sepharose	28.5 31.5 35.0	10.1 13.0 10.0	289.0 412.0 355.7	52.0 47.0 49.0	19.8 36.2 33.4	14.6 11.4 10.7	47.0 34.5 34.4
After Sephacryl	17.5 26.0 25.0	10.8 11.6 12.2	189.0 302.6 305.0	34.0 34.5 42.0	13.5 27.6 28.7	14.0 11.0 10.6	45.0 33.5 34.0
By amino acid analysis					8.1 13.3 17.3	23.4 22.7 17.6	
After Sephacryl when HTP hydroxyapatite is used	26.1 23.1 32.4	4.6 7.3 7.2	120.0 169.0 234.0	33.4 34.0 40.5	14.0 21.2 37.4	8.6 7.9 6.3	32.4 31.8 23.3
By amino acid analysis					8.6 10.2 22.4	13.9 16.6 10.4	

dissolved in 20 ml of 2 mM-sodium phosphate buffer, pH 7.0, and passed through a column (50 cm $\times$ 5 cm diam.) of Sephadex G-25 (medium grade) to lower the salt concentration of the enzyme solution. The fractions that showed alcohol dehydrogenase activity were pooled and passed through a column (8.5 cm $\times$ 2.5 cm diam.) of CM-Sephacryl CL-6B equilibrated with 10 mM-sodium phosphate buffer, pH 7.0. The column was washed with 2 bed volumes of buffer and eluted with a 0–1 M-NaCl gradient in buffer. The eluate fractions with activity were pooled and dialysed against five 2-litre volumes of 2 mM-sodium phosphate buffer, pH 7.5, to lower the salt concentration of the solution.

The dialysed preparation was passed through another column of CM-Sephacryl CL-6B, the difference being that the pH had been raised to pH 7.5. The fractions with alcohol dehydrogenase activity were pooled and dialysed first against two 2-litre volumes of 2 mM-Tris/HCl buffer, pH 7.5, and then against three 2-litre volumes of 20 mM-Tris/HCl buffer, pH 7.5. The dialysed preparation was passed through a column (7.5 cm $\times$ 2.5 cm diam.) of Blue Sepharose previously equilibrated with 20 mM-Tris/HCl buffer, pH 7.5. The sample was pumped into the column at 20 ml/h. The column was washed with 150 ml of 20 mM-Tris/HCl buffer, pH 7.5, and eluted with 1 mM-NAD⁺ in the same buffer at 12 ml/h. The fractions that showed alcohol dehydrogenase activity were pooled and precipitated by 70% saturation with (NH₄)₂SO₄, the slurry was centrifuged at

23 000 g for 30 min and the precipitate was re-dissolved in 0.2 M-Tris/HCl buffer, pH 8.6. Final purification was achieved by gel-permeation chromatography on a column (90 cm $\times$ 1.5 cm diam.) of Sephacryl. The fractions with activity were pooled, dialysed against double-distilled water and freeze-dried when required.

During purification of alcohol dehydrogenase from *D. virilis* the initial blending buffer as well as those for the Sephadex G-25 step and the first CM-Sephacryl CL-6B step were at pH 6.5. The second CM-Sephacryl CL-6B step was run at pH 7.0. All the other steps were identical.

For *D. melanogaster* the initial blending buffer as well as those for the Sephadex G-25 step and the first CM-Sephacryl CL-6B step had pH 6.0. The second CM-Sephacryl CL-6B step was run at pH 6.5. Also all the other steps were identical.

The results obtained in the three experiments are presented in Table 1 for *D. simulans*, *D. virilis* and *D. melanogaster*. Fig. 2 illustrates the elution profiles of the first and second CM-Sephacryl CL-6B steps for *D. simulans*. Fig. 3 illustrates the elution profile of the Blue Sepharose step for the three enzymes. Fig. 4 shows the pattern of the different purification steps and the purified preparation in polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate for *D. virilis*. The purified preparations, although at a high concentration (50 μ g) in all the gels, appear to be free from any other contaminant. This technique allows us also to

Fig. 2. Elution profile from the first (a) and the second (b) CM-Sephacryl CL-6B steps for alcohol dehydrogenase of *D. simulans*

Protein (% transmittance at 280 nm) (—) and enzyme activity (O—O) were determined for each fraction. ----, Conductivity. Experimental details are given in the text. The bar under the curve indicates the volume with alcohol dehydrogenase activity used in the next step.

Fig. 3. Elution profile from the Blue Sepharose CL-6B step for alcohol dehydrogenase of *D. simulans*, *D. virilis* and *D. melanogaster*

Experimental details are given in the text. ●, *D. simulans* enzyme; ○, *D. virilis* enzyme; ■, *D. melanogaster* enzyme. ----, NAD^+ concentration (% transmittance at 280nm).

Fig. 4. Sodium dodecyl sulphate/polyacrylamide-gel plate electrophoresis of alcohol dehydrogenase of *D. virilis*

The gel was prepared, electrophoresed and stained as described in the text. (a) and (g) are the standard proteins: bovine serum albumin (mol.wt. 68 750), ovalbumin (mol.wt. 45 000), carbonic anhydrase (mol.wt. 31 000), myoglobin (mol.wt. 17 800) and cytochrome *c* (mol.wt. 12 300). (b) After the salmine sulphate step; (c) after the first CM-Sepharose CL-6B step; (d) after the second CM-Sepharose CL-6B step; (e) after the Blue Sepharose CL-6B step; (f) after the Sephacryl step. The subunit molecular weight of alcohol dehydrogenase is 27 800.

calculate the subunit molecular weight of the pure enzymes. The regression line of the plot of molecular weight of standards versus migration distance (cm) is $1.98 = y + 0.106x$ and the correlation coefficient is $r = 0.98$. The subunit molecular weight of alcohol dehydrogenase for the three species is 27 800.

An alternative purification procedure was also devised. It involves a column (11 cm $\times$ 2.5 cm diam.) of Bio-Gel HTP hydroxyapatite instead of the first CM-Sepharose CL-6B run in the previous experiment. The Bio-Gel HTP hydroxyapatite was equilibrated with 10 mM-sodium phosphate buffer, pH 6.5 for *D. virilis*, pH 7.0 for *D. simulans* and pH 6.0 for *D. melanogaster*. The column was washed with 1 bed volume of buffer and eluted with a 0.01–0.3 M-sodium phosphate gradient. The fractions with activity were pooled and precipitated by 70% saturation with $(\text{NH}_4)_2\text{SO}_4$. A Sephadex G-25 column was used to lower the salt concentration of the sample, which was then passed through a column of CM-Sepharose CL-6B. The sodium dodecyl sulphate/polyacrylamide-gel electrophoresis during the purification of the alcohol dehydrogenase from the three species shows that any contaminant has been removed and the only band in the gel corresponds to the alcohol dehydrogenase enzyme.

The dry weight of 100 g frozen samples taken for each species differs considerably. In terms of total alcohol dehydrogenase activity per mg dry wt. the species with the highest activity is *D. melanogaster* and the lowest value is for *D. simulans*. The quantity of alcohol dehydrogenase (mg) per g dry wt. also varies with the species. *D. virilis* and *D. melanogaster* have comparable amounts, whereas *D. simulans* appears to have approx. 40% less enzyme when the yield for all three preparations is corrected to what it would have been if the 42% yield achieved with *D. virilis* had been obtained for all three. We repeatedly get the same values after a minimum of three purifications for each species. Immunogenetic experiments to quantify the alcohol dehydrogenase in *D. melanogaster* have been reported (Day *et al.*, 1974). We consider that during comparison of these species the purification data could also give an indication of the different amounts of enzyme synthesized.

U.v. spectra

The u.v. spectra of the three enzymes were recorded and the protein concentrations were calculated by amino acid analysis. The ϵ_{280} values calculated for those enzymes are given in Table 2.

Stability

Fig. 5 illustrates the stability of the alcohol dehydrogenase enzymes from the three species in the pure preparations. The alcohol dehydrogenase pre-

Table 2. Molar absorption coefficients of pure alcohol dehydrogenase from *D. melanogaster adh^S*, *D. simulans* and *D. virilis*

The molecular weight was calculated as described in the text.

	ϵ_{280} (litre $\cdot$ mol ⁻¹ $\cdot$ cm ⁻¹)
<i>D. melanogaster adh^S</i>	4.8×10^4
<i>D. melanogaster adh^{UF}</i>	$3.6 \times 10^{4*}$
<i>D. simulans</i>	5.4×10^4
<i>D. virilis</i>	4.0×10^4

* Determined by Thatcher (1977).

Fig. 5. Stability of alcohol dehydrogenase in purified extracts of *D. simulans*, *D. virilis* and *D. melanogaster* ●, *D. simulans* enzyme; ○, *D. virilis* enzyme; ■, *D. melanogaster* enzyme. The enzyme was purified as described in the text and was kept in solution (20 mM-Tris/HCl buffer, pH 8.6) at 4°C. The percentage of activity remaining was calculated at the various times indicated.

paration from *D. virilis* keeps 100% of the initial activity even after 3 months. The enzyme from *D. simulans* shows an increase of activity at the beginning and reaches a value of 86% after 3 months. The *D. melanogaster* enzyme shows a more rapid decrease in activity, although it retains more than 50% of the initial activity after 3 months.

Discussion

The purification procedure devised for alcohol dehydrogenase of three *Drosophila* species produces a very stable enzyme preparation and in good yield. It has been reported that this enzyme is very susceptible to proteolytic degradation (Jacobson & Pfuderer, 1970; Thatcher, 1977), and in our cases the pure preparations of alcohol dehydrogenase must be practically free from proteinases and peptidases. The sodium dodecyl sulphate-containing and the electrofocusing polyacrylamide gels as well as the N- and C-termini of the pure alcohol dehydrogenases and the amino acid analysis (E. Juan & R. González-Duarte, unpublished work) confirm that the contaminants, if any, must be at a very low concentration. Alcohol dehydrogenase enzymes differ in their stability in the pure preparations. The *D. virilis* enzyme resists inactivation more than the other two. The enzyme form *D. melanogaster* is the least stable, and differences among the different alleles could well exist. Thatcher (1977) reports that a pure preparation of the *adh^{UF}* alcohol dehydrogenase alloenzyme has a half-life of 200 h at 0°C. It is surprising that the difference in stability appears to be much greater between two alloenzymes than among enzymes of different species.

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