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Purification of Alcohol Dehydrogenase from *Drosophila* by General-Ligand Affinity Chromatography

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A method for the purification of alcohol dehydrogenase from *Drosophila melanogaster* is described. The method makes use of 8-(6-aminohexyl)amino-5′-AMP, immobilized on Sepharose 4B, as an affinity ligand. Since alcohol dehydrogenase from *Drosophila* shows weak affinity for this column, a novel technique was developed to separate alcohol dehydrogenase from both unbound proteins and more strongly bound enzymes. The purification procedure is simple to operate and gives a homogeneous preparation in good yield after only three steps.

General-ligand affinity chromatography (Mosbach *et al.*, 1972) has been used extensively in recent years as a highly efficient method of purification for many kinases and dehydrogenases (Andersson *et al.*, 1975; Lee *et al.*, 1977, 1978). One of the best studied enzymes in *Drosophila* biochemistry is alcohol dehydrogenase (EC 1.1.1.1) (Day *et al.*, 1974; Vigue & Johnson, 1973). There is as yet no purification method published that uses an affinity technique, although there are several classical schemes available (Sofer & Ursprung, 1968; Schwartz *et al.*, 1975; Elliot & Knopp, 1975). We have developed a method for the purification of alcohol dehydrogenase from *Drosophila melanogaster* that uses a general-ligand affinity column. This method may have considerable utility, because it employs the stable derivative 8-(6-aminohexyl)amino-5′-AMP (8-hexyl-AMP) as an affinity ligand (see Lee *et al.*, 1974).

Experimental

Materials

Drosophila melanogaster adults of a strain which carries the recessive markers *cn bw*; *ri e* were obtained from Dr. Y. Hiraizumi at the University of Texas, Austin, TX, U.S.A. This strain carries a fast allele at the *Adh* locus (Johnson & Denniston, 1964). The flies were grown in mass culture, harvested with a small vacuum cleaner, and frozen at −70°C until required. Dithiothreitol was obtained from Sigma (St. Louis, MO, U.S.A.), NAD^+ from Boehringer Mannheim (New York, NY, U.S.A.) and all other chemicals were from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

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Methods

Assays. Alcohol dehydrogenase activity was assayed in 0.1M-Tris base (pH9.8) which contained 1mM-NAD^+ and 5% (w/v) propan-2-ol at 25°C. One unit of enzyme activity was defined as that amount of alcohol dehydrogenase which caused the reduction of 1μmol of NAD^+/min under these conditions. Protein measurements were made by the method of Lowry *et al.* (1951), against a bovine serum albumin standard.

Electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out by using the adaptations by Studier (1973) of the method of Laemmli (1970). Gels were fixed in 45% (v/v) methanol/10% (v/v) acetic acid overnight, stained in the same solution containing 0.025% Coomassie Blue R and destained in 25% methanol/7.5% acetic acid.

Extract preparation. Crude homogenate was prepared by homogenizing frozen flies in approx. 5vol. (v/w) of 10mM-potassium phosphate buffer, pH6.5, which contained 1mM-dithiothreitol, by using a Teflon pestle. The homogenate was centrifuged at 27000*g* for 20min and the supernatant was filtered through glass wool. All procedures were carried out at 4°C.

Preparation of the affinity column was described elsewhere (Lee *et al.*, 1974). The column was equilibrated with 10mM-phosphate buffer which contained 1mM-dithiothreitol and regenerated with 2 column volumes of 6M-urea which contained 2M-NaCl.

Results

Alcohol dehydrogenase activity is not retained when a crude fly extract is passed over an 8-hexyl-AMP–Sepharose column. Fig. 1 illustrates how washing with buffer is sufficient to elute all the enzyme activity. The activity is eluted as two peaks under these conditions, the first of which is detected at the tail of the main protein peak. The second, major, peak of alcohol dehydrogenase activity is retarded by the column. We believe this peak to be caused by enzyme which interacts weakly with the immobilized ligand and is displaced, probably by enzymes with a stronger interaction. This explanation is supported by a study of the isoenzyme patterns of the two peaks. Alcohol dehydrogenase from *D. melanogaster* migrates on native gel electrophoresis as three major bands, which are controlled by a single genetic locus (Johnson & Denniston, 1964). These bands are termed ADH_1, ADH_3 and ADH_5 (Jacobson, 1968) in order of their speed of migration towards the anode, ADH_5 being the slowest. Whereas the first peak of enzyme activity shown in Fig. 1 gave all three bands on electrophoresis, the second peak gave only band ADH_5 (see the Discussion section).

To investigate the retardation of alcohol dehydrogenase by the 8-hexyl-AMP–Sepharose column, we passed two different volumes of crude homogenate, which contained the same amount of enzyme activity and total protein, over one 40ml 8-hexyl-AMP–Sepharose column. Both samples were eluted with buffer alone. Retardation of alcohol dehydrogenase activity is increased when sample volume is decreased (Fig. 1). We have applied these findings to the purification of *Drosophila* alcohol dehydrogenase on this affinity column.

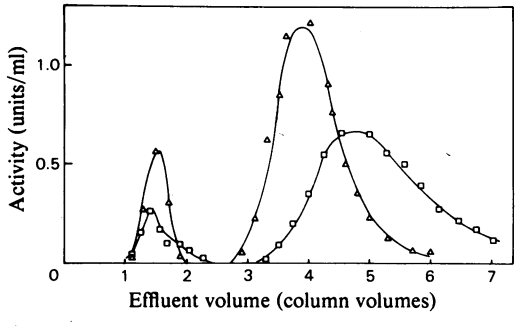

Fig. 1. *Elution profiles from an 8-(6-aminohexyl)amino-5′-AMP–Sepharose column on washing with buffer alone*
Crude homogenate from 10g of flies was prepared as described in the text. The extract from the $(NH_4)_2SO_4$ precipitation was divided into two equal parts, one of which was concentrated by ultrafiltration to a volume of 1ml and the other was diluted to 5ml. Both samples were applied to a 40ml 8-hexyl-AMP–Sepharose column and eluted with 10mM-phosphate buffer. Fractions were collected and assayed as described in the text. A total of 84units of alcohol dehydrogenase activity was applied in the 5ml volume, and 88units in the 1ml sample. Over 90% of the total initial activity could be accounted for after elution in each case. Alcohol dehydrogenase activity: △, 5ml input; □, 1ml input.

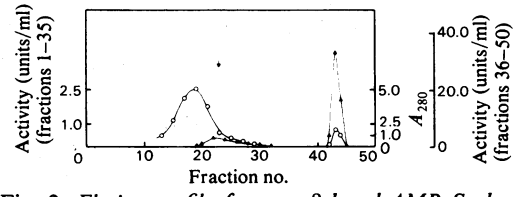

Fig. 2. *Elution profile from an 8-hexyl-AMP–Sepharose column by using the specific inhibitor NAD^+/pyrazole*
For details see the text. ○, Protein, measured by A_{280}; ▲, alcohol dehydrogenase activity. Arrow indicates start of elution with NAD^+/pyrazole. Note the change in activity scale after fraction 35.

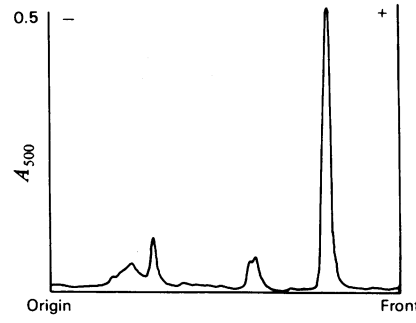

Fig. 3. *Gel scan of alcohol dehydrogenase peak fractions after elution from an 8-(6-aminohexyl)amino-5′-AMP–Sepharose column*
A sample (100μl) of the pooled peak fractions eluted from the affinity column was prepared and electrophoresed in a sodium dodecyl sulphate/polyacrylamide gel by the method of Laemmli (1970), as described in the text. The gel was scanned with a Gelman integrating scanner after staining, and the major band represents 68% of the total protein.

Purification

In a standard experiment, 5g of adult flies was homogenized in 30ml of buffer. The crude homogenate was adjusted to 40% saturation with $(NH_4)_2SO_4$ and equilibrated. The slurry was centrifuged at 27000*g* for 20min and the precipitate discarded. Then the supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$, equilibrated and centrifuged as before. The supernatant was discarded.

Table 1. *Data on the purification of Drosophila melanogaster alcohol dehydrogenase by affinity chromatography*
Details of methods are given in the text. Protein was assayed by the method of Lowry *et al.* (1951), against a bovine serum albumin standard.

Stage	Activity (units/ml)	Total activity (units)	Protein concn. (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Purity (%)	Yield (%)
Crude	19.0	380	14.0	280	1.35	0		
(1) (*a*) $(NH_4)_2SO_4$ precipitation	65.0	155	40.0	100	1.63			
(*b*) Concentration by ultrafiltration	122.4	97.9	116.0	92.8	1.05		10	25.7
(2) 8-(6-Aminohexyl)amino-5′-AMP–Sepharose	21.4	90.0	0.68	2.86	31.5	23.3	68	23.6
(3) DEAE-Sepharose column	4.8	60	0.138	1.73	34.7	25.7	Homogeneous	16

The precipitate was dissolved in the minimum quantity of buffer (2–3ml) and dialysed overnight against 1 litre of buffer.

After dialysis, the extract was concentrated to approx. 1ml by ultrafiltration. The concentrated extract was then applied to the top of an 8-hexyl-AMP–Sepharose column (1.5cm×28cm) which had been equilibrated previously with several column volumes of 10mM-phosphate buffer, pH6.5. Care was taken to avoid any dilution of the sample. The column was washed with buffer to elute the main protein peak. Fractions (2.1ml) were collected and assayed for alcohol dehydrogenase activity. When the minor peak of activity was detected, elution of the retarded enzyme was initiated by the addition of 0.2mM-NAD^+ and 5mM-pyrazole (Theorell & Yonetani, 1963) in 1 column volume of phosphate buffer. The elution profile shown in Fig. 2 and sodium dodecyl sulphate/polyacrylamide-gel pattern shown in Fig. 3 demonstrated that the enzyme was substantially purified by this procedure. The purity of the pooled peak fractions was estimated to be 68% on a Gelman gel scanner. Subsequent passage of these pooled fractions over a DEAE-Sepharose column pre-equilibrated at pH8.5 with 10mM-Tris/HCl was sufficient to give a homogeneous preparation after elution with a gradient of 0.01M-NaCl in the same buffer. The alcohol dehydrogenase had a specific activity of 34units/mg. Complete data on the purification are given in Table 1.

This method has been used to purify 800units of alcohol dehydrogenase from 30g of adult flies with 320ml of 8-hexyl-AMP–Sepharose. It has proved to be very suitable for large-scale preparative work.

Discussion

The technique used to purify *Drosophila* alcohol dehydrogenase represents an important application of the concept of 'progressively perpetuating effectiveness' (Cuatrecasas, 1972) to general-ligand affinity chromatography. With this and similar techniques, it is now possible to purify, in high yield, many more enzymes which show only weak affinity for immobilized ligands (Lee *et al.*, 1978). By concentrating the extract we have obtained sufficient retardation of the enzyme on the 8-hexyl-AMP–Sepharose column to separate it from unbound protein (Fig. 2). In order to avoid contamination of the enzyme with other dehydrogenases NAD^+/pyrazole, a specific inhibitor (Theorell & Yonetani, 1963), is used for elution. Preliminary studies indicate that the apparent K_i of *Drosophila* alcohol dehydrogenase for pyrazole is of the order of 3μM (A. J. Leigh Brown, unpublished work). The use of the inhibitor permits elution of all of the retarded alcohol dehydrogenase activity in one or two fractions, which results in a substantial purification without the need for extensive washing of the column (Table 1).

One possible area for improvement in our technique lies in the use of $(NH_4)_2SO_4$ precipitation to concentrate the crude homogenate. There is a considerable loss of activity at this step. Subsequently there is only an 8% loss on passage over the 8-hexyl-AMP–Sepharose column and a 32% loss in DEAE-Sepharose chromatography.

When alcohol dehydrogenase was eluted from the 8-hexyl-AMP–Sepharose column by washing with buffer, the isoenzyme pattern changed. Several studies have shown that alterations in the isoenzyme pattern result from an interaction between the enzyme and cellular metabolites, in particular NAD^+ (Jacobson, 1968; Jacobson *et al.*, 1972; Schwartz *et al.*, 1975). The most recent evidence suggests that the more anodally migrating isoenzymes contain one or more molecules of an NAD^+–carbonyl complex (Schwartz *et al.*, 1975). This explanation is consistent with our findings, which suggests that when an enzyme molecule interacts with the immobilized ligand such bound complexes must be displaced.

Classical methods for purification of *Drosophila* alcohol dehydrogenase have used lengthy procedures and give low yields (Sofer & Ursprung, 1968;

Elliot & Knopp, 1975; Schwartz *et al.*, 1975). It has been reported that such methods give a preparation which is contaminated with a proteinase which causes instability (Thatcher, 1977). Affinity chromatography appears to be a simple way to separate alcohol dehydrogenase from proteinase activity, since our preparation is stable indefinitely at 4°C. It is probable that the double-ternary-complex affinity method of Lange & Vallee (1976) would also be applicable to the *Drosophila* enzyme. Our general-ligand technique has certain advantages, however, especially with regard to the commercial availability and multiplicity of applications of the affinity ligand.

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