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Enzyme Instability and Proteolysis during the Purification of an Alcohol Dehydrogenase from *Drosophila melanogaster*

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The alcohol dehydrogenase of the *Drosophila melanogaster* adh^{UF} allele (alloenzyme with ultra-fast electrophoretic mobility) was unstable in crude or partially purified preparations. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that inactivation was probably due to proteolytic degradation, and a new method of purification of the enzyme was developed. After three steps, namely salmine sulphate precipitation, hydroxyapatite chromatography and Sephadex G-100 gel filtration, a 10-fold purified preparation was obtained. The enzyme produced was relatively stable compared with alcohol dehydrogenase purified by other methods, and was shown to be proteinase-free. The enzyme had a subunit mol.wt. of 24000 and had a single thiol residue per subunit available for titration with 5,5′-dithiobis-(2-nitrobenzoic acid). The amino acid composition and *C*-terminal amino acid sequence of the enzyme were determined. The substrate specificity of this alcohol dehydrogenase was also characterized. These results are discussed in relation to experiments on the evolutionary significance of thermostability at the *adh* locus.

The isolation of the 'ultra-fast' alcohol dehydrogenase alloenzyme of the adh^{UF} allele of *Drosophila melanogaster*, is reported. [Alcohol dehydrogenase alleles in *Drosophila* are described by the relative electrophoretic mobility of the alloenzymes at pH 8.0: fast (adh^{F}); slow (adh^{S}); ultra-fast (adh^{UF}) (Johnson & Dennison, 1964).] The enzyme was found to be particularly susceptible to proteolytic degradation, which was implicated as a major cause of instability in crude and partially purified preparations. It is therefore proposed that the thermostability of *Drosophila* alcohol dehydrogenases is not a simple or characteristic property of the gene, but is dependent on an interaction with the products of other structural genes.

Experimental

Materials

An isogenic strain of *Drosophila melanogaster* with the adh^{UF} allele was constructed by Dr. D. A. Briscoe (Department of Genetics, University of Edinburgh, Edinburgh, Scotland, U.K.) and Dr. R. Gonsalez (Department of Genetics, University of Barcelona, Barcelona, Spain) from a single female of a naturally occurring *Drosophila* population from La Mancha, Spain (J. M. Malpica & D. A. Briscoe, unpublished work). The flies were grown continuously in three Perspex cabinets (50cm×150cm), fitted with muslin tops. A modified vacuum cleaner was used to harvest the flies and a corn-meal molasses-based food was provided in 36cm×24cm trays. Flies were stored frozen at −15°C until required.

Acrylamide and *NN′*-methylenebisacrylamide were from Eastman Kodak Chemical Co., Rochester, NY 14650, U.S.A., and Bio-Gel HTP hydroxyapatite (batch no. 7288) was from Calbiochem, Hereford HR4 9BQ, U.K. Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, U.K., provided 5,5′-dithiobis-(2-nitrobenzoic acid), *N*-benzoyl-DL-arginine *p*-nitroanilide and phenylmethanesulphonyl fluoride. Mercaptoethanesulphonic acid was from Pierce Chemical Co., Rockford, IL, U.S.A. Carboxypeptidase A (batch no. 487-5) was from P–L Biochemicals, Milwaukee, WI, U.S.A. All other laboratory reagents and biochemicals were from BDH, Poole, Dorset BH12 4NN, U.K.

Methods

Spectrophotometric measurements. All spectrophotometric measurements were made on a SP. 1800 recording spectrophotometer (Pye-Unicam, Cambridge, U.K.) fitted with a SP. 876 temperature programme controller set at 30°C.

Alcohol dehydrogenase activities were quantified by measuring the rate of NAD^+ reduction at 340nm. In the standard assay 1μmol of NAD^+ (dissolved in 10μl of reaction buffer) were added to 1ml of 0.1M-Tris/HCl, pH 8.7. The reaction was initiated by the addition of 10μl of enzyme solution and the initial velocity recorded. Kinetic constants were defined by using the direct linear plot of Eisenthal & Cornish-Bowden (1974). To minimize

the number of assays to be performed, apparent Michaelis parameters were determined by varying alcohol concentrations in the presence of saturating concentrations of NAD^+. In a preliminary experiment the initial velocity of the enzyme was measured at several NAD^+ concentrations between 1 and 0.0375mM, while maintaining the propan-2-ol concentration at 10mM. A direct linear plot of these results gave a value of 0.3mM for the $K_{m(app.)}^{NAD^+}$ which is similar to the value obtained by Day *et al.* (1974) for the $K_m^{NAD^+}$ of the 'fast' alloenzyme of the *adh*^{F} allele of *D. melanogaster* with ethanol as substrate. Initial-velocity measurements on a range of alcohol substrates were therefore performed in the presence of 1mM-NAD^+, where $K_{m(app)}^{alcohol}$ will approach the true value.

Proteinase activities were determined by using a modified Kunitz assay (Northrop *et al.*, 1948), and also from the release of *p*-nitroaniline (ε_{410} = 8800 litre·mol^{-1}·cm^{-1}; Arnon, 1970) from the synthetic trypsin substrate *N*-benzoyl-DL-arginine *p*-nitroanilide. In the Kunitz assay, 1g of casein was boiled for 10min with 100ml of 0.1M-Tris/HCl buffer, pH8.7, and 1ml of this solution was incubated with 0–50μl of enzyme solution for 2h at 37°C. The reaction was terminated by the addition of 0.25ml of 10% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation at 50000*g* for 10min and the A_{280} of the supernatant read against an appropriate blank. Activities are expressed in terms of the supernatant A_{280}^{1cm} units after 1min. With the synthetic substrate, proteinase activity was measured by the addition of 1μmol of *N*-benzoyl-DL-arginine *p*-nitroanilide (dissolved in 10μl of dimethyl sulphoxide) to 1ml of enzyme solution. When proteinase activities were high the reaction could be followed directly on the spectrophotometer at 410nm. In dilute solution the proteinase activity present was determined by reading the A_{410}^{1cm} of the enzyme solution, containing 1mM synthetic substrate, after 2h incubation at 37°C.

The number of thiol groups per molecule available for reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) was calculated from the release of nitrobenzoate ion (ε_{412} = 13000 litre·mol^{-1}·cm^{-1} (Ellman, 1959). In each experiment 10μl of a solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (4mg/ml) in 0.1M-Tris/HCl, pH8.0, was added to 1ml of protein solution. The reaction was performed in either 0.1M-Tris/HCl, pH8.0, or in the same buffer, also containing 4M-guanidinium chloride.

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

Electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis was performed in a slab-gel apparatus (Raven Scientific, Haverhill, Suffolk, U.K.) by using the discontinuous Tris/glycine buffer of King & Laemmli (1971). Each gel contained a linear gradient of acrylamide polymer, from 10% (w/v) at the top to 35% (w/v) at the bottom. Protein samples were denatured by heating in the King & Laemmli (1971) sodium dodecyl sulphate-containing sample buffer for 15min at 105°C. Gels were first fixed in methanol/acetic acid/water (5:1:5, by vol.) before staining in 0.1% (w/v) Coomassie Blue. Destaining was done by soaking the gel in 10% (v/v) acetic acid and by using plastic-foam test-tube plugs to scavenge excess dye.

Amino acid analysis. The amino acid composition of the protein was determined by analysis of eight acid-hydrolysed samples of the enzyme. Six samples of native enzyme were hydrolysed in 6M-HCl at 105°C for 24, 48 or 96h. A sample of oxidized alcohol dehydrogenase was hydrolysed in the same way for 24h and a sample of native enzyme was hydrolysed in 3M-mercaptoethanesulphonic acid for 96h at 105°C. After hydrolysis the sulphonic acid was neutralized with NaOH and the volume doubled with 0.2M-sodium citrate buffer, pH2.2, containing 1μmol of γ-aminobutyric acid/ml as internal standard. The sample was then applied directly to the analyser resin. All protein hydrolysates were analysed on a Beckman 120C automatic amino acid analyser. Carboxypeptidase A digests were analysed on a Bio Cal BC 500 automatic amino acid analyser.

Carboxypeptidase A digestion. A 2mg/ml solution of carboxypeptidase A was prepared as described by Ambler (1972). Portions (5, 10, 25 and 50μl) of this solution were added to 100nmol samples of alcohol dehydrogenase, each dissolved in 100μl of 0.2M-*n*-ethylmorpholine acetate buffer, pH8.5. After incubation for 1h at 37°C, the digests were dried in a desiccator. Each sample was suspended in 1ml of 0.2M-sodium citrate buffer, pH2.2, containing 2nmol of norleucine/ml as internal standard. After centrifugation the sample was applied directly to a Bio Cal BC 500 amino acid analyser.

Purification procedures. All manipulations were performed in the cold-room at 4°C. The flies were blended in a Waring Commercial Blendor and the mixture centrifuged in a 6×250ml rotor in a MSE High-Speed 18 centrifuge. A Radiometer CDM 2E conductivity meter (calibrated with 0.1M-HCl) was used to measure the conductivity of enzyme solutions. Unless otherwise stated the buffer used during the purification was 0.1M-Tris/HCl, pH8.7.

Results

Stability

The thermostability of crude enzyme extracts was investigated by measuring the rate of loss of enzymic activity at various temperatures. Frozen flies (5g)

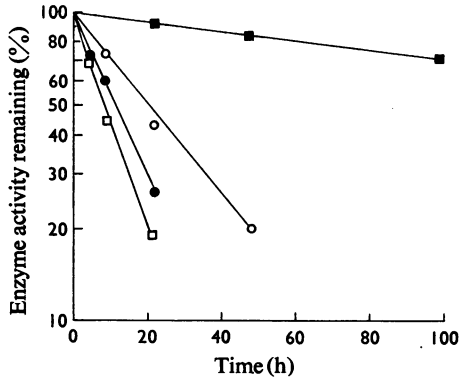


Fig. 1. *Stability of alcohol dehydrogenase in crude and partially purified extracts*

The enzyme was prepared as detailed in the text and incubated at 0 (■), 20 (○) and 30°C (●). The enzyme was also partially purified by $(NH_4)_2SO_4$ fractionation, Sephadex G-100 gel filtration and quaternary aminoethyl-Sephadex ion-exchange chromatography [essentially the method of Schwartz *et al.* (1975)] and incubated at 0°C (□). The percentage of activity remaining was then calculated at the various times.

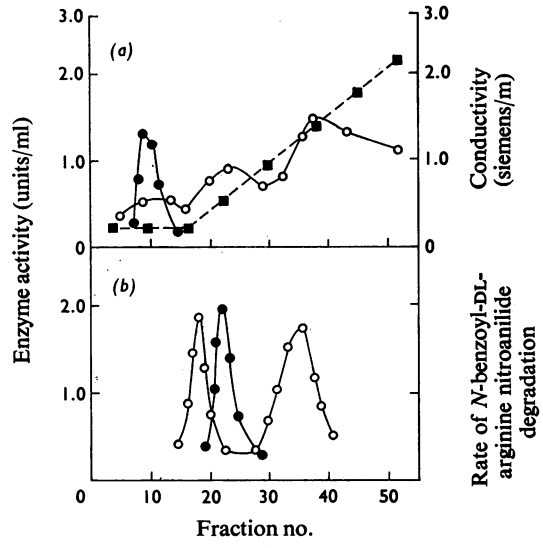


Fig. 2. *Separation of peptidase activity from alcohol dehydrogenase*

(*a*) Result of hydroxyapatite chromatography (details are given in the text); (*b*) Sephadex G-75 gel filtration of a crude fly homogenate. Alcohol dehydrogenase activity (●), rate of *N*-benzoyl-DL-arginine nitroanilide degradation (○) and conductivity (■) were measured. Fractions (5ml) were collected.

were homogenized with 10ml of 0.1M-Tris/HCl, pH8.7, containing 0.01% (v/v) 2-mercaptoethanol, in a 30ml glass tissue grinder. The slurry was then centrifuged at 30000*g* in a microcentrifuge. Portions (0.5ml) of the supernatant were stored at 0, 20 and 30°C. The enzymic activity of each sample was then determined at regular time-intervals. Inactivation displayed first-order kinetics (Fig. 1) and the rate of inactivation was unaffected by the presence of 1mM-phenylmethanesulphonyl fluoride, a serine proteinase inhibitor.

Purification rationale

Purification of the enzyme from flies carrying the adh^{UF} allele by published methods (Sofer & Ursprung, 1968; Schwartz *et al.*, 1975) produced preparations, which, although homogeneous by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis, were highly unstable (Fig. 1). In the worst preparations the half-life of the enzyme was about 4h at 4°C. This loss of enzymic activity was correlated with the disappearance of the alcohol dehydrogenase band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 1), suggesting that inactivation was largely due to degradation by an unknown *Drosophila* proteinase. In another case the enzyme preparation was apparently stable at 0°C (half-life 40h), (Thatcher *et al.*, 1975), but subsequent tryptic digestion of the enzyme released several peptides which lacked *C*-terminal arginine or lysine residues (D. R. Thatcher, unpublished work), again suggesting that the original enzyme preparation was contaminated by a proteinase.

Separation of proteinase and peptidase activity from the dehydrogenase was attempted in small-scale experiments with crude *Drosophila* extracts. Flies (10g) were homogenized as described in the experiments on thermostability (see under 'Stability') and the crude supernatant (12ml) was divided into two portions. One portion was made 60% saturated with respect to $(NH_4)_2SO_4$ and the precipitate removed by centrifugation at 23000*g* for 30min. The precipitate was then redissolved in Tris/HCl buffer and applied to a column (100cm × 2.5cm diam.) of Sephadex G-100. The other portion of the crude enzyme solution was dialysed against 0.02M-Tris/HCl, pH8.7, for 24h. The dialysed enzyme was then applied to a column (10cm × 2.5cm diam.) of hydroxyapatite (Bio-Gel HTP) and eluted with a linear gradient of 0–0.5M-sodium phosphate buffer, pH8.0. The elution profile obtained in this experiment and the profile obtained on elution of the Sephadex G-100 column are shown in Fig. 2. In both cases considerable separation of the dehydrogenase activity from the contaminating proteinases was achieved.

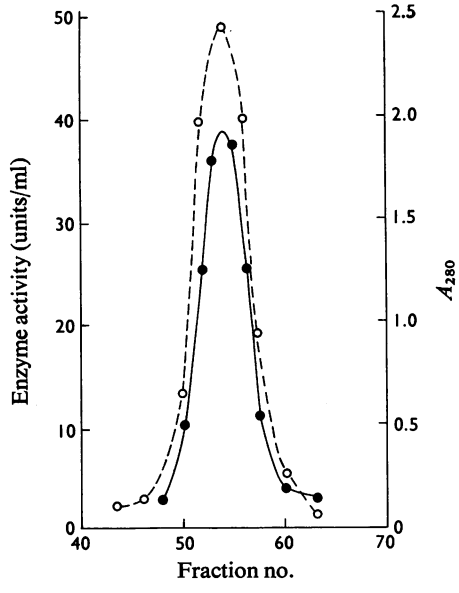


Fig. 3. *Final Sephadex G-100 gel filtration*
Enzyme activity (●) and protein (A_{280}) (○) were determined for each fraction. Fractions (12ml) were collected.

Large-scale purification of the enzyme

Frozen flies (442g) were blended with 800ml of 0.1M-Tris/HCl, pH8.7, containing 0.01% (v/v) 2-mercaptoethanol. After blending for three periods of 1min, interspersed with 2min periods during which the blender was cooled in ice, the slurry was clarified by centrifugation at 23000*g* for 30min. The supernatant was filtered through glass wool to remove fatty deposits and then treated with 100ml of 10% (w/v) salmine sulphate. A heavy precipitate formed, which was removed by centrifugation at 23000*g* for 30min. The supernatant was dialysed against 3×3 litres of water, lowering the conductivity of the enzyme solution from 0.82 to 0.1 siemens/m. A column (20cm×5cm diam.) of the hydroxyapatite (freshly soaked Bio-Gel HTP) was equilibrated with 0.05M-Tris/HCl, pH8.7, and the dialysed enzyme solution passed through. The column was then washed with 2 bed volumes of buffer and the eluate fractions were pooled. The enzyme was concentrated by precipitation with $(NH_4)_2SO_4$; by raising the $(NH_4)_2SO_4$ content of the enzyme solution to 60% saturation, a dense white precipitate formed, which was collected by centrifugation. After the precipitated enzyme had been redissolved in 20ml of 0.1M-Tris/HCl, pH8.7, final purification was achieved by gel-permeation chromatography on a column (100cm×4cm diam.) of Sephadex G-100 (Fig. 3).

Purification data obtained during this experiment are presented in Table 1, and Plate 1 shows the results of gradient polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate at each stage. The purified preparation was homogeneous by this criterion and appeared to be free of any contaminating proteinases. The enzyme could be stored at 25°C for 5h without the formation of any low-molecular-weight protein bands, and the half-life of the purified material was increased to 200h at 0°C (in the presence of 0.1M-Tris/HCl, pH8.7, containing 0.01% 2-mercaptoethanol).

Characterization of the purified enzyme

Chemical properties. The enzyme had a mol.wt. of 24000 as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 1) and its amino acid composition has been determined (Table 2). The amino acid composition obtained is largely in agreement with the results of Schwartz *et al.* (1975) except that values for glycine and phenylalanine were lower and four tryptophan residues per subunit were calculated and not two.

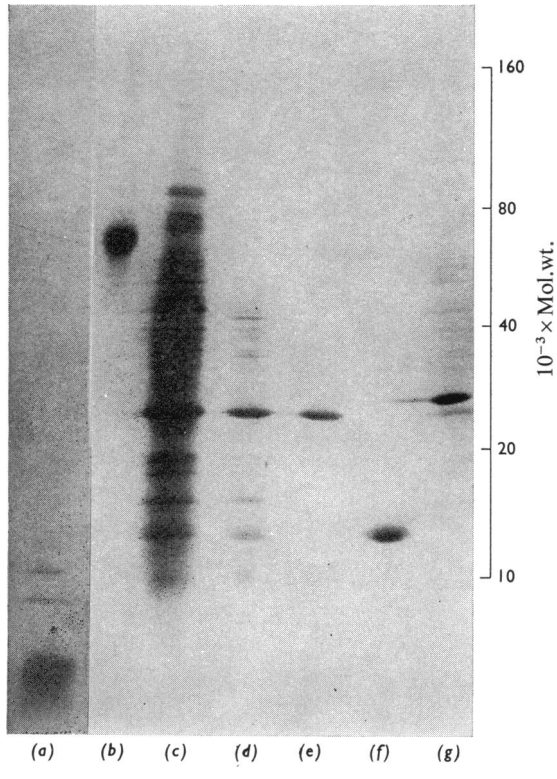
The u.v. spectrum of the enzyme was measured and a ε_{280} of 3.6×10^4 litre·mol^{-1}·cm^{-1} ($A_{280}^{1mg/ml}$ = 1.53) was calculated.

The alcohol dehydrogenase was titrated with excess of 5,5′-dithiobis-(2-nitrobenzoic acid) in the spectrophotometer. Under non-dissociating conditions 5.6nmol of enzyme subunit released 4.73nmol of nitrobenzoate ion over a period of 5min. The modified enzyme was totally inactivated. In 4M-guanidinium chloride the enzyme reacted instantaneously with an exact 1:1 stoicheiometry, 5.6nmol of protein subunit releasing 5.6nmol of nitrobenzoate ion. Native alcohol dehydrogenase therefore has a single thiol residue available for titration with 5,5′-dithiobis-(2-nitrobenzoic acid). On denaturation the stoicheiometry remains the same, but the reaction rate is increased considerably.

The results of carboxypeptidase A digestion are presented in Fig. 4. Different quantities of proteinase were incubated with 100nmol of alcohol dehydrogenase and the amounts of amino acids released determined by analysis. The data obtained are consistent with a *C*-terminal sequence:

$$-\begin{pmatrix}\text{Ala}\\ \text{-Leu-Thr}\\ \text{Gly}\end{pmatrix}\text{Ile-CO}_2\text{H}$$

This experiment is capable of distinguishing pure alcohol dehydrogenase from preparations contaminated with the *Drosophila* proteinase. Treatment of the impure enzyme with carboxypeptidase A



EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis of alcohol dehydrogenase in gradient gels

The gels were prepared, electrophoresed and stained as described in the text. The gels were then photographed in transmitted light. (*a*) Partially purified sample of the enzyme after 24h at 0°C; (*b*) bovine serum albumin (mol.wt. 68000); (*c*) alcohol dehydrogenase after salmine sulphate treatment; (*d*) alcohol dehydrogenase after hydroxyapatite chromatography; (*e*) alcohol dehydrogenase after Sephadex G-100 gel filtration; (*f*) cytochrome *c* (mol.wt. 12000); (*g*) penicillinase from *Bacillus cereus* (mol.wt. 28000).

Table 1. *Purification data on the isolation of alcohol dehydrogenase from the adh^{UF} allele*

Details of the techniques and assay methods used are given in the text. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Proteinase activity was measured against the synthetic substrate *N*-benzoyl-DL-arginine nitroanilide. N.D., Not detectable.

Purification step	Volume (ml)	Alcohol dehydrogenase activity (units/ml)	Total alcohol dehydrogenase activity (units)	Yield (%)	Proteinase activity (total units)	Kunitz assay (total units)	Total protein (mg)	Specific alcohol dehydrogenase activity (units/mg)
After salmine sulphate treatment	920	10.52	9678	—	15.33	4140	7084	1.37
After hydroxyapatite chromatography	1200	7.24	8688	90	N.D.	N.D.	2293	3.79
After Sephadex G-100 gel filtration	119	45.83	5454	56	N.D.	N.D.	392	13.92

Table 2. *Amino acid composition of alcohol dehydrogenase from the adh^{UF} allele*

Results from different samples were normalized to the same total content of aspartic acid, glutamic acid, proline, glycine, alanine, leucine and phenylalanine. The best value was obtained by averaging the values for the six samples hydrolysed with HCl, except for serine and threonine (extrapolated to zero hydrolysis time). To determine the number of residues per mol of alcohol dehydrogenase, the best-value results were divided by a factor (6.586), which was derived by summing the values for Asx, Glx, Pro, Gly, Ala, Leu, Phe, Lys, His and Arg and dividing by 144. Hydrolysis in 3M-mercaptoethanesulphonic acid was used to determine tryptophan concentrations, and performic acid-oxidized enzyme hydrolysed in 6M-HCl for 24h gave values for the cysteine content.

	Amino acids recovered after acid hydrolysis (nmol)						
	24h	48h	96h	Mercaptoethanesulphonic acid-hydrolysed	Performic acid-oxidized	Best value	Content (residues/mol)
Lys	105.6	106.2	105.5	104.7	109.1	105.8	16.1
His	23.4	22.8	24.4	20.6	23.5	23.5	3.6
NH_3	165.6	133.2	165.4	—	—	165.4	25.2
Arg	32.4	31.7	32.3	33.8	32.2	32.2	4.9
Asx	165.6	164.6	167.5	165.5	156.1	165.9	25.3
Thr	144.9	142.2	138.7	139.8	132.9	146.0	22.2
Ser	60.9	58.2	54.2	62.7	60.5	63.5	9.7
Glx	105.5	105.4	104.0	102.2	108.3	105.0	18.0
Pro	67.1	63.6	63.4	71.6	69.8	63.5	9.7
Gly	108.1	107.8	108.9	110.8	116.4	108.3	16.5
Ala	131.1	133.0	127.3	127.4	131.4	130.5	19.9
Val	117.4	121.4	122.8	101.7	124.2	125.5	19.1
Ile	110.3	118.4	121.0	107.5	112.3	122.0	18.6
Leu	157.5	158.5	158.8	155.2	152.1	158.3	24.1
Tyr	36.6	35.8	35.9	49.2	30.5	36.1	5.5
Phe	53.2	52.2	53.4	50.6	48.8	52.9	8.0
Cys	—	—	—	—	18.6	18.6	2.8
Trp	—	—	—	27.1	—	27.1	4.1

brought about a rapid release of phenylalanine, tyrosine, isoleucine, alanine and serine in a complex fashion which was difficult to interpret.

Substrate specificity of the enzyme. The activity of the purified alcohol dehydrogenase was assessed against a range of alcohol substrates. $K_{m(app.)}$ and $V_{max.(app.)}$ were determined for each alcohol at a saturating NAD^+ concentration of 1 μm (Table 3).

Discussion

Irreversible inactivation is often the major factor responsible for low yields during enzyme preparation. This inactivation may be caused by one factor or a combination of many factors. For example, major conformational changes may occur, leading to dramatic decreases in protein solubility (Tanford, 1968). Alternatively irreversible denaturation can occur by

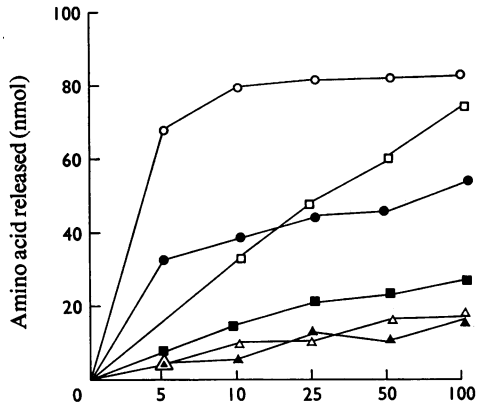


Fig. 4. *Carboxypeptidase A digestion of alcohol dehydrogenase*

The concentration of amino acids released after incubation of the enzyme with various concentrations of proteinase are shown. ○, Isoleucine; ●, leucine; □, threonine; ■, alanine; △, phenylalanine; ▲, glycine.

Table 3. *Substrate specificity of alcohol dehydrogenase from flies carrying the adh^{UF} allele*

Assays were performed as described in the text. The protein concentration used to calculate the specific activity of the enzyme was determined by automated amino acid analysis.

Alcohol Substrate	$10^2 \times V_{max.(app.)}$ (mol/min per mol of subunit)	$K_{m(app.)}$ (mM)
Pentan-1-ol	1.34	6.0
Butan-1-ol	1.86	15.0
Butan-2-ol	2.53	3.0
Propan-1-ol	0.89	9.0
Propan-2-ol	2.61	4.0
Ethanol	0.42	17.0

chemical modification (e.g. thiol oxidation) or by direct removal of the protein by proteolytic degradation. All three types of inactivation are influenced by temperature and together will define the thermostability of an enzyme. Although rates of enzyme aggregation and chemical denaturation are functions of the physical and chemical properties of an enzyme, rates of proteolytic degradation will also be dependent on the quantity and specificity of any proteinases present in the surrounding milieu.

An apparently thermolabile enzyme, alcohol dehydrogenase from *Drosophila melanogaster* carrying the *adh*^{UF} allele, has been purified in high yield. By designing a purification procedure that eliminated proteinases at an early stage, the stability of the purified enzyme has been improved. Stability, particularly in partially purified preparations, is not a simply physical property of the enzyme, but is also dependent on the activities of various proteinases presumably released on disruption of the fly's alimentary canal during sample preparation.

Evidence that the 'fast' alloenzyme is sensitive to proteinases, comes from the work of Jacobson & Pfuderer (1970). Their sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis results were interpreted as indicating a subunit mol.wt. of 7500. However, the multiple low-molecular-weight bands seen on these gels are more likely to be due to partial degradation by a contaminating proteinase.

Where thermolability is used to characterize a particular type of alcohol dehydrogenase, interpretation of data will be obscured by the effects of proteolytic degradation. The evidence of Grell *et al.* (1968), that the multiple forms of alcohol dehydrogenase that occur on electrophoresis at pH 8.0 differ significantly in thermostability, is therefore inconclusive.

Differential thermostability has, moreover, been implicated as a probable environmental factor influencing the relative frequencies of *adh* alleles in natural populations (Gibson, 1970; Vigue & Johnson, 1973). Also Milkman (1976) reports the isolation of a number of electrophoretically identical alcohol dehydrogenases, which differ significantly in their stability to heat treatment. These results were interpreted in terms of electrophoretically 'silent' amino acid substitutions, which, although having no effect on the mobility of the enzyme, led to changes in thermolability.

Drosophila alcohol dehydrogenase alloenzymes, as Day *et al.* (1974) point out, could well differ in their sensitivity to proteinase degradation, and this difference could be one of the variable properties through which selection maintains these polymorphisms in natural populations.

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