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PURIFICATION AND PARTIAL CHARACTERIZATION OF XANTHINE DEHYDROGENASE FROM *DROSOPHILA MELANOGASTER*

WILLIAM D SEYBOLD*

Laboratory for Developmental Biology, Swiss Federal Institute of Technology, Zurich (Switzerland)

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SUMMARY

The appearance of at least three enzymes in *Drosophila melanogaster*, xanthine dehydrogenase, aldehyde oxidase and pyridoxal oxidase, is under the control of two loci, maroon-like *(ma-l)* and low xanthine dehydrogenase *(lxd)*. These loci map to positions distinct from the putative structural genes for the enzymes. The purification of xanthine dehydrogenase is reported as a preliminary step in a study of the functions of the *ma-l* and *lxd* genes. $(NH_4)_2SO_4$ fractionation, heat precipitation and chromatography on DEAE-cellulose, hydroxyapatite and Sephadex G-200 have been utilized to produce a xanthine dehydrogenase preparation which is homogenous by the criteria of polyacrylamide gel electrophoresis and analytical electrofocusing. The enzyme has a molecular weight of approximately 300 000 as judged by gel filtration. Experiments with sodium dodecylsulphate gel electrophoresis indicate that the enzyme is composed of subunits with a molecular weight between 130 000 and 140 000.

INTRODUCTION

Two loci in *Drosophila melanogaster*, maroon-like (*ma-l*, 1-64.8) on the X chromosome, and low xanthine dehydrogenase (*lxd*, 3-33) on the third chromosome, affect the levels of at least three enzymes [1–5]. Xanthine dehydrogenase, aldehyde oxidase and pyridoxal oxidase activities are absent or substantially decreased in flies homozygous for mutations at either locus. Three other loci, each affecting levels of only one of the enzymes, are closely grouped on the third chromosome. One of these loci, rosy (*ry*, 3-52.0), appears to be a structural gene for xanthine dehydrogenase. The enzyme is absent in flies homozygous for mutations at this locus and electrophoretic variants map to the same position [1, 6]. Another locus, aldehyde oxidase (*aldox*, 3-56), appears to be a structural gene for aldehyde oxidase. Both electrophoretic variants and enzyme deficiencies map to this locus [7]. Flies homozygous for mutations at a third locus, low pyridoxal oxidase (*lpo*, 3-57) contain less than 5% of the pyridoxal oxidase activity observed in wild type flies [8].

Extensive biochemical and biological analyses of mutants at these five loci

* Present address, Department of Genetics, University of Alberta, Edmonton, Canada.

have revealed a complex pattern of interactions and pleiotropy. This work has been reviewed by Glassman [9, 10].

One approach to understanding the functions of the *ma-l* and *lxd* loci involves purification of the enzymes affected by these genes and analysis of the proteins in an attempt to find features common to the enzymes (such as common subunits or co-factors) which might be affected jointly by the two genes. Previous attempts to purify xanthine dehydrogenase have been reported by Glassman et al. [11] and by Parzen et al. [12]. Homogenous enzyme was not prepared in either case. The purification of aldehyde oxidase has been accomplished by Dickinson [7]. In this paper a technique for the purification of xanthine dehydrogenase is presented.

MATERIALS AND METHODS

Ultra pure $(NH_4)_2SO_4$ was obtained from Schwarz/Mann. The enzyme substrate, 2-amino-4-hydroxypteridine was purchased from K and K. Isoxanthopterin and bovine serum albumin were obtained from Fluka. NAD and dithiothreitol were from Sigma. All other chemicals were standard reagent grade.

D. melanogaster eggs of the Sevelen strain were collected and cultured according to the mass culture technique of Mitchell and Mitchell [13]. Adults were collected when less than three days old since enzyme activity declines in older flies [1].

Xanthine dehydrogenase activity was assayed by a modification of the method described by Glassman and Mitchell [1] which is based on the change in fluorescence when 2-amino-4-hydroxypteridine is oxidized to isoxanthopterin. The reaction mixture consisted of 1 ml of 0.2 M potassium phosphate (pH 7.2) containing 10 nmoles of 2-amino-4-hydroxypteridine, 0.2 μmole of NAD and 0.01 to 0.1 ml of enzyme. The increase in fluorescence was measured in an Eppendorf photometer fitted with a fluorimeter attachment. A 366-mm primary filter and a 400-nm secondary filter were used. A unit of enzyme activity is defined as that amount of enzyme which will cause the production of 1 μmole of isoxanthopterin per min in the system described above.

Disc electrophoresis on polyacrylamide gels was carried out according to the method of Davis [14]. Gels were stained overnight in 25% propanol, 10% acetic acid and 0.025% Coomassie brilliant blue followed by 6 h in 10% propanol, 10% acetic acid and 0.0025% Coomassie. Gels were destained by diffusion in 10% acetic acid.

Analytical gel electrofocusing was carried out according to the method of Wrigley [15]. Following the removal of carrier ampholytes with 10% trichloroacetic acid, gels were stained for protein as described above.

Xanthine dehydrogenase activity was located on gels by a method described by Brewer [16].

Sodium dodecylsulphate polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn [17]. Bovine serum albumin, chymotrypsinogen, catalase, ovalbumin and chicken xanthine dehydrogenase were used as standards. Gels were stained for protein as described above.

Protein solutions were concentrated by ultrafiltration under nitrogen pressure in an Amicon apparatus using a PM-30 membrane.

Protein concentrations were measured by the method of Lowry et al. [18] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

A crude homegenate was prepared by grinding 40 g of frozen 3-day-old adult flies to a smooth paste in a mortar and pestle with 20 g of sand and 80 ml of 0.1 M Tris–HCl, 1 mM dithiothreitol (pH 8.0). The paste was diluted with 320 ml of the same buffer and the homogenate was centrifuged at 14 600 × *g* for 20 min. The supernatant was filtered through nylon mesh. 10 g of Norite-A were added to the preparation to remove inhibitory substances (purines, pteridines, etc.). After 10 min the solution was centrifuged at 14 600 × *g* for 20 min and the supernatant was filtered through nylon mesh.

A saturated solution of $(NH_4)_2SO_4$ in 0.1 M Tris–HCl (pH 8.0) was added to the filtrate to 40% saturation and the resultant solution was centrifuged immediately at 14 600 × *g* for 30 min. The supernatant was brought to 60% saturated $(NH_4)_2SO_4$ by the addition of a saturated solution and the precipitate was collected by centrifugation at 14 600 × *g* for 30 min. The pellet was dissolved in 20 ml of the homogenization buffer. The yield of enzyme activity was 79% and the purification was 2.2-fold with respect to the charcoal-treated material.

Heat treatment of the $(NH_4)_2SO_4$ fraction provided a convenient method of further purification at this stage. The solution was added to two thick-walled 12-ml-glass Sorvall centrifuge tubes, immersed in a water bath at 68 °C for 5 min and then immediately cooled by immersion in ice–water. The precipitate was removed by centrifugation at 12 000 × *g*. The yield of enzyme activity was 82% and the purification was 5.5-fold for the step.

The heat treated preparation was dialyzed overnight against 2 l of 0.1 M Tris–HCl (pH 8.0) and loaded onto a 1.5 cm × 50 cm DEAE-cellulose (Whatman DE-52) column equilibrated with the same buffer. The column was eluted with a 500-ml linear gradient of 0.1–0.3 M NaCl in 0.1 M Tris–HCl (pH 8.0). Fig. 1 shows a typical elution

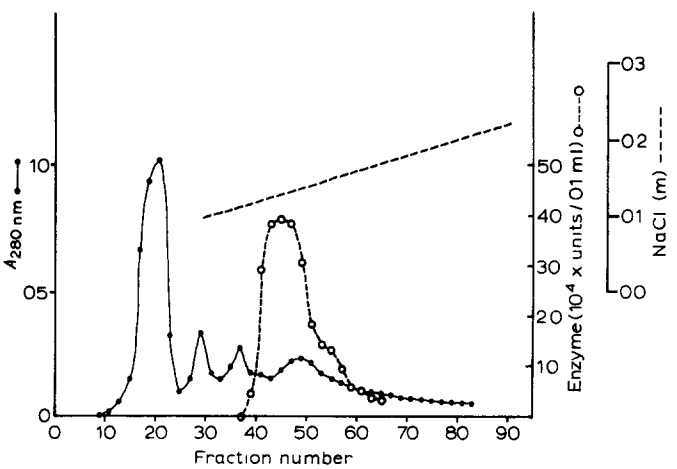

Fig. 1. DEAE-cellulose chromatography of xanthine dehydrogenase. The sample was an extract prepared from 40 g of adult *Drosophila* as described in the text. The column, 1.5 cm × 50 cm, was equilibrated with 0.1 M Tris–HCl (pH 8.0) and resolved with a 500-ml linear concentration gradient from 0.1 to 0.3 M NaCl in the same buffer. Tubes containing enzyme activity were pooled and concentrated. The closed circles represent absorbance at 280 nm and the open circles represent enzyme.

profile for this step. The yield was 66% and the purification was 9.7-fold with respect to the heat treated enzyme.

The peak fractions from the DEAE step were concentrated by ultra-filtration and loaded onto a 1.5 cm × 25 cm hydroxyapatite (Bio-Gel HTP) column equilibrated with 1 mM sodium phosphate (pH 6.7) and eluted with a 500-ml linear gradient of 1 mM to 0.25 M sodium phosphate (pH 6.7). A typical elution profile is shown in Fig. 2. The yield for this step was 48% and the purification was 1.7-fold.

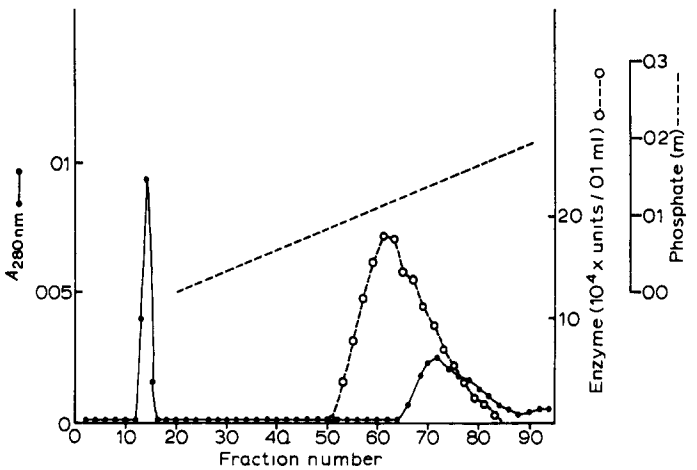

Fig. 2. Hydroxyapatite chromatography of xanthine dehydrogenase. The sample was prepared from the pooled and concentrated fractions of the DEAE column as described in the text. The column, 1.5 cm × 25 cm, was equilibrated with 1 mM sodium phosphate (pH 6.7) and resolved with a 500-ml linear concentration gradient from 1 to 0.25 M sodium phosphate (pH 6.7). Tubes containing enzyme activity were pooled and concentrated. The closed circles represent absorbance at 280 nm and the open circles represent enzyme activity.

The peak fractions from the hydroxyapatite column were concentrated to 3 ml by ultrafiltration and applied to a 2.5 cm × 100 cm Sephadex G-200 column equilibrated with 0.1 M Tris–HCl (pH 8.0) and eluted with the same buffer. The xanthine dehydrogenase activity was eluted from the column in a symmetrical peak at a position indicating a nominal molecular weight of approximately 300 000. This step resulted in a yield of 54% and a purification of 1.9-fold.

Yields and purification for the whole scheme are summarized in Table I. An overall yield of 11% and a purification of 406-fold with respect to the charcoal treated preparation were observed. It was not possible to measure accurately enzyme levels prior to the charcoal step due to the presence of inhibitory substances.

At a concentration of 1.0 mg/ml the preparation has a pale yellow color. At 0 °C the enzyme activity had a half life of approximately 12 h. Charcoal treatment of the preparation resulted in complete loss of activity. Treatment with 10 μM FMN or FAD were ineffective in restoring activity.

A sample of the enzyme prepared through the purification scheme described above was subjected to polyacrylamide disc electrophoresis. A single major protein component was observed coincident with the enzyme activity. A minor band of en-

TABLE I

PURIFICATION OF XANTHINE DEHYDROGENASE

Step	Protein (mg)	Activity (units × 10^4)	Specific activity (units/mg × 10^4)	Fold purification	Yield (%)
Charcoal	1337	5920	4.4	—	—
$(NH_4)_2SO_4$	472	4690	9.9	2.2	79 2
Heat	70	3840	54.8	12.4	64 9
DEAE	4.7	2540	534.7	120 7	42 9
Hydroxyapatite	1.3	1220	938.5	211.8	20.6
Sephadex G-200	0.36	650	1800.6	406.4	10.9

zyme activity was observed trailing the main band and coincident with a minor protein band.

Analytical gel electrofocusing was used to further test the degree of homogeneity of the enzyme preparation. On both pH 3–10 and pH 3–6 gels a single protein band was observed coincident with the enzyme activity.

Preliminary experiments with sodium dodecylsulphate gel electrophoresis indicate that the enzyme can be dissociated into subunits with a molecular weight between 130 000 and 140 000. Experiments to determine whether the enzyme is composed of two identical subunits and whether further dissociation is possible are in progress.

Further experiments comparing the structures of aldehyde oxidase and xanthine dehydrogenase are anticipated in an attempt to gain insight into the functions of the *lxd* and *ma-l* genes.

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