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# ISOLATION OF THE CYTOPLASMIC FORM OF MALATE DEHYDROGENASE FROM HONEY BEE (*APIS MELLIFERA*) LARVAE

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SUMMARY

A simplified system for the preparation of cytoplasmic MDH from honey bee larvae is presented. The study shows the enzyme to be a dimer with a subunit molecular weight of 34,000. The enzyme was found to have a lower specific activity than that found in *Drosophila melanogaster*.

## INTRODUCTION

Malate dehydrogenase (L-malate $NAD^+$ oxidoreductase E.C.1.1.1.37) has been extracted from a wide variety of sources (see 1 for a review). Only a single form of the enzyme has been observed in prokaryotes but it seems probable that all eukaryotes possess two distinct forms of malate dehydrogenase. One, soluble or cytoplasmic MDH (s-MDH), is found exclusively in the cytoplasm of eukaryotic cells; the other, mitochondrial MDH (m-MDH), is located within the mitochondria.

Malate dehydrogenases from several bee species have been separated by gel electrophoresis in genetic studies (2, 3, 4). These studies have indicated identical complements of the two forms in larval and adult life stages although an additional MDH isozyme of unknown origin is reported from honey bee pupae (2).

Several recent studies have described the isolation of s-MDH and m-MDH from *Drosophila melanogaster* (5, 6). This study is an application of the principles outlined in those studies to the preparation of s-MDH from honey bee larvae.

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## MATERIALS AND METHODS

Biological materials: Five to six day old larvae (mature but not capped) were removed from brood comb of a single colony at the Bee Research Facility at the University of California at Davis. One hundred grams of larvae were frozen and stored at -70°C for two weeks prior to enzyme purification.
Purification scheme: This is shown in detail in Figure 1. Elution profiles of chromatography columns employed are shown in Figures 2-4. The purification table for the preparation is shown in Table 1.
Enzyme assays: MDH activity was measured according to McReynolds and Kitto (7). ADH activity was also routinely screened throughout the preparation using the technique described by Chambers et al. (5) according to the methods of McDonald et al. (8). One unit of enzyme activity is defined as that which causes an increase or decrease in absorbance at 340 nm of 1.0 per minute in these assay streams.
Polyacrylamide gel electrophoresis: The system of Laemelli (9) was employed as modified by Chambers et al. (5).
SDS-urea gel electrophoresis: The system of Weber et al. (10) as modified by Snyder (11) was employed in molecular weight determinations.

## RESULTS

In dealing with enzyme preparations from large numbers of individuals it is important to consider possible genetic heterogeneity within the pooled sample. To test for genetic variation among the larvae collected from the test colony, 20 individuals were subjected to starch gel electrophoresis at pH 6.8 (12). This procedure gave two zones of MDH activity, an anodal zone and a cathodal zone. The anodal zone contains two bands, a major band and a minor band of slightly higher mobility. This pattern is identical to that observed for s-MDH in *Drosophila* (5, 7, 12) and many other eukaryotes. The cathodal band bears a somewhat smudged appearance as is commonly found for m-MDH. Both activity zones gave discrete bands with homogeneous mobilities as is found in population samples lacking genetic variation. This was expected as the haplo-diploid Hymenoptera generally possess little genetic variation at the enzyme level (3, 4, 13) although genetic variation at the s-MDH locus has been reported for honey bees (2). It was concluded from this analysis that the test colony was monomorphic for a single s-MDH allele and pooled samples of larvae were used in subsequent purifications.

Starch gels were also stained for ADH activity but activity of this enzyme could not be detected. Neither could ADH activity be detected by spectrophotometric assay on crude larval extract (detection limit 5 units activity/g wet weight of larvae, compare this to a detected MDH activity of 600 units/g).

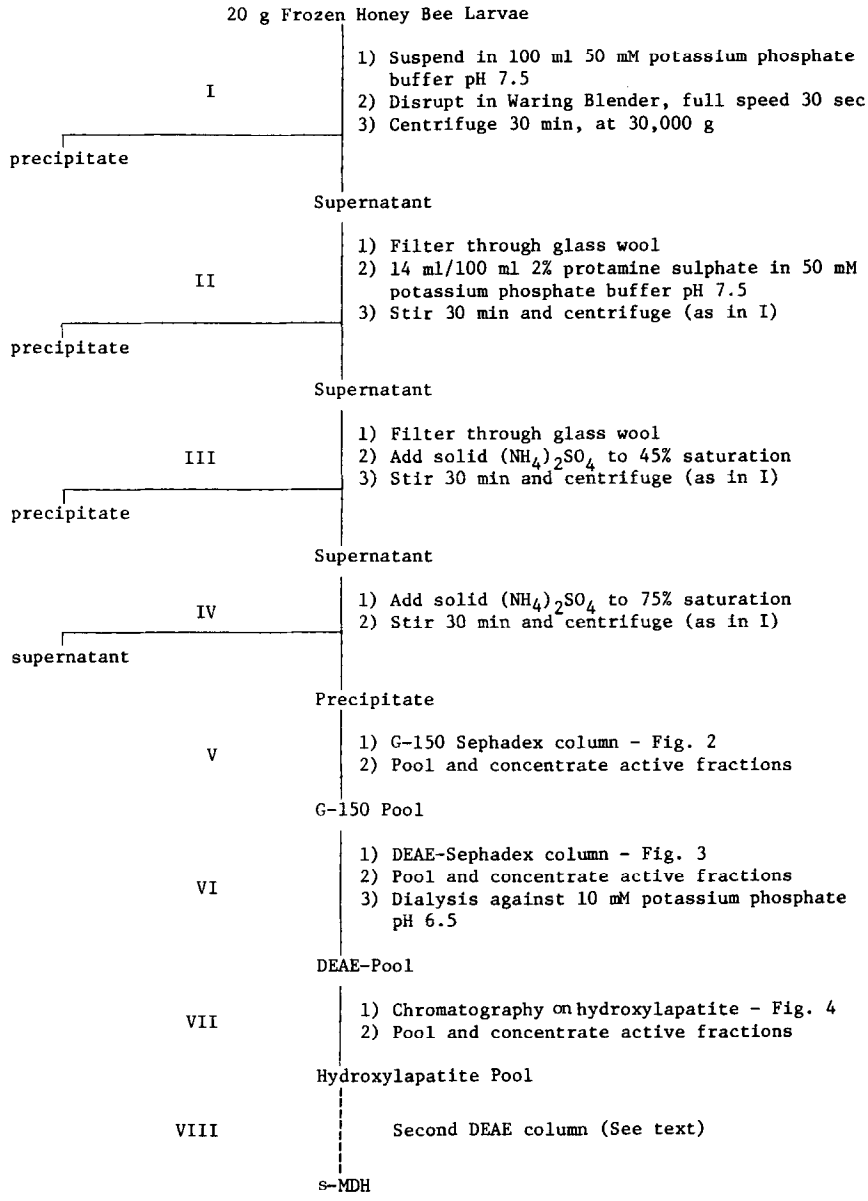


Figure 1. A detailed purification scheme for cytoplasmic MDH from honey bee larvae. Elution profiles from columns are shown in Figures 2-4. All buffers contained 1 mM β-mercaptoethanol and 1 mM EDTA. All steps were conducted at 0-4° C. Enzyme activity and protein concentration was measured at each step.

Mitochondrial MDH activity was lost subsequent to the first centrifugation (Figure 1) and was not again detected throughout the purification procedure. This loss will be discussed later.

TABLE 1.

Purification table for s-MDH from honey bee larvae

| Step | Vol. (ml) | MDH Activity (units/ml) | Protein (mg/ml) | Totals Activity (units) | Totals Protein (mg) | Sp. Activity (units/mg) | Yield (%) | Purity (fold) |
|---|---|---|---|---|---|---|---|---|
| 1. Crude extract | 113 | 105 | 10.2 | 11,865 | 1153 | 10.3 | 100 | 1 |
| 2. Protamine sulphate supernatant | 129 | 96 | 8.15 | 12,384 | 1051 | 11.8 | 104 | 1.14 |
| 3. 45% $(NH_4)_2SO_4$ supernatant | 145 | 80 | 5.25 | 11,600 | 761 | 15.2 | 98 | 1.48 |
| 4. 75% $(NH_4)_2SO_4$ pellet | 8.5 | 1100 | 54.5 | 9,350 | 463 | 20.2 | 79 | 1.96 |
| 5. Sephadex G-150 pool | 91 | 87 | 1.5 | 7,917 | 137 | 57.8 | 67 | 5.61 |
| 6. DEAE-Sephadex pool | 60 | 70 | 0.19 | 4,200 | 11.3 | 372 | 35 | 36.1 |
| 7. Hydroxylapatite pool | 50 | 65 | 0.05 | 3,250 | 2.52 | 1,290 | 27 | 125 |

All data were taken from the single purification illustrated in Figures 1-4 for a 20 g batch of frozen larvae.

The purity and properties of the prepared enzyme pool from the hydroxylapatite column (Figure 4) assayed by polyacrylamide gel electrophoresis revealed a single major protein band corresponding to s-MDH on an activity stained gel. A second minor protein band with slightly higher anodal mobility was observed also which showed MDH activity. This latter band corresponds to the minor band observed on starch gels run on crude larval extract. Several very minor protein contaminants (estimated 5% total protein) were also noted at the end of the purification procedure. Final purification was achieved by rechromatography on DEAE-Sephadex (bed volume 30 ml) with conditions as in Figure 2 but at a 7 ml/hr flow rate and an elution gradient up to 250 mM KCl. Although 90% of the protein applied was recovered, a 50% drop in specific activity was recorded. Electrophoresis revealed that all minor contaminants had been removed and the same two-banded protein-activity pattern described earlier was again observed. SDS-urea gels run on the final purified sample revealed a single band with an approximate molecular weight of 34,000.

## DISCUSSION

The procedure presented here is a simple technique for the purification of a malate dehydrogenase from honey bee larvae. The comparison of the properties of the prepared enzyme to those of other better characterized malate dehydrogenases suggest it is the cytoplasmic form of the enzyme. The elution volume from G-150 Sephadex columns suggests an oligomeric molecular weight of approximately 70,000. This together with the SDS-urea gel data indicated that the enzyme is a dimer. This has previously been suggested based on starch gel separation patterns of heterozygous individuals by Contel et al. (2).

The enzyme yield is about 2 mg from 20 g of starting material. This allows a calculation that the enzyme represents close to 1% of the total extractable larval protein. The specific activity of the enzyme obtained was 1290 units/mg compared to about 6000 units/mg for *Drosophila melanogaster* s-MDH. However, it must be mentioned that the assay system of McReynolds and Kitto (7) is adjusted to give maximal activity with the *Drosophila* enzyme (i.e. at pH optimum

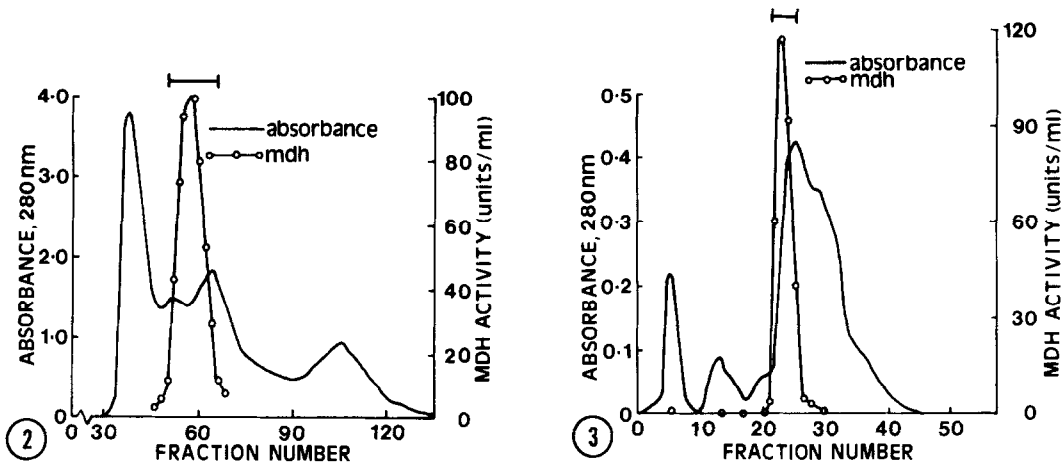


Figure 2. Elution profile of 75% $(NH_4)_2SO_4$ pellet, volume 8.5 ml, on G-150 Sephadex column (5 x 25 cm). The column was eluted with 50 mM Tris-HCl buffer pH 8.3 at 50 ml/hr. 5 ml fractions were collected.

Figure 3. Ion exchange chromatography of G-150 Sephadex pool on DEAE-Sephadex column (2.5 x 21 cm). The s-MDH pool from the G-150 column was concentrated by ultrafiltration to a final volume of 9.6 ml and applied to the DEAE-Sephadex column equilibrated with 50 mM Tris-HCl buffer pH 8.3. The column was washed with equilibration buffer to fraction number 13 (130 ml) and then a linear gradient to 500 mM potassium chloride in equilibration buffer was applied (total volume 600 ml). A flow rate of 40 ml/hr. was maintained throughout. Enzyme containing fractions (10 ml) were pooled as indicated by the bar.

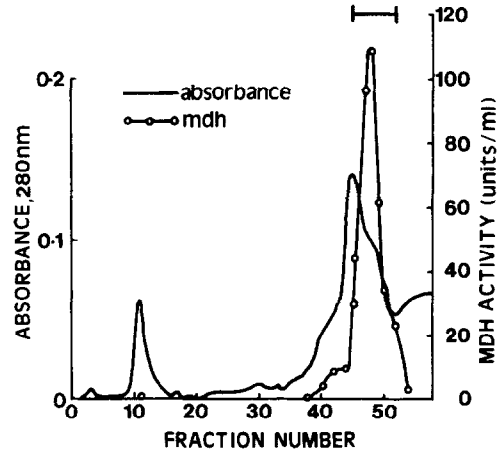


Figure 4. Chromatography of s-MDH DEAE pool on hydroxylapatite column (2.5 x 13 cm). The enzyme pool from the DEAE column was concentrated by ultrafiltration and dialysed against 10 mM potassium phosphate buffer pH 6.5 (final volume 5.5 ml). The sample was applied to the hydroxylapatite column equilibrated with the same buffer. The column was washed with this buffer until 18 fractions had been collected (110 ml) then a pH and concentration gradient from 10 mM potassium phosphate pH 6.5 to 250 mM potassium phosphate pH 7.5 (total volume 240 ml). Pooled fractions (6 ml each) are indicated by the bar.

and below inhibitory oxaloacetate concentrations). It is therefore possible that higher activities may be recorded under other assay conditions for the honey bee enzyme. This has not been investigated further than the finding that oxaloacetate reduction by honey bee MDH proceeds more rapidly at pH 7.5 than pH 8.6 (activity ratio 1.55) in which respect it does resemble its *Drosophila* counterpart.

In both purification and electrophoresis of material that had been frozen, disrupted and centrifuged, no mitochondrial MDH activity was detected. Several possible reasons may be suggested for this loss of activity although these have not been investigated in detail. It seems likely that the reason lies in the disruption and centrifugation procedure as larvae of *Apis mellifera* and other bee species have been frozen at similar temperature, disrupted gently in distilled water, and yet yielded both s-MDH and m-MDH. It must be pointed out that this activity loss in the m-MDH enzyme does not hold for *Drosophila* from which that enzyme is easily extracted by similar procedures after storage for months at -70°C.

The simplified procedure presented here should allow work to progress rapidly on comparisons of malate dehydrogenases among bees of different species and among related organisms.

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REFERENCES

1. Banaszak, L. J. and Bradshaw, R. A. (1975) The Enzymes, pp. 369-396, Academic Press, New York.
2. Contel, E. P. B., Mestriner, M. A., and Martins, E. (1977) Biochem. Genet. 15, 859-876.
3. Metcalf, R. A., Marlin, J. C., and Whitt, G. S. (1975) Nature (London) 257, 792-794.
4. Snyder, T. P. (1975) Evolution 28, 687-689.
5. Chambers, G. K., Fletcher, T. S., and Ayala, F. J. (1978) Anal. Biochem. (submitted).
6. Chambers, G. K., Fletcher, T. S. and Ayala, F. J. (1978) Biochem. J. (submitted).
7. McReynolds, M. S. and Kitto, G. B. (1970) Biochem. Biophys. Acta 198, 165-175.

8. McDonald, J. F., Chambers, G. K., David, J., and Ayala, F. J. (1977) Proc. Nat. Acad. Sci. USA 74, 4562-4566.
9. Laemelli, U. K. (1970) Nature 227, 680-685.
10. Weber, K., Pringle, J. R., and Osborn, M. (1972) Methods in Enzymology 26, 2-27, Academic Press, New York.
11. Snyder, T. P. (1977) Biochem. Syst. and Ecol. 5, 133-150.
12. Ayala, F. J., Powell, J. R., Tracey, M., Mourão, C., and Pérez-Salas, S. (1972) Genetics 70, 113-139.
13. Lester, L. J. and Selander, R. K. (1979) Genetics (in press).