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AFFINITY CHROMATOGRAPHY OF MALIC ENZYME FROM GRAPE BERRIES*

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Key Word Index—*Vitis vinifera* cv Raboso; Vitidaceae; grape berries; malic enzyme; affinity chromatography; gel-permeation.

Abstract—NADP-dependent malic enzyme from grape berries is associated with NAD-dependent malate dehydrogenase. A two step procedure, involving affinity chromatography on 2′,5′-ADP-Sepharose 4B, followed by gel-permeation on Bio-Gel A-1.5 m, was used to separate malic enzyme from malate dehydrogenase and other proteins. The yield was *ca* 60%. Malic enzyme and malate dehydrogenase migrated respectively as three bands and one band during disc electrophoresis in polyacrylamide gel. The MW resulting from gel-permeation was 220 000 for malic enzyme and 53 000 for malate dehydrogenase.

INTRODUCTION

Several NADP-dependent enzymes have been purified by affinity chromatography on an immobilized form of the inhibitor N^6-(6-aminohexyl)-adenosine 2′,5′-bisphosphate [1]. By the same procedure malic enzyme was purified from chicken liver by Kwok Kam Yeung and Carrico [2], and also the glucose-6-phosphate dehydrogenase from mouse and *Drosophila* by Chi-Yu-Lee *et al.* [3]. Malic enzyme (EC 1.1.1.40) from plant sources was found [4–6] to be often associated with malate dehydrogenase (MDH, EC 1.1.1.37). Even after affinity chromatography on 2′,5′-ADP-Sepharose 4B, NADP-dependent malic enzyme from grape berries was associated with NAD-dependent MDH and was devoid of allosteric properties [7]. The purpose of the present study was to improve purification of malic enzyme by using affinity chromatography followed by gel-permeation.

RESULTS

The final recovery of enzyme activity was *ca* 63% and represented a 700-fold increase in purity (Table 1). NAD-dependent MDH appeared at the bed volume, whereas the NADP-dependent malic enzyme together with a small amount of associated MDH were eluted from the column with the buffer containing mM NADP and M NaCl. Pooled fractions of NADP-dependent enzymes were submitted to gel-permeation; MDH and malic enzyme emerged from the gel each as a single peak. By electrophoresis in polyacrylamide gel and staining for protein, 3 bands from malic enzyme and one band from MDH were obtained. The corresponding bands in parallel electrophoresis experiments stained also for the enzyme activity. The results of gel-permeation runs of the two purified enzymes suggested that, in terms of enzyme activity, they behaved as a single component, with an MW of *ca* 53 000 for MDH and *ca* 220 000 for malic enzyme. These values agree with previous data obtained in experiments on animal and plant sources [3, 8, 9].

DISCUSSION

Malic enzyme from plant sources [5, 8, 10] has been purified *ca* 200-, 1200- and 2500-fold by conventional

Table 1. Purification of malic enzyme from grape berries

Fraction	Total units	Units/mg protein	Yield (%)	Increase in purity
Crude homogenate	19.2	0.016	100.0	—
$(NH_4)_2SO_4$ precipitate	17.3	0.240	90.0	15
Affinity chromatography	15.7	9.250	81.8	578
Gel-permeation	12.1	11.200	63.1	700

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methods with recoveries ranging from 1 to 14%. We obtained over 80% yield by affinity chromatography of the malic enzyme extracted from grape berries (Table 1).

Brodelius *et al.* [1] chromatographed a yeast extract on immobilized N^6-(6-aminohexyl)-adenosine 2',5'-bisphosphate and isolated several NADP-dependent dehydrogenases by development of the chromatogram with a gradient of NADP. Malic enzyme from animal sources was purified by affinity chromatography followed by biospecific elution on N^6-substituted 2',5'-ADP-Sepharose 4B [2] and on 8-substituted 2',5'-ADP-Sepharose 4B [3]. In our experiments, starting from a plant source, the biospecific elution of malic enzyme with either 2 mM and 10 mM NADP, or 2 mM and 10 mM NADPH, led to lower yields (20–30%). Malic enzyme could be desorbed with higher recovery by adding to the buffer mM NADP and M NaCl.

Nevertheless a small NAD-dependent MDH activity was still present. On the other hand, by using a 5'-AMP-Sepharose 4B column, which should be specific for isolation of NAD-dependent MDH, we obtained the same results. In this respect, MDH from grape berries behaved like cytoplasmic isoenzymes of MDH from watermelon and pig heart [11], which were not fixed by 5'-AMP-Sepharose 4B.

A very strong interaction between malic enzyme and 2',5'-ADP-Sepharose 4B could bring about the partial immobilization of MDH in association with malic enzyme. The above behaviour could be explained by differences in the conformation of the binding sites or by structural changes of MDH preventing its immobilization on the 5'-AMP-ligand. The alternative hypothesis of two separate enzyme activities located on the same protein is in contrast with the results of gel-permeation showing the presence of two enzymes with different MW.

EXPERIMENTAL

Mature grapes, *Vitis vinifera* cv Raboso, from a local vineyard, were washed, packed and stored according to the procedure of ref. [12].

Chemicals. NAD, NADP, malic acid, phenazine methosulfate, nitro blue tetrazolium were purchased from Sigma; Bio-Gel A-1.5 m, acrylamide and bis-acrylamide from Bio-Rad; 2',5'-ADP-Sepharose 4B and 5'-AMP-Sepharose 4B from Pharmacia; Coomassie Brillant Blue R 250 and standard enzymes from Serva.

Assay of enzymes and protein. Malic enzyme and MDH were evaluated by spectrophotometry at 340 nm and 35 of the reduced NADP and NAD respectively. The assays were carried out on 0.2 ml of enzyme extract in a reaction mixture (3 ml) containing 0.1 ml of 4.9 mM $MgCl_2$, 0.2 ml of 0.2 M K-L-malate, 0.1 ml of 3.9 mM NADP and 2.4 ml of 5 mM Tris-HCl pH 7.1, in the case of malic enzyme; 0.2 ml of 0.2 M K-L-malate, 0.1 ml of 4.5 mM NAD and 2.5 ml of 50 mM Tris-HCl pH 9.5, in the case of MDH. Protein was determined according to the method of ref. [13] using bovine serum albumin as standard.

Enzyme purification. The extraction procedure was as described in a previous paper [7]. Solid $(NH_4)_2SO_4$ was added to the crude extract up to 90% conc. After stirring for 1 hr at 4°, the ppt. was collected by centrifugation at 17000 *g* for 15 min, dissolved in 80 ml of 25 mM Tris-HCl pH 7.1, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol (standard buffer), and dialyzed overnight against 5 l. of the same buffer. The dialyzed soln was applied to a 1 × 15 cm column containing 12 ml of 2',5'-ADP-Sepharose 4B equilibrated at 4° with the same buffer. After washing with 70 ml of standard buffer, malic enzyme was eluted with 30 ml of 25 mM Tris-HCl pH 7.1, containing mM NADP and M NaCl. Fractions (2.2 ml) at 0.6 ml/min were collected and assayed for activity of both enzymes. The active fractions eluted from 2',5'-ADP-Sepharose 4B column were pooled, coned to a final vol of *ca* 4 ml in an Amicon ultrafiltration cell with Diaflo UM-10 membrane and applied to a 1.6 × 70 cm column of Bio-Gel A-1.5 m, previously equilibrated at 4° with standard buffer. Fractions (1.8 ml) at 0.36 ml/min were collected and submitted to enzyme assays. The MW of the purified enzymes was determined by gel-permeation on Bio-Gel A-1.5 m. The following commercially available enzymes were employed as standards: myoglobin (MW 17800), albumin (MW 67000), alkaline phosphatase (MW 110000), catalase (MW 240000) and ferritin (MW 480000).

Electrophoresis in 5.5% polyacrylamide gel was carried out at 3 mA/tube, according to the method of ref. [14]. Gels were stained for protein with Coomassie Brillant Blue R 250, and for malic enzyme activity by the method of ref. [15] with some modifications: Mg^{2+} instead of Mn^{2+}, and 25 mM Tris-HCl pH 7.4 instead of 0.1 M glycine. MDH activity was assayed in the same buffer pH 8.5 with NAD instead of NADP. Gels used as control were treated with H_2O at 100° for 10 min.

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REFERENCES

1. Brodelius, P., Larsson, P-O. and Mosbach, K. (1974) *Eur. J. Biochem.* **47**, 81.
2. Yeung, K. K. and Carrico, R. J. (1976) *Analyt. Biochem.* **74**, 369.
3. Lee, C. Y., Langley, C. H. and Burkhart, J. (1978) *Analyt. Biochem.* **86**, 697.
4. Johnson, H. S. and Hatch, M. D. (1970) *Biochem. J.* **119**, 273.
5. Krishnamurthy, S. and Patwardhan, M. V. (1971) *Phytochemistry* **10**, 1811.
6. Davies, D. D., Nascimento, K. H. and Patil, K. D. (1974) *Phytochemistry* **13**, 2417.
7. Spettoli, P., Bottacin, A. and Zamorani, A. (1980) *Vitis* **19**, 4.
8. Brandon, P. C. and van Boekel-Mol, T. N. (1973) *Eur. J. Biochem.* **35**, 62.
9. Wolfe, R. G. and Neilands, J. B. (1956) *J. Biol. Chem.* **221**, 61.
10. Davies, D. D. and Patil, K. D. (1974) *Biochem. J.* **137**, 45.
11. Walk, R-A. and Hock, B. (1976) *Eur. J. Biochem.* **71**, 25.
12. Arnold, W. N. (1965) *Biochim. Biophys. Acta* **110**, 134.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
14. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 405.
15. Frenkel, C., Klein, I. and Dilley, D. R. (1968) *Plant Physiol.* **43**, 1146.