SEPARATION OF THE MALATE DEHYDROGENASE ISOENZYMES OF COTTON SEEDS

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In preceding papers [1] we have reported the presence in cotton seeds of four isoenzymes with malate dehydrogenase activity. It is known from the literature that heterogeneous forms of malate dehydrogenase with molecular weights of 69,000, 275,000, and 70,000-145,000 may exist in one and the same plant simultaneously [2, 3]. The majority of workers consider that the isoenzyme with mol. wt. 60,000-70,000 is a dimer and that with 135,000-145,000 a tetramer. The iso forms of malate dehydrogenase from bacterial and animal sources generally have similar molecular weights [4, 5, 9-12].

We have studied the homogeneity of the molecular forms of malate dehydrogenase from cotton seeds using gel filtration. The ammonium-sulfate-precipitated and desalted protein was passed through a column of Sephadex G-100. Three protein fractions were found (Fig. 1). The malate dehydrogenase activity was determined in each tube, and only one active peak was obtained. This shows that the molecular weights of the isoenzymes of malate dehydrogenase present in cotton seeds are similar.

We then separated the isoenzymes using precipitation of the protein with ammonium sulfate. Two fractions were isolated: at 20-50% [1] and 50-100% saturation. An electrophoretic study with the determination of the position of localization of the activity in the gel showed that both fractions contained two iso forms (Table 1, Fig. 2).

After separation on a column of DEAE-cellulose, two active fractions were obtained, while two others showed traces of activity. All the protein fractions were studied by electrophoresis in polyacrylamide gel, their activities being determined in the gel. In each of fractions I and IV one colored band was found, and in each of fractions II and III two with weak colorations. For preparation IV, the fraction with the maximum activity, we found a molecular weight (by ultracentrifuging) of 145,000 [7] and determined some of its properties: dependence of the activity on the pH, on the substrate saturation, and on the amount of coenzyme added. The oxidation of NAD·H_2 was used to determine activities.

TABLE 1

Purification stage	Total volume, ml	Protein content, mg/ml	Malate dehydrogenase activity		Yield, %		Degree of purification
			specific	total	protein	activity	
Primary extract	66	39	2057	5 291 718	100	100	1
50-100% saturation fractions desalted on Sephadex G-25	10	28	3240	907 200	11,26	17	1,6
Separation on DEAE-cellulose							
I	48	0,855	12 353	506 473	1,6	9,6	6
II	48	0,817	850	58 150	1,5	0,62	—
III	28	0,49	1240	16 988	0,14	0,32	—
IV	36	0,08	60 000	168 000	0,11	3,2	30

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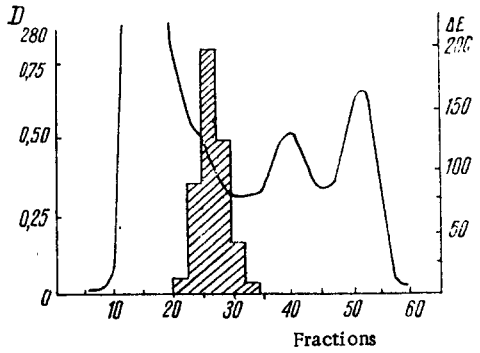

Fig. 1

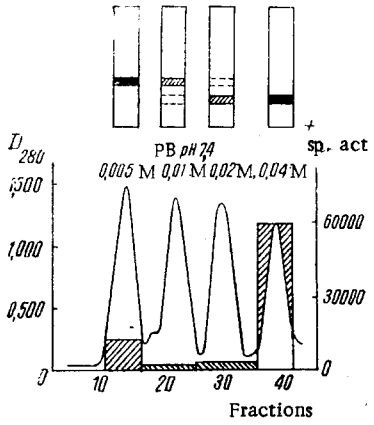

Fig. 2

Fig. 1. Separation of the combined fraction on Sephadex G-100.

Fig. 2. Separation of the total fraction for 50-100% saturation with ammonium sulfate on DEAE-cellulose and zymograms of the fractions obtained (for conditions, see text).

Fig. 3 shows the saturation curve of the enzyme with oxaloacetic acid (OAA), which is characteristic for the curve of inhibition of an enzyme by high concentrations of substrate. This acid has an inhibiting effect in concentrations greater than $1 \cdot 10^{-3}$ M. The value of K_M for OAA is $3 \cdot 10^{-4}$ M at pH 7.4 in a phosphate buffer. In addition, we found that the maximum activity is shown in a concentration of $1.91 \cdot 10^{-4}$ M NAD·H_2. Figure 3 also shows the dependence of the activity on the pH; it can be seen that the maximum activity appears in the pH range from 9 to 10.

EXPERIMENTAL

Determination of Protein. The amount of protein in the solutions was determined by the biuret reaction and by the Warburg–Christian method [13].

Extraction. Defatted cottonseed flour (8 g) was extracted with 80 ml (1:10) of phosphate buffer, pH 7.4, containing 10^{-3} M cysteine and 10^{-3} M EDTA. Extraction was performed with stirring at +4°C for 2-4 h. The mixture was centrifuged at 18,000 rpm for 15 min.

Determination of the Activity of the Enzyme. The activity of the malate dehydrogenase was determined by the spectrophotometric method with respect to the oxidation of NAD·H_2 and by the decrease in optical density at 340 nm.

That amount of enzyme which changed the optical density by 0.001 in 1 min at room temperature was taken as the unit of activity.

The specific activity is the number of activity units per mg of protein. In an activity determination, the experimental mixture consisted of 0.51 mmole of OAA neutralized with 2% KOH solution, 0.17 mmole of NAD·H_2, and 2-5 μg of enzyme. The total volume of the reaction mixture was made up to 3 ml with 0.1 M phosphate buffer, pH 7.4. The phosphate buffer was used as control. The rate of the reaction was measured every 30 sec for 3 min.

Gel Filtration through Sephadex G-100. A column with a porous bottom (45×2.2 cm) was filled with Sephadex G-100 that had been swollen in 0.1 M phosphate buffer, pH 7.4, and was eluted 8-10 h with the same buffer for compaction. The residue obtained by the extraction of the defatted cottonseed powder (5 g of defatted powder was extracted in 50 ml of phosphate buffer, pH 7.4, containing $1 \cdot 10^{-3}$ M EDTA and 10^{-3} M cysteine and was precipitated with ammonium sulfate in the range of concentrations from 0 to 100% saturation) was dissolved in the buffer and the solution was centrifuged at 18,000 rpm at 0-2°C for 15 min. The supernatant liquid was desalted in a column of Sephadex G-25 equilibrated with the above-mentioned buffer and was then transferred to a column of Sephadex G-100. Elution was performed with 0.1 M phosphate buffer, pH 7.4, containing 10^{-3} M EDTA and 10^{-3} M cysteine. The rate of elution was 20 ml/h, the fraction volume 5 ml, and the temperature 0-4°C.

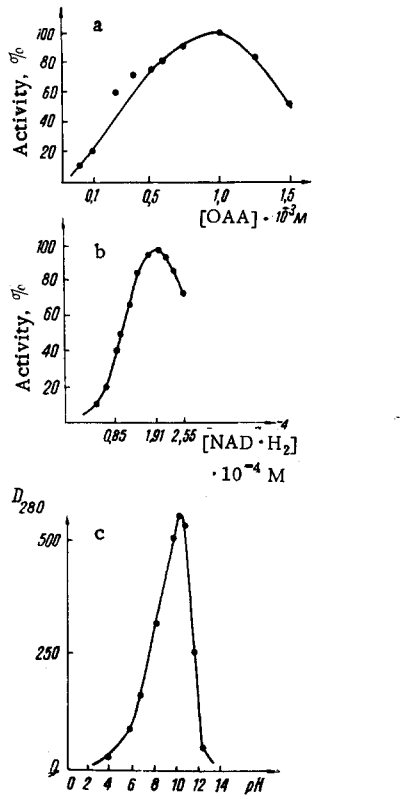

Fig. 3. Dependence of the activity of MDH on the concentration of OAA (the activity of MDH at a concentration of OAA of $1 \cdot 10^{-3}$ M is taken as 100%) (a); NAD · H_2 (the activity of MDH at a concentration of NAD · H_2 of $1.91 \cdot 10^{-4}$ M is taken as 100%) (b), and the pH of the medium.

Disc Electrophoresis in Polyacrylamide Gel. The electrophoresis tubes (5×60 mm) were fixed in a stand and filled with a polymerization mixture containing two-thirds part of 7.5% acrylamide in tris-glycine buffer (gel for separation) with pH 8.6 and the catalyst. Polymerization was performed in UV light for 30 min. Then one-quarter part of 2.5% acrylamide (gel for concentrating the protein solution) with pH 6.6 (tris-glycine buffer) was added, and this was polymerized in UV light for 30 min. The solution under investigation was deposited with the anticonvective substance (40% solution of sucrose in water and a few crystals of dye). Electrophoresis was performed for 2 h (voltage 400 V, current strength not more than 3.5 mA per tube). After electrophoresis, the gel was removed from the tubes and placed in a 0.1% solution of the dye Coomassie Blue in methanol – water – acetic acid (5:5:1). The dye residues were washed out with 4% acetic acid solution.

Determination of the Position of Localization of the Activity in the Gel after Electrophoresis. After the lapse of the time for electrophoresis, the gel was removed from the tubes and fixed with a 5% solution of trichloroacetic acid. After fixation, the gels were immersed in the substrate mixture in the dark at 37°C for 30 min. The composition of the incubation mixture was as follows: 6.5 mg of malic acid (neutralized), 3.5 mg of NAD, 0.6 mg of Nitrozolium Blue and 1 mg of phenazine methosulfate.

Fractionation with Ammonium Sulfate. With slow stirring, 7.12 g of ammonium sulfate was added to 65.5 ml of the total extract to give 20% saturation. The mixture was allowed to stand for 2 h and was centrifuged at 18,000 rpm for 30 min. The precipitate was discarded. With stirring, 11.6 g of ammonium sulfate was added to the supernatant liquid after the removal of the precipitate for 0-20% saturation to give 50% saturation, and the mixture was left for 2 h and was centrifuged at 18,000 rpm for 30 min. The precipitate was discarded and, with stirring, 21.18 g of ammonium sulfate was added to the supernatant liquid to give 100% saturation. After 2 h, the mixture was centrifuged at 18,000 rpm for 30 min. The precipitate was dissolved in the minimum amount of 0.005 M phosphate buffer, pH 7.4.

Desalting on a Column of Sephadex G-25. The protein solution obtained after salting out with ammonium sulfate was passed through a column of Sephadex G-25 (2.5×40 cm) previously equilibrated with the 0.005 M phosphate buffer, pH 7.4. The rate of elution was 40 ml/h, 5-ml fractions being collected.

Separation of the Fraction from 50-100% Saturation with Ammonium Sulfate on a DEAE-Cellulose Column. A desalted solution of 280 mg of the protein was deposited on a column of DEAE-cellulose (1.0×17 cm) equilibrated with the 0.005 M phosphate buffer, pH 7.4, containing 10^{-3} M cysteine and 10^{-3} M EDTA. The rate of elution was 14 ml/h. Elution was first performed with the buffer (15 tubes) and then with stepwise increasing concentrations of 0.01 M, 0.02 M, and 0.04 M phosphate buffer. Fractions of 3.5 ml each were taken in a collector. Their extinctions were determined on an SF-4A spectrophotometer at 280 nm. A graph of the spectrophotometric readings was plotted (see Fig. 2). The protein was eluted in four fractions. The protein content and activity of each fraction was determined. Fraction IV, eluted with the 0.04 M phosphate buffer was found to be the most active.

Ultracentrifuging was performed in an ultracentrifuge (model G-120 MOM), using 0.1 M phosphate buffer at room temperature. The molecular weight was found by the method of unestablished equilibrium, with calculation by W. Archibald's method.

SUMMARY

Gel filtration and ultracentrifuging have shown that the four isoenzymes of malate dehydrogenase present in cotton seeds have the same molecular weight.

In the case of the izoenzyme obtained at a high saturation with ammonium sulfate, the dependence of its activity on the pH and on the concentrations of substrate and coenzyme have been determined.

LITERATURE CITED

1. K. Davranov, M. A. Kuchenkova, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 5, 335 (1969); 7, 213 (1971).
2. Z. S. Bronovitskaya and V. P. Kretovich, Dokl. Akad. Nauk SSSR, 190, 461 (1970).
3. W. H. Murphey, G. B. Kitto, et al., Biochemistry, 6, 603 (1967).
4. I. P. Ting, Arch. Bioch. et Biophys., 126, No. 1, 1-7 (1968).
5. W. H. Murphey et al., J. Biol. Chem., 242, No. 7, 1548 (1967).
6. C. Woltenstein et al., J. Biol. Chem., 224, No. 23, 6415 (1969).
7. W. Archibald, J. Phys. Chem., 51, 1204 (1947).
8. K. Davranov, M. A. Kuchenkova, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 6, 545 (1970).
9. G. Sulebele and E. Silverstein, Arch. Bioch. Biophys., 133, No. 2, 425 (1969).
10. G. B. Kitto and N. O. Kaplan, Biochemistry, 5, 3966 (1966).
11. C. J. R. Thorne and N. O. Kaplan, J. Biol. Chem., 238, 1864 (1963).
12. C. J. R. Thorne and P. M. Cooper, Biochim. Biophys. Acta, 81, 397 (1963).
13. O. Warburg and W. Christian, Biochem. Z., 310, 384 (1941-2).