SOME PROPERTIES OF THE MALATE DEHYDROGENASE OF COTTON SEEDS

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UDC 577.158.4

In preceding papers we have reported that the four forms of malate dehydrogenase (MDH) [1-3] of dormant seeds of cotton of the variety 108-F are homogeneous in molecular weight as determined by gel filtration through Sephadex G-100 [4]. By precipitation with ammonium sulfate of the total protein extract, two fractions were obtained, at 20-50% and at 50-100% saturation. The purification process and some properties of the enzymes from 50-100% saturation have been described previously [4].

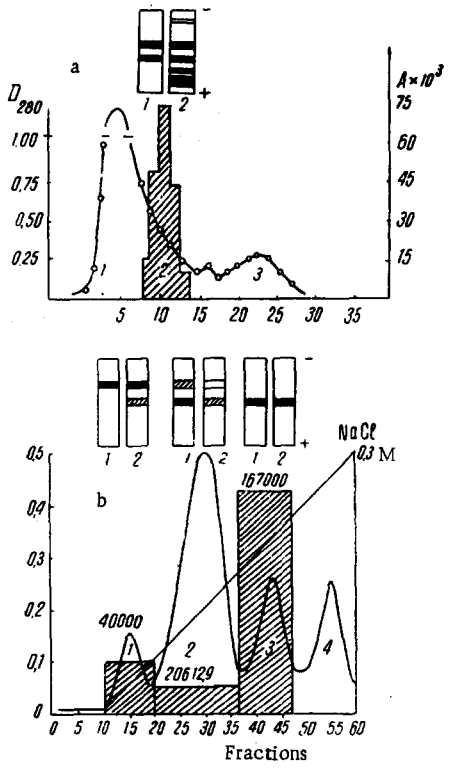

Fig. 1. Separation of the fraction corresponding to 20-50% saturation with ammonium sulfate (a) on Sephadex G-100 and of the active fraction (b) after Sephadex G-100 on DEAE-cellulose (for conditions, see text): 1) electrophoresis with determination of the malate dehydrogenase activity (zymograms); 2) electrophoresis in polyacrylamide gel.

In the present paper we give the results of a study of the enzymes in the fraction corresponding to 20-50% saturation with ammonium sulfate. The precipitated protein was dissolved in 0.005 M phosphate buffer (PB) containing 10^{-3} M cysteine and 10^{-3} M ethylenediaminetetraacetate (EDTA) and was desalted on a column of Sephadex G-25. Subsequent purification was performed by gel filtration through a column of Sephadex G-100 (Fig. 1a). The malate dehydrogenase and the aspartate transaminase (E. C. 2.6.11 – L-aspartate-2-hydroxyglutarate aminotransferase) activities were determined in the fractions obtained, since it is known from the literature that the latter partially accompanies MDH activity [5-12]. It was found that the aspartate transaminase is eluted from the column earlier than the MDH, which permits their complete separation.

The fraction with MDH activity was collected and studied by disc electrophoresis, with the determination of the positions of the protein bands and of the MDH activity in the gel.

It can be seen from a consideration of Fig. 1a (curves 1 and 2) that the fraction investigated is separated into five protein components, two of which possess MDH activity. The active forms of the MDH were separated from the accompanying proteins by using chromatography on DEAE-cellulose.

The active fractions after gel filtration on Sephadex G-100 were combined, concentrated, and deposited on a column of DEAE-cellulose equilibrated with 0.005 M phosphate buffer, pH 7.4. Elution was first performed with the initial buffer (10 test tubes) and then with a linear gradient of sodium chloride from 0 to 0.3 M, the protein being eluted in four fractions (Fig. 1b), three of which possessed MDH activity. The fractions were

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 374-380, May-June, 1972. Original article submitted November 10, 1971.

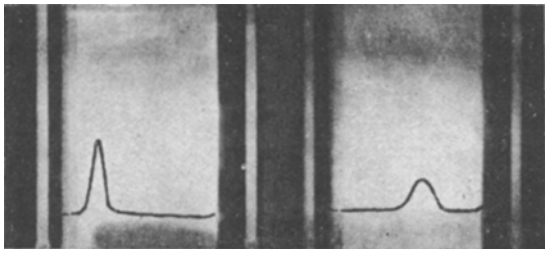

Fig. 2

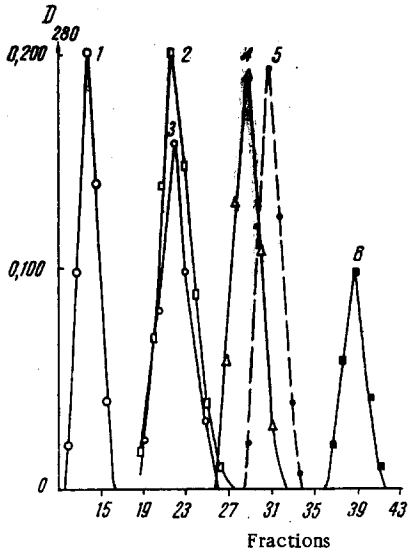

Fig. 3

Fig. 2. Sedimentation diagram of the rechromatographed fraction from DEAE-cellulose [concentration of protein 0.1% (10 mg/ml), solvent 0.1 M phosphate buffer, pH 7.5, time of sedimentation 40 min, set speed 50,000 rpm].

Fig. 3. Results of gel filtration on a column of Sephadex G-100 of the label proteins and of cottonseed malate dehydrogenase: 1) Dextran Blue; 2) lactate dehydrogenase from pig muscle; 3) malate dehydrogenase from cotton seed; 4) ox serum albumin; 5) malate dehydrogenase from pig cardiac muscle; 6) trypsin.

TABLE 1

Purification stage	Protein content, mg	MDH activity		Yield, %		Deg. of purification
		specific	total	in protein	in activity	
First extract in 0.005 M PB, pH 7.4	3800	3 600	$13,68 \cdot 10^6$	100	100	—
Fraction of 0-20% saturation	230	51	11 730	6,05	—	—
Fraction of 20-50% saturation after desalting on Sephadex G-25	1500	6 720	$10,08 \cdot 10^6$	39,5	73,68	1,87
Separation of the fractions on Sephadex G-100						
1	940	—	—	24,7	—	—
2	190	51 950	9 870 500	5	72,15	14,4
3	150	—	—	4	—	—
Separation of the fractions on DEAE-cellulose						
1	37	40 000	1 480 000	1	10,82	11
2	75	20 512,9	153 847,5	2	11,25	6
3	40	167 000	6 680 000	1	48,4	47
4	20	—	—	0,5	—	—
Rechromatography on DEAE-cellulose	17	210 000	3 570 000	0,44	26,09	60

studied by electrophoresis in polyacrylamide gel with a determination of the position of localization of the activity in the gel. Two components were found in the first fraction, one of which possessed MDH activity, two protein components with MDH activity were found in the second fraction, and one component, likewise with MDH activity, was found in the third fraction [see Fig. 1b (curves 1 and 2)].

The third fraction possessed the highest activity, and this was concentrated and rechromatographed on a column of DEAE-cellulose under the same conditions. Elution gave a single symmetrical protein peak. The fraction isolated has provisionally been given the designation MDH-I. Information on the purification of MDH-I is given in Table 1.

The homogeneity of this fraction was checked by ultracentrifugation (Fig. 2). Electrophoresis in polyacrylamide gel revealed only one band in it, which appeared again when the localization of the activity

in the gel was determined by the tetrazolium method. We found no lactate dehydrogenase or aspartate transaminase activity in the MDH-I. To determine the molecular weight of the purified MDH we used ultracentrifugation and gel filtration through a column of Sephadex G-100. The marker proteins used for calibrating the column were trypsin with mol. wt. 24,000 (Spofa), ox serum albumin with mol. wt. 67,000-70,300 (Nipdachu), malate dehydrogenase from pig cardiac muscle with mol. wt. 67,000 (Reanal), and lactate dehydrogenase from pig muscle with mol. wt. 142,000 ± 3600 (Reanal). The free volume of the column was determined with Dextran Blue (Pharmacia, Uppsala, Sweden). The results of gel filtration are shown in Fig. 3. It can be seen that the volumes in the elution of the MDH from cotton seeds and of lactate dehydrogenase from pig muscle coincide. Consequently, the molecular weight of the MDH-I from cotton seed must be taken as close to 145,000, as is confirmed by ultracentrifugation.

The amino-acid composition of the MDH-I given below was determined in samples of hydrolyzates of the malate dehydrogenase after hydrolysis for 24 h in 6 N HCl under vacuum at 105-110°C.

Amino acid	No. of moles of amino acid per mole of protein	Amt. of amino acid per 100 g of protein
Lysine	72	7,32
Histidine	23	2,46
Arginine	29	3,60
Aspartic acid	115	10,90
Threonine	70	5,23
Serine	80	5,98
Glutamic acid	93	9,70
Proline	69	5,66
Glycine	113	6,05
Alanine	107	6,87
Valine	90	7,52
Methionine	16	0,17
Isoleucine	64	5,26
Leucine	94	8,76
Tyrosine	25	3,33
Phenylalanine	39	4,68
Tryptophan*	2	0,21

These results show that the molecule of MDH-I contains considerable amounts of lysine, aspartic and glutamic acids, glycine, alanine, valine, and leucine and very little methionine and tryptophan, while cysteine is completely absent.

We have determined some physicochemical properties of the material obtained: the dependence of the activity on the pH, the temperature, the concentration of the substrate, and the amount of coenzyme. The optimum pH is 7.8-8.0, and the optimum temperature 30°C; oxaloacetic acid (substrate) in a concentration greater than $0.85 \cdot 10^{-3}$ M has an inhibiting action. The concentration of NAD · H_2 (enzyme) at which the maximum activity is observed is $1.70 \cdot 10^{-4}$ M.

In a comparison of the results obtained with those found for the isoenzyme isolated by precipitation with ammonium sulfate at 50-100% saturation it can be seen that there are appreciable differences only in the optimum pH (pH 9-10) [4].

EXPERIMENTAL

Extraction. The defatted flour from cotton seeds (50 g) was extracted with 500 ml of 0.005 M phosphate buffer, pH 7.4, containing $1 \cdot 10^{-3}$ M cysteine and $1 \cdot 10^{-3}$ M EDTA. Extraction was performed for 2-4 h with stirring at +4°C. The mixture was centrifuged at 18,000 rpm for 15 min.

Fractionation with Ammonium Sulfate. With slow stirring, 49.0 g of ammonium sulfate was added to 450 ml of the combined extract to give 20% saturation. The mixture was left for 2 h and was then centrifuged at 18,000 rpm for 30 min. The precipitate was removed. After the removal of the precipitate for 0-20% saturation, a further 80 g of ammonium sulfate was added to the supernatant liquid to give 50% saturation. The mixture was left for 4 h and was then centrifuged at 18,000 rpm for 30 min. The precipitate was then 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 (2.5 × 40 cm) of Sephadex G-25 previously equilibrated with 0.005

* The tryptophan content was determined by spectrophotometric methods [13, 14].

M phosphate buffer, pH 7.4. The rate of elution was 40 ml/h. The fractions were collected in an automatic collector in 5-ml amounts. The extinction of each fraction was measured on an SF-4A spectrophotometer at 280 nm.

Gel Filtration on Sephadex G-100. The desalted solution of 1500 mg of the protein was deposited on a column with a porous base (4.5 × 120 cm) filled with Sephadex G-100 and equilibrated with 0.005 M phosphate buffer, pH 7.4, containing $1 \cdot 10^{-3}$ M cysteine and $1 \cdot 10^{-3}$ M EDTA. Elution was performed with the same buffer. The rate of elution was 20 ml/h, the fraction volume 5 ml, and the temperature 0-4°C. The graph of the elution of the protein had two peaks (see Fig. 1a). The MDH activity in each test tube was monitored. The active fractions corresponded to the tailing side of the first peak.

Separation on DEAE-Cellulose. The active fraction (190 mg) after gel filtration (Sephadex G-100) was concentrated and deposited on a column of DEAE-cellulose (1.0 × 17 cm) equilibrated with 0.005 M phosphate buffer, pH 7.4, containing $1 \cdot 10^{-3}$ M cysteine and $1 \cdot 10^{-3}$ M EDTA. The rate of elution was 12 ml/h. Elution was performed first with the initial buffer (ten test tubes) and then with a linear gradient of sodium chloride from 0 to 0.3 M. The fractions were collected in an automatic collector in 3.0-ml portions. The extinction was determined on an SF-4A spectrophotometer at 280 nm. A graph of the spectrophotometric results was plotted (see Fig. 1b). Four peaks were obtained. The fractions relating to the individual peaks were combined, and their protein contents and activities were determined. The fraction of the third peak proved to be the most active.

Rechromatography on DEAE-Cellulose. The fraction of the third peak was dialyzed against 0.005 M phosphate buffer, pH 7.4, containing $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-3}$ M cysteine until the reaction for chloride ion was negative and was concentrated and deposited on a column of DEAE-cellulose equilibrated with the above-mentioned buffer. Elution was performed with a linear gradient of sodium chloride from 0 to 0.3 M. The elution graph showed only one symmetrical peak.

Determination of the Protein. The protein in the solutions was determined by the biuret method and by the Warburg–Christian method [15].

Determination of Enzyme Activity. The malate dehydrogenase and lactate dehydrogenase activities were measured spectrophotometrically with respect to the oxidation of NAD · H_2 and the decrease in the optical density at 340 nm as described previously [3, 4]. The aspartate transaminase activity was also determined by a method described previously [16].

Molecular-Weight Determination by Gel Filtration on Sephadex G-100. A column with a porous base (2.5 × 60 cm) was filled with Sephadex G-100 swollen in 0.1 M phosphate buffer, pH 7.4, and was washed with the same buffer for two days in order to compact it. The free volume of the column was determined by the passage of Dextran Blue (mol. wt. 300,000) through the column. The protein markers (for calibrating the column) were taken in an amount of 5 mg in 1 ml of buffer. The protein was eluted with 0.1 M phosphate buffer, pH 7.4. The rate of elution was 14 ml/h. The fractions were collected in 3.5-ml portions.

Ultracentrifugation was performed in a G-120 MOM ultracentrifuge using solutions prepared by dissolving 10 mg of protein in 1 ml of 0.1 M phosphate buffer, pH 7.4, at room temperature. The molecular weight determined by the sedimentation and the unestablished-equilibrium methods was 140,000 ± 5000, calculation being performed by Archibald's method [4] (see the sedimentogram of MDH from cotton seed in Fig. 3).

Amino-Acid Analysis. The malate dehydrogenase preparation (1 mg) was hydrolyzed in 6 N hydrochloric acid under vacuum at 105-110°C for 24 h. The amino acids were analyzed on a Biokal (GFR) type BC-200 amino-acid analyzer. The hydrolyzate from 1 mg of protein was dissolved in 4 ml of buffer with pH 2.2, and 0.5 ml of this solution was used for the analysis of the basic amino acids and 0.9 ml for the neutral and acidic amino acids (see Table 2).

Determination of the Tryptophan Content. A. The optical density of a solution of 2 mg of the enzyme in 4 ml of sodium hydroxide was measured at 280 and 294 nm on an SF-4A spectrophotometer. The molar ratio of tryptophan was calculated from the formula given by Goodwin and Morton [13].

B. To 2 mg of the enzyme were added 2 ml of acetic acid and a solution of $FeCl_3$ prepared in the following way: 0.27 g of $FeCl_3 \cdot 6H_2O$ was dissolved in 0.5 ml of water, the volume was made up to 1 liter with glacial acetic acid, and 2 ml of conc. H_2SO_4 was added. The mixture was shaken vigorously and was left for 15 min. Then the optical density of the solutions was measured at 545 nm. The amount of tryptophan in the protein was determined from a calibration curve [14].

Dependence of the MDH Activity on the Temperature. To 2.3 ml of 0.1 M phosphate buffer, pH 7.4, was added 0.1 ml of a solution of the enzyme (20 μg/ml). The mixture was kept at various temperatures (from 20 to 60°C in 5°C steps) for 10 min and was then rapidly brought to room temperature. The measurement of the activity began with the addition of the substrate and the coenzyme by the method described previously [3, 4].

Determination of the Optimum pH of the MDH. To determine the influence of the pH, various buffer solutions were prepared: citrate–phosphate buffers (pH 2-6.5), phosphate buffers (pH 6.5-8.0), and glycolic acid buffers (pH 8.5-12). The MDH activity was measured by the method described [3, 4], with the replacement of the 0.1 M phosphate buffer, pH 7.4, by the same amount (2.3 ml) of one of the buffers mentioned above.

Dependence of the Activity of MDH-I on the Concentration of Oxaloacetic Acid. The activity was measured by the method described [3, 4] at various concentrations of oxaloacetic acid (OAA). For this purpose, 4 mg of OAA was dissolved in 2 ml of distilled water and the solution was neutralized with 2% KOH solution. To the reaction mixture was added 0.01, 0.02, 0.03, 0.04, 0.05, 0.07, 0.09, 0.1, 0.12, 0.15, 0.2, 0.25, 0.3, and 0.35 ml of the prepared solution of OAA, the volume of the reaction mixture being kept constant (3 ml).

SUMMARY

1. From the seeds of cotton of variety 108-F a homogeneous preparation of malate dehydrogenase has been isolated. The molecular weight and amino-acid composition of the enzyme have been determined.

2. The optimum pH, the optimum temperature, and the dependence of the activity on the concentration of substrate and coenzyme have been determined for the purified enzyme.

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