Phytochemistry, 1974. Vol. 13, pp. 553 to 557. Pergamon Press. Printed in England.

SOLUBLE AND CHLOROPLAST MALATE DEHYDROGENASE ISOENZYMES OF *TRITICUM AESTIVUM**

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(*Revised Received* 15 *July* 1973. *Accepted* 24 *September* 1973)

Key Word Index—*Triticum aestivum*: Gramineae; wheat; isoenzymes of malate dehydrogenase; isolation; characterization.

Abstract—Three isoenzymes of malate dehydrogenase have been isolated from 9-day-old wheat shoots. The microbody (peroxisome) and chloroplast MDH are similar in their electrophoretic behaviour. The mitochondrial MDH, soluble MDH and chloroplast MDH differ in K_m values for malate and NAD. The activity of MDH isoenzymes with NAD^+-analogues as substrate was in the order 3-AP-NAD^+ > 3-AP-deam NAD^+ > NAD^+ > TN-NAD^+ and deam NAD^+. The thermal stabilities of the isoenzymes were significantly different: C-MDH > M-MDH > S-MDH.

INTRODUCTION

ISOENZYMES of malate dehydrogenase are known to be present in animals[1] and plants.[2] In both plant and animal tissues particulate (mitochondrial MDH) and non-particulate (soluble MDH) forms are known. The two enzymes are distinct, differing in amino acid composition,[3] immunologically,[4] structurally[5] and catalytically.[6] Also, there is considerable variation in the number of isoenzymes which have been reported for the same species. These range from two[7] to 10[8] in wheat, three[9] to 12[10] in maize, three[11] to four[12] in spinach, three[13,14] to five[15] in cotton. Again, a number of workers have found changes

* *Abbreviations*: 3-AP-deam-NAD^+—3-acetyl-pyridine-hypoxanthine-dinucleotide; 3-AP-NAD^+—3-acetyl-pyridine-adenine-dinucleotide; Deam-NAD^+—Nicotinamide-hypoxanthine-dinucleotide; TN-NAD^+—Thionicotinamide-adenine-dinucleotide; MDH, L-malate: NAD^+ Oxidoreductase, E.C. 1.1.1.37.

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in malate dehydrogenase isoenzymes during plant development[14,16–18] and that the enzymes are localized in specific cell organelles.[9–12,19–21] In the present work MDH isoenzymes have been studied in 9-day-old wheat shoots.

RESULTS

Intracellular localization of MDH isoenzymes

The homogenate prepared from nine-day-old shoots showed the presence of three isoenzymes of MDH having R_p 0·40, 0·44 and 0·50 on polyacrylamide gel electrophoresis (Fig. 1). The enzyme preparation from purified chloroplasts showed one band of MDH having

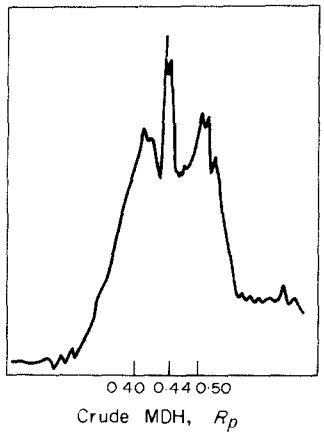

FIG. 1. DENSITOGRAM OF MDH ISOENZYMES.

R_p 0·40. The chloroplasts were isolated in the presence of polysaccharides, ficoll and dextran, which are known to prevent leaching because of their colloidal properties. It is therefore, evident that chloroplast contains only one MDH isoenzyme, designated as C-MDH.

Separation of soluble MDH isoenzymes

The MDH isoenzymes of the soluble fraction were purified and separated by initial ammonium sulphate precipitation, followed by DEAE-Sephadex chromatography. Most of the enzyme activity appeared in 40–80% ammonium sulphate fraction and this fraction was layered on a DEAE-Sephadex column. Stepwise elution showed a peak for microbody MDH (mb MDH) and a major peak with a shoulder (Fig. 2). Proteins from minor peak, upon electrophoresis showed one MDH band having R_p 0·40. The major peak was heterogenous; therefore, different fractions of this region were pooled and rechromatographed on DEAE-Sephadex. Gradient elution of the column resulted in one minor and two major peaks (Fig. 3), representing mitochondrial MDH (m-MDH) and soluble-MDH (S-MDH). Soluble MDH had a shoulder showing the presence of a second enzyme. On electrophoresis the fraction representing m-MDH showed one band of R_p 0·44 whereas fraction from

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S-MDH showed peak at R_p 0·50 and it had a shoulder again confirming the presence of second isoenzyme. However, these two could not be completely separated and all studies were done on pooled fractions.

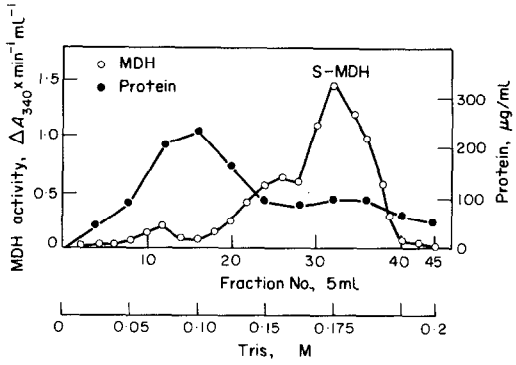

FIG. 2. DEAE-SEPHADEX A50 MDH ELUTION PROFILE. The column was eluted with a discontinuous gradient. For details see Experimental.

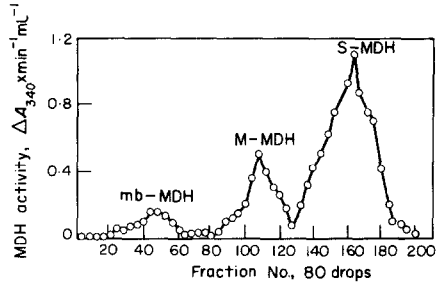

FIG. 3. DEAE-SEPHADEX A50 MDH ELUTION PROFILE. The column was eluted with a linear Tris-HCl gradient (0·075 → 0·2 M). For details see Experimental.

Characterization of MDH isoenzymes

All the three MDH isoenzymes of wheat were found to have pH optima of 8·8 and all the reactions have been studied in the direction of malate oxidation. All the three isoenzymes were NAD dependent and no NADP-MDH could be detected.

Effect of L-*malate and NAD concentration*

Double reciprocal plots were linear and Michaelis constants for malate for different isoenzymes differed slightly (Table 1). C-MDH showed more affinity for L-malate compared to S-MDH, which in turn had higher affinity than m-MDH. Michaelis constants for NAD^+ (Table 1) of the three isoenzymes did not differ significantly.

TABLE 1. MICHAELIS CONSTANTS FOR THE THREE NAD^+-MALATE DEHYDROGENASE ISOENZYMES IN *Triticum aestivum* SHOOTS*

Isoenzyme	Substrate	K_m†	Isoenzyme	Substrate	K_m†
m-MDH	L-malate	3·3	m-MDH	NAD^+	0·71
S-MDH	L-malate	2·0	S-MDH	NAD^+	0·66
C-MDH	L-malate	1·0	C-MDH	NAD^+	0·80

* Calculation from double-reciprocal plots.
† Constants expressed as mM concentration.

Effect of NAD^+-analogues

All the isoenzymes were less active in the presence of deam-NAD^+ and TN-NAD^+ than in the presence of NAD^+ (Table 2). Activity of m-MDH and C-MDH was almost 50% in the presence of TN-NAD^+ and deam-NAD^+, whereas the activity of S-MDH was found to be only 25%. 3-AP-NAD^+ and 3-AP-deam NAD^+ appeared to be better substrates for all the three isoenzymes than NAD^+. The activity was 3·5 times when 3-AP-NAD^+ was used as coenzyme, when 3-AP-deam-NAD^+ was used as coenzyme the activity was twice for m-MDH, thrice for C-MDH and 1·7 times for S-MDH. These data suggest

some kinetic differences among the isoenzymes. Effects of different NAD^+-analogue concentrations on different isoenzymes were determined (Fig. 4). For m-MDH, (Fig. 4a) the curves obtained with 3-AP-NAD^+ appeared to give a typical hyperbola, but curves obtained with 3-AP-deam NAD^+ and deam-NAD^+ were not hyperbolic. With deam-NAD^+ it was hyperbolic (Fig. 4b) for S-MDH. For C-MDH the plot with 3-AP-NAD^+ and 3-AP-deam-NAD^+ were normal hyperbolae, whereas that with deam-NAD^+ was not a normal hyperbola (Fig. 4c).

TABLE 2. RATE OF REACTION OF NAD^+-MALATE DEHYDROGENASE ISOENZYMES IN THE PRESENCE OF NAD^+-ANALOGUES

Analogue	Wave-length (nm)	m-MDH	S-MDH	C-MDH
NAD^+	340	1·00	1·00	1·00
Deam-NAD^+	340	0·51	0·27	0·67
TN-NAD^+	400	0·42	0·21	0·51
3-AP-NAD^+	365	3·68	3·52	3·84
3-AP-deam-NAD^+	365	2·11	1·73	2·81

Data are ratios of initial rates in the presence of an analogue relative to the initial rate in the presence of NAD^+.

Thermal stability

Chloroplast MDH was found to be more thermostable compared to m-MDH and S-MDH. Inactivation is linear when graphed semilogarithmically. Half times for inactivation were 1·3, 1·5 and 3·0 min for S-MDH m-MDH and C-MDH respectively.

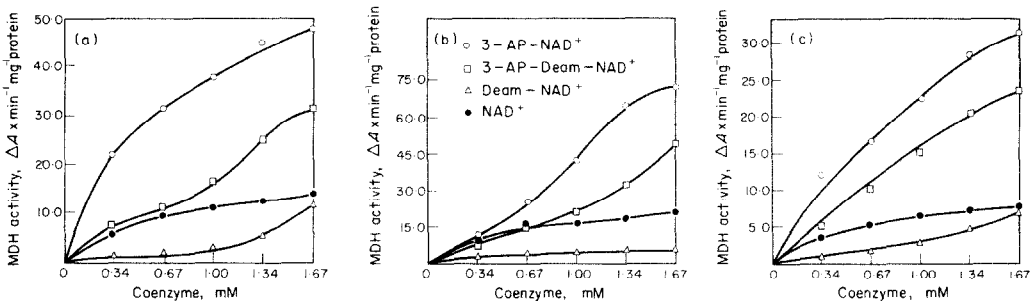

FIG. 4. EFFECT OF NAD^+-ANALOGUE CONCENTRATION ON MDH ISOENZYMES.

DISCUSSION

The physical properties of the wheat malate dehydrogenases are significantly different as indicated by their separation by polyacrylamide gel electrophoresis and DEAE-Sephadex anion exchange chromatography. The relative electrophoretic mobility order is S-MDH > m-MDH > C-MDH/mb-MDH. The chloroplast and microbody MDH were found to be similar in their electrophoretic properties. Also the soluble MDH was found to have a shoulder showing the presence of two soluble MDH isoenzymes.

The activities of isoenzymes in the presence of 3-AP-NAD^+ and 3-AP-deamino-NAD^+ are greater than with NAD^+ as cofactor. Identical results have been obtained for spinach[22] and corn tissue.[23] Thionicotinamide analogue of NAD^+ and deamino NAD^+ were not as effective cofactors for the MDH isoenzymes as NAD.[22,23]

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Our results are slightly at variance with those of Yamazaki and Tolbert,[11] in that they did not detect any chloroplast MDH in spinach and wheat. In the present investigation MDH isoenzyme has been detected in a purified chloroplast preparation. The C-MDH and mb-MDH have similar electrophoretic mobility. We have employed Ficoll and dextran, which prevent leaching from chloroplasts. It is already known that considerable leaching losses occur during chloroplast isolation and this could perhaps be responsible for the failure of some workers to detect C-MDH. This is further supported by our observation that chloroplast isolated and purified on sucrose medium instead of Honda medium show very low MDH activity. No NADP-MDH was detected in chloroplasts from wheat. The exact physiological role of C-MDH must await further experimentation. Differences in kinetic properties are not striking, despite the fact that they are demonstrable. Activity with NAD-analogues was quite similar; we assume that their biological functions are also similar.

EXPERIMENTAL

Raising of seedlings. Wheat seeds (*Triticum aestivum* L. cv Kalyan Sona) were grown at 25° in vermiculite for 9 days in a germinating chamber.

Isolation of chloroplast. Freshly harvested shoots from 9-day-old seedlings were chilled, cut and mixed with Honda medium (Ficoll 2·5%, Dextran 5%, sucrose 0·25 M, Tris 25 mM, $MgCl_2$ 1·0 mM, 2-mercaptoethanol 4·0 mM, pH 7·8) in a ratio of 1:2 (w/v) and homogenized in chilled pestle–mortar. The chloroplasts were isolated and purified on sucrose gradient in Honda Medium as described by Hadziyev *et al.*[24] All operations were carried out at 4° unless otherwise stated.

Extraction of chloroplast-MDH. MDH from purified chloroplasts was extracted by dispersing the pellet for 1 hr in hypotonic 10·0 mM Tricine buffer (pH 7·8) containing 2 mM dithiothreitol, and supernatant containing MDH was obtained by centrifugation at 10 000 *g* for 30 min.

Protein estimation. Proteins were estimated using Folin–Ciocalteu reagent[25] and crystalline bovine serum albumin as standard.

Fractionation of soluble MDH-isoenzymes. The supernatant after sedimenting chloroplasts was centrifuged at 20 000 *g* for 30 min and the supernatant concentrated by $(NH_4)_2SO_4$ precipitation (0·4–0·8 of saturation). The precipitated protein was resuspended in Tris-HCl buffer (0·05 M, pH 7·4) containing 4 mM 2-mercaptoethanol and dialysed overnight against three changes of the same buffer. Five millilitres (about 100 mg protein) were layered on a DEAE-Sephadex A50 column (2·5 × 25 cm) equilibrated with 0·04 M Tris-HCl buffer (pH 8·5). The column was eluted with 40 ml of each of 0·05, 0·1, 0·15, 0·175 and 0·2 M Tris-HCl buffer (pH 8·5) containing 4 mM 2-mercaptoethanol. Five-millilitre fractions were collected and assayed for MDH-activity. Three peaks of enzyme activity were obtained. One sharp peak representing mb-MDH and the two overlapping peaks were observed. Fractions from the two overlapped peaks were pooled and further chromatographed on a DEAE-Sephadex A50 column (2·5 × 30 cm) equilibrated with 0·05 M Tris-HCl (pH 8·5) buffer. The column was eluted with a linear Tris-HCl (pH 8·5) gradient (0·075, 0·2 M) containing 4 mM 2-mercaptoethanol. Fractions of 80 drops each were collected, and assayed for MDH activity.

Enzyme assay. MDH activity was measured following the reduction of NAD^+ at 340 nm at 37°. The reaction mixture contained (vol. 3 ml), $MgCl_2$ 1 mM, NaCN 1 mM, 2-mercaptoethanol 5 mM, EDTA 5·0 mM, NAD^+ 1·34 mM, L-malate, 10 mM, Tris 50 mM, pH 8·8. Activity of MDH has been expressed as $\Delta A_{340} \times min^{-1}\ mg^{-1}$ protein.

Electrophoresis. Electrophoresis was done in 7·5% acrylamide using an anionic system.[26] Isoenzymes of MDH were detected by zymogram staining technique using nitro blue tetrazolium and phenazine methosulphate.[27] Gels were scanned in Joyce–Loebl chromoscan.

Acknowledgements—The authors are grateful to Dr. N. P. Datta, Project Director, Nuclear Research Laboratory, Indian Agricultural Research Institute, and to the Indian Council of Agricultural Research for awarding a Senior Research Fellowship to H. S. N.

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