CAM　Mesembyanthemum crystallinum L.　NAD-

ISSN	00111848
	， ， 　， ， ，
／	64　4
	p. 760-766
	1995　12

Purification and Properties of NAD-Dependent Malate Dehydrogenase from *Mesembryanthemum crystallinum* L. Exhibiting Crassulacean Acid Metabolism

Kazuyuki SAITOU, Waichi AGATA, Takeshi KAWARABATA,
Yuko YAMAMOTO and Fumitake KUBOTA

(*Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812, Japan*)
Received October 31, 1994

Abstract : By DEAE-cellulose anion exchange column chromatography, three different forms of NAD-dependent malate dehydrogenase (EC 1.1.1.37) (NAD-MDH) were isolated from leaves of *Mesembryanthemum crystallinum* L. in the crassulacean acid metabolism mode. NAD-MDH that eluted last from an anion exchange column was purified to a specific activity of 1,096 units • (mg protein)$^{-1}$ using Blue Sepharose CL-6B chromatography. Citrate and adenosine 5′-triphosphate effectively inhibited the activity of NAD-MDH. The inhibition of the enzymatic activity by citrate was reversed by inorganic phosphate and the degree of reversal increased with increasing the concentration of oxaloacetate, the substrate of the reaction. The optimal pH for NAD-MDH activity was around 7.5. Citrate inhibited the enzymatic activity over a wide range of pH and caused a shift in the optimal pH. The enzymatic activity in the presence of citrate was increased by adding inorganic phosphate below pH 7.5. On the basic side, higher than pH 7.5, however, the inhibition by citrate was enhanced by adding inorganic phosphate.
Key words : Crassulacean acid metabolism, Enzyme purification, *Mesembryanthemum crystallinum* L., NAD-dependent malate dehydrogenase (EC 1.1.1.37).

CAM 型 *Mesembryanthemum crystallinum* L.の NAD-リンゴ酸脱水素酵素の精製と性質：斎藤和幸・縣和一・河原畑勇・山本優子・窪田文武（九州大学農学部）

要 旨：CAM 型光合成を行っている *Mesembryanthemum crystallinum* L.の葉身より NAD-リンゴ酸水素酵素 (EC 1.1.1.37) (NAD-MDH) を抽出し，DEAE-セルロース陰イオン交換カラムによりクロマトグラフィーを行ったところ，三つの異なった NAD-MDH が分離された．陰イオン交換クロマトグラフィーにより最後に溶出した NAD-MDH を Blue Sepharose CL-6 B を用いたアフィニティークロマトグラフィーによって 1,096 ユニット/mg タンパク質の比活性まで精製した．クエン酸及びアデノシン 3 燐酸は NAD-MDH 活性を効果的に阻害した．pH 7.0 でのクエン酸による NAD-MDH の活性阻害は無機燐酸により解除され，解除の程度は基質であるオキザロ酢酸の濃度の増加とともに大きくなった．NAD-MDH 活性の最適 pH は約 7.5 であった．クエン酸は広い範囲の pH で NAD-MDH 活性を阻害し，NAD-MDH 活性の最適 pH のシフトを引き起こした．pH 7.5 以下でクエン酸存在下の NAD-MDH 活性は無機燐酸の添加によって活性が高まった．しかし，pH 7.5 以上では，クエン酸存在下での NAD-MDH 活性は無機燐酸の添加によってさらに阻害された．

キーワード：NAD-リンゴ酸脱水素酵素 (EC 1.1.1.37)，酵素の精製，*Mesembryanthemum crystallinum* L，ベンケイソウ型酸代謝．

Higher plants contain various nicotinamide-adenine dinucleotide (NAD) dependent isoforms of malate dehydrogenase (L-malate : NAD oxidoreductase, EC 1.1.1.37) (NAD-MDH) that catalyze the reduction of oxaloacetate to malate. The NAD-MDH isoforms are involved in different metabolic functions, i.e. the glyoxylate pathway, the tricarboxylic acid cycle and the malate/aspartate shuttle[13]. The individual forms exhibit different molecular properties[24,25], and they can be separated by polyacrylamide gel electrophoresis, isoelectric focusing, or other techniques[23]. The association of the isoenzymes with different cellular compartments has been established by electrophoretic analysis of cell organelles which were purified by sucrose density gradient centrifugation[9].

We are using the halophyte *Mesembryanthemum crystallinum* L. Exposure of the plants to

---

high soil salinity causes a shift in the mode of carbon assimilation from that typical of C_3 plants to that typical of crassulacean acid metabolism (CAM) plants, which exhibit substantial net carbon gain at night[27]. In CAM plants, β-carboxylation of phosphoenolpyruvate catalyzed by PEPCase is the initiating reaction of CAM, followed by the reduction of oxaloacetate to malate by NAD-MDH[19]. Decarboxylation of malate via malic enzyme prevails in the light and malate synthesis during the light period is inhibited. Feed-back inhibition of PEPCase by malate is assumed to be one of the key factors regulating the day/night rhythm of acidification and deacidification in tissues exhibiting CAM[11,29]. It has been proposed that inorganic phosphate regulates malate accumulation through the promotion of the activity of PEPCase[21]. However, the relationship between properties of NAD-MDH and the physiology of CAM are poorly understood. The present report describes purification and properties of an NAD-MDH from leaves of *M. crystallinum* in the CAM mode. Special attention has been paid to the regulatory function of NAD-MDH in tissues exhibiting CAM.

Materials and Methods

1. Plant materials

Plants were grown from seeds of *Mesembryanthemum crystallinum* L. as described previously[17]. All plants were kept in a growth cabinet with a light period of 12 h, a photon flux density (400-700 nm) of 250 μmol m^{-2} s^{-1} on plant level, an air temperature of 25°C and a relative humidity of 70%. Six-week-old plants that had been maintained under hydroponic conditions were used for induction of CAM. CAM was induced by including 400 mM NaCl in the root medium for fourteen days[17]. The fully developed leaves were harvested 4 h before the end of the light period. The leaves were frozen with liquid nitrogen and stored at -55°C until use.

2. Purification of NAD-MDH

The following steps were carried out at 0 to 4°C. Leaves (about 300 g) of *M. crystallinum* in the CAM mode were chopped into fine pieces and then ground with a chilled mortar and pestle in 3 volumes of extraction buffer that consisted of 100 mM Tris-acetic acid (pH 8.0), 2.5 mM MgCl$_2$ and 12.5% (w/v) glycer-

ol. After filtration of the extract through four layers of gauze, the filtrate was centrifuged at 10,000×g for 30 min. To the supernatant fraction, crystalline ammonium sulfate was gradually added up to 55% saturation. After centrifugation at 10,000×g for 30 min, the supernatant was brought to 75% saturation with ammonium sulfate. After centrifugation at 10,000×g for 30 min, the pellet was resuspended in about 18 mL of buffer A that contained 20 mM Tris-acetic acid (pH 7.4), 2.5 mM MgCl$_2$ and 10% (w/v) glycerol, and centrifuged at 10,000×g for 10 min. The supernatant fraction was passed through a NAP column (Pharmacia Biotech, Uppsala, Sweden; 1.5 cm inside diameter×5 cm) which had previously been equilibrated with buffer A. The eluate was then applied to a column of DE 52 (Whatman, Maidstone, England; 1.6 cm i.d.×10 cm) that had previously been equilibrated with buffer A. The column was washed with 200 mL of the same buffer. The enzyme was eluted with a linear 200-mL gradient of KCl (0-200 mM) in buffer A. Fractions of 2 mL were collected at a flow rate of 180 mL $\cdot$ h^{-1}. The peak fractions with enzymatic activity were pooled and were passed through a NAP column which had previously been equilibrated with buffer A. The eluate was chromatographed on a column of Blue Sepharose CL-6B (Pharmacia Biotech; 1.6 cm i.d.×12 cm) that had previously been equilibrated with buffer A. The enzyme was eluted with a linear 200-mL gradient of NADH (0-0.4 mM) in buffer A. The most active fractions were pooled and stored at 4°C.

3. Assay of NAD-MDH and units of enzymatic activity

The enzyme was assayed by following the oxaloacetate-dependent oxidation of NADH at 340 nm[12]. The assay condition was modified from the refferred source to give optimum activities in extracts from *M. crystallinum*. The standard reaction mixture (1.0 mL) contained 100 mM TES-Tris (pH 7.5), 0.12 mM NADH and 0.5 mM oxaloacetate. The reaction was initiated by the addition of oxaloacetate and carried out at 30°C. One unit of enzyme was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADH per min under these conditions.

4. Quantitation of protein

Protein concentrations were determined

Table 1. Purification of NAD–MDH from leaves of *M. crystallinum* in the CAM mode.

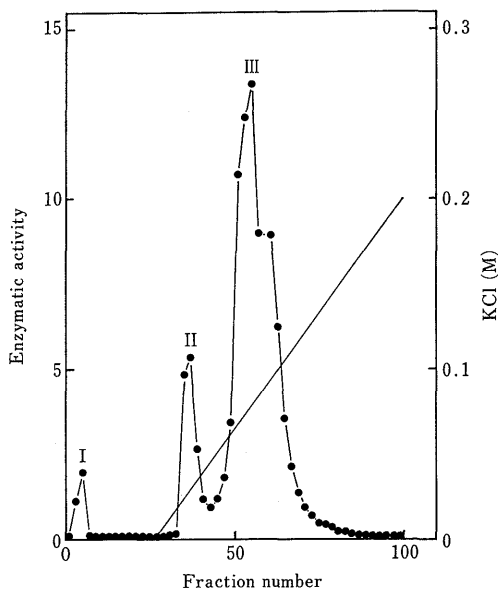| Purification step	Total volume (mL)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units mg^{-1})	Purification (fold)
Crude extract	540	297	5,296	100.0	17.8	1.0
Ammonium sulfate, 55-75% fraction	72	104	4,937	93.2	47.5	2.7
DE 52	42	6.48	1,851	35.0	285.6	16.0
Blue Sepharose CL-6B	36	0.24	263	5.0	1,095.8	61.6

Fig. 1. Elution profile of ion exchange chromatography on DE 52 of NAD-MDH from leaves of *M. crystallinum* in the CAM mode. Circles, activity of NAD-MDH; solid line, concentration of KCl. Note that enzymatic activity is expressed in arbitrary units.

spectrophotometrically at 595 nm by the method of Bradford[3] with Coomassie brilliant blue G-250 (Bio-Rad Co., Richmond, CA). A standard curve was established using bovine serum albumin.

Results

The purification of NAD-MDH from leaves of *Mesembryanthemum crystallinum* in the CAM mode is summarized in Table 1. The enzyme was predominantly recovered in the 55-75% ammonium sulfate precipitate. The enzyme was then applied to a column of DE 52. Peak I shown in Fig. 1 was not adsorbed onto the column of DE 52. The remaining adsorbed activity could be eluted with a linear gradient

of KCl (0-200 mM) in buffer A (Fig. 1). Two distinct NAD-MDH protein peaks were obtained. The peak III fractions were pooled and applied to a column of Blue Sepharose CL-6B. A single peak was eluted with a linear gradient of NADH (0-0.4 mM) (data not shown). The enzyme was purified 62-fold to a final specific activity of 1,096 units • (mg protein)$^{-1}$.

The effects of various organic acids on the activity of the purified NAD-MDH were investigated in the presence of 0.5 mM oxaloacetate at pH 7.0 (Table 2). Minimal effects were observed upon inclusion, at 2 mM, of the following compounds in the standard assay mixture: glucose-6-phosphate, fructose-6-phosphate, fructose-1, 6-bisphosphate, fructose-2, 6-bisphosphate, dihydroxyacetone phosphate, glycerate-3-phosphate, glycerate-2-phosphate, succinate, fumarate, adenosine 5'-monophosphate, inorganic phosphate, L-alanine and glutarate. Phosphoenolpyruvate, glycerate-2, 3-diphosphate, pyruvate, 2-ketoglutarate, glyoxylate, L-malate, acetyl-CoA and adenosine 5'-diphosphate caused 20-40% inhibition of the enzymatic activity. In the presence of citrate, the enzymatic activity was inhibited by 43%. ATP caused a 54% reduction of the enzymatic activity.

Because citrate and ATP inhibited the activity of NAD-MDH more than the other organic acids tested, a more detailed study of the inhibition by citrate and ATP was performed. The effect of the concentration of citrate or ATP on the activity of NAD-MDH from *M. crystallinum* are shown in Fig. 2. When the assays were carried out at pH 7.0 in the presence of 0.25 mM oxaloacetate, the inhibitory effects of citrate and ATP were increased with increasing concentrations of citrate and ATP.

The optimal pH for the activity of NAD-MDH was around 7.5 (Fig. 3). This value was

Table 2. Effect of various solutes on the relative activity of NAD-MDH from *M. crystallinum* in the CAM mode.

Additions*	Relative activity (%)**
Glucose-6-phosphate	90.4
Fructose-6-phosphate	90.4
Fructose-1,6-bisphosphate	87.6
Fructose-2,6-bisphosphate	93.2
Dihydroxyacetone phosphate	82.3
Glycerate-2,3-diphosphate	63.5
Glycerate-3-phosphate	87.6
Glycerate-2-phosphate	82.3
Phosphoenolpyruvate	66.6
Pyruvate	62.0
Citrate	57.4
2-Ketoglutarate	74.6
Glyoxylate	78.7
Succinate	90.4
Fumarate	100.0
L-Malate	78.1
Acetyl-CoA	67.3
Adenosin 5'-triphosphate	45.5
Adenosine 5'-diphosphate	64.5
Adenosine 5'-monophosphate	91.1
Inorganic phosphate	100.0
L-Alanine	90.4
L-Glutarate	96.6

*The concentration of various solutes was 2 mM.
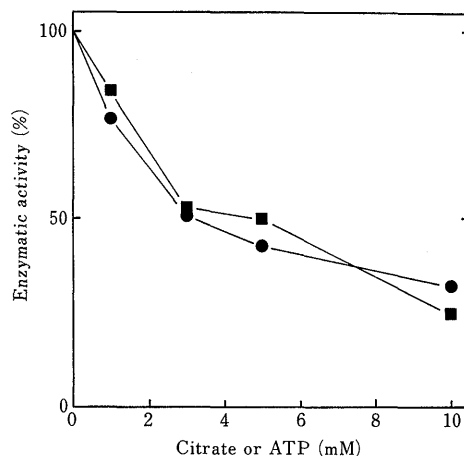**The activity in the absence of various solutes was taken as 100%.

Fig. 2. Effects of the concentration of citrate (●) or ATP (■) on the activity of NAD-MDH. Enzyme was assayed at pH 7.0 in the presence of 0.25 mM oxaloacetate.
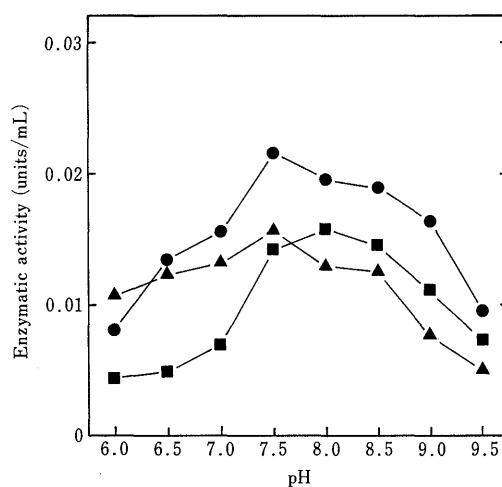
Fig. 3. Effects of pH on the activity of NAD-MDH in the presence of 3 mM citrate or 3 mM citrate plus 40 mM inorganic phosphate. Enzyme was assayed in the presence of 0.25 mM oxaloacetate. ●, Control assay; ■, 3 mM citrate; ▲, 3 mM citrate plus 40 mM inorganic phosphate.

consistent with the value reported for the analogous enzyme from pea seeds[26] and French bean[20]. Over a wide range of pH, citrate inhibited the activity of NAD-MDH. Citrate caused a shift in the optimal pH. The enzymatic activity in the presence of 3 mM citrate culminated at pH values around 8. The same result was obtained in the presence of 3 mM ATP (data not shown).

It was observed that the inhibition of the enzymatic activity by 3 mM citrate was completely reversed by 40 mM inorganic phosphate (Fig. 4 and see also Fig. 6). The inhibition of the enzymatic activity by 3 mM ATP was also completely reversed by 80 mM inorganic phosphate. When citrate and ATP were not included in the reaction mixture, however, inorganic phosphate did not affect the enzymatic activity at pH 7.0 in the presence of 0.25 mM oxaloacetate (Fig. 5). The enzymatic activity in the presence of citrate

was increased by adding inorganic phosphate below pH 7.5 (Fig. 3). On the basic side, higher than pH 7.5, however, the enzymatic activity in the presence of citrate was reduced by adding inorganic phosphate. The enzymatic activity in the presence of 3 mM citrate and 40 mM inorganic phosphate culminated at pH values around 7.5. These

Fig. 4. Effect of the concentration of inorganic phosphate on the activity of NAD-MDH in the presence of 3 mM citrate (●) or 3 mM ATP (■). Enzyme was assayed at pH 7.0 in the presence of 0.25 mM oxaloacetate.

Fig. 5. Effect of the concentration of inorganic phosphate on the activity of NAD-MDH. Enzyme was assayed at pH 7.0 in the presence of 0.25 mM oxaloacetate.
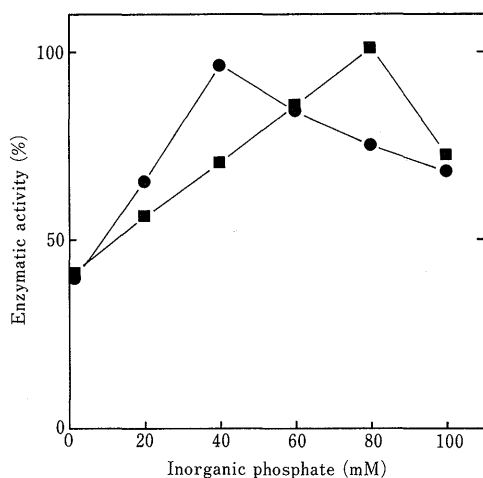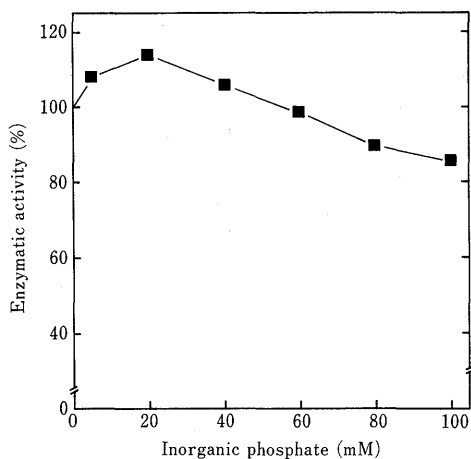
values were consistent with the optimal pH for the enzymatic activity.

When the initial velocity of the reaction was studied as a function of the concentration of oxaloacetate, in the presence of saturating concentration of NADH (0.12 mM), a hyperbolic response was obtained (Fig. 6). There was no apparent oxaloacetate substrate inhibition below the concentration of 0.5 mM at pH 7.5. The inhibition of the activity of NAD-MDH by citrate was not influenced by the
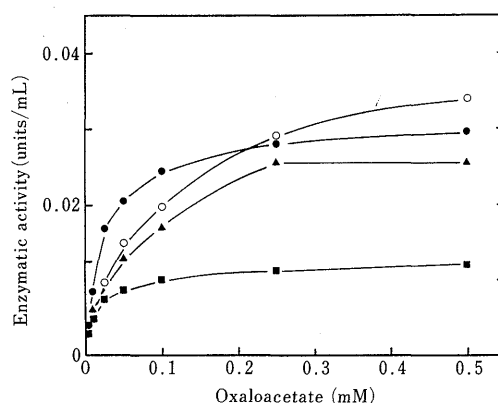

Fig. 6. Effect of the concentration of oxaloacetate on the activity of NAD-MDH at pH 7.0 in the presence of 3 mM citrate, 40 mM inorganic phosphate or 3 mM citrate plus 40 mM inorganic phosphate. ●, Control assay; ■, 3 mM citrate; ○, 40 mM inorganic phosphate; ▲, 3 mM citrate plus 40 mM inorganic phosphate.

concentration of oxaloacetate. The inhibition of the enzymatic activity by citrate was reversed by inorganic phosphate and the degree of reversal increased with increasing in the concentration of oxaloacetate; it was observed that NAD-MDH recovered about 100% of the enzymatic activity in the absence of citrate above the concentration of 0.25 mM oxaloacetate. The enzymatic activity was inhibited by inorganic phosphate below the concentration of 0.25 mM oxaloacetate, while slight stimulation of the enzymatic activity by inorganic phosphate was observed above the concentration of 0.25 mM oxaloacetate.

Discussion

Multiple forms of NAD-MDH have been reported in numerous plant tissues[1,2,14,15,16,18,30, 31,32]. It has been reported that spinach[32] and pea leaf tissue[31] and green stem tissue of *Opuntia ficus-indica*[14] contain at least three unique forms of the enzyme, one associated with the leaf microbodies, second form associated with the mitochondria, and third nonparticulate form was detected as the soluble or cytosol isoenzyme. The three NAD-MDH proteins were separated on a DEAE-cellulose column, and the cytoplasmic form was eluted from DEAE-cellulose column later than the microbody form and the mitochondrial form[14,32]. Three different NAD-MDH

isoenzymes were isolated from green leaf tissue of *M. crystallinum* in the CAM mode by DEAE-cellulose anion exchange column chromatography (Fig. 1). The present report describes the properties of NAD-MDH that was most strongly retained by DEAE-cellulose. Although the total activity of NAD-MDH in extracts from leaves in the C_3 mode was a third of that in extracts from leaves in the CAM mode, extracts from leaves in the C_3 mode exhibited almost identical behaviour to those from leaves in the CAM mode when chromatographed on a DEAE-cellulose column (data not shown).

NAD-MDH from leaves in the CAM mode was purified about 62-fold to a specific activity of 1,096 units • (mg protein)$^{-1}$. It has been reported the specific activities of purified mitochondrial and soluble isoenzymes of NAD-MDH from *Zea mays* to be 800 and 700 units • (mg protein)$^{-1}$, respectively[5]. NAD-MDH purified to apparent electrophoretic homogeneity from animal sources exhibited a range of specific activities from 300 to 1,400[6,7,8,10]. Thus, the purified NAD-MDH in this report appears to have a higher specific activity by comparison.

Cennamo et al.[4] reported that commercially obtained pig heart mitochondrial NAD-MDH was specifically inhibited by 3.3 mM fumarate and citrate, but the soluble form was not inhibited. Mukerji and Ting[14] reported that no significant differences were apparent in the inhibition patterns among NAD-MDH isoenzymes in green stem tissue of *Opuntia ficus-indica* and metabolites such as 2-ketoglutarate, *cis*-aconitate, citrate, DL-isocitrate, succinate, fumarate, aspartate, glutamate and maleate partially inhibited the isoenzymes. The isoenzymes were inhibited by 2-ketoglutarate more than the other metabolites tested. In the present study, the activity of NAD-MDH from leaves of *M. crystallinum* in the CAM mode was inhibited by citrate more than 2-ketoglutarate. It is likely that the properties of the enzymes differ depending on the source of enzyme, and that the difference in the properties is relevant for the difference in the regulation of enzymatic activity *in vivo*. The importance of such physiological effect remains to be determined.

In the present study, we found that the inhibition of the activity of NAD-MDH by

citrate was completely reversed by 40 mM inorganic phosphate (Figs. 4 and 6). *M. crystallinum* in the CAM mode accumulates substantial amounts of citrate and isocitrate in the mesophyll cells[28]. Accordingly, the activity of NAD-MDH might be inhibited in the mesophyll cells of *M. crystallinum* in the CAM mode. It has been reported that inorganic phosphate (appropriate concentration of 30 mM) restores malate-caused inhibition of the activity of PEPCase isolated from *M. crystallinum*[22], and that inorganic phosphate regulates malate accumulation through the promotion of the activity of PEPCase[21]. It is likely that inorganic phosphate regulates malate accumulation through the promotion of the activity of NAD-MDH as well as of PEPCase. Further experimentation is required to substantiate this hypothesis.

It was reported that NAD-MDH of spinach and pea showed oxaloacetate substrate inhibition at pH 6.0 and 7.5[16,31], and that these phenomena were absent in the purified enzyme[32]. NAD-MDH purified from leaves of *M. crystallinum* in the CAM mode did not show oxaloacetate substrate inhibition (Fig. 6). This difference could be mainly adjudged to the purification state of the enzyme as well as to the different experimental conditions.

References

1. Abou-Zamzam, A.M. and A. Wallace 1970. Some characteristics of the mitochondrial and soluble forms of malate dehydrogenase in lemon fruits. Biochim. Biophys. Acta 220 : 396—409.
2. Asahi, T. and M. Nishimura 1973. Regulatory function of malate dehydrogenase isoenzymes in the cotyledons of mung bean. J. Biochem. 73 : 217—225.
3. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72 : 248—254.
4. Cennamo, C., G. Montecuccoli and G. König 1967. Inhibition of mitochondrial malate dehydrogenase by citrate. Biochim. Biophys. Acta 139 : 514—516.
5. Curry, R.A. and I.P. Ting 1973. Purification and crystallization of three isoenzymes of malate dehydrogenase from *Zea mays* seed. Arch. Biochem. Biophys. 158 : 213—224.
6. Dupourque, D. and E. Kun 1969. Cytoplasmic and mitochondrial malate dehydrogenases from beef kidney. In Lowenstein J.M. ed., Methods in

Enzymology. Academic Press, New York. Vol. XIII : 116—122.

7. Englard, S. 1969. Extramitochondrial L-malate dehydrogenase of beef heart. In Lowenstein J.M. ed., Methods in Enzymology. Academic Press, New York. Vol. XIII : 123—129.

8. Englard, S. and L. Siegel 1969. Mitochondrial L-malate dehydrogenase of beef heart. In Lowenstein J.M. ed., Methods in Enzymology. Academic Press, New York. Vol. XIII : 99—106.

9. Hock, B. 1973. Kompartimentierung und Eigenschaften der Malatdehydrogenase-Isoenzyme aus Wassermelonenkeimblättern. Planta 112 : 137—148.

10. Kitto, G.B. 1969. Intra- and extramitochondrial malate dehydrogenases from chicken and tuna heart. In Lowenstein J.M. ed. Methods in Enzymology. Academic Press, New York. Vol. XIII : 106—116.

11. Kluge, M. and C.B. Osmond 1972. Studies on phosphoenolpyruvate carboxylase and other enzymes of crassulacean acid metabolism of *Bryophyllum tubiflorum* and *Sedum praealtum*. Z. Pflanzenphysiol. 66 : 97—105.

12. Lavergne, D., S. Bouraima, S. Tostain and M.-L. Champigny 1986. Thermal sensitivity of NAD-malate dehydrogenase isoforms from *Various pennisetum* ecotypes. Plant Sci. 45 : 87—94.

13. Mettler, I.J. and H. Beevers 1980. Oxidation of NADH in glyoxysomes by a malate-aspartate shuttle. Plant Physiol. 66 : 555—560.

14. Mukerji, S.K. and I.P. Ting 1969. Malic dehydrogenase isoenzymes in green stem tissue of *Opuntia*: isolation and characterization. Arch. Biochem. Biophys. 131 : 336—351.

15. Peak, M.J., J.G. Peak and I.P. Ting 1972. Isoenzymes of malate dehydrogenase and their regulation in *Euglena gracilisz*. Biochim. Biophys. Acta 284 : 1—15.

16. Rocha, V. and I.P. Ting 1971. Malate dehydrogenases of leaf tissue from *Spinacia oleracea*: properties of three isoenzymes. Arch. Biochem. Biophys. 147 : 114—122.

17. Saitou, K., Y. Nakamura, Y. Kawamitsu, M. Matsuoka, M. Samejima and W. Agata 1991. Changes in activities and levels of pyruvate, orthophosphate dikinase with induction of crassulacean acid metabolism in *Mesembryanthemum crystallinum* L. Jpn. J. Crop Sci. 60 : 146—152.

18. Ting, I.P. 1968. Malic dehydrogenase in corn root tips. Arch. Biochem. Biophys. 126 : 1—7.

19. Ting, I.P. 1985. Crassulacean acid metabolism. Annu. Rev. Plant Physiol. 36 : 595—622.

20. Vidal, J. and P. Gadal 1977. NADH and NADPH dependent malate dehydrogenases of *Phaseolus vulgaris*. Physiol. Plant. 39 : 190—195.

21. von Willert, D.J. 1975. Stomatal control, osmotic potential and the role of inorganic phosphate in the regulation of the crassulacean acid metabolism in *Mesembryanthemum crystallinum*. Plant Sci. Lett. 4 : 225—229.

22. von Willert, D.J. 1975. The role of inorganic phosphate in the regulation of the phosphoenolpyruvate carboxylase of *Mesembryanthemum crystallinum* L. Planta 122 : 273—280.

23. Walk, R.A. and B. Hock 1976. Separation of malate dehydrogenase isoenzymes by affinity chromatography on 5'-AMP-Sepharose. Eur. J. Biochem. 71 : 25—32.

24. Walk, R.A. and B. Hock 1977. Glyoxysomal and mitochondrial malate dehydrogenase of watermelon (*Citrullus vulgaris*) cotyledons. II. Kinetic properties of the purified isoenzymes. Planta 136 : 221—228.

25. Walk, R.A., S. Michaeli and B. Hock 1977. Glyoxysomal and mitochondrial malate dehydrogenase of watermelon (*Citrullus vulgaris*) cotyledons. I. Molecular properties of the purified isoenzymes. Planta 136 : 211—220.

26. Weimberg, K. 1967. Effect of sodium chloride on the activity of a soluble malate dehydrogenase from pea seeds. J. Biol. Chem. 242 : 3000—3006.

27. Winter, K. and D.J. von Willert 1972. NaCl-induzierter Crassulaceen-Säurestoffwechsel bei *Mesembryanthemum crystallinum*. Z. Pflanzenphysiol. 67 : 166—170.

28. Winter, K., G.E. Edwards and J.A.M. Holtum 1981. Nocturnal accumulation of malic acid occurs in mesophyll tissue without proton transport to epidermal tissue in the inducible crassulacean acid metabolism plant *Mesembryanthemum crystallinum*. Plant Physiol. 68 : 355—357.

29. Winter, K. 1982. Properties of phosphoenolpyruvate carboxylase in rapidly prepared, desalted leaf extracts of the crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. Planta 154 : 298—308.

30. Yamazaki, R.K. and N.E. Tolbert 1968. Malate dehydrogenase in leaf peroxisomes. Biochim. Biophys. Acta 178 : 11—20.

31. Zschoche, W.C. and I.P. Ting 1973. Malate dehydrogenases of *Pisum sativum*. Plant Physiol. 51 : 1076—1081.

32. Zschoche, W.C. and I.P. Ting 1973. Purification and properties of microbody malate dehydrogenase from *Spinacia oleracea* leaf tissue. Arch. Biochem. Biophys. 159 : 767—776.