

# Partial Purification and Characterization of NADP<sup>+</sup>-Isocitrate Dehydrogenase from Immature Pod Walls of Chickpea (*Cicer arietinum* L.)<sup>1</sup>

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## ABSTRACT

NADP<sup>+</sup>-isocitrate dehydrogenase (threo-DS-isocitrate: NADP<sup>+</sup> oxidoreductase [decarboxylating]; EC 1.1.1.42) (IDH) from pod walls of chickpea (*Cicer arietinum* L.) was purified 192-fold using ammonium sulfate fractionation, ion exchange chromatography on DEAE-Sephadex A-50, and gel filtration through Sephadex G-200. The purified enzyme, having a molecular weight of about 126,000, exhibited a broad pH optima from 8.0 to 8.6. It was quite stable at 4°C and had an absolute requirement for a divalent cation, either Mg<sup>2+</sup> or Mn<sup>2+</sup>, for its activity. Typical hyperbolic kinetics was obtained with increasing concentrations of NADP<sup>+</sup>, DL-isocitrate, Mn<sup>2+</sup>, and Mg<sup>2+</sup>. Their *K<sub>m</sub>* values were 15, 110, 15, and 192 micromolar, respectively. The enzyme activity was inhibited by sulphydryl reagents. Various amino acids, amides, organic acids, nucleotides, each at a concentration of 5 millimolar, had no effect on the activity of the enzyme. The activity was not influenced by adenylate energy charge but decreased linearly with increasing ratio of NADPH to NADP<sup>+</sup>. Initial velocity studies indicated kinetic mechanism to be sequential. NADPH inhibited the forward reaction competitively with respect to NADP<sup>+</sup> at fixed saturating concentration of isocitrate, whereas 2-oxoglutarate inhibited the enzyme noncompetitively at saturating concentrations of both NADP<sup>+</sup> and isocitrate, indicating the reaction mechanism to be random sequential. Results suggest that the activity of NADP<sup>+</sup>-IDH *in situ* is likely to be controlled by intracellular NADPH to NADP<sup>+</sup> ratio as well as by the concentration of various substrates and products.

NADP<sup>+</sup>-IDH<sup>2</sup> (threo-DS-isocitrate: NADP<sup>+</sup> oxidoreductase [decarboxylating]; EC 1.1.1.42), reported about four decades ago (3), is found both in prokaryotes and eukaryotes. It is present in plant cytosol (5, 8, 15, 18), mitochondria (1, 5), and chloroplasts (7, 11), thus indicating that it may have varying roles in the metabolism of the plant. The chloroplastic form of NADP<sup>+</sup>-IDH is believed to supply 2-oxoglutarate for glutamate synthesis (7), while the cytosolic enzyme probably functions in the transfer of reducing power between the mitochondria and cytosol (12). In addition to the various bacterial and mammalian species, this enzyme has been well studied from a number of plant sources

including etiolated seedlings (16), leaves (15, 18), germinating (21) and maturing seeds (5, 20), and root nodules (8).

Recent experiments conducted in our laboratory in the presence of labeled <sup>14</sup>CO<sub>2</sub> revealed that the first products of CO<sub>2</sub> fixation in pod wall tissue of chickpea, which utilizes PEP carboxylase for the reaction, are oxaloacetate and malate (23) rather than 3-phosphoglycerate, as is the case in the C<sub>3</sub> pathway. It has been postulated that the pod wall surrounding seed has the capacity for C<sub>4</sub> or CAM-like metabolism, thus helping in recapturing of respired or photorespired CO<sub>2</sub>. This recaptured CO<sub>2</sub> is subsequently released within the cell by the action of NADP<sup>+</sup>-malic enzyme and could either be reduced via the Calvin cycle or used in the generation of carbon skeletons (2-oxoglutarate) for amino acids (9, 10, 24). In the latter case, oxaloacetate formed in the carboxylation step condenses with acetyl CoA to give ultimately isocitrate via citrate. Isocitrate is then oxidatively decarboxylated by NADP<sup>+</sup>-IDH to 2-oxoglutarate. The released CO<sub>2</sub> is recycled to form oxaloacetate. In our earlier investigations, properties of two of the enzymes, *viz.* PEP carboxylase (22) and NADP<sup>+</sup>-malic enzyme (6), of the postulated sequence from chickpea pod wall were reported. We now describe the properties of partially purified NADP<sup>+</sup>-IDH from the same tissue.

## MATERIALS AND METHODS

**Plant Material.** Immature pods after removing seeds were collected, just before use, from chickpea (*Cicer arietinum* L.) plants grown in the field of the Department of Agronomy, Haryana Agricultural University, Hisar.

**Chemicals.** All biochemicals used were purchased from Sigma Chemical Co. All other chemicals were of analytical grade (B.D.H., India).

**Enzyme Purification.** Unless otherwise stated, all steps of enzyme purification were carried out at 0 to 4°C.

Fresh immature pod walls (20 g) were homogenized in a prechilled mortar and pestle using acid-washed quartz sand with 50 mM Na-phosphate (pH 7.5) containing 5 mM MgCl<sub>2</sub>, 2 mM EDTA, and 1% (w/v) soluble PVP. The resulting homogenate was passed through four layers of cheesecloth, and the filtrate was centrifuged at 10,000g for 30 min. The supernatant so obtained was referred to as crude extract. NADP<sup>+</sup>-IDH in the crude extract was precipitated between 50 and 80% saturation of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitate was then dissolved in 50 mM Na-phosphate (pH 7.2) containing 5 mM MgCl<sub>2</sub> and 0.5 mM EDTA (buffer A) and was desalted on a Sephadex G-25 column (14 × 2.0 cm) that had been previously equilibrated with buffer A.

**DEAE-Sephadex A-50 and Sephadex G-200 Chromatography.** The enzyme was then fractionated on a DEAE-Sephadex A-50 column (30 × 2.6 cm) equilibrated with buffer B (20 mM Na-phosphate [pH 7.2], containing 5 mM MgCl<sub>2</sub> and 0.5 mM EDTA).

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<sup>2</sup> Abbreviations: IDH, isocitrate dehydrogenase; PEP, phosphoenol pyruvate; PVP, polyvinyl pyrrolidone; *M<sub>r</sub>*, molecular mass.

The enzyme was eluted with a 0 to 0.3 N KCl gradient prepared in buffer B at a flow rate of 16 ml h<sup>-1</sup>, and fractions of 4 ml each were collected. The active fractions were pooled and concentrated by osmosis against solid sucrose. The concentrated enzyme was loaded onto a Sephadex G-200 column (60.8 × 1.9 cm) previously equilibrated with buffer A and eluted with the same buffer at a flow rate of 10 ml h<sup>-1</sup>. The active fractions eluted as a single peak were pooled and stored at 4°C.

**Enzyme Assay.** NADP<sup>+</sup>-IDH was assayed by following the rate of NADP<sup>+</sup> reduction at 340 nm using a Calbiochem Enzymometer. The standard assay mixture contained 50 mM Hepes (pH 7.5), 5 mM MgCl<sub>2</sub>, 0.25 mM NADP<sup>+</sup>, 4 mM DL-isocitrate, and an appropriate amount of the enzyme preparation in a final volume of 1.2 ml. The reaction carried out at 30°C was initiated by addition of DL-isocitrate. The enzyme activity has been expressed as nmol NADP<sup>+</sup> reduced min<sup>-1</sup> at 340 nm under the specified conditions. Kinetic studies were performed by varying assay conditions as indicated along with the individual experiments.

**Protein Estimation.** The protein content of various fractions obtained after chromatography was monitored by measuring the absorbance at 280 nm. Quantitative estimation of protein after each step of purification was done by the method of Bradford (2).

**Determination of Molecular Weight of the Enzyme.** The mol wt of the partially purified enzyme was estimated by gel filtration through a Sephadex G-200 column that had previously been calibrated with alcohol dehydrogenase (*M<sub>r</sub>* 150,000), albumin bovine (*M<sub>r</sub>* 66,000), carbonic anhydrase (*M<sub>r</sub>* 29,000), and lysozyme (*M<sub>r</sub>* 14,000).

## RESULTS AND DISCUSSION

**Enzyme Purification.** NADP<sup>+</sup>-IDH from pod walls of chickpea was purified by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation, ion exchange chromatography on DEAE-Sephadex A-50, and gel filtration through Sephadex G-200 (Table I). The final preparation possessed 192-fold higher specific activity than the crude extract with a recovery of about 23%. During purification, higher enzyme activity was recovered in the desalted 50 to 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction than in the crude extract. Such an increase has also been reported for bacterial (26) and lucerne nodule (8) NADP<sup>+</sup>-IDH. This increase was probably not due to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> stimulation, as addition of neither NH<sub>4</sub>Cl nor (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to the standard assay mixture for NADP<sup>+</sup>-IDH had any effect on the activity of the purified enzyme or crude preparation.

**Determination of Molecular Weight of the Enzyme.** The *M<sub>r</sub>* of the partially purified NADP<sup>+</sup>-IDH, as determined by gel filtration through Sephadex G-200, was about 126 kD and this is slightly higher than the values reported for the enzyme from *Pisum sativum* leaves (15), maize leaves (5), and *Escherichia coli* (19).

**Stability.** The purified enzyme was stable at 4°C for at least 1 month. Inclusion of glycerol and sulfhydryl reagent such as 2-mercaptoethanol in the extraction medium did not affect the stability of the crude enzyme preparation. No loss of activity of the purified enzyme occurred upon incubation for 15 min at 40°C. However, after 15 min incubation at 55°C, the enzyme

activity was reduced to 20% and the enzyme was completely inactivated when incubated at 60°C for 15 min. Henson *et al.* (8) reported that NADP<sup>+</sup>-IDH from lucerne nodule cytosol was stable for at least 30 min at temperatures below 35°C, and the deviation from linearity at higher temperatures was probably due to enzyme denaturation. Satoh (20) also reported a heat-stable NADP<sup>+</sup>-IDH in the crude extract from maturing castor bean endosperm; however, the partially purified enzyme was labile against heat treatment at 57°C. The latter could be stabilized against heat inactivation by the addition of Mg<sup>2+</sup> in the presence of isocitrate or NADP<sup>+</sup>. To the contrary, NADP<sup>+</sup>-IDH from *E. coli* was found to be cold labile (4).

**Effect of pH.** The activity of the enzyme was determined as a function of pH by buffering the reaction mixture with Tris-maleate, Hepes-NaOH, and Tris-HCl from pH 6.4 to 9.0. With Mg<sup>2+</sup> as the divalent cation, the optimum pH ranged from 8.0 to 8.6. The pH optima of NADP<sup>+</sup>-IDH from several plant tissues has been reported to range from 7.5 to 9.2 (8, 15, 16, 18, 20).

**Effect of Metal Ions.** NADP<sup>+</sup>-IDH exhibited an absolute requirement for a divalent cation. Although both Mg<sup>2+</sup> and Mn<sup>2+</sup> could fulfill this requirement, at limiting equimolar concentration, a much higher activity was obtained with Mn<sup>2+</sup> than with Mg<sup>2+</sup>. The *K<sub>m</sub>* values for these cations were 15 and 192 μM, respectively. At saturating concentration, Mg<sup>2+</sup> gave 76% activity of that with Mn<sup>2+</sup>. Almost negligible enzyme activity was obtained with chloride salts of other divalent cations, such as Ca<sup>2+</sup>, CO<sup>2+</sup>, Ni<sup>2+</sup>, Ba<sup>2+</sup>, Sr<sup>2+</sup>, and Cd<sup>2+</sup> when used at 5 mM each. These metal ions at 5 mM were inhibitory even in the presence of Mn<sup>2+</sup>. Higher activity at saturating concentrations of Mn<sup>2+</sup> than with Mg<sup>2+</sup> has also been reported for the enzyme from pea seedlings (16), lucerne nodules (8), and pea leaves (15). On the contrary, Cd<sup>2+</sup>, Ba<sup>2+</sup>, Co<sup>2+</sup>, Sr<sup>2+</sup>, and Ni<sup>2+</sup> have been shown to promote enzyme activity at 8.7 × 10<sup>-3</sup> M (20). Hensen *et al.* (8) indicated the metal effects due to either differences in the enzyme's affinity for the metal-isocitrate complex or differences in the association constants of the metal and isocitrate. The substrate has been shown to be a metal-isocitrate complex rather than free isocitrate and metal for pea stem NADP<sup>+</sup>-IDH (13).

**Effect of Substrates.** NADP<sup>+</sup>-IDH showed a hyperbolic response with increasing concentrations of either NADP<sup>+</sup> or DL-isocitrate in an otherwise standard assay mixture. The enzyme had no activity with NAD<sup>+</sup> as the coenzyme. The *K<sub>m</sub>* value for NADP<sup>+</sup> as calculated from the double reciprocal plot was 15 μM, which is slightly higher than that reported for the enzyme from lucerne nodules (8), castor bean seeds (20), and *Pisum sativum* leaves (15, 16), but lower than that from etiolated pea seedlings (16). *K<sub>m</sub>* for DL-isocitrate (110 μM) of the enzyme from pod walls of chickpea was substantially higher than that from other plants, suggesting that the enzyme is active at comparatively higher concentrations of this substrate.

**Effect of Sulfhydryl Reagents.** Sulfhydryl reagents like iodoacetate (5 mM), iodoacetamide (5 mM), and N-ethylmaleimide (2 mM) inhibited the enzyme activity by 85, 33, and 63%, respectively. The inhibition was reversed by cysteine hydrochloride in the assay mixture. This indicated a requirement of sulfhydryl groups for the enzyme activity. However, as inclusion of 2-

Table I. Purification of NADP<sup>+</sup>-Isocitrate Dehydrogenase from Immature Pod Walls of Chickpea

Fraction	Total Activity	Protein	Specific Activity	Recovery	Overall Purification
	nmol/min	mg	nmol/min/mg protein	%	-fold.
Crude extract	26667	245.3	108.7	100.0	1
50-80% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate desalted	27000	27.5	981.8	101.2	9.0
DEAE-Sephadex A-50	12750	1.6	7968.7	47.8	73.3
Sephadex G-200	6250	0.3	20833.3	23.4	191.7

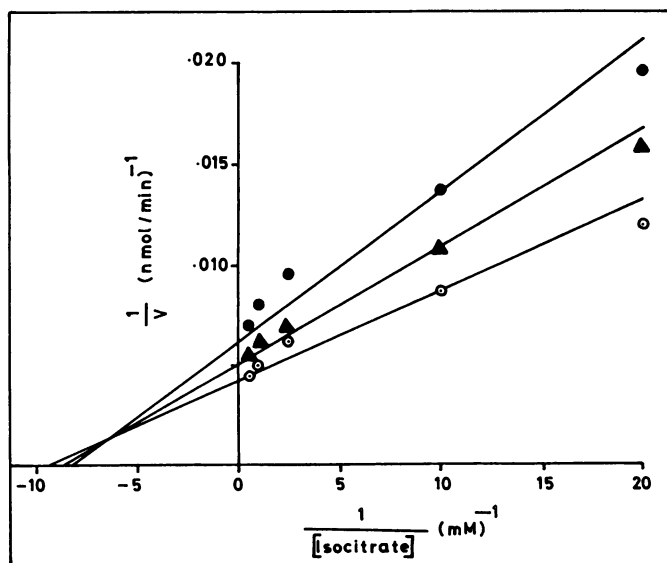


FIG. 1. Substrate interaction kinetics of NADP<sup>+</sup>-isocitrate dehydrogenase with respect to isocitrate in the presence of 0.01 (●), 0.04 (▲), and 0.16 (○) mM NADP<sup>+</sup>.

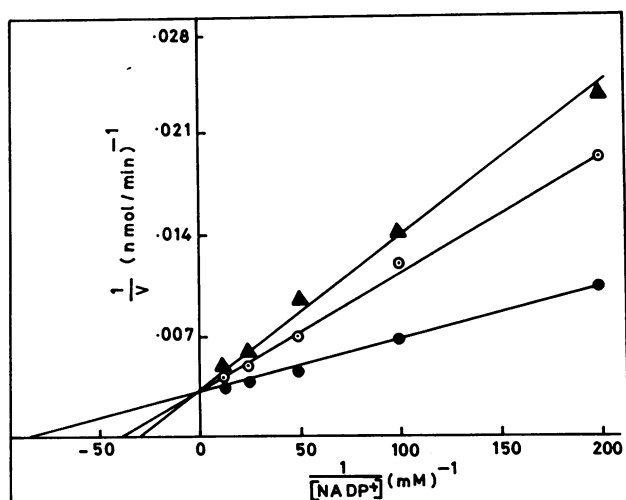


FIG. 2. Product inhibition of NADP<sup>+</sup>-isocitrate dehydrogenase from pod walls of chickpea by NADPH with NADP<sup>+</sup> as the varied substrate. The enzyme activity was determined as described in "Materials and Methods" except that the assay mixture contained the indicated concentrations of NADP<sup>+</sup> and 0 (●), 42 (○), and 84 (▲) μM NADPH. The isocitrate concentration was 4.0 mM.

mercaptoethanol in the extraction medium did not influence the enzyme activity or stability, it appears that the sulfhydryl groups were not susceptible to autoxidation. The inhibition of NADP<sup>+</sup>-IDH by *p*-chloromercuribenzoate also has been reported in pea seedlings (16) and castor bean seeds (20).

**Effect of Metabolites.** Metabolites including glycine, alanine, proline, cysteine, aspartate, asparagine, glutamate, glutamine, citrate, succinate, fumarate, malate, oxaloacetate, oxalate, phosphoenolpyruvate, pyruvate, fructose-6-phosphate, 3-phosphoglyceric acid, AMP, ADP, and ATP, each at a concentration of 5 mM, had no significant effect on the activity of the enzyme. However, enzyme activity was inhibited by NADH (0.17 mM) and the products of the reaction, *i.e.* 2-oxoglutarate and NADPH. NADH acted noncompetitively against NADP<sup>+</sup> with a  $K_i$  value of 0.50 mM as determined by Dixon's plot. In contrast, the enzyme from lucerne nodules (8) has been reported to be inhibited by citrate and that from pea seedlings (16) by oxaloacetate,

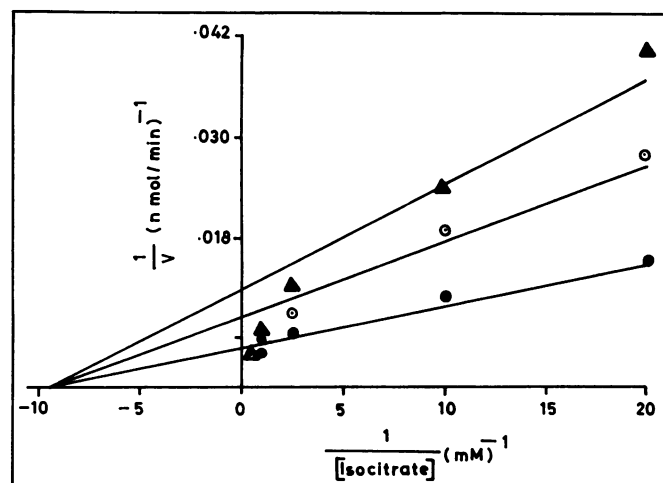


FIG. 3. Product inhibition of NADP<sup>+</sup>-isocitrate dehydrogenase by 2-oxoglutarate with respect to isocitrate. The standard reaction mixture in addition contained 0 (●), 1.0 (○), and 2.0 (▲) mM 2-oxoglutarate. NADP<sup>+</sup> concentration was 160 μM.

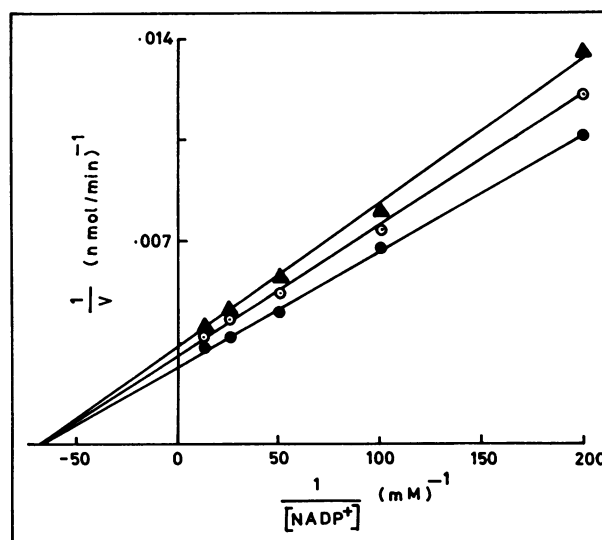


FIG. 4. NADP<sup>+</sup>-isocitrate dehydrogenase inhibition by 2-oxoglutarate with respect to NADP<sup>+</sup>. The standard assay mixture in addition contained 0 (●), 1.0 (○), and 2.0 (▲) mM 2-oxoglutarate. Isocitrate concentration was 4.0 mM.

nucleosides, and nucleotides. At nonsaturating isocitrate concentrations, glutamine was reported to act as a positive effector of the enzyme from lucerne nodules (8). The enzyme from pod walls of chickpea showed no response to glutamine either at saturating or nonsaturating isocitrate concentrations.

**Effect of Adenylate Energy Charge.** The enzyme activity was not influenced by adenylate energy charge over a range of 0 to 1.0 at a total adenylate pool concentration of 4 mM, indicating that the enzyme from pod walls of chickpea is insensitive to fluctuations in energy charge of the cell. However, in case of the enzyme from the protozoan *Crithidia fasciculata*, a possible regulation by ATP has been suggested (14).

**Effect of NADPH to NADP<sup>+</sup> Ratio.** At a constant concentration of NADP<sup>+</sup> (0.2 mM) and varying concentrations of NADPH, the enzyme activity decreased linearly when this ratio of NADPH to NADP<sup>+</sup> increased from 0 to 1.2 (data not shown). Omran and Dennis (16) also reported a linear decrease in the reaction rate with an increase in the mole fraction of reduced NADP<sup>+</sup> from 0 to 1.0. It thus appears that functioning of this enzyme might be

regulated by the relative concentrations of NADPH and NADP in the cell.

**Initial Velocity Studies.** The enzyme activity was determined at three fixed concentrations of one of the substrates, while the concentration of the other substrate was varied. Double reciprocal plots of varying concentrations of isocitrate *versus* velocity at three fixed concentrations of NADP<sup>+</sup> gave a set of lines which intersected to the left of the ordinate (Fig. 1). A qualitatively similar Lineweaver-Burk plot, with intersection of curves in between the two axes, was obtained when the enzyme activity was determined at three fixed concentrations of isocitrate with NADP<sup>+</sup> as the varied substrate. This indicates the kinetic mechanism to be sequential, which is in agreement with that of the NADP<sup>+</sup>-IDH from lucerne nodules (8). The point of intersection, in both cases being above the abscissa, indicates that binding of either isocitrate or NADP<sup>+</sup> lowered the apparent  $K_m$  for the varied substrate (17).

**Product Inhibition Studies.** These studies were carried out to ascertain whether binding of the substrates was ordered or random. NADPH inhibited the forward reaction competitively with respect to NADP<sup>+</sup> at the fixed saturating concentration of isocitrate (Fig. 2), indicating that the forward reaction mechanism may be either random or partially or fully ordered, where NADP<sup>+</sup> binds to the enzyme first and NADPH is the last product released from the enzyme.

When isocitrate concentration was varied at the fixed saturating concentration of NADP<sup>+</sup>, 2-oxoglutarate inhibition of NADP<sup>+</sup>-IDH was noncompetitive (Fig. 3). This would indicate that if the mechanism were ordered, the first product released would be 2-oxoglutarate. When NADP<sup>+</sup> was the varied substrate and isocitrate was saturating, 2-oxoglutarate inhibition of NADP<sup>+</sup>-IDH was noncompetitive (Fig. 4). This observation excludes the possibility of an ordered sequential mechanism because if the mechanism of the forward reaction were ordered, 2-oxoglutarate inhibition at varied NADP<sup>+</sup> concentration would be uncompetitive. From these data, we conclude that NADP<sup>+</sup>-IDH from pod walls of chickpea has a random sequential mechanism of reaction. Similar mechanism has been proposed for NADP<sup>+</sup>-IDH from pig heart (25) and lucerne nodules (8).

The results of the present investigation indicate that NADP<sup>+</sup>-IDH from pod walls of chickpea resembles the enzyme from other plants in its metal specificity, the thermostability, pH optimum, substrate interaction kinetics, and inhibition by products of the reaction. Though *in vitro* Mn<sup>2+</sup> is a more effective cofactor than Mg<sup>2+</sup> at equimolar concentrations, it is not known whether the enzyme *in vivo* functions with Mg<sup>2+</sup> or Mn<sup>2+</sup> as the activating ion. Our results also suggest that fluctuations in endogenous concentrations of NADPH and NADP<sup>+</sup> could be important in regulating the activity of this enzyme.

It is generally accepted that in developing fruits, the respired CO<sub>2</sub> is reassimilated. A pathway for its reassimilation in developing fruits has been proposed by Latzko and Kelly (10) and Singal *et al.* (24). According to this scheme, IDH is important in providing carbon skeletons (2-oxoglutarate) for the synthesis of amino acids, as has been suggested for chloroplastic NADP<sup>+</sup>-IDH from pea seedlings (18) and cytosolic NADP<sup>+</sup>-IDH from lucerne nodules (8). The inhibition of NADP<sup>+</sup>-IDH from pod walls of chickpea by products, *viz.* NADPH and 2-oxoglutarate, suggests that its activity is controlled by intracellular concentrations of these metabolites. It thus appears that the principle role

of NADP<sup>+</sup>-IDH in pod walls of chickpea is to furnish reducing power and 2-oxoglutarate for synthesis of glutamate. In this way, recapturing of respired CO<sub>2</sub> by PEP carboxylase could be effectively linked to the deposition of protein reserves in legume seeds.

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