JOURNAL OF PROTEINS AND PROTEOMICS
Vol. 1, No. 1, January-June 2010, pp. 25-32

Isolation and Characterization of NADP+-Linked Isocitrate Dehydrogenase in Germinating Urd Bean Seeds (*Phaseolus mungo*)

Pramod Kumar Srivastava*, Govind Kant Srivastava** and Siddhartha Singh***

Abstract: *Isocitrate dehydrogenase (EC 1.1.1.42) has been purified to homogeneity from germinating urd bean seeds. The enzyme $NADP^+$-linked isocitrate dehydrogenase is a tetrameric protein (molecular weight 130,000; gel filtration) made up of four identical monomers (sub unit molecular weight about 32,000-33,000; PAGE in presence of sodium dodecyl sulphate). Thermal inactivation of purified enzyme at 40 °C, 45 °C and 50 °C shows single exponential loss of enzyme activity suggesting that the inactivation of this enzyme follows simple first order kinetics (rate constants for purified enzyme 0.020, 0.043 and 0.077 min^{-1} at 40 °C, 45 °C and 50 °C respectively). Thermal inactivation in presence of glutathione and dithiothretol at 45 °C and 50 °C also follows simple first order kinetics, but the presence of these compounds protects the loss of enzyme activity. The enzyme shows optimum activity at pH 7.3-8.0. The variation of Vmax and Km at different pH values (6.5-8.0) suggests that proton behaves as an "Uncompetitive Inhibitor". A basic group is present at the active site of enzyme which is accessible for protonation in this pH range in the presence of substrate only, with a pKa equal to 6.8. Successive dialysis against EDTA and phosphate buffer, pH 7.5 at 0-4 °C gives an enzymatically inactive protein. Thermal inactivation of this protein at 45 °C and 50 °C shows an exponential loss of enzyme activity as in the case of untreated (native) enzyme. Full activity is restored on adding Mn^{2+} (3.75mM) to a solution of this protein. Addition of Mg^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} brings about partial recovery. Alkali metal ions bring about 75% inhibition at 4mM concentration. The inhibition is stronger at high concentration of Na^+ and K^+. Other metal ions are not effective.*

I INTRODUCTION

Isocitrate dehydrogenase (EC 1.1.1.42) is an interesting enzyme of cytoplasm which catalyzes the reversible oxidative decarboxylation of isocitrate to α-ketoglutarate via the formation of an enzyme bound intermediate (oxalosuccinate) in presence of a coenzyme NAD^+ or $NADP^+$ (as oxidant) and a divalent metal ion (Mg^{2+}/Mn^{2+}) .Most of the plants, microorganisms and tissues of animals and higher plants contains two types of Isocitrate dehydrogenase (ICDH). One of these requires $NADP^+$ and typically occurs in cytoplasm[1-3] with small proportion of activity present in mitochondria and exhibit normal hyperbolic kinetics [4] whereas, the other requires NAD^+ and is exclusively associated with mitochondria [5-7].This enzyme has been isolated from a variety of sources. The molecular weights of $NADP^+$ linked isocitrate dehydrogenase from various sources have been reported and lie in the range of 53×10^3 to 126×10^3. $NADP^+$ linked Isocitrate dehydrogenase from rat heart mitochondria [8],alkalophilic *Bacillus* [9],*Thermoleophillum minutam* [10]and rat liver cytosol [11] are reported to be dimeric protein. However, our enzyme isolated from urd bean (*Phaseolus mungo*.L) is a tetrameric protein. This paper deals with investigations of physico-chemical properties of $NADP^+$ linked isocitrate dehydrogenase.

In the present communication we describe some properties of this enzyme, which have direct bearing on the structural symmetry, characteristics and partial identification of the constituents of the active site.

II MATERIALS

Nicotinamide adenine dinucleotide phosphate sodium salt and DL-isocitric acid of analytical grade was purchased from Sisco Research Laboratory (SRL) Bombay; Magnesium chloride was from Qualigens Fine Chemicals, Bombay; Trichloroacetic acid, BSA and Folin Ciocalteau's phenol reagents were from SRL, Bombay; Sodium Carbonate, Copper Sulphate and Rochelle salt were from S.D. Fine Chemicals, Bombay; Ammonium Sulphate, Ammonia solution, acetic acid and DEAE Cellulose were from SRL, Bombay; Dialysis tube was purchased from Arthur H. Thomas, Philadelphia, P.A., U.S.A; Riboflavin, acrylamide, N,N-methylene-bisacrylamide,

TEMED,Sodiumlaurylsulphate, Coomassie blue, myoglobin, ribonuclease, amido black were from SRL,Bombay; Methanol was from Qualigens Fine

* Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi - 221005, India, Tel-91-0542-2575382, Mob. - 09415811822, E-mail- pksrivastavabhu@rediffmail.com

Chemicals, Bombay; Biogel P-200, ova albumin, glyceraldehyde-3-phosphate dehydrogenase, catalase and γ-globulin were from Sigma Chemicals Co., St Louis, U.S.A.; Ammonium persulphate was from BDH Chemicals, Bombay; DS(+) isocitric acid, DL–isocitric acid, Nicotinamide adenine dinucleotide phosphate disodium salt, glutathione, dithiothreitol and cysteine were from Sigma Chemicals Co., U.S.A.. A commercial rectified spirit (95% ethanol) was distilled twice before use. Other chemicals were of analytical reagent grade.

III METHODS

Purification of $NADP^+$ Linked Isocitrate Dehydrogenase from *Phaseolus Mungo* Seeds

All purification steps were performed at 0–4 °C unless specified otherwise.

(1) Extraction of Enzyme: The 24 hours germinated urd bean seeds were carefully washed with double distilled water and chilled. The seeds were crushed in 150 ml of chilled phosphate buffer (50mM, pH 7.5) in a kitchen blender for 1.0 minute. The resulting suspension was squeezed through a muslin cloth and centrifuged at 15,000 rpm for 20 minutes. The residue was discarded and clear supernatant (pH 6.7) was taken in clean beaker and brought to pH 7.5 by adding ten fold diluted ammonia solution. A total of 180 ml yellow coloured clear extract was obtained.

(2) Ethanol Fractionation: The above solution was cooled to a semi frozen state and precooled ethanol (–10°C) was added to extract with vigorous shaking at –10°C (15 ml of ethanol / 100 ml of extract).The resulting solution was allowed to stand for 5 minutes at 10°C and centrifuged for 15 minutes at 15,000 rpm at –10°C. The resulting supernatant was treated with more precooled ethanol added gradually with shaking at –10°C (40 ml of ethanol /100 ml of supernatant). The resulting precipitate was collected by centrifugation (10 minutes at 15,000 rpm), dissolved in phosphate buffer (50mM), stirred for half an hour and finally centrifuged to get a clear solution (18 ml).

(3) **Ammonium Sulphate Fractionation:** To the above solution, solid ammonium sulphate was gradually added with gentle stirring and bringing it to 45% saturation of $(NH_4)_2SO_4$. The pH was maintained at 7.5 by adding ten fold diluted ammonia solution. After 30 minutes stirring, the precipitated protein was discarded by centrifugation and supernatant was brought to 75% ammonium sulphate saturation by employing the above procedure. The enzyme proteins were precipitated in the range of 45–75% saturation of $(NH_4)_2SO_4$ and collected by centrifuging the solution of enzyme and dissolving in minimum volume of phosphate buffer 50 mM, 7.5 pH (10.2ml).

(4) Dialysis: After ammonium sulphate fractionation, the above fraction of enzyme was dialyzed against pre-chilled phosphate buffer (50mM, pH 7.5 at 0–4°C) with 5-6 repeated change of same buffer till the complete removal of ammonium ions (checked with Nessler's Reagent).

(5) DEAE Cellulose Column Chromatography: The above solution was loaded to DEAE cellulose column (25×2.5cm.) previously equilibrated with 50 mM phosphate buffer at pH 7.5. The enzyme was eluted with 0.2 M KCl prepared in phosphate buffer at a flow rate of 25 ml/ hour and fraction of 5 ml each was collected. The active fraction containing $NADP^+$– linked ICDH activity (25ml) were pooled together and entire protein was precipitated at 0–95% ammonium sulphate saturation. The precipitate was collected after centrifugation and dissolved in minimum volume (3.5ml) of 50 mM phosphate buffer at pH 7.5.

Protein Concentration

Protein concentration was estimated by the method of Lowry et. al. [12] using Folin-Ciocalteau's reagent [13]. The specific activity is expressed in terms of enzyme units per milligram protein.

Assay for Enzyme Activity

The enzyme activity has been measured by monitoring the rate of formation of NADPH at 366 nm. The NADPH is formed as a result of oxidation of isocitrate. The appropriately diluted enzyme solution (0.01ml) was added to 0.79 ml of reaction mixture containing isocitrate (2.25mM), $NADP^+$ (0.62 mM) and $MgCl_2$ (3.75 mM) in 2.5 ml assay buffer (50 mM phosphate buffer, pH 7.5). The rate of increase in absorbance was noted at 15 seconds intervals at 366 nm and initial rate of reaction was obtained graphically. The enzyme activity was calculated from the ϵ^{NADPH} value = $3.11\times10^3\ M^{-1}\ cm^{-1}$ at 366 nm, which was determined independently under these conditions.

An enzyme unit has been defined as the amount of enzyme which brings about the reduction of 1µmole of NADP to NADPH in one minute under our test conditions.

Physical Characterization

(1) Ultra-violet Absorption Spectrum: The absorption spectrum of purified $NADP^+$-linked isocitrate dehydrogenase of urdbean (*Phaseolus mungo* L.) was done in ultraviolet region.

(2) Polyacrylamide Gel Electrophoresis: Polyacrylamide gel electrophoresis of $NADP^+$-linked ICDH was performed as per the method used by Reisfeld et. al. [14] at pH 7.5.

(3) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis: The Polyacrylamide gel electrophoresis in presence of anionic detergent sodium dodecyl has also been carried out at pH 7.5.The subunit molecular weight for purified $NADP^+$-linked isocitrate dehydrogenase of urdbean (*Phaseolus mungo* L) has been determined by comparing its electrophoretic mobility in presence of sodium dodecyl sulphate (SDS) with mobilities of known proteins i.e. ribonuclease, myoglobin, bovine serum albumin, trypsin, ova albumin, glyceraldehyde-3-phosphate dehydrogenase, as the method given by Weber and Osborn [15].

(4) Gel Filtration: Molecular weight of purified $NADP^+$-linked isocitrate dehydrogenase of urdbean was determined by gel-filtration on Bio-gel by the method of Ezzeddine and Al-Khalidi [16]. By this method, Biogel P-200 is used as column media. The molecular weight of purified enzyme was calculated by comparing its dilution factors of known proteins, i.e. ribonuclease, myoglobin, ova albumin, bovine serum albumin and α-globulin.

IV INACTIVATION STUDIES

(1) Thermal Inactivation: The appropriate diluted enzyme solution was kept in a thermostat at desired temperature. Aliquots of 0.05ml were withdrawn at different intervals of time, chilled immediately and tested for enzyme activity at 30°C to a mixture of buffer, isocitrate, $MgCl^-_2$ and $NADP^+$ at 366 nm. The thermal inactivation in presence of glutathione and dithiothreitol were also carried out.

(2) Chemical Inactivation: SH-reagents (Iodoacetamide and NEM) prepared in assay buffer were added to the mixture of enzyme, $NADP^+$ and $MgCl_2$ in assay buffer at 30°C. The aliquots of 0.05 ml were withdrawn at different intervals of time and pipetted to the cuvette. The reaction was started by adding 0.1 ml of buffer aliquots and enzyme activity was estimated as usual at 30°C.

(3) Inhibition by Nucleotides and Metabolites: Stock solutions of different nucleotides and metabolites (ATP, ADP, AMP. oxaloacetate, succinate, α-ketoglutarate, maleic acid and citric acid) were prepared in assay buffer. Appropriately diluted enzyme $NADP^+$ and $MgCl_2$ were taken in assay buffer (total 1.0 ml) in the test cuvette. Reaction was started by adding 0.05ml of isocitric acid and monitored as usual at 366nm.

V RESULTS

(1) Purification: $NADP^+$– linked isocitrate dehydrogenase was purified from 40 hours germinated urdbean (*Phaseolus mungo* L.) seeds. A sample protocol of the purification of urd bean $NADP^+$-linked isocitrate dehydrogenase is given in Table-1. This procedure results in 169.5 fold purification with an overall recovery of 34.92%. The purified $NADP^+$ linked isocitrate dehydrogenase of urd bean shows typical absorption spectra in ultra violet region with maximum absorbance of 0.33 at 280nm. The absorbance ratio A_{280}/A_{260} ratio equal to 1.32 suggests that our enzyme preparation is free form any bound nucleotides and coloured pigments. The purified enzyme gives a single protein band on polyacrylamide gel electrophoresis at pH 7.5 [fig 1(A)] and a single and wide band is observed on gel electrophoresis in presence of SDS [fig 1(B)]. All purification steps produced an increase in specific activity despite the instability of the enzyme which diminishes the recovery. In fact, final recovery of activity was only 34.9%. The final specific activity of our enzyme is quite low (16.1 units/mg protein) than the earlier reported by Popova et.al, 1986 (63.5 units/mg protein) [17]; Al Ali et.al, 1989 (58.7 units/mg protein) [18] and Gupta & Singh, 1988 (20.8units/mg protein) [19] for this enzyme.

Table I
A Protocol of purification of NADP+ Linked Isocitrate Dehydrogenase Enzyme from 100g of 40 Hours Germinating urd Bean Seeds.

S.No	Step	Volume (ml)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg Protein)	Fold purification	%age Recovery
1.	Crude	180	378	3978	0.095	-	-
2.	Ethanol Fractionation (15-45% Saturation)	18.0	268	164	1.60	16.8	70.9
3.	Ammonium Sulphate (45-75% Saturation)	10.2	219	94.0	2.33	24.5	57.9
4.	Dialysis	10.4	201	-	-	-	53.2
5.	DEAE Cellulose Column chromatography	3.50	132	8.40	16.1	169.5	34.9

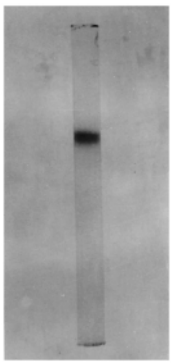

Figure 1(a): Polyacrylamide gel Electrophoresis of Purified NADP⁺- Linked Isocitrate Dehydrogenase in Urdbean at pH 7.5. 60µg Protein was Fed to the Gel.

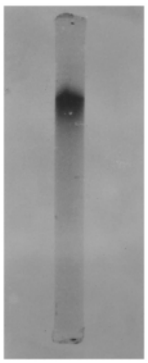

Figure 1(b): Polyacrylamide gel Electrophoresis of Purified NADP⁺- Linked Isocitrate Dehydrogenase in Urdbean at pH 7.5 in Presence of 2% Sodium Dodecyl Sulphate .20 µg Protein was Fed to the Gel.

(2) Molecular And Subunit Molecular Weight: The molecular weight of the enzyme has been estimated by comparing its dilution factor with those of some known proteins (fig2) and is found to be 130×10^3.A single although wide protein band is observed on polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate [fig1 (b)] .This suggests single molecular weight for all the monomers of $NADP^+$ linked isocitrate dehydrogenase of urdbean. The subunit molecular weight for purified $NADP^+$ - linked isocitrate dehydrogenase of urdbean (*Phaseolus mungo* L.) has been determined by comparing its electrophoretic mobility in presence of SDS with mobilities of some known proteins(fig3). The subunit molecular weight for $NADP^+$-linked isocitrate dehydrogenase is found to be 32×10^3 –33×10^3. Thus, the enzyme is a tetrameric protein made up of apparently identical monomers.

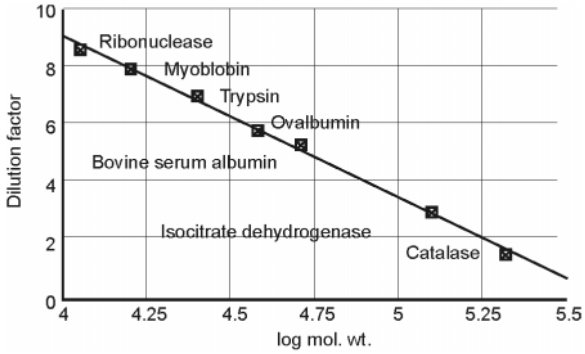

Figure 2: Molecular Weight Determination of Purified Urdbean NADP⁺-Linked Isocitrate Dehydrogenase. The Bio-Gel P-200 was Calibrated with 1mg/ml Protein.

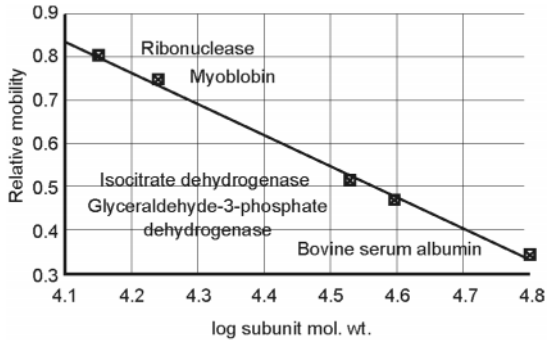

Figure 3: Subunit Molecular Weight Determination of Purified Urdbean NADP⁺-Linked Isocitrate Dehydrogenase. The Proteins were Incubated with 2% SDS in 50mM Phosphate Buffer (pH 7.5) for 4-5 Hours at Room Temperature. The Relative Mobility of Proteins has been Calculated with Respect to the Mobility of Tracking Dye.

3. **Thermal Inactivation:** The time dependent thermal inactivation of purified urd bean (*Phaseolus mungo* L.) $NADP^+$ - linked isocitrate dehydrogenase has been studied at 40°C, 45°C and50°C. The thermal inactivation of urd bean enzyme follows simple first order kinetics. This is more evident in the semi-log plot shown in fig.4. The first order kinetics of thermal inactivation of urdbean $NADP^+$–linked isocitrate dehydrogenase suggested that there is no site-site heterogeneity indicating that all the subunits behave in equal manner. Thus, the ICDH protein is a regular tetramer. Kinetics of the thermal inactivation of $NADP^+$–linked isocitrate dehydrogenase enzyme from other sources has not been reported. Therefore, it is not possible to compare the behaviour of our enzyme with similar preparations from other sources in this respect. The different thiol compounds such as dithiothreitol and glutathione decreases the time of inactivation of enzyme at 45°C and 50°C without affecting the overall kinetic pattern.

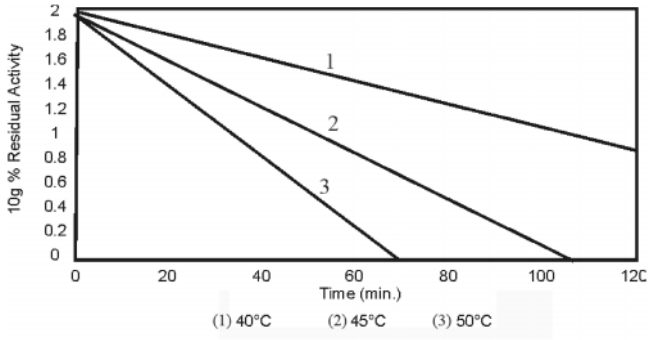

Figure 4: Kinetics of Thermal Inactivation of Urdbean NADP+- Linked Isocitrate Dehydrogenase at Different Temperatures. Enzyme Solution (0.8mg/ml) in 50mM Phosphate Buffer (pH 7.5) were Incubated at 40 °C, 45 °C and 50 °C. The Aliquots (0.05ml) were withdrawn at Different Intervals of Time and Assayed Activity Immediately at 366 nm.

4. **Effect of pH:** Using threo DS(+) and DL- isocitrate, the pH optimum of urd bean $NADP^+$ - linked isocitrate dehydrogenase is found to be 7.3-7.8, this value is close to the pH optima of the enzymes from alkalophilic *Bacillus* (pH 7.8-8.4) [20], *Diceutrarchus labrax* L. liver (pH 7.5) and from bull adrenal cortex (pH 8.0)[21]. The effect of pH on the K_m and V_{max} values of substrate has been investigated in the pH range 6.5–8.0 (fig 5). The double reciprocal plot shown in fig 5. gives a family of parallel straight lines with decreasing activity at lower pH. This suggests that proton behaves as "uncompetitive inhibitor" and appears to be protonation of some basic group of active site of enzyme below the pH 8.0 which leads to the loss of enzyme activity in reversible manner. The ''uncompetitive'' nature of inhibition further suggests that this group may be protonated in presence of substrate only. Thus it appears that a "masked" basic group is involved in the activity of urdbean isocitrate dehydrogenase enzyme. This active site group becomes accessible for protonation, only when the substrate is bound to the active site of the enzyme. From the data of fig 5, the pKa value of this "masked" basic group is found to be 6.8. On the basis of pKa value the "masked" basic group may be tentatively identified as the imidazole moiety of a histidine residue at the active site of urdbean isocitrate dehydrogenase.

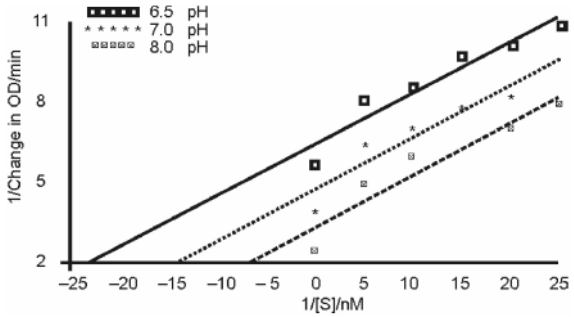

Figure 5: Influence of pH Variations on K_m and V_{max} Values . . . . . $^+$-linked Isocitrate Dehydrogenase. The pH of Assay System was Varied in the Range 6.5-8.0 with Respect to Different Fixed Concentrations of Substrate. The Enzyme Concentration was 20µg/ml. The Rate of Reaction is Expressed in Terms of O.D. Change at 366 nm.

5. **Role of Metal Ions:** The $NADP^+$ linked isocitrate dehydrogenase of purified urdbean reveals one-fourth activity of the maximum activity, in absence of externally added metal ions. EDTA has two types of effects. If EDTA is present in the activity assay medium, it functions as competitive inhibitor (fig 6). The inhibition may have possibly occurred due to removal of bound metal ions or by polyionic nature of EDTA as substrate. Dialysis against EDTA resulted into a protein which is enzymically inactive in absence of added $MgCl_2$. The activity of apo-isocitrate dehydrogenase has been tested in presence of various divalent metal cations. The results are given in Table-II. The result suggests that the full enzyme activity was restored on the addition of Mn^{2+} (3.75 mM). The partial activity

was found with Mg^{2+}, Zn^{2+} Co^{2+} and Cu^{+2} at same concentration as Mn^{2+}. Other metal ions such as Ni^{2+} and Ba^{2+} did not show any enzyme activity. The EDTA dialysed enzyme preparation which shows no activity in absence of Mg^{2+} ions shows the same kinetic parameters on thermal inactivation as the untreated native enzyme (fig.4).

Table-II
Effect of Different Divalent Metal Ions on the Activity of NADP⁺-linked Isocitrate Dehydrogenase of urd Bean. (The Enzyme Activity was Tested in 50mM Phosphate Buffer, pH 7.5at 30 °C by Adding Different Metal Ions to the "apo-isocitrate Dehydrogenase'')

S.NO.	Pre-Treatment	Specific Activity (Eu/mg protein)
1.	Before removal of metal ions	14.861
2.	Dialysis against EDTA and phosphates butter	0.00
3.	Asno.2+3.75 mMMn^{2+}	12.135
4.	Asno.2+3.75mM Mg^{2+}	6.348
5.	Asno.2+3.75mM Zn^{2+}	5.634
6.	As no.2+3.3.75mM Co^{2+}	3.158
7.	As no.2+3.75 mM Cu^{2+}	1.130
8.	As no.2+3.75 mM Ba^{2+}	NIL
9.	As no.2+3.75 mM Ni^{2+}	NIL

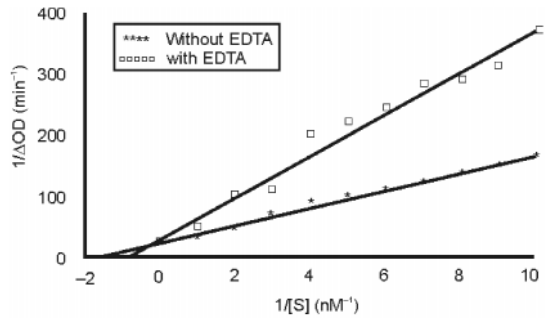

Figure 6: Kinetics of Inhibition of Urdbean NADP⁺-linked Isocitrate Dehydrogenase with EDTA(0.05mM).The Concentration of Substrate (isocitrate) was Varied Keeping other Constituents Same as in Assay System. The Concentration of "apo-enzyme" was 0.05mg/ml. The Rate of Reaction is Expressed in Terms of O.D. Change at 366 nm.

The inhibition studies with alkali metals were carried out in assay buffer. $NADP^+$ -linked isocitrate dehydrogenase is sensitive to Na^{+}- and K^+ ion but not affected by Li^+ ion. At 4mM concentration of alkali metal ion, enzyme activity was inhibited to 75%. Inhibition is stronger at high concentration of Na^+ or K^+ ions whereas Li^+ has no effect (Table-III).

S.No	Alkali Metal ions	Concentrations mM	% age Residual Activity	% age Inhibition
1.	Na^+	1	80	20
		2	60	40
		4	26	74
2.	K^+	1	72	28
		2	55	45
		4	30	70
3.	Li^+	2	0	0
		4	0	0

V DISCUSSION

$NADP^+$-linked isocitrate dehydrogenase was purified from the 40 hours germinated seeds of urd bean (*Phaseolus mungo* L.) .During purification higher enzyme activity was recovered in $(NH_4)_2SO_4$ fraction than the crude extract, such an increase has been reported by Henson et.al (1986) [22] in lucernae root nodules and Vasquez and Reeves (1979) [23] in bacteria. The final specific activity of our enzyme is quite low (16.1 unit/ mg. protein) than the earlier reported by Popova et.al (63.5 units/mg protein) [17]. Al Ali et al (58.7 units/mg protein) [18] and Gupta and Singh (20.8 units/mg protein) [19].

The molecular weight was determined to be 130×10^3 which is quite higher than the earlier reported by Gupta and Singh in immature wall of chickpea (126×10^3) [19] Shikata et.al.,in alkalophilic Bacillus (90×10^3) [9], Park et.al, in rat heart mitochondria (120×10^3) [8] and by Fatania et.al. in rat liver cytosol (94×10^3) [11]. Our enzyme is tetrameric protein (molecular weight of monomer 32×10^3 to 33×10^3) but the enzyme from rat heart mitochondria, rat live cytosol, alkalophilic Bacillus and *Thermoleophillum minutam* [10] are reported to be dimeric proteins (Molecular weight 120×10^3, 90×10^3, $94\times10^3$and 60×10^3).

Thermal inactivation of purified $NADP^+$-linked ICDH enzyme at different temperatures (40°C, 45°C and 50°C) shows a single exponential loss of enzyme activity, suggesting that the enzyme follows simple first order kinetics. Kinetics of thermal inactivation of $NADP^+$-linked ICDH enzyme from other sources has not been reported. Therefore, it is not possible to compare the behaviour of our enzyme with similar preparations from other sources in this respect.

The optimum pH for this enzyme is found to be 7.3 to 8.0 in phosphate buffer. The result is quite close to as reported by Medina-Puerta et.al (pH 7.8–8.4) [20] and in alkalophilic *Bacillus*, Taranda et.al, (pH 8.0) [21] in bull adrenal cortex. In the pH range 6.5-8.0, the substrate

inhibition sets in at lower values of substrate, so that K_m and V_{max} cannot be determined with adequate precision. In the pH range 6.5–8.0; double reciprocal plots for oxidative decarboxylation at different pH values give a family of parallel straight lines. Thus, proton behaves formally as an "uncompetitive inhibitor". It can therefore be concluded that a "masked" basic group is present at the active site and is important for catalytic action of the enzyme. This group becomes accessible for protonation in presence of substrate only. The pKa value of this group has been calculated and found to be 6.8. On the basis of pKa value, this group may be tentatively identified as the imidazole moiety of a histidine residue.

The inhibition of $NADP^+$-linked ICDH has been carried out at pH 7.5. The inhibition at 4mM concentration of alkali metals ion was upto 75%. Thus, the cell must possess some protective mechanism to counteract this inhibition. The inhibition is stronger at high concentration of Na^+ and K^+, therefore it appears that there is non-specific protective protein.

Dialysis against EDTA results in an enzymically inactive protein. Full activity was restored on addition of Mn^{2+} and partially restored in presence of Mg^{2+}, Zn^{2+} and Cu^{2+}. The thermal inactivation of this enzymically inactive protein also exhibits first order kinetics with high rate constants as compared to native enzyme suggesting that the removal of metal ions does not bring any major change in overall protein structure. But the stability of this enzyme decreases on removal of bound metal ions. Therefore, the endogenous metal ion (and possibly the externally added Mg^{2+} ions also) must be involved in catalysis of the reaction. Also the molecular symmetry apparent from single exponential loss of activity of thermal inactivation (native and "apo-enzyme") must be consequence of the regular tetrameric structure of protein and can not be said to be induced by the metal ions or any other effector or substrate etc. In this respect, our results are consistent with the concept of 'pre-existent symmetry" and not with the "induced fit" model (Koshland et al., 1966) [24].

Effect of adding different concentrations of Mn^{2+} and Mg^{2+} to the "apo-enzyme" on the rate of enzyme catalyzed reaction has been investigated. The data suggests the presence of single type of Mg^{2+} & Mn^{2+} binding site. EDTA appears to exert its influence on ICDH by two different mechanisms. In a slow process, it brings about inactivation of the enzyme, presumably by removal of endogenous metal ions. The resultant enzyme is inactive even when EDTA is removed, but can be fully reactivated on the addition of Mg^{2+}. There is also a strong and immediate competitive inhibition with K_i 0.048mm. This may be due to the polyionic nature of EDTA at pH 7.5, similar to the physiological substrate.

Several metabolites of Krebs' cycle have been tested for inhibitory action. We have observed that oxaloacetate, succinate and a-ketoglultarate inhibit the urd bean enzyme in competitive manner with K_i values equal to 7.27, 10.67, 6.67 mM respectively, whereas, citric acid and maleic acid inhibit non competitively with K_i values equal to 1.33 and 3.33 mM, respectively. Such type of inhibition of $NADP^+$ linked ICDH enzyme have been reported from different workers like Marr and Weber (1969) [25], Ozaki & Shiio (1968) [26] in pig heart, Leyland and Kelly (1991) [27] in photosynthetic bacterium and Popova (1993) [17] in maize scutellum.

It has been reported that the ICDH enzyme from *Azotobacter vinelandii* (Barrera & Jurtshuk, 1970) [28] and maturing castor bean (Satoh, 1972) [29] were found to be sensitive to PCMB. Seelig and Colman [30] suggested pseudo first order kinetics of inactivation enzyme from human heart by 0.01M iodoacetamide at pH 5.0. The total numbers of "SH-groups" were not reported.

Our enzyme has four reactive SH groups per tetrameric molecular (i.e. one SH-group per monomer, weight $32\times10^3 - 33\times10^3$) which react with DTNB more than 5.0 minutes. Denatured urd bean ICDH enzyme shows 8 SH-groups (i.e. 2 SH-groups per monomer). The SH groups appear to be important for enzyme activity as apparent from inactivation with iodoacetamide and N-ethyl maleimide with excess of any of these reagents, the enzyme loses activity showing first order kinetics. The rate constants were found to be 0.092, 0.099 and 0.173 min^{-1} with 1.0mM iodoacetamide and 0.4 and 4.0 mM NEM, respectively. The kinetics data suggest that all the SH groups react at the same rate and are independent of one another. Thus, there is no evidence for site-site interactions so far as the reactions with SH reagents are concerned.

Hence, the results of **: (i)** Kinetics of thermal inactivation of both native and "apo-enzyme", **(ii)** Binding of Mg^{2+} and Mn^{2+} to the "apoenzyme", and **(iii)** Kinetics of inactivation with SH-reagents, suggests that all the four monomers of tetrameric $NADP^+$-linked isocitrate dehydrogenase of urd bean, are identical chemically and functionally. The results of thermal inactivation of "apoenzyme" are significant because these help to distinguish between the "pre-existent symmetry" and 'induced fit models' in an unambiguous manner.

As the enzyme preparation is free from bound nucleotides or any known effector of enzyme, the molecular symmetry of monomers are observed under these conditions, must arise due to regular tetrameric

structure of the urdbean ICDH enzyme and can not be "induced" by any effector.

The thermal inactivation of native and "apo-enzyme" obeys the first order rate kinetics but the rate constants of "apo-enzyme" are higher than that of native enzyme, suggesting that the endogenous metal ions do not induce any change in the degree of symmetry of ICDH protein molecule but changes the stability of enzyme. Thus, these experiments indicate that the purified $NADP^+$–linked isocitrate dehydrogenase from urd bean, is a regular tetramer and all the monomers are equally reactive.

REFERENCES

(1) P.K. Srivastava, K Pathak, Om Prakash, *J. Plant Biochemistry & Biotechnology*, **13**(2004), 145-148.

(2) P. K. Srivastava, D S Singh, *Indian Journal of Biochemistry & Biophysics*, **38** (2001), 335-341.

(3) Jesus Palomo, Fernando Gallardo, Maria F. Suarez, Fransico M. Canovas, *Plant Physiol*. **118** (1998), 617-626.

(4) S C Sung, C H Hsii; *J. Formosan Med. Assoc*., **56** (1957), 103.

(5) Cecilia A. McIntosh, David J. Oliver, *Plant Physiol*. **100**(1) (1992), 69-75.

(6) C Bernofsky, M F Utter, *Biochem. Biophys. Acta*, **132**(1966),244.

(7) J A Hathway, D E Atkinson, *J. Biochem*. , **238**(1963) ,2875.

(8) Park, Kwang Kyun, Ahn Yong Ho, Hahm, Kyung Soo, Yoon Soo Kim, *Yanse Uidac Nonmunjip*, **17**(2) (1984),668-81.

(9) Shikata Shitsuw, Ozaki Katsuya, Kawai Shuji, Ito Susumu Okamoto Kikuhiko, *Biochem. Biophys. Acta*, **952**(3) (1988), 282-9

(10) Novotny James F., Perry Jerome, *J. Appl.Microbiol. Biotech*. **35**(4) (1991),461-65.

(11) Fatania Hasmukh, Al Nassar, Khaled Eid, SidhanVasanta, *FEBS Lett*. **320**(1) (1993),57-60.

(12) O. H. Lowry, N. J. Rosebrough, A .L. Farr, R.J. Randall, *J. Biol. Chem*., **193**(1951), 265-280.

(13) O Folin, V Ciocalteau, *J.Biol.Chem*., **73**(1927),627-650.

(14) R.A. Reisfeld, U.J. Lewis, D.E. Williams, *Nature*, **195**(1962), 281-283.

(15) K.Weber, M.Osborn, *J. Biol. Chem*., **244**(1969) ,4406-4412.

(16) S. Ezzeddine, U. Al-Khalidi, *Mol.Cell Biochem*., **45**(1982), 127-128.

(17) T N Popova, A N lzv, *Ser. Biol*, **5**, (1993) 652 - 659.

(18) A K Al-Ali, H Al-Husayni, A Al-Mutairy, R Saba, D M Power, (1989) Comp. *Biochem. Physiol. Comp. Biochem*. **92B**, 517-521.

(19) K Vijay Gupta, Randhir Singh, *Plant Physiol*., **87**(3), (1988), 741-4.

(20) Medina-Puerta, Mariadel Mar, Gallego-Iniesta, Milagrosa, Garrido-Pertierra, Amando, *Biochem. Int*. ,**17**(3) (1988), 489-98.

(21) N I Taranda, V V Vinogradov, S A Strumilo, *Ukr. Biokhim. Zh*., **59**(1) (1987), 24-9

(22) A Henson Cynthia, H D Stanley, C Michael, *Physiol. Plant*, **67**(1986), 538-540.

(23) Barbara Vasquez, C. Henry Reeves, *Biochem, Biophys. Acta*, **578**(1) (1979), 31-40.

(24) Jr.D.E. Koshland, G. Nemethy,D.Filmer, *Biochemistry*, **5**(1966),365-385.

(25) J.J.Marr, M.M. Weber, *J.B.C.* **244**(1969), 2503-9.

(26) Hachiro Ozaki,Shiio E. Samu *J. Biochem*., **64**(3),(1968).

(27) Mark L. Leyland, David J. Kelly, European *J. Biochemistry*, **202**(1), 85-93.

(28) C R Barrera, P Jurtshuk, *Biochem. Biophys. Acta* 220(1970), 416 - 429.

(29) Y. Satoh, *Plant Cell Physiol*. **13**(1972), 493 - 503.

(30) Seelig, Gail Foure, F. Colman Roberta, *Arch. Biochem. Biophys*., **188**(2) (1978), 394-409