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IDENTIFICATION OF ACTIVE SITE GROUPS IN MUNG BEAN $NADP^+$– LINKED ISOCITRATE DEHYDROGENASE

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Abstract

The purified $NADP^+$– linked isocitrate dehydrogenase shows optimal activity in the pH range 7.5-8.0 for threo-DS (+)-isocitrate as well as for DL-isocitrate. Influence of pH on the K_m and V_{max} values for physiological substrate has been investigated in the pH range 6.5-7.5. At pH below 7.5 or above 6.0; the inhibition of enzyme activity becomes more marked. The pKa value of the masked basic group is found to be 6.8 indicating that a imidazole moiety of histidine residue is involved in the reaction mechanism and accessible to protonation only to the enzyme-substrate complex and not to the free enzyme. The single exponential inactivation of enzyme activity in presence of methylene blue indicated that the imidazole moieties of histidine residue are equally reactive towards the photo-oxidation and in presence of NADPH $t_{1/2}$ of the photo-inactivation is increased.

Key words: mung beans, isocitrate dehydrogenase, $NADP^+$, ICDH, photo-oxidation.

Introduction

Isocitrate dehydrogenase (E.C.1.1.1.42) is an interesting enzyme of Krebs' cycle which catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate via the formation of an enzyme bound intermediate (oxalosuccinate) in the presence of a divalent metal ion (Mn^{2+}/Mg^{2+}) and a coenzyme (NAD^+ or $NADP^+$) as oxidant.

Most of the micro-organisms (1-7), plants (8), tissues of higher animals contain two types of isocitrate dehydrogenase (ICDH). One of these requires NAD^+ and the other $NADP^+$. The $NADP^+$-linked enzyme typically occurs in cytoplasm with a small proportion of the activity residing (9) in mitochondria and exhibits normal hyperbolic kinetics, whereas the NAD^+-linked isocitrate dehydrogenase is exclusively associated with mitochondria, modified by ADP (10,11) or AMP.

The kinetic properties of beef heart mitochondrial $NADP^+$-dependent isocitrate dehydrogenase were studied by Montani, et al., (1980). They reported optimum pH of 8.4 and

6.7 for forward and reverse reaction respectively, study of the dependence of K_m and V_{max} on pH indicated the presence of a ionizable group (pKa=6.0) for the forward reaction, i.e. synthesis of α–ketoglutarate and of 2 ionizable groups (pKa = 5.78 and 7.59) for the reverse reaction, *i.e.*, synthesis of isocitrate from α–ketoglutarate, in the enzyme-substrate complex. There is only much little information available about the involvement of catalytic group from plant sources.

This paper deals with the effect of pH on the rate of purified ICDH catalysed reaction, K_m and V_{max} values for substrate, and the photo-oxidation of the enzyme in the presence of methylene blue.

Materials

Threo DS(+) isocitric acid was from Sigma chemicals Co., St. Louis, USA. DL-isocitric acid, Nicotinamide adenine dinucloeotide phosphate disodium salt were from Sisco Research Laboratories Pvt. Ltd; Bombay. $NaH_2PO_4.2H_2O$, $Na_2HPO_4.2H_2O$, were G. R. grade of Sarabhai M. Chemicals. Methylene blue was from Glaxo Laboratories Ltd. Other biochemicals used were of analytical grade.

Methods

1. Assay for Enzyme Activity and Protein Concentration : $NADP^+$–linked isocitrate dehydrogenase from mung bean was isolated as described (8) and enzyme assay was done by determining the rate of formation of NADPH, which is produced as a result of oxidation of isocitrate. An aliquot (0.79ml) of 50 mM phosphate buffer (pH = 7.5), containing isocitrate (2.5 mM), $NADP^+$ (0.625 mM) and $MgCl_2$ (3.75 mM) was brought to 30°C. The reaction was started by adding 0.01 ml of suitably diluted enzyme and the rate of increase in optical density of reaction mixture was recorded at 366 nm in Eppendorf spectrophotometer. The enzyme activity was calculated from ϵ_{NADPH} value ($3.11 \times 103\ M^{-1}cm^{-1}$). A unit of enzyme activity was defined as the amount of enzyme required to transform one μ mole of $NADP^+$ to NADPH in one minute under the test conditions defined above. Protein was estimated by the method of Lowry et. al., with Folin Ciocalteau reagent calibrated with crystalline bovine serum albumin.(12)

2. Effect of pH on the Rate of Reaction and K_m and V_{max} Values of Substrate : Variation of the rate of ICDH catalyzed reaction in the presence of threo-DS (+) and DL–isocitrate with respect to different pH has been investigated. Influence of pH on the K_m and V_{max} values of substrate has also been investigated in the pH range 6.5-7.5.

3. Photo-oxidation of Mung Bean Isocitrate Dehydrogenase : For this experiment, the solution of enzyme (0.57 mg/ml) and methylene blue (10μM) in 50 mM phosphate buffer (pH = 7.5), were incubated in water thermostat, at 30°C and irradiated with visible light from a 200W tungsten bulb, kept at 10 cm away from the mixture. Similar mixture was kept in dark at room temperature (30°C). The enzyme solution of same concentration without methylene blue was incubated at 30°C in light and dark. The time dependent changes in the activity of enzyme, was monitored in all the samples.

Results

1. Effect of pH on the Rate of Reaction and K_m and V_{max} values of Substrate : The optimum pH is found to be 7.5-8.0 for threo- DS (+) as well as DL– isocitrate (Fig. 1).

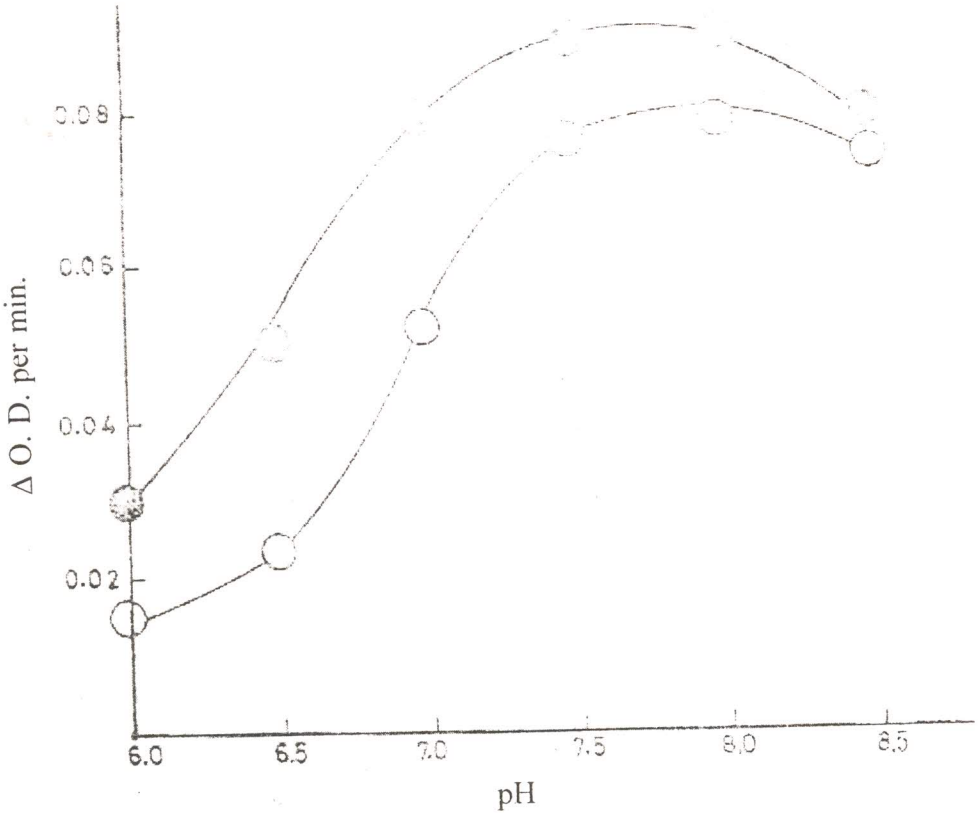


Figure 1. Influence of pH on the rate of oxidative decarboxylation of threo-DS(+) isocitrate (•) and DL-isocitrate (o) by mung bean ICDH in 50 mM phosphate buffer at 30°C in the presence of 3.75 mM Mg^{2+} and 0.625 mM $NADP^+$ The enzyme concentration was 0.007mg/ml. The substrate concentration was used as usual the assay mixture in both the cases.

Influence of pH on K_m and V_{max} values of substrate has been investigated in the pH range 6.5-7.5. The results are shown in Fig (2). At pH's below 7.5 or above 6.0 the inhibition becomes more marked.

Therefore, such experiments were not extended to those pH values. The double reciprocal plot shown in Fig 2, gives families of parallel straight lines. However, the data of Fig. 2, shows that the activity of mung bean enzyme decreases as pH is lowered. This suggests that at below the pH 7.5, proton behaves as an "Uncompetitive" inhibitor and seems to be protonate some of basic groups of the active site of the enzyme which leads to the loss of enzyme activity in reversible manner. The "Uncompetitive" nature of inhibition concluded that this group can be

protonated in the presence of substrate only. Thus a "masked" basic group is involved in the catalysis action to mung bean enzyme. This active site group becomes accessible for the protonation when the substrate is bound to the catalytic site of the enzyme.

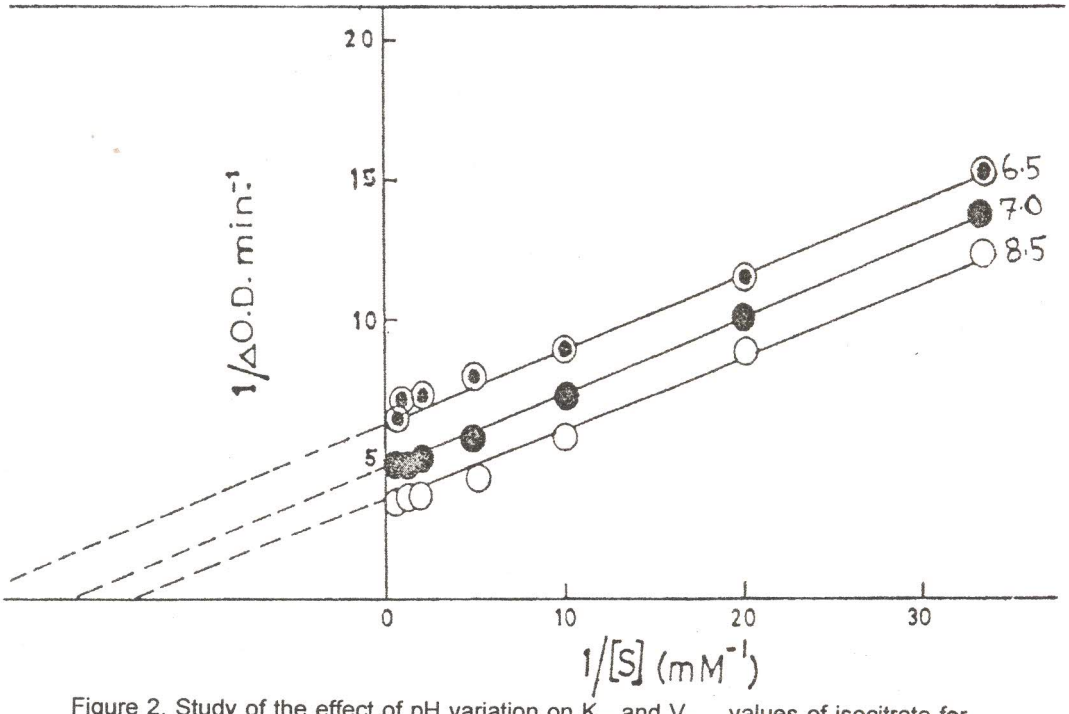


Figure 2. Study of the effect of pH variation on K_m and V_{max} values of isocitrate for the mung bean $NADP^+$-linked ICDH enzyme. The pH of assay system was varied in the range of 8.0-6.5 with respect to the different fixed concentration of substrate. The enzyme concentration was 7.0µg /ml. The rate of reaction is expressed in terms of rate of absorbance change at 366 nm.

The rate equation for uncompetitive inhibition was calculated to ESI complex but not to the free enzyme, is given below.

$$1/V_{max'} = 1/V_{max'} [1 + [I]/K_{si}]$$

where,

V_{max} : Maximal velocity in absence of inhibitor.

$V_{max'}$: Apparent maximal velocity in presence of inhibitor.

[I] : Concentration of inhibitor

K_{si} : Dissociation constant.

Therefore, $K_{si} = [ES] I] / [ESI]$

The relationship between the V_{max} and values at two different inhibitor concentrations is as follows:-

$$\frac{V_{max'}}{V_{max}} = \frac{[I] + K_{si}}{[I'] + K_{si}} \quad (1)$$

or $$K_{si} = V_{max'} [I'] - Vmax [I] / V_{max} - V_{max'} \quad (2)$$

where V_{max} : Maximal velocity

$V_{max'}$: Apparent velocity

[I'] and [I] : Inhibitor concentration

The K_{si} has been calculated and found to be 1.703×10^{-7} M. The K_{si} value apparently is the Ka value for the "masked" basic group. The pKa value is calculated by the following formula:

$$pKa = - \log ka. \quad (3)$$

Therefore, $$Ka = K_{si}$$

The pKa value 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 the mung bean isocitrate dehydrogenase.

2. Photo-oxidation of Mung Bean Isocitrate Dehydrogenase : Dye-sensitized photo-oxidation of enzyme for identification of critical residues, at the catalytic site of the enzyme was done. The irradiated and non-irradiated tubes, containing only enzyme solution and non-irradiated tubes containing mixture of enzyme and methylene blue do not show any change in the activity of mung bean enzyme, for a long period. The solution of enzyme (0.57mg/ml) and methylene blue (10μM), irradiated with visible light was inactivated with time (Fig 3). A semi-log plot of this data is found to be linear. This suggests that the inactivation of $NADP^+$–linked isocitrate dehydrogenase from mung bean, obeys simple first-order kinetics with a half-life period ($t_{1/2}$) equal to be 9.0 minute.

Addition of successive concentration of reduced NADPH brings about protection for the mung bean enzyme against the inactivation of photo-oxidation by increasing the value of half-life period ($t_{1/2}$) from 9.0 to 40 minutes and onwards, (Fig.4).

Photoxidation phenomena of SH-enzyme is found due to the significant role of specific amino acids i.e., tryptophenyl, tyrosol and cystein residue with imidazole moieties, this experiment with mung bean enzyme to be concluded that the involvement of imidazole moiety in the catalysis of isocitrate dhydrogenase of mung bean. The single exponential inactivation of the activity of this enzyme in the presence of cationic dye (methylene blue) suggested that imidazole moieties of histidine residues are equally reactive to photo-oxidation. This statement is strongly supported by the variation of K_m and V_{max} values with varying pH.

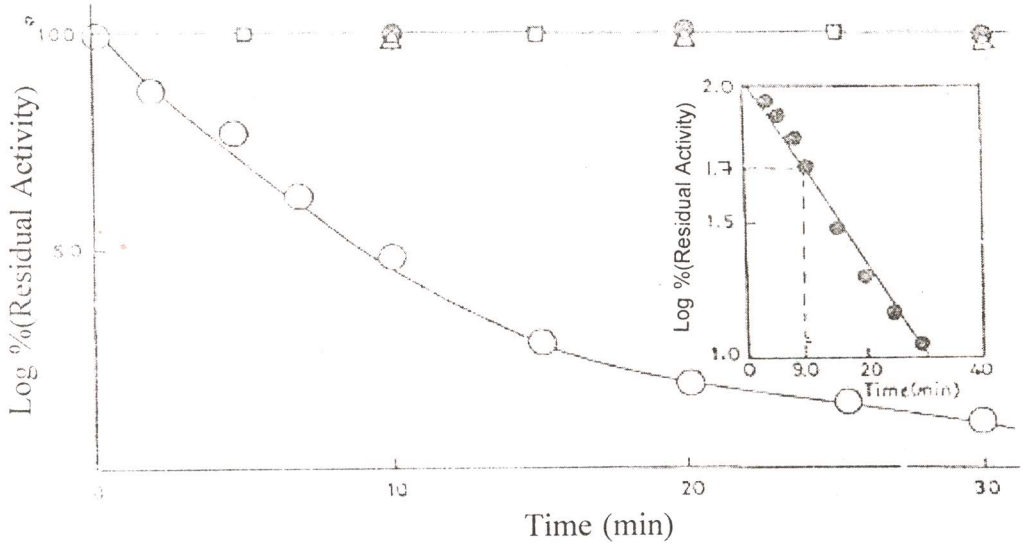


Fig. 3. Dye-sensitized photo-oxidation of purified mung-bean $NADP^+$–linked isocitrate dehydrogenase, in the presence of methylene blue. The enzyme (0.50 mg/ml) and methylene blue (10 μM) in 50 mM phosphate buffer (pH 7.5) were incubated at 30°C with a 200W tungsten bulb, kept at 10 cm away from the sample. Aliquotes were withdrawn at different time intervals and tested for the activity (O). Different controls were run simultaneously. These were-(i) Enzyme containing methylene blue (10 μM) kept in dark (•),(ii) Enzyme alone placed in dark (Δ), and (iii) Enzyme irradiated alone (□).

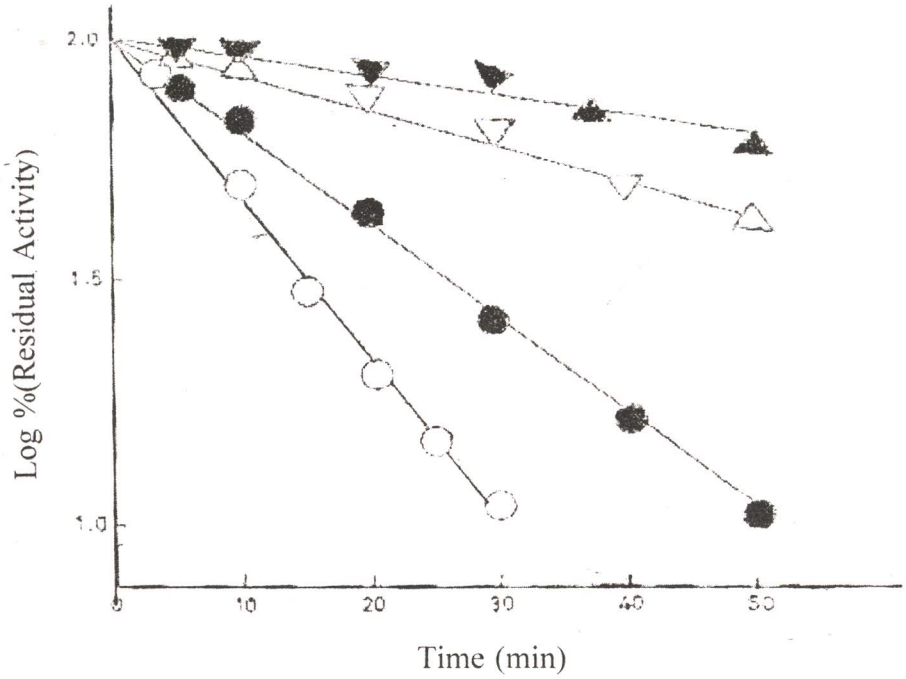
Discussion

The pH optimum for this enzyme is found to be 7.5-8.0 in phosphate buffer which is in agreement with that reported by Medina-Puerta, Maria del Mar, et al.(13), the pH optimum for the enzymes from Alkalophilic bacillus [pH 7.8-8.4; Shikata, Shit-Suw, et al; 1988 (14)]; *Diceutrarchus labrax* L. liver [pH 7.5 Medina-Puerta, Mara del Mar., et al. 1988(13)] and from bull adrenal cortex [pH 8.0, Taranda, N.I., et al 1987(15)]are close to the pH range of mung bean ICDH enzyme. On the other hand, the enzyme from the host cytosol of lucerne (*Medicago sativa*) root nodulated [pH range 7.5-9.0, Henson Cynthia A.et.al 1986(16)] shows broad pH range.

The pH dependence of K_m and V_{max} has been studied in the pH range 6.5-7.5 or 8.0. Above and below this pH range the substrate inhibition sets in at lower values of [S], so that K_m and V_{max} cannot be determined with adequate precision. In this pH range double reciprocal plots for oxidative decarboxylation at different pH values give a set 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 the catalytic action of the enzyme. This group becomes accessible for protonation in the 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 imidazole moiety of a histidine residue. Seelig, Gail Foure and Colman Roberta F. (17). reported the requirements of a basic form of an essential ionizable group in the enzyme-substrate complex, by the study of pH dependence of V. The basic group exhibited a pKa value of about 5.5. A histidine specific pH-dependent pattern was reported by the enzyme from Bovine parotid gland (18) on photo-oxidation.



(4) Study of photo-inactivation of mung bean isocitrate dehydrogenase and its protection by addition of NADPH. The solutions of enzyme and methylene blue (10 μM) in 50 mM phosphate buffer (pH 7.5) was incubated at 30°C and irradiated with visible light from a 200W tungsten bulb, kept at 10 cm away from the sample. Aliquotes were withdrawn at different time intervals and tested for the activity (O). Solutions of enzyme with different amount of NADPH, i.e., (i) 1.25 mM NADPH(•), (ii) 5.0 mM NADH (Δ),(iii)10μM NADPH (Δ), and methylene blue (10 μM) were irradiated with visible light as described above.

Four ethoxyformyl histidine residues were formed on treatment of this enzyme with Diehtylpyrocarbonate (DEPC), whereas only 2 histidine residues were lost during photo-oxidation. The photo-oxidation and DEPC-related inactivation of enzyme was prevented by the addition of NADP; but not by the addition of substrate. Thus, ≥ 2 active-site histidines may be associated with the NADP-binding site but not with the substrate-binding site. Thus, the same group is present on the active site of the mung bean ICDH, as the enzyme of Bovine parotid gland and human heart.

Exposure of light from 200W tungsten bulb placed at 10 cm away form the incubated mixture of enzyme and methylene blue led to the inactivation of $NADP^+$–linked isocitrate dehydrogenase activity at 30°C temperature, No such losses were observed in various controls which run simultaneously. With methylene blue the mung bean ICDH enzyme activity is lost in exponential decay manner.

On addition of different concentration of NADPH i.e. 1.25mM, 5.0 mM and 10.0mM, the half life time of this enzyme gradually increases from 9.0 to 40 minutes and onwards. From this, it is clear that NADPH protects the loss of ICDH activity against photo-oxidative reagent, methylene blue. On photo-oxidation, the inactivation of the mung bean isocitrate dehydrogenase suggested the involvement of imidazole moiety in the catalysis action of this enzyme. The single exponential inactivation of enzyme activity, in the presence of methylene blue is concluded that the imidazole moieties of histidine residues are equally reactive towards the photo-oxidation. It is evident from the variation of K_m and V_{max} values with pH change that the imidazole moiety of histidine residue plays significant role in the catalysis of this enzyme and its pKa value is found to be 6.8.

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