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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.