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ROLE OF SULFHYDRYL COMPOUNDS FOR THE STABILITY OF ISOCITRATE LYASE

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ABSTRACT

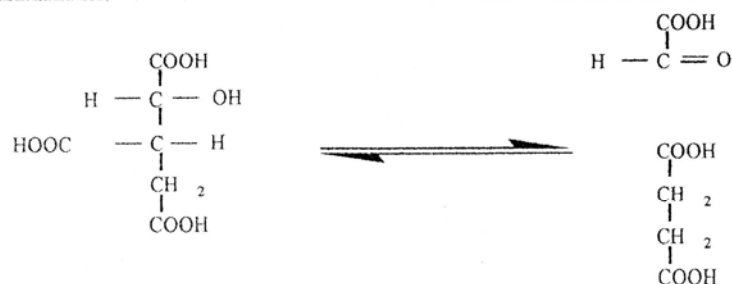
The important role of sulfhydryl compounds (dithiothreitol, 2-mercaptoethanol, glutathione, and cysteine) for the stability of isocitrate lyase purified from the glucose-grown basidiomycete *Fomitopsis palustris* was investigated. The enzyme was inactivated in the absence of any sulfhydryl compound, but nearly full activity was recovered by addition of 5 mM dithiothreitol, which was also able to keep the stability of isocitrate lyase during storage. Inhibition of enzyme activity by *p*-chloromercuribenzoate and inactivation of the enzyme by iodoacetate suggest that the thiol group is located at the active center of the enzyme and sulfhydryl compounds protect the enzyme against inactivation, which was further confirmed with the changes of the UV visible spectra of the enzyme.

Key words : isocitrate lyase, *Fomitopsis palustris*, sulfhydryl compounds.

INTRODUCTION

Isocitrate lyase, the first key enzyme of the anaplerotic glyoxylate cycle, has been shown to be present in a number of organisms including protozoa, bacteria, algae, fungi, plants, and some nematodes (for a review see Cioni et al. 1981) and in mammals (Davis et al. 1990; Povov et al. 1998). The enzyme firstly found by Campbell and his coworkers in *Pseudomonas aeruginosa* (Campbell et al. 1953) catalyzes reversible cleavage of isocitrate to glyoxylate and succinate as illustrated in the following figure. Isocitrate lyase has been purified to homogeneity from a wide variety of species (Vanni et al. 1990) and in most cases for microorganisms the enzyme is purified from C2-grown cultures.

The common quaternary structure of purified isocitrate lyase is a homotetramer and biochemical analyses have shown that the enzyme from different species have comparable catalytic properties and kinetic mechanisms each other and respond similarly to different



inhibitors (Vanni et al. 1990). Recently, a trimmeric isocitrate lyase has been reported for *Fomitopsis palustris* enzyme (Munir et al. 2002), which is the first isocitrate lyase, purified for any glucose-grown basidiomycete. Molecular analysis of the purified isocitrate lyase has shown that cysteine residue is located in a conserved region (KKCGHM) in the obtained cDNA fragment, which further suggests that cysteine residue plays an important role in catalytic function of *F. palustris* isocitrate lyase (Nishide 2002).

Characterization study has shown the dependence of isocitrate lyase activity on sulfhydryl compounds, including dithiothreitol, 2-mercaptoethanol, glutathione, and cysteine. This paper discusses the important role of sulfhydryl compounds to keep the stability of isocitrate lyase isolated from *F. palustris*.

MATERIALS AND METHODS

Materials

Enzyme Source

The brown-rot basidiomycete *Fomitopsis palustris* known to produce high amount of oxalic acid to the culture medium was used as the enzyme source. *F. palustris* has also been used as a Japanese Industrial Standard test fungus for wood preservative efficacy tests. The fungus was cultivated on glucose-containing medium as described in the previous paper (Munir et al. 2001).

Methods

Preparation of Mycelial Extracts and Enzyme Purification

All manipulations were carried out at 4°C, and all buffer solutions contained 1 mM ethylenediaminetetraacetate (EDTA) and 1 mM dithiothreitol (DTT). Mycelia were harvested by filtration through cheesecloth and rinsed with distilled water and 20 mM potassium phosphate (KPi) buffer (pH 7.0). Mycelial extracts were prepared

by homogenizing the collected mycelia resuspended in 50 mM KPi buffer (pH 7.0) in a cold mortar in the same way as previously reported (Munir et al. 2001).

Isocitrate lyase in the mycelial extracts was purified by several steps; fractionation with ammonium sulfate, hydrophobic (on TSK gel Phenyl-Toyopearl), weak anion exchange (on Protein-Pak DEAE), gel filtration (on HiLoad Superdex), and strong anion exchange (Cosmo gel QA) column chromatography, consecutively. Conditions and results for each purification step were detailed in other reports (Munir et al. 2002; Munir 2002).

Enzyme Assays

The enzyme activity was assayed by the continuous assay method described by Dixon and Kornberg (1959) with a slight modification. The standard reaction mixture comprised of 2 mM $MgCl_2$, 4 mM phenylhydrazine, 20 mM isocitrate (pH 7.0), and 40 mM KPi buffer (pH 7.0), in a final volume of 3 ml. The reaction was started with the addition of isocitrate. Formation of phenylhydrazone, a derivative of glyoxylate produced from isocitrate, was spectrophotometrically recorded at 324 nm. One unit of the enzyme activity is defined as the amount of enzyme that catalyzes formation of 1 mol of phenylhydrazone per minute under the conditions described. Specific activities were given as units of enzyme activities per mg of protein ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein). Protein contents were determined by the Bradford method using the protein assay kit with bovine serum albumin as a standard (Bradford 1976).

RESULTS AND DISCUSSION

Effect of Sulfhydryl Compounds on Isocitrate Lyase Activity

Table 1 shows the effect of sulfhydryl compounds on isocitrate lyase activity. Independent addition of sulfhydryl compounds, including DTT, 2-mercaptoethanol, glutathione, and cysteine, into the enzyme reaction elevated the enzyme activity to a different extent. The maximal activity was observed with the presence of DTT followed by 2-mercaptoethanol. Addition of 1 mM DTT increased the activity by 25%. This value, however, was obtained when 5 mM 2-mercaptoethanol was included to the reaction mixture. Addition of 1 mM glutathione and cysteine also activated isocitrate lyase activity by 11 and 17%, respectively, but relatively no effect on the activity when 5 mM of these compounds was used. These findings were consistent with general reported studies that most isocitrate lyases were activated by sulfhydryl compounds (Nakamura et al. 1989; Cioni et al. 1981).

Table 1. Effect of sulfhydryl compounds and EDTA on isocitrate lyase activity

Compounds added	Concentration (mM)	Relative activity (%)
Absence	–	100
DTT	1	125
	5	138
2-Mercaptoeyhanol	1	115
	5	125
Glutathione	1	111
	5	105
Cysteine	1	117
	5	104
EDTA	1	116
	5	39

On the hand,, *Pinus densiflora* isocitrate lyase was inhibited by these compounds at concentration of 1–4 mM (Tsukamoto et al. 1986). Nevertheless, a higher concentration of sulfhydryl compounds (10 mM) inhibited the catalytic activity of isocitrate lyase for all compounds tested (results not included in this table).

Furthermore, the results suggest that sulfhydryl compounds play an important role in maintaining the isocitrate lyase in a reduced state, which further indicate that one or more –SH groups are probably located at the active site domain of isocitrate lyase. Importantly, molecular analysis of amino acid sequence of this purified enzyme has shown that cysteine residue is located in a conserved region (KKCGHM) of the obtained cDNA fragment suggesting that cysteine residue plays an important role in catalytic function of *F. palustris* isocitrate lyase (Nishide 2002). In agreement with this finding, Cys195, the site of modification with 3-bromoyruvate, was also located in the active site enzyme of *Escherichia coli* isocitrate lyase from (Ko and McFadden 1990).

In addition, EDTA, a chelating compound, could substitute the role of sulfhydryl compounds in activation of isocitrate lyase at lower concentration (1 mM), but it strongly inhibited the activity at higher concentration (5 mM), probably by limiting available Mg^{2+} required for catalytic action of the enzyme. EDTA at a concentration of 0.2 mM also activated the enzyme from *Azotobacter vinelandii*; above 2 mM it has an inhibitory effect (Kennedy and Dilworth 1963).

Importance of Sulfhydryl Compounds for The Stability of Isocitrate Lyase

Because the purified enzyme used for the above study was at all time kept in buffer containing the sulfhydryl compound DTT, an effort has been done to investigate the effect of removing sulfhydryl compound on the enzyme stability. The originally added DTT was removed from the enzyme preparation by dialysis against 20 mM KPi buffer (pH 7.0) containing 1 mM EDTA. The buffer solution was changed twice and dialysis was continued for four hours.

Results (in Table 2) show that only 11% of the original activity remained after DTT had been removed. However, the enzyme activity was recovered to a different level with addition of sulfhydryl compounds, DTT, 2-mercaptoethanol, glutathione, and cysteine; the addition of 1 mM of each compound singularly recovered the activity by 65, 41, 35, and 45% of the original value, respectively. Interestingly, almost full activity (95 %) was recovered at the addition of 5 mM DTT, but only half of the original activity was recovered at the addition of 2-mercaptoethanol, glutathione, and cysteine.

Furthermore, 1 mM EDTA was also able to recover the activity by 31%, but not at higher concentration (5 mM). These evidences suggest that the presence of 1 mM DTT was sufficient for maintaining the enzyme stability during storage. Importantly, the findings have demonstrated reversible inactivation of isocitrate lyase after removing sulfhydryl compounds as also reported for the enzyme from *Haloferax volcanii* (Serrano et al. 1998). In addition, differences of the UV visible spectra of the enzyme before and after dialysis probably due to a change in conformational protein, as shown in Figure 1, further indicate the important of sulfhydryl compound (DTT) to maintain –SH group of the enzyme.

Table 2*. Recovery of isocitrate lyase activity after removing DTT

Compounds added	Concentration (mM)	Relative activity (%)
Before Absence	–	100
After dialysis	–	11
DTT	1	65
	5	95
2-Mercaptoeyhanol	1	41
	5	56
Glutathione	1	35
	5	50
Cysteine	1	45
	5	51
EDTA	1	31
	5	13

* This table is a modified form of the previously reported one (Munir et al. 2002)

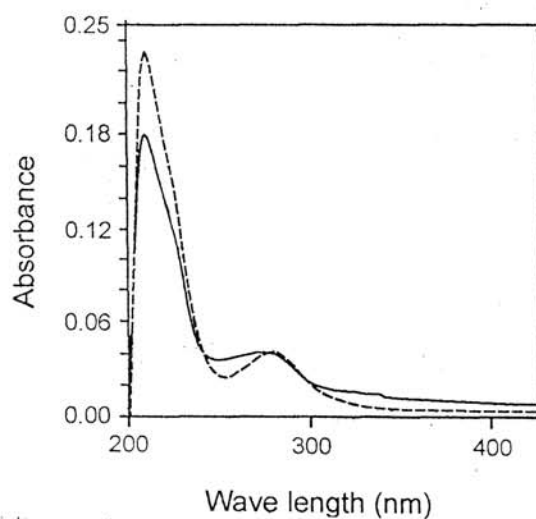


Figure 1. UV visible spectra of the purified isocitrate lyase
Dotted line, before dialysis; solid line, after dialysis (after DTT was removed).

Table 3. Inhibition of isocitrate lyase activity by -SH modifying reagents

Inhibitors	Concentration (mM)	Inhibition rate (%)
PCMB	0.0002	98
	0.001	100
Iodoacetate	1	2
	5	20
	10	33

Inhibition of isocitrate lyase activity by -SH modifying reagents

Inhibition of isocitrate lyase activity by -SH modifying reagents, p-chloromercuri-benzoate (PCMB) and iodoacetate was investigated. Results, Table 3, show that 1 mM PCMB completely inhibited the enzyme activity, whereas 5 mM iodoacetate gave 20% inhibition and a small increase in inhibition rate (33%) was obtained at 10 mM iodoacetate. Furthermore, inactivation of the enzyme by iodoacetate was found to be a time- and concentration-dependent manner and isocitrate fairly protected the enzyme inactivation, as shown in Figure 2.

When the enzyme was incubated for 30 minutes in the presence of 5 mM iodoacetate, the enzyme activity was inhibited by 35% of the original activity. At this incubation period, isocitrate did not show a protective effect, but when incubation was extended isocitrate partially prevented the enzyme from inactivation.

About 45% activity remained when the enzyme was cultivated in the presence of 5 mM iodoacetate and 2 mM isocitrate. However, the enzyme was almost completely in active of when it was incubated with iodoacetate alone at this incubation period. In agreement, the enzyme from *E. coli* was also protected by isocitrate against inactivation by 3-bromopyruvate (Ko and McFadden 1990) and diethyl pyrocarbonate (Agy and Paznokas 1985).

All together, the results clearly demonstrated the important of sulfhydryl compounds, with DTT as the best reduction, to keep the enzyme in a reduced state. Thus it has been proposed that *F. palustris* isocitrate lyase is a -SH enzyme. Importantly, cysteine residue was found to be located in a conserved region of the obtained cDNA fragment suggesting that

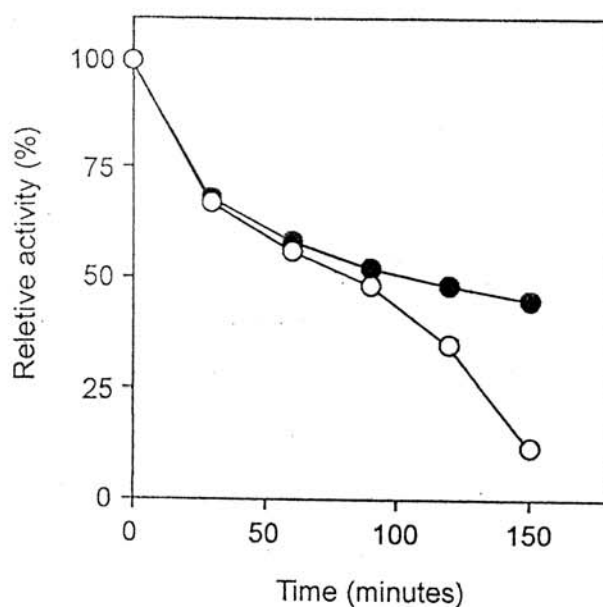


Figure 2. Inactivation of isocitrate lyase by iodoacetate.

Open circle, the enzyme was incubated in the presence of 5 mM iodoacetate; filled circle, the enzyme was incubated in the presence of 5 mM iodoacetate and 2 mM isocitrate, cysteine residue plays an important role in catalytic function isocitrate lyase (Nishide 2002). As a consequence, in order to keep the enzyme from oxidation, the sulfhydryl compound DTT was always included to the buffer solution.

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