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Purification and Characterization of Glyoxalase I from *Chlamydomonas reinhardtii*

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Abstract: Glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) from *Chlamydomonas reinhardtii* was purified to homogeneity by ammonium sulfate fractionation, anion-exchange chromatography, hydrophobic interaction chromatography, and affinity chromatography on *S*-hexylglutathione agarose. The purified enzyme was judged to be homogeneous on SDS-PAGE, and consisted of a single polypeptide chain with a relative molecular weight of 24,000. The enzyme was most active at 40℃ and pH 7.5. It was catalytically most active with methylglyoxal as substrate. A number of properties of the *Chlamydomonas* glyoxalase I enzyme, such as substrate specificity, molecular mass, kinetic parameters, pI, metal ion effect, have been determined and compared with those reported for preparations from other sources. It had somewhat different characteristics from mammalian enzymes.
Key words: *Chlamydomonas reinhardtii*, glyoxalase I, *S*-hexylglutathione, methylglyoxal.

Most methylglyoxal is metabolized by a glyoxalase system, composed of two enzymes, glyoxalase I and glglyoxalase II (Thornalley, 1990; Ranganathan *et al.*, 1995). Glyoxalase I catalyzes the formation of *S*-lactoylglutathione from hemithioacetal formed non-enzymatically from methylglyoxal and reduced glutathione (Murata *et al.*, 1989; Jacoby, 1990). Glyoxalase II catalyzes the hydrolysis of S-lactoylglutathione to D-lactic acid and regenerates the reduced glutathione consumed in the glyoxalase I catalyzed reaction. One of the suggested roles of the glyoxalase system is to remove cytotoxins such as methylglyoxal (Cooper, 1984; Carrington and Douglas, 1986; Thornalley, 1990). Although the physiological significance of glyoxalase I is well recognized in detoxifying 2-oxoaldehydes (Murata *et al.*, 1986; Ranganathan *et al.*, 1995), regulation of cell division and proliferation (Fraval and McBrien, 1980; Dudani *et al.*, 1984), and involvement in haem biosynthesis and its relationship with anticancer activity (Szent-Gyorgyi *et al.*, 1967; Gillespie, 1981), the general mode of the biological functions of glyoxalase I is not well established (Inoue *et al.*, 1987; Thornalley, 1990).

Glyoxalase I appears to be a ubiquitous enzyme (Thornalley, 1990), therefore, it has been purified from both eukaryotes (Arronson *et al.*, 1979; Penninckx *et al.*, 1983; Ranganathan *et al.*, 1995) and prokaryotes (Rhee *et al.*, 1986; Lu *et al.*, 1994). Recently, the activity of glyoxalase I in green algae was detected (Hwang *et al.*, 1994). Some properties of this enzyme were somewhat different from the known reported results (Murata *et al.*, 1989; Thornalley, 1990). In this work, we report the purification of *Chlamydomonas* glyoxalase I, and the kinetic properties of this enzyme compared with those of mammals or bacteria.

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Materials and Methods

Materials

C. reinhardtii 5177D mt^- was grown aerobically in TAP media at 25℃ for one week as previously described (Hwang *et al.*, 1994).

Methylglyoxal, reduced glutathione, *S*-hexylglutathione, DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B, and *S*-hexylglutathione agarose were purchased from Sigma (St. Louis, USA). All other reagents were analytical grade and obtained from Merck (Darmstadt, Germany).

A solution of *S*-hexylglutathione used to elute *S*-hexylglutathione agarose was freshly prepared by the method of Hayes (1988).

Enzyme purification

Glyoxalase I was purified from *C. reinhardtii* by the procedure of Rhee *et al.* (1986) with a minor modification. All the procedures were performed at 0℃ to 4℃ unless noted otherwise.

Wet cells (100 g) were washed twice with distilled water and resuspended in 2 volumes of buffer A (100

mM potassium phosphate buffer, pH 7.5, containing 1 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Cell disruption was performed using a Bead beater for 20 cycles of 20 sec stroking and 40 sec resting at 0℃. The cell extract was then clarified by centrifugation at 10,000×*g* for 45 min. The supernatant was fractionated by ammonium sulfate at 40% and 60% saturation (4℃) and pH 7.5, which precipitated the glyoxalase I. The precipitate was dissolved in 48 ml of buffer B (10 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM PMSF) and dialyzed against the same buffer. The dialysate was then loaded onto a DEAE-Sepharose CL-6B column (2.5×30 cm) equilibrated with buffer B. After the column was washed with equilibration buffer to remove unwanted protein, elution was carried out with a linear gradient of KCl in buffer B (500 ml), from 0 to 0.5 M, at a flow rate of 30 ml/h. The fractions that had glyoxalase I activity were pooled and dialyzed against buffer C (10 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM PMSF and 35% (w/v) ammonium sulfate). The dialysate was applied to a phenyl-Sepharose CL-4B column (2.5×15 cm) equilibrated with buffer C. After the column was washed with buffer C, the adsorbed enzyme was then eluted by a reverse linear gradient of ammonium sulfate in buffer C (400 ml), from 35% to 0% saturation at a flow rate of 35 ml/h. Enzyme-containing fractions were pooled, concentrated into 8 ml by Stirred Cell (Amicon, Danvers, MA) using PM10 membrane, and dialyzed against buffer D (10 mM Tris-HCl, pH 7.8, containing 0.1 mM PMSF and 0.2 M NaCl). The concentrated solution was applied to a *S*-hexylglutathione agarose column (0.7×10 cm) equilibrated with buffer D. The column was washed with the same buffer until the absorbance at 280 nm returned to the baseline. The column was then washed with 20 ml of buffer D containing 0.25 mM *S*-hexylglutathione. Glyoxalase I was eluted specifically by using a solution of 3 mM *S*-hexylglutathione in buffer D at a flow rate of 20 ml/h. The fractions having glyoxalase I activity were pooled, concentrated into about 2 ml by Stirred Cell using PM10 membrane, and dialyzed extensively against buffer B. The purified enzyme was stored at −70℃.

Glyoxalase I assay

The glyoxalase I activity was measured at 25℃ by monitoring the *S*-lactoylglutathione formation at 240 nm for 10 min in a reaction mixture (1 ml) containing 10 mM potassium phosphate buffer (pH 7.5), 2 mM methylglyoxal, and 2 mM reduced glutathione (Racker, 1951). One unit of glyoxalase I activity was equal to the release of 1 μM *S*-lactoylglutathione per min and mg of protein. The enzymatic activity as a function of pH was tested in the standard assay mixture but with the potassium phosphate buffer replaced in turn by 50 mM sodium citrate buffer (pH 3.0~6.0), potassium phosphate buffer (pH 6.0~8.0), or 50 mM Tris-HCl buffer (pH 7.5~9.5).

The enzymatic activity as a function of temperature over a range from 15 to 80℃ was also examined in the standard assay mixture.

Protein determination

Protein concentration was determined according to Smith *et al.* (1985) using the BCA Protein Assay Reagent (Pierce, Rockford, USA), with bovine serum albumin as a standard.

Determination of molecular weight

SDS-PAGE was performed according to the method of Laemmli (1970) and gels were stained with Coomasie brilliant blue G-250. The molecular weight of the purified glyoxalase I was estimated by analyzing the standard curve obtained from the following proteins as standard markers: phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,000), and α-lactalbumin (M_r 14,400).

The native molecular weight of the purified glyoxalase I was estimated by analyzing the standard curve obtained from three standard proteins on gel permeation chromatography using a Protein-pak I-125 column. The column was equilibrated and developed with 50 mM sodium phosphate buffer (pH 6.8) at a flow rate of 0.7 ml/min. The column was calibrated with the following standard proteins as markers: blue dextran (M_r 2,000,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 43,000), and horse heart cytochrome c (M_r 12,000).

Results

Enzyme purification

The results of a typical purification procedure are summarized in Table 1. The most effective purification step was represented by the affinity chromatography on *S*-hexylglutathione-Sepharose (Fig. 1). The high efficiency was due to the complete adsorption of the enzyme on the affinity column and to its specific elution with *S*-hexylglutathione.

The *Chlamydomonas* glyoxalase I was purified about 422-fold over the crude extract, with a specific activity of 33.24 units per mg of protein at 25℃. As judged by SDS-PAGE, the enzyme preparation appeared to be homogeneous (Fig. 2).

Table 1. Purification of glyoxalase I from *Chlamydomonas reinhardtii*

Steps	Total protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)	Yield (%)	Purification factor
Crude cell extracts	2881.5	226.3	0.078	100.0	1.0
Ammonium sulfate	953.8	196.0	0.21	86.6	2.6
DEAE-Sepharose	78.1	110.8	1.42	49.0	18.2
Phenyl-Sepharose	10.9	60.5	5.55	26.7	71.2
S-hexylglutathione agarose	0.37	12.3	33.24	5.4	426.2

[a]Protein was determined by BCA protein assay reagent (Smith *et al.*, 1985).
[b]One unit of the enzyme activity was defined as the amount (μmol) of produced S-lactoylglutathione per min by 1 mg of enzyme at the standard reaction condition.

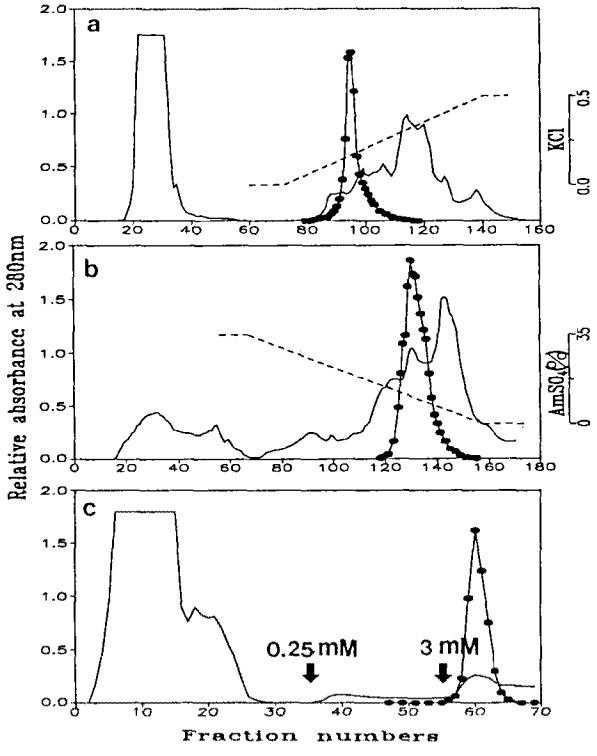

Fig. 1. The purification of glyoxalase I by column chromatographies. (A) Ion exchange chromatography of glyoxalase I on DEAE-Sepharose CL-6B. (B) Hydrophobic interaction chromatography of glyoxalase I on phenyl-Sepharose CL-4B. ($AmSO_4$; Ammonium sulfate). (C) Affinity chromatography of glyoxalase I on S-hexylglutathione agarose. This column was equilibrated with 10 mM Tris-HCl (pH 7.8), containing 0.1 mM PMSF and 0.2 M NaCl. This column was developed by step gradient of S-hexylglutathione. The solid line and the dashed line represent relative protein amount (absorbance at 280 nm) and gradient concentrations, respectively. The closed circles refer to glyoxalase I activity.

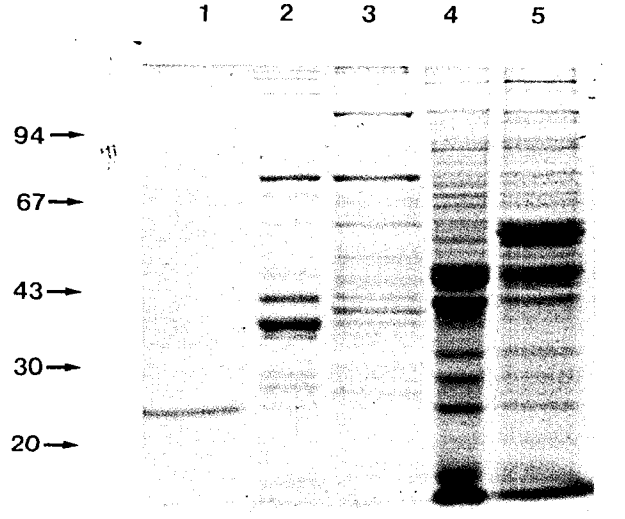

Fig. 2. SDS-polyacrylamide gel electrophoresis of proteins at the various steps of purification. Lane 1: samples from S-hexylglutathione agarose chromatography; lane 2: phenyl-Sepharose CL-4B chromatography; lane 3: DEAE-Sepharose CL-6B chromatography; lane 4: ammonium sulfate fractionation; lane 5: crude cell extracts. The following proteins were used as standard markers: phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000) and soybean trypsin inhibitor (M_r 20,000).

Determination of molecular weight

The purified glyoxalase I migrated to a molecular weight of 24,000 as a single band on SDS-PAGE (Fig. 2, lane 1).

The purified enzyme had a retention time on a HPLC gel permeation column (Protein-pak I-125 column) that corresponded to a M_r of 24,000 (data not shown). The subunit M_r was shown to be 24,000 by SDS-PAGE. These results imply that glyoxalase I of *Chlamydomonas* is a monomer with a M_r of 24,000. On the basis of the results from DEAE-Sepharose CL-6B column chromatography, *Chlamydomonas* glyoxalase I seems to have no other separable isoenzymes (data not shown).

Mammalian glyoxalase I has a M_r of about 43~48 kDa and is a dimer of two identical or similar subunits. Glyoxalase I from prokaryotic or eukaryotic micro-organisms is a monomer of about 20~36 kDa (Thornalley, 1990). Interestingly, *Chlamydomonas* glyoxalase I

exhibited a topology similar to those of eukaryotic micro organisms with monomeric subunit of a M_r 24,000.

Substrate specificity and kinetics

Table 2 shows the substrate specificities of the purified glyoxalase I. It was reported that glyoxalase I had a broad substrate specificity for 2-oxoaldehydes such as glyoxal, phenylglyoxal, methylglyoxal, 4,5-di-oxovalerate, hydroxypyruvaldehyde, and many other alkyl- and aryl-glyoxals (Thornalley, 1990). Among the aldehydes examined, *C. reinhardtii* glyoxalase I can recognize glyoxal, methylglyoxal, and 4,5-dioxovalerate, but not phenylglyoxal or formaldehyde, as substrate.

The specific activities for 4,5-dioxovalerate and glyoxal were 8.32 and 6.97 U/mg, respectively. When the specific activity for methylglyoxal was taken as 100%, those for 4,5-dioxovalerate and glyoxal were calculated to be 25.3 and 21.2%, respectively. This result indicated that glyoxalase I from *C. reinhardtii* has a relatively high specificity for methylglyoxal compared to that other sources (Table 3).

The kinetic parameters of *C. reinhardtii* glyoxalase I for methylglyoxal were determined at 25℃ by the standard assay method. The purified enzyme exhibited Michaelis-Menten kinetics for methylglyoxal with a K_m of 0.44 mM and a V_{max} value of 0.9 mol $min^{-1}mg^{-1}$ of protein, obtained from Lineweaver-Burk plot (data not shown).

Table 2. Substrate specificity of glyoxalase I

Substrate	Specific activity (U/mg)[a]	Relative activity (%)
Glyoxal	6.97	21.2
Methylglyoxal	32.89	100.0
Phenylglyoxal	n.d[b]	n.d
4,5-Dioxovalerate	8.32	25.3
Formaldehyde	n.d	n.d

[a]The activity was determined as described in the 'Materials and Methods'. The substrate concentration was 2.0 mM, except for phenylglyoxal, which was 0.2 mM. The activity for methylglyoxal was taken as 100%.
[b]n.d : Not detectable.

Effects of pH and temperature

The enzymatic activity *vs.* pH was determined over a pH range of 3.0~9.5. In the range of pH 7.0~8.0, glyoxalase I exhibited 90~100% activity, with a maximum at pH 7.5 (data not shown).

When enzyme activity was examined at various temperatures, it was found that the glyoxalase I had maximal activity at 40℃. In the range of temperature between 15℃ to 60℃, glyoxalase I had 80~100% activity, with an optimum temperature at 40℃ (data not shown).

Effects of metal ions and chelating agents

The effects of several divalent metal ions and chelating agents on the glyoxalase I activity were investigated (Table 4). Among the metal ions tested, Fe^{2+} showed

Table 3. Comparisoon of the properties of glyoxalase I from those of various sources

	Sheep liver	*Pseudomonas putida*	*Saccharomyces cerevisiae*	*Aspergillus niger*	*Chlamydomonas reinhardtii*
Mr	45,900	20,000	32,000	36,000	24,000
Subunit	2	1	1	1	1
Zn^{2+} content (per enzyme)	2	n.d[a]	1	n.d	n.d
pI	5.0	n.d	7.0	n.d	n.d
Inhibitor	EDTA[b]	Zn^{2+}	EDTA	EDTA	Ni^{2+}
	EGTA		Zn^{2+}	Zn^{2+}	
	o-PNTL				
Activator	n.d	n.d	Fe^{2+}	Fe^{2+}	Fe^{2+}
			Polyamines		
Substrate (K_m in mM)	MG(0.078)	MG(3.5)	MG(0.35)	MG(1.25)	MG(0.44)
	PhG(0.113)	PhG(n.d)	PhG(0.10)		
	DOVA(n.d)	DOVA(1.2)	DOVA(0.21)	DOVA(0.87)	DOVA(n.d)
References	Uotila and Koivusalo, 1975	Rhee *et al.*, 1986	Marmstal and Mannervik, 1979	Inoue *et al.*, 1987	This study

[a]not determined (n.d).
[b]Abbreviation used are: GSH, glutathione; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene bis-(oxyethylene-nitrilo)-tetraacetic acid; o-PNTL, o-phenanthroline; MG, methylglyoxal; PhG, phenylglyoxal; DOVA, 4,5-dioxovalerate.

Table 4. Effect of metal ions and chelating agents on glyoxalase I activity

Metal ion added	Specific activity (U/mg)	Relative activity (%)
None	32.89	100.0
Zn^{2+}	36.77	111.8
Mn^{2+}	38.15	116.0
Mg^{2+}	39.63	120.5
Fe^{2+}	165.30	502.6
Ni^{2+}	24.14	73.4
EDTA	37.89	115.2
EGTA	34.11	103.7

The activity was determined as described in the 'Materials and Methods'. The metal ionconcentration was 1.0 mM, except for Fe^{2+}, which was 0.25 mM. The enzyme activity without metal ion was taken as 100%.

a remarkable activation of *C. reinhardtii* glyoxalase I. At 0.25 mM of $FeSO_4$, the activity of the enzyme was 165.3 U/mg, 5 times the activity of the metal-free enzyme. At higher concentrations of Fe^{2+} over 0.5 mM, the initial velocity increased too rapidly to be monitored with the present assay method (data not shown). However, Zn^{2+}, which is well known as a potent inhibitor of microbial glyoxalase I, did not show any inhibitory effect on *C. reinhardtii* glyoxalase I activity. Interestingly Ni^{2+} reduced enzyme activity to about 27%. The addition of 1.0 mM of EDTA or 1.0 mM of EGTA showed no additional effect on enzyme activity.

Discussion

Glyoxalase I has been purified and characterized from various sources: yeast *Saccharomyces cerevisiae* (Murata *et al.*, 1986), mold *Aspergillus niger* (Inoue *et al.*, 1987), bacterium *Pseudomonas putida* (Rhee *et al.*, 1986; Lu *et al.*, 1994), rats (Elango *et al.*, 1978), sheep (Uotila and Koivusalo, 1975), and humans (Arronson *et al.*, 1979). As shown in Table 3, the properties of glyoxalase I from microbial cells are significantly different from those of mammalian systems, especially in molecular subunit components and the effect of Zn^{2+} and EDTA or EGTA. In terms of molecular weight, bacterial glyoxalase I from *P. putida* and mold glyoxalase I from *A. niger* are monomers with a M_r of 20,000 and 36,000, respectively. Yeast glyoxalase I also consists of a single polypeptide chain with a M_r of 32,000. However, mammalian glyoxalase I has two subunits per molecule with M_r ranging from 46,000 to 54,000 (Penninckx *et al.*, 1983; Thornalley, 1990). Secondly, in terms of enzyme inhibitors, microbial glyoxalase I is inhibited by Zn^{2+}, unlike mammalian glyoxalase I. On the other hand, glyoxalase I from yeast and mammals has constitutive zinc ion (Zn^{2+}) (Rhee *et al.*, 1986; Murata *et al.*, 1989), which is essential for the catalytic activity of these enzymes. Such enzymes are sensitive to chelating agents such as EDTA and EGTA. However, bacterial glyoxalase I from *P. putida* is not affected by chelating agents. Additionally, there is another difference between bacterial and eukaryotic microbial glyoxalase I. Eukaryotic microbial glyoxalase I is activated by ferrous ion (Fe^{2+}), but bacterial glyoxalase I is not.

The glyoxalase I purified from green algae, *C. reinhardtii*, is a monomeric enzyme with a Mr of 24,000, which is quite similar to that of a microbial enzyme. As shown in Table 3 and 4, *Chlamydomonas* glyoxalase I has a unique response to metal ions and chelating agents. Inhibition by Zn^{2+} has been known as a common characteristic of microbial glyoxalase I. However, *Chlamydomonas* glyoxalase I is not inhibited by Zn^{2+}; this is the case for the mammalian enzymes but not for the microbial ones. *Chlamydomonas* glyoxalase I is not inactivated by chelating agents such as EDTA and EGTA. This is the same phenomenon as in bacterial glyoxalase I. In mammals and in eukaryotic microbial cells, inhibition by chelating agents has been detected. This inhibition is attributed to the removal of catalytically essential Zn^{2+} from the enzyme through the chelating process. Therefore, it is clear that the *Chlamydomonas* glyoxalase I does not have a constitutive Zn^{2+} ion. Additionally, the potent activation by Fe^{2+} shown in *Chlamydomonas* glyoxalase I has been found in eukaryotic microbial cells such as yeast *S. cerevisiae* and mold *A. niger*. Finally, the glyoxalase I from *Chlamydomonas* is the first to show inhibition by Ni^{2+} ion.

These characterization studies of the glyoxalase I from the green algae *Chlamydomonas* successfully illustrate its unique features combining characteristics that are exhibited by bacterial and eukaryotic microbial glyoxalase I as well as mammalian enzymes. This feature also clearly represents the relative position of the unicellular eukaryote, *Chlamydomonas*, on the evolutionary pathway. The data obtained from our experiments may suggest another approach for systematic studies of glyoxalase I.

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