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ORIGINAL PAPER

Purification and characterization of a psychrophilic glutathione reductase from Antarctic ice microalgae *Chlamydomonas* sp. Strain ICE-L

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Abstract A psychrophilic glutathione reductase from Antarctic ice microalgae *Chlamydomonas* sp. Strain ICE-L was purified by ammonium sulfate fractionation and three steps of chromatography. The yield was up to 25.1% of total glutathione reductase in the crude enzyme extract. The glutathione reductase activity was characterized by the spectrophotometric method under different conditions. Purified glutathione reductase was separated by SDS-PAGE, which furnished a homogeneous band. The native molecular mass of the enzyme was 115 kDa. Apparent *K*m values for NADPH and NADH (both at 0.5 mmol L^{-1} oxidized glutathione) were 22.3 and 83.8 μmol L^{-1}, respectively. It was optimally active at pH 7.5, and it was stable from pH 5 to 9. Its optimum temperature was 25°C, with activity at 0°C 23.5% of the maximum. Its optimum ion strength and optimum Mg^{2+} were 50–90 and 7.5 mmol L^{-1}, respectively. Ca^{2+}, Mg^{2+}, and cysteine substantially increased the activity of the enzyme but chelating agents, heavy metals (Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, etc.), NADPH, and ADP had significant inhibitory effects. This glutathione reductase can be used to study the adaptation and mechanism of catalysis of psychrophilic enzymes, and it has a high potential as an environmental biochemical indicator under extreme conditions.

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Keywords *Chlamydomonas* sp. strain ICE-L · Glutathione reductase · Purification · Function characterization · Inhibitory effect

Abbreviations

DTT	Dithiothreitol
EGTA	1,2-Di-(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
NEM	*N*-ethylmaleimide
PVP	Polyvinylpyrrolidone
SDS	Sodium dodecyl sulfate

Introduction

Glutathione and glutathione-dependent enzymes are important in protecting cells against oxidative stress caused by free radicals and their products. Glutathione is the most abundant low-molecular-weight thiol; it is presenting millimolar quantities in cells and in the reduced form (GSH) has many biochemical functions in organisms. GSH is of central importance in detoxification mechanisms and is a cofactor for several enzymes (Pais and Schulz 1983). Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, EC.1.8.1.7; GR), a disulfide oxidoreductase flavoprotein, is the key enzyme of glutathione metabolism and is widespread in the tissues and cells of all organisms (Lascano et al. 2001). This enzyme catalyzes reduction of oxidized glutathione (GSSG) to reduced glutathione in the presence of NADPH and maintains a high intracellular GSH/GSSG ratio in organisms (Pais and Schulz 1983). Otherwise, high GSSG levels can inhibit several important enzyme systems,

including protein synthesis (Lascano et al. 2001). GR seems to be very important in the regeneration of ascorbate and glutathione and plays a pivotal role in scavenging active oxygen generated as a result of environmental stress, for example chilling, irradiation, heat, and heavy metals (Tanaka et al. 1994).

In higher plants GR is involved, by GSSG reduction, in the defense against oxidative stress caused by different factors. Most studies on the response of GR to exposure to heavy metals and other types of environmental stress have shown that GR activity increased as part of the defense against stress (Gratão et al. 2005). Production of larger amounts of GR by transgenic plants might increase the amount of reduced glutathione (Kouřil et al. 2003). The protective role of over-expressed GR against oxidative stress has been observed at both low temperatures and room temperature in transgenic poplar (Foyer et al. 1995). Overproduction of cytosolic or chloroplastic GR in transgenic tobacco plants resulted in lower susceptibility to stress factors compared with control plants (Aono et al. 1993). Expression of the GR gene of wheat increased noticeably during a week adapting to cold (Baek and Skinner 2003).

Results from previous studies of plant and algal GRs have suggested that GR should be conservative (Lascano et al. 2001; Serrano and Lobell 1993), and occurred as a result of the change from a reducing to an oxidizing atmosphere on the primitive Earth (Ondarza et al. 1983). Consequently, GR might be useful as a marker in classification and evolution of organisms.

Antarctic ice microalgae with special characteristics living in an extreme environment characterized by low temperature, high dissolved oxygen, the presence of ice, and strong seasonal changes in light intensity have been investigated increasingly in recent years. Previous studies (Ding et al. 2005, 2006) showed that GR activity in Antarctic ice microalgae was higher than that of mesophile microalgae, and that the activity increased markedly when Antarctic ice microalgae were exposed to the heavy metal cadmium or to low temperature.

Antarctic ice microalgae GR may be slightly different from other GRs because of their greater tolerance of low temperatures. GR has been purified from numerous organism sources, and the native enzyme and its subunits have several molecular forms of markedly different structure and molecular mass. Different GR isoforms from congeneric organisms have been described for some plants (Serrano and Lobell 1993; Tanaka et al. 1994). We are not aware of any other reports on GR of Antarctic ice microalgae except our results (Ding et al. 2005, 2006). The objective of this study was therefore to purify this GR and clarify its properties, and try to explain the high tolerance of low temperatures by Antarctic ice microalgae ICE-L.

Material and methods

Material

DEAE-Sephadex A50, 2′,5′-ADP Sepharose 4B, Sephadex G200, protein assay reagents, and chemicals for electrophoresis were purchased from Amersham Biosciences. GSSG and GSH were purchased from Kyowa, Japan. Low-molecular-mass protein markers were purchased from the Biochem Institute of Shanghai, China. All other chemicals used were of analytical grade and purchased from Sigma or Biomol.

Algal culture and preparation of enzyme extracts

A unialgal strain of Antarctic ice microalgae ICE-L (*Chlamydomonas* sp.) was obtained from the Antarctic pole region (68°30′E, 65°00′S; 71°45′E, 66°15′S) by the key laboratory of marine bioactive substance of the State Oceanic Administration of China and cultured in the Provasoli medium (Provasoli 1968). Flasks containing 1,200 mL medium were inoculated with 300 mL of a mother culture. The microalgae were grown at 6–8°C in the refrigerator under a 12 h:12 h light–dark cycle of 24–34 μmol m^{-2} s^{-1}. Every flask was shaken five times a day.

Fresh ICE-L microalgae (100 g) obtained by centrifugation were powdered in liquid nitrogen. These powdered materials were further homogenized in 4–5 times the volume of 0.1 mol L^{-1} potassium-phosphate buffer (pH 7.8, including 5 mmol L^{-1} GSH, 1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} DTT, 1% PVP) using quartz sand, filtered through four layers of gauze, then centrifuged at 17,000*g* for 15 min. (All operations for purifying GR were conducted at 4°C). Solid ammonium sulfate was added to the supernatant and the precipitate formed between 40 and 90% saturation was collected by centrifugation at 16,000*g* for 20 min (Tanaka et al. 1994). The precipitate was re-suspended in 40 mL buffer A (50 mmol L^{-1} potassium phosphate buffer containing 1 mmol L^{-1} EDTA, 5 mmol L^{-1} GSH, and 0.1 mmol L^{-1} DTT, pH 7.8) for subsequent chromatography.

Chromatography

DEAE-Sephadex A50 (10 g) in a 1.6 cm × 30 cm column was used for ion chromatography. The above sample was loaded on to the column then eluted with an nonlinear gradient of 0–0.5 mol L^{-1} KCl in buffer A. The flow rate was adjusted to 24 mL h^{-1}. Active fractions were collected and dialyzed against buffer B (50 mmol L^{-1} pH 7.8 potassium phosphate buffer containing 0.1 mol L^{-1} KCl, 1 mmol L^{-1} EDTA, and 0.1 mmol L^{-1} DTT). The active fractions were loaded on a 2′,5′-ADP Sepharose 4B affinity-chromatogra-

phy column (0.9 cm × 5 cm) and eluted with 5 mmol L^{-1} $NADP^+$ in buffer B. The flow rate was 15 mL h^{-1}. Active fractions were collected and dialyzed against buffer B. Finally the sample was loaded on a 1.6 cm × 90 cm Sephadex G200 column and eluted with buffer B at a flow rate of 15 mL h^{-1}. The active fractions were lyophilized and store at −78°C for the following test. The native molecular mass of the GR was determined by the same gel filtration chromatography with molecular markers including apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

GR activity determination and SDS-PAGE

GR activity was determined with a model 751 Spectrophotometer at 25°C for 10 min by the method of Guo et al. (1999). Protein concentration was determined at 595 nm by the method of Bradford (1976), using bovine serum albumin as a standard. To check the purity of the enzyme, SDS-PAGE was performed by the method of Laemmli (1970). The acrylamide concentrations in the stacking and separating gels were 5 and 12.5%, respectively. The gel was stained in 0.1% Coomassie Brilliant Blue R-250 and washed with distilled water. Separated protein bands were photographed.

Assessment of apparent Michaelis–Menten constants (*K*m)

The apparent *K*m for each of three possible substrates of the enzyme (GSSG, NADPH, and NADH) were determined by plotting enzymatic activity against concentration of one of the substrates while keeping that of the other component constant and saturating. Enzyme activities were assessed under the above standard conditions.

Effect of pH and temperature on GR

The effect of pH on GR activity was determined by the standard method as described above but at different pH from 5.0 to 11.0; pH 7.5 was used as the control. The pH stability of GR was determined under standard conditions after preincubation of GR for 30 min at different pH within the range 5.0–11.0 at the same time. The thermal dependence of GR activity was estimated by varying the temperature of the reaction mixture within the range 0–40°C; 25°C was used as the control. Apparent energy of activation (*E*a) of GR was estimated by use of the Arrhennius plot. Thermal stability was studied under standard conditions after preincubation of GR for 30 min at different temperatures (55, 65, 75, and 100°C). The preservation stability of the GR was also studied under standard conditions after preservation for several weeks in a refrigerator at 4, −20 and −78°C. Relative activity was expressed as a percentage of control enzyme activity.

Effect of metals and other factors on GR

The effect of ion strength on GR activity was estimated by adding 30–110 mmol L^{-1} KCl (final concentration) to the reaction mixture. The effect of 0–12.5 mmol L^{-1} Mg^{2+} on the GR was also studied. Enzyme activity of the control was assessed under standard conditions. Relative activity was expressed as a percentage of maximum enzyme activity. The effect of different ions and chelating agents on the GR was studied under standard conditions by preincubating 0.003% (*w*/*v*) enzyme with different reagents for 30 min. Relative activity was expressed as a percentage of control enzyme activity.

Results

Purification and molecular weight of GR

GR of ICE-L was purified 12,771.4-fold by four different steps, and its specific activity was 178.8 U mg^{-1} (Table 1). A single protein band was obtained by SDS-PAGE after the last purification step, which showed it was homogeneous. The subunit molecular weight determined was 54.6 kDa (Figs. 1, 2a). The native molecular mass of GR determined by gel-filtration chromatography was approximately 115 kDa (Fig. 2b).

Apparent Michaelis–Menten constants of GR

The apparent *K*m value for oxidized glutathione (at 0.1 mmol L^{-1} NADPH) was 66.0 µmol L^{-1}. *K*m for NADPH and NADH (both at 0.5 mmol L^{-1} oxidized glutathione) were

Table 1 Purification of GR from *Chlamydomonas* sp. Strain ICE-L

Steps	Total volume (mL)	Total activity (Units)	Specific activity (Units mg^{-1})	Purification times	Yield (%)
Crude extract	80	37.8	0.014	–	100.0
Ammonium sulfate	40	36.2	0.031	002.2	95.5
DEAE-Sephadex A50	10	11.8	0.203	014.5	31.2
2′,5′-ADP Sepharose 4B	10	10.8	117.0	8,359.6	28.4
Sephadex G200	2	9.5	178.8	12,771.4	25.1

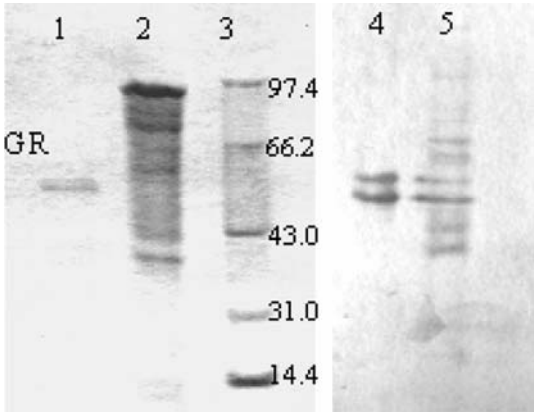

Fig. 1 SDS-PAGE analysis of the GR from *Chlamydomonas* sp. Strain ICE-L: *lane 1*, purified enzyme, *lane 2*, ion exchange chromatography on DEAE-Sephadex A50, *lane 3* molecular mass markers (*top* to *bottom* 97.4, 66.2, 43.0, 31.0, and 14.4 kDa), *lane 4* affinity chromatography on 2′,5′-ADP Sepharose 4B, *lane 5* ammonium sulfate precipitate

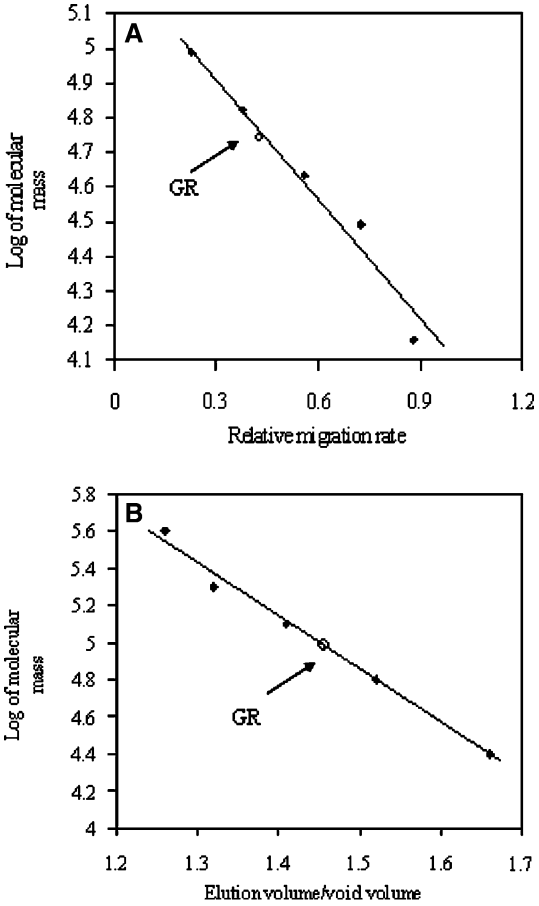

Fig. 2 Molecular mass estimation of GR. The molecular mass of GR subunits is indicated as 54.6 kDa (**a**). Native molecular mass estimation of GR by gel filtration with the markers of molecular mass 443, 200, 150, 66, and 29 kDa. The *arrow* indicates the position corresponding to the peak fraction of GR, the native molecular mass of which is 115 kDa (**b**)

22.3 and 83.8 μmol L^{-1}, respectively (Table 2). *V*max values for NADPH, NADH, and GSSG were 2.60 μmol L^{-1} (mg $min)^{-1}$, 0.60 μmol L^{-1} (mg $min)^{-1}$ and 0.45 μmol L^{-1} (mg $min)^{-1}$, respectively.

Effect of pH on GR

The highest GR activity was obtained between pH 7.5 and 8.5, and pH 7.5 was the optimum pH. Outside the pH range a marked reduction in GR activity was observed, especially between pH 9.0 and 11.0 (Fig. 3). Compared with the control (at pH 7.5) in the stability test, the enzyme lost only nearly 10% of its activity at pH 5.0 but activity decreased by more than 25% when pH was higher than 10 (Fig. 3). So GR activity is steady between pH 5.0–9.0, but unsteady above pH 10. Figure 3 also shows that GR was rather stable within the optimum pH range.

Effect of temperature on GR

GR activity increased steadily at least up to 25°C, and activity was also observed at 0 or 40°C (Fig. 4a). The relative activity at 0°C was 23.5% of the maximum (the control) at 25°C. The relative activity at 40°C was 35.3% of the

Table 2 Kinetic data for GR from *Chlamydomonas* sp. Strain ICE-L

GR	*K*m (μmol L^{-1})			Ref.
	GSSG	NADPH	NADH	
ICE-L GR	66.0	22.3	83.3	This study
Chlamydomonas reinhardtii GR1	50	7	ND	Serrano and Lobell 1993
Chlamydomonas reinhardtii GR2	50	28	ND	Serrano and Lobell 1993

ND, not determined

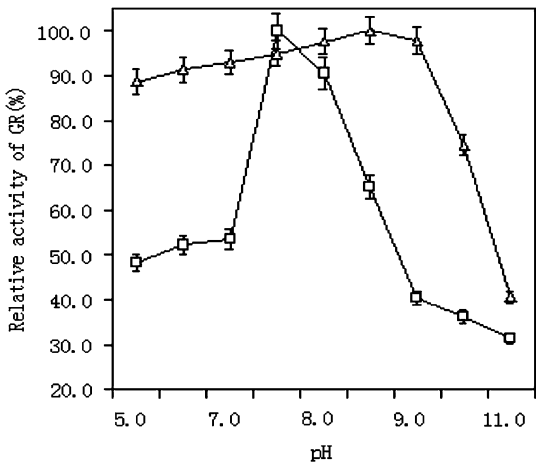

Fig. 3 Effects of pH on the activity of the purified GR from *Chlamydomonas* sp. Strain ICE-L. Values are means ± SE ($n = 3$). pH optimum (*open squares*), pH stability (*open triangles*)

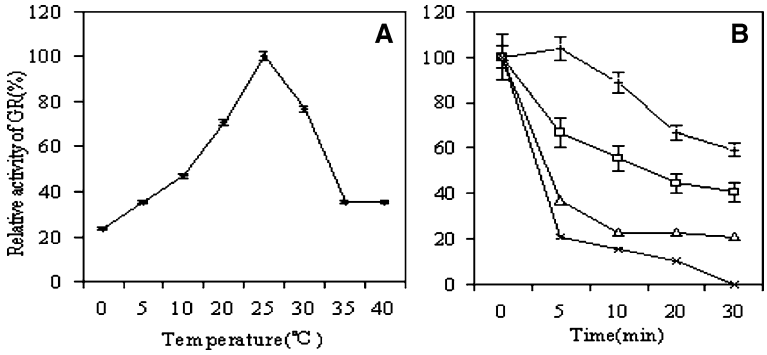

Fig. 4 Thermal dependence of the purified GR from *Chlamydomonas* sp. Strain ICE-L. Values are means ± SE ($n = 3$). Temperature optimum (**a**), Stability of the GR (**b**). 55°C (*plus symbols*), 65°C (*open squares*), 75°C (*open triangles*) and 100°C (*crosses*)

maximum. The results showed that *E*a of ICE-L GR was 3.7 kJ (mol K)$^{-1}$ (plot not shown).

The enzyme was found to be stable at 55°C for 5 min but it lost about 50% of its activity after approximately 10 min at 65°C and was completely inactivated after 30 min at 100°C (Fig. 4b). GR was stable; it lost only 2% of its activity when preserved at −78°C but lost 20% of its activity after 4 weeks at −20°C and lost approximately 50% of its activity after 2 weeks. It was almost completely inactivated after 4 weeks at 4°C (Fig. 5).

Effect of metals and other factors on GR

Figure 6 shows that GR activity was higher when the ion strength was between 50 and 90 mmol L^{-1}. There is probably no significant maximum here but a plateau of larger values. The relative activity of the control without KCl was 54.1% of the maximum (data not shown in Fig. 6). Mg^{2+} had remarkable effect on the activity of ICE-L GR. The maximum GR activity was obtained with 7.5 mmol L^{-1} Mg^{2+}, and approximately 60% of the activity was lost if Mg^{2+} was not present (Fig. 7).

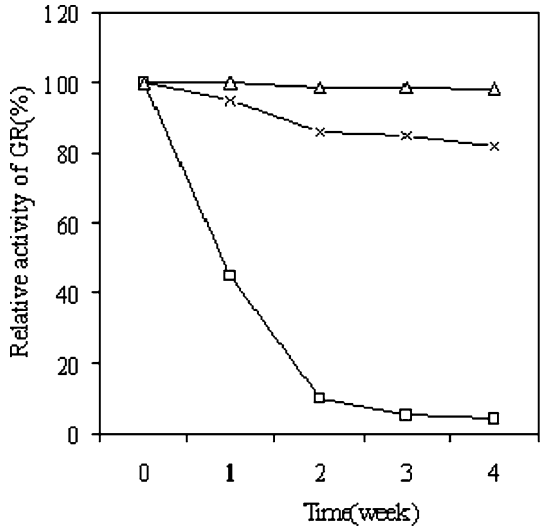

Fig. 5 Stability of GR from *Chlamydomonas* sp. Strain ICE-L when it was preserved at different temperatures. Values are means ± SE ($n = 3$). 4°C (*open squares*), −20°C (*crosses*), and −78°C (*open triangles*)

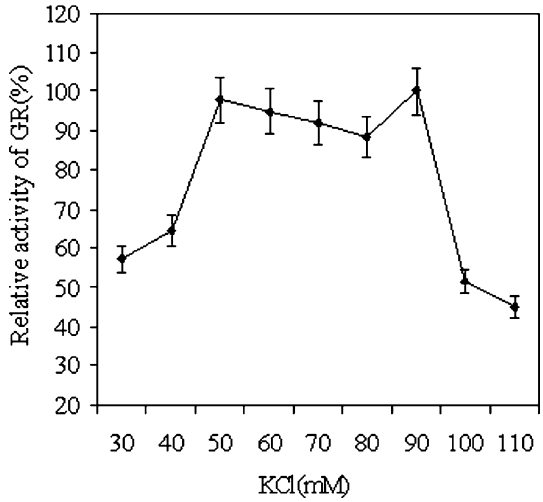

Fig. 6 The effect of ion strength on GR from *Chlamydomonas* sp. Strain ICE-L. Values are means ± SE ($n = 3$)

The enzyme was found to be sensitive to 1,2-di-(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraaetic acid (EGTA), which inhibited 100% of the activity. Compared with the

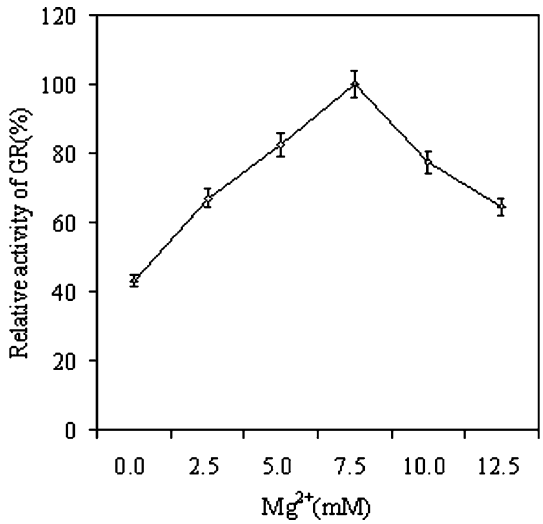

Fig. 7 The effect of Mg^{2+} on GR from *Chlamydomonas* sp. Strain ICE-L. Values are means ± SE ($n = 3$)

control, all bivalent and trivalent ions except Ca^{2+} and Mg^{2+} inactivated the enzyme. Some univalent ions had no marked effect on its activity (Table 3). It was evident that Ca^{2+} was a strong activator and that Cd^{2+}, Pb^{2+}, Cu^{2+}, Al^{3+}, and Zn^{2+} were strong inhibitors which inhibited 100% of the original activity.

It was also found that neither β-mercaptoethanol nor $NADP^+$ had any effect on the GR activity whereas *N*-ethylmaleimide (NEM), NADPH, and ADP significantly inhibited the activity. ATP, GSH, and, especially, cysteine (Cys) had a marked positive effect on GR (Table 3).

Discussion

The glutathione reductase of ICE-L studied in this work had properties similar to and different from those of other reported GRs (Halliwell and Foyer 1978; Serrano and Lobell 1993; Rendon and Hernandez 2001).

The molecular mass of the subunit of the GR (Fig. 2a) is similar to that of plasmodium GR (55 kDa) (Gutterer et al. 1999) but there are substantial differences between the molecular mass of this GR and those of *Chlamydomonas reinhardtii* GR (66 kDa) (Serrano and Lobell 1993) or grass GR (44 kDa) (Hakam and Simon 2000). The native molecular mass (115 kDa) of the GR was in the range of reported data (100–190 kDa) (Halliwell and Foyer 1978). These results suggest that the GR is a dimer, formed by two identical subunits. Most other studies concur with our results in considering GR to be a dimer (Halliwell and Foyer 1978).

It was shown that *K*m for NADH of ICE-L GR was much higher than that for NADPH (Table 2). This indicated that the GR could catalyze the reduction of GSSG using NADH as reducer, although the efficiency was low. Data from the literature indicate that kinetic data for the enzyme vary depending on the study and the source (Serrano et al. 1984). *K*m (NADPH) values of reported GRs range from 1.3 to 13 μmol L^{-1} and values for *K*m (GSSG) range from 7.5 to 200 μmol L^{-1} (Serrano et al. 1984). Compared with other purified GRs (Serrano et al. 1984), it seems that the affinity of ICE-L GR is low for NADPH and moderate for GSSG. Compared with purified *Chlamydomonas reinhardtii* GRs (Serrano and Lobell 1993), however, it is significant that the affinity of ICE-L GR is low for GSSG. Its affinity for NADPH is close to that of *Chlamydomonas reinhardtii* GR2 (a chloroplast GR) (Table 2) but inhibition was marked at high NADPH concentrations (Table 3). This may be the reason *K*m for NADPH of ICE-L GR was higher than other GRs (Serrano and Lobell 1993).

Reported pH optima of GRs vary between 7.0 and 9.0 (Halliwell and Foyer 1978; Kouřil et al. 2003), and pH optima for ICE-L GR also fell within the range. The narrow range of optimum pH for GR activity could effectively regulate its activity in the ICE-L cell. The optimum pH for ICE-L GR was slightly more acidic than that for spinach GR (8.5–9.0) (Halliwell and Foyer 1978) and *Cyanobacteria* GR (9.0) (Serrano et al. 1984); it was similar to those

Table 3 Effect of some reagents on GR from *Chlamydomonas* sp. Strain ICE-L

Reagent	Concentration (mmol L^{-1})	Relative activity of GR (%)	Reagent	Concentration (mmol L^{-1})	Relative activity of GR (%)
EGTA	0.9	0	Mn^{2+}	0.25	73.3 ± 6.0
EDTA	0.9	16.6 ± 1.4	Ag^{+}	0.25	53.3 ± 6.6
$EDTA(Na)_2$	0.9	25.3 ± 2.9	Hg^{2+}	24 μmol L^{-1}	24.6 ± 1.8
KNO_3	1 M	26.7 ± 2.3	Cd^{2+}	25 μmol L^{-1}	0
$(NH_4)_2SO_4$	1	106.7 ± 11.5	Co^{2+}	25 μmol L^{-1}	30.0 ± 3.5
	10	86.7 ± 9.4	Ni^{2+}	25 μmol L^{-1}	20.0 ± 2.6
NaCl	100	86.5 ± 7.8			
KCl	100	95.0 ± 8.7	NEM	1	26.7 ± 3.2
KI	2.5	73.3 ± 7.6	β-mercaptoethanol	5	100.0 ± 8.0
Fe^{3+}	1.25	26.7 ± 4.2	GSH	1	106.7 ± 11.3
Ca^{2+}	0.5	113.0 ± 9.6	Cys	1	133.3 ± 12.0
Mg^{2+}	2.5	140.0 ± 12.5	GSSG	0.5	106.0 ± 10.8
Pb^{2+}	0.25	0	DTT	5	106.0 ± 11.5
Al^{3+}	0.25	0	NADPH	0.5	60.0 ± 5.7
Cu^{2+}	0.25	0	$NADP^+$	1	100.0 ± 5.0
Ba^{2+}	0.25	33.3 ± 4.6	ATP	1	106.0 ± 9.8
Zn^{2+}	1.25	0	ADP	1	73.3 ± 6.8

Values were means ± SE (n = 3). Relative activity was expressed as a percentage of the enzyme activity of the control

for *Chlamydomonas reinhardtii* GR (7.0 or 8.5; Serrano and Lobell 1993), wheat GR (8.0; Lascano et al. 2001), and rice GR(7.9; Kouřil et al. 2003).

GR is important in alleviating the harm of reactive oxygen species (ROS) produced as a result of cold or freezing stress. It has been reported that GR of several plant species increased during cold acclimation (Leipner et al. 1999). GR affected the revival of soy seedlings at 5°C (Kocsy et al. 2001). Anti-cold species of maize and tomato had higher GR activity (Leipner et al. 1999). Our previous results suggested that the GR activity of ICE-L correlated positively with low temperature (Ding et al. 2006). So GR should be important in the adaption of ICE-L to the Antarctic environment. In this study the optimum temperature of ICE-L GR is approximately twenty degrees lower than that of mesophile GR (Lascano et al. 2001), and *E*a of ICE-L GR was less than that for the GR of cold-adapted grass (Hakam and Simon 2000). It is clear that the GR is tolerant of low temperatures with high activity. The optimum temperatures of psychrophilic enzymes tend to be low. A low temperature optimum has also been observed for an extracellular enzyme of Arctic bacteria (Huston et al. 2000). Enzymes adapted or acclimated to cooler climates should have lower activation energies than those adapted or acclimated to warmer climates (Hakam and Simon 2000). So it can be concluded that the GR is a psychrophilic enzyme with different thermal properties, which has evolved with acclimation of ICE-L to the Antarctic polar region. This low-temperature activity could be part of the responses of Antarctic ice microalgae to the cold. A relatively high value of GR activity at low temperatures would be most effective for maintaining the tolerance of low temperatures by ICE-L. So an increased defense system against active oxygen may be effective in maintaining the viability of ICE-L. This Antarctic ice microalga is one of major primary producers in the marine ecology system of the Antarctic polar region and is of primary importance to Antarctic ecology. It is, for example, one of the major food resources of Antarctic krill, the biomass of which is very high in Antarctica. So the GR could have an indirect ecological role in maintaining the viability of Antarctic microalgae.

The ion strength optimum of ICE-L GR was consistent with that of pea GR, which is in the 60–100 mmol L^{-1} range (Kalt-Torres et al. 1984). It is interesting that the residual enzyme activity was 26.7% on treatment with 1 mol L^{-1} KNO_3. This showed the GR is not sensitive to KNO_3. One explanation is that KNO_3 has little effect on the enzyme structure, surface charge, or active site, etc., of GR. But the real mechanism should be clarified by further research. Mg^{2+} is necessary for the ICE-L GR to catalyze the reduction of GSSG by NADPH. These results were helpful for optimizing the conditions for GR assessment. It is clear that EGTA, a special inhibitor of Ca^{2+}, has an opposite effect on the ICE-L GR. So Ca^{2+} is important in the GR. Exogenous inhibitors of the GR, including heavy metal ions, have also been identified and studied by some researchers (Schirmer et al. 1989; Ding et al. 2005). Redox inactivation of glutathione reductase could arise from blocking of the dithiol formed at the active site of the reduced enzyme by metal cations such as Zn^{2+} or Cd^{2+}. Our results showing the effects of Zn^{2+} and Cd^{2+} on the GR were similar to those from other studies. It was also observed that GR activity in the cells dropped substantially when ICE-L was cultured in medium containing Cd (Ding et al. 2005). These results can be used to assess the biological effect of heavy metal ions on Antarctic life. The GR, as a sensitive environmental molecular marker of chemical pollution, has high potential for monitoring of the marine Antarctic environment and evaluating the ecological state of Antarctic water and ice. It is possible that ADP can combine with the active site of the GR and inhibit the activity. The GR activity of yeast cell-free extracts was rapidly inactivated by low concentrations of NADPH (Peinado et al. 1991), which is consistent with our data. $NADP^+$ promoted rapid inactivation of freshly extracted GR (Peinado et al. 1991) but had a different effect in our work. The low concentration of $NADP^+$ may contribute to this phenomenon. The effects of inhibitors are therefore indicative of the possibility of involvement of thiols groups in the catalytic activity of ICE-L GR.

To the best of our knowledge, there have been no studies on the psychrophilic GR from Antarctic ice microalgae except for studies of the effects of temperature and cadmium on GR in cells of *Chlamydomonas* sp. Strain ICE-L (Ding et al. 2005, 2006). In the current paper some properties of purified ICE-L GR have been clarified; the results could be used to explain effectively the acclimation of Antarctic ice microalgae to freezing temperatures. This GR is potentially useful for obtaining antifreezing species by gene-transfer. As a cold-active enzyme, the GR is important for study of the adaptation and catalyzing mechanisms of psychrophilic enzymes. Enzyme activity is correlated with its molecular structure, configuration, and active site. Further structural study is necessary to characterize its differences.

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