Characterization and immunological properties of selenium-containing glutathione peroxidase induced by selenite in *Chlamydomonas reinhardtii*

Shigeru SHIGEOKA,* Toru TAKEDA and Tuyoshi HANAOKA

Department of Food and Nutrition, Kinki University, 3327-204 Nakamachi, Nara 631, Japan

The selenite-induced glutathione peroxidase in *Chlamydomonas reinhardtii* has been purified about 323-fold with a 10 % yield, as judged by PAGE. The native enzyme had an M_r of 67000 and was composed of four identical subunits of M_r 17000. Glutathione was the only electron donor, giving a specific activity of 193.6 $\mu\text{mol/min}$ per mg of protein. L-Ascorbate, NADH, NADPH, pyrogallol, guaiacol and *o*-dianisidine did not donate electrons to the enzyme. In addition to H_2O_2 , organic hydroperoxides were reduced by the enzyme. The K_m values for glutathione and H_2O_2 were 3.7 mM and 0.24 mM respectively. The enzyme reaction proceeded by a Ping Pong Bi Bi mechanism. Cyanide and azide had no effect on the activity. The enzyme contained approx. 3.5 atoms of selenium per mol of protein. On immunoprecipitation, *Chlamydomonas* glutathione peroxidase was precipitated and its activity was inhibited about 90 % by the antibody raised against bovine erythrocyte glutathione peroxidase. The antibody also cross-reacted with the subunits of *Chlamydomonas* glutathione peroxidase in Western blotting SDS/PAGE. In terms of enzymic, physico-chemical and immunological properties, the experimental results demonstrate clearly that *Chlamydomonas* glutathione peroxidase resembles other well-characterized glutathione peroxidases from animal sources that contain selenium.

INTRODUCTION

Organisms usually have catalase and electron-donor-specific peroxidases, glutathione peroxidase (GSHPOD) and L-ascorbate peroxidase (AsAPOD) capable of scavenging H_2O_2 , one of the toxic forms of oxygen [1]. Since catalase, with a low affinity for H_2O_2 , is located only in peroxisomes and cannot decompose H_2O_2 in other intracellular compartments and the lipid peroxides, AsAPOD and GSHPOD function as the first line of defence against the oxidative damage by H_2O_2 or lipid peroxides generated in energy-generating organelles [1,2].

On the basis of data on the occurrences of both GSHPOD and AsAPOD in living organisms, it is likely that the distribution of the peroxidases is distinct: AsAPOD occurs in plant tissues [3,4], *Euglena* [5,6], *Trypanosoma* [7], cyanobacteria [8] and also legume root nodules [9], while GSHPOD is only present in animal tissues [10]. Some investigators have reported that GSHPOD was absent from higher plants [1,11]. In contrast, GSHPOD has been detected in the blue-green alga *Cloecupsa* [12] and extracts from cultured plant cells [13]. Thus the existence of GSHPOD in photosynthesizing organisms has been debated. Recently, some studies have indicated the occurrence of a GSHPOD in the marine diatom *Thalassiosira pseudonana* [14] and insects [15], but the enzyme has not been further characterized.

We have found previously that culture of the green alga *Chlamydomonas reinhardtii* in a medium containing sodium selenite caused the activity of AsAPOD to disappear and GSHPOD to appear [16]. In this study, we purified GSHPOD from *Chlamydomonas reinhardtii* grown in the presence of sodium selenite, characterized the enzyme and studied its immunological properties. Here we also discuss the physiological function of the enzyme coupled with the redox cycle of glutathione in *Chlamydomonas*. Part of these results has been published already [17].

EXPERIMENTAL

Materials

The following were obtained from Sigma: alkaline phosphatase-coupled goat antibody against rabbit IgG, human erythrocyte GSHPOD and Protein A-Sepharose. Molecular markers for SDS/PAGE and nitrocellulose sheets were obtained from Pharmacia Fine Chemicals. Bovine erythrocyte GSHPOD was from Toyobo Co. Other chemicals used were of analytical grade.

Organism and culture

C. reinhardtii Dangeard was cultured aseptically with sodium selenite (3 mg/l) on Allen's medium [18] at 26 °C for 5 days under illumination at 240 $\mu\text{E/s}$ per m^2 and sterile air bubbled at a rate of 8 litres/min.

Enzyme assay

The activity of GSHPOD was monitored spectrophotometrically by coupling with the reduction of H_2O_2 by GSH oxidation with glutathione reductase as described previously [16]. AsAPOD was assayed according to Shigeoka *et al.* [19].

Purification of GSHPOD

All purification steps were done at 4 °C. The algal cells (wet wt. 14.5 g) were washed with 100 mM-Tris/HCl buffer, pH 8.3, containing 5 mM-GSH and 0.3 M-sucrose and disintegrated by passing them through a cooled French pressure cell at 400 kg/ cm^2 . This lysate was centrifuged at 10000 *g* for 20 min. The obtained crude extract was put on to a DEAE-cellulose column (2.2 cm $\times$ 40 cm) equilibrated with the above Tris/HCl buffer. The column was eluted with a 500 ml linear gradient (0–0.6 M) of KCl. Active fractions were combined and fractionated with $(\text{NH}_4)_2\text{SO}_4$, and the pellet precipitating between

Abbreviations used: AsA, L-ascorbate; AsAPOD, L-ascorbate peroxidase; GSHPOD, glutathione peroxidase.

* To whom correspondence should be addressed.

30 and 70% saturation was dissolved in 2 ml of the buffer. The enzyme solution was then chromatographed on a Sephadex G-150 column (2.2 cm × 90 cm) equilibrated with the Tris/HCl buffer. The active fractions were combined and put on to a column (1.6 cm × 10 cm) of DEAE-Sepharose CL-6B equilibrated with the Tris/HCl buffer, and the column was eluted with a 300 ml linear gradient (0–0.4 M) of KCl. The active fractions were combined and concentrated in dialysis tubing with poly(ethylene glycol) 20000 powder for 12 h. The concentrated solution was subjected to chromatography on a Sephadex G-15 column (1.1 cm × 15 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.8, containing 5 mM-GSH to remove KCl. Enzyme fractions were loaded on to a hydroxyapatite column (1.6 cm × 10 cm) equilibrated with 10 mM-phosphate buffer and eluted with a 160 ml linear gradient (10–100 mM) of the above phosphate buffer. The purified enzyme was collected, dialysed for 5 h against Tris/HCl buffer, pH 8.3, concentrated to a final volume of 2.5 ml and stored at –20 °C.

PAGE

Slab-gel electrophoresis in 7.5% polyacrylamide at pH 9.4 was performed as described previously [20]. Proteins in the gel were retained with Coomassie Brilliant Blue R-250 and destained in 5% acetic acid.

M_r estimation

The M_r of the native GSHPOD was evaluated by gel filtration on a Sephadex G-150 column (0.9 cm × 100 cm) equilibrated with 100 mM-Tris/HCl buffer, pH 8.3, containing 5 mM-GSH and 0.1 M-KCl. The column was calibrated with lactate dehydrogenase from pig heart (M_r 135000), malate dehydrogenase (NAD⁺) from pig heart (M_r 70000), BSA (M_r 67000), ovalbumin from egg white (M_r 43000) and cytochrome *c* from horse heart (M_r 12400). SDS/PAGE system of Laemmli [21] was used to run 15% acrylamide gels in a slab-gel format. The following proteins were used as standards: phosphorylase *b* from rabbit muscle (M_r 94000), BSA (M_r 67000), ovalbumin from egg white (M_r 43000), carbonic anhydrase from bovine erythrocytes (M_r 30000), trypsin inhibitor from soybean (M_r 20100) and α -lactalbumin from bovine milk (M_r 14400).

Selenium content analysis

The purified enzyme was subjected to a slab SDS/PAGE. The GSHPOD subunits were excised from the slab gels, and the proteins from several gels were eluted electrophoretically [22]. The eluted protein (27 μ g) was concentrated to a final volume of 0.25 ml in a Centricon-30 microconcentrator (Amicon). The solution obtained was used as the sample for selenium content analysis. Selenium content in GSHPOD subunits was assayed fluorimetrically using 2,3-diaminonaphthalene after digestion with HNO₃ and HClO₄ according to Bayfield & Romalis [23].

Preparation of antibody against GSHPOD from bovine erythrocytes

An antigen preparation consisting of 200 μ g of purified GSHPOD (Toyobo, Japan) in 0.5 ml of 10 mM-Tris/HCl buffer, pH 8.3, and an equal volume of Freund's complete adjuvant was injected subcutaneously into a male 6-month-old New Zealand White rabbit. The immunization was repeated on days 7 and 14. After 3 weeks, 200 μ g of the enzyme emulsified with incomplete adjuvant was injected as a booster. The rabbit was bled after 1 month. Before using sera, they were routinely treated to purify and concentrate the IgG using Protein A-Sepharose. Western blotting was conducted by a modification of the procedure of Towbin *et al.* [24].

Other methods

The pH-stability was determined by the purified enzyme pre-treated at the various pH values for 10 min at 35 °C. The thermal stability was determined by using the enzyme pre-incubated at various temperatures for 10 min at pH 8.3. Determinations of chlorophyll [25] and protein [26] were done by the methods in the references cited.

RESULTS

Purification of GSHPOD

Chlamydomonas GSHPOD was so unstable that it was almost inactivated after 3 days in 100 mM-Tris/HCl buffer, pH 8.3. However, the addition of 5–10 mM-GSH to the above buffer allowed the enzyme to retain 85% of the original activity for 20 days. In the presence of 1 mM-dithiothreitol or 2-mercaptoethanol, the enzyme activity was reduced to a level of 60–70% of the initial activity. Thus we included 5 mM-GSH in the Tris/HCl buffer through the purification. Table 1 summarizes the purification of GSHPOD from *Chlamydomonas reinhardtii*. A six-step procedure yielded a GSHPOD preparation purified approx. 323-fold over the crude enzyme, giving finally a 10% recovery of the peroxidase activity. This purification was repeated several times with similar results. SDS/PAGE showed only one detectable protein band (Fig. 1).

Table 1. Purification of GSHPOD from *Chlamydomonas*

Details of the purification are described in the Experimental section.

Step	Total protein (mg)	Total activity (μ mol/min)	Specific activity (μ mol/min per mg of protein)	Yield (%)
1. Crude extract	225.0	135.0	0.6	100
2. DEAE-cellulose	20.7	47.6	2.3	35.3
3. (NH ₄) ₂ SO ₄ (30–70%)	3.14	21.7	6.9	16.1
4. Sephadex G-150	0.64	19.3	30.1	14.3
5. DEAE-Sepharose CL-6B	0.16	15.4	96.4	11.4
6. Hydroxyapatite	0.07	13.6	193.6	10.1

Fig. 1. SDS/PAGE of GSHPOD from the final purification step

The purified protein (10 μ g) was subjected to electrophoresis in a slab gel as described in the Experimental section.

Fig. 2. Kinetic analysis of *Chlamydomonas* GSHPOD activity with GSH and H_2O_2 .

(a) Double-reciprocal plots of initial velocity against variable H_2O_2 concentrations at several fixed GSH concentrations. GSH concentrations were 0.5 mM (1), 0.6 mM (2), 0.7 mM (3), 1.0 mM (4) and 1.5 mM (5). (b) Re-plots of intercepts against the reciprocal of the GSH concentration. Each experimental point presents the mean for four assays (coefficient of variation < 5%).

M_r estimation

M_r of *Chlamydomonas* GSHPOD was determined by gel chromatography on the calibrated Sephadex G-150 column. The M_r of GSHPOD was estimated at 67000. The subunit M_r of GSHPOD deduced from SDS/PAGE was 17000, indicating that *Chlamydomonas* GSHPOD is a tetramer composed of identical subunits.

Some properties of GSHPOD

When the enzyme was pre-incubated at various temperatures for 10 min in the Tris/HCl buffer, pH 8.3, containing 5 mM-GSH, the activity of the enzyme was stable below 18 °C at between pH 7.8 and 8.7. The enzyme was inactivated with increasing temperatures over 18 °C and the activity was lost completely at 50 °C.

GSH was the sole electron donor; ascorbate, NADH, NADPH, guaiacol, pyrogallol and *o*-dianisidine did not substitute for GSH. The peroxidase reduced *t*-butyl hydroperoxide and cumene hydroperoxide with specific activities of 150.6 and 86.1 $\mu\text{mol}/\text{min}$ per mg of protein respectively. These values corresponded to 77.8 and 44.5% respectively of that found with H_2O_2 as the substrate.

In double-reciprocal plots of H_2O_2 concentration versus reaction velocity with different GSH concentrations, the enzyme system gave parallel lines (Fig. 2), indicating that the reaction proceeds by a Ping Pong mechanism. Secondary plots of intercepts allowed us to calculate the kinetic constants; the K_m values of GSHPOD for H_2O_2 and GSH were 0.24 mM and 3.7 mM respectively. When *t*-butyl hydroperoxide was used as the electron acceptor, the K_m values for GSH and *t*-butyl hydroperoxide were 3.1 mM and 0.026 mM respectively.

Mg^{2+} , Cu^{2+} and Ca^{2+} , all at 1 mM, inhibited the enzyme activity by 33, 44 and 67% respectively, whereas Hg^{2+} , Mn^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} and Fe^{2+} also completely inhibited the enzyme, showing that this enzyme is sensitive to bivalent cations. The reaction mixture for GSHPOD assay contained 0.1 mM- $NaNO_3$. Further addition of KCN up to 1 mM did not affect on the activity of GSHPOD. Thiol inhibitors such as *N*-ethylmaleimide and *p*-chloromercuribenzoate markedly inhibited the enzyme activity.

Selenium content

Chlamydomonas GSHPOD subunits were electrophoretically eluted from the slab gels as described in the Experimental

Fig. 3. Immunoprecipitation of antibody against bovine erythrocyte GSHPOD with GSHPODs from several sources

The reaction mixture contained the indicated amounts of antibody to bovine erythrocyte GSHPOD. The mixtures were left at 4 °C for 1 h and centrifuged at 10000 *g* for 10 min, and the supernatant was used to assay the activity of GSHPOD. Normal serum did not inhibit the enzyme activity. ○, *Chlamydomonas*; □, rat erythrocytes; ●, human erythrocytes; ■, bovine erythrocytes.

section. The selenium content of the GSHPOD subunits (27 μg) was found to be 0.41%. Since each subunit of GSHPOD had an M_r of 17000, as evidenced by SDS/PAGE, it would account for about 3.5 atoms of selenium per molecule of enzyme protein.

Immunological properties

GSHPODs in *Chlamydomonas* and erythrocytes from several sources were incubated with the indicated amounts of anti-GSHPOD antibody to examine whether each GSHPOD is quantitatively immunoprecipitated by the antibody. After incubation, the insoluble complex was removed by centrifugation and the remaining enzyme activity in the supernatant was determined. As shown in Fig. 3, GSHPODs from *Chlamydomonas* and rat, human and bovine erythrocytes were precipitated and their activities were inhibited by approx. 90, 60, 70 and 80% respectively, by antibody against bovine erythrocyte GSHPOD, showing that bovine GSHPOD has the same antigenic epitopes as those of GSHPODs from other sources.

On immunoblot analysis of the purified GSHPOD from

Fig. 4. Immunoblot analysis of antibody against bovine erythrocyte GSHPOD

Proteins were subjected to PAGE and transferred to a nitrocellulose sheet by Western blotting. The sheet was incubated with anti-(bovine GSHPOD) antibody, and the antibody-antigen reaction was detected by using alkaline phosphatase. Lanes: 1, *Chlamydomonas*; 2, bovine erythrocytes; 3, human erythrocytes; 4, rat erythrocytes.

Chlamydomonas, the antibody against GSHPOD from bovine erythrocyte recognized a single protein of M_r 17000, which corresponds to the subunit of the purified GSHPOD (Fig. 4). The antibody also cross-reacted with GSHPOD in bovine [27] erythrocytes and those of human [28] and rat [29].

DISCUSSION

We have demonstrated previously that GSHPOD was induced by growing *Chlamydomonas* in the presence of selenite [16].

GSHPOD has been purified here for the first time from *Chlamydomonas*, an organism capable of photosynthesis. A purified enzyme was obtained by a six-step procedure (Table 1). *Chlamydomonas* GSHPOD was so labile that only 3–4 % of the initial activity was recovered after two chromatographic steps without the reducing agents. Inclusion of 5–10 mM-GSH in the buffer was necessary to prevent the GSHPOD from inactivation during the purification. Reducing agents such as dithiothreitol and 2-mercaptoethanol had a similar protective effect on the enzyme activity. Thiol inhibitors markedly inhibited the enzyme activity. These results show that free thiol groups play an important role in the efficient operation of peroxidation of GSH.

Table 2 shows a comparison of some properties of GSHPOD from several sources. *Chlamydomonas* GSHPOD had an M_r of 76000 and consisted of four identical subunits with an M_r of 17000. Although this M_r value of a subunit is lower than that reported for the rat liver [30], rat lung [31], human placenta [32], bovine [27] and human [29] erythrocytes, *Chlamydomonas* GSHPOD is similar to the others in being a tetramer with the same M_r .

The total selenium content of GSHPOD was estimated at 0.41 %, which corresponds to about 3.5 atoms of selenium/mol of protein, indicating that *Chlamydomonas* GSHPOD is the same seleno-protein as has been reported in GSHPODs from mammalian sources [27,28,32], which contain 4 atoms of selenium/mol of protein. That cyanide and azide, specific inhibitors for a haemoprotein, had no effect on the GSHPOD activity supports this view.

The K_m values of the enzyme for H_2O_2 , t-butyl hydroperoxide and GSH were of the same order of magnitude as those reported for the animal enzyme [28]. The K_m value for GSH was relatively high, but the intracellular concentration of GSH in *Chlamydomonas* cells was approx. 3–4 mM (T. Takeda, S. Shigeoka & T. Mitsunaga, unpublished work), like those in photosynthetic organisms [33–35].

This enzyme used GSH as the sole natural electron donor, and reduced organic hydroperoxides as well as H_2O_2 . It is well known that the mammalian GSHPODs detoxify injurious lipid peroxides and maintain membrane integrity [2,10]. Accordingly, *Chlamydomonas* GSHPOD should work for cell-membrane protection. The kinetic behaviours of the purified GSHPOD followed a Ping Pong Bi Bi mechanism (Fig. 2): the enzyme first reduced

Table 2. Comparison of some properties of the purified GSHPODs from several sources

Property	<i>Chlamydomonas</i>	Rat liver [30]	Rat lung [31]	Human placenta [32]	Human erythrocytes [28]	Bovine erythrocytes [27]
M_r						
Native	67000	75000–76000	80000	85000	95000	84500
Subunit	17000	19000	20000	22000	A: 23000 B: 47000	21000
Se content (atoms/molecule of protein)	3.5	4	Present	4	3.5	4
K_m value (mM)						
GSH	3.10				A: 4.10	
t-Butyl hydroperoxide	0.026				A: 0.052	
GSH	3.70					
H_2O_2	0.24					
Peroxide specificity (%)						
H_2O_2	100		100	100		
t-Butyl hydroperoxide	77.8		84	67		
Cumene hydroperoxide	44.5		97	71		
Reaction mechanism	Ping Pong Bi Bi		Ping Pong Bi Bi			

the peroxide substrate to the corresponding hydroxyl compound, then was regenerated to the native form by GSH. The same mechanism has been reported for rat lung GSHPOD [31] and *Euglena* AsAPOD [6].

As shown in Fig. 3, the antibody against bovine erythrocyte GSHPOD precipitated *Chlamydomonas* GSHPOD and all the erythrocyte GSHPODs. On immunoblots, the antibody reacted with the purified *Chlamydomonas* GSHPOD subunit with an M_r of 17000 and the subunits of the enzyme from bovine, human and rat erythrocytes (Fig. 4). The results on immunological properties indicate that *Chlamydomonas* GSHPOD is similar in molecular structure to that of animal enzymes.

The results reported here allow us to conclude that the enzymic, physico-chemical and immunological properties of selenite-induced GSHPOD in *Chlamydomonas* closely resembles that from mammalian sources containing selenium. As described previously [16], high light intensity (240 $\mu\text{E/s per m}^2$) and low CO_2 concentration (0.03 %) in the atmosphere during the culture of *Chlamydomonas* cells were needed for the induction of GSHPOD with high activity. In addition, when selenite was fed to cells grown in the medium without selenium for 4 days, GSHPOD increased linearly to reach a peak after 24 h. Transfer of *Chlamydomonas* cells either from low CO_2 to high CO_2 (4–5 %) concentration or from the light to the dark, concurrent with the addition of selenite, significantly arrested the increase of GSHPOD (T. Takeda, S. Shigeoka & T. Mitsunaga, unpublished work). It has been suggested that *Chlamydomonas*, grown in low CO_2 concentrations, forms H_2O_2 by pseudocyclic electron transport to produce ATP, which is used to concentrate CO_2 or HCO_3^- in cells or chloroplasts from the surrounding medium [25,36]. From this we suggest that selenite-induced GSHPOD works mainly by scavenging H_2O_2 produced in large amounts when *Chlamydomonas* is concentrating CO_2 .

Within the rat hepatocyte, approx. 70 % of the total GSHPOD activity is accounted for in the cytosol, with 30 % localized within the mitochondrial matrix [37], showing that isozymes function to remove H_2O_2 and lipid peroxides generated in each compartment. In erythrocytes, GSHPOD was only distributed in the cytosol compartment, and constituted the oxidation–reduction cycle of glutathione in combination with glutathione reductase and NADP-dependent enzymes such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [38]. The subcellular localization of the *Chlamydomonas* GSHPOD, together with that of glutathione reductase, remains to be elucidated. However, on the basis of the evidence for a high GSHPOD activity corresponding to the rate of formation of H_2O_2 (100–150 $\mu\text{mol/h per mg}$ of chlorophyll) [25,36], and the rapid and drastic responses of the GSHPOD activity against changes in CO_2 concentration and light intensity during culture [16], it can be hypothesized that this enzyme might be located in the chloroplast compartment to detoxify effectively the H_2O_2 produced at high rates by pseudocyclic electron transport. H_2O_2 has inhibited CO_2 fixation at several defined sites in chloroplasts of higher plants [39] (glyceraldehyde-3-phosphate dehydrogenase, sedoheptulose biphosphatase, ribulose-5-phosphate kinase and fructose biphosphatase). This mode of inhibition is likely to occur in *Chlamydomonas* because this also has a C_3 -type CO_2 -fixation mechanism [40]. Chloroplasts in spinach (*Spinacia oleracea*) leaves contain the redox pairs of ascorbate-(mono)dehydroascorbate and GSH–GSSG, consisting of AsAPOD, (mono)dehydroascorbate reductases and glutathione reductase together with large concentrations of both ascorbate and GSH and with photosynthetically generated NADPH [41].

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