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A SELENIUM-INDUCED PEROXIDATION OF GLUTATHIONE IN ALGAE

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Key Word Index—*Dunaliella primolecta*; *Porphyridium* cruentum; unicellular marine algae; glutathione peroxidation; selenium.

Abstract—Two unicellular marine algae cultured in media containing sodium selenite were examined for glutathione peroxidase activity. The 400 *g* supernatant from disrupted cells of both the green alga *Dunaliella primolecta* and the red alga *Porphyridium cruentum* were able to enhance both the H_2O_2 and the *tert*-butyl hydroperoxide dependent oxidation of glutathione. The glutathione peroxidation activity of *D. primolecta* was reduced only slightly by heating the 400 *g* supernatant, a 30% decrease in the rate with H_2O_2 and 10% decrease in the rate with *t*-BuOOH being observed. Heating caused the H_2O_2 dependent activity in *P. cruentum* to be reduced by only 30%, but the activity with *t*-BuOOH was reduced by 90%. Freezing decreased the *t*-BuOOH dependent activity of *P. cruentum* by 90%, but did not lower the *t*-BuOOH dependent activity of *D. primolecta* or the H_2O_2 dependent activity of either alga. It was concluded that the heat and cold stable, glutathione peroxidation was non-enzymatic in nature. A variety of small molecules (ascorbate, $Cu(NO_3)_2$, selenocystine, dimethyldiselenide and selenomethionine) were shown to be able to enhance the hydroperoxide dependent oxidation of glutathione in the assay system employed in this study. Such compounds could be responsible for the activity observed in algae. The heat and cold labile *t*-BuOOH reductase activity of *P. cruentum* was possibly enzymatic, but was not attributable to the presence of glutathione-*S*-transferase. Both algae, when cultured in the presence of added selenite, displayed an approximate doubling of the non-enzymatic H_2O_2 and *t*-BuOOH dependent glutathione oxidase activities. The heat and cold labile *t*-BuOOH reductase activity of *P. cruentum* was unaltered when the alga was grown in the presence of added selenite. These observations are consistent with the hypothesis that selenium compounds present in the algae are responsible for the selenium induced glutathione peroxidation.

INTRODUCTION

In animals, selenium is now considered to be an essential micronutrient, among other reasons because of its role in the maintenance of normal cellular antioxidant defences [1]. Selenium, in the form of selenocysteine is covalently incorporated at the active site of the enzyme, glutathione peroxidase [2]. This enzyme catalyses the glutathione dependent reduction of H_2O_2 and organic hydroperoxides (including fatty acid hydroperoxides), thereby reducing hydroperoxides and protecting against oxidative damage to the cell.

The role of selenium in plants has yet to be clearly defined. Selenium is considered to be essential only among certain higher plants which are able to accumulate high levels of selenium, primarily in the form of selenoamino acids [3]. Glutathione peroxidase has not been detected in a number of higher plants [4], but algae have not been examined. Protection from oxidative damage in plant cells is conferred by the presence of high concentrations of soluble glutathione and ascorbate [5] as well as membrane associated carotenoids [6] and α-tocopherol [7]. Selenium has not been shown to function as an antioxidant in plants.

During a study of the effect of selenium on algae, we found that a sublethal quantity of selenite improves the growth of a variety of unicellular marine algae [8]. This observation prompted us to investigate the possibility that selenium may function as an antioxidant in algae. This report describes the results of experiments performed to determine whether glutathione peroxidase is present in algae cultured in media containing added selenite.

RESULTS AND DISCUSSION

Although glutathione (GSH) peroxidase has not been found in higher plants [4] algae have not been examined for the presence of this enzyme. The observation that culture in the presence of sublethal quantities of selenite slightly improves the growth of a wide variety of unicellular marine algae [8] prompted us to investigate the possibility that GSH peroxidase is present in algae. The direct measurement of GSH peroxidase activity in two markedly different algae was therefore investigated.

When the 400 *g* supernatant from *D. primolecta* grown in SeO_3^{2-} was examined for GSH peroxidation, the results depicted in Fig. 1 were obtained. Hydroperoxide dependent GSH oxidation with H_2O_2 or *tert*-butyl hydroperoxide (*t*-BuOOH) as substrate was observed. The specific activity using H_2O_2 as substrate is about twice as great as that with *t*-BuOOH. The GSH peroxidase from mammalian sources shows identical rates of reaction with

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✻ Deceased.

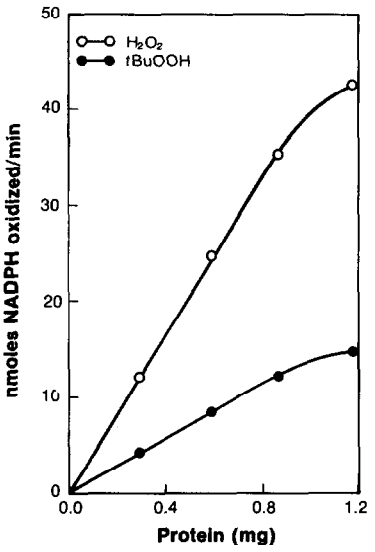

Fig. 1. GSH peroxidation activity as a function of protein in a 400 *g* supernatant from homogenized *D. primolecta* grown in the presence of selenite. Substrate is H_2O_2 (○), *t*-BuOOH (●). Points represent the average of duplicate determinations.

a variety of hydroperoxides (including H_2O_2 and *t*-BuOOH) under maximum velocity conditions [9]. Following heating of the *D. primolecta* 400 *g* supernatant in boiling water for 30 min, a 10% decrease in the specific activity with *t*-BuOOH as substrate and 30% decrease in H_2O_2-dependent activity was observed. In a control experiment, the GSH peroxidase activity of rat liver cytosol was completely eliminated by this heating. Iodoacetate and silver nitrate, inhibitors of GSH peroxidase [10], inhibited the activity towards H_2O_2 observed in both freshly isolated and boiled 400 *g* supernatants; 50% inhibition with iodoacetate and 68% inhibition with silver nitrate.

Dialysis (M_r cut off of 12 000–14 000) did not significantly reduce the specific activity towards H_2O_2; 42 nmol/min/mg before dialysis and 39 nmol/min/mg after dialysis. Freezing at −20° preferentially increased the activity with H_2O_2, such that this activity from frozen 400 *g* supernatant was three times as great as the activity with *t*-BuOOH as substrate. The activity with *t*-BuOOH as the substrate was unaffected by freezing.

These data furnish strong evidence that the observed activity is non-enzymatic. The presence of GSH peroxidase in *D. primolecta* is not indicated. In the assay system used, a number of small organic and inorganic molecules are able to enhance the same reduction (GSH-dependent reduction of hydroperoxides) which GSH peroxidase catalyses in mammals. Caldwell and Tappel have shown that diselenides are able to accelerate the oxidation of GSH in the presence of hydroperoxide [11]. Masukawa and Iwata have been able to demonstrate that selenite and selenate, in addition to diselenides, are capable of enhancing the GSH dependent reduction of methemoglobin [12]. Selenite also exhibits 'GSH peroxidase' activity [13]. In the current investigation copper(II), ascorbate, dimethyl diselenide, selenomethionine and selenocystine have all been found to enhance both H_2O_2 and *t*-BuOOH dependent GSH oxidation in the assay system used to measure algal GSH peroxidation (Table 1). This non-enzymatic activity is unaffected by heat and, in the case of selenium compounds, inhibited by iodoacetate and silver nitrate. All of these small molecules enhance the reduction of H_2O_2 to a greater extent than *t*-BuOOH. The only observation not consistent with the view that hydroperoxide reduction in *D. primolecta* is catalysed non-enzymatically by small molecules such as ascorbate is the inability to remove the activity by dialysis. However, the difficulty of removing selenium bound to proteins by dialysis is well established [14].

When the 400 *g* supernatant from *P. cruentum* grown in SeO_3^{2-} was examined for GSH peroxidation the results shown in Fig. 2 were obtained. Hydroperoxide dependent GSH oxidation with either H_2O_2 or *t*-BuOOH as the substrate was observed. In contrast with the results obtained for *D. primolecta*, the activity with *t*-BuOOH as substrate was somewhat greater than the activity with H_2O_2. When heated in boiling water for 30 min, the *P. cruentum* 400 *g* supernatant exhibited a 30% decrease in H_2O_2 reduction and 90% loss of the *t*-BuOOH

Table 1. Ability of several compounds to stimulate GSH peroxidation (nmol/min/nmol catalyst)

	Substrate	
Catalyst	H_2O_2	BuOOH
Selenocystine	458	296
Dimethyl diselenide	39.2	30.8
Selenomethionine	40.9	5.20
Ascorbate	0.0262	0.0108
$Cu(NO_3)_2$	0.466	*

*Not determined.

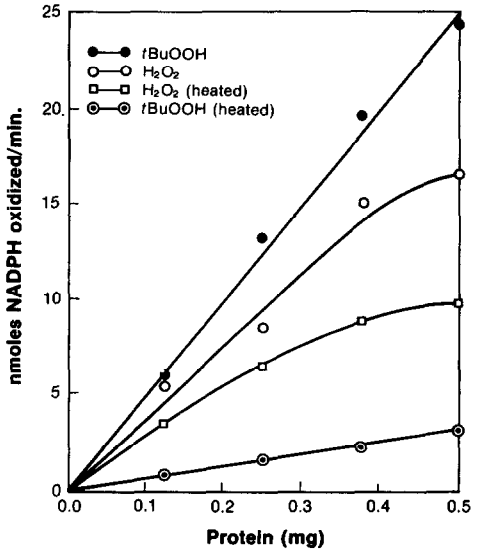

Fig. 2. GSH peroxidation as a function of protein in a 400 *g* supernatant from *P. cruentum* grown in the presence of selenite. Points represent the average of duplicate determinations.

activity. Following dialysis, the specific activity with either substrate was unaltered: 36 nmol H_2O_2/min/mg before dialysis and 37 nmol/min/mg after; 45 nmol *t*-BuOOH/min/mg before dialysis and 48 nmol/min/mg after. The activity with H_2O_2 as the substrate was increased slightly by freezing at −20° while *t*-BuOOH reduction was lowered to the level found in the boiled 400 *g* supernatant.

These results indicate that all of the GSH peroxidase activity observed with H_2O_2 as substrate and a portion of the activity with *t*-BuOOH as the substrate (the heat stable activity) is non-enzymatic. The heat and cold labile reduction of *t*-BuOOH may be enzymatic. However, no selenium induced (discussed later), enzymatic glutathione-dependent hydroperoxide reductase activity was observed. The presence of GSH peroxidase in *P. cruentum* has not, therefore, been demonstrated.

The enzyme GSH *S*-transferase catalyses the conjugation of GSH with a wide variety of hydrophobic, electrophilic compounds. It is present in many eukaryotes, including higher plants [15]. This enzyme in rat liver has been shown to have selenium-independent GSH peroxidase activity toward organic hydroperoxides [16]. H_2O_2 is not a substrate for the rat liver enzyme and its GSH conjugating activity is considerably higher than its organic peroxidase activity. It seemed possible, therefore, that the heat and cold labile, *t*-BuOOH dependent GSH oxidase activity in *P. cruentum* might be catalysed by GSH *S*-transferase.

When the 400 *g* supernatant from *P. cruentum* grown in SeO_3^{2-} was examined for GSH *S*-transferase activity using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the results shown in Fig. 3 were obtained. Very low levels of GSH-dependent GSH *S*-transferase activity were observed. However, the activity was stable to heating in boiling water for 30 min and stable to freezing at −20°. The rat liver enzyme lost all activity when heated at 60° for 10 min [17]. It is concluded, therefore, that the observed activity is non-enzymatic. The heat and cold labile *t*-BuOOH dependent GSH oxidase activity of *P. cruentum* is not considered to be due to the enzyme GSH *S*-transferase. This activity may be enzymatic, but it was not characterized further. The exact nature of the non-enzymatic GSH transferase activity remains unexplained. The observation that it is induced by selenium (Table 2) suggests that some selenium compound may be at least partially responsible.

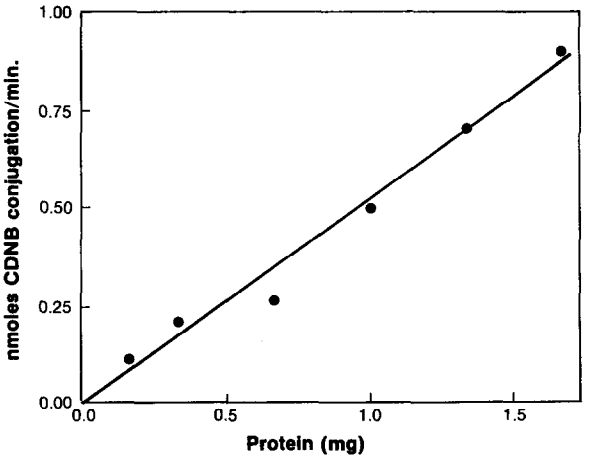

Fig. 3. GSH *S*-transferase activity as a function of protein in a 400 *g* supernatant from sonicated *P. cruentum* grown in the presence of selenite. CDNB is substrate. Points represent single determinations of a single experiment.

Although the presence of GSH peroxidase was not demonstrated in either of the algae, the possibility exists that the selenium compounds present in the algae may non-enzymatically increase the cellular antioxidant potential as measured by the hydroperoxide dependent ability to oxidize GSH. Glutathione peroxidation and GSH *S*-transferase activity of algae grown in the presence and absence of SeO_3^{2-} was therefore determined (Table 2). The presence of SeO_3^{2-} in the growth medium of both algae caused *ca* two-fold increase in the non-enzymatic H_2O_2 and *t*-BuOOH dependent GSH oxidase activities. The non-enzymatic GSH *S*-transferase activity of *P. cruentum* was also doubled. However, the heat and cold labile *t*-BuOOH dependent peroxidase activity of *P. cruentum* was unaltered. These results can be explained by the presence of selenium compounds which are able to enhance the GSH-dependent reduction of hydroperoxides within the algal cell.

This selenite induction of GSH peroxidation was observed a second time in *D. primolecta*. The selenite induction in both algae was observed whether activities were reported on the basis of the 400 *g* supernatant

Table 2. Effect of selenium on GSH peroxidation and GSH transferase activity of *D. primolecta* and *P. cruentum*

		D. primolecta		*P. cruentum*	
		(nmol/min/mg)		(nmol/min/mg)	
Activity	Substrate	−Se	+Se	−Se	+Se
Peroxidase	H_2O_2	29.9 ± 2.46*	76.3 + 12.7†	122 ± 29.0	233 ± 29.0‡
Heat stable peroxidase	*t*-BuOOH	9.83 ± 0.280	20.9 ± 1.88†	14.5 ± 1.62	45.1 ± 20.9§
Heat labile peroxidase	*t*-BuOOH	—	—	33.6 ± 4.51	35.7 ± 9.27
Transferase	CDNB	—	—	1.14 ± 0.224	2.09 ± 0.173†

*Standard deviation of the mean of three replicates.

†‡§Significantly greater than the activity of algae grown in the absence of added selenite as determined by the one-tailed Students *t*-test at the $p < 0.005$ (†), $p < 0.01$ (‡), $p < 0.1$ (§) level.

protein content (nmol/min/mg) or overall cell content (nmol/min/10^8 cells). The protein content of the 400 *g* supernatant from both algae was the same whether grown in the presence or absence of selenite. We observed for *P. cruentum*: 2.80 ± 0.68 mg/10^8 cells (minus selenite) and 3.32 ± 0.09 mg/10^8 cells (plus selenite); for *D. primolecta*: 2.19 ± 0.23 mg/10^8 cells (minus selenite) and 2.19 ± 0.86 mg/10^8 cells (plus selenite).

When *P. cruentum* grown in the absence of selenite were harvested and washed once with artificial sea water containing 10 ppm Se as selenite, the apparent GSH peroxidase activity with H_2O_2 as substrate (1.63 ± 2.82 nmol/min/mg) was still well below the activity measured for algae grown in the presence of selenite (22.1 ± 2.06 nmol/min/mg). This is strong evidence that the observed induction of apparent GSH peroxidase activity by selenium was not due to the presence during assay of selenite bound to the outside of the cells.

The observation that selenite is able to induce a non-enzymatic, hydroperoxide dependent GSH oxidase activity in algae furnishes an *in vitro* demonstration that selenium (compounds) is able to act as an antioxidant independently of GSH peroxidase. Although not proven, it appears likely that selenium compounds are responsible for the selenium induced peroxidation. Dimethyl diselenide and selenocystine, which are believed to be present in the algae [18] also have the ability to enhance the rate of GSH dependent hydroperoxide reduction. The possibility that selenium functions in this non-enzymatic manner as an antioxidant in animals was actively investigated [11, 19, 20] until the relation between selenium and GSH peroxidase was established, following which it was neglected.

The chloroplast of higher plants contains the 'Foyer–Halliwell' cycle to dispose of H_2O_2 generated during photosynthetic electron transport [21]. In this cycle, H_2O_2 is reduced by ascorbate in a reaction catalysed by ascorbate peroxidase. GSH non-enzymatically reduces the dehydroascorbate. Reduced GSH is regenerated *via* reducing equivalents from NADPH in the GSH reductase reaction. The observed GSH peroxidation of algae cultured in the absence of selenite (Table 2) may be attributed to the functioning of this cycle and is limited by the endogenous levels of ascorbate and ascorbate peroxidase (if present). The selenium induction of peroxidation (Table 2) may be a result of the selenium-catalysed, GSH dependent reduction of hydroperoxides, by-passing the ascorbate link in the cycle.

The present observations indicate that selenium compounds have the potential to enhance antioxidant defences of algal cells. Experiments reported elsewhere [22] were conducted in order to determine whether growth in selenite is able to improve the cells antioxidant defences *in vivo*.

EXPERIMENTAL

Algal culture. Dunaliella primolecta Butcher is a biflagellate unicellular marine green alga lacking a cell wall [8]. *Porphyridium cruentum* (S. D. Grey) is a non-motile, unicellular marine red alga containing an inner cellulosic wall surrounded by a continuously produced gelatinous sheath of sulphated polysaccharides [8]. Both algae were obtained from the University of Texas Culture Collection (Austin, TX, U.S.A.). Axenic cultures were grown photoautotrophically at 22° in Müller's artificial sea water (MASW) as described previously [8]. Sulphur at a concn of 850 ppm (Na_2SO_4) was present in all growth media. When added, selenium was present at a concn of 10 ppm as Na_2SeO_3. It should be noted that algae grown in the absence of added SeO_3^{2-} are not necessarily devoid of Se, since traces may contaminate salts in the medium. At 10 ppm Se, all algae exhibit slightly greater rates of growth as compared to algae cultured in the absence of SeO_3^{2-} [8]. No morphological changes in algae are observed under these conditions.

For studies in which the glutathione peroxidation of algae was characterized, *D. primolecta* were grown in 2 l. vol. and *P. cruentum* in 200 ml vol. Algae were pooled at harvest. When glutathione peroxidation of algae grown either in the absence of added selenite (−Se) or in the presence of selenite (+Se) was compared, *D. primolecta* and *P. cruentum* were cultured simultaneously in 250 ml, care being taken to provide a uniform inoculum to each flask. Inocula were derived from exponentially growing algae. Cells in these studies were harvested and analysed concurrently. All algae were harvested during the exponential stage of growth (10^6 cell/ml); *ca* 20 days of culture for *D. primolecta* and 27 days for *P. cruentum*. Cell growth was monitored by measuring the increase in turbidity at 680 nm. Algal cells were counted using a hemocytometer.

Assay of GSH peroxidation. Cells were harvested by centrifugation at 1500 *g* for 10 min. They were resuspended once in MASW lacking SeO_3^{2-} and recentrifuged. *D. primolecta* were then resuspended in 0.1 M Pi buffer, (pH 7 containing 0.1% Triton X-100) which had been purged with N_2 gas and were then homogenized in a glass tissue homogenizer. *P. cruentum* was resuspended in 0.1 M Pi buffer (pH 7 containing 1% Triton X-100) which had been purged with N_2 gas. Large quantities of *P. cruentum* were disrupted by cell cavitation following a 30 min equilibration in an atmosphere of N_2 at 71 bar. Small quantities of *P. cruentum* were disrupted by sonication for 1 min at a setting of 10 (65 watts) with Sonifier Cell Disruptor (Heat Systems Ultrasonics, Inc.). Following cell disruption, homogenates were centrifuged at 400 *g* for 10 min to sediment cell debris and the supernatant was removed for assay of GSH peroxidation.

Glutathione (GSH) peroxidation was assayed at 22° by the method of Wendel [10] using H_2O_2 and *t*-butyl hydroperoxide (*t*-BuOOH) as substrates. In this assay glutathione reductase is utilized to couple the hydroperoxide dependent rate of GSH oxidation to NADPH oxidation which is then measured at 340 nm. In all assays a slow background rate of NADPH oxidation was observed (due to non-specific NADPH oxidase activity and GSH oxidation). Therefore, the reported activity represents the hydroperoxide stimulated rate minus background NADPH oxidation, minus a blank lacking 400 *g* supernatants to correct for GSH reduction of hydroperoxide. The activity observed in algal extracts is dependent upon the presence of GSH and therefore does not represent enhancement of hydroperoxide oxidation of NADPH. Activity is reported as the amount of NADPH oxidized per min per mg of protein. GSH-*S*-transferase was assayed at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to ref. [15]. Activity is reported as the amount of CDNB conjugated with GSH per min per mg of protein at 22°. Protein was determined by the method of ref. [23] at 725 nm.

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