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Identification of a copper-sensitive ascorbate peroxidase in the unicellular green alga *Selenastrum capricornutum*

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Extracts from the unicellular green alga *Selenastrum capricornutum* exhibit high superoxide dismutase activity, but only traces of catalase activity. The excess hydrogen peroxide (H_2O_2) generated by the superoxide dismutase in *S. capricornutum* may be degraded by a unique peroxidase. This peroxidase has a high specificity for ascorbate as its electron donor. The enzyme has an optimum pH at 8, is insensitive to cyanide and is inhibited by oxine. Addition of low concentrations of copper to algal cultures stimulates the peroxidase activity threefold. This enzymatic system could be used as a sensitive bioindicator for copper in fresh water.

Keywords: alga, ascorbate peroxidase, biondicator, copper enzyme, *Selenastrum*

Introduction

The use of algae as bioindicators of changes in water quality is widely established (Miller *et al.* 1978). The rationale for using algae is related to their position as primary producers in the aquatic food chain (Odum 1971). Changes in algal biomass can affect the animals that use alga as a food source. In addition, algal populations can affect the dissolved oxygen concentration, pH, color, alkalinity, clarity and taste of surface waters (Parrish 1985).

The ability of a chemical to reduce biomass production by algae is often used as a criterion in toxicity testing (Thompson & Courture 1991). The unicellular green algae *Selenastrum capricornutum* is commonly used in aquatic toxicity testing (ASTM 1990) because it is easy to grow and because loss in biomass production is often related to the concentration of the toxicant. However, the usefulness of biomass analysis is limited because the effects of toxicants on cell yield are often complex (Goudey 1987). Physiological studies of *S. capricornutum* are limited. In particular, there have been few biochemical studies on stress responses in this organism. A single component enzymatic test may be a more sensitive indicator of exposure to certain toxicants than cell yield. Peroxidase in many plants has been shown to be very sensitive to environmental stress and has been used as a bioindicator (Morgan *et al.* 1966, Lee *et al.* 1976, Asada & Takahashi 1987). In this paper, we describe an ascorbate-specific peroxidase in *S. capricornutum* that was highly sensitive to copper. This peroxidase may be used as a bioindicator of copper in water.

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Materials and methods

Cell culture

S. capricornutum (UTEX 1648) was obtained from the Culture Collection of Algae, University of Texas at Austin. The alga was cultured in Bold's Medium (BM) at pH 7.2 as described by James (1978). The alga requires copper to grow and the concentration of copper sulfate in the BM was 20 μM. Low-copper BM differed from BM only in that copper was added to a final concentration of

5 μM instead of 20 μM. Cultures were shaken continuously, bubbled with air enriched with 1% CO_2, and illuminated under constant cool white light (GE F20T12/CW) at 28ºC. Cells were transferred (15% inoculum) at least twice to fresh medium before growing in 1 liter of fresh BM for testing. Cell growth was monitored by direct cell counting in a Petroff-Hausser counting chamber.

Cell-free extract preparation

Cells were collected by centrifugation at 3000 × *g* for 10 min and washed twice in 50 mM Tris-acetate buffer containing 1 mM EDTA, pH 8 (TAE buffer). Cells were suspended in lysing buffer, which contained TAE buffer, 330 mM sorbitol and 50 μM ascorbate. Cells were disrupted by being passed twice through a chilled French Pressure cell at 18 000 psi. The cell homogenate was centrifuged at 30 000 × *g* for 30 min. The supernatant was used for enzyme assays.

Enzyme assays

The K_m value of this ascorbate peroxidase was quite high (about 0.6 mM). Thus, high concentration of ascorbate was used to ensure accurate measurement of this enzyme. Although ascorbate oxidation can be measured at 290 nm with a mM extinction coefficient of 2.8 (Cheng & Asada 1989, Miyake *et al.* 1991), the high concentration of ascorbate used in our assay prevented the use of this wavelength for measurement. Instead, the rate of ascorbate oxidation was recorded at 305 nm. The enzyme was assayed in a final volume of 1 ml TAE buffer containing 3 mM H_2O_2 and 2 mM ascorbate. The reaction was initiated by the addition of 100–400 μl cell-free extract. Activities were calculated using an extinction coefficient of 0.18 mM cm^{-1}. One unit is defined as the amount of enzyme which can oxidize 1 μmole of ascorbate per min. The glutathione (GSH)-dependent peroxidase was assayed by the method described by Little *et al.* (1970). The NADPH, reduced cytochrome c-, guaiacol-, and pyrogallol-dependent peroxidation were assayed by methods described by Cheng & Asada (1989). All enzyme assays were performed in a temperature controlled cuvette compartment at 25ºC using a Varian model DMS 200 spectrophotometer (Sugarland, TX, USA).

Superoxide dismutase (SOD) was assayed using the procedure of Beauchamp & Fridovich (1971). One unit of superoxide dismutase was defined as that amount which causes 50% inhibition of formazan blue formation. Catalase was measured by the method of Beers & Sizer (1952). Alternatively, the enzyme was measured as the amount of O_2 released from H_2O_2 in the presence of the cell-free extracts. Oxygen release was detected by a Clark type oxygen electrode (Yellow Springs Instruments Model 53, Yellow Springs Instruments, OH, USA). To measure the rate of O_2 production, samples were assayed in the TAE buffer at reduced light at 30ºC. Reaction mixtures without cell-free extract, or with boiled sample, were used as controls and showed no non-enzymatic release of O_2. Protein concentration in the cell-free extracts was determined by the coommassie blue assay (Bio-Rad, Richmond, CA) with BSA as a standard.

Cell culture in the presence of metals

Fifty ml of a three-day old culture (optical density, O.D. at 540 nm = 0.19) grown in BM were transferred aseptically to duplicate sterilized flasks (250 ml). Each flask contained 1 or 2.5 μmoles each of the following compounds: NH_4MoO_3, CdCl, $CuSO_4$, $Co(NO_3)_2$, $MnCl_2$, $Pb(NO_3)_2$, $PbCl_2$ and $NiCl_2$. Culture was also transferred to two empty sterile flasks as controls. Cells were shaken continuously at 150 rpm under constant cool white light at 28ºC ± 2ºC for 32 h. After 32 h, cell growth was recorded and cells from each flask were used to prepare cell-free extracts for assay of peroxidase activity. We noticed that copper is needed for normal growth of *S. capricornutum*. Since copper also stimulated peroxidase production, the low-copper BM was used for detailed analysis of the effect of copper or lead on the peroxidase production. Fifty ml of a three-day old culture (O.D. at 540 nm = 0.12) in low-copper BM were transferred to empty sterile flasks containing a range (0.25–5 μmoles) of either copper sulfate or lead chloride in each flask, incubated for 32 h and assayed.

Time course of peroxidase production

Cells (2 l) were grown in BM for three days and divided evenly to two flasks. Copper sulfate was added to one of the flasks to a final concentration of 100 μM. Both flasks were placed under light and shaken at 150 rpm. At intervals, cells (50–25 ml) were removed from the flasks for cell-free extract preparation.

Results and discussion

Figure 1 shows the growth curve of *S. capricornutum*. Changes in the SOD, catalase and peroxidase activities in cell-free extracts from cells at the same growth period are also shown.

The alga exhibited high levels of SOD activity. The specific activity of SOD increased with the age of the culture and was highest when cells reached stationary phase. The high SOD activity in *S. capricornutum* suggested that a high concentration of H_2O_2 would be produced in the cells. Yet, initial experiments with glutathione, NADPH, reduced

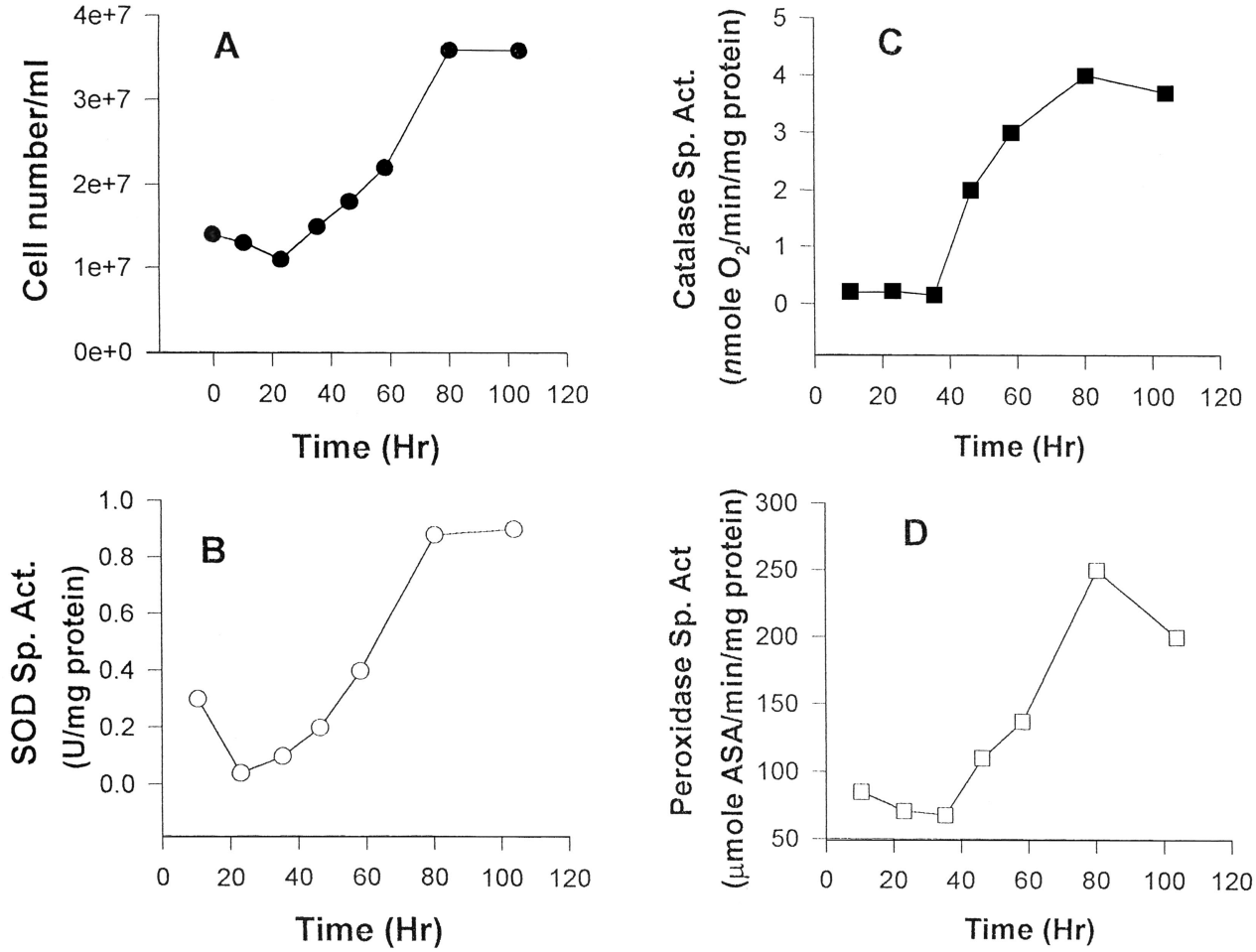

Figure 1. Quantification of superoxide dismutase (SOD), catalase and peroxidase of a growing culture of *Selenastrum capricornutum*. Growth of the culture was monitored spectrophotometrically (A). Cells were removed from the culture at intervals. The cell-free extracts were used to analyze for the activities of SOD (B), catalase (C) and ascorbate peroxidase (D).

cytochrome c, guaiacol, or pyrogallol as electron donor failed to detect any peroxidase activity in *S. capricornutum* extract. Furthermore, a catalase assay using the established procedure of Beers & Sizer (1952) for catalase detection failed to detect the presence of this enzyme in the cell-free extracts.

Subsequent studies showed that *S. capricornutum* exhibited a very low catalase activity. We were able to detect H_2O_2-dependent oxygen formation in the cell-free extracts only with a very sensitive oxygen probe. This suggested that the catalase in *S. capricornutum* was not the major enzyme for H_2O_2 removal.

A hydrogen peroxide-dependent ascorbate oxidation activity was detected in the cell-free extracts of *S. capricornutum*. The level of activity of this peroxidase increased in parallel with the SOD activity and was proportional to the age of the culture. This peroxidase was unusual because it was specific for ascorbate as electron donor. The peroxidase could not oxidize H_2O_2 with other electron donors such as NADH, NADPH, GSH, cytochrome c, guaiacol, pyrogallol, iodide, *o*-dianisidine, or 4-aminoantipyrene (data not shown). This explains the earlier failure to detect peroxidase in *S. capricornutum*.

Ascorbate oxidation occurred only when both H_2O_2 and an active cell-free extract were present in the assay mixture. Ascorbate was not oxidized if the cell-free extract was replaced with boiled cell-free extract. This enzyme showed an optimum pH of 8. At pH 7.0 or 9.2, the activity was approximately 50% of the maximum value. The enzyme was relatively insensitive to cyanide (50% inhibition at

2.0 mM), but was sensitive to oxine (50% inhibition at about 0.6 mM).

Some transition metals can generate free radicals by the metal-catalyzed Haber–Weiss reaction. Free radicals can damage cell components, leading to decrease in growth or even cell death. We reasoned that if these metals were present in the medium, *S. capricornutum* may increase its peroxidase activity to prevent cell damage. We compared the effects of several metals on growth and on the peroxidase level in *S. capricornutum*. Results are summarized in Table 1. Several metals, such as copper, cobalt, lead and nickel inhibited the growth of *S. capricornutum* and induced high levels of peroxidase in the cells. Others, such as molybdate and cadmium exhibited general inhibition of both cell growth and peroxidase activity. The low peroxidase activity in cultures with molybdate was likely due to elevated cell death in these cultures.

Biomass production is commonly used in aquatic toxicity testing (Thompson & Courture 1991). We noticed that the biomass production of algae can be affected by the kind of salt used, not by the metal itself. For example, lead nitrate stimulated cell growth in *S. capricornutum*. Subsequent studies on lead showed that the growth stimulation effect was due to the added nitrate in the medium. Replacing lead nitrate with lead chloride resulted in growth inhibition. This is different from the peroxidase response. The peroxidase response was a more specific method for the detection of certain metals than was biomass measurement. The peroxidase responses to nitrate and chloride salts of lead at similar concentrations were essentially the same.

The sensitivity of the peroxidase differed from metal to metal. Figure 2 shows the levels of the peroxidase in cultures grown on copper or lead at different concentrations. The enzyme increased in a linear fashion at low concentration (5–40 μM) of copper. The activity continued to increase up to about 150 μM copper. This was different from the effect of lead. Lead at low concentrations failed to stimulate the peroxidase. At higher concentrations (30–60 μM), lead stimulated the peroxidase rapidly. However, when the lead concentration was higher than 60 μM, it strongly inhibited growth, and the peroxidase activity decreased due to cell death.

The response of the peroxidase in *S. capricornutum* to metal was not immediate. Figure 3 shows a typical time course of the peroxidase response after copper addition to the culture. Addition of 100 μM $CuSO_4$ to the culture did not result in an immediate increase in enzyme activity. A three-

Table 1. Effects of metals on biomass and peroxidase activity in *Selenastrum capricornutum*. Results are the mean values of three independent experiments

Compound	μM	Relative biomass formed	Relative peroxidase level
Control	–	1.00	1.0
NH_4MoO_3	20	0.96	0.5
	100	0.50	0.5
$MnCl_2$	20	0.97	0.9
	100	0.98	0.9
CdCl	20	0.59	1.0
	100	0.45	N.D
$CuSO_4$	20	0.70	1.4
	100	0.50	2.2
$Co(NO_3)_2$	20	0.61	1.0
	100	0.38	2.2
$NiCl_2$	20	0.49	1.0
	100	0.45	1.8
$Pb(NO_3)_2$	20	0.98	1.0
	100	1.11	1.7
$PbCl_2$	20	0.79	1.0
	100	0.46	1.8

N.D. = no data available

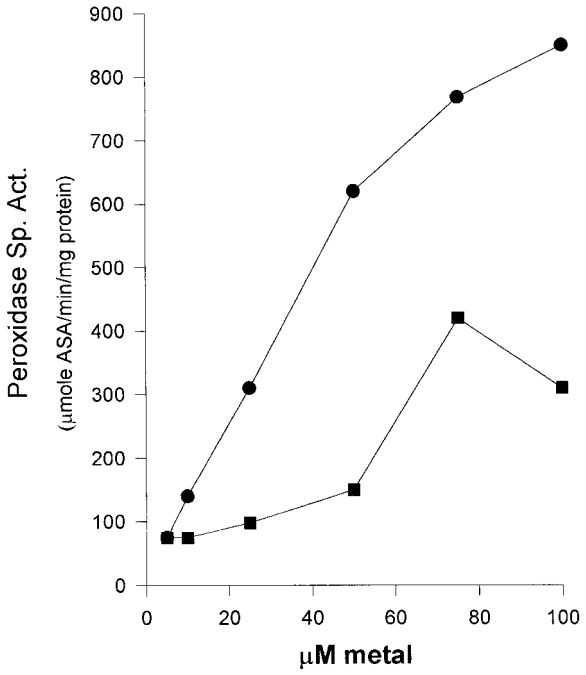

Figure 2. Responses of ascorbate peroxidase in *Selenastrum capricornutum* to $CuSO_4$ (●) and $PbCl_2$ (■). Fifty ml of three-day old culture grown in low-copper Bold's Medium were used. Cultures were exposed to the metals for 32 h before assay.

fold increase in peroxidase activity was observed 8 h later.

Ascorbate-dependent peroxidases have been demonstrated in algae (Shigeoka *et al.* 1980), and in cyanobacteria (Tel-Or *et al.* 1986, Miyake *et al.* 1991). Two types of ascorbate peroxidase have been recognized. One type has a high donor specificity for ascorbate but also shows significant activity with pyrogallol and guaiacol (Cheng & Asada 1989). This type is labile without ascorbate and has a broad pH optimum around 6 (Cheng & Asada 1989, Miyake *et al.* 1991). The other type of ascorbate oxidizing peroxidase is less specific for ascorbate and shows some activity with NADH (Cheng & Asada 1989). The latter peroxidase is stable even without ascorbate and has a pH optimum at 7. All absorbate peroxidases reported are heme-proteins and are therefore sensitive to cyanide (Gaspar *et al.* 1982).

The peroxidase reported herein from *S. capricornutum* has an absolute requirement for ascorbate as its natural electron donor. Although ascorbate was initially included in the lysing medium, subsequent studies showed that the enzyme was stable even without ascorbate. It was not likely that this ascorbate peroxidase was a heme-protein because the enzyme was insensitive to cyanide at physiological concentrations. Additionally, spectrophotometric analyses of the cell-free extracts failed to reveal a redox absorbency spectrum typical to hemoprotein. Conversely, the peroxidase was sensitive to oxine, a strong copper chelator (Welcher & Boschmann 1979), indicating the presence of a copper component.

Copper and a few transition metals were shown to increase peroxidase activity in *S. capricornutum*. The relative sensitivity to various metals at 100 μM concentrations ranked from Cu > Co > Pb > Ni. However, at very low concentration, cells responded only to copper in the medium. The increase in ascorbate oxidation was not caused by the metal's direct catalysis of H_2O_2 degradation because: (1) an incubation period was needed after the addition of metal to the culture to observe the increase in peroxidase activity; (2) the cells were well washed in buffer containing chelator (EDTA) to minimize carry-over of metal ions to the enzyme fractions; and (3) addition of copper or lead alone to the assay system did not cause ascorbate oxidation.

The high sensitivity of this peroxidase to copper, and its distinctive copper stimulation profile may be useful as a tool to detect this metal in natural waters. Research is in progress to explore this possibility to quantitate copper in some urban stormwaters, which are often high in copper.

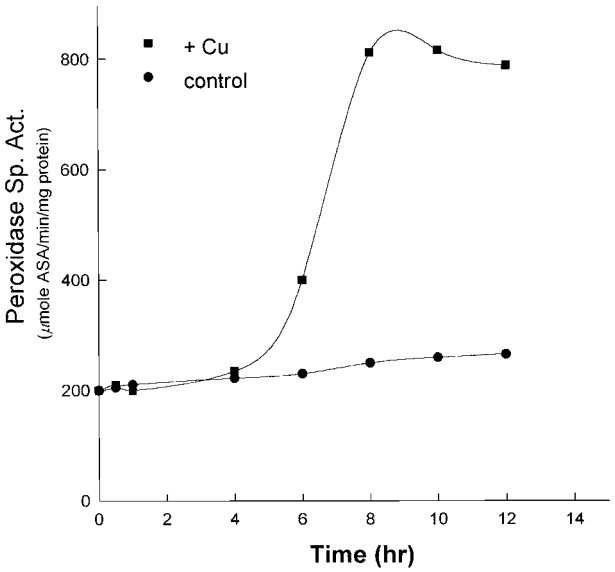

Figure 3. Induction of the ascorbate peroxidase in *Selenastrum capricornutum* by $CuSO_4$. Copper sulfate (0.1 mM) was added to a three-day old culture (■). At intervals, cells were removed from the culture and assayed. The control culture without copper added (●) showed little increase in enzyme activity during the same incubation period.

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