

Effect of copper on the activation of the acid phosphatase from the green algae *Pseudokirchneriella subcapitata*

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Abstract The presence of copper in water environment may have detrimental effects on aquatic organisms, including algae, where different enzymatic systems can be affected. Algae acid phosphatase plays important roles in metabolic processes such as decomposition of organic phosphate, autophagic digestive process, recycling cellular materials and zygote formation during reproduction. This work describes an in vitro activation effect of copper on the acid phosphatase of the green algae *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) under preincubation condition. Apparent Michaelis constant values of 1.21 and 0.37 mM, and activation energy values of 26.8 and 13.6 kJ mol⁻¹ were determined in the absence and in the presence of 0.2 mM Cu²⁺, respectively. The dissociation constant value for Cu²⁺ binding to the enzyme was determined to be 22.04 μM. The decrease of the apparent Michaelis constant (K_m) and activation energy values in the presence of Cu²⁺ correlates well with its activating effect on the acid phosphatase activity. This propriety could be used as

a sensitive bioindicator for copper in environmental samples.

Keywords Enzyme · Phytoplankton · Toxicity · Fungicide · Metal · *Selenastrum capricornutum*

Introduction

There has been an increasing interest in studying the action of metal on enzyme activity and in using their biochemical changes in the aquatic biota as an index of metal toxicity (Lan et al. 1995). In this context, copper, an essential metal ion, has been intensively studied as a potential toxic compound (Gill et al. 1992, Guasch et al. 2002). This element is widely used in agriculture to control fungal diseases and its increasing concentration in the environment has been attributed to mining operations, sewage sludge disposal and industrial discharges (WHO 1998). Copper sulphate has also been used in the control of natural phytoplanktonic communities (Larsen et al. 2003; Le Jeune et al. 2006). The increase of availability and potential toxicity of copper in water may have detrimental effects on aquatic organisms including algae, an important component of primary production and thus the entire aquatic food chain. Algae are able to bioconcentrate and to metabolize

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several aquatic pollutants like insecticides and polycyclic aromatic hydrocarbons (Warshawsky et al. 1995; Jonsson et al. 2001).

Pseudokirchneriella subcapitata (formerly *Selenastrum capricornutum*), an unicellular *Chlorophyceae* (green) algae present in the freshwater aquatic and terrestrial compartments (Keddy et al. 1995), has been widely used in studies of pollutants effects (Jonsson et al. 1998; Okamura et al. 2002) and is recommended by regulatory national (Gherardi-Goldstein et al. 1990; Jonsson and Maia 1999) and international (OECD 1984) agencies as a test organism.

Acid phosphatase (EC 3.1.3.2) plays important roles in the metabolism such as decomposing organic phosphates into free phosphates and organic compounds. Several functions have been attributed to algae acid phosphatases, such as autophagic digestive processes, hydrolysis of phospholipid materials (Cooper et al. 1974), fertilization (breakdown of plasmalemma and absorption of flagella) (Braten 1975), availability of inorganic phosphate from the extracellular medium (Sommer and Blum 1965), recycling of inorganic phosphate for its reassimilation (Theodorou et al. 1991), endomembrane recycling (Domozych 1989) and spore differentiation (Tsekos and Schnepf 1991). In the freshwater algae *S. tenue*, the enzyme was found in the cell wall, plasmalemma and vacuole (Michetti et al. 2006). The induction of acid phosphatase activity in unicellular green algae at low N and P concentrations has been observed by Kruskopf and Du Plessis (2004).

It has been reported that copper or other metal ion are needed for the catalytic action of different acid phosphatases. Vincent and Averill (1990) have observed that the oxidized form of some mammalian acid phosphatases exhibited a iron–phosphorus interaction indicating that phosphate, and presumably the phosphate esters, bound to one of both irons present in the protein. Reilly et al. (1999) demonstrated that the addition of copper restored nearly all the enzymatic activity of an acid phosphatase from *Haemophilus influenzae*. The acid phosphatases from the microorganisms *Mycoplasma fermentans*, *Pseudomonas aeruginosa* and *Yarrowia lipolytica* are enhanced in the presence of copper (Domenich et al. 1992; Noda et al. 1994; Ito et al. 2007). This last effect was also observed by Bounias et al. (1996) in the kinetic studies for alkaline and acid phosphatases present in a gut crude extract obtained from honeybees.

The activities of algal enzymes like esterase (Franklin et al. 2001), β -D-galactosidase (Peterson and Stauber 1996), nitrate reductase, urease, ATPase (Rai and Rai 1997) and antioxidants enzymes (Li et al. 2006) were affected in presence of the metal.

Previously, we reported the activator effect of copper on the activity of acid phosphatase from *P. subcapitata*. Also, we demonstrated that the metal behaved as a slight antagonist for the inhibitor effect of mercury on the enzyme activity (Jonsson and Aoyama 2007). In the present work, we studied the effect of copper ions on the enzyme activity from the algae. We analyzed the stability and determined dose effects and kinetic parameters in order to understand the magnitude and mechanism of metal ion–phosphatase interaction.

Materials and methods

Materials

p-Nitrophenylphosphate (*p*NPP) was obtained from Sigma Chemical Co. Stock solutions of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Merck) were prepared in Milli-Q water or in liquid medium (OECD 1984). All the other reagents were AR grade.

Organisms and growth conditions

Unicellular green algae *Pseudokirchneriella subcapitata* was maintained and subcultured in an inorganic liquid medium prepared as recommended by OECD (OECD 1984). Cultures were grown in 250 ml flasks sealed with cotton bungs and containing 200 ml of sterilized medium. The flasks were incubated in a controlled temperature chamber ($20 \pm 2^\circ\text{C}$) under a continuous white fluorescent light of 3,000–4,000 lux and manually shaken twice a day. Every 40–60 days, a new stock culture maintained at 4°C (in dark) was prepared by inoculating approximately 5×10^4 cells ml^{-1} (Jonsson and Aoyama 2007).

Harvesting and preparation of extracts

All centrifugation procedures were carried out at 4°C . Exponential phase organisms were harvested by centrifugation at 4,000 r.p.m. for 5 min in a Beckman J2-21 refrigerated centrifuge (rotor SER# 7644,

JA-20) and washed twice with 0.1 M sodium acetate buffer, pH 5.0. The algae pellet was suspended in 0.1 M sodium acetate buffer (1:4 w/v) and the cell suspension was submitted to the cell disruption procedure for phosphatase extraction as described previously (Jonsson and Aoyama 2007): The sample was frozen at -20°C , thawed at room temperature and submitted to a probe sonication at 0°C (ice bath) for 50 s followed by 20 s interval (1 cycle) with an amplitude of 70 (Vibra Cell, Sonics Materials Inc., 45 mm tipped probe). This procedure was repeated twice. The resultant cell disrupted suspension was centrifuged at 10,000 r.p.m. for 20 min and the supernatant fluid (extract) was used for acid phosphatase activity determination.

Assay of phosphatase activity

Acid phosphatase activity was routinely assayed at least in duplicate by incubating the enzyme with *p*NPP as substrate and measuring the *p*-nitrophenol (*p*NP) produced as previously described (Prazeres et al. 2004). The enzyme activity was determined in a final volume of 1 ml containing 0.1 M sodium acetate buffer (pH 5.0) and 10 mM substrate. After incubation for 40 min at 37°C , the reaction was terminated by the addition of 1 ml of 1 M NaOH. The *p*NP released was measured at 405 nm in a UNICAM 8625 UV/VIS spectrophotometer. For the initial velocity (*V*) determination, the amount of *p*NP produced was calculated using a molar extinction coefficient of $18,300\text{ M}^{-1}\text{cm}^{-1}$ (Chaimovich and Nome 1970). Units (U) of enzymatic activity are defined as $\mu\text{moles of } p\text{NP released per min.}$

Effect of Cu^{2+} concentration

The enzyme activity was determined in the absence (control) or in the presence of several copper concentrations in a range of 0.1–200 $\mu\text{M Cu}^{2+}$. Lineweaver–Burk plot was used to determine the Cu^{2+} -enzyme dissociation constant by plotting the velocity change (ΔV) versus the reciprocal of copper concentration ($1/[\text{Cu}^{2+}]$), as described by Dixon and Webb (1979).

Enzyme stability

The enzyme activity was determined after preincubation at 37°C for 0, 20, 40 and 60 min in the absence or in the presence of 0.2 mM Cu^{2+} .

Apparent Michaelis constant (K_m) and maximum velocity (V_{max}) determination

The enzyme was preincubated in the absence or in the presence of 0.2 mM Cu^{2+} , and after that the enzyme activity was determined, using *p*NPP as substrate, in a concentration range of 0.1–10.0 mM. The apparent K_m and V_{max} values were calculated from Lineweaver–Burk plot.

Activation energy determination

The enzyme activity was determined after preincubation at different temperatures (27, 32, 37, 42 and 47°C) in the absence or in the presence of 0.2 mM Cu^{2+} . The activation energy values were determined from the slopes of the Arrhenius plots ($\log V$ versus $1/T$ (Kelvin)), according to Dixon and Webb (1979).

Data analysis

The results were analyzed by a Simple Regression program with a Statgraphics® Plus Version 2 software package.

Results

In vitro effect of copper on *P. subcapitata* acid phosphatase activity

A remarkable increase in *p*NPP dephosphorylation was observed in the presence of Cu^{2+} up to a concentration of 0.05 mM, when this metal was previously incubated with the enzyme (Fig. 1). The dissociation constant ($K_d\text{Cu}^{2+}$), for the complex Cu^{2+} -enzyme was calculated from the double reciprocal plot to be 22.04 μM (Fig. 1, inset). In contrast to the results described in the Fig. 1, no significant enzyme activity changes were observed in the presence of Cu^{2+} without preincubation (results not shown).

Stability of acid phosphatase in the presence of copper

The enzyme was stable for at least 60 min at 37°C when preincubated in the presence of 0.2 mM Cu^{2+} . In contrast, a loss of acid phosphatase activity of

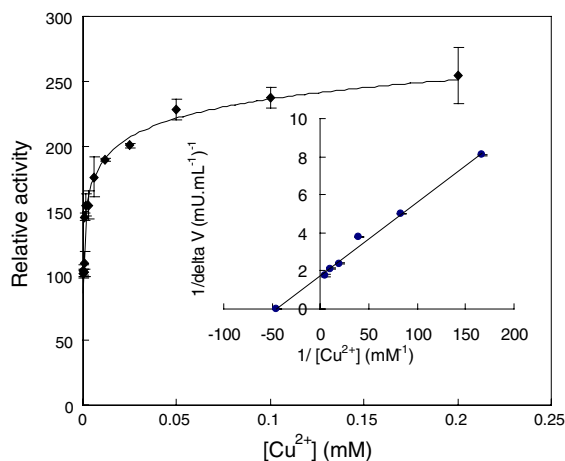


Fig. 1 Effect of copper concentration on acid phosphatase activity. The preincubation of the enzyme and Cu^{2+} (at different concentrations) was performed for 20 min at 37°C . At the end of this period, the reaction was initiated by the addition of *p*NPP 10 mM as substrate, as described in Methods. The activity in the absence of Cu^{2+} was considered as 100% (control) and the other values were determined as percentages of this value. Determination of dissociation constant for the interaction Cu^{2+} -acid phosphatase (inset). Bars represent the standard deviations of the averages of two replicates

about 60% was observed when the enzyme was preincubated in the absence of the metal ion (Fig. 2).

Effect of Cu^{2+} on the acid phosphatase kinetic parameters

The values of apparent K_m and $V_{\max}$ were determined from the double reciprocal plot of Lineweaver–Burk, and the activation energy from the Arrhenius plot (not shown). These results are shown in the Table 1. In the presence of 0.2 mM Cu^{2+} , the apparent K_m value decreased from 1.21 to 0.37 mM and the activation energy from 26.8 to 13.6 kJ mol^{-1} ; no significant difference was observed for the $V_{\max}$ values (0.55 and 0.75 mU ml^{-1}).

Discussion

The metallic ion Cu^{2+} has been generally used in enzymatic systems in order to inactivate the reactions by binding to essential sulfhydryl groups located at or near the active site. In the case of acid phosphatases, we have previously reported that the enzyme purified from bovine kidney (Granjeiro et al. 1997) and from

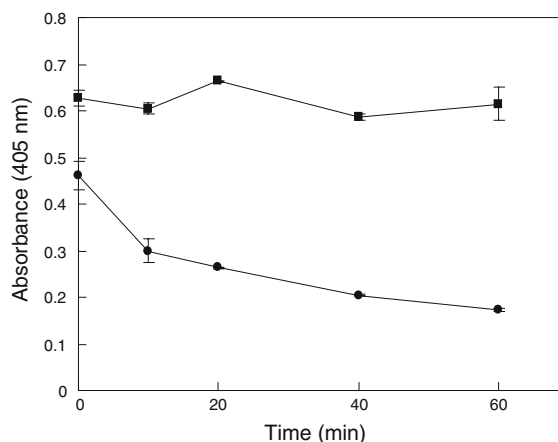


Fig. 2 Preincubation time course of acid phosphatase activity. The enzyme activity was determined at the indicated times by preincubation at 37°C in the absence (●) or in the presence (■) of 0.2 mM Cu^{2+} . Bars represent the standard deviations of the averages of two replicates

Table 1 Effect of Cu^{2+} on the kinetic parameters for *P. subcapitata* acid phosphatase^a

Parameter	[Cu^{2+}] (mM)	
	0.0	0.2
K_m (mM)	1.21 ± 0.42	0.37 ± 0.05
$V_{\max}$ (mU ml^{-1})	0.55 ± 0.16	0.75 ± 0.06
Activation energy (kJ mol^{-1})	26.8 ± 1.5	13.6 ± 3.9

^a The K_m and $V_{\max}$ values were determined from Lineweaver–Burk plot and the activation energy from the Arrhenius plot. The standard deviations ($\pm$) represent the averages of two replicates

castor bean seed (Granjeiro et al. 1999) were inhibited 97 and 93% in the presence of 10 mM and 1 mM Cu^{2+} , respectively.

In the present work we demonstrated that Cu^{2+} when preincubated with *P. subcapitata* acid phosphatase activated the *p*NPP-directed reaction. Lineweaver–Burk plot was used to determine the dissociation constant value for Cu^{2+} binding to the enzyme ($K_d\text{Cu}^{2+}$), as described by Dixon and Webb (1979) (Fig. 1, inset). Youngs et al. (2000) have also applied this method to determine the dissociation constant for the Cd–Mn-peroxidase complex. The low magnitude of the $K_d\text{Cu}^{2+}$ value (22.04 μM) obtained for the complex Cu^{2+} -acid phosphatase indicated a strong binding capacity of this metal to the enzyme.

Even after preincubation of Cu^{2+} , and enzyme, no activation effect was observed for the acid

phosphatase obtained from the algae *Ochromonas danica* (Patni and Aaronson 1974). However, others in vitro studies showed that acid phosphatases from crude extracts of *Rizopus delemar* (Tsekova and Galabova 2003) and honeybee guts (Bounias et al. 1996) were activated by Cu^{2+} .

In order to explain the reasons for the activation of the *P. subcapitata* acid phosphatase by Cu^{2+} , we performed three different experiments: enzyme stability study, determination of apparent K_m , and determination of activation energy, in the absence and in the presence of the metal.

The presence of Cu^{2+} promoted a protection of the *P. subcapitata* acid phosphatase from thermal inactivation at 37°C for at least 1 h (Fig. 2), which supports an enzyme– Cu^{2+} interaction. Protection of enzymes by metal ions in stability studies was also described for fungal laccase by Cu^{2+} (Baldrian and Gabriel 2002), fungal peroxidase by Cd^{2+} and Mn^{2+} (Youngs et al. 2000), and *E. coli* alkaline phosphatase by Cu^{2+} and other metals (Trotman and Greenwood 1971).

The kinetic parameter apparent K_m for *pNPP* decreased (3.3 fold) in the presence of Cu^{2+} (Table 1), suggesting an enhancement on the affinity enzyme-substrate by copper.

Bounias et al. (1996) also described a decrease of the K_m value for honeybee gut acid phosphatase contained in the crude extract in the presence of copper. The apparent K_m value of 0.37 mM, obtained in the presence of Cu^{2+} is similar to those reported for other algae species when the enzyme was assayed in a non-preincubated system and in the absence of the metal (Bennum and Blum 1966; Patni and Aaronson 1974). The decrease of the activation energy value from 26.8 to 13.6 kJ mol^{−1} in the presence of Cu^{2+} (Table 1), also contributed to the activating effect of Cu^{2+} on the *P. subcapitata* acid phosphatase activity.

Summarizing, different factors contributed to the increase in the acid phosphatase activity in the presence of Cu^{2+} : protection of the enzyme against thermal inactivation, increase in the enzyme-substrate affinity, and reduction of the activation energy. The high sensitivity of the enzyme crude extract to copper may be useful as a tool to detect this metal in natural waters and other environmental samples.

We hope that the results presented in this work can improve the understanding of the basic events of the impact of copper at biochemical levels in primary producer organisms. Moreover, they could provide a

sound basis for comparison with enzymatic processes affected by other pollutants.

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