

EXTRACTION, PARTIAL CHARACTERIZATION AND SUSCEPTIBILITY TO Hg²⁺ OF ACID PHOSPHATASE FROM THE MICROALGAE *Pseudokirchneriella subcapitata*

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ABSTRACT: *Pseudokirchneriella subcapitata* is a unicellular green algae widely distributed in freshwater and soils. Due to its cosmopolitan characteristic, its use is recommended by national and international protocols in ecotoxicity studies. The alteration of phosphatase activities by agriculture pollutants like heavy metals has been extensively used as a biomarker in risk assessment and biomonitoring. In this study, we compared the extraction of acid phosphatase from *P. subcapitata* by different procedures and we studied the stability, substrates specificity, kinetics and the effect of Hg²⁺ in the crude extract. The freezing and thawing technique associated with probe sonication was the most suitable method of extraction. The enzyme was stable when frozen at -20°C for at least six months, showed an optimum pH of 5 and a Km value of 0.27 mM for *p*-nitrophenylphosphate (*p*NPP) as substrate. Some natural organic substrates were cleaved by a similar extent as the synthetic substrate *p*NPP. Short term exposure (24 hours) to Hg²⁺ had little effect but inhibition of the specific activity was observed after 7 days with EC₅₀ (concentration of Hg²⁺ that promotes 50% decrease of specific activity) value of 12.63 μM Hg²⁺.

Key words: *Selenastrum capricornutum*, algae, enzyme, metal, toxicity

EXTRAÇÃO, CARACTERIZAÇÃO PARCIAL E SUSCEPTIBILIDADE AO Hg²⁺ DA FOSFATASE ÁCIDA DA MICROALGA *Pseudokirchneriella subcapitata*

RESUMO: *Pseudokirchneriella subcapitata* é uma alga verde unicelular amplamente distribuída em corpos d'água e solos. Devido a sua natureza cosmopolita, seu uso é recomendado por protocolos nacionais e internacionais na realização de estudos de ecotoxicidade. A alteração da atividade de fosfatases por agentes poluentes de origem agrícola, como metais pesados, tem sido largamente usada como um biomarcador na avaliação de risco e biomonitoramento. No presente trabalho foi comparada a extração da fosfatase ácida de *P. subcapitata* por diferentes métodos e estudada a sua estabilidade, especificidade por substratos, cinética e efeito do Hg²⁺ no extrato bruto. O congelamento e descongelamento, associado com ultrassom, foi o método que proporcionou maior rendimento de extração. A enzima, praticamente estável por armazenamento a -20°C, durante aproximadamente seis meses, demonstrou uma atividade ótima em pH 5 e um valor de Km para o *p*-nitrofenilfosfato (*p*NPP) de 0,27 mM. Alguns substratos naturais foram hidrolisados com uma intensidade semelhante à do substrato sintético *p*NPP. Diferentemente dos estudos de exposição a curto prazo (24 horas), observou-se inibição da atividade específica nas culturas expostas durante 7 dias, com um valor de CE₅₀ (concentração de Hg²⁺ que promove 50% de decréscimo da atividade específica) equivalente a 12,63 μM Hg²⁺.

Palavras-chave: *Selenastrum capricornutum*, alga, enzima, metal, toxicidade

INTRODUCTION

Pseudokirchneriella subcapitata (formely *Selenastrum capricornutum*) is a chlorophyceae algae present in freshwater and soil, which is widely used in studies of contamination by agriculture pollutants (Keddy et al., 1995; Baun et al., 2002; Okamura et al., 2002). The alteration of phosphatase activities by

chemical pollutants such as heavy metals has been used as a biomarker in risk assessment and biomonitoring (El Demerdash & Elagamy, 1999; Strmac & Braunbeck, 2002). In this context, *in vitro* and *in vivo* changes on aquatic and soil acid and alkaline phosphatases activities by contaminants have been reported (Verma et al., 1985; El Demerdash & Elagamy, 1999; Jonsson & Aoyama, 2007; Revoredo & Melo, 2007).

Acid phosphatases are a group of enzymes which catalyze the non-specific hydrolysis of phosphate monoesters in an acidic environment. Several functions have been attributed to algae acid phosphatases, such as autophagic digestive processes, availability of inorganic phosphate from the extracellular medium and recycling of inorganic phosphate for its reassimilation (Cooper et al., 1974; Jonsson & Aoyama, 2007). Some properties of purified (Rivoal et al., 2002) or crude extract (Granbom et al., 2004) algae enzymes have been described. However, up to now, there are few references reporting the extraction, characterization and sensibility to pollutants of acid phosphatase in relevant primary producers with cosmopolitan distribution and their use as test organisms in ecotoxicity risk assessment (OECD, 1981; CETESB, 1992).

Sewage sludge used as agriculture fertilizer (Mamais et al., 2000) and phosphate fertilizers (Oyedele et al., 2006) often contain considerable amounts of heavy metals, including mercury. This element, as well as other metals, can intensify the production of reactive oxygen species (ROS) in soil (Gratão et al., 2005) and in aquatic organisms (Verlecar et al., 2008). ROS toxicity can be avoided by antioxidant enzymes like ascorbate peroxidase, superoxide dismutase and catalase, which are associated to Hg²⁺ and Ni²⁺ stress in algae (Okamoto et al., 2001; Huang et al., 2006) and in plants (Pompeu et al., 2008), respectively. Mercury can modulate enzymatic activities of algae β -D-galactosidase (Peterson & Stauber, 1996), malate dehydrogenase (Fathi, 2002) and protochlorophyllide reductase (Assche & Clijsters, 1990) leading to detrimental effects in metabolic pathways.

We compared some methods of extraction of acid phosphatase from *P. subcapitata* and we investigated some aspects of the enzyme activity focusing on storage stability, pH range, substrate specificity and kinetic parameters. We also investigated the effect of the highly toxic heavy metal Hg²⁺ on the enzyme activity.

MATERIAL AND METHODS

Organisms and growth conditions

Unicellular green algae *P. subcapitata* (Chlorococcales, Chlorophyceae) was maintained in an axenic culture and was subcultured in an inorganic liquid medium prepared as recommended by OECD (OECD, 1981). 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°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 culture was prepared by inoculating approximately 5 \times 10⁴ cells mL⁻¹.

Harvesting and preparation of extracts

All centrifugation procedures were carried out at 4°C. Exponential phase organisms were harvested by centrifugation at 4,000 rpm 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 or algae suspension were prepared in 0.1M sodium acetate buffer at pH 5.0 (1:4 w/v) and submitted to the procedures described below:

a) Freezing (N₂)/maceration: the algae pellet was frozen with liquid N₂ in a mortar and macerated after adding portions of the acetate buffer. The volume was adjusted in order to obtain a 1:4 (w/v) suspension.

b) Freezing/thawing/probe sonication: the algae suspension was frozen at -20°C in a freezer and thawed at room temperature. The sample was sonicated (Vibra Cell, Sonics Materials Inc., 45 mm tipped probe, amplitude of 70) for 50 s followed by a 20 s interval (1 cycle). The sonication process was repeated twice. The process was performed in polypropylene tubes in ice.

c) Freezing/thawing/ultra-sound water bath sonication: the algae suspension was frozen at -20°C in a freezer and thawed at room temperature. The sample was sonicated during 20 min, at 4°C, in an ultra-sound water bath (Model T7; Thorthon-Inpec Eletrônica, Vinhedo, Brazil, potency set of 12).

The disrupted cell suspensions obtained by the above procedures were centrifuged at 10,000 rpm for 20 min. The supernatant (extract) was used for protein and acid phosphatase assays.

Characterization

Effect of pH - enzyme preparations were directly assayed in the pH range of 3.1-10.0, using 0.1M of the following buffers: citrate (pH 3.1), acetate (pH 4.2 and 5.0), bis-Tris (pH 6.2), imidazole (pH.7.3), Tris-HCl (pH 8.6) and carbonate-bicarbonate (pH 10.0).

Effect of storage at low temperatures - enzyme extracts were stored at -20°C (in a freezer) or at 5°C (in a refrigerator) and in both cases, the activities were followed for six months.

Substrate specificity - synthetic or natural compounds were tested as substrates at 10 mM final concentration. The enzyme activity was determined by measuring the released inorganic phosphate.

Kinetic parameters - enzyme activity was assayed in the concentration range from 0.05 to 10.0 of mM *p*-nitrophenylphosphate (*p*NPP) as substrate. Michaelis-Menten constant (K_m) and maximum velocity

(Vmax) were calculated from the Lineweaver-Burk plot. Activation energy (Ea) was determined from the slope of the Arrhenius plot (log V versus 1/T) obtained for five temperatures values, according to Dixon & Webb (1979). Analyses were carried out in duplicate.

Effect of Hg²⁺ - *in vitro* evaluation. Prior to the determination of enzyme activity, the enzyme was preincubated for 20 minutes at 37°C in the presence of the metal ion. The concentration that promotes 50% of enzyme inhibition (IC₅₀) and its 95% confidence limits were calculated by adjusting the regression curve data (% activity versus concentration) for enzyme activity at fifteen doses of Hg²⁺ (HgCl₂). Analysis was carried out in duplicate.

***In vivo* evaluation - algae cell suspensions** (~5 × 10⁶ cells mL⁻¹) were exposed to Hg²⁺ present in the algae medium at three or five concentrations. The assays were performed with a control (no pollutant) by incubating at 20°C (2,000-3,000 lux) for 24 hours or 7 days. The cells suspensions were harvested by centrifugation, suspended in 3 mL sodium acetate buffer (pH 5.0) and submitted to freezing/thawing/sonication followed by centrifugation as described above. The enzyme activity in the culture, the protein content in the extract, and the specific activity in the extract were expressed as mU mL⁻¹, µg mL⁻¹ and mU mg⁻¹ protein, respectively. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. The concentration that promoted 50% decrease in specific activity (EC₅₀) and its 95% confidence limits were calculated from the regression curve data (% specific activity versus pollutant concentration).

Data Analysis and Statistics - results were analyzed by Simple Regression and One Way ANOVA modules with a Statgraphics® Plus Version 2 software package. A *p* value < 0.05 was considered as significant.

Assay of phosphatase activity - was routinely assayed, at least in duplicate, by incubating the enzyme with *p*-nitrophenylphosphate (*p*NPP) as substrate, 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, 10 mM substrate

and the enzyme extract. The reaction medium was incubated for 40 min at 37°C and the reaction was terminated by the addition of 1 mL of 1 M NaOH. The *p*-nitrophenol (*p*NP) released was measured at 405 nm in a UNICAM 8625 UV/VIS spectrophotometer, using a molar extinction coefficient of 18,300 M⁻¹cm⁻¹.

Enzyme activity towards various phosphate esters used as substrates was determined by the liberation of Pi (Lowry & Lopez, 1945). Reactions were terminated by the addition of 1 mL 3% ammonium molybdate (in 200 mM sodium acetate buffer, pH 4.0), followed by the addition of 0.1 mL of 2% ascorbic acid (in 200 mM sodium acetate buffer, pH 4.0). The color was developed for 30 min and the absorbance was read at 700 nm.

RESULTS

Extraction

Table 1 shows that freezing with liquid N₂ followed by maceration yielded the highest enzyme extraction compared with the other procedures. The results demonstrated an increase of about 4-fold in the enzymatic activity in the samples submitted to freezing (N₂)/maceration or probe sonication in relation to those submitted to ultra-sound water bath.

Temperature stability and effect of pH

A loss of approximately 85% in the activity was observed when the enzyme was stored at 5°C for 177 days (Figure 1). However, the storage at -20°C maintained full enzyme activity. The enzyme activity was maximum at a pH value of 5.0, but above 6.2, the activity sharply decreased (Figure 2).

Substrate specificity

Fifteen compounds at 10 mM concentration were tested as substrates (Figure 3). The synthetic compounds *p*NPP and β-naphthyl phosphate, as well as the natural compounds flavine mononucleotide (FMN), DL-α-glycerol phosphate and fructose 1.6-biphosphate, were efficiently hydrolyzed at approximately the same rate. Other substrates like P-tyrosine, ribose 5-phosphate and 5'AMP were also hydrolyzed, but to a lesser extent (about 65%). When compared with *p*NPP, the hydrolysis of P-serine, P-threonine and α-D-glucose 1-phosphate was low.

Table 1 - Acid phosphatase activity of extracts from *P. subcapitata* obtained by different procedures. The absorbance in the method that rendered the highest extraction was taken as 100%. The other values were considered as percentages of this value. Each value was based on duplicate analysis.*denotes difference (*p* < 0.05) from the other methods.

Method	Yield of the extraction (% ± s.d.)
Freezing (N ₂)/Maceration	100 ± 5
Freezing (-20°C)/Thawing/Sonication (probe)	82 ± 19
Freezing (-20°C)/Thawing/Sonication (ultra-sound water bath)	23 ± 1*

Determination of Km and Ea

When the activity of the enzyme was measured at various concentrations of *p*NPP as substrate, a double-reciprocal plot gave a straight line (Figure 4) that allowed the determination of Km and Vmax values of 0.27 ± 0.02 mM and 0.037 ± 0.002 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, respectively. The results obtained from three batches of extract are shown in Table 2.

The activation energy (Ea) was determined from the Arrhenius plot by varying the incubation temperatures from 27.5 to 47.0°C (not shown). The activity increased by a factor of 1.6 for a 10°C increase in the temperature. The Ea value calculated from the slope of the Arrhenius plot was 38 ± 2 kJ mol⁻¹ (Table 2).

Susceptibility to Hg²⁺

In order to calculate the IC₅₀ values for Hg²⁺, the *in vitro* inhibition was performed at the concentration range of 0.0025 – 0.5 mM of this pollutant, with 10 mM *p*NPP as substrate. The data obtained were analyzed by linear regression to fit a logarithmic-x model curve ($y = a + b \ln x$) for Hg²⁺. The calculated IC₅₀ and 95% confidence limits value based on the experiments with the pollutant using two extract batches was 0.085 (0.064 - 0.117) mM.

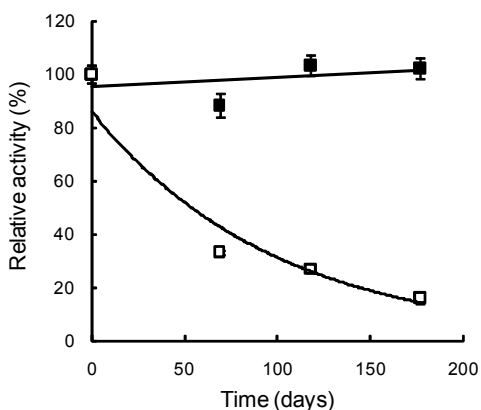


Figure 1 - Stability of acid phosphatase after storage. Aliquots of enzyme extract, stored at -20°C (■) and 5°C (□), were removed at different periods and the activities were determined, using *p*NPP as substrate, as described in Material and methods. The activity initially determined in the study was considered as 100%. Each point was based on triplicate analysis.

Table 2 - Kinetic parameters for *P. subcapitata* acid phosphatase.

Parameters	This work	Ref. ^[a]	Ref. ^[b]
Km (mM)	0.27 ± 0.02	0.5; 0.3	0.19
Vmax ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	0.037 ± 0.002	-	-
Ea (kJ mol ⁻¹)	38 ± 2	-	44.73

^aPatni & Aaronson (1974). ^bLien & Knutsen (1973).

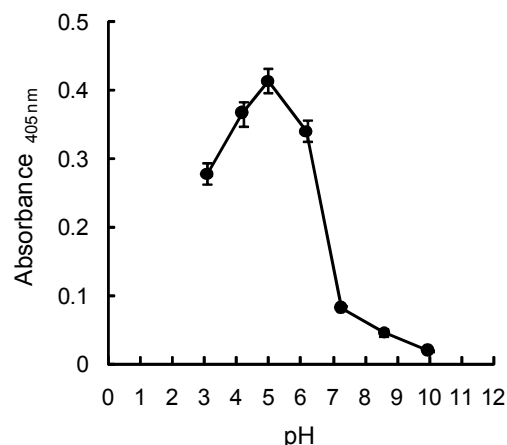


Figure 2 - pH-dependent activity of *P. subcapitata* acid phosphatase. Activities were measured in 0.1 M of the following buffers: citrate (pH 3.1), acetate (pH 4.2 and 5.0), Bis-Tris (pH 6.2), imidazole (pH 7.3), Tris-HCl (pH 8.6) and carbonate-bicarbonate (pH 10.0). Each point was based on triplicate analysis.

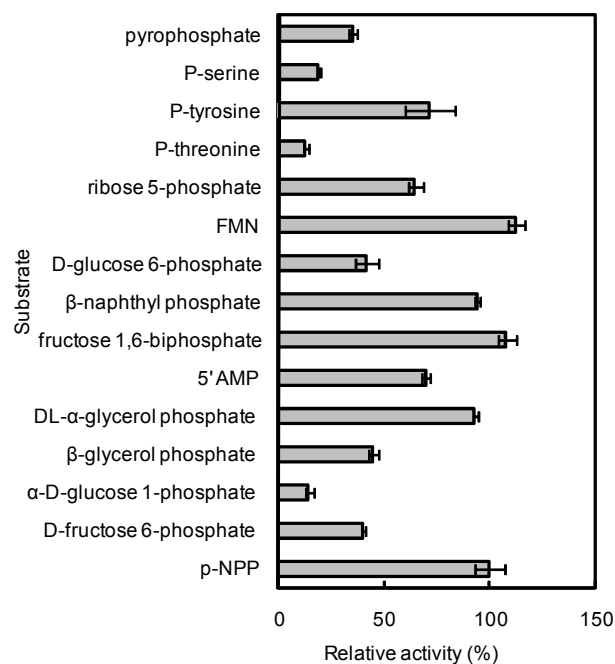


Figure 3 - Substrate specificity for acid phosphatase from *P. subcapitata*. The rate of Pi released by *p*NPP during hydrolysis was considered to be 100% and the other values were considered as percentages of this value. Each value of relative activity was based on triplicate analysis.

Table 3 - Biochemical parameters in cultures of *P. subcapitata* exposed to Hg^{2+} . Enzyme activity in the culture, protein concentration in the extract and specific activity in the extract were determined from cultures grown in OECD medium after 24 hours or 7 days. Average values and standard deviations (in parenthesis) from two experiments (24h exposure) and three experiments (7 d exposure) are shown. Analyses were carried out in duplicate. * denotes difference ($p < 0.05$) from the control.

Hg^{2+} μM	Activity mU mL^{-1}	Protein $\mu\text{g mL}^{-1}$	Specific Activity mU mg^{-1}
24 hours			
0	0.052 (0.012)	62.30 (27.37)	37.94 (9.23)
10	0.035* (0.009)	51.00 (17.74)	32.05 (6.59)
23	0.030* (0.005)	24.30* (1.11)	56.49 (11.53)
50	0.030* (0.005)	26.10* (6.11)	53.90 (15.35)
110	0.016* (0.001)	13.30* (0.53)	54.40 (2.59)
242	0.013* (0.0007)	12.20* (3.39)	68.50* (26.44)
7 days			
0	0.089 (0.029)	88.00 (4.58)	30.10 (8.50)
5	0.054* (0.009)	64.00* (2.50)	24.90 (3.34)
10	0.040* (0.009)	70.10 (24.90)	18.00* (3.10)
15	0.027* (0.012)	60.70* (6.90)	13.30* (4.67)

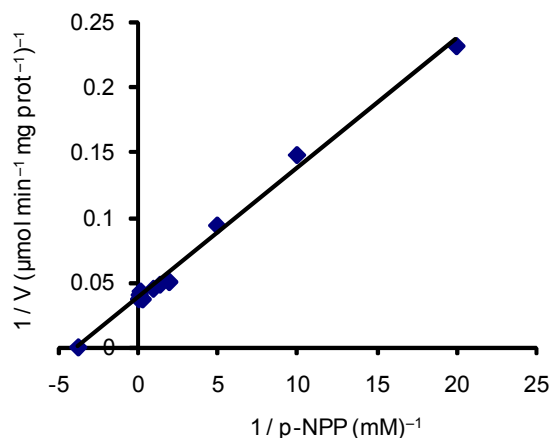


Figure 4 - Lineweaver-Burk plot for the determination of K_m and V_{max} of *P. subcapitata* acid phosphatase. Each point was based on duplicate analysis.

In the short term *in vivo* exposure study (24 h), cultures of *P. subcapitata* growing in OECD medium were exposed to Hg^{2+} (HgCl_2) with final concentrations in the range from 10 to 242 μM . The data in Table 3 shows a decrease in enzymatic activity which was concentration-dependent. In a concomitant manner with this activity decrease, a reduction in protein content was observed at 23 μM and higher concentrations of Hg^{2+} . An increase in the specific activity was observed between 23 – 242 μM Hg^{2+} , nevertheless it was statistically significant only at the highest tested concentration.

We also studied the *in vivo* effect of Hg^{2+} in the algae cultures exposed for seven days at three con-

centrations of the metal (Table 3). Forty and 70% reduction in enzyme activity were observed for the lowest and the highest Hg^{2+} concentration tested, respectively. A marginal decrease in the protein content in relation to the control was observed for all concentrations tested; however, this decrease was only significant at 5 and 15 μM . This slight protein reduction, together with a strong inhibition of the enzyme activity, resulted in a marked decrease in the specific activity between 10 and 15 μM . The EC_{50} calculated for this parameter was equivalent to 12.63 (9.78 - 17.61) μM .

DISCUSSION

Extraction

In this study, some procedures that promoted cell disruption with enzyme release were compared. Two methods of sonication were used in combination with a prior freezing/thawing cycle. The total enzyme activity was determined instead of the specific activity. The main objective was to compare the activity of the three extraction methods applied for the same extract, so the determination of protein for the specific activity calculation was unnecessary. Some authors expressed the enzyme activity by the absorbance or used this parameter to calculate the relative activity (%) in which the control is taken as 100% (Granjeiro et al., 1997; Demeke et al., 2001; Amani et al., 2005; Jonsson & Aoyama, 2007). In this study, we adopted similar assumptions for other experiments.

Cooper et al. (1974) studied enzyme release using the latter procedure and observed that three cycles on the whole homogenates of the algae *Polytomella caeca* promoted a gradual increment in acid phosphatase activity.

Simon & Helliwell (1998) compared the extraction efficiency of chlorophyll from *Selenastrum obliquus* and observed that the maceration in acetone was more effective than probe and bath sonication. Although we demonstrated that freezing with liquid N₂ followed by the biomass maceration promoted the highest yield of extraction, the disadvantage of this procedure was the loss of material observed during the maceration process. Therefore, we adopted the combination of freezing, thawing and probe sonication for our routine analyses of the enzyme.

The extraction of enzymes, including phosphatases, from *Selenastrum* (*Pseudokirchneriella*) genus has been previously reported. Kong & Chen (1995) extracted acid phosphatase and glucose-6-phosphate dehydrogenase from *Selenastrum capricornutum* with Tris-borate 0.1–0.3 M pH 7.5 buffer at 0°C for 10 min followed by centrifugation at 10,000 × g. However, Kong & Chen (1995) did not mention the cell disruption method employed. Rivoal et al. (2002) extracted the phosphoenolpyruvate carboxylase from *Selenastrum minutum* from frozen cells that were thawed in a buffer and passed through a French press at 18,000 p.s.i. The same method was employed by Theodorou et al. (1991) to extract eight enzymes from the same species.

Some other methods for algae cell disruption to obtain phosphatases or other enzymes were described in the literature. For example, Geoffroy et al. (2002) extracted four enzymes from *Scenedesmus obliquus* by maceration with sand. Maceration was also employed by Nakazato et al. (1997) for the extraction of acid phosphatase from the aquatic macrophyte *S. oligorrhiza*, although, this procedure was performed in liquid N₂.

Effect of storage and pH

The enzyme maintained its activity for at least six months when stored at when stored at -20°C. However, the enzyme was not stable when stored at 5°C. Different patterns of temperature inactivation were reported for acid phosphatases from some algae species. Approximately 70% of activity was lost by heating at 60°C the acid phosphatases from *Ochromonas danica* (Patni & Aaronson, 1974), *Chlamydomonas reinhardtii* (Matagne et al., 1976) and *Euglena gracillis* (Bennun & Blum, 1966) during 240, 1 and 17 minutes, respectively. We observed a similar percentage of inactivation of *P. subcapitata* acid phosphatase after storing at 5°C for about 100 days.

The enzyme is more stable in acidic solutions than in basic solutions (Figure 2). Similar optimum pH value for acid phosphatase has been reported for other algae species such as *Chlamydomonas reinhardtii* (Matagne et al., 1976) and *Ochromonas danica* (Patni & Aaronson, 1974).

Cleavage of substrates and phosphatase inhibition

The specificity of the enzyme in its ability to hydrolyze several compounds is very broad. Synthetic substrates such as pNPP and β-naphthyl phosphate are routinely used in the determination of phosphatase activities. Some substrates that are present in the cellular metabolism, or natural substrates, were hydrolyzed at similar rates as that of the synthetic one. The efficient rate of hydrolysis of the natural substrates β-glycerol phosphate and FMN found in this study was also observed for acid phosphatases extracted from *Chlamydomonas reinhardtii* (Matagne et al., 1976) and from bovine kidney (Granjeiro et al., 1997). Similar enzymatic activities were reported for the cleavage of D-glucose 6-phosphate, D-fructose 6-phosphate and 5'AMP by the acid phosphatase extracted from de algae *Enteromorpha linza* (Yamamoto, 1972). The acid phosphatases from *P. subcapitata* did not efficiently hydrolyze inorganic phosphates. This is in contrast with the results in the literature which showed that inorganic pyrophosphates are efficient substrates for the acid phosphatases extracted from the algae *Euglena gracilis* (Bennun & Blum, 1966) and from the aquatic macrophyte *Spirodela oligorrhiza* (Nakazato et al., 1997).

P-tyrosine, ribose 5-phosphate and 5'AMP can be considered as good substrates for *P. subcapitata* acid phosphatase. Phospho-tyrosine was the best non-synthetic substrate for the enzymes extracted from potato tuber (Gellatly et al., 1994) and AMP for the acid phosphatase extracted from some plant species (Duff et al., 1994). Among the three phosphorylated amino acids tested, only P-tyrosine exhibited a good rate of hydrolysis. A low rate of cleavage for P-serine was also reported for the enzyme extracted from the algae *Euglena gracilis* (Bennun & Blum, 1966) and *Enteromorpha linza* (Yamamoto, 1972).

Glycerol-phosphate, fructose 1,6-biphosphate and FMN are strong candidates as physiological substrates for acid phosphatases from *P. subcapitata*. Phospho-tyrosine (in proteins), ribose-5-phosphate and AMP should also be considered for this purpose. Furthermore, the substrate specificity is dependent on the enzyme isoforms. Abd Elaah & El Nagdy (1999) studied acid phosphatase isozymes from the aquatic fungal genera *Achlya* and observed a wide range of variability in relation to the substrates.

Kinetics studies

The apparent K_m value for *p*NPP (0.27 mM) obtained for the acid phosphatase from *P. subcapitata* was within the same magnitude as those observed for acid phosphatases from other algae species: 0.29 mM for *Euglena gracilis* (Bennun & Blum, 1966), 0.5 mM for *O. danica* (Patni & Aaronson, 1974), 0.19 mM for the unicellular green algae *Chlamydomonas reinhardtii* (Lien & Knutsen, 1973). Similar values were also observed for the K_m values for acid phosphatases extracted from fungi (Tsekova & Galabova, 2003) and from vegetables such as *S. oligorrhiza* (Nakazato et al., 1997), sweet potato tuber and wheat germ (Duff et al., 1994). The K_m value determined for an acid phosphatase from *Selenastrum minutum* using phosphoenolpyruvate as substrate (Theodorou et al., 1991) was close to that obtained for *P. subcapitata* in the present work.

The activation energy obtained in our experiments (37.94 kJ mol⁻¹) is similar to those described by Lien & Knutsen (1973) for phosphatases extracted from the green algae *C. reinhardtii* (44.73 kJ mol⁻¹) and for the acid phosphatases from other organisms, for example, bovine kidney (45.44 kJ mol⁻¹) (Granjeiro et al., 1997). The activation energy value obtained for the acid phosphatase in this work is also closed to the values reported for other algae enzymes, such as hydrogenase (Schnackenberg et al., 1993) and urate oxidase (Alamillo et al., 1991).

Effect of Hg²⁺

The degree of *in vitro* inhibition of phosphatases by Hg²⁺ has been described for some organisms. Approximately 50% inhibition of the acid phosphatase from the algae *Ochromonas danica* was reported at 1 mM HgCl₂ (Patni & Aaronson, 1974). IC₅₀ values of 0.5 mM and 0.025 mM were determined for fish liver (El Demerdash & Elagamy, 1999) and crab (Chen et al., 2000), respectively.

The observed enzyme inhibition by Hg²⁺ or other heavy metal can be explained by their interactions with essential -SH groups. The presence of such groups in the active site of enzymes or in the stabilization of the quaternary structure is essential for enzyme activity (Assche & Clijsters, 1990). Other mechanisms of inhibition may be the deficiency of an essential metal in metalloproteins or metal-protein complexes which results in the substitution of the toxic metal for the deficient element (Omar, 2002).

Hg²⁺ did not change the specific activity after short term exposures. However, a decrease in activity and protein content was observed in the extracts. The increased specific activity observed in Hg²⁺ treatments for 24 hours was associated with an increase of the

activity/protein ratio. Gill et al. (1991) assumed that this increment could be due to enzyme induction by the metal as part of the biochemical adaptation to meet increased metabolic needs under toxicant induced stress, and/or increased lysosomal liability. Enhanced acid phosphatase activity is often associated with increased lysosomal activity in the tissues undergoing cellular degeneration and necrosis due to exposure to toxic substances. Another factor is that algae seem to be capable of promoting metal binding to protein as a detoxification mechanism (Omar, 2002).

After seven days, the inhibitory effect caused by Hg²⁺ was similar in magnitude when compared to that observed for the green algae *Scenedesmus bijuga* acid phosphatase, where about 50% decrease in the activity was observed at 2.5 μM Hg²⁺ after the same period of exposure (Fathi, 2002).

In contrast to the specific activity depression observed after 7 days of exposure, this parameter did not change at 24 hours. The EC₅₀ value for *in vivo* Hg²⁺ effect at seven days exposure was about seven times lower than the IC₅₀ value for *in vitro* experiments, indicating a major vulnerability of the enzyme in the intracellular medium. The specific activity decrease together with the *in vitro* inhibition suggests that the exposure time of cells would be relevant to promote the passage of the chemical across the membranes, which interacts with the enzyme causing its inhibition. This phenomena was probably hindered at 24 hours of exposure where the alterations of enzyme activity in the extract were accompanied by loss of protein content. Thus, the above short term effect on phosphatase activity seems to be the result from other acute toxicant effects rather than the direct intracellular chemical interaction with the enzyme. In other words, the capability of Hg²⁺ to interact with protein and alter membrane permeability suggests that the metal ion acted as a general tissue poison rather than a highly specific inhibitor.

There is no obvious explanation for the alteration in the specific enzymatic activities after 7-day exposures, since a number of factors may be involved including, reduction of the synthesis of the protein or binding of the enzyme with the chemicals. However, based on the *in vitro* results, the observed depression in specific activity may actually represent a pollutant-enzyme interaction.

Our results corroborate the results of other studies which reported the *in vivo* acid phosphatase activity alteration by metals in unicellular green algae. For example, in *Chlorella vulgaris*, about 20% of activity was depressed by 0.15 mM Al³⁺ (Rai et al, 1998). The specific activity in *S. capricornutum* was decreased about 86 and 70% by 6 μM Al³⁺ and 4.6 μM Zn²⁺, re-

spectively (Kong & Chen, 1995). In *Scenedesmus obliquus*, this inhibition was approximately 40% by 0.12 mM Zn²⁺, while it was observed near 140% specific activity increase in *Scenedesmus quadricauda* exposed to this same condition (Omar, 2002).

Reduction in acid phosphatase specific activity was also reported for exposed fishes, where the pollutants effects were more prominent. The inhibition observed at the highest tested concentration in our work (7-days exposure), is similar to that observed with 0.44 µM Hg²⁺ in water on the enzyme extracted from gills (Verma et al., 1985). The estimated Hg²⁺ value for the analyzed biochemical parameters was higher than that reported for the classical growth inhibition test in algae (WHO, 1989). This suggests a higher sensibility of the latter physiological parameter in comparison with the enzyme inhibition.

The highest concentration of Hg²⁺ tested that did not promote significant effect on specific activity was 5,000 fold higher than the maximum allowed concentration in freshwater compartments, according to Brazilian legislation (CONAMA, 2005).

CONCLUSIONS

The combination of freezing, thawing and probe sonication demonstrated to be the more suitable method for acid phosphatase extraction due to the relatively low loss of material, when compared with the process using maceration. The extract could be stored at least six months at freezer conditions (-20°C) without loss of acid phosphatase activity; for the same period of time, the remaining activity was very low when the extract was stored in the refrigerator (5°C).

Some enzyme parameters such as optimum pH, Km and activation energy were similar to those described for other algae species. Several substrates could be recognized by *P. subcapitata* acid phosphatase. Considering the pNPP hydrolysis as 100%, P-tyrosine, ribose 5-phosphate, FMN, D- fructose 1.6-biphosphate, 5'AMP and DL-α-glycerol phosphate were hydrolyzed with more than 50% efficiency.

The addition of Hg²⁺ in the reaction system containing the extract decreased the enzyme activity. The decrease of enzyme activity was also observed on the extracts from algae grown in the presence of the metal. However, under these exposure conditions, the specific activity increased or decreased, respectively, after 24 hours or 7 days.

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