Effect of Copper on Acid Phosphatase Activity in Yeast

_Yarrowia lipolytica_

Hiroyasu Ito, Masahiro Inouhe, Hiroshi Tohoyama, and Masanori Joho*

Department of Biology, Faculty of Science, Ehime University, Matsuyama, 790-8577, Japan.
Fax: +81 (0)089-927-9625. E-mail: joho@sci.ehime-u.ac.jp

* Author for correspondence and reprint requests

_Z. Naturforsch. 62c_, 70–76 (2007); received July 3/August 10, 2006

Acid phosphatase (APase) activity of the yeast _Yarrowia lipolytica_ increased with increasing Cu\(^{2+}\) concentrations in the medium. Furthermore, the enzyme in soluble form was stimulated in vitro by Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\) and inhibited by Ag\(^{+}\) and Cd\(^{2+}\). The most effective ion was Cu\(^{2+}\), especially for the enzyme from cultures in medium containing Cu\(^{2+}\), whereas APase activity in wall-bound fragments was only slightly activated by Cu\(^{2+}\). The content of cellular phosphate involving polyphosphate was decreased by adding Cu\(^{2+}\), regardless of whether or not the medium was rich in inorganic phosphate. Overproduction of the enzyme stimulated by Cu\(^{2+}\) might depend on derepression of the gene encoding the APase isozyme.

Key words: Acid Phosphatase, Copper, _Yarrowia lipolytica_

Introduction

Acid phosphatases (APases) are non-specific enzymes with a pH optimum below 7.0 that catalyze the hydrolysis of monoesters resulting in the release of inorganic phosphate. APases are typically located near the cell walls and organelles of fungi (González et al., 1993). APases play important roles in the biosynthesis of yeast cell walls (Field and Schekman, 1980) and the enzyme is derepressed upon inorganic phosphate starvation (Moran et al., 1989; Galabova et al., 1993). The activity of APases is also increased by Cu\(^{2+}\) in _Aspergillus niger_, regardless of whether the medium is rich in inorganic phosphate or not (Tsekova et al., 2002). The bacterium _Citrobacter_ sp. accumulates heavy metals via the activity of an APase that produces inorganic phosphate (Jeong and Mackie, 1999). These findings suggest that enhanced APase activity participates in Cu\(^{2+}\) resistance, causing precipitation as a phosphate-metal complex. Both Cu\(^{2+}\) and Al\(^{3+}\) induce a phosphorus deficiency in some plants and thereby enhance the activity of APases (Hutková et al., 2002; Lee et al., 2005). Cultured cells of tobacco also acquired Al\(^{3+}\) tolerance during phosphate starvation (Yamamoto et al., 1996). Enzymatic APase activity is also influenced by various metal ions in vitro, being activated by Cu\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\), but inhibited by Al\(^{3+}\) and Hg\(^{2+}\) in potato tuber cells (Tu et al., 1988). In phosphate-starved tomato cell culture, the enzyme APase is activated by Mg\(^{2+}\) and Mn\(^{2+}\), but inhibited by Cu\(^{2+}\) and Zn\(^{2+}\) (Bozzo et al., 2002). The enzymatic responses of APases to metal ions differ among kinds of cell types and culture conditions.

_Yarrowia lipolytica_ is a strictly aerobic yeast. This dimorphic fungus has traditionally been used to produce amino or other organic acids due to its formidable excretory capacity (Antonucci et al., 2001; Barth and Gaillardin, 1997; Fickers et al., 2004). Furthermore, _Y. lipolytica_ can survive in extreme environments containing high concentrations of NaCl or heavy metals (Andreishcheva et al., 1999; Butinar et al., 2005; Strouhal et al., 2003; Zvyagilskaya et al., 2001). In preliminary studies we found that Cu\(^{2+}\) increases the APase activity in the yeast _Y. lipolytica_, which can grow in high CuSO\(_4\) concentrations. The increased activity of APase that occurs during culture under such conditions might reflect differences in the enzymatic activation by metal ions and/or in its increased production. However, little is understood about the response of APase to metal ions in _Y. lipolytica_. The present study examines the effect of metal ions on the activity of APase in _Y. lipolytica_, cultured in vitro in the presence or absence of Cu\(^{2+}\).
The dimorphic fungus *Y. lipolytica* assumes mycelia-producing fungal and/or yeast-like forms, depending on the culture conditions (Ruiz-Herrera and Sentandreu, 2002). The metabolic activities of the two forms differ (Gadd and Mowll, 1985). The cell wall composition of the yeast and mycelial forms of *Y. lipolytica* are qualitatively similar, but quantitatively different (Vega and Domínguez, 1986). Some transcription factors involved in the morphogenesis of dimorphic fungi are also activated by Cu^{2+} (Osiewacz and Nuber, 1996). The coexistence of morphologically different cell types in culture considerably complicates the quantitation of growth as well as other physiological responses. Therefore, we used a *Y. lipolytica* mutant that can only grow in the yeast-like form.

**Materials and Methods**

**Organisms and culture**

A mutant strain of *Yarrowia lipolytica mhy 1-1* (MAT A, ura3-302, leu2-207, lys8-11, mhy1-1) used this work was donated by Dr. Rachubinski, University of Alberta, Edmonton, Alberta, Canada (Hurtado and Rachubinski, 1999). The organism was kept at 4 °C by periodic transfer on nutrient agar slants containing the following ingredients (g l⁻¹): glucose (20); polypeptone (5); yeast extract (5); KH₂PO₄ (5) and MgSO₄ · 7H₂O (2). Yeast cultures were grown at 30 °C in 500-ml flasks containing 100 ml nutrient medium in a reciprocal shaker at 120 strokes min⁻¹. Filter-sterilized CuSO₄ · 5H₂O was added to liquid media to yield the various final concentrations.

**Cell permeabilization**

Cells were permeabilized as described by Galabova *et al.* (1996). Cells were harvested, washed twice with distilled water and then 9 ml of cell suspension (containing about 1 mg DW ml⁻¹) were mixed with 1 ml of Triton X-100. The cells were incubated at room temperature with intermittent shaking for 30 min and centrifuged at 3,000 × g for 10 min. The pellet was resuspended in 0.1 M sodium acetate buffer (pH 4.2). Permeabilized cells were used for the determination of total enzyme activities.

**Subcellular fractionation**

The harvested cells were washed twice with distilled water. Protease activity was protected by adding phenylmethylsulphonyl fluoride (PMSF, final concentration 1 mM) and then cultures were disrupted by vigorous vortex mixing with glass beads in 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) containing 1 mM EDTA at 4 °C. Intact cells were absent according to the observation by light microscopy. The disrupted cells were then centrifuged at 10,000 × g for 30 min. The pellet was washed twice with 50 mM PIPES buffer (pH 7.0) by centrifugation at 10,000 × g for 30 min. The supernatant (soluble) and pellet (mainly comprising cell wall) were used for the determination of enzyme activities and protein.

**Assay of APase activity**

We assayed APase activity in a mixture comprising 0.5 ml of enzyme (permeabilized cell suspension, soluble and wall fractions), 0.5 ml of 0.25 M sodium acetate buffer (pH 4.2), 0.5 ml p-nitrophenylphosphate (pNPP; final concentration, 1.9 mM) and 0.5 ml of metal ions or distilled water. The reaction was initiated by adding substrate and a 10 min incubation at 30 °C was terminated by adding 1 ml of 0.1 M NaOH. One unit of activity was defined as the amount of enzyme required to release 1 nmol of p-nitrophenol in 1 min at 30 °C.

**Cell dry weight and protein measurement**

The cells were dried for 48 h at 90 °C in glass centrifuge tubes and then weighed (dry weight, DW). Protein concentrations were determined using the Folin-Ciocalteau reagent with bovine serum albumin as the standard (Lowry *et al.*, 1951).

**Assay of inorganic phosphate**

Phosphate compounds were extracted from cells using a slight modification of the method of Okorokov *et al.* (1983). Free inorganic phosphate was extracted from cells stirred for 15 min with 0.5 M HClO₄ at 4 °C. After centrifugation, phosphate compounds (mainly polyphosphates) were extracted from the pellet with 1 M HClO₄ for 10 min at 100 °C. Total phosphate was determined by hydrolyzing yeast cells with 50% H₂SO₄ for 15 min at about 300 °C. Inorganic phosphate was determined using a modification of the procedure of Ames and Dubin (1960). A 1:1 mixture of fresh 10% ascorbic acid and 2.5% ammonium molybdate containing 0.1% ammonium potassium tar-
trate in 1 N H₂SO₄ (ascorbic-molybdate; 0.2 ml) was mixed with 5 ml samples at room temperature and then inorganic phosphate levels were compared 15 min later by spectrophotometry at 883 nm against a blank containing only water (Hitachi U-3000 type).

**Results**

*Effect of Cu²⁺ on cell growth and APase activity*

Fig. 1 shows the effect of Cu²⁺ on the growth and the APase activity of Y. lipolytica. When cultured in medium containing Cu²⁺ for 24 h, 5 mM Cu²⁺ were required to inhibit cell growth by 50%. In contrast, APase activity was proportionally increased with increasing concentrations of Cu²⁺. The enzyme activity of cells cultured in medium containing 6 mM Cu²⁺ was about 15-fold more than that of cells cultured without Cu²⁺. Fig. 2 shows increasing APase activity during the growth of Y. lipolytica. In medium containing Cu²⁺ at 2 mM, APase activity slightly decreased until 9 h and then increased to about 1.6-fold of the initial enzyme activity for 24 h (Fig. 2A). In contrast, APase activity did not significantly increase in Cu²⁺-free control medium for 24 h. The growth profiles in medium with or without 2 mM Cu²⁺ were similar (Fig. 2B). The APase activity was also immediately enhanced about 1.4-fold, when cells were transferred from control medium to medium containing 2 mM Cu²⁺.

*Effect of metal ions on APase activities in vitro*

To assess the effect of metal ions on APase activity, soluble enzyme isolated from cells cultured in medium with or without 2 mM Cu²⁺ was incubated with various metal ions for 10 min at 30 °C (Fig. 3). The enzymatic activities of cells cultured with or without 2 mM Cu²⁺ were 8.4 and 5.2 units mg⁻¹ protein, respectively (Fig. 3A). The specific activity of APase obtained after culture in medium containing 2 mM Cu²⁺ was about 1.6-fold higher than in Cu²⁺-free medium. Furthermore, in the presence of 0.05 mM Cu²⁺ APase activities of cells cultured in medium with or without Cu²⁺ were increased to 12.1 and 39.2 units mg⁻¹ protein, respectively. The APase was also significantly activated by adding Mn²⁺ and Co²⁺, but inhibited by Ag⁺ and Cd²⁺. Other cations such as Mg²⁺ and Ni²⁺ had no appreciable effect. Furthermore, Co²⁺ (1 mM) was the most effective metal ion among those added to the reaction mixture at the same concentration, stimulating APase activity 3.4- and 5.5-fold in cells cultured without and with Cu²⁺, respectively (Fig. 3B). The enzymatic activities were also significantly in-
Fig. 3. Effect of metal ions on acid phosphatase activity from *Y. lipolytica*. Cells were cultured in medium with or without 2 mM Cu^{2+} for 24 h at 30 °C and then enzyme activities were estimated without and with added metal ions at concentrations of 0.05 mM (A) or 1 mM (B). Permeabilized cells as described in Materials and Methods were used for determination of enzyme activities. Results are shown as averages of three replicates with standard error.

Fig. 4. Effect of Cu^{2+} on acid phosphatase from *Y. lipolytica*. Cells were cultured in medium with or without Cu^{2+} at 2 mM for 24 h at 30 °C and then disrupted with glass beads. The homogenate was centrifuged and separated into soluble and pellet fractions as described in Materials and Methods. Enzyme activities in soluble (A) and pellet (B) fractions were estimated at various concentrations of Cu^{2+}. The enzyme activity in the soluble form was proportionally increased by Cu^{2+} concentrations above 0.01 mM, reaching 2.8- and 3.8-fold in the presence of 0.05 mM Cu^{2+} in cells cultured both with and without 2 mM Cu^{2+} in the media, respectively (Fig. 4A). The APase activity in the wall-bound fragments of cells cultured with or without Cu^{2+} increased by about 1.2-fold in 0.05 mM Cu^{2+} solution (Fig. 4B). The amount of APase was almost equally distributed between the soluble and the wall-bound forms (data not shown) and the presence of 2 mM Cu^{2+} in the medium did not affect this distribution.

**Phosphate content**

To evaluate the effect of Cu^{2+} on cellular phosphate levels, cells were cultured for 24 h at 30 °C in the presence of various concentrations of Cu^{2+}. Fig. 5 shows that the cellular phosphate content in yeast cell extracts gradually decreased with increasing Cu^{2+} concentrations in the medium. The
总磷酸含量在6 mM Cu²⁺的培养基中，大约有60%的细胞来自
Cu²⁺-free medium。这种损失的总重量磷酸化合物在与
的总磷酸含量显著地随着Cu²⁺浓度的增加而减少。磷酸
磷酸含量占总磷酸的70%。我们假设细胞与Cu²⁺相
Simultaneous determination of acid phosphatase in Y. lipolytica cells was performed by a colorimetric procedure using p-nitrophenyl phosphate as substrate. The enzyme activity was determined at various concentrations of Cu²⁺ and the data were analyzed using a sigmoidal curve fitting program. The activity of APase in Y. lipolytica cells increased dose-dependently with Cu²⁺ concentration, with a saturation point at about 60 μmol g⁻¹ DW. We also found that the total phosphorus content of cells cultured with or without Cu²⁺ comprised about 0.4 μmol mg⁻¹ DW, which was about 6% of the total. The polyphosphate content significantly decreased with increasing concentration of Cu²⁺ in the medium. Polyphosphate accounted for about 70% of the total phosphate content of cells cultured with or without Cu²⁺.

Discussion

We found here that APase activity in the Cu²⁺-tolerant yeast Y. lipolytica increased dose-dependently according to the amount of Cu²⁺ in the culture medium. Derepression of the gene encoding the enzyme under inorganic phosphate deficient conditions results in APase synthesis (Moran et al., 1989; Galabova et al., 1993). Even when the medium is rich in inorganic phosphate, Cu²⁺ elicits increased APase biosynthesis in Aspergillus niger and Rhizopus delemar (Tseková et al., 2000, 2002). Gabrielli et al. (1989) also reported that Ni²⁺ increases APase activity in plants of the metal-tolerant Alyssum species, but not in metal-sensitive plants. The growth curve of Y. lipolytica in medium containing Cu²⁺ positively correlated with the increasing activity of APase (Fig. 2), indicating that increased enzyme activity is required for the growth in such medium.

When Acidithiobacillus ferrooxidans is cultured in medium containing high concentrations of heavy metal ions, Cu²⁺ stimulates polyphosphate degradation and phosphate efflux (Alvarez and Jerez, 2004). We also found that the total phosphorus content comprising mainly polyphosphate decreased after culture in medium containing Cu²⁺ (Fig. 5). Therefore, the increased activity of APase elicited by Cu²⁺ probably resulted from a decrease in intracellular phosphate, which caused derepression of the APase gene (Galabova et al., 1993). In addition, Al³⁺ also causes a phosphate deficiency in barely roots, by increasing APase activity (Huttová et al., 2002). An inorganic phosphate deficiency renders Al³⁺ tolerance of cultured tobacco cells through the decreased accumulation of metal ions (Yamamoto et al., 1996). In contrast, some microorganisms can accumulate heavy metals via enzymatically-mediated precipitation as insoluble metal phosphates and the APase activity varies according to the growth conditions (Roig et al., 1995; Turnau and Dexheimer, 1995). We found that the total cellular phosphate content was decreased by culture in the presence of Cu²⁺, indicating that excess accumulated Cu²⁺ was probably not sequestered as an intracellular metal-phosphate complex in Y. lipolytica. We supposed that decreased cellular phosphate and/or a subsequent increase in APase activity participates in the efflux of Cu²⁺ as metal-phosphate complexes in a Cu²⁺ tolerance mechanism of the yeast Y. lipolytica.

We also demonstrated that the increased activity of Y. lipolytica APase was due not only to production during growth in the presence of Cu²⁺, but also to stimulation by metal ions. The increase of APase activity induced by Al³⁺ was accompanied by an increase in the amount of APase isoform(s) (Huttová et al., 2002). The yeast Y. lipolytica also has some APase isozymes (Galabova et al., 1993; Tréton et al., 1992). These results indicate that the repressible enzyme(s) in Y. lipolytica has a metal activating nature like the metalloenzymes synthesized in the presence of metals in Zea mays (Tu et al., 1988) and Aspergillus niger (Mullaney and Ullah, 1998).

The responses of soluble and bound APase to Cu²⁺ differed, since metal ions slightly activated the enzyme bound to the wall (Fig. 4). Moran et al. (1989) reported that the kinetic behavior of Y. lipolytica APase is non-Michaelian, that is, the enzyme has multiple binding sites for its substrate. The purified Y. lipolytica enzyme shows size het-
erogenicity, indicating an apparent molecular weight in the range of 90,000–200,000 according to SDS-polyacrylamide gel electrophoresis (López and Domínguez, 1988). The activity of Y. lipolytica APase is also activated by increasing the ionic strength of the reaction mixture (González et al., 1993), whereas that of purified APase from sycamore cell walls is not similarly activated. However, the enzyme is activated when bound to small cell wall fragments of Acer pseudoplatanus (Noat et al., 1980). Therefore, the enzyme APase apparently hydrolyses its substrate in different cell types via a more complex mechanism. The present study found that APase activation by some metal ions might be caused by a slight modification of its molecular structure during growth in medium containing Cu\(^{2+}\) as well through the synthesis of its isoform. Further studies are required to determine whether metal-activating APase is a repressible enzyme and/or whether it plays a role in the Cu\(^{2+}\) tolerance of Y. lipolytica.

Acknowledgements

We thank Dr. R. Rachubinski, University of Alberta, Edmonton, Alberta, Canada for kindly donating the mutant yeast strain.


Mullaney E. J. and Ullah A. H. J. (1998), Conservation of the active site motif in Aspergillus niger (ficunum) pH 6.0 optimum acid phosphatase and kidney bean...