Copper Accumulation and Phosphatase Activities of *Aspergillus* and *Rhizopus*

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Copper accumulation and phosphatase activities of three *Aspergillus* species resistant to copper were compared to three copper-sensitive *Rhizopus* species. High level of acid phosphatases and decreased Cu^{2+}-uptake were found with resistant in contrast to sensitive strains. The presence of copper(II) ions in the medium increased the production of acid phosphatases in the resistant *A. niger* and decreased their activity in the sensitive *R. delemar*. Copper ions inhibited the activity of *A. niger* cellular acid phosphatase with a K_i of 8.9×10^{-4} M and slightly activated the *R. delemar* enzyme.

Introduction

Extensive study on the microbial utilization of heavy metals withing the past decade revealed that heavy metal resistance may be mediated by genetic factors (Silver and Waldehaug, 1992), changes in membrane permeability (Levine and Marzluf, 1989), immobilisation of metal ions within the cell wall (Cervantes and Gutierrezcorona, 1994), adsorption (Huang *et al.*, 1988), energy dependent ion efflux (Nies and Silver, 1995). The cellular mechanisms of overcoming or adapting to the toxic effects of metal ions may involve precipitation of metals as insoluble salts (Blake *et al.*, 1993), and biochemical transformation of metals (Williams and Silver, 1984). As reviewed by Gadd (1990), these processes may be metabolism independent (adsorption) or dependent on metabolic activity (transport).

The ability of some bacteria to accumulate heavy metals has been attributed to phosphatase overproduction as a detoxification mechanism with precipitation of metals away from the sensitive cellular sites (Macaskie *et al.*, 1988). Little information is available so far about the participation of phosphatases in mechanisms of heavy metal resistance in fungi and yeast. Acid and alkaline phosphatases belong to the group of enzymes that hydrolyse the phosphate esters thus providing inorganic phosphate for the cells (Metzenberg, 1979). While the location of alkaline phosphatases (ALPases) is connected with vacuoles and their membranes (Klionski and Emr, 1989), acid phosphatases (APases) are located in parts of the cells that undergo considerable changes due to the effect of external factors (Vasileva-Tonkova *et al.*, 1996) As periplasmic enzymes, a part of acid phosphatases exists in soluble form and may secrete into the culture medium while the other part is attached to the membrane. Despite the marked tolerance towards the metals and other environmental conditions (low pH, temperature), filamentous fungi have high capacities of metal binding to cell walls and low rates of intracellular metal uptake (Gadd, 1986). Since the AP – ases are located near the cell walls they may an participate role in these processes.

This paper describes copper uptake of six filamentous fungi, three of *Aspergillus* and three of *Rhizopus*. Based on the differences in their abilities to accumulate copper ions we attempted to define the role of cellular and extracellular phosphatases in processes of overcoming or adapting to the toxic effects of metal ions.

Materials and Methods

Microorganisms

Six fungal strains, three of *Aspergillus* strains (*A. niger, A. ussami, A. awamori*) and three of *Rhizopus* strains (*R. niveus, R. delemar, R. chi-*

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nensis) were used in this study. All strains were obtained from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences. The fungi were routinely maintained on potato – dextrose – agar slants and stored at 4 °C. The medium consisted of the following (grams per liter): potato infusion, 200; dextrose, 20; $CaCO_3$, 0.2; $MgSO_4.7H_2O$, 0.2; agar, 15 (pH not adjusted). Subcultures were made every 3 months.

Culture media and growth conditions

Fungi were grown in 500 ml flasks with 100 ml medium at 30 °C for 24 hours (the end of exponential growth), shaking at 220 rpm. The medium (100 ml) containing ($g.l^{-1}$): soluble starch, 30; corn step liquor, 40 and 0, 1 and 2 mM $CuSO_4$ was inoculated with 0.1 ml diluted spore suspension (about 10^6 spores per ml). The pH of the medium was adjusted to 5.0.

Copper uptake by fungal biomass

Cells (24 h incubated) were harvested by filtration through Buhner funnel. Fungal mycelia were washed twice with bi-distilled water and samples of 1 g wet weight were used to determine the Cu^{2+}-uptake of living cells. Parallel pretreatment step was used as follows: 1 g wet biomass was boiled for 15 min in bidistilled water, cooled and the suspension was centrifuged at 5000 g for 15 min to separate the biomass. The cells mass obtained was considered as dead cells and used as a biosorbent. Cells were resuspended in 100 ml 1 mM solution of $CuSO_4$ in bidistilled water (pH 5.0) and incubated in 250 ml flasks on orbital shaker at 220 r.p.m. for 60 min at 30 °C, when the equilibrium was reached. The exposed mycelia were removed through membrane filter with pore size 0.45 μm (Millipore) and the residual Cu^{2+} in the filtrate was determined using Perkin-Elmer 2380 atomic absorption spectrophotometer.

Uptake of metal ions was calculated from a metal mass balance yielding (Volesky, 1990): $q = V(C_i - C_f) / m$M where q is mmol metal ions / g dry biomass, V is the sample volume (l), C_i and C_f are the initial and residual metal concentrations (mg / l) respectively, m is the amount of dry biomass (g) and M is the relative molecular mass of the metal. Control samples with no biomass added were used as blanks.

The amount of Cu^{2+} in cells was expressed as mg Cu^{2+} per 1 g dry weight.

Preparation of cell-free extract

The harvested mycelia were washed twice with distilled water. The biomass was disrupted in a homogenizer three times for 1 min and centrifuged at 5000×*g* for 20 min. The supernatant was used for determining cellular acid (APase) and alkaline phosphatase (ALPase) activities.

Enzyme assay

The activity of extracellular and cellular phosphatase enzymes (in culture liquid and in cell-free extract) was assayed as described earlier (Galabova *et al.*, 1993) with *p*-nitrophenylphosphate (*p*NPP) as substrate. The reaction mixture contained 100 μl 3.8 mM pNPP, 100 μl 0.1 M buffer (sodium acetate buffer, pH 4.2 or Tris-HCl [tris(-hydroxymethyl)-aminomethane-HCl] pH 8.6, for acid and alkaline phosphatase determinations, respectively), and 100 μl enzyme solution (supernatant or cell-free extract).

One unit was defined as the amount of enzyme releasing 1 nmol *p*-nitrophenol per min at 30 °C.

Cell dry weight and protein measurement

Dry weight of the fungal biomass was determined after drying for 48 h at 85 °C in tared aluminium foil caps. Soluble protein in the culture liquid as well as in cell free extract was determined by the method of Bradford (1976) using bovine albumin as standard.

Results and Discussion

Effect of the copper on culture growth

As shown in Fig. 1 the inhibitory effect of Cu^{2+} on cell growth is almost identical within the genus and increases with higher Cu^{2+} concentration. Generally, the growth of *Aspergillus* species is less affected by the presence of Cu^{2+} than *Rhizopus* species. Most resistant strain to the copper ions inhibition was *Aspergillus niger*, while *Rhizopus delemar* was the most sensitive strain. Regarding their resistance to Cu^{2+} strains may be ordered as follows:

A. niger > *A. usamii* > *A. awamori* > *R. niveus* > *R. chinensis* > *R. delemar.*

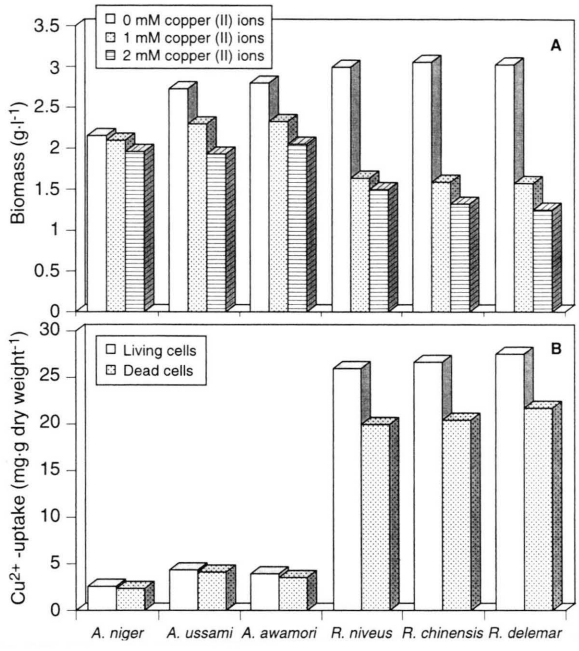

Fig. 1. A/ Effect of Cu^{2+}-ions on the yield of biomass of *Aspergillus* and *Rhizopus* species. Species were grown for 24 h (the end of exponential phase) at 30° C in presence and absence of copper in the medium, as described in Materials and Methods. Data (three determinations from each of two separate experiments) were pooled to give a mean ± S. D. of 10–15%.
B/ Cu^{2+}-uptake (mg.g cell dry weight $^{-1}$) by cells of *Aspergillus* and *Rhizopus* species. The cells were pre-incubated in the presence of 1 mM Cu^{2+} as described in Materials and Methods. The data shown are mean values of three experiments. Individual values deviate less than ±10% of the mean values.

Copper uptake by cells of Aspergillus and Rhizopus species

The results shown in Fig. 1 demonstrate the distinct difference in Cu $^{2+}$-uptake by cells of the metal – resistant *Aspergillus* and the metal – sensitive *Rhizopus* strains. The cells of *A. niger* have the lowest capacity to bind the copper ions while the cells of *R. delemar* show highest ability to accumulate Cu^{2+}.

On the other hand, the live fungal biomass of *Aspergillus* species showed the same values of Cu^{2+}-uptake as the dead cells. By contrast, *Rhizopus* species live biomass exhibited higher uptake of Cu^{2+} than the dead cells at the same conditions.

Phosphatase production of filamentous fungi

The assay of phosphatase activities revealed remarkable differences in phosphatase levels between the two genera tested (Fig. 2). Compared to data for *Rhizopus*, the *Aspergillus* species showed much higher values of total phosphatase activities. Very strong difference in the levels of acid phosphatases, particularly with cellular acid phosphatase was observed in both genera. As shown on Fig. 3, the metal-resistant species (*Aspergillus*) contain approximately 8 to 20 times as much acid phosphatase as the metal-sensitive *Rhizopus* species. Furthermore, the most Cu^{2+}-resistant strain tested in this study, *A. niger*, possesses much higher cellular acid phosphatase activity than extracellular in contrast to all *Rhizopus* species.

It is noteworthy that strains with well expressed acid phosphatase activity showed low capacity for bioacumulation of Cu^{2+} ions (Fig. 1). In contrast, low acid phosphatase activity was registered with the high accumulating strains.

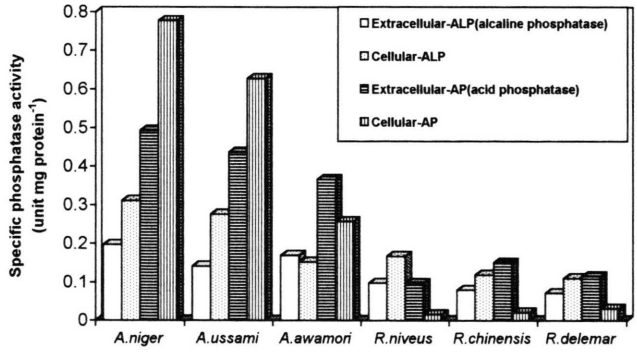

Fig. 2. Phosphatase production of *Aspergillus* and *Rhizopus* species tested. Phosphatase activities were determined in liquid (extracellular activities) and in cell-free extracts (cellular activities). Strains were grown on the standard medium in absence of Cu^{2+}. All values are means (n = 3) with a standard deviation < 10%.

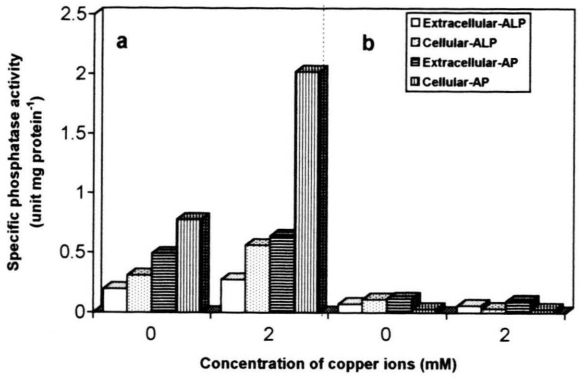

Fig. 3. Effect of Cu^{2+} on the phosphatase production of *A. niger* (a) and *R. delemar* (b). Cellular and extracellular activities were determined at pH 4.2 (0.1 M acetic acid/sodium acetate) and pH 8.6 (0.1 M Tris-HCl). Strains were grown on the standard medium in absence and in presence of 2 mM Cu^{2+} as described in Materials and Methods. Data (three determination of two separate experiments) were pooled to give a mean ± S. D. of within 10–15%; mean values are given.

The changes in alkaline phosphatase activities are smaller and the values of the ALP-ase for the most sensitive strain *R. delemar* was up to two-fold lower than for the most resistant *A. niger.*

Effect of copper ions on the phosphatase production

Two strains, metal – resistant and metal – sensitive, *A. niger* and *R. delemar* respectively, were grown in the presence of Cu^{2+} and their phosphatase activities were studied. The results obtained showed that the effect of the Cu^{2+} on phosphatase production of both strains is distinctly different. As shown on Fig. 3, the phosphatase activities of the metal – resistant *A. niger* increases in the presence of 2 mM Cu^{2+} in the medium and the increase for cellular APases is more clear than for the extracellular activities. In the opposite, cellular and extracellular phosphatase activities of the metal-sensitive *R. delemar* are decreased in the presence of Cu^{2+}, and the decrease of cellular alkaline phosphatase is most evident.

Effect of copper ions on the cellular acid phosphatase activity

The results obtained for the effect of Cu^{2+} concentration on the catalytic properties of cellular acid phosphatases of the two strains studied, the Cu^{2+}-sensitive *R. delemar*, and the resistant to Cu^{2+} A. niger, showed the following: While the presence of copper ions in the medium increases the level of the cellular acid phosphatase activity of *A. niger* (as is shown in Fig. 3), the presence of Cu^{2+} in enzyme reaction mixture causes a decrease of enzyme activity. The inhibition effect of copper ions on the *p*NPP – activity of *A. niger* acid phosphatase was determined from Dixon plots as shown by Fig. 4. Cu^{2+}-ions were an non-competitive inhibitor of this process with an apparent K_i of 8.9×10^{-4} M. In contrast, the presence of copper ions increases cellular acid phosphatase activity of the Cu^{2+}-sensitive *R. delemar.*

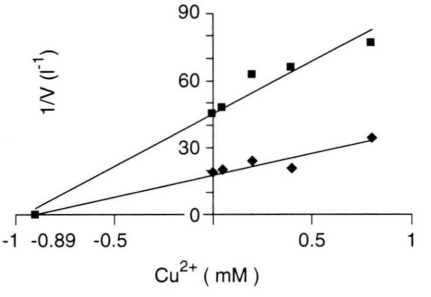

Fig. 4. Dixon plots of Cu^{2+}-ions inhibition effect on the *p*NPP-activity of *A. niger* acid phosphatase. APase activity was determined by *p*NPP hydrolysis at pH 4.2 in a presence of different concentrations of Cu^{2+}-ions. Substrate concentration: 3.8 mM (◆) and 2 mM (■). All data points are means of four experiments.

Fungi express a wide variety of potential accumulation sites on their cell walls, phosphoryl, carboxyl, sulfhydryl and amino groups. So, they have high capacities of metal binding to cell walls and also may have high values of intracellular accumulation (Gadd, 1986). The differences in the values of Cu^{2+}-uptake by dead cells of both genera are probably due to different content of ligand groups in their cell walls. However, the observed higher amount of copper binding by live cells of *Rhizopus* species compared to the dead biomass, probably, is not a result of different proportions of wall constituents only (Fig. 1). Recently it was found that four bacterial strains bound similar amounts of copper at the cell surface in contrast to the significant differences in binding of some other metals (Langley and Beveridge, 1999). Since copper did not easily form surface precipitates the authors hypothesised that copper ions were preferentially bound in sites which were common to all strains,

perhaps by phosphoryl groups in the core-lipid A region.

Our study revealed that resistant to copper *Aspergillus* strains posses up to 20 times higher phosphatase activities than sensitive *Rhizopus* species studied (Fig. 2). The presence of Cu^{2+} in the medium increases the activities, particularly of cellular acid phosphatases. Thus, the enhanced resistance of *A. niger* to metal ions (Fig. 1) may be due to the overproduction of acid phosphatases (Fig. 3). On the other hand, phosphatase activities of the sensitive strain *Rhizopus delemar* were low and the presence of Cu^{2+} decreases them additionally. Furthermore, the different effect of copper ions shows that the acid phosphatases of both, "resistant" and "sensitive" strains are quite different enzymes in terms of the catalytic properties studied. It seems that the high resistance and the low accumulative ability of the living cells of *A. niger* are connected in some way with the overproduction of cellular AP-ase and, on the other hand, with the inhibitory effect of copper on the enzyme activity. In contrast, with *R. delemar* a high degree of copper accumulation by cells and stable to copper inhibition acid phosphatase was observed. The enhanced Cu^{2+}-uptake may be due to the participation of AP-ase, causing precipitation of metal away from the sensitive cellular sites.

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