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Growth Phase-Dependent Antioxidant Enzyme Defense of *Humicola lutea* against Copper Stress

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Abstract

Copper is an essential element for the growth and development of the full range of living organisms, including filamentous fungi. At the same time, it is toxic for organisms when present in excess. The present study was conducted to assess the role of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) on the tolerance strategy of the fungal strain *Humicola lutea* 103 at different growth phases under enhanced Cu ions concentrations. We examined the changes in the growth, intracellular protein content and levels of antioxidant enzyme defense. The results revealed that the presence of Cu ions affected the duration of growth phases in a dose-dependent manner. The effect of Cu treatment (150 μg/ml Cu ions) depends on the age of the treated culture. The spores and cells from the stationary growth phase demonstrated higher resistance compared to the corresponding control due to the enhancement of SOD and CAT activity. The increased total SOD activity was largely due to the Cu/Zn-SOD isoform. The non-growing cells taken from cultures of different growth phases demonstrated also up-regulation of both antioxidant enzymes in response to oxidative stress imposed by the ROS-generating heavy metal. During the stationary-phase, *H. lutea* cells demonstrated higher resistance to Cu-induced oxidative stress compared with the exponential phase cells.

Key words: heavy metals, filamentous fungi, oxidative stress, superoxide dismutase, catalase, growth phase

Резюме

Медта е основен елемент за растежа и развитието на цялата гама от живи организми, включително филаментозни гъби. В същото време, високите концентрации на медни йони оказват силно токсично въздействие. Настоящото изследване е насочено към проучване значението на антиоксидантните ензими - супероксид дисмутаза (СОД) и каталаза (КАТ) за толерантността на култури от щам *Humicola lutea* 103 в различни фази на растеж към повишени концентрации на Cu-йони. Проследени са промените в растежа, съдържанието на вътреклетъчен белтък и нивата на антиоксидантната ензимна защита. Резултатите показват, че наличието на Cu йони променя продължителността на фазите на растеж и тези промени корелират с концентрацията на метала. Ефектът от въздействието с 150 мкг/мл медни йони зависи от възрастта на третираната култура. Едновременно с това се наблюдава повишената активност на двата антиоксидантни ензима – СОД и КАТ. Индукцията на изоензима Cu/ZnСОД е отговорна за повишената обща СОД активност. Спорите и клетките от стационарната фаза проявяват най-висока устойчивост към медните йони. В експериментите с нерастящи клетки от различни фази на растеж се отчита същата тенденция.

Introduction

Heavy metals are among the pollutants of greatest importance and concern in the world today (Luna *et al.*, 2015). Although they are naturally present in the soil, geologic and anthropogenic activities increase the concentration of these elements to amounts that are harmful to both plants and animals. Some of these activities include mining and smelting of metals, burning of fossil fuels, use of fertilizers and pesticides in agriculture, production of batteries and other metal products in

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industries, sewage sludge, and municipal waste disposal (Chibuike and Obiora, 2014). Based on their chemical and physical properties, three different molecular mechanisms of heavy metal toxicity can be distinguished: (a) production of reactive oxygen species by autoxidation and Fenton reaction; this reaction is typical of transition metals such as iron or copper, (b) blocking essential functional groups in biomolecules; this reaction has mainly been reported for non-redox-reactive heavy metals such as cadmium and mercury, (c) displacement of essential metal ions from biomolecules; the latter reaction occurs with different kinds of heavy metals (Schützendübel and Polle, 2002).

There is ample evidence that exposure of aerobic organisms to excess concentrations of redox active heavy metals such as Cu results in oxidative injury as a results of increased generation of reactive oxygen species (ROS) (Avery, 2001; Krumova *et al.*, 2009; Pócsi, 2011). These ROS include the superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$) and can cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately lead to cell death (Fridovich, 1998). At the same time, Cu serves an essential role in biological processes because of its catalytic and structural properties. Thus, the maintenance of Cu homeostasis at the cellular level is crucial for aerobic organisms (Rees and Thiele, 2004; Krumova *et al*., 2009; Lazarova *et al*., 2014). Antioxidant enzymes play a very important role in this balance (Luna *et al*., 2015).

In filamentous fungi, the effect of Cu stress on growth and physiological characteristics is currently under investigation. The published results concern mainly alterations in the antioxidant defense system in the presence of Cu ions regardless of the age of the culture.

In our previous studies we focused on assessing the Cu-induced cell response of the fungal strain *Humicola lutea* 103. The treatment was applied at the beginning of growth (Krumova *et al*., 2009). But the effect of Cu ions added to cultures at different growth stages has not been investigated. The aim of the present study was to gain insight into the influence of oxidative stress induced by Cu exposure on *H. lutea* cells of different age (0, 12, 18, 24, 36, and 48 h old). We examined the changes in growth and activities of SOD and CAT.

Materials and Methods

Fungal strain and culture conditions

The fungal strain, *H. lutea* 103 from the Mycological Collection at the Institute of Microbiology, Sofia, was used throughout and maintained at 4°C on beer agar, pH 6.3. For the submerged cultivation, both seed and productive media were used (Angelova *et al*., 1996). The cultivation was performed in 500 ml Erlenmeyer flasks or in 3 bioreactor ABR-09 (working volume 2 L) equipped with a pH and DO monitoring and control system. To prepare the inoculum, 80 ml of seed medium was inoculated with 5 ml of spore suspension at a concentration of 2 x10^8 spores ml^{-1} in 500 ml Erlenmeyer flasks. Cultivation was performed on a shaker (220 rpm) at 30°C for 24 h. The bioreactor cultures were performed with 8% (v/v) 24-h-old shake-flask inoculum and 1800 ml of the production medium. The fermentation parameters were: temperature 30°C, impeller speed, 600 rpm, and air flow, 1 vvm (1 volume air per 1 volume liquid per min).

For characterization of the fungal response to copper stress, a sterile stock solution of $CuSO_4$ was added to 6 h bioreactor cultures of *H. lutea* 103 to bring the final Cu ion concentration to 70, 150 and 300 μg/ml, respectively. To determine growth, samples were drawn every 6 h for up to 120 h dry weight estimation.

The impact of the growth phase on Cu-stress response was evaluated using growing and non-growing mycelia. For growing cultures, 150 μg/ml Cu ions were added after 0, 12, 18, 24, 36, and 48 h of incubation and cultivation continued until 120 h.

For experiments with non-growing mycelium, cells were cultivated for 0, 12, 18, 24, 36, 48, 60 and 72 h, respectively, in the seed medium as described above. Then 1 g of wet mycelium was added to 40 ml of medium III (KH_2PO_4 - 5 g/l and $MgSO_4.7H_2O$ - 2.5 g/l, pH 7.8) with or without 150 μg/ml Cu ions in 500-ml Erlenmeyer flasks, followed by incubation at 30°C on a shaker (220 rev/min) for 120 min.

Cell-free extract preparation

The cell-free extract was prepared as previously described (Krumova *et al.,* 2009). All of the steps were performed at 0–4°C.

Enzyme activity determination

SOD activity was measured in CFE by NBT reduction (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units

per mg protein (U/mg protein). KCN (5 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/Zn-SOD and the cyanide-resistant Mn-SOD. The Cu/Zn-SOD activity was obtained as total activity minus the activity in the presence of 5 mM cyanide. Catalase was assayed by the method of Beers and Sizer (1952), in which the decomposition of H_2O_2 was followed spectrophotometrically at 240 nm. One unit of catalase activity was defined as the amount of enzyme which decomposes 1 μmol H_2O_2/min at initial H_2O_2 concentration of 30 mM at pH 7.0 and 25°C.

PAGE electrophoresis

The SOD isoenzyme profile was visualised on polyacrylamide gels. Forty μg of total protein were applied to 10% nondenaturing PAGE and stained for superoxide dismutase activity, as described by Beauchamp and Fridovich (1971).

Other analytical methods

Protein was estimated by the Lowry procedure (1951), using crystalline bovine albumin as a standard. The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) No 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Results and Discussion

Aging of H. lutea cells in the presence of enhanced concentrations of Cu ions

The growth of *H. lutea* in medium supplemented with varied Cu ions concentrations at the beginning of cultivation is demonstrated in Fig. 1.

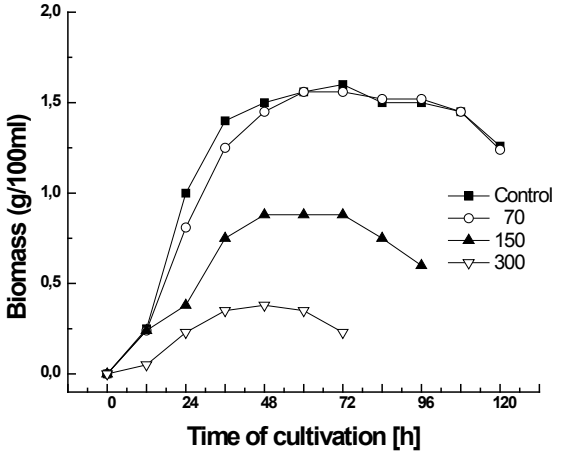

Fig. 1. Effect of Cu ion concentrations on the growth profile of *H. lutea* 103

Although the curves demonstrated typical fungal growth phases for each experiment, it can be seen that the presence of Cu ions affected the duration of growth phases in a dose-dependent manner. Supplementation of the medium with 70 μg/ml Cu ions did not have a significant influence on the growth rate and growth phase duration. There was a slight delay in the onset of the exponential phase, but in the process of active growth the culture rapidly reached the parameters of the control. It should be noted, however, that the stationary phase concluded about 12 hours earlier than the control.

On the other hand, the treatment with higher Cu concentrations (150 or 300 μg/ml) caused 40-45% reduction in the life span. Figure 1 demonstrates a prolonged lag-phase and shortened exponential and stationary growth phases, which was more significant at a dose of 300 μg/ml. Moreover, at copper concentrations of 150 or 300 μg/ml, *H. lutea* 103 accumulated about 50% and 80% less biomass (dry mass), respectively, than the control. Similar results on the growth and phase duration have been reported for *Aspergillus niger* (Tsekova and Todorova, 2002; Luna *et al*., 2015), *Cunninghamella elegans* (de Souza *et al*., 2005) etc. Our previous results revealed that the addition of copper ions 18 h after cultivation more slightly affected the growth phase and biomass content (Krumova *et al*., 2012). Probably, cultures in the exponential growth phase exhibited greater copper tolerance compared to those in the lag phase (current study). Anahida *et al*. (2011) found such relationship between the age of exposure and heavy metal tolerance of *Aspergillus* and *Penicillium* strains.

If we compare the effect of copper ions on the *H. lutea* growth with our previous results (Krumova *et al*., 2009), we have to note that copper stress imposes an oxidative burden, of which $^{\bullet}O_2^-$ would be a major component. According to Osiewacz and Stumpferl (2001), cellular copper levels play a significant role in the generation of oxygen metabolites by the fungus *Podospora anserina*. Other studies have examined copper-induced ROS in higher eukaryotes (Sharma *et al*., 2012; Shahid *et al*., 2014). Probably, the Cu-induced ROS generation in the cells plays a main role in the control of the life span of fungi (Borghouts *et al*., 2001).

Growth-phase-dependent response of H. lutea cultures against Cu stress

The results from the experiments with growing cells have revealed that the degree of Cu effect (150 μg/ml) on *H. lutea* depends on the age of the treated culture (Fig. 2). Spores demonstrated the highest resistance compared with other variants. The amount of biomass formed by treated spores

(albeit lower than the control) was about 20-40% higher than in the cultures from different growth-phases (Fig. 2). The cells of the early exponential phase (12th and 18th h) were most sensitive. They maintained dry weight about 40% of the control. The exposure of cells after 36 and 48 h of cultivation (the end of exponential phase and the beginning of stationary phase, respectively) led to a gradual increase in the biomass content, i.e. in the fungal resistance to Cu ions. At the end of the cultivation, the treated stationary growth cells showed 55% enhanced dry weight compared to those measured by culturing the Cu-stressed cells of exponential phase.

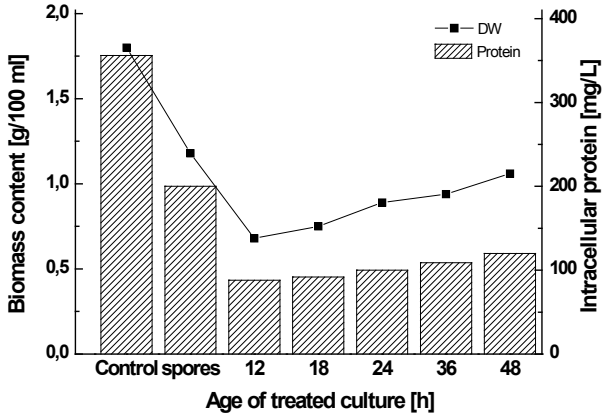

Fig. 2. Biomass and intracellular protein content in *H. lutea* cultures from different growth phases treated with 150 μg/ml Cu ions.

The same trend was shown for intracellular protein content, but the evaluated increase at the end of exponential phase and the beginning of stationary phase was less pronounced (Fig. 2). The content of total protein indicates that a significant effect on the protein content has not been found between the treated cultures from exponential and stationary phases. There was a decrease in comparison with the control, which might demonstrate the inhibitory effect of the copper content on the protein isolated from *H. lutea*.

A possible reason could be an age-dependent metal uptake due to qualitative changes in the cell wall structure when the cells pass from exponentially to stationary phase. Anagnostopoulos *et al.* (2011) reported the highest metal uptake for the exponential phase cells of *Saccharomyces scerevisiae, Kluyveromyces marxianus* and *Debaromyces hansenii*. Copper, as a redox metal, can directly generate oxidative injury via the Haber-Weiss and Fenton reactions, which leads to production of ROS, resulting in cell homeostasis disruption, DNA strand breakage, defragmentation of proteins, or cell membrane and damage to photosynthetic pigments, which may trigger cell death (Bellion *et al*., 2006; Luna *et al*., 2015; Oves *et al*., 2016). It has been reported that Cu induces plasma membrane disruption in fungi (*Azevedo et al*., 2007). The data on Cu effect on *A. niger* revealed that oxidative stress is involved in the mechanisms of copper toxicity and suggests that this fungus exhibits an increased level of lipid peroxides (Luna *et al*., 2015). In the mycelia of *Curvularia lunata* exposed to Ni, the levels of lipid peroxidation products increased and ranged between 156 and 823% over the control (Paraszkiewicz *et al*., 2010).

To compare the antioxidant response of the fungal cells to Cu toxicity and oxidative stress, altered cellular antioxidant enzyme defense was investigated in the presence of 150 μg/ml copper concentration (Fig. 3). The activities of SOD and CAT were simultaneously assayed in Cu-treated growing cells taken from different growth phases (see Materials and methods).

During the aging, the SOD and CAT activity increased in a time-dependent manner. The observed increase was from 1.4- to 2.5-fold above the activity of the controls. Entry in the stationary phase resulted in some attenuation of the response, but both enzyme activities were significantly higher than those of the untreated cells.

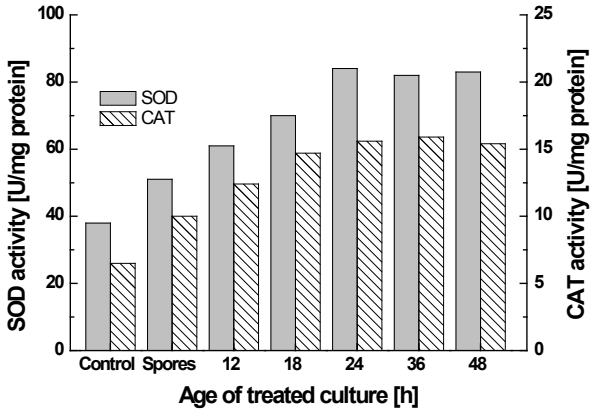

Fig. 3. SOD and CAT activity of *H. lutea* cultures from different growth phases treated with 150 μg/ml Cu ions

The increased total SOD activity was largely due to the Cu/Zn-SOD isoform, which showed a 1.7- to 2.5-fold increase as compared to the control culture; we found no change in the activity of Mn-SOD (data not shown). To confirm the levels of MnSOD and Cu/ZnSOD activity in the fungal cultures, we used the native gel technique. Figure

4 shows that Mn-SOD activity did not change with aging. In contrast, Cu/Zn-SOD activity was significantly increased in the cells taken after 24 and 48 h of cultivation compared with the cells from the beginning of exponentially phase (12th h).

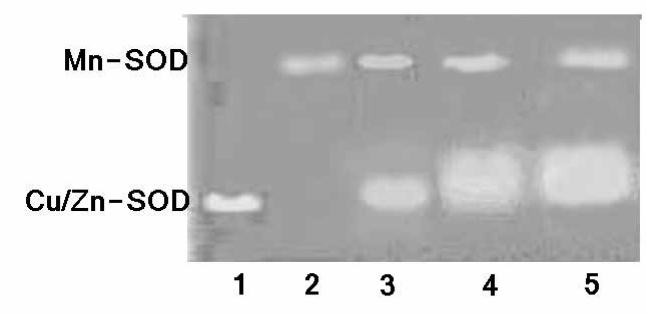

Fig. 4. Effect of copper treatment (150 mg/ml Cu^{2+}) on isoenzyme profiles of SOD in *H. lutea* cells. Mn- and Cu/Zn-SOD activity evaluated by polyacrylamide gel electrophoresis (10% gel) lane 1, standard Cu/Zn-SOD from bovine erythrocytes; lane 2, standard Mn-SOD from *E. coli*; lane 3, SOD in the cells of early exponentially phase (12 h); lanes 4, SOD in the cells taken after 24 h of cultivation; lane 5, SOD in the cells taken after 48 h of cultivation.

The present study also showed that copper-induced oxidative stress was indicated by enhanced antioxidant enzyme activities. Fungi, like all aerobic organisms, have a set of defense mechanisms to deal with oxidative stress. Enzymes, such as SOD and CAT, have been reported to be activated against ROS in several organisms under Cu stress (Tsekova *et al*., 2002; Azevedo *et al.*, 2007; Krumova *et al*., 2009; 2012). Both enzymes are crucial for cellular detoxification, controlling the levels of superoxide anion radical and hydrogen peroxide (Pócsi *et al*.**,** 2011). The effect of Cu ions depends on *H. lutea* growth stage. Fast enhancement of the antioxidant enzyme activity was observed in the cells from lag- and exponential phases. The experiments with cultures grown for 36 or 48 h did not demonstrate further increase in SOD and CAT activity. Taking into account that the level of Cu-induced ROS generation in *H. lutea* cells had a tendency to keep rising (Krumova *et al*., 2009), it can be assumed that these ROS caused also enzyme inhibition *via* denaturation and protein degradation (Gessler *et al*., 2007; Seto *et al*., 2017). Similar results were observed about plants treated with ROS-generated agents (Lijun *et al*., 2005; Soares *et al*., 2010). Cyrne *et al.* (2003) have described that oxidative stress modifies Cu/Zn-SOD and Mn-SOD gene expression in a complex way, at the transcriptional, posttranscriptional, translational, and posttranslational levels.

Antioxidant enzyme activity in non-growing H. lutea cells under conditions of Cu stress

The non-growing cells taken from cultures of different growth phases were used as cell systems for a rapid induction of antioxidant enzymes in the absence or presence of Cu ions. Figure 5 illustrates the activity of both antioxidant enzymes, SOD and CAT, as a result of treatment with 150 mg/ml Cu ions.

The growth phase-dependent alteration of SOD outlined a trend similar to those obtained with growing cells. Cultures from lag- and early exponential phases drastically enhanced SOD activity in the presence of Cu ions compared to the corresponding control. The results revealed that the cells from the 12-, 18-, 24- and 36-h cultures showed about a 4-fold increase in the enzyme activity during 120 min exposure to a heavy metal. It was also shown a phase-dependent up-regulation of antioxidant enzyme activity in cells of 48-, 60- and 72-h cultures. Cu exposure of *H. lutea* cells had a comparable effect on CAT profile and changes in the activity provide support for growth phase-dependent manner of enzyme induction. Although the older cells demonstrated a significant increase in SOD and CAT levels compared to the control, the activities were lower than those in the younger ones.

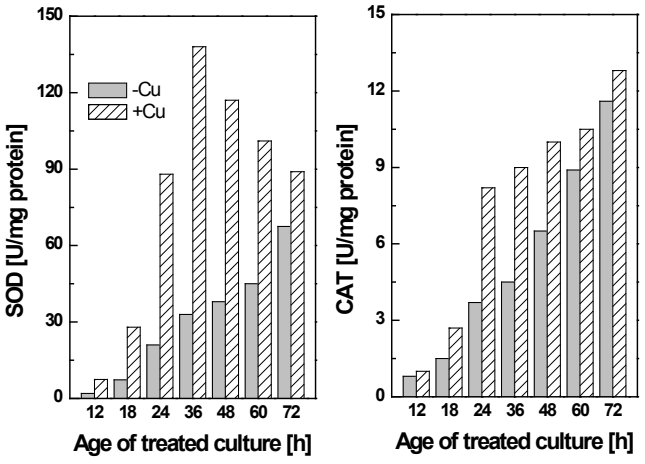

Fig. 5. Growth phase-dependent induction of SOD (A) and CAT (B) activity in non-growing cells of *H. lutea* 103 treated with Cu ions.

From the above experiments it is clear that there is a direct relationship between the antioxidant response and growth phase of *H. lutea* cultures. The influence of oxidative stress on the antioxidant enzyme activity has been found to be dependent on the

growth phase of pro- and eukaryotic cells (Michán and Pueyo, 2009; Radhika, 2013). *Candida albicans* and *C. glabrata* showed growth phase-dependent SOD and CAT induction by enhanced ROS generation (Cuéllar-Cruz *et al*., 2008). Hao *et al*. (2010) also reported that the antioxidant enzymes participate to different degrees in stage-dependent resistance to rice blast fungus *Magnaporthe oryzae*. A differential expression of SOD and CAT depending of the growth stage has been determined in *Phycomyces blakesleeanus* treated with H_2O_2, CAT being expressed in the exponential and SOD in the stationary phase (de Castro *et al*., 2013).

Furthermore, *H. lutea* cell response to oxidative stress generated by Cu ions increased SOD and CAT activity to a greater extent in the exponential phase than in the stationary growth phase. De Castro *et al.* (2013) found the opposite trend in *Phycomyces blakesleeanus* cultivated in the presence of H_2O_2. They suggested tha$_t$ oxidative stress increased the activity of SOD and glutathione-S-transferase to a greater extent in the stationary phase than in the exponential growth phase. Glucose starvation induced CAT activity in *P. blakesleeanus* during both phases (Rúa *et al.*, 2014). The high activity of CAT could be due to the induction of an isoform which would be expressed in these conditions, similar to CatD from *A*. *nidulans* induced during the late stationary phase by glucose starvation (Szilágyi *et al*., 2013).

Conclusion

Our results suggest the importance of both the Cu ion concentration and growth phase in how the fungal strain maintains its intrinsic redox state. The *H. lutea* cells up-regulate their defense system against oxidative stress imposed by the ROS-generating heavy metal. Antioxidant enzymes SOD and CAT were induced by Cu exposure and protected the fungus from further damage. The higher resistance of stationary fungal cultures to Cu stress is due to the activation of the antioxidant enzyme defense.

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