Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 66, No 4, 2013

BIOLOGIE

Microbiologie

REGULATION OF SUPEROXIDE DISMUTASE SYNTHESIS IN *HUMICOLA LUTEA* CELLS UNDER Cu^{2+} STRESS CONDITIONS

Ekaterina Krumova, Radoslav Abrashev, Jeni Miteva-Staleva, Svetlana Pashova, Maria Angelova

(*Submitted by Academician A. Galabov on December 13, 2012*)

Abstract

The superoxide dismutase (SOD) is the first enzyme to respond against oxygen radicals and to offer the greatest response to oxidative stress. The great interest in SODs is a result of their important physiological role and therapeutic potential. The fungal strain *Humicola lutea* 103 has been selected as an effective producer of Cu/Zn-SOD exhibiting protective effect against influenza virus infection and myeloid Graffi tumour. The present paper studies the role of control mechanisms induction and catabolite repression on SOD synthesis under copper stress conditions. Cu ions cause rapid and profound induction of enzyme activity by dose- and time-dependent manner. The deinduction experiments also demonstrate the induction model of SOD synthesis. The results suggest that SOD synthesis in the fungal strain *H. lutea* 103 treated with Cu ions is a subject to catabolite repression.

Key words: copper stress, regulation of SOD synthesis, induction, catabolite repression

Introduction. The superoxide dismutases (SODs) are ubiquitous components of cellular antioxidant systems that protect cells against the harmful effect of reactive oxygen species (ROS). SODs are generally classified according to the metal species which acts as redox-active centre to catalyze the dismutation of superoxide radicals ($^{\bullet}O_2^-$) to O_2 and H_2O_2. SODs have been shown to exhibit

This work was supported by Grant DO02-172/08 from the National Fund for Scientific Research, Republic of Bulgaria.

high levels of conservation and to fall within three main structural classes based upon metal specificity: copper-zinc, manganese or iron, or nickel [1].

The great interest in SODs is a result of their important physiological role and application as antioxidants [2]. There is accumulating evidence that exogenous SOD has therapeutic potential in critical care medicine for the treatment of ROS-mediated diseases. The large scientific and practical importance of SOD resulted in development of new technologies for the enzyme production from various sources – animal and human erythrocytes, plants and microorganisms. Microbial technologies are more effective and inexpensive, compared with the production of this enzyme by human and bovine erythrocytes.

Our previous study demonstrated both the biotechnological potential of the fungal strain *Humicola lutea* 103 as an effective producer of Cu/Zn-SOD and its protective effect against influenza virus infection and myeloid Graffi tumour [3]. At the same time, the addition of Cu ions to productive medium resulted in oxidative stress events and enhanced Cu/Zn-SOD activity [4].

Two mechanisms are known as essential in regulation of the enzyme biosynthesis in microorganisms, including fungi: induction and catabolite repression. Enzyme induction is a process where an enzyme is manufactured in response to the presence of a specific molecule named inducer. On the other hand, catabolite repression is a mechanism preventing transcriptional expression of genes required for the degradation of the less-preferred substrate in the presence of the preferred substrate [5, 6]. Until now, very few studies have been carried out on the role of these mechanisms in regulation of SOD synthesis [7–9]. Furthermore, there are no investigations on the relationship between heavy metal stress and regulation of SOD synthesis.

In this study, we examined the role played by control mechanisms induction and catabolite repression, which regulate the changes in SOD activity of *Humicola lutea* 103 cultivated under Cu-stress conditions.

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 submerged cultivation, both seed and productive media were used [9]. For the inoculum, 80 ml of seed medium was inoculated with 5 ml of spore suspension at a concentration of 2×10^8 spores ml^{-1} in 500 ml Erlenmeyer flasks. Cultivation was performed on a shaker (220 rpm) at 30 °C for 24 h. Then 6 ml of seed culture were transferred to 500 ml Erlenmeyer flasks containing 74 ml of production medium. The cultures were grown at 30 °C for 24 h.

For experiments, non-growing mycelium was used. In these cases, cells were cultivated for 24 h (mid-logarithmic growth phase) in the seed medium as described above. Then 1 g of wet mycelium was added to 40 ml of medium NK2 (KH_2PO_4: 5 g/l and $MgSO_4$_$7H_2O$: 2.5 g/l, pH 7.8) with or without stress-inducing agents in 500 ml Erlenmeyer flasks, followed by incubation at 30 °C on

a shaker (220 rev/min). For investigation of the effects of different Cu concentrations, cultures were incubated with various concentrations of $CuSO_4$ for 2 h in order to achieve 20, 50, 100, 150, 300 and 700 µg/ml Cu ions. Time-dependent SOD induction was provided in presence of 70 µg/ml Cu ions for 30, 60, 120, 180 and 240 min. Deinduction experiments were started with mycelium incubated in medium NK2 for 1 h. The washed mycelium was suspended in medium NK2 and deinduction culture was shaken at 30 °C for 15, 30, 45, 60, 120 and 180 min respectively. Catabolite repression experiments were provided with adding of 0.5; 2.0 and 6.0% glucose or 2.0% Glucose-6-Phosphate to incubation mix containing 70 µg/ml Cu ions in presence and absence of 25 mM exogenous cAMP.

Enzyme activity determination. Cell free extract was prepared as described earlier [9]. SOD activity was measured in CFE by NBT reduction [10]. One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of NBT by 50% (A560) and was expressed as units per mg protein (U/mg protein). The cytosolic HK (EC 2.7.1.1) activity was determined according to Bergmeyer et al. [11].

Polyacrylamide gel electrophoresis. The SOD isoenzyme profile was determined using polyacrylamide gels. Forty micrograms of total protein were applied to 10% nondenaturing PAGE and stained for superoxide dismutase activity as described by Beauchamp and Fridovich [10].

Results were evaluated from repeated experiments using three or five parallel runs.

Results. ***Time- and dose-dependent SOD induction.*** Exposure of non-growing *H. lutea* cells taken from late exponential phase to 70 µg/ml Cu ions resulted, after a brief lag period (5–10 min), in a rapid increase in SOD

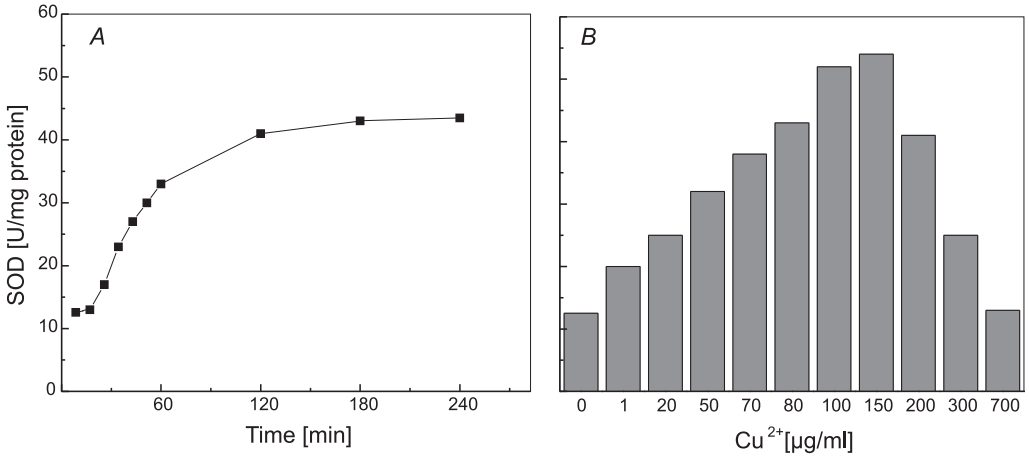

Fig. 1. SOD-induction in non-growing cells of *H. lutea* 103 under copper stress conditions. Time dependence (*A*), dose-dependence (*B*)

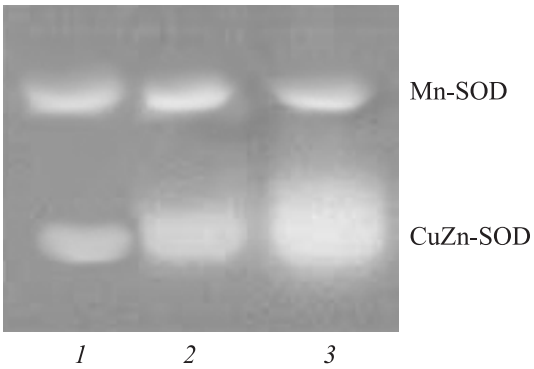

Fig. 2. Effect of Cu^{2+} on the synthesis of SOD isoenzymes in non-growing *H. lutea* cells incubated for 2 h in absence (lane *1*) or presence of 70 (lane *2*) and 150 (lane 3) µg/ml Cu ions

activity up to 2 h after the start of treatment (Fig. 1*A*). While the units of SOD calculating per mg extractable protein increased sharply, it could be suggested that Cu ions caused a disproportionate increase in the rate of biosynthesis of this enzyme, in comparison with other cell proteins. The lag period could be explained by the time needed for accumulation of effective intracellular level of Cu ions and for the heavy metal to accelerate ROS generation. The highest enzyme level was obtained at hour 2, recording 4-fold increase compared to the starting level. At longer incubation periods, the enzyme activity did not change exhibiting a saturation kinetic curve. One possible explanation for the shape of kinetic curve is the rapid Cu absorption by the non-growing cells in the first 15–30 min (data not shown). The subsequent slowdown in Cu consumption leads to a decrease in the rate of the true inducer formation (superoxide radicals) and hence a decrease in SOD activity.

The effect of varying Cu ions concentrations on SOD activity was demonstrated in Fig. 1*B*. The non-growing *H. lutea* cells were incubated for 2 h in medium, containing graded amounts of heavy metal. In the control variant, the enzyme activity was about 12 U/mg. The addition of Cu ions, even at concentration of 1 µg/ml, led to approximately 67% increase in SOD activity. Any further increase in the heavy metal concentration up to 150 µg/ml caused exponential increase in the activity; the maximum enzyme level was 55 U/mg. A significant reduction of SOD activity was reached with higher Cu concentrations, 40, 22 and 11 U/mg at 200, 300 and 700 µg/ml, respectively.

As it is shown in Fig. 2, the PAGE of crude extract of induced and non-induced mycelium reveals two bands, namely Cu/Zn- and Mn-containing SOD. The exposure to 70 and 150 µg/ml Cu ions (lanes *2* and *3*) strikingly enhanced the Cu/Zn-SOD isoenzyme, whereas Mn-SOD level was the same as in the control (lane *1*).

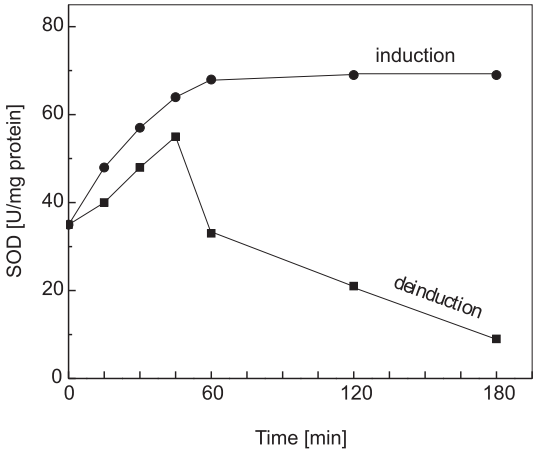

Fig. 3. Deinduction of SOD synthesis by removal of Cu ions

The very rapid increase in enzyme activity as a result of enhanced Cu content suggested an inducible regulation of SOD synthesis in *H. lutea*. This conclusion was confirmed by the experiments for deinduction (Fig. 3). The enzyme was optimally induced by exposing cells to 70 µg/ml Cu ions. After 2 h, the medium containing inducer was removed and replaced with normal growth medium in the absence of the heavy metal. The abrupt removal of Cu ions from previously-induced cells produced a sharp decline in enzyme activity after an initial 40-min lag period.

Effect of glucose on SOD production by H. lutea cells treated with Cu ions. Cu treated cultures were used to investigate SOD activity when cells were subjected to enhanced glucose concentration (Table 1). While the control culture demonstrated 12.5 U/mg SOD activity, addition of 70 µg/ml Cu^{2+} caused approximately 3-fold increase in the enzyme level. The simultaneous presence of glucose resulted in dose-dependent reduction in SOD activity; 23.3, 18.2 and 11.2 vs 36.7 at 0.5, 2, and 6% glucose, respectively. At the same time, cessation of the repression was established after removal of glucose. Moreover, our data indicate that glucose 6-phosphate is not the trigger of catabolite repression in fungal cells. Demonstration effect is typical of the control mechanism catabolite repression.

As it is known, in many cases catabolite repression in microorganisms is correlated with lowered levels of cAMP [8, 12, 13]. Our present results (Table 1) illustrate the reversal effect of cAMP on SOD synthesis by *H. lutea* cells in the presence of glucose. Addition of 25 mM cAMP increased SOD level in cells incubated in glucose-rich medium.

Published data suggest that extent of glucose repression in yeasts is inversely correlated with HK activity and hence with an adequate elevation of intracellular sugar phosphate levels [14]. In this work, we wanted to evaluate whether the

Table 1

Effect of catabolite repression on SOD synthesis in the presence of Cu ions

N	Variants	SOD [U/mg]		HK[a]
		– cAMP	+ cAMP	[U/mg]
1	Control[b]	12.5	11.9	1.42
	Addition of[c]:			
2	70 µg/ml Cu^{2+}	36.7	31.8	0.89
3	+ 0.5% glucose	23.3	41.5	1.26
4	+ 2.0% glucose	18.2	46.8	1.73
5	+ 6.0% glucose	11.1	49.2	2.17
6	removal of 2.0% glucose	29.7	26.1	ND
7	+ 2.0% G6P	31.2	33.8	1.14

[a]HK activity was determined in the variants without cAMP; [b]Control culture was incubated in the medium without both glucose and Cu ions; [c]For all other experiments, fungal cultures were incubated in the presence of 70 µg/ml Cu^{2+} and glucose or glucose-6-phosphate

activity of HK is altered in the fungal cultures treated by heavy metal in the presence and absence of glucose. It is clear from Table 1 that a high SOD activity corresponds to the low HK level. In contrast, glucose-repressed culture exhibits continuing increase of HK activity.

Discussion. Our results showed that Cu ions caused rapid and profound induction of SOD activity in non-growing *H. lutea* cells. Cu/Zn-SOD was mainly responsible for modulating total SOD activity. The presence of inducer seemed to be essential for initiating the process of increase in the enzyme level. Copper is a redox-active transition metal, known to catalyze ROS generation. Our previous results provided evidence that the Cu treatment resulted in increased formation of superoxide anion radicals ($^{\bullet}O_2^-$), the true inducer of SOD synthesis [4]. A similar effect of Cu ions on the intracellular ROS level in various organisms has been reported [15, 16]. Published data suggest that the induction of the sodA gene is a response to an increase in superoxide radical production mediated by different stress factors including heavy metals [17]. The deinduction experiments that demonstrated a lack of enzyme formation after 40 min also demonstrated the induction model of SOD synthesis by Cu ions. According to Blanchard et al. [18], microbial cells posses an intracellular signal response system that rapidly detects changes in superoxide levels and modulates gene expression of SOD.

The present study shows that glucose exerts a strong catabolite repression of SOD synthesis in *H. lutea* cells treated by Cu ions. This is in contrast with our previous report [8], which suggest that SOD synthesis in the fungal strain

H. lutea 110 is not a subject to catabolite repression. But changing the glucose concentration from low to high in presence of Cu ions caused significant decrease in SOD activity in *H. lutea* 103. The enzyme synthesis was restored by the addition of exogenous cAMP to glucose-contained medium. This suggests that cells incubated in glucose medium have lower levels of intracellular cAMP which results in a repression of SOD synthesis. Moreover, when glucose was replaced with G6P, a marked increase in SOD activity was immediately produced in Cu-treated cultures. The influence of G6P as an effector of catabolite repression has also been shown in bacteria and fungi [19]. Graham et al. [20] proposed that the signal giving rise to a change in gene expression of glucose repressed enzymes originates from the intracellular concentration of hexose sugars or the flux of hexose sugars into glycolysis. In addition, HK is involved in down-regulation of enzyme synthesis during catabolite repression [14].

In conclusion, regulatory mechanisms induction and catabolite repression are an important part of the control system of SOD synthesis under Cu-stress conditions. New information could be useful in elaboration of technology for SOD production by the fungus *H. lutea* 103.

REFERENCES

[1] Miller A. M. FEBS Letters, **586**, 2012, No 5, 585–595.

[2] El Shafey H. M., S. A. Bahashwan, A. A. Alghaithy, S. Ghane. In: Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology Microbiology Series N° 2, edited by A. Méndez-Vilas, 2010, 435–443.

[3] Angelova M., P. Dolashka-Angelova, E. Ivanova, J. Serkedjieva, L. Slokoska, S. Pashova, R. Toshkova, S. Vassilev, I. Simeonov, H.-J. Hartmann, S. Stoeva, U. Weser, W. Voelter. Microbiology (UK), **147**, 2001, No 6, 1641–1650.

[4] Krumova E. Z., S. B. Pashova, P. A. Dolashka-Angelova, Tz. Stefanova, M. B. Angelova. Process Biochemistry, **44**, 2009, No 3, 288–295.

[5] Ullmann A. Res. Microbiol., **147**, 1996, 455–458.

[6] Görke B., J. Stülke. Nat. Rev. Microbiol., **6**, 2008, 613–624.

[7] Hristozova Ts., T. Rasheva, T. Nedeva, A. Kujumdzieva. Z. Naturforsch., **57c**, 2002, Nos 3–4, 313–318.

[8] Angelova M., L. Genova, L. Slokoska, S. Pashova. Can. J. Microbiol., **41**, 1995, No 11, 978–983.

[9] Angelova M., L. Genova, S. Pashova, L. Slokoska. Can. J. Microbiol., **42**, 1996, No 12, 1197–1202.

[10] Beauchamp C., I. Fridovich. Anal. Biochem., **44**, 1971, No 1, 276–287.

[11] Bergmeyer H. U., M. Grassl, H.-E. Walter. In: Methods of enzymatic analysis (H.U. Bergmeyer, ed.), Verlag Chemie, Weinheim Deerfield Beach Basel, 3rd Ed., Vol. **2**, 1983, 222–223.

[12] Karimova G., D. Ladant, A. Ullmann. Research in Microbiology, **155**, 2004, No 2, 76–79.
[13] Milanesio P., A. Arce-Rodríguez, A. Muñoz, B. Calles, V. de Lorenzo. Environ Microbiol., **13**, 2011, No 2, 324–339.
[14] Fickers P., J. M. Nicaud, J. Destain, P. Thonart. Curr. Microbiology, **50**, 2005, No 3, 133–137.
[15] Ogita A., K. Fujita, M. Taniguchi, T. Tanaka. Planta Med., **72**, 2006, No 10, 875–880.
[16] Hureau C., P. Faller. Biochimie., **91**, 2009, No 10, 1212–1217.
[17] Sharma P., A. B. Jha, R. Sh. Dubey, M. Pessarakli. Journal of Botany, vol. **2012**, 2012, doi:10.1155/2012/217037 Article ID 217037, 26 pages.
[18] Blanchard J. L., W.-Y. Wholey, E. M. Conlon, P. J. Pomposiello. PLoS ONE `www.plosone.org`, November 2007, Issue 11, e1186.
[19] Fernandez J., J. D. Wright, D. Hartline, C. F. Quispe, N. Madayiputhiya, R. A. Wilson. PLoS Genetics, **8**, May 2012, Issue 5, e1002673.
[20] Graham L. A., K. J. Denby, Ch. J. Leaver. Plant Cell, **6**, 1994, No 5, 761–772.

Stephan Angeloff Institute of Microbiology
Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail: `ekrumova@abv.bg`
`mariange@microbio.bas.bg`