Agrovoc descriptors: candida, yeasts, chromium, stress, oxidation, damage, temperature

Agris category codes: Q01

COBISS code 1.01

Induction of Hsp104 by Cr(VI) in yeast *Candida intermedia*

Polona JAMNIK[1], Peter RASPOR[2]

Received: January 18, 2005; accepted: May 12, 2005.
Delo je prispelo 18. januarja; sprejeto: 12. maja 2005.

ABSTRACT

The synthesis of a polypeptide family named heat shock or stress proteins is one of the most conserved mechanisms of cellular protection in the case of changes in the environment. Among them Hsp104 is important, because it promotes survival of cells under extreme stresses such as high temperatures, severe oxidative damage and high concentrations of ethanol. The aim of our research was to investigate whether Hsp104 is induced in the yeast *Candida intermedia* exposed to Cr(VI). Namely, Cr(VI) belongs to redox active metals, which play an important role in the generation of reactive oxygen species in the cell and therefore they can lead to oxidative damages. Yeast cells were treated with Cr(VI) in the mid-exponential phase and after a defined time of incubation, Hsp104 induction was investigated by using Western blotting. Results showed that cultivation of yeast cells in the presence of 100 μM Cr(VI) caused induction of Hsp104, which is connected to formation of protein aggregates. Hsp104 assists their resolubilization and so contributes to cell survival. Its synthesis was detected 0.5 h after Cr(VI) addition and was later for up to 2 h more apparent. Therefore, we showed that Hsp104 plays an important role in the stress response of yeast *Candida intermedia* to Cr(VI).

Key words: yeasts, *Candida intermedia*, chromium, oxidative stress, stress proteins, Hsp104

IZVLEČEK

INDUKCIJA Hsp104 S Cr(VI) PRI KVASOVKI *Candida intermedia*

Sinteza družine polipeptidov, ki jih imenujemo proteini toplotnega šoka ali stresni proteini, je eden izmed najbolj ohranjenih mehanizmov celične zaščite pri spremembah v okolju. Med njimi je pomemben Hsp104, ki omogoča preživetje celic v ekstremnih stresnih pogojih, kot so visoke temperature, močne oksidativne poškodbe in visoke koncentracije etanola. Namen naše raziskave je bil preučiti, če Cr(VI) povzroči indukcijo sinteze Hsp104 v kvasovki *Candida intermedia*. Cr(VI) namreč sodi med redoks aktivne kovine, ki imajo pomembno vlogo pri nastanku reaktivnih kisikovih zvrsti v celici in lahko vodijo do nastanka oksidativnih poškodb. Celice smo izpostavili Cr(VI) v sredini eksponentne faze rasti in po določenem času inkubacije preverjali indukcijo Hsp104 z analizo proteinov western. Rezultati so pokazali, da

[1] Assist., Ph.D., University of Ljubljana, Biotechnical faculty, Food Science and Technology Department, Chair of Biotechnology, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

[2] Prof., Ph.D., University of Ljubljana, Biotechnical faculty, Food Science and Technology Department, Chair of Biotechnology, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

kultivacija celic v prisotnosti 100 µM koncentracije Cr(VI) povzroči indukcijo Hsp104, kar je povezano s tvorbo proteinskih agregatov. Hsp104 namreč pomaga raztopiti agregate in s tem celicam omogoči preživetje. Sinteza Hsp104 se je pojavila že 0,5 h po dodatku Cr(VI), kasneje tja do dveh ur pa se je še povečala. V raziskavi smo pokazali, da ima Hsp104 pomembno vlogo v stresnem odgovoru kvasovke na Cr(VI).

Ključne besede: kvasovke, *Candida intermedia*, krom, oksidativni stres, stresni proteini, Hsp104

1 INTRODUCTION

Changes in the chemical or physical conditions of the cell's environment that impose a negative effect on growth demand rapid cellular responses in order to survive (Mager and Hohmann, 1997). One of the most conserved mechanisms of cellular protection is the expression of a polypeptide family named heat shock or stress proteins (DeMaio, 1999). Many of stress proteins are present continuously and they appear to be necessary in the critical step of three-dimensional folding of new proteins while other proteins are stress inducible. They assist in the repair of denaturated proteins or promote their degradation after injury (Whitley et al., 1999). Among them Hsp104 has drawn a great attention. It belongs to highly conserved Hsp100 family (Piper, 1997). In yeast *Saccharomyces cerevisiae* Hsp104 promotes survival under extreme stresses such as heat and high concentrations of ethanol (Sanzhes and Lindquist, 1990; Sanzhes et al., 1992). It is responsible for tolerance to arsenite and long-term storage in the cold (Sanzhes et al., 1992). Hsp104 induction was also reported in cells *Saccharomyces cerevisiae* exposed to tetrachloroisophthalonitrile (Fujita et al., 1998) and cadmium (Lee and Ueom, 2001). It appears to enhance survival by promoting the solubilization and reactivation of protein aggregates (Parsell et al., 1994).

Cr(VI) has widespread industrial use. Consequently, it is contaminant in soils, sediments, ground water, drinking water sources and agricultural crops that presents a serious risk for organism life. It is well established as carcinogens and is potently genotoxic in a number of *in vitro* and *in vivo* studies (Losi et al., 1994; Shi et al., 1994). It belongs to redox active metals, which have a major part in the generation of reactive oxygen species in the cell (Mager and Hohmann, 1997).

In our previous study we reported that Cr(VI) causes increased intracellular oxidation (Jamnik and Raspor, 2003). It is also known, that cadmium and tetrachloroisophthalonitrile stress, which are also associated with oxidative stress, induce Hsp104 (Fujita et al., 1998; Lee and Ueom, 2001). According to these observations, we wanted to investigate wheather Hsp104 is induced in the yeast *Candida intermedia* exposed to Cr(VI). We used yeast as a model organism. It is known that yeast is appropriate model for study of oxidative stress on biochemical, molecular-biological and cellular level. Namely, oxidative damages to particular cell components as well as defense systems against oxidative stress are basically similiar in all eukaryotes (Sigler et al., 1999).

2 MATERIALS AND METHODS

Microorganism and cultivation

The yeast *Candida intermedia* - ZIM 156 was obtained from the culture collection of industrial microorganisms (ZIM) at the Biotechnical Faculty, Chair of Biotechnology, Ljubljana. Cells were cultivated in a CHEMAP bioreactor (3.5 l, 200 rpm, air supply: 2.5 l/min, T = 28 °C) in a chemical defined medium with the following composition: 10.0 g/l glucose (Kemika), 4.5 g/l ammonium sulfate (Merck), 0.100 g/l KCl (Kemika), 0.100 g/l NaCl (Merck), 0.500 g/l $MgSO_4$ x 7 H_2O (Kemika), 0.100 g/l $CaCl_2$ x 2 H_2O (Merck), 1.0 g/l KH_2PO_4 (Merck), 0.005 g/l boric acid (Merck), 0.002 g/l $CuSO_4$ x 5 H_2O (Merck), 0.002 g/l $FeCl_3$ x 6 H_2O (Kemika), 0.010 g/l $ZnSO_4$ x 7 H_2O (Kemika), 0.002 g/l Na-molybdate x 2 H_2O (Kemika), 0.001 g/l KI (Kemika), 0.004 g/l Ca-D-pantothenate (Fluka), 0.020 g/l myo-inositol (Sigma), 0.004 g/l thiamin·HCl (Aldrich), 0.004 g/l nicotinic acid (Sigma), 0.004 g/l pyridoxin·HCl (Sigma), 0.002 g/l riboflavin (Sigma), 0.05 x 10^{-3} g/l folic acid (Sigma), 0.05 x 10^{-3} g/l biotin (Fluka). The initial pH of the medium was 4.2.

Stress induction

In the mid-exponential phase, corresponding to an optical density (OD) of 1.0 (at 650 nm), $K_2Cr_2O_7$ (Sigma) was added into the medium to give Cr(VI) concentration of 50, 100, 300 and 500 μM. Samples were taken at 0, 1, 2 h after the addition of $K_2Cr_2O_7$ and were used to asses cell viability and to detect Hsp104.

Cell viability assay

Cell viability was assessed by counting the number of colony forming units (CFU) (Raspor et al., 1999). Both the control cells and cells exposed to Cr(VI) were after dilution seeded to YEPD agar with the following composition: 10.0 g/L glucose (Kemika), 5.0 g/L yeast extract (Biolife), 5.0 g/L pepton (Oxoid), 20.0 g/l agar (Biolife). The results were expressed as % CFU of treated yeast cells compared with the untreated control cells.

Western blot analysis

Samples were centrifuged at 4000 rpm for 5 min and washed twice with distilled water. 0.5 g cells (wet weight) was suspended in 2.5 mL 50 mM potassium phosphate buffer pH = 7.0 containing protease inhibitor cocktail (Roche) - 1 tablet per 10 mL of buffer. The cells were disrupted by vortexing with acid washed glass beads (Sigma, diameter: 425-600 microns) four times, 1 min each with 1-min intervals for cooling the mixure on ice. The cell homogenate was centrifuged at 20 000 x g for 20 min at 4 °C. The protein content in the cell extracts was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard. Proteins (6 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) on 10 % (w/v) running gel with 4 % (w/v) focusing gel. The protein samples were subsequently blotted to a PVDF membrane using semidry transfer blotter and immunoassayed using the anti-Hsp104 polyclonal antibody (Affinity Bioreagents) at a dilution of 1:1000 and a secondary antibody (Sigma, anti-rabbit IgG conjugated with alkaline phosphatase) at a dilution of 1:3000. Prestained MW markers (Biorad) were used (myosin, 203.0 kDa; β-galactosidase, 118.0; bovine serum albumin, 82.0; ovalbumin, 50.4 kDa; carbonic anhydrase, 33.4; soybean trypsin inhibitor, 26.7; lysozyme, 19.6 kDa, aprotinin, 7.4 kDa).

Statistical analysis

Cultivations of the yeast *Candida intermedia* - ZIM 156 in bioreactor were repeated three times. The results for cell viability are presented as mean ± S.D. of three independent measurements. In addition, Tukey HSD test was employed for further determination of the significance of differences between control and stressed cells. In the case of Hsp104 detection western blot analysis was repeated twice.

3 RESULTS AND DISCUSSION

The survival of living cells depends on their ability to sense changes in the environment and to respond rapidly and appropriately to the new situation (Mager and Hohmann, 1997). In our previous study (Jamnik and Raspor, 2003) we treated yeast cells with different Cr(VI) concentrations and demonstrated that the most significant changes after Cr(VI) addition were obtained specifically at 100 μM Cr(VI), where increased oxygen consumption was observed. Herein we show decreased cell viability in the presence of increasing amounts of Cr(VI). Incubation of the yeast cells with 100, 300 and 500 μM Cr(VI) for 1 h led to growth inhibition. The relative CFU were 62.5, 16.5 and 2.0 %, respectively. In contrast at 50 μM Cr(VI) relative CFU after 2-h exposure was still 83.5 % (Table 1). Therefore, simultaneous increased metabolic rate and growth inhibition indicate stress response induction. At higher Cr(VI) concentrations (300 and 500 μM) cell viability was too low to study stress response.

Table 1: Effect of different Cr(VI) concentrations and duration of exposure on yeast viability

Time of exposure to Cr(VI) (h)	Relative CFU[a] (%) at different Cr(VI) concentration				
	control	50 μM	100 μM	300 μM	500 μM
0	100.0[b] ± 10.0	100.0[b] ± 10.0	100.0[b] ± 10.0	100.0[b] ± 10.0	100.0[b] ± 10.0
1	100.0[b] ± 9.0	84.7[b] ± 9.0	62.5[c] ± 8.0	16.5[d] ± 2.0	2.0[e] ± 0.2
2	100.0[b] ± 11.0	83.5[b] ± 8.0	51.1[c] ± 5.0	ND[f]	ND[f]

[a] Results are expressed as relative values with respect to control assumed as 100% and they are mean values ± S.D., n = 3. Values for cell viability for control at 0, 1, 2 hr are 1.0 ± 0, 2.48 ± 0.68, 2.31 ± 0.57, respectively.

[b,c,d,e] Mean values marked with these different letters at the same time are statistical different between each other at $p < 0.05$.

[f] Not determined.

Stress responses are defined as molecular mechanisms, which induced in the cell after exposure to adverse conditions (Mager and Hohmann, 1997). They lead to accumulation of a number of proteins and other molecules involved in cellular survival and restoration of function (Attfield, 1997). Stress proteins have been recognized as being one of the primary defense mechanisms that are activated by the occurrence of denaturated proteins in the cell (Bierkens, 2000). We paid attention to Hsp104, which is a member of highly conserved heat shock protein family Hsp100 (Piper, 1997). Yeast Hsp104 responds to many forms of stress (Sanzhes and Lindquist, 1990; Sanzhes et al., 1992, Fujita et al., 1998; Lee and Ueom, 2001). Cultivation of yeast cells in the presence of 100 μM Cr(VI) caused induction of Hsp104. Its synthesis was detected 0.5 hr after Cr(VI) addition and was later for up to 2 h more apparent (Figure 1).

We demonstrated that Cr(VI) increases oxidant level in the cell measured by oxidation of 2`,7`-dichlorofluorescin, specifically in the cells exposed to 100 μM Cr(VI) (Jamnik and Raspor, 2003). Namely, Cr(VI) belongs to redox active metals, which have an important role in the generation of reactive oxygen species in the cell (Mager and Hohmann, 1997). Therefore, at 100 μM Cr(VI) oxidative damages to proteins might be quite intensive. Furthermore, the rate of protein denaturation might exceed the ability of other protective systems to prevent aggregation. Thus, Hsp104

induction for disassembling aggregates becomes critical. Simillary, Lee and Ueom (2001) suggested that Hsp104 induction by cadmium is due to the oxidative stress mainly consisting in formation of reactive oxygen species and more damaged proteins.

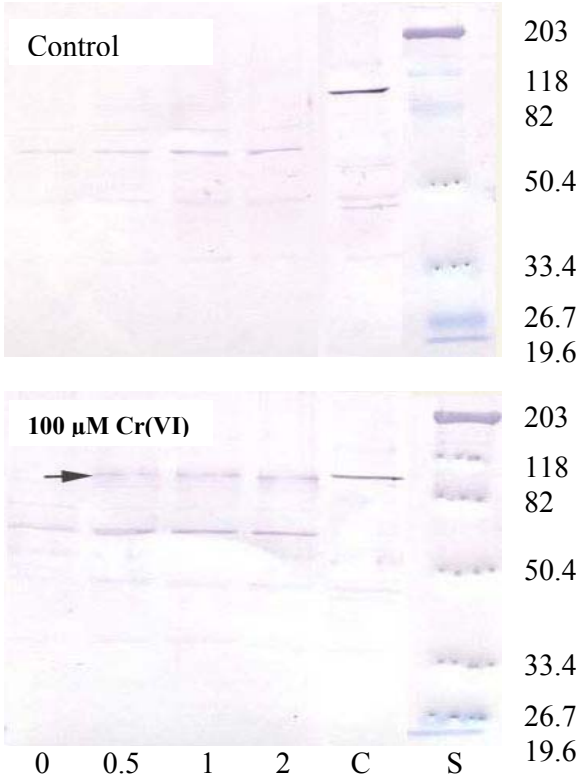

Figure 1: Detection of Hsp104 by western blot analysis in the yeast *Candida intermedia*. The yeast cells were treated with Cr(VI) in the mid-exponential phase (**0**) and incubated for 0.5 (**0.5**), 1 (**1**) and 2 h (**2**). **C**, proteins from cell extract from *Saccharomyces cerevisiae* grown at 25 ºC and heat shocked at 37 ºC for 30 min; **S**, MW markers (kDa). Hsp104 was detected with anti-Hsp104 polyclonal antibody at a dilution of 1:1000. Arrow indicated Hsp 104 induction.

4 CONCLUSIONS

Hsp104 may play an important role in stress response of yeast *Candida intermedia* to Cr(VI). It seems that Hsp104 could be a direct marker of cell exposure to heavy metals, such as cadmium and chromium(VI). Namely, stress proteins should have three characteristics to use them as biomarkers of pollution: (1) they are part of stress response, (2) their synthesis is likely to be induced by a large number of chemicals and (3) they are highly conserved in all organisms (Bierkens, 2000).

5 REFERENCES

Attfield, P.V. 1997. Stress tolerance: The key to effective strains of industrial baker´yeast. Nat. Biotechnol., 15: 1451-1357.

Bierkens, J.G.E.A. 2000. Applications and pitfalls of stress-proteins in biomonitoring. Toxicology, 153: 61-72.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72 :248-254

DeMaio, A. 1999. Heat shock proteins: facts, thoughts, and dreams. Shock, 11: 1-12.

Fujita K., Kawai R., Iwahashi H., Komatsu, Y. 1998. Hsp104 responds to heat and oxidative stress with diferrent intracellular localization in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun., 248: 542-547.

Jamnik, P., Raspor, P. 2003. Stress response of yeast *Candida intermedia* to Cr(VI). J. Biochem. Mol. Toxicol., 17: 316-324.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 22: 680-685.

Lee, K., Ueom, J. 2001. Protection of metal stress in *Saccharomyces cerevisiae*: Cadmium tolerance requires the presence of two ATP-binding domains of hsp 104 protein. Bull. Korean Chem. Soc., 22: 514-518.

Losi, M.E., Amrhein, C., Frankenberger, Jr.W.T. 1994. Environmental biochemistry of chromium. Rev. Environ. Contam. Toxicol., 136: 91-115.

Mager, W.H., Hohmann, S. 1997. Stress response mechanisms in the yeast *Saccharomyces cerevisiae*. In: Yeast stress responses. Hohmann, S., Mager, W.H. (eds.). New York, Springer-Verlag: 1-5.

Parsell, D.A., Kowal, A.S., Singer, M.A., Lidquist, S. 1994. Protein disagregation mediated by heat-shock protein Hsp104. Nature 372: 475-478.

Piper, P. 1997. The yeast heat shock response. In: Yeast stress responses. Hohmann, S., Mager, W.H. (eds.). New York, Springer-Verlag: 75-99.

Raspor, P., Batič, M., Jamnik, P. 1999. Measurement of yeast viability/mortality in the presence of chromium(VI). Food Technol. Biotechnol., 37: 81-86.

Sanzhes, Y., Lindquist, S.L. 1990. HSP104 required for induced thermotolerance. Science, 248: 112-115.

Sanzhes, Y., Taulien, J., Borkovich, K.A., Lindquist, S. 1992. Hsp104 is required for tolerance to many forms of stress. EMBO J., 11: 2357-2364.

Shi, X., Dong, Z., Dalal, N.S., Gannet, P.M. 1994. Chromate-mediated free radical generation from cystein, penicillamine, hydrogen peroxide, and lipid hydroperoxides. Biochim. Biophys. Acta, 1226: 65-72.

Sigler, K., Chaloupka, J., Brozmanova, J., Stadler, N., Höfer, M. 1999. Oxidative stress in microrganisms - I. Microbial vs. higher cells - damage and defenses in relation to cell aging and death. Folia Microbiol., 44: 587-624.

Whitley, D., Goldberg, S.P., Jordan, W. 1999. Heat shock proteins: A review of the molecular chaperones. J. Vasc. Surg., 29: 748-751.