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THE ROLE OF GLUTATHIONE IN DETOXIFICATION OF CHROMATE BY *HANSENULA (OGATAEA) POLYMORPHA* YEAST

Glutathione (GSH, γ -glutamyl-cysteinylglycine) is the most abundant non-protein thiol present at relatively high concentration in most living cells. It is the main reservoir of non-protein sulfur which protects cells from metabolic, oxidative and environmental stresses [1–3]. Due to the unique reductive and nucleophilic properties, GSH plays pivotal role in protecting cells against reactive oxygen species (ROS), xenobiotics and heavy metals, as well as in detoxification of endogenous toxic metabolites. GSH is the main cellular antioxidant, which can directly react with the reactive oxygen species and take part in detoxification of lipid peroxidation products, like malondialdehyde and 4-hydroxy-2-nonenal. It is known that GSH is involved in homeostasis of different metals in many organisms [3–5]. Microbial detoxification of metal ions is carried out by several mechanisms, which include the regulation of uptake, transformation to less toxic forms and intracellular immobilization [6]. Metal ions that are assimilated by cells generate ROS directly (redox-active metals Cu, Fe, Cr, V) or indirectly by the substitution of redox-active metals in their binding sites (Cd, Hg, Ni, Pb). GSH can directly bind some metal ions in six potential coordination sites for their binding. GSH complexes with metals may be generated spontaneously. Such complexes may have various metabolic functions, in particular they can help in metal transport through the cell membrane, be a source of cysteine and as cofactors in redox reactions.

The involvement of GSH in metabolism of cadmium, copper, iron is well studied [3–5]. In many organisms GSH is involved in reduction of chromate, which is mutagenic and carcinogenic. Cr^{6+} is reduced thus to the less toxic Cr^{3+} . This process is accompanied by the generation of ROS, that damage cellular phospholipids, proteins and

DNA. Although Cr^{6+} can be reduced non-enzymatically, GSH and GSH-dependent enzymes play an important role in the reduction of intracellular chromate. Also essential role in the detoxification of chromate in yeasts belongs to extracellular reduction of Cr^{6+} to Cr^{3+} , which forms kinetically inactive complexes with substances of unknown nature [7]. The role of GSH in chromate detoxification in yeasts is unclear. In the fission yeast *Schizosaccharomyces pombe* the decreased intracellular GSH level leads to increased sensitivity to chromate, and, according to the authors, this suggests that GSH may effectively protect cells against chromate toxicity by ROS scavenging [8]. Mutant strain with decreased GSH reductase activity was more resistant to chromate due to reduced generation of hydroxyl radicals by the action of chromate. The introduction of the gene encoding GSH reductase into genome of this mutant caused the loss of resistance to chromate, demonstrating the importance of the GSH reductase-NADPH system in chromate reduction. On the other hand, in the yeast *Saccharomyces cerevisiae* GSH is not involved in cell tolerance to chromate and other metals. However, reduction of GSH pool in cells led to decreased chromate adsorption [6].

To evaluate the role of GSH in chromate detoxification in yeasts we used recombinant strains of *Hansenula (Ogataea) polymorpha* with overexpressed *GSH2* (coding the first enzyme of GSH biosynthesis, gamma-glutamyl cysteine synthetase) and *MET4* (the central regulatory gene of sulfur metabolism) genes, as well as mutant with deleted *GSH2* gene.

The paper presents data showing that high level of GSH in cells of *H. polymorpha* slightly changed the sensitivity/tolerance to chromate, but increased the rate of reduction of Cr^{6+} and reduced

the amount of chromium accumulated in the cells. Deletion of *GSH2* gene, encoding the enzyme of the first reaction of GSH synthesis, increased sensitivity to chromate.

Materials and methods

Wild-type strain of *Hansenula (Ogataea) polymorpha* DL-1 and recombinant strains overexpressing *MET4* and *GSH2* genes, as well as *H. polymorpha* strain with deletion of *GSH2* gene from the collections of Institute of Cell Biology NAS of Ukraine, Lviv, Ukraine were used in this work. List of used strains is shown in Table 1.

Yeasts were grown on YNB medium (6.7 g/l YNB without amino acids, 20 g/l glucose) on a rotary shaker (200 rpm) at 37°C. Analysis of sensitivity of yeast cells to Cr⁶⁺ was performed after 3 days of incubation at 37°C on YNB medium with the addition of 0.1–1.5 mM Cr⁶⁺. In the case of *Δgsh2* mutant defective in GSH synthesis, the medium additionally contained 0.1 mM GSH. Cell

biomass was determined turbidimetrically on a spectrophotometer Helios Gamma UVG-1000105 ($\lambda = 590$ nm, cuvette 10 mm). Total GSH concentration (GSH + GSSG) was measured in culture medium and in cell-free extracts by means of the standard recycling assay based on DTNB (5,5'-dithiobis-2-nitrobenzoic acid) reduction in the presence of GSH reductase and NADPH [8]. The rate of the chromate reduction by yeasts was determined by measuring the chromate content in the culture medium [7]. Cell-free extracts were prepared by disintegration of yeast cells with glass beads and 0.1 M Tris-HCl buffer (pH 7.5) at 4°C for 20 min. Protein content in cell-free extracts was determined by the Lowry method with bovine serum albumin used as a standard [12]. Total chromium content in the culture medium and in mineralized cells was determined photometrically using Chromazurol S in the presence of anionic surfactant – sodium dodecyl sulfate (SDS) according to the method [7]. All experiments were repeated three times.

Table 1. Strains of *Hansenula polymorpha* used in this work

Name	Genotype	Bibliography
Wild type strain DL-1	<i>leu2</i> ⁻	
13	<i>MET4</i> [↑] <i>ura</i> ⁻	[10]
25k	<i>GSH2</i> [↑]	[9]
25a	<i>GSH2</i> [↑] - <i>MET4</i> [↑]	[10]
57	<i>Δgsh2</i>	[11]

Results and discussion

Since it is known that extracellular reduction of Cr⁶⁺ to Cr³⁺ plays an important role in the detoxification of chromate in yeasts [6] and GSH can form complexes with this cation, we studied influence of exogenous GSH (0.02 mM) on the sensitivity of the wild-type strain of *H. polymorpha* yeast to Cr⁶⁺. Yeast was incubated during 5 days in YNB medium with different concentrations of chromate (0.1–0.5 mM). Slight increase of yeast cells resistance to Cr⁶⁺ was detected only at very low chromate concentrations (0.1 mM) (data not shown). At higher chromate concentrations there were no difference in biomass accumulation depending on Cr⁶⁺ presence in the growth medium. These data suggest that exogenous GSH, apparently is not involved in the formation of extracellular Cr³⁺-chelate complexes like it was found in several species of yeast [13].

In order to clarify the role of intracellular GSH in chromate detoxification, *H. polymorpha*

strains with overexpressed *GSH2* (№ 25k) or *MET4* (№ 13) genes, and with co-overexpressed *GSH2* and *MET4* (№ 25 a) were used. GSH level in recombinant strains was several times higher than in the wild-type strain (Fig. 1). The highest GSH accumulation in yeast cells was observed on the second day of incubation. Culture medium of all studied strains contained only trace amounts of GSH. In the presence of chromate (0.3 mM) in the culture medium, the intracellular GSH content in recombinant strains overexpressing *GSH2* and *MET4* genes decreased on the first day of incubation to the level of 100–130 nmoles/mg protein, however it was still higher than in the wild-type strain (80–88 nmoles/mg protein).

The effect of chromate on the growth of *H. polymorpha* with different GSH amount in cells is shown on Fig. 2. Yeasts were grown on YNB medium supplemented with Cr⁶⁺ (0.1–1.5 mM).

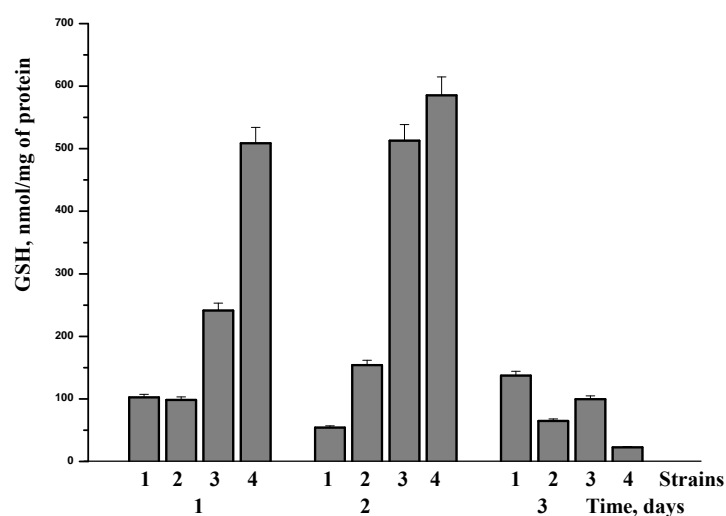


Fig. 1. GSH concentration in the cells of recombinant *H. polymorpha* strains as compared to the wild-type strain during three days incubation in YNB medium. 1 – wild-type strain, 2 – 13, 3 – 25k, 4 – 25a.

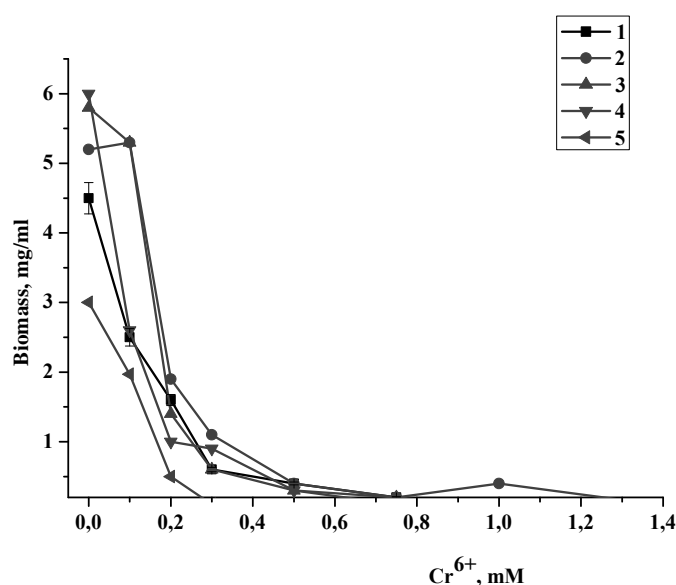


Fig. 2. Influence of chromate on the growth of *H. polymorpha* strains with different GSH amount in cells. 1 – wild-type strain, 2 – 13, 3 – 25k, 4 – 25a, 5 – *Dgsh2*.

It was found that strains overexpressing *GSH2* and *MET4* genes were more resistant to chromate in comparison with the wild-type strain only at its low concentration (0.1 mM). Mutant *Dgsh2* with impaired synthesis of GSH, as might be expected, was more sensitive to the action of chromate (Fig. 2).

The ability of *H. polymorpha* yeast strains with higher pool of intracellular GSH to chromate reduction was assessed by decreasing level of Cr⁶⁺

in culture medium during cells growth with 0.3 mM chromate (Fig. 3). The lower content of Cr⁶⁺ in culture medium was observed for all yeast strains with overexpressed *GSH2* and *MET4* genes as compared to the wild-type strain, indicating the more active reduction of this oxyanion.

Postulated protective effect of GSH against toxicity of metals is based, primarily, on the change in their concentration in the cell. To find out the correlation between GSH content and chromate

accumulation *H. polymorpha* strains with overexpressed *GSH2* and *MET4* genes were grown in the YNB medium with different chromate concentrations (0.3 mM and 0.5 mM). Chromium

content in yeast cells with higher level of GSH was much lower in comparison to the wild-type strain (Fig. 4)

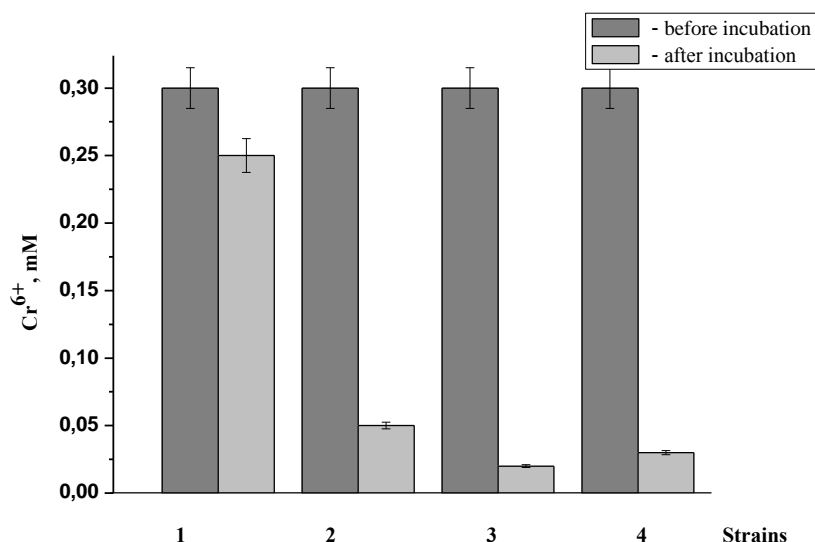


Fig. 3. Cr⁶⁺ content in the culture medium of *H. polymorpha* strains with overexpressed *GSH2* and *MET4* genes after 3-day incubation in medium with 0.3 mM of chromate. 1 – wild-type strain, 2 – 13, 3 – 25k, 4 – 25a.

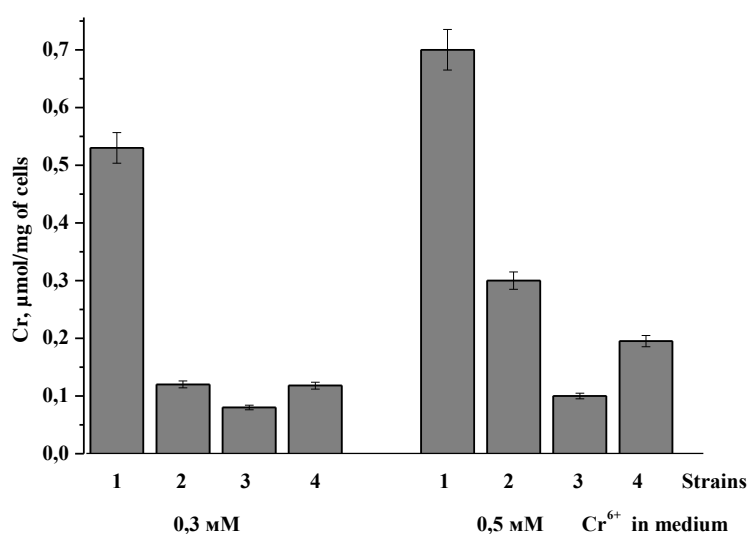


Fig. 4. Chromium content in *H. polymorpha* strains with overexpressed *GSH2* and *MET4* genes after 3-day incubation in the media with different chromate concentrations.

Conclusions

Elevated intracellular GSH level in yeast *H. polymorpha*, as a result of overexpression of *GSH2* and *MET4* genes, slightly changed the sensitivity/tolerance to chromate, but increased the rate of reduction of Cr⁶⁺ and reduced the amount of

chromium accumulated in the yeast cells. Deletion of *GSH2* gene led to the increased sensitivity of *H. polymorpha* to chromate.

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Aim. Chromate is very toxic, displays mutagenic and carcinogenic activity and its pollution poses a serious environment problem. The development of efficient methods for detoxification of this oxyanion is of great importance. For microbial cells, it is known that chromate transported in cells can be reduced to less toxic Cr³⁺. The most powerful chromate reductants could be glutathione (GSH) or cysteine. The role of GSH in detoxification of chromate by the yeast *Hansenula polymorpha* was evaluated. **Methods.** For this purpose recombinant strains with overexpressed *GSH2* and *MET4* genes and mutant defective in GSH biosynthesis were used. Profiles of GSH and chromium contents as well as rate of reduction of chromate were studied in relation to chromate resistance/sensitivity of the wild- type and mutant yeast strains. **Results.** High level of GSH in recombinant strains of *H. polymorpha* slightly changed the sensitivity/tolerance to chromate, but increased the rate of reduction of Cr⁶⁺ and reduces the amount of chromium accumulated in the cells. Deletion of *GSH2* gene, encoding the enzyme of the first reaction of GSH synthesis, leads to increased sensitivity to the action of chromate. **Conclusions.** GSH can be considered as an important part of chromate detoxification system in *H. polymorpha*.

Keywords: glutathione, chromium, yeast, chromate reduction, *Hansenula (Ogataea) polymorpha*.