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## The inhibition kinetics of yeast glutathione reductase by some metal ions

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### Abstract

Glutathione reductase (GR, type IV, Baker's yeast, E.C 1.6.4.2) is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). In this study some metal ions have been tested on GR; lithium, manganese, molybdate, aluminium, barium, zinc, calcium, cadmium and nickel. Cadmium, nickel and calcium showed a good to moderate inhibitory effect on yeast GR. GR is inhibited non-competitively by  $\text{Zn}^{2+}$  (up to 2 mM) and activated above this concentration.  $\text{Ca}^{2+}$  inhibition was non-competitive with respect to GSSG and uncompetitive with respect to NADPH. Nickel inhibition was competitive with respect to GSSG and uncompetitive with respect to NADPH. The inhibition constants for these metals on GR were determined. The chelating agent EDTA recovered 90% of the GR activity inhibited by these metals.

**Keywords:** *Saccharomyces cerevisia*, glutathione reductase, nickel, zinc, calcium, inhibition, kinetics, EDTA, yeast

### Introduction

Glutathione reductase (E.C 1.6.4.2) is a pivotal enzyme of the antioxidant system in the cells [1] which utilize molecular oxygen and generate highly reactive oxygen-derived free radicals. Endogenous cellular oxidants inactivate oxidant free radicals and protect aerobic cells from oxidant injury. Glutathione reductase (GR) and superoxide dismutase are key components of this antioxidant defence and inhibition of these antioxidant components would be expected to result in cell injury [2]. GR has a central role in glutathione (GSH) metabolism and as such is a potential target for chemotherapy [3]. Metal ions have diverse functions on organisms and a number of metals which act as a prosthetic group for enzymes and are activators and some are inhibitors.  $\text{Zn}^{2+}$  is a trace element known to be an essential nutrient for life and functions as a cofactor for numerous enzymes [4]. However, excess in  $\text{Zn}^{2+}$  the body interacts with free thiol groups on macromolecules, so blocking the active sites of enzymes, co-enzymes and membrane

receptors [5]. GR is an important factor in cellular zinc susceptibility.  $\text{Zn}^{2+}$  toxicity has been linked to decreased reduced GSH and increased GSSG contents, which might be caused by GR inhibition by  $\text{Zn}^{2+}$  [6]. 6-Phosphogluconate dehydrogenase, like many fungal dehydrogenases, was inhibited by  $\text{Zn}^{2+}$  [7].  $\text{Ca}^{2+}$  is the most abundant mineral in the body and regulates many cellular process and has important structural roles in living organisms [8]. However, overdoses of certain vitamins and minerals can produce toxic effects as they are inhibitors of some enzymes [9].  $\text{Ni}^{2+}$  and  $\text{Cd}^{2+}$  are carcinogenic to humans and/or animals, but the underlying mechanisms are poorly understood [10].  $\text{Ni}^{2+}$  is well-known inhibitor of Fe(II)/alpha-ketoglutarate (alphaKG)-dependent hydroxylases [11], yeast hexokinase [12], horseradish peroxidase [13] and microsomal epoxide hydrolase [14]. Inhibition of some enzymes by lithium, such as inositol monophosphatase and glycogen synthase kinase-3, probably results in its mood-stabilizing effects [15].  $\text{Mn}^{2+}$  is an important trace element and may be essential for some

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metalloenzymes [16]. Anaemia, one consequence of  $\text{Al}^{3+}$  toxicity, may be due to inhibition of delta-ALA dehydratase which occurs in the heme biosynthetic pathway [17]. The ability of  $\text{Al}^{3+}$  to inhibit ( $\text{Na}^+/\text{K}^+$ ) ATPase activity has been observed by several investigators [18].

The present study aims at investigating the influence of  $\text{Li}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Ni}^{2+}$  on the activity of *Saccharomyces cerevisiae* GR as well as the kinetic behaviour and type of inhibition of GR inhibition observed.

## Materials and methods

### Materials

Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), oxidized glutathione (GSSG), Baker's yeast glutathione reductase (specific activity 1.25 U/mg), barium acetate and calcium chloride were obtained from Sigma Chemical Co., MO, USA. Nickel sulphate was obtained from NEEDHAM project. Zinc sulphate was obtained as ANALAR (Hopkin & Williams Ltd). EDTA was obtained from SERVA Feinbiochemica GmbH & Co. Aluminium chloride, Lithium carbonate, were from Fischer; Manganese ( $\text{MnSO}_4$ ) from Merck and Molybdenum ( $\text{Na}_2\text{MoO}_4$ ) from BDH Chemicals Ltd.

### Assay of glutathione reductase activity

Glutathione reductase activity was determined according to the modified Stall method [19]. The incubation mixture contained 100 mM sodium phosphate buffer, pH 7.4, 1 mM GSSG, 200 mM NADPH and Baker's yeast glutathione reductase. Decrease in the absorbance of NADPH at 340 nm was monitored spectrophotometrically, at 37°C. Assays were carried out in duplicate and the activities were followed for 40 s. The reaction was linear during this time period.

A unit of activity (U) was defined as the amount of enzyme that catalyses the oxidation of 1  $\mu\text{mole}$  of NADPH in 1 min under these conditions. Specific activity is defined as units per mg of protein.

### Inhibition studies

Activities were measured after adding different concentrations  $\text{Li}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Ni}^{2+}$  to the assay mixture given above for glutathione reductase measurement. Assays of GR in the presence of heavy metal ions were performed without enzyme-inhibitor preincubation in that the reactions were initiated by adding enzyme to the substrate-inhibitor mixture.

### Recovery of glutathione reductase activity

EDTA (0–12 mM) concentrations were added to the above assay mixtures containing 2.5 mM  $\text{CaCl}_2$ , 2 mM  $\text{ZnSO}_4$ , and 1 mM  $\text{NiSO}_4$ , respectively, and the initial velocities were determined.

### Statistical analysis of kinetic data

The data were analyzed and the kinetic constants were calculated using the following equations [20] by means of a nonlinear curve-fitting program of Statistica.

Michaelis – Menten equation :

$$V = V_m[S]/K_m + [S] \quad (1)$$

Non-competitive inhibition :

$$V = (V_m S / (1 + I/K_i)) / (K_m + S) \quad (2)$$

Uncompetitive inhibition :

$$V = V_m S / (1 + I/K_i) / (K_m / (1 + I/K_i) + S) \quad (3)$$

Pure competitive inhibition :

$$V = V_m S / (K_s(1 + I/K_i) + S) \quad (4)$$

where  $V$  = Reaction rate,  $[S]$  = Substrate concentration,  $V_m$  = Maximum rate, and  $K_m$  = Michaelis-Menten constant (substrate concentration at half the maximal velocity ( $V_m$ )).

## Results

In this study we have investigated the effects of several metal ions on Baker's yeast glutathione reductase. The values of the kinetic parameters of GR in a non-inhibited reaction were determined as  $K_{m\text{GSSG}} 90 \pm 12 \mu\text{M}$ ,  $K_{m\text{NADPH}} 30 \pm 4 \mu\text{M}$ . In other kinetics studies, yeast GR  $K_{m\text{GSSG}}$  and  $K_{m\text{NADPH}}$  values were found to be 55  $\mu\text{M}$  and 3.8  $\mu\text{M}$  respectively [21]. The *E.coli* GR values were  $K_{m\text{GSSG}} 97 \pm 12 \mu\text{M}$  and  $K_{m\text{NADPH}} 22 \pm 2 \mu\text{M}$  [22], Cyanobacterium *Anabaena* sp. Strain 7119 GR values were  $K_{m\text{GSSG}} 210 \mu\text{M}$ ,  $K_{m\text{NADPH}} 9.4 \mu\text{M}$  [23] and rat liver values were  $K_{m\text{GSSG}} 56.7 \pm 0.4 \mu\text{M}$ ,  $K_{m\text{NADPH}} 7.9 \pm 0.6 \mu\text{M}$  [24].

The inhibition kinetics of *Saccharomyces cerevisiae* GR was studied without enzyme-inhibitor preincubation.

We found that some metals  $\text{Li}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$  had no effect the GR activity but  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Ca}^{2+}$  inhibited the enzyme in a concentration-dependent manner with  $\text{IC}_{50}$  values of 0.025, 0.8 and 5 mM respectively. Whereas zinc was both an inhibitor and activator of the enzyme. We established

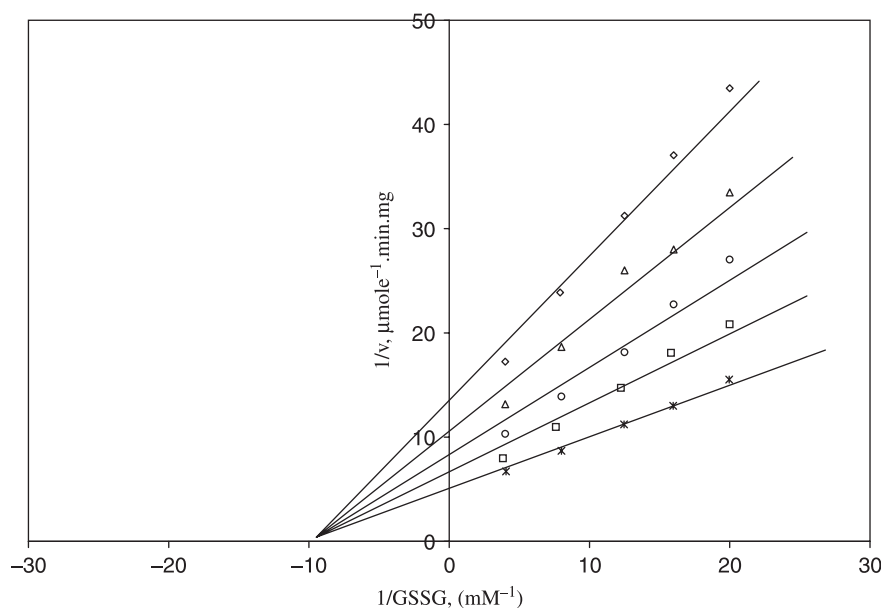


Figure 1. Lineweaver-Burk double reciprocal plot of initial velocity against GSSG as varied substrate and  $\text{ZnSO}_4$  (0.05–1 mM) as inhibitor at a fixed NADPH (0.1 mM) concentration. \*0.1 mM NADPH (constant);  $\square$  0.05 mM  $\text{ZnSO}_4$ ;  $\circ$  0.1 mM  $\text{ZnSO}_4$ ;  $\Delta$  0.5 mM  $\text{ZnSO}_4$ ;  $\diamond$  1 mM  $\text{ZnSO}_4$ .

that yeast GR is inhibited by  $\text{Zn}^{2+}$  (0.1–2 mM) and activated above this concentration (2.5–5 mM). The  $\text{IC}_{50}$  value of  $\text{Zn}^{2+}$  could not be determined because at 2 mM  $\text{Zn}^{2+}$  concentration the enzyme lost its activity by 36% and above this concentration GR was activated in a concentration-dependent manner (i.e. 3.5 fold at 5 mM  $\text{Zn}^{2+}$  concentration). Kinetic characterization of the inhibition effects of  $\text{Zn}^{2+}$  on GR from *Saccharomyces cerevisiae* have been investigated and no inhibitory effect was found with  $\text{Zn}^{2+}$  (30–90  $\mu\text{M}$  concentration) on this enzyme [25]. The obtained  $\text{IC}_{50}$  values of calcium are 5 mM,

and nickel 0.8 mM and Yeast GR is inhibited by much lower concentrations of  $\text{Cd}^{2+}$  ion than the other metals ( $\text{IC}_{50}$  of  $\text{Cd}^{2+}$  is 0.025 mM).  $\text{Cd}^{2+}$  is a very potent enzyme inhibitor; it inhibits many enzymes such as in our previous study we have reported that  $\text{Cd}^{2+}$  is also a potent inhibitor of glucose-6-phosphate dehydrogenase (G-6-PD) from lamb kidney cortex [26].

The kinetic characterization of the inhibitory effects of these metals on GR was also investigated. Kinetic characterization of the inhibition of  $\text{ZnSO}_4$  on GR is shown in Figures 1 and 2; the inhibition

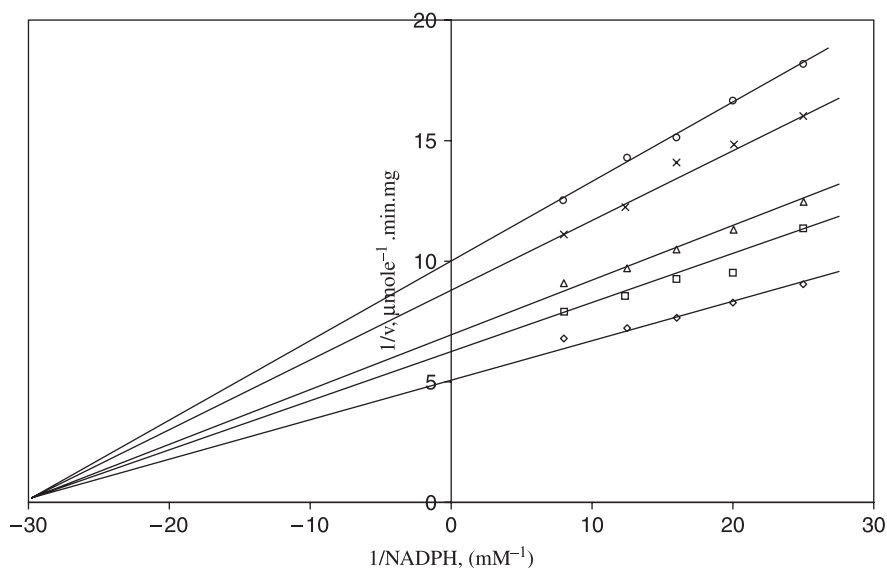


Figure 2. Lineweaver-Burk double reciprocal plot of initial velocity against NADPH as varied substrate and  $\text{ZnSO}_4$  (0.05–1 mM) as inhibitor at different fixed GSSG (0.7 mM) concentrations.  $\diamond$  0.7 mM GSSG (constant);  $\square$  0.05 mM  $\text{ZnSO}_4$ ;  $\Delta$  0.1 mM  $\text{ZnSO}_4$ ; \*0.5 mM  $\text{ZnSO}_4$ ;  $\circ$  1 mM  $\text{ZnSO}_4$ .

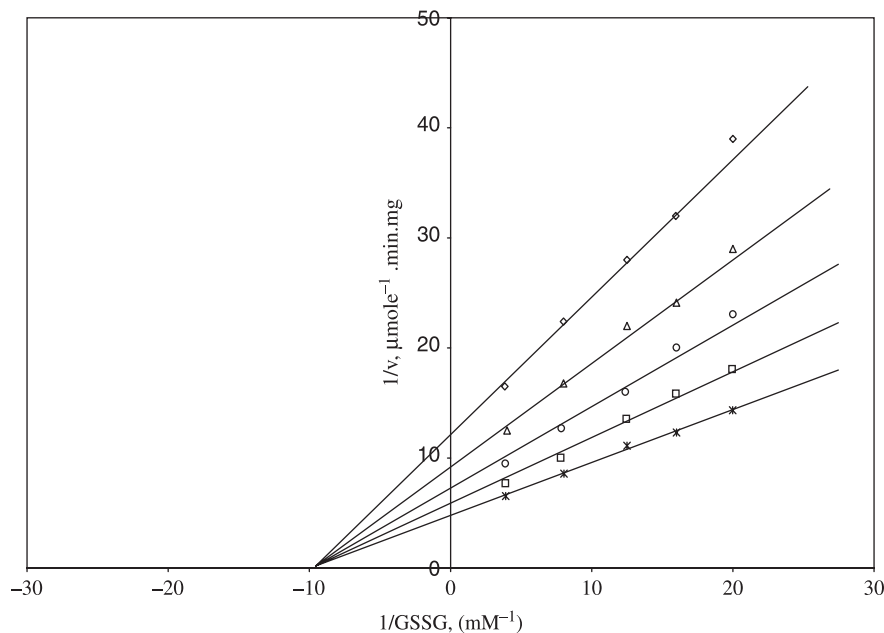


Figure 3. Lineweaver-Burk double reciprocal plot of initial velocity against GSSG as varied substrate and  $\text{CaCl}_2$  (0.8–1.6 mM) as inhibitor at a fixed NADPH (0.1 mM) concentrations. \*0.1 mM NADPH (constant);  $\square$  0.8 mM  $\text{CaCl}_2$ ;  $\circ$  1 mM  $\text{CaCl}_2$ ;  $\Delta$  1.2 mM  $\text{CaCl}_2$ ;  $\diamond$  1.6 mM  $\text{CaCl}_2$ .

is non-competitive with respect to both GSSG and NADPH with  $K_{i\text{GSSG}} 0.476 \pm 0.085 \text{ mM}$  and  $K_{i\text{NADPH}} 0.96 \pm 0.134 \text{ mM}$ .  $\text{Zn}^{2+}$ , at low levels, has several basic housekeeping functions in metallo-enzymes, transcription factors, immunoregulation, growth, and cytoprotection, displaying antioxidant, anti-apoptotic, and anti-inflammatory roles. At high levels, however, the metal can be highly toxic [27]. Toxic doses of  $\text{Zn}^{2+}$  inhibit intestinal alkaline phosphatase [28], mitochondrial cytochrome c

oxidase [29], glyceraldehyde-3-phosphate dehydrogenase [30], beta amylase [31] and G-6-PD from lamb kidney cortex [26].  $\text{Zn}^{2+}$  homeostasis in bacteria is achieved by export systems and uptake systems which are separately regulated by their own regulators. Three types of  $\text{Zn}^{2+}$  export systems that protect cells from high toxic concentrations of  $\text{Zn}^{2+}$  have been identified [32].

Kinetic characterization of the inhibition effects of  $\text{CaCl}_2$  on glutathione reductase is shown in Figures 3

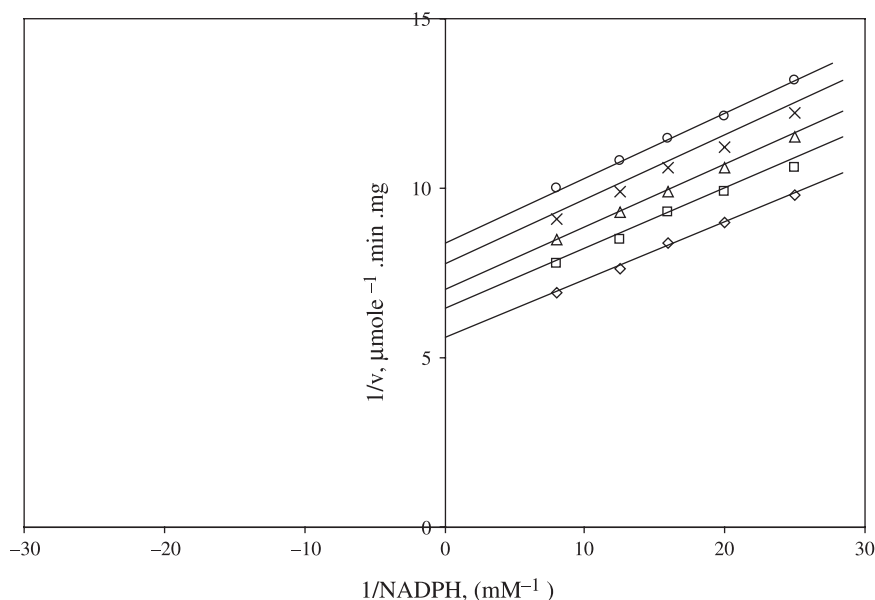


Figure 4. Lineweaver-Burk double reciprocal plot of initial velocity against NADPH as varied substrate and  $\text{CaCl}_2$  (0.8–1.6 mM) as inhibitor at different fixed GSSG (0.7 mM) concentrations.  $\diamond$  0.7 mM GSSG (constant);  $\square$  0.8 mM  $\text{CaCl}_2$ ;  $\Delta$  1 mM  $\text{CaCl}_2$ ; \*1.2 mM  $\text{CaCl}_2$ ;  $\circ$  1.6 mM  $\text{CaCl}_2$ .

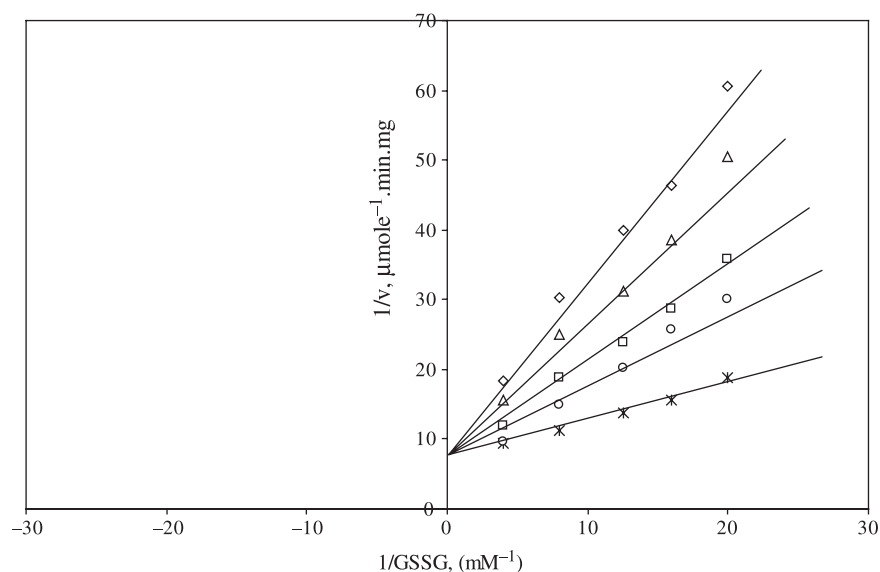


Figure 5. Lineweaver-Burk double reciprocal plot of initial velocity against GSSG as varied substrate and  $\text{NiSO}_4$  (0.1–0.4 mM) as inhibitor at different fixed NADPH (0.1 mM) concentrations. \* 0.1 mM NADPH (constant); ○ 0.1 mM  $\text{NiSO}_4$ ; □ 0.2 mM  $\text{NiSO}_4$ ; Δ 0.3 mM  $\text{NiSO}_4$ ; ◇ 0.4 mM  $\text{NiSO}_4$ .

and 4; the inhibition is non-competitive with respect to GSSG and uncompetitive with respect to NADPH with  $K_{\text{GSSG}} 1.476 \pm 0.195 \text{ mM}$  and  $K_{\text{NADPH}} 2.993 \pm 0.227 \text{ mM}$  respectively.  $\text{Ca}^{2+}$  is essential to maintaining total body health although high levels of  $\text{Ca}^{2+}$  may be harmful. Hypercalcemia may also provoke acute renal failure or hypertension, or aggravate tubular necrosis [33].

Nutrient minerals are essential yet are potentially toxic and homeostatic mechanisms are required to regulate their intracellular levels [34]. Although  $\text{Ni}^{2+}$

is an essential cofactor for a number of enzymatic reactions in prokaryotes and eukaryotes [35], this metal ion can inhibit GR in a concentration-dependent manner.  $\text{Ni}^{2+}$  is a widely distributed metal that is industrially applied in many forms. Accumulated epidemiological evidence confirms that occupational exposures to nickel compounds are mostly associated with increased nasal and lung cancer incidence [36]. DNA damage in the form of strand breaks and DNA-protein cross-links resulted *in vivo* following injection of nickel carbonate in rats

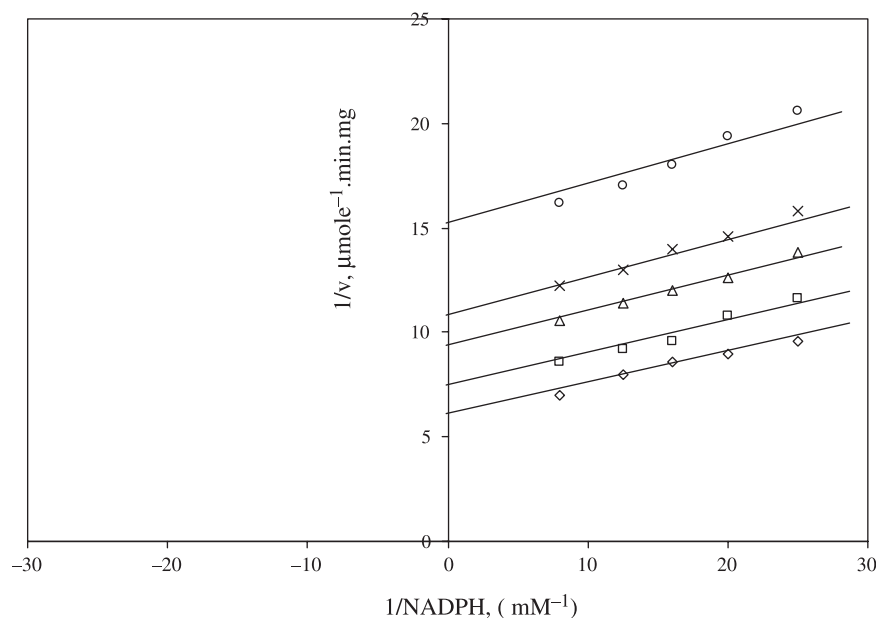


Figure 6. Lineweaver-Burk double reciprocal plot of initial velocity against NADPH as varied substrate and  $\text{NiSO}_4$  (0.1–0.4 mM) as inhibitor at different fixed GSSG (0.7 mM) concentrations. ◇ 0.7 mM GSSG (constant); □ 0.1 mM  $\text{NiSO}_4$ ; Δ 0.2 mM  $\text{NiSO}_4$ ; \* 0.3 mM  $\text{NiSO}_4$ ; ○ 0.4 mM  $\text{NiSO}_4$ .



[37]. The interaction between  $\text{Ni}^{2+}$  and yeast hexokinase has been studied and kinetic studies showed that  $\text{Ni}^{2+}$  caused a non-competitive inhibition when glucose was the variable substrate and competitive inhibition when ATP was the variable substrate [12]. Here, we found that  $\text{Ni}^{2+}$  was an inhibitor of GR giving to a competitive inhibition pattern when GSSG (Figure 5) was the varied substrate and uncompetitive pattern when NADPH (Figure 6) was the varied substrate.

EDTA is a chelating agent that binds divalent metal ions. When the divalent metals are chelated by the EDTA the toxic effects are lost. We suggest that EDTA has a recovering role in inhibition of yeast GR with metal ions and we found that when EDTA (0–12 mM) concentrations are added to the assay mixtures containing 2.5 mM  $\text{CaCl}_2$ , 2 mM  $\text{ZnSO}_4$  and 1 mM  $\text{NiSO}_4$ , respectively, GR activity was recovered by approximately 90%.

## Conclusions

We have found that  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Ni}^{2+}$  ions are potent inhibitor of baker's yeast GR. This is probably due to the interactions of the metal ions with aminoacids of the enzyme. GR was inhibited with  $\text{NiSO}_4$  competitively with respect to GSSG, so there may be a competition between substrate GSSG and  $\text{Ni}^{2+}$  ions for the active site of this enzyme. A non-competitive inhibitor may bind to a non-substrate binding site on a protein and distort it to the point of non-functionality [20]. The inhibition of GR with  $\text{ZnSO}_4$  was found non-competitive.

Glutathione reductase is a important enzyme that catalyzes the reduction of GSSG using NADPH as a cofactor. The enzyme is a major component of cellular defense mechanisms against oxidative injury [38] and is an attractive target for the development of antimalarial agents, agents to decrease malarial drug resistance and anticancer agents. In addition, inhibition of the enzyme has been employed as a tool in research for various purposes [39]. Investigation of the inhibitors of this enzyme is important for antimalarial and anticancer researches. Because of GR is a crucial enzyme in the antioxidant system, this study may be useful for understanding the mechanisms for oxidative damage associated with heavy metal toxicity.

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