Effect of Mercuric Chloride on Kinetic Properties of Horseradish Peroxidase

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Abstract

Mercury is one of the three major environmental metal poisons, and mercuric chloride is a highly reactive compound which can harm cells by a variety of mechanisms including direct interaction with sulphydryl groups of proteins and enzymes, therefore affecting the enzymatic activity. This study focused on the effect of Hg^{++} on horseradish peroxidase (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) (HRP) (Isoenzyme C) activity. In the presence of 88 mM hydrogen peroxide K_m for *o*-dianisidine oxidation was 0.05 millimolar and V_{max} was 8.5 $\mu M.s^{-1}$. Incubation of the enzyme with 1 to 100 millimolar mercuric chloride for 5-20- and 60 min resulted in progressive inhibition of the enzymatic activity. At low Hg^{++} concentrations the inhibition was reversible by excess substrate, while at high Hg^{++} concentration the inhibition was not reversible. Results also indicated that the type of inhibition depended on the duration of incubation of the enzyme with metal ion and on the Hg^{++} concentration. So we could conclude that the type of inhibition changed from noncompetitive to mix with increased incubation time and increased metal concentration.

Keywords: *Horseradish peroxidase, Mercuric chloride, Enzyme inhibition, O dianisidine*

Introduction

Metal- induced toxicity and carcinogenicity, is mediated by various modifications of biomolecules. For heavy metals such as mercury, cadmium and nickle the primary route for their toxicity is depletion of glutathione and bonding to sulfhydryl groups of proteins (1). The remarkable affinity of Hg^{2+} for aminoacids and proteins can cause structural and functional abnormalities in biomolecules (2).

Mercury is widely distributed in the earth's crust, sea, ground and rain water, and its toxic effects on biological systems through direct uptake as well as by accumulation in food chains is well known (1, 2).

Peroxidase is important detoxifying enzymes serving to rid cells of excess H_2O_2 under normal and stress conditions. Although peroxidases remain active in the presence of a number of metal ions, recent reports have also indicated their inhibition by metal ions (3-6).

In this study, the effect of increasing amounts of Hg^{2+} ion on horseradish peroxidase (HRP) activity was investigated in vitro. HRP is a member of the plant peroxidase superfamily, which utilizes hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds. This gives rise to a range of colorimetric, fluorimetric, chemiluminescent and electrochemical assays for HRP activity. HRP is very widely used as an indicator in immunoassays, non-isotopic DNA probes, cytochemistry, bienzyme systems, biosensors and chimeric cancer therapy prodrugs. This is owing to its stability, high catalytic rates, ease of conjugation to other molecules and wide choice of assays of activity. (7-10)

Furthermore this enzyme has good potentialities for removing toxic aromatic compounds like phenol from waste water of various industries, such as pulp and paper industry (11-13).

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Materials and Methods

HRP (isoenzyme c) was obtained from Sigma Chemical Co., as a freeze-dried powder (type XII); *o*-dianisidine dihydrochloride was also obtained from Sigma. Hydrogen peroxide (30% solution), mercury chloride and all the other chemicals used in this work were obtained from Merck and were of reagent grade.

HRP activity was measured by following the H_2O_2 dependent oxidation of *o*-dianisidine at 460 nm, using an extinction coefficient of 11.3 mM^{-1} cm^{-1} (14). *o*-dianisidine stock solutions (10 mM) were prepared by dissolving *o*-dianisidine in distilled water. H_2O_2 stock solutions (88 mM) were prepared daily by appropriate dilution of 30% H_2O_2 in distilled water. HRP solutions (1 mg/ml) were prepared by dissolving the enzyme in distilled water. Enzyme concentrations were determined spectrophotometrically using an extinction coefficient of 133 $mM^{-1}cm^{-1}$ at 404 nm and a molecular weight of 44,000. Stock solutions of $HgCl_2$ (1M) were prepared in distilled water. The assay was performed in 0.1 M citrate buffer, pH 4.0, since a preliminary pH profile indicated 4.0 as the optimum pH. The reaction was started by adding 30 µl of 0.088 M H_20_2. For assays done in the presence of Hg^{2+} ions, appropriate amounts of stock solution were mixed with 0.1 M citrate buffer, the final volume was always 3 ml and the concentration of ions varied from 1 to 100 mM. The enzyme (final concentration 3.6 nM) was added and incubated with the ions for 5, 20 or 60 min before addition of *o*-dianisidine. The reaction was then started by adding H_20_2 as usual.

All assays were carried out on ice using ultraspec 4000 spectrophotometer. Results were average of at least three separate experiments and were analyzed with swift software.

Results

The effect of Hg^{2+} on the oxidation of *o*-dianisidine by HRP in the presence of H_2O_2 was determined by following the formation of oxidized *o*-dianisidine at 460 nm, under steady - state conditions, and after 5-, 20- or 60-min preincubation of the enzyme with the metal ion.

Optimum pH for assay of catalytic activity of HRP was 4 which is shown in Fig. 1. In Fig. 2 the optimum enzyme concentration for assay of HRP catalytic activity is presented. Fig 3 and 4 are Michaelis- Menten and Lineweaver- Burk plots for HRP. The enzyme's V_{max} was 8.5 µMs-1 and its K_m was 0.06mM. Incubation of the enzyme with 1 to 100 millimolar mercuric chloride for 5-, 20- and 60 min resulted progressive inhibition of the enzymatic activity. In preliminary experiments 5 min incubation with 1 to 100 millimolar $HgCl_2$ in room temperature led to 27% to 63% inhibition while 60 min incubation with 100 millimolar $HgCl_2$ led to as much as 57% to 88% inhibition of the enzyme activity.

In order to reduce the variations of temperature, rest of the experiments were performed on ice. Table 1 shows inhibition percent of various concentrations of Hg^{2+} on HRP activity on ice.

Incubation of HRP (3.6 nM) with Hg^{2+} (1-100 mM) resulted in inhibition of the enzymatic activity; the type of inhibition depended on the length of incubation of the enzyme with the metal ion and on Hg^{2+} concentration. Inverse plots obtained after 5-min preincubation of HRP and Hg^{2+} at different Hg^{2+} concentrations with *o*-dianisidine as the varied substrate are shown in Fig 5. The plot was linear with a common intercept in the abscissa indication non-competitive inhibition for 1 mM concentration of $HgCl_2$. The enzyme's V_{max} decreased from 8.3±0.2 μMs^{-1} for the control to 6.6±0.2 μMs^{-1} for 1 mM Hg^{2+} while K_m remained unaffected. As concentration increased, the type of inhibition changed from noncompetitive to mix. For concentrations of 25 to 100 mM, the common intercept was no longer found on the abscissa but above it and to the left of the ordinate, indicating mixed inhibition of enzyme activity by Hg^{2+}. Similar results were found when HRP and Hg^{2+} were preincubated for 20 min (Fig. 6). For 1 mM Hg^{2+} concentration the enzyme's

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V_{max} decreased from 8.3±0.2 μMs^{-1} for the control to 6.2±0.2 μMs^{-1} for 1 mM, while its Km remained unaffected. For Hg^{2+} concentrations of 25 to 100 mM, mixed inhibition of HRP activity by Hg^{2+} was found and both V_{max} and K_m were affected. When HRP was preincubated with Hg^{2+} for an interval of one hour, noncompetitive inhibition was observed for 1 mM Hg^{2+} concentration, and for Hg^{2+} concentrations of 5 mM and higher mixed inhibition of the enzyme activity by Hg^{2+} was found (Fig. 7).

The values of K_m and V_{max} after incubation of HRP with increasing concentrations of Hg^{2+} for different times have been summarized in Table 2.

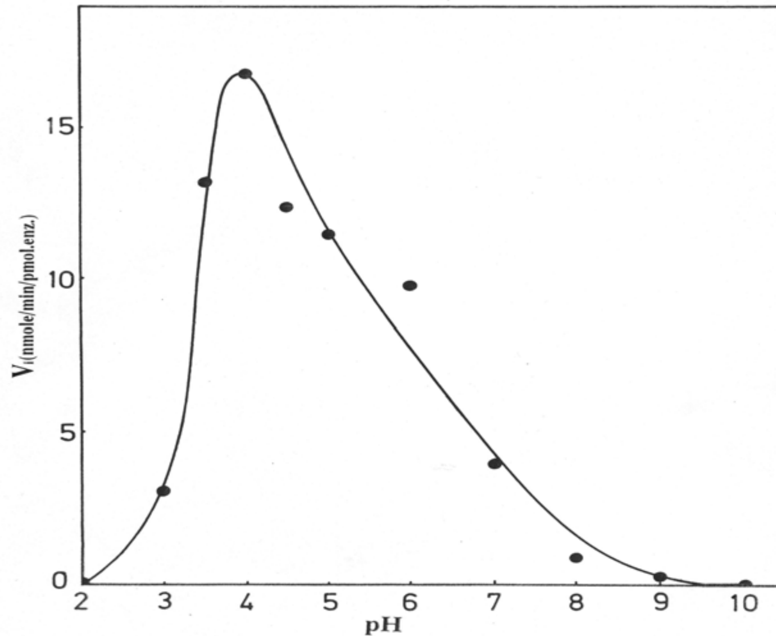

Fig. 1: pH profile for HRP

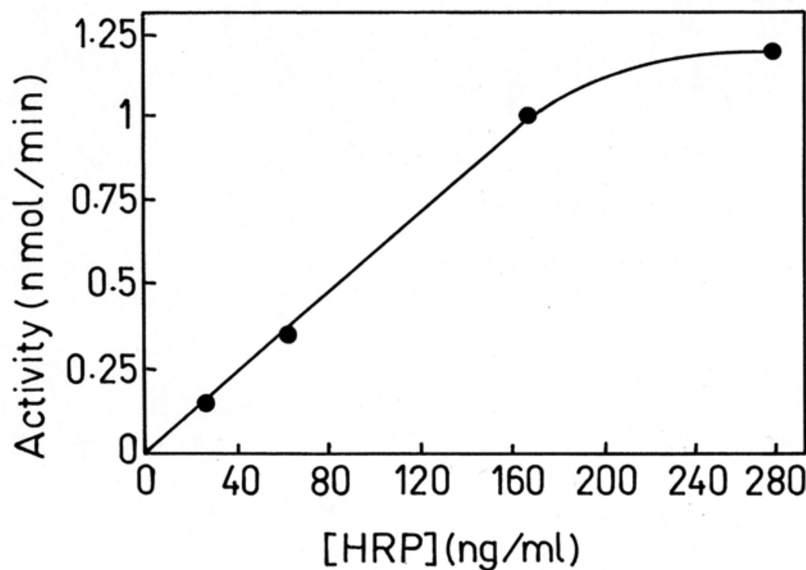

Fig. 2: The optimum enzyme concentration for assay of HRP catalytic activity.

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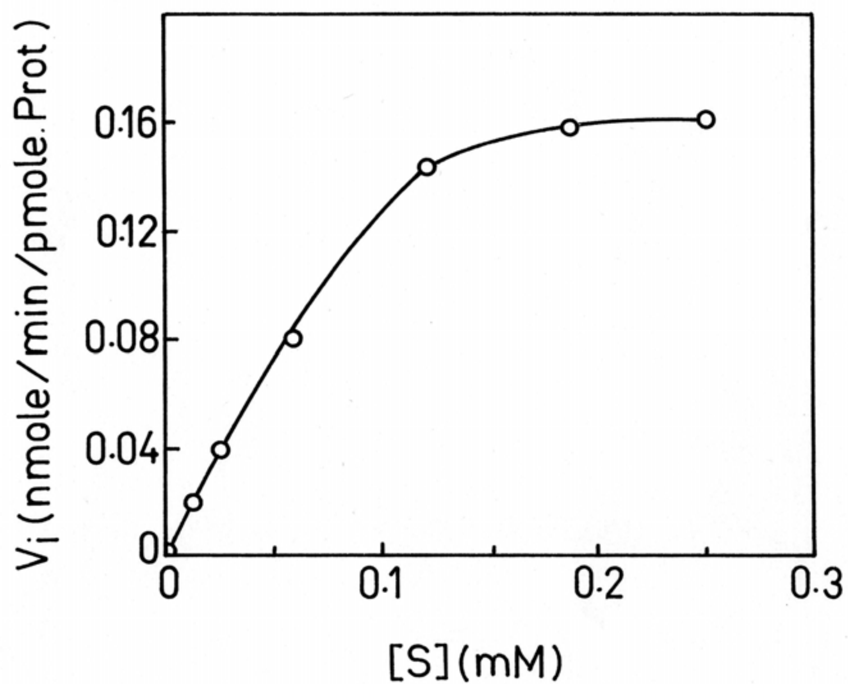

Fig. 3: The Michaelis - Menten plot for HRP to determine K_m and V_{max} of the enzyme.

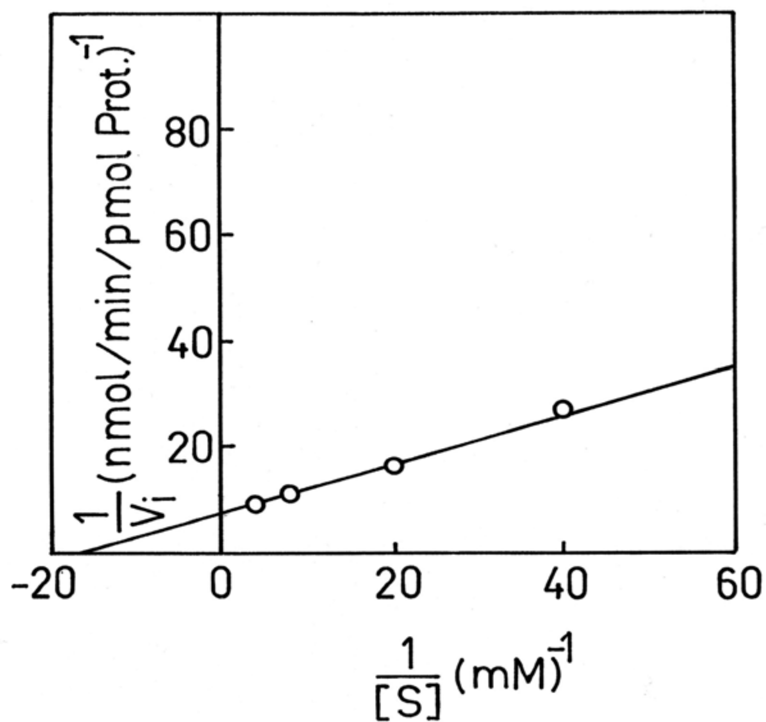

Fig. 4: The lineweaver- Burk plot for HRP to determine K_m and V_{max} of the enzyme.

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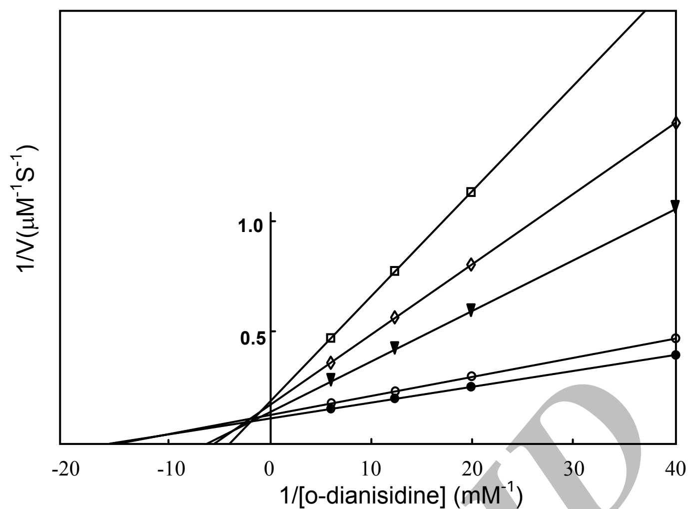

Fig. 5: Inverse Plots of 1/rate VS. 1/[o-dianisidine]. $HgCl_2$ concentratinos of 0 (●), 1 (○), 25 (▾), 50 (◊) and 100 (□) mM were used. HRP and $HgCl_2$ were preincubated for 5 minutes.

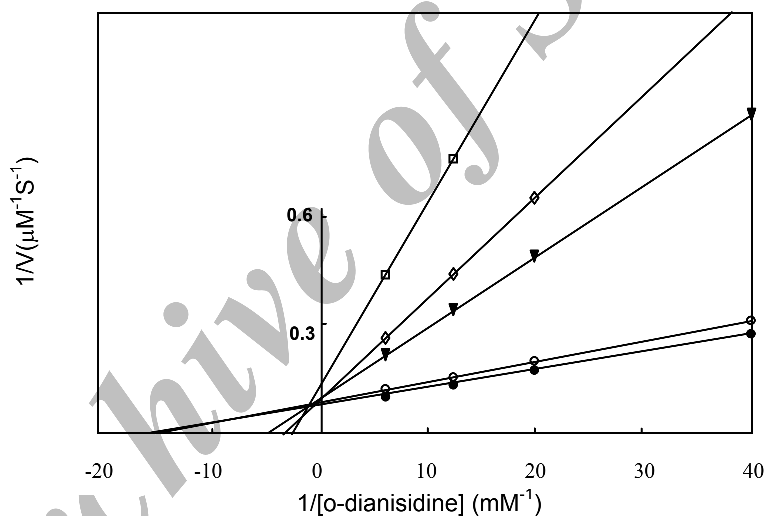

Fig. 6: Inverse Plots of 1/rate VS. 1/[o-dianisidine]. $HgCl_2$ concentratinos of 0 (●), 1 (○), 25 (▾), 50 (◊) and 100 (□) mM were used. HRP and $HgCl_2$ were preincubated for 20 minutes.

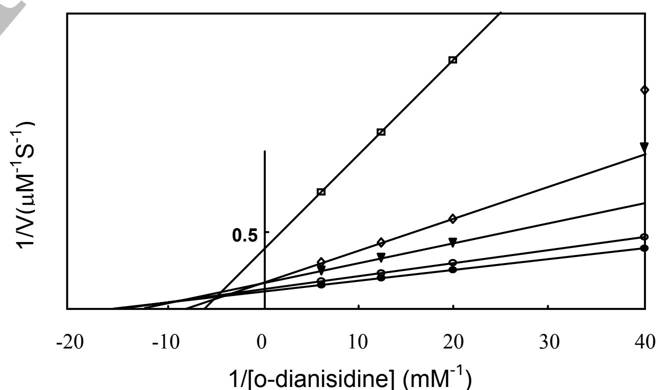

Fig. 7: Inverse Plots of 1/rate VS. 1/[o-dianisidine]. $HgCl_2$ concentratinos of 0 (●), 1 (○), 5(▾), 25 (◊) and 50 (□) mM were used. HRP and $HgCl_2$ were preincubated for 60 minutes.

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Table 1: Inhibition percent of various concentrations of Hg^{2+} on HRP activity

Incubation time \ Hg^{++} Concentration	1 mM (%)	25 mM (%)	50 mM (%)	100 mM (%)
5 min	10	13	16	27
20 min	18	20	33	48
60 min	23	25	52	57

Table 2: Values of Km and V_{max} after incubation of HRP in increasing concentrations of Hg^{2+} for different times

Incubation time	5 min		20 min		60 min	
$[Hg^{2+}]$ mM	K_m (mM)	V_{max} (μMS^{-1})	K_m (mM)	V_{max} (μMS^{-1})	K_m (mM)	V_{max} (μMS^{-1})
0	0.06 ± 0.02	8.3 ± 0.2	0.06 ± 0.02	8.3 ± 0.2	0.06 ± 0.02	8.3 ± 0.2
1	0.06 ± 0.02	6.6 ± 0.2	0.06 ± 0.02	6.2 ± 0.2	0.06 ± 0.02	7.2 ± 0.2
5	-	-	-	-	0.09 ± 0.02	6.8 ± 0.2
25	0.16 ± 0.02	6.6 ± 0.2	0.19 ± 0.02	6.4 ± 0.2	0.11 ± 0.02	5.4 ± 0.2
50	0.20 ± 0.02	5.8 ± 0.2	0.26 ± 0.02	5.7 ± 0.2	0.16 ± 0.02	2.5 ± 0.2
100	0.26 ± 0.02	5.2 ± 0.2	0.38 ± 0.02	4.4 ± 0.2	-	-

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Discussion

Mercury is of public health concern due to its toxic effects and widespread occurrence in the environment. Nowadays it is widely used in hundreds of applications. The distribution, absorption, metabolism, toxicity and excretion of metallic mercury have been examined extensively in recent years (15). But unfortunately past research has rarely focused on the effect of mercuric chloride on horseradish peroxidase(8). In this study the effect of metallic mercury on HRP has been investigated. HRP is an important heme-containing enzyme that has been studied for more than a century. It is an important analytical tool, and has been used as a reagent for organic synthesis and biotransformation as well as in coupled enzyme assays, chemiluminescent assays, immunoassays, the treatment of waste waters and targeted cancer therapy(8). Few plant enzymes are represented so widely in the scientific and patent literature as HRP (8).

There are several reports that show metal ions and organic molecules such as cadmium, cobalt, copper, P-aminobenzoic acid, thiouracils and phenylcyclopropylamine can inhibit HRP activity (16, 17). Also the effect of metallic mercury on different enzymes other than HRP has been investigated. For example it is reported that mercury is a noncompetitive inhibitor of glutathione peroxidase (18). Company et al. has shown Hg^{2+} has inhibitory effect on catalase activity in marine organisms (19).

But there are few reports about the effect of Hg^{2+} on HRP activity. In two different reports which have used luminol-enhanced chemiluminesence (LmCL) to study the in vitro effect of contaminants such as heavy metals, Ilyina et al.

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has shown that mercury inhibited HRP-mediated LmCL (20), though in another study mercury did not significantly affect the HRP mediated Lm CL (21). In yet another report, a methylene blue- mediated enzyme biosensor for the detection of mercury (II) was developed and $HgCl_2$ could irreversibly inactivate immobilized HRP (22).

The studies on enzymatic activity presented here showed that Hg^{2+} was a reversible inhibitor of *o*-dianisidine oxidation by HRP. Incubation of HRP with Hg^{2+} resulted in inhibition of the enzymatic activity; the type of inhibition depended on the length of incubation of the enzyme with the metal ion and on Hg^{2+} concentration. Inverse plots obtained after different times of HRP preincubation with Hg^{2+} showed two patterns of inhibition the plot was linear with a common intercept in the abscissa indicating noncompetitive inhibition for low concentrations of $HgCl_2$ or for short preincubation times. The type of inhibition changed from noncompetitive to mix with increased incubation time and increased metal concentration. Further research should be performed to determine the binding site and the stoichiometry of Hg^{2+} binding to the enzyme.

It was concluded that mercuric chloride was a potent reversible inhibitor of HRP and showed two different patterns of inhibition.

Acknowledgements

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