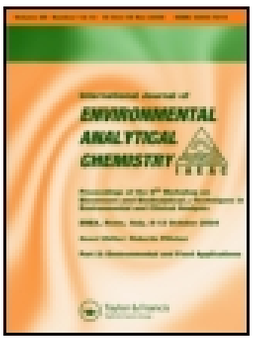

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ARTICLE

Inhibition assays of horseradish peroxidase by hexavalent chromium and other heavy metals

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ABSTRACT

In this study, individual and combinations of heavy metals were tested at different pH for their inhibition potential by horseradish peroxidase (HRP). At pH of 6.4, maximum velocities for a single substrate concentration of hydrogen peroxide was observed. The double reciprocal of Lineweaver-Burk plot gave the same values of K_m and decreased V_{max} represents the non-competitive type of inhibition by hexavalent chromium metal ions. The IC_{10} value of Cr^{6+} ion for free HRP was achieved at 0.55ppm, while inhibition for 1ppm of substrate concentration was 24.5%. For other tested heavy metals, no significant inhibition properties were observed except for Cd^{2+} and Mg^{2+} metal ions. However, when combined with Cr^{6+} ions, substantial changes were detected. Based on IC_{10} values, the sensitivity order for free HRP for individual metal ions was observed as $Cd^{2+} > Cr^{6+} > Mg^{2+} > Cu^{2+} > Cr^{3+} > Mn^{2+}$ while the combination with Cr^{6+} gave the order as $Cd^{2+} > Cu^{2+} > Cr^{3+} > Cr^{6+} > Mg^{2+} > Mn^{2+}$. The order of inhibition byquaternary combinations of metal ion were in the order $(Cr^{6+} + Cd^{2+} + Cu^{2+} + Cr^{3+}) > (Cr^{6+} + Cu^{2+} + Mg^{2+} + Cd^{2+}) \geq (Cr^{6+} + Cd^{2+} + Mg^{2+} + Cr^{3+}) \geq (Cr^{6+} + Cu^{2+} + Mg^{2+} + Cr^{3+})$, while the quinary combination $(Cr^{6+} + Cr^{3+} + Cu^{2+} + Mg^{2+} + Cd^{2+})$ was the most effective grouping. The studies shown excellent selectivity towards Cr^{6+} and suggest further use of HRP enzymes in the design and selection of biosensors for heavy metals and other pollutants.

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KEYWORDS
Chromium; horseradish peroxidase; non-competitive inhibition; sensitivity; heavy metals; enzyme kinetics

1. Introduction

Heavy metals are essential life sustainable elements for plants and animals in limited concentrations, while a higher concentration can serve as toxic for the same. Out of 35 heavy metals, chromium is the most readily available metal on earth and is widely used in textile, glass, stainless steel, leather, wood, and plastic industries [1,2]. Cr^{6+} stands-out over other heavy metal due to its high toxicity that is considered to be 500–1000 times higher than that of Cr^{3+}. Its strong oxidising properties, an occurrence as soluble oxyanions and ability to transform into other forms make it highly pernicious for plants, animals, and humans even at a very low concentration [3–5]. The hexavalent state of chromium (Cr^{6+}) is highly stable, reactive and can easily penetrate the living cell resulting in several forms of diseases such as skin lesions, stomach cancer, diarrhoea,

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Supplemental data for this article can be accessed here.

bronchospasm, pneumonia, nasal ulcer, respiratory tract cancer, lung cancer, tuberculosis and dermatitis [1,6,7]. Although the permissible limit of Cr^{6+} in drinking water, soil and the air are 0.05 mg/l [5], 10–15 mg/kg [2] and 5 µg/m^3 [8,9] respectively, the rapid expansion of mining activities and other industrial activities had increased the concentration of this heavy metal to several folds higher in real-time environmental conditions.

The necessity for a highly sensitive technique for multi-analytic detection of heavy metals from different sources is extremely critical for the safeguard of the food, environment and biomedical care [4]. Among the many available approaches used for chromium detection such as spectroscopy, chromatographic, voltammeter, electrochemical and polymer-based, enzymatic centred techniques have been the preferred once due to its sensitivity, easy extraction processes, cost-efficiency, and multiple applications. The most commonly used enzymes are urease, tyrosinase, acetylcholine esterase, glutathione, phytochelatins, N- ethylmaleimide reductase, L-proline dehydrogenase, and horseradish peroxidase (HRP) [1,3,8,10].

Peroxidase plays a crucial role in various physiological processes and serves as a bridge or messenger molecule for several enzymatic assays and their products in living bodies [11]. Peroxidases are a group of enzymes found in animal and plant tissues as well as in microorganisms functioning as catalysts in the redox reaction between H_2O_2 and various reductants. Besides its role as catalysts, peroxidase in plants also participates in many other functions like hormone regulation, defence mechanisms, indoleacetic degradation, and lignin biosynthesis [12,13]. Therefore, the detection of H_2O_2 is of considerable significance both in enzyme-based assays and in metabolism control. The binding of heavy metals with HRP changes the conformation of the active site and inhibits the enzyme activity even at low concentrations. Herein, the present work determines the inhibition of HRP activity by chromium metal ion along with other metals, namely Cu^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+} ions by exploiting the properties of hydrogen peroxide. The study will be immensely useful for designing an HRP based biosensor for detection of Cr^{6+} and other heavy metals in water samples.

2. Materials and methods

2.1. Chemicals and reagents

Commercial horseradish peroxidase (HiMedia, India) and laboratory-grade hydrogen peroxide were procured (Fisher Scientific, India). Hydrogen peroxide (1 mM) solution and other enzyme stock solution (1 µg/ml) were prepared in phosphate buffer (pH 6.4). Metal salts such as Potassium dichromate ($K_2Cr_2O_7$), Chromium chloride ($CrCl_3$), Copper sulphate ($CuSO_4$), Magnesium sulphate (Mg_2SO_4), Cadmium nitrate ($Cd(NO_3)_2$) and Manganese sulphate ($MnSO_4$) were used for the preparation of Cr^{6+}, Cr^{3+}, Cu^{2+}, Mg^{2+}, Cd^{2+} and Mn^{2+} solutions respectively. O- Dianisidine (ODA) (1%) was prepared by dissolving one gm in 100 ml of methanol.

2.2. Estimation of HRP activity and pH optimisation

Enzyme kinetics was studied from the reaction mixture of 25 µl ODA solution and different concentration of H_2O_2 solution (0.1 mM to 0.4 mM) prepared in 3 ml of phosphate buffer.

50 µl of HRP enzyme solution was then added to the reaction mixture and incubated for 3 min. Upon incubation, the absorbance was measured at 460 nm using a spectrophotometer. The enzyme kinetic parameters were estimated by plotting the experimental observations, as suggested in the Michaelis-Menten and Lineweaver-Burk plot. For the optimisation of pH of enzymatic reaction, the assays were performed at various pH buffer solutions such as 5.8, 6, 6.4, 7, 7.4, and 7.8.

The possible oxidation and reduction reactions can be expressed as in Equations (1) and (2):

$$H_2O_2 + ODA \xrightarrow{HRP} ODA_{ox} + H_2O \tag{1}$$

$$ODA_{ox} + 2e^- \rightarrow ODA_{red} \tag{2}$$

ODA_{ox} is the oxidised form (colour compound) and gives the maximum absorbancy at 460 nm. The enzyme activity of HRP (Equation (3)) was calculated by using the extinction coefficient of 11.3 mM^{-1} cm^{-1} [14].

$$Enzyme\ activity = \frac{Absorbance\ of\ the\ sample}{11.3 \times assay\ time} \tag{3}$$

2.3. HRP inhibition assays

HRP inhibition assays were performed by adding different metal ions (Cr^{6+}, Cr^{3+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+}) to the assay mixture at varying concentrations and combinations as shown in Table 1.

Table 1. Experimental design for inhibition assays of HRP by various metal ion concentrations.

Expt. No			Combination Type	Metal Ions Concentration (ppm)					
				Cr^{6+}	Cr^{3+}	Cu^{2+}	Cd^{2+}	Mn^{2+}	Mg^{2+}
1	(a)	**Single**	Cr^{6+}	0.01 − 1	–	–	–	–	–
	(b)		Cr^{3+}	–	0.01–1	–	–	–	–
	(c)		Cu^{2+}	–	–	0.01–1	–	–	–
	(d)		Cd^{2+}	–	–	–	0.01–1	–	–
	(e)		Mn^{2+}	–	–	–	–	0.01–1	–
	(f)		Mg^{2+}	–	–	–	–	–	0.01–1
2	(a)	**Binary**	Cr^{3+} + Cr^{6+}	1	0.01–1	–	–	–	–
	(b)		Cu^{2+} + Cr^{6+}	1	–	0.01–1	–	–	–
	(c)		Cd^{2+} + Cr^{6+}	1	–	–	0.01–1	–	–
	(d)		Mn^{2+} + Cr^{6+}	1	–	–	–	0.01–1	–
	(e)		Mg^{2+} + Cr^{6+}	1	–	–	–	–	0.01–1
3	(a)	**Ternary**	Cr^{6+} + Cu^{2+} + Cd^{2+}	0.01–1	–	1	1	–	
	(b)		Cr^{6+} + Cu^{2+} + Mg^{2+}	0.01–1	–	1	–	–	1
	(c)		Cr^{6+} + Mg^{2+} + Cd^{2+}	0.01–1	–	–	1	–	1
	(d)		Cr^{6+} + Cr^{3+} + Cd^{2+}	0.01–1	1	–	1	–	
	(e)		Cr^{6+} + Mg^{2+} + Cr^{3+}	0.01–1	1	–	–	–	1
	(f)		Cr^{6+} + Cu^{2+} + Cr^{3+}	0.01–1	1	1	–	–	
4	(a)	**Quaternary**	Cr^{6+} + Cd^{2+} + Mg^{2+} + Cr^{3+}	0.01–1	1	1	–	–	1
	(b)		Cr^{6+} + Cd^{2+} + Cu^{2+} + Cr^{3+}	0.01–1	1	1	1	–	
	(c)		Cr^{6+} + Cu^{2+} + Mg^{2+} + Cr^{3+}	0.01–1	1	1	–	–	1
	(d)		Cr^{6+} + Cu^{2+} + Mg^{2+} + Cd^{2+}	0.01–1	–	1	1	–	1
5	(a)	**Quinary**	Cr^{6+} + Cr^{3+} + Cu^{2+} + Mg^{2+} + Cd^{2+}	0.01–1	1	1	1	–	1

2.3.1. Inhibition assay of single metal ions and a combination of binary metal ions

For the inhibition study of HRP by Cr^{6+}, the concentrations of Cr^{6+} was started from 0.01 to 1 ppm in phosphate buffer (pH- 6.4) solution. 0.15 mM of H_2O_2 and 25 µl of ODA was added to each reaction mixture. To start the inhibition reaction, 50 µl of HRP from the stock (1 µg/ml) was added to the reaction mixture and incubated for 3 min, and the optical density (OD) was measured at 460 nm. Similarly, the inhibition assay for other metal ions (Cr^{3+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+}) between the concentration ranges of 0.01–1ppm was performed. For binary combination, metal ions of Cr^{3+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+} were taken in different concentrations (0.01–1 ppm), and 1 ppm of Cr^{6+} was added to each variant.

2.3.2. Inhibition assay of HRP by multiple mixtures of metal ions

The HRP inhibition assay was carried out through various mixtures of heavy metal ions, as shown in Table 1. Metal ions of Cr^{3+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+} in a concentration of 1ppm were added to each concentration of Cr^{6+} (0.01–1ppm). The reaction mixture was added with 25 µl of ODA, H_2O_2 (0.15 mM) and HRP (50 µl), and the final volume was made to 3 ml. After 3 min of incubation, the reading was taken at 460 nm using a spectrophotometer.

3. Results and discussion

3.1. HRP kinetics and pH optimisation

The sensitivity of the enzyme is highly dependent on the pH of its reaction solutions. The ideal velocity of the HRP enzymatic reaction, performed at different pH showed that the pH 6.4 produced the maximum velocity for a single substrate concentration (Figure 1). This observation is also supported by the studies of Bovaird et al. (1982), which reported that the HRP was more active above the pH of 5.8 in a phosphate buffer solution [15].

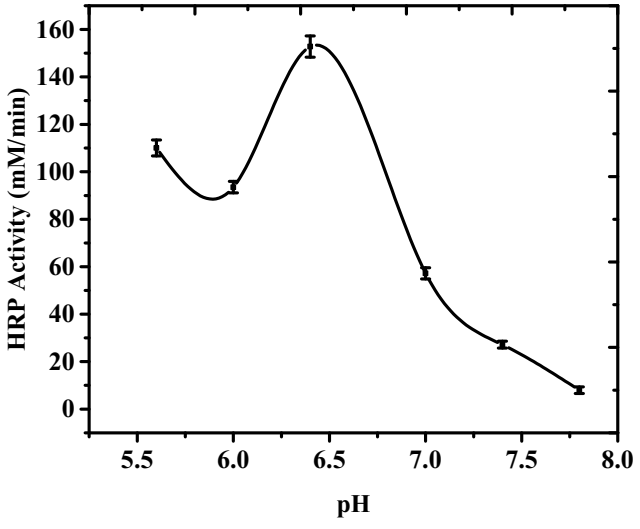

Figure 1. Optimisation of the ideal pH of HRP activity for determining the inhibition rate by metal ions.

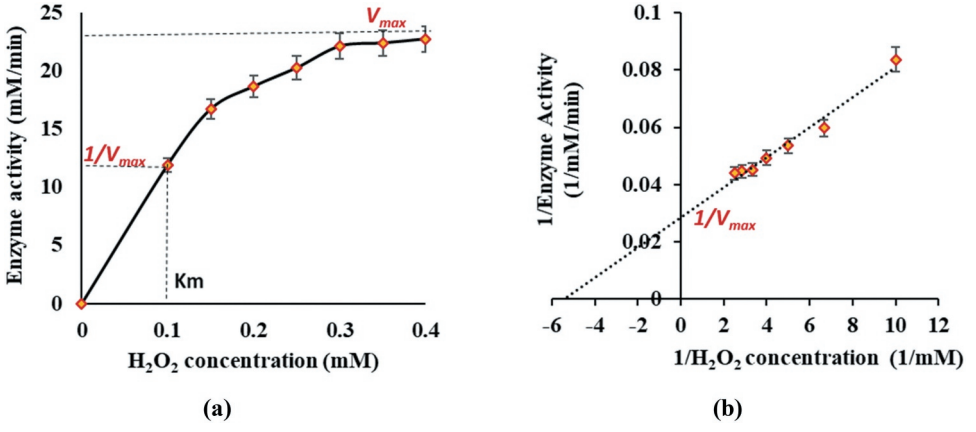

Figure 2. (a) Michaelis Menten plot and (b) Lineweaver Burk plot for HRP activity.

A hyperbolic curve was obtained by plotting the enzyme activity against substrate concentrations (Figure 2(a)), also known as the Michaelis–Menten curve. The approximate K_m and V_{max} values for HRP were found to be as 0.1 mM of H_2O_2 and 22.74 mM/min respectively. At the concentrations of 0.3 mM and above, the velocity of the reaction became almost constant. Figure 2(b) represents the Lineweaver-Burk plot that gives the accurate determination of kinetic parameters. The double reciprocal plot gives the values of K_m and V_{max} as 0.2 mM of H_2O_2 and 33.33 mM/min respectively.

The double reciprocal plot was plotted for both the free enzyme activity with and without the presence of Cr^{6+} (1ppm), in which Cr^{6+} acts as an inhibitor of HRP (Figure 3). The K_m values were found to be the same for both presence and absence of inhibitor. The V_{max} for HRP without inhibitor was 33.33 mM/min and with inhibitor was 28.57 mM/min. A decrease in the V_{max} shows the non-competitive type of inhibition by chromium metal ions, as the inhibitor (Cr^{6+}) binds to some other site of the enzyme, other than the active

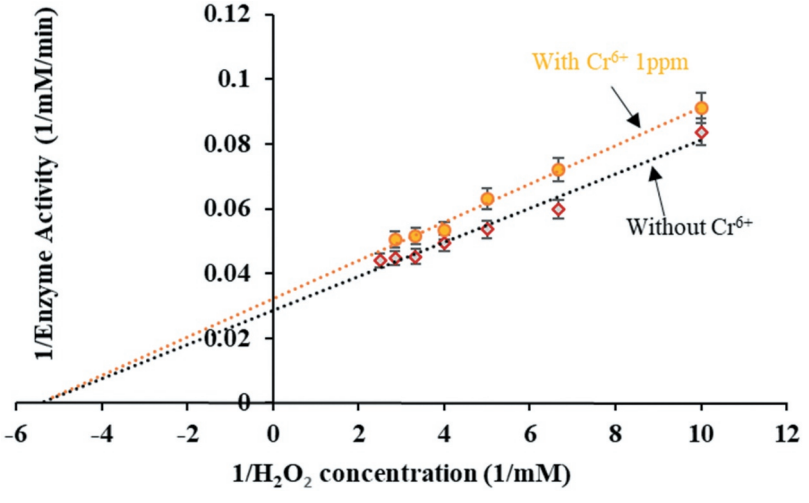

Figure 3. Lineweaver-Burk plot for HRP inhibition by Cr^{6+}.

site. It also inhibits $NADPH_{cyst}$ P_{450} reductase in a non-competitively manner similar to Cr^{3+}. Other works related to HRP and Cr^{6+} suggests mixed inhibition, a mixture of competitive and uncompetitive inhibition with Poly (neutral red) based HRP/carbon film electrode biosensors in the presence of H_2O_2 substrate based on Dixon plot and the dissociation constant of the enzyme-inhibitor substrate complex [8,16].

3.2. HRP inhibition assays

3.2.1. Inhibition by individual metals and binary combination of metal ions

The inhibition potential of Cr^{6+} on the oxidation of o-dianisidine (ODA) by HRP in the presence of H_2O_2 was determined by measuring the intensity of the brick red coloured solution (reaction mixture) spectrophotometrically at 460 nm. The inhibition reactions were found to be varied with the concentration of Cr ion and the incubation duration. At the concentration of 0.02 ppm, the inhibition was 2%, while a further increase in the concentration of Cr (0.07 ppm) shows a steady phase in the inhibition pattern (Figure 4(a)). However, with an increase in level up to 0.1ppm resulted in inhibition of over 5.3%. Gradual increase in Cr^{6+} concentration in the assay mixture ensued in an inclined pattern in inhibition, as observed in Figure 4(b). The IC_{10} value of Cr^{6+} ion for free HRP was obtained at 0.5–0.6 ppm, while inhibition of 24.5% was achieved at a concentration of about 1ppm.

For other tested heavy metals in the study, no significant inhibition properties were observed except for Cd^{2+} and Mg^{2+} metal ions. However, when combined with Cr^{6+} ions, considerable changes were detected (Table 2). Cr^{3+} in combination with Cr^{6+} showed an enhanced inhibition rate with the increase in its concentrations and has an IC_{10} value at 0.1ppm (Figure 5(a)). The binary combination of Cu^{2+} and Cr^{6+} demonstrate a promising inhibitory effect of 15% HRP enzyme compared to Cu^{2+} metal ions (Figure 5(b)). Though the blending of Mg^{2+} and Mn^{2+} with Cr^{6+} metal ions resulted in declined inhibition of HRP by 10–15%; no IC_{10} value could be obtained for both the metal ions (Figure 5(c,d)). Presence of Cd^{2+} reported the IC_{10} of HRP at concentration of 0.07 ppm approximately. When combined with $Cr^{6+,}$ the IC_{10} value was further reduced to <0.01ppm thereby, enhancing the inhibition capability of Cd^{2+} metal ions up to 32% (Figure 5(e)). A similar inhibitory experiment performed with heavy metals like Fe^{2+}, $Fe^{3+,}$ Co^{2+}, Sr^{2+}, Zn^{2+}, Hg^{2+}, Ni^{2+}, Al^{2+}, La^{3+} and Pb^{2+} reported much higher IC_{50} values of 12.58, 9.48, 12.59, 24.51, 13.57, 7.32, 10.57, 18.69, 12.0 and 6.00 mM respectively [17–20]. Such outcomes were attributed to several progressively induce conformational changes and interaction of heavy metal ions with some amino acids near or in the active site of HRP. Non-planarity of the porphyrin ring in the haem group of HRP molecule, binding with – SH group's and irregularities in HRP thermal inactivation pattern from first-order kinetics along with the extended period to exposure of active centre are the few driving factors for activation and inactivation effect of metal ions [21–24].

Based on IC_{10} values, the sensitivity order for free HRP for individual metal ions was observed as $Cd^{2+} > Cr^{6+} > Mg^{2+} > Cu^{2+} > Cr^{3+} > Mn^{2+}$. The combination of the metal ions with Cr^{6+} gave the order as $Cd^{2+} > Cu^{2+} > Cr^{3+} > Cr^{6+} > Mg^{2+} > Mn^{2+}$. The combination of Cu^{2+} and Cr^{3+} with Cr^{6+} enhances the inhibition rate compared to individuals. On contrary, the order of inhibition was reported earlier as $Cu^{2+} > Ni^{2+} > Al^{3+} > Mo^{2+} > Co^{2+} > Cd^{2+} > Pb^{2+}$ [13] and $Pb^{2+} > Cu^{2+} > Cd^{2+}$ [25].

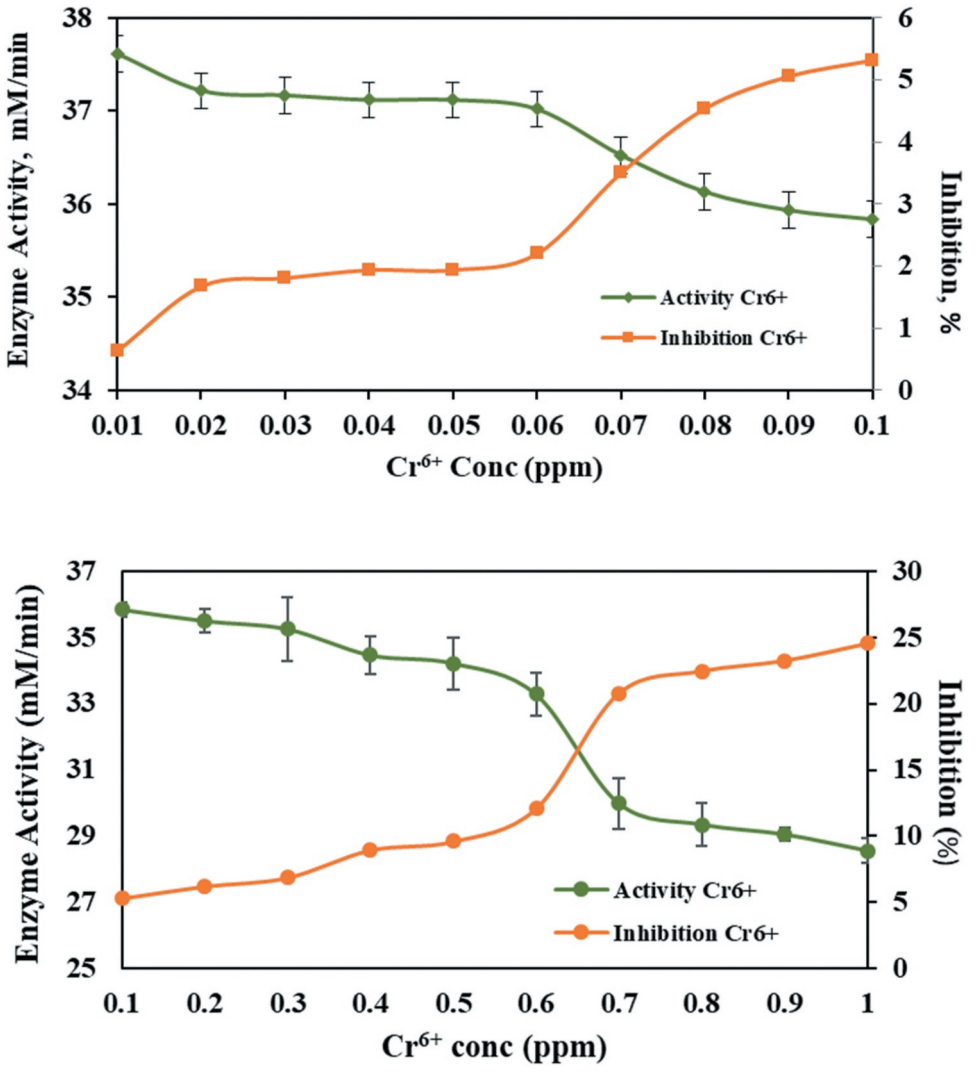

Figure 4. Effect on HRP activity by Cr^{6+} on the concentration range (a) 0.01–0.1 ppm and (b) 0.1–1 ppm.

3.2.2. Inhibition assay of HRP by multiple or intermediary metal ions

In the study, six sets of tertiary combination in different concentration were experimented (Table 1) to determine the inhibition potential of Cr^{6+} metal ion in the presence of other interference metal ions. It was found that set I, Cr^{6+} with Cu^{2+} and Cd^{2+} metal ions differ greatly from the individual Cr^{6+} inhibition pattern. Compare to individual Cr^{6+} activity, the combined form of metal ions resulted in an increased inhibition as the concentration of Cr^{6+} increases and offered the IC_{10} at 0.1ppm (Figure 6(a)).

The mixture of Cr^{6+} with Cu^{2+} and Mg^{2+} (Set II), though have an initial rise in the inhibition rate, with the addition of Cr^{6+} ions, the inhibition percentage followed a decreasing trend, and combined metals were less effective on HRP compare to individual Cr^{6+}. Further, Cr^{6+} with Mg^{2+} & Cd^{2+} (Set III) was found to have no inhibition effect till

Table 2. Comparison of IC_{10} values of HRP for different combinations of metal ions.

Metal Ion Combination		IC_{10} Values (ppm)	Metal Ion Combination		IC_{10} Values (ppm)
Single	Cr^{6+}	0.55–0.6( )	Ternary	Cr^{6+}, Cu^{2+} & Cd^{2+}	0.1
	Cr^{3+}	NA		Cr^{6+}, Cu^{2+} & Mg^{2+}	1
	Cu^{2+}	NA		Cr^{6+}, Mg^{2+} & Cd^{2+}	0.5
	Cd^{2+}	<0.1		Cr^{6+}, Cr^{3+}& Cd^{2+}	0.5
	Mn^{2+}	NA		Cr^{6+}, Mg^{2+}& Cr^{3+}	0.5
	Mg^{2+}	<1		Cr^{6+}, Cu^{2+}& Cr^{3+}	0.07
Binary	Cr^{3+} + Cr^{6+}	0.1	Quaternary	Cr^{6+}, Cd^{2+}, Mg^{2+} & Cr^{3+}	0.2
	Cu^{2+} + Cr^{6+}	<0.01		Cr^{6+}, Cd^{2+}, Cu^{2+} & Cr^{3+}	0.1
	Cd^{2+} + Cr^{6+}	< 0.01		Cr^{6+}, Cu^{2+}, Mg^{2+}& Cr^{3+}	0.07
	Mn^{2+}+ Cr^{6+}	NA		Cr^{6+}, Cu^{2+}, Mg^{2+} & Cd^{2+}	0.2
	Mg^{2+}+ Cr^{6+}	NA	Quinary	Cr^{6+}, Cr^{3+}, Cu^{2+}, Mg^{2+} and Cd^{2+}	0.03

the concentration of 0.1ppm, but as the volume of Cr^{6+} reaches over 0.5 ppm the combination suppresses the inhibition potential of individual Cr^{6+} and also had an IC_{10} value. Another blending of Cr^{6+} with Cr^{3+}& Cd^{2+} (Set IV) was observed to perform better at

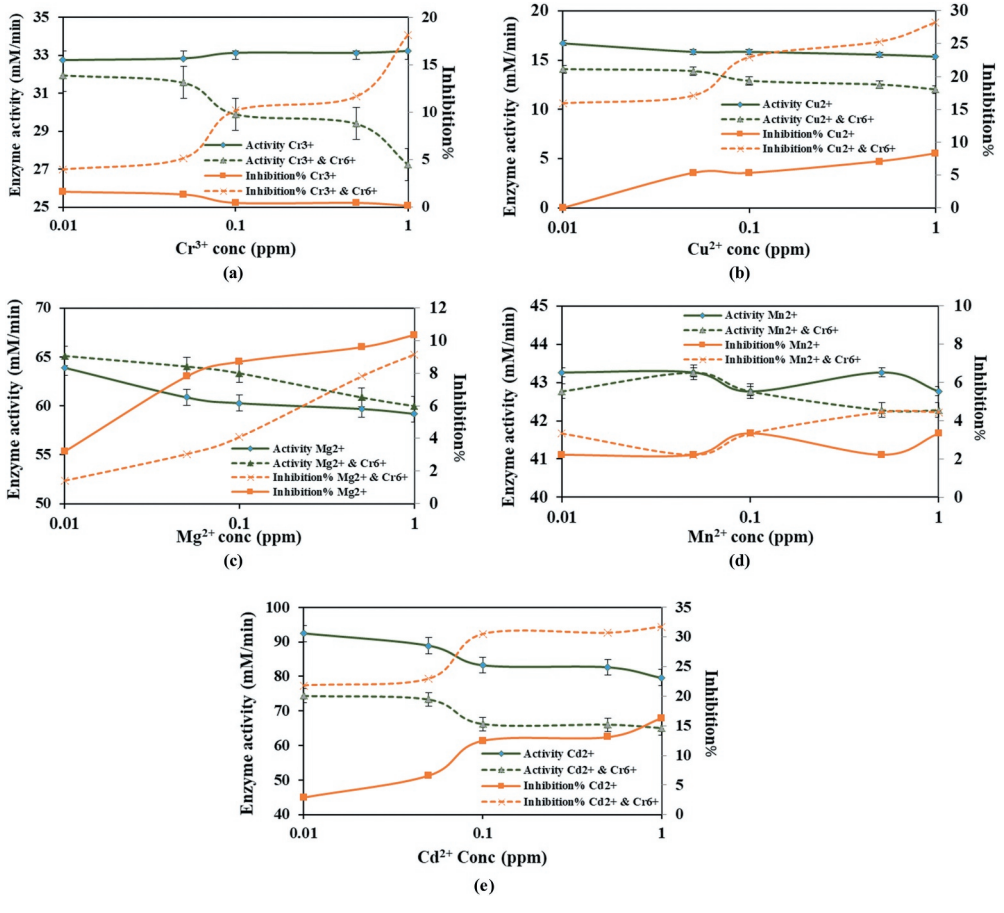

Figure 5. Effect of individual metals (a) Cr^{3+}, (b) Cu^{2+}, (c) Mg^{2+}, (d) Mn^{2+}, (e) Cd^{2+} and combination with 1 ppm of Cr^{6+} to each on HRP activity.

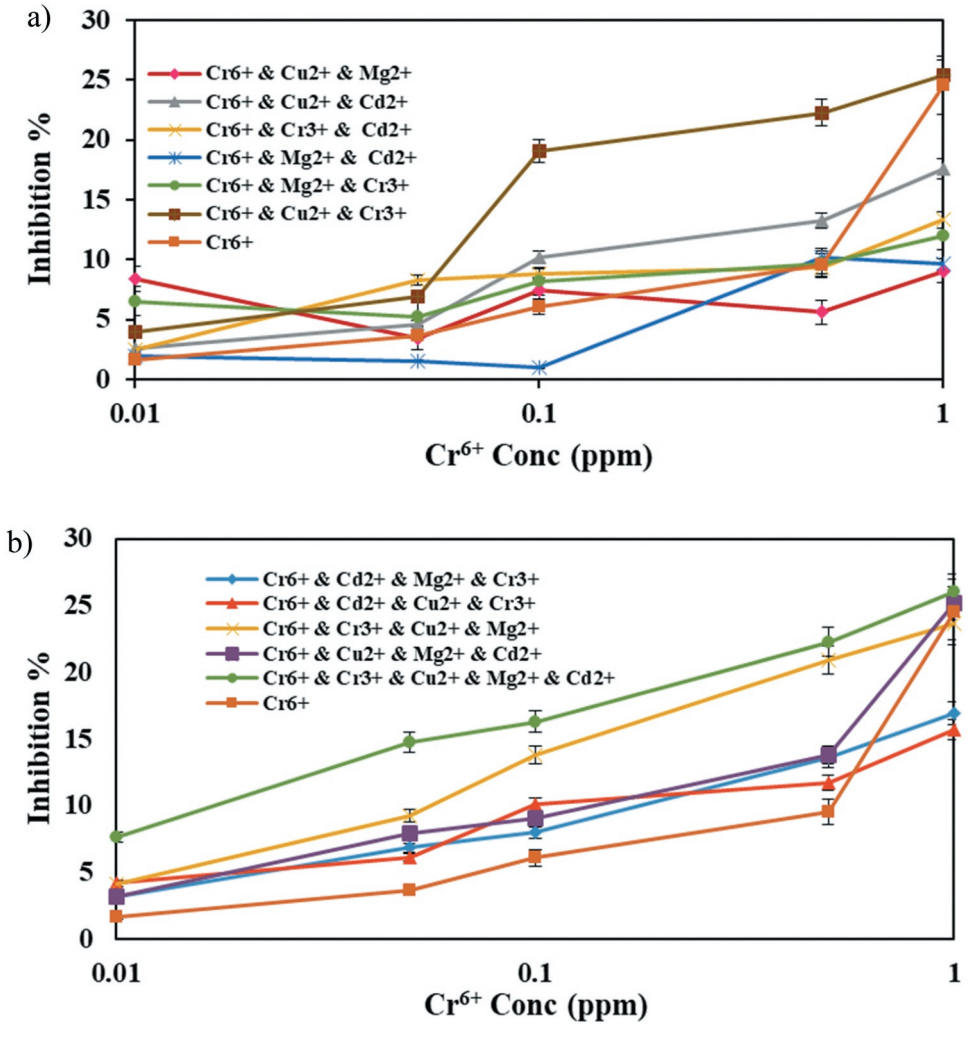

Figure 6. Inhibition assay of HRP by (a) ternary (b) quaternary and quinary combinations of heavy metal ions and comparison with the inhibition pattern of Cr^{6+}.

a lower concentration of Cr^{6+} (IC_{10} value at 0.5ppm) and as the amount of inhibitors increases the activity rate of the mixture decreases compared to their singular forms.

Grouping of Cr^{6+} with Mg^{2+} & Cr^{3+} (Set V) was more identical to the individual performance of Cr^{6+} as for both the top inhibition activity was at 0.5ppm. In this case, both combined metal and single Cr^{6+} gave the IC_{10} value. The sensitive of HRP was a bit more towards the combined metals than Cr^{6+}. The blending of Cr^{6+}, Cu^{2+} and Cr^{3+} (Set VI) was highly effective in HRP activity. It showed the best inhibition characteristic at the concentration of 0.07 ppm as shown in Figure 6(a). Upon reaching the IC_{10} value, the inhibition pattern further inclined and resulted in more than 25% of inhibition at the concentration of 1 ppm, which was more than the inhibition from individual Cr^{6+}. Several researchers had also worked on the aspect of using intermediary metal ions and determining the possible impact on the enzymatic activity of one metal ion by another. The

interference studies shown very good selectivity towards Cr^{3+} and Cr^{6+}, and no perceptible inhibition effect was detected in the presence of metal such as Zn^{2+}, Cu^{2+}, Cd^{2+}, and Pb^{2+}. However, Ni^{2+}, Co^{2+}, and Hg^{2+} were found to have some considerable influence, causing inhibition of the enzymatic activity of HRP [1,8,26].

3.2.3. Inhibition by quaternary and pentavalent combinations of metal ions

In this section, four metal ions were grouped, forming four different combinations, and their enzymatic activity were measured. In the first and second group, Cr^{6+} was added with Cd^{2+}, Mg^{2+}, Cr^{3+} and Cd^{2+}, Cu^{2+} and Cr^{3+}. The presence of three metals inhibits the HRP activity more than the individual Cr^{6+} as the IC_{10} achieved was much lower as 0.2 ppm and 0.1 ppm, respectively (Figure 6(b)). In another mixture, Cr^{6+} was mixed with Cu^{2+}, Mg^{2+}, and Cr^{3+} and at the lowest concentration of 0.01 ppm, the mixture gave 4% inhibition of HRP activity. Increasing the concentration from 0.05 ppm to 1 ppm results in increased inhibition of 23% and had IC_{10} at the concentration of 0.07 ppm. When Cr^{6+} was with Cu^{2+}, Mg^{2+}, and Cd^{2+}, HRP was highly sensitive to the combination of Cr^{6+}, Cu^{2+}, Mg^{2+}, and Cd^{2+}. The inhibition percentage raised with the increasing concentration of Cr^{6+} contrasting to the individual Cr^{6+}. This combination represented the highest inhibition value of 25% at the concentration of 1 ppm Cr^{6+}. At 0.2 ppm, the IC_{10} value was observed, as shown in Figure 6(b).

In the quinary combinations of metal ions (Cr^{6+}, Cr^{3+}, Cu^{2+}, Mg^{2+}, and Cd^{2+}), the enzymatic activity of HRP was highly sensitive. Inhibition rate of up to 8% was observed at the lowest concentration, and it increases to 26% with the increase in Cr^{6+}. The IC_{10} value was also found to occur at a relatively low concentration of 0.03 ppm. A complete list of all the IC_{10} values obtained in the reaction process of all individual and combinations of metal ions are presented in Table 2.

The overall order of inhibition activity was $(Cr^{6+} + Cu^{2+} + Cr^{3+}) > (Cr^{6+} + Cu^{2+} + Cd^{2+}) > (Cr^{6+} + Mg^{2+} + Cr^{3+}) \geq (Cr^{6+} + Cr^{3+} + Cd^{2+}) \geq (Cr^{6+} + Mg^{2+} + Cd^{2+})$. These four combinations highly influenced the enzyme activity. Mostly, the four combinations showed IC_{10} value in between 0.1 ppm to 0.2 ppm of Cr^{6+} concentration. The order of inhibition by four combinations of metal ion was $(Cr^{6+} + Cu^{2+} + Mg^{2+} + Cr^{3+}) > (Cr^{6+} + Cd^{2+} + Cu^{2+} + Cr^{3+}) > (Cr^{6+} + Cu^{2+} + Mg^{2+} + Cd^{2+}) \geq (Cr^{6+} + Cd^{2+} + Mg^{2+} + Cr^{3+})$, while the pentavalent combo $(Cr^{6+} + Cr^{3+} + Cu^{2+} + Mg^{2+} + Cd^{2+})$ was recorded to be the best for inhibition of peroxidase enzyme. The mechanism involved in the inhibition potential of the heavy metals can be attributed to the conformational changes in the protein structure [16]. Possibly, the binding of a first metal ion to HRP would move Arg^{38} away from the haem-group [27]. Arg^{38} maintains the distant between His^{42} and haem-H_2O_2, which was suitable for the reduction reaction [28]. Due to the removal of Arg^{38}, the confirmation got disturbed and affected the enzyme activity. On contrary, Arg^{38} Lue act as a wet enzyme sometimes and thus unfavoured for the initialisations of haem-H_2O_2 complex formation [29].

Several vegetables and plant species have been used to detect the presence of heavy metal in different aqueous solution [19,30,31]. The success with Acetylcholinesterase (AChE) based sensors was quite fascinating [32,33], yet there were numerous physicochemical parameters that were unanswered such as substrate concentration, optimal pH and temperature, the role of interfering heavy metals, kinetics etc. Pandey et al. (2019) [34] attained positive results on graphene oxide electrode modified with electrodeposited thionine and

horseradish peroxidase, however, the inhibition sensitivity after 20 min decreased rapidly and were unable to detect a lower concentration of Cr in the tested sample solutions. Presence of reduced mediator and a matrix of other heavy metals, high substrate concentration often leading to insensitive to inhibition by chromium ions were other potential hitches on the use of HRP based biosensors [8]. The extension of this study with HRP based biosensor provides a unique possibility for determining multiple combinations of heavy metal with respect to Cr in a single bioassay. Recently, biosensor by immobilising HRP on paper were reported to detect catechol and resorcinol in aqueous samples [35]. The embedded enzymatic matrix can easily be incorporated into any device of nano-scale, which further increases in efficiency and feasibility. The results of the study are easily replicable and can be used against different sources of industrial or mining waste. The future is to design or fabricate a hydrogen peroxide (H_2O_2) based low-cost microchip biosensor that can be useful to detect heavy metals in comparison with instrumental techniques even at a relatively low quantity.

4. Conclusion

The study was the first of its kind as it analysed a number of combinations of heavy metal ions with respect to Cr^{6+}. The optimal pH of inhibition of HRP was found to be at 6.4 phosphate buffer. The inhibition of HRP by Cr^{6+} was the non-competitive type of inhibition as the K_m value was constant with different V_{max}. Individually, Cr^{3+} and Mn^{2+} were found to be un-effective in inhibition of HRP activity compare to Cu^{2+}, Cd^{2+} and Mg^{2+}. The presence of heavy metals altered the activities of Cr^{6+} by acting either as uncompetitive or non-competitive inhibitors depending on the type of heavy metals. The order of inhibition by individual metal ions was Cd^{2+}> Cr^{6+}> Mg^{2+}> Cu^{2+}> Cr^{3+}> Mn^{2+} and when mixed with Cr^{6+} was Cd^{2+}> Cu^{2+}> Cr^{3+}> Cr^{6+}> Mg^{2+}> Mn^{2+}. Presence of Cr^{6+} enhances the inhibition ability of Cr^{3+}, Cu^{2+}, and Cd^{2+} while decreasing the inhibition rate of Mn^{2+} and Mg^{2+}. The combination of all five metal ions (Cr^{3+}, Cu^{2+}, Cd^{2+} and Mg^{2+} with Cr^{6+}) was highly inhibitory and had an IC_{10} value at 0.03 ppm inhibiting over 10% of HRP activity. Such formulation and combination will be highly effective for the design and selection of sensitive biosensors for the detection of heavy metals in different environmental sources.

Research highlights

- Free enzyme inhibition kinetics were evaluated for HRP on Cr^{6+} and other heavy metals
- Effect of various heavy metals on Cr^{6+} detection by HRP was outlined
- Sensitivity order for free HRP is Cd^{2+} > Cr^{6+} > Mg^{2+} > Cu^{2+} > Cr^{3+} > Mn^{2+}
- Non-competitive type of inhibition was observed for HRP on Cr^{6+}
- Knowledge of HRP kinetics on Cr^{6+} detection for biosensing applications were highlighted

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Disclosure statement

The authors declare that they do not have any conflict of interest. Further, no animals or human specimens were used in the experiment process.

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