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Research Article

Evaluation of the interaction of two phenothiazines with 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonate) and potassium iodide in horseradish peroxidase reaction

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ABSTRACT: The effect of two phenothiazines: promethazine and chlorpromazine on the initial velocity of horseradish peroxidase (HRP) oxidation of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) and potassium iodide (KI) was investigated. The phenothiazines enhanced the oxidation of ABTS and KI by HRP. Chlorpromazine (10 µM - 100 µM) produced a proportional increase in the initial velocity of HRP for oxidation of KI and ABTS. Increase in promethazine concentration within the range of 10 µM – 60 µM caused a linear increase in the activity of the enzyme. However, higher concentrations of promethazine (60 µM mM to 100 µM) resulted in a proportionate decrease in HRP activity. Unlike promethazine, chlorpromazine caused a linear increase in oxidation products within a range of 10 µM-100 µM. A comparative study of the phenothiazines showed that chlorpromazine was a better redox mediator of the enzyme than promethazine. Hence, the turnover of the enzymatic products depends on the structure of the phenothiazine.

KEYWORDS: Horseradish peroxidase, Promethazine, Chlorpromazine, ABTS, KI

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INTRODUCTION

Peroxidases are ubiquitous enzymes that catalyze a variety of oxygen-transfer reactions and are thus useful for industrial and biomedical applications. However, peroxidases are unstable and are readily inactivated by their substrate, hydrogen peroxide (Valderrama *et al*, 2002). Horseradish peroxidase (HRP) is isolated from horseradish roots (*Amoracia rusticana*) and belongs to the ferroprotoporphyrin group of peroxidases. HRP readily combines with hydrogen peroxide (H_2O_2) and the resultant [HRP–H_2O_2] complex can oxidize a wide variety of chromogenic hydrogen donors.

The catalytic cycle of peroxidases is described usually as a sequence of three consecutive reactions: Compound I (**E_I**), is two oxidizing equivalents above the ground oxidation state. It reacts with a substrate molecule (AH_2) and is converted into a secondary compound that has lost one oxidizing equivalent,

generally indicated as Compound II (**E_{II}**). A second substrate molecule (AH_2) recycles Compound II into the resting enzyme (**E**). The organic cation radical ($AH^{\cdot}$) produced by this oxidative process can initiate free radical reactions. A large excess of H_2O_2 converts Compound I and II into the inactive intermediate, Compound III (**E_{III}**).

$E + H_2O_2 ====> E_I + H_2O$ ----------(1)

$E_I + AH_2 ====> E_{II} + AH^{\cdot}$----------(2)

$E_{II} + AH_2 ====> E + AH^{\cdot} + H_2O$ ---------- ------(3)

In excess H_2O_2,

$E_{II} + H_2O_2 ====> E_{III} + H_2O$----------(4)

$E_I + H_2O_2 == ==> E_{III} + H_2O$----------(5)

The first three reactions dominate as the main reaction path in a mixture of aromatic substrate, hydrogen peroxide and peroxidase. The reaction of H_2O_2 with E and E_{II} is independent of the type of aromatic substrate (AH_2) used, but the relative rate of the consecutive one-electron transfer process depends on the structure and redox potential of AH_2 (Childs and Bardsley, 1975).

2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), is a synthetic nitrogen-substituted aromatic compound which allows the oxidation of non-phenolic lignin model compounds (Bourbonnais and Paice,1990) and the delignification of kraft pulp (Bourbonnais *et al* 1975) by laccase. Iodide ion (I^-) is a mild reducing agent and is easily oxidized to I_2 by powerful oxidizing agents. The phenothiazines are a group of compounds having excellent electron donating properties leading to the formation of relatively stable free radical cations (Levy *et al*., 1972). It has been hypothesised that even at physiological pH, other sulfur containing drugs e.g. phenothiazines could act as a pro-oxidant when such drugs become oxidized by peroxidases. The concept of redox mediation has been explored in some studies with the goal of improving the overall catalytic efficiency of peroxidase oxidation reaction (Olorunniji *et al*., 2000; Malomo *et al*., 2011).

This study aims at identifying the structural functions of two phenothiazines: chlorpromazine and promethazine in the HRP oxidation of ABTS and KI.

MATERIALS AND METHODS

Horseradish peroxidase, promethazine, chlorpromazine, ABTS, KI, sodium dihydrogen phosphate, disodium hydrogen phosphate, and hydrogen peroxide (30%) were of analytical grade and purchased from Sigma-Aldrich (Dorset, Poole, United Kingdom). All kinetic measurements were carried out using a UV-780 recording spectrophotometer.

Enzyme Preparation

HRP solutions were prepared by dissolving carefully weighed crystals of the pure enzyme in 100 mM sodium phosphate buffer, pH 7.0.

Determination of initial velocity of HRP

The initial velocity of HRP was determined by measuring the rate of oxidation of KI and ABTS at 25 ^{o}C in a 3.0 ml reaction mixture containing 2.3 ml of 400 mM sodium phosphate buffer (pH 5.4), 0.2 ml of 1000μM - 3000μM KI (or ABTS 25 μM –75 μM), 5 μg (in 0.1ml) of HRP, and 0.2 ml of varying concentrations of promethazine or chlorpromazine (10 μM - 100 μM). In all cases, 0.2 ml of 1 μM –1000 μM H_2O_2 were added last to initiate the reaction. Initial velocity of HRP for KI and ABTS oxidation was determined as a function of absorbance from the time course at 353 nm and 414 nm respectively.

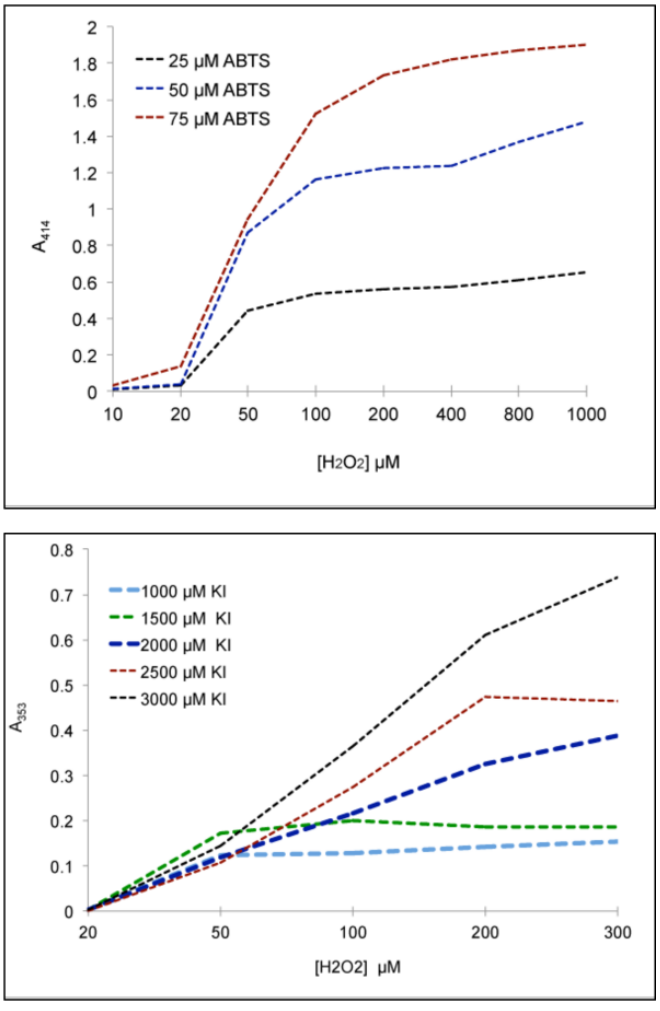

Figure 1: Effects of hydrogen peroxide concentration and donor substrates concentrations on HRP-catalysed oxidation of ABTS (1A) and KI (1B).

RESULTS

Figure 1A shows the effect of hydrogen peroxide concentration on change in absorbance due to HRP-catalysed oxidation of ABTS. Results show no significant amount of $ABTS^+$ formed by the enzyme when the concentration of H_2O_2 was less than 20 µM. However, a further increase in H_2O_2 concentration led to a steady appearance of $ABTS^+$. This steady appearance of $ABTS^+$ peaked at 300 µM of H_2O_2 concentration and a further increase in H_2O_2 above 400 µM did not result in any significant increase in the concentration of $ABTS^+$ radial when the ABTS concentration was 25 µM. The absorbance value of $ABTS^+$ at any concentration of H_2O_2 greater than 20 µM was proportional to the concentration of ABTS used at the start of the reaction.

Figure 1B shows the effect of varying H_2O_2 and potassium iodide concentrations on the absorbance change (at 353 nm) in the formation of triiodide. No visible reaction was observed when the concentration of H_2O_2 was less than 20 µM. However, increasing the concentration of H_2O_2 above 20 µM led to a steady formation of the triiodide radical. On the average, the concentration of the triiodide radical formed by HRP peaked when the concentration of H_2O_2 was within the range of 200 µM to 300 µM, after which a steady disappearance of the iodide radical was observed with further increase in H_2O_2 concentration.

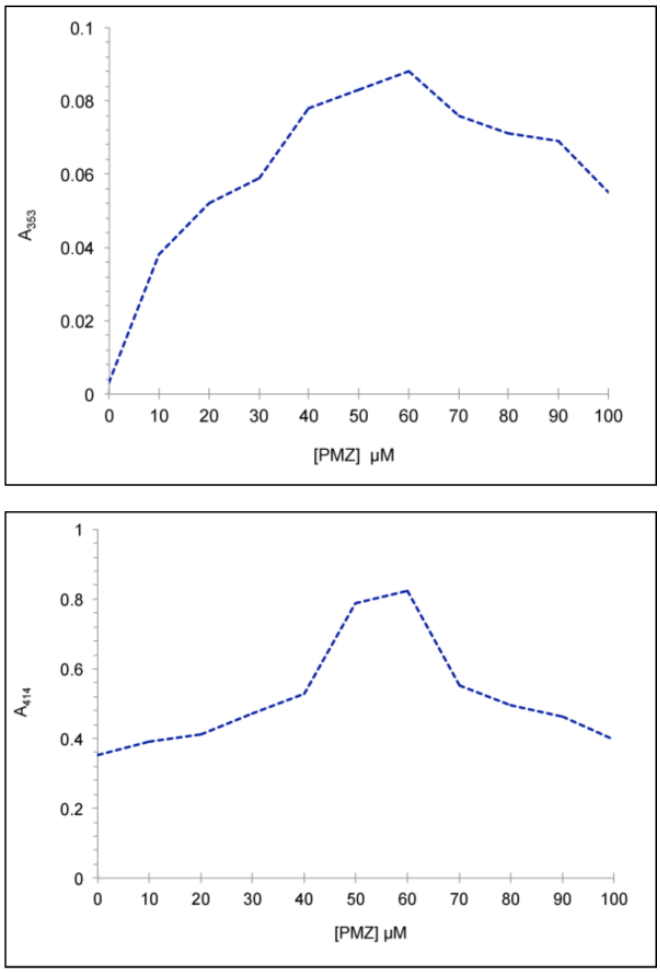

Figure 2: Effects of varying concentration of promethazine on HRP-catalysed oxidation of KI (A) and ABTS (B).

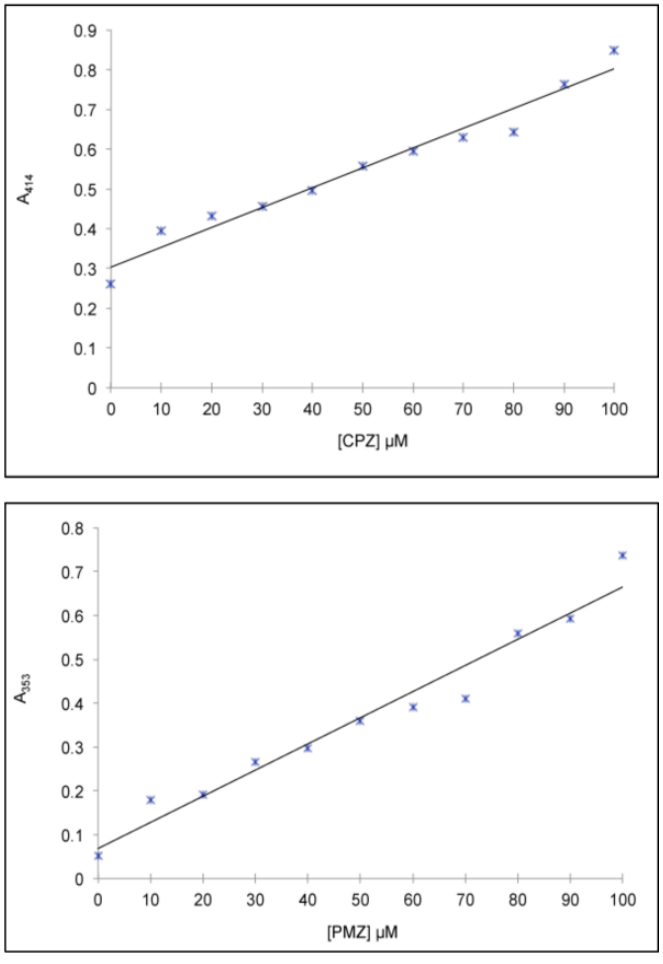

Figure 3: Effects of varying chlorpromazine concentration on HRP-catalysed oxidation of ABTS (A) and KI (B)

Figure 2A shows the effect of varying concentration of promethazine on the initial velocity of HRP-catalysed oxidation of KI. The results show an increased initial velocity as the concentration of PMZ was increased from 10 µM to 60 µM. Further increase in promethazine concentration above 60 µM resulted in the gradual decrease in the accumulation of the triiodide product. This trend of HRP activity observed within the range of promethazine concentration of 10 µM and 60 µM may be implicated in the formation of promethazine sulphoxide which enhances the oxidation of KI. However, further increase in promethazine concentration may lead to an increased formation of promethazine sulfoxide which consequently reacts faster with KI to form the triiodide radical. An increased formation of iodide radical may lead to a dissociation reaction of iodide radicals to form iodide and iodine. Consequently, the amount of available iodide radical at higher promethazine concentration reduces (Childs and Bardsley, 1975). A similar trend was observed when ABTS was used instead of KI (Figure 2B).

The reaction pattern observed when chlorpromazine was used as the cooxidant in the HRP-catalysed oxidation of KI and ABTS was different from that seen with promethazine. A steady increase in the accumulation of the reaction product was observed as chlorpromazine concentration was increased from 10 µM to 100 µM in its cooxidation with each of ABTS and KI respectively by HRP (Figures 3A and 3B)

DISCUSSION

Determination of optimal hydrogen peroxide concentration for HRP catalysis

Peroxidases are subject to inactivation by hydrogen peroxide and other hydroperoxides at relatively high concentrations (Dunford, 1982), despite the fact that these enzymes require the same oxidants to catalyse their reactions (Choi *et al*, 1999). Therefore, the balance of H_2O_2 concentration is one of the most important parameters in peroxidase enzymatic reaction. Hence, in order to determine the stoichiometric non-inactivating concentration of H_2O_2 to use for this study, the concentration of H_2O_2 was varied from 1µM to 1000 µM (Figures 1A and 1B).

Data presented in Figure 1A showed that visible oxidation of ABTS commenced when the concentration of hydrogen peroxide approached 20 µM irrespective of the concentration of ABTS. The rate of ABTS oxidation was proportional to hydrogen peroxide concentration up to 400 µM hydrogen peroxide concentration. This trend was same within a range of 25 µM and 75 µM ABTS concentration. This result is similar to that obtained by Kim *et al*. (2004) in a study of the peroxidase activity of cytochrome *c* using ABTS as a chromogenic substrate. It was observed that the initial rate of ABTS oxidation was linear with respect to the concentration of cytochrome *c* between 2.5-10 µM and of H_2O_2 between 100 µM and 500 µM.

Figure 1B showed that formation of triiodide increased as reflected in increased absorbance with increasing H_2O_2 concentration up to 200 µM and then decreased with further increase in H_2O_2 concentration.

The data presented in Figures 1A and 1B are in agreement with previous studies (Dunford, 1982). In an experiment with HRP, ABTS as the reductant, the initial rate decreased at H_2O_2 concentrations higher than 4000µM in an ABTS concentration range of 50 µM - 28000 µM. The kinetics of veratryl alcohol (VA) oxidation by lignin peroxidase (LIP) showed a similar pattern (Hu and Korus,1993). Inhibition of peroxidase activity appeared at H_2O_2 concentration above 400µM in a VA concentration range between 80 µM and 200 µM. However, studies on phenol oxidation demonstrated that the initial rate did not decrease and followed Michaelis-Menten type-kinetics when the phenol concentration was 5000 µM and the H_2O_2 concentration was above 10,000 µM (Vasudevan and Li, 1996). We conclude that under the conditions used in this study, the optimal concentration of hydrogen peroxide for HRP catalysis using ABTS and KI is 200 µM.

The effect of concentration of the phenothiazines on HRP activity

The inhibitory effect of promethazine concentration greater than 60 µM on HRP catalysis (Figure 2A) was similar to a study where EDTA was found to inhibit catalytic cooxidation of iodide by HRP in a concentration dependent manner (Bhttacharyya et al, 1989). The observed mechanism found in this reaction of EDTA could be the same as that observed in this HRP catalyzed cooxidation of promethazine and KI. EDTA inhibits the catalytic activity of HRP not by acting as a metal ion chelator but by acting as an electron donor. Kinetic studies indicated that EDTA competitively inhibits iodide oxidation (Baanerjee, 1989) suggesting that it acts as a co-substrate. EDTA also inhibits oxidation of guaiacol competitively and the effect was reversed by higher concentrations of the donor (Bhttacharyya *et al*, 1989)

The results shown in Figure 2A suggest that promethazine actually enhanced the oxidation of KI. This is due to the formation of $PMZ^{\cdot+}$ from promethazine which serves as the electron shuttle between the enzyme and potassium iodide. However, what appears to be an inhibition of iodide oxidation when the concentration of promethazine exceeded 60 µM may be due to a decomposition reaction of the oxidation product of potassium iodide: triiodide (I_3^-) which is formed in excess, to Iodine (I_2) and I^-. Consequently, I_3^- will be consumed as it is being formed. Another possible explanation is that protonated promethazine reacts with the triiodide forming highly stable and insoluble ion pair products, thus limiting the availability of I_3^-.

The pattern of HRP-catalysed cooxidation of chlorpromazine with ABTS and KI (Figures 3A and 3B respectively) was in contrast to previous studies in which chlorpromazine rapidly inactivated cholinesterase in the presence of HRP-H_2O_2 (Muraoka and Miura 2002). Incubation for 10 minutes caused

almost complete loss of cholinesterase activity, and chlorpromazine had no effect on cholinesterase in the absence of HRP-H_2O_2. The results indicated that cholinesterase activity was lost during oxidation of chlorpromazine by HRP-H_2O_2. Inactivation of cholinesterase was dependent upon the concentrations of chlorpromazine.

Results shown in Figures 3A and 3B in which chlorpromazine was used as the redox mediator suggest that increasing the concentration of chlorpromazine enhances ABTS and potassium iodide oxidation. The difference in the observed pattern between promethazine and chlorpromazine, both having the same phenothiazine backbone may be due to the attached functional group.

In a study on the peroxidase activity of hemoglobin in the sulfoxidation of chlorpromazine, it was found that only in the presence of H_2O_2 was chlorpromazine converted to chlorpromazine sulfoxide in significant amount (Kelder *et al*., 1989). Chlorpromazine enhanced the autoxidation of oxyhemoglobin, without being transformed itself (Kelder *et al*., 1989). This ability of chlorpromazine to cause auto oxidation reaction without it being transformed to its sulphoxide, could likewise account for the observed trend of initial velocity of HRP observed in Figures 3A and 3B. This is also similar to results obtained with the spectral change of chlorpromazine when its aerated solution in water (pH 4.7) is irradiated by 253.7nm light from a low pressure mecury lamp (Iwaoka and Kondo, 1974). The ultraviolet spectrum obtained after the irradiation up to 40 seconds was ascribed to chlorpromazine sulfoxide but a considerable amount of starting chlorpromazine still remained.

Studies on stopped flow kinetic investigations of one electron transfer reaction of phenothiazines and their radical cation in aqueous solutions reveal that among the phenothiazines investigated, chlorpromazine was found to be more reactive than promethazine (Venkatasubramanian and Maruthamuthu, 1989). The rate constant measured for the formations of CPZ^+ and PMZ^+ by two inorganic peroxides (peroxomonosulfate and peroxodisulfate) indicate that chlorpromazine reacts 60-70 times faster than promethazine. The difference in reactivity was probably due to the difference in redox potential of the phenothiazines (E_{CPZ}= 0.78V, E_{PMZ} =0.86V). In the same study, it was shown that although the two radical cations of promethazine and chlorpromazine individually oxidize ascorbic acid to revert back to their parent compound, only chlorpromazine radical was found to react with other reducing sulphur compounds, sulfites, thiosulfate and dithionite.

Our findings from this study suggest that phenothiazines, when used as redox mediators can enhance the turnover of some enzymatic products in HRP reactions. However, the catalytic activity of HRP depends on the structure of the phenothiazine and the donor substrate.

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