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The Comparison of the Protective Effect of Ascorbate and *O*-Dianisidine on the Inactivation of Peroxidase from *Gongronema latifolium*

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Abstract: Peroxidase from Gongronema latifolium was extracted and purified, on a two-step purification process of ammonium sulphate precipitation followed by dialysis the inactivation of peroxidase, by hydrogen peroxide over different periods of time and the comparison of the protective effect of 1mM ascorbate and 0.4mM o-dianisidine on the inactivation of peroxidase by different concentrations of hydrogen peroxide (0.1, 1, 5, 7.5 and 10mM) over a period of time was done. Peroxidase from Gongronema latifolium was purified,6.8 fold with a specific activity of 2.04 when o-dianisidine was used as substrate and purification fold of 2.49 with a specific activity of 3.71 when guaiacol was used as substrateThe inactivation of peroxidase from Gongronema latifolium by hydrogen peroxide was time dependent and it also showed a biphasic inactivation curve with the initial fast phase and a slower second phase. From our results, 1mM of ascorbate had above 20% protection on the inactivation of enzyme in all the concentrations of hydrogen peroxide while o-dianisidine had above 15% in all the concentrations of hydrogen peroxide.

Key weords: *O*-dianisidine · Ascobate and Hydrogen peroxide

INTRODUCTION

Peroxidases (donor: hydrogen peroxide oxidoreductases) are ubiquitous enzymes that catalyze the oxidation of substrate at the expense of hydrogen peroxide (H_2O_2) [1, 2, 3, 4, 5, 6]. The heme peroxidases have been classified into two distinct groups, termed the animal (found only in animals) and plant (found in plants, fungi and prokaryotes) superfamilies [7, 8, 9]. They are ubiquitous in nature and are involved in various physiological processes in plants. Studies have suggested that peroxidases play a role in lignification, suberization, cross-linking of cell wall structural proteins, auxin catabolism, self-defense against pathogens and senescence [10]. Becauseof their widespread distribution and substrate diversity, they are useful as catalysts in clinical biochemistry and enzyme immunoassays. Some of their new applications include treatment of waste water, bio-bleaching and synthesis of several aromatic compounds [11]. The function of all the forms of peroxidase in plants is thought to be the scavenging of the H_2O_2 that is continuously generated in cells [12]. For instance, in the chloroplasts of photosynthetic organisms superoxide (O_2^{o-}) is formed when insufficient CO_2 is available to balance electrons being generated by the photosystems; these excess electrons then reduce O_2 to O_2^{o-}. Additionally, in the mitochondria the electron transport chains can also produce O_2^{o-}. In both cases superoxide dismutase converts O_2^{o-} into H_2O_2 which peroxidase or catalase can then remove. The first step in the catalytic cycle of peroxidase is the reaction between H_2O_2 and the Fe(III) resting state of the enzyme to generate compound I, a high oxidation state intermediate comprising an Fe(IV) oxoferryl centre and a porphyrin-based cation radical. A transient intermediate (compound 0) formed prior to compound I has been detected in reactions between HRP C and H_2O_2 at low temperatures and described as an Fe(III)-hydroperoxy complex. Molecular dynamics simulations of these peroxide-bound complexes have been carried out [13 and 14]. In formal terms, compound I is two oxidising equivalents above the resting state. The first one-electron reduction step requires the participation of a reducing substrate and leads to the generation of compound II, an Fe(IV) oxoferryl species that is one oxidising equivalent above the resting state. Both compound I and compound II are powerful oxidants, with redox potentials estimated to be close to +1 V. The second one-electron reduction step returns compound II to the resting state of the enzyme. Reaction of excess hydrogen peroxide with the

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resting state enzyme gives compound III, which can also be prepared by several [15, 16 and 17], other routes that lead to the degradation of haem, the release of iron [6] and the formation of two fluorescent products and inactivation of the enzyme. This intermediate is best described as a resonance hybrid of iron(III)-superoxide and iron(II)-dioxygen complexes. A high-resolution crystal structure of 95% pure compound III published recently shows dioxygen bound to haem iron in a bent conformation[14]. *Gongronema latifolium,* most commonly utize in the South Eastern part of Nigeria. The parts commonly used are the leaves, stem and root [12, 13]. The origin of the plant is traced to Nigeria in West Africa.. It is a rainforest plant which has been traditionally used in the South Eastern part of Nigeria over the ages for the management of diseases such as diabetes and high blood pressure. *G latifolium* is a woody tropical plant. It has bitter taste and the ideal soil for growing it is red late rite soil. It is a slender climber, often 3-4 m long, but able to climb to the canopy of high trees, with woody base and fleshy roots, containing latex. The inactivation of peroxidase has been study in other vegetable but not in.

MATERIALS AND METHODS

Sample Collection and Location: Matured fresh leaves of *Gongronema latifolium* were purchased from Ogige Market in Nsukka Senatorial Zone of Enugu StateBovine serum albumin (BSA) (Merck, England.) Folin-Ciocalteau Phenol Reagent (Sigma-Aldrich, Germany.) Guaiacol Hydrogen peroxide (BDH pool, England.) *O*-Dianisidine.

Preparation of Enzyme Extract: The leaves of *G. latifolium* were left under room temperature to dry for 24 hours. A known weight, 35g was weighed out and finely ground with pestle and mortar. The powdered sample was put into a beaker, after which 200ml of 0.01M ice cold phosphate buffer (pH 6.5) was added. The mixture was stirred and the solution was filtered using cheese cloth. The filtrate was collected and centrifuged at 4000 rpm for 30minutes in order to remove chlorophyll. The supernatant was measured and found to be 160ml. The enzyme extract was stored in the refrigerator.

Peroxidase Assay Using *O*-Dianisidine as Substrate: Peroxidase activity was assayed using the modified method of Mclellan and Robinson (1987) and Eze *et al*., (2010). The change in absorbance at 460nm due to the oxidation of *o*-dianisidine in the presence of hydrogen peroxide and ezyme extract at 30°C was monitored using Jenway 6405 UV/VIS Spectophotometer. The standard assay solution contained 0.3ml of 0.1% *o*-dianisidine, 0.2ml of hydrogen peroxidase, 2.4ml of sodium phosphate buffer pH 6.5 and 0.1ml of enzyme extract in total of 3.0 ml.

One unit of enzyme activity was defined as the amount of enzyme that gave an absorbance change. = 0.1/min at 30°C.

Inactivation of Peroxidase by Hydrogen Peroxide: The same volume of enzyme and hydrogen peroxide of different concentrations (0.0M, 0.1M, 1.0M, 5.0M, 7.5M and 10M) were incubated at different time (from 0 minute to 10 minutes) . The incubated mixture served as the enzyme. The residual activity was assayed using an assay mixture of, 2.4ml of 0.1Mphosphate buffer, 0.1ml of enzyme, 0.3ml of *o*-dianisidine and 0.2ml of H_2O_2. The % residual activity was calculated using the relationship.

$$\% \text{ Residual activity} = \frac{At}{Ao} \times 100$$

where At is activity at present, Ao activity at time 0. The % residual activity was plotted against different times of incubation.

In a similar experiment the enzyme was incubated with either 0.4mM *o*-dianisidine or 1.0mM ascorbate with different concentrations of hydrogen peroxide (0.0M, 0.1M 1.0M, 5.0M, 7.5M and 10M) at different times (from 0 minute to 10 minutes). And the residual activity was assayed using *o*-dianisidine as the reducing substrate.

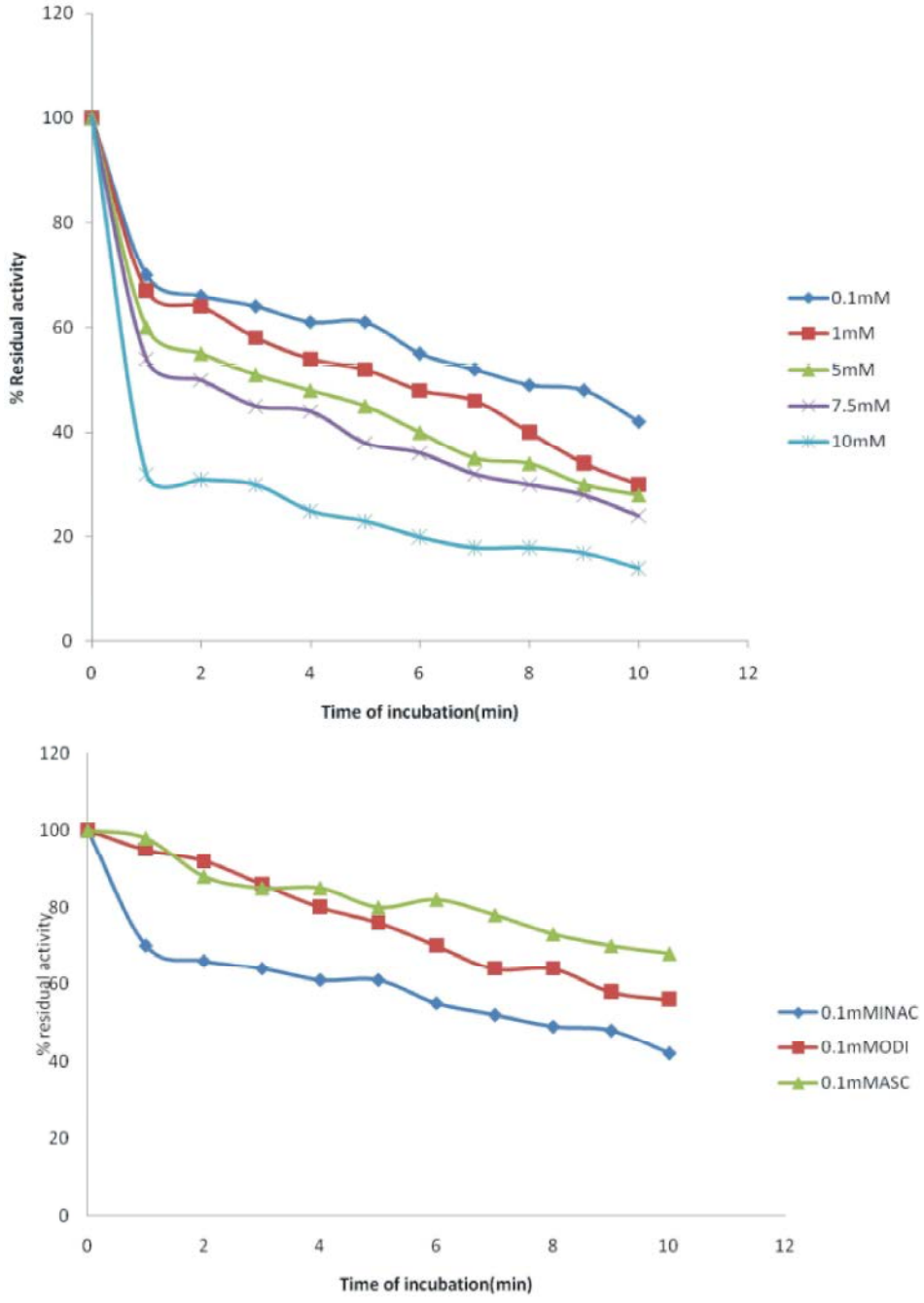
RESULTS

Purification of Peroxidase: Table 1 shows the purification profile of peroxidase from *Gongronema latifolium* on a two-step purification process of ammonium sulphate precipitation followed by dialysis.The enzyme was purified 2.49 fold with a specific activity of 3.71 when guaiacol was used as substrate and a purification fold of 6.8 and specific activity of 2.04 when *o*-dianisidine was used as the substrate

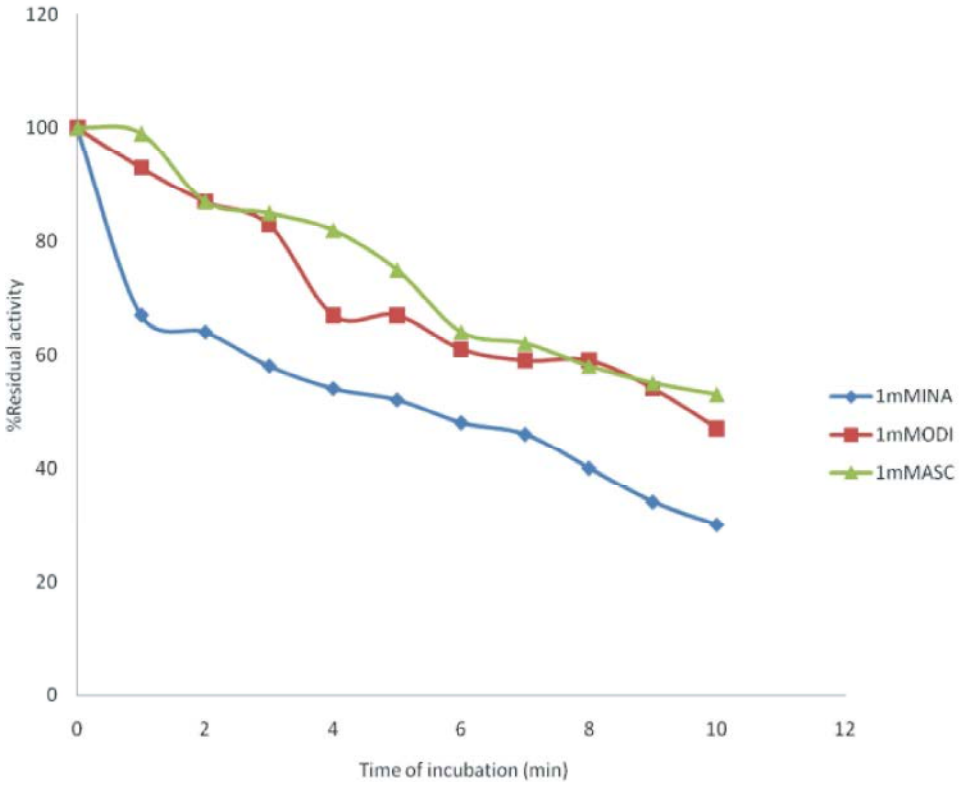
The Inactivation of Peroxidase by Hydrogen Peroxide: Figure 1 shows the inactivation of peroxidase, by hydrogen peroxide over different periods of time. The inactivation shows a biphasic inactivation curve with the initial fast phase and a slower second phase.

Table 1: Purification Table

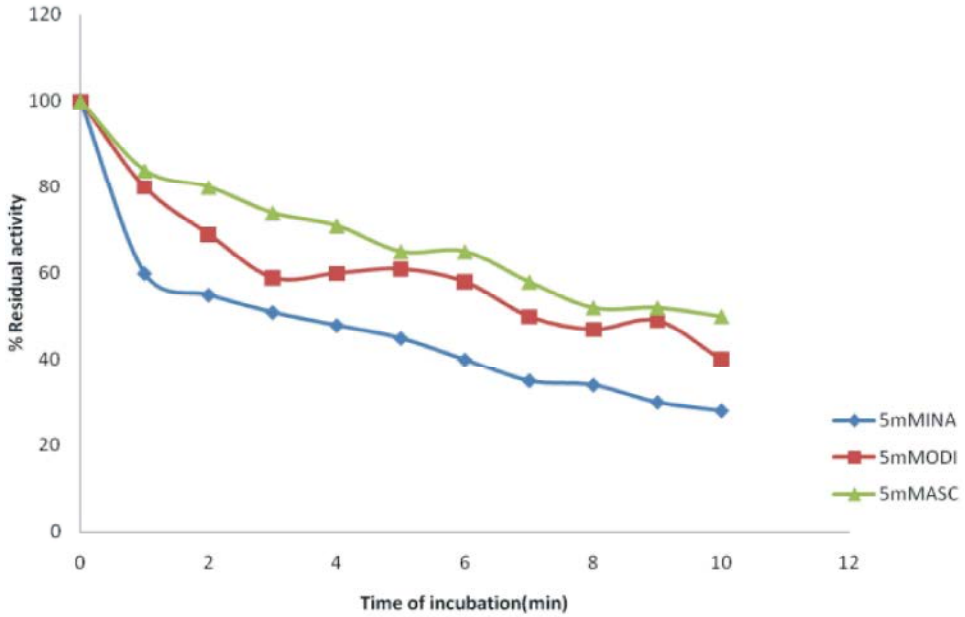
Procedure	Total Protein		Specific Activity		Total Activity		Activity yield		Purification factor	
S	A	B	A	B	α	B	A	B	α	B
Crude Enzyme	6.0	7.53	0.30	1.30	1.36	5.0	100	100	1.0	1.0
70% $NH_4(SO_4)_2$ ppt	6.0	31.09	1.02	1.23	196	230	34	9.45	3.4	0.95
Dialysed Enzyme	8.0	8.02	2.04	3.71	131	238	90	37.98	6.8	2.49



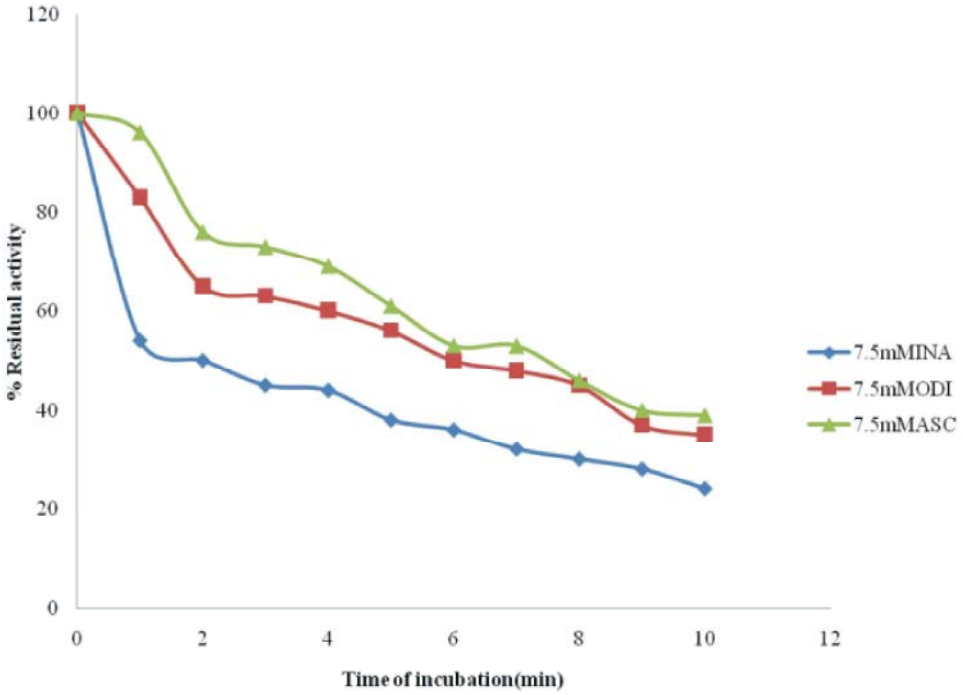
The Comparison of the Effect of Ascorbate and *O*-dianisidine on Inactivation of Peroxidase by 0.1mm Hydrogen Peroxide: From Figure 2, ascorbate had 26% protection at 1 minute of incubation and 28% at 10 minutes of incubation, while o-dianisidine had 14% at 1minute and 25% at 10 minutes of incubation respectively, against the inactivation of peroxidase by hydrogen peroxide.



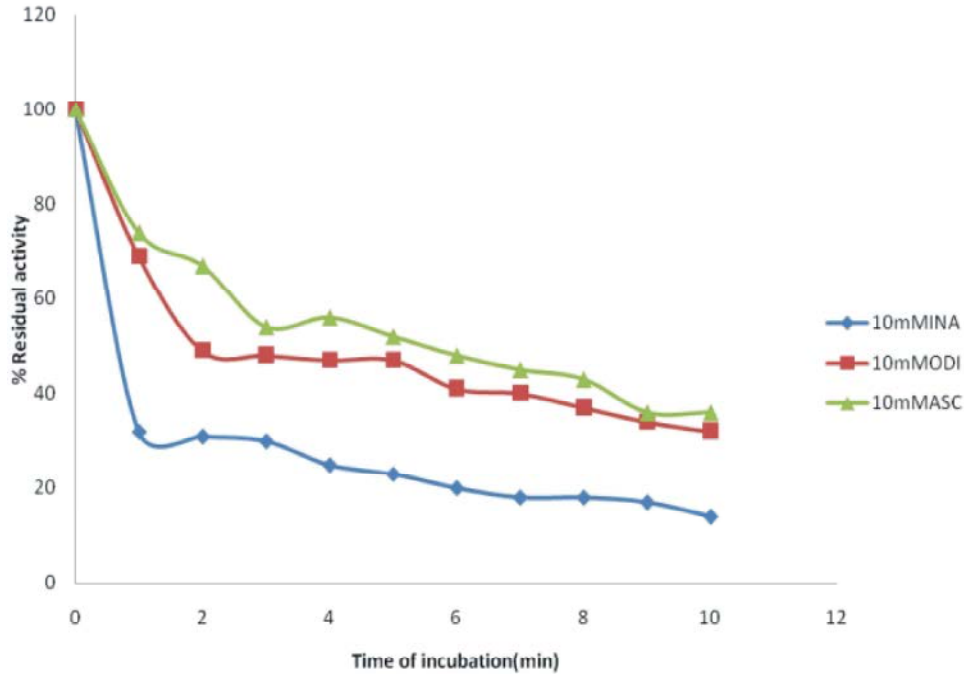
The Comparison of the Protective Effect of Ascorbate and *O*-Dianisidine on Inactivation of Peroxidase by 1mm of Hydrogen Peroxide: Figure 3 shows that ascorbate exhibited 22% protection and o-dianisidine 16%, at 1 minute of incubation. While at 10 minutes of incubation ascorbate and o-dianisidine show 32% and 26% protection respectively, against inactivation.



The Comparison of the Protective Effect of Ascorbate and *O*-Dianisidine on the Inactivation of Peroxidase by 5mm of Hydrogen Peroxide: From figure 4 there was 24% protection by ascorbate and 20% by o-dianisidine at 1 minute of incubation and at10 minutes 22% for ascorbate and 20% for o-dianisidine respectively, against inactivation.



The Comparison of the Protective Effect of Ascorbate and *O*-Dianisidine on the Inactivation of Peroxidase by 7.5mM of Hydrogen Peroxide: Figure 5 shows that at 1 minute of incubation there was 42% protection for ascorbate and 37% for o-dianisidine and at 10 minutes, 17% and 15% respectively for ascorbate and o-dianisidine. Also at 8 minutes of incubation the protection was the same for the two reducing substrates.



The Comparison of the Protective Effect of Ascorbate and *O*-Dianisidine on the Inactivation of Peroxidase by 10mM of Hydrogen Peroxide: From Figure 6, at 1 minute of incubation there was 42% protection by ascorbate and 37% by o-dianisidine while at 10 minutes, 22 % for ascorbate and 18% for o-dianisidine with ascorbate having the higher protective effect, against inactivation.

DISCUSSION

The purification profile of peroxidase from *Gongronema latifolium* on a two-step purification process of ammonium sulphate precipitation and dialysis yielded enzyme with 2.49 fold of purification and specific activity of 3.71 when guaiacol was used as a substrate, while a purification fold of 6.8 and specific activity of 2.04 was obtained when *o*-dianisidine was used as substrate. Yihong *et al*. [15] reported a purification fold of 17.92 on peroxidase from lettuce stems on a three-step purification of ammonium sulphate precipitation, G-100 filtration and concanavalin A affinity chromatography. Also Melda *et al*. [11] reported a purification fold of 9.7 with a three-step purification process of ammonium sulphate precipitation, dialysis and a CM Sephadex ion exchange chromatography on peroxidase from Turkish black radish. On a four-step purification process of Sephadex G-25, ammonium sulphate precipitation, DEAE Sepharose and cancanavalin A Sepharose, peroxidase from horseradish was purified 2692 fold. The low purification fold from this result was as a result of only two-step purification process used, when compared to the three or four-step purification reported on other sources of peroxidases.

The result obtained when the enzyme was incubated with different concentrations of hydrogen peroxide over different time, reveals that the inactivation of peroxidase from *Gongronema latifolium* by H_2O_2 was time dependent. The inactivation shows a biphasic inactivation curve with the initial fast phase and a slower second phase which is similar to the findings of [7] that ascorbate peroxidase inactivation is time dependent. Also, the inactivation of ascorbate-free peroxidase from *Leihmania major* was time dependent and an irreversible inactivation of the enzyme followed a pseudo-first-order kinetics [1]. The catalase-like reaction of H_2O_2 with HRP is the dominant pathway of enzyme turnover in the absence of reducing substrate and accounts for almost all the protection of HRP against inactivation by H_2O_2 [8].

In the chloroplasts of higher plant, ascorbate is formed at a concentration from 10 to 50mM [5]. In this study peroxidase was protected against inactivation by H_2O_2 in the presence of a fixed concentrations of reducing substrates, (0.4mM of o-dianisidine or 1mM of ascorbate) when added during the incubation time. From our results, 1mM of ascorbate had above 20% protection on the enzyme in all the concentrations of hydrogen peroxide while o-dianisidine had above 15% in all the concentrations of hydrogen peroxide. A similar effect has been seen with ascorbate peroxidase (APX), that was reported at 80% protection on the enzyme when 1µM of ascorbate or pyrogallol was incubated with the enzyme, 90% with 10µM and there was complete protection on the enzyme when 100µM of the reducing substrates were incubated [8]. Similar effect has been seen with HRP. There was complete protection when a large amount of reducing substrate was used [2]. The reduced protective effect by ascorbate and *o*-dianisdine from these results may be due to the low concentrations of the reducing substrates used in the study. From this work, it was observed that ascorbate has a higher level of protection on the enzyme, when compared with o-dianisidine. The protective effect of the enzyme by *o*-dianisidine is an evidence that the enzyme not only contains APX alone. APX has been known not to demonstrate any reaction with phenolic substrates.

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