THE EFFECT OF HYDROGEN PEROXIDE ON PEROXIDASE (EC 1.11.1.7) FROM GONGRONEMA LATIFOLIUM

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BY

OGANA, JOY
(PG/M.Sc/09/51015)

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF NIGERIA, NSUKKA

SUPERVISOR: DR S.O.O.EZE

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APPROVAL PAGE

Ogana, Joy, a postgraduate student of the Department of Biochemistry with Registration Number, PG/M.Sc/09/51015, has satisfactorily completed the requirements of research work for the degree of Master of Science (M.Sc.) in Biochemistry (Environmental Biotechnology). The work embodied in this dissertation is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

…………………………
…………………………

Dr S. O.O. Eze
(Supervisor)

Prof. L. U. S. Ezeanyika
(Head of Department)

…………………………

External Examiner
DEDICATION

This work is dedicated to God Almighty. And to my mother, Mrs M.N. Ekpenisi, who bore the burden of my sponsorship of this programme.
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ABSTRACT

Peroxidase (EC 1.11.1.7) extracted from Gongronema latifolium was purified, on a two-step purification process of ammonium sulphate precipitation followed by dialysis. The enzyme was purified 6.8 fold with a specific activity of 2.04 when o-dianisidine was used as substrate. When the enzyme was subjected to different concentrations of hydrogen peroxide and o-dianisidine, the peak activity was 17.75µ/ml at 5mM for hydrogen peroxide and for o-dianisidine the peak activity was 2.4µ/ml observed at 0.4mM. The optimum pH and temperature were at pH 7.0 and 30°C respectively. The Km and Vmax for hydrogen peroxide were 1.8mM and 20u/ml and o-dianisidine had Km of 0.12mM and Vmax of 3.3 μ/ml. The inactivation of peroxidase extracted 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. About 20% protection of the enzyme against inactivation was obtained when 1mM ascorbate was incubated in all the concentrations of hydrogen peroxide while o-dianisidine had above 15% in all the concentrations. Spectral studies, indicated the peak at soret band as 381 nm for the native enzyme, and when the enzyme was incubated with hydrogen peroxide, there was a shift in the soret band of the enzyme from 381nm to 389nm. Increases in the concentration of hydrogen peroxide lead to decreases in the absorbance peak at the soret band of the enzyme and also reduction of size of Soret band. There were elevations in the absorbance peak when 1mM ascorbate and 0.4mM o-dianisidine were incubated with the enzyme at different concentrations of hydrogen peroxide.
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CHAPTER ONE

INTRODUCTION

The super-family of haem peroxidases from plants, fungi and bacteria is a group of enzymes that utilize hydrogen peroxide to oxidize a second (reducing) substrate often aromatic oxygen donor. These enzymes share similar catalytic cycles where hydrogen peroxide reacts with the resting ferric enzyme to form the intermediate compound I (known as compound ES in cytochrome c peroxidase) which carries two oxidizing equivalents. Compound I is subsequently reduced by reactions with two reducing substrate molecules. The reaction of these reduction steps generate the intermediate, compound II, which is then further reduced back to the ferric enzyme (Hiner et al.,2000). Peroxidase forms part of the defense system of living organisms against radical-mediated peroxidation of unsaturated lipids. 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 protein, auxin catabolism and self –defense against pathogens and senescence (Hiraga et al., 2001). Currently,
industrial application of peroxidase in chemistry, pharmacology and biotechnology is well developed. Peroxidase is used in waste treatment in order to remove aromatic phenols and amine from aqueous solution in the presence of hydrogen peroxide. In this treatment, phenolic compounds are polymerized in the presence of hydrogen peroxide through a radical oxidation-reduction mechanism (Nazari et al., 2005). As hydrogen peroxide concentration increases, an irreversible mechanism-based inactivation process becomes predominant (Rodriguez-Lopez et al., 1997) and it leads to the degradation of haem, the release of iron and the formation of two fluorescent products (Gutteridge, 1986). At a low concentration of hydrogen peroxide below 0.1 mM, inactivation is predominately reversible, resulting to the formation and accumulation of catalytically inert intermediate compound III. This inactivation of peroxidase by hydrogen peroxide is dependent on the concentration of hydrogen peroxide. (Zheng et al., 2001) Inactivation reaction between hydrogen peroxide and the intermediate of the enzyme’s catalytic cycle that reduced the sensitivity and efficiency of peroxidase has been studied with different sources of peroxidase, but not that of Gongronema latifolium (utazi).

1.1 PEROXIDASES

Peroxidases are known to occur in different tissues and the pattern of expression and properties of these peroxidases vary between them. Peroxidases are haem-containing oxidoreductases (EC 1.11.1.7) that reduce peroxides, mainly hydrogen peroxide, to water and subsequently oxidize small molecules, often aromatic oxygen donors (Delannoy et al., 2006). 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 (Hiraga et al., 2001). Plant peroxidases contain two-calcium ions (Ca$^{2+}$), which are essential for the structural stability and thermal stability of the enzyme as well as its in vitro activation during analysis (Manu and Prasada Rao, 2009; Sticher et al., 1981). Peroxidases are widely used in clinical laboratories, industries and in environmental conservation (Lopez-Molina et al., 2003)

1.1.1 Functional roles
Most reactions catalysed by peroxidase especially horseradish peroxidase can be expressed by the following equation, in which AH$_2$ and $^0$AH represent a reducing substrate and its radical product respectively. Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates. 

\[
\text{H}_2\text{O}_2 + 2\text{AH}_2 + \text{POD} \rightarrow 2\text{H}_2\text{O} + 2^0\text{A} \]

---

Figure 1: Catalytic cycle of peroxidase (Villalobos and Buchanan, 2002)

During the catalytic cycle of peroxidase as shown in figure 1, the ground state enzyme undergoes a two electron oxidation by H$_2$O$_2$ forming an intermediate state called compound I (E). Compound I (E) will accept an aromatic compound (AH$_2$) in its active site and will carry out its one-electron oxidation, liberating a free radical ($^0$AH) that is released back into the solution, converting compound I (E) to compound II (Ei). A second aromatic compound (AH$_2$) is accepted in the active site of compound II (Ei) and is oxidized, resulting in the release of a second free radical ($^0$AH) and the return of the enzyme to its resting state, completing the catalytic cycle (Figure 1). The two free radicals ($^0$AH) released into the solution combine to produce insoluble precipitate that can easily be removed by sedimentation or filtration.

Various side reactions that take place during the reaction process are responsible for the enzyme inactivation (E) or inhibition (Eii) leading to a limited lifetime, but this form is not permanent since compound III (Eii) decomposes back to the resting state of peroxidase. Some peroxidases, like horseradish peroxidase (HRP), lead to a permanent inactivation state (P-670) when H$_2$O$_2$ is present in excess or when the end-product polymer adheres to its active site,
causing its permanent inactivation by causing changes in its geometric configuration (Villalobos and Buchanan, 2002).

1.2.0 The structure of peroxidase

1.2.1 The description horseradish peroxidase

Horseradish peroxidase comprises a single polypeptide of 308 amino acid residue, the sequence of which was determined by Welinder, (1976). The N-terminal residue is blocked by pyroglutamate and C-terminus is heterogeneous with some molecules lacking terminal residue Ser308. There are four disulphide bridges between cysteine residue 11-91, 44-49, 97-301, and 177-209 and a buried bridge between Asp99 and Arg123. Nine potential N-glycosylation site can be recognized in the primary sequence from the motif Asn-X-ser/Thr (where ‘X’ represents an amino acid residue) and of these, eight are occupied. A branched heptasaccharide accounts for 75 to 80% of the glycans, but the carbohydrate profile of HRP C is heterogeneous (Yang et al., 1996). These invariably contain two terminal GlcNAC and several mannose residue. A further complication is the variation in the type of glycan present at any of the glycosylation site. The total carbohydrate content of the HRP C is somehow dependent on the source of the enzyme and value of between 18 and 22% typically.
I. **His170** forms coordinate bond to haem Iron

II. **Asp242** carboxylate side-chain help to control imidazolate character of **His170** ring

III. **His170** Ala mutant undergoes heme degradation. When hydrogen peroxide is added and compound I and compound II are not detected, imidazole can bind to haem Iron in the artificially created cavity but full catalytic activity is not restored because the **His170** imidazole complex does not maintain a five coordinate state (His42 also binds to Fe)

IV. Aromatic substrates are oxidized at the exposed haem edge but do not bind to haem Iron

---

**Figure 2:** Haem component of horseradish peroxidase isoenzyme C (HRPC) (Veitch, 2004)

**Figure 3:** Calcium ions component of (HRPC) (Veitch, 2004)
For the distal o-donors Asp43, Asp50, Ser52, (side chain) Asp43, Val46, Gly48 (carbonyl) one structural water. For the proximal o-donors Thr171, Asp222, Thre225, Asp230, (side chain) Thr171, Thre226, Ile228 (carbonyl)

I. Structural water of distal calcium site hydrogen bonded to Glu64 which is itself hydrogen bonded to Asn70 and thus connect to the distal haem pocket
II. Distal and proximal Ca²⁺ ions are both seven-coordinate.
III. On calcium ions loss, enzyme activity decreases by 40%

Figure 4: Carbohydrate component of (HRPC) (Veitch, 2004)

I. Site of glycosylation are in loop regions of the structure, at Asn57, Asn13, Asn158, Asn186, Asn198, Asn214, Asn255 and Asn268.
II. The major glycan is shown here, there are also minor glycans of the form Manₘ GlcNAc₂
Arg38 Essential role in (i), the formation and stabilization of compound I, (ii) binding and stabilization of ligands and aromatic substrates (e.g. benzhydroxamic acid, ferulate etc.).

Phe41 Prevent substrate access to the ferryl oxygen of compound I.

His42 Essential role in (i), compound I formation (accept proton from H$_2$O$_2$), (ii) binding and stabilization of ligands and aromatic substrates.

Asn70 Maintains basicity of His42 side-chain through Asn70-His42 couple (hydrogen bond from Asn70 amide oxygen to His42 imidazole NH).

Pro139 Part of a structural motif, -Pro-X-Pro- (Pro139-Ala140-Pro141 in HRP C), which is conserved in plant peroxidases

Figure 5: Key amino acid residues in the haem-binding region of HRPC. (Veitch, 2004)

HRPC contains two different types of metal centre, iron III protoporphyrin IX (usually referred to as the haem group) and two calcium atoms. Both are essential for the structural and functional integrity of the enzyme. The haem group is attached to the enzyme at His170 (the proximal histidine residue) by a coordinate bond between, the histidine side-chain atom and the haem iron atom. The second axial coordination site (on the so called distal side of the haem plane) is unoccupied in the resting state of the enzyme, but available to hydrogen peroxide during enzyme turnover (Figure 5). Small molecules such as carbon II oxide, cyanide, fluoride
and azide bind to the haem iron atom at the distal site giving six-coordinate peroxidase complexes. Some bind only in their protonated forms, which are stabilized through hydrogen bonded interaction with the distal haem pocket amino acid side-chain of Arg38 (the distal arginine) and the His42 (the distal histidine) (Figure 5). The two calcium binding sites are located at positions distal and proximal to the haem plane and are linked to the haem-binding region by a network of hydrogen bonds. Each calcium site is seven-coordinate with oxygen-donor ligands provided by a combination of amino acid side-chain carboxylate (Asp), hydroxyl group (Ser, Thr), backbone carbonyls and a structural water molecules (distal site only) as shown in figure 2 to 4. Loss of calcium results in decrease in both enzyme activity and thermal stability (Haschke and Friedhoff, 1978) and to subtle changes in the haem environment that can be detected spectroscopically (Howes et al., 2001).

Figure 6: Three –dimensional representation of the x-ray crystal structure of HRPC (Brook haven accession code IH5A). (Veitch, 2004)

1.2.2 Three-dimensional structure of peroxidase
The first solution of the three-dimensional structure of HRP C using X-ray crystallography appeared in the literature relatively recently (Gajhede et al., 1997). The recombinant enzyme used as the source of crystals and heavy atom derivatives was produced by expression in *Escherichia coli* in non-glycosylated form (Smith et al., 1990). Previous attempts to obtain suitable crystals for diffraction were frustrated partly by the heterogeneity of plant HRP C preparations comprising multiple glycoforms. The structure of the enzyme is largely α-helical, although there is also a small region of β-sheet (Figure 6). There are two domains, the distal and proximal, between which the heme group is located. These domains probably originated as a result of gene duplication, a proposal supported by their common calcium binding sites and other structural elements (Welinder and Gajhede, 1993).

1.3.0 The mechanism of oxidation of peroxidase

1.3.1 Mechanisms of oxidation of indole-3-acetic acid with peroxidase

One of the most interesting reactions of peroxidase (HRP-C) occurs with the plant hormone, indole-3-acetic acid (IAA) as shown in figure 7. In contrast to most peroxidase–catalysed reactions, this takes place without hydrogen peroxide, hence the use of the term ‘indole acetic acid oxidase’ to describe this activity of HRP C in the older literature. More recent studies of the reaction at neutral pH indicate that it is not an oxidase mechanism that operates, but rather a peroxidase mechanism coupled to a very efficient branched-chain process in which organic peroxide is formed (Dunford, 1999). The reaction is initiated when a trace of the indole-3-acetate cation radical is produced. The major products of indole-3-acetic acid oxidation include indole-3-methanol, indole-3-aldehyde and 3-methylene-2-oxindole, the latter most probably as a result of the non-enzymatic conversion of indole-3-methylhydroperoxide. Conflicting theories have been proposed to explain the mechanism of reaction at lower pH (Dunford, 1999), in the formation of the ferrous enzyme, compound III and hydroperoxyl radicals must also be accounted for. The physiological significance of IAA metabolism by (HRP C) and other plant peroxidases is still an area of active debate. For example, studies on the expression of an anionic peroxidase in transgenic tobacco plants indicate that while overproduction of the enzyme favours defensive strategies (such as resistance to disease, physical damage and insect attack), it has a negative impact on growth due to increased IAA degradation activity (Lagrimini, 1996).
Thus peroxidase expression in plant tissues at different stages of development must reflect a balance between the priorities of defense and growth.

![Diagram of chemical reactions]

Figure 7: A mechanism proposed for the formation of 3-methylene-2-oxindole from horseradiperoxidase (HRP C) and indole-3-acetic acid (after Folkes et al., 2002). R represents a cellular nucleophile (e.g. sulphhydryl groups of enzymes or histone).

1.3.2 Mechanism of oxidation of small phenolic substrates (Ferulic acid) with peroxidase

Ferulic acid ((3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid. FA) is a phenolic cinnamic acid derivative that is abundant in nature and known to act as an in vivo substrate for peroxidases (Fry, 1986). FA enhances the rigidity and strength of plant cell walls by cross-linking with pentosans, arabinoxylans, and hemicelluloses, thereby making the cell walls less susceptible to enzymatic hydrolysis during germination. The compound is a dibasic acid that exhibits an extended resonance stabilization of the phenolate anion, hence slightly increasing its acidity relative to phenol. pKa values of 4.6 and 9.4 have been reported (Kenttamaa et al., 1970). Peroxidases have been reported to be the FA cross-linking catalyst (Markwalder and Neukom, 1976). The level of FA and its derivatives seems to be positively correlated with protection of the plant against insects (Suga et al., 1993), fungal, viral and avian attacks. In plants, FA is thought to arise from the conversion of cinnamic acid, and frequently it is esterified to hydroxyl groups of polysaccharides (Takahama and Oniki, 1996), flavonoids, hydroxycarboxylic acids and plant sterols. The initial step in the biosynthesis of lignin is the enzymatic dehydrogenation of monolignols to produce phenoxy radicals. The radicals can link up to form dimers, trimers, and higher oligomers.

Laccases and plant peroxidases have been proposed to be the in vivo generators of the phenoxy radicals. Peroxidase oxidation of compounds with a syringyl group can be enhanced by
esters of 4-coumaric acid and FA (Takahama et al., 1996). For these reasons, it is of interest to study the interactions between the cell wall component of ferulic acid and the well characterized horseradish peroxidase C. Peroxidases catalyze the oxidative coupling of phenolic compounds using H₂O₂ as the oxidizing agent as shown. The reaction is a three-step cyclic reaction by which the enzyme is first oxidized by H₂O₂ and then reduced in two sequential one-electron transfer steps from reducing substrates, typically a small molecule phenol derivative (the charges of heme propionates are ignored in scheme 1).

\[
\text{HRPC}[(\text{Fe}(\text{III}))\text{Porph}^{2-}]^+ + \text{H}_2\text{O}_2 \rightarrow \text{HRPC}[(\text{Fe}(\text{IV})=\text{O})\text{Porph}^0]^0+ + \text{H}_2\text{O} \quad \text{Reaction 2}
\]

Native state

\[
\text{HRPC}[(\text{Fe}(\text{IV})=\text{O})\text{Porph}^0]^0+ + \text{AH} \rightarrow \text{HRPC}[(\text{Fe}(\text{IV})=\text{O})\text{Porph}^2^+] + \text{H}^+ + \text{A}^0 \quad \text{Reaction 3}
\]

Compound I

\[
\text{HRPC}[(\text{Fe}(\text{IV})=\text{O})\text{Porph}^2^+] + \text{H}^+ + \text{AH} \rightarrow \text{HRPC}[(\text{Fe}(\text{III}))\text{Porph}^2^+]^+ + \text{H}_2\text{O} + \text{A}^0 \quad \text{Reaction 4}
\]

Compound II

\[
\text{SCHEME 1: Reactions 2–4}
\]

The oxidized phenolic radicals polymerize with the final product depending on the chemical character of the radical, the environment, and the peroxidase isoenzyme used (Frias et al., 1991). The oxidation of native enzyme by H₂O₂ is well understood, and numerous experiments have confirmed the general catalytic mechanism for this step first proposed by (Poulos and Kraut, 1980). The oxidation of phenolic substrates (reactions 4 and 4) is less well understood, but a histidine (His42 in HRPC) and an arginine (Arg38 in HRPC) (Rodriguez-lopez et al., 1997) have been shown to contribute significantly to enhance the rate of substrate oxidation.
First, the active site arginine (Arg38 in HRPC) donates a hydrogen bond to the phenolic oxygen of the reducing substrate. This hydrogen bond will assist proton transfer from the phenolic oxygen to active site histidine (His42 in HRPC) through an active site water molecule held in position by the backbone oxygen of a conserved proline residue (Pro139 in HRPC). The electron is transferred to the haem group via the C-18 methyl-C-20 haem edge. Then compound II reduction is assisted by a similar proton transfer. The proton can be transferred to the ferryl oxygen through the active site water molecule situated equidistant between the distal histidine and the expected position of the ferryl oxygen of compound II, regenerating the resting state enzyme and a water molecule (Henriksen et al., 1999)
1.4 Classes of peroxidases

Peroxidases, a class of enzymes in animals, plants and microorganisms, catalyze oxidoreduction between $\text{H}_2\text{O}_2$ and various reductants. Peroxidases fall into two major super families according to their primary sequence: animal and plant peroxidases (Table 1).

**Table 1: Classification of peroxidases** (Hiraga et al., 2001)

<table>
<thead>
<tr>
<th>CLASSES SUPERFAMILY</th>
<th>(EC NUMBER) MEMBER (PEROXIDASE)</th>
<th>ORIGIN</th>
<th>MOLECULARWEIGHT (KDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal peroxidase</td>
<td>Eosinophil peroxidase (EC1.11.1.7)</td>
<td>peroxidase</td>
<td>Animal</td>
</tr>
<tr>
<td></td>
<td>Lacto peroxidase (EC1.11.1.7)</td>
<td>peroxidase</td>
<td>Animal</td>
</tr>
<tr>
<td></td>
<td>Myclo peroxidase (EC1.11.1.7)</td>
<td>peroxidase</td>
<td>Animal</td>
</tr>
<tr>
<td></td>
<td>Thyroid peroxidase (EC1.11.1.9)</td>
<td>peroxidase</td>
<td>Animal</td>
</tr>
<tr>
<td></td>
<td>Glutathione peroxidase (EC1.11.1.7)</td>
<td>peroxidase</td>
<td>Animal</td>
</tr>
<tr>
<td>Catalase</td>
<td>Catalase (EC 1.11.1.6)</td>
<td>Animal</td>
<td>Plant Fungus and Yeast</td>
</tr>
<tr>
<td>Plant Peroxidase</td>
<td>Cytochrome C peroxidase (EC 1.11.1.6)</td>
<td>peroxidase</td>
<td>Bacterium and Yeast</td>
</tr>
<tr>
<td></td>
<td>Catalase peroxidase (EC1.11.1.6)</td>
<td>peroxidase</td>
<td>Bacterium</td>
</tr>
<tr>
<td></td>
<td>Ascorbate peroxidase (EC1.11.1.11)</td>
<td>peroxidase</td>
<td>Plant</td>
</tr>
<tr>
<td></td>
<td>Manganese-dependent peroxidase (EC1.11.1.13)</td>
<td>peroxidase</td>
<td>Fungus</td>
</tr>
<tr>
<td></td>
<td>Ligninase (EC1.11.1.14)</td>
<td>peroxidase</td>
<td>Fungus</td>
</tr>
<tr>
<td></td>
<td>Peroxidase(EC1.11.17POX)</td>
<td>Plant</td>
<td>28-60</td>
</tr>
</tbody>
</table>
1. 5.  **Plant peroxidase**

1.5.1 Plant peroxidases

Based on differences in primary structure, the plant peroxidase super family can be further divided into three classes (Table 1). The plant peroxidases, which share similar overall protein folds and specific features, (such as catalytically essential histidine and arginine residues in their active sites), have been subdivided into three classes on the basis of sequence comparison (Welinder, 1991). In class I are intracellular enzymes including yeast cytochrome c peroxidase, ascorbate peroxidase (APX) from plants, and bacterial gene duplicated catalase-peroxidases (Welinder, 1991). Class II consists of the secretory fungal peroxidases such as lignin peroxidase (LiP) from *Phanerochaete chrysosporium*, manganese peroxidase from the same source, and *Coprinus cinereus* peroxidase or *Arthromyces ramosus* peroxidase (ARP), which have been shown to be essentially identical in both sequence and properties (Kjalke *et al.*, 1992). The main role of class II peroxidases appears to be the degradation of lignin in wood. Class III contains the secretory plant peroxidases such as those from horseradish (HRP), barley and soybean. These peroxidases seem to be biosynthetic enzymes involved in processes such as plant cell wall formation, and lignifications as shown in (Figure 9)

1.5.2 Functions of plant peroxidase

Plant peroxidases have often been suggested to be involved in the biosynthesis of complex cell wall macromolecules such as lignin and suberin, both of which are synthesized by plant for mechanical strength, defense, restoring damaged tissues, and water transport (Vidali, 2001 and De Gara, 2004). Plant peroxidases (PODs) oxidise phenolic domains of feruloylated polysaccharides and tyrosine residues of cell wall structural proteins such as hydroxyproline-rich glycoproteins to form more complex and larger molecules in the cell wall, thereby restricting cell expansion and pathogen invasion. In tobacco, a positive correlation was found between PODs activity and resistance to tobacco wildfire disease. The roles of PODs in defense are considered as follows:

I. Reinforcement of cell wall physical barriers comprising lignin, suberin, feruloylated polysaccharides and hydroxyproline-rich glycoproteins.

II. Enhancement of reactive oxygen species production as signal mediators and antimicrobial agents.

III. Enhancement of phytoalexin production.
Generally, multiple PODs are induced by pathogen infection, suggesting that each POD is involved in a specific defense process (Hiraga et al., 2001 and Cosio and Dunand, 2009). Peroxidases from tobacco and HRP have shown higher specific activities to NADH, NADPH and IAA than to monolignols, suggesting their involvement in some cell wall biosynthetic processes other than polymerization of monolignols (Figure 9) (Delannoy et al., 2006; Cosio and Dunand, 2009).

Figure 9: The diverse function and role of class III peroxidase (Cosio and Dunand, 2009)

1.5.3 FUNCTIONS OF PEROXIDASE IN PHARMACOLOGY AND FINE CHEMISTRY

Recently, peroxidases have been used as reagents for organic syntheses and biotransformations, as well as in coupled enzyme assays, chemiluminescent assays and immunoassays.

I. Oxidative decarboxylation of auxin (IAA), a plant hormone that affects many physiological processes by PODs (from tobacco and HRP). PODs induce IAA inactivation, thereby
offering new potential for target cancer therapy. Studies reported that IAA is cytotoxic to human tumour cells in the presence of POD. The mechanism of toxicity involves 3-methylene-2-oxindole which is generated through IAA oxidation. Many other substituted indole-3-acetic acid derivatives have been tested for cytotoxicity in combination with HRP C in an attempt to place relationship between structure and activity on a predictive level. No simple correlation was found between levels of cytotoxicity of indole derivatives and their reactivity towards compound I; for example 5-fluoroindole-3-acetic acid is more cytotoxic towards tumour cells than IAA but less effective as a reductant of compound I (Folkes et al., 2002). Other factors such as the pKa of the indolyl radical cation and rates of decarboxylation and radical fragmentation may also be significant. One of the most cytotoxic indoles identified from in vitro screening is 6-chloroindole-3-acetic acid, a derivative with potential as a pro drug for targeted cancer therapies mediated by HRP C (Rossiter et al., 2002). The challenge now is to develop strategies to evaluate and implement this promising system in vivo. Indeed the combination of HRP C and indole-3-acetic acid or its derivatives offers several advantages for future antibody-, gene- or polymer-directed enzyme pro-drug therapies (Folkes and Wardman, 2001; Wardman, 2002). Among the favourable properties of HRP C are its good stability at 37°C, high activity at neutral pH, lack of toxicity and the ease with which it can be conjugated to antibodies and polymers. Furthermore, evidence available at present suggests that IAA does not show any adverse side-effects in humans. The fact that peroxide is not required as a co-substrate for the reaction with HRP C is also a significant advantage.

II. Some applications of HRP in small-scale organic synthesis include N- and o-dealkylation, oxidative coupling, selective hydroxylation and oxygen-transfer reactions:

III. Peroxidase-catalysed oxidative coupling of methyl-(E)-sinapate with the syringyl lignin-odel compound, 1-(4-hydroxy-3,5-dimethoxyphenyl) ethanol yielded a novel spirocyclohexadienone together with a dimerization side-product

IV. Coupling of catharanthine and vindoline to yield α-3, 4, - anhydrovinblastine. This reaction, catalysed by HRP, offers potential interest as it is a semisynthetic step in the production of the anti-cancer drugs vinblastine and vincristine from Catharanthus roseus (Vidali, 2001, Veitch, 2004).
V. Peroxidases have also shown an action on tyrosine, both as free amino acid and in peptides or proteins. After one electron oxidation and subsequent deprotonation, dityrosines and higher oligomers are produced.

VI. Ferulic acid and tyrosine are subject to peroxidase-mediated oligomerization. Such peroxidase-mediated hetero-coupling could provide an explanation for the occurrence of protein-carbohydrate complexes in plant cell walls and the incorporation of ferulic acid and other hydroxycinnamic derivatives into lignin and suberin tissues on a protein template. Recent studies have further explored the mechanism of hetero-adduct formation of GYG (Gly-Tyr-Gly) and FA. (Ahn et al., 2002).

VII. Reactive oxygen species (ROS) generated through abiotic and biotic stresses trigger programmed cell death (PCD) in mammalian cells, yeast and plants (Delannoy, 2005). In plants and yeast the PCD is induced by Bax proteins that cause organelle dysfunction by their localisation onto the outer mitochondrial membranes and formation of ion channels. Several enzymes have been reported to suppress Bax-induced cell death such as peroxidase, ascorbate peroxidase, peroxidase with glutathione transferase and phospholipid hydroperoxid glutathione peroxidase (Chen et al., 2004; De Gara, 2004).

VIII. Many studies have suggested an association of plant peroxidases with production and scavenging of hydrogen peroxide, porphyrin metabolism, senescence and organogenesis, indicating that PODs have diverse functions (Hiraga et al., 2001). Based on previous published works (El Agha et al. 2008; 2009; Osman et al.; 2008; Majdalany, 2008) for the exploitation and valorisation of crude POD from cheap vegetable sources.

1.5.4 The use of peroxidase for wastewater treatment

Although the use of enzyme in the waste water treatment was first proposed in the 1930s only as late as in the 1970s the concept of environmental biocatalysts that is, application of enzymes to destroy target pollutant was established. Enzyme may transform pollutant to diminish their toxicity, to increase water solubility and its subsequent removal from the industrial waste stream. Peroxidase was shown to be able to remove a variety of phenols and aromatic amines from an aqueous solution (Klibanov and Morris, 1981) and to decolorize phenolic and amines industrial effluents. It was shown that phenols are effectively removed by treatment with horseradish peroxidase in the presence of a coagulant. However, peroxidase quickly becomes inactivated during the reaction, and the coagulant prevents peroxidase inactivation and reduces
the amount of peroxidase required for phenol removal. Arseguel and Baboulne (1994) studied the removal of phenol using peroxidase in the presence of a mineral and showed that the mineral could prolong the catalytic action because of the adsorption of the reaction products. Enzyme immobilization is excellent due to its high storage stability and better control of the catalytic process (Tatsumi et al., 1994).

1.5.5 The use of peroxidase in textile industry

Most synthetic industrial dyes are complex aromatic compounds with an azo bond connected to various aromatic structures. Some, however, are polymeric structures containing metals. It is estimated that there are over 10,000 commercially available dyes and pigments of industrial use, representing an annual consumption of around 7 x 105 tonnes worldwide (Akhtar et al., 2005). However, about 10-15% of the synthetic dyes produced are discharged into industrial effluents (Spadaro et al., 1992), causing environmental problems. Then, dye contamination of water bodies is a great problem in many countries. Removal of dyes can be carried out by means of oxidative enzymes. Peroxidases, a versatile group of enzymes that catalyze the oxidation of a large number of aromatic structures through a reaction with hydrogen peroxide, being applied in the chemical, environmental, pharmaceutical and biotechnological industries (Spadaro et al., 1992).

1.5.6 The use of peroxidase in the dairy industry

Hydrogen peroxide has been use in the dairy industry as an effective bactericidal and bacteriostate agent, although its mechanism of action is unclear. The bacterial reduction by H₂O₂ depends on the initial quality of the milk (i.e. the bacterial count) (Nambudripad et al., 1949). And also H₂O₂ used as a preservation in the dairy industry to preserve the milk against microbial spoilage can lead to the destruction of the physical properties, chemical composition and original nutritional value. These H₂O₂ used in dairy industry either to preserve or to kill bacterial can be destroyed easily and quickly and completely through the use of peroxidase, after enzymatic treatment, the breakdown products, water and oxygen are normally undetected in milk and no toxic residue remains once H₂O₂ has been broken down.
1.6 Substrates

The enzyme peroxidase has two substrates, both of which must be present in order for the reaction to occur. One of the substrates is hydrogen peroxide, the other may vary, and depending on the cell or tissue the enzyme is found. The various substrates that can react with peroxide and their respective products is shown in Table 2 below.

Table 2: Various substrates that can react with peroxide and their respective products

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogallol</td>
<td>Purpurogallin</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>Tetraguaiacol quinone</td>
</tr>
<tr>
<td>Benzidine</td>
<td>o-Quinonediamide</td>
</tr>
<tr>
<td>Catechol</td>
<td>o-Quinone</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>Quinhydroine</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Yellow solution</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>Milky precipitate</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>Flesh-coloured solution</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>Green-solution</td>
</tr>
<tr>
<td>o-Dianisidine</td>
<td>Vivid purplish red</td>
</tr>
</tbody>
</table>

The general reaction is as shown in reaction 5:

\[
H - R - O - H + H - O - H \quad \overset{R = O + 2H - O - H}{\longrightarrow} \quad \text{Reaction 5}
\]

Oxygen atom is transferred to an acceptor molecule, which for example is the organic molecule guaiacol. This reaction is facilitated by the enzyme peroxidase, which is found in many plant tissues. Peroxidase is more in horseradishes rather than in turnips. Hydrogen peroxide and guaiacol are both substrates.
The guaiacol peroxide system is convenient because guaiacol changes from colourless to brown colour as it is oxidized shown in figure 10. The functional peroxidase will make a test tube containing reduced guaiacol and hydrogen peroxide turn increasingly brown over time. This change to brown colour is influenced by three substances, these substances: hydrogen peroxide, guaiacol and peroxidase must be present in the test tube. Guaiacol is a phenolic natural product first isolated from guaiac resin and the oxidation of lignin. Guaiacol is readily oxidized by the haem iron of peroxidase including the peroxidase of cyclooxygenase (COX) enzymes. It therefore serves as a reducing co-substrate for COX reactions.

The one electron oxidation product of guaiacol is a dimer absorbing at 460 nm with an extinction coefficient of 26,6000. Two moles of guaiacol are oxidized for each mole of hydrogen peroxide reduced by peroxidase. The resulting guaiacol chromophore can be used for the colorimetric determination of hydrogen peroxide activity.

Another substrate that can be used in place of guaiacol for plant peroxidase is o-dianisidine. o-dianisidine is not normally used because of its carcinogenic nature. The specific reaction of o-dianisidine is shown below.
Hydrogen peroxide and reduced o-dianisidine are the substrates, water and oxidized o-dianisidine are the products as shown in figure 11. In other cells, its function is to convert the potentially harmful peroxide into non-toxic compound, water.

1.7 Factors that affect peroxidase activity

1.7.1 pH

This is a measure of hydrogen ion activity of a solution and is defined as the negative logarithm of the hydrogen ion concentration. The rate of a chemical reaction or the enzyme activity is greatly influenced by the structure of the enzyme. Or in other words, a change in the structure of the enzyme affects the rate of reaction. When pH of a particular medium changes, it leads to alteration in the shape of the enzyme. pH level also affects the charge properties and shape of the enzyme. Within a narrow pH range, changes in the structural shapes of the enzymes may be reversible. But for a significant change in pH levels, the enzyme may undergo denaturation. Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has the optimum pH range where it is most active. Amino acid acid side chains contain groups, such as –COOH and –NH₂ that readily gain or loose H⁺ and eventually enough side chain will be affected so that the enzyme is disrupted. This is the summary of the effect on pH and on a combination of these factors:
1.7.2 Temperature

Temperature is one of the critical factors affecting enzyme-catalysed reactions, like other chemical reactions, the rate of an enzyme-catalysed reaction increases with a modest increase in temperature. This is true only over a strictly limited range of temperature. When the temperature of a reaction is raised, there is sufficient energy to overcome the energy barrier and so cause an increase in the number of collisions between the enzyme involved and its substrate. These result in an increase in the rate of the reaction to reach its maximum activity. Beyond optimum temperature, every further increase in temperature introduces vibrational energy that weakens the three-dimensional structure of the enzyme. Once the hydrogen bonds and hydrophobic bonds holding the native structure together are broken or disrupted, the enzyme is denatured and the reaction stops. The temperature range over which an enzyme is stable and catalytically active depends on the temperature of the cell in which the enzyme is found.

As temperature increases, the rate of reaction also increases, as is observed in many chemical reactions. However, the stability of the protein also decreases due to thermal degradation. Holding the enzyme at a high enough temperature for a long period of time may cook the enzyme or inactivate it. It was observed that the maximum temperature for peroxidase activity was between 30°C and 70°C in most vegetables and fruits that have been studied (Majed and Mohammad, 2005). Inactivation temperature of peroxidase has been reported to be 95°C in soybean seed coat peroxidase, 81.5°C in horseradish peroxidase C and that of Caprinus cinereus peroxidase (a class II POD from the fungus Caprinus cinerus with similar activity) is 65°C (McEldoon and Dordick, 1996). That of litch POD was 90°C for 10 minutes and 100°C for 1 minute.

1.8 Inhibition and inhibitors of peroxidase

Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of the substrate or its turnover number. Substances that reduce an
enzyme’s activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme’s substrate but either does not react or reacts very slowly compared to the substrate.

Inhibition of enzymes decreases yield of products and finally the affectivity of the process. There exists two prepositions concerning the mechanism of enzyme inactivation. The first one hypothesizes intermediates (radicals), a formation that reacts with active centre of enzyme (Chang et al., 1999). Following the second hypothesis microparticles adsorb enzyme (Masuda et al., 2001). However, the intrinsic mechanism of inactivation of absorbed enzyme is not understood.

1.8.1 **Inhibitor of peroxidases/ peroxidase suppressor**

Horseradish peroxidases are inhibited by thiol type inhibitor: mercaptoethanol (MCE) and mercaptoacetic acid (MCA) Mercaptoethanol (MCE) is a more potent inhibitor than mercaptoacetic acid using 4 – aminoantipyrine as a substrate. Other inhibitors are p-aminobenzoic acid, sodium azide (NaN\(_3\)), cyanide, cyclopropanone, L-cystine dichromate ethylenethiourea, hydroxylamine, sulfide, sulfite, vanadate and a number of divalent anions of Cd, Co, Cu, Fe, Mn, Ni, Pb.

1.9. **Inactivation of The enzyme**

1.9.1 **Inactivation of peroxidase**

The enzymes have a region (called the substrate binding site, the active site or the catalytic site) that is complementary in size, shape and chemical nature to the substrate molecule. Today, it is recognized that the active site, rather than a rigid geometrical cavity, is a very specific and precise spatial arrangement of amino acid residues R-groups that can interact with complementary groups on the substrate (Segel, 1993). Three main processes have been considered to be involved in the inactivation of peroxidase,

(1) Dissociation of prosthetic (heme) group from the holoenzyme (active enzyme system);
(2) Conformational change in the apoenzyme (protein part of the enzyme); and/or
(3) Modification or degradation of the prosthetic group (Lemos et al., 2000).
1.9.2 Inactivation of peroxidase by hydrogen peroxidase

The function of all the forms of peroxidase in plants is thought to be the scavenging of the H$_2$O$_2$ that is continuously generated in cells (Veitch, 2004). For instance, in the chloroplasts of photosynthetic organisms superoxide (O$_2^{\cdot-}$) 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$_2^{\cdot-}$. Additionally, in the mitochondria the electron transport chains can also produce O$_2^{\cdot-}$. In both cases superoxide dismutase converts O$_2^{\cdot-}$ into H$_2$O$_2$ which POD or catalase can then remove. The first step in the catalytic cycle of POD is the reaction between H$_2$O$_2$ 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$_2$O$_2$ at low temperatures and described as an Fe(III)-hydroperoxy complex. Molecular dynamics simulations of these peroxide-bound complexes have been carried out (Filizola and Loew, 2000). 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 resting state enzyme gives compound III, which can also be prepared by several (Veitch, 2004), other routes (Dunford, 1999) that lead to the degradation of haem, the release of iron (Gutteridge, 1986), 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 (Berglund et al., 2002). In scheme 2 models for the irreversible inactivation of APX by hydrogen peroxides have been developed.
**Scheme 2.** Mechanistic model of the reaction of peroxidase with H$_2$O$_2$ in the absence of other substrates (Hiner et al., 2000)

$E$ is native ferric peroxidase. $S$ is H$_2$O$_2$. $E'$ and $E''$ are the enzyme intermediates, compounds I, and II, respectively. $E'S$ and $E_L$ are complexes between the respective intermediates and H$_2$O$_2$, [compound IH$_2$O$_2$], and [compound IIH$_2$O$_2$]. $Ei$ is inactive peroxidase. $E_L$ is also a peroxidase species with modified specificity for reducing substrate.

1.10 Spectral studies

1.10.1 Spectral properties of protein

The peptide groups of the protein absorb light in the ‘far-UV range (180-230 nm). The aromatic side chain of tyrosine (Tyr) and tryptophan (Trp) absorbs light in this region and in addition, they absorb in the 240-300 nm region. This region is called the near-UV or the aromatic region. Disulfide bond that form between two cysteine residues also show an absorbance band near 250 nm (Schmid, 1990). The absorbance properties of the aromatic amimo acid are shown in (Table 3.) In the near-UV, the absorbance properties of phenylalanine ( alpha maximum 257nm) is much smaller than that of tryptophan and tyrosine and the spectrum of a protein between 240 and 300 nm is therefore dominated by the contribution from the tryptophan (Trp) and tyrosine (Tyr) side-chains. Phenylalanine residues contribute fine structure (wiggles) to the spectrum between 250 and 260 nm. The aromatic amino acids do not absorb above 310 nm and therefore the absorbance of solution containing only protein should be zero at wavelength greater
than 310 nm (Schimid, 1990) and solution containing only protein without Trp residues do not absorb above 300 nm.

Table 3: The absorption maximum of the aromatic amino acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{Max}}$ (nm)</th>
<th>$\Sigma_{\text{max}}$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Sigma_{280}$ (M$^{-1}$cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>280</td>
<td>5600</td>
<td>5500</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>275</td>
<td>1400</td>
<td>1490</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>258</td>
<td>200</td>
<td>-</td>
</tr>
</tbody>
</table>

(Schmid, 1990)

The absorption spectrum of the aromatic amino acids depends on the nature of the molecular neighborhood of various chromophores. This environmental sensitivity can result in broadening of bands, shifts in wavelength of maximal absorption and over- all change in intensity. In general, the shift in wavelength maximal absorption predominates. A red-shift of protein spectrum is observed when the polarity of the solvent increases (Schmid, 1990). For example, the maximum absorbance of tyrosine is blue- shifted by about 3 nm from 277 nm to 274 nm, when the solvent is changed from carbon tetrachloride to water. This spectral shift combined with minor changes in the strength of absorbance and in the fine structure of the spectrum, leads to maxima (prominent peak) in the difference spectra in the descending slope of the original spectrum, which is in the 285-288 nm region for tyrosine and 291-294 nm for tryptophan.

In folded native protein, some of the aromatic amino acid residues are buried within the hydrophobic core of the molecule. They become exposed to the aqueous solvent during unfolding, giving rise to absorbance in the 288 to 295 nm region. The difference in the absorption spectrum between the native and the unfolded states of a protein is generally small; nevertheless difference spectrum can be determined with good accuracy by difference spectroscopy.

The size and shape of the difference spectrum depends on the kind and number of aromatic amino acids, as well as on the degree of burial of their side chain in interior of the native protein. The contribution of phe residues to the difference spectrum is very small. It is
sometimes apparent as a ripple structure in the 250nm-260nm regions. Proteins that lack Trp display a Try difference spectrum with prominent positive peak at 287nm and a mirror peak of 278nm. Proteins that contain both Try and Trp show an additional prominent peak around 292nm that originates from the buried Trp residues in the folded protein. The difference spectra are usually too complex to sort out the contributions of individual amino acid; they are extremely useful as convenient means to monitor conformational changes of a protein. Also, measurement of spectral changes that accompany unfolding transition provides a very powerful technique to determine the stability of proteins and to follow the kinetics of conformational changes.

1.10.2 Spectra Properties of peroxidase

Peroxidases belong to the haem family, the haem group consists of a porphyrin ring with a ferrous or ferric iron co-ordinated centrally. The conjugated double bond system of the porphrin ring causes a strong absorption in haemprotein termed α β γ-bands. Typically, α-bond occur at the longest wavelength. γ-Bond at the shortest wavelength, also called Soret-band, after the swiss scientist who first examined the near UV region of cytochromes and the β band lie between.

Studies showed that different classess of peroxidase have different absorption spectrum on the Soret band and other bands. The UV-visible spectrum of ascorbate-free leishmania-peroxidase (Δ34 LmAPX) has shown the presence of a Soret band peak at 408 nm with the secondary peak at 500 nm and 640 nm. The addition of 5 molar excess of hydrogen peroxide to the resting state of enzyme produces oxyferryl compound II. [oxyferryl compound II is produced via compound I, a short lived ferryl heam iron with porphyrin π cation radical (Patterson et al., 1995) The Soret band region was shifted to 420 nm with high absorbance and the visible peak at 532 and 560. This spectrum is reminiscent of compound II of the other peroxidase. The compound II species returns to ferric state of the enzyme within 30 seconds. The enzyme was further studied to find out why the enzyme was inactivated in the presence of high concentration of hydrogen peroxide.

The native ascorbate-peroxidase (tAPX) has an absorption spectrum characteristic of a ferric (feIII) high-spin state, with a soret peak at 403 nm. On the addition of an equimolar amount of hydrogen peroxide to the native enzyme, the soret peak shifted to 415 nm as observed in the case of cyctochrome c peroxidase (Yonetani and Anni, 1987). When excess hydrogen peroxide was added to the solution of compound I, the size of the Soret peak decreased with time,
indicating the degradation of the haem moiety. Also, the rate of degradation of the haem of compound I increased as the concentration of hydrogen peroxides increased, as estimated from the decrease in absorbance at 415 nm after the addition of hydrogen peroxide (Miyake and Asada, 1996). It was also shown that the absorption spectrum of peroxidase A and B of tomato plant isoenzyme in the oxidized, reduced, CO-reduced, and alkaline were as follows, both had 405 nm maximum absorption at the Soret region in the oxidized and native form. Absorption maxima at 435 nm and 547 nm were evident for peroxidase A and B in the reduced state while both had absorption maxima at 424, 547, and 573 nm in the CO-reduced state (John, 1970). In the alkaline state, peroxidase A had an absorption maximum at 412 nm and B at 418 nm. The absorption spectrum of the pyridine haemochromogens for A and B at maxima occurs at 397, 420, 257 and 558 nm. The absorption spectrum of A and B peroxidase are similar to the absorption spectrum of other peroxidase (Gallagher and Elliotts, 1965).

For intestinal peroxidase the maximum absorbance for the Soret band was noted at 417 nm. In the range of visible light, absorption bands were observed at wavelength 490, 543, 596, and 642 nm. Significantly, the position of the absorption bands of intestinal peroxidase, as well as of its derivative (CN-peroxidase, reduced peroxidase, CN-reduced peroxidase and pyridine hemechromogen) are similar to the parameters of the absorption spectrum of lactoperoxidase and its derivative also with peroxidase of eosinophilic granulocytes. Table 4 shows a comparison of the absorption spectrum of intestinal peroxidase and its derivative with corresponding spectrum of lacto-peroxidase (Carlstrom, 1969) eosinophilic granulocytes and myelo-peroxidase.

Table 4: Absorption maxima for intestinal peorxidase, lacto-peroxidase, essinophilic peroxidase and myeloperoxidase with their derivatives

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>Oxidized-CN</th>
<th>Reduced-CN</th>
<th>Pyridine hemochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal peroxidase</td>
<td>417 490</td>
<td>543 596</td>
<td>448 565 596</td>
<td>429 555 596 650</td>
<td>435 536 569</td>
</tr>
<tr>
<td></td>
<td>596 642</td>
<td></td>
<td></td>
<td></td>
<td>423 530 568</td>
</tr>
<tr>
<td>Lacto-peroxidase</td>
<td>412 501</td>
<td>541 589</td>
<td>442 565 600</td>
<td>430 555 595</td>
<td>435 537 571</td>
</tr>
<tr>
<td></td>
<td>561 631</td>
<td></td>
<td></td>
<td></td>
<td>425 530 566</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>558 571</td>
</tr>
</tbody>
</table>
Eosinophili peroxidase | 415 505 640 | 445 566 595 | | 525 567
Myelo peroxidase | 430 570 625 690 | 475 590 637 | 458 634 | 438 590

(Olsen et al., 1982)

Also the native form of bromoperoxidase has maximum absorption spectrum at 403 and 616 nm with shoulders at 490 and 503 nm. Although the addition of Na₂SO₄ to the enzyme solution did not shift the Soret peak instead it caused loss of enzyme activity (Konch et al., 1989).

1.11 Gongronema latifolium (Utazi)

1.11.1 Gongronema latifolium (Utazi)

Gongronema latifolium is known as ‘utazi’ in the southeastern and ‘arokeke’ in the south-western part of Nigeria. Also Gongronema latifolium is called “madumaro” by Yoruba ethnic group in Nigeria. It is a perennial edible plant with soft and pliable stem. It is a tropical rainforest plant which belongs to the family of Aslepiadaceae (Ugochukwu and Babady, 2002 and Ugochukwu et al., 2003). It is a climber with tuberous base found in deciduous forest from Guinea Bissau and western Cameroons. Various parts of these plants, particularly the stems and leaves are used as chewing sticks or liquor and in places such as Sierra Leone they are also used as a decoction or cold infusion of pounded stem is used for colic and intestinal symptoms usually associated with worm (Deighton 1957) The liquor, usually obtained after the plant is sliced and boiled with lime juice or infused in water over three days is usually taken as a purge for colic and stomach pains as well as to treat symptoms connected with worm infections (Okafor, 1981). In Ghana the boiled fruit are used as laxative. In Eastern State of Nigeria, the leaves are used to prepare food for mother that have recently put to bed, where it is believed to stimulate appetite, reduce post-partum contraction and enhance the return of the menstrual cycle (Morebise et al., 2002).

The plant is also widely used in folk medicine as a spice and vegetable (Morebise et al., 2002) for maintaining healthy blood glucose levels (Okafor, 1981). Antibacterial activity of the leaf extract has also been reported (Nwinyi et al., 2008). The use of medicinal plants in curing
diseases is as old as man (Grabley and Thiericke, 1999, Abinu et al., 2007). The World Health organization (WHO) has long recognized and drawn the attention of many countries to the ever increasing interest of the public in the use of medicinal plants and their products in the treatment of various ailments. These plants which are found in our environment enjoy wide acceptability by the population and serve as cheaper alternatives to orthodox medicine (Sofowora, 1993; Akah and Nwabie., 1994). Gongronema latifolium is one of such medicinal plants whose therapeutic application has a folkloric background. The plant enjoys widespread reputation as a remedy for inflammation, bacteria, ulcer, malaria, diabetes and analgesic. Hence a scientific verification of its uses would be important in establishing a pharmacological basis for some of the claimed ethno medicinal uses.

1.11.2 Physiological Properties of Gongronema latifolium Plant

Gongronema latifolium, most common name is amaranth globe. The parts commonly used are the leaves, stem and root. 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 leaves are opposite, simple, softly hairy; petiole up to 4 cm long; blade ovate, 5–14 cm × 3–10 cm, base cordate, apex acuminate, margins entire. Inflorescence is cymose is composed of 2–3 primary branches divided dichotomously, each division ending in a 10–14-flowered umbel. The flowers are bisexual, small, regular, 5-merous, yellow-green; pedicel is 1 cm long; sepals elliptical-oblong, 2 mm × 1 mm; corolla tubular, with campanulate tube up to 4 mm long, lobes elliptical-oblong, is 2 mm long, spreading; corona lobes as long as stamens; stamens with deltoid to ovate anther appendages, connivent around the stout, roundish style apex. The fruit is a pair of leathery, pendent follicles, each one cylindrical, 10–15 cm× 4–8 mm, densely brown-grey hairy. Gongronema is a small genus comprising 5 species in Africa, much resembling Dregea.
Plate 1: The Leaves of *Gongronema latifolium*

Plate 2: Farm land showing *Gongronema latifolium* climbing on sticks
1.11.3 Chemical Composition of *Gongronema latifolium*

The chemical composition of Gongronema latifolium leaves has been determined using standard methods. The aqueous and methanol, crude protein, lipid extracts, ash, crude fibre and nitrogen free extractives obtained are: 27.2%, 6.07%, 11.6%, 10.8% and 44.3% dry matter respectively (Afolabi, 2007). Their potassium, sodium, calcium, phosphorus and cobalt contents are 332, 110, 115, 125 and 116 mg/kg respectively. The dominant essential amino acids are leucine, valine and phenylalanine. Aspartic acid, glutamic acid and glycine are 13.8%, 11.9% and 10.3% respectively of total amino acid content. Saturated and unsaturated fatty acids are 50.2% and 39.4% of the oil respectively. Palmitic acid makes up 36% of the total fatty acid. (Afolabi, 2007) Vitamin A, C, and E contents are 21.29, 2740, 3.19 u/100g respectively. Riboflavin, thiamine, niacin comprise 0.96, 0.18 and 0.81% respectively (Atangwho et al., 2009).

1.11.4 Microbial Studies on the *Gongronema latifolium*

*G. latifolium* extracts were tested against thirteen pathogenic bacterial isolates. The extracts show no activity against *E. faecalis, Y. enterolytica, E. aerogenes, B. cereus* and *E. agglomerans*. The methanol extracts were active against *S. enteritidis, S. cholerasius ser typhimurium* and *P. aeruginosa* with minimum inhibitory concentration (MIC) 1 mg; zone of growth inhibition 7, 6.5 and 7 mm respectively. The aqueous extracts show activity against *E. coli* (MIC 5 mg) and *P. aeruginosa* (MIC 1 mg) while methanol extracts are active against *P. aeruginosa* and *L. monocytogenes*. *G. latifolium* has potential food and antibacterial uses. (Afolabi, 2007)

1.11.5 Phytochemical compositions of *Gongronema latifolium*

Phytochemical analysis of *G. latifolium* showed that it contains alkaloids, acidic compounds, flavonoids, saponins, tannins, resins, steroids and essential oils as shown in table 5. These classes of compounds have some curative effect on micro-organisms induced disease. The natural products are good in several ways, flavonoids are found to be antimicrobial. It is used as a seasoner in food, which may be bitter or sweet or astringent (Uhegbu et al., 2011).
Table 5: Phytochemical And, anti-nutrient content (%) of *Gongronema latifolium*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>9.40</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>0.042</td>
</tr>
<tr>
<td>Saponin</td>
<td>2.70</td>
</tr>
<tr>
<td>Steroid</td>
<td>4×10⁻³</td>
</tr>
<tr>
<td>Hydrogen cyanide</td>
<td>5× 10⁻⁴</td>
</tr>
<tr>
<td>Tannin</td>
<td>6.10</td>
</tr>
<tr>
<td>Starch</td>
<td>ND</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>ND</td>
</tr>
</tbody>
</table>

(Uhegbu et al., 2011)

1.1.1.6 Uses of *Gongronema latifolium*

I. The antioxidant and antitussive properties of *Gongronema latifolium* used locally by Nigerian poultry farmers for the treatment of fowl cough was investigated (Essien et al., 2007). It was stated that the leaf extract significantly reduced the mortality rate of the broilers by 25% within 3 weeks of treatment and by 40% in 6 weeks of administration, when the broilers were 13 weeks old. The reduction in mortality coincided with reductions in the microbial loads in the trachea of the sick 7-week old broilers.

II. The anti-oxidative properties of *Gongronema latifolium* are being utilized in management of diabetes (Ugochukwu et al., 2003). Traditionally, the leaf is believed to stimulate appetite, reduce post-partum contraction, enhance the return of the menstrual cycle and used in controlling weight gain in lactating women (Nwanjo et al., 2006).

III. The spice has been used historically to improve the anti-oxidation and anti-malaria activity of food. *Gongronema latifolium* is one of the plants used as spice for flavouring, seasoning, and imparting aroma to food.

IV. *Gongronema latifolium* has been investigated to be nutritionally high in iron, zinc, vitamin, protein and amino acid, thus could complement the inadequacies of these substance in feed (Agbo et al., 2006).
V. Also the bitter principles when extracted may have potential in beer brewing (Adenuga et al., 2010).

1.12 Aim of the research

Peroxidase forms an important part of the defenses of the cell against oxidative stress. The study of the inactivation of this enzyme by one of the major products of such stress, namely H$_2$O$_2$, provides an indication of the limitation of resistance to attack by reactive oxygen species. This investigation on Gongronema latifolium aims to extend our knowledge of peroxidase inactivation by H$_2$O$_2$ by providing an additional and distinct example.

1.13 Objectives of the study

To extract peroxidase from Gongronema latifolium leaf.
To partially purify the enzyme extract via ammonium sulphate precipitation and dialysis.
To characterize the purified enzyme.
To study the stability of the enzyme in different concentration of H$_2$O$_2$
To study the substrate protection on the enzyme
To study the spectral properties of peroxidase inactivation
CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Sample collection and location

Matured fresh leaves of Gongronema latifolium were purchased from Ogige Market in Nsukka Senatorial Zone of Enugu State.

2.1.2 Apparatus and Instruments

Blender
Centrifuge (Fin lab-80-2B)
Cheese cloth
Dialysis bag
Dialysis tube
Electronic weighing balance (B2404-5 Mettle Toledo made in Switzerland)
Hot plate
Magnetic stirrer (AM-3250B surgifriend medical England.)

pH meter (model PHS-3C, Search Tech. instrument)
Refrigerator
UV/VIS spectrophotometer (Jenway 6405)
Weighing balance (Ohans-Dial-O-Gram. Ohans-co-oparation N J USA.)

2.1.3 Chemical / Reagents

Acetic acid (May and Baker,Dagenhan, England).
Ammoninm Sulphate
Bovine serum albumin (BSA) (Merck, England.)
Disodium hydrogen phosphate (BDH, Pools, England.)
Folin-Ciocalteau Phenol Reagent (Sigma-Aldrich, Germany.)
Guaiacol
Hydrochloric acid
Hydrogen peroxide (BDH pool, England.)
Methanol (Sigma Aldrich (Germany.))
O-Dianisidine
Phosphoric acid (BDH, pools England.)
Sodium acetate (Vickers Laboratories Ltd, West Yorkshire, London.)
Sodium carbonate (Merck, England.)
Sodium Dihydrogen phosphate (BDH, Pools, England.)
Sodium hydroxide (Avondale Laboratories)
Sodium hydroxide (Merck, Germany).
Sodium potassium tartrate (Merck, Germany).
Tris (hydroxymethytyl) aminomethane (May & Baker Dagenhan, England.)
Whatman NO 1 Filter paper (whatman international Ltd.) Maldstone England.

2.2 METHODS
2.2.1.0 Preparation of Buffer Solutions

2.2.1.1 Sodium phosphate buffer (stock solution)

In preparing 1M of sodium phosphate buffer of pH 6.5, 15.6g of sodium dihydrogen phosphate (conjugate base) was dissolved in 100ml of distilled water and 14.2g of disodium hydrogen phosphate were dissolved in 100ml of distilled water. The pH was adjusted to 6.5 with the conjugate acid.

2.2.1.2 Acetate buffer (stock solution)

To prepare 1M of acetate buffer, 13.6g of sodium acetate was dissolved in 100ml of distilled water. The pH was adjusted with acetic acid.

2.2.1.3 Tris-HCl buffer (stock solution)

Tris buffer (1M) was prepared by weighing 24.2g of Tris (hydroxymethyl) aminomethane and dissolving some in 100ml of distilled water, the pH was adjusted with hydrochloric acid
2.2.1.4 Preparation of working phosphate buffer

The working phosphate buffer (0.1M, pH 6.5) was freshly prepared when needed, measured and mixed with distilled water and refrigerated.

2.2.1.5 Preparation of reagent for protein standard curve

Protein content was estimated by the method of Lowry et al. (1951). The standard used was bovine serum albumin. The reagent used was prepared as follows

I. Solution 1: 2% of Na₂CO₃ was dissolved in 0.1N NaOH.
II. Solution 2: 1% of sodium potassium tartarate that is 1g of sodium potassium tartarate dissolved in 100ml of distilled water. Also 0.5g of Copper sulphate (CuSO₄) was dissolved in the 1% sodium potassium tartarate solution
III. Solution 3: 1ml of solution 2 was dissolved in 50ml of solution 1
IV. Solution 4: 1N Folin Ciocalteu, 1:1 dilution of 1N Folin Ciocalteu with distilled water was made
V. Solution 5: 0.50g of bovine serum albumin (BSA) was weighed and dissolved in 100ml distilled water

2.2.2 Measurement of protein content

I. Different concentrations of protein standard solution (BSA) were prepared, by preparing a ratio of protein :water in the order :10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9,10:1 in eleven different test tubes
II. To each test tube, 5ml of solution 3 was added (alkaline copper reagent) thoroughly mixed and left to stand for five minutes.
III. A known, 0.5ml of solution 4 (diluted Folin Ciocalteu solution) was added to the test tubes rapidly and thoroughly mixed.
IV. Solution was left for 30 minutes to incubate and the absorbance read at 750nm.
V. The final concentration of the protein was calculated using the equation C₁V₁=C₂V₂.

2.2.3 Extraction of enzyme (peroxidase)

Crude peroxidase from Gongronema latifolium leaf was extracted, following the modified method of Khali-Ur-Rehaman et al. (1999) and Eze et al. (2010).
2.2.3.1 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 30 minutes in order to remove chlorophyll. The supernatant was measured and found to be 160ml. The enzyme extract was stored in the refrigerator.

2.2.3.2 Preparation of substrate solution

a) 0.1% *o*-dianisidine solution. This was prepared by weighing 0.1g of *o*-dianisidine and dissolving in 100ml of methanol and filtering with Whatman no 1 filter paper to give 0.1% of *o*-dianisidine solution.

b) 0.3% H₂O₂: The stock H₂O₂ is 30%. This means that 1ml of hydrogen peroxide was dissolved in 100ml of water

c) Guaiacol. Prepared daily by the method of (Melda et al. 2010) was added and mixed vigorously, 0.1ml Guaiacol, 0.1ml H₂O₂ (30%), 99.8ml 0.1M phosphate buffer pH 6.5

2.2.3.3 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 enzyme 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.

The readings were taken for every 30 seconds for 5 minutes.

The variables below were calculated as follows using the method of (Segel,1993).
Reaction rate = \[ \frac{\text{Change in absorbance (OD) at 460nm}}{\text{Time interval}} \]

Specific activity (Unit/ml) = \[ \frac{\text{Reaction rate}}{\text{Protein concentration}} \]

Where, Total units = unit/ml \times \text{total volume of enzyme.}

Percentage yield or recovery = \[ \frac{\text{Total unit of purified enzyme}}{\text{Specific activity of crude enzyme}} \]

Purification fold = \[ \frac{\text{Specific activity of purified enzyme}}{\text{Specific activity of crude enzyme}} \]

2.2.3.4 Peroxidase assay using Guaiacol as substrate

Peroxidase activity was measured using the method reported by Melda et al. (2010). Peroxidase substrate solution was prepared daily by mixing 0.1 ml guaiacol, 0.1 ml hydrogen peroxide (30%) and 99.8 ml 0.1M sodium phosphate buffer (pH 6.5). Peroxidase assays were conducted by pipetting 0.12 ml of enzyme extract and 3.48 ml of substrate solution in the cuvette. The peroxidase activities were measured from the increase in absorbance at 470 nm using an UV/VIS spectrophotometer (Jenway 6406). The reaction was monitored for 5 min at 30sec intervals at 25°C. All experiments were run in triplicates.

2.2.4 Purification of peroxidase from Gongronema latifolium

2.2.4.1 Ammonium sulphate precipitation

To the remaining volume of the enzyme extract, 75.52g of the solid reagent, that is ammonium sulphate (\(\text{NH}_4\)\(_2\)\(\text{SO}_4\)) was added slowly to the enzyme extract in a beaker until it becomes 70% saturation. This was stirred slowly for 1 hour and then the solution was kept undisturbed at 4°C for 48 hours. Then the mixture was centrifuged at 4000rpm for 30 minute. The precipitated protein collected was re-dissolved in 0.1M sodium phosphate buffer pH 6.5. The enzyme activity and protein content were measured and determined. The remaining solution was kept and stored in refrigerator.
2.2.4.2 Dialysis

The supernatant was discarded and precipitate dialysed by pouring the remaining precipitate in a dialysis bag suspended in a beaker containing ice cold 0.01M sodium phosphate buffer pH 6.5. The buffer in the beaker was continuously stirred with a magnetic stirrer and kept for 48 hours. The buffer was changed for every 12 hours, the total volume of the dialysed enzyme was measured and recorded. The enzyme activity and protein contents of the dialysed protein were determined at 460nm using o-dianisidine, as a substrate and 470nm using guaiacol as a substrate and 750nm for protein respectively. The remaining was stored in the freezer.

2.2.5 Effect of H2O2 on peroxidase activity

Peroxidase was assayed with different concentrations (0.0M, 0.1M, 0.5M, 1.0M, 2.0M, 3.0M, 4.0M, 5.0M, 6.0M, 7.0M, 8.0M, 9.0M 10M and 12M) of hydrogen peroxide to determine the suitable concentration of hydrogen peroxide to be used for the assay. The assay mixture contained 2.4ml of 0.1M phosphate buffer pH 6.5, 0.2ml of different concentration of H2O2, 0.1ml of Enzyme, and 0.3ml of 1% o-dianisidine. The change in OD per minute was calculated and plotted against time with the different concentrations of hydrogen peroxidase.

2.2.6 Effect of o-dianisidine on peroxidase activity

Peroxidase activity was assayed using different concentrations of o-dianisidine (0mM, 0.1mM, 0.2mM, 0.3mM, 0.4mM, 0.5mM, 0.6mM 0.7mM,0.8mM and 0.9mM) . The assay mixture contained 2.4ml of 0.1M phosphate buffer pH 6.5, 0.1ml of enzyme, 0.2ml of H2O2, and 0.3ml of different concentrations of o-dianisidine. The change in OD per minute was calculated and plotted against time.

2.2.7 Characterization of Enzyme

2.2.7.1 Determination of Optimum pH

The activity of peroxidase was examined within the pH range of 4.0 - 9.0 using the following buffer systems: Sodium-acetate buffer (0.1M, pH 4 - 4.5); sodium phosphate buffer (0.1M, PH 5.0 – 7.5); Tris – HCl buffer (0.1M, PH 8 – 9.0) The residual activity was then determined using o-dianisidine as the reducing substrate for the assay method.
2.2.7.2 Optimum Temperature

The optimum temperature was determined at the optimum pH by measuring the activity of the enzyme in temperatures ranging from 10 to 90°C.

2.2.8 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 0.1M phosphate buffer, 0.1ml of enzyme, 0.3ml of o-dianisidine, and 0.2ml of H₂O₂. The % residual activity was calculated using the relationship

\[
\% \text{ Residual activity} = \frac{A_t}{A_0} \times 100
\]

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.

2.2.9 The absorption spectrum of inactivation of peroxidase by different concentrations of hydrogen peroxide

Different concentrations of hydrogen peroxide (0.1M, to 12M.) were incubated with a constant volume of enzyme (0.2ml) with 2.6ml of 0.1M sodium phosphate buffer for 30 minute at room temperature and the spectra readings were taken from 340nm to 800nm.

In a related experiment the enzyme was incubated with either 0.4mM o-dianisidine or 1mM ascorbate with the different concentrations of hydrogen peroxide and buffer as in the above experiment. And the spectra readings were also taken from 340nm to 800nm.
CHAPTER THREE

RESULTS

3.1 Purification of Peroxidase

Table 6 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.

3.2 The effect of different concentration of o-dianisidine on peroxidase

Figure 12 shows the effect of o-dianisidine on peroxidase activity. Peroxidase activity varies with time (of assay) and with concentration of the substrate used for the assay. At concentrations of 0.5mM to 0.9mM, the activity plot tends to stabilize after 1 minute while following the same pattern at concentration of 0.2, 0.3 and 0.4mM, the decrease in activity was sharper after 1 minute of assay. From the graph the peak activity was observed at 0.4mM at 1 minute of assay.

3.3 The effect of different concentration of hydrogen peroxide on peroxidase

Figure 13 shows the effect of hydrogen peroxide on peroxidase activity. Peroxidase activity varies with time of assay and concentration of hydrogen peroxide. All the concentrations tends to stabilize after 1 minute of assay. From the graph the peak activity was observed at 5mM at 1 minute of assay,
Table 6: Purification Table

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Total Activity</th>
<th>Activity yield</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>B</td>
<td>α</td>
<td>B</td>
<td>α</td>
</tr>
<tr>
<td>Crude Enzyme</td>
<td>6.0</td>
<td>7.53</td>
<td>0.30</td>
<td>1.30</td>
<td>1.36</td>
</tr>
<tr>
<td>70% NH₄(SO₄)₂ ppt</td>
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Where β represents guaiacol and α represents o-dianisidine
Figure 12: The effect of different concentration of o-dianisidine on peroxidase activity with time
Figure 13: Effect of different concentrations of hydrogen peroxide on peroxidase activity with time
3.4: Effect of pH on peroxidase activity

Figure 14 shows that peroxidase activity was pH-dependent, a maximum enzyme activity of 17.15 µ/ml was observed at pH 7.0, there were decreases in activity after pH 7.

3.5: Study on temperature optimum

From Figure 15, the optimum temperature was observed at 30°C with the highest activity of 17.15 µ/ml, after which the activity of the enzyme began to decrease with almost complete inactivation at 90°C

3.6: Variation of peroxidase activity with different concentrations hydrogen peroxide

Figure 16 shows the effect of different concentration of hydrogen peroxide (0.1, 0.5, 1, 2, 3, 4, 5, and 6 mM) on peroxidase activity. As the concentration of hydrogen peroxide increases, the activity of the enzyme also increases, until it reached 4mM. At that concentration, the activity became constant indicating hyperbolic curve of Michaelis-Menten plot.

3.7: Determination of Km and Vmax

Figure 17 is the Lineweaver-Burk plot from variation of peroxidase activity with hydrogen peroxide. From this plot the Vmax is 20 u/ml and the Km is 1.8mM

3.8: Variation of peroxidase activity with varying concentration of o-dianisidine.

Figure 18 shows the Michaelis–Menten plot of o-dianisidine on peroxidase. As the concentration of o-dianisidine increases the activity of peroxidase also increases until the concentration reached 0.3mM. At this point increase in the concentration of o-dianisidine does not lead to corresponding increase in the velocity of the reaction.

3.9 The Lineweaver-Burk plot of effect of different concentrations of o-dianisidine on peroxidase activity

Figure 19 shows the Lineweaver-Burk plot, from Michaelis-Menten plot of o-dianisidine on peroxidase. The Km is 0.12mM and Vmax is 3.3 u/ml.
Figure 14: Effect of pH on peroxidase activity
Figure 15: Temperature depandance of peroxidase activity
Figure 16: Michealis-Menten plot of peroxidase
Figure 17: Lineweaver-Burk plot of $\text{H}_2\text{O}_2$
Figure 18: Michealis-Menten plot of o-dianisinidine
Figure 19: Lineweaver-Burk plot of o-dianisidine
3.10 The inactivation of peroxidase by hydrogen peroxide

Figure 20 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.

3.11: The comparison of the effect of ascorbate and o-dianisidine on inactivation of peroxidase by different concentrations of hydrogen peroxide (0.1 to 10mM)

Figure 21a to 21e shows a 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.

3.11.1: The comparison of the effect of ascorbate and o-dianisidine on inactivation of peroxidase by 0.1mM hydrogen peroxide

From Figure 21a, ascorbate had 26% protection at 1 minute of incubation and 28% at 10 minutes of incubation, while o-dianisidine had 14% at 1 minute and 25% at 10 minutes of incubation respectively, against the inactivation of peroxidase by hydrogen peroxide.

3.11.2 The comparison of the protective effect of ascorbate and o-dianisidine on inactivation of peroxidase by 1mM of hydrogen peroxide

Figure 21b 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.

3.11.3 The comparison of the protective effect of ascorbate and o-dianisidine on the inactivation of peroxidase by 5mM of hydrogen peroxide

From Figure 21c there was 24% protection by ascorbate and 20% by o-dianisidine at 1 minute of incubation and at 10 minutes 22% for ascorbate and 20% for o-dianisidine respectively, against inactivation.
Figure 20: Inactivation of peroxidase by H$_2$O$_2$
Figure 21a: Comparison of the protective effect of ascorbate and o-dianisidine on inactivation of peroxidase by 0.1mM H₂O₂

Where INA represents inactivation without the two substrates, ODI represents the effect of o-dianisidine and ASC represents the effect of ascorbate.
Figure 21b: Comparison of the protective effect of ascorbate and o-dianisidine on inactivation of peroxidase by 1.0mM H₂O₂

Where INA represents inactivation without the two substrates, ODI represents the effect of o-dianisidine and ASC represents the effect of ascorbate.
Figure 21c: Comparison of the protective effect of ascorbate and o-dianisidine on inactivation of peroxidase by 5mM H$_2$O$_2$

Where INA represents inactivation without the two substrates, ODI represents the effect of o-dianisidine and ASC represents the effect of ascorbate.
3.11.4 The Comparison of the protective effect of ascorbate and o-dianisidine on the inactivation of peroxidase by 7.5mM of hydrogen peroxide

Figure 21d 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.

3.11.5 The comparison of the protective effect of ascorbate and o-dianisidine on the inactivation of peroxidase by 10mM of hydrogen peroxide

From Figure 21e, 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.
Figure 21d: Comparison of the protective effect of ascorbate and o-dianisidine on inactivation of peroxidase by 7.5 mM H₂O₂.

Where INA represents inactivation without the two substrates, ODI represents the effect of o-dianisidine and ASC represents the effect of ascorbate.
Figure 21e: Comparison of the protective effect of ascorbate and \(\alpha\)-dianisidine on inactivation of peroxidase by 10mM \(\text{H}_2\text{O}_2\)

Where INA represents inactivation without the two substrates, ODI represents the effect of \(\alpha\)-dianisidine and ASC represents the effect of ascorbate.
3.12.1 The spectra studies of native peroxidase

In the spectra properties of native peroxidase from Figure 22a, there was peak at 381 nm at the Soret band and other peaks observed are at 418, 498, 587, 653, 662, and 658 nm respectively.

3.12.2 Spectra properties of inactivation of peroxidase by different concentrations of hydrogen peroxide

Figure 22b shows the spectra of inactivation of peroxidase by different concentrations of hydrogen peroxide, the peak at the Soret band shifted from 381nm to 389nm and other peaks 418 498 587 653 662 and 658nm respectively remained the same. Also as the concentration of hydrogen peroxide increases there was decrease in the Soret peak absorbance.

3.12.3 Spectra properties of the protective effect of ascorbate on inactivation of peroxidase by different concentrations of hydrogen peroxide

Figure 22c shows the spectra changes of the protective effect of ascorbate on inactivation of peroxidase by hydrogen peroxide, the peaks remain the same with the inactivation studies at 389nm at the Soret peak and 418 498 587 653 662 and 657 nm respectively at other peaks respectively. There was increase in the Soret peak absorbance.

3.12.4 Spectra of the protective effect of o-dianisidine on inactivation of peroxidase by different concentrations of hydrogen peroxide

Figure 22d shows the spectra of the effect of o-dianisidine on inactivation of peroxidase by hydrogen peroxide. The peaks remain the same with the inactivation studies at 389nm at the Soret band and 418 498 587 653 662 and 657 nm respectively at other bands. There was increase in the Soret peak absorbance.
Figure 22a: Spectra properties of native peroxidase
Figure 22b: Spectra properties of inactivation of peroxidase by hydrogen peroxide
Figure 22c: Spectra of the protective effect of ascorbate against inactivation of peroxidase by hydrogen peroxide
Figure 22d: Spectra studies of the protective effect of o-dianisidine against inactivation of peroxidase by hydrogen peroxide
CHAPTER FOUR

DISCUSSION

4.1 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. (2012) 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. (2010) 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.

It was known that pH is a key factor for enzyme activity, as it changes ionization states of the protein and substrate (Gawlik-Dziki et al., 2008). Peroxidase from Gongronema latifolium has an optimum pH of 7.0. Mamounata et al. (2011) reported optimum pH of four different sources of peroxidase, pH 5.5 to 6.5 for Allium sativum, pH 6 for Ipomoea batatas, pH 5 for Raphanus sativus and pH 3.5 to 4 for Sorghum bicolor. Also the optimum pH range 6.5-7.5 has been reported previously by Khalil-Ur-Rehaman et al. (1999) on peroxidases from different kinds of vegetable. In the cases of horseradish peroxidase and beans cell peroxidase the pH optimum were 8.5 and 7.2 respectively (Bowell et al., 2002). The result of this study is consistent with the findings of Majed and Mohammad (2005) who reported that haem-peroxidase from palm tree leaves is stable over a broad pH range with optimum pH at pH 7.0. Also, Kim and Lee (2005) reported optimum pH of 5 from cauliflower bud peroxidase when guaiacol was used as a substrate, pH 4 when ABTS and catechol were used and pH of 7.5 when pyrogallol and 4-
methyl catechol were used as substrates. An optimum pH of 7.0 from this study will enable peroxidase from Gongronema latifolium to be applied widely in industrial processes.

Peroxidase is thought to be the most heat stable enzyme in plant, because plant peroxidases are glycosylated proteins. It was observed in this investigation that peroxidase from Gongronema latifolium has an optimum temperature of 30°C and the activity was at minimum at 90°C, showing inactivation of the enzyme. Optimum temperature 30°C correlated those of Civello et al. (1995) who reported maximum enzyme activity at 30°C. It is also interesting to note that Mamounata et al. (2011) also reported optimum temperature on different sources of peroxidase as follows, 40°C for Allium sativum and Sorghum bicolor, 30°C for Ipomoea batatas and Raphanus sativu (which are consistent with these result). Also Yihong et al. (2002) reported 45°C for peroxidase purified from lettuce stems. Optimum temperature varies among species of plant, also differences in optimum temperature may be as a result of different reducing substrates used for the assay. Most industrial applications of peroxidase use temperature ranges of 25 to 55°C; as such peroxidase from Gongronema latifolium with optimum temperature of 30°C can be applied in some of these processes.

The effect of the substrates (hydrogen peroxide and o-dianisidine) concentration on peroxidase activity showed that the activity of peroxidase increased with corresponding increase in substrates concentration until a saturation point of about 5mM for hydrogen peroxide and 0.3mM for o-dianisidine, indicating that the active sites are saturated with the substrates. The Km from these results was 1.12mM for o-dianisidine and 1.8mM for hydrogen peroxide and Vmax value of 3.3u/ml for o-dianisidine and 20µ/ml for hydrogen peroxide. Similar to these results is the observation of Kim and Lee (2005) that reported Km value of 1.18mM for o-dianisidine and 1.27mM for hydrogen peroxide, with Vmax 0.032 u/ml/min for o-dianisidine and 0.138 u/ml/min for hydrogen peroxide, for peroxidase from Raphanus sativus. Also, Melda et al. (2010) reported that peroxidase from Raphanus sativus had Km values of 0.036mM for guaiacol and 0.0084mM for hydrogen peroxide, with Vmax values of 3512.23u/ ml/min and 38728.17u/ml/min respectively. From these reports it is evident that the nature of different reducing substrates affect the Km and Vmax of peroxidase.

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$_2$O$_2$ 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 Hiner et al. (2000) that ascorbate peroxidase inactivation is time dependent. Also, the inactivation of ascorbate-free peroxidase from Leishmania major was time dependent and an irreversible inactivation of the enzyme followed a pseudo-first-order kinetics (Adak and Alok, 2005). The catalase-like reaction of H$_2$O$_2$ 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$_2$O$_2$ (Hiner et al., 2002).

In the chloroplasts of higher plant, ascorbate is formed at a concentration from 10 to 50mM (Foryer et al., 1983). In this study peroxidase was protected against inactivation by H$_2$O$_2$ 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 (Hiner et al., 2000). Similar effect has been seen with HRP. There was complete protection when a large amount of reducing substrate was used (Arnao et al., 1990). The reduced protective effect by ascorbate and o-dianisidine 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.

From these results native peroxidase has an absorption spectrum characteristic of a ferric (Fe$^{III}$) high-spin state, with a Soret peak at 381 nm and other secondary peaks at 496,653 and 658nm. Adak and Alok (2005) reported a Soret peak of 408nm and 500 and 640 at the visible peak from ascorbate-free Leishmania major peroxidase. Ascorbate peroxidase was reported to have a Soret peak of 403 (Miyake and Asada, 1996). Also Olsen et al. (1982) reported absorption maxima of intestinal peroxidase to be 417, 490, 543 596 and 642nm, lacto-peroxidase to be 412,501,541, 589, and 631nm and myelo peroxidase to be 430,570, 625,and 690nm
respectively, with the first value being the Soret peak. This low wavelength at the Soret band compared to the reported results may be as a result of impurity due to the fact that the enzyme was purified on a two-step purification. On the addition of different concentrations of H$_2$O$_2$ to a solution of native enzyme and incubating, there was a shift in the Soret band from 381nm to 389 nm with other secondary bands remaining the same in (Figure 25b). This result is consistent with the findings of Adak and Alok (2005), where a shift in the soret peak of the native enzyme from 408 to 420nm when equivalent amount of hydrogen peroxide was added to the native enzyme of Δ34 ascorbate-free LmAXP. Similar to this result are the findings of Miyaka and Asada (1996) where they reported a shift in Soret peak from 403nm to 415nm when equimolar amount of hydrogen peroxide was added to the native ascorbate peroxidase. Also the same was observed in the case of cytochrome c peroxidase, there was a shift in the Soret peak when hydrogen peroxide was mixed with the enzyme (Yonetani and Anni, 1987). However, on addition of H$_2$O$_2$ to the enzyme, no green colour was detected, this was the same with APX. Thus, the H$_2$O$_2$-oxidized intermediate of the enzyme seems to correspond to haem-Fe$^{IV}$=O with an amino acid residue radical. In contrast to Compound I of the enzyme and that of APX, it was reported recently that Compound I of cytosolic APX is green in color, with haem-Fe$^{IV}$=O and a porphyrin π cation radical, resembling Compound I of HRP (Patterson et al., 1995)

However, when the concentration of H$_2$O$_2$ was increased, the Soret peak absorbance decreased, indicating the degradation of the haem moiety in Figure 25b. The rate of degradation of the haem of Compound I increased as the concentration of H$_2$O$_2$ increased, as observed from the decrease in Soret absorbance at 389 nm in Figure 25b which corresponds to the result of Miyake and Asada (1996) with decrease in the Soret absorbance when excess concentration of hydrogen peroxide was added to the solution of the native enzyme. There was increase in the Soret absorbance when the enzyme and different concentrations of hydrogen peroxide were incubated with either 1mM of ascorbate or 0.4mM of o-dianisidine, with the secondary peaks remaining the same with that of inactivation in Figure 25b. This increase in Soret absorbance in Figure 25c and 25d was due to the protective effect of the substrates, ascorbate and o-dianisine. Compound I of peroxidase prefers a reducing substrate to hydrogen peroxide, as such in the presence of reducing substrates the enzyme was protected against degradation of the haem moiety of compound I of the enzyme.
4.2 Conclusion

The evidence presented in these studies have shown that peroxidase from *Gongronema latifolium* undergoes inactivation by hydrogen peroxide in a concentration dependent manner, but was highly protected by ascorbate and *o*-dianisidine. This suggests that ascorbate which is found in abundance within the chlorophyllous cells of the leaves offer protection to the plant against \( \text{H}_2\text{O}_2 \) which is a product of oxidative stress. In other words, the enzyme has been implicated in other cellular processes in plant in which its instability to hydrogen peroxide could be important.
REFERENCES


Appendix 1: Values for protein standard curve

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Appendix 2: Graph of protein standard curve

Appendix 3: Percentage residual activity of inactivation of peroxidase by different concentrations of hydrogen peroxide
### Time of incubation (min) vs Different Concentrations of Hydrogen Peroxide

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Appendix 4: Percentage residual activity of protective effect of ascorbate against inactivation of peroxidase by different concentrations of hydrogen peroxide
Appendix 5: Percentage residual activity of protective effect of o-dianisidine against inactivation of peroxidase by different concentrations of hydrogen peroxide

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