Isolation, Partial Purification, and Characterization of Peroxidase from *Ganoderma Lipsiense*

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CHAPTER ONE

1.0 Introduction

The ubiquitous heme-containing enzymes known as peroxidases (donor: hydrogen-peroxide oxidoreductase; E.C.1.11.1.7) belong to the family of oxidoreductases, catalyzes hydrogen peroxide as the electron acceptor and facilitate the oxidation of a wide array of substrates, from aromatic amines to polyaromatic hydrocarbons (PAHs). (Reina Prego 2016). Macro-fungi or mushrooms are species with a natural fruit body that can grow underground. The spores, produced by the fruiting body, are the unit of sexual and asexual reproduction and are responsible for fungi's spread. Fungi are eukaryotic and spore-bearing organisms with a life cycle divided into two phases: a growth phase and a reproductive phase. The macrofungi known as mushrooms have long been used as food and in conventional treatment. Research has demonstrated the value of mushrooms as nutrient-dense foods and as an effective ingredient in the pharmaceutical and medical industries (Elkhateeb et al., 2021; Elkhateeb et al., 2022). Apart from their uses in food, industry, and pharmaceuticals, the fungus kingdom is rich with members that play significant roles in many facets of human life, starting with penicillin, which saved millions of lives. Notably, among the vast diversity of mushroom species, numbering over 14,000, approximately 2000 are deemed edible, drawing significant scientific attention. Despite the relatively low incidence of toxicity among fungi, their richness in biologically active compounds, including peptides, terpenes, and flavonoids, underscores their pharmaceutical and industrial significance. As higher *Basidiomycetes* and *Ascomycetes*, mushrooms have fruit bodies, cultured mycelium, and cultured broth that contain secondary metabolites (Alkolaibe et al., 2021; Elkhateeb et al., 2021). The biological properties of ingredients and extracts derived

from mushrooms have been extensively documented in the literature (Elkhateeb et al., 2021). As a dietary supplement and nutraceutical, mushrooms are becoming more and more significant (Üstün et al., 2018; Atri et al., 2019). Most importantly, they are extensively utilized in pharmaceutical, cosmetic, and business domains because of the presence of antioxidants, carotenes, and secondary metabolites (Bulam et al., 2018).

1.1 Statement of the Problem

Horseradish Peroxidase is a widely and commercially used enzyme but it is highly expensive. Due to its high cost, there is a need for local sources like *Ganoderma lipsiense* to characterize a stable peroxidase. However, little or no research has been made on this study and also low knowledge-based how on its uses/applications. To fully harness the biotechnological capabilities of non-edible mushrooms, it is crucial to delve into isolation methods, refine partial purification techniques, and thoroughly elucidate the properties of the peroxidase extracted from the local sources.

1.3 Aim and Overall Objective of the Study

Peroxidases are multifunctional enzymes that find extensive uses in food processing, medicines, diagnostics, and environmental remediation. This study aims to explore alternative and sustainable enzyme reservoirs that are often overlooked. The overall objective is to isolate, purify and characterize peroxidase from non-edible mushroom (*Ganoderma Lipsiense*)

1.4 Specific Objectives of the Study

The specific objectives are:

i. To isolate peroxidase from non-edible mushroom (*Ganoderma Lipsiense*)

ii. To determine proximate composition of *Ganoderma Lipsiense*

iii. To carry out partial purification of peroxidase from *Ganoderma Lipsiense.* Techniques such as ammonium sulfate precipitation, and dialysis may be employed to achieve partial purification and enrich the peroxidase fraction.

iv. To determine the optimum pH and temperature of partially purified peroxidase from *Ganoderma Lipsiense*

v. To determine the decolourization capacity of partially purified *Ganoderma lipsiense* peroxidase;

vi. To determine the effect of Urea, Mercury ion (Hg^{2+}) and Nickel ion (Ni^{2+}) on partially purified *Ganoderma lipsiense* peroxidase;

vii. To determine kinetics of inhibition on partially purified *Ganoderma lipsiense* peroxidase using EDTA as an inhibitor.

1.5 Justification of the Study

Peroxidase are used in a wide range of biotechnological applications in food processing, pharmaceuticals, diagnostics, and environmental remediation, among other industries because they are a plentiful and little-studied source of enzyme. Non-edible mushrooms are excellent choices for biotechnological applications. In the forestry and agricultural sectors, non-edible mushrooms are frequently disregarded and treated as garbage. Although non-edible mushrooms provide a different and maybe more affordable option, peroxidase are traditionally obtained from plant and microbial sources, reducing reliance on scarce resources and improving resilience to supply chain disruptions are two benefits of diversifying the sources of peroxidase. Also, this study supports the circular economy by adding value to agricultural byproducts by using enzyme extraction to valorize non-edible mushroom biomass.

This strategy is in line with trash reduction and sustainable resource management. It improves the practical usability and commercial feasibility of active peroxidase as an enzyme by streamlining the recovery and concentration process.

CHAPTER TWO

2.0 Literature Review

2.1 *Ganoderma species* and their distribution

The well-known genus *Ganoderma* has been the subject of effective research in conventional eastern medicine. It has been possible to separate and identify bioactive elements of the several *Ganoderma* species, including polysaccharides, triterpenoids, steroids, and phenolic compounds (Costa et al., 2019). Microorganisms rich in components with biological activity are called fungi. Because of this, *Ganoderma* is a mushroom that has historically been used in oriental medicine. It is thought that there are more than 250 *Ganoderma* species, each with distinct biological properties worldwide. Few researchers have looked into the chemicals made by *Ganoderma lipsiense* in this literature (Costa et al., 2020). Some of these species are *Ganoderma* species from the Greater Mekong Subregion (GMS), encompassing tropical parts of Laos, Myanmar, Thailand, Vietnam, and temperate areas in Yunnan Province, and China namely, *G. adspersum, G. applanatum, G. australe, G. calidophilum, G. ellipsoideum, G. flexipes, G. gibbosum, G. heohnelianum, G. hochiminhense, G. leucocontextum, G. lucidum, G. multiplicatum, G. multipileum, G. myanmarense, G. orbiforme, G. philippii, G. resinaceum, G. sichuanense, G. sinense, G. subresinosum, G. williamsianum, and G. tsugae.* Natural bioactive substances with a wide molecular weight range, including polysaccharides, proteins, sterols, and triterpenoids, can be found in sufficient amounts in *Ganoderma* species. Numerous medicinal benefits, including antibacterial, antifungal, antiviral, anticancer, antitumor, anti-inflammatory, anti-hypotensive, and antioxidant characteristics, are known to be associated with these substances (Luanghan et al., 2021).

Table 1. Species of Ganoderma, host plants, diseases, and main regions of distribution within the Greater Mekong Subregion

Ganoderma Species	Host Plant	Diseases	Region
G. adspersum	*Pterocarpus* sp.	Wood decay	*Thailand*
	Mangifera indica	Wood decay	*Laos*
G. applanatum	*Acer* sp.		Vietnam
	Rhizophora apiculata, *Machilus yunnanensis*, and hardwoods	Wood decay, Butt rot	Thailand, China, Myanmar, Laos
G. applanatum	*Machilus yunnanensis*	Wood decay	Yunnan, China
	Artocarpus sp., and *Dipterocarpus* sp.	Wood decay	Thailand
G. australe	*Anisoptera costata* and *Shorea robtusa*	*Wood decay*, rotten wood	Laos, Thailand, and Myanmar
	Camellia sinensis var. assamica	*Root rot*	*Thailand*
G. calidophilum	*Castanopsis* sp. and *Machilus yunnanensis*	*Wood decay*	*Yunnan, China*
G. subresinosum	*Peltophorum pterocarpum* and *Castanopsis* sp.	Wood decay	Thailand
G. thailandicum	*Pinus merkusii*	Wood decay	Thailand
G. tropicum	*Dipterocarpus* sp.	Wood decay	Thailand
G. tsugae	*Larix* sp., *Picea* sp. and *Tsuga* sp.	*Butt rot*, Wood decay	*China*
G. williamsianum	Unknown	Wood decay	Myanmar

2.2 **Peroxidases and its structures**

Peroxidases, (EC number1.11.1.x) are a diverse group of oxidoreductases that are recognized for their ability to facilitate the oxidation of various inorganic and organic compounds through the utilization of H_2O_2 (Adewale & Adekunle 2018). These enzymes share a uniform catalytic process for breaking down H_2O_2, involving a two-electron oxidation-reduction mechanism with three clearly defined stages (Section 2.2). They participate in biological activities such as the degradation of toxins, detoxification of heavy metals, and regulation of hormones. Moreover, they serve as catalysts in different sectors, including clinical biochemistry, enzyme immunoassays, and the elimination of peroxides from industrial effluents, as well as

the production of diverse aromatic compounds (Dragana et al., 2017). When Linossier isolated peroxidase from pus in 1898, he gave it the first name. Research conducted up to 1918 revealed that peroxidase activity was broadly distributed throughout plants, occurring in a variety of locations such as seeds and the cell walls of woody plants. They can also be found in microbes and mammal (Nnamchi et al., 2021). The Peroxidases (PRXs) superfamily is divided into heme and non-heme Peroxidases in accordance with the structure. Peroxidase-containing heme, also known as heme PRXs, into which additional categories include animal and non-animal. The three classes of non-animal heme PRXs are Class I, II, and III. They have a heme group made up of protoporphyrin IX and Fe(III) and a comparable three-dimensional structure. Different PRX classes, however, have different amino acid sequences that enable them to carry out distinct reaction mechanisms and roles with various subcellular localizations (Kidwai et al., 2020).

Table 2. Peroxidase isoforms, their sources and industrial applications.

Enzyme	Substrate	Source	Application
Manganese peroxidase (MnP)	Lignin and other phenolic compounds	*Rigidoporous Lignosus*, *Ceriporiopsis subvermispora*, *Bacillus anthracis*, *Bacillus cereus*	Paper and pulp industry, textile industry, food industry, plastic degradation
Lignin peroxidase (LiP)	Halogenated phenolic compounds, polycyclic aromatic compounds	*P. chrysosporium*, *Chrysonilia sitophila*, *Streptomyces* sp., *Bacillus cereus*	Textile industry, paper and pulp industry, plastic degradation, bioremediation, biopulping
Versatile peroxidase (VP)	Methoxybenzenes and phenolic aromatic compounds	*P. chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*	Industrial biocatalyst, bioremediation
Glutathione peroxidase (GPx)	Lipid hydroperoxides, hydrogen peroxide	Grass carp, silver carp Southern bluefin tuna	Lignin degradation, dye decolorization, bioremediation
Dye-decolorizing peroxidase (DyP)	Phenols, hydroquinone, dyes, amines, aromatic alcohols and xenobiotic	*Bacillus subtilis*, *Phanerochaete chrysosporium*	Dye degradation, phenol degradation

2.3 Classification of Peroxidases

The existence or lack of a heme is the primary criterion for categorizing peroxidases (Pandey et al., 2017). RedOxiBase (formerly PeroxiBase, primarily because it now intends to consolidate most of the data) claims that Peroxidases can be either heme- or non-heme-containing proteins, according to the oxido-reductase superfamilies) database, which has been devoted to covering the peroxidase superfamilies from 2004 (RedOxiBase 2021). The heme group is divided into two main super families: the peroxidase-cyclooxygenase superfamily (PCOXS) and the peroxidase-catalase superfamily (PCATS). Approximately 80% of known peroxidases are heme peroxidases, while the remaining 20% are heme-free peroxidases, as indicated by the PeroxiBase database (Pandey et al., 2017). According to the RedOxiBase database,

the heme group has since split into numerous other aristocratic households containing the Di heme cytochrome C peroxidase superfamily, the Dyptype peroxidase superfamily and the catalase superfamily (heme, monofunctional), class I of the non-animal peroxidase superfamily, class II of the non-animal peroxidase superfamily, class III peroxidasecyclooxygenase superfamily (animal peroxidases) (Twala et al., 2020).

Peroxidase type	**Superfamily**
Heme peroxidases	Catalase superfamily (heme, monofunctional)
	Di-heme cytochrome C peroxidase superfamily
	Dyp-type peroxidase superfamily (Dye-decolorizing peroxidases)
	Non Animal peroxidase superfamily: Class I
	Non Animal peroxidase superfamily: Class II
	Non Animal peroxidase superfamily: Class III
	Peroxidase-Cyclooxygenase superfamily (Animal peroxidases)
Non-Heme peroxidases	Alkylhydroperoxidase D-like family
	Glutathione peroxidase family (Thiol peroxidase superfamily)
	Haloperoxidase superfamily
	Manganese catalase family (pseudocatalase)
	NADH peroxidase/oxidase family
	Peroxiredoxin family (Thiol peroxidase superfamily)

Exclusive fungal peroxidases, or Class II of the non-animal peroxidase superfamily, comprise significant fungal ligninases including lignin peroxidase (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs), among others (Nnamchi et al., 2019).

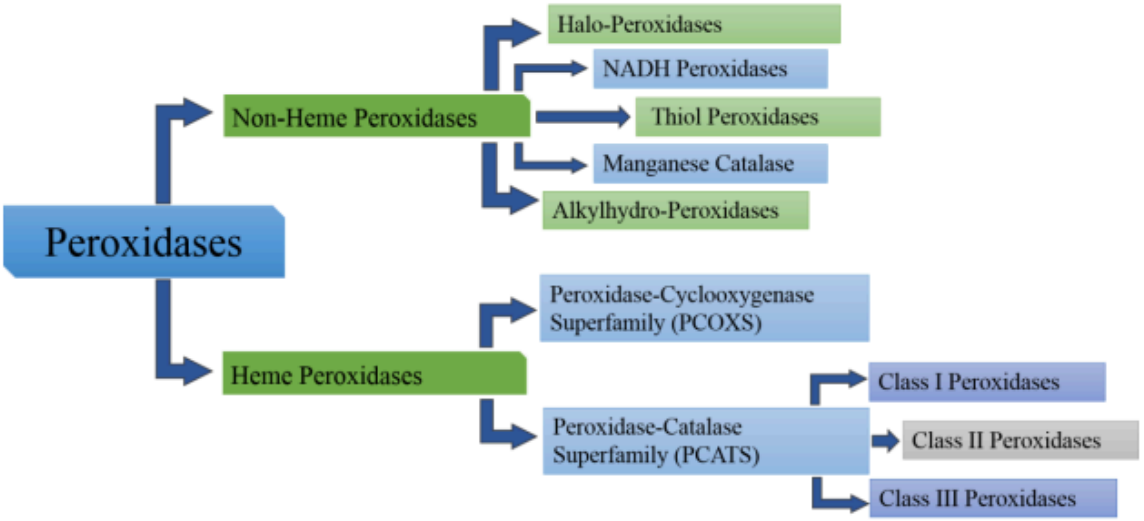

Figure 1. A schematic representation illustrating the classification of peroxidases

The superfamily encompasses plant peroxidases, heme-containing peroxidases from fungi and bacteria, and is categorized into three classes based on origin, amino acid homology, and metal-binding capability.

Class I peroxidases consist of prokaryotic and eukaryotic intracellular peroxidases from non-animal sources, such as cytochrome c peroxidase (CCP), ascorbate peroxidase (APX), and catalase-peroxidase (CP), which play a crucial role in preventing oxidative stress in bacteria by dismutating H_2O_2 and evolving molecular O_2.

Class II peroxidases are exclusively fungal peroxidases, including white-rot fungal LiP, MnP, and versatile peroxidases (VP), which are essential for lignin biodegradation.

Class III peroxidases are predominantly found in the plant kingdom, comprising extracellular plant peroxidases like horseradish peroxidase (HRP), soybean peroxidase (SBP), and Turpin peroxidase (TP), involved in various physiological processes such as cell wall metabolism, lignification, wound healing, auxin metabolism, and defense against pathogens. Unlike Class I peroxidases, which lack disulfide bridges, calcium, and an endoplasmic reticulum signal sequence at the structural level, both Class II and Class III peroxidases contain N-terminal signal peptides, four conserved disulfide bridges (differently located for each class), and calcium in their structure. The non-heme peroxidases, on the other hand, are not evolutionarily related and are classified into five independent families, namely thiol peroxidases, alkylhydro-peroxidase, non-heme halo-peroxidase, manganese catalase, and NADH peroxidase, with the thiol peroxidase family being the largest and having two subfamilies, glutathione peroxidases, and peroxy redoxins. (Pontsho et al., 2020)

2.4 Mechanism of action

Plants and animals utilize peroxidases to efficiently break down toxic peroxides. These peroxidases are a crucial component of the antioxidative defense system, playing a role in the decomposition of various substrates (such as ferricyanides and ascorbate) into harmless components by transferring O_2 from H_2O_2 or other peroxides.

The catalytic cycle of peroxidases involves a three-step process with distinct intermediate enzyme forms:

Step (i). Peroxidase + H_2O_2 → Compound I + H_2O

Step (ii). Compound I + PS → Compound II + FRP

Step (iii). Compound II + PS → Peroxidase + FRP + H_2O_2

2 Substrate (reduced) + H_2O_2 → 2 Product (oxidized) + 2 H_2O

Initially, the native ferric enzymes are oxidized by H_2O_2, leading to the formation of an unstable intermediate, Compound I. Compound I contains a heme structure of Fe IV = O-porphyrin -cation radical, resulting in the reduction of peroxide to water. In the next step, Compound I oxidizes an electron donor substrate (PS) to form Compound II, which then releases a free radical product (FRP). Finally, Compound II is further reduced by a second substrate molecule in the third step, regenerating the iron (III) state and producing another FRP (Shigeto & Tsutsumi 2016).

*PS: peroxidase substrate, FRP: free-radical product.

2.5 Physiological Roles of Plant Peroxidases

Plant Defense Mechanisms

Peroxidases play a vital role in plants' defense mechanisms against pathogens by aiding in the production of reactive oxygen species (ROS). These ROS help fortify cell walls and serve as antimicrobial agents. (Kidwai et al., 2020).

Lignin Biosynthesis

They are crucial in the biosynthesis of lignin, a fundamental process for the structural integrity of plants. Lignin reinforces cell walls and assists in water transportation. (Singh et al., 2024).

Hormone Regulation

Auxins and other plant hormones are regulated by peroxidases. They have the ability to oxidize the primary auxin, indole-3-acetic acid (IAA), which impacts plant growth and development. (Kidwai et al., 2020).

Oxidative Stress Response

Peroxidases such as myeloperoxidase (MPO) are involved in the immune response in animals. MPO is responsible for generating hypochlorous acid by catalyzing the reaction between hydrogen peroxide and chloride ions, resulting in the production of a powerful antimicrobial agent. (Mechanisms of myeloperoxidase in health and disease, Journal of Clinical Investigation, 2021).

Bioremediation

In bioremediation, peroxidases are employed to break down contaminants. Their versatility in oxidizing various organic substrates renders them beneficial for environmental remediation endeavors. (Gupta et al., 2023).

Wound Healing

Peroxidases play a role in the healing of wounds in animals. They influence the activity of growth factors and help build components of the extracellular matrix. (Polaka et al., 2022).

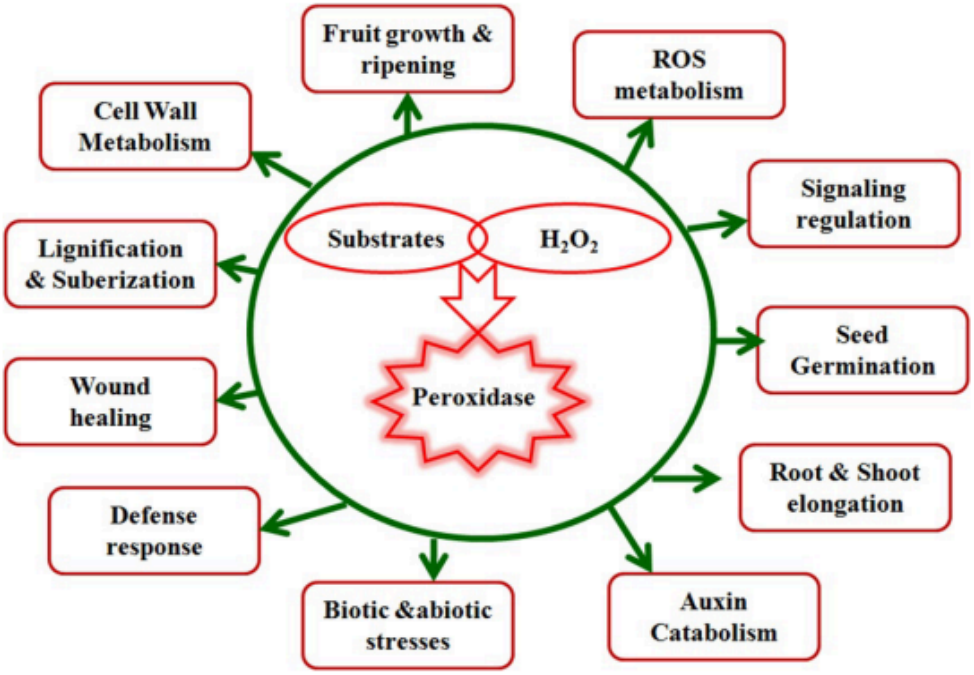

Figure 2. A schematic representation showing the physiological roles of plant peroxidases

2.6 Application of Peroxidases

In Industry

Peroxidases have the ability to catalyze reaction of a wide range of substrates and they play significant physiological and industrial application roles in nature. The subsequent sections discuss in depth the different roles peroxidase enzymes play.

Rubber degradation

Natural rubber, also known as cis-1,4-poly-isoprene, is a significant polymer with an annual production estimated at 12.5 million. It exhibits high temperature resistance and environmental persistence, but its recycling is challenging due to Sulphur cross-linkages formed during vulcanization. The discovery of enzymes and microorganisms capable of biodegrading rubber is crucial. Research revealed that *Bacillus pumilus* can utilize NR as its sole carbon source, leading to its degradation, with Laccase and MnP identified as key players in the process. (Nwadike & Akunna 2023).

Synthetic dye decolorization

Synthetic dyes are broadly used in many industries such as the textile, paper printing, color photography, and additives in petroleum products. Various studies have focused on the decolorization of synthetic dyes by white rot fungi since it can degrade various xenobiotic compounds through the release of enzymes such as LiP, MnP, and laccases. However, it has been proven that enzymatic treatment is simple and more flexible than the use of fungi cultures. Although most of the enzymes used in various industries to decolorize dyes are of fungal origin several bacteria have been used for decolorization of synthetic textile dyes such as *B. cereus* DC11, *B. subtilis*, *Sphingomonas paucimobilis* and *Staphylococcus epidermidis* (Shi et al., 2021).

Waste water treatment

Removal of phenolic compounds from contaminated water before discharge into any natural stream has become of great importance, as these compounds are toxic to aquatic organisms and humans. Various studies have been conducted to observe the removal of phenol contaminants using peroxidase enzymes, with the aim of

developing eco-friendly, economically yet effective biological methods of removing pollutants from wastewater (Petronijević et al., 2024).

In Biotechnology and Diagnostics

Clinical Diagnostics

Peroxidase enzymes, particularly horseradish peroxidase (HRP), are used in various diagnostic assays, including enzyme-linked immunosorbent assays (ELISA), where they facilitate the detection of target molecules through colorimetric changes (Li et al., 2019).

Biosensors

Peroxidase's ability to react with hydrogen peroxide makes it ideal for developing biosensors. These sensors can detect various analytes, including glucose, alcohols, and cholesterol, by coupling the peroxidase reaction with the production of hydrogen peroxide (Teymourian et al., 2020).

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials and Reagents

Dried mushrooms (*Ganoderma lipsiense*) whose general name is known as "Lipstick Bracket" or "Lipstick Fungus" were harvested from Thomas Adewumi University Premises (New Site) In Oko, Kwara State, Nigeria. Chemicals such as Sephadex G-75, Pyrogallol, ABTS, and Ammonium Sulphate (Salt) are used for the analytical grading. Other materials used were spectrophotometer, centrifuge, pH meter, electric blender, water bath, and analytical balance (Arise et al., 2016).

3.2 Methods

3.2.1 Proximate Analysis of the Non-Edible Mushroom (*Ganoderma Lipsiense*)

The samples of *Ganoderma lipsiense* were analyzed for moisture, ash, fat, protein, crude fiber, and carbohydrate at the central laboratory of the Federal University of Akure, Ondo State, Nigeria.

3.2.2 Preparation of the Crude Extract

After the harvested sample, it was then stored in a flask containing ice packs in the laboratory. The homogenate was produced by mixing 5g of the sample in 1200 ml of cold 100 mM Tris-HCl buffer (pH 7.5) in an electric blender. The process is called homogenization. Then, it was filtered through several layers of cheesecloth. The homogenate was then centrifuged for 20 minutes at 4 ˚C at 4000rpm. After that, the supernatant was separated and discarded from the pellet (sediment). then, the supernatant (crude extract) was stored in ice packs to avoid the denaturation of the enzyme (protein).

3.2.3 Estimation of Protein Concentration using Biuret Protein Assay

To selectively inactivate the contaminating traces of catalase moieties, the crude extract was heated at 65ºC for 3 minutes (As described by Arise et al., 2016) in a water bath and cooled promptly by placing it in an ice bucket for 30 minutes. Thermal activation and inactivation were done repeatedly to determine the protein concentration.

Procedure

A standard protein solution of known concentration such as bovine serum albumin (BSA) was prepared. A known amount of 2mg of standard protein (BSA) was dissolved in 2 ml of pH 7.5. Then, a series of dilutions of the BSA stock solution was also prepared to create a standard curve. 5 standard solutions with concentrations of 0.2, 0.4, 0.6,0.8, and 1 mg/ml were prepared respectively. It was then diluted using distilled water. 2 ml of biuret reagent was added to each sample. Also, 2ml of biuret reagent and 1ml of crude extract were mixed for thermal activation and inactivation respectively. This procedure was done repeatedly to ensure accuracy. The whole sample was incubated at room temperature for 30 minutes. During this time, the biuret reagent reacts with the peptide bonds in the protein, forming a purple-colored complex. The intensity of the purple color is directly proportional to the protein concentration in the sample. The spectrophotometer was tared to zero using a blank cuvette filled with distilled water. Then, the absorbance of the samples was measured at 540nm using a spectrophotometer. This experiment was done repeatedly to ensure good precision. Afterward, the absorbance values of the standard solutions against

their corresponding protein concentrations were plotted to create a standard curve using an Excel sheet as a tool.

3.3 Purification of the Isolated Enzyme from *G. lipsiense*

3.3.1 Preparation of 100% ammonium sulphate saturation

To prepare a 100% saturated ammonium sulphate solution, approximately 136g of ammonium sulphate crystals were weighed and gradually added to 250ml of distilled water, with occasional stirring until no more ammonium sulphate could dissolve. The solution was then filtered to remove the undissolved particles that had remained after several hours. The resulting saturated solution was stored in the refrigerator until needed (Farohunbi & Arise 2024).

3.3.2 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation is a widely used technique in biochemistry for protein purification. The principle behind this method is based on the solubility of proteins, which decreases in the presence of high salt concentrations, causing the proteins to precipitate out of solution (Putatunda et al., 2019).

Procedure

The supernatant was subjected to 75% ammonium sulphate precipitation by mixing 300ml of 100% saturated ammonium sulphate solution with 100ml of the supernatant. The precipitate obtained was allowed to settle at 4^{o}C overnight and then centrifuged at 4000xg for 20 minutes. The pellet obtained was collected by gently slanting the container and decanting the supernatant into another container. This experiment was

done repeatedly for 136g, 27g, 62g and 62g respectively. After adequate experiment was done each solution has 99ml, 32ml, 82ml, and 50ml respectively.

3.3.3 Dialysis

Dialysis is commonly used to remove unwanted small molecules such as salts from larger macromolecules like proteins, DNA, or polysaccharides. This process involves separating a sample and a buffer solution (known as the dialysate) with a semipermeable membrane (dialysis tube), which creates different diffusion patterns and allows for the separation of molecules in both the sample and dialysate. The membrane's pore size prevents large molecules, such as proteins, from passing through, restricting their diffusion from the sample chamber. On the other hand, small molecules can freely diffuse across the membrane and reach equilibrium throughout the solution volume, thereby altering the overall concentration of these molecules in the sample and dialysate.

Pretreatment of the Dialysis Tube

The dialysis tube was divided into two equal parts and prepared prior to dialysis. It underwent a soaking process in distilled water for around 30 minutes to eliminate glycerol. Subsequently, the tube was rinsed once more with distilled water, and to eliminate any metal residues, it was immersed in a 2% sodium bicarbonate solution for 3-4 hours. Finally, both the interior and exterior of the tube were washed with distilled water.

Procedure

Different concentrations of $(NH_4)_2SO_4$ ranging from 0% - 90% and exactly 50% were placed into a beaker that contained a tris buffer with a pH level of 7.5. The mixture was then placed on a magnetic stirrer overnight for a duration of 24 hours to eliminate the salt content in the sample, which will subsequently undergo analysis for peroxidase activity. The sample with the highest peroxidase activity was chosen for further purification. The protein concentration was determined using bovine serum albumin as a standard protein. Subsequently, the specific enzyme activity was computed, and the purification yield was also assessed.

3.4 Characterization of Purified Peroxidase

3.4.1 Determination of peroxidase activity of peroxidase from *G. Lipsiense*

The activity of *Ganoderma Lipsiense* in enzyme extract was assessed by measuring the absorbance changes at 590nm using a UV-vis spectrophotometer. The assay mixtures were composed of 0.96ml 12.5mg/ml pyrogallol, 0.48ml 147mM H_20_2, 0.3ml H_20, 0.3ml enzyme extract, and 0.96ml phosphate buffer pH 7.38, in a total volume of 3ml. Additionally, the enzyme activity was determined for concentrations of 25mg/ml, 50mg/ml, 75mg/ml, and 100mg/ml. The enzyme activity unit was defined as the amount of enzyme that resulted in an increase in absorbance of 0.001 unit/min at 50°C.

3.4.2 Substrate specificity of peroxidase of *G.Lipsiense*

The peroxidase activity was assessed by utilizing ABTS and pyrogallol as reducing substrates. For the ABTS assay, the reaction mixture consisted of 0.8ml of 1.67mM ABTS, 0.4ml of enzyme, 0.8ml of 150mM H202, 0.4ml of H20, and 0.1mM sodium

acetate buffer at pH 4.0 (0.4ml), making a total volume of 2.8ml. The change in absorbance was recorded at 415nm every minute for a duration of 3 minutes. On the other hand, the peroxidase assay using pyrogallol as a reducing substrate was conducted by preparing a reaction mixture containing 0.96ml of 5% pyrogallol, 0.48ml of 147mM H_20_2, 0.3ml of H_20, 0.3ml of enzyme extract, and 0.96ml of phosphate buffer at pH 7.38, in a total volume of 3ml. The change in absorbance was monitored at 590nm using a UV-vis spectrophotometer over a 3-minute period. The molar extinction coefficients used for calculating peroxidase activity with the different substrates were 36.0mM cm-1 for ABTS and 2.6mM cm-1 for pyrogallol. However, Pyrogallol was selected as the reducing substrate for this specific study.

3.4.3 Determination of kinetic and catalytic parameters of peroxidase from *G. Lipsiense*

Determination of pH optimum and stability of peroxidase

Buffers ranging from pH 2.0 to 12.0 were prepared, and the ideal pH was identified by exposing 0.3ml of the enzyme to various pH solutions for 10 minutes. Subsequently, the enzyme's activity was assessed. The pH stability was also evaluated by subjecting the enzyme to specific buffers with known pH values for 24 hours, followed by determining the enzyme's activity.

Determination of optimum temperature and thermal stability of peroxidase

The enzyme sample was incubated at a specific temperature for 10 minutes to determine the optimum temperature, with enzyme activity immediately assayed at that temperature. Temperature ranges of 40^{o}C-100^{o}C were studied, and thermal stability was also assessed at 40^{o}C, 60^{o}C, and 100^{o}C. Additionally, the enzyme sample was

heated to each of these temperatures over a 24-hour period. Enzyme activity was determined.

CHAPTER FOUR

4.0 Results

Isolation and Purification of *Ganoderma Lipsiense* Peroxidase

Ganoderma Lipsiense is a fungus that has been traditionally utilized in Eastern medicine due to its various therapeutic properties, including antiviral, anticancer, antitumor, and antioxidant effects. This research aims to transform these byproducts into valuable resources by identifying and isolating a significant commercial enzyme derived from *Ganoderma Lipsiense.* The purification process, which progresses from crude extract to dialysis, is summarized in Table 3. The yield percentage and purification fold achieved following the purification process were recorded at 16.04% and 1.41 respectively.

Table 3. Summary of the purification steps of *Ganoderma Lipsiense* peroxidase

Purification step	Volume	Total Protein	Total Enzyme Activity	Specific Enzyme Activity	Purification (Fold)	Yield (%)
Crude extract	600.00	3.55	65000.00	18309.86	1.00	100
$(NH_4)_2SO_4$	82.00	3.21	2219.23	691.35	0.04	3.41
Supernatant (Heated)	5.00	3.85	2626.92	682.31	0.99	4.04
Supernatant (Not heated)	5.00	3.70	2534.62	685.03	1.00	3.90
After dialysis	44.00	0.42	406.54	967.95	1.41	16.04

Optimum pH of *Ganoderma Lipsiense* peroxidase using pyrogallol

Fig. 3 depicts the influence of pH on the peroxidase activity of *G.lipsiense* when pyrogallol is employed as the reducing substrate. The optimal pH for peroxidase activity was determined to be 6.0, showing a sharp decrease in activity in alkaline conditions. In contrast, enzyme activity was significantly lower in acidic conditions as opposed to basic pH levels.

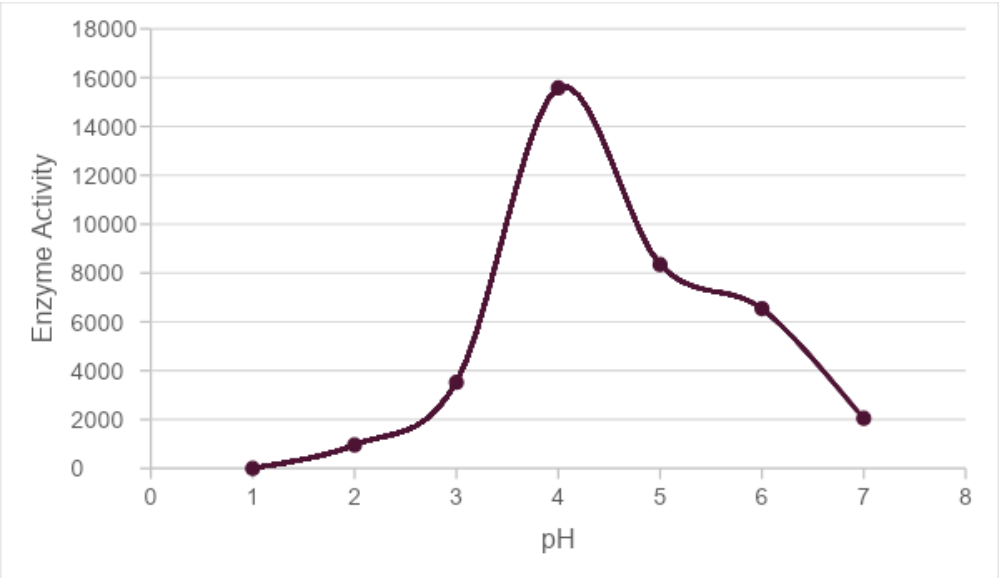

Fig.3 Optimum pH of *Ganoderma Lipsiense* peroxidase using pyrogallol as substrate

pH stability of *G.lipsiense* peroxidase

Fig. 4 depicts the influence of pH on the stability of *Ganoderma lipsiense* peroxidase after being exposed for 24 hours. The enzyme exhibits similar profile characteristics, with the highest enzyme activity observed at pH 6.4. This finding implies that a neutral pH level is optimal for peroxidase activity during prolonged exposure.

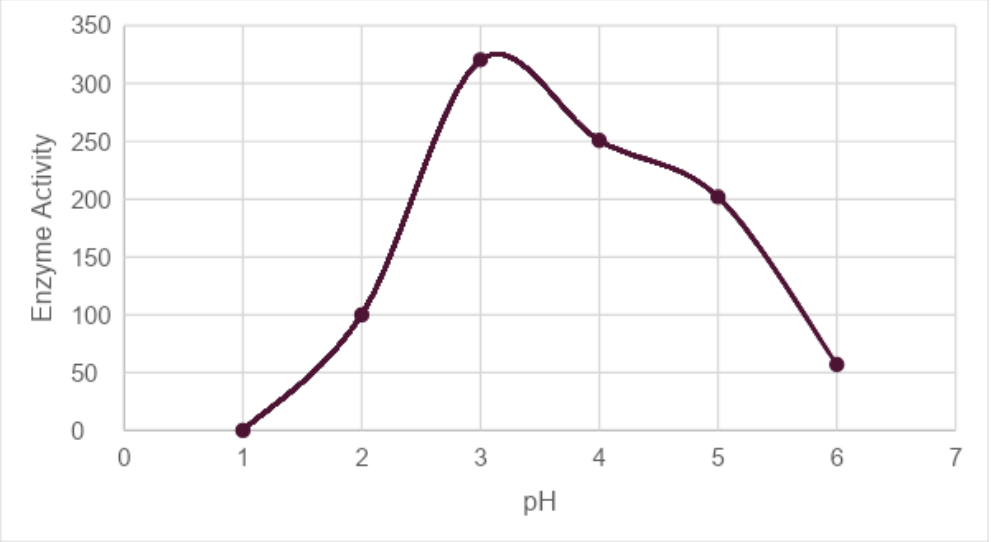

Fig. 4 pH stability of *G.lipsiense* peroxidase suing pyrogallol as substrate

Optimum temperature and thermal stability of *G.lipsiense* peroxidase

Fig. 5 illustrates the impact of temperature on *G.lipsiense* peroxidase activity. The most significant activity was displayed at a temperature of 60ºC, with a sharp decline in enzyme activity as the temperature rose. A rapid decrease in enzyme activity due to an increase in temperature was evident between 40ºC and 60ºC, followed by a period of relative stability despite variations in exposure time.

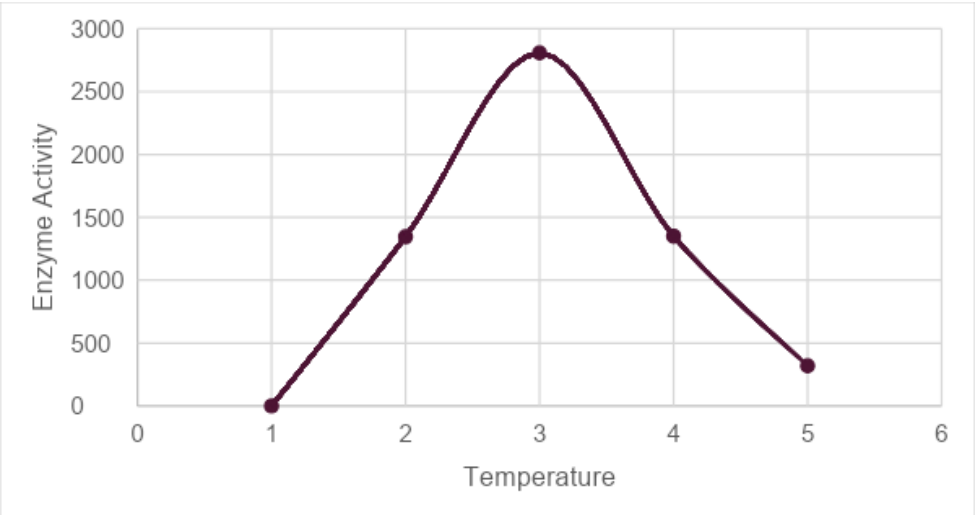

Fig. 5 Optimum temperature of *Ganoderma lipsiense* peroxidase

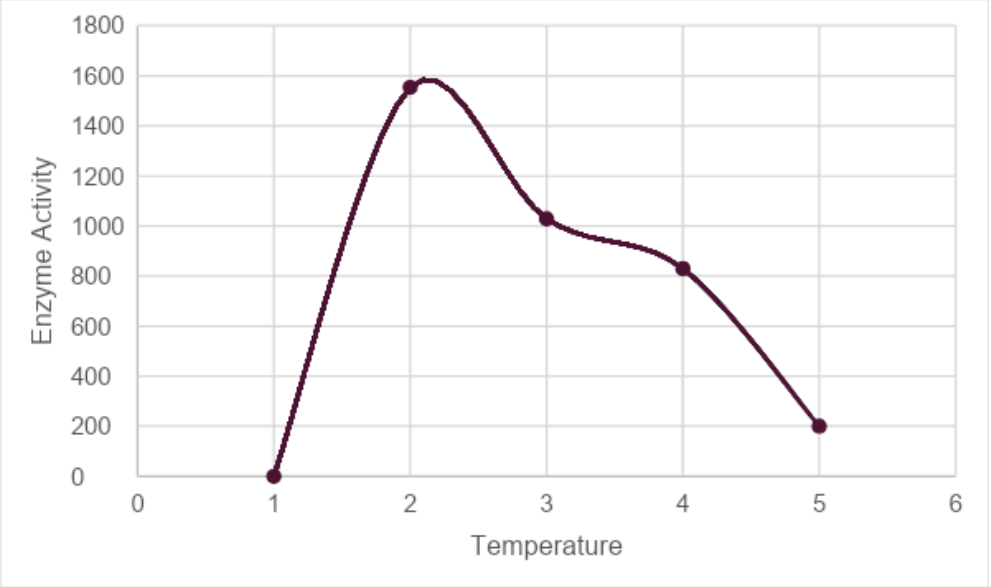

Fig. 6 Thermal stability of *G. lipsiense* peroxidase

4.1 Discussion

CHAPTER FIVE

5.0 Conclusion

Peroxidases are becoming more useful in clinical, environmental and industrial applications, but these enzymes are difficult to purchase and quite expensive. *Ganoderma lipsiense* is locally sourced and it is quite cheap which makes this study a unique one and targetting sustainable development goal 9 and 12. The partially purified *G.lipsiense* peroxidase showed moderate stability under some of the conditions observed in this study. Thus, *G.lipsiense* peroxidase has prospects for industrial applications. Also, there is great potential for developing enzyme biotechnology and tackling environmental issues with the isolation, partial purification, and characterization of peroxidase from *Ganoderma lipsiense*. This study aims to investigate the possible industrial uses of peroxidases generated from *Ganoderma lipsiense* while also adding to the body of scientific knowledge.

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Appendix