Enzymes with biocatalytic potential from *Sorghum bicolor*

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By

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ABSTRACT

Sorghum is a staple food in the semi-arid tropics of Asia and Africa, sustaining the lives of the poorest rural people. This project set out to improve the potential economic value of *Sorghum bicolor* as a crop. The task was undertaken by screening for selected enzymes in the plant that would have a potential market for use in industrial applications and in biotransformations, specifically proteases, polyphenol oxidases and peroxidases.

A survey was conducted using standard enzyme assays and crude plant extracts, to determine whether the selected enzymes were present. Grain tissue did not appear to have significant protease or polyphenol oxidase activity, but high levels of peroxidases were detected, with the young grain extracts showing more activity (4.63 U/mL) than ripe grain extracts (0.62 U/mL). Leaf tissue extracts contained low levels of protease activity, a considerable amount of polyphenol oxidase (0.127 U/mL), and peroxidase (4.7 U/mL) activities comparable with that found in grain tissue. Root tissue extract was found to contain the highest levels of peroxidase activity (7.8 U/mL) compared to the other extracts. Therefore, sorghum peroxidase from the root was isolated, purified, characterized and applied to biotransformation reactions.

Different sorghum strains, with varying grain colour, (Zimbabwe - bronze, Seredo - brown and Epurpur - cream/white) were investigated for the presence of polyphenol oxidase and peroxidase activities. Results of spectrophotometric analysis showed that the enzymes did not appear to be strain specific. However, gel electrophoresis analysis revealed differences in band patterns among the strains.

Partial purification of sorghum root peroxidase was achieved after centrifugation, extraction with polyvinylpolypyrrolidone (PVPP), ultrafiltration, and hydrophobic chromatography with phenyl Sepharose, followed by polyacrylamide gel electrophoresis (PAGE). The specific activity of the 5-fold purified enzyme was found to be 122.3 U/mg. After PAGE analysis, two bands with molecular weights of approximately 30 000 and 40 000 were detected, which compares well with horse radish peroxidase (HRP) which has a molecular weight of approximately 44 000. The colour intensity of the bands in the activity gels indicated that sorghum root peroxidase had apparently higher levels of peroxidase activity than commercial horseradish peroxidase (HRP).

Characterization experiments revealed that sorghum root peroxidase is active over a broad temperature range and remains active at temperatures up to 100°C. It also has a broad substrate range. The optimum pH of the enzyme was found to be pH 5 - 6. Under standardized assay conditions, the optimal substrate concentration, using *o*-dianisidine as substrate, was 50 mM, and the optimal H_2O_2 concentration under these conditions was found to be 100 mM.

Sorghum root peroxidase was applied in a preliminary investigation into the oxidative biotransformation of a number of aromatic compounds. The products obtained were comparable with those when the compounds are reacted with HRP which is the most commonly used commercial peroxidase and has been extensively studied. However, HRP is relatively costly, and the use of peroxidase from sorghum roots as an alternative source, appears to be promising. A patent has been provisionally registered, covering application of sorghum root peroxidase for biotransformations.

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List of Abbreviations

ABTS	2,2-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
ATPS	Aqueous two-phase system
APS	Ammonium persulfate
BADH	Betaine aldehyde dehydrogenase
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CMO	Cholinemonoxygenase
2,4-DCP	2,4-Dichlorophenol
DMA	*N*,*N*-dimethylaniline
ETDA	Ethylenediaminetetraacetic acid
GST	Glutathione *S*-transferase
HPLC	High pressure liquid chromatography
HNL	Hydroxynitrile lyase
HRP	Horseradish peroxidase
HRPC	Horseradish peroxidase isozyme C
L-DOPA	L-$-3,4-Dihydroxyphenylalanine
MES	2-[*N*-morpholino]ethanesulfonic acid
NMR	Nuclear magnetic resonance
OA	Osmotic Adjustment
PAGE	Polyacrylamide gel electrophoresiss
PEG	Polyethylene glycol
PPO	Polyphenol oxidase
PVPP	Polyvinylpolypyrrolidone
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylene diamine
TLC	Thin layer chromatography

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Chapter 1
LITERATURE REVIEW

1.1 INTRODUCTION

For millions of people in the semi-arid tropics of Asia and Africa, sorghum is one of the most important staple foods, and is grown mainly as a subsistence crop. The aim of this study was to investigate the potential use of sorghum as a cash crop, by detecting enzymes present in the plant which could be extracted and utilized as novel catalysts in industrial, medical and analytical applications. Because of their large biomass, plants present a potentially cheap source for large amounts of enzymes. Sorghum would be a particularly cheap source of enzymes since it is able to grow in harsh environments without the need for fertilizers and irrigation, yet it is relatively under-exploited at present.

Plants are known to contain numerous phenolic and polyphenolic compounds which suggests the presence of oxidative enzymes, because these oxidative enzymes play a role in the biosynthesis of the phenolics (Mayer and Harel, 1979; Robinson, 1991). Commercially, oxidative enzymes find application in biotransformations, bioremediation, in analytical biochemistry and as specific reagents such as bleaching agents. Horse radish peroxidase (HRP), for instance, is currently the most common peroxidase used in industry. However, HRP is costly and, therefore the introduction of a cheaper peroxidase could be appropriate. In view of the economic potential of oxidative enzymes, the detection and characterisation of polyphenol oxidase (PPO) and peroxidase in *Sorghum bicolor* were major objectives of the present study.

Proteases are also ubiquitous in plants, and therefore the proteases in *S. bicolor* were also identified as candidates for investigation. Plant proteases are commercially important in meat tenderizers and in other areas of food production including brewing and baking, in tanning and in pharmaceuticals as digestive-aid preparations and also have many industrial applications. Therefore, finding a novel protease in sorghum would enhance its potential as a cash crop.

An additional reason for selecting proteases was the possibility of using these enzymes to de-hull grains. In rural areas, grain is de-hulled by the strenuous method of pounding with a pestle and mortar, but it has been suggested that proteases might facilitate de-hulling (Hulse *et al.*, 1980). Thus, a protease in sorghum itself, might provide a readily available de-hulling aid even in low-technology or rural environments.

1.2. CHEMICAL COMPOUNDS FOUND IN SORGHUM

1.2.1. Polyphenols

Phenols are widely recognized as antioxidants because of their ability either to scavenge free radicals or in some cases to react directly with the oxidant (Howard, 1974). The functional group in phenols is a hydroxyl group attached to one of the carbon atoms of a benzene ring. The formula of the simplest member, phenol itself, is C_6H_5OH (Fig 1.1).

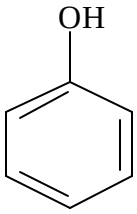
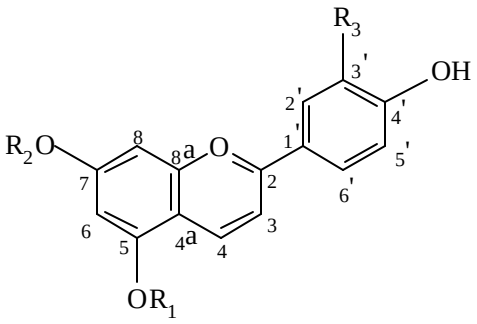


Figure 1.1. Phenol

Phenolic compounds in sorghum can be classified as phenolic acids, flavanoids and tannins (Bate-Smith, 1969; Gupta and Haslam, 1980). Sorghum's nutritional benefit is limited by the presence of tannins in some genotypes. These polyphenols give the grain an astringent taste. They are also involved in the formation of deep red to brown colour which some grains assume as they ripen, and may cause off-colour in products derived from sorghum grain. For example, during wet weather or during steeping they can migrate from the seed coats into the endosperm and cause starch to darken in colour, either by complex formation with trace metals or by oxidation.

3-Deoxyanthocyanidins extracted from the grains of *Sorghum caudatum* (L.) Moench (Poaceae) are known for the yellow or orange coloration they show in acidic solutions. Previous studies on the grains and the leaf sheaths have shown the presence of apigeninidin and luteolinidin derivatives (Nip and Burns, 1969; Nip and Burns, 1971; Sérémé *et al*., 1993; Kouda-Bonafos *et al*., 1994). In 1997, Pale *et al*. (1997) isolated and elucidated the structure of a new *O*-methylated 3-deoxyanthocyanidin. In their experiments, sorghum grains were extracted with ethanol under acidic conditions and they detected four anthocyanidins (Fig 1.2. I-IV).

One of the oldest suggestions of a physiological role of polyphenol oxidases in plants is that of synthesis of *o*-diphenols (Mayer and Harel, 1979) and these enzymes have been shown to hydroxylate naturally occuring phenolics, e.g. flavanoids in spinach beet (Vaughan *et al*., 1969). This confirms the likelihood of the presence of polyphenol oxidases in the sorghum plant.



R_1 = Glc, R_2 = H, R_3 = OH (I)
R_1 = R_2 = H, R_3 = OH (II)
R_1 = R_2 = R_3 = H (III)
R_1 = R_3 = H, R_2 = Me (IV)

Figure 1.2. Anthocyanidins

1.3. INDUSTRIAL ENZYMES

1.3.1. The discovery of enzymes and their industrial application through history

The first observation of an enzymatic degradation reaction was in 1783 by Spalazani (1729-1799), a priest and naturalist from Padua, Italy. After placing meat in small porous capsules, he examined the regurgitated pellets of hawks which had eaten this material and found the capsules to be empty, proving that the meat had been rapidly liquefied by the stomach juices of these birds of prey.

In 1857 Pasteur showed that fermentation is closely associated with live yeast. He distinguished between the actions of "organized ferments" (cellular) and the "unorganized ferments" (soluble). These soluble "ferments" which are not bound to the living cell, were labeled *enzymes* by Kühne (1878). This term is derived from the Greek *en zyme*, meaning "in sour dough". Concrete evidence for this assumption was provided by E. Buchner in 1897, as he showed that the cell-free extract from yeast cells could also produce alcohol from sugars (Buchner, 1897, 1898). The enzyme active in fermentation was termed *zymase*. The first book summarizing the "wonderful mechanism" of the enzymes known at that time and outlining the history of their discovery was written by Green (1901). Friederich Wilhelm Ostwald (1954) first coined the now-familiar definition of a catalyst, specifically, that a catalyst is a substance that alters the rate of a

chemical reaction without being present in the reaction products. Thus Ostwald recognized enzymes as catalysts.

The development of today's industrially important microbial enzymes began with Takamine who studied the production of enzymes from molds. In 1894, he obtained a process patent for making a diastatic enzyme preparation from fungi, (a mixture of carbohydrases and proteolytic enzymes) which he named *Takadiastase*. The production occurred either via a surface culture, a semisolid culture, or the *koji* process on moist wheat bran with nutrient salts and buffers added. The sterile culture medium, mixed with spores from *Aspergillus oryzae*, was poured onto metal trays to a thickness of a few centimeters and incubated in climate-controlled culture chambers. The culture media, permeated with fungal mycelia, are ground and extracted with water. In 1907 Otto Röhm discovered the effectiveness of pancreatic proteases in liming and bating of hides in leather manufacture (Röhm, 1907a, 1907b). Until then, slurries of dog feaces had been used in bating. In 1913 Röhm introduced the first commercial enzymatic detergent. In addition to pancreatic enzymes, it contained sodium carbonate and sodium bicarbonate, which maintained the pH in the wash water below 9. Wallerstein (1911) was the first to use papain, a plant protease, for stabilizing beer, preventing protein flocculation or hazing on refrigeration. In 1960 Novo Nordisk began production of alkaline bacterial proteases from *Bacillus licheniformis* for use in detergents, and these proteases are currently the most commercially important microbial enzymes (Uhlig, 1998).

1.3.2. General characteristics of industrial enzymes

While enzymes are being used increasingly in industrial processes, their biochemical nature requires certain conditions to be maintained in order for them to be efficient catalysts. Enzymes are large, three-dimensional protein molecules with an active site at a defined location on the folded surface that permits entry only to specific substrates for a reaction to occur. An enzyme is evaluated according to its activity which describes how much of a given substrate S is converted within a given time and under defined conditions into a corresponding product P. For maximum enzyme activity, enzyme and substrate must have a constant and

unimpaired contact. Generally, as long as the reaction conditions do not change, twice the yield of product will be generated in twice the time. The conversion rate is reduced when there is insufficient substrate available to saturate the enzyme or the enzyme is denatured because of an increase in temperature, resulting in inactivation (Uhlig, 1998).

Every enzyme has an optimal activity which is affected by pH and temperature. The temperatures at which enzymes are stable or labile is of great significance for many technical processes. In some processes, enzymes should be sufficiently labile so as to be completely inactivated at temperatures of 70-80^{0}C. On the other hand, enzymes that are active above 100^{0}C may be needed, e.g. for the modern production of glucose from starch. Thus, high enzyme stability may be of significant economic value. In addition to heavy metals, there are a number of chemicals and many natural substances that can inhibit or completely inactivate enzymes.

1.3.3. Applications of industrial enzymes

Major targets of modern enzyme technology continue to include preservation of foods and food components (e.g. vitamins), more efficient use of raw materials and improvement of food quality such as texture and taste. Other current objectives include the utilization of new raw materials for feeding humans and animals, the manufacture of dietetic foods and elimination of antinutritive substances from certain nutritional raw materials.

Biotechnological methods have replaced some of the traditional chemical processes. Examples of new biotechnological processes include the use of enzymes for recycling food wastes, in wastewater treatment, and in biotransformations (Shoemaker, 1986); the use of proteases and lipases as active components of detergents; the production of sweeteners, such as aspartame, using combined microbiological and enzymatic methods (Uhlig, 1998). This study contributes to the continuing search for novel enzymes with specific characteristics, for particular applications.

1.4. PEROXIDASES

Peroxidases are widely distributed in the plant and animal kingdoms and have been found in all higher plants that have been investigated. Oxygen toxicity is an inherent feature of aerobic life because of the highly reactive by-products produced by molecular oxygen. In chloroplasts, the photosynthetic activity generates superoxide radicals which are immediately disproportionated to hydrogen peroxide (H_2O_2) and dioxygen. The rapid scavenging of H_2O_2 is essential to keep the photosynthetic activity functioning. As a consequence, peroxidases serve to rid plant cells of excess H_2O_2 under normal and stress conditions (Laloue *et al*., 1997). Peroxidases are found mainly as haemoproteins and use hydrogen peroxide as the oxidizing substrate, although other more unusual peroxidases have been shown recently to contain either metal ions, such as selenium and vanadium, or a flavin prosthetic group (Table 1.1).

Table 1.1. Classification of peroxidases. From Robinson (1991)

Prosthetic group	*Common name*	*Source*	*Potential substrates*[a]
Haem	Peroxidases	Source	Test substrates including guaiacol and *o*-dianisidine
	Ascorbic acid peroxidase	Plants	Ascorbic acid
	Cytochrome c peroxidase	Plants	Cytochrome c
	Lactoperoxidase	Animals	Thiocyanate
	Lignin peroxidase	Plant + fungal cell walls	Lignin precursors including cinnamic and ferulic acids
	Myeloperoxidase	Animals	Test substrates and thiols
	Eosinophil peroxidase	Animals	Test substrates and thiols
	Thyroid Peroxidase	Animals	Phenols including tyrosine
Selenium	Glutathione peroxidase	Plants	Reduced glutathione
Vanadium	Bromoperoxidase	*Ascophyllum nodosum*	Halogens
Manganese	Mn-haem peroxidase	*Phanerochaete chrysosporium*	Lignin
Flavin	Flavoperoxidases	Micro-organisms	---

[a]The peroxidatic of the peroxidase action may not necessaritly be restricted to the named substrate.

Peroxidase (EC 1.11.1.7) is a member of a large group of enzymes called the oxidoreductases (Robinson, 1991). The majority of known peroxidases belong to the plant peroxidase superfamily, which is characterized by a central haem group sandwiched between a distal and a proximal protein domain. The plant peroxidase superfamily is subdivided into three classes based on structural divergence (Welinder, 1992a). Class I constitutes intracellular peroxidases of prokaryotic origin, and are found in yeast (cytochrome *c* peroxidase; CCP), plants (pea cytosolic ascorbate peroxidase) and bacteria. Class II and III peroxidases are found in fungi and plants respectively, and are largely extracellular. Both class II and class III peroxidases contain two conserved calcium ions, one in each of the distal and proximal domains. Class II includes the lignin-degrading manganese-dependent (MnP) lignin peroxidase (LiP) and *Coprinus* peroxidases (synonymous with *Arthromyces ramosus* peroxidase) (Poulos *et al*., 1993). Class III includes the classic horseradish peroxidase isoenzyme C (HRPC); peanut peroxidase (PNP), for which the first crystal structure of a class III peroxidase was solved (Schuller *et al*., 1996); and the major peroxidase from barley grain (BP 1) (Henriksen *et al*., 1997). Within class III, HRPC and PNP (peanut peroxidase) occupy distant branches of the phylogenetic tree and therefore provide a close structural representative for the many class III sequences currently identified, which should facilitate future rationalization of structure-function relationships (Gajhede *et al*., 1997). The molecular structure of HRPC is discussed in Section 1.4.3.

There have been numerous reports in the plant physiology literature of the specific involvement of peroxidases in lignin biosynthesis, oxidation of indoleacetic acid, ripening of fruits and protection against fungal and bacterial attack; peroxidases have been claimed to influence a large array of physiological functions. In harvested plant foods, peroxidases are believed to be responsible for loss of colour, flavour and texture as well as nutritional attributes (Robinson, 1991).

1.4.1. History of the study of peroxidases in plants

Products of the action of peroxidases in both horseradish and milk were first observed approximately 190 years ago; it was observed that "extract of guaiacum" turned blue in the presence of what we now know to be peroxidase. Fundamental investigations began in 1920 with the work of Onslow (1920) who

observed that the activity of oxidizing enzymes varied with the ripeness of fruit. During the 1930s, Keilin and co-workers observed the importance of the haem component (Keilin and Mann, 1937). Davis (1942) reported that the greatest peroxidase activity was in the inner seed coat in citrus fruits. With advances in spectral techniques and protein purification methods, Chance (1951) showed the existence of the peroxidase-hydrogen peroxide complexes, compound I and compound II. These are important intermediates formed between peroxidase and the oxidant in peroxidase-catalysed reactions (Chance, 1952) and will be discussed later.

1.4.2. Occurrence and distribution of peroxidases in plant cells

Isoperoxidases seem to be located in almost all parts and organelles of the cell, although they vary in number and activity with physiological changes; peroxidase activity has been detected in vacuoles, tonoplasts, plasmalemmae, mitochondria, microsomes and cell walls. The tissues of lupin (Ros Barcelo *et al*., 1987), petunia (Hendriks *et al*., 1985) and tobacco (Pang *et al*., 1989) have been used to study the transport and distribution of isoperoxidases in plant cells. It is accepted that peroxidases are mainly located in cell walls, cytoplasm and vacuoles, although there is little unanimity on the location of individual isoperoxidases in plant cells (Robinson, 1991).

Fractionation of intracellular membranes by isopynic sucrose density gradient contrifugation (Thomas *et al*., 1981) has indicated that lupin anionic isoperoxidases are associated principally with membranes of the endoplasmic reticulum and to a lesser extent with the Golgi apparatus and plasmalemma-derived membranes. These locations are in accord with the biosynthesis of peroxidases on polysomes attached to the rough endoplasmic reticulum and with the transport pathway: endoplasmic reticulum ÷ Golgi ÷ plasmalemma. Following biosynthesis and transport of peroxidases, it has been claimed (Gaspar *et al*., 1986) that the final locations of peroxidases are likely to be cell walls, intracellular free spaces and vacuoles. The subcellular location of membrane-bound acidic isoperoxidases in etiolated lupin hypocotyls has been reported (Ros Barcelo *et al*., 1988a). Doulis *et al.* (1997) have data that suggest that the ascorbate peroxidase activity in maize leaves is localized in the bundle sheath cells.

The exact localisation of bound peroxidases within whole cells has not been sufficiently investigated, although Naveh *et al*. (1981) claimed to have developed a chemiluminescent method for the quantitative analysis of peroxidases. Using an immunogold-labelling technique Hu *et al*. (1989) have claimed that the anionic isoenzyme of peanut cell cultures is predominantly located in the plasmalemma.

1.4.3. Molecular structure of peroxidases

Peroxidases in plants are mainly haem-containing enzymes where the prosthetic group is protophorphyrin IX. The haem moiety is involved in the active site of peroxidases. The secondary structure of yeast cytochrome c peroxidase has been determined by X-ray crystallography (Finzel *et al*., 1984) and has provided a reference point for comparing and matching amino acid sequences for active site regions in horseradish and turnip peroxidases. For cytochrome c peroxidase it has been shown that the arginine and histidine side chains in the distal pocket are configured in such a way as to be able to serve as catalytic sites for reaction with hydrogen peroxide. Nuclear magnetic resonance (NMR) studies have also been reported on the electronic structure and molecular structure specifically of the haem cavity in the horseradish peroxidase (HRP) (Thanabal *et al*., 1987). Using this technique Thanabal *et al.* (1987) have identified on the proximal side of the haem: His 170, Leu 237 and Tyr 185 and on the distal side: Arg 46, His 42 and Phe 41, as catalytically important residues in a cyano complex of HRP.

The size of plant peroxidases is similar to that of cyochrome c peroxidase, with approximately 300 amino acid residues per mole. Isoperoxidases do not differ significantly in size, with molecular weights generally in the range 40 000 - 50 000 (Robinson, 1991). Plant peroxidases differ from yeast cytochrome *c* peroxidases, in so far as they contain four disulfide bonds, bind two atoms of calcium per mole and are glycoproteins (Welinder 1985).

1.4.4. Peroxidatic reaction

Peroxidases are able to catalyse peroxidatic reactions, oxidatic reactions and hydroxylations, utilizing both

an oxidizing substrate and a reducing substrate. The oxidizing substrate is usually a peroxide or a peroxy acid, ROOH (Robinson, 1991). Typically, peroxidases can use hydrogen peroxide (H_2O_2), methyl-hydrogen peroxide and ethyl-hydrogen peroxide (Kermasha and Metche, 1988) as oxidizing agents in the peroxidatic reaction. The distinctive absorption spectra due to the haem prosthetic group exhibits transient changes on mixing with H_2O_2. The Soret band and visible spectra change markedly on the binding of peroxide and the consequent formation of compound I, which has a ferryl-type structure carrying an oxygen atom acquired from the peroxide. Peroxidases cause the heterolytic cleavage of the O-O bond. The iron-oxo compound is in the formal oxidation state of +5, the H_2O_2 having effectively removed two electrons from the iron atom of the enzyme. The second stage of the peroxidatic reaction involves the reduction of compound I by a reducing donor substrate. Table 1.2. lists some typical donor test substrates.

Table 1.2 Action of peroxidase-H_2O_2 on different substrates (Burnette, 1977)

Substrate	***Product***
Pyrogallol	Pupurogallin
Guaiacol	Tetraguaiacoquinone
Hydroquinone	Quinhydrone
Benzidine	*p*-Quinone di-imide
o-Phenylene diamine	Phenazine
Leucomalachite green	Malachite green
Catechol	*o*-Quinone
p-Cresol	Milky precipitate
o-Cresol	Green solution
m-Cresol	Flesh-colored solution
Tyrosine	Yellow solution
Adrenaline	Reddish solution

Hydrogen abstraction readily occurs from phenolic substrates because of the resonance stabilization of the resulting phenoxy radical.

Generally, peroxidases are defined as enzymes that catalyze the following overall peroxidatic reaction:

$$ROOH + AH_2 \rightarrow H_2O + ROH + A^{\cdot}$$

The first step of the initial oxidation of the donor substrate (AH_2) produces a free radical ($A^{\cdot}$).

The following reaction (Scheme 1) describes the action of horseradish peroxidase isozyme C (HRPC).

(i) $HRPC(Fe^{3+})Porphyrin + H_2O_2 \rightarrow HRPC(Fe^{4+}{=}O)Porphyrin^{\cdot+} + H_2O$

Compound I

(ii) $HRPC(Fe^{4+}{=}O)Porphyrin^{\cdot+} + AH \rightarrow HRPC(Fe^{4+}{=}O)Porphyrin + A^{\cdot}$

Compound II

(iii) $HRPC(Fe^{4+}{=}O)Porphyrin + AH \rightarrow HRPC(Fe^{3+})Porphyrin + A^{\cdot} + H_2O$

Scheme 1

The resting ferric enzyme reacts rapidly with H_2O_2 to form compound I, an oxy-ferryl species in which one electron has been withdrawn from the haem group to form a porphyrin B cation radical. This intermediate is reduced by a reducing substrate in two sequential one electron steps through compound II, in which the porphyrin cation radical has been reduced (Gajhede *et al.*, 1997). The overall reaction involves a cycle

of oxidation and reformation of the enzyme. The separate cationic free radicals ($A^{\cdot}$) derived from the donor substrate then react non-enzymatically with each other to form dimers or higher polymers. At high concentrations of donor substrates, the liberated free radicals interact with each other to form polymers of the A-A type as in the case of the oxidation of guaiacol or *o*-dianisidine (Robinson, 1991).

From the natural substrates, including the phenolic compounds, present in fruits and vegetables, a wide range of products may be formed through reactions involving free radicals. The specific effects of peroxidase action are difficult to identify, since they become apparent only during prolonged storage of fresh produce and they may be confused with polyphenoloxidase catalysed oxidations. Mono- and dihydroxyphenols and complex compounds such chlorogenic acid are potential substrates for peroxidases. Other substrates include the flavonoids from which it has been claimed that a large number of products are formed through the action of peroxidases (Schreier and Miller, 1985) and it is also suggested that flavonoids act as radical scavengers (Darimont *et al*., 1989). Other reducing agents for compound I include halides, the bisulfite ion and secondary aldehydes (Dunford, 1986).

1.4.5. Industrial applications of peroxidases

Elucidation of the mode of action and the availability of purified isoperoxidases increases the likelihood of their use for manufacturing purposes. The catalytic action of peroxidases is unusual in so far as these enzymes generate two well-defined chemically distinct oxidant enzyme-substrate complexes from hydrogen peroxide. Moreover, the substrates that are converted to free radical intermediates may polymerize or react with other substances to form further products. Also, for enzymes bound to solid supports, thermostability is frequently a sought-after attribute, and the natural occurrence in plants of thermostable isoperoxidases augers well for their use as reagents in manufacturing processes (Robinson, 1991).

Based on the knowledge of the action of peroxidases on phenolics, dihydroxy compounds, e.g. dihydroxyfumarate, and heterocyclics, e.g. indoleacetic acid, a likely commercial use of peroxidases is to provide sources of both simple and complex oxidised aromatic intermediates in the form of hydroperoxides

or hydroxy compounds. The degradation of chlorophyll, catalysed by peroxidases, also indicates a possible use in the degreening of, otherwise, unattractive leaf protein and other proteins of photosynthetic origin.

Some more unusual peroxidases are already known that catalyze the oxidation of halogens to form hypochlorous and hypobromous acids and there is recognized potential for their use as oxidising agents (Robinson, 1991).

Horseradish peroxidase (HRP), which is widely used in biochemical analysis, oxidizes phenols and aromatic amines (Saunders *et al*., 1964; Dunford and Stillman 1976) as well as low oxidation potential methoxybenzenes (Kersten *et al.*, 1990) and nonaromatic reductants such as dihydroxyfumaric acid, NADH, glutathione, cysteine and dithiothreitol (Halliwel and Rycker, 1978). McEldoon *et al*. (1995) have shown that soybean peroxidase is an effective catalyst for veratryl alcohol oxidation.

Prior oxidation of aromatics in waste effluents by peroxidases may also be very beneficial in reducing the oxygen demands that such products place on lakes and rivers (Robinson, 1991). Phenolic compounds are pervasive pollutants in the water environment as evidenced by the presence of 11 of them in the EPA 126 priority pollutant list (40 CFR 423.A., Code of Federal Regulations, 1991, p. 744-745) (Adler *et al.*, 1994). Peroxidases have been identified on the root surface of many plant species (Mueller and Beckman, 1978; Smith and O'Brien, 1979; Zaar, 1979; Albert *et al*., 1986) and have been shown to polymerize phenolic compounds *in vitro* (Klibanov *et al*., 1980; Alberti and Klibanov, 1981; Klibanov *et al*., 1983; Nakamoto and Machida, 1992) thereby removing them from solution by precipitation.

Adler *et al*. (1994) have experimented on the polymerization of phenolics *in vivo* in the tomato root system. After the tomato root system was exposed to 100 : g mL^{-1} guaiacol for 24 h, it turned brown, the color of tetraguaiacol which is a polymer of guaiacol. This demonstrates that the root system can polymerize guaiacol and possibly other phenolic compounds under natural conditions. They have thus suggested that plants may be utilized as a source of peroxidases for removal of phenolic compounds that

are on the EPA priority pollutant list. Plant roots may be viewed as columns with immobilized enzymes associated with them. If roots behave like other immobilized enzyme systems (Nannipieri and Bollag, 1991), root surface peroxidases will treat water as it flows by the root surface in aquatic systems, bioremediate rhizosphere soil in terrestrial ecosystems and minimize the absorption of phenolic pollutants and allelochemicals into plants by precipitating them at the root surface (Adler *et al.*, 1994).

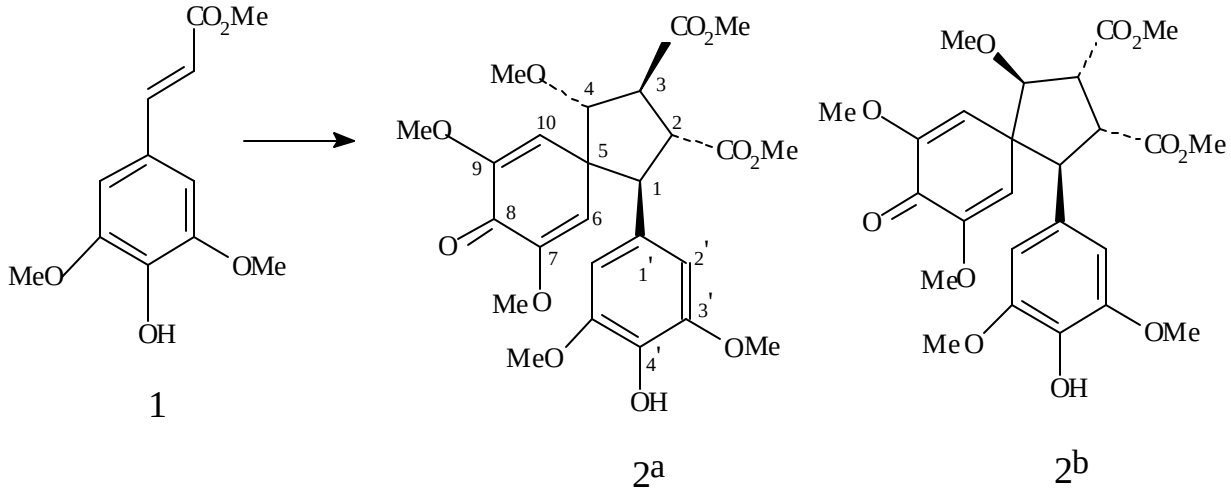
N-demethylation reactions by peroxidases have been studied by a number of investigators. In their *N*-demethylation studies of *N*,*N*-dimethylanilines (DMA) by HRP and peroxides, Kedderis and Hollenberg (1983) reported that there was a 1:1 ratio of formaldehyde formed to peroxide consumed. *N*-demethylation of DMA to formaldehyde and *N*-methylaniline by HRP immobilized on a graphite felt, and electrogenerated H_2O_2, has been reported by Chen and Nobe (1993a), for synthesis on a commercial scale. Electrogeneration provides a continuous supply of H_2O_2 to HRP at low concentrations, which extends reaction times and minimizes formation of undesirable products. The optimum temperature was increased from 28^0C for free enzymes to 55^0C for immobilized HRP. The thermal stability of immobilized HRP was greater than free enzyme; deactivation did not occur until 65^0C compared to 40^0C for free enzyme.

Recently, to replace benzoyl peroxide as a bread dough-bleaching agent, Gelinas *et al.* (1998) screened pure and commercial oxido-reductases (peroxidases, catalases, glucose oxidases, lipoxygenase and laccase) based on degradation of beta-carotene in a liquid system or dough. They found that peroxidases had the best bleaching actvity.

1.4.6. Medical applications of peroxidases

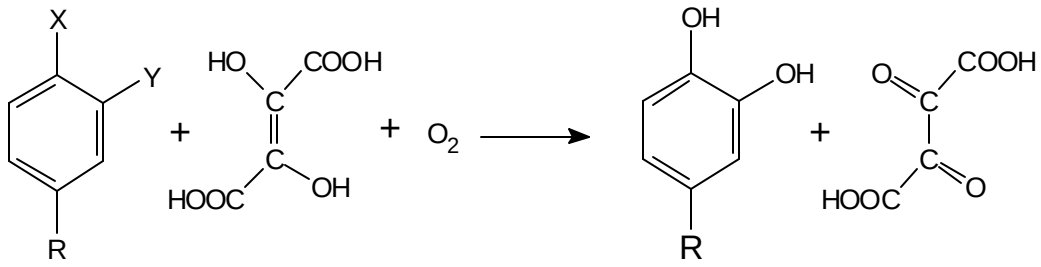
Aryltetralins are an important group of natural lignans with a 1,2-dihydro- or tetrahydro-naphthalene skeleton. Many of these compounds have been observed to have biological and pharmacological effects (Hostettler and Seikel, 1969). The oxidative coupling of phenols catalyzed by peroxidases is a very attractive method for preparing phenolic dimers of this type from phenolic cinnamates. The advantages of the enzymatic method are mild reaction conditions and fast reaction rates (Setätä *et al*., 1994).

The use of peroxidases in a preparatively useful manner is limited by low selectivity of the oxidative coupling and the complexity of the subsequent reactions. Setätä *et al*. (1994) managed to enhance the selectivity of this reaction type by carrying out the reaction at low pH since low pH-values favour the formation of dimers at the expense of polymeric products (Chioccara *et al*., 1993). In their experiment, the oxidative coupling of methyl sinaptate (1) with H_2O_2/HRP at pH 4 in the presence of methanol, the main products yielded were diastereoisomeric spiro compounds [2^a (32%) and 2^b (17%)] (Scheme 2).



Scheme 2

Selective hydroxylation of aromatic compounds is a difficult task in preparative organic chemistry particularly when the compounds to be hydroxylated are optically active, due to racemization and decomposition (Gunstone, 1965). Therefore, such hydroxylations are carried out by microbiological means (Sih and Rosazza, 1977) which are laborious, time consuming and usually provide relatively low yields (Klibanov *et al.*, 1981). Klibanov *et al*. (1981) have found that under certain conditions, the reaction in Scheme 3, catalysed by peroxidase, can be used for fast, convenient and selective hydroxylations which afford yields up to 70%.



I: R = $CH_2CH(NH_2)COOH$, X = OH, Y = H

II: R = $CH(NH_2)COOH$, X = OH, Y = H

III: R = $CH(OH)CH_2NHCH_3$ X = H, Y = OH

Scheme 3

Three important drugs have been produced as examples using this enzymatic hydroxylation: L-DOPA (used for the treatment of Parkinson's disease) from L-tyrosine (I, Scheme 3)), D-(-)-3,4-dihydroxyphenylglycine (II, Scheme 3) (a potential intermediate in the synthesis of semisynthetic antibiotics, including the cephalosporin-type antibiotics) from D-(-)-*p*-hydroxyphenylglycine, and L-epinephrine (adrenaline) from L-(-)-phenylephrine (III, Scheme 3).

Thus, there is a continual search for novel peroxidases for various applications. This project seeks to

find a novel peroxidase in sorghum.

1.5. POLYPHENOL OXIDASES

One reason for selecting to investigate the presence of polyphenoloxidase (PPO) (EC 1.14.18.1) in sorghum, for this study, was to ascertain whether it is involved in polyphenol formation in sorghum grain. Also, there is the possible use of sorghum PPO as a novel PPO for biotransformations. PPOs occur almost ubiquitously in plants (Robb *et al.*, 1984; Sherman *et al*., 1991). PPO has also been found in the common mushroom, *Agaricus bisporus*, and this has been used in the majority of biocatalytic studies, since it is commercially available (Burton, 1994).

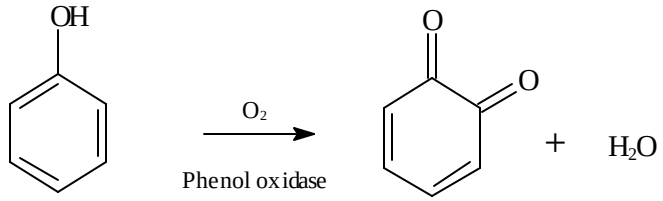
Half of the world's fruit and vegetable crops is lost due to postharvest deteriorative reactions. Browning results from both enzymatic (PPO) and non-enzymatic oxidation of phenolic compounds. Browning in fruit and in some vegetables, such as lettuce and potato, is initiated by the enzymatic oxidation of phenolic compounds by PPOs. The formation of shrimp black spot is another example of browning due to PPO activity. The initial products of oxidation are quinones, which rapidly condense to produce relatively insoluble brown polymers (melanins). Some non-enzymatic causes of browning in foods include the Maillard reaction, autooxidation reactions involving phenolic compounds and the formation of iron-phenol complexes (Martinez and Whitaker, 1995).

Based on previous work (Hulse *et al.*, 1980) it is clear that there is a correlation between seed colour and total phenol content in sorghum. White colored seeds have smaller amount of phenols than brown colored seeds. In this project, a variety of sorghum seeds with colors ranging from white, red-brown to bronze were examined for the presence of polyphenoloxidases.

PPO is a copper containing enzyme which is also known as catechol oxidase, catecholase, diphenol oxidase, *o*-diphenolase, phenolase and tyrosinase. PPO is present in some bacteria and fungi, in most plants, some arthropods and all mammals (Martinez and Whitaker, 1995). In all cases, the enzyme is

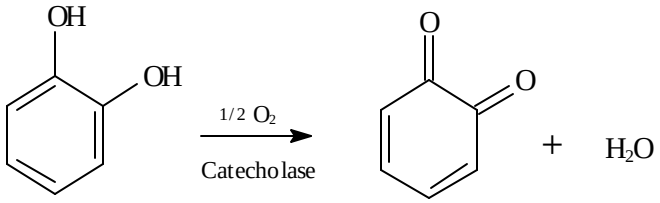
associated with dark pigmentation in the organism and seems to have a protective function (Mayer and Harel, 1991). PPO catalyses two distinct reactions:

(a) The insertion of oxygen in a position *ortho* to an existing hydroxy group, followed by oxidation of the diphenol to the corresponding quinone, often referred to as cresolase activity (Scheme 4):



Scheme 4

and (b) The oxidation, with hydrogen abstraction of *o*-diphenol (*o*-dihydroxybenzene or catechol) often referred to as catecholase activity (Scheme 5). Molecular oxygen participates in both reactions.



Scheme 5

1.5.1. Occurrence of PPOs in plant cells

PPO is an intracellular enzyme. It is membrane-bound, particularly in chloroplasts. Burton and Kirchman (1997) have identified a chloroplast membrane-bound PPO from tea (*Camellia sinensis*) and have optimized a detergent-based method for its extraction. It has also been reported to be

localized in mitochondria (Mayer and Friend, 1960; Mayer, 1961; Bonner, 1955; Demenyuk *et al*., 1974) and in peroxisomes and microsomes (Kato *et al*., 1976; Ruis, 1972). The strength of binding of PPO to membranes appears to vary depending on the tissue and the stage of development of the plant. Thus, in tobacco, washing with buffer suffices to release the enzyme from chloroplast lamellae (Hoffer, 1964). In most cases, more drastic conditions are required for the solubilization of membrane-bound catechol oxidaze, such as the use of detergents, e.g. digitonin (Harel *et al*., 1965; Alberghina, 1964), Triton X-100 (Harel *et al*., 1964; Harel and Mayer, 1971; Walker and Hulme, 1966) and SDS (Yamaguchi *et al*., 1969; Ben-Shalom *et al*., 1977). These treatments evidently cause changes in the enzyme's structure and/or conformation and are frequently accompanied by activation (Kenten, 1957; Robb *et al*., 1965; Mayer, 1966).

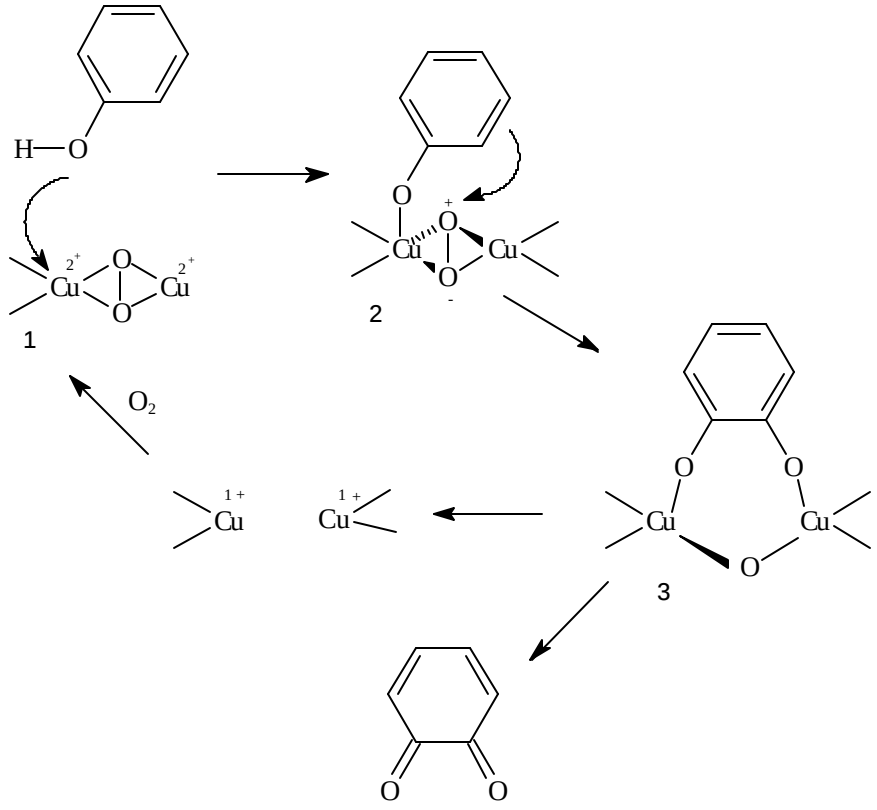
The compartmentalisation of the phenolic substrates of the enzyme, both in cells (Barnell and Barnell, 1945; Reeve, 1959; Mace, 1963) and within the cell have been reported. This results in the separation between the enzyme and the bulk of its phenolic substrates *in situ.*

1.5.2. Structure of polyphenol oxidases

No complete structure elucidations have been reported for polyphenol oxidases (PPO); the difficulties inherent in the purification of multi-subunit enzymes, and the multiplicity which PPOs exhibit, contribute to the lack of progress in defining their protein structures. The amino acid composition and sequences for *Neurospora* and *Streptomyces* tyrosinases have been published by Lerch *et al*. (1982). In the case of mushroom tyrosinase, the amino acid composition was determined by Jolley *et al*. (1965). Various investigations into the quaternary structure of mushroom tyrosinase have been carried out (Bouchilloux *et al*., 1963; Jolley, 1965). Chen *et al*. (1992) have also examined the multiple forms of potato and mushroom polyphenol oxidases. In the absence of crystals sufficiently pure for X-ray crystallographic analysis, the solid state structure of the proteins remains undefined.

1.5.3. Mechanism of oxidation by polyphenol oxidases

Much attention has been devoted to intensive investigations of the dinuclear copper binding site which is present in the group of "Type III" copper proteins, of which PPO is a member. These proteins are characterized by two anti-ferromagnetically coupled copper ions situated close together in the active sites, capable of binding dioxygen to form a dioxygen-dicopper (II) complex (**1** in Scheme 6) (Solomon *et al*., 1992). A mechanism proposed to explain the activity of tyrosinase, based on these considerations, is shown in Scheme 6 (Solomon *et al*., 1992). The phenolic substrate is suggested to coordinate initially from the axial position, and electron density is donated from the substrate into the lowest unoccupied molecular orbital (LUMO) of the oxy-dicopper unit **2** which is anti-bonding with respect to the oxygen-oxygen and copper-oxygen bonds. This initiates oxygen transfer to the *ortho* position of the phenyl ring, resulting in the formation of bound catechol, **3**. Electron transfer from the catechol to the copper atoms generates the deoxy site and releases the *o*-quinone.



Scheme 6 (Burton, 1994)

1.5.4. Physiological function of PPO

Any attempts to ascribe a physiological function to PPO in green plants, from the algae to higher plants must take into account a number of properties of the enzyme. These properties are: (1) its subcellular location - the enzyme may be particulate or soluble and it often appears in several subcellular fractions ; (2) the activation of the enzyme under certain conditions due to conformational changes; (3) the presence of native inhibitors; (4) the enormous variation in the enzyme level at different periods of growth and development; (6) the separation of the enzyme from most of its substrate due to compartmentation, cellular or subcellular (Mayer and Harel, 1979).

One of the oldest suggestions of a physiological role is that of synthesis of *o*-diphenols. This suggestion is based on the undoubted ability of many PPO preparations to oxidize monophenols to the corresponding *o*-quinone (Mayer and Harel, 1979). Although plant PPOs have been shown to hydroxylate naturally occurring phenolics, e.g flavonoids in spinach beet (Vaughan *et al*., 1969), the physiological significance of these observations was not established. In *Vicia faba*, it was shown that PPO is not involved in L-DOPA synthesis (Griffith and Conn, 1973).

A second suggested role of PPOs is in electron transport. Since quinones are powerful oxidizing agents it is easy to imagine a reaction in which a diphenol is oxidized by PPO and the quinone then reduces some cell constituent, such as a nucleotide, non-enzymatically. Kubowitz (1939) claimed to have demonstrated such a reaction, and since then PPO has been suggested to be involved one way or another in aerobic respiration (Arnon, 1950; Bonner, 1955).

The presence of phenolic compounds in plants, their oxidation following injury, either mechanical or due to infection and the relatively high toxicity of the oxidation products have long drawn attention. The possible relationship of these properties to plant resistance to disease has prompted many research workers to ascribe a role to PPO in disease resistance. Kosuge (1969) concluded that in most cases

there was inadequate evidence to show that PPO plays a significant role in disease resistance. From the review of Kosuge (1969), the following general conclusions can be drawn: PPO does increase in activity following infection by virus, bacteria, fungi or mechanical injury, and the increase may be due to activation of host latent enzyme by solubilization of host PPO which is normally particulate. Kosuge also concluded that generally speaking, quinones are quite toxic to extracellular enzymes produced by pathogens, due to their great reactivity. Thus PPO may function by producing such quinones.

1.5.5. Applications of Polyphenol oxidases

The application of polyphenol oxidase as a biocatalyst, in aqueous and/or organic systems has been shown to have significant synthetic potential. Mushroom tyrosinase has been observed to catalyse the transformation of phenols to *o*-quinone products (Burton, 1994). Dopamine and its analogs, e.g. "-methylnoradrenaline also serve as substrates for tyrosinase, and the transformation of dopamine has been proposed as a minor pathway for the oxidation of DOPA *in vivo* (Jimenez *et al*., 1984). Fluorinated substances have been investigated because fluoride has been observed to be released as a result of tyrosinase activity. This fluoride is toxic to cancer cells, and thus there is potential for treatment of tyrosinase-rich melanoma with these substrates (Rice *et al*., 1987).

Tyrosinase is also capable of hydroxylating and oxidizing tyrosine residues in peptides and proteins (Marumo and Waite, 1986). This is important in the production of moisture-resistant adhesives which imitate naturally occurring adhesives such as mussel glue, and tyrosinase has been used in the production of synthetic glues which are simpliar in composition to these (Yamamoto *et al*., 1992).

PPO (from mushroom, frog epidermis and grape) has been used in a simple but accurate method for calibration of an oxygen electrode, since the oxidation of *tert*-butylcatechol by the enzyme is oxygen-dependent and can also be monitored spectrophotometrically (Rodriguez-Lopez *et al*., 1992). One important application of PPO capitalises on the polymerising properties of *o*-quinones: the removal of phenols from polluted waters can be effected by tyrosinase-mediated oxidation and the subsequent

non-enzymic polymerization which leads to the formation of melanin-like precipitates (Burton, 1994). Because of the wide commercial applications of PPOs and the possibility of their presence in sorghum, investigation of novel PPOs in sorghum is justified, especially for the enhancement of the plant's economic value.

1.6. PROTEASES

Protease preparations are, on a commercial basis, the most important of the currently produced enzymes (Table 1.3).

Table 1.3. World-wide sales (1986 estimates) of industrial enzymes (Poldermans, 1989)

Market	***Enzyme***	***Sales (10^6\$) p.a***
Detergents	Protease	140
Starch	"-Amylase	40
	Amyloglucosidase	40
	Glucose isomerase	25
Dairy	Rennet	70
	Others	10
Beverages	(Hemi)cellulase	
	Pectinase	40
	"-Amylase	
	Protease	
Bakery	"-Amylase	20
	Protease and others	
Miscellaneous	...	40

They are obtained from plants, animal organs and microorganisms, with the majority obtained from bacteria. To achieve targeted and specific changes in proteins, commercial protease preparations with different specificities are used. Such preparations are currently employed to produce many foods in which enzymes can replace potentially carcinogenic or otherwise harmful chemicals. Table 1.4 shows

some processes used in the manufacture of foods in which proteases have specific applications which are discussed later.

Table 1.4. Application of Proetases in Food and Industrial Technology (Uhlig, 1998)

Product or Process	***Use***
Beer	To solubilize grain proteins; to stabilize beer
Cheese	To coagulate milk proteins and to ripen cheese
Meat tenderizing	To partially separate connective tissues
Bread	To increase gluten elasticity
Cookies and crackers	To improve crispness
Leather	To remove wool, hair and pigments: to soften skins
Laundry detergents	To remove protein stains

In ancient China and Japan man utilized microbial amylases and proteases, in particular, for the production of soy-derived foods. A famous Greek writer, Homer, describes the production of cheese by stirring milk with a twig of a fig tree which leaks a protease, ficin. Otto Röhm obtained his famous trypsin-in-detergent patent in 1913, but it was to take 50 years before the real breakthrough occurred. Proteases were first used in brewing in 1911.

1.6.1. Plant proteases

Plant proteases are ubiquitous, occurring mainly in tropical plants. Well recognized and characterized proteases are found in papaya (*Carica papaya*), pineapple (*Anana sativa*), figs (*Ficus carica, Ficus glabrata*), artichokes *(Cynera cardunculus*) and soybeans (*Soya hispidus*). All of these enzymes belong to the cysteine protease family characterized by the presence of a sulfhydryl group in the active site (Uhlig, 1998)

The main commercial source of plant proteases is green papaya, from which papain is obtained. In addition, bromelain is produced by alcohol precipitation from extracts of pineapple or pineapple stalk,

and ficin is prepared in the form of ficus latex, but is of limited commercial importance. The plant proteases are used as meat tenderizers and in other areas of food production, including brewing, beer stabilization, cookie baking and the production of protein hydrolysates. Other applications are in tanning and pharmaceuticals as digestive-aid preparations (Uhlig, 1998).

1.6.2. A model protease: subtilisin proteases

Subtilisin is among the most important industrial enzymes due to its use in laundy detergents. Being a small single domain serine protease (MW 27 500) with no cofactor or metal ion requirement for its function, subtilisin displays Michaelis-Menten kinetics and is secreted in large amounts by a wide variety of *Bacillus* species. Protein engineering strategies for subtilisin have focussed on a number of aspects, including catalysis, substrate specificity, and pH profile.

1.6.3. Applications of proteases

The detergent industry is the largest single market for enzymes, at 25 - 30% of total sales. Over half of all detergents presently available contain enzymes, in particular proteases and lipases. Besides improved washing efficiency, the use of enzymes allows lower temperatures and shorter periods of agitation to be employed, often after a preliminary period of soaking.

The leather industry is responsible for a significant proportion of the world's enzyme consumption. Skins are soaked initially to clean them and to allow rehydration. This latter process is aided by the addition of very low concentrations of proteases. Use of pancreatic trypsin is especially favoured, since contaminating lipases solubilize fats and gums, further improving water uptake. Dehairing is then carried out using alkaline proteases, such as subtilisin, in an alkaline bath. Alkaline conditions tend to swell the hair roots, so easing removal of the hair by allowing the proteases to attack, selectively, protein in the hair follicle. Relatively large amounts of enzyme are required (0.1 - 1% (w/v)) and thus a cheaper source of proteases, from sorghum, for instance, would decrease leather processing costs.

Fabrics are normally sized to increase strength during weaving by the application of adhesive starch. Subsequent desizing by liquefaction of the starch by bacterial amylases is carried out because the sized fabric is less absorbent to liquids, making operations such as dyeing and bleaching more difficult. Proteases (papain) have been used in the past to 'shrink-proof" wool and to give it a silky lustre. The method was abandoned some years ago, primarily for economic reasons, but it is not unreasonable to expect its use to be re-established, now that cheaper enzyme sources are available (Uhlig, 1998).

This study investigates the possibility of isolating another cheap protease enzyme from the sorghum plant for such applications.

1.7. OTHER ENZYMES REPORTED IN SORGHUM

For the sake of completion, this section summarizes the literature reporting other, more specialized, studies on enzymes found in sorghum, but not necessarily for direct investigations relevant to application in biotransformations.

1.7.1. $-Glucosidase (dhurrinase)

$-glucosidases ($-D-glucoside glucohydrolase; EC 3.2.1.21) catalyse the hydrolysis of aryl and alkyl $-glucosides, releasing Glc (glycone) and aglycone (Reese, 1977). These enzymes occur ubiquitously in plants, fungi, bacteria and animals (Woodward and Wiseman, 1982). The natural substrates of $-glucosidases include $-glucosides and $-glucosyl ceramides of mammals, cyanogenic and hydroxamic acid $-glucosides of plant secondary metabolism and $-linked oligosaccharides released from the digestion of plant cell walls during germination (Conn, 1981; Niemeyer, 1988; Beutler and Eaton 1992; Ceuvas *et al*., 1992; Leah *et al*., 1995). Most $-glucosidases display broad range specificity for the agycone moiety of their substrates but somewhat narrow specificity for the glycone moiety (Hösel *et al*., 1987; Hughes and Dunn, 1982; Babcock and Esen,1994). Babcock and Esen (1994) proposed that a hydrophobic aglycone group is required for cleaving the $-glycosidic bond between the glycone and the aglycone in maize.

1.7.1.1. Functions of $-glucosidases

The aglycones, the active group of glucosides, play important roles in plant defense, development and growth (Selmer *et al*., 1987; Poulton, 1990). Cyanogenic $-glucosides have long been known to be involved in the defense against some pathogens and herbivores, releasing the respiratory poison hydrogen cyanide (HCN) upon hydrolysis by $-glucosidase (Hruska, 1988; Poulton, 1993). In fact, many important crops, including sorghum (*Sorghum bicolor)*, cassava, lima beans, flax, white clover, rubber tree and stone fruits contain cyanogenic $-glucosides and corresponding $-glucosidases (Poulton, 1989). Upon damage to tissues, the enzyme and its substrate, which are compartmentalized in intact tissues, come into contact and release a toxic aglycone or a derivative (e.g HCN) (Kakes, 1985; Hösel *et al*., 1987; Selmar, 1993). Under these conditions, dhurrin is hydrolysed by an endogenous $-glucosidase (dhurrinase) to produce *p*-hydroxymandelonitrile, which subsequently disassociates to free HCN and *p*-hydroxybenzaldehyde (this process is described in section 1.7.2 where hydroxynitrile lyases (HNLs) are discussed). Khan *et al*. (1997) elucidated the cyanogenic glucoside biosynthesic pathway using sorghum as a model plant.

1.7.1.2. Sorghum $-glucosidases

Sorghum has two cyanogenic $-glucosidases, *Dhr1* and *Dhr2*, which were purified by Hösel *et al*. (1987) and shown to be made up of 57- and 62-kD monomers, respectively. Both enzymes exhibit high specificity for the physiological substrate dhurrin, as well as its structural analog sambunigrin (Hösel *et al*., 1987). However, neither enzyme shows any detectable activity toward any of the other natural or artificial substrates tested, except that *Dhr2* shows substantial reactivity toward the synthetic substrates 4-methylumbelliferyl-$-D-Glc and *p*-nitrophenyl-$-D-Glc (Cicek and Esen, 1998). As yet, because of the low activity levels displayed by these enzymes, they are not likely to have commercial use.

1.7.2. Hydroxynitrile lyases (HNLs)

HNLs are enzymes catalyzing the dissociation of cyanohydrins to HCN and the corresponding aldehyde or ketone. They are involved in the cyanogenisis of higher plants. There they cleave cyanohydrins released from cyanogenic glycosides by the action of specific $-glucosidases (Hösel and Nahrstedt, 1975) (discussed in section 1.2.2.1.). Two different groups of HNLs exist, namely one which contains FAD and another which lacks it (Kuroki and Conn, 1989). Flavoprotein HNLs are found only in members of the family Rosaceae (Gerstner *et al.*, 1968), whereas nonflavoprotein HNLs occur in several families. All flavoprotein HNLs are of similar molecular weight and all are glycosylated (Wajant and Mundry, 1993). Of the nonflavoprotein HNLs, which differ in size and substrate specificity (Kuroki and Conn, 1989; Bové and Conn, 1961; Smitskamp-Wilms *et al*., 1991; Xu *et al*., 1988; Carvalhò, 1981), only HNLs isolated from *Ximenia americana* L. (Kuroki and Conn, 1989) and *Sorghum bicolor* (Wajant and Mundry, 1993) are described as glycoproteins.

In organic chemistry the HNLs are used for the stereoselective synthesis of "-hydroxynitriles (Effenberger *et al*., 1987a; Effenberger *et al*., 1990). In organic solvents it is possible to add HCN to aldehyde or ketone enzymically with highly selective production of only one of the possible enantiomers, because the base-catalysed addition, which leads to racemates, is suppressed (Effenberger *et al*., 1987a).

1.7.2.1. Sorghum HNLs

Bové and Conn (1961) described a hydroxynitrile lyase of *Sorghum vulgare* (EC 4.1.2.11, *S*-oxynitrilase) which catalyzes the reactions of *S*-cyanohydrins, the natural substrate being *p*-hydroxymandelonitrile. The glycosylation of sorghum HNL is necessary for maintenance of catalytic activity. Sorghum HNL is a heterotetramer which exists in three isoforms and the molecular weights of the " and $ subunits are 30 and 25 kDa respectively (Wajant and Mundry, 1993). Because of the practical importance of (*S*)-HNL from *Sorghum bicolor* L. as a tool for the synthesis of (*S*)-cyanohydrins (Effenberger *et al*., 1990), a number of procedures for its purification have been

established (Smitskamp-Wilms *et al*., 1991; Jansen *et al*., 1992; Wajant and Mundry, 1993). Thus, the study of these enzymes is an area of academic interest. However, as yet, commercial applications have not been developed.

1.7.3 Glutathione *S*-transferase (GST)

GSTs (EC2.5.1.18) are dimeric enzymes found in mammals, insects, plants and microbes that catalyze nucleophilic attack by the thiolate anion of GSH on electrophilic centres of hydrophobic molecules (Mannervik and Guthenburg, 1981).

1.7.3.1 Plant glutathione *S*-transferases (GSTs)

1.7.3.1.1. Classification of plant GSTs

In general, plant GSTs have not been as well characterized as mammalian GSTs. Plant GSTs are members of the archaic 2-GST class, from which other GST classes evolved (Buetler and Eaton, 1992). It has been proposed that 2-GSTs originally evolved in prokaryotes to protect against oxidative stress (Pemble and Taylor, 1992). Plant 2-GSTs have been subdivided into three types (I, II and III) based on amino acid sequence identity and conservation of intron:exon placement (Droog *et al*., 1995).

1.7.3.1.2. Function of plant GSTs

The best-characterized function of plant GSTs is their role in the detoxification of certain herbicide classes such as the chloroacetanilides, thiocarbamates and *S*-triazines (Lamoureux and Rusness, 1989). Plant GSTs can be induced by biotic stimuli such as pathogen invasion and abiotic stimuli, such as herbicide safeners and heavy metals (Marrs, 1996). Very little is known about endogenous substrates and functions of plant GSTs. Certain plant GSTs bind auxins as nonsubstrate ligands (Bilang *et al.*, 1993; Zettl *et al*., 1994). A GST encoded by the maize *bronze*2 gene conjugates anthocyanin prior to transport into the vacuole via a tonoplast transporter (Marrs *et al*., 1995). There are also increasing numbers of reports of plant GSTs exhibiting GSH peroxidase activity (Williamson and Beverley, 1987,

1988; Bartling *et al*., 1993; Zettl *et al*., 1994; Flury *et al*., 1996), which suggests a role in protection against oxidative stress.

1.7.3.1.3. Sorghum GSTs

From etiolated sorghum roots, Gronwald and Plaisance (1998) have purified two GST isozymes: GST A1/A1, a constitutively expressed homodimer and GST B1/B2, a heterodimer induced by the herbicide, fluxofenim. Fluxofenim treatment of sorghum causes stress, as indicated by growth inhibition of developing seedlings (Fuerst and Gronwald, 1986). Although this induced GST B1/B2 exhibits activity with herbicidal concentrations of metolachlor, it is possible that the primary function of this isoenzyme, and perhaps others induced by the safener, is to protect against lipid peroxidation products generated by various forms of stress (Gronwold and Plaisance, 1998).

1.7.4. Betaine aldehyde dehydrogenase (BADH)

Glycine betaine (Gly betaine) is a quaternary ammonium compound that accumulates in a diverse array of prokaryotic (Csonka, 1989) and eukaryotic organisms (Rhodes and Hanson, 1993). The ability to synthesize and accumulate glycine betaine is wide-spread among angiosperms (Weretilnyk *et al*., 1989) and is thought to contribute to salt and drought tolerance (Grumet and Hanson, 1986). In plants, Gly betaine is sythesized by the two-step oxidation of choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) (Hansen, 1993). CMO is responsible for the oxidation of choline to betaine aldehyde and BADH converts betaine aldehyde to Gly betaine.

BADH is a pyridine nucleotide-dependent dehydrogenase (Weretilnyk *et al*., 1989) specific to betaine aldehyde (Weigel *et al*., 1986). BADH has been purified to homogeneity from the leaves of salinized spinach plants and is found primarily in the stromal fraction of the chloroplast (Arakawa *et al*., 1987; Weretilnyk and Hanson, 1989). The accumulation of Gly betaine in higher plants is induced by cold (Kishitani *et al*., 1994), drought (Ladyman, *et al*., 1980) and salinity (Hanson and Wyse, 1982). Gly betaine accumulates primarily in the leaves of stressed plants (Hanson, 1993). The stress-induced

accumulation is associated with increases in the activities of CMO (Broquisse *et al*., 1989) and BADH (Weretilnyk and Hanson, 1989).

1.7.4.1. Betaine aldehyde dehydrogenase (BADH) and its function in sorghum

The ability to modify essential metabolic processes is a key to plants' adaptation to adverse environmental conditions (Yancey *et al*., 1982). Plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components. In response to declining Ψ_w, (leaf water potential), many plant species experience a similar decline in Ψ_s (osmotic potential) (Morgan, 1984). The decrease in Ψ_s can be the result of either passive ion concentration, caused by a reduction in cell volume, or the active accumulation of solutes. The decrease in Ψ_s that results from the active accumulation of solutes is termed osmotic adjustment (OA) (Morgan, 1984). OA is a mechanism for maintaining turgor and reducing the deleterious effects of water stress on vegetative and reproductive tissues (Flower *et al*., 1990). The active accumulation of solutes compatible with cellular metabolism is thought to play a central role in OA (Yancey, 1994). Glycine betaine (Gly betaine) accumulates in response to osmotic stress, and its accumulation may represent an important adaptive response to drought stress (Rhodes and Hanson, 1993).

Sorghum is known to accumulate Gly betaine in response to salinity stress (Weinburg *et al.*, 1982; Grieve and Mass, 1984). Wood *et al*. (1996) have shown that levels of Gly betaine increased in response to water deficit and made major contributions to Ψ_s and OA. Gly betaine accounted for 3% (6% total) of the maximal measured OA. They also showed that the level of BADH1 and BADH15 mRNAs increased 2- to 3-fold under conditions of water deficit.

1.8. CONCLUSION

This literature review has demonstrated that the sorghum plant contains a number of enzymes with potential applications of commercial interest. However, relatively little or no information has been reported on the presence or nature of peroxidase, polyphenol oxidase, or protease activities in sorghum. In the present study, an investigation into these enzymes was initiated with the objectives of identifying novel enzymes which might have interesting characteristics and of developing opportunities for enhancing the value of sorghum as a cash crop.

If highly active, novel enzymes were found, they would have potential application in a number of biotransformation reactions, and hence the enzymes would possibly have commercial value.

1.9. OBJECTIVES

The objectives set at the start of the project were, thus, as follows:-

1) To conduct an investigation to determine whether the following enzyme activities were present in sorghum: peroxidase, polyphenol oxidase, protease.

2) To isolate and characterise the enzymes found to be present and active in sorghum extracts.

3) To demonstrate the potential of these enzymes in applications involving biotransformations.

Chapter 2
SURVEY TO DETECT SELECTED ENZYME ACTIVITIES

2.1. INTRODUCTION

A survey was conducted in order to detect proteases, polyphenoloxidases and peroxidases in *Sorghum bicolor*. These enzymes were selected for their potential in enhancing the economic value of sorghum, since they could have important applications in industry and medicine. Sorghum contains phenolic compounds and thus it is reasonable to expect the presence of oxidative enzymes, polyphenoloxidases and peroxidases, which are known to be involved in the synthesis of these compounds (Mayer and Harel, 1979; Robinson, 1991).

Proteases are common in plants and used extensively in industry. One of the reasons for selecting them was their potential use in grain de-hulling. In rural areas, grain hulls are removed using pestles and mortars, which is a strenuous exercise.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Sorghum bicolor seeds, (a gift from Mr. C. Muzariri, University of Zimbabwe and Dr. Kamugira of the Kawanda Agricultural Research Institure, Uganda)) were planted in the Grounds and Gardens Department of Rhodes University and the crops were used for all experiments described unless otherwise stated. Leaf, root and grain tissue was stored at -20^{0}C until used. Before homogenization, the tissue was powdered by liquidising in liquid nitrogen.

For the extraction of proteases the following chemicals were purchased:- 2-mercaptoethanol from Unilab Saarchem (Pty) Ltd, (Tris[hydroxymethyl]-aminomethyl), trichloroacetic acid, sulfanilamide-azocasein and Sephadex G-25 from Sigma Chemical Co. (USA), calcium chloride from Merck NT Laboratory Supplies (Pty) Ltd and hydrochloric acid from BDH chemicals. The following chemicals were purchased for the extraction and assay of peroxidase:- malonate, sodium hydroxide, ammonium sulfate and ETDA were purchased from Unilab Saarchem (Pty) Ltd, sodium- and calcium chloride from Merck NT Laboratory Supplies (Pty) Ltd, ascorbic acid from Seelze-Hannover, Triton-X100 from BDH Chemicals, sodium dihydrogen ortho-phosphate from Holpro Analytics (Pty) Ltd, 2-[*N*-Morpholino]ethanesulfonic acid (MES), polyvinylpolypyrrolidone (PVPP) hydrogen peroxide and *o*-dianisidine from Sigma Chemical Co. (USA), phenyl Sepharose Cl-4B and Sephadex G-75 from Pharmacia. For the extraction and assay of polyphenoloxidase, the following chemicals were purchased:- sodium hydroxide and sucrose from Saarchem, ascorbic acid from Seelze-Hannover, sodium chloride from Merck NT Laboratory Supplies (Pty) Ltd, Triton X-114 from BDH Chemicals, polyethylene glycol 6000 CP (PEG) from Associated Chemical Enterprises, L-$-3,4-dihydroxyphenylalanine (L-DOPA) and caffeic acid from Sigma and 4-methylcatechol from Aldrich.

Other materials included dialysis tubing (Sigma) (MWCO - 12 000) and Slide-A-Lyzer cassettes (Pierce) for dialysis. Centrifugation was conducted using a Beckman J2-J21 centrifuge. A Molecular/Por Stirred

Cell and a cellulose Molecular/Por filtration membrane (MWCO - 10 000) were used for ultrafiltration. A Shimadzu UV - 160 A spectrophotometer was used for spectrophotomeric assays. Polyacrylamide Gel Electrophoresis was done using a Hoefer Tall Mighty Small apparatus. Molecular weight markers were from Sigma Chemical Co. (USA).

2.2.2. Assay methods

2.2.2.1. Protease assays

The assay procedure was adapted from the method of Ilami *et al.* (1997). Sulfanilamide-azocasein (0.2% (w/v)) was dissolved in 50 mM Tris-HCl buffer pH 7.5 containing 10 mM $CaCl_2$. 800 :L of the azocasein solution was aliquoted into 12 eppendorf tubes (six sets of two duplicates) and 200 :L of crude enzyme solution was added to each tube to initiate the proteolytic reaction which was carried out at 37°C in a water bath. The reaction was terminated by denaturing the enzyme in one pair of tubes, with 100 :L of 50% (w/v) trichloroacetic acid, every 2 minutes for 30 minutes. The tubes were placed in a fridge and microfuged for 3 minutes. The absorbance of the supernatant, containing soluble fragments of the dyed protein, was determined at 336 nm against a blank containing azocasein and buffer. Proteolytic activity (units) was expressed by the amount of azo dye released per minute per 200 :L of enzyme, i.e. 1 unit = the amount of enzyme required for 1 :mole product to be formed per minute.

2.2.2.2. Polyphenol oxidase assay

Duplicate colorimetric assays were performed using a spectrophotometer. The substrates used were 10 mM caffeic acid, 20 mM 4-methylcatechol and 20 mM L-$-3,4-dihydroxyphenylalanine (DOPA) in potassium phosphate buffer (pH 6.8). To 0.9 mL of the appropriate aerated substrate, 0.1 mL of the enzyme mixture was added and the rate of absorbance (420 nm) of the mixture was monitored over a period of 30 minutes. 1 U of polyphenol oxidase activity was expressed as product formed (:mol) per min per mL of enzyme solution (Burton *et al.*, 1993).

2.2.2.3. Peroxidase assays

(1). Peroxidase assays (in duplicate) were adapted from Vágújfalvi and Petz-Stifter (1982). 50 µL of substrate, 50 mM *o*-dianisidine, was added to 6 mL sodium phosphate buffer, pH 6, containing 0.01 M H_2O_2. The peroxidatic reaction was initiated by adding 100 µL of enzyme solution to 2.9 mL of the substrate mixture in a plastic cuvette and monitored at 460 nm. Assays were conducted in duplicate.

(2). Peroxidase assays, adapted from Pütter (1975), using 0.02 M 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate, used the following reaction mixture: 500 µL enzyme extract, 500 µL 0.1 M sodium phosphate buffer, pH 6, 100 µL ABTS and 100 µL 0.01 M H_2O_2 which was added to initiate the reaction. The change in absorbance was monitored at 465 nm. Assays were conducted in duplicate.

(3). The reaction mixture, with 10 mM guaiacol as substrate, consisted of 100 µL of enzyme extract, 700 µL of guaiacol and 200 µL of 0.01 M H_2O_2 to initiate the reaction. The change in absorbance was monitored at 465 nm. Assays were conducted in duplicate.

(4). The reaction mixture, with 2,4-dichlorophenol (2,4-DCP), consisted of 100 µL enzyme extract, 200 µL of sodium phosphate buffer, pH 6, 200 µL 1 mM 4-aminoantipyrine, 300 µL 5 mM 2,4-DCP and 200 µL of 0.01 M H_2O_2 to initiate the reaction. The reaction was monitored at 510 nm. Assays were performed in duplicate.

Peroxidase activity was expressed as : mol of product formed per minute per mL enzyme. Equations for the calculations of enzyme units are given in Appendix 2.

2.2.2.4. Protein assay

Proteins assays were performed according to Bradford (1976). To prepare a protein standard curve, into 8 microfuge tubes, duplicate amounts of 5, 10, 15 and 20 :L of 0.5 mg/mL bovine serum albumin (BSA) were aliquoted. The volume in each tube was adjusted to 100 :L with 0.15 M NaCl. Blanks were prepared by aliquoting two microfuge tubes with 100 :L of 0.15 M NaCl. 1 mL of Coomassie Brilliant Blue solution (CBB) (100 mg CBB, G-250, in 50 mL 95 % ethanol, 100 mL 85% phosphoric acid and 850 mL H_2O) was added to the tubes and vortexed till thoroughly mixed. The solution was left to stand for 2 minutes at room temperature. The absorbances of all samples were determined at 595 nm. To determine the protein concentration of the enzyme extracts, duplicate amounts of 100 :L of extract (diluted if necessary) were assayed in the same method. The absorbance of the extracts were measured against the absorbance values of the protein standards in order to determine their concentrations. The standard curve and calculations of protein concentration are given in Appendix 1.

2.2.3. Extraction methods

2.2.3.1. Extraction of proteases

Crude enzyme extract was obtained by grinding leaf powder, young, newly harvested grain and old, dried grain in sodium phosphate buffer, pH 6, using a pestle and mortar, and filtering through cheese cloth. The extract was assayed for protease activity (Section 2.2.2.1), in comparison with commercial proteinase K and trypsin.

2.2.3.2. Extraction of polyphenol oxidase

2.2.3.2.1. Extraction of polyphenol oxidase from sprouted seeds

A variety of seeds (Zimbabwe, Seredo and Epurpur - the latter two strains donated by Kawanda Institute) were sprouted for ten days after being washed with water, drained and left in inverted jars, closed with cheese cloth. Seeds were washed and drained three times a day. The sprouts were homogenized in 0.1 M sodium phosphate buffer (1 g/3 mL), pH 6.8, containing 0.1 M ascorbic acid, using a pestle and

mortar and the homogenate was centrifuged at 28 000 x*g*, 15 min, 0°C). Experiments were carried out at 0°C. The supernatant was then assayed for polyphenol oxidase using 20 mM 4-methylcatechol, 10 mM caffeic acid and 10mM L-$-3,4-dihydroxyphenylalanine (DOPA) as substrates (assays described in Section 2.2.2.2.).

2.2.3.2.2. Extraction of polyphenol oxidase from grain

Harvested grain (ripe or immature grain), (1 g), was homogenized in 10 mL 0.1 M sodium phosphate buffer, pH 6, using a pestle and mortar, on ice. The homogenate was squeezed through cheese cloth and allowed to settle for 1 h. The supernatant was used as the enzyme extract and was assayed for polyphenol oxidase activity using 20 mM L-DOPA as a substrate.

2.2.3.2.3. Extraction of polyphenol oxidase from leaves

Method 1

Samples (7 g) of 25 day old leaf tissue, powdered in liquid nitrogen, were homogenized in 15 mL 0.1 M sodium phosphate buffer, pH 6.8, containing 0.1 M ascorbic acid using a pestle and mortar. Because the enzyme is thought to be membrane-bound, the detergent-based method of extraction, developed by Burton and Kirchman (1997), was used. The homogenate was squeezed through cheese cloth and centrifuged at 18 500 rpm for 15 minutes. The precipitate was resuspended in 20 mL Triton X-114 (1.5 % in 0.1 M sodium phosphate buffer) for 30 minutes with stirring, and centrifuged at 60 000 g for 15 minutes at 0°C. The supernatant was assayed for polyphenol oxidase activity using 20 mM L-DOPA, before being subjected to temperature phase partitioning. This process involved adding Triton X-114 to the supernatant to 4 % (w/v). This solution was kept on ice (15 min), warmed to 35°C (10 min) and centrifuged (5000 x*g*, 10 min, RT). The supernatant was dialysed and assayed for polyphenol oxidase activity using 20 mM L-DOPA as a substrate (Section 2.2.2.2) and protein content (Section 2.2.2.4) while the dark green detergent-rich phase was discarded.

Method 2

Based on the method of Meyer and Biehl (1980), leaf tissue was homogenised in high salt buffer (327 mM NaCl in 0.1 M sodium phosphate buffer pH 6) and high sucrose buffer (327 mM NaCl in 0.1 M sodium phosphate buffer, pH 6). Leaf powder (2 g) was homogenized in 5 mL of the relevant buffer using a pestle and mortar. The homogenate was squeezed through cheese cloth and centrifuged (17 000 x *g*, 10 min, 0°C). The supernatant was assayed for polyphenol oxidase activity using 20 mM L-DOPA as substrate (Section 2.2.2.2) and protein content (Section 2.2.2.4).

2.2.3.3. Extraction of peroxidase

2.2.3.3.1. Extraction of peroxidase from grain

The first grain to appear on the plant, at 3 months, was harvested and was green in colour. Grain (4.9 g) was homogenized in 25 mL (1 g /5 mL) of 0.1 M sodium phosphate buffer, pH 6, using a pestle and mortar. The homogenate was squeezed through cheese cloth and the filtrate was assayed for protein content and peroxidase activity (Section 2.2.2.3 - method 1) and analyzed using polyacrylamide gel electrophoresis (PAGE) (Section 3.2.4). Ripe grain (30 g) from 5 month old plants was harvested, subjected to the same extraction conditions and assayed for peroxidase activity. This grain was golden brown in color.

Units of peroxidase activity were expressed as U/mL and the calculation is given in Appendix 2.

2.2.3.3.2. Extraction of peroxidase from leaves

Method 1

The following experiment was performed using fresh and dried leaves of different *Sorghum bicolor* strains, (Zimbabwe, Seredo and Epurpur) to investigate whether peroxidase levels are strain specific. Leaf tissue was homogenised in 10 mL of 0.1 M Na phosphate buffer, pH 6, (1g/5mL) using a pestle and mortar and squeezed through cheese cloth. PVPP (polyvinylpolypyrrolidone) (1 g/10 mL) was added to remove

phenolics, the homogenate was stirred on ice (2 h) and centrifuged (10 000 x*g*, 10 min, 0°C). The supernatant (crude extract) was assayed for peroxidase activity (Section 2.2.2.3 - method 1) and protein content (Section 2.2.2.4) and analyzed by PAGE (Section 3.2.4). The rest of the sample was subjected to ammonium sulfate precipitation (80% w/v) and assayed for peroxidase activity and protein content. Leaf samples were harvested weekly, homogenized in 0.1 M sodium phosphate buffer, pH 6, centrifuged and the supernatant was assayed for peroxidase activity. Samples were also subjected to PAGE (Section 3.2.4).

Method 2

Nineteen day old leaves were extracted using an adaptation of the aqueous two-phase system (ATPS) of Srinivas *et al.* (1999). Fresh leaves (60 g) were homogenized in distilled H_2O (200 mL) with a Waring blendor for 3 min at RT. The extract was filtered through four layers of cheese cloth. The ATPS was prepared by adding solid PEG, ammonium sulphate and $NaCl_2$ to 80 mL of homogenate in amounts that would give 24%, 7.5% and 2%, respectively, when finally made up to 100 mL. The volume was adjusted to 100 mL using distilled H_2O. The solution was placed in a 100 mL separating funnel and the volumes of the two different phases obtained were measured. The salt-rich, bottom layer was dialyzed against distilled water to remove salts and concentrated to 6.5 mL by dialysis against 25 % PEG. This was used as the crude extract. The crude extract was subjected to gel filtration chromatography using Sephadex G-75, in 10 mM Na phosphate buffer, pH 6. 1 mL fractions were collected and their absorbance was measured at 280 nm. The samples containing the most protein were pooled and assayed for peroxidase activity (Section 2.2.2.3 -method 1), and protein content (Section 2.2.2.4). Crude and purified extracts were subjected to PAGE (Section 3.2.4).

2.2.3.3.3. Extraction of peroxidase from roots

Method 1

This method of extraction was adapted from the method of Kvaratskheliaet *al.* (1997). This involved grinding dry plant tissue to a powder using a Waring blendor. 100 g of powdered root tissue was homogenized, by stirring, in 250 mL of 50 mM MES buffer, pH 5.5, containing 1 M NaCl, 30mM ascorbic acid, 1 mM EDTA and Triton-X100. The homogenate was squeezed through two layers of cheese cloth and centrifuged (10 000 x *g*, 30 min, 0°C). The supernatant (crude extract) was then assayed for peroxidase enzymatic activity (Section 2.2.2.3 - method 1) and protein content (section 2.2.2.4). The buffer used in this method of extraction was modified by omitting Triton-X100 which interferes with protein determination. The crude extract was partially purified as described in Section 3.2.3.

Method 2

Sorghum root powder (20 g) was homogenized in 100 mL 0.1 M sodium phosphate buffer (1 g/ 5 mL), pH 6.8, using a pestle and mortar. The homogenate was squeezed through cheese cloth, assayed for peroxidase activity (Section 2.2.2.3 - method 1) and centrifuged (10 000 rpm for 15 minutes). The supernatant was assayed for peroxidase activity and ammonium sulfate cuts, from 20 - 70% saturation in 10% increments, were harvested. This involved dissoving the relevant amount of ammonium sulfate in the supernatant to give the desired percentage of ammonium sulfate saturation, with gentle stirring at 4°C. The mixture was left to stand on ice for 3 h and centrifuged (20 000 x *g*, 15 min, 0°C). The supernatant was dialyzed against several changes of half-strength Na phosphate buffer, pH 6, to remove salts, and assayed for peroxidase activity (Section 2.2.2.3 - method 1) and protein content (Section 2.2.2.4). The fractionation procedure was repeated at the next ammonium sulfate level and the precipitate was harvested, dialysed and assayed as before.

2.2.3.3.4. Effect of buffers used during extraction

A comparison of different extraction buffers was studied with 19 day old root tissue in order to determine what effect the solvent constituents had on the extraction process. Extraction buffers compared were 0.1 M Na phosphate buffer, pH 6, MES buffer, pH 5.5 and MES buffer, pH 5.5 with $CaCl_2$. Distilled water was used as a control extraction medium. The extraction procedure involved homogenizing the root tissue with a pestle and mortar on ice in the relevant buffer (1 g /5 mL) and squeezing the homogenate through cheese cloth. The filtrate was used as the crude enzyme extract.

2.3. RESULTS AND DISCUSSION

2.3.1. Extraction of proteases

An attempt was made to extract proteases from *Sorghum bicolor* by grinding the plant tissue in sodium phosphate buffer, using a pestle and mortar (Section 2.2.3.1), with the view to utilizing this enzyme for de-hulling grain. However, results presented in Table 2.1 show that there was little convincing evidence for high levels of protease activity in the plant extracts. It was expected that by increasing the reaction time between substrate and enzyme, there would be a linear increase of azo dye in the solution as the protease cleaved the dye from the substrate. Ripe grain and young grain tissue showed higher absorbance values than leaf tissue but there was a decrease in the absorbance values with time, after the initial two minute period. It is possible that any reaction was over after two minutes due to product inhibition or other interfering factors. One could add commercial proteases to detect the presence of inhibitors. Although the absorbance values in leaf tissue were low, there was an increase in absorbance as the reaction time increased, suggesting low levels of protease activity. These results are inconclusive because the trends observed are not typical as compared with those observed using the commercial enzymes studied.

Table 2.1. Protease activity in sorghum leaf tissue detected using azocasein assay

Time (min)	*Absorbance (336 nm)*		
	Ripe Grain	**Young grain**	**Leaf Tissue**
0	0	0	0
2	0.32	0.38	0.0195
4	0.37	0.37	0.027
6	0.41	0.31	0.034
8	0.30	0.29	0.039
10	0.33	0.20	0.033
20	0.28	0.27	0.048
30	0.31	0.22	0.057

The values shown are averages of the duplicate experiments.

Two commercial proteases were tested for protease activity using the same method and both were found to have higher activities than the leaf extract. Not only was there a linear increase in absorbance, but the actual absorbance readings proved to be much higher than those found with sorghum extracts. Table 2.2 shows the protease activity of proteinase K and trypsin.

Table 2.2. Protease activity with proteinase K and trypsin.

Time (min)	*Absorbance 336nm*	
	Proteinase K	**Tripsin**
0	0	0
2	0.83	0.22
4	1.09	0.38
6	1.19	0.48
8	1.29	0.68
10	1.33	0.63

30	1.39	0.94

The values shown are averages of the duplicate experiments.

In view of these unconvincing results as compared with commercial proteases, it was decided that the investigation of proteases should not be pursued in this study.

2.3.2. Extraction of polyphenol oxidase

Sorghum bicolor contains phenolic compounds and therefore it was expected that it would contain polyphenol oxidase (PPO) activity. The PPO activity was investigated in assays using various substrates, and with different strains of *Sorghum bicolor* which had varying colors. The sorghum strains studied were Zimbabwe (bronze colored), Seredo (brown) and Epurpur (cream white).

2.3.2.1. Extraction of polyphenol oxidase from sprouted seeds

A variety of 10 day old sprouted seeds (Zimbabwe, Seredo and Epurpur), extracted using sodium phosphate buffer containing ascorbic acid (section 2.2.3.2.1), were assayed for polyphenol oxidase activity using L-$-3,4-dihydroxyphenylalanine (L-DOPA), caffeic acid and 4-methylcatechol as substrates. Very little or no activity was detected (Table 2.3). In all strains showed no PPO activity was detected when DOPA was used as a substrate. Therefore there is no conclusive evidence that there is a correlation between grain color and the presence of PPO or indeed a correlation between PPO and the presence of phenolics. The extract of the brown coloured grain (Seredo) showed no activity while the extract of the cream/white coloured grain (Epurpur) had some activity. This is interesting because the colour of seeds implies the presence of PPO which probably plays a role in generating these colored complexes (Mayer and Harel, 1979).

These experiments were carried out with sprouted seeds and it is possible that PPO activity increases at a later developmental stage (Mayer and Harel, 1979). Thus, PPO activity was screened in the tissue of older plants.

Table 2.3. Polyphenol oxidase activity in extracts from a variety of *Sorghum bicolor* sprouted seeds.

Strain	***Substrate***		
	DOPA	**Caffeic Acid**	**4-Methylcatechol**
Zimbabwe	-	0.0014 U/mL	0.0019 U/mL
Seredo	-	-	-
Epurpur	-	-	0.002 U/mL

The values shown are averages of the duplicate experiments.

U/mL - Units of activity is expressed as ：mol product produced per mL of enzyme extract (Appendix 2).

2.3.2.2. Extraction of polyphenol oxidase from leaves

Using method 1 (Burton and Kirchman, 1997, section 2.2.3.2.3), 25 day old leaves were homogenized in buffer containing ascorbic acid. In order to ascertain whether the enzyme is membrane-bound, after centrifugation, the precipitate was subjected to a detergent based method of extraction using Triton X-114 (Burton and Kirchman, 1997). The crude extract and the extracts obtained after the temperature phase partitioning process were assayed for PPO activity (Section 2.2.2.2) using DOPA as the substrate. The results (Table 2.4) indicated the presence of considerable levels of PPO activity in 25 day old leaves. The detergent based method of extraction did not seem to effect release of PPO from membrane tissue since the specific activity of the final product of the detergent based method (supernatant - Table 2.4) was similar (0.224 U/mg) to that of the crude extract (0.281 U/mL).

Table 2.4. Polyphenol oxidase activity in extracts of sorghum leaf (Zimbabwe strain) extracted by temperature phase partitioning

	Crude Extract	*Temp. Phase Partitioning*	
		Precipitate	Supernatant
Protein (mg/mL)	0.441	0.334	0.347
Activity(：mol/min/mL)	0.127	0.006	0.078
Specific Activity (：mol/min/mg)	0.281	0.018	0.224

The values shown are averages of the duplicate experiments.

Specific activity is calculated as ：mols product produced per minute per mg protein (Appendix 2).

Using method 2, (Meyer and Biehl, 1980) (Section 2.2.3.2.3), leaf tissue was extracted using high salt and high sucrose buffers. Leaf powder was homogenized, filtered through cheese cloth, and after centrifugation, the supernatant (crude extract) was assayed for protein and enzyme activity using L-DOPA as a substrate. Table 2.5 shows that in leaf tissue (Zimbabwe strain), compared to sprouted seeds, considerably higher PPO activity was observed.

Table 2.5. Polyphenol oxidase activity in leaf tissue (Zimbabwe strain) after extraction in high salt and high sucrose buffers

	Buffer Used	
	High Salt Buffer	High Sucrose Buffer
Protein (mg/mL)	1.22	2.09
Activity (：mol/min/mL)	0.026	0.014
Specific Activity (：mol/min/mg)	0.021	0.007

The values shown are averages of the duplicate experiments.

Specific activity is calculated as ：mols product produced per minute per mg protein (Appendix 2).

Extraction in high salt and sucrose buffers resulted in relatively large amounts of protein being extracted. It is possible that the enzyme is ionically bound, which is the reason that these these high salt/sucrose solutions were used (Meyer and Biehl, 1980). However, the specific activity of PPO was still relatively low, with the high salt extract exhibiting a higher specific activity of 0.021 U/mg than the high sucrose extract which had a specific activity of 0.007 U/mg (Table 2.5).

2.3.2.3. Extraction of polyphenol oxidase from grain

Ripe grain and young grain, extracted using sodium phosphate buffer (Section 2.2.3.2.2), showed no PPO activity when assayed with L-DOPA as a substrate. Thus, PPO activity is not evident in the seed in *Sorghum bicolor*, which is surprising because one would have expected that at this stage of development (grain production) PPO activity would be relatively more abundant, than at other stages, for the synthesis of phenolics in order to protect the plant from predators. Possibly another constituent in the plant is more involved than PPO in the synthesis of phenolics in the plant. In fact, when grain extract was investigated for peroxidase activity, there was clear evidence of its presence, at the level of 4.9 U/mL of activity (section 2.3.4.1). It is likely then, that peroxidase is more involved in phenolic synthesis than originally thought and it was thus investigated in more detail. At this point, it is important to differentiate between the roles of PPO and peroxidase activities.

2.3.3. Differentiation between polyphenol oxidase (PPO) and peroxidase

The reaction of PPO with DOPA has been adapted for use in electron microscopy for determining the localization of the enzyme within cells. Cotton roots present a well defined system for determining the localization since the accumulation in endodermal cells can be followed by light and electron microscopy (Mueller and Beckman, 1976). However, Veech (1976) reported that he could not detect PPO activity in hypocotyls of the Deltapine-16 cultivar of cotton and that oxidation of phenolics in cotton was due to peroxidase. Further, it has been shown that peroxidase can participate in the oxidation of DOPA (Okun *et al.*, 1972).

Mueller and Beckman (1978) thus attempted to differentiate and localize PPO and peroxidase at the ultrastructural level in the roots and hypocotyls of cotton seedlings. PPO and peroxidase were differentiated and localized in the roots and hypocotyls of cotton seedlings at the ultrastructural level by means of the DOPA and 3,3'-diaminobenzedine tetrahydrochloride (DAB) reactions. They observed that two distinct reaction products were present and resulted from the action of two different enzyme systems. It was concluded that both PPO and peroxidase are present in cotton root and hypocotyl tissues, that the enzymes occur in distinct locations (PPOs in the thylakoids of plastids and peroxidases in the cell walls) and that in the root, the presence of PPO is correlated with phenolic synthesis. The failure of Veech to detect PPO could result from its relatively low concentration or because it is bound to the plastid thylakoids (Mueller and Beckman, 1978).

Thus a possible reason for detecting low concentrations of PPO in the sorghum extracts in this study could be due to the extraction methods being ineffective in isolating plastids which contain the thylakoids to which the PPO is bound. Further investigation, utilizing the staining techniques described above, might provide further information on the presence of PPO in sorghum.

However, peroxidase activity can also be distinguished from PPO in enzyme extracts, on the basis that addition of H_2O_2 is required for peroxidase activity, but not for PPO activity. On this basis, when the sorghum plant was investigated for peroxidase activity, relatively high amounts were detected in crude extracts. The results of the extraction of peroxidases are given below.

2.3.4. Extraction of sorghum peroxidase

2.3.4.1. Extraction of peroxidase from grain tissue

Peroxidase was extracted, using sodium phosphate buffer, from young grain that was just emerging, and from ripe grain in order to study the effect of ripening on peroxidase activity (Section 2.2.3.3.1). Young grain was harvested from 3 month old sorghum plants and was green in colour. Ripe grain was harvested from 5 month old plants and was golden brown in colour.

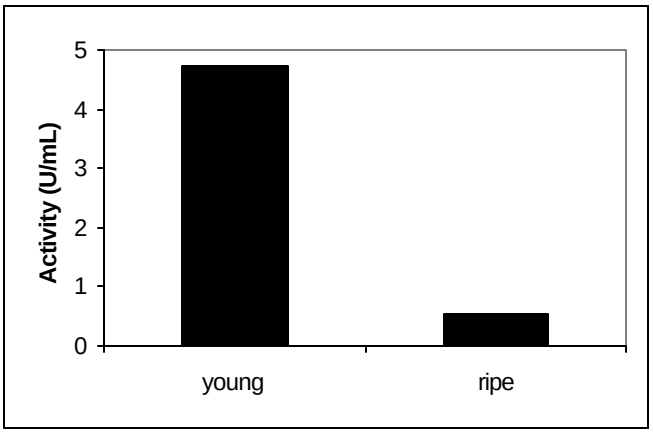


Figure 2.1. Peroxidase activity in grain tissue extracts. Units (U) of activity = :mol product produced per min per mL of enzyme extract. (The values are the average of duplicate experiments).

The results shown in Figure 2.1 indicated that the young grain had more activity (4.63 U/mL) than ripe grain (0.62 U/mL). Ricardo (1998) have shown that different peroxidase groups show differential patterns of activity during vegetative development and peroxidases have been implicated in plant development in numerous studies. Thus, the results obtained in this study are in accordance with this suggestion, since peroxidase activity was found to be most evident in young grain. The results from polyacrylamide gel electrophoresis (PAGE) analysis (section 3.2.4) of partially purified peroxidase from grain tissue are shown in Figure 3.5 (section 3.3.3.3) and confirm these results.

2.3.4.2. Extraction of peroxidase from leaf tissue

2.3.4.2.1. Weekly extraction of peroxidase from leaf tissue

Leaf samples were harvested weekly, extracted (Section 2.2.3.3.2) and the extracts were assayed for peroxidase activity. Figure 2.2 shows that leaves of different ages had different levels of peroxidase activity. Of the 16 weekly samples studied, the highest activity (6.5 U/mL) was found in 13 week old

leaves. The PAGE analysis (Section 3.2.4) of grain tissue extracts are shown in figure 3.3 (Section 3.3.3.1).

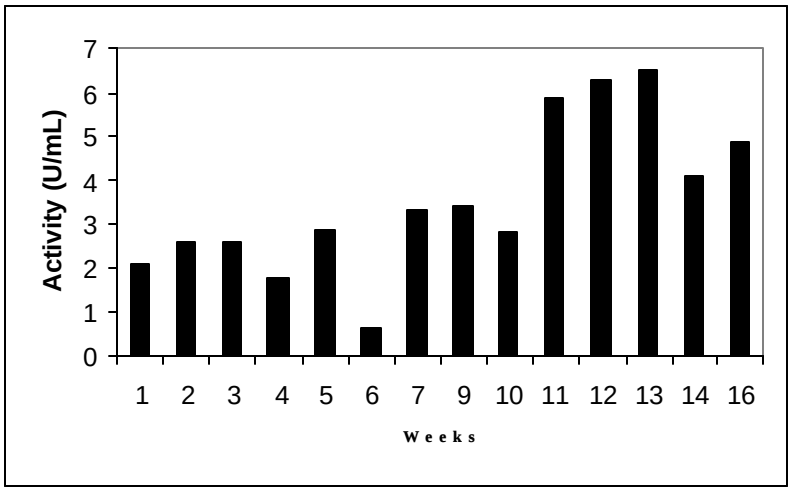


Figure 2.2. Peroxidase activity in weekly leaf samples. (The values are the average of duplicate experiments).

Peroxidases in plant tissues exist as groups of isoenzymes which can be separated and detected on gels after electrophoresis (Robinson, 1991). In the living plant the pH of cell walls may be modified from pH 4.5 to 7.5 during growth and this will clearly affect the binding of acidic isoperoxidases. The binding equilibrium as influenced by pH has been suggested to act as a control of both peroxidase activity and hydrogen peroxide production by peroxidases from NADH (Pedreno *et al.*, 1989). Thus protons may modulate the binding of acidic isoperoxi dases to cell walls and therefore the level of enzyme in the intercellular space as well as the pH-dependent activity of the enzyme. Significant changes in the Ca^{2+}:H^{+} ratio in cell walls would induce modifications of the cell wall pH and hence peroxidase activity (Ros Barcelo *et al*., 1988*b).* Thus, in this study, it is suggested that the varying levels of peroxidase activity in the leaves in sorghum also may depend on the pH of the cell walls which is modified at a specific stage of development, depending on when peroxidase is required in the plant. Assuming that sorghum peroxidase

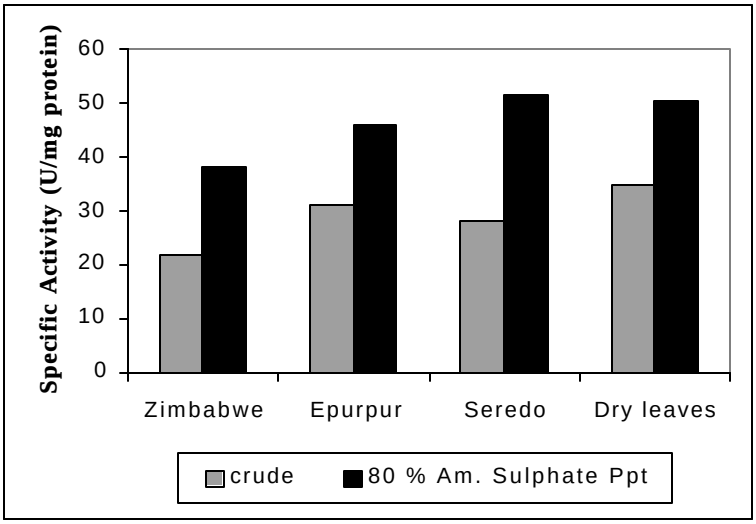
is acidic, it is possible that at 13 weeks, the pH of the cell walls was acidic (pH between 4.5 and 5.5) because, when the sodium phosphate buffer (pH 6, therefore more basic than the cell walls) was introduced during extraction, the enzyme was released, hence the high levels of peroxidase activity at 13 weeks.

2.3.4.2.2. Isolation of leaf peroxidase with ammonium sulphate

McLellan and Robinson (1981) suggested that peroxidases can be extracted from the pellet using high molarity salt solutions. Salts have the ability to trap water and expose the hydrophobic areas on the surface of enzyme molecules. The hydrophobic interaction between enzyme molecules results in aggregation, and consequently, in their precipitation. Salting out depends on hydrophobic interaction, alteration of pH or ionic strength, polarity and temperature (Ballesteros *et al.* 1994).

In this experiment, fresh leaf tissue from different *Sorghum bicolor* strains (Zimbabwe, Epurpur and Seredo) and dry leaves were studied (Section 2.2.3.3.2). The specific activities (the calculation shown in Appendix 2) of the samples were compared in order to determine whether peroxidase activity is dependent on strain, and dry leaves were studied to investigate latent peroxidase activity after plant death.

A high ammonium sulphate concentration seemed appropriate, initially, because previous experiments with leaf tissue showed that high salt buffers (Section 2.3.2.2) yielded high levels of enzyme activity. The 80% ammonium sulphate precipitation proved to be an effective way of partially isolating the enzyme. Figure 2.3 shows an increase in the specific activity of peroxidase after 80% ammonium sulphate was added to the crude extract. The results obtained from different strains of *Sorghum bicolor* indicated that peroxidase activity is not strain-specific, since similar levels of peroxidase occurred in each strain. Seredo peroxidase showed the highest specific activity in the 80% precipitation (57.7 U/mg) while the enzyme from the Zimbabwe strain had the lowest specific activity (38.4 U/mg). It is interesting to note that the dry leaves gave a high specific activity of 50.3 U/mg of peroxidase in the 80% ammonium sulphate precipitate. Results of PAGE analysis (Section 3.2.4) are shown in Figure 3.4 (Section 3.3.3.2).



Fresh and dried sorghum leaves

Figure 2.3. Specific activity of peroxidase in leaf extracts and driedleaves. (The values are the averages of duplicate experiments).

2.3.4.2.3. Isolation of Leaf Peroxidase Using the Aqueous Two-Phase System (ATPS)

Water soluble high molecular weight organic polymers, of which polyethylene glycol (PEG) is the most widely used, can be used for the precipitation of enzymes. These have the advantages of being non-toxic, non-inflammable and non-denaturing to proteins. The mechanism of precipitation is not fully explained but it is suggested that the polymers exclude the proteins from part of the solution and reduce the effective amount of water available for their solvation. In precipitation of enzymes with PEG, phase separation can occur, with one of the phases containing the enzyme (Ballesteros *et al.*, 1994).

The aqueous two-phase system (ATPS) separates enzymes and other biopolymers, as well as cell debris, according to their size, charge and surface characteristics. At low concentrations of PEG and $(NH_4)_2SO_4$, homogeneous solutions are obtained but at discrete concentration ratios, phase separation occurs, giving a PEG-rich upper phase and a high-salt lower phase. Each phase contains a high percentage of water,

forming a very favourable environment for biologically active proteins and even cell organelles. Partition of a compound between two phases is described by the relationship $K = C_u/C_i$, where K is the partition coefficient and C_u and C_i are the concentrations of the compounds at equilibrium in the upper and lower phases respectively. The location of a particular compound in different phases depends on nature, concentration and molecular weight of polymers, temperature, pH, ionic strength of the mixture, and the presence of polyvalent salts.

In this experiment, 19 day old leaf tissue (Zimbabwe strain) was homogenized in distilled water. Using the ATPS developed by Srivinas*et al.* (1999), the formation of two phases resulted (section 2.2.3.3.2-method 2). The lower, dark green, salt-rich phase contained a much higher peroxidase activity (4.44 U/mL) than the top, clear phase (0.89 U/mL) (calculation of enzyme activity as in Appendix 2). The salt rich layer was dialysed against distilled H_2O to remove salts and then concentrated by dialysis against 25 % PEG. This concentrated sample (the crude extract) was centrifuged (10 000 x*g*, 10 min, RT) and the supernatant was subjected to chromatography using Sephadex G-75. The degree of purification using the ATPS procedure is summarised in Table 2.6. (The equations used for the data presented in Table 2.6 are explained in Appendix 2).

The product after chromatography on Sephadex (G-75) showed a 1.5 fold purification, with a high specific activity of 70.3 U/mg. This method of purification proved to be more effective than ammonium sulphate precipitation which resulted in a specific activity of 38.4 U/mg for the Zimbabwe strain. Two important advantages of using ATPS over ammonium sulphate precipitation are that it is faster and each phase in ATPS contains a high percentage of water, forming a very favourable environment for enzymes.

Table 2.6. Purification of sorghum leaf peroxidase using the aqueous two phase system and chromatography

	Crude extract	***Product after chromatography***
Volume (mL)	6.2	2
Protein (mg/mL)	0.09	0.05
Activity (∶mol/min/mL)	4.7	3.32
Specific Activity (∶mol/min/mg)	47.5	70.3
Total Activity (∶mol/min)	29.3	6.65
Total Protein (mg)	0.62	0.09
Yield (%)	100	22.7
Relative Purification	1	1.5

The values shown are averages of the duplicate experiments.

2.3.4.3. Extraction of peroxidase from roots

Levels of peroxidase activity are subject to vegetative development (Jackson and Ricardo, 1998) and enzymes occur in distinct locations (Mueller and Beckman, 1978), and, therefore, root tissue was investigated for peroxidase activity.

2.3.4.3.1. Extraction of root peroxidase using different extraction buffers

Optically clear solutions containing peroxidase activity are generally obtained after relatively low-speed centrifugation (e.g. 800 *g*) (Robinson, 1991). Such a solution is normally designated the soluble or cytoplasmic peroxidase fraction, although the solution may still contain suspended intracellular membranes with bound peroxidases. However, for a complete extraction of peroxidase activity a high ionic strength is needed to solubilize ionically bound isoperoxidases (Robinson, 1981). The *800-g* pellet consists mainly of cell walls, possibly some large vacuoles, a few nuclei and partially broken cells. After extensive washing of the pellet the remaining ionically bound peroxidases can be extracted from the insoluble pellet using 1 M salt solutions (McLellan and Robinson, 1981; Moulding *et al*., 1987). These ionically bound

isoperoxidases are often of the cationic type and rarely contain a substantial quantity of anionic isoenzymes (Thomas *et al.*, 1981; McLellan and Robinson, 1983; Moulding *et al.*, 1987). This simple fractionation procedure has proved valuable for the rapid separation mainly of the cationic peroxidases which seem to be ionically bound to the crude cell wall material.

Both the anionic and cationic isoperoxidases are present in the soluble reaction, which would be expected to contain suspended intracellular endoplasmic reticulum Golgi membranes and microsomes. However, it is possible that the cationic peroxidases are not only located in the cell wall, since binding may arise during homogenization and their apparent location may then be an artefact of cell breakage (Schloss *et al.*, 1987; Goldberg *et al.*, 1989).

Binding of isoperoxidases to cell walls and other membranes may be influenced by the ionic strength and pH value of the extracting buffer, the isoelectric points of isoperoxidases and charged groups present in cell walls. For acidic isoperoxidases Ros Barcelo *et al.* (1988*b*) have shown that low pH (4.0 - 5.0) favours binding to cell walls while a neutral pH favours release. At neutral pH both the cell walls and the acidic isoperoxidases are negatively charged and therefore interactions are prevented. However, under these conditions the cationic isoperoxidases would be positively charged and therefore would be expected to bind to cell wall fragments during extraction.

Sorghum peroxidase was homogenized with a variety of buffers in order to determine the effect of reagents during extraction. These buffers were sodium phosphate buffer (pH 6), MES buffer (pH 5.5 and MES buffer pH 5.5 + calcium chloride. Distilled water was used as a control medium (Section 2.2.3.3.4). Figure 2.4 shows the results obtained; 2.67 U/mL of peroxidase in the distilled water extract, 3.82 U/mL in the sodium phosphate buffer (pH 6) extract, 7.23 U/mL in the MES buffer, pH 5.5 extract and 7.8 U/mL in MES buffer, pH 5.5 + calcium chloride extract.

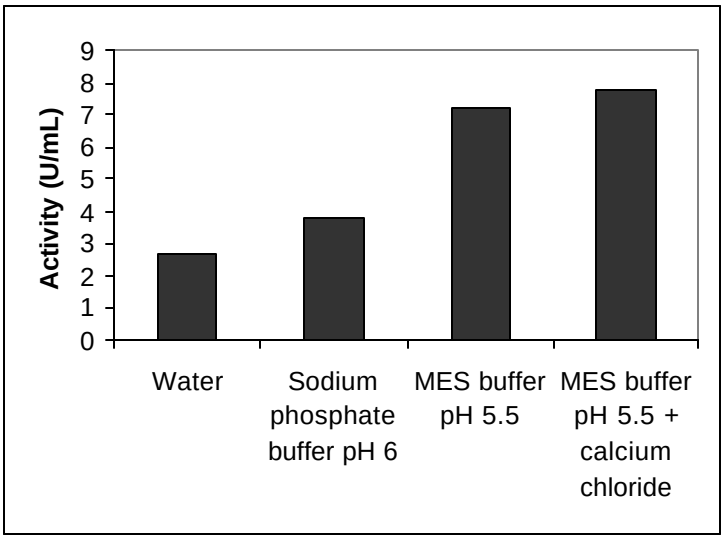


Figure 2.4. Extraction of peroxidase from roots using different extraction buffers. (Values shown are the average of duplicate expriments).

In the present study, as expected, distilled water was a relatively ineffective extraction medium, having a negligible ionic charge. However, it is more feasible that the pH, and not the ionic charge of the water contributed to this ineffectiveness, if it assumed that the peroxidase is cationic and therefore remained bound to cell wall fragments at a neutral pH during extraction (Ros Barceló *et al.* 1988*b*). Low pH values (4.0 - 5.0) were expected to favour the release of cationic isoperoxidases. Thus, MES buffer (pH 5.5) was shown to be an effective extraction medium for the release of peroxidase. However, further experimentation needs to be conducted in order to confirm whether the isolated sorghum peroxidase is cationic or indeed, if both cationic and anionic isoperoxidases are present.

When $CaCl_2$ was added to MES buffer at pH 5.5, there was only an increase of 0.52 U/mL in the peroxidase activity extracted. A higher increase of peroxidase activity was expected with the addition of calcium chloride because calcium appears to enhance secretion in the plant cells. Robinson (1991) and

other workers have confirmed that it intensifies release of the enzyme in culture cells (Bakarjieva*et al*, 1987; Welinder, 1985). Welinder (1985) suggests that for horse radish peroxidase (HRP), because calcium is an integral part of the molecule, it affects the conformational structure of the protein close to the haem group. It is not clear why only a slight increase in sorghum peroxidase activity occurred when calcium was added to the extract. It is possible, but perhaps unlikely, that calcium is not an integral part of this particular enzyme. The calcium induced increase in peroxidase activity in plants has also been attributed to an increase in the activity of the cationic isoperoxidases initiated by stimuli-induced release of potassium (Gaspar*et al*, 1985). Further experimentation with potassium could increase calcium-induced peroxidase activity in this study. It is also possible that the extraction was already effective even without addition of calcium.

When the different buffers were used for extraction of peroxidase from root tissue, the lag phase in the assay (the period from the time of addition of the enzyme extract to the substrate till a colorimetric change is detected) differed for each buffer (Figure 2.5). A possible explanation for this is the occurrence of chemical interactions between the enzyme and reagents, perhaps rendering the active site inaccessible for a period due to steric hindrance.

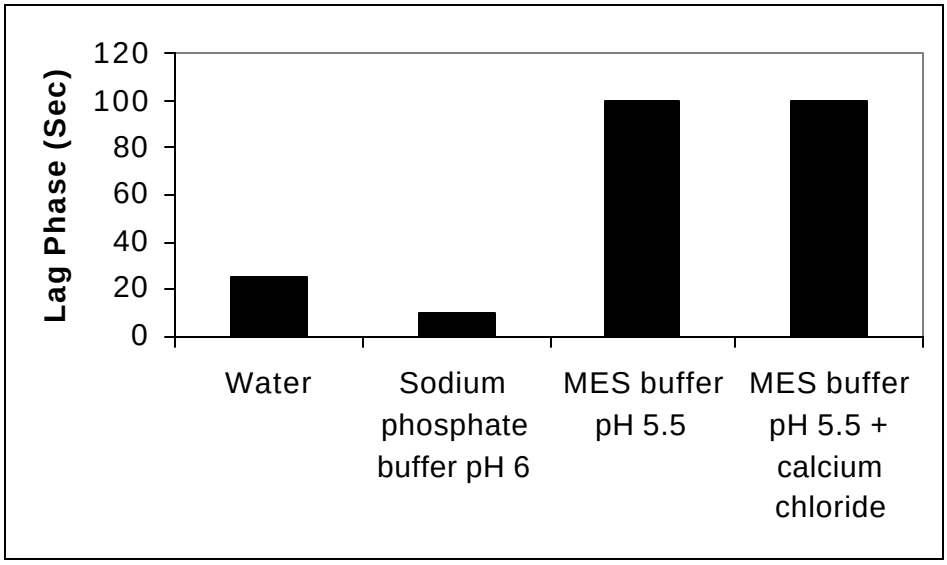


Figure 2.5. Lag phase in assay for sorghum peroxidase extracted using different extraction buffers. (Values are averages of duplicate experiments).

There is a need for further investigation as to what causes the change in lag phases when different extraction buffers are used. Such investigation would warrant experimentation with purified enzyme, and hence the purification of sorghum peroxidase from root tissue has been studied, as described in Chapter 3.

2.4. CONCLUSION

In this chapter, a survey was made of proteases, polyphenol oxidases and peroxidases in *Sorghum bicolor*. Sprouted seeds, leaf tissue and grain tissue were extracted and studied for the presence of these enzymes. Generally, extraction techniques involved homogenization in the relevant buffer using a pestle and mortar or a Waring blendor. Homogenization with a Waring blendor yielded better results than extraction with a pestle and mortar. The crude extract was then filtered through cheese cloth and assayed for enzyme activity and protein content and also subjected to polyacrylamide gel electrophoresis (PAGE).

Enzyme assay methods involved spectrophotometric analysis with the relevant substrate for each enzyme. Protein assays were conducted according to Bradford's method (Bradford, 1976). PAGE analysis of the extracts was conducted according to Laemmli's method (1970) (section 3.2.4) and the results are given in the following chapter.

Protease assays involved the hydrolyzation of azocasein by the protease to yield the colored complex, azo dye and casein. A high amount of azo dye after the reaction, detected spectrophotometrically, indicated protease activity. According to the spectrophotometric analysis, proteases were found to be present at very low concentrations in leaf tissue and to be absent in young and ripe grain extracts. Using the same assay method, two commercial proteases, proteinase K and trypsin showed evidence of much higher protease activity. Protease concentrations in sorghum were probably too low to elicit a significant reaction or proteases could be produced only at specific developmental stages.

Polyphenol oxidase (PPO) was extracted in sodium phosphate buffer using a pestle and mortar, and detected by spectrophotometrically monitoring its oxidation of L-DOPA, caffeic acid and 4-methylcatechol. Interestingly, PPOs did not feature as prominently as expected. *Sorghum bicolor* contains phenolics and PPO is thought to be involved in their synthesis (Mayer and Harel, 1979). Low levels of PPO activity (0.0014 U/mL for caffeic acid in the Zimbabwe extract; 0.0019 U/mL for 4-methyl catechol in the

Zimbabwe extract; 0.002 U/mL for 4-methylcatechol for the Epurpur extract) were detected in sprouted seeds and no PPO activity was evident in grain extract. Although a higher level of PPO was found in leaf tissue (0.127 U/mL in the sodium phosphate buffer extract; 0.026 U/mL in the high salt buffer extract; 0.014 U/mL in the high sucrose buffer extract), these levels were not sufficiently high to justify consideration of its use in biotransformation applications.

Peroxidase was extracted from grain and leaf tissue, in sodium phosphate buffer, using a pestle and mortar. An alternative method of extraction of leaf tissue involved using the aqueous two-phase system (ATPS) after homogenization with a Waring blender. Extraction of root tissue involved using two methods; homogenization in MES buffer (pH 5.5) with a Waring blender and homogenization with a pestle and mortar in sodium phosphate buffer, MES buffer (pH 5.5), MES buffer (pH 5.5) + calcium chloride, and distilled water. Peroxidase activity was detected by spectrophotometrically monitoring its oxidation of *o*-dianisidine in the presence of hydrogen peroxide. High levels of peroxidase activity were detected in sorghum grain (4.63 U/mL in young grain and 0.62 U/mL in ripe grain). Even higher levels of peroxidase activity were detected in leaf (6.5 U/mL in the sodium phosphate buffer extract; 4.7 U/mL in the ATPS extract) and, particularly, in root tissue (7.8 U/mL in MES buffer + calcium chloride extract). This led to the objective of purifying and characterizing sorghum peroxidase from the roots. The results of the purification and characterization experiments will be described in Chapter 3.

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Chapter 3
PURIFICATION AND CHARACTERIZATION OF SORGHUM ROOT PEROXIDASE

3.1. INTRODUCTION

The purification and characterization of a novel peroxidase that has been detected in *Sorghum bicolor* is described in this chapter. Horse radish peroxidase (HRP) is the most common commercial peroxidase used in industry today but it is costly, and thus a cheaper source of peroxidase from sorghum would be advantageous. Sorghum is a potential cheap source of peroxidase because it is able to grow in harsh environments, elimitating the expense of fertilizers and irrigation. Also, it is already grown as a food crop - but here, the root would be used which would otherwise wasted. Sorghum peroxidase has not been reported in the literature previously.

Plant peroxidases have very high specific activities and while it has been straightforward to detect peroxidases, it may be difficult to obtain sufficient purified enzyme to characterize the protein moiety. Some of the practical problems in the isolation of plant isoperoxidases have been mentioned by Aibara *et al*. (1982), *viz*., that purification procedures are time-consuming and frequently require expensive carrier ampholytes. Furthermore, claims of purity of enzymes depend on the adequacy and sensitivity of the methods available for detection of protein. During recent years more sensitive staining methods, using silver, have been developed to detect low levels of protein on zymograms. This method is particularly suitable for peroxidases, where either the isoenzymes or contaminating enzymes may not

stain with Coomassie Blue. Khan and Robinson (1991) have shown, for mango isoperoxidases, that contaminating proteins and the enzyme protein can only be observed after silver staining. Lagrimini *et al*. (1987) have also used silver staining to establish that a mixture of anionic tobacco isoperoxidases was free of contaminating proteins.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Materials used for the purification experiments are listed in section 2.2.1. The following chemicals were used for polyacrylamide gel electrophoresis (PAGE). Ammonium persulphate, glycerol, methanol, glycine, glacial acetic acid, hydrochloric acid and mercaptoethanol were from Unilab Saarchem (Pty) Ltd. Coomassie brilliant blue R-250 (CBB), Tris(hydroxymethyl)aminomethane), bromophenol blue, molecular weight markers (listed in appendix 3), silver stain kit, and horse radish peroxidase (HRP) were from Sigma Chemicals Co. (USA). Acrylamide was from BDH chemicals, tetramethylethylene diamine (TEMED) was purchased from Merck NT Laboratory Supplies (Pty) Ltd, and *N*,*N*-methylene-bis-acrylamide was from Boehringer Manneim. Sodium dodecyl sulphate was from Reidel-de Häen. For polyacrylamide gel electrophoresis (PAGE) experiments, the Hoefer Tall Mighty Small electrophoresis kit was used.

3.2.2. Partial purification of sorghum root extract with ammonium sulphate

Root powder was extracted using 100 mL 0.1 M sodium phosphate buffer (1g / 5mL), pH 6.8, using a pestle and mortar, the homogenate was filtered through cheese cloth, assayed for enzymatic activity and centrifuged (10 000 x *g*, 15 min, 0°C). The supernatant was assayed for peroxidase activity (Section 2.2.2.3 - method 1) before ammonium sulphate precipitation (Section 2.2.3.3.3 - method 2).

3.2.3. Purification of sorghum root peroxidase with alternative methods

Plant tissue was ground to a powder using a Waring blender. The powder (100 g) was homogenized by stirring in 250 mL 50 mM MES buffer, pH 5.5 containing 1 M NaCl, 30 mM ascorbic acid, and 1 mM EDTA. The homogenate was filtered through two layers of cheese cloth and centrifuged (10 000 x *g*, 30 min, 0°C). The supernatant (crude extract) was assayed for peroxidase activity (Section

2.2.2.3 - method 1) and protein content (Section 2.2.2.4). The first purification step involved blending 10% polyvinylpolyprrolidone (PVPP) in the crude extract using a Waring blendor operated at maximum speed for 1 minute. This mixture was centrifuged (10 000 x *g*, 30 min, 0°C). The supernatant was dialysed against a 25 mM malonate solution, pH 5.5, containing 10 mM of ascorbic acid and centrifuged (10 000 x *g*, 10 min, 0°C) to remove the solids. The supernatant was analysed by (PAGE) (Section 3.2.4), assayed for peroxidase activity and protein content, and ultrafiltered through a cellulose Molecular/Por filtration membrane (MWCO 10,000) at a pressure of 3000 Atm in a Molecular/Por Stirred Cell. A portion of the filtrate was used for to PAGE analysis (Section 3.2.4). Chromatography on phenyl Sepharose was used as the final purification step. The column was equilibrated with 0.1 M Na sodium buffer, pH 6, and the sample was applied and eluted in distilled water. The eluate was assayed for peroxidase activity (Section 2.2.2.3 - method 1) and a sample was analysed by PAGE (Section 3.2.4).

3.2.4. Polyacrylamide gel electrophoresis (PAGE) analysis

Polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). The Tall Mighty Small electrophoresis kit was set up according to the Hoefer Scientific Instruments manual (Catalog No. SE 280).

The denaturing polyacrylamide gel was prepared in the following manner:- the resolving part of the gel contained 13.5 mL 30% of acrylamide stock (2 g bis-acrylamide, 75 g acrylamide and 250 mL dH_2O), 15 mL of 1 M Tris-HCl pH 8.8, 9.25 mL dH_2O, 0.4 mL 10% sodium dodecyl sulphate (SDS), 0.3 mL 10% ammonium persulphate (APS) and 20 µL tetramethylethylene diamine (TEMED); the stacking part of the gel contained 2.0 mL acrylamide stock, 1.9 mL 1 M tris-HCl buffer pH 6.8, 9.25 mL dH_2O, 1.0 mL 80 % glycerol, 0.15 mL 10% SDS, 0.10 mL 10% APS and 20 µL TEMED. The non-denaturing gel was prepared in the same manner, except it did not contain 10% SDS.

Enzyme samples (30 µL) were denatured by boiling in eppendorf tubes for 5 min in 15 µL of dissociation buffer which contained 5 g (10%) SDS, 5 mL mercaptoethanol, 7.5 mL glycerol, 2.5 mL bromophenol blue, 6.3 mL 1 M Tris-HCl buffer, pH 6.8 and 28.7 mL dH_2O. 30 µL of the denatured

enzyme samples were loaded into the denaturing gels and electrophoresed, using bath buffer (10 times diluted stock of 30.3g Tris, 144.1 g glycine, 10.0g SDS and 1 L dH_2O) as a medium, for 3 hours at 100 V. 30 :l of undenatured enzyme samples (same enzyme run in denaturing gel but unboiled) (30 :L) were loaded into non-denaturing gels and subjected to the same electrophoretic conditions. Protein marker solution, containing proteins with known molecular weights (5 :L), was applied to the gel and electrophoresed along with the enzyme samples in order to determine the molecular weight of the enzymes.

After electrophoresis, the gels were soaked in Coomassie Brilliant Blue (CBB) staining solution which contained 45 mL methanol, 10 mL glacial acetic acid, 45 mL dH_2O and 0,2 g Coomassie Brilliant Blue R-250. This was done in order to stain all proteins with CBB. These gels were called the CBB gels. Duplicate gels (non denaturing and denaturing gels containing unboiled enzyme samples identical to the boiled ones) were soaked in a dilute substrate solution (100 :L of 50 mM *o*-dianisidine in 30 mL H_2O containing 50 :L H_2O_2), to detect the colour reaction between the peroxidase enzyme and substrate, at the position where the enzyme migrated. These gels were called the activity gels. The gels were photographed and analysed to determine the molecular weights of the enzyme. Gels were also stained for protein detection using the silver stain method as described in the silver stain kit booklet supplied by Sigma Chemicals Co. (USA).

3.2.5. Characterization experiments

3.2.5.1. Assay optimization

3.2.5.1.1. Temperature dependence of sorghum root peroxidase

Experiments to determine the optimal temperature for sorghum root peroxidase activity involved measuring the activity at varying temperatures (in duplicate). The assay mixture containing *o*-dianisidine as substrate, with H_2O_2, in sodium phosphate buffer (Section 2.2.2.3 - method 1), was adjusted to the relevant temperature, and the enzyme solution was added to initiate the reaction. Temperatures ranged from 20 - 60°C and the pH used was 6.

3.2.5.1.2. Thermostability of sorghum root peroxidase

Enzyme samples were incubated at the relevant temperature for a specific period in a water bath. The temperatures ranged from 40 - 100°C and peroxidase activity (Section 2.2.2.3 - method 1) was monitored hourly for a total of six hours. At 100°C, peroxidase activity was monitored every 2 minutes for a total of 8 minutes. The extract was removed from the water bath and allowed to equilibrate to room temperature for 15 minutes before assaying for activity.

3.2.5.1.3. Effect of hydrogen peroxide concentration on sorghum root peroxidase

A series of 0.1 M Na phosphate buffer solutions of pH 6 were prepared containing varying concentrations of hydrogen peroxide ranging from 0.005 to 0.9 M. Peroxidase assays (Section 2.2.2.3 - method 1) at room temperature.

3.2.5.1.4. Optimal substrate concentration for sorghum root peroxidase

This experiment involved assaying for peroxidase activity using different substrate (*o*-dianisidine) concentrations, according to the first method described in Section 2.2.2.3, at room temperature and pH 6. The substrate concentration ranged from 10 - 150 mM.

3.2.5.1.5. Substrate range for assay of sorghum root peroxidase

Assays were carried out using other substrates to determine the substrate range of sorghum peroxidase. The other substrates used to measure the activity of sorghum peroxidase were guaiacol, 2,4-

dichlorophenol and 2.2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).
The assay methods are described in Section 2.2.2.3 - methods 2-3.

3.2.5.1.6. Effect of $CaCl_2$ on sorghum root peroxidase

This experiment involved adding 10 µL of the appropriate $CaCl_2$ concentration to 100 µL samples of enzyme extracts and proceeding with the assay procedure described in method 1 of Section 2.2.2.3. The $CaCl_2$ concentration ranged from 0.001 to 10 M.

3.2.5.1.7. Optimal pH of sorghum root peroxidase

A series of 0.1 M sodium phosphate buffers with pH values ranging from 3 to 8 were prepared. The assay mixture was prepared using sodium phosphate buffer and *o*-dianisidine as substrate, at room temperature, and enzyme extract was added to initiate the reaction (Section 2.2.2.3 - method 1).

3.3. RESULTS AND DISCUSSION

The initial investigation to purify sorghum root peroxidase involved ammonium sulphate fractionation, then alternative methods of purification were employed. The resulting fractions were assayed for enzyme activity and protein content.

3.3.1. Purification of sorhum root peroxidase ammonium sulphate

Ammonium sulphate fractionation was used initially, in attempting to isolate sorghum root peroxidase. This involved homogenisation of the root powder in sodium phosphate buffer followed by the addition of ammonium sulphate, in increments ranging from 20 - 70 % (w/v), to the extract (Section 3.2.2 (method 1)). The results are shown in figure 3.1.

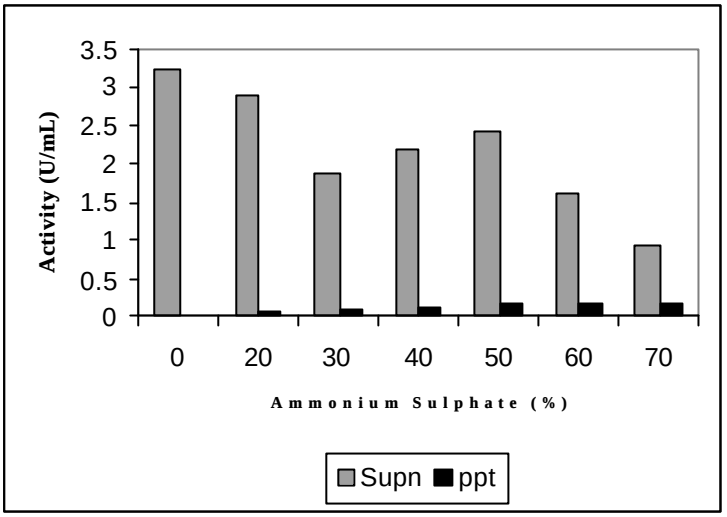


Figure 3.1. Activity of sorghum root peroxidase in ammonium sulfate precipitation fractions. (The values are averages of duplicate experiments).

Although an expected trend was noted, namely, a decrease of peroxidase activity in the supernatant fraction and an increase of activity in the precipitate fraction with increasing ammonium sulphate precipitation, there was no clear separation of peroxidase activity in one fraction. Thus, ammonium sulphate precipitation was not an effective means of isolating peroxidase from the root extract. The highest enzyme activity (0.17 U/mL) (calculations of enzyme activity are given in Appendix 3) in the precipitate was seen when ammonium sulphate was at 60% saturation. However, this activity was very low compared to the activity in the corresponding supernatant fraction (1.61 U/mL) at that ammonium sulphate saturation level. The gradual decrease in the activity of the supernatant could have been due to denaturation of the enzyme with increasing salt concentration or due to dilution during the dialysis step. Although there was a gradual increase in activity in the precipitate fractions, the values were too low to conclude that the enzyme was precipitated out of solution.

3.3.2. Partial purification using alternative methods

An alternative method for proxidase isolation was adapted from that of Kvaratskhelia *et al*. (1997) (Section 3.2.2 - method 2). This involved homogenisation using MES buffer containing sodium chloride, ascorbic acid and EDTA, PVPP treatment, dialysis and ultrafiltration, followed by

hydrophobic chromatography using phenyl Sepharose Cl-4B. Table 3.1 describes the successive purification steps. (The equations used for the calculation of enzyme activity units and the data presented in Table 3.1 are in Appendix 2).

After centrifugation of the crude extract, a 1.7-fold purification resulted, while the PVPP purification step gave a 2.3-fold purification, indicating the removal of interfering phenolic substances. Ultrafiltration separates macromolecules in the molecular weight range from 1000 to about 10 000. Ultrafiltration effected a 3-fold purification. The filtrate was clear, compared to the retenate which was orange-coloured and which became more intensely coloured when concentrated.

Chromatography on phenyl Sepharose CL-4B column gave a *ca*. 2-fold increase in specific activity but with the loss of *ca*. 75% of the total activity (Table 3.1). This process involves the separation of enzymes according to their size. Sepharose Cl-4B is a rigid, cross-linked agarose material. The separation depends on the different abilities of the particular protein molecules to enter the pores within the gel beads. Large molecules which cannot enter even the largest pores, pass through the column faster. Molecules are eluted in order of decreasing size.

Table 3.1 Partial purification of sorghum root peroxidase

Fraction	Crude	Centrifuged	PVPP*	Ultrafiltration	Chromatography
Vol (mL)	210	200	100	14	5
Protein (mg/mL)	0.053	0.029	0.025	0.079	0.034
Activity (:mols/min/mL)	1.24	1.19	1.4	5.4	4.11
Specific Activity (:mols/min/mg)	23.3	39.9	54.3	69.3	122.3
Total Activity (:mols/min)	260.4	238.0	140.0	75.6	20.6
Total Protein (mg)	11.13	5.96	2.58	5.20	0.17
Yield (%)	100	91.4	57.3	29.0	7.9
Relative Purification	1	1.7	2.3	2.9	5.2

* PVPP - polyvinylpolypyrrolidone

All values are averages of duplicate experiments.

3.3.3. Polyacrylamide gel electrophoresis (PAGE)analysis

A range of plant isoperoxidases have been detected by PAGE and PAGE zymography (Robinson, 1991) and an attempt was made to detect sorghum root isoperoxidases using PAGE analysis. This also allowed comparison of sorghum root peroxidase with horse radish peroxidase (HRP). Gels stained in Coomassie brilliant blue (CBB gels) and in substrate solution (activity gels), were used to visualize the banding pattern of the enzymes. PAGE gels and zymograms give only approximate indications of molecular weight, but are nonetheless useful for the sake of comparison.

3.3.3.1. PAGE analysis of sorghum peroxidase in leaf and root tissue

In each gel equal and equal mass (30 :g) of protein was loaded. CBB gels, stained in Coomassie Brilliant Blue, and activity gels, soaked in substrate solution, (Section 3.2.4), of the leaf and root

extracts, were analysed. The leaf extract was obtained using the aqueous two-phase system (ATPS) (Section 2.2.3.3.2) and the root extract was the purified extract after the hydrophobic chromatography step (Section 3.2.3). Figure 3.2 shows two CBB gels and one activity gel of the leaf and root extracts. The CBB gel (A) is the non-denaturing gel, the CBB gel (B) is a denaturing gel and gel (C) shows enzyme activity. Lane 1 (left) contained marker proteins (Appendix 3), lane 2 contained crude leaf extract, lane 3 contained purified leaf extract and lane 4 contained purified root extract.

In the undenatured gel (A), of the crude leaf extract (lane 2) one major band, with an approximate molecular weight of 77,000, was detected. When denatured, this protein was apparently partially fragmented into two fractions, of approximate molecular weights 77,000 and 63,000 (lane 2 of Gel B). Similarly, the purified leaf extract showed a single band with approximate molecular weight of 77,000 in gel A (lane 3) and, when denatured, a band of molecular weight, approximately 50,000 was seen in the denaturing gel (B).

Lane 4, in both gels A and B, contained the root extract but no bands were seen. However, in the activity gel (C, Figure 3.2), observation of the band intensities showed that the root extract (lane 4) contained higher activity than comparable amounts of the leaf extracts (lanes 2 and 3). This also indicated that very low amounts of root peroxidase, that could not be detected in the CBB gels, contained high levels of peroxidase activity.

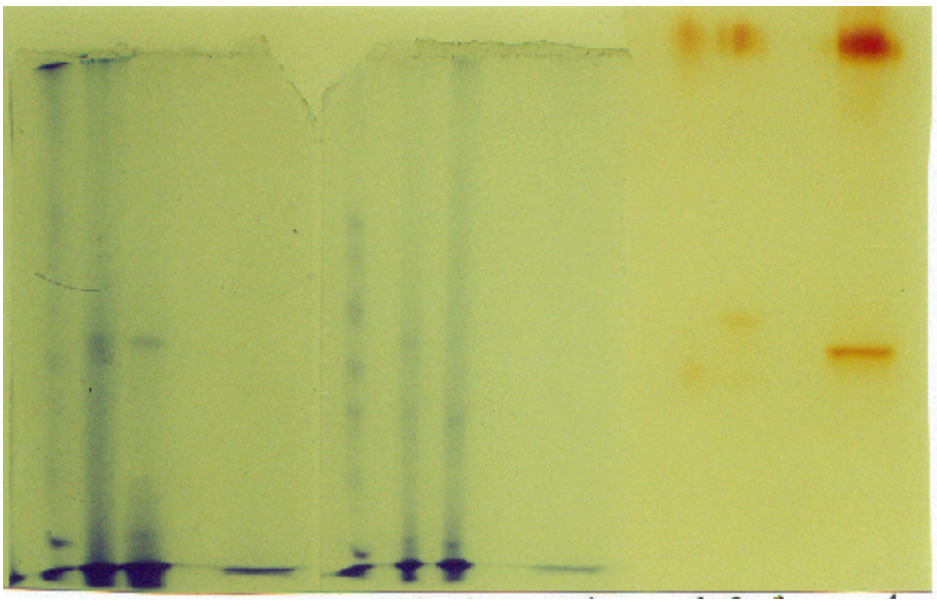
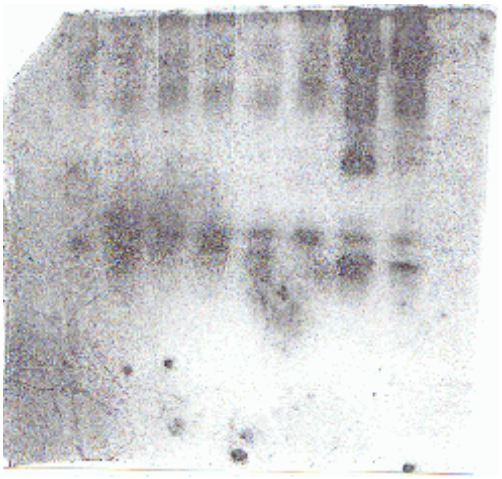


Figure 3.2. CBB gels (A and B) and an activity gel (C) of leaf and root tissue. Gel A: lane 1: marker; lane 2: undenatured crude leaf; lane 3: undenatured purified leaf; lane 4: undenatured purified root. Gel B and C: lane 1: marker; lane 2: denatured crude leaf; lane 3: denatured purified leaf; lane 4: denatured purified root. Each lane was loaded with 30 ∶g protein.

The activity gel (undenatured proteins) shown in Figure 3.3 confirms that root extract contained more peroxidase levels than leaf extract. Lanes 1 - 6 contained weekly extracts of leaf tissue (Section 2.2.3.3.2 - method 1) where leaf tissue was homogenized in buffer using a pestle and mortar. Lane 1 contained 1 week old leaf extract, lane 2, two week old leaf extract, etc. up to 6 weeks. Lane 7 contained 6 month old root extract and lane 8 contained 1 month old root extract. The root extracts (both 1 and 6 month old) appeared to have more peroxidase activity, since they gave a higher colour intensity than the leaf extracts. From the band patterns in lane 7 and 8, it appears that peroxidase in root extract of 6 months is different from that in root extract of 1 month, confirming that the development of peroxidase activity depends on age of plant tissue. The 6 month old root extract showed higher activity than 1 month old extract, for the same amounts (30 ∶g) of protein loaded on

the gels.

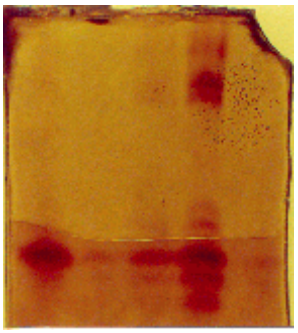


1 2 3 4 5 6 7 8

Figure 3.3. Activity gel of weekly leaf extracts (lanes 1 - 6) and 6 month old root extract (lane 7) and one month old root extract (lane 8). (30 ：g protein loaded in each lane).

3.3.3.2. PAGE analysis of sorghum peroxidase in leaf tissue of different strains

Prior to the demonstration of high sorghum peroxidase activity in root tissue, the leaf tissue of different sorghum strains (Zimbabwe, Seredo and Epurpur) was subjected to PAGE to compare banding patterns for the different strains. Leaf tissue was obtained by homogenization with a pestle and mortar in buffer. The activity gel shown in Figure 3.4 showed that different sorghum strains displayed different peroxidase activity band patterns. The Zimbabwe strain gave 1 band which appears to have the highest peroxidase activity, judging visually from its intensity. The 45 day old Zimbabwe strain had the same band but at a lower intensity, indicating that younger leaf tissue has higher peroxidase activity. The Epurpur strain had three bands, suggesting the presence of three isoenzymes. The Seredo strain had 6 bands, suggesting the presence of several isoenzymes. The dried leaf extract showed 1 band at the same position as the 4th band of the Seredo extract but at a lower intensity. This experiment shows that sorghum peroxidase is strain dependent, with Seredo and Zimbabwe showing the highest levels of activity.



1 2 3 4 5

Figure 3.4. Activity gel of leaf peroxidase in different sorghum strains. Lane 1: Zimbabwe (25 days); lane 2: Zimbabwe (45 days); lane 3: Epurpur (cream coloured); lane 4: Seredo (brown coloured); lane 5: dried leaves. (30 ：g protein loaded in each well).

3.3.3.3. PAGE analysis of sorghum root peroxidase in grain tissue

Peroxidase activity appears to depend on the vegetative development of the plant, thus, peroxidase activity in grain tissue was studied. Grain tissue (young and ripe) was homogenized in buffer using a pestle and mortar. 30 ：g of each extract was loaded in the gel which was soaked in substrate (*o*-dianinsidine). 30 ：g of horse radish peroxidase (HRP) was loaded in the gel for comparison with grain peroxidase. Figure 3.5 shows the results. Lane 1 contained the HRP, lane 2 contained the young grain extract and lane 3 contained the ripe grain extract. The commercial HRP showed much higher levels of peroxidase actvity than the grain extracts. Judging from the band intensity, young grain appeared to have higher levels of activity than ripe grain.

1 2 3

Figure 3.5. An activity gel showing a comparison of peroxidase activity between commercial HRP (lane 1) and grain tissue (lane 2: young grain; lane 3: ripe grain). (30 μg of protein loaded in each lane).

3.3.3.4. A comparison of HRP and sorghum root peroxidase using PAGE analysis

Figure 3.6. shows a denaturing CBB gel and an activity gel stained with *o*-dianisidine (Section 3.2.1) of commercial HRP and sorghum root extracts (Section 3.2.3). The CBB gel (A) contained the following extracts: lane 1 - crude root extract; lane 2 - freeze dried root extract after the ultrafiltration step (Section 3.2.3); lane 3 - freeze dried root extract after the phenyl sepharose step (Section 3.2.3); lane 4 - commercial HRP; lane 5 - marker proteins (Appendix 3). The activity gel (B) contained the same extracts, loaded in the same pattern as extracts in gel A, except marker proteins.

The results showed that the crude root extract contains 6 bands corresponding to molecular weights of 33 000, 34 000, 38 000, 44 000, 54 000 and 97 000. Lane 2 had no visible bands, while lane 4 (the phenyl sepharose-purified root extract) had one band corresponding to a molecular weight of 35 000. Although overloaded, the HRP (lane 4) showed a molecular weight of 44 000 which is in accordance with the literature (Welinder, 1979). The molecular weight of the other HRP component was found to be 64 000. Thus the apparant molecular weight of the sorghum root peroxidase fraction obtained by the purification procedure is 35 000. However, in the crude root extract, one of the bands (3rd band from the top in lane 1) indicated a molecular weight of approximately 44 000.

Interestingly, the activity gel (B) reveals that the root extracts (lanes 2 and 3) (which are hardly noticeable in the CBB gel), appeared to contain higher levels of peroxidase activity than the commercial HRP (lane 4), judging from the intensity of the bands. It appears also, in the activity gels, that the HRP bands and root extract bands appeared in slightly different positions from each other, indicating the presence of different isoenzymes. The crude root extract (lane 1) had a very light band, indicating low levels of activity.

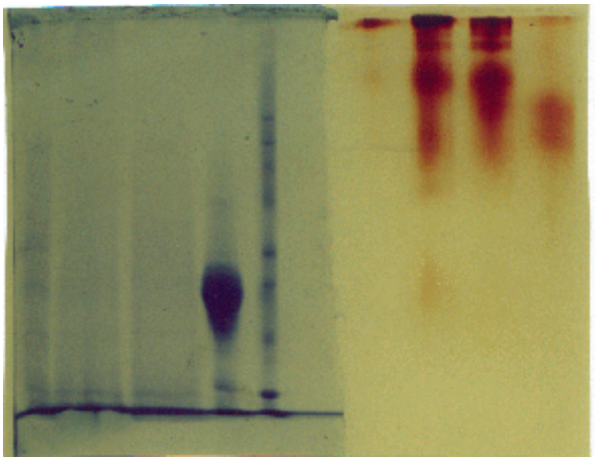


Figure 3.6. PAGE analysis: comparison between commercial HRP and sorghum root peroxidase. **Lane 1: crude root extract; lane 2: root extract after ultrafiltration; lane 3: root extract after hydrophobic chromatography; lane 4: commercial HRP.** (30 ∴ g protein loaded).

The purification of root peroxidase (Section 3.2.3) was repeated and another CBB gel was run to confirm these results (Figure 3.7), showing major bands corresponding to molecular weights of 64 000 and 44 000 for HRP (Gel B: lane 2), 46 000 for phenyl sepharose-purified root extract (Gel B: lane 3, Figure 3.7), and 64 000 for the root extract after ultrafiltration (Gel B: lane 4, Figure 3.7) which is very similar to the denatured HRP component. The crude root extract contained 5 components with molecular weights of 33 000, 34 000, 46 000, 63 000 and 64 000 (Gel B, lane 5, Figure 3.7). These

results, indicate that sorghum root peroxidase is similar to HRP in having bands corresponding to the molecular weights of 44 000 and of 64 000. The results also confirm that sorghum peroxidase is difficult to detect using the CBB staining procedure.

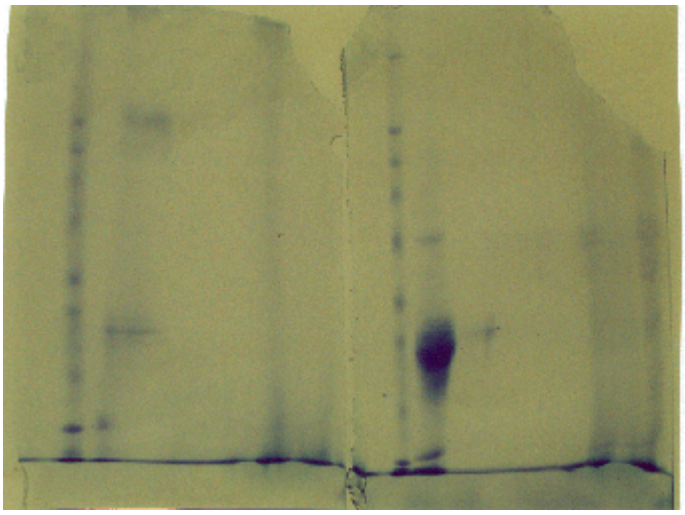


Figure 3.7. Non-denaturing (A) and denaturing (B) CBB gels showing a comparison between HRP and sorghum root peroxidase. Loading pattern in gels A and B: lane 1: marker protein; lane 2: HRP; lane 3: freezed-dried root extract after chromatography with phenyl Sepharose; lane 4: freezed-dried root extract after ultrafiltration; lane 5: crude root extract. (30 ∶g protein loaded).

Generally, peroxidases are not easily stained with CBB (Robinson, 1991), and thus, in recent years, more sensitive staining methods, using silver have been employed. In the following experiment (Figure 3.8), sorghum peroxidase (lane 1), HRP and lignin peroxidase (LiP) were electrophoresed and the gels stained with silver stain, with marker proteins in lane 4.

The same amount of protein was loaded (30 ∶g) was loaded, but since silver stain is more sensitive, the gel appeared overloaded. Nevertheless, it was observed that sorghum peroxidase (lane 1, Figure 3.8) comprised two major bands with molecular weights of approximately 30 000 and 40 000. HRP

(lane 2, Figure 3.8) contained two major bands with molecular weights of approximately 74 000 and 44 000. In the same lane, two larger bands, which were not detected in the same gel stained in CBB (Figure 3.9), were seen above the 77 000 band. This confirms that silver staining is a more sensitive method of detecting proteins in gels. Lane 3 contained commercial lignin peroxidase (LIP) which has sub-units with molecular weights of 44 000 and 50 000. This method needs refining to give improved results.

1 2 3 4

Figure 3.8. Silver stain of a denaturing gel with sorghum peroxidase (lane 1), commercial HRP (lane 2), commercial lignin peroxidase (lane 3) and marker proteins (lane 4).

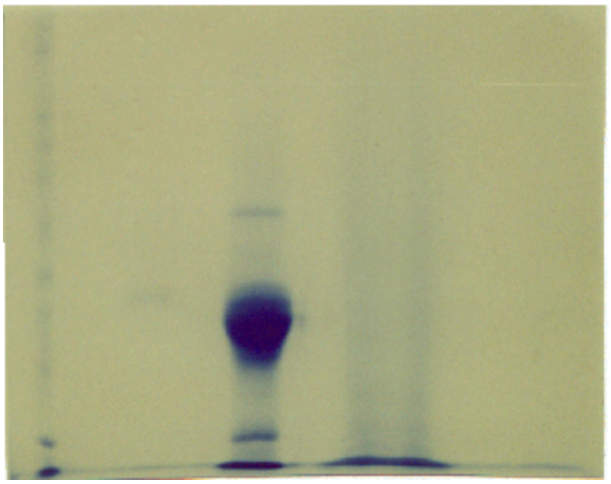


Figure 3.9. CBB stained denaturing gel with marker proteins (lane 1), commercial lignin peroxidase (lane 2), commercial HRP (lane 3) and sorghum root peroxidase (lane 4).

3.3.3.5. A comparison of HRP and sorghum root peroxidase using spectrophotometric analysis

Solutions of commercial HRP and partially purified sorghum root peroxidase, containing equal protein concentrations, were compared, using the peroxidase assay where the enzyme solution was added to the assay mixture containing, substrate (*o*-dianisidine) dissolved in sodium phosphate buffer, pH 6, containing H_2O_2, at room temperature (Section 2.2.2.3 - method 1). HRP was found to have an activity of 10.3 U/mL and sorghum root peroxidase had an activity of 3.5 U/mL.

In the assay, HRP showed no lag phase and the reaction was completed within 10 seconds while sorghum root peroxidase had a lag phase of 90 seconds, with the enzymatic conversion taking 1 minute. Thus, the HRP had apparently higher activity than sorghum root peroxidase. However, the activity gel (Figure 3.6) indicated that sorghum peroxidase had higher levels of activity than HRP. Confirmatory experiments thus need to be performed. A long lag phase is a feature common to impure enzymes. Thus it was clear that sorghum root peroxidase needed to be purified to a greater degree than it has been thus far. However it was decided that characterization was more important at that point for comparison with HRP in order to determine whether sorghum root peroxidase would be useful for biotransformations. Complete purification is not always necessary for an enzyme to be used in

biotransformation, and indeed, if this can be avoided, the process may be more economically viable.

3.3.4. Characterization of root sorghum peroxidase

An attempt was made to characterize sorghum root peroxidase for its applications in biotransformations and to determine its optimal functioning conditions during biotransformations.

3.3.4.1. Assay optimization

In industrial applications it is relevant to know the optimal conditions in which an enzyme functions. An enzyme-catalyzed conversion can be influenced by a number of factors, including the type and concentration of the participating substances, temperature, pH value and ionic strength, and natural properties of the enzyme such as its catalytic specificity or its stability under the given conditions. Peroxidases are also influenced by the amount of H_2O_2 present in the system, and therefore, its effect also has to be studied. Thus, optimization experiments were carried out to determine temperature dependence, optimal pH, substrate and H_2O_2 concentrations of sorghum peroxidase. The effect of calcium chloride on enzyme activity, the substrate range and thermostability of sorghum peroxidase were also investigated.

3.3.4.1.1. Temperature dependence

Enzyme activity is dependent on temperature, thus, experiments were performed to determine how sorghum root peroxidase is affected by temperature. In this experiment, enzyme assays were carried out at temperatures varying from 20 - 60 °C, by equilibrating the assay mixture (*o*-dianinsidine in sodium phosphate buffer, pH 6, containing H_2O_2) to the relevant temperature, and initiating the reaction with the enzyme solution (Section 3.2.5.1.1). Figure 3.10 shows that peroxidase activity increased as the temperature of the assay mixture increased. At 60°C the activity was 3.5 U/mL while the activity was 2.4 U/mL at 20°C.

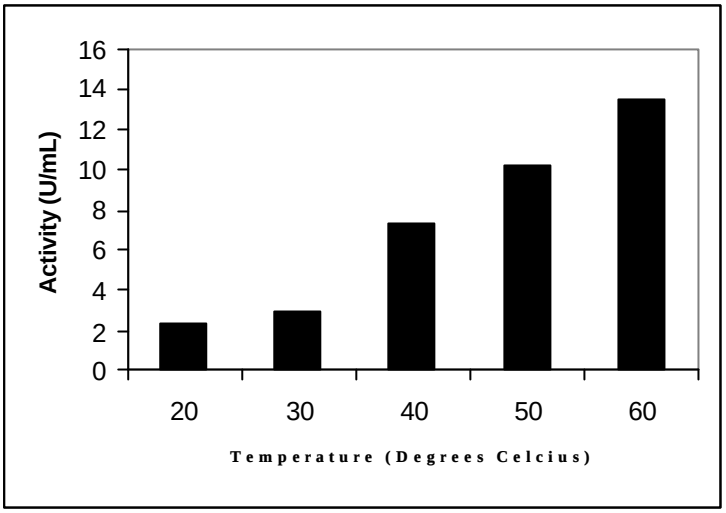


Figure 3.10. Effect of temperature on sorghum root peroxidase activity. Units of activity are in U/mL (Appendix 2). (The values are the average of duplicate experiments).

The rate of a chemical reaction depends on temperature, and this dependence can be described by the empirical Arrhenius equation:

$$K = Ae^{-E/RT}$$

where K is the rate constant, E is the activation energy of the reaction and A is the "action constant". In the case of enzymes, the rate of the catalysed reaction increases regularly with increasing temperature (Ballesteros *et al*., 1994). Generally, the enzymatic conversion rate doubles on a 10°C temperature increase. This holds true from about 10 - 40°C (Uhlig, 1998). The results in this experiment show that sorghum peroxidase activity more than doubled when the temperature was increased from 30 - 40°C, and the enzyme remained active at high temperatures, indicating its potential value in biotransformations.

3.3.4.1.2. Thermostability of sorghum root peroxidase

In a number of technical processes, high enzyme stability is of significant economic value especially in processes operated at high temperatures (Uhlig, 1998). In this experiment, sorghum root peroxidase was subjected to incubation at a series of high temperatures in order to determine its thermostability.

The experimental procedure involved heating the enzyme extract to the relevant temperature for a specific period of time, allowing it to equilibrate to room temperature for 15 minutes, and then assaying for enzyme activity at room temperature (Section 3.2.5.1.7).

Figures 3.11 and 3.12 reveal that sorghum peroxidase is relatively stable for 6 h at 40 and 50°C. At 40°C for a period of 6 h the activity varied slightly around 6 U/mL. At 50°C, the activity remained constant at approximately 4.5 U/mL over 6 h. At 60°C the activity remained constant at approximately 4.0 U/mL for 2 h, then gradually decreased to 2.34 U/ ml over the remaining 4 h. At 70°C peroxidase activity was very low (1.67 U after 1 h) and gradually decreased to 0.37 U/mL at the end of 6 h. Traces of activity (0.6 U/mL) were detected when the enzyme was incubated for 1 h at 80°C. At this temperature, activity was detected up to 3 h (0.005 U/mL) and there was no activity after 4 h. Even at 100°C, the sorghum root peroxidase activity remained at 2 U/mL for a total of 8 min (Figure 3.15). This indicates significant thermostability which is a useful characteristic for industrial applications. This suggests further investigation of sorghum root peroxidase for use in organic, non-aqueous media.

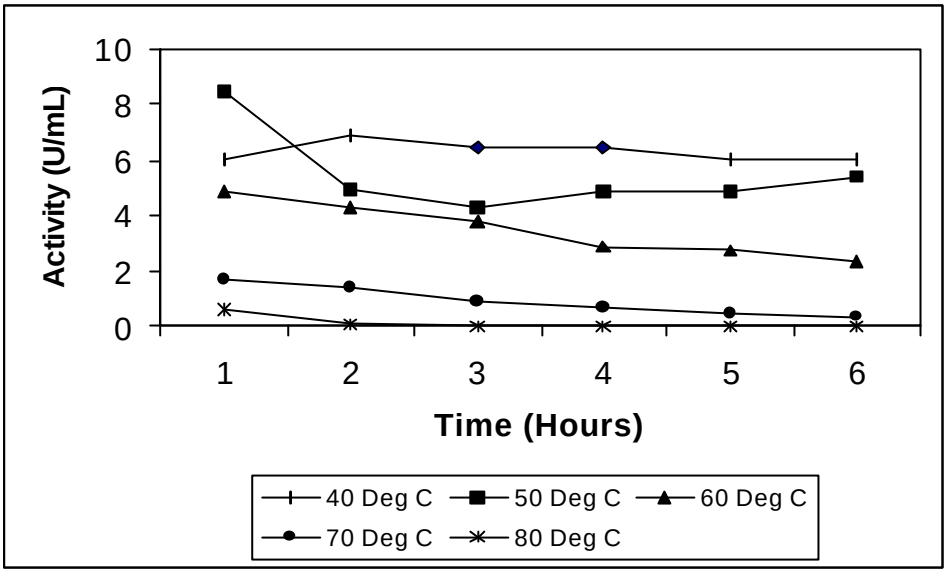


Figure 3.11. Thermostability of sorghum root peroxidase. Enzyme units are in U/mL (Appendix 2). (The values are averages of duplicate experiments).

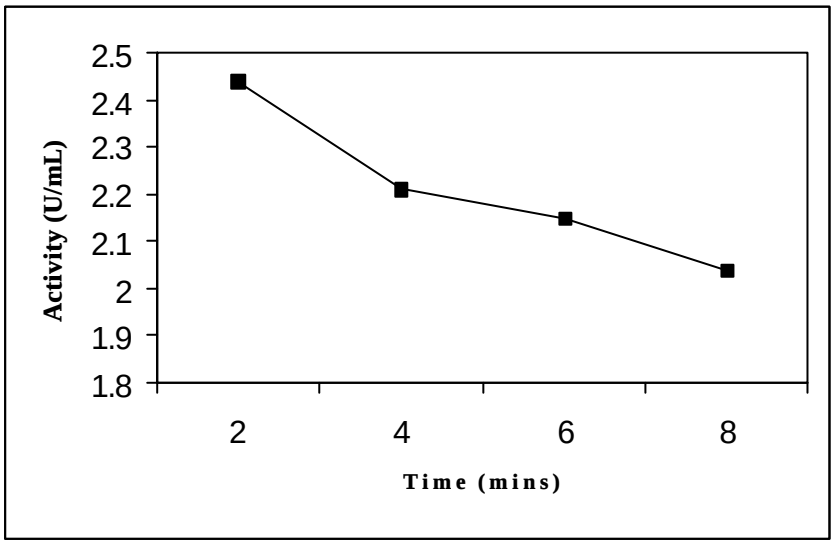


Figure 3.12. Thermostability of sorghum peroxidase at 100°C. (Enyzme units are in U/mL Appendix 2). (Values are averages of duplicate experments).

3.3.4.1.3. Effect of hydrogen peroxide concentration on sorghum root peroxidase

Peroxidases are affected by the levels of hydrogen peroxide during assays, with the optimum concentrations of H_2O_2 resulting in optimal peroxidase activity and high H_2O_2 concentrations inhibiting activity. Enzyme assays were carried out at room temperature, in 0.1 M sodium phosphate buffer, pH 6, containing varying concentrations of H_2O_2 (Section 3.2.5.1.3). Figure 3.13 shows that the optimal hydrogen peroxide concentration for sorghum peroxidase activity was 0.01 M where the activity was 3.7 U/mL. Only very low levels of hydrogen peroxide, 0.005 M, were needed to elicit a reaction (where the activity was 2.4 U/mL). Saturation was observed with increasing hydrogen peroxide concentrations. However, high levels of hydrogen peroxide (e.g. 0.9 M where the activity was 2.3 U/mL) did not dramatically decrease peroxidase activity. It is not clear why these results were observed, but it is clear that, hydrogen peroxide levels affect peroxidase activity. The shoulder of the curve suggests that a second enzyme with a different pH dependence may also active (side activity) (Uhlig, 1998) and this observation needs to be confirmed.

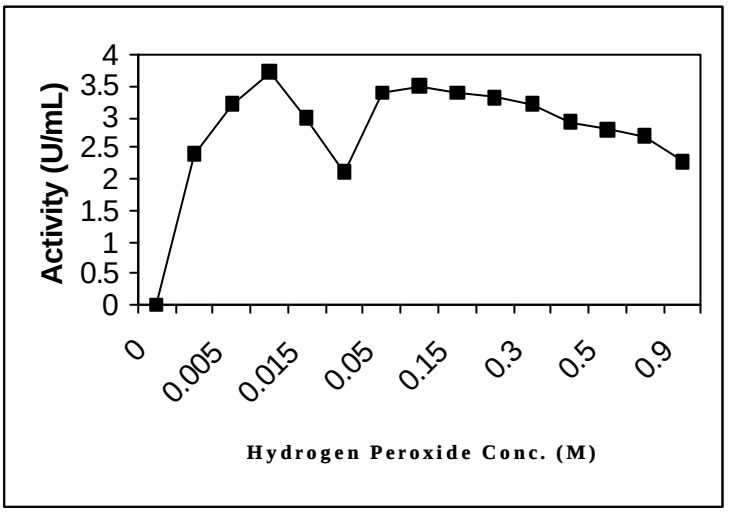


Figure 3.13. Effect of hydrogen peroxide concentration on sorghum root peroxidase actvity. Enzyme units are in U/mL (Appendix 2). (The values are averages of duplicate experiments).

3.3.4.1.4. Optimal substrate concentration

During assays, enzymes are affected by the concentration of the substrate and they function best at an optimal substrate concentration. Experimental procedures to determine the optimal concentration of substrate for sorghum root peroxidase were carried out by conducting assays at room temperature in 0.1 M sodium phosphate buffer, pH 6, with varying concentrations of *o*-dianisidine (Section 3.2.5.1.4). Figure 3.14 shows that the optimal concentration of *o*-dianisidine for peroxidase activity was 50 mM, where the activity measured was 7.0 U/mL. Doubling the substrate concentration to 100 mM led to a slight decrease in activity to 5.8 U/mL while tripling the substrate concentration to 150 mM led to a significant decrease in activity to 1.6 U/mL, indicating substrate inhibition effects. Thus, peroxidase activity is dependent on substrate concentration for optimal activity. At a relatively low concentration (10 mM) of substrate, lower activity levels (4.6 U/mL) were detected. This is presumably due to insufficient substrate available for the oxidation reaction.

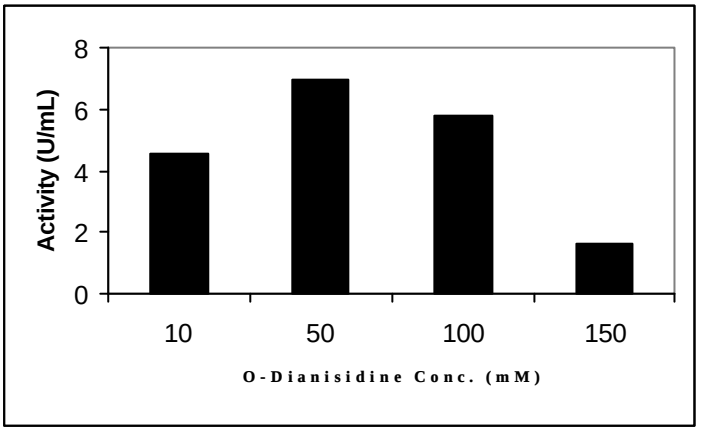


Figure 3.14. Optimal substrate concentration for sorghum root peroxidase activity assay using *o*-dianisidine. Units of activity are in U/mL). (The values are the average of the duplicate experiments).

3.3.4.1.5. Assay of sorghum root peroxidase with different substrates

There are many naturally occurring phenolic compounds in all plant tissues, and non-natural phenolics, which can be oxidised by peroxidase. Due to the diversity of compounds which are susceptible to oxidations catalyzed by peroxidases, the range of products formed is very extensive

(Robinson, 1991). This is an important characteristic for industrial applications where there is a constant search for economically valuable compounds. Three different substrates were investigated in this experiment, for oxidation by sorghum peroxidase. The enzyme solution (500 ：L) was added to 100 ：L 0.2 M ABTS in sodium phosphate buffer, pH 6 and the reaction was initiated by the addition of H_2O_2 (Section 2.2.2.3: method 2). With guiacol as substrate, 100 ：L of enzyme was added to 700 ：L 10 mM guaiacol and the reaction was initiated with H_2O_2 (Section 2.2.2.3: method 3). When 2,4-DCP was used as a substrate, 100 ：L of enzyme solution was added to 300 ：L 5mM 2,4-DCP solution and the reaction was initiated with 100 ：L of 0.1 M H_2O_2 (Section 2.2.2.3: mehod 4).

Figures 3.15 and 3.16 show the activity of the enzyme with different molarities of 2,4-dichlorophenol and guaiacol respectively. Using 2,4-DCP, the highest level of peroxidase activity was seen (2.48 U/mL) when 5 mM substrate was used. The optimal concentration of guaiacol for peroxidase activity was 10 mM, where the activity was 3.7 U/mL. ABTS was also oxidized by the enzyme - a colourless ABTS solution turned green and eventually purple when enzyme extract was added to it. This experiment supports the known fact that peroxidases can act on a wide range of substrates.

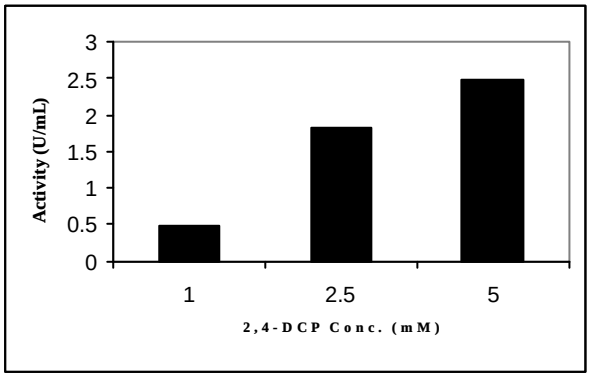


Figure 3.15. Oxidation of 2,4-dichlorophenol (2,4-DCP) by sorghum root peroxidase. Units of activity are U/mL (Appendix 2). (The values are average of the duplicate experiments).

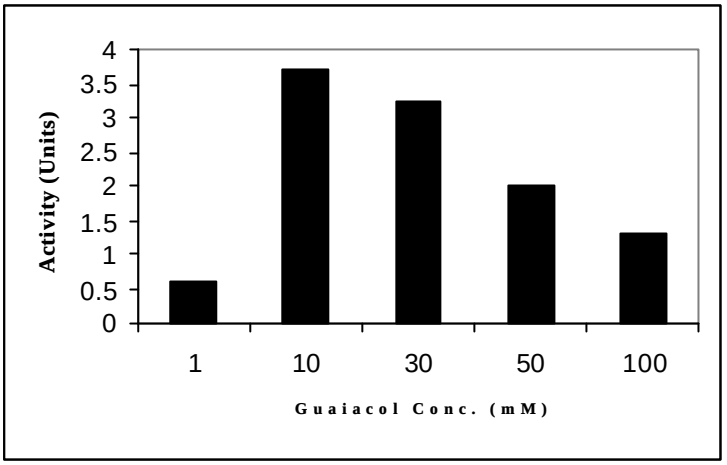


Figure 3.16. Oxidation of guaiacol by sorghum root peroxidase. Enzyme units are in U/mL, Appendix 2. (The values are average of the duplicate experiments).

3.3.4.1.6. Effect of $CaCl_2$ on sorghum root peroxidase activity

In this experiment, 10 ∶L of varying $CaCl_2$ concentrations were added to 100 ∶L of sorghum root peroxidase and assayed for enzyme activity (Section 3.2.5.1.6). Figure 3.17 shows that a high molarity (10 M) of $CaCl_2$ increased peroxidase activity slightly.

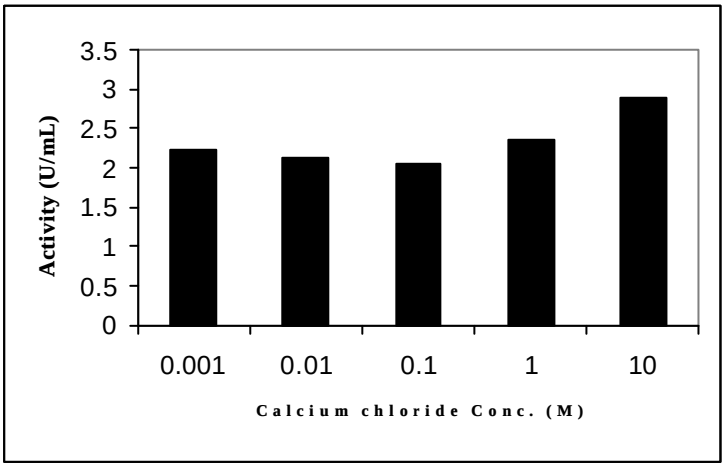


Figure 3.17. Effect of calcium chloride on peroxidase activity. Enzyme units are in U/mL (Appendix 2). (The values are averages of duplicate experiments).

These results are interesting considering that when Rasmussen *et al*. (1998) studied the effect of calcium ions on barley peroxidase (BP 1) reacted with hydrogen peroxide, it was found that, in the absence of Ca^{2+}, the reaction was slow and strictly monophasic whereas it became biphasic in the presence of Ca^{2+}, with a fast phase approximately 100 times faster than the slow phase observed in the absence of Ca^{2+}. Furthermore, the fast phase constituted approximately 20% of the total change of enzyme absorbance in the presence of 1 mM Ca^{2+} and 90% in the presence of 50 mM Ca^{2+}. The absorption spectrum of B1 changed very little upon Ca^{2+} binding in the absence of Cl^-. Consequently, the near haem environment was not changed directly on Ca^{2+} binding. The results of the present study show that $CaCl_2$ was unlikely to have such a significant effect on sorghum peroxidase activity since very high levels of $CaCl_2$ elicited a slight increase in activity. It is not clear why this is so, and further investigation of this effect could be useful.

3.3.4.1.7. Optimal pH of Sorghum root peroxidase

Enzyme assays were carried out in 0.1M sodium phosphate buffer with varying pH, at room temperature (Section 3.2.5.1.2). According to the results shown in figure 3.18 the optimal pH of sorghum peroxidase was 6, where an activity of 2.38 U/mL was seen. This value was virtually the same as the activity at pH 5 where the activity was 2.36 U/mL. It is acknowledged that ionic strength would vary in the buffers used for this experiment. However, for practical purposes, the optimal pH range for sorghum peroxidase activity was taken to be between 5 and 6. At a pH of 3, the enzyme was inactivated while at a basic pH of 8, the activity was very low (0.048 U/mL).

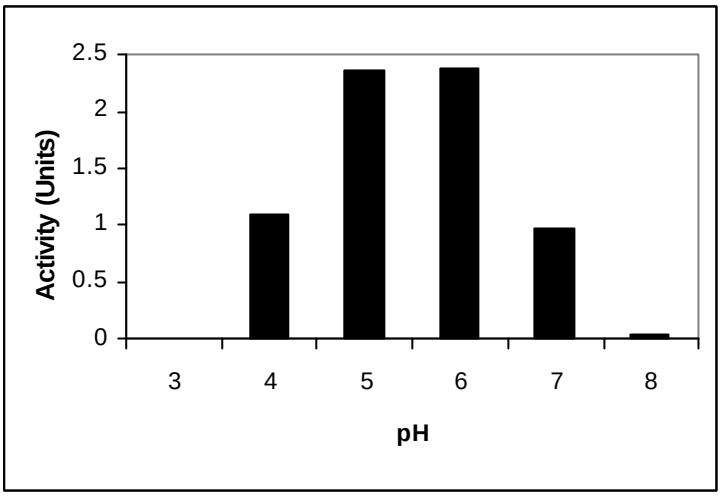


Figure 3.18. Effect of pH on activity of sorghum root peroxidase. Units of activity are in U/mL (Appendix 2). (These values are the average of duplicate experiments.)

The pH dependence of the activity of an enzyme is the result of a fundamental effect of the H^+ ion concentration. pH can exert its effects by changing the ionisation of groups in the active site, as well as on the gross conformation of the protein. Fortunately, large inactivating conformational changes occur usually in pH regions far from the pH optimum of the activity (Ballesteros, 1994). In the case of sorghum root peroxidase, the pH values at which deactivation of the enzyme occurs (pH 3 and 8) are far from the optimal pH at which the enzyme functions (pH 6). This is important because enzyme inactivation through pH-mediated conformational changes would, therefore, not cause difficulties in the industrial application of the peroxidase.

3.4. CONCLUSION

Sorghum root peroxidase was partially purified and characterized. A five-fold purification of the enzyme resulted after hydrophobic chromatography with sepharose CL-4B. Using polyacrylamide gel electrophoresis, the molecular weight of the enzyme was found to be approximately 40 000 which is similar to that of HRP (44,000). According to the colour intensity of the band in the activity gel, sorghum root peroxidase appeared to have a higher activity than HRP.

A number of characteristics of sorghum peroxidase make it an attractive candidate for industrial applications, and a cheaper substitute for HRP. 1) It is stable and active at high temperatures, a characteristic of particular importance in industrial applications which operate at high temperatures. In assay conditions that ranged from temperatures of 20 - 60°C, sorghum root peroxidase maintained activity and increased in activity as the temperature increased as was expected. When the enzyme was incubated at high temperatures (40 - 100°C), although it decreased with increasing temperature incubations, it maintained its activity for 6 h at 40°C and 50°C; 2 h at 60°C; 1 h at 70°C, and for 8 min at 100 °C. 2) The optimal pH of sorghum peroxidase is between 5 and 6 and the enzyme retains some activity at pH 4 and 7. However, in extreme acidic (pH 3) conditions it is denatured and in basic (pH 8) conditions it exhibits very low activity. 3) It has a wide substrate range and this is a particularly advantageous feature in industry. Having a wide substrate range confirms that sorghum root peroxidase is applicable in biotransformations. The biotransformation of a number of substrates by sorghum peroxidase is discussed in Chapter 4.

Chapter 4

APPLICATIONS OF SORGHUM PEROXIDASE

4.1. INTRODUCTION

In the previous chapter, the purification and characterization of sorghum peroxidase from *Sorghum bicolor* was discussed. Sorghum peroxidase was shown to have properties similar to HRP. Thus, sorghum root peroxidase is a potential substitute of horse radish peroxidase (HRP), which is commonly used in a wide variety of industrial and analytical applications, but which is relatively costly.

In the field of biotransformations, peroxidases are particularly useful, because they have the potential to oxidize a wide range of substrates, and have the advantage that no cofactor is required. The nature of the products produced by the reactions of known plant peroxidases is relatively well documented (Robinson, 1991), and one aim of this study was to compare the activity of sorghum peroxidase with that of HRP. The reaction typically involves oxidation of an electron donating substrate by H_2O_2 and involves the overall transfer of two electrons, although most reactions catalyzed by HRP and other peroxidases occur in sequential one electron steps.

In addition to application in biotransformation reactions, peroxidases can be utilized for bioremediation. While a number of peroxidases enzymes obtained from plant, animal and microbial sources have been

investigated for their ability to catalyze the removal of aromatic compounds from waste waters, the majority of studies have focused on using commercially available pure HRP (Adler *et al.*, 1994). However, removal of phenolics by this enzyme is not cost effective, and it is susceptible to inactivation by various side reactions of the treatment process. This suggests an additional use for sorghum root peroxidase.

This chapter reports a preliminary investigation, in collaboration with Dr. Koteshwar (Post-Doctoral Fellow, Rhodes University), of the reactivity of sorghum peroxidase in biotransformation of a range of model aromatic substrates. These substrates were selected on the basis of their presence in industrial residues which provide an inexpensive source of starting materials for industrial processes. Thus, biotransformation of these substrates could be developed as the basis for commercialisable processes, yielding economically valuable products. Characterization of products was achieved using analytical techniques such as UV-Visible absorption spectroscopy, nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), and HPLC-mass spectroscopy.

4.2. MATERIALS AND METHODS

4.2.1. Biotransformation reactions

Sorghum peroxidase was used as the biocatalyst in a series of biotransformation reactions. The reaction mixture consisted of equal volumes of buffer and ethyl acetate, with 1 mM substrate and partially purified enzyme extract added (prepared as described in Section 3.2.3). The reaction was initiated by addition of 1 mM H_2O_2 and this addition was repeated periodically. After 24 hours the reaction mixture was allowed to separate into two layers and the organic layer was separated, washed with brine, dried, and evaporated. The formation of the reaction products from a given phenolic substrate was determined by C18 reverse phase HPLC with UV detection. Any product eluting before the parent compound was assumed to be more polar than the parent compound.

4.2.1.1. Biotransformation reactions with *p*-dianisidine

p-Dianisidine (100 mg) was dissolved in a biphasic system (0.1 M phosphate buffer, pH 6/ ethylacetate, 1:2) and agitated for 5 - 10 minutes. To this, 1 mL of enzyme extract was added and the mixture was stirred for another 5 - 10 minutes. 100 μL of 100 mM H_2O_2 was added to initiate the reaction. After 3 hours, the reaction was allowed to settle into two phases. The aqueous phase was removed and the organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum. 160 mg of a reddish-brown to brick red product formed.

4.2.1.2. Biotransformation reactions with phenol

Phenol (94 mg) was dissolved in a biphasic system (0.1 M phosphate buffer, pH 6/ethylacetate, 1:2). To this mixture, 1 mL of enzyme was added and the reaction was initiated with 100 μL of 100 mM H_2O_2. After 16 hours a colour change was observed. The product was extracted with ethylacetate, dried over anhydrous Na_2SO_4 and evaporated under vacuum.

4.2.1.3. Biotransformation reactions with *p*-cresol

p-Cresol (105 mg) was reacted in the biphasic system described in section 4.2.2.1. The product was analysed using HPLC, with conditions as follows:- mobile phase: H_2O/CH_3OH, 70:30; flow rate 0.8 mL/min; detection by UV at 290 nm.

4.2.1.4. Biotransformations with *m*-cresol (*m*-methylphenol)

m-Cresol (100 mg) was dissolved in the following biphasic system - (0.1 M phosphate buffer, pH 6/ethylacetate, 1:3). The product was analysed using HPLC, with conditions as described in Section 4.2.1.3.

4.2.1.5. Biotransformations with guaiacol (*o*-methoxyphenol)

Guaiacol (250 mg) was dissolved in the biphasic system (0.1 M phosphate buffer, pH 6/ ethylacetate, 1:2)

and reacted with the enzyme. HPLC analysis was carried out using the same conditions as described in Section 4.2.1.3.

4.2.1.6. Biotransformation reactions with *p*-methoxyphenol

Biotransformation with 124 mg methoxyphenol was carried out as described in Section 4.2.1.5. The product was analysed using HPLC, with conditions as follows:- mobile phase: H_2O/CH_3CN, 60:40; flow rate 1mL/min; detection by uv at 270 nm.

4.2.1.7. Biotransformation reactions with veratryl alcohol

Veratryl alcohol (100 mg) was dissolved in the biphasic system buffer described in Section 4.2.1.5. After reacting with the enzyme, the product was analysed using the following HPLC conditions:- mobile phase:- H_2O/CH_3CN, 60:40; flow rate1 mL/min; dectection with uv at 250 nm for alcohol and 310 nm for aldehyde.

4.2.1.8. Biotransformation reactions with 2,4 diaminophenol

2,4-Diaminophenol (394 mg) was dissolved in the biphasic system described in Section 4.2.1.5. and reacted with 0.5 mL of enzyme extract. The product was analysed using the HPLC conditions described in Section 4.2.1.3.

4.2.1.9. Biotransformation reactions with procatechuic acid (3,4-dihydroxybenzoic acid)

Procatechuic acid (0.154 mg) was dissolved in the biphasic system described in Section 4.2.1.5. and reacted with 0.3 mL of enzyme extract. The product was analysed using the HPLC conditions described on Section 4.2.1.3.

4.2.1.10. Biotransformations with ferulic acid (4-hydroxy-3-methoxy-2-propenoic acid)

Ferulic acid (100 mg) was dissolved in the biphasic system buffer described in Section 4.2.1.5. After reacting with enzyme, the product was analysed using the HPLC conditions described in Section 4.2.1.3.

4.2.1.11. Biotransformation reactions with *p*-coumaric acid (4-hydroxycinnamic acid)

p-Coumaric acid (164 mg) was dissolved in the biphasic system (buffer/ethylacetate, 1:3). To this mixture, 0.4 mL sorghum peroxidase was added and 100 :L of 100 mM H_2O_2 initiated the reaction. The product was analysed with HPLC (same conditions as in Section 4.2.1.3)and thin layer chromatography (TLC), on silica gel plates with a mobile phase of $CHCl_3$/MeOH, 60:40.

4.2.1.12. Biotransformations with 4-hydroxyphenylacetic acid

4-Hydroxy phenyl acetic acid (0,152 mg) was dissolved in the biphasic system described in Section 4.2.1.5 and reacted with 0.3 mL of enzyme extract. The product was analysed using the HPLC conditions described in Section 4.2.1.3 and by TLC (as above).

4.3. RESULTS AND DISCUSSION

4.3.1. Biotransfomation reactions of sorghum root peroxidase

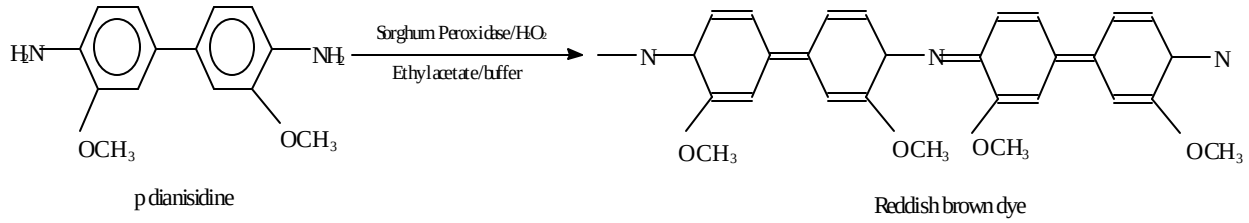
The activity of sorghum root peroxidase in biotransformation reactions was investigated by conducting a series of reactions using a range of different substrates. These substrates have all been reported to be oxidized by HRP, and thus comparisons could be drawn between sorghum root peroxidase reactions and HRP reactions. In addition, the substrates used are components of industrial residues, which provides an inexpensive source of the substrates. The products of these peroxidase oxidation reactions have potential use as organic synthons, in industrial processes, or as analytical products.

4.3.1.1. Biotransformation of *p*-dianisidine by sorghum root peroxidase

The *p*-amino groups on aromatic rings are prone to oxidation and form a diazo linkage between the

molecules, which leads to a reddish brown colour and finally the solid separating out. In the reaction between sorghum root peroxidase and *p*-anisidine, the yield was 161 mg (70%). The HPLC analysis of this product showed a single peak, detected at 300 nm, with retention time 3.6 min, which confirms that a single product was formed. This product is similar to that produced by HRP, and could have application as a dye (Vágújfalvi and Petz-Stifter, 1982) (see scheme a).

Scheme a



4.3.1.2. Biotransformation of phenol by sorghum root peroxidase

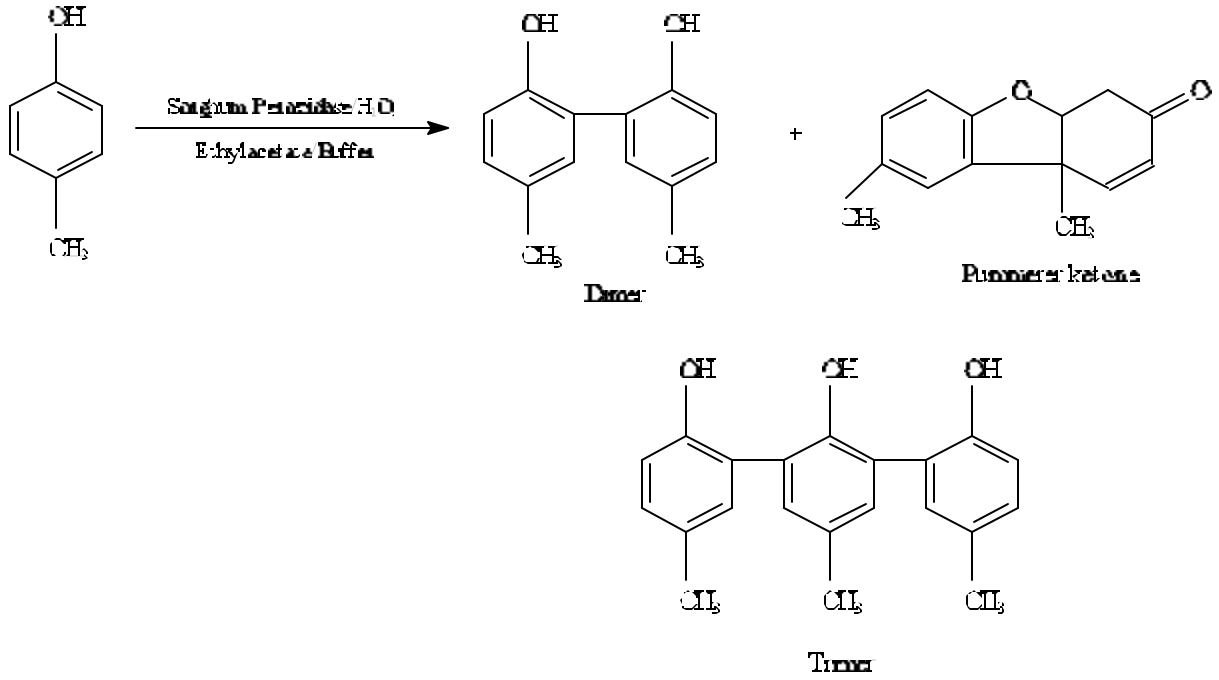
Generally, when phenol reacts with a peroxidase in the presence of H_2O_2 it gives polymeric products which are insoluble in aqueous systems and which deactivate the enzyme (Adler, 1994). When a biphasic system was used, however, the polymeric materials remained in the organic phase, and no evidence of enzyme inactivation was observed. HPLC analysis showed the starting material peak, observed at 6.3 min, and two other products at 8.2 and 10.8 min which may be polymeric products (Dunford, 1986).

4.3.1.3. Biotransformation of *p*-cresol by sorghum root peroxidase

This reaction has been thoroughly investigated with HRP (Pietikäinen and Aldercreutz, 1990; Hewson and Durnford, 1976)), and *p*-cresol yields Pummerer's ketone with other polymeric compounds, such as the dimer and trimer, in the presence of the H_2O_2/peroxidase system (scheme b). In the presence of sorghum peroxidase and H_2O_2 the formation of the same compounds was observed. This was confirmed by using HPLC analysis and HP-mass spectral analysis. The HPLC retention times were observed at 4.3 min (*p*-

cresol), 6.46 min (Pummerer's ketone) > 5%, 8.3 min (dimer) < 5% and 13 min (trimer) (very little), which match exactly with information in the literature describing the HRP reaction under the same conditions (Pitiekäinen and Aldercreutz, 1990). From HP-mass spec analysis, the molecular ion peak 214 m/z was observed for the component with retention time 6.46 min, which confirms the identity of the Pummerer's ketone product, and also confirms the similarity between sorghum root peroxidase and HRP.

Scheme b

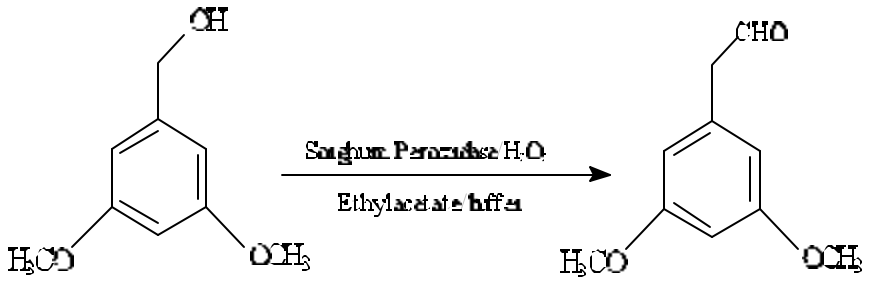


4.3.1.4. Biotransformation of *m*-cresol by sorghum root peroxidase

No reaction was observed with *m*-cresol. The same has also been reported when HRP was used (Uyama *et al.*, 1995).

4.3.1.5. Biotransformation of guaiacol by sorghum root peroxidase

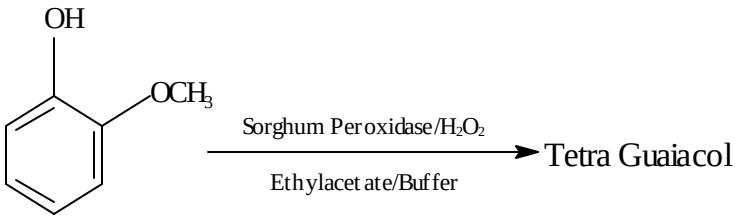
During the guaiacol reaction with sorghum peroxidase in the presence of H_2O_2, the solution, which was initially colourless, turned yellow and later became pink. This indicated that *o*-quinone formed first and underwent rapid polymerization leading to the formation of tetraguaiacol (scheme c). This is a recognised reaction of peroxidases with guaiacol, and is used as an analytical assay of peroxidases (Biles and Abeles, 1991).



Scheme c

4.3.1.6. Biotransformation of *p*-methoxyphenol by sorghum root peroxidase

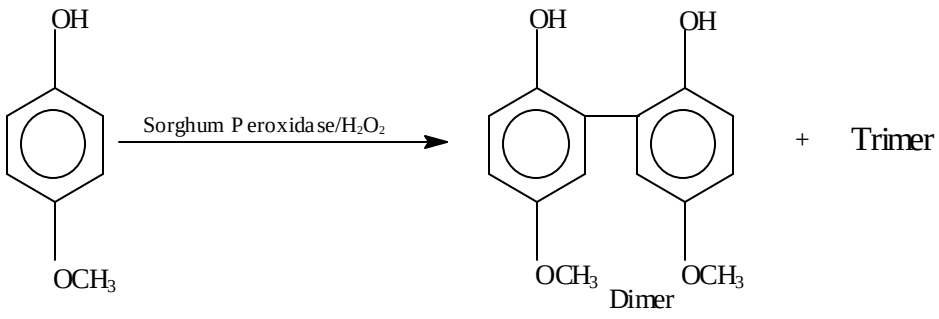
It was observed from HPLC analysis that *p*-methoxyphenol reacted with peroxidase and H_2O_2, yielding two different products apart from the starting material, where one peak was more major than the other. The retention times were 5.54 min (*p*-methoxyphenol), 11.89 min and 20.74 min. These peaks may indicate the dimer and trimers products as shown in scheme d. Biotransformation of *p*-methoxyphenols with HRP have been found to yield the same results (Setti *et al*., 1998). The results need to be confirmed by mass spectrophotometric analysis.



Scheme d

4.3.1.7. Biotransformation of veratryl alcohol by sorghum root peroxidase

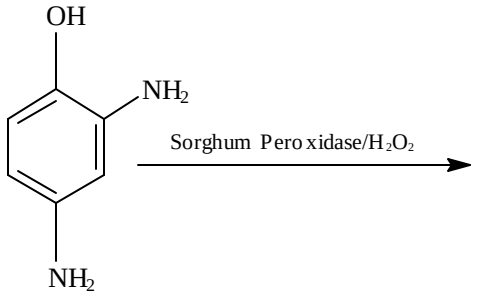
3,5-Dimethoxybenzyl alcohol (veratryl alcohol), with peroxidase and H_2O_2, reacted to give 3,5-dimethoxybenzaldehyde (scheme e), which has a maximum absorbance at wavelength 310 nm, while the alcohol absorbs at 280 nm. HPLC analysis indicated that the oxidized product, the aldehyde, is less polar than the parent compound, since it eluted later. The retention times are 5.391 min (aldehyde) and 3.6 min (alcohol). Veratryl alcohol is used in the standard assay for peroxidases and reactions with HRP confirm the results obtained here (Aitken *et al.*, 1993).



Scheme e

4.3.1.8. Biotransformation of 2,4-diaminophenol by sorghum root peroxidase

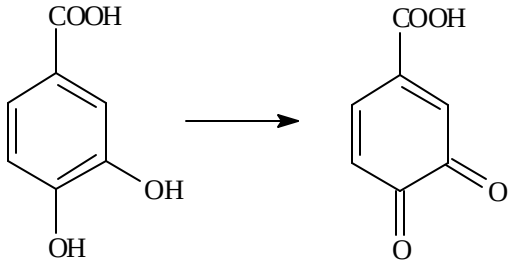
In the reaction of 2,4-diaminophenol with sorghum root peroxidase and H_2O_2, as the reaction progressed, a black precipitate separated out (scheme f), which may be due to the formation of a diazo linkage between the molecules as discussed in section 4.3.1.1. Klibanov *et al*. (1981) have reported the biotransformation of diaminophenol by HRP and reported the same results.



Scheme f

4.3.1.9. Biotransformation of procatechuic acid by sorghum root peroxidase

3,4-Dihydroxybenzoic acid, in the presence of H_2O_2 and peroxidase, gave a colourless solution which turned yellow, possibly due to formation of the *o*-quinone (absorbed at 380 nm - 420 nm), in organic phase, and reddish brown in aqueous phase. As the reaction progressed, the yellowish organic phase turned brown. TLC showed that the product was very similar in polarity to the parent compound. UV spectrophotometric analysis showed a bathochromic shift in wavelength from 290 nm - 310 nm. The possible reaction scheme is shown (scheme g). This reaction



distinguishes peroxidase from polyphenol oxidase which is inhibited by this compound (Burton, 1994).

Scheme g

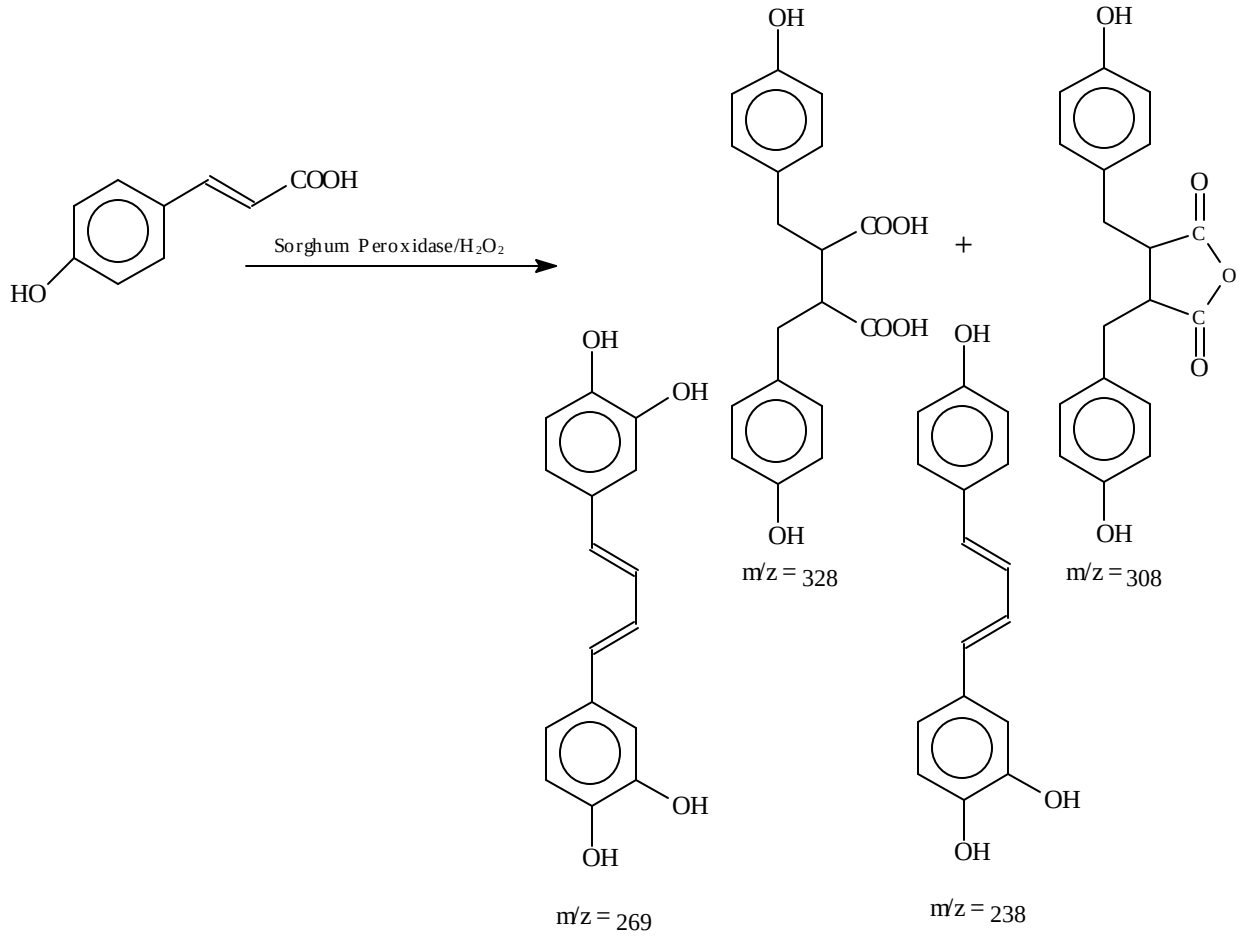
4.3.1.10. Biotransformation of ferulic acid by sorghum root peroxidase

During the reaction, the solution changed from light brown to yellow. HPLC analysis showed peaks with retention times 4.9 min (ferulic acid) and a product at 18.2 min. HPLC-Mass spec analysis showed major ion peaks at 297, 358, 360 and 390 as major ones, corresponding to the structures shown below (scheme h). This correlates with data reported by Rosazza *et al*., 1995, on the reaction of HRP.

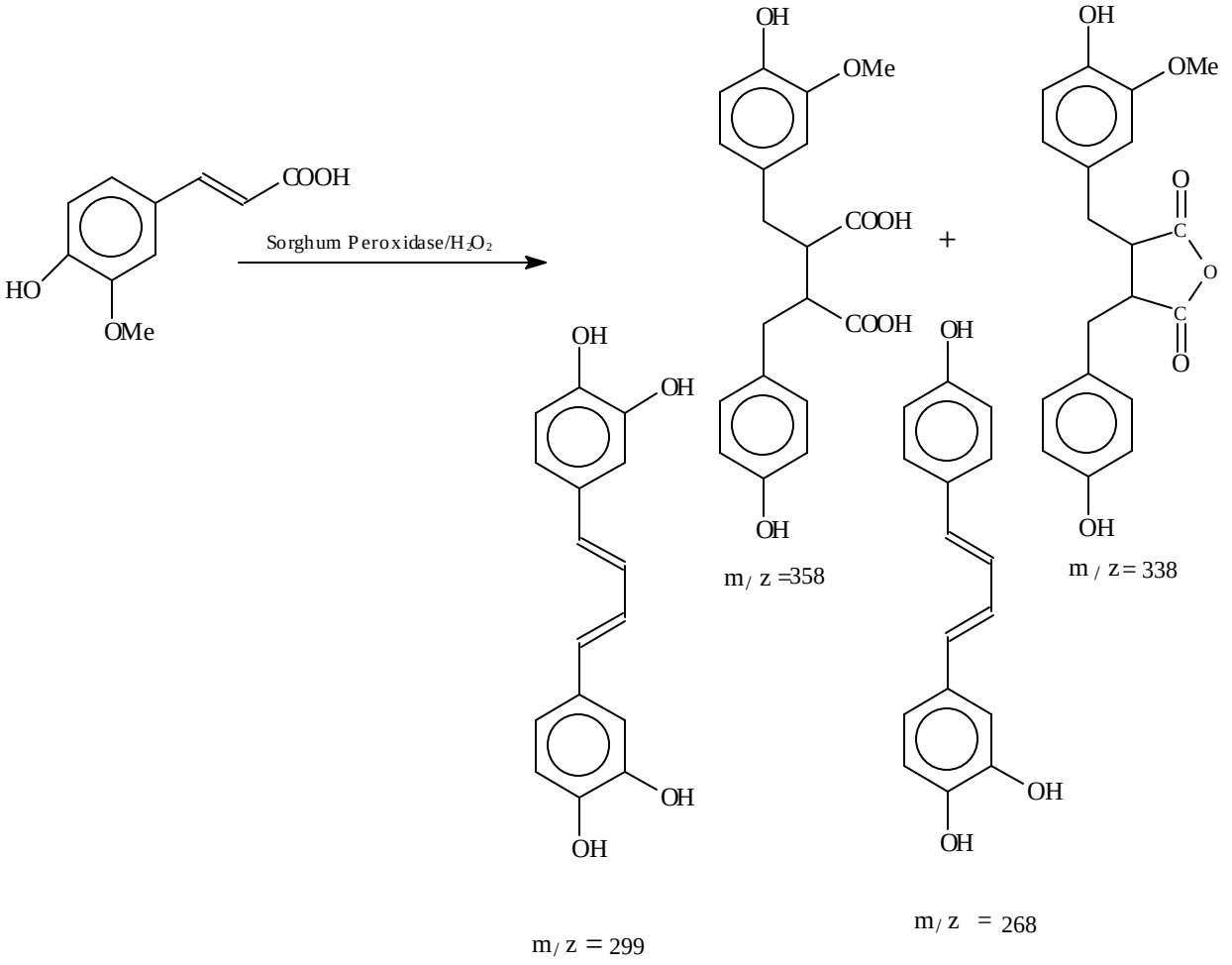
Scheme h

4.3.1.11. Biotransformation of *p*-coumaric acid by sorghum root peroxidase

HPLC analysis showed a product peak at 4.9 min and TLC analysis showed three different spots apart from starting material. The products shown (scheme i) are suggested to be formed.



Scheme i



4.3.1.12. Biotransformation of 4-hydroxyphenyl acetic acid by sorghum root peroxidase

A change in wavelength of absorption was observed indicating product formation. The aqueous solution was acidified and extracted with ethyl acetate. From the NMR analysis of the compound, no acid remained after the reaction. TLC analysis showed that the product was more polar than the parent compound, and

was found to be the same as benzaldehyde on the TLC. Again, this showed a similar reaction to HRP (Uyama *et al.*, 1995).

4.4. CONCLUSION

The work reported here represents a preliminary study, and much of the analysis of the products must still be confirmed. However, it is clear that sorghum peroxidase is a highly active enzyme and that its reactions are comparable with those of HRP. It is thus feasible to suggest the use sorghum peroxidase in bioremediation and biotransformation reactions.

The substrates selected for trial biotransformation reactions are representative of the aromatic compounds found in common industrial residues, and in particular, olive waste. The fact that they were transformed by sorghum peroxidase indicates that the beneficiation of industrial residues using peroxidases in general, and with sorghum peroxidase, in particular, is feasible.

This research will need to be further developed to fully characterize the nature of the products. In addition, subsequent investigations will be required to establish which of the constituents of the specific effluents are the most promising candidates for biotransformation to make marketable products, and whether extraction of substrates prior to bioconversion, or after it, will be the most efficient process to develop.

The potential value of sorghum root peroxidase has been recognized by the Water Research Commission of SA who have supported the registration of a provisional patent for the application of sorghum root peroxidase in biotransformation.

Chapter 5
CONCLUSION

The objectives of this project were to conduct an investigation to determine whether peroxidase, polyphenol oxidase and protease enzyme activities are present in *Sorghum bicolor*, to isolate and characterize the enzymes found to be present and active, and to demonstrate the potential of these enzymes in applications involving biotransformations.

Only low levels of protease were found and it was therefore decided not to proceed with their isolation. Polyphenol oxidases were found to be present in the grain and leaf tissues but at levels which did not suggest the feasibility of application in biotransformation. High levels of peroxidases were found in grain, leaf and root tissue.

Extraction of sorghum peroxidase proved to be relatively straightforward, with the crude extracts yielding large amounts of peroxidase activity. Peroxidase was isolated from young and ripe grain tissue in sodium phosphate buffer using a pestle and mortar. Young grain appeared to have more peroxidase activity than ripe grain. Peroxidase levels are subject to plant growth and development (Jackson and Ricardo, 1998) and this was confirmed in this study. Two methods were employed for the isolation of peroxidase from the leaves; homogenization using a pestle and mortar in sodium phosphate buffer, followed by ammonium sulphate precipitation, and using the aqueous two-phase system (ATPS) after homogenization in a Waring blender. The ATPS method of leaf peroxidase isolation proved to be more effective than precipitation with ammonium sulphate, since this method yielded higher peroxidase specific activities than the precipitation method. Isolation of peroxidase from the roots was carried out

using two methods. One method involved homogenization with a Waring blender in MES buffer, and the other method involved homogenization with a pestle and mortar in sodium phosphate buffer followed by ammonium sulphate fractionation. The most effective method of isolating peroxidase was with a Waring blender in MES buffer, pH 5.5.

Because the roots appeared to have the highest levels of peroxidase activity, sorghum root peroxidase was purified from the roots. Purification steps included centrifugation, treatment wtih PVPP, ultrafiltration and hydrophobic chromatography. A 5-fold purification was achieved.

Purification of the enzyme proved to be challenging, as the enzymes were not readily stained by protein stain, Coomassie Brilliant Blue (CBB), making them barely identifiable in CBB gels after PAGE. However, they showed high levels of activity in the activity gels after PAGE separation. These results also suggested that sorghum root peroxidase had higher activity than the most commonly used commercial peroxidase, horse radish peroxidase (HRP). From their molecular weights, two bands observed in CBB gels after SDS-PAGE, appeared to be very similar in molecular weight to HRP which has a molecular weight of 44 000. Another possible sub-unit or isoenzyme of sorghum peroxidase was identified, which had a molecular weight of 64 000. Further work needs to be done to confirm these results.

Characterization of sorghum root peroxidase activity revealed that it has considerable thermal stability and it still has activity at 100°C for about 8 minutes. Its optimal pH range was found to be between 5 and 6. Like other plant peroxidases, it was found to have a wide substrate range. For the assays, *o*-dianisidine was used as a substrate at an optimal concentration of 50 mM, while the optimal concentration of H_2O_2 during the assay was found to be 100 mM.

Sorghum root peroxidase was applied in biotransformation reactions with a number of compounds found in common industrial residues. The reactions observed were comparable with those of HRP, and thus it is feasible to consider the use of sorghum root peroxidase in biotransformation applications.

Thus, sorghum root peroxidase, which has been isolated and partially purified from *Sorghum bicolor*, appears to have similar properties to HRP, making it a cheaper alternative to HRP which is relatively costly.

Sorghum bicolor is a plant that sustains the lives of rural people in the semi-arid tropics of Africa and Asia. By isolating a commercially valuable enzyme from the plant, its economic value can be enhanced, thereby providing the potential to improve the quality of the lives of the people who live on it.

5.1. FUTURE WORK

Future work would involve the complete purification of sorghum root peroxidase in order to isolate a single band during SDS-PAGE analysis. Thus far, the characterization of sorghum root peroxidase is incomplete, because only the level of purification required for biotransformations was carried out.

It is possible to induce peroxidase production. Environmental stresses, especially drought and salt-stress, alter the steady-state level of mRNA coding for peroxidases, and accordingly the level of their activity in the cells (Eshdad *et al*., 1997; Gangopadhyay *et al.*, 1996). Nkang, (1995), has shown that in seeds of *Guilfoylia monostylis*, cyanide pretreatment caused a sharp increase in cotyledonary peroxidase activity. Kwak *et al*. (1996), found that stress-related plant hormones, abscisic acid or ethephon, enhanced peroxidase activity by 50% in suspension cultures of sweet potato. Thus, subsequent work could involve the enhancement of peroxidase in the plant using methods similar to these.

Future work could also entail applying sorghum root peroxidase in bioremediation of waste water contaminated with aromatic compounds, since it has been found to have biotransformation properties similar to HRP.

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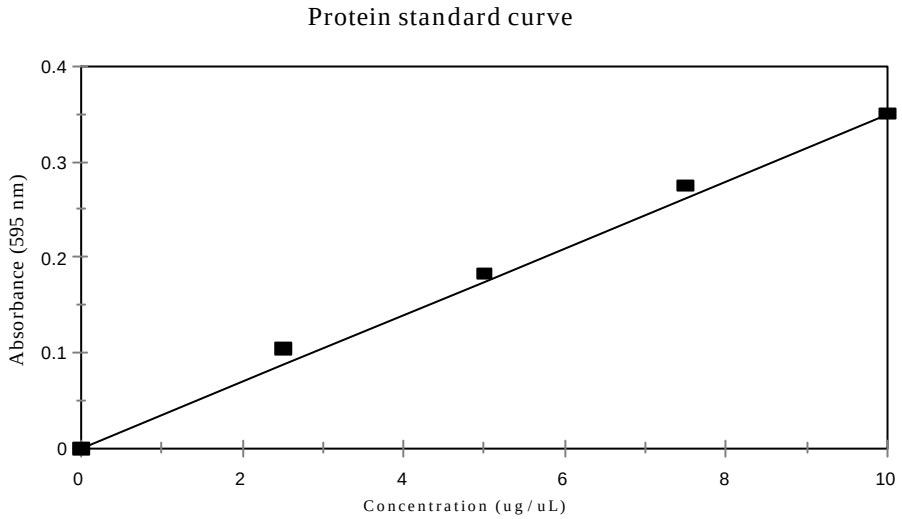
Appendix 1

Protein standard curve and calculation of protein concentration

The protein standard curve, using Bradford's method, was prepared according to the method in section 2.2.2.4 and is shown below. A standard curve was prepared for each experiment.



1. Calculations of concentrations in the standard curve.

BSA stock (0.5 mg/mL) - 50 ∶g in 100 ∶L

therefore, 2.5 ∶g in 5 ∶L

5 ∶g in 10 ∶L

7.5 ∶g in 15 ∶L etc

But, the volume was brought up to 100 ∶L, therefore, the concentration becomes:

2.5 ∶g/100∶L , 5 ∶g/100∶L, 7.5 ∶g/100∶L, etc.

2. Conversion from ∶g/100∶L to mg/mL protein

Say the unknown protein concentration is calculated to be 1.8 ∶g/100∶L from the std. curve.

i.e 0.0018 mg in 100 ∶L

therefore, 0.018 mg in 1000 ∶L (mL)

Appendix 2

1. Calculation of enzyme units

The units (U) of enzyme activity are expressed as the amount of product formed per minute, during the assay. Product formed is expressed in : mols/min/mL using the following equation:

$$\frac{\Delta A \times y \times 1000}{x \times 1\text{cm} \times t \times \varepsilon} = \text{mol/min/mL} = \text{Units (U)}$$

where $\Delta A/\Delta t$ = change in absorbance / minute

y = total assay volume (mL)

x = volume of enzyme extract (mL)

ε = extinction coefficient (expressed as $M^{-1}.cm^{-1}$) of the substrate (listed below)

multiply the value obtained by 1000 to make the calculation a function of mL

hence U/mL

Substrate	**ε ($M^{-1}.cm^{-1}$)(extinction coefficient)**
o-dianisidine	11,300
ABTS	18,600
guaiacol	6390
2,4-DCP	1.36×10^4
L-DOPA	1460

2. Purification table

The values in the purification tables are expressed as follows:

a) Activity - explained in section 1

b) Specific activity - U/mg protein (: mols/min/mg)

c) Yield (%) - Total activity of the purified fraction / Total activity of the crude extract

d) Relative purification - Specific activity of the purified fraction / specific activity of the crude extract

Appendix 3

1) Table of molecular weights of protein standards

Protein standard	Molecular Weight
Myosin	205,000
$-Galactosidase, *E. coli*	116,000
Phosphorylase b, rabbit muscle	97,000
Fructose-6-phosphate kinase, rabbit muscle	84,000
Albumin, bovine serum	66,000
Glutamic dehydrogenase, bovine liver	55,000
Ovalbumin, chicken egg	45,000
Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36,000

2) Standard curve of protein standards versus distance migrated during electrophoresis

For each gel that was electrophoresed, a new standard curve was prepared.