CHAPTER I

INTRODUCTION

1.1 Cassava

Cassava (*Manihot esculenta* Crantz) is the fourth most important food crop in the tropics. It is grown predominantly for its strachy roots. Roughly 85% of the dry matter of these root is starch. This high starch content makes cassava a major source of carbohydrates for 500 million people in the world. Cassava leaves are also eaten as a vegetable by humans and used as animal feed (1).

Cassava can be grown throughout the year since it has no critical period of propagation and harvesting. Cassava is propagated easily by using stem cuttings and requires little attention during growth. Therefore, its cultivation is relatively inexpensive. Cassava was initially brought into Southern Thailand from Malaysia, and had since spread to other parts of the country.

Nowadays, the major areas for cassava cultivation in Thailand are the east and the northeast which together account for about 90% of the country's cassava production (2). In addition to being used for human consumption and as animal feed, cassava is also used in industries to make such products as alcohol, sorbitol, dextrin, soap, candy, powder and pelletized medicine (3).

Moreover, cassava storage roots are uniquely deficient in proteins, which comprise only 1-2% of the dry weight (1). The overall quality of cassava root proteins is further limited by sulphur-containing amino acids, which can cause protein deficiency in people consuming cassava as a staple food without protein supplement. Cassava leaves contain up to 10 times more proteins than the storage roots, but are still deficient in the essential

amino acids, cysteine and methionine. Root peel discarded in food preparation has a higher level of protein than the edible parenchyma. Therefore, the peels of low-cyanide varieties are often used as a nourishing animal feed (1).

1.2 Physiological deterioration in cassava

The rapid development of primary or physiological deterioration in cassava has been strongly associated with mechanical damage which occurs during harvesting and handling operations (4). Frequently, the tips are broken off as the roots are pulled from the ground and severance from the plant necessarily creates further wounding. In addition, transport from sites of storage cause tissue damage which is initially observed as discoloration of the vascular tissue often referred to as vascular streaking. Initial symptoms are rapidly followed by a more general discoloration of the storage parenchyma.

In most plants, tissue damage results in a cascade of wound responses that quickly result in the defence of the wounded tissue and the subsequent sealing of exposed tissue by regeneration of a protective barrier (periderm formation).

Cassava roots when stored at high relative humidities (RH) of around 80 to 90% show a typical wound-healing response with periderm formation occurring in seven to nine days at 35°C and 10 to 14 days at 25°C (5). This response is notably slower than in the other tropical root crops, such as yam, which form a periderm in four to five days at 35°C (5). However, it was demonstrated that periderm formation in cassava roots occurred around small v-shaped cuts within four to seven days at 35°C and the magnitude of the wound sustained can affect the time required for periderm formation.

In cassava the formation of a wound periderm (curing) has been found to suppress the development of physiological deterioration (4).

In common plant wound responses, phenolic compounds from the phenylpropanoid pathway are acted upon by a range of peroxidases to produce wound-repair substances in the immediate vicinity of the site of tissue damage. The enzymes involved in these processes are synthesized in response to wounding after a time lag which is dependent on the nature of the stimulus.

In cassava, cytochemical tests for peroxidase gave increased staining responses during the development of a localized wound response (curing) and during the later stages of physiological deterioration (5,6). Biochemical analysis of peroxidase in wounded cassava roots showed that increase in activity occurred after a lag period of about one day and continued to increase with the development of physiological deterioration (7).

The rapid post-harvest deterioration of cassava restricts the storage potential of the fresh root to a few days. In addition to direct physical loss of the crop, post-harvest deterioration causes a reduction in root quality, which leads to price discounts and contributes to economic losses.

1.3 Peroxidase

Peroxidase (donor: H_2O_2 oxidoreductase; EC. 1.11.1.7) belongs to a large family of enzymes capable of catalyzing the oxidation of several different substrates in the presence of H_2O_2. Higher plants possess several peroxidase isozymes. These isozymes are differentially expressed in various tissues and organs in response to environmental

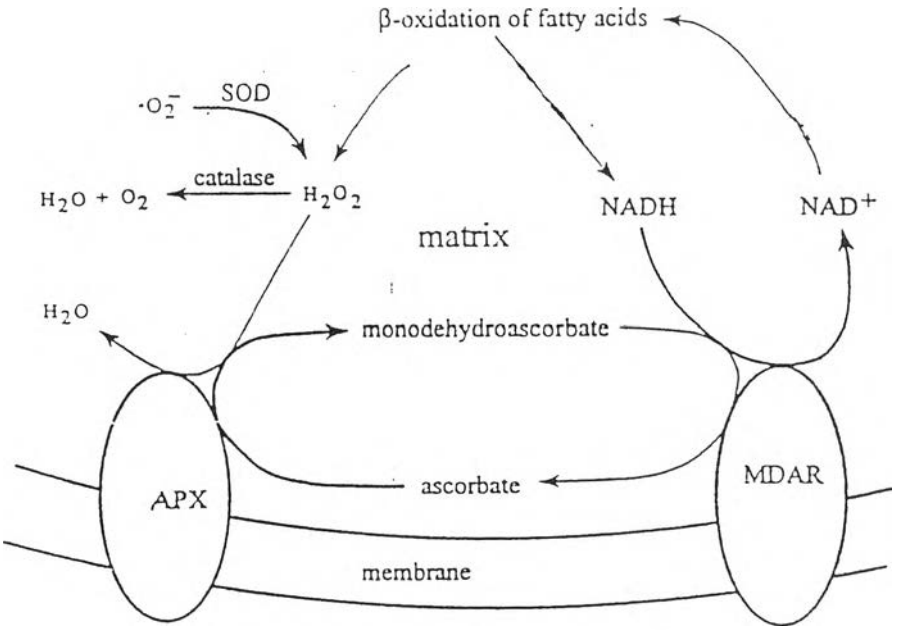
conditions. According to current classification, plant peroxidases are divided into two types: ascorbate peroxidase and classical peroxidases (8).

1.3.1 Ascorbate peroxidase (APX) (EC 1.11.1.11)

Plants have APX to rid themselves of excess hydrogen peroxide under normal and stress conditions such as extreme salinity and sandy soil. Reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$), H_2O_2, hydroxyl radical ($OH^{\bullet}$) which are the inevitable by-products of cell metabolism. Under stressful conditions, the formation of ROS may be excessive. These ROS attack lipids and proteins causing lipid peroxidation and protein denaturation (9). To prevent the damage, plant cells are equipped with an antioxidative system consisting of low-molecular-weight antioxidants such as ascorbate, glutathione and protective enzymes, ascorbate peroxidase (10). APX has a high perference for ascorbate as an electron donor. The enzyme catalyzes the reaction:

$$2 \text{ ascorbate} + H_2O_2 \longrightarrow 2 \text{ monodehydroascorbate} + 2H_2O \quad (11)$$

Cellular regeneration of ascorbate is accomplished through the direct reduction of monodehydroascorbate (MDA) to ascorbate by MDA reductase (MDAR) using NADH as the electron donor (Fig. 1)



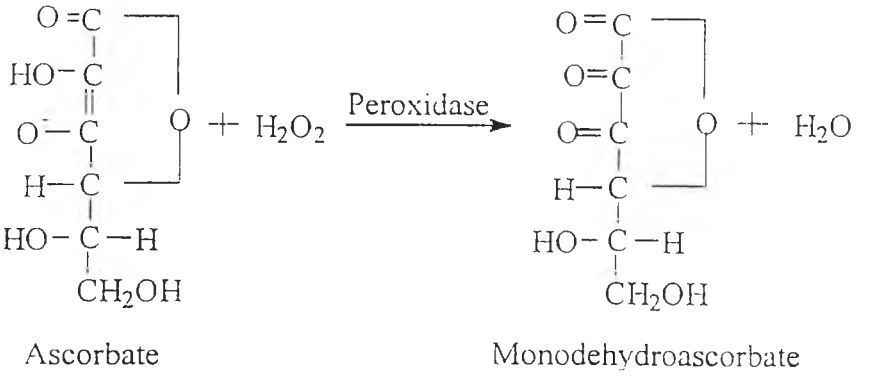


Fig 1. Proposed model illustrating H_2O_2 scavenging under excess H_2O_2 or stress condition (12)

There are two types of APX in plants (13), one of which is associated with the chloroplasts while the other is localized in non-photosynthetic tissues (cytosolic isozyme). However, the chloroplastic and cytosolic isozymes of ascorbate peroxidase differ from each other as follows (14): a) the chloroplastic isozyme has a very short life time in an ascorbate-depleted medium; b) the sensitivity of the chloroplastic isozyme to inhibitors such as thiol reagents, p-chloromercurisulfonic acid, *p*-hydroxymercuri-benzoate are higher than the cytosolic isozyme; c) the chloroplastic isozyme is more specific to ascorbate as the electron donor than the cytosolic isozyme, and d) the chloroplastic isozymes has a narrow pH optimum.

APX is distinct from guaiacol peroxidase with respect to the properties shown in Table 1 (15). Examples of ascorbate peroxidases are:

1. Pea cytosolic ascorbate peroxidase is a homodimer with molecular weight of 57,500, composed of two subunits with molecular weight of 29,500. The enzyme has a high stability in the presence of ascorbate, a broaden pH optimum and the capability to utilize alternate electron donors such as pyrogallol, guaiacol (11).

2. Ascorbate peroxidase in roots of Japanese radish is monomeric with molecular mass of 28 kD and was stabilized by ascorbate. The enzyme has a narrow optimum around pH 6. It cannot use guaiacol, 2,2'-diaminobenzidine or pyrocatechol. The enzyme is labile in absence of ascorbate (16).

3. Ascorbate peroxidase in potato tubers is labile in the absence of ascorbate. It has a molecular weight of 30 kD, a pH optimum of 7. Ascorbate is specifically required as the electron donor. The enzyme is inhibited by thiol reagents (17).

1.3.2 Classical peroxidase

Classical plant peroxidases can be distinguished by their non-specific use of phenolic derivatives as substrates and their involvement in polymerization reactions (18). One of the common substrates that can be utilized by classical peroxidase is guaiacol. Classical peroxidases are distinct from APX as shown in Table 1.

Examples of classical peroxidase are:

1. Horseradish peroxidase (HRP) EC (1.11.1.7) is the well-known classical peroxidase. HRP consists of more than 30 multiforms (19) which can be classified into 3 groups: acidic, neutral and basic isozymes by their isoelectric points. HRP is found mainly in the roots of horseradish. It is a hemoprotein catalyzing the oxidation by H_2O_2 of a number of substrates. It has a molecular weight of 40 kD and reversibly inhibited by 5-10 M cyanide and sulfide (20).

2. Peroxidase in vacuoles of pea (*Vicia faba*) leaves is a basic peroxidase with molecular weight of 49 kD. It is hemoprotein and has a pH optimum at pH 5. Cyanide and azide inhibit its activity. The phenolic compounds such as quercetin, kaempferol and rutin have been found in the vacuoles of *Vicia faba* leaves and they can be oxidized by the peroxidase in vacuoles in the presence of H_2O_2 (21).

3. Lignin peroxidase

Lignin is a polymer of phenylpropane units (monolignol precursors), such as p-coumaryl, coniferyl, and sinapyl alcohols (25). The lignin synthesis, a phenolic hydrogen atom is removed from coniferyl alcohol enzymatically (Fig. 2). The phenoxy radical thus formed is in resonating state among several structures, and a radical reacts with another radical to form lignin.

Table 1 Comparison between ascorbate peroxidases and classical peroxidases

Properties	Ascrobate peroxidases	Classical peroxidases
Glycoprotein	No	Yes
Heme	Yes/No	Yes
Ca^{2+} binding	No	Yes
Substrate	Ascorbate	Guaiacol or phenolic compounds: DAB, o-dianisidine, pyrogallol, coniferyl alcohol
Inhibition by thiol reagents	Yes (14)	No (14)
Physiological roles	To scavenge excess H_2O_2 formed in plant cell under normal and stress conditions	Biosynthetic process (36) - lignin polymerization (37) - pathogen defense - wound-healing - suberization - involved in the biosynthesis and polymerization of extension - phenol oxidation - the oxidative deterioration of vegetables

Summarized from (15) and other sources as indicated.

Peroxidase participates in the biogenesis of lignin. The enzyme can convert coniferyl alcohol into a lignin-like material which is called the dehydrogenative polymerization product (DHP) (25).

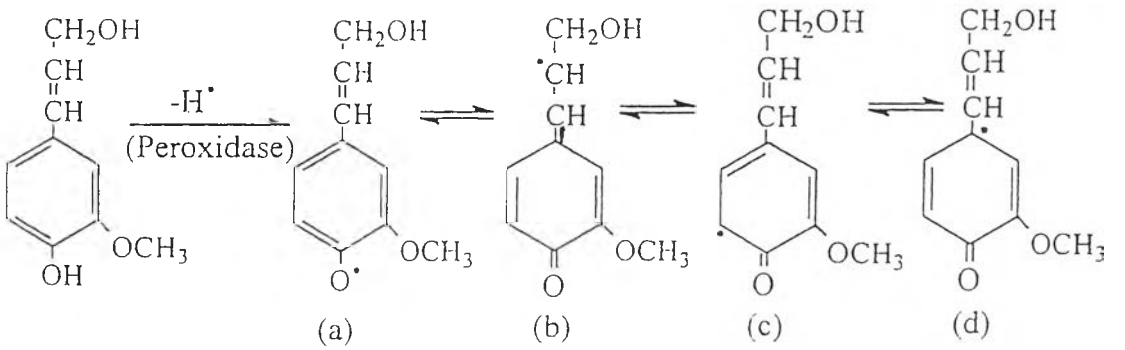


Fig 2. Scheme of dehydrogenative polymerization products of coniferyl alcohol (29). (a) – (d) are the resonant forms

4. Peroxidase found involved in plant cell wall synthesis three different classes of primary cell wall structural proteins (22) in plants are hydroxyproline-rich glycoproteins (extensins), proline-rich glycoproteins and glycine-rich proteins. Extensins are synthesised as soluble precursors but they are immobilized in the cell wall be a hydrogen peroxide/peroxidase-mediated process (23). Enhanced deposition and cross-linking of extensins in the plant cell wall lead to a more resistant barrier against pathogen infection. Induction or inhibition of extensins deposition can result in increased or decrease pathogen resistance respectively (24).

5. Peroxidase secreted in response to wounding in Hevea bark, peroxidase activity is induced by wounding from tapping injury. The enzyme can convert wound-induced latex phenols into phenolic polymers. Thus the Hevea bark peroxidase may have an opposing effect on the latex flow (27).

1.4 Cassava peroxidase

In our research group, peroxidases have been studied in many cassava tissues:

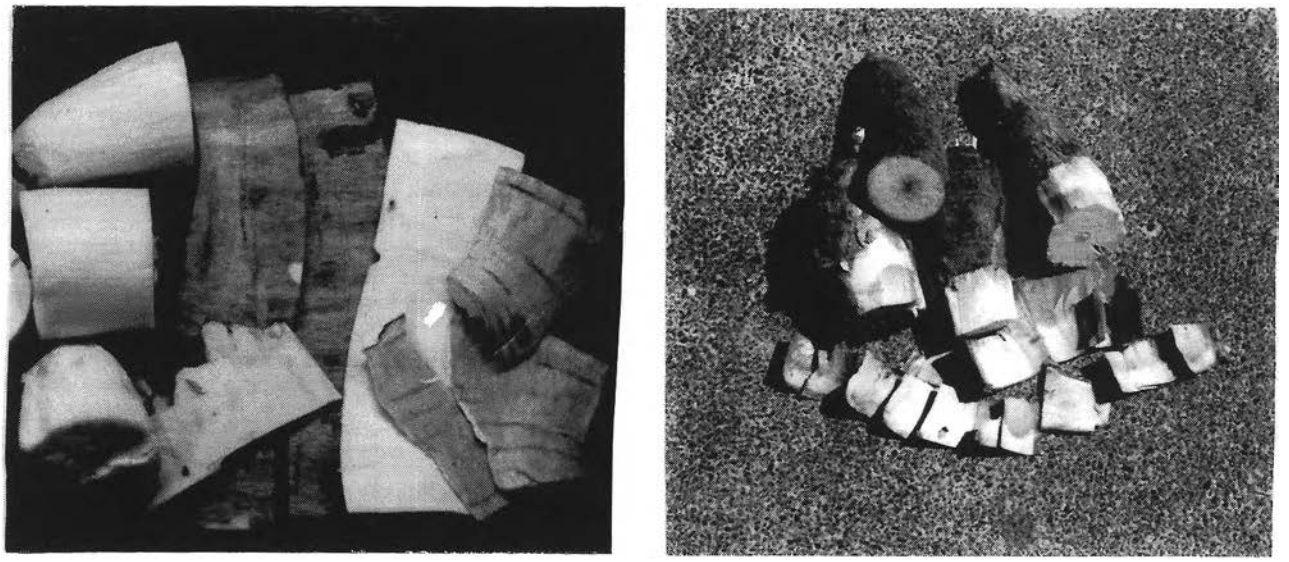
1.4.1 In roots of cassava plantlet, peroxidase activity was found to be higher than in cassava tubers (cassava roots). This isozyme showed pH optimum at 4 and pH stability at 6.5 and exhibiting the Km of 0.07 μM for 3-3′ Diaminobenzidine tetrahydrochloride (DAB) and 0.33 mM for hydrogen peroxide (71).

1.4.2 In cassava tubers, peroxidase were separated into 3 groups; A, B, C according to their mobilities in nondenaturating PAGE (Fig. 5). In post-harvest deterioration (Fig. 3), the activity of peroxidase isozyme A from the parenchyma and cortex of cassava increases while isozyme B and C decreases significantly (Figs. 4-5).

1.4.3 In cassava leaves, 3 forms of peroxidase were detected on ND-PAGE, with the slowest migrating band appeared at similar position as isozyme A in deteriorated cassava tuber. It was found to be hemoprotein with molecular weight of 112 Kd. It can be separated into 2 forms on IEF gel with pI values of 6.4 and 6.25.

The enzyme was stable in a broad pH range of 4-11 and its optimum pH was 6. The cassava leaf peroxidase can catalyze the oxidation of the following substrates: coniferyl alcohol (K_m = 0.003 mM), o-dianisidine (K_m = 0.037 mM), quercetin (K_m = 0.054 mM), syringaldazine (K_m = 0.077 mM) 3.3′-diaminobenzidine (K_m = 0.022 mM),

pyrogallol (K_m = 0.89 mM) and guaiacol (K_m = 5.52 mM). KCN, NaN_3 and thiourea were inhibitory to the enzyme (56).



A B

Fig. 3 Cortex and parenchyma of cassava tuber

A. fresh cassava (0 day)

B. storage cassava at room temperature (7 days)

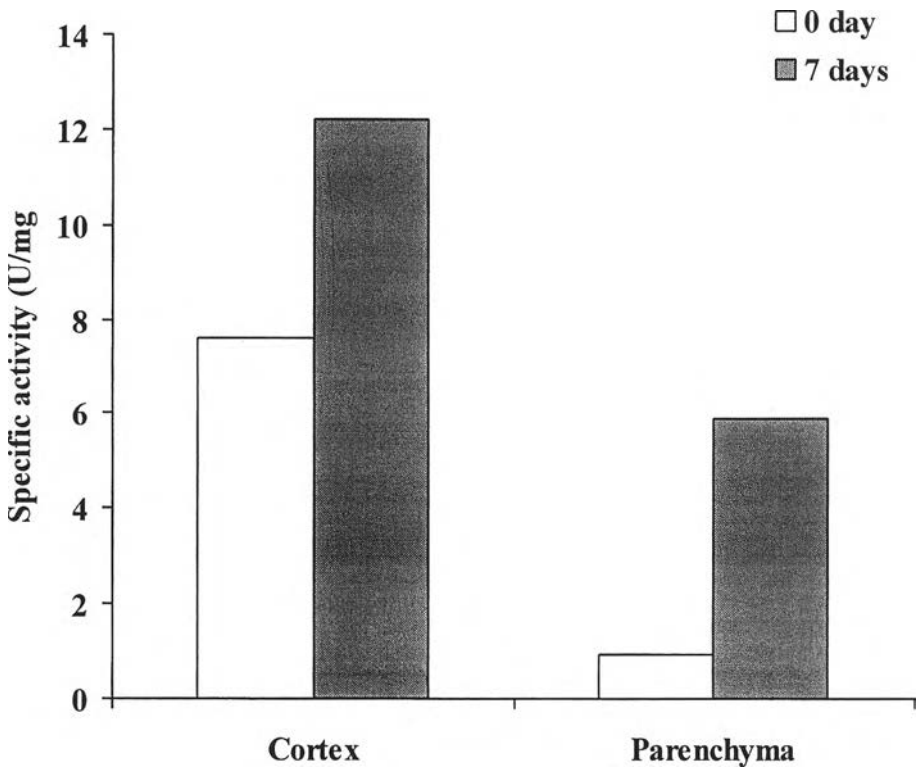


Fig 4. Chromatograms of peroxidase activity in cassava tubers

Fresh cassava tubers 0 day

Storaged cassava tubers 7 days

One unit was defined as a change of one absorbance unit at 465 nm per min.

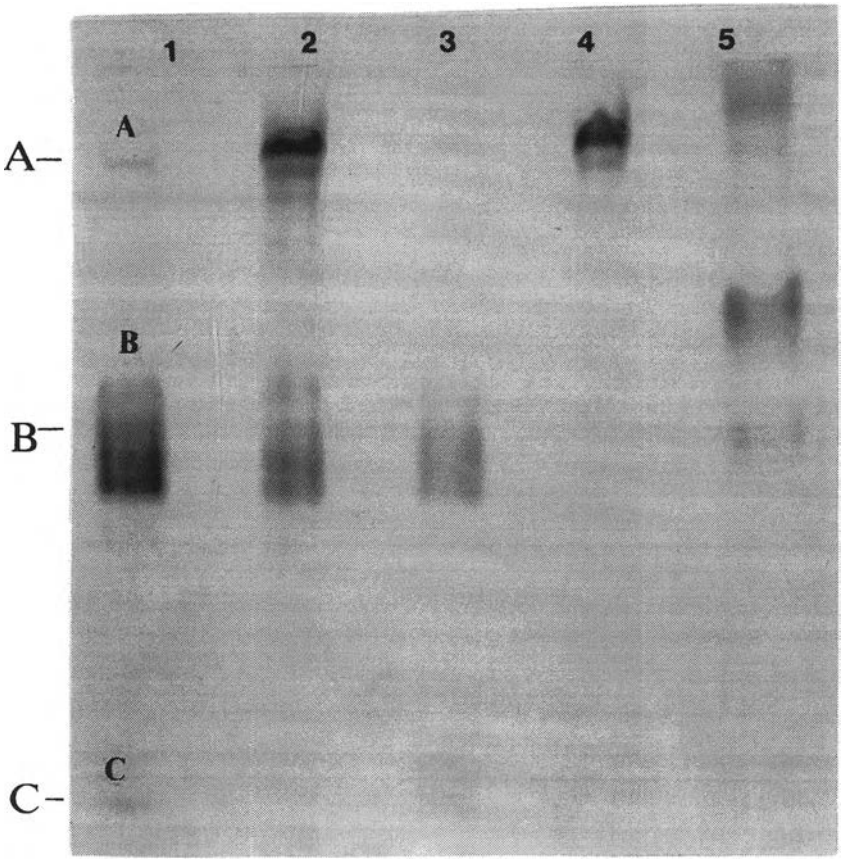


Fig 5. Peroxidase activity stain of nondenaturing-PAGE of cassava tubers (crude extracts)

Lane 1 = Cortex 0 day

Lane 2 = Cortex 7 days

Lane 3 = Parenchyma 0 day

Lane 4 = Parenchyma 7 days

Lane 5 = HRP

Amount loaded in each lane = 0.2 U

The gel (5%) was stained for peroxidase activity

A, B, C = stained for groups of peroxidases

1.5. Peroxidase reactions

Peroxidase catalyzes the oxidation of a electron donor (AH) using hydrogen peroxide as the hydrogen acceptor. The stable oxidation product (A) is produced by the reaction. The overall reaction mechanisms is shown below (28).

$$\text{Native Enzyme} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + AH \longrightarrow \text{Compound II} + A$$

$$\text{Compound II} + AH \longrightarrow \text{Native Enzyme} + A + 2H_2O_2$$

$$\text{Sum: } H_2O_2 + 2AH \longrightarrow 2A + 2H_2O_2$$

Oxidation of the substrate (AH) involves the activation of ferric peroxidase by H_2O_2. In the process, two electrons are removed from the native enzyme, producing ferryl (IV) porphyrin cation radical (or protein radical) of the enzyme known as compound I. Compound I is an active form of enzyme. It is unstable and has a characteristic green color. It can oxidize organic compounds (AH), and itself is reduced by an electron to compound II. Compound II is also an active form. It is quite stable and has a characteristic red color. A subsequent one-electron oxidation returns the peroxidase to its ferric state. The excess of H_2O_2 converts compound II into compound III. Compound III is known as oxyperoxidase which is an inactive from of the enzyme as shown the following equation.

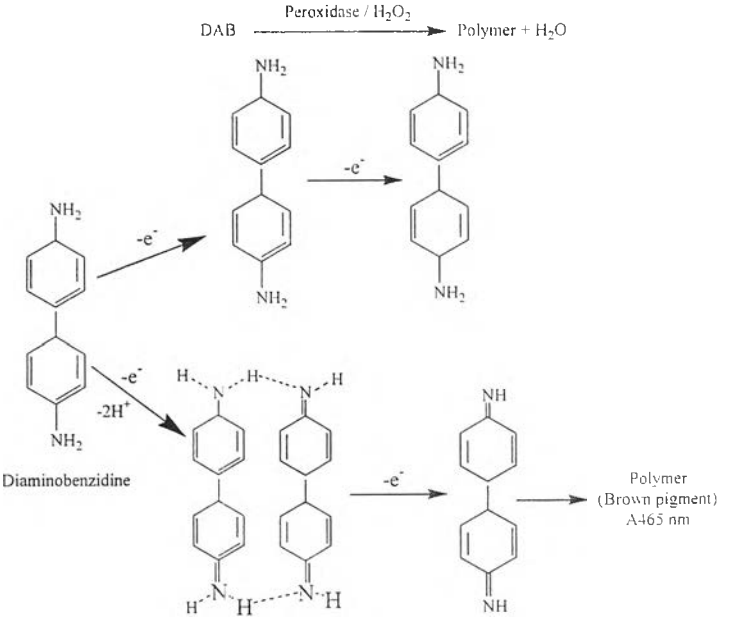
$$\text{Compound II} + H_2O_2 \longrightarrow \text{Compound III, inactive}$$

The reaction are generally considered as irreversible change. The rate-determining step in peroxidation is the return of compound II back to the native enzyme (32).

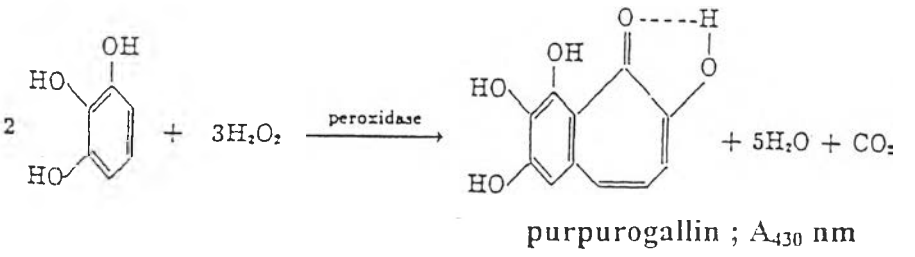
A number of substrates can be used as hydrogen donors in peroxidase reaction, eg. phenols 3',3-diaminobenzidine (DAB), coniferyl alcohol, guaiacol, pyrogallol and o-dianisidine. Their reactions are shown in Figure 6 . The DAB assay provides a suitable procedure for quantitation and biochemical characterization of peroxidases, immunochemical studies using peroxidase-labeled antigen-antibody reactions and histochemical staining for peroxidase.

The reactions using some selected substrates of peroxidases are shown below:

(a) Diaminobenzidine (32)



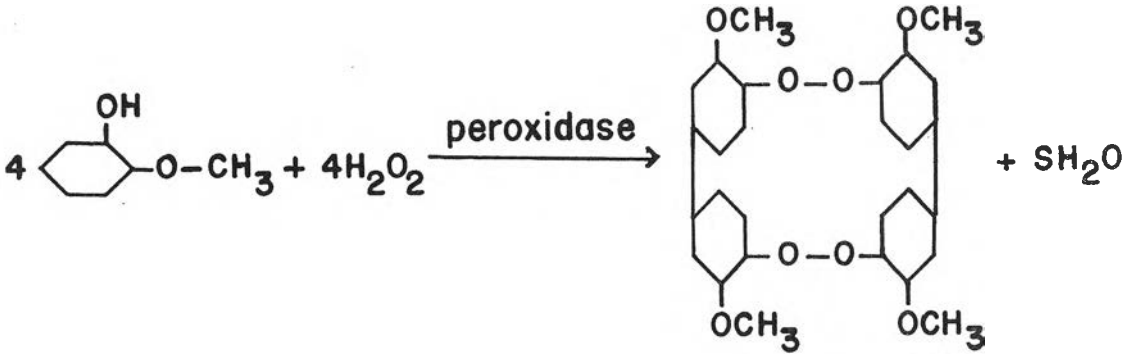
(b) Pyrogallol (33)



purpurogallin ; A_{430} nm

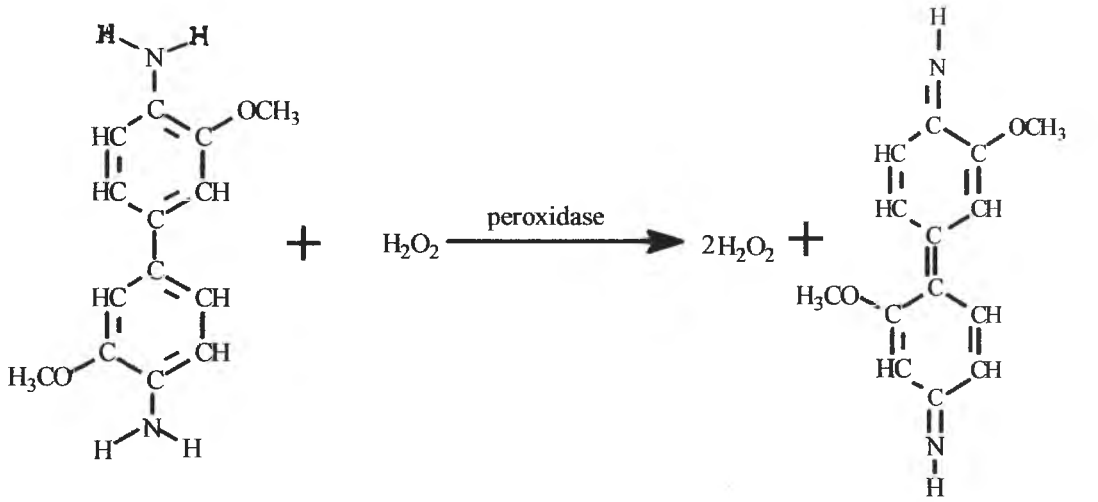
Fig. 6 The reaction of peroxidase using selected substrates

(c) Guaiacol (**34**)



tetraguaiacol ; A_{470} nm

(d) o-Dianisidine (**35**)



Oxidized O-dianisidine A_{460}

Fig. 6 (cont'd) The reaction of peroxidase using selected substrates

1.6 Application of peroxidases

Peroxidases have been used mainly analytical in bioscience. Selected popular applications, are below:

1.6.1 Peroxidase-conjugated antibody for enzyme-linked immunosorbant assay (ELISA)

The use of enzyme-labelled antibody in immunochemistry and ELISA is popular because of their simplicity and sensitivity (30). This technique allows detection and quantitation of small amounts of antigen or antibody in biological fluid. Many different enzymes have been used. Among these, horseradish peroxidase is widely employed.

In the ELISA, an antibody specific for protein of interest is attached to the bottom of a well in a plastic tray, to which biological fluid are added. If any protein is present, it will bind to the antibodies. The well is then washed, and a second antibody also specific for the protein also is added. This second antibody is linked to an enzyme peroxidase that catalyzes the conversion of a colorless substrate to a colored one; the amount of colored compound produced provides a sensitive measure of the amount of protein present.

1.6.2 Electronmicroscopic staining

Immunoelectron microscopy is a technique characterized by the association of various electron-dense markers with antibody or other immunoreactive macromolecules. Under electron microscope, it is possible to identify the antigen-antibody complexes at the ultrastructural level.

Histochemical methods for peroxidase localization in tissues or cells was first developed in 1966 by Graham and Karnovsky. The visualization of the enzyme localization is based on the use of DAB. The polymer end products form the precipitate, insoluble and electron-opaque product at the site of the peroxidase reaction (31). Tissue was dehydrated through a graded series of ethanol, and embedded in paraffin. Specimens were cut into small pieces in thickness and then stained immunohistochemically by incubation with diluted anti-peroxidase antiserum, the tissue sections was sequentially exposed to anti-rabbit immunoglobulins and HRP peroxidase-conjugated streptavidin. The staining reaction was visualized with DAB.

1.6.3 Enzyme coupled assays

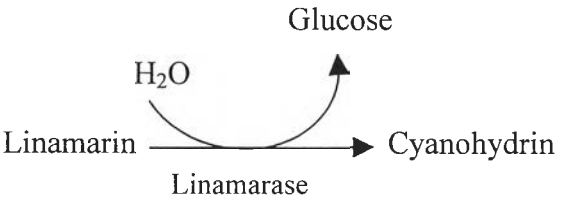
Hydrogen peroxide is produced by enzymic conversion of a number of clinically significant compounds. One of the methods to follow the formation of H_2O_2 is by using a phenolic compound to form a colored product in the presence of a peroxidase.

For example, in uric acid assay, chromogen ABTS (2,2'-azinodi(3-ethylbenzthiazoline-6-sulfonate) can be used.

$$\text{Uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantonin} + H_2O_2 + CO_2$$

$$H_2O_2 + ABTS_{red} \longrightarrow ABTS_{ox} + 2H_2O \qquad (38)$$

A new chemiluminescent assay of linamarin consists of coupled reactions. The first reaction is the conversion of linamarin to glucose by linamarase. The second reaction is the conversion of glucose to H_2O_2 by glucose oxidase. Utimately, H_2O_2 is assayed by adding luminol solution containing of HRP to give 3-aminophthalate with the emission of light (39).



$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{luminol} \xrightarrow{\text{HRP}} \text{3-aminophthalate} + \text{light}$$

1.7 Deglycosylation of peroxidase

Enzyme deglycosylation glycoproteins usually used with are the endoglycosidasse N-glyconase, endo M, and endo F (40). These endoglycosdases had no effect on HRP. Chemical deglycosylation using trifluoromethanesulfonic acid (TFMS) have been employed by several groups (40). Peroxidases have been reported to be glycoproteins (20). Deglycosylation studies have been performed on peroxidase of peach seeds (41) and avocado (42).

Partial deglycosylation of an anionic peroxidase purified from peach seeds using N-glycosidase (PNGase F) resulted in reduced activity against selected substrates, a higher Km for H_2O_2, higher values for Arrhenius activition energy and enthalpy, and a decrease in enzyme stability at 4°C. Partial deglycosylation of an avocado peroxidase using PNGase F also caused a decrease in the K_m for reductor substrates and a decrease in thermostability. Together, these experiments suggest that the glycan is important for enzyme activity and stability.

1.8 Aim of thesis

The aim of this study is to purify and characterize the peroxidase form A from parenchyma of post-harvested cassava roots including comparative study of the deglycosylated and native enzymes.