CHAPTER 1

INTRODUCTION

I. Introduction

Peroxidase enzymes are ubiquitous in living organisms. The primary biological function of peroxidases is to oxidize a variety of hydrogen donors at the expense of peroxide or molecular oxygen in oxidative reactions (Welinder, 1992).

Enzymatic oxidation has been called the technology of the future, because as environmental needs and regulations continue to become more severe, destructive treatment technologies consistent with waste minimization must be developed. In addition, the ideal oxidant must be inexpensive and readily available.

Many oxidative enzymes have been studied and described in terms of isolation, characterization and potential applications. However, the use of these enzymes is restricted by requirements of other cellular components as cofactors, which can only be efficiently regenerated inside the cells or in the case of membrane enzymes, in the presence of intact cellular membranes. Peroxidases are oxidative enzymes which do not need any other cellular components to work and have a broad substrate specificity (Torres *et al*., 2003). These enzymes, which are widely distributed in nature, have been studied for many years i.e. their potential uses as biocatalysts in pulp and paper bleaching (Archibald *et al*., 1997), waste-water treatment (Durán and Esposito, 2000), soil remediation (Machado *et al*., 1996), on-

site waste destruction and medical diagnosis (Kaplan *et al*., 1988). The other areas where peroxidases would have an immediate use and economic impact are formaldehyde formation, food application as indicator of oxidative species formed (Cluck *et al*., 1996), and synthesis of polyelectrolyte complexes (Sakharov *et al*., 2003). Recently, the combination of peroxidase and indole-3-acetic acid (IAA) has been introduced as a novel cancer therapy (Kim *et al*., 2004). The sales of peroxidase for medical diagnosis could reach four million dollars per year, and novel applications in the food industries have enormous potential for growth (Bhopale and Naik, 2000). The peroxidase produces polyelectrolyte complex, polyaniline, which does not produce a waste stream (Sakharov *et al*., 2003). In each of these areas, peroxidase has advantages over other technologies currently in use. The natural catalyst peroxidase offers significant improvements over traditional processes by minimizing the use of toxic chemicals, lowering capital costs, producing cleaner products, increasing cost efficiency, and producing unique chemical products (Bhopale and Naik, 2000). However, an important aspect must be kept in mind, namely that peroxidases degrade over time along with their raw materials degradation. Superior temperature stability, long shelf life and increased sensitivity are really required.

In an isozyme electrophoretic study of peroxidase in *Hevea brasiliensis* using leaves and C-serum of rubber latex, higher peroxidase activity was found in leaves (Suvachittanont and Pongpaiboon, 1994). The purified peroxidase from *Hevea* leaves showed three bands in non-denaturing polyacrylamide gel electrphoresis (ND-PAGE) and two bands in SDS-PAGE, with the MW of the enzyme being 204,000 daltons by gel filtration. These peroxidases are acidic glycoproteins, with a pI of 3.6 and an absorbance at 403 nm, indicating the presence of a heme in the enzyme. They also

exhibited great thermostability and long shelf life (Rattanapumee, 2000). In this study, peroxidase from *Hevea* leaves was purified and some characteristic properties were examined. Its potential applications as a coupled enzyme in clinical diagnosis and the advantage of peroxidase-antibody conjugates were also verified. The prepared peroxidase-antibody conjugates were used as second antibodies in detecting protein of interest in various samples. Genes for peroxidase from *H. brasiliensis* were also cloned and sequenced.

II. Review of Literature

1. *Hevea* botanical aspects and distribution

Hevea brasiliensis was originally described under *Hevea* by Jean Mueller – Argoviensis. Taxonomic classification is as following:

Class: Angiospermae

Subclass: Dicotyledoneae

Order: Euphobiales

Family: Euphobeaceae

Genus: *Hevea*

Species: *brasiliensis*

H. brasiliensis is found in the natural tropical forests in the Amazon region, in particular in the low lands of Brazil, Bolivia, Colombia, Ecuador, French Guiana, Guyana, Peru, Surinam and Venezuela. Under optimal conditions, natural *H. brasiliensis* is one of the tallest species in the genus, growing up to 40 m in height.

All the clones cultivated today, called Wickham clones, come from a few seeds collected in 1876 by H. Wickham in Brazil. From the original ‘Wickham gene

pool' with a productivity range of 200-300 kg dried rubber/ha/yr, breeders have developed clones with a production potential of above 3000 kg/ ha/ yr (Mercykutty *et al*., 2002). At present, a good number of high yielding clones are available for commercial plantations (Figure 1). In order to obtain high-yielding rubber clones, correct identification of each rubber clone is necessary.

Identification of clones is rather difficult and not always reliable when the trees are immature. Techniques like electrophoresis and the use of molecular markers have also been employed to differentiate between clones. Walujono and Agung Suseno (1973) proposed the use of a gel pattern obtained by electrophoresis of latex serum as a mean to identify clones, but this method is suitable only for mature trees and also appears too elaborate for practical purposes. Applications of the isozyme technique to distinguish germplasm materials (Chevallier, 1988) and budwood gardens (Leconte *et al.,* 1994) have also been reported. Genetic fingerprinting using RFLP (Restriction Fragment Length Polymorphism) by Besse *et al.,* (1994) and the possible application of RAPD (Random Amplified Polymorphic DNA) markers by Varghese *et al.,* (1997) have proved more effective than isozymes in identification of *Hevea* clones.

Figure 1. A plantation of cultivated rubber trees (*Hevea brasiliensis*).

The most foolproof method to identify trees of *Hevea* clones is by observing the morphology of seeds. However, without having the correct seeds in hand for comparison, morphological properties have to be used. Trees of *Hevea* clones are described based on the morphological characteristics, for examples: stem shape, virgin bark colour, appearance, texture, thickness and leaf scars, branching habit, crotch height, crown appearance and shape, foliage colour and density, fruit size, colour of latex and growth characters. These characters are generally applicable to trees of all ages (Mercykutty *et al*., 2002).

2. *Hevea brasiliensis* (rubber tree) in Thailand

H. brasiliensis was taken from the Amazon to South Asia (Sri Lanka) and South East Asia (Singapore and Malaysia) by the British Colonial Office where it was grown experimentally and later on plantations. Subsequently, cultivation spread to Vietnam, Indonesia and Thailand, and then Africa.

Hevea trees in Thailand are called Para-rubber trees or rubber trees. They were introduced in Thailand from Malaysia by Praya Ratchadanupradit (Coresimbee) Na Ranong in 1899 to Trang Province, Southern Thailand. It later spread to Chantaburi Province, in Southeastern Thailand in 1928. The rubber tree growing areas are currently mainly found in Southern Thailand, where the rubber plantations comprise 88.4% of the total rubber holdings in Thailand. Some 9.8% is in Southeast, and 1.8% is located in the Northeast (Kangpitsadan, 2004). Natural rubber produced from rubber latex is one of the major export commodities of Thailand. In fact, Thailand has been the world's largest producer natural rubber since 2001, when it overtook Indonesia and Malaysia. The natural rubber production of the world's five top-countries is shown in Table 1 (Kerdvongbundit *et al*., 2003).

The source of rubber leaves used in this study is the RRIM 600 clone, one of the recommended clones belonging to the first category variety of Rubber Research Institute of Thailand. Its main morphological characters are shown in Figure 2.

Table 1. The world's five top-countries for natural rubber production (million tons) (Kerdvongbundit *et al*., 2003)

Country	1999	2000	2001	2002	2003	2004*
Thailand	2.15	2.35	2.32	2.62	2.65	2.70
Indonesia	1.60	1.50	1.61	1.63	1.65	1.67
Malaysia	0.77	0.62	0.55	0.59	0.60	0.61
China	0.46	0.45	0.46	0.47	0.48	0.49
Vietnam	0.23	0.29	0.32	0.37	0.39	0.42
Other	1.61	1.52	1.93	1.59	1.58	1.56
Total	6.82	6.73	7.19	7.27	7.35	7.45

*Estimated

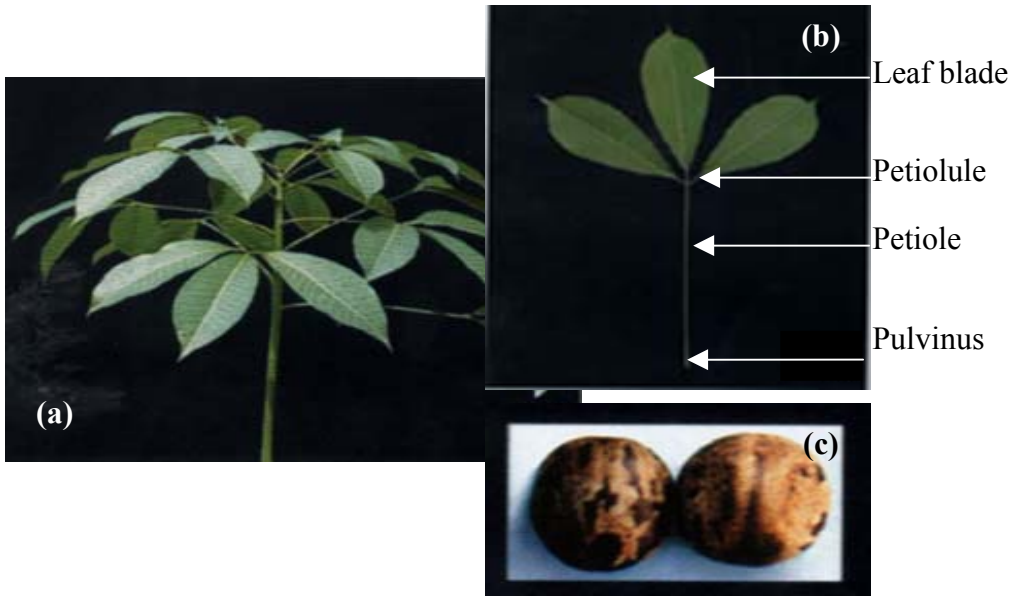


Figure 2. Morphological characters of RRIM 600 clone a) fully grown leaf storey, b) single leaf and c) seed-dorsal (left) and ventral (right) sides.

3. *Hevea* latex

H. brasiliensis latex is a specialized cytoplasm of the laticiferous tissue and contains 30-40% rubber, which chemically is cis-1,4-polyisoprene. In general, the latex has a biological function in herbivore defense. At the present day, it is an economic product in the world market. Latex is obtained by wounding the bark by a process termed tapping. The fresh latex is a suspension of rubber and non rubber particles in an aqueous medium (Frey-Wyssling, 1929; Southorn, 1961 and Archer *et al*., 1969). Using high speed centrifugation at 59,000 *x g*, Cook and Sekhar (1953) separated latex into four fractions. These are: an upper white fraction of rubber cream, an orange or yellow layer containing Frey-Wyssling complex, a colourless serum named C-serum and a grayish yellow gelatinous sediment, the 'bottom fraction' consisting mainly of lutoids (Dickenson, 1969). The production of rubber is largely dependent upon (a) volume and type of laticiferous tissues in which latex is stored, (b) capacity of storage vessels, (c) physiological and biochemical processes controlling latex flow and (d) capacity of the tree to resynthesize latex and other organic constituents within the drained area (Jacob and Prevot, 1992). The organic non-rubber constituents such as proteins, carbohydrates, lipids, phospholipids and nucleic acids play a significant role in the resynthesis of latex to replace that which has been drained off through tapping. The organic non-rubber constituents of fresh latex are presented in following chart (Figure 3) (Pakianathan *et al*., 1992).

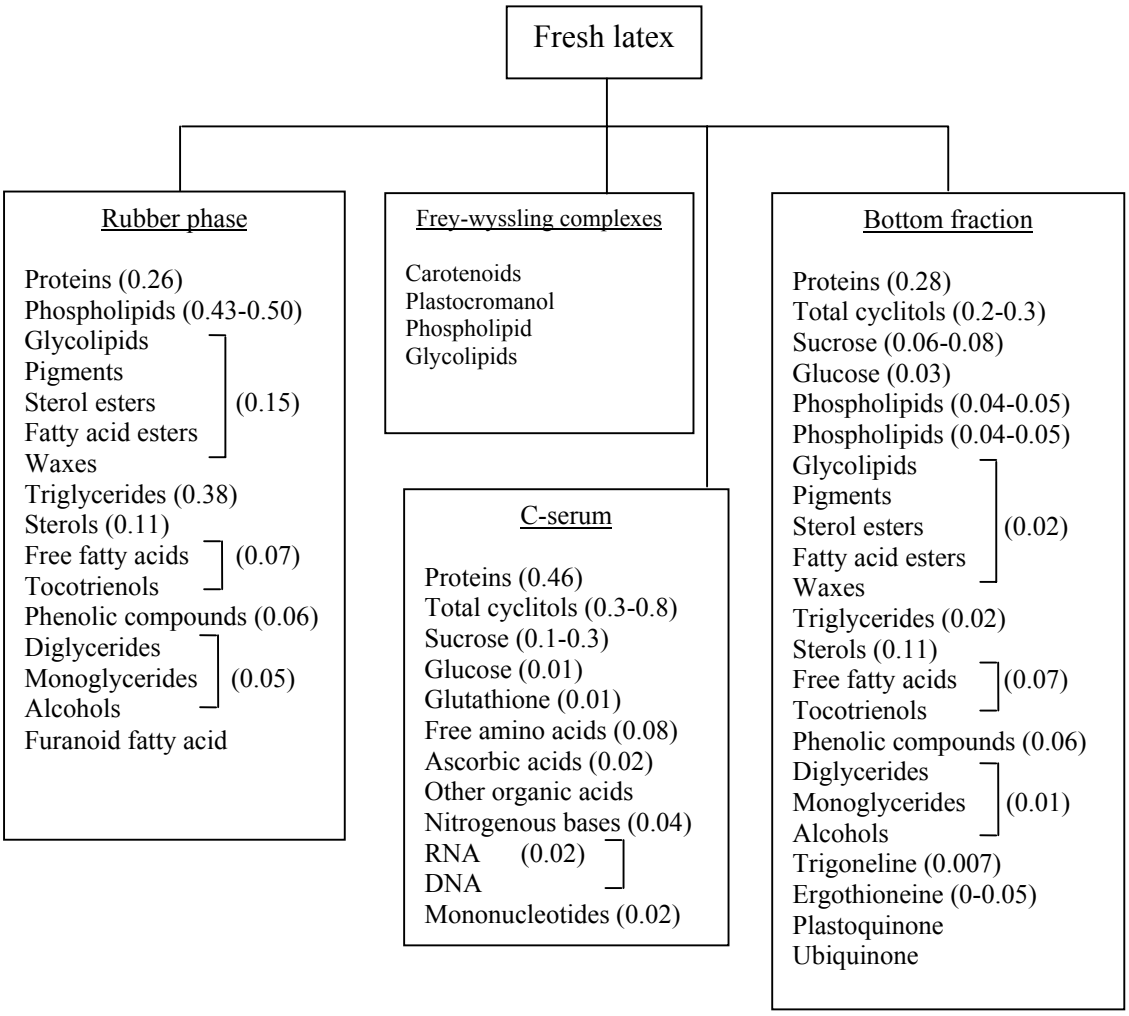


Figure 3. Organic non-rubber constituents of latex with approximate concentration in g/100 g latex (Pakianathan *et al*., 1992).

4. *Hevea* leaves

Leaf of *Hevea* is palmately compound, which is folded back at emergence, produced in storey or whorl between the length of the bar stem. It consists of pulvinus, petiole, (leaf stalk) and three leaflets, each of which comprises a petiolule and a lamina/leaf blade. Extrafloral nectarines, the nectar secreting glands, are present in the distal end of petiole (Figure 2) (Mercykutty *et al*., 2002). In young plants, leaves of each whorls form a distinct layer on the stem, producing two to four whorls every year. Leaf characters are of special significance for description of clones. Besides their functions of transpiration and respiration, the leaves are the factories in which the organic plant food, sucrose, is formed and transported to the laticiferous cells (Jacob and Prevot, 1992).

Recently, not only rubber latex but also other parts from the rubber tree are used to make high value export products, such as home furnitures from rubberwood. Rubber leaves are also used in the production of ornamental flowers. Moreover, some valuable substances have been extracted from the tree, like peroxidase enzyme from rubber leaves. It is well known that peroxidases are used in many industrial sectors, as for instance peroxidase-antibody conjugates in clinical and research diagnosis. Most of this important biochemical substance is currently extracted from horseradish roots and exported world-wide by Europeans.

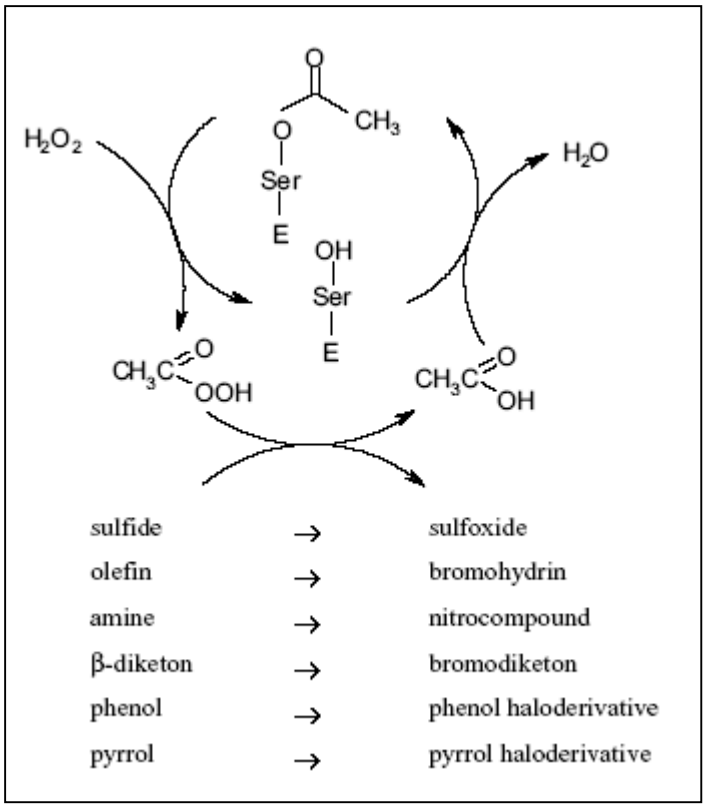
5. Classification of peroxidases

Peroxidases (E.C. 1.11.1.-; donor : hydrogen-peroxide oxidoreductase) are enzymes which catalyze the oxidation of various electron donor substrates (AH_2: phenols, aromatic amines) by hydrogen peroxide. They are classified into three

groups based on the specific active center in their molecules by Welinder (1992) as follow:

5.1 Nonheme (or metal free) peroxidases

The peroxidases in this group are enzymes without heme and metal as a prosthetic group at the active site. They use functional acetate or propionate derived from serine in their reactions. An example is bacterial haloperoxidase from *Serratia marcescens*, which is capable of incorporating halogen atoms into the molecules of organic substrates under the influence of hydrogen peroxide and halide ions (Preobrazhenskaya *et al*., 2003). It catalyses the halogenation reaction under the influence of acetate ions. A key position in its active site is the Asp-His-Ser catalytic triad (Hofmann *et al*., 1998). Acetate ions bind covalently with the nucleophilic serine residue and the thus obtained ester is then hydrolyzed by hydrogen peroxide to form peracetic acid. Being a strong oxidizing agent, this is able to oxidize halide ions to the corresponding hypohalous acid, which readily incorporates the halogen atom into organic substrates as in the following reaction (Preobrazhenskaya *et al*., 2003).



5.2 Vanadium peroxidase

Vanadium peroxidase was first discovered in the brown algae *Ascophyllum nodusum* in 1984 (Boer and Wever, 1988), and after that in lichen *Xanthoria parichina* and some fungi. Vanadium peroxidases contain a vanadate ion (VO_4^{3-}) at their active site and are most commonly found in marine plants such as seaweeds (Wever *et al*., 1991). It also oxidizes halides in the presence of hydrogen peroxide to the corresponding hypohalous acids according to:

$$H_2O_2 + X^- + H^+ \longrightarrow H_2O + HOX$$

or in the absence of a suitable substrate followed by the oxidation of H_2O_2 into singlet oxygen and water (Griffin, 1991). In addition, it has been shown that the chloroperoxidase (CPO) secreted by the fungus *Curvularia inaequalis* is also a

vanadium enzyme. CPO forms HOCl as a product which is a strongly bactericidal and oxidizing agent (Littlechild, 1999). It has been suggested that the enzyme and product of the fungus are used to oxidize plant cell walls to facilitate penetration of the fungus into the host. The tertiary structure of the enzyme from *C. inaequalis* is mainly helical with two four-helix bundles (α-helices B, C, E and F and α-helices K, L, N and O) as main structural motifs (Figure 4). The vanadium center is located on top of the second four-helix bundle (Messerschmidt and Wever, 1996).

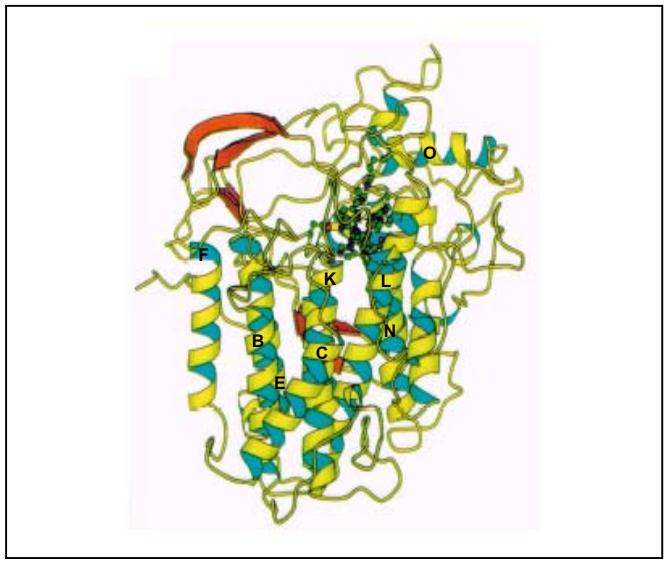


Figure 4.Three-dimensional structure of vanadium peroxidase from *C. inaequalis*. Shown are the main structural motifs : the two four-helix bundles (α-helices B, C, E and F and α-helices K, L, N and O) and the vanadium center (green) (Messerschmidt and Wever, 1996).

5.3 Heme peroxidases

Heme peroxidases are heme containing enzymes that use hydrogen peroxide (H_2O_2) as the electron acceptor to catalyse a number of oxidative reactions (Duker, 1997). Active sites of all heme peroxidases contain the prosthetic group, ferriprotoporphyrin IX, containing iron (III), and made up of four pyrrole rings joined by methane bridges with iron (III) centering the molecule. All known heme peroxidases have the *b*-type heme (Figure 5).

Heme peroxidases can be categorized into two superfamilies on the basis sequence similarity : fungal, plant and bacterial peroxidases form one superfamily and animal enzymes form another (Welinder, 1992).

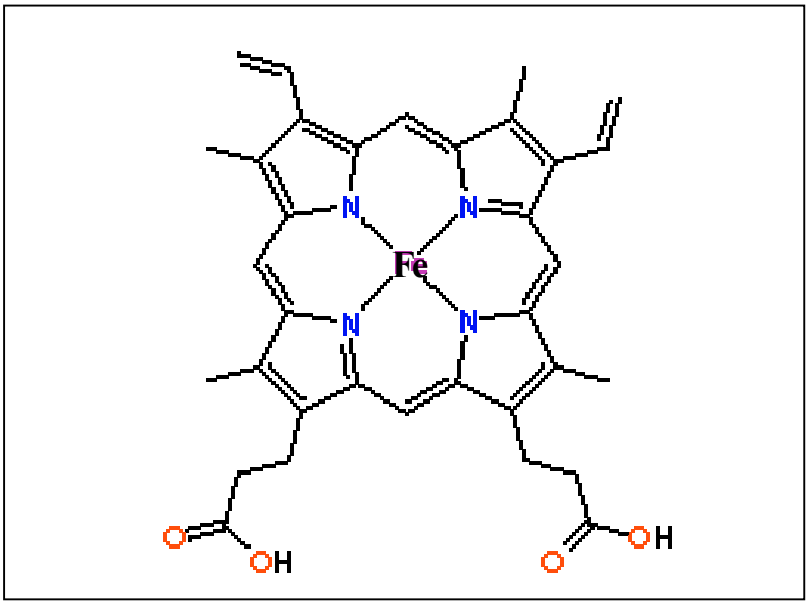


Figure 5. The *b*-type heme of peroxidase containing ferriprotoporphyrin IX as a prosthetic group (www.metallo.scripps.edu/PROMISE/HEAMCPO).

5.3.1 The animal peroxidase superfamily

Animal peroxidases include lactoperoxidase, thyroid peroxidase, myeloperoxidase, eosinophil peroxidase and glutathione peroxidase (Welinder, 1992). These enzymes may have halides or pseudo-halides as electron donors. Myeloperoxidase is one of the well-known enzymes in this superfamily and is localized in mammalian neutrophils. It participates in a highly complex antimicrobial response (Zeng and Fenna, 1992). The phagocytosis of pathogenic microbes undergoes a "respiratory burst", resulting in the production of toxic oxygen species, including H_2O_2. Within the phagosome, the enzyme catalyzes the hydrogen peroxide mediated peroxidation of halide ions and the pseudo halide thiocyanate in the reaction:

$$H_2O_2 + Cl^- + H_3O^+ \longrightarrow HOCl + 2H_2O$$

Products of the reaction and their secondary metabolites are responsible for killing phagocytosed bacteria and viruses (Griffin, 1991).

5.3.2 The plant peroxidase superfamily

There are also three classes of peroxidase in the plant peroxidase superfamily, based on their sequence homologies (Welinder, 1992).

Class I. Peroxidase of prokaryotic origin.

Class I peroxidases lack carbohydrates, cysteine bridges, calcium ions, and signal peptides for secretion (Welinder, 1992). These peroxidases include yeast cytochrome *c* peroxidase, chloroplast, and cytosolic ascorbate peroxidases. The amino acid sequence and crystal structures show a certain similarity with pea

cytosolic ascorbate peroxidase and with yeast cytochrome *c* peroxidase (Schuller *et al*., 1996). Yeast cytochrome *c* peroxidase and ascorbate peroxidases also have high sequence similarity to bacterial catalase-peroxidases (Vainshtein *et al*., 1986). Therefore, bacterial catalase-peroxidases are also classified as part of the class I family of plant peroxidases. A search of the public sequence database for all known ascorbate peroxidases, results in five cytosolic ascorbate peroxidases, three of them membrane-bound, and two types of chloroplast ascorbate peroxidase. Ascorbate peroxidase was also detected in soybean root nodules and in cyanobacteria. The role of ascorbate peroxidase as a scarvenger of photosynthetically generated hydrogen peroxide is now clear. It catalyses the removal of hydrogen peroxide by ascorbate in the following reaction:

$$H_2O_2 + 2\ \text{Ascorbate} \longrightarrow H_2O + 2\ \text{Monodehydroascorbate}$$

This monodehydroascorbate is a fairly unreactive free radical, which explains its protective role against oxidative damage. Its formation may remove a much more reactive free radical (Dalton, 1991). The other two class I crystal structures are those of the yeast and pea cytosolic enzymes. The pea cytosolic enzyme is dimeric, whereas the chloroplast enzymes are monomeric (Bosshard *et al*., 1991).

Class II. Secreted fungal peroxidases.

Enzymes in this class are the lignin peroxidases (LiP) and manganese peroxidases (MnP) of *Phanerochaete chrysosporium* and the ink cap mushroom peroxidase from *Corpinus cinerius*. They have an amino terminal signal peptide sequence for secretion through the endoplasmic reticulum. They consist of about 5%

carbohydrate, two calcium ions, and four conserved disulfide bridges (Welinder, 1992).

Lignin is second to cellulose in abundance and plays a very important role in the carbon cycle. Its degradation is accomplished aerobically by a narrow array of microbes, which have their own hydrogen peroxide system (Reddy, 1993). It is now known that there are at least eight isozymes of LiP, (Schuller, 1996). Three isoforms of LiP, I, II and III, were isolated and shown to be glycoproteins, and their amino acid sequences were also analyzed (Rothschild *et al*., 2002). The dominant form was isozyme II. There was one heme per protein and that heme was ferriprotoporphyrin IX. The heme of lignin peroxidase is exceptionally sensitive to degradation by hydrogen peroxide, as the heme is the site of the electron-transfer reaction (Martinez, 2002). A preferred substrate of lignin peroxidase is veratryl alcohol, which was shown to reduce compound I to compound II and then compound II to native enzyme, suggesting that the alcohol plays a role in preventing enzyme degradation. The reaction of lignin peroxidase using veratryl alcohol as substrate is shown below (Reddy and D'Souza, 1994).

$$\text{Compound I} + \text{Veratryl alcohol} + H^+ \longrightarrow \text{compound II} + \text{Cation}^{\cdot}$$

$$\text{Compound II} + \text{Veratryl alcohol} + H^+ \longrightarrow \text{Native LiP} + \text{Cation}^{\cdot} + H_2O$$

$$\text{Cation}^{\cdot} \longrightarrow \text{Benzyl}^{\cdot} + H^+$$

$$\text{Cation}^{\cdot} + \text{Benzyl}^{\cdot} \longrightarrow \text{Veratryl alcohol} + \text{Veratryl aldehyde} + H^+$$

Class III. Secreted plant peroxidases.

The class III peroxidases contain two types of metal centers, namely iron (III) protoporphyrin IX and two calcium atoms (Figure 6). Both are essential for the structural and functional integrity of the enzymes. The class III peroxidases are N-linked glycoprotein. The glycosylation corresponds to the MW ranges from 0% in tea leaf peroxidase (Kvaratskhelia *et al*., 1997) to 26% in barley leaf peroxidase (Saeki *et al*., 1986). Soybean seed coat peroxidase contains 18% glycans, which are known to be heterogeneous (Gray *et al*., 1996). Its seven glycosylation sites have been observed (Welinder and Larsen, 2003). The most studied isoenzyme C from horseradish root (HRP C) is also a heterogeneous glycoprotein, comprising a single polypeptide of 308 amino acid residues (Welinder, 1976). It has nine N-glycosylation sites recognized in the primary sequence from the motif N-X-S/T (where 'X' represents an amino acid residue) and of these, eight are occupied at N13, N57, N158, N186, N198, N214, N255 and N268 (Veitch, 2004). Its total carbohydrate content (18 and 22%) depends on the source of the enzyme.

Most peroxidases are single polypeptides. MW of peroxidases from different plants varies depending on the polypeptides and glycans values in their molecule from low to high MW. Examples for low MW peroxidases are found in Korean radish (30.1 kDa), cucumber (35.2 and 36.5 kDa), *Arabidopsis* (36.3 kDa), turnip (39.3 kDa) and horseradish (42 kDa) (Lee and Kim, 1994; Battistuzzi *et al*., 2001; Kavita, *et al*., 2004; Agostini *et al*., 2002). High MW peroxidases are found in mung bean, potato and coconut with 152, 170 and 196 kDa, respectively (Chabanet *et al*., 1993; Decedue *et al*., 1984; Mujer *et al*., 1983). Mung bean hypocotyl peroxidase and coconut

endosperm peroxidase were found to consist of three polypeptides with MW of 48 and 55 kDa, respectively.

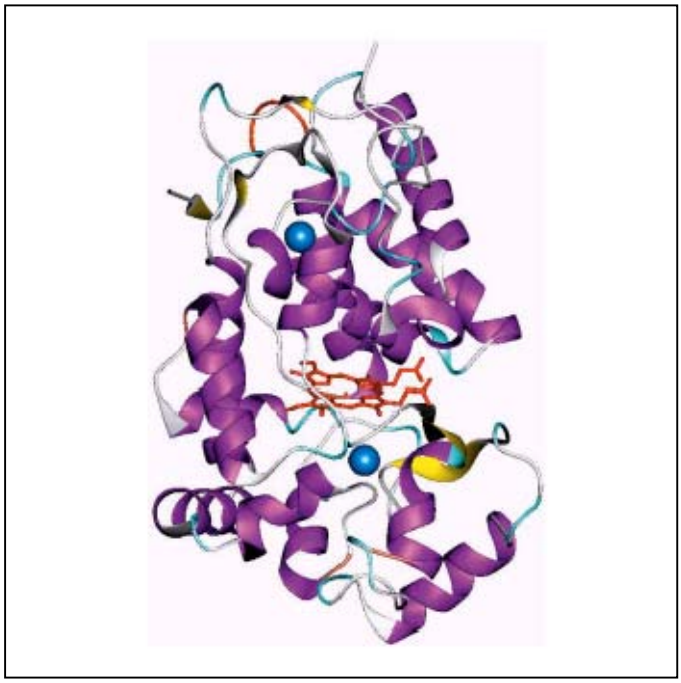


Figure 6. Three-dimensional structure of HRP isoenzyme C. The heme group (red) is located between the distal and proximal domains, and which each contains one calcium atom (blue spheres). A-Helical and β-sheet regions of the enzyme are shown in purple and yellow, respectively (Veitch, 2004).

Substrate specificity of peroxidases also varies (Chabanet *et al*., 1993; Converso and Fernandez, 1995). This character is sometimes used to identify heme peroxidase, as in ascorbate peroxidase from tea leaves, because its activity determined

by using ascorbate as substrate was higher than that using guaiacol (Kvaratskhelia *et al*., 1997). Most heme peroxidases can use guaiacol as substrate, and are called guaiacol-type-peroxidase. The level of peroxidase activity in many plants has been shown to fluctuate depending on various environmental factors such as stress, salt, and temperature (Siegel *et al*., 1982).

Peroxidases in various plants have several physiological functions. The enzymes are widely believed to catalyze the last enzymatic step in biosynthesis of lignin (van Huystee and Zheng, 1993; Christen *et al*., 1998). Three of the five isolated peroxidases (PXP3, 4 and 5) from poplar xylem could oxidize the lignin monomer analog syningaldazine. These isoenzymes are also specifically expressed in poplar xylem (Christen *et al*., 1998). In another study, a cationic cell wall peroxidase from poplar callus showed a strong substrate preference for sinapyl alcohol and syringaresinol, which are found in plant lignification (Sasaki *et al*., 2004). Therefore, these peroxidases were suggested to be involved in lignification of plant cell wall. Sources of higher plant peroxidases from various functional organs and their specific substrates are listed in Table 2.

HRP C and other plant peroxidases are involed in metabolism of indole-3-acetic acid (IAA) (Cohen and Bandurski, 1978; Veitch, 2004). The reaction of HRP C with IAA is interesting in that it takes place without hydrogen peroxide added, unlike most peroxidase-catalyzed reactions (Veitch, 2004). The physiological significance of IAA metabolism by plant peroxidases is still an area of active debate. Peroxidases may play a role in the defense mechanism of the plant (Riquelme and Cardemil, 1993) as well as in responses to wounding (Lagrimini and Rothstein, 1987). Low temperature stress at 1 ^{o}C induced increased protein and mRNA levels of

ascorbate peroxidase in potato tubers (Kawakami *et al*., 1999). In rubber trees, peroxidase was found in newly excised bark strip, possibly in response to wounding, i.e. the excision (Wititsuwannakul *et al*., 1997). In sun-flower leaves, it may also be involved in leaf senescence since the activity increases during senescence (Hazell and Murray, 1982).

Table 2. Sources of higher plant peroxidases, functional organs and their specific substrates.

Plant	Functional organ	Substrate	Reference
Artichoke (*Cynara scolymus*)	flower	ferric acid	López-Molina *et al*., 2003
Araucaria araucana	seed (embryo)	*O*-phenylen-diamine	Riquelme and Cardemil, 1993
Arabidopsis thaliana	leaf	ABTS	Kavita *et al*., 2004
Barley (*Hordeum vulgare*)	leaf	*o*-dianisidine	Saekie *et al*., 1986
Bean (*Phaseolus vulgaris*)	hypocotyl	guaiacol	Xue *et al*., 1998
Bean (*Phaseolus vulgaris*)	leaf blade, pulvinus, petiole	guaiacol	McManus, 1994
Cassava *(Manihot esculenta)*	tuber (cortex, parenchyma)	guaiacol	Sornwatana and Chulavatanatol, 1996
Coconut (*Cocos nucifera*)	endosperm	*o*-dianisidine	Mujer *et al*., 1983
Cucumber (*Cucumis sativus*)	seedling	guaiacol	Decedue *et al*., 1984

Table 2. (continued)

Plant	Functional organ	Substrate	Reference
Flax (*Linum usitatissimum*)	stem	tetramethyl-benzidine	McDougall, 1993
Korean radish (*Raphanus sativus*)	root	*o*-dianisidine	Lee and Kim, 1994
Horseradish (*Armoracia rusticana*)	hypocotyl, root, petiole	guaiacol	Shannon *et al*., 1996
Indian Radish (*Raphanus sativus*)	root	ABTS	Aruna and Lali, 2001
Ipomoea palmetta	leaf	guaiacol	Srinivas *et al*., 1999
Japanese radish (*Raphanus sativus*)	root	ascorbate	Ohya *et al*., 1997
Litchi (*Litchi chinensis*)	fruit (pericarp)	guaiacol	Underhill and Critchley, 1995
Lupin (*Lupinus albus)*	root, leaf, seed	guaiacol	Jackson and Ricardo, 1998
Maize (*Zea mays*)	root, cytoplasm	guaiacol, 3-amino-9-ethylcarbazole	Grison and pilet, 1985
Maize (*Zea mays*)	cell-suspension, membrane	guaiacol	Myton and Fry, 1995
Maize (*Zea mays*)	seedling, leaf, root	guaiacol	Anderson *et al*., 1995
Mung bean (*Vigna radiata*)	hypocotyl, phloem, epidermis	PPD-PC	Chabanet *et al*., 1993

Table 2. (continued)

Plant	Functional organ	Substrate	Reference
Norway spruce (*Picea abies*)	seedling	guaiacol	Polle and Junkermann, 1996
Palm tree (*Elaies guineensis*)	leaf	guaiacol	Deepa and Arumugham, 2002
Pepper (*Capsicum annuum)*	fruit (pericarp, placenta)	capsaicin	Pomar *et al*., 1997
Pinus pinaster	seedling, hypocotyl	ferulic acid	Sanchez *et al*., 1996
Poplar (*Populus xeuramericana*)	bark, xylem, pholem	PPD-PC	Baier *et al.,* 1993
Poplar (*Populus xeuramericana*)	xylem	ABTS, DAB, SYR	Christensen *et al*., 1998
Poplar (*Populus xeuramericana*)	xylem	ABTS	Sasaki *et al*., 2004
Rice (*Oryza sativa*)	leaf	guaiacol	Young *et al*, 1995
Rubber tree (*Hevea brasiliensis*)	bark	*o*-dianisidine	Wititsuwannakul *et al*., 1997
Rubber tree (*Hevea brasiliensis*)	leaf	*o*-dianisidine	Ratthanapumee, 2000
Sphagnum magellnicum	apical of gametophytes	*o*-dianisidine	Tutschek, 1997

Table 2. (continued)

Plant	Functional organ	Substrate	Reference
Soybean (*Glycine max*)	plasma membrane	NADH, *o*-dianisidine	Zancani *et al*., 1995
Stinging nettle (*Urtica dioica*)	stem, leaf	ABTS	Douroupi and Margaritis, 1999
Turnip (*Brassica napus*)	root	ABTS	Agostini *et al*., 2002

The most intensively studied example of plant peroxidases is horseradish peroxidase isoenzyme C (Veitch, 2004). The catalytic cycle of peroxidase in the presence of hydrogen peroxide is shown in Figure 7. The heme group of the resting enzyme first reacts with one molecule of hydrogen peroxide to produce compound I, an oxoiron-porphyrin (Fe^{IV}) radical. This occurs in a two electron oxidation/reduction reaction where H_2O_2 is reduced to water and the enzyme is oxidized. Compound I then oxidizes the reduced substrate (AH_2) to give a substrate radical (·AH), which is then reduced to compound II, which contains an oxyferryl center coordinated to a normal (dianionic) porphyrin ligand. Both compounds I and II react with the same AH_2. Therefore, once the reaction between compound I and the AH_2 starts, compound II is produced and simultaneously reacts with a second molecule of AH_2. As a result, compound II is reduced back to the ferric state, corresponding to the native form of the enzyme, with concomitant oxidation of a second molecule of substrate and production of water (Silaghi-Dumitrescu, 1999) (Figure 7).

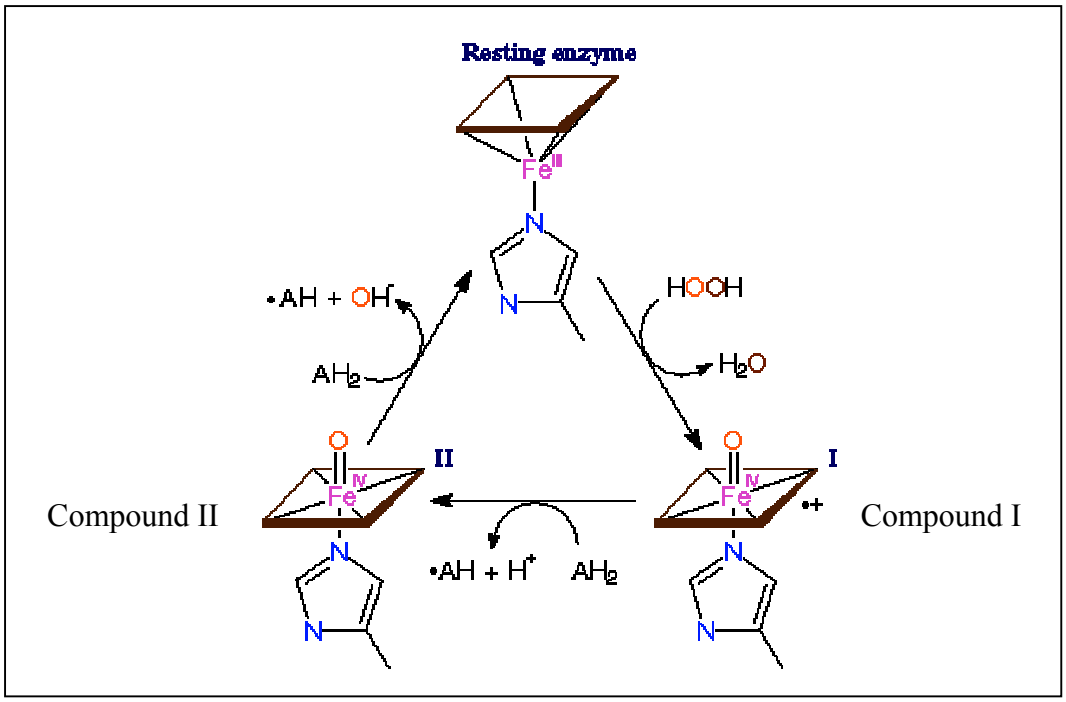


Figure 7. Catalytic cycle of heme-containing peroxidase in the presence of hydrogen peroxide (Silaghi-Dumitrescu , 1999)

6. Molecular biology of plant peroxidase genes

6.1 Plant peroxidase genes

Plant peroxidases are heme-containing glycoproteins, and are classified as acidic, neutral, or basic, according to their isoelectric points. Most higher plants possess a large number of peroxidase isoforms, which are encoded by multigene families. In commercial preparations of horseradish peroxidase, 42 isozymes have been found (Hoyle, 1977). The multiplicity of peroxidase genes was observed in peanut (Buffard *et al*., 1990), horseradish (Welinder, 1992), spinach (Simon *et al*., 1993), and in the model plant *Arabidopsis thaliana* (Kjaersgard *et al*., 1997; Justesen *et al*., 1998; Tognolli *et al*., 2002). Four genomic DNAs (*prxC1a*, *prxC1b*, *prxC2* and *PrxC3*) (Fujiyama *et al*., 1990) and three cDNAs (*prxC1a*, *prxC1b* and *prxC1c*)

encoding HRP have been isolated (Fujiyama *et al*., 1988). More than 100 expressed sequences tags (ESTs) encoding different peroxidase isozymes are found in *Arabidopsis*. According to recent studies there are 73 full-length genes for class III plant peroxidases in the *Arabidopsis* genome (Tognolli *et al*., 2002; Welinder *et al*., 2002). Of these, 71 are predicted to encode for stable enzymes folded similar to HRP C (Welinder *et al*., 2002). Amino acid sequence identities among the predicted peroxidases range from 28 to 94% (Veitch, 2004). Alignment of these sequences has been used as a basis for comparisons with other plant peroxidases and the identification of potentially similar enzymes. The cloning, expression and analysis of a number of cDNAs and genomic DNAs encoding peroxidases in higher plants are summarized in Table 3.

Table 3. Principle plant peroxidase genes, expression and functional analysis.

Plant	Gene	Expression and/or functional analysis	Reference
Populus	*prxA3a, prxA4a*	Stem	Osakabe *et al*., 1995
Populu	*PXP1, PXP11, PXP22, PXP3-4*	Xylem of stem and root	Christensen *et al*., 2001
Populus	*prxCa, prxEa, prxCb*	Promoter activity in transgenic plant	Intapruk *et al*., 1994
Populus	Nine *prxr* genes	All organs	Capelli *et al*., 1996
Arabidopsis thaliana	*ATP1a, ATP2a*	All organs	Kjaersgard *et al*., 1997
Arabidopsis thaliana	*ATP15a, ATP24a, ATP2a*	Wound inducible	Cheong *et al*., 2002
Arabidopsis thaliana	*ATPA2*	Promoter activity in transgenic plant	Østergaard *et al*., 2000
Picea abies	*SPI2*	Pathogen inducible	Fossdal *et al*., 2001
Gossypium hirsutum (cotton)	n.d.[a]	Bacterial inducible	Assigbetse *et al*., 1999
Cucumis sativa (cucumber)	*Cuper2*	Ethylene inducible	Morgens *et al*., 1990
Cucubitar pepo (zucchini)	*APRX*	Cell-wall-bound	Carpin *et al*., 2001
Armoracia rusticana (horseradish)	*prxC1*	Promoter activity in transgenic plant	Fujiyama *et al*., 1988

n.d.= not determined

Table 3. (continued)

Plant	Gene	Expression and/or functional analysis	Reference
Armoracia rusticana (horseradish)	*prxC2*	Wound inducible.	Fujiyama *et al*., 1990
Ipomoea batatas (sweet potato)	*Swpa2, Swpa3*	Promoter activity in transgenic plant	Huh *et al*., 1997
Ipomoea batatas (sweet potato)	*POX22.3, POX8.1, POX5*	Pathogen inducible	Chittoor *et al*., 1997
Lycopersicon esculentum (tomato)	*TAP2*	Wound inducible, antisense analysis	Sherf *et al*., 1993
Triticum aestivum (wheat)	*pox1, pox2, pox3, pox4*	Powdery mildew induced (*pox2*)	Baga *et al*., 1995
Oryza sativa (rice)	*prxRPN , prxRPA*	Wound and ethephon inducible	Ito *et al*., 1994
Oryza sativa (rice)	*prxRPN*	Promoter activity	Ito *et al*., 2000
Lycopersicon esculentum (tomato)	*TPX2*	Transgenic analysis	Amaya *et al*., 1999
Lycopersicon esculentum (tomato)	*TAP1*	Promoter (wound), antisense analysis	Mohan *et al*., 1993
Glycine max (soybean)	*SPOD4.1, Prx2, Ep*	Seed coat	Huangpu *et al*., 1996
Stylosanthes humilis	*Shpx6a, Shpx6b*	Pathogen and jasmonate inducible	Curtis *et al*., 1997
Arachis hypogaea (peanut)	*prxPNC1,*	Wound inducible	Breda *et al*., 1993

Table 3. (continued)

Plant	Gene	Expression and/or functional analysis	Reference
Solanum tuberosum (potato)	*Stprx2*	Wound inducible	Collinge and Boller, 2001
Medicago sativa (alfalfa)	*Msprx1A, Msprx1b, Msprx1C*	Pathogen inducible	El-Turk *et al*., 1996

6.2 Regulation of gene expression

Expression of plant peroxidases genes is regulated at different times and places by various kinds of biotic and abiotics stressors (Yoshida *et al*., 2002). Specific peroxidase genes, *prxC1a* and *prxC1b*, isolated from cultures of HRP, are expressed both in stems and roots, while *prxC2* and *prxC3* are mainly expressed in roots. All of these genes consist of four exons and three introns and have identical splice site positions, a feature common to other plants peroxidase genes (Fujiyama *et al*., 1988; 1990). Three highly homologous cDNAs (*prxC1a*, *prxC1b* and *prxC1c*) have also been isolated using an oligonucleotide probe corresponding to the primary sequence region from His40 to Ala51 of HRP C (Fujiyama *et al*., 1988). Expression of the *prxC2* gene is induced by wounding and its transcriptional regulation has been investigated (Kaothien *et al*., 2000). The expression of other plant peroxidases by physical wounding were also found in rice *poxA* and *poxN* (Ito *et al*., 2000), tobacco *tpoxN1* (Hiraga *et al*., 2000), sweet potato *swpa1*, *swpa2*, *swpa3* and *swpn1* (Huh *et al*., 1997; Kim *et al*., 1999), and tomato *tap1/tap2* (Mohan *et al*., 1993). Analysis of the rice *poxA* promoter in transgenic tobacco plants has revealed UV and wound-

responsive *cis* elements within 144 bp of the *poxA* translation start site (Ito *et al*., 2000). Promoter analysis in a heterologous host confirmed wound- and fungus-responsive activity of the legume *Shpx6b* promoter, which contains a motif similar to the methyl jasmonate (MeJA) responsive element (Curtis *et al*., 1997). Cooling at 4 ^{o}C induces the expression of sweet potato *swpa1*, swpa2 and swpa3, but reduces the expression of sweet potato *swpn1* (Huh *et al*., 1997; Kim *et al.*, 1999).

6.3 Peroxidase gene expression and function of the enzyme.

Information about the tissue specificity of peroxidase gene expression may reveal the function of a specific peroxidase isozyme encoded by that gene. Some specific plant peroxidases are thought to be important in the biosynthesis of lignin, a compound with great commercial value. For example, expression of an acidic peroxidase isoenzyme from *A. thaliana* (AtPA2) with 95% amino acid sequence identities to HRP A2 (an HRP isoenzyme of unknown function) was found to coincide with lignification (Østergaard *et al*., 2000). The three-dimensional structure solved for recombinant AtPA2 by X-ray crystallography reveals a substrate binding site that can accommodate monolignols such as *p*-coumaryl and coniferyl alcohols. Both enzymes catalyse the oxidation of monolignols and phenolic acids (Nielsen *et al*., 2001). These studies support the role of plant peroxidases in lignin biosynthesis (Østergaard *et al*., 2000).

Recombinant DNA technology has been used to investigate the precise physiological functions of plant genes. Some plant peroxidase genes have been expressed transgenically and the phenotypes of these transgenic plants yield information about their functions. For example, the *NtpoxAN* gene, which encodes an anionic peroxidase in tobacco (*N. tabacum*), was placed under the control of the

CaMV 35S promoter and introduced back into tobacco (Lagrimini *et al*., 1990; 1997). The transgenic tobacco plants exhibited peroxidase activity 2- to 10-fold higher than that of the wild-type plants and displayed chronic severe wilting through loss of turgor in leaves, initiated at the time of flowering. Indole acetic acid (IAA) metabolism by overexpression of anionic peroxidase was not significantly different. The transgenic plants expressing higher peroxidase activity and root elongation were insensitive to exogeneously applied IAA. Lagrimini *et al* (1997) proposed that the overexpression of the tobacco anionic peroxidase results in diminished root mass from fewer root branches, which contributes to wilting.

Overexpression of the *TPX2* gene which encodes a cell wall-associated peroxidase involved in modifying cell wall architecture in tomato (*Lycopersicon esculentum*), had no effect on wild-type development under normal growth conditions. However, the germination rate of the transgenic plants was increased greatly under high salt (250 mM NaCl) or osmotic stress (470 mM mannitol) conditions. Thermoporometry calculations indicated a lower mean pore size in the walls of transgenic seeds. Therefore, they speculated that the salt-tolerant phenotype seen in the transgenic tobacco is due to a higher water-retaining capacity in transgenic seeds.

Anionic (*swpa1*) and neutral (*swpn1*) peroxidases from sweet potato (*Ipomoea batatas*) were overproduced in transgenic tobacco plants under the control of the CaMV 35S promoter. Leaves of transgenic plants with either the *swpa1* or *swpn1* gene showed higher peroxidase activity than those of nontransgenic control plants (Yun *et al*., 2000). When tobacco leaf discs were treated with 10 μM methyl viologen (MV; paraquat), *swpa1*-transgenic plants showed a reduction in membrane damage of

about 25% compared to *swpn1*-transgenic or untransformed control plants. Leaves of the *swpn1*-transgenic and the control plants were also bleached more than those of the *swpa1*-transgenic by 1 μM MV treatment. These results indicate that the increased H_2O_2-scavenging capacity provided by overproduction of *swpa1* peroxidase (a guaiacol-type peroxidase) confers increased oxidative-stress tolerance on the transgenic plants (Yun *et al*., 2000).

Overexpression of two HRP genes showed significant growth stimulation to tobacco plant (*N. tabacum* L. cv. W-38). The study was performed by separately introducing the chimeric genes of either the 1.1 kb *prxC1a* cDNA ligated to the CaMV 35S or to the HRP *prxC2* promoters into tobacco plants. Crude extracts of leaves from ten independent transgenic plants showed 2- to 10-fold higher peroxidase activity than that of the wild-type plants (Kawaoka *et al*., 1994). In a related study, the self-pollinated seeds of these transformed tobacco plants were harvested and the phenotypes of about 50 seedlings were subjected to statistical analysis. After being grown for 3 months in a greenhouse, the transgenic plants with the CaMV 35S promoter-*prxC1a* construct were 20% taller than control plants and northern blot analysis showed that the fast-growing plants also expressed *prxC1a* at a higher level (Yoshida *et al*., 2002).

Peroxidase has become a model enzyme for studying the molecular mechanisms of vesicular transport accompanied with glycosylation. The amino acid sequence deduced from horseradish *prxC1a* contains a hydrophobic leader sequence as well as a C-terminal extension which may be responsible for vacuolar targeting. The recombinant AtPA2 (as expressed in *E. coli*) is non-glycosylated, whereas plant HRP A2 is highly glycosylated with one bulky glycan attached close to residues at the

entrance of the substrate binding site, however, small differences in their peroxidase activity were found (Veitch, 2004).

The whole picture of how peroxidase genes regulate plant metabolism is still unclear. However, the information accumulated so far is a promising starting point for further studies to identify their functions and to apply them to the molecular breeding of useful plants and trees, and, in particular, to rubber trees.

7. Applications Overview

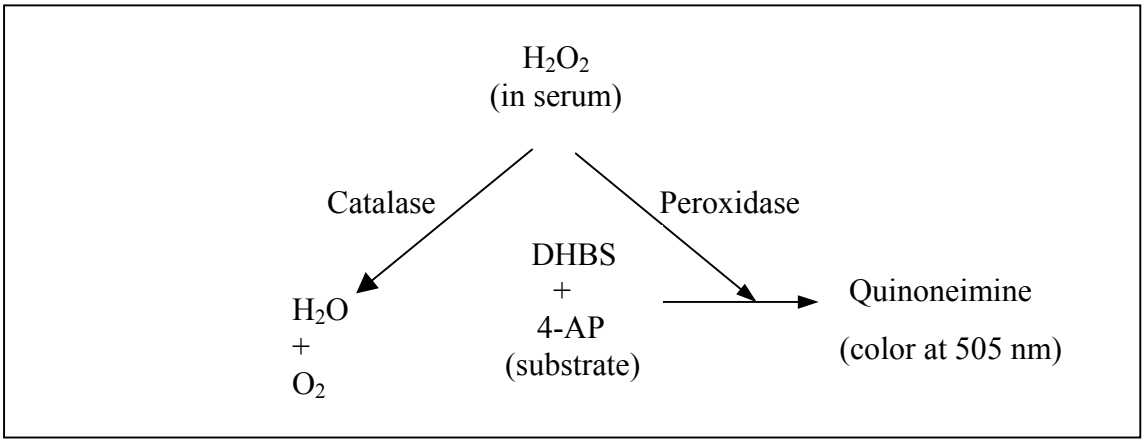
7.1 Research and clinical diagnosis involving peroxidases

Peroxidase is used in coupled enzyme assays. An enzymatic method of monitoring H_2O_2 concentration is widely used by modern automated analyzers to assay the activity of a variety of interesting enzymes and analytes, including glucose, cholesterol, urea, triglyceride, oxalate and creatinine (Barham and Trinder, 1972; Fossati *et al*., 1980; Fossati and Prencipe, 1982; Fossati *et al*., 1983; Petrarulo *et al*., 1994; Agostini *et al*., 1999). These assays typically use peroxidase to catalyse the oxidation by enzymatically produced H_2O_2 of a chromogenic dye.

Peroxidase is used in catalase activity determination. Catalase is a primary component of the antioxidant system that defends against oxidative stress that is ubiquitously associated with pathologic conditions. Serum catalase is increased in a wide variety of diseases, including pancreatic, liver, hemolytic, renal, skin, and respiratory diseases as well as acquired immunodeficiency and oxidant-mediated vascular diseases (Hosono *et al*., 1996; Goth, Meszaros and Nemeth, 1987; Goth, Meszaros and Nemeth, 1988; Goth, 1989; Leff *et al*., 1992a; Leff *et al*., 1992b; Leff *et al*., 1993 and Oka *et al*., 1999). Conversely, serum catalase is decreased in

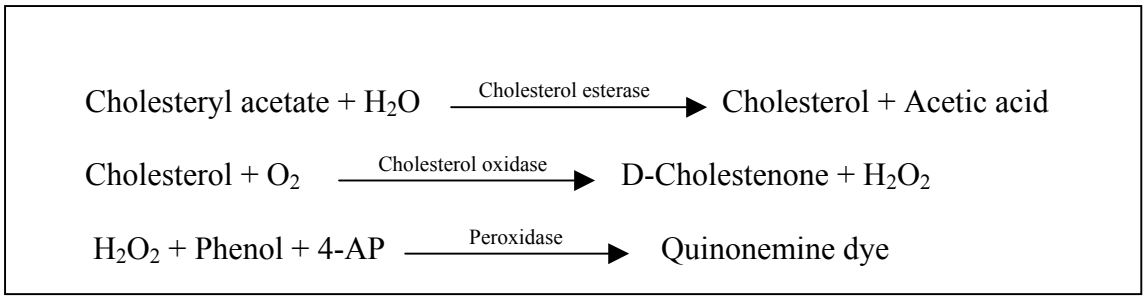
thalassaemia, alcoholism, cancers and psychiatric disease (Vitai and Goth, 1998; Tarasova, 1998).

Catalase activity assay is effectively based on competition between peroxidase and catalase for H_2O_2 to produce color or inhibit its development, respectively. Both catalase and peroxidase use H_2O_2 as substrate, but peroxidase activity produces a colored product (Goth *et al*., 1984; Goth, 1989). The activity of peroxidase was, therefore, adjusted to provide sufficient sensitivity toward catalase such that inhibition of color formation shows a linear relationship over a wide range of catalase activities. A scheme for a catalase activity assay using peroxidase as the second enzyme in coupled enzymatic reaction as developed and described by Slaughter and O'Brien (2000), is shown next.



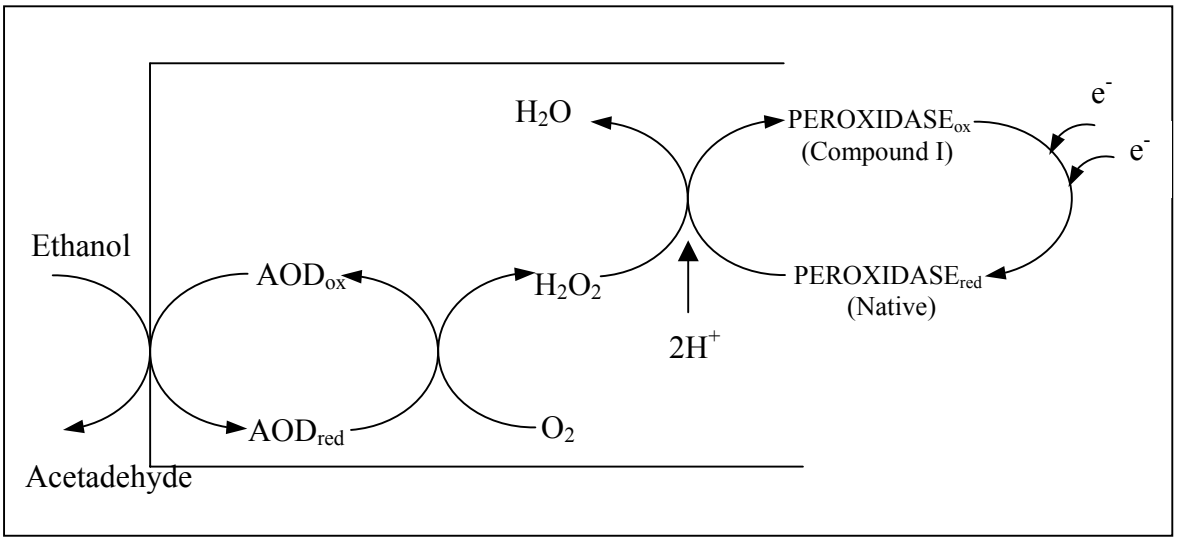
Peroxidase is used in cholesterol determination. Cholesterol is a fatty substance found in blood, bile and brain tissue, and it is mainly found in esterified form. It serves as a precursor of bile acids, steroids and vitamin D. The determination of serum cholesterol is known to be clinically important for diagnosis and classification of lipemias, heart diseases, arteriosclerosis, cerebral thrombosis,

hepatic thyroid diseases, anemias and diabetes (Kumar *et al*., 2000; Nauck *et al*., 2002; Punder, 2003). Cholesterol esters are hydrolyzed to produce cholesterol. Among the various methods available for determination of total cholesterol, the colorimetric method employing cholesterol esterase, cholesterol oxidase and peroxidase is simple, sensitive and specific, and thus is suitable for routine analysis. The spectrophotometric determination of cholesterol is usually based on the colour of the quononeimine dye produced by the reaction of 4-aminoantipyrine and phenol with the hydrogen peroxide generated by the oxidation of cholesterol catalyzed by cholesterol oxidase, in the presence of a second enzyme, peroxidase. Related reactions are shown in the following scheme (Allain *et al*, 1974; Richmond, 1992).



Peroxidase is also used in alcohol determination. Quantitative determination of alcohol is important in many sectors, including in the pulp industry, in the food and beverage industry from market classification to quality control, and so on. In clinical chemistry, ethanol is an aliphatic alcohols, which is the most common social drug. When abused, the necessity arises for a fast determination of acute intoxication, as well as of suspected drunk-driving. High sensitivity and inexpensive methods are required in order to distinguish alcohol intoxication. Pharmacological effects are

obtained at blood ethanol levels of approximately 10 mM, whereas lethal levels are 10 times this concentration (Rang and Dale, 1987). The principle of alcohol detection using peroxidase as a second enzyme in a coupled enzymatic reaction is based on production of hydrogen peroxide by alcohol oxidase (AOD) (in its oxidation step). The hydrogen peroxide reacts with native peroxidase in a single two-electron transfer step forming water and an oxidized form of the enzyme (compound I). The reduction of compound I back into native peroxidase is made by donation of electrons provided by a donor molecule. A coupled enzymatic system using peroxidase for the detection of some aliphatic alcohols is shown in the reaction scheme below (Lidén *et al.*, 1998).



Peroxidase is used to accurately determine glucose concentration in a variety of samples, including foods, beverages, fermentation media and serum, and to monitor the release of glucose by any glucosidase enzyme. Glucose is present in the majority of fruit beverages in relatively high concentration (up to 100 mM in orange juice and up to 300 mM in grape juice (Nagy *et al*., 1993; Ashurst, 1995). Glucose

oxidase is a highly specific enzyme (for D-glucose), which catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone using molecular oxygen and releasing hydrogen peroxide. Hydrogen peroxide generated in the reaction can be removed by treatment with either peroxidase or catalase, which converts it to water and molecular oxygen. In the food industry, glucose oxidase derived from the fungi *Aspergillus niger* and *Penicillium*, is applied for the removal of either glucose or oxygen from foodstuffs in order to improve their storage properties. Therefore, glucose contents can be determined by exploiting this well-known enzymatic reaction (Kaplan *et al*., 1988):

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-glucono-1,5-lactone} + H_2O_2$$

$$2H_2O_2 + (\text{ reduced dye}) \xrightarrow{\text{Peroxidase /or catalase}} (\text{oxidized dye}) + 2H_2O + O_2$$

A major application of the glucose oxidase/ peroxidase system is in the determination of glucose in serum (Barham and Trinder, 1972; Guo *et al*., 1999).

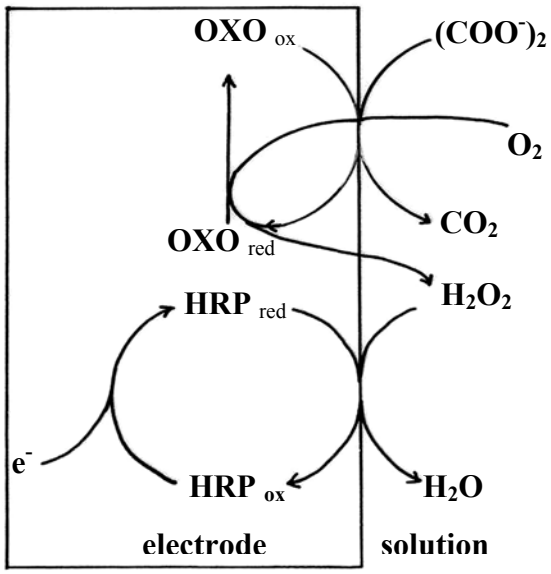
Peroxidase is used in uric acid determination. It is done following the reactions described below (Agostini, 1999).

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2$$

$$H_2O_2 + \text{4-amine phenazone} + \text{phenol} \xrightarrow{\text{peroxidase}}$$

$$\text{4-}p\text{(benzoquinone)} \quad \text{monoamine phenazone} + H_2O$$

Peroxidase is used in oxalate determination. Oxalate has great importance in food industries and clinical analysis. There are several sources of oxalate like spinach, mushrooms and beet leaves (Pérez-Ruiz *et al*., 1995). It is formed through glyoxylic and ascorbic acid oxidation. Its biological function is unclear (Ilarslan *et al*., 1997). For plants, it is believed that oxalate is an important source of calcium (Azarashivili *et al*., 1996) as its metabolism generates hydrogen peroxide associated with the cell wall mechanism (Kotsira and Clonis, 1997). In spite of this, oxalate is damaging to humans. Its excess in the diet may cause some certain diseases. It affects human health in two different ways: (a) before absorption by the body, oxalate reduces the Ca^{2+} concentration by making an insoluble compound with Ca^{2+} ions, which may lead to some disorders; (b) after absorption oxalate complexes with Ca^{2+} in the blood and can be precipitated in organs, causing diseases like renal calculus (Nelson *et al*., 1997). In addition, oxalate is known to be a byproduct of the ozonation of drinking water treatment and it is susceptible to bacterial regrowth, so its quantification is also important in this case (Peldszus *et al*., 1998; Petrarulo *et al.*, 1994). Oxalate determination is based on its oxidation by oxalate oxidase (OXO), resulting hydrogen peroxide that is further oxidized by peroxidase according to the reaction below (Pérez-Ruiz *et al*., 2001).



7.2 Peroxidase-antibody conjugate preparation

Peroxidase is widely used to prepare peroxidase-antibody conjugate. Conjugation is the chemical cross-linking reaction routinely employed in the diagnostic industry. Preparation of well-defined conjugates such as enzyme-antigen or enzyme-antibody conjugates is vital to the success of an immunoassay production process. Conjugation reactions must preserve the immunogenicity of these molecules, i.e., its antigenic determinant, when linked to the carrier protein, as well as the nature of the antibody and of enzymes in terms of a stable conjugate with high retention of both immunoreactivity and enzymatic activity. In a conjugation process, the proteins are linked together by using their available reactive groups and cross-linking reagents.

The chemical cross-linking methods in conjugation procedures have been derived from the field of peptide chemistry and chemical modification of proteins, because of their importance either as carrier immunogens or as enzyme labels. Generally, only these reactive groups located on the protein surface and thus exposed to the aqueous environment are available for chemical modification and conjugation

purposes. In proteins, there are essentially eight side chains that are chemically active. These side chains and their functional groups are as follows (Wong, 1993 and Tijssen, 1993):

1) Amino group of N-terminal amino acid and ε-amino groups of lysine
2) Sulhydryl group of cysteine
3) Thioether group of methionine
4) Carboxyl group of C-terminal amino acid and the β- and γ- carboxyl group of aspartic and glutamic acids, respectively
5) Phenolic group of tyrosine
6) imidazolyl group of histidine
7) Guanidinyl group of arginine
8) Indolyl group of tryptophan.

Of these, the first five groups are chemically the most reactive. These are also normally exposed on the protein surface and form the major targets for conjugation (Deshpande, 1996). Several proteins also contain carbohydrates that provide a useful site for chemical modification and cross-linking of proteins. Most chemical modification reactions involving the functional group of amino acid side chain are nucleophilic substitution reactions. In terms of protein modification, the relative chemical reactivity is basically a function of nucleophilicity of the amino acid side chains (Wong, 1993), whereas the cross-linkers must contain characteristics for the nucleophilic attack (Deshpande, 1996).

Modification reactions commonly used in the field of enzyme-antibody conjugate preparation are activation of carbohydrates and interconversion of functional groups (Tijssen, 1993). Cross-linking is essentially a chemical

modification process in which specific functional groups within a molecule or between two different molecules are linked together using bifunctional cross-linkers with or without a spacer arm that contains two groups-specific reagents. Chemical cross-linkers are widely used for the preparation of enzyme-antibody conjugates and other labeled protein reagents.

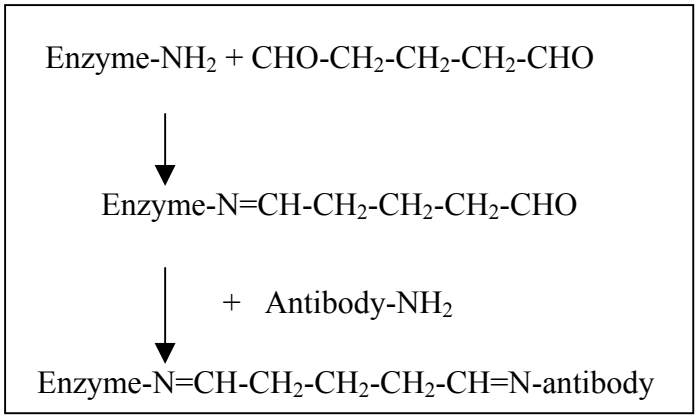
7.3 Cross-linking reactions for enzyme-antibody conjugation.

Protein-protein coupling reactions can be divided into four categories: one-step, two-step, three-step, and multi-step reactions. The basic characteristics of these reactions are described in the following sub-sections:

7.3.1 One-step reactions.

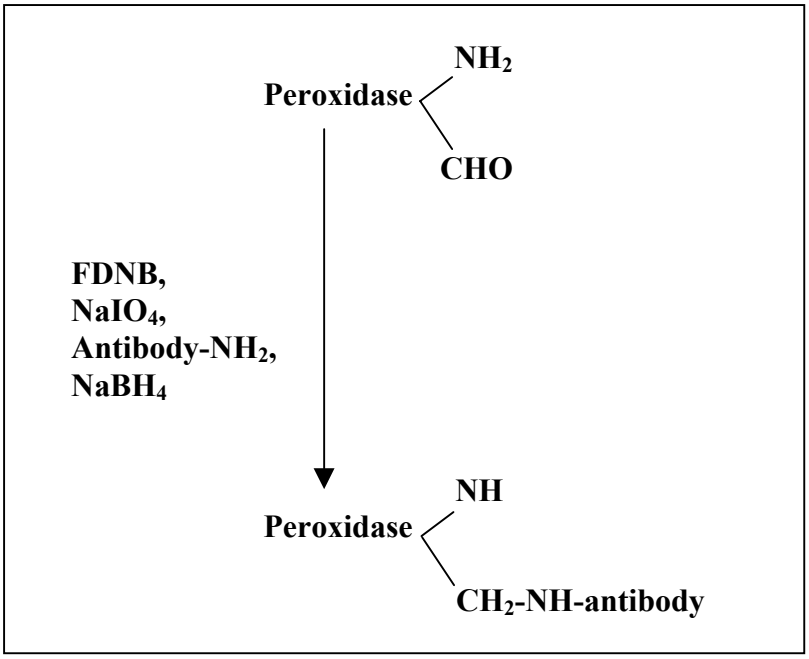
These are the simplest and the oldest of all conjugation reactions in which the bifunctional cross-linker is added to a mixture of the conjugation species. In the preparation of enzyme-antibody conjugates, one-step reactions, however, are often undesirable, as they yield both homo- and heteropolymers of the reacting agents. Moreover, the reactivity of the conjugating agents may not be the same to different cross-linking reagents, thereby resulting in a selective homopolymerization reaction with one of the conjugating species (O'Sullivan and Marks, 1981). The most popular technique employed to prepare enzyme-antibody conjugate are the one-step glutaraldehyde method and the periodate oxidation method which use glutaraldehyde and periodate as the cross-linkers, respectively.

In the one-step glutaraldehyde method, the enzyme is activated by the aldehyde group of glutaraldehyde, the activated enzyme then reacts with the amino group of the antibody via Schiff's base formation as illustrated.



In one-step conjugation reactions, the rate of addition of the cross-linking reagent greatly influences the yield and efficiency of conjugation (Modesto and Pesce, 1971). Slow addition of the cross-linker over a period of time, rather than its addition all at once, often increases the yield of coupled proteins (Deshpande, 1996).

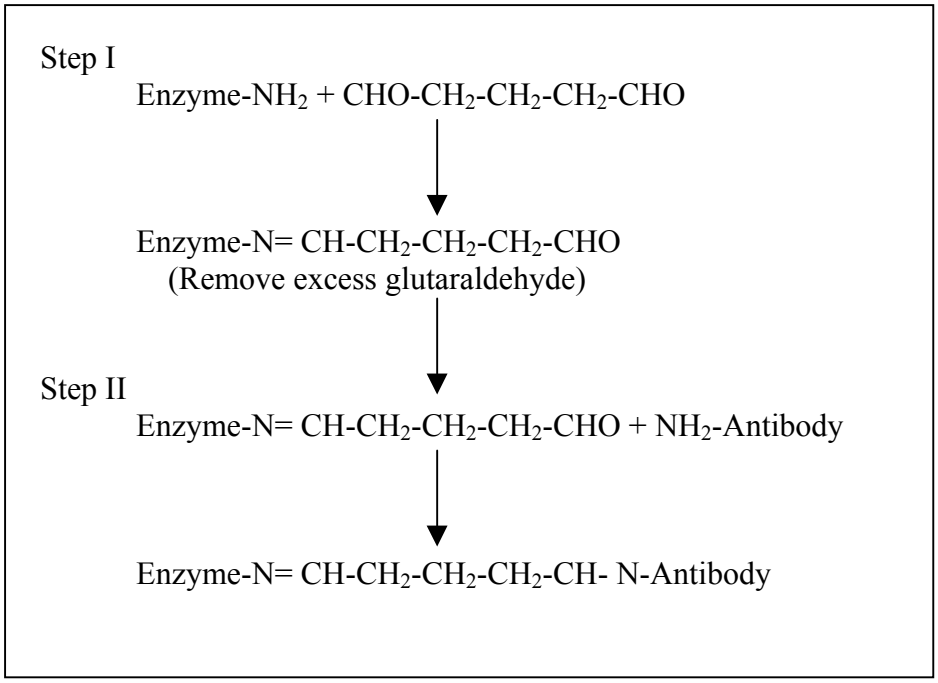
The periodate oxidation method has mainly been applied to horseradish peroxidase because this enzyme is a glycoprotein. Here the carbohydrate moiety is not required for enzyme activity and can be coupled to the antibody by using sodium periodate ($NaIO_4$) as a cross-linker. The reaction involves (a) blockage of the amino groups of the enzyme by reaction with fluorodinitrobenzene (FDNB) to prevent self-coupling of the enzyme, (b) periodate oxidation of the carbohydrate residues of the enzyme to form aldehyde groups, (c) reaction between these aldehyde groups and the free amino groups of the antibody, and (d) stabilization of the cross-link by reduction with sodium borohydride ($NaBH_4$). Conjugates prepared by this method generally provide very high sensitivity (Wilson and Nakane, 1979; Tijssen, 1993; Tijssen and Kurstak, 1984). The reaction is illustrated in the scheme below (O'Sullivan and Marks, 1981).



7.3.2 Two-step reactions.

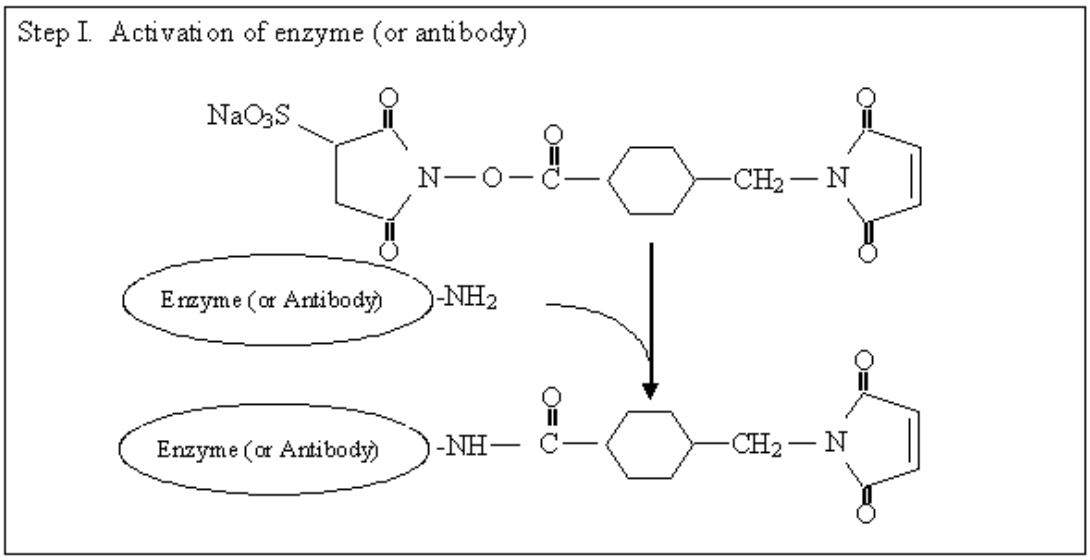
In two-step reactions, one of the reactants to be conjugated is first activated with the cross-linker. The unreacted reagent is then removed prior to addition of the second reactant. This method has the advantage of using the different reactivities of functional groups in heterobifunctional reagents as well as the differential selectivity of homobifunctional reagents on the two conjugates to be coupled (Wong, 1993).

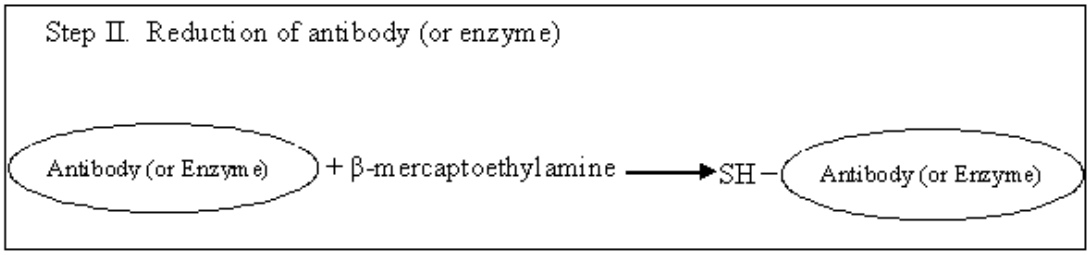
Example for the two-step reaction is the two-step glutaraldehyde method. In this technique, the enzyme is activated but not extensively polymerized by glutaraldehyde. After removal of excess glutaraldehyde, the activated enzyme is allowed to react with antibody. This technique has been used to prepare HRP-antibody conjugate (Avrameas *et al.*, 1978) and also commercial HRP-anti-IgG conjugate by Sigma Chemical Company, St. Louis, MO, USA. The reaction scheme is illustrated below:

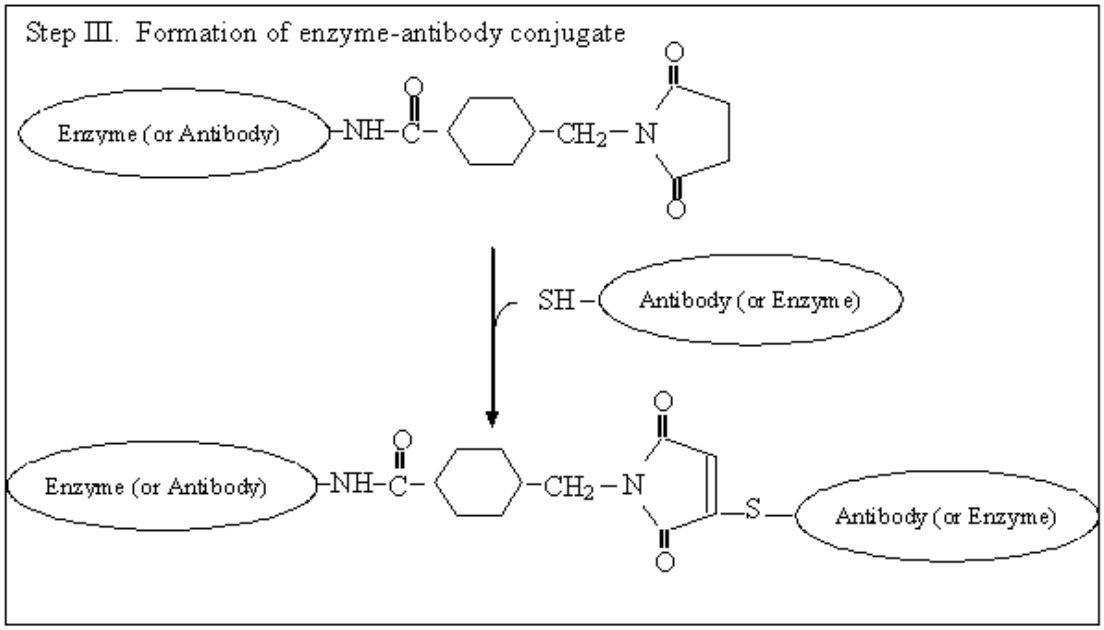


7.3.3 Three-step reactions.

This procedure involves an extra step for the preparation of proteins to be coupled. An example of this type of reaction is the labeling of antibody with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (Liu *et al*., 2000). Sulfo-SMCC is a novel water soluble heterobifunctional cross-linker for enzyme-antibody conjugate preparation. Three reactions are involved, activation of enzyme (or antibody) by sulfo-SMCC, reduction of antibody (or enzyme), and formation of enzyme-antibody conjugate is illustrated as follows (modified from Pierce instruction manual, Rockford, Il, USA):


Step I. Activation of enzyme (or antibody)
NaO3S
N—O—C—
CH2—N
Enzyme (or Antibody)-NH2
Enzyme (or Antibody)-NH—C—
CH2—N


Step II. Reduction of antibody (or enzyme)
Antibody (or Enzyme)
+ β-mercaptoethylamine
SH—
Antibody (or Enzyme)


Step III. Formation of enzyme-antibody conjugate
Enzyme (or Antibody)-NH-C-
-CH2-N
SH—
Antibody (or Enzyme)
Enzyme (or Antibody)-NH-C-
-CH2-N
S—
Antibody (or Enzyme)

In this procedure, the sulfo-NHS ester in sulfo-SMCC first reacts with free amino groups of the enzyme (or antibody) to form reactive maleimide-labeled enzyme (or antibody), then the excess sulfo-SMCC is removed by size exclusion chromatography. In the second step, the antibody (or enzyme) is reduced with β-mercaptoethylamine to produce antibody with free sulhydryl group. At the final step, the maleimide group reacts with free sulhydryl group in enzyme (or antibody) to form enzyme-antibody conjugate. This technique is also recommended for peroxidase-antibody conjugate and other glycoprotein conjugate preparations (Pierce instruction manual).

7.3.4 Multi-step reactions.

Multi-step reactions, although sometimes used for the preparation of immunotoxins, are not very common in the diagnostic industry. These reactions require preparation of protein procedures prior to the actual coupling process (Deshpande, 1996).

7.4 Purification of the conjugate.

Non-labeled antibody should be removed from the conjugate, as it lowers the sensitivity of the assay. Removal of free enzyme from the conjugate is not essential because it can be washed out in the washing procedure in most immunoassay techniques. However, when large amounts of free enzyme are present, the washing procedure is more critical and the precision of the assay may suffer (O'Sullivan and Marks, 1981). Purification methods that have been employed include size-exclusion chromatography on Sephadex G-200 (Liu *et al*., 2000) and Sephacryl S-200 (Agostini

et al., 1999) and affinity chromatography on Con-A Sepharose (Tijssen and Kurstak, 1984) and benzhydroxamic acid-agarose (Husereau and Suresh, 2001).

7.5 Applications of peroxidase–antibody conjugate

More than 25 different enzymes have been used as enzyme-antibody conjugates in immunoassays. However, only three enzymes, namely peroxidase, alkaline phosphatase (ALP), and *E. coli* β-D-galactosidase (BG) have been extensively used in both research applications and in commercial diagnostic kits. For enzyme-labeled assays, horseradish peroxidase (HRP) is used in about 50% of all tests, and alkaline phosphatase in about 25% (Deshpande, 1996).

Recently, perxoidase conjugate has become widely used to amplify the diagnostic signal in immunoassays. Among the various techniques, such as dot blot, western blot, reverse rocket electrophoresis, enzyme-linked immunosorbant assay (ELISA), and blot overlay (Mulvey and Ohlendieck, 2003), the most common technique is ELISA. It is preferred for routinely quantifying and qualifying analysis of large samples. Many methods of ELISA have been developed since the 1960s, such as direct ELISA, indirect ELISA, capture ELISA and competitive or blocking ELISA methods. However, the basic concept of these methods is the immunological detection and quantification of single or multiple antigens (Ag) or antibodies (Ab) in a sample (usually serum) (Kemeny and Challacombe, 1988). 'Indirect' ELISA and the similar 'capture' ELISA method are popular in clinical diagnosis. Indirect ELISA detects an antigen after it has adsorbed onto a solid phase (eg. a membrane or polystyrene microwell or dipstick). The first or primary antibody is incubated with the antigen, then the excess is washed off. An enzyme-antibody conjugate used as a

secondary antibody is then incubated with the samples. The excess is again removed by washing and enzyme substrate is added to develop the color which refers to the specific Ag-Ab interaction and can be detected by using a spectrophotometer. The principle of the indirect ELISA method using peroxidase-antibody conjugate as a secondary antibody is shown in Figure 8.

Detection of the herbicide atrazine for environmental monitoring was developed into a new technique, the polyelectrolyte ELISA, by using atrazine-peroxidase conjugate as a specific secondary antibody. Immune interaction of polycation-polyanion in microplate prior the formation of protein A-antibody-atrazine-peroxidase complexes is significantly more rapid than the traditional ELISA. This technique is aimed at optimization of the assay regime and application for detection of another herbicide, simazine (Yazynina *et al*., 1999).

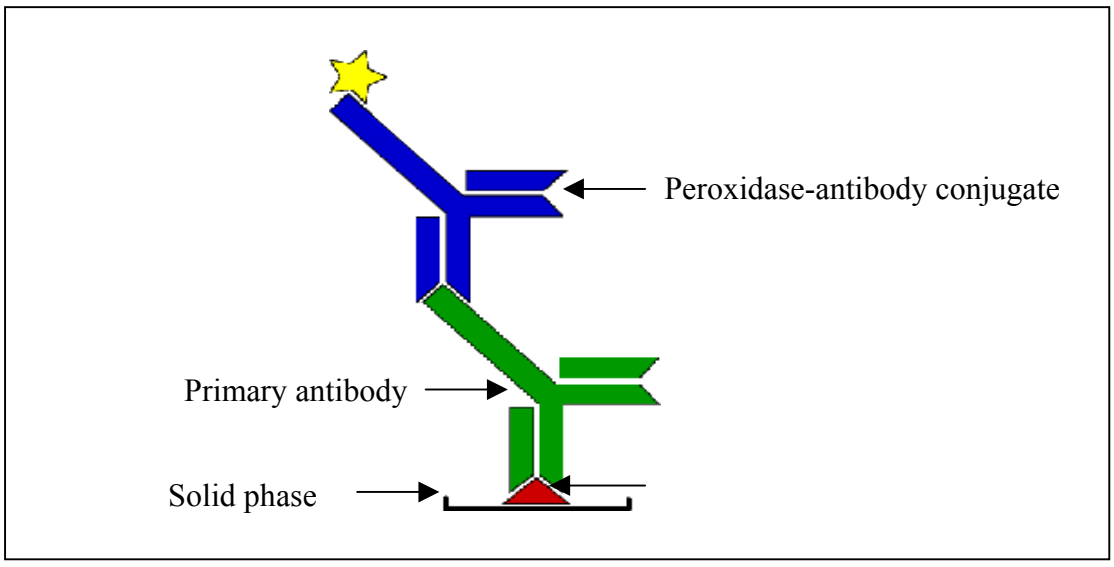


Figure 8. The principle of indirect ELISA. The antigen (red) is bound to a solid phase, and then incubated with primary antibody (green) and secondary antibody (peroxidase (yellow)-antibody (blue) conjugate), respectively.

The peroxidase-antibody conjugate is a useful tool to improve new techniques for immunoassay systems. It is widely used in routine laboratories to detect blood-borne infections. These include human immunodeficiency virus (HIV), human T lymphotropic virus (HTLV), hepatitis, and many others viruses. (Baumeister, *et a*l., 2000; Carneiro-Proietti *et al*., 1998; Dhawan, 2002).

In the past, the diagnosis of human liver disease was routinely detected by measuring the activity of hepatic enzymes, such as aspartate aminotransferase and alanine aminotransferase. To date, the method has been changed to human liver-type arginase (a specific marker) detection by an ELISA system using a peroxidase-antibody conjugate as the secondary antibody (Ikemoto *et al*., 2001).

Soybean seed coat peroxidase (SP)-mouse monoclonal antibody conjugate coupled to soybean seed coat peroxidase (SPAP) and used as the third antibody does improve the sensitivity and specificity in a three-tiered sandwich ELISA configuration (Figure 9). The SPAP system has a higher sensitivity and a lower background signal than ELISA and dot blot and achieves a significant improvement over the standard ELISA protocol (Vierling *et al*., 1999).

A new indirect competitive ELISA for quantitating taxol, a new therapeutic anticancer indicator, was improved by using a horseradish peroxidase-mouse monoclonal antibody conjugate to generate a highly specific and sensitive signal. Starting with mouse-anti taxol antibodies, bound to solid phase coated 7-succinlytaxol-bovine serum albumin, the antigen, and adding the diluted horseradish peroxidase–mouse monoclonal antibody conjugate works well to detect active taxol in the solution phase. This technique is able to detect the anticipated taxol in plasma and

saliva at a minute concentration level of less than a picogram per milliliter (Svojanovsky *et al*., 1999).

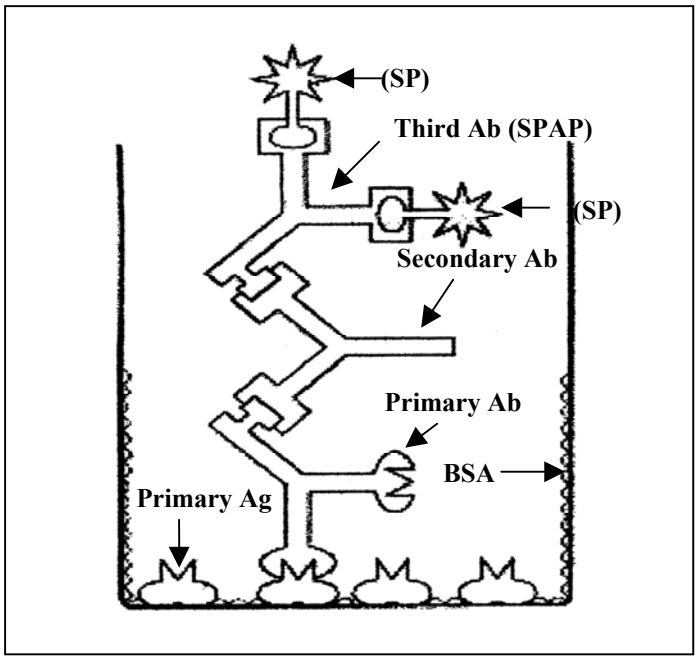


Figure 9. A three-tiered sandwich ELISA. Soybean seed coat peroxidase (SP)-mouse monoclonal antibody conjugate coupled to soybean seed coat peroxidase (SPAP) used as the third antibody.

7.6 Application of peroxidase-antibody conjugate in DNA diagnosis

DNA analysis has become an important area in molecular biology and biotechnology studies. Fields of interest for DNA diagnosis are forensic and personal identification, biomedical research, toxicology and pharmacology-targeted drug design, biomass diversity, bioremediation, industrial processing, and monitoring of biological processes (MacGall, 1997). Conventional methods for analysis of specific gene sequences are based on either direct sequencing or DNA hybridization. DNA hybridization is commonly used in the diagnostic laboratory because of its simplicity

(Christopoulos, 1999). Many techniques are applied to provide detectable signals for DNA hybridization, for example: Southern blot (Pryce *et al*., 1999), dot-blot, slot-blot (Pividori *et al*., 2001) or colorimetric detection described by Thermo Labsystems, Finland.

Peroxidase-antibody conjugate is introduced for signal generation in a microplate method for specific, fast and simple detection and differentiation of amplified DNA. In this assay, the PCR products are specifically bound to the well of coated microplate. In the well, DNA is rendered single-stranded by short alkali treatment and the hybridization is performed using type specific antigen labeled probes. Finally, enzyme-antibody conjugate and colorimetric substrate are added for signal generation. Herpes Simplex virus DNA detected by this method using peroxidase-antibody conjugate gives a higher sensitivity than the analysis by gel electrophoresis and ethidium bromide staining. Colour detection of DNA using peroxidase-antibody conjugate is shown in Figure 10 (Thermo Labsystems, Finland, http://www.chemical.com/resource/l:tlibrary/).

7.7 Waste-water treatment

Most studies on waste-water treatment with oxidoreductive enzymes are focused on the characteristics of the treated effluent and its toxicity. Additionally, the implementation of more stringent environmental regulations on hazardous waste has led to the necessity of finding innovative and environmentally friendly treatment technologies. Many oxidative enzymes including peroxidases have been described that could potentially help to remove hazardous compounds from the environment. Peroxidases have a broad substrate specificity and are able to transform a wide range of toxic compounds.

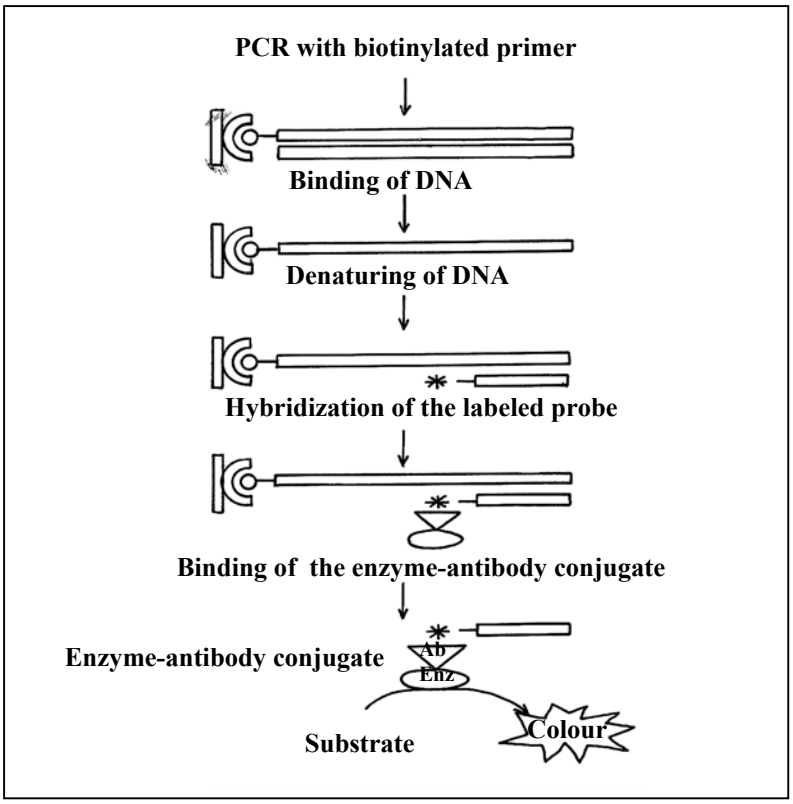


Figure 10. The principle of colorimetric detection of DNA by using peroxidase-antibody conjugate.

The polymerization of phenolic compounds by peroxidases causes their insolubilization and therefore they can precipitate out and give a less toxic waste-water (Miland *et al*., 1996; Guerra *et al*., 1997, Guerra *et al*., 1998; Aitken *et al*., 1994). Peroxidases also have been studied for many years due to their potential use as biocatalysts in pulp and paper bleaching, soil remediation, and on-site waste destruction (Durán and Esposito, 2000; Bosco *et al*., 2002).

Horseradish peroxidase (HRP), lignin peroxidase and chloroperoxidase have been reported for removing toxic compounds from the environment (Durán and Esposito, 2000). Examples of such toxic compounds oxidized by these enzymes are:

poly-cyclic aromatic hydrocarbon (PAHs) such as pyrene, benzo(a)pyrene, phenanthrene, anthracene and fluoranthene; phenolic compounds such as phenols, chlorophenols, dimetoxiphenols and nitrophenols; organophosphorus pesticides, such as parathion, terbufos, dichlofenthion, chlorpyrifos, azinphos-methyl, and phosmet; and azo dyes containing *p*-diphenylenediamine and benzidine moieties (Torres *et al*., 2003).

HRP is suitable for waste-water treatment because it retains its activity over a broad pH and temperature range (Karam and Nicell, 1997). Many studies were carried out and considerable efforts have been made to optimize the HRP-catalyzed removal of phenols from aqueous solutions. For instance oxidation of 2,4,6-trichlorophenol generating 2,6-dichloro-1,4-benzoquinone (Durán and Esposito, 1997) and removal of phenol and chlorophenol from waste-water (Zhang *et al.*, 1998). Many techniques for HRP applications in waste-water treatment have been reported: plug-flow reactors and continuous-low-stirred tank reactors (Buchanan *et al*., 1998), stopped flow techniques (Zahida *et al*., 1998), continuous flow systems (Ibrahim *et al*., 1999) and enzyme immobilization (Ferrer *et al*.,1991; Bodzek *et al*., 1994; Tatsumi *et al*., 1996; Peralta-Zamora *et al*., 1998a; Peralta-Zamora et al., 1998b; Horak *et al*., 1999; Shen and Tu, 1999). Rubber peroxidase probably is suitable for waste-water treatment because of its great thermostability from 30-70 ^{o}C over a pH range from 4.5-6.9 (Rattanapumee, 2000).

7.8 Other applications of peroxidases

It has been reported that the combination of indole-3-acetic acid and horseradish peroxidase (HRP) is cytotoxic to mammalian cells and, as such, it has

been proposed as a novel cancer therapy (Folkes *et al*., 1998). The results showed that IAA/HRP treatment induces apoptosis- programmed cell death- in G361 human melanoma cells, whereas IAA or HRP alone had no effect. It is known that IAA produces free radicals, which are oxidized by HRP. The oxidative stress could induce apoptosis, therefore this mechanism may lead to the apoptosis of human melanoma cells via both death receptor-mediated and mitochondrial apoptotic pathways (Kim *et al*., 2004). In addition, 5-fluoroindole-3-acetic acid is also oxidized by HRP compound I, but ten-fold slower than indole-3-acetic acid, and is much more cytotoxic towards V79 hamster fibroblasts in the presence of peroxidase than the unsubstituted indole. The fluorinated prodrug/peroxidase combination also shows potent cytotoxicity in human and rodent tumor cell lines. The high cytotoxicity of 5-fluoroindole-3-acetic acid after oxidative activation calls for further evaluation as a prodrug for targeted cancer therapy involving antibody-, polymer-, or gene-directed delivery of horseradish peroxidase (Folkes *et al.*, 2002).

Peroxidase is introduced as a new catalyst to synthesize valuable industrial products. In medicinal industry, it catalyses oxidative coupling of catharanthine with vindoline yielded α-3′,4′-anhydrovinblastine as a semisynthetic step in the production of the anti-cancer drugs vinblastine and vincristine from *Catharanthus roseus* (Sottomayor *et al.*, 1997). In paper industry, peroxidase catalyses oxidative coupling of methyl-(E)-sinapate with the syringyl lignin-model compound, 1-(4-hydroxyl-3,5-dimethoxyphenyl) ethanol and yields a novel spirocyclohexadienone together with a dimerization side-product, both being commercially valuable compounds in lignin synthesis (Setälä *et al*., 1999).

The royal palm tree peroxidase is a catalyst to synthesize polyelectrolyte complexes of polyaniline (PANI) and sulfonated polystyrene (SPS) (Sakharov *et al*., 2004). The production of PANI is of tremendous interest because of its high environmental stability and promising electronic properties. Its potential use is in a wide range of applications including organic lightweight batteries, light-emitting diodes, optical displays, and bio-analysis (Tatsuma *et al*., 2001). However, the chemical polymerization of its monomer aniline is carried out under strong acid conditions (Roy *et al*., 2002). This problem can possibly be overcome by the enzymatic production of PANI by using palm tree peroxidase, which can be performed under environmentally friendly conditions because the enzyme shows unusual stability over pH range of 1.0-7.0 (Sakharov *et al*., 2004).

III. Aims of Study

Rubber trees in Thailand provide natural rubber latex, the main raw material for the rubber industry, which is used to produce a range of products from car tires to medical gloves and condoms. Rubber peroxidase (RBP) and RBP-antibody conjugate may offer economic and scientific benefits for Thailand, because RBP can be obtained from leaves of rubber trees as a by-product from rubber plantations. This is depending on whether the cost of RBP and RBP-antibody conjugate preparation at an industrial scale is competitive with that of the industrial production of peroxidase from horseradish. It is therefore important to study at a lab scale techniques which may lead to an efficient industrial preparation of peroxidase and peroxidase-antibody conjugate. The aims of this study are as follows:

1. To purify the RBP from *H. brasiliensis* leaves.
2. To prepare RBP-antibody conjugate by using three different cross-linking methods.
3. To study the applications of RBP and RBP-antibody conjugate in comparison with the commercial peroxidase and peroxidase-antibody conjugate.
4. Molecular characterization of a cDNA encoding RBP should also be studied in order to know the primary structure of the RBP.