

# Purification of Natural Plant Peroxidases and their Physiological Roles

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## ABSTRACT

The plant peroxidase superfamily comprises heme-containing glycoproteins that differ in their structure and catalytic properties. POX has been isolated and characterized from a large number of plant sources like fruits, leaves, tubers and grains, and the major source of commercially available peroxidase is horseradish roots. However, availability of POX with higher stability and different specificity would improve immunoenzymatic analytical kits and promote the development of new analytical methods and potential industrial processes. Therefore, extensive investigations of several peroxidases of different origins have been reported. The diversity of reactions catalyzed by plant peroxidases (POX) accounts for the implication of several isoenzymes in a broad range of physiological processes including indole-3-acetic acid metabolism, pathogen resistance, response to oxidative and chemical stresses involving reactive oxygen species, and lignin and suberin biosynthesis. In addition, the recent description of the hydroxylic cycle which leads to the formation of various radical species, opens a new range of implications for these enzymes. A major limitation for the widespread use of POX is the current high cost of production of the enzyme. A cost-effective purification technology and alternative sources with high peroxidase activity can help bring down the cost of this enzyme production.

**Keywords:** affinity chromatography, antioxidant defense, lignification, peroxidase catalysis, peroxidase functions, peroxidase isoenzymes  
**Abbreviations:** ATPE, aqueous two phase extraction; CoI, CoII, CoIII, compounds I, II, and III, respectively; ConA, concanavalin A; E<sup>2+</sup>, ferrous form of peroxidase; E<sup>3+</sup>, native ferric peroxidase; HRP, horseradish peroxidase; HRP C, HRP isoenzyme C; IAA, indole-3-acetic acid; IAA<sup>•+</sup>, IAA cation radical; IAAOOH, exogenous IAA hydroperoxide; In-CH<sub>2</sub>O, indole-3-epoxide; In-CH<sub>2</sub>O<sub>2</sub><sup>•</sup>, skatole peroxide radical; In-CH<sub>2</sub>OOH, skatole hydroperoxide; PEG, polyethylene glycol; POX, peroxidase; ROS, reactive oxygen species

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## INTRODUCTION

Peroxidase (POX) is a ubiquitous enzyme found in animals, plant tissues and microorganisms, which reduces hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in the presence of an electron donor. Plant peroxidases (E.C. 1.11.1.7) often designated as class III

peroxidases, are heme-containing glycoproteins whose activity was first described in 1855, and whose purification took a few decades to achieve (Theorell 1951). Generally, they are encoded by a large number of paralogous genes; more than 73 different POX expressed sequence tags (ESTs) are found in *Arabidopsis* (Welinder *et al.* 2002),

while Passardi *et al.* (2004) found 138 POX genes distributed among the 12 rice chromosomes.

Class III POX can oxidize a large variety of aromatic hydrogen donors such as phenolic compounds, lignin precursors or secondary metabolites. They can also oxidize the growth hormone auxin, as well as other substrates to produce reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> (Blee *et al.* 2001), and hydroxyl radicals (Chen and Schopfer 1999) involved in oxidative burst and in cell elongation (Lizkay *et al.* 2003). Due to the diversity of reactions catalyzed by plant peroxidases, they have been implicated in a broad range of physiological processes.

Since POX is relatively stable at a high temperature (generally no activity loss after heating at 60°C for 10 min; Duarte-Vázquez *et al.* 2001, 2003b), and its activity is easily measured using simple chromogenic reactions, it has been used as a model enzyme to study protein structure, enzyme reactions and functions, and it has been used for several practical applications.

POX is widely used in clinical biochemistry and enzyme immunoassays (Johnstone and Thorpe 1987), in the treatment of waste water containing phenolic compounds (Duarte-Vázquez *et al.* 2003a), for the construction of enzyme electrodes (Ruzgas *et al.* 1996) and in organic synthesis for biotransformation of various chemicals and drugs (Adam *et al.* 1999; Colona *et al.* 1999).

Although peroxidase is widely distributed in nature, the one isolated from horseradish (HRP) is the most available and commonly used peroxidase. Currently, numerous investigations (Johri *et al.* 2005; Rudrappa *et al.* 2005; Dicko *et al.* 2006) are testing alternative sources more locally available and are studying their properties as possible substitutes for HRP.

A major limitation for the widespread use of POX is the current high cost of production of the enzyme. A cost-effective purification technology and alternative sources with high peroxidase activity can help bring down the cost of enzyme production.

This review will summarize the characteristics and reported physiological functions of POX, the main sources from which POX has been isolated as well as the methods commonly employed for its purification.

## PEROXIDASES CLASSIFICATION AND STRUCTURE

Based on structure and catalytic properties peroxidases are divided into three superfamilies (Welinder 1992; **Table 1**). The first superfamily consists of POX found in animals, including a partial region of prostaglandin endoperoxide syn-

thase (EC 1.14.99.1). Although glutathione peroxidase has been categorized into the animal peroxidase superfamily, its activity has also been detected in plants (Churin *et al.* 1999). The second peroxidase superfamily consists of catalases (EC 1.11.1.6) found in animals, plants, bacteria, fungi and yeast.

The third superfamily also known as plant peroxidases comprise heme-containing POX and has been divided into three classes based on amino acid sequence comparisons (Welinder 1992). POX belonging to this family has been found in plants, fungi, bacteria and yeast. Class I comprises intracellular peroxidases, while Class II comprises the secretory fungal POX, such as manganese peroxidase (EC 1.11.1.13) and lignin peroxidase (EC 1.11.1.14). Finally, class III consists of the secretory plant peroxidases (EC 1.11.1.7), which were originally described as peroxidases, that are secreted outside the cells or transported into vacuoles. Horseradish peroxidase is included among the later class, from which isoenzyme c (HRP C) is one of the most widely studied because of its stability and easy isolation from horseradish (*Armoracia rusticana*) roots. Consequently, most of the structure-function relationship studies within the peroxidase family have been made using HRP C as the archetypical class III peroxidases.

Higher plants possess the class I ascorbate peroxidase, and a number of isoenzymes belonging to class III peroxidases. In contrast to stringent substrate specificities of ascorbate peroxidase, class III peroxidases oxidize several substrates ranging from small molecules to macromolecules.

The plant peroxidase superfamily comprises proteins that differ in their structure and catalytic properties. The amino acid sequences among members of this superfamily have shown less than 20% identity in most divergent cases. However, their three-dimensional structures are remarkably similar, being invariably constituted by ten  $\alpha$ -helices, a small amount of  $\beta$ -sheets and a single heme located in the cavity between two antiparallel  $\alpha$ -helices. Class II peroxidases have an additional sequence of 40 to 60 amino acid residues in their C-termini when compared to peroxidases from other classes (Welinder 1992). Class III peroxidases key amino acid residues and protein size, are highly conserved between orthologs and paralogs (Welinder 1992). These proteins are characterized by some invariant residues which play important structural functions and/or are involved in the catalytic process. This is the case of proximal histidine residue interacting with the heme group (for HRP His 170), and eight cysteine residues forming disulfide bridges, as well as the adjacent arginine and aspartic acid hydrogen bonded to the proximal histidine. The distal histidine together with the invariant residue of arginine participates in

**Table 1** Classification of heme peroxidases (adapted from Welinder 1992; Chen *et al.* 1992; Ridout *et al.* 1995; Dunford 1999; Koga *et al.* 1999; Apitz and van Pée 2001; Gabaldón *et al.* 2006).

Superfamily	Class <sup>a</sup>	Member (EC number)	Origin	Molecular weight (kDa)
Animal peroxidase		Eosinophil peroxidase (EC 1.11.1.7)	Animal	50-75
		Glutathione peroxidase (EC 1.11.1.9)	Animal and plant	75-112
		Myeloperoxidase (EC 1.11.1.7)	Animal	79-150
		Thyroid peroxidase (EC 1.11.1.7)	Animal	90-110
		Prostaglandin-F synthase (EC 1.1.1.188)	Animal	17-66
		Lactoperoxidase (EC 1.11.1.7)	Animal	78-85
		Prostaglandin endoperoxide synthase (EC 1.14.99.1)	Animal	115-140
Catalase		Catalase (EC 1.11.1.6)	Animal, plant, fungus and yeast	140-530
Plant peroxidase	I	Cytochrome c peroxidase (EC 1.11.1.5)	Yeast and bacterium	32-63
		Catalase-peroxidase (EC 1.11.1.6)	Bacterium and fungus	150-240
		Ascorbate-peroxidase (EC 1.11.1.11)	Plant	30-58
	II	Manganese-dependent peroxidase (EC 1.11.1.13)	Fungus	43-49
		Lignin peroxidase	Fungus	40-43
	III	Peroxidase (EC 1.11.1.7)	Plant	28-60
		Syringyl peroxidase (EC 1.11.1.7)	Plant	33-35
		Chloroperoxidase <sup>b</sup> (EC 1.11.1.10)	Fungus	40-46
		Di-heme cytochrome c peroxidase <sup>b</sup> (EC 1.11.1.5)	Bacterium	36.5-45
		Peroxidase <sup>b</sup> (EC 1.11.1.7)	Bacterium	220->300

<sup>a</sup> Established only for plant peroxidase

<sup>b</sup> Peroxidases that do not follow the Welinder classification because of their unusual structures and properties.

the cleavage of  $\text{H}_2\text{O}_2$  during the catalytic cycle (Rodríguez-López *et al.* 1996). Both distal and proximal histidine residues are present in all known heme-containing peroxidase sequences.

In spite of this conservation, the isoelectric points of plant POX largely differ even within the same source (anionic to cationic; Duarte-Vázquez *et al.* 2000).

Class III plant peroxidases are glycoproteins that contain N-linked oligosaccharide chains, such as HRP (Welinder 1985), and peanut POX (Hu and van Huystee 1989). Some authors (Nie *et al.* 1999; Duarte-Vázquez *et al.* 2003b) have shown that the carbohydrate moieties of peroxidase contribute to the high stability of the POX. The glycosylated sites are found in the consensus N-glycosylation sites, Asn-X-Ser/Thr. However, the number and location of potential glycosylation sites are not highly conserved among plant peroxidases. In anionic flax cationic peroxidase there is only one potential glycosylation site located at Asn 71 (Omman *et al.* 1994); HRP C1 is glycosylated at eight Asn residues (13, 57, 158, 186, 198, 214, 255 and 268; Welinder 1976), while three potential N-glycosylation sites were found in HRP C2 (Asn 57, 186, and 214) and HRP C3 (Asn 57, 188, and 214) (Fujiyama *et al.* 1990).

The number of glycosylation sites varies thoroughly among plant peroxidases. One glycosylation site was found for peanut peroxidase (Buffard *et al.* 1990), two for barley peroxidase (Kristensen *et al.* 1999), while six glycosylation sites were found for soybean peroxidase (Chen and Vierling 2000). The variability in number and location of N-linked oligosaccharide chains may be important for modulating the physicochemical properties of the glycoproteins but also in mediating their biological activity.

The glycosylation site occurring at Asn 185 has been recognized as a conserved region in the amino acid sequence of some plant peroxidases (Fujiyama *et al.* 1990; Omann *et al.* 1994). Lige *et al.* (2001), using site directed replacement of each of the three glycosylation sites of cationic peanut peroxidase, demonstrated that the glycan linked to Asn 185 was important for thermal stability, although its removal had no effect on enzyme activity. This study also demonstrated that the three glycosylation sites are important for a stable peroxidase conformation. A POX showing high thermal stability may have wider applications in biosensors, diagnostic kits, or in other relatively high temperature processes (Wang *et al.* 2001; Alpeeva *et al.* 2005; Sakharov *et al.* 2006).

### CLASS III PLANT PEROXIDASES CATALYTIC CYCLE

These peroxidases catalyze the oxidation of a wide variety of electron donor substrates (e.g. phenols, aromatic amines) using  $\text{H}_2\text{O}_2$  or other peroxides (Dunford 1999). The peroxidase catalytic cycle involves distinct intermediate enzyme forms (Wong, 1995). In the initial step the native ferric enzyme resting form is oxidized by  $\text{H}_2\text{O}_2$  to form an unstable intermediate called compound I (CoI), which has a heme structure of  $\text{Fe}^{\text{IV}}=\text{O}^-$  porphyrin  $\pi$ -cation radical, and the consequent reduction of peroxide to water. Then, CoI oxidizes an electron donor substrate to give compound II (CoII) (same oxyferryl structure but protonated) releasing a free radical. CoII is further reduced by a second substrate molecule regenerating the iron (III) state and producing another free radical product (Fig. 1).

While the above reactions dominate the overall process, a number of side reactions could also take place. A significant portion of compound II could be oxidized by  $\text{H}_2\text{O}_2$  to form compound III (CoIII) in a hydroxylic cycle (Fig. 1). CoI could also react with  $\text{H}_2\text{O}_2$  producing the inactive form, verdohemoprotein (P-670). These two reactions are usually observed at rather high concentrations of  $\text{H}_2\text{O}_2$ . However, it should be noted that some peroxidases, such as those isolated from palm tree show improved stability in presence of hydrogen peroxide (Sakharov 2004).

The diversity of reactions catalyzed by plant peroxi-

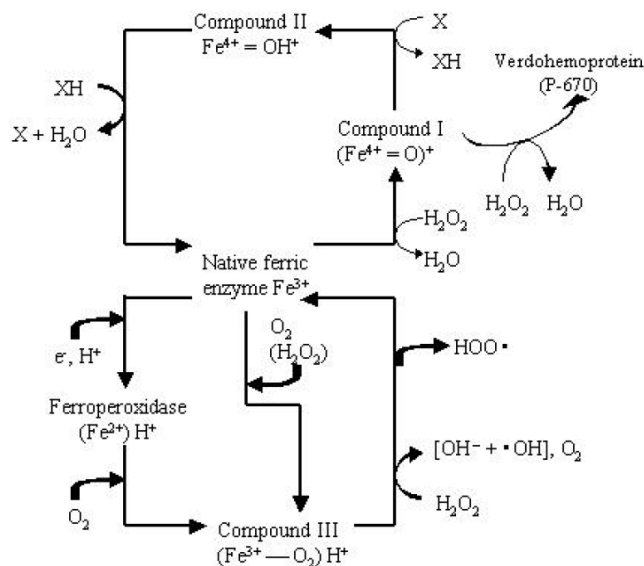


Fig. 1 Peroxidative and hydroxylic catalytic cycles of peroxidase.

dases accounts for the implication of these proteins in a broad range of physiological processes (Fig. 1). In addition, the recent description of the separate hydroxylic cycle, which leads to the formation of various radical species, opens a new range of implications for the peroxidases (Pasardi *et al.* 2004).

### PLANT PEROXIDASES: SOURCES AND PURIFICATION METHODS

#### Sources of peroxidase

POX is widely distributed among higher plants, generally showing several isoenzyme forms differing in molecular and catalytic properties. These characteristics have made difficult to assign an *in vivo* function for a particular isoenzyme.

Peroxidases are used commercially as catalysts for phenolic resin synthesis (Dordick *et al.* 1994), as an indicator for ROS formed during food processing and as a component of kits for research, medical diagnosis (Trivedi *et al.* 1978; Ternaux and Chamoin 1994; Elekes *et al.* 1995) and bioremediation (Adam *et al.* 1999). Peroxidase is the enzyme most frequently used in the manufacture of enzyme immunoassay kits and medical diagnosis kits. In fact, more than 90% of the immunoenzymatic kits are prepared using peroxidase as label enzyme to generate immune conjugates. It is used in the enzymatic determination of glucose plasma concentration (Krell 1991), and to quantify a number of compounds using ELISA tests, such as therapeutic drugs (Ternant *et al.* 2006; Zhang *et al.* 2006b), viruses (Zhuang *et al.* 2001; Zhang *et al.* 2006a) and some grain toxins (Sibanda *et al.* 2000; Güll *et al.* 2007). This enzyme can also be employed in biosensors, the transformation of drugs, the production of chemicals, degradation of aromatic compounds, environmental control and in processes for making a wide variety of chemical intermediates, as well as formaldehyde-free phenolic resins (Regalado *et al.* 2004), and synthesis of conducting polyaniline (Liu *et al.* 1999b; Caramishev *et al.* 2005).

Peroxidase has been isolated and characterized from a large number of plant sources like fruits, leaves, tubers and grains. However, nowadays the major source of commercially available peroxidase is horseradish roots, where it occurs as multiple isoenzymes (Shannon *et al.* 1966). On the other hand, availability of peroxidases with higher stability and different specificity would improve immunoenzymatic analytical kits and promote the development of new analytical methods and potential industrial processes. Therefore, extensive investigations of several peroxidases of different

**Table 2** Sources and purification schemes of some plant peroxidases, from 1995 to December 2006.

Source	Purification scheme	Main properties of purified enzyme	Reference
Sorghum grain	Acetone precipitation followed by conventional chromatographic techniques (gel filtration and cation exchange).	Glycoprotein, Mr weight about 35 kDa, N-terminal fragment has high identity with barley, rice, wheat and maize peroxidases. Catalyzes more efficiently the oxidation of hydroxycinnamates.	Dicko <i>et al.</i> 2006
Wheat grass	Temperature and ammonium sulfate precipitation followed by isoelectric chromatofocusing.	At optimal reaction conditions, it oxidized aromatic amine substrate (OPD) more efficiency than phenolic substrates.	Lai <i>et al.</i> 2006
Buckwheat ( <i>Fagopyrum esculentum</i> Moench var. Kitawasesoba) seeds	AS precipitation followed by cation exchange and gel filtration chromatographies.	Two isoperoxidases were purified with a molecular weight ranging from 46.1 to 58.1 kDa. Both peroxidases oxidized quercetin, o-dianisidine and guaiacol.	Suzuki <i>et al.</i> 2006
Olive fruit ( <i>Olea europea</i> L., cv. Douro)	From an acetone powder was used as source. Differential AS precipitation followed by sequential cation and anion exchange chromatographies.	Anionic fraction with isoelectric point of 4.4, with optimum pH and temperature of 7.0 and 34.7, respectively. Characterized as a pectin binding peroxidase.	Saraiva <i>et al.</i> 2006
Korean radish seeds ( <i>Raphanus sativus</i> )	Immunoaffinity purification using polyclonal anti-peroxidase C3 antibodies covalently attached to a solid-phase.	Cationic glycoprotein, Mr about 44 kDa, high activity for coumaric acid, ferulic acid, and scopoletin. Thought to play an important role in plant cell wall formation during germination.	Kim and Lee 2005
Marula fruits ( <i>Sclerocarya birrea</i> subsp. Caffra)	Combination of temperature induced phase separation, DEAE-ion exchange and Sephadex G100 gel filtration.	Mr about 70 kDa and pI 5.4. Maximal activity at pH 4.0. Bi-phasic curves for thermal deactivation.	Mdluli 2005
<i>Withania somnifera</i> roots	Four isoperoxidases were purified from cell free extracts by FPLC using ion exchange, affinity and hydrophobic columns.	Monomeric glycoproteins with Mr between 34 and 48 kDa, and pI between 3.6 and 4.8. Mostly indole-3-acetic acid oxidase activity, did not require H <sub>2</sub> O <sub>2</sub> for the activity.	Johri <i>et al.</i> 2005
<i>Arabidopsis thaliana</i> leaves	Affinity chromatography through a column filled with Ca <sup>2+</sup> -polygalacturonate polyacrylamide gel, followed by preparative isoelectric focusing.	Pectate-binding peroxidase (AtPrx34) identified by N-terminal amino acid microsequencing.	Shah <i>et al.</i> 2004
Corn steep water ( <i>Zea mays</i> )	Combination of acetone and AS precipitations, and sequential chromatography on CM-cellulose, phenyl-Sepharose, and Sephadex G-7.	Nonglycosylated peroxidase with Mr about 33 kDa and pI > 10. Optimum pH depended on substrate. Active on ABTS, guaiacol, ferulic acid, o-dianisidine, o-phenylenediamine, and pirogallol. In the presence of 1 mM Ca <sup>2+</sup> POX stability is increased.	Gray and Montgomery 2003
<i>Aloe barbadensis</i> leaves	Combination of hydrophobic and ion-exchange chromatographies.	One cationic (pI 9.0) and one anionic (pI 4.5) peroxidases were purified. The two enzymes showed a Mr = 40 kDa. Apparent K <sub>M</sub> values for phenolic compounds were higher in the cationic than in the anionic isoenzyme.	Esteban-Carrasco <i>et al.</i> 2002
Royal palm tree ( <i>Roystonea regia</i> ) leaves	AS precipitation, extraction of colored compounds by DEAE-toyopearl column and consecutive chromatographies on phenyl-Sepharose, Sephacryl S100 and DEAE-toyopearl.	Monomeric protein with Mr = 51 kDa, and pI of 3.5 Substrate specificity was highest for ferulic acid. Palm peroxidase was highly stable over the pH-range 4-11. It showed unusual thermal stability (1 h at 70°C).	Sakharov <i>et al.</i> 2001
Iranian winter radish ( <i>Raphanus sativus</i> ) roots	Purification achieved using Zn <sup>2+</sup> precipitation followed by ATPE with a polyethylene glycol (PEG)/phosphate system.	A novel stable peptide (Mr= 5.85 kDa), showing peroxidase activity was isolated.	Omumi <i>et al.</i> 2001
Strawberry ( <i>Fragaria ananassa</i> cv. Chandler) callus culture	Cold acetone precipitation followed by sequential CM-Cellulose, Con A Sepharose 4B and Sephacryl S-200 chromatographies.	Monomeric glycoprotein with Mr = 35 kDa and pI 9.2. Highly stabilized by bovine serum albumin (BSA), Ca <sup>2+</sup> , and hematin.	López-Arnaldos <i>et al.</i> 2001
Cucumber ( <i>Cucumis sativus</i> ) peelings	AS precipitation and sequential DEAE-cellulose, Q-Sepharose, Phenyl Sepharose, Con A-Sepharose and Sephacryl S-100 chromatographies.	Two heme peroxidases of 35.2 and 36.5 kDa were isolated. Both proteins existed as a mixture of high spin and low spin ferri forms in a 2:1 molar ratio. Efficiently catalyzed the oxidation of guaiacol and pirogallol, and ascorbate to a lower extent.	Battistuzzi <i>et al.</i> 2001
Melon ( <i>Cucumis melo</i> L.)	AS precipitation followed by hydrophobic and anion exchange chromatographies.	Anionic (pI 3.7) peroxidase not active on ascorbic acid, but oxidized guaiacol at a high rate. Salinity induced levels of melon POX.	Rodríguez-López <i>et al.</i> 2000
Turnip ( <i>Brassica napus</i> L. var <i>esculenta</i> D.C.) roots	Differential AS precipitation followed by anion exchange (DEAE-cellulose), and HPLC-ion exchange (Resource Q) chromatographies.	Two acidic (pI 3.0) and one basic (pI 8.5) isoenzymes were purified. The Mr ranged from 39.2-42.5 kDa. The ABTS K <sub>M</sub> values were 5 times lower than that reported for acidic commercial HRP. Activation energies for inactivation of the three isoenzymes were higher or comparable to other peroxidases.	Duarte-Vázquez <i>et al.</i> 2000
Brussels sprouts ( <i>Brassica oleracea</i> var. <i>gemmifera</i> )	Precipitation with AS followed by gel filtration (Sephadex G-100), cation exchange (SPTrisacryl) and anion exchange (Q-Sepharose) chromatographies.	Two cationic and two anionic isoperoxidases were purified. The Mr ranged 26.8-48.3 kDa. It was 95% identical to drought/salt stress induced POX from turnip and 43% to drought-induced proteins from radish and rape.	Forsyth and Robinson 1998
Tea leaves	Cation exchange (SP-Sepharose, Mono S), gel filtration (Superdex 200), and hydroxyapatite (Bio-Scale CHT2-1) chromatographies.	Monomeric isoenzyme purified to homogeneity. Specific activity 150 μmol min <sup>-1</sup> mg <sup>-1</sup> ; Mr 34.6 kDa, and pH optimum 4.5-5.0. It is a class III peroxidase showing high specificity to ascorbate (K <sub>M</sub> = 470 μM).	Kvaratskhelia <i>et al.</i> 1997

Table 2 (cont.)

Source	Purification scheme	Main properties of purified enzyme	Reference
Barley grain ( <i>Hordeum vulgare</i> L., mutant <i>Risoe 1508</i> )	AS precipitation followed by cation exchange (CM 52 cellulose, CM Sephadex C-50, CM Sepharose CL 6B), and affinity (Con A-Sepharose) chromatographies	The peroxidase was stable from pH 3 to 11. Melting temperature of 75 °C at pH 6.6.	Rasmussen <i>et al.</i> 1997
Wheat germ	AS precipitation followed by anion exchange (DEAE-Cellulose), cation exchange (CM-Trisacryl, and mono S) chromatographies.	Three isoperoxidases were purified. They are glycoproteins with very similar Mr values (35 kDa).	Converso and Fernández 1995

AS, ammonium sulfate; ATPE, aqueous two phase extraction; Con A, Concanavalin A; Mr, molecular weight; POX, peroxidase.

origins have been reported. Peroxidase from soybean (*Glycine max*) seed coat (SBP) has a pI of 4.1 (single isoenzyme) and properties characteristic of glycoprotein such as high thermal stability, since the enzyme could tolerate heating for 10 min at 75°C (Liu *et al.* 1999a). An ideal enzyme for large-scale biocatalysis would be one that is readily abundant, possesses wide substrate specificity and remains stable over a wide range of pH and temperatures. Peroxidase from soybean hulls has better temperature and solvent stability, higher redox potential and is more cost-effective than HRP for waste water treatment. Moreover, the activity, stability, long shelf-life, and lower rate of emission decay by luminol oxidation, make SBP an adequate replacement of HRP in immunoassays and diagnosis (Liu *et al.* 1999a; Sakharov *et al.* 2006).

Peroxidase is highly distributed in plants that belong to the *Cruciferae* family such as Brussels sprouts, broccoli, turnip roots and radishes which are extensively produced in North America and México, and have been tested as alternative source of peroxidase. Duarte-Vázquez *et al.* (2001) purified to homogeneity and partially characterized a neutral peroxidase (TNP; pI 7.2) from turnip roots. This enzyme showed a low ABTS  $K_M$  value and high specific activity (1930 ABTS units/mg) and high catalytic efficiency (500  $M^{-1} s^{-1}$ ). These properties make TNP an enzyme with high potential as alternative to HRP in various applications. Later, Duarte-Vázquez *et al.* (2003b) purified a thermally stable acidic peroxidase (TAP) from the same source, and studied its spectral changes during thermal reversible inactivation. These authors clarified the role of the heme group and calcium domains in thermal inactivation and reactivation of TAP.

Within the Brassica family other plants have been tested to obtain peroxidase. Regalado *et al.* (1999) purified 503-fold an isoenzyme from Brussels sprouts through ammonium sulfate and acetone fractionation, and successive chromatography on DEAE-cellulose, Sephadex G-100 and Mono-S columns. The catalytic and stability properties of this POX might lead to a potential application in immunoassays as an alternative to HRP.

López-Molina *et al.* (2003) have examined the peroxidase activity of several plants among them artichoke (*Cynara scolymus* L.), which is widely cultivated in Mediterranean countries. From this source they purified a basic peroxidase (AKPC) highly reactive with nitric oxide, suggesting its potential use for quantitative spectrophotometric determination of NO and as a component of novel NO sensitive electrodes.

Considering the high diversity of tropical flora around the world, peroxidases from tropical plants have a good potential. Oil palm (*Elaeis guineensis* Jacq.) leaf is one of the richest sources of peroxidases, however it has not been fully studied. The crude extract of oil palm leaves has shown peroxidase specific activity of 590 guaiacol U/mg (Sakharov *et al.* 2000). In addition, comparative screening of different palm tree leaves as a source of peroxidase was reported by the same group (Sakharov *et al.* 2001).

Later, a single peroxidase from oil palm leaves was purified 429-fold to homogeneity with a recovery of 54%. It showed an optimum pH of 5 and exhibited a very high resistance even at temperatures as high as 80°C (Deepa and

Arummughan 2002). From the same source Sakharov *et al.* (2002), purified an acidic peroxidase that exhibited an unusually high thermal stability, besides being more stable in organic solvents than HRP. The unique stability of this peroxidase together with the relatively high abundance of oil palm leaves, make this source a good alternative to produce POX useful for analytical applications as well as for large-scale applications.

Some other tropical plants have shown high peroxidase activity, among them sweet potato (*Ipomoea batatas*) tubers. It has been previously reported that cultured cells of sweet potato exhibit higher peroxidase activity than that of horseradish roots (Kwak *et al.* 1995). Using these cells as a source, three anionic peroxidases have been purified and partially characterized. Castillo Leon *et al.* (2002) demonstrated that the major peroxidase activity is present in the peel of sweet potato. From this source, sweet potato peroxidase (SPP) was purified to homogeneity and partially characterized. The substrate specificity of SPP is distinct from that of other plant peroxidases. This new POX source with alternative substrate specificity could be of interest for the development of new electrochemical biosensors for bioanalytical applications (Lindgren *et al.* 2000). Other reports showing purification of POX from plant sources are shown in Table 2.

### Conventional purification methods

A major limitation for the widespread use of peroxidase is the current high cost of its production. It has been estimated that the bioseparation steps for final product recovery may account for 50-80% of the overall production costs (Wheelwright 1989). The enzyme cost can be addressed either by reducing the production cost and/or by reducing its recovery and purification cost. Chromatographic separation of proteins which can be applied to peroxidase purification, involve processing costs which can dramatically increase depending on the number of purification stages and type of chromatography (Bonnerjea *et al.* 1986). In addition, chromatographic procedures are limited in many cases by the scale of operation and the cost of resins.

From previous reports (Kula 1980; Dekker and Lesser 1994; Miranda *et al.* 1998), it seems that liquid-liquid extraction processes being potentially scalable to large scale biotechnological separations, show one of the most economically feasible alternatives for plant peroxidase purification. One purification scheme is reverse micellar extraction, which is selective, with high recovery and activity yields, and can be adequately modeled. However, high interfacial mass transfer resistance during back extraction with a second aqueous phase may delay continuous processes and individual proteins must be tested to obtain economical feasibility of an industrial process (Dekker *et al.* 1991). Aqueous two phase systems is another purification scheme, which has been successfully applied to plant peroxidases, incorporating polyvinylpyrrolidone in a second extraction stage to provide a relatively pure enzyme in two purification steps (Miranda and Cascone 1997), with potential application for large scale economical processing.

Another alternative is a two step precipitation with ammonium sulfate, followed by a three phase separation pro-

**Table 3** Purification fold and yield of some plant peroxidases purified using conventional techniques.

Peroxidase from	Purification fold	Yield (%)	Reference
<b>Sorghum grain</b>	107.1	28.0	Dicko <i>et al.</i> 2006
<b>Broccoli</b>			Thongsook and Barret 2005
Neutral POX	92.5	9.8	
Acidic POX	173	20.1	
Basic POX	30.6	2.6	
<b>Turnip roots</b>			
TNP	200	13.5	Duarte-Vázquez <i>et al.</i> 2001
TAP	315	20.3	Duarte-Vázquez <i>et al.</i> 2003b
<b>Cabbage roots</b>			Wang <i>et al.</i> 1999
A1	21.73	3.73	
A2	13.04	1.86	
<b>Brussels sprouts</b>			Forsyth and Robinson 1998
A1	26.0	0.19	
A2	8.0	0.04	
C1	18.0	0.14	
C2	13.0	0.15	
<b>Buckwheat seed</b>			
POXI	6.6	0.10	Suzuki <i>et al.</i> 2006
POXII	67.4	1.14	
<b>Corn steep water</b>			Gray and Montgomery 2003
F1	ND	2.4	
F2	31033	12.5	
F3	19166	8.2	
<b>Sweet potato tubers</b>	117	15	Castillo Leon <i>et al.</i> 2002
<b>Royal palm tree leaves</b>	73.0	12.5	Sakharov <i>et al.</i> 2001
<b>Soybean hulls</b>	57.1	16.4	Liu <i>et al.</i> 1999a
<b>Tobacco</b>	10.0	80.0	Gazaryan and Lagrimini 1996

POX, Peroxidase; TAP, turnip acidic peroxidase; TNP, turnip neutral peroxidase.

cess of peroxidase using *t*-butanol and aqueous phases, which has been tested on a pilot scale and only involves three liquid-liquid extraction processes to obtain a purified enzyme, probably involving a low purification cost (Singh and Singh 2003).

Many methods for plant peroxidase purification have been reported. After homogenizing the crude material with a buffer or water, the homogenate is filtered, concentrated by precipitation, centrifuged, and the enzyme purified by applying different chromatographic steps. Following this scheme peroxidase preparations with a relatively high grade of purity and yield have been obtained (**Table 3**).

The above mentioned laboratory procedures produce a low yield. There is a clear need to develop simpler methods for the large-scale purification of this industrially important enzyme.

### Membrane affinity chromatography

The formation of the association between lectins and the carbohydrate moiety of a glycolipid or a glycoprotein has been widely used in affinity separations. Among the lectins, Concanavalin A (ConA) is the one mostly used because of its simplicity of handling in classical column affinity chromatography.

Affinity membrane chromatography is another technique of using ConA which offers some advantages over classical column chromatography, such as higher flow rate, faster binding rate, lower pressure drop, higher productivity and easier scale-up (Charcosset 1998).

ConA was covalently coupled to a cellulose membrane via three activation and immobilization steps involving triazine, glutaraldehyde and diazotization. The absorption behavior of the affinity membranes for various feed concentration and flow rates was evaluated by using two common glycoproteins (ovalbumin and  $\gamma$ -globulin; Guo and Ruckenstein 2003). The diazotization method provided the best ConA affinity membranes showing high ligand density and

adsorption capacity. These authors used the affinity membranes for the separation and purification of POX. The peroxidase from an acetone precipitated extract of horseradish could be separated with 24.5% recovery and 142-fold enrichment, while purification of a commercial sample could achieve 71% recovery with 2.3-fold purification.

The separation of plant peroxidases by column chromatography usually includes one or more ion-exchange processes, before the affinity separation (López Arnaldos *et al.* 2001; Sakharov *et al.* 2001). On the other hand affinity membrane processes involve dialysis as the only previous step, resulting in lower losses of enzyme activity because of fewer purification steps.

### Aqueous two-phase extraction (ATPE)

Some of the problems encountered during the isolation, extraction and purification of plant enzymes are due to the presence of a rigid cell wall, phenolic compounds, and sometimes a high extract viscosity. The use of aqueous two-phase extraction systems in downstream processing has been focused on the extraction, separation and concentration of various biomolecules included enzymes from plant sources (Vilter 1990). However, many times ATPE has been used as a primary purification technique to reduce the bulk of the processing stream, followed by more selective final purification steps such as chromatography, electrophoresis, etc.

Conventional initial purification steps such as ammonium sulfate precipitation and acetone are laborious and time consuming. The advantages of ATPE are low process time, low energy consumption and biocompatible environment to the biomolecule because of the aqueous extraction system.

Following this approach many extraction procedures have been developed to purify plant peroxidases. Purification of POX from African oil palm leaves involved homogenization and extraction of pigments using an ATP system. Initially, the polyethyleneglycol/K<sub>2</sub>HPO<sub>4</sub> system was used, however, the replacement of K<sub>2</sub>HPO<sub>4</sub> with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> allowed direct application of the salt phase, rich in peroxidase, on a Phenyl-Sepharose column. Final purification was carried out by chromatographic separation using Sephacryl S200 and DEAE-toyopearl. This purification scheme resulted in a 112-fold purification producing nearly homogenous enzyme with about 40% activity recovery (Sakharov *et al.* 2000).

Mdluli (2005) purified a peroxidase from marula fruit using a combination of temperature induced phase separation in Triton X-114, DEAE-ion exchange and Sephadex G-100 gel filtration. Peroxidase was purified 19-fold with 25% recovery.

Srinivas *et al.* (1999) reported the use of polyethylene glycol (PEG)/ammonium sulfate ATP system coupled with gel filtration on Sephadex G-100 to purify POX from *Ipomoea palmata* leaves. The PEG/ammonium sulfate system gave a purification factor of 2.18, volume reduction of 57.5% and bottom phase recovery of 91.5%. The enzyme was finally purified about 49-fold by gel filtration chromatography, with a recovery of 75.3%.

Another variant to conventional ATPE is the use of metal affinity partitioning. Metal ions are potentially useful to selectively extract proteins which have histidine, cysteine and tryptophan residues on their surfaces by affinity partitioning in a simple and rapid procedure with good applicability to scale-up and may replace some of the chromatographic steps in downstream processing. The main advantages using metals as ligands are: (1) many times recycling with insignificant loss in performance, (2) high metal loading and therefore high protein capacities, (3) product elution and ligand regeneration are achieved with relative ease, (4) low cost of the metals used, (5) interactions with target molecules are reasonable specific and reversible under mild conditions. Combining these concepts da Silva and Franco (2000) developed a two-step liquid-liquid extraction process to purify peroxidase from a crude extract of soybean seeds.

PEG 4000 was activated using thionyl chloride, covalently linked to iminoacetic acid (IDA), followed by Cu<sup>2+</sup> attachment to the PEG. In the first step the system comprised 14% (w/w) PEG 4000-IDA-Cu<sup>2+</sup> and 8% (w/w) Na<sub>2</sub>SO<sub>4</sub>, and POX partitioned mainly to the top phase. In the second step, a 4% PEG 4000 and 10% phosphate system was used to revert POX partitioning, this time to the bottom salt-rich phase, thereby achieving a 64% recovery. The purification factor of the POX collected in the phosphate phase was 145. Three main bands appeared after electrophoresis, indicating that this material was purer than the commercial standard soybean peroxidase.

ATPE has also been used in combination with ultrafiltration for purification of *Ipomoea* peroxidase (Srinivas *et al.* 2002). Optimum conditions were achieved using PEG-1550/KH<sub>2</sub>PO<sub>4</sub> system, having 2% NaCl. Extraction, enrichment, and purification of POX was performed on a large scale in combination with ultrafiltration to obtain about 76% recovery, 5.9-fold purity and 9.7-fold activity. The major point of this approach was the achievement of differential partitioning to separate POX from cell debris and color compounds.

### Peroxidase purification using reverse micelles extraction

Reverse micelles are thermodynamically stable surfactant aggregates with polar cores, which are formed spontaneously when certain types of surfactants are dissolved in organic solvents. Water and host macromolecules can be solubilized within those cores, protected from direct contact with the organic solvent. Proteins can be selectively extracted by manipulating parameters such as surfactant concentration, salt concentration in the aqueous solution, water acidity, or aqueous:organic phase ratio. Electrostatic interactions have been identified as the main driving force of protein solubilization.

Several groups have investigated the process of peroxidase incorporation into reversed micelles. Huang and Lee (1994) purified peroxidase from horseradish roots. A single-step extraction at pH 11 was used to remove contaminant proteins. A purification factor of 24 indicated a partly purified peroxidase, but no evidence was provided regarding the purity achieved. Regalado *et al.* (1996) purified the same enzyme by a two-stage reverse-micellar extraction from the dialyzed extract. The HRP was obtained with a purification factor of 80 and yield of 46%. SDS-PAGE showed two overlapping bands, with HRP corresponding to that of 43.8 kDa. Image analysis on isoelectric focusing gels showed that the HRP was 80% pure. Ion exchange liquid chromatography showed that most of the specific activity was due to the basic isoenzyme with pI 8.5.

Pérez-Arvizu *et al.* (1999) purified peroxidase from broccoli using 0.2 M surfactant cetyl trimethyl ammonium bromide (CTAB) reverse micelles in isoctane/*n*-pentanol (95:5) with 60% yield and a purification factor of 6.7. Later, Regalado *et al.* (2003) used reverse micelles to purify peroxidase from turnip roots, Brussels sprouts and radishes. Purification using CTAB reverse micelles produced a turnip peroxidase extract having specific activity (173 ABTS units/mg) suitable for the analysis of glucose and cholesterol, where high purity is not important. A two-step forward extraction with reverse micelles of 0.20 M CTAB in isoctane/hexanol (90:10) produced about 8 times the purification factor achieved with ammonium sulfate with similar activity yield. Since the purification and activity yield compared favorably with the results obtained by precipitation methods, it was concluded that the process of prepurifying peroxidase involving dialysis and reverse micellar extraction is an economic alternative to salting-out procedures. The following purification stage would be ion exchange chromatography.

An affinity-based reverse micellar extraction for the purification of peroxidase from soybean hulls was proposed (Paradkar and Dordick 1993). High purification factor with

nearly pure peroxidase was obtained after regeneration of the CoA ligand. Unfortunately, the ligand could not be completely recovered, thus adding to the cost of the process.

## PHYSIOLOGICAL ROLES

Peroxidases are found in cell walls and vacuoles and they have been implicated in many physiological processes. Interesting insights concerning the physiological roles of peroxidases have been obtained by *in vitro* data or by correlating the induction of specific isoforms to particular external stimuli. By using these approaches it has been suggested that POX play an important role in several physiological processes including indole-3-acetic acid metabolism (Gazaryan and Lagrimini 1998), pathogen resistance (Sasaki *et al.* 2004), response to stress conditions (Kim *et al.* 2000; Yannarelli *et al.* 2006) and lignin and suberin biosynthesis (de Obeso *et al.* 2003; Gabaldón *et al.* 2005).

### Lignin and suberin biosynthesis

Lignin is present in vascular plants and is mainly synthesized in cells to form part of the transport system. Suberin is synthesized in the endodermis and exodermis parts of the root, where it strengthens the cell wall and contributes to control water movement. In aerial parts suberin is also considered to be a component of the plant defense response induced by pathogens and wounds (Mohan *et al.* 1993).

Lignins are three-dimensional, amorphous heteropolymers that result from oxidative coupling of monolignols, *p*-hydroxycinnamyl, *p*-coumaryl, coniferyl and sinapyl alcohols (Kolattukudy 1981). The initial step of lignification is synthesis of monolignols through the phenylpropanoid pathway. Monolignols are transported to cell walls, and polymerized to form lignin on site. Of these three steps, the final reaction of monolignols polymerization is thought to be executed mainly by POX (Boerjan *et al.* 2003). For a long time, peroxidases have been considered the unique enzymes responsible for this reaction, although further investigations suggest a role for laccase in lignin biosynthesis (Bao *et al.* 1993). Laccase activity or gene expression was observed in lignifying tissues, such as those from *Liriodendron tulipifera* (LaFayette *et al.* 1999) and *Populus trichocarpa* (Ranocha *et al.* 1999).

Cell wall associated POX have two roles in the lignification of plant cells. First, they oxidize cinnamyl alcohols in a reaction that requires H<sub>2</sub>O<sub>2</sub> to form mesomeric phenoxy radicals that polymerize to form lignin. Second, they can oxidize NADH to form H<sub>2</sub>O<sub>2</sub>, which activate xylem peroxidases, and also play a regulatory role during programmed cell death, which characterizes xylem differentiation (Fukuda 1997; Drury and Gallois 2006).

A more precise understanding of the role of specific plant POX isoenzymes may be obtained by applying a molecular analysis approach and the production of transgenic plants. In that way, POX associated to lignification have already been described in several plants, such as wheat (Baga *et al.* 1995), poplar (Christensen *et al.* 2001), and pine (Charvet-Candela *et al.* 2002).

Tomato POX has been proposed to participate in the last steps of both lignin and suberin synthesis (Quiroga *et al.* 2000), since the oxidation of cinnamyl alcohols prior to their polymerization is a common process to synthesize both heteropolymers.

The identification of two presumably full-length and a truncated cDNA encoding for corn peroxidases named *ZmPox1*, *ZmPox2*, and *ZmPox3*, respectively, were reported by de Obeso *et al.* (2003). These cDNA clones were isolated by differential screening from a corn cDNA library. *In situ* hybridizations indicated that *ZmPox2* mRNA was located in vascular tissues and the epidermis. Although mRNAs *ZmPox1* and *ZmPox2* were both located in root tips, *ZmPox1* mRNA was only detected in the epidermis but not in the vascular tissue of young roots, suggesting that its function is not correlated to lignification. Earlier, only one



corn peroxidase, *ZmAP1*, had been well characterized and was related to the suberization process (Teichman *et al.* 1997).

Cells of *Zinnia elegans*, a flowering plant belonging to the Asteraceae family have been widely used as a model for studying the last step of lignin biosynthesis, due to the simplicity and duality of the lignin pattern shown by stems and hypocotyls (Pesquet *et al.* 2006). These cells are also characterized by showing a pattern which is almost completely restricted to the presence of basic isoperoxidases (López-Serrano *et al.* 2004). Furthermore, *Z. elegans*, and recently, *Arabidopsis thaliana* offer the unique possibility of working with cell cultures that resemble differentiating xylem cells and may provide exceptionally useful models for monitoring the expression of enzymes from the lignin biosynthetic pathway, especially the segment that is concerned with the phenylpropanoid backbones (Milloni *et al.* 2002).

Using *Z. elegans* cell culture, Gabaldón *et al.* (2005) purified and cloned the major basic peroxidase (ZePrx) involved in lignin biosynthesis which was composed of two isoforms with Mr of 34,700 (ZePrx34.70), and 33,400 (ZePrx33.40). Full-length cDNAs coding for these peroxidases were cloned and sequenced, and they only differed in the 5'-untranslated region. Sinapyl alcohol was the best substrate for these POX isoforms, and the endwise polymerization of this alcohol by both enzymes yielded highly polymerized lignins with a degree of polymerization  $\geq 87$ . Western blots showed that ZePrx33.40 was expressed in the tracheary elements (TEs), roots, and hypocotyls, while ZePrx34.70 was only expressed in roots and young hypocotyls.

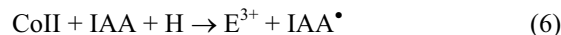
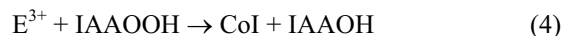
Sato *et al.* (2006), in an attempt to elucidate the regulatory mechanism of vessel lignification, isolated and characterized the novel peroxidase gene *ZPO-C*. The expression and function of this gene is closely associated with lignification during TE differentiation. A transient accumulation of the *ZPO-C* transcript was observed at the time of secondary wall thickening of TEs in xylogenic cultures. *ZPO-C* was expressed in cultured tobacco cells, and the purified peroxidase showed significant oxidizing activity on coniferyl and sinapyl alcohols.

The  $H_2O_2$  used by xylem peroxidase to polymerize cinamyl alcohol is apparently supplied by a NADPH oxidase-like enzyme present in lignifying xylem cells. Ros-Barceló *et al.* (2002) and Pedreño *et al.* (2006) showed that  $H_2O_2$  production by lignifying xylem was stimulated by the sulfhydryl group blockers, N-ethyl-maleimide and N-pyrenyl-maleimide. This stimulation was inhibited by imidazole (inhibitor of the phagocytic plasma membrane peroxidase), suggesting that it was performed by a NADPH oxidase-like enzyme.

### Indole-3-acetic acid (IAA) catabolism

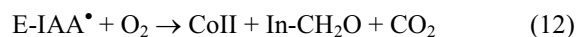
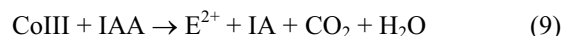
IAA is a plant hormone that regulates growth and development, and earlier studies indicated that HRP was capable to oxidize IAA (Hinman and Lang 1965). Transgenic tobacco plants either developed to overproduce tobacco anionic peroxidase or silencing expression of this enzyme using antisense RNA have supported a role for peroxidase in the metabolism of IAA. Transformed plants underproducing anionic peroxidase grew taller and flowered sooner than non-transformed plants, while transformed plants overproducing anionic peroxidase grew slower and flowered later than nontransformed plants (Lagrimini *et al.* 1997).

However, there is no consensus about the reaction mechanism because it is significantly complicated by numerous radical reactions. Two schemes have been proposed for the peroxidase-catalyzed oxidation of IAA. One of them is based on a common peroxidase cycle including compounds I and II generated by hydroperoxides derived from IAA-oxygen reactions (Eqs. 1-6) (Krylov and Dunford 1996):



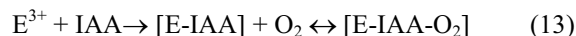
where  $E^{3+}$ , is the native enzyme, IAAOOH is an exogenous hydroperoxide derived from IAA, IAAOH is the corresponding alcohol,  $IAA^{\bullet}$  and  $IAAO_2^{\bullet}$  are IAA radical and the corresponding peroxide radical.

An alternative proposed mechanism assumes independent oxygenase and peroxidase cycles and involves only Compound III and ferric enzyme (Eqs. 7-12; Ricard and Job 1974):

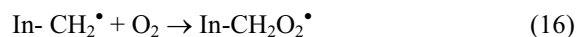
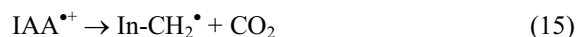


where,  $E^{3+}$ ,  $E^{2+}$  are ferric and ferrous enzyme forms, respectively;  $IAA^{\bullet+}$  is the IAA cation radical,  $E-IAA^{\bullet}$  is an enzyme-radical complex generated in the course of catalysis, IA is indole-3-aldehyde and In- $CH_2O$  is indole-3-epoxide.

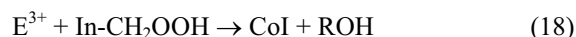
There have been some modifications to the above proposed reactions. Gazaryan *et al.* (1996) demonstrated that no reaction (7) occurred between the enzyme and IAA in the absence of oxygen. Therefore, the initial mechanism proposed by Krylov and Dunford (1996), was extended by Gazaryan *et al.* (1998, 1999), following the identification and isolation of skatole hydroperoxide as a reaction intermediate (Eqs. 13 to 21). The reaction cycle is initiated by the formation of a ternary complex between peroxidase, IAA and oxygen. This complex yields an IAA cation radical and a superoxide radical. These intermediates are assumed to remain bound close to the active site of horseradish peroxidase and react according to Eqs. (13) and (14) to form compound III (Gazaryan *et al.* 1998):



The IAA cation radical decarboxylates yielding skatole radical (Eq. 15) which reacts with oxygen (Eq. 16) and is subsequently converted by reaction with a second molecule of IAA into skatole hydroperoxide (Eq. 17).



where  $In-CH_2^{\bullet}$ ,  $In-CH_2O_2^{\bullet}$  and  $InCH_2OOH$  are skatole radical, skatole peroxide radical and skatole hydroperoxide, respectively. Skatole hydroperoxide switches on the peroxidase cycle generating two IAA radicals for each molecule of skatole hydroperoxide consumed (Eqs. 18 to 20).







Hu and Dryhurst (1997), showed that HRP catalyzed the oxidation of IAA producing oxindole-3-acetate which is a major IAA metabolite found in plants. However, *in vitro* indole-3-methanol and indole-3-aldehyde have been identified as the main degradation products (Gazaryan *et al.* 1998).

### Response to pathogen infection

Levels of peroxidase expression and its isoenzyme patterns increase in response to a variety of chemical, physical and biological stresses in several plant systems (Yannarelli *et al.* 2006).

The roles of peroxidase in defense mechanisms are considered to be as follows: (1) reinforcement of cell wall physical barriers comprising lignin (Hammerschmidt and Kuc 1982), suberin (Espelie *et al.* 1986), cross linking of structural cell wall proteins (Bradley *et al.* 1992), and dimerization of ferulate esters (Ikegawa *et al.* 1996), (2) enhanced production of reactive oxygen species as signal mediators and antimicrobial agents (Passardi *et al.* 2005), (3) enhanced production of phytoalexins (Kristensen *et al.* 1999).

Peroxidases are activated in response to pathogen attack. Infections of *Nicotiana tabacum* plants with tobacco mosaic virus induced two moderately anionic isoenzymes in the leaves where the virus was applied. However, POX was also systemically induced in leaves, of the same plant, which were not inoculated with the virus (Lagrimini and Rothstein 1987). Kristensen *et al.* (1999) studied the accumulation of neutral peroxidase (pI 7.3, Prx7) in tissue and subcellular organelles of barley leaves after inoculation with powdery mildew spores. Peroxidase accumulated predominantly in the epidermis, apparently in vacuoles, and appeared to be exclusively a pathogen-induced vacuolar peroxidase. These authors found that peroxidase (Prx7) was probably responsible for the biosynthesis of antifungal compounds known as hordatins, which accumulate abundantly in barley coleoptile.

*Orobanch* sp. (broomrape) is one of the most important agricultural pests in several major crop systems worldwide. Infection of *A. thaliana* with *Orobanch* *ramose* induced a gene coding for a class III peroxidase. Expression of this early induced gene was transient, and the transcript accumulated during the first 24 h after infection (Vieira dos Santos *et al.* 2003).

Sasaki *et al.* (2004) analyzed the expression profile of 22 rice POX genes after infection with rice blast fungus. After fungal inoculation of both compatible and incompatible rice hosts, expression of 10 among the 22 POX genes were observed. Six to seven h after fungus inoculation the incompatible hosts expressed 7 POX genes at levels higher than in the compatible host.

Some reports showed that POX genes have been activated by infection with bacteria (Bestwick *et al.* 1998) and viroids (Vera *et al.* 1993). Therefore, POX may be classified within the pathogenesis-related (PR) protein-9 family (van Loon *et al.* 1994).

### Wounding-response

During pathogenic attack, plant tissues can be damaged leading to wounding which can also occur through meteorological adversities or animals. Plants respond by activating defense systems for healing wounded tissues, and for protection from subsequent pathogens invasion. These responses include cell wall repair by suberization (Dean and Kollattukudy 1976), cross-linking (Bradley *et al.* 1992), or enhancement of phenylpropanoid synthesis (Collinge and Slusarenko 1987). Plants can also activate the systemic induction of defense-related proteins such as the basic type PR (Niki *et al.* 1998). A plethora of stress- and disease-acti-

vated enzyme systems are activated (reviewed extensively in Teixeira da Silva 2006).

Lagrimini and Rothstein (1987), trying to determine the peroxidase isoenzyme involved in stress response, subjected plants from *N. tabacum* to wounding. This triggered the expression of several cationic isoenzymes in leaves and both cationic and anionic isoenzymes in pith tissue. Maximum enzyme activity was detected 72 h after wounding, and cycloheximide treatment prevented this induction. *TpxoN1* is a unique wound-inducible and possible wound-healing gene which is rapidly expressed in stems and petioles, and remains for a long time in the vascular system (Sasaki *et al.* 2004). This gene was expressed within 1 h after tobacco leaves were wounded (Hiraga *et al.* 2001).

Peroxidases together with polyphenol oxidases are also induced by herbivore attacks (Ruuholta and Yang 2006). These enzymes catalyze the conversion of plant diphenols, commonly found as chlorogenic acid, to highly reactive quinones. These bind to electron-rich moieties of amino acids and proteins, decreasing their assimilation in the herbivore digestive tract and also leading to malnutrition in developing insects.

### Ultraviolet radiation stress

The effect of UV-B radiation (280-320 nm) on free radical production and scavenging, and on cell membranes in plants, has been well documented (Costa *et al.* 2002). This radiation produces oxidative stresses by increasing reactive oxygen species (ROS) such as singlet oxygen ( $^1\text{O}_2$ ), superoxide anion ( $\text{O}_2^{\bullet-}$ ),  $\text{H}_2\text{O}_2$ , and hydroxyl radicals ( $\text{OH}^{\bullet}$ ), which diffuse across biological membranes and cause cellular damage. Plants show an efficient enzymatic antioxidant defense system to counteract oxidative stress, which operates with the sequential and simultaneous performance of superoxide dismutase, peroxidase, catalase and ascorbate peroxidase. Superoxide dismutase catalyzes the disproportionation of two  $\text{O}_2^{\bullet-}$  radicals to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$ .  $\text{H}_2\text{O}_2$  is then eliminated by antioxidant enzymes such as catalase and peroxidase. It could be speculated that peroxidase contributes to UV tolerance by removing  $\text{H}_2\text{O}_2$ .

In *Nicotiana sylvestris*, increased activity of an anionic peroxidase correlated with increased tolerance to UV radiation (Jansen *et al.* 2001). In *A. thaliana*, the enhancement of peroxidase activity specific to conyferil alcohol, and the synthesis of several new isoforms suggested that UV radiation might have enhanced the synthesis of secondary metabolites (Rao *et al.* 1996). In sunflower cotyledons subjected to 30 kJ m<sup>-2</sup> of UV-B radiation, and further recovery in the dark or in white light, a general induction of enzymes involved in  $\text{H}_2\text{O}_2$  breakdown took place (Yannarelli *et al.* 2006).

### Chemical stress

Oxidative stress-generating ROS has been shown to produce tissue damage after the exposure of plants to heavy metals, various organic chemicals, and air pollutants like  $\text{O}_3$  and  $\text{SO}_2$  (Clijster *et al.* 1999; Chernikova *et al.* 2000). An excess of iron produces oxidative stress in *Nicotiana plumbaginifolia* and sunflower leaves (Gallego *et al.* 1996), while excessive amounts of cadmium in soil, commonly induce oxidative stress changing the activity of various antioxidant enzymes (Dong *et al.* 2006).

Plants can protect themselves from the toxic effects of ROS by the enzymatic antioxidant system already described in the UV-radiation stress. Many studies have shown that heavy metals, salt stress and other abiotic conditions induce activity alteration of several representatives of the enzymatic antioxidant defense system on which peroxidase plays an important role (Mittova *et al.* 2002; Dias *et al.* 2006).

Another possible function of peroxidases, specifically in the treatment of heavy metals is their contribution to accumulate them in plants reducing their toxic effects. For example, the waterlily *Nymphaea* probably uses peroxidases to produce phenolic polymers that trap Cd and isolate it in

the form of Ca-Cd crystals in specific glands situated on the aquatic side of its leaves (Lavid *et al.* 2001).

## Embryogenesis

Early evidence that POXs play a role during the early stages of somatic embryogenesis was provided by Corde-wener *et al.* (1991). They showed that somatic embryogenesis in carrots is inhibited by the glycosylation inhibitor tunicamycin, suggesting that protein glycosylation is necessary for embryogenesis. This inhibition was reversed by the administration of functional cationic peroxidases of the type secreted during normal somatic embryogenesis.

Cultured asparagus (*Asparagus officinalis*) cells induced to regenerate into whole plants through somatic embryogenesis secreted a number of proteins newly detected, one of these being AoPOX1 (Asparagus peroxidase; Take-da *et al.* 2003). AoPOX1 transcripts were particularly abundant during early somatic embryogenesis. These authors also suggested that POX located in cell walls played a role in the activation of cell division and differentiation in asparagus cultures, catalyzing the dimerization of coniferyl alcohol in the synthesis of neolignan (a dimer of monolignol). Neolignans produced by this mechanism participate in the activation of cellular division and differentiation in asparagus cultures (Hirai *et al.* 1994).

## Cellular growth

It has been widely assumed that peroxidases are involved in growth inhibition (Fry 1986), but there are now indications that they could also promote growth. Transformed tobacco plants overexpressing the horseradish peroxidase *prxC1a* gene under the control of the cauliflower mosaic virus 35S promoter and HRP *prxC2* promoter, grew significantly faster than control plants (Kawaoka *et al.* 2003). In these experiments, the flowering time of the transformed plants was reduced by approximately 20% in comparison with the control plants. The underexpression of the homologous anionic peroxidases genes using antisense RNA, suppressed endogenous enzymatic activity which affected the growth rate (Lagrimini *et al.* 1997). Kawaoka *et al.* (2003) introduced the *prxC1a* gene into hybrid aspen (*Populus sieboldii* × *Populus grandidentata*) trees. Overproduction of *prxC1a* stimulated vegetative growth rate and conferred resistance to oxidative stress.

The mechanism associated with the rapid growth of plants containing HRP gene *prxC1a* is still unclear. Dunand *et al.* (2003) found a clear correlation between the expression of an anionic peroxidase gene (*APRX*) and growth in zucchini (*Cucurbita pepo* L., cv. 'Black Beauty'). Gene expression was generally related to cell elongation and inversely correlated to lignin deposition. The most accepted mechanism for growth stimulation by POX overproduction is related to changes in cell wall internal structure achieved by the action of POX through its peroxidative and hydroxylic cycles. Peroxidases can favor cell elongation by regulating the local concentration of H<sub>2</sub>O<sub>2</sub> and generating hydroxyl radicals (Dunand *et al.* 2003).

## Cellular cross-linking

Composition of the plant cell wall can be markedly altered by environmental stimuli, especially in response to biological stress, or just as part of normal cell wall development during growth, differentiation, and senescence. POX quickly responds to various stress producing agents and actively joins the lignification process, controlling the availability of H<sub>2</sub>O<sub>2</sub> in the cell wall, which is a prerequisite for the cross-linking of phenolic groups.

Cell wall rigidification is a result of the peroxidase-mediated cross-linking of several compounds. POX has the ability to effectively join intermolecular linking processes encompassing various plant cell wall components, such as cellulose, xylans and pectins (Brownleader *et al.* 2000).

The biochemical implication of POX in such mechanism is related to the peroxidative cycle, where substrates such as tyrosine residues, monolignols, suberin units, and ferulic acid are oxidized. The radicals produced by the peroxidative cycle can form cross-links between cell wall polymers and proteins implicated in the defense reactions, and during elaboration of the cell wall network. Using H<sub>2</sub>O<sub>2</sub> as oxidant, POX can generate monolignol phenoxy radicals that spontaneously form lignin polymers. POX may also act on the phenolic moiety of tyrosine, and some speculate that it may also act on lysines, hence creating Tyr-Tyr or Tyr-Lys bonds (Schnabelrauch *et al.* 1996), contributing to the formation of a very uniform mesh within the cell wall structure.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Despite peroxidase being discovered more than 150 years ago, in recent years there have been many new advances concerning its chemistry and biology. Current understanding of the complex functions of plant peroxidases has been derived from the development of immunohistochemical techniques, and by associating the onset of specific processes with changes in activity of a given group of isoperoxidases.

Physiological functions of peroxidases have also been ascertained from cDNA clones of isoperoxidases, and using them as molecular probes to study the expression of each peroxidase group. Class III plant peroxidases are present in all land plants, where the occurrence of multimolecular forms of the enzyme and their remarkable catalytic versatility have explained the participation of peroxidase in a broad range of physiological and developmental processes. Cell wall associated plant peroxidases have been shown to be involved in the cross-linking of cell wall constituents, lignin and suberin biosynthesis, processes which are important both in cell wall differentiation and in plant resistance against pathogenic attack. Recently Gabaldón *et al.* (2006) have found that plant cell wall lignification is controlled by the concentration of H<sub>2</sub>O<sub>2</sub>, but the sites of monolignols coupling may be saturated. Although peroxidase promotes the formation of reactive oxygen species which potentially participate in cell damage processes, at the same time it can also act together with catalase and superoxide dismutase to provide protection against biological oxidation.

It is known that peroxidase also acts upon the catabolism of the growth hormone IAA. However, the manner and extent on which this reaction influences plant growth is still unclear, and the clarification of this point will probably be the reason for further research. In addition, we are sure that new discoveries will involve peroxidase participation in plant functions where it was not previously related, derived from studies of the molecular mechanisms and catalytic properties of the different peroxidase isoenzymes. Structural studies on the heme pocket and its interactions with the polypeptide chain and substrates may provide a better understanding of peroxidase activity under different environmental conditions. Genetic manipulation of the different types of peroxidase isoenzymes may provide new insights of peroxidase functions as well as the opportunity to obtain genetically engineered peroxidases which may be of interest for a variety of practical purposes. Peroxidases isolated from plants are widely employed as a reagent for many applications, including organic synthesis and biotransformation, coupled enzyme assays in biosensors, chemiluminescent assays, immunoassays, and detoxification of wastewaters.

Therefore, current and further research on this field should derive in the development of new easy scale up methods of peroxidase purification. In addition, there should be an improvement in the desirable qualities of peroxidase, such as good stability and enhanced catalytic properties in aqueous and non aqueous solvent systems. This may be achieved by chemical modification, or the production of genetically engineered peroxidases by site directed mutagenesis or directed evolution techniques.

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