Current Topics in Biochemical Research
Vol. 13, 2 2011

Review

Palm peroxidases: The most robust enzymes

Nazaret Hidalgo Cuadrado[1], Juan B. Arellano[2], Juan J. Calvete[3], Libia Sanz[3], Galina G. Zhadan[4], Larissa C. Textor[5], Igor Polikarpov[5], Sergey Bursakov[6], Manuel G. Roig[1,7], and Valery L. Shnyrov[4,*]

[1]Departamento de Química Física, Facultad de Ciencias Químicas, Universidad de Salamanca, 37008 Salamanca, [2]Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), 37008 Salamanca, [3]Instituto de Biomedicina de Valencia (CSIC), 46010 Valencia, [4]Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Salamanca, 37007 Salamanca, Spain. [5]Instituto de Fisica de São Carlos da Universidade de São Paulo, 13566-590 São Carlos, Brasil. [6]Departamento de Protección Ambiental, Estación Experimental del Zaidin (EEZ-CSIC), 18008 Granada, [7]Centro de Investigación y Desarrollo Tecnológico del Agua (CIDTA), Universidad de Salamanca, 37007 Salamanca, Spain

ABSTRACT

Peroxidases are ubiquitous enzymes that catalyze a variety of oxygen-transfer reactions and are thus potentially useful for industrial and biomedical applications. Over the last decade, several studies have shown that peroxidases isolated from the leaves of different kinds of palm trees such as the royal palm (*Roystonea regia*), the date palm (*Phoenix dactylifera*), the African oil palm (*Elaeis guineensis*), the ruffle palm (*Aiphanes cariotifolia*) and the windmill palms (*Trachycarpus fortunei* and *Chamaerops excelsa*) exhibit higher activity and stability than commercially available peroxidases isolated from, for example, horseradish roots (*Armoracia rusticana*) and soybean (*Glycine max*). Here, the structure, thermal denaturation, and the catalytic cycle of peroxidases from palm trees are reviewed and compared with those of other plant peroxidases. In addition, we report the biotechnological potential of palm peroxidases and their implications in cellular aging and diseases, such as Refsum's and Alzheimer's diseases. This paper summarizes the main characteristics of the palm peroxidases studied.

KEYWORDS: palm peroxidase, crystal structure, molecular mechanism of palm peroxidase activity, stability, biotechnological applications

ABBREVIATIONS

AOPTP, African palm tree peroxidase; aPrx, Anionic peanut peroxidase; CEP, palm tree (*Chamaerops excelsa*) peroxidase; cHRP, Isoenzyme c horseradish peroxidase; PNP, peanut peroxidase; RPTP, royal palm tree (*Roystonea regia*) peroxidase; SBP, soybean peroxidase; WPTP, windmill palm tree peroxidase

INTRODUCTION

Palm peroxidases are involved in cell-wall formation, plant hormone regulation, defense mechanisms, and stress- and pathogen-induced responses [1]. They are recruited to overcome adverse environmental conditions, under which the production of reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$) increases. These enzymes catalyze the oxidation of organic substrates while reducing H_2O_2 or other peroxides to water in a three-step catalytic cycle known as the Poulos-Kraut mechanism,

*To whom correspondence should be addressed:
V. L. Shnyrov, Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Salamanca, Plaza de los Doctores de la Reina, s/n, E-37007 Salamanca, Spain.
shnyrov@usal.es

which involves different intermediate enzyme forms [2]. For most peroxidases, the typical substrates are small aromatic molecules such as phenols and aromatic amines; however, it is difficult to envisage which substrates are physiologically relevant for plant peroxidases owing to the ability of peroxidases to oxidize a broad variety of organic and inorganic substrates [3].

Palm tree peroxidases belong to the family of secretory plant peroxidases (class III). They are constitutive, soluble enzymes that contain glycan groups covalently bound to several asparagine side-chains of a single polypeptide chain with about 300 residues. Their catalytic site harbours two calcium ions, and a haem prosthetic group located in the cleft created by two anti-parallel α-helices. Surrounding the haem plane, there are several invariant or highly conserved amino acid residues that also are essential for the catalytic properties of peroxidases [4, 5].

Recently, peroxidases from the leaves of some tropical palm trees have been isolated and characterized [5-10]. These enzymes showed very high stability at elevated temperatures, within a broad range of pH values, and in the presence of hydrogen peroxide and chemical denaturants [11-14], making them intriguing catalysts for industrial applications. Owing to their high thermal stability, high reactivity and stability at low pH values, several plant peroxidases have been used in development of biosensors for analytical applications [15, 16]. Among the class III peroxidases that have been characterized structurally and biochemically, the palm tree peroxidases (RPTP, CEP, AOPTP, WPTP) have been found to be the most stable ones [6, 8, 11, 17].

1. Superfamily of palm peroxidases

Welinder (1992) classified plant peroxidases in three groups on the basis of their different structural features. Here we pay special attention to peroxidases Class III and readers are referred to a previous work [18] for comparative analyses among peroxidase groups. Class III peroxidases have a secondary structure rich in α-helices separated by unordered loops and turns of variable length. These peroxidases (EC 1.11.1.7) have two calcium ions, one peptide signal at the N-terminus for secretion, four conserved disulphide bridges, a set of helices that play an important role in substrate access to the active site, a level of glycosylation ranging between 0 and 25%, and a propeptide at the C-terminus responsible for driving peroxidases towards vacuoles (Table 1).

From the structural point of view, palm peroxidases have a series of additional helices that are not found in other Class III peroxidases. In the RPTP, and in preliminary studies of CEP, two additional short helices, B' and B", appear inserted between the B and C helices (Fig. 1). Like other Class III peroxidases, palm tree peroxidases have two short anti-parallel β-strands, β_1 and β_2, that flank the large insertion between helices F and G, which contains the F' and F" helices. There are four

Table 1. Characteristics of class III peroxidases and palm tree peroxidase superclass.

Superfamily of plant peroxidases		
	Class III	**Palm peroxidase superclass**
Haem group	Yes	Yes
Carbohydrates	0-25%	24-25%
Metal ions	Ca^{2+}	Ca^{2+}
Disulphide bridges	4	4
Peptide signal for secretion	Extreme N-terminal	Extreme N-terminal
Propeptides at the C-terminal	Yes	Yes
Additional helices	D' between D-E F' and F" between F-G	B' and B" between B-C D' between D-E F' and F" between F-G
Examples	cHRP, PNP, BP1, TcPX, RPPX	RPTP, CEP

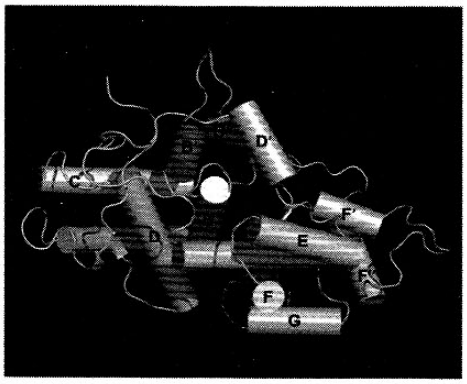

Fig. 1. Ribbon diagram of CEP and RPTP. Conserved helices appear in grey and the helices unique to CEP and RPTP peroxidase are highlighted in dark grey.

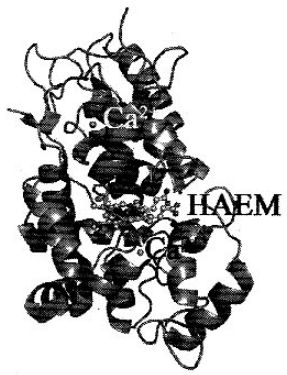

Fig. 2. Three-dimensional representation of the X-ray crystal structure of palm peroxidase (RPTP PDB code 1HDL). The haem group (represented in centre of structure) is located between the distal and proximal domains each of which contains one calcium atom (shown as small spheres above and below the haem group).

disulfide bridges based on the invariant Cys residue pairs that keep the overall structure rigid [19]. Based on the comparison between different classes of peroxidases [18], it may be seen that one of the main characteristics of Class II and Class III is the presence of additional helices. Class II has an additional B' helix between B-C, and Class III has D' between D-E, and F' and F" between F-G and the propeptides at the C-terminal. In sum, palm tree peroxidases exhibit all the characteristics of peroxidases belonging to the Class III group, together with two additional helices. We thus propose that they form a new group of peroxidases: the superfamily of palm peroxidases.

2. Structural components

The number of crystal structures of peroxidases that have been solved by X-ray diffraction is low [18]. A significant problem for the crystallization of any peroxidase is the high level of glycosylation of this group of enzymes. This drawback evidently includes palm tree peroxidases, whose degree of glycosylation is even higher in comparison with other peroxidases. The three-dimensional structure of RPTP shows nine N-glycosylation sites [19] and preliminary crystallographic studies on CEP also show the same number of sites. In contrast HRP-C, SBP and PNP have seven, five and three glycan chains, respectively. It has been shown that glycosylation increases the solubility of peroxidase in water solutions and improves peroxidase stability against proteolysis [20].

Like other Class III peroxidases, palm peroxidases have a polypeptide chain with about 300 amino acid residues, an active site with one prosthetic group where one Fe(III) ion is coordinated, two calcium ions (Fig. 2), and eight sulfhydryl groups forming four invariant disulphide bridges between Cys 11-91, 44-49, 97-299 and 176-208 [19]. Iron and calcium ions are essential for the structural and functional integrity of the enzyme. The glycosylation sites are on the surface of the loops connecting the α-helices and are always at the outer edge of the protein.

The secondary structure is mainly composed of α-helices and a small number of short β-sheets. Palm peroxidases have ten α-helices (A-J) occupying very similar topological sites in all Class III peroxidases. A significant difference between Class III and the new Class for palm peroxidases is that the latter contain two additional α-helices (B' and B") with a yet unknown role in protein stability. The structural integrity of this region of the protein is maintained by the disulphide bridge between Cys44 and 49.

It is possible that the different residues of the B' α-helix play an important role in accessing and binding the substrate to active sites.

Like other Class III peroxidases, palm peroxidases have two domains - the distal and the proximal - the haem group lying in between (Fig. 2).

2.1. The active site

As is the case of other Class III peroxidases, palm peroxidases contain two different types of metal center: Fe (III) protoporphyrin IX, usually referred to as the "haem group" and two calcium ions (Table 1, Fig. 2). Both are essential for the structural and functional integrity of the enzyme.

The haem-containing active site of palm tree peroxidases closely resembles those of the anionic HRP-C and the cationic SBP peroxidases, and shows that the iron atom is not displaced from central haem plane and is coordinated to a hydrogen peroxide molecule. The peroxide oxygen-oxygen bond distance corresponds to a single bond and supports the view that the crystal structures of palm peroxidases do not define their resting state. The resting state of the enzyme contains a Fe(III) ion with a weakly bound water ligand that is replaced by a H_2O_2 molecule. Thus, the three-dimensional crystal structure of palm peroxidases corresponds to a hydroperoxide complex state, which, according to the reaction mechanism proposed by Poulos and Kraut [2], is a transient intermediate formed before compound I.

The conserved residues in Class III peroxidases responsible for maintaining the structure of the haem group are as follows. In the distal domain of the haem plane, the NH_2 group of Arg38 assists in charge stabilization through a nearby water molecule during the formation of the peroxidase: H_2O_2 complex; Phe41 hides the access to the haem iron; the Nε2 atom of His42 acts as a proton acceptor, while H_2O_2 binds to the vacant sixth coordination site of the haem iron and Asn70, which is an invariant residue in Class III peroxidases. In the proximal domain of the haem plane, both His169, coordinated to the iron atom through its side-chain Nε2 atom, and Asp248, whose carboxylate group forms a hydrogen bridge with the Nδ1H of His169, play an important role in the stabilization of the oxidized states of the iron (Fig. 3). In palm peroxidases, the peroxide O_2 atom forms hydrogen bonds with the Nε2 atom of His42, with the Nε atom of Arg38, and with the water molecule w1 which is hydrogen-bonded to

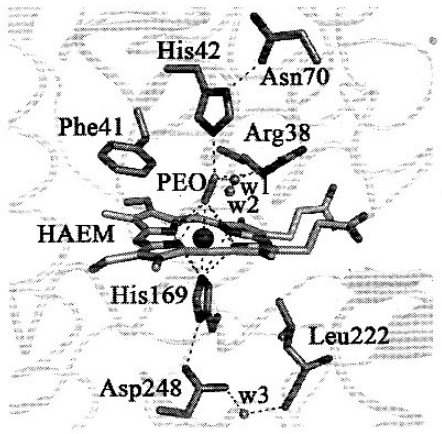

Fig. 3. Key amino acid residues in haem-binding region of palm peroxidases. The haem group and haem iron atom are shown in grey and black respectively; the remaining residues are in a stick representation. Three conserved water molecules are shown as small spheres. The hydrogen-bonding interactions are shown as dashed black lines.

the NH_2 side-chain of Arg38 and another water molecule w2. The two water molecules w1 and w2 are conserved in the distal cavity of haem-containing class III peroxidases (Fig. 3) and they play an important role as acid-base catalytic molecules [21].

A structural comparison of all the X-ray solved plant peroxidases shows that there is only one distinct haem propionyl-protein interaction. In HRP-C, a glutamine residue (Q176) is hydrogen-bonded to the haem propionyl situated near the entrance of the substrate access channel. However, in palm peroxidases, the same propionate is hydrogen-bonded to His175 and Ser178 (structurally equivalent to the HRP-C Phe179), and to a conserved water molecule (w7) that is found in the 3D structures of both HRP-C and palm peroxidases. Interestingly, the orientation of the haem propionates in the anionic palm peroxidases and SBP structures are twisted, whereas in both cationic peroxidases (HRP-C and PNP) the charged propionates are oriented parallel to each other. These differences in the haem propionyl-protein interactions among class III peroxidases could influence the stability and catalytic properties of these enzymes [19].

Thus, haem propionates play an important role in the stability of the haem cavity and in the formation of catalytic intermediates. Palm peroxidases also contain both a distal and proximal calcium-binding sites that are identical to other class II and class III peroxidases [21-27].

2.2. Glycans

Class III peroxidases are glycoproteins with a percentage of glycosylation of 0-25%. For palm tree peroxidases, the total carbohydrate content is 24%. Glycans are essential for maintaining protein stability and activity; they increase hydrophobicity, and protect the protein from proteolysis. Glycans are composed of five different sugars -N-acetyl glucosamine, xylose, mannose, fucose and galactose- with a length of 7 up to 14 sugars (176 Da being the average sugar mass). The linkage points for glycans in the protein are the Asn residues. Each peroxidase has its own percentage of glycosylation.

Like many other class III peroxidases, native palm tree peroxidases are highly glycosylated and their three-dimensional structures show that all the N-glycosylation sites are found within the surface turns or loop regions connecting the helices to the glycan chains protruding into the solvent. Studies on RPTP [19] and preliminary studies of CEP have shown that these peroxidases have nine N-linked glycans covalently bonded to Asn8, Asn28, Asn114, Asn144, Asn154, Asn211, Asn256 and Asn298, while cHRP has 8 glycosylation sites located in loops at Asn13, Asn57, Asn157, Asn186, Asn198, Asn214, Asn255 and Asn268 [28]. The glycan chains attached to palm peroxidase protein contain N-acetyl-D-glucosamine (GlcNAc), mannose (Man), fucose (Fuc), and xylose (Xyl). There are three glycan chain sequences that have been determined previously as Man-[Xyl]Man-Man-GlcNAc-[Fuc]GlcNAc, GlcNAc-Man-[Man] Man-GlcNAc-[Fuc]GlcNAc and [Man]Man-Glc-NAc-[Fuc]GlcNAc. Thus, the most abundant glycan chain found in the RPTP X-ray structure was determined as Man-[Xyl]Man-Man-GlcNAc-[Fuc]GlcNAc, which was attached to Asn8 [19]. The highly glycosylated form of RPTP or other peroxidases hinders crystallization, but in contrast the hydrophilic glycans improve their solubility and protect them from proteolysis. Furthermore, the distribution among the glycoforms is normally non-homogeneous.

3. Stability of peroxidases

After the discovery of the high stability of HRP, an increasing number of biotechnological applications appeared and research efforts were directed to determining the structural components responsible for such high stability. Nevertheless, over the last 15 years, several peroxidases from the leaves of tropical plants have been found to be much more stable than HRP. This is the case of a group of peroxidases found in the leaves of palm trees [6].

Several studies that have explored the effects of the different structural elements on the stability of peroxidases have concluded that Ca^{2+} cations, disulphide bridges, glycans and several amino acid residues at the active site are essential factors for the thermal and chemical stabilities of this type of enzyme [18].

The most detailed investigation of the thermal stability of different peroxidases was done at pH 3 because of the tremendous interest in the production of conducting polymers, whose process of synthesis is most effective at a pH below 4 [29-31].

The thermal denaturation of peroxidases at this pH value gave rise to well defined transitions whose parameters were dependent on the temperature scan rate. Under these experimental conditions, the denaturing process was always irreversible since no thermal effect was observed in a second heating of the enzyme solution. All this clearly indicates that the observed thermal transitions characterize an irreversible kinetically controlled process. Analysis of DSC transitions for palm tree peroxidases was accomplished on the basis of a simple two-state irreversible model $N \xrightarrow{k} D$, in which only the native (N) and final (irreversibly denatured) (D) states are significantly populated, and in which the conversion from N to D is determined by a strongly temperature-dependent, first-order rate constant (k) that changes with temperature, as given by the Arrhenius equation. The highest likelihood values of the activation energy of the denaturation process E_A and temperature, at which k is equal to 1 min^{-1} (T^*), obtained with the nonlinear least squares minimization procedure for fitting experimental data to a two-state irreversible model, are shown in Table 2.

Changes in the CD spectrum of proteins at around 222 nm correspond to changes in their secondary structure. This CD band is related to the peptide bonds and to aromatic residues. Changes in the CD spectrum of peroxidases at around 400 nm (Soret band) are related to changes in the tertiary structure around the haem group. With CD it is possible to estimate the content of the secondary structural components of a protein. The Table 3 shows the secondary structure (%) for intact and denatured selected Class III peroxidases and palm peroxidases. The secondary structure components of native palm peroxidases at pH 3.0 are around 39±1% α-helices, 9±2% β-strands, 21±1% β-turns and 30±3% unordered structures. However, at the same pH, the thermally denatured protein contains 10±2% α-helices, 24±4% β-strands, 15±1% β-turns and 51±4% unordered structures. In the case of palm peroxidases (CEP and RPTP), large differences are observed with respect to other haem peroxidases in the quantity of β-strands. The increase in the quantity of β-strands that also occurs in denatured proteins indicates that these forms of the enzymes undergo some aggregation, most probably of an intramolecular character. The values obtained for intact CEP and RPTP are typical of different haem peroxidases [21]. It should be noted here that the estimates for the β-sheet and β-turn are usually worse than the estimate for the α-helix because the intensities of the β structure elements are lower than those of the α-helix [32]. Upon heating the CEP and RPTP up to the denaturing temperature, the shape of the spectrum changed [13, 14], pointing to an increase in the unordered structure, mainly at the expense of the α-helical structure (Table 3). The increase in the quantity of β-strands that also occurs in denatured palm peroxidases indicates that this form of the enzyme undergoes some aggregation, most probably of an intramolecular nature because we failed to detect an increase in turbidity in the denaturing process.

Evidently, the leaves of palm trees are the best source of the most structurally and functionally stable peroxidases, pointing to their great potential for different biotechnological applications.

Table 2. Arrhenius parameter estimates for the two-state irreversible model of the thermal denaturation of some peroxidases at pH 3.

	T^* (K)	E_A (kcal mol^{-1})	Ref.
AOPTP	347.5 ± 0.3	103 ± 6	[12]
CEP	345.0 ± 0.2	105.1 ± 1.7	[14]
RPTP	342.0 ± 0.2	129.1 ± 0.8	[13]
HRP	344.4 ± 0.5	38.2 ± 0.5	[68]

4. Steady state kinetics

Peroxidases catalyze the oxidation of organic substrates while reducing H_2O_2 (or other peroxides) to water in a three-step catalytic cycle that involves different intermediate enzyme forms [2]:

$$E + H_2O_2 \xrightarrow{k_1} EI + H_2O_2 \quad (1)$$

$$EI + AH_2 \xrightarrow{k_2} EII + AH^{\bullet} \quad (2)$$

$$EII + AH_2 \xrightarrow{k_3} E + H_2O + AH^{\bullet} \quad (3)$$

where E is the native enzyme. The monoelectronic oxidation of the native state E (Eqn. 1) gives rise to an intermediate state termed compound I (EI),

Table 3. Secondary structure elements (%) determined from analysis of the CD spectra for intact and denatured some peroxidases at pH 3.

	AOPTP		CEP		RPTP		HRP		aPrx	
	Int.	Denat.	Int.	Denat.	Int.	Denat.	Int.	Denat.	Int.	Denat.
α Helix	15	12	38	10	41	8	42	27	47	30
β Strand	38	23	7	28	12	20	11	17	6	9
β Turn	20	14	22	15	20	16	21	18	27	23
Unordered	40	50	33	47	27	56	26	38	20	37

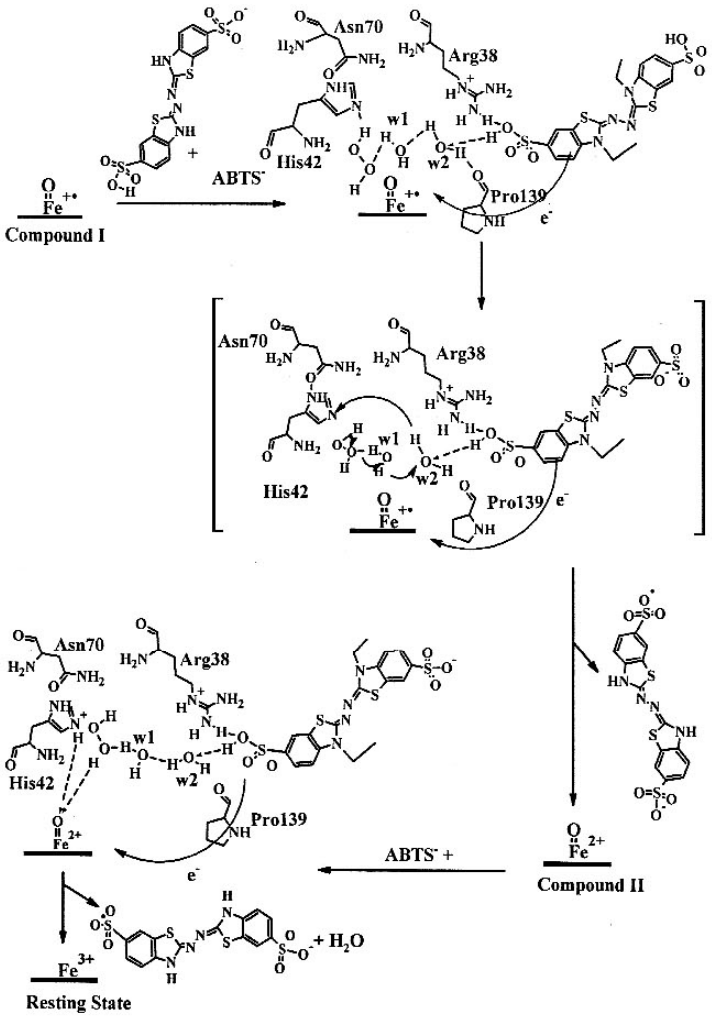

Fig. 4. Mechanism of the distal site. Based on the known compound I (formed through the mechanism of Poulos & Kraut) at the distal side and taking into account the links stored in the structure, the substrate (ABTS·) will enter through the second water molecule (w2), giving a proton to w2 (which is stabilized by Pro139 and hydrogen-w1). Also, the substrate is stabilized by the hydrogen bonds formed between the conserved Arg38 in the distal part, which will give an electron in a redox process characteristic of Class III peroxidases. It will produce a reaction intermediate, which will result in a sigmatropic rearrangement of H belonging to the molecule w2, which will then be transferred to the imidazole ring His42, so that it can acidify. Thus, the w2 water molecule accepts hydrogen from the substrate molecule and allows this hydrogen to come in the form of a radical (as we show for the mechanisms proposed for Class III peroxidase), leading to the formation the so-called compound II. Then, another molecule of ABTS (on the same side) enters and through a rearrangement of H this will allow the acid H imidazole ring of His42 to be the oxygen yielded to the iron active site thus regenerating the iron in its initial oxidation state and forming a water molecule and another molecule of substrate radical.

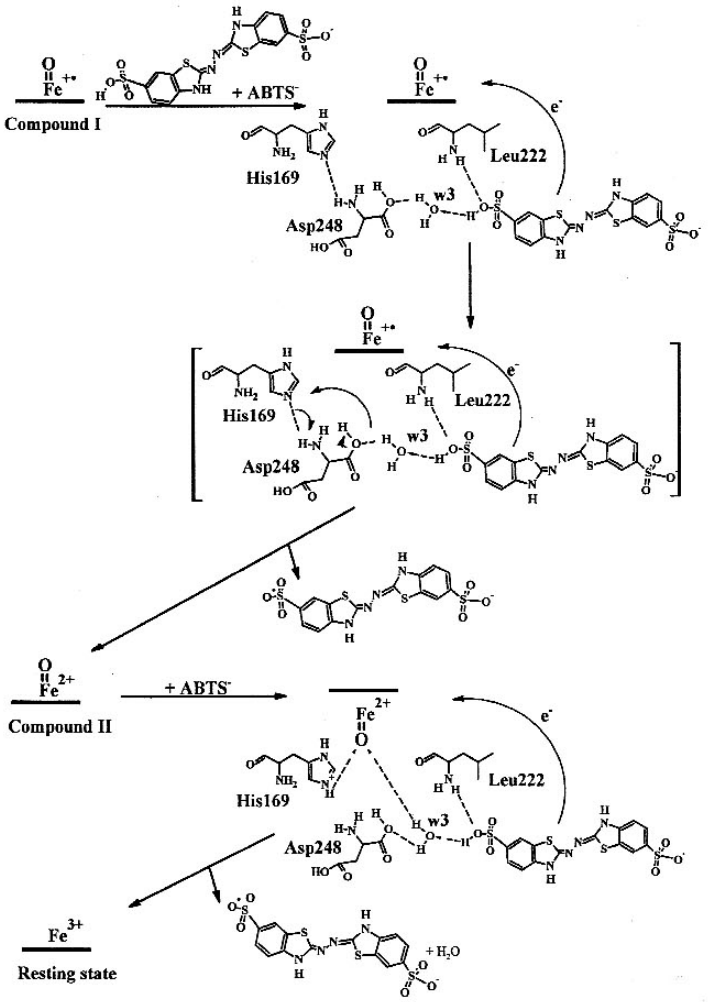

Fig. 5. Mechanism of proximal site. At the proximal site a process analogous to the process that occurs in the distal side takes place. Here the substrate molecule, when it enters, is stabilized by hydrogen bonds with Leu222 and the H-SO_3H group of ABTS, which will be donated to the water molecule w3. This water molecule (w3) is stabilized by hydrogen bonds with Asp248, conserved in the structure, forming bridges with the proximal His169 NH by a rearrangement of the H.

described as an oxyferryl prophirin π-cation radical. EI accepts one electron and one proton from a reducing substrate (AH_2) to yield the corresponding free radical ($AH^{\bullet}$) and the oxyferryl haem intermediate known as compound II (EII). The subsequent one-electron reduction of EII by a second molecule of reducing substrate yields the ferric form of peroxidase (E). The microscopic constant k_1 (constant of EI and a second free radical ($AH^{\bullet}$) formation) indicates the reactivity of

the enzyme towards hydrogen peroxide and k_3 (constant of EII reduction) the reactivity of the enzyme towards the reducing substrate.

Here, we propose a mechanism to explain the cycle of palm peroxidase (Figs. 4 and 5), in which the decomposition of hydrogen peroxide is achieved through the heterolytic cleavage of H_2O_2 to form water. The cycle is presented for the ABTS substrate, which exhibits the highest affinity for the enzyme.

Following Cleland's nomenclature [33], this catalytic cycle is an irreversible Ping-Pong mechanism. When the reaction is irreversible, the reaction rate of the compound is much higher than its dissociation rate. Apparently, the global reaction seems to have no limits to its rate: the higher the substrate concentration, the higher the reaction rate.

Recently, studies of CEP and RPTP have shown that the mechanism of palm peroxidades is a Bi-Bi Ping-Pong mechanism [34, 35]. The microscopic constant values for k_1 and k_3 of CEP and RPTP (Table 4) point to greater reactivity toward ABTS, the chromogen of choice for the assay of peroxidase in cervical mucus and for diamine oxidase activity [36, 37], and less reactive toward guaiacol, although better than other Class III peroxidases. In contrast, both SBP and PNP peroxidases are more reactive toward guaiacol than toward aromatic amines [38]. The microscopic constant of palm tree peroxidases indicates that they show greater affinity at acid pH than other peroxidases of Class III. This again shows that this kind of peroxidases is more stable than the other peroxidases.

5. Suicide inactivation

Although there are distinguishing characteristics among peroxidases, a common inactivation mechanism comprising several stages has been put forward for all of them. In the absence of substrate, or when exposed to high concentrations of hydrogen peroxide, peroxidases show the kinetic behavior of suicide inactivation, where hydrogen peroxide is the suicide substrate that converts Compound II into a highly reactive peroxy-iron(III) porphyrin free radical called Compound III [39]. Compound III is not part of the cycle, but is produced under excessive exposure of protonated compound II to oxidative species in a reaction partially mediated by free superoxide radicals [39-41]. ESR spin trapping and spectral analyses have demonstrated the occurrence of this species after the oxidative treatment of HRP [40] and other classes of peroxidases [42, 43, 45].

The kinetic models for the hydrogen peroxide-mediated inactivation of HRP [44] and RPTP [35] are similar in that they are time-dependent and show saturation kinetics. In every case, the addition of a reducing substrate protects the enzyme from inactivation. From a stoichiometric analysis of suicide inactivation, it was concluded that approximately 250 molecules of hydrogen peroxide were required per active site to generate the inactive form of HRP, whereas one order of magnitude greater was required for palm peroxidase [35]. Another parameter to consider in inactivation is the inactivation constant, which are higher in the case of palm peroxidases [35] unlike HRP [44]. In view of the values obtained, it may be seen

Table 4. Kinetic parameters obtained from the initial rate of oxidation of different substrates catalyzed by RPTP and CEP.

	CEP				RPTP			
	ABTS	Guaia-col	o-diani-sidine	o-phenyl-endiamine	ABTS	Guaia-col	o-diani-sidine	o-phenyl-endiamine
$K_m^{H_2O_2}$ (M)	$7.1 \cdot 10^{-5}$	$3.6 \cdot 10^{-3}$	$3.2 \cdot 10^{-3}$	$9.3 \cdot 10^{-4}$	$1.8 \cdot 10^{-3}$	$2.6 \cdot 10^{-3}$	$4.2 \cdot 10^{-4}$	$1.4 \cdot 10^{-3}$
$K_m^{AH_2}$ (M)	$6.2 \cdot 10^{-4}$	$9.2 \cdot 10^{-3}$	$6.8 \cdot 10^{-3}$	$3.0 \cdot 10^{-3}$	$4.9 \cdot 10^{-4}$	$14.4 \cdot 10^{-3}$	$6.0 \cdot 10^{-5}$	$9.2 \cdot 10^{-4}$
k_{cat} (s^{-1})	$1.2 \cdot 10^{3}$	$9.1 \cdot 10^{3}$	$3.5 \cdot 10^{3}$	$5.5 \cdot 10^{3}$	$3.3 \cdot 10^{3}$	$2.0 \cdot 10^{3}$	$2.4 \cdot 10^{3}$	$5.0 \cdot 10^{2}$
k_1 ($\mu M^{-1} s^{-1}$)	1.29	0.45	0.15	0.19	1.30	0.31	0.15	0.15
k_3 ($\mu M^{-1} s^{-1}$)	4.35	0.01	0.27	0.05	4.80	0.07	1.15	0.17

that palm peroxidases have greater resistance to inactivation by suicide substrate than HRP, which suggests that its application in biotechnological processes would be more effective than the peroxidases used so far.

6. Biotechnological applications

HRP has found a broad range of biotechnological applications. It has been used as a reagent for organic synthesis [45], biotransformations [46-53], and several types of chemiluminescent, immunological [54] and enzyme assays [55, 56]. However, very few studies aimed at biotechnological applications have used palm tree peroxidases, even though their thermal and chemical stabilities are higher than those of other peroxidases.

One biotechnological application for palm tree peroxidases has been found in the development of enzyme electrodes. Peroxidases are enzymes widely used to make enzyme electrodes by immobilizing the enzyme on the surface of an electrode made of graphite, gold, viologens, modified gold, or platinum. Comparison of the parameters of these H_2O_2-sensitive biosensors has shown that RPTP-built electrodes show higher operational and storage stabilities in combination with higher sensitivity and a broader linear range. Furthermore, the RPTP-built electrode can be used under acidic conditions due to the high pH-stability of this peroxidase. These results illustrate that the RPTP is apparently the best of the known peroxidases for developing applications at neutral and acidic conditions. This unique property is very promising for the determination of H_2O_2 during fermentation and other food industrial applications [16].

Another application for palm peroxidases is the synthesis of organic polymers. Polyaniline (PANI) is commonly synthesized by the chemical polymerization of aniline under strongly acidic conditions at 0°C and below, using ammonium persulphate as the initiator of polymerization. Owing to its remarkable electronic, optical, redox and mechanical properties and higher stability in air, a number of practical applications has been found in rechargeable and light-weight batteries, chemical sensors, electrical and electrochemical devices, electrochromic displays, bioanalysis, sensors for volatile organic compounds, electrochemical separation of racemic mixtures using chiral polyaniline, etc. [57-60]. The first peroxidase used for the synthesis of PANI was HRP, but this enzyme was found to exhibit a low affinity for aniline and to lose some of its catalytic activity at pH values lower than 4.5 [61, 62]. Thus, using a pH-stable peroxidase isolated from royal palm tree leaves, the enzymatic approach to the production of the electroactive form of polyelectrolyte complex of PANI and sulfonated polysterene (SPS) was developed [63, 64]. The advantage of this approach is that the stable peroxidase catalyzes the polymerization of aniline, which proceeds under environment-friendly conditions and is easier to control.

7. Overview of applications

Protein oxidation is considered to be a cause or contributory factor to many diseases, such as Refum's disease and Alzeheimer's disease, and it has also been related to the aging process [65-69]. Moreover, regarding the global effects of oxidative damage to proteins, as evidenced by the overall increase of protein carbonyl content [54], certain enzymes have been found to be specifically inactivated by the irreversible oxidation of catalytically important residues [70-74]. For example, Cys residues, once oxidized, disrupt the overall protein structure and facilitate further damage [75]. Additionally, protein glutathione adducts can be formed through Cys residues [76]. In particular, the reversible oxidation of methionine residues has been suggested to play an important role in the regulation of several cellular process [77, 78]. Although protein oxidation is generally independent of the catalytic activity of any given protein, the oxidative inactivation of haem peroxidases is mechanism-based. Owing to the high resistance to inactivation by H_2O_2, analyzed by means of different substrates, as compared with the other peroxidases studied to date and used in different applications, palm peroxidase can be said to be an excellent candidate for use in processes involving its oxidation, as mentioned above.

Another point is targeted cancer therapy using indole-3-acetic acid (IAA) and palm peroxidase in combination, which may offer new potentials for this application according to work by Wardman *et al.* [79-81]. Those authors observed that IAA is cytotoxic towards mammalian cells, including

human tumor cells, in the presence of cHRP. We used palm peroxidase for the present experiments because it exhibits high affinity, more than cHRP, for H-bond acceptor atom substrates. For example, we have recently demonstrated some substrates [34] and IAA have three H-bound acceptor sites (www.chemexper.com), which makes palm peroxidase an excellent candidate for this kind of application.

REFERENCES

1. Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., and Matsui, H. 2001, Plant Cell Physiol., 42, 462.
2. Poulos, T. L. and Kraut, J. 1980, J. Biol. Chem., 255, 8199.
3. Savitsky, P. A., Gazaryan, I. G., Tishkov, V. I., Lagrimini, L. M., Ruzgas, T., and Gorton, L. 1999, Biochem. J., 340, 579.
4. Feng, J. Y., Liu, J. Z., and Ji, L. N. 2008, Biochemie, 90, 1337.
5. Ryan, B. J., Carolan, N., and O'Fágáin, C. 2006, Trends Biotechnol., 24, 355.
6. Shakharov, I. Y. 2004, Palm tree peroxidases. Biochemistry (Moscow), 69, 823.
7. Shakharov, I. Y., Castillo Leon, J., Ariza, J. C., and Galaev, I. Y. 2000, Bioseparation, 9, 125.
8. Sakharov, I. Y., Vesga, M. K., Galaev, I. Y., Sakharova, I. V., and Pletjushkina, O. Y. 2001, Plant Sci., 161, 853.
9. Watanabe, L., Nascimiento, A. S., Zamorano, L. S., Shnyrov, V. L., and Polikarpov, I. 2007, Acta Cryst., F63, 780.
10. Onsa, G. H., bin Saari, N., Selamat, J., and Bakar, J. 2004, Food Chem., 85, 365.
11. Shakarov, I. Y. and Sakharova, I. V. 2002, Biochim. Biophys. Acta, 1598, 108.
12. Rodriguez, A., Pina, D. G., Yélamos, B., Castillo Leon, J. J., Zhadan, G. G., Villar, E., Gavilanes, F., Roig, M. G., Sakharov, I. Y., and Shnyrov, V. L. 2002, Eur. J. Biochem., 269, 2584.
13. Zamorano, L. S., Pina, D. G., Arellano, J. B., Bursakov, S. A., Zhadan, A. P., Calvete, J. J., Sanz, L., Nielsen, P. R., Villar, E., Gavel, O., Roig, M. G., Watanabe, L., Polikarpov, I., and Shnyrov, V. L. 2008, Biochemie, 90, 1737.
14. Zamorano, L. S., Vilarmau, S. B., Arellano, J. B., Zhadan, G. G., Hidalgo Cuadrado, N., Bursakov, S. A., Roig, M. G., and Shnyrov, V. L. 2009, Int. J. Biol. Macromol., 44, 326.
15. Regalado, C., Garcia-Almendarez, B. E., and Duarte-Vanquez, M. A. 2004, Phytochem. Rev., 3, 243.
16. Alpeeva, I. S., Niculescu-Nistor, M., Leon, J. C., Csöregi, E., and Sakharov, I. Y. 2005, Biosensors and Bioelectronics, 21, 742.
17. Caramyshev, A. V., Firsova, Y. N., Slastya, E. A., Tagaev, A. A., Potapenko, N. V., Lobakova., E. S., Pletjushkina, O. Y., and Sakharov, I. Y. 2006, J. Agric. Food Chem. 54, 9888.
18. Zamorano, L. S., Roig, M. G., Villar, E., and Shnyrov, V. L. 2007, Curr. Top. Biochem. Res., 9, 1.
19. Watanabe, L., de Moura, P. R., Bleicher, L., Nascimiento, S. A., Zamorano, S., Calvete, J. J., Sanz, L., Pérez, A., Bursakov, S., Roig, M. G., Shnyrov, V. L., and Polikarpov, I. 2010, J. Struct. Biol., 169, 226.
20. van Huystee, R. B., Roig, M. G., Shnyrov, V. L., and Sakharov, I. Y. 2004, Phytochem. Rev., 3, 19.
21. Banci, L. 1997, J. Biotech., 53, 253.
22. Gadjhede, M. 2001, Biochem. Soc. Trans., 29, 91.
23. Poulos, T. L. 2006, Peroxidase and catalases, Bertini, I., Gray, H. B., Stiefel, E. I., and Valentine, J. S. (Eds.), Sausalito, 343.
24. Poulos, T. L. and Fenna, R. 1994, Met. Biol., 30, 25.
25. Ryan, B. J., O'Connell, M. J., and O'Fágáin, C. 2006, Trends Biotech., 24, 355.
26. Veitch, N. C. 2004, Phytochem. Rev., 3, 3.
27. Welinder, K. G. 1992, Curr. Opin. Struct. Biol., 2, 388.
28. Veitch, N. C. 2004, Phytochemistry, 65, 249-259.
29. Lui, W., Kumar, J., Tripathy, S., Senecal, K. J., and Samuelson, L. 1999, J. Am. Chem. Soc., 121, 71.
30. Nagarajan, R., Liu, W.; Kumar, J., Tripathy, S., Bruno, F. F., and Samuelson, L. 2001, Macromolecules, 34, 3921.
31. Caramyshev, A. V., Evtushenko, E. G., Ivanov, V. F., Ros Barceló, A., Roig, M. G., Shnyrov, V. L., van Huystee, R. B., Kurochkin I. N., Vorobiev, A. K., and Sakharov, I. Y. 2005, Biomacromol., 6, 1360.

32. Venyaminov, S. Y. and Yang, J. T. 1996, Determination of Protein Secondary Structure, Fasman, G. D. (Ed), Plenum Press, New York, 69.
33. Cleland, W.W. 1970, The Enzymes, 3rd ed., Boyer, P. D. (Ed.), Academic, New York, 1.
34. Hidalgo Cuadrado, N., Arellano, J. B., Calvete, J. J., Sanz, L., Zhadan, G. G., Polikarpov, I., Bursakov, S., Roig, M. G., and Shnyrov, V. L. 2012, J. Mol. Cat. B: Enz., 49, 1078.
35. Zamorano, L. S. 2009, Caracterización Químico-Física de la peroxidasa de palma real (*Roystonea regia*) una enzima de elevada estabilidad. PhD Thesis, Salamanca University, Spain.
36. Shindler, J. S., Childs, R. E., and Bardsley, W. G. 1976, Eur. J. Biochem., 65, 325.
37. Childs, R. E. and Bardsley, W. G. 1975, Biochem. J., 145, 93.
38. Gazaryan, I. G., Reshetnikova, I. A., Doseeva, V. V., and Bekker, E. G. 1995, Biochemistry (Moscow), 60, 776.
39. Nakajima, R. and Yamakazi, I. 1987, J. Biol. Chem., 262, 2576.
40. Adediran, S. A. and Lambeir, A. M. 1989, Eur. J. Biochem., 186, 571.
41. Keilin, D. and Mann, T. 1937, Proc. R. Soc. B., 122, 119.
42. Wariishi, H. and Gold, M. H. 1990, J. Biol. Chem., 265, 2070.
43. Wu, G., Wei, C., Kulmacz, R. J., Osawa, Y., and Tsai, A. 1999, J. Biol. Chem., 274, 9231.
44. Hiner, A. N., Hernandez-Ruiz, J., Garcia-Canovas, F., Smith, A. T., Arnao, M. B., Acosta, M. 1995, Eur. J. Biochem., 234, 506.
45. Colonna, S., Gaggero, N., Richelmi, C., and Pasta, P. 1999, Trends Biotechnol., 17, 163.
46. Regalado, C., García-Almendárez, B., and Duarte-Vázquez, M. A. 2004, Phytochem. Rev., 3, 243.
47. Caza, N., Bewtra, J. K., Biswas, N., and Taylor, K. E. 1999, Water Res., 33, 3012.
48. Duarte-Vázquez, M. A., Ortega-Tovar, M. A., García-Almendárez, B. E., and Regalado, C. 2003, J. Chem. Technol. Biotechnol., 78, 42.
49. Duarte-Vázquez, M. A., García Almendárez, B. E., Regalado, C., and Whitaker, J. R. 2001, J. Agric. Food Chem., 49, 4450.
50. Cooper, V. A. and Nicell, J. A. 1996, Water Res., 30, 954.
51. Kinsley, C. and Nicell, J. A. 2000, Biores. Technol., 73, 139.
52. Chivukula, M., Spadaro, J. T., and Renganathan, V. 1995, Biochemistry, 34 7765.
53. Bunhia, A., Durati, S., and Wangikar, P. 2002, Biotechnol. Bioeng., 72, 562.
54. Rammakrishna, N., Lacey, L., Candish, A. A. G., Smith, J. E., and Goodbrand, I. A. 1990, J. Assoc. Off. Anal. Chem., 73, 71
55. Ruzgas, T., Csöregi, E., Emnéus, J., Gorton, L., and Marko-Varga, G. 1996, Anal. Chim. Acta, 330, 123.
56. Ruzgas, T., Csöregi, E., Katakis, I., and Gorton, L. 1996, J. Mol. Recognit., 9, 480.
57. MacDiarmid, A.G. 1997, Synth. Met., 84, 27.
58. MacDiarmid, A. G. 2002, Synth. Met., 125, 11.
59. Heeger, A. J. 2002, Synth. Met., 125, 23.
60. Raitman, O. A., Katz, E., and Buckmann, A. F. 2002, J. Am. Chem. Soc., 124, 6487.
61. Cattopadhya, K. and Mazumdar, S. 2000, Biochemistry, 39, 263.
62. Pina, D. G., Shnyrova, A. V., Gavilanes, F., Rodriguez, A., Leal, F., Roig, M. G., Shakarov, I., Zhadan, G. G., Villar, E., and Shnyrov, V. L. 2001, Eur. J. Biochem., 268, 120.
63. Sakharov, I. Y., Vorobiev, A. C., and Castillo Leon, J. J. 2003, Enzyme and Microbial Technology, 33, 661.
64. Mazhugo, Y. M., Caramyshev, A. V., Shleev, S. V., Sakharov, I. Y., and Yaropolov, A. I. 2005, Appl. Biochem. Microbiol., 41, 283.
65. Raha, S. and Robinson, B. H. 2000. Trends Biochem. Sci., 25, 502.
66. Muller, F. 2000, J. Am. Aging Asoc., 23, 227.
67. Anson, R.M., and Bohr, V.A. 2000, J. Am. Aging Asoc., 23, 199.
68. Ruan, H., Tang, X. D., Chen, M.-L., Joiner, M. A., Sun, G., Brot, N., Weiddbach, H., Heinemann, S. H., Iverson, L., Chun-Fang, W., and Hoshi, T. 2002, Proc. Natl. Acad. Sci. USA, 99, 2748.
69. Chevion, M., Berenshtein, E., and Stadtman, E. R. 2000, Free Radic. Res., 33, S99.
70. Allen, J. D., Bentley, D., Stringer, R. A., and Lowther, N. 1999, Int. J. Pharm., 187, 259.
71. Liu, J. L., Lu, K. V., Katta, V., Wescott, K. R., Narhi, L. O., and Lu, H. S. 1998, Pharm. Res., 15, 632.

72. Taggart, C., Cervantes-Laurean, D., Kim, G., McElvaney, N. G., Wehr, N., Moss, J., and Levine, R. L. 2000, J. Biol. Chem., 275, 27258.
73. Milzani, A., Rossi, R., Di Simplicio, P., Giustarini, D., Colombo, R., and Dalle Donne, I. 2000, Protein Sci., 8, 430.
74. De Paz, R. A., Barnett, C. C., Dale, D. A., Carpenter, J. F., Gaertner, A. L., and Randolph, T. W. 2000, Arch. Biochem. Byophys., 384, 123.
75. Kim, B. M., Schultz, L. W., and Raines, R. T. 1999, Protein Sci., 8, 430.
76. Hanson, S. R. A., Chen, A. A., Smith, J. B., and Lou, M. F. 1999, J. Biol. Chem., 274, 4735.
77. Levine, R. L., Moskovitz, J., and Stadtman, E. R. 2000, IUBMB Life, 50, 301.
78. Levine, R. L., Berlett, B. S., Moskovitz, J., Mosoni, L., and Stadtman, E. R. 1999, Mech. Ageing Dev., 107, 323.
79. Folkes, L. K. and Wardman, P. 2001, Biochem. Pharmacol., 61, 129.
80. Greco, O., Rossiter, S., Kanthou, C., Folkes, L. K., Wardman, P., Tozer, G. M., and Dach, G. U. 2001, Mol. Cancer Therapeutics, 1, 151.
81. Wardman, P. 2002, Current Pharmaceutical Desing., 8, 1363.