

Horseradish peroxidase: a modern view of a classic enzyme

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Abstract

Horseradish peroxidase is an important heme-containing enzyme that has been studied for more than a century. In recent years new information has become available on the three-dimensional structure of the enzyme and its catalytic intermediates, mechanisms of catalysis and the function of specific amino acid residues. Site-directed mutagenesis and directed evolution techniques are now used routinely to investigate the structure and function of horseradish peroxidase and offer the opportunity to develop engineered enzymes for practical applications in natural product and fine chemicals synthesis, medical diagnostics and bioremediation. A combination of horseradish peroxidase and indole-3-acetic acid or its derivatives is currently being evaluated as an agent for use in targeted cancer therapies. Physiological roles traditionally associated with the enzyme that include indole-3-acetic acid metabolism, cross-linking of biological polymers and lignification are becoming better understood at the molecular level, but the involvement of specific horseradish peroxidase isoenzymes in these processes is not yet clearly defined. Progress in this area should result from the identification of the entire peroxidase gene family of *Arabidopsis thaliana*, which has now been completed.

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1. Introduction

The horseradish (*Armoracia rusticana* P.Gaertn., B.Mey. & Scherb.; Cruciferae) is a hardy perennial herb cultivated in temperate regions of the world mainly for the culinary value of its roots (Fig. 1). These are also a rich source of peroxidase, a heme-containing enzyme that utilises hydrogen peroxide to oxidise a wide variety of organic and inorganic compounds. Production of peroxidase from horseradish roots occurs on a relatively large scale because of the commercial uses of the enzyme, for example as a component of clinical diagnostic kits and for immunoassays. Although the term horseradish peroxidase is used somewhat generically, the root of the plant contains a number of distinctive peroxidase isoenzymes of which the C isoenzyme (HRP C) is the most abundant. This has been the subject of much of the published work on horseradish peroxidase, which comprises many thousands of papers in the scientific literature. Some major advances in our understanding of the structure and function of HRP C have

been achieved relatively recently, and were initiated by the successful production of recombinant enzyme (Smith et al., 1990). From this work came the long-awaited solution of the three-dimensional structure of HRP C by X-ray crystallography and more recently, a high resolution description of the intermediates in the catalytic cycle of the enzyme (Gajhede et al., 1997; Berglund et al., 2002). Many physiological roles have been assigned to horseradish peroxidase isoenzymes including indole-3-acetic acid metabolism, lignification, cross-linking of cell wall polymers, suberin formation and resistance to infection. It is surprising therefore that so little is known about their specific functions in the plant. New insights into this problem may result from comparative studies of the peroxidases of the ‘model plant’, *Arabidopsis thaliana* (L.) Heynh., which is also a member of the Cruciferae. Two groups have now described the entire peroxidase gene family of *A. thaliana*, which comprises no less than 73 full-length genes, the majority of which are predicted to code for stable enzymes (Tognolli et al., 2002; Welinder et al., 2002). It seems therefore, that each plant species contains a ‘suite’ of peroxidase isoenzymes with the potential to carry out a range of different functions. This is one of the issues that will be highlighted in the present paper, which aims to provide an overview of recent advances in our

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Fig. 1. An early illustration of the horseradish plant, from the 1633 edition of Gerard's celebrated 'Herball, or generall historie of plantes'. According to Gerard 'the root is long and thick, white of colour, in taste sharpe, and very much biting the tongue like mustard'. These properties are due to volatile isothiocyanates released by the hydrolysis of constituent glucosinolates when the roots are crushed, a reaction that is catalysed by myrosinase. The same roots are also one of the most important sources of the heme-containing enzyme, peroxidase. Reproduced with permission. Copyright 2003, Royal Botanic Gardens, Kew.

understanding of horseradish peroxidase and a summary of its most important characteristics and uses.

2. Historical perspectives

The first recorded observation of a reaction catalysed by horseradish peroxidase appears in a note published by Louis Antoine Planché (1776–1840) describing the analysis of 'jalap' resin imported to France for medicinal use, probably from the Caribbean (Planché, 1810). A series of tests was carried out to determine whether a particular consignment was adulterated with 'gaiac' resin (also known as 'guaiacum', the powdered heartwood of the Caribbean trees, *Guaiacum officinale* and *G. sanctum*). During these investigations Planché noted that a tincture of gaiac resin became a beautiful blue colour when a piece of fresh horseradish root was placed in it. The reaction observed was probably the peroxidase-catalysed oxidation of 2,5-di-(4-hydroxy-3-methoxyphenyl)-3,4-dimethylfuran (a minor constituent of the resin and formerly referred to as α -guaiaconic acid) to give the corresponding bis-methylenequinone, a

blue product also known as 'guaiacum blue' (Kratochvíl et al., 1971). Although the term 'peroxidase' came into use towards the end of the nineteenth century it was the work of Robert Chodat (1865–1934) and Alexei Nikolaevich Bach (1857–1946) at the University of Geneva during the early years of the twentieth century that initiated further research on peroxidase from the horseradish root and other plant sources (Bach and Chodat, 1903). Some of their observations stimulated a vigorous debate on the nature of oxidases and peroxidases which was later recounted in a book by David Keilin (1887–1963), another distinguished contributor to the peroxidase field (Keilin, 1966). Although Bach and Chodat obtained a crude preparation of horseradish peroxidase it was through the subsequent studies of Richard Willstätter (1872–1942) and Hugo Theorell (1903–1982) that pure enzyme was finally isolated. Other advances in the period from approximately 1920 to 1945 included the demonstration of heme and carbohydrate as components of horseradish peroxidase, the first observation of the catalytic intermediates known as compounds I and II and the first kinetic analysis of the reaction with hydrogen peroxide. Detailed commentaries on the history and development of peroxidase are available in the literature (Saunders et al., 1964; Paul, 1986). These emphasise the important contribution that research on horseradish peroxidase has made to our understanding of peroxidase enzymes in general.

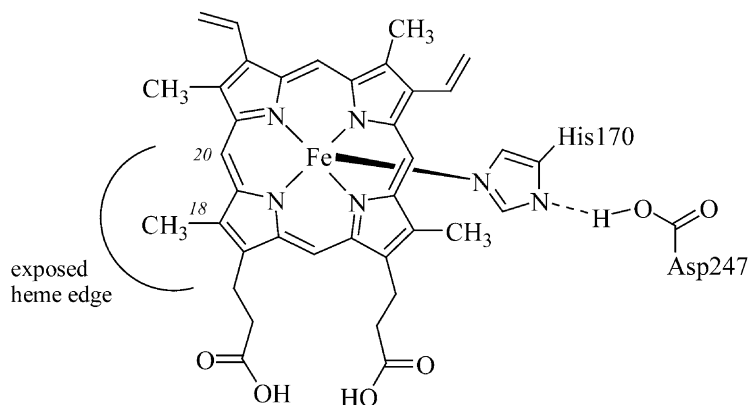
3. Description of the enzyme

3.1. General features

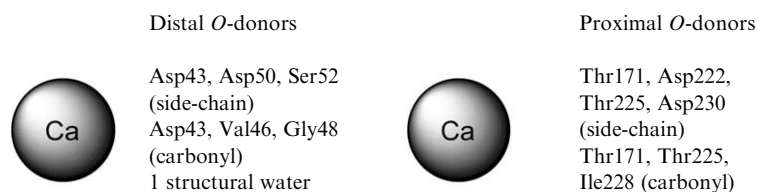
Horseradish peroxidase isoenzyme C comprises a single polypeptide of 308 amino acid residues, the sequence of which was determined by Welinder (1976). The N-terminal residue is blocked by pyroglutamate and the C-terminus is heterogenous, with some molecules lacking the terminal residue, Ser308. There are 4 disulphide bridges between cysteine residues 11–91, 44–49, 97–301 and 177–209, and a buried salt bridge between Asp99 and Arg123. Nine potential *N*-glycosylation sites can be recognised in the primary sequence from the motif Asn-X-Ser/Thr (where 'X' represents an amino acid residue) and of these, eight are occupied. A branched heptasaccharide accounts for 75 to 80% of the glycans (Table 1) but the carbohydrate profile of HRP C is heterogeneous, and many minor glycans have also been characterised (Yang et al., 1996). These invariably contain two terminal GlcNAc and several mannose residues. A further complication is the variation in the type of glycan present at any of the glycosylation sites. The total carbohydrate content of HRP C is somewhat dependent on the source of the enzyme and values of between 18 and 22% are typical.

Table 1

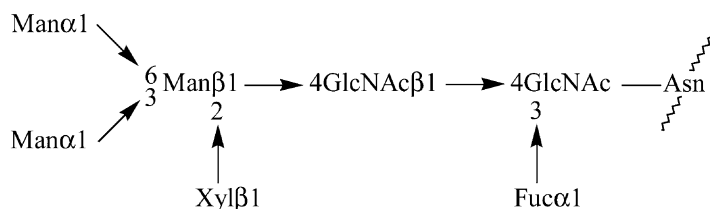
Anatomy of a peroxidase. Some essential structural features of horseradish peroxidase isoenzyme C (HRP C)

Heme

- (i) His170 forms coordinate bond to heme iron atom.
- (ii) Asp247 carboxylate side-chain helps to control imidazole character of His170 ring.
- (iii) His170Ala mutant undergoes heme degradation when H_2O_2 added and compounds I and II are not detected; $k_1 = 1.6 \times 10^1 \text{ M}^{-1} \text{ s}^{-1}$. Imidazoles can bind to heme iron in the artificially created cavity but full catalytic activity is not restored because the His170Ala-imidazole complex does not maintain a five-coordinate state (His42 also binds to Fe).
- (iv) Aromatic substrates are oxidized at the exposed heme edge but do not bind to heme iron.

Calcium

- (i) Distal and proximal Ca^{2+} ions are both seven-coordinate.
- (ii) On calcium loss enzyme activity decreases by 40%.
- (iii) Structural water of distal Ca site hydrogen bonded to Glu64 which is itself hydrogen bonded to Asn70 and thus connects to the distal heme pocket.

Carbohydrate

- (i) Sites of glycosylation are in loop regions of the structure, at Asn13, Asn57, Asn158, Asn186, Asn198, Asn214, Asn255 and Asn268.
- (ii) The major glycan is shown here; there are also minor glycans of the form $\text{Man}_m\text{GlcNAc}_2$ ($m = 4$ to 7) and $(\text{Xyl})_x\text{Man}_m(\text{Fuc})_f\text{GlcNAc}_2$ ($m = 2, 4, 5, 6$; $f = 0$ or 1 ; $x = 0$ or 1).

Amino acid residues

Arg38	Essential roles in (i), the formation and stabilization of compound I, (ii) binding and stabilization of ligands and aromatic substrates (e.g. benzhydroxamic acid, ferulate etc.).	Arg38Leu mutant; $k_1 = 1.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$; K_d (BHA) $12.1 \pm 0.7 \text{ mM}$.
Phe41	Prevents substrate access to the ferryl oxygen of compound I.	Phe41Ala/Leu/Thr mutants show improved rates of thioanisole oxidation (oxygen-transfer chemistry).
His42	Essential roles in (i), compound I formation (accepts proton from H_2O_2), (ii) binding and stabilization of ligands and aromatic substrates.	His42Leu mutant; $k_1 = 1.4 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$; K_d (BHA) $2.9 \pm 0.5 \text{ mM}$.
Asn70	Maintains basicity of His42 side-chain through Asn70-His42 couple (hydrogen bond from Asn70 amide oxygen to His42 imidazole NH).	Asn70Val mutant; $k_1 = 6.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.
Pro139	Part of a structural motif, '-Pro-X-Pro-' (Pro139-Ala140-Pro141 in HRP C), which is conserved in plant peroxidases.	Crystallographic evidence indicates role for Pro139 in substrate oxidation and binding. Note that residues Ala140, Phe142 and Phe143 are also part of the ferulate binding site shown in Fig. 5.

Original references for the results described in this table can be found in Veitch and Smith (2001). The rate constant for compound I formation (k_1) for wild-type enzyme is $1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The apparent dissociation constant (K_d) for complex formation between resting state HRP C and benzhydroxamic acid is $2.1 \pm 0.2 \text{ } \mu\text{M}$ (pH 7.0, 25 °C).

HRP C contains two different types of metal centre, iron(III) protoporphyrin IX (usually referred to as the 'heme group') and two calcium atoms (Fig. 2 and Table 1). Both are essential for the structural and functional integrity of the enzyme. The heme group is attached to the enzyme at His170 (the proximal histidine residue) by a coordinate bond between the histidine side-chain N ϵ 2 atom and the heme iron atom. The second axial coordination site (on the so-called distal side of the heme plane) is unoccupied in the resting state of the enzyme but available to hydrogen peroxide during enzyme turnover (Fig. 3). Small molecules such as carbon monoxide, cyanide, fluoride and azide bind to the heme iron atom at this distal site giving six-coordinate peroxidase complexes. Some bind only in their protonated forms, which are stabilized through hydrogen bonded interactions with the distal heme pocket amino acid side-chains of Arg38 (the distal arginine) and His42 (the distal histidine) (Fig. 3).

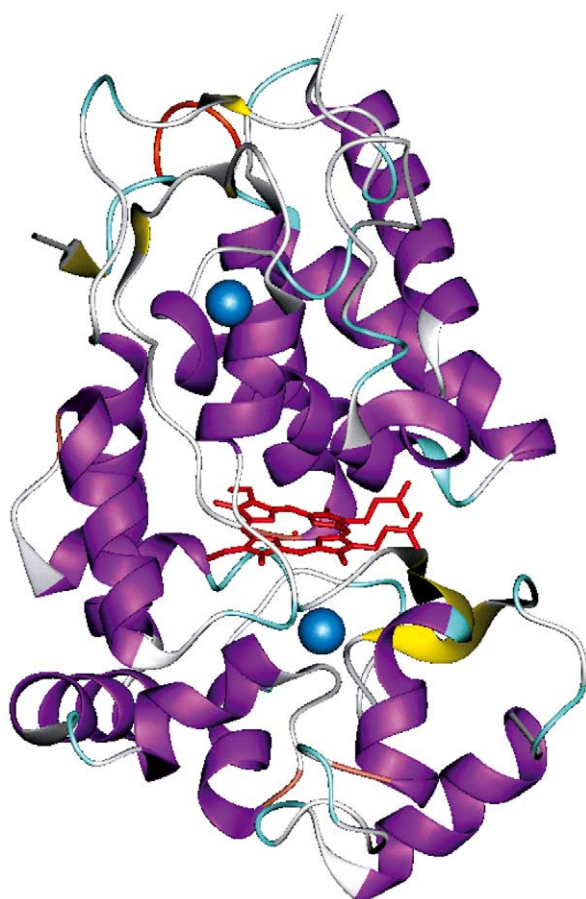


Fig. 2. Three-dimensional representation of the X-ray crystal structure of horseradish peroxidase isoenzyme C (Brookhaven accession code 1H5A). The heme group (coloured in red) is located between the distal and proximal domains which each contain one calcium atom (shown as blue spheres). α -Helical and β -sheet regions of the enzyme are shown in purple and yellow, respectively. The F' and F'' α -helices appear in the bottom right-hand quadrant of the molecule.

The two calcium binding sites are located at positions distal and proximal to the heme plane and are linked to the heme-binding region by a network of hydrogen bonds. Each calcium site is seven-coordinate with oxygen-donor ligands provided by a combination of amino acid side-chain carboxylates (Asp), hydroxyl groups (Ser, Thr), backbone carbonyls and a structural water molecule (distal site only) (Table 1). Loss of calcium results in decreases to both enzyme activity and thermal stability (Haschke and Friedhoff, 1978) and to subtle changes in the heme environment that can be detected spectroscopically (Howes et al., 2001).

3.2. Three-dimensional structure

The first solution of the three-dimensional structure of HRP C using X-ray crystallography appeared in the literature relatively recently (Gajhede et al., 1997). The recombinant enzyme used as the source of crystals and heavy atom derivatives was produced by expression in *Escherichia coli* in non-glycosylated form (Smith et al., 1990). Previous attempts to obtain suitable crystals for diffraction were frustrated partly by the heterogeneity of plant HRP C preparations comprising multiple glycoforms. The structure of the enzyme is largely α -helical although there is also a small region of β -sheet (Fig. 2). There are two domains, the distal and proximal, between which the heme group is located. These domains probably originated as a result of gene duplication, a proposal supported by their common calcium binding sites and other structural elements (Welinder and Gajhede, 1993).

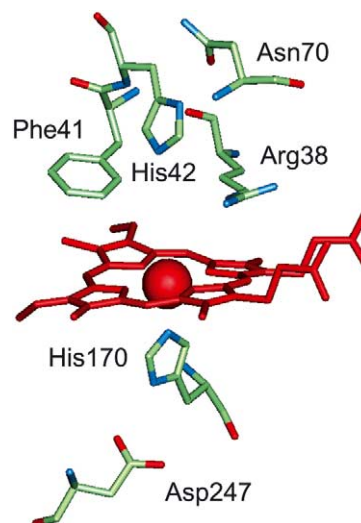


Fig. 3. Key amino acid residues in the heme-binding region of HRP C. The heme group and heme iron atom are shown in red, the remaining residues in atom colours. His170, the proximal histidine residue, is coordinated to the heme iron atom whereas the corresponding distal coordination site above the plane of the heme is vacant. Specific functions for the amino acid residues shown here are given in Table 1.

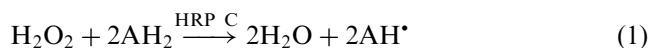
3.3. HRP and the plant peroxidase superfamily

Horseradish peroxidase isoenzymes belong to class III ('classical' secretory plant peroxidases) of the plant peroxidase superfamily, which includes peroxidases of bacterial, fungal and plant origin (Welinder, 1992a). The remaining two classes comprise yeast cytochrome *c* peroxidase, gene-duplicated bacterial peroxidases and ascorbate peroxidases (class I), and fungal peroxidases (class II). This classification was based originally on comparisons of amino acid sequence but is well-supported by more recent three-dimensional structural data obtained for enzymes from each of the classes. Many structural elements are conserved among peroxidases of the three classes leading to the definition of a 'core' peroxidase fold. The structures of HRP C and of other class III plant peroxidases contain three α -helices that are additional to this core peroxidase fold (Gajhede et al., 1997). Two of these (helices *F'* and *F''*) are located in a long insertion that shows great variation in both sequence and number of residues (Fig. 2). The overall integrity of the structure in this region is maintained by the disulphide linkage from Cys177 to Cys209. What is particularly interesting in the case of HRP C is the location of residues in helix *F'* that are involved in substrate access and binding. Some authors have speculated that this structural region is important for stabilization or retention of radical species produced in reactions catalysed by plant peroxidases (Gajhede et al., 1997). A comparison of structural and functional characteristics of peroxidases from the three classes of the plant peroxidase superfamily has been published (Smith and Veitch, 1998).

4. Mechanism of action

4.1. Functional roles

Most reactions catalysed by HRP C and other horseradish peroxidase isoenzymes can be expressed by the following equation, in which AH_2 and AH^\bullet represent a reducing substrate and its radical product, respectively. Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates.



The conversion of hydrogen peroxide to water is not the primary function of class III plant peroxidases such as HRP C. Other enzymes, including ascorbate peroxidase (class I), are used by plants to regulate levels of intracellular hydrogen peroxide (Mittler, 2002; Shigeoka et al., 2002). Little is known about the ascorbate peroxidases of horseradish, but in a recent study the accumulation of H_2O_2 in *Arabidopsis thaliana* plants deficient in cytosolic

ascorbate peroxidase (*Apx1*) was demonstrated (Pnueli et al., 2003). An interesting observation in the knock-out-*Apx1* plants was the induction of transcripts encoding for two class III plant peroxidases, presumably to compensate for the reduction in peroxide-scavenging ability.

The formation of radical products in HRP-catalysed reactions gives an indication of some possible *in vivo* functions in the plant. These may involve cross-linking reactions such as the formation of diferulate linkages from polymer-attached ferulate groups of polysaccharides or pectins, the formation of dityrosine linkages, the cross-linking of phenolic monomers in the formation of suberin and the oxidative coupling of phenolic compounds as part of the biosynthesis of lignin. Peroxidases that catalyse cross-linking reactions may be expressed in response to external factors such as the wounding of plant tissue. Water loss and invasion by pathogens can thus be limited by the formation of a protective polymeric barrier such as suberin. Although *in vivo* roles for HRP isoenzymes have not yet been determined, it is known that peroxidase activity is induced when horseradish leaves are wounded (Kawaoka et al., 1994).

4.2. Catalytic mechanism

The mechanism of catalysis of horseradish peroxidase and in particular, the C isoenzyme, has been investigated extensively (reviewed in Dunford, 1991, 1999; Veitch and Smith, 2001). Some important features of the catalytic cycle are illustrated in Fig. 4 with ferulate as reducing substrate. The generation of radical species in the two one-electron reduction steps can result in a complex profile of reaction products, including dimers,

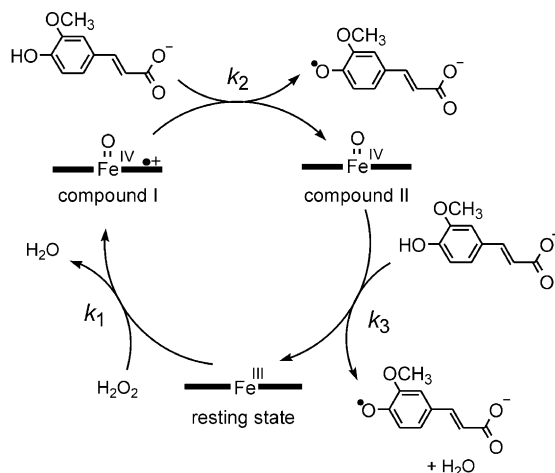


Fig. 4. The catalytic cycle of horseradish peroxidase (HRP C) with ferulate as reducing substrate. The rate constants k_1 , k_2 and k_3 represent the rate of compound I formation, rate of compound I reduction and rate of compound II reduction, respectively.

trimers and higher oligomers that may themselves act as reducing substrates in subsequent turnovers.

The first step in the catalytic cycle is the reaction between H_2O_2 and the Fe(III) resting state of the enzyme to generate compound I, a high oxidation state intermediate comprising an Fe(IV) oxoferryl centre and a porphyrin-based cation radical. A transient intermediate (compound 0) formed prior to compound I has been detected in reactions between HRP C and H_2O_2 at low temperatures and described as an Fe(III)-hydroperoxy complex. Molecular dynamics simulations of these peroxide-bound complexes have been carried out (Filizola and Loew, 2000). In formal terms, compound I is two oxidising equivalents above the resting state. The first one-electron reduction step requires the participation of a reducing substrate and leads to the generation of compound II, an Fe(IV) oxoferryl species that is one oxidising equivalent above the resting state. Both compound I and compound II are powerful oxidants, with redox potentials estimated to be close to +1 V. The second one-electron reduction step returns compound II to the resting state of the enzyme. Reaction of excess hydrogen peroxide with resting state enzyme gives compound III, which can also be prepared by several other routes (Dunford, 1999). This intermediate is best described as a resonance hybrid of iron(III)-superoxide and iron(II)-dioxygen complexes. A high-resolution crystal structure of 95% pure compound III published recently shows dioxygen bound to heme iron in a bent conformation (Berglund et al., 2002). Models for the irreversible inactivation of HRP C by peroxides have been developed in studies with *m*-chloroperoxybenzoic acid (Rodriguez-Lopez et al., 1997).

Detailed mechanisms showing the role of the distal heme pocket residues Arg38 and His42 (conserved in all members of the plant peroxidase superfamily) in the formation of compound I have been described in the literature but are beyond the scope of this review. High resolution crystal structures of the oxidised intermediates of HRP C confirm the importance of Arg38 and His42 for peroxide catalysis (Berglund et al., 2002). For example, the ferryl oxygen of compound I is hydrogen bonded to Arg38 N ϵ H and a water molecule that is also hydrogen bonded to both Arg38 and His42. A mechanism for the reduction of compounds I and II by ferulate has been proposed based on new information from the crystal structure of a ternary complex formed between ferulic acid and cyanide-ligated HRP C (Henriksen et al., 1999).

4.3. Aromatic substrate binding sites

Substrate oxidation by HRP C occurs at the 'exposed' heme edge, a region comprising the heme methyl C18 and heme meso C20 protons (Table 1). This interaction site was identified in enzyme inactivation experiments

with alkyl- and phenylhydrazines, sodium azide and other reactive agents (Ortiz de Montellano, 1992). The free radical species generated when these are incubated with HRP C and H_2O_2 give adducts such as C18-hydroxymethyl and C20-meso-phenyl heme derivatives. What is particularly interesting is the contrast between the behaviour of HRP C and other heme proteins such as the globins and cytochromes P450, where modification to the heme iron atom and pyrrole nitrogens occurs. Substrate access to the oxoferryl centre of HRP C appears to be hindered by the local protein environment, an example of 'closed' heme architecture. This is one reason why peroxidases are much less effective as catalysts of oxygen-transfer reactions than cytochromes P450. Attempts to improve this aspect of the chemistry of HRP C have been made by site-directed mutagenesis. In these experiments, substrate access to the oxoferryl centre of compound I was increased by substituting the distal residues Phe41 and His42 with smaller amino acid residues (Newmyer and Ortiz de Montellano, 1995; Ozaki and Ortiz de Montellano, 1995).

Stable, reversible 1:1 complexes with a variety of aromatic molecules are formed both by resting state HRP C and some of its ligand-bound derivatives (e.g. cyanide-ligated HRP C) in the absence of H_2O_2 . Spectroscopic and crystallographic studies have revealed a detailed picture of the site where these aromatic substrates are located (Veitch and Smith, 2001). For example, when ferulic acid binds to cyanide-ligated HRP C its aromatic ring is located in a distal site close to the exposed heme edge and its phenolic acid side-chain is oriented towards the entrance of the binding site (Henriksen et al., 1999). There are hydrogen-bonded interactions between Arg38 N η H₂ and the phenolic and methoxyl oxygen atoms of ferulic acid and between the phenolic oxygen and an active site water molecule. The latter is also hydrogen bonded to the backbone carbonyl of Pro139 (an invariant residue in the plant peroxidase superfamily) and the nitrogen atom of the cyanide ligand. There are hydrophobic interactions with amino acid side-chains (Phe68, Gly69, Pro139, Ala140, Phe142 and Phe179) as well as heme methyl C18H₃ and the heme meso proton C20H (Fig. 5). The structure and dynamics of benzhydroxamic acid complexes of HRP C have also been investigated in detail with site-directed mutants used to explore the contribution of binding site residues to substrate affinity (Veitch and Smith, 2001).

5. The isoenzyme question; horseradish and *Arabidopsis* peroxidases

5.1. The horseradish peroxidase isoenzyme family

Fifteen peroxidase isoenzymes have been isolated from horseradish root using classical protein purification

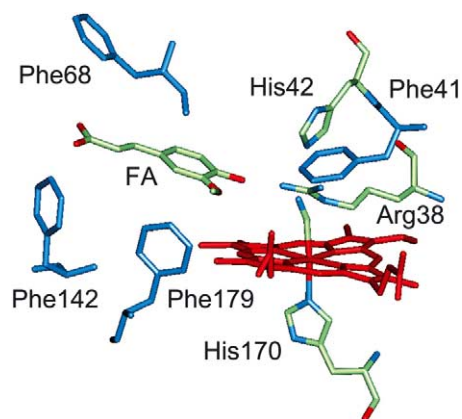


Fig. 5. The ferulic acid binding site of horseradish peroxidase (HRP C). This representation is taken from the X-ray crystal structure of the ternary complex of ferulic acid (FA) and cyanide-ligated HRP C (Brookhaven accession code 7ATJ). The heme group is shown in red and ferulic acid, the cyanide ligand (bound to heme iron at the vacant distal coordination site) and the catalytic residues Arg38, His42 and His170 are in atom colours. Several phenylalanine side-chains that contribute to the binding site are shown in blue. Note that the ferulic acid side-chain is located towards the entrance of the binding site.

methods and are generally referred to by codes based on their isoelectric point value as HRP A1–A3 ('acidic'), B1–B3 and C1–C2 ('neutral' or 'neutral-basic') and D and E1–E6 ('basic'). An even greater number of peroxidase 'isoforms' can be detected by isoelectric focusing but their identity has proved controversial as some may be artifacts produced by deamidation, cross-linking or other processes. The fact that amino acid sequences determined for HRP A2 and E5 show only 54 and 70% identity, respectively, to HRP C, is one piece of evidence for the existence of distinct peroxidase isoenzymes in horseradish root (Welinder, 1992b).

Four genomic DNA clones encoding neutral and neutral-basic HRP isoenzymes have been isolated and characterised from cultured cells of *Armoracia rusticana* (Fujiyama et al., 1988, 1990). Two of these peroxidase genes (*prxC1a* and *prxC1b*) are expressed both in stems and roots and two mainly in roots (*prxC2* and *prxC3*). All consist of four exons and three introns and have identical splice site positions, a feature common to other plant peroxidase genes. Three highly homologous cDNAs (*prxC1a*, *prxC1b* and *prxC1c*) have also been isolated using an oligonucleotide probe corresponding to the primary sequence region from His40 to Ala51 of HRP C (Fujiyama et al., 1988). The amino acid sequence deduced from *prxC1a* is identical to that of HRP C but also contains a hydrophobic leader sequence as well as a C-terminal extension which may be responsible for vacuolar targeting. Expression of the *prxC2* gene is induced by wounding and its transcriptional regulation has been investigated in detail (Kao-thien et al., 2000). One further cDNA has been cloned and sequenced from horseradish corresponding to a

neutral isoenzyme (HRP N) that has not yet been isolated from the plant (Bartonek-Roxå and Holm, 1999).

5.2. *Arabidopsis thaliana* peroxidases

The sequencing of the genome of the 'model' plant, *Arabidopsis thaliana*, and the availability of expressed sequence tag (EST) databases of cDNA clones has provided the opportunity to investigate an entire family of class III peroxidases from one plant for the first time, with striking results. According to recent studies there are 73 full-length genes for class III plant peroxidases in the *Arabidopsis* genome (Tognolli et al., 2002; Welinder et al., 2002). Of these, 71 are predicted to encode for stable enzymes folded similarly to HRP C (Welinder et al., 2002). Amino acid sequence identities among the predicted peroxidases range from 28 to 94%. Alignment of these sequences has been used as a basis for comparisons with other plant peroxidases and the identification of potentially similar enzymes. For example, expression of an acidic peroxidase isoenzyme from *A. thaliana* (AtPA2) with 95% amino acid sequence identity to HRP A2 (a horseradish peroxidase isoenzyme of unknown function) was found to coincide with lignification (Østergaard et al., 2000). The three-dimensional structure solved for recombinant AtPA2 by X-ray crystallography reveals a substrate binding site that can accommodate monolignols such as *p*-coumaryl and coniferyl alcohols (Nielsen et al., 2001). This structure is an excellent model for HRP A2, which has not yielded crystals suitable for crystallographic analysis. Both enzymes will catalyse the oxidation of monolignols and phenolic acids although small differences in their reactivity can be detected. These may have their origin in the levels of glycosylation of the two enzymes; recombinant AtPA2 (as expressed in *E. coli*) is non-glycosylated, whereas plant HRP A2 is highly glycosylated with one bulky glycan attached close to residues at the entrance of the substrate binding site.

The presence of similar peroxidase isoenzymes in taxa from two genera of the same plant family may not seem surprising, but analysis of *Arabidopsis* peroxidases indicates that enzymes from species in quite unrelated plant families may also share high amino acid sequence identity, for example, *Arabidopsis* peroxidase AtP1 (Cruciferae) and a peroxidase from *Gossypium hirsutum* (Malvaceae). Duroux and Welinder (2003) have used the peroxidase gene family of *A. thaliana* as a basis for a comprehensive survey of evolutionary relationships among class III plant peroxidases from angiosperms, gymnosperms, ferns, mosses and liverworts. They suggest that the class III plant peroxidase gene family appeared when plants colonised the land and that adaptive advantages were conferred through peroxidase functionality (e.g. in cell-wall metabolism and defence). Phylogenetic analysis confirms that the class III peroxidases of *A.*

thaliana represent a highly diverse gene family. Peroxidases from eudicots cluster with the different groups present in *Arabidopsis*, indicating that peroxidase diversity was extant before the radiation of eudicots. In contrast, peroxidases from monocots cluster in specific groups that have not been found in eudicots. It appears therefore that some groups of peroxidases evolved independently in monocots and eudicots or that some were lost. What is remarkable is the inference that each plant species contains a relatively large number of distinctive peroxidase enzymes. The challenge for the future lies in the determination of their specific functions.

6. Protein engineering and directed evolution of HRP C

Several expression systems for the production of recombinant HRP C have been developed since the early 1990s and are compared in a recent review (Veitch and Smith, 2001). Active recombinant HRP C in yields of 5–10 mg/l can be produced in a baculovirus system although the enzyme is highly glycosylated. Yields are lower (typically 2–4 mg/l) when *Escherichia coli* is used as the expression system because the enzyme must be recovered from inclusion bodies. Essential steps in this process are the solubilization of the inclusion bodies, controlled reoxidation of the reduced, denatured enzyme and *in vitro* refolding in the presence of heme and calcium (Smith et al., 1990). One advantage of *E. coli* expression is that active recombinant enzyme is obtained in non-glycosylated form, a factor crucial to the success of subsequent crystallisation studies and the solution of the three-dimensional structure of the enzyme. Site-directed mutants of the enzyme have been generated in both baculovirus and *E. coli* expression systems. Areas of interest addressed in these studies include the role of distal heme pocket residues in peroxide binding and catalysis, the identity of structural determinants of substrate oxidation, characterisation of small molecule and substrate binding sites, the significance of calcium binding sites and the rational design of artificial binding sites. A comprehensive survey of work published on site-directed mutants of HRP C up to the end of 1999 is available (Veitch and Smith, 2001) and some results are summarised in Table 1.

Directed evolution techniques have been applied to HRP C more recently in an attempt to improve qualities of the enzyme that are important for biotechnological and diagnostic applications, such as thermal stability and resistance to inactivation by peroxides. In these studies the host chosen for expression of HRP C was *Saccharomyces cerevisiae* rather than *E. coli*, as active enzyme can be produced without the need for *in vitro* refolding. However, expression levels in *S. cerevisiae* are

low (approximately 50 µg/l) and the secreted enzyme is hyperglycosylated. A 40-fold increase in the HRP C activity of *S. cerevisiae* culture supernatant was achieved in three rounds of directed evolution using random point mutagenesis and screening (Morawski et al., 2000). The majority of the mutations were to amino acid residues in either loop or surface regions of the enzyme. Although activity was improved, thermal stability showed a small decrease with respect to wild-type enzyme. However, the combination of an HRP C mutant evolved for higher activity with a single site mutation at Asn175 (N175S) gave significant improvements in thermal stability (Morawski et al., 2001). Expression of this mutant in *Pichia pastoris* (giving sufficient amounts of the enzyme for purification and characterisation) yielded recombinant enzyme showing a 3-fold improvement in thermal stability at 60 °C and pH 7.0 and increased resistance to inactivation by hydrogen peroxide and other additives. This recombinant enzyme ('HRP13A7-N175S') comprised one synonymous mutation (A85) and three amino acid substitutions (N175S, N212D and Q223L). In contrast, directed evolution of a fungal peroxidase from *Coprinus cinereus* yielded site-directed mutants with up to 190-fold increases in thermal stability and 100 times greater oxidative stability than the wild-type enzyme (Cherry et al., 1999). These dramatic improvements must be considered in the light of the greater thermal and oxidative stability already possessed by wild-type HRP C as compared to *Coprinus* peroxidase and the much larger number of mutants screened for the latter.

7. Horseradish peroxidase and indole-3-acetic acid

7.1. Mechanisms of oxidation

One of the most interesting reactions of HRP C occurs with the plant hormone, indole-3-acetic acid (IAA). In contrast to most peroxidase-catalysed reactions, this takes place without added hydrogen peroxide, hence the use of the term 'indole acetic acid oxidase' to describe this activity of HRP C in the older literature. More recent studies of the reaction at neutral pH indicate that it is not an oxidase mechanism that operates, but rather a peroxidase mechanism coupled to a very efficient branched-chain process in which organic peroxide is formed (Dunford, 1999). The reaction is initiated when a trace of the indole-3-acetate cation radical is produced. Major products of indole-3-acetic acid oxidation include indole-3-methanol, indole-3-aldehyde and 3-methylene-2-oxindole, the latter most probably as a result of the non-enzymatic conversion of indole-3-methylhydroperoxide. Conflicting theories have been proposed to explain the mechanism of reaction at lower pH (reviewed by Dunford, 1999), in which

the formation of the ferrous enzyme, compound III and hydroperoxyl radicals must also be accounted for. The physiological significance of IAA metabolism by HRP C and other plant peroxidases is still an area of active debate. For example, studies of the expression of an anionic peroxidase in transgenic tobacco plants indicate that while overproduction of the enzyme favours defensive strategies (such as resistance to disease, physical damage and insect attack) it has a negative impact on growth because of increased IAA degradation activity (Lagrimini, 1996). Thus peroxidase expression in plant tissues at different stages of development must reflect a balance between the priorities of defence and growth.

7.2. Targeted cancer therapy

Use of indole-3-acetic acid and HRP C in combination may offer new potential for targeted cancer therapy, according to recent work by Wardman and colleagues (Folkes and Wardman, 2001; Greco et al., 2001; Wardman, 2002). These authors observed that IAA is cytotoxic towards mammalian cells, including human tumour cells, in the presence of HRP C. The primary mechanism of toxicity is thought to involve 3-methylene-2-oxindole, a known product of the reaction between HRP C and IAA that shows high reactivity towards cellular nucleophiles such as glutathione and the thiol groups of proteins or histone (Fig. 6). However, the fact that 2-methylindole-3-acetic acid shows greater cytotoxicity in combination with HRP C than IAA suggests that there may be other mechanisms of toxicity which do not involve oxindole intermediates (Wardman, 2002). Many other substituted indole-3-acetic acid derivatives have been tested for cytotoxicity in combination with HRP C in an attempt to place relationships between structure and activity on a predictive level. No simple correlation was found between levels of cytotoxicity of indole derivatives and their reactivity towards compound I; for example 5-fluoroindole-3-acetic acid is more cytotoxic towards tumour cells than IAA but less effective as a reductant of compound I (Folkes et al., 2002). Other factors such as the pK_a of the indolyl radical cation and rates of decarbox-

ylation and radical fragmentation may also be significant. One of the most cytotoxic indoles identified from *in vitro* screens is 6-chloroindole-3-acetic acid, a derivative with potential as a prodrug for targeted cancer therapies mediated by HRP C (Rossiter et al., 2002). The challenge now is to develop strategies to evaluate and implement this promising system *in vivo*. Indeed the combination of HRP C and indole-3-acetic acid or its derivatives offers several advantages for future antibody-, gene- or polymer-directed enzyme:prodrug therapies (Folkes and Wardman, 2001; Wardman, 2002). Among the favourable properties of HRP C are its good stability at 37 °C, high activity at neutral pH, lack of toxicity and the ease with which it can be conjugated to antibodies and polymers. Furthermore, evidence available at present suggests that IAA does not show any adverse side-effects in humans. The fact that peroxide is not required as a cosubstrate for the reaction with HRP C is also a significant advantage.

8. Applications overview

Horseradish peroxidase (predominantly HRP C) is used as a reagent for organic synthesis and biotransformation as well as in coupled enzyme assays, chemiluminescent assays, immunoassays and the treatment of waste waters (for reviews see Ryan et al., 1994; Veitch and Smith, 2001; Krieg and Halbhuber, 2003). Improvements to desirable qualities of the enzyme such as its relatively good stability in aqueous and non-aqueous solvent systems are actively sought as an outcome of chemical modification, site-directed mutagenesis and directed evolution studies (Ó'Fágáin, 2003). Some applications of HRP C in small-scale organic synthesis include *N*- and *O*-dealkylation, oxidative coupling, selective hydroxylation and oxygen-transfer reactions. Site-directed mutagenesis at Phe41 and His42 of HRP C has been used to improve the enantioselectivity of arylmethylsulfide oxidations (Newmyer and Ortiz de Montellano, 1995; Ozaki and Ortiz de Montellano, 1995). However, Van de Velde et al. (2001) make the general point that scale-up of peroxidase-

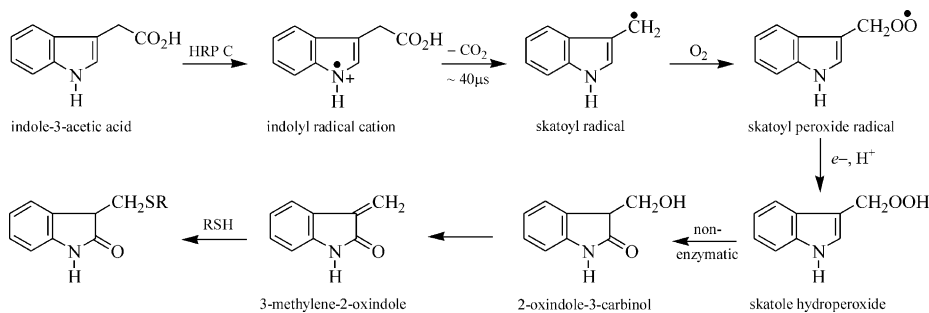


Fig. 6. A mechanism proposed for the formation of 3-methylene-2-oxindole from horseradish peroxidase (HRP C) and indole-3-acetic acid (after Folkes et al., 2002). R represents a cellular nucleophile (e.g. sulphydryl groups of enzymes or histone).

catalysed enantioselective oxidations to industrial level will require a substantial reduction in the price of enzyme per unit product. Solutions to this problem may include better process management of hydrogen peroxide to avoid enzyme inactivation and use of engineered enzymes with improved stability and catalytic efficiency.

Several examples of the use of HRP C in natural product synthesis or biotransformation have appeared in the literature. For example, peroxidase-catalysed oxidative coupling of methyl-(*E*)-sinapate with the syringyl lignin-model compound, 1-(4-hydroxy-3,5-dimethoxyphenyl)ethanol yielded a novel spirocyclohexadienone together with a dimerization side-product (Setälä *et al.*, 1999). Coupling of catharanthine and vindoline to yield α -3',4'-anhydrovinblastine is a reaction catalysed by HRP C of potential interest as a semisynthetic step in the production of the anti-cancer drugs vinblastine and vincristine from *Catharanthus roseus* (Sottomayor *et al.*, 1997). An enzyme with α -3',4'-anhydrovinblastine synthase activity that shows many features characteristic of a plant peroxidase has now been purified from *C. roseus* leaves (Sottomayor *et al.*, 1998).

9. Bibliography of horseradish peroxidase

Few plant enzymes are represented so widely in the scientific and patent literature as horseradish peroxidase. This has limited the number of primary sources that can be cited in the present report and a bias towards more recent papers has been adopted. Review articles with more extensive lists of references are quoted where appropriate. Two important monographs on peroxidase enzymes have been published by Dunford (1999) and Saunders, Holmes-Siedle and Stark (1964). The latter contains approximately 1500 references covering the period from 1810 to 1960. Two volumes of reviews and literature compilations on peroxidase covering the decades 1970–1980 and 1980–1990 were published by the University of Geneva and contain more than 5000 references (Gaspar *et al.*, 1982, 1992). Other general reviews on peroxidase are listed in Veitch and Smith (2001). Two review articles that deal specifically with horseradish peroxidase are available in the literature (Dunford, 1991; Veitch and Smith, 2001).

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