Horseradish and Soybean Peroxidases: Comparable Tools for Alternative Niches?

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Horseradish and soybean peroxidases (HRP and SBP, respectively) are useful biotechnological tools. HRP is often termed the classical plant heme peroxidase, and although it has been studied for decades our understanding has deepened since its cloning and subsequent expression, which has enabled numerous mutational and protein engineering studies. SBP, however, has been neglected until recently; despite offering a real alternative to HRP that actually outperforms it in terms of stability. SBP is now used in numerous biotechnological applications, including biosensors. Review of both is timely. This article summarises and discusses the main insights into the structure and mechanism of HRP, with special emphasis on HRP mutagenesis, and outlines its use in a variety of applications. It also reviews current knowledge and applications to date of SBP, particularly biosensors. The final paragraphs speculate on the future of plant heme-based peroxidases, with probable trends outlined and explored.

Key Words: Horseradish Peroxidase, Soybean Peroxidase, Review, Biosensors, Biocatalysis, Mutagenesis.

[http://www.dcu.ie/biotechnology/index.shtml]
[http://www.dcu.ie/~ncsr/]

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Abbreviations:
ABTS, 2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid); **Co(bpy),** Tris-2,2'-dipyridylocbalt(III) perchlorate trihydrate; **CV**, Cyclic voltammogram. **DAB**, 3,3'-diaminobenzidine tetrahydrochloride. **DMPC**, Dimyristoylphosphatidylcholine epoxidised olefins. **DTT**, dithiothreitol; **ELISA,** Enzyme-Linked Immunosorbent Assay. **GOx**, Glucose oxidase; **GP,** Graphite Powder; **H_2O_2**, Hydrogen peroxide; **HQ**, hydroquinone; **IPTG**, iso-propyl-β-thiogalactoside; **MET**, mediated electron transfer; **mm**, millimetre. **PEGDGE**, Poly(ethylene glycol) (400) diglycidylether. **PelB**, pectate lyase B fragment. **PDB**, Protein Data Bank; **POs-EA**, $Os(bpy)_2Cl^{+/2+}$ poly(4-vinylpyridine) quaternerised with 2-bromoethylamine. **Pt,** Platinum**. PVA**, Polyvinyl alcohol. **PVP**, Polyvinyl pyridine. **PVP-Os^{n+}**, polyvinyl pyridine-osmium complex. **POCT**, point-of-care testing; **Px**, peroxidase. **Px_{ox},** oxidised peroxidase. **rHRP**, recombinant HRP; **TTCA**, poly-5,2':5',2''-terthiophene-3'-carboxylic acid.

Introduction.

Peroxidase enzymes span the bioscience and biotechnology spectra, ranging from bioremediation [1] and biocatalysis [2] through diagnostics [3] and biosensors [4] to recombinant protein expression [5], transgenics [6], bioinformatics [7], protein engineering [8] and even to therapeutics [9]. This article contrasts two key heme-containing plant peroxidases, horseradish (HRP) and soybean (SBP), with special emphasis on mutagenesis studies and biosensor applications. HRP is a 'traditional' enzyme, whereas SBP emerged in the 1990s. A comparison of these key biotechnological tools is timely.

Brief biochemistry of horseradish and soybean peroxidase:

All heme peroxidases (E.C. 1.11.1.7) have a ferriprotoporphyrin IX prosthetic group located at the active site [10]. Both HRP and SBP are classified as Class III Classical Secretory plant peroxidases [7 & 11] and as such share common features (Table 1). Their catalytic mechanism involves a two-electron oxidation of the heme moiety to an intermediate known as Compound I. Successive one-electron reductions return the enzyme to its resting state via a second intermediate, Compound II [6].

Determining the *in vivo* function of peroxidases is complex owing to the numbers of isoenzymes in[E1] the family [12]. Interestingly, despite the several *in vitro* uses of HRP, its actual *in vivo* role has never been elucidated. Several suggestions have been proposed based on the known roles of other plant peroxidases. Peroxidases are usually found in the cell wall, vacuoles, transport organelles and the rough endoplasmic reticulum, and have noted roles in lignification, wound healing and auxin catabolism [4]. SBP has been isolated from that plant's seed coat and its presence prevents premature germination[E2] [13]. Plant peroxidases can use lignin and other plant compounds as reducing substrates. Indeed, SBP has been noted to polymerise coniferyl alcohol, indicating that it can efficiently catalyse reactions involving lignin precursors [14]. Therefore, it is possible that peroxidases are involved in the lignification or suberisation processes of plants [15].

Recombinant Peroxidase Expression.

Recombinant hemoprotein expression has been plagued by inclusion body formation, most notably in recombinant HRP expression. Several general methods have been suggested to reduce the formation of inclusion bodies, including reducing cultivation temperature and altering inducer composition and concentration [16]. Other, more peroxidase-specific, methodologies have been cited, including use of specific *E. coli* strains [17], inclusion of chaperones [18] and use of leader sequences [19]. Another major obstacle in the recombinant expression of hemoproteins is the limited availabilty of heme and iron within a bacterial cell. Bacterial cells each contain 10^5 to 10^6 iron ions, which are essential for many metabolic pathways [20]. Culture supplementation with the heme precursor δ-aminolevulinic acid has been suggested [21]; but Goodwin and co-workers have recently developed an elegant co-expression system incorporating a membrane heme receptor, allowing the use of exogenous heme as an iron source [22]. Jung and co-workers [5] noted an increased ratio of holoprotein to apoprotein with less-intense induction conditions, suggesting that slow recombinant hemoprotein production appears to allow easier incorporation of the available heme into the apoprotein [5]. Developments in HRP expression in both prokaryotic and eukaryotic systems are outlined in the supplementary online material.

HRP: Cloning and Expression.

The gene coding for the HRP protein was first synthesised by Smith and co-workers [23] based on the protein sequence published by Welinder [24]. This 940 basepairs synthetic gene was designed using commonly used codons in *E. coli* to minimise protein truncation owing to codon bias [25]. Recombinant HRP was over-expressed by induction but this led to the formation of misfolded apoprotein and the requirement to disrupt these aggregates, refold the protein correctly and add the heme centre. Disruption involved addition of EDTA to chelate ions, lysozyme and DNAse to reduce viscosity of the bacterial cell lysate, urea to solubilise the protein, and dithiothreitol (DTT) to break disulphide bonds. Refolding required slow exchange of disrupting reagents with folding facilitators such as calcium (for structural integrity), oxidized glutathione (to reform disulphide bridges) and hemin (to provide the prosthetic heme group) [23].

SBP: Cloning and Expression.

The gene encoding SBP was first derived from a soybean plant cDNA library screened with a peroxidase-specific probe [26]. The open reading frame for the SBP protein was cloned into the pET-34b (+) expression vector; however, induction of rSBP led to inclusion body formation and *E. coli* cell death. Active SBP was achieved by a refolding strategy similar to that of HRP. Unlike rHRP refolding, however, inclusion of oxidised glutathione in the refolding medium decreased active SBP recovery, possibly due to the formation of mispaired disulphides [26]. Henriksen and co-workers [15] also developed a recombinant SBP for crystallisation studies based on previous cDNA work, in which they successfully refolded SBP from inclusion bodies using a cocktail that included both oxidized and reduced glutathione [27].

There have been several other recent examples of recombinantly expressed plant peroxidases including Hushpulian and co-workers' [28] work on tobacco anionic peroxidase.

Mutagenesis of HRP.

Mutagenic studies on HRP began following successful cloning of a synthetic HRP gene [23]. Before the elucidation of the crystal structure [29], most mutations focused on ascertaining the key residues in the active site. Before 1997, researchers based their assumptions on crystal structures of closely related peroxidases, such as cytochrome *c* peroxidase, which suggested positions 38 through 42 as key catalytic residues. Mutations of Arg 38, Phe 41 and His 42 led to dramatic decreases in peroxidase catalytic activity. However, some Phe 41 mutants revealed an augmented thioether sulfoxidation activity owing to increased access channel area [30].

Asn 70 was also noted as an important residue in HRP catalysis: although it lies some distance from the heme iron atom, it is hydrogen-bonded to the side chain of the distal His 42. [31] Mutations in this region showed a decrease in HRP activity and a re-orientation of active site residues. Mutation of Phe 221 altered the heme iron of the resting enzyme to a quantum mixed-spin state [32]. Substitutions of Trp 117 revealed this residue's role in protein folding and electron transfer [33]. Mutations within the active site entrance revealed the key role of Phe 142 in binding aromatic molecules [34], whilst mutations within the proximal region (the area below the heme plane) disclosed the parts played by Phe 179 in aromatic molecule binding [35] and by His 170 in heme group anchorage [36]. Table 2 lists the various site directed HRP mutants. Recent examples of site directed mutants of HRP have been fewer [37], but Colas and de Montellano [38] identified the key role of carboxylate side chain amino acids in HRP protein-heme interactions.

To date, there have been few reports of HRP random mutagenesis. Arnold and co-workers directionally evolved HRP with the aim of increasing activity and stability. Development of a stabilised recombinant HRP is of great importance to increase and consolidate the range of peroxidase applications. Three rounds of random mutagenesis improved expression in yeast, yielding a nine-position HRP mutant displaying an 85-fold increase in activity over the parental molecule. One round of random mutagenesis was also carried out to improve stability, resulting in three mutants more stable than the parent in relation to temperature and H_2O_2 tolerance (supplementary online material) [8, 19, 39, & 40]. Recent publications suggest that targeted, "semi-rational", evolution of enzymes might yield superior mutants in less time [41 and references within]. Mendive and co-workers developed a rapid screening methodology for

150 random mutants displaying increased peroxidase activity, using DAB as substrate.
151 Whereas Arnold and co-workers expressed HRP in *E. coli*, *S. cerevisiae*. and *P.*
152 *pastoris*, Mendive and co-workers utilised a baculovirus expression system [42].
153 No mutagenesis studies on recombinant SBP have been reported to date, and the
154 authors believe that this requires urgent attention. For example, mutagenesis could
155 reveal which residues in SBP endow it with its enhanced stability *vis a vis* HRP [15].
156 Also, similar to HRP, SBP could be subjected to focussed directed evolution to
157 increase the number of substrates accepted.
158
159 **HRP in Biosensors**
160 One of the most common uses of HRP is in biosensors. A biosensor is *"an analytical*
161 *device that brings together an immobilised biological sensing material* [often HRP]
162 *and a transducer to produce an electronic signal that is proportional to the*
163 *concentration of the target chemical substance"* [43]. Although reports of SBP-based
164 biosensors are emerging, HRP biosensor research dominates and has continued to
165 develop through many forms, from the traditional voltammetric- and amperometric-
166 based methods of detection, to nano-sized devices. Real time quantification of
167 hydrogen peroxide continues to be one of the main reasons for sensor development
168 [44], although other diverse applications include the detection of glucose [45], ethanol
169 [46] and tumour markers *in vivo* [47]. Enzyme-based biosensors require rapid and
170 uniform transfer of electrons generated at the enzyme active site to the transducer.
171 The distance between the active site and the transducer can hinder electron transfer;
172 often, posttranslational modifications such as glycosylation increase this distance.
173 Recombinant HRP, devoid of glycans, offers a shorter path for electron transfer and
174 numerous reports of rHRP-based sensors have appeared [48 and references
175 within].While a detailed review of HRP-based sensors is beyond the scope of this
176 article, we outline some of the emerging trends in HRP-based biosensor development.
177 Biosensors, including immunosensnors [49] and electrosensors [50], incorporating
178 organic solvents have developed as an expanding area of peroxidase research,
179 primarily due to insolubility of many analytes in aqueous solutions. Recently, Konash
180 and Magner [51] developed a HRP-immobilised, mediated H_2O_2 sensor, which
181 demonstrated good catalytic activity in 2-butanone and ethyl acetate. Organic solvent
182 compatible bi-enzyme peroxidase sensors have also been cited in the literature [52].

Size reduction remains a pivotal area in sensor research. The use of nanoparticles offers increased surface area for enzyme immobilisation, whilst simultaneously reducing apparatus size [53]. Currently, HRP-based nano-sensors are at the forefront of biosensor research [54 and Table 3].

SBP in biosensors.

Although HRP is the classical heme peroxidase, there is increasing interest in SBP. SBP has advantages over HRP in terms of catalytic activity and stability [55]; these can be exploited in biosensors. Also, unlike HRP, SBP is active in the pH range 2-6, offering a greater range of potential biosensing applications [56]. The first SBP biosensor was reported in 1995 by Vreeke and co-workers [57] as a thermostable wired enzyme electrode using an osmium-based mediator, which aids electron transfer from the active site to the electrode, modified by an epoxide. Kenausis and co-workers [58] also used a poly(4-vinylpyridine) polymer, complexing the pyridine nitrogens to the osmium-based mediator, quaternised with 2-bromoethylamine. Until the use of SBP by Heller and Vreeke [59], no peroxidase-based sensors could be used at 37°C for an extended period (~100 hours). Monitoring of glucose "*in vivo*" for diabetes mellitus, and of lactate for confirmation of hypoxia and ischemia, are vital in patient management; use of thermostable SBP immobilised into a mediator enables this [59].

The typical electrochemical reactions of a H_2O_2 sensing, peroxidase based, osmium-mediated electrode system are outlined in Box 1 [60]. In addition to conveying electrons, generated at the active site, to the electrode surface, the pyridin-N-ethylene groups of the osmium-containing mediator also increase the hydration and provide primary amines for cross-linking [58].

Table 3 summarizes reports to date of SBP-based biosensors, most of which use a mediator. H_2O_2 can be electrochemically detected by its electrooxidation on a Pt (or other inert Pt group metal) electrode [59]. Utilisation of an enzyme/mediator system produces a multi-step mediated electron-transfer (MET) process, in which each step transports the electron a small distance [61]. However, use of a mediator with a redox enzyme can create its own problems: the mediator-enzyme film can, depending upon its thickness, obstruct substrate diffusion [62]. Chemical modification of redox enzymes with an electron relay moiety can increase multi-step MET by decreasing the

electron transfer distance. This leads to improved electrical communication between the enzyme's redox centre and its external environment [61].

Peroxidase based Micro- and Nano-Systems

An emerging field in peroxidase research is the use of micro- and nano- sized structures in diagnostic and biosensing fields. Enzyme microreactors, for example, permit chemical and biochemical reactions to be carried out on a microscale. HRP has previously been used as a model microreactor system to monitor HRP catalytic activity and as a diagnostic tool; however, the development of this field will be determined by the ability to immobilise peroxidases onto suitable support structures [63]. Miniaturisation of enzymatic processes is also evident in the diagnostics sector, in "Lab on a Chip" and "Point of Care Testing"(POCT) research. The classical application of HRP in POCT is Clinistix^{TM}; however, recently Cho and co-workers [64] have applied HRP to a portable sequential cross flow immunoanalytical device. Cross flow imumunoassays are capable of introducing the antigen-antibody complex to the flow cell whilst sequentially extracting the catalytic signal, thus simplifying the complex traditional ELISA procedure. This device demonstrates many advantages over ELISA-based analytical methodologies including a rapid, sensitive and inexpensive in-situ diagnosis for the presence of Hepatitis B surface antigen in a sample. Miniaturisation of peroxidase-based devices also features in other research fields, including the use of micro-crystals for oxidoreductase-based catalysis in organic solvent [65], nano-immobilisation techniques for peroxidase based wastewater treatment [66], and a more widespread use of nano-structures for peroxidase based sensors [67 & 68]. Recently Yan and co-workers [69] described a microcantilever based biosensor, modified with HRP, for H_2O_2 detection. In this system the enzyme-functionalised microcantilievers deflected irreversibly in response to H_2O_2 concentrations in the nanomolar range. The deflection was caused by conformational change within the HRP molecule as it underwent oxidation by the H_2O_2; the irreversibility was due to the absence of a second, reducing substrate required for reversion of HRP to the resting state. This technique may also provide a sensitive tool for investigating protein structural change. HRP has also been utilised as the functional component of self-assembled three-dimensional (3-D) nano-structures. Rauf and co-workers [70] utilised self-assembly layer-by-layer technology to

construct controlled 3-D catalytically active nano-structures. This method of peroxidase immobilisation allows for increased catalytic activity per unit area, and will aid in the miniaturisation of biosensors, biochips and immobilised biocatalysts. With increasing sophistication of support structures on the micro- and nano-scale, miniaturisation of peroxidase-based devices will continue to develop in the future, particularly in the fields of POCT and biosensing.

Peroxidase based Biocatalysis.

A major shortcoming of all heme-dependent peroxidases is their low operational stability, owing to oxidative degeneration of the heme group [71]. Operational stability of SBP can be increased by generating H_2O_2 *in situ* from glucose and O_2. When co-immobilized with glucose oxidase in a polyurethane foam, SBP could act as a peroxygenase to convert thioanisole to its sulphoxide (i.e. by inserting an oxygen atom). Here, SBP uses the H_2O_2 generated *in situ* by glucose oxidase; it formed no sulphoxide with free, exogenous H_2O_2 [71]. Such an arrangement avoids excessive initial H_2O_2 concentrations and, hence, formation of compound III (a reversible dead-end complex formed from compound II in the presence of an excess H_2O_2, which slowly reverts to native enzyme; [10]) and/or irreversibly inhibited SBP. HRP has been subjected to intense experimentation, including a large body of work focussing on site directed mutants (see above and Table 2). Now that the key catalytic residues are known, researchers have begun to use site directed mutagenesis to alter the function of the HRP molecule [48], e.g. by construction of an improved luminol binding site [72]. Directed evolution stabilised HRP against thermal denaturation [39] and has endowed it with increased H_2O_2 tolerance and increased catalytic activity [40]. Further targeted directed evolution, focussing on the substrate access channel and binding pocket, could allow HRP to accept an increased variety of substrates [73], and promote further diversification of HRP applications in organic synthesis [41]. Peroxidase catalysis in organic solvents, both aqueous and anhydrous, offers a huge advantage to organic chemists, as difficult asymmetric oxidation and reduction reactions can take place rapidly and with high specificity [74]. The major problems of substrate solubility and unwanted side reactions promoted by water are also overcome during organic solvent based synthesis. Additionally, in some anhydrous solvents peroxidase (HRP and SBP) activity was actually increased [75], with additional

methods, such as salt activation [76] and excipient aided lyophilisation [77] also resulting in increased peroxidase activity. However, in some low water solvents, peroxidases can lose their confirmational structure [78]; although recent advances in peroxidase encapsulation in amphiphilic matrices [79], the use of reverse micelles [80] and oil emulsions [81] allow for peroxidase activity in an extended range of anhydrous solvents. Reactions carried out in these solvents include hydroxylations, N-demethylations and sulphoxidations [2]. An interesting recent environmental application of SBP polymerisation in organic solvents is the production of polycardanol in as a potential anti-biofouling agent [82], whilst recently it has been noted that HRP requires a mediator to catalyse the same substrate [83]. The interested reader is directed to a recent review of this area [74].

Peroxidase based Bioremediation.

Highly expressed, stabilized, recombinant HRP [39, 40] could be very useful for wastewater cleanup, provided the recombinant enzyme can be produced cost effectively and in sufficient quantities. Phenol cleanup by HRP has been widely reviewed [1] but several drawbacks limit its widespread application, including intolerance of high concentrations of the primary substrate H_2O_2 [84], low enzymatic reusability, and financial costs. Plant heme peroxidase expression in *E.coli* can be frustrating, but advances in peroxidase expression, without formation of inclusion bodies, may pave the way for increased production of recombinant peroxidases (See supplementary online material). SBP has proven itself a worthy alternative peroxidase: it displays superior stability and activity characteristics to the classical HRP. However, research into this enzyme lags far behind HRP. SBP can effectively cleanup phenolic wastewater, yet recent publications cite HRP as being a superior, albeit less stable, catalyst than SBP for phenol cleanup [85]. Development of an enhanced catalytic SBP mutant would provide a powerful tool for wastewater treatment. Bódalo and co-workers [85] noted that the choice of peroxidase for wastewater treatment also depends on effluent characteristics, operational requirements and costs. SBP has been shown to outperform HRP in oxidative dye removal [86]. SBP, possibly owing to its larger substrate access channel, and, hence, greater exposure of the catalytically important delta heme edge, can accept more substrates than HRP [87].

HRP: an Unlikely Therapeutic

An exciting application of HRP is as a novel cancer treatment via gene-directed enzyme/prodrug therapy. It has been noted that the non-toxic HRP substrate, indole-3-acetic acid (IAA), forms a radical that is toxic to cancer cells upon HRP catalysis. The exact mechanism of toxicity remains unclear: it is believed to involve lipid peroxidation induced by the free radical formation [88]; however, in human melanoma cells, death receptor-mediated and mitochondrial apoptotic pathways are known to be involved also [89]. Leaving aside the actual reason(s) for toxicity, inactive IAA can be introduced to the body; and then becomes activated by HRP at the region of interest. Localisation of the HRP molecule is achieved via its conjugation to an antibody specific to an extracellular tumour antigen. This approach has become the focus of much research and numerous clinical trials, due to several attractive features: these include the robust nature of the activating enzyme and the low toxicity of the prodrug [90]. HRP has been shown to activate other pro-drugs including ellipticine [91] and halogenated IAA derivates [92]. The interested reader is directed to the excellent recent review of Dachs et al. [93].

Conclusion and Future Directions:

As outlined, peroxidases are widely studied and very important enzymes, with many applications in the life sciences and beyond. They remain pivotal to advancing biotechnology, and as such, we present two clearly distinct, yet similar members of this classical family. Continued research into the "traditional" HRP, has been accompanied by the slow, but steady progression of SBP. Crude SBP, isolated from waste soybean hulls, offers a cheap bulk peroxidase catalyst for applications such as wastewater treatment and organic synthesis, whilst the more costly peroxidase alternatives (plant HRP and recombinant HRP and SBP) will prove themselves in higher value niches, such as diagnostics and therapeutics. With improved understanding of the catalytic and stability characteristics, the detection of new substrates and the increasing use of implantable devices in the medical field, SBP will rapidly develop its own high value market niche. As noted for HRP, use of recombinant SBP would also benefit the biosensor field by permitting more rapid electron transfer, due to the lack of protein glycosylation. Improvement of these two peroxidases, by rational mutation and "focussed" directed evolution, will widen their applications and expand their roles as key biotechnological tools in the future.

Acknowledgements

We thank the following for financial support: the Irish Research Council for Science, Engineering and Technology (Embark Initiative postgraduate scholarship, BJR) the National Centre for Sensors Research and School of Biotechnology, Dublin City University (NC) and the Research Advisory Panel of Dublin City University (Albert College Award, C.Ó'F.) (The National Centre for Sensors Research was established under the Programme for Research in Third Level Institutions of the Higher Education Authority)

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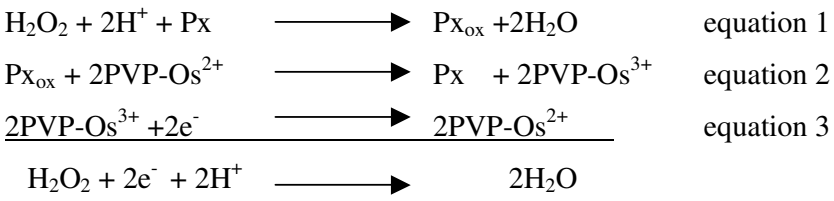

Box[E4] **1:** Typical biosensor based on a mediated peroxidase (Px) reaction scheme. Upon the addition of H_2O_2, Px catalyses the reaction forming water; in the process, Px goes through its catalytic cycle. This causes the mediator to go from its resting state of Os^{2+} to Os^{3+}. The osmium species is seen as a one-electron donor, used as the mediator to assist in electron transfer from the active site of Px to the electrode surface. Px_{ox} corresponds to the catalytic intermediate Compound I, formed by a two-electron oxidation. The individual one-electron reduction steps that take place on the enzyme itself (formation of catalytic intermediate Compound II and reversion to resting enzyme, Px) have been omitted from equation 2 for the sake of clarity. Adapted from *Analytica Chimica Acta.* **418**, Li W. et al. (2000). Fabrication of multilayer films containing horseradish peroxidase and polycation-bearing Os complex by means of electrostatic layer-by-layer adsorption and its applications as a hydrogen peroxide sensor. 225-232. [60] Copyright 2000, with permission from Elsevier.

Table 1: Comparison of the biochemical and structural properties of HRP and SBP. Key references are noted.

	Horseradish Peroxidase	**Soybean Peroxidase**
Species Name	*Armoracia rusticana* [23]	*Glycine max*[14]
Number of Amino Acids	306 [23]	306 [94]
Enzyme Classification	1.11.1.7 [29]	1.11.1.7 [15]
PDB Accession Number	1ATJ	1FHF
Molecular Weight	44,100 Da [6]	40,660 Da [15]
Carbohydrate	7,580 Da [6]	7,400 Da [15]
Heme Group	550 Da [6]	550 Da [15]
Calcium Ions	80 Da [6]	80 Da [15]
Glycosylation Sites	Asn: 13, 57, 158, 186, 198, 214, 255, 268, 316 [95]	Asn: 185, 197, 211, 216 [96]
pI	9.0 [24]	4.1 [14]
pH Activity Range	4-8 [97]	2-10 [15]
Secondary Structure	13 α-helices 3 β-sheets [29]	13 α-helices 2 β-sheets [15]
Disulphide Bridges	11-91, 44-49, 97-301, 177-209 [29]	11-91, 44-49, 97-299, 176-208 [15]
In vivo localisation	Roots, cell wall, vacuoles [6]	Hourglass cells, seed coat [14]

Table 2: Summary of HRP site directed mutants[E5]

Mutations		Conclusions
Active Site. [98, 99, & 100]		
R38A	F41T	Arg38 and His42 are key residues in enzyme catalysis.
R38E	F41V	Arg38 and His42mutations decrease Compound I formation.
R38G	F41W	Mutants affect reactivity towards reducing substrates. NB- Morimoto ref.
R38H	H42A	Arg 38 and His42 are acid base partners. Arg 38 stabilises His 42.
R38K	H42E	Arg38 and His42 operate in concert to distally bind BHA.
R38L	H42L	Arg38 and His42 are dioxygen-heterolytic cleavers
R38S	H42Q	Arg38 plays a role in H_2O_2 binding and cleavage.
F41A	H42R	Hydrophobicity of active site region is critical in enzymatic catalysis.
F41H	H42V	Space creating active site mutants alter substrate specificity.
F41L		Phe41 acts as hydrophobic barrier between Arg38 and His42.
Active Site Entrance. [101]		
S35K	F143E	Phe 142 plays a critical role in aromatic substrate binding.
F142A	F176E	Charged residues are important at the active site entrance.
F143A		Luminol binds to active site via electrostatic interactions in binding area.
Proximal Region. [102 & 36]		
F179A	H170A	Phe 179 is crucial for aromatic substrate binding.
F179H	F172T	His 170 tethers heme moiety in position. Prevents distal His coordination.
F179S		His 170 maintains heme moiety in penta-coordinated state.
Asparagine 70. [103]		
N70V	N70D	Asn70 hydrogen bonds to His42, mutations alter distal heme orientation. Mutant protein displays increased redox potential.
Tryptophan 117. [33 & 104]		
W117F		Mutants displayed increased acid stability. Trp 117 is important in internal electron transfer and protein unfolding.
Threonine 171. [37]		
T171S		Proximal structural alteration, affects proximal pocket hydrogen bonding.
Phenylalanine 221. [105 & 32]		
F221M	F221W	Mutants display decreased stability in alkaline conditions. Trp introduction destabilises protein, due to unfavourable surroundings.

Footnote:
Mutants are grouped into active site, active site entrance, proximal heme region, aspargine 70, tryptophan 117 and Phe 221 mutations. Mutants were expressed in a variety of hosts including *E.coli*, *Trichoplusia ni*, and *Spodoptera fruigiperda* cell lines. Key references only are noted for each collection of mutants; further references may be found within these.

Electrode type	Size of Electrode	Enzyme	Method of immobilisation	Mediator	Analyte measured	Method of measurement	Reference
Soybean Peroxidase							
Glassy carbon	4mm	SBP	Adsorption	PVA/PVP	H_2O_2	Amperometry	[106]
Glassy carbon	4mm	SBP	Entrapment	Sol-gel	H_2O_2	Amperometry	[56]
Glassy carbon	3mm	SBP	Entrapment	Pos-EA, PEGDGE	H_2O_2	Amperometry	[59]
Pyrolitic graphite (Rotating disk)	0.2mm	SBP	Entrapment	DMPC	H_2O_2	Amperometry /CV	[107]
Glassy carbon	3mm	GOx/SBP	Entrapment	Pos-EA	Glucose	Amperometry	[58]
Horseradish Peroxidase							
Gold	-	HRP	Adsorption	DNA	H_2O_2	Amperometry	[108]
Glassy Carbon	3mm	HRP	Adsorption	HQ	H_2O_2	Amperometry	[53]
Carbon Ceramic	6mm	HRP	Covalent	Nano Au	H_2O_2	Amperometry	[109]
Carbon Paste	7mm	HRP	Entrapment	GP	Biogenic Amine	Amperometry	[110]
Titanium	0.8mm	HRP	Adsorption	Thionine	H_2O_2	Amperometry	[111]
Platinum	1mm	HRP	Entrapment	Co(bpy)	H_2O_2	Amperometry	[112]
Carbon Fibre	3mm	Cho/HRP	Adsorption	Os- PVP	Choline	Amperometry	[113]
Platinum	7mm	Cho/HRP	Adsorption	TTCA	Choline	Amperometry	114
Recombinant HRP							
Gold	0.15mm	rHRP	Adsorption	Direct	H_2O_2	Amperometry	[115]
Graphite Rotating disk	3mm	rHRP	Adsorption	Direct	H_2O_2	Amperometry	[116]
Gold	0.3mm	CytC / rHRP	Adsoprtion	Direct	Superoxide Anion radical	Amperometry	[117]
Gold	0.15mm	L-LO / rHRP	Adsorption	Direct	L-lysine	Amperometry	[118]

Table 3: Some SBP, HRP and rHRP biosensors, and their properties, from the literature.