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Review

Potential applications of peroxidases

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ABSTRACT

Peroxidases are oxidoreductases produced by a number of microorganisms and plants. Peroxidases catalyse a variety of reactions in the presence of peroxides such as hydrogen peroxide. Oxidative polymerisation of phenols and aromatic amines, conducted by horseradish peroxidase (HRP) in water and water-miscible organic solvents, may lead to new types of aromatic polymers. Peroxidase has a potential for soil detoxification, while HRP, as well as soybean and turnip peroxidases have been applied to the bioremediation of waste waters contaminated with phenols, cresols and chlorinated phenols. Lignin peroxidase (LiP) and manganese peroxidase (MnP) may be successfully used for biopulping and biobleaching in the paper industry and can produce oxidative breakdown of synthetic azo dyes. Peroxidase-based biosensors have found use in analytical systems for determination of hydrogen peroxide and organic hydroperoxide, while, co-immobilized with hydrogen peroxide-producing enzyme, they can be used for determination of glucose, alcohols, glutamate and choline. Peroxidase has also been used for practical analytical applications in diagnostic kits, such as quantification of uric acid, glucose, cholesterol, lactose, etc. Enzyme-linked immunosorbent assay (ELISA) tests, in which peroxidase enzyme is the most common enzyme used for labelling an antibody, are a simple and reliable way of detecting toxins, pathogens, cancer risk in bladder and prostate, and many other analytes.

Contents

1. Introduction

Peroxidases (EC 1.11.1.7) are haem proteins and contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. They have a molecular weight ranging from 30,000 to 150,000 Da. These are a group of oxidoreductases that catalyse the reduction of peroxides, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds. The term peroxidase represents a group of specific enzymes, such as NADH peroxidase (EC 1.11.1.1), glutathione peroxidase (EC 1.11.1.9), and iodide peroxidase (EC 1.11.1.8), as well as a

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group of nonspecific enzymes that are simply known as peroxidases.

Peroxidase activity has been identified in plants, microorganisms and animals, where peroxidases play important roles. In plants they participate in the lignification process (Wakamatsu & Takahama, 1993) and in the mechanism of defence in physically damaged or infected tissues (Biles & Martyn, 1993).

Peroxidases catalyse the oxidation of a wide variety of substrates, using H_2O_2 or other peroxides. The peroxidase catalytic cycle involves distinct intermediate enzyme forms (Wong, 1995; Chung, Kirkovsky, Kirkovsky, & Purcell, 1997; Mantha, Biswas, Taylor, & Bewtra, 2002). In the initial step the native ferric enzyme is oxidised by hydrogen peroxide to form an unstable intermediate called compound I (CoI), which has a haem structure of Fe IV = O-porphyrin π-cation radical, with consequent reduction of peroxide to water. Then CoI oxidises electron donor substrate to give compound II (CoII), releasing a free radical. CoII is further reduced by a second substrate molecule, regenerating the iron (III) state and producing another free radical.

Peroxidases are widely used in clinical biochemistry and enzyme immunoassays (Vamos-Vigyazo, 1981; Lin, Chen, & Zhang, 1996). Some novel applications of peroxidases suggested include treatment of waste water containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from materials such as foodstuffs and industrial wastes (Agostini et al., 2002). Horseradish root tubers are commonly employed as a commercial source for peroxidase production (Kim & Yoo, 1996; Yamada et al., 1987; Saitou, Kamada, & Harada, 1991). However other cultivated species may provide peroxidases exhibiting similar or better properties, especially recombinant species (Egorov, Reshetnikova, Fechina, & Gazaryan, 1995).

Reduction of peroxides at the expense of electron-donating substrates makes peroxidases useful in a number of industrial and analytical applications. Peroxidase is probably the most well-suited enzyme for the preparation of enzyme-conjugated antibodies, which are used in enzyme-liked immunosorbent assay (ELISA) tests, due to its ability to yield chromogenic products at low concentrations (Krell, 1991). Peroxidase coupled with other enzymes in polyenzymatic systems producing hydrogen peroxide is also used in the determination of many compounds, such as glucose in blood. Because of the oxidative nature of peroxidase there are several areas where it could replace current chemical oxidant techniques. A number of processes using peroxidases have been described in the following sections.

2. Applications

2.1. Removal of phenolic contaminants and related compounds

Aromatic compounds, including phenols and aromatic amines, constitute one of the major classes of pollutants. They are found in the waste waters of a wide variety of industries, including coal conversion, petroleum refining, resins and plastics, wood preservation, metal coating, dyes and other chemicals, textiles, mining and dressing, and pulp and paper (Nicell, Al-Kassim, Bewtra, & Taylor, 1993). Most aromatic compounds are toxic and must be removed from waste waters before they are discharged into the environment. Enzymatic treatment has been proposed by many researchers as a potential alternative to convential methods. Firstly, enzymes are highly selective and can effectively treat even dilute wastes (Aitken, 1993). Secondly, they are less likely to be inhibited by substances which may be toxic to living organisms and their cost could eventually be less than that of other methods, if commercially available enzymes are produced in bulk quantities. Moreover, enzymes operate over a broad aromatic concentration range and require low retention times with respect to other treatment methods (Siddique, St. Pierre, Biswas, Bewtra, & Taylor, 1993).

Peroxidases that have been used for treatment of aqueous aromatic contaminants and decolorization of dyes (Selvam, Swaminathan, & Chae, 2003) include horseradish peroxidase (HRP), lignin peroxidase (LiP) and a number of other peroxidases from different sources.

Most applications have focussed on the treatment of phenolic contaminants in the presence of H_2O_2 (Klibanov, Alberti, Morris, & Felshin, 1980; Klibanov, Tu, & Scott 1982; Klibanov, 1983; Nicell, Bewtra, Biswas, & Taylor, 1993; Buchnan & Nicell, 1997; Varon et al., 1998; Mantha et al., 2002; Filizola & Loew, 2000; Kim, Lee, Cho, Shim, & Moon, 2005; Mohan, Prasad, Rao, & Sarma, 2005; Cheng, Yu, & Zuo, 2006). The use of HRP for the treatment of contaminants including anilines, hydroxyquinoline and arylamine carcinogens, such as benzidines and naphthylamines, has also been demonstrated in the laboratory (Klibanov, Alberti, Morris, & Felshin, 1980; Klibanov & Morris, 1981). In addition, HRP has the ability to co-precipitate certain difficult-to-remove contaminants, including non-substrate of HRP, along with the more easily removable compounds, by inducing the formation of mixed polymers that behave similarly to the polymeric products of easily removable products (Klibanov et al., 1980). This phenomenon has an important practical implication for waste waters, which usually contain many different pollutants. An extension of this principle to hazardous wastes was demonstrated when it was observed that polychlorinated biphenyls could be removed from solution through coprecipitation with phenols (Klibanov, 1983).However this particular application of HRP does not appear to have been pursued in any subsequent research.

Considerable efforts have been aimed at optimising the HRP-catalysed removal of phenols from aqueous solutions. Improvements in the useful life of the enzyme and thereby a reduction in treatment cost, have been accomplished through selection of an appropriate reactor configuration (Nicell, Bewtra, Biswas, St. Pierre, & Taylor, 1993), enzyme immobilization (Siddique et al., 1993; Bodzek, Bohdziewicz, & Kowalska, 1994), the use of additives such as sodium borate, gelatin and poly (ethylene glycol) to protect the enzyme from entrapment in the precipitating polymers (Nakamoto & Machida, 1992; Wu, Taylor, Bewtra, & Biswas, 1993; Nicell, Saadi, & Buchanan, 1995), and the addition of adsorbents, such as talc, which protect the enzyme from inhibition by oxidation products (Arseguel & Baboulene, 1994).

Lignin peroxidase (LiP, EC unknown) was first reported in 1983 (Aitken, Massey, Chen, & Heck, 1994). It is part of the extracellular enzyme system of the white-rot fungus *Phanerochaete chrysosporium* (Aitken & Irvine, 1989; Venkatadri & Irvine, 1993). LiP was shown to mineralise a variety of recalcitrant aromatic compounds and to oxidise a number of polycyclic aromatic and phenolic compounds (Aitken, Venkatadri, & Irvine, 1989; Aitken et al., 1994). The role of LiP's in lignin depolymerisation has also been confirmed (Aitken & Irvine, 1989; Aitken et al., 1989; Hammel, 1989; Cornwell, Tinland-Butez, Tardone, Cabasso, & Hammel, 1990). Its mechanism is very similar to that of HRP (Aitken et al., 1989).

The stability of LiP, which influences greatly the economic and technical feasibility of the enzyme's application in waste treatment, was studied by Aitken and Irvine (1989). They reported that LiP was readily inactivated at low pH. The enzyme's stability was improved by increasing the pH, increasing enzyme concentration or incubating the enzyme in the presence of its substrate veratryl alcohol. It was also found that optimised conditions for phenolics removal included high enzyme concentration, a pH above 4 and controlled addition of H_2O_2 (Aitken & Irvine 1989). Cornwell et al. (1990) reported an immobilization of LiP on porous ceramic supports did not adversely affect its stability and showed a good potential for degradation of environmentally-persistent aromatics.

Venkatadri and Irvine (1993) developed a silicon membrane reactor that could be used for hazardous waste treatment and LiP production.

Manganese peroxidase (MnP; EC unknown), produced by *P. chrysosporium* has also been observed to catalyse the oxidation of several monoaromatic phenols, and aromatic dyes, but these reactions depend on the presence of both divalent manganese and certain types of buffers (Aitken & Irvine, 1989). In fact, MnP catalyzes the oxidation of Mn(II) to Mn(III) in the presence of Mn(III)-stabilizing ligands. The resulting Mn(III) complexes can then carry out the oxidation of organic substrates (Aitken et al., 1994).

The use of a microbial peroxidase from *Coprinus macrorhizus* as an alternative to HRP for the removal of aromatic compounds from waste water was recently investigated (Al-Kassim, Taylor, Nicell, Bewtra, & Biswas, 1994). Its performance was found to compare favourably to HRP, in that it could catalyse the same reactions, although it was noticeably more easily inactivated.

The majority of reports on detoxification of waste water contaminated with phenols, cresols and chlorinated phenols have used HRP. Recently, however, peroxidase from other sources such as soybean (Caza, Bewtra, Biswas, & Taylor, 1999; Kinsley & Nicell, 2000; Kennedy, Alemany, & Warith, 2002) and turnip (Duarte-Vazquez, Ortega-Tovar, Garcia-Almendarez, & Regalado, 2003), have been suggested as alternatives to horseradish. The peroxidase treatment process is still in the experimental stage. Characteristics of this process, such as the effect of pH, substrate concentration, reaction time and effects of some additives have been studied, to optimise reaction conditions.

Optimum pH for the removal of aqueous phenols has been reported for many peroxidases (Klibanov, 1983; Dec & Bollag, 1990; Bewtra, Biswas, Henderson, & Nicell, 1995; Wright & Nicell, 1999; Duarte-Vazquez et al., 2003). Using HRP, Klibanov and Morris (1981) found that phenol in coal conversion waste water could be precipitated in a wide range from pH 3 to 12 with an optimum at pH 9, which was close to the waste water pH of 8.5. Bewtra et al. (1995) reported that the optimum pH for removal of 2,4-dichlorophenol was 6.5 with a working pH range of 3–10. Kennedy et al. (2002), obtained an optimum pH of 8.2 for 2,4-dichlorophenol removal, using soybean peroxidase. Even at pH 2.5, 1 unit/ml of soybean peroxidase removed nearly 90% of 2,4-dichlorophenol, indicating that soybean peroxidase can function in more extreme acidic conditions than HRP.

Duarte-Vazquez et al. (2003), studied the efficiency of turnip roots peroxidase extract to remove several different phenolic compounds: phenol, 2-chlorophenol, 3-chlorophenol, *o*-cresol, *m*-cresol, 2,4-dichlorophenol and bisphenol. At least 85% of the phenolic compounds studied were removed over the pH range 4–8 with an optimum removal at pH 5–7. Wright and Nicell (1999), and Caza et al. (1999) found similar results but using soybean peroxidase. A decrease in removal efficiency close to pH 10 has been attributed to the phenol conjugated base, since the pK_a of phenol at 25 °C is 10 (Budavari, 1989), and the basic form does not permit phenolic compounds to act as hydrogen donors.

The removal efficiency is also affected by the hydrogen peroxide concentration. Caza et al. (1999) obtained a maximum removal efficiency of phenol and bisphenol A from synthetic waste water treated with soybean peroxidase, using an $[H_2O_2]/[\text{phenolic}]$ molar ratio of 1.2, similar to that reported by Duarte-Vazquez et al. (2003) for turnip peroxidase extracts (1.6). Flock, Bassi, and Gijzen (1999), using soybean peroxidase, found that higher H_2O_2 concentrations produced a continuous decrease in removal efficiency. Duarte-Vazquez, Garcia-Almendarez, Regalado, and Whitaker (2001), reported that H_2O_2 concentrations > 1.2 mM acted as peroxidase inhibitor by irreversible oxidation of the enzyme ferrihaem group, essential for activity.

During the removal process, a decrease in peroxidase activity has been observed. It may occur through stabilization of the phenoxy radical by a π-conjugation with the cation radical (CoI). Inactivation could also be due to the adsorption of the enzyme molecules on the end-product polymer, limiting the substrate diffusion to the active site (Nakamoto & Machida, 1992). Additives, such as PEG or gelatin, have been studied to improve removal efficiency by protecting the enzyme. The additives might exert their protection by interacting with the reaction products (Kinsley & Nicell, 2000).

Cooper and Nicell (1996) showed that PEG addition increased removal efficiency of a foundry waste water using HRP. Addition of PEG-3350 or PEG-8000 to soybean peroxidase, increased the removal efficiency of 2,4-dichlorophenol by a factor of 10 or 50, respectively (Kennedy et al., 2002). At an HRP dose of 0.04 U/ml, the catalyst lifetime improved from 5.2×10^4 turnovers without PEG to 2.1×10^5 turnovers with 32 mg/l PEG, an improvement of 400% (Nicell et al., 1995). Crude HRP extract achieved better phenol removal than pure HRP at the same enzyme concentration (Cooper & Nicell, 1996); the enzyme requirement for almost 100% removal using crude HRP decreased to one half of that required using pure enzyme. Masuda, Sakurai, and Sakakibara (2001), using *Coprinus cinereus* peroxidase (CIP), found that enzyme impurities improved removal efficiency. Crude CIP extract was more efficient in removing phenol than purified CIP; the requirement ratio of crude CIP to purified CIP was about one-sixth.

High-molecular-weight PEG (Nakamoto & Machida, 1992; Wu et al., 1993; Wu, Taylor, Biswas, & Bewtra, 1997), gelatin, milk casein, bovine serum albumin and polyvinyl alcohol (Nakamoto & Machida, 1992) have been effective in suppressing the inactivation of HRP. Some cationic polymer coagulants also reduced inactivation of HRP (Tatsumi, Ichikawa, & Wada, 1994). More acidic and higher molecular weight proteins were more effective in suppressing the inactivation of CIP (Masuda et al., 2001).

A batch fermentation of the white-rot fungus *P. chrysosporium* ATCC 24725 was used for phenols removal, when added to the culture after LiP synthesis (96 h). Complete phenol removal (5.3 mM) took 30 h, whereas only 62% of added 4-chlorophenol (1.6 mM) was removed after 10 days (Manimekalai & Swaminathan, 2000). The effect of pH, enzyme and co-catalyst concentrations, were tested on the removal of phenolic compounds by a purified LiP from the same fungal strain. The removal efficiency of *o*-cresol was 37–69% at pH 4.75 while by continuous addition of hydrogen peroxide (130 nmol ml^{-1} h^{-1}) a 98% removal was achieved (Aitken et al., 1989).

The potential of peroxidase for soil and water detoxification constitutes a possible basis for the development of bioremediation technologies. For instance, the herbicide atrazine was biotransformed to the less toxic compounds deethylatrazine and hydroxyatrazine (*N*-alkylated and hydroxylated metabolites, respectively) by *P. chrysosporium* in a liquid culture. The active production of LiP and MnP coincided with the degradation of atrazine (Mougin et al., 1994). A soil sample containing a triazine (hexahydro-1,3,5-trinitro-1,3,5 triazine) was biodegraded by a bacterial strain. The resulting ring cleavage product (4-nitro-2,4-diazabutanal) was used as a substrate by *P. chrysosporium*, leading to its removal with the release of nitrous oxide. Further experiments showed that MnP was responsible for this biodegradation (Fournier et al., 2004).

2.2. Decolorization of synthetic dyes

Dyes used for paper printing, colour photography, textile dyeing and as an additive in petroleum products have a synthetic origin and complex aromatic molecular structure. About 10–15% of the synthetic dyes produced are discharged into industrial effluents (Spadaro, Gold, & Renganathan, 1992), causing environmental

problems. Some dyes are recalcitrant and toxic substances; they are resistant to biological degradation, not being easily degraded in waste water treatment plants (Liu, Wang, & Ji, 2006). Many of the synthetic dyes are not amenable to conventional biological waste water treatment because of their structure and the treatment of effluents containing dyes usually involves physicochemical methods. Although currently available methods, such as chemical oxidation, reverse osmosis and adsorption, are highly efficient, they suffer some disadvantages. The limitations include high cost, limited applicability and high energy input, and usually these treatments may result in the production of toxic by-products. Therefore, interest is now growing in the use of microbial degradation of dyes, since this process is less expensive and represents a less intrusive alternative (An et al., 2002; De Souza, Forgiarini, & De Souza, 2007).

Some authors have shown that oxidative destruction of coloured compounds is significantly stimulated by oxidative enzymes (Shin & Kim, 1998; Bhunia, Durani, & Wangikar, 2002; Yang et al., 2003), and may be of practical interest for decolorization of synthetic dyes. Enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP), both associated with lignin degradation, are involved in the decolorization of synthetic azo dyes, such as Acid Orange II, and others (Thurston, 1994; Chivukula, Spadaro, & Renganathan, 1995).

Horseradish peroxidase (HRP) is known to degrade certain recalcitrant organic compounds, such as phenol and substituted phenols, *via* free radical polymerisation mechanisms (Tatsumi, Wada, & Ichikawa, 1996). Recently Bhunia et al. (2002) showed that HRP was effective in degrading and precipitating industrially important azo dyes, such as Ramazol blue. This industrially-used dye contains at least one aromatic group in its structure making it a possible substrate of HRP. Bhunia et al. (2002) evaluated the specificity of HRP toward different dyes, such as Ramazol blue and Cibacron red. They obtained the pH for optimal dye degradation, the dyes which inhibited HRP activity, the kinetic constants for degradation of selected dyes and HRP inactivation in the presence of dyes and H_2O_2. They found that at pH $\geqslant$ 6.0, the rates of enzymatic dye degradation were very low, but increased with decreasing pH. Ramazol blue was degraded the fastest, followed by Cibacron red, but both were slow, compared to the use of phenol as substrate. Inactivation of HRP by the dyes was the major limitation for a potential commercial application of this technique in effluent treatment for the dye manufacturing industry. De Souza et al. (2007) reported that HRP in its free form is effective for decolorization of textile dyes and effluents, as well as for achieving a reduction in the toxicity of the effluent after the enzyme treatment.

The whole cultures or unpurified preparations of the ligninolytic system of the fungi have been tested for degradation of dyestuffs. Shin, Oh, and Kim (1997), showed that the edible macroscopic fungi *Pleurotus ostreatus* produced an extracellular peroxidase that can decolorize Ramazol brilliant blue. In a later report, Skin and Kim studied the efficiency of a *Pleurotus ostreatus* peroxidase on the decolorization of dyes belonging to several structurally different groups including triarylmethane, heterocyclic azo, and polymeric. All dyes tested were partially decolorized; the best decolorization (98%) was achieved for bromophenol blue, while heterocyclic dyes, methylene blue and toluidine blue O, were least decolorized (10%).

A purified peroxidase produced by *Geotrichum candidum* Dec I, was involved in decolorization of dyes (Kim & Shoda, 1999). This peroxidase was produced under aerobic conditions as a secondary metabolite in the stationary phase. Nine of the 21 dyes decolorized by the cells of *G. candidum* Dec I, were also decolorized by the purified peroxidase, in particular anthraquinone dyes. Few studies have shown enzymatic degradation of anthraquinone dyes, which are xenobiotic chemicals similar to azo dyes but with a different structure (Knapp, Newby, & Reece, 1995; Vyas & Molitoris, 1995). The optimal temperature for peroxidase activity was 30 °C and was stable after incubation at 50 °C for 11 h.

Recently, Yang et al. (2003) reported the isolation and enzyme production of two yeasts (*Debaryomyces polymorphus* and *Candida tropicalis*) and two filamentous fungi (*Umbelopsis isabellina* and *Penicillium geastrivorus*), capable of decolorizing Reactive black 5.

2.3. Problems encountered during the application of peroxidase for treatment of phenolic compounds

Utilization of peroxidase for the treatment of phenolic compounds is efficient and cost-effective, as compared to other conventional methods (Taylor, Al-Kassim, Bewtra, Biswas, & Taylor, 1996; Chung et al., 1997; Mantha et al., 2002). However, there is an important problem in the peroxidative removal of phenol from aqueous solutions; peroxidase is inactivated by free radicals, as well as oligomeric and polymeric products formed in the reaction, which attach to the enzyme and inactivate it (Wu et al., 1993). This is known as the suicide-peroxide inactivation of peroxidase, which needs to be solved in order to achieve an efficient and successful treatment (Nazari, Esmaeili, Mahmoudi, Rahimi, & Massavi-Movahedi, 2007). Sucide-peroxide inactivation has been shown to reduce the senestivity and efficiency of peroxidase (Arnao, Acosta, Del Rio, Varon, & Garcia-Canovas, 2001; Hiner et al., 2002). Suitable techniques has been introduced to reduce the extent of suicide inactivation, and to improve the lifetime of the active enzyme (Nazari et al., 2007).

Immobilization offers some protective effect against inactivation of horseradish peroxidase (HRP) (Gomez et al., 2006). Dalal and Gupta (2007), used immobilized HRP enzyme by bioaffinity layering, to convert the phenol into free radicals, during the first 10 min of the reaction; after that it was removed and polymerisation allowed to continue in the absence of enzyme. By using this approach, immobilized bioaffinity-layered HRP prepration could bring about complete conversion of *p*-chlorophenol from synthetic waste water. Bioaffinity-layered HRP prepration (1 IU ml^{-1}) could be used five times successfully, with 100% conversion of *p*-chlorophenol.

Cheng et al. (2006) observed that the poly (ethylene glycol) (PEG) sac improved the efficiency of phenol removal, by forming a protective layer in the vicinity of the active centre of HRP, to restrict the attack of free phenoxy radicals formed in the catalytic cycle; also PEG has a greater affinity with the polymer product than the enzyme.

Similar results were reported for the effect of PEG on HRP stability (Buchanan & Nicell 1998; Kinsley & Nicell, 2000; Duarte-Vazquez et al., 2003; Kennedy et al., 2002; Villabolos & Buchanan, 2002; Bodalo, Gomez, Gomez, Bastida, & Maximo, 2006).

Nazari et al. (2007) reported a mechanism to prevent and control the suicide-peroxide inactivation of horseradish peroxidase by means of the activatory and stabilization effects of Ni^{2+} ion, which was found to be useful in processes, such as phenol removal and peroxidative conversion of reductant substrates, in which high concentration of hydrogen peroxide may lead to irreversible enzyme inactivation. Metal ions can coordinate to oxidative site residues leading to activation of enzymes (Louie & Meade, 1999). Mahmoudi, Nazari, Mohammadian, and Moosavi-Movahedi (2003) reported HRP activatory effects in the presence of low concentrations of metal ions.

2.4. Organic and polymer synthesis

HRP has been used to polymerise phenolic and aromatic amine compounds, while new types of aromatic polymers have been

synthesized in water and in water miscible organic solvents (Oguchi, Tawaki, Uyama, & Kobayashi, 1999).

Cardanol is a phenol derivative having a C_{15} unsaturated alkyl chain with 1–3 double bonds at its *meta* position (Ikeda, Tanaka, Uyama, & Kobayashi, 2000). It is obtained by thermal distillation of cashew nut shell liquid and has been used as a raw material to produce resins and friction linings. In a study reported by Kim, An, Song, Kim, and Chelikani (2003), soybean peroxidase catalysed the oxidative polymerisation of cardanol, using methanol, ethanol, 2-propanol, *t*-butyl alcohol or 1,4-dioxane as solvents. Highest yield (62%) was obtained using 2-propanol as solvent. However, the use of HRP resulted in inefficient cardanol polymerisation.

The production of conducting polymers has remarkable interest because of their wide range of applications, including anticorrosive protection, optical display, light-emitting diodes etc. (Raitman, Katz, Buckmann, & Willner, 2002). Polyaniline is one the most extensively investigated conducting polymers because of its high environmental stability and promising electronic properties. Currently, polyaniline is synthesized by oxidising monomer aniline under strongly acidic conditions and low temperature using ammonium persulfate as the initiator of radical polymerisation (Rannou et al., 1998). Chemical methods of polyaniline synthesis have some disadvantages. First the reaction is a radical polymerisation and hence is not kinetically controlled and second, the reaction is not environmentally friendly because it is carried out at very low pH. For these reasons enzymatic polymerisation of aniline is an attractive alternative to the chemical synthesis of polyaniline. Horseradish peroxidase has been used in the synthesis of polyelectrolyte complex polyaniline (Liu, Kumar, Tripathy, Senecal, & Samuelson, 1999). However, HRP shows low activity toward aniline and low stability at pH below 4.5 (Chattopadhyay & Mazumdar, 2000). In nature, there are peroxidases that show good stability at low pH, making them a good alternative for polymerising aniline under acidic conditions. Recently, Sakharov, Vorobiev, and Leon (2003), using anionic peroxidase purified from the African oil palm tree, developed an enzymatic synthesis of the polyelectrolyte complex of polyaniline and also sulfonated polystyrene. The template polymerisation of aniline was carried out in aqueous buffer at pH 3.5. Production of the electroactive form of polyaniline sulfonated polystyrene complex was confirmed by visible spectroscopy and electron paramagnetic resonance spectroscopy (EPR). Sakharov et al. (2003) also evaluated the effect of aniline concentration on polymerisation rate, by varying the substrate concentrations of aniline; no inhibition of palm peroxidase was observed. However, at high H_2O_2 concentration (20 mM), aniline polymerisation did not proceed.

Enzymatic polymerisation of substituted and unsubstituted phenols and anilines was catalysed by HRP, using a template which could be a micelle, a borate-containing electrolyte or lignin sulphonate (Nagarajan et al., 2003). Conditions were adjusted to align the monomer along the template to produce a polymer-template complex which may be used in biological sensors and anti-static and anti-corrosive coatings amongst other uses. Lignin is the second most abundant biopolymer on earth; it has potential application in production of polymeric dispersants, soil-conditioning agents, phenolic resins or adhesives, and laminates. In the presence of H_2O_2, peroxidase catalyzes the oxidation of phenols that eventually give rise to higher molecular weight polymers (Nicell & Wright, 1997). This characteristic can be used as an attractive alternative to the conventional formaldehyde method used for the production of lignin-containing phenolic resins.

White-rot basidiomycetes such as *P. chrysosporium, Phleba ratia, Pleurotus* sp., amongst others, secrete the lignin-degrading enzyme, MnP. This enzyme oxidises phenolic compounds directly, *via* phenoxy radicals, which represent the first step in the degradation of lignin. Alternatively, radicals undergo condensation to give polymerisation products (Wariishi, Valli, & Gold, 1991). MnP produced by the basidiomycete *Bjerkandera adusta* was used by Iwahara, Hirata, Honda, Watanabe, and Kuwahara (2000), for acrylamide polymerisation, in the presence of 2,4-pentanedione as a radical initiator. The yield of polyacrylamide was 96% against added acrylamide. The decomposition of polyacrylamide started at 254 °C and was completed at 520 °C, suggesting that polyacrylamide could be used as a thermoplastic resin.

A patent has been filed in relation to *in situ* cross-linking of proteins, including collagen, using HRP to form biocompatible semi-solid gels. This material can be used as wound sealant, delivery vehicle or as binding agent in food product applications (Miller, Tizard, Keeton, & Prochaska, 2003).

HRP catalysed the oxidative coupling of methyl-sinapate with a syringyl lignin model compound 1-(4-hydroxy-3,5-dimethoxyphenyl)ethanol in the presence of hydrogen peroxide. The main product was a novel spirocyclohexadienone, which is of importance in understanding the biosynthesis of lignin in the cell walls of woody plants by oxidative cross coupling of different phenolic precursors (Setala, Pajunen, Rummakko, Sipila, & Brunow, 1999).

Another application of plant peroxidases in the field of organic and polymer synthesis is related to the coupling of catharanthine and vindoline to yield α-3′,4′-anhydrovinblastine. This compound is believed to be the metabolic precursor of vinblastine and vincristine, which are part of most curative regimes used in cancer chemotherapy (Sottomayor, Lopez-Serrano, Di Cosmo, & Barcelo, 1998).

2.5. Deodorization of swine manure

Odorant compounds such as phenols, indoles, volatile fatty acids, ammonia, hydrogen sulfide and mercaptans are either initially present in manure or result from anaerobic transformation of animal waste (Hobbs, Pain, & Misselbrook, 1995; Zahn et al., 1997). Elevated odour level in confinement buildings can reduce livestock growth rates, increasing the outbreaks of infections and adversely affecting farm workers (Hardwick, 1985). Treatments, such as dietary management, to intense aeration or zone treatment and the application of manure additives have been used to decrease or eliminate odorous compounds. (Hobbs et al., 1995; Wu et al., 1999; McCrory & Hobbs, 2001). These methods are expensive and require specialist knowledge.

Recently HRP has been proven an effective alternative for deodorization of manures. Minced horseradish with calcium peroxide reduced the concentration of phenol by 70% and for VFAs by 45%. The horseradish batch could be used five times as an enzymatic source in the deodorization of swine slurry (Govere et al., 2007). A 100% reduction in the concentration of phenolic odorants without reoccurrence within 72 h was achieved by using HRP and minced horseradish roots (Govere et al., 2005).

2.6. Application in the paper pulp industry

White-rot fungi can attack lignin and simultaneously degrade wood components to carbon dioxide and water (Arana, Tellez, Gonzalez, & Gonzalez, 2002). Some of them selectively and efficiently degrade lignin rather than cellulose and hemicellulose (Li, 2003). There are drawbacks to the direct use of microorganisms for breaking down lignocellulosic materials, including degradation of cellulose fibres (Jimenez, Martinez, Perez, & Lopez, 1997) and long reaction times, extending to several days (Katagiri, Tsutsumi, & Nishida, 1995).

Biopulping is a process where extracellular enzymes (hydrolytic and oxidative) produced by a white-rot fungus remain adsorbed on the wood chips, degrading lignin (de Souza-Cruz, Freer, Siika-Aho, & Ferraz, 2004). After the pulping process, about

10% of the lignin appears as modified lignin, which is responsible for a characteristic brown colour. Modified lignin can be enzymatically degraded, using a biobleaching process (Antonopoulos, Hernandez, Arias, Mavrakos, & Ball, 2001). Major lignin-degrading enzymes from basidiomycetes include MnP, laccase and to a lesser extent LiP (Hatakka, Hakacla, Lundell, Horichter, & Maijala, 2002). MnP, in the presence of Mn(II) chelated with an organic acid, Tween 80, and an H_2O_2-generating system, depolymerised milled pine wood (Hatakka, Lundell, Hofrichter, & Maijala, 2003). A key element in the delignification system is Mn(III) a strong oxidising agent that is generated by MnP (Feijoo, Moreira, & Lema, 2002).

Wood pulp may be delignified enzymatically with good results, using LiP in the absence of peroxide, when the enzyme is firstly chemically modified to avoid its adsorption to the pulp (Gysin & Griessmann, 1994). Enzyme pulping using MnP and laccase of *Polyporus* sp., and pectinases from *Rhizopus* sp. 26R significantly reduced the amount of NaOH used in an alkaline pulping process (Poonpairoj, Peerapatsakul, & Chitadron, 2001).

More studies are needed to determine if selective lignin degradation and efficient biopulping require a proper balance between lignin and cellulose degradation.

2.7. Peroxidase biosensors

Electrochemical biosensors offer a great potential for peroxidase application. Recently, peroxidase-based electrodes have had widespread use in analytical systems, for determination of hydrogen peroxide and organic hydroperoxides (Jia et al., 2002). When co-immobilized with a hydrogen peroxide-producing enzyme they may be exploited for determination of glucose, alcohols, glutamate and choline (Ruzgas, Csoregi, Katakis, Kenausis, & Gorton, 1996).

As described in the introduction, the peroxidase catalytic cycle involves reaction of the active site with hydrogen peroxide as given in Eq. (1)

$$Fe^{III} + H_2O_2 \rightarrow \left[\left(Fe^{IV} = O\right) - R^{+*}\right] CoI + H_2O \quad (1)$$

Then, CoI oxidises a substrate to give a substrate radical and CoII, which is reduced by a second substrate molecule, regenerating the native ferric enzyme.

When an electrode substitutes the electron donor substrate in a common peroxide reaction cycle, the process is denominated as direct electron transfer (Freire, Pessoa, Mello, & Kubota, 2003). Enzymes immobilized on an electrode can be oxidised by hydrogen peroxide (Eq. (1)) and then reduced by electrons provided by an electrode (Eq. (2))

$$\left[\left(Fe^{IV} = O\right) - R^{+*}\right] CoI + 2e^- + 2H^+ \rightarrow Fe^{III} + H_2O \quad (2)$$

When an electron donor (A) is present in a peroxidase electrode system, the direct process can occur simultaneously with reduction of the oxidised donor, A^*, by the electrode (Eq. (3)) (Liu & Ju, 2002).

$$A^* + e^- + H^+ \rightarrow AH \quad (3)$$

During direct electron transfer, electrons act as the second substrate for enzymatic reaction, resulting in a shift of the electrode potential, with measured current being proportional to the H_2O_2 concentration (Everse, Everse, & Grisham, 1991). This technique can also be used to quantify other metabolites, especially when combined with another oxidase enzyme.

Peroxidase can also interact with an electrode by mediated electron transfer, where a mediator (an electron donor, A) is transporting the electron between the enzyme and the electrode. In this system the enzymatically oxidised donor (A^*) is thus electrochemically reduced by the electrode.

When H_2O_2 and an aromatic electron donor (A) are present at a peroxide electrode, both direct and mediated electron transfer can occur simultaneously. The phenomenon has been used to develop a method for determination of phenols and aromatic amines down to the nanomolar range (Munteanu et al., 1998).

2.8. Application in analysis and diagnostic kits

Horseradish peroxidase is the most commonly used enzyme for practical analytical applications. However, peroxidases from other sources appear to be good as substitutes for HRP.

Lactose contained in milk and milk products is determined by various analytical methods requiring trained persons and sophisticated instruments such as a spectrophotometer, biosensor, etc. Sharma, Sehgal, and Kumar (2002) developed a simple and economical biostrip technology for estimation of lactose, by immobilizing β-galactosidase, galactose oxidase and HRP onto a polymeric support, the β-galactosidase the key enzyme to detect lactose. The biostrip was dipped in milk or a milk product and colour developed from an added chromogen; it was used to estimate lactose in the concentration range <20−100 g/l.

Due to the ability of peroxidase to yield chromogenic products in low concentrations and it's relatively good stability it is well suited for the preparation of enzyme-conjugated antibodies and application in diagnostic kits (Krell, 1991).

Agostini et al. (2002) purified various peroxidase isoenzymes from roots and hairy root cultures of turnip (*Brassica napus*). They developed a diagnostic test kit for determination of uric acid. This assay was based on the following reaction:

$$\text{uric acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2 \quad (4)$$

$$H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} p\text{-(benzoquinone)monoamine-phenazone} \quad (5)$$

In analytical applications the enzyme must be present in saturated amounts, to make sure that the H_2O_2 produced in the test is stoichiometrically converted into a coloured substance (Krell, 1991). The concentration of turnip peroxidase giving a linear response with time and increasing uric acid concentration was 30 mM, Analysis of uric acid in human serum from ten different patients using either the kit containing turnip peroxidase or a commercially available kit, gave the same results. This confirmed that the cationic peroxidase isoenzyme from turnip hairy roots could be used as a reagent for clinical diagnosis, as part of a kit where H_2O_2 is generated.

In humans, an increase as well as decrease in serum cholesterol levels, is associated with some diseases (Hirany, Li, & Jialal, 1997), and, thus there is a need for its quantification. An enzymatic colorimetric method has been developed for total cholesterol determination, showing accuracy, precision selectivity and rapidity of assay (Ragland, Kourad, Chaffin, Robinson, & Hardy, 2000). This method employs cholesterol oxidase, cholesterol esterase and peroxidase but the enzymes, stability and cost have restricted its application for routine uses. Alternatively, Malik and Pundir (2002) employed the same enzymes individually immobilized, resulting in high selectivity and increased stability. In this method cholesterol ester is hydrolysed by cholesterol esterase to free fatty acid and cholesterol, which is oxidised by cholesterol oxidase to cholestenone and H_2O_2. H_2O_2 is determined using HRP following the reaction shown in Eq. (5) where the resulting quinoneimine is measured at 520 nm. The lower detection limit was 42.8 mg/l for individually immobilized enzymes and 21.4 mg/l for co-immobilized enzymes. A good correlation ($r = 0.99$) was found between total serum cholesterol obtained by this method and from a commercial kit using free enzymes.

HRP has also been used in several diagnostic applications in medicine such as the detection of 8-hydroxydeoxyguanosine and its analogues in urine, to identify bladder and prostate cancer risks (Chiou et al., 2003). An enzyme-linked HRP secondary antibody was used in an enzyme immunoassay (EIA), based on an antidouble-stranded DNA primary monoclonal antibody, to detect the cystic fibrosis delta F508 mutation in blood (Hopfer, Makowski, Davis, & Aslanzadeh, 1995). A solid-phase EIA was developed to measure human tumour necrosis factor alpha (hTNF-alpha) in clinical research, using bispecific antibodies hTNF-alpha and HRP, with a detection limit of 1 ng/ml (Berkova et al., 1996). HRP and the plant hormone indole-3-acetic acid (IAA) have been used in gene-directed therapy. HRP/IAA represented an efficient system for enzyme/prodrug-based anticancer approach (Greco et al., 2001).

2.9. Enzyme immunoassays

Enzyme-linked immunosorbent assays (ELISA), also known as EIA, are tests designed to detect antigens or antibodies, by producing an enzyme-triggered change of colour. To this end, an enzyme-labelled antibody, specific to the antigen, is needed, as well as a chromogenic-substrate, which in the presence of the enzyme changes colour. The amount of developed colour is proportional to the amount of antigen in the test specimen.

HRP is probably the most common enzyme used as a reporter (enzyme-labelled antibody) in enzyme immunoassays.

ELISA tests on which peroxidase is used for labelling an antibody, have been developed for screening monoclonal antibodies against mycotoxins (Kawamura et al., 1989). Mycotoxins are dangerous by-products of several species of fungi, such as *Aspergillus* and *Pencillium* (ochratoxins) and *Fusarium* species (T-2 toxins, trichothecenes) amongst others. They are known to be hepatotoxic, nephrotoxic, teratogenic and mutagenic to a wide variety of mammalian species (Clarke et al., 1993). Mycotoxins are frequently found in cereals and their detection is essential to avoid risk of consumption (Ramakrishna, Lacey, Candlish, Smith, & Goodbrand, 1990).

Mycotoxins can be detected using HPLC, GCMS or TLC; these techniques are expensive, time-consuming and require complicated sample cleanup. Immunoassays such as ELISA have some advantages, including simplicity, low cost, reliability, low requirements for technical skills and simple equipment.

2.10. Emerging strategies of fungal peroxidases for biofuel production

Ethanol and other biofuels produced from lignocellulosic biomass represent a renewable, more carbon-balanced alternative to both fossil fuels and corn-derived or sugar-cane-derived ethanol. Unfortunately the presence of lignin in plant cell walls impedes the breakdown of cell wall polysaccharides to simple sugars and subsequent conversion of these sugars to useable fuel. To achieve an optimal biological conversion of lignocellulosic biomass to biofuel, lignin must be physically removed from plant tissue before saccharification (Weng, Li, Bonawitz, & Chapple, 2008). Lignin is chemically difficult to degrade because of the free radical coupling mechanism (Boerjan, Ralph, & Baucher, 2003) and due to its four stereoisomers (Ralph, Peng, Lu, Hatfield, & Helm, 1999).

Owing to the physical properties of lignin and its incorporation into the cell wall, current approaches used for its removal from biomass are sufficiently expensive and energy-intensive to make large cellulosic biofuels production unfeasible (Hamelinck, van Hooijdonk, & Faaij, 2005).

One of the most common fates of lignin in nature is to be metabolized by lignin peroxidases (LiPs), manganese peroxidases (MnPs) and closely-related enzymes of white rot basidiomycetes (Martinez et al., 2005; Chen & Dixon, 2007; Hammel & Cullen, 2008). These organisms are responsible for initiating the depolymerisation of lignin and are able to oxidise lignin to carbon dioxide (Weng et al., 2008).

Schoemaker and Piontek (1996) described the mechanism by which lignin peroxidase (LiP) interacts with lignin polymer, in which veratryl alcohol (valc), a secondary metabolite of white rot fungi, acts as a cofactor for the enzyme. It was observed that, in the depolymerisation with fungal cultures, both the presence of LiP and veratryl alcohol stimulated the degradation of lignin:

$$\mathrm{Lip} + \mathrm{H_2O_2} \rightarrow \mathrm{H_2O} + \mathrm{LiPI} \quad (\text{Compound I}) \tag{6}$$

$$\mathrm{LiPI} + \mathrm{valc} \rightarrow \mathrm{valc^{+\cdot}} + \mathrm{LiPII} \quad (\text{Compound II}) \tag{7}$$

$$\mathrm{LiPII} + 2\mathrm{H^+} \rightarrow \mathrm{valc^{+\cdot}} + \mathrm{H_2O} + \mathrm{LiP} \quad (\text{Native enzyme}) \tag{8}$$

LiPI will oxidise the first molecule of veratryl alcohol to the corresponding radical cation ($\mathrm{valc^{+\cdot}}$), which is liberated from the active site. Subsequently, the second substrate molecule is oxidised by LiPII to form a second $\mathrm{valc^+}$. In the process, LiPII is converted to native enzyme.

Schoemaker and Piontek (1996) also described the *in vivo* lignin biodegradation process. In the process fungal hyphae grow within the lumen of the woody cells, excreting both ligninolytic and (hemi-)cellulolytic enzymes. Also, during ligninolysis the fungus produces an extracellular mucilaginous sheath, closely associated both with the hyphae and with the decaying wood. At the early stages of decay the ligninolytic enzymes, like LiP, are found at the surface of the lignified wall, unable to penetrate it. At later stages, LiP is found within the degraded regions. Lignin is insoluble in water; its degradation *in vivo* takes place in a polysaccharide gel. $\mathrm{H_2O_2}$, the oxidant for LiP, is present in low concentrations in fungal cultures. In the oxidative depolymerisation process, phenolic compounds are formed and are prone to polymerise again. Manganese peroxidase (MnP) or – in other classes of white rot fungi–laccases, convert the phenolic LiP breakdown products to form quinones, which are further metabolized in a process that most probably involves reduction to the corresponding hydroquinones.

3. Conclusions

No doubt peroxidases have many possibilities for application but commercial processes based on these enzymes have been confined to diagnostic kits and antibody labelling. The inactivation of peroxidases by peroxides through oxidation is one of the major reasons. Another important reason for the limited application of peroxidase is the low water solubility of the substrates of interest. Peroxidase activity is also greatly affected by elevated temperature, limiting its application to processes at relatively low temperatures.

The stability of peroxidase is a severe and difficult to solve problem. In spite of its low operational stability, peroxidase can be an efficient biocatalyst for the production of industrially relevant compounds. Therefore, a mutated peroxidase produced from different plant sources with improvements in its stability and activity, would be useful for a number of industrially relevant applications.

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