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Structure, Function and Applications of a Classic Enzyme: Horseradish Peroxidase

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Abstract: An oxidation-reduction catalyzing enzyme i.e. peroxidases that are produced by numerous plants and microorganism are widely known in today's world. This enzyme can catalyze peroxide based reactions. Novel types of aromatic polymers may result from the oxidative polymerization of aromatic amines and phenols by horseradish Peroxidase (HRP) present in water or water-soluble organic solvents. Besides, playing a potential role by the peroxidases in the soil detoxification, HRP, turnip and soybean peroxidases have also shown their significance in the bioremediation of cresols, phenols and chlorinated phenols contaminated water. Other types like manganese peroxidase (MnP) and Lignin Peroxidase (LiP) are of important use in paper industry for performing bio-bleaching and bio-pulping. These enzymes can also oxidatively breakdown the synthetic azo dyes. One of the major use of peroxidases are in analytical systems where peroxidase based biosensors are used for organic hydroperoxide and hydrogen peroxide determination. On the other hand, if co-immobilized with H_2O_2–producing enzyme, it can be used for determination of range of compounds e.g. alcohols, choline, glucose and glutamate. Furthermore, peroxidases are simple and trustworthy way of detecting pathogens, toxins and antigens in various analytes by labeling a pre applied antibody like in ELISA (enzyme immuno sorbent assay) and quantification of chemicals like glucose, lactose, uric acid and cholesterol that are its practical analytical applications in diagnostic kits.

Keywords: Horseradish Peroxidase, Biochemical Assays, Bioremediation, Bio-Bleaching, Bio-Pulping

1. Introduction

Being oxidoreductases, peroxides (EC 1.11.1.7) catalyze both oxidation and reduction of variety of compounds and peroxides respectively. These are heme proteins having molecular weight ranging 30 to 150 Kilo Dalton (KD) and has a specific prosthetic group i.e. ferriprotoporphyrin IX also known as iron (III) protoporphyrin IX. The term "Peroxidase" not only indicates a set of specific enzymes like glutathione peroxidase, NADH peroxidase and iodide peroxidase (EC 1.11.1.9), (EC 1.11.1.1) and (EC 1.11.1.8) respectively but it also applies to the set of generally known nonspecific peroxidases.

In living organisms specifically in animals, microorganisms and plants, important roles of peroxidases activity has been unraveled since 90s of the 20th century. It contributes in the lignification [1] and provides assistance in the recovery mechanism for plant tissues that are either infected or bodily damaged [2]. Peroxidase catalyzes the peroxides oxidation and a variety of other substrates too but this catalytic round engages distinctive forms of intermediate enzymes [3-5]. Firstly, during reaction, an unstable

compound 1 (CoI) resulted when hydrogen peroxide oxidizes the native ferric enzyme. The CoI contains a heme structure of Fe IV=O-porphyrin π-cation radical. Secondly, compound 2 (CoII) which releases free radical is produced upon oxidizing electron donor substrate. Finally, CoII regenerates iron III state when reduced by a second substrate molecule and also results in the formation of another free radical.

Uses of peroxidases in immuno assays and clinical biochemistry are many fold. Some of the novel and effective uses of peroxidases encompass phenolic compound containing waste water treatment, removal of peroxide contamination and debris from the food stuffs as well as industrial wastes and synthesis of the aromatic chemicals and compounds [6, 7]. The tubers of horseradish roots are mostly considered and employed for the commercial and large scale production of peroxidases [8] but some of the recombinant species having better properties are also been used peroxidase production [9].

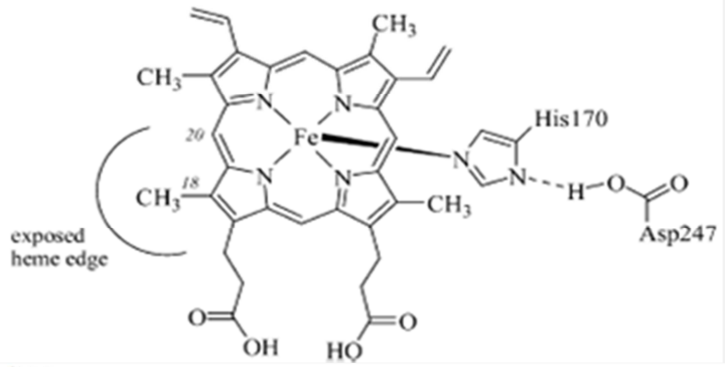
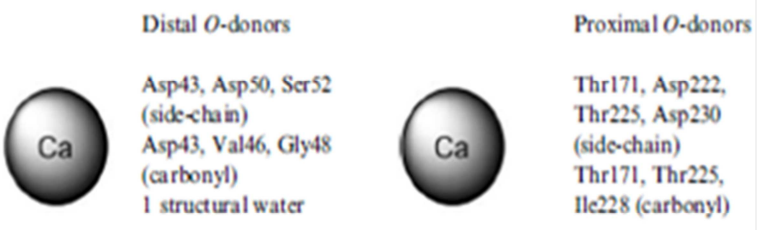
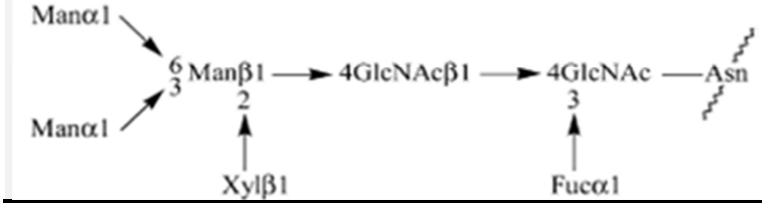
Peroxidases ability to get reduce by the electron- donating substrates makes it very fruitful in numerous analytical and industrial applications. It is one of the majorly well-matched enzymes for the generation of enzyme-conjugated antibodies that are used in ELISA tests and has the caliber to yield chromogenic products at minimum concentrations [10]. Peroxidases when coupled with in polyenzymatic systems can produce H_2O_2 that can be used for the detection of glucose level in blood. In addition, as this enzyme has oxidative nature, it can revolutionize chemical oxidant techniques in many ways. In this review, structure, chemistry and mechanism of action of HRP isoenzyme C will be discussed. Moreover, applications of the enzyme in biotechnological based industries will be described in the light of gene or protein level modifications that have been brought in the peroxidases for the sake of better and efficient performance.

2. Structure Chemistry and Mechanism of Action

2.1. Description of the Enzyme

2.1.1. General Features

Table 1. *Anatomical dissection of HRP C enzyme: An overview of some crucial structural features.*

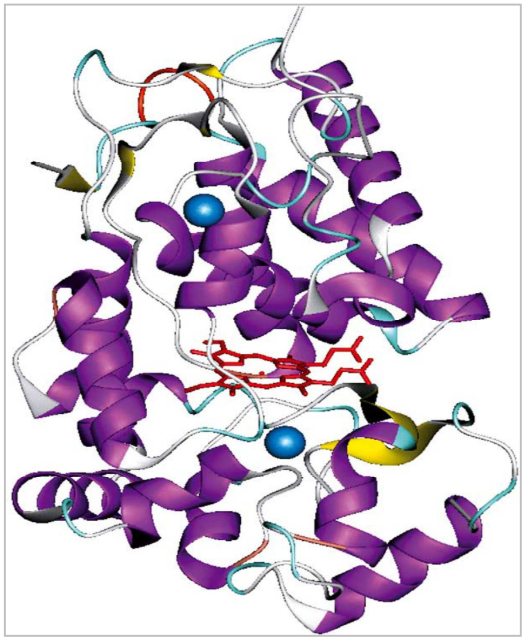
Region	Relation of Important residue/s with structural features and functional roles
Heme 	Coordinate bond between heme iron atom and His170. Imidazolate character of His170 ring is controlled with the help of Asp247 carboxylate side chain. His170Ala mutant undergoes heme degradation upon hydrogen peroxide addition and also compound I and II are not detected. Exposed heme edge is responsible for oxidation of aromatic substrates.
Calcium 	Ca^{2+}ions both proximal and distal are seven coordinate. Calcium loss can reduce the enzyme activity by 40%. 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 	Sites of glycosylation are in loop regions of the structure, at Asn13, Asn57, Asn158, Asn186, Asn198, Asn214, Asn255 and Asn268. The major glycan is shown here; there are also minor glycans of the form $Man_mGlcNAc_2$ (m = 4 to 7) and $(Xyl)_xMan_m(Fuc)_fGlcNAc_2$(m = 2, 4, 5, 6; f = 0 or 1; x = 0 or 1).

Adapted from Nigel C. Veitch 2004

Welinder determined the HRP isoenzyme c sequence for the first time in late 70s of the 19th century [11]. The enzyme is a single polypeptide comprising of only 308 amino acid residues. Structurally, the protein N terminal is blocked by pyroglutamate in contrast to the c terminus which is heterogeneous with some molecules lacking ser308, a terminal residue. Besides buried salt bridge between Arg123 and asp 99 there are 4 disulphide bridges between cysteine residues present at 11-91, 44-49, 97-301 and 177-209. A conserved motif Asn-X-Ser/Thr where X represents an aa residue can be easily noted in the primary sequence and 9 potential glycosylation sites of which 8 are engaged can be recognized from this signature. The major glycan which accounts for almost 80% of the HRP C is branched heptasaccharide (Table 1) but many slight glycans have also been described indicating heterogeneous profile of HRP C [12].

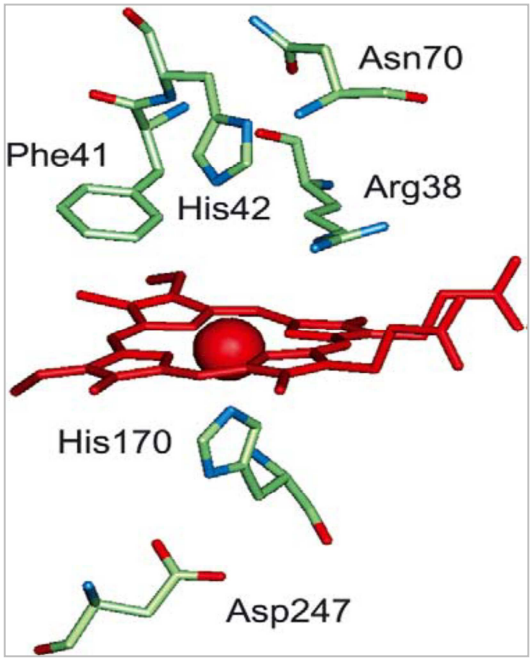
These consistently contain numerous mannose residues along two terminals GluNAc. The variations in the complication are because of presence of the different glycan at any of the glycosylation sites. In HRP C, total carbohydrate contents are to some extent dependent on the source of enzyme and the typical values are between 18% and 22%.

Structurally, HRP C is composed of two dissimilar types of metal centre which are both indispensable for the structural and functional role of the enzyme (Figure1 and Table 1). These metal centers are iron (III) protoporphyrin IX and two calcium atoms. A coordinate bond is usually present between the side-chain NE2 atom of the proximal histidine residue i.e. His 170 and the heme iron atom. The secondary axial coordination site located at the distal end of the heme plate is vacant when the enzyme is inactive state but accessible to the substrate like H_2O_2 during HRP C turnover (Figure. 2). In addition, six coordinate complexes of peroxidases can be produced when small molecules like fluoride and cyanide, azide or carbon monoxide binds to the distal site of the heme iron atom. Some of these only bind when they are protonated that are stabilized via hydrogen bounded interactions with the side chains of Arg 38 and His 42 which are the distal residues of the heme pocket (Figure. 2). Both calcium binding sites are situated at positions proximal and distal to the heme plane and are linked to the heme binding region through a hydrogen bonds network. Both of these calcium sites are seven coordinate with oxygen-donor ligand that are provided by a combination of amino acids side chains like carboxylates (Asp), hydroxyl (Thr and Ser) backbone carbonyls and a distal sited structural (Table 1). Thermal stability and enzyme activity both decreases upon loss of calcium [13] and bring subtle alterations in the heme environment which can be spectroscopically detected [14].



Adapted from Nigel C.V (2004)

Figure 1. *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). a-Helical and b-sheet regions of the enzyme are shown in purple and yellow, respectively. The F 0 and F 00 a-helices appear in the bottom right-hand quadrant of the molecule.*



Adapted from Nigel C.V (2004)

Figure 2. *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.*

2.1.2. Three-Dimensional Structure

Almost two decades ago, using X-ray crystallography the earliest solution of the HRP C 3D structure emerged in the scientific literature [15]. Smith et al used the recombinant enzyme as the source of crystals and non- glycosylated form of heavy atom derivatives was generated using Eschershia Coli expression system. Previous to this refined structure of the HRP C, numerous attempts were failed in order to obtain the appropriate crystals for diffraction analysis and major reason was the heterogeneity of HRP C preparations consisting multiple glycoforms. Largely, the HRP C is composed of alpha-helical structure and a little region is confined to the beta-sheets. Heme group is located in between the distal and proximal domains of the enzyme. It is suggested that gene duplication probably result in the domain origination and the proposal is supported by the common calcium binding sites and other structural elements [16].

2.1.3. HRP and the Plant Peroxidase Super Family

HRP isoenzymes belong to a super family of the plant peroxidases also called class III that includes fungal and bacteria alongside plant originated peroxidases. Class I and Class II peroxidases include gene-duplicated bacterial peroxidases, ascorbate peroxidases yeast cytochrome C peroxidases and fungal peroxidases respectively. Usually a peroxidase fold termed as a core is conserved among classes of peroxidases super family along many other structural elements. For instance, Gajhede et al revealed three alpha helices in the HRP C and class III of plant peroxidases besides the core peroxidase fold mentioned above. From the structure, it is evident that two of these helices named as F0 and F00 are sited in an elongated insertion and exhibits a colossal variation not only in the number of residues but also in the sequence (Figure 2). The reason behind the overall structural integrity in this region is the disulphide linkage between Cys residues located at position 177 and 209. In case of HRP C, the localization of certain residues in the F0 helix is of great interest for the scientific community that how they are involved in the substrate accessing and binding. Infact, this region is crucial as speculated by some authors besides Gajhede et al., for the retention of produced radical species as a result of plant peroxidase catalyzed reaction. Furthermore, structural and functional characterization of all the three classes of plant peroxidases along with their comparison has been published afterwards [17].

2.2. Mechanism of Action

2.2.1. Functional Roles

The following equation (given below) indicates the HRP C and isoenzymes catalysed reaction. Reducing substrates which are mostly phenolic acids, aromatic phenols, sulfonates and amines and their radical substrates are represented by AH2 and AH respectively.

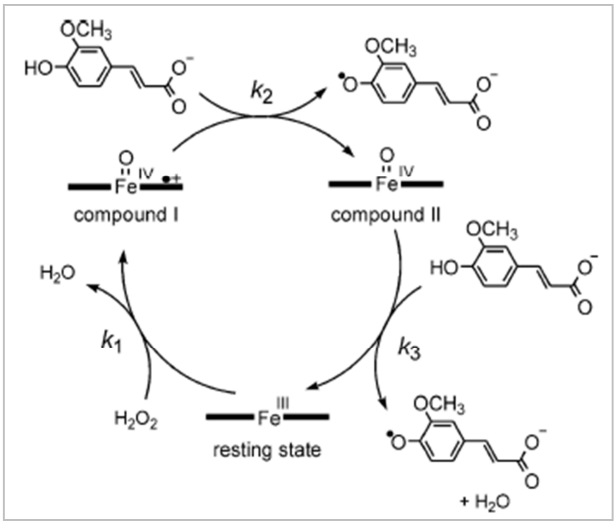
$$H2O2 + 2AH2 \xrightarrow{\text{HRP C}} 2H20 + 2AH^{*}$$

$$\text{Hydrogen peroxide} + \text{reducing substrates} \rightarrow \text{Water} + \text{radical substrate}$$

The primary function of HRP C is not only to convert the H_2O_2 into water. For instance, enzyme of class 1 family like ascorbate peroxidase is utilized by many plants for the regulation of H_2O_2 at intracellular level, however, still very less is known about this enzyme [18, 19]. Research on HRP enzymes for the past two decades revealed some very interesting facts. Presumed in vivo functions of HRP enzymes in plants can be indicated from the radical products produced as the result of these enzyme catalyzed reaction. These functions might include involvement in the cross-linking reactions for the diferulate linkage formation from pectin or polysaccharides that having polymer coupled ferulate groups, formation of suberin by cross linking the phenolic monomers, di-tyrosine linkages formation and facilitation in lignin biosynthesis through oxidative coupling of phenolic compounds. Most of these functions are performed by HRP enzymes when the plants are in stress conditions or expose to the external factors like loss of water, pathogen invasion, wounds formation etc.

2.2.2. Catalytic Mechanism

The catalytic mechanism for HRP and C isoenzyme has been widely studied since late 90s of the 20th century. The catalytic cycle (Figure. 3) indicates ferulic acid as a reducing substrate in the reaction. Some other important features of the catalytic mechanism are also demonstrated in the Figure 3. The combination of reaction products like dimmers, trimers and oligomers results from the production of radical species during one-electron reduction steps. These multipart reaction products later on in succeeding turnovers act as reducing substrates.



Adapted from Nigel C.V (2004)

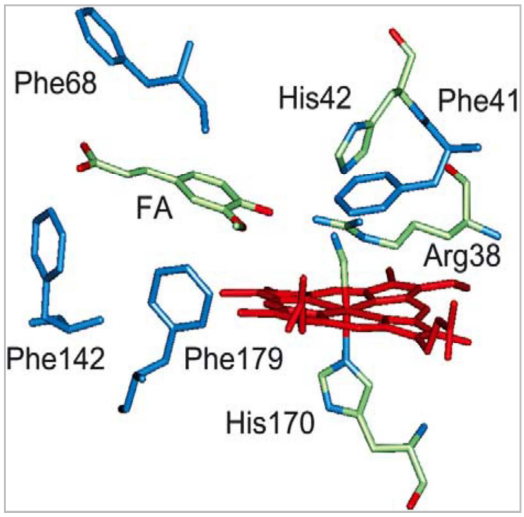
Figure 3. *The catalytic cycle of horseradish peroxidase (HRP C) with ferulate as reducing substrate. The rate constants k1, k2 and k3 represent the rate of compound I formation, rate of compound I reduction and rate of compound II reduction, respectively.*

Formation of compound 1 is the primary step in the whole catalytic cycle when hydrogen peroxide reacts with the Fe (III) resting state of the HRP C. Compound 1 is an intermediate characterized with a high oxidative state and composed of Fe (IV) oxoferryl center and a cation radical which is porphyrin based. During low temperature, H_2O_2 and HRP C catalysed reaction leads to Fe (III)-hydroperoxy complex formation which is also termed as compound 0, a transient intermediate. Filixole and Loew 2000 have carried out the molecular dynamics simulation of these peroxide-coupled complexes. Generally, participation of a reducing substrate is necessary for the first half of the one-electron reduction step that leads to the production of Fe (IV) oxoferryl species which is comparable to one of the two oxidizing equivalents above the resting state. Compound 1 and 2, both are strong oxidants and their redox potentials are estimated to be close to +1 V. Afterwards, the compound II get return by the second one-oxidizing reduction step to the enzyme resting state. The intermediate, Compound III results when the excess H_2O_2 reacts with the resting state enzyme and is demonstrated as resonance hybrid of Fe (III) superoxide and Fe (II) dioxygen complexex. Berglund et al., 2002 published a high resolution crystal structure of compound III of 95% purity and showed that di-oxygen and heme iron bound in a curved conformation [18]. Almost 20 years ago, models for HRP C irreversible inactivation have been developed in numerous studies with m-chloroperoxybenzoic acid. [19]. In addition, in depth mechanisms underlying the crucial role of far heme pocket amino acids like Arg 38 and His 42 in the generation of compound 1 have been deeply discussed in the literature. These crucial amino acids of the distal heme pocket are very important for peroxide catalysis as confirmed via high resolution structural studies of the oxidized intermediates of horseradish peroxidase [20]. For instance, Arg 38 NEH interacts with ferryl oxygen of compound 1 via hydrogen bonding. Both Arg38 and His42 through hydrogen bonds bind with a water molecule. Crystallographic studies also unraveled a new mechanism for the reduction of both compound 1 and 2 through ferulate when a ternary complex formed between cyanide-ligated HRP C and ferulic acid [21].

2.2.3. Aromatic Substrate Binding Sites

HRP C can bind with the range of substrates. The interaction of the enzyme with it substrates has been identified almost 26 years ago when Ortiz de Montellano documented the HRP C interaction site using enzyme inactivation experiments with reactive reagents like alkyl and phenyl hydrazines sodium azide [22]. The HRP C heme edge which is exposed and composed of methyl C18 and hememeso C20 proton (Table 1) is the site where substrate oxidation usually occurs. Products like C18-hydroxymethyl and C20 meso phenyl which are heme derivatives are produced upon generation of free radical species when HRP C and hydrogen peroxide are incubated with reactive agents mentioned above. The point of interest is the contrast between the manners of horse radish peroxidase C and other heme proteins like cytochrome P450 and globins where pyrrole nitrogen and heme iron atom undergo modification. It is seemed to be rather difficult for the substrate to reach oxoferryl core of the HRP C as local protein environment hindered this centre. It can also be termed as closed heme architecture, one of the reasons behind the peroxidase inefficiency to use as catalysts for oxygen transfer reactions than cytochrome P450.

Site-directed mutagenesis has been carried out by number of people to improve this feature of HRP C [23, 24]. These experiments were performed with the aim to increase the substrate accessibility to the oxoferryl core of the compound 1. Substituting the residues like Phe 41 and His42 with smaller residues resulted in improved substrate access to the heme center. Aromatic molecules were used for the generation of 1:1 complexes which are both stable and reversible in the absence of hygrogen peroxide and are produced by using resting state HRP C as well as its ligand-bound derivatives like cyanide ligated HRP C. For instance, the aromatic ring is spotted in a far site near to the exposed heme edge when cyanide ligated HRP C binds to the ferulic acid molecules. In contrast, the phenolic acid side chain is slanted towards the opening of the binding site [25]. Usually, the Arg38 NZH2 interacts with the phenolic and the methoxyl oxygen atoms of ferulic acid through hydrogen bonds. In addition, hydrophobic interactions also exist with the side chain residues like Phe68, Phe143, Phe179, Gly69, Ala140 and Pro139 as well as with hememeso proton C20H and heme methyl C18H3 (Figure. 4). Furthermore, other complexes including benzhydroxamic acid complex of HRP C have also been widely studied for structure and dynamics. The relation between crucial residues at the binding site with the substrate affinity has also been deeply investigated using site directed mutants [26].



Adapted from Nigel C.V (2004)

Figure. 4. *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.*

3. Applications

3.1. Peroxidase Biosensors

The applications of peroxidase are many fold. For instance, it can offer a great potential for electrochemical biosensors. In recent years, electrodes based on peroxidase enzyme have proved its extensive use in the analytical systems e.g. for the H_2O_2 and organic hydro peroxide determination [27]. In addition, if immobilized with enzyme that produces H_2O_2, the peroxidases can be used for the determination of certain chemical levels like glucose, choline alcohols and glutamate. Peroxidase interacts with the electrodes through both direct and mediated electron transfer mechanism. In the former way, when an electron donor specie is in the vicinity of peroxide electrode system, the direct process occurs concurrently and the oxidize donor is reduced. However, in the later way, a mediator, electron donor specie is responsible for transporting the electron between electrode and the enzyme. This is how the electrode electrochemically reduced the enzymatically oxidized electron donor. In a condition when both aromatic electron donor and H_2O_2 are present at a peroxide electrode, both mediated as well as direct electron transfer mechanism can occur at the same time. This observable fact has been used to build a method for the quantification of aromatic amines and phenols down to the nanomolar level [28].

3.2. Application in Analysis and Diagnostic Kits

It is evident that peroxidase from other sources could be a good substitute for HRP but still horse radish peroxidase is the practically widely used enzyme for analytical applications. Peroxidase also follows simple mechanism for these investigative procedures. For instance, in comparison to the use of sophisticated instruments like spectrophotometer and various biosensors for the determination of lactose level in milk and dairy products is replaced by a simpler and economical biostrip technology developed by Sharma et al in 2002 where HRP and enzymes like galactose oxidase and Beta galatosidase are immobilized on the polymeric support for the purpose to detect the lactose level. Simply by dipping the biostrip in the diary product or milk can determine the level of lactose present through developing the colour from the added chromogen.

Peroxides has generally good ability to yield chromogenic products even in low concentration and that is why it is used for the preparation of enzyme conjugated antibodies and has a wide applications in diagnostic kits. A diagnostic kit for determining the level of uric acid has been developed by Agostini et al in 2002 by using purified peroxidases isoenzymes from the turnip roots and hairy root cultures [7]. The crucial factor in the peroxidase catalysed reaction is the presence of enzyme in a saturated amount so that the chromogenic signal generated is stiochiochemically relevant to the amount of H_2O_2 produced during the reaction. A linear response with time has usually been observed for the concentration of turnip peroxidase when uric acid concentration is increased to 30 mM. Same results were obtained when a kit containing turnip peroxidase was used for the analysis of uric acid from ten different individuals serums which indicates that cationic peroxidase isoenzyme from turnip hairy roots could be used as a reagent for clinical diagnosis kit where hydrogen peroxide is generated.

Aberrant level of cholesterol is associated with some kind of diseases and thus its quantification is essential. Cholesterol determination is achieved by an enzymatic colorimetric method already developed that shows accuracy and precision selectivity along with assay rapidity [29]. The method encompasses the use of cholesterol esterase and oxidase along with peroxidase. However, the cost and stability of enzymes has confined this method applicability in routine practices. Same enzymes were separately immobilized by Malik and Pundir (2000) successfully and achieved high selectivity and increased stability for the enzymes [30]. This method is characterized with the hydrolysis of cholesterol ester by cholesterol esterase that free fatty acid and cholesterol that is later oxidized by cholesterol oxidase to cholestenone and hydrogen peroxide. The resulting quinoneimine is measured at 520nm.

3.3. Enzyme Immunoassays

For testing or detecting antigens or antibodies, a system was developed known as enzyme linked immunosorbent assays shortly termed as ELISA. These tests detect antigens by producing enzyme triggered change of colour. An antigen, antibody and a chromogenic substrate is required for this assay. The chromogenic substrate in the presence of HRP changes colour and thus detected. ELISA tests have been developed for detecting monoclonal antibodies against mycotoxins, a fatal by-product of numerous fungal species like asergillius and pencillium. These toxic chemicals are categorized as teratogenic, mutagenic, hepatotoxic or nephrotoxic to a range of higher class animals i.e. mammalian species. It is necessary to detect mycotoxins in the food as it is regularly found and it presence can cause serious health issues. Usually these mycotoxins can be screened through various biochemical techniques like GCMs, HPLC or TLC however; these techniques are either expensive or time consuming and sometimes need sophisticated sample cleanup. In contrast, ELISA has many advantages over the detecting techniques mentioned above like cost effectiveness, reliability, simplicity, less expertise requirement and simple instrumentation.

4. Conclusion

It is evident that peroxidases have numerous promising roles in the commercial processes and have wide potential for range of applications but unfortunately the enzyme is restricted only to be utilized for labeling antibodies and diagnostic kits. The ultimate reason behind this is the oxidation of peroxidases by peroxides. Second reason behind the confined application of peroxidase is the less water solubility potential of the substrates. Moreover, high temperatures can greatly affect the peroxidases abilities and restricting its use at low temperature only. Furthermore, it is

difficult to encounter the stability problem of the peroxidases and hard to solve this problem. Despite of the reduction and less operational stability, peroxidase is still a proficient biocatalyst for the industrially produced mass products. Therefore, structurally altered and genetically mutants peroxidase if generated from varied plant sources will upgrade its stability and enzymatic potentials. This new version of peroxidases would be certainly valuable for numerous industrially relevant applications in future for longer time.

Conflicts of Interest

All the authors do not have any possible conflicts of interest.

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