Role of Peroxidase in Clinical Assays: A Short Review

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Abstract

Development of sensitive enzymatic methods for hydrogen peroxide is key for the quantification of several bioconstituents such as glucose, triglycerides, creatinine, and uric acid and so on. Hydrogen peroxide released by the oxidase enzymes are quantified by peroxidase enzyme involving spectrophotometry, fluorimetry, chemiluminescence, potentiometric sensing, amperometric, coulometric and such others. Authors in this line, present a short review on the assay of peroxidase. The entire review is divided into three different sections; first the importance of peroxidase clinically, secondly peroxidase chemistry with hydrogen peroxide and finally its role in the assay of bioconstituents.

Keywords: Peroxidase; Clinical importance; Oxidase enzymes; Glucose assay; Nano sensors

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Peroxidase: A Clinically Important Enzyme

Peroxidases are widely distributed in nature especially in animal and plant cells. Peroxidases comprise of three major categories; plant peroxidases, animal peroxidases and catalases. These enzymes utilize hydrogen peroxide to catalyze the oxidation of variety of organic and inorganic compounds.

Over the years the development in clinical and diagnostic techniques, redox natured reactions are gaining vital importance. Biochemical reactions that are redox are gaining prominence in basic as well as applied research. Peroxidases are the driving force among these as most of the clinically important assays such as glucose; creatinine and uric acid determinations cannot be carried out without peroxidases. Immobilization of peroxidase enzyme has received much attention in the construction of biosensors due to economical and easy viability in enzyme immunoassays and enzyme linked immunosorbent assays. Keeping the views in logical frame work, the authors discuss the important role of peroxidase in clinically important assays, its chemistry behind hydrogen peroxide reaction extending to assay of diabetically important glucose and finally providing a tabulated physiological range of biochemical components in blood.

Peroxidase: A Friend to Oxidase Enzymes

The reactions catalyzed by peroxidases can be grouped into oxidative dehydrogenation, oxygen transfer, oxidative halogenations and hydrogen peroxide dismutation. Among these, oxidative dehydrogenation has greater application in analytical biochemistry.

Peroxidases are having great potential application as they can be used in a diagnostic kit for hydrogen peroxide, glucose and oxidase enzyme determination [1]. In particular, HRP are widely used in research areas such as enzymology, biochemistry, medicine, genetics, physiology, histo and cyto chemistry because of easy availability, economical and high catalytic activity [2]. The commercial production of peroxidase has increased due to its analytical diagnostics particularly biosensing, in immunosensors and in nucleic acid detection. Heme proteins named peroxisomes are present in high concentration in cell compartments preventing excessive accumulation of peroxide, a powerful oxidizing agent. HRP meets the entire requirements for successful analytical enzymology because of its specificity, flexibility in assay, stability, sensitivity of detection, and availability in pure form. HRP on coupling with oxidase enzyme can be used for analysis of wide range of analytes such as glucose, cholesterol, lactic acid, choline, xanthine, uric acid, bilirubin, and creatinine.

Combination of peroxidase and indole-3-acetic acid is currently being used as a cancer therapeutic agent [3]. The therapeutic
procedure involves two steps; first decoding of enzyme in the
tumor cells, second administering specific prodrug, indole-3-
acetic acid which gets converted into cytotoxic drug by the
enzyme expressed in the target tumor. The decarboxylated form
of radical cation indole-3-acetic acid can conjugate with DNA
and other biological nucleophiles [4]. Some other anticancer
strategies adopted include antibody and polymer directed
enzyme/prodrug therapy.

Chemistry of Reaction between Peroxidase and Hydrogen Peroxide

Peroxidases can catalyze a variety of organic substrates such as
phenols, and aromatic amines with two step three intermediate
cyclic processes. The process is as under

**Step I:** The formation of HRP-I with the incorporation of oxygen
by the reaction with hydrogen peroxide (oxoferryl). The oxidation
state of iron is +4 along with porphyrins-r-radical cation.

\[
\text{R} \quad \text{Fe}^{3+} + \text{H}_2\text{O}_2 \quad \rightarrow \quad \text{R} \quad \text{Fe}^{4+} = \text{O} + \text{H}_2\text{O}
\]

\[
\text{HRP} \quad \text{HRP-I}
\]

**Step II:** This HRP-II has a tendency to oxidize organic substrates
by single electron transfer mechanism

\[
\text{HRP-I} + \text{AH}_2 \quad \rightarrow \quad \text{HRP-II} + \text{AH}
\]

**Step III:** The conversion of HRP to native state by second substrate
molecule.

\[
\text{HRP-II} + \text{AH}_2 \quad \rightarrow \quad \text{HRP} + \text{AH}
\]

The initial steps of the reaction between peroxidase and hydrogen
peroxide involves the basic amino acid residue (B), in the reaction
[5] is depicted as:

\[
\text{Protein} \quad \text{Fe}^{3+} \quad \text{H}_2\text{O}_2 \quad + \quad \text{B} \quad \text{Protein} \quad \text{Fe}^{4+} \quad \text{OOH}^- \quad + \quad \text{BH}^+
\]

\[
\text{Protein} \quad \text{Fe}^{4+} \quad \text{OOH}^- \quad + \quad \text{BH}^+ \quad \rightarrow \quad \text{Protein} \quad \text{Fe}^{3+} \quad \text{O} \quad + \quad \text{H}_2\text{O} + \quad \text{B}
\]

The structures of oxoferriyl species as confirmed by different
research groups.

The intermediate of the reaction is highly unstable with spectrum
has been reported in cry solvents at temperatures less than -16°C
[6,7] (Scheme 1).

The initial interaction between peroxidase and hydrogen peroxide
consist the formation of two electron bonds between iron (III)
and one of the peroxide oxygen’s. The basic amino acid residue
acts as a mediator in the transfer of proton between α and β
oxygen which subsequently reacts and loses a molecule of water
along with the release of basic amino acid residue. The final oxy-
ylene structure consists of either a double bond or a dative
bond with both electrons originating from an oxygen atom. This ferryl
intermediate is responsible for the formation of hydroxyl free
radical which in turn causes tissue damage in vivo suggesting that
the oxygen may dissociate easily from the heme protein.

The oxyhemoglobin model [5] includes linear, bent, ring, met-
superoxide model and two electron oxidative additions as listed
in Table 1.

Glucose: An Often Difficult Species for Quantification

The two different structures glucose are closed loop and open
chain. The closed loop α and β structures are in equilibrium
with each other. The open chain structure has an aldehydic
reactive group, which can undergoes oxidation resulting in the
formation of gluconic acid with the concominant formation of
hydrogen peroxide. The gluconic acids are difficult to quantify
by the conventional colorimetric methods as carboxylic acids
are least accessible for colorimetric methods. The blessing in
disguise is the formation of hydrogen peroxide as a byproduct
in the oxidation of glucose by glucose oxidase. The hydrogen
peroxide liberated is active in various instrumental methods
such as spectrophotometric, fluorimetric, potentiometric,
ampferometric and enzymatic approaches. Enzymes that
use hydrogen peroxide as a substrate include catalase and
peroxidase. Catalase decomposes hydrogen peroxide into water
and oxygen. Oxygen can be determined by monometric or UV-Vis
spectrophotometric or electrochemical methods. Peroxidase converts
hydrogen peroxide into a highly reactive hydroxyl radical, a
powerful oxidant. Hydroxyl radical can oxidize various benzene
centered species into a highly reactive radical intermediate
which in turn can couple with secondary reagents such as phenol
or amine to result in colorimetric signal. Enzymes that oxidize
glucose as a principal substrate include glucose dehydrogenase,
Quinoprotein glucose dehydrogenase, glucose-1-oxidase, and
glucose-2-oxidase and glucose oxidase. Glucose oxidase is the
only enzyme used in the oxidation of glucose because glucose

![Scheme 1] Formation of ferryl species in cryosolvents.
Fe
Fe+++ O-
O

Determination.

Table 2

<table>
<thead>
<tr>
<th>Nano substrate</th>
<th>Linearity range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nanoprism</td>
<td>0.2-100 μM</td>
<td>[10]</td>
</tr>
<tr>
<td>Multicorel carbon nanotube</td>
<td>0-5 mM</td>
<td>[11]</td>
</tr>
<tr>
<td>CuS nanoparticle</td>
<td>2-1800 μM</td>
<td>[12]</td>
</tr>
<tr>
<td>ZnFe2O4</td>
<td>1.25-18.75 μM</td>
<td>[13]</td>
</tr>
<tr>
<td>Mn doped ZnS Quantum dots</td>
<td>10-0.1 mM and from 0.1-1 mM</td>
<td>[14]</td>
</tr>
<tr>
<td>Fe3O4</td>
<td>3.0-1000 μM</td>
<td>[15]</td>
</tr>
<tr>
<td>Cupric oxide</td>
<td>0.1-8.0 mM</td>
<td>[16]</td>
</tr>
<tr>
<td>Carbon nitride dots</td>
<td>1-0.1 mM</td>
<td>[17]</td>
</tr>
<tr>
<td>Prussian nanoparticles</td>
<td>0.1-50.0 μM</td>
<td>[18]</td>
</tr>
</tbody>
</table>

dehydrogenase and quinoprotein glucose dehydrogenase are specific for β form of glucose with high turnover rates but the former is less soluble while the latter is unstable. Glucose-2-oxidase also oxidizes other carbohydrates such as xylose and glucolactone but lack of specificity overrules its use [8]. The glucose oxidase can be isolated from variety of sources among these Aspergillus niger is very often used. It is more stable in the physiological pH at which quantitative analysis can be carried out. The most commonly used substrates are o-dianisidine, tetrymethylbenzidine, ABTS, 4-aminoantipyrine and phenol, Guaiacol, pyrogallol, cyanine dyes. Our research group has proposed different analytical probes for the assay of hydrogen peroxide involving HRP which include PPDD-DMA, PPDD-NEDA and DMA [9]. Nano, a dwarf technology with one of its dimension measured in nanometer, are also not left behind in the analysis. Nanoparticles mimic the behavior of peroxidase enzyme, and they can be used as substitutes for peroxidase in the assay of glucose in the physiological range. Use of nanoparticles help to overcome certain important constraints like; control of temperature in enzymatic assay, involving several steps to immobilize the enzyme on the solid support thereby reducing the enzyme activity, waste of expensive enzymes because of its non-utilization completely, reduce the cost. Nanoparticles attracted scientific and technological interest because of ease of preparation, biocompatibility, and large surface to volume ratio collective oscillations of the surface electrons emitting light in the visible range. Table 2 lists some nanosensors used in glucose determination.

Glucose Monitoring Methods: Invasive and Non-Invasive

The normal physiological range of glucose is 3.3-6.6 mM (60 mg/dL to 120 mg/dL). Frequent glucose determination in diabetic patients is required for the maintenance of glucose in blood. The invasive methods require regular lancing and finger bleeding. The noninvasive methods overcome pain in blood extraction, exposure to sharp objects, biohazard materials and the potential for increased frequency of testing [19]. The dual enzymatic colorimetric method involves glucose oxidase and peroxidase. The glucose oxidase catalyses the conversion of β-D-glucose by molecular oxygen producing gluconic acid and hydrogen peroxide. Glucose oxidase requires redox co-factor, FAD, which gets reduced to FADH2. The co-factor is regenarated by molecular oxygen with the formation of hydrogen peroxide. Furthermore, hydrogen peroxide can be monitored by spectrophotometry, fluorimetry, chemiluminescence, potentiometric sensing, amperometric, coulometric and such others.

The spectrophotometric, fluorimetric and luminescence methods involve the formation of the colored or luminescence active product. In potentiometry, amperometry and coulometric, the electrons liberated/consumed is directly proportional to the number of glucose molecules as shown in Scheme 2.

Biosensor is in the modification process which was initially proposed by Clark and Lyon of Children hospital in Cincinnati. The glucose biosensor was composed of oxygen electrode coated with a thin layer of glucose oxidase and an outer dialysis membrane. Decrease in the concentration of oxygen is taken as a measure of glucose concentration in the blood or urine sample. The major disadvantage in this is operational potential for hydrogen peroxide and oxygen deficit in the samples. The high operating potential of biosensors was interfered by ascorbic acid, uric acid and drugs like acetaminophen. These problems led to the development of second generation sensors such as ferrocene, ferricyanide, quinines, tetrathialfulvene, tetracyanoquinodimethane, thionine and methylene blue by the electron mediators to get improved performance. The use of mediators was overcome in the third generation biosensors, which have direct electron transfer reaction between the enzyme and the electrode.

Other non-invasive approaches include polarimetry [20] Raman spectroscopy [21], photoacoustics [22], optical coherence tomography [23], and photothermal deflectometer enhanced by total internal reflection [24].

As per the World Health Organization diagnosis should be ASSURED: Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment free and Delivery to end users [25]. Point care testing, microfluidic paper based analytical devices is rapid, less expensive, and more multiplexed than the instrumental analysis. They require small volume of liquid with little or no extra instrument for the quantification of biocomponent, a visual differentiation is only sufficient in making the near conclusion.

Table 2: Glucose linearity range of some nanosensors.
Enzyme immunoassay describes a broad range of assays to qualify and quantify antigens or antibodies. A typical assay involves immobilization of antigen with antibody on to a solid support. A secondary antibody labeled with peroxidase provides a detectable signal in the presence of colorimetric indicator. These are extensively used in enzyme linked immuno sorbent assays, western blotting and immuno-histochemistry techniques. Table 3 provides physiological range of bioconstituents in blood.

**Conclusion**

Peroxidase has a prominent place in biotechnology and associated areas such as microbiology, histochemistry, medicine, genetics, and clinical chemistry due to its versatility, viability, and economical nature. The major problems with peroxidase enzyme are suicide killing with the concomitant release of hydroxyl radical and lack of analysis at high temperature. Hence mutated peroxidase from different plant sources with improved stability and activity will be better contender for bioconstituents application. The use of nanomaterials which strongly influences the properties like stiffness, elasticity, and biocompatibility environment for oxidase enzymes can avoid peroxidase in the dual enzymatic systems.

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![Scheme 2](http://clinical-nutrition.imedpub.com/archive.php) General scheme for the determination of glucose.
Secondary enzyme | Substrate for secondary enzymes | Coenzyme/cofactor | Secondary product | Optimal pH | Normal physiological range of substrates* | References
---|---|---|---|---|---|---
Glucose oxidase | β-D-glucose | FAD | D-glucono-δ-lactone | 5.5 | 60-120 mg/dL | [27]
Alcohol oxidase | Primary alcohol | NAD⁺ | Aldehyde | 7.5 | - | [28]
Lactate oxidase | Lactic acid | FMN | Pyruvic acid | 7.2 | 4-20 mg/dL | [29]
Choline oxidase | choline | FAD | Betaine | 9-10 | - | [30]
D-aminoacid oxidase | D-aminoacid | FAD | α-ketoacid | - | 30-50 mg/dL | [31]
L-amino acid oxidase | L-aminoacid | FMN | 2-oxoacid | - | 30-50 mg/dL | [32]
Xanthine oxidase | Hypoxanthine | FAD | Uric acid | 7.5 | - | [33]
Uricase | Uric acid | Copper | Allantoin | 6.5-7.5 | 3.0-7.0 mg/dL | [34]
Cholesterol oxidase | Cholesterol | FAD | Cholest-4-en-3-one | 7.0-9.0 | 150-200 mg/dL | [35]
Polyamine oxidase | N₁-acetylspermine | FAD | N₁-acetylspermidine and 3-aminopropanal | 7.4 | - | [36]
Bilirubin oxidase | Bilirubin | - | biliverdin | 8.5 | 0.2-2.0 mg/dL | [37]
Glutamate oxidase | L-glutamate | FAD | 2-oxoglutarate | 7 | 8.0-10.0 mg/dL | [37]

Note: Optimum pH depends on the nature of substrates, co-substrates and methodology adopted in the determination.
References


