Research Article

A Spectrophotometric Method for the Assay of Peroxidase Using Para-phenylenediamine dihydrochloride and Iminodibenzyl as Chromogenic Reagents: Applications in Some Plant Sources

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Abstract

A kinetic model for measurement of rate reaction for peroxidaseactivity using para-enylenediaminedihydrochloride (PPDD) and iminodibenzyl (IDB) is presented. Under KH_2PO_4/K_2HPO_4 buffer (pH 7.0), PPDD entraps the free radical and gets oxidized to electrophilic diimine, which couples with IDB to give an intense green colored product with λ_{max}750nm at 25°C. The peroxidase assay is achieved from 0.379 to 6.064nM and the linearity for H_2O_2 was 3.5 to 164 μM. Catalytic efficiency and catalytic power of commercial peroxidase are 3.720×10^4 μM^{-1}min^{-1} and 2.251×10^{-3} min^{-1}, respectively. Catalytic constant (k_{cat}) and specificity constant (k_{cat}/K_m) at saturated concentration of co-substrates were 0.032 min^{-1} and 3.717×10^{-4} μM^{-1}min^{-1} respectively. Michaelis–Menten constants for H_2O_2, PPDD and IDB were $K_m^{H_2O_2}$ = 85 μM, K_m^{PPDD} = 69 μM and K_m^{IDB} = 92 μM respectively. The method was successfully tested peroxidase activity in some plant extracts.
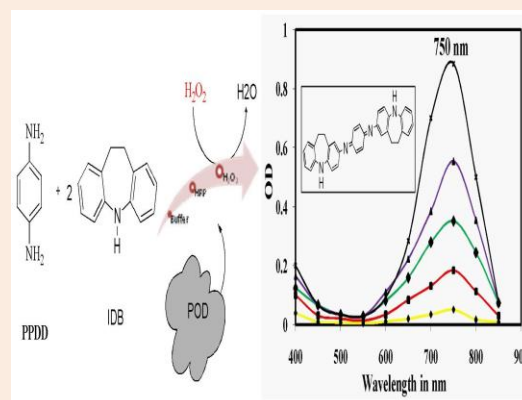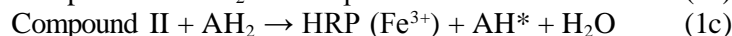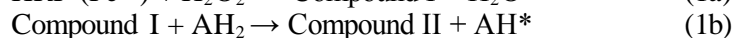
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Introduction

Horseradish peroxidase (EC 1.11.1.7; donor-H_2O_2 oxidoreductase) is one of the heme peroxidases and it is defined as oxidoreductase, which acts on peroxide as electron donor. HRP has been extensively studied and frequently used to exemplify the peroxidase reaction cycle. HRP catalyses the reduction of peroxides with oxidation of the heme group in the active site of the enzyme and the reaction mechanism can be represented as follows [1–4].

$$HRP\ (Fe^{3+}) + H_2O_2 \rightarrow Compound\ I + H_2O \qquad (1a)$$
$$Compound\ I + AH_2 \rightarrow Compound\ II + AH* \qquad (1b)$$
$$Compound\ II + AH_2 \rightarrow HRP\ (Fe^{3+}) + AH* + H_2O \qquad (1c)$$

Peroxidase catalyzes a variety of oxidative transformations of organic and inorganic co-substrates by using hydrogen peroxide as a substrate [5, 6]. The reduction of peroxides at the expense of electron donating substrates make peroxidase useful in a number of industrial, analytical and biotechnological applications [7]. Peroxidase is a ubiquitous enzyme in plants, microorganisms and animals, where it plays important roles. In plants, it participates in the lignification process and the mechanism of defense in physically damaged or infected tissues [8]. In food industry, peroxidase has been widely used as an indicator of the capability of vegetable blanching due to its high thermal

stability and wide distribution [9]. It is used in various analytical applications in diagnostic kits, such as quantification of important biomarkers such as glucose, uric acid, cholesterol, glutamate and choline [5]. Of later peroxidase based biosensors have found application in analytical systems for the quantification of hydrogen peroxide and organic hydro peroxides, while co-immobilized along with H_2O_2- producing enzymes they can also be used in the quantification of above stated biomarkers. Due to its ability to convert colorless substrates into chromogenic products, peroxidase is well suited in the preparation of enzyme-conjugated antibodies, which are used in enzyme-linked immunosorbent assay (ELISA) tests [10, 11]. Peroxidase can be used in the detoxification of various phenols and aromatic amines present in polluted water [12-14]. More recently reports have appeared on the decolorization and removal of fabric dyes from polluted water and dyeing effluents by using soluble and immobilized peroxides [15, 16].

Several methods are available for the quantification of peroxidase activity, which include the spectrofluorimetry and luminescence [17]. However, they have their own limitations, as the instruments used in fluorometry and luminescence is too expensive and less flexible. Another disadvantage of the luminescence is its poor selectivity [18]. The electro-analytical system involves several steps to immobilize the enzyme on a solid support, which may reduce the enzyme activity. During the assimilation of the enzyme within the solid support, a large amount of enzyme is not utilized, which results in the wastage of an expensive biocatalyst. Spectrophotometers are cost-effective, easy to handle, and the reagents used are generally economical. The unfussiness, rapidity, facile and inexpensive properties of spectrophotometry has made it an exemplary method to determine the activity of HRP [19]. In spectrophotometric method a wide range of hydrogen donors (e.g., caffeic acid and 2, 2-azino-bis (3-ethylbenzothiazolin- 6-sulfonate)) have been utilized in peroxidase assay systems including methods which use potentially carcinogenic and mutagenic materials [20, 21]. With the objective of utilizing the non-toxic chemicals in order to reduce the amount of formation of hazardous products, new chromogenic reagents which are less toxic when compared to other co-substrates for the assay of HRP has been proposed.

In the proposed method a detailed study on the horseradish peroxidase and hydrogen peroxide an enzyme-substrate compound is presented. A new kinetic mathematical model has been developed to evaluate the Michaelis–Menten constants. In the method PPDD-IDB couple serves as hydrogen donor for horseradish peroxidase assay and it is an elegant, highly sensitive spectrophotometric method for the determination of HRP activity and micro molar quantity of hydrogen peroxide. The coupled green coloured product is stable and has maximum absorbance at 750 nm. The absorption at longer wavelengths allows it to avoid the background interference caused by the crude extracts. The linearity range for the assay of peroxidase makes the method more considerate than the guaiacol method. The method has been tried in the quantification of peroxidase activity in crude extracts of some medicinal plants.

Experimental

Chemicals and reagents

All chemicals used in the assay were of Analytical grade. PPDD and IDB were purchased from Sigma-Aldrich and Merck, Germany, respectively. Peroxidase (EC 1.11.1.7, 100 units/mg) was purchased from Himedia Laboratories, Mumbai, India. H_2O_2 (30%) was purchased from E-Merck, Mumbai, India. Guaiacol was obtained from Loba Chemie, India. Double-distilled water was used throughout the experiment. PPDD (16.56 mM) solutions were prepared by dissolving requisite quantity in distilled water. IDB (51.2 mM) was initially dissolved in 5 μL of acetic acid and made up to 5 mL with distilled water. H_2O_2 stock solution (100 mM) was prepared daily and standardized by potassium permanganate method. Working standard solutions were prepared from the stock solution by dilution. Peroxidase stock solution was prepared by dissolving 2 mg in 10 mL of 100 mM potassium KH_2PO_4/ NaOH buffer at pH 6.0. Further dilution with the same buffer was made when required.

Equipment

All absorbance measurements were carried out with a JASCO model UVIDEC-610 ultraviolet–visible (UV–Vis) spectrophotometer with matched cuvettes of 1cm thickness. Water bath shaker (NSW 133, New Delhi, India) used to maintain constant temperature. EQ-614 pH meters (model, Equip-tronics, Mumbai, India) are used to adjust pH.

Plant crude extract preparation

As a source of peroxidase, crude extracts were prepared using leaf or stem portion of *Mimosapudica, Costus igneus, Yucca gloriosa or a Palm lilly, Ixora chinensis, Moringa oleifera, Aegle marmelos, Limonia acidissima* and *Syzygium cumini* which were collected from the local gardens and transported at 4 °C to the laboratory and stored at -20 °C until used. The extracts were prepared by carefully observing enzyme activity in different buffer solutions. In the buffer solution of potassium dihydrogen phosphate/sodium hydroxide at pH 6.0 the crude extract showed more activity. Hence all plant extracts were prepared using the same buffer solutions. Samples (5 g) were washed with distilled water and homogenized in a blender using 50 mL of 100 mM buffer at pH 6.0. The extract was passed through cheese cloth and centrifuged at 12000 g for 15 min, and the supernatant was labeled as crude extract. Stock enzyme solutions were stored at 4°C and warmed to room temperature immediately prior to use.

Recommended procedure for the assay of peroxidase activity

The reaction mixture used contained 23 μM PPDD, 17.06 μM IDB, and 164 μM H_2O_2 in 100 mM dihydrogen orthophosphate/dipotassium hydrogen orthophosphate buffer of pH 7.0. Reaction was initiated by adding 100 μL of varying concentrations of peroxidase. Change in absorbance was continuously recorded against the corresponding control containing all reagents except peroxidase at 25 °C. The initial velocity was recorded by the absorbance-time curve. The linear relationship between the initial velocity and the concentration of enzyme from rate method was 0.379 to 6.064 nM. In the fixed time method, the reaction mixture was incubated for 5 min at 25 °C and the peroxidase was assayed in the concentration range of 0.076 to 1.516 nM. The linear relationships by the rate and fixed time methods are shown in **Figure. 1.**
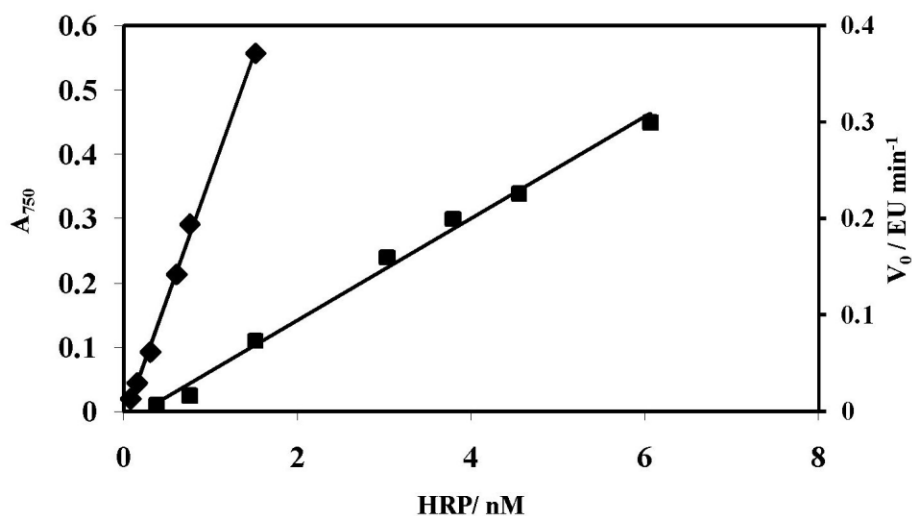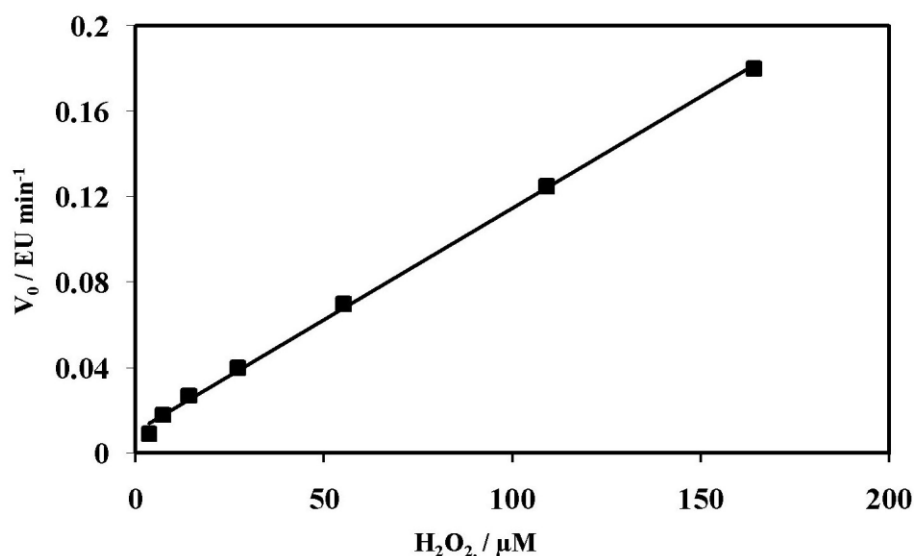

Figure 1 Calibration graph for the quantification of horseradish peroxidase by rate method (■) and fixed time method (♦)

Recommended procedure for quantification of H_2O_2

The concentration of H_2O_2 was determined in 3 mL of the solution, which contained 23 μM PPDD, 17.06 μM IDB, and 6.064 nM peroxidase in 100 mM dihydrogen orthophosphate/dipotassium hydrogen orthophosphate acid buffer at pH 7.0. Reaction was initiated at 30 °C by adding 100 μL of different concentrations of H_2O_2 within the linear range. The change in the absorbance was continuously recorded at 750 nm. The initial rate was plotted against the concentration of H_2O_2 to get the calibration graph. The linearity of the graph lies between 3.5 and 164 μM H_2O_2. The

calibration graph for the quantification of H_2O_2 is presented in **Figure. 2.** The value of $K_m^{H_2O_2}$ (85 μM) was determined from kinetic model and V_{max} (0.1916 min^{-1}) for the H_2O_2 from the plot of rate *verses* concentration of the substrate.

Figure 2 Calibration graph for the quantification of H_2O_2 by rate method

Protein assay

The total protein concentration was determined in triplicate by the Lowry [22] method, using bovine serum albumin as standard.

Results and discussion

Effect of PPDD and IDB

The effect of varying concentrations of PPDD and IDB showed that the rate increased on increasing the concentration of PPDD from 6 to 23 μM beyond which there was no considerable increase in the rate. Hence for all assays PPDD concentration of 23 μM was selected. Similarly, the effect of IDB concentration on the reaction rate was studied from 2.1 to 170.1 μM. The linearity prevailed up to 17.06 μM, above this concentration there was no effect on the rate. Hence 17.06 μM of IDB was selected as the optimized concentration for further analysis.

Effect of temperature

The stability and activity of the enzyme are influenced by the temperature. This can be clearly observed while studying enzyme thermal inactivation. Enzyme activity increases with temperature but enzyme stability decreases. These opposite trends make temperature a critical variable in any enzymatic process and demand the need for optimization. Temperature sensitivity and optimization were determined by pre-incubating the optimized concentration of reaction mixture for 5 min at varying temperatures (0 - 80 °C). The activity of the enzyme was registered as a function. of the absorbance of the colored solution. The maximum activity was observed at 30 °C.

Effect of pH and concentration

pH is known to alter the activity of enzymes as it affects ionization state of side chains of enzymatic proteins. The buffers of different concentrations studied for the assay include, citric acid/potassium citrate at pH 3.6-5.6,

acetate/acetic acid at pH 3.6-5.6, potassium dihydrogen phosphate/sodium hydroxide at pH 6.0-8.0, and potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate at pH 6.0-7.5. The activity of the enzyme was highest in 100 mM dihydrogen orthophosphate/dipotassium hydrogen orthophosphate buffer of pH 7.0. Hence, all studies were carried out at this pH.

Effect of non targeting species

The effect of various non-target species in the determination of HRP was investigated. Under the reaction conditions used for the coupling reaction, the suitability of the method for application in medicinal plant samples was studied by taking various cation, anions and other commonly accompanying amino acids in solutions containing 164 μM H_2O_2. The result indicated that there was no interference by any of the ions tested. It can be seen that the method is highly selective and that some compounds except ascorbic acid, Cu^{2+}, Fe^{3+}, L- Tryptophan, L- Tyrosine, L- Cystine, L-cysteine, Fe^{2+}, Zn^{2+}, L- serine, Citric acid and Uric Acid none of the other foreign species studied interfered in the quantification. The tolerance ratios are tabulated in **Table 1**.

Table 1 Effect of diverse foreign ions on the spectrophotometric determination of peroxidase activity
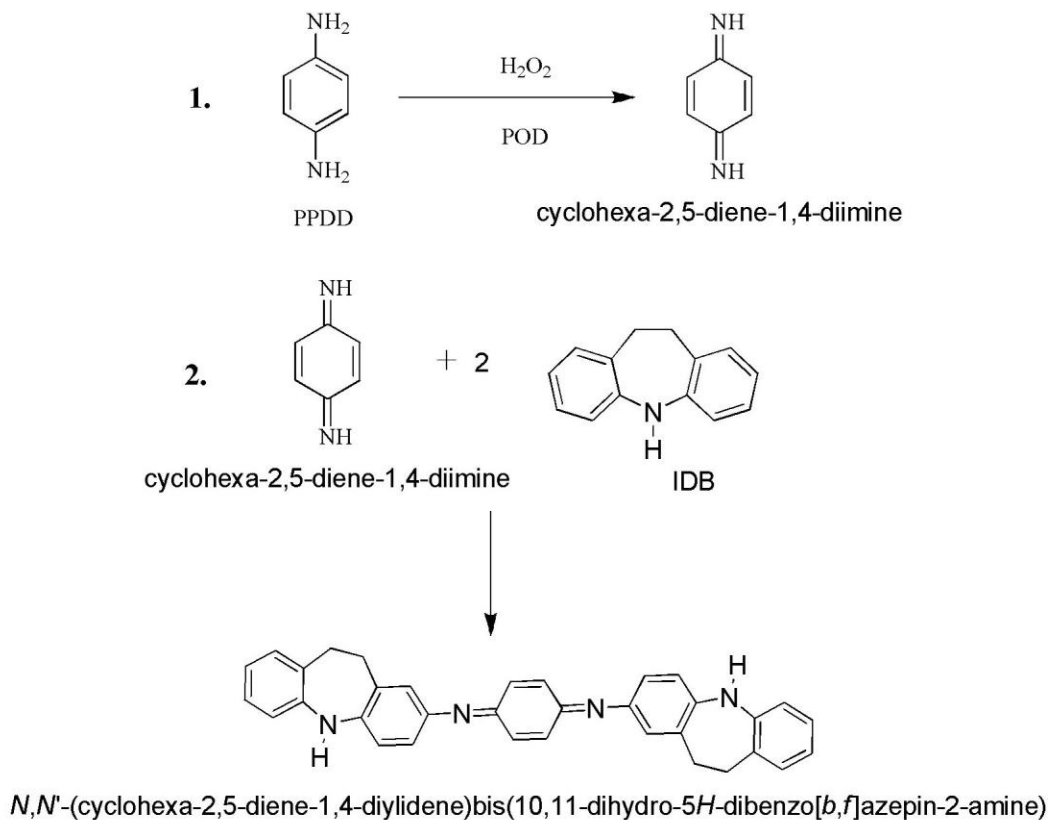
Foreign Species	Tolerance ratio[a]
Ascorbic acid	0.00065
Cu^{2+}	0.15
Fe^{3+}, L- Tryptophan	0.35
L- Tyrosine, L- Cystine, L- Cysteine	0.95
Fe^{2+}	1.21
Zn^{2+}, L- serine, Citric acid, Uric Acid	1.82
Oxalic acid, DL- Methionine	6.21
F^-	8.60
D- Asparagine, NO_2^-	10.11
L- Histidine, Isoleucine	15.23
DL- Threonine	48.23
K, Cl	50.23
NH_4^+	65.25
Urea	85.78
Na^+	70.45
SO_2^-, Lactose	100.55
Glycine	210.14
Galactose	200.50
Sucrose	500.21
Glucose	1000.00

[a] Tolerance ratio corresponds to ± 3 % error in the recovery

Reaction pathway for the enzyme activity response

The enzymatic mechanism of peroxidase in the presence of H_2O_2 for PPDD and IDB is similar to that suggested by Ngo and Lenhoff [11] for HRP-catalyzed oxidative coupling of 3-methyl-2-benzothiazolinonehydrazone hydrochloride and aromatic amines with the formation of indamines dye. The mechanism for the peroxidase-catalyzed reaction of PPDD and IDB is proposed in Scheme 1. The free radical is released by the oxidation of H_2O_2

through a ferryl intermediate (Fe IV = O-porphyrin π-cation radical) of the peroxidase.[5] Under the reaction conditions examined, PPDD loses two electrons and two protons upon enzymatic oxidation in the presence of H_2O_2, forming electrophillic 1, 4-diimine, which may be postulated as the oxidative coupling species.[16] The 1, 4-diimine undergoes electrophillic substitution with IDB in the free para position to the N, N-dimethylamino group, forming an intense green-colored product showing strong absorption at 750 nm.

1.

PPDD   →(H_2O_2, POD)→   cyclohexa-2,5-diene-1,4-diimine

2.

cyclohexa-2,5-diene-1,4-diimine   + 2   IDB

↓

N,N'-(cyclohexa-2,5-diene-1,4-diylidene)bis(10,11-dihydro-5H-dibenzo[b,f]azepin-2-amine)

Scheme 1 Suggested reaction for the formation of green coloured product

Mathematical representation of the determination of Michaelis constants, catalytic constants and substrate specificity

The kinetic constants were studied and the peroxidase activity was measured under optimal buffer pH. The Michaelis constant (K_m) and maximal velocity (V_{max}) of the peroxidation of various hydrogen donors and H_2O_2 by the purified peroxidase were determined at various substrate concentration ranges. The saturation concentration for each substrate was first determined. The K_m and V_{max} of H_2O_2 were calculated using the plot of reaction rate versus substrate concentration. K_m values from the kinetic model and Michaelis Menten plot are found to be same.

The catalytic constants of co-substrates were calculated by following the assay procedure and this can be carried out in three steps.

Step 1: Concentration of H_2O_2 was varied in the order, 3.4, 7.0, 14.0, 27.0, 55.0, 109.0 and 164.0 μM, with constant 0.017 mM IDB and 0.046 mM PPDD concentrations. Assuming that the initial rate of the reaction as V_0 and the initial concentration of H_2O_2, PPDD and IDB as H_0, P_0 and I_0, respectively the general equation for the reaction is written as,

$$\frac{H_0 P_0 I_0}{V_0} = \frac{K_H P_0 I_0}{V_{max}} + \left(\frac{P_0 I_0}{V_{max}} + \frac{K_I P_0}{V_{max}} + \frac{K_P M_0}{V_{max}} \right) H_0 \tag{1}$$

By plotting the graph of $H_0 P_0 I_0 / V_0$ verses the concentration of H_2O_2 we get,

$$Y = 3.738x + 373.6 \tag{2}$$

Step 2: Concentration of IDB was varied in the order: 2.1 μM, 4.3 μM, 8.5 μM, 17.0 μM and 34.0 μM with different H_2O_2 concentrations by maintaining a constant 46.0 μM PPDD concentration. The general equation can be written as,

$$\frac{H_0 P_0 I_0}{V_0} = \frac{K_I H_0 P_0}{V_{max}} + \left(\frac{H_0 P_0}{V_{max}} + \frac{K_H P_0}{V_{max}} + \frac{K_P H_0}{V_{max}} \right) I_0 \tag{3}$$

The plot of $H_0 P_0 I_0 / V_0$ verses the concentration of I_0 gives,

$$Y = 46.820x + 35.628 \tag{4}$$

Step 3: Concentration of PPDD was varied in the order: 9.2 μM, 18.4 μM, 27.6 μM, and 36.8 μM with different H_2O_2 concentrations by keeping a constant 17.0 μM IDB concentration. The general equation can be written as,

$$\frac{H_0 P_0 I_0}{V_0} = \frac{K_P H_0 I_0}{V_{max}} + \left(\frac{H_0 I_0}{V_{max}} + \frac{K_H I_0}{V_{max}} + \frac{K_I H_0}{V_{max}} \right) P_0 \tag{5}$$

The plot of $H_0 P_0 I_0 / V_0$ verses the concentration of P_0 gives,

$$Y = 13.783x + 58.936 \tag{6}$$

The slope of equations 1, 3 and 5 is,

$$m_1 = \frac{P_0 I_0}{V_{max}} + \frac{K_I P_0}{V_{max}} + \frac{K_P I_0}{V_{max}} \tag{7}$$

$$m_2 = \frac{H_0 P_0}{V_{max}} + \frac{K_H P_0}{V_{max}} + \frac{K_P H_0}{V_{max}}. \tag{8}$$

$$m_3 = \frac{H_0 I_0}{V_{max}} + \frac{K_H I_0}{V_{max}} + \frac{K_I H_0}{V_{max}} \tag{9}$$

The equations 7, 8 and 9 can be rearranged to give,

$$M_1 = \left(m_1 - \frac{P_0 I_0}{V_{max}} \right) = K_I \left(\frac{P_0}{V_{max}} \right) + K_P \left(\frac{I_0}{V_{max}} \right). \tag{10}$$

$$M_2 = \left(m_2 - \frac{H_0 P_0}{V_{max}} \right) = K_H \left(\frac{P_0}{V_{max}} \right) + K_P \left(\frac{H_0}{V_{max}} \right). \tag{11}$$

$$M_3 = \left(m_3 - \frac{H_0 I_0}{V_{max}} \right) = K_H \left(\frac{I_0}{V_{max}} \right) + K_I \left(\frac{H_0}{V_{max}} \right).$$

(12)

From the above equations, let us consider the values for PPDD as A_1, IDB as A_2 and H_2O_2 as B_2 that is,

$$A_1 = \frac{P_0}{V_{max}}$$

(13)

$$A_2 = \frac{I_0}{V_{max}}$$

(14)

$$B_1 = \frac{H_0}{V_{max}}$$

(15)

By calculating the values of A_1, A_2 and B_1, it can be expressed in the form of determinants as shown below,

$$D = \begin{vmatrix} 0 & A_1 & A_2 \\ A_1 & 0 & B_1 \\ A_2 & B_1 & 0 \end{vmatrix} \qquad D_1 = \begin{vmatrix} M_1 & A_1 & A_2 \\ M_2 & 0 & B_1 \\ M_3 & A_2 & 0 \end{vmatrix} \qquad D_2 = \begin{vmatrix} 0 & M_1 & A_2 \\ A_1 & M_2 & B_1 \\ A_2 & M_3 & 0 \end{vmatrix} \qquad D_3 = \begin{vmatrix} 0 & A_1 & M_1 \\ A_1 & 0 & M_2 \\ A_2 & B_1 & M_3 \end{vmatrix}$$

For H_2O_2 the Michaelies – Menten constant can be determined by,

$$K_H = \frac{D_1}{D}$$

$$K_H = \frac{-B_1^2 M_1 + A_1 M_3 B_1 + A_2 M_2 B_1}{2 A_1 A_2 B_1}$$

For IDB the K_I value can be determined by,

$$K_I = \frac{D_2}{D}$$

$$K_I = \frac{A_2 M_1 B_1 + A_2 A_1 M_3 - A_2^2 M_2}{2 A_1 A_2 B_1}$$
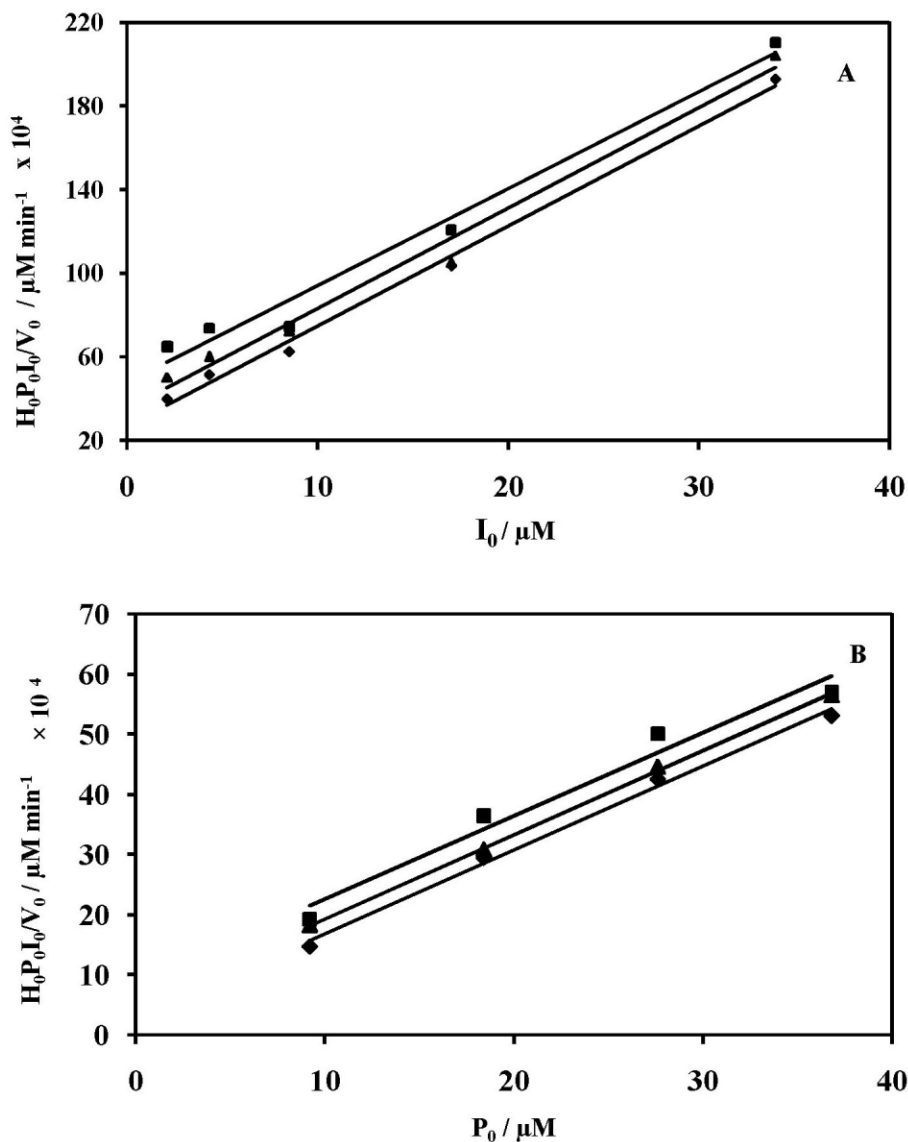
For PPDD, K_P value is determined by,

$$K_P = \frac{D_3}{D}$$

$$K_P = \frac{-A_1^2 M_3 + A_1 A_2 M_2 + M_1 A_1 B_1}{2 A_1 A_2 B_1}$$

From the above equations the K_H, K_I and K_P values for the substrate and co – substrates are 85 μM, 65.78 μM and 91.93 μM respectively.

The plot of $H_0P_0I_0/V_0$ verses the concentration of H_2O_2; $H_0P_0I_0/V_0$ verses the concentration of I_0 and $H_0P_0I_0/V_0$ verses the concentrations of P_0 are shown in the **Figure. 3.**

Figure 3 Kinetic behavior of two substrate reactions for the pure HRP (6.064 nM). (A) The plot of

$$\frac{H_0P_0I_0}{V_0}\; versus\,[I_0] \qquad \frac{H_0P_0I_0}{V_0}\; versus\,[P_0]$$

And (B)

Application to the crude medicinally valued plant tissues

Extraction of peroxidase from crude plant extracts was carried out with the buffer to tissue ratio ranging from 3:1 to 12:1 ml g^{-1}. Highest specific activity however was observed at 9:1 ml g^{-1} ratio. Hence, the extraction was carried out at this ratio for a better performance of the system. **Table 2** shows the peroxidase activity determined by the proposed and by guaiacol methods. The relative half-saturation point of the proposed method with reference to the guaiacol method is less than 1, indicating greater interaction between the active site, heme group of peroxidase, and PPDD–

IDB co-substrates. The quantification results obtained by PPDD–IDB suggest that the sensitivity of the method is equal to that of guaiacol. The kinetic parameters like catalytic power, relative catalytic power and relative catalytic efficiency for the crude plant extracts are tabulated in **Table 3.**

Table 2 Peroxidase activity of crude plant extracts as per the proposed and standard guaiacol methods

Sample	Activity in Units[a]		Protein [mg]	$\dfrac{K_m^{PI}}{K_m^{G}}$
	PPDD - IDB	**Guaiacol**		
Mimosapudica	17.18 ± 1.1	14.38 ± 1.3	280.02	0.9067
Costus igneus	59.01 ± 1.3	55.71 ± 1.5	119.25	0.0806
Yucca gloriosa	10.22 ± 1.0	33.5 ± 1.2	171.28	0.2806
Ixora chinensis	14.5 ± 1.7	15.2 ± 1.4	260.75	0.8383
Moringa oleifera	46.21 ± 1.9	41.54 ± 1.6	108.74	0.2402
Aegle marmelos	26.11 ± 2.01	24.31 ± 2.1	180.21	0.9371
Limonia acidissima	36.21 ± 1.85	30.21 ±1.8	200.21	0.1464
Syzygium cumini	19.85 ± 1.73	21.12 ± 1.93	190.21	0.7191

[a] Mean (n = 3 replicate determinations) ± R.S.D.

Table 3 Kinetic parameters for crude plant extracts from the PPDD – IDB and standard Guaiacol

Sample	PPDD – IDB method			Guaiacol method			Relative catalytic power $\left(\dfrac{K_{pow}^{PI}}{K_{pow}^{G}}\right)$
	K_m µM	V_{max} EU min^{-1}	K_{pow} x 10^{-3} min^{-1}	K_m µM	V_{max} EU min^{-1}	K_{pow} x 10^{-5} min^{-1}	
Mimosapudica	300.4	0.366	1.218	331.3	0.022	6.851	17.8
Costus igneus	32.4	0.070	2.188	401.9	0.028	7.040	31.08
Yucca gloriosa	119.8	0.116	0.973	427.0	0.034	7.984	12.19
Ixora chinensis	260.1	0.301	1.157	310.2	0.021	6.864	16.86
Moringa oleifera	46.1	0.085	2.635	192.1	0.065	8.654	30.45
Aegle marmelos	268.2	0.289	2.085	286.2	0.112	5.562	37.49
Limonia acidissima	58.0	0.158	3.124	396.2	0.056	6.965	44.85
Syzygium cumini	169.5	0.563	2.266	235.7	0.356	8.102	27.97

Conclusions

No work has been published so far on the coupling of PPDD with IDB for the quantification of peroxidase and hydrogen peroxide. These co-substrates are efficient, versatile, economical, having high catalytic power and the coupled product absorbs at a higher wavelength region. The kinetics of the system showed instantaneous color formation, the procedure requires only small quantities of colorimetric reagents. Optimization of the reaction conditions allowed the determination of H_2O_2 as low as 3.5 µM, which is more sensitive than our previous work [23, 24] and also unattainable by the guaiacol method. The linearity ranges for peroxidase assay by some of the reported

analytical methods are 0.0227–1.136 nM for chemiluminescence [25], 5.4×10^{-4} nM to 0.1088 nM for electrochemical [26] method, these require more dilution of the crude extract which may lead to dilution effect. The HRP-catalyzed oxidative coupling of PPDD and IDB in the presence of peroxide allowed the determination of HRP assay achieved within the linearity range of 0.379 – 6.064 nM and 0.076 -1.516 nM from the kinetic and fixed time methods, respectively. This linear dependence between the concentration of peroxidase and the absorbance is also an important feature for the practical application of the assay procedure in minimizing the dilution effect. The broad range of linearity of the reference guaiacol method made the determination uncomfortable and caused bigger errors. Thus, the proposed method serves as an appropriate replacement to guaiacol method for the assay of peroxidase. The kinetic study shows that the Km values for $K_m^{H_2O_2}$, K_m^{PPDD} and K_m^{IDB} is 85, 92 and 69 μM respectively, which is lesser than guaiacol method and also our previous work DMA ($K_m^{H_2O_2}$ - 301 μM) [27] and PPDD- NEDA ($K_m^{H_2O_2}$ - 550 μM) [24] method for the assay of peroxidase. The catalytic power (K_{PI}^{pow} = 2.251×10^{-3} min^{-1}) was more than that of PPDD-NEDA and DMA methods which have values of 3.38×10^{-4} min^{-1} and 4.59×10^{-4} min^{-1} respectively. Due to the low Michaelis–Menten constant value and higher catalytic power of the proposed method it is more efficient for the assay of peroxidase in crude plant extracts.

Acknowledgement

References

[1] T. Ruzgas, J. Emnéus, L. Gorton, and G. Marko-Varga, *Anal. Chim. Acta*. **1995**, *311*, 245.
[2] T. Ruzgas, E. Csoregi, J. Emnéus, L. Gorton and G. Marko-Varga, *Anal. Chim. Acta.* **1996**, *330,* 123.
[3] S.S. Rosatto, L.T. Kubota and G. Oliveira Neto, *Anal. Chim. Acta*. **1999,** *390,* 65.
[4] P.J. O'Brien, *Chem.–Biol. Interact.* **2000,** *129,* 113.
[5] J.H. Marjon, V. Haandel, M.J.M. Claassens, N.V. Hout, G.M. Boersma, J. Vervoort, M.C.M.I. Rietjens, *Biochim. Biophys. Acta.,* **1999**, *1435,* 22.
[6] A. Bodtke, W.D. Pfeiffer, N. Ahrensb and P. Langerc, *Tetrahedron.,* **2005,** *61,* 1092.
[7] B. Tang, Y. Wang and L. Ma, *Anal. Bioanal. Chem.*, **2004,** *378,* 523.
[8] M.A. Azevedo, C.V. Martins, M.F. DuartePrazeres, V. Vojinovic, M.S. Joaquim Cabral and P.L. Fonseca, *Biotechnol. Annu. Rev*., **2003**, *9,*199.
[9] C. Regalado1, B.E. Garc´ýa-Almend´arez and M.A. Duarte-V azquez, *Phytochemistry Reviews,* **2004**, *3,* 243.
[10] H.W. Krell, J. Lobarzewsky, H. Greppin, C. Penel, T. Gaspar (Eds.), 1991, Biochemical, Molecular and Physiological Aspects of Plant Peroxidases, University M. Curie/University of Geneva, Lublind Poland/Geneva, Switzerland, 469.
[11] A.J. Schutz, M. Winklmair, M.G. Weller and R. Neissner, *SPIE.*, **1997**, *3105*, 332.
[12] J.A. Nicell, J.K. Bewtra, K.E. Taylor, N. Biswas and C. St. Pierre, *Water Sci. Technol., 25* **1992,** 157.
[13] Q. Husain and U. Jan, *J. Sci. Ind. Res*. **2000,** *59,* 286.
[14] N. Duran and E. Esposito, *Appl. Catal. B: Environ.*, **2000,** *28,* 83.
[15] T.S. Shaffiqu, J.J. Roy, R.A. Nair and T.E. Abraham, *Appl. Biochem. Biotechnol.*, **2002**, *102,* 315.
[16] A. Bhunia, S. Durani and P.P. Wangikar, *Biotechnol. Bioeng.*, **2001,** *72,* 562.
[17] M. Hamid, Khalil-ur-Rehman, *Food Chem.*, 2009**,** *115,* 1177.
[18] C. Rodrigo, M. Rodrigo, A. Alvarruiz and A. Frigola, *J. Food Prot.*, **1997,** *60*, 961.
[19] A.C. Maehly, B. Chace, 1954, The Assay of Catalases and Peroxidases in Methods of Biochemical Analysis, Interscience Publishers, New York, 1357
[20] Q. Huang and W.J. Weber, *Environ. Sci. Technol.*, **2005,** *39,* 6029.
[21] S. Muthu and S. Renuga, *Spectrochim. Acta A.*, **2013,** *114,* 1.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr and Randall, *R.J.J. Biol. Chem.*, **1951**, *193,* 265.

[23] P. Nagaraja, A. Shivakumara and A.K. Shrestha, *Anal. Sci.,* **2009,** *25,* 1243.
[24] A. Shivakumara, P. Nagaraja, N. Aradhana and K. Avinash,, **2010,** *93*, 1227.
[25] X. Yang, Y. Guo and Z. Mei, *Anal. Biochem.*, **2009,** *393,* 56.
[26] K. Jiao and W. Sun, *Microchem. J.*, **2002,** *72,* 123.
[27] H. Krishna, P. Nagaraja, A. Shivakumar, N.A. Chamaraja and N. Aradhana, *Spectrochim. Acta A.,* **2013,** *102*, 75.