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Data Article

Spectrophotometric determination of peroxidase using N, N-diethyl-p-phylenediamine sulphate and 3-Aminophenol as a chromogenic reagent: Application of the method to seeds of some fruits

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ABSTRACT

A biocatalatic pathway for the determination of peroxidase using N, N–diethyl–p-phylenediamine sulphate (DPD) and 3-amino phenol (3-AP) is presented. The assay is based on the enzymatic consumption of hydrogen peroxide using DPD- 3AP system to give an intense blue colored compound with absorbance maxima at 660 nm. The increase in absorbance is proportional to the concentration of peroxidase in the range from 1.5–15.15 nM and 0.47 and 15.15 nM from rate and fixed time method respectively. The assay was adapted for the measurement of H_2O_2 at concentrations of 3.5–120 µM. The kinetic parameters like catalytic power, catalytic efficiency, catalytic constant (k_{cat}) and specificity constant (k_{cat}/K_m) was found to be 9.78×10^{-5} μM^{-1} min^{-1}, 1.483×10^{-3} min^{-1},

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0.0245×10^3 min^{-1} and 0.0445 μM^{-1} min^{-1} respectively. The applicability and thermal properties of the method has been tested in different seeds of fruits extracts that showed peroxidase activity.

Specifications table

Subject area	*Analytical Biochemistry*
Compounds	–
Data category	*Analtical procedure, Spectrophotometric data*
Data acquisition format	*UV visible spectrophotomeric, Kinetic measurement*
Data type	*Analyzed*
Procedure	*Development of rate and kinetic method*
Data accessibility	*Data is with this article*

1. Rationale

Peroxidase (E.C.1.11.1.7) is a typical heme enzyme found in many plants. Peroxidase is essential to many biochemical assays for their application in biomedicine or environmental monitoring. It contains glycoprotein with approximately 18% of its weight due to the covalently bound carbohydrate moiety. The native enzyme consists of single polypeptide chain with 308 amino acid residues; the relative molecular mass of POD is 44,000 g/mol [1–4]. They oxidize a wide range of substrates and are implicated in various physiological processes including pathogen defense, stress response and lignin polymerization.

POD is commonly occurs in animals, plants, fungi and microorganisms that act as oxidoreductase. It is a is major H_2O_2 decomposing enzymes which catalyze the oxidation of wide range of substrates at the expense of H_2O_2 [5], only a few reports of peroxidase from actinomycetes are available [6–9]. The mechanism of peroxidase is based on the formation of enzyme–hydrogen donor complexes [10]. It catalyses the oxidation of many aromatic compounds by hydrogen peroxide through on oxidation process that involves a cycle of changes in the oxidation state of an iron atom located at the catalytic site of the enzyme [11]. POD is considered as one of the thermal stable enzyme used as index of blanching. Peroxidase usually having heat resistant and heat labile isoenzymes, due to this it loses its activity in two phases. Owing to this property it is used in polymer synthesis especially for phenolic resin synthesis, nucleic acid analysis, biosensors, bioremediations, and other biotechnological processes [12–17]. POD can act on many hydrogen donors including chromogenic and luminescent compounds. This leads to its widespread use in detection and probe systems [18]. Due to potential application in wide spread areas, it is necessary to find and quantify the amount of peroxidase. The literature survey shows that several analytical techniques are available to quantify the HRP, such as fluorescence [19], Chemiluminescence [20], Electrochemical [21], Magneto elastic sensors [22], Amperometric [23], Flow injection analysis [24], Potentiometric assay [25], Radiometric assay and Coulometric biosensor techniques [26]. The instruments used in these are either very pricey, less flexible, use of radioactive substances and they are susceptible to interference from compounds that either absorb light in the excitation or emission range of the assay or that are themselves fluorescent resulting in false negatives [27]. In electrochemical assay enzyme activity (15%) gets reduces and resulting in the spoil of expensive biocatalyst [28].

To overcome the above drawbacks, we designed an innovative spectrophotometric method for the quantification of HRP and H_2O_2 using DPD and 3- AP. Spectrophotometers are economical, easy to handle and the co- substrates used are generally less pricey, water soluble and stable under lab conditions. The narrow linearity for the assay of H_2O_2 makes the method more significant in the assay of peroxidase. The proposed method is rapid, selective, and highly sensitive. Moreover, the absorption at longer wavelengths allows it to avoid the background interference caused by the biological constituents.

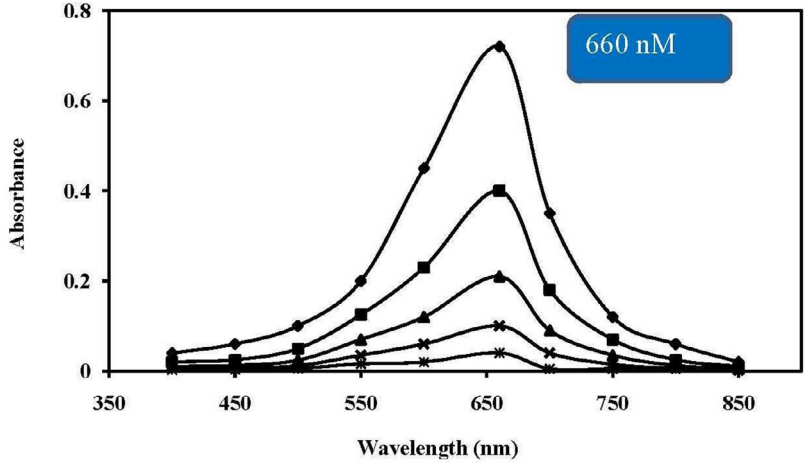


Fig. 1. Absorption spectrum for different concentration of H_2O.

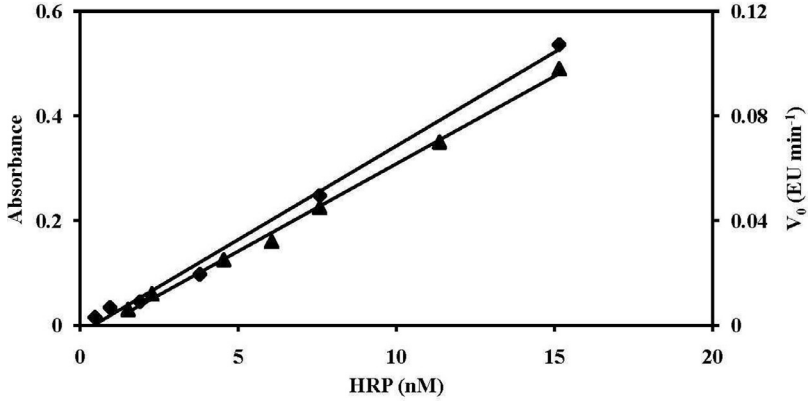


Fig. 2. Calibration graph for the quantification of rate (▲) and fixed time (♦) methods for the quantification of HRP.

2. Procedure

2.1. Chemicals

All of the chemicals used in the assay were of analytical grade. DPD and 3-AP 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. DPD (16.56 mM) and 3AP (51.2 mM) solutions were prepared by dissolving a requisite quantity in water. A 100 mM H_2O_2 stock solution was prepared daily and standardized by a potassium permanganate method. The peroxidase stock solution was prepared by dissolving 2 mg in 10 mL of 100 mM KH_2PO_4/K_2HPO_4 buffer at pH 5.93.

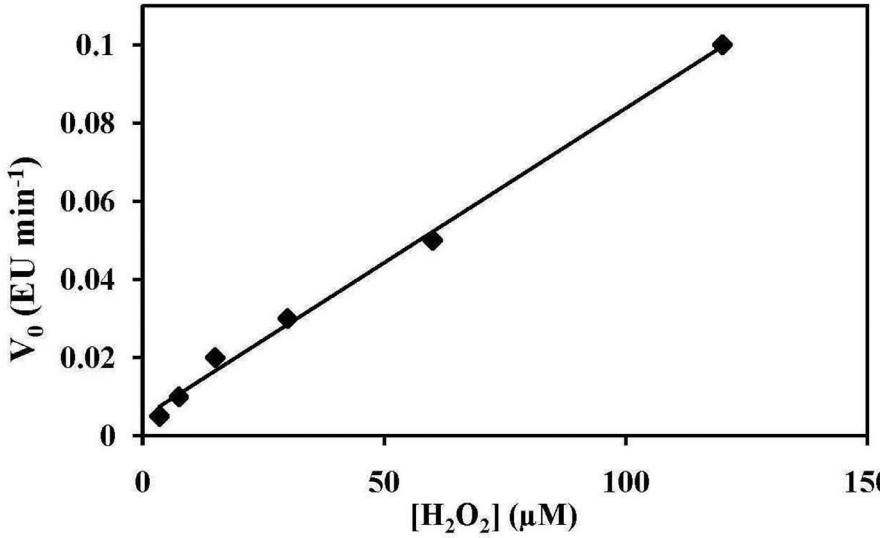


Fig. 3. Calibration graph for the quantification of H_2O_2.

2.2. Instruments

Absorbance measurements were recorded using Systronics spectrophotometer model 106 with 1-cm matched glass cell. Analytical scales (Sartorius, Germany), a pH-meter, EQUIP-TRONICS Model EQ-614 was employed for measuring pH.

2.3. Enzyme extraction

As a source of peroxidase, *Malus domestica, Pyrus communis, V. vinifera, Z. jujuba and C. × sinensis* were collected from the local markets. Seeds were separated from fruits, dried and soaked in 100 ml of 0.1 M phosphate buffer of pH 6.0 over night and thoroughly homogenized by blending for 15 to 20 min. The contents were centrifuged at 10,000 g for 15 min to remove cell debris. The supernatant was removed carefully from the sediments and filtered through Whatman No 1 filter paper to get more clarity of the crude enzyme extract. This stored at 4 °C until used.

2.4. Spectral characteristics

The proposed method involved the formation of blue-colored species. The wavelength of maximum absorbance of the colored species was identified by scanning the sample over the range of 400–800 nm for different concentrations of H_2O_2. The absorption spectra at different time intervals revealed that only one absorption peak, with no remarkable shift, was detected in this wavelength range at λ_{max} of 660 nm. The optimum wavelength for maximum absorbance was found to be at 660 nm, at which the reagent blanks showed slight absorbance or no absorbance as shown in Fig. 1.

2.5. Assay of peroxidase activity

The activity of the peroxidase can be determined by the reaction mixture containing 127 μM DPD, 95 μM 3-AP, and 120 μM H_2O_2 in 100 mM dihydrogen orthophosphate/dipotassium hydrogen orthophosphate buffer of pH 5.93. The reaction was initiated by adding 100 μL of varying concentrations of peroxidase enzyme. The change in the absorbance was continuously recorded against the corresponding control containing all of the reagents, except peroxidase at 30 °C. The initial velocity

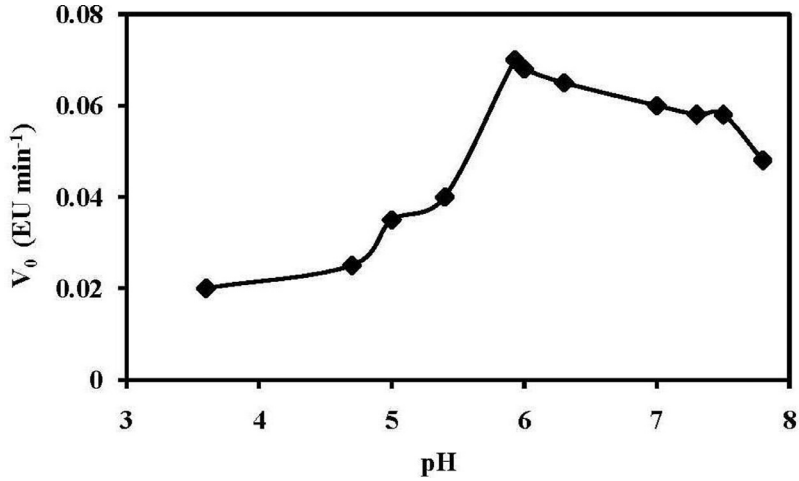


Fig. 4. Effect of pH on the enzyme activity.

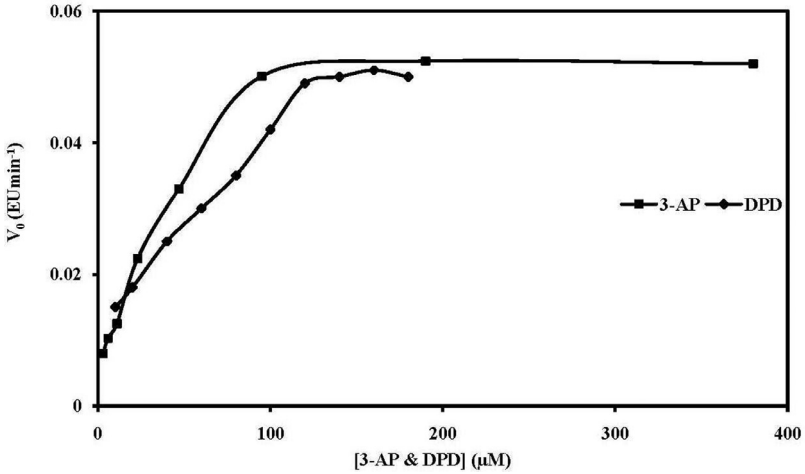


Fig. 5. Effect of DPD and 3-AP was studied by taking the various concentrations of DPD & 3-AP with 120 μM H_2O_2, and 15.15nM peroxidase in 100 mM KH_2PO_4 /K_2HPO_4 buffer at pH 5.93.

was recorded by the absorbance-time curve. The range for the linear relationship between the initial velocity and the concentration of enzyme was 1.51–15.15 nM. From the one time assay method, 5 min of incubation of the reaction mixture at 30 °C allows the peroxidase to be assayed in the concentration range of 0.47 –15.15 nM. The linear relationship by the rate and fixed time methods is shown in Fig. 2.

2.6. Quantification of H_2O_2

The concentration of H_2O_2 was determined in 3 mL of the solution containing optimized concentration of DPD, 3-AP, buffer and 15.15 EU peroxidase. The 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 660 nm. The initial rate was then plotted against the concentration of H_2O_2

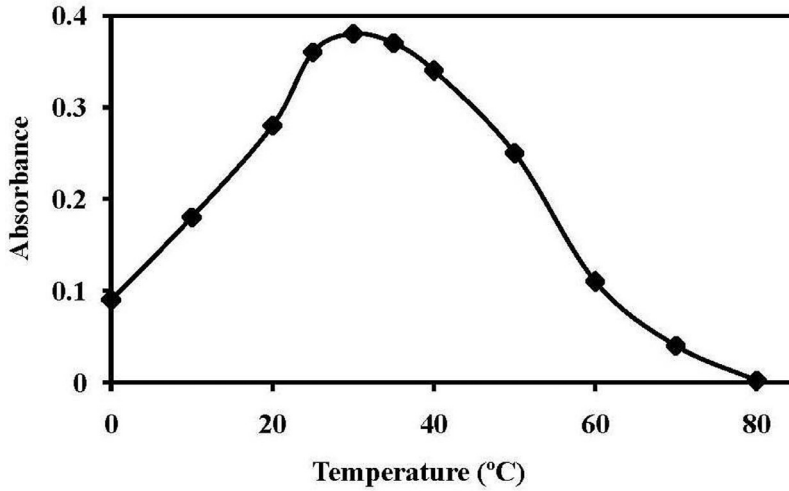


Fig. 6. Effect of incubation temperature on the reaction.

to obtain the calibration graph. The linearity of the graph lies between 3.5 and 120 µM H_2O_2. The calibration graph for the quantification of H_2O_2 is shown in Fig. 3.

2.7. Total protein assay

The total protein concentration was determined in triplicate by the Lowry [29] method, using bovine serum albumin as a standard.

2.8. Results and discussion

Optimum Experimental Condition

Investigators were carried out optimization of the reagents to ascertain the most favorable conditions to attain maximum color.

2.8.1. Effect of pH on absorbance

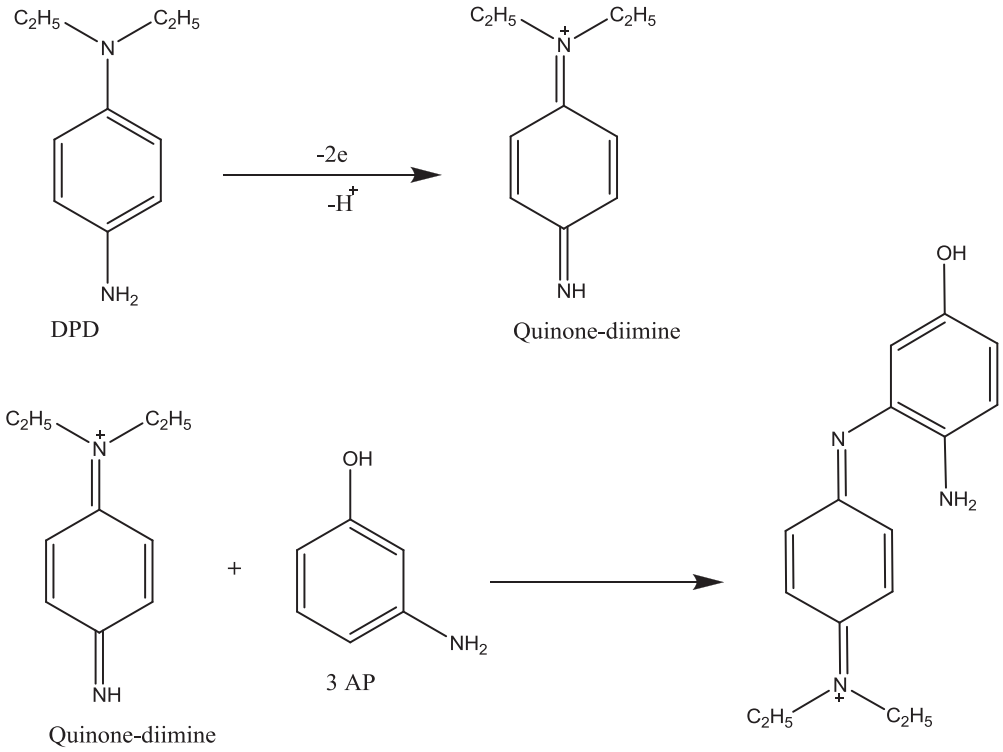
pH is known to alter the activity of enzymes as it affects ionization state of side chains of enzymatic proteins. The following buffers of 100 mM were studied for the assay namely, citric acid/potassium citrate at pH 3.6–5.6, acetate/acetic acid at pH 3.6–5.6, KH_2PO_4/NaOH of pH 6.0–8.0, and KH_2PO_4/K_2HPO_4 at pH 6.0–7.5. The highest activity of the enzyme was observed in KH_2PO_4/K_2HPO_4 buffer of pH 5.93. Hence, further studies were carried out at this pH. The effect of pH on the rate of reaction shown in Fig. 4

2.8.2. Study of the DPD and 3-AP concentration

The effect of varying concentrations of DPD and 3-AP was studied and the results showed that the rate increased on increasing the concentration of DPD from 16 µM to 127 µM beyond which there is no considerable increase in the rate. Hence for all further assays DPD concentration of 127 µM was selected. Similarly, the effect of 3-AP concentration on the reaction rate was studied from 3 µM to 305 µM. The linearity was observed up to 95 µM. Hence 95 µM was selected as the optimized concentration for all further analysis. The optimization of DPD and 3-AP are shown in Fig. 5.

2.8.3. Effect of reaction temperature

It is well established that temperature has pronounced effects on the activity of enzymes. Temperature sensitivity was determined by pre-incubating 3 ml of reaction mixture containing optimized



Scheme 1. Suggested reaction mechanism for the formation of green coloured product.

concentration of DPD, 3-AP, H_2O_2, and 15.15 nM peroxidase in 100 mM KH_2PO_4 /K_2HPO_4 buffer at pH 5.93 for 5 min at various temperatures (0–80 °C). The activity of the enzyme was registered as a function of the absorbance of the colored solution. The activity initially increased up to 30 °C and decreased thereafter. The effect of temperature on the rate of reaction is shown in Fig. 6.

2.8.4. Mechanistic approach for the enzyme activity response

The mechanism for the peroxidase-catalyzed reaction of DPD and 3-AP is proposed in Scheme 1. DPD is oxidized by H_2O_2 in presence of HRP to give reactive diethylbenzoquinone-diimine, which couples with the nucleophillic site of 3- AP to form leuco-dye, which is oxidized to an indo-dye, showing a strong absorption at 660 nm. The kinetic mechanism in which catalytic coupling between DPD and 3-AP involving different Michaelis-Menten values cannot overrule the activation of both by a bio-catalytic mechanism. Both DPD and 3-AP could be activated on the same or different catalytic sites through reduction, finally involving the reaction between the two activated reactants. The bimolecular reaction catalyzed by an enzyme involves the formation of an intermediate with any one of the reactant, followed by the reaction of the intermediate with another reactant. Either of these could be a rate-determining step. The catalytic cycle of two substrates based on the modified ping-pong mechanism suggested by Dunford [30] is below,

$$HRP + H_2O_2 \rightarrow E - (I) + H_2O$$

$$E - (I) + DPD \rightarrow E - (II) + [DPD]^*$$

$$E - (II) + 3\text{-}AP \rightarrow HRP + [3\text{-}AP]^*$$

$$[DPD]^* + [3\text{-}AP]^* \rightarrow \text{Product.}$$

Table 1
Effect of non targeting species.

Foreign Species	Tolerance ratio[a]
Cu^{2+}	0.055
L-Ascorbic acid	0.0916
Fe^{3+}, citric acid	0.875
Fe^{2+}	1.02
L- Tyrosine	1.52
Zn^{2+}, L- serine, Citric acid, Uric Acid	2.03
Oxalic acid, DL- Methionine	3.76
Fluoride	9.2
D- Asparagine, NO_2^-	9.85
L- Histidine, Isoleucine	18.02
DL- Threonine	23.05
NH_4^+	42.32
K^+, Cl^-	52.16
Na^+	70.45
Urea	85.78
Glycine	110.3
SO_2^-, Lactose	124.25
Galactose	220.15
Sucrose	650.23
Glucose	700.52

[a] Tolerance ratio corresponds to the ratio of limit of inhibiting species concentration to that of concentration of peroxidase used.

Where E is the resting enzyme, [DPD]* and [3-AP]* are activated reactants. The enzymatic reaction of HRP follows a bi-bi substrate mechanism [6–9]. The probable reaction is shown in below Scheme. 1.

2.8.5. Interferences studies

The effect of various non-target species in the determination of HRP was investigated, by examining the effect of various ions at μ gml^{-1} levels on the determination of HRP by the recommended method. The tolerance limit of interfering species was established at the concentrations that do not cause error more than ±3% in absorbance values of hydrogen peroxide at 1 μgml^{-1} level. Any precipitate formed during interference studies was removed by centrifugation. The results of the interference study are shown in Table 1.

3. Data, value and validation

3.1. Kinetic expressions based on the enzyme reaction mechanism for the determination of Michaelis constants, catalytic constants and substrate specificity

The catalytic constants of the co-substrates are calculated by taking, KH_2PO_4/K_2HPO_4 buffer of pH 5.93 (100 mM), and the enzyme concentration was 15.15 nM per 3 ml of reaction mixture. This can be carried out in three steps of experiments:

Step 1: Concentrations of H_2O_2 was varied in the order: 60, 80 and 120 μM, respectively, with a constant 190 μM 3-AP and 127 μM DPD concentration. Assuming the initial rate of the reaction asV_0, the general equation for the reaction is written as,

$$\frac{H_0P_0A_0}{V_0} = \frac{K_HP_0A_0}{V_{max}} + \left(\frac{P_0A_0}{V_{max}} + \frac{K_AP_0}{V_{max}} + \frac{K_PA_0}{V_{max}}\right)H_0 \quad (1)$$

By plotting the graph of $H_0P_0I_0/V_0$ verses the concentration of H_2O_2 we get

$$Y = 3.738x + 373.6 \quad (2)$$

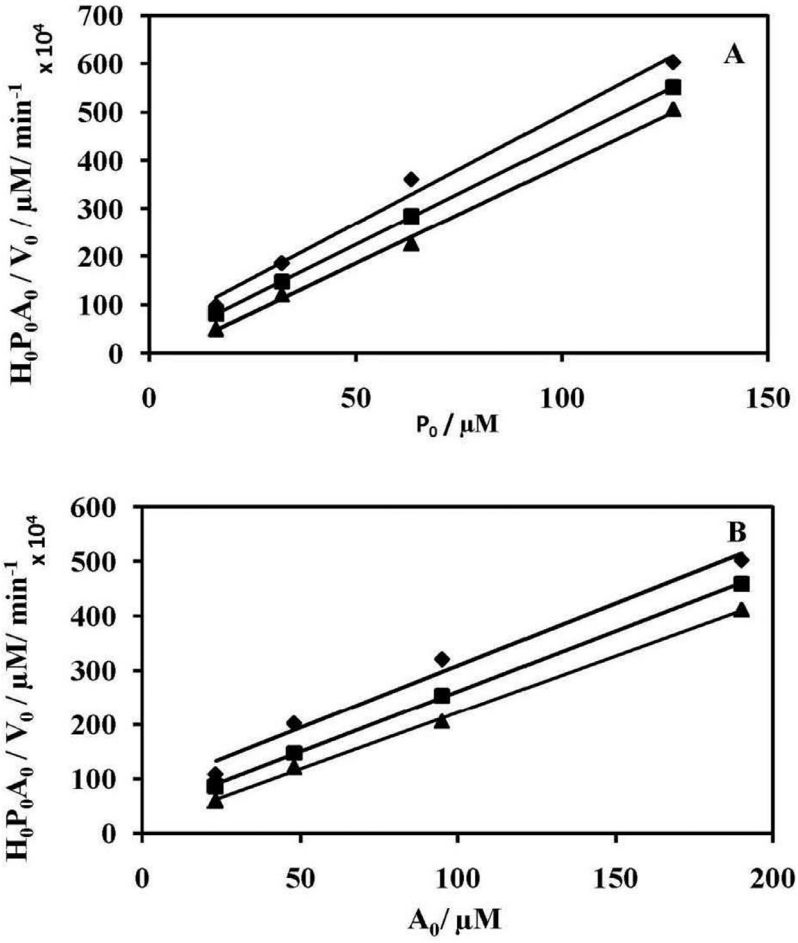


Fig. 7. Kinetic behavior of two substrate reactions for HRP (6.064 nM). (A) The plot of $\frac{H_0P_0A_0}{V_0}$ versus[I_0] and (B) $\frac{H_0P_0A_0}{V_0}$ versus[P_0].

Step 2: Concentrations of 3-AP was varied in the order: 23 µM, 48 µM, 95 µM, and 190 µM with different H_2O_2 concentration by maintaining a constant 127 µM DPD concentration. The general equation can be written as,

$$\frac{H_0P_0A_0}{V_0} = \frac{K_AH_0P_0}{V_{max}} + \left(\frac{H_0P_0}{V_{max}} + \frac{K_HP_0}{V_{max}} + \frac{K_PH_0}{V_{max}}\right)A_0 \quad (3)$$

The plot of $H_0P_0I_0/V_0$ verses the concentration of I_0 gives,

$$Y = 46820x + 35628 \quad (4)$$

Step 3: Concentrations of DPD was varied in the order: 16 µM, 32 µM, 63.5 µM, and 127 µM with different H_2O_2 concentration by keeping a constant 190 µM 3-AP concentration. The general equation can be written as,

$$\frac{H_0P_0A_0}{V_0} = \frac{K_PH_0A_0}{V_{max}} + \left(\frac{H_0A_0}{V_{max}} + \frac{K_HA_0}{V_{max}} + \frac{K_AH_0}{V_{max}}\right)P_0 \quad (5)$$

The plot of $H_0P_0I_0/V_0$ verses the concentration of P_0 gives,

$$Y = 13783x + 58936 \tag{6}$$

The slope of the above Eqs. (1), (3) and (5) is,

$$m_1 = \frac{P_0A_0}{V_{max}} + \frac{K_AP_0}{V_{max}} + \frac{K_PA_0}{V_{max}} \tag{7}$$

$$m_2 = \frac{H_0P_0}{V_{max}} + \frac{K_HP_0}{V_{max}} + \frac{K_PH_0}{V_{max}} \tag{8}$$

$$m_3 = \frac{H_0A_0}{V_{max}} + \frac{K_HA_0}{V_{max}} + \frac{K_AH_0}{V_{max}} \tag{9}$$

The Eqs. (7)–(9) can be rearranged to give,

$$M_1 = \left(m_1 - \frac{P_0A_0}{V_{max}}\right) = K_A\left(\frac{P_0}{V_{max}}\right) + K_P\left(\frac{A_0}{V_{max}}\right) \tag{10}$$

$$M_2 = \left(m_2 - \frac{H_0P_0}{V_{max}}\right) = K_H\left(\frac{P_0}{V_{max}}\right) + K_P\left(\frac{H_0}{V_{max}}\right) \tag{11}$$

$$M_3 = \left(m_3 - \frac{H_0A_0}{V_{max}}\right) = K_H\left(\frac{A_0}{V_{max}}\right) + K_A\left(\frac{H_0}{V_{max}}\right) \tag{12}$$

From the above equations, let us consider the values for DPD as A_1, 3-AP as A_2 and for H_2O_2 as B_2 that is,

$$A_1 = \frac{P_0}{V_{max}} \tag{13}$$

$$A_2 = \frac{A_0}{V_{max}} \tag{14}$$

$$B_1 = \frac{H_0}{V_{max}} \tag{15}$$

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

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

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

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

$$K_H = \frac{-B_1^2M_1 + A_1M_3B_1 + A_2M_2B_1}{2A_1A_2B_1}$$

For IDB the K_I value can be determined by,

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

$$K_A = \frac{A_2M_1B_1 + A_2A_1M_3 - A_2^2M_2}{2A_1A_2B_1}$$

For DPD K_P value is determined by,

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

Table 2
Peroxidase activity of crude fruit seed extracts as per the proposed and standard guaiacol methods and catalytic parameters.

Sample [a]	Units [b]		Protein[b] (mg)	Specific activity (Units/ mg of protein)		Relative catalytic efficiency	$\frac{K_m^{pl}}{K_m^{G}}$
	Proposed method	Reference method		Proposed method	Reference method		
Malus domestica	62.12 ± 1.8	48.02 ± 1.1	221.12	0.28 ± 1.74	0.21 ± 1.96	4.21	0.9067
Pyrus communis	48.23 ± 1.1	36.54 ± 1.6	149.21	0.32 ± 1.64	0.24 ± 2.21	2.06	0.0806
V. vinifera	21.07 ± 1.5	23.5 ± 1.8	191.08	0.11 ± 2.10	0.12 ± 1.54	1.12	0.2806
Z. jujuba	50.14 ± 1.6	51.23 ± 1.5	114.25	0.43 ± 1.72	0.44 ± 1.87	2.17	0.8383
C. × sinensis	46.21 ± 1.4	51.54 ± 1.9	98.86	0.46 ± 1.11	0.52 ± 1.55	2.12	0.2402

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

From the above equation KH, KA and KP values for the co - substrates is 57 μM, 29 μM and 67 μM respectively. Kinetic behavior of two substrate reactions for the pure HRP is shown in Fig. 7.

3.2. Application to the crude fruit seed extracts

The developed method is successfully applied to the assay of peroxidase in seeds of apple, pear, grapes, jujube, and orange. The peroxidase activity was calculated using the below relation. Unknown concentration in EU = $\frac{Rate_{CRUDEextract}}{Rate_{s\,tan\,dard}} \times Std_{concentration}$

The assay results showed that apple seed extract gave more activity of peroxidase than remaining seed peroxidase; hence, the apple seed is a rich natural source of peroxidase as compared to other seeds. However, the suitability of peroxidase for biotechnological applications can be investigated through its kinetic characterization. The k_{pow} of all the crude extracts was compared with that of the guaiacol method. The results obtained shown in Table 2.

4. Conclusion

The proposed spectrophotometric method is simple, economical, sensitive, temperature independence and stability of the colored product are the advantages of this method. No work has been published thus far on the coupling of DPD with 3-AP for the quantification of peroxidase. These co-substrates are versatile, economical, water-soluble, have high catalytic efficiency and a high molar extinction coefficient, and the coupled product absorbs at a higher wavelength region. The kinetics of the system showed “instantaneous” color formation. The HRP-catalyzed oxidative coupling reaction allowed spectrophotometric determination of the HRP within the linearity range of 1.51–15.15 nM and 0.47–15.15 nM from the kinetic and fixed time methods, respectively. This linear dependence between the concentration of peroxidase and the absorbance over a narrow range is also an important feature for the practical application of the assay procedure. The method has high acceptable limit for interfering substances that are generally associated with HRP, which is an additional advantage of the proposed method (Table 2).

Acknowledgments

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