Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section A: Chemical Sciences

CODEN (USA): JCBPAT Research Article

Evaluation of Horseradish Peroxidase Activity Using 3-Methyl-2-Benzothiazolinone Hydrazone Hydrochloride Monohydrate and N-(1-Naphthyl) Ethylenediamine Dihydrochloride as Co-Substrates; Application In Vegetable Extracts.

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Received: 16 November 2015; **Revised:** 02 November 2015; **Accepted:** 09 December 2015

Abstract: The present investigation was done to evaluate the enzymatic activity of peroxidase and hydrogen peroxide concentration, this method is based on the intermolecular coupling of 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) and N-(1-Naphthyl) ethylenediamine dihydrochloride(NEDA). In CH_3COOH/CH_3COONa buffer of pH 5 MBTH was oxidized to MBTH radical cation in presence of H_2O_2 and POD, this was further coupled with NEDA to form intense blue coloured product which showed maximum absorbance at 620nm. This method was successfully applied to Horseradish peroxidase (HRP) and H_2O_2. The linearity of HRP was determined by rate method and fixed time method which lied between 0.0468-5.9 nM and 0.0234-1.4995nM respectively and that of H_2O_2 showed between 4.6090 µM-

82.96 µM and 0.5761 µM-18.4362 µM respectively. The Michaelis–Menten constant (Km) and Vmax for the reaction was found to be 172.4137µM and 1137min-1 respectively. The kinetic parameters like catalytic power (Vmax/ Km) and catalytic efficiency(Keff) =1/ slope $[E]^0$were found to be $0.6594\times10^{-3}min^{-1}$ and $1.1533\times105\ M^{-1}$ min^{-1} respectively. Applicability of the method was tested for peroxidase activity in some vegetables and was compared with the standard method.

Keywords: Catalytic power, catalytic efficiency, Michaelis–Menten constant, Peroxidase assay.

INTRODUCTION

The peroxidases (POD, E.C. 1.11.1.7; oxidoreductase) are a large family of ubiquitous isozymes that catalyze a wide range of substrates mainly H_2O_2, reactive oxygen species and many organic metabolites, which catalyze a variety of oxidative transformations of organic and inorganic substrates by hydrogen peroxide[1]. Peroxidases may contain a heme factor in their active sites, or alternately redox-active cysteine or selenocysteine residues. Peroxidase is extensively distributed in nature and can be easily extracted from most of the plant cells, some animal organs and tissues[2, 3] which have been widely used in analytical biochemistry because of their rapidity and high selectivity. The HRP catalysed reaction is one of the most widely used enzymatic reactions in bio analytical chemistry[4, 5]. HRP has also been used for analytical applications in diagnostic kits for the quantification of clinically important biomarkers such as uric acid, glucose, cholesterol, and lactose [6, 7, 8.] A variety of enzymes including peroxidase, alkaline phosphatase, urease and D-galactosidase etc. have been used in ELISA kits,among which peroxidase is widely used to prepare ‘antibody-enzyme’ or ‘antibody-enzyme conjugates for ELISA due to its high turnover rate, rapid availability, ease of conjugation and better sensitivity[9].

Peroxidase is an enzyme commonly found in vegetables which bind to hydrogen peroxide and produce an activated complex that can react with a wide range of donor molecules and cause off-flavors and colors in raw and un-blanched frozen vegetables[10]. Inhibition of the enzyme activity in fruits and vegetables is generally achieved using physical or chemical treatments such as heating (blanching), lowering pH or adding chemical additives .In general, Peroxidases are the enzymes which are stable to high temperature compared to other enzymes present in plants and do not show thermal inactivation. Therefore, POD activity is used in many food industries as a whitening index or value. Plant peroxidases are found in tonoplast and plasmalemma, inside and outside the cell wall[11]. Generally in the redox reactions catalyzed by peroxidases, the hydrogen peroxide acts as the electron acceptor to liberate oxygen from many kinds of substrates. Reduced cytochrome-C which is a electron donor present in plants, first reacts[12] with H_2O_2

Peroxidase can be used for treatment of industrial waste waters. For example: Phenols, which are important pollutants, can be removed by enzyme-catalyzed polymerization using horseradish peroxidase, phenols are oxidized to phenoxy radicals, which participate in reactions where polymers and oligomers are produced that are less toxic than phenols. It can also be used to convert toxic materials into more harmless substances. Furthermore, peroxidases can be made out of the fecal matter of rats and chickens and eaten off human skin[13] there are many investigations about the use of peroxidase in many manufacturing processes like adhesives, computer chips, car parts, and linings of drums and cans. Other studies have shown that peroxidases may be used successfully to polymerize anilines and phenols in organic solvent matrices[14]. In recent years, various methods for H_2O_2 determination have been proposed,

including the use of enzymatic assays. Applications that involve peroxidases reported in literature are based on colorimeter[15], chemiluminescence[16], fluorescence[17] and amperometric measurements[18]. The simplicity, rapidity, facile and inexpensive properties of spectrophotometer has made it a popular method to determine activity of HRP[19].

In the proposed method the catalytic spectrophotometric method for the determination of HRP and H_2O_2 is developed using MBTH-NEDA as substrates. Among the number of methods reported in the literature for the determination of hydrogen peroxide and peroxidase activity.In our earlier studies, three approaches have been made for the quantification of peroxidase activity using paraphenylene diamine dihydrochloride (PPDD), 2,5-dimethoxyaniline (DMA), 2,4-dimethoxyaniline (DMA) and para-acetylaminophenol were used as the common co- substrates[20,21,22,] these reagents have some limitations like carcinogenicity, auto-oxidation, and solubility problem etc. And results obtained by proposed method was compared with reported methods.

The main advantages of the proposed method over the reported methods are, the lower value of Michaelies-Menten (K_m) constant indicates that there is a stronger affinity of active site of enzyme with substrate molecules means reaction is fast. The substrates used for the proposed assay is found to be best, compared to the reported methods, due to their higher value of catalytic power (V_{max}/K_m) and linearity obtained by H_2O_2 assay and peroxidase assay is more sensitive over reported methods. The validity of the method is assessed by comparing the results with standard guaiacol method.

This method was successfully applied in the quantification of peroxidase activity in the crude extracts of vegetable sources such as *Coccinia grandis*(Ivy gourd), *Benincasa hispida*(ash gourd), *Sechium edule*(chayote squash), *Luffa*(ridge gourd), *Momordica charantia*(bitter gourd), Solanum melongena(brinjal). It was found that bitter gourd has more peroxidase compared to other crude extracts examined. After an exhaustive literature survey, we noticed that, for the first time, this paper describes the presence of peroxidase activity in *Luffa* (ridge gourd), *Sechium edule*(chayote squash) and *Luffa*(ridge gourd).

MATERIALS AND METHOD

(i)Apparatus: A Jasco model UVIDEC-610 ultraviolet–visible (UV–Vis) spectrophotometer with 1.0-cm matched cells was used for all absorbance measurements. A water bath shaker (NSW 133, New Delhi, India) was used to maintain constant temperature for color development. All pH measurements and adjustments were done by a digital pH meter (model EQ-614, Equip-tronics, Mumbai, India), centrifuge and Remi cyclo rotator.

(ii) Reagents: All chemicals used were of analytical-reagent or higher grade. De-ionized water was used throughout. A 0.2g ml^{-1}stock solution of HRP was prepared by dissolving 2 mg in 10 mL of 0.1 mol/L KH_2PO4/NaOH buffer (pH 6.0). H_2O_2 stock solution (1.0%, v/v) was prepared by diluting the commercial reagent (30%, v/v, E. Merck, Mumbai, India), and standardized by titration with secondary standard KMnO4. MBTH and NEDA (E. Merck, Mumbai, India) were prepared by dissolving an appropriate amount of the reagent with distilled water

(iii)Crude extract preparation: As a source of peroxidase, Edible portion of vegetables namely *Coccinia grandis*(Ivy gourd), *Benincasa hispida*(ash gourd), *Sechium edule*(chayote squash), *Luffa*(ridge gourd), *Momordica charantia*(bitter gourd), Solanum melongena(brinjal) were collected from local

agricultural fields, transported at 4°C to the laboratory, and stored at -20°C until used. Samples (1 g) were washed with distilled water and homogenized in a blender using 10 ml of 0.5 M KH_2PO_4/NaOH buffer of pH 6**.** The extracts were passed through a cheese cloth, centrifuged at 12000×g for 20 min, and the supernatants were used as crude extracts.

Evaluation of kinetic constants for MBTH, NEDA and H_2O_2: Separate experiments with varying H_2O_2 concentration were performed along with varying concentrations of MBTH and NEDA. The H_2O_2, MBTH, NEDA concentration varied in the range of 2.3045 to 294.98 µM, 1.1093 to 568µM and 2µM to 256µM respectively. Finally, about 3 ml was used for each kinetic study.

The pH and temperature were kept constant. The ping-pong mechanism followed by HRP was confirmed by the double-reciprocal plot of the rate versus MBTH and NEDA concentrations at different H_2O_2 concentrations. Assuming the initial rates (Vo), a general equation for the mechanism in the forward direction is given as a function of all substrate concentrations

$$1/V_o=1/V_{max}+ K_H/H_oV_{max}+ K_M/M_oV_{max} +K_N/N_oV_{max} \quad (1)$$

The initial velocities were determined as a function of substrates concentration. The Michaelis-Menten constant was evaluated by varying one and keeping the other two constant. Keeping H_o and N_o as constants, eqn 1 yielded a constant slope and intercept given by

$$\text{Intercept} =1/V_{max} +K_H/H_oV_{max}+ K_N/N_oV_{max} \quad (2)$$

$$\text{Slope}= K_M/V_{max} \quad (3)$$

Keeping H_o and P_o as constants, eqn 1 yielded a constant slope and intercept given by

$$\text{Intercept} =1/V_{max}+ K_H/H_oV_{max} +K_M/M_oV_{max} \quad (4)$$

$$\text{Slope}= K_N/Vmax \quad (5)$$

Replots of the intercepts of the both kinetic eqn 2 and 4 versus the 1/Ho concentration produced a straight line with a constant slope and intercept as

$$\text{Intercept} =1/V_{max} + K_N/N_oV_{max} \quad (6)$$

$$\text{Intercept} =1/V_{max}+K_M/M_oV_{max} \quad (7)$$

$$\text{Slope}= K_H/V_{max} \quad (8)$$

The V_{max} of the catalytic reaction was ascertained by saturating the reaction system with MBTH, NEDA and H_2O_2. The constants K_M, K_N, K_H were determined from the eqn 3, 5, and 8, respectively.

From Lineweaver–Burk plot K_m and V_{max} of the enzyme was found to be 172.4137 µM and 0.1137 µM min^{-1} is shown in fig.5. By plotting slope v/s intercept on Y-axis gives constant slope, this helps in determination of K_M and K_N.

RESULTS AND DISCUSSIONS

Optimization of experimental variables and experimental conditions such as effect of substrates, co-substrates, pH, temperature and incubation period, which affect enzyme assay, have been studied

Effect of order of addition of Reagents: Addition of reagents in different order had an influence on the reaction. The experimental results indicated that it was optimum when solutions were added in the following order: MBTH, buffer, NEDA, H_2O_2, water and peroxidase. So this order was optimized.

Effect of temperature on the rate of reaction: The temperature effect on the enzyme assay was studied by adding solutions containing, 284 µM of MBTH, 64 µM of NEDA, 82.96 µM H_2O_2, 4.6 nM peroxidase and 100 mM acetate/acetic acid buffer of pH 5. The reaction mixture was incubated for 10 min at various temperatures, in the range of 10°C –80°C. A proportional increase in the absorbance values was observed with increase in temperature up to 30°C as shown in Fig.1 and the values decreased thereafter. Therefore, 30°C was chosen as optimum temperature for the assay.

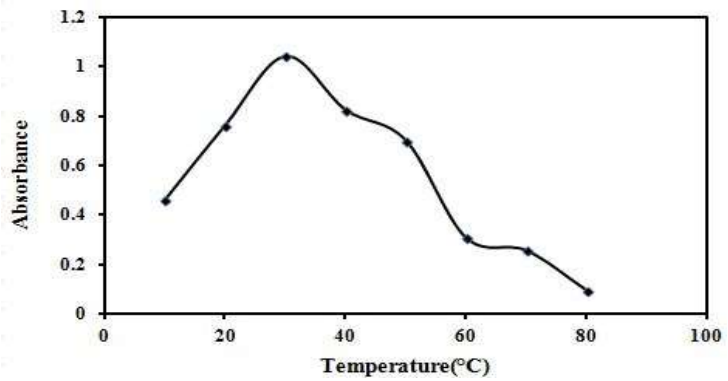

Fig 1: Effect of temperature on the sensitivity of enzyme assay containing 284 µM of MBTH, 64 µM of NEDA, 82.96 µM H_2O_2, and 4.6 nM peroxidase in a 100 mM acetate/acetic acid buffer of pH 5 in a final 3 mL of the reaction mixture.

Effect of pH and concentration of buffer solutions: The effect of pH on the enzyme activity was studied by using different buffers of concentrations ranging from 0.1 M to 2.0 M such as acetic acid/sodium acetate buffer (pH 3.6–5.6), citric acid/potassium citrate buffer (pH 3.6–5.6), KH_2PO_4/NaOH buffer (pH 3.6–8.0) and KH_2PO_4/K_2HPO_4 buffer (pH 3.6–7.5).

The activity was maximum at pH 5 in a 100 mM acetic acid/sodium acetate buffer solution. Hence, acetate buffer of pH 5 was selected for further assay procedures. The effect of pH on the rate of reaction is shown in Fig 2.

Effect of MBTH and NEDA on enzyme activity: Solutions containing MBTH concentrations ranging from 1.1093 to 568µM were added to reaction mixture containing, 128 µM NEDA, 2.2125mM H_2O_2, 4.6 nM peroxidase, and 100 mM acetate/acetic acid buffer of pH 5.0, for the study of co-substrate MBTH effect on enzyme assay. It was found that the rate of reaction increased, with increase in concentration of MBTH up to 284µM beyond which no considerable increase in enzyme activity was observed. Therefore 284 µM of MBTH was chosen as optimum concentration of co-substrate. Solutions containing NEDA concentrations ranging from 2µM to 256µM were added to reaction mixture containing, 284 µM MBTH, 2.2125mM H_2O_2, 4.6 nM peroxidase, and 100 mM acetate/acetic acid buffer of pH 5.0, for the study of co-substrate NEDA effect on enzyme assay, rate of reaction increased, with increase in concentration of NEDA up to 64µM beyond which no considerable increase in enzyme activity was observed. Therefore, 64 µM of NEDA was chosen as optimum concentration of co-substrate

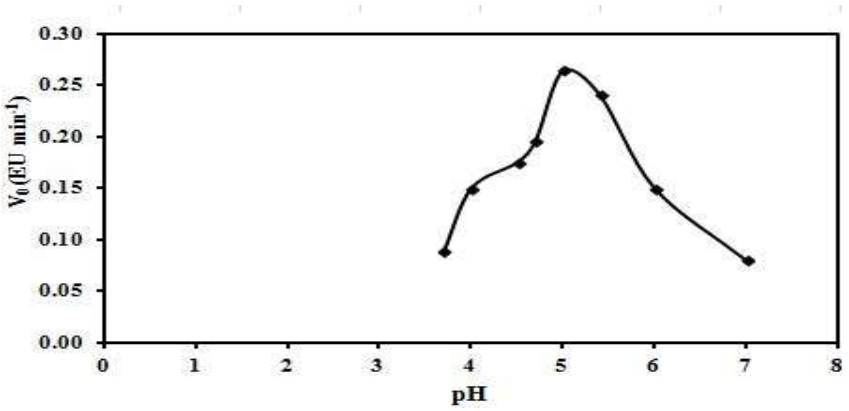

Figure 2: Effect of pH on the reaction

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Quantification of H_2O_2: Prior to use, the H_2O_2 stock solution was standardized by titration with secondary standard KMnO4, and accurate dilutions were made with distilled water to make a range of working standard solutions the linearity for the assay of H_2O_2 was examined in a 3 mL of the reaction mixture containing 284 µM MBTH, 64 µM NEDA, 4.6 nm peroxidase and 100 mM CH_3COOH/CH_3COONa buffer at pH 5 The H_2O_2 concentrations used were in the range of 2.3045–294.98 µM. The change in the absorbance was continuously recorded at 620 nm for 5 minutes, at 1 min interval. The initial rate was plotted against the concentration of H_2O_2 to obtain calibration graphs.The linearity for the assay of H_2O_2 was found to be in the range of 4.6090-82.96 µM by rate method and 0.5761 µM - 18.4362 µM by fixed time method and the calibration graph for the quantification of H_2O_2 by rate and fixed time method is shown in Fig3.

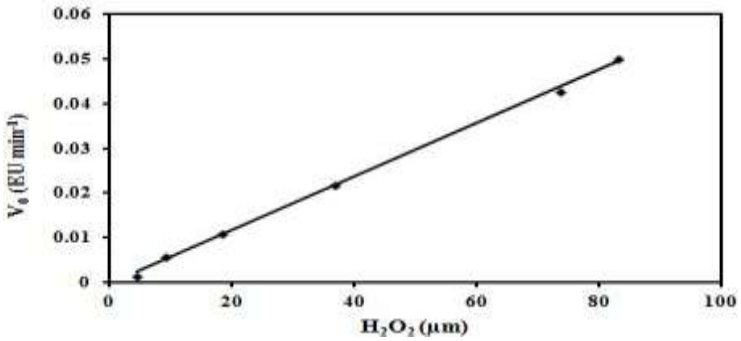


Figure 3 (A): Calibration graph for the quantification of H_2O_2 (4.6090 µM-82.96 µM from the rate method.

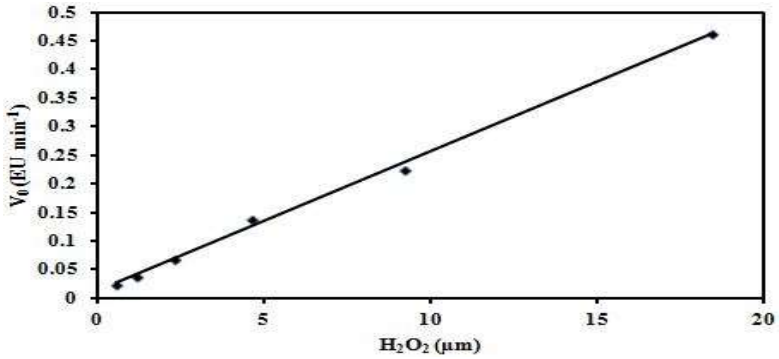


Figure 3(B). Calibration graph for the quantification of H_2O_2 (0.5761 µM-18.4362 µM) from the fixed time method.

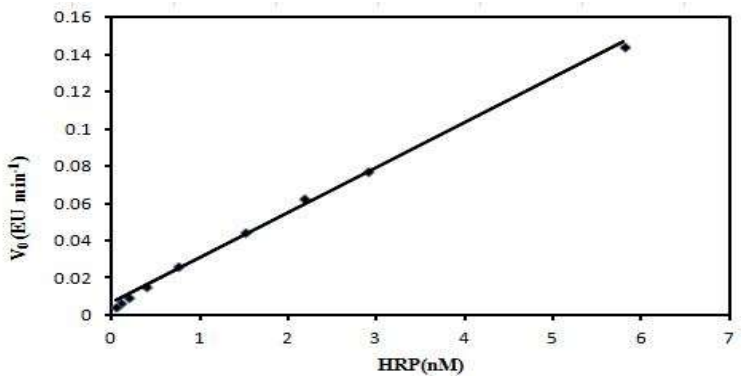


Figure 4 (A): Calibration graph for the quantification of horseradish peroxidase (0.0468nM - 5.9 nM) from the rate method.

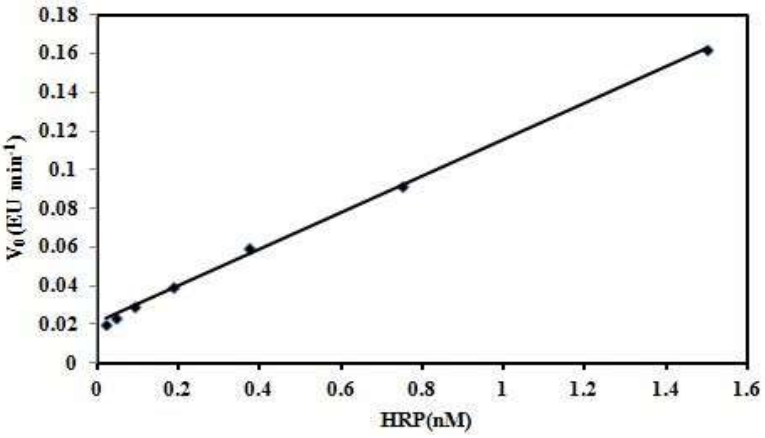


Figure 4 (B): Calibration graph for the quantification of horseradish peroxidase (0.0234- 1.4995 nM) from the fixed time method.

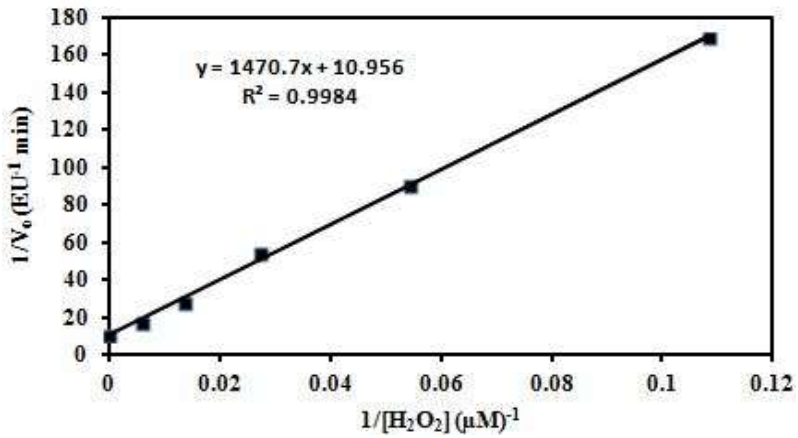


Figure 5: Lineweaver–Burk plot for horseradish peroxidase by the proposed method

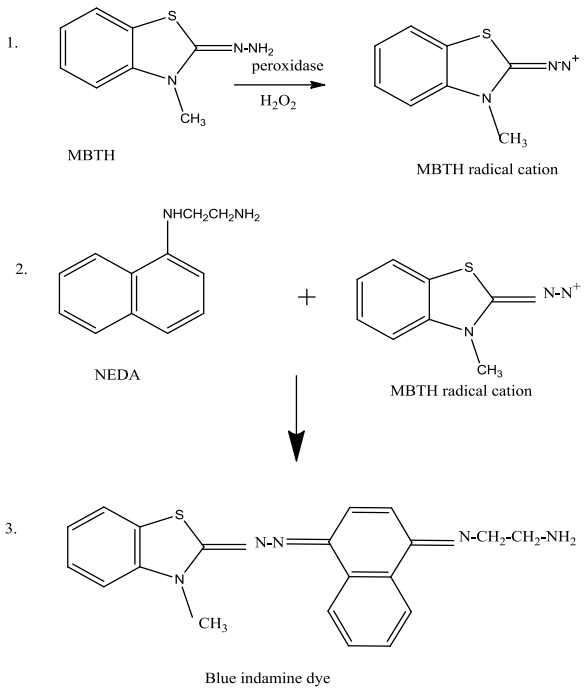
Quantification of peroxidase: The linearity of the HRP was determined in a reaction mixture containing 285.2 µM MBTH, 64 µM NEDA and 84 µM H_2O_2 and 100 mM CH_3COOH/CH_3COONa buffer of pH 5 and 100 µl of varying concentrations of POD ranging between 0.0113 nM to 11.6 nM. It was measured for 5 mins in the interval of 1min for the quantification of peroxidase. The linearity was found between 0.0452 to 5.8 nM and 0.0234 to 1.4499nM by rate and fixed time method respectively.

Stability of the enzyme activity: 3 mL of the reaction mixture containing 284 µM MBTH, 64 µM NEDA, 84 µM H_2O_2, 5.8 nm peroxidase and 100 mM CH_3COOH/CH_3COONa buffer at pH at 5. The enzyme activity was found to be stable up to 10 minutes, after that a small increase in absorbance was observed. Therefore, 10 minutes was chosen as optimum fixed time for the assay.

Evaluation of kinetic parameters for the enzyme assay: Kinetic parameters for the enzyme assay were studied under the optimized experimental conditions. A Lineweaver–Burk plot was used for the evaluation of Michaelis–Menten constants of H_2O_2 concentration ranging from 4.6090–82.96 µM. The values of K_m and V_{max} for the peroxidase enzyme from the Lineweaver–Burk plot were found to be 172.4137 µM and 0.1137 µM min^{-1}, respectively, which are lower when compared to our earlier work[16-18]. The catalytic constant K_{cat} and specificity constant was found to be 0.0196min^{-1} and 1.1368×10^{-4}µ$M^{-1}min^{-1}$ respectively. The catalytic efficiency K_{eff}= 1/slope[E]o, and catalytic power $K_{pow}=V_{max}/K_m$ of the proposed method is 1.1533 × 10^5µ$M^{-1}min^{-1}$ and 6.5946×10^{-4} EUµM^{-1} min, respectively and by standard guaiacol method, it was K_{eff}= 0.6580× 10^4µ$M^{-1}min^{-1}$and K_{pow}=2.001810^{-4} EUµM^{-1} respectively. This shows that proposed method is more efficient than the guaiacol method. The lower value of K_m which was 172.4137 µM for MBTH, NEDA and HRP, may be due to a stronger affinity of active site of HRP in presence of MBTH and NEDA to that of H_2O_2 molecules which signifies the extent of selectivity and specificity of the proposed reaction. Also, these observations suggest that MBTH and NEDA can be a better proton donor than guaiacol for the HRP assay.

Discussion of proposed reaction pathway for the intermolecular coupling of MBTH and NEDA in response to the enzymatic activity: The probable reaction mechanism involved is based on the intermolecular coupling of MBTH-NEDA in presence of strong oxidizing agent like H_2O_2 in presence of HRP to give a blue-colored product having maximum absorbance at λ_{max} = 620 nm. The above mentioned reaction is analogous to HRP-catalyzed synthesis of polyaniline as suggested by Caramyshev

et al[23]. The free radical is released by the oxidation of H_2O_2 through a ferryl intermediate (FeIV = O-porphyrin p-cation radical) of the peroxidase[24]. Under the assay conditions, MBTH loses 2 electrons and one proton on oxidation with hydrogen peroxide in the acidic medium of pH 5 forming the π-cation radical[25] which acts as a active coupling species. This intermediates reacts with amines like NEDA by electrophilic attack on the most nucleophilic site on the aromatic ring of amines (i.e., ortho or para positions, if para positions to amine is substituted). The resulting intermediate is spontaneously oxidized in presence of oxidant to form blue coloured product. The peroxidase-catalyzed reaction of MBTH-NEDA is as shown in Scheme 1.



Scheme 1. Proposed reaction for the peroxidase catalyzed reaction of MBTH with NEDA.

Application of the proposed assay for the assessment of peroxidase activity in vegetable extracts: The assessment of peroxidase activity in various vegetable samples was performed by analyzing the effect of different buffers of pH 4.0-7.5 and considering buffer to tissue ratio from 5:1 to 15:1 mL/g. The maximum peroxidase activity was observed in the buffer solution of KH_2PO_4/NaOH at pH 5 and highest specific activity was observed at 10:1 mL/gratio, this crude extracts (100 µL) were taken for the quantification of peroxidase. The results obtained were compared to the guaiacol method. The relative half-saturation point of the proposed method with reference to the guaiacol method is less than 1, indicating the greater sensitivity of the method. The quantification results obtained by this method suggest that the method is more sensitive than that of guaiacol method. The vegetables *Momordica charantia*(Bitter gourd) and *Sechium edule* (Chayote squash) extracts showed high peroxidase activity as determined by the proposed method and guaiacol method whereas *Coccinia grandis* (ivy gourd) showed less peroxidase activity . The catalytic power of all of the crude extracts was significantly greater than the reference guaiacol method.

Assessment of Peroxidase Activity in Crude Plant Extracts.

Table 1. Assessment of peroxidase activity in vegetable extracts

Sample	Activity in Units		K_m/K_G	K_{pow}
	MBTH-NEDA	GUAIACOL		
Coccinia grandis	1.5172	1.3587	0.1818	8.2×10^{-4}
Benincasa hispida	4.7954	3.2822	0.0931	3.0×10^{-4}
Sechium edule	7.8619	5.4644	0.4104	8.5×10^{-4}
Luffa	2.9823	2.5675	0.7125	9.03×10^{-4}
Momordica charantia	9.812	10.5675	0.5869	4.1×10^{-4}
Solanum melongena	4.4810	2.8712	0.0643	1.1×10^{-4}

CONCLUSION

No work has been published so far on the coupling of MBTH with NEDA for the quantification of peroxidase and H_2O_2. These co-substrates are versatile, economical, water-soluble, have high catalytic efficiency, catalytic power and less Michaelis–Menten constant(Km) and the coupled product absorbs at a higher wavelength range. The kinetics of the system showed "instantaneous" color formation. The procedure requires only small quantities of colorimetric reagents. Optimization of the reaction conditions from the enzymatic oxidation allowed for the determination of H_2O_2 as low as 4.6075µM, The Michaelis–Menten constant(Km) and Vmax for the reaction was found to be 172.4137µM and 1137min^{-1} respectively. The kinetic parameters like catalytic power (V_{max}/ K_m) and catalytic efficiency(K_{eff}= 1/ slope $[E]_0$ were found to be $0.6594\times10^{-3}min^{-1}$ and 1.1533×10^{5} M^{-1} min^{-1} respectively. From the Lineweaver–Burk plot, it was found that HRP shows a strong affinity towards the active sites of MBTH & NEDA to that of H_2O_2 molecules which signifies selectivity and specificity of the proposed reaction. Regarding superiority of the method, it is suggested that MBTH, NEDA method can be a better hydrogen donor for HRP assay than guaiacol system as evident from kinetic data. This is not possible by the standard guaiacol method. The proposed method serves as an appropriate replacement to the standard guaiacol method[26] for the assay of peroxidase.

ACKNOWLEDGMENT

The authors would like to thank the financial support given by the UGC, Centre with Potential for Excellence in Particular Area (CPEPA) project and University of Mysore for providing the research laboratory facilities.

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