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Peroxidase-Catalyzed Oxidative Coupling of Paraphenylenediamine with 3-Dimethylaminobenzoic Acid: Application in Crude Plant Extracts

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This paper presents a novel spectrophotometric method to measure peroxidase activity using paraphenylenediamine dihydrochloride (PPDD) and 3-dimethylaminobenzoic acid (DMAB). The PPDD traps free radicals and becomes oxidized to electrophillic 1,4-diimine, which couples with DMAB to give an intense green-colored chromogenic species with maximum absorbance at 710 nm. This assay was adopted for the quantification of hydrogen peroxide between 5 and 45 μM. From the kinetic data, a two-substrate ping-pong mechanism of peroxidase was established. The catalytic efficiency and catalytic constant (k_{cat}) of the proposed assay were 0.54×10^6 M^{-1} min^{-1} and 0.0436×10^3 min^{-1}, respectively. As a simple, rapid, precise, and sensitive technique, PPDD−DMAB stands as a potential replacement for the traditional guaiacol method. Application of this method in plant extracts opens its relevance in the field of biochemical analysis.

KEYWORDS: Horseradish peroxidase; PPDD; DMAB; catalytic parameters; crude extracts

INTRODUCTION

Peroxidases (EC 1.11.1.7) are widely distributed in nature. Most of them are heme proteins that contain iron(III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. Peroxidase catalysis is associated with four types of activity namely, peroxidic, oxidative, catalytic, and hydroxylation (1). It plays an important role in the plant defense system by the conversion of phenolic compounds to quinones, which are toxic to pathogenic organisms and pests. It is one of the key enzymes that control the plant differentiation and development. Moreover, it is also involved in quality deterioration in most of the fruits and vegetables and is accountable for lowering the nutritional value of foods (2).

The reduction of peroxides at the expense of electron-donating substrates is useful in a number of industrial and analytical applications. The peroxidase-coupled assay in the polyenzymatic systems producing H$_2$O$_2$ is used in the quantification of clinically important biomarkers such as glucose, cholesterol, and uric acid (3). Commercially available peroxidase is widely employed for removal of phenols and amines from industrial wastewater, bleaching of industrial dye stuff, lignin degradation, fuel and chemical production from wood pulp and in various organic syntheses (4, 5). The largest application of the peroxidase is in enzyme-linked immunosorbent assays (ELISAs) as an enzyme label of immunological reagents (6). It is also used in the detection of nucleic acid (7).

Multiple methods are available for the quantification of enzymatic activity, which includes fluorimetry and luminescence (8). However, they have such constraints as the instruments used in fluorimetry and luminescence are too expensive and less versatile. The selectivity of luminescence is poor (9). Spectrophotometers are economical, easy to handle, and the reagents used are not costly. Some of the common co-substrates employed in the colorimetric method include guaiacol (10), 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (11), o-dianisidine, benzidine, p-phenylenediamine (12), pyrogallol (13), 3,3′,5,5′-tetramethylbenzidine (TMB) (14), and o-phenylenediamine and catechol (15), but these reagents have some limitations, such as carcinogenicity and mutagenicity of o-dianisidine and benzidine (16) and broader linearity range of pyrogallol (17) and guaiacol (10). TMB has initial problems, such as stability and poor solubility in aqueous buffer solution (18). The inactivation of peroxidase has been observed by some of the assay techniques, which in turn results in the reduction of sensitivity and efficiency of the enzyme for its reactivity toward H$_2$O$_2$ (3).

This paper proposes peroxidase-catalyzed coupling of co-substrates paraphenylenediamine dihydrochloride (PPDD) and 3-dimethylaminobenzoic acid (DMAB) in the presence of H$_2$O$_2$ to give an intense green-colored product, which shows a strong absorption at 710 nm. The narrow linearity range for the assay of peroxidase makes the method more significant than the guaiacol method. The method has been applied in the quantification of peroxidase activity in the crude extracts of plant sources, such as *Nicotiana tabaccum*, *Alternanthera sessilis*, *Tinospora cardifolia*, *Petroselinum crispum* var. *neapolitanum*, *Brassica oleracea* var. *capitata*, and *Lactuca sativa*. It was found that *N. tabaccum* has more peroxidase and *B. oleracea* var.

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capitata has the highest specific activity 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 *A. sessilis* and *P. crispum* var. *neapolitanum*. The relative catalytic efficiency claims superiority of the proposed method.

MATERIALS AND METHODS

Apparatus. All of the absorbance measurements were made using spectrophotometer 117 (Systronics, Ahmedabad, India) with 1 cm matched quartz cells. The temperature was controlled using a constant temperature cell holder with a stirrer (Shimadzu, Japan, Model 206-88950-93). The pH measurement and adjustments were performed by a digital pH meter (Equip-tronics, Mumbai, India, Model EQ-614).

Chemicals. All of the chemicals used in the assay were of analytical grade. PPDD and DMAB 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 (1.1 mM) and DMAB (1.90 mM) solutions were prepared by dissolving a requisite quantity in water. DMAB was initially dissolved in a small quantity of dilute hydrochloric acid and made up with water. A 100 mM H_2O_2 stock solution was prepared daily and standardized by a potassium permanganate method. Working standard solutions were prepared from the stock solution by dilution. The peroxidase stock solution was prepared by dissolving 2 mg in 10 mL of 100 mM potassium dihydrogenphosphate/sodium hydroxide buffer at pH 6.0. Further dilution with the same buffer was made when required.

Sample and Crude Extract. As a source of peroxidase, the leaf/stem portion of *N. tabaccum*, *A. sessilis*, *T. cardifolia*, *B. oleracea* var. *capitata*, *P. crispum* var. *neapolitanum*, and *L. sativa* were collected from the local agricultural fields, transported at 4 °C to the laboratory, and stored at −20 °C until used. Samples (5 g) were washed with distilled water and homogenized in a blender using 50 mL of 100 mM phosphate buffer at pH 6.0. The extract was passed through a cheesecloth and centrifuged at 12000*g* for 15 min, and the supernatant was labeled as crude extract.

Protein Determination. The total protein concentration was determined in triplicate by the Lowry (*19*) method, using bovine serum albumin as a standard.

Kinetic Constants for PPDD, DMAB, and H_2O_2. In the proposed method, separate experiments for each H_2O_2 concentration were performed at varying concentrations of PPDD and DMAB.

Michaelis−Menten constants for PPDD and DMAB were determined at concentrations from 4.6 to 36.80 μM and from 20 to 200 μM, respectively. The H_2O_2 concentrations of 10, 20, 40, and 60 μM in the final volume of 3 mL were used for each kinetic study. The pH and temperature were kept constant. The ping-pong mechanism followed by HRP can be confirmed by the double-reciprocal plot of the rate versus PPDD and DMAB concentrations at different H_2O_2 concentrations. Assuming the initial rates (V_o), a general equation for the mechanism in the forward direction is given as a function of all substrate concentrations

$$\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K_H}{H_o V_{max}} + \frac{K_p}{P_o V_{max}} + \frac{K_D}{D_o V_{max}} \qquad (1)$$

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 constants.

Keeping H_o and D_o as constants, eq 1 yielded a constant slope and intercept given by

$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_H}{H_o V_{max}} + \frac{K_D}{D_o V_{max}} \qquad (2)$$
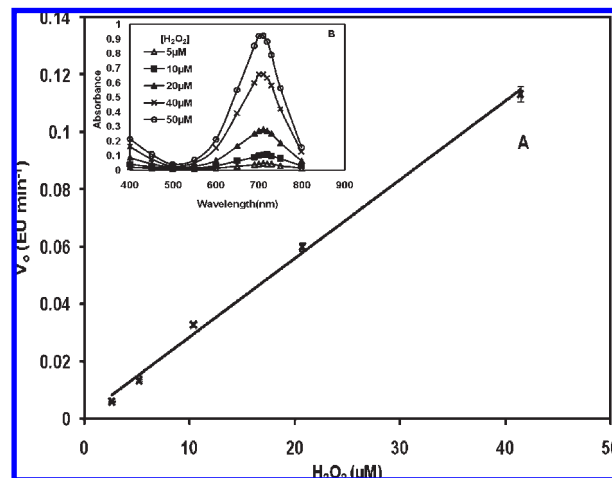
$$\text{slope} = \frac{K_p}{V_{max}} \qquad (3)$$

Figure 1. (A) Calibration graph for the quantification of H_2O_2 from the rate method. Means of triplicates with error bars indicating the standard deviation are shown. (B) Absorption spectrum of H_2O_2: 36 μM PPDD + 63 μM DMAB + 7.5 nM peroxidase in 100 mM acetate/acetic acid at pH 5.5 with varying concentrations of H_2O_2 (5−50 μM). The spectrum was recorded at a scan rate of 2 nm/s after incubating the reaction mixture for 5 min at 30 °C. The spectrum containing neither H_2O_2 nor enzyme shows negligible absorption.

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

$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_H}{H_o V_{max}} + \frac{K_p}{P_o V_{max}} \qquad (4)$$

$$\text{slope} = \frac{K_D}{V_{max}} \qquad (5)$$

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

$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_D}{D_o V_{max}} \qquad (6)$$

$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_p}{P_o V_{max}} \qquad (7)$$

$$\text{slope} = \frac{K_H}{V_{max}} \qquad (8)$$

The V_{max} of the catalytic reaction was ascertained by saturating the reaction system with PPDD, DMAB, and H_2O_2. The constants K_p, K_D, and K_H can be determined from the eqs 3, 5, and 8, respectively.

RESULTS AND DISCUSSION

Quantification of H_2O_2. The concentration of H_2O_2 was determined in 3 mL of the solution containing 36 μM PPDD, 63 μM DMAB, and 7.5 nM peroxidase in 100 mM acetate/acetic acid buffer at pH 5.5. The reaction was initiated at 25 °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 710 nm. The initial rate was then plotted against the concentration of H_2O_2 to obtain the calibration graph. The value of K_H and V_{max} for the peroxidase enzyme from the Lineweaver−Burk plot was found to be 80 μM and 0.3277 EU min^{-1}, respectively. The linearity of the graph lies between 5 and 45 μM H_2O_2. The calibration graph for the quantification of H_2O_2 is shown in **Figure 1A**.

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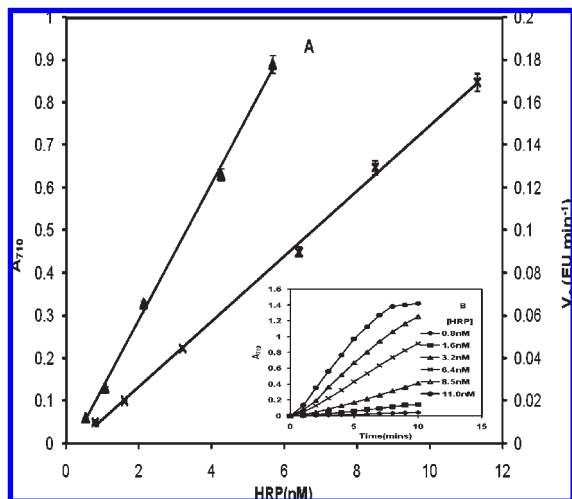*J. Agric. Food Chem.,* Vol. 57, No. 12, 2009   **5175**

Figure 2. (A) Calibration graph for the quantification of horseradish peroxidase from the (×) rate and (▲) fixed time methods. Means of triplicates with error bars indicating the standard deviation are shown. (B) Absorbance—time curves for varying concentrations of horseradish peroxidase.
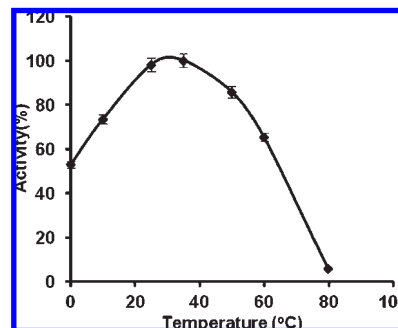

Figure 3. Effect of the incubation temperature on the reaction. Means of triplicates with error bars indicating standard deviation are shown.
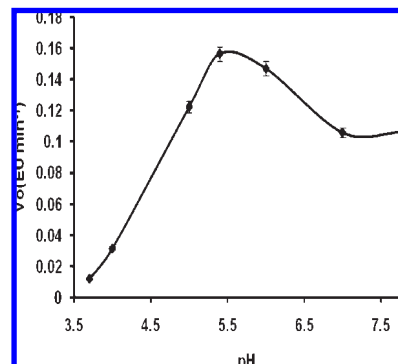

Figure 4. Effect of pH on the reaction. Means of triplicates with error bars indicating standard deviation are shown.

Quantification of Peroxidase Activity. A total of 3 mL of the reaction mixture containing 36 μM PPDD, 63 μM DMAB, and 85 μM H_2O_2 in 100 mM acetate/acetic buffer at pH 5.5 was taken. 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 25 °C. The initial velocity was recorded by the absorbance—time curve. Absorbance—time curves of the catalytic system in the presence of different concentrations of HRP are presented in **Figure 2B**. The range for the linear relationship between the initial velocity and the concentration of enzyme was 0.8—11.0 nM. From the fixed time method, 5 min of incubation of the reaction mixture at 30 °C allows the peroxidase to be assayed in the concentration range of 0.5—6.0 nM. The linear relationship by the rate and fixed time methods is shown in **Figure 2A**.

Optimum Experimental Condition. *Temperature Sensitivity.* Temperature sensitivity was determined by pre-incubating 36 μM PPDD, 63 μM DMAB, 50 μM H_2O_2, and 7.5 nM peroxidase in 100 mM acetate/acetic acid buffer at pH 5.5 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. **Figure 3** shows the percentage activity at different temperatures with reference to the enzyme activity at 30 °C.

pH for Maximum Activity. 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, 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 highest activity of the enzyme was observed in acetate/acetic acid buffer of pH 5.5. Hence, further studies were carried out at this pH. The response of the enzymatic activity with reference to pH is shown in **Figure 4**.

Evaluation of Kinetic Constants. Equation 1 was used to ascertain the ping-pong mechanism and to obtain the Michaelis—Menten constant values for all of the substrates (*1*). The initial velocities (V_o) were determined as a function of all of the substrate concentrations (H_o = H_2O_2, P_o = PPDD, and D_o = DMAB). Within one experiment, H_o and D_o were kept constant when P_o was changed, while in another experiment, H_o and P_o were kept constant

when D_o was changed. More experiments were conducted for both PPDD and DMAB at different concentrations of H_2O_2. The constant slope obtained in a double-reciprocal plot of V_o versus P_o and D_o (panels **A** and **B** of **Figure 5**) at different concentrations of H_2O_2 substantiate the ping-pong mechanism of HRP. The replots of the intercepts of panels **A** and **B** of **Figure 5** versus the reciprocal concentration of H_2O_2 also give a constant slope (figure not shown). The K_P and K_D were found to be 30 and 40 μM, respectively.

Catalytic Parameters. The K_H value of the coupling reaction was 80 μM, and this value is less than guaiacol oxidation by peroxidase, which is 5665 μM. The relative half-saturation point against the guaiacol method is 0.015, which reflects the higher number of interactions between PPDD—DMAB and the heme group at the active site (*20*). The catalytic efficiency of the proposed method calculated by a general kinetics procedure using 7.5 nM peroxidase was 0.54×10^6 M^{-1} min^{-1}, which is 20 times more competent than the guaiacol assay, taken as a reference method (*10*). **Figure 6** shows the Lineweaver—Burk plot for a comparison of catalytic efficiency of two co-substrates. The catalytic constant (k_{cat}) of the proposed assay corresponding to the point of saturation of all of the substrates was 0.0436×10^3 min^{-1}.

Mechanistic Approach for the Enzyme Activity Response. A similar oxidative product involving ferric chloride or peroxidase in the presence of H_2O_2 for PPDD and DMAB suggests that the enzymatic mechanism is analogous to that suggested by Ngo and Lenhoff (*16*) for the HRP-catalyzed oxidative coupling of 3-methyl-2-benzothiazolinonehydrazone hydrochloride and aromatic amines with the formation of indamine dye. The mechanism for the peroxidase-catalyzed reaction of PPDD and DMAB 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
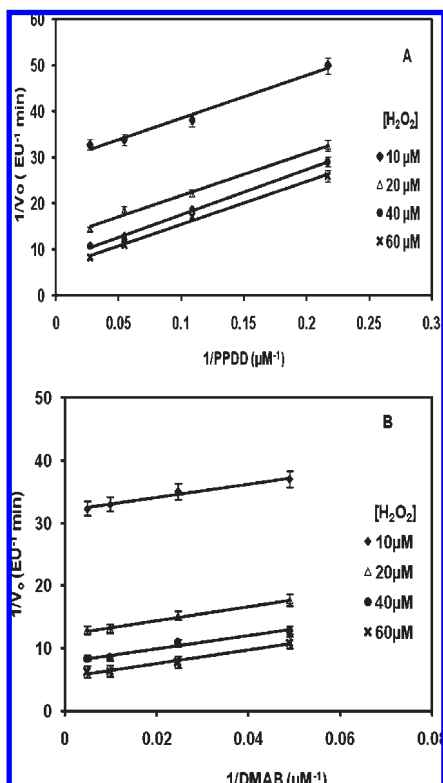
Figure 5. Kinetic behavior of the two substrate reactions for pure HRP (7.5 nM). Plot of the substrate−velocity relationship, according to the eq 1 for (A) PPDD and (B) DMAB. Means of triplicates with error bars indicating standard deviation are shown.
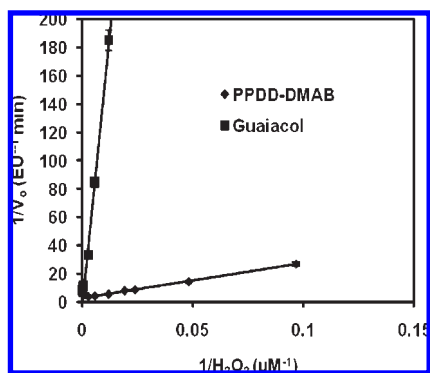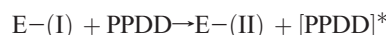

Figure 6. Lineweaver−Burk plots for HRP by PPDD−DMAB and the guaiacol method. The kinetic study was carried out for 7.5 nM peroxidase, as discussed in the general procedure. Means of triplicates with error bars indicating standard deviation are shown.
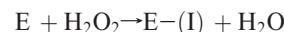
electrophillic 1,4-diimine, which may be postulated as the oxidative coupling species (*21*). The 1,4-diimine undergoes electrophillic substitution with DMAB in the free para position to the *N,N*-dimethylamino group, forming an intense green-colored product showing a strong absorption at 710 nm (**Figure 1B**). The kinetic mechanism in which catalytic coupling between PPDD and DMAB involving different Michealis−Menten values cannot over-rule the activation of both by a biocatalytic mechanism. Both PPDD and DMAB 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.

Scheme 1
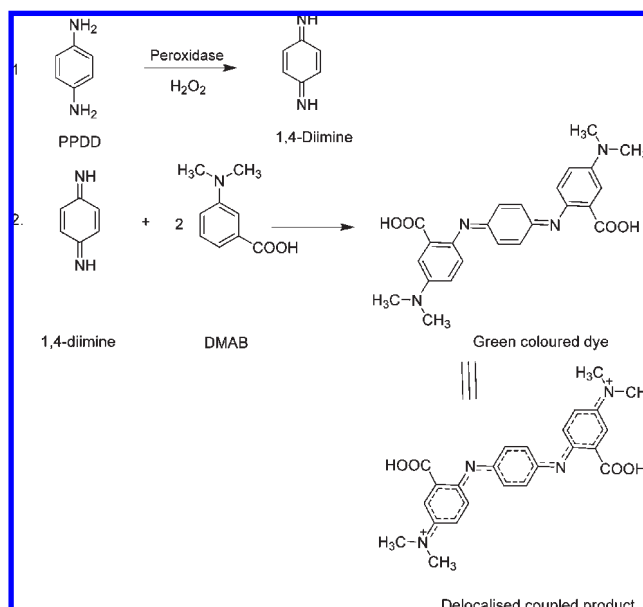
The reaction of two substrates based on the modified ping-pong mechanism suggested by Dunford (*22*) is below

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

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

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

$$[PPDD]^* + [DMAB]^* \rightarrow product$$

where E is the resting enzyme and [PPDD]* and [DMAB]* are activated reactants.

Applications to Plant Extracts. The buffer/tissue (b/t) ratio is an important factor in the preparation of peroxidase from the plant. In this study, b/t ratios from 5:1 to 15:1 mL g^{-1} were examined and the highest specific activity for each was obtained at 10:1 mL g^{-1} ratio.

The effect of pH on extraction of peroxidase from the plant sources was also investigated in the pH range of 5.0−7.5, and the highest enzymatic activity was obtained at pH 6.0. This crude extracts (100 μL) were taken for the quantification of peroxidase. The results obtained were compared to the guaiacol method (**Table 1**). The leaves of *T. cardifolia* and *B. oleracea* var. *capitata* extract showed the lowest and highest specific activities, respectively. The catalytic efficiency of all of the crude extracts was significantly greater than the reference guaiacol method. The quantification results obtained were analogous to the reference method, which proved that the proposed method is equally efficient and has the advantage of higher sensitivity and a rapid detection rate. Besides, it is for the first time that we detected the presence of peroxidase activity in the leaves of *A. sessilis* and *P. crispum* var. *neapolitanum.*

No work has been published thus far on the coupling of PPDD with DMAB 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 procedure requires only small quantities of colorimetric

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Table 1. Determination of the Peroxidase Activity in Plant Extracts

	activity (units)[a]		specific activity (units/mg)		
source	PPDD−DMAB	reference method (10)	PPDD−DMAB	reference method (10)	relative catalytic efficiency
N. tabaccum[b]	253.12 ± 1.5	252.00 ± 1.9	0.858	0.854	13.0
A. sessilis[b]	46.84 ± 2.4	47.00 ± 1.7	0.3969	0.3983	5.0
P. crispum var. *neapolitanum*[b]	28.51 ± 1.9	27.35 ± 2.3	0.4288	0.4114	12.0
T. cardifolia[c]	9.70 ± 1.5	9.25 ± 1.9	0.2319	0.2211	9.0
T. cardifolia[b]	8.96 ± 1.2	8.6 ± 2.0	0.0689	0.0661	8.0
B. oleracea var. *capitata*[b]	54.87 ± 2.5	53.3 ± 1.4	1.900	1.846	7.5
L. sativa[b]	6.52 ± 1.3	5.95 ± 1.9	0.1618	0.1476	12.0

[a] Mean, n = 5 determination. [b] Leaves. [c] Stem.

reagents. Optimization of the reaction conditions from the enzymatic oxidation allowed for the determination of H_2O_2 as low as 5 μM, which is unachievable by the guaiacol method.

The HRP-catalyzed oxidative coupling of PPDD and DMAB in the presence of peroxide allowed spectrophotometric determination of the HRP assay achieved within the linearity range of 0.8−11.0 and 0.5−6.0 pmol/mL 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 broad range of linearity of the guaiacol method made the determination discommodious and caused bigger errors. Thus, the proposed method serves as a suitable alternative to guaiacol for the assay of peroxidase.

ABBREVIATIONS USED

PPDD, paraphenylenediamine dihydrochloride; DMAB, 3-dimethylaminobenzoic acid; K_H, Michaelis−Menten constant for H_2O_2; K_p, Michaelis−Menten constant for PPDD; K_D, Michaelis−Menten constant for DMAB; V_{max}, maximum velocity; V_o, initial velocity; HRP, horseradish peroxidase.

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