Kinetic Study of Peroxidase-Catalyzed Coupling of Benzene-1,4-diamine and *N*-(2-Aminoethyl)naphthalen-1-amine: Development of Micromolar Hydrogen Peroxide Reaction System

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A novel chromogenic method to measure the peroxidase activity using *para*-phenylenediamine dihydrochloride (= benzene-1,4-diamine hydrochloride; PPDD) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (= *N*-(2-aminoethyl)naphthalen-1-amine; NEDA) is presented. The PPDD entraps the free radical and gets oxidized to electrophilic diimine, which couples with NEDA to give an intense red-colored chromogenic species with maximum absorbance at 490 nm. This assay was adopted for the quantification of H_2O_2 between 20 and 160 μM. Catalytic efficiency and catalytic power of the commercial peroxidase were found to be 4.47×10^4 M^{-1} min^{-1} and 3.38×10^{-4} min^{-1}, respectively. The catalytic constant (k_{cat}) and specificity constant (k_{cat}/K_m) at saturated concentration of the co-substrates were 0.0245×10^3 min^{-1} and 0.0445 μM^{-1} min^{-1}, respectively. The chromogenic coupling reaction has a minimum interference from the reducing substances such as ascorbic acid, L-cystein, citric acid, and oxalic acid. The method being simple, rapid, precise, and sensitive, its applicability has been tested in the crude vegetable extracts that showed peroxidase activity.

Introduction. – Horseradish peroxidase (EC 1.11.1.7), a heme protein containing iron(III)protoporphyrin IX (ferriprotoporphyrin IX) as prosthetic group, is a widely distributed enzyme in nature [1]. Its catalysis is associated with four types of activities, namely peroxidic, oxidative, catalytic, and hydroxylation [2]. Peroxidase and polyphenoloxidase act synergistically in the enzymatic browning. Peroxidase plays an important role in the plant defense mechanism by the oxidation of endogeneous phenolic compounds, which are toxic to the invading pathogens and pests [3]. Many physiological roles have been assigned to peroxidase that include indole-3-acetic acid metabolism, lignification, cross-linking of cell-wall polymers, submerin formation, and resistance to infection [4].

The reduction of peroxides at the expense of electron-donating substrates is useful in a number of industrial and analytical applications. The peroxidase reaction has acquired an additional advantage in the field of analytical biochemistry, because H_2O_2 produced by the oxidase reaction can be used to oxidize a chromogen to give a specific and accurate coupled assay for clinically important biomarkers such as glucose, lactose, cholesterol, uric acid, and others [5]. The combination of horseradish peroxidase and indole-3-acetic acid or its derivatives is currently being evaluated as an agent to be used in targeted cancer therapies [6]. Commercially available peroxidase is widely employed for removal of phenols and amines from industrial waste water, bleaching of industrial dye stuff, lignin degradation, fuel and chemical production from wood pulp, and in

various organic syntheses [7][8]. The largest application of peroxidase is in enzyme-linked immunosorbent assays as enzyme label of immunological reagents [9]. It is also used in the detection of nucleic acid [10].

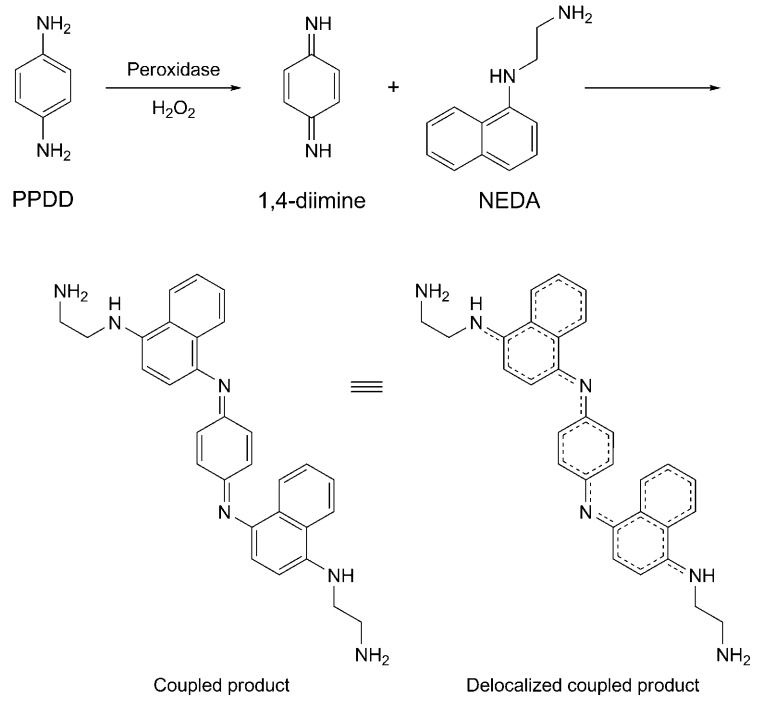
Many methods are available for the quantification of enzymatic activity that include fluorimetry, luminescence [11], and electroanalytical techniques [12]. However, they have some constraints, *e.g.*, the instruments used in fluorimetry and luminescence are too expensive and less versatile. The selectivity of luminescence is poor. The electroanalytical techniques need several steps to immobilize the enzyme on the solid support, which may ultimately reduce the enzyme activity. During incorporation of the enzyme within electropolymerized polymers or carbon paste, a large portion of enzyme will not be utilized, which results in the waste of expensive biocatalyst. Spectrophotometers are economical, easy to handle, and the reagents used in the assay are not expensive. Some of the common co-substrates employed in colorimetric method include guaiacol [13], 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) [14], *o*-phenylenediamine [15], tetramethylbenzidine, diaminobenzidine, and *p*-phenylenediamine [16], pyrogallol [17], 4-aminoantipyrine-phenol [18], catechol [19], 4-aminoantipyrine-aniline [20], 3-methyl-2-benzothiazolinonehydrazone hydrochloride(MBTH)-dimethylaminobenzoic acid [21], 4-amino-5-(*p*-aminophenyl)-1-methyl-2-phenylpyrazol-3-one with *N*-ethyl-*N*-sulfopropyl aniline sodium salt [22], *p*-phenylenediamine-dimethylaminobenzoic acid [23], and MBTH-10,11-dihydro-5*H*-benz[*b*, *f*]azepine [24]. However, these reagents have some limitations such as carcinogenicity of *o*-dianisidine and benzidine, and broader linearity range of pyrogallol and guaiacol due to their auto-oxidation. Tetramethylbenzidine has initial problems like stability and poor solubility in aqueous buffer solution. 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 towards H_2O_2 [5].

In this work, attempt has been made for the peroxidase-catalyzed coupling of co-substrates, *para*-phenylenediamine dihydrochloride (= benzene-1,4-diamine hydrochloride; *PPDD*) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (= *N*-(2-aminoethyl)naphthalen-1-amine; NEDA), in the presence of H_2O_2 and to measure the absorption at 490 nm with product as shown in the *Scheme*. The method will be applied for the quantification of peroxidase activity in the crude extracts of vegetable sources.

Evaluation of the Catalytic Parameters of the PPDD – NEDA – Peroxidase with Reference to the Guaiacol – Peroxidase. The term k_{cat}/K_m is often used as specificity constant to compare the relative rates of reaction of each pair of substrates, in which each is catalytically transformed by an enzyme [25]. This is because, if K_m is used on its own as the indicator of specificity, the effect of the 'better' substrate will be strongly manifested mainly at the values of $[S]/K_m \ll 1$. As $[S]/K_m$ increases above this value, k_{cat} becomes a parameter that describes well the substrates which are better. In general, for an enzyme acting simultaneously on two substrates, $[S]_{PN}$ and $[S]_g$ at rates V_{PN} and V_g are given by *Eqn. 1*.

$$\frac{V_{PN}}{V_g} = \frac{\left(k_{cat}^{PN}/K_m^{PN}\right)[S]_{PN}}{\left(k_{cat}^{g}/K_m^{g}\right)[S]_g} \tag{1}$$

Scheme. *Proposed Reaction Pathway for the Formation of the Intense Red-Colored Product*



If $[S]_{PN} = [S]_g$, the [S] terms in the equation get cancelled, and the k_{cat}/K_m value of each co-substrate is equal to the relative rate at which the enzyme catalyzes the transformation of each substrate.

The combined plot of the rate–substrate profile shows the way in which the rate of the reaction by guaiacol overtakes the PPDD–NEDA at a particular concentration of the substrate, [S], which can be evaluated by the equation concerning the rate of the enzymatic reaction.

$$[S] = \frac{k_{cat}^{PN} K_m^g - k_{cat}^g K_m^{PN}}{k_{cat}^g - k_{cat}^{PN}} \tag{2}$$

The equation was derived from the *Michaelis–Menten* equations for two co-substrates by setting $E_0^g = E_0^{PN}$, $V_g = V_{PN}$, and solving [S].

$$V_{PN} = \frac{k_{cat}^{PN} E_0^{PN} [S]}{K_m^{PN} + [S]} \tag{3}$$

$$V_g = \frac{k_{cat}^g E_0^g [S]}{K_m^g + [S]} \tag{4}$$

At $K_m \ll [S]$, the ratio $\frac{V_g}{V_{PN}}$ reduces to

$$\frac{V_g}{V_{PN}} = \frac{k_{cat}^{g}}{k_{cat}^{PN}} \tag{5}$$

At $K_m \gg [S]$, the ratio $\frac{V_{PN}}{V_g}$ reduces to

$$\frac{V_g}{V_{PN}} = \frac{k_{cat}^{g} K_m^{PN}}{k_{cat}^{PN} K_m^{g}} \tag{6}$$

The ratio of rates, $\frac{V_g}{V_{PN}}$, inverts at a particular substrate concentration [S], varying from <1 below [S] to >1 above [S], and the crossover occurs, because the ratio depends upon $\frac{K_m^{PN}}{K_m^{g}}$, which is less at low substrate concentration, and at high substrate concentration it depends on the ratio $\frac{k_{cat}^{g}}{k_{cat}^{PN}}$.

Here, k_{cat}^{g} and k_{cat}^{PN} are catalytic constants of guaiacol and PPDD–NEDA, respectively.

Similarly, K_m^{g} and K_m^{PN} are *Michaelis–Menten* constants of guaiacol and PPDD–NEDA, respectively.

Results and Discussions. – *Proposed Reaction Pathway for the Enzyme-Activity Response.* A similar oxidative product involving $FeCl_3$ or peroxidase, in the presence of H_2O_2, for PPDD and NEDA suggests that the enzyme mechanism is analogous to that suggested by *Ngo* and *Lenhoff* [21] for the horseradish peroxidase (HRP)-catalyzed oxidative coupling of MBTH and aromatic amines with the formation of indamine dyes. The mechanism of the peroxidase-catalyzed reaction of PPDD and NEDA is proposed in the *Scheme*. The free radical is released by the oxidation of H_2O_2 through ferryl intermediate ($Fe^{IV}=O$-porphyrin π-cation radical) of the peroxidase. Under the reaction conditions examined, PPDD loses two electrons and two protons upon enzymatic oxidation in the presence of H_2O_2, forming electrophilic 1,4-diimine, which may possibly be the coupling species [26]. The 1,4-diimine undergoes electrophilic substitution with NEDA in the free *para*-position to the substituent group, forming an intense red-colored product showing strong absorption at 490 nm, as shown in *Fig. 4*, *b* (see below).

Temperature Sensitivity. Temperature sensitivity was determined by pre-incubating 3 ml of the reaction mixture containing 92 μM PPDD, 771 μM NEDA, 330 μM H_2O_2, and 4.7 nM peroxidase in 0.1M potassium dihydrogen orthophosphate/NaOH at pH 6.0 for 5 min at a temperature range of 0–80°. The activity of the enzyme was registered as a function of absorbance of the colored solution. The activity initially increased up to 30° and decreased thereafter. *Fig. 1* shows the activity at temperatures ranging from 0 to 80°.

Effect of pH and Buffer Concentration. The following 0.1M buffers having different pH ranges were used for the assay: citric acid/citrate at pH 3.6–5.6, acetate/AcOH at pH 3.6–5.6, potassium dihydrogen orthophosphate/NaOH at pH 6.0–8.0, and potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate at pH 6.0–7.5. Enzyme activity was highest in potassium dihydrogen orthophosphate/NaOH at

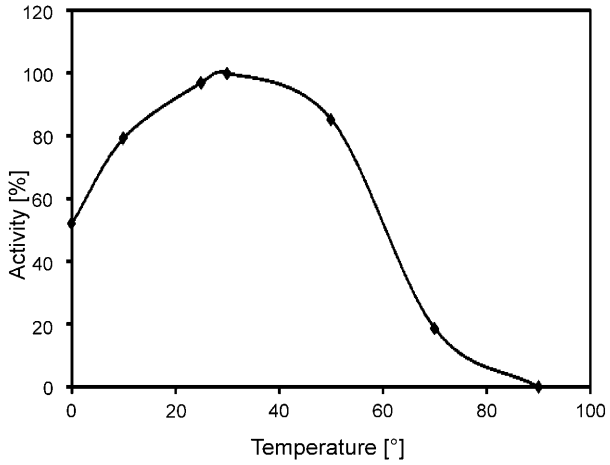


Fig. 1. *Effect of incubation temperature on the reaction*

pH 6.0. Hence, all further studies were carried out at this pH. The response of the enzyme activity with reference to pH is shown in *Fig. 2.*

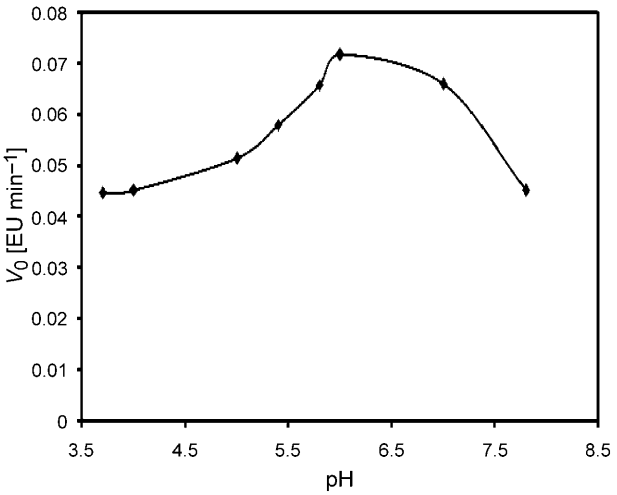


Fig. 2. *Effect of pH on the reaction*

Catalytic Parameters. The K_m^{PN} value of PPDD – NEDA reaction was found to be 550 μM, and this value is less than guaiacol oxidation by peroxidase, K_m^g of which is 14350 μM. The relative half-saturation point against the guaiacol method is 0.038, which reflects the higher number of interactions between PPDD – NEDA and heme group at the active site. The catalytic power and catalytic efficiency were found to be 3.38×10^{-4} min^{-1} and 4.47×10^4 M^{-1} min^{-1}, respectively, by the PPDD – NEDA method, which are 1.5 times more efficient than the guaiacol assay procedure. The catalytic constant, k_{cat}, and specificity constant, k_{cat}/K_m, at saturated concentration of the

substrates were found to be 0.0245×10^3 min^{-1} and 0.0445 μM^{-1} min^{-1}, respectively. The concentration of the substrate at which the rate of the reaction of the guaiacol [13] overcomes the PPDD–NEDA reaction was 315 μM. *Fig. 3,a*, shows the *Lineweaver–Burk* plot for the comparison of catalytic parameters of the two co-substrates. The plot of relative rate of the reaction *vs.* substrate concentration is shown in *Fig. 3,b*.

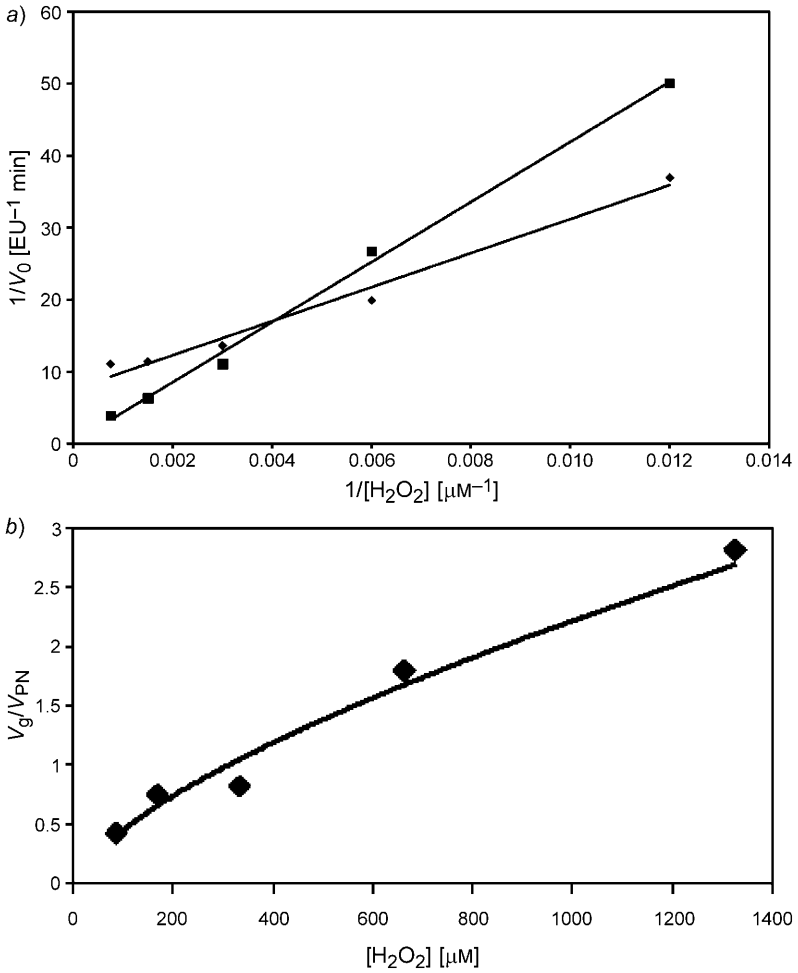


Fig. 3. a) Lineweaver–Burk *plot of the horseradish peroxidase-catalyzed reaction by two different co-substrates, namely, guaiacol* (◆) *and PPDD–NEDA* (■). b) *Ratio of the rates of the two reactions* (V_g/V_{PN}) *as a function of substrate concentration.*

Effect of Foreign Species on the Quantification. Under the reaction conditions used for the coupling reaction, the suitability of the method for application in vegetable samples was studied by taking various cations, anions, and other commonly accompanying amino acids in solutions containing 80 μM H_2O_2. Except ascorbic acid, Cu^{2+}, Fe^{3+}, L-tryptophan, L-tyrosine, L-cystine , L-cysteine, and Fe^{2+}, none of the other

foreign species studied interfered in the quantification. The tolerance ratio are tabulated in *Table 1*, which corresponds to the concentration of diverse species that cause an interference of ±3% in its quantification. The result showed that large number of ions, amino acids, and carbohydrates examined did not interfere in the assay.

Table 1. *Effect of Foreign Ion Species on the Quantification of Peroxidase Activity*

Foreign species	Tolerance ratio[a])
Ascorbic acid	0.00066
Cu^{2+}	0.0589
Fe^{3+}, L-Tryptophan	0.20
L-Tyrosine, L-Cystine, L-Cysteine	0.500
Fe^{2+}	1.272
Zn^{2+}, L-Serine, Citric acid, Uric acid	2.30
Oxalic acid, DL-Methionine	5.75
F^-	6.69
D-Asparagine, NO_2^-	9.00
L-Histidine, Isoleucine	13.50
DL-Threonine	47.26
KCl	53.87
NH_4^+	60.08
Urea	66.96
Na^+	67.92
SO_4^{2-}, Lactose	90.00
Glycine	196.80
Galactose	276.24
Sucrose	528.89
Glucose	1000.00

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

Application to Vegetable Extracts. The buffer-to-tissue ratio is an important factor in the preparation of peroxidase from the vegetable extracts. Maximum peroxidase activity was obtained at 2:1 buffer-to-tissue ratio.

The effect of pH on the extraction of peroxidase from the vegetable sources was also investigated in the pH range of 4.0–8.0, and the highest enzymatic activity was obtained at pH 6.0. The crude extracts (100 µl) from vegetables were taken for quantification of peroxidase activities. *Table 2* shows the peroxidase activity determined by the proposed method. The relative half-saturation point of the proposed method with reference to the guaiacol method is less than 1, indicating the greater interaction between the active site, heme group of peroxidase, and PPDD–NEDA co-substrates. The relative catalytic efficiency is significantly greater than that of the guaiacol method. The quantification results obtained by PPDD–NEDA suggest that the method is as sensitive as that of guaiacol, and detection rate is also similar to that of the latter.

Conclusions. – The proposed method is versatile and economical, and uses water-soluble chromogenic reagent in the quantification of peroxidase in crude vegetable extracts. These co-substrates are as sensitive as the guaiacol, and the method has similar

Table 2. *Determination of Peroxidase Activity in the Crude Vegetable Extracts*

	PPDD – NEDA[a])	Guaiacol[a])	Protein [mg][a])	$\frac{K_m^{PN}}{K_m^{g}}$
D. carota	10.6 ± 1.0	12.9 ± 1.2	36.7 ± 1.2	0.020
P. vulgaris	87.6 ± 1.5	227 ± 1.8	41.9 ± 1.0	0.147
B. oleracea var. *gongylodes*	1226 ± 1.5	1343 ± 2.0	48.8 ± 1.9	0.041
S. lycopersicum	86.5 ± 1.4	130 ± 1.3	372 ± 1.5	0.015
B. oleracea var. *capitata*	2824 ± 1.6	1846 ± 1.4	63.7 ± 1.1	0.117
R. sativus	613 ± 1.9	482 ± 1.3	81.0 ± 1.0	0.009

[a]) Mean ± SD, $n = 5$ determination.

detection rate as evidenced by kinetics study. Optimization of the reaction conditions allowed the quantification of H_2O_2, as low as 20 μM. The narrow *Beer*'s law linear range of the PPDD – NEDA is another important analytical feature for the detection of H_2O_2 and peroxidase. The broad linearity of guaiacol due to the auto-oxidation is disadvantageous and caused more errors in the assay procedure by the guaiacol method. Thus, the proposed method is as sensitive as the guaiacol method, and, as such, it can be used for routine analytical purposes.

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Experimental Part

Instrumentation. Absorbance measurements: *JASCO* model *UVIDEC-610 UV-VIS* spectrophotometer with 1.0-cm matched cells. A water-bath shaker (*NSW 133*, New Delhi, India) was used to maintain a constant temp. for color development. pH Measurements: digital pH-meter (*Model EQ-614*, *Equiptronics*, Mumbai, India).

Chemicals and Reagent Preparation. H_2O_2 Stock soln. (0.3% (*v*/*v*)) was prepared by diluting the commercial reagent (30% (*v*/*v*), *E. Merck*, Mumbai, India), its concentration was standardized by titration with secondary standard, $KMnO_4$. Working standard solns. were prepared from the stock soln. by dilution. PPDD and NEDA were purchased from *Sigma – Aldrich*. PPDD (1.38 mM) and NEDA (11.57 mM) were prepared by dissolving 2.5 and 30 mg, resp., in 10 ml of double dist. H_2O. Guaiacol was obtained from *Loba Chemie*, India. Peroxidase (100 U mg^{-1}) was purchased from *Himedia*, Mumbai, India, and the soln. was prepared by dissolving 2 mg in 10 ml of 0.1M potassium dihydrogen orthophosphate/NaOH buffer of pH 6.0. Further dilutions with the same buffer were performed as and when required. All chemicals used in the assay were of anal. grade.

Sample and Crude-Extract Preparation. As a source of peroxidase, the vegetables used included *D. carota*, *P. vulgaris*, *B. oleracea* var. *gongylodes*, *S. lycopersicum*, *B. oleracea* var. *capitata*, and *R. sativus.* These were collected from the local super markets, transported at 4° to the laboratory, and stored at − 20° until used. Samples (25 g) of vegetables were washed with dist. H_2O and homogenized in a blender using 50 ml of 0.1M phosphate buffer at pH 6.0. The extract was passed through cheesecloth and centrifuged at 12000*g* for 15 min, and the supernatant was used as crude extract.

Protein Determination. The total protein concentration was determined in triplicate by the method of *Lowry et al.* [27], using bovine serum albumin as a standard.

Quantification of H_2O_2. The concentration of H_2O_2 was determined in 3 ml of the soln. containing 92 μM PPDD, 771 μM NEDA, and 7.5 nM peroxidase in 0.1M potassium dihydrogen orthophosphate/NaOH buffer at pH 6.0. The reaction was initiated at 25° by adding 100 μl of different concentrations of

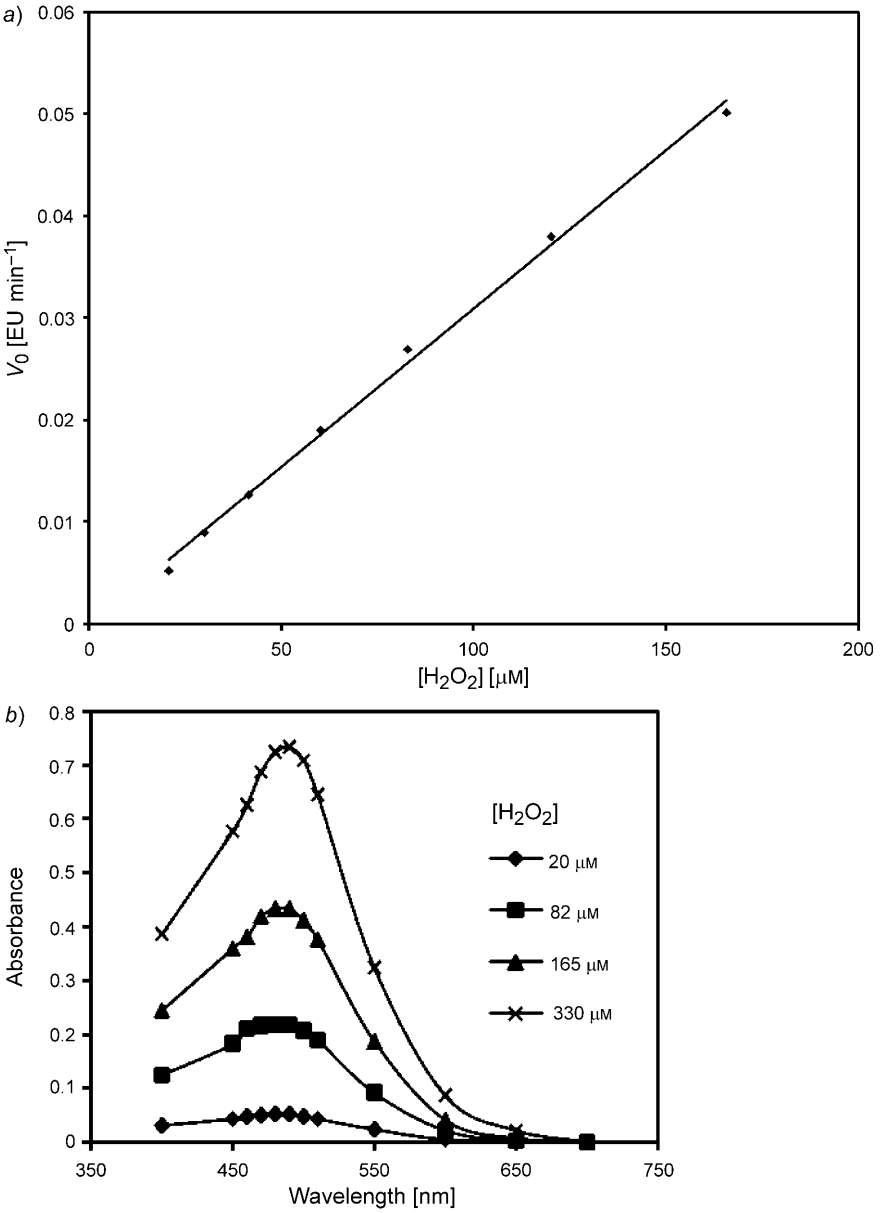

Fig. 4. a) *Calibration graph for the quantification of H_2O_2 by rate method.* b) *Absorption spectrum of H_2O_2: 92 μM PPDD + 771 μM NEDA + 7.5 nM peroxidase in 0.1M potassium dihydrogen orthophosphate/ NaOH of pH 6.0 with varying concentrations of H_2O_2* (20–330 μM). *The spectrum containing neither H_2O_2 nor enzyme shows negligible absorption.*

H_2O_2 within the linear range. The change in the absorbance was continuously recorded at 490 nm. The initial rate was then plotted against the concentration of H_2O_2 to obtain the calibration graph. The values

of K_H and V_{max} for the peroxidase enzyme from the *Lineweaver–Burk* plot were found to be 550 μM and 0.1861 EU min^{-1}, resp. The linearity of the graph remained between 20 and 160 μM H_2O_2. The calibration graph for the quantification of H_2O_2 is shown in *Fig. 4,a.*

Quantification of Peroxidase Activity. A total of 3 ml of the reaction mixture containing 92 μM PPDD, 771 μM NEDA, and 500 μM H_2O_2 in 0.1M potassium dihydrogen orthophosphate/NaOH at pH 6.0 were 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 the reagents except peroxidase at 25°. The initial velocity was recorded by the absorbance–time curve. Absorbance–time curves of the catalytic system in the presence of different concentration of horseradish peroxidase (HRP) are presented in *Fig. 5,b.* The range for the linear relationship between the initial velocity and concentration of the enzyme was between 1.18 and 37.87 nM. By the fixed-time method, 5 min of incubation of the reaction mixture at 30° allowed the peroxidase to be assayed in the concentration range from 2.36 to 18.93 nM. The linear relationship by the rate and fixed time methods is shown in *Fig. 5,a.*

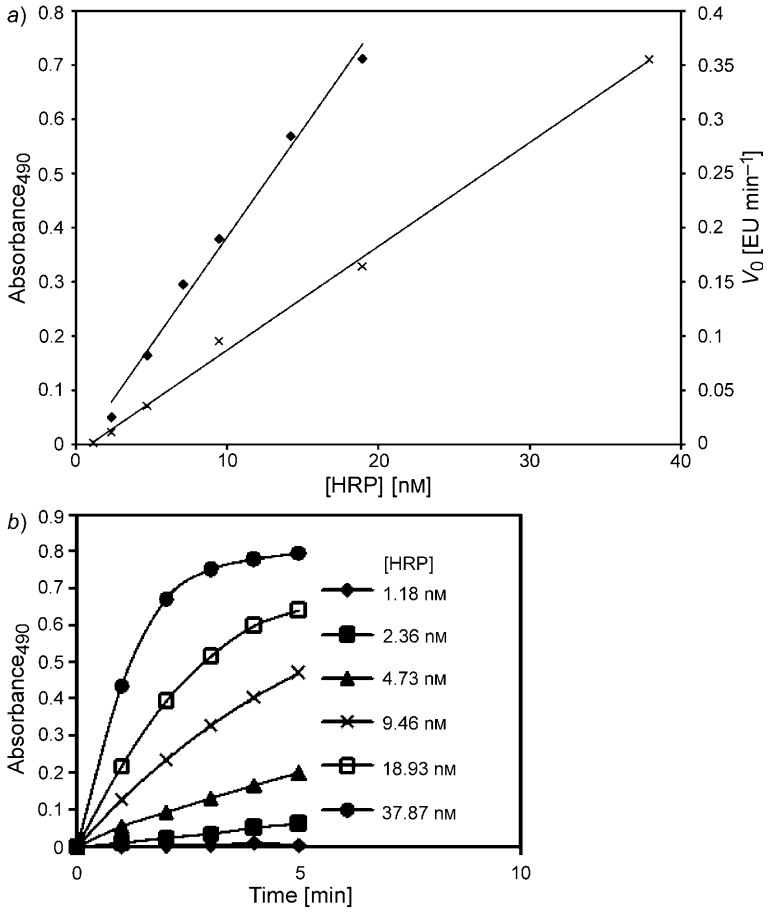

Fig. 5. a) *Calibration graph for the quantification of horseradish peroxidase by rate method* (×) *and by fixed-time method* (◆). b) *Absorbance–time curves for varying concentrations of horseradish peroxidase.*

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