RASĀYAN *J. Chem.*
Vol. 15 | No. 4 |2993-2998| October - December | 2022
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

DEVELOPMENT AND QUANTIFICATION OF HORSERADISH PEROXIDASE USING PARAPHENYLENEDIAMINE AND ALPHA NAPHTHOL: APPLICATIONS IN MEDICINAL PLANT SOURCES

Supriya B S and Avinash Krishnegowda✉
Department of Chemistry, ATME College of Engineering, Mysuru, India, 570028
✉Corresponding Author: avinashkchemistry@gmail.com

ABSTRACT

In this work, a unique calorimetric system was presented for the determination of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). This HRP-catalyzed system is based on the formation of electrophilic 1,4 –diimine by the oxidation of Paraphenylenediamine (PPD), this intermediate undergoes coupling with the free para position of Alpha naphthol (AN) to generate the stable intense purple-colored radical with λ_{max} 520nm had good linear with peroxidase concentration 0.08-1.32 nm and 0.02-0.66 nm by kinetic and one-time detection method and H_2O_2 amid 0.6 – 38.7 µM from the kinetic and 0.07- 4.83 µM by fixed time method, respectively. Moreover, the applicability of the proposed PPD-AN system has been successfully used to detect HRP activity in medicinal plants with crude extract as the source of enzyme and the outcomes were compared with the reference method. Overall, the present system can be adopted as a candidate system for the determination of H_2O_2.

Keywords: Hydrogen Peroxide, Alpha Naphthol, Paraphenylenediamine, Peroxidase.
RASĀYAN *J. Chem.*, Vol. 15, No.4, 2022

INTRODUCTION

Horseradish Peroxidases (EC 1.11.1.7) is a ubiquitous heme protein, catalytically active Fe (III) state, that catalyzes many oxygen transfer reactions with H_2O_2.[1] Peroxidase is extensively distributed as an oxidoreductase in animals, plants, microorganisms, and fungi. Peroxidase is extensively used as an index of blanching due to its heat thermal stability.[2] Peroxidases are involved in the conversion of toxic phenolic compounds into quinones.[3] Peroxidase catalysis is grouped into four classes namely, oxidative, catalytic, peroxidic, and hydroxylation[4]. It is also used in the removal of amines and phenols from industrial wastewater and the detection of nucleic acid.[5] Peroxidases play an important role in diagnostic assay, polymer synthesis, biosensors, and various organic syntheses.[6] The peroxidase reaction has widespread applications in the field of biochemistry due to the formation of H_2O_2 by the oxidase reaction employed in the study of some clinical significance biomarkers such as cholesterol, uric acid, and $C_6H_{12}O_6$. In plants, peroxidase is responsible for building cell walls and growth regulation due to the metabolism of the auxin hormone.[7] Because of its significance, many chromogenic reagents, and many analytical techniques are designed for the determination of HRP activity, namely amperometry, HPLC[8], spectrofluorimetric[9], electrochemical[10], cyclic voltammetry[11], Raman scattering[12], luminescence[13], and potentiometric assay.[14] However, they have some demerits, too expensive, less versatile, poor sensitivity, multi-step, high incubation period, and need for expensive biocatalysts. Some of the reagents used for the quantification of peroxidase are 2,4-DMA, PPD/mequinol[15], Para-acetylamino phenol[16], iminodibenzyl/PPD[17], N,N-diethyl-p-phenylenediamine sulphate/3-AP[18], PPD/3-Dimethylaminobenzoic acid[19], 2,5-DMA[20], 8 HQ/PPD.[21] Some of these reagents used are low sensitivity, toxicity, carcinogenic, poor solubility, and Mutagenicity.
Hence, the purpose of this work was to (i) construct an innovative catalytic spectrophotometric system for the determination of peroxidase and H_2O_2 based on enzyme-catalyzed oxidation of PPD, and (ii) Investigate the kinetic parameters (such as reaction rate, the effect of co-substrates, pH, temperature, substrates, and buffer, (iii) Investigate the stability of PPD-AN system, (iv) Applying the PPD-AN system to analyze peroxidase concentration in plants crude extracts. To validate the proposed system, the results were compared with the results obtained from the reference guaiacol method.[22]

http://doi.org/10.31788/RJC.2022.1548091

EXPERIMENTAL

Instrumentation and Chemicals

The absorbance measurements were recorded on a UV-Vis spectrophotometer (UVIDEC-610, Jasco model) using 1 cm quartz cuvettes. The pH measurements were measured on a pH digital meter (Equip-Tronics version, India). A water bath shaker (Version-206-88950-93, Japan) with a temperature controller was used for the color development of working solutions. All reagents used in the PPD/AN system were of analytical reagent or higher grade. Throughout the experiment ultrapure water was used. HRP (100 units/mg), PPD, AN procured from Sigma Aldrich Chemicals Pvt Ltd, India. PPD (616.4 µM) was prepared by dissolving 20mg using double distilled water finally making up to 10ml. AN (57.8 µM) was initially dissolved in a small amount of alcohol and makeup up to 10ml using Double distilled water. H_2O_2 (1mm) working solution was prepared daily by diluting (30%, Hi media laboratories, India) with Double distilled water. HRP working solution was made by liquefying the required quantity in a phosphate buffer of pH 5.93.

Crude Extract Sample Preparation

Disease-free leaf/stem portions of *Plectranthus amboinicus* (Mexican mint)*, Tinospora cordifolia* (Moonseed)*, Centella Asiatica* (Gotu kola), *Cymbopogon* (lemongrass) are selected from the local farmland as a source of HRP and carry to the research laboratory at 4 ^{0}C temperature and kept at -20 ºC till used. The crude extract was made by blending 5g of the sample with 100 mL of phosphate buffer (pH 5.93) using a blender. The plant crude extract was sifted using a muslin cloth and later used a centrifuge rotor to separate the filtrate and the clear liquid was collected as a plant extract.

Proposed experimental protocol

Hydrogen Peroxide and Peroxidase Assay

The H_2O_2 concentration was studied using 616.4 µM PPD, 57.8 µM AN, KH_2PO_4/K_2HPO_4 buffer of pH 5.93, 1.32nm peroxidase, and 0.1 ml of different H_2O_2 concentrations was used (0.6-77.4µM). The absorbance was noted at 520 nm at a 1min time interval. The λ_{max} linearity range of the H_2O_2 lies between 0.6-38.7 µM and 0.07- 4.83 µM from the kinetic and fixed time method. The effect of the enzyme on the reaction rate was measured in a 3ml reaction mixture containing 616.4 µM PPDA, 57.8 µM AN, KH_2PO_4/K_2HPO_4 buffer of pH 5.93, 19.3 µM H_2O_2, and 0.1 ml of different HRP concentrations varying in the range from 0.01-1.32nm. The linearity range of the peroxidase by the one-time detection method was studied by pre-incubating the working solution at optimum temperature (30 ^{0}C) for 5 minutes. The quantification of the H_2O_2 and HRP calibration graph by the kinetic method is detailed in Fig.-1 and 2. The relative absorbance time curves for ascertaining linearity for different concentrations of HRP and H_2O_2 by the kinetic method are displayed in Fig.-3.

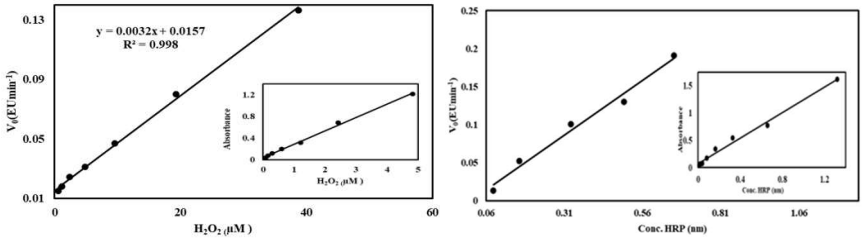


Fig.-1 and 2 Effect of H_2O_2 and HRP by the kinetic Method. The Inset Shows the Calibration Graph by One-Time Detection Method

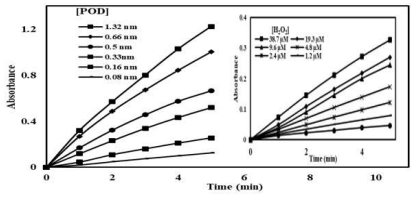


Fig.-3: Relative Absorbance Time- Graph for Different Concentrations of HRP. The Inset Shows the Relative Absorbance Time Graph for H_2O_2

Effect of Analytical Parameters on Enzyme Assay

Study of PPD and AN On-Enzyme Activity

The influence of co-substrates, PPD, and AN concentration on the reaction rate was examined, using 231.2 µM AN, 38.7 µM H_2O_2, 1.32nm peroxidase, 0.1M KH_2PO_4/K_2HPO_4 buffer of pH 5.93 and PPD concentrations ranging from 19.26-924.6 µM. The enzyme activity increased with increasing the concentration of PPD from 19.26-616.4 µM. For optimized concentration of AN, the reaction mixture containing 616.4 µM PPDA, 38.7 µM H_2O_2, 1.32nm peroxidase, 0.1M KH_2PO_4/K_2HPO_4 buffer of pH 5.93, and varying concentrations of AN in the range of 7.22-462.5 µM. there is linearity in the range of 3.61-57.8 µM, therefore 57.8 µM of AN and 616.4 µM PPD was chosen for all subsequent assays.

Temperature and pH Sensitivity

For this study, the reaction mixtures containing 616.4 µM PPD, 57.8 µM AN 38.7 µM H_2O_2, 1.32nm peroxidase, 0.1M KH_2PO_4/K_2HPO_4 buffer of pH 5.93 was pre-incubated at varying temperatures (0-80 ^{0}C) for 10 mins. The enzyme activity increased up to 30^{o}C and thereafter decreased. Hence 30 ^{0}C temperature was selected for further assays. The influence of pH has a pronounced effect on the reaction rate as quantified by using the following different buffers such as CH_3COONa/CH_3COOH (3.6-5.6) and $C_6H_8O_7$/pot. citrate buffer (3.6–5.6), KH_2PO_4/NaOH (6.0–8.0), KH_2PO_4/K_2HPO_4 (5.93-7.5), Tris- HCl (pH-9.8). The experimental result depicts that the highest enzyme activity was detected at pH 5.93 using KH_2PO_4/K_2HPO_4 buffer. Hence KH_2PO_4/K_2HPO_4 buffer of pH 5.93 was selected as the optimized pH.

Evaluation of Kinetic Variables

Kinetic variables were analyzed under experimental optimum conditions. Figure-4 depicts the Line weaver-Burk plot (L-B plot) used to calculate the Michaelis -Menten constant (K_m) for the projected method with a correlation coefficient of 0.9993. The Km was witnessed to be 4.4503 µM, the Km value shows the affection between the enzyme active site and substrate. The K_m value of the proposed method is low as compared to earlier reported methods[15,16,19-25], The maximal velocity (V_{max}) of the projected reaction is found to be 0.0694 EU min^{-1}. The specificity constant (k_{sp}) and Catalytic constant (k_{cat}) of the assay were found to be 0.0236 µm^{-1}min-1 and 0.1051 min^{-1}. The catalytic efficiency(k_{cat}) and catalytic power (K_{pow}) was ascertained to be 0.0236 µm^{-1}min^{-1} and 0.0155 EU µm^{-1}min^{-1}.

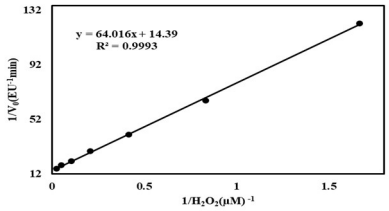


Fig.-4: Lineweaver- Burk Plot for POD

Method Validations

Analytical Performance

The proposed assay reveals that the enzyme activity increases with increasing the H_2O_2 concentration up to 38.7 µM. The excellent linearity range of H_2O_2 lies between 0.6-38.7 µM and 0.07- 4.83 µM from the rate and fixed time method from the calibration graph. The LOD (detection limit) and LOQ (quantification limit) were 0.18 µM and 0.60 µM, respectively. The peroxidase assay reveals that the enzymatic reaction rate increased up to 1.32 nm concentration, and the linearity range of peroxidase lies between 0.08-1.32nm and 0.02-0.66 nm by rate and fixed time methods, respectively. The LOD and LOQ of peroxidase were 0.0042 nm and 0.014 nm, respectively.

Study of Interferences

To verify the proposed assay, the study of interferences was done under the experimental optimized conditions using several foreign materials. The interference study was conducted by exercising 0.60 µM H_2O_2 throughout the procedure, the interfering species threshold limit value was calculated as tolerance ratio which means no error beyond ±3%. The experimental outcome showed that excluding ascorbic acid,

Mo (VI), Iron (III), L-tyrosinase, other carbohydrates, cations, anions, and amino acids did not show any interference in the proposed assay, the concentration of foreign materials, and their tolerance ratios are tabulated in Table-1.

Table-1: Effect of Interfering Species

Foreign species	Tolerant ratio*	Foreign species	Tolerant ratio*
Ascorbic acid	0.0031	Cl^-, oxalic acid, K^+	22.51
Fe (III), L-Tyrosinase	0.3336	Mg (II), DL-methionine	27.6744
Mo (IV)	0.4796	Citric acid, NH_4	34.8577
Cu^{2+}	0.6913	Sulfate, L-serine	53.82
L-Tryptophan, fructose, L-Cysteine	3.3475	CO, mannose, fructose	75.4383
L-Cystine, Lactose	5.4912	Zn (II)	85.6434
L-Histidine, Isoleucine	9.3814	Urea	100.39
D-asparagine, galactose	14.0588	Sodium, Glycine	117.9755
Maltose, sucrose, uric acid	17.6943	Acetone	2478.99

*Tolerant ratio for the measurement of 0.60 µM H_2O_2

Accuracy and Precision Studies

The accuracy and precision of the proposed assay were quantified by studying various solutions with known concentrations of hydrogen peroxide. The experimental outcome shows that intra and inter-precision of the proposed system were 0.77- 1.6% (n=10) and 1.3-2.44% (n=10), respectively. The accuracy range of [H_2O_2] at 4.83, 9.67 µmol/L was 90-95% and 93-97%, and for 19.35 µmol/L was 95-102%, respectively. results are exposed in Table-2.

Table-2: Inter and Intra Precision as Per the Proposed Method

Inter precision			Accuracy range %	Intra precision			Accuracy range %
H_2O_2 (µM)	SD (n=10)	CV	-	H_2O_2 (µM)	SD (n=10)	CV	-
4.83	0.000181	1.6	91.42-94.23	4.83	0.000143	2.44	90.34-94.16
9.67	0.000220	1.09	93.84-96.97	9.67	0.000561	1.97	93.40-95.12
19.35	0.000662	0.77	95.21-101.46	19.35	0.00191	1.3	96.16-100.86

Evaluation of HRP Activity in Crude Extracts

The application of the proposed assay was investigated by using medicinal plants as a source of peroxidase such as *Plectranthus amboinicus* (Mexican mint)*, Tinospora cordifolia* (Moonseed)*, Centella Asiatica* (Gotu kola), *Cymbopogon* (lemongrass). The evaluation of HRP activity in crude extracts was analyzed using different buffers with varying pH ranges and 5:1 to 15;1mL/g buffer to tissue ratio. The HRP activity was highest in the Phosphate buffer of pH 6.0, hence, this was chosen as the optimum pH for further assays. Of these, *Centella Asiatica* and *Tinospora cordifolia* crude extract were found to give maximum HRP and specific activity in the projected and reference method whereas *Cymbopogon* crude extract was found to be the least HRP activity. The obtained results are tabulated in Table-3.

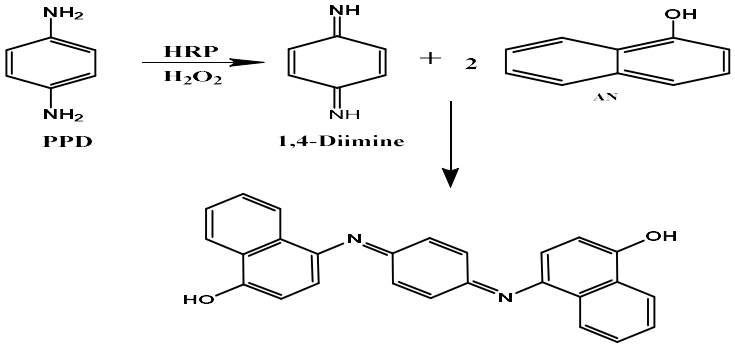
Table-3: HRP Activity in Plant Extracts

Crude samples	Enzyme activity (EU)[a]		$\frac{K_m^{PPD}}{K_m^G}$
	PPD-AN	Guaiacol	
Plectranthus amboinicus	46.84	48.12	0.0756
Tinosporacordifolia	67.40	61.32	0.7302
Centella Asiatica	86.13	79.11	0.9068
Cymbopogon	18.26	20.46	0.2383

The Mechanistic Approach Between PPD and AN in Response to Peroxidase Activity

The possible catalytic mechanism for the PPD/AN system is presented in Scheme-1. Under enzymatic oxidation, PPD loses 2 e^- and 2 H^+ in the presence of H_2O_2, resulting in the formation of 1,4-diimine

radicals.[26,27] Later the imine radical couples with AN to generate a purple-colored reaction product that had λ_{max} at 520nm.



Scheme-1: Possible Reaction Mechanism for Purple-Colored Chromogenic Species

CONCLUSION

The substantial literature analysis verified that no work was promulgated by the coupling of PPDA and AN for the assay of peroxidase. The reagents are inexpensive, versatile, steady, and have no technical risks. The higher catalytic efficiency, molar absorptivity, the lower limit of detection and relative standard deviation values, and the resulting purple-colored intermediate had maximum absorbance claims the superiority of the proposed assay. the lower value of K_m (4.4503 µM) and V_{max} (0.0694 EU min^{-1}) indicate the specificity and selectivity of the proposed system compared to the reported assay systems. The interference studies show that the reaction interferes with very few foreign materials and the evidence from the kinetic parameters shows that the PPDA-AN assay comparatively better substitute when compared with the reference assay.[22]

ACKNOWLEDGMENT

The authors thank ATME College of Engineering, Mysuru for providing laboratory facilities.

REFERENCES

1. P. Vasudev , K. S. Chandrashekar , Aravind Pai , Anuraag Muralidharan, M. Manjunath Setty, *Rasayan journal of Chemistry*, 83(2021), http://doi.org/10.31788/RJC.2021.1456602
2. A. L. Jangala, K. Pulipati, *Rasayan journal of Chemistry,* **13(3),** 1531(2020), http://dx.doi.org/10.31788/ RJC.2020.1335665
3. K. Prasannath, *Plant defense-related enzymes,* **33(1)**, (2017), http://doi.org/10.4038/agrieast.v11i1.33
4. S. Upadhayay, N. Sharma, A. K. Mantha and M. Dhiman, *Rasayan Journal of Chemistry*, **13(2)**, 1042(2020), http://dx.doi.org/10.31788/RJC.2020.1325603
5. R. Singh, M. Kumar, A. Mittal and P. K. Mehta, *Microbial enzymes*, **6(174)**, (2016), https://doi.org/10.1007/s13205-016-0485-8
6. N. Saptarini, D. Rahayu, S. Kusuma, *Rasayan Journal of Chemistry*, **12(4)**, 2074(2019), http://dx.doi.org/10.31788/RJC.2019.1245319
7. V. Jyothirmai, S. M. R. Sharmila, S. Arun, *Rasayan Journal of Chemistry*, **13(4)**. 2274(2020), http://dx.doi.org/10.31788/ RJC.2020.1346074
8. O. Tantawi, A. Baalbaki, R. El Asmar, A. Ghauch, *Science of The Total Environment*, **654**, 107(2019), https://doi.org/10.1016/j.scitotenv.2018.10.372
9. Q. Li, D. Yang and Y. Yang, *Molecular and Biomolecular Spectroscopy*, **244**, 118882(2021), https://doi.org/10.1016/j.saa.2020.118882
10. C. L. Manzanares-Palenzuela, N. de-Los-Santos-Álvarez, M. J. Lobo-Castañón, B. López-Ruiz, *Biosensors and Bioelectronics*, **68**, 259(2015), https://doi.org/10.1016/j.bios.2015.01.007
11. P. Martinkova and M. Pohanka, *International Journal of Electrochemical Science*, **10**, 7033(2015).
12. Z. Yu, Y. Park, L. Chen, B. Zhao, Y. M. Jung, Q. Cong, *Acs Applied Materials & Interfaces*, **7(42)**, 23472(2015), https://doi.org/10.1021/acsami.5b08643
13. S. Polacco, P. Wilson, M. Illes and T. Stotesbury, *Forensic science international*, **290**, 36(2018), https://doi.org/10.1016/j.forsciint.2018.06.026
14. M. Dhruvaraj, *Journal of Clinical Nutrition*, **3(2)**, 14(2017), https://doi.org/10.4172/2472-1921.100048

15. P. Nagaraja, A. Shivakumar, A. K. Shrestha, *Analytical Sciences*, **25**, 1243(2009), https://doi.org/10.2116/analsci.25.1243
16. N. A. Chamaraja, P. Nagaraja, H. Krishna. *Journal of Biotechnology*, **155(4)**, 406(2011), https://doi.org/10.1016/j.jbiotec.2011.07.035
17. P. Nagaraja, N. A. Chamaraja, A. Shivakumar, H. Krishna, *Chemical Science Review and Letters,* **3(12)**, 1068(2014)
18. N. Chamaraja, A. Shivakumar, C. Kumar, N. KumaraSwamy, *Chemical Data Collections*, **11**, 84(2017), https://doi.org/10.1016/j.cdc.2017.08.005
19. P. Nagaraja, A. Shivakumar, A. K. Shrestha, *Journal of agricultural and food chemistry*, **57**, 5173(2009), https://doi.org/10.1021/jf900696p
20. H. Krishna, P. Nagaraja, A. Shivakumar, N. A. Chamaraja, N. Aradhana, *Molecular and Biomolecular Spectroscopy*, **102**, 75(2013), https://doi.org/10.1016/j.saa.2012.10.024
21. R. A. Roopa, K. Mantelingu, K. S. Rangappa, *Chemical Data Collections*, **3**, 46(2016), https://doi.org/10.1016/j.cdc.2016.07.001
22. I. da Cruz Vieira, *Analyst*, **123**, 1809(1998), https://doi.org/10.1039/A803478H
23. A. Shivakumar, D. Rangappa, H. Krishna, P. Nagaraja, *Enzyme and microbial technology*, **47**, 243(2010) https://doi.org/10.1016/j.enzmictec.2010.07.012
24. N. Chamaraja, C. P. Kumar, N. Vedamurthy, H. Kavya, D. Sahana, B. Megha, U. Bhavani, N. Kumaraswamy, *Indian Journal of Advances in Chemical Science S2*, **38**, 41(2017), https://doi.org/10.1016/j.cdc.2017.08.005
25. N. Chamaraja, A. Shivakumar, C. Chidan Kumar, C. Praveen Kumar, N. KumaraSwamy, *Chemical Data Collections*, **11**, 84(2017), https://doi.org/10.1016/j.cdc.2017.08.005
26. K. Jiao, W. Sun, S.-s. Zhang, G. Sun, *Analytica chimica acta*, **413**, 71(2000), https://doi.org/10.1016/S0003-2670(00)00820-5
27. T. Ngo and H. M. Lenhoff, *Analytical biochemistry*, **105**, 389(1980), https://doi.org/10.1016/0003-2697(80)90475-3

[RJC-8091/2022]