***SUMMARY* on the**

"*ANALYTICAL METHODOLOGY IN THE EVALUATION OF ENZYME BASED SUBSTRATE ASSAYS*"

A MINOR RESEARCH PROJECT WITH THE FINANCIAL ASSISTANCE FROM UNIVERSITY GRANTS COMMISSION GOVERNMENT OF INDIA, NEW DELHI

Principal Investigator

Dr. A. SHIVAKUMAR
Assistant professor in Chemistry
St. Philomena's college
Mysore-570015

The project submitted to the
UNIVERSITY GRANTS COMMISSION, GOVERNMENT OF INDIA
NEWDELHI

Summary of the research findings:

Preamble of the proposed research work:

Enzymes are biocatalysts that effectively increases the rate of the reaction without involving a physical or chemical change. The project work involves the quantification of enzymes and substrates by secondary substrates such as *p*-phenylene diamine, *o*-phenylene diamine, phenols and such others by the native enzyme and its mimicking nanoparticles with reference to metals such as Silver, gold, cerium and so on.

Native enzymes such as peroxidase and catalase and its enzyme mimicking nanoparticles such as gold, cerium, graphene were selected in the assay

Planned work: Development of new assay methodology in the evaluation of enzymes and substrates using enzymes, nanoparticles and graphene.

1. Introduction:

Peroxidases are widely distributed in nature especially in animal and plant cells. Peroxidases comprise of three major categories; plant peroxidases, animal peroxidases and catalases. These enzymes utilize hydrogen peroxide to catalyze the oxidation of variety of organic and inorganic compounds.

Over the years the development in clinical and diagnostic techniques, redox natured reactions are gaining vital importance. Biochemical reactions that are redox are gaining prominence in basic as well as applied research. Peroxidases are the driving force among these as most of the clinically important assays such as glucose, creatinine and uric acid determinations cannot be carried out without peroxidases. Immobilization of peroxidase enzyme has received much attention in the construction of biosensors due to economical and easy viability in enzyme immunoassays and enzyme linked immunosorbent assays. Keeping the views in logical frame work, the authors discuss the important role of peroxidase in clinically important assays, its chemistry behind hydrogen peroxide reaction extending to assay of diabetically important glucose and finally providing a tabulated physiological range of biochemical components in blood. The reactions catalyzed by peroxidases can be grouped into oxidative dehydrogenation, oxygen transfer, oxidative halogenations and hydrogen peroxide dismutation. Among these, oxidative dehydrogenation has greater application in analytical biochemistry.

Peroxidases are having great potential application as they can be used in a diagnostic kit for hydrogen peroxide, glucose and oxidase enzyme determination (*1*). In particular, HRP are widely used in research areas such as enzymology, biochemistry, medicine, genetics,

physiology, histo and cyto chemistry because of easy availability, economical and high catalytic activity (*2*). The commercial production of peroxidase has increased due to its analytical diagnostics particularly biosensing, in immunosensors and in nucleic acid detection. Heme proteins named peroxisomes are present in high concentration in cell compartments preventing excessive accumulation of peroxide, a powerful oxidizing agent. HRP meets the entire requirements for successful analytical enzymology because of its specificity, flexibility in assay, stability, sensitivity of detection, and availability in pure form. HRP on coupling with oxidase enzyme can be used for analysis of wide range of analytes such as glucose, cholesterol, lactic acid, choline, xanthine, uric acid, bilirubin, and creatinine. Combination of peroxidase and indole-3-acetic acid is currently being used as a cancer therapeutic agent (*3*) . The therapeutic procedure involves two steps; first decoding of enzyme in the tumor cells, second administering specific prodrug, indole-3-acetic acid which gets converted into cytotoxic drug by the enzyme expressed in the target tumor. The decarboxylated form of radical cation indole-3-acetic acid can conjugate with DNA and other biological nucleophiles (*4*). Some other anticancer strategies adopted include antibody and polymer directed enzyme/prodrug therapy.

2. Work done so far:

2.1 Enzyme assay:

A peroxidase catalysed reaction in the assay of hydrogen peroxide involving co-substrates such as *p*-phenylenediamine and 3-aminophenol has been developed which is based on the enzymatic coupling with absorbance maximum at 660 nm. The linearity range that has been achieved lies in between 1.5 -15.15 nM and 0.47 -15.15 nM by rate and fixed time methods, respectively. The assay has been adopted with hydrogen peroxide linearity in the range 3.5 – 120 μM. The kinetic parameters such as catalytic power, catalytic efficiency, catalytic constant and specificity constant have been evaluated.

The kinetic constants evaluated by the matrix method for hydrogen peroxide, p-phenylenediamine and 3-aminophenol were found to be 57 μM, 29 μM and 67 μM, respectively. The results of the work have been published in the Chemical data Collections, an Elsevier journal, **details are attached in the end of the report.**

2.2 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 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 to 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 to 15.15 nM.

2.3 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 to obtain the calibration graph. The linearity of the graph lies between 3.5 and 120 µM H_2O_2.

2.4. Data, value and validation

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.

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.

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.

3. Analytical Probe for the Assay of Amino Acids and Proteins:

A sensitive method has been proposed for the quantification of amino acids and proteins using ninhydrin and sodium molybdate as chromogenic substrates in citrate buffer of pH 5.6. A weak molybdate-hydrindantin complex plays the role in the formation of Ruhemann's purple. The linear response for the amino acid, amino acid mixture and Bovine serum albumin is between 0.999 and 66.80 µM , 1.52 and 38 µM and 5 and 100 µg/L, respectively.

The molar absorptivity of the individual amino acid by the proposed reaction extends from 0.58×10^4 to 2.86×10^4 $M^{-1}cm^{-1}$. The linearity equations for the proposed ninhydrin-molybdate for amino acid mixture is Abs = 0.021 × Conc (μM) - 0.002. The applicability of the proposed method has been justified in food and biological samples in conjunction with Kjeldahl method. The so reported method for the assay of aminoacid and protein can be applied in the determination of protein in the enzyme extract during the purification process.

4. Additional works

Further, we have synthesised different types of Nanozymes such as silver and graphene functionalised by the use of rich antioxidant sources of plant origin. The synthesised Nanoparticle has been characterised which is being tested for Nanozymic property. The characterisation has been carried out by IR and XRD analysis which has been included subsequently in the report. Further the anti bacterial and antifungal studies have been carried out.

5. A review article has been accepted for publication in "**Critical Reviews in Analytical Chemistry**", a Taylor and Francis Journal.

The cellular electrophysiology refers to the performance of living organism, essential for normal functioning of any live organism. Electrophysiological systems are prone to release free radicals for functioning of biological system with proper balancing of antioxidant-prooxidant ratio for establishing a healthy living system. The biostress condition releases different reactive oxygen species such as hydroxyl, alkoxyl, superoxide, hydrogen peroxide, hydroperoxyl, ozone, singlet oxygen, hypochlorus acid, thiyl radical, etc. This review tries to discuss the general aspects of the antioxidant assay methodology which are currently used for the detection of antioxidant property. The entire review has been divided into three different components. The first section deals with the release of free radical by mitochondrial dysfuctioning and its curbing action by local antioxidants. The second and third sections discuss the general procedure adopted and reaction mechanism involved in the assay procedure along with the limitations and advantages

Conclusion: The following works has been successfully completed

- Peroxidase catalysed reaction in the assay of hydrogen peroxide involving co-substrates such as *p*-phenylenediamine and 3-aminophenol
- Analytical probing colorimetric system for aminoacids and proteins
- Synthesis of silver nanoparticle involving action of surfactant

Publication related to the project work:

Accepted:

1. 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. *Chemical Data Collections* **2017,** *11–12*, 84-95.http://www.stphilos.ac.in/wp-content/uploads/2018/10/Peroxidase-PPDD-and-3-AP.pdf

2. Ninhydrin-sodium molybdate Chromogenic analytical probe for the assay of amino acids and proteins, *Spectrochimica Acta Part A: Molecular and biomolecular spectroscopy*, 173 **(2017)** 897-903.http://www.stphilos.ac.in/wp-content/uploads/2018/10/NSM-amino-acids-assay.pdf

3. Critical Review on the Analytical Mechanistic Steps in the Evaluation of Antioxidant Activity. Critical reviews in Analytical Chemistry, 48 (**2018)** 214-236

4. Role of peroxidase in clinical assays: A Short review, *Journal of Clinical Nutrition and dietetics*, 3 **(2017)** xxx.http://www.stphilos.ac.in/wp-content/uploads/2018/10/role-of-peroxidase-in-clinicalassays-a-short-review.pdf

5. A Birds Eye View of Nanotechnology in Medicine. *J Clin Nutr Diet* **2017,** *3*, 20 http://www.stphilos.ac.in/wp-content/uploads/2018/10/A-birds-eye-view-of-nanotechology-in-medicine.pdf

Manuscript under preparation:

1. Synthesised Silver Nps by surfactant action

Literature cited:

1. Song, Y.; Qu, K.; Zhao, C.; Ren, J.; Qu, X., Graphene Oxide: Intrinsic Peroxidase Catalytic Activity and Its Application to Glucose Detection. *Advanced Materials* 2010, *22*, 2206-2210.
2. Sanz, V.; de Marcos, S.; Castillo, J. R.; Galbán, J., Application of Molecular Absorption Properties of Horseradish Peroxidase for Self-Indicating Enzymatic Interactions and Analytical Methods. *Journal of the American Chemical Society* 2005, *127*, 1038-1048.
3. Veitch, N. C., Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry* 2004, *65*, 249-259.
4. Azevedo, A. M.; Martins, V. C.; Prazeres, D. M. F.; Vojinović, V.; Cabral, J. M. S.; Fonseca, L. P., Horseradish peroxidase: a valuable tool in biotechnology. In *Biotechnology Annual Review*, Elsevier: 2003; Vol. 9, pp 199-247.