WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 8, 737-742. Research Article ISSN 2277– 7105

# PURIFICATION AND CHARACTERIZATION OF PEROXIDASE FORM PHYLLANTHUS AMARUS LEAVES

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Article Received on
18 February 2018,

Revised on 11 March 2018,
Accepted on 2 April 2018,

DOI: 10.20959/wjpr20188-11832

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## ABSTRACT

Peroxidase is an iron enzyme that catalyzes many oxidation reduction reactions using hydrogen peroxide as electron acceptor. It is universally present in bacteria, fungi, plants and animals. Plant peroxidase belongs toclass III which mainly contribute to removal of hydrogen peroxide from cell organelles; oxidation of toxins; biosynthesis of the cell wall and defense against wound. Apart from cellular function, peroxidase can be used for treatment of industrial wastes specially for removal and detoxification of phenols from effluent. Manufacturing of adhesives, computer chips and car parts are some other industrial applications of peroxidases. The present work was designed to extract peroxidase from plant source. Different parts of *Phyllantus amarus* was screened for peroxidase using optimized buffer and pH. Extracted enzyme was purified using ammonium sulfate and immobilized on calcium alginate beads.Crude enzyme extracted from sample was 84.52 U/mg of protein which increases to 148.09 U/ml after purification. Optimization of purification process exhibited increase in enzyme recovery. Further enzyme immobilized on calcium alginate. Immobilized enzyme showed remarkable stability and reusability with after immobilization which suggested the suture applicability.

**KEYWORD:** Oxidoreductase, peroxidase, immobilization, wound healing

## INTRODUCTION

Peroxidase is one of the very useful enzymes with lots of pharmaceutical and industrial applications. These enzymes are heme proteins, employed for the oxidation of a wide variety of organic and inorganic compounds. It catalyzes the oxidation-reduction reaction in presence of hydrogen peroxide as electron acceptor. The term peroxidase in a broad term includes

group of specific enzymes such as NAD peroxidase, fatty acid peroxidase as well as a group of very non-specific enzymes from different sources simply known as peroxidases. Out of diverse group, plant peroxidases are special group of enzymes that removes of hydrogen peroxide from cell organelles. It contributes to biosynthesis of the cell wall, defense against wound enable and in overcoming the oxidative stress.[1,2,3]

Plant peroxidases (POX) (EC 1.11.1.7) are exist in different isoforms with enormous diverse profile on the basis different amino acid sequence, suggest their applications in various physiological processes.[4] Removal and degradation of phenols and other pollutants from various industrial effluents is one of such important applications.[5] *Phyllanthus amarus*, commonly known as Bhumi amlakiis a wide spread tropical plant widely distributed in Jharkhand, Bihar, and Chhattisgarh. It is commonly known for its therapeutic applications for the preventionof jaundice, diabetes, dyspepsia, ulcer and restriction the infection of hepatitis B virus.[6] In present work, *Phyllanthus amarus* is screened for peroxidase enzyme production. After keeping its applications, enzyme is partially purified and immobilizedon calcium alginate.

## MATERIALS AND METHODS

### Collection of plant parts

Fresh samples of leaves stem and roots were collected from the garden and nearby areas of Ranchi University Campus, Ranchi, Jharkhand.

### Preparation of Crude Enzyme Solution

Sample was weigh and 20 gm of samples were washed properly under running water. Then samples were cut it into small pieces and crushed with the help of mortar pestle. Samples were homogenized in 0.1M potassium phosphate buffer (pH 7). For the preparation of crude enzyme, samples were filtered through mushling cloth (cheese cloth) and filtrate was centrifuged at 10,000rpm for 10 minutes at 40$^{o}$C. The supernatant was collected and used as source of crude enzyme and stored in into deep freezer for further use.[7]

### Estimation of Peroxidase activity

Peroxidase activity is measure by using 20mM guaiacol as substrate. Peroxidase enzyme oxidizes guaiacol solution, prepared in distilled water. Reduction in enzyme activity was recorded after every 3-4 seconds.[8]

"The change in absorbance was measured at 660nm by using colorimeter. The reduction in absorbance 0.01 in 3 to 7 second indicates the presence of 1 unit of enzyme."

For the estimation of specific enzyme activity protein estimation was done by colorimetric method.[9]

**Purification of Protein Precipitation by Ammonium Sulfate**

Peroxidase enzyme obtained from leaves was purified by ammonium sulfate precipitation. This is the most common salt used for enzyme precipitation. Different concentrations of salts were used for enzyme purification and after every step enzyme activity was determined.

In, however, as the salt concentration increased, a point of maximum protein solubility is usually reached. Further increase in salt concentration implies that there is less and less water available to solubilize to protein. Finally protein starts to precipitate when there is no sufficient water molecule to interact with the protein molecule. This phenomenon of protein precipitation in the presence of excess salt is known as salting out.[10]

**Enzyme Immobilization**

To increase the reusability and effective delivery, enzyme was immobilizedon calcium alginate prepared by sodium alginate and 0.2M $CaCl_2$. After immobilization, enzyme activity was recorded.[11]

## RESULTS AND DISCUSSION

**Sample collection**

For enzyme extraction different parts of plant sample of *Phyllanthus amarus* was collected from garden and nearby area of Ranchi University Campus, Jharkhand. For enzyme extraction fresh samples were used.

Samples were crushed, filtered and screened for enzyme activity (Table 1).

**Table 1: Screening of plant parts for enzyme activity.**

| Plant parts | Specific activity (U/mg) |
|---|---|
| root | 28.61 |
| Stem | 9.96 |
| Leaves | 84.52 |

In comparison to roots and stem, leaves exhibited higher enzyme activity hence selected for further work.

Buffer provide appropriated environment for enzyme stability and activity. During extraction different buffers were screened (Table 2).

**Table 2: Screening of buffer for enzyme activity.**

| Name of buffer | pH | Specific activity (U/mg) |
|---|---|---|
| Potassium phosphate | 7.0 | 83.23 |
| Citrate | 4.0 | 38.91 |
| Tris | 8.0 | 60.21 |
| Glycine – NaOH | 9.0 | 15.41 |

For enzyme extraction, potassium phosphate buffer at physiological pH showed maximum peroxidase activity hence it was considered for further work.

**Partial purification of peroxidase**

For analyze it's applications, enzyme was partially purified immobilized. Purification of enzyme was done by ammonium sulfate precipitation method. Salt was used in different concentrations and maximum enzyme was precipitated at 20% saturation of $NH_2SO_4$ followed by 30%, 40%, 50%, 60% and 70%. The whole protein of *Phyllanthus amarus* showed precipitation till 60% saturation point (Table 3 & 4).

**Table 3: Precipitation of Protein from Plant Extract by Ammonium Sulfate.**

| Name of plants | Protein Precipitation at different saturation of Ammonium Sulfate | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20% | 30% | 40% | 50% | 60% | 70% | 80% | 90% | 100% |
| ***P.amarus*** | +++ | ++ | ++ | + | + | - | - | - | - |

(-) absent; (+) low amount; (++) moderate amount; (+++) abundant amount

**Table 4: Estimation of Peroxidase Activity of *Phyllanthusamarus.***

| Sl. No. | Different saturation point | Absorbance (660nm) | | | | | | Change in absorbance every 4 sec (Average ±SD) | Specific activity (U/mg) |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 sec | 4 sec | 8 sec | 12 sec | 16 sec | 20 sec | | |
| 1. | 20% | 0.98 | 0.90 | 0.84 | 0.78 | 0.74 | 0.71 | 0.054±0.019 | 86.4±1.52 |
| 2. | 30% | 0.89 | 0.80 | 0.73 | 0.66 | 0.61 | 0.58 | 0.062±0.022 | 75.15±1.76 |
| 3. | 40% | 0.85 | 0.78 | 0.73 | 0.68 | 0.65 | 0.63 | 0.044±0.019 | 45.13±1.52 |
| 4. | 50% | 0.69 | 0.63 | 0.59 | 0.55 | 0.53 | 0.52 | 0.034±0.019 | 25.44±1.52 |
| 5. | 60% | 0.61 | 0.57 | 0.54 | 0.52 | 0.51 | 0.49 | 0.024±0.011 | 14.77±0.88 |
| 6. | 70% | 0.59 | 0.58 | 0.58 | 0.58 | 0.58 | 0.58 | 0 | 0 |
| 7. | 80% | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0 | 0 |
| 8. | 90% | 0.49 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0 | 0 |
| 9. | 100% | 0.47 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 | 0 | 0 |

It is clear that with increase in concentration of protein amount of enzyme precipitated reduced and highest amount of enzyme precipitated was in 20% saturation point. Obtained

precipitate was dialyzed overnight by using nitrocellulose membrane. Enzyme activity may differ with purification steps hence specific activity was considered.

**Table 5: Enzyme purification steps and recovery.**

| Steps | Protein (mg) | Enzyme (Unite) | Specific activity (U/mg) | Fold purification | Yield (%) |
|---|---|---|---|---|---|
| Crude | 571 | 48750 | 85.38 | 0 | 0 |
| $(NH_4)_2SO_4$ precipitation | 359 | 39916 | 111.19 | 1.30 | 81.89 |
| Dialysis | 232 | 34356 | 148.09 | 1.73 | 70.47 |

After purification yield of enzyme obtained was 70% with 1.73 purification folds.

**Enzyme immobilization**

The enzyme was immobilized on calcium alginate. Amount of enzyme immobilized and enzyme activity calculated to determine the immobilization efficiency. In present work immobilization efficiency recorded was 65.23±1.76 U/mg. As compared to crude enzyme activity of immobilized enzyme reduced may be due to loss of some enzyme during immobilization and restricted interaction between enzyme and substrate.

## DISCUSSION

Peroxidase is an iron containing oxido-reductase enzyme which uses hydrogen peroxide as electron acceptor.[12] In plants, peroxidase plays important role in defense and cell wall biosynthesis. *Phyllanthus amarus* is an important plant of Euphorbiaceae family well known for its medicinal properties and pharmaceutical applications such as ailments of stomach, genitourinary system, liver, kidney and spleen. In present work peroxidase enzyme was extracted from *Phyllanthus amarus* leaves, partially purified and immobilized. Highest enzyme activity was recorded as 148.09 U/mg after purification. In earlier study aqueous extract of *Phyllanthus amarus* was used for studying its antioxidant potential. It has been found that is aqueous extract is devoid of genotoxicity and improve defense against oxidative stress.[13]

## CONCLUSION

Peroxidases enzyme was recovered from the leaves with potassium phosphate buffer. Enzyme was recovered and partially purified and immobilized on calcium alginate with 93% immobilization efficiency. After purification 70% yield was recorded with 1.73 purification folds. Further efforts can be invested for application of immobilization enzyme. Present work suggested the addition of pharmaceutical applications.

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