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Purification and Characterstics of Peroxidase from Two Varities of Tulsi and Neem

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ABSTRACT

Peroxidase was extracted from different varieties of tulsi and neem and its purification by gel filteration. Medicinal plants are premier adaptogen helping the body and mind to adopt and cope with a wide range of physical, emotional, chemical and infectious stresses and restore physiological and psychological functions to a normal healthy state. The isoenzyme, peroxidase stands responsible for their antioxidant capacity which in turn contributes towards their medicinal properties. *Ocimum tenuiflorum* , *Ocimum gratissimum* varieties of tulsi and *Azadirachta indica, Melia azadirachta* varieties of neem were studied for peroxidase analysis.. The optimum pH and temperature of peroxidase were found to be 6.5 and 40ºC. This was studied to understand the variation between single species at protein level. Protein profiling was done in both Native PAGE and SDS PAGE.

Keywords: Tulsi, Neem, Peroxidase, Protein purification, SDS PAGE

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INTRODUCTION

Peroxidases(EC1.11.1.7) are widely distributed in nature and are produced by a wide variety of plant species, the chief commercial source being Horseradish (*Armoracia rusticana*) and Soyabean(*Glycine max*)[1]. Peroxidases are heme containing enzymes that oxidize a wide variety of organic and inorganic substrates by reducing hydrogen peroxide and peroxides[2]. Peroxidase is a heat stable enzyme, preferring the preparation of enzyme conjugated antibodies and other sensitive analytical techniques [3]. They play critical roles in physiological functions such as cell metabolism,plant resistance and auxin catabolism[4-6].This also commonly used in pharmaceutical industry especially glucose estimation drugs [7].

Two varieties of Neem viz. *Azadirachta indica* and *Melia azadirachta* and two varieties of Tulsi viz. *Ocimum tenuiflorum* and *Ocimum gratissim w*ere considered for the work.. The chemical composition of Tulsi is highly complex and contains many nutrients and biologically active compounds. The chemical compounds that have been identified as volatile oil,terpenoids,eugenol, thymol estragole and ursolic acid. Tulsi is used as an expectorant,bronchitis, ringworm and other cutaneous diseases, stomachic, gastric disorders and earache of children and also reduces the toxicity. It also enhances general health and well-being, having positive overall effects on the body and mind [8].

Neem has anti-bacterial properties that help in fighting against skin infections such as acne, psoriasis, scabies, eczema, etc. Neem extracts also help in treating diabetes, AIDS, cancer, heart disease, herpes, allergies, ulcers, hepatitis and several other diseases. Neem oil, leaves and neem extracts are used to manufacture health and beauty care products. Neem leaf tablets to increase immunity, insect repellents, nematicide, contraceptives, pet care products and various disease control[9,10].

The main objective of this work was to isolate peroxidase from two varieties of commonly found medicinal plants Neem and Tulsi and its partial purification by SDSPAGE .The isoenzyme patterns were identified by Native PAGE .The affinity chromatography was used for complete purification and its properties. .

MATERIALS AND METHODS

Plant Material

Tulsi varieties were identified by botanists Rama and Krishna Tulsi variety (*Ocimum tenuiflorum),*Vana Tulsi variety(*Ocimum gratissimum*), Neem varieties(*Azadirachta indica*, and *Melia azadirachta* were collected. The leaves of all the varieties were used for the estimation and isolation of peroxidase.

Preparation of Crude Extract

500 mg of all the four leaf samples were weighed and grinded with the addition of 1 ml of Phosphate buffer (pH7).This was then centrifuged at 10000 rpm for 15 minutes at 4ºC and

the supernatant was passed through filterpaper[1,11]. It was heated in a waterbath at 65ºC for 3 minutes to inactivate catalase in the extract and cooled promptly by placing in ice bucket for 10 min.

Purification of Peroxidase

The enzyme extract were subjected to ammonium sulphate precipitation by the method of Evans[12] and the precipitate was subjected to gel filteration in the form of desalting column.

Desalting Column

Desalting columns are pre-packed, ready to use columns for group separation between high and low molecular weight substances. The Desalting columns are prepared by packing size exclusion matrix. The martrix is beds of cross linked dextran with epichlorohydrin. The fractionation range for globular proteins is between 1000-5000 Da.

The desalting columns are made of of biocompatible polypropylene which is non interactive with biomolecules.The top and bottom frits are made of porous polyethylene. The column is fitted with top and bottom caps. The use of the eluant containing a buffer salt for substances carrying a charged groups. The column had a void volume of 3.5 ml. This column was equilibrated by first washing with 10 ml of distilled water followed by washing with 20 ml of Phosphate buffer(pH 7)(approximately 6 times the volume of the void volume). Special care was taken to prevent the liquid level from going below the column front. This was done in refrigerator condition. After purification by gel filtration, assay of peroxidase was performed

Assay of Peroxidase Activity

Assay of peroxidase activity and substrate specificity were performed according in the method by Koksal and Gulcin (2008). 50µl ofenzyme solution was added to the mixture of 1 ml260mM hydrogen peroxide and 50µl of 20 Mm guaiacol and the mixture was adjusted to 2ml by adding 0.1Mm sodium phosphate buffer(pH6.0).The absorbance of colored complex was read at 470nm after 3min reaction interval (Ambreen et.al,2000). Protein content of the enzyme extract at all steps were measure by bradford method[13].

SDS- Page and Native Page

Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoreseis was performed according to the Laemmli's sytem [14] using 12.5% polyacrylamide gel .For Native PAGE, the sample extract is electrophoresed in starch or polyacrylamide buffered(non denaturating) stack gel at a low temp.(4-8)^{0}C .Each lanes should be loaded with equal amount of proteins after normalizing the proteins content in exact in as small volume as possible (25-50µl). Later electrophoresis the gel is incubated in a solution containing all the necessary components for

enzyme reaction. The colored reaction product stains the gel where the enzymes were located. The separation of native (non denatured) proteins in this method was based on both the charge and size of the proteins. Since the native proteins are large and exist in quaternary structure, a gel of low percentage of acrylamide was used [15].

Effect of PH And Temperature on Peroxidase Activity

The optimum pH was determined by assaying enzyme activity at different pH levels. The assay was performed by taking buffers at different pH such as 0.1 M sodium acetate buffer (pH3.0 to5.0) and 0.1 M sodium phosphate buffer(pH5.5 to 6.5).The optimum temperature of peroxidase was determined by assaying the enzyme activity with different range of temperature from 20ºC to60ºC.The thermostability of peroxidase was measured by incubating the enzyme at 60ºC and the activity was measured every 10 min. Peroxidase activity was assayed by using guaiacol substrate.

RESULTS AND DISCUSSIONS

After gel filteration of two varieties of tulsi and neem the protein content of the crude and the purified samples were estimated by Bradfords methodology using standard BSA (Bovine serum albumin) graph was used as the reference for the estimation of the protein content. Table 1 which shows the amount of protein present in the different samples it was seen that the crude extract was found to be higher protein content than the purified sample.

Table 1: Partial purification of peroxidase of *Azadirachta indica*

Purification step	Total Volume(mL)	Protein content(mg/mL)	Enzyme activity(U/mL)	Specific activity(U/mg)	Fold purification
Crude extract	500	15.86	18.68	2.58	1
Ammonium sulphate precipitation	280	8.35	13.56	5.63	2.78
Gel filtered extract	25	4.67	10.9	12.34	8.99

Table 2: Partial purification of peroxidase of *Melia azadirachta*

Purification step	Total Volume(mL)	Protein content(mg/mL)	Enzyme activity(U/mL)	Specific activity(U/mg)	Fold purification
Crude extract	500	8.67	12.5	2.14	1.2
Ammonium sulphate precipitation	235	5.78	9.65	5.76	2.9
Gel filtered extract	20	0.78	6.34	14.56	7.86

Protein content of *Azadirachta indica* was found to be higher(15.86mg/mL) for the crude extract and 4.67mg for the purified protein when compared with *Melia azadirachta*

(Table.1,2). To purify the enzyme the crude extract was subjected to ammonium sulphate to remove unwanted proteins. This is the most commonly used reagent for salting out of proteins because of its high solubility permits the achievements of the solution with high ionic strength[16,17]..The degree of purification of desalted enzyme was increased by applying into gel filtration. The maximum specific activity of the peroxidase extracted from *Melia azadirachta* was 14.56 U/mg. The results of our findings which exhibited maximum fold of purification(8.99)and maximum enzyme activity was also observed in *Azadirachta indica.*(Table1,2)

Table 3: Partial purification of peroxidase of *Ocimum tenuiflorum*

Purification step	Total volume(mL)	Protein content(mg/ml)	Enzyme activity(U/mL)	Specific activity(U/mg)	Fold purification
Crude extract	500	9.8	13.43	2.45	1.1
Ammonium sulphate	254	5.5	9.87	5.34	2.3
Gel filtered extract	25	0.5	3.45	8.65	7.5

Table4: Partial purification of peroxidase of *Ocimum gratissimum*

Purification step	Total Volume(mL)	Protein content(mg/ml)	Enzyme activity(U/mL)	Specific activity(U/mg)	Fold purification
Crude extract	500	6.58	10.3	1.67	1.03
Ammonium sulphate precipitation	245	4.87	7.68	3.89	2.76
Gel filtered extract	23	0.36	3.45	6.08	5.8

Among the two varieties of neem plants,the maximum protein content of *Ocimum tenuiflorum* was found to be 9.8mg for the crude extract when compared with *Ocimum gratissimum* .The maximum specific activity of peroxidase(8.65U/ mg) and high degree of protein purification were found in *Ocimum tenuiflorum*(Table3,4).

Gel filtration which proved efficient as a fraction showed maximum (8.99) fold purification in *Azadirachta indica* and high specific activity (14.56 U/mg) was found in *Melia azadirachta* among four different samples. Zia (2002) found 18.644 fold purification after applying horse radish peroxidase to gel filtration chromatography. The protein and isoenzyme pattern of peroxidase were confirmed by SDS-PAGE (not shown) and NATIVE PAGE.(Fig 1)

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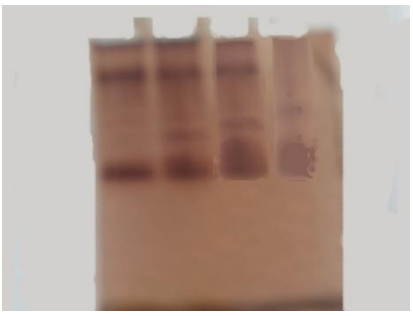

Lane1 :*Ocimum tenuiflorum,* Lane2: *Ocimum gratissimum,* Lane 3:*Azadirachta indica* Lane 4: *Melia azadirachta ,*
Figure 1:Native PAGE of peroxidase from two varieties of Tulsi and Neem

The zymogram(fig1) obtained showed the migration of the enzyme peroxidase to different levels under the electric field. The protein molecules migrate to different levels in accordance of their charge and molecular weight, thus from the zymogram it was seen that the peroxidase isolated from *Azadirachta indica* was having a higher molecular weight than *Melia azadirachta.* By similar observation ,it was found that the peroxidase isolated from*Ocimum tenuiflorum* had more molecular weight than *Ocimum gratissimum* .

It was known that pH and temperature were the key factor for enzyme activity ad it changes ionization state of protein and substrate[18]. The optimum pH was found to be around 5 and 6.5 in tulsi and neem varieties. The peroxidase shows optimum activity between pH6 and 8.5[19]. Altunkaya and Gokman (2011) suggested that the purified isoenzyme showed pH below7.The optimum temperature was found to be in the range of 40ºC-45ºC[20]. The optimum pH and temperature in chick pea were 5.5 and45ºC[21]; turnip-6.0 and 50ºC[22];lettuce-5.0 and30ºC[(23,25]..Plant peroxidases are glycosylated proteins and considered as a heat stable enzymes([24]. They are heat stable upto 55ºC but rapidly inactivated at 60ºC.[25].

CONCLUSION

Peroxidase was isolated and estimated from the two Neem varieties *Azadirachta indica, Melia azadirachta* and the two Tulsi varieties *Ocimum tenuiflorum, Ocimum gratissimum.*From this study it was found that the protein content and its enzyme activity was different for varieties within the same species .This study was helpful in understanding the varietal difference within the same species. Local availability of these plants and reasonably high specific activity of the enzymes isolated from these medcinal plants, makes it a better choice for the production of peroxidase for its use as anti oxidant.

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