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Research Article

Characterization of Tyrosinase enzyme from the tubers of *Amorphophallus paeoniifolius* (Dennst.) Nicolson, (Araceae)

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ABSTRACT

Tyrosinase, a copper-containing metalloprotein, catalyzes the oxidation of tyrosine, in particular L-DOPA to L-Dopaquinone, precursors of brown pigments in the wounded tissues. Tyrosinase, one among the Polyphenol oxidases, is found to possess excellent capacity for oxidizing phenolic compounds. In the present investigation, elephant yam (*Amorphophallus paeoniifolius*) has been identified as the source and enzyme has been extracted. The extracted enzyme was characterized by SDS PAGE and the isolated tyrosinase protein depicted the molecular weight of around 40 kDa. The protein band obtained was confirmed to be tyrosinase using zymography with L-DOPA as substrate. The enzyme activity was determined to be 0.04 min^{-1} mM^{-1}. Studies of kinetic parameters were carried out for tyrosinase under standard conditions. The K_m value of *A. paeoniifolius* was found to be 3.6 mM and V_{max} value was determined to be 0.1 s^{-1}. Their optimum pH value was found to be 7.2. The optimum temperature value for maximum enzyme activity was found to be at 25°C.

Keywords: Tyrosinase, *Amorphophallus paeoniifolius*, Michaelis-Menten parameters, Lineweaver-Burk plot, enzyme kinetics

INTRODUCTION

Tyrosinase (E.C: 1:14:18:1) is an enzyme system that is capable of oxidizing tyrosine using atmospheric oxygen leading to the production of a black pigment. It oxidizes various phenols to coloured compounds and form oxidation products with pyrogallol and dihydroxy phenylalanine. This dihydroxy phenylalanine is then converted into a black pigment called melanin, similar to that produced from tyrosine[1]. Along with catechol oxidase (E.C: 1:10:3:1) and laccase (E.C: 1:10:3:2), it is collectively called Poly Phenol Oxidase (PPO).

Polyphenol oxidases are copper containing metallo-proteins that catalyze the oxidation of broad spectrum of phenolic compounds by utilizing molecular oxygen. The enzyme catalyzes oxidation of catechol and related phenolic compounds referred as catecholase activity and many other PPO exhibit monophenol monooxygenase activity catalyzing the oxidation of L-tyrosine to 3, 4 dihydroxy-L-phenylalanine (L-DOPA) referred as cresolase or tyrosinase activity. Cresolase activity is generally considered to be a property of fungal and mammalian tyrosinase enzyme. Tyrosinase is found in both prokaryotes and eukaryotes and it is involved in the formation of pigments of polyphenolic origin such as melanins in animals, tannins in plants, cuticles in arthropods. By using hemocyanin structure as a template, a proposed structure model for the active site of tyrosinases was obtained[2].

Tyrosinase is the reason behind the blackening of many vegetables and fruits and also the blackening of blood from many insects when exposed to air[3]. The activity of tyrosinase was first noticed in crustacean blood in the late 19th century. In 1892, black granules in the blood of crustacean that are soluble in mineral acids producing brown colour were observed and it was thought that it formed by the action of tryptic ferment on some protein present in the blood[4].

After the enzyme product being noted as black granules in crustacean blood, the enzyme itself was first observed in fungi. This enzyme was later found to be a component of the enzyme system present in almost all plants, which turn brown on injury. The tyrosinase enzyme was found in microbes like *Streptomyces glaucescens*[5]. The tyrosinase of *Neurospora crassa* exists in two forms that differ in their thermal stability[6]. Being an intracellular enzyme in A*spergillus oryzae*, the mould mycelium was used for the biochemical conversion of L-tyrosine to L-DOPA[7].

Tyrosinase was also extracted from various plant sources like mushroom, tobacco root, *Nicotiana glutinosa* leaves[8]. Highly purified preparations of Tyrosinase have been obtained from *Podospora anserina*[9], broad beans[10], *Amanita muscaria*[11], *Portulaca grandiflora*[12] and *Beta vulgaris*[13].

Extraction, purification and characterization of plant tyrosinase have been focused on many fruits and vegetables because of the enzymatic browning in post-

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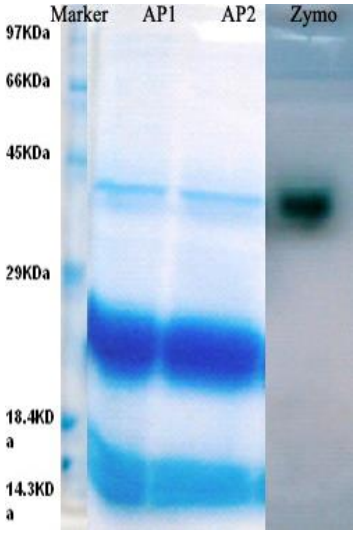

Fig-1 Protein bands of Tyrosinase enzyme extracted from tubers of *A. paeoniifolius* using SDS-PAGE & Zymography

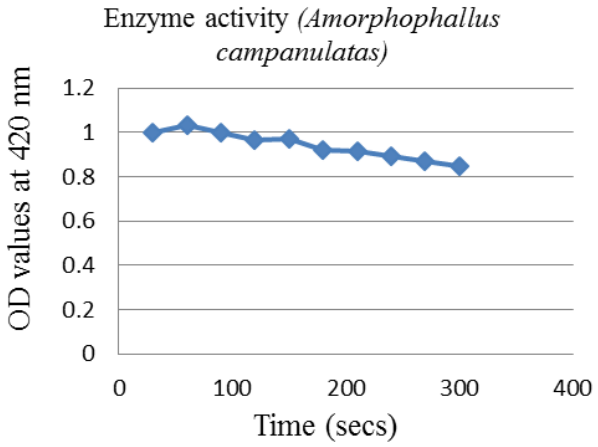

Fig-2 Effect of time in enzyme activity of tyrosinase extracted from *A. paeoniifolius*

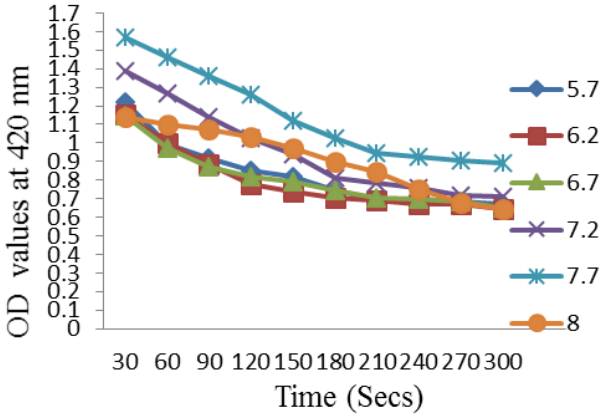

Fig-3a Effect of pH in enzyme activity of tyrosinase extracted from *A. paeoniifolius*

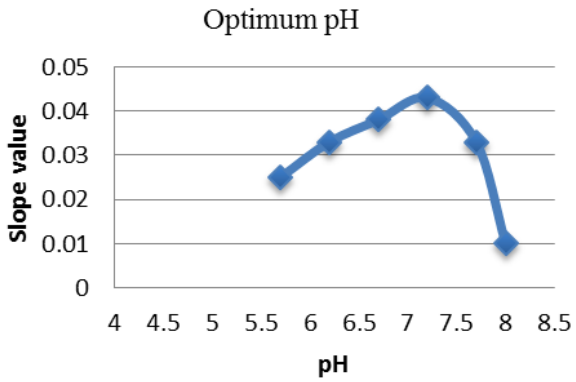

Fig 3b Determination of optimum pH for maximum enzyme activity of tyrosinase extracted from *A. paeoniifolius*

harvest physiology and food technology[14]. Its role in fruit and vegetable alteration during processing and during the storage of processed food is the object of numerous investigations. Elephant foot yam (*Amorphophallus* spp.) is basically an underground stem tuber and is gaining popularity due to its yield potential and culinary properties. It is rich in nutrients and is a delicacy as a food. The tubers are also used for preparing many ayurvedic preparations. It is a popular tuber crop and is grown as a vegetable in many parts of India.

In this study we focused on extracting tyrosinase from *Amorphophallus paeoniifolius*, its optimal pH and temperature conditions, characterizing it in SDS-PAGE and zymography for its activity confirmation.

MATERIALS AND METHODS

Fresh tubers of *A paeoniifolius* was collected freshly from agriculture field nearby Sivagangai and used as enzyme source. Sodium Phosphate buffer (0.2 M, pH-7.0), Tyrosine (Analytical Grade), different pH buffers, distilled water and other common reagents used for present studies. All the chemicals are purchased from SD – Fine chemicals.

Isolation of crude enzyme

The tubers were washed with distilled water and skin peeled off. The inner tissue is sliced and it is used as source of enzyme. Five grams of tuber tissue was taken into mortar and pestle and homogenized with 20 ml of 0.2 M sodium phosphate buffer and then centrifuged at 5000 rpm for 15 minutes. After centrifugation, supernatant was collected and precipitated with 4 times volume of ice cold (-20°C) acetone. This precipitate is resuspended in homogenization buffer and taken for SDS-PAGE for separation from other proteins

Characterization of Tyrosinase

The crude enzyme extract of *A paeoniifolius* was mixed with an electrophoresis sample buffer (20 % glycerol, 4 % sodium dodecyl sulphate, 0.125 M Tris-HCL (pH-6.8), 2 % β-mercaptoethanol and 0.02 % bromophenol blue) and then heated in boiling water (95°C) for 10 minutes. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% polyacrylamide resolving gel stacked with a 5% polyacrylamide stacking gel. Electrophoresis was started with a 50 V for first 30 minutes and then was increased to 100 V after the protein samples had entered the resolving gel. The gel was stained for proteins with Coomassie staining solution. To determine the molecular weight of protein bands,

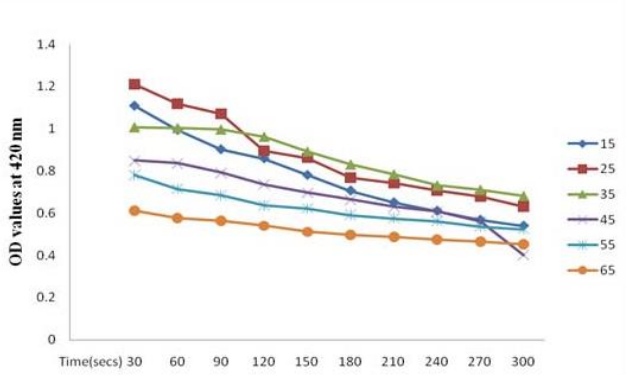

Fig-4a Effect of temperature in enzyme activity of tyrosinase extracted from *A. paeoniifolius*

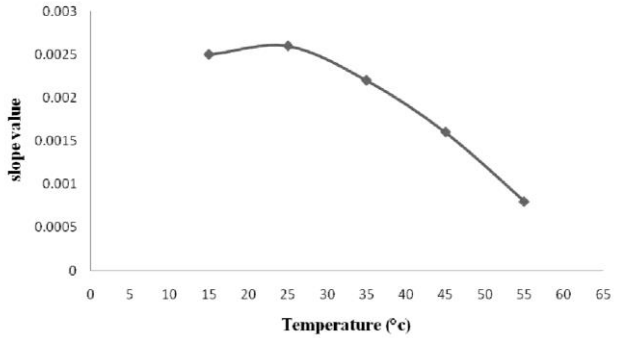

Fig-4b Determination of optimum temperature for maximum enzyme activity of tyrosinase extracted from *A. paeoniifolius*

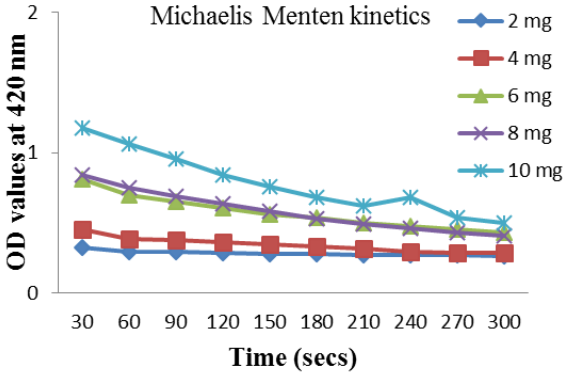

Fig-5 Determination of Michaelis- Menten kinetics of tyrosinase enzyme extracted from *A. paeoniifolius*

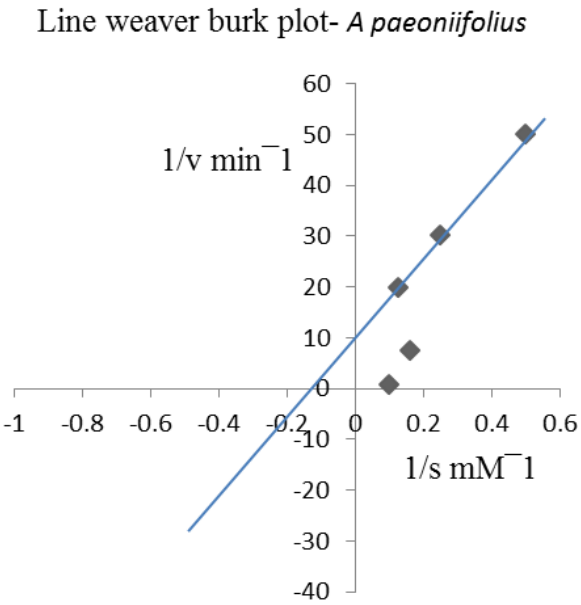

Fig-6 Lineweaver-Burk plot of kinetics tyrosinase enzyme extracted from *A. paeoniifolius*

electrophoresis was done for a protein standard of nine marker proteins (14 -100kDa) together with the tyrosinase sample. By comparing with the Zymogram, the corresponding band was for tyrosinase was identified and its molecular weight was estimated from the migration distance of standard proteins.

Zymogram Analysis of Tyrosinase

The SDS PAGE gel containing tyrosinase protein collected after electrophoresis was carefully rinsed with distilled water and was then transferred to a renaturation solution (2.5% Triton X in 50mM Tris, pH-8), rocked for 30mins at 37˚C at gel rocker. After rinsing, the gel was developed using developing buffer (0.2% L-DOPA in 0.1M Sodium Phosphate buffer, pH-6.8), till the dark brown colour appears.

Enzyme activity assay

The protein from SDS-PAGE band was eluted from gel and is used for further kinetic assays. Kinetic assays were performed by continuous spectrometric rate determination method in which the appearance of the product (L-DOPA) in the reaction medium is measured using UV-VIS spectrophotometer. The reaction mixture contained 1 ml of 1% tyrosine substrate, with 0.5 ml of the enzyme. Final volume of reaction solution was made to 4.5 ml using homogenization buffer. Blank solution contained all the components except the substrate. The activity was determined by measuring the absorbance at 420 nm from 30 sec to 300 sec.

Effect of pH and temperature

Enzyme activity was determined at extraction buffer pH values ranging from 5.7–8.0 at 25°C. The optimum pH value for the maximum tyrosinase activity was obtained with 1% tyrosine as substrate. Tyrosinase activity was also measured at varying temperatures from 15 to 65°C by using 1% tyrosine as substrate and 0.2M sodium phosphate buffer (pH-7) as extraction buffer. Substrate solution was brought to the test temperature by incubating at the corresponding temperature for 30 minutes immediately after the enzyme addition and the activity were determined.

Enzyme kinetics

The reaction rate of Tyrosinase at a series of substrate concentrations was determined. 1 ml of tyrosine at varying concentrations (2, 4, 6, 8, 10 mg/ml) was taken with 3 ml

of sodium phosphate buffer (pH-7). Immediately after the addition of 0.5 ml of the enzyme extract the absorbance readings were noted. Reaction rate in 0–30 sec corresponding to each substrate concentration was calculated. The absorbance versus time, and the reaction rate versus substrate concentration, were plotted to fit the Michaelis-Menten equation, and to derive the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}).

RESULTS AND DISCUSSION

Isolation, SDS-PAGE & Zymogram analysis of Tyrosinase

The present research is on isolation, characterization and enzyme kinetics study of tyrosinase enzyme extracted from tubers of *A. paeoniifolius.* Analysis of the crude tyrosinase enzyme extract of *A. paeoniifolius* by SDS-PAGE method revealed more distinct band corresponding to the molecular weight of 40 kDa (Fig.1). Molecular weight of different plant tyrosinase has been reported in previous studies from which cabbage, V*itis vinifera* (grapes) crystallized protein[15], *Hibiscus*, Tyrosinases were found to have molecular weight of 39 kDa and Pawpaw fruit showed tyrosinase of molecular weight 38.3 kDa which were similar to that of elephant yams tyrosinase. Molecular weight of tyrosinase of some other species is as follows: sweet potato 65.7 kDa, basil 54 kDa[14]. The obtained band was confirmed to be that of tyrosinase by zymogram with L-DOPA as substrate along with the gel (Fig.1).

Enzyme activity assay

The absorbance values were recorded at 420 nm for every 30 seconds of reaction time till 300 seconds using UV-VIS spectrophotometer. Since the L-DOPA formed is readily oxidised to dopachrome in presence of air, the absorbance values decreases with respect to time indicating the conversion of L-DOPA into Dopachrome. The OD values were found to be decreasing till 180 sec and then there is a small rise indicating the product inhibition of Dopachrome in the conversion from L-DOPA and again it starts decreasing. The slope value from the graph OD versus time depicted the value of enzyme activity. The enzyme activity was found from the enzyme eluted from SDS-PAGE and the activity of tyrosinase from *A. paeoniifolius* was determined to be 0.04 min^{-1} mM^{-1} (Fig.2).

Effect of pH

Most plant tyrosinases show maximum activity at or near neutral pH value. As reported earlier the optimum pH for maximum tyrosinase activity in plants varies depending on the extraction method, the substrates used for assay, and the localization of the enzyme in the plant cell[14]. It has been stated that optimum pH for potato tyrosinase activity was 6.4 while it was 7.0 for the other fruits tyrosinase. When the eluted enzyme was mixed with the substrate, the solution instantly

turned brown, which was absorbed strongly at 420 nm. The absorbance values were found to decrease with time initially and then found to remain steady approximately above 210 s (Fig 3a).

Among the six different selected pH values (5.7, 6.2, 6.7, 7.2, 7.7, and 8.0) of the extraction buffer, the *A. paeoniifolius* showed optimal activity at pH-7.2. From the previous studies it has been known that kiwi fruit and jongo red apple[16] tyrosinase has shown optimal pH same as that of *A paeoniifolius*. The variation of tyrosinase activity due to various buffer pH values followed a bell-shaped curve, and optimal enzyme activity was seen around pH 7.0 and this pH value was used in other determinations (Fig 3b).

Effect of temperature

Effect of temperature on tyrosinase enzyme from *A. paeoniifolius* was estimated using assay solution containing tyrosine as the substrate and 0.2 M sodium phosphate buffer maintained at six different temperature points ranging from 15 to 65 °C (15, 25, 35, 45, 55 and 65°C). Tyrosinase enzyme was added to the assay solution after maintaining the attained temperature value and the OD values were recorded at 420 nm using UV-VIS spectrophotometer (Fig. 4a). On addition of the enzyme, visible colour change into brown solution was noted. Recorded OD values were found decreasing linearly with time and entire tyrosinase activity diminished at temperature greater than 65°C. Values were plotted temperature versus enzyme activity and variation of tyrosinase activity with varying temperature values depicted a bell shaped curve graphically from which the optimum temperature for *A paeoniifolius* was found to be 25°C (Fig. 4b). From the early studies it has been found that grapes tyrosinase with catechol as substrate showed optimal enzyme activity at the temperature range exactly as same as that of the elephant yam tyrosinase.

Previous research has shown the optimal temperature values of 25°C for thymus tyrosinase, 30°C for Bramley's seedling apple and banana peel[17]. Also lettuce with pyrogallol as substrate[14], mango pulp[18] showed optimum temperature 30°C. Litchi, Pawpaw, dog rose, and sweet basil tyrosinase with catechol as substrate showed optimum temperature 20°C[14,19]. Thus it is stated that optimum temperatures for tyrosinase are quite species and substrate-dependent[14].

Kinetic parameters determination

Varying substrate (1% tyrosine) concentrations ranging from 2 mg to 10 mg/ml was taken to determine the enzyme kinetics. Immediately after addition of the tyrosinase extract the OD values for every 30 seconds of reaction time till 360 seconds was recorded at 420 nm using UV-Vis spectrophotometer (Fig. 5). The OD values were found increasing with the increase in the substrate concentration also the enzyme activity was found decreasing along with the time. Recorded values were plotted to generate Lineweaver-Burk plot (1/S versus $1/V_{max}$) to obtain Michaelis Menten parameters. *A paeoniifolius* tyrosinase enzyme activity fitted with Lineweaver equation producing linear line from which the Km and V_{max} values were determined. The Km value of *A paeoniifolius* was found to be 3.6 mM and V_{max} values were determined to be 0.1 s^{-1} (Fig. 6). V_{max} value of *A paeoniifolius* tyrosinase was comparable with Pawpaw fruit tyrosinase[19].

CONCLUSION

The crude tyrosinase enzyme from elephant yam (*A. paeoniifolius*) was isolated, purified by SDS-PAGE and its

biochemical characteristics were studied using tyrosine as the substrate. Tyrosinase enzyme from source was found to be more or less similar in their characterization aspects with previous research. The elephant yam tyrosinase enzyme revealed an exact optimum temperature of 25°C and optimum pH around 7.0. Kinetic parameters of tyrosinase enzyme showed in V_{max} values of 0.1 s and the K_m value 3.6 mM respectively. Based on these Michaelis-Menten parameters, tyrosinase from *A. paeoniifolius* can be used in further analytical studies and industrial uses.

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