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Extraction And Partial Purification Of Tyrosine Ammonia Lyase From *Santalum album* Linn A Possible Solution For Hyperpigmentation

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Abstract: **Tyrosine ammonia lyase (TAL) is an enzyme that deaminates the amino acid L- Tyrosine to p-coumaric acid. This enzyme was extracted from the plant *Santalum album* Linn. This study is aimed to determine the role of TAL enzyme for the treatment of hyperpigmentation in man by using Zebra fish embryo as test animal. Extraction of TAL was based on the principle of separation of proteins on treatment with an organic solvent acetone followed by the standardization of enzyme assay for the detection of the enzyme TAL. The presence of the enzyme was confirmed by the formation of p- coumaric acid which was detected using UV- Visible spectrophotometer at 333 nm. The crude extract was partially purified using ammonium sulphate. The activity of TAL was found to be hindered after partial purification. Hence zebra fish embryos were treated with crude enzyme extract of TAL. There was considerable reduction in the pattern of pigment formation in Zebra fish embryos. Hence *Santalum album* was found to be a good source of TAL and thereby can be used to treat Hyperpigmentation in man as tyrosinase enzyme in man is responsible for the conversion of tyrosine to melanin pigment. The enzyme Tyrosine ammonia lyase converts tyrosine to p-coumaric acid and thereby reduces the formation of melanin pigment resulting in hypopigmentation. Recent report suggest that there is syntenic relationship between zebra fish and human genomes.**

***Keywords*: Tyrosine ammonia lyase, *Santalum album*, Hyperpigmentation, Zebra fish, UV- Visible spectrophotometer.**

I. INTRODUCTION

Skin colour is one of man's major concerns. Dark spot, scar or a dull patch is inevitable especially with sun becoming harsher day by day. Our skin tone, hair colour and colour of iris, is determined by the pigment Melanin, a substance produced by special cells called Melanocytes. When these cells overproduce the amount of melanin, hyperpigmentation ensues. This skin condition is caused by excessive sun exposure, hormonal changes, genetic factor or drug reaction. Sometimes, another skin condition called acne vulgaris (severe acne/pimples) also leads to hyperpigmentation which is a common dermatological condition that is seen in all skin types but is most prominent in skin colour.

The intricate cellular and molecular interactions between melanocytes and keratinocytes, which together compose the epidermal melanin unit, are responsible for pigmentation of the skin. Melanocytes contain specialized cellular vesicles called melanosomes that convert aromatic amino acid tyrosine to melanin pigment giving skin its colour (Funan,H. U. 1968).

Mammals synthesize tyrosine from the aromatic amino acid phenylalanine, which is derived from food. The enzyme tyrosinase is responsible for the convertion of tyrosine to melanin pigment (C. J. Ellaway et al 2001).

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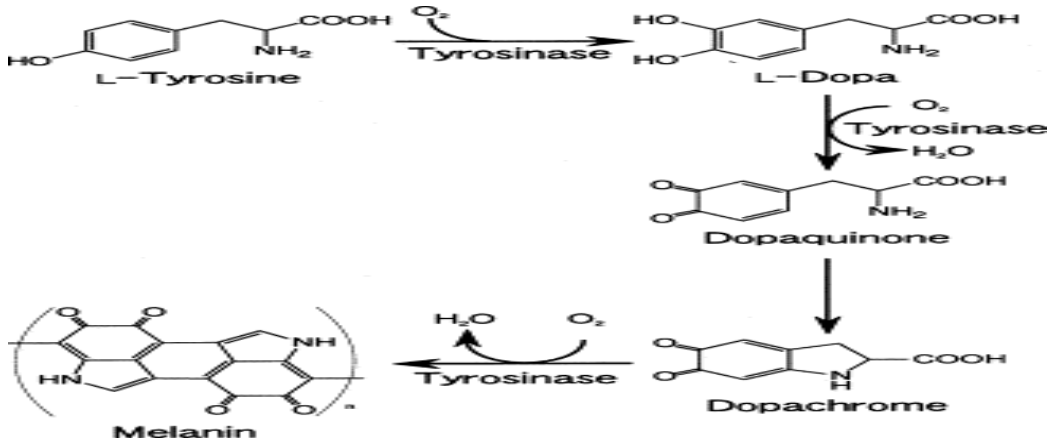
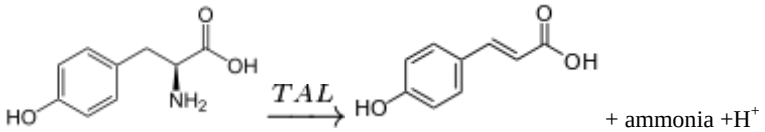


Fig.1 Pathway of melanin synthesis

For the treatment of hyperpigmentation, an enzyme of plant origin, tyrosine ammonia lyase (TAL) is used. TAL is involved in the conversion of L-tyrosine to p-coumaric acid (B.E.Ellis et al., 1973), an organic acid with the release of ammonia. This is an irreversible reaction. p-coumaric acid formed in this reaction catalyzed by TAL is a hydroxycinnamic acid with many physiological actions, including antioxidant, antimicrobial, antimutagenic, anxiolytic, analgesic, sedative, and immunoregulatory activities (S.Y. Ou et al 2012). TAL is found in very few plants (M R Young et al., 1966) and it is one of the major enzymes involved in phenol biosynthesis pathway of plants.



Tyrosine p- coumaric acid

Fig.2 TAL catalyzed reaction

Fig. 3 Santalum album

In the present investigation TAL was extracted from Santalum album, a small evergreen glabrous tree, belonging to the family Santalaceae. It is a native of the highlands of southern India mainly Coorg, Chennai and Mysore.

Sandalwood is mainly used as coolant, and also sedative effect and astringent activity, making it useful as disinfectant in genitourinary and bronchial tracts, diuretic, expectorant and stimulant. The sweet powerful and lasting odour makes Sandalwood oil useful in perfume industry. The same is also used as tonic for heart, stomach, liver, anti-poison, fever, and memory improvement and as a blood purifier. Various uses mentioned in Ayurveda system about sandalwood are in treatment of various other ailments like diarrhoea with bleeding intrinsic haemorrhage bleeding piles, vomiting, poisoning, and hiccoughs, initial phase of pox, urticarial, eye infections and inflammation of umbilicus. Plant leaves were used for the present piece of work. Fresh enzyme extract was prepared using plant leaves for the study.

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The test animal used in the present work, zebrafish is a tropical freshwater fish belonging to the minnow family (Cyprinidae) of order Cypriniformes. Native to the Himalayan region, it is a popular aquarium fish, frequently sold under the trade name zebra danio. The zebrafish is also an important vertebrate model organism in scientific research. It is particularly notable for its regenerative abilities and transparency during embryo development (Bradley, W. B. L., et al 2007). In the present investigation zebrafish was used as model organism to treat hyperpigmentation.

II. MATERIALS AND METHOD

Requirements:

Chilled distilled water, acetone, Tris HCl, 0.1N HCl, L-tyrosine ammonia lyase, 0.1N NaOH.

Other Requirements:

Centrifuge, mixer, refrigerator, Incubator, UV Spectrophotometer, mortar and pestle, magnetic stirrer, pH meter. Muslin cloth, fresh clean dried leaves of *Santalum album* (Indian sandalwood)

III. METHODOLOGY

Extraction Of The Tyrosine Ammonia Lyase Enzyme:

Leaves of ***Santalum album*** were collected, washed, cleaned and dried. 100g of these leaves were considered for extraction.The leaves were ground using 300ml of chilled distilled water and then filtered using a muslin cloth. The residue was collected and mixed with equal volume of acetone. This mixture was subjected to precipitation at -20^0C overnight. The precipitate was crushed using a mortar and pestle and then filtered using a muslin cloth. This was rinsed twice with acetone to remove the excess pigments which would otherwise hinder the assay. The precipitate was dried completely and weighed. 50ml of 0.025M Tris HCl of pH 8.2 was added to 2g of the protein extract. The mixture was subjected to centrifugation at 5000 rpm for ten minutes. The supernatant which served as a source of crude enzyme was collected for subsequent enzyme assay.

Enzyme Assay:

The reaction mixture was prepared using 0.8ml of 0.1M Tris HCl (pH 8.9), 0.2 ml of 1mM L-tyrosine and 1ml of enzyme extract and incubated at 37°C for 30 minutes. 0.2ml of 0.1N HCl was added after incubation to stop the reaction. TAL deaminates L-Tyrosine to give p-coumaric acid, ammonia, and H^+ ion which was quantitatively estimated using UV-VIS Spectrophotometer at 333nm.

Partial Purification Of TAL By Ammonium Sulphate Method And Enzyme Assay:

3 ml of enzyme source was made upto 10ml with extraction buffer and centrifuged at 5000 rpm for ten minutes. The supernatant was collected and 3.13g of ammonium sulphate was added to 2ml of supernatant, stirred and incubated for 15 minutes in ice cold condition. Centrifuged at 5000 rpm for 5 minutes. The pellet was dissolved in 3ml of 0.1M Tris HCl of pH 8.9. This was used as enzyme source for enzyme assay. Assay was conducted and the absorbance was read at 333 nm using UV-Spectrophotometry.

Estimation of Protein by Bradford Method (Bradford, 1976):

Estimation of the protein in TAL was done using Bradford method.

Preparation of Bradford Reagent:

0.05g of Coomassie Brilliant Blue G- 250 was dissolved in 50ml of 95% ethanol and 100ml of 85% phosphoric acid was added. The mixture was then diluted to 1 litre when the dye was completely dissolved. Vertical serial filtration was carried out by placing the funnel one below the other using Whatman #1 filter paper just before use. The Bradford reagent was light brown in colour.

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Preparation of stock solution using BSA (bovine serum albumin):

100mg of BSA was weighed and dissolved in distilled water and the volume was made up to 100ml in a standard flask. This solution of 1mg/ml concentration was used as the stock solution.

Preparation of Working Standard:

10 ml of the stock was taken and further diluted to 100 ml in a standard flask with 100 µg/ml concentration. Working solution was pipetted out in aliquots of 0.2-1ml (20-100µg) and the volume was made upto 1ml. 5ml of bradford reagent was added and vortexed gently and allowed to stand for ten minutes for colour development. The absorbance was taken using a spectrophotometer before completion of ten minutes at 595 nm against reagent blank. The values were plotted using standard graph.

Melanin Pigment Inhibiting Property by TAL Enzyme:

Melanin inhibiting property by TAL enzyme was checked by selecting Zebra fish as a model organism because of its unique characteristics such as, transparency and quick embryo development. Initially the work was started by selecting one pair of zebra fish which was allowed to mate. 24 hours after mating embryos were collected in petriplates. The collected embryos were incubated at laboratory conditions for further development at 37^0. At the 48^{th} hour of the embryo development, the embryos were treated with different concentration of enzyme source (50 µl, 100 µl, 200 µl) and they were kept under incubation. Every one hour the embryos were microscopically observed for the change in the pigment patterns.

IV. RESULT

TABLE 1 Crude activity of TAL

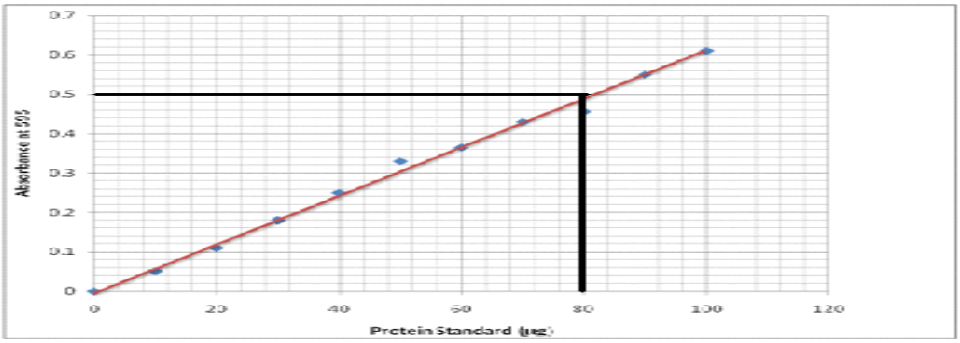
Trial	Absorbance at 333 nm	Enzyme activity
Blank	0.00	0.000
Test 1	0.50	0.16
Test 2	0.56	0.17

TABLE 2 Activity of TAL after partial purification on adding $(NH_4)_2SO_4$

Trial	Absorbence at 333 nm	Enzyme activity
Blank	0.00	0.000
Test 1	0.058	0.018
Test 2	0.058	0.018

Specific Activity Of Crude Enzyme Extract: *02 IU*

Graph1: A Standard graph depicting the protein standard vs absorbance at 595nm.



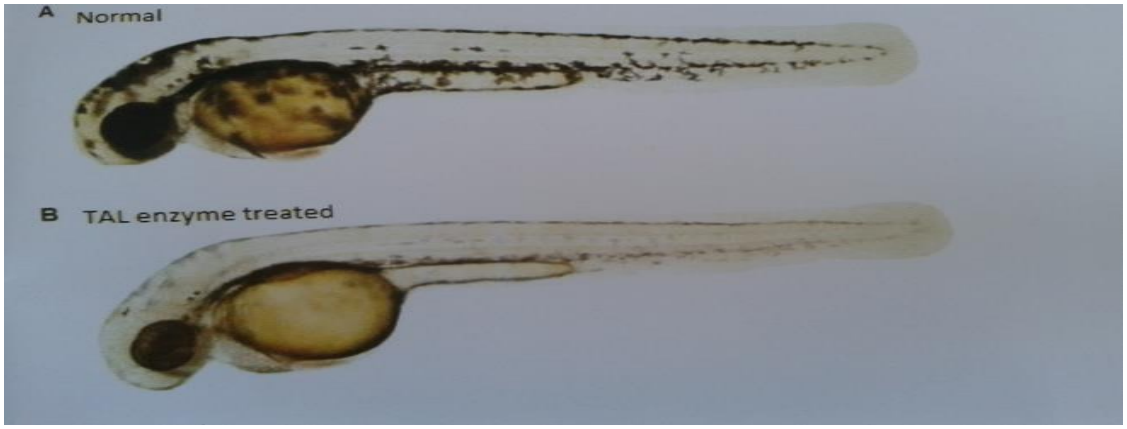


Fig 4: Reduction in pigment formation in the embryo of Zebra fish treated with TAL

V. CONCLUSION

Tyrosin is a parent amino acid for skin, hair and eye pigments. The spectrophotometry of the enzyme extract showed positive results for the presence of the enzyme TAL in the plant *Santalum album* Linn.The concentration of the same was obtained by Bradford's graphical method for estimation of proteins. The concentration of the crude enzyme from *Santalum album* was found to be 80µg and specific activityof TAL was found to be 02IU. Further the enzyme was partially purified by ammonium sulphate precipitation.

On incubating the partially purified enzyme extract, excessive precipitation reduced the concentration of the required enzyme. Hence, it was inferred that the purification step would obstruct the assay. Reduction in the pigment pattern formation was observed in embryos of Zebra fish treated with TAL enzyme extract.

Thus the enzyme TAL extracted from ***Santalum album*** served as a good source for the reduction of hyper pigmentation in Zebra fish. This enzyme can be used to reduce hyper pigmentation in Humans as the tyrosine pathway is similar in production of melanin pigment.

Santalum album showed good activity of TAL, which has similar function to that of tyrosinase enzyme found in humans and hence this enzyme can be used to treat hyper pigmentation in man.

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