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TYROSINE AMMONIA LYASE EXTRACTED FROM *CLITORIA TERNATEA LINN.* - ITS IMPORTANT ROLE IN METABOLISM OF HUMANS AND REACTION WITH DIFFERENT METAL IONS

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ABSTRACT

Tyrosine ammonia lyase (TAL; EC 4.3.1.23) is an enzyme required for deamination of the amino acid *L*-tyrosine to *p*-coumaric acid (B.E. Ellis *et al.,* 1973).The enzyme TAL was extracted from the plant *Clitoria ternatea linn*. This enzyme can be used in the treatment of an autosomal recessive metabolic disorder tyrosinemia type II where tyrosine is accumulated in excess amounts in the body. The extraction of this enzyme was based on two techniques: Firstly, the separation of proteins using an organic solvent, acetone. In the second step, the presence of enzyme is to be detected hence standardization of the enzyme assay was accomplished. The presence of the enzyme was confirmed by the formation of *p*-coumaric acid which was detected by using UV-Visible spectrophotometer at 333nm. Also, the activity of the enzyme was enhanced using an activator –nickel sulphate, an inorganic salt.

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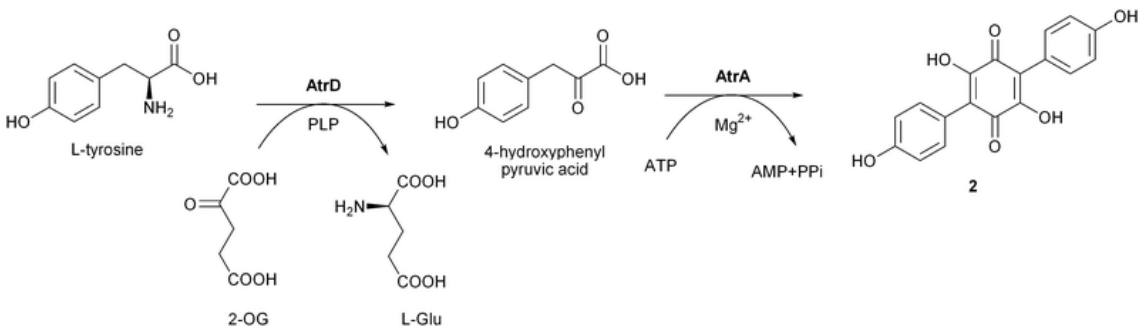
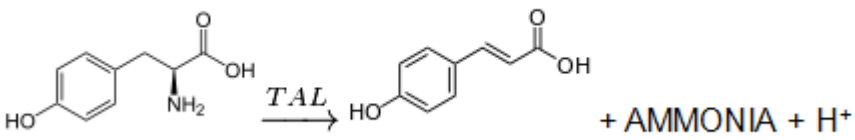
INTRODUCTION

Tyrosine is a semi-essential aromatic amino acid in infants (Imura K, Okada A, 1998) and a non essential amino acid in adults which can be synthesized in the body from Phenylalanine. Under normal conditions, tyrosine is converted into 4-hydroxyphenylpyruvate by the enzyme tyrosine amino transferase (TAT; EC 2.6.1.5) (shown in figure 1A) and the deficiency of this hepatic tyrosine amino transferase causes a severe disorder known as tyrosinemia type II (Natt E *et al.,* 1992). TAT is a dimer which consists of two identical active sites i.e., Lys280 attached to pyridoxal phosphate (PLP). It is a transamination reaction (J L Hargrove et al., 1989). TAT gene is located on the long arm of chromosome 16 in the position 22.1-q22.3 (Ernst Natt *et al.,* 1987) and spans a length of 10.9Kb, which includes 12 exons (Rettenmeier R *et al*., 1990). The mutation in TAT gene results in the deficiency of this enzyme. As a result, tyrosine is accumulated in the body in excess amounts. It is an autosomal recessive metabolic disorder (Steven C. Kazmierczak, 1993). This disorder is also known as oculocutaneous tyrosinemia. TAT enzyme is important for the transamination reaction to occur, because it is the first enzyme in the 5-step process of breakdown of tyrosine into small and harmless molecules which are excreted by the kidneys. Therefore, the deficiency of this enzyme cannot break down tyrosine which is accumulated in the body and it affects eyes, skin and mental development. The age of onset of this disorder is neonatal. The symptoms of this disorder include: excessive tearing and redness in the eyes, photophobia (sensitivity to light) and skin lesions on hands and feet (William D James *et al*., 2011). For the treatment of this disorder, an enzyme of plant origin, tyrosine ammonia lyase (TAL) is used. TAL is involved in the biotransformation of *L*-tyrosine to *p*-coumaric acid (B.E.Ellis *et al*., 1973), an organic acid with the release of ammonia (shown in figure 1B). This is an irreversible reaction.TAL is found only in plants (M R Young *et al*., 1966) and it is one of the major enzymes involved in phenol biosynthesis pathway of plants.

FIGure 1- (A)



(B)



(A) Conversion of L-tyrosine to 4-hydroxyphenylpyruvic acid by TAT in liver (Zhong Yu Zhou, Ji Kai Liu, 2010)
(B) Conversion of L-tyrosine to p-coumaric acid using TAL (Louie G V et al., 2006)

This paper throws a limelight on the management of tyrosinemia type II by extraction of the enzyme TAL from *Clitoria ternatea linn* (shown in figure 2*)* which is a perennial herb commonly known as butterfly-pea, blue-pea or cordofan-pea (English), Aparajit (Hindi) Shankpushpi (Sanskrit) (Manju Lata Zingare *et al*., 2013). It belongs to the family *Fabaceae* (Pulok K Mukherjee *et al*., 2008) and sub-family Papilionaceae (Manju Lata Zingare et al., 2013). This plant has vivid deep blue flowers;solitary; with light yellow marking and some varieties also yield white flowers.

FIGure 2
The plant Clitoria ternatea linn from which TAL was extracted

This plant is native to Caribbean, Central America and Mexico but it is also seen in Indian subcontinent and other parts of tropical Asia like Philippines and also Madagascar (Arumugam M, Panneerselvam R, 2012). Since ancient times, this plant has been used in the preparation of ayurvedic medicines and till today this plant has been used in preparation of about 40 medicines (Arumugam M, Panneerselvam R, 2012). The leaves and roots are used in the treatment of a number of aliments including body aches, infections and as an anthelmintic and antidote to animal stings (Nirmal *et al*., 2008). Also, the roots of this plant are helpful in improving memory power (Pulok K Mukherjee *et al.,* 2008). This plant is also known to possess tranquilizing property, analgesic activity, anti inflammatory, antipyretic, immunomodulatory activities (Mukherjee *et al.,* 2008), antioxidant, antidiabetic and hepatoprotective activities (Manju Lata Zingare *et al*., 2013).

MATERIALS

Instruments

Centrifuge, Centrifuge tubes, Mixer, Refrigerator, Incubator, UV-Spectrophotometer, Mortar and Pestle, pH meter.

Reagents

Chilled distilled water, Acetone, TRIS-HCL, 0.1N HCL, 5N NaOH, Tyrosine, $CaCl_2$, $MgCl_2$, $MnSO_4$, $CuSO_4$, $NiSO_4$, $ZnSO_4$, $FeCl_3$, Na_2MoO_4.

Other requirements

Muslin cloth, fresh and clean leaves of *Clitoria ternatea linn.*

METHODS

Extraction of the enzyme, tyrosine ammonia lyase (Guruprasad et al., 2013)

Leaves of *C. ternatea linn* were collected, washed and wiped with a tissue paper to remove excess water. 100g of this were taken for extraction (shown in figure 3).They were ground using 400ml of chilled distilled water and then filtered using muslin cloth .The residue was collected and mixed with equal volume of acetone .This mixture was kept undisturbed for precipitation at -20ºc overnight .The precipitate was crushed using mortar and pestle and then filtered using muslin cloth. This was rinsed thrice with acetone to remove excess pigments. The precipitate was dried completely and weighed (shown in figure 4). To 1g of this acetone powder, 13ml of 0.025M TRIS-HCl (pH-8.2) was added. The mixture was then centrifuged at 5000 rpm for 10min. The

supernatant which serves as a source of the crude enzyme was collected for consequent enzyme assay (shown in figure 5).

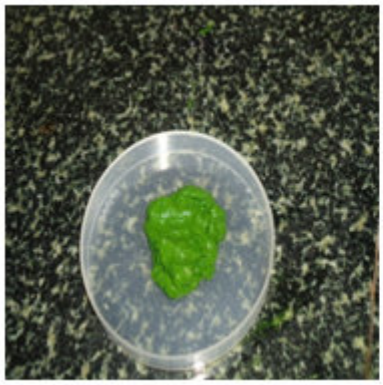


FIGure 3-Plant extract before

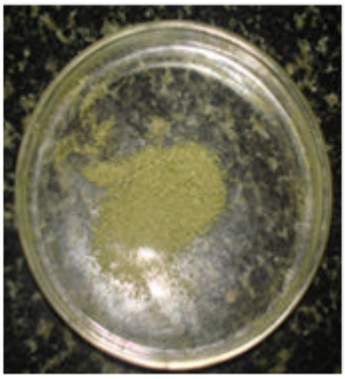


FIGure 4-Acetone powder

FIGure 5- Enzyme acetone treatment source

Enzyme assay

A reaction mixture was prepared using 0.8ml of 0.1M TRIS-HCl (pH-8.9), 0.2ml of 0.025M *L*-tyrosine and 1ml of enzyme extract and incubated at 37ºc for 30 min. 0.5ml of 1N HCl was added after incubation to arrest the reaction. TAL deaminates *L*-tyrosine to give *p*-coumaric acid with the release of ammonia, which is quantitatively measured using UV-Visible spectrophotometer at 333nm.

Estimation of proteins by Bradford method (Bradford, 1976)

Preparation of Bradford reagent

0.05g of coomassie brilliant blue G-250 was dissolved in 30ml of 95% ethanol. To this, 50ml of 85% of phosphoric acid was added .The mixture was then diluted to 500ml when the dye was completely dissolved. Filtration was carried out two to three times using Whatmann #1 filter paper just before use.

Preparation of stock solution using BSA (Bovine Serum Albumin)

100mg of BSA F-V was weighed and dissolved in distilled water and this solution was made up to 100ml using a standard flask. 1ml of this solution is equal to 1mg of protein. This solution serves as a stock solution.

Preparation of working standard

10ml of the stock was taken and further diluted to 100ml using another standard flask. So, 1ml of this solution is equal to 100µg. Therefore, 0.1ml of this contained 10µg. Standard solution containing a range between 10µg to 100µg were prepared and volumes made up to one tenth ml of the stock was taken and further diluted to 100ml using a standard flask. 1ml of this solution was equal to 100µg. Therefore 0.1ml contained 10µg.The standard graph is in the figure 6.

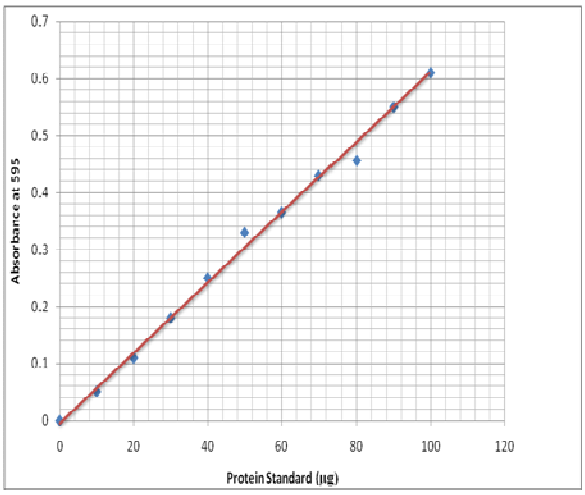


FIGure 6
A Standard graph depicting the protein standard vs absorbance at 595nm

Action of different metal ions

Along with the chemicals added for enzyme assay, □□□ of different metal ion solutions obtained by serial dilutions were added in different aliquotes to 1ml of the enzyme sample separately and the OD was read at 333nm.The readings were recorded and compared with the results of the standard enzyme assay. Finally, Nickel sulphate was found to be a potent activator of the enzyme (shown in Table 1) and the same result is shown in figure 7.

RESULTS

The concentration of the enzyme was found to be 340□g .The total activity of the enzyme is 393628.71pKat. One katal is defined as the catalytic activity that raises the rate of a chemical reaction by one mole per second. The activity is expressed as Pico katal (10^{-12} kat=pkat) enzyme. Enzyme source: acetone powder from *Clitoria ternatea linn* prepared as described in materials and methods. Enzyme was assayed as per the standard conditions. Protein was estimated by Bradford method as described in materials and method.

TABLE 1
Effect of different metal ions on TAL activity

Sample	Activity %
Crude	100
$CaCl_2$	82.04
$MgSO_4$	72.55
$NiSO_4$	115.58
$MnSO_4$	46.86
$CuSO_4$	46
$FeCl_3$	58.15
$ZnSO_4$	43.51
Na_2MoO_4	58.10

A constant amount of enzyme was pre-incubated with different metal ions for 10 min. TAL activity was determined under standard conditions.The activity was expressed as a percentage of control.

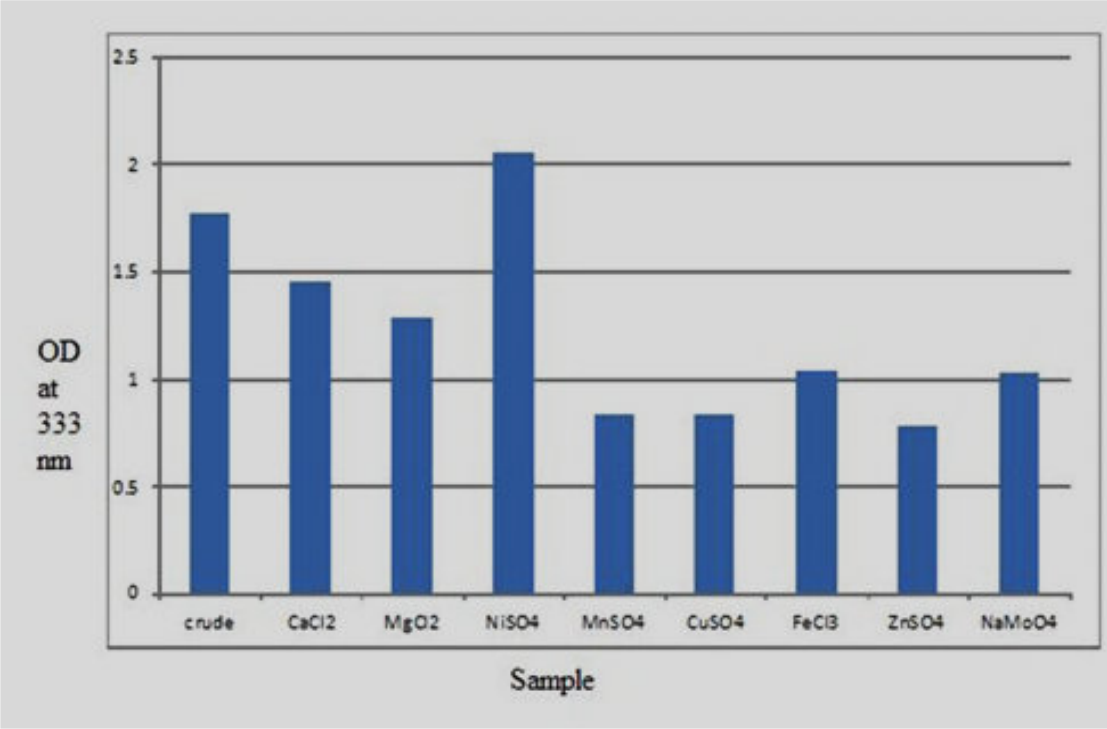


FIGure 7
Action of different metal ions on the activity of the enzyme TAL

DISCUSSION

The spectrophotometric analysis of the enzyme extract showed positive results for the presence of the enzyme TAL from the plant *Clitoria ternatea linn* .The concentration of the same was obtained by Bradford's graphical method of estimation of proteins. The concentration of the enzyme from C. *ternatea linn* was found to be 340µg. Further, we partially purified the enzyme extract by adding 1ml of Manganese sulphate to 10ml of the crude enzyme. Manganese sulphate serves as an excellent medium for the precipitation of unwanted biomolecules such as nucleic acids so that they do not interfere with the substrate. Also, we found precipitation on reduced concentration of the required enzyme on incubating the partially purified enzyme

extract. Hence, it was concluded that the purification step would help in obtaining good results. Therefore, to the original crude extract we added metal ions like Calcium chloride, Zinc sulphate, Nickel sulphate etc. and subjected it to UV-Visible spectrophotometric analysis and found an increase in the absorbance on addition of Nickel sulphate. Thus, it was inferred that nickel sulphate is a potent activator of the enzyme. As Phenylalanine ammonia lyase (PAL) is used for the treatment for Phenylketonuria (Sarkissian CN *et al*. 1999), the enzyme TAL extracted from *C. ternatea linn* can serve as an effective treatment for the disorder Tyrosinemia Type II.Studies till date have shown that the patients suffering from this disorder have to follow a strict diet. They should consume the food products which are devoid of the amino acids phenylalanine and tyrosine (Robert Tomaino, 2004).

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