

Potent activator *N*-arylenamine-3-chloro-4-fluoroaniline for Phenylalanine Ammonia Lyase extracted from *Plectranthus amboinicus*

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ABSTRACT

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is an enzyme required for the deamination of the amino acid L-phenylalanine to trans-cinnamic acid. We have extracted the enzyme PAL from the plant *Plectranthus amboinicus*. This enzyme is used for the treatment of an autosomal recessive metabolic disorder Phenylketonuria. Extraction was based on the principle of separation of proteins on treatment with acetone followed by the standardization of enzyme assay for the detection of the enzyme PAL. The presence of the enzyme was confirmed by the formation of trans-cinnamic acid using UV-visible spectrophotometer at 290nm. Increase in activity of the enzyme was studied using a potent activator *N*-arylenamine-3-chloro-4-fluoroaniline shows gradual increase in the activity of the enzyme with increase in the concentration of the activator keeping the concentration of the enzyme and substrate constant. Docking studies has been done using the program Autodock and other tools of bioinformatics. Thus the enzyme PAL extracted from *Plectranthus amboinicus* can serve as a very good treatment for phenylketonuria.

Key Words : PAL, PKU, Phenylalanine.

INTRODUCTION

Phenylalanine (Phe) is an essential aromatic α -amino acid consumed in the form of L-Phenylalanine by humans. In normal conditions the body changes phenylalanine to L-Tyrosine with the help of the enzyme Phenylalanine hydroxylase (PAH). PAH is an iron-dependent, tetrahydrobiopterin (BH₄)-dependent monooxygenase that catalyses the rate limiting step in the catabolism of Phe[1]. PAH converts Phe into tyrosine by hydroxylating Phenylalanine, using BH₄ as a reducing agent (shown in figure 1A). The PAH gene is located on the q arm of the 12th chromosome in the 22-24 position consisting of 13 exons spanning 90 kilobases [2]. The bio-transformations are given as follows:

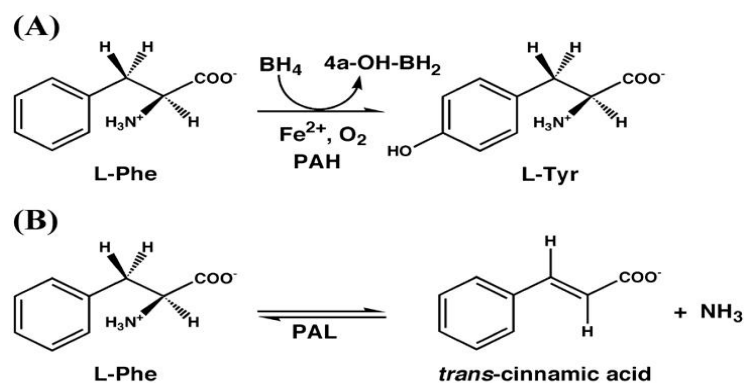


FIGURE 1:

(A) Conversion of L-Phenylalanine to L-Tyrosine using PAH.

(B) Conversion of L-Phenylalanine to trans-cinnamic acid using PAL [3].

A rare congenital autosomal recessive metabolic disorder Phenylketonurea occurs in people who lack this enzyme PAH which the body needs to breakdown L-phenylalanine [4]. PKU occurs due to the inability of the body to breakdown phenylalanine to tyrosine because of the absence of the enzyme PAH owing to which the amino acid will get accumulated in the blood stream (Hyperphenylalaninemia) in the form of phenylalanine acetate, phenylalanine pyruvate etc. This inadequacy is because of the point mutation in the long arm of the above specified chromosome. PKU results in an irreversible mental retardation if it is not diagnosed in the early days of the child birth. To overcome this issue a plant origin enzyme Phenylalanine ammonia lyase (PAL) also sometimes produced by algae and fungi is used as a substitute for the enzyme PAH.

Phenylalanine ammonia lyase is involved in the biotransformation of L-phenylalanine to trans-cinnamic acid releasing ammonia [5] which is a reversible reaction (shown in figure 1 B). It is one of the major enzymes involved in phenyl propanoid pathway of plants. PAL does not need any cofactor for its action on phenylalanine. The product trans-cinnamate does not show any cytotoxic effect on the body. The trans-cinnamic acid is excreted in the form of hippurate in the urine as seen in rats. This project aids the management of PKU by extraction of the enzyme PAL from *Plectranthus amboinicus* (Fig-2). *P. amboinicus* is a large succulent perennial herb [6] commonly known as Country Borage (English),



FIGURE 2: Plectranthus amboinicus plant

Cuban oregano (Cuba), Patta ajavain (Hindi) etc. It belongs to the family *Lamiaceae*. It is highly aromatic pubescent herb with distinctive smelling leaves. The plant is distributed throughout in India, cultivated in the gardens. The leaves of this plant have been used in malarial fever, hepatopathy, renal and vesicle calculi, cough, chronic asthma, hiccup, bronchitis, anthelmintic, colic and convulsions.

N-arylenamine-3-chloro-4-fluoroaniline as an activator

The title activator Figure 3 belongs to the family of fluorine substituted benzylidene anilines, screened for anti-bacterial and anti-inflammatory and analgesic activity. It has been pointed out that organo fluorine has low proton affinity and hardly accepts hydrogen bonds. Introduction of fluorine in a variety of aromatic compounds increases the molecular volume and causes photodimerisation. Fluorine can be found in drugs and drug intermediates, since it is actually a good hydrogen mimic adding only limited extra steric demands at receptor site. Fluorination can also aid hydrophobic interaction between the drug and binding sites on receptors or enzyme. In light of the above aspects present activator has been identified.

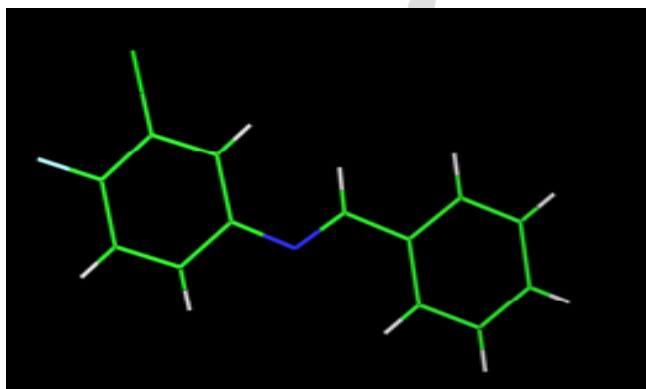


FIGURE 3 N-arylenamine-3-chloro-4-fluoroaniline

MATERIALS AND METHODS

Instruments and Reagents

Centrifuge, Centrifuge tubes, Mixer, Refrigerator, Incubator, UV Spectrophotometer, Computers, Autodock, and Pymol softwares. Chilled distilled water, Acetone, TRIS-HCL, 0.1N HCL, Phenylalanine, N-arylenamine-3-chloro-4-fluoroaniline, Cinnamic acid.

Extraction of the enzyme, Phenylalanine ammonia-lyase

Leaves of *P.amboinicus* were collected, washed, cleaned and dried. 200g of these leaves were considered for extraction. They were grinded 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°C 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 is dried completely and weighed. To 1g of this acetone powder, 80ml of 0.025M tris-HCl (pH- 8.2) was added. The mixture was subjected to centrifugation at 10,000rpm for 10min. The supernatant which serves as a source of the crude enzyme was collected for consequent enzyme assay.

Enzyme Assay

A reaction mixture was prepared using 0.8ml of 0.1M tris-HCl (pH-8.9), 0.2ml of 0.001M l-phenylalanine and 1ml of enzyme extract and incubated at 30°C for 30min. 0.5ml of 1N HCl was added after incubation to stop the reaction. PAL deaminates l-phenylalanine to give trans-cinnamic acid which is quantitatively estimated using a UV-VIS Spectrophotometer at 290nm. Results of which are mentioned in table 1.

1) Estimation of Protein by Bradford method [7]

Ten mL of the stock was taken and further diluted to 100 mL using another standard flask. So 1 mL of this solution was equal to 100 μg . Therefore, 0.1 mL of this contained 10 μg . Standard solution containing a range between 10 μg to 100 μg were prepared, and volumes made up to 1 mL. Apart from this 1 mL distilled water and reagent blank were taken in two separate test tubes. Bradford reagent (5 mL) was added and vortexed gently and allowed to stand for 10 min for color development. The absorbance was taken using a spectrophotometer before completion of 10 min at 595 nm against a reagent blank. The standard graph is in Fig. 4.

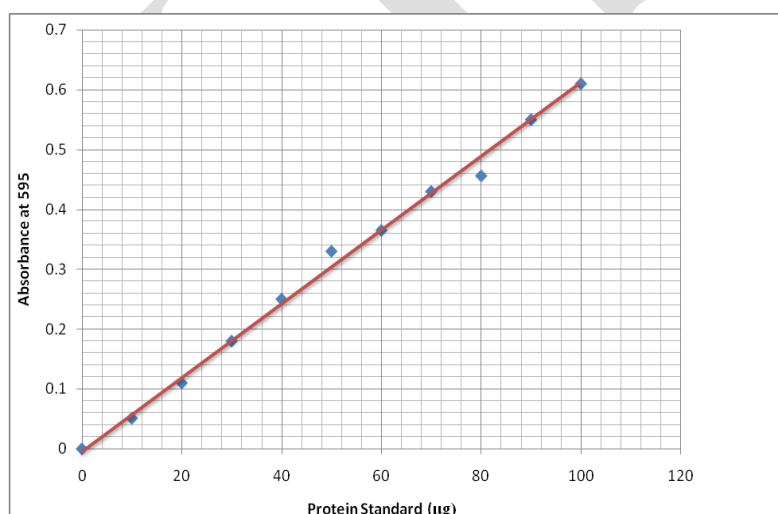


FIGURE 4 Standard graph

Ten mL of the stock was taken and further diluted to 100mL using a standard flask. One mL of this solution was equal to 100 μg ; therefore 0.1 mL contained 10 μg .

Docking Studies

Crystal information file (Cif) of the activator N-arylenamine-3-chloro-4-fluoroaniline was downloaded from Acta crystallographica [8] and docked with enzyme PAL of *Petroselinum crispum* PDB ID- 1w27 taken from Protein Data Bank [9] using Autodock [10].

N-arylenamine-3-chloro-4-fluoroaniline as activator

A constant amount of enzyme (35.42 pkat) was pre-incubated with N-arylenamine-3-chloro-4-fluoroaniline for 10 min. PAL activity was determined under standard conditions. The activity was expressed as a percentage of control show in Table 3.

RESULT

The concentration of the enzyme PAL in the plant was found using the following readings :

Table 1: Absorbance of the product (cinnamic acid) formed by PAL

STEP	VOLUME mL	TOTAL PROTEIN mg/mL	ACTIVITY *pkat/mL	TOTAL ACTIVITY pkat	SPECIFIC ACTIVITY pkat/mg protein	YEILD %	FOLD Purification
Enzyme from <i>P.amboinicus</i>	80	0.24	50.15	1013.14	2108.75	100	1

*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=pkcat) enzyme.

Enzyme source: acetone powder from *Plectranthus amboinicus* 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 **methods**.

Docking Result

N-arylenamine-3-chloro-4-fluoroaniline was docked with PAL protein by using auto dock software. Docking result shows the best 9 binding sites (Table 2). The complex of protein ligand interaction shown in Figure 5.

Table 2: Autodock showing the Binding affinity on phenyl alanine ammonia- layes for N-arylenamine-3-chloro-4-fluoroaniline

Mode	Affinity (kcal/mol)	Distance from best mode Rmsd l.b.	Rmsd u.b.
1	-4.9	0.000	0.000
2	-4.7	3.531	7.799
3	-4.7	3.998	7.153
4	-4.6	14.140	15.588
5	-4.5	13.651	14.977
6	-4.5	10.620	11.326
7	-4.3	13.667	15.141

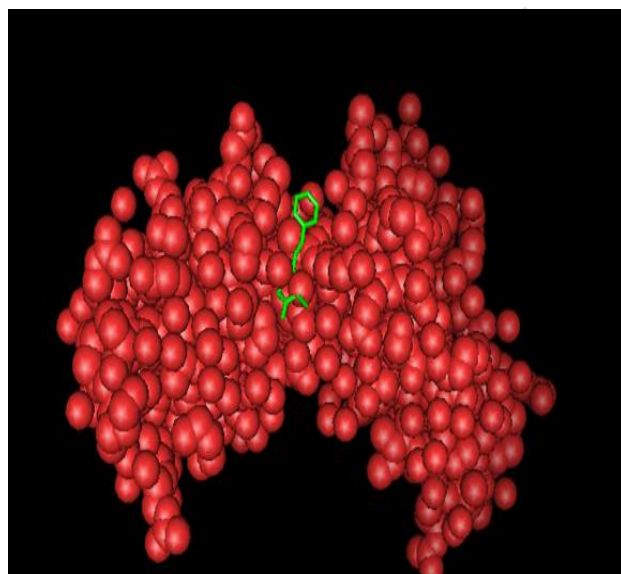


FIGURE 5 PAL and N-arylenamine-3-chloro-4-fluoroaniline as an activator (after docking)

Table 3: Effect of N-arylenamine-3-chloro-4-fluoroaniline activity

0.2μM N-arylenamine-3-chloro-4-fluoroaniline (ml)	Activity %
1	110
2	115
3	145
4	147

DISCUSSION

The spectrophotometry of the enzyme extract showed positive results for the presence of the enzyme PAL in the plant *P. amoinicus*. The concentration of the same was obtained by Bradford's graphical method for estimation of proteins. The concentration the enzyme from *P.amboinicus* was found to be 16μg. Further we tried to purify the enzyme extract by adding 1ml of Manganese sulphate to 10ml of the crude enzyme. Manganese sulphate precipitates the unwanted materials like nucleic acid so that only the enzyme can react with the substrate. On incubating the partially purified enzyme extract we found excessive precipitation on reduced concentration of the required enzyme. Hence we inferred that the purification step would obstruct the assay. To the original crude extract we then added N-arylenamine-3-chloro-4-fluoroaniline in different aliquots. On subjecting it to UV-spectrophotometry we found a gradual increase in the absorbance which concluded that N-arylenamine-3-chloro-4-fluoroaniline is a potent activator of the enzyme. We also saw that on addition of 0.3ml of 2μM of N-arylenamine-3-chloro-4-fluoroaniline, the enzyme showed maximum activity. Thus the enzyme PAL extracted from *Plectranthus amboinicus* serves as a very good treatment for phenylketonuria. Recent studies have shown that oral administration of tetrahydrobiopterin(a natural cofactor for DOPA synthesis and for the degradation or oxidation of phenylalanine) can reduce blood levels of this amino acid in the bodies of patients suffering from PKU. Sapropterin dihydrochloride (Kuvan) a form of tetrahydrobiopterin was first manufactured by Bionarin Pharmacueticals which acts as an analogue of BH₄. Kuvan is the first drug that helps BH₄ responsive PKU patients lower the Phe levels to great extents. Patients who respond to this drug may also be able to increase the amount of natural protein intake.

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