Extraction of Phenyl alanine ammonialyase (PAL)from *Ficusreligos*;itsactivatorsfrom *Lantana camara*and different metalions for the treatment of phenylketonuria

Simran Jain,Monica Samal,PoojaDasgupta, PoornimaVenkatesh,Guruprasad R,
DurgaFemto Technologies and Research,
Bangalore, Karnataka, India.

Abstract

Phenylketonuria (PKU) is an inborn, autosomal recessive disorder. It is causes due to the deficiency of the enzyme phenylalanine hydroxylase[PAH;E.C.1.14.16.1]. PAH helps in conversion of phenylalanine to tyrosine, due to the deficiency of the enzyme,phenylalanine is not converted to tyrosine and instead is converted to phenylketones. Phenylalanine ammonia lyase [PAL; E.C.4.3.1.] is the enzyme found in plants which performs the similar function as PAH. PAL was extracted from *ficusreligosa* (peepal) and the main aim was to find an activator which would increase the activity of the enzyme, the activatorsfrom different metal ions and also extracted from *Lantana camara.Compouns were*characterized by GC-MC and docking studies were done with PAL and different activators by using autodock software.

Introduction

Phenylketonuria is a human inborn error of metabolism for which the major biochemical defect has been found out and is an autosomal recessive disorder[1].It is caused due to deficiency of the enzyme called phenylalanine hydroxylase [PAH;E.C.1.14.16.1] which converts phenylalanineto tyrosine(Fig1).This enzyme is present in humans. Phenylalanine ammonia lyase(PAL; E.C.4.3.1)is an enzyme found in plants, which performs the similar function as PAH. The enzyme is known to catalyze biotransformation reaction which converts L-phenylalanine into trans-cinnamic acid and ammonia (Fig 2).

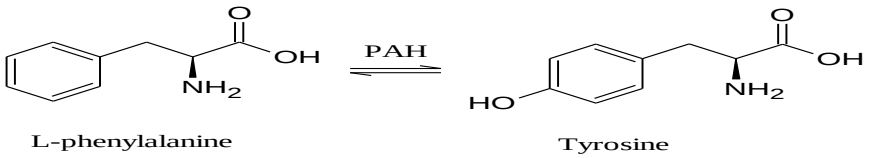


Fig1:Conversionof phenylalanine to tyrosine

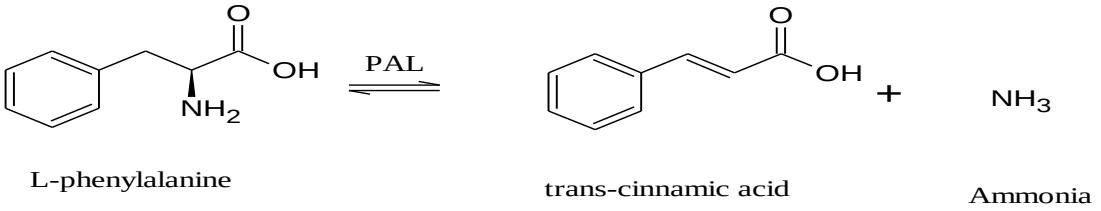


Fig2:Conversion of L-phenylalanine into trans-cinnamic acid and ammonia

Koukol and Conndiscovered the PAL enzyme in 1961[2]. PAL belongs to the family of ammonia lyase which cleaves carbon nitrogen bonds[3]. The enzyme has been found to play a catabolic role in micro-organisms[4]. It also plays an important role in phenyl propanoid pathway[5]. It is required for the synthesis of various polyphenol compounds like flavonoids, lignin and so on in plants[6].The molecular mass of the enzyme is 270-330 kDa[7]. The enzyme has various clinical, biochemical and biotechnological applications. Apart from that, it has also been used in the generation of L-phenylalanine by reversing normal physiological reaction. Aspartame (L-phenylalanyl-L-aspartylmethyl ester), a non-calorific sweetener is formed from L-phenylalanine[8].It has the natural ability to break down L-phenylalanine, and hence is effectively used in enzyme therapy[9].The enzyme may find use in the treatment of a certain type of mouse neoplastic tumor.Phenylketonuria is also known as hyperphenylalaninemia. The concentration of phenylalanine increases in the blood as it does not get converted to tyrosine due to deficiency of the enzyme and results in the formation of Phenyl ketones such as phenyl pyruvate, phenyl lactate and phenyl acetate which are excreted in the urine[10].When all the three phenyl ketones are present in the blood, it inhibits the synthesis of a neurotransmitter, serotonin; thus resulting in severe mental retardation. Patients suffering from PKU are also seen to have a very light skin pigmentation, unusual gait, stance and sitting posture. They even suffer from a frequency of epilepsy[11].The adverse effects of phenylketonuria are seen to diminish after a certain period of time, if the level of phenylalanine in blood is maintained within normal limits for the first 5-10 years of life. Urinary phenyl pyruvate can be detected using FeCl3, even though the screening test is not very reliable.The Peepal (Ficusreligosa) tree called “ashvattha” in Sanskrit is a very large tree with a light grey and smooth bark. The leaves are heart shaped and have a long tapering end. Peepal is a sacred with a long life.It has wide applications in the field of Ayurveda.. Its powder form is said to have healing power, as it has been used to treat wounds for years. The leaves have medicinal properties.The root bark is useful for treatment of stomatitis, clean ulcers and promotes granulation.

Materials and methods

Reagents: - Chilled acetone, chilled distilled water, Tris HCL, HCL (1 N), NaOH (0.1 N), L – Phenylalanine, Bradford reagent, BSA, Salts – $CaCl_2$,$ZnSO_4$,$CuSO_4$

Instruments: - Chilled grinder, Cooling centrifuge, UV spectrophotometer, Computer, Incubator, Refrigerator, pH Meter.

Other requirements: - Glass wares, Mortar & pestle, Centrifuge tubes, Muslin cloth, Fresh leaves of *Ficus religosa*

Extraction of the enzyme (PAL)

First, leaves of *Ficusreligosa*were collected and washed. They were wiped using tissue paper to remove the excess amount of water. 100g of these leaves were taken and ground using 350ml of chilled distilled water in a mortar and pestle. It was then filtered with the help of muslin cloth. The filtrate was measured and an equal amount of acetone was added to it. This was kept undisturbed at -20°C overnight for precipitation. It was then centrifuged for 10 minutes at 10000rpm. Acetone was added to the pellet to remove pigments. After drying completely, the amount of protein was measured. 1g of the powder was taken to which 20ml of 0.025M Tris-HCl (pH 8.2) was added and centrifuged for 10 min at 5000rpm. The supernatant obtained after centrifugation serves as the source of enzyme.

Enzyme assay

A reaction mixture using 0.8ml of 1M TrisHCl (pH-8.2), 0.2ml of 0.001M l-phenylalanine and 1ml of enzyme source was prepared and incubated for 30 minutes at 37°C. 0.5ml of 1N HCl was added after incubation to arrest the reaction. PAL undergoes deamination producing trans-cinnamic acid and liberating ammonia which is quantitatively, measured uv-spectrophotometer at 280nm.

Estimation of proteins by Bradford method[11]

Preparation of Bradford reagent

0.05g of comassive brilliant blue G-250 was dissolved in30ml of 95% ethanol. 50ml of 85% phosphoric acid was added to this. When the dye was completely dissolved, the mixture was then diluted to 500ml with distilled water. Whatmann #1 filter paper was used to carry out filtration two to three times, just before use.

Preparation of stock solution using BSA (Bovine Serum Albumin)

50mg of BSA F-V was weighed and dissolved in 50ml of distilled water and this solution was made upto 100ml using a standard flask. 1ml of this solution equals 1mg of protein. This solution serves as stock solution.

Preparation of working standard

10ml of the stock was taken and further diluted to 100ml with distilled water using another standard flask. 1ml of this solution equals 100ug of protein. Hence, 0.1ml of this solution contained 10ug of protein. Standard solution ranging from 10ug to 100ug was prepared and the volumes made upto one tenth ml of the stock was taken and further diluted to 100ml using a standard flask.

Action of metal ions

In addition to the chemicals added during enzyme assay, different metal ion solutions obtained by serial dilutions were added in different aliquots to 1ml of the enzyme sample separately and the OD was read at 280 nm.

GC-MS analysis

The GC-MS analysis of methanol extract of*Lantana camara* was done to know the compounds present in this plant. The column oven temperature of 60^{o}C and injection temperature of 250^{o} C with split mode of injection was used to run the GC-MS. The pressure of 57.4kpa is applied to give the column flow of 1.00ml/min and linear velocity of 36.5 cm/Sec, with a purge flow of 3.0ml/min and split ratio is 10.0. Different parameters ion source temperature, interface temperatures were set at 2000^{0}C and 3000^{o}C respectively with 2.00min solvent cut time. The mass spectra taken in the intervals of 0.50Sec, with scan range of 40- 600 m/z and scan speed of 1250. The total time consumed is 34.00 min

Molecular docking

The structure of the enzyme phenylalanine ammonia lyase(PDB ID-1W27) was downloaded from PDB. Using chemsketchthe compound structure ofoctanoicacidwas drawn and converted to PDB by using openbabble (fig 3). The rotatable bonds of the ligand were analyzed. It was then saved in PDBQT format;the PAL protein was also converted to PDBQT formatand interaction between the receptor protein and ligand were analyzed by autodock software.

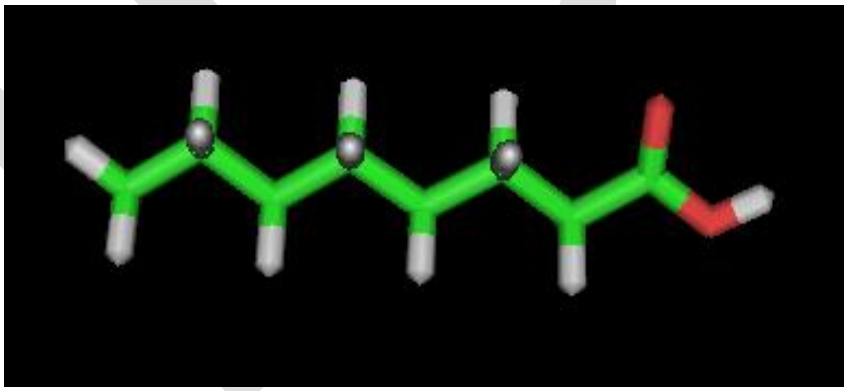


Fig 3:Structure of octanoic acid

Action of methanol extract of *Lantana camara on PAL*

In addition to the chemicals added during enzyme assay, different serial dilutions of extract of *Lantana camara* were added in different aliquots to 1ml of the enzyme sample separately and the OD was read at 280 nm

Results

The presence of PAL from *Ficusreligiosa* was confirmed by assay and concentration of PAL was found; shown in table 1

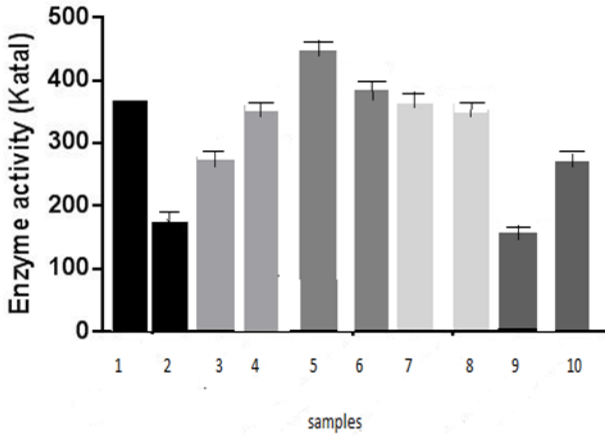
Table 1: Absorbance of the product (cinnamic acid) formed by PAL

STEP	VOLUME mL	TOTAL PROTEIN mg/mL	ACTIVITY *pkat/mL	TOTAL ACTIVITY pkat	SPECIFIC ACTIVITY pkat/mg protein	YEILD %	FOLD Purification
Enzyme ***Ficus religos***	40	0.34	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=pkat) enzyme.
Enzyme source: acetone powder from ***Ficus religos*** 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.**

Action of metal ions

The readings were recorded and compared with the crud enzyme assay. Zinc Sulphate showed highest activity among other metal ions snow in figure 4and considered as a potent activator of the enzyme



1	Crude
2	$NiSO_4.6H_2O$
3	$CuSO_4.5H_2O$
4	$Cacl_2$
5	ZnSO4
6	$(NH_4)_2SO_4$
7	$FeSO_4$
8	$K_4[Fe(CN)_6]$
9	$KMnO_4$
10	$CdCl_2$

Fig 4: Effects of different metal ions on enzyme activity was presented as mean ± SD. Among this $Znso_4$shows maximum activity

GC-MS analysis

The methanol extract of *Lantana camara* was subjected to GC-MS analysis and it shows 21 different compounds. The complete details of GC-MS chromatogram was shown in figure 5. The list of 21compounds along with the Retention time and the area was shown in the table 2

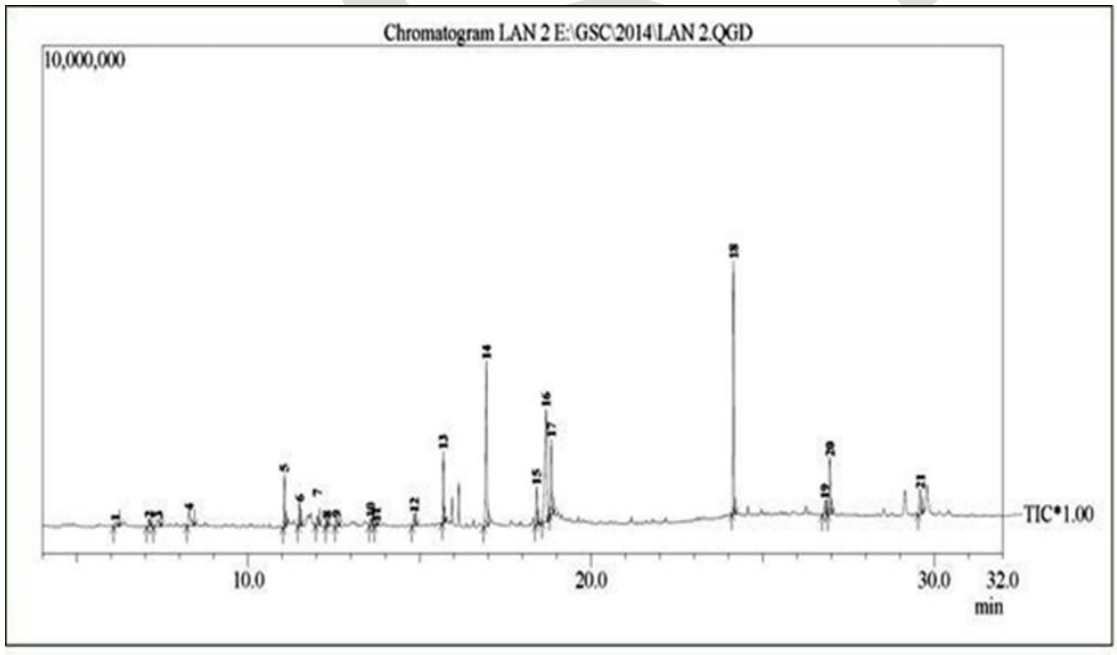


Fig 5: GC-MS chromatogram showing peaks at different time intervals.

Peak	R. Time	Area	Area%	Name
1	6.126	368490	0.72	Melamine
2	7.112	427678	0.83	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
3	7.373	861290	1.68	Octanoic acid
4	8.275	1424808	2.78	5-Oxymethylfurfurole
5	11.057	2161305	4.22	Caryophyllene
6	11.496	963621	1.88	Alpha.-Humulene
7	12.019	612669	1.20	(+)-Cycloisosativene
8	12.294	319390	0.62	Delta.-Cadinene
9	12.565	441992	0.86	Dodecanoic acid
10	13.570	413359	0.81	2-methyl-4-(2,6,6-trimethyl-cyclohex-1-enyl)-but-2-en-1-ol
11	13.734	159253	0.31	(+)-Aromadendrene
12	14.838	661695	1.29	Tetradecanoic acid
13	15.690	2598880	5.07	Neophytadiene
14	16.943	8709808	17.00	Hexadecanoic acid
15	18.409	1679953	3.28	trans-Phytol
16	18.685	10142781	19.80	cis-9-Octadecenal
17	18.833	3191193	6.23	Octadecanoic acid
18	24.147	9731916	19.00	Supraene
19	26.820	956067	0.87	Stigmast-5-en-3-ol, oleat
20	26.953	3467593	6.77	Alpha-tocopheryl-beta-d-mannosid
21	29.589	1927935	3.76	gamma.-Sitosterol

Table 2: List of compounds identified in methanol extract of *Lantana camara* during GC-MS analysis

Molecular docking analysis

All 21 different compoundsextract of *Lantanacamara* was subjected to docking with PAL receptor.Among 21 different compounds; octanoic acid showed maximum affinity shown in figure 6 and values in table 3.

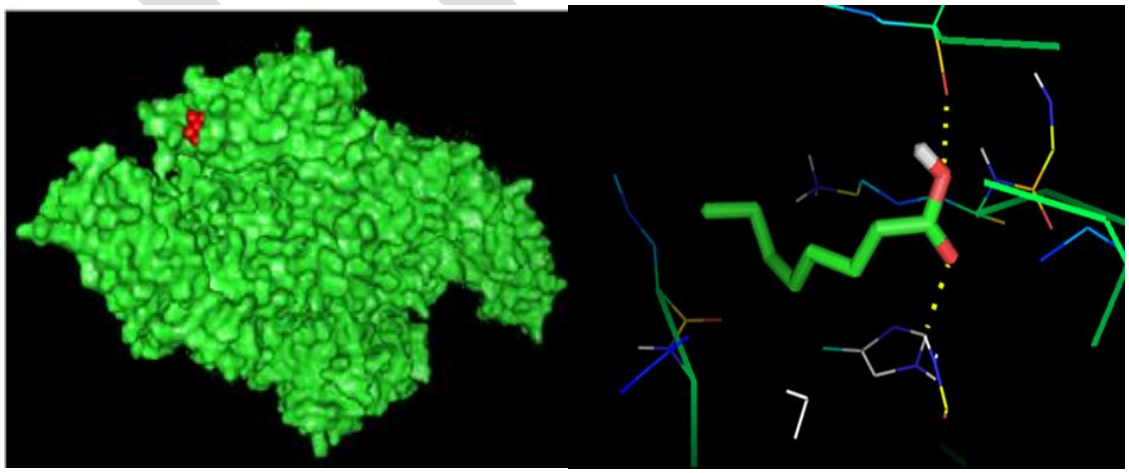


Fig 6:**The binding site of PAL with octanoic acid**

Mode	Affinity (kcal/mol)	Distance from best mode (rmsd l.b)	Distance from best mode (rmsd u.b.)
1	-3.4	0.000	0.000
2	-3.1	16.304	17.475
3	-3.1	16.911	18.292
4	-3.1	16.354	17.564
5	-3.1	19.446	20.076
6	-2.9	1.917	2.235
7	-2.9	22.125	23.317
8	-2.8	29.568	30.646
9	-2.8	16.909	17.570

Table 3:Represents the docking result of octanoicacid to PAL.

Analysis of Plantextract on PAL

Lantanacamara extract added to the assay has shown increasing in the activity of pal as show in fig 7.

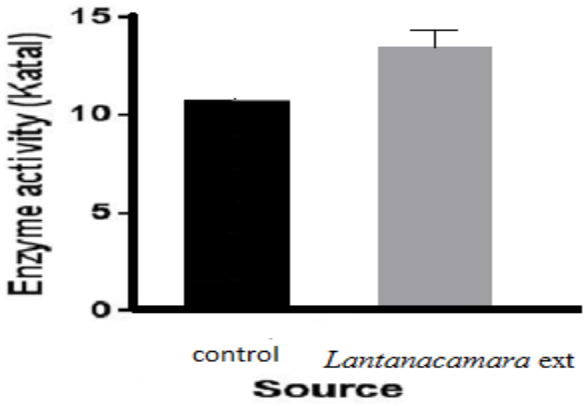


Fig 7: The enzyme activity of control and*Lantana camara*extract presented as mean ±SD.

Discussion

Positive results for the presence of the enzyme phenylalanine ammonia lyase (PAL) extracted from the plant *ficusreligosa* wasconfirmedby spectrophotometric analysis. Adding of metal ions to the crude enzyme;it was found an increase in the activity with addition of zinc sulphate. Thus, we could infer that zinc sulphateand methanol extracts of *Lantana camara*as a potent activators of the enzyme.

Conclusion

The treatment of phenylketonuria(PKU) is necessary to avoid death rate in newborns. The PALand its activators is one of the solutionsfor treating the PKU. This investigation successfully

screened the presence of PAL in *Ficusreligos* and potent PAL activator from the*Lantanacamara*extract and metal ions. As studies suggest, patients suffering from this disorder need to follow a strict diet. They are advised to consume diet which is devoid of phenylalanine[12]

Reference

1. James, William D.; Berger, Timothy G. Andrew. Diseases of the Skin: clinical Dermatology. Saunders Elsevier. ISBN 0-7216-2921-0 (2006)

2. Koukol and Conn .The journal of biochemistry, (1961)

3.Camm, Edith L; Towers, G.H.Neil.“Phenylalanine ammonia lyase”.Phytochemistry 12(5): 961-973 (1973)

4.Marusich W.C, Jensen R.A, Zamir L.O. Induction of L-phenylalanine ammonia lyaseduringutilization of phenyl alanine as a carbon or nitrogen source in *Rhodotorulaglutinis*,. J Bacteriol, 146: 1013-9 (1981)

5. Fritz, R.R; Hodgins, D.S; AbellC.W“Phenylalanine ammonia lyase. Induction and purification from yeast and clearance in mammals” The journal of biological chemistry 251(15): 4646-4650 (1976)

6.Tanaka, Y; Matsuoka M; Yamanoto N; Ohashi, Y; Kano-murakami, Y; Ozeki, Y “Structure and characterization of a cDNA clone for phenylalanine ammonia lyase from cut injured roots of sweet potato.” Plant Physiology 90(4): 1403-1407 (1989).

7.Appert, C; Logemann E; Hahlbrock K; Schmid J; Amrhein, N. “Structural and catalytic properties of the phenylalanine ammonia lyaseenzymes from parsley (Petroselinum crispumNym.).”European journal of biochemistry/ FEBS 225(1):491-499 (1994).

8.Sarkissian, Christineh N.; Gámez, Alejandra. "Phenylalanine ammonia lyase, enzyme substitution therapy for phenylketonuria, Molecular Genetics and Metabolism 86: 22–26 (2005).

9. Gonzalez, Jason; Willis, Monte S. "Ivar AsbjornFolling Discovered Phenylketonuria (PKU)". Lab medicine 41 (2): 118–119 (Feb 2010).

10.Fliano, James J. "Neurometabolic diseases in the newborn". Clinics in Perinatology 33 (2): 411–47910.1016.(31 May 2006).

11. Bradford, M M; AnalyticalBiochemistry.**72**. 248-254 (1976).

12. Robert Tomaino; MySpecialDiet, MetabolicConditions – Tyrosinemia.(2004)