Isolation, bioassay development and applications of tyrosine hydroxylase

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ABSTRACT

Objective: Isolation and purification of tyrosine hydroxylase. Isolated enzyme used for development of bioassay by using specific substrate L-tyrosine.

Materials and method: Tyrosine hydroxylase found in various tissues in animals. Enzyme isolated from caudate nuclei of sheep brain using various parameters like temperature, pH, Ionic strength etc. Enzymatic activity assessment protocol was developed based on specific substrate for tyrosine hydroxylase. The enzyme purity and concentration were determined by developing gel permeation chromatographic technique

Result: Maximum enzyme isolated at 37^0C using pH 5.8 buffer solution and 50% saturated ammonium sulphate solution. The enzyme was purified by using sephadex G 75 and assay performed by using L-tyrosine.

Conclusion: The isolated enzyme was used for development of bioassay design and tested for bioactivity assays of some of drugs for cardiovascular disorders.

Keywords- Tyrosine Hydroxylase, Bioassay, Gel permeation chromatography, cardiovascular diseases

INTRODUCTION

Cardiovascular diseases are still the major factor leading to death worldwide. Atherosclerotic lesion formation which leads to not only myocardial infarction but also stroke due to weaken endothelial function and proceeding with inflammation of the vessel. This may be caused due to increased level of blood pressure, diabetes, smoking, obesity, etc. Increased level of blood pressure leads to 13% CVD, tobacco resulting in 9% as well as diabetes leading to 6% deaths. Even lack of exercise and obesity leads to 6% and 5% fatalities. Major risk factor associated with coronary artery disease, heart failure, peripheral vascular disease, impaired vision, stroke and kidney disease is increased level of blood pressure.[1]

CVDs are expected to impose every domain of population regardless of age, economic status, gender, and locality. Newer drugs are being discovered and designed.[2] Research has identified many molecular targets playing role in controlling cardiovascular pathophysiology. They are guanylate cyclase stimulators which are soluble, tyrosine kinase inhibitors, EGF receptor blockers, Rho-kinase inhibitors, dopamine β hydroxylase, HIF hydroxylase,[3-4] 21-hydroxylase, 11 β hydroxylase, soluble epoxide hydroxylase,[5] tyrosine hydroxylase,[6] etc. Clinically, hydroxylases present a challenging but unique rewarding target for drug therapies. Hydroxylases are an important component of oxygen discerning proteins. Tyrosine hydroxylase structure shows tetramers with four identical subunits so it is called as homo tetramer. Each subunit is further divided into three domains. At the carboxyl end of the peptide chain, tetramerization is allowed by short alpha helix domain which is present there. The central 300 amino acids compose of a catalytic core, within which all the residues essential for catalysis are

present, along with a non-covalently bound iron atom.[7] Two histidine residues and one glutamate residue hold the iron in place. Thus, making it a non-iron-sulfur, non-heme iron-containing enzyme. ~150 amino acids amino terminal frame a regulatory domain, which controls the access substrates to the corresponding active site. In humans, it is thought to contain regulatory domain with four varying versions, and thus four versions of the enzyme, which rely on alternative splicing, though none of their structures have yet been properly determined. It has been put forward that this regulatory domain might be an intrinsically unstructured protein, which has no clearly defined tertiary structure, but also, there has been no evidence presented assisting this claim.[8] For catalyzing the transformation of L-tyrosine amino acid to L-3, 4-dihydroxyphenylalanine i.e. L-DOPA, tyrosine 3-monooxygenase or tyrosine hydroxylase is responsible.[9] It not only uses molecular oxygen, but also iron and tetrahydrobiopterin as cofactors. L-DOPA which is a precursor for dopamine, which is a precursor for important neurotransmitters, nor-epinephrine (nor-adrenaline) and epinephrine (adrenaline). In the catecholamine biosynthetic pathway, tyrosine hydroxylase is generally applicable as a rate limiting enzyme.[6]

MATERIALS AND METHODS

L- Tyrosine obtained from oxford lab reagent, Bovine serum Albumin (Sigma Aldrich), Triton-X-100 (S.D. lab), Sephadex G -75 (Sigma Aldrich), Hydroxylamine hydrochloride (Sigma Aldrich)

INSTRUMENT AND SOFTWARE SPECIFICATION

UV-Spectrophotometer (Jasco V-630, Japan), Ultra sonicator (Spectra lab Mumbai), Gel permeation chromatography (Waters 2414), V Life MDS 4.4(V Life sciences and technology Pvt. Ltd), Cold centrifuge (Remi)

EXPERIMENTAL:

ENZYME ISOLATION

The sheep brain was collected from slaughter house. Caudate nuclei were dissected and homogenized immediately in nine volumes of ice cold 0.25M dextrose solution at 0-4°C [10] Centrifugation of the homogenate was carried out for 15min in cold centrifuge machine at 5000 RPM and 4° C. The sediment was suspended in half the original volume of dextrose solution and re-centrifuged. Supernatants collected were combined. Enzyme was solubilized by treatment of 0.2% triton X-100 followed by centrifugation at 9000 RPM for 1.5 hours in cold centrifuge at 4°C. Supernatant collected was used for enzyme isolation at various pH conditions, temperature and ionic strength. From that parameter, one parameter was selected with greatest enzyme yield.[11-13]

ISOLATION OF ENZYME REGULATING PH, TEMPERATURE VALUES, IONIC STRENGTH

Tyrosine hydroxylase was isolated at various pH ranges such as 5.6, 5.8, 6.0, 6.2, and 6.4 respectively. During isolation, pH of dextrose solution was adjusted to above mentioned pH respectively with 0.1N HCl and 0.1N NaOH; the further enzyme isolation at that pH was carried out. Also, at various temperature ranges and ionic strengths such as 0°C, 25°C,37°C and 10%, 20%, 30%, 40%, 50%, and 60% respectively using ammonium sulphate. After studying the entire variables, one from each variable with maximum enzymatic activity was selected by performing enzymatic assay using specific substrate.

GROSS QUANTIFICATION OF TOTAL PROTEIN EXTRACTED

Gross quantification of total protein extracted was done by using Biuret method for total protein estimation. For estimating protein concentration, calibration of BSA i.e. bovine serum albumin was carried out. Calibration of bovine serum albumin was used to determine unknown concentration of protein in enzyme sample. BSA concentration 0 (blank), 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml were taken in different glass tubes.

BSA stock solution 10mg/ml was prepared using distilled water. From this stock solution, 0 ml, 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml were added respectively. 1 ml, 0.9 ml, 0.8 ml, 0.7 ml, 0.6 ml, 0.5 ml water was added respectively to above solutions. To each test tube, 2 ml of biuret reagent was added. All the samples were incubated at 25°C for 10min and then the absorbance was recorded at 542nm using UV visible spectrophotometer (Jasco V630).

ENRICHMENT OF ISOLATED PROTEIN

The enzyme tyrosine hydroxylase after centrifugation was not in purified form. It was necessary to carry out purification process to eliminate other enzymes other than target enzyme. Purification of the enzyme was carried out by dialysis, column chromatography process and ammonium sulphate precipitation. The supernatant was collected and stirred using magnetic stirrer continuously at 25°C and addition of saturated ammonium sulfate was slowly carried out to bring the final concentration to 10%, 20%, 30%, 40%, 50%, and 60% respectively. Stirring was stopped once final concentration was reached and the solution was kept overnight for precipitation. The solution was centrifuged at 2000 RPM for 10 minutes on the next day so that precipitate can settle down. Pellets were collected after discarding the supernatant which contains targeted precipitated protein. By ammonium sulfate precipitation, the targeted protein gets precipitated out by altering their solubility in presence of high salt concentration. This precipitated protein was loaded in dialysis bag having 25.4 mm diameter and 42.44 mm width. The dialysis bag was kept overnight against 0.2 M sodium acetate buffer with continuous stirring by maintaining 25°C temperature and 5.8 pH conditions. The dialysis buffer was replaced after every 2 hours with freshly prepared buffer. By dialysis using a membrane which is semi permeable membrane, unwanted and small compounds from macromolecules get removed from solution by passive as well as selective diffusion. In dialysis bag, the pure targeted protein was left out which was further purified by column chromatography.

ENRICHMENT OF PROTEIN BY COLUMN CHROMATOGRAPHY

Sephadex G-75 column was prepared by using 0.25M dextrose solution. It was equilibrated for 24 hours at 25°C. The above collected precipitate was re-suspended in sodium acetate buffer and passed through the column. The flow rate of 0.5ml/min was maintained. The eluent fractions were collected at 10 minutes interval each. The collected fractions were used for determination of enzyme by performing assay.[14]

CALIBRATION OF L-TYROSINE

Enzymatic activity assay protocol was developed on the basis of tyrosine hydroxylase specific substrate i.e. L-tyrosine. Enzymatic activity was determined indirectly by measuring the concentration of L-tyrosine. The Maximum wavelength of L-tyrosine having concentration 2mg/ml was determined in sucrose solution which was found to be 275nm. For determining unknown L-tyrosine concentration, calibration of L-tyrosine was plotted using 2,4,6,8,10 µg/ml concentration in 5.6 pH,5.8 pH,6.0 pH,6.2 pH,6.4 pH values.

ENZYMATIC ACTIVITY ASSAY

For determination of enzymatic activity and L-tyrosine content, the dialyzed enzyme and purified enzyme were taken by column chromatography using various parameters like pH of 5.6 ,5.8 ,6.0 ,6.2 ,6.4, at temperature conditions 0°C ,25°C,37°C and ionic strength of 10%, 20%, 30%, 40%, 50%, and 60% respectively. For preparation of sample, 200 µl enzyme + 200 µl L- tyrosine solution in sucrose (100 µg/ml) + 2.6 ml sucrose solution (0.25M) were taken. For blank sample, 200 µl enzyme + 200 µl sucrose solution (0.25M) + 2.6 ml

sucrose solution (0.25M) were taken. Using UV-visible spectrophotometer, the absorbance was recorded at 275 nm (Jasco V-630).

GEL PERMEATION CHROMATOGRAPHIC METHOD

GPC i.e. gel permeation chromatography is totally new method for detection of tyrosine hydroxylase. Enzyme runs were given at every step of purification like crude enzyme, dialyzed enzyme, purified fraction of enzyme having 15 min run time. 100µg/ml l-tyrosine was prepared in sucrose and incubated with crude enzyme , dialyzed enzyme, purified fraction of enzyme for 30 mins at 37°C and runs were given having runtime of 15min using Ultrahydrogel column (7.8 x 300 mm), refractive index detector and 0.1% sodium nitrate solution (w/v) mobile phase using HPLC grade water at 25°C -30°C column temperature. The flow rate was maintained to 1ml/minute throughout the analysis. The GPC chromatograph was analyzed by using BREEZE 2 software.

INSILICO MODELING AND INTERACTION ANALYSIS OF ENZYME AND LIGANDS

The 3-D coordinates in the X-ray crystal structure of tyrosine hydroxylase using PDB ID- 4J6S was selected as the receptor model in the docking simulations. Removal of water molecules and addition of hydrogen atoms was carried out to each protein atom. The incomplete residues in the tyrosine hydroxylase were completed and the possible ligand binding cavities present on PDB were identified using Biopredicta module. Docking library containing compounds which were selected with the drug-like filters. This selection is carried out only on the compounds with physicochemical properties of potential drug candidates and without reactive functional group(s). Ligands were designed and drawn using 2D draw on Vlife Molecular Design Suite 3.5 by using Vlife Engine module. The 2D structures were then converted to 3D and their geometry was optimized by energy minimization using MMFF force field till gradient of 0.001Kcal/mol was reached**.** By using Batch GRIP docking method of Bio predicta module, the designed molecules were docked into active site of tyrosine hydroxylase enzyme. After analyzing docking interactions, one with best interaction score was selected for synthesis.[15]

SYNTHESIS OF LIGAND

The synthesis was carried out using solution of 0.01 mol of aryl aldehyde in ethanol at room temperature and 10ml of 10% sodium hydroxide was added. The reaction mixture was further stirred. To this mixture 0.01 mol of the appropriate acetophenone was added dropwise with constant stirring. The stirring was continued until the final product precipitated. Filtration of the product was carried out followed by washing with water and recrystallization from ethanol to get the respective α, β-unsaturated carbonyl compounds. Characterization of synthesized product was carried out by determining melting point, chemical test results and IR interpretation.

COLOUR TEST FOR CARBONYL GROUP:

Procedure-

Reagent A- 5gm of Hydroxylamine hydrochloride was added in 1000 ml of 95% ethanol. To this solution 3 ml of Bogen universal indicator was added.

Then 0.5 to 1 ml of reagent A was added in test tube along with few crystals of compound A5B1. A change in colour from orange to red occurs which indicates presence of carbonyl group.

RESULT AND DISCUSSION

Calibration of BSA was carried out in distilled water. The absorbance was recorded at 542nm. BSA at various concentrations such as 1,2,3,4, and 5 mg/ml showed absorbance at 542nm whose absorbance values are 0.03591, 0.03598, 0.06391, 0.07788 and 0.1309 respectively. The coefficient of correlation of calibration curve was found to be 0.8832. The L-tyrosine maximum absorbance was found at 275nm. **(figure no.1).** The enzymatic assay was determined by using various pH, temperature, and ionic strength. Protein obtained from sample was estimated for various parameters like pH, temperature, and ionic strength**. (Table No. 1).**

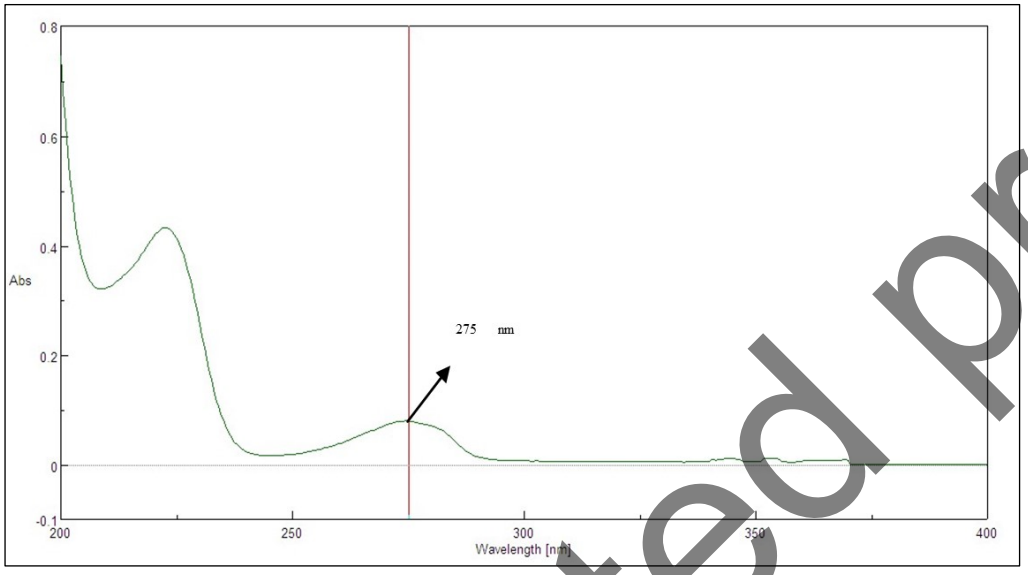


Figure 1. Maximum UV absorbance of L-Tyrosine

Table 1. Protein content at various parameters

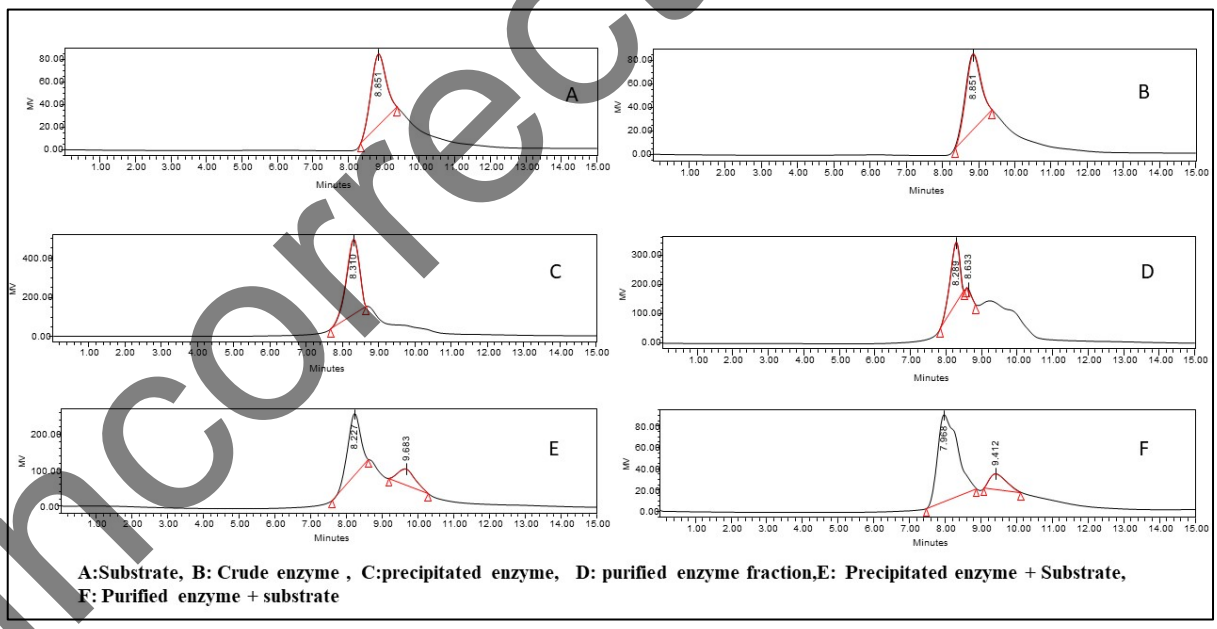
pH	Protein content	Ionic strength	Protein content	Temperature	Protein content
5.6	7.33	10%	4.32	0°C	1.27
5.8	9.15	20%	7.74	25°C	9.15
6.0	4.32	30%	4.83	37°C	19.96
6.2	5.34	40%	6.67	-	-
6.4	6.72	50%	9.16	-	-
-	-	60%	9.11	-	-

The maximum protein content was found at pH 5.8 at 37°C and 50 % Ammonium sulphate precipitation method. The maximum protein content of above parameters at various purification step like Ammonium sulphate precipitation, dialysis, column chromatography**. (Table No. 2).**

Table 2. Optimized parameters and L-tyrosine content at various purification step

Parameters	**Values**	**Protein content**	**L-tyrosine content (after dialysis)**	**L-tyrosine content (after column chromatography)**
pH	5.8	9.15	1.77	2.21
Temperature	37°C	19.96	3.89	2.19
Ionic strength	50%	9.16	2.100	1.25

The purified protein sample was determined by gel permeation chromatography. The Maximum amount of purified enzyme was found in third fraction of column chromatography. The purified enzyme showed highest peak i.e. Peak D height and area greater than crude enzyme and dialyzed enzyme peak B and C respectively. The interaction with L-tyrosine and dialyzed enzyme showed inhibition of enzyme by reducing the height and area of dialyzed enzyme shown in peak E. The interaction of L-tyrosine and purified enzyme showed area and height inhibition of purified enzyme shown in peak F. **(figure 2). (Table 3)**



A:Substrate, B: Crude enzyme , C:precipitated enzyme, D: purified enzyme fraction,E: Precipitated enzyme + Substrate, F: Purified enzyme + substrate

Figure 2. GPC chromatogram

Table 3. Interpretation of GPC chromatogram

Sample	**Retention time (Minutes)**	**Area**	**% Area**	**Height (mV)**

L-Tyrosine	8.677	3297561	100.00	102291
Crude enzyme	8.851	1850818	100.00	63554
Dialyzed enzyme	8.421	1895688	100.00	66825
Purified enzyme	8.289	3960529	98.87	208088
	8.633	45189	1.13	6286
Dialyzed enzyme+ L-tyrosine	9.683	1485447	26.07	44212
	8.227	4211777	73.93	170804
Purified enzyme+ L-Tyrosine	9.412	484894	13.53	14644
	7.968	3099989	86.4	81154

Amongst all designed molecules Ligand A5B1 showed highest negative docking score than others. Hence, it was selected for analyzing interactions and was synthesized further. From interaction analysis, it was observed that compound A5B1 showed the hydrogen bond interaction with amino acid residue LYS88 with distance 2.350,1.962 and 2.266 **(Table 4).** The hydrogen bonding was observed between O atom of compound and H atom of amino acid residue. Hence, this compound served as hydrogen bond acceptor. (**Figure no. 3)**

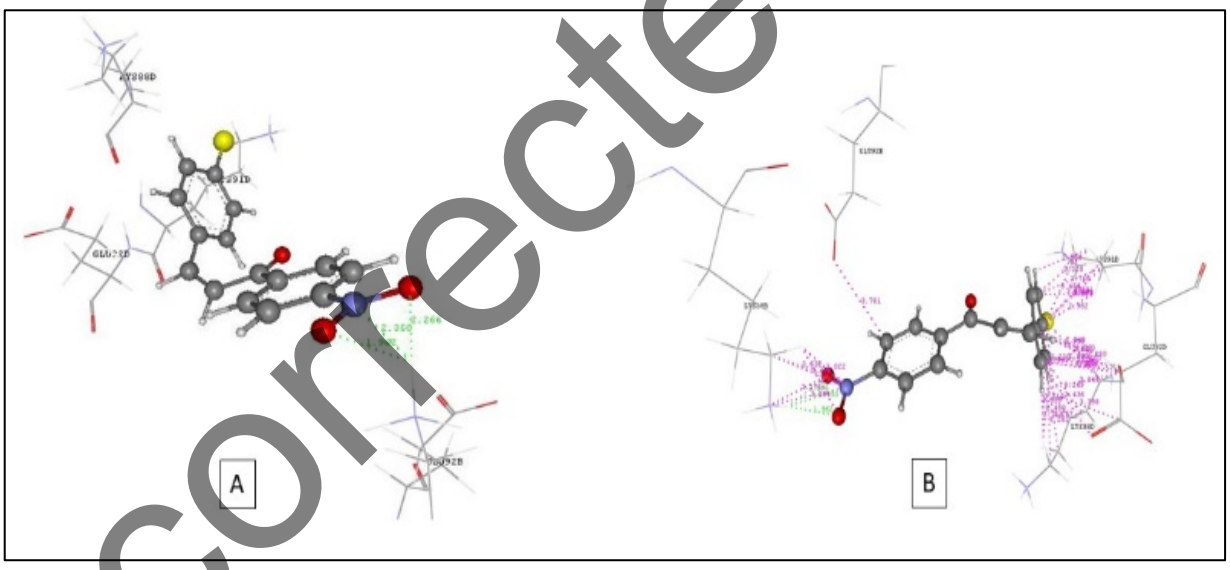
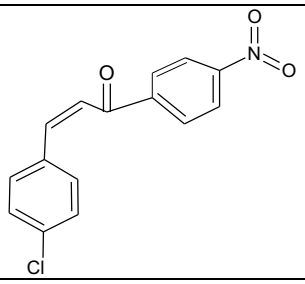
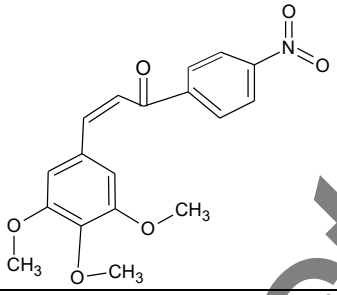
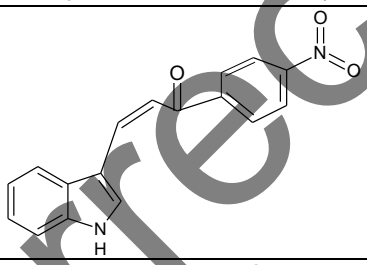
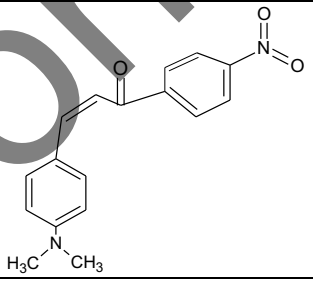


Figure 3. Docking interaction between Ligand and Enzyme

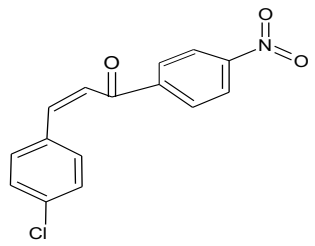
A: Hydrogen bond interaction, B: van derwaals interaction

Table 4: Interpretation of Insilico study

Sr. No.	Molecule code	Structure	Binding energy	Hydrogen bond	Distance
1	A5B1		-43.408876	LYS88 LYS88 LYS88	2.350 1.962 2.266
2	A5B2		-36.949678	LYS88 LYS88	2.460 2.335
3	A5B3		-40.046750	ARG56 ARG56 ARG56	2.012 2.947 2.899
4	A5B4		-39.596575	ARG56 LYS88	2.558 1.788

CHARACTERIZATION OF SYNTHESIZED LIGAND

Structure of A5B1 Ligand:



Molecular weight of A5B1: 287.70 mol/gm

Chemical test for carbonyl function in A5B1

Chemical test for carbonyl functional group was performed for synthesized ligand A5B1. The change in colour from orange to red confirmed the presence of carbonyl group in synthesized ligand.

IR spectra interpretation of A5B1 was carried out which confirmed presence of NH (3342 cm^{-1}), C-H (2857 cm^{-1}), C=O (1666 cm^{-1}), CH=CH (1542cm^{-1}), NO_2 (1564 cm^{-1}) in synthesized compound. **(Figure No. 4)**

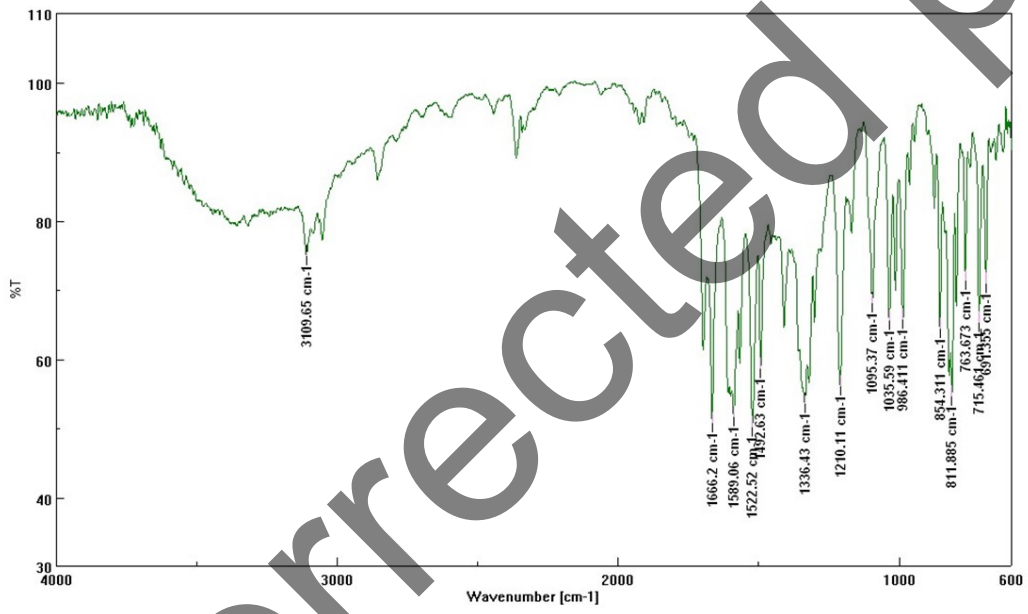


Figure 4. IR spectra of Ligand A5B1

CONCLUSION

The enzyme tyrosine hydroxylase was isolated efficiently from sheep brain caudate nuclei by regulating various parameters like pH, temperature and ionic strength. Enzymatic activity assessment protocol was developed on the basis of specific substrate for tyrosine hydroxylase. The developed GPC method has been proven to be effective method for tyrosine hydroxylase estimation. The method is economic, time saving, accurate and highly sensitive. The isolated enzyme can be effectively used for bioactivity assays of various drugs for cardiovascular disorders. Thus, the developed bioassay will limit the use of animals for experimental studies.

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Tables and Figures legends: