Isolation, Purification, Characterization of Tyrosinase from Button Mushroom

A
Dissertation Report
Submitted in the partial fulfilment of the Requirements
For the Award of the Degree of
Master in Science
In
Biotechnology

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Certificate

This is to certify that **Miss Himani Sethi** (MSc Biotechnology) from Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, has worked as a dissertation student under my supervision on project entitled **"Isolation, Purification, Characterization of Tyrosinase from Button Mushroom "**. She has actively carried out her work in my laboratory at Jamia Millia Islamia (Central University), New Delhi, from 2/01/2019 to 20/06/2019. The report has reached the requisite standards for submission to the best of my knowledge, understanding and belief.

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CERTIFICATE

This is to certify that the thesis entitled **"Isolation, Purification, Characterization of Tyrosinase from Button Mushroom"** being submitted by **Miss Himani Sethi (Roll No-301701011)** in partial fulfilment of the requirements for the award of the degree of Master in Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a Bonafede work carried out under the supervision of Dr. Manoj Baranwal and no part of the thesis has been submitted for the award of any other degree.

Dr. Manoj Baranwal (Internal Supervisor)

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DECLARATION

I, hereby declare that the project with entitled **" Isolation, Purification, Characterization of Tyrosinase from Button Mushroom"** in a partial fulfilment of the requirement for the award of degree of MSc (Biotechnology), Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work carried out during the period of January to June, 2019 under the guidance of Dr. Asimul Islam (Assistant Professor) at Jamia Milia Islamia (Central University) Delhi.

It is declared further that to the best of my knowledge, this work is an original contribution with existing knowledge and faithful record of research carried out by me.

I further declared that I have checked the thesis thoroughly and to the best of my knowledge it does not contain any plagiarized material.

Place Delhi

Date: 10-7-2019

Himani Sethi

Himani

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Himani Sethi

Abstract

A key enzyme Tyrosinase, responsible for melanin production in human, it is the black pigment of skin, eye and synthesis occurs in melanosomes present in melanocytes. *Agaricus bisporus* is the common edible mushroom from which tyrosinase has been extracted. Tyrosinase enzyme was purified by several techniques i.e. ammonium sulphate precipitation, dialysis followed by ion-exchange chromatography on DEAE cellulose. Fractions of protein obtained from ion-exchange chromatography were concentrated and then applied to gel filtration chromatography to get pure protein. The purity and molecular mass of protein was confirmed by SDS-PAGE. The enzyme was purified and give 3.59% yield. An enzyme activity assay performed which confirmed that the enzyme tyrosinase is present. Then purified protein was characterized by Circular Dichroism, Fluorescence spectroscopy. Secondary structure of protein was characterized by CD in the UV- far region and it indicated that it is a β-sheet containing protein. To estimate the stability of protein against heat and denaturing agent was monitored by CD and Fluorescence spectroscopy. Denaturation curve analysis gave values of 2.88±0.12 kcal mol^{-1} and 4.11±0.09M for $\Delta°G_D$ (Gibbs free energy change at 25°C) and C_m (midpoint of denaturation), respectively. GdmCl denaturation curve gives values which showed that the purified protein is stable.

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Introduction

Melanin Biosynthetic pathway is the pathway in which melanin pigment is formed. Tyrosinase enzyme is responsible for the production of melanin and plays a very significant role in melanogenesis. Tyrosinase has been isolated from number of sources like human, mammals, plants, fungi etc. Scientists were looking for a natural source from which tyrosinase has been isolated. As we know tyrosinase has been used in cosmetic industry as well in food industry. During the treatment of albinism, vitiligo like pigmentation disorders, tyrosinase enzyme plays a significant role. Browning of the fruits and vegetables, which is referred to as enzymatic browning is generally caused by tyrosinase and this takes place during the post harvesting period when any of them (fruit or vegetable) are damaged due to handling or senescence. Here, we choose button mushroom (*Agaricus bisporus*) to study the enzyme and its properties for human welfare.

There is wide variety of mushroom but we choose white button mushroom because it is easily available in market, cheap in cost, and also it has its nutritional values like high content of protein, carbohydrates, fibres and low content of fat present. Mushroom have been consumed by human in ancient times. There is a growing demand of mushrooms having therapeutic properties, in comparison to the conventional medicines as these medicines have a wide variety of side effects. Therefore, mushroom is considered to have great medicinal value from pre-historic times and a number of researchers has already performed detailed studies on its crude extract but there is no report available which highlights the stability factor of enzyme i.e. tyrosinase which is isolated from mushroom.

Taking these challenges into consideration we have started to purify protein by different chromatographic techniques. We have successfully isolated and purified the protein tyrosinase and do some biophysical characterisation to confirm the stability and structural factors of protein. Earlier, in our lab, scientists have purified number of proteins and performed detailed structure analysis and this study may provide essential basis for development and discovery of several modern therapeutics.

Review of Literature

2.1 Tyrosinase

Tyrosinase (EC 1.14.18.1) encoded as TYR in human. It is the rate limiting enzyme and is responsible for controlling the production of melanin [1]. It is a protein which is responsible for the pigmentation in human as it provides colour to the skin, hair, eye. Melanin synthesis is a two-step process, i.e. first step includes the hydroxylation of monophenol and the second step is completed when conversion of diphenol takes place [2]. This enzyme tyrosinase catalyzes production of melanin and other pigments from tyrosine by oxidation.

2.2 Structure of Tyrosinase

Tyrosinase is a ubiquitous enzyme and a part of type 3 copper enzyme family. The enzyme tyrosinase crystallized as a dimer in asymmetric unit. The two Copper (Cu) ions, acts as a major co-factor between the active site and these cofactors are eventually coordinated with six of the conserved histidine (His) residues. The copper ions linked with atmospheric oxygen catalysis two reactions which lead to the production of melanin, i.e. first one is o-hydroxylation of monophenols and second one is oxidation of di-phenols to o-quinones [3].

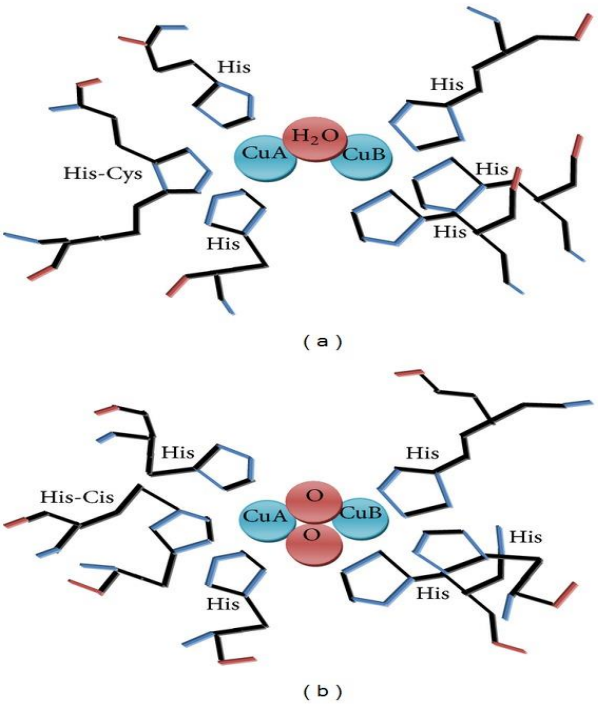


Figure 1: *Crystal Structure of Tyrosinase*

2.3 Human Tyrosinase

In homo-sapiens, tyrosinase is encoded by the gene TYR. This is a cuproenzyme, which is known to play an important function in the formation of pigments such as melanin and other phenolic compounds. Tyrosinase catalyzes steps in the melanin and catecholamine synthesis pathways. Formation of L-DOPA (L3,4 dihydroxyphenyl alanine) is also catalyzed by enzyme tyrosinase. Oxidation of monophenols and diphenols also catalysed by tyrosinase [4]. The molecular weight of human tyrosinase is approximately 60KDa. Human tyrosinase plays a significant role in study enzyme kinetics and screen for inhibitors. Below is crystal structure of human tyrosinase related protein 1.

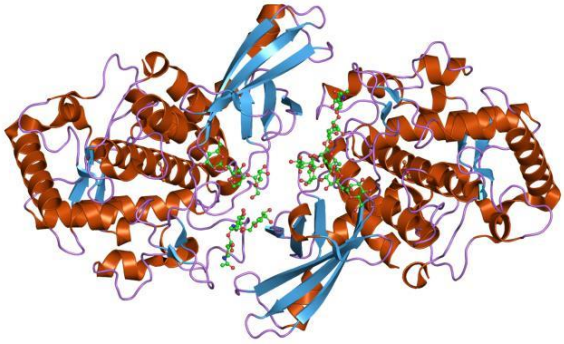


Figure 2: *Human Tyrosinase*

2.4 Mushroom Tyrosinase

In mushroom, Tyrosinase is encoded by PPO (polyphenol oxidase) and is mainly involved in synthesis or production of melanin. It is an oxidase. It has activity for both catechols and cresols reactions. It has been reported that tyrosinase consists of two different binding sites for the aromatic substrates with a different site for binding to oxygen copper. Tyrosinase also been used in a study to investigate the oculocutaneous albinism phenotype in a western population [5]. The molecular weight of mushroom tyrosinase is about 128 KDa. The pH range is about 6-7. The isoelectric point is 4.7-5.

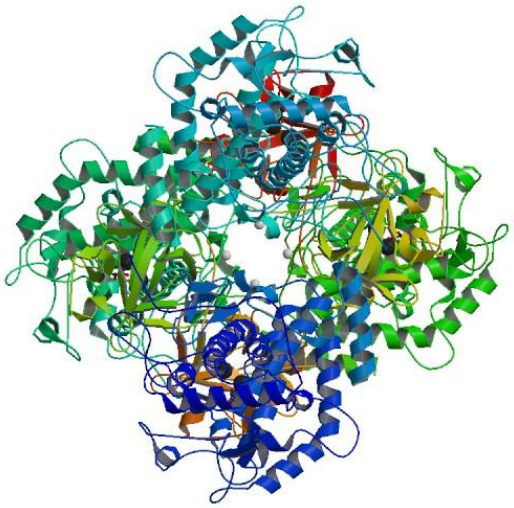


Figure 3*: Mushroom Tyrosinase*

2.5 Tyrosinase Sources

Tyrosinase could be generated from several organisms such as bacteria, fungi, mammals, plants and these sources for its extraction are studied for the specific function.

Sources	Species
Bacteria	Rhizobium Pseudomonas putida
Mammals	Homo sapiens (Humans) Mus musculus (Mouse) Rana esculanta (Frog)
Plants	Ipomoea batatas(Sweet Potato) Musa acuminate (Banana)
Fungi	Agaricus bisporous (Mushroom)

Table No. 1: *Sources of Tyrosinase from different sources*

2.6 Melanin Biosynthetic pathway

Melanogenesis is a process taking place in the specialized cells referred to as melanocytes, which are present within the membrane bound organelles called as melanosomes. The pathway of melanogenesis is shown in **(Figure 4.)** in which tyrosine is converted DOPA with the help of tyrosinase enzyme and this give rise to melanin pigment i.e. eumelanin (black and brown form), pheomelanin (yellow to red). It has been seen that the eumelanin has ability to convert light energy to heat energy while pheomelanin is a phototoxic pro-oxidant.

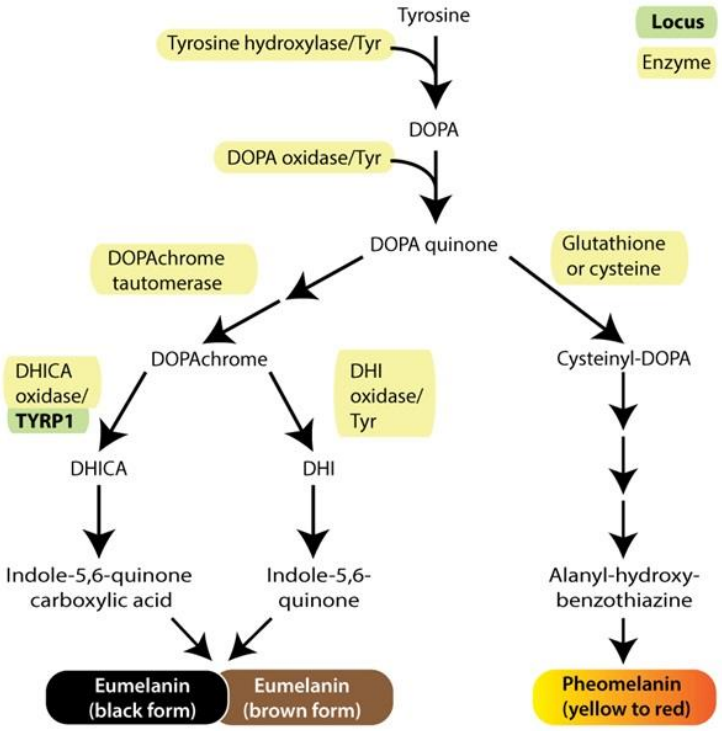


Figure 4: *Melanogenesis pathway*

2.7 Diseases Associated

Tyrosinase, a key enzyme plays a significant for curing diseases like vitiligo, albinism. Vitiligo can be called as depigmenting disorder, because of the presence of white patches on the skin [6]. White patches can be occurred anywhere on the body, like hands, legs, face etc. This disease can be of two types i.e. segmental and non-segmental vitiligo [7]. Segmental vitiligo begins in childhood and mainly occurs on face whereas non-segmental vitiligo can be beginning in childhood but onset is very common and occurs on chest, thighs etc. Vitiligo can be defined as a disease in which the melanocytes are destroyed from the body parts [8]. It is

likewise referred to be known as an autoimmune disorder. In this kind of the disorder the immune system of an individual misguidedly attacks its own body structure [9]. Several genetic factors involved in the cause of the vitiligo disease. It can be treated by using cosmetic supplements [10].

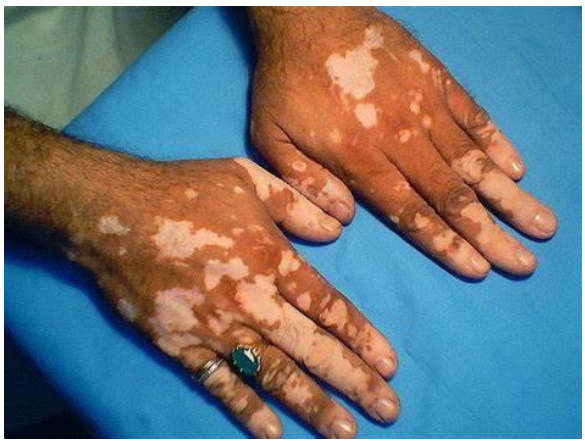


Figure 5: *An example of Vitiligo*

Albinism is a disease which is characterized by lower rate of melanin production. It is an inherited disease [11]. Albinism is generally caused by a result of mutation. People suffering from the disease albinism, tend to have lighter coloured skin and hair in respect to the other family members. [12].

There are different gene defects which help to characterize the numerous types of albinism. The different types of the albinism are as follows:

- **Oculocutaneous albinism (OCA)**

OCA is an abnormality disorder which affects the hair, skin etc

- **OCA1**

OCA1 is a subtype disorder which is mainly affected by absence of the enzyme tyrosinase. Two types of OCA1 are given below:

  - **OCA1a**: In OCA1a, melanin is completely absent. People with such subtype mutation have characteristics which include pale skin, white hair and light eyes [13].

- **OCA1b**: In the subtype OCA1b melanin is present but in very small amount. People with this subtype have light coloured eyes, hair, skin. Their colouring may increase as they age.

- **OCA2**

Individuals affected with OCA2 are born with light colouring and skin. Their hair colour could be yellow and light brown. OCA2 is commonly found in decent Africans and Native American [14].

- **OCA3**

It is a disorder of TYRP1 gene which is mutated. It involves people with a darker skin. Individuals having this type of disorder have reddish hair and hazel or brown eyes, reddish-brown skin.

- **OCA4**

Disorder in which protein SLC45A2 is defected. Melanin production takes place but in small amounts. [15]. It is very similar with OCA2.

- **X-Linked ocular albinism**

It occurs as a result of mutation, mutation in the X-chromosome of males can be called as X-linked ocular albinism. Mainly affects eyes of a person.

- **Chediak-Higashi syndrome**

It is caused by the mutation of the CHS1/LYST genes. The symptoms of this disorder are very similar to the above-mentioned OCA disorder expect the fact that their skin look slightly grey hair can appear silvery [16].

Figure 6: *An example of Albinism*

2.8 Mushroom

Mushrooms are a large group of organisms i.e. ***Agaricus bisporus*** plays a significant role in the environment. Mushroom consumed as a food product in daily life. Mushrooms has been found as a highly nutritional food stuff. In today's world, there is a wide variety of mushrooms available. In most of the countries, edible mushrooms are consumed as food. As there is variety of mushrooms available, it attracts people by their texture, aroma, taste etc. They are the source of essential nutrients, proteins, carbohydrate, vitamins. Medicinal mushrooms are broadly used for the treatment of several diseases such as cardiovascular diseases, cancer or oncology diseases and high blood pressure.

Figure 7: *Button Mushroom*

Objectives

1. **To isolate and purify the enzyme by different Chromatographic methods.**
2. **To check the activity of enzyme tyrosinase by UV-Spectrophotometer.**
3. **To characterise the enzyme tyrosinase by different biophysical methods.**

4.Materials and Method

Table No. 2: *Tabulated forms of all the materials used in experiment*

S. No	Materials Used	Source/Company
1.	Tyrosinase Enzyme	Mushroom
2.	Ethylene Diamine Tetraacetic acid (EDTA)	Merck (India).
3.	Phenyl methane sulfonyl fluoride (PMSF)	Sigma Aldrich Pvt Ltd. (USA)
4.	Dithiothreitol (DTT)	Sigma Aldrich Pvt Ltd. (USA)
5.	Polyphenolpyrollidine (PVP)	Sigma Aldrich Pvt Ltd. (USA)
6.	Tyrosine	Sigma Aldrich Pvt Ltd. (USA)
7.	Hi-Trap DEAE-Sepharose FF	GE Healthcare (USA)
8.	Supurdex 200pg	GE Healthcare (USA)
9.	Dialysis tubings	Sigma Aldrich Pvt Ltd. (USA)

4.1 Preparation of Buffers

Preparation of Tris Buffer

Tris is one of the most frequently buffer used in cell and molecular biology experiments. The Molecular weight of Tris is 121.14g/mol. It has a pKa value of 8.07 at 25^{o}C. Milli-Q-Water is being used in the preparation of buffer. The pH of the buffer is adjusted to 8.0 by addition of small amounts of HCL. The buffer solution can be stored at room temperature for several weeks.

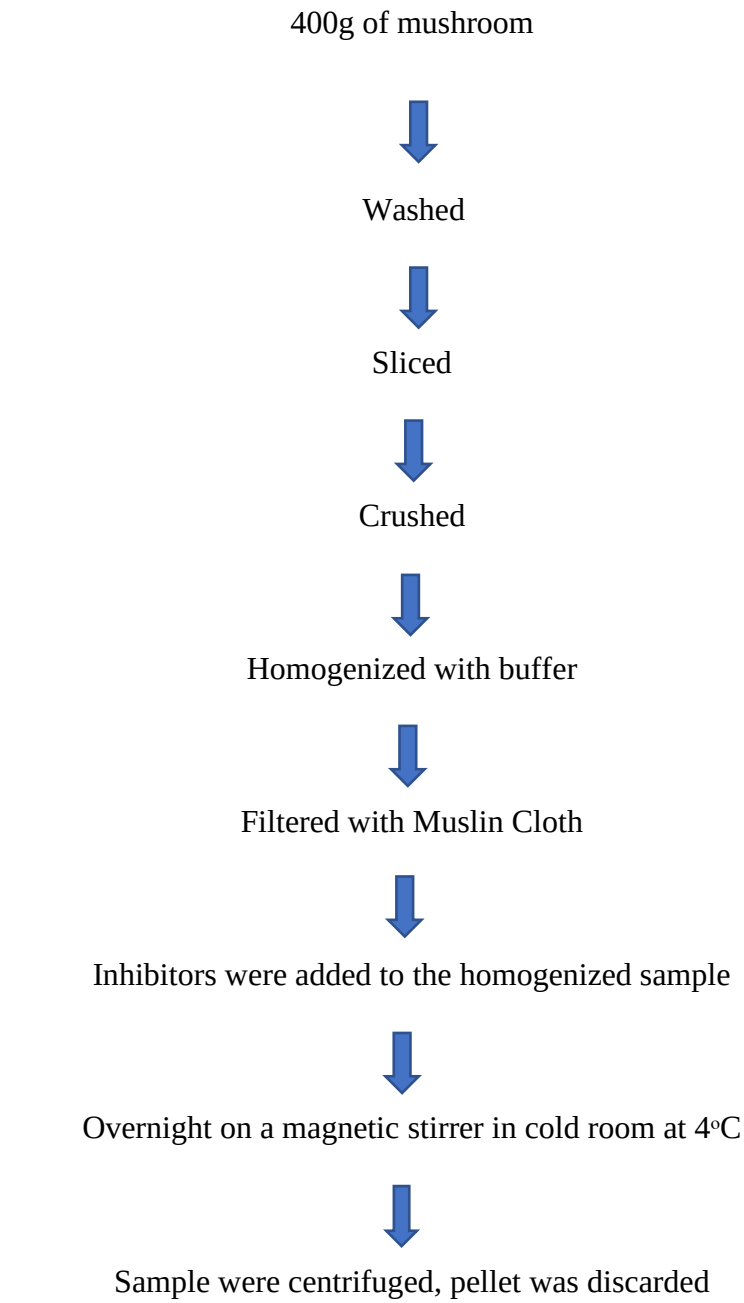
Preparation of Elution Buffer (Buffer B)

The molecular weight of NaCl is 58.44g/mol. Milli-Q-Water is being used in the preparation of buffer. The pH of buffer is adjusted to 8.0 by addition of Tris in small amounts. The buffer solution used in Ion-Exchange Chromatography for various purposes.

Preparation of Dialysis tubing

The dialysis tubes (5-6 inches) were essentially formulated according to the procedure recommended and followed by Dr. Mcphie. The tubing's were simmered in 50 % ethanol for one hour and then thoroughly washed with $NaHCO_3$and EDTA in the ratio 10:1 mM. The tubing's were soaked for 30-40 minutes at 60^{o}C. The tubing was then washed thoroughly with distilled water. This treatment removes all the impurities from the cellophane tubes which contain glycerin and plasticizers traces of sulphurous compounds and heavy metals. These were thoroughly washed with dialysate before placing protein solution in them. Since the wet tubing's were prone to attack by cellulolytic microorganism, they were stored at low temperature. To avoid alteration of pore size due to shrinkage, the tubing's were kept soaked in distilled water and stored at low temperature for further ruse.

4.2 Isolation of Tyrosinase



Mushrooms were taken, they were crushed in mixer grinder and homogenised with buffer and filtered with muslin cloth, inhibitors (DTT, PVP, EDTA) were added. Each purification step was conducted at 4°C. Homogenised sample centrifuged at 12000 rpm for 20 minutes and the supernatant was collected. It was subjected to ammonium sulphate precipitation. Supernatant retrieved after 60% of the saturation was collected and applied for further process.

4.3 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation is a tool to purify proteins. As it is cheap, water soluble, and is able to become much more hydrated than any other ionic solvent. It was done at 4°C by using finely grounded ammonium sulphate [17]. In this fractionation, different steps were carried out to achieve the best. Samples were centrifuged at 12000 rpm for 20 minutes [18].

Supernatant were subjected to 30%, 60%, 90%. It was placed on magnetic stirrer for several hours. After salting out the protein by salt, sample was again centrifuged at 12000 rpm for 20 minutes. Pellet of the different samples were collected and were than dissolved in 50mM Tris-HCL buffer maintaining a pH of 8.0. Post centrifugation, supernatant was collected and it was than precipitated with an increase in saturation by 30% to 60% by adding ammonium sulphate salt (to salt out the protein).

4.4 Dialysis

The Dialysis tubing were formulated with reference to procedure which was recommended by Dr. Mcphie. The molecular weight cut-off range for dialysis membrane is about 5000-8000Da.The concentration of the buffer was maintained (50mM tris with pH 8.0).

The Dialysis membrane is than methodically wash away with water (distilled) just before pouring the solution of protein into the membrane. To achieve complete desalting, protein sample dialyzed with no. of times exchange of buffer. [19].

4.5 Tyrosinase Activity

A number of tyrosinase assays has been performed. Different methods based on electrochemical, oximetric, spectrophotometric and several other techniques have come up to be employed for this purpose [20]. Spectrophotometric techniques are widely used because they are convenient, inexpensive and allow a sequence of reactions that could be studied continuously [21]. The enzyme assay performed spectrophotometrically at 25°C with absorbance of 280nm of wavelength [22]. We prepared samples, control in which enzyme is not present, blank in which substrate is absent and samples in which both the protein solution and substrate is present [23]. Thus, we analysed one by one which is more prominent and in which sample the colour changed [24]. Below is the reaction given. In the given **Figure 8**, Tyrosinase enzyme reaction shown, in which monophenol is converted into quinone.

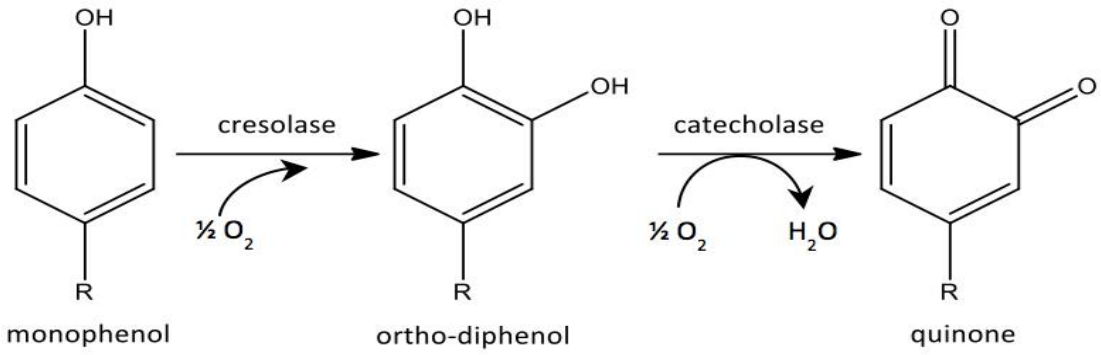


Figure 8: *Tyrosinase reaction*

4.6 Ion-Exchange Chromatography

The most prominent method for the purification of proteins and other charged molecules is ion-exchange method. There are two types of chromatography performed i.e. cationic and anionic chromatography [25]. In this, we choose anion exchange chromatography because of the pI (isoelectric point) of the sample. Dialyzed sample followed by ammonium sulphate precipitation and dialysis are applied to weak anion DEAE sepharose column. This was pre-equilibrated with 50mM Tris-HCL buffer with a flow rate of 0.5ml/min. The unbound proteins were wash away from buffer until the absorbance of proteins ranges to zero. The proteins bound to column were eluted with a gradient of 0 to 1.0 M NaCl.

4.7 Gel filtration chromatography

Gel Filtration chromatography, a type of size exclusion chromatography, separates protein based on their molecular size. It can be used to separate compounds such as small molecules, protein, protein complexes, nucleic acids. By using a porous matrix, protein separation is achieved, as molecules have different degree of access. Molecules which are smaller in size have huge access whereas molecules which are large in size are left out from porous matrix. Therefore, proteins are eluted in the decreasing order of size from the column [26].

After Ion-exchange chromatography, size exclusion chromatography performed, protein sample injected to superdex 200 pg column which is pre-equilibrated with Tris-HCL buffer through flow rate of about 0.5mL/min. Unicorn manager was employed for the analysis of elution profile at 280nm of absorbance. Protein Fractions eluted from column and they were analysed separately to obtain the pure protein

4.8 SDS-PAGE

Sodium do-decyl sulphate polyacrylamide gel electrophoresis is an analytical method for the separation of charged molecules based upon their molecular weight in an applied electric field. After purification by ion-exchange method, SDS performed to confirm the purity and molecular mass of protein. The PAGE system consists of two gel stacks i.e. the lower one and the upper one. The lower one is resolving gel and the other one is stacking gel. The only difference between these two gel stacks is the composition of gels. The composition of resolving gel is about 12% and stacking gel is about 5%. About 20µg of protein sample was loaded in each well. Along with sample loading dye also loaded which contains Glycerol, DTT, BPB. At constant current of 50mA and 100volts, the gel was run. Coomassie brilliant blue (G-250), was used as a staining dye. Protein ladder of size 10KDa to 180KDa was also run for the conformation of molecular weight.

Table No. 3: *Composition of Resolving Gel (12%)*

S.No.	**Reagents**	**Volume (For 10ml)**
1.	Distilled Water	3.2 ml
2.	Acrylamide/Bis acrylamide (30%)	4.8 ml
3.	1.5M Tris-HCL (pH8.8)	2.95 ml
4.	10% SDS	100µl
5.	10% Ammonium persulfate	100µl
6.	TEMED	10 µl

Table No. 4: *Composition of Stacking Gel (5%)*

S.No	Reagents	Volume For (4ml)
1.	Distilled Water	2.7 ml
2.	Acrylamide/Bis-acrylamide (30%)	670µl
3.	0.5M Tris-HCL (pH6.8)	500µl
4.	10% SDS	40µl
5.	10% Ammonium persulfate	50µl
6.	TEMED	5µl

4.9 UV/Vis Spectrophotometer

Ultraviolet-visible spectrophotometry is basically referred to the absorption spectroscopy in which, lights used in near and adjacent ranges. It is one of the quantitative methods used for the determination of organic compound and transition metal ion. The spectral region is in between 340-240nm. The cuvette used for UV is 1cm of path length.

4.10 Fluorescence Spectroscopy

Fluorescence spectrometry is one of those technique which is fast, simple and inexpensive. It is used to determine the concentration of the analyte in the solution based on its fluorescent properties. Fluorescence is mainly used for measuring compounds in solution. It measures the emission energy when, after excitation, an electron returns back to the ground state from the first excited state. Proteins consisting of aromatic amino acids residues (phenylalanine, tryptophan, tyrosine), when excited at 250-300 nm, emit fluorescence in the range of 300-400nm, with intensity order: Trp >> Tyr >> Phe. Since the excitation and emission spectra of these residues are dependent on their respective surroundings, they are good probes for investigating protein's local environment and providing localized information on its structure.

Fluorescence reveal information on microenvironment, influenced by folding of proteins. Tryptophan residue will fluoresce at maximum 325-330 nm range while other fluoresce 290-320 nm range. The advantage of fluorescence spectroscopy is its greater sensitivity when compared to other spectroscopic techniques.

4.11 Circular Dichroism (CD)

Circular Dichroism studies are generally done to characterize the secondary structure of the protein. For the study of chiral compounds of varying size and type, CD is extensively used. In addition, Far-UV CD studies are done in Jasco Spectropolarimeter (model J-715) equipped with Peltier-type temperature controller (PTC-348 WI) and is interfaced with personal computer. It is frequently used to study macromolecules structure. Secondary structure of protein is sensitive to pH, temperature, environmental conditions therefore CD is used to analyse the change in protein secondary structure with respect to environmental factors or when the protein molecules interact with each other. The range of spectra for Far-UV is between 190-250nm. Cuvette size used for CD is normally equal to 0.1cm of path length.CD instrument is calibrated initially with d-camphor sulphonic acid. The data and the spectra of the native protein was stored and the study was carried out using the software J700, provided by Jasco. Using the relation: $\mathbf{[\theta]_\lambda = M_0\theta_\lambda / 10\ lc}$, where,

$\mathbf{[\theta_\lambda]}$ - stands for the ellipticity in millidegrees at wavelength($\boldsymbol{\lambda}$),

$\mathbf{M_0}$- stands for the mean residue weight of the protein,

c- stands for the protein concentration(gm/cm^3) and

l – stands for the path length of the cell (cm).

4.12Sample preparation for GdmCl denaturation

The solution of Protein Stock was prepared and then filtered by means of 0.22 µm (Millipore) filter. With the help of Molar absorption coefficient, protein concentration was determined. By the help of refractive index method, GdmCl stock concentration was determined. However, the samples for optical measurements were made in Tris HCl buffer(50mM)

5. Results and Discussion

The isolation of tyrosinase from mushroom is somewhat difficult due to the presence of small amount of tissue present in mushroom taken and higher amount of melanin in these tissues. Having a great amount of melanin present in the tissues plays a very significant in cosmetic industry for treatment of hyperpigmentation diseases. This is the reason people were looking for natural source for the diseases associated with melanin's.

5.1 Purification of Enzyme Tyrosinase

The pellet obtained after 60% precipitation by ammonium sulphate salt, then after dialysis, the sample was poured into ion exchange chromatography. In **figure 9**, The proteins which were bound to the column were eluted into different fractions (four peaks in chromatogram)**.** Whereas, unbound proteins were eluted out of the column and the bound proteins which were eluted showed the purity of given protein (blue line). In second line, there is a gradient of NaCl (0-100%) buffer B, with concentration 1.0 M NaCl in 50 mM Tris buffer in addition to the buffer A which is a tris buffer(50mM), (green line).

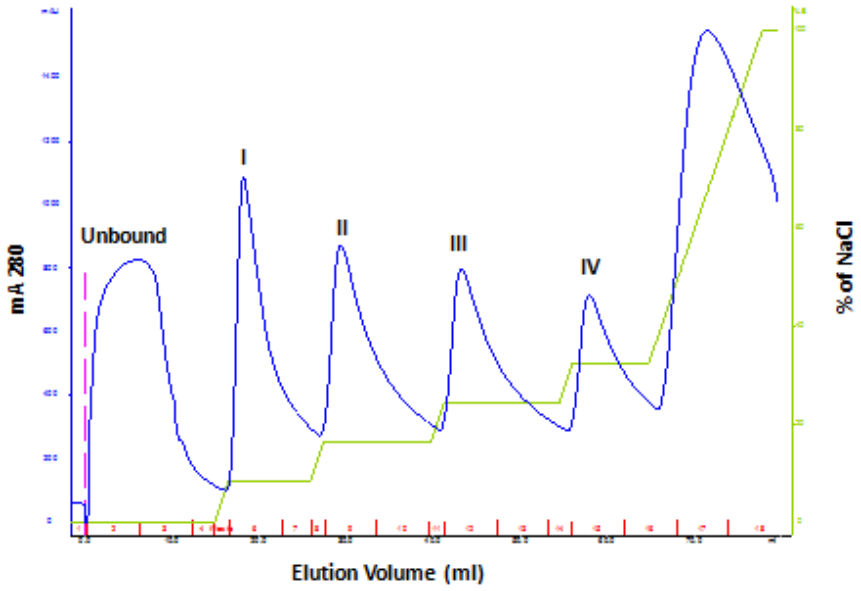


Figure 9: *Showing the elution profile of anion exchange chromatography using DEAE weak anion exchanger Hi-Trap CM-Sepharose.*

This fraction after anion-exchange chromatography, was concentrated and collected and applied on the gel filtration column. Hence, I observed two fractions of eluted proteins from the column of gel filtration **(Figure 10).** Proteins that were larger in size eluted out at the first position due to the fact that these molecules do not have a to pass through pores, whereas

small molecules get easily eluted out of the column. Protein eluted with the flow rate of 0.5 ml/min.

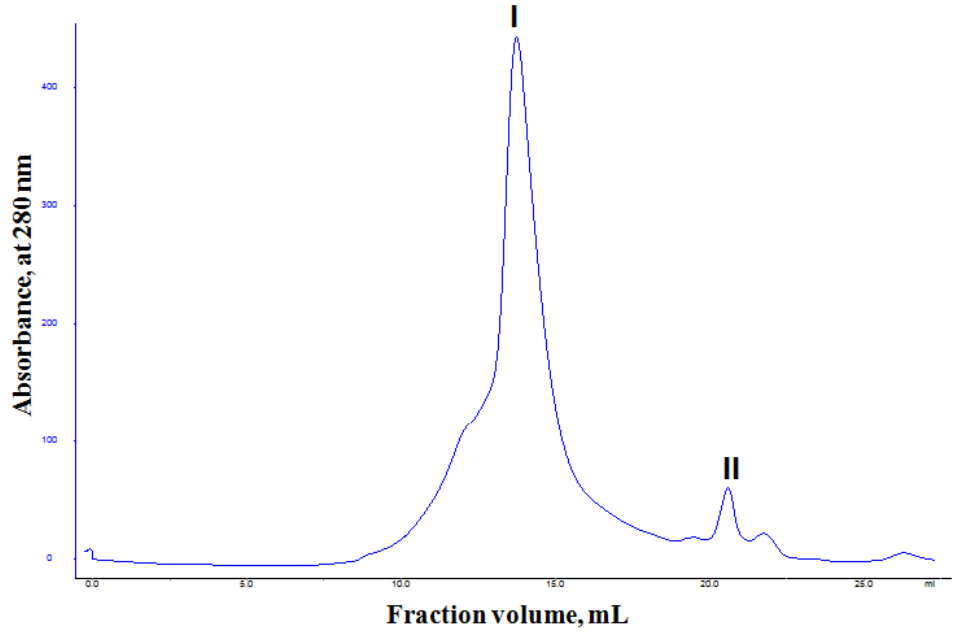


Figure 10: *Gel filtration chromatography-elution profile using superdexpg 200 column*

After chromatography, protein sample loaded on the SDS-PAGE on confirm the purity and molecular mass. Protein ladder (10-180 kDa) shown in Lane I, loading sample is in lane II 60% ppt. sample (crude sample), lane III is first peak of anion exchange chromatogram, lane IV is the peak obtained after size permeation chromatography indicating purified protein of 50 kDa size.

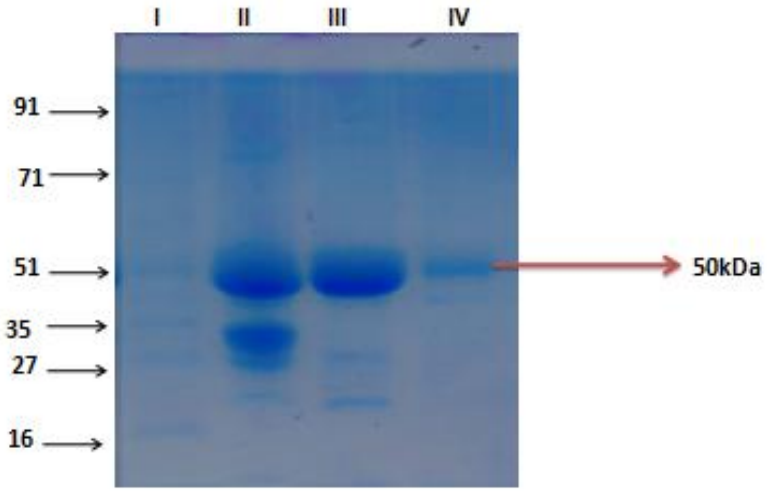


Figure 11: *Profile of SDS-PAGE.*

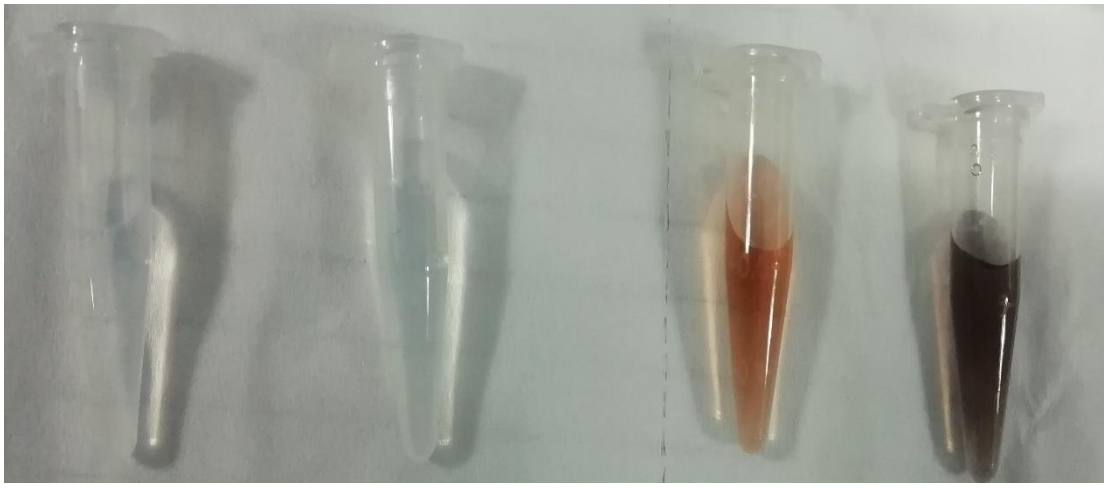
SDS-PAGE of each fraction is illustrated in the **figure 11.** The peak 1 of GF displayed a only a single band of 50 kDa (lane IV) which depicts the total purity of protein. As shown in the **figure 11**. By using Bradford protein estimation assay, amount of protein was calculated at each step. Summary is shown in **table no. 5**. Table shown the amount of protein obtained at each step, protein concentration and protein percentage yield.

Table No 5: *Summary of purification of Tyrosinase protein*

S. No	Step of purification	Volume of protein sample (ml)	Protein concentration (mg/ ml)	Total amount of protein (mg)	Percentage yield
1	Sample extract	810	1.65	1336	100
2	Ammonium sulphate precipitation (60% cut-off)	80	0.72	88	6.62
3	Ion-exchange chromatography	180	0.35	63	4.71
4	Gel-filtration chromatography	150	0.32	48	3.59

5.2 Enzyme Activity Assay

In this assay L-Tyrosine is converted into L- DOPA (Red Coloured Compound) in the presence of enzyme Tyrosinase. **Figure 12** represents the enzyme reaction in which, (A) represents Blank (B) Control (in which enzyme is absent) (C) Sample in which substrate and enzyme both present, as sample is heated up to 10-12 min at 25^{o}C (D) Sample is heated up to 1 hour at 25^{o}C. In **Figure12**, C shows that enzyme tyrosinase is present in it.



(A) (B) (C) (D)

Figure 12:*Enzyme reaction*

5.3 Biophysical Characterization of Tyrosinase

Characterization of Structure

Purified content of the enzyme Tyrosinase, was used to screen its secondary structure content using CD. However, Far-UV CD spectrum of Tyrosinase provides information regarding its secondary structure at 25^{o}C pH 8.0. **Figure13** shows spectra of Tyrosinase which shows a negative peak at 218 nm, and represent the presence of β-sheet structure. K2D2 software used for the analysis of secondary structure of protein. The α-helix content in Tyrosinase was about 10%. Whereas, in β-stand the amount of Tyrosinase was 43%. Hence, indicated that our Tyrosinase is mainly β-stand containing protein.

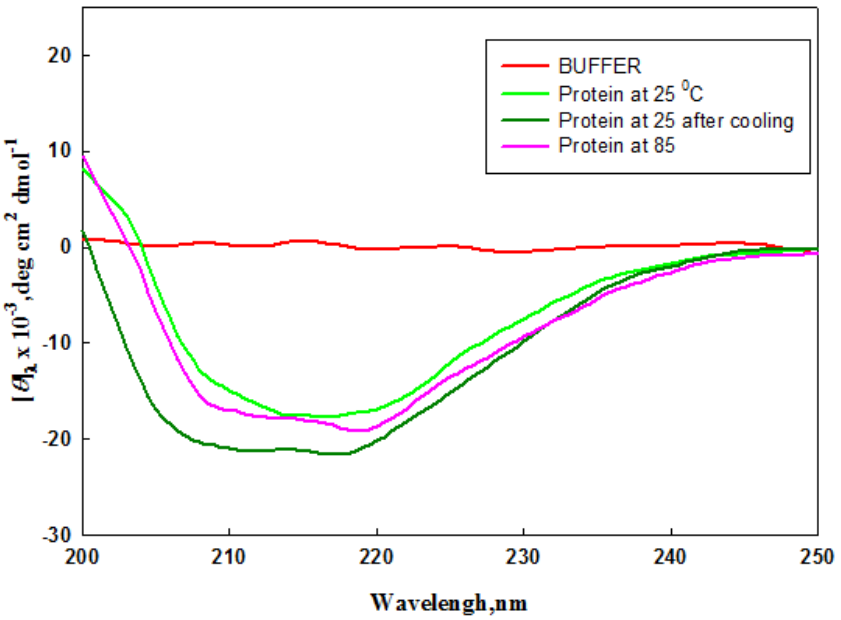


Figure 13*: Tyrosinase in Far-UV CD spectra at 25°C and 85°C at pH 8.0, representing thermal stability of protein.*

Likewise, to observe the consequence of temperature, protein sample was heated upto 85°C and we noticed that Tyrosinase was not denatured even at 90°C temperature. We also checked the reversibility of tyrosinase but we found out that the native protein spectrum was not retained after heating followed by its cooling again to 25°C, instead we have noticed little increase in the CD signal or increase in the secondary structure in Tyrosinase after cooling **Figure 13**.

This observation suggested that our Tyrosinase obtained from *Agaricus bisporus* was thermally stable.

Thermal-induced denaturation of Tyrosinase:

We have performed thermal-induced denaturation of Tyrosinase in two different probes that is UV-visible spectrophotometer as well as in circular dichroism (CD), with temperature varies from 20°C to 85°C. We have not observed any transition, while heating the Tyrosinase i.e. during heating there was no noticeable change in the absorption in UV-visible spectrometer at 280nm, (**Figure 14**) as well as no significant change in the CD signal in case

of circular dichroism performed at 222 nm (**Figure 15**). Thus, signifying the thermostability of Tyrosinase even upto very high 90^{o} C temperature.

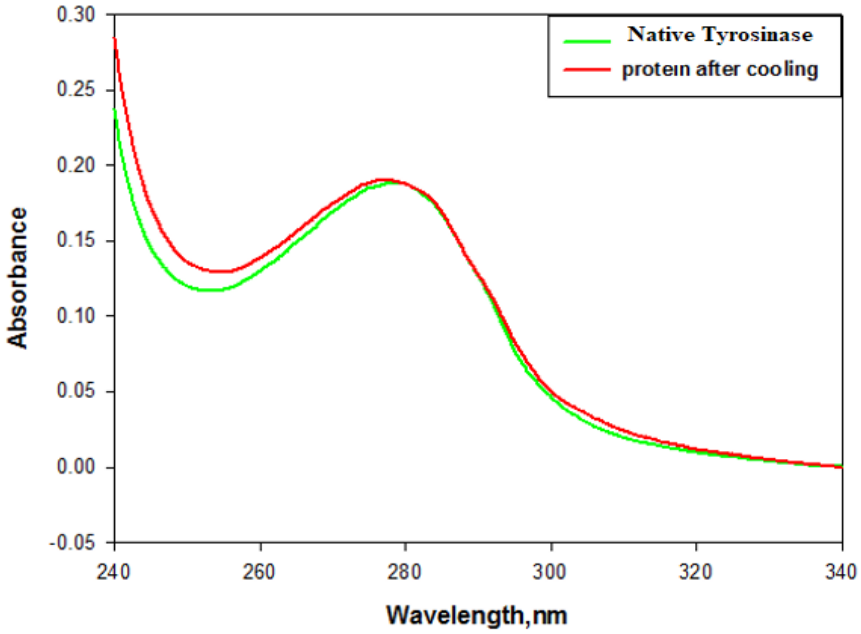


Figure 14: *UV-vis spectra of Tyrosinase before & after thermal.*

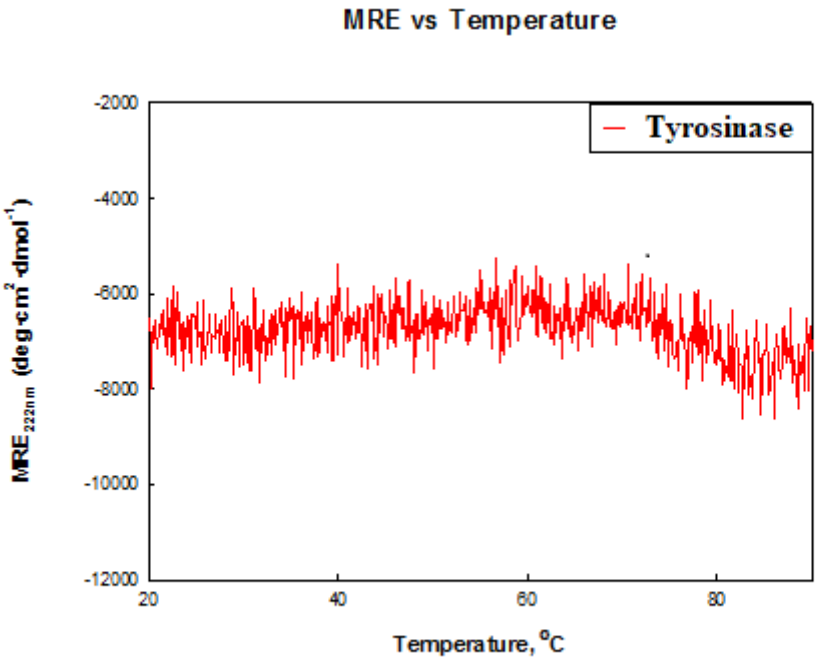


Figure 15: *Thermal-induced denaturation Tyrosinase, monitored by CD.*

We also have monitored thermal-induced denaturation of Tyrosinase in UV-visible spectrometry as a probe. In this case also, no significant change in the absorbance at 280 nm as well as in molar extinction coefficient was observed, while heating the protein even upto90^{o}C (**Figure16)**.

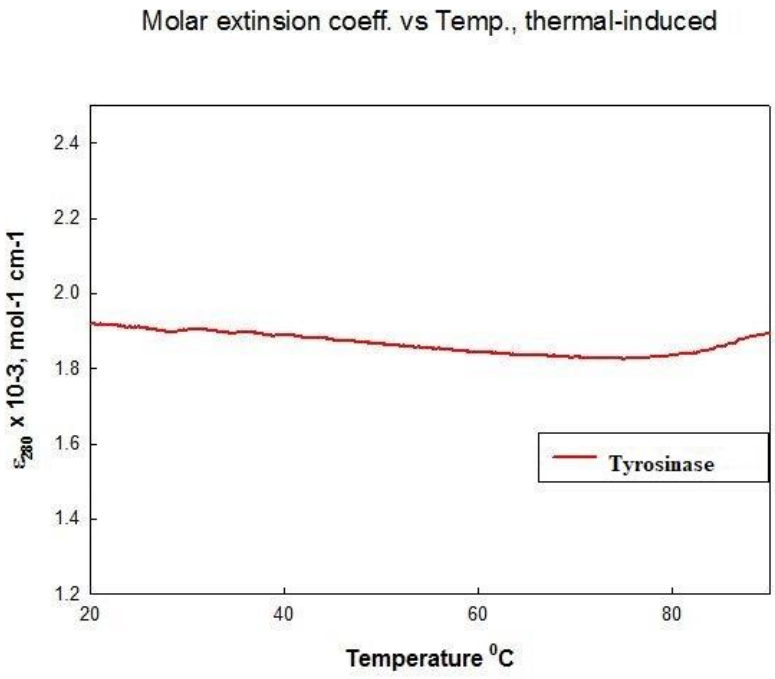


Figure 16: *Thermal-induced denaturation of Tyrosinase, monitored by UV*

5.4 GdmCl-induced denaturation examined by CD

To study the effect of denaturant (GdmCl) on the structural stability of tyrosinase, we did the denaturation experiment which is examined by CD.

$y(g) = y_N(g) + y_D(g)*\text{Exp}[-(\Delta G_D^o + m[g])/RT]/(1+\text{Exp}[-(\Delta G_D^o + m[g])/RT])$ - Equation 1

where, ***y*(g)** stands for the observed optical property,

$y_N(g)$ and $y_D(g)$ stands for the optical properties of the native and denatured protein molecules respectively,

ΔG_D^o stands for the Gibbs free energy change,

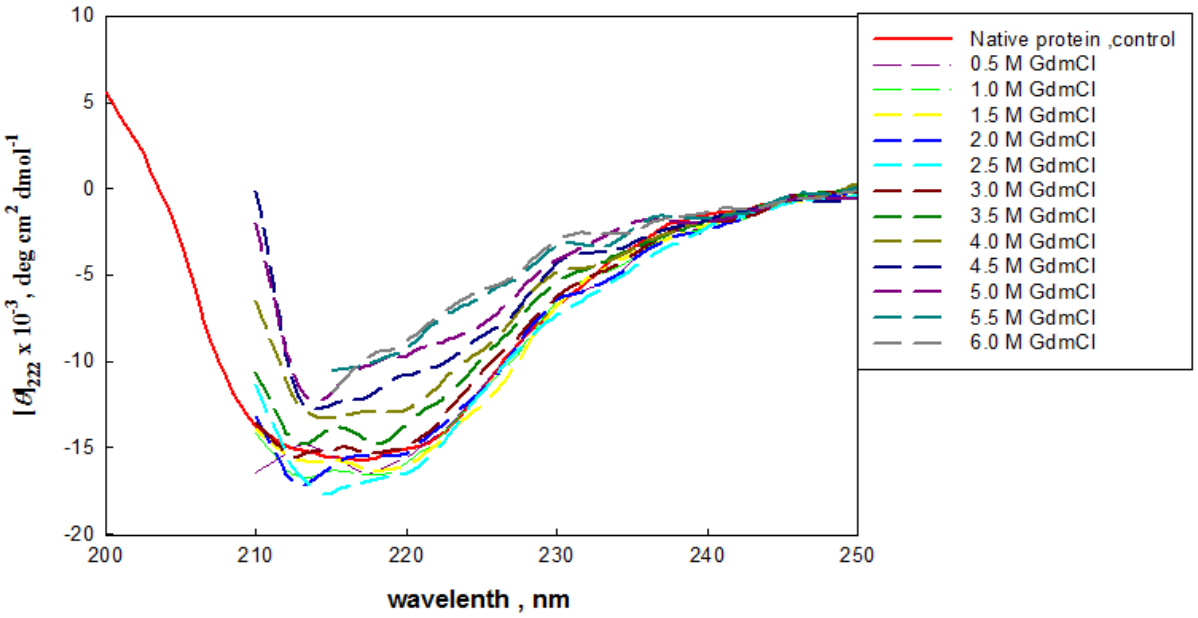
m represents the slope ($\partial\Delta G_D/\partial[g]$),

R stands for the universal gas constant, and

T stands for the temperature in Kelvin.

From (equation1), it is assumed, that a plot of ΔG_D versus [GdmCl] is linear, and also the dependencies of y_N and y_D on [GdmCl] are also linear i.e., $y_N(g) = a_N + a_N[g]$, and $y_D(g) = a_D + b_D[g]$, where *a* and *b* stands for the [g]-independent parameters, The denaturation curve gave values of ΔG_D^o, *m* and C_m (= $\Delta G_D^0/m$). The values are shown in **Table 6**. The calculated values of C_m and ΔG_D^o are 4.11±0.09 M and 2.88±0.12 kcal mol^{-1} respectively. This indicates that the Tyrosinase is highly stable towards denaturant. The value of *m* (slope) measures the cooperativity of denaturation. The unfolding pattern was accessed by change in the MRE (mean residual ellipticity). In **Figure 17**, we noticed that a significant decrease in dichroic signal seen on increasing the denaturant concentration.

(A)



(B)

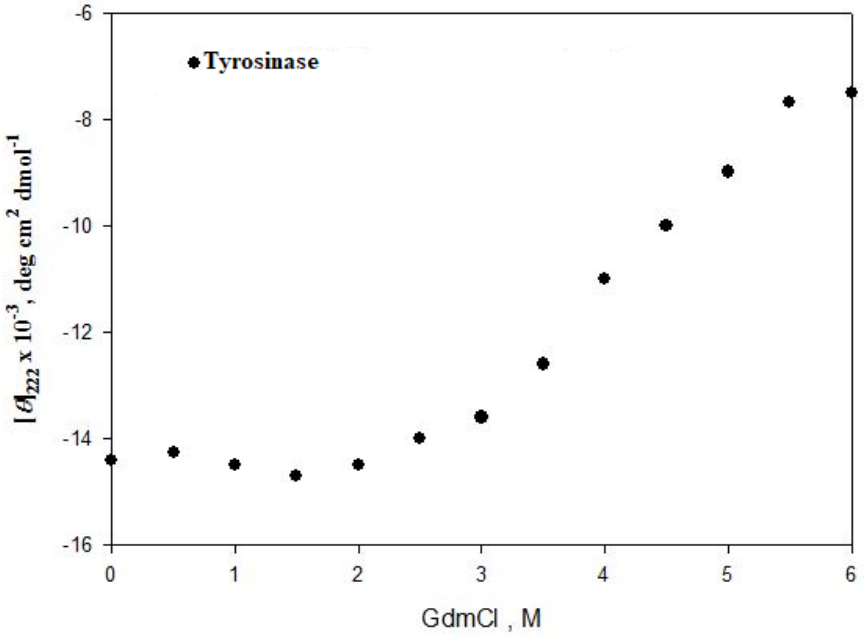


Figure 17:*(A).* *Far-UV CD spectra of Tyrosinase at [GdmCl] from 0 to 6 M.* ***(B)*** *GdmCl induced denaturation of Tyrosinase was monitored by CD.*

TableNo.6: *Secondary structure content and GdmCl induced denaturation of Tyrosinase*

Secondary structure	Secondary Structure Using K2D2 (%)	GdmCl-induced denaturation	
α-helix	10	ΔG_D^0(kcal mol^{-1})	2.88 ± 0.12
β-strand	43	*m* (kcal mol^{-1} M^{-1})	0.70 ± 0.06
Random coil	47	C_m (M)	4.11± 0.09

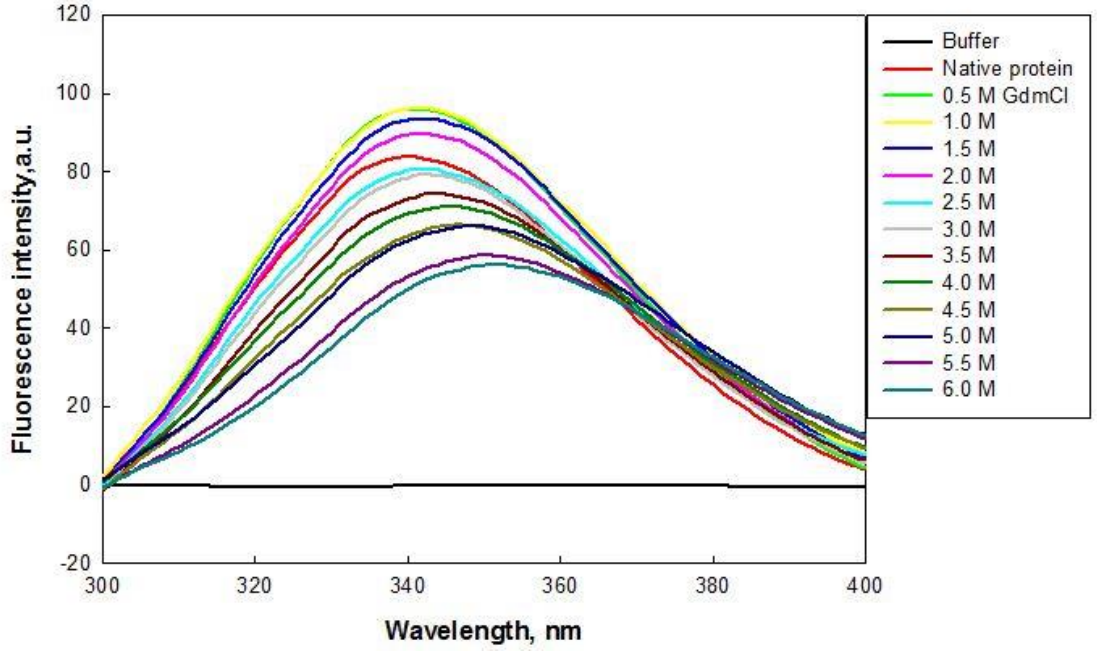
5.5 GdmCl-induced denaturation examined by fluorescence spectroscopy:

Tyrosinase contains tryptophan residues and Tyrosine residues, these amino acids are buried (partially or fully) in hydrophobic region of proteins. To study the structural integrity of tyrosinase, we used the intrinsic fluorescence. As we have seen the measurements of CD, emission spectrum of Tyrosinase changes as GdmCl concentration is increased, (**Figure 18 a)** shows that Tyrosinase undergoes denaturation with increase in GdmCl concentration, it was also seen that the fluorescence intensity decreases and λ_{max} of tryptophan residues was shifted towards the red shift i.e. longer wavelength (355 nm). These explanations clearly indicate that residues are transferred from non-polar environment to polar environment as it is shown that GdmCl exposes the buried tryptophan residues to the more polar conditions. F346 is a model which ensures that the milieu of residues changes. Results shown in **Figure 18**, were analysed to estimate thermodynamic parameters ΔG_D^0, *m* and *Cm*.The values of these parameters are shown in **Table 7.**

Table No. 7: *Thermodynamic parameters monitored by fluorescence probe, GdmCl-induced denaturation of Tyrosinase.*

GdmCl-induced denaturation	
ΔG_D^0(kcal mol^{-1})	3.305 ± 0.12
m (kcal mol^{-1} M^{-1})	0.909 ± 0.06
C_m(M)	3.635 ± 0.09

(A)



(B)

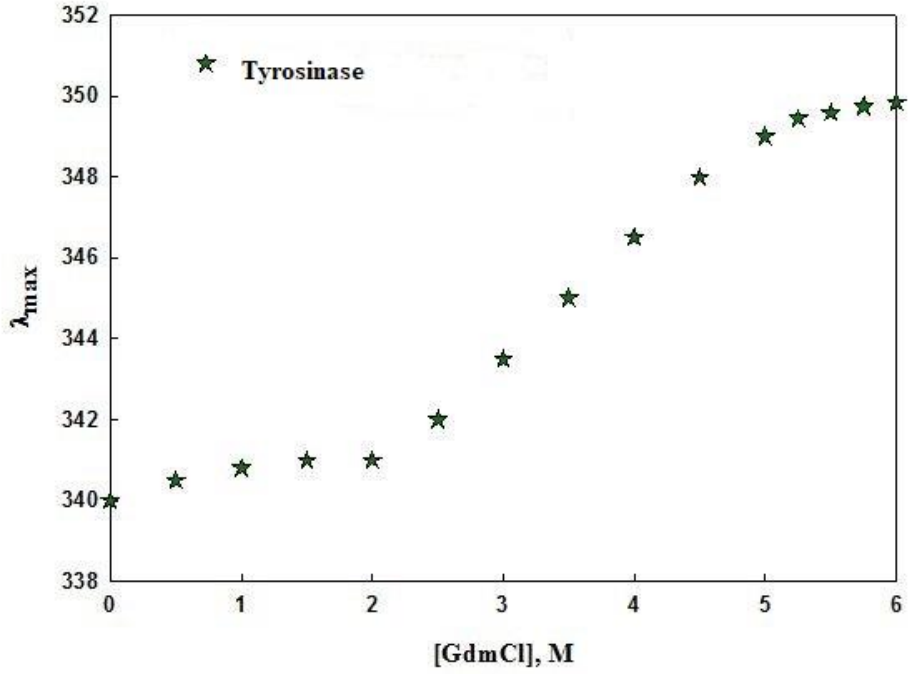


Figure 18. *(**A**) Fluorescence emission spectra of Tyrosinase were recorded as a function of increasing concentration of GdmCl (0.0–6.0M). (**B**) Denaturation curve of Tyrosinase.*

Conclusion

There are number of sources from which tyrosinase has been isolated. In the current study, tyrosinase has been isolated from button mushroom (*Agaricus bisporus*). In today's world specially in western areas, sun exposure like UV rays and skin inflammation affects the generation of world, it leads to various skin disorders like hyperpigmentation and dyspigmentation diseases. Melasma, Albinism, and Vitiligo are the examples of skin disorders which is caused by UV rays, genetic defect etc. A number of agents have been described to treat these disorders having various mechanism of action. Tyrosinase mainly responsible for the production of melanin. Here, we presented a quick and a novel procedure to purify a protein. Here, purified the tyrosinase by using various techniques and it results in the high yield and purity of protein obtained. It will be very useful to carry out in further research on function and modelling of protein.

We also performed the biophysical studies to understand the structural, functional, thermodynamic properties of Tyrosinase. Several methods employed to confirm the stability of tyrosinase. UV/Vis spectrometry and circular dichroism are the techniques by which we confirmed the stability of tyrosinase. Our results of GdmCl showed that the enzyme is highly stable. By using K2D2, It was also confirmed the α- helix content was about 10%, where as in β-sheet it was about 42%. This also confirms that Tyrosinase is β rich protein.

The characterization of tyrosinase by Fluorescence spectrometry and circular dichroism results showed its the similarity to tyrosinase (TYR) present in *Homo-sapiens*. This depicted that the purified and the mushroom characterized with tyrosinase can be used as a wealthy source for therapeutics, specifically in melanin related disorders

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