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Isolation, purification and characterization of tyrosinase from *Allium sativum*

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Abstract

Alzheimer's and Parkinson's are neurological disorders. Alzheimer's is a progressive neurodegenerative disorder that destroys memory with important mental functions. Parkinson's affects the central nervous system, it impairs movement, often including tremors for which there is no cure. Currently dopamine, a neurotransmitter is used in the treatment of these disorders. Tyrosinase (E.C 1.14.18.1) is an enzyme that catalyzes L- Tyrosine into L- DOPA a precursor for the synthesis of dopamine. Tyrosinase is present in sources like fungi, plants, animals. Very few work on tyrosinase has been reported in plants. In our present investigation tyrosinase was isolated and purified from Allium sativum (Garlic) and enzyme activity was carried out using UV - visible spectroscopy and the enzymatic activity was found to be 180 U/ ml. The kinetic parameters were studied and the optimum temperature was found to be 30ºC and the optimum pH was found to be pH 7. Saturation with increasing substrate concentration was found to be at 1200 U/ml. The enzyme was subjected to a series of purification steps that includes ammonium sulphate precipitation, dialysis, ion-exchange chromatography, and gel filtration chromatography. The maximum purification fold of the enzyme was found to be 97.58. SDS - PAGE was performed to determine the molecular weight and was reported to be 98 KDa. Hence through this research work, we have tried to identify a novel source of tyrosinase in Allium sativum.

Keywords: Tyrosinase, Allium sativum, Ion exchange chromatography, gel filtration, SDS-PAGE.

Introduction

Tyrosinase (E.C 1.14.18.1) is an enzyme that is a type of polyphenol oxidase classified as metalloenzymes containing a type-3 copper centre occurring in many organisms including plants, animals, fungi, arthropods, and bacteria[1]. Tyrosinase catalyzes the production of melanin from tyrosine by oxidation[2]. It also converts L-tyrosine into L-DOPA (L-3,4-dihydroxyphenylalanine) or levodopa which is a precursor for the synthesis of dopamine[3]. Therefore tyrosinase is used in the synthesis of L- DOPA, a precursor of the neurotransmitter dopamine.

Dopamine is used in the treatment of Alzheimer'sand Parkinson's neurodegenerative disorder[4,5]. It improves blood flow to the kidneys[6]. L-DOPA modulates motor cortex excitability in Alzheimer's disease patients[7]. L-DOPA is used in the treatment of Parkinson's neurodegenerative disorder[8]. Since tyrosinase synthesizes melanin it can be used in the production of self - tanning agents[9]. It can be used to increase the colour intensity of beverages like tea and coffee[10]. Tyrosinase is used in cross-linking dairy proteins and wool fibres[11]. It improves the gelation and crumb structure of bread[12,13]. It is employed in wastewater treatment and in biosensors to detect toxic phenolic compounds[14].

Very little work has been carried out on Isolation, Purification and Characterization of tyrosinase from plant sources. Enzymes extracted from plant sources are easily available and they can be extracted within a short period of time, unlike microbial sources which require longer duration for microbial culture and extraction. The current research reports about the Isolation, Purification and Characterization of tyrosinase from *Allium sativum.*

Materials and methods

Isolation: 100g of *Allium sativum* (Garlic) was homogenized using phosphate buffer. The extract was filtered using a sieve and the solution was centrifuged at 6000rpm for 10 mins.

Purification of tyrosinase enzyme: Salt Precipitation: The supernatant obtained was subjected to ammonium sulphate precipitation. 70% saturated ammonium sulphate was used for purification. 44g of ammonium sulphate was weighed and it was dissolved in 100ml of supernatant which was added pinch by pinch in an ice-cold condition using a magnetic stirrer for 4 hours. The contents were allowed to stand for 1 hour to make sure the precipitation was complete. Later salt suspension was stored at 4ºC overnight.

Dialysis: The salt suspension was centrifuged at 10000 rpm for 10 minutes and pellets were collected using 10m Mtris HCl (10ml). 1ml of pellets were taken for dialysis. Dialysis was carried out using nitrocellulose acetate membrane.

Activation of the dialysis bag: 100ml of distilled water was kept for boiling using a heating mantle. Nitrocellulose acetate

membrane was inserted into the boiling water bath for ten minutes with the addition of sodium hydrogen carbonate pinch by pinch. The nitrocellulose acetate membrane was removed and it was transferred to another boiling water bath for 10 minutes with the addition of disodium hydrogen carbonate (detergent) for pore activation thorough elimination of dirt, oils, and fats. The flask used as a water bath was removed from the heating mantle it was allowed to cool. The next day the solution within the flask was replaced with distilled water. The ammonium sulphate precipitated solution was filled into the activated dialysis bag. Dialysis bag was tied in a vertical hanging position which was partially immersed into the beaker filled with water. A magnetic bead was placed inside the beaker and the solution was stirred using a magnetic stirrer. The distilled water was replaced three times for every time interval of 2 hours.

Anion Exchange Chromatography: The principle involved is the separation of molecules based on their charges. DEAE cellulose was used as a stationary phase to isolate negatively charged protein molecules. Elution buffer was used as the mobile phase. The elution buffer was prepared for gradient elution with NaCl linear gradient with increasing salt concentration and TrisHCl solution.

Table-1: Mobile phase elution buffers concentration.

Elution buffer	TrisHCl Concentration	NaCl Linear Gradient
E 1	25mM	25mM
E 2	25mM	25mM
E 3	25mM	50mM
E 4	25mM	75mM
E 5	25mM	100mM
E 6	25mM	125mM
E 7	25mM	150mM

The flow of the column was checked employing a sonicator for cleansing the impurities within the column and 10ml of water was added which was subjected to suction using a vacuum motor. DEAE cellulose Anion exchange column (1.4×19cm) was used. DEAE - Cellulose (R-52) resin was added into a column which was allowed to stand for a period of 30 minutes. DEAE- Cellulose matrix was equilibrated using activation buffer (E1). Dialyzed enzyme solution was added to the column. Gradient elution was carried out with different fractions with respect to the linear NaCl gradient. The eluate was collected in seven different eluate test tubes (E1 to E7) from which the amount of protein was determined using Lowry's method of protein estimation[15]. The enzyme assay was carried for tyrosinase[16].

Gel filtration: The purified sample obtained from anion exchange chromatography was subjected to gel filtration. G-75 Sephadex gel bead matrix was poured into the clean sonicated gel filtration column. The matrix was allowed to settle. 100mM phosphate buffer (pH 7.5) was used as an elution buffer. Elutions were collected at a flow rate of 1ml/minute. Eppendorf microfuge tubes (E1 - E30) were used to collect the 30 different eluted samples. Protein estimation was carried out using 280 method[17]. The enzyme assay was carried out for all the eluates.

Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE): Cleaned and dried spacers and glass plates were assembled accurately. Clips were used to hold the setup in an erect position. Bioline petroleum jelly was used to seal the edges which was applied around the spacer edges. Separating gel was prepared by mixing the gel contents. To the periphery of the gel, a distilled water layer was made and the gel was made to settle for 30 minutes. The distilled water from the surface was eliminated and a small amount of stacking gel was used for this purpose. TEMED - 10µl, APS-50µl, SDS-100µl was added to the gel for the initiation, catalyzation of polymerization, addition of negative charge to the protein respectively. The comb was removed after the polymerization of the stacking gel without deforming the created wells. Sample and (2x) Laemmli loading buffer were boiled in a water bath at 95ºC for 5minutes. Crude, salted, dialyzed, ion-exchanged, gel filtered enzyme samples and sea blue marker with standard proteins were loaded in each well from left to right sequentially from crude to gel filtered enzyme samples in a volume of 50µl with a concentration of 50µg. The gel was transferred onto the electrophoretic apparatus. Electrode buffer was added to the electrophoretic apparatus.50V of voltage was applied and the setup was run for 6 hours.

Staining procedure: After the electrophoresis, the gel was placed in a plastic tray that contained a staining solution. The tray was placed on a gel rocker to stain the proteins and the process was carried out for 2 hours. The gel was washed to remove the excess stain with a destaining solution. The destaining solution and gel was placed within a tray on a rocking table for 2 hours. The gel was washed 3 times with a time interval of 2 hours. After destaining the gel it was photographed for documentation. The bands were visualized under UV - transilluminator.

Enzyme Assay: A reaction cocktail was prepared which was composed of deionized water, reagent A (Buffer), reagent B (Tyrosine) in 9:10:10 ratio respectively. A 2.9ml reaction cocktail was added to test (salted out sample, dialysate sample, anion exchange chromatography eluate and gel filtration chromatography eluate) and control tubes at room temperature. The enzyme assay was carried out photometrically using UV-VIS spectrophotometer at 280nm. The enzyme activity was calculated which was expressed in units/ml (U/ml).

Characterization of Protein: Temperature, pH, and substrate concentration kinetics were determined.

HPLC Analysis: L- DOPA was synthesized using L- tyrosine as the substrate and the purified tyrosinase enzyme[18]. High-performance liquid chromatography (HPLC) was performed to detect L-DOPA. The mobile phase used was a mixture of methanol and dihydrogen phosphate in a ratio of 95:5 with a volume of 285ml:5ml respectively. pH was adjusted to pH 2.5 using o - phosphoric acid. The; mobile phase was filtered using a Buchner funnel and it was stored in a brown bottle. 100µl of L- DOPA was used as an analyte. 900µl of the mobile phase was used for HPLC analysis and UV detection wavelength was set to 230nm. The flow rate was found to be 1ml/minute. Purging was 1ml/minute for up valve and 3ml / min for the down valve. Purging was performed to eliminate the air bubble. Iris 32 lite software was used for HPLC analysis. L-DOPA standard and synthesized L-DOPA from our sample were compared. The results were obtained as three distinct graphs which were namely standard of L-DOPA, sample, and overlay graph of both standard and synthesized L- DOPA. The graphs were later analyzed to determine synthesized L-DOPA.

Results and discussion

Tyrosinase was extracted as a crude enzyme which was further subjected to a series of purification steps which includes salt precipitation, dialysis, anion exchange chromatography and gel filtration chromatography. Amount of protein was determined and enzyme assay was performed after each step. Specific activity and purification fold increased with the purification of the enzyme. The molecular weight of tyrosinase was found to be 98KDa using SDS-PAGE. Enzyme kinetics were determined utilizing purified samples and results were graphically represented. L-DOPA was detected by HPLC.

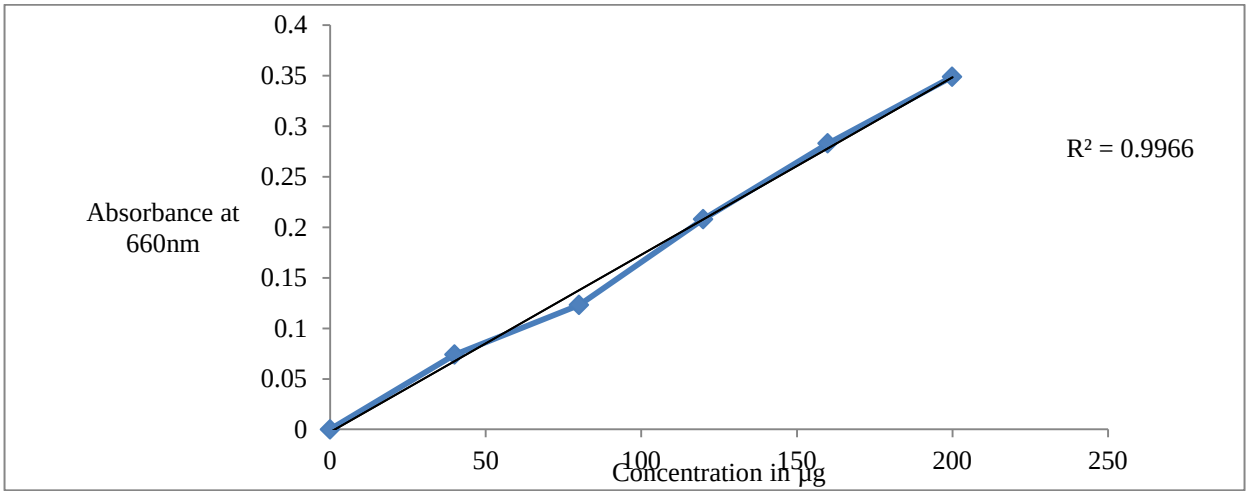

Figure-1: Estimation of protein by Lowry's method.

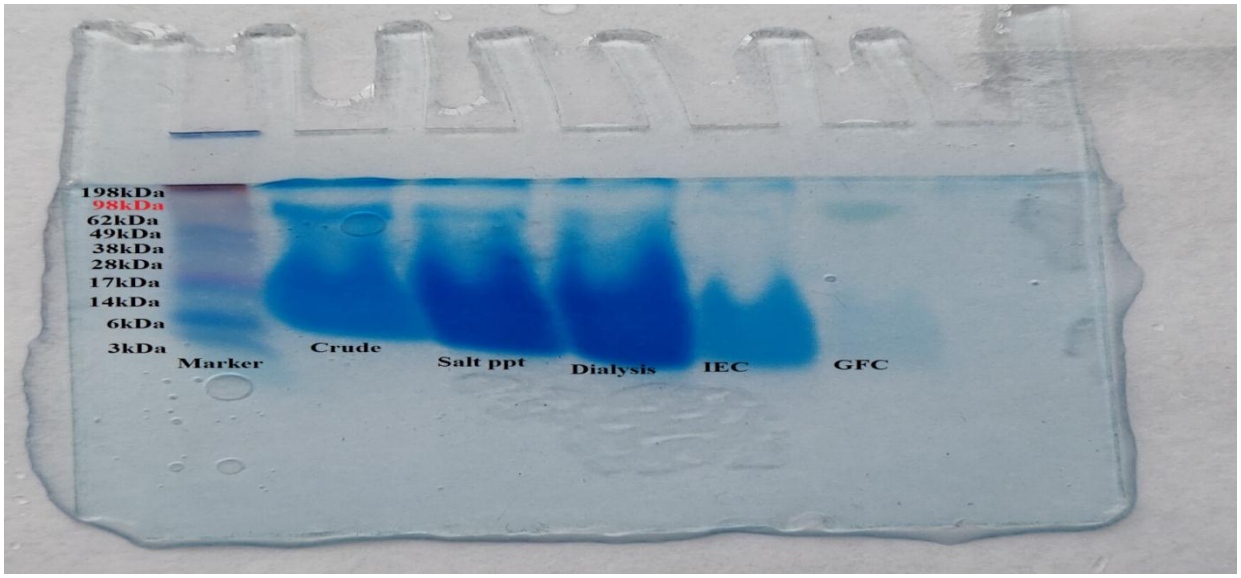

Figure-2: Molecular weight determination using SDS – PAGE.

Table-2: Purification table of tyrosinase.

Sample	Protein content (mg/ml)	Enzyme activity (U/ml)	Specific activity (units/mg)	Fold Purification	Yield (%)
Crude	17.76	520	29.279	1	100
Salt	1.765	300	169.97	5.8	57.69
Dialysis	1.176	230	195.57	6.67	44.23
Ion Exchange Chromatography	0.576	200	347.22	11.85	38.46
Gel Filtration	0.063	180	2857.14	97.58	34.61

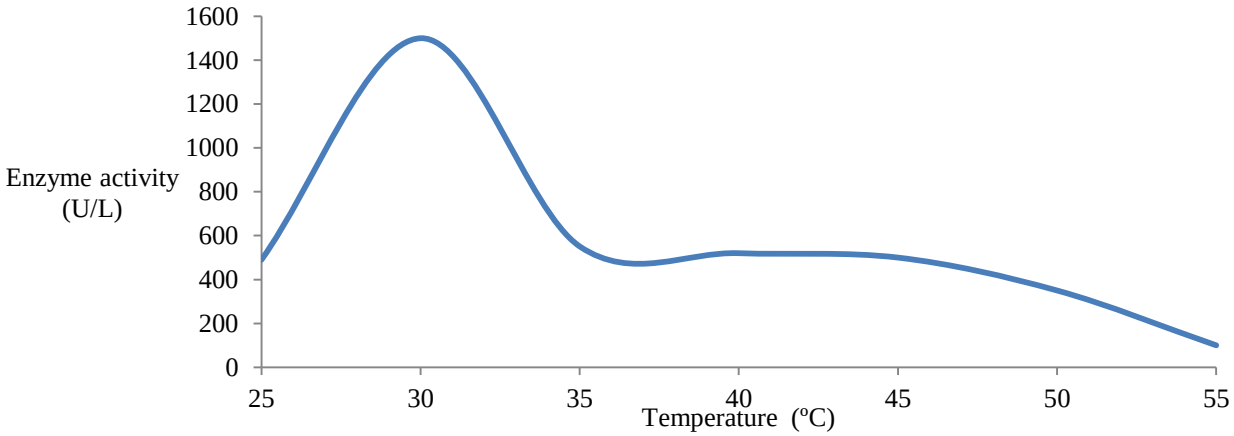

Figure-3: The optimum temperature of the tyrosinase enzyme in *Allium sativum* was found to be 30℃.

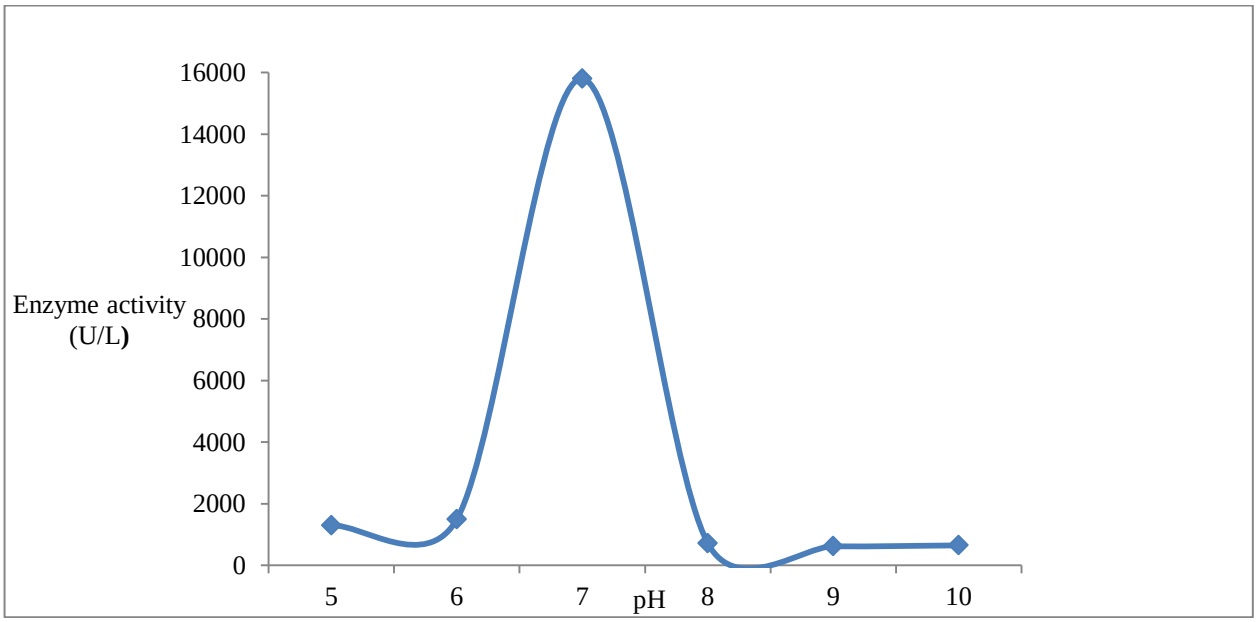

Figure-4: The optimum pH of the tyrosinase enzyme in *Allium sativum* was found to be pH 7.

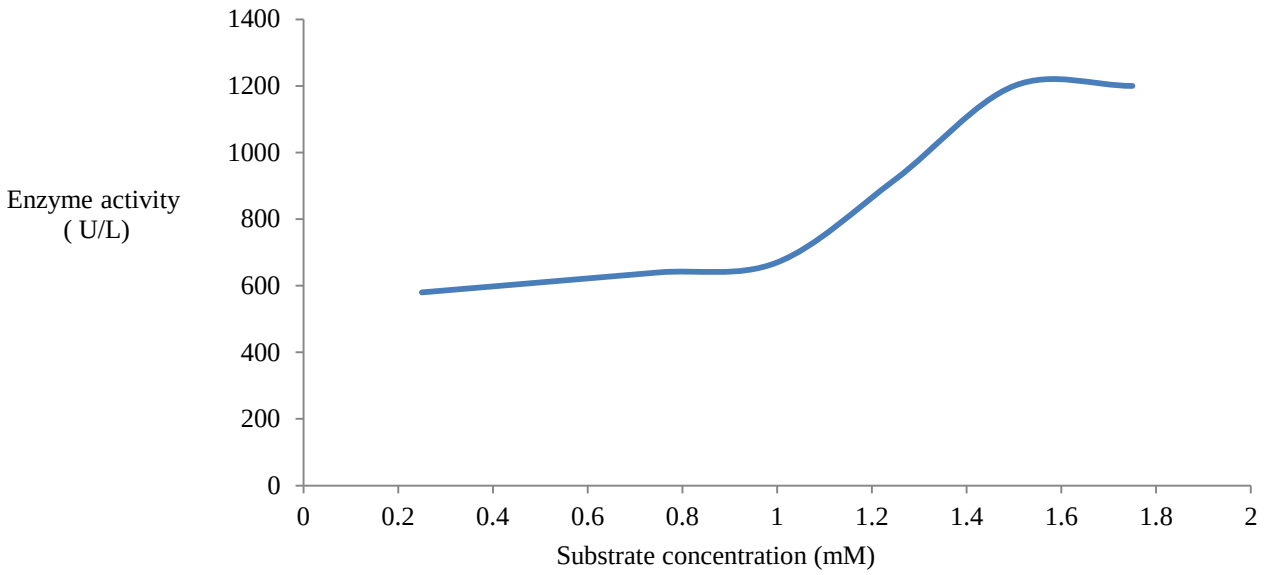

Figure-5: Tyrosinase enzyme saturation with increasing substrate concentration in *Allium sativum* was found to be at 1200 U/ml.

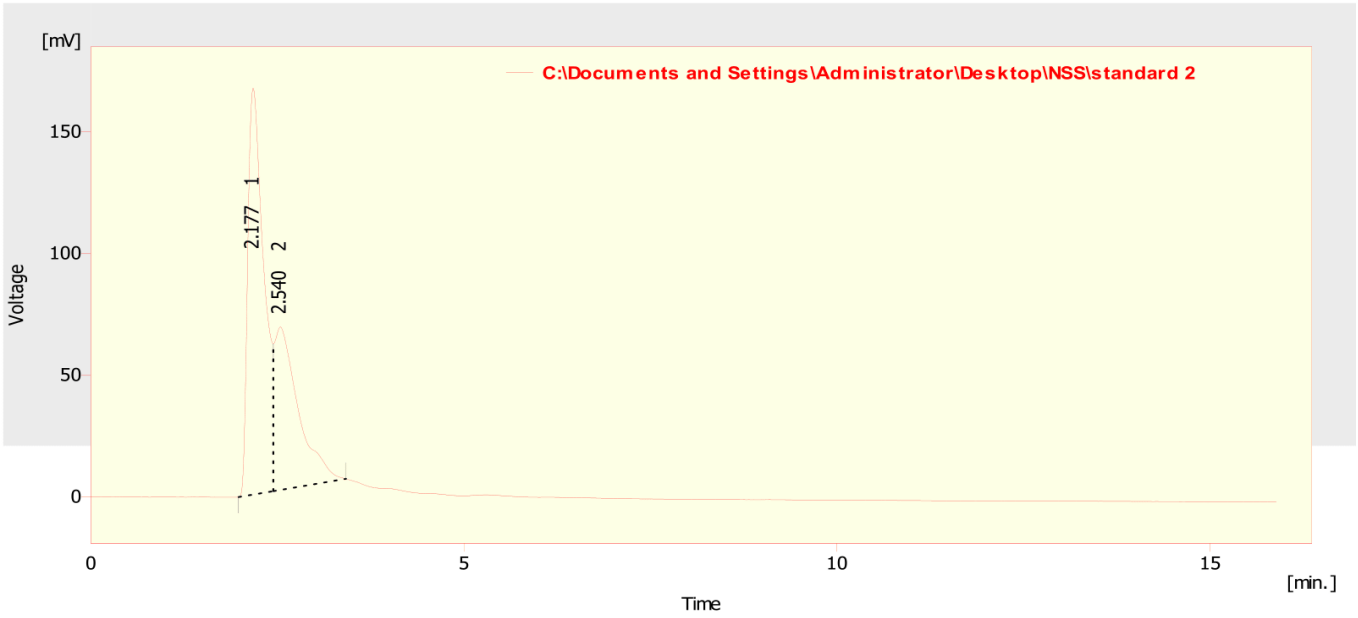

Figure-6: Standard of L – DOPA.

Table-3: Result of standard L – DOPA.

Retention Time [min]	Area [mV.s]	Height [mV]	Area (%)	Height [%]	W 05 [min]
2.177	2503.792	167.026	62.5	71.4	0.25
2.540	1504.232	66.974	37.5	28.6	0.33
	4008.024	234.000	100.0	100.0	

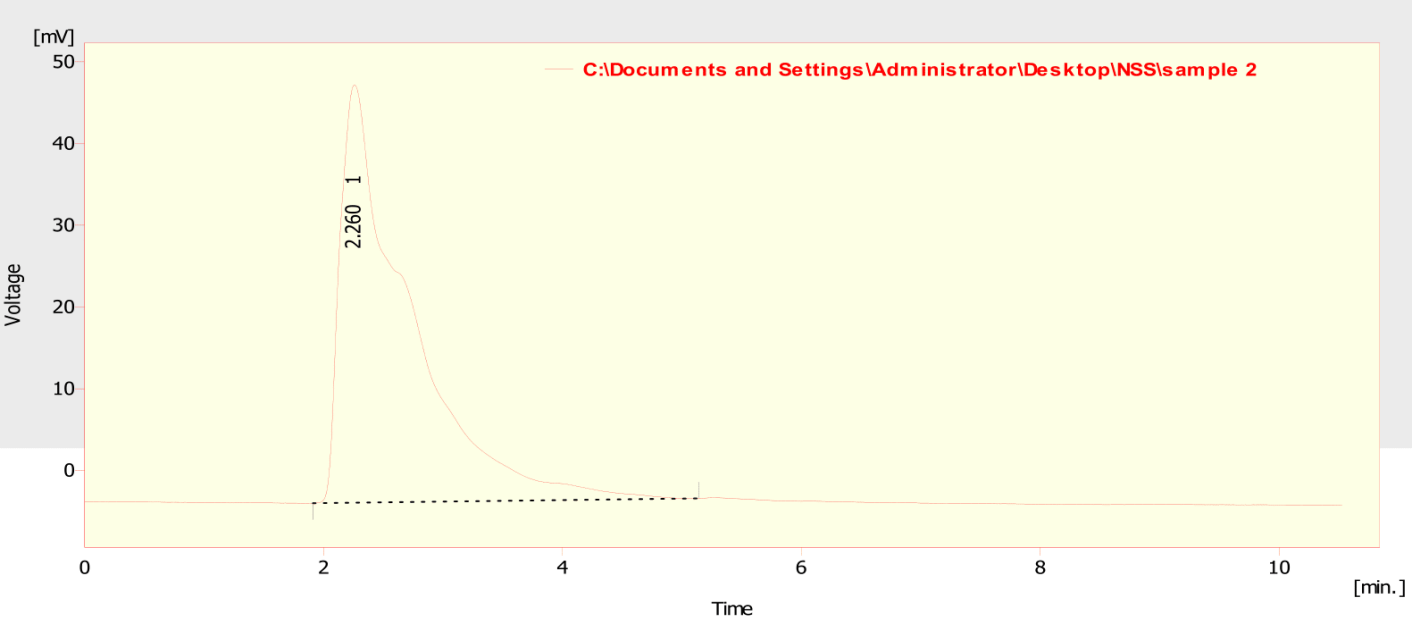

Figure-7: L-DOPA sample.

Table-4: Result of L – DOPA sample.

Retention Time [min]	Area [mV.s]	Height [mV]	Area (%)	Height [%]	W 05 [min]
2.260	2035.923	51.099	100.0	100.0	0.60
Total	2035.923	51.099	100.0	100.0	

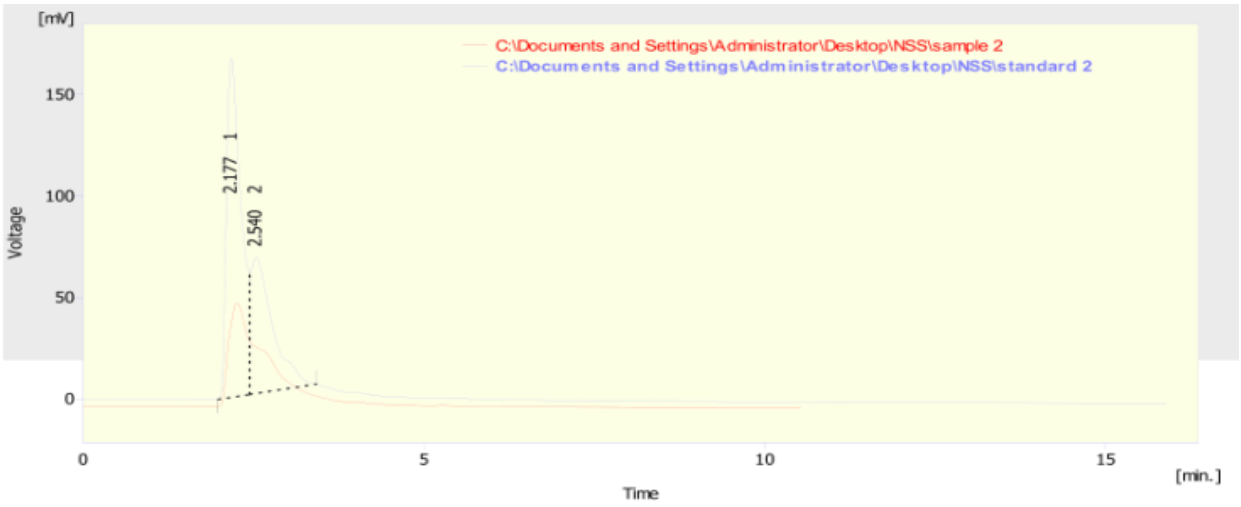

Figure-8: Overlay of standard and sample L-DOPA.

Table-5: Result of standard L-DOPA overlay.

Retention Time [min]	Area [mV.s]	Height [mV]	Area (%)	Height [%]	W 05 [min]
2.260	2035.923	51.099	100.0	100.0	0.60
Total	2035.923	51.099	100.0	100.0	

Table-6: Result Table L – DOPA sample overlay.

Retention Time [min]	Area [mV.s]	Height [mV]	Area (%)	Height [%]	W 05 [min]
2.177	2503.792	167.026	62.5	71.4	0.25
2.540	1504.232	66.974	37.5	28.6	0.33
Total	4008.024	234.000	100.0	100.0	

The bulb extract of *Alliumsativum* showed tyrosinase enzyme activity and therefore purification steps were carried out on crude extract. Tyrosinase enzyme activity was found to be 180U/ml and specific activity was 2857.14U/mg. Purification fold increased to 97.58 and the percentage yield was 34.61%. Optimal enzyme kinetics with respect to temperature was 30ºC and pH was 7. Saturation with increasing substrate concentration was 1200U/ml. L-DOPA, a precursor of dopamine, was synthesized and detected under HPLC analysis and was found to be 3.252mg in 1000µl.

Conclusion

Enzyme assay and characterization confirmed the production of tyrosinase in Allium sativum. Since purified tyrosinase has a neutral pH it can be optimal in neutral solvents such as water. Tyrosinase has the potential to synthesize L-DOPA, a precursor of dopamine. Therefore it may be effective in the treatment of Alzheimer's and Parkinson's neurological disorders. Further research could be carried to study its structure and therapeutic potential.

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