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Research Article

Screening Of Polyphenol oxidase and Peroxidase Enzymes from Brinjal (Solanum melongena) With Emphasis on Inhibition by Organic (edible) Inhibitors

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ABSTRACT:
Background: Polyphenol oxidase (PPO) and Peroxidase (POD) enzymes produce dark color to vegetables by oxidizing phenolic substrates with oxygen and thus converting the di-phenol into melanin pigments. Therefore, the enzymatic browning can be overcome by utilizing antioxidants, commercial synthetic inhibitors and organic edible inhibitors. **Findings:** PPO and POD enzymes were extracted from *Solanum melongena* by 30% and 70% (NH4)2SO4 saturation followed by Gel chromatographic purification by Sephadex G-300 Colum. The estimated molecular weight of PPO was found 100 kDa. The highest activity of the enzymes was measured at 50°C and above 50 °C, enzyme was deactivated. The enzyme showed temperature stability in the range of 30–70 °C. The optimum pH of PPO of *Solanum melongena* was determined as 7.2 showing stability in the range of 6.2–8.2. Diverse commercial synthetic compounds as well as organic edible compounds were used to inhibit the enzymatic activity in *Solanum melongena*. **Conclusion:** Coffee and cinnamon powder among edible organic compounds and acetic acid and ascorbic acid among synthetic compounds were proved to be more efficient inhibitors for PPO enzymatic activity.

KEYWORDS:
Brinjal (Solanum melongena) PPO, partial purification, characterization

1 | INTRODUCTION

The nuclear-encoded enzyme polyphenol oxidase (PPO) (EC 1.14.18.1) catalyses the conversion of phenolic compounds into quinones (coloured pigments) by oxidation. [1]. Two types of oxidation reactions are catalyzed, causing the hydroxylation of monophenols into o-diphenols" and the 'oxidation" of o-diphenols into o-quinones that form brown or black pigments. All plants have the PPO enzyme, an enzyme that contains copper. [2]. The enzyme is an enormously widespread that relates to the enzymatic browning in plants and production of melanins in animals[3.] Another heme protein containing enzyme is peroxidase (POD) E.C. 1.11.1.7 belonging to the oxidoreductase family. In reaction to Hydrogen Peroxide, POD is usually associated with wounds (injuries). It also exists in all living organisms, e.g., animals, microorganisms, and plants [4.] The polyphenol oxidase enzyme disturbs the quality of foodstuffs, so it is significant to industry. The PPOs and PODs are utilized in biosensors and in pharmacological drug production, and they are also used in the deprivation of many phenolic compounds [5.] The PPO and POD control activities have an important role in the handing out of fruits, and vegetables [6.] The Phenolic compounds have a significant role in food's visual form. The phenolic composition of plants is also affected by ecological conditions, i.e., water availability, soil composition, post-harvest factors and handling conditions [7]. The PPO oxidizes the phenolic compound when oxygen is there, while the peroxides, oxidize the phenolic compound in the presence of H2O2 [8.] The daily life vegetable, such as Brinjal(Solanum *melongena*) is cultivated in a tropical climate as well as subtropical areas [9]. Solanum melongena exposed a high hydrophilic O2 absorbance capability, which was linked to the occurrence of

phenolic compounds (delphinidin) as a key component in peel [10]. in the statement that the PPO enzyme causes browning of vegetables. Through heat or by reducing pH or an extra unit below the optimum pH, the enzyme could be denatured, which inhibits enzymatic browning.In the course of the research, the primary goals were to extract PPO and POD enzymes from a selection of vegetables, investigate their kinetic properties, and conduct a comparative inhibitory investigation of synthetic and organic substances on the enzymatic activity of PPO enzyme inhibition.

2 | MATERIAL AND METHODS

2.1 | Chemicals

4-methyl Catechol, Cold acetone, Potassium phosphate buffer, Ammonium sulfate, Tyrosine, and Catachol

2.2 | Source of Vegetable

A mature vegetable, Solanum *melongena* (brinjal), was purchased from the local market. Care was taken to choose healthy and mature samples for the study.

2.3 | Measurement of Enzymatic Activity

The activities of the enzymes were measured spectrophotometrically using specific substrates and following established protocol described by Ying and Zhang (2008) [11]. The assay conditions were optimized to ensure accurate and reproducible measurements. Catechol was used as a substrate. The measurement was done at 420 nm wavelength by using UV- spectrophotometer. The enzyme assay mixture contained 2.3 ml of phosphate buffer with pH-7 and 500 µl of catechol. After 15 minutes of incubation at 25 °C, 200 µl of samples were added to the mixture and incubated for another 15 minutes. After 15 minutes of incubation at 25 °C, enzymatic activity was measured through a spectrophotometer at different intervals.

2.4 | Crude PPO Extracting

Homogenizing the sample was done by mixing 20 gm slices of plant with 250 ml of cold 0.2M phosphate buffer pH 7.0. Then after filtration sample was by the centrifuged at 7000 rpm for 15 minutes. Cold acetone (-4 to -5) was added in 1.5 volumes with slight stirring for about 50 to 60 minutes to precipitate the enzyme from the supernatant. The mixture was then kept for centrifugation at 7000 rpm, and after 30 minutes, a crude extract was obtained.

2.5 | 30-70% (NH4)2SO4 Precipitation

Thirty percent (30%) $(NH_4)_2SO_4$ was added to the crude extract and stirred for 1 hour. After 30 minutes centrifugation at 7000 rpm, and the supernatant was taken. Then 70% $(NH_4)_2SO_4$ was added to the supernatant and stirred for 1 hour, then centrifuged for 30 minutes at 7000 rpm, and the palate was taken to dissolve in phosphate buffer and preserve the solution for the next step of purification.

2.6 | Gel Chromatography

The 70% $(NH_4)_2SO_4$ saturated sample was then subjected to the Sephadex G-300 column with the elution rate of 5 ml/min with extraction buffer. Fractions with highest enzymatic activity peak were collected and concentrated by ultrafiltration.

2.7 | SDS-PAGE

The homogeneity and purity of the isolated enzyme were estimated by SDS-PAGE.

Health Sciences Journal
EISSN: 2959-2259; PISSN:2959-2240

2.8 | Characterization

2.8.1 | Optimum Temperature

10 to 80 °C temperature ranges were used for the measurement of PPO activity to measure the optimum temperature.

2.8.2 | Temperature Stability

The sample (enzyme) was put in a water bath at 10-80 °C temperatures range. After the incubation period, the activity of the sample was measured at 24 °C.

2.8.3 | Optimum pH

The enzyme activity of PPO was checked with an altered pH solution ranging from 3.6 to 9.0.

2.8.4 | pH Stability

The enzyme activity of PPO was studied at diverse pH solutions in 3.6 to 9.0 range. Acetate buffers in the pH range of 3.6–5.6 were used, while Tris buffers in the range of 7.4–9.0 were used. The enzyme solution was incubated overnight at various pH levels. Afterward incubation, residual PPO activity was measured as usual.

2.9 Inhibitors

2.9.1 | Commercial Chemical Inhibitors and Organic (Edible) Inhibitors

The effect of inhibition of selected commercial chemical inhibitors and organic (edible) inhibitors was measured. Sodium chloride, Citric acid, ascorbic acid, oxalic acid, honey, black tea, Cinnamon powder, Coffee, and green tea were applied to inhibit the browning of vegetables.

3 | RESULTS

The oxidative enzyme PPO was purified by salt fractionation upto 30% and 70% level and then by G-300 gel chromatography. The highest PPO enzymatic activity for Solanum *melongena* was obtained as 8 U/ml. The result indicated that 333 folds purification of PPO from Solanum *melongena* was achieved with a recovery of 13.5% and 10 U/mg specific activity (table 1).

Table 1 PPO enzyme purification scheme from Solanum melongena

Step	Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific activity (U/mg)	Purification Factor	Recovery %
Extract	0.445	120	53.40	15.0	1800	0.03	1	100
30% $(NH_4)_2SO_4$ saturation	0.642	70	44.94	7.0	490	0.09	3	84.2
70% $(NH_4)_2SO_4$ saturation	0.827	10	8.27	1.31	13.1	0.63	21	15.5
Sephadex G-300	0.9	8	7.20	0.09	0.72	10	333	13.5

3.1 | Sephadex G-300 Column Chromatography.

PPO enzymatic activity was measured at 450nm through Sephadex G-300 column chromatography. The protein measurement of each fraction was done spectrophotometrically at 280nm. The enzyme was purified in the fractionating

range eluting out all unpurified fractions (Figure 1).

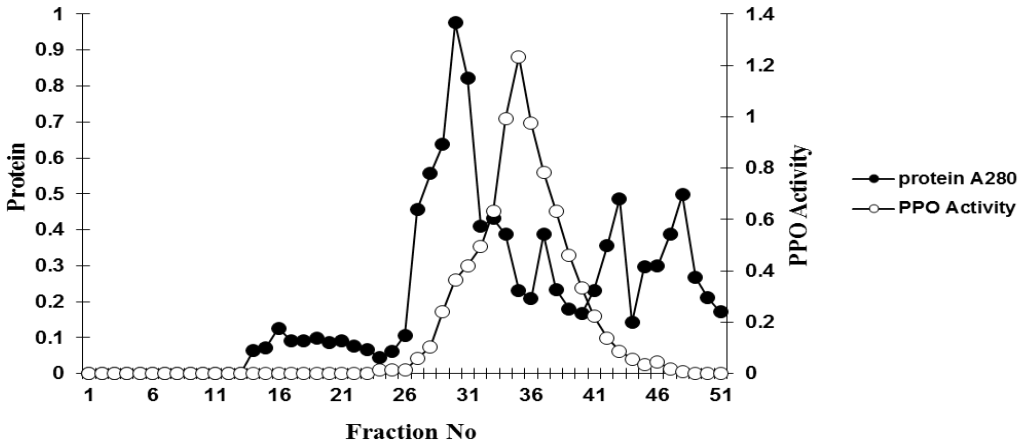

Figure 1: Gel filtrations through Sephadex G-300 column chromatography for PPO from Solanum *melongena.*

3.2 | Sodium Dodecyl Polyacrylamide Gel Electrophorese

The purity and homogeneity of the enzyme was assessed through SDS-PAGE by comparing with standard marker proteins. The molecular weight of PPO isolated from Solanum *melongena* was estimated as 100 kDa.

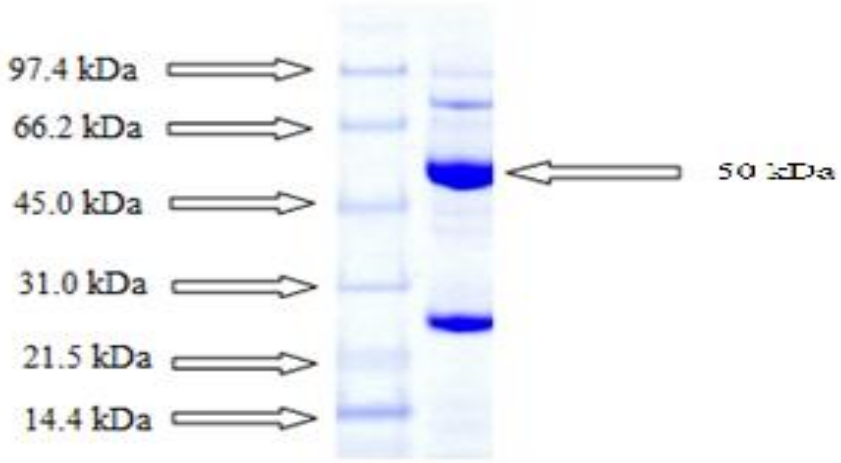

Figure 2: SDS-PAGE. of PPO enzyme of Solanum melongena.

3.3 | Optimum Temperature

The optimal temperature for PPO activity was measured at a range of 10–80 oC.For PPO enzymes, the optimum temperature was 50 oC for Solanum melongena (Figure 3).

Health Sciences Journal
EISSN: 2959-2259; PISSN:2959-2240

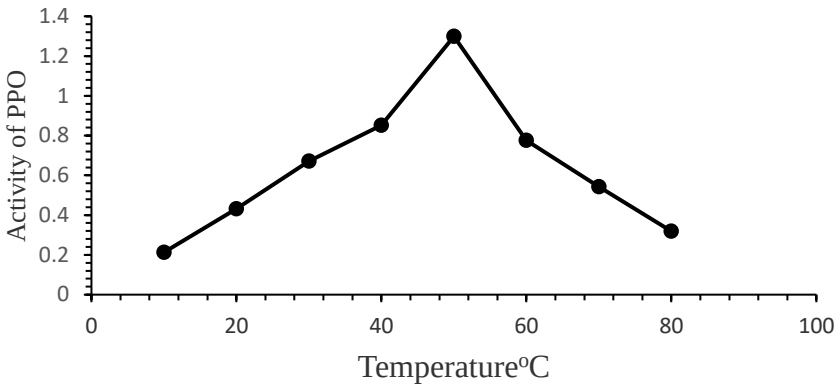

Figure 3: Optimum temperature of Solanum melongena

3.4 | Temperature Stability

In order to determine the degree to which the enzyme PPO in Solanum melongena is stable at varying temperatures, a variety of temperatures were utilized. For Solanum melongena, the results for temperature stability were between 30 and 70 degrees Celsius.

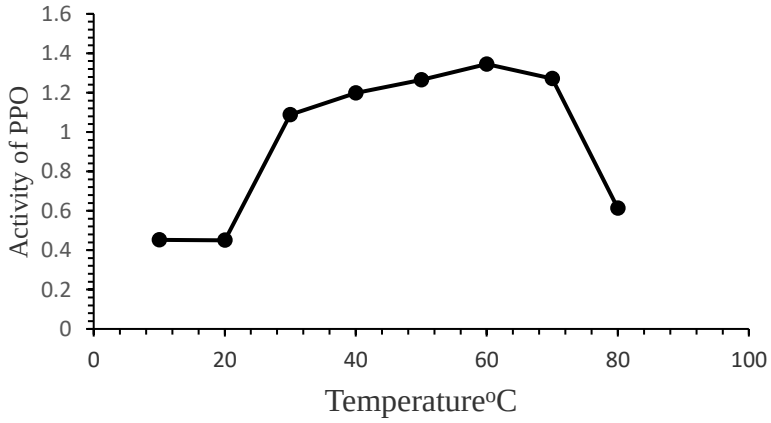

Figure 4: Temperature stability of *Solanum melongena*

3.5 | Optimum pH

The optimal pH for PPO extracted from *Solanum melongena* was discovered to be 7.2 using catechol as a substrate. 6.5 was the ideal pH for the enzyme's activity when 4-methylcatechol was utilised as the substrate.

Health Sciences Journal
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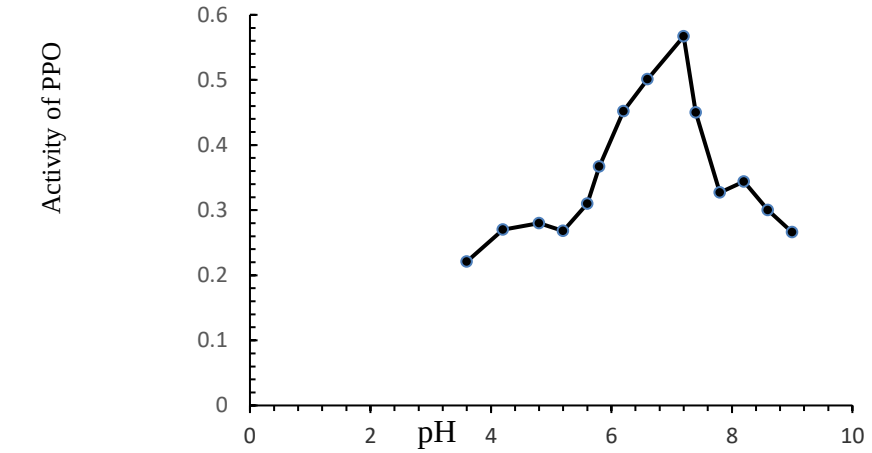

Figure 5: Optimum pH of PPO from *Solanum melongena*

3.6 | pH Stability

The PPO enzyme from *Solanum melongena* was measured for pH stability using a range of pH values. The pH range of 30 to 70 was stable for the enzyme in *Solanum melongena.*

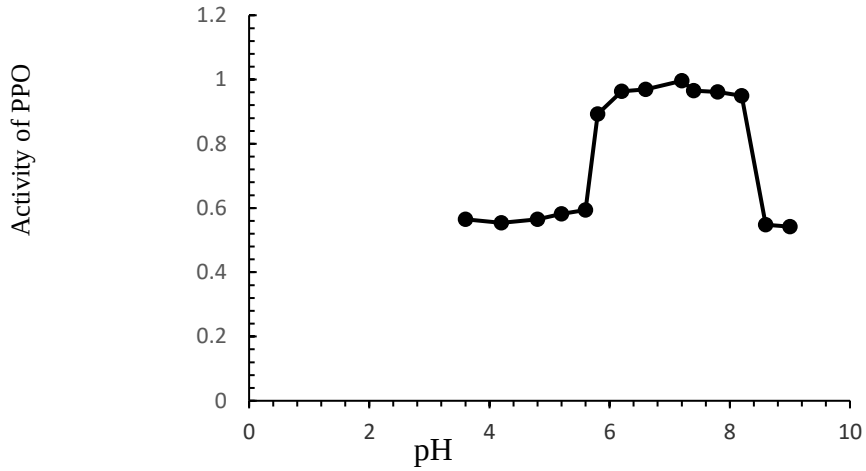

Figure 6: pH stability of *Solanum melongena*

3.7 | Effect of Incubation Time on PPO Activity

PPO from Solanum *melongena* was tested for enzymatic activity at intervals of 5, 10, and 15 minutes. The incubation period of 15 minutes resulted in the highest measured enzymatic activity. The highest activity by using catechol as substrate for Solanum melongena was 1.210 U/ml, while the activity through 4-methyl catechol was too great at 15 minutes of incubation. The maximum activity by using 4 methyl catechol for Solanum melongena was 0.453 U/ml; using tyrosine as substrate, the maximum activity for Solanum melongena was 0.473 U/ml (table 2).

Health Sciences Journal
EISSN: 2959-2259; PISSN:2959-2240

Table 2: Effect of incubation time on PPO activity from solanum melongena

Substrate	Sample	5 mint U/ml	10 mint U/ml	15 mint U/ml
Catechol	Extract	0.625	0.816	0.884
	30%	0.645	0.846	0.910
	70%	0.827	1.045	1.210
4-methyle	Extract	0.302	0.329	0.399
catechol	30%	0.352	0.415	0.428
	70%	0.422	0.430	0.453
Tyrosine	Extract	0.208	0.318	0.387
	30%	0.311	0.358	0.399
	70%	0.400	0.424	0.473

3.8 | Inhibitors

Different edible inhibitors (cinnamon powder, coffee, black tea, green tea, and honey) were used to inhibit PPO enzymatic activity and reduce the browning of the vegetable. In Solanum melongena, the PPO activity by using honey was 0.492, cinnamon powder was 0.241, coffee was 0.220, black tea was 0.35, and green tea was 0.302 as compared to control (0.9). Inhibition was shown by honey due to their protein components forming a complex with polyphenolic honey. Four different commercial chemical inhibitors—oxalic acid, NaCl, acetic acid, and ascorbic acid—were used to inhibit the PPO enzymatic activityisolated from the vegetable. In Solanum *melongena,* the activity was inhibited by oxalic acid up to 0.453, ascorbic acid 0.272, acetic acid 0.124, and NaCl 0.450 as compared to control (0.9). Enzymatically generated o-quinones were reduced by ascorbic acid and then polymerized to form brown pigments that stabilised the browning process without affecting the enzyme's activity. Because the ascorbic acid was permanently oxidised by the reaction with dye intermediates and endogenous enzymes, the ascorbic acid impact was only thought to be transient (Table 3).

Table 3: Effect of Inhibitors on PPO activity

Sample	Control	Organic Edible Inhibitors				Commercial Chemical Inhibitors			
Solanum melongena	**Extract**	**Honey**	**Coffee**	**Black tea**	**Cinnamon powder**	**Oxalic acid**	**Ascorbic acid**	**Acetic acid**	**Na Cl**
Activity	0.9	0.492	0.220	0.35	0.241	0.453	0.272	0.124	0.450

4 | DISCUSSION

O-diphenol is oxidised by the enzyme polyphenol Oxidase (PPO) to quinones, which are then converted into dark pigment. In the context of PPO enzyme catalysis, where drying operations pose the primary challenge, the browning response is deemed an insignificant characteristic of the final product 12. Peroxidase (POD) is an oxidoreductase enzyme that catalyzes the oxidation of organic and inorganic substrates in the presence of H2O2 13. In organisms, peroxidases are typically distributed. Catalases as well as POD are the two major systems for the enzymatic removal of H2O2. Cell walls, which are peroxidative harm, are forbidden by the effect of the anti-oxidative Peroxidase enzymatic system 14. Vegetable ripening was also associated with POD activity, which also produced browning, an additional or helpful effect of polyphenol oxidase activity. A new accurate inference of the POD mechanism is a significant step toward extra-competent control of reactions that are unwanted, mostly in handling heat products, which frequently contain residual peroxidase activity [15] The crude polyphenol and peroxidase were isolated and then partially purified through the 30%, 70%, and G-300 column saturations. For Solanum *melongena*, the best PPO enzyme activity was found to be 0.9 U/ml. In contrast to Senthil Kumar Rathnamsamy et al. (2014)'s findings, the PPO activity of turnip and cabbage The results for turnip were 1.54U/ml and cabbage was 0.9U/ml.

Health Sciences Journal
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The enzymatic activity was checked by using a change in time interval of 5, 10, and 15 min. In the case of PPO derived from Solanum melongena, the best level of enzyme activity was discovered to be 0.884 after 15 minutes of incubation with catechol. On the other hand, the maximum activity for tyrosine was 0.387, while the maximum activity for 4 methyl catechol was 0.399. The optimal incubation period for bananas, according to the study, was one minute at 981 U/mg with specific PPO activity. However, after half that time, the enzyme activity decreased to 688.4 U/mg, with the low delivery of enzyme from banana peels being the cause. In contrast, less activity was seen at 502.9, 455.3, and 430.4 U/mg after 3, 5, and 7 minutes, respectively 16. Purified enzymes seemed to form a precise band on SDS-PAGE. The apparent enzyme was evaluated in Solanum melongena (50 kDa) by using SDS-PAGE. Their outcomes exhibited that purified enzymes are dimers. PPO dimers were discovered in banana pulp, and 17 reports that using gradient ultra-centrifugation to separate the sucrose revealed that their molecular weight was approximately 62000. The different banana kinds that were used could be the cause of the molecular weight difference. The molecular weight is lower than that of the PPO enzyme from pears (Japanese) and chlorogenic acid oxidase from apples (Tono et al., 1986), which were found to be approximately 65000 and 56000, respectively 18.

When 4-methylcatechol was used as a substrate, the ideal pH of Solanum melongena was determined to be 6.5 and 7.8, respectively. The ideal pH range for longan extract with a variety of substrates was found to be 4.0 to 7.0. At 7.0, pH was more constant in Longan 19. Research showed that the ideal pH for apricot, apple, and aubergine was 7.0, while the pH for potatoes and aubergine was measured at 6.5 and 6.4, respectively. Apple and Apricot PPOs showed the highest enzymatic activity in alkaline and neutral pH and were significantly reduced in acidic pH. Additionally, potatoes exhibited decreased activity in an alkaline pH environment and greater activity in an acidic pH situation 20. The majority of plant polyphenol oxidases often exhibited their highest activity at a pH of neutral 21. PPOs' ideal pH is determined by a number of variables, including the source of the enzyme, the isolation technique, the substrate, temperature, and buffer concentration 22.

The measurement of PPO activity was conducted in a temperature range of 10–80 °C, which was shown to be ideal. 50°C was the ideal temperature for Solanum melongena's PPO enzyme. In previous studies, the activity of apricot potato, apple, and eggplant for the enzyme PPO was assayed at temperatures in the 10 to 30°C range. The activity was found to be very influential. The PPO enzyme obtained from apple as well as apricot exhibited maximum activity at 21°C and potato and eggplant at 20°C. Further than these temperature values, the activity was reduced rapidly 23. The optimal temperature changed due to the type of substrate, that was used. It was investigated the effect of 7 dissimilar substrates on Ankara pear at the optimum temperature. They originated the idea that optimum temperatures ranged from 20°C (catechol) to 54°C (D-tyrosine) 24.

In another experiment, the temperature stability of the enzyme PPO was measured at different temperatures. The thermal stability of the enzyme (PPO) was 30–70 °C for Solanum melongena. According to the literature, in potatoes, the enzyme PPO was found to be more constant than in apples, eggplant, and apricots. Following an hour of incubation at 60 °C, they set aside 35%, 22%, 14%, and 63% of their activities, respectively. The enzymes from potatoes and eggplants showed 41% and 26% activity after 30 minutes at 64 °C, respectively, but the PPOs from apples and apricots were totally denatured in the same conditions. It is generally recognised that fruit PPOs (apple and apricot) have less temperature stability than vegetable PPOs (potato and eggplant). Fruits were denatured by the heat treatment (65°C for fruits and 75°C for vegetables) for 15 minutes, and enzymatic browning was stopped, which was placed over the rising and crop storage. PPO from varied sources has diverse temperature stabilities 25. The longan PPO enzyme has sensible thermal stability. Its half-life was at 50°C for 25 minutes 26. PPO was mostly stable in bananas, and it retained its whole activity after 30 minutes of incubation at 55°C 27. The activity dropped at various temperatures, which led to a change in the enzymatic structure that postponed the enzyme's active sites' availability and may have induced denaturation. increasing temperature for the PPO enzyme's activity due to the mixer effect. A decrease in activity at elevated temperatures may alter the enzyme's denaturation process, which breaks down the active sites 28. The stability value of PPO enzymes isolated from Solanum melongena was measured at different pHs. PPO in Solanum melongena remained stable between pH 3.0 and 7.0. Results were compreable with other PPOs isolated from other vegetables and fruits. The crude PPO that was extracted from apricots required an hour at pH 4–9 using an appropriate buffer solution. PPO enzyme activity peaked at pH values between 7 and 7.5, and it demonstrated notable stability between 5 and 9, where it retained 75% of its activity. Apple enzyme was largely constant within the range of 6–9. PPO in the aubergine exhibited strong stability over a wide pH range of 5–8.5. After an hour of incubation, it still exhibits 90% of its initial activity. The ideal pH for potatoes was seven, and the range between 5.5 and 7.5 had the highest values of pH stability. The alkaline pH significantly hampered its stability 29. In order to clarify the influence that pH has on enzyme activity and its stability, it is necessary to make use of the information that the protein structure of an enzymatic molecule was infected by the acidity or alkalinity of the combination, as its diversity of amino acid remainders were in different

forms of ionization 30. The enzyme revealed a range of configurations from various sources that result in various pH stabilities. The four distinct investigations all shared the same finding, which was that PPOs were less stable at pH values below 5, which is the range where enzyme molecules denaturate quickly. The same result was found with Chinese chestnut water, indicating that the freshly cut fruit's attraction to the acidic solution hampered PPO and stopped browning. The kind, source, and clarity of the enzyme were among the variables that affected its stability 31. In Longan's fruit, the PPO stability was first raised at pH values between 4.0 and 7.0 and then lowered between pH values of 7.0 and 8.0 32. The enzymatic activity was measured with several substrate types. There were three kinds of substrates used: tyrosine, 4-methylecatechol, and catechol. Using substrate as a catechol, PPO demonstrated action in Solanum melongena up to 1.210 U/ml. However, the PPO activity of Solanum melongena was 0.430 U/ml with 4-methyl catechol. In Solanum melongena, the tyrosine activity was 0.675 U/ml. Additionally, the distinct actions of PPO derived from aubergine were examined in relation to two substrates: catechol and 4-methyl catechol. O-dihydroxyphenyls and monohydroxyl phenolic compounds were examined in the substrate specificity test. 4-Various aubergine formulations were used to oxidise methylcatechol and catechol, however L-tyrosine did not cause any response. The findings indicate that a diphenol oxidase should be the aubergine PPO enzyme. Bartlett pears and Kiwi fruit showed comparable outcomes 33. It was discovered that 4-methyl catechol for cultivar II and catechol for cultivar I were the most efficient substrates 34.

Various dietary inhibitors were employed to suppress the enzymatic activity of PPO. Various agents, including coffee, honey, black tea, and cinnamon powder, were employed to prevent the vegetable from browning. The PPO enzyme was suppressed more effectively by coffee and cinnamon powder than by honey and black tea. Marketly important edible plant foods were shown to be "prone to unpleasant browning responses containing fruits and vegetables such as apples 35, cucumbers 36, bananas, grapes 37, and avocados 38, according to certain research. Apple juice has already been amplified with success using honey. Because honey's protein components formed a compound with polyphenolic tannins, honey exhibited inhibition 39. Honey was used in grape juice, in apple slices to keep them from browning (Lee, 1990), and in grapes to make light raisins 40. Various commercial chemical inhibitors were employed to impede the vegetable's enzymatic activity. Four distinct inhibitors, including acetic acid, ascorbic acid, oxalic acid, and NaCl, were employed. Both ascorbic acid and acetic acid were more effective in lowering the enzymatic activity of PPO. The results of previous research demonstrated that L-cysteine was an effective PPO inhibitor. L-cysteine was able to prevent the browning process by reacting with o-quinones, which resulted in the production of colorless and stable molecules. However, large concentrations of L-cysteine would result in an unpleasant odor. There was also a study that citric acid was able to efficiently block the activity of PPO. It was the copper chelation that was located at the PPO's active site that was responsible for their inhibitory impact, which also resulted in a decrease in the pH value 41.The reduction in pH and the chelation of copper at the "active site" of the PPO were the respective factors that led to their inhibitory effect.41. Enzymatically generated o-quinones were reduced by ascorbic acid and then polymerized to form brown pigments that stabilised the browning process without affecting the enzyme's activity. Because ascorbic acid was permanently oxidised by the reaction with dye intermediates and endogenous enzymes, the ascorbic acid effect was only thought to be transitory 42.

5 | CONCLUSIONS

The results obtained from the enzyme assay and its kinetic characterization provide valuable insights into the activity and expression of the oxidative enzyme in Solanum *melongena*. The findings of the current study revealed that browning activity of the PPO enzyme could be inhibited more readily by using organic inhibitors than synthetic inhibitors. The most adequate inhibitors of the PPO were cinnamon powder, coffee and honey. The findings of this research have potential to offer practical applications in the agriculture and food industry, ultimately benefiting both producers and consumers.

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