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Comparison of the biochemical properties and enzymatic synthesis of theaflavins by soluble and membrane-bound polyphenol oxidases from tea (*Camellia sinensis*) leaves

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Abstract

Polyphenol oxidase (PPO) plays a key role in tea processing. It catalyzes the conversion of tea polyphenols into theaflavin and its derivatives. PPO was partially purified and characterized in both its soluble form (sPPO) and membrane-bound form (mPPO) from tea (*Camellia sinensis*) leaves. Both forms were purified by three-phase partitioning and membrane ultrafiltration. sPPO and mPPO showed high activity against diphenols as substrate and had the highest affinity for catechol and caffeic acid. The optimum temperatures and pH for enzyme activity were different. mPPO was more stable than sPPO in the acidic pH range. Among the chemical inhibitors studied, oxalic acid exerted the highest inhibitory effect on mPPO, whereas EDTA showed the highest inhibitory effect on sPPO. Both sPPO and mPPO showed similar enzymatic synthesis rates in the formation of theaflavin-3'-gallate and theaflavin-3,3'-gallate. At high concentrations of the substrate, the synthesis of theaflavins was inhibited due to the presence of high levels of ester catechins. However, mPPO showed stronger stability against inhibition by ester catechins than sPPO.

Keywords: *Camellia sinensis*; polyphenol oxidase; membrane-bound; enzymatic properties; theaflavins.

Practical Application: Two forms polyphenol oxidase can be involved in the quality formation of black tea.

1 Introduction

Tea (*Camellia sinensis*) leaves contain multiple secondary metabolites, such as polyphenols, caffeine, and amino acids, which are beneficial to human health (Tao et al., 2016; Xia et al., 2017). According to the manufacturing process, tea can be divided into three major types: non-fermented green tea, semi-fermented oolong tea, and fully fermented black tea. Black tea is the most commonly consumed tea beverage worldwide, and a key step in the manufacturing process of this tea type is "fermentation", which leads to the peculiar flavor of black tea (Stodt et al., 2014). Interestingly, during the processing of black tea, polyphenol oxidase (PPO) plays an active role in oxidizing tea polyphenols to o-quinones and later form theaflavins (TFs), thearubigins (TRs), and theabrownins (TBs) (Pereira-Caro et al., 2017). Catechins are the main components of tea polyphenols in green tea or fresh tea leaves and include catechin (C), epicatechin (EC), gallocatechin (GC), catechingallate (CG), epigallocatechin (EGC), gallocatechin gallate (GCG), epicatechingallate (ECG), and epigallocatechin gallate (EGCG). They possess a wide variety of biological activities, such as antioxidant activity, anti-inflammatory activity, and prevention of cardiovascular and cerebrovascular diseases (Isemura, 2019; Liu & Yan, 2019). TFs are the reddish-orange pigments present in black tea and are responsible for its color, and taste. So far, more than 20 types of TFs have been identified and the four main TFs are theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3'-gallate (TF-3'-G), and theaflavin-3,3'-gallate (TFDG) (Takemoto & Takemoto, 2018). However, black tea contains only 2-20 g/kg of TFs, and thus, it is important to focus on enhancing the TFs contents during tea processing.

PPO is commonly found across all phylogenic scales and contains copper in its structure (Mishra et al., 2012). It is encoded and controlled by multiple nuclear genes, which vary in number and type among different genetic resources (Zeng et al., 2019). PPOs play important physiological and metabolic roles in tea plants and are involved in enzymatic oxidation. They also affect various tea characteristics, including color, aroma, and taste, through the inhibition or enhancement of enzyme activities during tea processing (Guo et al., 2021; Zhang et al., 2020). In plants, PPO can exist in a membrane-bound (mPPO) or active soluble (sPPO) form. The spontaneous release of mPPO due to activation or ripening and progression senescence may result in the sPPO form (Zaini et al., 2013). Most reports on tea PPO have focused on the isolation, identification, and functional characterization of the soluble form (Ke et al., 2021; Öztürk et al., 2020; Zeng et al., 2019). Our research team mainly focused on the enzymatic activity of PPO isozymes and their enzymatic synthesis of theaflavins, and has published some related studies on tea PPO isozymes (Teng et al., 2017; Teng et al., 2021). However, research on mPPO in tea leaves is still scarce. Furthermore, three-phase partitioning (TPP) is a method for extracting, purifying, and concentrating proteins by using *t*-butanol and ammonium sulphate. It is a simple, rapid, and inexpensive technique that can be applied in protein purification processes (Dong et al., 2020). This study aimed to investigate the purification and characterization of sPPO and mPPO extracted from fresh tea leaves using the TPP method. The enzymatic properties and theaflavin synthesis by these enzymes were also explored.

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2 Materials and methods

2.1 Materials

One bud and two leaves of *C. sinensis* cv. Longjing No. 43 were picked from the experimental tea garden of Jiangxi Agriculture university (Nanchang, Jiangxi Province, China). The harvested leaves were immediately frozen in liquid nitrogen and were stored at −80 °C.

Catechins and theaflavin derivatives standard [(+)-catechin, C; (−)-gallocatechin, EC; (−)-epigallocatechin, EGC; (−)-epicatechin gallate, ECG; (−)-epigallocatechin gallate, EGCG; (−)-catechin gallate, GCG; theaflavin (TF); theaflavin-3-gallate (TF-3-G); theaflavin-3'-gallate (TF-3'-G), and theaflavin-3, 3'-digallate (TFDG) were obtained from Sigma-Aldrich (St. Louis, Mo., USA). Ultrafiltration membranes (15 kDa cut-off) were purchased from Merck Millipore Co. (Billerica, MA, USA). The Modified BCA Protein Assay Kit was purchased from Sangon Biotechnology Co., Ltd (Shanghai, China). All chemicals and organic solvents used were of either HPLC or analytical grade.

2.2 Crude enzyme extraction

The method described by Han et al. (2019) was used to extract and purify the two PPO forms. Tea leaves (20 g) were homogenized in cold 50 mmol/L disodium phosphate-citric acid buffer (pH 6.8) containing 2% crosslinked polyvinylpyrrolidone (PVPP), 30 mmol/L ascorbic acid, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) in a ratio of 1 : 2 (w/v) for 3 min. The resulting slurry was centrifuged at 11,000 × g for 20 min at 4 °C. The collected supernatant contained crude sPPO. The residue was rinsed 5 times with deionized water and then solubilized in 50 mmol/L Tris-HCl buffer (pH 6.8) containing 0.25% Triton X-100; the mixture was stirred for 1 min. The homogenate was ultrasonicated (SBI-54DT ultrasonic equipment, Ningbo Xingzhi Biotechnology Co., Ltd., Zhejiang Province, China) for 10 min, incubated at 4 °C for 1 h, and centrifuged at 11,000 × g for 15 min. The supernatant was then subjected to temperature-induced phase partitioning at 25 °C and centrifuged at 11,000 × g for 15 min. The clear supernatant contained crude mPPO.

2.3 The TPP for sPPO and mPPO purification

The purification of PPO by TPP was performed according to the method described by Alici & Arabaci (2016) with some modifications. Initially, 15% (w/v) saturated ammonium sulphate was added to the PPO crude enzyme extract at 25 °C. The pH of the reaction mixture was adjusted to 6.5 and then *t*-butanol was added to the mixture at a ratio of 1 : 1 (v/v). The mixture was vortexed gently for 50 s and then incubate at 25 °C for 60 min. The mixture was centrifuged at 5000 × g for 15 min to promote phase separation. After removing the upper *t*-butanol layer and the lower water layer, the PPO-containing precipitate at the interface was collected. The interface precipitate was dissolved in 0.1 mol/L phosphate buffer and the pH was adjusted to 7.0. The PPO activity and total protein content of these two phases were then analyzed by spectrophotometry. After the first cycle of TPP, it was observed that most of the PPO enzyme was enriched in the water phase. Therefore, a second TPP cycle similar to the first cycle was performed to collect the PPO enzyme at the interface. The PPO enriched phases were pooled and concentrated in a single fraction using membrane ultrafiltration (molecular weight cut-off of 15 kDa) driven by centrifugal force of 4500 × g at 4 °C.

2.4 Determination of enzyme activity and protein concentration

sPPO and mPPO activity was determined by a colorimetric method (Teng et al., 2017) with some modifications. The total volume (250 µL) of the reaction mixture consisted of 0.1 mol/L phosphate-citric acid buffer (pH 5.6), 1.0% catechol in a ratio of 10 : 3 (v/v), and 50 µL of the crude enzyme solution. After incubating at 37 °C for 10 min, PPO activity was calculated; one unit of PPO enzymatic activity was defined as an increase in absorbance at 410 nm by 0.001 per minute.

Protein content was determined on a microplate reader using the Modified BCA Protein Assay Kit (Sangon Biotechnology Co., Ltd, Shanghai, China) in accordance with the manufacturer's instructions.

2.5 Native and Sodium Dodecyl Sulphate (SDS)-polyacrylamide gel electrophoresis

Polyacrylamide gel (7%) and sodium dodecyl sulphate polyacrylamide gel (12%) were prepared for native-PAGE and SDS-PAGE, respectively. After electrophoresis, the gel was dyed with Coomassie Brilliant Blue R-250 for SDS-PAGE and incubated in 10 mM catechol solution at 35 °C for 30 min for native-PAGE. The apparent molecular weight of the enzyme was estimated using pre-stained molecular weight markers (Solarbio, Beijing, China).

2.6 Substrate specificity and kinetic parameters

To determine sPPO and mPPO Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}), catechol, gallic acid, guaiacol, and caffeic acid were used as substrates at various concentrations in the range of 10-50 mmol/L. Maximum absorption wavelengths were determined under the optimum reaction conditions by performing UV spectrophotometric scanning. K_m and V_{max} were calculated using the Michaelis-Menten equation and the Lineweaver-Burk plot (Equations 1-2):

$$v = V_{\max}[S]/(K_m + [S]) \quad (1)$$

$$1/v = K_m/[S]V_{\max} + 1/V_{\max} \quad (2)$$

where v is the reaction rate and [S] is the substrate concentration.

2.7 Optimum temperature and thermal stability

To determine the optimum temperature for the enzyme activity of sPPO and mPPO, their activity was measured using the method described in section 2.4 at a temperature range of 25 °C to 75 °C. The temperature at which the maximum enzyme activity was observed was considered the optimum and equalized to 100%. The relative enzyme activities at other temperatures

were subsequently calculated based on that recorded for the optimum. The assays were performed in triplicates.

To establish thermal stability of PPO, 1.0% catechol was dissolved in 250 μL phosphate-citric acid buffer (pH 5.6) and 50 μL-samples of sPPO or mPPO solutions were subsequently added. The reaction mixtures were incubated in a water bath at various temperatures (25-75 °C) for different times between 10 min to 60 min. Then, the enzyme activity was measured, and the residual activities with respect to the original activity were calculated. The assays were performed in triplicates.

2.8 Optimum pH and enzyme stability at different pH ranges

The optimum pH for sPPO and mPPO was determined based on the activity measured at various pH values in the range of 3.0 to 9.0. The pH of the reaction mixture was adjusted using different buffer solutions at 50 mmol/L concentration (acetate buffer, pH 3.0-5.5; phosphate buffer, pH 6.0-7.5; and Tris-HCl buffer, pH 8.0-9.0), in which 1.0% catechol was dissolved. The reaction mixtures consisted of 250 μL of these buffer solutions and 50 μL of the partially purified PPO. After incubating at 37 °C for 10 min, sPPO and mPPO activities were measured. The pH at which the maximum enzyme activity was observed was considered the optimum and equalized to 100%, and the relative enzyme activities under other pH values were subsequently calculated based on that recorded for the optimum. The assays were performed in triplicates.

To assess the enzyme stability at different pH values, 50 μL sPPO or mPPO solutions were incubated at various pH values adjusted by the buffer solutions mentioned above. After incubating the mixtures at 4 °C for 12 h, enzyme activities were measured, and the residual activities with respect to the initial activity were calculated. The assays were performed in triplicates.

2.9 Effect of inhibitors

Effects of various inhibitors on the activity of sPPO and mPPO were determined according to a previously described method (Han et al., 2019) with some modifications. The inhibitors tested were ascorbic acid, EDTA, citric acid, oxalic acid, sodium chloride, potassium iodide, and trisodium azide; these compounds were added to the reaction mixture at the following final concentrations: 0.1, 1, 3, 5, and 10 mmol/L. Their effects on sPPO and mPPO activity were examined separately. The percentage inhibition was calculated according to the following expression: $[(A_0-A_i)/A_0] \times 100$, where A_0 is the initial activity of sPPO or mPPO without the inhibitor and A_i is the activity of mPPO or sPPO with the inhibitor.

2.10 sPPO and mPPO enzymatic synthesis of theaflavins

Purified sPPO and mPPO solutions were mixed with various catechins substrate concentration as reaction solution. The reaction substrate was prepared at different ratios of 1 : 50, 1 : 40, 1 : 30, 1 : 20, 1 : 10, and 1 : 1 [catechins substrate (Table S1): 0.1 mol/L phosphate buffer, v/v]. After the enzymatic reactions proceeded for 40 min at the optimal pH and temperature, the reaction mixtures were placed in boiling water for 10 min to terminate the reaction. The reaction mixtures were centrifuged (3500 × g, 4 °C, and 10 min) and the supernatants were subjected to HPLC analysis to detect theaflavins. For the detection of theaflavins, a Shimadzu LC-20A HPLC (Kyoto, Japan) supplied with an ODS C18 column (4.6 mm × 250 mm, 5 μm; Shimadzu, Kyoto, Japan) was used. The eluate was monitored at 280 nm at a column temperature of 40 °C. A gradient elution system was followed using solvent A (Milli-Q water with 2.0% acetic acid) and solvent B (7:3 of acetonitrile: ethyl acetate, v/v). The following gradient method was used: 0-30 min, 18% to 30% B, followed by 18% for 10 min. The concentration of each sample was calculated based on the retention time and the area of the standard.

2.11 Statistical analysis

All data were subjected to analysis of variance using SPSS (21.0, SPSS Inc., Chicago, IL, USA). The statistical analyses were performed using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test and Duncan's test.

3 Results and discussion

3.1 sPPO and mPPO isolation and partial purification

Table 1 summarizes the results of the overall purification of sPPO and mPPO from tea leaves. Using the TPP and membrane ultrafiltration methods, sPPO was purified to 9.58-fold with a recovery yield and specific activity of 31.04% and 1595.26 U/mg, respectively. Approximately 4.01 mg of protein was purified from 123.77 mg of crude sPPO protein. Similarly, mPPO was purified to 9.05-fold with a recovery yield and specific activity of 23.39% and 2104.35 U/mg, respectively. Approximately 2.53 mg of protein was purified from 97.85 mg of crude mPPO protein. Different isolation and purification protocols have been reported, and the purification fold depends on the methods used, localization of enzyme, and species (Panadare & Rathod, 2018). PPO from borage plants was purified to 3.59-fold using the TPP purification technique (Alici & Arabaci, 2016); PPO from *A. paeoniifolius* was purified to 5.54-fold using unbound DEAE anion exchange chromatography (Singh & Wadhwa, 2017); mPPO from Fuji apple was purified to 64.29-fold using the temperature-induced phase partitioning and ion exchange chromatography. However, crude sPPO from Fuji apple was purified to 52.89-fold using 50-80% ammonium sulphate precipitation method (Liu et al., 2015).

3.2 Molecular weight

The SDS-PAGE analysis revealed that the major protein constituents of purified sPPO was distributed in a rather wide range of molecular weight about 30-150 kDa (Figure 1a). A single characteristic band was obtained with a molecular weight of about 78 kDa. Similarly, purified mPPO was also distributed in a rather wide range of molecular weight about 70-175 kDa (Figure 1a). A single characteristic band was obtained with a molecular weight of about 90 kDa. Despite these similarities in an SDS-PAGE analysis, sPPO and mPPO protein profiles differed in native-PAGE. sPPO was separated as three bands, while mPPO was separated as two bands (Figure 1b). The Native-PAGE gel of sPPO and mPPO was stained with catechol. Catechin oxidation

Table 1. Summary of purification procedure of sPPO and mPPO from tea leaves.

	Step	Total Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Activity yield (%)
sPPO	Crude extract	12.0	20612	123.77	166.53	1.00	100.00
	TPP	4.0	15214	32.67	465.69	2.80	73.81
	Ultrafiltrate	1.5	6397	4.01	1595.26	9.58	31.04
mPPO	Crude extract	12.0	22758	97.85	232.58	1.00	100.00
	TPP	4.0	18149	33.66	539.19	2.32	79.75
	Ultrafiltrate	1.5	5324	2.53	2104.35	9.05	23.39

TPP: three-phase partitioning.

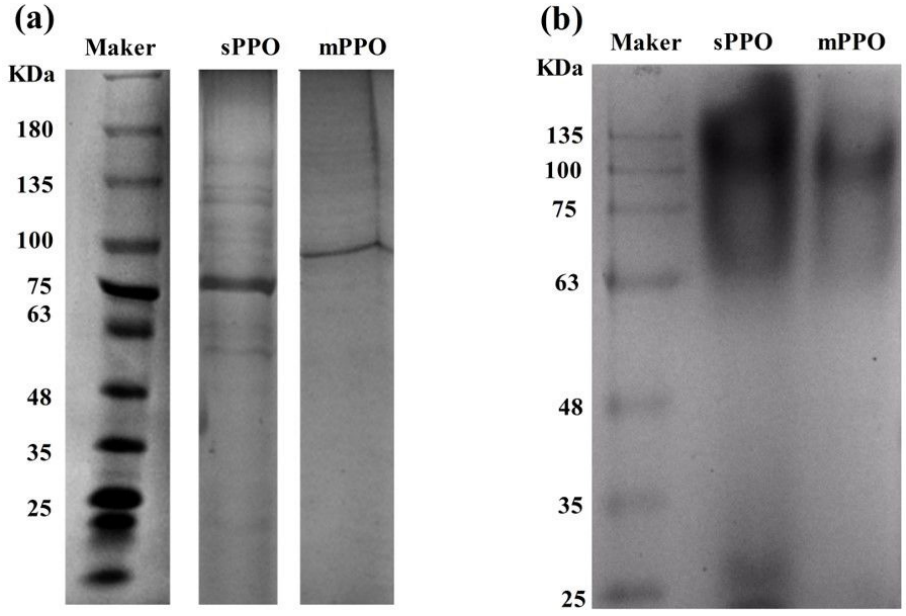

Figure 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of (a) purified soluble polyphenol oxidase (sPPO) and membrane-bound polyphenol oxidase (mPPO) from tea leaves. Native-PAGE of (b) sPPO and mPPO stained with catechol.

by peroxidases requires the presence of hydrogen (Sang et al., 2004). However, catechol is most possibly oxidized by PPO and their catalytic ability does not depend on the presence of cofactors or hydrogen peroxide. Therefore, cofactors were not added to the reaction mixtures in this study. Our results revealed that tea sPPO and mPPO exist in different isoforms. The molecular weights of sPPO isoforms were in the range of 35-180 kDa. This observation was similar to the previously reported molecular weight for PPO isozymes (42 kDa and 85 kDa) (Teng et al., 2017). The third sPPO isoform was reported to have a molecular weight greater than 135 kDa, possibly corresponding to protein aggregates reported in the previous analysis on black tea (Ke et al., 2021). mPPO primarily appeared as a band of approximately 135 kDa, characteristic of protein aggregates.

3.3 Substrate specificity and kinetic parameters

The Michaelis-Menten plots for sPPO and mPPO activity in the presence of catechol (diphenol), gallic acid (triphenol), guaiacol (monophenol), and caffeic acid (diphenol) as substrates are shown in Figure S1a and Figure S1b. A higher sPPO activity was observed when the substrate was catechol. However, mPPO exhibited a substrate preference for both catechol and caffeic acid. In addition, sPPO and mPPO showed a very low activity when guaiacol was the substrate. These results are similar to those reported in previous studies on the activity of PPO from apricot (Derardja et al., 2017) and snake fruit (Zaini et al., 2013).

Linear regression analysis results of the reciprocal of enzyme activity versus the reciprocal of substrate concentration for sPPO and mPPO are presented in Figure S1c, and Figure S1d, respectively. K_m, V_{max}, and K_{cat} for different substrates were determined by plotting the activities at optimum pH and temperature as a function of substrate concentration. As shown in Table 2, the K_m values of sPPO for different substrates were in the range of 42-214 mM and for mPPO, they were in the range of 21-98 mM. The smaller the K_m value, the greater the affinity for the substrate (Şener & Ünal, 2011). Hence, these results suggest that mPPO showed greater substrate affinity than sPPO. Additionally, sPPO and mPPO showed the greatest affinity for catechol and caffeic acid, respectively. These results are similar to those previously reported, demonstrating that K_m values for sPPO and mPPO vary according to the substrate. For instance, mPPO showed a higher K_m value than sPPO when 4-*tert*-butylcatechol was used as the substrate (Cabanes et al., 2007).

Table 2. Substrate specificity of sPPO and mPPO from tea leaves.

Substrates	Form	K_m (mmol/L)	V_{max} (mM min^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
Catechol	sPPO	42.07 ± 3.6	$2.23 \times 10^3 \pm 120$	$10.6 \times 10^5 \pm 46$	$2.52 \times 10^4 \pm 74$
	mPPO	64.07 ± 4.2	$3.16 \times 10^3 \pm 135$	$18.8 \times 10^5 \pm 73$	$2.94 \times 10^4 \pm 61$
Gallic acid	sPPO	95.37 ± 1.3	$0.56 \times 10^3 \pm 44$	$3.53 \times 10^5 \pm 31$	$0.37 \times 10^4 \pm 48$
	mPPO	39.53 ± 3.3	$1.82 \times 10^3 \pm 117$	$14.1 \times 10^5 \pm 68$	$3.57 \times 10^4 \pm 69$
Guaiacol	sPPO	214.05 ± 12.0	$0.45 \times 10^3 \pm 48$	$5.88 \times 10^5 \pm 77$	$0.27 \times 10^4 \pm 50$
	mPPO	98.36 ± 4.8	$0.16 \times 10^3 \pm 65$	$2.35 \times 10^5 \pm 44$	$0.23 \times 10^4 \pm 77$
Caffeic acid	sPPO	61.85 ± 3.5	$0.44 \times 10^3 \pm 90$	$4.71 \times 10^5 \pm 69$	$0.76 \times 10^4 \pm 45$
	mPPO	21.47 ± 0.6	$0.11 \times 10^3 \pm 15$	$5.88 \times 10^5 \pm 50$	$2.74 \times 10^4 \pm 53$

All data were expressed as the mean values ± standard deviation (n = 3).

In beet root, the K_m value for sPPO was higher than that obtained for mPPO when *L*-3, 4-dihydroxyphenylalanine was used as the substrate (Gandía-Herrero et al., 2004). mPPO from loquat fruit showed a higher K_m value when chlorogenic acid was used as the substrate (Sellés-Marchart et al., 2006). The catalytic efficiency of an enzyme, which is the ratio of k_{cat} to K_m, is commonly used to compare the relative rates at which an enzyme acts on alternative substrates under different conditions. The higher the value of the k_{cat}/K_m ratio, the better the enzyme reacts with that substrate (Alici & Arabaci, 2016). The highest K_{cat}/K_m ratio for sPPO was obtained with catechol as the substrate suggesting that it is the most suitable substrate. Both sPPO and mPPO showed the highest V_{max} values with catechol as the substrate. However, a higher reaction velocity was observed mPPO ($V_{max} = 3.16 \times 10^3$ mM/min) than for sPPO ($V_{max} = 2.23 \times 10^3$ mM/min) with catechol as the substrate.

3.4 Optimum temperature and thermal stability

Temperature is an important factor that significantly affects the catalytic activity of PPO. The effect of temperature in the range of 20-75 °C on the relative activity of sPPO and mPPO is shown in Figure 2a. Optimum temperatures for the activity of sPPO and mPPO were 30 °C and 25 °C, respectively. With the increase in temperature, the relative activities of sPPO and mPPO gradually decreased. PPO was almost completely deactivated at 75 °C. These results are similar to the previously reported for 35 °C optimum temperature for PPO from tea leaves (Ke et al., 2021).

The thermostability profiles of tea sPPO (Figure 2b) and mPPO (Figure 2c) in the range of 25-75 °C were assessed. sPPO and mPPO showed high stability after 60 min of incubation at 25 °C and 35 °C. Additionally, mPPO retained 50% of its original activity when incubated at 45 °C, 55 °C, and 65 °C for 30 min. However, sPPO retained 50% of its original activity when incubated at 45 °C and 65 °C for 40 min. Higher thermostability for sPPO than mPPO was also reported in apple (Liu et al., 2015). The fact that mPPO is an immature precursor of sPPO supports this observation.

3.5 Optimum pH and pH stability

The effect of pH on sPPO and mPPO activity was determined over a pH range of 3.0-9.0 (Figure 2d). Both sPPO and mPPO had the same optimum pH value (5.5) and approximately 10% of the maximal activity was detected for both enzyme forms when the pH was above 7.0. A similar result was reported for the optimum pH of PPO activity (pH 6.0) and a high enzyme activity over a broad pH range (4.0-7.0) in tea leaves (Ünal et al., 2011). However, mPPO showed another pH optimum of 6.5. Different pH optima for PPO activity suggests the presence of PPO isozymes. Similar results have been reported in other studies. The optimum pH for PPOI and PPOII isozymes was reported to be 4.5-5.0 and 5.5 in sprouts, respectively (Sikora et al., 2019).

The stability of sPPO and mPPO at various pH values was determined. As shown in Figure 2e, the relative activity of mPPO was over 50% only at a pH value under 4.0. Therefore, this observation suggests that mPPO is stable in the acidic pH range. However, sPPO was more stable than mPPO under alkaline conditions, with over 50% of relative activity. These results indicate that mPPO was less stable over a broad pH range than sPPO.

3.6 Effect of inhibitors

Different compounds were selected to determine their inhibitory effect against sPPO and mPPO (Table 3). The most effective sPPO inhibitor was EDTA ($IC_{50} = 4.354 \pm 0.67$ mmol/L); however, mPPO activity increased with EDTA. Similar findings were reported for apple (Liu et al., 2015) and apricot (Derardja et al., 2017). In addition, oxalic acid, trisodium azide, citric acid, and ascorbic acid had inhibitory effects on sPPO activity at concentrations below 10 mmol/L. However, mPPO activity was inhibited by oxalic acid, citric acid, and ascorbic acid only at high concentrations (IC_{50} = 12.08 ± 1.66 mmol/L, 20.63 ± 2.83 mmol/L, and 41.36 ± 2.76 mmol/L, respectively). Sodium chloride and potassium iodide had no inhibitory effects on the activity of sPPO and mPPO. Previously research also found that sodium chloride might not be good inhibitors for ginger PPO as low inhibition percentage was obtained (Lim & Wong, 2018). In this study, mPPO was more resistant to inhibitors than sPPO.

Ascorbic acid and citric acid were reported to show the highest inhibitory effect on snake fruit (Zaini et al., 2013) and apricot (Derardja et al., 2017) mPPOs. This indicates that o-quinone produced by the activity of PPO is reduced by ascorbic acid to

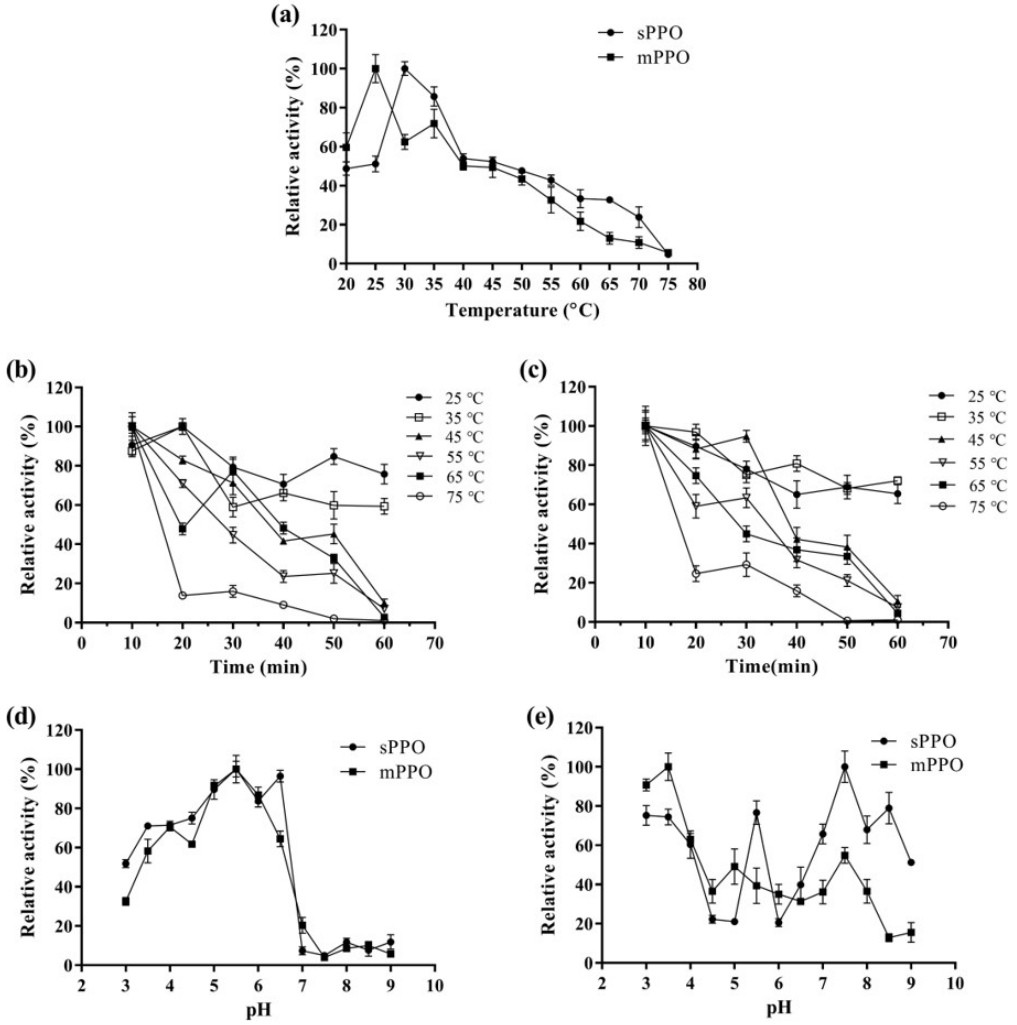

Figure 2. Effect of temperature and pH values on the activity and stability of soluble polyphenol oxidase (sPPO) and membrane-bound polyphenol oxidase (mPPO). (a) Determination of optimal temperature. Effect of temperature on the stability of (b) sPPO and (c) mPPO. Determination of (d) optimal pH and (e) pH stability of sPPO and mPPO.

Table 3. Effects of various inhibitors on the activity of sPPO and mPPO.

Inhibitor	sPPO		mPPO	
	IC_{50} (mmol/L)	R^2	IC_{50} (mmol/L)	R^2
Reducing agents				
Ascorbic acid	8.94 ± 0.79^{a}	0.91	41.36 ± 2.76^{a}	0.84
Chelating agents				
EDTA	4.35 ± 0.67^{d}	0.94	Activated	/
Citric acid	7.03 ± 0.79^{b}	0.97	20.63 ± 2.83^{b}	0.90
Oxalic acid	4.78 ± 0.68^{cd}	0.98	12.08 ± 1.66^{c}	0.95
Halides				
Sodium chloride	/	/	/	/
Potassium iodide	/	/	/	/
Sodium azide	6.07 ± 0.82^{bc}	0.92	/	/

/: no inhibition detected even at a concentration of 100 mmol/L. Activated: activity of mPPO increased at an EDTA concentration of 50 mmol/L. All data were expressed as the mean values ± standard deviation (n = 3); different letters correspond to significant differences at a level of $P < 0.05$.

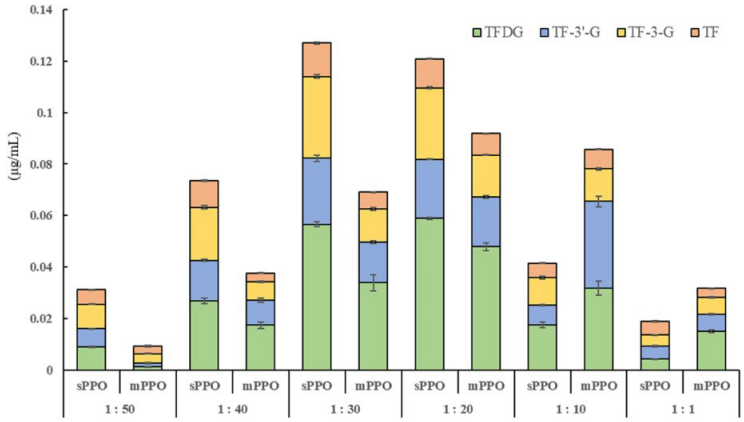

Figure 3. Effects of different concentrations of catechins on the enzymatic formation of theaflavins by soluble polyphenol oxidase (sPPO) and membrane-bound polyphenol oxidase (mPPO). TF: theaflavin; TF-3'-G: theaflavin-3'-gallate; TF-3-G: theaflavin-3-gallate; TFDG: theaflavin-3,3'-gallate. Each value is expressed as mean ± standard deviation (n = 3).

phenolic compounds before undergoing a secondary reaction that leads to browning (Gawlik-Dziki et al., 2008). In addition, chelating agents such as citric acid and oxalic acid probably inhibit PPO activity by the removal of copper from the enzyme (Benaceur et al., 2020).

3.7 sPPO and mPPO enzymatic synthesis of theaflavins

PPO-mediated synthesis of theaflavins was assessed using an *in vitro* oxidation experiment, in which various catechins at different concentrations were assayed (Figure 3). sPPO and mPPO also could catalyze the synthesis of TF, TF-3-G, TF-3'-G, and TFDG at different concentrations of catechins substrates. Both sPPO and mPPO could catalyze the synthesis of TF-3'-G and TFDG to higher concentrations. According to a study by Hua et al. (2021), the presence of a high ratio of EGC and EGCG among the catechins substrates could contribute to a higher synthesis of these TF products. It was observed that sPPO and mPPO have similar effects of PPO synthesis TFs. With an increase in the concentration of substrate, the total synthesis of the four TFs increased initially, followed by a decrease in their synthesis. For sPPO and mPPO, the peak levels of TFs formation were obtained at 1 : 30 and 1 : 20 ratio of catechins reaction substrate, respectively. These results are very similar to the reports on the inhibitory effects of ester catechins at high concentrations on the activity of enzymes (Teng et al., 2017). Surprisingly, at a high concentration of the substrate, mPPO could synthesize a higher content of TFs than sPPO. With the increase of the catechins substrate concentration in the reaction solution, the content of ester catechins increased more than that of non-ester catechins. This suggests that mPPO has stronger stability against inhibition by ester catechins. However, further studies are required to establish this.

4 Conclusion

The present study sheds light on the functional characterization of sPPO and mPPO enzymes isolated from tea leaves and the biochemical similarities and differences between them. Both the enzymes showed increased activity with diphenols as substrates and reduced activity with monophenols as substrates. The optimum pH for catalytic activity of both enzymes was 5.5, and they exhibited similar sensitivity to various inhibitors. The differential response of both forms to EDTA (sPPO inhibition and mPPO activation) and greater stability of mPPO in the acidic pH range, and its higher affinity for the tested substrates are the major differences between sPPO and mPPO. In addition, both sPPO and mPPO have similar rates of theaflavin formation by enzymatic synthesis. However, mPPO showed stronger stability against inhabitation by easter catechins. Briefly, PPOs are essential for tea fermentation during black tea manufacturing, and the accumulated amount of mPPO in fresh tea leaves and biochemical properties of this enzymatic form may be crucial for the fermentation process. Our study provides valuable information on tea mPPO, which could help optimize the fermentation process and allow increased synthesis of theaflavin *in vitro*.

Conflict of interest

All authors declared that there is no conflict of interest.

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Supplementary material

Supplementary material accompanies this paper.

Table S1. Catechins component content as substrate in sPPO and mPPO mediated enzymatic synthesis of theaflavins.

Figure S1. Michaelis-Menten plots for the activity of (**a**) soluble polyphenol oxidase (sPPO) and (**b**) membrane-bound polyphenol oxidase (mPPO) with catechol, gallic acid, guaiacol, and caffeic acid as substrates. Lineweaver-Burk plots for the oxidation of catechol, gallic acid, guaiacol, and caffeic acid by (**c**) sPPO and (**d**) mPPO.

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