Research Article

APPLIED FOOD BIOTECHNOLOGY, 2020, 7(3): 183-193
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Partial Purification, Characterization and Investigation of Inhibitory Effects of Organic Compounds on *Cinnamomum verum* Polyphenoloxidase Enzymes

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Abstract

Background and objective: Polyphenol oxidase enzyme catalyzes oxidation of *o*-diphenol to *o*-quinone using molecular oxygen, while the final product unacceptably includes brown pigments. Therefore, inhibition of polyphenol oxidase is essential for the preservation of food products and vegetables. The enzyme is clinically beneficial for the treatment of dermal disorders with links to unusual darkening of the skin (hyper pigmentation) and is helpful in development of skincare products. The present study describes characterization and inhibition kinetics of the polyphenol oxidase from *Cinnamomum verum* fruit coat.

Material and methods: Purification and quantification of polyphenoloxidase were carried out using $(NH_4)_2SO_4$ precipitation, dialysis with Sephadex G-100 column chromatography. The molecular weight was reported using SDS-PAGE. The *Km* and *Vmax* values were calculated using Lineweaver-Burk plot. The optimum pH, temperature and freeze-thaw were studied. Effects of several organic compounds on polyphenol oxidase activity were tested and IC_{50} values were calculated.

Results and conclusion: Polyphenol oxidase of *Cinnamomum verum* fruit coat has partially been purified as nearly 3.75-fold with an improvement of 4.58% using catechol as the substrate. The enzyme showed a single band with a molecular weight of approximately 66 kD. Optimal pH and temperature included 6.0 and 37°C, respectively. The *Km* and *Vmax* values included 1.67 Mm and 64.57 $\Delta A\ min^{-1}$, respectively. Inhibition type of cinnamic acid and ascorbic acid were uncompetitive while propyl benzoic acid showed a mixed type of inhibition. Thiol and chelating agents were strong inhibitors of enzyme activity. In conclusion, polyphenol oxidase can be used for the development of biosensors to detect and degrade phenolic compounds in industrial waste water.

Article Information

Article history:
Received 09 Apr 2020
Revised 26 May 2020
Accepted 14 June 2020

Keywords:
- Catechol
- Chemical agents
- *Cinnamomum verum*
- Polyphenoloxidase
- Purification

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How to cite this article

Laad S, HG P, Mirjankar M, Mulla S, Pujari N, Kamanavalli C. Partial Purification, Characterization and Investigation of InhibitoryEffects of Organic Compounds on Cinnamomum verum Polyphenoloxidase Enzymes. Appl Food Biotechnol 2020; 7(3): 183-193.
http://dx.doi.org/10.22037/afb.v7i3.29854

1. Introduction

Polyphenol oxidases (EC 1.10.3.1) (PPOs) represent a large family of copper-containing enzymes and are extensively scattered in microbes and animal and plant tissues. The PPOs have been studied in several vegetables, fruits and crustaceans [1]. These enzymes include various biochemical and anatomical functions in plants and other living organisms. The PPOs are chief enzymes in food processing industries due to their participation in enzymatic blackening of the plant parts. Using oxygen molecules, the enzymes activate oxidation of *o*-diphenol to *o*-quinone. However, the eventual polymerized products unacceptably include brown and/or black pigments [2]. Activity of PPOs

in various plant tissues demonstrates various substrate specificities to inhibitors [3]. Therefore, control of PPOs is important in inhibition of browning reactions. Additionally, PPO inhibitors are clinically beneficial in treatment of dermal disorders, associating to unusual darkening of the skin [4] and in skincare products [5]. Up-to-date, large numbers of potential PPO inhibitors have been synthesized from natural, synthetic and semi-synthetic sources. However, a few reported chemicals are used in clinical and skincare products because of their lower activities or serious side effects [6,7]. Therefore, it is necessary to investigate novel PPO inhibitors with higher activities and lower side effects. Industries usually release contaminated water containing hydroxy aromatics and their derivatives. Removal of hydroxy aromatics and their derivatives using enzymes is fascinating as enzymes include advantages over traditional biological treatments. In fact, PPO enzymes can be used for the development of biosensors to detect hydroxy aromatic compounds for various purposes. Furthermore, ability of PPOs to act on hydroxy aromatic compounds can help breaking of hydroxy aromatics in industrial waste water [8].

In this study, PPO activity in *Cinnamomum* (*C.*) *verum* fruit coat was investigated. Indeed, *C. verum* has been used since ancient times as a culinary spice and as ingredients of medicines for sore throat and cough. Cinnamon plant is commonly grown in tropical and subtropical regions of America, Australia, Asia, Oceania and Australasia, including southern parts of Western Ghats, India [9]. The plant belongs to Lauraceae family with more than 250 species consisting of tree-like shrubs. Flowers in January and fruits ripen from May to August. Color of the fruits is green, which becomes dark purple after ripening. Fruits possess a sweet spicy aroma and are one-seeded with ellipsoidal berries of 1.25-2.50 cm long. Cinnamon fruits include a large central seed covered with a thick peel and no pulps between the fruit coat (peel) and the seed. The *C. verum* plant includes three types of oils from the leaf (eugenol), stem bark (cinnamaldehyde) and root bark (camphor) [10]. In general, 34 compounds have been identified in cinnamon fruit oil, with (E)-cinnamyl acetate and (E)-caryophyllene as the essential compounds [11]. These compounds are used in perfume industries because of their good receptive and preservative properties. Water extracts of the cinnamon fruits contain high profiles of hydroxy aromatics such as protocatechuic acid, uroligniside, rutin and quercetin-3-O-A-L-rhamnopyranoside [12]. Cinnamon fruits include a large number of antioxidants, especially phenols to prevent damaging effects of free radicals, showing protection against mutagenesis [13]. Nearly all parts of the cinnamon plant include therapeutic uses and are used for the treatment of several diseases. However, the enzyme activity of cinnamon fruits has not been studied [14]. Literature studies include no reports on *C. verum* fruit coat PPOs. Understanding advantages and disadvantages, the current study purified PPOs from *C. verum* fruit coat and characterized inhibition kinetics of these enzymes.

2. Materials and methods

2.1. Materials

Catechol (1,2-benzenediol) was purchased from Merck, India. Sodium dodecyl sulphate (SDS) was purchased from Sigma, India. Coomassie brilliant blue R-250, acrylamide, methylene-bis-acrylamide, ammonium sulphate, sodium dihydrogen phosphate (NaH_2PO_4), potassium chloride**,** ethylenediaminetetraacetic acid, magnesium chloride, copper sulphate, sodium azide, ferrous sulphate, ferric chloride, 1,10-phenanthroline, dimethylglyoxime, β-mercaptoethanol, tannic acid, dithiothreitol, cinnamic acid, propyl benzoic acid, mercuric chloride, kojic acid, ascorbic acid, glutathione, polyvinylpyrrolidone were purchased from Merck, India. All chemicals included chemical grade.

2.2. Preparation of polyphenoloxidases

Unripe fruits of *C. verum* were collected from the southern part of Western Ghats (Mangalore, India). The outer coat of the fruits was peeled out, washed and air-dried. The crude was extracted using a method by Huang et al. [15]. Briefly, 25 g of the fruit coat were homogenized in 10 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% of polyvinylpyrrolidone (phenol scavenger) to prevent enzymatic phenol oxidation and polymerization of the PPO enzymes. The homogenate was centrifuged (Remi R-4C Laboratory Centrifuge, USA) at 5000 ×g for 15 min at 4°C. The clear filtrate was used for further studies.

2.3. Extraction, purification and quantification of the enzyme

Cinnamon PPO was prepared as previously described [16,17]. Supernatant of the enzyme was precipitated with $(NH_4)_2SO_4$ at 20-60% saturation for 1 h with gentle agitation. Residue of the enzyme was achieved by centrifugation at 15,000 ×g for 20 min. The enzyme residue was dissolved in 50 mM phosphate buffer (pH 7.0) and dialyzed at 4°C against 10 mM phosphate buffer (pH 7.0). Approximately, 10 ml of dialyzed extract were loaded in to Sephadex G-100 column (1.2 × 70 cm, 0.5 ml min^{-1}). Eluted fractions (2 ml) were achieved and investigated for the PPO activity and proteins. Fractions with PPO activity were pooled, dialyzed and concentrated [18,19]. The PPO activity was assessed using catechol as the substrate. The reaction mixture contained 20 µl of the purified enzyme sample, 1.6 ml of 60 mM catechol solution and 0.4 ml of 0.2 M sodium phosphate buffer (pH 6.0) at 37°C. This was incubated for 5 min and changes in absorbance were measured at 420 nm using spectrophotometric method. Generally, 1 U of enzyme

activity was measured as the quantity of enzyme that increased the absorbance of 0.001 per minute [16,20].

2.4. Denaturing SDS-PAGE and native-PAGE

Molecular weight of the purified enzyme was calculated using 10% SDS-PAGE (Biotech Model Code 05-01, India) based on the method of Davis and Laemmli [21,22]. Separating and stacking gels included 12 and 14%, respectively. Furthermore, 0.05 M of phosphate buffer at pH 7.2 (as the electrode tray buffer) and 0.02% bromophenol blue (as the tracking dye) were used. Electrophoresis of the purified enzyme from cinnamon fruit coat was carried out at 100 V for nearly 5 h. Standard protein markers (catalase, bovine serum albumin, ovalbumin, soybean trypsin and lactoglobulin) were electrophoresed as well. Native PAGE and SDS-PAGE gels were stained with 0.025% Coomassie brilliant blue R-250 in methanol-acetic acid-water (4:1:5 v v^{-1}). Gels were subsequently decolorized with methanol-acetic acid-water (4:1:5 v v^{-1}). The substrate staining of the native gel was carried out using 60 mM of the catechol substrate in 0.2 M of phosphate buffer at pH 6.0 for 3 h at ambient temperature.

2.5. Effects of pH and temperature

Effects of pH on the PPO enzyme activity were assessed using a modified method by Gulcin and Yildirim [23] and Gulcin and Koksal [24] in the range of 4.0-9.0. In general, 0.2 M citrate buffer was used at a pH range of 4.0-6.0 and 0.2 M phosphate buffer at 6.0-9.0 with 60 mM catechol as the substrate in both buffers. The PPO activity was assessed using standard reaction mixture with a different buffer. Effects of pH on PPO stability was assessed incubating 20 µl of the enzyme solution in 0.4 ml of various 0.2 M buffers with a pH range of 4.0-9.0 for 15 min at 37°C. Optimal temperature of the PPO activity was assessed by adding 1.6 ml of 60 mM catechol solution and 0.2 M of phosphate buffer at pH 6.0 to the enzyme solution. Before adding enzymes, mixture was pre-equilibrated for 5 min at various temperatures (15-75°C). Then, 20 µl of the enzyme solution were mixed with the solution and incubated at 37°C for 5 min and the enzyme residual activity was assessed. In thermal stability studies, enzyme solution was incubated at various temperatures from 15-75°C for 30 min and quickly cooled down using ice bath at 4°C for 5 min. Then, 0.1 ml of the enzyme was added to 1.6 ml of 60 mM catechol solution. The residual PPO activity was analyzed as described previously.

2.6. Effects of freeze and thaw

Effects of freeze and thaw on PPO activity were systematically assessed using crude extract. The extract was stored at 10, -20 and -40°C and thawed at regular intervals for a week. Residual PPO enzyme activity was assessed spectrophotometrically at 420 nm and compared to initial activity of the PPO enzyme.

2.7. Substrate specificity studies

For studies of maximum velocity (*Vmax*) and Michaelis-Menten constant (*Km*) values of the PPO, the enzyme activity was analyzed using various concentrations of catechol (0.3-15 mM) as the substrate under optimal pH and temperature conditions [25]. A plot of 1 $[V]^{-1}$ *vs* 1 $[S]^{-1}$ was shown based on the L-B plot [26] to calculate *Km* and *Vmax* values of the cinnamon PPO for catechol.

2.8. Effects of inhibitors on the enzyme activity

Cinnamic acid, propyl benzoic acid and ascorbic acid were dissolved in dimethyl sulfoxide and used for the experiment after a 30-fold dilution. The final concentration of dimethyl sulfoxide was 3.3% in the test solution. Then, 0.1 ml of dimethyl sulfoxide was added to the dilution with 1.6 ml of the substrate solution (60 mM catechol in 0.4 ml of 0.2 M sodium phosphate buffer, pH 6.0) with various concentrations of the inhibitors. Then, 20 µl of enzyme were added to this mixture and the enzyme residual activity was assessed. The rate of inhibition was expressed as the percentage necessary for 50% inhibition (IC_{50}) by addition of the sample [27].

2.9. Effects of various chemical agents

Effects of various chemical agents such as Fe^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Na^{+} and Cu^{2+} (0.5 mM) and potent chelating agents (0.5 mM) such as ethylenediaminetetraacetic acid, 1,10-phenanthroline, dimethylglyoxime, sodium azide and iodoacetamide were assessed on PPO. Thiol compounds such as β-mercaptoethanol, glutathione and dithiothreitol were assessed as well. Furthermore, effects of phenolic compounds such as kojic acid and tannic acid were investigated on the enzyme activity. The enzyme residual activity was assessed spectrophotometrically at 420 nm under standard assay conditions [28]. The enzymatic activity was reported as relative values (IC_{50}) with reference to PPO activity from the cinnamon fruit coat.

2.10. Statistical analysis

All experiments were carried out in three independent replicates. Analysis of data was carried out using one-way analysis of variance and SPSS Software v.7.5. Data were reported as means ±SE (standard errors). Values were significantly different when $P \leq 0.05$ based on Duncan's test [29].

3. Results and discussion

In this study, activity of the PPO enzyme *C. verum* fruit coat and seed extract was assessed using catechol as the substrate. The enzyme activity was shown in the fruit coat

extract but not in the seed. However, the seed extract contained higher protein (3.4-fold) and phenolic (6.84-fold) contents, compared to that the fruit coat extract did.

3.1. Enzyme extraction and quantification

Partial purification steps of the PPO enzyme are presented in Table 1. First, proteins were precipitated using $(NH_4)_2SO_4$ from 20-60% in the crude extract and all the fractions were characterized for the PPO activity. Only 40% of the fractions showed PPO activity. The purified enzyme was 3.75-fold with 4.58% yield. Then, fractions with partially purified enzyme were transferred into a Sephadex G-100 column; then, enzyme was collected and its activity was assessed using catechol as the substrate. All the fractions possessing PPO activity were mixed, concentrated, and loaded on 10% SDS-PAGE to assess the enzyme purity. In 10% SDS-PAGE, only a single protein band formed at nearly 66 kD (Fig. 1a). The single band of the enzyme of nearly 66 kD in SDS-PAGE included a monomeric protein type. Reports have been published on PPOs from Muscat bailey A [30] and Lychee fruit (*Litchi chinensis*) [31] with molecular weights of approximately 60 and 75.6 kD, respectively. Most of the highlighted PPOs have been reported as monomeric proteins [32]; similar to the present study. In this study, an activity band was observed when the native PAGE of PPO was carried out using the crude extract of PPO. The band appeared nearly at the same position as in SDS-PAGE (Figs. 1b and 1c). Enzyme with a molecular weight of approximately 66 kD was selected for purification.

3.2. Effects of pH

In the present study, the highest enzyme activity was observed at pH 6 when assessing PPO activity of *C. verum* fruit coat at pH 4-9 using catechol (Fig. 2). By shifting pH from acidic to neutral, activity of PPO increased and then decreases after pH 8. The optimal pH for PPO using catechol has been studied from *Solanum aethiopicum* [33], Koshu grape (*Vitis vinifera*) [34] and *Amorphophallus* corm *(Amorphophallus paeoniifolius*) [35]. Moreover, there is a report [35], which has shown the optimum pH of PPO as 8.2, 7.2, 5.6, 5.0 and 4.8, for pyrogallol, 4-methyl catechol, D-tyrosine, p-cresol and L-DOPA as the substrates. The optimum pH for PPOs ranges from acidic to alkaline (pH 4-8), majorly depends on the enzyme sources and substrates. The optimal pH of PPOs may vary, depending on the native of the materials, methods of preparation, ripening of the fruits and the substrates [36].

Table 1. Partial purification of polyphenoloxidases extracted from *Cinnamomum verum* fruit coat

Steps	Total activity (U ml^{-1})	Total protein (mg)	Specific activity (U mg^{-1} protein)	Fold Purification	% Yield
Crude extraction	21466	2456	8.74	1	100
Ammonium sulphate (20-60%)	12044	1050	11.47	1.31	56.1
Gel filtration	2832	105	26.97	3.08	13.19
Ion exchange (Sephadex G-100)	985	30	32.82	3.75	4.58

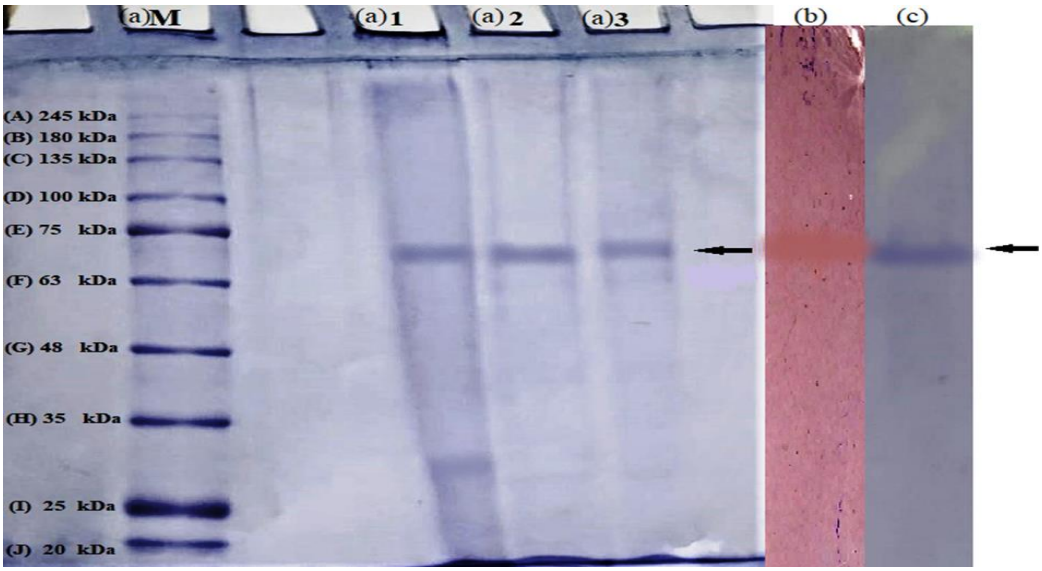

Figure 1. SDS-PAGE, activity staining and native PAGE of polyphenol oxidase activity of the *Cinnamomum verum* fruit coat. (a) 10% SDS-PAGE molecular mass standards: Lane 1, crude enzyme; Lanes 2 and 3, 40% ammonium sulphate precipitation of partially purified PPO; (b) activity staining of partially purified PPO; (c) 10% native-PAGE of partially purified polyphenol oxidases**.** PPO= Polyphenol oxidases

The optimal activity of PPOs from butter lettuce (*Lactuca sativa* var. *capitata* L.) (PH 5.5) [37], Barbados cherry (*Malpighia glabra*) (pH 6.3) [38], wheat bran (pH 5.5-6) [39], Muscat bailey A grape juice (pH 4.5) [30], loquat fruit (*Eriobotrya japonica*) (pH 5) [40], Henry chestnuts (*Castenea henryi*) (pH 6) [41], taro (*Colocasia antiquorum*) (pH 6.5) [42], artichoke (*Cynara scolymus*) (pH 5-7) [38] and bayberry (*Myrica rubra* Sieb. et Zucc.) (PH 7.2) has been studied using catechol [43]. The PPO enzyme from *C. verum* fruit coat is stable at pH 4.5-8 for 15 min. A significant loss of activity was seen in the enzyme at pH above 8, suggesting possible inactivation of the enzyme (Fig. 3). Methods of pH-induced inhibition are coupled to protonation of catalytic groups crucial for catalysis, irreversible denaturation of the proteins, structural changes in active sites and decreases in stability of the substrates as a function of pH [44].

3.3. Optimum temperature and thermal stability

The optimal temperature of PPO from *C. verum* fruit coat was 37°C. Above this temperature, enzyme activity decreased slowly. The optimal temperature of PPOs differs for individual plant origin as well as the substrate used in the analysis. The optimal temperature of PPOs totally increased; similar to that (35°C) reported for the PPOs from Elephant foot yam (*Amorphophallus* corm) [34], Chinese parsley (*Coriandrum sativum*) [45] and Mamey (*Pouteria sapota*) [46] against the catechol as the substrate. Temperature stabilityffects of PPOs are shown in Fig. 4. The reaction mixture of PPOs and catechol was stored at various temperatures (15, 30, 45, 60 and 75°C) for 2 h and the enzyme activity was assessed at various time intervals. Increases in enzyme activity could be observed at 15 and 30°C. The reaction mixture stored at 45°C showed increased activity for 5 min, but decreased later. The reaction mixture showed only basic enzyme activity at 60°C. Complete inactivation of the enzyme within 25 min was observed when the enzyme in reaction mixture was stored at temperatures above 75°C. Results suggest that the cinnamon fruit coat PPOs are temperature stable enzymes. The enzyme inactivation initiated at nearly 45°C and complete inactivation occurred later at nearly 75°C.

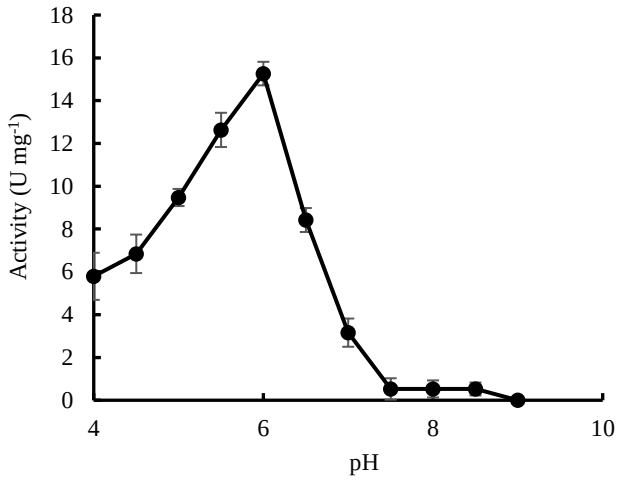

Figure 2. Effects of pH on *Cinnamomum verum* fruit coat polyphenoloxidases. All the values, which represent mean ±SE (standard error) for each experiment, are significantly different ($P \leq 0.05$) from other values based on Duncan's test.

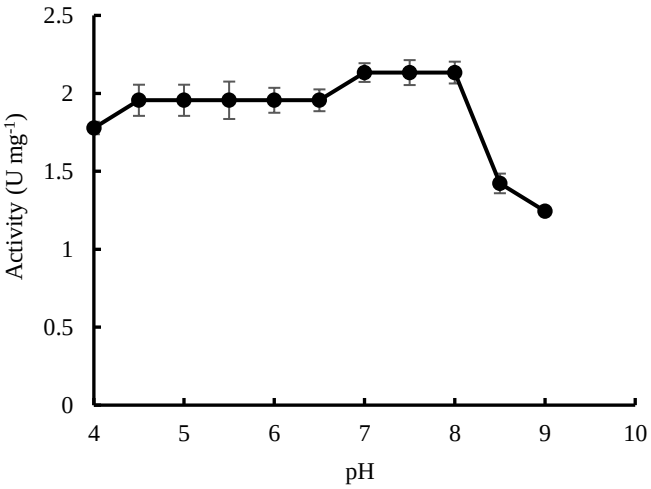

Figure 3. Stability of *Cinnamomum verum* fruit coat polyphenoloxidases as a function of pH. All the values, which represent mean ±SE (standard error) for each experiment, are significantly different ($P \leq 0.05$) from other values based on Duncan's test.

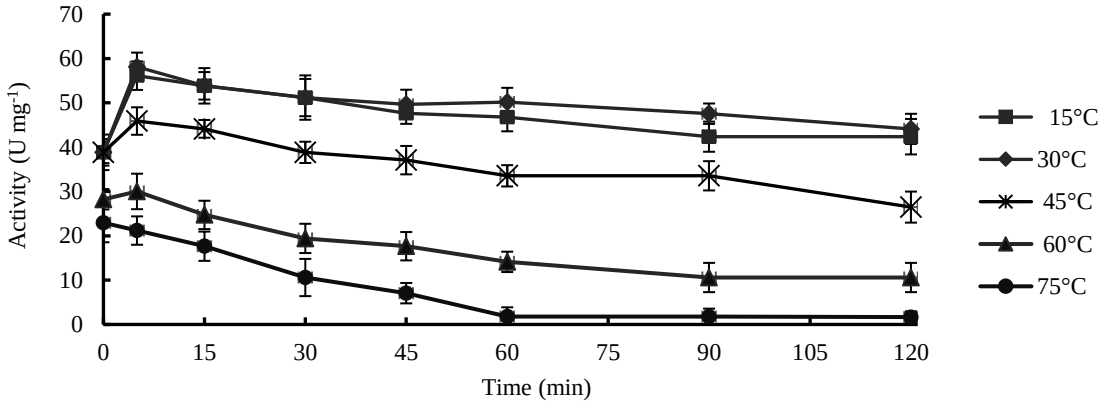

Figure 4. Stability of *Cinnamomum verum* fruit coat polyphenoloxidases as a function of temperature. All the values, which represent mean ±SE (standard error) for each experiment, are significantly different ($P \leq 0.05$) from other values based on Duncan's test.

3.4. Enzyme kinetics

The *Km* and *Vmax* values were calculated from L-B [26] plots by following assay conditions at optimal pH and temperature. The *Km* and *Vmax* values of PPOs for catechol substrate included 2.53 mM and 81 $\Delta A\ min^{-1}$. The *Km* value is a measure of the affinity of enzymes for the substrates. Lesser *Km* values mean larger affinities of the enzymes for the substrates and vice versa. The lower *Km* value of PPOs from *C. verum* fruit coat demonstrated the highest affinity for catechol. Affinity of plant PPOs for hydroxy aromatic substrates is comparatively less. Reported *Km* values have included 1.76, 0.94, 1.32 mM for lentil sprouts [47] and 3.9 in banana (*Musa sapietum*) anterior fruit segments [48]. In the present study, the *Km* value was in the range of reported values.

3.5. Effects of freeze and thaw

Effects of freeze and thaw on PPO activity of the cinnamon fruit coat were assessed using various temperatures of 10, -20 and -40°C at regular intervals within seven days (each cycle for 24 h). Each thawing cycle temperature included 37°C. A gradual decrease was seen in PPO enzyme activity for the initial three cycles. After the fourth cycle of freeze and thaw, nearly 40% of the activity decreased at 10, 40% at -40 and 32% at-20°C (Fig. 5). However, 90% of the activity decreased after eight cycles.

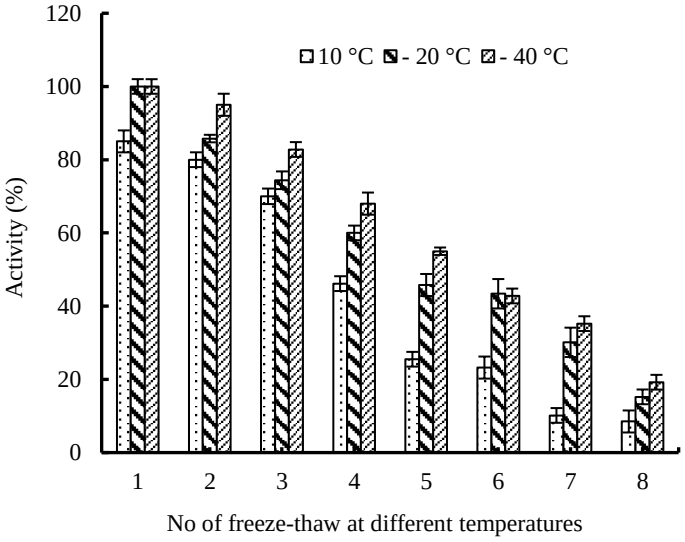

Figure 5. Effects of freeze-thaw cycle on *Cinnamomum verum* fruit coat polyphenoloxidases. Error bars represent standard errors. Differences within the mean values are determined using Duncan's multiple range tests at $P<0.05$.

3.6. Effects of cinnamic acid, propyl benzoic acid and ascorbic acid on activity of polyphenoloxidases from *Cinnamomum verum* fruit coat

Figure 6 (a, b and c) represents effects of ascorbic acid, cinnamic acid, and propyl benzoic acid on activity of the PPOs of *C. verum* fruit coat using catechol as the substrate. The three chemicals included the strongest inhibitors of PPO activity. Comparatively, propyl benzoic acid was less effective as an inhibitor.

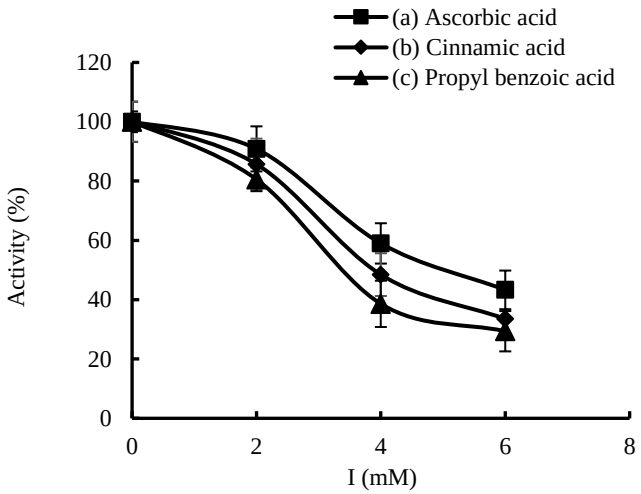

Figure 6. Effects of (a) ascorbic acid, (b) cinnamic acid and (c) propyl benzoic acid on *Cinnamomum verum* coat fruit polyphenoloxidases using catechol as the substrate. All the values, which represent mean ±SE (standard error) for each experiment, are significantly different ($P \leq 0.05$) from other values based on Duncan's test.

Cinnamic acid and ascorbic acid demonstrated the plots of 1 $[V]^{-1}$ vs 1 $[S]^{-1}$ resulted in a parallel straight line with slightly similar slopes (Figs. 7a and 7b). Increases in concentrations of the inhibitors decrease *Vmax* as well as *Km*, revealing the compound as an uncompetitive inhibitor of the *C. verum* PPOs. Cinnamic acid and ascorbic acid act as inhibitors only for the enzyme-substrate complex with no further acts when the additional substrate is added. Similarly, Lineweaver-Burk plot showed the mixed type inhibition mechanism of propyl benzoic acid on *C. verum* PPO activity (Fig. 7c). The double reciprocal plot yielded a series of lines with various slopes and intercepts, intersecting on each other on X axis.

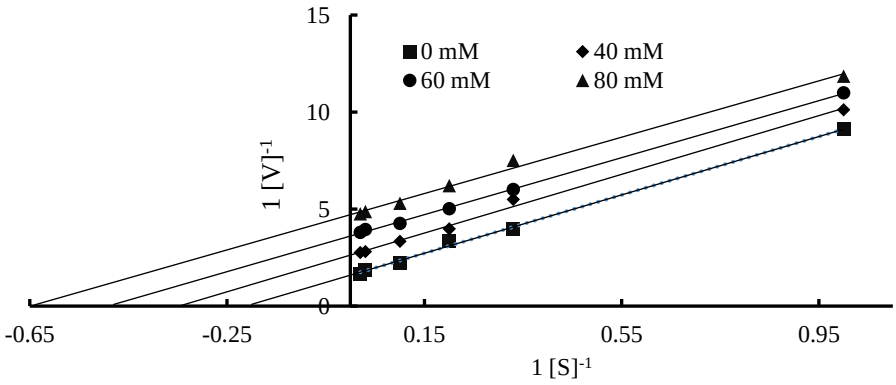

Figure 7a. Lineweaver-Burk plots showing inhibition of *Cinnamomum verum* fruit coat polyphenoloxidases by ascorbic acid using catechol as the substrate.

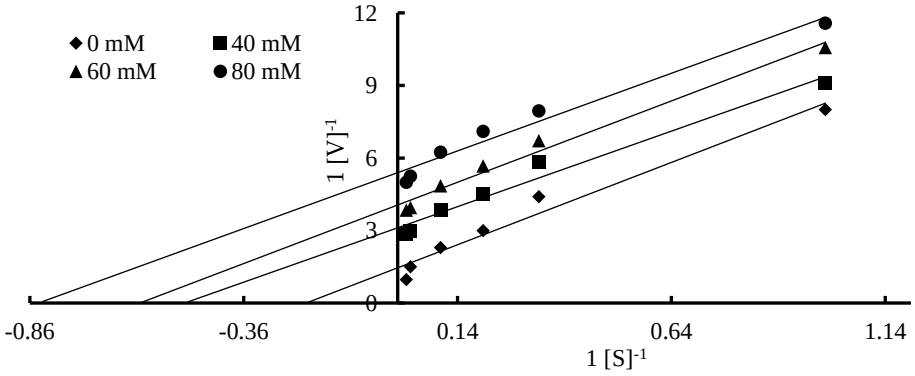

Figure 7b. Lineweaver-Burk plots showing inhibition of *Cinnamomum verum* fruit coat Polyphenoloxidases by cinnamic acid using catechol as the substrate.

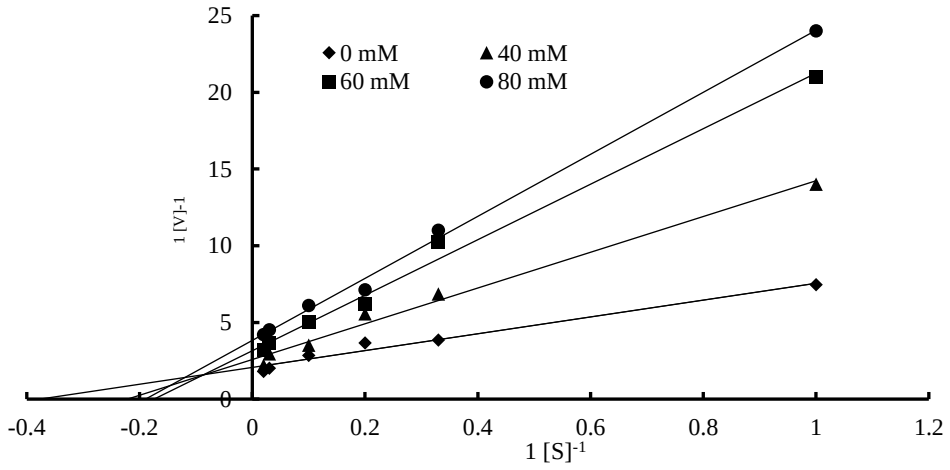

Figure 7c. Lineweaver-Burk plots showing inhibition of *Cinnamomum verum* fruit coat polyphenoloxidases by propyl benzoic acid using catechol as the substrate.

3.7. Effects of the chemical agents

Effects of various chemical compounds on PPO enzyme activity were studied using catechol as the substrate. Metal ions such as Hg^{2+}, Mg^{2+} and Na^{+}, phenolic compound such as tannic acid and chelating agent such as ethylenediaminetetraacetic acid did not affect PPO activity, whereas Fe^{2+} and Fe^{3+} ions moderately inhibited the enzyme activity. Similarly, β-mercaptoethanol, dithiothreitol, glutathione, 1,10-phenanthroline, dimethyl-glyoxime and sodium azide strongly inhibited the enzyme activity (Table 2). Although, effectiveness of the inhibitors against various PPOs can notably differ; therefore, certain controlling measures for personal systems are required. Since PPOs cause browning that decreases the food values, further research on potential inhibitors of PPOs can lead to prevention of food browning in food industries.

Table 2. Inhibition effects of various chemical (organic, inorganic, thiol and chelating) agents on *Cinnamomum verum* fruit coat polyphenoloxidases

Inhibitor	IC_{50} value (µM)
β-Mercaptoethanol	$9.2 \times 10^{-5} \pm 0.01$
DTT	$5.5 \times 10^{-2} \pm 0.1$
GSH	0.18 ± 0.01
Kojic acid	0.21 ± 0.01
1,10-Phenanathrolin	0.96 ± 0.03
Dimethyl glyoxime	1.20 ± 0.05
Sodium azide	5.91 ± 0.02
Ferric chloride	696 ± 0.08
Ferrous sulphate	214 ± 0.01

DTT= dithiothreitol
GSH= glutathione

4. Conclusion

In conclusion, *Cinnamomum verum* fruit coat is able to release stable polyphenoloxidase enzymes of industrial importance with 0.1% polyvinylpyrrolidone. The polyphenol oxidases can be used for the preparation of biosensors to detect hydroxy aromatics for various uses. Capability of polyphenol oxidases to act on hydroxy aromatic compounds can be used for the breaking of hydroxy aromatics in industrial waste water. However, polyphenol oxidases include possibilities of treating large diversifications of phenolic compounds in a wide range of pH and temperature.

5. Acknowledgements

The authors thank DST, New Delhi, for providing financial assistance (Grant no. PURSE-Phase-2/3 (G), SR). The authors also thank Dr. Avila D'souza, Euchem Biologicals, Santhekatte Udupi, for providing laboratory facilities and Dr. P. S. Subrahmanya Bhat, Dept. of English, Karnatak University's College, Dharwad, for critically reading the manuscript.

6. Conflict of interest

The authors report no conflicts of interest.

References

1. Murata M, Kurokami C, Homma S. Purification and properties of chlorogenic acid oxidase from apple (*Malus pumula*). Biosci Biotech Biochem. 1992; 56(11): 1705-1710. doi:10.1271/bbb.56.1705

2. Oliveira CM, Ferreira ACS, De-Freitas V, Silva AM. Oxidation mechanisms occurring in wines. Food Res Int. 2011; 44(5): 1115-1126. doi:10.1016/j.foodres.2011.03.050

3. Saeidian S. Partial purification and characterisation of polyphenol oxidase from tomatoes (*Solanum lycopersicum*). Int J Adv Biol Biomed Res. 2013; 1(6): 637-648.

4. Shiino M, Watanabe Y, Umezawa K. Synthesis of *N*-substituted *N*-nitrosohydroxylamines as inhibitors of mushroom tyrosinase. Bioorg Med Chem. 2001; 9(5): 1233-1240. doi:10.1016/S0968-0896(01)00003-7

5. Khan KM, Maharvi GM, Khan MTH, Jabbar Shaikh A, Perveen S, Begum S, Choudhary MI. Tetraketones: A new class of tyrosinase inhibitors. Bioorg Med Chem. 2006; 14(2): 344-351. doi:10.1016/j.bmc.2005.08.029

6. Chang TS. An updated review of tyrosinase inhibitors. Int J Mol Sci. 2009; 10(6): 2440-2475. doi:10.3390/ijms10062440

7. Kim YJ, Uyama H. Tyrosinase inhibitors from natural and synthetic sources: Structure, inhibition mechanism and perspective for the future. Cell Mol Life Sci. 2005; 62(15): 1707-1723. doi:10.1007/s00018-005-5054-y

8. Duran N, Esposito E. Potential application of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: A review. Appl Catal B Environ. 2000; 28(2): 83-99. doi:10.1016/S0926-3373(00)00168-5

9. Braun L. Cinnamon. J Compl Med. 2006; 5(5): 67-68

10. Jarvill-Taylor KJ, Anderson RA, Graves DJ. A hydroxyl-chalcone derived from cinnamon functions as a mimetic for insulin in 3T3-L1 adipocytes. J Am Coll Nutr. 2001; 20(4): 327-336. doi: 10.1080/07315724.2001.10719053

11. Jayaprakasha GK, Rao LJ, Sakariah KK. Chemical composition of the volatile oil from the fruits of *Cinnamomum zeylanicum* blume. Flavour Frag J. 1997; 12(5): 331-333. doi:10.1002/(sici)1099-1026(199709/10)12:5<331::aid-ffj663>3.0.co;2-x

12. Jayaprakasha GK, Ohnishi-Kameyama M, Ono H, Yoshida M, Jaganmohan Rao L. Phenolic constituents in the fruits of *Cinnamomum zeylanicum* and their antioxidant activity. J Agric Food Chem. 2006; 54(5): 1672-1679. doi:10.1021/jf052736r

13. Jayaprakasha GK, Negi PS, Jena BS, Rao LJM. Antioxidant and antimutagenic activities of *Cinnamomum zeylanicum* fruit extracts. J Food Compos Anal. 2007; 20(3-4): 330-336. doi:10.1016/j.jfca.2006.07.006

14. Hajja G, Bahlouli A. Medicinal plants in the prevention and treatment of rheumatoid arthritis. MOJ Bioequiv Availab. 2018; 5(1): 60-64. doi:10.15406/mojbb.2018.05.00084

15. Huang H, Zhu Q, Zhang Z, Yang B, Duan X, Jiang Y. Effect of oxalic acid on antibrowning of banana (*Musa* spp., AAA group, cv. 'Brazil') fruit during storage. Sci Hortic. 2013; 160: 208-212. doi:10.1016/j.scienta.2013.05.041

16. Bevilaqua JV, Cammarota MC, Freire DMG, Sant'Anna JRS. Phenol removal through combined biological and enzymatic treatments. Braz J Chem Eng. 2002; 19(2): 151-158. doi:10.1590/S0104-66322002000200010

17. Edalli VA, Kamanavalli CM. Removal of phenolic compounds by mushroom polyphenol oxidase from *Pleurotus* species. The Bioscan 2010; 4: 89-92.

18. Flurkey A, Cooksey J, Reddy A, Spoonmore K, Rescigno A, Inlow J, Flurkey WH. Enzyme, protein, carbohydrate, and phenolic contaminants in commercial tyrosinase preparations: Potential problems affecting tyrosinase activity and inhibition studies. J Agric Food Chem. 2008; 56(12): 4760-4768. doi:10.1021/jf800109a

19. Hamed M, Shora EI, Metwally M. Use of tyrosinase enzyme from *Bacillus thuringiensis* for the decontamination of water polluted with phenols. Biotechnol. 2008; 7(2): 305-310. doi:10.3923/biotech.2008.305.310

20. Hou MF, Tang XY, Zhang WD, Liao L, Wan HF. Degradation of pentachlorophenol by potato polyphenol oxidase. J Agric Food Chem. 2011; 59(21): 11456-11460. . doi:10.1021/jf202236c

21. Davis BJ. Disc electrophoresis- II method and application to human serum proteins. Ann Ny Acad Sci. 1964; 121(2): 404-427. doi:10.1111/j.1749-6632.1964.tb14213.x

22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259): 680-685. doi:10.1038/227680a0

23. Gulcin I, Yildirim A. Purification and characterization of peroxidase from *Brassica Oleracea* var. acephala. Asian J Chem. 2005; 17(4): 2175-2183.

24. Koksal E, Gulcin I. Purification and characterization of peroxidase from cauliflower (*Brassica Oleracea L*. var. botrytis) buds. Protein Pept. Lett. 2008; 15(4): 320-326. doi:10.2174/092986608784246506

25. Oktay M, Kufreviolu I, Kocacaliskan I, Saklrolu H. Polyphenoloxidase from amasya. Apple J Food Sci.1995; 60(3): 494-496. doi:10.1111/j.1365-2621.1995.tb09810.x

26. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc. 1934; 56(3): 658-666. doi:10.1021/ja01318a036

27. Chaisakdanugull C, Theerakulkait C, Wrolstad RE. Pineapple juice and its fractions in enzymatic browning inhibition of banana [*Musa* (AAA Group) Gros Michel]. J Agric Food Chem. 2007; 55(10): 4252-4257. doi:10.1021/jf0705724

28. Siddiq M, Sinha NK, Cash JN. Characterization of polyphenoloxidase from stanley plums. J Food Sci.1992; 57(5): 1177-1179. doi:10.1111/j.1365-2621.1992.tb11292.x

29. Duncan DB. Multiple range and multiple f tests. Biometrics 1955; 11(1):1-42. doi:10.2307/3001478

30. Tohru O, Aisak GP, Kazuhito F, Koki Y. Purification and characterization of polyphenol oxidase from muscat bailey, a grape Juice. Am J Enol Vitic. 1999; 50(2): 137-143. doi: 10.1016/S1389-1723(99)89002-1

31. Jiang Y. Role of anthocyanins, polyphenol oxidase and phenols in lychee pericarp browning. J Sci Food Agric. 2000; 80(3): 305-310. doi:10.1002/1097-0010(200002)80:3<305::aid-jsfa518>3.0.co;2-h

32. Mishra BB, Gautam S. Polyphenol oxidases: Biochemical and molecular characterization, distribution, role and its control. Enzyme Eng. 2016; 5(1): 141-149. doi:10.4172/2329-6674.1000141

33. Bello AB, Sule MS, Alhassan AJ. Optimum pH and pH stability of crude polyphenol oxidase (PPO) extracted from five fruit samples commonly consumed in Kano State, Nigeria. Bajopas 2011; 4(1): 26-31. doi:10.4314/bajopas.v4i1.5

34. Nakamura K, Amano Y, Kagami M. Purification and some properties of a polyphenol oxidase from koshu grapes. Am J Enol Vitic.1983; 34:122-127.

35. Singh A, Wadhwa N. Biochemical characterization and thermal inactivation of polyphenol oxidase from elephant foot yam (*Amorphophallus paeoniifolius*). J Food Sci Technol. 2017; 54(7): 2085-2093. doi:10.1007/s13197-017-2647-z

36. Emine Z, Sule P. Purification and characterization of pear polyphenol oxidase. Turk J Chem. 2004; 28: 547- 557.

37. Gawlik-Dziki U, Zlotek U, Swieca M. Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var. caitata L.). Food Chem. 2008; 107(1): 129-135. doi:10.1016/j.foodchem.2007.07.068

38. Anil Kumar VB, Kishor Mohan TC, Murugan K. Purification and kinetic characterization of polyphenol oxidase from barbados cherry (*Malpighia glabra* L.). Food Chem. 2008; 110(2): 328-333. doi:10.1016/j.foodchem.2008.02.006

39. Yoshiki Y, Haruyoshi K, Kazuhiko N. Polyphenol oxidase from wheat bran is a serpin. Acta Biochim Pol. 2008; 55(2): 325-328. doi:10.18388/abp.2008_3079

40. Ding CK, Chachin K, Ueda Y, Mochioka R. Changes in polyphenol concentrations and polyphenol oxidase activity of loquat (*Eriobotrya japonica* Lindl,) fruit in relation to browning. J Jpn Soc Hortic Sci. 1998; 67(3): 360-366. doi:10.2503/jjshs.67.360

41. Jinsen X, Tianling Z, Sadatoshi M, Shinsaku K. Purification and characterization of polyphenol oxidase from henry chestnuts (*Castanea henryi*). J Wood Sci. 2004; 50(3): 260-265. doi:10.1007/s10086-003-0554-6

42. Ahmet Y, Mehmet O, Bekir C. Some characteristics of polyphenol oxidase and peroxidase from taro (*Calocasia antiquorum*). Turk J Agric For. 1999; 23(4): 425-430.

43. Zhongxiang F, Min Z, Yunfei S, Jingcai S. Polyphenol oxidase from bayberry (*Myrica rubra* Sieb. et Zucc.): Its role in anthocyanin degradation. Food Chem. 2007; 103(2): 268-273. doi:10.1016/j.foodchem.2006.07.044

44. Ruhiye Y, Maurice RM. Physiological properties and function of plant polyphenol oxidase: A review. J Food Biochem. 2003; 27(5): 361-422. doi:10.1111/j.1745-4514.2003.tb00289.x

45. Lin H, Ng AWR, Wong CW. Partial purification and characterization of polyphenol oxidase from Chinese parsley (*Coriandrum sativum*). Food Sci Biotechnol. 2016; 25(1): 91-96. doi:10.1007/s10068-016-0103-x

46. Palma-Orozco G, Ortiz-Moreno A, Dorantes-Alvarez L, Sampedro JG, Najera H. Purification and partial biochemical characterization of polyphenoloxidase from mamey (*Pouteria sapota*). Phytochem. 2011; 72(1): 82-88. doi:10.1016/j.phytochem.2010.10.011

47. Sikora M, Swieca M, Franczyk M, Jakubczyk A, Bochnak J, Zlotek U. Biochemical properties of polyphenol oxidases from ready-to-eat lentil (*Lens culinaris* Medik.) sprouts and factors affecting their activities: A search for potent tools limiting enzymatic browning. Foods. 2019; 8(5): 154. doi:10.3390/foods8050154

48. Chikezie PC, Akuwudike AR, Chikezie CM, Ibegbulem CO. Fractional purification and kinetic parameters (*Km* and *Vmax*) of polyphenoloxidase extracted from three segments of *Solanum melongenas* and *Musa sapietum* fruits. Am J Plant. Physiol. 2013; 8(2): 84-92. doi:10.3923/ajpp.2013.84.92

Research Article

APPLIED FOOD BIOTECHNOLOGY, 2020, 7 (3): 183-193
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

خالص‌سازی نسبی، ویژگی‌ها و بررسی خواص مهاری ترکیبات آلی آنزیم‌های پلی‌فنل‌اکسیداز *Cinnamomum verum*

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چکیده

سابقه و هدف: آنزیم پلی‌فنول‌اکسیداز اکسایش اورتو-دی‌فنول به اورتو- کوئینون توسط اکسیژن مولکولی را کاتالیز می‌کند، در حالی که محصول نهایی رنگدانه‌های قهوه‌ای نامطلوبی دارد. بنابراین، مهار پلی‌فنول‌اکسیداز برای حفظ سبزی‌ها و مواد غذایی ضروری است. این آنزیم با اتصال با نواحی غیرمعمول تیره پوست (حاوی مقادیر زیاد رنگدانه) در درمان اختلال‌های پوستی اثر درمانی سودمندی دارد و در تولید محصولات مراقبت از پوست مفید است. مطالعه حاضر ویژگی‌ها و سینتیک مهار پلی‌فنول‌اکسیداز موجود در پوشش میوه *Cinnamomum verum* را بیان می‌کند.

مواد و روش ها: خالص سازی و تعیین میزان پلی‌فنول‌اک سیداز با ا ستفاده از ر سوب $(NH_4)_2SO_4$، دیالیز با ستون کروماتوگرافی سفادکس G-100 انجام شد. وزن مولکولی با استفاده از SDS-PAGE[1] تعیین شد. مقادیر Km[2] و $Vmax$[3] با ا ستفاده از طرح Lineweaver-Burk محا سبه شد. بهینه سازی pH، درجه حرارت و یخزدایی مورد مطالعه قرار گرفت. اثرات چند ترکیب آلی بر فعالیت پلی‌فنول‌اکسیداز بررسی و اندیس IC_{50}[4] محاسبه شد.

یافته‌ها و نتیجه‌گیری: خالص‌سازی نسبی پلی‌فنول‌اکسیداز پوشش میوه *Cinnamomum verum* حدود ۳/۷۵ برابر بود و با استفاده از کاتکول، به عنوان سوبسترا، به ۴/۵۸ برابر افزایش یافت. آنزیم یک اتصال با وزن مولکولی حدود ۶۶ کیلودالتون داشت. pH و درجه حرارت بهینه به ترتیب ۶/۰ و ۳۷ درجه سلسیوس بود. مقادیر Km و $Vmax$ به ترتیب ۱/۶۷ Mm و ۶۴/۵۷ ΔA به دست آمد. نوع مهار سینامیک اسید و آسکوربیک اسید غیر رقابتی بود، در حالی که در مورد پروپیل بنزوییک اسید نوعی مخلوط بود. تی‌اُل و چنگالنده‌ها[5] مهارکننده‌ها قوی برای فعالیت آنزیمی بودند. در نتیجه، پلی‌فنول‌اکسیداز می‌تواند برای توسعه زی‌حسگرها[6] در حذف و تجزیه ترکیبات فنولی در پساب صنعتی مورد استفاده قرار گیرند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۹ آپریل ۲۰۲۰
داوری ۲۶ می ۲۰۲۰
پذیرش ۱۴ژوئن ۲۰۲۰

واژگان کلیدی

- کاتکول
- عوامل شیمیایی
- *Cinnamomum verum*
- پلی‌فنول‌اکسیداز
- خالص‌سازی

***نویسنده مسئول**

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[1] Sodium dodecyl sulfate polyacrylamide gel electrophoresis
[2] Michaelis constant
[3] The reaction rate when the enzyme is fully saturated by the substrate, indicating that all binding sites are permanently reoccupied.
سرعت واکنش هنگامی‌که سوبسترا کاملا اشباع باشد، به‌عبارت دیگر سایت‌های اتصال تماما اشغال شده باشند.
[4] The half maximal inhibitory concentration (IC50) is a measure of the substance effectiveness on inhibition of a specific biological or biochemical function
نصف غلظت بیشینه مهاری، اثربخشی ماده‌ای در مهار عملکرد زیستی یا بیوشیمیایی را اندازه‌گیری می‌کند.
[5] Chelating agents
[6] Biosensors