Characterization of Tyrosinase- and Polyphenol Esterase-Catalyzed End Products Using Selected Phenolic Substrates

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The oxidative end products that result from the biocatalysis of tyrosinase (PPO) and/or a polyphenol esterase (PPE) extract have been investigated simultaneously in model systems containing selected phenolic compounds as substrates. The spectrophotometric scanning of brown color, formed in the presence of both PPO and PPE, showed a decrease in the absorbance compared to that obtained with PPO only. Graphical analyses of the iterative spectra of oxidized phenolic end products by PPO confirmed the presence of, at least, three kinetically related absorbing species. HPLC analyses of the end products, obtained by the biocatalysis of PPE or PPO activity, indicated the presence of two main groups of compounds: colored ones of λ_{max} at 294−324 nm and colorless products of λ_{max} at 264−290 nm. PPE produced both compounds when selected substrates were used as substrates, whereas PPO produced only one type of oxidation product. However, when both enzymes were incubated together, the nature of the end products was similar to that obtained with PPE only.

Keywords: *Tyrosinase; polyphenol esterase; enzymatic end products; characterization*

INTRODUCTION

Enzymatic browning is a phenomenon related to the oxidation of phenolic compounds by polyphenol oxidase (EC 1.14.18.1; PPO), which is also known as tyrosinase (Whitaker, 1995). Oxidative browning is mainly due to the hydroxylation of monophenols to *o*-diphenols and the oxidation of colorless *o*-diphenols to highly colored *o*-quinones (Janovitz-Klapp et al., 1990). Tyrosinase shows a wide specificity toward different phenolic substrates where the brown pigments, generated from the oxidation of these compounds, differ widely in color intensity (Nicolas et al., 1993). The *o*-quinones are highly reactive in aqueous media, especially at higher pH (Rouet-Mayer et al., 1990; Guyot et al., 1995). The *o*-quinone formed by enzymatic or coupled oxidation can also react with a hydroquinone to yield a condensation product (Singleton, 1987). The condensation of quinones with the hydroquinone or quinone of phenolic acids and catechin generates dimers and oligmers (Cheynier et al., 1988). The *o*-quinones of different monophenols and *o*-diphenols are converted into more stable compounds by the addition of water or an external nucleophile. The chemical pathways from these *o*-quinones and the factors that can have an effect on them are not well understood (Richard-Forget et al., 1992). A variety of techniques, including spectrophotometric and high-performance liquid chromatography (HPLC) analyses, have been used for the characterization of phenolic compounds and their *o*-quinone products (Singleton, 1987; Oszmiasky and Lee, 1990; Kermasha et al., 1995a,b).

Recently, Madani et al. (1997) reported on the "esterase-like" activity of a partially purified polyphenol esterase (PPE) obtained from *Aspergillus niger*, which was shown to inhibit the formation of brown color by tyrosinase activity. The objective of this work was to investigate the characteristics of the end products, generated in the presence of PPE and mushroom tyrosinase using selected phenolic substrates, in terms of spectroscopic analysis and HPLC chromatographic profiles.

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MATERIALS AND METHODS

Chemicals and Reagents. Catechol, 4-methylcatechol, and 3,4-dihydroxyphenylacetic and caffeic acids (Sigma Chemical Co., St. Louis, MO) were used as substrates. Trifluoroacetic acid (ACP Chemicals Inc., St-Leonard, PQ) and acetonitrile of HPLC grade (Fisher Scientific Ltd., Negean, CA) were used for HPLC analyses.

Enzyme Sources. A commercial grade purified mushroom tyrosinase (Sigma Chemical Co.), with an activity of 3400 units/mg, was used throughout this study; 1 unit of enzyme activity is defined as an increase in absorbance, at 280 nm, of 0.001 per minute at pH 6.5 and 25 °C in a reaction mixture containing L-tyrosine. The recovered crude enzymatic extract of PPE from the biomass of *A. niger* was partially purified by ammonium sulfate precipitation at 80−100% of saturation according to the procedure described previously by Madani et al. (1997).

Enzyme Assays. The tyrosinase activity was determined as described by Madani et al. (1997). For the enzymatic oxidation, 20 μL of mushroom tyrosinase (5.3 enzyme units) was added to 500 μL (5 mM) of catechol, 4-methylcatechol, 3,4-dihydroxyphenylacetic acid, and caffeic acid. The total volume of the reaction mixture was adjusted to 1 mL with phosphate citrate buffer solution (pH 6.0, 0.1 M). The reaction mixture was then incubated for 10 min at 25 °C, and the enzymatic reaction was halted by the addition of 1 mL of 1:1 (v/v) of 50% acetonitrile and 3% HCl.

PPE activity was assayed by HPLC analyses by determining the production of caffeic acid that resulted from the incubation of PPE with chlorogenic acid as substrate (Madani et al., 1997). The specific activity of PPE was 0.133 unit/mg of protein; 1 unit of PPE activity is defined as micromoles of caffeic acid produced per minute.

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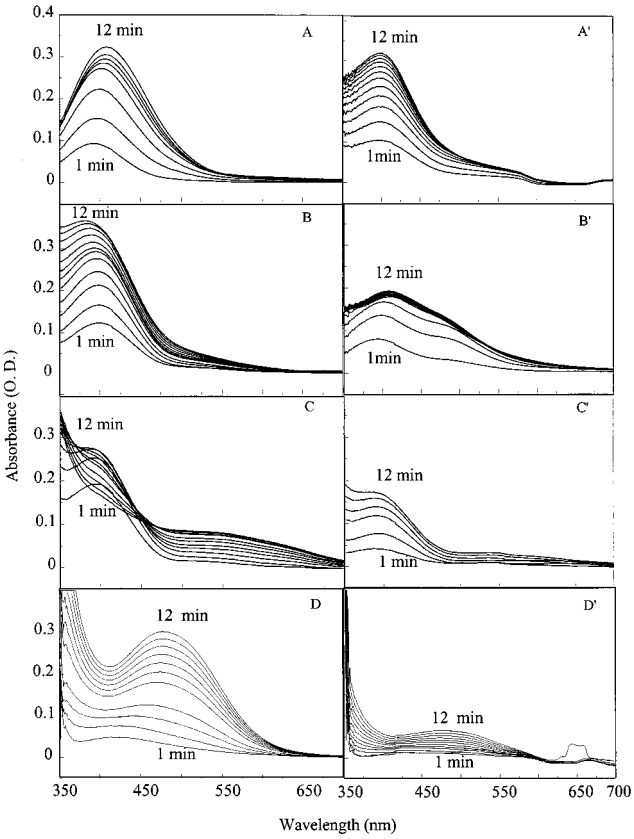

Figure 1. Spectroscopic scanning, at periodic intervals of 1 min, of enzymatic end products obtained after the incubation of (A) 4-methylcatechol and tyrosinase; (A′) 4-methylcatechol, tyrosinase, and PPE; (B) catechol and tyrosinase; (B′) catechol, tyrosinase, and PPE; (C) 3,4-dihydroxyphenylacetic acid and tyrosinase; (C′) 3,4-dihydroxyphenylacetic acid, tyrosinase, and PPE; (D) caffeic acid and tyrosinase; and (D′) caffeic acid, tyrosinase, and PPE.

To study the inhibitory effect of PPE on tyrosinase activity, 100 μL of PPE (2.10, 2.30, 3.50, and 0.96 mg of protein) was used with 4-methylcatechol, catechol, 3,4-dihydroxyphenylacetic acid, and caffeic acid, respectively.

Characterization of Tyrosinase- and PPE-Catalyzed End Products. *Spectrophotometric Analyses.* The formation of the brown compounds was followed spectrophotometrically at 350−700 nm during 12 min, at periodic intervals of 1 min of the reaction, using the tyrosinase assay procedure described above. A control solution, containing all of the components minus the tyrosinase, was run in tandem with the enzymatic trials. Graphical matrix analyses of the iterative spectra were carried out by the application of tests for two and three absorbing species in solution, with stoichiometric restriction (Coleman et al., 1970).

HPLC Analyses. Tyrosinase and PPE reaction end products were subjected to gradient HPLC analyses, using a Beckman HPLC system (Beckman Instruments Inc., San Ramon, CA) equipped with a diode array detector. The HPLC column was a Spherisorb ODS-2 reversed-phase silica-based column (150 × 4 mm i.d., 5 μm) (Alltech Associates Inc., Deerfield, IL). The analytical conditions were as follows: injection volume, 20 μL; flow rate, 0.75 mL/min; solvent A, 0.05% trifluoroacetic acid in distilled deionized water (Milli Q plus, Millipore Water System, Millipore, Milford, MA); solvent B, acetonitrile; linear gradient elution from 5 to 40% of solvent B in 20 min and from 40 to 80% of solvent B in 5 min, followed by washing and reconditioning of the column. Samples were filtered prior to the HPLC analyses using a 0.22 μm cellulose acetate syringe filter (Micro Filtration Systems, Dublin, CA). The UV−visible spectra of the reaction products were performed from 190 to 600 nm. The maximum absorbance of end products of the PPE reaction were measured simultaneously at two wavelengths, 280 and 320 nm. Calibration curves were performed for all substrates by duplicate injection of a known amount of each standard (0−500 μM). Quantification of the reaction products was based on the peak area at 280 nm. The reproducibility of the method was determined by the injection of eight replicates of caffeic acid (100 mM), with a standard deviation of 4.1.

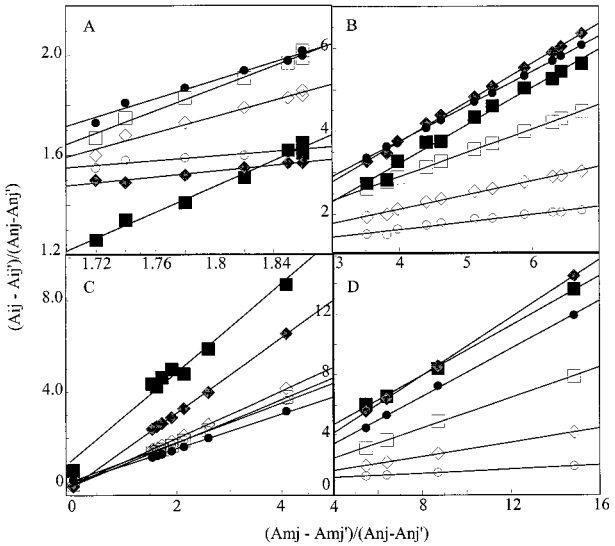

Figure 2. Graphical analyses of the spectra scanning of (A) catechol, (B) 4-methylcatechol, (C) caffeic acid, and (D) 3,4-dihydroxyphenylacetic acid, reported in Figure 1. Test for three species with restrictions was applied. A_{ij} = absorbance value at wavelength i when tracing j (recording). Selected wavelengths were m = 400 nm for all substrates except caffeic acid (480 nm) and n = 520 nm (two reference wavelengths); i (nm) = 360 (◆), 380 (●), 420 (■), 440 (□), 460 (◇), and 500 (○); j' = first recording (1 min).

RESULTS

Analyses of the PPE- and Tyrosinase-Catalyzed End Products. *Spectrophotometric Analyses.* The phenolic substrates showed different profiles (Figure 1) when monitored from 350 to 700 nm. The formation of brown products was determined at the λ_{max} of each compound. Two isosbestic points, at λ_{355} and λ_{450} nm, were obtained for the spectrum of the products generated from the bioconversion of 3,4-dihydroxyphenylacetic acid by tyrosinase activity (Figure 1C). In addition, one shoulder at λ_{490} nm was obtained for the spectrum of the reaction products resulting from the incubation of catechol, PPE, and tyrosinase (Figure 1B′). These findings suggest the presence of two or more different reaction end products. Graphical analyses of the iterative spectra of the oxidized phenolic substrates by tyrosinase activity, with or without PPE, over a period of time confirmed the presence of at least three kinetically related species in the end products of catechol (Figure 2A), 4-methylcatechol (Figure 2B), 3,4-dihydroxyphenylacetic acid (Figure 2D), and caffeic acid (Figure 2C).

Despite the fact that the λ_{max} remained constant in the presence of tyrosinase or of both tyrosinase and PPE, the addition of PPE reduced the rate of formation as well as the amount of brown compounds. The maximum decreases in absorbance were 1.2-, 1.7-, 2.0-, and 6.8-fold for 4-methylcatechol (after 5 min; Figure 1A′), catechol (8 min; Figure 1B′), 3,4-dihydroxyphenylacetic acid (1 min; Figure 1C′), and caffeic acid (5 min; Figure 1D′), respectively.

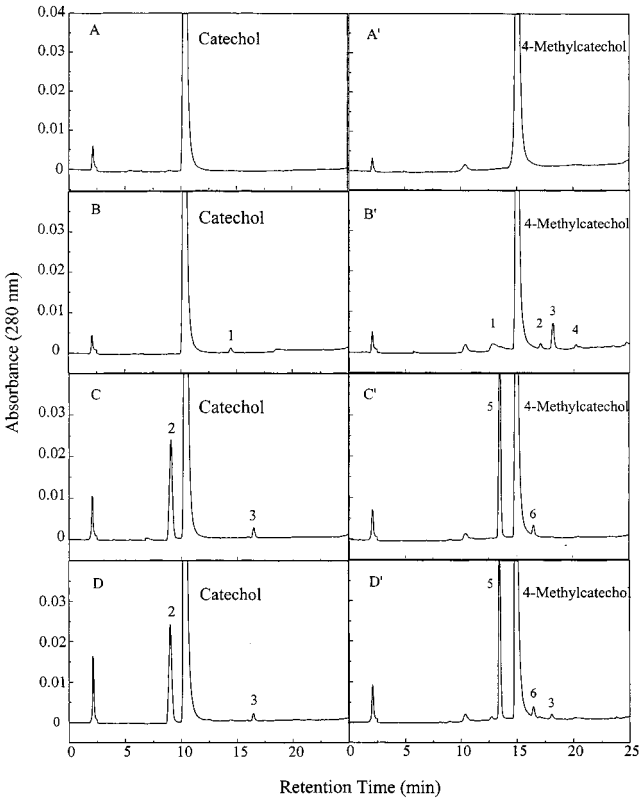

Figure 3. HPLC analyses of (A) catechol, (B) catechol with tyrosinase alone, (C) catechol with PPE alone, (D) catechol with both tyrosinase and PPE, (A′) 4-methylcatechol, (B′) 4-methylcatechol with tyrosinase alone, (C′) 4-methylcatechol with PPE alone, and (D′) 4-methylcatechol with both tyrosinase and PPE.

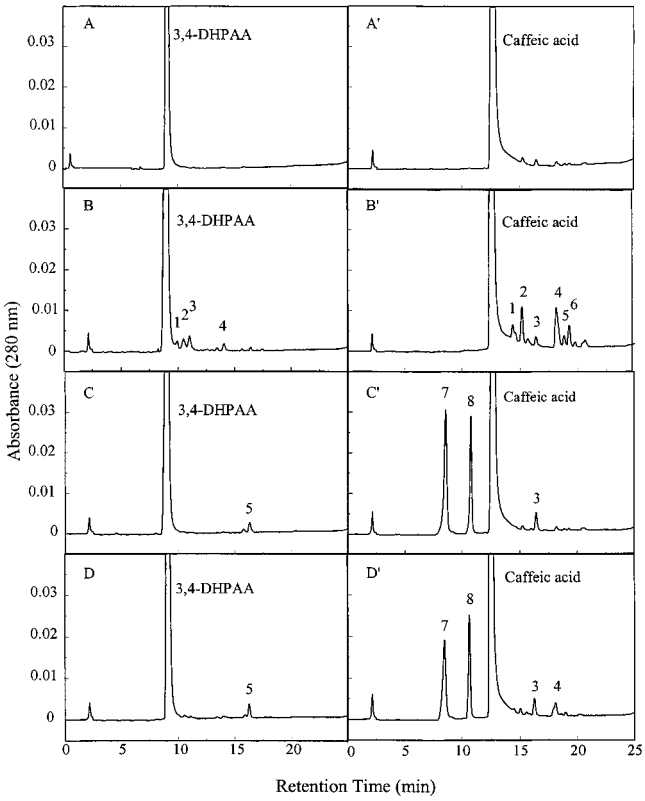

Figure 4. HPLC analyses of (A) 3,4-dihydroxyphenylacetic acid, (B) 3,4-dihydroxyphenylacetic acid with tyrosinase alone, (C) 3,4-dihydroxyphenylacetic acid with PPE alone, (D) 3,4-dihydroxyphenylacetic acid with both tyrosinase and PPE, (A′) caffeic acid, (B′) caffeic acid with tyrosinase alone, (C′) caffeic acid with PPE alone, and (D′) caffeic acid with both tyrosinase and PPE.

HPLC Analyses. The formation of enzymatic end products resulting from the bioconversion of catechol, 4-methylcatechol, and 3,4-dihydroxyphenylacetic and caffeic acids by tyrosinase, PPE, and both enzymes was monitored by HPLC analyses. Although HPLC analyses were performed simultaneously at 280 and 320 nm (data unshown), the experimental results indicated that the maximum absorbance of end products appeared to be at 280 nm.

The results (Figure 3B) show that the oxidation of catechol by tyrosinase resulted in the appearance of one end product (peak 1 of 8 μM; Figure 3B); however, two end products (peaks 2 and 3 of 175 and 12 μM, respectively) were observed when PPE was used alone (Figure 3C) or with both tyrosinase and PPE (Figure 3D). The retention time of the elution of the main product (peak 2; Figure 3C,D) suggests the presence of a compound of more polarity than that of catechol; this peak shows a catechol-related spectrum (λ_{max} at 270 nm). On the other hand, peak 3 shows a λ_{max} at 300 nm (Figure 3C) and 324 nm (Figure 3D); however, the chromatogram (Figure 3D) shows the disappearance of the oxidation product (peak 1) in the presence of PPE.

The oxidation of 4-methylcatechol by tyrosinase (Figure 3B′) resulted in three peaks, 1, 2, and 3, of 28, 8, and 34 μM, respectively, with λ_{max} values at 270, 276, and 290 nm, respectively; the incubation of PPE alone with this substrate produced a major product (peak 5 of 256 μM) with a λ_{max} at 270 nm and a minor product (peak 6 of 7 μM) with two λ_{max} values at 294 and 328 nm (Figure 3C′). The bioconversion of the 4-methylcatechol in the presence of both PPE and tyrosinase (Figure 3D′) resulted in a profile similar to that obtained when the substrate was incubated with PPE alone; however, the main difference was the 7-fold decrease in the relative peak area of the oxidation peak 3. The total amounts of the end products were 70, 263, and 223 μM when the substrate was incubated with tyrosinase, PPE, and both enzymes, respectively.

The enzymatic oxidation by tyrosinase of 3,4-dihydroxyphenylacetic acid (Figure 4B) produced several colorless compounds (peaks 1, 2, and 3 of 7, 17, and 18 μM, respectively), with λ_{max} at 280 nm, and a colored compound (peak 4 of 11 μM), with λ_{max} at 290 and 324 nm; however, using the same substrate, the PPE generated only one main peak (peak 5 of 12 μM) with λ_{max} at 290 and 324 nm (Figure 4C). When both enzymes were incubated with this substrate (Figure 4D), only peak 5 (17 μM) appeared and the total amount of reaction products decreased by 3.2-fold compared to that obtained with the presence of tyrosinase alone.

The oxidation of caffeic acid by tyrosinase resulted (Figure 4B′) in the formation of six major colored end products (peaks 1, 2, 3, 4, 5, and 6 of 4, 18, 5, 30, 4, and 10 μM, respectively) of similar spectra (λ_{max} at 290 and 324 nm), but with higher retention times as compared to that for caffeic acid. The use of PPE alone with caffeic acid (Figure 4C′) resulted in two main colorless reaction products (peaks 7 and 8 of 84 and 54 μM, respectively) with a single λ_{max} at 264 or 278 nm, respectively, and of a shorter retention time than that for the original substrate. An additional reaction product (peak 3 of 9 μM; Figure 4C′) was also present. The incubation of caffeic acid with both PPE and tyrosinase resulted in an HPLC profile (Figure 4D′) similar to that obtained

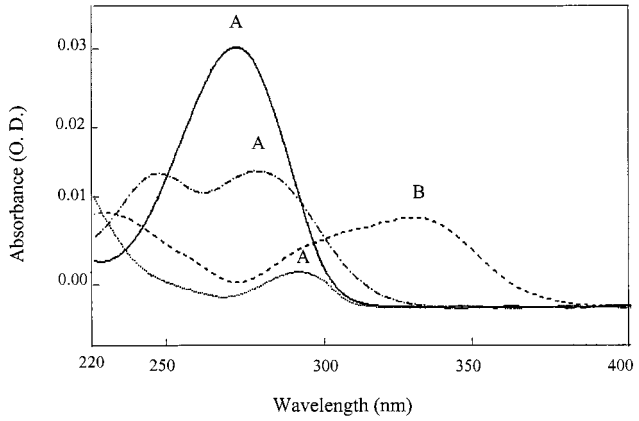

Figure 5. Overlay of the UV−visible diode array spectra of major end products formed by both PPE- and tyrosinase-catalyzed reactions of selected phenolic compound solutions: (A) colorless products with λ_{max} at 264, 280, or 290 nm; (B) colored products with λ_{max} at 294−324 nm.

with PPE alone; however, the only difference was the presence of the oxidation product (peak 4; Figure 4D′), and there was a decrease in the total amount of reaction products by 4.2-fold.

DISCUSSION

The *o*-quinones are the primary products of the enzymatic oxidation of phenolic substrates by PPO; they are very unstable compounds, especially as the pH increases (Richard-Forget et al., 1992).

The bioconversion of phenolic substrates, catalyzed by tyrosinase and/or PPE, generates complex mixtures in which the nature and the amount are substrate-dependent. Phenolic oxidation products could be separated, with regard to their UV−visible spectra, into two main classes (Figure 5): colored compounds (λ_{max} = 294−324 nm) and colorless products (λ_{max} = 264, 280, or 290 nm).

The incubation of PPE alone with many of the substrates resulted in the production of colored compounds; this fact may indicate the importance of the conjugated hydroxyl group in the reaction mechanism that leads to the formation of colored compounds and underscores the wider specificity of PPE in comparison to tyrosinase. In addition, PPE showed the ability to produce colorless end products, when caffeic acid was used as substrate; this ability was also present when tyrosinase was incubated with PPE. The results of this combined enzymatic reaction show a decrease from 70 to 17 μM in the amount of the colored end products. On the other hand, the unique ability of PPE to react with catechol and 4-methylcatechol resulted in an increase from 0 to 15 μM of the colored compounds. The spectra of certain peaks resulting from the oxidation of caffeic acid by tyrosinase were similar; these findings suggest that the oxidation products might be structurally analogous (Cilliers and Singleton, 1991).

The HPLC analyses of the reaction end products, obtained from the oxidation of caffeic acid and 3,4-dihydroxyphenylacetic acid, suggest that the *trans*-alkene (cinnamate) side-chain conjugation of caffeic acid is important for the enzymatic oxidation and related condensation that leads to the formation of colored end products. The production of oxidation products with retention times higher than that obtained for caffeic acid suggests that these compounds could be caffeic acid oligomers formed by polymerization reactions, which may involve the side chain of at least one of the caffeic acid molecules (Cheynier and Moutounet, 1992). The presence of a single absorption maximum in certain caffeic acid products suggests the loss of the conjugated side chains in the process of their formation, whereas the shorter retention time may indicate that the oxidation products are not ring-opened products (Cheynier and Moutounet, 1992).

As the authors are aware, there is little information on the structure of different end products sufficient to describe the mechanism of their formation. However, some authors (Cilliers and Singleton, 1991; Cheynier and Moutounet, 1992; Richard-Forget et al., 1992) proposed certain hypotheses. On the basis of these hypotheses, we suggest certain mechanisms to explain the reported results. First, because many peak spectra did not show a 40−80 nm bathochromic shift typical of *o*-quinone compounds, it is believed that the end products of tyrosinase and PPE activity are not *o*-quinones but are likely regenerated phenolic compounds. Second, some secondary oxidation products might be trihydroxylated colorless compounds, generated by the reaction of *o*-quinones with water. These products could be the intermediate products of the reaction pathways that lead to the formation of colored compounds; these peaks were eluted at shorter retention times compared to those of the original substrates and showed a single λ_{max} at 264, 280, or 290 nm (Figure 5). Finally, the *o*-quinones, which were initially formed, may be involved in several coupling reactions that led to the formation of various colored oligomers; these peaks eluted at higher retention times compared to those of the original substrates and showed a λ_{max} at 294−324 nm (Figure 5).

CONCLUSION

The results gathered in this study indicated that PPE inhibited tyrosinase by reacting with the phenolic substrates. The nature and the amounts of the end products formed by PPE and the combination of PPE and tyrosinase are substrate-dependent. Both colored and colorless end products were obtained by the oxidation/bioconversion of these substrates in the presence of tyrosinase and/or PPE only. The matrix analyses of the spectrophotometric scan of the end products, resulting from the oxidation of the selected phenolic substrates, indicated the presence of three species.

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