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Gel electrophoresis of polyphenol oxidase with instant identification by *in situ* blotting☆

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Abstract

Polyphenol oxidase (PPO) or tyrosinase is an important and ubiquitous enzyme responsible for browning in plants and melanization in animals. The molecular size of the plant PPO is varied among the species and its activity can be enhanced by a variety of anionic detergents. In the present study, we developed a simple method for the first-step identification of PPO in fruit and vegetable extracts. First, 3 mm chromatographic paper was immersed in 0.5% (w/v) catechol solution as an immobilized PPO substrate. After running the extract with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), one side of the glass plate was removed. The plate was immediately laid on top of the dried catechol-paper. A dark-brown band corresponding to PPO was visualized within 1 min and was further confirmed by a conventional Western blot using an antibody prepared against mushroom PPO. It also reveals that some vegetation (such as tomato, radish, and oriental melon) with low or no detectable activity in a conventional enzyme assay actually possessed marked levels of PPO activity when assessed by PAGE-blot. We propose that an inhibitor is associated with PPO in some plants; the inhibitor, however, is dissociated during the electrophoresis. Therefore, in addition to identify the molecular form of PPO, the present technique may explore the existence of PPO inhibitor(s) in plants. The detail of the method with respect to its relevance for searching a natural PPO inhibitor is described and discussed.

Keywords: Polyphenol oxidase; Tyrosinase; Catechol; Immobilized-paper; Browning reaction; Natural PPO inhibitor

1. Introduction

Plant polyphenol oxidase (PPO), also known as tyrosinase in animals (EC 1.14.18.1), is an enzyme containing copper that catalyzes two different reactions using molecular oxygen, hydroxylation of monophenols to *o*-diphenols and oxidation of the *o*-diphenols to *o*-quinones [1]. This enzyme, responsible for melanization in animals and for browning in fruits and vegetables, is widely distributed in microorganisms, animals, and plants.

Localization of PPO in the plant cells depends on the species, age, and maturity [2–4]. In green leaves, a considerable part of PPO activity is localized in the choloroplasts [5]. It is also present in the soluble fraction in different fruits and vegetables [6–9]. Since colorless polyphenols are converted into dark brown quinones, the browning reaction impairs the texture, flavor, and nutritional values of fruits and vegetables. Prevention of undesirable browning has traditionally been accomplished by various chemicals, including the antioxidant compounds (ascorbic acid, citric acid, flavonoids, and sulfites) [10] and some PPO inhibitors (tropolone, kojic acid, resorcinols and benzaldehyde) [11–14].

Designing a PPO inhibitor to suppress its enzyme activity, therefore, becomes an essential subject of challenge either in preventing the browning reaction in fruits or in intervention of darkening and aging processes for the cosmetic industry. In the present study, we developed a rapid gel-electrophoretic blotting technique to identify the possible molecular form of PPO on a dried chromatographic paper that was immobilized with a colorimetric substrate catechol. The method also allows us to identify potentially potent inhibitors from natural products.

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2. Experimental

2.1. Fruits and vegetables

Fresh fruits and vegetables at commercial maturity were purchased from a local market. They were then placed in a 4 °C ice-packed box and immediately used for the study.

2.2. Preparation of extract from fruits and vegetables

Approximately 150–200 mL (at 4 °C) of a phosphate buffered solution containing 0.02 M sodium phosphate, pH 7.4 (PB), were added to each 100 g of chopped fruit or vegetable to make a final volume of 200 mL or 50% (w/v) solution. The mixture was homogenized thoroughly by a blender at a maximal speed over a period of 5 min with 5 stops. The homogenate was sonicated for 15 s and centrifuged at 3000 × *g* for 30 min at 4 °C to remove the pellets. The supernatant was then passed through a 3 mm filter paper. The pass-through extract stored at 4 °C for less than 12 h was used for conventional PPO enzyme assay and for SDS-PAGE blot.

2.3. Determination of PPO activity in solution

To monitor the PPO activity, 200 μL of 4 mM catechol substrate in PB were added to 10 μL of the plant extract in a microtiter well [15]. The reaction mixture was incubated at room temperature (∼24 °C) over time. The enzyme activity was monitored by reading increased absorbance at 415 nm over a period of 30 min using a plate reader equipped with an automatic printer.

2.4. Preparation of catechol-immobilized paper

Unless otherwise mentioned, 3 mm chromatographic papers (Whatman® 3 mm Chr, Cat no. 3030-917, Maidstone, England) were briefly soaked into a 0.5% (w/v) catechol solution. After a brief blot on an absorption paper, the "catechol-paper" was dried at 37 °C for 5 min. The dried paper is stable for at least 60 days at room temperature when kept in a dark environment. Since the Km of PPO (mushroom, for example) is approximately 0.1–1 mM [16] and the concentration of catechol immobilized was in excess at 40 mM (or 0.5%), the maximal rate of PPO of tested extract could be achieved on the catechol-paper.

2.5. Detection of PPO activity following SDS-PAGE and densitometry

Electrophoresis was conducted in a vertical slab gel unit (Mini Protein® 3 Cell, Bio-Rad, Hercules, CA) equipped with a PAC 300 power supply (Bio-Rad, Hercules, CA). Sample extract 10–15 μL equilibrated in 10 mM Tris–HCl and 0.1% SDS, pH 6.8, without heat was loaded onto a 10% SDS-PAGE gel according to procedures described previously [17,18]. Prestained protein markers were used for the molecular weight index. Gel run at a constant current of 20 mA for 60 min was immediately blotted *in situ* (at room temperature) for PPO activity by pressing it onto the top of a dried catechol-paper without adding buffered solution (see Section 3). Quantum One Imaging Software (Bio-Rad) was used to measure the image density on the PAGE-blot.

2.6. Preparation of mushroom PPO

A gel-filtration of high performance liquid chromatography (HPLC) column Sephadex 200 was used for further purification of mushroom PPO purchased from Sigma–Aldrich (St. Louis, MO). HPLC was conducted at a flow rate of 0.5 mL/min and monitored at 280 nm when using PB as a mobile phase [19]. Tubes corresponding to PPO activity were pooled and stored at −20 °C prior to use.

2.7. Test of endogenous activity of PPO inhibitor

To 0.5 mL of plant extract (described in Section 2.2), 5 mL of 98% alcohol were added and vortexed for 5 min. The reaction mixture was then centrifuged at 3000 × *g* for 10 min to remove the pellets. The supernatant was dried under nitrogen and reconstituted to 0.5 mL of PB. To test the inhibitory activity for PPO, various amounts of the reconstituted extract were aliquoted into the standard PPO assay as described above (Section 2.3.).

2.8. Preparation of antibody against PPO

Female Balb/c mice (5–7 weeks of age) were immunized according to the method previously described [20]. In brief, 200–300 μg of purified mushroom PPO in 0.5 mL of a sterilized buffered solution containing 0.12 M NaCl and 0.02 M phosphate, pH 7.4 (PBS), was mixed and homogenized with 0.5 mL of Complete Freund's Adjuvant (Sigma–Aldrich) by a three-way stopcock. Each mouse was initially given a total emulsion of 0.5 mL with six subcutaneous injections onto the back and one intraperitoneal injection. At day 7, a same dose with complete adjuvant was given intraperitoneally, followed by two intramusclar injections without adjuvant at day 14. At day 21, blood was collected in 0.1% (w/v) EDTA to obtain plasma.

2.9. Western blot

Western blot analysis was performed according to the procedures described previously [21]. In brief, protein (typically 10 μg) or plant extract resolved by SDS-PAGE was transferred onto a nitrocellulose membrane and blocked by a 5% (v/v) skim-milk in PBS. Following washes, 10 mL of mouse PPO antiserum (1:2000 dilutions) were added, incubated for 1 h, and washed. A commercially available secondary antibody (goat anti-mouse IgG conjugated with HRP) (Sigma–Aldrich) was then added, incubated for 1 h, washed, and developed by 3,3-diaminbenzidine containing 0.01% peroxide.

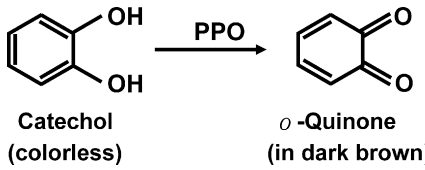
3. Results and discussion

3.1. Enzymatic reaction of PPO using catechol as a substrate

Oxidation using catechol as a polyphenol substrate for PPO activity and formation of *o*-benzoquinone product is given

below:

2catechol + O_2 → 2*o*-benzoquinone + $2H_2O$



Because of the chromogenic property of *o*-benzoquinone (dark brown), catechol [22] or its derivative L-3,4-dihydroxyphenylalanine (L-Dopa) [23] has been conventionally used for the PPO activity assay.

3.2. Rationale for the use of SDS gel electrophoresis in identification of PPO

PPO is widely distributed in all the plants. The molecular weight of PPO, however, is varied among the species [2–4]. Identification of the molecular form of an unknown PPO in plant via a purification procedure is time consuming. An alternative method is to use Western blot analysis, but the procedure is also tedious and the antibody employed must recognize or crossly react with the PPO of an unknown species. For this reason, we sought a new method for rapid identification of the plant PPO.

3.3. Evaluation of PPO activity on catechol-immobilized paper

Catechol is a specific substrate being widely used for measuring the PPO activity of a given plant species. We tested whether catechol could be immobilized on a paper for directly detecting the PPO activity. First, 3 mm chromatographic paper was immersed into a catechol solution as described in the Section 2. After brief blotting and drying at 24, 37, or 60 °C, mushroom PPO was then spotted on the "catechol-paper" to develop the chromogenic product *o*-benzoquinone (if any). A dark brown spot was clearly observed. There was no difference in PPO induced chromogeneity among the papers dried at 24–60 °C, although the shape of the paper was somewhat distorted when dried at 60 °C (data not shown). The catechol-paper dried at 37 °C for 5 min was, therefore, used for the subsequent studies and was found to be stable for at least 60 days at room temperature. The optimal concentrations of catechol immobilized were found to be between 0.5 and 1% (data not shown).

3.4. Determination of mushroom PPO following a SDS-PAGE

Since PPO activity is not adversely affected by SDS [24], separation of PPO from the plant extract using SDS-PAGE is possible. Fig. 1A depicts the present technique step by step for visualizing the PPO activity from the whole mushroom extract. After electrophoresis, conventional procedures including gel removing, washing, transferring, electroblotting, or using soluble reagents were not required in identifying the PPO. One single band corresponding to PPO of mushroom extract was

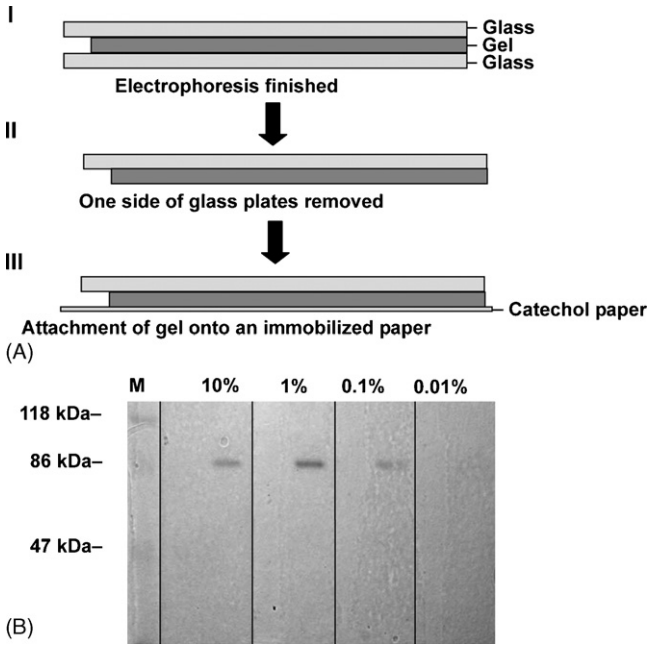


Fig. 1. Instant identification of PPO activity following a 10% SDS-PAGE: (A) Following electrophoresis (step I), one side of the glass plates (7 cm × 8 cm) was removed (step II). The resolving gel was immediately placed onto the top of a catechol-paper (step III). (B) Top view of visualized PPO activity (5 μL of mushroom extract) with different concentrations of immobilized catechol (0.1–10%).

instantly observed in less than 1 min, when laying the gel onto the catechol-paper (Fig. 1B). The optimal concentration of catechol immobilized was about 1%. Notably, 1% catechol-paper gave a sharp chromogeneity, but prolonged blotting (>30 min) should be avoided to prevent "over exposure" of the catechol-paper. This over exposure is a result of the *in situ* diffusion of PPO in the polyacrylamide gel. In some cases, decreasing immobilized catechol concentration in the paper to 0.5% may reduce this effect.

Fig. 2 shows the molecular form of PPO in the mushroom extract superimposed to that of purified PPO standard. To

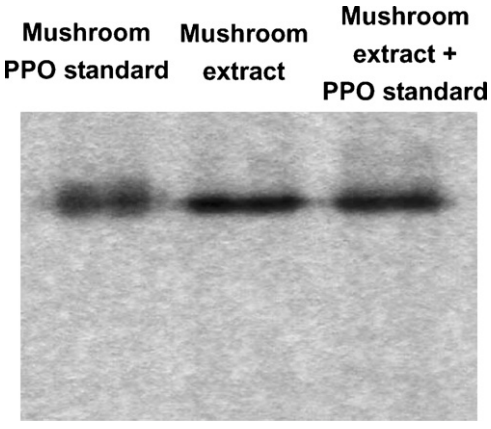


Fig. 2. Identification of PPO activity between the isolated PPO standard of mushroom and its extract on SDS-PAGE blot. One single band corresponding to PPO standard (60 units) or to that in mushroom extract (10 μL) was observed. The molecular form of PPO in the extract (5 μL) spiked with the purified PPO (30 units) was superimposed to that of purified PPO standard.

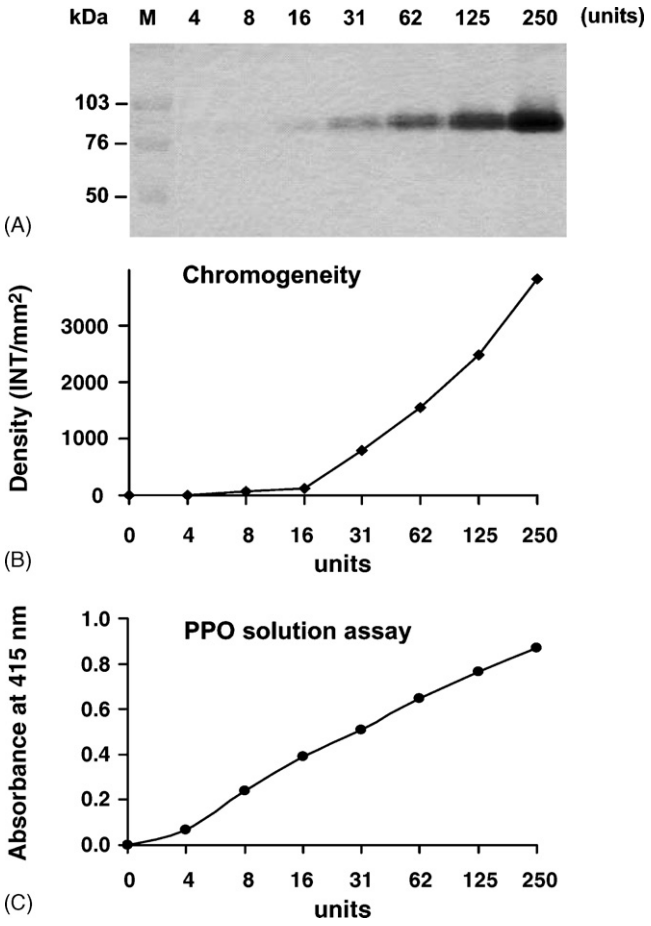


Fig. 3. Comparison of the PPO activity between SDS-PAGE bolt and conventional solution assay: (A) Serial dilutions of purified mushroom PPO (4–250 units) were run for SDS-PAGE. Following electrophoresis, the gel was placed onto the catechol-paper allowing the development of *o*-quinone product. (B) The intensity of developed bands was determined by an image densitometry. (C) Serial dilutions of purified mushroom PPO with which the activity was determined by a conventional solution assay. The linear relationship between the PPO dose and activity is observed in both methods.

evaluate the sensitivity and linearity of SDS-PAGE blot, various amounts of purified mushroom PPO were applied for electrophoresis. Fig. 3 reveals the chromogeneity developed on the paper to be linear to the amount of PPO loaded (16–250 units), which also correlates to PPO activity determined in a solution assay. The lower limit of detected PPO activity on PAGE-blot was approximately 31 units.

To test the feasibility of SDS-PAGE blot against other species, we randomly chose a total of 12 different fruits and vegetables for the identification of PPO. Some typical examples are shown in Fig. 4A, in which tomato possessed two molecular forms of PPO at about 58 and 59 kDa. These values are consistent to that recently reported [25]. Interestingly, the molecular forms of PPO identified in oriental melon, tomato, and radish were identical as identified by a Western blot analysis (Fig. 4B). The antibody used for this immunoblot was prepared against a mushroom PPO. It is capable of cross-reacting with the PPO of selective plant species. The data further indicate that the band corresponding to the PPO enzyme activity on the PAGE-blot to be a PPO.

Table 1
PPO activity in different fruits and vegetables as evaluated by conventional PPO and PAGE-blot assay

Species	Conventional assay[a] (percentage of maximal activity)	PAGE-blot[b] (density, INT/mm^2)
Peach	100.0	300
Mushroom	71.3	2000
Lettuce	13.5	3100
Oriental melon	5.9	2000
Cucumber	3.8	2200
Radish	2.7	3100
Cauliflower	2.1	100
Tomato	0.2	3600
Orange	0	2100
Papaya	0	2000
Pineapple	0	0
Guava	0	0

[a] PPO activity was determined using a conventional solution assay.
[b] PPO activity (arbitrary density) was determined by PAGE-blot as described in Fig. 4. Quantum One Imaging Software was employed to evaluate the image-intensity over the specific PAGE–blot bands.

3.5. Conventional PPO activity assay versus PAGE-blot

In the next experiment, we measured the PPO activity using a conventional solution assay in those randomly chosen fruits and vegetables. Table 1 shows that most of their PPO activity, including the tomato, was low or not detectable, except that of mushroom and peach. It should be noticed that the low PPO activity does not necessarily correspond to the actual PPO level in some species. For example, a recent study shows that an abundant amount of PPO is present in tomato [24]. Interestingly, more than half of the species that possessed the "low" PPO activity in the solution assay had revealed significant levels of enzyme activity on our SDS-PAGE blot. The overall results of the enzyme activity identified in PAGE-blot and solution assay are given in Table 1. We therefore speculated that there was an endogenous PPO inhibitor in the presence of the extract. The inhibitor (if any) might be dissociated from the PPO while running PAGE.

3.6. Presence of endogenous inhibitor of PPO in plant

In the next experiment, we addressed the existence of natural PPO inhibitors in some tested fruits and vegetables. First, using tomato and radish, we demonstrated that their extracts were able to inhibit the PPO activity of mushroom in a dose-dependent manner when assessed on catechol-paper (Fig. 3, inserts). Second, using alcohol to remove the proteins from the tomato and radish extracts, we found the inhibitor to be present in the alcohol-soluble fraction with a dose-dependent inhibitory activity by a PPO solution assay (Fig. 5). Gandia-Herrero et al. have shown that an aldehyde derivative of cucumber, 2,6-nonadienal, can inhibit mushroom PPO with a K_i of 3.4 mM [26]. In a preliminary study, we passed the alcohol-soluble fraction through an Amicon membrane of 3000-kDa cut-off (Millipore, Cork, Ireland). The pass-through was then heated at 95 °C for 10 min, of which the inhibitory activity was totally retained (data not

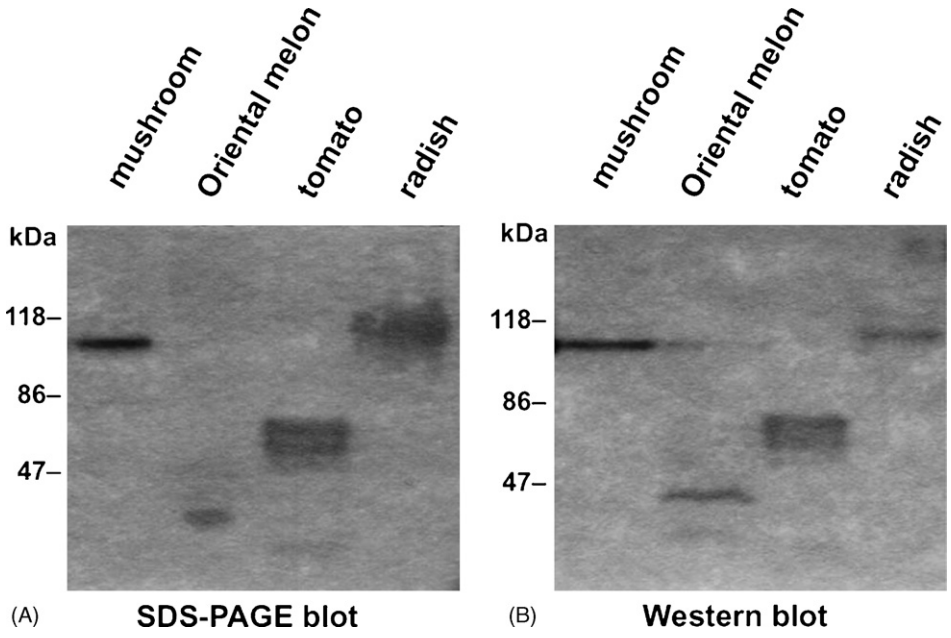


Fig. 4. Typical examples of PPO identified by PAGE-blot and Western blot: (A) PPO activity of plant extracts showing different molecular forms was identified by a SDS-PAGE blot. (B) PPO proteins were identified by a Western blot using a mouse polyclonal antibody prepared against mushroom PPO. This antibody could cross-react with the PPO of oriental melon, tomato, and radish, but not with all the tested species.

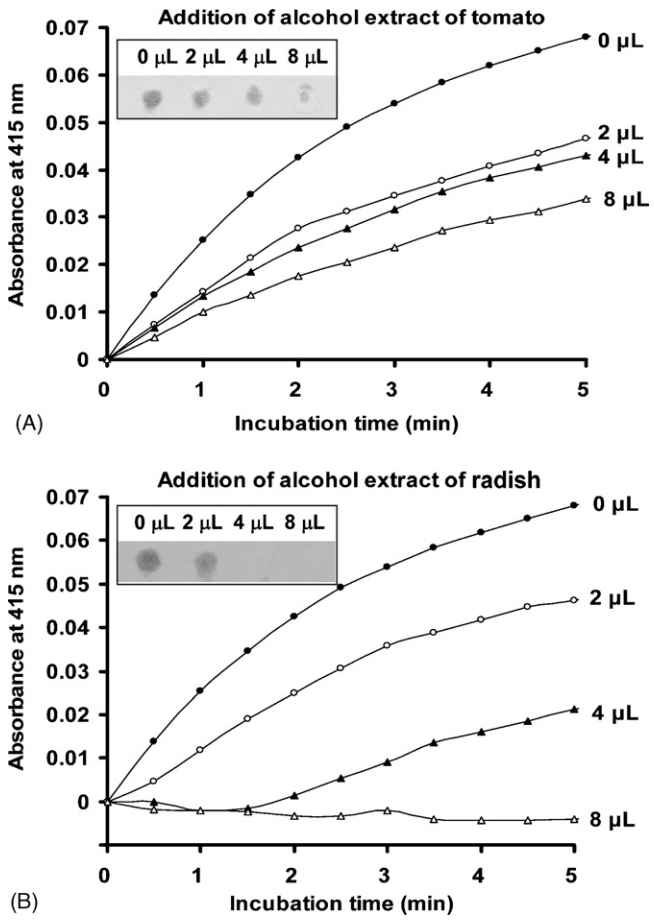


Fig. 5. Inhibitory effect of alcohol soluble-fraction from tomato and radish extract on mushroom PPO activity. Each fraction was incubated with 10 µL of purified mushroom PPO at 24 °C for 10 min prior to the solution assay (see Section 2). Insert: 5 µL of the reaction mixture were spotted on a catechol-paper.

shown). It suggests that the inhibitor is heat stable with a molecular weight less than 3000 kDa. Whether the chemical nature of the inhibitors found in our study is also an aldehyde analog deserves further study. Nonetheless, this PAGE-blot technique in combining with the PPO solution assay could also be used for an initial search for a possible natural inhibitor from plants (Table 1).

4. Conclusion

Instead of a Western blot, the ready-for-use catechol-paper can be used for an initial identification of PPO after a gel electrophoresis without any additional liquid reagents for instant visualization. It is also convenient to monitor the PPO fraction while isolating PPO over a column chromatography. For example, Fig. 6 shows a one-step isolation procedure for mushroom PPO on HPLC Sepharose G-200, in which the fractions containing PPO are exhibited on the catechol-paper. If desired, the catechol can be co-immobilized with 20 mM proline to produce a dark purple prolyl-quinone adduct for increased sensitivity (data not shown). The latter procedure has been used in PPO solution assay [27].

We demonstrated the use of this electrophoretic-blot technique for instant identification of PPO in fruits and vegetables. Because PPO is denatured at high temperature, pre-heating the sample for routine SDS-PAGE should be avoided.

Furthermore, in view of recent studies indicating that tyrosinase is responsible for hyperpigmentation in humans, tyrosinase inhibitors have become increasingly important in medical [28] and cosmetic products [29]. A widely used method in the food and beverage industries to control browning is to add reducing agents such as sulfites, which chemically reduce the *o*-quinones to less reactive colorless diphenols. These

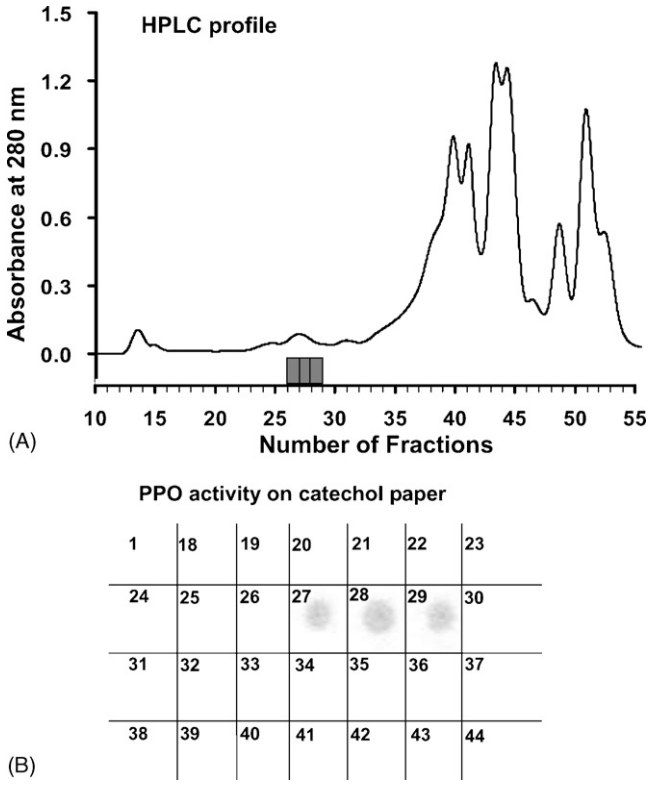


Fig. 6. One-step purification of mushroom PPO on HPLC Sepharose G-200. About 250 μL of mushroom extract were applied onto a Sepharose column (1.0 cm × 30 cm), while PB was used as a mobile phase at a flow rate of 0.5 mL/min (A). Each fraction contains 0.5 mL aliquot, in which 5 μL were used for identifying the PPO activity on the catechol-paper (B).

compounds, however, may be detrimental to the health and can also react with other components in the food system, resulting in unwanted effects [10]. The need for a safe and effective PPO inhibitor remains to be a subject of challenge [29]. For this reason, this electrophoretic-blot technique may contribute an initial exploring for the presence of a potent natural inhibitor in fruits and vegetables.

Acknowledgements

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