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資訊庫編號：953066

計畫名稱：**荔枝褐化酵素(PPO)結構與保鮮之應用** (第1年/全程1年)

(英文名稱) **Investigation the PPO in litchi pericarp and its application in browning protection**

計畫編號：95農科-1.3.2-糧-Z7

全程計畫期間：95 年 1 月 1 日至 95 年 12 月 31 日

本年計畫期間：95 年 1 月 1 日至 95 年 12 月 31 日

計畫主持人：毛仁淡

執行機關：國立交通大學

一、中文摘要：

荔枝褐化過程中發現，荔枝表面結構的變化對荔枝褐化具有決定性的結果，當其表面結構空洞後，褐化反應便會快速進行，因此再其表面覆蓋一層保護膜可以有效的防止其表面結構鬆散導致褐化反應快速進行，在這工作上，尋找全球應用於食品水果保鮮的塗蠟技術，將可食性的蠟均勻的覆蓋在其上，維持其表面完整，近一步的保持水分及防止內生性的PPO抑制劑散失，以SEM掃描其果皮表面證實表面結構完整，進而延緩和化反應產生。

荔枝褐化反應，主要是原由荔枝表結構改變破壞，導致荔枝內生性PPO活性的表現，另外，我們也純化出荔枝的PPO，找出其蛋白質分子特性(包含其最佳pH、溫度、KM、Vmax)，也得知荔枝本身含有內生性的抑制物，可以當荔枝未成熟及未被採收的情況下使內生性的PPO為非活性狀態，並且對採收後荔枝快速變黑、抑制劑的消失，都有充分的了解，然而荔枝本身的抑制劑具有揮發性，不易純化收集，因此致力尋找有效抑制PPO活性的抑制劑與如何防止降低荔枝表皮結構的改變是我們計劃執行的重點。

由農糧署支持的計畫, 在植物褐變酵素(PPO)活性測試的技術平台， 發展出來的廣泛性檢驗植物PPO 存在的測試人工皮膚 並預測 荔枝PPO 抑制劑存在的方法 "Gel electrophoresis of polyphenol oxidase with instant identification by in situ blotting" 已被國際知名期刊 "Journal of Chromatography B" (doi:10.1016/j.jchromb.2006.08.046)所接受並將付印。

二、英文摘要：

Polyphenol oxidase (PPO) is a copper-containing enzyme that catalyzes the chain-oxidation from monophenol or polyphenols to o-diphenols and subsequent o-quinones. The enzyme reflects the browning reaction in fruits.

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 initial characterization of PPO in fruit and vegetable extracts. First, 3MM chromatographic paper was immersed in 0.5% (W/V) catechol solution as an immobilized PPO substrate. After running each sample extract with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), one side of the glass plate was removed and immediately laid on top of a catechol-paper. A dark-brown band corresponding to the PPO was instantly visualized within one min. The PPO band was further confirmed by a conventional Western blot using antibody prepared against mushroom PPO. This instant PAGE-blot shows that some subjects (such as tomato, radish, and oriental melon) with low or no detectable activity determined by a conventional enzyme assay actually possessed high levels of PPO activity. We propose in some plants that an inhibitor is associated with PPO and masks the endogenous PPO activity. Therefore, in addition to characterize the molecular form of PPO, the present technique allows us to explore the existence of inhibitor(s) present in the subjects.

In the present study, we investigated the oxidation activity of PPO in litchi pericarp and the mechanism by which PPO instantly makes pericarp browning.

PPO of litchi pericarp was initially extracted and isolated through gel filtration chromatography and then eluted directly from SDS-PAGE. Two molecular forms of litchi PPO were identified as 86 and 66 kDa, and thereafter named as PPO-86 and PPO-66, respectively. The Km and Vmax were determined as 66 mM and 382 mM/min for PPO-86 and 102 mM and 290 mM/min for PPO-66, respectively. Most importantly, PPO-86 could trigger the 3,3 -diaminobenzidine and 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) oxidation, which was not found in other plant PPO. Thus, the unique PPO activity of litchi might account for its superior rapid-browning reaction. In addition, we show the presence of a potent volatile inhibitor(s) for PPO in litchi pericarp. The surface of post-harvesting litchi pericarp revealed an opening ultra structure under the scanning electron macroscopic examination, therefore allowing an instant evaporation of PPO inhibitor. As such, the PPO oxidation was proceeded. The novel finding clarifies the mechanism involved in the rapid browning phenomenon of post-harvesting litchi pericarp. Since the PPO is also know existed in human as tyrosinase responsible for the formation of "darkening spots" on skin, the finding of evaporation of potent PPO inhibitor may be potentially used as a strategy in developing a novel cosmetic product.

三、計畫目的：

樹上的荔枝呈現的是豔麗鮮紅色，一旦採收後2~3之內逐漸褐化同時失去口感。荔枝褐變一直都是降低其產值的重要問題。造成荔枝及其他花卉、水果(例如:玉蘭花、蘋果)褐化的酵素機制是相同的。造成荔枝快速褐化的成因是由於內生性之多酚氧化酶(PPO)產生黑色素(quinones)之代謝產物所致。因此透過褐化酵素結構及機制的研究，有助於對荔枝防褐的研究及應用。若是採收後仍能長時保持鮮豔顏色以及維持品質，使能夠延長採收後的存放時間，延長櫥架壽命，將對荔枝價格穩定有莫大助益。

四、重要工作項目及實施方法：

本計劃計畫的目標及實施方法與步驟如下：

(一) 抑制劑分子的純化及鑑定及抑制效果的比較。

將多元組成內生性的揮發性小分子抑制劑純化成個別分子並以氣相層析質譜儀(GC-MS)建立其分子結構。純化出抑制最佳的分子之後，據以由人工合成的方式，嘗試大量製造。

(二) 用人工合成的方法將揮發性抑制劑修飾成不具揮發性。

揮發性抑制劑不利收穫後產銷，保存的應用。因此，化學修飾使其變成不具揮發性的類似物抑制劑是必須的。

(三)果臘的塗抹技術的搜尋及研發。

從荔枝褐化過程中表皮結構變化的研究顯示，保濕對褐化的延遲有決定性的貢獻。因此，搜尋現有全球用於保鮮的塗蠟技術並修正、研發適用於荔枝的方法，在採收後的果實表皮能塗上一層食臘將對其保鮮有所助益。

(四) 測試製備的抑制劑對防治荔枝褐變的效果。

實際應用在採收後的荔枝。抑制劑塗裹的方式、效率、保存的時間。保鮮的時間，預期在外銷時加上船運的時間，到消費者手中時仍然是有如剛離枝荔枝的鮮艷。

(五) 儲運到消費者手中的時間，防止腐敗及細菌生長的效果。

模擬保鮮處理後，儲運的時間、環境、過程，達成不發霉長菌的條件。

本計畫的方法同時參閱附加檔(1),(2)

五、結果與討論：

從荔枝褐化過程中發現，荔枝表面結構的變化對荔枝褐化具有決定性的結果，當其表面結構空洞後，褐化反應便會快速進行，因此再其表面覆蓋一層保護膜可以有效的防止其表面結構鬆散導致褐化反應快速進行，在這工作上，尋找全球應用於食品水果保鮮的塗蠟技術，將可食性的蠟均勻的覆蓋在其上，維持其表面完整，近一步的保持水分及防止內生性的PPO抑制劑散失，以SEM掃描其果皮表面證實表面結構完整，進而延緩和化反應產生。

荔枝褐化反應，主要是原由荔枝表結構改變破壞，導致荔枝內生性PPO活性的表現，另外，我們也純化出荔枝的PPO，找出其蛋白質分子特性(包含其最佳pH、溫度、KM、Vmax)，本身含有內生性的抑制物，可以當荔枝未成熟及未被採收的情況下使內生性的PPO為非活性狀態，並且對採收後荔枝快速變黑、抑制劑的消失，都有充分的了解，然而荔枝本身的抑制劑具有揮發性，不易純化收集，因此有效抑制PPO活性的抑制劑與如何防止降低荔枝表皮結構的改變將是我們計劃執行的重點結果。

本計畫的結果與討論同時參閱附加檔(1),(2)中的"Results and Discussion"

六、結論

從荔枝褐化過程中發現，荔枝表面結構的變化對荔枝褐化具有決定性的結果，當其表面結構空洞後，褐化反應便會快速進行，因此再其表面覆蓋一層保護膜可以有效的防止其表面結構鬆散導致褐化反應快速進行，在這工作上，尋找全球應用於食品水果保鮮的塗蠟技術，將可食性的蠟均勻的覆蓋在其上，維持其表面完整，近一步的保持水分及防止內生性的PPO抑制劑散失，以SEM掃描其果皮表面證實表面結構完整，進而延緩和化反應產生。

計畫的結論同時參閱附加檔(1),(2)中的"Conclusion"

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附錄一

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

Tsai-Mu Cheng[a], Pei-Chen Huang[a], Ju-Pin Pan[b,c], Kuan-Yu Lin[a], Simon J.T. Mao[a,d,*]

[a] *College of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu, Taiwan, ROC*
[b] *Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[c] *School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*
[d] *Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan, ROC*

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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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* Corresponding author. Tel.: +886 3 571 2121x56948; fax: +886 3 572 9288.
E-mail address: mao1010@ms7.hinet.net (S.J.T. Mao).

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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]. Pre-stained 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

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below:

$$2\text{catechol} + O_2 \rightarrow 2o\text{-benzoquinone} + 2H_2O$$

Catechol (colorless) — *o*-Quinone (in dark brown)

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

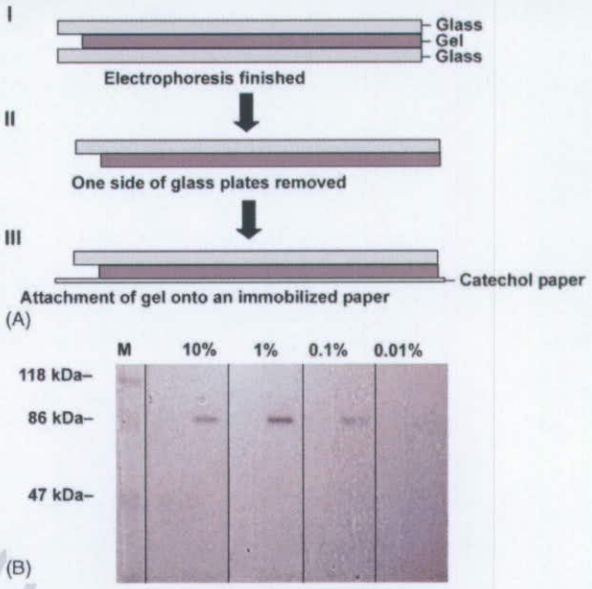

Fig. 1. Instant identification of PPO activity following a 10% SDS-PAGE: (A) Following electrophoresis (step I), one side of the glass plates (7 cm × 8 cm) was removed (step II). The resolving gel was immediately placed onto the top of a catechol-paper (step III). (B) Top view of visualized PPO activity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

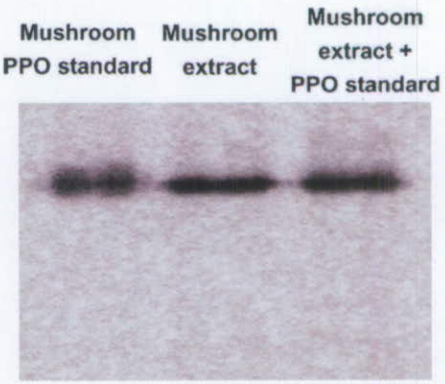

Fig. 2. Identification of PPO activity between the isolated PPO standard of mushroom and its extract on SDS-PAGE blot. One single band corresponding to PPO standard (60 units) or to that in mushroom extract (10 μL) was observed. The molecular form of PPO in the extract (5 μL) spiked with the purified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

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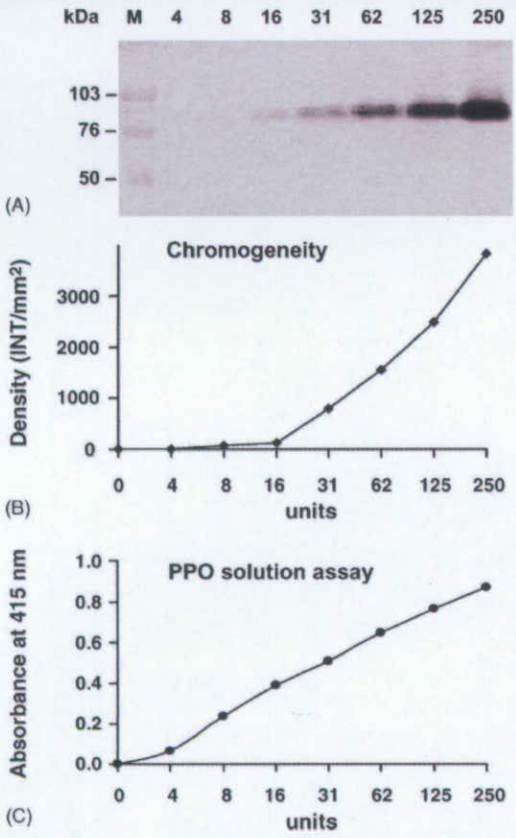

Fig. 3. Comparison of the PPO activity between SDS-PAGE bolt and conventional solution assay: (A) Serial dilutions of purified mushroom PPO (4–250 units) were run for SDS-PAGE. Following electrophoresis, the gel was placed onto the catechol-paper allowing the development of *o*-quinone product. (B) The 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.

uate 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

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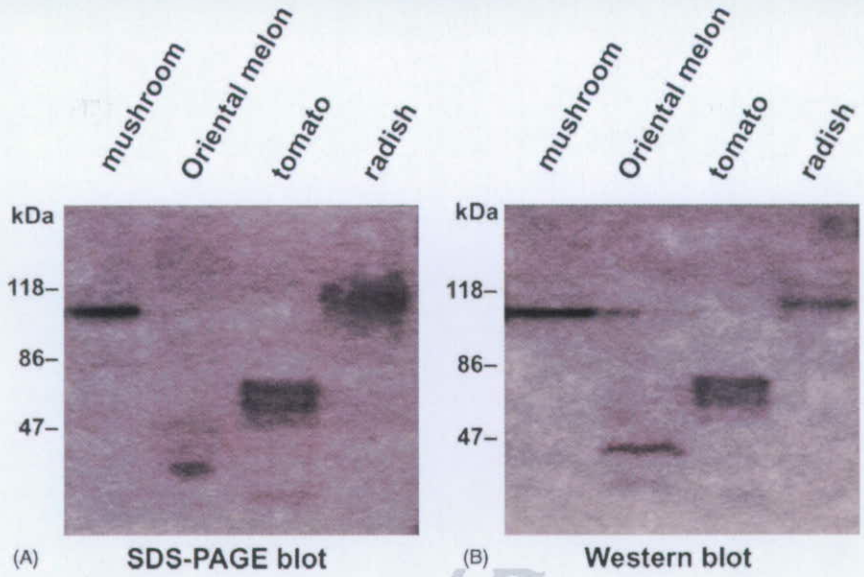

Fig. 4. Typical examples of PPO identified by PAGE-blot and Western blot: (A) PPO activity of plant extracts showing different molecular forms was identified by a SDS-PAGE blot. (B) PPO proteins were identified by a Western blot using a mouse polyclonal antibody prepared against mushroom PPO. This antibody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

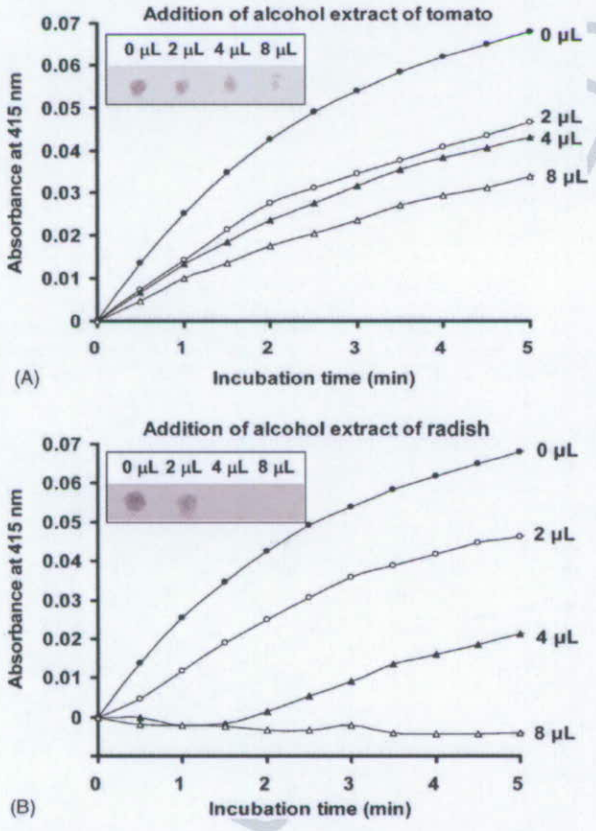

Fig. 5. Inhibitory effect of alcohol soluble-fraction from tomato and radish extract on mushroom PPO activity. Each fraction was incubated with 10 µL of purified mushroom PPO at 24 °C for 10 min prior to the solution assay (see Section 2). Insert: 5 µL of the reaction mixture were spotted on a catechol-paper.

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 compounds, how-

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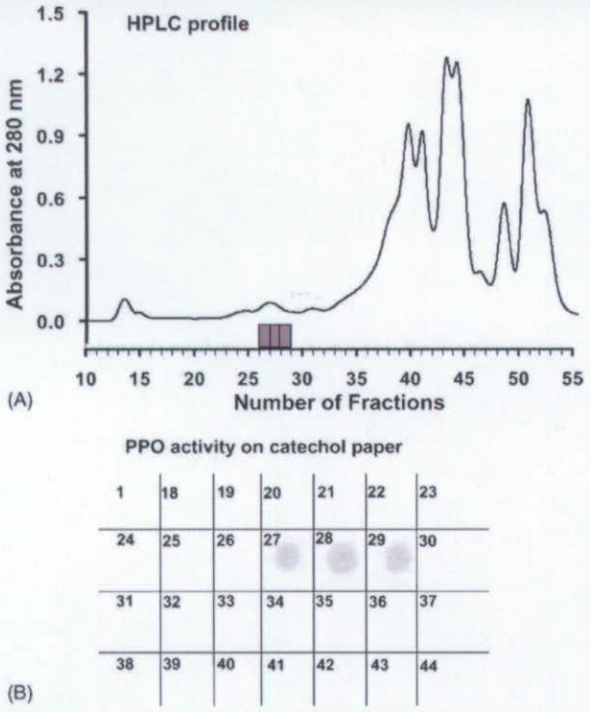

Fig. 6. One-step purification of mushroom PPO on HPLC Sepharose G-200. About 250 μL of mushroom extract were applied onto a Sepharose column (1.0 cm × 30 cm), while PB was used as a mobile phase at a flow rate of 0.5 mL/min (A). Each fraction contains 0.5 mL aliquot, in which 5 μL were used for identifying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

ever, 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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附錄二

An excited and novel mechanism involved in the skin darkening of litchi. An implication for cosmetic development.

Pei Chen Huang[1], Tsai Mu Cheng[1], Hsiao Han Hsu[1], Wie Yan Lai[1], Jerwei Hsieh[2], Simon J.T. Mao[1*]

1. Research Institute of Biochemical Engineering, College of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, ROC
2. Research & Development Division, Precision Instrument Development Center, National Science Council the Executive Yuan, Hsinchu, Taiwan, ROC

Short title:

*Correspondence to:
Simon J. T. Mao, Ph.D.
Dean and Professor
College of Biological Science and Technology
National Chiao Tung University
75 Po-Ai Street
Hsinchu, Taiwan, ROC

Phone: 886-3-571-2121 ext. 56948
FAX: 886-3-572-9288
E-mail: mao1010@ms7.hinet.net

Abstract

Polyphenol oxidase (PPO) is a copper-containing enzyme that catalyzes the chain-oxidation from monophenol or polyphenols to *o*-diphenols and subsequent *o*-quinones. The enzyme reflects the browning reaction in fruits. In the present study, we investigated the oxidation activity of PPO in litchi pericarp and the mechanism by which PPO instantly makes pericarp browning.

PPO of litchi pericarp was initially extracted and isolated through gel filtration chromatography and then eluted directly from SDS-PAGE. Two molecular forms of litchi PPO were identified as 86 and 66 kDa, and thereafter named as PPO-86 and PPO-66, respectively. The Km and Vmax were determined as 66 mM and 382 μM/min for PPO-86 and 102 mM and 290 μM/min for PPO-66, respectively. Most importantly, PPO-86 could trigger the 3,3'-diaminobenzidine and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) oxidation, which was not found in other plant PPO. Thus, the unique PPO activity of litchi might account for its superior rapid-browning reaction. In addition, we show the presence of a potent volatile inhibitor(s) for PPO in litchi pericarp. The surface of post-harvesting litchi pericarp revealed an opening ultra structure under the scanning electron macroscopic examination, therefore allowing an instant evaporation of PPO inhibitor. As such, the PPO oxidation was proceeded. The novel finding clarifies the mechanism involved

in the rapid browning phenomenon of post-harvesting litchi pericarp. Since the PPO is also know existed in human as tyrosinase responsible for the formation of "darkening spots" on skin, the finding of evaporation of potent PPO inhibitor may be potentially used as a strategy in developing a novel cosmetic product.

Keywords: litchi; polyphenol oxidase; tyrosinase; browning reaction; natural inhibitor; cosmetic product; skin darkening.

Introduction

Polyphenol oxidase (PPO), also known as tyrosinase (EC 1.14.18.1), is a copper containing enzyme that catalyzes two different reactions using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols and the oxidation of the *o*-diphenols to *o*-quinones [1]. PPO are ubiquitous plant enzymes containing a dinuclear copper center. In the wound-response mechanism of the plant they catalyze the oxidation of a broad range of *ortho*-diphenols to the corresponding *o*-quinones coupled with the reduction of oxygen to water [2]. PPO action has been implied in protecting plants against pathogens and herbivores and regulation of the rate of deleterious photo-oxidative reactions in chloroplasts [3-7]. This enzyme is widely distributed in microorganisms, animal, and plants and is responsible for melanization in humans and for browning in fruits and vegetables.

Browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during post harvest handling and processing. The mechanism of browning in food is well characterized and is mainly enzymatic in origin [8]. This phenomenon is mediated by endogenous enzymatic activities, such as polyphenol oxidase (PPO). Enzymatic browning of fruits and vegetables is mostly related to the oxidation of endogenous phenolic compounds. This process ultimately leads to the formation of dark brown

polymers of a quinoidal nature [9].

Litchi deteriorates once harvested, resulting in browning of the pericarp surface [10]. Browning has been attributed to rapid degradation of the red pigments and oxidation of phenolics by polyphenol oxidase (PPO), producing brown-colored by-products [11,12]. In this study, the objective was aimed to explore the mechanism by which PPO instantly makes pericarp browning.

Materials and Methods

Plant materials

Litchi (*Litchi chinensis* Sonn.) at commercial maturity, where maintaining at about 10℃, were purchased from a local market. They were then placed in a -20℃ ice-packed box and immediately used for the study.

Purification of Litchi pericarp Polyphenol Oxidase

Litchi pericarp was separated from whole fruits, placed in phosphate-buffered saline (pH 7.4) to a final concentration of 50% w/v, and homogenized in an ordinary domestic blender. The homogenized mixture was then centrifuged for 30 min. at 15000xg at 4℃. Then, the supernatant was filtered through 90mm filter paper to eliminate any residual solids. Solid ammonium sulfate was added to the supernatant and the 50-80% saturation precipitate collected for 30 min. centrifugation at 15000xg at 4℃., redissolved in phosphate-buffered saline (pH 7.4), and dialyzed overnight against the same buffter.

A gel-filtration high performance liquid chromatography (HPLC) column Sephadex 200 was used to partially purify PPO from Litchi pericarp extract at a flow rate of 0.5 mL/min using PB as a mobile phase [13]. The obtained fractions,

monitored at 280 nm, containing PPO activity were pooled and stored at -20 ℃ prior to the use.

Determination of PPO activity

A microtiter plate containing 96-wells was used for monitoring the PPO activity. To 200 μl of 100 mM catechol substrate containing phosphate-buffered saline (pH 7.4) 10 μL of the extracts were added. The PPO enzyme activity was determined by reading the absorbance at 415 nm for the first 30 min using a microplate reader equipped with an automatic printer over time.

Detection of PPO activity following SDS-PAGE

Electrophoresis was conducted in a vertical slab gel unit (Mini PIII, Bio-Rad) equipped with a PAC 300 power supply (Bio-Rad). Sample extract 15 μL (50%; W/V) equilibrated in 10 mM Tris-HCl and 0.1% SDS, pH 6.8, was loaded for a 10% SDS-PAGE according to procedures described previously [14,15]. The electrophoresis was conducted without β-mercaptoethanol and heat to avoid the deteriation of PPO activity. Prestained marker was used for the molecular weight index. Gels were run at a constant current of 20 mA. After 60 min, the electrophoresis was stopped and PPO activity was in situ blotted by pressing gel onto

the catechol-immobilized paper.

Detection of endogenous volatile PPO inhibitor

Browning reaction was observed over time when litchi fruit was placed in normal air, which was enhanced by increasing air flow, the reaction was attenuated while covering with petroleum jelly, and enhanced by increasing inhibitor evaporate under the vacuum for 10 min and placed in normal air.

Preparation of endogenous volatile PPO inhibitor

PPO inhibitor separated from Litchi pericarp crude extract used 5 kDa centricon (VIVASCIENCE, Germany), centrifuged for 30 min at 3,000 xg, 4℃. Path rough are small molecule inhibitor, stored at 4℃ prior to the use.

Scanning Electron Microscopy (SEM)

The structure of Litchi pericarp (before and after interposition between a pair of Pt electrodes and a dc electrical field of 20 V for 1 min) was evaluated by electron microscopy. A HITACHI S-800 scanning electron microscope, specimens were washed for 2 hr in a phosphate-buffered saline (pH 7.4) solution with 5% glutaraldehyde (Merck) for fixation. Then the samples were washed five times for 10

additional min, each time with the phosphate-buffered saline (pH 7.4). The samples were later washed for 15 min in each of an ascending gradient of ethanol baths (25%, 50%, 75%, 95%), and twice in 100% ethanol for 30 min, and Acetone overnight. They were then dried with CO_2 and gold-coated before inspection and characterization.

Immunolocalization of Litchi pericarp PPO

The sections mounted on slides were dewaxed in phosphate-buffered saline (pH 7.4). The sample was immersed in 1% skin milk for 1 h. Following 3 washes with PBS for 5 min, Primary antibody (polyclonal anti-litchi PPO-86, Mouse plasma) diluted 1:100 and (polyclonal anti-litchi PPO-66, Mouse plasma) diluted 1:50 in wash buffer(1% skin milk +0.05%Tween20), in the latter blocking solution was added to the sections in a moist chamber for 1 to 1.5 h, secondary antibody (Fluorescein(FITC)-conjugated Goat anti-Mouse IgG, Jackson) diluted 1:100 in wash buffer was added, incubated for 2h, and washed as in the previous step. The control (non-immune serum and omission of the primary antibody) did not show background level.[16]

Results and Discussion

Purification of Litchi pericarp PPO

Litchi pericarp PPO was partially purified by ammonium sulfate, the enzyme being most stable at pH 7.4 [17], we used 50%～90% ammonium sulfate partially purified Litchi pericarp PPO, initially extracted and isolated through gel filtration chromatography Sepharose G-200 and then eluted directly from SDS-PAGE (Fig.1), purify higher then Y.M. Jiang *et al.* (1997). In process, we detect two molecular forms of litchi PPO were identified as 86 and 66 kDa, and thereafter named as PPO-86 and PPO-66, respectively (Fig.2). Purified PPO-86 and PPO-66 yield were 3.1% and 1.2%, respectively.

Characterization of Litchi pericarp PPO

PPOs (polyphenol oxidases, or benzenediol:oxygen oxidoreductase (EC 1.10.3.2)) are copper metaloproteins that catalyze the oxidation of *o*-dihydroxy phenols to quinones. In order to understand of Litchi pericarp PPO a role as to impersonate in browning reaction, therefore used purified PPO to produce Characterization. Detection of Uniqueness of PPO-86 could trigger the 3,3'-diaminobenzidine and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) oxidation (Fig.3), which was not found in other plant PPO. Thus, the unique PPO

activity of litchi might account for its superior rapid-browning reaction.

Analysis of endogenous inhibitors of PPO

Mature litchis with attractive bright red color would rapidly turn brown after harvest (Fig.4), resulting in a significant loss of commercial value. To find out a way to stop from browning reaction, we wrapped up litchis with petroleum jelly and then it did work. According to our previous research and natural volatile inhibitors of cucumbers that have been proved [18], we proposed the hypothesis that litchi contains endogenous volatile inhibitors of PPO.

Fig.5 initially proved our hypothesis. The litchi pericarp turns brown after about 16 hours in room temperature. The evaporation of volatile endogenous inhibitors of PPO caused rapid-browning reaction in blowy condition. It successfully protected litchis from browning by using petroleum jelly to wrap. Furthermore, after lyophilized, the litchi pericarp turned brown immediately in room temperature. The result supports the assumption that litchi inhibitors against PPO are volatile.

The inhibitory effect of litchi volatile inhibitors on PPO activity

Previous papers indicate that papaya has PPO inhibitors with M_r of 650 Da [19] and inhibitors of cucumber are small molecules [20]. The M_r of PPO in litchi pericarp

is over 60 kDa so we separated enzymes and inhibitors by different molecule weights. To demonstrate the inhibitor as volatility, the removal of inhibitors by speed vacuum resulted in a great loss of inhibition against PPO (Fig.6 B). On the contrary, non-evaporated inhibitors can still stop the browning reaction of PPO (Fig. 6 A).

For further evidence to the hypothesis that the PPO inhibitor is endogenous and volatile, crude extract of litchi pericarp were evaporated by speed vacuum with time course. Then the enzyme activity was more active as more evaporation of inhibitors (Fig. 7). The result fully supports our hypothesis.

Effect of Litchi pericarp surface structure

After proving the existence of the volatility inhibitor of litchi, we want further whether understands the surface structure of litchi pericarp in order to influence the inhibitor to volatilize the fast factor, so we are by Scanning electron microscope (SEM) and observing the surface structure of litchi pericarp, SEM observe in Fig. 8, the longer the time with put in air is, its surface structure has been turned levelly and smoothly and closely into a coarse hole in a utensil and changed large, impelling the inhibitor to volatilize the more fast, the stronger PPO function is and produces and melts the more seriously browning.

Immunolocalization of litchi PPO

PPO was detected immunochemically near the cell walls with use of anti-litchi PPO antibodies, PPO are expressed at Litchi pericarp surface (Fig.9), since it is a membrane-bound enzyme, the intracellular localization of fruit PPO has been shown in chloroplasts, particularly on the inner face of thylakoids, In some cases, PPO activity has also been obtained from mitochondria in olive and apple, or partly associated with the cell wall in banana [21]. In mature apple fruits, where vacuoles occupy most of the cells, PPO was detected immunochemically near the cell walls with use of anti-apple PPO antibodies [22]. The position where PPO exists is all in the area of outside, so the inhibitor is instead of combining in the outside area, it is easy to volatilize and disappear to cause the inhibitor。

Conclusion

In view of recent studies indicating that tyrosinase is responsible for hyperpigmention in humans. Tyrosinase inhibitors have become increasingly important in medical and cosmetic products [23]. A large number of chemical compounds are characterised by particular fruit or flower fragrances, and therefore they are widely used in the food and perfume industry, molecular structures of chemical compounds were treated as molecular graphs, where the nodes represent atoms and the edges represent chemical bonds and whose definition was presented elsewhere [24]. 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 compounds, however, may be detrimental to the health and can also react with other components in the food system, resulting in unwanted effects [25]. The need for a safe and effective PPO inhibitor remains to be a subject of challenge [20, 26-28].

In this study, we detect two molecular forms of litchi PPO were identified as 86 and 66 kDa, and thereafter named as PPO-86 and PPO-66, respectively. Interesting, Most importantly, PPO-86 could trigger the 3,3'-diaminobenzidine and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) oxidation, which was not found in other plant PPO.

The surface of post-harvesting litchi pericarp revealed an opening ultra structure under the scanning electron macroscopic examination, therefore allowing an instant evaporation of PPO inhibitor. As such, the PPO oxidation was proceeded. The novel finding clarifies the mechanism involved in the rapid browning phenomenon of post-harvesting litchi pericarp.

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Figures and legends

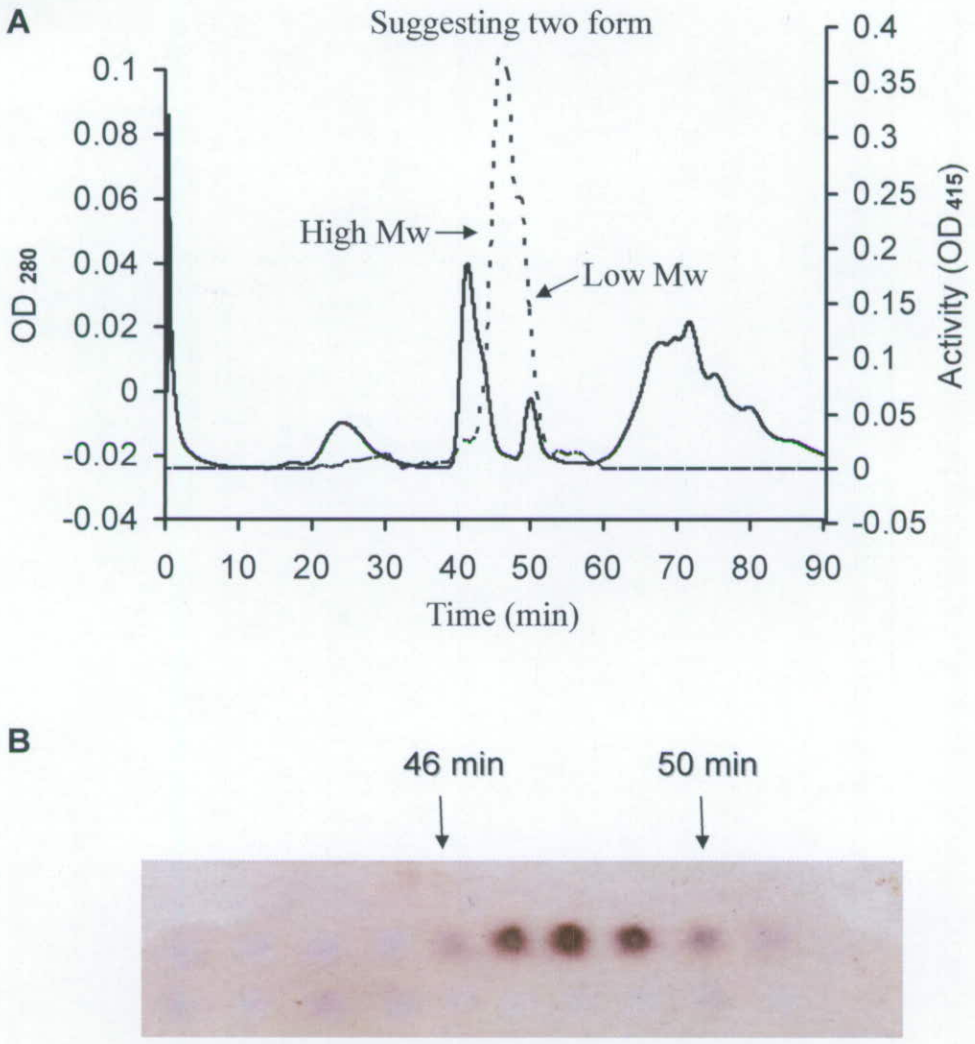


Figure 1. One-step purification of litchi pericarp PPO by HPLC (Sepharose G-200). Elution profiles of PPO activity on HPLC Sepharose G-200. About 250 μL of litchi extract were directly applied to the column at a flow rate of 0.3 mL/min using PBS, pH 7.4. Absorbance at 280 nm(—); Absorbance at 415 nm(---).

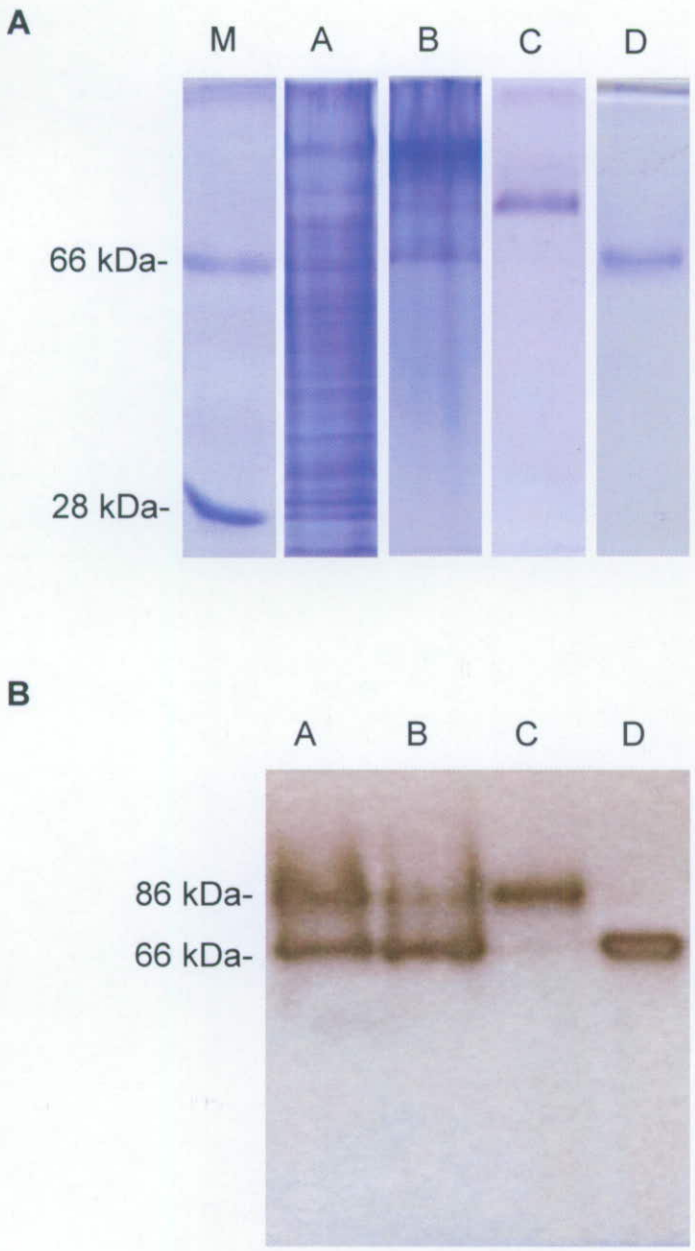
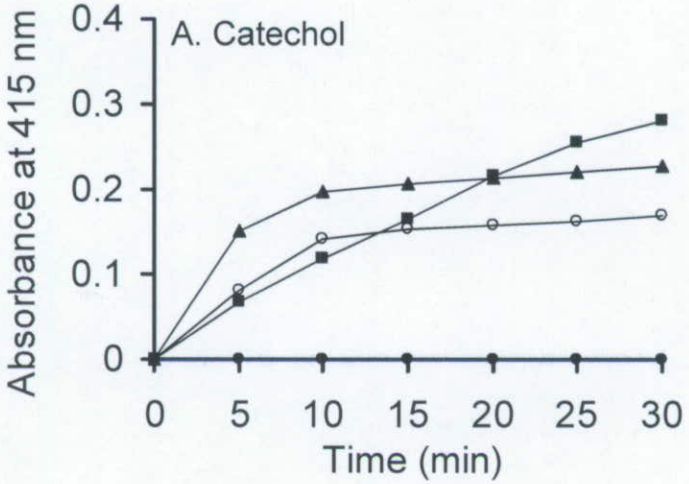
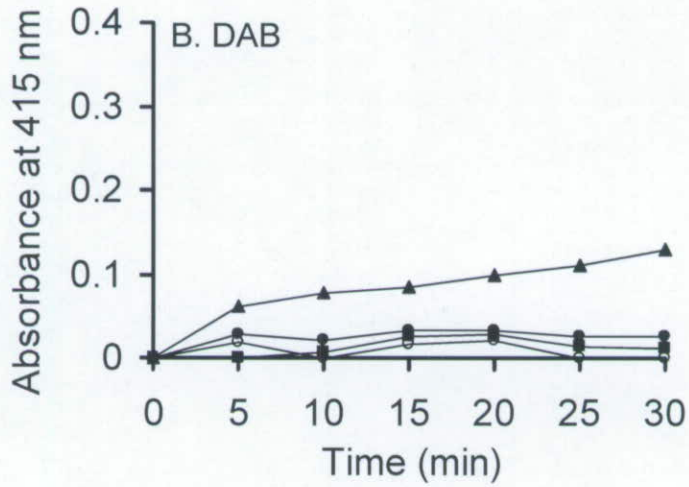


Figure 2. Purification of polyphenol oxidase from Litchi pericarp. A: Non-reducing SDS PAGE of litchi pericarp extract. B: SDS PAGE of PPO activity of litchi pericarp. Lane A: Total extract. Lane B: partially purified by ammonium sulfate precipitation. Lane C: purified PPO of higher Mw. Lane D: purified PPO of lower Mw.


A. Catechol
Absorbance at 415 nm
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25 30
Time (min)


B. DAB
Absorbance at 415 nm
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25 30
Time (min)

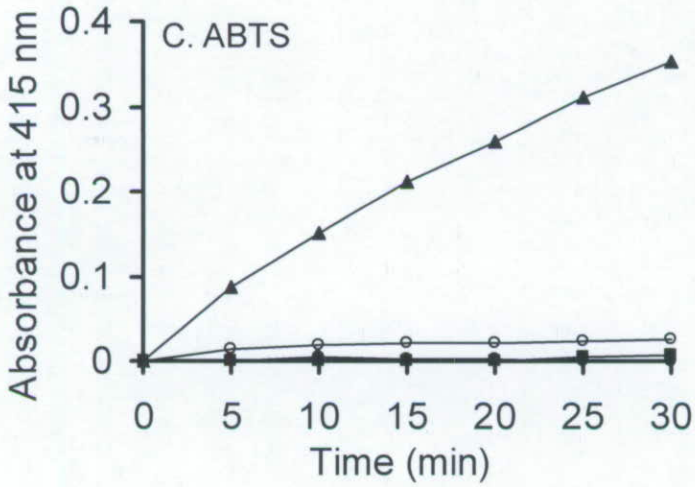


Figure 3. The PPO-86 of litchi pericarp has ability of special catalysis substrate. Add all kinds of enzymes (● HRP, ■ Tyrosinase, ▲ litchi pericarp PPO-86 , ○ litchi pericarp PPO-66) in 96 well plates separately, and incubated with different substrate (catechol , DAB , ABTS) of 200 μL and react separately in PBS (pH 7.4) at room temperature, measurement of 5 minutes under wavelength 415 nm. Fig. A: catechol; Fig. B: DAB; Fig. C: ABTS.

Figure 4. Browning reaction in litchis. Naturally grown (left) and harvested (right) litchi.

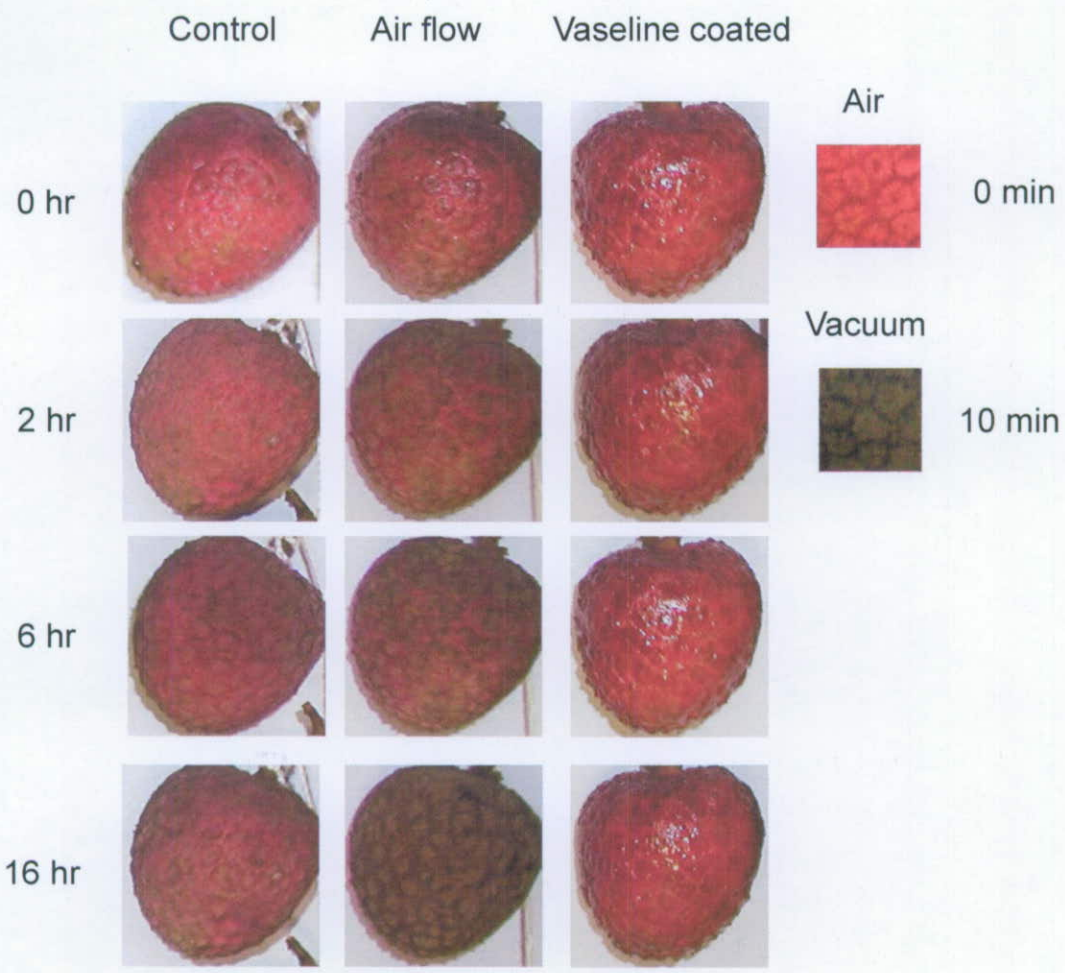


Figure 5. Demonstration of volatile inhibitor in litchi. Browning reaction was observed over time when litchi was placed in normal air (left), which was enhanced by increasing air flow (middle). The reaction was attenuated while covering with petroleum jelly (right). Top right: under the vacuum for 10 min.

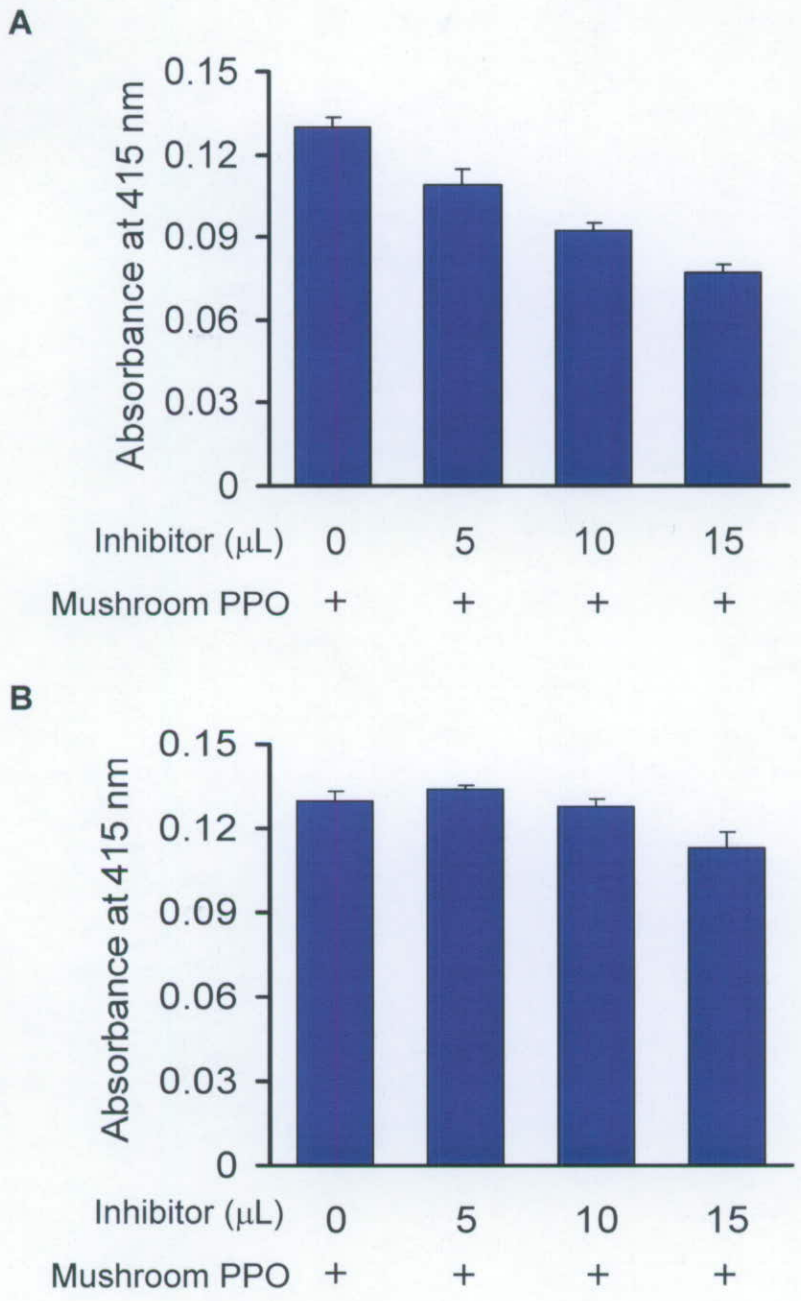


Figure 6. Inhibition of endogenous inhibitor extracted from litchi pericarp. A: Dose inhibition of extracted inhibitor. B: Reversed activity when inhibitor that was evaporated. Amicon filter was utilized to separate small molecules of inhibitor for PPO and demonstrated that the inhibitor was volatile in nature. Each fraction was incubated with mushroom PPO for 10 min at room temperature prior to the activity assay, in which 4 mM catechol were used as a substrate.

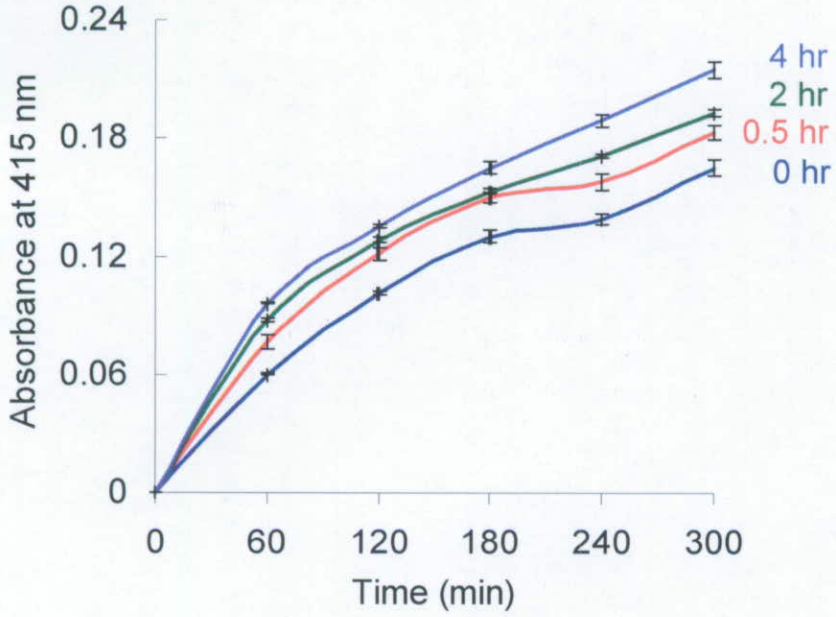


Figure 7. Increase in kinetics of PPO after evaporation of inhibitor. Time course of volatile inhibitor evaporated by speed vacum. To 200 μL of 100 mM catechol on microtiter well, 10 μL of sample extract were added. The reaction mixture was monitored at 415 nm over time.

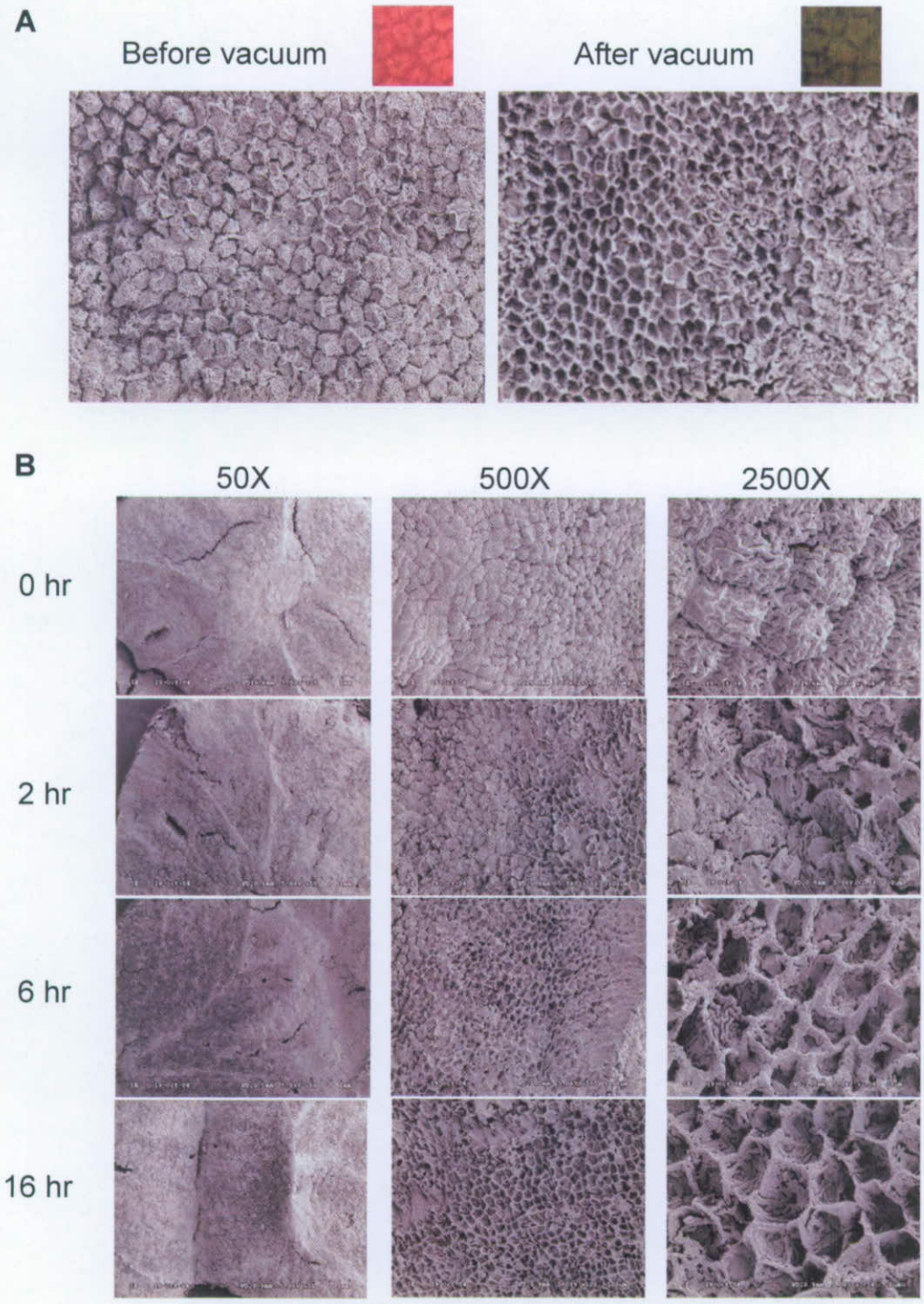
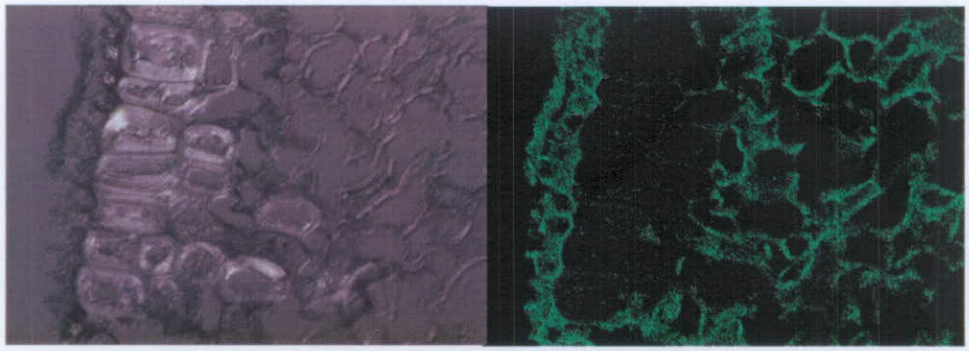


Figure 8. Scanning electron microscope (SEM) photographs of ultra structure of litchi pericarp surfaces. A: fresh area (left), and browning area (right) on the surface of litchi pericarp. B: Time-course following air blow. Scale bar in 50X, 500X, or 2500X represents 1 mm, 100 μm, or 20 μm in thickness length, respectively.

A

B



C

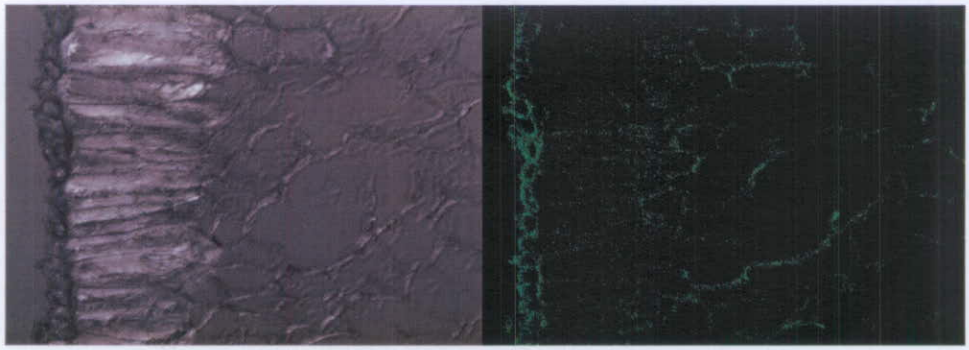


Figure 9. Immunolocalization of Litchi PPO. A: negative control, B: anti-litchi PPO-86, C: anti-litchi PPO-66. Bright-field microscopy was shows before exciting the light source of Litchi pericarp slices (left), Dark-field microscopy was used secondary antibody (Fluorescein(FITC), the green fluoresc only shows the position that PPO exists (right). Magnification 200X.