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Recovery of Active Polyphenol Oxidase and Peroxidase from Plant Tissues with High Phenolics and Chlorophylls

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Abstract

Extraction of active polyphenol oxidase and peroxidase from a plant rich in phenolics and chlorophylls in the post-harvest browning syndrome is described. Initially, general optimization using conventional enzyme extractions was performed. However, along with membrane-bound proteins, chlorophylls and phenols were also released with Triton X (TTX). With a view to obtaining high enzymatic activity, removal of the released chlorophylls and phenols by formation of TTX-114 micelles in the detergent rich phase after high-temperature induced phase separation was tested.

Introduction

Polyphenol oxidase (PPO) and peroxidase (POD) are key enzymes involving post-harvest browning disorder of horticultural produce [1-3]. Their latent stage together with high content of endogenous phenolic compounds and chlorophylls largely interfere enzymatic activity studied such as that of Backhousia myrtifolia tissues [4,5]. To overcome this problem, samples were extracted with different phenol removing agents in sodium phosphate buffer (pH 6.8) [4]. PPO was activated by the addition of detergents (e.g. TritonX-100) and POD was extracted with mild treatments (e.g. sucrose solution). For partial enzyme purification, high temperature induced phase separation was used. The inclusion of 5% Poly Vinyl Poly Pyrrolidone (PVPP) in the extraction buffer was effective in removing polyphenols. Latent PPO in crude extract from B. myrtifolia leaves was activated using 2% v/v TTX-114, resulting in an almost 30-fold increase in activity [4-6]. The same optimized extraction protocol also improved POD activity. While the phase separation step improved enzyme activity, it failed to maintain total protein content. Fresh extract of B. myrtifolia leaf tissue was, therefore, initially concentrated by filtering with an Amicon® PL-10 unit. However, concentrated endogenous phenolics interfered with enzyme activity [4].

Experimental

This protocol was conducted towards characterizing PPO and POD in the post-harvest browning syndrome of Backhousiamyrtifolia, an Australian native ornamental plant cultivated for cut flower production [7-9]. Leaf and floral tissues of this particular species are known for high phenolic and chlorophyll contents which is the interferences for enzymatic analyses [10,11]. Initially, general optimization using conventional enzyme extractions was performed. However, along with membrane-bound proteins, chlorophylls and phenols were also released with TTX. With a view to obtaining high enzymatic activity, removal of the released chlorophylls and phenols by formation of TTX-114 micelles in the detergent rich phase after high-temperature induced phase separation was tested.

Reagents and chemicals

Poly Vinyl Poly Pyrrolidone (PVPP), Poly Ethylene Glycol (PEG), Sodium phosphate buffer, 0.1 M, TritonX-114 (TTX) was purchased from Sigma-Aldrich Co., St Louis, MO, USA. Bio-Rad protein assay kit with Bovine Serum Albumin (BSA) was from Bio-Rad Laboratories, Inc., CA, and USA). 4-MethylCatechol (4-MC) Hydrogen Peroxide (H_2O_2), Calcium Chloride ($CaCl_2$), Ethylene Di amine Tetra Acetic Acid (EDTA), Boric acid, Tris, Guaiacol was also of Sigma-Aldrich Co., St Louis, MO, USA. Lastly, Native PAGE™ running buffer was supplied from Life Technologies CO., CA and USA.

Extraction and partial purification of the enzymes

Extraction was performed on 1.0 g samples ground in a mortar and a pestle with 5.0 mL of various concentration of PVPP or Poly Ethylene Glycol (PEG) saturated overnight in 0.1 M sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 10,000 × g for 25 min at 4°C and the supernatant used to analyze enzymatic activity and total phenolic content. It was found that incorporation of 5% (w/v) PVPP in the phosphate buffer removed most phenolic compounds and therefore, it was used for subsequent studies. By adding 2% (v/v) TTX-114 with the chosen phenol absorbing ager, the highest enzymatic activity was achieved. The rational for this is that latent or integral membrane proteins such as PPO require detergents to activate or loose from the membrane during extraction [12-14]. Further purification by temperature-induce phase separation with TTX-114 was as follows:

The supernatant was removed to a fresh tube and TTX-114 was added to a final concentration of 8% (v/v). The solution was kept at 4°C for 15 min and then transferred to a water bath at 30°C for 10 min. The solution became cloudy due to the formation of large mixed micelles of detergent, hydrophobic proteins, and chlorophylls [15,16]. This solution was centrifuged at 5,000 × g for 15 min at room temperature. The clear supernatant was used to measure both PPO and POD enzyme activity.

Protein extraction for electrophoresis studies

To improve the yield of protein with a view to maximizing enzymatic activity of the extracts, the extracts were filtered through an Amicon® ultra unit (PL-10, Millipore, and USA). Ten milliliters of the extracts was pipette into a filter unit chamber, capped and then centrifuged at 4°C and 5,000 × g for 10 min. The retentive (~ 5 mL) remaining in the filter chamber and the elute (~ 5 mL) in the receiving chamber were tested for enzymatic activity and protein content. Protein content was also determined spectrophotometrically using the Bio-Rad protein assay kit as a standard [17]. Enzymatic activity assays were carried as described herein.

The extract (100 µL) was added into a 1 cm semi-micro cuvette containing 250 µL 10 mM 4-methylcatechol (4-MC) solution and 650 µL 100 mM sodium phosphate buffer at pH 6.8 adapted from Jiang [18]. After mixing, the cuvette was immediately transferred into the spectrophotometer and absorbance at 410 nm was recorded every 30s for 3 min at room temperature using Pharmacia LKB-Utrospec III, spectrophotometer. An increase in absorbance indicated the formation of brown pigment (o-quinone). PPO activity was calculated as the slope of absorbance against time [19]. Similarly, POD activity was measured by adding 100 µL of the extract into a mixture of 500 µL sodium phosphate buffer (200 mM, pH 5.8), 200 µL of 350 mM hydrogen peroxide (H_2O_2) and 200 µL 2 mM guaiacol adapted from Dan and Deverall [20]. The increase in absorbance was recorded at 470 nm at room temperature for 3 min. The reaction assay with 200 µL deionizer water instead of H_2O_2 was also analyzed. POD activity was calculated by subtracting the activity without H_2O_2 (PPO activity) from the activity with H_2O_2 [21]. One unit of activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 units per min.

Native gel electrophoresis for PPO and POD activity

Non-denaturing gel electrophoresis (native) of protein extract was performed with the X Cell sure Lock™ Mini-cell electrophoresis (Nitrogen™, Canada). Mini-cell electrophoresis and the following protocol were followed. Briefly, samples (~ 10 µg) were loaded into Pre-cast NativePAGE™Novex®3-12% Bis-Tris gel (1.0 mm, 10 well) and electrophoreses using the Native PAGE™ running buffer. The gels were run at 150 V constantly for 115 min at room temperature. The native polyacrylamide gels were stained for PPO activity by immersion in 0.1 M catechol solution containing 1.5 g $CaCl_2$, 0.2 g EDTA, 0.15 g boric acid and 2.0 g Tris for 15 min. The same gels were then incubated in 100 mM H_2O_2 for a further 15 min for POD activity [22-23].

Results and Discussion

Cut stems of Backhousia myrtifolia or Cinnamon myrtle bearing clusters of small flowers with white to lime-green sepals are used in flower arrangements as filler flowers. However, extensive flower and leaf browning has become a problem for this Australian native cut flower. The pre and postharvest browning syndrome [7-9] leaves a lasting loss of confidence within its market places, mainly in Japan [10,11]. Browning in plant tissues with phenolic content, like B. myrtifolia, is typically the result of enzymatic browning reactions. PPO and POD from B. myrtifolia leaf and floral tissues were recoveries during the extraction with different phenol removing agents in sodium phosphate buffer (pH 6.8). PPO was activated by the addition of detergents (e.g. TritonX-114) and POD was extracted with mild treatments (e.g. sucrose solution). For partial enzyme purification, high temperature induced phase separation was used. The inclusion of 5% Poly Vinyl Poly Pyrrolidone (PVPP) in the extraction buffer was effective in removing polyphenols (Figure 1).

Latent PPO in crude extract from B. myrtifolia leaves was activated using 2% v / v TTX-114, resulting in an almost 30-fold increase in activity. The same optimized extraction protocol also improved POD activity (Figure 2). While the phase separation step improved enzyme activity, it failed to maintain total protein content. Fresh extract of B. myrtifolia leaf tissue was, therefore, initially concentrated by filtering with an Amicon® PL-10 unit. However, concentrated endogenous phenolics interfered with enzyme activity (Table 1).

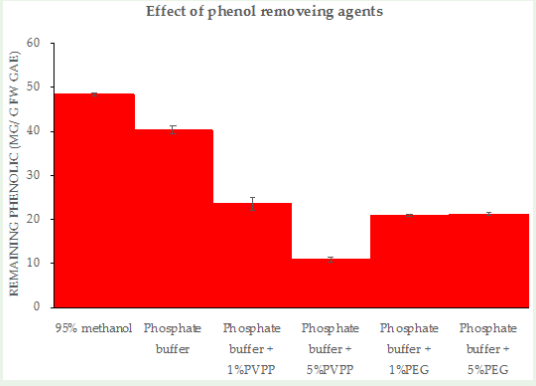

Figure 1: Total phenolic concentrations in crude extracts for B. myrtifolia leaves using various extraction solutions [9]. The buffer used was 0.1 M sodium phosphate buffer pH 6.8. Values are means (n = 3) ± Standard Error (SE).

Table 1: Effects on PPO and POD activities of partial purification by temperature-induce phase separation with TTX-114for leaf and floral tissue extract from b. myrtifolia.

Extraction	PPO (units/mL)	POD(units/mL)	Protein content(mg/mL)	Specific PPO (units/mgprotein)	Purification (x – fold)	Specific POD (units/mgprotein)	Purification (x – fold)
Leaf tissue							
A	117 ± 7.1	15 ± 0.0	0.6 ± 0.11	196 ± 12.0	**1**	25 ± 0.0	**1**
B	343 ± 24.0	78 ± 0.3	0.75 ± 0.14	460 ± 32.0	**2.5**	107 ± 0.4	**4.3**
C	182 ± 8.2	88 ± 35.0	1.8 ± 0.29	101 ± 4.5	**0.5**	48.4 ± 19.0	**2.4**
Floral tissue							
A	n/a	n/a	0.48 ± 0.12	n/a	**1**	0	**1**
B	73 ± 1.8	8.9 ± 1.1	0.23 ± 0.03	319 ± 7.8	**300**	39 ± 4.7	**40**
C	103 ± 25.0	22 ± 3.9	0.56 ± 0.01	183 ± 45.0	**200**	39 ± 7.0	**40**

A = Crude enzyme extract with 5% PVPP and 2% TTX-114 in sodium phosphate buffer.

B = Crude extract (A) subjected to temperature induced phase separation with 8% TTX-114.

C = Concentrated protein of extract (B) by an Amicon® Filter Unit (AFU).

Data is as presented in Sommano [4], n/a = no activity.Values are means (n = 3) ± SE.

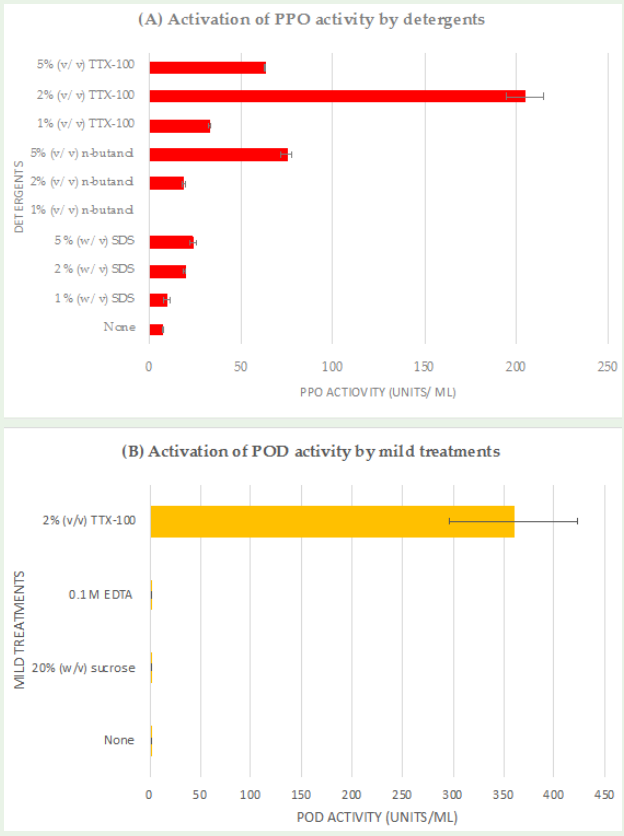

Figure 2: Effect of detergents (A) and mild extractants (B) on leaf PPO and POD activity for B. myrtifolia leaves. All detergents were mixed with 0.1 M sodium phosphate buffer pH 6.8 for PPO activity, while for POD detection the 0.1 M sodium phosphate buffer pH 5.8 was used except that of 2% TTX-100 (pH 6.8).Data is as presented in Sommano [4], n/d = not detected. Values are means (n = 3) ± SE.

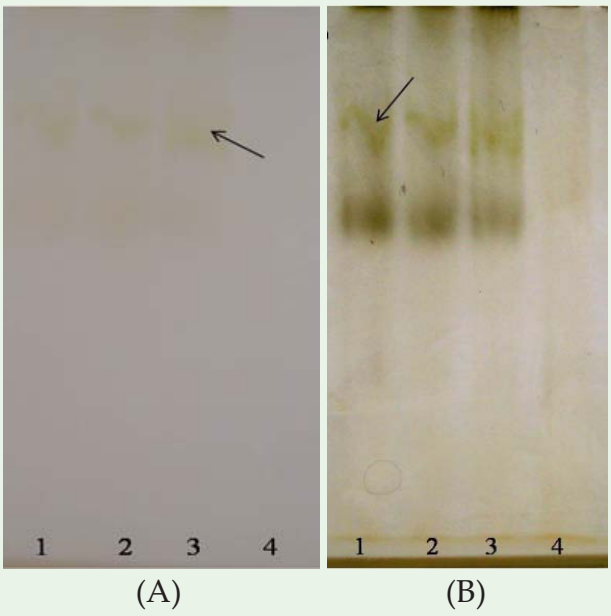

Figure 3: Native PAGE analysis for crude B. myrtifolia leaf and flower extracts with staining for PPO activity (a) and POD activity (b): Lane 1, fresh leaf extract (protein = 9.4 µg); lane 2, freeze dried leaf extract (protein = 11.4 µg); lane 3, fresh flower extract (protein = 9.7 µg); and, lane 4, freeze dried flower extract (protein = 10 µg) [4]. Arrows indicate chlorophyll (interference).

Upon staining in 0.1 M catechol solution for PPO activity, a single activity band was found in each lane for crude leaf, crude freeze-dried leaf and crude flower extracts (Figure 3A). However, no activity was present for the freeze-dried extract of floral tissue. No POD activity band was detected in the same gel post-stained in a 100 mM H_2O_2 solution (Figure 3B). Phenols and chlorophylls were evident in the lanes as greenish-brown pigments. Enzyme activities of crude and concentrated protein extracts (AFU retentive) were also compared on Native PAGE (Figure 4). No POD activity band was detected in any extracts, even with concentration through the AFU (data not shown).

Figure 4: Native PAGE analysis comparison of crude extract and concentrated protein extract for B. myrtifolia leaves and flowers obtained using an Amicon®PL-10 unit (AFU) and upon staining for PPO activity: Lane 1, fresh leaf extract (protein = 37.4 µg); lane 2, fresh flower extract (protein = 27.2 µg); lane 3, concentrated leaf extract (protein = 52.8 µg); and, lane 4, concentrated flower extract (protein = 30.5 µg).

Conclusion

In studying PPO and POD in plant phenolic rich like B. myrtifoliatissues, a combination of PVPP and high temperature-induced phase separation effectively improved enzymatic activity, including activity bands on native electrophoresis.

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