 doi:10.20944/preprints201810.0758.v1

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

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Abstract: The present protocol described extraction of active polyphenol oxidase and peroxidase from a plant rich in phenolics and chlorophylls in the post-harvest browning syndrome of *B. myrtifolia*. Initially, general optimisation 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.

Keywords: latent PPO; optimisation; peripheral membrane POD; total phenol content

1. Introduction

Backhousia myrtifolia (Cinnamon myrtle) is a native Australian plant cultivated for its cut flowers. Harvested stems 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 novel product. The pre and postharvest browning syndrome {1-3] leaves a lasting loss of confidence within its market places, mainly in Japan [4,5]. Browning in plant tissues high in phenolic content, like *B. myrtifolia*, is typically the result of enzymatic browning reactions.

Polyphenol oxidase (PPO) and peroxidase (POD) are key enzymes involving post-harvest browning disorder of horticultural produce [6-8]. Their latent stage together with high content of endogenous phenolic compounds and chlorophylls largely interfere enzymatic activity studied [9,10]. To overcome this problem, samples were extracted with different phenol removing agents in sodium phosphate buffer (pH 6.8) [9]. 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% polyvinylpolypyrrolidone (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 [9-11]. The same optimised 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 [9].

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2. Experimental Design

This protocol was conducted towards characterising PPO and POD in the post-harvest browning syndrome of *Backhousia myrtifolia,* an Australian native ornamental plant cultivated for cut flower production. Leaf and floral tissues of this particular species are known for high phenolic and chlorophyll contents which is the interferences for enzymatic analyses. Initially, general optimisation 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.

2.1. Materials

- Polyvinylpolypyrrolidone (PVPP)
- Polyethylene glycol (PEG)
- Sodium phosphate buffer, 0.1 M
- TritonX-114 (TTX)
- Bio-Rad protein assay kit with Bovine Serum Albumin (BSA) (Manufacturer's name, city, state if applicable, country)
- 4-methylcatechol (4-MC)
- Hydrogen peroxide (H_2O_2)
- Guaiacol
- NativePAGE™ running buffer
- Calcium chloride ($CaCl_2$)
- Ethylenediaminetetraacetic acid (EDTA)
- Boric acid
- Tris

2.2. Equipment

- Amicon® ultra unit (PL-10, Milipore, USA).
- Spectrophotometer (Pharmacia LKB-Utrospec III)
- XCellsureLock™ Mini-cell electrophoresis (Invitrogen™, Canada)
- Pre-cast NativePAGE™Novex®3-12% Bis-Tris gel (1.0 mm, 10 well)

3. Procedure

3.1 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 polyethylene 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 4ºC and the supernatant used to analyse 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:

1. The supernatant was removed to a fresh tube and TTX-114 was added to a final concentration of 8% (v/v).

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2. 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].
3. 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.

3.2 Protein extraction for electrophoresis studies

To improve the yield of protein with a view to maximising enzymatic activity of the extracts, the extracts were filtered through an Amicon® ultra unit. Ten millilitres of the extracts was pipetted into a filter unit chamber, capped and then centrifuged at 4°C and 5,000 × *g* for 10 min. The retentate (~ 5 mL) remaining in the filter chamber and the eluate (~ 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.

1. 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] }.
2. After mixing, the cuvette was immediately transferred into the spectrophotometer and absorbance at 410 nm was recorded every 30 s for 3 min at room temperature.
3. An increase in absorbance indicated the formation of brown pigment (*o*-quinone). PPO activity was calculated as the slope of absorbance against time [19].
4. 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 Dann and Deverall [20]}.
5. The increase in absorbance was recorded at 470 nm at room temperature for 3 min.
6. The reaction assay with 200 µL deionised water instead of H_2O_2 was also analysed.
7. POD activity was calculated by subtracting the activity without H_2O_2 (PPO activity) from the activity with H_2O_2 [21].
8. One unit of activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 units per min.

3.3 Native gel electrophoresis for PPO and POD activity

1. Non-denaturing gel electrophoresis (native) of protein extract was performed with the XCellsureLock™ Mini-cell electrophoresis and the following protocol was followed:
2. Samples (~ 10 µg) were loaded into Pre-cast NativePAGE™Novex®3-12% Bis-Tris gel (1.0 mm, 10 well) and electrophoresed using the NativePAGE™ running buffer.
3. The gels were run at 150 V constantly for 115 min at room temperature.
4. 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.

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5. The same gels were then incubated in 100 mM H_2O_2 for a further 15 min for POD activity [22-23].

4. Expected Results

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% polyvinylpolypyrrolidone (PVPP) in the extraction buffer was effective in removing polyphenols (Table 1).

Table 1. Total phenolic concentrations in crude extracts for *B. myrtifolia* leaves using various extraction solutions.

Extraction	Total phenolics (mg/g FW GAE)	± SE
95% methanol	48.4	0.02
Phosphate buffer[a]	40.3	0.09
Phosphate buffer[a] + 1%PVPP	23.7	0.14
Phosphate buffer[a] + 5%PVPP	11.0	0.06
Phosphate buffer[a] + 1%PEG	21.0	0.38
Phosphate buffer[a] + 5%PEG	21.2	0.27

As presented in Sommano [9]

[a]0.1 M sodium phosphate buffer pH 6.8.

Values are means (n = 3) ± standard error (SE).

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 optimised extraction protocol also improved POD activity (Table 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 3).

Table 2. Effect of detergents and mild extractants on leaf PPO and POD activity for *B. myrtifolia* leaves.

Phosphate buffer pH	Phenol absorbing Agent	Detergents/ mild treatments	Enzymatic activity (units/ mL)
PPO activity			
pH 6.8	5 % (w/v) PVPP	none	7.67 ± 0.3
pH 6.8	5 % (w/v) PVPP	1 % (w/v) SDS	10.0 ± 1.7

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pH 6.8	5% (w/v) PVPP	2 % (w/v) SDS	19.3 ± 0.7
pH 6.8	5 % (w/v) PVPP	5 % (w/v) SDS	24.0 ± 1.5
pH 6.8	5 % (w/v) PVPP	1% (v/v) *n*-butanol	n/d
pH 6.8	5% (w/v) PVPP	2% (v/v) *n*-butanol	19.0 ± 0.6
pH 6.8	5 % (w/v) PVPP	5% (v/v) *n*-butanol	74.7 ± 3.2
pH 6.8	5 % (w/v) PVPP	1% (v/v) TTX-100	33.0 ± 1.0
pH 6.8	5% (w/v) PVPP	2% (v/v) TTX-100	205 ± 9.9
pH 6.8	5 % (w/v) PVPP	5% (v/v) TTX-100	63.3 ± 0.3
POD activity			
pH 5.8	5 % (w/v) PVPP	None	0.0089 ± 0.0005
pH 5.8	5 % (w/v) PVPP	20% (w/v) sucrose	0.0093 ± 0.005
pH 5.8	5% (w/v) PVPP	0.1 M EDTA	0.003 ± 0.001
pH 6.8	5 % (w/v) PVPP	2% (v/v) TTX-100	359.8 ± 63.8

As presented in Sommano [9]

n/d = not detected.

Values are means (n = 3) ± SE.

Table 3. Effects on PPO and POD activities of partial purification by temperature-induce phase separation with TTX-114 for leaf and floral tissue extract from *B. myrtifolia*.

Extraction	PPO (units/mL)	POD (units/mL)	Protein content (mg/mL)	Specific PPO (units/mg protein)	Purification (x – fold)	Specific POD (units/mg protein)	Purification (x – fold)
Leaf							
A	117 ± 7.1	15 ± 0	0.6 ± 0.11	196 ± 12	1	25 ± 0	1
B	343 ± 24	78 ± 0.3	0.75 ± 0.14	460 ± 32	2.5	107 ± 0.4	4.3
C	182 ± 8.2	88 ± 35	1.8 ± 0.29	101 ± 4.5	0.5	48.4 ± 19.0	2.4

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Flower							
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	22 ± 3.9	0.56 ± 0.01	183 ± 45	200	39 ± 7.0	40

As presented in Sommano [9]

n/a = no activity.

Values are means (n = 3) ± SE.

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).

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 1. a). 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 1. b). Phenols and chlorophylls were evident in the lanes as greenish-brown pigments. Enzyme activities of crude and concentrated protein extracts (AFU retentate) were also compared on Native PAGE (Figure 2.). No POD activity band was detected in any extracts, even with concentration through the AFU (data not shown).

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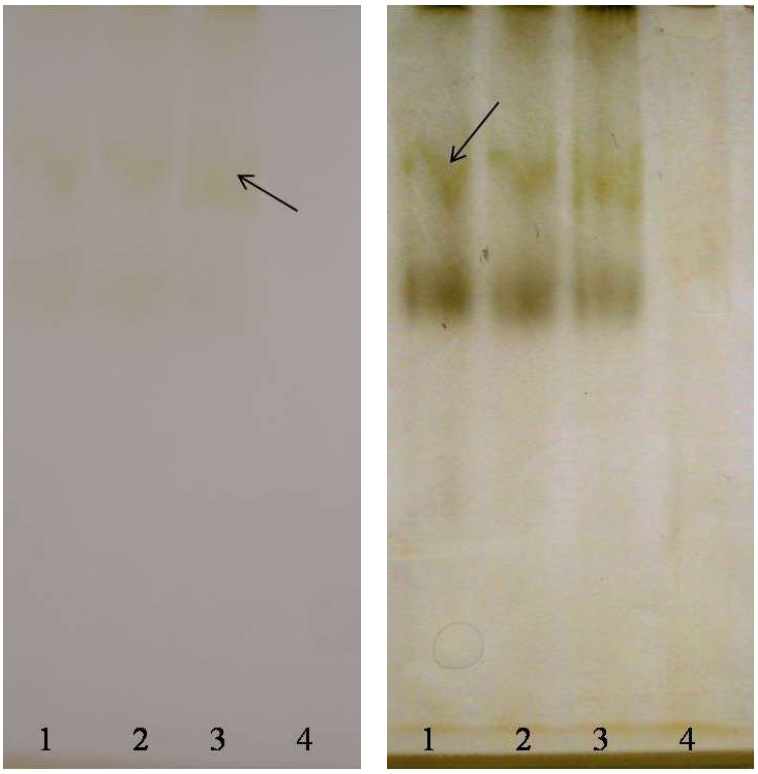

(**a**) (**b**)

Figure 1. 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) [9]. Arrows indicate chlorophyll (interference).

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Figure 2. 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).

5.Conclusion

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

Funding: This was part of research financially supported by School of Agriculture and Food Sciences, The University of Queensland, Australia (Prof. D. Joyce and Dr. B. D'Arcy).

Acknowledgments: The author also thank the School of Agriculture, Food and Wine at the University of Adelaide's Waite Campus (Dr. A. Able) and BSES Limited (Dr. P Joyce) for hosting part of the work describe herein.

Conflicts of Interest: The authors declare no conflict of interest.

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