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Polyphenol oxidase from bayberry (*Myrica rubra* Sieb. et Zucc.) and its role in anthocyanin degradation

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Abstract

Polyphenol oxidase (PPO) was extracted from bayberry (*Myrica rubra* Sieb. et Zucc. cv. *Biqi*), and its partial biochemical characteristics were studied. Stable and highly active PPO extracts were obtained using insoluble polyvinylpolypyrrolidone (PVPP) in sodium phosphate, pH 7.0, buffer. The highest PPO activity was observed in the ripe fruits. Optimum pH and temperature for bayberry PPO activity were pH 6.0 and $T = 30$ °C with 0.1 M catechol as substrate. PPO showed activity using the substrates of catechol, gallic acid and protocatechuic acid, but no activity with the substrates (+)-catechin, *p*-coumaric acid or caffeic acid. K_m and V_{max} values were 313 mM and 3.26ΔOD/min/g FW, respectively, with catechol as the substrate. Bayberry PPO did not act directly on cyanidin 3-glucoside but the rate of anthocyanin degradation was stimulated by the addition of gallic acid.

Keywords: Bayberry; *Myrica rubra* Sieb. et Zucc.; Polyphenol oxidase (PPO); Anthocyanin; Degradation

1. Introduction

Bayberry (*Myrica rubra* Sieb. et Zucc.) is one of six *Myrica* species native to China, belonging to the genus *Myrica* in the family *Myricaceae* (Chen, Xu, Zhang, & Ferguson, 2004). Bayberry is noted for its attractive red colour and exquisite flavours and is praised as a "precious southern Yangtze fruit of early summer" (Chen et al., 2004; Li, Zhang, & Chen, 1992). Unfortunately, bayberries are very perishable fruits, partly due to ripening in the hot and rainy season of June or July and with high respiration rates. The non-epicarp flesh exposed to the atmosphere is susceptible to bruising and mold rot. Most bayberry cultivars can only be stored with commercial value for 3 days at 20–22 °C and 9–12 days at 0–2 °C (Xi & Zheng, 1993). When stored at 1.0 ± 1.0 °C and chlorine dioxide is released slowly to the packaging bag, bayberries have a marketable life of 21 days (Li & Ma, 2004), which is the longest storage time reported for these fruits. Bayberry juice is an alternative product for longer consumption in which the *Biqi* cultivar has always been used as the major material because of its large production and excellent quality. However, the colour instability and haze formation during juice processing and storage are major problems facing food technologists (Chen, Su, & Ye, 1994; Li, Cen, & Joyce, 2002; Zhong, 2002).

Polyphenol oxidase (E.C. 1.14.18.1; PPO) in berry fruits plays an important role in the colour qualities and commercial properties of the fruits and their derived products. PPO and D-catechin together caused the loss of 50–60% of the strawberry anthocyanin pigments after 24 h at room temperature with the formation of a precipitate (Wesche-Ebeling & Montgomery, 1990a). PPO could even act directly on the blueberry anthocyanin degradation in crushed fresh blueberries, and the addition of chlorogenic acid stimulated browning reactions and pigment destruction (Kader, Rovel, Girardin, & Metche, 1997). Skrede, Wrolstad, and Durst (2000) demonstrated that endogenous PPO in blueberry fruits was responsible for anthocyanin

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degradation during juice processing. Yokotsuka and Singleton (1997) also showed that PPO was involved in anthocyanin destruction during grape juice processing.

The major anthocyanin in bayberry fruits has been identified as cyanidin 3-glucoside, which represents over 95% of the total pigments (Bao, Cai, Sun, Wang, & Corke, 2005; Ye, Chen, & Su, 1994). One of the degradation mechanisms of this *o*-diphenolic anthocyanin is enzymatic oxidation, including PPO catalyzing *o*-diphenol to *o*-quinone and then acting with cyanidin 3-glucoside to generate cyanidin 3-glucoside *o*-quinone with partial regeneration of the *o*-diphenolic cosubstrate (Kader, Haluk, Nicolas, & Metche, 1998; Peng & Markakis, 1963). Phenolic compounds with *o*-dihydroxy, such as chlorogenic acid (Kader et al., 1997), caffeoyltartaric acid (Sarni, Fulcrand, Souillol, Souquet, & Cheynier, 1995), catechol (Peng & Markakis, 1963), gallic acid (Prabha & Patwardhan, 1986) and catechin (Wesche-Ebeling & Montgomery, 1990a) are good substrates for PPO. In our previous study (Fang, Zhang, & Wang, 2007), gallic acid and protocatechuic acid were detected in the bayberry fruits. However, little is known about the PPO in bayberry fruits and its involvement in anthocyanin degradation. The purpose of the present work was to develop a procedure for the extraction of PPO from bayberry fruits. Its partial characterization, the variation of its activity during bayberry maturity, and its role in anthocyanin degradation were also studied.

2. Materials and methods

2.1. Plant material

The bayberry cultivar *Biqi* was used in the experiment since it is the dominant cultivar in industry. Fruits of unripe, ripe and overripe stages were harvested on June 18, 23, and 28, 2005, respectively. Fruits of different maturity were obtained from the same orchard in Cixi, Zhejiang province, China, and transported to our laboratory in 5 h. On arrival, 1 kg of undamaged fruits was homogenized using a blender and the pulps were used immediately for physicochemical determinations. Another 2 kg of each mature stage fruits was immediately quickly frozen and stored at −20 °C until PPO activity analysis was performed. Unripe and overripe bayberry fruits were only used in evaluation of the influence of maturity on PPO activity. All determinations were conducted within one month of arrival of the fruits.

2.2. Chemicals

Pure standards of protocatechuic acid, (+)-catechin, *p*-coumaric acid, caffeic acid and cyanidin 3-glucoside were purchased from Sigma (St. Louis, MO, USA), Fluka (Buchs, Switzerland) and Extrasynthèse (Genay, France). Insoluble polyvinilpolypyrrolidone (PVPP) was obtained from BASF Inc., Philippines, and pretreated as described by Wesche-Ebeling and Montgomery (1990b). Catechol and gallic acid were purchased from Shanghai Chemical Reagent Company, China.

2.3. pH and titratable acidity

A 10 g portion of bayberry flesh was crushed and blended with 40 ml of deionized water in a mortar. The pH was measured at this temperature, using a Toledo 320 S pH-meter (Mettler-Toledo Instruments (Shanghai) Ltd., China) calibrated with pH 4 and 7 buffers. After determination of pH, the solution was titrated with 0.1 N NaOH up to pH 8.1 (AOAC, 1984). The results were expressed as g citric acid per 100 g fresh weight (g/100 g FW).

2.4. Soluble solids

A Spoif refractometer (Shanghai Precise Instrument Corp. Ltd. Shanghai, China) was used for soluble solids analysis. Results were reported as Brix degrees.

2.5. Total solids and moisture content

The AOAC (1984) vacuum oven method was modified, employing a microwave oven operating at 650 W for 25–30 min.

2.6. Total pectic substances

Total pectic substances were analyzed by a colorimetric determination of galacturonic acid, produced by alkaline hydrolysis of pectic compounds (Dische, 1947).

2.7. Enzyme extraction

Enzyme extractions were modified according to the method of Cano, Lobo, de Ancos, and Galeazzi (1996), so that PPO activity was determined at the highest level (Table 1). In all assays, 20 g of fruit pulp was crushed with quartz sand for 10 min in a pre-cooled mortar and mixed with 20 ml of extraction buffer for 2 h at 4 °C in the dark. The mixture was centrifuged in a GL-22M centrifuge (Saite Xiangyi centrifuge Ltd., Hunan, China) at 16,000*g* for 30 min at 4 °C. The supernatants were filtered through Whatman No. 1 filter paper and kept on ice until used.

2.8. PPO activity

The PPO activity was determined at 25 °C by measuring the initial rate of increase in absorbance at 420 nm, as described by González, de Ancos, and Cano (1999). Unless otherwise stated, activity was assayed in 3 ml of reaction mixture, consisting of 2.7 ml of 0.1 M catechol in 0.2 M sodium phosphate buffer (pH 6.0) plus 0.3 ml of prepared enzyme, with a Vis-723 spectrophotometer (Shanghai Precise Instrument Corp. Ltd., Shanghai, China). The enzyme

Table 1
Influence of extraction buffer composition on bayberry PPO activity[a]

Buffer composition	PPO activity[b] (ΔOD/min/g FW)
0.05 M sodium phosphate + 1% (w/v) insoluble PVP	0.111 ± 0.008
0.05 M sodium phosphate + 1% (w/v) insoluble PVP + 1 M NaCl	0.118 ± 0.003
0.05 M sodium phosphate + 1% (w/v) insoluble PVP + 0.5% (w/v)Triton X-100	0.125 ± 0.011
0.05 M sodium phosphate + 1% (w/v) insoluble PVP + 1 M NaCl + 0.5% (w/v)Triton X-100	0.121 ± 0.002
0.2 M sodium phosphate + 1% (w/v) insoluble PVP	0.206 ± 0.006
0.2 M sodium phosphate + 1% (w/v) insoluble PVP + 1 M NaCl	0.213 ± 0.016
0.2 M sodium phosphate + 1% (w/v) insoluble PVP + 0.5% (w/v)Triton X-100	0.225 ± 0.007
0.2 M sodium phosphate + 1% (w/v) insoluble PVP + 1 M NaCl + 0.5% (w/v)Triton X-100	0.221 ± 0.004

[a] pHs for all extraction buffers are 7.0.
[b] Activity values are averages of three independent determinations ± standard deviation.

activity was determined by measuring the slope of the reaction line at zero time (initial rate). The enzyme activity unit was defined as the change in absorbance/min/mg protein or the change in absorbance/min/g FW.

2.9. Protein determination

Protein concentrations of the extracts were determined using the dye-binding method of Bradford (1976) with bovine albumin as the standard, measuring optical density (OD) at 595 nm.

2.10. Substrate specificity

The substrates used for specificity study are listed in Table 2. All compounds were prepared in 0.2 M sodium phosphate buffer at pH 6.0.

2.11. Effect of pH

The effect of pH on bayberry PPO activity was determined by catechol oxidation in 0.2 M sodium phosphate buffer at different pH values, ranging from 3.0 to 8.0.

2.12. Effect of temperature

The effect of temperature on the activity of bayberry PPO was investigated by equilibrating the substrate in a water bath (10–90 °C) for 10 min before introduction of the enzyme. PPO activity was measured at different temperatures, in the range 10–90 °C, using 0.1 M catechol in 0.2 M sodium phosphate buffer at pH 6.0.

Table 2
Effect of substrate on bayberry soluble PPO activity[a]

Substrate (0.1 M)	PPO activity (ΔOD/min/g FW)
Catechol	0.509 ± 0.011
Gallic acid	0.023 ± 0.008
Protocatechuic acid	0.018 ± 0.006
(+)-catechin	ND[b]
p-coumaric acid	ND
Caffeic acid	ND

[a] Activity values are averages of three independent determinations ± standard deviation.
[b] ND, not detectable.

2.13. Effect of substrate concentration

Solutions of catechol varying in concentration from 40 mM to 0.2 M were employed to study the effect of substrate concentration (González et al., 1999). In a quartz cell, 0.3 ml of enzyme solution was mixed with 2.7 ml of catechol at different concentrations in 0.2 M sodium phosphate buffer at pH 6.0. Michaels constant (K_m) and maximum velocity (V_{max}) of PPO were calculated from a plot of 1/activity versus 1/substrate concentration by the method of Lineweaver and Burk (1934).

2.14. Assay for cyanidin 3-glucoside degradation

A modified model solution was prepared to assay the bayberry anthocyanin degradation, as described by Kader et al. (1997). The reaction mixture contained 2.4 ml of 0.2 M sodium phosphate buffer at pH 3.5, 0.3 ml of 0.1 M gallic acid, 0.15 ml of cyanidin 3-glucoside (0.6 mg/ml) in the same buffer and 0.15 ml of the enzyme extract. The anthocyanin degradation was initiated by PPO addition and followed at 510 nm.

2.15. Data analysis

Values are averages of three determinations. The results were analyzed for variation (ANOVA) and statistical significance by *t* test with the software of SPSS.

3. Results and discussion

3.1. Selection of conditions for enzyme assay

For selection of the most suitable buffer compositions to extract PPO from bayberry tissue, several were employed, as described by Cano et al. (1996). The increase of molar concentration of sodium phosphate buffer increased the extraction of PPO activity nearly twofold. However, the increase of ionic strength by addition of sodium chloride and the addition of detergent Triton X-100 gave only slight increase of PPO activity in extracts of both low and high

concentration buffers (Table 1). The *t* tests were not statistically significant ($p > 0.05$) for these two factors. Therefore, for convenience and economy, a 0.2 M sodium phosphate buffer containing only 1% (w/v) insoluble PVPP was employed for all enzyme assays. The use of insoluble PVPP has been reported to produce good results in PPO extraction from different plant tissues, such as strawberry (Wesche-Ebeling & Montgomery, 1990b), papaya (Cano et al., 1996) and raspberry (González et al., 1999).

There are two main problems found in the optimization of the extraction conditions of PPO: the difficulty in obtaining full solubilization of the membrane-bound PPO, and avoiding phenolic oxidation during and after extraction (Rocha & Morais, 2001). The use of a detergent, such as Triton X-100, depends on the strength of PPO binding to membranes and, in most cases, increases the extraction of the enzyme (Galeazzi & Sgarbieri, 1981; Zhou, Smith, & Lee, 1993). To prevent the reaction of phenols with PPO, phenolic substrate must be removed prior to the assay with phenol-binding agents such as soluble and insoluble polyvinylpyrrolidone (PVP) and PVPP (Galeazzi & Sgarbieri, 1981; Mayer & Harel, 1979). In the study of Cano et al. (1996) and González et al. (1999), PVPP combined with Triton X-100 increased the PPO activities of the papaya and raspberry extracts, but this is not the case in our investigation. Using the PVPP alone is sufficient for PPO extraction from bayberry fruit tissue.

3.2. Characteristics of bayberry fruits of different maturity and their effects on bayberry PPO activity

Bayberry fruits at different stages of maturity exhibited the initial characteristics shown in Table 3. The ripe bayberry fruits had higher soluble solids, total solids, protein and total pectins than had the unripe fruits, while the titratable acidity was lower; thus, the ripe fruits had a higher sugar/acid ratio and better taste. When bayberry fruits were overripe, these changes were reversed. Although the above changes were not significantly different according to statistical analysis, PPO activity in the ripe fruits was almost 3 times and 1.7 times higher than those in the unripe or overripe fruits (Table 3), respectively. The highest PPO activity observed in the ripe fruits implies that the ripe bayberry fruits are susceptible to enzymatic reactions during storage and processing.

3.3. Substrate specificity

The total soluble enzymatic extract was used to study the substrate specificity, in which a number of *o*-diphenols were tested as substrates (Table 2). Bayberry PPO showed the highest activity with catechol and the lowest with protocatechuic acid. However, enzyme activity was not detectable with catechin, *p*-coumaric acid or caffeic acid. These results indicated that bayberry PPO contains catecholase activity. The number of hydroxyl groups and their position in the benzene ring of the substrate affected oxidase activity. Sherma and Ali (1980) reported that the active site of the other possible PPO activity, cresolase activity, was more labile than that of catecholase and was easily inactivated during the extraction process. Catechol was used for all bayberry PPO assays because of its less expense and better reproducibility.

Gallic acid and protocatechuic acid are naturally occurring phenolic compounds in bayberry tissues (Fang et al., 2007). Although the activities are relatively low (Table 2), the results revealed that these phenolics were substrates for bayberry PPO, which implies that bayberries have a potential for enzymatic browning during postharvest handling or during processing and storage of bayberry products.

3.4. Effect of pH

The effect of pH on the activity of bayberry PPO was investigated in the 3.0–8.0 pH range (Fig. 1). When catechol was the substrate, the maximum activity was at pH 6.0. The enzyme activity decreased rapidly at pHs below or above the optimum (6.0). However, the pH of *Biqi* bayberry tissue ranged from 3.15 to 3.31, depending on the fruit maturity (Table 3). In this pH range, bayberry PPO was not effectively active but, according to our practical experience, when the PPO activity was not inhibited during bayberry processing and storage and the bayberry products stored for a long time (over one month at room temperature), browning reactions could still occur.

Table 3
Physicochemical characteristics and PPO activities of bayberry with different maturities[a]

Characteristics[a]	Unripe	Ripe	Overripe
pH	3.15 ± 0.03[b]	3.28 ± 0.02	3.31 ± 0.02
Titratable acidity (g citric acid/100 g FW)	0.93 ± 0.02	0.86 ± 0.02	0.83 ± 0.04
Soluble solids (°Brix at 20)	9.22 ± 0.12	9.51 ± 0.13	9.23 ± 0.05
Moisture content (g/100 g FW)	90.28 ± 0.14	89.86 ± 0.24	90.14 ± 0.21
Total solids (g/100 g FW)	9.72 ± 0.07	10.14 ± 0.15	9.86 ± 0.14
Protein (mg/100 g FW)	456 ± 0.57	466 ± 0.68	452.13 ± 0.87
Total pectins (g/100 g FW)	0.58 ± 0.02	0.64 ± 0.03	0.62 ± 0.05
PPO activity (ΔOD/min/g FW)	0.178 ± 0.020a	0.509 ± 0.011b	0.306 ± 0.012c

[a] Different letters in the same row indicate significant differences ($p \leqslant 0.05$).
[b] Values are averages of three independent determinations ± standard deviation.

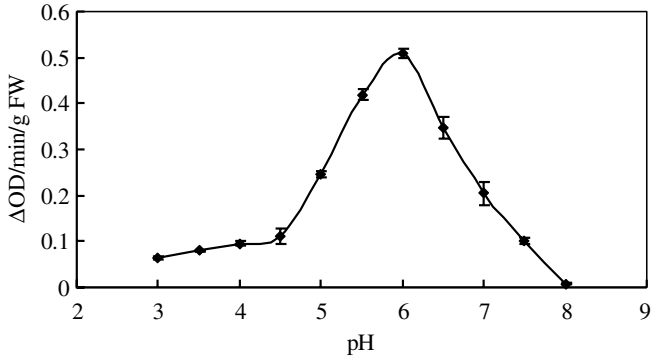

Fig. 1. Effect of pH on the enzymatic activity of bayberry PPO.

3.5. Effect of temperature

The optimum temperature for maximum bayberry PPO activity was at 30 °C (Fig. 2). It has been reported that the optimum temperatures for PPO of peach (Jen & Kahler, 1974), Victoria grape (Rapeanu, Loey, Smout, & Hendrickx, 2006), muscadine grapes (Lamikanra, Kirby, & Musingo, 1992), medlar fruits (Dincer, Colak, Aydin, Kadioglu, & Güner, 2002), and plum (Siddiq, Sinha, & Cash, 1992) were 20, 25, 30, 35 and 37 °C, respectively. Below and above 30 °C, the enzyme activity gradually decreased. At 60 °C, approximately 60% of PPO activity was lost and the enzyme was completely inactivated at 90 °C. It appears that crude PPO is sensitive to the increase in assay temperature.

3.6. Effect of substrate concentration

The effect of catechol concentrations ranging from 40 mM to 0.2 M on bayberry PPO activity was investigated. The K_m and V_{max} values for the PPO were determined from Lineweaver–Burk plots (Fig. 3). The K_m value was 313 mM and the V_{max} value was 3.26ΔOD/min/g FW. Rapeanu et al. (2006) have reported a K_m of 52.6 mM and a V_{max} of 653 $OD_{400\ nm}$/min for Victoria grape PPO with catechol as substrate. González et al. (1999) also found K_m values of 664 and 84.2 mM and V_{max} values of 4.47 and 1.28ΔOD/min/g FW for Heritage and Ceva raspberries, respectively, using catechol for PPO reaction.

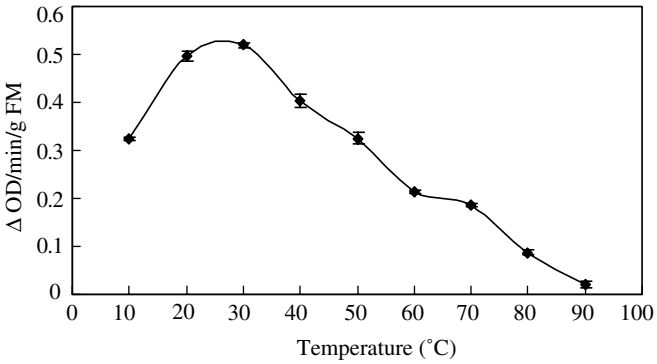

Fig. 2. Effect of temperature on the enzymatic activity of bayberry PPO.

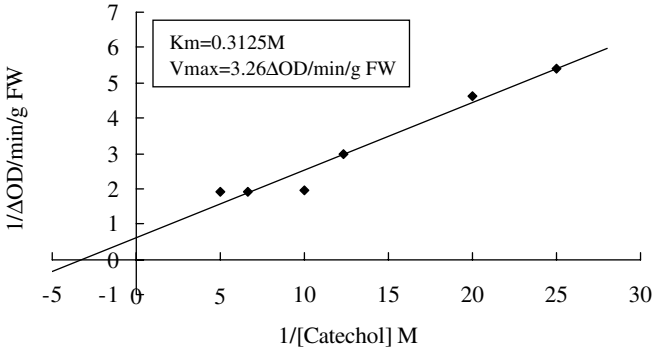

Fig. 3. Effect of substrate concentration (catechol) on bayberry PPO activity (Lineweaver–Burk plot).

3.7. Assay for cyanidin 3-glucoside degradation

Because over 95% of the total pigment in bayberry fruits was identified as cyanidin 3-glucoside (Bao et al., 2005; Ye et al., 1994), it was used in anthocyanin degradation assay. In the absence of gallic acid, the rate of cyanidin 3-glucoside discoloration (pH 3.5) was almost the same as when enzymatic extract was inactivated by heating and gallic acid was present (Fig. 4). This indicated that bayberry PPO alone could not react with cyanidin 3-glucoside directly, but this was not found by Kader et al. (1997), who reported that about 29% of the blueberry anthocyanins was oxidized by the blueberry PPO after 20 min of reaction. Mathew and Parpia (1971) also demonstrated that PPO could act directly on anthocyanins, though they remained fairly poor substrates.

The pH of *Biqi* bayberry tissue ranged from 3.15 to 3.31, depending on the fruit maturity (Table 3). At pH 3.5, bayberry PPO was not very active (Fig. 1) but, after 3 h of reaction at room temperature (25–30 °C), the presence of gallic acid drastically increased the rate of anthocyanin degradation (Fig. 4). This indicated that, after a relatively longer time of reaction (3 h compared with 20 min of reaction of blueberry PPO reported by Kader et al. (1997)), bayberry PPO could oxidize gallic acid, and then the oxi-

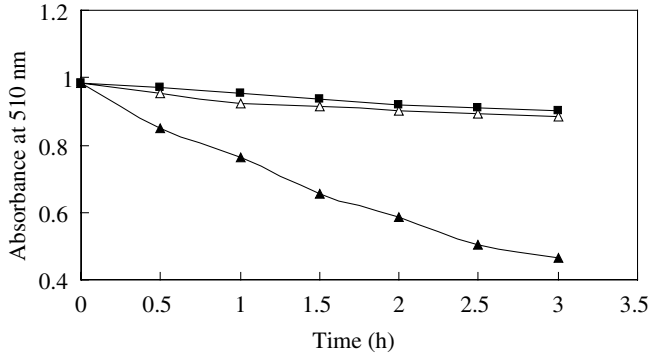

Fig. 4. Changes in absorbance of bayberry anthocyanin at 510 nm during its degradation by bayberry PPO at pH 3.5 in the presence (—▲—) and absence (—△—) of gallic acid and after thermal denaturation of the PPO in the presence of gallic acid (—■—).

dized products from gallic acid stimulated the anthocyanin degradation. Sarni et al. (1995) proposed that anthocyanins with an *o*-diphenolic B ring were oxidized via the enzymatically-generated *o*-quinone by a coupled oxidation mechanism, whereas non-*o*-diphenolic anthocyanins formed adducts with the same *o*-quinone. The results of the bayberry PPO assay accord with the anthocyanin-coupled oxidation mechanism. The results also suggested that inhibition of PPO during bayberry processing and storage is critical to the colour quality of bayberry products.

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