A Simple Method for Assaying Total Protein, Polyphenol Oxidase and Peroxidase Activity from 'Kaimana' *Litchi chinensis* Sonn.

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ABSTRACT

Polyphenol oxidase (PPO) and peroxidase (POD) are two well known enzymatic reactions involved in the browning process of fruits. To gain a better understanding of the browning process in lychee (*Litchi chinensis* Sonn.), effective methods to extract PPO and POD must be developed. Pericarps from thawed 'Kaimana' lychee were suspended in 100 mM potassium citrate buffer pH 4.0 and homogenized. The soluble extract was assayed for total protein, PPO and POD enzymes. For fresh fruit, 0.16 ± 0.02 mg protein/mL of extract was measured which is equivalent to an average of 0.81 ± 0.11 mg protein/g of peel. PPO specific activity was 167 ± 44 U/mg protein (or an average of 130 ± 31 U/g peel) and POD activity was 13.1 ± 1.8 U/mg protein (or an average of 10.1 ± 0.3 U/g peel). Use of 4-methylcatechol for PPO activity and guaiacol for POD activity are described for assaying lychee pericarps.

KEYWORDS: *Litchi chinensis* Sonn, Kaimana, Mauritius, lychee, polyphenol oxidase, peroxidase, pericarps.

INTRODUCTION

Senescence and/or tissue damage cause fruits and vegetables to turn brown by enzymatic and non-enzymatic biochemical reactions. Polyphenol oxidase (PPO) and peroxidase (POD) are two well known enzymatic reactions involved in the browning process. Reducing these browning reactions greatly improves the market life of lychee fruits. Currently, growers slow the browning process through refrigeration and packaging, which prevents desiccation and limits oxygen availability. To gain a better understanding of the browning process in lychee, effective methods to extract PPO and POD must be developed. Monitoring these enzymatic activities will greatly assist researchers in developing postharvest storage techniques to slow pericarp browning reactions in lychee.

Several assay procedures have been described for PPO and POD from lychee. PPO activity can be monitored by oxygen consumption (polarographic method; (Janovitz-Klapp, et al. 1990)) or spectrophotometrically using a variety of substrates such as pyrogallol (Munoz, et al. 2006) (Munoz, et al. 2006, Yue-Ming, et al. 1997, Robert, et al. 1995), pryocatechol (Munoz, et al. 2006), 4-methylcatechol (Zauberman, et al. 1991, Yue-Ming, et al. 1997, Robert, et al. 1995, Goutte, et al. 2001, Munoz, et al. 2006), L-DOPA (Chen, et al. 2002), 3,4-dihydroxyphenylacetic acid (Munoz, et al. 2006), 4-*tert*-butylcatechol (Munoz, et al. 2006), and chlorogenic acid (Munoz, et al. 2007). It has been shown that polarographic and spectrophotometric methods will give similar results only when true initial velocities are measured (Whitaker 1995).

Spectrophotometric assays for POD activity, utilizing guaiacol, have been described as well (McClune 1961, Underhill, et al. 1995, Zhang, et al. 2005).

Here we describe a simple and effective procedure for soluble protein extraction from lychee pericarp and two assays for PPO and POD activity. This study describes the measurement of PPO activity using 4-methylcatechol, which is converted to 4-methly-*o*-diquinone (Fig. 1A), and POD activity using guaiacol (and hydrogen peroxide) which is converted to 3,3'-dimethoxy-4,4'-biphenoquinone (Fig. 1B).

A point of clarification needs to be made regarding guaiacol oxidation by peroxidases measured by spectrophotometry. Earlier studies stated that tetraguaiacol is the product of guaiacol oxidation by peroxidases with a molar absorptivity of 2.66 x 10^4 $M^{-1}cm^{-1}$ (Maehly 1954, Chance, et al. 1955). In 1997, however, Doerge, et al. determined that 3,3'-dimethoxy-4,4'-biphenoquinone is the product of the peroxidase reaction (Doerge, et al. 1997). These authors did not determine the molar extinction coefficient for 3,3'-dimethoxy-4,4'-biphenoquinone so it is assumed here that the value of 2.66 x 10^4 $M^{-1}cm^{-1}$ applies to 3,3'-dimethoxy-4,4'-biphenoquinone rather than tetraguaiacol. In the same study Doerge, et al. also determined that phenolic compounds inhibit POD-catalyzed guaiacol oxidation. Therefore, phenolic compounds must be removed in order to obtain accurate enzyme activities.

MATERIALS AND METHODS

Kaimana litchi. Pericarps from freshly harvested mature fruit (obtained from a local producer in Hilo, Hawaii) were stored at -18 ºC until extraction.

Reagents. All buffers and assay reagents (including Bradford Reagent) were purchased from Sigma and hydrogen peroxide (3%) was obtained from a local pharmacy.

Pericarp extraction. Thawed peels from 3 fruits (15 to 35 g) were placed in a food processor (Black and Decker) and chopped for 2 or 3 min until the peels were a uniform size. Five grams of the chopped pericarps were removed to a 50 mL conical centrifuge tube containing 25 mL of 100 mM potassium citrate at pH 4.0 and 0.50 g of polyvinylpyrrolidone (PVP) to remove phenolic compounds. The centrifuge tube was incubated on ice for 30 min. During incubation, the pericarps were homogenized for 3 min at 35,000 rpm using a BioSpec Tissue-Tearor homogenizer. Homogenization resulted in peels of consistent size. Tubes were centrifuged at 15,000 x *g* (for 20 min 4 ºC in an Eppendorf 5804 R benchtop centrifuge) in pre-chilled rotor (4 ˚C). After centrifugation, tubes were kept in ice until protein and enzyme assays were performed.

Protein assay. Protein content of pericarp extracts were determined by the Bradford method (Bradford 1976). A BSA standard (0 to 1.0 mg/mL) was prepared from dilution of a 2 mg/mL stock solution. Bradford Reagent (3.00 mL, undiluted) was mixed with 0.10 mL of extract and allowed to develop for five min at room temperature before absorbance measurements at 595 nm. A blank was prepared by using 0.10 mL of extraction buffer.

Polyphenol oxidase assay. The assay mixture contained 1.45 mL of 100 mM potassium phosphate buffer, pH 6.8 (assay buffer) and 0.50 mL of 100 mM 4-methylcatechol (4-MC) prepared daily by dissolving 124.1 mg 4-MC in 10 mL of assay buffer. The final 4-MC concentration in the 2.00 mL assay was 25 mM. The assay mixture was allowed to incubate for 3 min at room temperature in a disposable polystyrene cuvette. Five- to ten-fold diluted extract (50 μL) was then added to the cuvette containing the assay mixture. The cuvette was placed into a Genesys 5 or Shimadzu UV-1700 spectrophotometer and the reaction monitored at 412 nm for

two minutes at room temperature. Enzyme activity was reported as enzyme units (U), defined as the quantity of enzyme required to produce 1 micromole of product per minute. This was based on a molar absorptivity of 1010 M^{-1} cm^{-1} for 4-methyl-*o*-quinone (Waite 1976).

Peroxidase assay. The assay mixture contained 2.70 mL of assay buffer (see PPO assay above), 0.10 mL of 3% hydrogen peroxide, and 0.15 mL of 4% guaiacol (freshly prepared). The 4% guaiacol was prepared by dispensing 0.4 mL guaiacol in 9.6 mL of deionized water and shaken vigorously to produce an emulsion before use. The final concentrations of hydrogen peroxide and guaiacol were 0.10 and 0.20 %, respectively, in a total volume of 3.00 mL. The assay mixture was incubated for 3 min at room temperature. After incubation, 50 μL of diluted (5 to 10 fold) extract was added and POD activity was monitored at 470 nm for two minutes at room temperature. POD activity was expressed as Units of enzyme activity (U). Units were calculated using a molar absorptivity of 2.66 x 10^4 M^{-1} cm^{-1} for tetraguaiacol (or 3,3'-dimethoxy-4,4'-biphenoquinone) (Maehly 1954).

RESULTS AND DISCUSSION

Protein content. The Bradford protein assay of 'Kaimana' pericarp extracts yielded 0.16 ± 0.02 mg protein/mL of extract or 0.81 ± 0.11 mg protein/g peel when correlated to pericarp mass. Protein measurements were similar for either pH 4.0 or pH 6.8 extraction buffers.

Activity of PPO and POD. Initial rates were calculated from the linear portion (initial velocity) of the reaction progress curve which occurred during the first 10 to 30 seconds. The average specific activity of PPO was 162 ± 40 U/mg protein and when correlated to pericarp mass, an average of 125 ± 29 U/g peel was obtained. The average specific activity of POD was 13.1 ± 1.8 U/mg protein and 10.1 ± 0.3 U/g peel. The relative contribution of PPO and POD in the overall browning process of lychee has never been evaluated. Our results show that PPO has approximately 12 times more activity than POD (162 vs. 13.1 U/mg protein) indicating that PPO has a larger role than POD in the browning process of 'Kaimana' litchi. Therefore, strategies to impede browning in 'Kaimana' litchi should include methods to inhibit PPO activity.

It should be noted that the PPO reaction produces a precipitate as part of the reaction. The quantity of precipitate depends upon the enzyme level present in the extract. This is most likely due to nonenzymatic coupling of 4-methly-*o*-benzoquinone, the product of the reaction, with the 4-MC substrate (Richard-Forget, et al. 1992). The precipitation reaction were minimized or eliminated by diluting the extracts 5- to 10-fold.

'Kaimana' litchi peels may contain greater protein levels and PPO activity compared to Mauritius variety. Yue-Ming et al. measured the specific activity of PPO from 'Mauritius' litchi pericarps using similar protein and PPO assay procedures (POD activity was not measured) (Yue-Ming, et al. 1997). A comparison of protein content and PPO specific activities values from our work with 'Kaimana' litchi and the results from Yue-Ming et al. with 'Mauritius' litchi are presented in Table 1. The protein concentration and the milligrams of protein per gram of peel from 'Mauritius' litchi are 25- and 54-fold less, respectively, than those presented here for 'Kaimana' litchi. Also, the specific activity of PPO is 46-fold higher in the 'Kaimana' variety. These differences are probable due to higher protein and enzyme yields attained by the extraction method we have described here. Yue-Ming et al. did not provide a detailed extraction procedure for 'Mauritius'; therefore it is difficult to access these differences. In addition, both 'Mauritius' and 'Kaimana' have similar shelf life (pericarp color change). Clearly, the Bradford reagent (total protein), 4-methylcatechol (PPO), and guaiacol (POD) provide significantly higher yields

compared to previous procedures and are excellent for conducting enzymatic browning studies on 'Kaimana' litchi.

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LITERATURE CITED

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Chance, B. and A.C. Maehly. Assay of catalases and peroxidases. In: *Methods Enzymol.*, vol. 2 (pp. 764-775), 1955.

Chen, Q.-X. and I. Kubo. 2002. Kinetics of mushroom tyrosinase inhibition by quercetin. J. Agric. Food Chem. 50, 4108-4112.

Doerge, D.R., R.L. Divi and M.I. Churchwell. 1997. Identification of the colored guaiacol oxidation product produced by peroxidases. Anal. Biochem. 250, 10-17.

Goutte, J.T.d.l., J.A. Khan and E.N. Vulfson. 2001. Identification of novel polyphenol oxidase inhibitors by enzymatic one-pot synthesis and deconvolution of combinatorial libraries. Biotechnol. Bioeng. 75, 93-99.

Janovitz-Klapp, A.H., F.C. Richard, P.M. Goupy and J.J. Nichols. 1990. Kinetic studies on apple polyphenol oxidase. J. Agric. Food Chem. 38, 1437-1441.

Maehly, A.C. In: Methods of Biochemical Analysis. (Glick, ed.), p. 358. New York, Intersciences Publishers, 1954.

McClune, D.C. 1961. Miltiple peroxidases in corn. Ann. N. Y. Acad. Sci. 94, 723-730.

Munoz, J., F. Garcıa-Molina, R. Varon, J.N. Rodriguez-Lopez, P.A. Garcia-Ruiz, F. Garcıa-Canovas and J. Tudela. 2007. Kinetic characterization of the oxidation of chlorogenic acid by polyphenol oxidase and peroxidase. Characteristics of the *o*-quinones. J. Agric. Food Chem. 55, 920-928.

Munoz, J.L., F. Garcıa-Molina, R. Varon, J.N. Rodriguez-Lopez, F. Garcıa-Canovas and J. Tudela. 2006. Calculating molar absorptivities for quinones: Application to the measurement of tyrosinase activity. Anal. Biochem. 351, 128-138.

Richard-Forget, F.C., M.-A. Rouet-Mayer, P.M. Goupy, J. Philippon and J.J. Nicolas. 1992. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by apple polyphenol oxidase. J. Agric. Food Chem. 40, 2114-2122.

Robert, C.M., F.R. Cadet, C.C. Rouch, M. Pabion and F. Richard-Forget. 1995. Kinetic study of the irreversible thermal deactivation of palmito (*Acanthophoenix rubra*) polyphenol oxidase and effect of pH. J. Agric. Food Chem. 43, 1143-1150.

Underhill, S.J.R. and C. Critchley. 1995. Cellular localisation of polyphenol oxidase and peroxidase activity in *Litchi chinensis* Sonn. pericarp. Aust. J. Plant Phys. 22, 627-632.

Waite, J.H. 1976. Calculating extinction coefficients for enzymatically produced *o*-quinones. Anal. Biochem. 75, 211-218.

Whitaker, J.R. Polyphenol Oxidase. In: D. W. S. Wong, In: *Food Enzymes: Structure and Mechanism* (pp. 271-307). New York: Chapman and Hall, 1995.

Yue-Ming, J., G. Zauberman and Y. Fuchs. 1997. Partial purification and some properties of polyphenol oxidase extracted from litchi fruit pericarp. Postharvest Biol. Tech. 10, 221-228.

Zauberman, G., R. Ronen, M. Akerman, A. Weksler, I. Rot and Y. Fuchs. 1991. Post-harvest retention of the red colour of litchi fruit pericarp. Scientia Horticulturae 47, 89-97.

Zhang, Z., X. Pang, D. Xuewu, Z. Ji and Y. Jiang. 2005. Role of peroxidase in anthocyanin degradation in litchi fruit pericarp. Food Chem. 90, 47-52.

Table 1. Comparison of protein and enzyme data from ‘Kaimana’ and ‘Mauritius’ litchi.

Lychee Variety	Protein Conc. (mg/mL)	Protein Content (mg/g peel)	PPO Specific activity (units[a]/mg protein)
Kaimana	0.16	0.81	87250
Mauritius[b]	0.0063	0.015	1900

[a] One unit of enzyme activity is defined as the amount enzyme causing a change in absorbance of 0.001 per minute (Yue-Ming, et al. 1997)

[b] Data from Yue-Ming et al.

Figure 1. Enzyme assay reactions catalyzed by A) polyphenoloxidase (PPO) and B) peroxidase (POD)