ELSEVIER

PII: S0308-8146(97)00144-1

Food Chemistry, Vol. 62, No. 1, pp. 49–52, 1998

Printed in Great Britain
0308-8146/98 $19.00+0.00

Inhibition of polyphenol oxidase and the browning control of litchi fruit by glutathione and citric acid

Yueming Jiang[a]* & Jiarui Fu[b]

[a]*South China Institute of Botany, Academia Sinica, Guangzhou 510650, People's Republic of China*
[b]*Department of Biology, Zhongshan University, Guangzhou 510275, People's Republic of China*

(Received 15 February 1997; revised version received and accepted 25 June 1997)

Polyphenol oxidase (PPO, EC 1.10.3.2) from litchi peel was partially purified by ammonium sulfate fractionation and gel filtration, and a 16-fold purification of PPO achieved. The use of 10 mmol litre^{-1} glutathione and 100 mmol litre^{-1} citric acid was found to give good control of the browning of litchi fruit and 80–85% inhibition of PPO observed. Application of glutathione in combination with citric acid is recommended as a way of slowing the browning of litchi fruit. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The litchi fruit (*Litchi chinensis* Sonn.) is native of subtropical China and is being grown as a commercial crop in subtropical Asia, South Africa, Australia, Hawaii and Israel. After harvest, the fruits are very perishable and quickly to lose their bright red skin colour (Akamine, 1960; Huang and Scott, 1985; Jaiswal *et al.*, 1987). Browning has been attributed to a rapid degradation of the red pigments by polyphenol oxidase, producing brown-coloured byproducts (Mayer and Harel, 1979; Tan and Li, 1984; Huang *et al.*, 1990).

Enzymatic browning can be prevented by bisulphite (Golan-Goldhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986), ascorbic acid and its analogies (Sapers and Ziolkowski, 1987; Hus *et al.*, 1988) and cysteine (Walker and Reddish, 1964; Dudley and Hotchkiss, 1989; Richard-Forget *et al.*, 1992). Although the bisulphites are effective, they can be dangerous to human health, especially in asthmatic patients (Taylor and Bush, 1986). So alternative chemicals without toxic effects are needed.

Langdon (1987) showed that the combination of ascorbic acid and citric acid prevented enzymatic browning of sliced potatoes. Santerre *et al.* (1988) confirmed that combinations of ascorbic acid, erythorbic acid and citric acid were efficient in preventing browning of sliced apples. According to Pizzocaro *et al.* (1993) sliced apples could be protected from browning using a mixture of ascorbic acid and calcium chloride. In the present work the litchi PPO was extracted and partially purified, and one good combination of glutathione and citric acid was adopted to examine its effect on the control of the browning of litchi fruit.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials and treatment

Reagents (reduced glutathione and citric acid) were purchased from Shanghai Biochemical Co., China. *Litchi chinensis* cv. Huaizhi (a major cultivar in China) was obtained on July 7, 1994 from a commercial orchard in Guangzhou, P.R. China. Fruits were selected for uniformity of shape, colour and size, and any blemished or diseased fruits discarded. A sub-sample of fruit (20 kg) was stored at −20°C until polyphenol oxidase was extracted and purified. A second sub-sample (12 kg) was dipped in water containing 10 mmol litre^{-1} glutathione (reduced form) and 100 mmol litre^{-1} citric acid for 5 min within 3 h after harvest before being air-dried and packed in units of 20 fruit into polyethylene bags (0.03 mm thick), sealed with a rubber band and stored at 25 ± 1°C for progressive assessments. We used the treatment with 10 mmol litre^{-1} glutathione and 100 mmol litre^{-1} citric acid, for the combination of 10 mmol litre^{-1} glutathione and 10 mmol litre^{-1} citric acid showed the best inhibition of PPO activity *in vitro* (data no shown). The fruit dipped only with water for 5 min and stored (from the experiment described above) was used as a control.

Browning assessment

Browning was estimated by measuring the extent of the total browned area on each fruit pericarp on the following scale (Jiang and Chen, 1995): 1 = no browning (excellent quality); 2 = slight browning; 3 = < 1/4 browning; 4 = 1/4–1/2 browning. 5 = > 1/2 browning (poor quality). The browning grade was calculated using the following formula:

$$\Sigma \text{ (browning scale} \times \text{percentage of corresponding fruit within each class)}$$

The fruit evaluated at higher than 3.0 (browning grade) were considered to be unacceptable for marketing.

Extraction and purification of polyphenol oxidase

All steps were carried out at 4°C. Litchi peel (800 g) was homogenised with 2500 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 80 g of polyvinglpyrrolidone (insoluble) using a Ultra-AII (Guangdong, China) homogeniser (5000 rpm) for 60 s. After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 20 000 *g* for 20 min (Beckman J2-21), and then the supernatant was collected as the crude enzyme. The enzyme solution was fractionated with $(NH_4)_2SO_4$(50–80% saturation), and the precipitate was collected by centrifugation at 20 000 *g* for 20 min, redissolved in 40 ml of 0.01 mol litre^{-1} sodium phosphate buffer (pH 6.8) and dialysed overnight against the same buffer on a magnetic stirrer (Model CJJ-1, China). After the dialysed solution was lyophilised and redissolved in small volume of 0.01 mol litre^{-1} sodium phosphate buffer (pH 6.8), a 2.0 ml portion of the sample was applied to a Sephadex G-100 (Pharmacia Co., Sweden) column (2.5×84 cm), preequilibrated with 0.01 mol litre^{-1} sodium phosphate buffer (pH 6.8), and eluted with the same buffer. The fraction with the highest enzymatic activity was pooled, freeze-dried, and redissolved in 10 ml of 0.01 mol litre^{-1} sodium phosphate buffer (pH 6.8).

Enzyme assay and protein determination

PPO activity was assayed with 4-methylcatechol as a substrate according to a spectrophotometric procedure (Zauberman *et al.*, 1991). The assay was performed using 0.5 ml of 100 mmol litre^{-1} 4-methylcatechol, 1.0 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 0.5 ml of the crude enzyme or 1.45 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 0.05 ml of the enzyme solution purified by $(NH_4)SO_4$ fractionation, or 1.49 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 0.01 ml of the enzyme solution purified by both $(NH_4)_2SO_4$ fractionation and Sephadex G-100 column. The increase in absorbance at 410 nm at 25°C was recorded automatically for 5 min (Beckman DU-7). One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.01 in absorbance per minute. Protein content was determined according to the dye-binding method of Bradford (1976) with bovine serum protein as the standard.

Effect of the treatment with glutathione and citric acid on PPO activity

PPO activity was measured 2, 4 and 6 days after storage of litchi fruit. Six grams of litchi peel from six fruit was ground with 30 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 0.6 g of polyvinylpyrrolidone (insoluble). After centrifugation at 20 000 *g* for 20 min, the supernatant was collected as the crude enzyme. The assay of the enzyme activity was performed using 1.0 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8), 0.5 ml of 100 mmol litre^{-1} 4-methylcatechol, and 0.5 ml of the crude enzyme solution according to the method described above.

Measurements of reduced and oxidized glutathione (GSH and GSSG)

Ten grams of litchi peel and 30 g of pulp from 12 fruit were homogenised on an ice bath using a Polytron homogeniser, respectively. The solution used for homogenisation consisted of 50 ml of 0.1 mol litre^{-1} sodium phosphate-0.005 mol litre^{-1} EDTA buffer (pH 8.0) and 10 ml of 25% HPO_3, which was used as a protein precipitant. The total homogenate was centrifuged at 4°C at 100 000 *g* for 30 min to obtain the supernatant for the assay of GSSG and GSH. Determination of GSH was performed by the method of Hilf and Hissin (1976). To 0.5 ml of the 100 000 *g* supernatant, 4.5 ml of the phosphate–EDTA buffer (pH 8.0) was added. The final assay mixture (2.0 ml) contained 0.1 ml of the diluted supernatant, 1.8 ml of the phosphate–EDTA buffer, and 0.1 ml of *o*-phthalaldehyde (OPT) solution, containing 0.1 mg of OPT. After through mixing and incubation at 25°C for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm using a Perkin–Elmer fluorescence spectrophotometer (Model MPE-3). For GSSG assay, a 0.5 ml portion of the original 100 000 *g* supernatant was incubated at 25°C with 0.2 ml of 0.04 mol litre^{-1} *N*-ethylmaleimide (NEM) for 30 min to interact with GSH present in the sample. To this mixture, 4.3 ml of 0.1 mol litre^{-1} NaOH was added. A 0.1 ml portion of this mixture was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that 0.1 mol litre^{-1} NaOH was employed as diluent rather than phosphate–EDTA buffer. The recovery of GSH was estimated in the following way. Equal amounts of peel or pulp were homogenized in three different tubes. In the first tube, prior to homogenisation, a known amount of GSH, usually 0.1 mg, was added. To the second tube, after homogenisation, 0.1 mg of

GSH was added. The third tube acted as the control and no addition of GSH was made prior to or after homogenisation. The three homogenates were subsequently handled identically for GSH measurement. Estimation of GSSG recovery was performed in a similar manner.

Statistical analysis

Effects of the treatment with 10 mmol litre^{-1} glutathione and 100 mmol litre^{-1} citric acid on PPO determined activity and browning of postharvest litchi fruit were determined three times with similar results and data were analysed using Duncan's multiple range test (< 0.05).

RESULTS AND DISCUSSION

Under experimental conditions, a 16-fold purification of PPO was achieved (Table 1) that was higher than the 8-fold purification reported by Tan and Li (1984) using acetone precipitation and ammonium sulfate fractionation.

Glutathione was adopted to carry out treatment of postharvest litchi fruit, for glutathione markedly inhibited PPO activity *in vitro* and it is safe to human health. Although glutathione (10 mmol litre^{-1}) in combination with citric acid (10 mmol litre^{-1}) showed good inhibition of PPO activity *in vitro*, it was not very effective in controlling browning of litchi fruit (data not shown). When 100 mmol litre^{-1} of citric acid (instead of 10 mmol litre^{-1}) was used, the PPO inhibition increased to 80% and the browning grade decreased to 2.1, whereas the control was higher than 4.0 at 25 ± 2°C, 6 days after storage (Table 2). The treatment was efficient; the treated fruit remained bright red in colour, while the initial red colour of the control fruit had largely disappeared by the end of the experiments. Browning has been studied in other fruit, and discoloration correlated well with PPO activity and phenolic concentration (Coseteng and Lee, 1987; Martinez-Cayuela *et al.*, 1988). Consequently brown pigmentation has been attributed directly to PPO action (Underhill and Critchley, 1993).

Table 1. Purification of polyphenol oxidase of litchi peel

Step	Volume (ml)	Specific activity (U mg^{-1} protein)	Purification (fold)
Crude	2100	29	1.0
$(NH_4)_2SO_4$	50	75	2.6
Sephadex G-100	4	467	16.1

Enzyme activity was assayed by using 0.5 ml of 100 mmol litre^{-1} 4-methylcatechol and 1.0 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6. 8) and 0.5 ml of the crude enzyme or 1.45 ml of sodium phosphate buffer (pH 6.8) and 0.05 ml of the enzyme solution purified by $(NH_4)_2SO_4$ fractionation, or 1.49 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 0.01 ml of the enzyme solution purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 column.

Table 2. Effect of glutathione (10 mmol litre^{-1}) + citric acid (100 mmol litre^{-1}) on PPO activity and browning of litchi fruit

Days of storage	Browning grade		PPO activity (U mg^{-1} protein)		
	Control	Treatment	Control	Treatment	(% of control)
2	1.7^a	1.3^b	39^a	6^b	15
4	2.7^a	1.6^b	53^a	9^b	17
6	4.2^a	2.1^b	40^a	8^b	20

Enzyme activity was assayed by using 0.5 ml of 100 mmol litre^{-1} 4-methylcatechol, 1.0 ml of 0.1 mol litre^{-1} sodium phosphate buffer (pH 6.8) and 0.5 ml of the crude enyme (pH 6.8). Corresponding means within a line between control and treatment followed by the same letter are not significantly different at the 5% level.

Glutathione residue in the treated fruit was highest immediately after the treatment with 10 mmol litre^{-1} glutathione and 100 mmol litre^{-1} citric acid (0.042 mg g^{-1} FW (GSH) and 0.138 mg g^{-1} FW (GSSG) of peel and 0.008 mg g^{-1} FW (GSH) and 0.022 mg g^{-1} FW (GSSG) of pulp), and then decreased to 0.018 mg g^{-1} FW (GSH) and 0.118 mg g^{-1} FW (GSSG) of peel and 0.003 mg g^{-1} FW (GSH) and 0.019 mg g^{-1} FW (GSSG) of pulp, respectively. Most of the residue was located in the inedible skin. In addition, it was observed that the content of glutathione of the fruit, treated with 10 mmol litre^{-1} glutathione and 100 mmol litre^{-1} citric acid, was markedly higher than that of the fruit treated with glutathione alone or in combination with 10 mmol litre^{-1} citric acid, especially in the content of GSH in litchi peel (data not shown). Among these treatments, the content of glutathione of the fruit treated only with glutathione was the lowest. These differences may suggest that the PPO inhibition was attributed, not only to its acid nature, but also to the higher content of glutathione in litchi peel after the addition of 100 mmol litre^{-1} citric acid, resulting in the delay in browning and better maintenance of the appearance of the litchi fruit.

ACKNOWLEDGEMENT

The financial support provided by International Foundation for Science (E/2265-1), Stockholm, Sweden is greatly appreciated.

REFERENCES

Akamine, E. K. (1960) Preventing the darkening of fresh lychees prepared for export. *Hawaii Agriculture College Experimental Station Technical Reports* **127**, 1–17.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemisty* **72**, 248–254.

Coseteng, M. Y. and Lee, C. Y. (1987) Changes in apple polyphenol oxidase and polyphenol concentrations in relation to the degree of browning. *Journal of Food Science* **52**, 985–989.

Dudley, E. D. and Hotchkiss, J. H. (1989) Cysteine as an inhibitor of polyphenol oxidase. *Journal of Food Biochemistry* **13**, 65–75.

Golan-Goldhirsh, A. and Whitaker, J. R. (1984) Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. *Journal of Agriculture and Food Chemistry* **32**, 1003–1009.

Hilf, R. and Hissin, P. J. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry* **74**, 214–226.

Huang, P. Y. and Scott, K. J. (1985) Control of rotting and browning of litchi fruit after harvest at ambient temperatures in China. *Tropical Agriculture (Trinidad)* **62**, 2–4.

Huang, S., Hart, H., Lee, H. and Wicker, L. (1990) Enzymatic and color changes during post-harvest storage of lychee fruit. *Journal of Food Science* **55**, 1762–1763.

Hus, A. F., Shieh, J. J., Bills, D. D., White and K., (1988) Inhibition of mushroom polyphenol oxidase by ascorbic acid derivatives. *Journal of Food Science* **53**, 765–767.

Jaiswal, B. P., Sah, N. L. and Prasad, U. S. (1987) Regulation of color break during *Litchi chinensis* Sonn. ripening. *Indian Journal of Experimental Biology* **25**, 66–72.

Jiang, Y. M. and Chen, F. (1995) A study on polyamine change and browning of fruit during cold storage of litchi (*Litchi chinensis* Sonn.). *Postharvest Biology and Technology* **5**, 245–250.

Langdon, T. T. (1987) Preventing of browning in fresh prepared potatoes without the use of sulfiting agents. *Food Technology* **41**, 64–67.

Martinez-Cayuela, M., Sanchez de Medina, S., Faus, M. J. and Gill, A. (1988) Cherimoya (*Annona cherimola* Mill) polyphenoloxidase: monophenolase and dihydroxyphenolase activities. *Journal of Food Science* **53**, 1191–1194.

Mayer, M. A. and Harel, E. (1979) Review: polyphenol oxidase in plants. *Phytochemistry* **18**, 193–215.

Pizzocaro, F., Torreggiani, D. and Gilardi, G. (1993) Inhibition of apple polyphenol oxidase (PPO) by ascorbic acid, citric acid and sodium chloride. *Journal of Food Processing and Preservation* **17**, 21–30.

Richard-Forget, F. C., Goupy, P. M. and Nicolas, J. J. (1992) Cysteine as an inhibitor of enzymatic browning. 2. kinetic studies. *Journal Agriculture Food Chemistry* **40**, 2108–2113.

Santerre, C. R., Cash, J. N. and Vannorman, D. J. (1988) Ascorbic acid/citric acid combinations in the processing of frozen apple slices. *Journal of Food Science* **53**, 1713–1716.

Sapers, G. M. and Ziolkowski, M. A. (1987) Comparison of erythorbic and ascorbic acids as inhibitors of enzymatic browning in apple. *Journal of Food Science* **52**, 1732–1733.

Sayavedra-Soto, T. A. and Montgomery, M. W. (1986) Inhibition of polyphenoloxidase by sulfite. *Journal of Food Science* **51**, 1531–1536.

Tan, X. J. and Li, Y. B. (1984) The partial purification and properties of polyphenol oxidase from the pericarp of litchi (*Litchi chinensis*). *Acta Phytophysiology Sineca* **10**, 339–345.

Taylor, S. L. and Bush, R. K. (1986) Sulfites as food ingredients. *Food Technology* **40**, 47–52.

Underhill, S. J. R. and Critchley, C. (1993) Lychee pericarp browning caused by heat injury. *Horticultural Science* **28**, 721–722.

Walker, J. R. L. and Reddish, C. E. S. (1964) Note on the use of cysteine to prevent browning in apple products. *Journal of Science and Agriculture* **15**, 902–904.

Zauberman, G., Roren, R., Akerman., I., Weksler, A., Rot, I. and Fuch, Y. (1991) Postharvest retention of the red color of litchi fruit pericarp. *Scientia Horticulture* **47**, 89–97.