H_2O_2-Mediated Pigment Decay in Strawberry as a Model System for Studying Color Alterations in Processed Plant Foods

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Phenolic and pigment (anthocyanin) stability in processing-ripe strawberries in response to aging under mildly oxidizing conditions, provoked by exogenous application of H_2O_2, has been studied to design a simplified model system to study color alterations (pigment decay) that occur in strawberry-derived foods during processing and storage. The results showed that phenolic metabolism in strawberry slices during aging under mildly oxidizing conditions may be either oxidative (independent of exogenous H_2O_2) or peroxidative (dependent on exogenous H_2O_2), and that feeding strawberry slices with H_2O_2 stimulates the oxidative phenomena which take place in their absence, such as the processes of anthocyanin and catechin degradation. The results also showed that because both (+)-catechin and anthocyanin levels in strawberry slices fall as H_2O_2 increases, both *p*-hydroxybenzoic acid and brown polymeric compounds are formed. Comparison of these results with controls in the absence of H_2O_2 suggests that peroxidase may play an important role in catechin consumption and in anthocyanin degradation and brown polymer formation during the aging of strawberry slices under mildly oxidizing conditions.

Keywords: *Aging; H_2O_2-induced; pigment stability; phenolics; strawberries*

INTRODUCTION

Fruits are very susceptible to undesirable alterations such as changes in texture, flavor, and color as a consequence of injuries suffered during storage, handling, and processing (Watada et al., 1990). One of the most important causes of color deterioration is enzymatic browning, a phenomenon that is caused by the oxidation of polyphenolic compounds by both polyphenol oxidase and peroxidase. This process ultimately leads to the formation of dark brown polymers of a quinoidal nature (Lee, 1992), accompanied by changes in the native color of the processed food, which may lead to substantial economic loss due to the unacceptability of the product by consumers. Enzymatic browning is a significant problem in a number of important commodities, especially fruits such as apples, pears, peaches, bananas, and strawberries. In the case of processed strawberries, they show a marked and undesirable change in color pigmentation, characterized by the decay of anthocyanin pigments and the appearance of a weak browning reaction (López-Serrano and Ros Barceló, 1995b, 1998).

Pigment (anthocyanin) instability in canned syrup strawberries has been associated in part with oxidative phenomena provoked by a basic peroxidase isoenzyme that remains active after the common process of appertization (López-Serrano and Ros Barceló, 1996). This isoenzyme is located in the concentric array of the vascular bundles and in the vascular connections with the seeds (López-Serrano and Ros Barceló, 1995a), and it is capable of efficiently oxidizing phenolics (i.e., catechin) at extremely low hydrogen peroxide concentrations (López-Serrano and Ros Barceló, 1997). This peroxidase, together with other phenol oxidases found in strawberries (Cano et al., 1997; Civello et al., 1995; Espin et al., 1997; Spayd and Morris, 1981), may be responsible for pigment decay in strawberry-derived foods.

Traces of H_2O_2, which is necessary for activating strawberry peroxidase (López-Serrano and Ros Barceló, 1996), may be formed in processed strawberries by a polyphenol oxidase-mediated catechin oxidation (Jiang and Miles, 1993; Richard-Forget and Gauillard, 1997). In this case, H_2O_2 is apparently formed by the dismutation of the O_2^- generated during the reaction of the catechin phenoxy radicals with oxygen (Jiang and Miles, 1993). Besides this enzymatic mechanism, there are other nonenzymatic mechanisms that produce H_2O_2 and which may be of great importance, especially in processed plant foods. One of these is the oxidation of ascorbic acid (AA), a commonly used antioxidant that occurs naturally in strawberries, through the Haber–Weiss reaction (Mahoney and Graf, 1986):

$$AA + Fe^{3+} \rightarrow AA^{\bullet} + Fe^{2+} \quad (1)$$

$$O_2 + Fe^{2+} \rightarrow O_2^{\bullet -} + Fe^{3+} \quad (2)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

This reaction involves the reduction of molecular oxygen to superoxide radical by ascorbic acid in the presence of traces of certain transition metals, such as iron.

In such a scenario, the aim of the present work was to study the effect of exogenous H_2O_2 on pigment (phenolic) decay in strawberries to design a simplified model system to study the color alterations (pigment decay) that occur in strawberry-derived foods during processing and storage.

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MATERIALS AND METHODS

Plant Material. Strawberries (*Fragaria* × *ananassa* var. Oso Grande) were grown in a field at Huelva (Spain) and harvested at the processing-ripe stage. The harvested fruits were frozen at −20 °C until use.

Chemicals. (+)-Catechin was purchased from Sigma Chemical Co. (Madrid, Spain). *p*-Hydroxybenzoic acid and *p*-coumaric acid were from Fluka (Madrid, Spain). Quercetin and quercetin-3-rhamnoside were from Extrasynthese (Genay, France). The other chemicals and solvents used in this study were of the maximum purity available.

Strawberry Incubation Media and Phenolic Extraction. Fifty grams of frozen strawberries was cut in slices and incubated for 24 h at 30 °C in the dark in 250 mL flasks containing 50 mL of either 1.0, 5.0, or 10 mM H_2O_2 in water. Controls in the absence of H_2O_2 were performed. No microbial growth was observed for these times in strawberry incubations. After this time, 100 mL of acetone was added to the strawberry-containing flask, and the mixture was homogenized with a mechanical blade (Sorvall Omnimixer 230). The homogenate was filtered through filter paper, and acetone was removed in vacuo. The aqueous phase obtained was acidified to pH 1.0 with HCl and extracted twice with 50 mL of ethyl acetate. The acidified aqueous phase was directly used to measure the anthocyanin content. The ethyl acetate phase was evaporated in vacuo and the phenolic residue dissolved in 1.0 mL methanol. The methanolic samples were diluted with 5 mL of 2.5% (v/v) acetic acid and prepurified by absorption on Sep-Pack C18 cartidges (Millipore Corp., Waters Chromatography). Elution was first with 2.5% acetic acid and then with methanol. The methanolic fractions were recovered and dried under an N_2 stream before being made up in 1.0 mL methanol and directly analyzed by HPLC.

Anthocyanin Quantitation. Total anthocyanins (mainly composed of pelargonidin-3-glucoside) were estimated by a pH differential method, as described by Wrolstad (1976), and expressed as pelargonidin-3-glucoside equivalents.

HPLC Analyses. HPLC analyses were carried out on a 4.6 mm i.d. × 25 cm Waters Spherisorb S5-ODS2 column (Millipore Corp., Waters) using a Waters system comprising a model 600 controller, a model 600 pump, a Rheodyne 7725i manual injector, and a Waters 996 photodiode array detector. Elution was performed at a flow rate of 1.0 mL/min at room temperature, using as solvent A 2.5% (v/v) acetic acid in water and as solvent B acetonitrile, by means of a linear gradient from 0% B to 10% B for 5 min, from 10% B to 30% B for 20 min, and from 30% B to 50% B for 20 min.

Due to the different maximum wavelengths of the different phenolics analyzed, chromatograms were obtained at the maximum wavelength of each peak (λ_{max} chromatogram). For this, the data were processed with Waters Millenium 2010 LC version 2.10 software. Quantification of known peaks (catechin, *p*-hydroxybenzoic acid, and *p*-coumaric acid) was performed using HPLC calibration curves for these compounds. Quercetin-3-glucoside and peak 12 in chromatograms were quantified using a quercetin-3-rhamnoside calibration curve. Other unknown peaks were quantified using the peak area at maximum wavelength.

RESULTS AND DISCUSSION

Canned syrup strawberries show strong pigment decay after several months of storage, which hinders the marketability and acceptability of strawberry-derived foods. This pigment decay may in part be due to oxidative phenomena catalyzed by a thermostable peroxidase that remains active after the process of appertization (López-Serrano and Ros Barceló, 1996). To simulate the postprocessing changes that occur in strawberries, a simplified experimental bath was designed, in which strawberry slices were incubated with increasing H_2O_2 concentrations to shorten the time required for complete pigment decay and, in this way, to know how phenolics change in response to mildly oxidizing conditions.

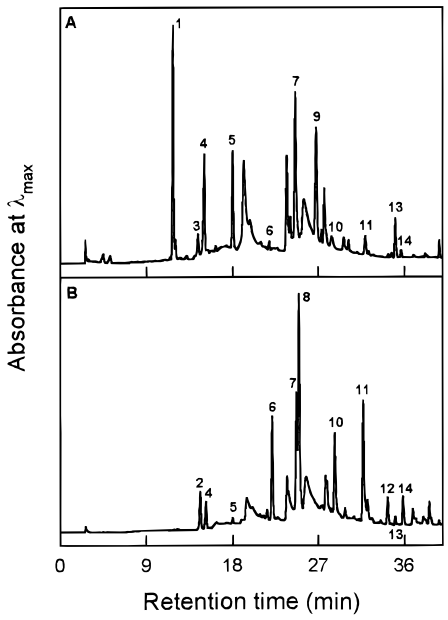

Figure 1. HPLC profiles (at λ_{max}) of phenolics extracted from processing-ripe strawberries at time zero (A) and after 24 h of incubation in the presence of 10 mM H_2O_2 (B). Retention times and UV spectroscopic properties for each of the unidentified peaks are given in Table 1 and Figure 2.

Table 1. Retention Time and UV−Vis Spectroscopic Properties of the Main Phenolics Present in Processing-Ripe Strawberries Corresponding to the HPLC Profiles Shown in Figure 1

peak	retention time (min)	λ_{max} (nm)	compound
1	11.73	289	
2	14.03	256	*p*-hydroxybenzoic acid
3	14.25	236, 279	(+)-catechin
4	15.05	234, 315	
5	18.14	278	
6	21.82	235, 309	*p*-coumaric acid
7	24.58	256, 355	quercetin-3-glucoside
8	24.82	263	
9	26.92	242	
10	28.73	256, 355	
11	31.71	263	
12	34.06	239, 356, 404	
13	34.92	234, 284	
14	35.61	277	

Incubation of strawberry slices with 10 mM H_2O_2 for 24 h at 30 °C resulted in a change in the phenolic pattern when monitored by HPLC (Figure 1), which was accompanied by a weak browning reaction. Thus, feeding strawberry slices with 10 mM H_2O_2 provoked a decrease in the level of certain phenolic compounds in the chromatograms (peaks 1, 3−5, 9, and 13), which was accompanied by an increase in the level of other phenolic compounds (peaks 2, 6, 8, 10−12, and 14). Attempts to identify these phenolic compounds were made using known phenolic standards on the basis of both their retention time in this HPLC system and their UV−vis spectrum. This enabled us to identify peak 2 as *p*-hydroxybenzoic acid, peak 3 as (+)-catechin, peak 6 as *p*-coumaric acid, and peak 7 as quercetin-3-glucoside. Other peaks remained unidentified, the retention time and the UV characteristics being listed in both Table 1 and Figure 2. Of all these unidentified compounds, it is worth noting the presence of peak 12 in the chromato-

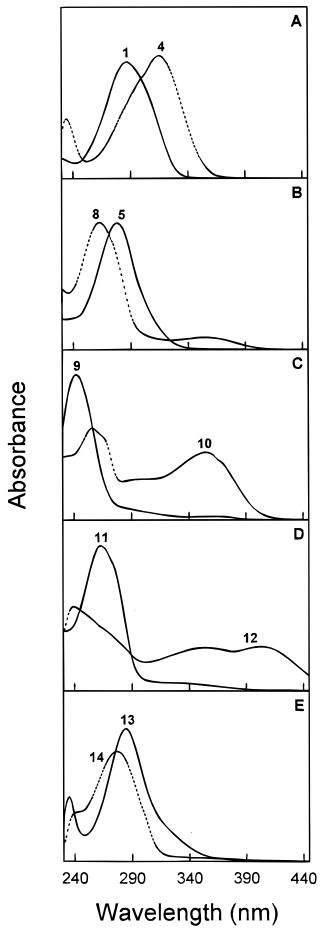

Figure 2. UV−visible spectra of unidentified compounds in the HPLC profiles of Figure 1 on diode array detector. The spectrum numbers correspond to each peak number in Figure 1.

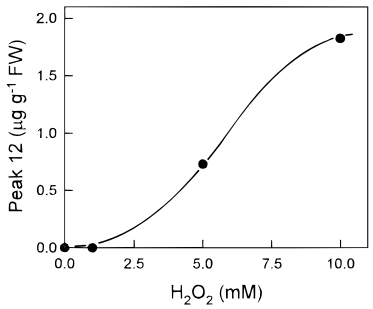

Figure 3. Dependence on H_2O_2 concentration of the formation of the quinone-type compound corresponding to peak 12 in chromatogram of Figure 1 responsible for the browning reaction. Quantification of this compound was performed using quercetin-3-rhamnoside as standard.

grams, a compound with a quinone-like spectrum (λ_{max} = 256 and 404 nm) (Figure 2), the formation of which may be responsible for the observed browning reaction and which was formed only when strawberry slices were fed with the higher H_2O_2 concentrations. In fact, the quantification of this compound using quercetin-3-rhamnoside as standard revealed (Figure 3) that its formation shows a dependence on H_2O_2 concentration similar to that of the browning reaction. Attempts to identify this compound have been unsuccessful to date but continue to be made.

Using the above-described experimental system, the strawberry phenolics that disappeared in response to

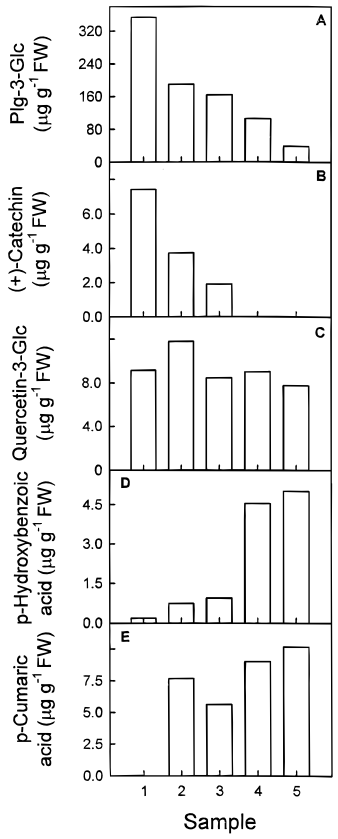

Figure 4. Changes in anthocyanins (A), (+)-catechin (B), quercetin-3-glucoside (C), *p*-hydroxybenzoic acid (D), and *p*-coumaric acid (E) of processing-ripe strawberries in response to mildly oxidizing conditions provoked by incubation of fruit slices for 24 h in the presence of 1 mM (sample 3), 5 mM (sample 4), and 10 mM (sample 5) H_2O_2. Values for controls at time zero (sample 1) and at 24 h in the absence of H_2O_2 (sample 2) are also shown.

H_2O_2-induced aging were classified into two main groups according to their response to these mildly oxidizing conditions. The first group comprised those phenolics that were degraded both in the absence and in the presence of exogenous H_2O_2, although the addition of H_2O_2 slightly stimulated the amount of oxidized phenol. In this first group may be included anthocyanins and catechin (but also peaks 1, 4, 5, 9, and 13 of chromatograms, see Figure 1), which were extremely labile under these oxidizing conditions.

A detailed analysis of the dose−response curves at various H_2O_2 concentrations showed that a 50% decay of anthocyanins was observed in the absence of H_2O_2 (Figure 4A, sample 2), whereas the addition of H_2O_2 increased the extent of anthocyanin oxidation to ∼85% (Figure 4A, samples 3−5). This was also true for (+)-catechin (Figure 4B) which, although oxidized 50% in the absence of H_2O_2 (Figure 4B, sample 2), was totally removed from strawberry slices fed with high (5.0−10.0 mM) H_2O_2 concentrations (Figure 4B, samples 4 and 5).

The second group comprised those phenolics that were stable in the absence and in the presence of H_2O_2, their levels being unaffected under these H_2O_2-induced mildly oxidizing conditions. This was exemplified by the case of quercetin-3-glucoside. For this compound, no oxidation was observed either in the absence of H_2O_2 (Figure 4C, sample 2) or in the presence of H_2O_2 (Figure 4C, samples 3−5).

The degradation of anthocyanins and catechin was not the only phenomenon observed for strawberry

phenolics under the H_2O_2-induced mildly oxidizing conditions, and some other phenolics increased in response to these oxidizing conditions. As in the case of the phenolics that were degraded, the phenolics which increased in response to mildly oxidizing conditions could also be classified into two main groups. The first group comprised those phenolics for which increases were totally dependent on the supply of H_2O_2. To this group belonged *p*-hydroxybenzoic acid (but also peaks 8, 11, and 12 in chromatograms of Figure 1), the accumulation of which in strawberry slices took place only when strawberry slices were fed with the higher (5.0−10 mM) H_2O_2 concentrations (Figure 4D, samples 4 and 5).

The second group comprised those phenolics for which increases were not dependent on the supply of H_2O_2, as in the case of *p*-coumaric acid (Figure 4E) (but also peak 14 in chromatograms of Figure 1). This compound suffered a rapid increase as a consequence of an H_2O_2-independent process during strawberry aging, which was only weakly stimulated when strawberry slices were fed with a 10 mM H_2O_2 concentration (Figure 4E, sample 5).

These results suggest that phenolic metabolism in strawberry slices during aging under mildly oxidizing conditions may be either oxidative (independent of exogenous H_2O_2) or peroxidative (dependent on exogenous H_2O_2) and that feeding strawberry slices with H_2O_2 stimulates the oxidative phenomena which take place in their absence, such as the processes of anthocyanin and catechin degradation (Figure 4A,B). Whether these oxidative processes independent of exogenous H_2O_2 are mediated by either strawberry phenol oxidases or peroxidases that are acting by using endogenous H_2O_2 deserves further attention.

The above results also suggest that because both (+)-catechin (Figure 4B) and anthocyanin levels (Figure 4A) in strawberry slices fall as H_2O_2 increases, both *p*-hydroxybenzoic acid (Figure 4D) and brown compounds (Figure 3) are formed. Comparison of these results with controls in the absence of exogenous H_2O_2 (samples 2 in Figure 4) suggested that peroxidase may play an important role in catechin consumption, in anthocyanin degradation, and in brown polymer formation. Moreover, the detection of increasing amounts of *p*-hydroxybenzoic acid suggests a coupled mechanism for glycosidase and peroxidase in the oxidation of pelargonidin-3-glucoside, the main anthocyanin present in processing-ripe strawberries, which is in accordance with the suspected philosophy of the peroxidase-mediated process of anthocyanin degradation (Ros Barceló et al., 1994; Ros Barceló and Muñoz, 1998). This metabolic process starts with the removal of the sugar moiety at C3 by glycosidases, the aglycon liberated being subsequently oxidized by peroxidase (Calderón et al., 1992; Ros Barceló et al., 1994). In this last oxidative step, it is assumed that the aglycons are oxidized until phloroglucinol-type structures and hydroxybenzoic acid-derived compounds are formed (Barz and Koster, 1981).

LITERATURE CITED

Barz, W.; Koster, J. Turnover and degradation of secondary (natural) products. In *The Biochemistry of Plants*; Conn, E. E., Ed.; Academic: New York, 1981; Vol. 7, Chapter 3.

Calderón, A. A.; García-Florenciano, E.; Muñoz, R.; Ros Barceló, A. Gamay grapevine peroxidase: its role in vacuolar antocyani(di)n degradation. *Vitis* **1992**, *31*, 139−147.

Cano, M. P.; Hernández, A.; De Ancos, B. High pressure and temperature effects on enzyme inactivation in strawberry and orange products. *J. Food Sci.* **1997**, *62*, 85−88.

Civello, P. M.; Martínez, G. A.; Chaves, A. R.; Añon, M. C. Peroxidase from strawberry fruit (*Fragaria ananassa* Duch.): Partial purification and determination of some properties. *J. Agric. Food Chem.* **1995**, *43*, 2596−2601.

Espin, J. C.; Ocho, M.; Tudela, J.; García-Cánovas, F. Monophenolase activity of strawberry polyphenol oxidase. *Phytochemistry* **1997**, *45*, 667−670.

Jiang, Y.; Miles, P. W. Generation of H_2O_2 during enzymic oxidation of catechin. *Phytochemistry* **1993**, *33*, 29−34.

Lee, C. Y. Enzymatic oxidation of phenolic compounds in fruits. In *Phenolic Compounds in Food and Their Effects on Health I*; Ho, C. T., Lee, C. Y., Huang, M. T., Eds.; American Chemical Society: Washington, DC, 1992; pp 305−317.

López-Serrano, M.; Ros Barceló, A. Peroxidase in unripe and processing-ripe strawberries. *Food Chem.* **1995a**, *52*, 157−160.

López-Serrano, M.; Ros Barceló, A. Peroxidase and browning reaction in strawberries. In *Current Trends in Fruit and Vegetables Phytochemistry*; García-Viguera, C., Castañer, M., Gil, M. I., Ferreres, F., Tomás-Barberán, F. A., Eds.; Consejo Superior de Investigaciones Científicas: Madrid, 1995b; pp 167−170.

López-Serrano, M.; Ros Barceló, A. Purification and characterization of a basic peroxidase isoenzyme from strawberries. *Food Chem.* **1996**, *55*, 133−137.

López-Serrano, M.; Ros Barceló, A. Unusual kinetic properties of (+)-catechin oxidation by a basic peroxidase isoenzyme from strawberries. *J. Food Sci.* **1997**, *62*, 676−679 and 723.

López-Serrano, M.; Ros Barceló, A. Peroxidase-mediated browning reactions in plant foods. *Rec. Res. Dev. Agric. Food Chem.* **1998**, *2*, 549−563.

Mahoney, J. R.; Graf, E. Role of alpha-tocopherol, ascorbic acid, citric acid and EDTA as oxidants in model systems. *J. Food Sci.* **1986**, *51*, 1293−1296.

Richard-Forget, F.; Gauillard, F. A. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combination of pear (*Pyrus communis* cv. Williams) polyphenol oxidase and peroxidase: a possible involvement of peroxidase in enzymatic browning. *J. Agric. Food Chem.* **1997**, *45*, 2472−2476.

Ros Barceló, A.; Muñoz, R. Metabolic plasticity of plant peroxidases. In *Plant Physiology, Biochemistry, and Plant Molecular Biology*; Hemantaranjan, A., Ed.; Scientific Publishers: Jodhpur, 1998; in press.

Ros Barceló, A.; Zapata, J. M.; Calderón, A. A. Towards a role for the vacuolar grapevine basic peroxidase isoenzyme B_5 in anthocyanin turnover and degradation. *Plant Peroxidase Newsl.* **1994**, *4*, 35−39.

Spayd, S. E.; Morris, J. R. Physical and chemical characteristics of puree from once-over harvested strawberries. *J. Am. Soc. Hortic. Sci.* **1981**, *106*, 101−105.

Watada, A. E.; Abe, K.; Yamuchi, N. Physiological activities of partially processed fruit and vegetables. *Food Technol.* **1990**, *44*, 116−122.

Wrolstad, R. E. Color and pigment analyses in fruit products. Oregon State Univeristy Agricultural Experiment Station Bulletin 624, 1976.

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