Comparative study of the banana pulp browning process of 'Giant Dwarf' and FHIA-23 during fruit ripening based on image analysis and the polyphenol oxidase and peroxidase biochemical properties

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Abstract

This work presents a novel method to associate the polyphenol oxidase (PPO) and the peroxidase (POD) activities with the ripening-mediated color changes in banana peel and pulp by computational image analysis. The method was used to follow up the de-greening of peel and browning of homogenized pulp from 'Giant Dwarf' (GD: Musa AAA, subgroup Cavendish) and FHIA-23 (tetraploid hybrid, AAAA) banana cultivars. In both cultivars, the color changes of peel during the ripening process clearly showed four stages, which were used to group the fruit into ripening stages. The PPO and POD were extracted from pulp of fruit at these ripening stages, precipitated, and partially purified by gel filtration chromatography. Moreover, the pulp browning was digitally monitored after homogenization for a span time of up to 120 min. The browning level was higher for GD than FHIA-23 tissues. This fact correlated with an 11.7-fold higher PPO activity in the GD cultivar, as compared with that of FHIA-23. POD activity was 8.1 times higher for GD as compared that that of FHIA-23.

Keywords Banana · Browning · Image analysis · Peroxidase · Polyphenol oxidase · Peel color

Introduction

Bananas are one of the most traded fruits worldwide (Aurore et al. 2009). Cavendish bananas account for 47% of global banana production (FAO 2003), with cultivar 'Giant Dwarf' (GD; Musa AAA, subgroup Cavendish) being the most

common cultivar in the market of dessert bananas. GD fruit is highly preferred by consumers due to its organoleptic characteristics (flavor and aroma) and important contribution to the daily intake of sugars, fiber, vitamins, and minerals (i.e., potassium) (Mohapatra et al. 2011; Kiyoshi and Wahachiro 2003; Wall 2006; Escalante-Minakata et al. 2013). However, GD is highly susceptible to fungal infections, including black Sigatoka (*Mycosphaerella fijiensis*) (Hoss et al. 2000; Chillet et al. 2009). This fungus causes crop banana losses of up to 50% in much growing areas (Barekye et al. 2011). Thus, the Honduran Agricultural Research Foundation (FHIA, for its acronym in Spanish) developed the banana hybrid FHIA-23, which showed advantages over the commercial cultivars in terms of productivity and resistance to fungal infections, including black Sigatoka (Hernández and Pérez 2001; Smith et al. 2014). In addition, the datasheet of the fruit provided by FHIA described to the FHIA-23 cultivar as a tetraploid hybrid with low susceptibility to browning, as compared with Cavendish (FHIA 2017). However, such cultivar has not been characterized clearly in this regard yet. Cultivars showing less browning might improve the color and appearance of many banana products, including banana juice (Ibarra-Junquera et al. 2014).

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Although bananas are commonly consumed as fresh fruit, they are used to obtain several agroindustrial products (Kyamuhangire et al. 2002; Lee et al. 2006a, b; López-Nicolás et al. 2007; Mohapatra et al. 2011; Chávez-Rodríguez et al. 2013). However, any use of this fruit is strongly limited by its fast ripening rate and low tolerance to refrigeration, which can cause external and internal browning and, consequently, compromise the acceptability of the fruit by consumers as well as suitability for processing (Sagu et al. 2014). Tissue browning is consequence of the enzymatic oxidation of phenolic compounds by PPO (EC 1.14.18.1) and POD (EC 1.11.1.7) (Yang et al. 2004; Fortea et al. 2009). This phenomenon typically occurs in cells from ripe fruits, where the loss of compartmentalization allows the contact between phenols and their oxidative enzymes (Su et al. 2005). Although several alternatives have been proposed to avoid fruit browning, the simplest consists in using cultivars with low activity of these enzymes. PPO has been studied in several fruits and vegetables to get information helpful to develop technological strategies to reduce enzymatic browning and, consequently, fruit losses (Yang et al. 2004; Fortea et al. 2009). The PPOs from different varieties of banana, including *Musa acuminata*, *M. cavendishii*, and *M. paradisiaca*, have been previously characterized (Chaisakdanugull and Theerakulkait, 2009; Galeazziet al. 1981; Palmer 1963; Sojo et al. 1998; Ünal 2007; Yang et al. 2000). However, there is a discrepancy in the number of active PPO isoenzymes and their molecular weights. Although there are PPO kinetic studies as a function of the ripening stage of fruits (Lee 2007), there are no reports for either the molecular characteristics of PPO or the POD activity in banana as a function of the ripening process.

In this study, the activities of PPO and POD were determined in fruit of two banana cultivars (FHIA-23 and GD) at different ripening stages and related to the postharvest color changes, which were determined by digital image analysis. The molecular characteristics of the enzymes (molecular weight and number of active isoforms) were also determined.

Materials and methods

Biological material

Fruit of ‘Giant Dwarf’ (*Musa* AAA, subgroup *Cavendish*) and the tetraploid hybrid (AAAA) FHIA-23 banana cultivars were used in this research. They were cultivated under identical conditions for irrigation, fertilization, and soil at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Forestales (INIFAP, for its acronym in Spanish) in

Tecomán, Colima, Mexico (latitude: 18.9671266, longitude: – 103.8417515, altitude: 297 m). Banana bunches were harvested according to the local practices. Bunches contained only mature-green fruits. Fruit was allowed to at 18 °C and a relative humidity of 80%. Periodically, 5 fruits were removed from the storage and evaluated for external color by image analysis. Then, the pulp homogenized to puree and evaluated for color (browning), PPO and POD activities, and characteristics. The obtained data were grouped according to the external color of fruits. Each group represented a different ripening stage. Finally, color of peel and pulp (browning) was related to PPO and POD activities.

Image acquisition

The evaluation of peel and pulp color was performed by digital images analysis. The images were taken with a DSC-W350 (Sony, USA) digital camera. The camera was externally adapted to a Bio-Rad ChemiDoc XRS system. The Bio-Rad ChemiDoc XRS system provided a photoset with controlled luminosity to capture the banana peel and pulp photographs. The system used two sources of light (18 W/965 lx) of 30-cm length, each one placed at 30 cm from the center of the bottom convergence, with an incidence angle of 45°.

Digital image analysis

An algorithm in Matlab (R2010a, MathWorks, USA) was designed to evaluate the color variables L^* (values from 0 to 100), a^* (values from – 60 to 60), and b^* (values from – 60 to 60) in peel and pulp from the digital images previously taken. The algorithm included two phases: (1) segmentation of the banana digital image (BDI); and (2) color evaluation in the CIELab system, eliminating defects and stains or spots in peel, such that the color evaluation is performed only in the most homogeneously colored peel area. The first step in the color analysis of the BDI (Fig. 1) was the BDI transformation to a grayscale image and the segmentation of fruit from the background. Then, three squared regions were identified inside of fruit perimeter to enable data acquisition (L^* , a^* and b^*) at each square. Then, the pixels presenting the highest levels of darkness were discarded of color analysis.

The digital image was, indeed, an $M \times N \times P$ array, where $M \times N$ represented the image dimension, in pixels, while P is the number of color planes, 3 in this case, corresponding to the matrices R' , G' , and B' . To convert an $R'G'B'$ digital image to an $L^*a^*b^*$ color space (CIELab), the Matlab object ColorSpaceConverter was used. In this point, the color values for each pixel inside the selected squares on the banana

Fig. 1 Digital image processing to image segmentation and determine $L^*a^*b^*$ values. **a** Digital image in JPEG format. **b** Transformation of the image to grayscale. **c** Contour extraction from binary image for

determining the fruit contour. **d** Square region inside of the fruit contour that was selected for calculating banana peel color. **e** Resultant binary image showing the spot-bruise detection on the banana peel

surface where available, allowing a detailed characterization of the banana image and thus providing a more precise way to evaluate and classify the fruit in different ripening stages. Figure 1 shows the segmentation and image analysis process. In all cases, color changes were determined according to the following equation (Larraín ET AL. 2008): $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

Banana puree preparation

The banana fruits were washed in 0.2% (v/v) sodium hypochlorite aqueous solutions for 5 min, peeled, and the edible portion cut into pieces. Then, pulp pieces were homogenized to puree in an electrical blender. The puree was placed in Petri dishes and the images were captured after 0, 15, 30, 45, 60, and 120 min of pulp homogenization.

PPO and POD extraction

Fifty grams of banana pulp were homogenized for 30 s in 100 mL of precooled 0.1-M sodium phosphate (pH 6.5) buffer containing 1% (w/v) of polyvinylpyrrolidone and 0.5% Triton X-100. Then, the samples were centrifuged at 11,000×g at 4 °C for 30 min (MacDonald and Schaschke 2000; Chaisakdanugull and Theerakulkait 2009). The supernatant was subjected to 0–40, 40–60, and 60–80% ammonium sulfate precipitation followed by centrifugation

at 11,000×g for 15 min. Each precipitate was re-suspended in a minimum volume of sodium phosphate buffer (0.1 M, pH 7.0) and dialyzed through a 12-kDa cut-off membrane in 2.5-L phosphate buffer at 4 °C with two buffer changes. Subsequently, the PPO and POD activity was independently measured for each recollected fraction. Fractions showing the highest activity were considered as a semi-purified extract. The selected dialyzed fraction (approximately 20 mL) was concentrated by freeze concentration to 2 mL, according to Virgen-Ortíz et al. (2012). Then, the concentration was subjected to gel filtration chromatography. The protein concentration was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

PPO and POD purification by gel filtration chromatography

A column (2.5 × 60 cm) packed with Sephacryl S-200 was used. The column was previously equilibrated with 0.1-M sodium phosphate buffer (pH 7.0). The empty volume of the column was determined with Dextran Blue (2000 kDa) (SIGMA) and calibrated with a set of standards: thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa) (BIO-RAD.). Sample proteins were eluted

at a flow rate of 0.5-mL/min and 2.5-mL samples were collected with a fraction collector (model 2110, Bio-Rad). PPO and POD activities were determined in each fraction. The fractions with high activity were pooled and used for molecular characterization by zymography.

Determination of PPO activity

The activity of PPO was assayed according to Terefe et al. (2010), with slight modifications. The reaction mixture consisted of 200 μ L of enzyme extract, 200 μ L of 0.05-M catechol solution, and 1.5 mL of 0.1-M phosphate buffer (pH 7.0). In the blank reaction, the enzyme extract was replaced with 200 μ L of phosphate buffer (pH 7.0). The absorbance of the mixture was measured against the blank at 410 nm every 15 s for 10 min (Spectrophotometer Lambda 25 UV/Vis, Perkin Elmer Inc., Waltham, MA, USA). The activity of PPO was expressed as the change in absorbance (ΔA_{410})/min/g fresh weight of sample.

POD activity determination

The activity of POD was determined using the absorbance change at 414 nm after the catalysis of ABTS [2,2'-azino-bis-(3-ethylbenzotiazolin-6-sulfonic acid)] oxidation (Fortea et al. 2009). The final reaction mixture contained 200 μ L of the enzyme extract, 1.2 mL of 2-mM ABTS, 2-mM H_2O_2 , and 0.2-mM tropolone dissolved in 10-mM sodium acetate buffer (pH 4.5). Tropolone was used to inhibit any contribution of PPO to the reaction (Fortea et al. 2009). The absorbance was measured at 414 nm at 15-s intervals for 10 min and the activity of PPO was calculated as the change of absorbance (ΔA_{414})/min/g fresh weight of sample.

Zymogram analysis

Zymogram analysis was used to identify active PPO isozymes. Activity staining of PPO was performed in gels prepared according to the Laemmli method (1970) on a 10% polyacrylamide gel. Protein samples were mixed with an equal volume of 2 $\times$ Laemmli sample buffer, but without addition of β -mercaptoethanol, and the samples were not heated prior to being loaded into the gel. The electrophoresis was run at 4 $^{\circ}$ C and at a constant power of 50 V. After electrophoresis, the lane with molecular weight markers (Precision Plus protein standards, Bio-Rad) was cut and separately stained using Coomassie blue G-250,

Fig. 2 Behavior of a^* parameter (CIELab) throughout ripening process for **a** 'Giant Dwarf' and **b** FHIA-23 cultivar

while the remainder of the gel was washed twice in 0.1-M sodium phosphate buffer (pH 7.0) containing 1% (v/v) Triton X-100 to remove any remaining SDS. Afterwards, PPO bands were detected by immersing the gel in a solution containing 0.2-M catechol dissolved in sodium phosphate buffer (0.1 M, pH 7.0). Color development occurred within 15 min, and as soon as bands appeared, the gel was rinsed with distilled water and fixed in 5% (v/v) acetic acid. The molecular weights were estimated by comparison to molecular weight markers.

Statistical analysis

All quantitative determinations were performed in triplicate. Statistically significant differences ($p < 0.05$) were determined by ANOVA. All the statistical analyzes were performed using MATLAB.

Results and discussion

Determination of fruit ripening stages by image analysis

The L^* and b^* values measured in peels did not change consistently during fruit ripening. However, a^* values gradually increased during ripening in both cultivars. Mendoza and Aguilera (2004) stated that the changes in a^* were consequence of the ripening-mediated decomposition of chlorophyll in banana peels. Based on the significant differences for a^* values, four ripening stages were identified for each cultivar (Fig. 2). The ripening rate was different for tested cultivars. GD and FHIA-23 were fully ripe after 13 and 20 days. However, it was possible to identify four well-defined ripening stages in each cultivar, allowing the comparison of the ripening process for both cultivars. To date, the ripening process of FHIA-23 based on changes of peel color had not been determined. This algorithm can easily be integrated into a conveyor belt, and be used for sorting fruits by ripening stage.

Pulp browning

The browning process for pulp of GD and FHIA-23 bananas is shown in Fig. 3. The results showed that L^* is a valuable parameter to characterize pulp browning, as concluded previously by Quevedo et al. (2009) in GD bananas. In our study, browning in pulp from both cultivars increased as L^* values decreased (Fig. 4). Moreover, the statistical analysis

for L^* values showed significant differences (ANOVA, $p < 0.5$) associated with ripening stage of fruit and exposition time. In fruit of both cultivars at advanced ripening stages, L^* values decreased at a higher rate as compared with other stages. On the other hand, the browning rate was significantly higher in GD than in FHIA-23 fruits, confirming that FHIA-23 fruits are highly desirable for processing given its low susceptibility to browning.

Biochemical properties of partial purified banana PPO and POD

As shown in Fig. 5a, the specific activity of PPO in pulp of both cultivars decreased concomitantly as the ripening stage of fruit progressed. However, the activity of PPO in GD was always higher than that of FHIA-23 in all tested ripening stages. The activity of PPO in GD fruit at the first stage of ripening was 11.7-fold higher than that of FHIA-23. The previous works have reported similar trends for PPO activity in other fruits. Murata et al. (1995) reported that the specific activity of PPO in apples decreased during the ripening process. In agreement with our results, Lee (2007) also observed that the PPO activity decreased as progressed the ripening process of bananas (*Musa acuminata*). Our value of PPO activity in GD at ripening stage 4 was similar to that obtained by Yousaf et al. (2006) in ripe yellow ‘Berangan’ and ‘Mas’ bananas.

On the other hand, the activity of POD increased continuously during the ripening process of both cultivars (Fig. 5b). The POD activity of GD ripe fruit (group 4) was 51.7-fold higher than that observed for the unripe fruit

Fig. 3 Visualization of browning in banana puree at different exposition periods for FHIA-23 and ‘Giant Dwarf’ cultivars

Fig. 4 Comparison of browning in banana puree from each ripening stage (1–4) for both cultivars; the letter **a** corresponds to the ‘Giant Dwarf’ cultivar, while the letter **b** corresponds to the cultivar ‘FHIA-23’

(group 1). The activity of POD was higher in GD than FHIA-23 at tested ripening stages. In both cultivars, the activity of POD was always lower in groups 1, 2, and 3 as compared with that of group 4. The trends observed in this study for POD activity in both cultivars during ripening were similar to that described by López-Miranda et al. (2011) in seedless grapes during ripening. They (López-Miranda et al. 2011) stated that levels of H_2O_2 increased with fruit ripening, evidencing the existence of the necessary conditions for an increased POD activity. To the best of the author’s knowledge, the activity of POD and PPO during ripening had not been evaluated in fruit of FHIA-23 cultivar. This research shows the potential of the FHIA-23 cultivar for industrial processing to produce juice or puree, since it presents less browning effects and possible occurrence of unpleasant tastes by action of POD.

The zymographic studies showed that the PPO isolated from GD fruit at different ripening stages showed distinct bands on the gel (Fig. 6). All ripening groups showed a band with an apparent molecular weight of approximately 25 kDa, but this band was unique for active PPO observed for the first ripening group. Nevertheless, along the ripening process, a new band appeared in groups 2 and 3 with molecular weight of about 35 kDa, making a total of two bands for these groups, the first of 25 kDa, similar to that present in the earlier stage of ripening, plus a 35-kDa band. While group 4 displayed at least six active PPO isoforms with estimated molecular weights at 25, 35, 49, 66, 95, and about 199 kDa (lane 4 of Fig. 6). Multiple forms of PPO with different electrophoretic mobility have been observed in a large variety of plants and fruits, with PPO molecular weights ranging from 12 to over 200 kDa (Yoruk and Marshall 2003). Possible

Fig. 5 **a** Behavior of polyphenol oxidase activity from each ripening stage (1–4) for both cultivars ‘Giant Dwarf’ (yellow bars) and FHIA-23 (blue bars). **b** Behavior of peroxidase activity from each ripening stage (1–4) for both cultivars ‘Giant Dwarf’ (yellow bars) and FHIA-23 (blue bars)

reasons for this multiplicity include the attachment of phenolic oxidation products or carbohydrates, proteolysis, conformational changes, oligomerization, and the presence of different distinct genes (Cheema and Sommerhalter 2015). On the other hand, for FHIA-23, only a single protein of 27 kDa with in situ PPO activity was identified, suggesting PPO polymorphism between GD and FHIA-23.

Conclusions

Through digital image analysis of the fruit peel, four ripening stages were detected in both cultivars, based on statistically significant differences in the CIELab color parameter

a^* . The polyphenol oxidase and peroxidase activities were associated with banana peel and pulp puree color changes, during fruit ripening. By the usage of zymograms, molecular differences in the PPO of GD were identified and were associated with a state of ripening. The PPO activity and isoform distribution depended of the ripening stage of the fruit. Both PPO and POD activities were higher in GD than FHIA-23 cultivar. In summary, the FHIA-23 cultivar has potential in industrial processes, since it presents less browning effects.

Fig. 6 Detection by zymography of the active PPO isoforms extracted from banana fruit ('Giant Dwarf') at each stage of ripening (1–4). Lane M: protein molecular weight marker, this section (I) of the gel was stained with Coomassie blue G-250. Section II was activity stained with catechol

Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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