Polyphenol Oxidase from Spanish Hermaphrodite and Female Papaya Fruits (*Carica papaya* Cv. Sunrise, Solo Group)

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A partial characterization of polyphenol oxidase (PPO) in papaya fruits is described in this work. Differences among papaya fruits from hermaphrodite and female flowers in terms of PPO activity are also presented. Total soluble PPO activity in female fruits was higher than in hermaphrodite fruits during all ripening proccesses, and greater PPO values were obtained from green papayas. In this ripening stage, female fruits exhibited 30% more PPO activity than hermaphrodite fruits. Specific PPO activity also underwent this evolution. Female green papayas showed a significantly higher PPO activity. This difference became insignificant in ripe fruits (10 days at 14 °C). Native PAGE experiments showed the presence of six PPO isoenzymes in hermaphrodite papayas whereas PPO from female fruits separated into only four bands (revealed with 0.003 M gallic acid). Assays of incubation with 0.003 M gallic acid followed by ethanolic washings produced a slightly different PPO pattern (five PPO active bands for hermaphrodite green fruits and four PPO active bands for female green ones). Through ripening, the PPO isoenzyme pattern did not undergo significant changes; only a band ($R_f = 0.28$) disappeared in the PPO extracts of mature-green, ripe, and over-ripe hermaphrodite fruits.

Keywords: *Polyphenol oxidase; papaya fruit; hermaphrodite; female; ripening*

INTRODUCTION

Papaya is an important crop in several tropical regions; for examples, Hawaii, India, etc. In Spain, this fruit is extensively harvested in the Canary Islands together with bananas (Rodriguez and Galan, 1992). Papaya belongs to the climateric class of fruits. The fruit ripen while still attached to the tree, exhibiting an increase in ethylene and carbon dioxide production (Salunke, 1984). The tolerance of papayas to temperatures of <10 °C varies with the initial maturity of the fruit and the duration and temperature of exposure (Chen and Paull, 1986). The characteristic features of papaya ripening are changes in texture, color, and sweetness.

Some enzymes from papaya fruits have been detected and partially characterized as peroxidase (EC 1.11.1.7; Silva et al., 1990), invertase (Chan and Kwok, 1976), and catalase (Chan et al., 1978). But, papaya polyphenol oxidase (PPO) was only reported by Cano et al. (1995) in a recent study together with peroxidase during post-harvest ripening and freezing process of commercial hermaphrodite fruits.

Papaya is an herbaceous plant of rapid growth and short life span. In papaya plantations, plants with hermaphrodite, female, and male flowers coexist but only hermaphrodite and female fruits can reach full development. Hermaphrodite papaya fruits are usually the commercial fruits for export. However, in the Canary Islands, female papaya fruits are consumed in the local markets. These female fruits have different characteristics than hermaphrodite fruits. Female papayas are small in size and spherical and have no seeds in their inner cavity.

Literature concerning the enzyme PPO was reviewed by Okuse et al. (1981), Vamos-Vigyazo (1981), and Mayer and Harel (1979). There are two reactions catalyzed by this enzyme. Cresolase catalyzes the oxidation of monohydric phenol, such as tyrosine and *O*-cresol to form hydroxyl group at the ortho position. Catecholase involves the removal of two hydrogen atoms from *o*-diphenol, such as catechol, chlorogenic acid, or 3,4-dihydroxyphenylalanine, to form the corresponding *o*-diquinone. There are not any previously published papers reporting the presence of PPO in papaya fruits. Only a recent study of Cano et al. (1995) described the changes in PPO activity in papaya tissue during ripening and after a freezing/thawing process. Although PPO or cathecol oxidase is apparently not ubiquitous in plants (Mayer and Harel, 1979), there are few fruits and vegetables in which it has not been found when looked for. Lack of enzyme activity in a particular variety of tissue, or a certain development stage, cannot be taken as proof of the absence of gene(s) coding for cathecol oxidase or even the absence of protein itself (Mayer and Harel, 1990). A survey of recent reports on PPO in fruits and vegetables and their products adds several new species to the already long list of those reported to exhibit enzyme activity (Mayer and Harel, 1979; Mayer, 1987). The results of the present study contribute to the evidence of the presence of this enzyme (PPO) in papaya tissues, in addition to previous report of PPO in other "exotic" species such as annona (Sanchez de Medina et al., 1987), dates (Hasegawa and Maier, 1980), and strawberry (Pilando et al., 1985). The purposes of this study were to partial characterize the PPO of papaya fruits and to establish the possible differences between hermaphrodite and female fruits in terms of this enzyme during post-harvest ripening.

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Table 1. Influence of Extraction Buffer Composition on Papaya PPO Activity

buffer composition	PPO activity (ΔOD/min/g lyophilized)[a]
0.05 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP	76.25 ± 2.34
0.05 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP + 1 M NaCl	82.36 ± 2.18
0.05 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP + 0.5% (w/v) Triton X-100	117.56 ± 1.65
0.05 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP + 1 M NaCl + 0.5% (w/v) Triton X-100	118.34 ± 2.41
0.2 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP	120.51 ± 1.75
0.2 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP + 1 M NaCl	122.82 ± 2.65
0.2 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP + 0.5% (w/v) Triton X-100	121.98 ± 1.77
0.2 M sodium phosphate (pH 7.0) + 1% (w/v) insoluble PVP 1 M NaCl + 0.5% (v/w) Triton X-100	123.74 ± 2.13

[a] Activity values are average of three independent determinations ± standard deviation.

MATERIALS AND METHODS

Fruit Ripening. Hermaphrodite and female papaya fruits (cv. Sunrise, Solo group) were harvested at preclimateric stage and obtained from commercial orchards in Tenerife (Canary Islands, Spain). They were brought to Instituto del Frío (Madrid) by air-shipment within 24 h after harvest. On arrival, undamaged fruits, free from infection (visually detected), were selected and stored at 14 °C and 90−95% relative humidity, conditions recommended by Salunke (1984). At each storage interval, 1 day (green), 5 days (green-mature), 10 days (ripe), and 15 days (over-ripe), 10 fruits were removed at random. These fruits were peeled, and the cheeks of each fruit were cut off and sliced. Papaya slices were combined, and three subsamples were removed for analysis.

Preparation of Lyophilized Samples. At each storage interval, papaya slices were inmediatelly frozen in liquid nitrogen and lyophilized with Telstar model Lioalfa equipment for 48 h. Lyophilized samples were stored at −24 °C until analysis.

Total Pectic Substances. The analysis of total pectic substances was carried out by a colorimetric determination of galacturonic acid produced by alkaline hydrolysis of pectic compounds (Dische, 1947).

Total Carotenoids. The amount of total carotenoids were determined after addition of individual carotenoid compounds by the HPLC method reported by Cano et al. (1996).

Enzyme Extraction. Enzymatic extracts were prepared so that PPO activity was determined at the highest level (Table 1). In all assays, 0.5 g of lyophilized powder was homogenized for 1-min intervals of mixing with 20 mL of extraction buffer, using an Omni-mixer Sorvall model 17106 with external cooling. The homogenates were centrifuged in a Sorvall model RC-5B refrigerated superspeed centrifuge for 30 min at 18000*g* and 4 °C until assayed for PPO activity.

PPO Activity. The PPO activity was determined at 25 °C by measuring the initial rate of increase in absorbance at 420 nm. Unless otherwise stated, activity was assayed in 3 mL of reaction mixture, consisting of 2.75 mL of 0.05 M catechol in 0.05 M sodium phosphate buffer (pH 6.5) with 0.25 mL of prepared enzyme, with a Perkin-Elmer spectrophotometer model Lambda 15. The enzyme 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 extrated (specific activity) or the change in absorbance/min/g lyophilized tissue.

Protein Determination. Protein concentrations of the extracts were measured by the Bradford (1976) method, measuring optical density (OD) at 595 nm, with bovine albumin as a standard.

Substrate Specificity. The substrates used for the specificity study are listed in Table 2. All compounds were prepared in 0.05 M sodium phosphate buffer (pH 6.5).

Effect of pH. A study was made of the effect of pH on the catechol oxidation by papaya PPO. Enzyme activity was determined in 0.05 M sodium phosphate buffer at different pH values, ranging from 5.0 to 7.5.

Effect of Substrate Concentration. Solutions of cathecol and 4-methylcathecol varying in concentration from 3 mM to 0.1 M for cathecol and from 0.3 to 1.0 mM for 4-methylcatechol were employed to study the effect of substrate concentration. In a cuvette, 0.25 mL of enzyme solution was mixed with 2.75 mL of cathecol or 4-methylcatechol at different concentrations in 0.05 M sodium phosphate buffer at pH 6.5. Michaelis constants (K_m) and maximum velocities (V_{max}) of PPO were calculated from a plot of 1/activity versus 1/substrate concentration by the method of Lineweaver and Burk (1934).

Table 2. Effect of Substrate on Papaya Total Soluble PPO Activity

substrate	PPO activity (DOD/min/g lyophilized)[a]
0.5 M catechol	119.81 ± 1.56
0.06 M 4-methylcatechol	56.97 ± 1.09
0.015 M L-dopa	7.66 ± 1.23
0.003 M catechin	ND

[a] Values are average of three independent determinations ± standard deviation.

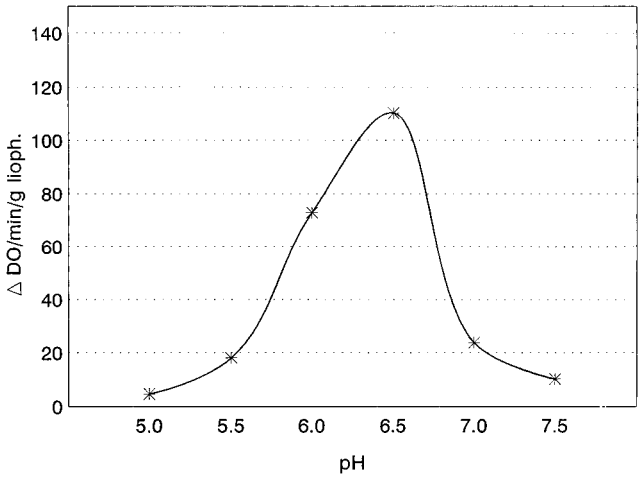

Figure 1. Effect of pH on the enzymatic activity of PPO. Enzymatic extracts were obtained from lyophilized powder of ripe fruit.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on a Miniprotean II dual slab cell unit (Bio-Rad). Runs were performed at constant current intensity (35 mA per plate), with cooling to ~4 °C for 30 min. Polyacrylamide gels (10%) were prepared according to Laemmli (1970) without SDS (native conditions). After running, gels were incubated at 10 g/L in a 0.003 M gallic acid solution in 0.05 M sodium phosphate buffer (pH 6.5) for 15 min. Also, duplicate gels were washed several times with 40% ethanol for an additional 5 min after the standard staining with gallic acid.

Data Analysis. Values are the average of three determinations. These results were analyzed for variance (ANOVA) and statistical significance by *t* test with Statgraphics and/or InStat software packages.

RESULTS AND DISCUSSION

Selection of Conditions for Enzyme Assay. Several buffer compositions were employed to select the most suitable to extract PPO from papaya tissue (Table 1). The increase of molar concentration of sodium phosphate buffer increases the extraction of PPO activity near 2-fold. Addition of detergent Triton X-100 produced a significant increase of PPO activity in

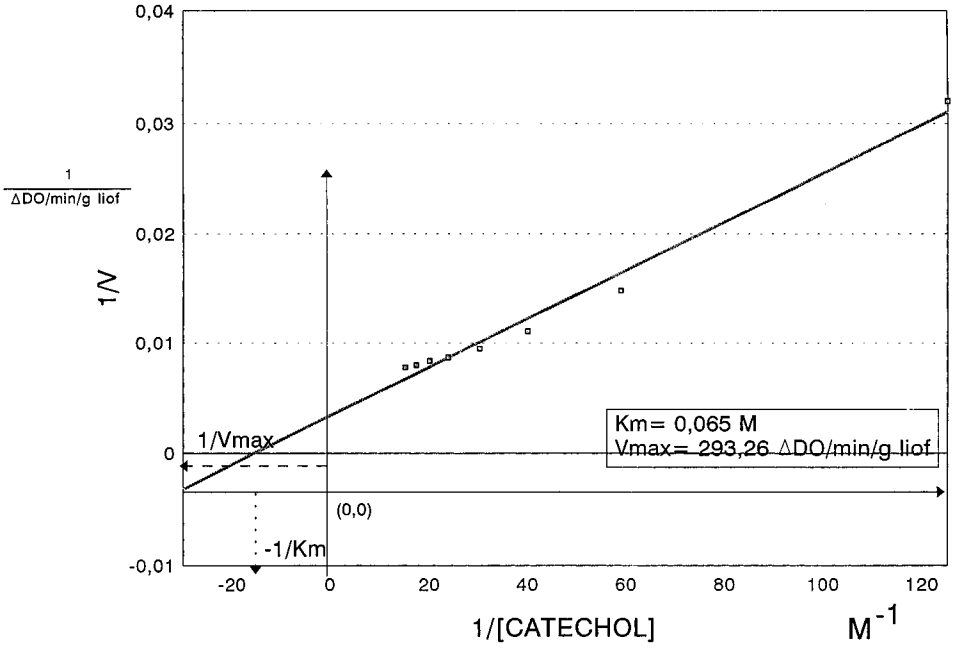

Figure 2. Effect of substrate concentration (cathecol) on papaya PPO activity (Lineweaver−Burk plot).

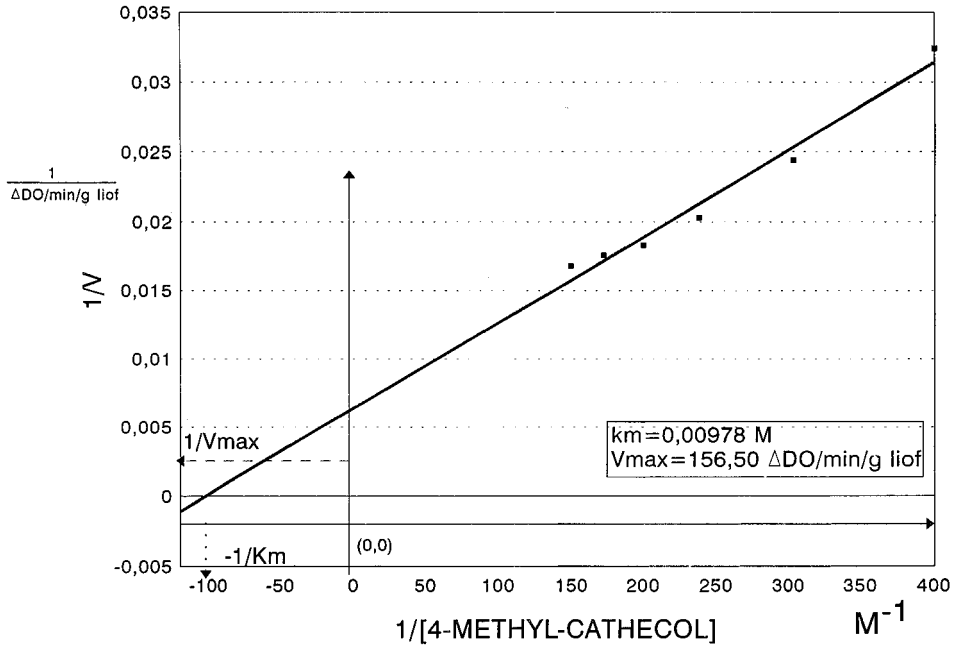

Figure 3. Effect of substrate concentration (4-methylcathecol) on papaya PPO activity (Lineweaver−Burk plot).

extracts made from low concentrated buffer (0.05 M sodium phosphate). However, the addition of Triton X-100 did not produce any significant advantage in high concentrated buffer (0.2 M). The increase of ionic strength by addition of sodium chloride only produced a slight increase in PPO activity when extracts were made from low concentrated buffer. This salt also did not produce a significant increase in the activity with the high concentrated buffer (0.2 M; Table 1). Therefore, a 0.2 M sodium phosphate buffer containing only 1% (w/v) insoluble polyvinylpyrrolidone was employed for all enzyme assays. The use of insoluble polyvinylpyrrolidone has been reported to produce good results in PPO extraction from different plant tissues, such as banana (Galeazzi et al., 1981) and strawberry (Wesche and Montgomery, 1990).

Tanning reactions during enzyme extraction can cause partial inactivation of the enzymes. To avoid these reactions, reducing agents are often added during extraction, but must be removed before assay. Alternatively, phenolic substrates must be removed prior to the assay with insoluble polyvinylpyrrolidone (Mayer and Harel, 1979).

Substrate Specificity. For studing substrate specificity, a total soluble enzymatic extract was used. A number of ortho-diphenols were used to test the substrate specificity (Table 2). Papaya PPO activity was more active with cathecol and at low levels with 4-methylcathecol. However, PPO activity was very low with L-dopa substrate and not detectable with cathechin. These results revealed that papaya PPO contains catecholase activity. The number of hydroxyl groups and their position in the benzene ring of the substrate determined the 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 cathecolase. Cresolase was easily inactivated during the extraction process.

Table 3. Characteristics of Spanish Papaya Fruits (*Carica papaya*, Solo Group), cv. Sunrise

characteristic[a]	unripe fruits		ripe fruits	
	hermaphrodite	female	hermaphrodite	female
Physical Parameters				
weight (g)	415.34 ± 1.23	205.37 ± 3.12	410.13 ± 2.65	203.27 ± 3.28
firmness (N/g)	33.29 ± 1.09	20.58 ± 0.87	3.75 ± 0.65	4.30 ± 0.89
color *L*	31.64 ± 0.32	38.47 ± 0.53	32.96 ± 0.49	34.12 ± 0.38
aL	6.51 ± 0.65	7.55 ± 0.23	8.11 ± 0.42	9.19 ± 0.27
bL	30.41 ± 1.21	26.65 ± 0.67	20.56 ± 0.95	19.30 ± 0.82
Physicochemical Parameters				
pH	5.5 ± 0.26	5.41 ± 0.31	5.46 ± 0.27	5.59 ± 0.17
titratable acidity (g citric acid/100 g FW)	0.13 ± 0.05	0.13 ± 0.03	0.12 ± 0.01	0.11 ± 0.02
soluble solids (°Brix at 20 °C)	13.05 ± 0.23	13.63 ± 0.19	14.10 ± 0.32	14.55 ± 0.17
total solids (%)	14.18 ± 0.35	15.15 ± 0.29	15.04 ± 0.38	15.95 ± 0.42
moisture content (%)	85.82 ± 0.35	84.85 ± 0.29	84.96 ± 0.38	84.05 ± 0.42
Chemical Parameters				
total carotenoids (g/100 g FW)	8.76 ± 1.02	8.25 ± 0.98	8.84 ± 0.87	10.26 ± 0.76
total pectins (g/100 g FW)	0.35 ± 0.07	0.34 ± 0.05	0.42 ± 0.04	0.45 ± 0.06

[a] Values are average of three independent determinations ± standard deviation.

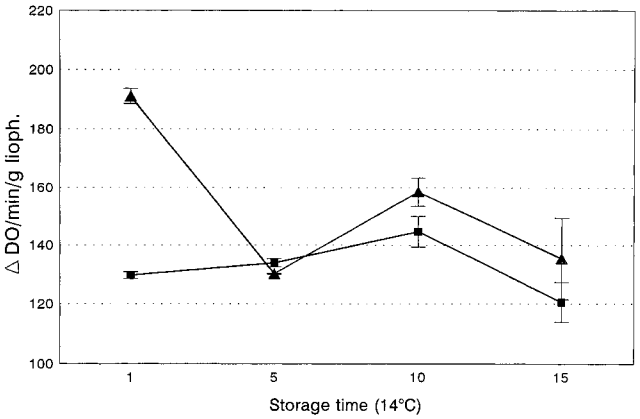

Figure 4. Changes in total soluble PPO activity in hermaphrodite (■) and female (▲) papaya fruits during ripening (storage at 14 °C, 90−95% RH).

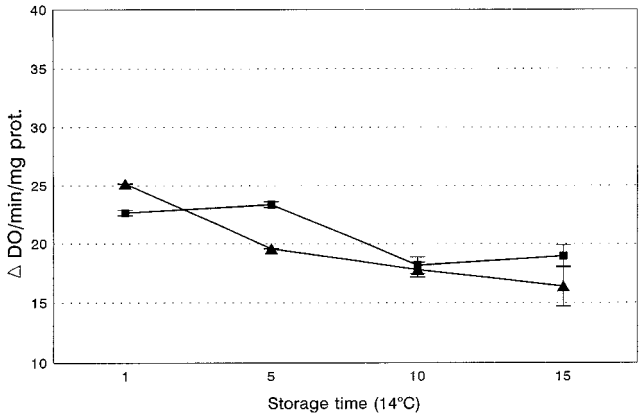

Figure 5. Changes in specific PPO activity on hermaphrodite (■) and female (▲) papaya fruits during ripening (storage at 14 °C, 90−95% RH).

Effect of pH. When cathecol was the substrate, the optimum activity was at pH 6.5 (Figure 1). This study was conducted in the 5.0−7.5 pH range. Reactions conducted at pHs above the optimum (6.5) produced a great decrease in PPO activity (nearly 5-fold). The pH of papaya tissue is 6.41 or 6.17, for hermaphrodite or female papaya, respectively. The papaya natural pH is close to the optimum pH for the PPO activity. Therefore, papaya tissue could be suceptible to severe browning during storage or/and processing; but, this fact is limited by the low phenol concentration in this fruit.

Effect of Substrate Concentration. The effect of cathecol and 4-methylcathecol concentrations on PPO activity were investigated. Cathecol concentrations ranging from 3 mM to 0.1 M and 4-methylcathecol concentrations ranging from 0.3 to 1.0 mM were employed. The K_m and V_{max} values for the PPO were determined from Lineweaver−Burk plots (Figures 2 and 3). The K_m values for cathecol and 4-methylcathecol were 65 and 9.7 mM, respectively. The V_{max} values were 293.26 156.5 OD/min/g protein for cathecol and 4-methylcathecol, respectively.

PPO Activity during Ripening. Papaya fruits exhibited the initial characteristics shown in Table 3. These values are related to green fruits at the preclimateric stage of the ripening process. Female papaya total soluble PPO activity showed a significant decrease during the first 5 days of storage, whereas PPO activity of hermaphrodite fruits remained almost constant until the climateric of the fruits (Figure 4). Female papaya PPO activity showed a maximum when the fruit reached ripeness. From this date, both types of papaya fruits exhibited a significant loss in PPO activity. Female papaya fruits have the highest PPO activities during ripening. In contrast, specific PPO activity (ΔOD/min/g protein) did not show any maximum at the climateric stage of the fruits (Figure 5). The differences between female and hermaphrodite papayas in terms of specific PPO activity were not significant in ripe and over-ripe fruits. Specific female PPO diminished continously throughout ripening, with almost a 40% decrease from green to ripe fruit.

Electrophoresis. To determine the active isoenzymes of PPO, solutions of different substrates were assayed: 0.03 and 0.07 M cathecol; 3 and 7 mM 4-methylcathecol, and 3 and 7 mM gallic acid in 0.05 M sodium phosphate buffer (pH 6.5). Staining the gels for 10 min was enough to visualize the PPO bands. The cathecol and 4-methylcathecol solutions produced staining of the gel and the PPO isoenzymes. In contrast, gallic acid clearly stained only the PPO bands. After gallic acid staining, a duplicate of the gels was washed several times with ethanol to produce a partial gel dehydration that brought out the PPO isoenzyme bands. This protocol was previously reported by Galeazzi et al. (1981) in banana PPO. The washing with ethanol produced new PPO bands that were only slightly stained with gallic acid.

The electrophoretic pattern of total soluble PPO stained with gallic acid from hermphrodite and female papaya fruits during ripening is shown in Figure 6. In green hermaphrodite fruits, PPO revealed six bands with the R_f values (A) 0.19; (B) 0.28; (C) 0.37; (D) 0.46; (E) 0.55; and (F) 0.64. In these fruits, band C lost

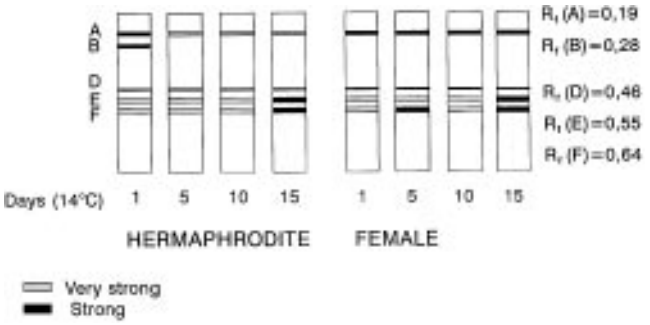

Figure 6. Electrophoretic patterns of total (soluble + bound) PPO from hermaphrodite and female papaya fruits during ripening (storage at 14 °C, 90−95% RH). 1 day (green fruits); 5 days (mature-green fruits); 10 days (ripe fruits); 15 days (over-ripe fruits). Staining of gels was with 0.003 M gallic acid.

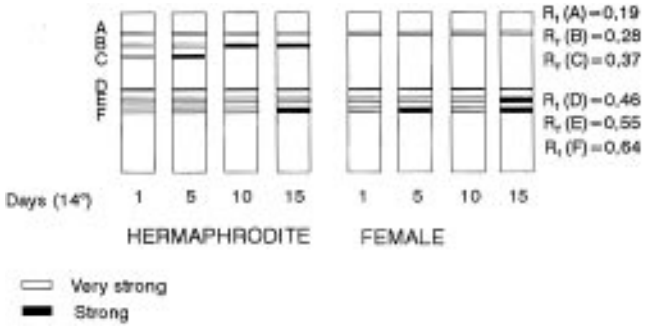

Figure 7. Electrophoretic patterns of total (soluble + bound) PPO from hermaphrodite and female papaya fruits during ripening (storage at 14 °C, 90−95% RH). 1 day (green fruits); 5 days (mature-green fruits); 10 days (ripe fruits); 15 days (over-ripe fruits). Staining of gels was with 0.003 M gallic acid and ethanol.

intensity at 5 days of storage (green-mature fruits) and it disappeared at 10 days (ripe fruits). Also in ripe fruits, band B lost some intensity. Bands A, D, and E were not modified throughout ripening, but band F lost some activity in over-ripe fruits.

Female papayas showed four PPO isoenzymes (A, D, E, and F). Isoenzymes B and C did not appear at any stages of ripening. In these fruits, band F (R_f, 0.64) also decreased in intensity at 5 and 15 days of storage, whereas band C (R_f, 0.37) lost only a slight amount of activity at 15 days of storage (over-ripe fruits). Gallic acid-stained gels washed with ethanol exhibited a stronger intensity of band A (R_f, 0.19) than gels not submitted to ethanolic washing (Figure 7). In addition, ethanol washing brought out band C (R_f, 0.37) in the green and green-mature hermaphrodite papayas. Also, isoenzyme B appeared at 10 (ripe) and 15 (over-ripe) days of storage in these fruits. The ethanol treatment may have produced some concentration of the isoenzymes (bands) by the partial dehydration of the gel, thus causing the isoenzymes to be more visible.

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