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Characterization and changes in polyphenol oxidase from eggplant fruit (*Solanum melongena* L.) during storage at low temperature

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Abstract

Polyphenoloxidase (PPO) from eggplant fruit was characterized, and its catecholase activity studied during storage of fruit at 0 and 10 °C. The high catecholase activity was observed using 4-methylcatechol as substrate at pH 6 and 30 °C and activation with sodium dodecyl sulphate (SDS) was unnecessary. The soluble PPO fraction was the most thermostable, as well as the most active, form of the enzyme. Fruits stored at 10 °C were undamaged, whereas those kept at 0 and 5 °C experienced chilling injury from days 6 and 8, respectively, as indicated by the decrease of lightness (L_0) of pulp tissue. During exposure of fruits at 10 °C, the activities of soluble and insoluble PPO fractions increased, whereas, at 0 °C, PPO activity of both fractions decreased and maintained lower levels when browning of pulp tissue was observed. Soluble PPO activity at 0 °C was directly related to the value for L_0.

Keywords: Eggplant fruit; *Solanum melongena* L.; Polyphenol oxidase; Chilling injury; Storage

1. Introduction

Most fruits and plants originating in tropical and subtropical regions are prone to physiological injury when exposed to temperatures below 12.5 °C but above their freezing point. Therefore, chilling injury (CI) is a serious problem in postharvest handling of these fruits (Wang, 1994). Eggplant is a tropical fruit and its sensitivity to CI is generally associated with problems in storage and processing. One of these symptoms is the darkening of seeds and pulp tissue. More severe symptoms include pitting and browning of skin or surface scald (Cantwell & Suslow, 1999).

Biochemical and nutritional characteristics of fruits may change due to the presence of brown pigments. Moreover, browning, after mechanical or physiological injury during harvest, processing or cold storage, affects consumer acceptability and palatability because of unpleasant appearance and concomitant off-flavour development (Das, Bhat, & Gowda, 1997; Valero & García-Carmona, 1998). In general, browning is caused by enzymatic oxidation of natural phenolic compounds, and polyphenol oxidase (PPO; EC 1.14.18.1) is a key enzyme in this degradation. In the presence of molecular oxygen, PPO catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity), and further oxidation of *o*-diphenols to *o*-quinones (catecholase activity). *O*-quinones are very unstable and rapidly react with amino acids or proteins, generating brown pigments by polymerization (García-Carmona, Valero, & Cabanes, 1988), and these reactions are more important in fruits with high phenol contents such as eggplants (Bajaj, Kaur, & Chadha, 1979; Sakamura & Obata, 1963). Although PPO from eggplant fruit has been partially characterized (Fujita & Tono, 1988; Perez-Gilabert & Garcia-Carmona, 2000), no references on changes in its activity during cold storage have been published.

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The aim of the present study was to analyze the changes in catecholase activity during the cold storage of eggplant (*Solanum melongena* L., cv Money Maker No. 2). Furthermore, PPO activity of this fruit was characterized.

2. Materials and methods

2.1. Plant material and storage

Eggplant fruits (*Solanum melongena* L. cv Money Maker No. 2) harvested at commercial maturity were provided by farmers from La Plata region, Argentina. This variety is highly susceptible to chilling and browning (Cantwell & Suslow, 1999). Undamaged fruits were chosen and they were used within 10 h of harvest. They were washed to remove dirt, drained to remove the excess of water, and finally dried. Eggplant fruits were randomly distributed in groups of 6. Each group was packed in a perforated low density polyethylene (LDPE) bag and finally stored at 0, 5 and 10 °C for 12 days. Determinations were made at the beginning of storage and after 2, 6, 8 and 12 days. On each sampling day, 12 fruits of each storage condition were visually observed and the CI index calculated as mentioned in the Section 2.2. Then, all these fruits were peeled, fractionated, frozen in liquid nitrogen and stored at −20 °C until used for PPO extraction and assay.

2.2. Chilling injury development

On each sampling day, CI symptoms in pulp tissue and peel of eggplant fruits were visually evaluated on a subjective scale. Injury level was expressed according to a modification of the scale proposed by Lederman, Zauberman, Weksler, Rot, and Fuchs (1997) and the CI index was calculated as follows:

$$\text{CI index} = \frac{\sum(\text{Injury level} \times \text{Number of fruits on the level})}{(\text{Total number of fruits in the treatment})}$$

The numerical injury level of the CI index is: 5 = severe damage, 4 = moderate damage, 3 = regular damage, 2 = low damage, 1 = no damage.

2.3. Browning of pulp tissue

The colour parameter L^* indicates the lightness of colour (0 = black and 100 = white). A Minolta Colorimeter model CR-300 was used to determine L^*, and the readings were taken soon after slicing the central section of each fruit (thickness = 0.5 cm). All measurements were done on three fruits from each condition, and results were expressed as L_0.

2.4. Enzyme extraction

2.4.1. General

Three enzymatic crude extracts from eggplant fruit pulp tissue were prepared according to Serradell, Rozenfeld, Martínez, Civello, Chaves, and Añón (2000). Each extraction was carried out in duplicate at 4 °C.

2.4.2. Soluble extract in phosphate buffer

Two grammes of frozen tissue were crushed in a mill (model A10 Janke & Kunkel – IKA Labortechnik), and then blended in 10 ml of phosphate buffer (0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, pH 6.0) supplemented with 30 g/l of polyvinylpolypyrrolidone (PVPP). The suspension was shaken for 30 min and centrifuged in a Sorvall RC 5B Plus at 11,200 *g* for another 15 min. The supernatant, containing the soluble PPO fraction, was separated, vacuum-filtered, fractionated, and stored at −80 °C for further assays. In turn, the pellet was resuspended in 10 ml distilled water, and centrifuged under the same conditions to separate the supernatant which was fractionated and stored at −80 °C until use. Both extracts were named "soluble PPO fraction".

2.4.3. Insoluble extract in phosphate buffer

The pellet obtained in the previous step was resuspended in 10 ml phosphate buffer (0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, pH 6.0) supplemented with 0.1% v/v Triton X-100. The suspension was shaken for 60 min, centrifuged at 11,200 *g* for 15 min and the supernatant fractionated and stored at −80 °C until use. This extract was named "insoluble PPO fraction".

2.4.4. Total extract

From the pulp frozen in liquid nitrogen, portions of 2 g were powdered and blended in 10 ml of phosphate buffer (0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, pH 6.0) supplemented with 0.1% v/v Triton X-100 and 30 g/l PVPP. The suspension was shaken for 60 min, centrifuged at 11,200 *g* for 15 min, and the supernatant separated, fractionated, and stored at −80 °C until use.

2.5. Enzyme assay

PPO activity was determined spectrophotometrically in a Beckman DU 650 instrument according to Yue-Ming, Zauberman, and Fuchs (1997) and Shatta and El-Shamei (1999), with modifications. Unless otherwise stated, the reaction medium (500 μl) was composed of 12 mM 4-methylcatechol (Sigma Chemical, St. Louis, USA) in phosphate buffer (0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, pH 6.0) and 75 μl of the enzyme extract (15% v/v). Reference blanks were prepared by mixing all components with the corresponding boiled enzyme extract. Reactions were carried out at 30 °C and the change in absorbance at 410 nm with time was recorded.

The enzymatic activity was calculated from the slope of the linear portion of the curve. The assay mixture was prepared in triplicate, using the same stock of crude enzyme extract.

2.6. Protein determination

Protein content of the different extracts was determined by the Bradford method (1976), using bovine serum albumin (BSA; Sigma Chemical, St. Louis, USA) as standard protein.

2.7. Evaluation of enzyme properties

2.7.1. Substrate specificity

Catecholase activity of PPO was tested using total crude extract and two substrates: 4-methylcatechol (4-MC; Sigma Chemical, St. Louis, USA) and pyrocatechol (PC; Sigma Chemical, St. Louis, USA). Both, the reaction medium and the determination of enzymatic, activity accorded with the procedures described in the previous section, although, in this case, the concentration used for both substrates was 10 mM. All measurements were done in triplicate.

2.7.2. Substrate and enzyme concentration

PPO activity was evaluated at 410 nm by mixing the total enzymatic crude extract with several 4-MC (Sigma Chemical, St. Louis, USA) concentrations, in the range of 2–12 mM. In another group of experiments, the concentration of 4-MC was fixed at 12 mM, and the enzymatic activity was studied by varying the total crude extract in the range 5–35% v/v, with respect to the total reaction volume. Triplicate determinations were carried out, and the apparent Michaelis constant (K_m) and maximum velocity (V_{max}) parameters were calculated by the Lineweaver and Burk method (1934).

2.7.3. Optimum pH

pH studies were carried out using Mc-Ilvaine buffer between pH 3 and 7. Enzymatic activity of the total crude extract was determined according to the procedure described in Section 2.5. All assays were performed in triplicate.

2.7.4. Optimum temperature

PPO activity of the total crude extract was measured between 0 and 60 °C. All assays were performed in triplicate, and the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached.

2.7.5. Thermal stability

One-ml samples of soluble and insoluble PPO extracts were incubated for 30 and 60 min at 0, 5, 10 and 20 °C. The residual enzymatic activity was measured under standard conditions (pH 6 and 30 °C). All assays were performed in triplicate.

2.7.6. Activation by SDS

Enzymatic extract was incubated with sodium dodecyl sulphate (SDS; Sigma Chemical, St. Louis, USA) for 5 min. The concentration of SDS in the medium varied from 0 to 2 mM. The enzymatic activity was measured as mentioned above. The reaction was initiated by the addition of 75 μl of the treated enzyme to the reaction medium.

2.8. Statistical analysis

Results were processed by ANOVA and the means compared by LSD test with a significance of 0.05 using the SYSTAT software package.

3. Results and discussion

3.1. PPO extraction and activity

In plants, PPO is predominantly located in plastids or chloroplasts of intact cells (Mayer & Harel, 1979). Murata, Tsurutani, Hagiwara, and Homma (1997) have detected PPO, mostly in plastids from immature fruits cells, and it may be solubilized and proteolyzed during ripening and storage. Therefore, PPO activity can be found in plant cells in both forms, even at the same time (Mayer & Harel, 1979).

In the present work, PPO was extracted from frozen fruits in the presence and absence of Triton X-100 to obtain either insoluble or soluble protein.

After measuring protein contents of these extracts (Table 1), about 65% of the total protein content was found in the soluble form. On the other hand, all fractions exhibited catecholase PPO activity (Table 1) when measured under the conditions as described in Section 2.5. The specific activity of the soluble PPO fraction was twice as high as the value reached by the insoluble PPO fraction. This result suggests that most of the eggplant PPO would not be membrane-associated.

Table 1
Protein content and specific PPO activity in eggplant fruit (*Solanum melongena* L., *cv* Money Maker No. 2)

Extract	Protein ($g\,prot\,kg\,fw^{-1}$[a])	Specific activity[b] ($\Delta OD\,min^{-1}\,mg\,prot^{-1}$)
Soluble	1.59 ± 0.12	5.64 ± 0.77
Insoluble	0.62 ± 0.03	2.86 ± 0.13
Total	2.50 ± 0.01	9.02 ± 0.82

[a] $kg\,fw^{-1}$ = kilogramme of fresh weight.
[b] Specific activity was tested spectrophotometrically at 410 nm with 0.012 M 4-MC, in 0.1 M phosphate buffer, pH 6.0, at 30 °C.

Table 2
Substrate specificity of PPO from eggplant fruit (*Solanum melongena* L., *cv* Money Maker No. 2)

Substrate (0.01 M)	Activity (ΔOD min^{-1})	Relative activity (%)	Specific activity[a] (ΔOD min^{-1} mg prot^{-1})
PC	0.030 ± 0.002	37	4.04 ± 0.21
4-MC	0.081 ± 0.0003	100	11.0 ± 0.03

[a] Specific activity was tested spectrophotometrically at 410 nm with 0.01 M substrate, in 0.1 M phosphate buffer, pH 6.0, at 30 °C.

3.2. Selection of conditions for the enzyme assay

3.2.1. Substrate specificity

The oxidizing ability of PPO from eggplant fruit was determined using 4-MC and PC. The enzyme showed a significantly ($P < 0.05$) higher specific activity when 4-MC was used as substrate (Table 2), and therefore, it was selected as substrate for further assays. PPO was reported to have a wide range of substrate specificity. Other authors have also used 4-MC as enzyme substrate (Onsa, Saaei, Seelamat, & Bakar, 2000; Perez-Gilabert & Garcia-Carmona, 2000; Trejo-Gonzalez & Soto-Valdez, 1991; Yue-Ming et al., 1997).

3.2.2. Effect of substrate concentration

The effect of the concentration of 4-MC on PPO activity was investigated. Apparent K_m and V_{max} values were 3.24 mM and 0.10 ΔOD min^{-1}, respectively. According to these results, the concentration of 4-MC was set to 12 mM for further assays. The value for K_m obtained was similar to those reported for another variety of eggplant (Perez-Gilabert & Garcia-Carmona, 2000) and for palmito stem (Robert, Rouch, Richard-Forget, Pabion, & Cadet, 1996), but lower than values found for pear (Gauillard & Richard-Forget, 1997) and blackberry (González, Begoña de Ancos, & Cano, 2000).

3.2.3. Effect of enzyme concentration

PPO activity, as a function of the percentage of enzymatic crude extract (enzyme concentration), was determined. Using 4-MC as substrate, the specific activity showed a linear increase with enzyme concentration until a maximum was reached at 25% v/v (data not shown). Further experiments were carried out, setting the enzyme concentration at 15–20% v/v, corresponding to the linear portion of the curve.

3.2.4. Effect of pH

pH is a key factor affecting the enzyme activity, therefore the pH profile of PPO activity, using 4-MC as substrate, is presented in Fig. 1. A wide range of optimum pH (4.8–6) was found for the total PPO fraction. The enzymatic activity was characterized by a rapid and significant ($P < 0.05$) decrease of activity below pH 4.8 and a slight decrease from pH 6. To gain more insight of the effect of pH, the enzyme activity of soluble and insoluble crude extracts were analyzed using the same conditions described earlier. The results indicated that activity of the soluble PPO fraction (Fig. 1) had a similar range as the corresponding total PPO fraction, whereas a maximum pH of 5.7 was observed for activity of the insoluble PPO fraction. Other authors obtained an optimum pH of 4 for total PPO activity from blueberry (Kader, Rovel, Girardin, & Metche, 1997) and 5.3 from strawberry (Serradell et al., 2000) while, for soluble PPO activity, optimum pH reported for apple (Trejo-Gonzalez & Soto-Valdez, 1991) was 5.4, and an interval from pH 6 to 7 for pineapple (Das et al., 1997).

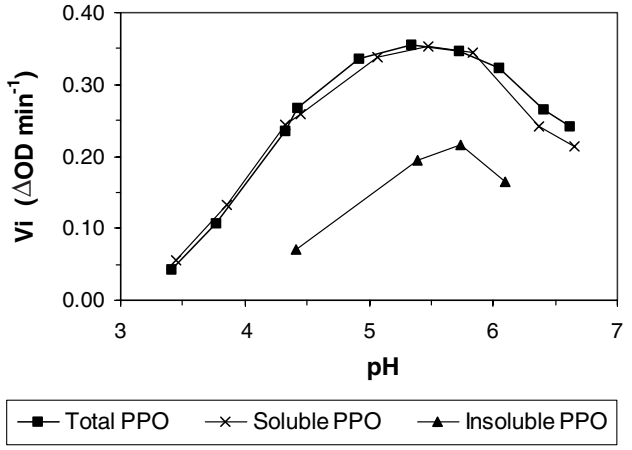

Fig. 1. Effect of pH on enzymatic activity of soluble, insoluble and total PPO from eggplant fruit. $LSD_{0.05} = 0.03$.

3.2.5. Effect of temperature

PPO activity was studied between 0 and 60 °C. As the aim of this work was to evaluate changes of PPO activity during low temperature storage of eggplant, the enzymatic activity was studied with special emphasis on chilling temperatures. Fig. 2 shows the effect of temperature on the activity of total PPO. The highest activity under the described conditions was obtained at 30 °C, and this value was considered as 100% of specific activity. Between 0 and 40 °C, the relative activity was above 80%. At the temperatures of eggplant refrigerated storage employed in this work, only 18% of relative PPO activity was lost at 0 °C, while, at 5 °C, losses were 12%. Enzymatic activity began to decrease more strongly above 35 °C but, even at 60 °C, the enzyme still maintained a relative activity of 48%. Consequently, 30 °C was considered as the optimum temperature measurement of eggplant PPO activity. Optimal temperatures for fruit PPO activity were reported by other authors (Ding, Chachin, Ueda, & Imahori, 1998; Trejo-Gonzalez & Soto-Valdez, 1991) and the values were between 30 and 40 °C.

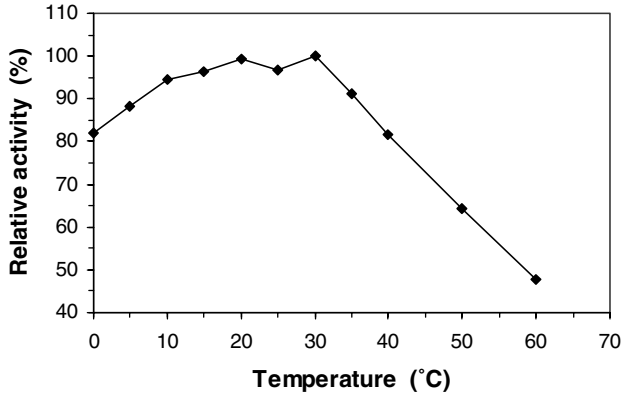

Fig. 2. Effect of temperature on enzymatic activity of total PPO from eggplant fruit. Each data point represents the relative activity expressed as a percentage of the maximum activity (at 30 °C), and they are the averages of at least three determinations. $LSD_{0.05} = 2.30$.

3.2.6. Thermal stability of PPO

Given the importance of low temperatures for fruit quality, the stability of PPO under these conditions was analyzed. To determine the form most affected, soluble and insoluble PPO fractions were studied. Results obtained after thermal treatment are shown in Fig. 3.

After 30 and 60 min at 5, 10 and 20 °C, the residual soluble PPO activity (Fig. 3) remained above 90%, while, after exposure for 30 and 60 min at 0 °C, the soluble PPO fraction retained more than 80% of its initial activity. The insoluble PPO fraction, upon heating for 30 min at 0, 5, 10 and 20 °C, underwent great changes of enzymatic activity. It is noteworthy that, in all cases, the residual activity was close to 40% (Fig. 3). Furthermore, no differences were observed between the effects of incubation times of 30 and 60 min.

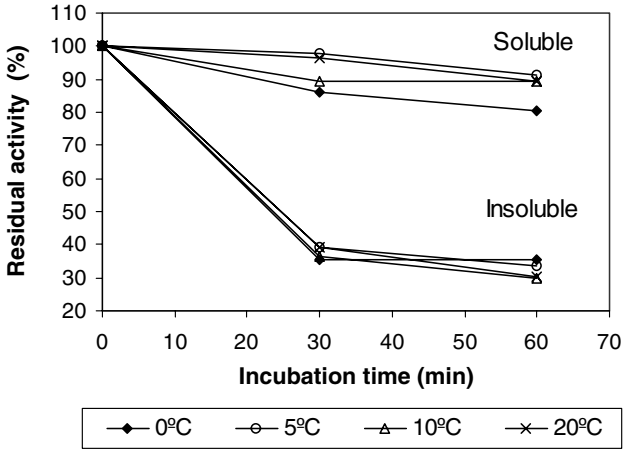

Fig. 3. Thermal stability of soluble and insoluble PPO fractions with respect to incubation time at different temperatures. Values represent the relative activity expressed as a percentage of the initial activity. $LSD_{0.05} = 0.65$.

As observed, the eggplant soluble PPO fraction was more stable than the insoluble fraction at the temperatures and under the conditions employed in this work. It is noteworthy that the thermal stability of the soluble PPO fraction was still high on incubation at 0 °C.

3.2.7. Effect of SDS

A study of enzyme activation was carried out using the detergent SDS, because PPO activity is assayed either without SDS (Murata, Kurokami, & Homma, 1992; Zhou, Smith, & Lee, 1993) or in the presence of SDS (Gauillard & Richard-Forget, 1997; Robinson, Loveys, & Chacko, 1993). Its effect in the activation process results from a conformational change, due to the binding of a small amount of SDS to the active site (Moor & Flurkey, 1990). The exposure time and concentration of SDS employed in this work were similar to those used to activate the enzyme PPO in sago log (Hassan Onsa, Bin Saari, Selamat, & Bakar, 2000), lettuce (Chazarra, Cabanes, Escribano, & García-Carmona, 1996), table beet (Escribano, Cabanes, & García-Carmona, 1997) and pear (Gauillard & Richard-Forget, 1997). No changes were found after the incubation of the enzyme with different concentrations of SDS (data not shown). These results indicate that eggplant PPO was fully active, and so it was not necessary to activate any latent form.

3.3. Effect of storage at low temperature

3.3.1. Chilling injury development

No symptoms of CI were found in eggplants stored at 10 °C, i.e., the lightness of a recently sliced fruit (L_0) remained constant along storage time and similar to the initial value, denoting no browning evolution (data not shown). In turn, during storage at 0 and 5 °C, the CI symptoms and corresponding index and L_0 values are shown in Table 3. The CI indices of fruits stored at 0 °C were higher and increased more rapidly than at 5 °C. Correspondingly, values of L_0 decreased with storage time and this was more noticeable at 0 °C ($P < 0.05$), showing a high rate of pulp tissue browning. By following the evolution of L_0, browning of seeds and pulp tissue was seen to begin after 6 days at 0 °C and 9 days at 5 °C.

For CI symptoms (Table 3), temperature and storage time are observed to be important factors affecting quality properties of eggplant fruit.

3.3.2. Changes in PPO activity during storage

Soluble and insoluble crude extracts from fruits exposed to low temperatures were analyzed to comparatively assess the possible effects of storage on soluble and membrane-bound PPO. For these experiments, 10 and 0 °C were chosen as control and chilling temperature, to follow catecholase PPO activity in both soluble and

Table 3
Chilling injury index (CI), lightness (L_0) values of pulp tissue and symptoms for eggplant fruit (*Solanum melongena* L., cv Money Maker No. 2) stored at 0 and 5 °C during 12 days

	CI Index	L_0	Symptoms
Days at 0 °C			
0	1.0	88.98	
2	1.4	88.52	Peel discoloration and loss of brightness
6	2.8	81.96	Pitting and incipient browning of seeds
8	3.4	78.92	Browning of pulp tissue with a few scalds on the peel
12	4.5	73.87	Fruit to discard
Days at 5 °C			
0	1.0	88.98	
2	1.4	88.63	Discoloration of the peel
6	2.2	86.54	Loss of brightness
8	3.0	83.27	Incipient pitting and slight browning of seeds
12	4.0	78.87	Incipient browning of pulp tissue with few scalds
	LSD = 0.25	LSD = 1.86	

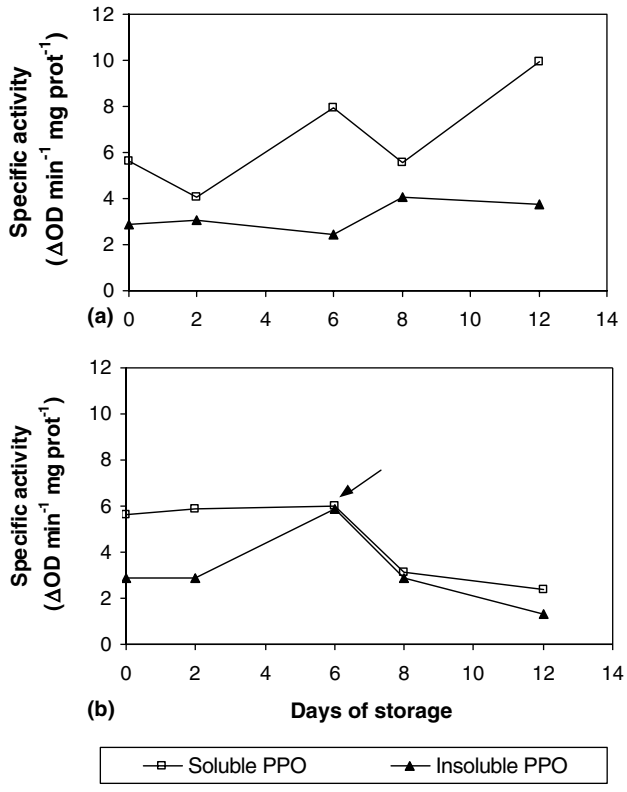

Fig. 4. Specific activity of soluble and insoluble PPO fractions from eggplant fruit during storage at 10 °C (a) and 0 °C (b) for 12 days. Each data point represents the average of at least three determinations. The arrow indicates the beginning of browning. $LSD_{0.05} = 0.76$.

insoluble crude extracts during all the storage periods. Soluble PPO activity was twice as high as insoluble PPO at the beginning of storage.

At 10 °C (Fig. 4a), soluble PPO activity remained with little variations until day 8 and finally increased by 75% at day 12 of storage. In turn, insoluble PPO activity levels were close to the initial value during the first 6 days, then increased by 30% from then. In general, soluble PPO activity was higher than was the insoluble activity over the storage period at 10 °C.

During the first 6 days of storage at 0 °C (Fig. 4b) no variations in the specific activity of soluble PPO fraction were observed, but two days later (day 8), it decreased by 50%. At the same temperature, the specific activity of the insoluble PPO fraction did not vary during the first 2 days, but then experienced a two-fold increase at day 6, and finally decreased to about half the initial value. Soluble and insoluble PPO activity were similar from day 6 on.

At the end of the experiments, the activities of both PPO forms were similarly affected by temperature. In both PPO forms, the activity increased at 10 °C and decreased at 0 °C. Moreover, between days 8 and 12 of storage, both PPO forms exhibited lower activity in cold-damaged than in undamaged fruits. Martinez-Tellez and Lafuente (1997) showed similar results, working with tangerine fruit.

PPO is capable of oxidizing phenolic compounds and this process has been related to fruit deterioration (Das et al., 1997). When tissue structure is altered, the compartments separating cellular polyphenol oxidase and polyphenolic substrates are generally disorganized, and consequently, enzyme and polyphenols are put together and rapidly form polymeric quinones by enzymatic oxidation. In the present work, at 0 °C, soluble and insoluble PPO activity began to show a marked decrease from day 6 on, precisely the time at which browning of pulp tissue appeared. At this temperature, a linear correlation between the activity of soluble PPO and L_0 values was obtained ($r = 0.867$; $P = 0.057$): the higher the soluble enzyme activity, the higher the values for L_0. Similar results were previously reported by Coseteng and Lee (1987) working with apple, and Cheng and Crisosto (1995) with peach.

Characterization results, previously shown in this work, demonstrated that PPO activity was still high at

low temperatures, especially at 0 °C. It was also observed that the soluble PPO fraction was thermostable at this temperature. Therefore, low PPO activity of fruit stored for 8 and 12 days at 0 °C could be attributed to structural changes of the enzyme or decreased PPO content. Besides, since our determinations were made on a total protein basis, it was not possible to detect variations in PPO content. Further experiments are required to obtain more evidence on this point.

4. Conclusion

In the present work, PPO activity was characterized and measured during storage of eggplant at different temperatures and times. The results showed that the best conditions to measure catecholase PPO activity were: 12 mM 4-MC, 15% v/v of enzyme concentration, pH 6, 30 °C and activation with SDS was not necessary. Soluble PPO was the most thermally stable form of the enzyme, so its activity was higher than that of the insoluble form, even at temperatures in the range of those used in storage. During exposure of fruits at the control temperature (10 °C), the activities of both PPO fractions increased whereas at the chilling temperature (0 °C), PPO activity of both forms decreased and were maintained at lower levels when browning of pulp tissue was observed. Therefore, at the chilling temperature, the evolution of PPO activity correlated with L_0 values in this variety of eggplant. Further specific studies are needed to understand PPO behavior during the browning reaction of chilled eggplant.

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