Effect of Wounding on Phenolic Enzymes in Six Minimally Processed Lettuce Cultivars upon Storage

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The effect of wounding on polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia-lyase (PAL) was studied in six minimally processed lettuce (*Lactuca sativa* L.) cultivars upon storage for 7 days at 5 °C (Iceberg Mikonos (IM), I. Green Queen (IGQ), I. Asdrúbal (IA), Little Gem Sandra (LGS), Romaine Cazorla (RC), and R. Modelo (RM)). Wounding of lettuce tissue midribs (because of minimal processing) caused an exponential increase in PPO activity due to the activation process from latent to fully active PPO by following first order kinetics in the time range from 3.7 days (LGS) to 6.3 days (RC). However, total PPO activity (active plus latent) remained constant. Isoform pattern of PPO changed upon storage probably because of posttranslational processes. POD activity linearly increased with induction of new POD isoenzymes. PAL activity presented a typical bell-shaped induction pattern in four cultivars. Only IM and IGQ showed a second induction response which has not been previously described in the literature. IM was the cultivar most susceptible to browning and RC was the cultivar least susceptible. However, no clear correlation was observed between browning and any of the biochemical and physiological attributes investigated (PPO, PAL, and POD activities, total and individual phenols accumulation, and ascorbic acid content).

Keywords: *Enzymatic browning; minimally processed lettuce; peroxidase; phenylalanine ammonia-lyase; polyphenol oxidase*

INTRODUCTION

Minimally processed lettuce has become popular because of the increased consumption of fast food and prepared salads. However, the shredding or cutting of lettuce provokes wound-induced physiological and biochemical reactions that shorten storage life of the product (Saltveit, 1997). The lettuce tissue with the highest susceptibility to enzymatic browning is the "white" tissue: the so-called midrib. This browning is a major problem which arises during minimal processing and further storage of lettuce midribs (Loaiza-Velarde et al., 1997; Tomás-Barberán et al., 1997a).

The process that is proposed to lead to the browning of minimally processed lettuce midribs has been previously pointed out. Phenylalanine ammonia-lyase (EC 4.3.1.5; PAL) is the entrypoint enzyme into phenylpropanoid metabolism. PAL is a wound-induced enzyme that increases phenolic compounds concentration after cell injury such as cutting or shredding of lettuce (Hyodo et al., 1978; Ke and Saltveit, 1989a). The accumulation of phenolic compounds could increase the browning susceptibility because they are natural substrates of oxidative enzymes such as polyphenol oxidase (EC 1.14.18.1; PPO) and peroxidase (EC 1.11.1.7; POD). Both enzymes are present in lettuce (Fujita et al., 1991; Heimdal et al., 1994; Gómez-Tena et al., 1994). PPO is the key enzyme involved in the melanogenesis pathway (Prota, 1988). Peroxidase is mainly involved in lignification processes but it can also form melanins as previously described (Richard-Forget and Gauillard, 1997). The activity of both PPO and POD activities can lead to a significantly diminished quality of foodstuffs by the appearance of melanins which change both sensory properties (color, aroma, texture, etc.) and nutritional quality of products (Whitaker, 1995). Therefore, the prevention of enzymatic browning has always been a challenge to food scientists (Dawley and Flurkey, 1993; Loaiza-Velarde et al., 1997; Espín et al., 1999a).

The browning susceptibility, as well as the biochemical and physiological attributes related to quality attributes and storage life of minimally processed lettuce, have been previously approached (Couture et al., 1993; Heimdal et al., 1995; Castañer et al., 1999). However, there is disagreement regarding the possible contribution of the enzymes PAL, PPO, and POD in the browning of minimally processed lettuce midribs upon storage. Previous reports stressed a correlation between the increase of PPO activity and the browning process (Couture et al., 1993; Castañer et al., 1999), whereas other works reported the lack of this correlation (Heimdal et al., 1995; Ke and Saltveit, 1989b). Ke and Saltveit reported the increase of POD activity because of wounding in leaf tissue and the correlation between the increase of POD activity and russet spotting (RS) development in one of the six lettuce cultivars analyzed (Ke and Saltveit, 1989a and 1989b, respectively). However, Hyodo et al. (1978) reported no correlation between RS and POD. PAL activity has been proposed as a possible index for determining browning potential of minimally processed lettuce cultivars (Hyodo et al., 1978; Ke and Saltveit, 1989b; Couture et al., 1993) although Castañer et al. (1999) did not find any correlation between browning and PAL activity in both "Baby" (Little Gem) and Romaine lettuces.

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Taking into account that there is a lack of basic studies which explain the possible contribution of the enzymes PAL, PPO, and POD in the browning of minimally processed lettuce, the aim of the present study is to characterize the evolution of PPO, POD, and PAL activities of six minimally processed lettuce cultivars upon storage (Iceberg Mikonos (IM), I. Green Queen (IGQ), I. Asdrúbal (IA), Little Gem Sandra (LGS), Romaine Cazorla (RC), and R. Modelo (RM)). Other biochemical and physiological attributes will be also studied (total and individual phenolic compounds accumulation, ascorbic acid depletion, and color development).

MATERIALS AND METHODS

Reagents. Ascorbic acid (AA), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), benzamidine chloride, catalase (H_2O_2:H_2O_2 oxidoreductase E. C. 1.11.1.6), 3,4-dihydroxyphenyl propionic acid (DHPPA), *N*,*N*′-dimethylformamide (DMF), L-phenylalanine (Phe), phenylmethylsulfonyl fluoride (PMSF), hydrogen peroxide (H_2O_2) (30%, v/v), 3-methyl-2-benzothiazolinone hydrazone (MBTH), sodium dodecyl sulfate (SDS), Triton X-114 (TX-114), and tropolone were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade and supplied by Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Plant Material and Midribs Preparation. Lettuce (*Lactuca sativa* L.; 15 heads of each cultivar: IM, IA, IGQ, LGS, RC, and RM) was kindly supplied by the commercial corporation Cota 120 (Torre Pacheco, Spain). Lettuces were grown under the same conditions in the same orchard and randomly harvested at commercial maturity stage. Lettuces were stored at 5 °C for 2 days until they were processed in order to mimic industrial processing at the factory.

Cap leaves were removed until uninjured leaves with white midrib tissue were observed. Midribs from the lower half of the leaf were carefully excised into 2-cm × 2-cm pieces and placed on ice until the entire sample was processed. Midribs were dipped in a 100-ppm chlorine bath for 1 min at 4 °C and then hand centrifuged to remove surface moisture. Cut midribs were immediately placed in polypropylene perforated bags (33 perforations/dcm^2, diameter 2 mm) and stored at 5 °C for 7 days.

Enzymes Extraction. PPO and POD were extracted using the method of Espín et al. (1995a) with some modifications. Fresh lettuce midribs (30 g) were homogenized in 20 mL of cold 0.1 M sodium phosphate buffer (PB) (pH 7) containing 2% TX-114, 1 mM PMSF, 5 mM benzamidine, and 50 mM AA. The homogenate was incubated at 35 °C for 15 min, filtrated through three gauze layers, and then centrifuged at 12 000*g* for 20 min at 25 °C. The clear (pigment-free) supernatant was used as enzymatic extract with both PPO and POD activities.

PAL was extracted from 4 g of frozen midribs in 16 mL of borate buffer (pH 8.5) according to the method of Ke and Saltveit (1986).

The extraction protocols (for the extraction of PPO, POD, and PAL activities) were repeated three times and then the supernatants were mixed. The final supernatants were used as enzyme sources to perform kinetic assays.

Kinetic Assays. PPO activity was determined according to the method of Espín et al. (1995b, 1997). This assay method is based on the coupling reaction between enzymatic generated *o*-quinones and the nucleophile MBTH. The formed adduct is stable and has a high molar absorptivity (ϵ). Adduct accumulation was followed at 467 nm (ϵ = 20 000 M^{-1} cm^{-1}). DMF (2%, v/v) was added to improve adduct solubility with no effect on PPO activity as previously described (Espín et al., 1995a,b; 1997) Unless otherwise stated, the standard reaction mixture for determining PPO activity contained 50 mM PB pH 6.5, 2% DMF, 5 mM MBTH, 1 mM DHPPA, and 1 μg protein of enzymatic extract (25 μL of extract). When PPO was assayed, 10 μg/mL bovine catalase was routinely added to the medium to remove possible traces of hydrogen peroxide so that PPO activity was accurately measured without the possible interference of POD activity. To discriminate between latent and active PPO, 0.1% SDS was added in the reaction medium. In the presence of SDS, total PPO activity was measured (Moore and Flurkey, 1990; Espín and Wichers, 1999b). One unit of PPO was defined as the amount of the enzyme that produces 1 μmol of MBTH−DHPPA−*o*-quinone adduct per minute (Espín et al., 1995b). The final assay volume was 1 mL.

POD was determined according to the method of Rodríguez-López et al. (2000) with some modifications by measuring the accumulation of the ABTS radical cation (ABTS$^{\bullet+}$) at 414 nm (ϵ = 31 300 M^{-1} cm^{-1}). Unless otherwise stated, the standard reaction mixture for determining POD activity contained 50 mM sodium acetate buffer (AB) (pH 4.5), 2 mM ABTS, 2 mM H_2O_2, 0.2 mM tropolone, and 1 μg/mL protein of enzymatic extract. No POD activity could be measured in the absence of H_2O_2 in the assay mixture. Tropolone, a specific PPO inhibitor (Kahn, 1985), was included in the medium to determine specifically POD activity. One unit of POD was defined as the amount of the enzyme that produces 1 μmol of ABTS$^{\bullet+}$ per minute. The final assay volume was 1 mL.

PAL activity was determined by following the accumulation of cinnamic acid at 290 nm, using Phe as PAL substrate, according to the method of Ke and Saltveit (1986). One unit of PAL was defined as the amount of the enzyme that produces 1 μmol of cinnamic acid per minute. The final assay volume was 3 mL.

The spectrophotometric assays for determining PPO, POD, and PAL activities were recorded in an UV-1603 Shimadzu spectrophotometer (Tokyo, Japan). Temperature was controlled either at 25 °C (for both PPO and POD activities) or at 40 °C (for PAL activity) with a CPS 240 Shimadzu temperature controller, checked using a precision of ± 0.1 °C. Figures show the mean of three separate kinetic assays.

Kinetic Data Analysis. PPO, POD, and PAL activities were determined by linear regression fitting of the spectrophotometric recordings. Curves for the activation process of PPO were determined by nonlinear regression fitting of experimental data to an uniexponential equation $PPO = (PPO)_a\,(1 - e^{-\lambda t}) + A_0$, where PPO is the active PPO at any time, PPO_a the PPO which is activated in the activation process, A_0 the initial active PPO, and λ is the first-order apparent constant which describes the velocity of the activation process upon storage. Therefore, $PPO_a + A_0$ = active PPO at any storage time; and 100% − ($PPO_a + A_0$) = % latent PPO remaining in the tissue at any storage time. Initial latent PPO is 100% − A_0. The parameter $t_{1/2}$ was also calculated. This parameter ($t_{1/2}$) gives the elapsed time to achieve 50% of activation and can be obtained by using the expression $t_{1/2} = \ln 2/\lambda$. The parameter t_{100} gives the elapsed time to achieve 100% activation and was obtained from the data arisen after the above nonlinear regression fitting. The fitting was carried out by using a Gauss−Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994).

Isoelectric Focusing (IEF) Experiments. Isoelectric points (IEP) of both PPO and POD were determined in the electrophoresis unit Phast System (Pharmacia, Sweden). Isoelectric focusing gels (PhastGel from Pharmacia) with IEP ranges of 4−6.5 (for PPO experiments) and 3−9 (for POD experiments) were used. Conditions for running IEF experiments were those suggested by the manufacturer. Low and broad IEP kit makers from Pharmacia were used in PPO and POD isoelectric focusing experiments, respectively. After focusing, the gels were rinsed in either 50 mM PB pH 6.5 (for PPO) or 50 mM AB pH 4.5 (for POD) then transferred to a Petri dish for activity staining. Solutions to develop PPO activity contained 10 mM DHPPA, 5 mM MBTH, 50 mM PB pH 6.5, and 10 μg/mL catalase. When "active" PPO isoforms appeared, 0.1% SDS was added to develop "latent" PPO isoforms. To develop POD activity, the solution contained 10 mM catechol, 5 mM MBTH, 50 mM AB pH 4.5, 1 mM tropolone, and 2 mM H_2O_2. ABTS was not chosen as POD

Table 1. Velocity and Intensity of Browning in Minimally Processed Lettuces upon Storage[a]

cultivar	a* values	velocity of browning (Δa* value/day)	r^2
I. Mikonos	4.53 ± 0.1	1.3 ± 0.01	0.98
I. Green Queen	3.46 ± 0.2	1.2 ± 0.04	0.97
L. G. Sandra	2.16 ± 0.2	1.3 ± 0.04	0.99
I. Asdrúbal	1.75 ± 0.3	0.9 ± 0.09	0.94
R. Modelo	1.19 ± 0.2	0.7 ± 0.04	0.97
R. Cazorla	−1.58 ± 0.2	0.4 ± 0.04	0.95

[a] Conditions are detailed in Materials and Methods. Entries under a* values represent the browning intensity. Entries in the r^2 column are the regression coefficients for the linear regression fitting of experimental a* values upon storage.

substrate because the reaction product ($ABTS^{\bullet+}$) is very soluble. For this reason, catechol was chosen as POD substrate. For developing both PPO and POD activities, DMF was not added in order to facilitate precipitation of MBTH-adducts in the gel (red-pink color). In both cases, washing the gel in water and drying it stopped the staining. Isoelectric experiments were repeated four times.

Color Measurement. The protocol for color measurement was adapted from Ke and Saltveit (1989a) with some modifications. Midrib aliquots (5 g) were homogenized in 5 mL of MeOH/NaF (4:1, v:v) in a blender for 30 s at 4 °C. The homogenates (three mixed different extracts) were filtered through four layers of cheesecloth. The residue was placed into 1.8 cm diameter plastic wells, pressed slightly with a glass rod, and the color was measured. CIELAB color parameters (L*, a*, and b* values) were determined (three replicates) with a Minolta Spectrophotometer CM-508i through the bottom of the plastic well. The supernatant was then recovered and filtered using a Millipore filter (pore diameter 0.45 μm) prior to HPLC analysis. Velocity of browning was estimated by linear regression fitting of experimental a* values.

HPLC Analysis of Phenolic Compounds. A sample of 20 μL of the supernatant obtained in the protocol for color measurement was analyzed using a Merck-Hitachi HPLC system with a pump model L-7100 and a diode array detector Merck-Hitachi 7455. Separations were achieved on a Licrochart column (Merck) (RP-18, 12 × 0.4 cm; 5-μm particle size). The mobile phase was water-formic acid (A) (19:1, v:v) and MeOH (B) in a linear gradient from 5 to 40% B in A in 25 min. The flow rate was 1 mL/min and detection was recorded at 320 nm. HPLC experiments were repeated three times.

Phenolic Identification and Quantification. Phenolic compounds were identified as previously described (Ferreres et al., 1997). Individual phenolic acids were quantified by comparison with an external standard of CGA from Sigma.

Ascorbic Acid Determination. Ascorbic acid (AA) and dehydroascorbic acid (DHA) were determined (two replicates) by HPLC (with the equipment above-described) according to the procedure of Zapata and Dufour (1992).

Protein Determination. Protein content was determined by using the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Browning of Lettuce Midribs. Generally speaking, the quality loss of minimally processed lettuce is due to the enzymatic "browning" process. However, the most significant color present after 7 days of storage was the red color. Therefore, enzymatic browning of midribs was estimated by using a* Hue angle values which measure the change from green to red color. These values positively correlated the appearance of red color in minimally processed lettuces (Castañer et al., 1996). The higher the a* value is, the deeper the red color is in the tissue. The classification of browning order in the six cultivars was according to the highest a* value reached after 7 days of storage (Table 1): IM > IGQ > LGS > IA > RM > RC. This classification was in agreement with their visual aspects. Values of a* increased linearly upon storage (Figure 1). This allowed us to calculate the "velocity of browning" which was estimated by linear regression fitting of experimental a* values upon storage and the order was (Table 1) IM = LGS > IGQ > IA > RM > RC. To explain the limiting or critical factor for the different browning susceptibilities among the six lettuce cultivars assayed, the evolution of PPO, POD,

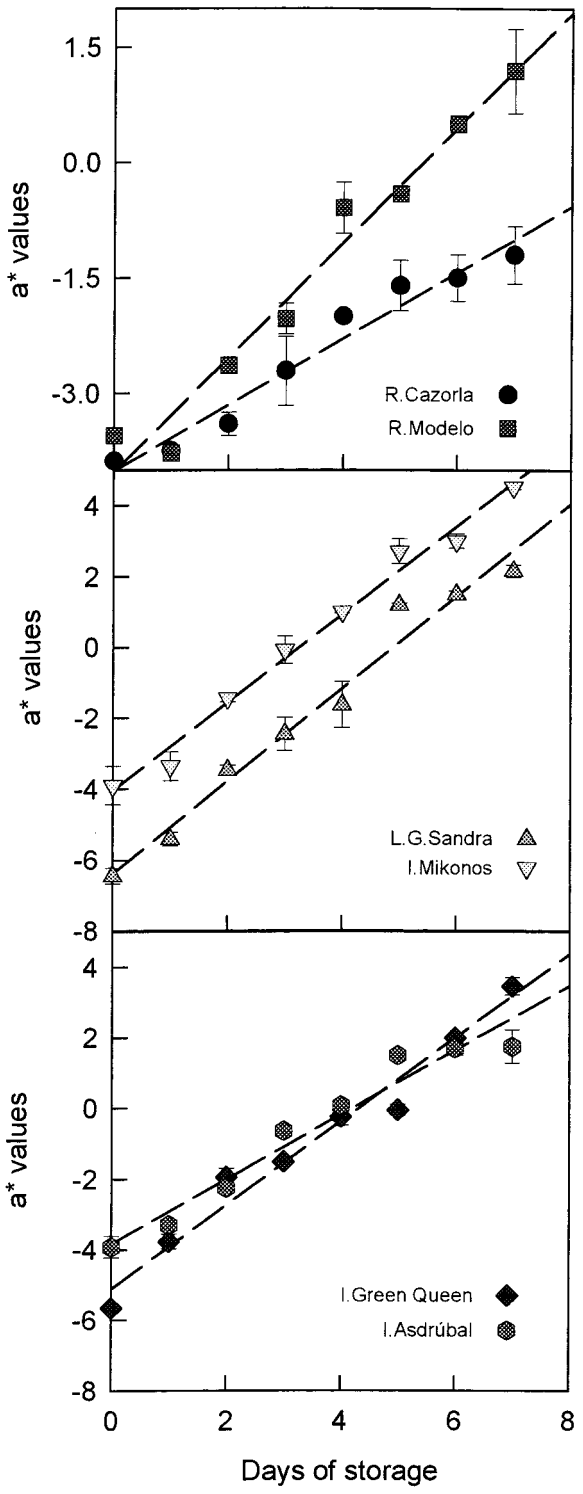

Figure 1. Velocity and intensity of browning of minimally processed lettuce midribs upon storage. Assay conditions are detailed in Materials and Methods.

and PAL activities as well as other attributes were studied.

Polyphenol Oxidase (PPO). Both extraction protocol and enzymatic assay conditions were performed daily to determine PPO and POD activities. We observed that PPO was rendered inactive after frozen storage of the lettuce midribs. Therefore, lettuce midribs could not be frozen for further analysis. This meant that a rapid and accurate protocol would have to be followed in order to determine PPO activity from fresh midribs. The protocol used (see Materials and Methods) allowed us to obtain an enzymatic extract with both PPO and POD activities in one easy step.

PPO can be found in the cell either in soluble form or membrane-bound or in both states at the same time (Mayer and Harel, 1979). The proportion of soluble or membrane-bound PPO in lettuce midribs was determined by using the detergent TX-114 in the protocol extraction. In the absence of TX-114, only soluble PPO was obtained. In the presence of TX-114, the entire PPO (soluble plus membrane-bound) was extracted (Espín et al., 1995a). The difference between both protocols yielded the membrane-bound enzyme. PPO was ca. 50% soluble and 50% membrane-bound with slight variations among cultivars. After 7 days of storage of the wounded lettuce midribs (because of minimal processing) at 5 °C, there was only a slight increase in the release of PPO from membranes to render soluble PPO (Table 2). Therefore, the degree of browning susceptibility among cultivars was not due to "solubilization" of PPO in wounded tissue upon storage.

PPO can be found in either a latent form or an active form. It is also possible that both forms occur at the same time (Whitaker, 1995). Sometimes, it can be difficult to determine the proportion of initial active/latent PPO in some sources. This can be due to the use of drastic extraction protocols which can activate latent PPO by a number of processes such as acid shocking, endogenous proteases which operate during the extraction procedure, etc. (Van Gelder et al., 1997). To minimize this "artifact", a simple, fast, gentle, one-step extraction protocol was approached which allowed us to obtain a pigment-free supernatant with the entire PPO and POD activities. To avoid PPO activation by endogenous proteases during the extraction procedure, PMSF and benzamidine (protease inhibitors) were used (Salvesen et al., 1989).

Initial active PPO (day 0, immediately after cutting of midribs) ranged from 28% (RC) to 42.5% (IM) of total PPO activity (Table 2). Wounding of tissue midribs caused an exponential increase in PPO activity due to the activation process from latent to fully active PPO by following first-order kinetics in the range of time from 3.7 days (LGS) to 6.3 days (RC). To our knowledge, this is the first report which shows this PPO activation pattern upon storage. However, total PPO activity (active plus latent) remained constant (Figure 2, open symbols in inset graphs). The fact that total PPO remained constant meant that, apparently, there was no de novo PPO synthesis upon storage due to initial wounding. A de novo synthesis would have implied an increase of total PPO activity, which did not occur (Figure 2, inset graphs, open symbols). Tissue wounding involves the decompartmentalization of cellular components with the subsequent release of proteases which could involve a cascade of events such as the activation of latent PPO (Espín et al., 1999). PPO is up-regulated

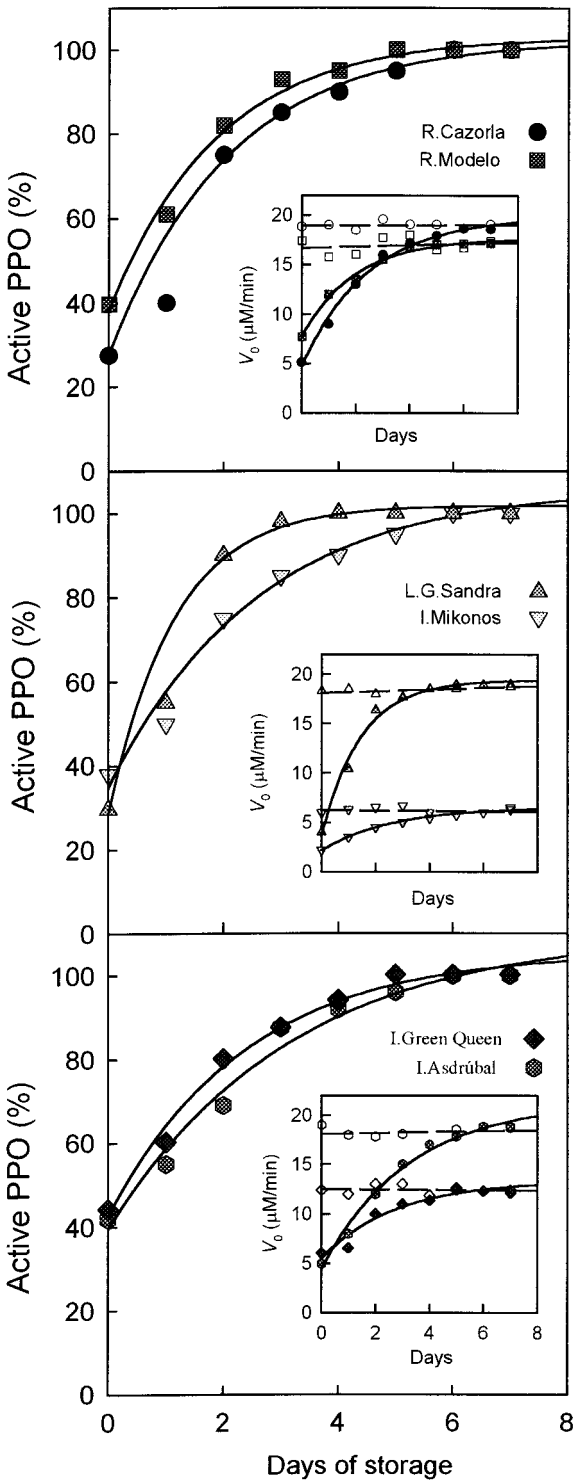

Figure 2. Activation process of latent PPO of wounded midrib lettuce upon storage. Solid lines are nonlinear regression fittings of experimental data to the uniexponential equation detailed in the Materials and Methods section. (Inset graphs). Difference between initial and total PPO activity upon storage. Open symbols designate total PPO activity (assayed in the presence of SDS). Dashed lines (insets) are linear regression fittings of total PPO activity experimental data. Assay conditions are those described in Materials and Methods.

in wounded tissue in apple (Boss et al., 1995) and systemic wound induction of PPO has been found in potato (Thipyanpong et al., 1995). In both cases, PPO mRNA accumulation was detected. Maybe in lettuce this induction process could also take place. However, no

Table 2. Effect of Wounding on Both Solubilization and Activation of PPO in Lettuce Midribs upon Storage[a]

cultivar	λ (day^{-1})	$t_{1/2}$ (days)	t_{100} (days)	[b]A_0 (%)	A_f (%)	S_0 (%)	MB_0 (%)	S_f (%)	MB_f (%)
I. Mikonos	0.42 ± 0.02	1.65 ± 0.04	5.4	42.5	100	30	70	50	50
I. Green Queen	0.38 ± 0.01	1.82 ± 0.01	5.8	35.0	100	50	50	60	40
L. G. Sandra	0.88 ± 0.03	0.79 ± 0.09	3.7	38.3	100	60	40	60	40
I. Asdrúbal	0.22 ± 0.01	3.15 ± 0.01	5.8	41.1	100	40	60	50	50
R. Modelo	0.53 ± 0.02	1.31 ± 0.04	5.2	28.4	100	50	50	60	40
R. Cazorla	0.48 ± 0.02	1.44 ± 0.04	6.3	27.7	100	60	40	70	30

[a] Conditions are detailed in the Materials and Methods section. [b] A_0, initial active PPO; A_f, final active PPO; S_0, initial soluble; MB_0, initial membrane-bound; S_f, final soluble; MB_f, final membrane-bound.

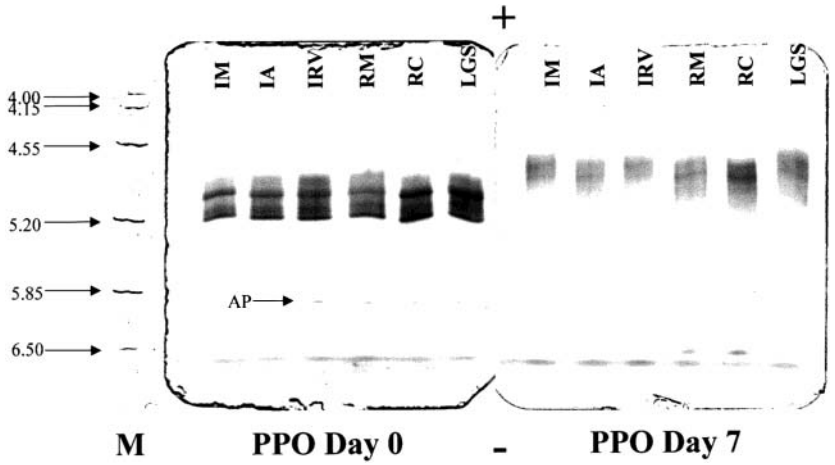

Figure 3. Isoelectric pattern of PPO isoforms from minimally processed lettuce midribs at days 0 and 7 (M, markers; AP, application point). Bands with IEP 6.5 were visible in all the cultivars in the original gels. Bands with IEP 5.36 were not visible after scanning of the gel. Assay conditions are specified in Materials and Methods.

Table 3. Effect of Wounding on Both Solubilization and Induction of POD in Lettuce Midribs upon Storage[a]

cultivar	[b]ΔI (μM/min/day)	ΔV (μM/min)	r^2	S_0 (%)	IB_0 (%)	S_f (%)	IB_f (%)
I. Mikonos	0.57 ± 0.04	4.1 ± 0.2	0.96	100	0	100	0
I. Green Queen	0.34 ± 0.01	2.4 ± 0.1	0.97	55	45	100	0
L. G. Sandra	0.34 ± 0.01	2.4 ± 0.1	0.99	67	33	100	0
I.Asdrúbal	0.41 ± 0.01	2.9 ± 0.1	0.96	50	50	100	0
R. Modelo	0.44 ± 0.04	3.1 ± 0.2	0.98	50	50	100	0
R. Cazorla	0.41 ± 0.04	2.9 ± 0.2	0.97	45	55	50	50

[a] Conditions are detailed in the Materials and Methods Section. [b] ΔI, velocity of induction; ΔV, variation of velocity after 7 days; S_0, initial soluble; IB_0, initial ionically bound; S_f, final soluble; IB_f, final ionically bound. Values in the r^2 column are the regression coefficients for linear regression fittings in Figure 4.

further translation of this possible induced mRNA occurred. This can be explained because, in our assay system, total lettuce PPO remained constant. Therefore, in wounded lettuce midribs, if PPO mRNA is induced, it is not further translated into protein.

A full PPO activation took place in the six cultivars (Figure 2), and it occurred (t_{100}) from 3.7 days for LGS to 6.3 days for RC (Table 2). The most susceptible cultivar to browning (IM) presented the lower amount of PPO and the least susceptible to browning (RC) was one of the cultivars with higher PPO activity (Figure 2).

Figure 3 shows the isoelectric pattern of PPO at days 0 and 7. It is worthwhile to note the presence, at day 0, of two remarkable isoforms: a latent one with IEP of ca. 5 and another active isoform with IEP of 5.2. Four more faint active bands were observed initially with IEPs of 4.95, 5.14, 5.17, and 5.36. After 7 days of storage of wounded (processed) lettuce midrib, no latent bands were observed but only active bands with IEPs of 4.69, 4.8, 6.5, and one more band with IEP higher than 6.5. To our knowledge there is only a single previous report regarding IEP of lettuce PPO. This study was carried out by Heimdal et al. (1994) who reported an IEP of 3.9 for iceberg lettuce (cv. Pennlake).

Therefore, browning susceptibility was not positively correlated with any of the biochemical features investigated regarding PPO (degree and velocity of activation, initial active−latent PPO content, isoform pattern, etc.; Table 2; Figures 2 and 3). These results agree with Heimdal et al. (1995) and Ke and Saltveit (1989b) who did not find a positive correlation between lettuce browning and PPO activity.

Peroxidase (POD). Traditionally, the involvement of POD in enzymatic browning has been questioned mainly because of the low hydrogen peroxide content in vegetable tissues and the relatively high catalytic power of PPO for its natural substrates. However, Richard-Forget and Gauillard (1997) reported the possible implication of peroxidase in enzymatic browning through a synergistic effect PPO−POD. These authors demonstrated the generation of H_2O_2 in PPO-catalyzed reactions and also the use by POD of semiquinonic intermediates of PPO-catalyzed reactions as oxidizing substrates (replacing the hydrogen peroxide). Moreover, Bestwick et al. (1997) described a high accumulation of H_2O_2 after pathogen attack in lettuces. Therefore, the findings of all of these previous studies together prompted us to study the possible correlation between lettuce browning and peroxidase activity.

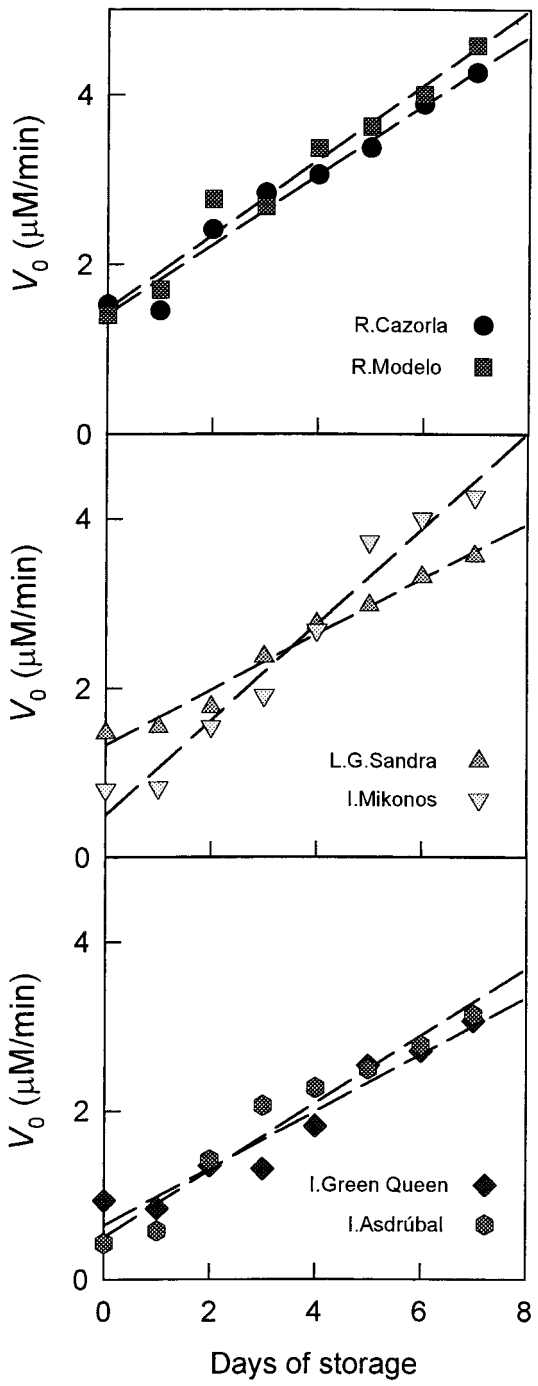

Figure 4. POD induction in wounded lettuce midribs upon storage. Conditions are detailed in the Materials and Methods section.

Gómez-Tena et al. (1994) reported that a basic peroxidase isoenzyme of crisphead lettuce was both located in the soluble fraction and attached to tonoplast membranes. Extraction protocol in the presence and absence of TX-114 yielded peroxidase that was present at day 0, immediately after wounding, with approximately 50% in soluble form in all the cultivars, except for that in IM which was 100% soluble (Table 3). At day 7, 100% of peroxidase was found to be soluble in all the cultivars, except that in RC which remained approximately unchanged (Table 3). However, the solubilization of per-

Table 4. Phenolic Compounds Accumulation in Minimally Processed Lettuce upon Storage[a]

	iceberg Mikonos		iceberg Asdrúbal		iceberg Green Queen		little gem Sandra		romaine Cazorla		romaine Modelo	
storage(days)	0	7	0	7	0	7	0	7	0	7	0	7
total phenolics	6.44 ± 1.4	24.02 ± 5.2	8.04 ± 0.9	4.30 ± 1.4	6.47 ± 0.4	15.65 ± 3.4	12.94 ± 1.2	21.44 ± 4.8	6.53 ± 0.4	12.39 ± 3.0	2.83 ± 0.5	8.71 ± 1.9
CGA[b]	1.12 ± 0.3	9.68 ± 2.2	1.35 ± 0.3	2.62 ± 0.5	0.90 ± 0.2	9.24 ± 1.7	1.01 ± 0.2	11.98 ± 2.4	1.08 ± 0.0	6.14 ± 0.7	0.36 ± 0.0	3.56 ± 0.3
ICGA	0.00 ± 0.0	6.85 ± 1.9	0.00 ± 0.0	0.41 ± 0.2	0.00 ± 0.0	1.88 ± 0.7	0.00 ± 0.0	1.20 ± 0.5	0.00 ± 0.0	0.78 ± 0.0	0.00 ± 0.0	1.48 ± 0.3
CAFTA	0.47 ± 0.1	0.85 ± 0.1	0.25 ± 0.0	0.29 ± 0.1	0.00 ± 0.0	0.74 ± 0.1	0.26 ± 0.0	1.58 ± 0.3	0.69 ± 0.0	0.6 ± 0.0	0.00 ± 0.0	0.62 ± 0.2
DCAFTA	1.49 ± 0.9	5.88 ± 0.9	1.67 ± 0.1	0.72 ± 0.5	0.50 ± 0.2	3.08 ± 0.6	1.39 ± 0.3	5.12 ± 1.5	0.34 ± 0.0	2.14 ± 0.5	0.00 ± 0.0	2.38 ± 0.7

[a] Conditions are detailed in the Materials and Methods section. Data at days 0 and 7 are shown. [b] CGA, chlorogenic acid; ICGA, isochlorogenic acid; CAFTA, caffeoyl tartaric acid; DCAFTA, dicaffeoyl tartaric acid.

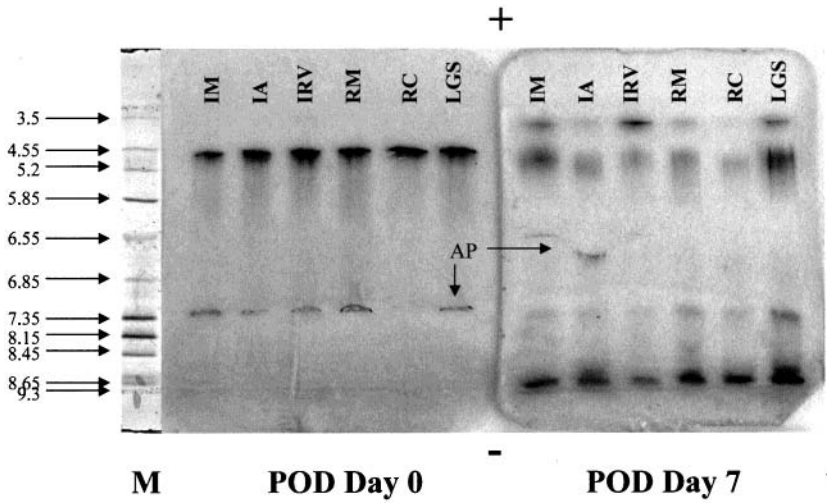

Figure 5. Isoelectric pattern of POD isoenzymes from minimally processed lettuce midribs at days 0 and 7 (M, markers; AP, application point). Bands with IEPs 3.5 and 8.7 were visible in all the cultivars in the original gels. Assay conditions are specified in Materials and Methods.

oxidase was not correlated with the degree of browning. Peroxidase activity linearly increased (Figure 4) upon storage after wounding, probably because of a de novo peroxidase isoenzymes synthesis (Figure 5). The velocity of induction of POD activity ranged from 0.34 μM/min/day for both LGS and IGQ to 0.57 μM/min/day for IM. The net increase of POD activity from days 0 to 7 ranged from 2.4 μM/min for LGS and IGQ to 4.1 μM/min for IM (Table 3). A single isoenzyme with IEP of 4.68 was observed at day 0 (Figure 5). After 7 days of storage of wounded lettuce midrib, more isoenzymes were detected with IEPs of 3.5, 4.68 (already present at day 0), 7.6, 8.7, and 8.85 (the most patent band). This induction of new peroxidase isoenzymes could be related with lignification processes to repair cell walls after tissue wounding.

From the above results concerning POD activity it was not possible to establish a positive correlation between midrib browning and POD activity. These results agree with Hyodo et al. (1978) who reported no correlation between russet spotting (RS) and POD activity. However, our results do not agree with Ke and Saltveit (1989a,b) who reported positive correlation between RS and POD in entire leaf tissue, not only in midribs.

Phenylalanine Ammonia-Lyase (PAL). PAL activity was determined from frozen midribs because PAL was not rendered inactive with storage below 0 °C. PAL activity presented a typical bell-shaped induction pattern in four lettuce cultivars (Figure 6) (Bernards and Ellis, 1994; Sarma et al., 1997; Castañer et al., 1999). Only IM and IGQ showed a second induction response which has not been previously described in the literature. To find a possible explanation for this unusual response for IM and IGQ cultivars, inhibition experiments with chlorogenic acid (CGA, the main phenolic accumulated) were carried out. The inhibition of PAL by product accumulation (CGA) has been previously reported (Sarma et al., 1997). However, no differences were found in the inhibition of PAL by CGA among the different cultivars which could justify the above second-induction response (results not shown). A possible explanation could be the different de novo synthesis of a previously described inactivating factor of PAL (Ritenour and Saltveit, 1996). Anyway, browning susceptibility was not correlated with either the induction pattern or with the amount of induced PAL (Figure 6). These results agree with Castañer et al. (1999) who did not find correlation between PAL activity and browning of "Baby" (Little Gem) and Romaine lettuces. However, our study does not agree with Hyodo et al. (1978), Ke and Saltveit (1989b), and Couture et al. (1993) who did find this correlation and even suggested PAL activity as a possible index for estimating lettuce browning.

Phenolics and Ascorbic Acid Content. To probe deeper the rate-limiting factor and/or step in the enzymatic browning of minimally processed lettuce midribs, individual and total phenolics and ascorbic acid contents were determined.

Caffeoyl tartaric (CAFTA), chlorogenic (5′-caffeoylquinic; CGA), dicaffeoyl tartaric (chicoric; DCAFTA), and isochlorogenic (3,5-dicaffeoyl quinic; ICGA) acids were identified (Table 4). CGA was the main phenolic compound that accumulated after wounding of lettuce midrib in the six cultivars (Table 4). The accumulation of phenolics was concomitant to the induction of PAL activity, which was in accordance with previous studies (Tomás-Barberán et al., 1997a,b). There was no positive correlation that could explain the different browning susceptibility on the basis of the amount and type of phenolics wound-induced (Table 4).

Heimdal et al. (1995) reported that dehydroascorbic (DHA) acid levels in lettuce positively correlated with browning. The content of AA in lettuce is rather low (Barry-Ryan and O'Beirne, 1999). The midribs of the six lettuce cultivars studied here lacked ascorbic acid (AA) at day 0 and only DHA could be detected. DHA content was determined but no positive correlation was found between DHA and browning (results not shown).

This study tried to standardize as much as possible the samples and assay conditions. To this purpose, lettuce harvesting at the orchard, midribs processing, extraction protocols, enzymatic assays, and determinations of other attributes were carefully considered to minimize possible uncontrolled variables. However, it is of note that there was a great variability observed in the bags stored for 7 days. Lettuces of the same cultivar, grown together at the same orchard, in the same maturity stage, etc., presented, in some cases, quite different browning susceptibility. Therefore, this study demonstrates, through complementary studies regarding PPO activation, POD induction, etc., carried out

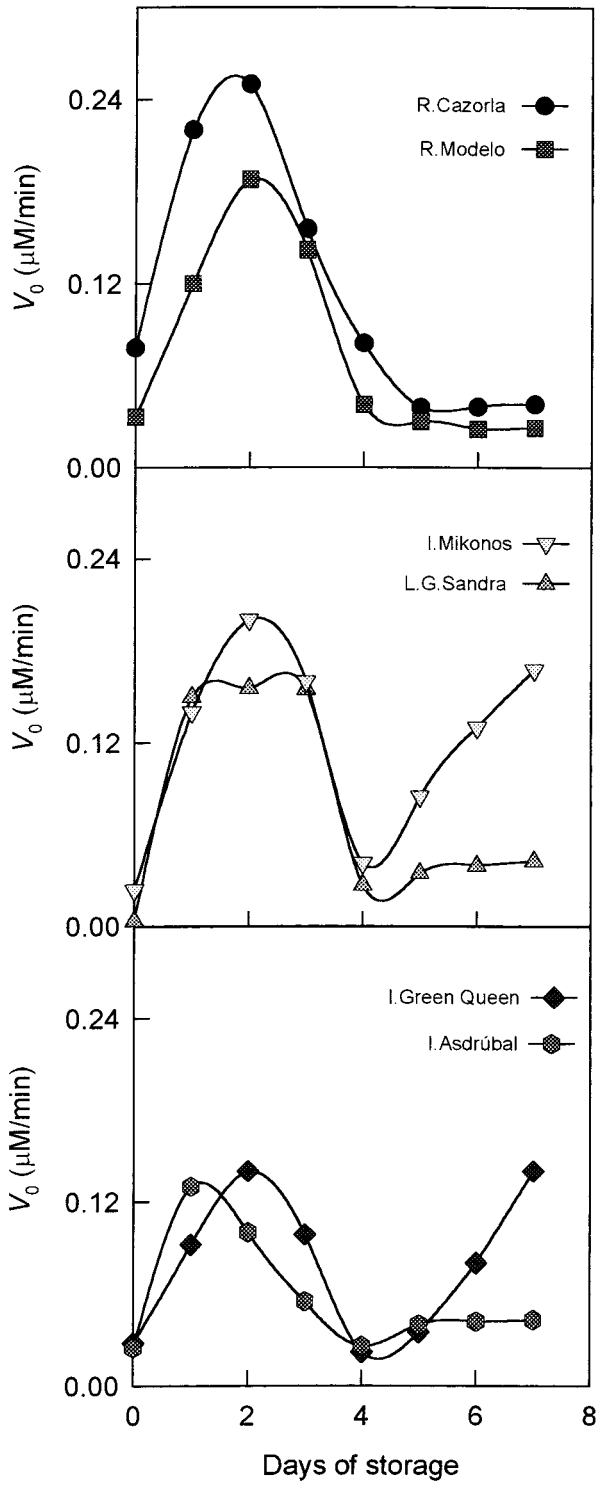

Figure 6. PAL activity in minimally processed lettuce midribs upon storage. Assay conditions are detailed in Materials and Methods.

from a point of view never previously approached, that the limiting factor for browning susceptibility in lettuce remains unknown, at least in our assay conditions and with the six lettuce cultivars randomly chosen. From this study we can conclude that the Romaine lettuce cv. Cazorla could be convenient for preparing fast salads with relatively low browning. However, this cannot be considered as a general rule. Some other factors may be involved which determine the different browning susceptibility in lettuce. Maybe complex and unknown reactions must occur between phenolic substrates, enzymes such as PPO, POD, PAL, and others, chemical polymerization, antioxidants, etc., which can render possible synergistic effects to provoke enzymatic browning in a nonsimple relationship. Our results suggest that enzymatic browning of minimally processed lettuce can be induced by only a minimal activity of oxidative enzymes such as PPO or POD. Furthermore, a minimal concentration of phenolic compounds induced by PAL could be enough to serve as substrates for oxidative reactions. The heterogeneity of results previously published regarding dependencies of lettuce browning with different attributes or factors (PPO, POD, PAL, AA, phenolics, etc.) could be due to the different lettuce cultivars studied, different lettuce tissues (photosynthetic, white midribs, etc.) within each lettuce, soil composition of the orchard, exact maturity stage, watering status, etc., and many other variables with unexpected interactions and effects on lettuce browning. The investigation on the effect of agronomic practices (irrigation, fertilizers, herbicides, pesticides, hormones to promote growth, etc.) on the activity of phenolic enzymes might shed some light on the critical step which promotes enzymatic browning of minimally processed lettuce, as well as on the different browning susceptibilities among different cultivars.

Abbreviations Used. AA, ascorbic acid; AB, sodium acetate buffer; ABTS, 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); CAFTA, caffeoyl tartaric acid; CGA, chlorogenic acid; DCAFTA, dicaffeoyl tartaric acid; DHPPA, 3,4-dihydroxyphenil propionic acid; DMF, *N*, *N*′-dimethylformamide; ICGA, isochlorogenic acid; IEF, isoelectric focusing; IEP, isoelectric point; IA, iceberg cv. Asdrúbal; IGQ, iceberg cv. Green Queen; IM, iceberg cv. Mikonos; LGS, little gem cv. Sandra; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MeOH, methanol; PAL, phenylalanine ammonia-lyase; PB, sodium phosphate buffer; PMSF, phenylmethylsulfonyl fluoride; POD, peroxidase; PPO, polyphenol oxidase; RC, romaine cv. Cazorla; RM, romaine cv. Modelo; SDS, sodium dodecyl sulfate; tropolone, 3-hydroxy-2,4,5-cycloheptatrien-1-one; TX-114, Triton X-114.

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LITERATURE CITED

Barry-Ryan, C.; O'Beirne, D. Ascorbic acid retention in shredded iceberg lettuce as affected by minimal processing. *J. Food Sci.* **1999**, *64*, 498−500.

Bernards, M. A.; Ellis, B. E. Phenylalanine ammonia-lyase from tomato cell cultures inoculated with *Verticillium alboatrum*. *Plant Physiol.* **1991**, *97*, 1494−1500.

Bestwick, C. S.; Brown, I. R.; Bennet, M. H. R.; Mansfield, J. W. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola*. *Plant Cell* **1997**, *9*, 209−221.

Boss, P. K.; Gardner, R. C.; Janssen, B. J.; Boss, G. S. An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. *Plant Mol. Biol.* **1995**, *27*, 429−433.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *210*, 727−735.

Castañer, M.; Gil, M. I.; Artés, F.; Tomás-Barberán, F. A. Inhibition of browning of harvested head lettuce. *J. Food Sci.* **1996**, *61*, 314−316.

Castañer, M.; Gil, M. I.; Ruiz, M. V.; Artés, F. Browning susceptibility of minimally processed Baby and Romaine lettuces. *Eur. Food Res. Technol.* **1999**, *209*, 52−56.

Couture, R.; Cantwell, M. I.; Ke, D.; Saltveit, M. E. Physiological attributes related to quality attributes and storage life of minimally processed lettuce. *HortScience* **1993**, *28*, 723−725.

Dawley, R. M.; Flurkey, W. H. 4-Hexylresorcinol, a potent inhibitor of mushroom tyrosinase. *J. Food Sci.* **1993**, *58*, 609−610.

Espín, J. C.; Wichers, H. J. Slow-binding inhibition of mushroom (*Agaricus bisporus*) tyrosinase isoforms by tropolone. *J. Agric. Food Chem.* **1999a**, *47*, 2638−2644.

Espín, J. C.; Wichers, H. J. Activation of a latent mushroom (*Agaricus bisporus*) tyrosinase by sodium dodecyl sulfate (SDS). Kinetic properties of the SDS-activated isoform. *J. Agric. Food Chem.* **1999b**, *47*, 3518−3525.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Verdedoncella apple. *J. Agric. Food Chem.* **1995a**, *43*, 2807−2812.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. A continuous spectrophotometric method for determining the monophenolase and diphenolase activities of apple polyphenol oxidase. *Anal. Biochem.* **1995b**, *231*, 237−246.

Espín, J. C.; Morales, M.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1997**, *45*, 1084−1090.

Espín, J. C.; van Leeuwen, J.; Wichers, H. J. Kinetic Study of the Activation Process of a Latent Mushroom (*Agaricus bisporus*) Tyrosinase by Serine Proteases. *J. Agric. Food Chem.* **1999**, *47*, 3509−3517.

Ferreres, F.; Gil, M. I.; Castañer, M.; Tomás-Barberán, F. A. Phenolic metabolites in red pigmented lettuce (*Lactuca sativa*). Changes with minimal processing and cold storage. *J. Agric. Food Chem.* **1997**, *45*, 4249−4254.

Fujita, S.; Tono, T.; Kawahara, H. Purification and properties of polyphenol oxidase in head lettuce (*Lactuca sativa*). *J. Sci. Food Agric.* **1991**, *55*, 643−651.

Gómez-Tena, M.; Pedreño, M. A.; Ros-Barceló, A.; Ferrer, M. A. Subcellular localization of a basic peroxidase isoenzyme in crisphead lettuce. *J. Am. Soc. Hortic. Sci.* **1994**, *119*, 1276−1278.

Heimdal, H.; Larsen, L. M.; Poll, L. Characterization of polyphenol oxidase from photosynthetic and vascular lettuce tissues. *J. Agric. Food Chem.* **1994**, *42*, 1428−1433.

Heimdal, H.; Kühn, B. F.; Poll, L.; Larsen, L. M. Biochemical changes and sensory quality of shredded and MA-packaged Iceberg lettuce. *J. Food Sci.* **1995**, *60*, 1265−1276.

Hyodo, H.; Kuroda, H.; Yang, S. F. Induction of phenylalanine ammonia-lyase and increase in phenolics in lettuce leaves in relation to the development of russet spotting caused by ethylene. *Plant Physiol.* **1978**, *62*, 31−35.

Jandel Scientific. *Sigma Plot 2.01 for Windows*. Jandel Scientific: Corte Madera, CA, 1994.

Kahn, V. Tropolone. A compound that can aid in differentiating between tyrosinase and peroxidase. *Phytochemistry* **1985**, *24*, 915−920.

Ke, D.; Saltveit, M. E. Effects of calcium and auxin on russet spotting and phenylalanine ammonia-lyase activity in iceberg lettuce. *HortScience* **1986**, *21*, 1169−1171.

Ke, D.; Saltveit, M. E. Wound-induced ethylene production, phenolic metabolism and susceptibility to russet spotting in iceberg lettuce. *Physiol. Plant.* **1989a**, *76*, 412−418.

Ke, D.; Saltveit, M. E. Developmental control of russet spotting, phenolic enzymes, and IAA oxidase in cultivars of iceberg lettuce. *J. Am. Soc. Hortic. Sci.* **1989b**, *114*, 472−477.

Loaiza-Velarde, J. G.; Tomás-Barberán, F. A.; Saltveit, M. E. Effect of intensity and duration of heat-shock treatments on wound-induced phenolic metabolism in iceberg lettuce. *J. Am. Soc. Hortic. Sci.* **1997**, *122*, 873−877.

Marquardt, D. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431−441.

Mayer, A. M.; Harel, E. Polyphenol oxidase in plants. *Phytochemistry* **1979**, *18*, 193-215.

Moore, B. M.; Flurkey, W. H. Sodium dodecyl sulfate activation of a plant polyphenol oxidase. *J. Biol. Chem.* **1990**, *265*, 4982−4988.

Prota, G. Progress in the chemistry of melanin and related metabolites. *Med. Res. Rev.* **1988**, *8*, 525−556.

Richard-Forget, F.; Gauillard, F. A. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations 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.

Ritenour, M. A.; Saltveit, M. E. Identification of a phenylalanine ammonia-lyase inactivating factor in harvested head lettuce (*Lactuca sativa*). *Physiol. Plant.* **1996**, *97*, 327−331.

Rodríguez-López, J. N.; Espín, J. C.; del Amor, F.; Tudela, J.; Martínez, V.; Cerdá, A.; García-Cánovas, F. Purification and kinetic characterization of an anionic peroxidase from melon (*Cucumis melo*, L.) cultivated under different salinity conditions. *J. Agric. Food Chem.* **2000**, *48*, 1537−1541.

Saltveit, M. E. Physical and physiological changes in minimally processed fruits and vegetables. In *Phytochemistry of Fruits and Vegetables*; Tomás-Barberán, F. A., Robin, R. D. Eds. Oxford University Press: Oxford, 1997; pp 205−220.

Salvesen, G.; Nagase, H. Inhibition of proteolytic enzymes. In *Proteolytic Enzymes: A Practical Approach;* Beynon, R. J., Bond, J. S., Eds. IRL Press: Oxford, 1989; pp 83−104.

Sarma, A. D.; Sreelakshmi, Y.; Sharma, R. Differential expression and properties of phenylalanine ammonia-lyase isoforms in tomato leaves. *Phytochemistry* **1997**, *49*, 2233−2243.

Thipyapong, P.; Hunt, M. D.; Steffens, J. C. Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry* **1995**, *40*, 673−676.

Tomás-Barberán, F. A.; Gil, M. I.; Castañer, M.; Artés, F.; Saltveit, M. E. Effect of selected browning inhibitors on phenolic metabolism in stem tissue of harvested lettuce. *J. Agric. Food Chem.* **1997a**, *45*, 583−589.

Tomás-Barberán, F. A.; Loaiza-Velarde, J.; Bonfanti, A.; Saltveit, M. E. Early wound- and ethylene-induced changes in phenylpropanoid metabolism in harvested lettuce. *J. Am. Soc. Hortic. Sci.* **1997b**, *122*, 399−404.

Van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **1997**, *45*, 1309−1323.

Whitaker, J. R. Polyphenol oxidase. In *Food Enzymes Structure and Mechanism*; Wong, D. W. S., Ed.; Chapman Hall: New York, 1995; pp 271−307.

Zapata, S.; Dufour, J. P. Ascorbic, dehydroascorbic and isoascorbic simultaneous determination by reverse phase ion interaction HPLC. *J. Food Sci.* **1992**, *57*, 506−511.

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