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Effect of Heat Shock on Browning-Related Enzymes in Minimally Processed Iceberg Lettuce and Crude Extracts

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The effects of heat shock on PPO and POD activity in minimally processed Iceberg lettuce was examined during storage (10 days). The results were compared with the effect of temperature on crude extracts of these enzymes (*in vitro* analysis). Fresh-cut lettuce washed at 50 °C showed significantly lower PPO and POD activity throughout storage than lettuce washed at 4 °C and 25 °C. These results were consistent with a sensory analysis in which the panellists found the lowest browning scores in those samples treated at 50 °C.

When PPO and POD were analysed *in vitro*, the samples treated at 50 °C showed a rapid loss of POD activity and a similar but slower loss of PPO activity in all tissues, while incubation at 4 °C and 25 °C showed no significant loss of activity. While heat shock did not lead to significant loss of activity it did repress the synthesis of PPO and POD during storage.

Key words: heat shock; Iceberg lettuce; polyphenol oxidase; peroxidase; vascular tissue

The consumption of minimally processed Iceberg lettuce has significantly increased in recent years. Its crispy texture and green colour are the most important attributes associated with its increased popularity relative to other lettuce varieties such as butterhead or romaine.

Lettuce is highly susceptible to enzymatic browning, thereby causing economic loss to the producer. This loss is greater if browning occurs closer to the consumer in the processing scheme, due to storage and handling costs prior to this point. The control of browning from harvest to consumer is therefore critical for minimising loss to the producer.

Both the shelf life and organoleptic properties of minimally processed lettuce are limited by the enzymatic browning reaction. The origin of the brown pigment is complex and not fully understood, but is known to involve the oxidation of polyphenols by peroxidase (POD) and polyphenol oxidase (PPO) enzymes.[1–3] The appearance of a brown colour dramatically decreases the appeal of the lettuce for the consumer.

The increased use of minimally processed lettuce and increased restriction on chemical treatment to prevent browning has created the need to understand the browning reactions and to find simple, natural treatments to control it. Enzymatic browning has been slowed by use of modified-atmosphere packaging.[4] Other authors have used various treatments to inhibit browning such as organic acid washing, antioxidant treatments, natural essential oils and the use of inhibitors of phenolic biosynthesis with varying degrees of success.[5–7]

More recently, several authors have shown that heat shock inhibits the progress of the browning reaction in Iceberg lettuce.[7–11] It is a simple treatment, relatively inexpensive and does not involve the addition of chemicals that might be toxic or influence the nutritional value of the vegetable. Heat shock normally involves washing freshly cut lettuce in water at 45–50 °C for a period of 1–2 minutes.[9,10,12]

Inhibition of the browning reaction caused by a heat shock treatment may be due to direct inactivation of browning-related enzymes or may be due to a general diversion of protein synthesis to the production of heat shock proteins.[8] The inhibition of browning has been correlated with repressing the induction of phenylalanine ammonia-lyase (PAL) activity.[8–11] Salveit and Murata *et al.* have reported a decrease in the total phenolic content after heat shock.[9,11] However, Fukumoto *et al.* have found that even though heat shock decreased the phenolic content, this was not consistently correlated with browning (Fig. 1).[10] The author found that Iceberg lettuce tissues with lower phenolic content showed a higher browning level. Thus, it was not clear that phenolic accumulation was directly related to browning, and Fukumoto suggested that other enzymes, *i.e.* POD, might be important in the browning process.

Many studies have examined vegetables treated by heat shock, but most have been focused on the effect of

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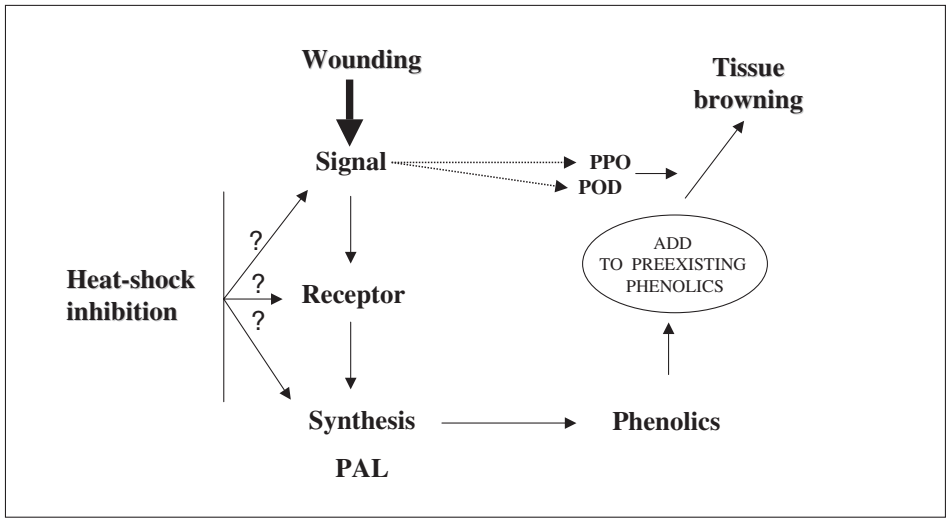

Scheme adapted from Salveit, 2000

Fig. 1. The Process of Enzymatic Browning.
The effect of heat shock on PAL synthesis is indicated.

this treatment on PAL activity. Figure 1 shows the process contributing to browning in Iceberg lettuce. Heat shock might interrupt this process at several points. However, the observation by several authors that the PAL activity was decreased by heat shock[9–11] has lead to the focus on PAL as the main determinant of the extent of browning. Few authors have considered that the possibility that heat shock might affect PPO and POD either by direct heat inactivation or by inhibition of their synthesis during storage. Fukumoto found that heat shock decreased the POD activity, giving lower browning, even though tissue studies had lead him to conclude that higher POD activity was a protection against browning due to its recycling of ascorbate.[10] Moreover, Fukumoto has pointed out that the association between POD activity and browning has been reported to have both a positive correlation and no correlation.[2,13]

Thus, the objective of this study is to analyse the effect of a heat shock treatment of minimally processed lettuce on PPO/POD and browning during storage. This data is compared with the effect of temperature on PPO and POD in crude extract (*in vitro* analysis), in order to understand the effect of heat shock on these enzymes.

Materials and Methods

Raw material. Iceberg lettuce (*Lactuca sativa sp.*) was grown in Ireland, purchased from a local grocery market and stored at 4 °C until further processing (within 24 hours).

Experimental design. Two parallel studies were conducted (Fig. 2). First, the effect of heat shock on browning in minimally processed Iceberg lettuce was analysed. Polyphenol oxidase and the peroxidase activity in minimally processed lettuce was measured for this study and compared with the sensory evaluation of browning. The second study involved an *in vitro* analysis of the effect of incubation temperature on PPO and POD in crude extracts from segregated tissues (photosynthetic and vascular). Finally, a first-order model was created in order to predict the loss of activity for both PPO and POD. All experiments were conducted three times, and each trial was carried out in duplicate.

Minimally processed lettuce.

Minimal processing treatment. The two outer leaves (damaged or wilted) were removed, and the core was excised with a stainless steel knife. The lettuce was cut in half, and each half was cut into four equal pieces. The washing treatment was performed by immersing the fresh-cut lettuce in a distilled water solution at 4 °C, 25 °C or 50 °C. The vegetable was dipped for 1 min with constant agitation. After this, the minimally processed lettuce was dried in a spinner for 5 min.

The processed fresh-cut lettuce was packed in bags (200 × 320 mm) made of 35-μm-thick oriented polypropylene (OPP; Amcor Flexibles, Barnwood, Gloucester, United Kingdom). The bags were chilled at (−20 °C) and stored at 4 °C for 10 days.

Polyphenol oxidase (EC. 1.10.3.1.) and peroxidase (EC. 1.11.1.7.) enzymatic activity. To prepare a homogenate, 10 grams of lettuce was placed in a Polytron homogeniser in a 1:2 (w:v) ratio with 0.5 M sodium phosphate buffer at pH 6.50 in the presence of 50 g/l of polyvinylpyrrolidone (PVPP; Sigma, St. Louis, U.S.A.) and centrifuged at 12,720 × *g* for 30 minutes. Homogenization was carried out at 4 °C and 5500 rpm for a total of 2 minutes, with a break of 3 minutes after each 30 second pulse, in order to avoid heating the sample. The

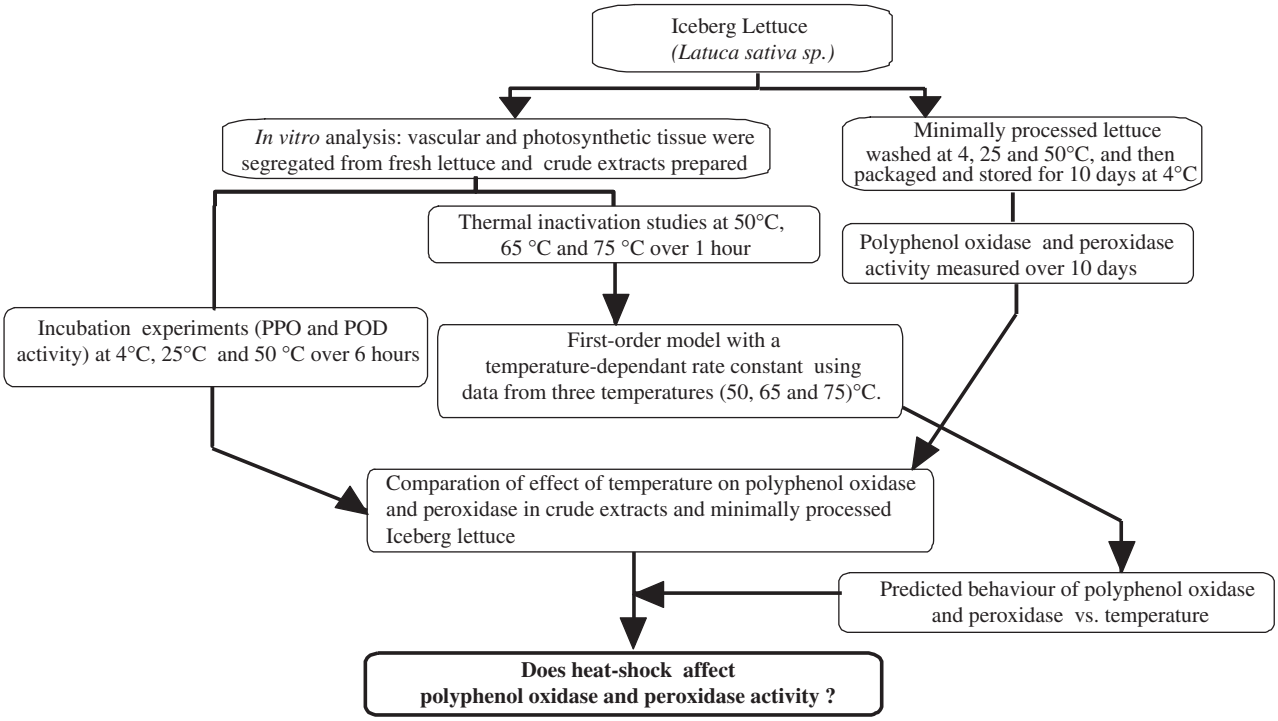

Fig. 2. Experimental Overview: Flow Diagram Summarizing the Experiments Carried Out in This Study.

homogenate was filtered through filter paper (Rundfilter MN 640w, 11 cm; Düren, Germany) and the supernatant collected in graduated 25 ml cylinders. The homogenate was used without further purification. The polyphenol oxidase (PPO) activity was assayed spectrophotometrically with a modification to the methods of Galeazzi *et al.* and Tan and Harris.[14,15] The reaction mixture contained 0.1 ml of the homogenate and 2.9 ml of a substrate solution (0.020 M of catechol as the substrate added to 0.05 M of a sodium phosphate buffer at pH 6.50). The reference cuvette contained only the substrate. The rate of oxidation of catechol was monitored at 400 nm at a temperature of 25 °C for 2 minutes. One unit of enzyme activity is defined as an increment of 0.1 in absorbance per minute at the maximum rate of increase.

The activity of peroxidase (POD) was quantified by using aliquots of 0.2 ml each at pH 6.5 in 2.7 ml of a 0.05 M phosphate buffer containing 100 µl of hydrogen peroxide at (1.5% v/v) as the oxidant and 200 µl of *p*-phenylendiamine as the hydrogen donor. The oxidation of *p*-phenylendiamine was measured with a spectrophotometer at 485 nm and 25 °C. The enzymatic activity was determined by measuring the maximum slope of the reaction line (one unit of enzyme activity is defined as an increment of 0.1 in absorbance per minute). The spectrophotometer used had a thermostat-controlled cell holder that maintained the temperature of the cuvette at 37 °C.

The PPO and POD activity assays were checked at two different pH values (6.50 and 7.00) to select the best pH for the assay. At pH 6.50, the results showed higher sensitivity for POD and PPO activities, so pH 6.50 gave better resolution for the assay. The activity of PPO and POD was measured in three independent trials to check the repeatability of the enzymatic assays. A linear regression equation ($R^2 > 0.95$) was calculated from the data for PPO and POD activity in lettuce homogenates at different concentrations (data not shown).

Sensory analysis. A sensory analysis was carried out on the lettuce samples during 10 days of storage by an untrained panel with an age range of 25–40 years. Browning of the samples was scored on a hedonic scale of 0 to 5. The sensory panel (10 persons) was selected from among the school members, and the evaluation was carried out in the sensory evaluation laboratory. The design of the sensory evaluation and the data analysis was carried out with Compusense® Five software (release 4.4, Ontario, Canada). Three independent trials were carried out.

Crude extracts.

Tissue segregation. The fresh-cut whole lettuce was separated into two parts, the upper half (Top) and the lower part (Bottom). Each part was further separated into two tissues, photosynthetic (Leaf) and vascular (Rib).

Thermal incubation. The homogenates to undergo the thermal treatment were prepared from fresh lettuce tissues. Aliquots of each homogenate (made from different parts of the lettuce (Top/Bottom) and different tissues (Rib and Leaf) were transferred to Eppendorf

plastic tubes (1 ml). The samples were heated in a circulating water bath (Lauda E-300, Könisghofen, Germany) at a temperature of 50 °C, 65 °C or 75 °C. The extracts treated at 4 °C and 25 °C were stored in an environmental test chamber (MLR-350; Sanyo, Japan), the temperature being verified with a calibrated mercury thermometer.

For homogenates incubated at 4 °C, 25 °C and 50 °C, activity measurements were carried out over a six hour time period. Aliquots were removed from incubations at 1 hour time intervals and cooled rapidly in ice before the determination of PPO and POD enzyme activity. Similarly, for the homogenates incubated at 50 °C, 65 °C and 75 °C activity measurements were carried out over a 50 minute time period (aliquots were withdrawn for PPO and POD activity assays at 0, 1, 5, 10, 20, 30, 40 and 50 minutes).

Polyphenol oxidase (EC. 1.10.3.1.) and peroxidase (EC. 1.11.1.7.) enzymatic activity. The methodology was the same as that used for the minimally processed lettuce (see above).

First order model. The activation energy (*Ea*) for PPO and POD was determined by using a first-order model with Arrhenius temperature dependence for data from the three temperatures (50, 65 and 75 °C). PPO and POD inactivation was adjusted to the following first-order model:

$$Enz = R_0 e^{e^{\mathrm{lrc}} e^{-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_r}\right)}_{\mathrm{time}}} \quad (1)$$

where *Enz* is the PPO or POD activity, R_0 the activity at time zero, *Ea* the activation energy (KJ mol^{-1}), *R* the gas constant (8.3144 10^{-3} KJ mol^{-1} K^{-1}), T_r the reference temperature (333.15 K), *T* the experimental temperature (K), lrc the logarithm of the reaction rate constant (log(sec^{-1})), and time the experimental time (sec).

Statistical analysis. The experimental results were analysed by a multifactor analysis of variance (ANOVA) to determine significant differences ($P < 0.05$) during storage and according to treatment, using Statgraphics software (version 2.1; Statistical Graphics Co., Rockville, U.S.A.). *R* software (*R* Development Core Team, 2004) was used to model the PPO and POD inactivation behaviour.

Results and Discussion

Minimally processed lettuce.

The browning-related enzymes, PPO and POD, were analysed in minimally processed lettuce during 10 days of storage after different washing temperature treatments.

PPO activity in minimally processed lettuce washed at 50 °C (heat shock) had significantly ($p < 0.05$) lower values than those samples washed at 4 °C or 25 °C (Fig. 3(1)). These lower values were particularly apparent from day 6 to day 10, whereafter the PPO activity level stayed constant. In contrast, an increase in activity was observed after washing at 4 °C and 25 °C. These findings are in agreement with Loaiza-Velarde *et al.* who reported that heat shock did not eliminate the enzymes involved in tissue browning.[12)]

This increase in PPO activity in the samples washed at 4 °C and 25 °C during days 6–10 is also in agreement with the results of other authors who have reported that the maximum PPO value was observed in minimally processed lettuce at the end of the shelf-life.[13)]

Salveit has suggested that there were many points in the sequence of reactions from wounding to browning where the process could be interrupted by heat shock (50 °C).[9)] All of these points were referenced only to PAL synthesis. He thus suggested the use of salts to maintain membrane integrity and the use of chemical inhibitors to control the PPO activity and reduce browning. This author did not consider that heat shock by itself could control the PPO activity. If heat shock could not affect PPO synthesis, an increase in activity would be expected during storage of the 50 °C-treated samples. However, the PPO values were found to be reduced in the tissue after heat shock (Fig. 3(1)).

Since the decrease in PPO activity was not immediate (there was no difference on day 2 between treatments), it seems that the decrease was not caused by a direct effect on the synthesis of PPO and POD by blocking a receptor that might regulate its synthesis (Fig. 3(1)), but could have been due to the feedback produced inhibition by the lack of phenolics.

The POD activity showed similar behaviour to that of PPO; the samples treated at 50 °C showed the lowest activity during storage (Fig. 3(2)).

The visual browning of fresh-cut lettuce was analysed by an untrained panel after washing at the different temperatures during 10 days of storage (Fig. 4). The sensory panel found significantly ($p < 0.05$) lower browning in those samples treated at 50 °C than in those treated at the lower temperatures and in a chlorine-washed (120 ppm, 25 °C) control.

Differences in the browning scores were apparent from day 4 to day 10, when lettuce washed at 4 °C and 25 °C increased in browning, while the lettuce treated at 50 °C maintained significantly lower browning until the end of the storage period. These data clearly show a correlation between the browning reduction and lower PPO activity.

In vitro analysis

The second objective of this study was to directly analyse the effect of incubation temperature on the stability of PPO and POD in crude extracts in order to avoid any secondary effects. First, the stability of PPO and POD was compared by using crude extracts from segregated tissues incubated at 4 °C, 25 °C and 50 °C. Second, the thermal stability of both enzymes was determined at 50 °C, 65 °C and 75 °C. These data were

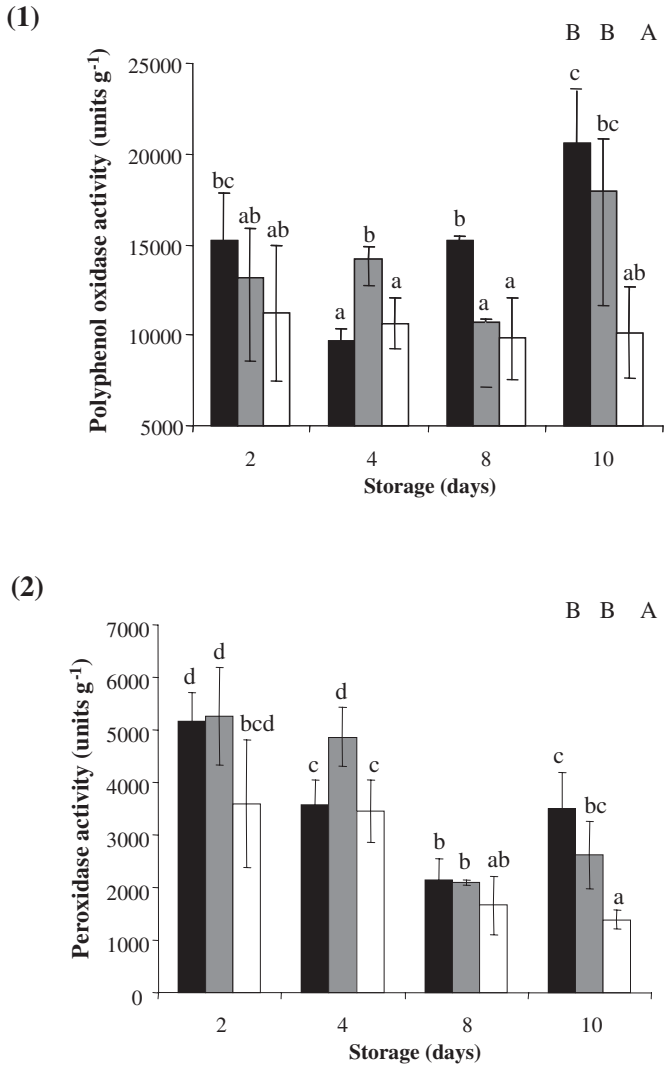

Fig. 3. Effect of Washing Temperature Treatments at 4 °C (■), 25 °C (▒), and 50 °C (□) on the Polyphenol Oxidase (1) and Peroxidase (2) Activity in Minimally Processed Fresh-Cut Iceberg Lettuce during Storage for 10 Days at 4 °C.

Points designated by the same letter are not significantly different ($p > 0.05$). Lower-case letters are used for comparisons during storage, and upper-case letters for treatment comparisons.

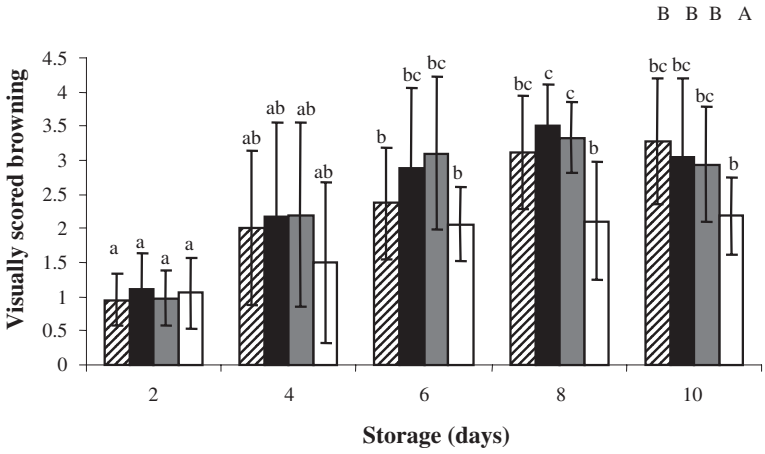

Fig. 4. Effect of Washing Treatment Temperature on Visual Browning over a 10-Day Storage Period.

Washing temperatures were 4 °C (■), 25 °C (▒) and 50 °C (□), with a chlorine wash at 25 °C (▨) as a control. Points designated by the same letter are not significantly different ($p > 0.05$). Lower-case letters are used for comparisons during storage, and upper-case letters for treatment comparisons.

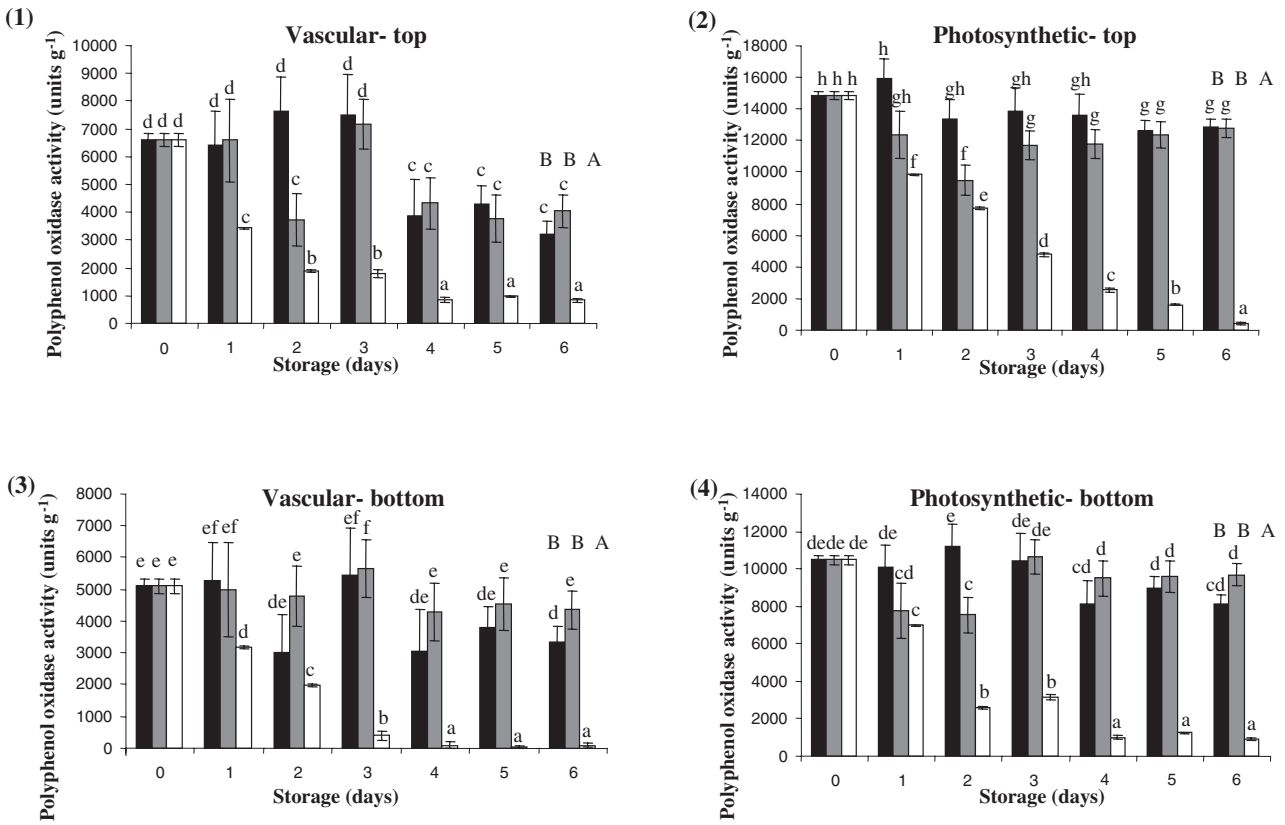

Fig. 5. Time-Course Characteristics of Polyphenol Oxidase Activity as a Function of the Incubation Temperature for Different Tissues and Parts of the Head of Lettuce.

The figure shows the effect of incubation temperature at 4 °C (■), 25 °C (▒) and 50 °C (□) on the PPO activity in crude extracts isolated from photosynthetic and vascular tissues: (1) top vascular (2) top photosynthetic (3) bottom vascular and (4) bottom photosynthetic. Points designated by the same letter are not significantly different ($p > 0.05$). Lower-case letters are used for comparisons during storage, and upper-case letters for treatment comparisons.

used to model the activity of both enzymes as a function of temperature. Finally, the thermal stability data were compared with the PPO and POD activity in extracts of minimally processed whole lettuce during storage after the different washing treatments (4 °C, 25 °C and 50 °C).

The effect of temperature on the enzymes in the crude extracts most closely approximated to the effect of temperature on the whole lettuce. We examined the PPO and POD activity in crude extracts from the top and bottom halves of the head of lettuce and in the vascular and photosynthetic tissues from each half (Figs. 5 and 6). Significantly higher values were found ($p < 0.05$) in those extracts from the bottom half of the head of lettuce than from the top half for both enzymes.

It was also observed that the PPO and POD activity was significantly higher ($p < 0.05$) in the photosynthetic tissue (leaf) than in the vascular tissue (rib) (Figs. 5 and 6). This latter finding is consistent with the data of Heimdal *et al.* and Vamos-Vigyazo, who attributed the higher activity in photosynthetic tissue to the location of PPO and POD in the thylakoid membrane of the chloroplasts.[16,17]

PPO and POD lettuce tissue extracts incubated at 50 °C for 6 hours (Figs. 5 and 6) showed different behaviour from the extracts incubated at 4 °C and 25 °C, regardless of the type of tissue (leaf/rib) and the position in the head (top/bottom).

PPO activity at 50 °C gradually decreased over the 6-hour incubation period in all the samples during storage. Only small residual activity (~3%) remained at the end of the 6-hour storage period. Thus, prolonged exposure to an elevated temperature caused loss of PPO activity in the crude extracts.

The same experiment was carried to monitor the POD activity (Fig. 6) over a six-hour period, and a similar pattern was observed. Thus, at 50 °C, the loss of activity is much faster than that at 4 °C and 25 °C. However, the POD activity declined more rapidly than the PPO activity at 50 °C, losing more than 95% of the activity in the first hour of incubation. Thus, POD was a more heat-labile enzyme than PPO in the crude extracts. Interestingly, for those samples incubated at 4 °C and 25 °C, the POD activity actually increased up to 4 hours, and then started to decrease to the initial value or below (Fig. 7). At 50 °C, in contrast, the POD activity decreased from the beginning.

Extracts incubated at 50 °C, 65 °C and 75 °C showed a percentage of PPO activity remaining after 1 hour of

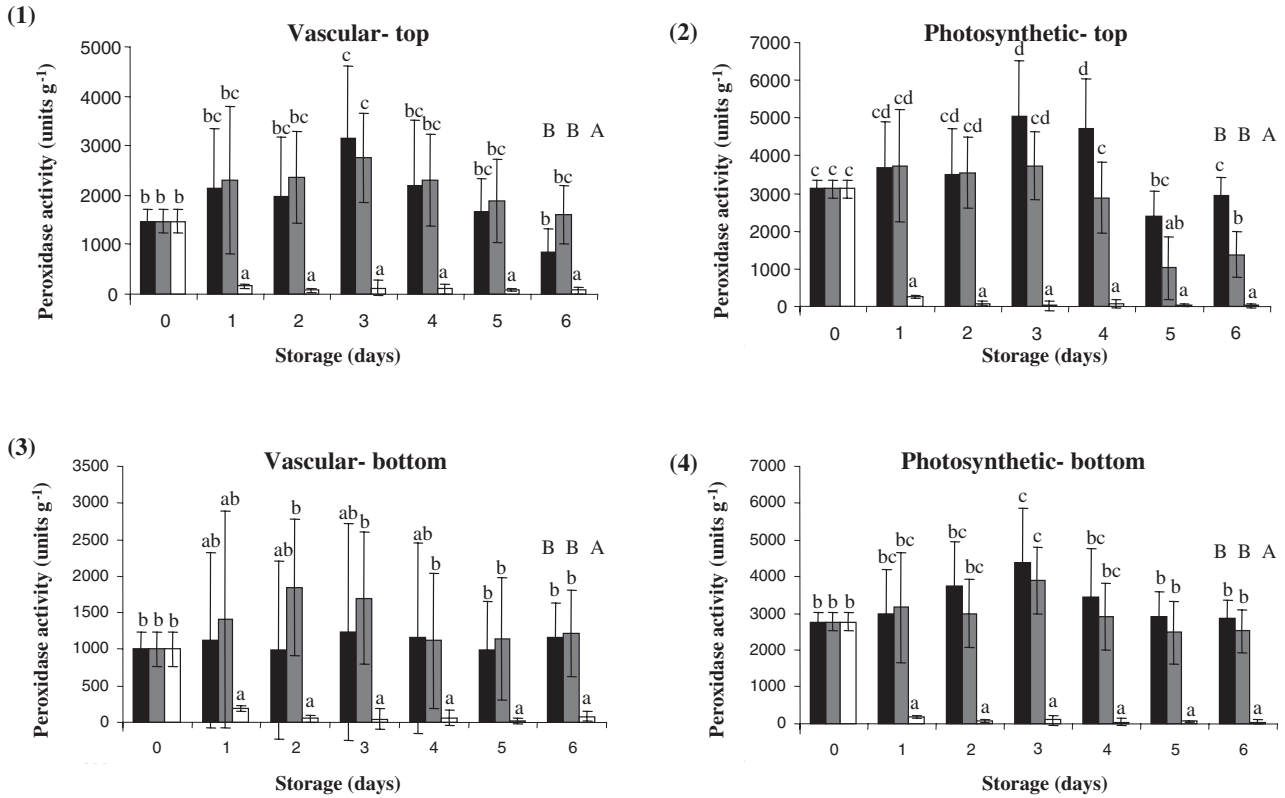

Fig. 6. Time Course Characteristics of Peroxidase Activity as a Function of Incubation Temperature for Different Tissues and Parts of the Head of Lettuce.

The figure shows the effect of incubation temperature at 4 °C (■), 25 °C (▩) and 50 °C (□) on the POD activity in crude extracts isolated from photosynthetic and vascular tissues: (1) top vascular (2) top photosynthetic (3) bottom vascular (4) bottom photosynthetic. Points designated by the same letter are not significantly different ($p > 0.05$). Lower-case letters are used for comparisons during storage and upper-case letters for treatment comparisons.

72%, 31% and 0% respectively (Fig. 7(1)). With all temperatures, an initial rapid decrease in activity was observed, followed by a slower loss of activity with time. The enzyme lost activity more rapidly with increasing incubation temperature. This initial rapid loss of activity followed by a slower decline could have been due to the presence in the extract of different isoenzyme forms of PPO with different thermolability profiles.[16,17] Alternatively, it could have been due to the formation of a less-active (damaged) form of PPO as a result of the heat treatment.

The same thermal stability study was carried out for POD (Fig. 7(2)). The extracts incubated at 50 °C, 65 °C and 75 °C for 1 hour showed more rapid loss of enzyme activity than for PPO. More than 90% inactivation was observed within 2000 seconds for all temperatures. The bulk of the POD activity was lost within 5 minutes, even at the lowest treatment temperature (50 °C). It is thus clear that POD was much more sensitive to an elevated temperature than PPO. These thermal stability data confirm the findings on the sensitivity of POD to heat treatment (see Figs. 4 and 5).

It is of interest to note that both PPO and POD (Fig. 7) showed a transient activation of approximately 10–15% at the beginning of the incubation, followed afterwards by a decline in activity. This activation was reproducible over 16 replicates and was more pronounced for POD than for PPO. This behaviour has been explained by the existence of an activation period for the restoration of essential thiol groups on the enzyme.[18]

The data in Fig. 7 were modeled by using a first-order relationship in which the rate constant is temperature dependant (see equation 1 in the Materials and Methods section). A parameter for the energy of activation (Ea) for the thermal inactivation process was inserted in the model in order to predict the remaining activity for a range of temperatures and times. The exponential model showed that PPO ($Ea = 106.458 \pm 11.917\,\mathrm{KJ\,mol^{-1}}$) was much less sensitive to high temperature than POD ($Ea = 52.371 \pm 11.510\,\mathrm{KJ\,mol^{-1}}$).

The exponential model (Fig. 8) was used to calculate the residual activity of PPO and POD incubated at 50 °C for 1 minute. These results indicate that the effect of a one-minute heat shock treatment in reducing the browning reaction cannot be directly ascribed to complete or even substantial inactivation of PPO and POD.

This observation is based on data derived from crude

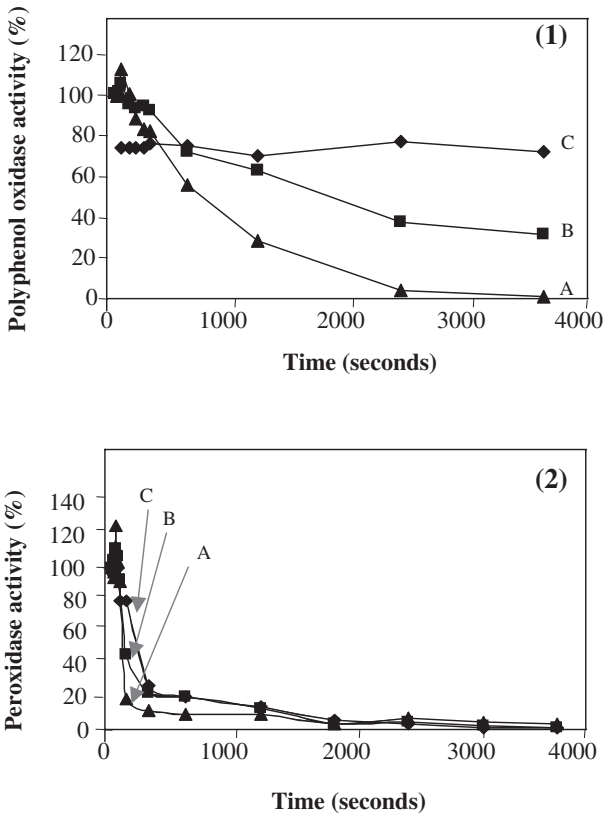

Fig. 7. Thermal Inactivation Profiles of Polyphenol Oxidase (1) and Peroxidase (2) Activity (%) at 50 °C (◆), 65 °C (■), and 75 °C (▲) in Crude Extracts over 1 Hour (3600 seconds).

Points designated by the same letter are not significantly different ($p > 0.05$). Upper-case letters are used for treatment comparisons during storage.

extracts. However, the stability of these enzymes in whole tissues is likely to be even higher than in crude extracts.

Conclusion

The main conclusion from these studies is that heat shock treatment of minimally processed lettuce caused a reduction of PPO and POD during storage. The thermal stability studies show that heat shock did not cause direct inactivation of PPO or POD by denaturation, since a heat shock for 1 minute at 50 °C only caused a 6% loss of activity for both enzymes. The reduced synthesis of PPO and POD could have been due to different effects: a possible indirect effect caused by feedback inhibition from the lack of phenolic compounds (substrate) or a direct effect on a receptor (unknown) implicated in the synthesis of PPO and POD, or perhaps to the diversion of protein synthesis to the production of heat shock proteins as suggested by Salveit[9] for PAL. However, these are speculations and further biochemical studies will be necessary in order to investigate the causes of the inhibition of PPO and POD activity.

Acknowledgments

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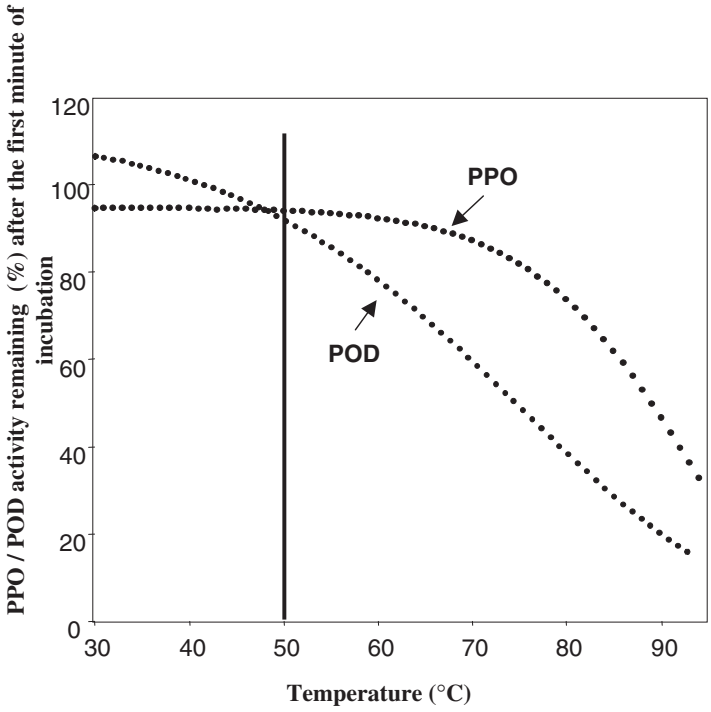

Fig. 8. Percentage of Residual Activity *vs.* Temperature for Polyphenol Oxidase (PPO) and Peroxidase (POD) after One Minute of Incubation at Different Temperatures.

Simulation was obtained by using the first-order model explained in the Materials and Methods section.

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