Thermal Inactivation Kinetics of Peroxidase and Lipoxygenase from Broccoli, Green Asparagus and Carrots

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ABSTRACT: Thermal inactivation curves for peroxidase (POD) and lipoxygenase (LOX) in broccoli (florets), green asparagus (tip and stem), and carrots (cortex and core) extracts were determined in the range of 70 to 95 °C for 0 to 600 s. The capillary tube method was used to obtain quasi-isothermal conditions. The kinetics of both enzymes showed a biphasic first-order model, while at 70 °C, LOX in asparagus showed a monophasic first-order behavior. LOX activity was not detected for carrots. Kinetic parameters, *k* and *Ea*, were determined for heat-labile and heat-resistant isoenzyme fractions. Additionally, initial and residual activities for both enzymes within tissue sections showed a different distribution and heat stability.

Keywords: peroxidase, lipoxygenase, thermal inactivation, kinetic parameters, blanching

Introduction

CARROT, BROCCOLI, AND GREEN ASPARAGUS CROPS AND RELATED freezable plants are important economic products in the southern part of Chile. Peroxidase (POD) and lipoxygenase (LOX) are 2 enzymes commonly found in vegetables and are often utilized as an index for blanching adequacy, since the inactivation of these enzymes increases the shelf-life of vegetables during frozen storage (Williams and others 1986; Barret and Theerakulkait 1995). Recently, Ramirez and Whitaker (1998) proposed to use cystine lyase (CL) as a blanching indicator in broccoli since it is the main enzyme responsible for off-aroma production (Lim and others 1989). However, different research groups have found that CL is more heat labile than POD and LOX (Barret and others 2000; Kawaguchi and others 2000).

LOX is related to off-flavor development and color change. The latter is due to hydroperoxide and radical formation by oxidation of lipids, which can destroy chlorophyll and carotenes during frozen storage (Vámos-Vigyázó 1981; Adams 1991; Robinson 1991; Zhuang and others 1994).

In vegetables, POD is located in soluble form (POD_{sol}) in the cell cytoplasm, and in insoluble form as ionically bound (POD_{ion}) and as covalently bound (POD_{cov}) to the cell wall (Gkinis and Fennema 1978; McLelland and Robinson 1981; Vamós-Vigyázó 1981). Previous studies in POD blanching inactivation in vegetables have been made with the POD_{sol} fraction (Halpin and Lee 1987; Kermasha and others 1988; Günes and Bayindirh 1993). Other groups such as Ganthavorn and others (1991) and Rodrigo and others (1996) worked with total POD which includes the soluble and the bound forms. Walker (1964) suggested that POD has a partial role in chlorophyll degradation and Kampis and others (1984) proposed that POD_{sol} is responsible for color changes in frozen green vegetables during long storage at –18 °C. Additionally, a carotene bleaching system related to POD activity was observed in the water-soluble fraction of tomato extracts (Blain and others 1968) and in the protein fraction of red pepper extracts (Kanner and others 1977). Later, Bubicz and others (1990) proposed that inactivation of POD_{sol} in blanched French beans led to markedly better carotenoid retention after their storage at −20 °C for 12 mo. The literature reports a higher proportion of the POD_{sol} fraction in the majority of vegetables (Liu and Lamport 1974; McLellan and Robinson 1981; Kampis and others 1984). Only in green beans it was shown there were identical levels of POD_{sol} and POD_{ion} (Gkinis and Fennema 1978). In all cases, POD_{cov} showed little activity. Thus, it seems that future studies on the thermal stability of POD in blanched and frozen vegetables should focus mainly on the soluble fraction.

All of the POD fractions are present in plant tissues as a combination of various isoenzymes with different thermal stability (Vámos-Vigyázó 1981; Adams 1997). For LOX there are apparently 3 to 4 isoenzymes in different vegetable products which differ in their thermal stability among other properties (Nicholas and others 1982; Engeseth and others 1987; Hildebrand 1989; Shiba and others 1991). The differences in heat resistance of the isoenzymes vary considerably with the vegetable source and origin; therefore, it is important to work with enzymatic extracts of the tested product (Vámos-Vigyázó 1981; Whitaker 1994).

Thermal inactivation kinetic studies in POD and LOX enzymes in the range of 70 to 100 °C have clearly shown biphasic curves which are thought to depend on the presence of isoenzymes with different thermal stabilities (Wang and Luh 1983; Powers and others 1984; Ganthavorn and others 1991; Sarikaya and Özilgen 1991; Günes and Bayindirh 1993; Forsyth and others 1999). An inactivation biphasic model was proposed by Ling and Lund (1978) to describe the inactivation thermal kinetics of an enzyme system formed by a heat-labile fraction and a heat-resistant fraction, both with first-order inactivation kinetics. The differences between kinetic parameters for heat-labile and heat-resistant isoenzyme fractions from several sources (Ling and Lund 1978; Günes and Bayindirh 1993) indicate the need and importance of determining the kinetics of POD and LOX in different vegetable extracts. The latter is important since the residual enzyme

activity is exponentially related to the activation energy (*Ea*) and to the inactivation rate constant (*k*). Thus, small errors in the calculations of these parameters or inappropriate values can have a big impact on residual enzyme activity predictions (Arabshahi and Lund 1985).

The design of efficient blanching treatments requires knowledge of critical factors such as enzymatic distribution within the tissue, inactivation kinetic parameters and relative proportions of heat-labile and heat-resistant fractions (Adams 1991). This type of information usually is not available in the literature and is unique to each vegetable, species, cultivar, and environmental condition, among other factors (Vámos-Vigyázó 1981; Kushad and others 1999).

Our main objective was to determine the inactivation kinetics of POD and LOX present in different tissue parts of broccoli, green asparagus, and carrots at conventional blanching temperatures. This information will aid in modelling blanching process of these vegetables for optimum thermal treatments.

Materials and Methods

Vegetable samples

Fresh broccoli heads (*Brassica oleracea* L., var. *Italica*, *cv* unknown) were purchased from a local market and analyzed within 24 h. Broccoli heads were cut into florets with dia and stems ranging from 3 to 4 cm and 6 to 8 g.

Green asparagus (*Asparagus officinalis* L., *cv* UC-72) and carrots (*Daucus carota* L., *cv Chantenay*) were harvested from the "Santa Rosa" Agricultural Experimental Station of the Austral Univ. of Chile. Asparagus was harvested in the early morning hours, classified, sized, stored at 2 °C and used within 24 h. Selected asparagus spears had a gauge of 12 to 14 mm (measured to 10 cm from the tip) and a length of 16 cm. For the study, 2 zones of the asparagus spears were defined: a tip section (first 4 cm from the tip) and a stem section (below 4 cm from the tip). Harvested carrots were washed, classified in the range of 200 to 300 g, stored at 5 ± 0.5 °C and utilized within 5 d. For carrots, 2 zones were also defined in this study: the cortex and core sections.

Moisture content determination

Moisture content for each sample vegetable and section tissue was determined following the vacuum oven method (Ranganna 1977). Samples were first predried in an oven at 55 °C until they reach apparent dryness. Afterwards, the predried samples were dried at 70 °C with a vacuum oven at a pressure not more than 100 mm Hg (Squaroid Duo-Vac Oven, Model 3628; Lab Line Instruments Inc., Melrose Park, Ill., U.S.A.) until constant weight was obtained.

Chemicals and buffer

Hydrogen peroxide (30%), linoleic acid (> 97%), guayacol (99.5%) and Tween 20 were purchased from Merck (Darmstadt, Germany). Other chemicals used (sodium hydroxide, monopotassium phosphate, and dipotassium phosphate) were reagent grade. Distilled water was used for all assays.

The buffer system, ionic strength, and pH were selected according to Chen and Whitaker (1986), Sheu and Chen (1991), and Theerakulkait and Barret (1995) with some modifications based on preliminary assays to get maximum enzyme activity. Potassium phosphate buffer was prepared with monopotassium phosphate and dipotassium phosphate in distilled water obtaining a molar concentration of 0.2 mol/L and pH 6.5. The buffer solution was cooled at 4 °C until used.

Preparation of enzyme extracts

Preliminary experiments were performed to determine the ratio between sample weight and extracting buffer solution volume (g sample: mL buffer) for optimal reproducibility and linearity between enzyme concentration and observed activity.

To obtain POD vegetable extracts, samples were cut to smaller size (green asparagus), shredded (broccoli), or grated (carrots) to increase the overall contact surface. Samples were then mixed with the cold (4 °C) potassium phosphate buffer in different proportions (g sample: mL buffer). For broccoli, green asparagus, and carrots, the proportions were 1.5:100 w/v, 30:100 w/v and 20:100 w/v, respectively. To obtain LOX vegetable extracts, samples were treated similarly. However, for broccoli the proportion used was 30:100 w/v.

Each sample-buffer mixture was homogenized in a Stomacher Lab blender, Model 400 (Seward Medical Limited Co., London, England) for 3 min at maximum (HIGH) load speed within a special disposable plastic bag. The suspensions were filtered using 2 layers of linen cloth to remove solid particles. To eliminate remaining turbidity the homogenates were centrifuged in a Beckman centrifuge model J2-HS with a JA-20 rotor (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) at 18,000 × *g* for 30 min and 4 °C using polycarbonate tubes. The supernatants were filtered through medium-fast filter paper and kept on ice until analyzed. Enzyme extracts were prepared in triplicate to check reproducibility of total initial enzyme activity readings from the vegetable samples.

Protein determination

Protein content was determined by Lowry's method (Lowry and others 1951) and by measuring absorbance at 750 nm. Bovine serum albumin (BSA) was used as standard.

Thermal inactivation experiments

Some researchers have proposed to use capillary or glass tubes to obtain "isothermal" conditions for a suitable determination of *k* and *Ea* parameters (Resende and others 1969; Wang and Dimarco 1972; Adams 1978; Bhirud and Sosulski 1993; Rodrigo and others 1997).

In this study heat inactivation experiments of POD and LOX extracts were conducted following the capillary tube method as described by Haas and others (1996a, 1996b). In order to achieve a quasi-isothermal condition, the heating-up time must be as short as possible.

Capillary tubes of 2.5 mm internal dia and 15 cm length (Soviquim Glass Factory Ltda, Santiago, Chile) with 0.5 mL of enzyme extract were first immersed in an oil bath with controlled temperature (± 0.5 °C), $T_A \approx T_H + 20$ °C. The capillary tubes were removed just before the solution reached the holding temperature (T_H) and moved to a holding water bath with a precision of + 0.1 °C (Model 1285PC, VWR Scientific Products Co., South Plainfield, N.J., U.S.A.). Heat inactivation was studied for holding temperatures ranging from 70 to 95 °C at exposure times between 15 and 600 s. To end the thermal treatments, capillary tubes were put in ice water (0 °C) until about 2 °C was reached. Thermal treatments of enzymatic extracts were done in triplicate.

The temperature within the capillary tubes was monitored using a 12-channel scanning thermocouple thermometer,

DIGI-SENSE, Model 92800-15 (Cole Parmer Instrument Co., Vernon Hills, Ill., U.S.A.) and stainless steel-type T hypodermic thermocouples 316SS (0.4318 mm needle dia; copper-constantan and 1 s time constant) (Cole Parmer Instrument Co.) connected to an IBM/PC computer.

Quasi-isothermal conditions would prevail when enzyme inactivation during the heating-up and cooling-down times can be neglected. Previous experiments showed that no significant inactivation of POD and LOX were detected before 50 °C in the early exposure times. The temperature recorded showed that come-up time for heating and come-down time for cooling took only a few seconds and can be considered negligible. Thus, no corrections were made and the times necessary to inactivate enzymes were obtained directly from the experimental values.

Determination of enzyme activities

POD was assayed according to the method of Hemeda and Klein (1990), Sheu and Chen (1991), Weng and others (1991), and Saraiva and others (1996) with some modifications. Preliminary experiments established that optimum conditions for analysis of POD activity were a pH value of 6.5 and a buffer of ionic strength of 0.1 mol/L. POD substrate solution was prepared daily by mixing 0.1 mL guayacol (99.5%), 0.1 mL hydrogen peroxide (30%), and 99.8 mL potassium phosphate buffer (0.1 mol/L; pH 6.5). This buffer solution was prepared from dilution of 0.2 mol/L potassium phosphate buffer having the same pH with distilled water. The substrate solution was homogenized in a Fisher vortex, Model 58 (Fisher Scientific Co., Springfield, N.J., U.S.A.) for 3 s. Afterwards, the substrate solution was left to settle for a few min. POD activity assays were conducted by mixing in glass tubes 0.120 mL of enzymatic extracts with 3.48 mL of substrate solution and homogenized with a vortex for 3 s. POD activities were measured from the initial increase in absorbance at 470 nm. The reaction was monitored for 20 min using a blank prepared with 0.120 mL distilled water and 3.48 mL POD substrate solution.

LOX was assayed using the method of Chen and Whitaker (1986) and Sheu and Chen (1991) with some modifications. The LOX substrate solution was prepared daily by mixing 8 mL linoleic acid, 8 mL Tween 20 and 10 mL distilled water, and clarified by adding 1 mL sodium hydroxide (1 mol/L). Before assay, the stock solution was diluted with potassium phosphate buffer (0.2 mol/L, pH 6.5) to obtain 100 mL substrate solution volume. The solution was mixed by shaking for 2 min and was left to settle in the dark for 10 min before adding the enzyme. For LOX activity assays, 0.120 mL of enzymatic extracts were mixed in glass tubes with 3.48 mL of substrate solution and homogenized with a vortex for 3 s. LOX activities were measured from the initial increase in absorbance at 234 nm. The reaction was monitored for 3 min using a blank prepared with 0.120 mL distilled water and 3.48 mL LOX substrate solution.

The quantity of enzyme extract assayed was determined according to substrate concentration and to obtain an absorbance increase between 0.000 and 1.000, which is the suitable range for enzyme activity readings.

Enzymatic activity measurements were done in triplicate using 10-mm-path-length quartz cuvettes (Starna, London, England) and a UV/vis, Spectronic spectrophotometer model Genesys 5 (Spectronic Instruments Inc., Rochester, N.Y., U.S.A.). Enzyme activities are reported in enzymatic units (U) and defined as the amount of enzyme that produced a change in absorbance of 1.0/min under the assay conditions, 25 °C (1 U = $\Delta A\ min^{-1}$; A = absorbance at 470 nm and 234 nm for POD and LOX, respectively).

Total remaining enzyme activities obtained after each time-temperature treatment (U_t) were expressed as percentage of the total initial activity (U_0) and graphically reported as:

$$\text{Enzyme activity } (\%) = \frac{U_t}{U_0} x100$$

Total initial enzyme activities (U_0) were related to 1 mL crude extract and to 1 g dry matter of each vegetable sample. Also, total initial enzyme activities were expressed as specific activity (U_0/mg of protein).

Biphasic model

Ling and Lund (1978) proposed a simple method to analyze the thermal inactivation kinetics of an enzyme system formed by 2 groups differing in their thermal stability, a heat-labile fraction and a heat-resistant fraction. The 2-fraction model assumes that each fraction of enzyme follows first-order kinetics and mathematically is expressed as follows:

$$\text{Enzyme activity } (\%) = \frac{\left[K_L E_{L0} e^{-k_L t} + K_R E_{R0} e^{-k_R t}\right]}{\left[K_L E_{L0} + K_R E_{R0}\right]} x\,100 \quad (1)$$

where t is the time treatment at constant temperature; E_{L0} and E_{R0} are the initial concentrations of the heat-labile and heat-resistant isoenzyme fractions, respectively; k_L and k_R are the first-order rate constants for thermal inactivation of the heat-labile and heat-resistant isoenzyme fractions, respectively; and, K_L and K_R are the reaction rate constants for the respective isoenzyme fractions with the substrate.

As indicated by Ling and Lund (1978) to simplify interpretation of thermal inactivation data, 2 limiting conditions can be applied to Eq. 1 since a vast difference between *k* values corresponding to heat-labile and heat-resistant fractions is always found. The same authors, on the basis of thermal inactivation studies on commercially available horseradish peroxidase, demonstrated that at long heating times normally less than 1% of the heat-labile isoenzyme fraction would still be active. This makes acceptable the assumption that all heat-labile fraction is inactiviated. A similar criterion was applied by Rodrigo and others (1997) to estimate the initial activity of the heat-resistant fraction of commercial horseradish peroxidase. Then, when heating time t is long, $e^{-k_L t}$ will close to zero and Eq. 1 becomes:

$$\text{Enzyme activity } (\%) = \left[\frac{K_R E_{R0} e^{-k_R t}}{K_L E_{L0} + K_R E_{R0}}\right] x\,100 \quad (2)$$

Afterwards, linearizing Eq. 2 gives,

$$\log\left[\text{Enzyme activity } (\%)\right] = \log\left[\frac{K_R E_{R0}}{K_L E_{L0} + K_R E_{R0}} x100\right] - \frac{k_R}{2.303} t \quad (2a)$$

During the short heating period the heat-resistant fraction would undergo some thermal inactivation. However, according to Ling and Lund (1978) the difference between the theoretical activity (assuming no inactivation of heat-resistant fraction) and the measured activity was less than 3.6%. This overestimation would be small compared to experimental uncertainties. Then, when the heating time (t) is short, little of the heat-resistant fraction is inactivated and Eq. 1 can be approximated and rearranged as:

$$\text{Enzyme activity } (\%) = \frac{K_R E_{R0}}{K_L E_{L0} + K_R E_{R0}} \times 100 = \left[\frac{K_L E_{L0} e^{k_L t}}{K_L E_{L0} + K_R E_{R0}} \right] \times 100 \quad (3)$$

Also, linearizing Eq. 3 gives,

$$\log\left[\text{Enzyme activity } (\%) - \frac{K_R E_{R0}}{K_L E_{L0} + K_R E_{R0}} \times 100\right] = \log\left[\frac{K_L E_{L0}}{K_L E_{L0} + K_R E_{R0}} \times 100\right] - \frac{k_L}{2.303} t \quad (3a)$$

The total initial activities in percentage of the heat-resistant $[(U_{0R}/U_0) \times 100]$ and heat-labile $[(U_{0L}/U_0) \times 100]$ fraction of the enzyme systems are equal to the values of the antilogarithm of the intercepts of Eqs. 2a and 3a, respectively. Thus, the following mathematical expression is verified:

$$\left[\frac{K_R E_{R0}}{K_L E_{L0} + K_R E_{R0}} \times 100\right] + \left[\frac{K_L E_{L0}}{K_L E_{L0} + K_R E_{R0}} \times 100\right] = 100 \quad (4)$$

If $K_L \approx K_R$, the values of the left side of Eq. 4 would represent the relative proportions of the heat-resistant $[E_{R0}/(E_{L0} + E_{R0})]$ and heat-labile $[E_{L0}/(E_{L0} + E_{R0})]$ isoenzyme fractions, respectively .

More details of the biphasic model as well pictorial representation can be found in the original source (Ling and Lund, 1978).

Temperature-dependence of the rate constants

The first-order rate constants (k_L and k_R) of thermal inactivation for the respective isoenzyme fractions were related to temperature using the Arrhenius equation as follows:

$$k_L = k_{0L} e^{-\frac{Ea_L}{RT}} \quad (5)$$

$$k_R = k_{0R} e^{-\frac{Ea_R}{RT}} \quad (6)$$

where k_{0L} and k_{0R} are Arrhenius constants, R is the gas constant (R = 8.31 J/ mol K), and T is the absolute temperature (K). Ea_L and Ea_R are the activation energy constants for thermal inactivation of the the heat-labile and heat-resistant isoenzyme fractions. Linearizing Eqs. 5 and 6 gives:

$$\text{Ln } k_L = \text{Ln } k_{0L} - \frac{Ea_L}{R} \cdot \frac{1}{T} \quad (7)$$

$$\text{Ln } k_R = \text{Ln } k_{0R} - \frac{Ea_R}{R} \cdot \frac{1}{T} \quad (8)$$

The estimation of kinetic parameters were obtained by the 2-step linear regression method. In the first regressions, k_R and k_L constants at each temperature were estimated from the slopes of Eqs. 2a and 3b, respectively. In the second regressions Ea_L *and* Ea_R values were calculated from the slopes of Eqs. 7 and 8, respectively.

Results and Discussion

Total initial enzyme activities

To provide a model describing a complex reaction, it is necessary to report all data pertaining to the conditions of the reaction. One of the most important conditions, not often reported, is the initial concentration of reactants (Arabshahi and Lund 1985). Also frequently not reported is the moisture content of samples, which is used for enzyme activity calculations. Initial moisture content for broccoli florets, for green asparagus tip and stem section, and for carrot cortex and core sections are shown in Table 1. Then, sample dry matter was determined by difference.

Total initial POD and LOX activities (U_0/g dry weight) are also shown in Table 1. The total initial enzyme activities and moisture contents were determined for at least 10 samples of each fresh product.

Broccoli had the largest initial POD activity in this study and is considered to be one of the largest among vegetables (Kampis and others 1984; Barret and others 2000). The large activity of LOX for broccoli and asparagus compared to other vegetables would explain the increased color losses and off-flavor development during frozen storage of nonblanched or underblanched products (Halpin and Lee 1987; Lee and others 1988; Sheu and Chen 1991). For carrots, LOX activity was not detected with the technique utilized. This could be due to the nonpresence of the enzyme in the carrot tissue, to activities too low to be detected (Baardseth and Slinde 1980; Schaller and Vámos-Vigyázó 1986; Günes and Bayindirh 1993; Pizzocaro and others 1993) or to the presence of high amounts of natural antioxidants such as carotenoid compounds (Pinsky and others 1971; Adams 1991).

Our results indicate that enzymes are distributed differently within the product. This was more evident for POD activities in carrots while less notorious for LOX activity in green asparagus. POD activity in asparagus stem sections was double that of tip sections. For carrots, POD initial activity in cortex sections was 4 times higher than in core tissues. Vora and others (1999) found that POD activity in carrot surface cortex tissues was double that of core tissues. Differences could be due to cultivars and environmental factors.

Thermal inactivation kinetics

Conventional blanching processes involve temperatures ranging from 70 to 95 °C and times usually not higher than 10 min (Lund 1977). Then in order to find inactivation kinetics, thermal inactivation experiments were carried out in the same time-temperature range.

Experimental enzyme activities (%) as a function of heating time were plotted on a semilog scale, as shown in Figures 1 and 2, obtaining nonlinear curves for each POD and LOX extract. For each product and for both enzymes, the inactivation rate kinetics increase with temperature.

In general, the plots show a drastic reduction in activity during the first 30 s. For longer heating times (above 60 s), there is a pronounced change in slope and another linear re-

Table 1—Summary of total initial enzyme activity calculations

		Carrot		Green asparagus	
	Broccoli florets	Core	cortex	tip	stem
Moisture content (% w.b.)	89.0 ± 0.2	90.9 ± 0.2	89.3 ± 0.3	90.5 ± 0.5	93.4 ± 0.2
Protein content					
mg/mL enzyme extract	0.067 ± 0.007[a]				
	1.334 ± 0.10[b]	0.728 ± 0.03[a,b]	1.054 ± 0.09[a,b]	1.538 ± 0.11[a,b]	1.271 ± 0.09[a,b]
mg/g dry weight basis	40.74 ± 8.98	47.35 ± 10.35	52.72 ± 13.10	52.71 ± 5.99	66.12 ± 11.88
POD activity					
U_0/mL enzyme extract	0.328 ± 0.067	0.055 ± 0.016	0.304 ± 0.133	0.110 ± 0.017	0.140 ± 0.05
U_0/mg protein	4.888 ± 1.11	0.076 ± 0.023	0.289 ± 0.126	0.072 ± 0.011	0.110 ± 0.039
U_0/g dry weight	199.12 ± 43.9	3.57 ± 0.78	15.21 ± 3.78	3.74 ± 0.66	7.28 ± 1.63
LOX activity					
U_0/mL enzyme extract	2.515 ± 0.109	ND[c]	ND	3.65 ± 0.19	2.267 ± 0.51
U_0/mg protein	1.885 ± 0.145	—	—	2.375 ± 0.123	1.784 ± 0.401
U_0/g dry weight	76.79 ± 5.92	—	—	124.28 ± 6.45	117.99 ± 15.95

[a] For POD extract
[b] For LOX extract
[c] ND—not detected

duction in activity but in smaller amounts, when the residual activity apparently tends to stabilize. Thus, the curves showed an initial steep straight line, an intermediate curved portion and a final straight line with a shallow slope. These results can be described with the biphasic first-order model proposed by Ling and Lund (1978) based on the presence of 2 isoenzyme groups with distinct thermal stabilities, a heat-labile fraction that inactivates rapidly and a heat-resistant fraction which cannot be inactivated completely. The biphasic pattern is also known as the 2-fraction model (Weng and others 1991; Saraiva and others 1996; Rodrigo and others 1997). Our results agree with those reported by Wang and Luh (1983), Powers and others (1984), Sarikaya and Özilgen (1991), Bhirud and Sosulski (1993), Günes and Bayindirh (1993), Pizzocaro and others (1993), Saraiva and others (1996) among others.

However, for the case of LOX activity in green asparagus at 70 °C, our results showed first-order monophasic kinetics with k values of 18.4×10^{-4} s^{-1} ($R^2 = 0.818$) and 9.2×10^{-4} s^{-1} ($R^2 = 0.833$) for tip and stem sections, respectively (Figures 2b and 2c). This would indicate that LOX at 70 °C behaves like only one group of enzymes. Similarly, Ganthavorn and others (1991) reported monophasic behavior for thermal inactivation of partially purified raw LOX extracts from green

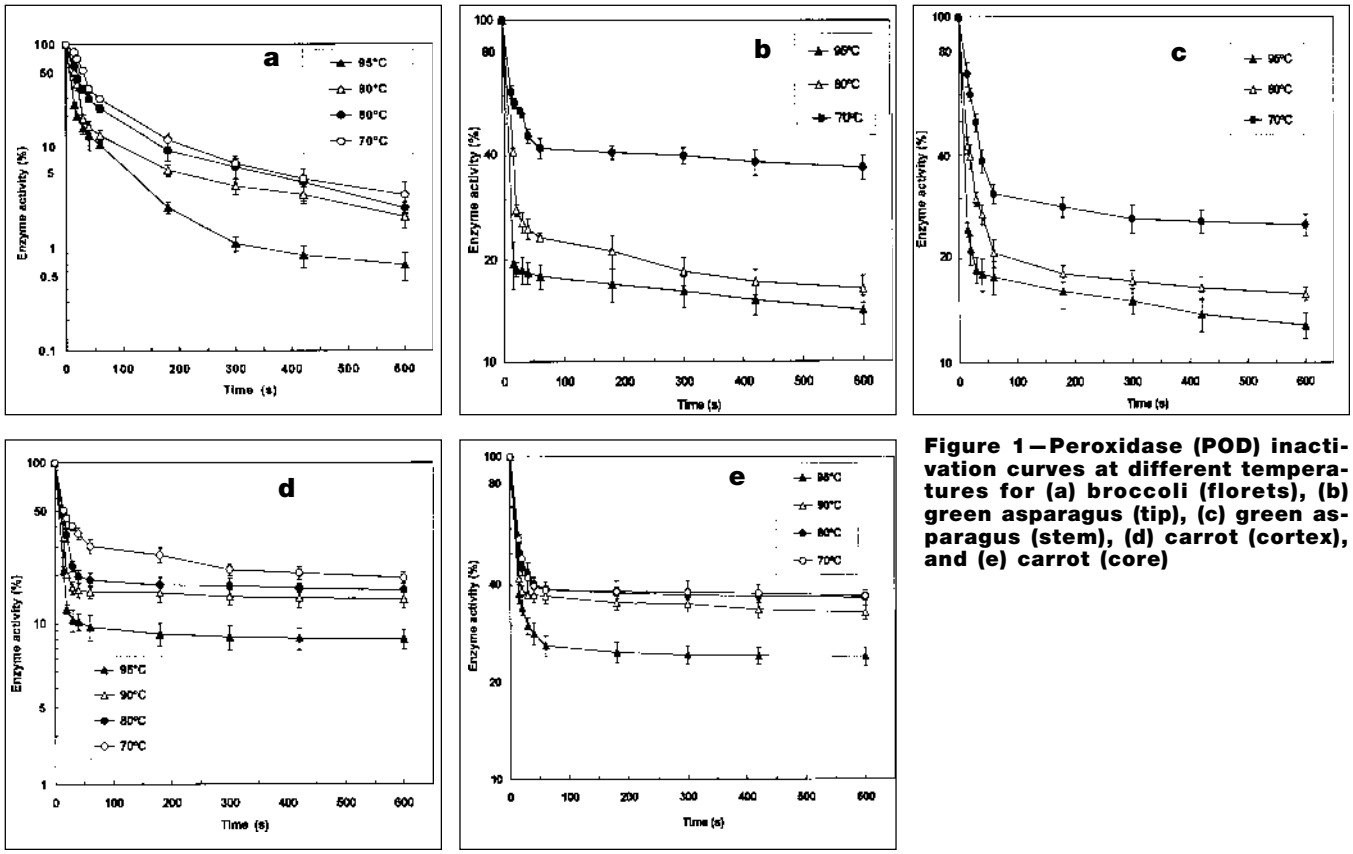

Figure 1—Peroxidase (POD) inactivation curves at different temperatures for (a) broccoli (florets), (b) green asparagus (tip), (c) green asparagus (stem), (d) carrot (cortex), and (e) carrot (core)

asparagus at 70 °C. Monophasic behavior of purified LOX for soybeans at 65 to 77.5 °C (Shibasaki and others 1973) and for potatoes at 50 to 60 °C (Park and others 1988) have also been reported. Ganthavorn and others (1991) reported that partially purified raw POD extracts from green asparagus closely responded to monophasic behavior at 50 and 60 °C. They also reported a biphasic behavior at 70 °C. Saraiva and others (1996) reported biphasic patterns of horseradish POD thermal inactivation in sodium phosphate buffer solutions for the temperature range of 70 to 95 °C.

Our results (Figures 1 and 2) show that enzyme activities for heat-labile and heat-resistant fractions are dependent on temperature and heating time. This dependency would show large enzyme activity differences for both fractions at higher temperatures, but it would be less evident at lower temperatures. Thus, we propose that the observed monophasic behavior for Asparagus LOX at 70 °C is likely due to a mathematical/graphical artifact that allows one to calculate only 1 rate constant for both enzyme fractions.

Further research is needed to clarify the mechanism of thermal inactivation of POD and LOX extracts. Thus, it will be necessary to perform substantial electrophoretic and/or chromatographic analyses on enzyme evaluated, as well as kinetic studies on different vegetable tissues and at wider ranges of temperature (50 to 100 °C).

Kinetic parameters

Based on the biphasic behavior we consider that during the first stage it is mainly the most labile fraction of the enzyme which is inactivated, while in the final stage only the most resistant fraction remains active. To determine k_L values the short heating period was fitted to include the first 30 s. To calculate k_R values a time of 60 s, or 180 s in some cases, was established as the beginning of a long heating period. The limiting conditions were established to avoid the intermediate curve portion of the biphasic model. Thus, inactivation rate constants (k_L and k_R) were calculated from slopes of the straight line portions (Eqs. 2a and 3a) of the curves considered for each product, temperature and enzyme type and fractions (Tables 2 and 3).

In general, k values increase with temperature and those for heat-labile fractions (k_L) are several times (from 10 to 1500 for POD and from 50 to 280 for LOX) larger than those for heat-resistant (k_R) fraction. This explains the higher decrease in enzyme activity during the first 60 s of thermal treatment (Figures 1 and 2). Large differences between k_L and k_R values indicate that limiting conditions employed to simplify data analysis (Eq. 2 and 3) were justified.

POD values of k_L and k_R calculated in this study fall in the range for those reported by Ling and Lund (1978) for commercial horseradish POD and for temperature inactivation of 76.7 to 87.2 °C which ranged from 183×10^{-4} to 768×10^{-4} s^{-1} and 5.75×10^{-4} to 14×10^{-4} s^{-1} for heat-labile and heat-resistant fractions, respectively. However, our results in some cases differ largely from previous reports on rate constants of POD and LOX (k_L and k_R) for temperature range of 70 to 96 °C (Günes and Bayindirh 1993). The observed differences could be due to the methods used in calculating the k_L and k_R parameters. The latter authors cited used a computerized iteration method of trial and error, adjusting the residual enzyme activity as a function of time experimental data in small vegetable slices (green beans, 5 cm length; sliced carrots, 2 cm dia and 1 cm thickness; peas, 1 cm dia). With this method, k values were determined for each enzyme fraction and temperature. However, it was not mentioned if corrections were done due to the come-up period in reaching isothermal conditions in the sliced vegetables studied.

The temperature-dependence of the inactivation rate constant (k) is explained with the concept of activation energy. Calculated Ea values for POD and LOX are shown in Tables 4 and 5, respectively. In general, for both enzymes, the activation energies of heat-labile fractions are slightly higher than heat-resistant fractions. Our results agree with those of Ling and Lund (1978) and Weng and others (1991) in studies on thermal inactivation kinetics of horseradish peroxidase. The first authors reported Ea values of 14.2×10^4 J/mol and 8.7×10^4 J/mol for heat-labile and heat-resistant fractions for temperatures ranging from 76.6 to 87.2 °C. The second authors reported Ea values of 22.6×10^4 J/mol and 9.6×10^4 J/mol for heat-labile and heat-resistant fractions and for temperatures between 65 °C and 98 °C.

However, Günes and Bayindirh (1993) reported that Ea values of POD and LOX for heat-labile fractions are lower than heat-resistant fractions for temperatures ranging between 70 and 96 °C. This was observed for POD in peas (4.1×10^4 J/mol against 7.5×10^4 J/mol), green beans (5.7×10^4 J/mol against 7.7×10^4 J/mol), and carrots (5.2×10^4 J/mol against 5.7×10^4 J/mol). Similar responses were observed for LOX in peas (4.6×10^4 J/mol against 20.7×10^4 J/mol) and

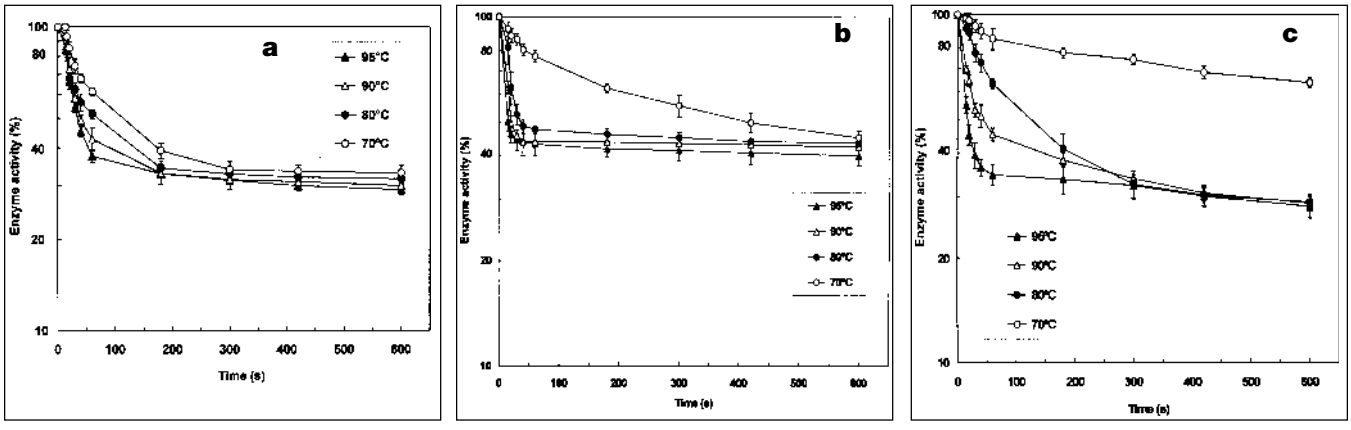

Figure 2—Lipoxygenase (LOX) inactivation curves at different temperatures for (a) broccoli (florets), (b) green asparagus (tip), and (c) green asparagus (stem)

Table 2—Paramaters of the biphasic first-order model for peroxidase (POD) heat-labile and heat-resistant fractions

Temperature (°C)	$\frac{K_R E_{R0}}{K_L E_{L0} + K_R E_{R0}} \times 100$	$k_R \times 10^4$ (s^{-1})	R^2	$k_L \times 10^4$ (s^{-1})	R^2
Broccoli (florets)					
70	23.54	24.0	0.99	250.0	0.99
80	21.12	35.7	0.99	496.0	0.96
90	19.24	67.9	0.95	1123.0	0.95
95	15.95	96.7	0.95	1439.0	0.96
Asparagus (tip)					
70	43.25	2.74	0.99	575.0	0.97
80	24.07	5.30	0.99	1199.8	0.97
95	19.44	7.90	0.96	2835.0	0.99
Asparagus (stem)					
70	31.63	6.86	0.98	449.0	0.99
80	21.72	10.70	0.98	737.0	0.99
95	18.59	23.03	0.98	1490.0	0.99
Carrot (cortex)					
70	24.90	1.25	0.93	446.0	0.93
80	19.80	1.38	0.92	750.0	0.99
90	16.17	2.99	0.94	1670.0	0.94
95	9.77	4.60	0.99	2880.0	0.99
Carrot (core)					
70	39.10	0.68	0.99	925.0	0.99
80	38.63	0.69	0.96	930.0	0.96
90	36.50	1.57	0.98	2550.0	0.95
95	26.14	1.98	0.91	3198.0	0.95

Table 3—Paramaters of the biphasic first-order model for lipoxygenase (LOX) heat-labile and heat-resistant fractions

Temperature (°C)	$\frac{K_R E_{R0}}{K_L E_{L0} + K_R E_{R0}} \times 100$	$k_R \times 10^4$ (s^{-1})	R^2	$k_L \times 10^4$ (s^{-1})	R^2
Broccoli (florets)					
70	34.83	0.69	0.97	128.9	0.94
80	34.82	1.26	0.95	223.4	0.95
90	34.74	1.90	0.96	423.0	0.98
95	34.68	2.70	0.98	546.0	0.96
Asparagus (tip)					
80	49.53	2.74	0.97	630.0	0.99
90	44.62	4.60	0.97	1301.0	0.96
95	44.18	6.90	0.98	1808.0	0.99
Asparagus (stem)					
80	39.43	6.90	0.99	350.0	0.94
90	39.01	11.52	0.98	697.8	0.97
95	36.38	13.82	0.99	1050.0	0.98

Table 4—Activation energies (E_a) for temperature-dependence of the thermal inactivation rates of peroxidase (POD) heat-labile and heat-resistant fractions

Temperature (°C)	Heat-resistant fraction		Heat-labile fraction	
	E_a (J/mol)	R^2	E_a (J/mol)	R^2
Broccoli (florets)	5.8×10^4	0.98	7.5×10^4	0.98
Asparagus (tip)	4.3×10^4	0.95	6.7×10^4	0.98
Asparagus (stem)	5.3×10^4	0.96	6.1×10^4	0.96
Carrot (cortex)	8.6×10^4	0.98	9.5×10^4	0.98
Carrot (core)	8.3×10^4	0.98	9.7×10^4	0.96

Table 5—Activation energies (E_a) for temperature-dependence of the thermal inactivation rates of lipoxygenase (LOX) heat-labile and heat-resistant fractions

Temperature (°C)	Heat-resistant fraction		Heat-labile fraction	
	E_a (J/mol)	R^2	E_a (J/mol)	R^2
Broccoli (florets)	5.5×10^4	0.98	6.1×10^4	0.98
Asparagus (tip)	6.5×10^4	0.98	7.6×10^4	0.98
Asparagus (stem)	5.6×10^4	0.99	7.8×10^4	0.99

green beans (5.7×10^4 J/mol against 19.8×10^4 J/mol). Sarikaya and Özilgen (1991) also reported larger *Ea* values for heat-resistant fraction compared to heat-labile fraction of POD in potatoes at blanching temperatures of 65 to 80 °C (10.4×10^4 J/mol against 8.3×10^4 J/mol). In general, the absolute values of *Ea* in this study are in the same range reported for other products; however, differences between *Ea* values of heat-labile and heat-resistant enzyme fractions could be due to experimental conditions and the methodology used to determine the kinetic parameters. It is possible that the trial-and-error adjustment procedure used previously may introduce errors in calculating *Ea* and *k* parameters.

Based on the kinetic parameters obtained for broccoli, carrots (cortex and core), and asparagus (tip and stem) we can observe 2 isoenzyme groups with distinct thermal behaviors. Kinetic parameters also differ when they are evaluated in different parts of the tissue; such was the case of carrots and green asparagus. Thus, our results show that kinetics largely depend on the time-temperature relationship and enzyme source.

Small or large differences in the kinetic patterns found between the different experimental studies cited above, including our results, can be explained by the presence of different isoenzymes depending on origin of vegetables as influenced by physiological stage and environmental conditions (Haard and Tobin 1971; Vámos-Vigyázó 1981; Rothan and Nicolas 1989; Whitaker 1994; Civello and others 1995) and tissue parts coming from vegetables or plants (Lee and Hammes 1979; Wakamatsu and Takahama 1993; Kushad and others 1999). Also, kinetics may be influenced by initial enzyme concentration (Arabshahi and Lund 1985; Saraiva and others 1996) and/or test conditions mainly related to differences in ionic strength and pH of buffer solutions (Saraiva and others 1996).

Thermal stability

Comparing the kinetic parameters *k* for the POD heat-resistant fractions, we observed that broccoli would be the most heat-sensitive; while carrot core section would be one of the most heat-stable (Table 2). For LOX, the thermal stability of the heat-resistant isoenzyme fraction, according to *k* values (Table 3), would be as follows: broccoli > asparagus tips > asparagus stems. These results indicate that the residual enzyme activities observed (Tables 6 and 7) may not be explained solely on the thermal stability of the heat-resistant fractions. It appears that the relative proportions of the isoenzyme heat-labile and heat-resistant fractions, and the total initial enzyme activities (U_0/g), may play a major role.

According to Wang and Dimarco (1972) and Ling and Lund (1978), to obtain the exact proportion of both isoenzyme fractions, it is necessary to determine the reaction rate constants K_L and K_R. However, to have an idea of the relative proportion of heat-resistant isoenzymes, $[E_{RO}/(E_{L0} + E_{R0})] \times 100$, we can use the procedure described by Yamamoto and others (1962). They assumed $K_R \approx K_L$ for the reaction rate constants of the isoenzymes with substrate. Thus, the values of the intercept antilogarithm of Eq. 2a, $[(K_R E_{R0})/(K_L E_{L0} + K_R E_{R0})] \times 100$, shown in Tables 2 and 3, would approximately indicate the relative proportion of the heat-resistant fraction. Afterwards, using Eq. 4 it is pos-

Table 6—Peroxidase activities (U_t/g dry weight) as function of heating times at 95 °C

Vegetable	Heating times			
	0.5 min	1 min	3 min	10 min
Broccoli (florets)	30.07 ± 3.78 (15.1 ± 1.9%)[a]	20.75 ± 0.85 (10.4 ± 0.5%)	5.02 ± 0.54 (2.5 ± 0.3%)	1.57 ± 0.65 (0.8 ± 0.2%)
Asparagus (tip)	0.70 ± 0.06 (18.6 ± 1.7%)	0.67 ± 0.05 (17.8 ± 1.5%)	0.63 ± 0.07 (16.8 ± 1.9%)	0.54 ± 0.05 (14.4 ± 1.4%)
Asparagus (stem)	1.36 ± 0.12 (18.7 ± 1.6%)	1.29 ± 0.15 (17.7 ± 2.0%)	1.17 ± 0.13 (16.1 ± 1.8%)	1.05 ± 0.08 (12.8 ± 1.1%)
Carrots (core)	1.07 ± 0.06 (29.9 ± 1.8%)	0.93 ± 0.07 (26.0 ± 2.0%)	0.89 ± 0.06 (24.8 ± 1.8%)	0.86 ± 0.05 (24.0 ± 1.6%)
Carrots (cortex)	1.60 ± 0.24 (10.5 ± 1.6%)	1.45 ± 0.26 (9.5 ± 1.7%)	1.30 ± 0.21 (8.6 ± 1.4%)	1.22 ± 0.17 (8.0 ± 1.1%)

[a] Values in parenthesis indicate percentage of the initial activity.

Table 7—Lipoxygenase activities (U_t/g dry weight) as function of heating times at 95 °C

Vegetable	Heating times			
	0.5 min	1 min	3 min	10 min
Broccoli (florets)	41.69 ± 1.46 (54.3 ± 1.9%)[a]	28.82 ± 1.41 (37.5 ± 1.9%)	25.25 ± 1.21 (32.9 ± 1.6%)	22.35 ± 0.76 (29.1 ± 1.0%)
Asparagus (tip)	55.30 ± 3.72 (44.5 ± 3.0%)	53.66 ± 2.14 (43.2 ± 1.7%)	51.94 ± 2.48 (41.8 ± 2.0%)	49.84 ± 2.61 (40.1±2.1%)
Asparagus (stem)	46.49 ± 4.10 (39.4 ± 3.3%)	40.95 ± 2.71 (34.7 ± 2.3%)	39.68 ± 3.77 (33.6 ± 3.2%)	34.33 ± 2.36 (29.1 ± 2.0%)

[a] Values in parenthesis indicate percentage of the initial activity.

sible to obtain the proportion of the heat-labile fraction, $[E_{L0}/(E_{L0} + E_{R0})] \times 100$.

When $K_L \approx K_R$ the relative proportion of the heat-resistant POD for broccoli (19.9 ± 3.2%) is lower than that for carrot core section (35.1 ± 6.1%) and similar for cortex section (17.7 ± 6.4%). This could explain the higher relative residual POD activities (%) for carrot core sections compared to broccoli after the thermal treatments (Figures 1a, 1d, and 1e). For green asparagus we also observed high relative residual POD activities (%) for the different temperature-time conditions (Figures 1b and 1c), which could also be explained in part by the high relative proportions of the heat-resistant fraction (tip section, 28.9 ± 12.6% and stem section, 24.0 ± 6.8%).

The relative proportions of LOX heat-resistant fractions when $K_L \approx K_R$ present a different trend, where green asparagus stems > green asparagus tips > broccoli. These larger values of relative proportions of LOX heat-resistant fraction in asparagus (tip section, 46.1 ± 2.9%; stem section, 38.3 ± 1.7%), and broccoli (34.7 ± 0.5%) could explain the higher relative residual LOX activities (%) after each thermal treatment (Figures 2a, 2b, 2c).

Our results suggest that residual enzyme activities of POD and LOX are a result of the thermal stability of the isoenzyme heat-resistant fraction and depend on the relative proportions of the heat-resistant fraction, temperature and heating time.

Another important factor would be the total initial enzyme activity (U_0/g), since a low percentage of residual enzyme activity could still represent a significant enzyme activity (U_t/g) if the initial one was high enough. This could be the case of POD in broccoli (Table 6). According to this we propose to use enzyme activities expressed as U/g instead of percentage of initial activity when necessary to establish the minimal residual enzyme activities to guarantee an appropriate product shelf-life during frozen storage.

Finally, our results would indicate that the pattern of the biphasic first-order model can be explained considering that the initial period of inactivation would be predominantly affected by the kinetics of the component with the highest initial proportion (heat-labile fraction), then the inactivation curve gradually will deviate from the straight line when the proportion of the heat-labile fraction would not be so predominant. Afterwards, as time increases, the inactivation pattern would be affected by the kinetics of the component whose rate constant is the lowest (heat-resistant fraction). Similar characteristics were observed by Fujikawa and Itoh (1996) using a multicomponent first-order model to calculate, by simulation, the thermal inactivation of a mixture of microorganisms.

Conclusions

EXPERIMENTAL RESULTS INDICATE THAT THE BIPHASIC FIRST-order model provides an adequate description for the nonlinear thermal inactivation curves of soluble POD and LOX systems from broccoli, green asparagus, and carrots at 70 to 95 °C. In carrots no LOX activity was detected. For asparagus at 70 °C the LOX inactivation kinetics showed a monophasic behavior of first order. It seems that this phenomenon or behavior at lower temperatures would be a graphical/mathematical artifact.

For successful predictions of the residual enzyme activities, it is necessary to know the kinetic parameters (*k* y *Ea*), the total initial enzyme activities and proportion of the heat-labile and heat-resistant isoenzyme fractions and the enzyme distribution in plant tissue. Finally, these results in combination with a heat penetration model could be used to optimize the blanching process in vegetables, reducing the process time and thus minimizing the loss of nutritional and sensory properties.

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