Kinetic Parameters for the Thermal Inactivation of Peroxidase and Lipoxygenase in Precooked Frozen Brassica Species

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Abstract: Thermal inactivation of peroxidase (POD) and lipoxygenase (LOX), both enzymes present in broccoli and Brussels sprouts, is required before freezing, to obtain high-quality precooked frozen vegetables. Rate constants of a 1st-order biphasic model for the heat-labile and heat-resistant POD and LOX isoenzymes were determined at different temperatures (75, 80, and 90 °C) and the corresponding activation energies were estimated using nonlinear regressions. In the case of Brussels sprouts, the activation energies for the resistant and labile fractions were 56.3 and 62.5 kJ/mol for POD and 63.7 and 65.8 kJ/mol for LOX, respectively. For Brussels sprouts, different precooking times were tested to analyze the effect of residual enzyme activity on quality parameters and sensory attributes, after a frozen storage of 4 mo at −20 °C. A significant reactivation of enzyme activity after frozen storage was observed (especially in the case of POD) for short precooking times (<6 min) leading to low-quality parameters at the interior zone of the vegetable. A precooking time of 6 min at 90 °C allowed an adequate inactivation of LOX and POD obtaining a high-quality final frozen vegetable. A sensory analysis confirmed the global acceptability of the product. The obtained results are relevant to define the precooking stage conditions in the production of frozen cruciferous vegetables.

Keywords: lipoxygenase, peroxidase, precooked frozen Brassicas, quality attributes, thermal inactivation kinetics

Practical Application: Broccoli and Brussels sprouts contain compounds beneficial to health. A current trend in the global "ready to eat" food market is to consume frozen precooked vegetables due to their nutritional characteristics. The industrial processing requires a precooking stage to inactivate enzymes responsible for the deterioration during frozen storage and to optimize quality attributes. Peroxidase and lipoxygenase are enzymes found in these vegetables whose presence is associated with quality-sensory deterioration. Color and sensory attributes were assessed to optimize the process. In this study, results show the necessary processing times during the precooking stage in order to obtain a high-quality frozen product.

Introduction

The insufficient consumption of vegetables is one of the top 10 risk factors that contribute to the mortality (WHO 2003); therefore, there is an increasing tendency of incorporating vegetables to the human diet in a quick and easy presentation compatible with modern life (Danesi and Bordoni 2008). The frozen vegetables are an attractive solution and they constitute a major sector in the global frozen food market. Broccoli (*Brassica olareacea* L. *Italica*) and Brussels sprouts (*Brassica olareacea* L. *gemmifera*) are rich sources of glucosinolates, which are known to have anticarcinogenic properties (Kuroiwa and others 2006). Freezing allows the preservation of vegetables for long storage times. A current trend in the global "ready to eat" food market is to consume frozen precooked vegetables that only requires a minimal reheating process before consumption. Therefore, the industrial process requires a precooking stage not only to inactivate enzymes (blanching) responsible for the deterioration during frozen storage but also to improve sensory quality.

Peroxidase (POD) and lipoxygenase (LOX) are enzymes present in Brassicas and they are often utilized as an index for blanching, because they remain active below 0 °C causing detrimental effect in the quality attributes (Viña and others 2007). LOX is related to off-flavor development and color change whereas POD is the most thermal-stable enzyme in vegetables, facilitating the quantification of the enzyme throughout the thermal processing.

POD (EC 1.11.1.7) belongs to the oxidoreductase class and catalyzes the reaction of hydrogen peroxide or other organic peroxides, which are reduced, whereas an electron donor (AH_2) is oxidized. LOX (EC 1.13.11.12) is an oxidoreductase enzyme which catalyzes the hydroperoxidation of linoleic acid and other polyunsaturated fatty acids containing a *cis*, *cis*-1,4-pentadiene double bond system. Products formed during the enzymatic reaction can degrade hydroperoxides that could react with proteins, peptides, and amino acids producing off-flavors. The production of frozen vegetables includes a thermal processing stage prior to freezing, which should be controlled in order to maintain color, texture, flavor, and the nutritional composition of the Brassicas (Barrett and others 2008; Aires 2015). In order to properly assess the thermal processing stage, the kinetics that describes the enzyme inactivation of POD and LOX in these vegetables should be

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determined. Previous studies indicate that broccoli exhibits a biphasic inactivation curve that depends on the presence of isoenzymes with different thermal stabilities for POD and LOX (Güneş and Bayindirli 1993; Morales-Blancas and others 2002; Anthon and Barrett 2003; Thongsook and Barrett 2005; Anese and Sovrano 2006; Lin and others 2012; Wawire and others 2016). Therefore, it is important to perform experiments to verify the presence of heat labile and heat resistant enzymatic fractions and to propose the corresponding models to obtain the kinetic parameters of both isoenzymes (inactivation rate constants [k] and activation energies [E_a]). Information about the inactivation rate of LOX and POD in Brussels sprouts is scarce in literature. The knowledge of the enzyme inactivation kinetic parameters is very important to establish values of residual activity (after a given precooking treatment) that leads to an acceptable quality during frozen storage of cruciferous. Considering that thermal inactivation of enzymes may be reversible (Bahçeci and others 2005), it is important to analyze if certain reactivation of the enzymes is produced during frozen storage; besides, it is relevant to relate quality attributes, such as color and sensory parameters of the vegetables with the residual enzyme activity (EA) after the thermal treatment and frozen storage.

The objectives of the present work were: to experimentally determine the thermal inactivation kinetics and residual concentrations of LOX and POD in broccoli and Brussels sprouts during the precooking process as affected by temperature and time; to calculate the parameters of a model according to the experimentally observed kinetics for LOX and POD enzymes; to use the obtained information in order to determine adequate time–temperature precooking conditions of Brussels sprouts that are frozen and stored at −20 °C; to analyze enzymes reactivation after frozen storage; to relate residual enzymatic activity of LOX and POD in this vegetable with color measurement and sensory analysis after frozen storage

Materials and Methods

Vegetable samples

Brussels sprouts (*Brassica oleracea* L. *var gemmifera*) and Broccoli (*Brassica oleracea* L. *var italica*) were purchased from agricultural producers of field-grown in the horticultural belt of La Plata city (Buenos Aires Province, Argentina). Broccoli is available year round; Brussels sprouts are a seasonal vegetable and they were obtained between May and June. Assays were carried out along 2 yr. Broccoli inflorescences were used; they were cleaned of leaves and divided into florets; in the case of Brussels sprouts, the external leaves were discarded.

Proximate composition

Moisture content was determined by the indirect method of drying the sample in a vacuum oven LiTekvo model DZF-6030A (LiTekvo Instruments, Minhang District, Shanghai, China) at 70 °C and reduced pressure of 100 mmHg to a constant dry weight (AOAC 2010). The crude protein content was determined by the Kjeldahl method (AOAC 2010) using an appropriate conversion factor for these vegetables (6.25) to convert total nitrogen to crude protein (Lisiewska and others 2009). Fat was determined using the Soxhlet extraction method with ethyl ether (AOAC 2010). Ash content was obtained by calcining the samples in a muffle at 550 °C for 8 h (AOAC 2010). Carbohydrates were determined by difference.

Thermal inactivation of LOX and POD in broccoli and Brussels sprouts

Chemical reagents and buffer. For the POD assays, a substrate solution was prepared using 0.1 mL of hydrogen peroxide (30 g/100 mL solution), 0.1 mL guaiacol (99 g/100 mL solution), and 99.8 mL of potassium phosphate buffer solution (pH 6.5). In the case of LOX, 78.6 μL linoleic acid (>98 g linoleic acid linoleic/100 g solution) were mixed with 78.6 μL of Tween 20 and 10 mL distilled water. The solution was then clarified by adding 1 mL NaOH (1 mol/L) and diluted up to a total volume of 100 mL phosphate buffer (final concentration of linoleic acid 2.5 mmol/L). The buffer solution used for POD and LOX substrate was prepared as suggested by Morales-Blancas and others (2002) using monopotassium and dipotassium phosphate in distilled water. For the POD assays, the buffer concentration of the substrate was 0.1 mol/L and for LOX 0.2 mol/L. The buffer was refrigerated at 4 °C until used.

Enzyme extracts. To obtain the vegetable extracts, the samples were cut and grated to small pieces, in order to increase the surface contact area; then they were mixed with a buffer solution of monopotassium and dipotassium phosphate having a molar concentration of 0.2 mol/L. For both tested vegetables, the proportions were 1.5:100 w/v (g sample/mL buffer) for POD assay. In the case of LOX, the proportions were 20:100 and 30:100 w/v (g sample/mL buffer) for broccoli and Brussels sprouts, respectively. A Stomacher Lab blender model 400 (Seward Medical Limited Co., London, U.K.) was used to homogenize the sample-buffer mixture during 5 min using a special plastic bag. Afterward, the solution was filtered using a single linen cloth separating the solids from the solution; the latter was centrifuged at 18000 × g using a Beckman Avanti model J-25 (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) rotor JA-2550 for 30 min at 4°C in polycarbonate tubes. The supernatants were filtered through filter paper and kept on ice until use.

Enzyme inactivation experiments. Aliquots of extracts were placed in thin-walled polypropylene microtubes (volume < 0.2 mL) often used in PCR analysis. The microtubes were selected in order to attain uniform temperature profiles within the tube and a rapid thermal response; these are conditions suitable for kinetic parameter determination since the heating up time is minimized by the small thickness and dimension of the tubes. The microtubes were purchased from Científica Nacional SA, La Plata-Argentina.

Thermal treatments and enzyme inactivation assays were carried out by immersing the samples in a thermostatic water bath Techne Model FTE-10DDC (Bibby-scientific Ltda, Stone Staffordshire, U.K.) at 3 different temperatures (75, 80, and 90 °C), with contact times ranging between 50 and 400 s. Afterward, samples were immediately plunged into an ice-water bath (0 °C) to stop the enzyme inactivation process, minimizing the cooling time.

POD and LOX enzyme activities (EA) were assessed spectrophotometrically according to the method described by Morales-Blancas and others (2002) by mixing 0.120 mL of enzyme extract with 3.5 mL of the substrate solution. Immediately after mixing the extract with the substrate, the final solution was homogenized using a vortex Arcano model Hx-2000-1 (Arcano, Zhejian, China) before measuring the EA. The EA was measured in duplicate in a Hitachi spectrophotometer model U-1900 (Hitachi High-Technologies Co., Tokyo, Japan). POD activity was measured monitoring the change in absorbance during 400 s at 470 nm. LOX activity was measured monitoring the change in absorbance at 234 nm (UV region) using quartz cuvettes.

The EA was defined as the amount of enzyme necessary to produce an increase of one unit in the value of absorbance during 1 min. The residual activity was determined as EA/EA_0, where EA is the average EA after thermal processing and EA_0 is the initial enzyme activity (fresh vegetable). Enzyme activity at $t = 0$ corresponds to the enzyme activity of extracts without thermal treatment.

Mathematical modeling of enzyme inactivation

Enzyme inactivation kinetics of Brassicas vegetables was analyzed assuming the presence of heat-labile and heat-resistant fractions (Ling and Lund 1978). First-order inactivation kinetics was assumed for each isoenzyme (Gonçalves and others 2009; Zheng and Lu 2011).

The percentage of enzyme activity (%EA) after thermal inactivation in enzyme systems consisting of heat labile and heat-resistant fractions is given by

$$\%EA = \frac{Co_R e^{-k_R t} + Co_L e^{-k_L t}}{Co_R + Co_L} \quad (1)$$

where Co_L and Co_R are the initial concentrations of heat-labile and heat-resistant enzyme fractions, and k_L and k_R are the rate constants for heat-labile and heat-resistant isoenzymes, respectively.

According to the method of Ling and Lund (1978), the labile enzyme fraction is completely inactivated at long heating times. Therefore, the term $e^{-k_L T}$ in Eq. (1) approaches 0, obtaining Eq. (2):

$$\%EA = \frac{Co_R}{Co_R + Co_L} e^{-k_R t} \quad (2)$$

The initial fraction of the heat-resistant enzyme (α_R) obtained by extrapolating the heat resistant curve to zero time (Yamamoto and others 1961) is given by

$$\alpha_R = \frac{Co_R}{Co_R + Co_L} \quad (3)$$

However, for short heating times ($t \rightarrow 0$), the initial resistant fraction can be considered intact, therefore, the term $e^{-k_R T}$ in Eq. (1) tends to one obtaining the following equation:

$$\%EA = \frac{Co_R + Co_L e^{-k_L t}}{Co_R + Co_L} = \alpha_R + \alpha_L e^{-k_L t} \quad (4)$$

The temperature effect on rate constants was described by the Arrhenius law:

$$k = k_{REF} e^{\frac{-E_a}{R}\left(\frac{T_{REF}-T}{T_{REF}\times T}\right)} \quad (5)$$

where k_{REF} is the reaction rate constant at a reference temperature (T_{REF}), R is the universal gas constant ($R = 8.31$ J/mol K), E_a is the activation energy, and T the absolute temperature (K). The reference temperature used for the calculations was the average value of the tested range ($T_{REF} = 353$ K).

To calculate the inactivation rate constants of the resistant isoenzyme (k_R) at each temperature (75, 80, and 90 °C) and the fraction of the heat-resistant enzyme (α_R), a nonlinear regression of Eq. (2) was implemented. The heat labile rate constants were calculated using Eq. (4) by nonlinear regression introducing the previously determined parameter α_R. The obtained parameters at each temperature were α_L and k_L.

The Arrhenius equation (Eq. (5)) was introduced into Eq. (2) and (4); after applying natural logarithms, the following equations were obtained:

$$\mathrm{Ln}(\%EA) = \mathrm{Ln}(\alpha_R) - t k_{R_{REF}} e^{\frac{-E_{aR}}{R}\left(\frac{T_{REF}-T}{T_{REF}\times T}\right)} \quad (6)$$

$$\mathrm{Ln}(\%EA - \alpha_R) = \mathrm{Ln}(\alpha_L) - t k_{L_{REF}} e^{\frac{-E_{aL}}{R}\left(\frac{T_{REF}-T}{T_{REF}\times T}\right)} \quad (7)$$

The activation energy for the heat-resistant fraction was obtained by nonlinear regression of Eq. (6) using all the obtained experimental data at different temperatures and heating times. The dependent variable was Ln(%EA) and the independent variables were temperature and time. For each enzyme, the activation energy of the heat-labile fraction was calculated by nonlinear regression of Eq. (7), where Ln(%EA-α_R) was the dependent variable. The heat-resistant fraction α_R, $k_{R,REF}$, and $k_{L,REF}$ are known parameters previously determined.

Taking into account that the traditional method for the calculation of the activation energies using kinetic coefficients determined at 3 different temperatures led to high errors, the method suggested by Arabshahi and Lund (1985) was applied in order to obtain accurate activation energy (E_a) values within a smaller confidence interval. The use of a nonlinear regression in one step using all enzyme activity values (%EA) as a function of both independent variables time and temperature data increases the number of degrees of freedom, therefore, reducing the standard deviation.

Frozen storage of Brussels sprouts

Brussels sprouts samples were selected in order to have a uniform size for the experiments. The average weight of the sprout heads was 17.8 ± 0.9 g and their mean dimensions were 30.1 ± 1.1 mm width and 44.2 ± 1 mm height. The precooking of Brussels sprouts was carried out in a thermostatic water bath Techne Model FTE-10DDC (Bibby-scientific Ltda, Stone Staffordshire, U.K.) at 90 °C. Samples were removed from the bath at 3, 6, and 10 min and immediately plunged in a water/ice bath. Afterward, they were kept frozen during 4 mo at −20 °C. Enzyme activity (%) of LOX and POD after the thermal treatment and after the final frozen storage time were determined at the apical zone (core) and external layers of the vegetable using a number of samples $n = 6$.

Additionally, quality parameters (color and sensory attributes) were analyzed to correlate these values with the residual enzyme activity. To better visualize the quality of the final products at the core and external layers, digital photographs of the samples were taken.

Color measurements. The surface color of Brussels sprouts was determined using a Minolta colorimeter CR 400 Series (Konica, Osaka, Japan). The Standard illuminant C that corresponds to average daylight (not including ultraviolet wavelength region) with a correlated color temperature of 6774 K, and the 2-degree standard observer angle (that closely matches CIE-1931 standard observer) were used.

The CIE$L^*a^*b^*$ scale was used, and lightness (L^*) and chromaticity parameters: a^* (red–green) and b^* (yellow–blue) were measured. The parameters L^*, a^*, b^* were used to describe the Browning index (BI; Tavera-Quiroz and others 2014) and the Hue angle ($h°$; Mazzeo and others 2011). The Browning index

(BI), which represents the purity of brown color was calculated by Eq. (8) and (9) introducing the parameters (L^*, a^*, and b^*) as follows:

$$\mathrm{BI} = \frac{[100\,(x - 0.31)]}{0.172} \tag{8}$$

where x is defined as:

$$\mathrm{x} = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)} \tag{9}$$

The Hue angle ($h°$) which comes from the polar representation of the (b^*, a^*) cartesian system can be calculated using Eq. (10) in the case when $a < 0$ and $b > 0$:

$$h^\circ = 180 + \tan^{-1}\left(\frac{b^*}{a^*}\right) \tag{10}$$

The $h° = 90°$ is equivalent to a yellow hue taking the CIE scale, as the angle increases the objects are more green (maximum 180°).

Sensory analysis. For sensory evaluation, a 30 member expert panel was selected from the personnel of CIDCA, in La Plata, Argentina. An acceptability test was carried out using a 9-point hedonic scale to determine the degree of liking of the final product (Peryam and Girardot 1952). The data from the 9-point scale are assigned the values 1 to 9 (1 for dislike extremely and 9 for like extremely). Analysis of variance followed by comparisons of means for more than 2 products (Lawless and Heymann 2010) was applied to analyze the information. Sensory analysis was based on the evaluation of 3 samples: S = cooked fresh vegetable, CF1 and CF2 = cooked-frozen samples having different thermal treatment times. The sample (S) consisted in fresh vegetables which were washed, rinsed and then cooked in the water bath (90 °C) during 10 min. The frozen Brussels sprouts precooked 6 min (CF1) and 10 min (CF2) and stored for 4 mo were reheated before consumption. All the samples were analyzed for their global acceptability, evaluating the following attributes: general acceptability, interior and exterior color, texture, and aroma. Samples were presented in disposable trays that contained the 3 samples.

Statistical analysis

The nonlinear regressions to obtain the kinetic parameters and the statistical analysis were performed using the software Origin Pro 8 (Origin Lab Corporation, Northampton, Ma., U.S.A.) and SYSTAT 12.0 (Systat Software, Evanston, Ill., U.S.A.). The error of the parameters were evaluated by standard deviations and confidence interval at 95%. The quality of the regressions were verified taking into account the adjusted coefficient of determination (R^2), the randomness and normal distribution of the residuals, thus allowing to obtain the parameters of the model that achieved the best fit. The analysis of variance (ANOVA) was conducted to assess significant differences between the samples, means were compared by a Fisher LSD test using a 95% confidence level (significant difference, $P \leq 0.05$).

The statistical analysis to detect the effect of precooking conditions on color differences was carried out independently for each vegetable region (apical and external layers). Values of %EA were calculated with reference to the initial activity before thermal treatment.

Results and Discussion

Proximate composition, initial enzyme activities, and inactivation curves

The chemical composition of the vegetables (broccoli and Brussels sprouts) and the total initial enzyme activity (EA_0) expressed in different units are shown in Table 1.

Figure 1A and B show the inactivation curves of POD in broccoli and Brussels sprouts as a function of time at 3 different temperatures (75, 80, and 90 °C). Similarly, Figure 1C and D show the inactivation curves of LOX in both vegetables. The inactivation curves for POD and LOX enzymes in the tested vegetables clearly show a biphasic behavior with isoenzymes having different thermal stabilities, with thermo-labile and thermo-resistant fractions. This behavior has been previously reported in literature for broccoli (Morales-Blancas and others 2002); however, no information was found in literature concerning Brussels sprouts. The biphasic 1st-order model was also proposed for the inactivation of POD in several crops such as tomatoes, potatoes, carrots, asparagus, green beans, green peas, and butternut squash (Yemenicioğlu and others 1998; Kuroiwa and others 2006; Agüero and others 2008; Wawire and others 2016).

The observed lines in Figure 1A, B, C, and D correspond to the fitting of the nonlinear regressions (E and (7)). In the case of POD (Figure 1A and B), it can be observed that during the first 150 s, inactivation of the thermo-labile fraction occurred in both vegetables. At longer times, the residual enzyme activity of the thermo-resistant fraction was less than 20% in both vegetables. For LOX (Figure 1C and D) it can be observed that the thermal inactivation of the thermo-labile isoenzyme takes place during the first 100 s for both vegetables. The residual enzyme activity of the resistant fraction of LOX was approximately 24% for broccoli ranging between 20% and 37% for Brussels sprouts, without showing marked changes during the heating time. These results are in agreement with the residual enzyme activity of LOX of 30% reported in literature for broccoli by Morales-Blancas and others (2002). As can be observed, LOX was not completely inactivated during the thermal process in these vegetables; similar behavior was observed in green asparagus (tip) showing a residual LOX activity of 40% (Morales-Blancas and others 2002). The behavior of the LOX resistant fraction is different from that of POD for both tested vegetables, which would explain why POD is a better indicator of the enzyme inactivation over time.

Table 1–Proximate composition of the vegetables and total initial enzyme activity (EA_0) of the fresh vegetables expressed in different units.

Proximate composition	Broccoli	Brussels sprouts
Moisture (%)	87.06 (1.88)	83.54 (0.55)
Protein (%)	3.16 (0.09)	3.20 (0.09)
Lipid (%)	1.27 (0.02)	0.36 (0.02)
Ash (%)	0.94 (0.02)	1.19 (0.02)
Carbohydrate (%)[a]	7.57	11.74
Total initial enzyme activity		
POD initial activity		
EA_0/g dry weight	345 (20)	457 (65)
EA_0/mL enzyme extract	0.714 (0.04)	1.13 (0.16)
EA_0/mg protein	1.452 (0.081)	2.35 (0.33)
LOX initial activity		
EA_0/g dry weight	69.4 (7.5)	44.9 (4.2)
EA_0/mL enzyme extract	1.7 (0.2)	2.22 (0.21)
EA_0/mg protein	0.274 (0.028)	0.231 (0.022)

Values are reported as means and standard deviations (SD) between parentheses ($n = 6$).
[a]Carbohydrate was determined by difference.

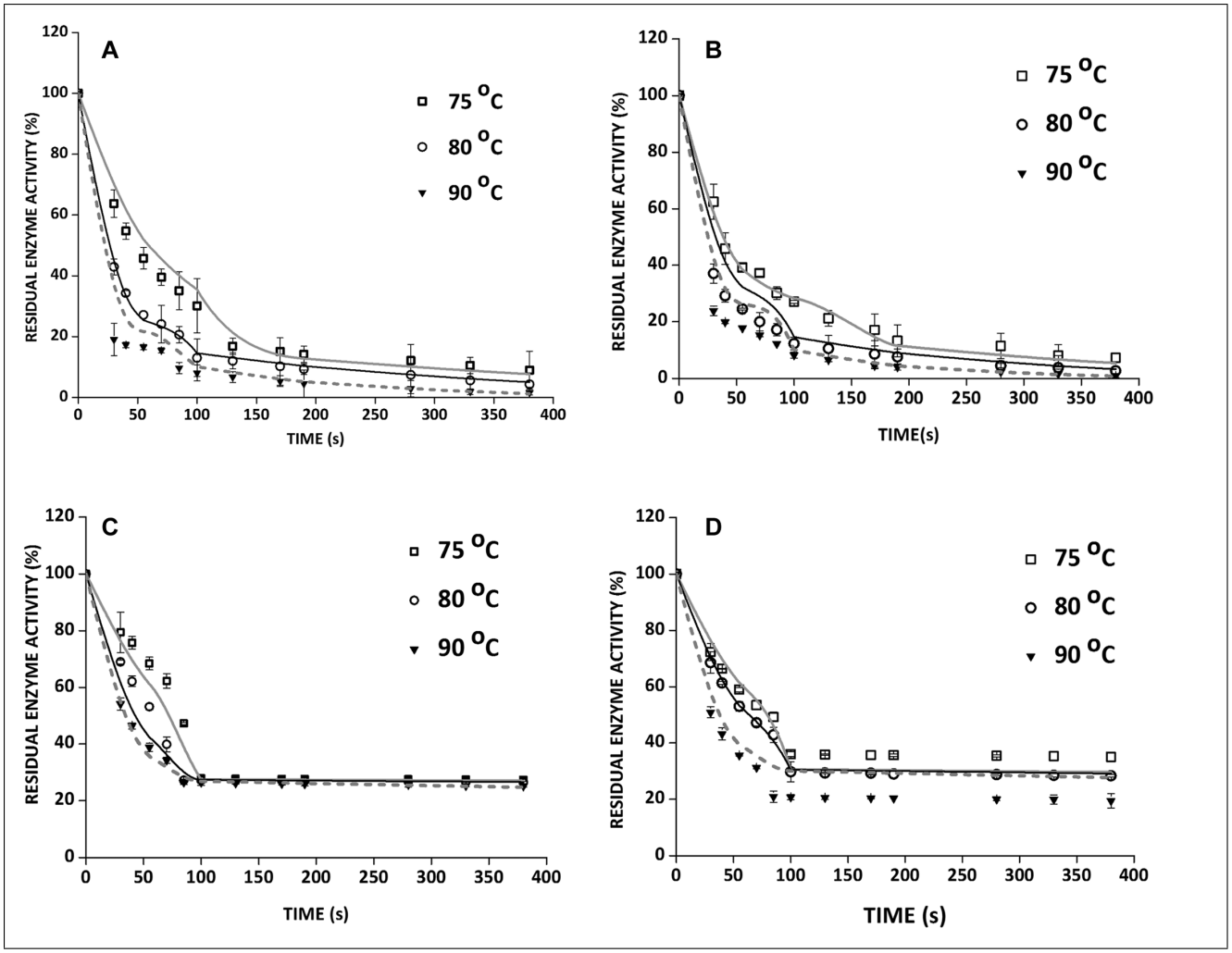

Figure 1–Inactivation curves at different temperatures for peroxidase (POD) in Broccoli (A) Brussels sprouts (B) and for lipoxygenase (LOX) in broccoli (C) Brussels sprouts (D). The error bars show the confidence interval (CI) at 95% of the experimental data obtained in duplicates. Symbols represent experimental values, and lines correspond to the fitting of the non-linear regressions (Eq. (6) and (7)).

Thermal inactivation of POD and LOX: Kinetic coefficients and activation energies

The kinetic rate constants at each assayed temperature are given in Table 2 for POD and LOX, including the corresponding standard deviations and the determination coefficient adjusted for degree of freedom of the nonlinear regressions. A good agreement between the data reported in the presented work and those published by Morales-Blancas and others (2002) for broccoli was observed. In the case of POD, Morales-Blancas and others (2002) reported $k_L = 4.96 \times 10^{-2}$ s^{-1} and $k_R = 3.57 \times 10^{-3}$ s^{-1} at 80 °C and for LOX; $k_L = 2.23 \times 10^{-2}$ s^{-1} and $k_R = 1.26 \times 10^{-4}$ s^{-1} at the same temperature. Gonçalves and others (2009) reported for broccoli a kinetic rate constant of $k = 0.54 \times 10^{-3}$ s^{-1} at 80 °C for POD; however, the authors used a 1st-order monophasic

Table 2–Kinetic inactivation rate constants of POD and LOX at different temperatures (T).

	POD						LOX	
T (°C)	k_L x 10^2 (s^{-1}) $n = 10$	R^2	k_R x 10^3 (s^{-1}) $n = 14$	R^2	k_L x 10^2 (s^{-1}) $n = 8$	R^2	k_R x 10^4 (s^{-1}) $n = 16$	R^2
Broccoli								
75	2.38 (0.09)	0.98	2.41 (0.13)	0.97	1.66 (0.21)	0.99	0.49 (0.5)	0.89
80	5.39 (0.46)	0.95	3.83 (0.19)	0.97	2.89 (0.29)	0.93	1.07 (0.43)	0.98
90	6.66 (0.93)	0.88	6.15 (0.18)	0.99	3.26 (0.03)	0.99	1.79 (0.78)	0.97
Brussel sprouts								
75	3.11 (0.21)	0.95	3.85 (0.36)	0.93	1.85 (0.02)	0.99	0.80 (0.04)	0.98
80	4.24 (0.42)	0.92	5.37 (0.15)	0.98	2.05 (0.02)	0.99	1.61 (0.16)	0.89
90	7.87 (0.18)	0.99	7.47 (0.24)	0.98	2.80 (0.06)	0.98	2.14 (0.18)	0.90

Note: Reported values correspond to the heat-resistant (k_R) and heat-labile (k_L) fractions. Standard deviations (SD) of the constants are given between parentheses and the determination coefficient adjusted for degree of freedom (R^2) are reported.

Table 3–Activation energy of the heat-resistant (E_{a_R}) and heat-labile (E_{a_L}) fractions of POD and LOX and initial fraction of the heat-resistant enzyme (α_R).

Vegetable	Enzyme	E_{a_L} (kJ/mol)	R^2	E_{a_R} (kJ/mol)	R^2	α_R%
Broccoli	POD	66.9(3.6) $n = 30$	0.88	71.2(2.5) $n = 38$	0.94	21.36 (1.03)
Brussels sprouts		62.5(1.9) $n = 30$	0.97	56.3(3.0) $n = 38$	0.92	25.02 (1.04)
Broccoli	LOX	34.6 (9.3) $n = 26$	0.91	108.3 (5.0) $n = 36$	0.85	27.66 (1.00)
Brussels sprouts		65.8 (4.5) $n = 26$	0.97	63.7 (2.8) $n = 20$	0.96	30.97 (1.03)

Note: The values shown are the result of nonlinear regressions; the corresponding standard deviations (SD) are given between parentheses and the determination coefficient adjusted for degree of freedom (R^2) are reported.

kinetic behavior, assuming that there were no difference in the thermostability of the enzymes studied.

Table 3 shows the activation energies of thermal resistant and labile fractions of POD and LOX along with the α_R% for both vegetables. To our knowledge, there are no data in the literature regarding the thermal inactivation kinetic parameters of POD and LOX in Brussels sprouts. The resistant fraction α_R% of both enzymes (LOX and POD) was higher in Brussels sprouts than in broccoli (Table 3). For broccoli, Morales-Blancas and others (2002) reported α_R% = 21.12 for POD and 34.82 for LOX (from data at 80 °C) and Połata and others (2009) estimated α_R% = 26.4 for POD.

With reference to the activation energy values, Sarikaya and Ozilgen (1991) determined E_{a_R} = 104.3 kJ/mol and E_{a_L} = 83.3 kJ/mol for the resistant and labile fractions of POD in potatoes, in the range of temperature between 65 and 80 °C. Güneş and Bayindirli (1993) reported activation energy values for POD heat-labile and heat-resistant fractions in a temperature range of 70 to 96 °C of E_{a_L} = 41.3 kJ/mol, E_{a_R} = 75.3 kJ/mol in peas; in the case of green beans E_{a_L} = 57.3 kJ/mol, E_{a_R} = 77.3 kJ/mol, and for carrots E_{a_L} = 52.3 kJ/mol, E_{a_R} = 57 kJ/mol. Wawire and others (2016) obtained for African cowpea E_{a_R} = 109.67 and E_{a_L} = 256.9 kJ/mol) . In the case of LOX, the obtained values were: E_{a_L} = 46.3 kJ/mol, E_{a_R} = 207.3 kJ/mol in peas and E_{a_R} = 57.3 kJ/mol, E_{a_L} = 198.3 kJ/mol in green beans. Garrote and others (2001) reported E_a = 160.7 kJ/mol for LOX in cut green beans. Morales-Blancas and others (2002) published similar values to those obtained in the present work for POD (E_{a_R} = 58 kJ/mol and E_{a_L} = 75 kJ/mol) and LOX (E_{a_R} = 55 kJ/mol and E_{a_L} = 61 kJ/mol) in broccoli. Anese and Sovrano (2006) reported activation energy values of LOX at 80 °C in tomato extracts (E_{a_R} = 60.8 kJ/mol and E_{a_L} = 147 kJ/mol). In the case of fruits, Lopes and others (2014) obtained E_a = 97.2 kJ/mol for POD in Jubileu clingstone peach; Deylami and others (2014) reported E_a = 35.06 kJ/mol for POD inactivation in mangosteen pericarp.

Effect of the residual enzyme activity on the quality of precooked frozen Brussels sprouts

The overheating prior to freezing leads to quality and nutritional losses. According to Bottcher (1975), the absence of POD indicates overblanching. Williams and others (1986) suggested that for best quality products a residual POD activity in the range of 7.5% to 11% for Brussels sprouts is recommended. For LOX, there is limited information concerning the values of optimum residual activity in cruciferous vegetables.

Table 4 shows %EA of LOX and POD and color parameters of Brussels sprouts that were precooked in a water bath temperature of 90 °C during different times (3, 6, and 10 min) and then frozen and stored at −20 °C for 4 mo.

The vegetable submitted to 6 and 10 min to 90 °C showed at the apical and external layers an adequate residual enzyme activity to obtain a stable product because the heat penetration was sufficient to inactivate enzymes (apical zone before frozen storage: POD %EA = 0.92 and LOX %EA = 19.5). The POD activity before and after frozen storage with 6 and 10 min heat treatment showed no significant differences in %EA for both regions (apical zone and external layers). In contrast, at the apical zone with a thermal treatment of 3 min, the process resulted insufficient (POD %EA = 7.3, LOX %EA = 24.01) leading to changes in color that decreased the quality. The core of the Brussels sprouts constitutes the target zone to be analyzed in terms of %EA and quality parameters, because it represents the coldest point of the system during the thermal treatment. As can be observed in Table 4 at the apical zone and external layers after a precooking time of 3 min, the %EA of POD were 7.3% and 2.7% respectively, being these values below 11% as recommended by Williams and others (1986). In contrast, it can be observed that the sample located at the apical zone and external layer after storage at −20 °C during 4 mo increased the %EA POD to 83.5% and 17.8%, respectively; this result can be attributed to the reactivation of the enzymes in both regions.

Thermal inactivation of enzymes (especially in POD) is reversible and the enzyme can recover the activity under given conditions (Bahçeci and others 2005). The reactivation of POD in broccoli has been previously reported in literature for purified extracts with different characteristics acidic, neutral, and basic (Neves and Lourenço 1998; Thongsook and Barrett 2005; Thongsook and others 2007). It can be pointed out that the main differences between the reported results and this work, lies in the experimental conditions and model system selected to study the enzyme behavior. In this work, the reactivation was measured in vegetables (Brussels sprouts) that were frozen and stored at −20 °C during 4 mo, and then enzyme extracts were obtained from the apical and external layers. In the case of Thongsook and Barrett (2005), the reactivation of the enzyme was measured by using initial extracts from broccoli stems that were first heated, then cooled in ice-water bath, and later incubated at room temperature. The ability of POD to reactivate after it is denatured by heat, depends on several factors: treatment conditions, the species of vegetable, and type of isoenzymes of the same species. The broccoli POD isoenzymes show variations in their reactivation behavior. In Brassica species, the soluble POD activity which contains mainly the acidic isoenzyme has generally been shown to be more heat stable than that associated with the basic isoenzymes extracted from ionically bound fractions (Robinson 1991). The findings of Thongsook

Table 4–Percentage of enzyme activity (%EA) of LOX and POD in Brussels sprouts immediately after the thermal treatment (precooked at a water bath temperature of 90 °C) to different times (3, 6, 10 min) and after frozen storage during 4 mo; $n = 6$ in 2 regions of the vegetable (apical and external layers).

			%EA		COLOR PARAMETERS					
Zone	Pre-cooking Time(min)	Condition of the analysis	LOX	POD	Picture AFS	L*	a*	b*	hº	BI
Apical	3	BFS[1]	24.01 a (1.1)	7.3 a (0.9)		48.1 a (5.5)	−12.7 a (1.2)	23.5 a (0.7)	118.4a (2.4)	41.9 a (5.0)
		AFS[2]	30.6 b (6.3)	83.5 b (1.6)		67.1 b (3.8)	−2.5 d (0.7)	28.7 b (0.6)	95.1 b (1.4)	51.2 b (4.1)
	6	BFS	19.5 c (2.2)	0.92 c (0.05)		44.0 a (0.3)	−10.6 b (3.3)	17.9 c (1.2)	119.9a (7.1)	30.3c,d (6.6)
		AFS	22.9 a (1.0)	1.4 c (0.2)		68.9 b,c (3.4)	−5.9 c (0.6)	24.2 a (2.0)	103.8c (1.2)	35.4 d (4.7)
	10	BFS	17.2 c (1.5)	0.12 c (0.03)		44.1 a (4.0)	−10.1 b (1.2)	17.3 c (1.7)	120.2a (2.9)	29.9 c (7.0)
		AFS	18.4 c (1.1)	0.37 c (0.03)		71.9 c (2.5)	−6.1 c (1.6)	22.9 a (3.1)	105.3c (4.4)	30.7c,d (6.6)
External	3	BFS	23.9 a,b (2.7)	2.7 a (0.3)		45.7 a,b (2.9)	−12.1 a (1.4)	21.5 a (1.1)	119.3a (2.2)	38.5 a (4.1)
		AFS	29.9 c (8.5)	17.8 c (1.1)		47.2 b,d (2.8)	−9.6 b (1.0)	22.9 b (1.2)	112.7b (1.4)	46.6 b (3.3)
	6	BFS	21.9 a (2.0)	0.78 a,b (0.07)		43.2 c (1.6)	−15.1 c (1.6)	22.8a,b (5.6)	126.5c (2.8)	36.6 a (9.5)
		AFS	26.8 b,c (1.2)	1.2 a,b (0.2)		45.6 a,b (2.5)	−15.1 d (1.6)	23.0 b (1.2)	123.4 d (2.3)	37.6 a (4.2)
	10	BFS	20.3 a (0.9)	0.11 b (0.01)		41.8 c (1.1)	−17.6 c (0.7)	18.2 c (0.9)	134.2e (1.7)	17.5 c (3.3)
		AFS	21.3 a (1.1)	0.34 b (0.03)		43.8 a,d (1.8)	−15.2 d (1.5)	17.8 c (2.4)	130.7f (4.5)	20.3 c (8.4)

Note: The color parameters (L^*, a^*, b^*, BI = Browning index, and h° = hue angle; $n = 10$) of the vegetable correspond to conditions before and after frozen storage. Values between parentheses are standard deviations. Digital photographs show the apical and external zone of the vegetable after frozen storage. Different letters in the same column for each zone (apical and external layers) indicate significant differences between the samples ($P \leq 0.05$).
[a]BFS, before frozen storage.
[b]AFS, after frozen storage.

and Barret (2005) show that enzyme reactivation is poor when treatments were at low temperatures for long times in all types of POD extracted from broccoli. The reactivation is therefore a problem in terms of quality loss for high-temperature short times (HTST) processed foods (Schwimmer 1944; Lu and Whitaker 1974; Adams 1978; Rodrigo and others 1996), which is the target objective when maximizing nutrient retention in precooked frozen "ready to eat" vegetables. Thongsook and others (2007) reported that for broccoli and horseradish peroxidases (HRP) the highest reactivation temperature was 90 °C for acidic POD and HRP, whereas it was approximately 70 and 80 °C for basic and neutral PODs, respectively. The reactivation of POD has been suggested to be influenced by changes in the secondary and tertiary structure of the protein and it depends on several factors. Thongsook and others (2007) detected using circular dichroism the percentage of helical structure during thermal treatment and after cooling and incubation at 25 °C. The decrease in the helical structure was regained upon cooling and incubation at 25 °C, but to a lower extent; refolding of the protein structure after cooling is related to an increment in enzyme activity. The Soret band absorbance at 403 nm allows to determine the tertiary structure around the heme active site of the protein. In this case, the authors detected the increase in absorbance upon cooling in thermally treated POD at 403 nm indicating the re-association of the heme to the protein moiety, and this was also correlated with an increase of POD activity. The renaturing of POD was detected in a greater extend by different techniques when the thermal treatment was HTST with respect to lower temperatures longer times.

With reference to LOX, Table 4 shows a small increase of this enzyme activity after frozen storage for 4 mo at −20 °C, but it was less marked compared to POD. The highest %EA of LOX after frozen storage were observed in samples precooked during 3 min at 90°C; these values were 30.6% at the apical zone and 29.9% at the external layers (Table 4). For samples preheated for 6 min, the

Table 5–Sensory analysis results for the fresh vegetables cooked for 10 min in thermostatic water bath at 90 °C (S).

	General acceptability	Interior color	Exterior color	Aroma	Texture
S	6.7a (2.1)	7.1a (2.4)	6.8a (2.4)	7.0a (2.1)	5.8a (2.6)
CF1	6.6a (1.1)	7.2a (2.2)	7.4a (2.2)	6.5a (1.1)	5.8a (2.1)
CF2	6.9a (2.4)	7.4a (2.3)	7.6b (1.2)	7.3a (1.3)	6.9a(2.6)

Note: Brussels sprouts precooked during 6 min (CF1) and 10 min (CF2) and then kept frozen for 4 mo (samples were reheated before consumption). Values between parentheses correspond to standard deviations. Different letters in the same column indicate significant differences between the samples ($P \leq 0.05$).

LOX activity was lower but showed significant differences before and after storage at both tested zones. Only in the case when the thermal treatment took 10 min, the LOX activity showed no differences between the samples before and after frozen storage.

The internal browning of the vegetable can be related with enzyme activity and/or reactivation after denaturing. The color change (browning) at the apical zone can be determined by calculating the browning index (BI) parameter (Table 4). For samples preheated for 6 and 10 min, the BI indicator showed no significant differences before and after frozen storage at the apical and external layers. On the contrary, a thermal treatment for 3 min led to a marked increase of BI after freezing and frozen storage with respect to the initial condition (after precooking); therefore, 3 min was not enough to inactivate enzymes. This fact can be evidenced by the brown color in the photograph of the apical zone. At the apical zone the lightness (L^*) increased as thermal time increased which indicates an adequate inactivation of the enzymes for 6 and 10 min.

For the external layers of Brussels sprouts, as the precooking process time increased, the $h°$ parameter also increased and this behavior was previously observed and reported in literature (Canet and others 2004; Viña and others 2007; Mazzeo and others 2011). The change in greenness can be attributed to different factors; some authors indicate that there is a modification in the Chlorophylls (Chl *a* and Chl *b*) compounds (Kidmose and others 2002; Bahçeci and others 2005), others report that color change is due to air removal from around the cells (Tijskens and others 2001; Canet and others 2004; Mazzeo and others 2015).

A sensory analysis was conducted to further determine the acceptability of the pre-cooked frozen vegetables. Considering that the samples submitted to a precooking time of 3 min deteriorated during frozen storage, they were discarded from the sensory tests.

Table 5 shows the results of the sensory test in which the following attributes were evaluated: general acceptability, interior and exterior color, aroma, and texture in cooked fresh vegetable (S), and cooked-frozen samples having thermal treatment times of 6 min (CF1) and 10 min (CF2).

All the attributes tested, with the exception of the external color, did not show significant differences among the 3 samples ($P > 0.05$), thus indicating that consumers did not detect differences between the precooked frozen vegetables (CF1, CF2) and the fresh vegetable (S).

The score was in the range of 5.8 to 7.6, therefore, according to the panelists, the 3 samples show an acceptable taste. Because LOX has been related to off-flavor development, as well as color change, the sensory test was conducted to detect whether the amount of residual enzyme activity after storage would negatively affect these attributes. Results show a residual LOX activity around 23% did not affect the sensory characteristics; therefore, panelists did not detect aromatic compounds due to the degradation of polyunsaturated fatty acids (Stephany and others 2016). Considering that CF1 and CF2 samples did not differ in the general acceptability from the fresh vegetable (S), a precooking time of 6 min (corresponding to CF1) was selected as an adequate thermal treatment time that could retain more nutrients. The residual enzyme activities (at apical zone) that correspond to the best thermal processing time are $EA_{POD} = 0.92\%$ and $EA_{LOX} = 19.5\%$.

Conclusions

Thermal inactivation curves of POD and LOX enzymes during precooking process (prior to freezing) were experimentally measured in broccoli and Brussels sprouts at different temperatures (ranging between 75 and 90 °C). The parameters of a 1st-order biphasic model were calculated according to the experimentally observed kinetics for LOX and POD. The rate constants for the heat-labile and heat-resistant POD and LOX isoenzymes and their corresponding activation energies were estimated using nonlinear regressions and statistical tools, for minimizing the parameter errors.

For Brussels sprouts, different precooking times were tested to analyze the effect of residual enzyme activity on quality parameters and sensory attributes, after a frozen storage of 4 mo at −20 °C. A reactivation of POD after short thermal treatments was detected at the apical zone of the vegetable, even though exterior enzyme activity and quality parameters were in an acceptable range. For Brussels sprouts, a pre-cooking time of 6 min at 90 °C allowed an adequate inactivation of LOX and POD obtaining a high-quality final frozen product. A sensory analysis confirmed the global acceptability of the vegetable.

These results are useful to optimize the production of precooked frozen cruciferous vegetables, which constitutes an attractive alternative to increase the consumption of healthy and nutritional foods.

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Author Contributions

J. Peréz- Calderón collected the experimental work, analyzed the results, and drafted the manuscript. M.V. Santos designed the experimental work, analyzed and interpreted the results, drafted and reviewed the manuscript. A. Califano reviewed the manuscript. N. Zaritzky designed the experiments, analyzed and interpreted data, and reviewed the manuscript.

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