

Thermostability of Soluble and Bound Peroxidases from Artichoke and a Mathematical Model of Its Inactivation Kinetics

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Summary

Soluble peroxidases (SP), and ionically (IBP) and covalently (CBP) bound peroxidases were extracted from leaves and edible part of artichoke heads (heart). The peroxidase (POD) forms showed a characteristic electrophoretic pattern; in particular SP and IBP forms showed nearly the same pattern, while CBP was quite different. Several basic and acid POD isoforms were present in SP and CBP forms, whereas in IBP only basic components were found. The thermal stability of different POD forms was tested at different temperatures (70, 80, 90 and 100 °C). The leaf POD forms showed greater heat stability compared to the head ones. The heat sensitivity of three POD forms was different: the bound forms were characterized by a greater heat stability than the soluble form. A series-type mathematical model has been developed to describe the inactivation kinetics of artichoke POD. The activation energies for the three POD forms of artichoke leaves and hearts were estimated by an Arrhenius-type relationship. The second step of the inactivation requires more energy to be carried out in all POD forms, especially in SP and IBP.

Key words: artichoke, peroxidase, blanching, thermal inactivation, kinetic parameters, mathematical model

Introduction

Most vegetables and fruits which are preserved by canning, freezing or even dehydration are thermally treated in order to inactivate endogenous enzymes held responsible for off-flavour and off-colour in raw and unblanched cold-stored vegetables (1). Blanching is the common term used to describe a heat inactivation of enzymes naturally present in vegetables and always involves short time heating for periods of 2–3 min at temperatures between 70 and 100 °C.

Peroxidase (POD) activity is often used as an index of blanching (1) since it is considered one of the most thermostable enzymes. These proteins usually contain a ferriprotoporphyrin IX prosthetic group and oxidize several substrates in the presence of hydrogen peroxide (2).

In higher plants, the number of POD isoenzymes may be extremely high, coded by up to 40 genes for each plant, and several other isoforms generated by post-transcriptional and post-translational modifications (3). POD isoenzymes vary in substrate specificity, heat stability, molecular mass, isoelectric point and immunological properties (4,5). PODs have been essentially classified in three classes, supported in the first instance by comparison of amino acid sequence data and confirmed by more recent crystal structure data: class I, intracellular prokaryotic PODs; class II, extracellular fungal PODs; and class III, secretory plant PODs (6).

Thermal inactivation of PODs for a few minutes of blanching at selected temperatures shows non-linear kinetics (7–10).

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Typically, non-linear curve consists of approximately three phases: an initial steep, nearly straight line for a relatively short period, an intermediate curved portion and a third phase with a low, almost horizontal line.

Several mechanisms have been proposed to explain the observed deviation from linear first order kinetics. In many instances, as described by Ling and Lund (11), it is highly probable that mixtures of heat-resistant and heat-labile isoenzymes could be present. Alternatively, it has been suggested that the heat-inactivation process may involve the formation of thermostable aggregates (12) or the process may follow series-type inactivation kinetics with the formation of partially inactivated intermediates (13). It is suggested that this may be due to microheterogeneity in covalently bound oligosaccharide residues at the molecular level (10). Moreover, enzyme is a complex molecule with a three-dimensional structure and it is indeed expected that heat-inactivation may not follow the first order kinetics. For PODs, which also contain a haem subconstituent, it seems more likely that different types of intrinsic conformational and chemical changes will continually take place during the early stages of heat-inactivation.

Adams and Lock (14) showed that haem fragments were formed during inactivation of a commercial preparation of horseradish POD and that its non-linear kinetics could be due to recovery and regeneration of POD activity.

In the crude extracts, the non-linearity of heat inactivation may be due to the presence of a number of isoperoxidases with different thermostability. Forsyth *et al.* (15) found that the anionic isozymes in *Brassica* sp. showed greater capacity in enzymatic activity regeneration after heat treatment, while in potato Boucoiran *et al.* (16) and in orange Clemente (17) found that the more thermostable isoperoxidases were the cationic ones.

After certain heating conditions, inactivated PODs such as horseradish, kohlrabi, tobacco, orange or pears regain their enzymic activity (regeneration) during storage of the extract (1,8,18,19), while no POD reactivation was observed in palm tree, potato, cauliflower, turnip, mango or grape (9,10,18,20) due to the modifications such as digestion, aggregation, loss of prosthetic group, *etc.* (21). The variability in regeneration, depending on the extent of haem polymerization, has been proposed as the major cause of the non-linear first-order kinetics of inactivation; further support for this proposal was provided by the observation that simple first-order inactivation kinetics was followed when no regeneration occurred (7). The study of POD inactivation kinetics could be very useful to optimize blanching parameters utilized to avoid POD regeneration, which causes off-flavour and off-colour formation.

In plant extracts, POD is present in soluble (SP), ionically bound (IBP) and covalently bound (CBP) forms; the first one is distributed within both intra- and extracellular environment (22), while the bound ones are considered to be associated with plant cell walls and possibly with certain organelles, for example mitochondria.

All three POD forms are involved in several physiological processes. In potato, Chaman *et al.* (23) found that pathogen infestation induced an increase in the ac-

tivity of the three POD forms and that a new different isozyme in IBP appeared. The same response was observed by Thomas *et al.* (24) during the development of tomato fruit.

The soluble and bound forms differ among themselves in heat stability and regeneration properties. The ionically bound POD in orange was found to be more heat-stable than the soluble form (19), on the contrary, in apple the soluble POD showed to be the most heat-stable (25). McLellan and Robinson (26) observed that the heat-sensitive ionically bound PODs in Brussels sprouts had a greater ability to regenerate.

In the literature few studies about biochemical properties of artichoke PODs were found (27,28). Moreover, no data were available about thermostability of soluble and bound POD forms, except little information published by Di Venere *et al.* (29).

The main objective of this work is to study the kinetics of the inactivation of SP, IBP and CBP from leaves and hearts of artichoke (*Cynara cardunculus* L. subsp. *scolymus* (L.) Hayek Fiori). A comprehensive mathematical model was developed from experimental data to better understand the kinetic mechanism of the process. Moreover, it is useful to identify the kinetic order of reactions and to determine the basic process parameters and the concentration of all reactive components, including those not subjected to direct experimentation. It is also possible to make predictions for durations of heat treatment which, because of technical difficulties, cannot be measured.

Materials and Methods

The trials were performed on leaves and heads of artichoke (cv. Violetto di Provenza) grown in an experimental field in Policoro (Matera, Southern Italy). Leaves with different physiological stage were used; very old outer leaves and any damaged tissue were not included in the sample. Heads of about 120 g were deprived of external bracts and only the edible part (heart), representing about 40 % of the total mass, was used for the analysis. Enzyme extraction was performed according to Boucoiran *et al.* (16) with minor modifications as mentioned below. Artichoke leaves and hearts (25 g of both) chopped into pieces of approximately 1 cm³ in size were homogenized for 3 min with respectively 100 and 60 mL of Na-acetate buffer (50 mM, pH=5.6) and 2 % polyvinylpyrrolidone (PVP), using a Waring blender. The homogenate was centrifuged (17 400×g, 30 min, 4 °C), giving the soluble peroxidase (SP) as supernatant. The residue from centrifugation was washed three times with Na-acetate buffer (50 mM, pH=5.6), washed twice with cold distilled water (4 °C) and then resuspended with 3 M NaCl and centrifuged (17 400×g, 30 min, 4 °C) to give the ionically bound peroxidase (IBP). To extract the covalently bound peroxidase (CBP), the pellet was incubated overnight with 0.2 M Na-acetate buffer (pH=5.6), pectinase 0.4 g and cellulase 0.2 g, at 35 °C. The solution was centrifuged and the supernatant separated. The extracts of the three POD forms were held at -18 °C until analysis.

Isoenzymatic patterns of soluble and bound PODs were obtained in a vertical gel apparatus (Mini-Protean, Amersham Pharmacia Biotech, USA) by means of discontinuous polyacrylamide gel electrophoresis (SDS-PAGE). The 1-cm stacking (3 %) and separating (10 %) polyacrylamide gels were prepared, as described by Laemmli (30) with some modifications. For the electrophoretic analysis, the different POD forms were first precipitated with acetone and then solubilized in the sample buffer (0.1 M Tris HCl, 0.1 % β-mercaptoethanol, pH=7.2). Before loading on the gel, the IBP and CBP extracts were dialysed against water to remove salts and then concentrated on AMICON YM1 membrane. Samples containing approximately 30 µg of protein were applied to the gel.

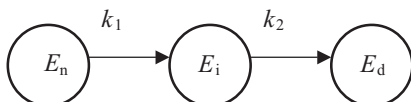
Isoelectrofocusing (IEF) of different POD forms was performed on a 0.4-mm polyacrylamide gel containing 5 % (by mass per volume) acrylamide cross-linked with bis-acrylamide and 5 % ampholyte (pH=3.5–10.0, Amersham Pharmacia Biotech, USA), using a Multiphor II (Pharmacia Corp., USA) system according to the manufacturer’s protocol. The isoelectric point (pI) markers (Pharmacia Corp., USA), ranging from pI=3.5 to pI=9.3, were used as pI standards.

After the SDS-PAGE and IEF runs, the gels were incubated in a solution containing pyrocatechin, *o*-phenylenediamine and H₂O₂ for the detection of enzyme activity (31).

The thermal inactivation was performed by placing Eppendorf tubes in triplicate, filled with 1.5 mL of enzymatic extract, in a thermostatic water bath, previously equilibrated at the required temperatures of inactivation. Crude extracts of the three enzymatic forms were inactivated at different temperatures (70, 80, 90 and 100 °C) for enough time to lower the activity below 1 % of the initial value. After the reaction time, the Eppendorf tubes were immediately cooled in a water-ice mixture to stop the inactivation process. The residual POD activity was determined immediately in triplicate by the change in absorbance at 470 nm due to guaiacol oxidation in the presence of H₂O₂ and POD enzyme (32). The assay measurements were done until the inactivation curves reached an asymptotic straight line.

Data analysis

It is well known (11,33) that some mathematical models may be suggested in order to adequately represent enzyme deactivation kinetics. In particular the following series-type enzyme deactivation mechanism has been repeatedly suggested (34,35):



In this model k_1 and k_2 are deactivation rate constants, E_n is the specific activity of the initial enzyme state, while E_i and E_d represent the specific activities of the single intermediate and final enzyme states, respectively. A kinetic mathematical model of two coupled first order differential equations is introduced:

$$\frac{dE_n}{dt} = -k_1 E_n; \quad \frac{dE_i}{dt} = k_1 E_n - k_2 E_i \quad /1/$$

assuming initial conditions $E_n(0)=E_0, E_i(0)=E_d=0$.

According to Henley and Sadana (34), a weighted function of the specific activities of the two enzyme states may be introduced:

$$A(t) = \frac{E_n(t) + \alpha E_i(t)}{E_0} \quad /2/$$

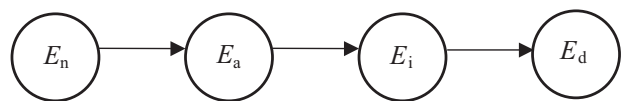
where $A(t)$ is the total activity at the time of inactivation (t). By solving the Eq. 1, the function $A(t)$ explicitly gives:

$$\frac{A(t)}{A_0} = \left(1 - \frac{\alpha k_1}{k_1 - k_2}\right) \exp(-k_1 t) + \frac{\alpha k_1}{k_1 - k_2} \exp(-k_2 t);$$

$$A(0) = A_0 \quad /3/$$

where $\alpha = \frac{E_i}{E_n}$ is the ratio between the specific activities of the intermediate and native forms.

In our experiments a little activation was observed after few seconds of inactivation at high temperatures (90–100 °C). Therefore, in these cases, the validity of the series-type model, with two partially inactivated intermediates, was tested:



Solving the differential equation system, the total activity $A(t)$ results are given by:

$$A(t) = \frac{E_n(t) + \alpha_1 E_a(t) + \alpha_2 E_i(t)}{E_0}; \quad \alpha_1 = \frac{E_a}{E_n}, \quad \alpha_2 = \frac{E_i}{E_n}$$

and explicitly:

$$\frac{A(t)}{A_0} = \left(1 - \frac{\alpha_1 k_1}{k_1 - k_2} + \frac{\alpha_2 k_1 k_2}{(k_1 - k_2)(k_1 - k_3)}\right) \exp(-k_1 t) +$$

$$+ \left(\frac{\alpha_1 k_1}{k_1 - k_2} - \frac{\alpha_2 k_1 k_2}{(k_1 - k_2)(k_2 - k_3)}\right) \exp(-k_2 t) +$$

$$+ \left(\frac{\alpha_2 k_1 k_2}{(k_1 - k_3)(k_2 - k_3)}\right) \exp(-k_3 t); \quad A(0) = A_0 \quad /4/$$

The results of our analysis gave very similar values of k_2 and k_3 , differing only in the fourth decimal. Moreover, α_1 assumed values between 1.4 and 2.1 and α_2 showed very low values, in the order of 10^{-3} .

In conclusion, the mathematical model given in the Eq. 3 represented well the POD thermic deactivation kinetics also when a rise in activity with time occurred if we assumed that $\alpha > 1$ (34). The experimental data were analyzed by non-linear regression technique with Lab Fit (Curve Fitting) Software.

It is well established that the rate constant values increase as the temperature rises. Quantitatively, the relationship between the reaction rate increase and the process temperature is regulated by the Arrhenius equation:

$$\ln(k_i) = -E_{ai}/RT + \text{constant}; \quad i=1, 2 \quad /5/$$

which was used to calculate activation energies.

In the Eq. 5, k_1 and k_2 are the kinetic parameters estimated by fitting the experimental data for each given value of the temperature, E_{ai} is the activation energy, R is the universal gas constant (8.314 J/mol K) and T is the temperature in Kelvin degrees. The Eq. 5 was estimated by using a standard software for linear regression.

Results and Discussion

Three POD forms: SP, IBP and CBP were extracted from artichoke hearts and leaves. The sum of SP, IBP and CBP activities in hearts was about 40 % of the total POD activity found in leaves. All three POD forms were present in different amounts in both hearts and leaves. In artichoke hearts SP was found to be 6 times more abundant than IBP and 33 times more than CBP, whereas in artichoke leaves SP was found to be 5 times more abundant than IBP and about 200 times than CBP (29).

The POD forms showed very similar SDS-PAGE patterns in hearts and leaves; in particular SP and IBP forms were similar, with two groups of bands very evident at about 60 and 35 kDa and another zone at 100 kDa with light intensity. On the contrary, CBP pattern was different; it showed two groups of bands with molecular mass between 60 and 90 kDa (Fig. 1).

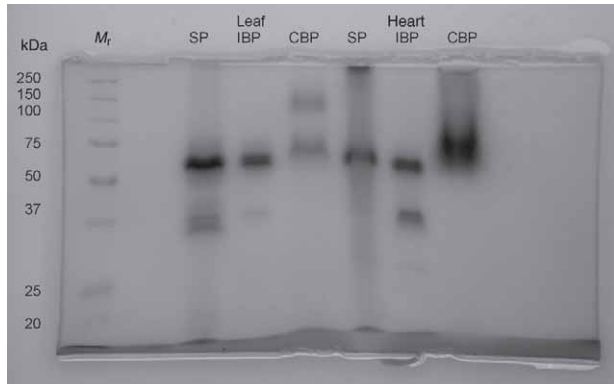


Fig. 1. Specific staining of SP, IBP and CBP forms from artichoke leaf and heart after modified SDS-PAGE; pre-stained molecular mass markers (M_r)

The isoelectric points of the isoenzymes obtained by thin layer isoelectric focusing (IEF) are shown in Fig. 2. The IEF separation of POD forms was also found very similar in hearts and leaves. A very basic isoform (pI=9.5) was found to be present in all POD forms, but IBP from hearts also showed another basic isoform with slightly lower pI. SP and CBP forms showed one isoform with pI=3.5 and a group of bands with pI ranging between 4.5 and 6, whereas acidic isoforms were not present in IBP. This also happens in fruits such as grapes (9) and vegetables such as cabbage and Brussels sprouts (36).

The not very good resolution obtained in the SDS-PAGE and IEF separation of POD was probably due to high percentage of glycosilation of this enzyme (6).

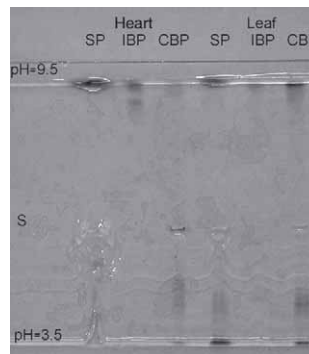


Fig. 2. Specific staining of SP, IBP and CBP forms from artichoke leaf and heart after IEF (S=application point)

The three POD forms, extracted from artichoke hearts, showed different behaviour when inactivated at several temperatures (Fig. 3). The IBP showed to be, at any temperature, the most heat-resistant POD compared to the other forms. This result is similar to those obtained by Moulding *et al.* (8) in peel pears and by McLellan and Robinson (19) in orange juice and albedo, while in other species like Brussels sprouts the behaviour was the opposite (26).

About 50 % of residual activity was found in IBP after 60 seconds of heating at 100 °C; while in SP and CBP the residual activity was 20 and 40 %, respectively. The three POD forms reached, within 2 min at 90 °C, residual activity values between 10 and 15 %. After 10 min at 80 °C residual POD activities reached very low values (10 % or less), while a 10-minute treatment at 70 °C reduced the activity to 32 % in SP, 56 % in IBP and 26 % in CBP.

Heat treatments at higher temperatures (90–100 °C) produced in some cases a considerable enzyme activation (about 10–20 %) within the first 15 s. This phenomenon was observed in all three POD forms. It was reported that in kohlrabi (*Brassica oleracea*) heat inactivation of POD was also preceded by an increase in the activity. This phenomenon was attributed to the transformation of the native enzyme, prior to inactivation, into a transient active form with an activity different from the initial, which was then, in turn, inactivated (18).

POD forms from leaves showed higher stability to heat inactivation compared to those from hearts. This result is always true for the SP at any temperature, whereas a little reversal trend was found in IBP at 80 °C, CBP at 70 and 80 °C and during the first seconds at higher temperatures (90–100 °C).

These results show that there is a threshold of temperature (80 °C) below which the process of heat inactivation of POD does not occur efficiently. Indeed, heat treatments at 70 °C for 10–20 min reduce the enzymatic activity no more than 40–50 %. This is a very important outcome from a technological point of view; in fact our results suggest that it could be very difficult to perform a good blanching of artichoke hearts at temperatures below 80 °C.

At 100 °C, the blanching time is a very critical factor; a few seconds can produce different effects on each POD form. Therefore, in evaluating the efficiency of blanching treatments, it is very important to consider the be-

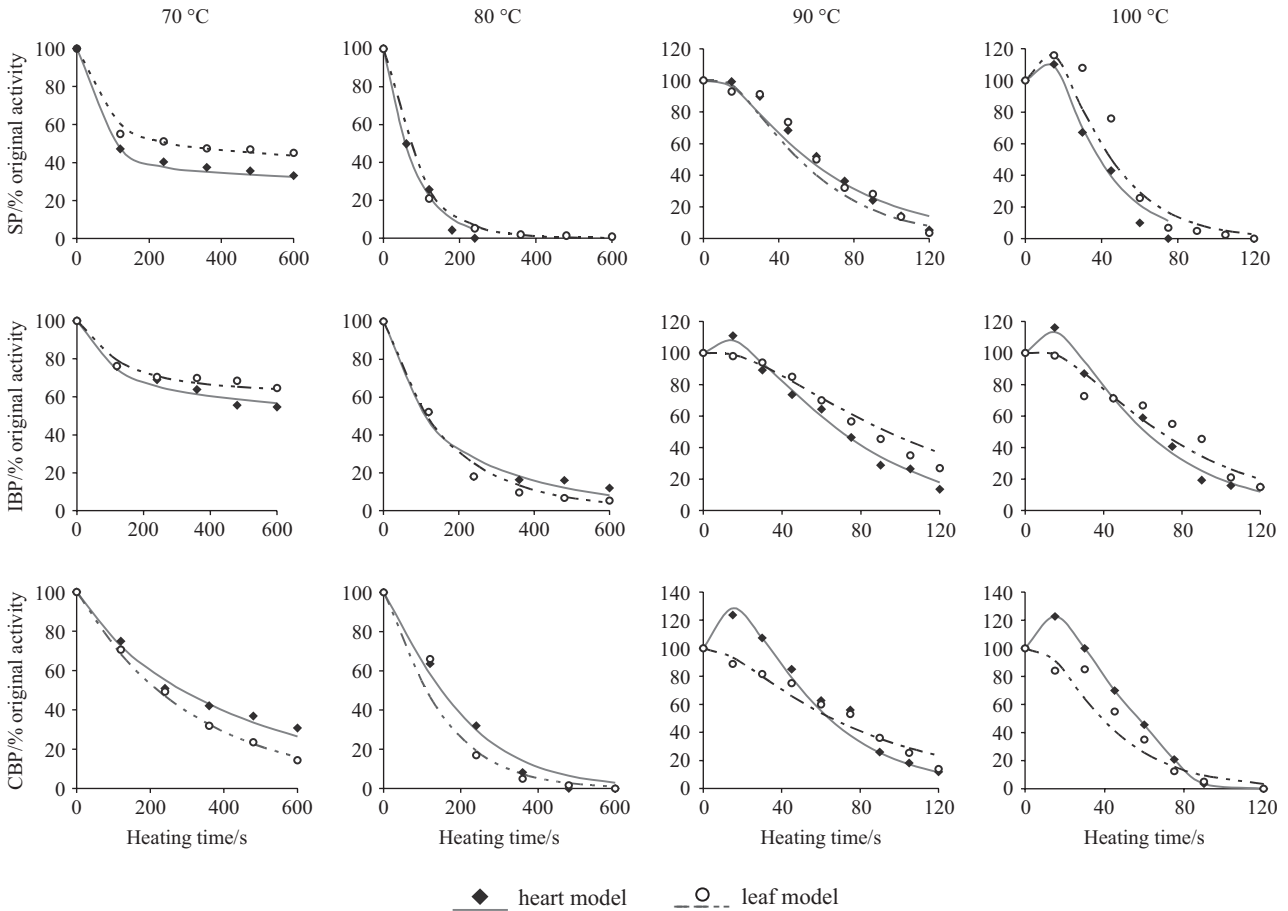


Fig. 3. Heat inactivation kinetics of SP, IBP and CBP forms from artichoke leaf and heart at 70, 80, 90 and 100 °C plotted against model output. Data are average values of $N=3$ experiments (maximum standard error, $SE=2.3\%$)

haviour of each POD form; although the SP form is totally inactivated, the other forms could still be active and produce oxidation phenomenon in frozen artichokes.

A numerical analysis of the adopted model was performed and all the kinetic parameters were estimated

(Table 1). The predicted activities were calculated and compared with the experimental data as reported in Fig. 3. The dashed and dotted lines express the predicted behaviour of activities under different conditions, while experimental data are represented by points.

Table 1. Kinetic parameters (and their 95 % confidence intervals) calculated by the model for SP, IBP and CBP forms from artichoke leaf and heart at 70, 80, 90 and 100 °C

		Leaf			Heart		
		$k_1/(10^{-2}s^{-1})$	$k_2/(10^{-2}s^{-1})$	α	$k_1/(10^{-2}s^{-1})$	$k_2/(10^{-2}s^{-1})$	α
SP/°C	70	1.300±0.196	0.030±0.004	0.510±0.0196	1.500±0.110	0.030±0.005	0.380±0.028
	80	1.700±0.098	1.100±0.196	0.330±0.0196	1.800±0.098	1.400±0.190	0.340±0.020
	90	3.000±1.960	5.000±0.450	1.350±0.0860	2.000±0.098	7.000±0.490	1.400±0.196
	100	4.000±0.249	8.000±1.560	2.450±0.1960	4.400±0.530	9.500±0.650	2.400±0.345
IBP/°C	70	0.800±0.210	0.010±0.002	0.670±0.096	1.000±0.260	0.028±0.011	0.650±0.034
	80	1.000±0.100	0.480±0.058	0.370±0.013	1.400±0.390	0.330±0.100	0.450±0.092
	90	1.300±0.190	3.200±0.560	1.200±0.143	2.200±0.190	4.300±1.490	1.650±0.457
	100	2.000±0.098	4.000±0.686	1.300±0.147	2.500±0.098	6.500±0.490	2.300±0.196
CBP/°C	70	0.700±0.058	0.300±0.078	0.540±0.049	0.980±0.078	0.200±0.039	0.700±0.025
	80	1.100±0.057	0.900±0.098	0.500±0.149	1.000±0.098	0.700±0.058	0.650±0.039
	90	1.400±0.190	7.500±1.490	1.100±0.113	2.700±0.196	7.000±0.882	3.100±0.098
	100	3.400±0.290	8.000±1.960	1.400±0.392	3.100±0.294	9.000±0.588	3.000±0.098

Fig. 4 shows the satisfactory adequacy of the model in describing the experimental data. The scatter plot of experimental *vs.* predicted activity values evidences a convergence quantified by $R^2=0.983$.

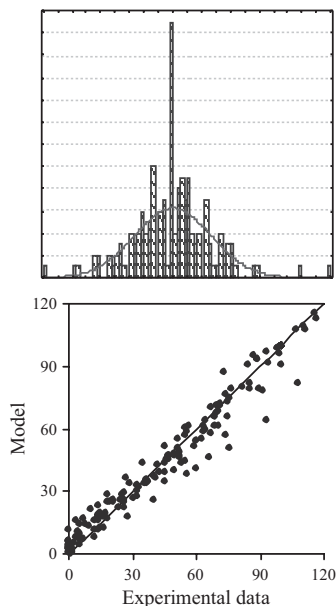


Fig. 4. Global diagnostics of the model fitting: experimental *vs.* calculated values. The small plot at the top is the error distribution. The coefficient of determination R^2 of the experimental *vs.* calculated values linear fit is 0.986

Table 1 indicates the values of k_1 , k_2 , and α with their 95 % confidence intervals that were estimated in the different experimental conditions. The values of the rate constants, k_1 and k_2 , increased with the increase of inactivation temperatures in all experimental conditions. In particular, the rate constant k_1 assumed values greater than k_2 in the 70–80 °C range for SP, IBP and CBP from leaf and heart extracts, while in the 90–100 °C range the rate constant k_2 assumed values greater than k_1 in all cases. Therefore, we had two opposite kinetic behaviours: in the range 70–80 °C the reaction $E_n \xrightarrow{k_1} E_i$ proceeded faster than $E_i \xrightarrow{k_2} E_d$, while the opposite happened in the range 90–100 °C.

Moreover, as it is possible to observe in Table 1, when a slight reactivation in the first phase of the inactivation curves occurred, the values of α increased.

The activation energies (E_a) for three POD forms from artichoke leaves and hearts estimated by an Arrhenius plot are reported in Table 2. E_{a2} values are mark-

Table 2. Estimated activation energies (\pm standard error of regression with probability of 95 %) of SP, IBP and CBP forms from artichoke leaf and heart

	Leaf		Heart	
	$E_{a1}/(\text{kJ/mol})$	$E_{a2}/(\text{kJ/mol})$	$E_{a1}/(\text{kJ/mol})$	$E_{a2}/(\text{kJ/mol})$
SP	41.89 \pm 2.330	196.21 \pm 25.79	35.10 \pm 6.33	202.95 \pm 29.17
IBP	31.90 \pm 2.160	213.52 \pm 29.83	34.16 \pm 2.28	202.33 \pm 18.29
CBP	52.74 \pm 5.414	127.92 \pm 14.33	47.23 \pm 7.27	146.54 \pm 14.17

edly higher than E_{a1} , reaching values 4–6 times higher in SP and IBP, while in the CBP E_{a2} is three times higher than E_{a1} . These results suggest that the second step of the inactivation reaction requires more energy than the first one.

Conclusions

This research is a contribution to the characterization of the SP, IBP and CBP peroxidase from leaves and hearts of artichoke. The results show that the three POD forms have different heat sensitivity; the bound forms (IBP and CBP) are characterized by a greater heat stability than the soluble one. This information could have a great importance from the biochemical as well as technological point of view. To perform a good blanching it is important to inactivate all POD forms to avoid oxidation phenomenon in frozen artichoke, avoiding the overblanching that might impair the quality of the canned or frozen product, causing deterioration of colour, consistency and flavour, as well as losses of valuable components, *e.g.* vitamins, proteins, amino acids, *etc.* In addition, in order to better understand the heat inactivation processes, the separation and purification of different isoperoxidases from artichoke are now in progress to study their contribution to thermostability.

The adopted series-type mathematical model adequately describes the inactivation of artichoke POD and helps to understand the kinetic mechanism of the studied process. Although a series-type heat-inactivation model can be formulated so that it includes more than one irreversibly inactivated intermediate, a single intermediate model leads to a satisfactory agreement between calculated and observed data, as was also reported in other studies (34).

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