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Red Cabbage (*Brassica oleracea*) as a New Source of High-Thermostable Peroxidase

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ABSTRACT: Soluble and membrane-bound peroxidases (PODs) were extracted from red cabbage using Triton X-114. Optimum activity was obtained at pH 4.0 for both enzymes, and both were inactivated by sodium dodecyl sulfate (SDS). The K_M and V_m values for H_2O_2 were found to be 0.98 mM and 8.1 μM/min, respectively, for soluble POD and 0.82 mM and 6.1 μM/min, respectively, for membrane-bound POD. When the 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid (ABTS) concentration was increased, maintaining a steady concentration of H_2O_2, the activity was inhibited at the highest ABTS concentrations in soluble POD. Ascorbic acid was found to be the most active modulator of POD activity. The effect of cyclodextrins was also studied, and the complexation constant between ABTS and hydroxypropyl-β-cyclodextrins (HP-β-CDs) was calculated (K_c = 312 M^{-1}). Membrane-bound POD is more thermostable than soluble POD, losing >90% of relative activity after 5 min of incubation at 76.6 and 30.2 °C, respectively.

KEYWORDS: *Peroxidase, kinetic parameters, thermal inactivation, SDS, reducing agent, cyclodextrin*

INTRODUCTION

Red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*) is a native vegetable of the Mediterranean region and southwestern Europe that is now grown all over the world. Red cabbage belongs to the family Brassicaceae, whose annual world production is 70 million tons.[1]

Although different brassicaceous crops are cultivated under various climatic conditions, they are generally cool-weather crops, with an optimum temperature that varies between 14 and 21 °C depending upon the variety and minimum and maximum temperatures of 4−30 °C.[2] Consequently, brassicaceous crops are produced in both temperate regions and the colder seasons or high altitudes in tropical and subtropical regions. They generally prefer deep, well-drained, fertile, friable, sandy or silty loam soils, and an approximately neutral pH. The optimum pH is 6.5 for mineral soils and 5.8 for organic soils.[3]

There is increasing evidence that a high consumption of *Brassica* vegetables, for example, broccoli, red cabbage, kale, mustard greens, Brussels sprouts, and cauliflower, reduces the risk of several types of cancer.[4,5] The anticarcinogenic effect of these vegetables has been attributed to glucosinolate decomposition products, indoles, isothiocyanates,[6] and phenolic compounds. Red cabbage is a rich source of phenolic compounds, particularly flavonoids. These can be classified into at least 10 chemical groups. Of these, flavones, flavonols, flavanols, flavanones, anthocyanins, and isoflavones[7] are the most abundant types in red cabbage. These phenolic compounds are subjected to oxidation, a process that involves peroxidase (POD) and polyphenol oxidase (PPO) enzymes.

PPO (EC 1.14.18.1) is a copper enzyme, which, in the presence of oxygen, catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to their corresponding *o*-quinones (catecholase activity).[8] These, in turn, are polymerized to undesirable brown, red, or black pigments.[9] In plants, PPO is predominantly located in the chloroplast thylakoid membranes, and its phenolic substrates are mainly located in the vacuoles, but upon any cell-damaging treatment, the enzyme and substrates may come in contact, leading to rapid oxidation of phenols.[10] These reactions lead to changes in physical, chemical, or nutritional characteristics.[11] PPO has been intensively studied in several plant tissues, such as grape,[12] peach,[13] persimmon,[14] iceberg lettuce,[15] and pineapple.[16]

PODs (EC 1.11.1.7) are haem proteins that contain iron(III) and protoporphyrin IX as the prosthetic group. These are a group of oxidoreductases that catalyze the reduction of peroxides, such as hydrogen peroxide, coupled to the oxidation of a variety of organic and inorganic compounds.[17] The enzyme is reported to exist in both soluble and membrane-bound forms[18] and may be found in vacuoles, tonoplast, plasmalemma, and inside and outside the cell wall.

PODs are ubiquitous in nature and have been implicated in a broad range of physiological functions in plants. Lignin biosynthesis and degradation in cell walls, defense against pathogens and the environment, and metal stress response are some of the proposed functions.[19] POD shows wide substrate specificity, a characteristic that makes it useful in a number of industrial and analytical applications, clinical and biochemical analysis of biomarkers,[20] and commercial production.[21] PODs are used commercially as catalysts for phenolic resin synthesis and components of medical diagnosis kits.[22]

The involvement of POD in browning processes has been reported by many researchers. However, this POD activity is

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limited by the availability of electron-acceptor compounds, such as superoxide radicals, hydrogen peroxide, and lipid peroxides. POD is involved in these processes because diphenols may function as reducing substrates in this reaction.[18]

The browning damage caused in the tissues of fruits and vegetables during postharvest handling and processing is one of the main causes of quality loss. POD, which is responsible for a multitude of quality and flavor alterations in fruits and vegetables,[23] may be associated with losses in color, flavor, and the nutritional values of raw and processed food.[24]

To prevent undesirable reactions, heat treatment, which inactivates the enzyme, is usually used for the preservation of many foods. However, the application of heat treatment is limited by the alterations in sensory characteristics and loss of nutrients, which may be caused by this process.[25] POD is the most thermally resistant enzyme in vegetables, and for this reason, it is usually used as an indicator of heat treatment in food processing. Consequently, POD has been widely used as a biological indicator of the effectiveness of blanching.[26]

The aim of this work was to extract and characterize soluble and membrane-bound PODs from red cabbage and to determine their kinetic parameters and thermal stability to maximize the quality and minimize the economic and nutritional loss induced by this oxidative enzyme during storage or processing.

Moreover, another objective of this paper was to obtain a novel and cheaper POD alternative to horseradish POD with high thermostability.

MATERIALS AND METHODS

Plant Material. Fresh red cabbage was obtained from a local market in Murcia and washed with distilled water. Only the leaves were used for POD purification. The samples were stored at 4 °C until use.

Reagents. Reagents were purchased from Sigma (Madrid, Spain) and used without purification. Triton X-114 was obtained from Fluka and was condensed 3 times, as described by Bordier,[27] using 100 mM sodium phosphate buffer (pH 7.3). The detergent-rich phase of the third condensation had a concentration of 25% Triton X-114 (w/v). Hydroxypropyl-β-cyclodextrins (HP-β-CDs) were from TCI (Europe).

The hydrogen peroxide solutions were freshly prepared every day, and their concentrations were calculated[28] using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$.

Protein Determination. The protein content was determined according to Bradford's dye binding method,[29] using bovine serum albumin (BSA) as a standard. Analyses were performed in triplicate for each sample.

POD Extraction. Red cabbage POD was extracted using the method described by Núñez-Delicado et al.[30] All extractions were performed in triplicate, as explained below.

Red cabbage (50 g) was washed and homogenized for 5 min with 50 mL of 100 mM sodium phosphate buffer (pH 7.3). The homogenate was filtered through four layers of gauze and centrifuged at 4000*g* for 15 min. The supernatant was used as a source of enzyme soluble, and the precipitate was extracted with 20 mL of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3). The mixture was subjected to temperature-induced phase partitioning, kept at 4 °C for 15 min, and then warmed to 37 °C for 15 min. At this time, the solution became spontaneously turbid because of the formation, aggregation, and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins, anthocyanins, and phenolic compounds.[31] This turbid solution was centrifuged at 10000*g* for 10 min at 25 °C. After centrifugation, the detergent-rich phase was discarded and the clear detergent-poor supernatant, which was used as a source of membrane-bound enzyme, was stored at −20 °C.

Enzymatic Activity. The POD activity was followed spectrophotometrically in a Shimadzu model UV-1603 spectrophotometer at the absorption maximum of the 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid (ABTS) radical cation[32] of 414 nm ($\varepsilon_{414} = 31.1\ mM^{-1}\ cm^{-1}$). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of ABTS radical per minute.

The standard reaction medium (kept at 25 °C) contained 100 mM sodium acetate buffer (pH 4.0), 20 μg/mL protein, 2 mM ABTS, 4 mM H_2O_2, and 0.2 mM tropolone, in a final volume of 1 mL.

Optimum pH. The optimum pH profile was obtained in the standard reaction medium, which consisted of 100 mM sodium acetate buffer (pH 3.0−5.0), 100 mM sodium phosphate buffer (pH 6.0−7.5), and 100 mM sodium borate buffer (pH 8.0−9.0), in the absence and presence of 1.5 mM sodium dodecyl sulfate (SDS) for membrane-bound POD and 0.5 mM SDS for soluble POD.

In the SDS standard assay, samples contained the above mixture and increasing concentrations of SDS in the cuvette.

In the standard assay using reducing agents, samples contained the above mixture and increasing concentrations (0−3.5 mM) of ascorbic acid, L-cysteine, or metabisulfite in the cuvette.

To determine the kinetic parameters of POD, the effects of ABTS and H_2O_2 concentrations on the enzymatic activity were studied. To study the effect of the ABTS concentration, it was increased from 0 to 5 mM for membrane-bound POD and from 0 to 12 mM for soluble POD, in the standard reaction medium. In the case of H_2O_2, its concentration was increased from 0 to 7 mM for both membrane-bound and soluble PODs in the standard reaction medium, at a fixed ABTS concentration (2 mM).

Enzyme Activity in the Presence of Cyclodextrins (CDs). The CD standard medium at 25 °C contained 100 mM sodium acetate buffer (pH 4.0), 20 μg/mL protein, 4 mM H_2O_2 for both membrane-bound and soluble PODs, and increasing concentrations of HP-β-CDs (0−35 mM) and ABTS (1, 2, or 4 mM) for membrane-bound POD and increasing concentrations of HP-β-CDs (0−40 mM) and ABTS (0.5, 2, or 4 mM) for soluble POD.

Optimum Temperature. The optimum temperature was determined by measuring the enzymatic activity between 5 and 70 °C at 5 °C steps using the optimum pH. The temperature was controlled using a TCC controller, Shimadzu Corporation (model TCC-240A).

Thermal Stability. The enzyme solutions (in Eppendorf tubes) were incubated in a circulating water bath, Julabo Shake Temp SW 22, at different temperatures (50, 60, 70, and 80 °C for membrane-bound POD and 30, 40, 50, 70, and 80 °C for soluble POD) for different times. After heating, the samples were cooled in ice water and assayed immediately at 25 °C.

The statistical analysis was performed. Each sample was assayed in triplicate, and the mean ± standard deviation was plotted.

RESULTS AND DISCUSSION

Enzyme purification in plant extracts is a difficult process because of the presence of a large variety of secondary products that can bind strongly to the enzymes and change their characteristics.[33] To overcome this problem, different methods have been developed, such as using detergents. In this paper, POD was extracted by a phase-partioning method with the detergent Triton X-114.[34] This detergent has a cloud point at 23 °C, which allows it to be used in a temperature range compatible with protein stability. During the phase separation induced by incubation at 37 °C, most of the detergent, plastidic pigments, phospholipids, phenolics, and very hydrophobic membrane proteins migrate to the lower detergent-rich phase, whereas polar phenolics and many other membrane proteins migrate to the upper aqueous detergent-poor phase.[13] The removal of phenols by Triton X-114 was sufficient to avoid browning of the enzyme solution, even after many cycles of freezing and thawing or months of storage at −20 °C.

The enzymatic activity of POD from different vegetable sources has been studied using different substrates, such as guaicol in horseradish roots[21] and lentils,[20] ABTS in turnips,[35] *p*-chlorophenol and *o*-cresol in Indian turnip roots.[36] In this paper, red cabbage was studied using ABTS as the hydrogen donor and H_2O_2 as the hydrogen acceptor. The pH is a determinant factor in the expression of enzymatic activity. Figure 1 shows the pH profile for the oxidation of ABTS by

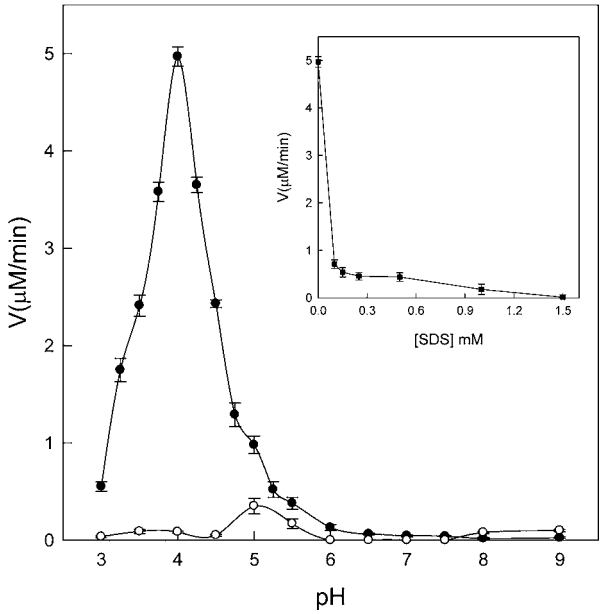

Figure 1. Effect of pH on membrane-bound POD activity in the (●) absence or (○) presence of 1.5 mM SDS. (Inset) Effect of the SDS concentration.

membrane-bound POD (●) in a pH range from 3.0 to 9.0. The optimum pH was found to be 4.0 (Table 1), which is similar to that obtained for marula fruit[37] and lower than that obtained for melon,[38] pepper,[24] or red alga.[17]

Table 1. Kinetic Characterization and Thermal Inactivation Parameters Obtained for *B. oleracea* PODs

		membrane-bound POD	soluble POD
Kinetic Characterization			
K_M (mM)	H_2O_2	0.82	0.98
	ABTS	0.2	0.61
V_m (μM/min)	H_2O_2	6.1	8.1
	ABTS	5.2	8.9
K_{si} (mM)	ABTS		24.3
optimum pH		4.0	4.0
optimum temperature (°C)		45	30
Thermal Inactivation			
inactivation range (°C)		50−80	30−80
E_a (kJ/mol)		143.1	38.5
D value of 5 min (°C)		76.6	30.2

The activating or inhibiting effect of SDS on different enzymes, including PPO and POD, has been widely described. In the case of POD, SDS showed an inhibitory effect in many cases.[39,40]

The pH profile of membrane-bound POD was analyzed in the presence of 1.5 mM SDS (○ in Figure 1) when the enzyme was found to be inactivated at all of the studied pH values. A similar effect has previously been described for horseradish POD,[40] crimsom seedless table grape POD,[39] and red alga *Mastocarpus stellatus* POD.[17]

In addition, the effect of SDS depended upon the surfactant concentration, as shown in the inset of Figure 1. The enzymatic activity decreased as the SDS concentration increased, with total inactivation being reached at 1.5 mM SDS. This inactivation was due to the conformational changes produced in the protein by the interaction with the detergent molecules. These results contrast with those described for PPO obtained from a variety of sources, where the changes produced by the SDS interaction had an activating effect.[13,30,39]

In the case of soluble POD from red cabbage, Figure 2 shows the pH profile for the oxidation of ABTS by POD (●) in a pH

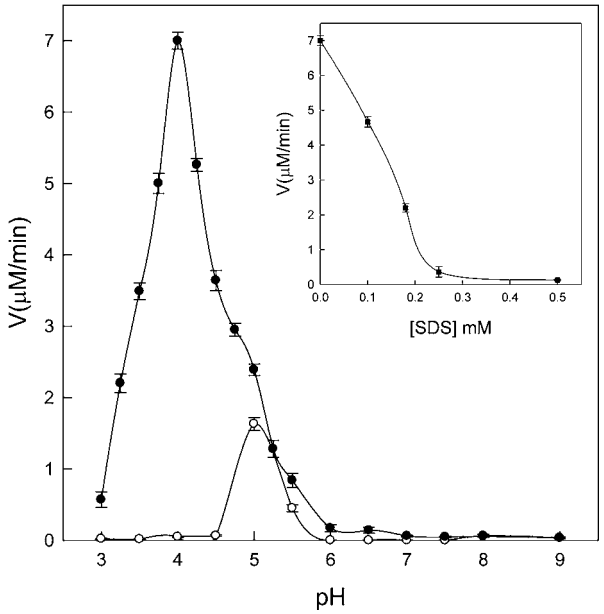

Figure 2. Effect of pH on soluble POD activity in the (●) absence or (○) presence of 0.5 mM SDS. (Inset) Effect of the SDS concentration.

range from 3.0 to 9.0, with 4.0 being the optimum (Table 1). This result is similar to that found for membrane-bound POD (Figure 1). Moreover, the effect of SDS on the pH profile of soluble POD was analyzed in the presence of 0.5 mM SDS (○ in Figure 2). The enzyme was inactivated in all pH values, although in the case of pH 5.0, a small activation in the presence of SDS was observed. The degree of SDS inactivation obtained at pH 5.0 for soluble POD was only 1.5-fold, whereas in the case of membrane-bound POD, it was 2.8-fold.

When the effects of the SDS concentration on both membrane-bound and soluble PODs from red cabbage were compared, an inhibitory effect was observed in both cases. This effect was more acute on soluble POD, where the total inactivation was achieved with 0.5 mM (inset of Figure 2).

To further characterize membrane-bound and soluble PODs from red cabbage, a detailed study of their kinetic parameters was carried out. The apparent kinetic parameters (V_m and K_M) of membrane-bound and soluble PODs were determined by fitting the experimental points to the Michaelis−Menten equation using the data obtained at pH 4.0. Figure 3A shows the variation in initial velocity versus substrate concentration for membrane-bound POD. When the H_2O_2 concentration was increased at a fixed, saturating concentration of ABTS (2 mM),

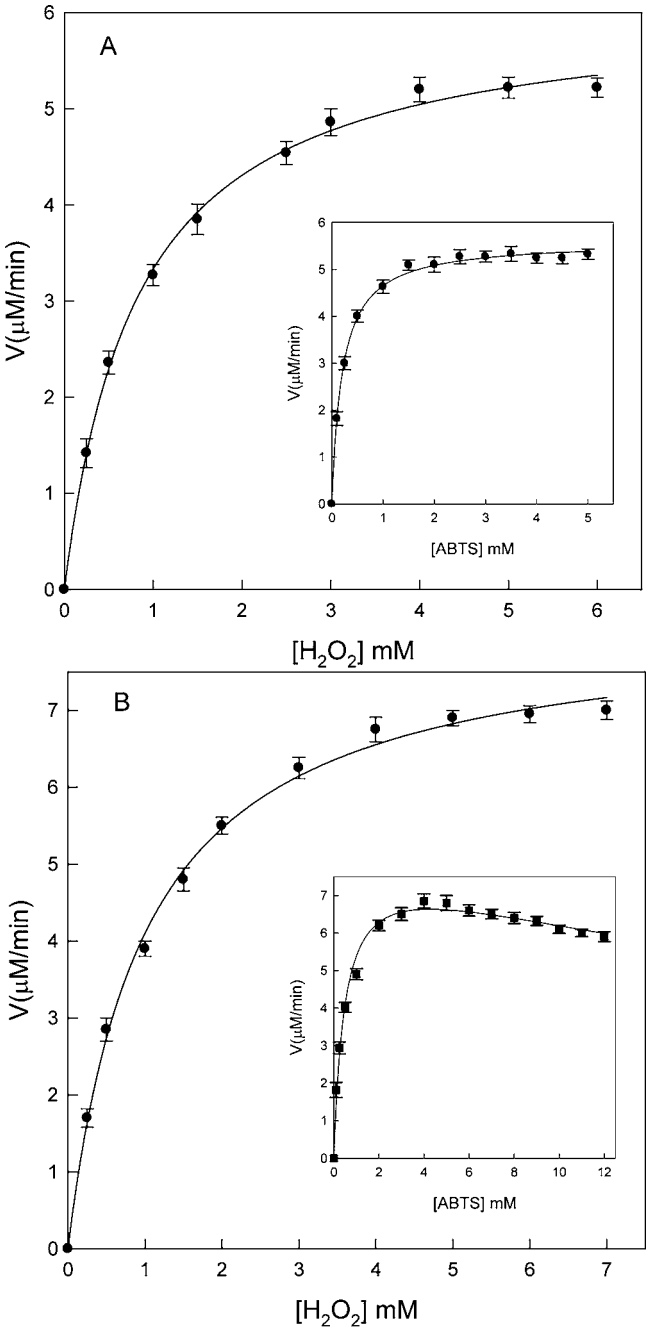

Figure 3. (A) Effect of the H_2O_2 concentration on membrane-bound POD activity. (Inset) Effect of the ABTS concentration. (B) Effect of the H_2O_2 concentration on soluble POD activity. (Inset) Effect of the ABTS concentration.

the activity increased to reach saturation. The K_M value for H_2O_2 was 0.82 mM, and the V_m value was 6.1 μM/min (Table 1). This K_M value obtained for H_2O_2 was similar to that described for turnip POD[41] (0.8 mM), lower than that obtained for pepper POD[24] (1.3 mM), and higher than those described for grape[39] (0.4 mM) or melon[38] (0.2 mM). This kinetic profile contrasts with that obtained for horseradish, asparagus, turnip, and red alga PODs,[17,41,42] in which an inhibition by the substrate concentration was described in the case of H_2O_2.

The inset of Figure 3A shows the kinetic profile for ABTS in membrane-bound POD. The K_M value was 0.2 mM, and the V_m value was 5.2 μM/min (Table 1). The K_M value for ABTS was similar to that described for Brussels sprouts POD[43] and lower than those obtained for recombinant turnip[35] (0.33 mM), acidic turnip[41] (0.47 mM), pepper[24] (0.5 mM), native turnip[35] (0.56 mM), horseradish[42] (4 mM), or red alga[17] (13 mM).

In the case of soluble POD, the ABTS oxidation rate is dependent upon the concentration of peroxide needed to reach saturation (Figure 3B). The K_M value for H_2O_2 was found to be 0.98 mM, and the V_m value was 8.1 μM/min (Table 1). This K_M value observed for H_2O_2 was similar to that obtained for turnip POD,[41] lower than that observed for pepper POD,[24] double that described for table grape POD,[39] and 5 times higher than that observed for melon POD.[38]

In addition, when the ABTS concentration was increased from 0 to 12 mM (inset of Figure 3B) at a fixed concentration of H_2O_2 (4 mM), the activity increased at first before falling gradually at higher concentrations. The inset of Figure 3B shows a typical kinetic profile of substrate inhibition, which can be kinetically analyzed by nonlinear regression fitting of the experimental points to the following equation:[44]

$$v = \frac{V_m[S]}{K_M + [S] + [S]^2/K_{si}} \quad (1)$$

where K_{si} is the substrate inhibition constant. The kinetic parameters V_m, K_M, and K_{si} were 8.9 μM/min, 0.61 mM, and 24.3 mM, respectively (Table 1). The K_M value obtained for ABTS was higher than those described for Brussels sprouts POD[43] (0.2 mM), turnip POD[41] (0.47 mM), and pepper POD[24] (0.5 mM) and lower than those observed for horseradish POD[42] (4 mM) or red alga POD[17] (13 mM). The kinetic profile of inhibition by a high concentration of ABTS observed for soluble POD was not observed in the case of membrane-bound POD.

To complete the study of red cabbage POD, its inhibition by reducing agents (ascorbic acid, L-cysteine, and metabisulfite) was studied in soluble POD (Figure 4) and membrane-bound POD (inset of Figure 4). In this way, ascorbic acid appeared to be the most effective modulator in both cases (▲ in both Figure 4 and its inset), acting as an antioxidant rather than an enzyme inhibitor because it reduces the ABTS radical to its original form. The inhibition produced by metabisulfite was lower in both cases (■ in both Figure 4 and its inset), perhaps as a result of an additional reaction taking place with the ABTS radical to form stable products and/or binding to the active center of the enzyme as in the case of metabisulfite in grape PPO[45] and pepper POD.[24] The inhibition produced by L-cysteine in the case of membrane-bound POD (inset of Figure 4) was higher than that obtained in the case of soluble POD (Figure 4).

Furthermore, the effect of substrate sequestrants, such as CDs, was studied. CDs are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven, or eight glucose residues linked by $\alpha(1 \rightarrow 4)$ glycosidic bonds in a cylinder-shaped structure, designated α-, β-, and γ-CDs, respectively. The central cavity of these molecules is hydrophobic, whereas the rims of the surrounding walls are hydrophilic. This hydrophobic cavity forms inclusion complexes with a wide range of organic guest molecules, including phenols.[46] It has been sugested that CDs may moderate the enzymatic browning of different fruits and vegetables.[47] This effect was also observed during the oxidation of phenols by

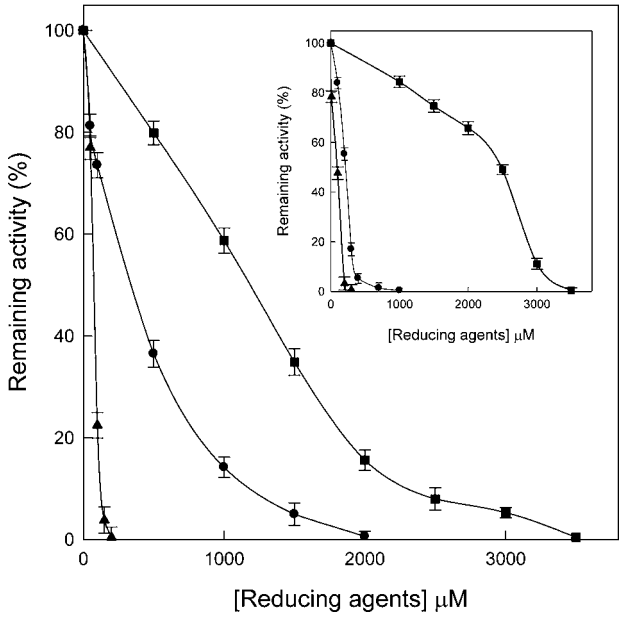

Figure 4. Inhibitory effect of reducing agents in soluble POD. The standard reaction medium at 25 °C contained 100 mM sodium acetate buffer (pH 4.0), 2 mM ABTS, 4 mM H_2O_2, 0.2 mM tropolone, 20 μg/mL enzyme, and reducing agent concentrations ranging from 0 to 3.5 mM [(▲) ascorbic acid, (●) L-cysteine, or (■) metabisulfite]. (Inset) Inhibitory effect of reducing agents in membrane-bound POD.

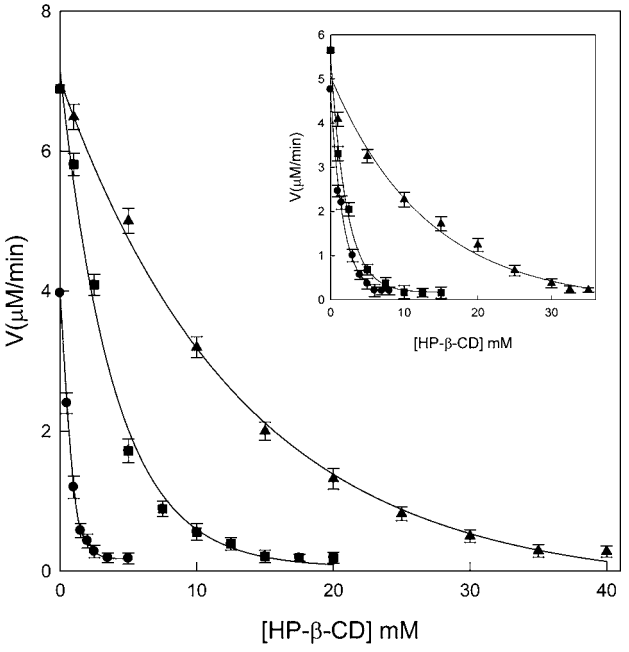

Figure 5. Effect of the HP-β-CD concentration on the enzymatic activity of soluble POD. Reaction medium at 25 °C contained 100 mM sodium acetate buffer (pH 4.0), 20 μg/mL protein, 4 mM H_2O_2, and increasing concentrations of HP-β-CDs (0–40 mM) and ABTS [(●) 0.5 mM, (■) 2 mM, or (▲) 4 mM]. (Inset) Effect of the increasing concentrations of HP-β-CDs (0–35 mM) and ABTS [(●) 1 mM, (■) 2 mM, or (▲) 4 mM] on the enzymatic activity of membrane-bound POD.

lipoxygenase,[48] in which CDs act as secondary antioxidants in synergism with ascorbic acid.

ABTS is a compound that could enter in the hydrophobic cavity of the CDs, forming an inclusion complex, and this fact has been previously described by our group.[49] This complexation explains the decrease in enzymatic activity of membrane-bound (inset of Figure 5) and soluble (Figure 5) red cabbage PODs when increasing concentrations of HP-β-CDs were used in the reaction medium. Using the following mathematical equation for determining the free substrate concentration in the presence of CDs[48]

$$[\mathrm{ABTS}]_t = -([\mathrm{CD}]_t K_c - [\mathrm{ABTS}]_t K_c + 1 + (([\mathrm{CD}]_t K_c - [\mathrm{ABTS}]_t K_c + 1)^2 + 4K_c[\mathrm{ABTS}]_t)^{1/2}) /2K_c \quad (2)$$

the complete Michaelis–Menten equation can be expressed as

$$v = [V_m[-([\mathrm{CD}]_t K_c - [\mathrm{ABTS}]_t K_c + 1 + (([\mathrm{CD}]_t K_c - [\mathrm{ABTS}]_t K_c + 1)^2 + 4K_c[\mathrm{ABTS}]_t)^{1/2}) /2K_c]]/[K_M + [-([\mathrm{CD}]_t K_c - [\mathrm{ABTS}]_t K_c + 1 + (([\mathrm{CD}]_t K_c - [\mathrm{ABTS}]_t K_c + 1)^2 + 4K_c[\mathrm{ABTS}]_t)^{1/2}) /2K_c]] \quad (3)$$

Equation 3 shows a nonlinear relationship between v and the total CD concentration ($[\mathrm{CD}]_t$), as depicted in Figure 5. When data presented in Figure 5 was fit to eq 3 by nonlinear regression, a value of 312 M^{-1}, for the inclusion constant (K_c) between ABTS and HP-β-CDs was obtained, using both membrane-bound and soluble PODs.

The optimum temperature was determined for membrane-bound and soluble PODs (Table 1). The optimum temperature for membrane-bound POD was 45 °C (● in Figure 6), which

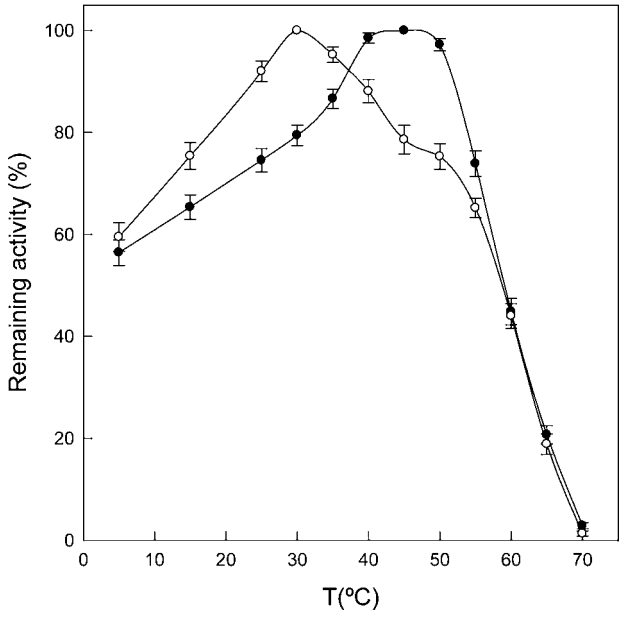

Figure 6. Optimum temperature for (●) membrane-bound and (○) soluble PODs.

agrees with those obtained in "Charantais" melon[50] and recombinant turnip[35] PODs. Temperatures higher than 45 °C in the assay medium caused a gradual decrease of the activity. The optimum temperature for soluble POD was 30 °C (○ in Figure 6), a result that agrees with that described in "Amarillo" melon POD.[50] Temperatures higher than 30 °C caused a

progressive decrease of the activity until 60% inactivation was observed at 60 °C.

To complete the study of red cabbage PODs, the thermal stability of both enzymes was studied. The semi-log plots of the residual activity of membrane-bound POD (Figure 7A) and soluble POD (Figure 7B) versus heating time were linear at all temperatures studied, which is consistent with inactivation by means of a simple first-order process. The fact that the lines all extrapolate back to a common point indicates that the inactivation of the only isoenzyme is being measured in each case (membrane-bound POD in Figure 7A and soluble POD in Figure 7B). These results are in accordance with those obtained for other PODs from potato or carrot,[51] pepper,[24] grape,[39] and red alga.[17] From the slopes of these lines, the inactivation rate constants (k) were calculated by linear regression according to the following equation:[51] 4

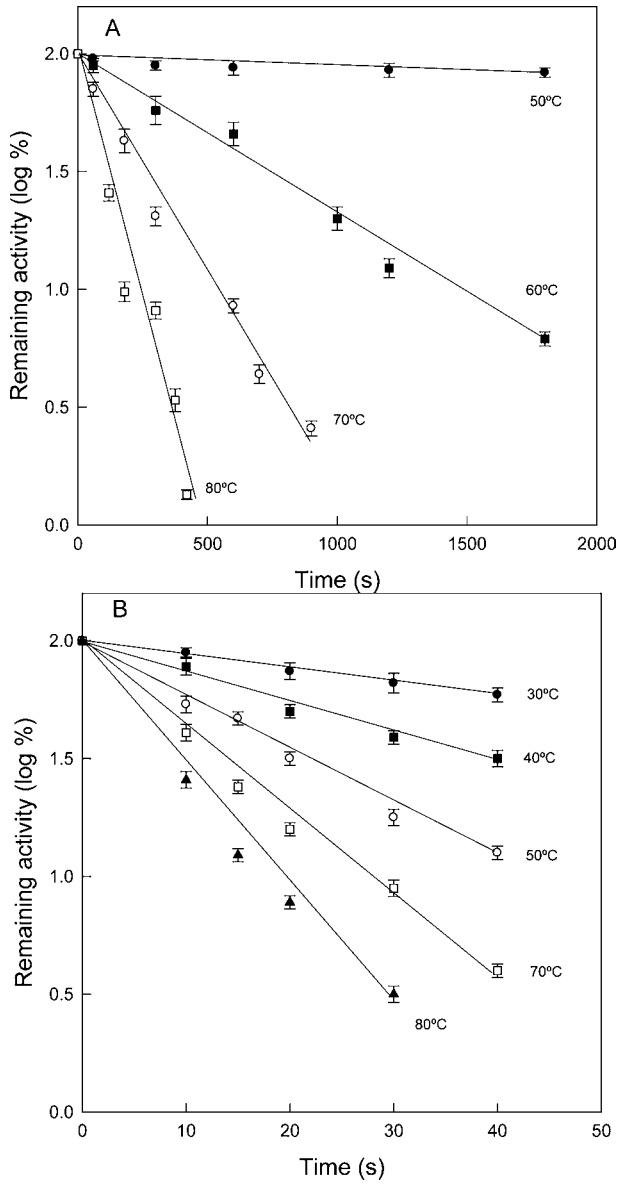

Figure 7. (A) Heat inactivation of membrane-bound POD. Remaining POD activity versus heating time at (●) 50 °C, (■) 60 °C, (○) 70 °C, and (□) 80 °C. (B) Heat inactivation of soluble POD. Remaining POD activity versus heating time at (●) 30 °C, (■) 40 °C, (○) 50 °C, (□) 70 °C, and (▲) 80 °C.

$$\log\left(\frac{A}{A_0}\right) = -\left(\frac{k}{2.303}\right)t \tag{4}$$

where A_0 is the initial enzyme activity and A is the activity after heating for time t. When the k values obtained were plotted in an Arrhenius plot (Figure 8), a simple linear fit was obtained for membrane-bound POD (● in Figure 8) and soluble POD (○ in Figure 8). The activation energies (E_a) were calculated from the slopes of the Arrhenius plot by the following equation:[51] 5

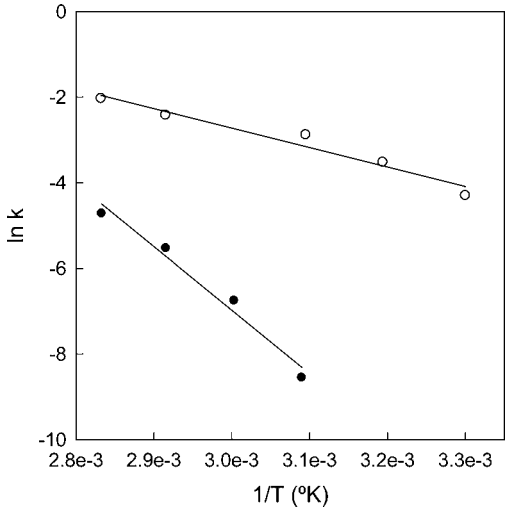

Figure 8. Arrhenius plots of inactivation rates from (●) membrane-bound and (○) soluble PODs.

$$\ln(k) = -\frac{E_a}{RT} + c \tag{5}$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature in K. This linear fit contrasts with the curvature obtained for other vegetables, including potato and carrot PODs,[51] green bean lipoxygenase,[52] pea seed lipoxygenase,[53] and grape POD,[39] but agrees with that obtained for pepper POD[24] and red alga POD.[17] The explanation for this linearity in the Arrhenius plot is that the inactivation in both membrane-bound and soluble PODs occurs through a one temperature-dependent mechanism, such as protein unfolding. However, when a curvature in the Arrhenius plot is observed, the explanation is that the inactivation occurs through more than one mechanism, each with its own temperature dependence, and the overall temperature dependence is simply the sum of the individual processes.

In the case of membrane-bound POD, the value obtained for E_a (143.1 kJ/mol) was higher than those obtained for "Charantais" melon POD[50] (86 kJ/mol) and "Elsanta" and "Madame Moutout" strawberry PODs[54] (96 and 74 kJ/mol, respectively), about half that obtained for grape POD[39] (271.9 kJ/mol), and 3 times lower than those obtained for potato and carrot PODs[51] (478 and 480 kJ/mol, respectively). However, it was similar to those obtained for "Amarillo" melon POD[50] (160 kJ/mol), pepper POD[24] (151 kJ/mol), and red alga POD[17] (121.6 kJ/mol).

For soluble POD, the value obtained for E_a (38.5 kJ/mol) was about half that obtained for "Charantais" melon POD[50] (86 kJ/mol) and "Elsanta" and "Madame Moutout" strawberry PODs[54] (96 and 74 kJ/mol, respectively), 3 times lower than that observed for red alga POD[17] (121.6 kJ/mol), 4 times lower than those obtained for "Amarillo" melon POD[50] (160 kJ/mol) or pepper POD[24] (151 kJ/mol), 7 times lower than that observed for grape POD[39] (271.9 kJ/mol), and 12 times lower than those obtained for potato or carrot PODs[51] (478 and 480 kJ/mol, respectively).

The range of temperatures required for the inactivation of membrane-bound POD was 50−80 °C (Figure 7A), which is higher than that required for red alga POD[17] (30−50 °C) and pepper POD[24] (30−60 °C) but similar to that required for grape POD[39] (60−80 °C), strawberry POD[54] (50−80 °C), and melon POD[50] (40−70 °C). These results indicated that red cabbage membrane-bound POD is more thermostable than red alga and pepper PODs but similar to grape, strawberry, and melon PODs. The range of temperatures required for the inactivation of soluble POD was 30−80 °C (Figure 7B), which is higher than that required for red alga POD[17] (30−50 °C) and pepper POD[24] (30−60 °C) but similar to melon POD[50] (40−70 °C).

In some cases, inactivation is expressed as the D value, the time required to reduce the enzyme activity to 10% of its original value. The temperature required for a D value of 5 min for membrane-bound POD was 76.6 °C, which is higher than that observed for red alga POD[17] (48.4 °C) and pepper POD[24] (44.5 °C) and similar to those obtained for grape POD[39] (75 °C), potato or carrot POD[51] (80 °C), melon POD[50] (70 °C), and strawberry POD[54] (80 °C). The temperature required for a D value of 5 min for soluble POD was 30.2 °C, which is lower than obtained for red alga POD[17] (48.4 °C), pepper POD[24] (44.5 °C), grape POD[39] (75 °C), potato or carrot POD[51] (80 °C), melon POD[50] (70 °C), and strawberry POD[54] (80 °C).

Table 1 shows the substantial differences observed between membrane-bound and soluble PODs. The E_a, D value, and the range of temperatures required for the inactivation obtained for membrane-bound POD are higher than those obtained for soluble POD. These results indicate that the membrane-bound POD is more thermostable than the soluble POD.

In conclusion, this paper presents, for the first time, a detailed kinetic study of membrane-bound and soluble PODs from red cabbage isolated using Triton X-114. Both enzyme forms were inactivated by the anionic detergent SDS. The reducing agents ascorbic acid, L-cysteine, and metabisulfite also inhibited these enzymatic forms. The use of substrate sequestrant agents, such as CDs, has an antibrowning effect because of the entrapment of phenolic compounds in the hydrophobic cavity of CDs. Food-grade CDs could be an interesting alternative compared to restricted-use compounds. A thermal stability study showed that membrane-bound POD is a more thermostable enzyme than soluble POD extracted from red cabbage. The thermostability of POD is very important, because this enzyme may have very negative effects on the color and flavor of vegetables during storage.

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Notes

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