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Asparagus Byproducts as a New Source of Peroxidases

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ABSTRACT: Soluble peroxidase (POD) from asparagus byproducts was purified by ion exchange chromatographies, and its kinetic and catalytic properties were studied. The isoelectric point of the purified isoperoxidases was 9.1, and the optimum pH and temperature values were 4.0 and 25 °C, respectively. The cationic asparagus POD (CAP) midpoint inactivation temperature was 57 °C, which favors its use in industrial processes. The K_m values of cationic asparagus POD for H_2O_2 and ABTS were 0.318 and 0.634 mM, respectively. The purified CAP is economically obtained from raw materials using a simple protocol and possesses features that make it advantageous for the potential use of this enzyme in a large number of processes with demonstrated requirements of thermostable POD. The results indicate that CAP can be used as a potential candidate for removing phenolic contaminants.

KEYWORDS: *peroxidase, asparagus byproduct, phenols, bioremediation*

INTRODUCTION

Soluble peroxidases (PODs) are capable of catalyzing the oxidation of a wide range of substrates at the expense of H_2O_2. This property confers numerous applications in both clinical analysis and industry, such as enzymatic assay kits for the enzyme biosensors glucose, cholesterol, triglycerides, and uric acid. It is also used for the preparation of bakery products, paper bleaching, the denim clothing industry, and degradation of pollutant synthesis of chemicals, etc.[1]

An effective treatment of industrial wastewaters has become an increasing cause of concern in recent decades. Among the different types of pollutants present in waste streams from industrial sources, phenols and their derivatives deserve special attention. Effluents containing phenolic compounds are a mixture of various products from industrial activities such as chemicals, pharmaceuticals, and paper, whose concentrations range from parts per million to 1 g/L.[2]

Phenols and their derivatives are characterized by possessing a high toxicity and bactericidal properties. In fact, at concentrations of 50 mg/L, they can inhibit the proliferation of microorganisms, and at concentrations above 1 g/L, they can destroy the flora of the sewage plant. As a result of these characteristics, industrial effluents containing these compounds must be treated before the disposal of waste into the environment or sending them to conventional biological treatment plants.[3] Therefore, alternative methods for the removal of phenols from wastewaters have been developed. Among them, enzymatic polymerization using peroxidase enzymes has been suggested as a new method for the treatment of phenol contaminants.[4]

The use of POD for bioremediation is based on the oxidation of aromatic compounds by hydrogen peroxide. The free radical oxidation products have limited solubility in water and tend to precipitate from solution, so they could be easily removed by flocculation and sedimentation. This application was first reported by Klibanov and Morris in 1981[5] and there are a wide range of enzymes and genetically modified microorganisms that are used to oxidize the phenols. Horseradish (*Armoracia rusticana*) root is the primary source of commercial POD production; however, due to the high cost of this enzyme, considerable effort has been devoted to finding an alternative source of POD that is more effective in catalyzing a particular process, more resistant to inactivation, and with a higher quality/cost ratio.

Asparagus is a continuously growing crop, with Spain being the fourth largest producer in the world with around 63 000 tons per year.[6] Only 50% of the spear is used for human consumption and the rest is considered a byproduct that has been traditionally used for animal feeding and low-value products. However, several studies showed that asparagus byproducts are an important source of high biological activity compounds,[7,8] which increases the value of the waste generated by the industry. Furthermore, asparagus (*Asparagus oficinalis L.*) spears are known to increase their hardening during postharvest storage and this hardening is mainly located in the lower portions of the white spears.[9]

In previous research we concluded that the mechanism of toughening takes place due to the formation of cell-wall complexes through phenolic dimers catalyzed by POD.[10] For this reason, we have hypothesized that asparagus byproducts are an alternative source for obtaining POD. A possible application for the purified enzymes could be in the removal of phenols and other derivatives of different industrial effluents.

The aims of this study were (1) to extract, purify, and characterize CAP in terms of its catalytic activity and (2) to study the performance of CAP and assess its potential

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application for the treatment of industrial wastewaters containing phenolic contaminants.

MATERIALS AND METHODS

Plant Material. White asparagus byproducts were obtained from local producers (Seville, Spain) and were frozen with liquid nitrogen until further use.

Asparagus POD Extracts. White asparagus byproducts (500 g) were homogenized in a Janke & Kunkel Ultraturrax T-25 (IKA-Labortechnik, Staufen, Germany) at top-speed for 3 min with 500 mL of 50 mM Tris/HCl buffer, pH 7.0, containing 1 g/L ascorbic acid. The solid residue was recovered by filtration through nylon, and the extraction procedure was repeated twice to maximize the extraction yield. The supernatants were pooled to get the soluble POD extract.

Assay for the POD Activity. POD activity was determined with ABTS as the reducing substrate in a total volume of 0.2 mL. The assay mixture contained 20 μL of a 1 M acetate buffer with a pH of 5.0, 20 μL of 1 mM ABTS, and 20 μL of 0.3% H_2O_2 and variable amounts of H_2O and enzyme preparations to reach the final assay volume. The oxidation of ABTS was followed by monitoring the increase in absorbance at 415 nm and using $\varepsilon_{415\ nm} = 31.1\ mM^{-1}\ cm^{-1}$ for ABTS cation radical formation.[11] One unit of activity (UA) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute at pH 5.0 and 25 °C. A Bio-Rad iMark microplate reader was used for spectroscopic measurements.

Protein Determination. Protein was quantified on the basis of the dye-binding method of Bradford using bovine serum albumin (BSA) as standard.[12]

Purification of POD. The extract of POD was dialyzed against deionized water, centrifuged at 21 214*g* for 2 h at 4 °C, and filtered prior to purification. The chromatography purification steps were carried out at room temperature.

Anion Exchange Chromatography. To carry out the chromatography, Hi-Trap Q-Sepharose (Amershan Pharmacia Biotech) columns were used and equilibrated with a 10 mM imidazole/HCl buffer at pH 7.0 at flow of 3.0 mL/min. The sample was dialyzed against the above buffer and it was injected into the column at a flow rate of 1 mL/min. The sample was eluted with imidazole/HCl buffer followed by a linear gradient of 0−1 M NaCl in the above buffer. Fractions of 5 mL were collected and the POD activity and protein content were determined.

Cationic Exchange Chromatography. The unretained fraction from the anionic exchange columns was dialyzed against 10 mM of acetate buffer at pH 5.0 and loaded into the top of a Hi-Trap SP-Sepharose (Amershan Pharmacia Biotech) column at a flow rate of 1 mL/min. Afterward, the sample was eluted with 10 mM of acetate buffer at pH 5.0 and the retained protein was eluted at the same flow rate using a linear gradient of 0−1 M NaCl in the above buffer. Fractions of 5 mL were collected and the POD activity and protein contents were determined.

Isoelectric Focusing. Preparative isoelectric focusing was carried out using a Rotofor preparative IEF cell (Bio-Rad). The protein samples were dialyzed against deionized water overnight, supplemented with 2% (v/v) Biolytes (Bio-Rad) of 3.0−10.0 pH range, and then loaded into the Rotofor cell. The isoelectric focusing, without prerunning, was done according to the manufacturer's instructions. The power supply was set at a constant power of 12 W. Once focusing was completed, the electrofocusing cell was fractionated into 20 aliquots ranging from acidic to basic isoelectric point (p*I*) proteins. POD activity and protein contents were determined for each aliquot.

Gel Electrophoresis. SDS−PAGE of the crude extract and purified fraction was performed on a 12% acrylamide minigel in a Bio-Rad protein II electrophoresis cell set at 150 V for 120 min according to the method of Laemmli.[13] Protein band detection was conducted by silver staining as previously described by Rabilloud.[14] Molecular weights of the bands were calculated using Labimage software v2.7.1 (Kapelan Bio-Imaging). Native gel electrophoresis was performed on a 10% acrylamide minigel at 80 V for 300 min according to the method of Thomas and Hodes.[15] POD active isoforms were detected on X-ray films (CL-XPosure Film, Pierce) using the enhanced chemiluminescence detection kit (Supersignal West Pico Chemiluminescent Substrate, Pierce).

Effect of pH on POD. POD activity as a function of pH was established by incubating the purified POD in 1 M sodium acetate buffer (pH 3.0−5.0), 1 M sodium phosphate buffer (pH 5.0−7.0), and 0.05 M Tris-HCl buffer (pH 7.0−9.0). Analysis conditions were the same as those described for the POD activity assay.

Determination of Optimum Temperature and Thermal Stability. The optimum temperature and thermal stability of POD were assayed using a water bath (Precis-Term P-Selecta) with the optimum pH at temperatures ranging from 15 to 80 °C, for 10 min prior to substrate addition. After heating, the solutions were rapidly cooled in ice water, and the POD activity was immediately determined. The percent residual activity was plotted against different temperatures. Thermal stability was also tested at varying temperatures in the range of 50−75 °C with exposure times ranging from 1 to 20 min.

To calculate the kinetic inactivation, rate constants (k) were obtained for POD inactivation in samples treated at specific temperatures above the CAP midpoint inactivation temperature (T_m), defined as the temperature at which 50% of enzyme activity is lost, and different times. Enzyme deactivation kinetics is often first-order and k can be obtained from the slope of a plot of log A (% original activity) versus time of heat exposure. From the slopes of the straight lines obtained using the Arrhenius plots of $\ln(k)$ versus $1/T$, the energy for the inactivation of POD values (E_a) was calculated.

Kinetic Constants for ABTS and H_2O_2. Kinetic parameters were calculated from Lineweaver−Burk plots for the two-substrate mechanisms followed by POD[16] using H_2O_2 and ABTS concentrations from 0.2 to 3.0 mM, respectively. Temperature and pH were kept constant in the optimum values determined previously. Taking into account the reciprocal of both sides of the Michaelis−Menten equation leads to the Lineweaver−Burk relationship

$$1/\nu_0 = K_a/A_0V_{max} + K_b/B_0V_{max} + 1/V_{max}$$

where ν_0 is the initial reaction velocity, V_{max} is the maximum reaction velocity, K_a and K_b are K_m values, the Michaelis−Menten constant for substrate A (H_2O_2) and B (ABTS), respectively, and A_0 and B_0 are concentrations of substrate A and B. The ν_0 were determined as a function of both substrate concentrations. A plot of $1/\nu_0$ vs $1/B_0$ for varying values of A_0 will give a series of parallel lines, each of slope K_b/V_{max}. A plot of intercept vs $1/A_0$ will give a line of slope K_a/V_{max} and intercept $1/V_{max}$. Hence K_a, K_b, and V_{max} may be determined.

Removal of Phenolic Compounds. We evaluated the catalytic activity of the purified POD, using different model compounds such as monoaromatic phenolic type 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP) as a model of the disubstituted substrate, and 2-(3,4-dihydroxyphenyl)ethanol [hydroxytyrosol (HT)], a polyphenol present in olive mill wastewater. The assays were performed in a reactor at room temperature. All substances were dissolved in 1 M acetate buffer at pH 4.0. The optimum ratio of CAP and H_2O_2 concentration for phenol removal was investigated using H_2O_2 concentrations between 1 and 20 mM. In order to study the influence of CAP, H_2O_2, and their combination on the removal of phenols, we followed different incubation conditions: (a) a control solution that consisted of 2 mM phenol, (b) 2 mM phenol and H_2O_2, (c) 2 mM phenol and CAP, and (d) 2 mM phenol, H_2O_2, and CAP. The course of the reaction was monitored by taking 1 mL of sample, and the concentration of the phenol was analyzed until the value was constant.

Phenol Quantification. The removal of each phenol was monitored by high-performance liquid chromatography (HPLC) and an ultraviolet detector. The aliquots were taken from model wastewaters at the indicated times. The reaction was stopped by the addition of 10 μL of 20% trichloroacetic acid (TCA). After centrifugation, 20 μL was injected into a MediterraneaSea C18 (Teknokroma), 5 μm, 25 × 0.6 reversed phase HPLC column. The initial mobile phase consisted of 70:30 (v/v) water:acetonitrile with 0.1% formic acid, which was brought to 0:100 (v/v) in a linear gradient lasting for 20 min, held for another 10 min, and then 35−40 min of 30% acetonitrile. Phenol concentrations were determined from

a straight-line standard calibration obtained using known concentrations (standards) of each compound. The results are expressed as removal efficiency, which is defined as the percentage of phenols removed from the solution under established experimental conditions.

Statistical Analysis. The mean ± SD of three replicates was calculated. All data were analyzed using multivariate analysis of variance (MANOVA) followed by the Fisher−LSD multicomparison test. The level of significance was $p < 0.05$.

RESULTS AND DISCUSSION

Extraction and Purification. In order to extract the soluble POD from asparagus byproduct tissues, we have found that the most efficient conditions were using a 50 mM phosphate buffer at pH 7.0. The use of a protease inhibitor cocktail (CLAP) or a phenolics scavenger like polyvinylpirrolidine (5% PVP) did not have any effect on the extraction.

The POD activity in the crude extract of white asparagus byproducts was characterized by a higher POD activity, 5.31 units/g of fresh asparagus, than those reported for other potential sources of POD such as broccoli (3.50 units/g of fresh material).[17] The method to purify the soluble POD consisted of a combination of chromatographic techniques including anion and cationic exchange. First, we loaded the extract into the anionic ion exchange columns of Q-Sepharose. Figure 1A showed the elution profile in which two distinct fractions containing POD activity could be detected. The first fraction consisted of nonretained isoperoxidases apparently corresponding to cationic POD and it was named A0 fraction. The second fraction was eluted when we applied high ionic strength. This fraction contained a mixture of neutral and anionic isoenzymes, and it was named A1 fraction. A small amount of protein (2%) that eluted with A0 fraction coincided with most of the POD activity (90%). As shown in Table 1, the A0 fraction was characterized by a high specific activity. However, the A1 fraction was discarded due to its high content of proteins with a relatively low specific activity value, which meant a poor degree of purification for POD.

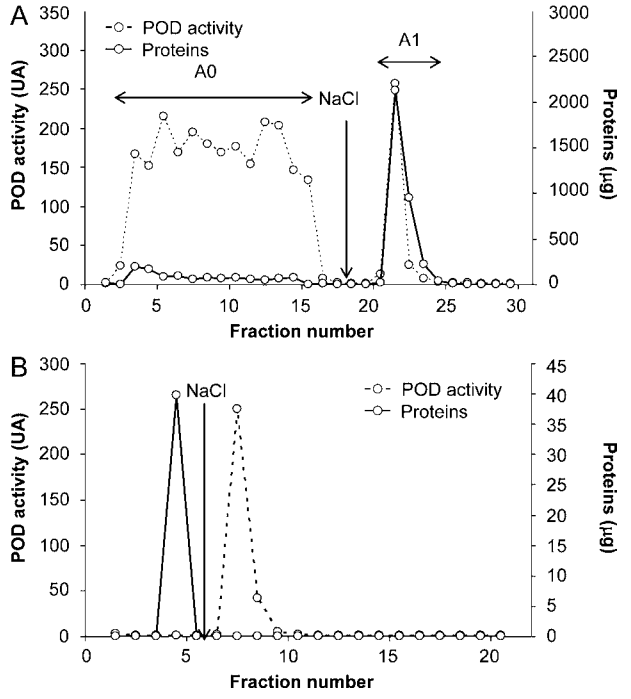

Figure 1. (A) Typical elution profile for anionic exchange chromatography for crude extract peroxidase from asparagus byproducts. (B) Typical elution profile for cationic exchange chromatography for A0 fraction.

Second, the A0 fraction was dialyzed against an acetate buffer at pH 5.0 and then it was loaded into a cationic exchange chromatography column using Hi Trap SP-Sepharose. We obtained the POD activity as a single peak (bound to the SP-Sepharose column) separated from the protein fraction (Figure 1B). This purification step allowed us to obtain pure cationic isoforms of POD with important values of specific activity, activity yields, and purification factor. At this end-step in the purification, a final purification factor of 273 was obtained, with a yield of 50% (Table 1). This purification process was very easy and economically feasible in order to obtain a new source of CAP from asparagus byproducts.

Isoelectric Focusing. The isoelectric point (p*I*) of the crude extract and purified isoperoxidases was studied. Figure 2A shows a typical pattern of crude soluble isoperoxidases from asparagus. POD found in higher plants include isoenzymes with anionic, neutral, and cationic p*I*. White asparagus byproduct extract constituted mainly of isoenzymes of clearly cationic character, where 72% have p*I* above 9.0. The profiles of the purified cationic isoenzymes showed that POD activity migrated to the negative electrode, resulting in a fast method of concentrating proteins into two main peaks (p*I* 9.1 and 8.6) and eliminating proteins outside of a p*I* range of interest (Figure 2B). Other basic POD found in turnip hairy roots have a p*I* 9.6,[18] garlic cloves have a p*I* 8.9[19] and in green asparagus the p*I* was determined as 7.83.[20]

Partial Characterization of CAP. SDS−PAGE electrophoresis, after 5 min of exposure to the developer (Na_2CO_3), revealed three major bands with molecular weights of 23, 27, and 43 kDa, approximately (Figure 3A). The 23 kDa band was the first to appear in the gel, as shown in Figure 3B (1 minute of exposure to Na_2CO_3), which probably corresponded to the most concentrated fraction of proteins with high p*I* which also have high POD activity, as shown in Figure 2B. In addition, native gel electrophoresis showed two bands with marked differences of intensity that are likely to belong to the proteins with higher p*I*, which presented the highest POD activities. These two bands of activity were undetectable in the crude extract. The relative amount of POD proteins in the total crude extract might be very low for detecting them by this method.

Table 1. Summary of Purification of CAP from Asparagus Byproducts

purification step	total activity (UA)	total protein (mg)	specific activity (UA/mg)	recovery activity (%)	purification factor
crude extract	547 ± 5	148 ± 2	3.70	100 ± 1	1
dialyzed extract	621 ± 8	151 ± 9	4.11	111 ± 4	1.1
Q-Sepharose[a]	559 ± 12	3.6 ± 0.4	155	88 ± 2	42
SP-Sepharose	313 ± 9	0.31 ± 0.06	1009	56 ± 1	273

[a]Values corresponding to AO fraction.

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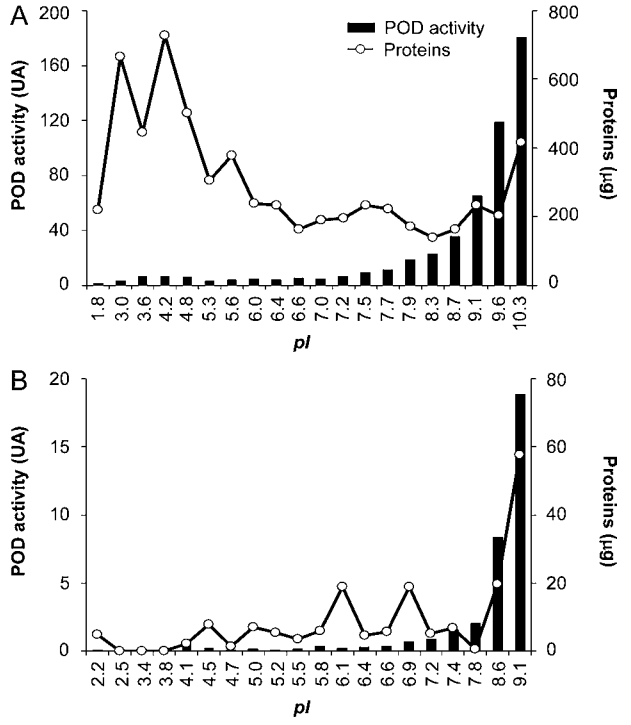

Figure 2. Typical isoelectric profile for soluble peroxidases from asparagus byproducts: (A) crude extract and (B) purified cationic peroxidase.

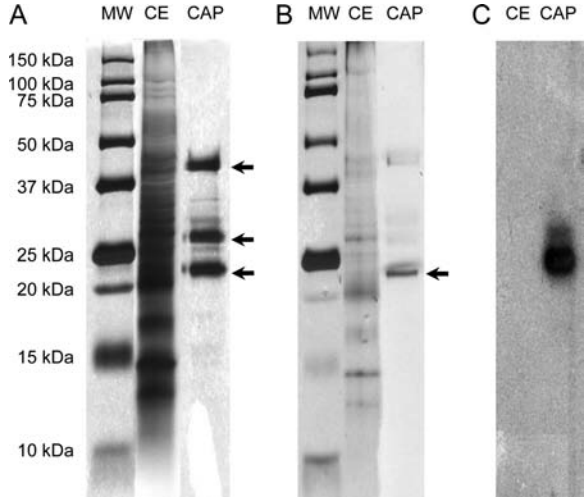

Figure 3. SDS–PAGE electrophoresis after 5 min (A) and 1 min (B) of exposure to the developer (Na_2CO_3) and native gel electrophoresis (C) of the crude extract (CE) and purified CAP. Molecular weights (MW) are 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa.

These are the first soluble cationic POD described in white asparagus, and they have molecular weights in the range of several cationic POD described in other vegetables.[18,19] Soluble POD from green asparagus was described previously.[20] Wang and Luh detected three major bands of 33, 43, and 48 kDa by SDS electrophoresis. We have determined a 43 kDa band, too. However, none of the proteins in green asparagus had a p*I* above 7.87. This p*I* is far from what we have measured for the most active POD in white asparagus. According to this, soluble extracts of green asparagus also lack the two low molecular weight POD. Taken together, these data suggest that white asparagus has different cationic POD than green asparagus. The structure and oligomerization of these isoenzymes is unknown and needs further research.

Optimum pH. POD activity is strongly affected by pH. Indeed, the prosthetic group of the enzyme is sensitive to pH change.[21] The optimum pH for any POD depends on the hydrogen donor substrate and the buffer solutions used in the activity assay. Using ABTS as a hydrogen donor, CAP showed a broad range of pH profiles from 3.0 to 6.0 where it retained more than 80% of its maximum activity (Figure 4A). The

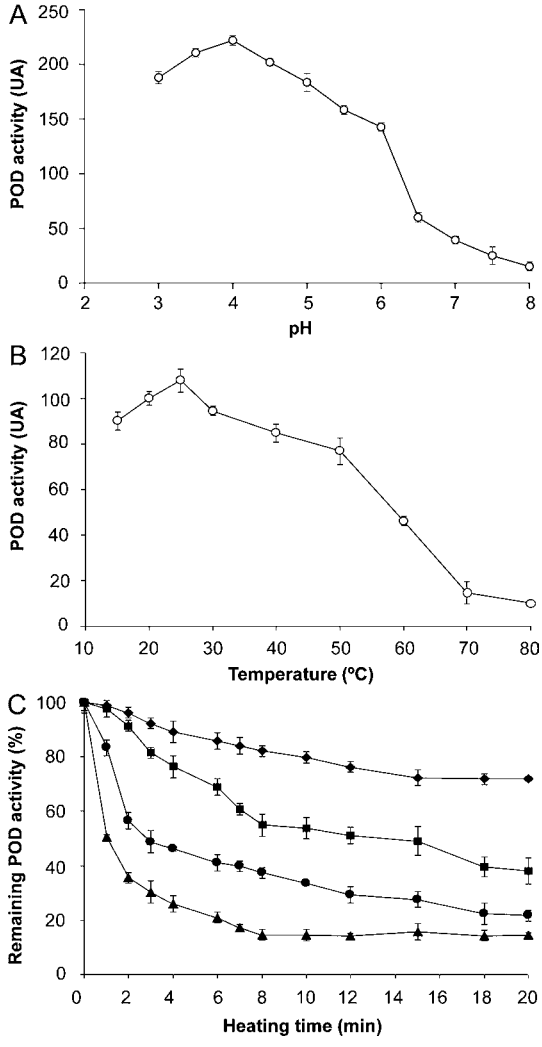

Figure 4. Optimum pH (A) and temperature (B) for the enzymatic activity of purified CAP using ABTS as substrate. Values are means of triplicate ± SD. (C) Thermal inactivation of purified CAP at the following temperatures: 55 °C (◆), 60 °C (■), 65 °C (●), and 75 °C (▲). Values are means of triplicate ± SD.

optimum pH for POD activity under these conditions was 4.0. This value agrees with those reported for green asparagus POD[21] and for several PODs purified from other sources with their optimum pH included in 3.5–5.5 values.[22−25] However, at pH above 6.0, the stability of the CAP drastically decreased, thereby losing its activity. This phenomenon may be attributed to the dissociation and modification of the heme prosthetic group and protein denaturation.[26,27]

Thermal Stability of CAP. POD is reported to be one of the most heat-stable enzymes in plants. However, its heat-resistance depends on the source of the enzyme and the

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conditions of the assay. When CAP was assayed at optimum pH in temperatures ranging between 15 and 80 °C for 10 min, the enzyme remained active up to 60 °C with maximum activity at 25 °C (Figure 4B). The activity of the enzyme was significantly reduced at 60 °C and difficult to be detected from 70 °C and above. Other studies have reported a wide range of optimum temperature variability for POD from different sources. Buckwheat seed POD showed an optimum temperature of 10−30 °C,[28] whereas olive and green asparagus PODs have an optimum temperature of 35 and 56 °C, respectively.[29,20] The value of T_m for CAP was 57 °C (Figure 4B).

Kinetic inactivation parameters of the CAP were investigated by incubating the enzyme at different temperatures for 20 min. The residual activities (in percent) at different time intervals are represented in Figure 4C. The results clearly show that the residual activity gradually decreases as the temperature increases, yielding nonlinear curves. The curves showed an initial steep straight line, an intermediate curved portion, and a final straight line with a shallow slope, where the residual activity apparently tends to stabilize. These results can be described with the biphasic first-order model proposed by Ling and Lund[30] on the basis of the presence of two isoenzyme groups with distinct thermal stabilities, a heat labile fraction that inactivates rapidly, and a heat-resistant fraction that cannot be inactivated completely. Our results agree with those reported by other authors for green asparagus.[20,31,32] In fact, Ganthavorn[33] reported that partially purified raw POD extracts from green asparagus closely corresponded to monophasic behavior at 50 and 60 °C, and a biphasic behavior at 70 °C was observed. In order to determine the k for both isoenzymes, the biphasic model assumes that each fraction of enzyme follows first-order kinetics and, as described previously, k_L and k_R are the first-order rate constants for the thermal inactivation of the heat-labile and heat-resistant isoenzyme fractions, respectively. To determine k_L values, the short heating period was fitted to include the first 5 min, while k_R values were established at longer heating times. The data showed that CAP activities for heat-labile and heat-resistant fractions are dependent on temperature and heating time. The reaction rate constants for both fractions of CAP were determined by linear regression of the straight portions of the resulting curve by plotting the logarithms of the residual activities as a function of time of heat treatment (Table 2). In general, k values increase with temperature, and those for heat-labile isoenzymes are higher than those for the heat-resistant fraction. The values obtained for k_L and k_R were in agreement with those reported for commercial POD; however, greater differences were found for green asparagus POD at similar inactivation temperatures.[33] It could be due to the fact that these kinetic values depend on the concentration of the enzyme and the assay conditions mainly relate to differences in ionic strength and the pH of buffer solutions.

Table 2. Kinetic Parameters for Thermal Inactivation Obtained for CAP

temp (°C)	$k_L \times 10^3$ (min^{-1})	R^2	$k_R \times 10^3$ (min^{-1})	R^2
55	30.17	0.966	14.51	0.904
60	78.30	0.989	39.84	0.925
65	231.50	0.973	45.83	0.976
75	320.1	0.893	223.16	0.980

The activation energy (E_a) values for thermal inactivation processes of the heat resistant and labile fractions of CAP were determinated from the corresponding Arrhenius plot. E_a values of heat resistant and heat labile fractions were found from the slopes of the curves as 123.3 kJ/mol (R^2 = 0.962) and 111.76 kJ/mol (R^2 = 0.870), respectively. The E_a values were similar to those obtained for other cationic PODs[11,26] considered, such as high-thermostable POD, which suggests that CAP has potential for use in various industrial applications.

Kinetic Constant for ABTS and H_2O_2. The efficiency of the catalysis of the POD depends on the chemical nature of the phenolic substrates and on the source of the enzyme.[34] In order to calculate the K_m value for each substrate, double-reciprocal plots done according to the Materials and Methods are shown in Figure 5A. The parallel lines are compatible with the two-substrate ping-pong mechanism generally accepted for peroxidases. The calculated y-intercepts from Figure 5A were plotted against $1/A_0$ and resulted in a straight line (Figure 5B). From this figure we achieved the V_{max} = 1.33 mM ABTS min^{-1}. The K_m value for H_2O_2 was found to be 0.21 mM. This value was lower than those reported by turnip POD (0.8 mM)[18] and table grape POD (0.4 mM).[35] The K_m value for ABTS was 0.551 mM, lower than those reported for turnip POD (0.71 mM),[26] table grape POD (0.79 mM),[35] and red alga POD (13 mM)[36] but higher than those described for Brussels sprouts POD (0.2 mM).[37] These results showed a promising use of CAP for biotechnological applications requiring low amounts ABTS and H_2O_2.

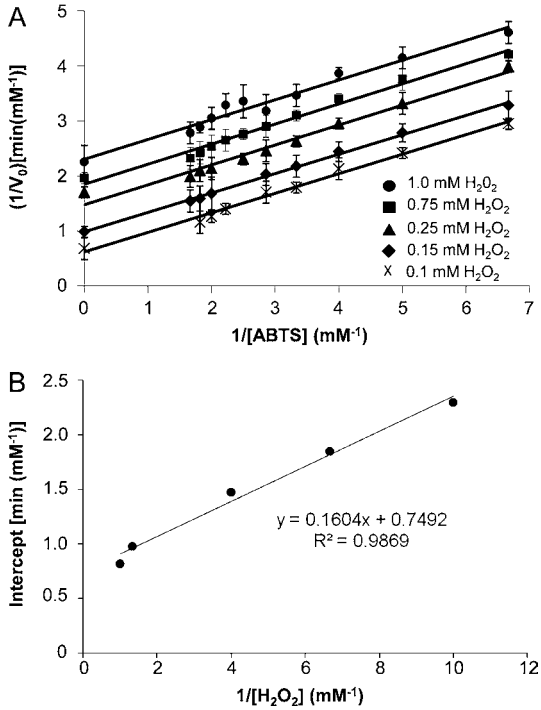

Figure 5. Kinetic behavior of the two-substrate reactions for purified CAP: (A) plot of the 1/[substrate] versus 1/velocity relationship at a fixed dose of CAP (15 UA mL^{-1}) . (B) plot of the y-intercepts of the lines of part A versus 1/[H_2O_2].

Application of CAP for Phenolic Compound Removal. Wastewaters containing phenolic compounds are highly toxic. Specifically, chlorophenols are generated through various industrial processes involved in the leather industry,[38] pulp

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and paper industry,[39] petrochemical industry,[40] and pesticides.[41] In particular, the principal use of the 4-CP and 2,4-DCP have been as intermediates for the production of higher chlorinated phenols and in the production of herbicides.[42] On the other hand, olive oil production generates large amounts of highly polluted wastewater that represent one of the most contaminating effluents among those produced by the agrofood industries. These wastewaters show a wide variability in the amount of polyphenols and secoiridoids, but HT is the main polyphenol present.[43] In this research a bioremediation treatment for the removal of phenolic aromatic pollutant models from different wastewaters is described.

As H_2O_2 is able to inactivate POD activity at high amounts, different concentrations of H_2O_2 were tested to ensure that an optimum ratio CAP/H_2O_2 was used, minimizing inactivation while maximizing the removal efficiency. The removal of three model phenolic compounds contained in wastewaters was investigated as a function of CAP dose fixed using 1-h-stirred batch reactions. All of the experiments were carried out at the optimum pH and temperature for CAP. Figure 6A shows the results of the experiments that used various amounts of hydrogen peroxide, 1.2 mM phenol, and 20 UA mL^{-1} CAP. At the lowest concentration used (2 mM) the removal efficiency appeared consistent but was quite slow. With the 4 mM concentration, not only was the greatest quantity of phenol removed but also the reaction rate was much faster. In fact, maximum removal was observed using 4 mM H_2O_2. However, increasing the concentration further resulted in less removal. These results show the optimum relationship of CAP/H_2O_2 for the removal of phenols is 5 UA CAP mL^{-1} $mmol^{-1}$ H_2O_2. Once doses and efficiency incubation assays were optimized, several trials were developed, as described previously in the Materials and Methods. The series a and b were used to evaluate the phenol auto-oxidation, phenol loss by evaporation, as well as the possible reaction between both substrates. Control (c) was used in order to analyze other enzymatic processes, such as oxidation by phenol oxidases like laccases and tyrosinases or the oxidation catalyzed by POD with endogenous peroxide. As the extracts obtained could contain other enzymes that oxidize phenols without H_2O_2, such as polyphenol oxidases (PPO), we performed the phenol removal assay in the absence of H_2O_2 with both extracts.

For each phenol model, there was no removal in the controls containing only phenol (a) or phenol with H_2O_2 after incubation (b), indicating that phenol losses during the reaction time were negligible and no chemical reaction between both reactants occurred, respectively. Similarly, no changes were found in the phenol concentration in the presence of CAP after 120 min (data not shown). However, the presence of H_2O_2 and CAP greatly reduced the concentration of every phenol in the reaction. The time courses of the different phenols removed are shown in Figure 6B. The greatest decreased in the concentration of 2,4-DCP took place during the first 10 min of reaction. This result suggests that CAP was especially active toward this phenol. Longer times were necessary to observe an important oxidation of the monophenol 4-CP and the HT. This confirms that the efficiency of CAP for the removal of phenols is strongly dependent on the chemical nature of the substrate. Lopez-Molina et al.[44] reported artichoke POD to be especially active for monophenols like 4-CP. So, the botanical source of POD plays an important role in the efficiency of the reaction. In addition, for the three phenols tested, at times up to 60 min, small changes in the

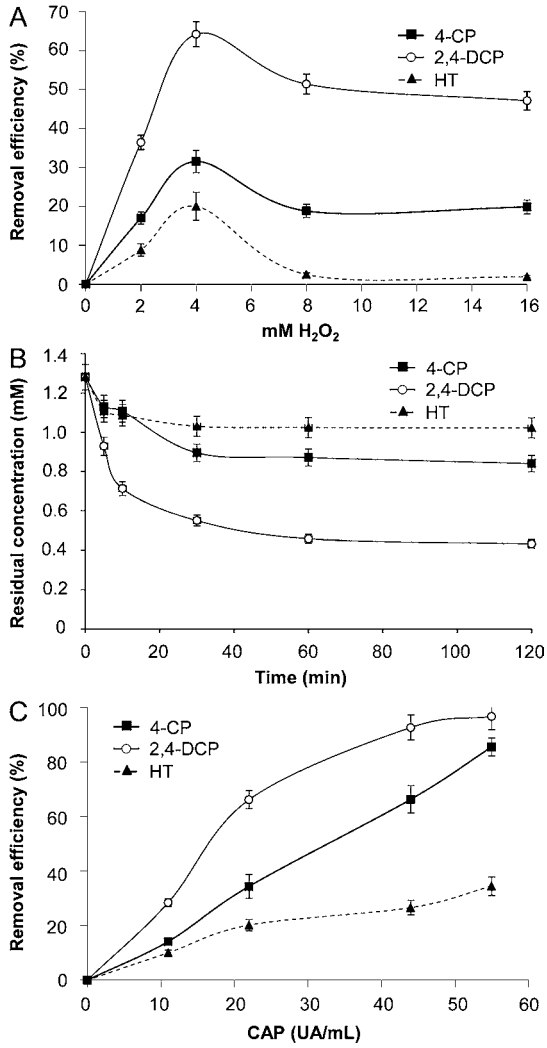

Figure 6. Optimum reagent doses. (A) Effect of H_2O_2 concentration on phenol reduction. Conditions: 2 mM phenol, 20 UA mL^{-1} of CAP after 1 h. (B) Course of the oxidation with time of 4-chlorophenol, 2,4-dichlorophenol, and hydroxytyrosol at pH 4.0 and 5 UA CAP mL^{-1} $mmol^{-1}$ H_2O_2. (C) Removal efficiency of CAP extract for phenol reduction. Conditions: 1.3 mM phenol, 5 UA CAP mL^{-1} $mmol^{-1}$ H_2O_2 after 1 h. Values are the means of triplicate ± SD.

concentration were detected over the time of the experiment. This suggested that CAP is inactivated by the phenoxy radical or by the phenolic polymers produced in the enzymatic reaction.

In order to compare the efficiency of CAP to degrade phenolic compounds, the samples were incubated with increasing amounts of CAP while the ratio CAP/H_2O_2 dose previously found (5 UA CAP mL^{-1} $mmol^{-1}$ H_2O_2) was maintained. The relationship between the phenols removed as a function of CAP dose in the optimum ratio CAP/H_2O_2 is shown in Figure 6C. The degree of removal achieved by a fixed dose depended on the phenolic compound being treated. The removal efficiency was as follows: 2,4-DCP > 4-CP > HT. Increases in CAP induced proportional effects on the removal of 4-CP and 2,4-DCP, and we could obtain a total elimination of them. However, the efficiency for removing HT was not increased, but concentrations of CAP were significantly higher. In fact, the maximum removal was 25% and this profile of efficiency of the degradation indicated that CAP may be fully

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inactivated faster by the degradation products from HT during the catalysis.

These results showed that CAP is a good alternative for removal of chlorinated aromatic compounds, with an efficacy near 70% with feasible conditions of treatment. However, although the results are better than those reported for the PODs of others vegetables,[45] the low affinity of CAP for the removal of HT cannot certainly be regarded as a principal process that could efficiently remediate oil mill waste waters. Sergio et al.[46] studied the use of artichoke extracts for the removal of some phenols present in olive mill wastewater. They achieved a reduction of almost 100% of HT but with large reaction time. Now the efforts are being focused on reducing concentrations of high molecular mass polyphenols.

In conclusion, CAP is obtained economically from low raw materials using a simple protocol, becoming available in large quantities for scientific research and industrial use. This novel CAP might exert its activity in an acidic environment, and the high thermostability of CAP also represents a great advantage in the potential use of this enzyme for the broad number of processes with demonstrated requirements of thermostable POD, including wastewater treatments. In fact, CAP shows a high efficiency for the removal chlorinated aromatic compounds. New advances in this sense would be useful to optimize commercial applications for these enzyme extracts, making their use more competitive.

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ABBREVIATIONS USED

A, percent original activity; A_0, substrate A concentration; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); B_0 , substrate B concentration; BSA, bovine serum albumin; CAP, cationic asparagus peroxidase; CE, crude extract; CLAP, protease inhibitor cocktail; 4-CP, 4-chlorophenol; 2,4-DCP, 2,4-dichlorophenol; E_a, activation energy for thermal inactivation; HPLC, high-performance liquid chromatography; HT, hydroxytyrosol; IEF, isoelectric focusing; k, inactivation rate constant; k_L, the first-order rate constants for thermal inactivation of the heat-labile isoenzyme fraction; k_R, the first-order rate constants for thermal inactivation of the heat-resistant isoenzyme fractions; K_a, Michaelis−Menten constant for substrate A; K_b, Michaelis−Menten constant for substrate B; K_m, Michaelis−Menten constant; pI, isoelectric point; POD, peroxidase(s); PVP, polyvinypirrolidine; T_m, midpoint inactivation temperature; TCA, trichloroacetic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; UA, units of activity; V_{max}, maximum reaction velocity; ν_0, initial reaction velocity.

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