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Screening of Postharvest Agricultural Wastes as Alternative Sources of Peroxidases: Characterization and Kinetics of a Novel Peroxidase from Lentil (*Lens culinaris* L.) Stubble

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ABSTRACT: Aqueous crude extracts of a series of plant wastes (agricultural, wild plants, residues from sports activities (grass), ornamental residues (gardens)) from 17 different plant species representative of the typical biodiversity of the Iberian peninsula were investigated as new sources of peroxidases (EC 1.11.1.7). Of these, lentil (*Lens culinaris* L.) stubble crude extract was seen to provide one of the highest specific peroxidase activities, catalyzing the oxidation of guaiacol in the presence of hydrogen peroxide to tetraguaiacol, and was used for further studies. For the optimum extraction conditions found, the peroxidase activity in this crude extract (110 U mL^{-1}) did not vary for at least 15 months when stored at 4 °C (k_{inact} = 0.146 $year^{-1}$, $t_{1/2\ inact}$ = 4.75 year), whereas, for comparative purposes, the peroxidase activity (60 U mL^{-1}) of horseradish (*Armoracia rusticana* L.) root crude extract, obtained and stored under the same conditions, showed much faster inactivation kinetics (k_{inact} = 2.2 × 10^{-3} day^{-1}, $t_{1/2\ inact}$ = 315 days). Using guaiacol as an H donor and a universal buffer (see above), all crude extract samples exhibited the highest peroxidase activity in the pH range between 4 and 7. Once semipurified by passing the crude extract through hydrophobic chromatography on phenyl-Sepharose CL-4B, the novel peroxidase (LSP) was characterized as having a purity number (RZ) of 2.5 and three SDS-PAGE electrophoretic bands corresponding to molecular masses of 52, 35, and 18 kDa. The steady-state kinetic study carried out on the H_2O_2-mediated oxidation of guaiacol by the catalytic action of this partially purified peroxidase pointed to apparent Michaelian kinetic behavior ($K_m^{app H_2O_2}$ = 1.87 mM; $V_{max}^{app H_2O_2}$ = 6.4 mM min^{-1}; $K_m^{app\ guaicol}$ = 32 mM; $V_{max}^{app\ guaicol}$ = 9.1 mM min^{-1}), compatible with the two-substrate ping-pong mechanism generally accepted for peroxidases. Finally, after the effectiveness of the crude extracts of LSP in oxidizing and removing from solution a series of last-generation dyes present in effluents from textile industries (1) had been checked, a steady-state kinetic study of the H_2O_2-mediated oxidation and decolorization of Green Domalan BL by the catalytic action of the lentil stubble extract was carried out, with the observation of the same apparent Michaelian kinetic behavior (K_m^{appGD} = 471 μM; V_{max}^{appGD} = 23 μM min^{-1}). Further studies are currently under way to address the application of this LSP crude extract for the clinical and biochemical analysis of biomarkers.

KEYWORDS: *agricultural wastes, screening, crude extracts, lentil stubble peroxidase, kinetics, guaiacol, Green Domalan textile dye*

INTRODUCTION

Peroxidases are enzymes widely distributed throughout the animal and plant kingdoms. They catalyze the oxidation of a large variety of organic and inorganic substrates, using hydrogen peroxide as an electron-accepting molecule. As their active site, all peroxidases have a type b heme group (protoporfirin IX) with pentacoordinated Fe(III) as a prosthetic group. These enzymes are classified as peroxidases from mammals, catalases, and the superfamily of plant peroxidases. The latter have been classified on the basis of their different structural features as class I peroxidases from prokaryotes, such as ascorbate peroxidases and cytochrome *c* peroxidase; as class II from fungi, such as lignin peroxidase and manganese peroxidase; and as class III from higher plants, such as horseradish peroxidase and barley peroxidase. Owing to their strong enzymatic activity, stability, and broad industrial, analytical, and environmental applications,[2,3] class III plant peroxidases have been studied in some depth.[4−8]

Peroxidases have mainly been isolated from sources such as horseradish, turnip, and soybean. Currently, the most widespread commercial peroxidase (HRP) is obtained from horseradish (*Armoracia rusticana* L.) roots, accounting for 90% of world production.[9−12] However, HRP has certain problems with regard to stability and inactivation under certain conditions, hence the interest in finding novel plant peroxidases with greater stability and similar applicability. In this sense, novel peroxidases from different plant sources are currently being characterized biochemically, biophysically, and structurally in a search for increasingly stable enzymes. The problems in the extraction of enzymes of plant origin are due to the hardness of the tissues and the presence of polyphenols. This is

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justification for the cloning of any new peroxidase.[13] However, in many cases these enzymes are distributed in the parts of plants that to date have been considered waste. This could be considered an advantage in attempts aimed at optimizing the economics of the extraction and purification of the corresponding cheap and stable peroxidases, also providing high added value to a range of crops, waste products, and agricultural practices.

The main goal of this work was to identify and study the peroxidase activity in aqueous crude extracts obtained from agricultural, wild, garden, and sports plant wastes involving at least 16 different plant species that are abundant on the Iberian peninsula with the aim of obtaining novel and cheaper peroxidase alternatives to horseradish peroxidases that will exhibit the same or better catalytic and stability characteristics and the same and/or further potential biotechnological applications with the same or increased performances. In this sense, the Scientific Committee of the European Union has defined peroxidases as the group of proteins of greatest biotechnological interest in the 21st century. Among other reasons, this definition is based on the potential contribution of these enzymes to the conservation of the environment because they are potential substitutes of other polluting industrial catalysts.

The use of crude extracts is attractive when, as in this case, they exhibit high stability and high enzyme activity and are easy to obtain and very cheap in comparison with the pure enzymes. There is an array of fresh green biomasses discarded after harvesting, such as the green pods of harvested legumes (peas, beans), the stubble from lentil plants, and the leaves of sugar beet. Other green raw biomaterials considered in this work are those from traditional agricultural practices, such as the pruning of vines. To date, the main uses of this type of waste are as fertilizers on crop fields and as livestock feed.

Considering the potential amount of available waste biomass, we first considered the fresh and green postharvest crop residues from peas, beans, lentils, sugar beet, and grapevines. Furthermore, common wild plant species of the Iberian peninsula, such as Scotch and Spanish broom and thistles, which are usually plucked from the soil and removed (burned) owing to the problems caused to breeders and farmers deriving from their tendency to invade pastures and owing to modifications in the soil pH produced by their metabolic action, were investigated. Finally, grass wastes from golf courses and public gardens (collected for composting) were analyzed as potential and abundant raw materials for the production of peroxidases. For comparative purposes, in some cases, for crops such as chickpeas, sunflower, and corn, which are harvested when the plant (leaves, pods, etc.) is dried, samples from the green plants were collected and assayed for their peroxidase activity. Preharvest green wastes from grapevines were also checked. Samples of horseradish roots, the most common source of commercial peroxidase, were also tested for comparative purposes.

Several procedures can be implemented to obtain enzymatic crude extracts.[14−16] The scalable liquid−liquid extraction procedure developed by our group is simple, fast, and efficient, and the peroxidase activity in the extract persists for long periods of time (see below). Of the extracts studied, lentil (*Lens culinaris* L.) stubble crude extract was found to afford one of the highest specific peroxidase activities and had a broad optimum pH operational range. For these reasons, apart from strategic considerations such as its better availability, the amount of wastes produced, and the potential economic interest in the agricultural sector of the Spanish region of Castile and Leon, the crude extract from lentil stubble was selected for semipurification of this novel peroxidase, preliminary kinetic characterization, and feasibility studies for applied processes such as the oxidation/removal of dyes commonly found in contaminated effluents from textile industries.

MATERIALS AND METHODS

Sample Collection and Storage. Prior to sampling, we contacted several Agricultural Regulatory Boards of Appellations of Origin (CRDO), such as Armuña lentils, chickpeas from Fuentesaúco, beans from Barco de Avila, wineries from the Ribera del Duero and Rueda Appellations of Origin, pea processing plants, and companies managing public gardens and golf courses, as well as farmers from different areas of Castile and Leon (Spain). All of them were asked to provide recommendations about the calendars of the different crops and about the associated agricultural and processing practices that generate fresh green wastes. Counting on their cooperation, we visited their factories, fields, and farms for the direct collection of plant wastes generated in preharvest, harvest, and postharvest agricultural practices and the processing of wastes, etc. Samples of different wild plants were also collected in the field. The amount of sample collected was 100−500 g, which was packed in sample bags marked with the corresponding reference and kept cool at 0 °C during transportation to the laboratory, where they were stored at −20 or −80 °C. The calendar of sample collections was as follows: chickpea (harvest, August), sunflower (harvest, October), pea (harvest, June), beans (harvest, October), butter beans (harvest, October), lentil (harvest, June−July), corn (harvest, September), sugar beet (preharvest, September; harvest, postharvest, December), horseradish roots (harvest, November), grapevine (preharvest, June; harvest, August; postharvest October), thistle (August), ryegrass turf (June), annual meadow (*Poa annua*) grass (June), 1-day-, 1-month-, and 1-year-old composts (June), Scotch broom (November), and Spanish white broom (February, just before flowering). Horseradish roots were purchased from Kart Imp. (Palau, Austria).

Selection and Preparation of Crude Extracts. In this work, crude extracts of plants such as chickpea (dry pods, plant) (*Cicer arietinum* L.), sunflower (flowers, leaves) (*Helianthus annuus*), pea (fermented, discarded pods, discarded grains, pods, leaves) (*Pisum sativum*), beans (pods, leaves) (*Vicia faba*), butter beans (pods) (*Phaseolus vulgaris*), lentil (green, threshed plant, stubble) (*Lens culinaris*), corn (cobs, leaves) (*Zea mays*), sugar beet (leaves, roots) (*Beta vulgaris*), horseradish roots (*Armoracia rusticana*), grapevine (Muscat, Sauvignon leaves, ripening grapes) (*Vitis vinifera*), thistle (*Cynara cardunculus*), ryegrass turf (*Lolium perenne*), annual meadow grass (*Poa annua*), 1- day-, 1-month-, and 1-year-old composts, Scotch (*Cytisus scoparius*), and Spanish white- (*Cytisus multiflorus*) and yellow-flowered (*Cytisus striatus*) broom (leaves, stems) were obtained following the same extraction procedure. As a representative example, the following describes the aqueous extraction,[17−20] performed immediately after harvesting of lentil stubble peroxidase (LSP). Green leaves and stems were milled and incubated in distilled water with constant stirring for 22−24 h at room temperature. The homogenate obtained was vacuum-filtered and centrifuged (10000*g*, 277 K, 20 min). Pigments were extracted by phase separation over 20−22 h at 277 K after the addition of solid PEG 10000 MW at 14% (w/v) and solid ammonium sulfate at 10% (w/v) to the supernatant. Two phases were formed after the addition of ammonium sulfate: an upper polymer phase (dark red in color), which contained pigments, phenols, polyphenols, oxidized phenols, and PEG, and a lower aqueous phase (yellow in color) containing peroxidase. Each phase consisted of 50% of the initial volume. These phases were separated, and the aqueous phase, containing peroxidase activity, was centrifuged (10000*g*, 277 K, 15 min). The polymer upper phase showed 0−3% of the peroxidase activity measured at the aqueous bottom phase.[21] The same partition coefficient for peroxidase activity has been shown

previously by the authors for a series of different palm tree leaf extracts.[17−20]

The clear supernatant, containing peroxidase activity, was the final extract in which peroxidase activity was measured and was used for semipurification of the enzyme and for the experiments addressing the oxidation of guaiacol and the industrial textile dye Green Domalan BL.

Reagents. Analytical or extra-pure grade polyethylene glycol (PEG), guaiacol, ammonium sulfate, sodium phosphate, and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were used without further purification. H_2O_2 was from Merck (Darmstadt, Germany). Green Domalan BL dye was a gift from the Dyes Alçada & Pereira Ltd. textile factory (Covihlâ, Portugal). Domalan dyes are manufactured by M. Dohmen GmbH & Co. KG (Korschenbroich, Germany). Phenyl-Sepharose CL-4B columns were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Cellulose membrane tubing for dialysis (average flat width = 3.0 in.) was purchased from Sigma Chemical Co.; slide A-lyzer dialysis cassettes (extra-strength, 3−12 mL capacity, 10000 MWCO) were from Pierce Biotechnology, Inc. (Rockford, IL, USA), and filter devices (Amicon Ultra Cellulose 30000 MWCO, 15 mL capacity) were from Millipore Corp. (Billerica, MA, USA). All other reagents were of the highest purity available. The water used for preparing the solutions was double-distilled and then subjected to a deionization process.

Semipurification of LSP. To proceed further in the purification of LSP, its crude extract was titrated with ammonium sulfate to a conductivity value of 326 mS cm^{-1} and was applied to a phenyl-Sepharose CL-4B column (1.5 × 35 cm) equilibrated with 100 mM phosphate buffer, pH 6.5, with 1.7 M ammonium sulfate, which has the same conductivity as the sample. The enzyme was eluted with 100 mM phosphate buffer, pH 6.5, plus 0.2 M ammonium sulfate at a flow rate of 1 mL min^{-1}. Twelve milliliter fractions were collected, and those showing peroxidase activity were dialyzed against 10 mM Tris buffer, pH 8.1, with constant stirring for 24 h at 277 K.[17−20] After centrifugation (10000*g*, 277 K, 20 min), these fractions were membrane-concentrated six times (Amicon, 30 kDa cutoff).

Characterization of LSP. The peroxidase purity number or Reinheitszahl (RZ) value was determined by UV−visible spectrophotometry between 200 and 700 nm.

Gel Electrophoresis. The purity of the LSP was determined by SDS-PAGE as described by Fairbanks et al.[22] on a Bio-Rad minigel device using a flat block with a polyacrylamide gradient of 4−12%. Gels were prefixed and stained using the method of Merril et al.[23] The Novex Sharp Prestained Protein Standard (Life Technologies, Carlsbad, CA, USA), containing 12 proteins, was used to estimate molecular weights of LSP.

Protein Determination and Peroxidase Activity. Total protein concentrations were determined by using the Bradford assay,[24] with bovine serum albumin as standard. Peroxidase activity was assessed with a colorimetric method using H_2O_2 as substrate and guaiacol as chromogenic substrate. The assay was performed at 25 °C by adding 20 μL of the enzyme solution to 2.0 mL of 20 mM $H_2PO_4^-/HPO_4^{2-}$ buffer, pH 6.0, containing 4.9 mM H_2O_2 and 18 mM guaiacol. The rate of H_2O_2 consumption was estimated by measuring the absorbance of a colored product (tetraguaiacol) at 470 nm, using a molar absorptivity of 5200 M^{-1} cm^{-1}.[25] Furthermore, following a similar experimental procedure, peroxidase activity at pH 4.0 (20 mM acetic acid/acetate buffer) was found in the H_2O_2-assisted oxidation of Green Domalan BL, an anthraquinone and azoic acid-dispersed dye with a confidential formulation. This peroxidase activity was measured at 25 °C, following the removal kinetics of the dye from solution at 600 nm, where the dye shows its absorbance maximum. At this wavelength, the corresponding Beer−Lambert correlation between absorbance and dye concentration afforded a molar absorptivity of 8.6 mM^{-1} cm^{-1}, which was used to estimate the dye concentration.[1] The assays were performed at 25 °C using a Beckman DU-7 UV−vis spectrophotometer. One unit of peroxidase activity (U) was defined as the amount capable of oxidizing 1 μmol of guaiacol (2-methoxyphenol) per minute under standard conditions.

For the measurement of the peroxidase activity of crude extracts versus pH, 20 μL of the extract was added to a universal buffer (10 mM acetic acid/sodium acetate, 10 mM boric acid/sodium borate, and 10 mM $H_3PO_4/H_2PO_4^-/HPO_4^{2-}/PO_4^{3-}$) containing 4.9 mM guaiacol and 18 mm H_2O_2. The initial pH of the universal buffer was 3.2, such that by adding 0.1 M NaOH the different study pH values between 3 and 9 were reached. Three replicates were carried out for each peroxidase activity assay.

Analysis of Kinetic Data. Despite the heterogeneity of the purified LSP (see below), we wished to know the apparent rate equation to which it would be possible to fit the initial rates of guaiacol oxidation at 25 °C measured at (1) different H_2O_2 concentrations (0.005−13.0 mM) and a fixed 18.0 mM guaiacol concentration and (2) different guaiacol concentrations (0.45−45.0 mM) and a fixed 4.9 mM H_2O_2 concentration under the above assay conditions. For study of the oxidation kinetics of Green Domalan BL by the LSP crude extract obtained, the fixed concentration of H_2O_2 was 0.3 mM and the range of dye concentrations studied was 10.8−270 μM. Any attempts to conduct a rigorous analysis of the steady-state kinetic data for mechanistic purposes or to calculate the values of the microscopic constants would be presumptuous and premature, given the lack of homogeneity of the biocatalysts used.

The rate equation for a two-substrate (H_2O_2 and AH) ping-pong kinetic mechanism, in the absence of products and at noninhibitory substrate concentrations, seen for peroxidases,[26,27] is given by

$$v_0 = \frac{V_{max}[H_2O_2][AH]}{K_m^{H_2O_2}[AH] + K_m^{AH}[H_2O_2] + [H_2O_2][AH]} \quad (1)$$

The initial rates (v_0) were measured as a function of both substrate (H_2O_2 and guaiacol) concentrations at fixed concentration of the cosubstrate. If the concentration of guaiacol (AH) is maintained constant at 18.0 mM, eq 1 could be written as an apparent Michaelis−Menten rate equation:

$$v_0 = \frac{V_{max}^{appH_2O_2}[H_2O_2]}{K_m^{appH_2O_2} + [H_2O_2]} \quad (2)$$

Thus, the double reciprocal of eq 2 is given by

$$\frac{1}{v_0} = \frac{1}{V_{max}^{appH_2O_2}} + \frac{K_m^{appH_2O_2}}{V_{max}^{appH_2O_2}[H_2O_2]} \quad (3)$$

Similarly, when the concentration of H_2O_2 is fixed at 4.9 mM, eq 1 could be written as

$$v_0 = \frac{V_{max}^{appAH}[AH]}{K_m^{appAH} + [AH]} \quad (4)$$

and the corresponding double reciprocals as

$$\frac{1}{v_0} = \frac{1}{V_{max}^{appAH}} + \frac{K_m^{appAH}}{V_{max}^{appAH}[AH]} \quad (5)$$

Generally, the double reciprocals or Lineweaver−Burk plots, in this case following eqs 3 and 5, distort the v_0 measurements at low substrate concentrations, and this can lead to somewhat inaccurate estimates of the V_{max} and K_m parameters.[28,29] Accordingly, the Hanes−Woolf linearizations of eq 1, which can be obtained by multiplying eq 3 by $[H_2O_2]$ and eq 5 by [AH], were used to fit the kinetic data. Thus, the $[H_2O_2]/v_0$ versus $[H_2O_2]$ and $[AH]/v_0$ versus [AH] kinetic data were fitted by linear regression to obtain, from the corresponding slopes and *y* intercepts, the apparent kinetic parameters $K_m^{appH_2O_2}$, $V_{max}^{app\ H_2O_2}$, and $K_m^{app\ guaicol}$, $V_{max}^{app\ guaicol}$, respectively.

RESULTS AND DISCUSSION

Peroxidase Activity of Crude Extracts. As can be seen in Figure 1, of the crude extracts obtained from agricultural crops, green lentil leaves show the highest peroxidase activity (135 U mL^{-1}), followed by green postharvest lentil stubble (110 U mL^{-1}), horseradish roots (60 U mL^{-1}), postharvest pea leaves (45 U mL^{-1}), chickpea green leaves (preharvest) (35 U mL^{-1}),

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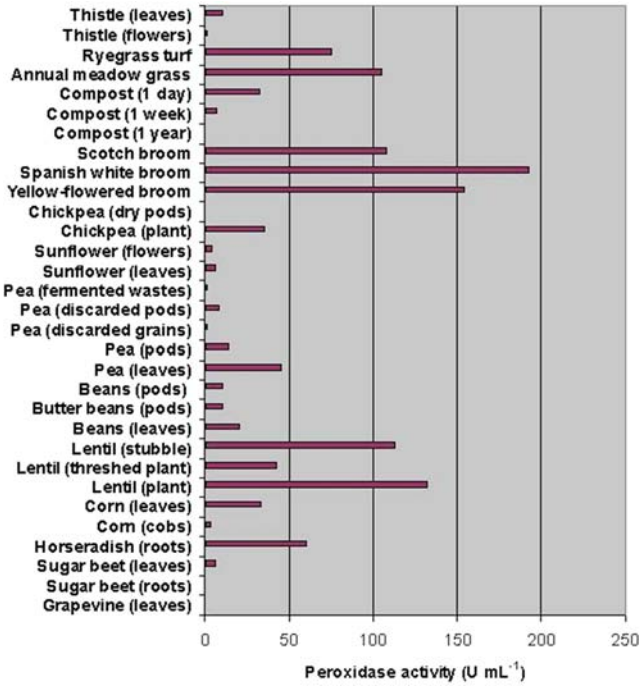

Figure 1. Peroxidase activity of aqueous crude extracts obtained from a series of different plant wastes (agricultural, wild plants, residues from sports activities (grass), ornamental residues (gardens)).

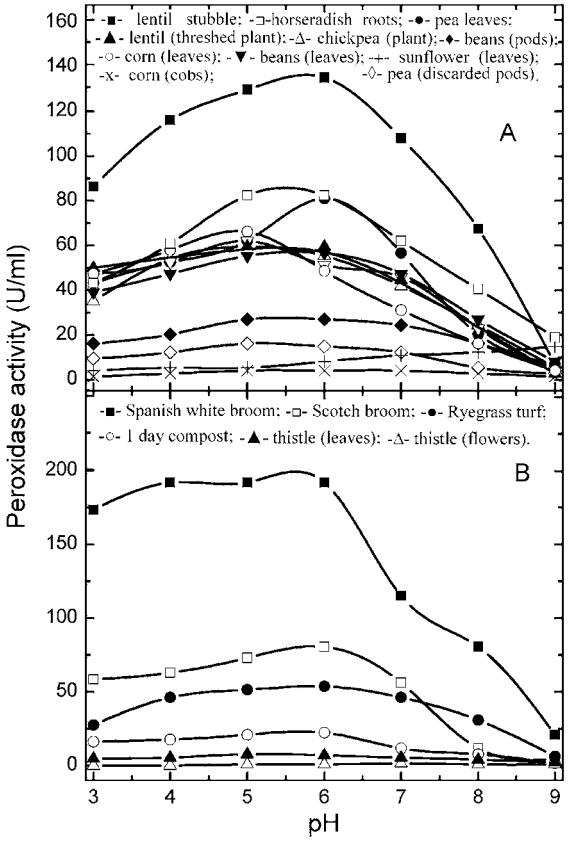

Figure 2. Effect of pH on peroxidase activity of crude extracts from plant wastes (agricultural (A) and wild plants and golf grass (B)) with guaiacol as H donor. See text for experimental conditions. Each point represents the mean of three separate experiments.

preharvest corn leaves (33 U mL^{-1}), green bean leaves (20 U mL^{-1}) and pods (10 U mL^{-1}), butter bean pods (postharvest) (10 U mL^{-1}), sugar beet leaves (postharvest) (6 U mL^{-1}), sunflower leaves and flowers (harvest) (6–4 U mL^{-1}), and corn cob (preharvest) (3 U mL^{-1}). However, the samples of green and fresh leaves from grapevine showed no peroxidase activity, a surprising finding that is currently under investigation to determine whether the phenomenon might be due to some artifact or some kind of interference in the extraction process used.

Among the gardening and sports samples, the crude extracts from two types of grass from golf course greens, annual meadow grass (*Poa annua*) (105 U mL^{-1}) and ryegrass (*Lolium perenne*) (75 U mL^{-1}), showed higher peroxidase activities, followed by public garden compost at 1 (32 U mL^{-1}) and 7 (7 U mL^{-1}) days old.

Of the samples of wild plants studied, the findings for the two types of broom were quite surprising owing to the high peroxidase activity found in their crude extracts, especially in the samples of Spanish white broom (*Cytissus multiflorus* L.) (192 U mL^{-1}), the yellow-flowered broom (*Cytisus striatus*) (154 U mL^{-1}), and the Scotch broom (*Cytisus scoparius*) (108 U mL^{-1}). These activities depend on the date of collection, being highest just before flowering (February–March). Thistle leaves and flowers showed 10 and 1 U mL^{-1} peroxidase activities, respectively. These wild species are considered damaging shrubs by farmers and stockbreeders because they grow spontaneously and abundantly and invade crop and pasture fields in many areas of Spain.

Storage Stability of Crude Extracts. The storage stabilities of the crude extracts showing the highest peroxidase activities were measured for the optimum extraction conditions found. Peroxidase activity was measured monthly over several years with three replicates in 20 μL aliquots taken from the crude extracts stored at 4 °C. The processes of enzyme inactivation involve the unfolding of the protein, which is an intramolecular process, and hence the kinetics of inactivation of the enzymes is first-order. Thus, the semilogarithmic plots of the data of enzyme activity versus time are linear, with slopes measuring the values of $-k_i$, that is, the inactivation rate constant (a function of temperature, medium conditions, etc.). Furthermore, the half inactivation time ($t_{1/2}$), that is, the time required for the enzyme activity to reach 50% of its initial value, can be determined from the ln $2/k_i$ ratio.

Whereas most of the crude extracts from the different species studied have a high storage stability (for example, Scotch broom extract exhibits k_{inact} = 1.46 $year^{-1}$ and $t_{1/2\ inact}$ = 0.474 year), the crude extracts from lentil stubble, besides their remarkable peroxidase activities (see above), show the highest and most surprising stability, with no significant variation in activity over at least 15 months when stored at 4 °C (k_{inact} = 0.146 $year^{-1}$, $t_{1/2\ inact}$ = 4.75 year). However, for comparative purposes, horseradish (*Armoracia rusticana* L.) root crude extract, obtained and stored under the same conditions, displayed much faster inactivation kinetics (k_{inact} = 2.2 × 10^{-3} day^{-1}, $t_{1/2\ inact}$ = 315 days).

Optimum pH for the Peroxidase Activities of Crude Extracts. The optimum pH for any peroxidase depends on the hydrogen donor and buffer solution used in the activity assay. Using guaiacol as an H donor and a universal buffer (see above), all crude extract samples exhibited the highest peroxidase activity in the pH range between 4 and 7 (Figure 2), values similar to the optimum pH found for other plant

Table 1. Purification Steps of LSP

procedure	vol (mL)	protein (mg)	total activity (U)	specific activity (U/mg)	purifn factor	yield (%)
1, homogenate	1040	9161	64480	7	1	100
2, PEG + $(NH_4)_2SO_4$	490	1332	63700	48	7	98.8
3, phenyl-Sepharose	10	17	17650	1038	47	27.4

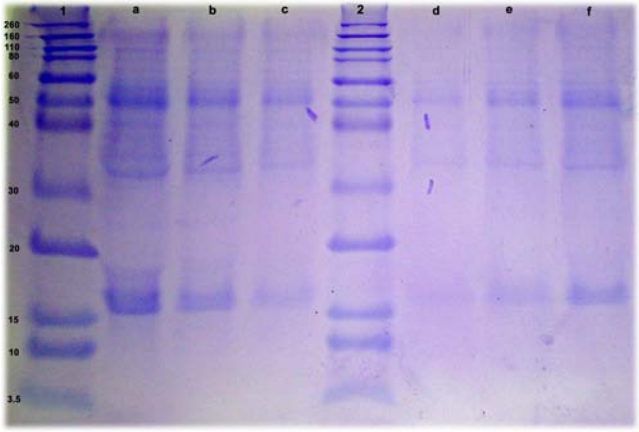

Figure 3. SDS-PAGE of semipurified LSP samples (lanes a (87.6 μg), b (43.8 μg), c (21.9 μg), d (8.7 μg), e (17.5 μg), and f (35.0 μg)). The Novex Sharp Prestained Protein Standard (Life Technologies), containing 12 proteins as markers (lanes 1 and 2), was used to estimate molecular weights of LSP.

peroxidases, such as pea peroxidase,[30] and the peroxidases from strawberry fruit,[31] radish and turnip,[32] tomato and horseradish,[33] tea leaves,[34] wild cabbage leaves,[35] cotton leaves,[36] artichoke leaves,[37] Za'atar shrub leaves,[38] leaves of the Copaiba oil tree,[39] marula tree,[40] morning glory creeper,[41] sweet potato,[42] etc.

Also noteworthy is that the majority of the crude extracts studied also display a remarkable level of peroxidase activity between pH 3.0 and 8.0, which enables them to act effectively as biocatalysts in a number of biotechnological processes that take place within this broad pH range.

Semipurification of LSP). LSP was partially purified in high yield from postharvest lentil stubble. The purification steps and their efficiencies are summarized in Table 1. The Reinheitszahl value (RZ = A_{403}/A_{280} = 2.5 ± 0.1), a measure of hemin content of the peroxidase, was determined by UV−visible spectrophotometry. Normally, higher RZ values indicate higher purity.

In SDS-PAGE, the semipurified peroxidase migrated as three main bands, corresponding to molecular masses of 52, 35, and 18 kDa (Figure 3). This molecular mass range is characteristic of certain other plant peroxidases. Thus, 59 kDa is the molecular mass of the anionic peroxidase from the leaves of the Copaiba oil tree (*Copaifera langsdorffi* L.);[39] 35 kDa is the molecular mass of the hemeperoxidase from tea (*Camellia sinensis*) leaves;[34] that of sorghum (*Sorghum vulgare*) grain peroxidase is 38 kDa,[43] and the molecular mass of peanut (*Arachis hypogaea*) anionic peroxidase is 40 kDa.[44]

LSP Kinetic Studies. The initial rate versus [substrate] data of the H_2O_2-assisted oxidation of guaiacol through the catalytic action of LSP seem to fit a hyperbola-type saturation equation similar to the Michaelis−Menten rate equation with respect to both substrates. To check that the kinetic data were indeed consistent with an apparent Michaelian kinetic behavior, the

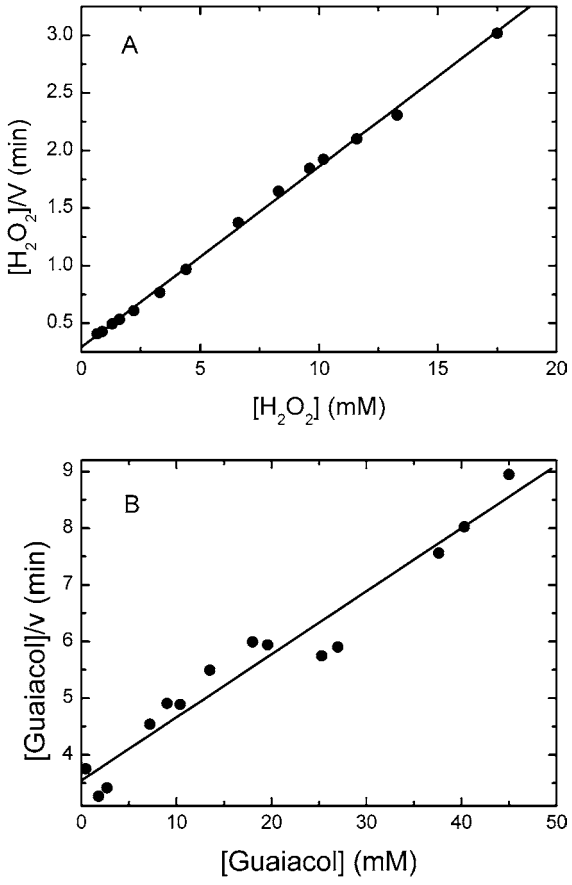

Figure 4. Apparent kinetic behavior of the two-substrate reactions for LSP (87.6 μg mL^{-1}): Hanes−Woolf plot of (A) initial rates of oxidation of guaiacol versus $[H_2O_2]$ at constant guaiacol concentration and (B) initial rates of oxidation of guaiacol versus [guaiacol] at constant H_2O_2 concentration. See text for experimental conditions. Means of triplicates are shown.

data were rigorously fitted to the Hanes−Wolf linearization of the Michaelis−Menten equation (Figure 4).

The corresponding apparent Michaelian kinetic parameters obtained were $K_m^{app\ H_2O_2}$ = 1.87 mM, $V_{max}^{app\ H_2O_2}$ = 6.4 mM min^{-1}, $K_m^{app\ guaicol}$ = 32 mM and $V_{max}^{app\ guaicol}$ = 9.1 mM min^{-1}. The $K_m^{app\ guaicol}$ (32 mM) was higher than those found for guaiacol oxidation by peroxidase from carambola or starfruit (20.3 mM),[45] royal palm tree (15.2 mM),[46] Korean radish roots (6.7−13.8 mM),[47] green peas (10.2 mM),[30] *Chamaerops excelsa* palm tree (9.25 mM),[4] tomato (5−10 mM),[48] turnip (3.7 mM), [9] and the Copaiba oil tree (0.04 mM).[39] The $K_m^{app\ H_2O_2}$ (1.87 mM) was also higher than that reported for the peroxidase from the Copaiba oil tree (0.39 mM)[39] but lower than that of carambola or starfruit (12.8 mM),[45] *Chamaerops excelsa* palm tree (3.58 mM),[4] and royal palm tree (2.7 mM).[46] These studies used the same substrates (guaiacol and hydrogen peroxide) and similar chemical concentrations, and their results can therefore be compared.

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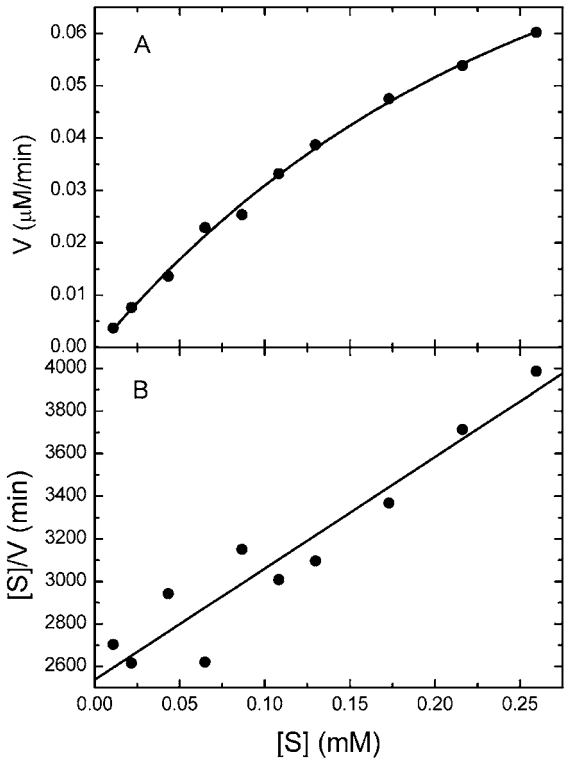

Figure 5. Apparent kinetic behavior of the oxidation of the textile dye Green Domalan BL by lentil stubble crude extract: direct (A) and Hanes–Woolf (B) plots of initial rates of oxidation versus [dye] at constant H_2O_2 concentration. See text for experimental conditions. Means of triplicates are shown.

H_2O_2-Assisted Oxidation of Green Domalan BL by LSP. Dyes can be removed by means of oxidative enzymes.[49−51] Peroxidases are versatile enzymes that catalyze the oxidation of a large number of aromatic structures through a reaction with hydrogen peroxide, and they are applied in the chemical, environmental, pharmaceutical, and biotechnological industries. Peroxidases can act on specific recalcitrant pollutants either through free radical oxidative polymerization and consequent settling or by transformation into other products.[2] HRP in the presence of H_2O_2 is known to be effective in the removal of a broad spectrum of aromatic compounds (phenols, biphenols, and anilines) via a free radical oxidative polymerization mechanism,[2,52−55] and it is also involved in the degradation and precipitation of industrial dyes.[56−59]

One of the objectives of our current research on peroxidases with regard to applications was to check the feasibility of the peroxidase activity of fresh postharvest lentil stubble, and (implicitly) of its H_2O_2-mediated oxidation mechanism for the oxidation/removal of a series of dyes commonly found in contaminated effluents from textile industries.[1] Such dyes, with complex structures, contain several aromatic groups (phenol, aniline), making them possible substrates for LSP. Screening experiments designed to assess dye degradation in the presence of H_2O_2 and LSP afforded positive results. The effects of parameters such as pH, H_2O_2, enzyme and dye concentrations, contact and centrifugation times, the temperature of decolorization, and the detoxification of the dye solutions were investigated. The dye solutions were found to be stable upon exposure to H_2O_2 alone and to the enzyme extract alone. Thus, dye precipitation is the result of an H_2O_2-dependent enzymatic reaction, possibly involving free radical formation followed by polymerization and precipitation.[1]

Finally, with Green Domalan BL, the last-generation dye most efficiently removed (90%) by H_2O_2-assisted oxidation catalyzed by lentil stubble crude extract, a preliminary kinetic study of v_0 versus [dye] was carried out in 0.02 M acetic acid/acetate, pH 4.0−4.7, the buffer present in the effluents from the Portuguese textile industry. The range of dye concentration studied was 21.5−258 μM, the H_2O_2 concentration was constant (0.3 mM), and the peroxidase activity in the reaction samples was 2.26 U mL^{-1}. Not knowing the exact chemical formula of the dye because of trade secrets legislation, the molar mass of Green Domalan BL was measured by mass spectroscopy, proving to be 930 g/mol. This preliminary kinetic characterization points to an apparent Michaelian kinetic behavior (Figure 5), with the following kinetic parameters: K_m^{appGD} = 471 μM; V_{max}^{appGD} = 23 μM min^{-1}.

Further studies addressing the application of this LSP crude extract to the clinical and biochemical analysis of biomarkers and the purification and characterization of Spanish white broom peroxidase are currently under way.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CRDO, Agricultural Regulatory Boards of Appellations of Origin; GD, Green Domalan BL; HRP, horseradish peroxidase; LSP, lentil stubble peroxidase; PEG, polyethylene glycol; RZ, ratio of A_{403}/A_{280}; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane; UV–vis, ultraviolet–visible.

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