This article has been published in the

International Journal of Plant Biology and Research

ISSN: 2333-6668

Study on extraction, purification and characterization of a novel peroxidase from white Spanish broom (*Cytisus multiflorus*)

Patricia Pérez Galende[1], Cándido García de María[2], Juan B. Arellano[3]*, Manuel.G. Roig[4] and Valery L. Shnyrov[5]

[1] Centro de Investigación y Desarrollo Tecnológico del Agua (CIDTA), Universidad de Salamanca, 37007 Salamanca, Spain

[2] Departamento de Química Analítica, Nutrición y Bromatología, Universidad de Salamanca, 37008 Salamanca, Spain

[3] Departamento de Estrés Abiótico. Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), 37008 Salamanca, Spain

[4] Departamento de Química Física, Universidad de Salamanca, 37007 Salamanca, Spain

[5] Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, 37007 Salamanca, Spain

Corresponding author: Juan B. Arellano. Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de merinas 52, 37008 Salamanca, Spain. Phone: +34 923 219 606. Fax: +34 923 219 609. Email: juan.arellano@irnasa.csic.es

Abstract

Peroxidases (EC 1.11.1.7) are a large group of enzymes widely distributed in the plant kingdom. The present work describes a study on the isolation, purification and some features of a novel peroxidase from white Spanish broom (*Cytisus multiflorus*), a tree legume very abundant in the northern half of Spain and Portugal. Optimal conditions are proposed for enzyme extraction, removal of phenolic compounds and enzyme purification by consecutive hydrophobic, ion-exchange and size-exclusion chromatographies. Peroxidase from *Cytisus multiflorus* (CMP) was found to have a molecular weight of 49 kDa. The spectrum of CMP showed a Soret band at 403 nm with a R_z factor of 3.3. Substrate specificity and the effect of some variables on the activity of CMP with guaiacol as cosubstrate have also been investigated.

Keywords: peroxidase, plant, purification, chromatography.

Introduction

Peroxidases (EC 1.11.1.7) 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. The following three-step mechanism has been proposed [1], where E is a peroxidase, Cpd I and Cpd II are intermediates of the oxidized peroxidase and AH_2 is the electron donor substrate which is converted to the radical $AH^{\bullet}$ in an one-electron oxidation:

$$E + H_2O_2 \longrightarrow \text{Cpd I} + H_2O \quad (1)$$

$$\text{Cpd I} + AH_2 \longrightarrow \text{Cpd II} + AH^{\bullet} \quad (2)$$

$$\text{Cpd II} + AH_2 \longrightarrow E + AH^{\bullet} + H_2O \quad (3)$$

Peroxidases have several proposed functions in plants such as biosynthesis of lignin in cell walls, defense against pathogens and metal stress response [2, 3]. They are classified in three classes. Class I includes prokaryotic and plant intracellular enzymes from mitochondria and chloroplasts. Class II includes extracellular fungal peroxidases such as manganese peroxidase. Class III consists of peroxidases secreted by higher plants [4] and they are single-chain proteins, often glycosilated, which exist in multiple isoforms with some differences in their function, substrate specificity or optimum pH [5]. These enzymes are metalloproteins containing a heme center (Fe porphyrin IX) which is proposed to be stabilized by some metal ions such as Ca^{2+} [6]. In the native forms of these enzymes, a Fe^{3+} ion is linked to four

nitrogens of the heme center and to a nitrogen of a histidine residue, the sixth position remaining free [7].

Because of their high enzymatic activity and stability and their widespread industrial, analytical and environmental applications, Class III peroxidases have been studied in some depth [8]. Peroxidases are used commercially as catalysts for the synthesis of phenolic resins [9], in the treatment of waste waters containing phenolic compounds or aromatic amines [10], as labeling enzymes in immunochemistry [11], in chemiluminescence and as components of kits for medical diagnosis [12]. The Scientific Committee of the European Union has defined peroxidases as the group of proteins of greatest biotechnological interest in the 21st century. This definition is based on the potential contribution of these enzymes to the conservation of the environment as they are potential substitutes of other polluting industrial catalysts [13].

The most widely used and commercially available peroxidase is Horseradish peroxidase (HRP), from root of horseradish (*Amoracia rusticana* L.). Although accounting for 90% of the world production of peroxidases [14], HRP has drawbacks affecting its stability and inactivation under certain conditions. In search for increasingly stable enzymes to overcome these problems, peroxidases from different plants are being biochemically and biophysically characterized.

Different techniques have been used to purify peroxidases from their native sources [15, 16]. Most of them include an initial solid-liquid extraction where a homogeneous extract containing proteins in buffered aqueous medium is obtained. Additives such as salts or surfactants are commonly added to improve the extraction and stabilization of membrane-bound proteins [17]. Along with peroxidases, other non-protein species, such as phenolic compounds, are extracted. Phenolic compounds can inhibit the peroxidase activity [18] and must be removed in the first purification stage. Subsequent stages of the purification processes are focused on separating the desired peroxidase from other proteins, retaining its structure and biological activity. These stages exploit chemical, structural or functional differences between the target peroxidase and other proteins of the crude extract.

The peroxidase activity of some agricultural wastes and wild plants from the Spanish region of Castilla y León has recently been investigated [13]. The highest peroxidase activities have been observed in extracts of lentil plant (*Lens culinaris* Medikus) and white Spanish broom (*Cytisus multiflorus*). The present paper deals with the extraction, purification and characterization of the peroxidase from *Cytisus multiflorus* (CMP), an abundant tree legume in the northern half of Spain and Portugal.

Materials and methods

Materials

Analytical or extra-pure grade polyethyleneglycol (PEG), guaiacol (2-methoxyphenol), ammonium sulphate and sodium chloride were supplied by Sigma Chemical Co (Madrid, Spain). Hydrogen peroxide, H_2O_2, 30 w/v %, analytical grade**,** was from Panreac Quimica S.L.U. (Barcelona, Spain). All other reagents were of the highest purity available. Universal PAB buffer solutions were prepared with equimolar amounts of sodium hydrogen phosphate, sodium acetate and sodium orthoborate; the final pH was fixed with hydrochloric acid or sodium hydroxide and PAB concentration was the sum of phosphate, acetate and borate in the final solution. All laboratory solutions were prepared in double-distilled deionized water. Columns for separations by hydrophobic interaction (Phenyl-Sepharose CL-4B), cation exchange (HiTrap^{TM} SP HP) and size exclusion (Superdex-200) were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Anion exchange column (TSKgel-DEAE) was purchased from Tosoh Corporation (Tokyo, Japan). Cellulose membrane tubing for dialysis (avg. flat width 3.0 in.) was from Sigma Chemical Co.; slide-A-lyzer dialysis cassettes (extra-strength, 3-12 mL capacity, 10.000 MWCO) were from Pierce Biotechnology, Inc. (Rockford, IL, USA); and centrifuge filter devices (Amicon Ultra Cellulose 10.000 MWCO, 15 mL capacity) were supplied by Millipore Corp. (Billerica, Massachusets, USA).

Plant extracts

Plants of white Spanish broom were collected from different areas in the province of Salamanca (Castilla y León) during different seasons (see Table 1). Unrooted plants (leaves and stems) were cleaned, milled and incubated at room temperature with continuous agitation in 100 mM PAB buffer containing sodium chloride or a surfactant. The homogenate was vacuum-filtered and centrifuged for 30 min at 10000 g and 4 ºC. To remove interfering phenolic compounds, solid polyethylene glycol (PEG 10000MW) and solid ammonium sulphate were added to the resulting supernatant. Once stirred for a few minutes, the mixture was left for 8 h at room temperature. Then, the two phases formed were separated by decantation and the bottom aqueous phase, containing the peroxidase, was centrifuged for 15 min at 10000 g and 4 ºC, giving the final plant extract. To evaluate the extraction efficiency, the peroxidase activity and the total protein concentration were measured in both the final extract and the remaining PEG top phase.

The effects of incubation time, pH of buffer, concentration of sodium chloride, presence of surfactants, and amounts of ammonium sulphate and PEG on the extraction efficiency were investigated. In all the experiments, 500 mL of PAB buffer per 100 g of milled plants were used.

Enzyme activity

Peroxidase activity was measured by photometry at 25 ºC with guaiacol and hydrogen peroxide as substrates. A double beam T80+ PG UV/Vis spectrophotometer (Instruments Ltd, Leicester, UK) fitted with standard 1 cm quartz cells and a water-recirculation thermostat was used. In the reaction cell, 20 μL of enzyme solution were added to 2.0 mL of 20 mM sodium phosphate buffer, pH 6, containing 18 mM guaiacol and 5 mM H_2O_2. The blank solution was prepared in the same way in the reference cell, adding 20 μL of buffer instead of the enzyme solution. Absorbance at 470 nm was then monitored for 2 min. From the increase in absorbance over this time (ΔA), the activity was calculated in units per millilitre, one unit (U) being the amount of enzyme that causes the oxidation of 1 μmol of guaiacol (ε_{470} = 5200 L $mol^{-1}cm^{-1}$) per minute, under the working conditions.

Total protein

Total protein concentration was achieved by absorbance measurements at 595 nm, according to Bradford´s method [19]. Bovine serum albumin was used as standard.

CMP purification

CMP from the plant extracts was isolated and purified by consecutive hydrophobic, ion-exchange and size-exclusion chromatographies.

First, separation was performed by hydrophobic chromatography in a Phenyl-Sepharose column (1.4 x 35 cm). The effect of pH and ionic strength on protein retention was studied. The ionic strength of plant extracts at different pH was varied with solid ammonium sulphate. The extract sample volume used was 100 mL in all cases and the column was equilibrated with 100 mM PAB buffer, the same pH as the sample extract, containing 1.7 M ammonium sulphate. Elution was carried out by reducing the concentration of ammonium sulphate to 200 mM in the same buffer. Peroxidase activity and total protein concentration were monitored in the fractions obtained. For optimum pH and ionic strength, fractions with

high peroxidase activity were collected, mixed and divided into smaller fractions which were dialyzed against 5 mM PAB buffer at different pH.

As a second stage in the purification process, the possible separation of the protein of the dialyzed fractions by ion-exchange chromatography was studied. The effect of pH on the retention of protein in cation-exchange (HiTrap^{TM} SP HP) and anion-exchange (TSKgel-DEAE) columns, connected to an ÄKTA-purifier system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), was considered. The columns were connected to a multi-wavelength UV-Vis detector where absorbances at 403 and 280 nm were simultaneously monitored. In all cases, elution was performed with a linear sodium chloride gradient (0 to 1 M) in 5 mM PAB buffer, at the same pH as the starting fraction and a flow rate of 1 mL min^{-1} for 40 min. As in the previous stage, peroxidase activity and total protein concentration were monitored in the new fractions. For optimum pH, fractions with high peroxidase activity were collected, mixed and dialyzed against 5 mM PAB buffer.

Finally, the dialyzed mixture obtained in the preceding stage was brought to a size-exclusion column (Superdex-200) connected to an ÄKTA-purifier system. The column was equilibrated with 5 mM PAB buffer containing 150 mM sodium chloride and at the same pH as the starting mixture. It was connected to the multi-wavelength UV-Vis detector and absorbances at 403 and 280 nm were monitored. Elution was carried out at a flow rate of 0.5 mL min^{-1}. Both peroxidase activity and total protein concentration were once again measured in the new fractions, and those with peroxidase activity were collected, mixed, dialyzed against 5 mM PAB buffer and stored at 4˚C.

Molecular weight

The molecular weight of purified CMP was determined by sodium dodecyl sulphate--polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [20]. CMP was prepared in 20 mM Tris-[hydroxymethyl]aminomethane (TRIS) buffer, pH 8.3, with 10 % glycerol, 2 mM EDTA, 0.2 % sodium SDS, 4% 2-mercaptoethanol and 0.001 % bromophenol blue. The electrophoresis buffer was a 25 mM TRIS solution with 250 mM glycine and 0.1 % SDS at pH 8.3. A dual colour calibration kit (Fermentas UK Ltd., Cambridge, UK) was used. After electrophoresis, proteins were stained with Coomassie Brilliant Blue and then washed-out overnight in a solution containing 10% methanol and 7.5 % acetic acid.

Substrate specificity

Different compounds were tested as hydrogen donor substrates of CMP: guaiacol, o-dianisidine, pyrogallol, ferulic acid, o-phenylenediamine, catechol and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The effects of pH and concentration of buffering medium on the activity of CMP was studied for each substrate. The experimental conditions proposed earlier [21] to measure the activity of other peroxidases with these substrates were used.

Variables affecting the activity of CMP with guaiacol.

The effects of pH, concentrations of guaiacol and hydrogen peroxide, ionic strength, temperature and presence of some potential effectors were investigated. All these effects were evaluated using the procedure described in section 2.3 (Enzyme activity) with the changes detailed below.

The effect of co-substrates guaiacol and H_2O_2 on the activity of CMP was studied by varying the concentration of each of them, the other being fixed at the concentration of saturation.

To study the effect of ionic strength, the concentration of sodium chloride in the reaction cell was varied between 0 and 5 M.

To fix the temperature of the reaction and reference cells, water at the working temperature was recirculated through the cell holder. The temperature was checked before the addition of the enzyme solution.

Potential activity effectors were added to enzyme solutions with identical content of CMP. After 30 min incubation at room temperature, the peroxidase activities of these solutions were measured and compared with that of a solution containing only CMP.

Thermal stability

Aliquots of purified CMP were kept for 15 min at different pH and temperatures in a water thermostatic bath. Then, once the temperature was readjusted, the peroxidase activity of each solution was measured by the method with guaiacol described above, at pH 6 and 25 ˚C.

CMP spectrum and R_z factor

The UV-Vis absorption spectrum of a solution of purified CMP was registered in a double beam T80+ PG UV/Vis spectrophotometer (Instruments Ltd, Leicester, UK) fitted with standard 1 cm quartz cells and a water-recirculation thermostat. The R_z value (Reinheitszhal value; ratio of absorbances at 403 and 280 nm, A_{403}/ A_{280}) calculated from the

spectrum was used to estimate the purity of CMP. Depending on the protein, a R_z value of 2.5 - 4.2 is consider to indicate a high purity degree for heme proteins [22].

Results and Discussion

CMP Extraction

Extractions at pH from 3 to 9 were performed, but no differences of peroxidase activity were observed between the resulting extracts. All subsequent extractions were performed at pH 7.

Incubation times up to 24 h were tested. The maximum peroxidase activity was achieved for an incubation time of 8 - 10 h. For longer times, the activity decreased, probably due to the inactivation of the enzyme by co-extracted phenolic compounds [23]. An incubation time of 8 h was used in all subsequent extractions.

Peroxidase activity increased as sodium chloride was added to the extraction medium. A maximum of activity was observed for sodium chloride close to 250 mM. This concentration of salt was incorporated into the extraction medium in all subsequent experiments.

No change in activity was observed when 0.1% Triton-XD was added to the extraction medium.

The ratio of solid ammonium sulphate in the supernatant of the first centrifugation varied from 8 to 20 w/v %, with solid polyethylene glycol (PEG) fixed at 14 w/v %. Similarly, the ratio of solid PEG varied from 10 to 25 w/v %, with solid ammonium sulphate fixed at 12 w/v %. The peroxidase activities of extracts for these experiments were compared and results indicated maximun activity, and therefore maximum removal of phenolic compounds, for contents of ammonium sulphate and PEG of 12 w/v % and 17 w/v %, respectively.

After phase separation, the upper PEG-phase contained phenolic compounds while the bottom aqueous phase contained the extracted peroxidase. The volume of each phase was approximately 50 % of the initial homogenate.

The peroxidase activity of the extracts was found to be very different depending on the origin and age of plants and collection season. Table 1 shows the calculated activity in units per gram of plant material for the samples subjected to extraction under the proposed experimental conditions (Table 2). Plants, especially young plants, collected in the area of Almendra during the flowering period (from February to May) showed the highest peroxidase

activity. All subsequent chromatographic experiments were performed with extracts of plants collected in this area.

CMP Purification

The effects of pH and ionic strength on the retention of CMP in the hydrophobic chromatography column were studied with extract samples at pH from 6 to 9. For each pH, the ionic strength of samples was increased with different amounts of solid ammonium sulphate so that the resulting conductivity ranged from 150 to 250 mS cm^{-1}. Optimal conditions for enzyme retention were pH 7 and 230 mS cm^{-1}. Values of pH below 7 resulted in poor protein retention and conductivities above 230 mS cm^{-1} caused turbidity due to protein precipitation.

The possible retention of CMP in two ion-exchange columns, HiTrap™ SP HP and TSKgel-DEAE, was investigated at pH ranging from 5 to 7. Retention was only observed in the cation-exchange column used (HiTrap™ SP HP) and was maximum at pH 5.5. At this pH, the fractions with high peroxidase activity were collected, mixed and dialyzed against 5 mM PAB buffer. As CMP was not retained therein, the anion-exchange column (TSKgel-DEAE) was used to clean the samples before entering the HiTrap™ SP HP column.

The dialyzed mixture from the HiTrap™ SP HP column was load onto the size exclusion column (Superdex-200) and eluted with 5 mM PAB buffer, pH 5.5, containing 150 mM sodium chloride. Figure 1 shows the elution profiles of absorbance and peroxidase activity under the proposed conditions. The overlapping of peaks of absorbance and activity indicate that elution of purified CMP occurs at elution volumes around 10 mL. The absorbance peaks at volumes next to 8 and 11 mL indicate the presence of other separated compounds, most likely proteins with sizes different to CMP but without peroxidase activity. Fractions with high peroxidase activity were collected, mixed, dialyzed against 5 mM PAB buffer, pH 5.5, and stored at 4˚C. Subsequent characterization processes were made from these solutions of purified CMP.

The optimal conditions for the different chromatographic stage in the purification of CMP are summarized in Table 2. Table 3 shows the values of some significant parameters after each stage. The overall CMP purification yield from the first plant extract was 38%.

CMP characterization

The separation process by SDS-PAGE showed (Figure 2) that CMP migrated as a single band corresponding to a molecular weight of 49 ± 2 kDa.

CMP showed peroxidase activity with substrates other than guaiacol, such as o-dianisidine, pyrogallol, ferulic acid, o-phenylenediamine, catechol and ABTS. The effect of pH on the activity of CMP with these substrates is shown in Table 4. The peroxidase-catalized oxidation of many of these substrates was optimum at pH between 4.5 and 6.0. ABTS and catechol were better oxidized in more acidic media, pH close to 3.5. The maximum peroxidase activity was observed in all cases at PAB buffer concentrations below 100 mM and for most substrates investigated at concentrations lower than 50 mM (Table 5). Differences in activity at different concentrations of buffer suggest that the substrates studied bind to different parts of the enzyme molecule [23]. The strongest dependence of activity on the concentration of buffer was observed for ABTS. A similar effect has been reported for this substrate with royal palm tree peroxidase [24] and sweet potato peroxidase [23].

As indicated in Tables 4 and 5, the maximum activity of CMP with guaiacol occurs at pH 6 (Figure 3A) with a buffer concentration of 40-50 mM.

The effect of guaiacol on the activity of CMP is shown in Figure 3B. The concentration of guaiacol was varied from 0 to 60 mM with hydrogen peroxide fixed at 4.9 mM. The activity increased with increasing the concentration of guaiacol up to values close to 18 mM. At higher concentrations, an apparent gradual loss of activity was observed. This loss could be due to competitive inhibition by the reductor cosubstrate but it could also be caused by a change in the optical properties of the measured product, supposedly a tetramer of oxidized guaiacol [25]. Under the reaction conditions, the solutions with high concentrations of guaiacol went spontaneously reddish and cloudy, even when CMP was not present. Such changes point at a further polymerization of guaicol to give finally an insoluble product.

The effect of hydrogen peroxide on the activity of CMP is shown in Figure 3C .The concentration of hydrogen peroxide was varied from 0 to 20 mM with guaiacol fixed at 18 mM. The activity increased with increasing the concentration of hydrogen peroxide up to values close to 5 mM. Higher concentrations led to a linear loss in activity, this being due to suicide substrate inhibition, a process widely reported for class III peroxidases [26].

The effect of ionic strength on the activity of CMP is shown in Figure 3D. The concentration of sodium chloride in the reaction medium varied from 0 to 5 M. With increasing the concentration of sodium chloride, the activity first increased slightly, it reached a maximum at concentrations close to 0.5 M and then gradually decreased to a substantially stable value of 65% for concentrations above 3 M.

The effect of temperature on the activity of CMP was studied in the range of 15 to 90 ^{o}C. As shown in Figure 3E, no change of activity was observed for temperatures from 15 to

37 ºC, approximately. At higher temperatures, the activity decreased drastically surely due to the enzyme denaturation.

Table 6 shows the comparative activities of CMP in the presence of some metal ions and organic compounds. The concentration of the potential effector was 10 mM in all cases. $Hg^{2+}$and especially Cu^{2+} have a strong inhibitory effect on the activity of CMP. Hg^{2+} is known to irreversibly inhibit the activity of many enzymes by binding to thiol groups next to the active site [27]. In the presence of Ca^{2+} and especially Fe^{3+} a strong increase in activity was observed, up to 120 % and 156 %, respectively. Ca^{2+} is known to be a cofactor that serves to maintain the structure of the active site [28]. Results with metal ions are similar to those obtained with other peroxidases such as vanilla bean peroxidase [27] or black gram peroxidase [29]. Sodium azide, thiourea and oxalic acid had a strong inhibitory effect on the activity of CMP.

Thermal stability of enzymes depends on the assay conditions, especially incubation time and pH. Peroxidases show highly variable thermal stability which is attributed to their particular enzyme structure [27]. Some peroxidases exhibit high thermal stability probably because of the presence of sugars moiety in their structure. The main process involved in the thermal denaturation of peroxidases is the modification or degradation of the prosthetic group [29]. The thermal stability of CMP was investigated at pH 6, 7 and 9 (Figure 4). The activity of aliquots of purified enzyme solution was measured at 25 ºC and pH 6 after incubation for 15 min at pH 6, 7, or 9 and temperatures of 15 - 90 ºC. At pH 6, no differences were observed in the activity of aliquots incubated between 15 and 40 ºC. Higher incubation temperatures led to an irreversible loss of activity; the higher the temperature the greater the inactivation. Aliquots incubated at temperatures above 80 ºC lost completely their activity. A similar behaviour was observed at pH 7, although at this pH the incubated enzyme underwent a strong irreversible inactivation, close to 50 % in comparison with the activity at pH 6. Incubation at pH 9 rendered the enzyme totally inactive.

The UV-Vis absorption spectrum of CMP in 5 mM PAB buffer at pH 5.5 and 25 ºC is shown in Figure 5. The Soret band, characteristic of heme proteins, can be observed at 403 nm. Bands at 507 and 648 nm are typical of plant peroxidases [29]. The calculated R_z value was 3.3, indicating high purity of the CMP obtained.

Conclusions

In the present work, a method for purifying a class III peroxidase from white Spanish broom was developed. Due to its properties, the purified peroxidase may have a potential

application in biotechnology, as a biocatalyst in development of new biotechnological processes or as a substitute for other commonly used peroxidases.

Acknowledgements

This work was partially supported by project SA052A10-2, funded by Consejería de Educación de la Junta de Castilla y León (Spain).

References

[1] Dunford, H.B. Heme peroxidases, Wiley-Vch, New York, USA 1999.

[2] Passardi, F. et al. Peroxibase: the peroxidase database. Phytochem. 2007, 68: 1605-1611.

[3] Cosio, C.; Dunand, C. Specific functions of individual class III peroxidase genes. J. Exp. Bot. 2009, 60: 391-408.

[4] Passardi, F.; Cosio, C.; Penel, C.; Dunand, C. Peroxidases have more functions than a swiss army knife. Pant Cell Repo. 2005, 24: 255-265.

[5] Sergio, L.; Pieralice, M.; Di Venere, D.; Cardinali, A. Thermostability of soluble and bound peroxidases from artichocke and a mathematical model of its inactivation kinetics. Food Technol. Biotechnol. 2007, 45: 367-373.

[6] Almagro, L.; Gómez Ros, L.V.; Belchi-Navarro, S.; Bru, R.; Ros Barceló, A.; Pedreño, M.A. Class III peroxidases in plant defence reactions. J. Exp. Bot. 2009, 60: 377-390.

[7] Banci, L. Structural properties of peroxidases. J. Biotechnol. 1997, 53: 253-263.

[8] Hamid, M.; Khalil-ur-Rehman. Potential applications of peroxidases. Food Chem. 2009, 115:1177-1186.

[9] Dordick, J.S.; Marletta, M.A.A.; Klibanov, A.M. Polymerization of phenols catalyzed by peroxidase in nonaqueous media. Biotechnol. Bioeng. 1987, 30: 31-36.

[10] Bódalo, A.; Gómez, J.L.; Gómez, E.; Bastida, J.; Máximo, M.F.. Comparison of commercial peroxidases for removing phenol from water solutions. Chemosphere. 2006, 63: 626-632.

[11] Alexander, P.W.; Maitra, C. Enzyme linked immunoassay of human immunoglobulin G with the fluoride-selective electrode. Anal. Chem. 1982, 54: 68-71.

[12] Pérez Galende, P.; Manzano Muñoz, T.; Roig, M.G.; de María, C. G. Use of crude extract of lentil plant (*Lens culinaris* Medikus) in peroxidase-based analyses: fast kinetic determination of hydrogen peroxide and sarcosine in urine. Anal Bioanal Chem. 2012, 404: 2377-2385.

[13] Hidalgo-Cuadrado, N.; Pérez Galende, P.; Manzano, T.; de María, C.G.; Shnyrov, V.L. ;Roig, M.G. Screening of postharvest agricultural wastes as alternative sources of peroxidases: characterization and kinetics of a novel peroxidase from lentil (*Lens culinaris* L.) stubble. J. Agric. Food Chem. 2012, 60: 4765-4772.

[14] Duarte-Vázquez, M.A.; García-Almendárez, B.E.; Regalado, C.; Whitaker, JR. Purification and properties of a neutral peroxidase isoenzyme from turnip (*Brassica napus* L. var. purple top white globe) roots. J. Agric. Chem. 2001, 49: 4450-4456.

[15] Mika, A.; Lüthje, S. Properties of guaiacol peroxidase activities isolated from corn root plasma membranes. Plant Physiol. 2003, 132: 1489-1498.

[16] Da Silva, M.E.; Franco, T.T. Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. J. Chromatogr. B Biomed Sci. Appl. 2000, 743: 287-294.

[17] Mader, M. Plant Peroxidases 1980–1990 in: C. Penel, T. Gaspar, H. Greppin (Eds.), Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects. University Geneva, Geneva, 1992, 37–46.

[18] Michalak, A. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress polish. J. of Environ. Stud. 2006, 15: 523-530.

[19] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72: 248-254.

[20] Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 277: 680-685.

[21] Sakharov, I.Y.; Vesga Blanco, M.K.; Sakharova, I.V. Substrate specificity of African oil palm tree peroxidase. Biochem. 2002, 67: 1043-1047.

[22] Shannon, L.M.; Kay, E.; Lew, J.Y. Peroxidase isozymes from horseradish roots, I. Isolation and physical properties. J. Biol. Chem. 1966, 241: 2166- 2172.

[23] Castillo Leon, J.; Alpeeva, I.S.; Chubar, T.A.A.; Galaev, I.Y.; Csoregi, E.; Sakharov, I.Y. Purification and substrate specificity of peroxidase from sweet potato tubers. Plant Sci. 2002, 163: 1011-1019.

[24] Sakharov, I.Y.; Vesga, M.K.; Galaev, I.Y.; Sakharova, I.V.;.Pletjushkina, O.Y Peroxidase from leaves of royal palm tree *Roystonea regia*: purification and some properties. Plant Sci. 2001, 161: 853-860.

[25] Sakharov, I. Y.; Castillo, L.J.; Areza, J.C.; Galaev, I.Y. Purification and stability of peroxidase of African oil palm *Elais guineensis*. Bioseparation. 2000, 9:125-132.

[26] Cleland, W.W. The enzymes. Academic Press, New York, 1970.

[27] Márquez, O.; Waliszewski, N.; Oliart, R.M.; Pardio, V.T. Purification and characterization of cell wall-bound peroxidase from vanilla bean. LWT- Food Sci. Technol. 2008, 41: 1372-1379.

[28] Adams, J.B.; Harvey, A.; Dempsey, C.E. Regenerated and denatured peroxidase as potential lipid oxidation catalysts. Food Chem. 1996, 57: 505-514.

[29] Ajila, C.M.; Prasada Rao, U.J.S. Purification and characterization of black gram (*Vigno mungo*) husk peroxidase. J. Mol. Catal. B: Enzym. 2009, 60: 36-44.

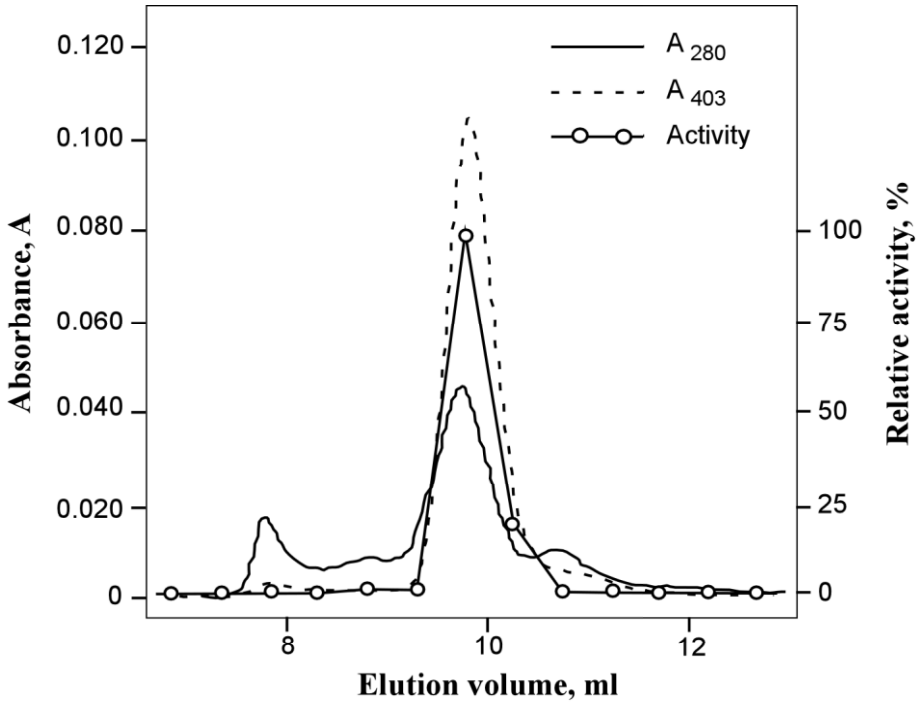

Figure 1

Absorbance signals and activity profile in the size-exclusion stage.

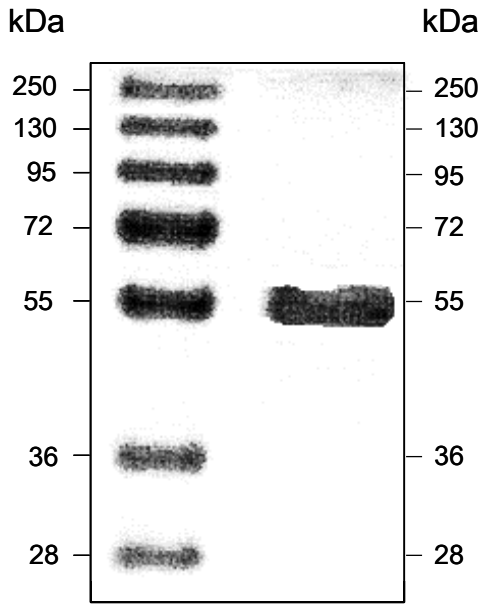

Figure 2

SDS-PAGE of CMP.

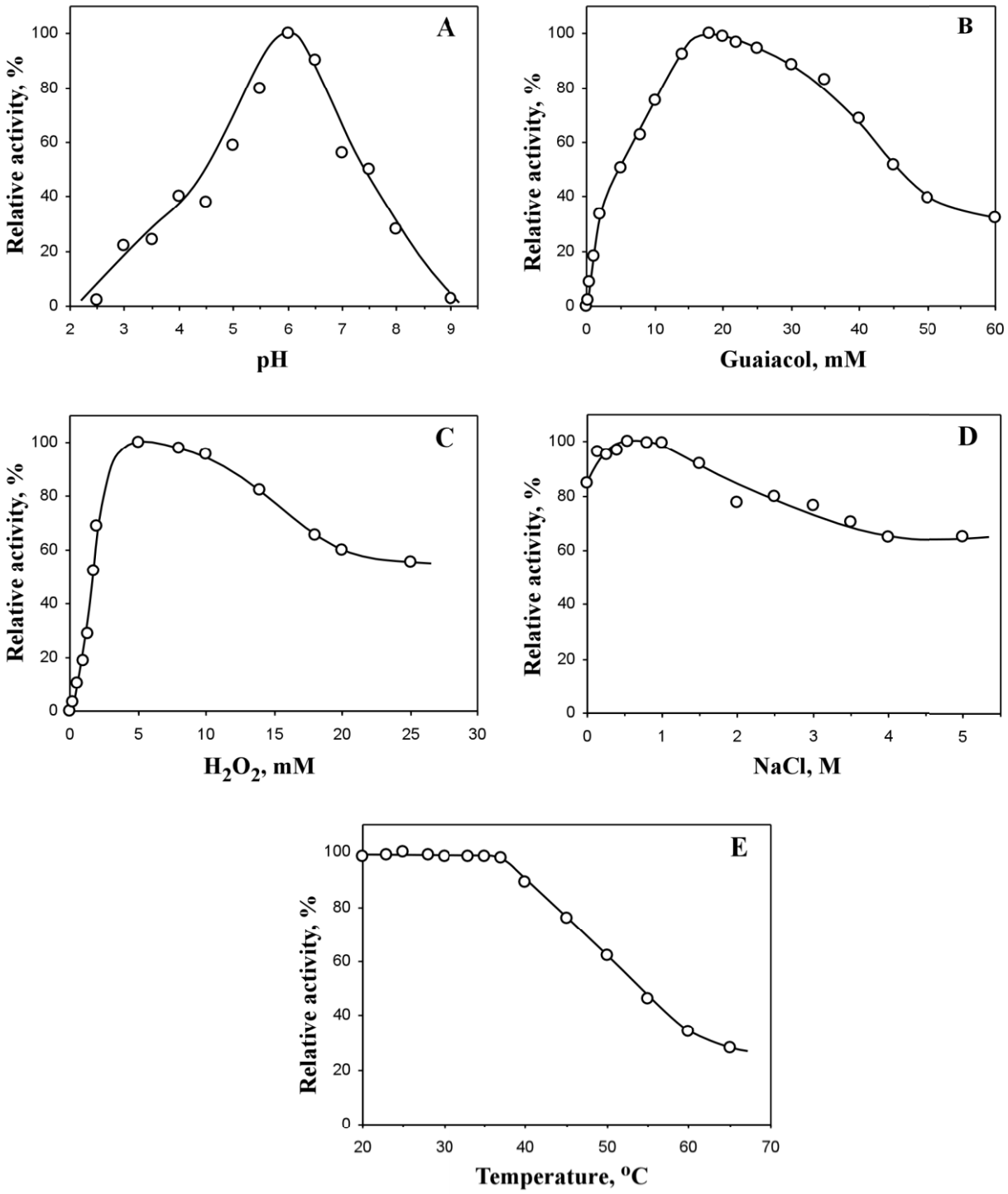

Figure 3

Effects of pH (**A**), guaiacol (**B**), hydrogen peroxide (**C**), ionic strength (**D**) and temperature (**E**) on the activity of CMP.

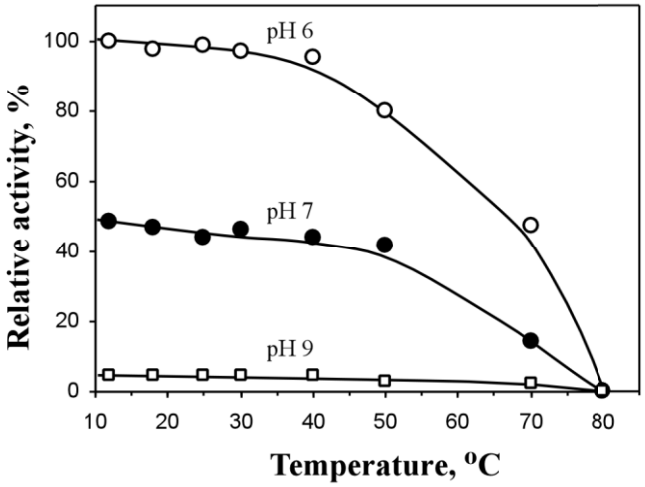

Figure 4

Thermal stability of CMP at different pH.

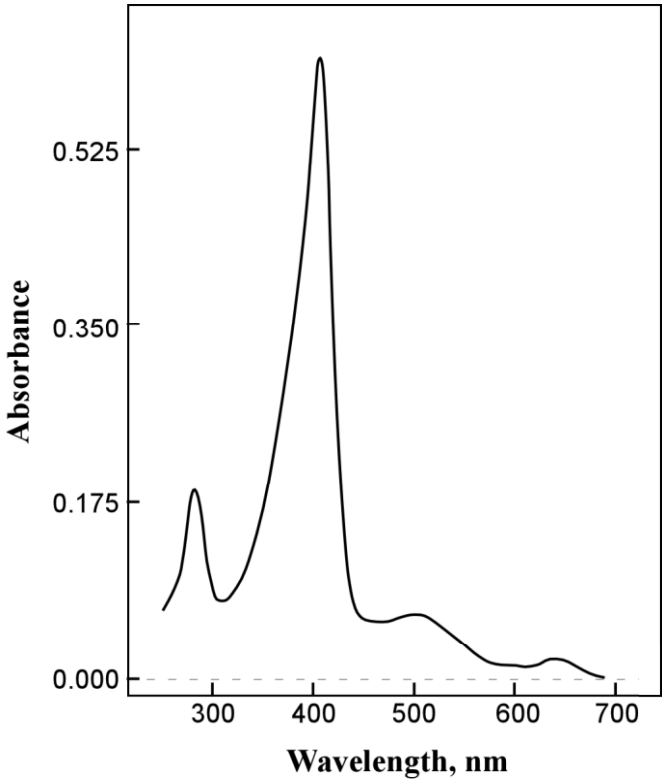

Figure 5

UV-Vis absorption spectrum of CMP.

Tables

Table 1. Peroxidase activity for plants of white Spanish broom collected in different areas and season

Sampling area Sample	**Plant description** Y: young; M: mature	**Collection date**	**Activity** U g^{-1}
Merladet[a] **(Barruecopardo)**			
M1	Y	28/05/2010	58.0
M2	Y	13/11/2010	13.5
M3	M	28/05/2010	10.5
M4	M	13/11/2010	9.5
Almendra[b]			
A1	Y	27/02/2008	570
A2	Y	13/11/2010	10.5
A3	Y	12/03/2011	665
A4	M	13/11/2010	6.0
A5	M	12/03/2011	535
A6	M	10/05/2011	400
A7	M	25/02/2012	360
A8	M	01/07/2012	21.0
Saelices[c]			
S1	Y	28/05/2010	92.0
S2	M	28/05/2010	27.0
C. Framontanos[d]			
Z1	Y	07/03/2011	209
Z2	Y	21/03/2011	224
Z3	M	03/05/2010	132
Z4	M	07/03/2011	288
Z5	M	21/03/2011	348

[a]41º04´21´´N 6º39´46´´O [b]41º16´01´´N 6º18´28´´O

[c]40º40´06´´N 6º38´03´´O [d]41º01´35´´N 6º35´25´´O

Table 2. Proposed experimental conditions for the extraction and purification of CMP

Stage/conditions
Extraction
➢ PAB buffer: 100 mM, pH 7, 250 mM NaCl
➢ Incubation time: 8 h, room temperature
Removal of phenolics
➢ Ammonium sulphate: 12 w/v %, PEG: 17 w/v %
➢ 8 h for phase separation
Hydrophobic chromatography
➢ Column: phenyl-sepharose CL-4B equilibrated with 100 mM PAB buffer, pH 7, 1.7 M $(NH_4)_2SO_4$
➢ Retention: samples in 100mM PAB buffer, pH 7, $(NH_4)_2SO_4$ (230 mS cm^{-1})
➢ Elution: 100 mM PAB buffer, pH 7, 200 mM $(NH_4)_2SO_4$
Ion-exchange chromatography
➢ Column: cation-exchange, HiTrap™ SP HP equilibrated with 5 mM PAB buffer, pH 5.5
➢ Retention: samples in 5 mM PAB buffer, pH 5.5
➢ Elution: 0-1 M NaCl linear gradient
➢ Cleaning column: anion-exchange TSKgel-DEAE equilibrated with 5 mM PAB buffer, pH 5.5
Size-exclusion chromatography
➢ Column: Superdex-200 equilibrated with 5 mM PAB buffer, pH 5.5, 150 mM NaCl
➢ Elution: 5 mM PAB buffer, pH 5.5, 150 mM NaCl, 0.5 mL min^{-1}

Table 3. Significant parameters after the stages of extraction and purification of CMP

Stage	Total protein [a] mg g^{-1}	Total activity [a] U g^{-1}	Specific activity[b] U mg^{-1}	Purification factor[c]	Yield[d] %
Extraction	34.4	530	15	1	100
Removal of phenolics	2.71	332	123	8	63
Hydrophobic chromatography	0.300	309	1029	67	58
Ion-exchange chromatography	0.054	229	4222	275	43
Size-exclusion chromatography	0.011	200	17962	1168	38

[a] Per gram of plant material

[b] Specific activity = Total activity/Total protein

[c] Purification factor= Specific activity of each stage/specific activity of the extraction stage

[d] Yield = Total activity of each stage/Total activity of the extraction stage

Table 4. Effect of pH on the activity of CMP with different substrates

pH	Relative activity*, %						
	Guaiacol	**ABTS**	**Pyrogallol**	**o-Dia**	**o-Phen**	**Ferulic acid**	**Catechol**
2.5	2	65,1	5	0	2	19	6
3.0	22	80	27	4	5	41	59
3.5	24	**100**	25	20	16	63	**100**
4.0	40	90,5	31	40	43	**100**	84
4.5	38	87,7	38	60	54	**100**	58
5.0	59	83	62	89	86	96	36
5.5	80	78,9	62	**100**	**100**	76	19
6.0	**100**	66	**100**	90	95	61	12
6.5	90	7	93	70	76	38	6
7.0	56	0	86	42	53	21	0
7.5	50	0	79	10	39	9	0
8.0	28	0	75	0	21	3	0
9.0	3	0	0	0	12	0	0

ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ; o-Dia: o-dianisidine ; o-Phen: o-phenylenediamine.

* For each independent substrate in 50 mM PAB buffer.

Table 5. Effect of the PAB buffer concentration on the activity of CMP with different substrates

mM	Relative activity*, %						
	Guaiacol	**ABTS**	**Pyrogallol**	**o-Dia**	**o-Phen**	**Ferulic acid**	**Catechol**
0	0	0	81	50	19	81	80
10	90	**100**	**100**	**100**	54	94	79
20	90	94	98	**100**	54	**100**	83
30	93	85	50	**100**	60	96	89
40	**100**	83	52	**100**	54	87	92
50	99	80	45	**100**	69	86	95
75	90	57	31	99	87	86	**100**
100	79	44	29	80	93	81	93
150	80	37	21	76	**100**	79	90
200	80	20	15	73	96	65	87
300	10	0	0	65	73	62	83
400	0	0	0	59	59	56	83
500	0	0	0	54	55	55	81

ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ; o-Dia: o-dianisidine ; o-Phen: o-phenylenediamine.

* For each independent substrate at its optimum pH.

Table 6. Effect of some metal ions and organic compounds on the activity of CMP with guaiacol

Compound 10 mM	**Activity %**	**Compound 10 mM**	**Activity %**
	100		100
$ZnCl_2$	100	$FeCl_3$	156
LiCl	100	$AlCl_3$	91
$MgCl_2$	97	KCl	87
$BaCl_2$	99	$FeCl_2$	56
$MnCl_2$	93	$HgCl_2$	49
$CuCl_2$	10	Sodium azide	5
$CdCl_2$	89	Thiourea	10
$CaCl_2$	120	Oxalic acid	22