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A haem-peroxidase from the seeds of *Araujia sericifera*: Characterization and use as bio-tool to remove phenol from aqueous solutions

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ABSTRACT

Plants seeds are an important source of enzymes with biotechnological interest. In particular, plant peroxidases, because of their enzymatic activity and stability, are used for the synthesis of phenolic resins, in the treatment of waste waters, or as labelling enzymes and in food industry. Thus, these enzymes can give a potential substitute of other polluting industrial catalysts to the conservation of the environment. In the present work, a novel plant peroxidase (named As-sP) was purified and characterised from seeds of *Araujia sericifera*. This enzyme (~40 kDa) exhibits a maximum activity at pH 5.0 and is a haem-peroxidase, basing on both the spectroscopic properties and amino acid composition. As-sP activity increased by Ca^{2+}, Cu^{2+}, Mg^{2+}; while in presence of EDTA, it is lost. On the other hand, As-sP activity is stable in a range of 40–60 °C, while decreased at higher temperature (70–80 °C). Furthermore, the activity has been tested in presence of natural (chlorogenic acid, pyrogallol and guaiacol) or synthetic (ABTS) substrates, finding that chlorogenic acid (Km 38.6 ± 2.4 μM and Vmax 92.6 ± 3.9 μM min^{-1}) was the most suitable. Finally, this enzyme was able to remove phenol from water solutions, and this capacity increases in presence of Ca^{2+} and polyethylene glycol.

This novel peroxidase, due to its enzymatic properties and heat stability, is a novel tool for bioremediation and can be used as a beneficial candidate for industrial applications, such as in decontamination of phenol-polluted waters.

1. Introduction

Peroxidases (EC number 1.11.1.x) play a central role in many biological processes, catalysing oxidation-reduction reactions (Grisham et al., 1991). Generally, these enzymes use as optimal substrate hydrogen peroxide but various are more active with organic peroxides (Wong, 1995) and contain a haem cofactor in their active sites (Vlasits et al., 2010), while in some cases flavin (Stehle et al., 1993), manganese (Janusz et al., 2013), vanadium (Butler, 1998) or specific reactive cysteinyl or selenocysteinyl residues (Toppo et al., 2009), for which these enzymes are classified into haem and non-haem peroxidase (Passardi et al., 2007). Peroxidases are found in all living organisms (bacteria, fungi, plants and animals) and are grouped into two major superfamilies commonly known as "non-animal peroxidases" retrieved in bacteria, fungi and plants and "animal peroxidases" mainly found in animals (Passardi et al., 2007). Non-animal peroxidases can be further subdivided into three classes: i) Class I, are intracellular prokaryotic and plant enzymes, localized in mitochondria and chloroplasts; ii) Class II are found in fungi and contain haem and/or manganese as cofactor; iii) Class III consisting of secreted peroxidases produced by higher plants (EC 1.11.1.7, POD), known as plant peroxidases (Hiraga et al., 2001).

Plant peroxidases, hereafter PODs, play crucial roles in plant life cycle (Cosio and Dunand, 2009) and are related to leading physiological processes such as cell wall metabolism, lignification, suberization, auxins reactive and oxygen species (ROS) metabolism, fruit growth, stress tolerance and defence against pathogens (Shigeto and Tsutsumi, 2016). PODs are single-chain proteins, often glycosylated, which exist in multiple isoforms with some differences in their function, substrate specificity or optimum pH. Moreover, these enzymes are haem-proteins, with conserved disulphide bridges and structural metal ion sites, such as for Ca^{2+} (Longu et al., 2004).

Research on PODs has long been focused not only on understanding their physiological role in plants but also on their possible

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biotechnological applications, because of their high enzymatic activity and stability (Hamid and Khalil ur, 2009). Nowadays, PODs are used for the synthesis of phenolic resins (Dordick et al., 1987), in the treatment of waste waters containing phenolic compounds or aromatic amines (Bodalo et al., 2006), as labelling enzymes in immunochemistry, in chemiluminescence or as components of kits for medical diagnosis (Perez Galende et al., 2012; Shumiantseva et al., 2010) and in food industry. Thus, these enzymes can give a potential substitute of other polluting industrial catalysts to the conservation of the environment.

In this framework, the present work has foreseen the research for a novel POD from seeds of *Araujia sericifera* Brot. (Asclepiadaceae, Magnoliophyta) an invasive vine plant of Mediterranean region (Coombs and Peter, 2010), also known as cruel vine or silk plant in Italian regions.

A. sericifera is an evergreen climbing plant from South America, introduced in southern Italy at the beginning of last century as ornamental plant. Today, this invasive plant is considered adventitious and a noxious weed. In Italy, *A. sericifera* is known for its large grey-green pear-shape fruit that contains hundreds of fluffy white seeds, which are easily dispersed by wind when the fruit splits open. Furthermore, ripe fruits have also a particular silky fuzz used in artefacts, for which it is known in Italy as “silk plant” (Di Noto and Castellano, 2010).

Considering of seeds availability in Caserta territory, our group decided to purify and characterize the POD found in *A. sericifera* seeds, named As-sP (peroxidase from seeds of *A. sericifera*). Taking into account the possible novel molecular tool for biotechnological applications, we have tested phenol polymerization capacity *in vitro* of this novel POD, since the use of enzymes in biodegradation has economic and environmental advantages.

2. Materials and methods

2.1. Plant material

A. sericifera seeds, harvested at the end of October 2017 in Marcianise (Caserta, Campania Region of Southern Italy; geographical coordinates: 40°58′N 14°15′E), were stored at −80 °C until use. *A. sericifera* seeds were manually separated from the pod and washed with distilled water.

2.2. Material and reagents

ABTS [2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)], guaiacol, chlorogenic acid, pyrogallol, phenol and Coomassie Brilliant Blue R-250 were purchased from Sigma-Aldrich S.r.l. (Milan, Italy). All chemicals were of analytical grade. Materials for chromatography were described elsewhere (Guida et al., 2011; Guida et al., 2014a) and purchased from GE Healthcare (Milan, Italy).

The following buffers in MilliQ water (Millipore Co., Milan, Italy) were used: buffer A: 5 mM Na-phosphate, pH 7.2 contanining 0.14 M NaCl; buffer B: 10 mM Na-acetate, pH 4.0; buffer C: 5 mM Na-phosphate, pH 7.2; and buffer D: 5 mM Na-phosphate pH 7.2, containing 0.30 M NaCl.

2.3. Purification of POD

Seeds (100 g) were homogenized in 1 L (w : v; 1 : 10) of buffer A in a Waring blender (Waring Products; Torrington, USA). The homogenate was stirred for 12 h at 4 °C, filtered through Miracloth paper (Inalco, Milan, Italy), and centrifuged at 15,000 *g* (Centrifuge Avanti J-25, Beckman Coulter, CA, USA) at 4 °C for 1 h. The pH of the crude extract was adjusted to pH 4.0 with glacial acetic acid, stirred at 4 °C for 1 h, and then centrifuged at 15,000 *g* (Centrifuge Avanti J-25) at 4 °C for 1 h. The supernatant, further filtered through Miracloth paper, was loaded onto a Streamline™ SP column (5 × 15 cm) equilibrated in buffer B at 2.0 mL/min flow-rate. After sample loading, the column was sequentially washed in buffer B and buffer C until the absorbance at 280 nm was below 0.01 optical density (OD). The bound basic proteins were eluted with buffer C containing 1 M NaCl, monitoring the absorbance of the eluate at 280 nm. Factions (10 mL) with POD activity were pooled and concentrated in an Amicon cell concentrator (200 mL) fitted with a PM-10 membrane (MWCO 10,000 Da). The resulting protein sample (15 mL) was loaded onto a Sephacryl S-100 (1–100 kDa separation range; 3.0 × 120 cm) column equilibrated with buffer D. Proteins were eluted with the same buffer at a flow-rate of 22 mL/h. Fractions (5 mL) with elution time corresponding to a molecular weight of about 45 kDa (major peak) with POD activity were pooled and loaded onto SP-Sepharose (2 × 10 cm) column, equilibrated with buffer D at a flow-rate of 1 mL/min. The column was washed thrice with same buffer and the retained proteins eluted using a stepwise elution with 0.50 M NaCl in buffer C (fractions 4 mL). Proteins eluted from SP-Sepharose with POD activity were pooled, dialyzed against buffer C and purified by FPLC (AKTA Purifier System, GE Healthcare), using a Source 15S PE 4.6/100 column. Source 15S was equilibrated in buffer C at a flow-rate of 1 mL/min and eluted applying a linear gradient from 0 to 60% of buffer C containing 0.6 M NaCl over 60 min, collecting fraction of 1 mL. The peak with POD activity was pooled and dialysed against MilliQ water and stored at −20 °C until use. During purification procedure, absorbance was measured at 280 nm, monitoring it also at 409 nm for haem detection, and the protein homogeneity was verified by SDS-PAGE under reducing conditions.

Raw soluble proteins from seeds for phenol removal (see paragraph 2.11) were obtained homogenizing seeds (1g) in 10 mL (w : v; 1 : 10) of 10 mM Na-acetate, pH 5.0 by using a T 25 digital ULTRA-TURRAX (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The homogenate was stirred for 4 h at 4 °C, filtered through Miracloth paper, and centrifuged at 15,000 g at 4 °C for 1 h. Soluble proteins were estimated by using the Bradford method.

2.4. Enzyme activity assay and analytical methods

Peroxidase activity was determined spectrophotometrically according to previously reported procedures (Guida et al., 2011). In particular, the assays were performed in 10 mM Na-acetate, pH 5.0 with 1 mM ABTS and 5 mM H_2O_2, at 25 °C. Hereafter, we refer to these conditions as “standard conditions”. One enzyme unit (U) is defined as the amount of enzyme that oxidizes 1.0 μmol (μmol) of ABTS per minute. Total protein content was estimated by using the Bradford method with bovine serum albumin as standard. Homogeneity of isolated proteins was determined by SDS-PAGE with a Mini-Protean II mini-gel apparatus (Bio-Rad; Milan, Italy), using 6% (w/v) stacking polyacrylamide gel and 12% (w/v) separation gel with or without reducing conditions. The dot blot analysis for glycoprotein detection was performed as previously reported applied onto the nitrocellulose blotting membrane (pore size 5 μm; Sartorius AG, Göttingen, Germany) (Leach et al., 1980). The separation of apo-As-sP peroxidase from the haem group was carried out by reversed-phase high-performance liquid chromatography (RP-HPLC), and mass spectrometric analysis was performed on a MALDI-TOF micro MX instrument (Waters Co., Manchester, UK) as previously reported (Guida et al., 2014a; Pizzo et al., 2006).

2.5. Amino acid analysis

Amino acid analyses were performed as previously reported (Landi et al., 2017). To detect cysteines, it was subjected to oxidation with performic acid. Protein samples were essentially treated as previously reported (Aitken and Learmonth, 2002). Briefly, 100 μg of protein was hydrolysed in a glass tube and 400 μL of performic acid were added. After incubation at 0 °C for 60 min, 200 μL of cold HBr were added. Samples were taken to dryness in a desiccator, rinsed with water, and then the hydrolysed was correctly analysed. All experiments were performed in triplicate and the standard deviations were < 5%.

The amino acid composition of As-sP was used as query to screen the proteins in the Swiss-Prot and TrEMBL databases (https://web.expasy.org/aacompident/) by using AACompIdent tool, choosing Constellation 2. This constellation is for 16 amino acids (Asx, Glx, Ser, His, Gly, Thr, Ala, Pro, Tyr, Arg, Val, Met, Ile, Leu, Phe, Lys), does not consider Cys and Trp, and calculates Asn and Asp together as Asx, and Glu and Gln together as Glx.

2.6. Enzyme activity

The influence of pH on the As-sP activity with 1 mM ABTS and 5 mM H_2O_2 was determined at 25 °C by using buffer solutions with different pH values. The buffer systems (10 mM final concentration) were the following: Na-citrate (pH 3.0); Na-acetate (pH 4.0, 4.5, 5.0 and 5.5); Na-phosphate (pH 6.0 and 7.0); Tris•Cl (pH 8.0 and 9.0) and Na_2CO_3 (pH 10.0) (Di Maro et al., 2010; Guida et al., 2011). Subsequently, the activity of As-sP was determined with and without EDTA, and in presence of different metal divalent cations (Ca^{2+}, Mg^{2+} and Cu^{2+}) to verify ions dependence. For these assays, As-sP was used at a concentration of 11.3 ng/mL (282 pM), in standard conditions. All experiments were performed in triplicate.

2.7. Heat inactivation of peroxidase

The enzyme (11.3 ng/mL (282 pM) was incubated at 40–80 °C up to 60 min in standard conditions and then the residual activity was measured. The activity of POD without pre-incubation was defined as 100%.

2.8. Suitable substrate for the peroxidase

Suitable substrate was determined using different substrates, such as: ABTS (λ: 414 nm; ε, 36 $mM^{-1}cm^{-1}$), *p*-guaiacol (λ: 470 nm; ε: 26.6 $mM^{-1}cm^{-1}$), chlorogenic acid (λ: 410 nm; ε: 2.3 $mM^{-1}cm^{-1}$) and pyrogallol (λ: 430 nm; ε: 2,47 $mM^{-1}cm^{-1}$). All substrates were used with a concentration of 1 mM by following the standard conditions. All experiments were performed in triplicate.

2.9. Determination of kinetic parameters

The Michaelis-Menten constant (Km) and maximal velocity of the reaction (Vmax) were determined using ABTS (0.1–10 mM) or chlorogenic acid (2.5–20 μM) as a substrate in the presence of 11.3 ng/mL (282 pM), in standard conditions, using a Lineweaver-Burk plot (Lineweaver and Burk, 1934).

2.10. Spectroscopic measurements and circular dichroism

Resting UV–visible spectra of purified As-sP enzyme in 10 mM Na-phosphate, pH 7.2 were recorded at 25 °C using a Cary 50 UV–Vis Spectrophotometer (Agilent Technologies Italia S.P.A., Cernusco s/N, Milan, Italy). Far-UV CD spectrum was obtained at 25 °C on a Jasco J-815 dichrograph [Jasco Europe, Cremella (LC) Italy]. Far UV spectrum measures were performed with a protein concentration of 0.28 mg/mL (6.3 μM) in 10 mM Na-phosphate, pH 7.2, using a 0.1 cm path-length quartz cuvette. DichroWeb (on-line analysis for protein Circular Dichroism spectra; http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) was used to estimate the percentages of secondary structural elements (Whitmore and Wallace, 2008).

2.11. Phenol removal

Experiments were conducted in 0.5 mL total volume and all chemicals were added in Na-acetate buffer 10 mM, pH 5.0 at 25 °C. Firstly, phenol (2 mM) as well as Ca^{+2} (10 mM) and polyethylene glycol 3350 (PEG 3350; 100 mg/L) were mixed, then the enzyme solution (5 μg; 0.25 μM) and hydrogen peroxide (5 mM) were added to start the reaction (Bodalo et al., 2006). Phenol, hydrogen peroxide and As-sP were kept constant; while incubation times were increased in a range from 0 to 120 min. The reactions were stopped by adding 10 μL Tris 1 M. Finally, samples were centrifuged (14,000 rpm), filtrated and the supernatant was analysed by RP-HPLC. Phenol removal was determined in the same conditions, also with raw soluble proteins extracted from seeds.

Phenol concentration was analysed and quantified by using HPLC Hewlett Packard 1100 Series HPLC System (Agilent Technologies Italia S.P.A.) loaded onto a C-18 column (i.d. 4.6 × 150 mm, 5.0 μm; Alltech, Sedriano, Milan, Italy). The higher resolution was achieved, at constant flow rate of 1 mL/min, with an elution concentration gradient made of 1% glacial acetic acid (solvent A) and methanol (solvent B) as mobile phase, whose composition started at 25% B for 5 min, followed by a linear increase in B to 95% over 5 min. Finally, a wash step was performed at 95% B for 5 min. A return step to the initial conditions for 15 min was necessary before the next run. The detection of phenol was performed with an UV diode array detector (DAD) at λ 272 nm, corresponding to the maximum of phenol absorbance. The calibration curve of phenol was obtained by plotting peak area *vs* known inject amounts of phenol, from 10 nmol to 200 nmol (range 5.0–100 mM) with a linear regression coefficient of $r^2 = 0.99$.

3. Results

3.1. Purification of POD

The purification protocol to obtain the homogenous peroxidase from seeds of *A. sericifera* consists in: phosphate saline buffer extraction, acid precipitation, and several chromatography steps based on charge and molecular weight of proteins (i. e.: Streamline SP cationic-exchange chromatography, Sephacryl S-100 gel-filtration, S-Sepharose step-wise chromatography). In particular, from the last purification step on cation exchange chromatography on S-Source, one protein peak was obtained (Fig. 1), and all peak fractions showed POD activity. Each fraction of the active peak was analysed by SDS-PAGE (*data not shown*) and the homogeneous samples were pooled for further experiments. Details about peroxidase activity yield as well as fold enrichment during the purification steps are reported in Table 1.

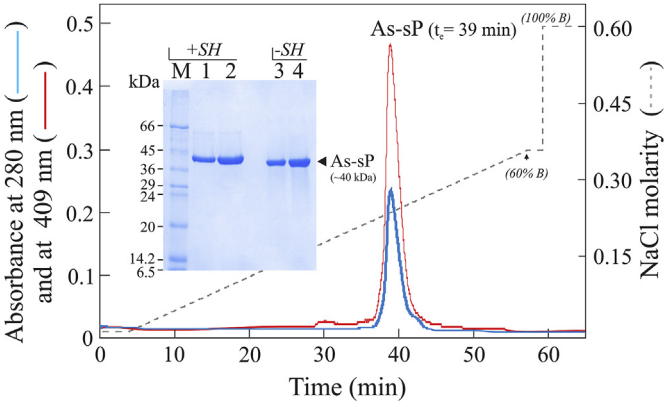

Fig. 1. FPLC elution profile of As-sP on an AKTA purifier system from cation exchange chromatography using a Source 15S PE 4.6/100 column. The end of linear gradient applied is indicated by a black arrow. In the inset is reported the SDS-PAGE analysis of the purified As-sP with or without β-Mercaptoethanol (lanes 1–2 and 3–4; 1.5 and 3 μg, respectively; M, protein markers). SDS-PAGE was carried out in 12% polyacrylamide separating gel.

Table 1
Purification of peroxidases from *A. sericifera* seeds (calculated per 100 g of seeds).

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Activity yield (%)
Crude extract	2965	129	0.04	1	100
Acid precipitation	1051	88	0.08	2	68
StreamLine SP	364	116	0.32	7	90
Sephacryl S-100	41	24	0.58	13	18
SP-Sepharose	6.0	24	4.19	96	19
S-Source	1.0	34	33.34	163	26

Values are means calculated from purifications performed in triplicate.

3.2. Molecular weight, spectroscopic properties and amino acid composition of As-sP

As-sP was analysed by SDS-PAGE with and without reducing agent, which revealed the presence of a highly homogeneous protein band with a molecular mass of ~40 kDa (see inset of Fig. 1). Moreover, the experimental relative molecular weight (M*r*) of As-sP peroxidase after RP-HPLC determined by MALDI-TOF mass spectrometry is 40,689.

Subsequently, spectrophotometric parameters of As-sP were determined. The UV/visible spectrum (Fig. 2A) of As-sP contains the Soret band with a maximum at 402 nm and two lower bands at 499 and 634 mm (see inset of Fig. 2A).

The amino acid analysis of As-sP peroxidase was carried out to determine its amino acid composition (Table 2) after acid hydrolysis.

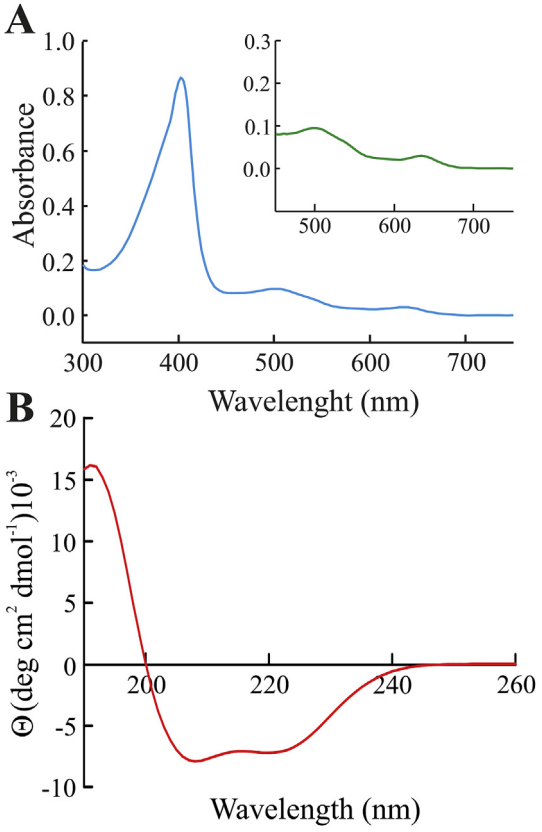

Fig. 2. (**A**) Resting state UV/Visible absorption spectrum for purified As-sP. The spectrum was recorded in 10 mM Na-phosphate, pH 7.2 at 25 °C. The Soret maximum band is at 402 nm, while the inset is the magnified region of 450–750 nm showing the charge transfer bands at 634 and 499 nm. (**B**) Far-UV CD spectrum of purified As-sP obtained as described in Materials and methods. In the inset is reported the near-UV CD.

Table 2
Amino acid composition of peroxidase from seeds of *A. sericifera* (As-sP) obtained after acid hydrolysis. Amino acid composition of both horseradish (*A. rusticana*, AC: P59121) and peroxidase 67 (*A. thaliana*; AC: Q9LVL2) peroxidases is reported as reference. Residues are expressed as number of residues per mole of protein.

Amino acid	As-sP	*A. rusticana* P59121	*A. thaliana* (PER67)
Asx[a]	43	43	30
Thr[#]	31	22	15
Ser[§]	33	23	30
Glx[b]	4	15	14
Gly	33	19	23
Ala	30	25	24
Val	22	19	20
Cys	7	8	8
Met	7	4	9
Ile	12	14	17
Leu	36	33	32
Tyr[#]	11	5	5
Trp	nd	1	1
Phe	25	21	17
Lys	21	7	17
His	6	3	4
Arg	29	26	25
Pro	17	18	14

nd, not determined. For [§], [#], values were increased of 5%; and 10%, respectively, due to their decomposition during acid hydrolysis. [a] and [b], indicate aspartate plus asparagine and glutamate plus glutamine, respectively, due to deamination during acid hydrolysis.

Asx (aspartic acid + asparagine; 43 residues per mole of As-sP) was the most abundant among As-sP hydrolysate, followed by leucine (36 residues), serine (33 residues) and threonine (31 residues), which represent about 39% of the As-sP amino acid content, not considering the tryptophanyl residues, lost during the acid hydrolysis. In addition, the amount of cysteinyl residues found is 7 residues per mole of As-sP (~1.9%), that is in agree with than previously reported for other plant peroxidases (generally 8 cysteinyl residues) involved in disulphide bridges necessary to stabilize these enzymes (Hiraga et al., 2001). Moreover, the amino acid composition of As-sP was used as query to the identification of similar sequences in protein databases. In particular, the analysis showed that horseradish peroxidase (AC: P59121) and peroxidase 67 (AC: Q9LVL2) sequences from *A. rusticana* and *A. thaliana*, respectively, are the most similar and their amino acid composition is reported in comparison with As-sP in Table 2.

Finally, a study on As-sP secondary structure was performed by circular dichroism (CD) analysis (Fig. 2B). The far UV circular dichroic spectrum of As-sP suggested that the periodic secondary structure of the protein is partially dominated by the α-helix with a predicted percentage more than 50% (~5% β-strand).

3.3. Effects of pH, H_2O_2 concentration on As-sP activity and possible suitable substrates

The As-sP activity was observed in a close pH range (4.5–5.5) with an optimum enzyme activity at pH 5.0 (Fig. 3A). Subsequently, the peroxidase activity was evaluated in 10 mM Na-acetate supplemented with different H_2O_2 concentrations at the optimum pH (Fig. 3B). The As-sP in presence of H_2O_2 showed an increase of activity from 0 to 5.0 mM with an optimum enzyme activity in presence of 5.0 mM H_2O_2, while the loss of activity was observed in a range from 5.0 to 100 mM H_2O_2.

3.4. Heat inactivation of peroxidase

The enzyme was thermally treated in a range of 40–80 °C to test its heat inactivation (Fig. 3C) since high temperatures and incubation

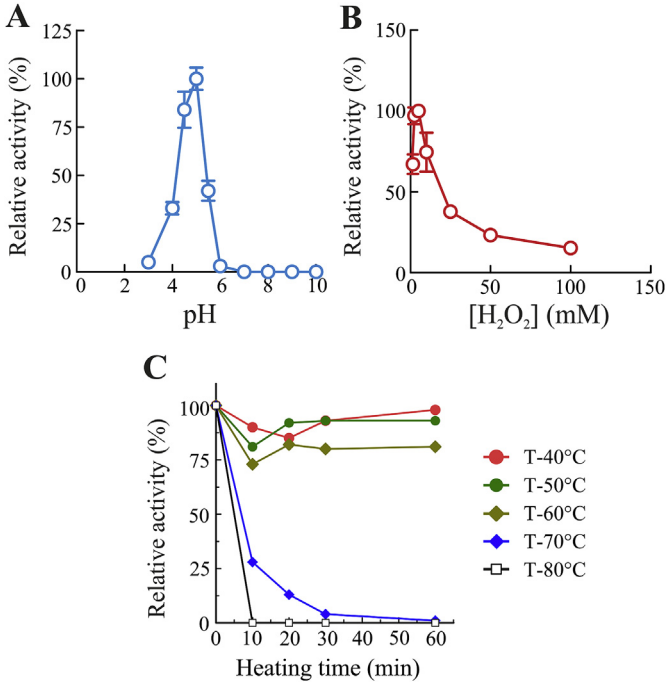

Fig. 3. Graphical representation of the effects of pH (**A**), H_2O_2 concentration (**B**) and the effect of heat treatment (**C**) on the As-sP enzymatic activity. Data represent the mean ± SD of three experiments performed in triplicate.

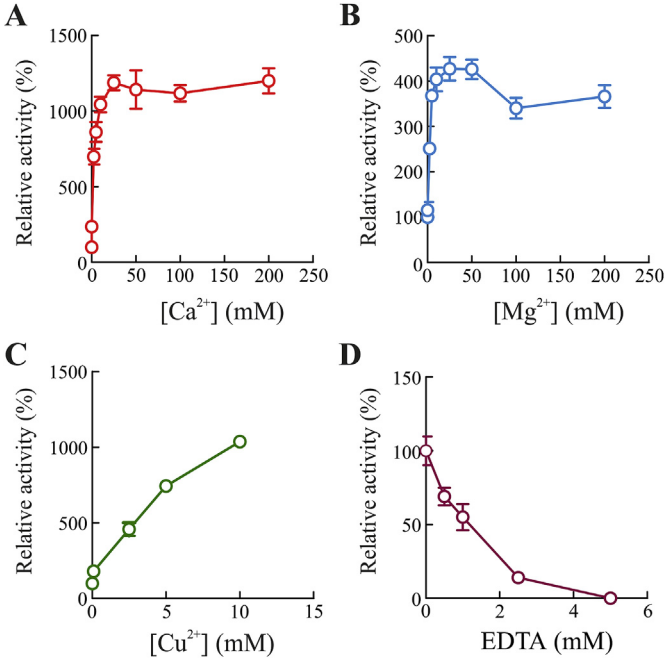

Fig. 5. Graphical representation of the effects of Ca^{2+} (**A**), Mg^{2+} (**B**) and Cu^{2+} (**C**) as well as EDTA (**D**) on the As-sP enzymatic activity at the optimum pH. The assays were performed under the standard conditions (see Materials and methods) by using ABTS as substrate. Data represent the mean ± SD of three experiments performed in triplicate.

times are commonly used in enzymes biotechnological application. As-sP was quite stable between 40 and 50 °C for 1 h retaining ~95% of its activity, while retained ~80% of its residual enzymatic activity after 1 h at 60 °C. Finally, the enzyme was quickly inactivated between 70 and 80 °C after 20 or 10 min of incubation, respectively.

3.5. Substrate specificity and kinetic parameters

In the presence of hydrogen peroxide, different compounds are reported as possible substrates for PODs enzymes, particularly for those involved in lignin biosynthesis such as chlorogenic acid (Boerjan et al., 2003). In standard conditions, As-sP displayed a moderately greater oxidizing activity with either pyrogallol or guaiacol (about 2.2-fold or 1.8-fold, respectively as compared to ABTS). A significantly greater oxidizing capacity was observed *versus* chlorogenic acid (about 9-fold with respect to ABTS), Fig. 4A. In this framework, the kinetic behaviour of the As-sP during the oxidation of chlorogenic acid or ABTS as synthetic substrate was compared (Fig. 4B and C). Km in presence of chlorogenic acid was 38.6 ± 2.4 μM (Vmax of 92.6 ± 3.9 μM min^{-1}), whereas in presence of ABTS, the Km value was 0.97 ± 0.06 mM (Vmax of 0.024 ± 0.002 mM min^{-1}). Therefore, data show that the affinity of this enzyme for chlorogenic acid is ~25-fold greater with respect to ABTS, considering Km values in standard conditions.

3.6. Peroxidase activity in presence of different bivalent cations

As-sP activity was investigated in the presence of Ca^{2+}, Mg^{2+} and Cu^{2+} ions at different concentrations. Among the tested metal ions, As-sP activity was significantly activated in the presence of Ca^{2+} followed by Cu^{2+} and Mg^{2+}, Fig. 5. In particular, results show that the addition of Ca^{2+} up to 25 mM causes a roughly 12-fold increase of the peroxidase activity, while higher Ca^{2+} concentrations, keep these activity values unaltered (Fig. 5A). A similar behaviour is observed in presence of Mg^{2+} in the range from range 0–25 mM, although lower activity values are observed compared to Ca^{2+} (roughly 4-fold increased); furthermore at concentrations above 25 mM the As-sP activity slightly decreases (Fig. 5B). Finally, low concentrations of Cu^{2+} (range 0–10 mM) increased the activity of As-sP (Fig. 5C), while higher concentrations were not explored since they interfere with the linearity of enzymatic assay. *Vice versa* EDTA decreased the enzymatic activity (Fig. 5D), completely abolished in presence of 5.0 mM EDTA,

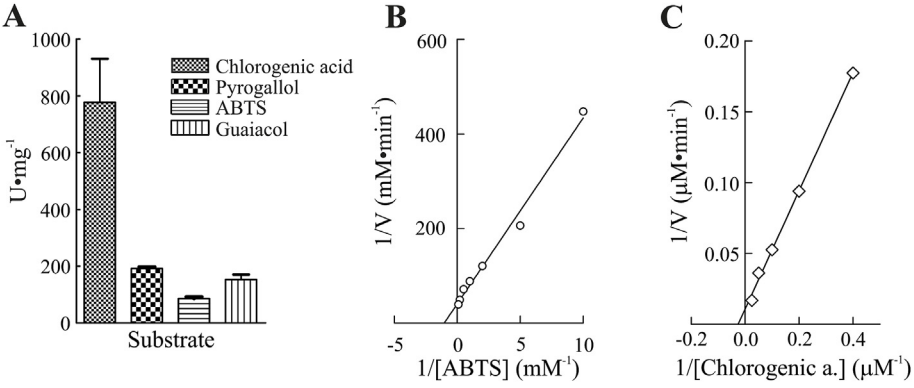

Fig. 4. (**A**) Catalytic activity of As-sP in standard conditions for different natural substrates and synthetic ABTS. (**B**) and (**C**) Use of Lineweaver-Burk plots to determine the kinetic parameters of peroxidase activity with ABTS or chlorogenic acid as substrates, respectively. Data represent the mean ± SD of three experiments performed in triplicate.

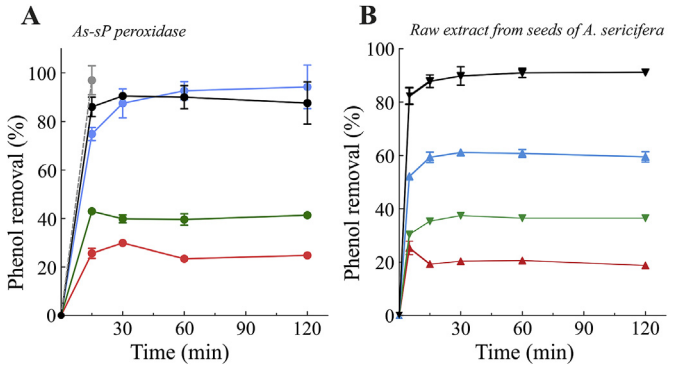

Fig. 6. (**A**) Phenol removal efficiency at different incubation times using 0.25 μM of As-sP enzyme with the exception of the curve in black (50 nM). Curves represent enzyme alone (●), enzyme with 10 mM Ca^{2+} (●), enzyme with PEG-3550,100 mg/L (●), enzyme with 10 mM Ca^{2+} and PEG-3550 100 mg/L (●). The curve in black (●) is obtained with 56 nM of enzyme with 10 mM Ca^{2+} and PEG-3550 100 mg/L. (**B**) Phenol removal efficiency at different incubation times using 20 μg (▲), 40 μg (▼), 80 μg (▲) and 160 μg (▼) amounts of raw soluble proteins from seeds of *A. sericifera* with 10 mM Ca^{2+} and PEG-3550 100 mg/L. Data represent the mean ± SD of three experiments performed in triplicate.

confirming that As-sP requires bivalent metal ions as cofactors, as largely described for other peroxidases (Plieth and Vollbehr, 2012).

3.7. Phenol removal

Peroxidases are well known for their capability to catalyse the oxidation of many phenol compounds (Hamid and Khalil ur, 2009; Guida et al., 2014b; Bodalo et al., 2006). The time course of As-sP activity was determined *in vitro* by using 2 mM phenol and standard conditions (Fig. 6A, red line) measuring the residual phenol after different incubation times at 15, 30, 60 and 120 min. The enzyme alone promotes phenol removal up to 25% after 30 min, but higher incubation times, does not influence the percentage of residual phenol. On the other hand, in presence of 10 mM Ca^{2+}, the degree of phenol oxidation is increased up to 40% given the enzyme stabilization (Fig. 6A, green line) following the same trend of the enzyme alone. Moreover, it has previously been reported that PEG (average molecular weight of 3350 g/mol), has stimulatory effects in phenol removal (Bodalo et al., 2006). Therefore, we have added 100 mg/L of PEG-3350 in the reaction mixture (Kurnik et al., 2015). When As-sP was used in the removal process, we observed a very high efficiency of phenol removal (Fig. 6A, blue line). Indeed, after 30 incubation minutes, the percentage of phenol removal is about 87%. This value is 2.9-fold higher with respect to the enzyme alone. Finally, when As-sP was incubated in standard conditions with 10 mM Ca^{2+} and 100 mg/L PEG-3550, the complete phenol removal from the solution was observed at 15 min in presence of 5 μg of enzyme (0.25 μM in assay) (Fig. 6A, dotted grey line). The same trend was also observed with 1 μg enzyme (50 nM in assay) after 30 incubation minutes (Fig. 6A, black line).

However, considering the difficulties of obtaining large quantities of purified As-sP, and *vice versa,* a great availability of *A. sericifera* seeds we have tested the removal of phenol also by using the raw soluble proteins (see paragraph 2.3, Materials and methods) in presence of 10 mM of Ca^{2+} and 100 mg/L of PEG-3550 (Fig. 6B). Data display a decrease of phenol concentration dose dependent and the almost complete removal was observed with 160 μg of raw extract after 15 incubation minutes (Fig. 6B, black line).

4. Discussion

Plants and their seeds are important research subjects, being a rich source of proteins and polypeptides with interesting biological activities. In addition, non-cultivated invasive plants could represent a source of possible enzymes with biotechnological interest. In this framework, the purification and characterization of a novel peroxidase, As-sP, from *A. sericifera* seeds, the first enzyme isolated from this plant, was carried out.

The purification protocol from seeds of *A. sericifera* based on a classical approach for protein purification allows us to obtain a pure homogenous peroxidase with a yield of 26% and a specific activity of 33.34 U/mg per 100 g of seeds. As-sP exhibited a molecular mass of ~40 kDa and its spectral characteristics are typical of a haem protein or other known haem peroxidases (Jones et al., 1998; Longu et al., 2004; Nnamchi et al., 2016) and the horseradish peroxidase from *Armoracia rusticana* (Al-Azzam et al., 2002). This data was confirmed with the amino acid composition that is similar to horseradish peroxidase (AC: P59121) and peroxidase 67 (AC: Q9LVL2) isolated from *A. rusticana* and *A. thaliana*, respectively. The content of secondary structure achieved by CD analyses (50% of α-helix and 5% of β-strand) is similar to than reported for horseradish peroxidase from *A. rusticana* (45% α-helix and 2% β-strand), the prototype of plant peroxidase family (Al-Azzam et al., 2002). The optimum pH of As-sP (pH 5.0) is in according to the values obtained for peroxidases isolated from different sources (range of 4.5–6.5). Indeed, three turnip (*Brassica napus* L.) peroxidase has an optimum pH of about 5.0 (Duarte-Vázquez et al., 2000), black gram husk (*Vigna mungo* L.) peroxidase pH 5.5 (Ajila and Prasada Rao, 2009), while the pH optimum of horseradish peroxidase is in the range of 6.0–6.5 (AA.VV, 1994). Generally, the loss of peroxidase activity is strongly dependent of pH, since the release of haem group from the enzyme active site was pH dependent and occurred most rapidly at lower and higher pH and lead to the loss in activity (Lopez and Burgos, 1995). As-sP is heat stable in a range between 40 and 50 °C for 1 h retaining ~95% of its activity, while this enzyme shows high oxidizing capacity *versus* chlorogenic acid with a Km of 38.6 ± 2.4 μM (Vmax of 92.6 ± 3.9 μM min^{-1}). Moreover, the presence of bivalent ions in the solution modulates the activity of many peroxidases, maintaining protein structure in the haem vicinity by controlling the specific structural situation of the proximal and distal histidinyl residues (Morishima et al., 1986; Plieth and Vollbehr, 2012; Nadaroglu et al., 2013). In this framework, As-sP activity increases in presence of Ca^{2+}, Cu^{2+} and Mg^{2+}, while decreases by adding EDTA. This behaviour is in agreement with than reported for other haem-peroxidases that require metal ions as cofactors (Plieth and Vollbehr, 2012; Morishima et al., 1986).

Finally, we have investigated the possible use of this novel peroxidase for phenol removal from aqueous solution since it was reported that haem peroxidases have the capability to catalyse the oxidation of many phenol compounds (Hamid and Khalil ur, 2009; Guida et al., 2014b; Bodalo et al., 2006). As-sP, shows the capacity to remove phenol from water solution; in particular, this capability increases in presence of Ca^{2+} and PEG. This last can be used for decreasing the adsorption of polymers onto the active site of the enzyme, thus reducing the precipitation of enzyme in presence of the polymeric products (Wu et al., 1997; Bodalo et al., 2006). On the other hand, considering the difficulties to obtain large quantities of As-sP purified, we have tested also phenol removal capability by using the raw soluble proteins extracted from *A. sericifera* seeds. Data display that also raw soluble proteins decrease the phenol in water solution in a dose dependent manner.

Overall, purified As-sP peroxidase or raw protein extract from seeds of *A. sericifera* represent a novel bio-tool for removed phenolic compounds from environment. Indeed, it is known that several phenolic compounds have different levels of toxicity, and are considered as carcinogenic and dangerous (Damborsky and Schultz, 1997). This finding is of interest for several industries (e. g.: coal mining, petroleum refining, pulp and paper, synthetic resins and plastics, dyes and textiles) that discharge these compounds into the environment that could use an evolving technology. This green technology is based on peroxidases (Bodalo et al., 2006; Guida et al., 2011; Kurnik et al., 2015; Hamid and Khalil ur, 2009) instead of many conventional treatment methods

including activated carbon/resin adsorption, wet air oxidation and ozonation/advanced oxidation (Villegas et al., 2016).

5. Conclusion

A novel haem peroxidase has been purified and characterised as possible bio-tool for bioremediation, such as in decontamination of phenol-polluted water. On the other hand, also raw extracted soluble proteins from the same plant show this ability and therefore data obtained for both the purified haem-enzyme and the raw extract from *A. sericifera* put in a different light this plant, not as an invasive plant but as a possible source of biocatalysts at lower costs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101215.

Conflicts of interest

The authors declare that they have no conflict of interest.

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