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Purification and enzymatic properties of a peroxidase from leaves of *Phytolacca dioica* L. (Ombú tree)

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A peroxidase (PD-cP; 0.47 mg/100 g leaves) was purified from autumn leaves of *Phytolacca dioica* L. and characterized. PD-cP was obtained by acid precipitation followed by gel-filtration and cation exchange chromatography. Amino acid composition and *N*-terminal sequence of PD-cP up to residue 15 were similar to that of *Spinacia oleracea* (*N*-terminal pairwise comparison showing four amino acid differences). PD-cP showed a molecular mass of approx. 36 kDa by SDS-PAGE, pH and temperature optima at 3.0 and 50.0°C, respectively and seasonal variation. The Michaelis-Menten constant (K_M) for H_2O_2 was 5.27 mM, and the velocity maximum (V_{max}) 1.31 nmol min^{-1}, while the enzyme turnover was 0.148 s^{-1}. Finally, the presence of Ca^{2+} and Mg^{2+} enhanced the PD-cP activity, with Mg^{2+} 1.4-fold more effective than Ca^{2+} [BMB reports 2011; 44(1): 64-69]

INTRODUCTION

Peroxidases (EC 1.11.1.7) belong to a large family of ubiquitous enzymes in fungi, plants, and vertebrates. These proteins usually contain ferriprotoporphyrin IX as a prosthetic group and oxidize several substrates in the presence of hydrogen peroxide (1, 2). According to the current classification, the plant peroxidase (hereafter POD) super-family includes prokaryotic enzymes (bacterial catalase-PODs, yeast cytochrome c POD, plant ascorbate PODs), fungal enzymes (lignin and manganese PODs and plant-type enzymes) as well as plant enzymes. Peroxidases differ significantly from each other in their primary structures, although the crystal structure motifs and catalytic centres are very similar (3-6). Isoenzymatic forms of PODs have different thermal stability, pH optimum, substrate specificity, amino acid composition and physiological roles (7). Moreover, PODs can be found in vacuoles, tonoplast, plasmalemma and inside and outside the cell wall. These enzymes are reported to be involved in plant hormone regulation (8), defence mechanisms (9), control of cell elongation, polymerization of extensin (10), cross-linkage of cell wall polysaccharides (11), lignin biosynthesis (12) and suberization processes (13). In addition, it is generally accepted that the pattern of peroxidase isoenzymes changes during plant development.

In biotechnology, PODs have a prominent position and following their activity remains the main test to evaluate the heat processing of vegetables. The characterization of these enzymes may be of interest, not only for their negative effects on colour, flavour and nutritional values, but also for their positive effects in food (14, 15). POD is used to develop reliable methods for hydrogen peroxide determination, which is of great importance in both biological and industrial fields. Furthermore, POD has an economical impact.

As it is used in diagnostic kits for enzymatic determination of glucose, uric acid, cholesterol and many other metabolites in biological fluids and it is also an important enzyme in ELISA systems. The application of plant and microorganism peroxidases to catalyze the polymerization and precipitation of aqueous phenols and decolourization of bleach plant effluent by hydrogen peroxide is potentially promising, because PODs from new sources are less expensive than horseradish peroxidase (16), which has been the focus of most wastewater research.

Because of the crucial role in biotechnological applications, we isolated POD from Ombú tree (*Phytolacca dioica* L.) leaves, normally used by our research group to isolate type 1 ribosome-inactivating proteins (17, 18). The purified peroxidase (hereafter PD-cP: *P. dioica* cationic peroxidase) was characterized by determining amino acid composition, N-terminal sequence up to residue 15, thermal stability and kinetic parameters.

RESULTS AND DISCUSSION

Purification of *P. dioica* peroxidase

During the purification of type 1 ribosome-inactivating proteins from *P. dioica* leaves (19-21), we obtained different chromatographic profiles between summer (June) and autumn

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(October) leaves. In particular, the protein pattern of the last purification step (CM-52 chromatography) of autumn leaves showed three new protein peaks eluted at high NaCl concentration (Fig. 1). Analysed by SDS-PAGE, the fractions of these peaks showed a protein band with an apparent molecular mass of 36 k. To identify the protein, we determined, by microsequencing analysis, its *N*-terminal sequence up to residue 15, blotting the band on PVDF (polyvinylidene) membrane, as described in the Materials and Methods section. The amino acid sequence $1HLASNFYSST_{10}$ XPKAL15 was used for a Blast search that showed a significant degree of identity (66.63%)/ similarity (86.6%) with the *N*-terminal sequence of *Spinacia oleracea* peroxidase [$1QLSPNFYSST_{10}$ CPNAL15 (AC: CAA 71492.1)]. *N*-terminal pairwise comparison showed that identical residues were at 10 positions 2, 5, 6, 7, 8, 9, 10, 12, 14 and 15, while differences were four (Q1H, S3A, P4N and N13K). Position 11 was not assigned. It is reported to be a cysteinyl residue in *S. oleracea* peroxidase, horseradish peroxidase and related secretory plant peroxidases (NBCI Conserved Domains, cd 00693). To confirm the comparison result, we ran peroxidase assays on the protein peaks 4-6 of Fig. 1, which confirmed that they contained the enzyme suggested by the Blast search. On the basis of its *N*-terminal sequence, the protein can be included in the secretory peroxidases belonging to class III plant heme-dependent peroxidase family (9). After identifying the nature of the protein, we assayed the crude extract of *P. dioica* leaves with the peroxidase activity assay (see Materials and Methods section), and then started a purification procedure from autumn leaves. These contained a higher PDO activity than leaves harvested during spring, summer and winter (data not shown). Total proteins were extracted from *P. dioica* leaves in phosphate-buffered saline and acid precipitated with acetic acid (pH 4.0). Soluble proteins were fractionated by: i) cation exchange chromatography on Streamline SP; ii) gel-filtration on Sephacryl S-100 HR and, iii) cation exchange chromatography on CM-52 (Fig. 1). As already shown (Fig. 1), six main protein peaks were obtained from this last purification step. Peak 6, representing about 70% of the total peroxidase activity, was subjected to gel-filtration (Fig. 2A) on Superdex^{TM} 75 10/300. A single peak was obtained with a molecular mass of approx 36 k. This was analysed by SDS- PAGE with and without 2-mercaptoethanol, and native PAGE to reassess its peroxidase activity (Fig. 2B, C). Fig. 2B showed that the protein had an high degree of purity and a molecular mass of 36 kDa coincident with the gel filtrated form, while the native PAGE showed minor bands with PDO activity. The *N*-terminal sequence was again determined on this purified peroxidase and the results were the same as reported above. The PD-cP amount of purified peroxidase was estimated to be about 0.47 mg/100 g fresh leaves. The activity yield and purification factor after the last gel-filtration step were 49.7% and 200-fold, respectively. The PD-cP amount (0.47 mg/100 g) is similar to that reported for the cationic peroxidase from artichoke leaves [0.2 mg/100 g; (22)] and for broccoli processing wastes [1.02 mg/100 g; (23)], but lower than that from horseradish (*Armoracia rusticana*) roots [8.9 mg/100 g; (24)].

Finally, the PD-cP was also purified from leaves of *P. dioica* collected in spring (May), summer (July) and winter (January)

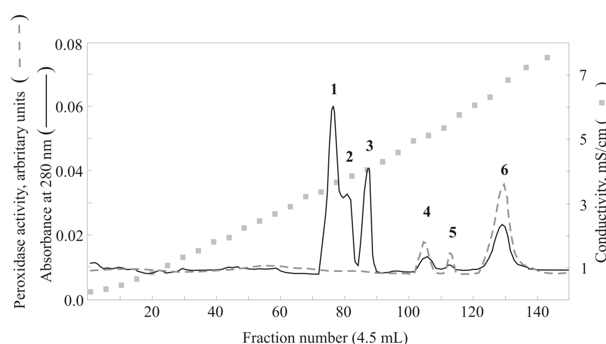

Fig. 1. Purification of peroxidases from *P. dioica* autumn leaves. The elution profile from the CM-52 chromatography showed three active peaks (4-6) eluted at higher ionic strength than type 1 ribosome inactivating proteins (peaks 1-3).

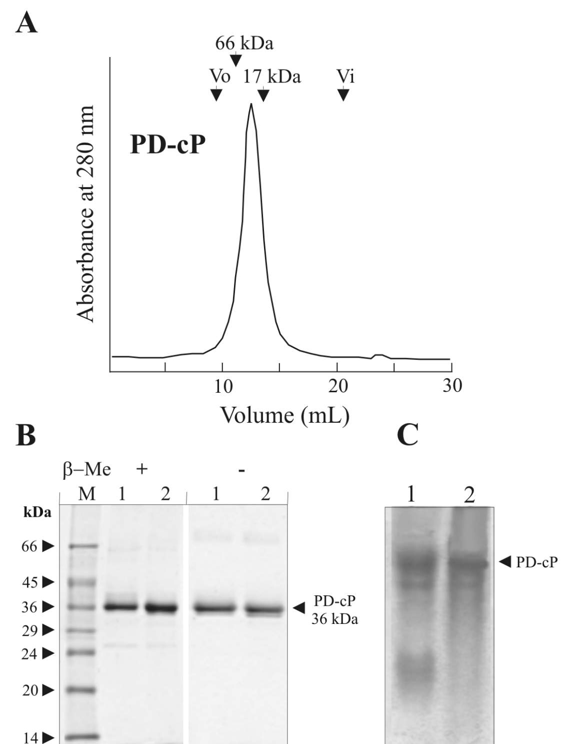

Fig. 2. (A) Chromatographic profile of PD-cP from the gel-filtration. (B) SDS-PAGE in the presence or absence of 2-mercaptoethanol. M, molecular weight markers; lines 1-2 (1.5 and 3 μg, respectively), PD-cP pool from the gel-filtration. (C) Same samples of B subjected to peroxidase assay on native polyacrylamide gel. Lane 1, horseradish-peroxidase (HPR, 2 μg); lane 2, purified PD-cP (10 μg).

using the same protocol reported in the Materials and Methods section. The POD activity was detected, but no protein peak was present in the CM-52 eluate. Seasonal variation in peroxidase levels has been reported for a number of woody species: i) in walnut peroxidase the amount was higher in the late growth to early dormant period, as reported by Nelson, who suggested that the dormant period was the time of heartwood formation for both walnut and cherry (25); ii) in crude extracts of poplar xylem tissue, Sagisaka and Asada found low peroxidase activity from June to September, which increased three-fold from October to May (26); iii) the results of Imberty et al. also suggested seasonal fluctuations in the phloem and xylem peroxidase activity (27). Moreover, in several plants variation of peroxidase levels was observed in response to infection (9).

Amino acids composition

The amino acid analysis, carried out on the PD-cP peroxidase from the last purification step, revealed an amino acid composition similar to *S. olearea* peroxidase (AC: CAA71492.1; Table 1).

Biochemical characterization of PD-cP

PD-cP was active in the pH range 2.0-5.0, with a pH optimum ≥3.0 (Fig. 3A). Peroxidases purified from various sources have their optimum pH mostly in the region of 4.5-6.5. Usually, the pH optimum of HRP is in the range of 6.0 to 6.5 (28). Only few papers report a pH optimum value lower than pH 4.0 (29). The temperature range of the PD-cP activity was 30-60°C with optimum at 50°C (Fig. 3B). Moreover, PD-cP thermostability was also evaluated by determining the residual peroxidase activity after heat treatment for 10 min at various temperatures. PD-cP exhibited low thermal stability, because 70% of the initial activity was lost after heating at 55°C for 10 min, pH 3.0 (insert Fig. 3B). The PD-cP midpoint inactivation temperature (T_m) defined as the temperature at which 50% of enzyme activity is lost, was about 50°C. After drastic heat treatment (85°C) the enzyme was inactivated (insert Fig. 3B). PD-cP is less thermostable than a heat labile HPR isoenzyme [A1; (30)] and commercial HPR (type XII), which lost 50% activity after 8 min of heating at 60°C and pH 6.0 (31).

The presence of 25 mM EDTA enhanced its activity; while 50 mM EDTA decreased it (Fig. 3C). The enzyme activity was also investigated in presence of different concentrations of Ca^{2+} and Mg^{2+}. PD-cP showed an increased of activity in presence of Ca^{2+} 0-50 mM (Fig. 3D). Instead, the presence of Mg^{2+} (0-50 mM) increased the activity of PD-cP 5-fold (Fig. 3 D). PD-cP activity increased in the presence of Mg^{2+} (1.4-fold with respect to Ca^{2+}).

The POD activity was determined using different substrates (ABTS, guaiacol, *p*-coumaric acid and indole-3-acetic acid). PD-cP was active on ABTS and guaiacol, but not on p-coumaric acid and indole-3-acetic acid. The activity values for ABTS and guaiacol were 67.5±0.004 and 4.25±0.003 μmol/min/

Table 1. Amino acid composition of PD-cP from acid hydrolyzate, compared with that of *Spinacia olearea* sequence [accession number: CAA71492.1 (see text)]

Amino acid	PD-cP[a] from *P. dioica*	Peroxidase from *S. oleracea*
Asx	40.86 (41)	36
Thr*	12.30 (12)	15
Ser*	34.98 (35)	26
Glx	27.72 (28)	19
Gly	21.94 (22)	22
Ala	26.18 (26)	25
Val	16.04 (16)	17
1/2 Cys	*n.d.*	8
Met	4.00 (4)	3
Ile	14.10 (14)	15
Leu	26.01 (26)	23
Tyr*	8.88 (9)	8
Phe	17.08 (17)	17
Lys	17.12 (17)	22
His	5.86 (6)	7
Arg	14.23 (14)	20
Pro	12.33 (12)	11
Total	299	295

[a]Residues are expressed as number of residues/mol of protein. The closest integers of the calculated number of residues are given in parentheses; n.d., not determined. *a correction factor was applied for threonine (5%), serine (10%) and tyrosine (5%) for losses during hydrolysis.

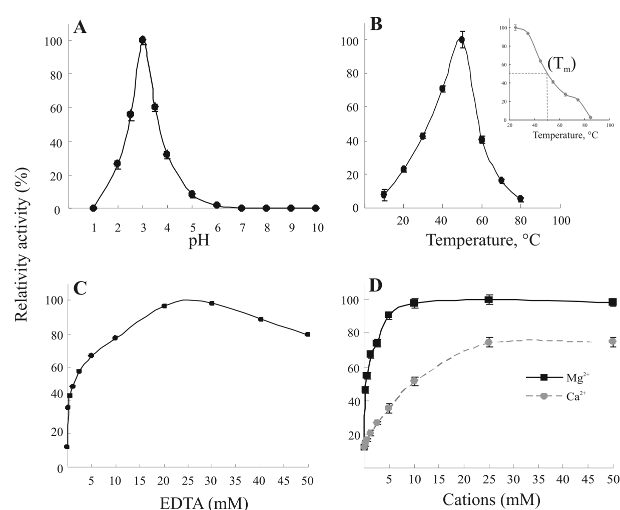

Fig. 3. Graphical representation of the effects of pH (A), and temperature (B) on the enzymatic activity on the PD-cP. In the insert B, residual peroxidase activity after heat treatment for 10 min at different temperatures. (C, D) effect of EDTA and Ca^{+2}/Mg^{+2}, respectively on the peroxidase activity. The assay were performed under the standard conditions (see Method) using ABTS as substrates.

mg, respectively.

Kinetic properties

Kinetic parameters for PD-cP were determined using H_2O_2 as substrate. The V_{max} value was found to be 1.31 nmol min^{-1}, the K_M value 5.27 mM and the enzyme turnover 0.148 s^{-1}. These values were in good agreement with those previously reported for other peroxidases (13). In particular, the K_M for H_2O_2 (5.57 mM) is similar to that reported for POD from marula fruit [1.77 mM; (32)] and turnip peroxidase isoenzyme [0.2 mM; (33)], and lower than 11.4 and 6.2 μM reported for Brussels sprouts peroxidase isoenzyme A1 and A2 (34). These studies used the chemical concentration and therefore may be compared.

MATERIALS AND METHODS

Chemicals and reagents

Fully expanded leaves (harvested in Spring, Summer, Autumn and Winter) were collected from an adult *P. dioica* plant growing in the garden of Via Vivaldi (Caserta, Italy). Materials for chromatography were described elsewhere (19). ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid], guaiacol, *p*-coumaric acid and indole-3-acetic acid were purchased from Sigma-Aldrich (Milan, Italy). Reagents for automated Edman degradation were supplied by Applied Biosystem (Monza, Italy).

The following buffers and solvents were used: buffer A: 5 mM Na-phosphate, pH 7.2, containing 0.14 M NaCl; buffer B: 10 mM Na-acetate, pH 4.5 and buffer C: 5 mM Na-phosphate pH 7.2.

Protein purification

The purification procedure has been previously reported (19, 21). Leaves (100 g) were homogenized in 500 ml of buffer A by 20 s bursts at full power using a Waring blender (Waring Products; Torrington, USA). The supernatant was adjusted to pH 4.0 with glacial acetic acid and then subjected to three sequential chromatographies: (i) a Streamline SP column (i.d. 3 × 10 cm, GE Healthcare, Milan, Italy) equilibrated in buffer B and eluted with buffer C, containing 1 M NaCl; (ii) a Sephacryl S-100 column (i.d. 2.6 × 120 cm, GE Healthcare), equilibrated and eluted with 0.35 M NaCl in buffer C and, (iii) a CM-52 column (i.d. 1.4 × 25 cm, GE Healthcare) equilibrated in buffer C and eluted with a NaCl gradient up to 0.10 M (solvent A, 500 ml; solvent B, 500 ml; total volume 1 L). Single eluted fractions were assayed for peroxidase activity using ABTS as substrate. Fractions with peroxidase activity were pooled and further purified by analytical gel-filtration on Superdex™ 75 10/300 column (GE Healthcare, separation range 70-3 kDa), equilibrated in buffer C, containing 0.3 M NaCl. The purification was monitored after each step by SDS-PAGE and native gel electrophoresis to highlight peroxidase activity.

Enzymatic activity

Peroxidase activity was determined spectrophotometrically according to previously reported procedures (23). The reaction (1.5 ml, final volume) was carried out on appropriate dilutions of PD-cP (generally 5.33 ng) in 20 mM K-phosphate buffer, pH 3.0 at 25°C, using 750 μl of 2 mM (1 mM, final concentration) ABTS as hydrogen donor, 150 μl of 50 mM (5 mM, final concentration) H_2O_2 and 600 μl milliQ water. Hereafter, we refer to these conditions as "standard conditions". The absorbance changes due to the ABTS oxidation (ε 414 = 36 mM^{-1}cm^{-1} were read at 414 nm) for 4 min using a double beam UV-Vis spectrophotometer (Shimadzu, Milan, Italy). Enzyme activity was expressed as POD activity units (UA), corresponding to the change of one absorbance unit for 1 min taking into account the dilution factors. The activity of peroxidase on various substrates was measured as increase in absorbance using the same reaction mixture and assay conditions, but with ABTS replaced by guaiacol (A_{470}; ε_{470} = 16.6 mM^{-1}cm^{-1}), p-coumaric acid (A_{310}; ε_{310} = 26.6 mM^{-1}cm^{-1}) and indole-3- acetic acid (A_{261}; ε_{261} = 3.2 mM^{-1}cm^{-1}). The activity was expressed as μmol of substrate transformed per min by 1 mg of enzyme.

SDS-PAGE and peroxidase activity on native gel electrophoresis

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 15% (w/v) separation gel (38). For the native gel electrophoresis the same conditions were used with modifications. In particular, the SDS was not used and the electrophoresis was run for three hour.

Dried samples were dissolved with 15-25 ml of sample buffer (65 mM Tris·Cl, pH 8.8, 20% glycerol, and 0.002% bromophenol blue). Lower and upper-gel were 10 and 4%, respectively. The run conditions of native gel electrophoresis were: 25 mA for 15 min, then 35 mA for 145 min, at 4°C. At the end of the electrophoresis the gel was stained with o-toluidine as reported (39).

Amino acid sequencing and amino acids analysis

PD-cP, separated by SDS-PAGE, was transferred onto PVDF membrane and directly subjected to Edman degradation as previously described (40). Amino acid analyses were performed as previously reported (41), adapting the Stein and Moore procedure (42).

Homology studies

The search for sequence similarity was carried out by using BLASTp. The program was available at the NCBI (National Center for Biotechnology Information) web site. The family data were obtained from the NBCI Conserved Domain Database (10).

Enzyme activity

The influence of pH on the PD-cP activity with ABTS was determined at 25°C in buffer solutions whose pH was adjusted to the desired value. The buffer systems (25 mM final concentration) were the following: Na-citrate (pH 3,0); Na-acetate (pH 4.0 and 5,0); Na-phosphate (pH 6,0 and 7.0); Tris·Cl (pH 8,0 and 9,0) and Na_2CO_3 (pH 10,0) (43). The peroxidase activity was also determined by varying the EDTA concentration (0-50 mM). The dependence of the peroxidase activity from divalent cations was determined in presence of Ca^{2+} and Mg^{2+} concentrations (0-50 mM). Thermophilicity was evaluated in the temperature range 10-80°C. Thermostability of PD-cD was measured for 10 min in a water bath at various temperatures (15, 25, 35, 45, 55, 65, 75 and 85°C) and immediately cooled on ice (30). Activity was assayed after 5 min using ABTS as substrate.

For these studies PD-cP was used at a concentration of 5.33 ng/ml, under standard conditions.

Enzyme kinetics for H_2O_2

Kinetic constants were calculated as described in the preceding paragraph, using 8 ng of protein in 1.5 ml reaction volume, at the optimum pH. K_M value was determined using the Lineaweaver-Burk plot for the two-substrate mechanism followed by peroxidase (23). The Michaelis-Menten constant K_M and maximum velocity V_{max} values for H_2O_2 were determined as the reciprocal absolute values of the intercepts on the x- and y-axes, respectively, of the linear regression curve.

CONCLUSION

Here we have reported the isolation for the first time of peroxidase from the Phytolaccaceae plant family. The amino acid composition, the *N*-terminal sequence and activity on guaiacol of PD-cP allowed us to include this protein in the secretory peroxidases, belonging to class III of the plant heme-dependent peroxidase family [guaiacol-type peroxidases (9)].

Seasonal variation and the higher PD-cP protein expression in autumn might suggest a role for this peroxidase in *P. dioica* leaves, but this is not supported by evidences.

The literature is replete with information regarding the role of Ca^{2+} on the peroxidase activity. It is essential for maintaining the heme structure, enzymatic activity and thermal stability (35-37). This is also true for the PD-cP activity when assayed in presence of the Ca^{2+}. Higher enzymatic activity was observed with Mg^2 than with Ca^{2+} (1.4-fold). POD activity enhancement with Mg^{2+} has been previously reported (29).

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