

Original article

Purification of two peroxidase isoenzymes of *Aloe barbadensis* which oxidize *p*-coumaric acid

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Received 8 June 2001; accepted 24 September 2001

Abstract

Using a combination of hydrophobicity and ion-exchange chromatography methods, one cationic (*pI* 9.0) and one anionic (*pI* 4.5) peroxidase (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) isoenzymes of *Aloe barbadensis* have been purified (the cationic peroxidase to homogeneity as judged by SDS-PAGE analysis and microsequencing). This allowed us to initiate the investigation of individual catalytic properties to be related to their respective functions *in vivo*. The two peroxidases have an optimal activity at pH 6.0. Apparent affinities for H₂O₂ range between 0.01 and 0.14 mM depending on the phenolic substrate and the isoenzyme. The apparent *K_m* values for the phenolics (*p*-coumaric acid and hydroquinone) are some 25-fold lower in the anionic (around 0.02 mM) than in the cationic (around 0.77 and 0.34 mM, respectively) isoenzyme. The possible functions of the activities are discussed. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: *Aloe barbadensis*; Hydroquinone; Peroxidase; *p*-coumaric acid; Purification; Reactive oxygen species

1. Introduction

Peroxidases (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) oxidize a wide range of molecules with H₂O₂ and are involved in several physiological roles in plant tissues, including lignin biosynthesis [8], wound healing [3] and defense reactions [6]. Various anionic and cationic isoforms of peroxidases with a *pI* ranging from 3.5 to 9.5 have been found in all plant species studied [1]. One main question is the specific function of each peroxidase isoenzyme. In order to advance in this field, the purification and characterization of individual peroxidase isoenzymes (together with their localization in the cell) are required. A peroxidase activity has been described in leaf aqueous parenchymatous tissue of the *Aloe* spp. [5,17,18]. Peroxi-

dase activity, which oxidizes endogenous phenolic compounds, has been found in mesophyll tissues of *Aloe* [6].

Aloe phenolics such as barbaloin have been proposed as a part of the defense mechanisms against herbivores [9,10]. Phenolics may be toxic for herbivores and contribute, after reactions mainly initiated by peroxidases and polyphenol oxidases (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1), to sealing the injured tissue.

The main phenolic compounds described in the *Aloe* species are anthrone, chromone and phenyl pyrone type [6,15]. Among them, only chromones are good substrates of the peroxidase (probably by the presence of the *p*-coumaryl group in the molecule), but not of endogenous polyphenol oxidase present in mesophyll tissues [6]. Furthermore, *p*-coumaric acid (*p*-CA) inhibits polyphenol oxidase activity [16].

The complex transformation of phenolics in healthy and injured tissues requires the characterization of potential enzymes involved in the defense processes. For this reason, *p*-CA may be a good substrate to screen peroxidases acting on endogenous phenolics of *Aloe*. In this work, we describe the purification and characterization of two peroxidase

Abbreviations: HQ, hydroquinone ; IEF, isoelectric focusing ; MN, 4-methoxy- α -naphthol ; *p*-CA, *p*-coumaric acid ; TMB, 3,3',5,5'-tetramethylbenzidine

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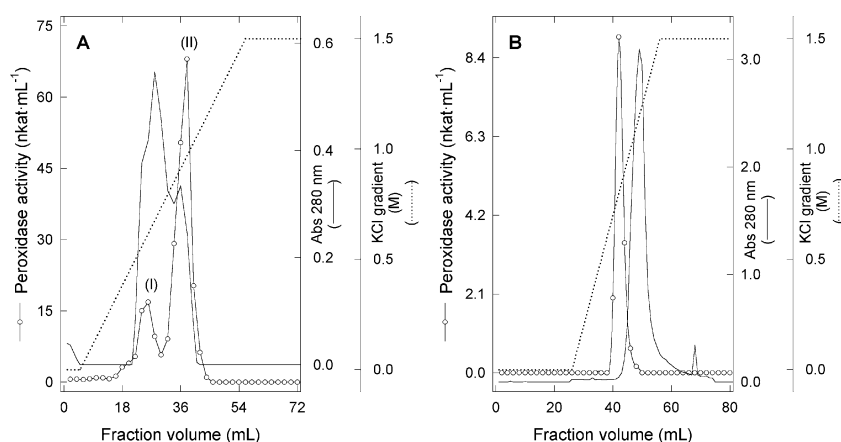


Fig. 1. Chromatography profiles of SP-Sepharose (A) and DEAE-Sephacel (B). The chromatographic conditions are described in 'Methods'. (○), peroxidase activity (with TMB as substrate); (—), protein elution profile (UV at 280 nm). More details in the text.

isoenzymes from *Aloe barbadensis* outer mesophyll leaf cells. The kinetic properties of the activities with *p*-CA and HQ are reported.

2. Results and discussion

2.1. Screening of the *Aloe peroxidase* isoenzymes oxidizing *p*-coumaric acid

The peroxidase isoenzymes oxidizing *p*-CA were revealed after native PAGE by a staining method based on the reactivity of the dye-product with 4-aminoantipyrine [19]. When extracts from the leaf mesophyll of *A. barbadensis* were separated by electrophoresis, the peroxidase isoenzyme banding pattern with *p*-CA as substrate was identical to those found with 4-methoxy- α -naphthol (data not shown), the latter being a peroxidase specific substrate, not oxidized by other heme-proteins [7]. Similar results were obtained using commercial horseradish peroxidase (type X) or when hydroquinone (HQ) is used as substrate. Hence, very probably, the spectrophotometric and zymographic determinations of peroxidase with *p*-CA or HQ as substrate described below correspond to genuine peroxidase activities and not to other heme-protein-catalyzed side reactions.

2.2. Purification of the *Aloe barbadensis* peroxidase isoenzymes

In order to avoid the difficulties associated with carbohydrate and polyphenolics present in *A. barbadensis* meso-

phyll tissues, extracts were prepared after homogenization of leaf green tissues in acetone at -20°C , in a method previously developed [6]. The buffer-resuspended acetone powder had a peroxidase specific activity of 5.1 nkat mg^{-1} . After centrifugation of the ammonium sulfate precipitate, the supernatant had a specific activity of $54.3 \text{ nkat mg}^{-1}$. The fraction eluted after the Phenyl-Sepharose batch treatment had a specific activity of $160.5 \text{ nkat mg}^{-1}$. It was concentrated, dialyzed and loaded on the anionic ion exchange column of SP-Sepharose. Firstly, the column was washed with 20 mM Bis-Tris buffer (pH 6.5) and, then, a linear gradient of KCl (0–1.5 M) was applied. The bulk of bound protein eluted between fractions 20 and 30 (at some 0.6 M KCl) (Fig. 1A) included a peak (I) of peroxidase activity. However, most of the peroxidase activity eluted with about 0.8 M KCl as a single peak (II) from which, fraction 38 with high specific activity (some $455.4 \text{ nkat mg}^{-1}$) was used for further assays. This enzyme must be positively charged at the pH 6.5 and we named it cationic peroxidase. As Table 1 shows, 3.3% of the total peroxidase activity detected in acetone powder was recovered as purified cationic peroxidase in fraction 38 (Fig. 1A). The proteins not bound to the SP-Sepharose column (eluted with washing Bis-Tris buffer) contain peroxidase activity and were loaded onto a second ion-exchange column of DEAE-Sephacel. The peroxidase activity eluted as a single peak at about 0.75 M KCl before the bulk of protein (Fig. 1B). Fractions 40–45 were pooled rendering a purified anionic peroxidase with a specific activity of $20.2 \text{ nkat} \cdot \text{mg}^{-1}$.

Isoelectric focusing (IEF) of the various fractions of the peroxidase purification (Fig. 2) showed that the fraction

Table 1

Summary of purification of cationic peroxidase from *A. barbadensis* leaf mesophyll. Indicated activity was assayed with TMB as substrate

Purification step	Volume (ml)	Total activity (nkat)	Protein (mg ml^{-1})	Specific activity (nkat mg^{-1})	Recovery (%)
Crude extract	530	2055	0.762	5.1	100.0
Supernatant SA 1.7 M	595	2531	0.0783	54.3	123.2
Phenyl-Sepharose	142	1338	0.0587	160.5	65.1
SP-Sepharose	1	68	0.1494	455.5	3.3

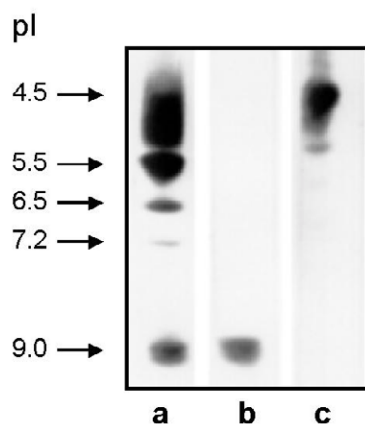


Fig. 2. IEF of *A. barbadensis* peroxidase. Lanes a, fraction obtained from Phenyl-Sepharose chromatography; b and c, peroxidase isoenzymes purified from SP-Sepharose and DEAE-Sephacel, respectively. Peroxidase bands were revealed with MN as substrate.

isolated with Phenyl-Sepharose presents a complex pattern of at least five peroxidase isoenzymes (Fig. 2, lane a). Only one band of pI 9.0 was recovered in the cationic peroxidase fraction (Fig. 2, lane b), corresponding to the peak II of SP-Sepharose chromatography (Fig. 1A). A majority peroxidase band of pI 4.5 was detected (Fig. 2, lane c) in the anionic peroxidase fraction purified by DEAE-Sephacel chromatography. The peroxidases of intermediate pIs (lane a of Fig. 2) were lost during treatments following the Phenyl-Sepharose step. According to the pIs, the purified peroxidases are different from the *Aloe* glutathione peroxidase described in the leaf aqueous parenchyma of *Aloe* [17].

Some fractions of the peroxidase purification were analyzed by SDS-PAGE followed by silver-staining and western blotting with horseradish peroxidase antibody (Fig. 3). The fractions of the cationic (Fig 3, lane b) and anionic (Fig 3, lane c) peroxidase contain essentially one polypeptide of 40 kDa similarly to horseradish peroxidases. Similarly also to horseradish peroxidase (Fig 3B), a polypeptide of 40 kDa was detected with the antibody in Phenyl-Sepharose (Fig 3, lane a), cationic (Fig 3, lane b) and anionic (Fig 3, lane c) peroxidase fractions.

The amino terminal sequencing of the 40 kDa bands of the cationic peroxidase allows us to detect only one three amino acid unambiguous sequence: Ser Gly Gly, probably because of the difficulties related to the glycoprotein nature of the peroxidases.

IEF, SDS-PAGE analysis and microsequencing (for cationic isoenzyme) strongly suggest that the two peroxidase isoenzymes have been essentially purified, which will allow us to investigate the catalytic properties of each isoenzyme. Thus, the combination of the Phenyl-Sepharose treatment with ion-exchange chromatographies seems appropriate for the purification of at least some peroxidase isoenzymes.

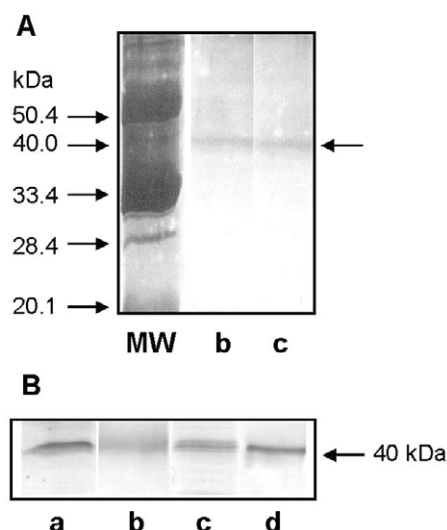


Fig. 3. SDS-PAGE and western blot of the *A. barbadensis* peroxidase isoenzymes. After SDS-PAGE, bands were silver-stained (A) or western-blotted and analyzed with antibody raised against peroxidases from horseradish (B). Lanes a, fraction obtained from Phenyl-Sepharose chromatography; b and c, cationic and anionic peroxidase isoenzymes, respectively; d, commercial horseradish peroxidase (type X). MW lane: molecular mass markers. Each lane was loaded with 50 μ g proteins.

2.3. Characterization of the oxidation of *p*-coumaric acid by *Aloe barbadensis* peroxidase isoenzymes

The oxidation of *p*-CA by peroxidase can be followed spectrophotometrically, since the end-product has a characteristic absorption band in the region 225–325 nm. Appropriate controls of chemical oxidation by H_2O_2 did not show significant changes. In addition, the peroxidase activity was inactivated by 5 min incubation in a water bath at 100 °C. The appearance of an isosbestic point at $\lambda = 250$ nm in the consecutive spectra suggested that there was a constant formation of end-product (*A* increase at 240 nm) from *p*-CA (*A* decrease at 285 nm). Similar results were obtained using commercial horseradish peroxidase (type X).

In order to characterize the peroxidative oxidation of *p*-CA by the purified *A. barbadensis* isoenzymes, the oxidation rate (estimated from the increases in *A* at 240 nm) was determined at various concentrations of H_2O_2 and *p*-CA and at various pHs. The variation in the pH of a 50 mM Tris–acetate buffer in the 3.0–7.0 range showed that both cationic and anionic peroxidases reached a maximum of activity at pH 6.0. Activity strongly decreased at lower and higher pHs (Fig 4).

Except for progressive inhibitions at high substrate concentrations, the dependence of the *p*-CA oxidation rate on a variation in the H_2O_2 and *p*-CA concentrations showed Michaelis–Menten-type kinetics for the two isoenzymes. Although the valid K_m values cannot be defined for oxidations catalyzed by peroxidases, since these reactions show no sign of reversibility of the complex enzyme–substrate formation [4], apparent K_m values were calculated from the Lineweaver–Burk (double reciprocal) plots of four indepen

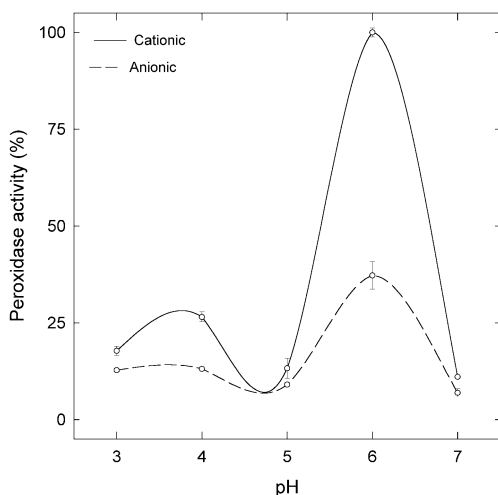


Fig. 4. The effect of pH on the cationic (—) and anionic (---) peroxidase activities from *A. barbadensis*. Results are expressed as the relative activity with respect to the maximum activity. Each point represents the mean \pm SE of the three independent experiments.

dent experiments not showing significant differences. As Table 2 shows, the anionic isoenzyme showed lower apparent K_m values for *p*-CA (0.02 mM) than the cationic isoenzyme (0.77 mM). With *p*-CA as substrate, the anionic isoenzyme showed higher apparent K_m values for H_2O_2 (0.08 mM) than cationic isoenzyme (0.02 mM).

The two isoenzymes oxidized HQ with H_2O_2 . As can be seen in Table 2, lower apparent K_m values were found in the anionic (0.02 mM for HQ and 0.01 mM for H_2O_2) than in the cationic (0.34 mM for HQ and 0.14 mM for H_2O_2) isoenzyme. At the standard assay conditions, the activities relative to the various substrates were similar for the two enzymes: 3,3',5,5'-tetramethylbenzidine, 100%; *p*-CA, 54%; HQ, 38%; and MN, 25%. The differences in the apparent affinities for phenolics and H_2O_2 could be related to different function and cell localization [13] which will be investigated in the future.

An anionic isoenzyme showing high affinity for phenolic seems suited to be involved in the defense mechanism of the plant against injuries [11]. The response to injury may consist in the production of H_2O_2 and other reactive oxygen species that are toxic. Later, the oxidation with H_2O_2 of the phenolic compounds by peroxidase (mainly certain chromones containing the *p*-coumaryl group) firstly produces more toxic species, such as radicals and quinones, and finally, polymers, tannin or melanin-type which can protect against injury by their toxic properties and by sealing the affected area [6]. In addition, the high affinity for H_2O_2

suggests that the investigated peroxidases are scavengers of H_2O_2 under oxidative stress conditions.

3. Conclusion

Two peroxidases of respective *pI* 9.0 (cationic) and 4.5 (anionic) have been purified from the *A. barbadensis* mesophyll cells by hydrophobicity and ion exchange chromatography. The two purified preparations show a single 40 kDa polypeptide in SDS-PAGE. The cationic peroxidase preparation also shows a single band of activity in IEF whereas the anionic peroxidase preparation shows, in addition to a main band, a minor band of activity in IEF. The Ser Gly Gly sequence has been identified in the purified cationic peroxidase. The two purified peroxidases show maximum activity at pH 6.0 and oxidize *p*-CA and HQ. The apparent K_m values for the phenolic are higher in the cationic (0.77 and 0.34 mM for *p*-CA and HQ, respectively) than in the anionic (0.02 mM for *p*-CA and HQ) peroxidase, which suggests that the latter may be involved in defense reaction after injuries by oxidizing the *p*-coumaryl-containing chromones of Aloe.

4. Methods

4.1. Plant material and chemicals

Aloe barbadensis Miller (1-year-old plants) was purchased from a local garden center. Plants were cultured in pots with universal substrate, regularly watered and maintained in the greenhouse (around 20–30 °C) for no more than one additional year. Extracts were prepared from the oldest basal leaves of uniform plants. Hydroquinone was obtained from BDH Chemicals; *p*-CA was purchased from Sigma-Aldrich Chemical and its purity was checked by HPLC. The chromatography products were obtained from Amersham Pharmacia Biotech and the rest of the reagents were of analytical grade.

4.2. Ion-exchange fractionation of the peroxidase isoenzymes

The peroxidase activity was extracted from the *A. barbadensis* mesophyll tissue essentially as described in Esteban-Carrasco et al. [6]. Acetone powder from around 200 g of fresh tissue (about two basal leaves) was resuspended with 500 ml of buffer 50 mM Tris-HCl (pH 7.5), 1 M KCl and 0.1 M $CaCl_2$. After centrifugation at 20 000 g for 20 min, ammonium sulfate was added to the supernatant up to a 1.7 M concentration and the new supernatant was mixed with Phenyl-Sepharose equilibrated in 50 mM Tris-HCl (pH 7.5) and 1.7 M ammonium sulfate. Proteins (including peroxidase activities) were recovered by washing the precipitate with 50 mM Tris-HCl (pH 7.5) and were

Table 2
Kinetic properties of *A. barbadensis* peroxidase isoenzymes

	K_m <i>p</i> -coumaric acid (mM)		K_m <i>p</i> -hydroquinone (mM)	
	<i>p</i> -CA	H_2O_2	<i>p</i> -CA	H_2O_2
Anionic	0.02	0.08	0.02	0.01
Cationic	0.77	0.02	0.34	0.14

dialyzed overnight against 20 mM Bis–Tris buffer (pH 6.5) (bis [2-hydroxyethyl] imino–tris [hydroxymethyl] methane) at 4 °C. The dialyzed proteins were applied to an SP-Sephacrose Fast Flow (22 × 1.5 cm) previously equilibrated with 20 mM Bis–Tris buffer (pH 6.5). After washing with two bed volumes of buffer, bound positively charged proteins were eluted with a linear gradient of 0–1.5 M KCl with a flow rate of 1 ml min⁻¹. Fractions of 1 ml were collected. The fraction showing the highest enzymatic activity was dialyzed overnight against 50 mM Tris–HCl buffer (pH 7.5) at 4 °C. Fractions not retained by the column were also pooled and directly loaded onto a DEAE-Sephacel column (15 × 1.5 cm) equilibrated with 20 mM Bis–Tris buffer (pH 6.5). After washing with two bed volumes of buffer, bound negatively charged proteins were eluted with a linear gradient of 0–1.5 M KCl with a flow rate of 1 ml min⁻¹. Fractions of 1 ml were collected. Six fractions with the highest activity were dialyzed overnight against 50 mM Tris–HCl buffer (pH 7.5) at 4 °C. The two dialyzed fractions constituted the purified enzyme preparations. Isolation was performed three times starting from different plants and rendering similar results.

4.3. Enzyme assays and protein determination

The spectrophotometric assays of the peroxidase (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) were performed at 30 °C in a final volume of 1 ml containing 50 mM Tris–acetate buffer usually at pH 6.0. Unless otherwise stated, the combinations of the phenolic and H₂O₂ concentrations were: (i) 0.1 mM *p*-CA and 0.1 mM H₂O₂; (ii) 0.5 mM TMB and 0.5 mM H₂O₂; (iii) 0.5 mM HQ and 0.1 mM H₂O₂ [19]; and (iv) 1.0 mM MN and 0.3 mM H₂O₂ [7]. After mixing, the enzymatic reaction was initiated by adding 0.5 nkat of enzyme (expressed with MN as substrate). The increase in *A* was always linear with respect to time. The oxidation rates were expressed in nkat using: $\epsilon_{240} = 17\,900\text{ M}^{-1}\text{ cm}^{-1}$ for *p*-CA, $\epsilon_{652} = 39\,000\text{ M}^{-1}\text{ cm}^{-1}$ for TMB, $\epsilon_{250} = 19\,000\text{ M}^{-1}\text{ cm}^{-1}$ for HQ and $\epsilon_{593} = 21\,000\text{ M}^{-1}\text{ cm}^{-1}$ for MN. The indicated ϵ_{240} for the product of *p*-CA oxidation was calculated from a calibration curve of *p*-CA oxidized in the presence of an excess (molar ratio 1:5) of H₅IO₆. Specific activities are referred to mg of protein. The protein concentration was measured by the method of Bradford [2] with a Protein Assay Kit (Bio-Rad) using bovine serum albumin as standard.

4.4. Zymographic assays

SDS–PAGE and native PAGE (without SDS) gels were prepared as described by Laemmli [12] with a 10% acrylamide gel. The proteins of SDS–PAGE were silver-stained as described by Oakley et al. [14]. The molecular mass of the peroxidases was estimated by SDS–PAGE using the low range molecular mass standards (Bio-Rad). The IEF of the *A. barbadensis* peroxidase isoenzymes was performed on

3.5–10.0 pH gradients as described [5]. The isoelectric point of the Aloe peroxidase bands was determined by measuring the local pH along the IEF gel. Peroxidase isoenzymes of the IEF and native PAGE were revealed with MN as substrate [7] or with *p*-CA by using an incubation medium identical to that described above for the kinetic assay. The reaction was performed at 30 °C for 30 min. After removal of the *p*-CA-containing reaction media, the staining reaction was developed by adding 50 mM of 4-aminoantipyrine in 0.1 M HCl. The red-wine colour of the product was seen 10 min after the initiation of the coupling reaction, and was stable for at least 30 min.

4.5. Immunoblot assays

After SDS–PAGE, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore). After incubation with the primary antibody peroxidase from *Armoracia rusticana* (horseradish, P-7899, Sigma), immunodetection was performed using alkaline phosphatase conjugated goat Antirabbit Ig G (Sigma). Immunoblot assays were reproducibly performed at least six times.

4.6. Peptide microsequencing

The peroxidase protein spot in one band by SDS–PAGE was cut for microsequencing in an Applied Biosystems Procise Sequencer (Spanish CSIC facilities).

Acknowledgements

This work was supported by a Grant from the Spanish DGICYT (BFI2000-0781). Matías López-Serrano holds a postdoctoral fellowship from Fundación Séneca, Centro de Coordinación de la Investigación (Murcia, Spain).

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