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Biochemical and ecological characterization of two peroxidase isoenzymes from the mangrove, *Rhizophora mangle*

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ABSTRACT

This study examines phenolic peroxidase (POX) in *Rhizophora mangle* L. leaves in order to assess its role in phenolic manipulation and H_2O_2 scavenging. Sun-exposed and understorey leaves experiencing varying degrees of nutrient stress were analysed from an oligotrophic cay off the coast of Belize. POX activity was unaffected by growth environment, but increased throughout leaf development and persisted through senescence and after abscission. Histochemical analyses indicated POX activity throughout leaf tissues, especially in the apoplast. Phenolics were similarly broadly distributed. Two isoenzymes of POX were partially characterized with pIs of 4.1 and 6.3 and masses of 65.5 and 54.3 kDa, respectively. The larger, more acidic isoenzyme showed especially high heat stability, showing no reduced activity after 24 h at 60 °C. *Rhizophora mangle* POX oxidized quercetin preferentially, and, to a lesser extent, coniferyl alcohol, caffeic acid, chlorogenic acid, and *p*-coumaric acid. It did not oxidize ascorbate, but ascorbate could act as a secondary electron donor in the presence of a phenolic substrate and H_2O_2. However, because quercetin and other aglycones were not present in *R. mangle* leaves, and because POX showed no activity with the most abundant leaf flavonoid, rutin, it was concluded that detoxification of H_2O_2 is secondary to the other roles of POX in manipulation of phenolics.

Key-words: Belize; hydrogen peroxide; photoprotection; red mangrove; Rhizophoraceae.

Abbreviations: APX, ascorbate peroxidase; Asc, ascorbate; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; POX, phenolic peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TMB, 3,3′,5,5′-tetramethylbenzidine.

INTRODUCTION

The rhizophoracean mangroves, including *Rhizophora mangle* L. in the neotropics, are characterized by their tolerance of environmental extremes, including concomitant high temperature, high irradiance (including UV), high salinity, low nutrients and prolonged periods of inundation. In previous studies, we reported that photosynthetic rates in three species in this family were light saturated at less than one quarter of full sunlight, regardless of the conditions in which the leaves developed (Cheeseman *et al*. 1991, 1997; Cheeseman & Lovelock 2004). Those studies also indicated that chloroplast electron transport rates at high light were significantly higher than combined rates of electron consumption by CO_2 fixation and photorespiration. Although such an imbalance could lead to photoinhibition and photodamage, in *Rhizophora stylosa*, it was accompanied by a high level of cyanide-sensitive superoxide dismutase (SOD; EC 1.15.1.1). This suggested that the Mehler reaction, or the Mehler–ascorbate peroxidase cycle (Asada 1999), could play a photoprotective role in this species. However, the CuZn–SOD activity also exceeded that of ascorbate peroxidase (APX; EC 1.11.1.11) by 25 times (Cheeseman *et al*. 1997). Qualitatively similar results – an eight-fold discrepancy – were obtained using greenhouse grown *R. mangle*. In both species, we hypothesized that this indicated that the problem of excess electrons was translated to one of excess H_2O_2, with the problem of photoprotection, in turn, being translated into one of H_2O_2 detoxification.

Contemporaneously, a model relating phenolic peroxidase (POX; EC 1.11.1.7) to the scavenging of H_2O_2 resulting from excess electron flow in the photosynthetic light reactions was proposed (Takahama & Oniki 1997; Yamasaki, Sakihama & Ikehara 1997). In this model, H_2O_2 detoxification involves cyclic oxidation of flavonoids, producing phenoxy radical intermediates, which are then re-reduced by ascorbate. This scheme exploits the relatively high mobility of H_2O_2, compared with less stable reactive oxygen species (ROS), enabling delocalization of its reduction to vacuoles and walls throughout a leaf. Since its formulation, this model has received support from a number of studies (e.g. Yamasaki & Grace 1998; Pérez & Maureira 2003).

The Takahama/Yamasaki model represented a departure from classical studies of phenolic peroxidases, and of H_2O_2 detoxification; previously, H_2O_2 removal had been ascribed to catalase (EC 1.11.16), which reduces H_2O_2 without a hydrogen donor (Willickens *et al*. 1995), and APX (APX,

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Asada 1999), especially in chloroplasts. Studies involving POX, on the other hand, were usually linked to the manipulation of the phenolic electron donors. POX, as an electron transfer enzyme, has been shown to be involved in the biosynthesis of lignin (Halliwell 1978; Faivre-Rampant *et al*. 1998), cell wall crosslinking (Lamport 1986; Brisson, Tenhaken & Lamb 1994), suberization (Bernards 2002), oxidative turnover of free phenolics and flavonols (Takahama, Egashirira & Wakamatsu 1989; Ros Barceló *et al*. 2003), and IAA degradation (Kawano 2003). It has also been associated with plant defence, including response to pathogens (Caruso *et al*. 2001) and insects (Bi & Felton 1995), often as part of a co-ordinated response known as the oxidative burst (Kawano 2003).

A number of factors led us to consider the role of POX in mangroves. First, each of the environmental stress characteristics of the mangrove habitat is known to induce oxidative stress in plants (e.g. Mishra, Fatma & Singhal 1995; Rao, Paliyath & Ormrod 1996; Hernández *et al*. 2000; Takemura *et al*. 2000; Larkindale & Knight 2002). Although the Mehler reaction in chloroplasts was emphasized as the source of reactive oxygen species (ROS) in development of the Takahama/Yamasaki model, leakage of electrons to molecular oxygen in mitochondria, peroxisomes, and at cellular membranes is also inevitable and enhanced by stresses (Alscher, Donahue & Cramer 1997; Bolwell 1999). Thus, high production rates of ROS in mangrove leaves, eventually translating to H_2O_2, would be expected. Finally, the most reactive, physiologically available, POX substrates have been reported to be simple phenolics such as coniferyl alcohol, caffeic acid and *p*-coumaric acid, polyphenolics such as the flavonol aglycones, quercetin and myricetin, and flavonol glycosides such as rutin (Takahama & Egashira 1991). In *R. mangle,* these account for more than 5% of leaf dry weight (Benner, Hatcher & Hedges 1990; Kandil *et al*. 2004).

Thus, the objectives of this study were to examine the relationship between growth environment and POX activity in *R. mangle* in the field, to biochemically characterize the enzyme purified from mangrove leaves, including its suitability for a hot, saline environment, and to assess its potential role in phenolic manipulation and H_2O_2 scavenging. Our results suggest that while H_2O_2 detoxification may accompany other POX activities, the major role of the enzyme is manipulation of phenolics for other defensive and structural activities.

MATERIALS AND METHODS

Field sites and procedures

All samples for this study were collected at Twin Cays, Belize, unless otherwise noted. Twin Cays is a highly oligotrophic, peat-based archipelago, located 19 km off the coast and approximately 3 km inside the barrier reef (Rützler & Feller 1996; Middleton & McKee 2001). The red mangrove, *Rhizophora mangle* L. (Rhizophoraceae), is the dominant tree throughout the islands.

For consideration of the relationship between growth environment and POX activity, sun and shade leaf samples were collected from the fringe zone (the zone of 3–5 m trees along the main channels between the east and west islands of the group), and from sun leaves in the interior dwarf zone (with 0.5–2 m trees). Because of the low leaf area indices of dwarf trees (0.7, Cheeseman & Lovelock 2004), all dwarf tree leaves were fully exposed to the sky and there were no shade leaves. Additional samples were collected at Hidden Lake (88.10132° W, 16.82733° N) from trees which were part of a multiple year fertilization study (Feller *et al*. 1999), with emphasis, here, on P-fertilized trees, and from a site (88.07667° W, 16.80167° N) along a slightly elevated ridge which had much taller trees (approximately 5 m) than the adjacent dwarf zone (approximately 1.5 m). The influence of growth environment was also considered by collecting samples at two outlying, eutrophic sites. Man-O-War Cay (88.10418° W, 16.88385° N), approximately 3.5 km north of Twin Cays, is a roosting site with a large and permanent population of magnificent frigate birds (*Fregata magnificens*) and brown-footed boobies (*Sula leucogaster*). Sittee River (88.27135° W, 16.81722° N) is a mainland estuarine site with terrigenous nutrient inputs, including input from agriculture, and lower salinity (approximately $0.25 \times$ seawater). In all cases, one or two leaves were collected from each tree, with at least three trees from any site or treatment. Leaves were harvested and placed in plastic bags in the dark for transport to the Smithsonian laboratory facilities at Carrie Bow Caye. All samples were extracted the day they were collected.

For age and senescence studies, samples were taken at a site designated as Boa Flats (88.10500° W, 16.82556° N). This is one of the more highly oligotrophic parts of the archipelago and is characterized by more or less permanent flooding and evaporation-induced hypersalinity. Trees at this site rarely reach 2 m, have few branches, and only three to four pairs of active leaves on any branch at a given time (Feller 1995; Feller *et al*. 1999, 2003). The large number of closely packed leaf scars and the high angles of leaves on all branches were taken as further indicators that this site was experiencing the greatest overall stress levels on the island. In these analyses, 'young' designates the most recently fully expanded leaves, with an average specific leaf mass (SLM) of 530 ± 20 g m^{-2}. 'Old' leaves were the oldest fully green leaves – usually the third or fourth pair – with an average SLM of 910 ± 20 g m^{-2}. 'Fallen' leaves had senesced naturally on the trees, and were recovered from the surface of the peat. They were in the initial stages of fungal and bacterial colonization associated with fragmentation; that is, still intact enough to be handled, rinsed and transported. To remove as much of the microbial material as possible, these leaves were rinsed in fresh water and the surfaces allowed to dry in air in a dark room prior to use. Attached, senescing leaves were separated into senescence 'classes' prior to analysis: Sn1 were green with incipient yellowing, Sn2 were yellow with approximately 10–20% remaining green, Sn3 were clearly and uniformly yellow, Sn4 were yellow with approximately 50% reddish colora-

tion, Sn5 were quintessentially red, and Sn6 were totally brown but on the tree. In the results presented here, only classes Sn3 to Sn6 are considered. POX sampling was supplemented by larger samples for quantification and characterization of total polyphenolics (see Kandil *et al*. 2004). These bulk samples were also used for purification and biochemical characterization of POX (see below).

Sample extraction and enzyme purification

Samples collected at Twin Cays were transported to the Smithsonian laboratory at Carrie Bow Caye for initial processing, where subsamples of known leaf area were weighed, finely chopped, and extracted with 80% acetone until the solid material was no longer green. The decanted acetone solvent was used for chlorophyll determination (Graan & Ort 1984). After air drying, insoluble material ('acetone powder') was stored at –20 °C until needed for assays or enzyme purification.

To purify phenolic peroxidase (POX; EC 1.11.1.7) approximately 100 g of acetone powder was extracted with 5 mM Tris/MOPS buffer (pH 7.0) at a ratio of 8 mL buffer per gram. The suspension was filtered through three layers of Miracloth (Calbiochem, San Diego, CA, USA) and rinsed twice with 200 mL of the buffer. Sorbitol (100 mM) was added to the combined filtrates and the solution was clarified by centrifugation ($6700 \times g$, 60 min). The supernatant was decanted and lead acetate was added to approximately 200 mM in order to precipitate flavologlycans (Neilson, Painter & Richards 1986). Excess lead was then removed by addition of 250 mM K_2HPO_4 until no further precipitation occurred, followed by centrifugation ($7000 \times g$, 60 min). It was determined separately that this treatment had no effect on POX activity. The supernatant was then extracted in a two-phase polymer system (Saitoh *et al*. 1995) with 30% (w/v) ammonium sulphate (AS) and 14% polyethylene glycol (PEG; MW 10 000), followed by centrifugation. The POX activity was in the lower, AS layer; the PEG layer, containing polyphenolics, pigments and other impurities, was discarded. The AS solution was then passed through a phenyl-sepharose CL-4B column (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with 30% AS. The proteins were eluted with 5 mM ammonium acetate (pH 5.2) with AS decreasing stepwise from 2 M to 0 M. The fractions at 0.5–0.25 M AS contained essentially all the POX activity; these were combined, and desalted using Sephadex G-25 equilibrated with 25 mM Tris/HCl (pH 9.0). Active fractions were concentrated to a final volume of 850 μL using a 30-kDa MWCO Vivaspin 20 filter (VivaScience, Hannover, Germany). Using quercetin as the standard (see below), the final POX activity was 105 U μL^{-1}. The purified sample was stored at –20 °C.

Protein quantification, electrophoresis and isoelectric focusing

The protein concentration in the purified enzyme samples was determined using the Non-Interfering Protein Assay (Geno Tech, St. Louis, MO, USA) with bovine serum albumin as the standard. Sodium dodecyl sulphate (SDS)-polyacrylamide gell electrphoresis (PAGE) electrophoresis was conducted using basic, 8–16% Tris-Glycine GeneMate Express gels (ISC BioExpress, Kaysville, UT, USA) and pH 8.3 SDS running buffer. Gels were stained with GelCode Blue stain reagent, based on Coomassie G-250 (Pierce, Rockford IL, USA) and photographed under white light. For isoelectric focusing (IEF), precast pH 3–10 gels (Bio-Rad 161–1183; Bio-Rad, Hercules, CA, USA) were used. The current was maintained at 8 mA. POX activity was localized by staining with 10% w/v 3,3′,5,5′-tetramethylbenzidine (TMB) or 0.04% v/v guaiacol with 2 mM H_2O_2 in 0.1 M Na-acetate buffer (pH 5.2).

POX assays

For POX activity assays, acetone powders were ground in a mortar and pestle at a ratio of 0.1 g leaf material to 3 mL of extraction buffer (0.1 M K-phosphate, 0.5 mM Na_2EDTA and 1% PVP-40, pH 7.0), and clarified by centrifugation. Assay mixtures included 1% (v/v) tissue extract and 30 μM phenolic substrate in 0.095 M Na-acetate (pH 5.2) unless otherwise stated. The reactions were started by addition of H_2O_2 to a final concentration of 0.1 mM. H_2O_2-dependent oxidation of the phenolic substrate was measured using an Ocean Optics UV/Vis Chem 2000 spectrometer (Ocean Optics Inc, Dunedin, FL, USA). For initial characterization (Fig. 1), guaiacol was used as the substrate; the absorption increase was followed at 470 nm and rates were calculated based on an extinction coefficient, $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$ (Caruso *et al*. 2001). Activities were calculated on the basis of leaf fresh weight, leaf area and leaf chlorophyll content. For more detailed characterization of the *R. mangle* POX, quercetin was used as the standard phenolic substrate. Oxidation was monitored at 360 nm using an extinction coefficient, $\varepsilon = 19.6\ mM^{-1}\ cm^{-1}$. Operationally, we defined one unit of activity as the amount of enzyme which would oxidize quercetin at a rate of 1 nmol min^{-1} with initial quercetin and H_2O_2 concentrations of 30 and 100 μM, respectively, at pH 5.2 and 23 °C.

Because the Takahama/Yamasaki model was formed partly around the observation that ascorbate (Asc) can re-reduce flavonoid radicals following oxidation by H_2O_2/POX (Takahama & Oniki 1992; Yamasaki *et al*. 1997), the effect of added Asc (25 μM) was determined after initiation of the reaction with quercetin or other phenolic substrates. The Asc oxidation rate was calculated based on $\varepsilon = 9.8\ mM^{-1}\ cm^{-1}$ at 265 nm. As the observations were similar in form to those reported for grapevine leaves (Pérez, Villegas & Mejia 2002), the experiment will be described here without illustration. At time 0, the reaction was initiated with quercetin (or alternative substrates) and H_2O_2. Phenolic oxidation was monitored at its characteristic wavelength until sufficient data points were acquired to allow determination of the linear rate (approximately 1 min). At that point, ascorbate (25 μM) was added, and its oxidation monitored at 265 nm. During this phase, phenolic oxidation stopped.

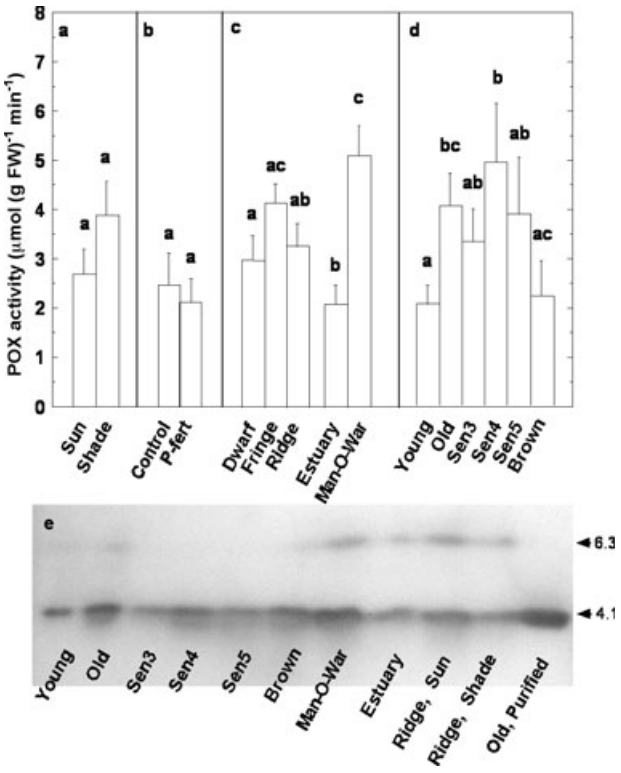

Figure 1. POX activity in crude leaf extracts from Twin Cays. Results are grouped as a function of sun/shade exposure of leaves from the mid-island ridge (a), of P-fertilization treatment for dwarf zone trees (b), of habitat from which the leaves were collected (c), and of leaf age and developmental state (d). With the exception of (a), all data are for sun leaves. Activity was measured using guaiacol as a substrate. Different letters within a panel indicate significant differences ($P < 0.05$). Error bars are means ± SEM. (e) Isoelectic focusing gel stained for peroxidase activity with guaiacol/H_2O_2. For this analysis, acetone powders were re-extracted in buffer, concentrated and desalted using 10000 MWCO Vivaspin filters, and applied to the gels. Activities are shown for six leaf developmental stages (dwarf-vertical leaves), two outlying habitats, sun and shade leaves from the mid-island ridge, and purified enzyme from dwarf-vertical 'old' leaves. pI values for the individual bands are shown on the right. Approximately 0.6 units of enzyme were loaded in each lane. With the exception of the last lane, all samples were re-constituted from crude-extract acetone powders. The last lane is the purified dwarf zone old leaf enzyme (see Fig. 2).

Thus, even though quercetin (for example) absorbed at the same wavelength, it did not interfere with calculation of the Asc oxidation rate. Once all the Asc was consumed, phenolic oxidation resumed. Asc was not oxidized by POX without both a phenolic electron donor and H_2O_2, and phenolic substrates were not oxidized in the absence of H_2O_2. The addition of KCN (5 mM) as a POX inhibitor immediately stopped the oxidation of both phenolics and Asc (data not shown). With the exception of guaiacol, whose oxidation product bleached completely in a few seconds after addition of either Asc or KCN, phenolic substrates, once oxidized, were not re-reduced following the addition of Asc.

pH optima and heat stability determinations

Both crude and purified enzyme preparations were assayed for POX activity using quercetin as a phenolic substrate across a pH range of 2.3–9.0 in 0.2 M citrate/phosphate buffer. In all cases, the pH optimum was 5.2 (data not shown) and that value was used in all other assays.

Enzyme heat stability was determined based on activity of preparations heated in a 60 °C water bath for periods from 5 min to 24 h. The ability of mangrove POX to alter the heat stability of horseradish peroxidase (HRP; EC 1.11.1.7 – Type IV-A; Sigma, St. Louis MO, USA) was determined by mixing the enzymes prior to heating. Activity was determined using quercetin as the substrate, after cooling samples to room temperature. Note that 1.0 unit of Sigma-specified HRP activity based on the 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay corresponded to 17.6 units of activity using quercetin as the substrate (defined above); for these experiments, 1.2 Sigma-specified units (20.8 quercetin units) were mixed with 6.2 quercetin units of mangrove POX. In this way, the majority of the activity, and thus also the change due to HRP inactivation, was from HRP.

Substrate specificity determinations

The characteristic absorption peaks and extinction coefficients in the assay buffer were determined by spectrum analysis using the Ocean Optics UV/Vis Chem 2000 spectrometer, and rate calculations were based on these parameters.

Histochemistry

Leaf sections for microscopy were prepared from both fixed and fresh leaf material. For studies using fresh tissue, leaves from greenhouse-grown trees were hand-sectioned with a razor blade and viewed under a light microscope. For thin-sectioning, mangrove leaves were collected in the greenhouse or in Belize, cut into approximately 5 mm × 15 mm pieces, and infiltrated under partial vacuum with 2.5% gluteraldahyde fixative solution in 0.1 M cacodylate buffer. Upon return to the home laboratory, samples were dehydrated stepwise, embedded in Epon 812/Spurr VCD low-viscosity resin, and sectioned to a thickness of 1–3 μm on an ultramicrotome for light microscopy. All microscopy supplies were obtained from Electron Microscopy Sciences (Hatfield, PA, USA).

For general tissue structure (Fig. 4a), sections were stained with hematoxylin and eosin. Phenolics were localized using equal proportions of 50 mM ferric chloride in 0.1 M HCl and 8 mM $K_3Fe(CN)_6$. Phenolics appeared blue after a 15-min incubation at ambient temperature (Lavid *et al.* 2001). Fresh sections were stained for POX activity by a modification of the method used by Lavid *et al.* (2001) using guaiacol in 0.1 M acetate buffer (pH 5.2). H_2O_2 (10 mM) was added to the buffer to initiate the reaction. For lignin localization, fresh sections were stained with

10% phloroglucinol followed by reaction with concentrated HCl (Gahan 1984).

Statistics

All statistical analyses were performed using StatView 5.0.1 statistical software (SAS Institute, Cary, NC, USA).

RESULTS

Environmental and developmental effects on peroxidase activity

As a first step in examining the applicability of the Takahama/Yamasaki model for the involvement of phenolic peroxidase in photoprotection via the detoxification of H_2O_2, we examined the relationship between POX activity in leaves and the environment in which they were growing. Figure 1 compares POX activity in crude enzyme extracts based on sun/shade exposure, growth environment, and leaf development and senescence. Figure 1a compares leaves developed in sun and shade from the interior central ridge of the island. Similar results were obtained when sun and shade leaves from the fringe zone were compared, but the non-significant 'trend' was reversed. Figure 1b compares leaves of dwarf, unfertilized trees with those which had been fertilized with superphosphate. This treatment dramatically stimulated height growth, branching and leaf production (Feller *et al*. 1999; Cheeseman & Lovelock 2004), but the POX activities were the same. The effect of growth habitat was considered further by comparing leaves from the dwarf, fringe and ridge zones, and leaves from two outlying areas: a mainland estuary and a densely populated bird rookery (Fig. 1c). Within Twin Cays, there were no differences between any of the zones. Both the outlying areas were significantly different from Twin Cays, but the differences are not completely explicable. Both sites had higher nutrients than Twin Cays (data not shown) and the estuarine site had lower salinity.

Finally, leaf age effects were considered using samples from the dwarf zone (Fig. 1d). Because of the canopy architecture of these trees, all leaves were fully exposed to the sky, precluding any shading effects. Old leaves had approximately double the POX activity of young leaves. There were no clear trends during senescence, and although activity declined after abscission, it was still equal to that in young leaves. Interestingly, POX activity remained high in the leaf litter through the initial stages of decomposition.

Although the results presented in Fig. 1 are expressed on the basis of leaf fresh weight, the patterns were similar when expressed on the basis of leaf area or chlorophyll content, with the exception of the leaf age comparison. The ratio of activities in old versus young leaves was twice as great when expressed per unit area or chlorophyll, as that shown in Fig. 1d. This difference was consistent with leaf structure and histochemistry (see below).

Characterization of *R. mangle* POX enzymes

As many plants express multiple forms of the enzyme at any one time and throughout development, and as the activity measured in fallen leaves could have come from the fungi and bacteria colonizing them, extraction and purification of the mangrove peroxidase was carried out on two samples, one from the highly stressed, Boa Flats dwarf trees ('old' leaves in Fig. 1d) and the other from the rapidly growing trees on the central ridge of the island (see Materials and methods). The results are shown in Fig. 2. SDS-PAGE (Fig. 2a) showed two dominant protein bands. A 65.5 kDa peptide was present in both samples, whereas a second, 54.3 kDa peptide was present only in ridge leaves. A second, smaller band (49.6 kDa) was also present in the 'old' leaves. Isoelectric focusing (Fig. 2b) and activity staining for POX revealed two acidic isoenzymes with pIs of 4.1 and 6.3. The pI 4.1 isoenzyme was present in both samples, whereas the pI 6.3 isoenzyme was only present in ridge leaves, suggesting that they are distinct isoenzymes, not biochemically active degradation products. Based on the IEF results, we concluded that the more acidic form in the ridge leaves was the 65.5 kDa peptide, and that the 49.6 kDa band in the old leaves was either a degradation product of the 54.3 kDa isoform, or an unrelated protein which co-purified with the POX. This was further supported by activity staining of non-denaturing gels: the ridge leaf preparation yielded two stained bands whereas the old leaf preparation yielded only one (data not shown).

The two purified isoenzymes were also those found in the samples used for Fig. 1. As shown in Fig. 1e, the larger,

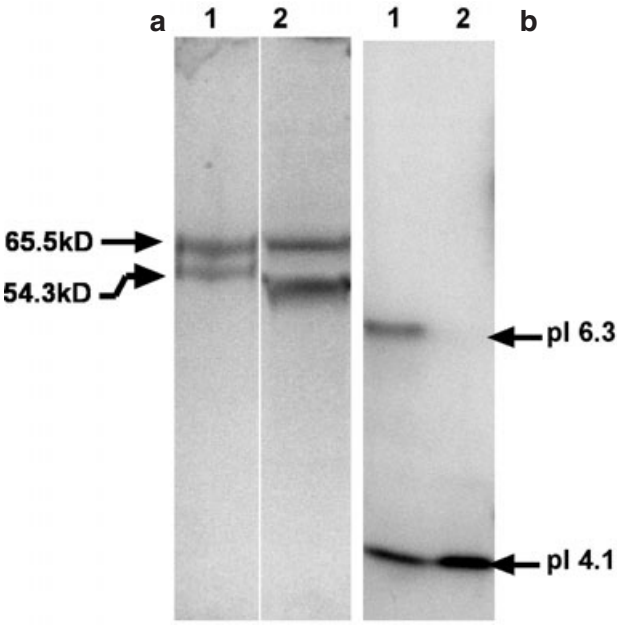

Figure 2. Electrophoretic characterization of purified POX from *R. mangle*. (a) SDS-PAGE electrophoresis stained with Coomassie G-250. POX from mid-island ridge sun leaves (1) and dwarf-vertical-old-leaf (2). (b) Isoelectric focusing stained with guaiacol/H_2O_2.

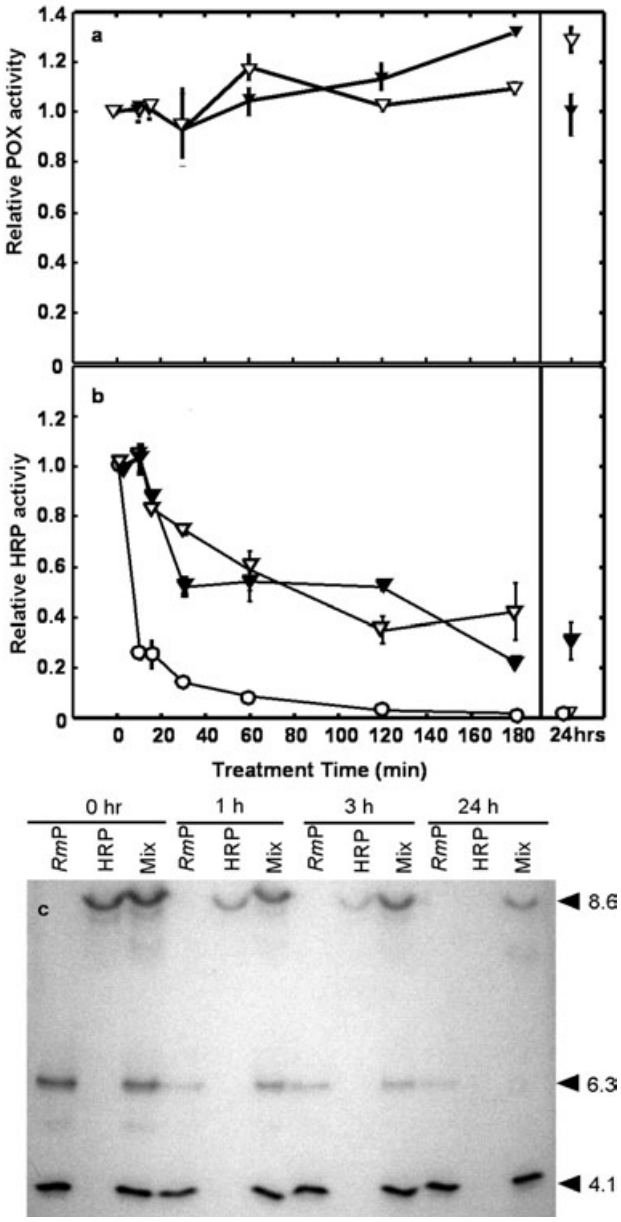

Figure 3. Effect of incubation at 60 °C on activity of *R. mangle* POX (a) and HRP (b). After heating for the indicated times, activity was measured immediately after cooling to room temperature, with quercetin as the electron donor. (a) *R. mangle* POX – crude extracts (▼), purified enzyme from 'old' leaves (▽). (b) HRP alone (○), coincubated with crude *R. mangle* extract (▼), or coincubated with purified enzyme from old leaves (▽). Error bars denote means ± SEM ($n = 2$). HRP activity in mixtures was calculated by subtracting POX activity from the total before plotting; (c) Isoelectric focusing gel stained for peroxidase activity with guaiacol/H_2O_2. Activities are shown for four incubation times using purified *R. mangle* POX from rapidly growing trees on the mid-island ridge (*RmP*), horseradish peroxidase (HRP), and a mixture of the two. pI values for the individual bands are shown on the right.

more acidic isoenzyme was present in all samples, but the smaller, less acidic form was absent at all developmental stages in leaves from the dwarf trees. The similarity of the isoelectric point of the isoenzyme from the fallen leaves indicates that it was the same plant enzyme present in other developmental stages, and not a fungal or bacterial form appearing only as decomposition began.

Heat stability

This apparent stability of the mangrove POX (Fig. 1d & e) was further examined by prolonged incubation of the enzyme at 60 °C. Figure 3a shows that POX activity in both the crude and purified enzyme extracts was unaffected, or even increased, by incubation for as long as 24 h. By comparison, horseradish peroxidase (HRP) lost 80% of its activity over the first 15 min of incubation and 98% of its activity within 3 h (open circles, Fig. 3b). Interestingly, both crude mangrove extracts and purified POX (see below) partially stabilized HRP. In mixed enzyme incubations, at most 20% of the HRP activity was lost over 15 min and 25% was retained over 3 h. The crude POX preparation (closed symbols) conferred greater stability on HRP, with approximately 30% of its activity remaining after 24 h.

IEF confirmed the stability and protective activity of the mangrove enzyme. As shown in Fig. 3c, heat treatment resulted in loss of staining in the pI 8.6 band associated with the dominant isoenzyme in the Type IV-A HRP, but the loss was greatly reduced when the HRP and mangrove POX were co-incubated. Interestingly, the less acidic form of POX was also lost during the treatment whereas the activity of the pI 4.1 isoenzyme appeared unchanged or enhanced, consistent with the upward trend in activity with time shown in Fig. 3a.

Substrate specificity

The activity of *R. mangle* POX with a variety of phenolic substrates is summarized in Table 1. The activity was greatest with quercetin, based both on direct measurement of its oxidation and on the coupled oxidation of Asc. By comparison, the activity was immeasurable with rutin, the major quercetin glycoside in the leaves, as the substrate. (+)-Catechin, the flavanol with the same hydroxyl configuration as quercetin in the B-ring and the major monomer on which mangrove tannins are based (Kandil *et al*. 2004), was also an ineffective substrate. Of the other compounds tested, *p*-coumaric acid (the first phenolic compound and a key branch point in the phenylpropanoid pathway), caffeic acid (a diphenol formed by hydroxylation of *p*-coumarate and an early precursor in lignin biosynthesis), coniferyl alcohol (an immediate precursor to lignins and lignans), and chlorogenic acid (an ester of quinic and caffeic acid with strong antioxidant activity), were also found to be potential substrates based on direct and Asc-coupled POX assays. Compounds toward which POX showed no activity included phenylalanine and *t*-cinnamic acid (the initial compounds in the phenylpropanoid pathway), gallic acid [a polyphenol which inhibits TMB oxidation by HRP (Metelitza *et al*. 2004), but which also acts as an antioxidant], and phloroglucinol (1,3,5-trihydroxybenzene, used in staining lignin).

Table 1. Activity of potential POX substrates determined directly by loss of absorbance at the wavelength of maximal absorbance (λ) and by coupling to oxidation of ascorbate

Substrate	λ (nm)	Extinction coefficient of pure substrate (mM^{-1} cm^{-1})	POX activity with the substrate [nmol min^{-1} (unit POX)$^{-1}$]	POX activity with ascorbate [nmol min^{-1} (unit POX)$^{-1}$]	Ratio of ascorbate to substrate activity	Adjusted extinction coefficient of substrate ($mM^{-1}cm^{-1}$)
Quercetin	360	19.6	1.00 ± 0.02	1.73 ± 0.03	1.73	11.3
Rutin	350	4.6	ND	ND		
(+)-Catechin	277	4.0	ND	ND		
Caffeic acid	305	14.9	0.11 ± 0.01	0.23 ± 0.02	2.14	7.0
Chlorogenic acid	325	32.0	0.10 ± 0.01	0.28 ± 0.03	2.81	11.4
Coniferyl alcohol	264	7.8	0.33 ± 0.02	UR		
p-Coumaric acid	285	32.0	0.02 ± 0	0.14 ± 0.01	8.41	3.8
t-Cinnamic acid	269	20.9	ND	ND		
Phenylalanine	257	1.9	ND	ND		
Gallic acid	260	10.6	ND	ND		
Phloroglucinol	265	0.7	ND	ND		

The ratio of these activities was used to calculate an effective extinction coefficient accounting for the effect of absorption by oxidized substrates (discrepancies between ratios and tabulated activities are due to rounding). ND, not detectable, calculated activity less than 0.01 nmol min^{-1} (unit POX)$^{-1}$. UR, unnreadable due to overlap of phenolic and Asc spectra. Data are means ± SEM.

Table 1 also shows the ratio of the activities based on the direct and ascorbate-linked assays. In all cases, the ascorbate-linked rates were higher. This disparity resulted from the use of extinction coefficients for the phenolic substrates in calculating activity without taking into account the extinction coefficients of their oxidation products; as previously reported (Takahama & Hirota 2000) and confirmed in our studies (data not shown), the absorption spectra for the substrates and their oxidation products significantly overlapped, leading to underestimation of rates. Because Asc oxidation produced no interfering compounds, the ratio of rates based on its oxidation and that of the substrate could be used to calculate operational extinction coefficients for the effective substrates (last column of Table 1).

Leaf structure and POX-related histochemistry

To put the results of these studies into the context of the leaves themselves, and our previous quantification of tannins and phenolics in *R. mangle,* we examined leaf structure and the localization of tannins, phenolics, lignin and peroxidase activity (Fig. 4). The overall structure of a *R. mangle* leaf is shown in Fig. 4a. Notable features include the large number of stomates in any section (data not shown), very diffuse spongy mesophyll, small size of the palisades chlorenchyma cells, and multiple layers of epidermis, especially on the adaxial surface. With age, leaves thickened, and most of the change was due to expansion of the adaxial epidermal cells (data not shown). Eventually, these occupied more than half the volume of the old leaves (Fig. 4d), while the photosynthetic mesophyll thickness (and chlorophyll content per unit area) remained largely unchanged.

Figure 4a, stained with haematoxylin and eosin, also shows the accumulation of tannins in the outer layers of both the upper and lower epidermis and in parenchyma cells surrounding the vascular bundles. This was confirmed using toluidine blue and by electron microscopy (data not shown). Between the upper epidermis and the palisades, a layer of large, pear-shaped cells stained with a distinctive pattern (except in very young leaves where the cells appear empty). These have been called 'slime' cells by Roth (1992). Presumably, these are the major site of the flavologlycans which appear characteristic of this genus (Neilson *et al.* 1986).

POX was localized in fresh leaf sections by staining with guaiacol/H_2O_2 (controls without one or both substrates are not shown). As shown in Fig. 4b, the activity was distributed throughout the leaf, including the epidermal layers, and especially in the cell walls. Returning to the interpretation of the developmentally related changes in POX activity in Fig. 1d, this means that absolute POX activity (the numerator) and leaf fresh weight (the denominator) increased in parallel with age. In contrast, chlorophyll content per unit area (the alternate denominator) changed much less, such that changes in activity per unit chlorophyll appeared larger.

Figure 4c and d show the distribution of non-tannin phenolic compounds and lignin. Like POX, phenolics were distributed throughout the leaves except in large, inner layers of the adaxial epidermis, although even in that region, they appeared to be present in the cell walls. Lignin was present only in vascular tissue and occasional sclerids (not shown). There was no identifiable lignin accumulation in epidermal and mesophyll cell walls, even in the oldest, thickest leaves.

DISCUSSION

In this paper, we have presented a partial characterization of the phenolic peroxidase from *R. mangle*, including both ecological and biochemical aspects of its biology. It has long

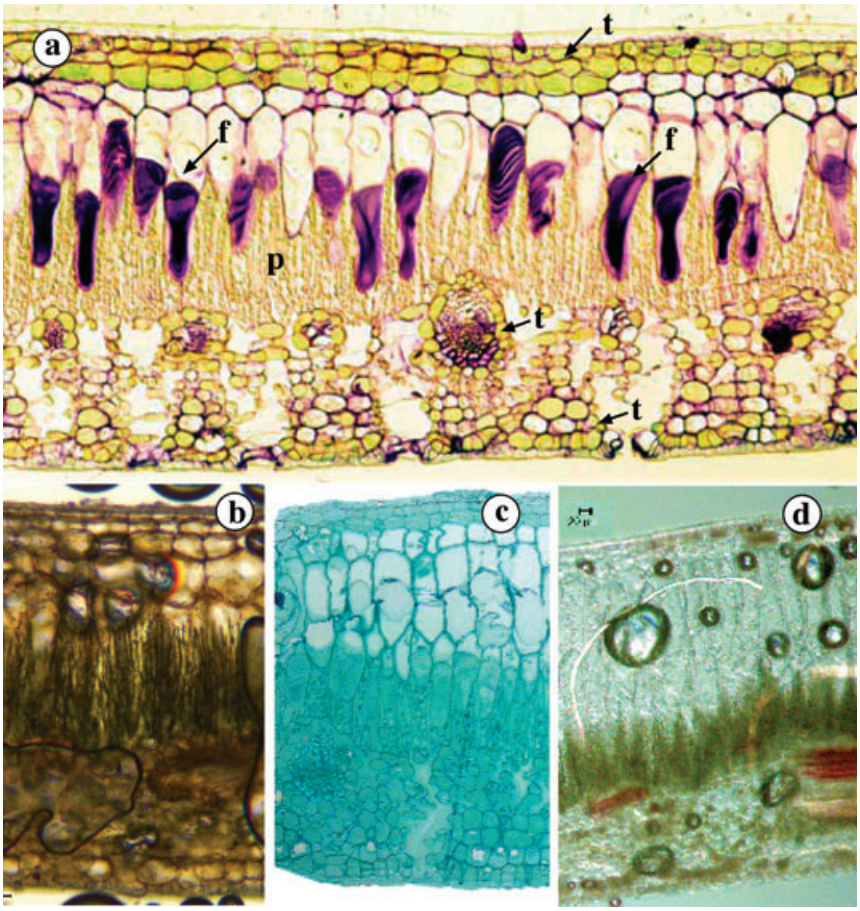

Figure 4. Anatomy and POX-related histochemistry of *R. mangle* leaves. (a) Anatomical overview. Mature leaf stained with haematoxyln and eosin: t = tannins, f = flavologlycans ('slime'), p = palisades mesophyll; (b) peroxidase (brown) stained with guaiacol/H_2O_2; (c) non-tannin phenolics (blue) stained with acidified $FeCl_3$ and $K_3Fe(CN)_6$; (d) lignin (red) stained with phloroglucinol followed by reaction with HCl.

been discussed that functional differences exist between acidic and basic POX isoenzymes (Boyer & De Jaeger 1986), and unlike the basic POX isoenzymes previously studied as potential H_2O_2 scavengers (Takahama & Oniki 1997; Yamasaki & Grace 1998; Pérez *et al*. 2002; Pérez & Maureira 2003), the two constitutively expressed POX isoenzymes obtained from *R. mangle* had acidic pIs (Fig. 2). The larger and more acidic isoenzyme was present in all samples, while the less acidic form was absent in dwarf zone samples, possibly reflecting its lower heat stability (Fig. 3). A comparable, constitutive POX (57 kDa, pI of 3.8) has been reported from the African oil palm, *Elaies guineensis* (Sakharov *et al*. 2000). It is interesting that in both these tropical species, the number of isoenzymes was low compared to that of other species based on IEF (e.g. Curtis, Howell & Kremer 1976; Faivre-Rampant *et al*. 1998). Moreover, higher plants typically have 8–15 POX gene families (Welinder 1992); whether *R. mangle* is depauperate in this regard is yet to be determined.

The heat stability of both the *R. mangle* and oil palm peroxidases contrasts sharply with the temperature sensitivity of horseradish peroxidase (Fig. 4, see also Tams & Welinder 1998), perhaps reflecting an evolutionary adaptation to high air and leaf temperatures. Its overall stability was also indicated by its sustained activity during leaf senescence (Fig. 1). Interestingly, the co-incubation of *R. mangle* POX with HRP conferred some stability to HRP (Fig. 3b), possibly reflecting protein aggregation or interactions between the carbohydrate units of these glycoproteins, although it has previously been reported that de-glycosylation of HRP had no effect on its heat stability (Tams & Welinder 1998).

We originally began this study to consider the relevance to *R. mangle* of the Takahama/Yamasaki model for photoprotective scavenging of H_2O_2. That model proposed that a potentially continuous supply of H_2O_2 (produced by the Mehler reaction and chloroplast SOD activity) is detoxified by the cyclic oxidation of flavonoids by POX and re-reduction of flavonoid radicals by ascorbate. In *Rhizophora* mangroves, this model seemed consistent with observed activities of SOD (both chloroplast and cytosolic forms) which substantially exceeded the activity of APX, with an apparent imbalance of photosynthetic electron transport rates and Calvin cycle electron consumption (Cheeseman *et al*. 1997), with high levels of leaf polyphenolics (Kandil *et al*. 2004), and with the constitutive and ubiquitous distributions of both POX and polyphenolics in the leaves (Fig. 4).

In practice, however, our results point to one major problem: evidence supporting the model has largely been based on the activity of aglycones such as quercetin and kaempferol, often with HRP as the model enzyme (Takahama & Oniki 1997; Yamasaki *et al*. 1997; Pérez *et al*. 2002), but aglycones are actually uncommon constituents in plant

tissues (Wollenweber & Dietz 1981). In *R. mangle,* quercetin, the major aglycone on which the flavonoids are built, is present at detectable levels only in senescent leaves (Kandil *et al*. 2004), and while flavonol glycosides are effective non-enzymatic antioxidants (Rice-Evans, Miller & Paganga 1996), they are ineffective as POX substrates (Table 1, see also Yamasaki *et al*. 1997). Clearly, the model could be modified to substitute simpler, but still active, monophenols (Table 1) for flavonoids. Monophenol oxidation by peroxidase is generally regarded as an extracellular POX activity (Siegel & Siegel 1986), however, operative in lignification, suberization, and cell wall strengthening, and requiring the radicals generated by POX activity (Ogawa, Kanematsu & Asada 1997). Non-enzymatic re-reduction of radicals by Asc at that point would be in competition with enzymatic processes needed for leaf development. Similarly, the model could be reconfigured for detoxification of H_2O_2 from any source, not just the Mehler reaction. However, based on the high concentrations of rutin, catechin and other good-antioxidant-but-poor-POX-substrate polyphenolics in leaves, the need for enzyme involvement at all remains uncertain.

What, then, are the roles of POX in *R. mangle*? How, if at all, might they differ from those in mesophytic species, or reflect the special demands of the mangrove ecosystem? In other plants, POX is a multifunctional enzyme (see Ros Barceló *et al*. 2003), and it is unlikely that its overall functions will be different in these trees. Rather, differences in the performance of those functions will most likely reflect differences in its expression, stability, and environment.

One alternative function of POX is cell wall protein cross-linking involving the oxidation of simple phenolics (Brisson *et al*. 1994), effectively supplementing the chemical protection against herbivores by polyphenolics with mechanically impaired palatability. In mangroves, this function is suggested by progressive changes in leaf structure. As mangrove leaves age, they become brittle; when bent, they crack rather than fold. Their rigidity, however, is neither a reflection of cuticular strength nor of progressive lignification (Fig. 2d). As cross-linked proteins are less susceptible to enzymatic digestion, this may also explain why isolation of protoplasts from mangrove leaves is problematic at best (data not shown, see also Brisson *et al*. 1994).

A second role for POX, especially constitutively expressed, is control of mechanical damage. When *R. mangle* leaves are broken or wounded, the exposed surface browns and seals very quickly, reducing water loss and preventing microbial invasion. As this requires oxidation of aglycones, wounding must be accompanied by the release of glycosidases (as yet uncharacterized in mangroves), liberation of aglycones, and POX-mediated production of quinones (Takahama & Hirota 2000). Again, this has consequences for the experimentalist, making it very difficult to vacuum infiltrate leaves or to enhance gas exchange by exposing cut edges.

A third possible function for mangrove POX is as part of H_2O_2-related signalling. In rhizophoracean mangroves, stomatal conductance collapses and intercellular CO_2 increases toward ambient within minutes of clamping a gas exchange chamber on a leaf (Cheeseman *et al*. 1991, 1997), suggestive of a mechanically induced oxidative burst (Yahraus *et al*. 1995) beginning a signal cascade leading to stomatal closure (Pei *et al*. 2000; Mori *et al*. 2001), and eventually, perhaps in the inhibition of Rubisco (Badger *et al*. 1980). Preliminary studies of H_2O_2 levels in mangrove leaves indicate that steady state concentrations are in the low millimolar range, and that both production and consumption are highly dynamic (Cheeseman, unpublished results).

Finally, a constitutive, stable and persistent POX, able to function throughout senescence, may play an important role in allowing *R. mangle* trees to persist and dominate in their highly oligotrophic and often anoxic ecosystem. In this environment, turnover of nutrients is critical, as is prevention of litter accumulation which would further limit O_2 diffusion into the substrate ('soil') (Cohen & Spackman 1977; Middleton & McKee 2001). A role for POX, here, is mediation, before leaf-drop, of the breakdown of simple phenolics, which can inhibit root metabolism at low levels in soil solutions (Baziramakenga *et al*. 1997), and of polyphenolics, which interfere with fragmentation and decomposition (Kandil *et al*. 2004).

CONCLUSION

The phenolic peroxidase from *Rhizophora mangle* leaves occurs in two isoforms, both acidic. The larger, more acidic isoenzyme is highly stable, both with temperature and through all phases of leaf development and senescence, even after abscission. The mangrove POX oxidizes a variety of phenolic substrates, with greatest activity toward the flavonol aglycone, quercetin, and ascorbate can re-reduce the intermediate free radicals before oxidation is complete. The lack of activity of POX to the most prevalent flavonoid (rutin) and the absence of quercetin in green leaf tissue, however, argues against the importance of detoxification of H_2O_2 as a major role for the enzyme. On the other hand, roles in cell wall protein cross-linking, closing of wound surfaces, metabolic signalling, and phenolic turnover during and after senescence are functions well suited to this constitutive, stable and persistent enzyme.

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