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Peroxidase-mediated transformation of hydroxy-9,10-anthraquinones

Daniel Arrieta-Baez[a,b], Rosa Roman[c], Rafael Vazquez-Duhalt[c], Manuel Jiménez-Estrada[a,*]

[a]*Facultad de Química, UNAM, Ciudad Universitaria, Coyoacán, D.F. 06860, CP 04510, Mexico*
[b]*Instituto de Química, UNAM, Ciudad Universitaria, Coyoacán, D.F. 06860, CP 04510, Mexico*
[c]*Instituto de Biotecnología, UNAM. Apdo. Postal 510-3 Cuernavaca, Morelos 62271, Mexico*

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Abstract

A peroxidase (EC 1.11.1.7) has been isolated and purified from *Senna angustifolia*. The enzyme was purified by ion-exchange chromatography on high Q and high S columns. SDS–PAGE electrophoresis showed that the protein has a molecular mass of approximately 70 kDa. Hydroxy-anthraquinones and hydroxy-anthracenones were evaluated as substrate of *S. angustifolia* and horseradish peroxidases. Both peroxidases catalyzed the oxidation of alizarin and purpurin anthraquinones to the corresponding 3,3′-bializarin and the new compound 3,3′-bipurpurin, respectively, as well as the formation of 2,2′-biquinizarin from quinizarin anthracenone. The K_{Mapp} and V_{max} values for alizarin and purpurin were 97 and 95 μM, and 1.5 and 2.1 μM min^{-1} mg $prot^{-1}$, respectively. The results suggest that peroxidase may participate in the biogenesis of anthraquinones.

Keywords: *Senna angustifolia*; Leguminosae; Bianthraquinones; Anthracenones; Phenolic oxidation; Kinetic properties; Horseradish peroxidase

1. Introduction

Since hypericin, a polycyclic quinone isolated from natural sources, was studied with regard to its inhibitory effects on different retroviruses, including HIV (Kraus et al., 1990; Barnard et al., 1995), bianthraquinones have been considered potential anti-viral agents. Many bianthraquinones, isolated from *Rheum*, *Aloe*, *Rhamnus* and *Senna*, also exhibit laxative (Lemli, 1986; Gupta et al., 2000), antibiotic and anti-tumor activities (Kitanaka and Takido, 1994; Koyama et al., 1997, 2001; Gupta et al., 2000). Studies using tracer techniques and specific incorporation of labelled compounds have suggested that monomeric anthraquinones are derived from octaketide, and in some cases have pre-anthraquinone precursors, namely anthracenones (Steglich and Oertel, 1984; Gill and Steglich, 1987; Anderson et al., 1990; Anderson and Lin, 1993; Gill, 1994; Zhi-Gang et al., 1995). There is some evidence that anthraquinones and pre-anthraquinones act as precursors to several dimeric anthraquinones, which may be formed by oxidative coupling (Ohnishi et al., 1991; Okamura et al., 1993). However, it is still unclear whether bianthraquinones are either artifacts of isolation or true natural products.

Oxidative phenolic coupling contributes to the biogenesis of several natural products including alkaloids, flavonoids, lignins and antibiotics (vancomycin). It is possible that this enzyme-mediated mechanism could be involved in the biogenesis of bianthraquinones as well.

Peroxidases (EC 1.11.1.7) catalyze the oxidation of a large number of aromatic structures at the expense of H_2O_2. These enzymes have a variety of functions, as evidenced by several isoenzymes being found in different plant cell compartments. They are involved in plant hormone regulation, defense mechanisms, control of cell elongation, lignin biosynthesis and suberization processes (Morales and Ros Barceló, 1997; Wititsuwannakul et al., 1997). Two main groups of peroxidases have been distinguished, acidic and basic, with pI′s ranging from ca 3.5 to 9.5 (Pomar et al., 1997). The precise roles of

* Corresponding author. Tel.: +52-55-5622-4430; fax+52-55-5616-2203.
E-mail address: manuelj@servidor.unam.mx (M. Jiménez-Estrada).

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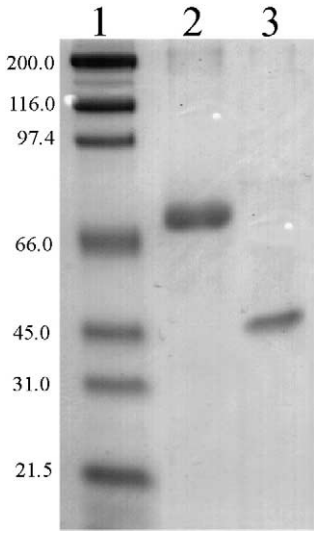


Fig. 1. SDS–PAGE electrophoresis of purified peroxidase from *Senna angustifolia* (2) and horseradish peroxidase type X (3).

individual isoforms of peroxidase remain unclear due to the lack of information on their localization and in vivo substrate specificities. Thus, it is still unknown whether peroxidases participate in bianthraquinone biosynthesis.

The aim of this work was to study the oxidative phenol coupling reaction of hydroxy-9,10-anthraquinones and two hydroxy-anthracenediones by a peroxidase purified from *Senna angustifolia* (SAP) and horseradish peroxidase (HRP). The results suggest that specific peroxidases may play a role in the biogenesis of bianthraquinones as well.

2. Results and discussion

The reactivity of phenolic compounds makes them subject to oxidation, substitution and coupling reactions mediated by peroxidases. In addition to a wide variety of phenolic compounds, hydroxy-anthraquinones can also be used as substrates for peroxidases. To determine the role of peroxidases in the oxidative formation of bianthraquinones, a peroxidase from *S. angustifolia* was purified and assayed with hydroxy-anthraquinone **1–5** and hydroxy-anthracenone **6** and **7** as substrates. Salt precipitation and chromatographic purification of the soluble crude peroxidase gave a highly pure preparation. In SDS–PAGE electrophoresis, the peroxidase showed a 70-kDa protein band (Fig. 1).

Anthraquinones **1–5** were incubated separately in the presence of 1 mM H_2O_2 and purified *S. angustifolia* peroxidase. The reaction was stopped after 10 min and the products were analyzed by HPLC (Table 1). While reactions with HRP or SAP produced the same products, fungal laccase from *Coriolopsis gallicans* did not produce any products under the same reaction conditions.

Enzymatic oxidative phenolic coupling of **3** produced bianthraquinone **8**. Since **3** is only slightly soluble in the buffer medium, we used 20% acetonitrile in H_2O as co-solvent in the reaction mixture. After 1 h, the main product **8** was isolated as a dark yellow-red powder. ($R_f = 0.82$; $CHCl_3$:MeOH; 9:1 v/v). Compound **8** showed IR absorptions due to carbonyl groups (1670, 1618 cm^{-1}) and EI–MS showed a molecular ion at m/z 480 and fragments at m/z 452, 424, 396, and 240, which strongly suggest the presence of an alizarin dimer. Its ^1H NMR (300 MHz, $CDCl_3$) spectrum revealed two double doublets (8.29 and 7.9 ppm), assigned to the 5, 5′, 6, 6′, 7, 7′, 8 and 8′ protons. The presence of a singlet aromatic proton at δ 7.77 (2H) indicated the presence of an alizarin dimer with C3–C3′ coupling, which was confirmed by a HETCOR-NMR experiment. Thus, the analytical data suggest the formation of 1,1′,2,2′-tetrahydroxy[3,3′-bianthracene]-9,9′,10,10′-tetrone or compound **8** (3,3′-bializarin) (Scholl, 1919).

The peroxidase-catalyzed reaction of **5** (in 60 mM phosphate buffer pH 6.1, without a co-solvent, at room temperature for 1 h) produced dimer **9**. Compound **9** ($R_f = 0.71$; $CHCl_3$:MeOH; 9:1 v/v), showed IR absorption due to carbonyl groups (1673, 1623 cm^{-1}), and FAB^+–MS gave a molecular ion at m/z 510 with fragments at m/z 481, 452, 386, and 256, which suggested the presence of a purpurin dimer. The ^1H NMR (300 MHz, $CDCl_3$) spectrum of the reaction product indicated the presence of two double doublets (8.14 and 7.68 ppm), that were assigned to the 5, 5′, 6, 6′, 7, 7′, 8 and 8′ protons. The absence of a singlet at δ 6.65, which

Table 1
Oxidative phenolic coupling (%) of hydroxy-anthraquinones by peroxidase- and laccase-mediated reactions

	Rhein (**1**)[b]	Emodin (**2**)[b]	Alizarin (**3**)[a]	Quinizarin (**4**)[b]	Purpurin (**5**)[b]
Horseradish peroxidase	NR	10%	98%	10%	97%
Peroxidase from *S. angustifolia*	NR	5%	90%	NR	85%
Laccase	NR	NR	NR	NR	NR

NR: no reaction.

[a] 800 μl of 60 mM phosphate buffer, pH 6.1, 200 ml of acetonitrile contains 1 mM H_2O_2, 4 μM anthraquinone and peroxidase (HRP or *S. angustifolia* peroxidase).

[b] 1000 μl of 60 mM phosphate buffer, pH 6.1, 1 mM H_2O_2, 4 μM anthraquinone and peroxidase (HRP or *S. angustifolia* peroxidase).

Table 2
Oxidative phenolic coupling (%) of anthracenones by peroxidase- and laccase-mediated reactions

	Alizarin-anthracenone (**6**)[a]	Quinizarin-anthracenone (**7**)[a]
Horseradish peroxidase	98%	80%
Peroxidase from *S. angustifolia*	97%	74%
Laccase	NR	NR

NR: no reaction.
[a] 1000 μl of 60 mM phosphate buffer, pH 6.1, contains 1 mM H_2O_2, 4 μM anthraquinone and peroxidase (HRP or *S. angustifolia* peroxidase).

was present in the substrate purpurin (H-3), indicated that the dimer has C3–C3′ coupling. The analytical data suggest the formation of the new compound 1,1′,2,2′,4,4′-hexahydroxy[3,3′-bianthracene]-9,9′,10,10′-tetrone **9** (3,3′-bipurpurin).

We also examined the peroxidase-catalyzed reaction of two anthracenones. Substrates **6** and **7** were efficiently obtained from alizarin (**3**) and quinizarin (**4**), respectively, in the presence of sodium dithionite dissolved in DMF in neutral solution, and the products were readily identified spectroscopically (Prinz et al., 1996). The peroxidase-catalyzed reactions of these compounds with SAP and HRP were performed at room temperature for 1 h (Table 2). The alizarin anthracenone (**6**) reaction was monitored at 255 nm by HPLC. In this reaction, the first peak (2.6 min) appeared as an important product, and disappeared as the reaction continued while a second peak (2.8 min) appeared (data not shown). These results suggest that the first product is an intermediate of the enzymatic transformation that yields the second peak. The final product had the same retention time as 3,3′-bializarin (**8**) and was confirmed by EI–MS.

Quinizarin anthraquinone (**4**) appears to be a poor substrate for this peroxidase (Table 1), while quinizarin-anthracenone (**7**) is extensively converted to the dimer in its presence (Table 2). A transient intermediate was also detected in the quinizarin-anthracenone peroxidase-catalyzed reaction (data not shown). The final product **10** showed IR absorptions due to carbonyl groups (1670, 1618 cm^{-1}), and the EI–MS showed a molecular ion at m/z 480 with fragments at m/z 452, 424, 396, and 240, strongly suggesting the presence of a quinizarin dimer compound. Thus, the analytical data suggest the formation of 1,1′,4,4′-tetrahydroxy[2,2′-bianthracene]-9,9′, 10,10′-tetrone **10** (2,2′-biquinizarin) (Laatsch, 1986).

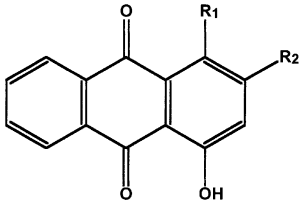
Only anthraquinones with a 1,2-hydroxy system were used as substrates by HRP and SAP, whereas anthracenones were used more efficiently to produce bianthraquinones (Tables 1 and 2). Even though the bianthraquinones reported in this work were not found in vivo and have not been isolated from *S. angustifolia*, their structures are analogous to others reported in the literature, i.e. the C–C bond present in dimers occurs at the position *ortho*- to the OH. Even though laccases and peroxidases could be involved in the same oxidative phenolic coupling reaction, no laccase-mediated reaction was found with these substrates (Tables 1 and 2).

The oxidation of anthraquinones by *S. angustifolia* peroxidase follows Michaelis–Menten-type kinetics, with substrate inhibition observed at higher concentrations. K_{Mapp} (H_2O_2, 1 mM) was 97 and 95 μM, and V_{max} was 1.5 and 2.1 μM min^{-1} mg $prot^{-1}$, for alizarin (**3**) and purpurin (**5**) respectively (Zapata et al., 1992; Rasmussen et al., 1995).

Our results suggest that HRP and the *Senna angustifolia* peroxidase oxidize alizarin (**3**) and purpurin (**5**) (1,2-dihydroxy anthraquinones) efficiently to produce the respective bianthraquinones. The ability of the purified peroxidase to synthesize bianthraquinones from anthracenones or anthraquinones suggests that both intermediates could be used to produce these dimers. The data suggest that peroxidase plays a role in the biosynthesis of bianthraquinones.

Structures of anthracenones

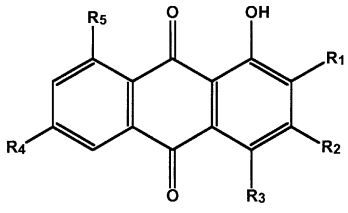
	R1	R2
6: Alizarin-anthracenone	H	OH
7: Quinizarin-anthracenone	OH	H



anthracenones

Structures of anthraquinones

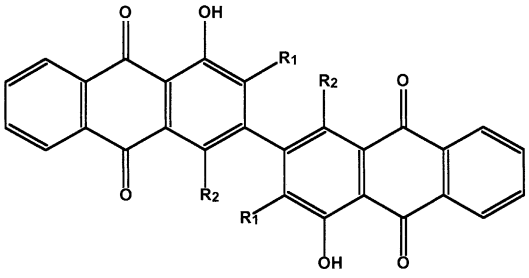
	R1	R2	R3	R4	R5
1: Rhein	H	COOH	H	H	OH
2: Emodin	H	OH	H	CH_3	OH
3: Alizarin	OH	H	H	H	H
4: Quinizarin	H	H	OH	H	H
5: Purpurin	OH	H	OH	H	H



anthraquinones

Structures of bianthraquinone products

	R1	R2
8: 3,3-bializarin	OH	H
9: 3,3-bipurpurin	OH	OH
10: 2,2-biquinizarin	H	OH



bianthraquinones

3. Experimental

3.1. Plant material

Leaves of *Senna angustifolia* were collected from the University campus in México City. A voucher specimen has been deposited at the National Herbarium of the Institute of Biology, UNAM (MEXU).

3.2. Chemicals

Rhein, emodin, quinizarin, purpurin, alizarin and horseradish peroxidase (HRP) were obtained from Sigma Chemical Co. (St. Louis, MO). Buffer salts and H_2O_2 were purchased from J.T. Baker (Phillipsburg, NJ). HPLC-grade solvents (MeOH, THF and EtOAc) were obtained from Merck (Darmstadt, Germany). THF was distilled in the presence of $FeSO_4$ prior to use to eliminate peroxides.

3.3. Senna angustifolia enzyme purification

All steps were performed at 4 °C. Fresh leaves (500 g) of *Senna angustifolia* were frozen using liquid nitrogen and ground in a mortar with acid-washed fine quartz. The powder was extracted three times with 1 l of 60 mM Pi buffer (pH 6.1). After filtration through four layers of nylon gauze, the homogenate was centrifuged at 10,000 rpm for 5 min. To the resulting peroxidase-containing supernatant was slowly added solid $(NH_4)_2SO_4$ with stirring to 40% saturation, and the ppt. was removed by centrifugation at 10,000 rpm for 15 min. The peroxidase-containing supernatant was again treated with solid $(NH_4)_2SO_4$ to 80% saturation and the ppt. was collected by centrifugation at 10,000 rpm for 15 min. The pellet was dissolved in a minimum volume of 60 mM Pi buffer (pH 6.1), and dialyzed extensively against the same buffer for 12 h. The concentrated extract was applied to an anion-exchange column (diethylaminoethyl cellulose DE52, Whatman), pre-equilibrated with 10 mM Na–Pi buffer (pH 6.0) and eluted with a 0–1.0 M NaCl gradient in the same buffer. Most of the pigment was removed on this column. Fractions containing peroxidase activity were pooled, concentrated and dialyzed by ultrafiltration. The peroxidase fraction was subjected to further chromatography using two successive columns (16×2.3 cm): High Q (strong anionic interaction) and High S (strong cationic interaction), equilibrated with 10 mM Na–Pi (pH 6.0) and eluted with a 0–1.0 M NaCl gradient in the same buffer.

The purity of the enzymatic preparation was checked by SDS–PAGE using 8% acrylamide gel and detected with Coomassie blue. Myosin (200,000), β-galactosidase (116,250), phosphorylase B (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and trypsin inhibitor (21,500) were used as standard proteins (Fig. 1).

3.4. Enzyme assay

The peroxidase activity of the enzyme was monitored during purification by spectrophotometric measurement of the products of guaiacol oxidation at 470 nm ($\varepsilon_{470} = 26.6\ mM^{-1}.cm^{-1}$). The reaction was performed at 25 °C in 1.3 ml reaction mixture containing 60 mM Pi buffer (pH 6.1), 1 mM H_2O_2, 16 mM substrate and suitable amounts of enzyme. The reaction was started by adding H_2O_2 (Shannon et al., 1966).

3.5. Protein determination

Protein concentrations were estimated using the Bradford reagent. The absorbance of the reaction mixture was measured at 595 nm, and compared with a standard curve of BSA (Bradford, 1976).

3.6. Oxidative phenol coupling assays

Peroxidase oxidative coupling reactions of anthraquinones and anthracenones were performed at 25 °C in 1.3 ml reaction mixture containing 60 mM phosphate buffer (pH 6.1), 1 mM H_2O_2, 4 μM anthraquinone and 20 μl of HRP (1.8 u/mg) or 30 μl of *S. angustifolia* peroxidase (1.5 u/mg). The reaction was monitored by HPLC (Perkin-Elmer) with a reversed-phase column (150×3.9 mm) (Resolve C_{18}, Millipore), eluted with a NaOAC acetate buffer (100 mM, pH 3):THF (50:50, v/v) mixture. The extent of the enzymatic reaction was determined by measuring the decrease in the peak area of phenolic substrate at A_{255}. All reactions were performed in triplicate. To obtain enough material for structural identification, separate 500-ml reaction mixtures containing 50 mg of each anthraquinone or

anthracenone were separately treated with HRP and H_2O_2. Complete conversion of substrates into products (monitored by HPLC) was achieved by two successive additions of HRP and H_2O_2 at 30-min intervals.

3.7. Product analysis

The products of peroxidase-catalyzed reactions were extracted three times with EtOAc. The extracts were dried over Na_2SO_4 and concentrated under vacuum. Two systems were used for product purification: preparative TLC with $CHCl_3$/MeOH (9:1, v/v) and preparative HPLC with NaOAc buffer (100 mM, pH 3):THF (50:50, v/v). The main purified product was analyzed by spectroscopic techniques.

3.8. 1,1′,2,2′-Tetrahydroxy[3,3′-bianthracene]-9,9′,10,10′-tetrone or 3,3′-bializarin (**8**) (42 mg, 84%)

Dark yellow-red powder. (R_f = 0.82; $CHCl_3$:MeOH; 9:1 v/v). ν_{max}^{MeOH} cm^{-1}: 1670, 1618 (>C=O). ^1H NMR (300 MHz, $CDCl_3$): δ 8.29 (*m*, 4H), 7.9 (*m*, 4H), 7.77 (2H, *s*, H-4, H-4′). EI–MS 70 eV, *m*/*z* (rel. int.): 480 $[M]^+$, 452 $[M\text{-}28]^+$, 424 $[M\text{–}CO\text{-}28]^+$, 396 $[M\text{–}CO\text{–}CO\text{-}28]^+$, 240 $[M\text{-}240]^+$. (Scholl, 1919).

3.9. 1,1′,2,2′,4,4′-Hexahydroxy[3,3′-bianthracene]-9,9′,10,10′-tetrone or 3,3′-bipurpurin (**9**) (40 mg, 80%)

Dark yellow-red powder. (R_f = 0.71; $CHCl_3$:MeOH; 9:1 v/v). ν_{max}^{MeOH} cm^{-1}: 1673, 1623 (>C=O). ^1H NMR (300 MHz, $CDCl_3$): δ 8.14 (*m*, 4H), 7.68 (*m*, 4H). FAB^+-MS *m*/*z* (rel. int.): 510 $[M]^+$, 481 $[M\text{-}29]^+$, 452 $[M\text{–}CO\text{-}29]^+$, 386, 256 $[M\text{-}255]^+$. HRMS–EI (70 eV) *m*/*z* calc. for $C_{28}H_{14}O_{10}$ 510.0587, found 510.0600.

3.10. 1,1′,4,4′-Tetrahydroxy[3,3′-bianthracene]-9,9′,10,10′-tetrone or 3,3′-biquinizarin (**10**) (30 mg, 64%)

Dark yellow-red powder. (R_f = 0.79; CHCl3:MeOH; 9:1 v/v). ν_{max}^{MeOH} cm^{-1}: 1675, 1620 (>C=O). EI–MS 70 eV, *m*/*z* (rel. int.): 480 $[M]^+$, 452 $[M\text{-}28]^+$, 424 $[M\text{–}CO\text{-}28]^+$, 396 $[M\text{–}CO\text{–}CO\text{-}28]^+$, 240 $[M\text{-}240]^+$ (Laatsch, 1986).

3.11. Kinetic analysis

The oxidations of alizarin (**3**) and purpurin (**5**) by purified *S. angustifolia* peroxidase were monitored by measuring the decrease in the peak area of phenolic substrate at A_{255} at 25 °C during the first 15 min of the reaction. The reaction was monitored by HPLC (Perkin-Elmer) with a reversed-phase column (150×3.9 mm Resolve C_{18}, Millipore). All reactions were performed in triplicate. To calculate the kinetic constants (V_{max} and K_{Mapp}) for the oxidation of alizarin (**3**) and purpurin (**5**) anthraquinones, the saturating concentrations of phenols and H_2O_2 were determined. The rates of oxidation were estimated for substrate concentrations ranging between 0.05 and 0.2 mM (and 1 mM for H_2O_2). The reaction rates were obtained by HPLC at different times. When data were plotted using the Michaelis–Menten integrated equation, $(2.303/t)\log(S_o/S) = -(P/K_{st}) + (V_m/K_s)$, straight lines were obtained and kinetic parameters were acquired (Zapata et al., 1992; Rasmussen et al., 1995).

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