FEBS Letters 376 (1995) 202–206 FEBS 16345

Laccase-mediated formation of the phenoxazinone derivative, cinnabarinic acid

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Received 23 October 1995

Abstract The phenoxazinone chromophore occurs in a variety of biological systems, including numerous pigments and certain antibiotics. It also appears to form as part of a mechanism to protect mammalian tissue from oxidative damage. During cultivation of the basidiomycete, *Pycnoporus cinnabarinus*, a red pigment was observed to accumulate in the culture medium. It was identified as the phenoxazinone derivative, cinnabarinic acid (CA). Laccase was the predominant extracellular phenoloxidase activity in *P. cinnabarinus* cultures. In vitro studies showed that CA was formed after oxidation of the precursor, 3-hydroxyanthranilic acid (3-HAA), by laccases. Moreover, oxidative coupling of 3-HAA to form CA was also demonstrated for the mammalian counterpart of laccase, the blue copper oxidase, ceruloplasmin.

Key words: Pycnoporus cinnabarinus; Basidiomycete; Cinnabarinic acid; 3-Hydroxyanthranilic acid; Blue copper oxidase; Ceruloplasmin; Lignin degradation

1. Introduction

Upon oxidation, *o*-aminophenols are converted into their corresponding *o*-quinone imines; however, because of their high reactivity, these *o*-quinone imines often couple spontaneously to form derivatives, such as the cyclic phenoxazinones. The phenoxazinone ring structure has been identified in a number of different biological systems. The *o*-aminophenol, 3-hydroxyanthranilic acid (3-HAA), a metabolite of the kynurenine pathway, is the precursor of the phenoxazinone derivative, cinnabarinic acid (CA). Conversion of 3-HAA into CA can proceed non-enzymatically through the action of active oxygen species and various transition metals [1,2], but it can also be mediated enzymatically. Thus, CA formation was catalyzed by compound I of peroxidase or catalase as well as tyrosinase [3,4].

The conversion of 3-HAA to CA has received considerable attention in clinical studies because 3-HAA is produced in relatively large amounts by interferon-γ-primed mononuclear phagocytes [5] and has been shown to act as a powerful scavenger of reactive oxygen species [1]. CA is one of the major products of the peroxyl radical-mediated oxidation of 3-HAA suggested to prevent oxidative damage in mammalian tissues [6]. In certain antibiotic-producing bacteria, such as *Streptomyces antibioticus*, 3-HAA serves as a precursor for another phenoxazinone derivative, actinomycin [7]. The enzyme catalyzing 3-HAA oxidation in *S. antibioticus*, phenoxazinone synthase, was recently proposed as a new member of the blue copper oxidase family [8]. Phenoxazinone chromophores are also used as pigments by such diverse oganisms as insects, fungi and Australian marsupials [9–11].

CA and two of its derivatives, cinnabarin and tramesanguin, are known to impart a bright orange-red color to the fruiting bodies of members of the genus *Pycnoporus* [12–14] (Fig. 1). However, synthesis of CA in these fungi has not received further attention. In our effort to study the ligninolytic system of *Pycnoporus cinnabarinus*, we identified laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2) as the predominant extracellular phenoloxidase in cultures of this wood-degrading, white-rot fungus. Laccases are known to oxidize a wide range of substituted phenols and arylamines, including aminophenols [15].

In this paper, we report on the production of 3-HAA and CA in cultures of *P. cinnabarinus* and describe for the first time the formation of CA by laccase-mediated oxidation of 3-HAA. Interestingly, the mammalian protein, ceruloplasmin, which, like laccase, is a member of the blue copper oxidase class of enzymes, also catalyzed 3-HAA coupling to CA.

2. Materials and methods

2.1. Chemicals

3-HAA, 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) and bovine ceruloplasmin were purchased from Sigma. CA was synthesized as described [1]. *Coriolus hirsutus* laccase was obtained from Calbiochem (San Diego, CA) (120 U/mg dry weight). Organic solvents were HPLC grade, while all other chemicals were of analytical grade and purchased from either Fisher or Sigma.

2.2. Organism and culture conditions

P. cinnabarinus, strain PB, was maintained on 2% (w/v) malt extract agar plates grown at 24°C and kept at 4°C. For preparing spore suspensions, the fungus was cultivated on parboiled rice (60 g/1-l Erlenmeyer flask) for 10 days at 30°C. Spores were harvested by filtration through cheesecloth and washed extensively with 0.9% (w/v) NaCl. They were kept at 4°C in the dark and used within 2 weeks of storage. Spore suspensions at a final concentration of $3.75 \cdot 10^5$ spores/ml were used to inoculate culture media [16]. The pH of the medium was adjusted to 4.5 with 1 N NaOH. Cultures were incubated at 30°C on a rotary shaker (125 rpm; φ 18 mm).

2.3. Enzyme assays

After removing the mycelium by centrifugation (10 min at 5000 × *g*), laccase activity was determined by monitoring the oxidation at 420 nm of 500 μM ABTS buffered with 50 mM sodium tartrate buffer (pH 4.0). Enzyme units are given in μmol of product formed per minute ($\varepsilon_{max} = 3.6 \cdot 10^4$ $M^{-1}cm^{-1}$). Peroxidase activity was measured by adding H_2O_2 to 100 μM final concentration to the laccase assay solution (pH 3.5) and subtracting the previously determined laccase activity. For detection of tyrosinase activity, 0.3 mM tyrosine was used as the substrate in 50 mM sodium phosphate buffer (pH 6.5) [17].

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Abbreviations: 3-HAA, 3-hydroxyanthranilic acid; CA, cinnabarinic acid.

SSDI 0014-5793(95)01274-5

2.4. Laccase purification

Laccase was purified to apparent homogeneity from 6-day-old cultures of *P. cinnabarinus* [18]. After removal of the fungal mycelia by centrifugation (10 min at 5000 × *g*), the spent medium was concentrated in stirred cells against a PM-10 ultrafiltration membrane (Amicon). The following purification steps were performed on an FPLC system (Pharmacia) at 4°C. They included DEAE-anion exchange chromatography (DEAE-M Toyopearl, TosoHaas, Mongomeryville, PA) using a linear gradient of 25 mM to 100 mM sodium acetate buffer (pH 5.0), hydrophobic interaction chromatography on a Butyl-Toyopearl column using a linear gradient of 1.7 mM ammonium sulfate to 0.0 mM ammonium sulfate in 25 mM sodium acetate buffer (pH 5.0) and a final gel filtration step. Laccase was eluted from the gel filtration column (Sepharose 12, Pharmacia) in 25 mM sodium acetate buffer.

2.5. Gel electrophoresis and staining

SDS-PAGE was performed according to Schägger and von Jagow [19]. Protein bands were visualized by staining with Coomassie Brilliant Blue and compared with broad-range molecular weight markers (Bio-Rad). For activity staining after SDS-PAGE, electrophoresis was carried out without prior boiling of the samples and the gels were immersed in 50 mM sodium tartrate buffer (pH 4.0) containing diaminobenzidine (0.6 mg ml^{-1}) [20].

2.6. In vitro oxidation of 3-hydroxyanthranilic acid (3-HAA)

For 3-HAA oxidation studies, either laccase purified from *P. cinnabarinus* (20 μg) or commercially available *C. hirsutus* laccase (20 μg) was added to a solution of 1 mM 3-HAA in 1 ml of 50 mM sodium tartrate buffer (pH 4.0) and incubated at 30°C. Reaction conditions were identical for studies of 3-HAA oxidation by ceruloplasmin (120 μg), except that 50 mM sodium phosphate (pH 5.5) was used as the buffer system. Oxidation of 3-HAA was monitored spectrophotometrically and scans were taken at intervals as indicated in the text. For product analysis, reaction mixtures were incubated for 6 h at 30°C.

Enzyme kinetics. The K_m values for 3-HAA and guaiacol oxidation by *P. cinnabarinus* laccase were obtained from Lineweaver-Burk plots using substrate concentrations in the range of 10 μM to 5 mM. Reactions were performed at 30°C in 50 mM sodium tartrate buffer (pH 4.0).

2.7. Identification of 3-HAA and CA

Thin-layer chromatography (TLC). Analysis of 3-HAA and CA by TLC was performed on silica gel plates (GF grade, Uniplate, Analtech, Newark, DE) (200 mm × 200 mm) using butanol/acetic acid/water (4:2:1) as the eluant system. For identification of compounds in reaction mixtures, R_f values were compared with those of the pure compounds.

UV-visible spectroscopy. All spectrophotometric measurements were performed on a Varian UV-Vis DMS 200 spectrometer (Varian, Sugarland, TX).

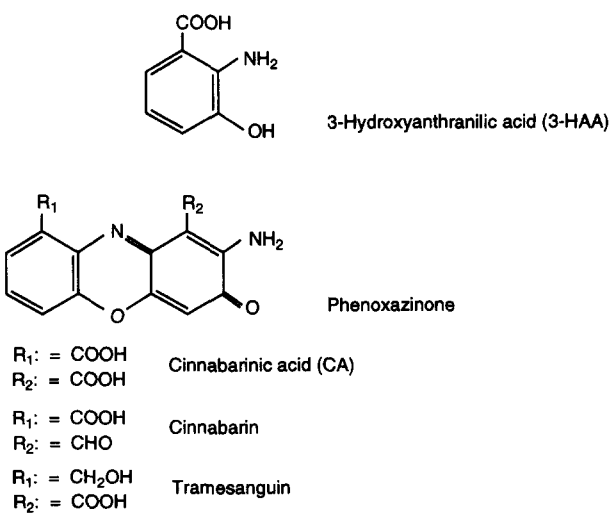

Fig. 1. Structures of 3-hydroxyanthranilic acid and major phenoxazinone derivatives isolated from *Pycnoporus* species.

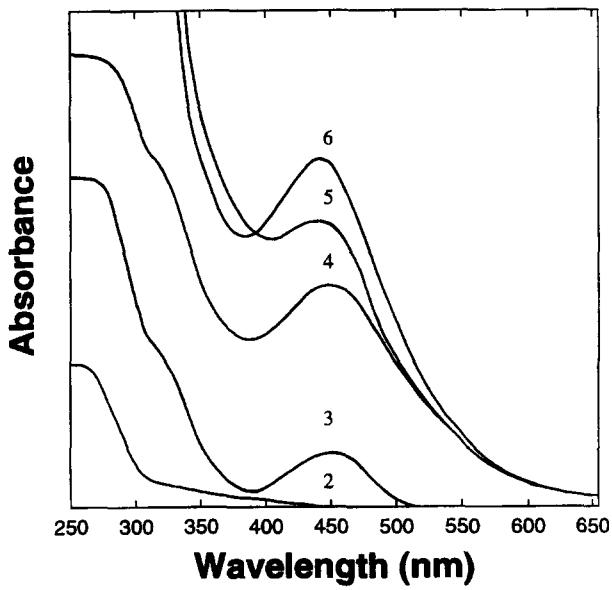

Fig. 2. Absorbance spectra of samples from the cell-free medium of *P. cinnabarinus* grown in shake-flasks (3 g/l glucose; pH 4.5). Spectra were recorded at daily intervals from days 2 to 6.

HPLC analysis. Reaction products were separated by HPLC on a C-18 reversed-phase column (Intertsil ODS-2, 4.6 mm × 250 mm, 5 μM; Alltech, Deerfield IL) using a linear gradient of 5% to 100% methanol in 0.086% (v/v) phosphoric acid. The flow rate was 1 ml/min at room temperature and the eluant was monitored at 280 nm. Since filtration has been reported to significantly reduce the recovery of CA [21], samples were only centrifuged (10 min, at 7000 × *g*) prior to HPLC injection. Peaks corresponding to CA and 3-HAA, were identified by co-elution with their respective reference compound.

Mass spectrometry. Following HPLC separation, peaks showing an identical retention time as authentic CA were collected and analysed by electron spray ionization mass spectrometry (EI-MS). Mass spectra were acquired on a Sciex (Ontario, Canada) API III mass spectrometer by introducing the sample solution into the API source at a flow rate of 5 μl/min with a syringe pump. The ion spray needle was held at 5 kV and the orifice voltage was 70. Reported values were the averages over four runs.

3. Results

P. cinnabarinus was grown at 30°C in shake flask cultures with glucose (3 g/l) as the sole carbon source. Over the time course of cultivation, a color change in the initially clear medium was noted. After 3 days, the culture fluid turned pale yellow and subsequently dark red. Samples of the cell-free culture fluid taken over a period of 6 days, revealed an increasing absorbance around 450 nm (Fig. 2). This increase leveled off after day 4 of cultivation.

The absorption maximum at 450 nm exhibited by samples from the *P. cinnabarinus* cultures was in good agreement with spectra reported previously for CA [3,4] as well as with the absorbance spectrum of CA chemically synthesized in our laboratory. TLC analysis of the culture samples found bands with R_f values identical with authentic CA (0.51 ± 0.02). Reversed-phase HPLC revealed a peak whose retention time was identical with that of CA and fractions containing this peak were subjected to mass spectrometric analysis. EI-MS found a predominant mass signal at 300, identical with the molecular mass of CA. A minor peak (<9%), corresponding to a molecular

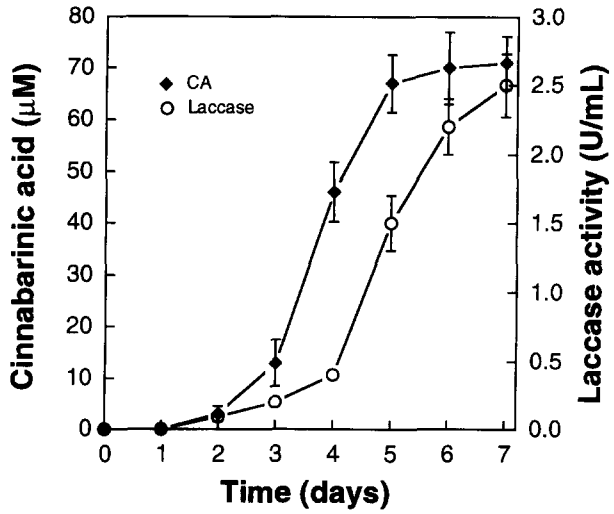

Fig. 3. Laccase and cinnabarinic acid production during growth of *P. cinnabarinus*. Error bars show the SD of the sample for 5 replicate cultures.

mass of 287, was also detected and most likely represented the phenoxazinone derivative, cinnabarin (cf. Fig. 1).

Because the *o*-aminophenol, 3-HAA, has been demonstrated to be a precursor for CA in other biological systems, excretion of this kynurenine metabolite by *P. cinnabarinus* cultures was measured. Samples of the cell-free culture supernatant were separated by TLC and bands detected by autofluorescence co-migrated with authentic 3-HAA (R_f = 0.82 ± 0.01). With exposure to atmospheric oxygen these colorless bands turned reddish brown over time, suggesting auto-oxidative formation of CA. HPLC analysis of the culture samples revealed a peak having a retention time identical with authentic 3-HAA. After 2 days of cultivation, 3-HAA was present in the culture medium at concentrations of about 20 μM, as judged by HPLC. After 3–4 days of cultivation, 3-HAA was no longer detectable, most likely due to conversion of 3-HAA to CA. The concentration of CA in culture medium reached a maximum of about 70 μM after 5 or 6 days of cultivation.

Under alkaline conditions, 3-HAA is readily auto-oxidized [1]. However, when 3-HAA was added to uninoculated medium, the compound was very stable. Thus, auto-oxidation did not significantly contribute to CA formation during growth of the fungus.

Under our growth conditions, laccase was found to be the predominant extracellular phenoloxidase produced in *P. cinnabarinus* cultures. Peroxidase activity in *P. cinnabarinus* cultures contributed only slightly to the total extracellular phenoloxidase activity (< 1 U/l) and no tyrosinase activity could be detected in the culture fluid. Laccase activity appeared after 2 days and reached a maximum of about 2500 U/l on day 7 (Fig. 3). The time course of laccase activity during growth of *P. cinnabarinus*, thus, paralled the accumulation of CA.

In vitro oxidation of 3-HAA by laccase. To determine whether laccase could be responsible for the conversion of 3-HAA to CA, the enzyme was purified to apparent homogeneity from liquid cultures of *P. cinnabarinus* as described [18]. The purified enzyme migrated as a single band on SDS-PAGE gels and reacted with diaminobenzidine after electrophoretic separation under non-denaturing conditions (data not shown). The purified laccase (20 μg) was incubated with 1 mM 3-HAA in 50 mM sodium tartrate buffer (pH 4.0) at 30°C and the reaction was monitored spectrophotometrically over a time course of 60 min (Fig. 4). Rapid conversion of 3-HAA to CA was observed, as evidenced by a decrease in the 3-HAA absorbance peak at 325 nm and an increase in CA absorbance at 450 nm. Conversion was essentially quantitative and the reaction was complete after 90 min. A similar reaction profile was obtained when laccase from *C. hirsutus* (20 μg) was incubated with 3-HAA under the same reaction conditions (data not shown). Less than 0.2% of 3-HAA was oxidized under these reaction conditions in the absence of enzyme, suggesting that auto-oxidation of 3-HAA was of minor importance.

TLC analysis of the products after a 6-h incubation of 3-HAA with *P. cinnabarinus* laccase exhibited a single predominant band with an R_f value (0.51 ± 0.02) identical with that of authentic CA. For HPLC analysis, samples were taken after reaction times of 60 min and 6 h, centrifuged and separated on a C-18 column (Fig. 5). After 6 h, 3-HAA was completely oxidized and the major product corresponded to CA (R_t = 40.2 min). A number of unidentified aromatic compounds resolved by HPLC most likely represented intermediates of the laccase-mediated 3-HAA oxidation. Unlike CA, these compounds were found to decrease over time. The fraction corresponding to CA was further analysed by EI-MS, and a mass signal of 300 confirmed that CA was the major product of 3-HAA oxidation by laccase.

The K_m for 3-HAA oxidation by *P. cinnabarinus* laccase was 0.67 (±0.02) mM, while that for guaiacol was 0.75 (±0.03) mM. An almost identical K_m was observed for laccase from *C. hirsutus*.

In vitro oxidation of 3-HAA by ceruloplasmin. Laccase and ceruloplasmin have previously been shown to oxidize many of the same substrates [22]. Because 3-HAA oxidation has been

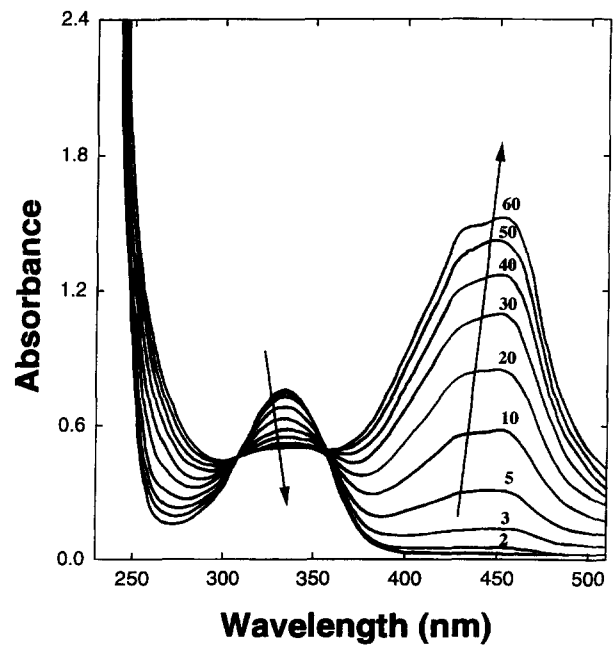

Fig. 4. Changes in the UV-visible spectrum during the oxidation of 3-hydroxyanthranilic acid by *P. cinnabarinus* laccase. Conditions: 50 mM sodium tartrate buffer (pH 4.0), 1 mM substrate, and 20 μg enzyme at 30°C in a 1-ml reaction volume. Time intervals are given in minutes.

reported to play an important physiological role in mammals [1,5], we tested the ability of ceruloplasmin to catalyze CA formation. Oxidation of 3-HAA by ceruloplasmin was slower than for the laccase-mediated reaction. However, after 60 min, about 30% of the substrate was oxidized and the product displayed the characteristic absorption spectrum for CA (λ_{max} at 450 nm) (data not shown). The reaction product was confirmed to be CA by TLC, HPLC and EI-MS. Interestingly, the reaction stopped before 3-HAA was completely oxidized, possibly as a result of inactivation of ceruloplasmin by radical or quinone intermediates. Oxidative coupling of 3-HAA to tyrosine residues in proteins was recently demonstrated to occur during the tanning of proteins [23].

4. Discussion

This paper reports an investigation into the formation of the phenoxazinone derivative, cinnabarinic acid, catalyzed by laccase in liquid cultures of the white-rot fungus, *P. cinnabarinus*. A red pigment (λ_{max} 450 nm) and laccase activity were found to accumulate in parallel in liquid cultures of *P. cinnabarinus*. The predominant pigment was identified as the phenoxazinone derivative CA by spectrophotometry, TLC, HPLC and EI-MS. As no tyrosinase or significant peroxidase activity was detected under the growth conditions examined, the results suggested that laccase catalyzed formation of CA in *P. cinnabarinus* cultures.

In vitro studies with purified laccases clearly demonstrated the ability of laccase to catalyze the formation of CA by oxidative coupling of 3-HAA. The K_m value obtained for 3-HAA was in the same range as other common laccase substrates, such as guaiacol. Tyrosinase from *Neurospora crassa* has been reported to convert 3-HAA into CA and the K_m of 0.7 mM for that enzyme was very similar to that determined for *P. cinnabarinus* laccase [4].

A characteristic feature of the three members of the genus *Pycnoporus* (Kars.), *P. cinnabarinus*, *P. sanguineus* and *P. coccineus*, is the production of pigments which contain the phenoxazinone ring structure. It is worth noting that, like *P. cinnabarinus*, *P. sanguineus* and *P. coccineus* are known to be prodigious laccase producers [24,25]. The three major pigments isolated from these species, CA, cinnabarin and tramesanguin, differ only in the oxidation state of their substituents (Fig. 1).

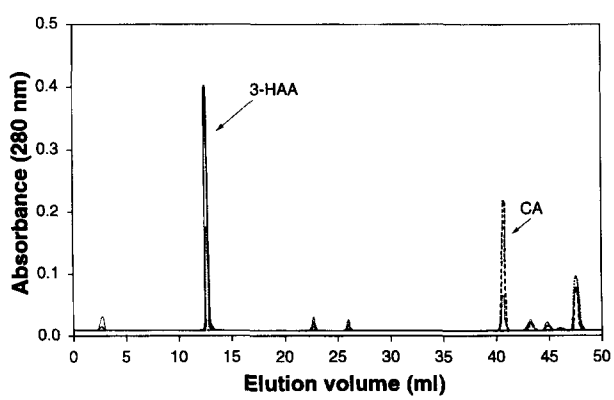

Fig. 5. HPLC-profile of the reaction products separated after 0 min (solid line), 60 min (dotted line) and 6 h (dashed line) incubation of 3-HAA with laccase. Peaks corresponding to authentic 3-HAA and CA are marked by arrows.

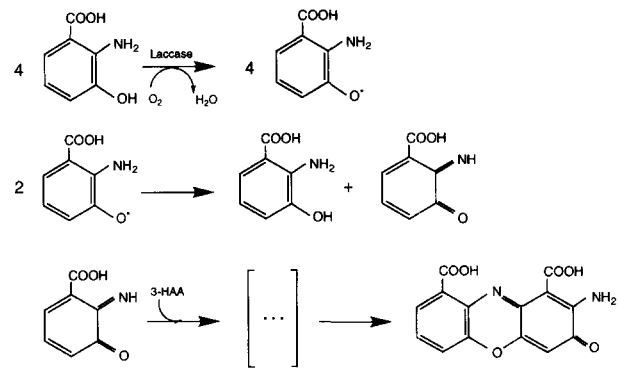

Fig. 6. Hypothetical reaction mechanism for laccase-mediated conversion of 3-hydroxyanthranilic acid into cinnabarinic acid (adapted from [3,4]).

This variation may represent oxidative coupling of different *o*-aminophenol precursors, having either the alcohol, aldehyde or carboxyl group as substituents. Production of these precursors could be strain specific or may depend on the culture conditions. Upon oxidation, a series of *o*-aminophenols form colored products with UV-visible spectra similar to that of CA [26].

Differences in culturing conditions for this study might explain why CA was identified as the major pigment in liquid cultures of *P. cinnabarinus*, strain PB, whereas cinnabarin contributed less than 10% to the phenoxazinone chromphores detected. Isolation of pigments from fruiting bodies of *P. cinnabarinus* previously yielded cinnabarin as the major product [12,13].

It seems highly likely that oxidative coupling of 3-HAA by laccase progresses along the same pathway that has been proposed for tyrosinase and peroxidase [3,4] (Fig. 6). The *o*-quinone imine is the first intermediate after a one electron abstraction from 3-HAA. The last step of the 6-electron oxidation leading to CA was demonstrated to proceed spontaneously. However, the intermediate reactions of this proposed pathway need verification.

Although ceruloplasmin has been shown to oxidize a number of *o*- and *p*-phenols [27], this is, to our knowledge, the first report of CA formation by ceruloplasmin. Our findings suggest that ceruloplasmin should be considered a possible catalyst of 3-HAA oxidation in mammalian tissues. Considering the abundance of the enzyme in mammalian serum (0.2 to 0.5 g/l) and its proposed antioxidative function [28], this reaction could potentially be of physiological importance.

Acknowledgements: We wish to thank Dr. Ron Orlando and Dr. V.S.K. Kolli at the Center for Complex Carbohydrate Research Center, University of Georgia for acquisition of mass spectra. One of us (C.E.) was supported by a grant from the Alexander von Humboldt-Stiftung. Further funding was received from the National Science Foundation (MCB-9507331) and the University of Georgia Biotechnology Grant Program.

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