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Running title: Benzo[a]pyrene oxidation by laccase

Key words: polycyclic aromatic hydrocarbons, biodegradation, filamentous fungi, purified enzymes

Summary

The biotransformation of benzo[a]pyrene by purified extracellular laccase of *Pycnoporus cinnabarinus* was investigated in bench scale reactors. The reaction required the presence of exogenous mediator ABTS. Most of 95% of the substrate was converted within 24 hours. The enzyme preparation oxidised the substrate mainly to benzo[a]pyrene 1,6- 3,6- and 6,12-quinones in a 2/1/1 ratio after 24 hours incubations.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants widely disributed in terrestrial and aquatic environments. They represent a health risk to animals and humans because of toxic, mutagenic and carcinogenic properties (Shaw and Connell, 1994). This is especially the case of benzo[a]pyrene, a five ring hydrocarbon accumulated in industrial sites polluted by PAHs. Bioremediation is a cost-effective process used for the treatment of soils polluted by organic xenobiotics. Altough benzo[a]pyrene was often described as recalcitrant to biodegradation by bacteria, it is well degraded by filamentous fungi belonging to zygomycetes (Cerniglia and Gibson, 1979) and white rot basidiomycetes (Field *et al*., 1992). Fungal oxidases have been identified among P-450 monooxygenases (Ghosh *et al*., 1983), ligninases (Haemmerli *et al*., 1986) and laccases (Collins *et al*., 1996). Laccases (EC 1-10-3-2) are copper-containing enzymes catalysing a direct oxidation mechanism of benzo[a]pyrene, but also an indirect mechanism involving the exogenous mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate (ABTS), or a putative natural mediator (Collins *et al*., 1996). Nevertheless, the metabolites formed have not been described yet.

The knowledge of all the metabolites formed from fungal metabolism is a key requirement to validate soil bioremediation. In a previous paper, we showed that laccases producing strains such as *Trametes versicolor* and *Pycnoporus cinnabarinus* were efficient degraders of PAHs in wet sand cultures mimicking soil (Rama-mercier *et al*., in press). We investigated in this study the enzymatic biotransformation of benzo[a]pyrene by laccase in bench scale reactors.

MATERIALS AND METHODS

Chemicals

Unlabelled and [7-^{14}C]benzo[a]pyrene (980 MBq mmol^{-1}), as well as ABTS, were obtained from Sigma. Radiochemical purity of the hydrocarbon was 99%.

Laccase production

The organism used for laccase production was *Pycnoporus cinnabarinus* I-938 belonging to UBCF and deposited in the Collection Nationale de Cultures de Microorganismes (Institut Pasteur, France). Inoculum was obtained from precultures grown for 10 days at 30°C in Roux flasks, which contained 200 ml synthetic medium composed of: 20 g maltose, 1.84 g diammonium tartrate, 2.3 g disodium tartrate, 1.33 g KH_2PO_4, 0.1 g $CaCl_2$ $2H_2O$, 0.5 g $MgSO_4$ $7H_2O$, 0.07 g $FeSO_4$ $7H_2O$, 0.046 g $ZnSO_4$ $7H_2O$, 0.035 g $MnSO_4$ H_2O, 0.007 g $CuSO_4$ $5H_20$, 1 g yeast extract, 1 ml vitamin solution according to Tatum et al. (1950), per litre. Mycelium from two flasks was collected, mixed with 100 ml sterile water and blended. For laccase production, 1 ml sample of this suspension was inoculated into the medium. Incubation was carried out at 30°C in 250 ml baffled Erlenmeyer flasks, containing 100 ml medium and shaken at 120 rev min^{-1} on a rotary shaker. Stimulation of the laccase production was achieved by adding 0.5 mM ferulic acid to the medium after 24 h of incubation.

Laccase purification

After 8 days of growth, the laccase-containing extracellular culture fluid was separated from the mycelium by filtration through glass wool, and then successively on glass and cellulose acetate filters with decreasing pore diameters (0.8 to 0.2 µm). Concentration of the filtrate was obtained by two successive ultrafiltrations on Amicon YM10 membranes. The enzyme concentrate was then applied on a DEAE-M ion-exchange column equilibrated with a 25 mM sodium acetate buffer pH 5. The laccase was eluted with a linear gradient of 0 to 1 M NaCl. The active fractions were pooled, concentrated and diafiltered on YM10 membrane. A second purification step involved gel filtration on Sephacryl S-400 in the same buffer. This purified enzyme solution was stored at -20°C in 30% (v/v) glycerol before use.

No lignin peroxidase or manganese peroxidase were found in the supernatants within 8 days. Purity control of the purified enzyme solution by SDS-PAGE gave a single band corresponding to a molecular mass of 74 Kda (data not shown).

Enzyme assay

Laccase activity was determined by monitoring the increase in A_{420} in 100 mM phosphate-citrate buffer pH 3, with 1 mM ABTS as a substrate (ε_{420} 36 000 M^{-1} cm^{-1}) according to Wolfenden and Wilson (1982). The enzyme activity was expressed in international units (U). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per min.

Laccase treatment of benzo[a]pyrene and analytical procedures

The reactor used for hydrocarbon biodegradation contained 2 liter 0.1 M citrate-phosphate buffer pH 3 supplemented with 1% (v/v) Tween 20, 2 mM ABTS and 0.1 mM benzo[a]pyrene comprising 50 kBq labelled chemical. The hydrocarbon was dissolved in 5 ml acetone. The reaction was started by adding 8000 U laccase and incubated at 30°C under light stirring for 24 hours with a continuous bubbling of air.

The extent of benzo[a]pyrene transformation was determined by extracting 20-ml aliquots of the medium with four fractions of 10 ml dichloromethane at the beginning and after 1, 2, 3 and 24 h of incubation. The organic extracts were dried over anhydrous Na_2SO_4, pooled, concentrated under light vacuum to dryness, and then dissolved in 8-ml acetonitrile.

HPLC analysis was performed by using both the analytical column and solvent system previously described (Mougin *et al*., 1997). Radioactivity and UV A_{254} of the column eluate were monitored.

Metabolite identification

After 24 hours of incubation, the culture medium was extracted with dichloromethane and the organic extract was concentrated to 20 ml. It was purified on silica gel 60 (0.040-0.063 mm) and eluted with ethyl acetate/cyclohexane (10/90 then 20/80, v/v).

After concentration under reduced pressure, the separated fraction were submitted to semi-preparative HPLC equipped with a diode array detector (DAD Waters 990, 190-600 nm) and an Hewlett Packard column C_{18} P/N 84176 with the similar solvent system as used above, at a flow rate of 3 ml min^{-1}. The interesting compounds were collected separately and submitted to spectroscopic analysis.

The molecular weight and mass spectra of the products were obtained by chemical ionisation (CI-MS) using NH_3 as a reactant gas, and electron impact (EI-MS, 70 eV)

analysis, using a Nermag R10-10C quadrupole spectrometer with sample introduction by direct insertion probe.

Nuclear magnetic resonance (NMR) data (^{1}H: 300 MHz, ^{13}C: 75.5 MHz) are recorded on a Varian Gemini 300 instrument. NMR spectra are obtained in $CDCl_3$.

Ultraviolet and visible spectra (190-600 nm, UV-Vis) of compounds dissolved in 95% ethanol were recorded on a Beckman BU70 spectrophotometer.

RESULTS

Transformation of benzo[a]pyrene

Benzo[a]pyrene concentration was strongly decreased within 3 hours and was less than 5% of the initial radioactivity after 24 hours incubations in the presence of purified laccase and ABTS (Figure 1). A few numbers of radioactive products were observed in the reaction mixture. The main product (called metabolite I, RT 20.5 min on the analytical column) accumulated along the incubation and corresponded to 95% of the initial radioactivity after 24 hours. A second less polar compound (metabolite II, RT 22.1 min) was formed between 1 and 2 hours of incubation and amounted to 18% of the radioactivity. It was then totally transformed (likely to metabolite I) during the following hour.

Isolation and identification of benzo[a]pyrene metabolites

The incubation medium was extracted after 24 hours incubation. Five coloured fractions were successively eluted from the concentrated extract following silica gel chromatography, and submitted to HPLC-DAD. The first eluted fraction contained residual benzo[a]pyrene. The two following fractions contained low amounts of chemicals which were poorly ^{14}C labelled. Finally, fraction 4 contained two main radioactive products (a: RT 24.5 min on the semi-preparative column; b: RT 20.7 min), whereas fraction 5 was formed from one chemical (c: RT 22.2 min) and minor components (a and b). Compound b, a and c had comparable UV-Vis spectra, and were formed approximately in a 2/1/1 ratio (based on A_{254}).

MS-CI established that the molecular weight of the three compounds was 282 (MH^+ *m/z* 283) possibly corresponding to benzo[a]pyrene quinones (Cerniglia and Gibson,

1979). EI-MS data gave important ions at m/z 282, 254 and 226 with different relative abundance according to the chemicals. The major compound b was submitted to NMR analysis. Data confirmed the presence of ten hydrogen atoms (^{1}H) and two carbonyl groups (^{13}C) in accordance with two structures: benzo[a]pyrene 1,6- or 3,6-quinones. Finally, definitive identification of a, b and c was established by UV-Vis analysis as benzo[a]pyrene 6,12-, 1,6- and 3,6-quinones, respectively (Table 1) by comparison with spectra previously reported (Haemmerli *et al*., 1986).

DISCUSSION

Our results showed that purified laccase from the white rot fungus *Pycnoporus cinnabarinus* I-938 efficiently transformed the hydrocarbon benzo[a]pyrene, as laccases from *Trametes versicolor* also did (Collins *et al*., 1996). In a similar way, the reaction required ABTS as a mediator. Spectroscopic analysis established that the pollutant was oxidised essentially to benzo[a]pyrene 1,6- 3,6- and 6,12-quinones. These metabolites have been already described by Cerniglia and Gibson (1979) and Haemmerli *et al*. (1986) who identified them in whole cultures of *Cunninghamella elegans* and incubations with purified ligninases of *Phanerochaete chrysosporium*. The isomer ratios obtained with the two oxidases (laccases and ligninases) were quite similar, suggesting the occurrence of similar mechanisms. The compound detected in the medium after 2 hours incubations could be probably 6-hydroxy-benzo[a]pyrene, rapidly oxidised further by the enzyme. Such as mechanism has been proposed with ligninases (Haemmerli *et al*., 1986) and with rat liver homogenate (Lesko *et al*., 1975).

Transformation of benzo[a]pyrene leads to the formation of quinoid free metabolites which are potentially more toxic for animals and more water-soluble than the parent compound (Sutherland, 1992). Further studies are under progress to evidence their own biodegradation pathway in liquid conditions. Yet in soils, these chemicals present another behaviour. They are substrate for microbial populations and are mineralised to carbon dioxide. They may also undergo polymerisation and become part of the humus pool (May *et al*., 1997). Accordingly, they are thought to be non-bioavailable and have lost any toxicological hazard.

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Figure 1. Transformation of benzo[a]pyrene (●) to compounds I (■) and II (σ) by purified laccase of *Pycnoporus cinnabarinus* in the presence of the mediator ABTS.

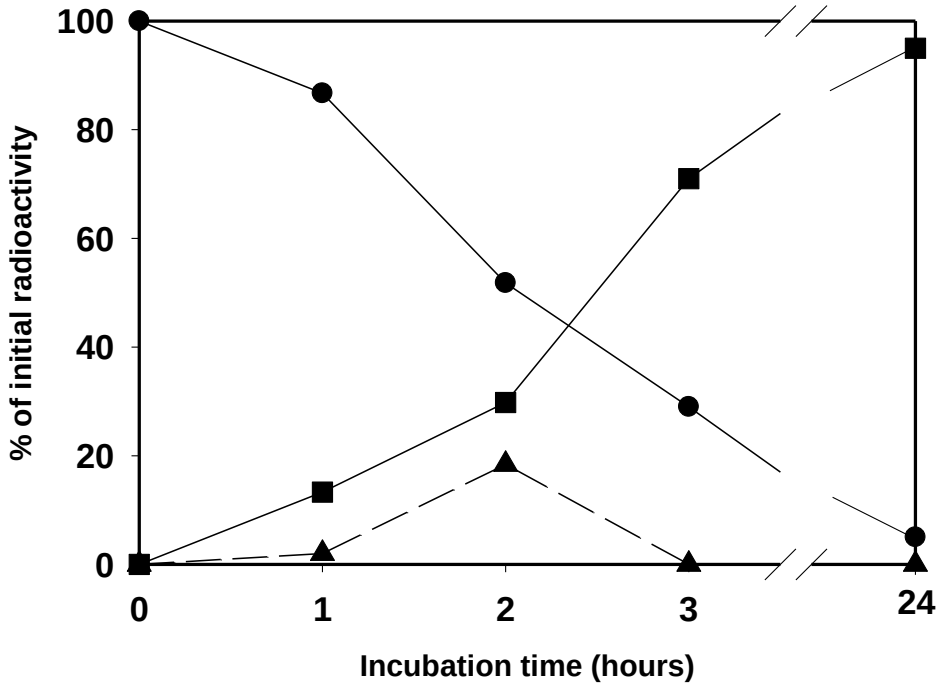

Table 1. UV-Vis absorption spectra of compounds b, c and a obtained from transformation
of benzo[a]pyrene by laccases of *Pycnoporus cinnabarinus*, and spectra of corresponding benzo[a]pyrene 1,6-, 3,6- and 6,12-quinones, respectively.

			Benzo[a]pyrene quinone		
b	c	a	1,6-	3,6-	6,12-
		λ_{max} *(nm)*			
260	287	285	261	287	284
302	315	298	300	318	298
438	332	349	440	332	350
461	469	366	464	470	366
		432			425

Data corresponding to benzo[a]pyrene quinones were from Heammerli *et al*. (1986).