Breakdown products on metabolic pathway of degradation of benz[a]anthracene by a ligninolytic fungus

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Received 29 June 2005; received in revised form 3 November 2005; accepted 3 November 2005
Available online 3 January 2006

Abstract

Cultures of the ligninolytic fungus Irpex lacteus incubated in a nutrient liquid medium degraded more than 70% of the initially applied benz[a]anthracene within 14 days. At the first step of metabolization, benz[a]anthracene was transformed via a typical pathway of ligninolytic fungi to benz[a]anthracene-7,12-dione (BaAQ). The product was further transformed by at least two ways, whereas one is complied with the anthracene metabolic pathway of I. lacteus. Benz[a]anthracene-7,12-dione was degraded to 1,2-naphthalenedicarboxylic acid and phthalic acid that was followed with production of 2-hydroxymethyl benzoic acid or monomethyl and dimethylesters of phthalic acid. Another degradation product of BaAQ was identified as 1-tetralone. Its transformation via 1,4-naphthalenedione, 1,4-naphthalenediol and 1,2,3,4-tetrahydro-1-hydroxynaphthalene resulted again in phthalic acid. None of the intermediates were identified as dead-end metabolites. Metabolites produced by ring cleavage of benz[a]anthracene using the ligninolytic fungus are firstly presented in this work.

Keywords: Biodegradation; Ring-fission; PAHs; Metabolic pathway; Irpex lacteus

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of serious environmental pollutants often possessing high mutagenic and carcinogenic potential. One way for removing PAHs from the environment is biodegradation using various types of organisms (Cerniglia, 1992; Kästner, 2000), including ligninolytic fungi (Cerniglia, 1997; Pointing, 2001; Bhatt et al., 2002). These fungi produce extracellular enzymes with low substrate specificity that enable degradation of different compounds with structures similar to lignin, including PAHs. Besides extracellular ligninolytic system, monoxygenase of cytochrom P-450 may be also involved in degradation of PAHs transforming them to hydroxysterivates or diols (Bezalel et al., 1997). Ligninolytic enzymes support a non-specific one-electron radical oxidation, producing cation radicals from PAHs followed by the appearance of quinones (Vyas et al., 1994; Bogan and Lamar, 1995). Although bacterial degradation of several PAHs has been already elucidated (e.g. Moody et al., 2004), information about fungal degradation products is scarcely available (Kästner, 2000). Using 14C-labeled compounds it was proved that ligninolytic fungi are able to mineralize PAHs completely to carbon dioxide (Bezalel et al., 1996a; Wolter et al., 1999). However, intermediates arisen from ring-cleavage reactions that were provoked by fungi are hardly described. Previously, Hammel and co-workers (1991) showed that Phanerochaete chrysosporium was able to decompose anthracene into phthalic acid that was identified as ring-fission product. Bezalel et al. (1996b) presented the mechanism of 2,2'-diphenic acid production from phenanthrene. These authors suggested that cytochrom P-450 of Pleurotus ostreatus was responsible for the attack on phenanthrene enabling further ring opening reactions. Moen and Hammel (1994) reported formation of 2,2'-diphenic acid from phenanthrene after lipi
peroxidation by manganese peroxidase. Majcherczyk and co-workers found several ring-cleavage products of ace-naphthylene and acenaphthene after incubation with laccase as well as a laccase-mediator system of *Trametes versicolor* (Johannes et al., 1998; Majcherczyk et al., 1998). In a previous study (Cajthaml et al., 2002), the degradation of phenanthrene, anthracene, fluoranthene and pyrene by *I. lacteus* provided several metabolites that pointed to ring-cleavage processes during degradation. Structures of some of the compounds suggested involvement of both enzymatic systems, P-450 and ligninolytic, respectively.

Benz[a]anthracene typically detected in soils that are contaminated with PAHs belongs to suspected carcinogens that can be activated via cytochrome P-450 monooxygenase and epoxid hydrolase with formation of biologically active products. Whilst low-weight PAHs possessing two or three aromatic rings are usually easily degradable, higher condensed PAHs including benz[a]anthracene are significantly more recalcitrant. This PAH was shown to be biodegraded by several species of ligninolytic fungi (Bogan and Lamar, 1996; Bhatt et al., 2002). However, to our knowledge, the only identified fungal metabolite was benz[a]anthracene-7,12-dione (Bogan and Lamar, 1996).

Structure elucidation of PAH-degradation products is a prerequisite for selection/application of microorganisms in remediation to avoid accumulation of dead-end metabolites or to prevent the formation of intermediates that might be even more dangerous than the parent compounds (Pothuluri et al., 1992; Belkin et al., 1994; Andersson and Henrysson, 1996).

The investigation presented provides further insight into the mechanism of benz[a]anthracene degradation by *I. lacteus* marked particularly by ring cleavage reactions. Metabolites and the degradation sequence were elucidated.

2. Materials and methods

2.1. Organism and cultivation

The ligninolytic fungus *I. lacteus* (Fr.: Fr.) Fr., strain 617/93, was grown stationary in 250 ml Erlenmeyer flasks as described elsewhere (Novotný et al., 2000). The medium (malt extract-glucose) was inoculated with 5% suspension of homogenized one-week grown mycelium. As major intermediates of benz[a]anthracene (BaA) transformation benz[a]anthracene-7,12-dione (BaAQ), 1-tetralone and 1,4-naphthalenedione were detected. These compounds were also used as substrates in separate degradation experiments. The respective compounds dissolved in 150 µl of dimethylformamide (0.5 mg per sample = 25 µg ml⁻¹ culture solution) were added into the medium at time of inoculation. However, 1,4-naphthalenedione was applied with only 0.2 mg per flask (10 µg ml⁻¹) due to its highly toxic effect towards *I. lacteus*. The study of degradation behaviour of the individual intermediates should confirm the proposed decomposition pathway of BaA. During incubation the samples were analyzed up to 49 days. The abiotic controls were one-week grown mycelia killed in an autoclave; the biotic control (non spiked parallels) was included to eliminate the possibility of natural origin of intermediates.

2.2. Chemicals

Benz[a]anthracene (99%), benz[a]anthracene-7,12-dione (97%), 1-tetralone (97%) and 1,4-naphthalenedione (97%) were used as substrates for degradation experiments and were purchased from Aldrich, Germany. Other chemicals used as standards were pure or of higher quality and were supplied by Fluka, Aldrich or Merck-Schuchardt. All solvents were purchased from Merck (Darmstadt, Germany) of p.a. quality, trace analysis quality or gradient grade.

2.3. Chemical analysis

The whole content (mycelium with medium) of each culture was homogenized with Ultraturrax and acidified to pH 2. The samples (total volume 20 ml) were then extracted five times with 10 ml portions of ethyl acetate. Afterwards the extracts were dried with sodium sulphate and concentrated using a rotary evaporator.

Samples were analyzed by gas chromatography coupled with mass spectrometry (GC–MS, GCQ, Thermo, USA). Electron impact and chemical ionization mass spectrometry as well as MS–MS techniques were used for structure elucidation. The GC instrument was equipped with split/splitless injector and a DB-5MS column. Mass spectra were recorded under electron impact at 70 eV. The excitation potential for the MS/MS product ion mode was 0.5 V, and 0.9 V. Methane was used as reagent gas for chemical ionisation (CI). The extracts were directly injected without any derivatization. Moreover, the samples were trimethylsilylated with aliquots of N,O-bis(trimethylsilyl)trifluoroacetamide (60 min, 60 °C). Sample preparation and analysis were carried out in accordance with the experiments published elsewhere (Cajthaml et al., 2002).

3. Results and discussion

*Irpex lacteus* degraded 0.5 mg/flask of benz[a]anthracene efficiently despite the compound is hardly soluble in the nutrient medium used (Fig. 1). The intermediates and products merely appeared at trace level (µg ml⁻¹) and did not show any accumulation during degradation. In all cases, excepting BaAQ, the compounds were detected below 1% of the stoichiometric concentration expected for their total degradation. The maximum BaAQ concentration corresponded to approx. 10% of transformed BaA. Therefore, additional degradation experiments of individual major intermediates were performed to obtain higher amounts of metabolites. This way proved to be useful to elucidate the sequence of BaA degradation.

Recoveries of degraded BaA and of two major metabolites, BaAQ and 1-tetralone, are shown in Fig. 1. The
values are expressed as percentage of the originally applied amount (0.5 mg per sample). Degradation results of 1,4-naphthalenedione are not shown because the compound disappeared completely from culture medium after one week (0.2 mg per sample). Cultures of I. lacteus incubated in nutrient liquid medium degraded 74% of benz[a]anthracene within 14 days. The compound was removed below the analytical detection limit between 28 and 49 days.

The fungus metabolized 73% of BaAQ and 55% of 1-tetralone, respectively within 49 days. Although the rates of the individual degradations are not comparable due to different water solubility, it is evident that BaAQ, 1-tetralone and 1,4-naphthalenedione are not dead-end products, an important fact, particularly, in case of the toxic 1,4-naphthalenedione (Yen et al., 2002).

The degradation intermediates were identified by comparing the mass spectra with data of the NIST 98 library, and independently by interpreting the fragmentation pattern. The structures of metabolites were explored using MS/MS (product ion scan) to clarify the fragmentation sequence and chemical ionization was employed to find the molecular weight of unknown metabolites. Most of the compounds were later confirmed with authentic chemical standards.

Table 1 lists retention data and mass spectral characteristics of the detected degradation products. A possible degradation sequence is given in Fig. 2. Some metabolites were confirmed by GC–MS of trimethylsilylation products: 4-hydroxy-tetralone (No. 4), phthalic acid (No. 5), phthalic acid monomethylester (No. 6). Phthalic acid, 1,2-naphthalenedicarboxylic acid and 2-hydroxymethyl benzoic acid (No. 7) were detected without derivatization as dehydrated forms, i.e. anhydrides and lactone, respectively. The structures signed with asterisks were later confirmed with respective chemical standards. The detailed MS/MS characteristic of 1,2-naphthalenedicarboxylic acid was published in our previous work (Cajthaml et al., 2002).

The structural suggestion of the metabolite 4-hydroxy-1-

Table 1
Retention data and electron impact mass spectral characteristics of BaA metabolites

<table>
<thead>
<tr>
<th>Product no.</th>
<th>t_R (min)</th>
<th>MW (CI)</th>
<th>m/z of fragment ions (relative intensity)</th>
<th>Structural suggestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.81</td>
<td>258</td>
<td>258 (100), 230 (41), 202 (47.2), 174 (4.5), 150 (4.6)</td>
<td>Benz[a]anthracene-7,12-dione^a</td>
</tr>
<tr>
<td>2</td>
<td>7.43</td>
<td>146</td>
<td>146 (75.9), 131 (13), 118 (100), 104 (3.4), 90 (26.9), 77 (8.7)</td>
<td>1-Tetralone^a</td>
</tr>
<tr>
<td>3</td>
<td>7.14</td>
<td>148</td>
<td>148 (8.4), 147 (20.1), 130 (100), 119 (42.4), 105 (20.2), 91 (25), 77 (5.9)</td>
<td>1,2,3,4-Tetrahydro-1-hydroxynaphthalene^a</td>
</tr>
<tr>
<td>4</td>
<td>9.34</td>
<td>162</td>
<td>162 (18.6), 145 (17), 134 (68.2), 115 (15.8), 105 (100), 77 (24.1), 51 (15.8)</td>
<td>4-Hydroxy-1-tetralone</td>
</tr>
<tr>
<td>5</td>
<td>13.15</td>
<td>234</td>
<td>219 (100), 189 (8.8), 115 (10.2)</td>
<td>4-Hydroxy-1-tetralone-TMS</td>
</tr>
<tr>
<td>6</td>
<td>6.74</td>
<td>148</td>
<td>148 (2.3), 104 (100), 76 (41.2), 50 (20.4)</td>
<td>Phthalic anhydride^a,b</td>
</tr>
<tr>
<td>7</td>
<td>10.23</td>
<td>310</td>
<td>310 (3.7), 295 (57.6), 265 (6.4), 221 (27.5), 193 (3.8), 147 (100), 73 (53.1)</td>
<td>Phthalic acid di-TMS^a</td>
</tr>
<tr>
<td>8</td>
<td>8.95</td>
<td>180</td>
<td>163 (15.4), 149 (60.7), 136 (14.2), 104 (100), 92 (19.5), 76 (96.7)</td>
<td>Monomethyl phthalic acid^a</td>
</tr>
<tr>
<td>9</td>
<td>9.15</td>
<td>252</td>
<td>252 (2.2), 237 (100), 163 (50), 133 (7.5), 89 (77.7)</td>
<td>Monomethyl phthalic acid-TMS^a</td>
</tr>
<tr>
<td>10</td>
<td>7.07</td>
<td>134</td>
<td>134 (12.7), 105 (100), 77 (40.9), 51 (9.0)</td>
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</tr>
<tr>
<td>11</td>
<td>7.89</td>
<td>194</td>
<td>194 (3.0), 163 (100), 133 (15.8), 77 (9.7)</td>
<td>Dimethyl phthalic acid^a</td>
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<td>12</td>
<td>7.78</td>
<td>158</td>
<td>158 (100), 130 (38.9), 104 (62.9), 102 (60.5), 76 (45.1)</td>
<td>Naphthalene-1,4-dione^a</td>
</tr>
<tr>
<td>13</td>
<td>8.35</td>
<td>160</td>
<td>160 (85.4), 131 (21.5), 104 (100), 76 (41.5)</td>
<td>1,4-Dihydroxynaphthalene^a</td>
</tr>
<tr>
<td>14</td>
<td>12.15</td>
<td>198</td>
<td>198 (80.1), 154 (87.9), 126 (100)</td>
<td>1,2-Naphthalic anhydride^a</td>
</tr>
</tbody>
</table>

^a Structures were later identified with authentic standard.
^b Dehydrated form of the metabolite.
tetralone (No. 4) was not quite unambiguous, but the position of hydroxyl group proposed fits the presented pathway.

Most of the intermediates were found directly after degradation of BaA, excepting 1,2,3,4-tetrahydro-1-hydroxynaphthalene and 1,4-naphthalenediol (Nos. 3 and 10) that were detected only after degradation of BaAQ (No. 1) and 1-tetralone (No. 2), respectively. The brackets with arrows indicate that the precursor of the products is not absolutely clear defined. None of the metabolites were detected neither in heat-killed (abiotic) nor in biotic controls.

Benz[a]anthracene was metabolized by I. lacteus via typical pathway of ligninolytic fungi (Cerniglia, 1992; Vyas et al., 1994), when the product (BaAQ) was further transformed by at least two ways. One of the pathways is similar to anthracene metabolic pathway (Hammel et al., 1991; Cajthaml et al., 2002). Benz[a]anthracene-7,12-dione (No. 1) was degraded to phthalic acid (No. 5) that was followed with production of 2-hydroxymethyl benzoic acid (No. 7) or phthalic acid monomethyl- and dimethylesters (Nos. 6 and 8). Products of methylation by I. lacteus (Nos. 6 and 8) were found during degradation of BaA in accordance with our previous studies (Cajthaml et al., 2002). Probably, the availability of a methylating system prevents futile redox cycling of peroxidases and allows oxidation of the aromatic ring (Harvey and Palmer, 1990). On the other hand, methylation decreases water solubility of potentially toxic compounds thus, it can be also assumed as an auto-protection mechanism of the organism.

The appearance of 1-tetralone (No. 2) could be a result of a complementary or completely different pathway. This compound is either a transformation product of a residuum after phthalic acid split off or a result of 1,2-naphthalenetricharboxylic acid degradation.

Degradation of 1-tetralone resulted into 1,2,3,4-tetrahydro-1-naphthalenol (No. 3), phthalic acid (No. 5) and 1,4-naphthalenedione (No. 9). 1,4-Naphthalenedione was further hydrogenated to 1,4-dihydroxynaphthalene (No. 10) and this intermediate was probably a precursor of 4-hydroxy-1-tetralone (No. 4). The same compound was identified after degradation of 1,4-naphthalenedione by the fungus Cunninghamella elegans (Sutherland, 1992). Metabolites Nos. 3, 10 and 4 are clearly formed by...
hydrogenation whereas intracellular quinone oxidoreductase could take a role especially in the hydrogenation of 1,4-naphthalenedione (Buswell et al., 1979).

4. Conclusion

This investigation advances the knowledge about degradation of PAHs such as benz[a]anthracene by ligninolytic fungi. The studied fungal species I. lacteus is able to degrade benz[a]anthracene partially via a pathway similar to anthracene. The product benz[a]anthracene-7,12-dione can be degraded via two ways, once to phthalic acid pathway and twice into 1,2-naphthalenedicarboxylic acid. Another metabolite, 1-tetralone, probably arising from 1,2-naphthalenedicarboxylic acid, is transformed into several two-ring intermediates and gives phthalic acid as well. None of the detected intermediates were accumulated as a dead-end metabolite. Several new metabolites of benz[a]anthracene biodegradation using a ligninolytic fungus could be identified. Furthermore, this work presents a rather detailed pathway for biodegradation of a recalcitrant and potentially hazardous polycyclic aromatic hydrocarbon, benz[a]anthracene, including specific ring cleavage strategies.

Acknowledgement

This work was supported by grant KJB6020308 of the Grant Agency of the Academy of Science of the Czech Republic and by Institutional Research Concept No. AV0Z50200510.

References