Abstract  Hemoglobin (Hb) is a member of heme-protein that can perform catalytic non-specific chain reaction in the presence of hydrogen peroxide (H$_2$O$_2$). Catalytic ability of Hb to degrade pyrene was demonstrated using soil contaminated with $^{14}$C pyrene and 10 mg pyrene/kg soil. The composition of soil was similar to previously used soil except that it had lower organic carbon content. Bench scale laboratory tests were conducted in the presence of buffer only, H$_2$O$_2$ only, or Hb with H$_2$O$_2$ for 24 h. After 24 h reaction, 0.1 and 1.3% of $^{14}$C pyrene in contaminated soil were mineralized with H$_2$O$_2$ only or Hb plus H$_2$O$_2$. No mineralization to $^{14}$CO$_2$ was detected with buffer only. Approximately 12.2% of pyrene was degraded in the presence of H$_2$O$_2$ only while 44.0% of pyrene was degraded in the presence of Hb plus H$_2$O$_2$ during 24 h of catalytic reaction. When degradation intermediate products were examined, two chemicals were observed in the presence of H$_2$O$_2$ only while 25 chemicals were found in the presence of Hb plus H$_2$O$_2$. While most degradation products were simple hydrocarbons, four of the 27 chemicals had aromatic rings. However, none of these four chemicals was structurally related to pyrene. These results suggest that Hb catalytic system could be used to treat pyrene-contaminated soil as an efficient and speedy remediation technology. In addition, intermediate products generated by this system are not greatly affected by composition change in soil organic matter content.

Keywords  Hemoglobin · Hydrogen peroxide · Mass balance · Pyrene · Remediation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organopollutants that do not break down easily in the environment. PAHs are very hydrophobic. Thus, they are adsorbed onto non-aqueous entity of soil particles [1-2]. In addition, PAHs are composed of chemicals that are hard to be bioremediated. Among various means of remediation, bioremediation via microbes could be performed economically to decontaminate PAHs in soil [3-4]. For example, white rot fungus Phanerochaete chrysosporium can transform PAHs using various enzymes including extracellular enzymes. However, degradation of PAHs with over four benzene rings cannot be easily achieved by such treatments, leading to their persistence in the environment [5-6].

Among various PAHs, pyrene with four aromatic rings is one of the most persistent PAHs in the environment. Although it is not genotoxic, it is quite carcinogenic. It has been commonly used as an indicator to monitor PAHs contaminated soil [7]. Chemical and biological processes or technologies are nowadays preferred means for remediation of PAHs-contaminated sites [8-12].

Hemoglobin (Hb) can efficiently oxidize organic chemicals such as PAHs. It is known that Hb can acts as a biocatalyst, similar to lignin peroxidases and other heme-containing peroxidases [11-12]. Thus, organic radicals are generated during the catalytic cycle of Hb in the presence of H$_2$O$_2$. These organic radicals might participate in chemical reactions such as oxidation and polymerization, during which various intermediate products can be formed during detoxification of contaminants [10-15]. In our recent study with Hb-catalyzed reaction, when pyrene in liquid solution is oxidatively removed, hydrophilic degradation intermediates are formed [15]. Thus, it is important to show that intermediates formed during the degradation process are less toxic or non-toxic.
The objective of this study was to investigate intermediate products during degradation of pyrene in soil by hemoglobin-catalyzed reaction. Especially, we aimed to examine degradation intermediates in another loam soil with lower organic carbon content than that used in our previous study [17].

Materials and Methods

Soil preparation
We collected loam soil from Mt. Eunbong in Hongchun of Kwangwon, Korea. In our previous study, we have confirmed that the area is not contaminated with organopollutant such as PAHs [17]. The property of the soil was found to be a sandy clay loam with 45% sand, 26% silt, 29% clay, organic carbon content of 9.9%, and water holding capacity of 50%. The soil was treated and prepared as described previously [17].

Soil phase experiment
The prepared soil (2 g) described above received 1 mL of pyrene stock solution (20 mg pyrene/L of acetonitrile) and 0.5 mL of 14C pyrene. Pyrene stock solution was made with pyrene from AccuStandard (New Haven, CT, USA). The contaminated soil was then air-dried in the dark for 1 day. Initial pyrene concentration in soil was found to be 10.0 mg/kg by LS-6500 liquid scintillation counter (Beckman) as described previously [17]. After treatment, 50% water holding capacity was kept. Three trials were performed for every sample. Student’s t test was used to compare differences between samples.

Degradation of pyrene by hemoglobin-catalyzed reaction
The extent of 14CO2 evolution during treatment was measured by LS-6500 liquid scintillation counter (Beckman) as described previously [13-14]. After 14CO2 evolution experiment, 14C remaining in soil samples was extracted with dichloromethane for 24 h using Soxhlet procedure. The extracted solvent was concentrated to be solvent-exchanged with acetonitrile. The sample solvent was then concentrated to have a final volume of 2 mL for further analysis as follows. One mL of 2 mL solvent sample was used to measure 14C extent of sample solvent by LS-6500 liquid scintillation counter. For the remaining soil after Soxhlet procedure, 14C extent of solvent non-extractable minerals was determined [11,17].

Pyrene degradation intermediates
The other 1 mL of 2 mL sample solvent was subjected to HPLC. A part of HPLC eluent was used to measure the extent of radioactivity with a Beckman LS-6500 liquid scintillation counter [13,14]. The other part of HPLC eluent was extracted with hexane to be concentrated to 1 mL with a solvent concentrator. Mixture of the resulting 1 mL plus additional 15 mL acetonitrile was concentrated again to have a final volume of 200 μL which was subject to gas chromatography mass spectrometry (GC/MS) analysis to obtain information of pyrene and its degradation intermediates as described previously [17].

Results and Discussion

Along with two controls, the treated soil received 10 mg pyrene/kg of soil and 14C pyrene, after which it was subjected to treatment. Change in extent of 14CO2 evolution for 24 h was measured for the two controls and soil treated with Hb and H2O2 (i.e., treatment) (Table 1). Control with buffer only showed no detectable 14CO2 evolution. Control with H2O2 only showed 0.1% of 14CO2 evolution. However, the soil treated with Hb and H2O2 had about 1.3% mineralization after 24 h. In our previous study, we have shown that mineralization to 14CO2 is not a major degradation reaction for soil treated with Hb and H2O2 [11,17]. To further trace down the fate of pyrene, mass balance study was performed by observing the extent of 14C distribution among solvent and soil (Table 1). Extent of total 14C radioactivity recovery for the soil treated with Hb plus H2O2 (i.e., Hb-dependent catalytic reaction) was 67.9%, among which 66.2 and 1.6% of radioactivity were found in acetonitrile solvent and soil phase, respectively. Thus, the total recovery for the soil treated with Hb and H2O2 was 69.1%, including 14CO2 gas phase. Total recoveries of 14C radioactivity in the two controls were over 85.9 and 81.1% for control with buffer only and control with H2O2 only, respectively. Extent of total 14C recovery decreased in the order of control with buffer only, control with H2O2 only, and soil treated with Hb and

| Treatment | Extent (%) of radioactivity in | Soil | 14CO2 | Total |
|-----------|-------------------------------|------|-------|-------|
| Buffer only | 85.5±8.4 | 0.4±0.0 | 0.0±0.0 | 85.9 |
| H2O2 only | 80.4±3.7 | 0.6±0.1 | 0.1±0.0 | 81.1 |
| Hb + H2O2 | 66.2±2.6 | 1.6±0.0 | 1.3±0.1 | 69.1 |

*Initially added radioactivity of 14C pyrene was about 5,500,000 dpm.
**Acetonitrile
H$_2$O$_2$ (Hb-dependent catalytic reaction). This result suggests that most pyrene and its degradation products in soil could be extractable with acetonitrile after 24 h of treatment with Hb-dependent catalytic reaction. This kind of trend has been observed in our previous study [14]. We expected that pyrene, a carcinogenic component of PAHs, might be rapidly degraded by Hb-dependent catalytic reaction. Upon treatment with Hb and H$_2$O$_2$, pyrene concentration was decreased from 10.0±0.2 to 6.6±0.7 mg/kg soil, resulting in 44.0% of pyrene degradation in soil after 24 h. In contrast, about 12.2% of pyrene was degraded for control with H$_2$O$_2$ only (Fig. 1). No degradation was observed for control with buffer only. These results indicate that treatment with Hb and H$_2$O$_2$ can be employed to remediate pyrene in soil and possibly other PAHs-contaminated soil. Of note, the catalytic activity of treatment with Hb and H$_2$O$_2$ is due to hemoglobin heme group that reacts with H$_2$O$_2$ to complete the catalytic cycle [10-14].

Acetonitrile solvent fractions from three different treatments were analyzed to observe pyrene and its degradation products (Fig. 2). HPLC eluent was collected every 1 min to measure the extent and distribution of radioactivity due to pyrene and its degradation products. Variations in the extent of absorbance and radioactivity for three treatments are shown in Fig. 2. Pyrene peaks at 13-14 min matched with radioactivity peak at 13-14 min. The size of pyrene peaks decreased in the sequence of control with buffer only, control with H$_2$O$_2$ only, and treatment with Hb and H$_2$O$_2$ (Hb-dependent catalytic reaction). We expected that degradation

![Fig. 1 Pyrene concentration after 24 h of hemoglobin catalytic reaction under different experimental conditions](image)

![Fig. 2 HPLC chromatogram of acetonitrile extract after pyrene treatment with buffer only, H$_2$O$_2$ only, and with Hb plus H$_2$O$_2$](image)

| GS/MS analyses of pyrene degradation products for control with H$_2$O$_2$ |
|---|
| HPLC fraction time (min) | Compound name | Chemical structure | Chemical formula | Major ion (m/z) |
| --- | ----- | --- | --- | --- |
| 5 | 5-Methyl-1-phenyl-4-hydrocyclopenta[3,2-b]pyrrole | ![chemical structure image] | C$_{14}$H$_{13}$N | 195 |
| 13 | Tetradecane | ![chemical structure image] | C$_{14}$H$_{29}$ | 57 |
intermediates would appear before pyrene peaks at 13 min because intermediates are supposed to be less hydrophobic than pyrene. Some HPLC fractions having peaks of absorbance (254 nm) and $^{14}$C radioactivity were observed before 13 min for both control with H$_2$O$_2$ only and treatment with Hb and H$_2$O$_2$ (Fig. 2). It is currently unclear whether these two kinds peaks coincide due to complexity of the chromatogram.

We intended to examine pyrene degradation intermediate products in another loam soil with lower organic carbon content than that used in our previous study during Hb-catalyzed reaction. To identify what kind of degradation intermediates were produced for control with H$_2$O$_2$ only and treatment with Hb and H$_2$O$_2$, HPLC eluents were collected each min and analyzed by GC/MS to examine differentially specific chemicals produced for each treatment. In case of control with Hb only, no specific degradation product was observed. Table 2 reveals that two chemicals were observed at 5 and 13 min for control with H$_2$O$_2$ only. Table 3 revealed that 18 chemicals of mostly low molecular weight were observed until 12 min. Some chemicals of high molecular weight were found at 13 min. Table 3 revealed that 4 of 25 compounds had aromatic ring during Hb-dependent catalytic reaction. Possible toxicity of these four aromatic chemicals was examined. However, none of these chemicals had noticeable carcinogenicity after we searched the database of toxic chemicals (SHEDS models, US EPA, Washington DC, USA). Additionally, these four chemicals with aromatic ring have no structural characteristics of pyrene, suggesting that they might not be directly derived from pyrene. Other chemicals are aliphatic hydrocarbons that might not

| HPLC fraction time (min) | Compound name                  | Chemical structure | Chemical formula | Major ion (m/z) |
|-------------------------|---------------------------------|--------------------|-----------------|---------------|
| 3                       | 3,6-Dimethyldecane              | C$_{12}$H$_{26}$    | 57              |
| 3                       | Tridecane                      | C$_{13}$H$_{28}$    | 57              |
| 3                       | Pentadecane                    | C$_{15}$H$_{32}$    | 57              |
| 3                       | 1-Nonadecene                   | C$_{19}$H$_{38}$    | 57              |
| 3                       | 2-Methyl-octadecane            | C$_{19}$H$_{40}$    | 57              |
| 3                       | 2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene | C$_{25}$H$_{42}$    | 69              |
| 3                       | Carbonic acid, eicosyl vinyl ester | C$_{23}$H$_{44}$O$_{3}$ | 57      |
| 4                       | Cetene                         | C$_{16}$H$_{32}$    | 41              |
| 4                       | 1,2-Benzenedicarboxylic acid, dibutyl ester | C$_{16}$H$_{22}$O$_{4}$ | 149             |
| 4                       | Tricosane                      | C$_{23}$H$_{44}$    | 57              |
| 5                       | 1-Octadecene                   | C$_{19}$H$_{38}$    | 43              |
| 5                       | 7,9-Dimethylhexadecane         | C$_{19}$H$_{38}$    | 57              |
| 6                       | 1-Octadecanol                  | C$_{19}$H$_{40}$O$_{2}$ | 83           |
| 9                       | 2-Methyloctacosane             | C$_{20}$H$_{40}$    | 57              |
be toxic in general, especially at low concentration, indicating that there might be almost no toxic chemicals or residues remaining after treatment with Hb and H$_2$O$_2$. All these results are contrasted with previous studies in which numerous degradation intermediates of PAHs were observed [2,16]. Four additional chemicals such as 1-octadecanol, 2-methyloctacosane, pentatriacontane, and phthalic acid butyl hexyl ester were observed in the present intermediate analysis compared with previous analysis [17]. Of note, the two soils used for this present and previous studies had very similar soil texture, although they were different in organic content. Overall, only limited difference was observed in the number of observed chemicals possibly due to the difference in organic carbon content. Here, we found that a little bit more number and kind of organic intermediates were generated from soil with lower organic carbon content, possibly less interaction of reactive intermediates with organic matter.

Fenton reaction could be performed in aqueous systems as well as soil [18]. Hb-catalytic system could also be employed for degradation of pyrene in aqueous system and soil as shown in our previous study [15,17]. We also tried to investigate degradation product in soil with much lower organic content (i.e., <10%). However, it is very difficult to obtain soil with similar composition but with much less organic content. We also expect that different soil compositions might affect the process of organopollutants. Regardless of organic content degradation, it is of some importance to have information on the kind of degradation intermediates because we need to have information on whether any hazardous intermediates are generated during the degradation process.

Table 3 Continued

| HPLC fraction time (min) | Compound name | Chemical structure | Chemical formula | Major ion (m/z) |
|-------------------------|---------------|--------------------|-----------------|----------------|
| 12                      | 2-Methyldodecane | ![Image](image1.png) | C$_{12}$H$_{25}$ | 43             |
| 12                      | 4,6-Dimethyldodecane | ![Image](image2.png) | C$_{14}$H$_{30}$ | 57             |
| 12                      | 1-Pentadecene   | ![Image](image3.png) | C$_{15}$H$_{30}$ | 41             |
| 12                      | Pentatriacontane | ![Image](image4.png) | C$_{35}$H$_{72}$ | 57             |
| 12                      | Phosphonic acid, dioctadecyl ester | ![Image](image5.png) | C$_{36}$H$_{75}$O$_3$P | 55             |
| 13                      | 2-Bromododecane | ![Image](image6.png) | C$_{12}$H$_{25}$Br | 57             |
| 13                      | 2,6,10,14-Tetramethylpentadecane | ![Image](image7.png) | C$_{26}$H$_{50}$ | 57             |
| 13                      | 1,2-Benzenedicarboxylic acid, dioctyl ester | ![Image](image8.png) | C$_{26}$H$_{50}$O$_4$ | 149            |
| 13                      | Iron, tricarbonyl[N-(phenyl-2-pyridinylmethylene)benzenamine-N, N’]- | ![Image](image9.png) | C$_{34}$H$_{36}$FeN$_2$O$_5$ | 57             |
| 13                      | Tetratetracontane | ![Image](image10.png) | C$_{44}$H$_{90}$ | 57             |
| 13                      | Phthalic acid, butyl hexyl ester | ![Image](image11.png) | C$_{38}$H$_{60}$O$_4$ | 149            |
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