Detection and identification of dyes showing AhR–binding affinity in treated sewage effluents

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Abstract A bioassay using the YCM3 recombinant yeast strain was utilised to investigate the presence of dioxin-like compounds that activate the aryl hydrocarbon receptor (AhR) in treated sewage effluents. AhR ligand activity was detected in the concentrated extracts of effluent samples collected in March, June and October 2004 from Kyoto city, Japan. HPLC fractionation was carried out using C18 reversed-phase columns, and possible AhR ligands were further isolated and purified. By using LC/MS/MS, one weak AhR ligand was identified to be rhodamine B base, a fluoran dye. In addition, two other coloured ligands were postulated to be disperse anthraquinone dyes or their metabolites because of their UV spectra and HPLC retention times. The AhR-binding affinities of 12 commercial dyes with different chemical structures were also studied. Among the dyes tested, hydrophobic anthraquinone dyes exhibited higher AhR ligand activity, but azo dyes or hydrophilic acid dyes showed no or very low AhR ligand activity. Rhodamine B base and disperse anthraquinone dyes were suggested to be potential xenobiotic AhR ligands. Future research regarding their contamination in aquatic environments and toxicological information is necessary.
Keywords Aryl hydrocarbon receptor; dyes; treated sewage effluents

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Introduction

Dyes are made to be resistant to many physical or chemical reactions, such as light, heat, acid and so forth. Some dyes, dye metabolites and dye plant effluents have been reported to be toxic, carcinogenic or mutagenic (Hildenbrand *et al.*, 1999; Gottlieb *et al.*, 2003; Moawad *et al.*, 2003; Schneider *et al.*, 2004; Umbuzeiro *et al.*, 2004), but conventional biological treatment processes are incapable of treating many refractory dyes satisfactorily (Hutton *et al.*, 1996). Advanced chemical treatment processes may show better efficiency in removing colour or COD of dyeing wastewater. However, mutagens converted from dyes by chlorination have been detected in river water (Nukaya *et al.*, 1997; Shiozawa *et al.*, 1998), and ozonation has also been indicated to increase the toxicity in synthetic dye wastewater (Hitchcock *et al.*, 1998; Kunz *et al.*, 2002).

In Kyoto city, there are many dyeing factories and their wastewaters are treated by sewage treatment plants. Treated sewage effluents are discharged into the Yodo River system, in which 2-phenylbenzotriazole-type mutagens generated from azo dyes and oestrogenic contaminants have been detected (Nukaya *et al.*, 1997; Oguri *et al.*, 1998; Kawanishi *et al.*, 2004). Since the Yodo River system serves as the major drinking water source for residents living in the nearby area, monitoring of pollutants discharged into the river system is important to provide basic information for hazard assessment and reduction. In this study, dioxin-like compounds were chosen as the target substances for investigation. It has been indicated that the toxic and biological effects of dioxins are mediated by the aryl hydrocarbon receptor (AhR) (Denison and Heath-Pagliuso, 1998). Thus, an AhR-dependent yeast bioassay (Miller, 1999) in combination wih HPLC

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fractionation was used as a screening tool to detect the presence of dioxin-like compounds (hereafter referred to as AhR ligands) in the treated sewage effluents from Kyoto city. Rhodamine B base, a fluoran dye that showed weak AhR binding affinity, was isolated and identified by LC/MS/MS. Two other coloured ligands were postulated to be disperse anthraquinone dyes or their metabolites based on their UV spectra and HPLC retention times. In addition to effluent samples, three acid dyes, one basic dye, one direct dye, five disperse dyes and two reactive dyes were also investigated for their AhR-binding affinities, and hydrophobic anthraquinone dyes such as Disperse Blue 56 were suggested to be potential xenobiotic AhR ligands.

Materials and methods

Materials

Rhodamine B base was purchased from Sigma-Aldrich (USA). Other commercial dyes were provided by Nippon Kayaku (Japan) and used without purification. Sep-pak C18 environmental cartridges were purchased from Waters (USA). Blue rayon was purchased from Funakoshi (Japan) or synthesised as described (Hayatsu, 1992).

Sampling and extraction

Treated effluents of one sewage treatment plant located in Kyoto city, Japan, were collected in March, June and October 2004. Two litres of effluent samples were filtered on 0.45-μm glass fibre filters, and passed through Sep-pak C18 environmental cartridges. After extraction, each cartridge was washed with 10 mL of water and then eluted with 10 mL of methanol. The eluents were combined, evaporated to dryness, redissolved in 400 μL of dimethyl sulfoxide (DMSO), and then subjected to the yeast bioassay.

In order to collect a sufficient quantity of target compounds for chemical identification analysis, 20 bags (5 g/bag, 100 g in total) of blue rayon were put in a net and hung in the sampling site for approximately 24 h in June and October 2004. The extraction method was similar to that described in Nukaya *et al.* (1997). Blue rayon was washed with water, dried by paper towels, and then stirred in 3 L of methanol:ammonia water (50:1, v/v) for 3 h at least two times. The extracts were evaporated to dryness and redissolved in DMSO. Blue rayon extracts were subjected to HPLC fractionation for isolating potential AhR ligands.

Yeast bioassay

AhR ligand activity was measured by a yeast bioassay using the YCM3 recombinant yeast strain, a yeast containing the human AhR and aryl hydrocarbon receptor nuclear translocator (Arnt) expression construct, with a pTXRE5-Z (*LacZ*) reporter plasmid responding to ligand-induced AhR complexes (Miller, 1999). The bioassay was carried out as described in Adachi *et al.* (2001). Briefly, the yeast was grown in a synthetic glucose medium lacking tryptophan at 30 °C. After 14–18 h, 1 μL of test sample was mixed with 5 μL of the saturated culture and 200 μL of the synthetic galactose medium, and subsequently incubated at 30 °C. Cell density was determined by reading the absorbance at 595 nm after 18 h of incubation. In order to start the reaction, 10 μL of the cell suspension was mixed with 140 μL of Z-buffer and 50 μL of *O*-nitrophenyl-β-D-galactopyranoside (4 mg/mL solution made in Z-buffer), and the absorbance at 405 nm was read after incubating at 37 °C for 60 min. The β-galactosidase activity (reported as *LacZ* units) was calculated as the following formula: (absorbance at 405 nm)/(absorbance at 595 nm × mL of cell suspension added × minutes of reaction time). An equivalent concentration of β-naphthoflavone (β-NF) was calculated from a dose-response curve obtained from the bioassay.

HPLC fractionation, ligand isolation and purification

Fifty microlitres of the Sep-pak cartridge extract were injected into a C18 HPLC reversed-phase column (Shim-pack FC-ODS, 150 × 4.6 mm, Shimadzu, Japan), and eluted in a linear gradient of 10–100% methanol in water within 20 min followed by 100% methanol held for another 20 min at a flow rate of 1 mL/min. Fractions were collected every minute for 30 min, evaporated to dryness, redissolved in 50 μL of DMSO and then subjected to the yeast bioassay. The blue rayon extracts were fractionated by another reversed-phase column (Wakosil-II 5C18HG, 20 × 50 mm, Wako, Japan) with a linear gradient of 75–100% methanol in water within 5 min followed by 100% methanol held for another 25 min at a flow rate of 2.5 mL/min. Fractions were collected every 30 sec from 7 to 13 min, and potential AhR ligands were further isolated by both of the HPLC columns. All HPLC experiments were undertaken at the ambient temperature.

HPLC/ESI-MS/MS

Experiments were carried out using a Micromass Quattro Ultima Pt mass spectrometer (Waters, USA) equipped with a Shim-pack FC-ODS column eluted in an isocratic mode with 100% methanol at a flow rate of 0.5 mL/min. Nitrogen was used as the sheath gas; desolvation gas flow rate was set at 700 L/h and desolvation gas temperature was 380 °C. The ion source temperature was 130 °C. The capillary voltage was set at 3.5 kV, and the cone voltage was 35 V. The collision energy was 25 eV for the compounds tested. Data acquisition was performed in positive ion mode.

Results and discussion

Detection of AhR ligand activity

AhR ligand activity was detected in the concentrated extracts of treated sewage effluents collected in different months. In order to isolate potential AhR ligands, HPLC fractionation was carried out to reduce the complexity of extracts. Figure 1 shows the HPLC chromatogram (absorption wavelength: 540 nm) and the corresponding AhR ligand activity of HPLC fractions of the effluent sample collected in October 2004. Higher AhR ligand activity was detected in the 23rd, 24th, and 25th fractions (fractions collected from 22 to 25 min), and the 23rd fraction elicited the highest activity. A similar pattern was also observed in the effluent samples collected in March and June 2004 (data not shown). According to the HPLC chromatogram, one major peak, named as Red 1 according to its colour, was detected in the 23rd

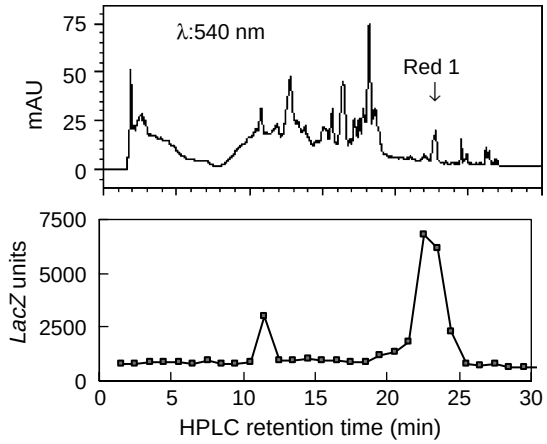

Figure 1 HPLC chromatogram and the AhR ligand activity of fractions of the 5,000-fold Sep-pak cartridge extract

fraction and several small peaks were observed in the 24th and 25th fractions. The UV spectrum and HPLC retention time of Red 1 is shown in Figure 2.

Isolation and identification of dyes as potential AhR ligands

Blue rayon, which can selectively adsorb aromatic compounds having three or more fused rings (Hayatsu, 1992), was used to collect target compounds for chemical identification analysis. In this study, 100 g of blue rayon was capable of collecting 500–1,000 L equivalent AhR ligands in the treated sewage effluents. Red 1 was isolated from the blue rayon extract and then subjected to LC/MS/MS analysis. According to the HPLC retention time, UV, MS and MS/MS spectra, Red 1 was confirmed to be rhodamine B base, a solvent fluoran dye used as the colorant in ballpoint pen inks and so forth (Green, 1991). Its MS/MS spectrum and chemical structure are shown in Figure 3.

Although rhodamine B base was the major peak in the 23rd fraction, it only showed weak AhR-binding affinity in the yeast bioassay (Figure 4). Therefore, other compounds in the same fraction were investigated by further separating the fraction into three subfractions, subfraction 1 (SF-1, which contained the compounds having the retention time earlier than rhodamine B base), subfraction 2 (SF-2, which contained rhodamine B base) and subfraction 3 (SF-3, which contained the compounds having the retention time later than rhodamine B base). As shown in Figure 5, the AhR ligand activity of the concentrated subfractions SF-1 and SF-3 were higher, and SF-3 elicited the highest activity. It can be suggested that the AhR ligand activity of the 23rd fraction was mostly contributed by the compounds contained in these two subfractions. The UV spectra of the

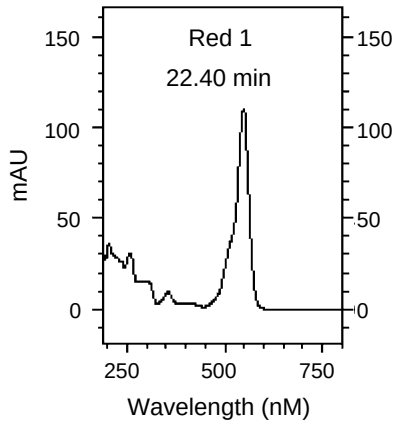

Figure 2 UV spectrum and HPLC retention time of Red 1

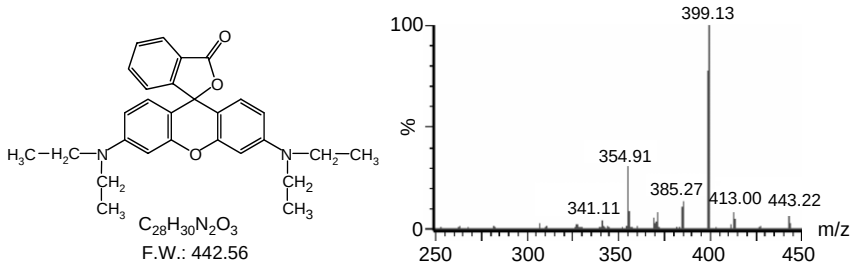

Figure 3 The chemical structure and MS/MS spectrum of rhodamine B base

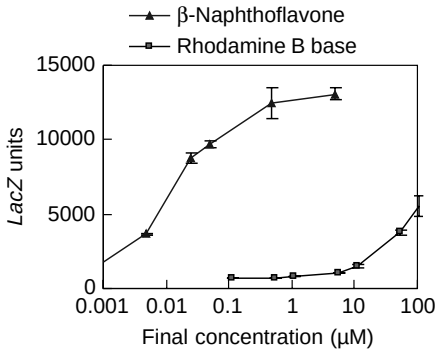

Figure 4 AhR ligand activity of rhodamine B base and an archetypal AhR ligand, β-naphthoflavone

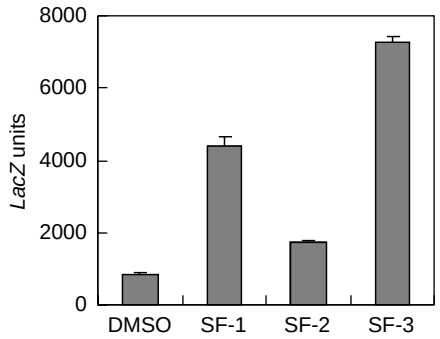

Figure 5 AhR ligand activity of the subfractions of the 23rd fraction

main compounds in SF-1 and SF-3 are shown in Figure 6. The identification of these compounds was not undertaken in this study.

Two coloured ligands named as Red 2 and Violet 1 were isolated from the 25th Fraction. Figure 7 shows the UV spectra and HPLC retention times of Red 2, Violet 1, compared with two commercial disperse anthraquinone dyes, Disperse Red 164 and Disperse Blue 56. The HPLC retention times of Red 2 and Violet 1 were close to those of the disperse anthraquinone dyes, and their UV spectra were also similar. Therefore, Red 2 and Violet 1 were postulated to be disperse anthraquinone dyes or dye metabolites. Their MS spectra showed the $[M + H]^+$ ions at m/z: 497 and 432, respectively.

AhR-binding affinity of commercial dyes

Twelve commercial dyes (Table 1) with different chemical structures were investigated for their AhR-binding affinities by using the yeast bioassay. As shown in Figure 8,

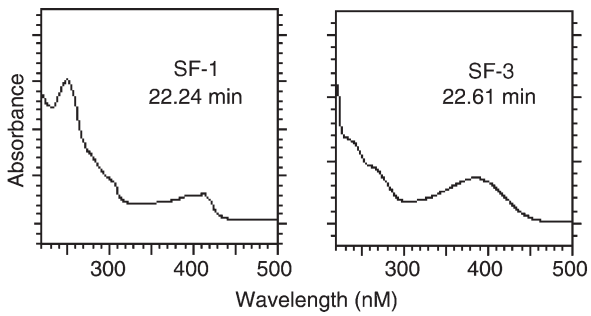

Figure 6 UV spectra and HPLC retention times of the main compounds in SF-1 and SF-3

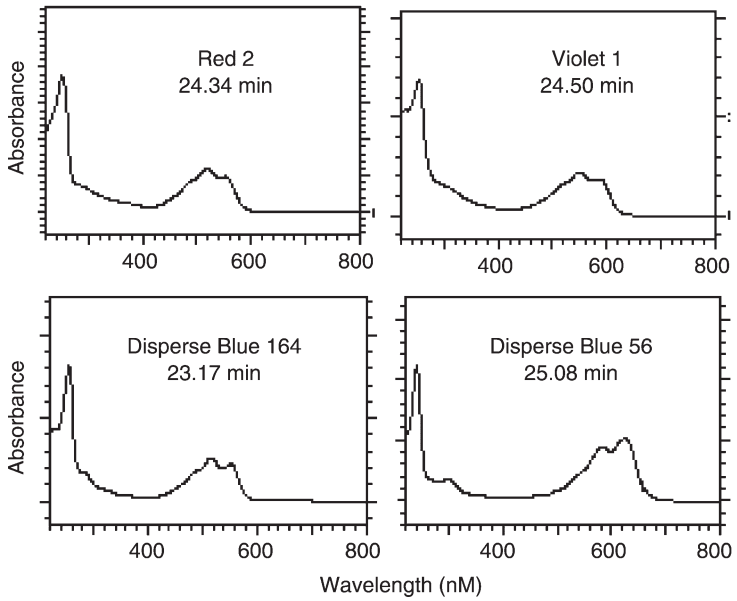

Figure 7 UV spectra and HPLC retention times of Red 2, Violet 1, Disperse Red 164 and Disperse Blue 56

Table 1 Commercial dyes used and their structure

C.I. No.	Structure
Disperse Blue 56 (D.B.56)	Anthraquinone
Disperse Blue 81:1 (D.B.81)	Anthraquinone
Disperse Red 164 (D.R.164)	Anthraquinone
Direct Green 80 (Dire.G.80)	Monoazo-anthraquinone
Reactive Blue 4 (R.B.4)	Anthraquinone
Disperse Blue 291 (D.B.291)	Monoazo
Acid Red 114 (A.R.114)	Disazo
Disperse Blue 341 (D.B.341)	Monoazo
Acid Green 25 (A.G.25)	Anthraquinone
Acid Blue 129 (A.B.129)	Anthraquinone
Reactive Violet 8 (R.V.8)	Monoazo
Basic Blue 75 (B.B.75)	Oxazine

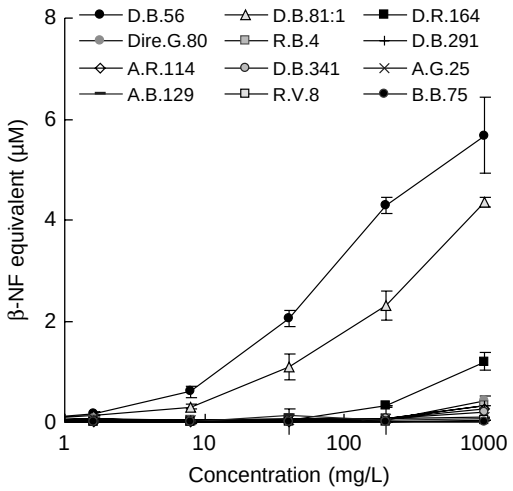

Figure 8 AhR ligand activity of commercial dyes

disperse anthraquinone dyes elicited higher AhR ligand activity, but hydrophilic acid anthraquinone dyes or azo dyes showed very weak or no AhR ligand activity. The results corresponded to the nature of classical AhR ligands that are planar, aromatic and hydrophobic compounds. Hydrophobic disperse anthraquinone dyes were suggested to be potential xenobiotic AhR ligands.

Conclusions

In this study, AhR ligand activity was detected in treated sewage effluents, and several coloured AhR ligands were isolated and postulated to be dyes. One red compound was identified to be rhodamine B base, but it only showed weak AhR-binding affinity and was not the most potent ligand in the 23rd fraction that elicited the highest AhR ligand activity. In addition, two other AhR ligands separated from the 25th fraction were suggested to be disperse anthraquinone dyes or their metabolites because of their UV spectra and HPLC retention times.

The AhR-binding affinities of 12 commercial dyes with different chemical structures were also investigated, and hydrophobic disperse anthraquinone dyes such as Disperse Blue 56 elicited higher AhR ligand activity in the yeast bioassay. Rhodamine B base and disperse anthraquinone dyes are hydrophobic compounds which may be absorbed to sediments or aquatic biota after being discharged into aquatic environments. Future research concerning the contamination and ecotoxicological information of these xenobiotic AhR ligands is necessary.

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