Isolation and Identification of a New AhR Ligand 3'-Hydroxybenzo[b]quinophthalone in Dyeing Wastewater

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Introduction

Numerous natural compounds and environmental contaminants have been reported to activate the aryl hydrocarbon receptor (AhR)[1]. Among them, natural dye indigo and its by-product indirubin were isolated from human urine and presumed to be endogenous AhR ligands[2].A principal azo-dye methyl yellow and its halogenated derivatives were also shown to be potential xenobiotic AhR ligands, although their use has been prohibited due to the carcinogenicity[3]. It can be assumed that there are also other anthropogenic dyes which may activate the AhR, and that their residues or derivatives in industrial wastewater may cause adverse effects to aquatic environments.

In this study, a method for investigating potential AhR ligands in dyeing wastewater is presented, and 3'-hydroxybenzo[b]quinophthalone, a yellow dye chemical, was isolated and identified to be a weak AhR ligand. This method includes the applications of HPLC fractionation, a yeast bioassay[4], HPLC-diode array detector (LC-DAD), and HPLC-tandem mass spectrometry (LC-MS/MS). The analytical technique is particularly facilitated by the use of LC-MS/MS because of its high sensitivity and selectivity, which has been demonstrated to characterize dyes[5] and their derivatives[6] in environmental samples successfully.

Materials and Methods

Sampling and Extraction– Dyeing wastewater was taken from an open channel in Kyoto city, Japan, in October 2004. 1500 mL of the wastewater was filtered on 0.45 µm glass fiber filters, and passed through Sep-Pak® Vac C18 cartridges (10 g, Waters). After extraction, the cartridges were washed by water, and then eluted with 60 mL of 50% methanol in water, 60 mL of 75% methanol in water, and 120 mL of 100% methanol. The three extracts collected with respect to different eluents were evaporated to dryness in a centrifugal vacuum concentrator, and then redissolved in dimethyl sulfoxide (DMSO).

Yeast Bioassay for AhR Ligand Activity– AhR ligand activity was detected by an AhR-dependent yeast bioassay using the recombinant yeast YCM3 strain as described[2-4]. The yeast strain was grown in a synthetic glucose medium lacking tryptophan at 30℃. After 14 to 18 hours, 1 µL of the test sample was mixed with 5 µL of the saturated culture and 200 µL of the synthetic galactose medium, and subsequently incubated at 30℃. Cell density was determined by reading the absorbance at 595 nm after 18 hours of incubation, and reaction was started by throughly mixing 10 µL of the cell suspension with 140 µL of Z-buffer and 50 µL of O-nitrophenyl-β-D-galactopyranoside (4 mg/ml solution made in Z-buffer). The absorbance at 405 nm was read after incubating at 37℃ for 60 minutes. The β-galactosidase activity (reported as LacZ units) was calculated by the following formula: (absorbance at 405 nm×1000) / (absorbance at 595 nm×ml of cell suspension added×minutes of reaction time).

HPLC Fractionation, Ligand Isolation, and Purification– An aliquot of extract was injected into a C18 HPLC reversed-phase column (Shim-pack FC-ODS, 150×4.6 mm, Shimadzu), and eluted in a linear gradient of 10% to 100% methanol in water within 20 minutes followed by 100% methanol held for another 20 minutes at a flow rate of 1 mL/min. Fractions were collected every minute for 36 minutes, evaporated to dryness, redissolved in DMSO and then subjected to the yeast bioassay. After the yeast bioassay was carried out, potential AhR ligands were isolated from fractions showing AhR-mediated activity and then further purified by reversed-phase columns (Wakosil-II 5C18HG, 20×50 mm, Wako; Shim-pack FC-ODS, 150×4.6 mm, Shimadzu).

Synthesis of 3'-Hydroxybenzo[b]quinophthalone – 3'-Hydroxybenzo[b]quinophthalone was synthesized according to

the method described[7]. Equimolar amount of 2,3-naphthalenedicarboxylic acid anhydride and 3-hydroxy-2-methyl-4-quinolinecarboxylic acid were dissolved in nitrobenzene, stirred and heated overnight under reflux. Nitrobenzene was removed by distillation under reduced pressure, and then the target compound was purified by silica gel column chromotagraphy (eluent: hexane/chloroform) and recrystallization from methanol. Further purification was carried out by using reversed-phase HPLC columns.

LC/ESI-MS/MS– Experiments were carried out using the Micromass Quattro Ultima Pt mass spectrometer (Waters) 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/hr and desolvation gas temperature was 380℃. The ion source temperature was 130℃. The capillary voltage was set at 3.5 kV, and the cone voltage was 35 V. The collision energy was 30 eV for acquiring the MS/MS spectrum of synthesized 3'-hydroxybenzo [b]quinophthalone. Data acquisition was performed in the positive ion mode over thte range of m/z 50-350.

Results and Discussion

Detection of AhR Ligand activity– Compounds extracted by Sep-Pak cartridges were roughly separated into three extracts according to their polarity by using different eluents. Each extract was subjected to the yeast bioassay, and only the most hydrophobic extract (eluent: 100% methanol) elicited AhR ligand activity. 10 µL of the extract was injected into a Shim-pack FC-ODS column. Figure 1 shows the HPLC chromatogram at wavelength 456 nm and the AhR ligand activity of corresponding HPLC fractions (final concentration factor: about 5-fold of the relevant environmental concentration). The fractions collected in the 24th and 34th minutes elicited higher AhR ligand activity. According to the HPLC chromatogram, there was one major peak in the 34th fraction, which was named Yellow 1 due to its color, and its UV spectrum is shown in Figure 2. The rest of the extract was fractionated by the Wakosil-Ⅱ 5C18HG column to isolate this compound, and further purification was carried out by using the Shim-pack FC-ODS column.

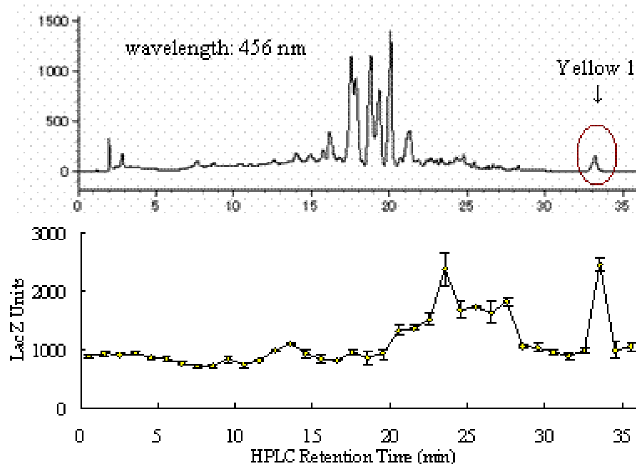

Fig 1. HPLC chromatogram and AhR ligand activity of HPLC fractions

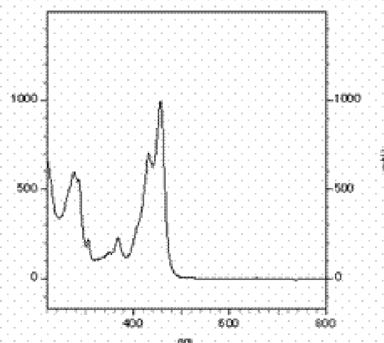

Fig 2. UV spectrum of yellow 1

Identification of Yellow 1– Purified Yellow 1 was subjected to LC/MS/MS analysis in order to obtain more information for identification. Yellow 1 exhibited a molecular ion peak at m/z 340 in the MS spectrum. Its mass was suggested to be 339 that corresponded to the molecular weight of a yellow dye, 3'-hydroxybenzo[b]quinophthalone (3'-HB[b]QP) found in a handbook of pigments (written in Japanese) [8]. 3'-HB[b]QP was synthesized as described[7], and the HPLC retention time, UV, MS and MS/MS spectra of synthesized 3'-HB[b]QP were consistent to those of Yellow 1 . Thus, Yellow 1 was confirmed to be 3'-hydroxybenzo[b]quinophthalone.

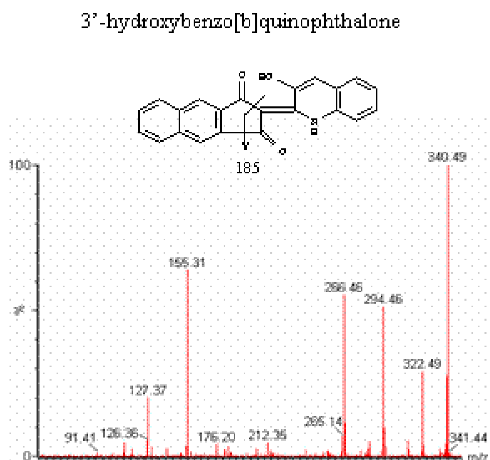

Fig 3. MS/MS spectrum of 3'-HB[b]QP

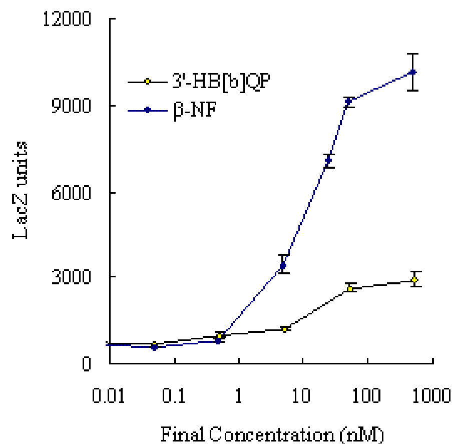

Fig 4. AhR ligand activity of 3'-HB[b]QP

Properties of 3'-Hydroxybenzo[b]quinophthalone – The MS/MS spectrum and the structure of 3'-HB[b]QP are shown in Figure 3. Figure 4 shows the dose-response curve of the AhR ligand activity of 3'-HB[b]QP comparing to an archetypal AhR ligand, β-naphthoflavone (β-NF). Although 3'-HB[b]QP only showed weak AhR ligand activity, it was able to induce signaling in the YCM3 strain at nanomolar concentrations.

3'-HB[b]QP and its brominated derivatives were studied as potential substitutes for the toxic dye cadmium yellow in Japan during 1970s. In this study, the brominated derivatives of 3'-HB[b]QP were not detected in the dyeing wastewater. However, brominated or other halogenated derivatives of 3'-HB[b]QP may also show AhR ligand activity, and sometimes the halogen-substitution might even enhance the AhR ligand activity by altering the size or planarity of the original compound[3]. Further research is necessary for monitoring the presense of these dyes in the environment as potential xenobiotic AhR ligands.

Acknowledgement

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