Mixture effects using the DR-CALUX assay and HPLC fractionation: time course of AhR-binding activity

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Introduction

Non-additive increases of CYP1A expression or EROD activity among various compounds have been reported elsewhere.[1,2] However, if a synergistic effect occurs in environmental samples, it is more important to investigate the mechanism of non-additive CYP1A increase and assess the risk associated with it. Our previous study[3] prepared HPLC fractions from night soil sludge (NSS) compost with an octadecyl silica (ODS) column and evaluated mixture effects by co-exposure of each HPLC fraction and 2,3,7,8-TCDD to the cells using the CALUX assay (DR-CALUX®: Dioxin-Responsive Chemical-Activated Luciferase gene eXpression). Results indicated that the experimental CALUX activity was higher than the predicted CALUX activity for some fractions. Some compounds in the compost sample interacted synergistically with 2,3,7,8-TCDD in the CALUX activity. However, we found that it was difficult to identify the cause of a synergistic effect in a crude extract of NSS compost using only HPLC with an ODS column.

This study specifically examined co-existing stable compounds such as HAHs and labile compounds such as PAHs. We investigated the cause of mixture effects in a crude extract of the NSS compost sample. First, we confirmed that HPLC on a nitrophenylpropylsilica (NITRO) column was capable of separating CYP1A-inducing PAHs and HAHs[4] and then established the fractionation procedure for the sample. Subsequently, we applied HPLC fractionation combined with the CALUX assay to five compost samples (including NSS compost). We compared the CALUX activity of the crude extract with the arithmetical sum of the CALUX activities of all fractions separated by HPLC at various exposure durations. We observed changes in mixture effects.

Materials and Methods

Sample preparation: Sampling procedures of NSS compost have been described elsewhere.[3] NSS/Cow manure (CM) compost, CM compost, bark compost, and fish waste (FW) compost were obtained in Hokkaido, Japan. All compost samples were extracted using ASE 200 with acetone/*n*-hexane (v/v=1/1). After removing elemental sulfur with activated copper, extracts were subjected to alkali digestion. After extraction with *n*-hexane, a portion of *n*-hexane layer was extracted with DMSO. The DMSO fraction was diluted with water and was followed by re-extraction with *n*-hexane. After evaporation, the residue was redissolved in nonane and stored at 4°C for subsequent HPLC fractionation.

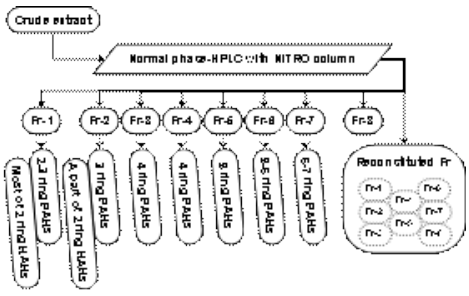

Fig. 1. Fractionation scheme for crude extracts of a compost sample.

Normal-phase HPLC fractionation: Figure 1 shows the fractionation scheme determined in compliance with a previous study.[4] Cut-off points of the fractionation procedure providing eight fractions were determined according to the retention time of standard compounds. Standard compounds of 62 polyaromatic compounds (PACs) and samples were separated on a NITRO column (Nucleosil 5u NO_2, 4.6 × 250 mm, 5 µm; Phenomenex Inc.) and a guard column (4.6 × 30 mm, 5 µm) at 20°C, and eluted with *n*-hexane at a flow rate of 2 ml/min. Standard compounds were identified using a photodiode array detector (Agilent Technologies, Inc.). Peaks detected at 210–360 nm in the UV absorption spectrum were used to fix a retention time for each compound. For HAHs, peaks detected by HRGC/HRMS were used to fix the retention time. Furthermore, recovery rates of PAH and HAH

standard compounds were calculated respectively using HPLC on an ODS column and HRGS/HRMS. A 10-µl sample was injected and then fractionated from 2.5 min to 100 min. Furthermore, to consider the amount of sample lost through fractionation, all fractions were recombined to form a reconstituted fraction (Fig. 1). All fractions were evaporated. Then the residue was taken up in DMSO and assessed using the CALUX assay.

CALUX assay: The rat hepatoma H4IIE cell line, stably transfected with an AhR-regulated luciferase gene construct, was obtained from BioDetection Systems B.V. (Amsterdam, Netherlands). Conditions for cell culture and a procedure for the CALUX assay have been described in detail elsewhere.[3] Toevaluate a change of CALUX activity during exposure time, the CALUX activity was measured respectively after 6, 12, 24, 48, and 72 h of exposure to samples. The CALUX-TEQ of HPLC fractions and reconstituted fractions was determined by interpolation from the fitted 2,3,7,8-TCDD calibration curve. All measurements were conducted in three wells. Experiments for the HPLC fraction and the reconstituted fraction were repeated independently two or three times.

Results and Discussion

Development of a NP-HPLC fractionation method that separates stable compounds from labile compounds: We investigated elution characteristics of 42 HAHs and 20 PAHs by NP-HPLC on a NITRO columntodetermine cut-off points on HPLC fractionation. Figure 1 shows that NITRO-HPLC fractionation separated the CYP1A-inducing 4-6 ring PAHs and HAHs.[5] In addition, 29 HAHs and 13 PAHs were used for determination of recovery rates. The recovery rates were 52–116%, which were similar to results reported by Brack*et al*.[4] Moreover, the coefficients of variation between three experiments for calculating recovery rates were 1–25%, which showed good reproducibility. The results suggest that NITRO-HPLC fractionation can evaluate the influence to mixture effects of different metabolic rates among compounds through combination with CALUX assay.

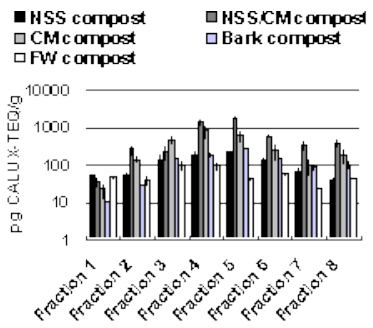

Fig. 2. The CALUX activity pattern obtained from a crude extract after 24 h exposure. NSS, Night soil sludge; CM, Cow manure; FW, Fish waste.

Mixture effects and CALUX activity of each HPLC fraction after 24 h exposure duration: The CALUX activity patterns in the crude extract of five compost samples after 24 h exposure are shown in Fig. 2. The third to sixth fractions obtained from all samples showed higher activity (Fig. 2). Taking the elution results for the standards into consideration, we estimated that the contribution of the CYP1A-inducing 4–6 ring PAHs[5] was relatively high (Fig. 1). The CALUX activity of the reconstituted fraction (see Fig. 1.) was compared with the arithmetical sum of the CALUX activities of all fractions separated by HPLC to determine whether or not the activity of HPLC fraction obtained from composts increased additively. The CALUX activity of the reconstituted fraction (see Fig. 1) would be equal to the sum of the CALUX activities of all HPLC fractions if compounds in HPLC fraction interact additively. After 24 h exposure, the CALUX activity of the reconstituted fraction (see Fig. 1) obtained from the NSS compost was twice the CALUX activity sum of all fractions. On the other hand, the CALUX activities of reconstituted fractions (see Fig. 1) obtained from the other four composts were roughly equal to the arithmetical sum of the CALUX activity of all separated fractions (data not shown). Our results confirmed the respective non-additive and additive increases of CALUX activity in the NSS compost and the other four composts.

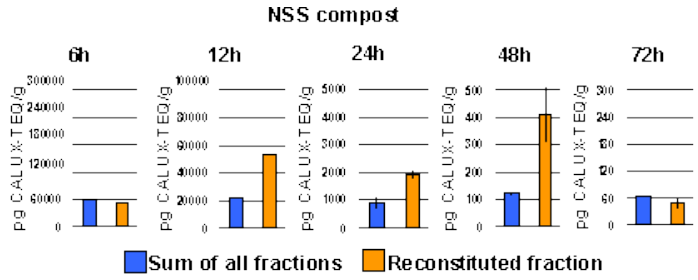

Fig. 3. The CALUX activity of the reconstituted fraction and the sum of the CALUX activity of the separated fractions (sum of all fractions) obtained from a crude extract of NSS compost at different exposure durations. NSS, Night soil sludge.

Change of mixture effects among compounds at various exposure durations: To investigate changes in mixture effects in a crude extract of NSS compost during exposure time, the CALUX activity of each HPLC fraction and reconstituted fraction were measured at 6, 12, 48, and 72 h exposure time durations. The mixture effects in a crude extract of NSS compost were evaluated at various exposure durations (Fig. 3). After 12 h and 48 h exposure duration, the respective CALUX activities of the reconstituted fractions (see Fig. 1) obtained from NSS compost were 2.5 and 4.0 times the CALUX activity sum of all fractions (Fig. 3). On the other hand, the CALUX activities of the reconstituted fractions (see Fig. 1) were roughly equivalent to the arithmetical sum of the CALUX activity of all separated fractions after 6 h and 72 h exposure durations (Fig. 3). As a result, additive increases of CALUX activity were observed at 6 and 72 h exposure durations and non-additive increases of activity were apparent at 12, 24, and 48 h exposure duration.

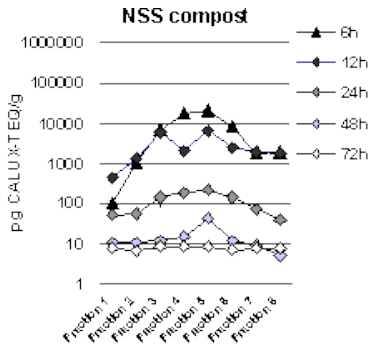

Fig. 4. The CALUX activity profiles obtained from a crude extract of NSS compost at different exposure durations. NSS, Night soil sludge.

Relation between the CALUX activity profiles and the mixture effects in the crude extract: The time course changes of CALUX activity profile of each fraction were shown for the NSS compost in Fig. 4. A non-additive increase of CALUX activity was observed when the CALUX activity for HPLC fractions showed a remarkable reduction, resulting in a change of activity profiles (see Figs. 3 and 4). In detail, this was observed at exposure durations between 6 and 12 h, 12 and 24 h, 24 and 48 h. In contrast, additivity was observed at 72 h exposure duration when the CALUX activity of HPLC fractions showed neither remarkable reduction nor a change in profile (see Fig. 4., it was indicated between 48 and 72 h exposure duration). For the other four composts, it was observed between 24 and 48 h, and 48 and 72 h exposure durations (Fig. 5). Previous studies have pointed out that the reduction of CALUX activity on the course of exposure time occurred because of the metabolic decomposition of CALUX active compounds.[6,7] Therefore, our results suggest that mixture effect is relevant to the metabolic decomposition of compounds in a crude extract of the NSS compost.

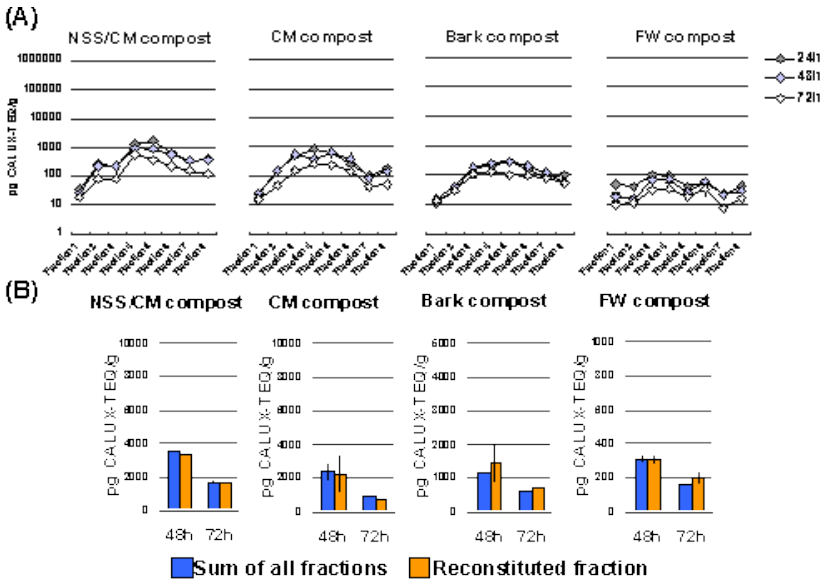

Fig. 5. Comparison of (A) CALUX activity patterns obtained from crude extract of four composts at 24–48 h exposure durations, and (B) CALUX activity of the reconstituted fraction and the sum of the CALUX activity of the separated fractions (sum of all fractions) at 48 and 72 h exposure durations. NSS, Night soil sludge; CM, Cow manure; FW, Fish waste.

Our results suggest that the difference in the metabolic decomposition of a compound affects the mixture effects in a crude extract from NSS compost.

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