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Phase-II conjugation ability for PAH metabolism in amphibians: characteristics and inter-species differences

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Abstract

The present study examines amphibian metabolic activity—particularly conjugation—by analysis of pyrene (a four ring, polycyclic aromatic hydrocarbon) metabolites using high-performance liquid chromatography (HPLC) with fluorescence detector (FD), a mass spectrometry detector (MS) system and kinetic analysis of conjugation enzymes. Six amphibian species were exposed to pyrene (dissolved in water): African claw frog (*Xenopus laevis*); Tago's brown frog (*Rana tagoi*); Montane brown frog (*Rana ornativentris*); Wrinkled frog (*Rana rugosa*); Japanese newt (*Cynops pyrrhogaster*); and Clouded salamander (*Hynobius nebulosus*); plus one fish species, medaka (*Oryzias latipes*); and a fresh water snail (*Clithon retropictus*), and the resultant metabolites were collected. Identification of pyrene metabolites by HPLC and ion-trap MS system indicated that medaka mainly excreted pyrene-1-glucuronide (PYOG), whilst pyrene-1-sulfate (PYOS) was the main metabolite in all amphibian species. Pyrene metabolites in amphibians were different from those in invertebrate fresh water snails. Inter-species differences were also observed in pyrene metabolism among amphibians. Metabolite analysis showed that frogs relied more strongly on sulfate conjugation than did Japanese newts and clouded salamanders. Furthermore, urodelan amphibians, newts and salamanders, excreted glucose conjugates of pyrene that were not detected in the anuran amphibians. Kinetic analysis of conjugation by hepatic microsomes and cytosols indicated that differences in excreted metabolites reflected differences in enzymatic activities. Furthermore, pyrenediol (PYDOH) glucoside sulfate was detected in the Japanese newt sample. This novel metabolite has not been reported previously to this report, in which we have identified unique characteristics of amphibians in phase II pyrene metabolism.

Keywords: Amphibians, pyrene, conjugation, polycyclic aromatic hydrocarbons (PAHs), metabolism, inter-species difference

1. Introduction

Over 70% of the world's amphibian species are experiencing declines (Stuart et al., 2004). Although the phenomenon of amphibian declines was first recognized and publicized in the early 1990s (Barinaga, 1990; Blaustein and Wake, 1990; Vitt et al., 1990; Beebee, 1992), they appear to have started earlier. There are many causes for global amphibian decline: pathogens, environmental pollutants, atmospheric change, habitat modification, and invasive species (Blaustein and Kiesecker, 2002). However, the causes of decline in many species are still unknown. Some chemicals may be causing unexpected effects. To protect amphibians from environmental pollutants, advanced knowledge of their xenobiotic metabolism is essential.

Polycyclic aromatic compounds, which include polycyclic aromatic hydrocarbons (PAHs), are a widely studied class of contaminant. Many polycyclic aromatic compounds are toxic to humans and other organisms (Neff, 1979, Hertel et al., 1998). They are released into the environment both naturally and through human activities, such as in the combustion of fossil fuel (Lima et al., 2005). PAHs are cytotoxic and genotoxic (Babich and Borenfreund, 1991). In the presence of sunlight, some PAHs—such as anthracene, benzo[a]pyrene, and fluoranthene—can be extremely toxic to amphibians at environmentally achievable levels (Fernandez and L'Haridon, 1992; Hatch and Burton, 1998; Monson et al., 1999).

The metabolism of PAHs can lead to strong carcinogenic activity (Shimada and Fujii-Kuriyama, 2004) and is classified into three phases: phase I is oxidation, phase II is conjugation, and phase III is excretion. The phase I process is an oxidative reaction occurring mainly through the cytochrome P450 (CYP) family at specific sites on the PAH where oxidation can occur (Zamek-Gliszczynski et al., 2006). The phase II process attaches biomolecules to xenobiotics or phase I products to make them hydrophilic and easy to excrete. Phase II is mainly performed by conjugation enzymes including

UDP-glucuronosyltransferase (UGT), sulfotransferases (SULT), and glutathione-S-transferase (GST). Phase III involves excretion of metabolites from cells by membrane proteins, such as ATP binding cassette (ABC) transporters (Cole and Deeley, 1998; Keppler et al., 1998). The toxicity of PAHs is linked to an activated phase I metabolite (Lambert et al., 1995; Incardona et al., 2006) and this type of intermediate is also associated with DNA adduct formation (Stowers and Anderson, 1985), for example benzo[a]pyrene. It is thus important to know the metabolic pathways for PAHs, including the conjugation and excretion of activated phase I metabolites. Metabolism of PAHs in fish or mammals has been well-studied (Stickland et al., 1994; Tuvikene, 1995; Luthe et al., 2002) but there is little information about PAH metabolism in amphibians.

In this study, amphibians were exposed to pyrene, a tetracyclic PAH commonly studied as a model compound because it occurs as a major constituent in mixtures with other, more harmful, PAHs (Rossbach et al., 2007). In fish and mammals, pyrene's major phase I metabolite is 1-hydroxypyrene (PYOH), which is conjugated by UGT or SULT (Luukkanen et al., 2001; Ma et al., 2003). Pyrene is converted to pyrene-1-glucuronide (PYOG) and pyrene-1-sulfate (PYOS) following phase II conjugation (Luthe et al., 2002). Other studies showed major phase II metabolites among invertebrates to be PYOS and pyrene-1-glucoside (PYOg) (Ikenaka et al., 2006; Beach et al., 2009). The few studies of amphibian conjugation reactions *in vivo* have shown differences in interspecies sensitivity to drugs or toxins to be derived from variations in metabolizing enzymes (Alexandrie et al., 2000; Furlong et al., 2005).

In this report, we characterized amphibian conjugation reactions by the analysis of pyrene metabolites and measured the kinetics of amphibian conjugation enzymes (liver microsomal UGT and liver cytosolic SULT) against PYOH. This study reveals new information about xenobiotic metabolism in amphibians.

2. Materials and Methods

2.1. Chemicals

Pyrene was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). 1-Hydroxypyrene, sulfatase (from limpets Type V; 34 units/mg), β -glucuronidase (from bovine liver, Type B-1; 1240 units/mg), β -glucosidase (from almonds; 3.4 units/mg), bovine serum albumin, and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); UDP-glucuronic acid (UDP-GA) and UDP-glucose (UDP-Glu) were purchased from Wako (Osaka, Japan). All chemicals used for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) (HPLC grade or MS grade) were obtained from Kanto Chemical Co., Inc.

2.2. Animals

We used six amphibian species, one fish species, and one gastropod species. African claw frog (*Xenopus laevis*, n = 6) was obtained from Xenopus hanshoku kyozai (Ibaraki, Japan). Tago's brown frog (*Rana tagoi*, n = 1), Montane brown frog (*Rana ornativentris*, n = 1), and Wrinkled frog (*Rana rugosa*, n = 1) were collected in Gifu prefecture. Japanese newt (*Cynops pyrrhogaster*, n = 20) was collected in Miyagi prefecture. Clouded salamander (*Hynobius nebulosus*, n = 2) was collected in Shiga prefecture. Medaka (*Oryzias latipes*, n = 10) and fresh water snail (*Clithon retropictus*, n = 10) were purchased from a local fish farm in Sapporo, Japan. Animals had been reared in water at room temperature (around 23° C) under a 12/12 h light/dark photoperiod.

Treatment of all animals was in accordance with the policies of the Institutional Animal Care and Use Committee of Hokkaido University (No. 15099 and No. 08-0331).

2.3. Pyrene exposure

Animals were exposed to pyrene for 24 h. Pyrene (500 ppm; dissolved in ethanol) was added to distilled water at 3 pm and the water was collected at 3 pm the following day. Since large amounts of metabolites were needed to determine the structure of each metabolite, the volume of pyrene added for exposure (250 µg/l) was more than its solubility (135 µg/l). We confirmed that pyrene was sufficiently dissolved in water and that pyrene entered the animals. The water volume used was determined by the size of the animal. In the case of *X. laevis*, 1 ml of 500 ppm pyrene solution was added to 2 L of water. Oral administration was also performed and the same metabolites were detected in both cases. The effect of solvent (ethanol) and stress on animals by oral administration is thought to be higher. Furthermore, percutaneous absorption is likely to be an important route of exposure in amphibians. Therefore, the pyrene was dissolved in water for this study. Exposure and sampling was repeated three times to confirm reproducibility.

2.4. Extraction of pyrene metabolites

After 24 h pyrene exposure, we extracted pyrene metabolites from the water by filtering with a glass filter (GF/C; Whatman, Int. Ltd., Kent, UK). The filtered water was passed through a solid-phase cartridge containing styrene-divinylbenzene copolymer (PS@Liq; Showa Denko, Tokyo, Japan) and eluted with 10 ml of 70% (v/v) methanol. We confirmed that the metabolite profiles did not change during the extraction procedure by using HPLC with a Fluorescence Detector (FD). For preliminary experiment, we also gathered pyrene metabolites from bile and urine of *X. laevis*. Bile and urine were mixed with 70% (v/v) methanol and the supernatants of centrifugation at $5,000 \times g$ for 5 min were used for analysis.

2.5. Analysis of pyrene metabolites

Pyrene metabolites were determined by HPLC (20A series; Shimadzu, Kyoto, Japan) with FD (RF-10AXL; Shimadzu) equipped with an ODS column (ODS-120T 4.6 mm × 300 mm; Tosoh, Tokyo, Japan). The HPLC protocol followed the method of Beach et al. (2009) with a slight modification. Mobile phases consisted of A = 10 mM ammonium acetate buffer (pH 5.0), B = methanol: acetonitrile: water (38:57:5, v/v/v). The solvent gradient was 35 min in length with 10% B from 0–2 min, followed by a linear gradient to 38% B from 2–11 min, to 56% B from 11–20 min, and to 100% B from 20–27 min with an 8 min hold at 100% B to 35 min. An injection volume of 10 µl, a flow rate of 500 µl/min, and a column temperature of 45°C were used throughout. Excitation (Ex) and emission (Em) wavelengths for FD were 343 and 385 nm, respectively.

2.6. Identification of pyrene metabolites formed by amphibians

The HPLC with an electrospray ionization ion-trap mass spectrometry detector (ESI/ion-trap/MS, LTQ Orbitrap; Thermo Fisher Scientific, MA, USA) was used to identify pyrene metabolites produced by amphibians. The ESI conditions were full scan (m/z 80 to m/z 800), negative mode, with an ion source voltage of -5.0 kV and an ion source temperature of 300°C.

2.7. Deconjugation

Deconjugation was performed using the method described by Ikenaka et al. (2007). Sulfatase, β -glucuronidase, and β -glucosidase were briefly dissolved in 0.1 M sodium acetate buffer (pH 5.0) to concentrations of 10, 4000, and 17 units/ml, respectively. The methanol solution, containing the pyrene metabolites, was concentrated under a gentle stream of

nitrogen, dissolved in 300 μ l of 0.1 M sodium acetate buffer (pH 5.0) and then 200 μ l of each deconjugation enzyme was added. In the control treatment, 200 μ l of bovine serum albumin (1mg/ml) was added in place of deconjugation enzyme. After 8 h of incubation at 37 °C, 500 μ l of methanol was added to stop the reaction. The deconjugated compounds were analyzed by HPLC/FD.

2.8. *Preparation of microsomes and cytosol from liver*

Livers were extirpated from *X. laevis* and *C. pyrrhogaster* for analysis of enzyme activities. Livers of *C. pyrrhogaster* were so small that we used five livers as one pooled sample. The livers were homogenized in 40 ml of homogenization buffer (0.1 M phosphate buffer containing 10% glycerol, 2 ppm pepstatin A, and 2 ppm leupeptin). Microsomal and cytosolic fractions were prepared at 4°C by differential centrifugation: the supernatant of the first centrifugation at $9,000 \times g$ for 20 min was further centrifuged at $100,000 \times g$ for 70 min to obtain the microsomal and cytosolic fractions. The cytosols were used to measure SULT activity; microsomal pellets were resuspended in resuspension buffer (0.1 M phosphate buffer containing 20% glycerol, 2 ppm pepstatin A, and 2 ppm leupeptin) to provide a protein content of 10 mg/ml and used to determine UGT activities. Protein concentrations of each fraction were measured using the Lowry method (Lowry et al., 1951) with modification.

2.9. *SULT activity*

The SULT activity for PYOH was determined by the method of Lee et al. (2007) with a slight modification. Of 400 μ g/ml hepatic cytosolic protein solution, 100 μ l was mixed with 53 μ l of 100 mM Tris-HCl buffer (pH 7.4), 20 μ l of 50 mM MgCl₂, 20 μ l of 50 mM Na₂SO₃, and 2 μ l of PYOH. The mixtures were pre-incubated at 25 °C for 5 min. The reaction was initiated with 5 μ l of 1 mM PAPS in a final volume of 200 μ l. After incubation at 25 °C for

10 min, the reaction was stopped by adding 800 μ l of ice-cold methanol. Reaction samples were then placed on ice for 10 min prior to centrifugation at $750 \times g$ for 10 min. The resultant supernatant was injected into a HPLC system. Kinetics parameters were determined by the Michaelis-Menten equation using Graph Pad Prism 5 (GraphPad Software, CA, USA).

2.10. UGT activities

The UGT activity of PYOH was determined as described below. Initially, 80 μ l of the hepatic microsome solution (10 mg/ml) was mixed with 10 μ l of 1% sodium cholate. Next, the microsome mixture was mixed with 110 μ l of 500 mM phosphate buffer (pH 7.4) and left on ice for 30 min. After the treatment, 50 μ l of the microsome solution was mixed with 123 μ l of 500 mM phosphate buffer (pH 7.4), 20 μ l of 50 mM $MgCl_2$, and 2 μ l of 1-OHP, and then pre-incubated at 25 $^{\circ}C$ for 5 min. The reaction was started with 5 μ l of 50 mM UDP-GA, UDP-Glu in a final volume of 200 μ l. After incubation at 25 $^{\circ}C$ for 10 min, the reaction was stopped by adding 800 μ l of ice-cold methanol. The reaction samples were then placed on ice for 10 min before centrifugation at $750 \times g$ for 10 min. The resultant supernatant was then analyzed using the same HPLC conditions as in the measurement of PYOS. Kinetic parameters were determined using the Michaelis-Menten equation, as above.

3. Results and Discussion

3.1. Identification of pyrene metabolites formed in each species

The HPLC/FD chromatograms of pyrene metabolites are shown in Fig 1. Peaks were not detected from control treatment samples (without animals or without pyrene). (A) shows the pyrene and PYOH standards. Two major peaks were seen in medaka (B; peaks -h and -i) and fresh water snail (C; peaks -i and -k) samples, while three or more characteristic peaks (peaks a–j) were detected in amphibian samples (D–I). These peaks were seen in the pyrene exposure treatment group, but not detected before pyrene exposure (data not shown). Therefore, these peaks were considered to be pyrene-derived substances and revealed the main pyrene metabolite of amphibians (peak-i) to be different from that of fish (peak-h). The results also reveal that amphibians produce several pyrene metabolites that are not observed in medaka and fresh water snail samples. In our experiments, it was thought that bacteria break down pyrene metabolites such as PYOG, so we also analyzed pyrene metabolites using bile and urine samples of *Xenopus laevis*. However, the results of metabolite profiles were almost identical amongst bile, urine and exposed water samples. It was very difficult to analyze the metabolites from bile and urine samples of small animals such as Japanese newt; we chose to analyze water soluble pyrene metabolites from water body in our study.

We attempted to identify pyrene metabolites produced by medaka, fresh water snail, and amphibians according to retention time (min), MS (m/z), MS² (m/z) spectrum measured by the HPLC/MS system, and from deconjugation treatments (Table 1). Sulfatase was added to the extracted *X. laevis* sample. In Fig. 2, a typical example of deconjugation treatment, peak-i became smaller after treatment with sulfatase and a new PYOH peak appeared. However, peak-i was not completely extinguished by sulfatase treatment, which implied that peak-i included two or more metabolites. Although production of pyrenediol (PYDOH) was predicted, we could not find any peaks corresponding to PYDOH. However, we could detect

the peaks corresponding to PYDOH by changing FD setting (Ex/Em: 385/425 nm). This setting is according to the data from Pinyayev et al. (2010). Also, by using MS, we could detect PYDOH (m/z 233) in samples after deconjugation.

Peaks -a and -b are thought to be PYDOH disulfate because MS was m/z 393, MS^2 was m/z 313 (PYDOH sulfate) and m/z 233 (PYDOH), and sulfatase made the peaks shift or disappear. Though β -glucuronidase also affected peak-b, we place more weight on the MS result because β -glucuronidase is also thought to have sulfatase activity. According to the HPLC/MS system, peak-c corresponds to m/z 475. Fig. 3 shows the construction and MS^2 spectrum (m/z 475 was the precursor ion). The value m/z 395 corresponds to PYDOH glucoside and m/z 233 corresponds to PYDOH. In deconjugation experiments, peak-c disappeared with sulfatase, β -glucuronidase, and β -glucosidase. Therefore, we estimate that peak-c was PYDOH glucoside sulfate. This is a novel metabolite, unreported before this report. Peaks -d, -e and -f are considered to be PYDOH sulfate because MS was m/z 313, MS^2 was m/z 233 (M-80) (PYDOH), and sulfatase made them disappear. Since they were eluted at different times, they are, presumably, sulfate conjugates of PYDOH where the OH groups are at different ring positions. Peak-g is thought to be pyrene glucuronide sulfate because MS was m/z 473, MS^2 was m/z 393 (M-80) (PYOG) and 217 (M-256) (PYOH), and peak-h was seen after sulfatase treatment. Peak-h was considered to be PYOG because MS was m/z 393, MS^2 was 217 (M-176) (PYOH), and the retention time (RT) changed to 33.6 min by β -glucuronidase treatment. Peak-i, the main metabolite of amphibians, is thought to be PYOS because MS was m/z 297, MS^2 was 217 (M-80) (PYOH), and the RT changed to 33.6 min by sulfatase treatment. We found peak-j included in peak-i because peak-k was seen after sulfatase treatment in the *Hynobius nebulosus* sample. Peak-j was considered to be PYOH glucoside sulfate because MS was m/z 459, MS^2 was m/z 379 (M-80) (PYOg) and 217 (M-242) (PYOH), and the result happened after sulfatase treatment. This interest metabolite,

glucoside sulfate conjugate of PYOH, was also detected from aquatic crustaceans, decapoda (Ikenaka et al. 2007). Peak-k is considered to be PYOg because MS was m/z 439, MS^2 was m/z 217 (M-222) (PYOH), and the result came after β -glucosidase treatment. Reportedly, PYOg was seen as m/z 439 because of acetate adducts (Ikenaka et al. 2007).

These results show that amphibian conjugation reactions to pyrene are significantly different from those of medaka. Other studies have described PAH metabolism in various fish species (Tuvikene, 1995; Luthe et al. 2002). While PYOH mainly conjugates with glucuronide in fish, PYOS is the principal conjugated metabolite in amphibians. Mammals—including humans—exposed to pyrene, excrete PYOG as the main metabolite (Stickland et al. 1994). Huang and Wu (2010) have also shown enzymatic differences between amphibians and other vertebrates, revealing *X. tropicalis*, medaka, and humans to be in clearly different groups by phylogenetic analysis of UGT1A. These reports support the idea that amphibians metabolize various environmental chemicals differently to other vertebrates.

In addition, conjugates derived from PYDOH formed a large percentage of metabolites excreted from amphibians. Interspecies differences in pyrene hydroxylation (phase I) were also seen in this study. Interspecies differences in CYP were reported by Suzuki and Iwata (2010), who suggested that numbers of dioxin-response elements (DREs) and their localization in upstream CYP1 gene regions differ between amphibians and other vertebrates; these differences accounting for variations in TCDD resistance among amphibian species.

3.2. Comparison of pyrene metabolizing ability among amphibian species

We found interspecies differences in pyrene metabolism. In medaka, PYOG was the main conjugated metabolite. Although amphibians vary in their metabolism of toxins, pyrene in amphibians was mainly conjugated with sulfate.

Unlike frogs, Japanese newt and clouded salamander conjugated pyrene with glucose (peak-c and peak-j). Moreover, frogs (especially *X. laevis*), rely on sulfate conjugation to metabolize pyrene. Therefore, we measured conjugation enzyme activities (UGT and SULT) and their relationships to these differences. We chose *X. laevis* and *C. pyrrhogaster* to compare Anura with Urodela because they showed large species differences in excreted metabolites of pyrene *in vivo*. We measured enzyme activities *in vitro* using liver cytosols and microsomes to observe sulfation, glucuronidation, and glucosidation activities to PYOH.

The sulfation activity of PYOH was higher in the *X. laevis* cytosolic fraction than that of *C. pyrrhogaster* (Fig. 4A). Kinetics parameters are shown in Table 2. However, unlike the SULT activity, *X. laevis* microsomes showed lower PYOH glucuronidation activity than did those of *C. pyrrhogaster* (Fig. 4B). The range of substrate concentration was small due to the solubility of PYOH, so we could not obtain clear V_{max} and K_m ; estimated parameters are shown in Table 2. However, we saw clear differences between the two species in this result.

We also measured PYOH glucosidation activity. In accordance with the results of experiments *in vivo*, *C. pyrrhogaster* microsomes showed PYOH glucosidation activity, although the observed activity was very low. On the other hand, microsomes of *X. laevis* did not display PYOH glucosidation activity (Fig. 4C). We could not obtain clear V_{max} and K_m and estimated parameters are shown in Table 2.

In *X. laevis*, which excreted little PYOG, hepatic SULT activity was higher and hepatic UGT activities were lower than in *C. pyrrhogaster*. In *C. pyrrhogaster*, which excreted PYDOH glucoside sulfate, glucosidation activity was detected. In this study, metabolic enzyme activities critically affected, and were coincident with, the quantity of excreted metabolites.

From our results, both *in vivo* and *in vitro*, glucose conjugation was observed in the Urodela group. It has been reported that glucose conjugation is catalyzed by UGT, as is

glucuronide conjugation. Generally, conjugation with glucose has been well-known in invertebrates and plants (Luthe et al., 2002; Ikenaka et al. 2006). Furthermore, only recently, various conjugates including glucoside were found in detail metabolite profiles analysis of anticancer agent in human and the major experimental animals such as mouse, rat and dog (Mial et al., 2009). Although glucose conjugation is not a major metabolic pathway in vertebrates, it seems to be partially used in some cases. However, there seems to be various interspecies differences in glucuronidation activity. In this study, in amphibians, UGTs only in Urodela group are considered to have glucosidation activity, but not in Anura group. Similarly, an earlier study on amphibian CYP suggested that there was a clear difference between Anura and Urodela (Nakayama et al., 2009).

From these results, we could clearly identify the species differences in Anura (frogs and toads) from Urodela (newts and salamanders) in this study by their profiles of pyrene conjugation and the metabolism enzymatic activity.

4. Conclusion

This study showed three new findings:

First, amphibians mainly conjugate hydroxylated pyrene metabolites with sulfate, which has not been reported as a major pathway in other vertebrates.

Second, pyrene metabolism differs among amphibians. Frogs rely strongly on sulfate conjugation, but are poor at glucuronidation in pyrene metabolism, while newts and salamanders can conjugate pyrene with glucose. We confirmed that metabolic enzyme activities are related to these differences.

Finally, we found pyrene glucoside sulfate in Japanese newt excretion. This novel metabolite has not been reported in other studies.

We showed inter-species differences in phase II conjugation ability for PAH in amphibians. The differences in metabolic pathway directly cause the difference in the sensitivity to xenobiotics. Further studies for metabolic pathway of xenobiotics are needed to understand the toxicological effects of environmental pollutant to amphibians.

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Tables

Table 1

Characteristics of pyrene metabolites estimated by HPLC/FD, MS, and deconjugation.

	RT (min)	MS (<i>m/z</i>)	MS ² (<i>m/z</i>)	Deconjugation			Metabolites
				Sulfatase	β-glucuronidase	β-glucosidase	
Peak-a	16.2	393	313, 233	++	-	-	PYDOH disulfate
Peak-b	18.2	393	313, 233	++	++	-	PYDOH disulfate
Peak-c	19.1	475	395, 233	++	++	++	PYDOH glucoside sulfate
Peak-d	20.6	313	233	++	-	-	PYDOH sulfate
Peak-e	21.2	313	233	++	-	-	PYDOH sulfate
Peak-f	22.9	313	233	++	-	-	PYDOH sulfate
Peak-g	24.3	473	393, 217	+	-	-	PYOH glucuronide sulfate
Peak-h	25.2	393	217	-	++	-	PYOG
Peak-i	27.8	297	217	++	-	-	PYOS
Peak-j	(27.8)	459	379, 217	++	-	-	PYOH glucoside sulfate
Peak-k	28.5	439	379, 217	-	++	++	PYOG
PYOH	33.6	217	-	-	-	-	
Pyrene	37.2	-	-	-	-	-	
++	Shifted or disappeared						
+	Decreased						
-	Unchanged						

Table 2

Kinetics parameters of conjugation enzymes in amphibians.

	V_{max} (pmol/min/mg protein)	K_m (μ M)	V_{max}/K_m (pmol/min/mg protein/ μ M)
Sulfation			
<i>X. laevis</i>	2563	13.77	186
<i>C. pyrrhogaster</i>	663.9	39.76	16.7
Glucuronidation			
<i>X. laevis</i>	440.7	131.2	3.36
<i>C. pyrrhogaster</i>	6138	784.7	7.82
Glucosidation			
<i>X. laevis</i>	Not detected	Not detected	Not detected
<i>C. pyrrhogaster</i>	62.47	170.1	0.367

The parameters show averages calculated from Figure 4 using the Michaelis-Menten equation.

X. laevis: n = 1

C. pyrrhogaster: pooled from five livers

Figure legends

Fig. 1. HPLC/FD chromatogram of pyrene metabolites. (A) Chromatogram of standards (PYOH and pyrene). (B–I) Chromatogram of *Oryzias latipes*, *Clithon retropictus*, *Xenopus laevis*, *Rana rugosa*, *Rana tagoi*, *Rana ornativentris*, *Cynops pyrrhogaster* and *Hynobius nebulosus*, respectively. Ten characteristic peaks (peaks a–k) were observed in pyrene-exposed water. (j) of (I) means that peak-i of (I) contains two metabolites. Excitation (EX) and emission (EM) wavelengths for FD were 343 nm and 385 nm, respectively.

Fig. 2. Chromatogram of *X. laevis* sample with and without sulfatase treatment. After sulfatase treatment, peaks -a, -b, -d, and -f disappeared, peak-h stood, peak-i fell, and the peak for PYOH rose. The peak for PYDOH was not detected.

Fig. 3. Electrospray ionization MS² spectra of peak-c. The ESI conditions were full scan (m/z 150 to m/z 620), negative mode. The metabolite of peak-c contained a major ion at m/z 475 (MS: not shown) with product ions of m/z 395 and 233 (MS²). *This figure indicates one possibility for the structure of the metabolite (peak-c).

Fig. 4. SULT (A) or UGT (B and C) activity versus differing concentrations of PYOH in *X. laevis* and *C. pyrrhogaster*. Assay method for activities is described in the text. Data were fitted to nonlinear regression curves using the Michaelis-Menten equation. The plots show the means (\pm SD) of triplicate procedures.

Fig. 1

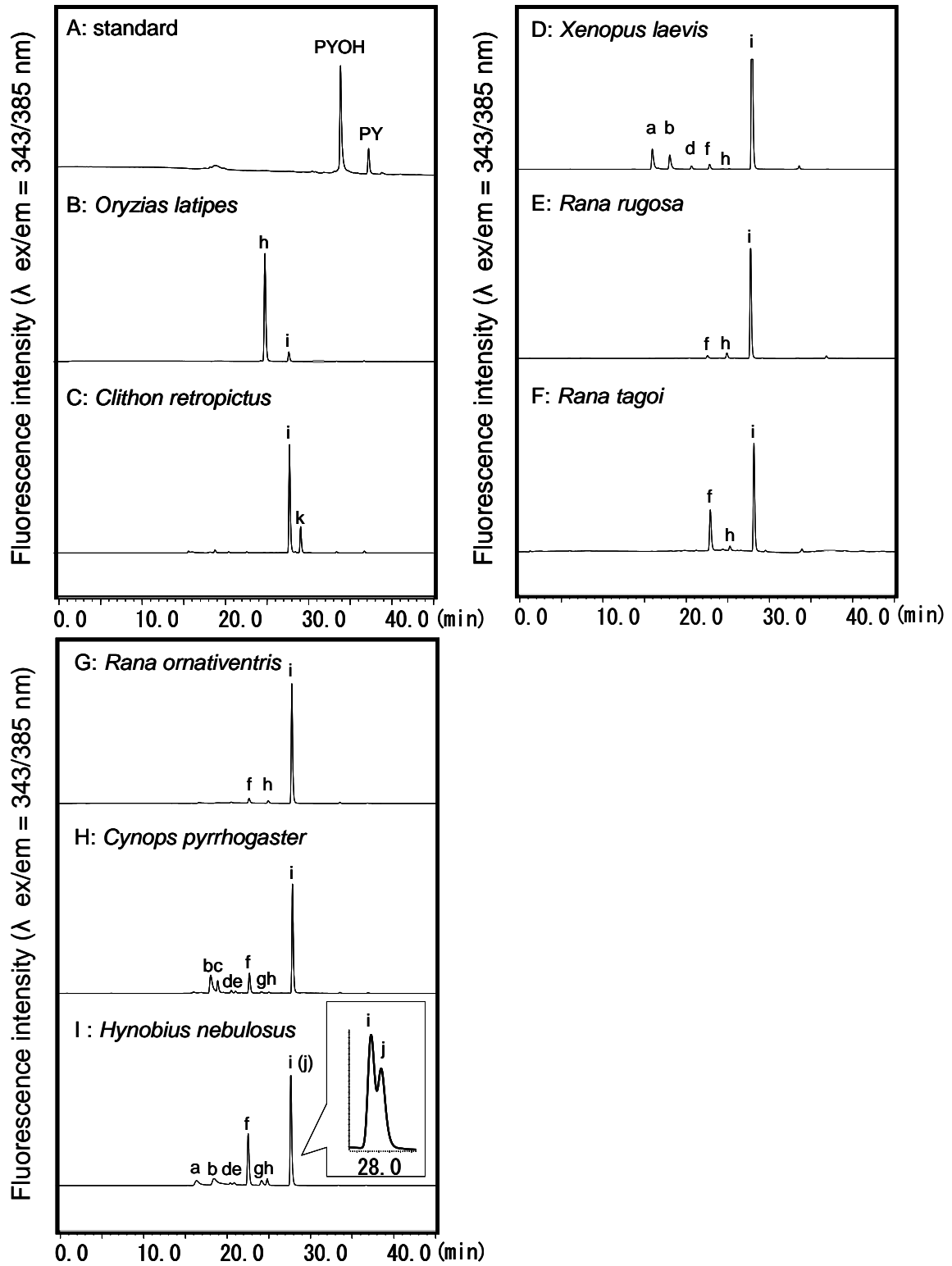


Fig. 2

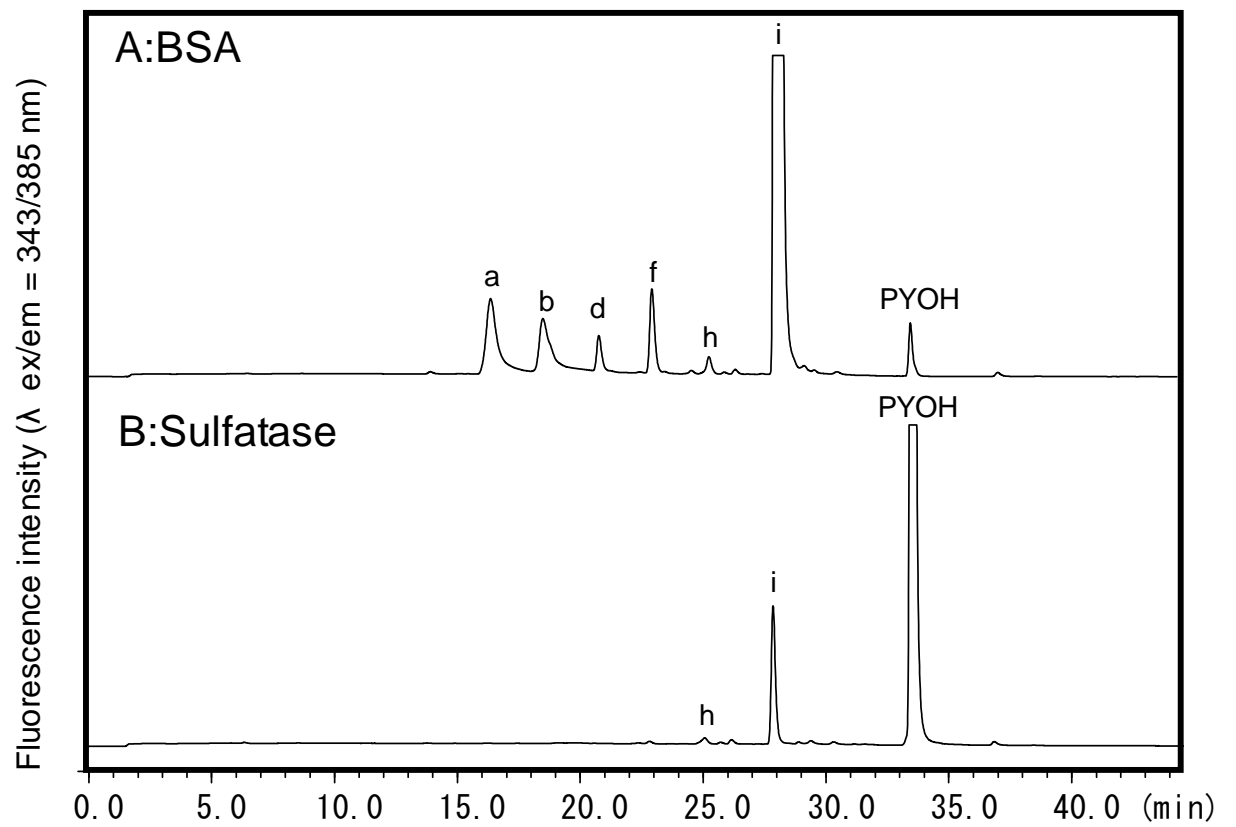
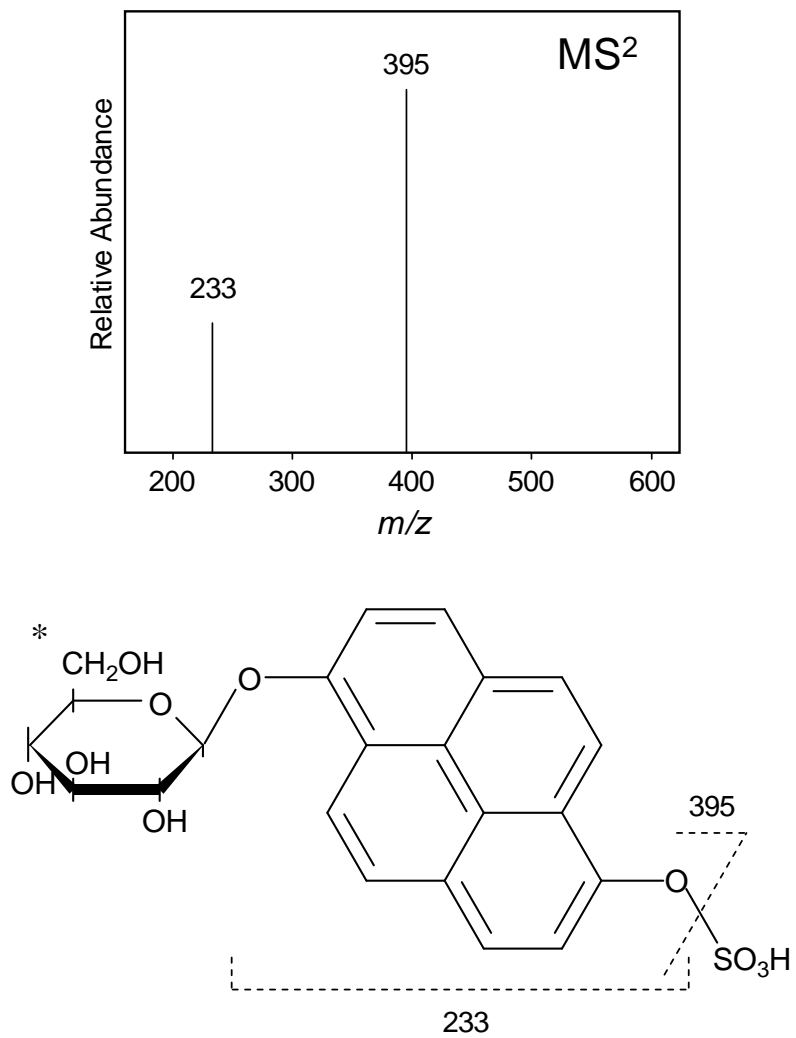


Fig. 3



MW = 476: pyrenediol glucose sulfate

Fig. 4

