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Identification of perfluorooctane sulfonate binding protein in the plasma of tiger pufferfish *Takifugu rubripes*

Masato Honda [a], Akemi Muta [a], Taiki Akasaka [b], Yoshiyuki Inoue [c], Yohei Shimasaki [a], Kurunthachalam Kannan [d], Nozomu Okino [e], Yuji Oshima [a,*]

[a] Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan
[b] Center for Advanced Instrumental and Educational Supports, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan
[c] Chemical Biotesting Center, Chemicals Evaluation and Research Institute, Bunkyo-ku, Tokyo 112-0004, Japan
[d] Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany, Albany, NY 12201-0509, USA
[e] Laboratory of Marine Resource Chemistry, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan

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ABSTRACT

It is well known that perfluorooctane sulfonate (PFOS) preferentially accumulates in the plasma of wildlife and humans. Although earlier studies have suggested that this was due to binding of PFOS to a plasma protein, definite characterization of the protein in *in vivo* exposure studies was not conducted thus far. In this study, we conducted both *in vitro* and *in vivo* experiments to identify PFOS binding protein in the plasma of fish. For the *in vivo* studies, PFOS was administered intraperitoneally to tiger pufferfish, *Takifugu rubripes*, and the plasma was separated by ammonium sulfate fractionation. High concentrations of PFOS were found in the 65–70 percent ammonium sulfate fraction (190 ng/mL). After SDS-PAGE and N-terminal amino acid sequence analysis, the PFOS-binding protein was identified as an apolipoprotein A–I, which was confirmed on the basis of a significant correlation to the PFOS concentration in each fraction. The plasma samples fractionated by ammonium sulfate from untreated pufferfish were subjected to PFOS binding assay by the equilibrium dialysis method. The results further confirmed that the 60–65 percent ammonium sulfate fraction showed a high PFOS-binding ratio, similar to that found from *in vivo* studies. We demonstrated that PFOS is likely bound to an apolipoprotein A–I in the plasma of tiger pufferfish in *in vivo* and *in vitro* studies.

1. Introduction

Because of the high energy carbon-fluorine bond and amphipathic property obtained from hydrophilic (–SO_3H) and hydrophobic (CF_3–$(CF_2)_7$–) moieties, perfluorooctane sulfonate ($C_8HF_{17}O_3S$, PFOS) is a stable compound and possesses superior properties such as chemical and abrasion resistance, and surfactant characteristics (Giesy and Kannan, 2002). In industrial and commercial applications, PFOS is widely used all over the world as a mist suppressant, leveling agent, aqueous film forming foam, surfactant, and coating agent (Giesy and Kannan, 2002). Following widespread use for over five decades, PFOS was identified as a global environmental pollutant in 2001 (Giesy and Kannan, 2001; Harada and Koizumi, 2009; Taniyasu et al., 2003). Several studies have reported that PFOS is widely distributed in seawater, fish, sea birds, marine mammals, and polar bears (Bossi et al., 2005; Hölzer et al., 2011; Houde et al., 2011; Smithwick et al., 2005; Yamashita et al., 2008). PFOS is also an ubiquitous contaminant in human blood (Kannan et al., 2004; Olsen et al., 2003; Zhao et al., 2012).

In 2009, PFOS was banned or regulated in many countries, and was designated as a persistent organic pollutant (POP) by the Stockholm Convention (Lindstrom et al., 2011). In Japan, PFOS was banned from industrial use in 2009. However, several countries continue to use PFOS and because of its persistent and bioaccumulative properties, its harmful effects as an environmental pollutant will continue in the near future (Zareitalabad et al., 2013).

PFOS concentrations in wildlife, especially fish, are higher in plasma and liver than in other tissues (Martin et al., 2003; Ahrens et al., 2009). For example, in rainbow trout, *Oncorhynchus mykiss*, PFOS accumulated in blood at higher levels than in liver and other tissues (Martin et al., 2003); in Japanese scad, *Trachurus japonicas*, the mean PFOS concentrations were 170 ng/mL and 9 ng/g in blood and liver, respectively (Taniyasu et al., 2003). Reports from the National Health and Nutrition Examination Survey (NHANES) of the United States indicate that PFOS was detected in human serum at concentrations ranging from below the limit of detection to 435 ng/mL, and PFOS was detected in 99.9 percent of 2094 specimens analyzed (Calafat et al., 2007). In rats, urinary and fecal

* Corresponding author. Fax: +81 92 642 2904.
E-mail address: yoshima@agr.kyushu-u.ac.jp (Y. Oshima).

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excretion was suggested as the primary clearance route for PFOS (Cui et al., 2010), and the existence of a PFOS-binding substance in serum was suggested to contribute to the slow elimination of this compound; *i.e.*, cynomolgus monkeys ca. 200 days (Seacat et al., 2002) and human 5.4 years (Olsen et al., 2007). PFOS binding might play an important role in the accumulation, distribution, and elimination of this compound in fish. However, earlier studies have not characterized proteins that bind to PFOS in fish.

The mechanisms of accumulation of PFOS in plasma/serum and the biological consequences are still unclear. It is reported that PFOS had a weak affinity for serum albumin like protein in carp and bound strongly to bovine serum albumin, in an *in vitro* assay (Jones et al., 2003). PFOS was reported to be associated with albumin in cows (D'Alessandro et al., 2013), humans (Salvalaglio et al., 2010), and liver fatty acid-binding proteins in mammals (Luebker et al., 2002). Albumin is known to bind and transport several metabolites, drugs, and organic compounds (Curry et al., 1998). However, these studies were conducted in *in vitro* systems, and prior to this study, no *in vivo* studies have been performed. Furthermore, most of the earlier studies were focused on mammals, and only a few studies were performed on fish, which indicated a link with PFOS contamination in humans (Falandysz et al., 2006).

Tiger pufferfish (*Takifugu rubripes*) is an excellent marine fish model for studies in biology and environmental toxicology. This fish species has been cultured in East Asia, and its whole genome analysis is characterized. Therefore, it is possible to identify proteins associated with genes in this species and this fish species can make an excellent ecotoxicological animal model. This study was performed to identify PFOS-binding proteins in the plasma of tiger pufferfish using both *in vitro* and *in vivo* tests.

2. Materials and methods

2.1. Chemicals and standards

PFOS sodium salt was purchased from Strem chemicals (> 97 percent; Newburyport, MA, USA), and PFOS potassium salt was purchased from National Metrology Institute of Japan (9.93 ± 0.15 mg/kg, Tsukuba, Japan). The internal standard, MPFOS (sodium perfluoro-1-[1,2,3,4,-$^{13}C_4$] octanesulfonate), was purchased from Wellington laboratories (50.0 ± 2.5 μg/mL; Guelph, ON, Canada). All organic solvents were LC/MS grade, and other chemicals were analytical grade. Ultra-pure water was prepared using a Simplicity UV system (Millipore, Billerica, MA, USA).

2.2. In vivo experiments with pufferfish

Three cultured tiger pufferfish (average body weight: 453 ± 55 g, average fork length: 24.7 ± 0.8 cm) were purchased and acclimatized for 2 days in a 500-L tank under flow-through conditions with filtered seawater. All fish were administered intraperitoneally with 0.5 mg/mL PFOS in 0.9 percent (w/v) saline at dose of 0.5 mg PFOS/kg bodyweight. The fish were maintained in the tank at ambient temperature for normal day length hours. After 14 days, the fish were dissected and blood was drawn from the hepatic portal vein using a syringe precoated with sodium heparin. The blood was then centrifuged at 1110 × *g* for 10 min at 4 °C and the upper layer was collected and pooled as the plasma sample. The plasma was stored at − 80 °C until analysis. No mortality was observed during the exposure period. Experiments were conducted in accordance with the Japanese Ministry of Education, Culture, Sports, Science and Technology and Kyushu University guidelines for the protection of animal welfare.

2.3. In vivo experiments with plasma protein fractionation

Ten milliliters of pooled plasma were placed in a beaker and finely milled ammonium sulfate was added gradually with continuous stirring for 30 min to precipitate protein. The concentration of ammonium sulfate was increased at 0, 20, 30, 35, 40, 45, 50, 55, 60, 65, 70, 80 and 100 percent saturation. After precipitation, the sample was centrifuged at 17750 × *g* for 30 min at 4 °C. The supernatant was removed and subject to further fractionation. The pellets were resuspended in 3 mL of phosphate buffered saline (PBS, pH 7.4) and stored at − 30 °C until analysis. All operations were performed at < 4 °C. Protein concentrations in fractionated samples were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Each suspension was analyzed for its PFOS concentration.

2.4. In vivo experiments: SDS-PAGE

To confirm the composition of protein in resuspended samples, sodium dodecyl sulfate polyaclylamide gel electrophoresis (SDS-PAGE) was conducted as follows. The acrylamide solution for the running gel composed of 10 percent (w/v) acrylamide, 0.27 percent (w/v) bisacrylamide, 375 mM Tris–HCl (pH 8.8), 0.1 percent (w/v) SDS, 0.1 percent (w/v) ammonium persulfate (APS), and 0.04 percent (w/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). The stacking gel composed of 5 percent (w/v) acrylamide, 0.13 percent (w/v) bisacrylamide, 125 mM tris–HCl (pH 6.8), 0.1 percent (w/v) SDS, 0.1 percent (w/v) APS, and 0.001 percent (w/v) TEMED. The electrode buffer composed of 192 mM glycine, 25 mM tris–HCl, and 0.1 percent (w/v) SDS. The sample buffer composed of 10 percent (v/v) 2-mercaptoethanol, 10 percent (v/v) sucrose, 0.01 percent (w/v) bromophenol blue, 125 mM tris–HCl (pH 6.8), and 4 percent (w/v) SDS. The plasma samples, adjusted to 0.5 mg protein/mL using PBS, were mixed at 1:1 (v/v) with sample buffer and heated for 5 min at 95 °C. The samples were run at 7.5 mA in an electrophoresis cell (AE-6500 Dual Mini Slab Kit; ATTO, Tokyo, Japan) at room temperature until the dye front reached the bottom of the stacking gel, and then at 15 mA until the dye front reached about 0.5 cm above the bottom of the running gel. The molecular weights of the protein bands were calculated by referring to the standard protein markers (Broad range protein molecular markers; Promega, Madison, WI, USA). After electrophoresis, the gels were fixed using a fixing solution composed of 10 percent (v/v) acetic acid and 40 percent (v/v) methanol and stained with Quick-CBB solution (Coomassie Brilliant Blue; Wako, Osaka, Japan).

2.5. In vivo experiments: Image analysis

Protein bands in the gels were imaged and evaluated as the intensity of expression using a CS Analyzer ver 3.0 (ATTO). The correlation between the intensity of the protein band on SDS-PAGE and PFOS concentrations per protein (ng PFOS/mg protein) was evaluated by simple linear regression analysis (R software, 2.15.3, Ihaka and Gentleman, 1996). The level of significance was set at $p < 0.05$.

2.6. In vivo experiments: N-terminal amino acid sequencing

The protein fraction that was significantly related to PFOS concentration as separated by SDS-PAGE was then electroblotted onto polyvinylidene fluoride membranes (Trans-Blot; Bio-Rad, Hercules, CA, USA) by the semi-dry method under the following conditions. The sample was run at 2 mA/cm^2 of membrane for 90 min using a semidry transblot apparatus (AE6675; ATTO). The buffer was composed of 192 mM glycine, 100 mM tris–HCl, and 0.1 percent (w/v) SDS. N-terminal amino acid sequencing of the blotted sample was performed by Edman degradation with a PPSQ-31A protein sequencer (Shimadzu, Kyoto, Japan). The N-terminal amino acid sequence was determined by using Basic Local Alignment Search Tool (BLAST) to compare it against the non-redundant protein sequences database as provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/, Bethesda, MD, USA).

2.7. In vitro tests: Plasma specimens

Four cultured tiger puffer fish (no PFOS treated, average body mass: 825 ± 20 g, average fork lengths: 26.8 ± 0.9 cm) were dissected and plasma was collected as described previously. The pooled plasma was stored at − 80 °C until analysis.

2.8. In vitro experiments: Equilibrium dialysis binding assay

The ammonium sulfate fractionation of plasma was performed as described previously. The concentration of ammonium sulfate was increased at 0, 20, 30, 40, 45, 50, 55, 60, 65, 70, 80, and 100 percent saturation. The pellets were resuspended in 3 mL of PBS and stored at − 30 °C until analysis. The protein concentration of the fractionated samples was determined, and adjusted using PBS to 0.1 mg protein/mL. The equilibrium dialysis binding assay was conducted using a multiple equilibrium dialysis cell (Sanplatec Co., Osaka, Japan). The visking dialysis membrane (molecular weight cut-off at 14 kDa; Sanplatec Co., Osaka, Japan) was rinsed in PBS, soaked in PBS for 1 h, and then placed in the multiple equilibrium dialysis cell. The 2-mL aliquot of resuspended sample and the 2-mL aliquot of PFOS solution in PBS (0.1 μM) were introduced inside the chamber and both sides of the outside chambers, followed by incubation of the apparatus at 15 °C for 24 h to reach equilibrium. After 24 h, the sample solutions in each chamber were collected, and the concentrations of PFOS in the samples were measured. The binding ratio was calculated as A/B; where A was the PFOS concentration in the sample solution in the inside chamber and B was the PFOS concentration in the outside chambers.