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Purification and Characterization of Tributyltin-binding Protein Type 2 from Plasma of Japanese Flounder, *Paralichthys olivaceus*

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We used gel filtration chromatography, anion-exchange chromatography and polyacrylamide gel electrophoresis to purify tributyltin-binding protein type 2 (TBT-bp 2) from plasma of Japanese flounder (*Paralichthys olivaceus*) injected intraperitoneally with TBT (5.0 mg/kg body weight). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that the molecular mass of TBT-bp 2 was approximately 48 kDa, and isoelectric focusing–polyacrylamide gel electrophoresis indicated that the isoelectric point was approximately 3.0. TBT-bp 2 contained 40% *N*-glycan. The complete cDNA nucleotide sequence and the genome sequence of TBT-bp 2 were determined by means of rapid amplification of cDNA ends of liver tissue of Japanese flounder and a genome-walking technique, respectively. The 216 amino acid sequence of TBT-bp 2 showed 47% identity to the sequences of puffer fish (*Takifugu pardalis*) saxitoxin- and tetrodotoxin-binding protein but only 27% similarity to the sequence of TBT-bp 1. Analysis of the motif sequence of the amino acid sequence and the structure of the gene encoding TBT-bp 2 suggested that this protein belongs to the lipocalin superfamily.

Key words: detoxification, glycoprotein, Japanese flounder, lipocalin superfamily, serum protein.

Abbreviations: AGP, α1-acid glycoprotein; cds, coding sequences; dNTP, deoxyribonucleotide triphosphate; EST, expressed sequence tag; GSPs, gene-specific primers; GRE, glucocorticoid-response element; IEF–PAGE, isoelectric focusing–polyacrylamide gel electrophoresis; MT, metallothionein; native-PAGE, native-polyacrylamide gel electrophoresis; PFAs, perfluorinated alkylated substances; PFOS, perfluorooctanesulfonate; PSTBP, saxitoxin- and tetrodotoxin-binding proteins of the puffer fish (*T. pardalis*); RACE, rapid amplification of cDNA ends; TBT, tributyltin; TBT-bp, tributyltin-binding protein; TBTCl, tributyltin chloride; TPhTCl, triphenyltin chloride.

When xenobiotics are absorbed by an aquatic organism, they generally accumulate in the liver and adipose tissues because of their high hydrophobicity. However, tributyltin (TBT), perfluorooctanesulfonate (PFOS), and perfluorinated alkylated substances (PFAs), which are marine pollutants, accumulate in the blood, as indicated by analysis of blood samples from fish collected during field and exposure studies (*1–4*).

In a previous work, we demonstrated that accumulation of high levels of TBT in the blood of the cultured Japanese flounder (*Paralichthys olivaceus*) is attributable to the presence of TBT-binding protein 1 (TBT-bp 1) (*5*). TBT-bp 1 is a glycoprotein with 42% *N*-glycan content, and its molecular mass and isoelectric point are 46.5 kDa and 3.0, respectively. The large amount of *N*-glycan and the amino acid sequence suggest that TBT-bp 1 is a homologue of α1-acid glycoprotein (AGP), which belongs to the lipocalin superfamily. AGP is one of the major serum glycoproteins found in the blood of mammals, and it has the ability to bind and transport various drugs and steroid hormones. Three genetic variants of human AGP have been reported, and only one of the three can be induced by acute-phase stimuli. In addition, the human protein is reported to have various immunomodulatory effects and may also have anti-inflammatory effects (*6–8*).

The proteins involved in the binding and accumulation of xenobiotics *in vivo* among fishes are fairly well understood. One such protein, metallothionein (MT), is known to bind mainly to heavy metals. The affinity of MT for TBT is unclear; however, organotin compounds can increase the MT content in the liver and pancreas of red sea bream (*Pagrus major*) and red carp (*Cyprinus carpio* Linné) (*9*). In this study, we found a novel TBT-binding protein (TBT-bp 2) which was induced in the blood of Japanese flounder intraperitoneally injected

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB277758 and AB277759.
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with TBT. We purified this novel TBT-bp 2 from the fish and analysed its genome sequence and predicted its physiological function.

MATERIALS AND METHODS

Fish—Cultured Japanese flounder (*P. olivaceus*) were obtained from Kumamoto Prefecture Fisheries Research Center in October 1996. Tributyltin chloride (TBTCl; Tokyo Kasei Kogyo, Japan) was dissolved in corn oil (10 mg/ml) and intraperitoneally injected into 15 flounder [average body weight (b.w.) 646 ± 120.6 g] at a single dose of 5.0 mg/kg b.w. Seven days after the injection, blood from the caudal vessel of each fish was collected with a heparinized syringe. The blood was centrifuged at 3,000 r.p.m. for 10 min, and the plasma was separated. The resulting plasma was stored at 4°C.

For gene analysis, one flounder (b.w. 600 g) was obtained in September 2004 and another one was obtained (b.w. 620 g) in February 2005 from Heisei Suisan (Fukuoka Prefecture, Japan). Whole-blood suspension was collected as described above and stored at −80°C. Liver tissue was collected and stored in Trizol (Invitrogen, Carlsbad, CA, USA) at −80°C.

Purification of TBT-Binding Protein Type 2—The plasma of flounder treated with TBT was filtered through a filter with a pore size of 0.5 μm (LCR 13-CH, Millipore, Bedford, MA, USA), and fractionated by gel filtration chromatography with a HiLoad 16/60 Superdex 200 pg (prep grade) column (GE Healthcare, Little Chalfont, Buckinghamshire, England). The gel was equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 5 mM ethylenediaminetetraacetate (EDTA). The sample was eluted with the same buffer at a flow rate of 1 ml/min, and 1 ml fractions were collected. The protein in each fraction was detected by monitoring the absorbance at a wavelength of 280 nm. The TBT concentrations in each fraction were analysed on a gas chromatograph equipped with a flame photometric detector, according to a method in the literature (*10*), with a slight modification in that extraction of TBT was carried out with a methanol solution of 1 N HCl rather than a tetrahydrofuran solution of 1 N HCl. The fractions containing TBT were combined and centrifuged at 10,000 r.p.m. for 10 min to remove precipitates. The molecular mass of the substance containing the TBT was estimated by gel filtration chromatography using bovine serum albumin (MW 66,000) and carbonic anhydrase (MW 29,000) as molecular mass markers.

The combined above sample was passed through an albumin affinity column (Blue Sepharose CL-6B, GE Healthcare) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl and 5 mM EDTA for removal of albumin from the sample. Serum albumin binds this affinity support in a less specific manner by electrostatic and/or hydrophobic interactions and was eluted by competitive elution with free cofactor, NaCl or KSCN, in elution buffer. The sample was eluted with 50 ml of 200 mM NaCl and then stepwise with 25 ml portions of 300 mM, 500 mM and 1 M NaCl and 500 mM KSCN in the same buffer. The flow rate was maintained at 2 ml/min, and 5 ml fractions were collected. The fractions containing TBT were combined, concentrated, desalted with Centriplus concentrators (Millipore), and then filtered through a 0.5 μm pore-size filter.

The resulting sample was loaded onto an anion-exchange column (Resource Q, GE Healthcare) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 5 mM EDTA, and was eluted with a linear salt gradient (0–500 mM NaCl for 25 min). The flow rate was maintained at 1 ml/min, and 1 ml fractions were collected.

Gel Electrophoresis—The purity of the TBT-bp 2 in the Resource Q fractions was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), native-polyacrylamide gel electrophoresis (native-PAGE) and isoelectric focusing–polyacrylamide gel electrophoresis (IEF–PAGE), followed by Coomassie blue staining.

SDS–PAGE was conducted as follows. The acrylamide solution for the running gel was composed of 10% (w/v) acrylamide, 0.27% (w/v) bisacrylamide and 375 mM Tris–HCl (pH 8.8). The stacking gel was composed of 4.5% (w/v) acrylamide, 0.12% (w/v) bisacrylamide and 60 mM Tris–HCl (pH 6.8). The electrode buffer was composed of 192 mM glycine, 25 mM Tris–HCl (pH 8.3) and 0.1% (w/v) SDS. The sample buffer was composed of 12% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 100 mM Tris–HCl (pH 6.8) and 4% (w/v) SDS. The samples were mixed 1:1 (v/v) with sample buffer and heated for 2 min at 90°C. The samples were run at 7.5 mA in an electrophoresis cell (AE-6400, 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 the bottom of the running gel.

Native-PAGE was conducted under the conditions used for SDS–PAGE but without denaturation of samples by SDS, 2-mercaptoethanol or heating at 90°C. No SDS and 2-mercaptoethanol were also contained in the electrode buffer and gel.

IEF–PAGE was conducted as follows. The buffer in the samples was replaced by distilled water. The resulting samples were run at 100 V for 15 min, 200 V for 15 min and 450 V for 60 min in a Model III mini IEF cell (Bio-Rad, Hercules, CA, USA). The gel was composed of 4.85% (w/v) acrylamide, 0.15% (w/v) bisacrylamide, 5% (v/v) glycerol and 2% carrier ampholytes (pH 3.5–9.5) (Bio-Lyte, Bio-Rad).

The purified TBT-bp 2 was enzymatically digested with a De-*N*-glycosylation set (Takara, Tokyo, Japan), and the deglycosylated sample was analysed by SDS–PAGE.

N-Terminal Amino Acid Sequencing and Determination of cDNA—TBT-bp 2 separated by SDS–PAGE was electroblotted onto polyvinylidene fluoride membranes (Trans-Blot, Bio-Rad) by the semi-dry method (*11*) under the following conditions. The sample was run at 2 mA/cm^2 of membrane area for 90 min with a semi-dry transblot apparatus (AE6675, ATTO). The buffer was composed of 192 mM glycine, 100 mM Tris–HCl (pH 8.3) and 0.02% (w/v) SDS. N-terminal amino acid sequencing of the blotted sample was performed by Edman degradation (*12*) with a PSQ-1 protein sequencer (Shimadzu, Kyoto, Japan).

The cDNA sequence for TBT-bp 2 was determined by means of a homology search of the determined N-terminal amino acid sequence by using the FASTA program against the expressed sequence tag (EST) database in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/, Shizuoka Prefecture, Japan).

RNA Isolation and Rapid Amplification of cDNA Ends (RACE)—Total RNA was extracted from liver tissue by using Trizol reagent according to the manufacturer's instructions (Invitrogen). Extracted RNA was diluted to a standardized RNA concentration of 1 μg/μl and stored at −80°C.

To obtain the complete 5′ ends of TBT-bp 2 cDNA, 5 μg of RNA was used as a template for adaptor-ligated cDNA synthesis by using a GeneRacer Kit (Invitrogen). For 5′ RACE, gene-specific primers (GSPs) were designed from the sequence of the identified cDNA. Nested PCR amplifications were performed by using the two GSPs: 5′ RACE-R1 (5′-TGTCGACAGTGATCCGAGA-3′; outer) and 5′ RACE-R2 (5′-CTAAACATGCTCTCGCCGAG-3′; inner). A 50-μl primary PCR reaction contained 5 μl of 10×PCR buffer, 4 μl of dNTP (2.5-mM each), 1.5 μl of GSP (10 μM), 4.5 μl of adaptor primer (10 μM), 1 μl of adaptor-ligated cDNA library and 0.5 μl of Ex *Taq* (5 U/μl, TaKaRa). The amplification profile consisted of heat denaturation as follows: 94°C for 2 min; 5 cycles at 94°C for 30 s and 72°C for 1 min; 5 cycles at 94°C for 30 s and 70°C for 1 min; 25 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 1 min; 72°C for 10 min.

The secondary PCR reaction was carried out as described for the primary PCR, except that 1/50 diluted primary PCR product was used for the template, and 1-μl of each of the appropriate primers was used. The amplification profile consisted of heat denaturation as follows: 94°C for 2 min; 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 2 min; and 72°C for 10 min. For 3′ RACE, RT–PCR was performed with oligo$(dT)_{20}$ primer and a SuperScript III First Strand Synthesis System (Invitrogen) in accordance with the manufacturer's instructions. Nested PCR amplifications were performed with the two GSPs: 3′ RACE-F1 (5′-ACATCACCAGTTCCCACGTC-3′; outer) and 3′ RACE-F2 (5′-ACATGTCGTCGTCCTCTGTG-3′; inner). The PCR reaction and the amplification profile for primary and secondary PCR were the same as for 5′ RACE PCR.

The resulting PCR products were purified and cloned by using a TOPO TA Cloning Kit (Invitrogen) and sequenced with a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Homology and Motif Search—Homologies of the cDNA sequence encoding TBT-bp 2 and the amino acid sequence deduced from the cDNA sequence were searched by means of the FASTA and BLAST programs against the nucleotide sequence database in the DNA Data Bank of Japan and the Swiss-Prot protein sequence database (http://www.expasy.ch/sprot). Motifs of the amino acid sequence of TBT-bp 2 and other known proteins were searched by using GenomeNet (http://www.genome.jp/).

DNA Sequencing Analysis—The complete genome sequence of TBT-bp 2 was determined with a Universal GenomeWalker Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, four genomic libraries were constructed by digestion of *P. olivaceus* genomic DNA extracted from a whole-blood suspension (25×10^7 cells) with *Dra*I, *Eco*RV, *Pvu*II and *Stu*I and subsequent ligation of an adaptor to the ends of the genomic fragments. Nested PCR amplifications were performed with the adaptor-specific primers AP1 (5′-GTAATACGACT CACTATAGGGC-3′) and AP2 (5′-ACTATAGGGCACGC GTGGT-3′), in combination with two GSPs designed from the cDNA sequence. To isolate the 5′ end of the genomic sequence, GSP-R1 (5′-TGTCGACAGTGAATCCGAGA-3′) and GSP-R2 (5′-GATTTGTACGAGAGCGGCTC-3′) were used in the first and second rounds, respectively. The 3′ end of the genomic sequence was isolated by using GSP-F1 (5′-ACATGTCGTCGTCCTCTGTG-3′) as the outer primer and GSP-F2 (5′-GTGGAGAAGGACGG AGTTGT-3′) as the inner primer. A 50-μl PCR reaction contained 5 μl of 10× PCR buffer, 1 μl of dNTP (10 mM), 1 μl of GSP (10 μM), 1 μl of adaptor primer (10 μM), 1 μl of adaptor-ligated genomic library (for primary PCR) or 1/30 diluted primary PCR product (for secondary PCR) and 1 μl of 50× BD Advantage 2 Polymerase Mix (BD Biosciences Clontech). The amplification profile consisted of heat denaturation as follows: 95°C for 1 min; 30 cycles of 95°C for 30 s, 67.7°C for 1 min (for primary PCR) or 72°C for 3 min (for secondary PCR); and 72°C for 10 min. The resulting PCR products were cloned and sequenced by the method used for the RACE PCR products.

The known consensus transcription factor binding sequences were searched by using the GENETYX program (Software Development Co., Tokyo, Japan).

RESULTS

Purification of TBT-bp 2—TBT-bp 2 was purified by a three-step procedure from plasma of the Japanese flounder injected intraperitoneally with TBT. A single TBT peak (total 22,300 ng), which reached a maximum value (465 ng TBT/ml) in fraction number 80, was observed in fractions 75–83 during the elution of plasma from the Japanese flounder by gel filtration (Fig. 1). The peak for TBT concentration corresponded with the fourth peak of absorbance at 280 nm. By using molecular mass markers, we estimated the molecular mass of the substance corresponding to this peak to be approximately 50 kDa (data not shown). This value indicates that the TBT in the plasma was bound to a substance of high molecular mass, because the molecular mass of TBTCl is only 325 Da.

Figure 2 shows the elution profile of the TBT-binding substance as well as TBT concentration in the eluate obtained by albumin affinity chromatography. A high concentration of TBT was detected in the initial pass-through fraction, indicating that the TBT-binding substance was not albumin, because little TBT was detected in other high-salt fractions.

Figure 3 shows the elution profile of the TBT-binding substance by anion-exchange chromatography. TBT was detected in fractions 17–23 (total TBT amount 19,000 ng).

The purity of TBT-bp 2 (fraction 21 of the Resource Q eluate) was confirmed by the results of three types of electrophoresis. A single band stained with Coomassie blue was observed by native-PAGE (Fig. 4A) and IEF–PAGE (Fig. 4C), although an additional, minor fragment was observed by SDS–PAGE (Fig. 4B). The molecular mass of TBT-bp 2 was estimated to be 48 kDa by SDS–PAGE, and its pI was estimated to be 3.0 by IEF–PAGE. The molecular mass of the substance was almost the same as that ascertained by gel filtration. These results demonstrate that TBT-bp 2 is some type of protein having anionic character.

As shown in Table 1, the recovery of TBT after the three chromatographic steps was high (89%). The concentration of TBT (4,300 ng/mg protein) in fraction 21 from anion-exchange chromatography was 86 times the concentration in the plasma.

The purified TBT-bp 2 was enzymatically digested with peptide *N*-glycosidase F. After digestion at 37°C for 20 h, SDS–PAGE showed that the molecular mass of TBT-bp 2 was reduced from 48 to 29 kDa owing to complete release of the *N*-linked sugar chains (Fig. 5). This result indicates that TBT-bp 2 consists of at least 40% *N*-glycan.

The N-terminal amino acid sequence of TBT-bp 2 was determined to be Val-Val-Glu-Glu-Thr-Asn-X-Leu-Phe-X-Val-X-Pro-Thr, where X represents an amino acid that could not be identified in this study.

cDNA Sequencing and Homology Search—To determine the cDNA sequence encoding TBT-bp 2, we performed a homology search of the EST database using the obtained N-terminal amino acid sequence for TBT-bp 2. The homology search revealed that the amino acid sequence predicted by the cDNA showed 72% identity to the detected 11 amino acid sequences for TBT-bp 2 (Genbank accession no. C23296). Consequently, this cDNA was determined to be a partial sequence for TBT-bp 2.

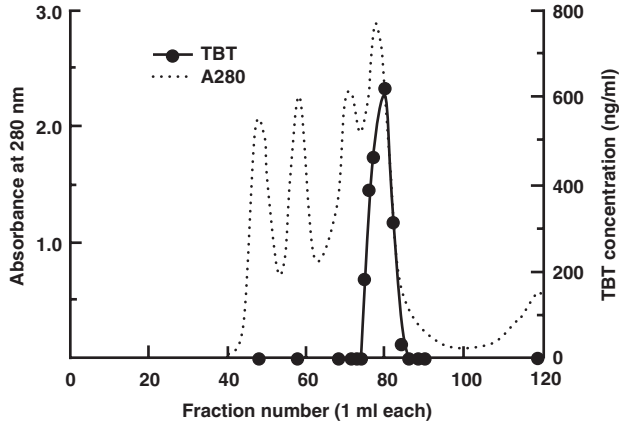

Fig. 1. **Elution profile of plasma from Japanese flounder with a Superdex 200 gel filtration column.**

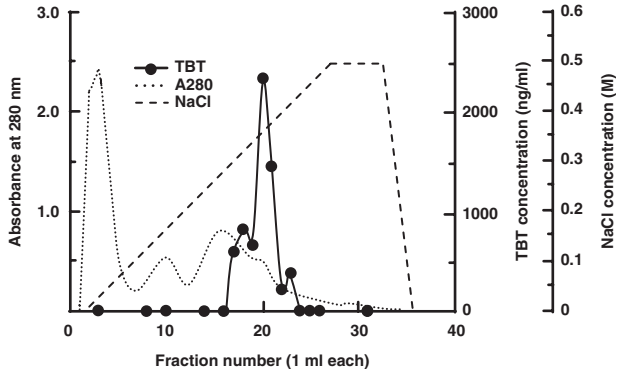

Fig. 3. **Profile of TBT-binding substance after elution in a Resource Q anion-exchange column.**

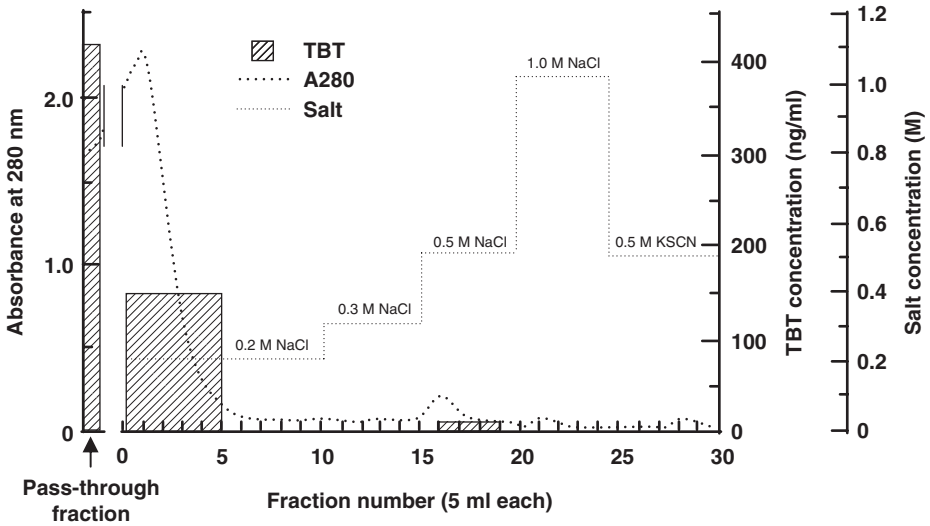

Fig. 2. **Elution profile of TBT-binding substance with a Blue Sepharose CL-6B albumin affinity column.** The volume of the pass-through fraction was *ca.* 50 ml; the volume of the other fractions was 5 ml.

The complete cDNA sequence for TBT-bp 2 was determined using 5′ and 3′ RACE of the total RNA extracted from the liver tissue of Japanese flounder (Genbank accession no. AB277759). Figure 6 shows the alignment of the complete cDNA sequence with the amino acid sequence deduced from the cDNA sequence. TBT-bp 2 full-length cDNA is 849-bp long and contains an open reading frame of 651 bp, a 5′-untranslated region (UTR) of 21 bp, and a 3′-UTR of 187 bp. A single polyadenylation signal sequence (AATAAA) is located 20 bp upstream from the poly (A) tail. The mature protein deduced from the cDNA is composed of 194 amino acids. The molecular mass of TBT-bp 2 containing 194 amino acids was calculated to be about 22 kDa, which is less than that of 29 kDa determined by SDS–PAGE after the deglycosylation treatment of TBT-bp 2. The cDNA for TBT-bp 2 was inferred to have four putative *N*-linked sugar chain binding sites and to encode an *N*-glycoprotein. Since both TBT-bp 2 and TBT-bp 1 have the same number of putative *N*-linked sugar chain binding sites, TBT-bp 2 must have a longer mature amino acid sequence and a higher molecular mass than TBT-bp 1, which has 191 amino acids.

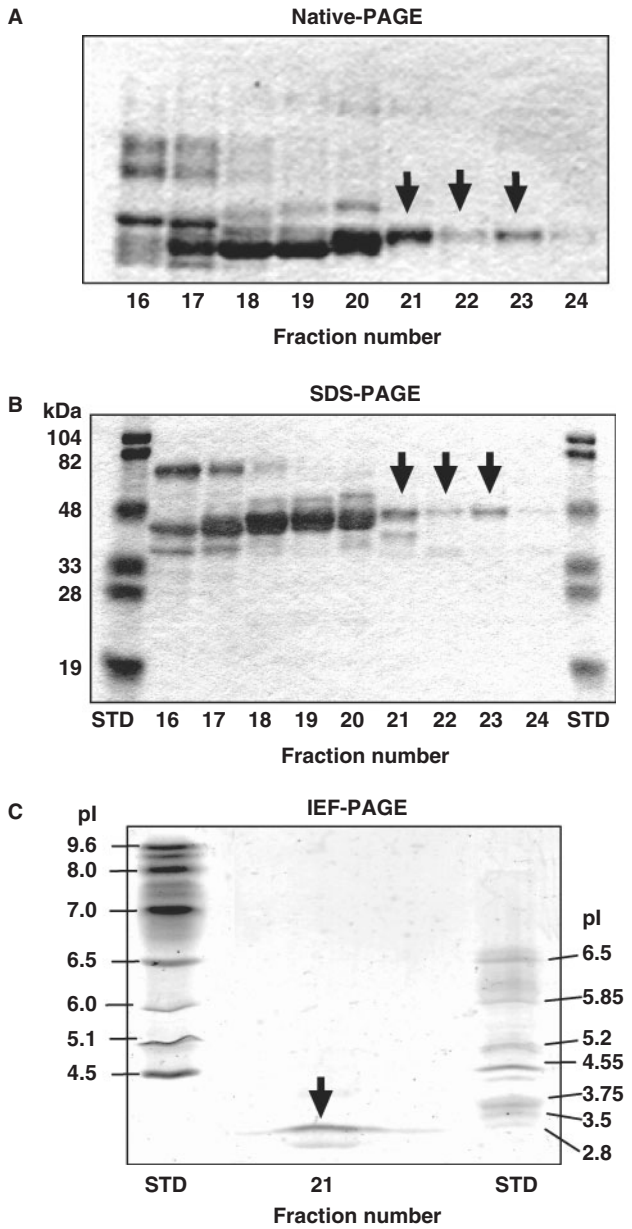

Fig. 4. **(A) Native-PAGE, (B) SDS–PAGE and (C) IEF–PAGE of Resource Q fractions.** Arrows point to TBT-bp 2. STD, standard. Two kinds of pI standards were used in IEF–PAGE.

Tables 2 and 3 show the results of homology searches of the amino acid sequence and complete cDNA sequence for TBT-bp 2. The amino acid sequence showed the highest identity (47%, 186 amino acid overlap) with puffer fish (*T. pardalis*) saxitoxin- and tetrodotoxin-binding protein 1 and 2 (PSTBP1 and PSTBP2). The sequence also showed 27% similarity with the amino acid sequence deduced for TBT-bp 1; 26% similarity with Chromosome 12 SCAF14993, a whole-genome shotgun sequence from *Tetraodon nigroviridis* (green puffer); and 25% similarity with TBT-bp (Fragment) from *Fundulus heteroclitus* (killifish, mummichog). The cDNA sequence for TBT-bp 2 showed the high similarity with complete mRNA cds of PSTBP 1 and 2.

Analysis of the Structure and Transcription Factor of the Gene Encoding TBT-bp 2—The complete genome sequence of TBT-bp 2 (GenBank accession no. AB277758) was determined by a genome-walking technique. Figure 7 shows the alignment of the sequences of the genome with the amino acid deduced from the cDNA sequence, as well as the exon/intron organization. The gene is composed of 2897 bp, and the 6 exons are interrupted by 5 introns. The 'intron phase' refers to the location of the intron within the codon. The positions of the intron within the codon are as follows: phase 0, an intron lies between two codons; phase I, an intron lies after the first nucleotide of a codon; and phase II, an intron lies after the second nucleotide. The positions of the introns are believed to be important for evaluating intron evolution (*13–15*). On the basis of the above description, the gene encoding TBT-bp 2 shows an intron phase of 0-II-I-I-I. In the 578 bp of the obtained 5′-flanking sequence, we found a TATA box (TAATA at nt. positions −28 to −24) and the sequence TGAACAGA TGTTCT at positions −273 to −260, which is similar to the sequence for a glucocorticoid-response element (GRE; AGAACANNNTGTTCT). In the 3′-flanking region, we found a single polyadenylation signal sequence (AATAAA at nt. positions 2201 to 2206).

DISCUSSION

In this study, a high concentration of TBT (21,300 ng/ml) was detected in the plasma of flounder 7 days after injection of TBT at a dose of 5.0 mg/kg b.w. In our previous study, the high concentration of TBT (1,139 ± 88 ng/ml) was detected in the serum of Japanese flounder intraperitoneally injected with TBT at a dose of 2.5 mg/kg b.w. (*16*). Shim *et al*. (*2*) reported that high concentrations of TBT accumulate in the serum of Japanese flounder exposed to TBT under 30-day static-renewal conditions (3.65, 36.5, 365 ng Sn/l

Table 1. **Purification of TBT-bp 2 from plasma of Japanese flounder.**

Purification step	Total TBT (ng)	Total protein[a] (mg)	TBT conc. (ng/mg protein)	Yield (%)	Purification (-fold)
Plasma	21,300	424	50	100	1
Superdex 200	22,300	100	223	105	4.5
Blue Sepharose	28,100	67	419	132	8.4
Resource Q	19,000	9	2,100	89	42
Fraction number 21	4,300	1	4,300	20	86

[a]Protein conc. (mg/ml) was calculated from absorbance at a wavelength of 280 nm.

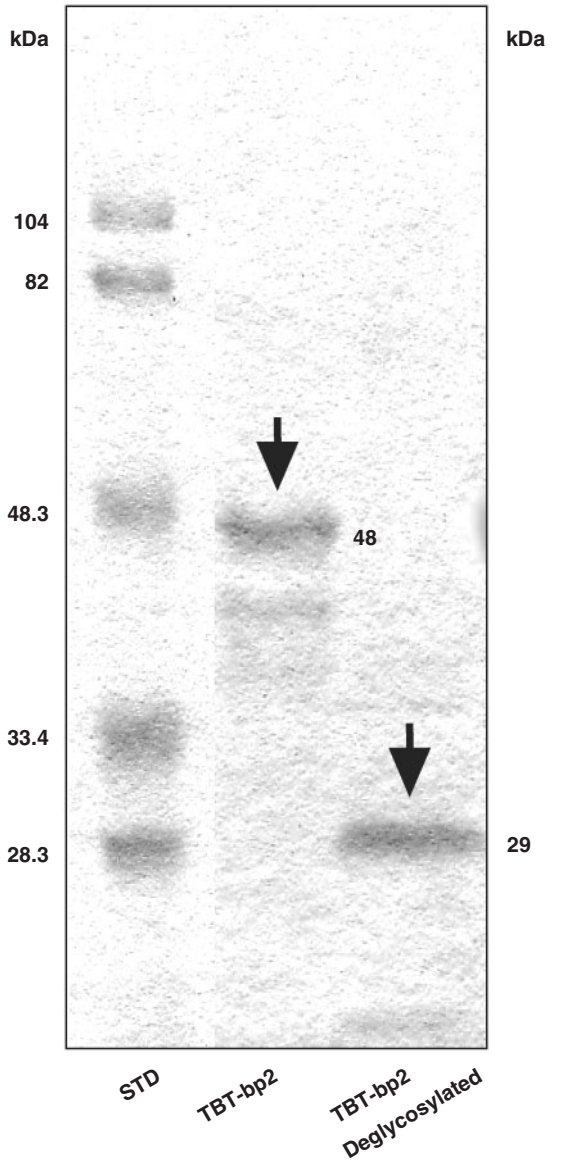

Fig. 5. **SDS–PAGE of purified and deglycosylated TBT-bp 2.** Arrows point to TBT-bp 2. STD, standard.

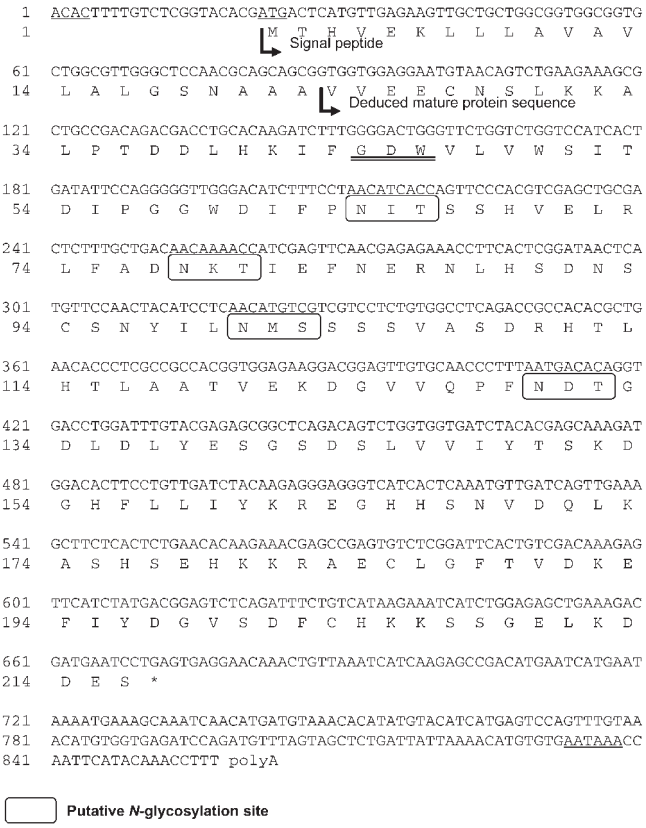

Fig. 6. **Alignment of the complete cDNA and the deduced amino acid sequence for TBT-bp 2.** Transcription start site (ACAC), start codon (ATG) and polyadenylation signal (AATAAA) are underlined. Asterisk indicates the stop codon. The first motif core sequence of the lipocalin superfamily (GDW) is double underlined.

in 800 l). Furthermore, they found a positive relationship between serum TBT concentrations in fine-spotted flounder (*Pleuronichthys cornutus*) and TBT concentrations in sediment collected from each-fish collection sites. These results suggest that blood is the best tissue for monitoring TBT pollution. We therefore purified and characterized a novel TBT-binding protein (TBT-bp 2) in the blood of flounder exposed to TBT.

From the result of gel filtration, the TBT peak was observed in the fraction corresponding 50 kDa, which was approximately 150-times larger than free TBT. Concerning the molecular mass of TBTCl (325 Da), the free TBT molecules might be eluted at later fractions. The result of Resource Q also suggests that TBT was bound to an anionic substance, because TBT is a cationic substance. These lines of fact indicate that TBT was bound to TBT-bp 2. The purified TBT-bp 2 contained 4,300 ng of TBT per milligram protein, therefore the molar TBT: TBT-bp 2 ratio was calculated to be 3:5.

Table 2. **Result of homology search of amino acid sequence for TBT-bp 2, showing 8 high-ranking proteins.**

Protein name	Identity	*E*-value	Accession no.
Saxitoxin- and tetrodotoxin-binding protein 1 [Precursor] from *Takifugu pardalis* (panther puffer)	47% identity in 186 amino acid overlap	2e-42	Q90WJ7
Saxitoxin- and tetrodotoxin-binding protein 2 [Precursor] from *Takifugu pardalis* (panther puffer)	47% identity in 186 amino acid overlap	4e-42	Q90WJ9
Tributyltin-binding protein type 1 from *Paralichthys olivaceus* (Japanese flounder)	27% identity in 190 amino acid overlap	3e-06	Q1XG02
TBT-binding protein [Fragment] from *Paralichthys olivaceus* (Japanese flounder)	27% identity in 190 amino acid overlap	3e-06	Q8UW87
Chromosome 12 SCAF14993, whole genome shotgun sequence from *Tetraodon nigroviridis* (green puffer)	26% identity in 202 amino acid overlap	3e-04	Q4RUP8
Dof zinc finger protein DOF2.5 from *Arabidopsis thaliana* (mouse-ear cress)	26% identity in 123 amino acid overlap	0.46	Q9ZPY0
Isoform 2 of Q9ZPY0 [DOF2.5] from *Arabidopsis thaliana* (mouse-ear cress)	26% identity in 123 amino acid overlap	0.46	Q9ZPY0-2
TBT-binding protein [Fragment] from *Fundulus heteroclitus* (killifish; mummichog)	25% identity in 140 amino acid overlap	1.8	Q645P8

Table 3. **Result of homology search of complete cDNA sequence for TBT-bp 2, showing 4 high-ranking genes.**

Gene name	*E*-value	Accession no.
Sparus aurata contig 63 unknown mRNA.	4.8e-34	AY550941
Takifugu pardalis PSBP1 mRNA for saxitoxin-binding protein1, complete cds.	1.5e-17	AB055707
Takifugu pardalis PSBP2 mRNA for saxitoxin-binding protein2, complete cds.	1.1e-16	AB037569
Sciaenops ocellatus clone SOC 442 microsatellite sequence.	2e-06	AY161038

Shimasaki *et al*. (*5*) reported that TBT was bound to TBT-bp 1 purified from cultured Japanese flounder. In addition, PFOS is reported to bind to albumin and to have affinity for binding to β-lipoproteins (*17*, *18*), albumin and liver fatty acid–binding protein (*19*). However, whether native protein binds to PFOS is unknown, because these studies were based on the results of *in vitro* tests.

The gene encoding TBT-bp 2 is composed of 6 exons and 5 introns, and its intron phase (0-II-I-I-I) is similar to the intron phases of members of the lipocalin superfamily (Fig. 8). Lipocalins are known to bind to hydrophobic low-molecular mass molecules. Generally, lipocalins show low levels (<20%) of overall sequence conservation, but they share highly conserved protein structures and the exon/intron gene structure among the family members. In particular, the exon/intron organization of TBT-bp 2 corresponds to that of AGP, which belongs to the lipocalin superfamily. Lipocalins also share three conserved sequence motifs, and the first motif (Gly-Xaa-Trp residues close to the N-terminus) is consistently conserved in all the lipocalin proteins (*20–22*). AGP is considered to belong to the outlier lipocalins, because it has only the first motif (*23–25*). This first motif was identified in the amino acid sequence of TBT-bp 2 (Fig. 6). Thus, TBT-bp 2, like AGP, may be categorized as an outlier lipocalin.

The molecular mass of TBT-bp 2 is 48 kDa, its pI is 3.0 and its *N*-glycan content is 40%. The characteristics of TBT-bp 2 are highly similar to those of AGP. For example, human AGP is a 41–43-kDa serum glycoprotein with a pI of 2.7–3.2 and a single chain of 183 amino acids. The *N*-glycan content makes up 45% of the molecular mass. AGP is an acute-phase protein, and its serum concentration rises several fold during an acute-phase response to, for example, inflammation, burn injury or infection. AGP is one of the major drug-binding proteins containing at least two distinct binding-sites, and it has various immunomodulatory effects and may have anti-inflammatory effects (*6–8*). Expression of the gene encoding AGP is controlled mainly by glucocorticoids and a cytokine network (*6*).

The gene encoding TBT-bp 2 may be regulated by a glucocorticoid, because the gene bears a putative glucocorticoid response element (GRE-like element) in its 5′-flanking region. TBT is well-known to have high cytotoxicity and trigger apoptosis (*26*, *27*). Thus, toxic stress due to TBT administration may induce expression of the gene encoding TBT-bp 2 in flounder. If TBT-bp 2 has immunomodulatory effects or anti-inflammatory effects as an acute-phase protein, the production of TBT-bp 2 may exert immunomodulating effects or anti-inflammatory effects.

The molecular mass, pI, and *N*-glycan content of TBT-bp 2 are close to those of TBT-bp 1(*5*). In both TBT-bp 1 and 2, the molecular mass determined by SDS–PAGE after the deglycosylation treatment was approximately 6 kDa larger than which of deduced amino acid. Causes of these differences are unknown but some modification, e.g. *O*-linked sugar chain, may contribute to it. However, the similarity of the deduced amino acid sequences of TBT-bp 1 and 2 was only 27%. Therefore, TBT-bp 2 and TBT-bp 1 are expected to have functional differences, although those differences are not yet known. In the present study, TBT-bp 2 was purified from plasma of TBT-injected flounder, whereas TBT-bp 1 was purified from serum of untreated flounder. This difference indicates that administration of TBT may induce the production of TBT-bp 2. At low TBT exposure levels, at which fish suffer no stress, TBT binds to TBT-bp 1 in blood. However, with exposure to high levels of TBT, glucocorticoid receptor or some unknown factor

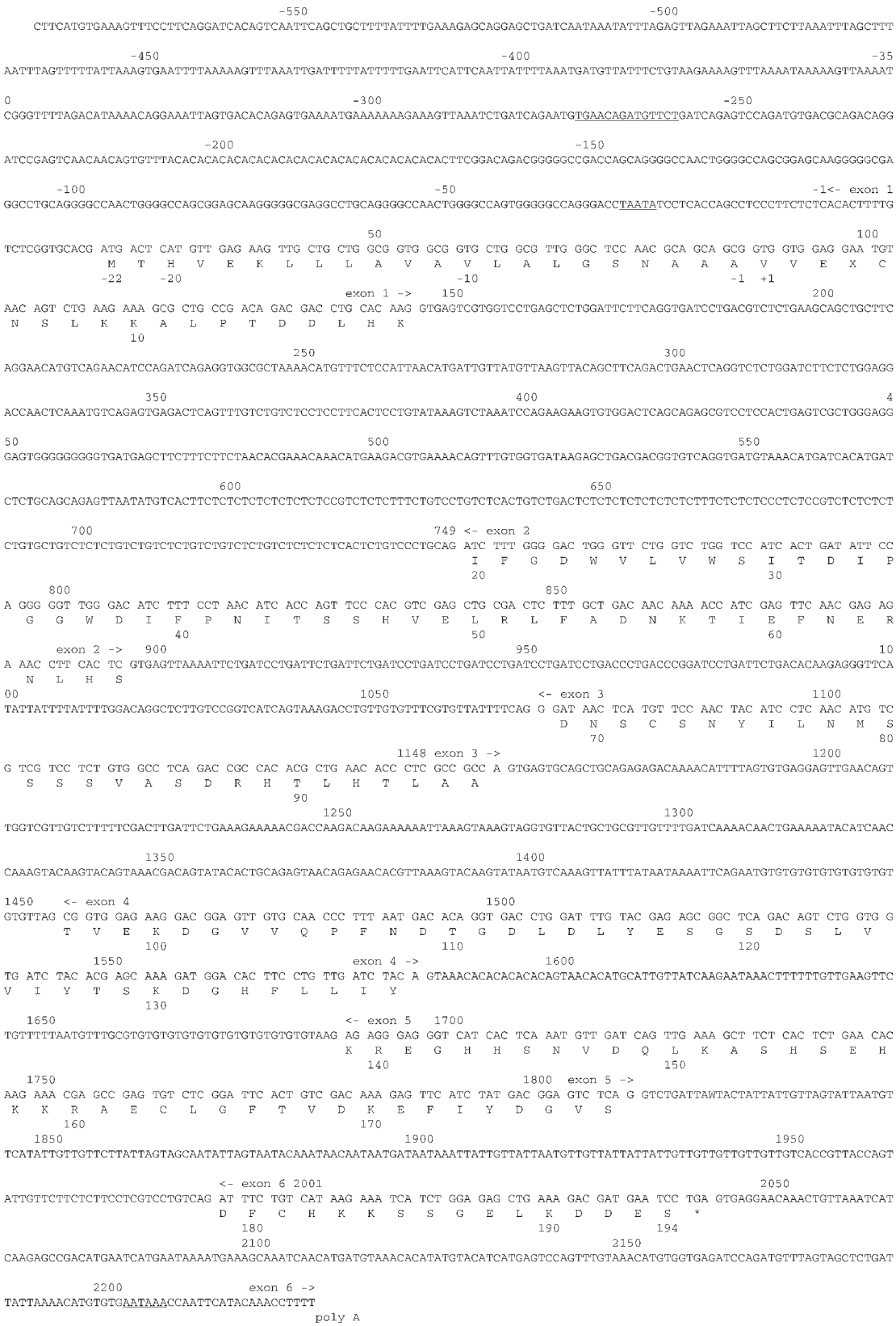

Fig. 7. **Complete genome sequence for TBT-bp 2 showing exon–intron structure and alignment with the deduced amino acid sequence.** Underlined sequences indicate a sequence similar to glucocorticoid response element (positions −247 to −260), a TATA box (position −28 to −24) and a single polyadenylation signal sequence (positions 2201 to 2206). Asterisk indicates the stop codon.

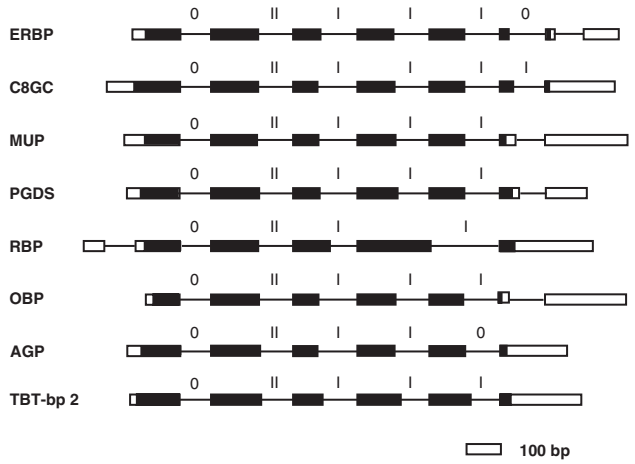

Fig. 8. **Exon/intron organizations of typical lipocalins and tributyltin-binding protein type 2.** Exons are shown as boxes; coding regions are black. Lines represent intron insertions (not drawn to scale). Phase 0, an intron lying between two codons; phase I, an intron lying after the first nucleotide of a codon; phase II, an intron lying after the second nucleotide. Abbreviations: ERBP, epididymal retinoic acid-binding protein (mouse); C8GC, complement C8γ subunit (human); MUP, major urinary protein (mouse); PGDS, prostaglandin D synthase (rat); RBP, retinol-binding protein (rat); OBP, odorant-binding protein (mouse); AGP, α1-acid glycoprotein (human); TBT-bp 2, tributyltin-binding protein type 2 (flounder).

released by the stress of exposure may increase the expression of TBT-bp 2; TBT might then bind mainly to TBT-bp 2. TBT-bp 2 may therefore play a transient but important role in detoxification and accumulation of TBT.

The amino acid and cDNA sequences of TBT-bp 2 showed homology with those of the puffer fish (*T. pardalis*) saxitoxin- and tetrodotoxin-binding proteins (GenBank accession nos. Q90WJ7 for PSTBP 1 and Q90WJ7 for PSTBP 2). PSTBPs are thought to bind to saxitoxin and tetrodotoxin involved in the accumulation or excretion of toxins in puffer fish (*28*). Additionally, the amino acid sequence of TBT-bp 2 showed identity with the deduced amino acid sequence from genome sequence of *T. nigroviridis* (Genbank accession no. Q4RUP8). These similarities might be the key to understanding the evolution of the proteins that bind to saxitoxin and tetrodotoxin. Also, high sequence identity (E-value: 4.8e-34) with the unknown mRNA of gilthead seabream (*Sparus aurata*; Genbank accession no. AY550941) was confirmed. These proteins may have a similar function in binding xenobiotics in fishes.

A few studies on xenobiotic-binding proteins in fish have been performed. The intracellular protein MT is known to bind mainly with heavy metals. TBT-bp 2 seems to be unrelated to MT, on the basis of amino acid sequence comparison and the extremely different molecular masses. MTs have been reported in many vertebrates, including fish, and in wild aquatic invertebrates; they are thought to be involved in the homoeostatic control of essential metals and in the detoxification of excess amounts of both essential and non-essential trace metals. Production of MT can be induced by metals, glucocorticoids, hydrogen peroxide, interleukin-6 and a variety of other stimuli in the kidney and liver (*29*). Kawano *et al*. (*9*) reported that the administration of TBTCl and triphenyltin chloride (TPhTCl) increases the content of MT in the liver and pancreas of red sea bream (*P. major*) and red carp (*C. carpio* Linné). However, it has not been determined that MT binds to TBT. We purified TBT-bp 2 from the blood of flounder injected with TBT at a high concentration. Tawaratsumita *et al*. (submitted for publication) report that the production of TBT-bp 2 is induced in flounder injected with TBT at a dose of 11.6 μg/fish (average b.w. 15 ± 1.9 g). We believe that TBT administration induces the production of TBT-bp 2 and that this protein reduces the toxicity of TBT by binding to it.

The deduced 216 amino acid sequence of TBT-bp 2 showed 47% identity to the sequences of PSTBP 1 and 2, although the similarity to the sequence of TBT-bp 1 was only 27%. Analysis of the motif sequence of the deduced amino acid sequence and the gene structure of TBT-bp 2 suggests that this protein belongs to the lipocalin superfamily. Thus, TBT-bp2 may play an important role in detoxification of injected TBT.

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