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- REVIEW ARTICLE-

Tetrodotoxin binding protein in the marine puffer fish

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Abstract

Marine pufferfish generally involve a potent neurotoxin, tetrodotoxin (TTX), which might be the leading cause for many human intoxications. It blocks nervous impulses' conduction along nerve fibers and axons during the act, and the LD50 for the mouse is 10 nanograms. Being much larger than the sodium ion, TTX acts as a cork of a bottle, prevents sodium from flowing until it diffuses slowly. The TTX expanse appears to be species-specific in pufferfish bodies. The toxin is thought to bioaccumulate via the marine food based on the observations that marine pufferfishes that are cultured are not toxic, and non-toxic cultured pufferfishes become toxic when they feed on TTX-containing artificial diets. TTX-bearing animals show incredibly high resistance to TTX, and therefore TTX presumably retains or accumulates as a biological defense substance. These animals carrying TTX can accumulate toxins in their bodies despite not killing themselves is an object of interest. Fort his reason, and it is argued that TTX is wrapped in a particular protein and does not bind directly to the target's side-sodium channel, and therefore does not induce intoxication. The pufferfish TTX-binding protein (PSTBP) was first isolated as a potential TTX-carrier protein from the plasma of the marine pufferfish *Takifugu niphobles*. This protein is discovered to be a dimeric glycoprotein and formed a non-covalent dimer.

Keywords:
pufferfish**,** tetrodotoxin (TTX), TTX-binding protein
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Introduction

Marine pufferfishes from the Tetraodontidae family are commonly considered significant threats to consumers due to the involvement of a potent neurotoxin called tetrodotoxin (TTX) can be lethal for humans. It is estimated that the minimum lethal dose in an adult human is 2–3 mg, but this number can vary depending on age, health. In some particular tissues such as the liver, ovary, and

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skin, TTX blocks voltage-gated sodium channels, which cause paralytic poisoning and ultimately human fatality due to respiratory insufficiency and coronary failure. (Narahashi et al., 1967; Halstead, 1988; Narahashi, 2001; Geffeney & Ruben, 2005; Soong & Venkatesh, 2006; Lee & Ruben, 2008; Walker et al., 2012;).

TTX is a weak base organic compound that is non-protein, colorless, odorless, thermostable, and acid-stable (Figure 1). The TTX molecule is formed by a moiety of guanidinium connected to a highly oxygenated skeleton of carbon which has a portion of 2,4-dioxa adamantane with five groups of hydroxyl (Isbister & Kiernan, 2005; Zhang et al., 2018**;** Pinto et al., 2019). TTX's guanidinium moiety is essential for its toxicity and initially serves as an excellent target to predict the molecule's biosynthesis because of its secondary metabolite rarity. The guanidinium moiety binds to the voltage-gated sodium channels; it forms a salt bridge between the hydroxyl groups in the sodium channels (Lipkind & Fozzard, 2005; Lee & Ruben, 2008) and thus, for TTX to be bound correctly to the receptor, it is vital. Ironically, many other species such as xanthid crabs, *Atergatis floridus*, and puffer fish, *Takifugu oblongus* and *Fugu pardalis*, which are believed to be sources of TTX, were also identified with saxitoxin production (Arakawa et al., 1995; Jang & Yotsu-Yamashita, 2006; Ngy et al., 2009). The possibility of saxitoxin and tetrodotoxin biosynthesis involving similar mechanisms is reasonable. At least 26 analogs of the TTX were identified to occur naturally (Bane et al., 2014).

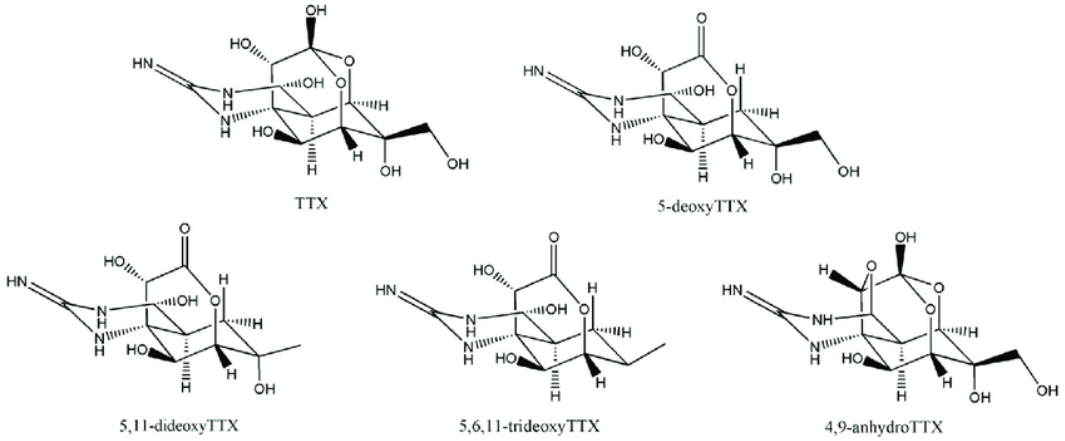

Figure 1. Chemical structure of dominant tetrodotoxin (TTX) analogues (Chau et al., 2011).

A common consensus now indicates that marine pufferfish produce TTX by bioaccumulating across the aquatic food chain (Noguchi & Arakawa, 2008), relying on the results which showed that cultured marine pufferfish is not toxic (Matsui et al., 1981; Noguchi et al., 2006) but instead of feeding on artificial TTX-containing diets, non-toxic pufferfish becomes toxic (Yamamori et al., 2004; Honda et al., 2005).

Pufferfish accumulate too high TTX concentrations with no adverse effects. Based on researchers findings (Koyama et al., 1983;Saito et al., 1985; Hwang et al., 1992; Arakawa, 2001), it is quite evident that TTX-bearing animals must be endowed with high tolerance to TTX in order to accumulative for a certain level of TTX in their bodies. Hence, in this paper, it is suggested that if TTX is wrapped in a particular protein, it does not bind directly to the target side-sodium channel and does not induce intoxication (Hwang et al., 2007).

Distribution of TTX in Pufferfish

The majority of the Tetraodontidae family pufferfish have a potent neurotoxin, tetrodotoxin (TTX). In addition to pufferfish, TTX formation and TTX content vary by several marine organisms, including certain species of gobies, poultry, gastropods, octopuses, crabs, marine flatworms, and ribbon worms (Jang & Yotsu-Yamashita, 2006; Noguchi & Arakawa, 2008; Jang et al., 2010). Moreover, it is stressed that liver and ovary use exhibits a higher accumulation of TTX and its analogs rather than muscle and testis (Bane et al., 2014). Several tissues, such as reproductive organs, liver, skin, muscle, and intestines, have been found to be spread (Jang & Yotsu-Yamashita, 2006; Jang et al., 2010). Gao et al. (2020) suggested that TTX in pufferfish (*Takifugu rubripes*) is being transferred and accumulated from exocrine pancreatic cells to hepatic parenchymal cells in the liver, from the connective tissue to the basal cells in the skin, and from the villi epithelial cells through the lamina propria to the intestinal muscle layer. Many findings indicate that pufferfish's toxification is exogenous and derived from a food chain that begins with marine bacteria (Noguchi & Arakawa, 2008). The toxicity of pufferfish, which is too great to be explained by the endogenous factor, i.e., the divergence in physiological conditions between individual puffers (Kanoh, 1988), often supports the exogenous intoxication of pufferfish, usually seen in broad individual and regional variations. (Noguchi et al., 2006).

The accumulation of this toxin along the pufferfish body and its analogs are based upon marine and freshwater habitat, sex, maturation,individual growth,species, feeding and season.

Marine or freshwater habitat

In marine pufferfish species, the level of toxicity is observed to be high in the liver and ovary; as for the brackish water and freshwater species toxicity, it is higher in the skin (Noguch & Arawaka, 2008; Bane et al. 2014; Gao et al., 2019).

Sex

Female pufferfish accumulates most of TTX within the liver and is proved to cause a higher amount of toxicity (Noguchi et al., 2011). According to Köşker et al. (2016)'s findings, *L. sceleratus* female gonads caught in the autumn and the skin of *L. suezensis* caught in the spring had the highest level of TTX. Nevertheless, no quantifiable TTX levels were detected for *L. spadiceus.* The results show that female individuals have higher TTX levels being compared to male pufferfishes during all seasons apart from summer (*Torquigener flavimaculosus*) (Köşker et al., 2018)

Maturation

In the natural habitat, *T. alboplumbeus, Takifugu pardalis,* and *Takifugu flavipterus* (formerly known as *Takifugu poecilonotus*) usually have high levels of TTX concentration in the liver and skin. However, during the ripening process, females accumulate TTX primarily in the ovary and skin, and males accumulate TTX mostly in the skin and liver, which concludes with a higher total TTX amount in females (Ikeda et al., 2010; Itoi et al., 2016; Gao et al., 2018). The findings of Wang et al. (2011), stated that hybrid specimens produced by crossbreeding *T. rubripes* with *T. Alboplumbeus,* which ripens earlier than *T. rubripes* are applied TTX in an intramuscular way; first being absorbed in the liver and then being transferred to and accumulated in the females' ovary and the males' skin.

Individual Growth

Another factor affecting the distribution of TTX within the pufferfish body is the individuals' growth. The toxication level of wild adult *T. Rubripes* is generally high in the liver and ovary, again, the skin, muscle, and testis are non-toxic (Noguchi & Arakawa, 2008), but the skin is the principal toxin-accumulating tissue in wild young fish (Ikeda, 2009; Tatsuno, 2012). Tatsuno et al. (2013a) performed a TTX administration experiment on *T. rubripes* of different ages, *in vivo* oral gavage. Results detected that the transfer of administered TTX was mostly spread to the young fish's skin, which is 6 months old); while the majority of it was accumulated and transferred to the liver of adult fish, which is 15 months old. Most of the TTX is transferred to the skin in TTX administration experiments that use non-toxic cultured young *T. Rubripes* (Honda et al., 2005; Ikeda et al., 2009). Some studies recommend that wild *T. Rubripes'* liver toxicity increases in parallel to fish's age (Kanoh et al., 1984; Fuchi et al., 1986). Hence, the toxin transfer/accumulation profiles within the pufferfish body may differ according to the stage of growth.

Species

They all are from the Tetraodontidae family. On the contrary, *L. gloveri* and *Logocephalus wheeleri,* which belong to the same family, despite showing weak toxicity occasionally, are usually declared as non-toxic species (Hwang et al., 1992). The whole species of the Diodontidae and Ostracidae family are non-toxic (Tani, 1945). *Takifugu rubripes* and *T. xanthopterus* adult fish, one of the 25 species of Takifugu genus, usually exposes high levels of toxicity both in the liver and ovaries skin, muscle, and testes are mostly non-toxic. Contrarily, the rest of the *Takifugu* species, counting *T. Porphyreus, release high levels of toxicity over the skin and* within the liver and ovaries (Tani, 1945).

Feeding

TTX derives from marine bacteria (Magarlamov et al., 2017). When artificially grew via non-toxic diets, pufferfishes like *Takifugu alboplumbeus* (formerly known as *Takifugu niphobles*) and *Takifugu rubripes* and become non-toxic (Matsui et al., 1982; Noguchi et al., 2006). If mentioned non-toxic pufferfishes are administered TTX orally, they become toxic (Honda et al., 2005; Yamamori et al., 2004). Even though the TTX in pufferfishes is considered to originate from the food chain, the body distribution of TTX diversifies among species (Noguchi et al., 2006), starting with TTX-producing marine bacteria (Noguchi & Arakawa 2008). The origination of aforesaid toxin has been fixed in an endo-symbiotic bacterium in pufferfish, TTX accumulating within their bodies through the food chain and being potential vectors of toxins (Yu et al. 2004; Noguchi & Arakawa, 2008; Bane et al., 2014). If puffer consumes TTX-containing foods, it is understood that the first place that the toxin goes to is the liver, then towards the skin/gonad and rest of the organs. This information helps clearly understand why the continual increase in the amount of toxin in the liver and skin. Further, the TTX degradation in gonads is prolonged, indicating the reason for fluctuations being limited in the toxin levels in gonads (Bane et al., 2014).

Season

During different seasons, Akbora et al. (2020) examined *L. sceleratus'* TTX levels. For TTX levels, 80 tissues were examined, of which about 40% were discovered to be toxic (>2.2 μg/g). During

spring and summer, mostly at mature fishes, the toxicity levels were higher. It can be seen that from autumn to summer, there is a regular increase in the liver and over the skin when analyzing the seasonal distributions of TTX in the tissues. Intestinal toxicity is observed to be increasing before the summer. According to the findings of Kösker et al. (2018), it was winter season when the highest TTX level was seen, as for the autumn, the TTX levels were at the lowest in various parts of pufferfishes (*Torquigener flavimaculosus*) being compared to all seasons.

Pufferfish TTX binding protein (PTBP)

How these animals carrying TTX can accumulate toxins in their bodies without killing them is still one of the unsolved mysteries that science is curious about. Consequently, it is suggested that TTX is wrapped in a particular protein and does not bind directly to the target side-sodium channel, and therefore does not induce intoxication (Hwang et al., 2007). One of the propositions about this subject is as follows; TTX is wrapped in a different protein that does not bind directly to the sodium channel as its primary target side, so it can not induce poisoning. Generally, animals carrying TTX were found to be much more resistant to the lethal effects of TTX than those without TTX. It is not fully understood how the puffer fish's TTX accumulation mechanism works in specific tissues, especially the liver, skin, and ovary. Several proteins have recently been reported that cause toxicity to this group. The process of gaining TTX resistance within skeletal muscle and neuronal voltage Na+ channels in pufferfishes, for example, happens through amino acid substitution in the protein P-loop region (Venkatesh et al., 2005; Soong & Venkatesh, 2006).

Proteins binding TTX were present in several marine species, including electric eels (Miller et al., 1983), gastropods (Hwang et al., 2007), and shore crabs (Nagashima et al., 2002), but were studied and described most extensively in pufferfishes (*Takifugu* spp.) (Matsui et al., 2000; Yotsu-Yamashita et al., 2001; Matsumoto et al., 2007; Yotsu-Yamashita et al., 2013). These proteins, which play an auxiliary role in the accumulation and transport of TTX, also bind free toxins in the host organism's plasma and tissues, toxic organisms, thereby preventing side effects from toxins (Hashiguchi et al., 2015).

As the majority of studies showed, the TTX binding protein (TBP) has been found to be common in various types of toxic pufferfish, such as following: *Arothron nigropunctatus, A. hispidus, A. manilensis, Chelonodon patoca* (Yotsu-Yamashita et al., 2018), *Fugu niphobles* (Matsui et al., 2000), *Fugu pardalis (*Yotsu-Yamashita et al., 2001; Yotsu-Yamashita et al., 2010; Yotsu-Yamashita et al., 2013), *Takifugu rubripes* (Matsumoto et al., 2010; Tatsuno et al., 2013). In addition, Hashiguchi et al., (2015) suggest that PSTBPs have a vital role in toxicity formation in Takifugu pufferfishes, but they are not a factor in toxicity formation of non-toxic pufferfish species other than Takifugu, as they do not have PSTBPs. Apparently, PSTBPs may not be necessary for nontoxic species. Besides, a toxic species called *T. nigroviridis* is predicted to have some unidentified structures other than PSTBPs to accumulate and transfer TTX. Again, Yotsu-Yamashita et al. (2018) conducted research on the presence of PTBP analogs in other toxic pufferfish species (namely, *A. hispidus, A. manilensis, Arothron nigropunctatus, and Chelonodon patoca*) except the Takifugu genus. TTXs which bind to high-molecular-weight-compounds in the species of Takifugu, similarly bind to high-molecular-weight compounds in pufferfish plasma of the three *Arothron* species and *C. Patoca*, but the binding is preferably partially in them (Yotsu-Yamashita et al. 2001; Yotsu-Yamashita et al., 2002).

Yotsu-Yamashita et al. (2010), found that pufferfish often have the same type of glycoproteins that are similar to the proteins that bind the puffer fish's saxitoxins and tetrodotoxins (PSTBP), yet N-glycan sizes are claimed to be specific for each species. Also, they suggested that PTBPS in the blood could help move TTX and STX from one organ to another, and this protein could also be included in the toxin secretion system in pufferfish skin. The immunohistochemical staining of PSTBP in *T. pardalis* tissues, which are the subject of research conducted by Yotsu-Yamashita et al. (2013), can be seen in Figure 2 and Figure 3. They claimed that the tetrodotoxin of PSTBP, which is supposed to be a carrier protein, can help transfer the tissues between the liver, ovary, and skin, especially *T. Pardalis*.

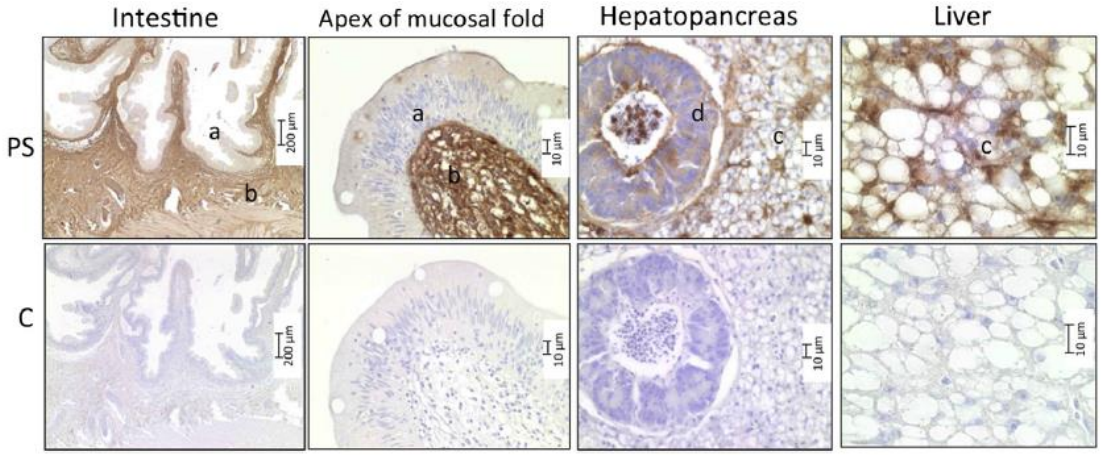

Figure 2. Light micrographs of representative intestine and liver sections of *Takifugu pardalis.* The positive stain (PS) to PSTBP-antibody results in brown color. C: negative control sections. Alphabetical letters indicate a, mucosal epithelium; b, lamina propria mucosae; c, hepatocytes; d, pancreatic cells. (Yotsu-Yamashita et al.,2013)

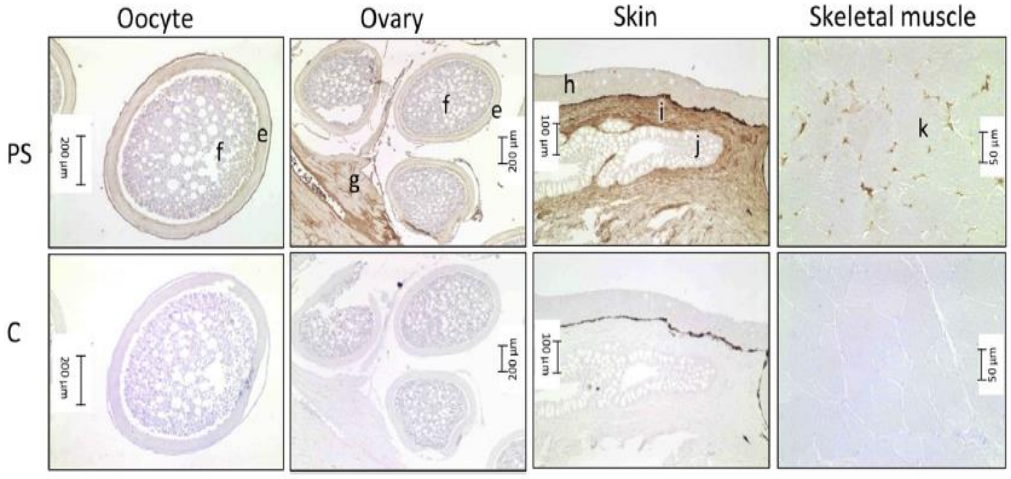

Figure 3. Representative light micrographs of the ovarian, skin and skeletal muscle sections of *Takifugu pardalis* are presented. The Brown color is derived from the positive stain (PS) to PSTBP-antibody. C negative control sections. Alphabetic letters respectively: e, standing for vitellin wave; f standing for egg yolk; g, for ovarian wall; h, standing for epidermis; i, referring to dermis; j, for toxin-secreting gland; k, standing for myofiber (Yotsu-Yamashita et al., 2013).

Matsui et al. (2000) were the first to investigate the emergence of a TTX binding protein (TBP) from pufferfish (*Fugu niphobles*) plasma, which acted within the TTX transfer and transport process and report on the purification of this protein. In laboratory binding assays, they demonstrated its reversible binding affinity to TTX and named TTX binding protein (TBP), which was 116 kDa by SDS_PAGE and 91 kDa light mass spectrometric time (Figure 4). Subsequently, Yotsu-Yamashita et al. (2001) purified TTX and saxitoxin (STX) binding protein from pufferfish plasma (*Fugu pardalis*) and sequenced two PTSBP isoforms (PTSBP1 and PTSBP2) having 93 percent amino acid sequencing identity, with 208 kDa molecular mass.

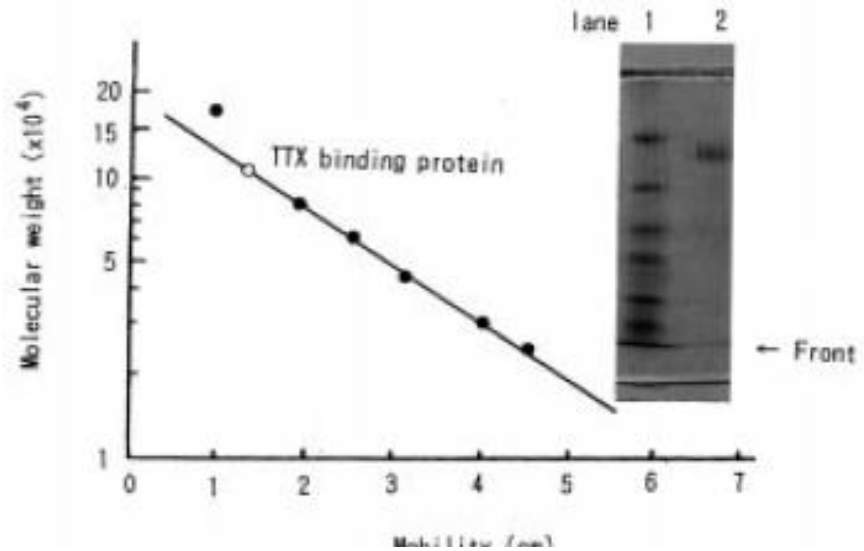

Figure 4. SDS-PAGE binding protein purified from TTX. With 7.5 percent gel, the protein was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. They used molecular weight markers to measure the protein 's apparent molecular weight. Lane 1, Protein marker (New England Biolabs), MBP-b- Galactosidase (175,000), MBP-Paramyosin (83,000), Glutamic dehydrogenase (62,000), Aldolase (47,500), Triosephosphate isomerase (32,500), and b-Lactoglobulin A (25,000) (from top to bottom); lane 2, puri®ed protein (Matsui et al., 2000).

PSTBPs are fusion proteins consisting of two tandem repeated tributyltin-binding protein type 2 (TBT-bp2) domains. PSTBPs are fusion proteins consisting of two tandem repeated tributyltin-binding protein type 2 (TBT-bp2) domains. STBPs are fusion proteins consisting of two tandem repeated tributyltin-binding protein type 2 (TBT-bp2) domains. STBPs are fusion proteins consisting of two tandem repeated tributyltin-binding protein type 2 (TBT-bp2) domains.

Studies in recent years argue that PSTBP should be classified as one of the lipocalin members (Tatsuno et al., 2013) and two tandem repeated tributyltin binding protein type 2 (TBT-bp2) domains compose its fusion proteins (Yotsu-Yamashita et al., 2001; Oba et al., 2007; Hashıguchı et al., 2015). These protein types are highly toxic to aquatic organisms (Shimasaki et al., 2002) and are fish alpha 1-acid glycoprotein-like lipocalin proteins (Fournier et al., 2000) that bind to tributyline (TBT). This type of protein is a dimeric glycoprotein that forms a non-covalent dimer (Matsui et al., 2000; Yotsu-Yamashita et al., 2001). According to Yotsu-Yamashita et al., (2001)'s findings, glycopeptidase F completely deglycosylated the binding protein (PTBP) of pufferfish tetrodotoxin (TTX) in the puffer fish's blood plasma (*Fugu pardalis*), while producing a single band at 42 kDa. The PSTBP monomer is composed of a 42 kDa protein

and an N-glycan of 62 kDa in their analysis. Moreover, it has also been found in other species belonging to the genus *Arothron,* with the molecular masses of 163 kDa in *A. nigropunctatus,* 118 kDa *in A. hispidus,* and 130 kDa *in A. Manilensis* by Yotsu-Yamashita et al., (2018). However, the findings of this analysis were more significant than those of PSTBP reported by Matsui et al., (2000) at *Takifugu pardalis* (108 kDa as the monomer). The molecular masses of these bands after the treatment with glycopeptides F were as follows, respectively: the molecular masses decreased to about 86 kDa, 71 kDa, and 67 kDa for *A. nigropunctatus, A. hispidus, and A. manilensis,* also more generous than *Takifugu* PSTBP (43 kDa) (Figure 5).

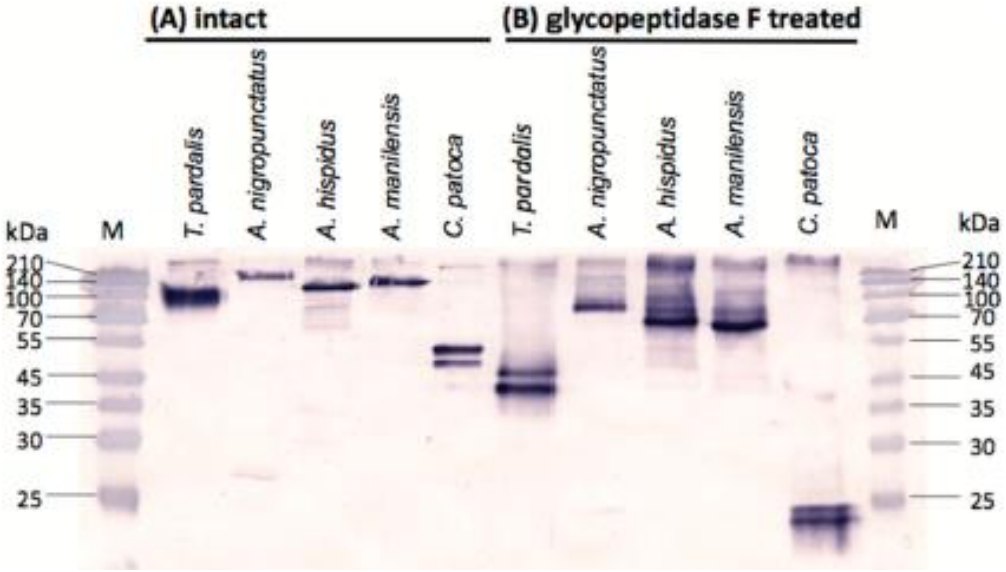

Figure 5. Western blot analysis (15% SDS-PAGE separation gel) of (**A**) intact plasma from five species of pufferfish (1 μg protein per lane); and (**B**) those after treatment with glycopeptides F (1 μg protein per lane), detected with anti-pufferfish saxitoxin and tetrodotoxin binding protein (PSTBP) IgG. (Yotsu-Yamashita et al., 2018)

Conclusion

One of the proteins involved in TTX accumulation in toxic pufferfish is the pufferfish tetrodotoxin (TTX) binding protein (PTBP). Through studies to this date, the role and mechanism of TTX binding proteins in pufferfish have been partially explained by the mechanism of TTX accumulation. The details of other proteins included in TTX storage are expected to be clarified in the future.

Author Contributions

All author contributions are equal for the preparation research in the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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