5. シガテラ毒

サンゴ礁が発達した熱帯・亜熱帯の海域では，本来は無毒な多数の魚が毒化し，シガテラと呼ばれる特異な食中毒の原因となることがある．

中毒症状としては，下痢や嘔吐などの消化器系障害，知覚異常や筋肉痛や関節痛そして掻痒感などの神経系症状，脈拍数や血圧の低下などの循環器障害が認められる．とくに，冷たいものに触れたときに，電気的刺激のような痛みを感じる温度感覚異常はドライアイスセンセーションと呼ばれ，最も特徴的な症状である．軽症の場合は，神経症状のみを認めることが多い．致命率は低いが，神経症状が長期間持続することが多い．世界的には毎年2～5万人の患者が発生していると推定されている．わが国では，沖縄県や鹿児島県奄美地方を中心に毎年発生が見られる．それ以外の地域では，熱帯・亜熱帯産の魚が持ち込まれて発生した事例が大部分であるが，沿岸魚による食中毒も散発している．毒かます（ドクカマス，オニカマス）については，通知により食品衛生法第6条第2号に該当するとして食用禁止措置が取られている[解説注1)]．毒の本体はシガトキシンとその類縁体であり，熱安定な脂溶性の環状ポリエーテル化合物である[解説注2)]．筋肉中の濃度より内臓の濃度が高いが，通常の食中毒は筋肉の摂食によって起きている．

なお，毒力が0.025 MU/gを超えるものは食用不適とされている．

マウス毒性試験法（参考法）

① 機器・試薬

(a) 機器・器具

細砕器（ホモジナイザー）

ロータリーエバポレーター

ブフナー漏斗

分液漏斗（300 mLまたは500 mL，200 mL）

ナス形フラスコ（2 L，500 mL，100 mL）

目盛付き試験管

ボルテックスミキサー

注射器（ツベルクリン用26 Gなど）

(b) 試薬

アセトン（特級）

エーテル（特級）

n-ヘキサン（特級）

メタノール（特級）

1% Tween 60 液（Tween 60 を 1% になるように生理食塩水に溶解）

② 試験液の調製

皮や骨を除いた筋肉 240 g を包丁で細切して細砕器に入れ，アセトン 700 mL を加えて 3 分間抽出を行う．ブフナー漏斗を用いて減圧ろ過する．ろ紙上のフィルターケーキを，同様の操作で再度抽出する．抽出液を合わせて減圧濃縮する．アセトンが留去されて水層表面に油が分離し始めると，非常に発泡しやすくなるので注意する．液量が 100 mL 以下になったと思われる時点で濃縮を止め，濃縮液を分液漏斗（300 mL または 500 mL 容）に移す．濃縮フラスコに付着した油を，総量 200 mL のエーテルを数回に分けて洗い込みながら分液漏斗に加え，分配によりエーテル層を得る．水層を分液漏斗に戻し，エーテル 200 mL で再び抽出し，エーテル層を 1 回目の抽出液と合わせて減圧乾固する．分液漏斗を激しく振ると，頑固なエマルジョンを形成するので注意する．エマルジョンのために 2 層に分離しないときには，飽和食塩水を適量加えてもう一度振り直す．エーテル層を濃縮すると，含まれていた水が残り，表層に油が分離して発泡しやすくなる．いったん減圧を切り，エタノールを加えて水を共沸で除くとよい．減圧乾固したエーテル抽出物に 50 mL の 90% 含水メタノールと 100 mL の *n*-ヘキサンを加え，200 mL 容の分液漏斗に移し，分配を行う．下層の含水メタノール層をフラスコに移し，減圧乾固する．最後の段階で発泡しやすいので，エタノールを加えるとよい[解説注3)]．

含水メタノールを乾固して得た粗毒画分に，2 ～ 3 mL の 1% Tween60 液を加え，ボルテックスミキサーなどを用いてエマルジョンとし[解説注4)]，10 mL の目盛り付き試験管に移し，全量を 6 mL にして試験液とする．試験液 1 mL は検体魚筋肉の 40 g に相当する．

③ マウス腹腔内投与[解説注5)]

試験には ddY または ICR 系の雄で体重が 17 ～ 20 g の範囲にあるマウスを用いる．1 投与量に対しては 1 群 3 尾のマウスを用いる．まず，試験液 1 mL ずつを 3 尾のマウス腹腔内に注射する．次いで，0.5 mL ずつを第 2 群のマウス 3 尾に投与する．試験液は静置すると直ちに水層と油層に分離するので，毎回よくかくはんし，均一なエマルジョンにして注射器に採取する．目盛付き試験管内に残った試験液に 3 倍量の Tween 60 液を加えてエマルジョンとし，4 倍希釈試験液とする．希釈試験液の各 1 mL と 0.5 mL ずつを用いて，それぞれ 3 尾ずつのマウスの腹腔内に注射する．必要に応じて，16 倍，64 倍希釈液を同様に調製し，マウスに投与する．注射してから 24 時間後のマウスの生死を観察し，3 尾ともすべて，あるいは 3 尾中少なくとも 2 尾のマウスが死亡する最小濃度を求める[解説注6)]．

④ 毒力の計算と表示

毒力の表示はフグ毒や貝毒の場合と同じく，検体 1 g 中に含まれる毒量（MU/g）で行う．1 マウス単位（MU）は，供試マウス 1 尾を 24 時間で死亡させる毒量と定義され，シガトキシン-1B（CTX1B）7 ng に相当する．マウス毒性試験の結果から，表 7-7 に従って検体の毒力を算出する．検体の毒力が 0.025 MU/g を超えた場合は，食用に不適と判定する[解説注7, 8)]．

表 7-7　投与量と毒力の関係（シガテラ）

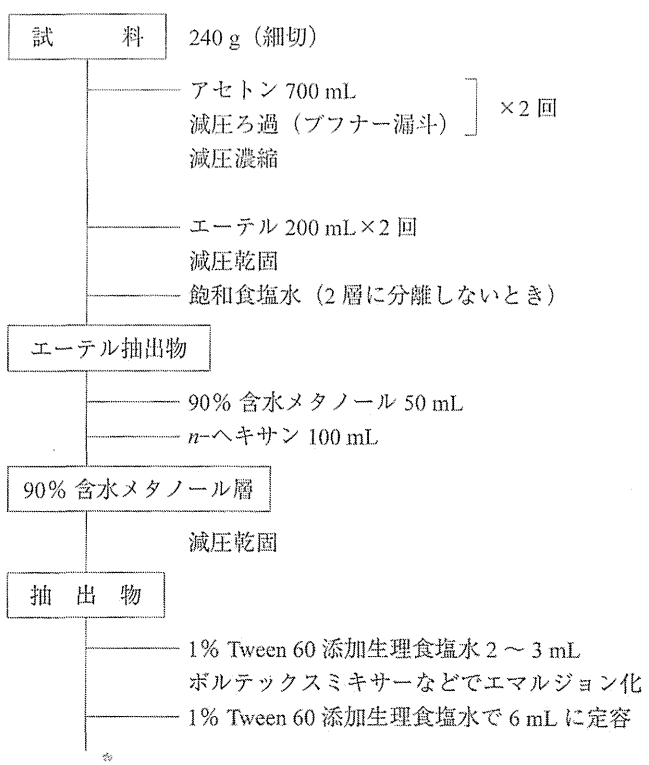
試験液	投与量 (mL)	投与量相当検体量 (g)	検体毒力 (MU/g)
原　液	1.0	40	0.025
原　液	0.5	20	0.05
4 倍希釈	1.0	10	0.1
4 倍希釈	0.5	5	0.2

解説

❶ 試験法の概要

魚肉中に含まれるシガトキシン類（CTXs）は微量であるため，抽出物を濃縮し，40 g 魚肉相当量/mL の試験液を調製する．脂溶性の CTXs をマウス腹腔内投与するために，乳化剤（Tween 60）を添加した生理食塩水でエマルジョン化し試験液を調製する．マウスへの投与後 24 時間の生死で判定する．

❷ 操作のフローチャート



試　験　液

ddY 系または ICR 系マウス（17 ～ 20g，オス）3 尾（各投与量につき）に腹腔内注射
24 時間後の生死を観察

毒力（MU/g）計算

❸ 注解および留意点

1）「毒かます」について（昭和 28 年 6 月 22 日，衛環発第 20 号）

「毒かます」（俗名おにかます）は，太平洋，印度洋の熱帯及び亜熱帯海岸にせい息している魚類であるが，最近我国の漁場の拡大にともない，漁獲され，一般鮮魚とともに市場に販売されるむきがあり，また，これによる中毒の発生も二，三起こっている．「毒かます」は神経系を侵す固有の有毒物質を有し，食品衛生法第 4 条第 2 号（現第 6 条第 2 号）に該当するものとみられるので，今後これが食用に供せられることがないよう販売そのほかについて十分処置されたい．特に，毒かますの分布地域の魚類の陸揚げされる漁港のある都道府県においては十分注意され，食品衛生の万全を期せられたい．

2）太平洋からは骨格の異なるシガトキシン-1B（CTX1B）群（図 7-6）とシガトキシン-3C（CTX3C）群（図 7-7）が，また，カリブ海からはカリビアン・シガトキシン（C-CTX）群など，これまで 20 以上のシガトキシン類（CTXs）が報告されている[文献1, 2]．最近，南西諸島における原因物質は CTX1B 群であることが明らかになった[文献3]．それ以外の海域では，CTX3C 群もしくは，CTX1B 群と CTX3C 群との混合型が知られている[文献3]．

ナンヨウブダイから検出されてスカリトキシンと称された成分は，シガトキシン 4A と 4B の混合体であることが明らかとなっている[文献2]．小型藻食魚のサザナミハギからはマイトトキシンなどの毒が得られているが，わが国沿岸のこれらの魚種での中毒事例はない．

本来は無毒な魚が毒化する機構は，藻類付着性の有毒渦鞭毛藻（*Gambierdiscus toxicus*）を起点とする食物連鎖で説明される．CTX1B のマウス腹腔内投与による最小致死量は 0.35 μg/kg である[文献1]．

3）濃縮液に濁りがでてきたタイミングでエタノールを数 mL 加えるとよい．必要に応じて，この操作を繰返す．

4）まず，ナス形フラスコの壁面についている抽出物をエマルジョン化し，徐々に底面のほうへ進めていくとよい．最初の 2 ～ 3 mL で全抽出物をエマルジョン化し，

試験管に回収する．残物は 0.5 ～ 1 mL の Tween 60 液でナス形フラスコとピペットを洗いこみながら回収する．

5)　検体の量が試験法に定める 240 g に達しない場合は，試験法に記された抽出溶媒の量や試験液量を，比例的に減じる．マウス毒性試験は試験液（原液）のみで行い，食用の適，不適を判定する．内臓の毒含量は，筋肉より数倍高いことが多いので，筋肉より少量の試料で試験を行うことが可能である．

6)　通常，食用の対象にされる筋肉のシガトキシン含量は低いので，濃度と致死時間との関係を用いて定量を行うことはできない．シガトキシンを投与したマウスでは，下痢や唾液の分泌亢進が見られることが多い．しだいに不活発になり，呼吸の異常と後肢の麻痺が認められる．呼吸が困難になると，嘔吐に似た動作を示す．死亡直前には，激しくけいれんし飛び跳ねることが多い．

7)　アメリカでは，魚肉中に含まれるシガトキシン類の指針値として，太平洋産は 0.01 ppb CTX1B 相当量（約 0.0014 MU/g），カリブ海産は 0.1 ppb C-CTX1（図 7-8）相当量が示されている[文献4]．マウス法でこのような低濃度の測定を達成するのは困難である．

8)　CTXs の分析法として，アメリカや仏領ポリネシアでは，マウス神経芽細胞を用いた細胞毒性試験，マウスシナプトソームへの親和性を利用したレセプターバインディング法などが採用されている[文献5]．最近では，ELISA 法[文献6]や LC-MS/MS 法[文献3]による分析法の開発が進められており，とくに LC-MS/MS 分析法による定量結果は，マウス毒性試験法と一致している．

天然試料から CTXs を単離することはきわめて困難であるため，分析用 CTXs 標品を所有する機関は，国立医薬品食品衛生研究所など少数の機関に限られている．なお，有機合成された CTX3C（100 ng）が，本稿執筆時点で和光純薬工業から購入可能である．

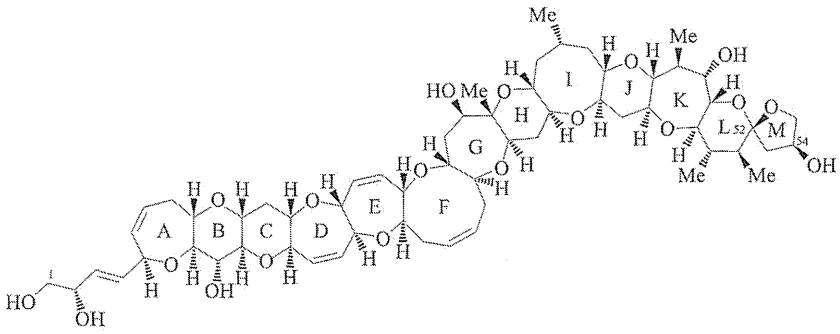


図 7-6　シガトキシン（シガトキシン-1B，CTX1B）

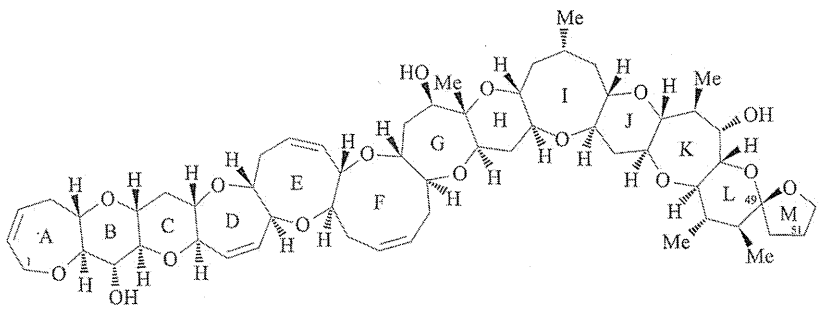


図 7-7　シガトキシン-3C（CTX3C）

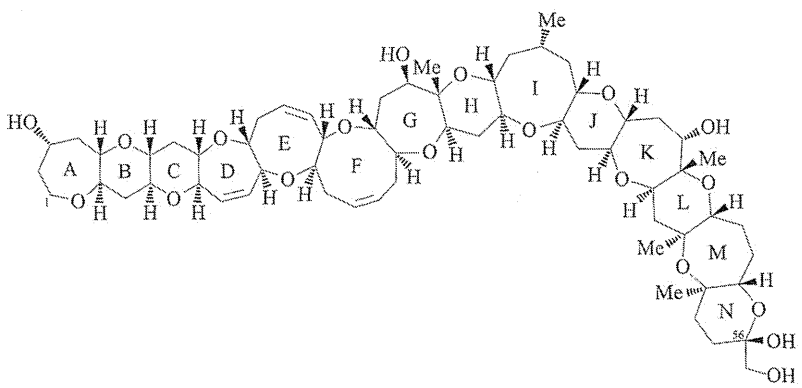


図 7-8　カリビアン・シガトキシン-1（C-CTX1）

[参考文献]

1) T. Yasumoto：*Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.*, **81**, 43 (2005)

2) 大城直雅：食品衛生研究，**60**, 36 (2010)

3) K. Yogi, *et al.*：*Anal Chem*, **83**, 8886 (2011),
K. Yogi, *et al.*：*J. AOAC Int.*, **97**, 398 (2014)

4) Fish and Fishery Products Hazards and Controls Guidance Fourth Edition (2011), FDA
http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/seafood/

SPECIAL GUEST EDITOR SECTION

Quantitative ELISA Kit for Paralytic Shellfish Toxins Coupled with Sample Pretreatment

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A new ELISA kit to quantitate the level of paralytic shellfish poisoning (PSP) toxins in crude shellfish extracts was developed. A conjugate for preparing antigen and a novel antibody used in the ELISA was prepared based on the unique reactions between C11-O-sulfate toxins such as gonyautoxins 2 and 3 (GTX2,3) and various thiol compounds, followed by coupling to keyhole limpet hemocyanin. The compounds necessary for competitive ELISA, labeled toxin and an artificial standard toxin to replace saxitoxin in the analysis, were also produced by the same techniques. The resulting ELISA recognized all the toxin components tested; however, carbamoyl-N-sulfate derivatives such as B and C toxins and N1-OH toxins such as neoSTX and GTX1,4 showed low affinity to the antibody. The difference in the reactivity of the antibody observed among the toxin components prevents accurate quantification of the toxin amounts in shellfish extracts. To address this problem, the former toxin components were transformed to corresponding carbamate toxins by mild HCl treatment according to a conventional method. The reduction of N1-OH of the latter toxins to N1-H was performed by our original method using hemin as a catalyst. We report here the new ELISA kit coupled with the pretreatment process to transform the toxin components favorable for the quantitative analysis of PSP toxins.

During a bloom of toxic plankton, filter feeding bivalves become toxic by ingesting them. Human consumption of toxic bivalves causes food poisonings, often including lethal cases. Several types of shellfish poisonings are known. Among them, paralytic shellfish poisoning (PSP) is the most dangerous because of the potentially fatal paralytic shellfish toxins (PSTs) and their global occurrence. In the areas where PSTs are found, their levels in shellfish are regularly monitored. When their total toxicity exceeds the quarantine limit, shellfish harvesting in that area is closed to avoid the poisoning. This system of harvest level monitoring is applied in various countries that have PST-producing algal blooms. With the help of this system, severe poisonings from commercial shellfish have been avoided. Traditionally, shellfish toxicity has been analyzed by mouse bioassay, or MBA (1), a simple and reliable means of detecting the PSTs. MBA-based monitoring has protected public health, since in the areas where shellfish toxicity has been monitored by MBA, no significant poisoning has been reported (2). However, the MBA has negative aspects since it requires the use of laboratory animals. First, mice used for the assay are limited to healthy males of a single strain, with weight between 18 to 23 g, and this requirement causes practical problems since mice grow quickly and so it is difficult to maintain them in the conditions necessary for the assay. In addition, use of the MBA has been recently criticized from the standpoint of animal ethics. Thus, there is a compelling need for finding methods to replace the MBA.

Several excellent methods for the analysis of PSTs offer alternatives to the MBA (3). Among these, ELISA is the primary candidate for a simple and convenient method. Johnson et al. (4) developed a specific antibody against saxitoxin (STX) by immunizing the antigen as a conjugate, in which the guanidinium-N of STX was bound to amino residues on the surface of a protein using formaldehyde as a linker, opening the door to the development of ELISAs for PSTs. Using the method of Johnson et al. (4), several groups have developed their own antigens and prepared antibodies against PSP toxins using various conjugated antigens (5–10). These antibodies showed specific affinity to PSTs with high sensitivity. However, their affinity showed remarkable differences among the toxin components. It is quite interesting that N1-OH toxins such as neoSTX always showed low affinity to the antibody (11–13). Cembella et al. (11) also tried to develop the antibody by developing a new antigen in which carrier proteins were replaced with artificial poly-lysine with alanine as spacers, expecting a new antibody with high affinity to various toxin components. However, these trials were not successful in solving the affinity problems since antibodies were reported to show the similar characteristics reported before.

We are addressing the affinity issues described above by applying unique chemical reactions between PSP toxins and thiol compounds we had previously discovered in our PST metabolism studies, i.e., the 11α-O-sulfate PSP derivatives such as gonyautoxin 2 (GTX2) react with thiol compounds to form stable conjugates in which C11 of toxins couple with the sulfur atom of thiols covalently, and that further treatment of the conjugates with excess amounts of thiols gives C11-O-sulfate-free toxins (14, 15). These reactions allow us to easily produce various compounds conjugated with PSTs. We report here a

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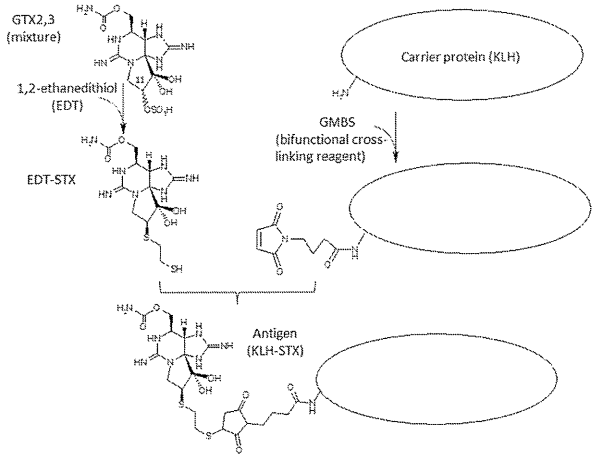

Figure 1. Preparation process and estimation structure of the KLH-STX antigen.

simple, rapid, and quantitative assay for PSTs, a novel ELISA using a new antibody, and a simple two-step sample pretreatment procedure.

Experimental

Molluscan Shellfish

Scallops (*Patinopecten yessoensis*) were collected from a culture farm in Ofunato Bay, Iwate Prefecture, Japan, before, during, and after a bloom of *Alexandrium tamarense*. Oysters (*Crassostrea gigas*) were collected from a culture farm in Senzaki Bay, Yamaguchi Prefecture, Japan, during a bloom of *Gymnodinium catenatum*. Mussels (*Perna viridis*) harvested during a bloom of *Pyrodinium bahamense* var. *compressum* were obtained from Manila Bay, Luzon, the Philippines. The crude extracts of bivalves were prepared according to the standard protocol for MBA (1).

Isolation of PSP Toxin Components

Equilibrated toxin mixtures such as GTX1 and GTX4 (GTX1,4) and GTX2 + GTX3 (GTX2,3) were purified from the crude toxic extracts of toxic scallops described above by column chromatography on activated charcoal (Wako, Osaka, Japan), Bio-Gel P-2 (200–400 mesh; Bio-Rad, Hercules, CA), and Bio-Rex 70 (200–400 mesh, Bio-Rad) successively. STX, neoSTX, GTX5 (B1), and GTX6 (B2) were also purified from the crude extracts of toxic oysters and mussels in a same way. An equilibrated mixture of C1 and C2 (C1,2) was purified from toxic oysters by column chromatography on activated charcoal, Bio-Gel P-2, and Toyopearl SuperQ-650M (Tosoh, Tokyo, Japan). Purified toxin components thus obtained were dissolved in diluted HCl and kept in a freezer until use.

Chemical Analysis of PSP Toxins in Crude Extracts of Shellfish

Toxin components in the crude extracts of toxic shellfish collected from different areas of Japan were quantitated by postcolumn derivatization fluorometric HPLC according to Oshima (16) using toxin standards kindly provided by Yasukatsu Oshima (Tohoku University, Faculty of Agriculture, Sendai, Japan).

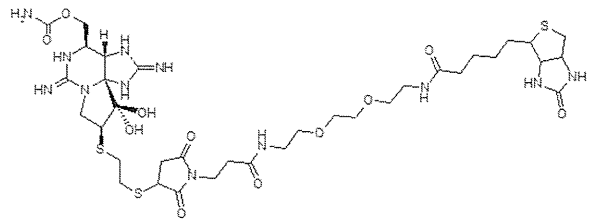

Figure 2. Estimation structure of biotin-STX, the competitive labeled antigen.

Preparation of Antibody Against STX

Using the reaction between toxin components with 11α-O-sulfate and thiol compounds (15), GTX2 was treated with 1,2-ethanedithiol (EDT; Aldrich, St. Louis, MO) to obtain an EDT-STX complex. Freeze-dried GTX2 (100 μmol) was dissolved in 200 mL of 0.1 M ammonium phosphate buffer (pH 7.4) and mixed with 50 mL tetrahydrofuran containing 2 mL EDT. After stirring gently overnight at room temperature, the reaction mixture was extracted four times with an equivalent volume of ethyl acetate to remove remaining EDT and tetrahydrofuran. The EDT-STX conjugate in the aquatic phase was isolated by Bio-Gel P-2 column chromatography.

Separately, 20 mg keyhole limpet hemocyanin-HG (KLH, Wako, CAS. No. 9013-72-3) dissolved in 2.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) was mixed with 10 mg N-(4-maleimidobutyryloxy) succinimide (GMBS, Dojindo), CAS. No. 80307-12-6) dissolved in 150 μL *N*-dimethylformamide. The mixture was left at room temperature for 30 min and applied to a column of Sephadex G-25 (GE Healthcare, Uppsala, Sweden), fine, 1.5 × 35 cm, and eluted with 0.1 M sodium phosphate buffer (pH 6.0). Monitoring the eluate by absorption at 280 nm, fractions containing the KLH-GMBS conjugate were collected. After the pH of combined KLH-GMBS fractions was adjusted to 7.2 by addition of 0.1 M sodium hydroxide, the solution was mixed with 20 μmol EDT-STX described above. The reaction mixture was left at 5°C for 2 days and dialyzed four times against 1 L phosphate buffered saline without Ca^{++} and Mg^{++} [PBS (–)]. Antigen thus obtained was immunized to rabbits and goats using complete and incomplete adjuvants.

Quantitation of STX Coupled with KLH by 2-Mercaptoethanol (ME)-Treatment

The amount of STX coupled with KLH was estimated from STX released from the conjugate by ME treatment (14, 15). Briefly, an aliquot of the solution containing thiol-STX conjugate such as EDT-STX and the antigen, in which the C11 position of toxin couples with sulfur atom of the thiol moiety, was diluted 10 or 100 times with 10% ME in 0.1 M sodium phosphate buffer (pH 7.2) and heated in a boiling water bath for 5 min. Under these conditions, the conjugates release STX quantitatively. The amount of STX coupled with thiol at the C11 position can be

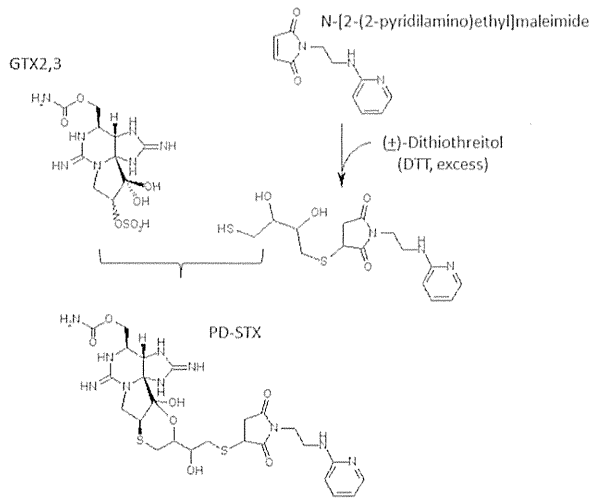

Figure 3. Preparation process and the estimation structure of PD-STX, the substitute standard of STX in ELISA.

quantitated by comparison of the amount of GTX2, GTX3, and STX in the solution before and after ME treatment.

Immunization of Animals

The conjugate of KLH-STX was immunized to rabbits and a goat. In the first month, the antigen was immunized to animals biweekly with complete adjuvant. After 1 month, animals were immunized with the antigen mixed with incomplete adjuvant biweekly. One or 2 mL of blood was collected from each animal before immunization, and antibody activity against STX was analyzed.

Monitoring of the Antibody Activity During Immunization

During immunization, the antibody activity of test animals was monitored as follows. A 50 μL portion of serum collected over time was mixed with 50 μL of 50 μM STX and left at 5°C for 1 h. Then the mixture was filtered through an Ultrafree-MC filter (NMWL 5000, EMD Millipore, Billerica, MA), and STX in the filtrate was analyzed by HPLC fluorometric analysis according to Oshima (16). The antibody activity of the serum was evaluated by the amount of STX trapped by the antiserum. The reaction mixture in which the antiserum was replaced with PBS(–) was analyzed in the same way as a control.

Preparation of Biotin-STX, a Competitive Labeled Toxin in ELISA

Biotin-labeled STX (biotin-STX), a competitive labeled toxin in ELISA, was prepared as follows. Four μmol EDT-STX dissolved in 2 mL of 0.1 M sodium phosphate buffer (pH 7.4) was mixed with 4.8 mg maleimido-polyethylene glycol-biotin (Thermo Scientific, Rockford, IL), and stirred for 2 h at room temperature. The biotin-labeled STX formed in the reaction mixture was purified by column chromatography on Bio-Gel P-2.

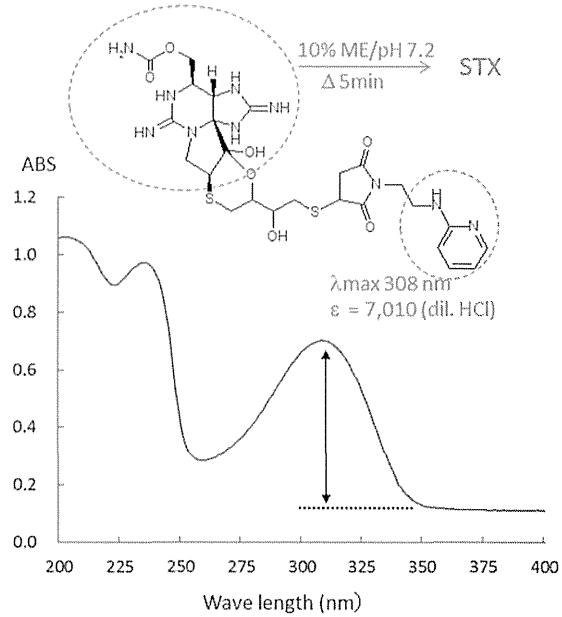

Figure 4. UV spectrum of PD-STX.

The concentration of biotin-labeled STX was quantitated as the amount of STX released by ME treatment as mentioned above.

Novel Fluorescent-Labeled STX, an Alternative Standard Replaceable with STX in ELISA

A 100 mg amount of N-[2-(2-pyridylamino) ethyl] maleimide hydrochloride (PAEM, Wako), a water-soluble thiol-reactive fluorescent reagent, was dissolved in 30 mL of 0.01 M phosphoric acid and mixed with 300 mg of (±)-dithiothreitol (DTT; Wako). After the pH of the solution was adjusted to 7.3 by addition of 1% ammonium hydroxide, the solution was gently stirred at room temperature for 2 h. The reaction mixture was then applied to a column of Bio-Rex 70 (H^+ form, 200–400 mesh, 2.5 × 6 cm). After the column was washed with 200 mL water, adsorbed substances containing PAEM-DTT conjugate were eluted with 200 mL of 0.5 M acetic acid. After freeze drying, the residue dissolved in a small volume of water was mixed with 20 μmol freeze-dried GTX2,3 in 20 mL of 10 mM ammonium phosphate buffer (pH 7.4) and then left at room temperature for 1 day. The conjugate of PAEM-DTT-STX (PD-STX) formed in the reaction mixture was isolated by successive column chromatography on Bio-Gel P-2 and Bio-Rex 70. During purification, the concentration of PD-STX was monitored by the absorption at 308 nm and the amount of STX released by ME treatment.

Direct 1 Step ELISA for PSP Toxins

Direct 1 step competitive ELISA was designed for the analysis of PSP toxins as follows. The antiserum from the goat that showed relatively high antibody activity was stored in a deep freezer (–80°C). Using a part of the antiserum, the immunoglobulin G fraction was prepared by conventional ammonium sulfate precipitation. The obtained fraction was then dissolved in the same volume of the original antiserum by PBS(–) and used as the stock antibody solution in the present study. A part of the stock solution was then diluted 2000 times with PBS(–) and added to a

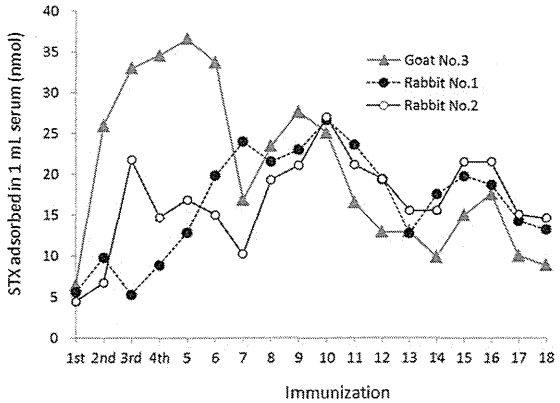

Figure 5. Enhancement of antibody activity in a goat and rabbits during immunization.

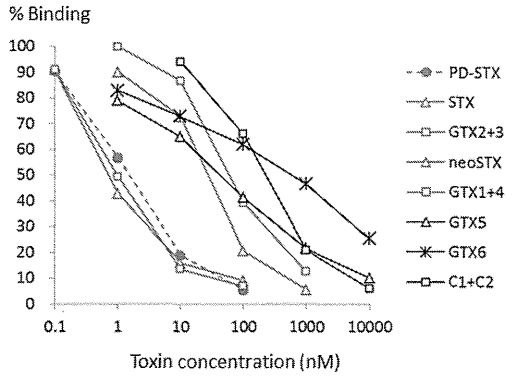

Figure 6. Comparison of the reactivity to the antibody of isolated toxins by ELISA. PD-STX, an artificial standard, is also analyzed.

96-well ELISA plate (Maxisorp, Nunc, Roskilde, Denmark) for coating. The plates were then treated with N101 (Nihon Yushi Co. Ltd, Tokyo, Japan) for blocking and used for the analysis.

In ELISA, the sample solution, biotin-STX solution (2 nM), and horseradish peroxidase (HRP)-labeled streptavidin (Funakoshi, Tokyo, Japan) solution diluted 2000 times with PBS(–) were added to each well in this order and reacted on the plate. After washing the plate, orthophenylenediamine (OPD)-hydroperoxide (H_2O_2) solution was added for coloring. Samples were analyzed in triplicate. The assay procedure is summarized as follows:

(**a**) Add 50 μL PD-STX reference (0.1, 1, and 10 nM) or sample solutions to each well of the ELISA plate coated with the antibody. [*Note*: Crude extract of shellfish prepared according to AOAC protocol is diluted 400 times with 0.1 M sodium phosphate buffer (pH 7.4).]

(**b**) Add 50 μL biotin-STX to each well.

(**c**) Add 50 μL HRP-streptavidin to each well.

(**d**) Incubate the plate at 37°C for 30 min.

(**e**) Discard the reaction mixture and wash the wells three times with PBS containing 0.1% Tween 20.

(**f**) Add 100 μL coloring reagent (OPD-H_2O_2) to each well.

(**g**) Incubate the plate at 37°C for 5 min.

(**h**) Add 100 μL HCl (2 M) to each well.

(**i**) Measure 492 nm absorbance of each well with a plate reader (iMark, Bio-Rad).

(**j**) Calculate PSP concentration of the sample solution using the calibration curve obtained from PD-STX references.

Chemical Conversion of PSP Toxins in Shellfish Extracts

As described in the results, the antibody of the present study recognized all the PSP toxin components tested. However, a big difference was observed in the affinity of toxin components to the antibody. These facts show that the quantitative toxin analysis of the shellfish extracts is not possible by this ELISA, because the toxic shellfish extracts are usually mixtures of several toxin components. In order to use this ELISA as a tool for quantitative analysis of the mixture of toxin components, it is necessary to transform the toxin components to those with the same affinity to the antibody before analysis. Carbamoyl-N-sulfate of the toxins such as C toxins is well known to be removed by mild HCl treatment (17). C11-O-sulfate of the toxins could be removed by ME treatment. We have recently found that N1-OH toxins are easily reduced to N1-H toxins by mild reductant such as ascorbic acid under the presence of heme compounds as a catalyst (18). Therefore, toxin components except decarbamoyl derivatives can be converted to corresponding carbamate N1-H toxins such as GTX2,3 and STX by mild HCl and heme/reductant treatments. Thus, an aliquot of crude extract prepared from toxic scallops according to the AOAC protocol (1) was mixed with the same volume of 0.3 M HCl and heated in boiling water for 5 min. A 1 mL amount of the HCl-treated extract corresponding to 0.25 g shellfish tissue was combined with 1 mL of 80 mM ascorbic acid (Wako) containing 0.4 mM hemin (Wako) in 0.5 M potassium phosphate buffer (pH 7.4). The mixture was heated again in a boiling water bath for 5 min. The ascorbic acid/hemin treated extract was diluted 100 times with 0.1 M sodium phosphate buffer (pH 7.4) and subjected to the ELISA as mentioned above. Toxin composition of the crude extract, HCl-treated extract, and ascorbic acid/hemin-treated extract were analyzed by fluorometric HPLC according to Oshima (16).

Results and Discussion

PSTs

GTX1,4, GTX2,3, C1,2, neoSTX, GTX5, GTX6, and STX were successfully purified from toxic shellfish extracts. After the concentrations of purified toxins were determined by HPLC-fluorometric analysis (16), these toxins were kept in a freezer until use.

Conjugate of KLH and STX Used for Antigen

Figure 1 shows the estimation structure of antigen (KLH-STX conjugate). A 21.3 mg amount of antigen was obtained as a precipitate after dialysis against PBS(–). When a 2.13 μg equivalent portion was suspended in 1 mL of 10% ME (w/w) in 0.1 M sodium phosphate buffer (pH 7.2) and heated in a boiling water bath for 5 min, 0.67 nmol of STX was detected by fluorometric HPLC analysis, indicating that 9.4% (w/w) of STX was coupled with KLH.

Conjugate of Biotin and STX

The conjugate of biotin and STX (biotin-STX; Figure 2) was prepared by the reaction of EDT-STX and maleimide-PEG2-

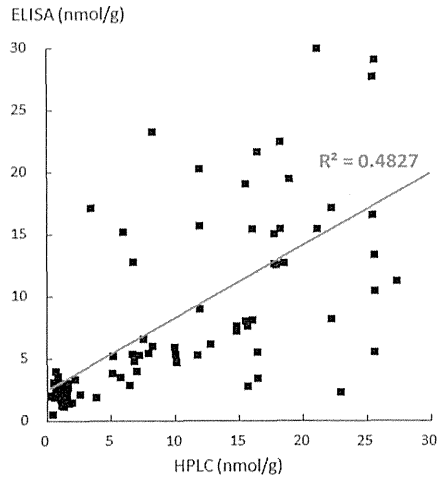

Figure 7. Comparison of the toxin concentration data obtained from ELISA with those from HPLC-fluorometric analysis. X-axis: crude extracts of shellfish were analyzed by a fluorometric HPLC system according to Oshima (16); Y-axis: crude extracts of shellfish were diluted with 0.1 M sodium phosphate (pH 7.2) and analyzed by ELISA. Regression line: Y = 0.58X + 2.39 > [ELISA (nmol/g)] = 0.58 × [HPLC (nmol/g)] + 2.39.

biotin. The conjugate in the reaction mixture was purified by Bio-Gel P-2 column chromatography. A 2.9 mg amount of biotin-STX was obtained as a white powder. A part of the conjugate thus obtained was analyzed for confirmation by a quadrupole mass spectrometer (API-2000; AB Sciex, Framingham, MA), positive Q1 scan mode). The data showed significant peaks at *m/z* 917.8, *m/z* 900.0, and *m/z* 459.5, corresponding to the pseudo-molecular ion peaks of $[M + H]^+$, $[M + H - H_2O]^+$, and $[M + 2H]^{++}$ of the target compound, respectively.

PD-STX Conjugate

Spectrophotometric analysis of PAEM in 10 mM HCl showed a characteristic absorbance at 308 nm with the molar extinction coefficient (ε) 7010. By Bio-Rex 70 column chromatography of the reaction mixture, PAEM-DTT-STX (PD-STX) conjugate was purified in diluted HCl fraction and obtained as a yellowish solid (12.4 mg). Figure 3 shows the estimation structure of PAEM-DTT-STX (PD-STX) conjugate. A quadrupole mass spectrum (positive Q1 scan mode) of the isolated material showed ion peaks at *m/z* 651.6, *m/z* 326.4, and *m/z* 217.9, which correspond to the pseudo-molecular ion peaks of $[M + H]^+$, $[M + 2H]^{++}$, and $[M + 3H]^{+++}$, respectively. Figure 4 shows a UV spectrum of isolated PD-STX in acidic solution, indicating that UV absorption of PD-STX is due to the PAEM moiety. Thus, a part of purified PAEM-DTT-STX was dissolved in 0.01 M HCl. The concentration of the solution was estimated to be 84.5 μM by UV absorption (308 nm). Separately, the same solution treated with ME was analyzed for STX by HPLC-fluorometric analysis gave 83.1 ± 1.9 μM. These results support the estimation structure of PD-STX conjugate and that its concentration can be easily determined spectrometrically.

Development of Antibody Against STX

High antibody activity usually appeared after immunization for 1 month (Figure 5). No significant difference in antibody formation was observed between rabbits and goats. Because of the greater yield of antibody due to animal size, the goat antisera showing relatively high activity were combined and used for ELISA.

Response of the Antiserum to Various PSP Toxin Components

Direct 1 step ELISA was prepared using the goat antibody described above. Biotin-STX described above was used as a labeled toxin. Various toxin components such as neoSTX, GTX5, GTX6, and the equilibrated mixtures such as GTX1,4, GTX2,3, and C1,2 were dissolved in PBS(–) and analyzed by ELISA together with artificial toxin standard (PD-STX) described above. As shown in Figure 6, STX and GTX2,3 showed the highest and almost similar affinity to the antibody. Interestingly, the artificial reference, PD-STX, also showed similar affinity to the antibody as STX and GTX2,3. This means that PD-STX could be used as the substitute standard of STX in ELISA. In contrast, other toxin components such as N1-OH toxins and carbamoyl-N-sulfate toxins showed much lower affinity to the antibody than the N1-H toxins such as STX and GTX2,3. Poor correlation (R^2 = 0.48) was observed between the ELISA data and those of HPLC-fluorometric analysis on the crude extracts of toxic shellfish collected in Japan (Figure 7), indicating that some additional improvement is necessary to quantitate the toxin level in the crude extracts of shellfish by ELISA.

Figure 8 shows the fluorometric HPLC chromatograms of diluted HCl extract of toxic scallops. The chromatogram of the untreated extract is shown on the left: the intact extract. In addition to the peaks of GTX2 and 3, a significantly higher peak of GTX4 and a small peak of GTX1 are observed. On the right in Figure 8 is the chromatogram of the same extract after reduction with ascorbic acid in the presence of hemin. Note that peaks due to the N1-OH toxins GTX1 and 4 in the extract have disappeared, and the peaks arising from the N1-H toxins GTX2 and 3 have been increased by the treatment. These results show that most of the N1-OH toxins in the crude extract are transformed to N1-H toxins such as GTX2 and 3 (and STX) following the reduction reaction.

Thus, the shellfish extracts described above were treated with diluted HCl in boiling water to transform the carbamoyl N-sulfate

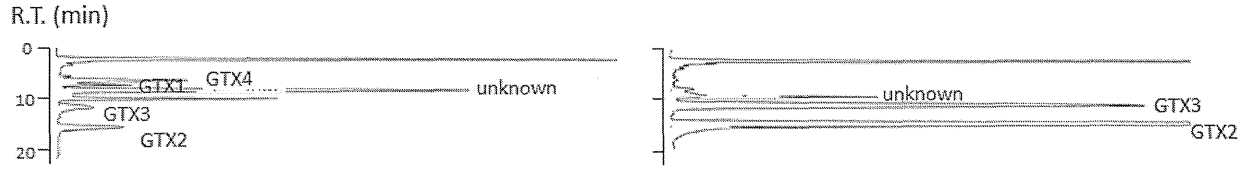

Figure 8. Chromatograms showing selective reduction of N1-OH of GTX1,4 to N1-H of GTX2,3 by ascorbic acid under presence of hemin as a catalyst. Left: before reduction. Right: after reduction. R.T. = retention time.

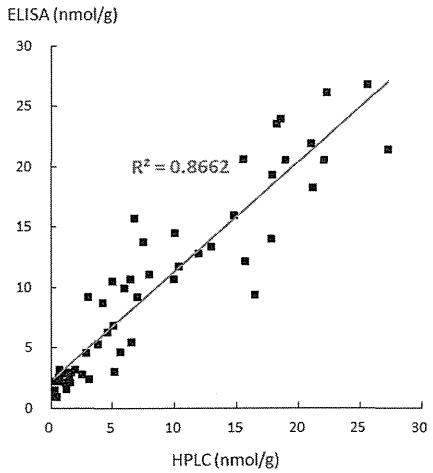

Figure 9. Comparison of the toxin concentration data of ELISA after transformation of toxin components by HCl treatment and selective reduction in the presence of hemin as a catalyst with those from HPLC-fluorometric analysis. X-axis: toxin level of crude extracts of shellfish analyzed by a fluorometric HPLC system according to Oshima (16); Y-axis: toxin level of crude extracts of shellfish analyzed by ELISA after the sample treatments with mild HCl and then selective reduction by ascorbic acid in the presence of hemin as a catalyst. Regression line: $Y = 1.07X + 1.44$ > [ELISA (nmol/g)] = 1.07 × [HPLC (nmol/g)] + 1.44.

toxins to corresponding carbamate toxins. Then, the extracts were treated with ascorbic acid in the presence of hemin to reduce N1-OH toxins to N1-H toxins. In Figure 9, the amounts of PSP toxins of the shellfish extracts treated with ascorbic acid in the presence of hemin analyzed by ELISA are shown against those analyzed by HPLC-fluorometry. Both data show good correlation ($R^2 = 0.87$), indicating that the ELISA of the present study could be available for a simple and rapid quantitative analysis of PSP toxins when combined with transformation procedures of toxins using HCl treatment and reduction of N1-OH.

As shown in Figure 6, PD-STX reference, STX, and GTX2,3 show almost the same response in the ELISA of the present study. By the pretreatment of the samples, toxin components other than STX and GTX2,3 in the samples are transferred to STX and GTX2,3 and expressed as a total nmols of the toxins. STX has the highest specific toxicity (2483 MU/μmol) among the PSP toxins accumulated in shellfish (16). If shellfish contains STX only as the toxic principle, 1.6 nmol of STX in 1 g shellfish tissue corresponds to the regulation limit, 80 μg STX equivalent/100 g internationally, or 4 MU/g in Japan. Usually, however, contaminated shellfish contains more than one toxin component. Therefore, the toxicity of total toxin concentration (1.6 nmol/g) is always lower than the corresponding regulation limit on toxicity because STX and GTX2,3 are the most toxic components. Thus, the ELISA of the present study could be available for at least the first screening even though all the toxin components in the sample are the mixture of STX and GTX2,3.

On the other hand, it has been pointed out that the analysis method of PSP toxins currently applied for food safety possesses some problems. One of them is the presence of N21-sulfo toxins with low toxicity in the shellfish extracts. These toxins easily transform to N21-sulfo-free components with high toxicity by mild HCl treatment. Currently, special care is taken for pH of the extract during preparation of the test solution. If the safe consumption of shellfish is evaluated by dose of toxins such as nmole/g instead of the toxicity (MU/g), the cost and labor of the monitoring will be much reduced. In this case, the current safe consumption level (80 μg of toxins/100 g of shellfish meat) is too severe because the specific toxicity of most of the toxin components contained in the shellfish is much lower than that of STX. Further discussion will be necessary.

Acknowledgments

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References

(1) *Official Methods of Analysis* (1995) 16th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) Ogata, T., Kodama, M., Fukuyo, Y., Inoue, T., Kamiya, H., Matsuura, F., Sekiguchi, K., & Watanabe, S. (1982) *Bull. Japan. Soc. Sci. Fish.* **48**, 563–566. http://dx.doi.org/10.2331/suisan.48.563

(3) Etheridge, S.M. (2010) *Toxicon* **56**, 108–122. http://dx.doi.org/10.1016/j.toxicon.2009.12.013

(4) Johnson, H.M., Frey, P.A., Angelloti, R., Campbell, J.E., & Lewis, K.H. (1964) *Proc. Soc. Exp. Biol. Med.* **117**, 425–430. http://dx.doi.org/10.3181/00379727-117-29599

(5) Davio, S.R. (1985) *Toxicon* **23**, 669–675. http://dx.doi.org/10.1016/0041-0101(85)90371-X

(6) Davio, S.R., Hewetson, J.F., & Beheler, J.E. (1985) in *Toxic Dinoflagellates*, D.M. Anderson, A.W. White, & D.G. Baden (Eds), Elsevier, New York, NY, pp 343–348

(7) Chu, F.S., Huang, X., & Hall, S. (1992) *J. AOAC Int.* **75**, 341–345

(8) Chu, F.S., Hsu, K.H., Huang, X., Barrett, R., & Allison, C. (1996) *J. Agric. Food Chem.* **44**, 4043–4047. http://dx.doi.org/10.1021/jf960244w

(9) Cembella, A.D., & Lamoureux, G. (1993) in *Toxic Phytoplankton Blooms in the Sea*, T.J. Smayda & Y. Shimizu (Eds), Elsevier, Amsterdam, The Netherlands, pp 857–862

(10) Usleber, E., Burk, R.D.C., Schneider E., & Martlbauer, E. (2001) *J. AOAC. Int.* **84**, 1649–1656

(11) Cembella, A.D., Parent, Y., Jones, D., & Lamoureux, G. (1990) in *Toxic Marine Phytoplankton*, E. Graneli, B. Sundstrom, L. Edler, & D.M. Anderson (Eds), Elsevier, New York, NY, pp 339–344

(12) Chu, F.S., & Fan, T.S.L. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 13–16

(13) Carlson, R.E., Lever, M.L., Lee, B.W., & Guire, P.E. (1984) in *Seafood Toxins, ACS Symposium Series 262*, E.P. Ragelis (Ed.), American Chemical Society, Washington DC, pp 181–192

(14) Sakamoto, S., Sato, S., & Kodama, M. (2000) *Fish. Sci.* **66**, 136–141. http://dx.doi.org/10.1046/j.1444-2906.2000.00020.x

(15) Sato, S., Sakai, R., & Kodama, M. (2000) *Bioorg. Med. Chem. Lett.* **10**, 1787–1789. http://dx.doi.org/10.1016/S0960-894X(00)00332-2

(16) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

(17) Koehn, F.E., Hall, S., Wichmann, C.F., Schnoes, H.K., & Reichardt, P.B. (1982) *Tetrahedron Lett.* **23**, 2247–2248. http://dx.doi.org/10.1016/S0040-4039(00)87312-8

(18) Sato, S., & Kodama, M. (2006) *Patent Abstracts of Japan*, Publication No. 2006-98293, Industrial Property Digital Library, http://www.ipdl.inpit.go.jp

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Article

DNA Microarray Analysis on the Genes Differentially Expressed in the Liver of the Pufferfish, *Takifugu rubripes*, Following an Intramuscular Administration of Tetrodotoxin

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Abstract: Pufferfish accumulate tetrodotoxin (TTX) mainly in the liver and ovary. This study aims at investigating the effect of TTX accumulation in the liver of cultured specimens of torafugu *Takifugu rubripes* on the hepatic gene expression by microarray analysis on Day 5 after the intramuscular administration of 0.25 mg TTX/kg body weight into the caudal muscle. TTX was detected in the liver, skin and ovary in the TTX-administered individuals. The total amount of TTX accumulated in the body was 67 ± 8% of the administered dose on Day 5. Compared with the buffer-administered control group, a total of 59 genes were significantly upregulated more than two-fold in the TTX-administered group, including those encoding chymotrypsin-like elastase family member 2A, transmembrane protein 168 and Rho GTP-activating protein 29. In contrast, a total of 427 genes were downregulated by TTX administration, including those encoding elongation factor G2, R-spondin-3, nuclear receptor activator 2 and fatty acyl-CoA hydrolase precursor. In conclusion, our results demonstrate that the intramuscular administration of TTX changes the expression of hepatic genes involved in various signaling pathways.

Keywords: tetrodotoxin; pufferfish *Takifugu rubripes*; microarray analysis; gene expression; intramuscular administration; accumulation; toxification

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin, which binds to voltage-gated sodium channels with a very high affinity, and it is generally accepted that TTX is accumulated at high levels in specific tissues, such as the liver, ovary and skin, of *Takifugu pufferfish* [1,2]. The hypothesis that pufferfish themselves are unable to synthesize TTX is now widely accepted, which is mainly supported by the fact that cultured specimens are non-toxic, but become toxic by feeding TTX-containing artificial diets [3–7]. TTX was also found in various wild marine animals, such as worms, annelids, snails, starfish and crabs [8]. In addition, TTX-producing marine bacteria were isolated from pufferfish, xanthid crab and red calcareous alga, as well as from marine environments [9–14]. These results support that TTX is an exogenous substance for TTX-bearing organisms, and the toxification occurs via the food chain, bacterial parasitism or symbiosis [15,16].

We investigated the *in vivo* pharmacokinetics of TTX in cultured specimens of torafugu *T. rubripes* given a single intravenous and gastrointestinal administration [17,18]. TTX was well introduced into the systemic circulation from the gastrointestinal tract by a saturable mechanism and rapidly taken up into the liver. In addition, we developed tissue models of *in vitro* accumulation/uptake of TTX in the liver, revealing the involvement of the carrier-mediated transport system in the TTX uptake mechanism of torafugu *T. rubripes* [19–21]. These results strongly indicate that pufferfish have a special function to actively accumulate TTX in the liver at high concentrations.

We previously investigated the genes related to the accumulation of TTX in the liver by comparing mRNA expression patterns in the wild marine pufferfish, *T. chrysops* and *T. niphobles*, which have different concentrations of TTX in the liver using mRNA arbitrarily-primed (RAP) RT-PCR [22].

Briefly, RAP RT-PCR provided a 383-bp cDNA fragment, and its transcripts were higher in toxicity than non-toxic pufferfish liver. Its deduced amino acid sequence was similar to those of fibrinogen-like proteins reported for other vertebrates. The cDNA fragment of 383 bp was composed of at least three fibrinogen-like protein (flp) genes (flps), flp-1, flp-2 and flp-3, in the liver of *T. chrysops* and *T. niphobles* containing high concentrations of TTX, and the relative mRNA levels of these genes showed a linear correlation with TTX levels in the liver of the two species. The gene encoding flp-1 in the liver of *T. niphobles* located in scaffold 628 of the Fugu Genome Database, and the amino acid sequence in a C-terminal region of flp-3 in *T. chrysops* liver was homologous to hepcidin precursors of the spotted green pufferfish, *Tetraodon nigroviridis*, European sea bass *Dicentrarchus labrax*, mouse and human. In addition, we also examined the hepatic gene expression profile in cultured torafugu by suppression subtractive hybridization (SSH) at 12 h after the intramuscular administration of 0.50 mg TTX/kg body weight into the caudal muscle [23]. The intramuscular administration of TTX increased the transcripts encoding acute-phase response proteins, such as hepcidin, complement C3, serotransferrin, apolipoprotein A-1 and high temperature adaptation protein Wap65-2 in the liver at 12 h after administration. Very recently, we performed DNA microarray analysis with total RNAs from toxic and non-toxic wild pufferfish [24]. The mRNA levels of 1,108 transcripts were more than two-fold higher in toxic than in nontoxic specimens, and the expression levels of nine genes were upregulated more than 10-fold in toxic ones. It was noted that proteins encoded by these genes are related to vitamin D metabolism and immunity. It is unclear, however, whether the transcripts of these genes are involved in TTX disposition and how they function in pufferfish. Thus, the biological and physiological significance of TTX in pufferfish remains unknown.

In this study, we performed DNA microarray analysis on the liver of marine pufferfish *T. rubripes* at five days after the intramuscular administration of 0.25 mg TTX/kg body weight into the caudal muscle to investigate the effect of TTX accumulation into the liver on the hepatic gene expression and to identify the genes possibly related to TTX accumulation in the liver. This study would answer questions beyond just the transcriptomic changes that may help drive TTX accumulation in the liver and also contribute to better understanding how the transcriptomic response can limit the toxicity of TTX to pufferfish.

2. Experimental Section

2.1. Materials

Experimental marine pufferfish *T. rubripes* specimens (18 months old, approximately 1 kg body weight), cultured by the flow-through aquaculture system that efficiently utilized the underground seawater from the Kanmon Tunnel at the Aquaculture Station, Kawaku Co., Ltd. in Shimonoseki, Yamaguchi Prefecture, Japan, were used in the present study. The temperature of the seawater was constant at around 20 °C throughout the year, and fish were fed arbitrarily with commercial diets. TTX used in the administration study was purified from the ovary of wild pufferfish *T. rubripes* collected at the coastal area of the Genkai-nada Sea in Japan by a combination of ultrafiltration and a series of column chromatographic separations, as reported previously [21]. Crystalline TTX (Wako Pure

Chemicals Industries, Osaka, Japan) was used as a standard for the liquid chromatography-fluorescence detection (LC-FLD) analysis. All other reagents were of analytical grade.

2.2. TTX Administration and Sample Preparation

The administration experiments were carried out at Kawaku Aquaculture Station in September, 2010. TTX was dissolved in modified Hank's balanced salt solution buffer (160 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, adjusted to pH 7.4 with NaOH solution), and five pufferfish specimens (0.99 ± 0.06 kg body weight) received an intramuscular injection of 0.25 mg TTX/500 μL/kg body weight into the caudal muscle and were maintained in a 1000-L circular culture tank using the flow-through aquaculture system for 5 days at 20 °C (the TTX-administration group, city, state, country). On Day 5 after administration, these fish were removed from the circular culture tank, and their tissues were dissected. The liver was cut into 5-mm pieces, immediately stored in RNAlater solution (Applied Biosystems, Foster City, CA, USA) and stored at −80 °C until use. For the control group, five pufferfish specimens (1.02 ± 0.06 kg body weight) were given an intramuscular injection of the buffer (500 μL/kg body weight) that did not contain TTX, and the liver samples were prepared as described above. The remaining liver samples and other tissues (ovary, skin, and muscle) were stored at −20 °C for TTX determination by LC-FLD.

2.3. TTX Extraction and Quantification

TTX was extracted from the tissue samples with 0.1% acetic acid by heating in a boiling water bath for 10 min after ultrasonication for 1 min according to the standard assay procedures for TTX [25]. TTX quantification was performed by LC-FLD analysis according to the methods of Nagashima *et al.* [26] and Shoji *et al.* [27] with some modifications. Briefly, the analytical column was a Wakopak Navi C30-5 (4.6 mm i.d. × 250 mm, 5 mm particle size, Wako Pure Chemical Industries, Osaka, Japan) and maintained at 25 °C. The mobile phase consisted of 5 mM sodium heptanesulfonate in 10 mM ammonium formate (pH 5.0) containing 1 vol% acetonitrile and was eluted at a flow rate of 1.0 mL/min. The eluates were heated at 105 °C with 4 N NaOH (flow rate 1.0 mL/min) in a Teflon tubing (0.5 mm i.d. × 20 m). The reaction products were cooled by flowing through the stainless tube (0.46 mm i.d. × 0.3 m) kept in ice-cold water and detected by an FP-2020 fluorescence detector (JASCO, Tokyo, Japan) with excitation at 365 nm and emission at 510 nm.

2.4. RNA Extraction for Microarray Analysis

Total RNAs were extracted from the liver samples of each group using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), as described in the manufacturer's instructions. Total RNA concentrations were measured using a NanoPhotometer (IMPLEN, Munich, Germany), and the quality of total RNA was analyzed by agarose gel electrophoresis.

2.5. Preparation of Fluorescently-Labeled cRNA and Microarray Analysis

A custom 44k oligonucleotide microarray was designed using the Agilent eArray application (Agilent Technologies, Santa Clara, CA, USA) [28] based on the predicted cDNA data (FUGU version 4) of the genome assembly [29]. A 700-ng aliquot of total RNAs extracted from the liver sample each of TTX-administered and control groups was mixed with One-Color Spike-Mix (Agilent Technologies), reverse-transcribed and labeled with Cy3 using the Quick Amp Labeling Kit (Agilent Technologies). Cy3-labeled cRNA was purified using the RNeasy Mini Kit (Qiagen) and fragmented using the Gene Expression Hybridization Kit (Agilent Technologies). The samples were mixed with an equal volume of hybridization buffer and transferred on the microarray slide glass, which was subsequently incubated at 65 °C for 17 h. After hybridization, the microarray glass slides were washed with gene expression wash Buffer 1 at room temperature for 1 min and rinsed with gene expression wash Buffer 2 at 37 °C for 1 min. The slide glasses were then dried with nitrogen gas and scanned immediately using a GenePix4000B scanner (Axon Instruments, Foster City, CA, USA). The scanning image files were converted into expression data using Feature extraction software version 10.7.3 (Agilent Technologies) [30]. Microarray data analysis was performed using GeneSpring GX software version 11.0 (Agilent Technologies) [31]. The raw expression values were normalized, and gene expression ratios were calculated by normalizing the TTX-administered *versus* control group. Differentially expressed genes in the TTX-administered group were selected based on a >2.0-fold change.

2.6. Quantitative Real-Time PCR

The differential expression of chymotrypsin-like elastase family member 2A (cela2a), which was the highest upregulated gene in the TTX-administered group, was further validated by quantitative real-time PCR. Briefly, total RNAs were extracted from the liver samples by the above protocol and treated with DNase I (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs were constructed using oligo-dT20 primers and SuperscriptTM III reverse transcriptase (Invitrogen), as described in the manufacturer's instructions. Real-time PCR was performed in a 20-μL reaction mixture containing 2 μL of cDNA (1:20 dilution), 10 μL of SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), 0.4 μL of ROX reference dye (Takara Bio), 0.8 μL of 10 μM gene-specific forward primer and 0.8 μL of 10 μM gene-specific reverse primer on an ABI7300 Real-Time PCR System (Applied Biosystems). Reactions were as follows: 95 °C for 30 s; then 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The relative fold change of the cela2a mRNA expression level was determined by the comparative delta threshold cycle (ΔCt) method for relative quantification based on the beta actin 1 mRNA (Accession Number U37499.1) expression level. The gene-specific primers were designed using Primer Express software version 3.0 (Applied Biosystems) [32], and the sequences are as follows: 5'-CTCTTCCAGCCATCCTTCCTT-3' (forward) and 5'-GACGTCGCACTTCATGATGCT-3' (reverse) for beta actin 1 (Accession No. U37499.1) and 5'-GGCACCACACCTTCAATCCT-3' (forward) and 5'-GGCTGGGAACAGATGGA ATG-3' (reverse) for cela2a (Ensemble FUGU ID, ENSTRUT00000045544).

2.7. Data Analysis and Statistics

Data from the quantitative real-time PCR are expressed as the mean ± standard error (SE), and the Student's *t*-test was used to analyze the significance of differences among the means at the level of $p < 0.05$.

3. Results

3.1. TTX Determination

TTX in the tissues of pufferfish *T. rubripes* on Day 5 after administration was analyzed by LC-FLD. TTX was detected only in the tissues from the TTX-administered group, but not from the control group (<0.15 μg TTX/g tissue). The concentration and total amounts of TTX in the tissues are summarized in Table 1. The concentration was highest in the liver followed by the skin and ovary, whereas TTX in the muscle was not at the detectable level. The ratio of the total amount of TTX accumulated to that administered was quite high (about 70%).

Table 1. Tetrodotoxin (TTX) concentrations and contents in the tissues of *Takifugu rubripes* specimens in the TTX-administered group.

No.	Sex	Body weight (kg)	Dose (μg)	TTX concentration (μg/g)				Total amount (μg)	Accumulation (% of dose)
				Liver	Skin	Ovary	Muscle		
1	F	1.14	285	0.48	0.30	5.43	N.D.[1]	145	51
2	F	0.84	210	0.68	0.97	5.30	N.D.	162	77
3	F	1.00	250	1.12	0.62	4.97	N.D.	206	82
4	F	1.10	275	0.54	0.33	4.51	N.D.	124	45
5	M	0.86	215	1.35	0.79	N.D.[1]	N.D.	176	82
Mean ± SE		0.99 ± 0.15	247 ± 15	0.84 ± 0.17	0.60 ± 0.13	5.05 ± 0.21	N.D.	163 ± 14	67 ± 8

[1] N.D.: not detected (<0.15 μg TTX/g tissue).

3.2. Gene Expression Analysis

To identify the differentially expressed genes between the TTX-administered pufferfish *T. rubripes* liver and control, the cDNA microarray analysis was performed using the Agilent eArray platform. A total of 59 genes were found to be upregulated more than two-fold with a *p*-value <0.05 in the TTX-administered group, as shown in Figure 1. The genes upregulated three-fold and more were extracted and are listed in Table 2. The highest upregulated gene was chymotrypsin-like elastase family 2A, and its fold change (FC) value was 37.6. The upregulated genes were assigned to have major molecular functions of the putative translated proteins based on their gene ontology information and the Gene Ontology Database [33] As shown in Figure 2, genes involved in enzyme and cofactors (metabolism) and transcription accounted for 27.1% and 15.3%, respectively, whereas those involved in receptor activity, protein binding and metal ion binding accounted for 13.6%, 10.2% and 8.5%, respectively.

Figure 1. Fold change analysis of 486 genes differentially expressed in the liver of *Takifugu rubripes*. The histogram shows the fold change values for 427 downregulated genes (**left**) and 59 upregulated genes (**right**) in the liver of *T. rubripes* on Day 5 after the intramuscular administration of TTX.

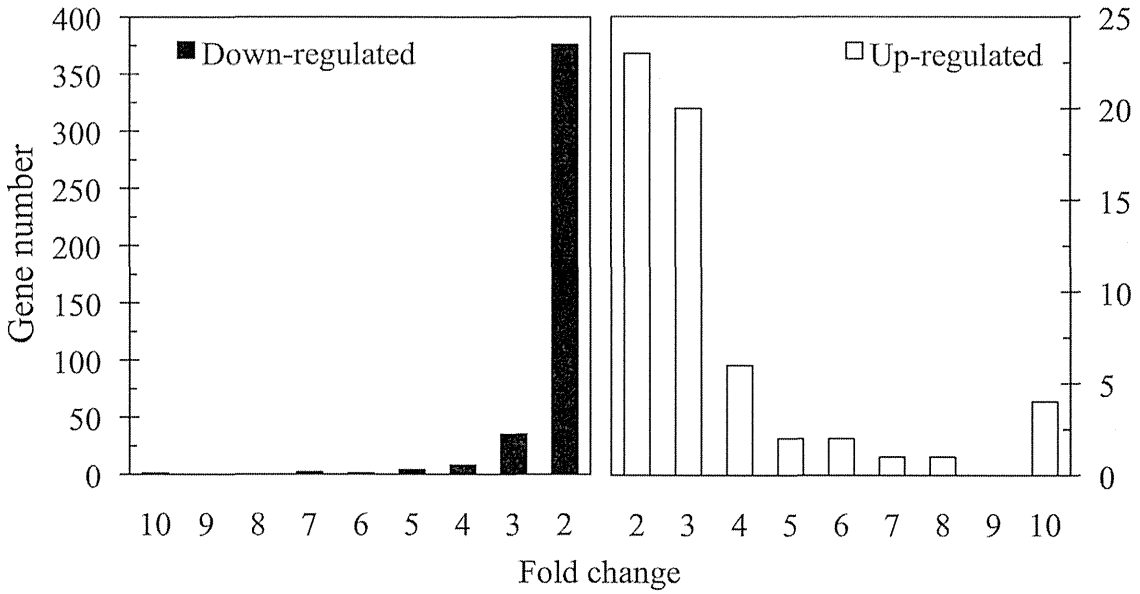

Figure 2. Functional classification of 59 genes significantly upregulated in the liver of *Takifugu rubripes* in the TTX-administered group.

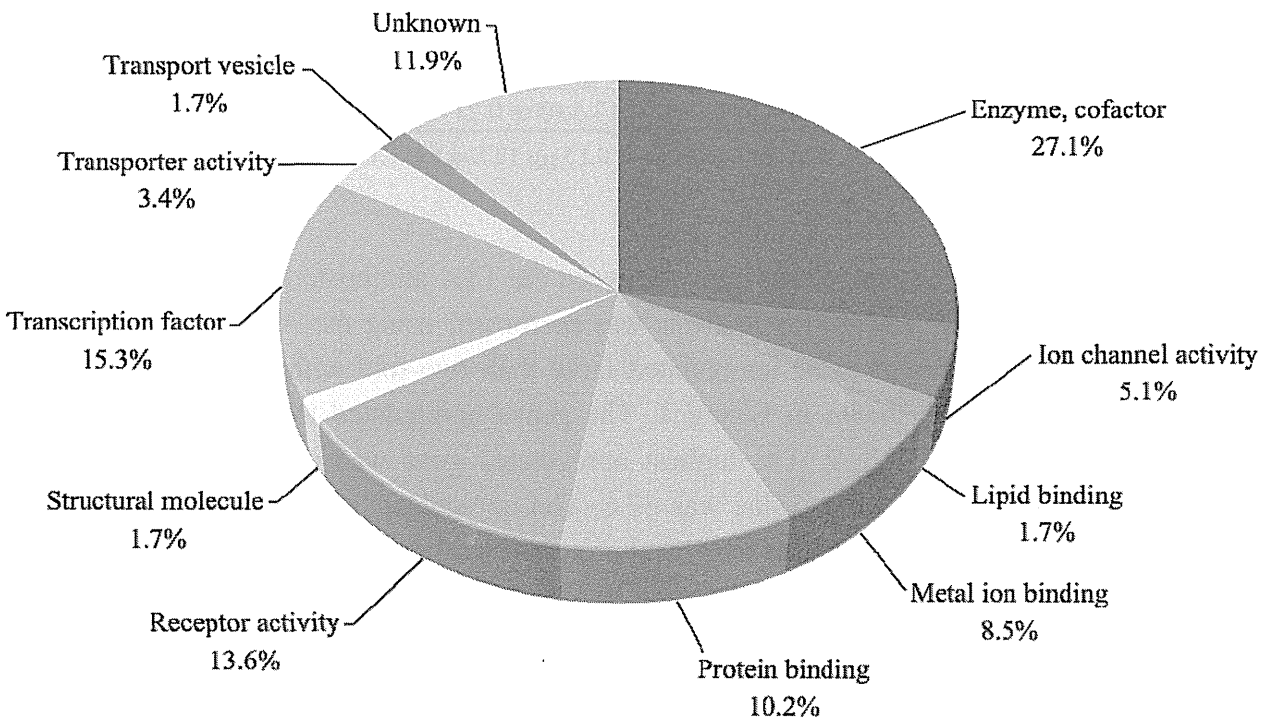

Table 2. List of genes upregulated in the liver of the TTX-administered pufferfish *Takifugu rubripes* group compared to those in the buffer-administered control group (FC [1] >3.0).

Ensemble ID	Gene name	Predicted description	Functional classification	FC
ENSTRUT00000045544	Cela2a	Chymotrypsin-like elastase family member 2A	Enzyme, cofactor	37.6
ENSTRUT00000041722	Tmem168	Transmembrane protein 168	Transport vesicle	20.0
ENSTRUT00000006023	Arhgap29	Rho GTPase-activating protein 29	Enzyme, cofactor	12.1
ENSTRUT00000034782	Kcnma1	*T. rubripes* calcium channel alpha-1 subunit homolog (AF026198.1)	Ion channel activity	10.4
ENSTRUT00000004724	Chrna9	*T. rubripes* nicotinic acetylcholine receptor alpha 9d subunit (AY299471.1)	Ion channel activity	8.1
ENSTRUT00000038610	Csmd1	CUB and sushi domain-containing protein 1	Protein binding	7.0
ENSTRUT00000036792	Serinc4	Serine incorporator 4	Transporter activity	6.8
ENSTRUT00000026094	C14orf135	Pecanex-like protein C14orf135	Unknown	6.6
ENSTRUT00000046072	Lrp2	Low density lipoprotein receptor-related protein 2	Receptor activity	5.4
ENSTRUT00000046718	Col27a1	Collagen alpha-1(XXVII) chain A-like	Structural molecule	5.2
ENSTRUT00000000397	Klhl32	Kelch-like protein 32	Protein binding	4.5
ENSTRUT00000000173	Elk4	ELK4, ETS-domain protein	Transcription factor	4.3
ENSTRUT00000025902	Zbtb45	Zinc finger and BTB domain-containing protein 45	Transcription factor	4.3
ENSTRUT00000020252	Spsb1	SPRY domain-containing SOCS box protein 1	Receptor activity	4.3
ENSTRUT00000032342	Dysf	Dysferlin	Lipid binding	4.1
ENSTRUT00000003310	Scn2b	Sodium channel beta-2 subunit	Ion channel activity	4.0
ENSTRUT00000011167	Loxl2	Lysyl oxidase homolog 2	Enzyme, cofactor	3.9
ENSTRUT00000017057	Dmbx1	Diencephalon/mesencephalon homeobox protein 1	Transcription factor	3.9
ENSTRUT00000023088	Clk2	Dual specificity protein kinase CLK2	Enzyme, cofactor	3.9
ENSTRUT00000045827	Myo3b	Myosin-IIIb	Enzyme, cofactor	3.8
ENSTRUT00000025143	Megf11	Multiple epidermal growth factor-like domains protein 11	Metal ion binding	3.7
ENSTRUT00000003539	Slc44a1	Choline transporter-like protein 1	Transporter activity	3.7
ENSTRUT00000017798	Cc4	Carbonic anhydrase 4	Enzyme, cofactor	3.5
ENSTRUT00000006962	OR4563-2	*T. rubripes* odorant receptor (DQ306241.1)	Receptor activity	3.5
ENSTRUT00000024316	Pitpnm2	Membrane-associated phosphatidylinositol transfer protein 2	Metal ion binding	3.4
ENSTRUT00000030952	Ptafr	Platelet-activating factor receptor	Receptor activity	3.4
ENSTRUT00000038903	Hepacam2	HEPACAM family member 2	Unknown	3.4
ENSTRUT00000043658	Gpr22	Probable G-protein coupled receptor 22	Receptor activity	3.3
ENSTRUT00000031306	Ptpn2	Tyrosine-protein phosphatase non-receptor type 2	Enzyme, cofactor	3.3
ENSTRUT00000019629	Eps15l1	Epidermal growth factor receptor substrate 15-like 1	Receptor activity	3.2
ENSTRUT00000036590	Zdhhc8	Membrane-associated DHHC8 zinc finger protein (NM_001078596.1)	Enzyme, cofactor	3.2
ENSTRUT00000031045	Rheb	GTP-binding protein Rheb	Enzyme, cofactor	3.2
ENSTRUT00000047583	Agap2	Arf GAP with GTPase domain, ankyrin repeat and PH domain 2	Enzyme, cofactor	3.0
ENSTRUT00000017824	Ppargc1a	Peroxisome proliferator activated receptor gamma coactivator 1 alpha (DQ157766.1)	Receptor activity	3.0
ENSTRUT00000027961	Pfkp	6-phosphofructokinase type C	Enzyme, cofactor	3.0
ENSTRUT00000029743	E2f2	Transcription factor E2F2	Unknown	3.0

[1] FC is the average fold change of the TTX-administered (n = 5) compared to the buffer-administered control group (n = 5).