明らかとはなっていない.

われわれは前報[20]で，奄美群島の加計呂麻島でCFPが潜在的に高い頻度で発生していることを明らかにした．さらに同島で2008年に発生した食中毒2事例について，患者の症状，原因魚種の同定およびMBAにより，原因魚肉中に中毒量のCTXsが含まれることを示した．今回，これらの食中毒試料についてLC-MS/MSにより毒組成を解析し，同時に漁獲された近海魚についても分析を実施した．また，同年に奄美大島で発生したCFPが疑われる事例の原因魚肉を入手し，魚種同定，MBAおよびLC-MS/MSにより解析したので報告する．今回分析対象としたCFP 3事例の概要をTable 1に示す.

実験方法

1. 試料および魚種同定

2008年9月に鹿児島県大島郡瀬戸内町（加計呂麻島）沿岸で漁獲され，同島でCFP[20]の原因となったバラハタ *Variola louti*（試料1，事例1），イッテンフエダイ *Lutjanus monostigma*（試料2，事例2），事例1で原因魚と同時に摂食された魚肉（試料3），およびこれらと同海域で同時に漁獲されたヒメフエダイ *Lutjanus gibbus* 2試料（試料4, 5），オジロバラハタ *Variola albimarginata*（試料6），オオグチイシチビキ *Aphareus rutilans*（試料7），オオメカマス *Sphyraena forsteri* 2試料（試料8, 9）の筋肉を用いた．さらに，同年8月に鹿児島県奄美市（奄美大島）で食中毒の原因となった同島近海産の魚肉を用いた（試料10，事例3）.

なお，魚種については，試料1および2は遺伝子解析により，試料4～9は外部形態により同定し報告した[20]．患者らが摂食したにもかかわらずMBA陰性であった試料3および今回新たに得られた試料10について，遺伝子解析による種同定を行った．既報[20),21)]に準じて，ユニバーサルプライマー（16S arLおよび16S brH）によりミトコンドリアDNA中の16S rRNA遺伝子部分領域をPCR法により増幅した．PCR産物を精製後，ダイレクトシーケンスにより塩基配列を決定した.

なお，魚種の和名は文献22の標準和名に従った.

2. 試　　薬

抽出用溶媒にアセトン，ジエチルエーテル，メタノール，ヘキサンの特級を使用した．固相抽出およびLC移動相にはメタノール，酢酸エチル，アセトニトリル，蒸留水，ギ酸のHPLC用グレードを用い，ギ酸アンモニウムは特級を使用した（いずれも和光純薬工業(株)製）.

標準品は，天然試料から単離・同定[1),23)]されたCTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B*, CTX4A, CTX4B, M-*seco*-CTX4A/B*, CTX3C, 49-*epi*CTX3C*, 2-hydroxyCTX3C*, 2,3-dihydroxyCTX3C, 51-hydroxy-CTX3C, M-*seco*-CTX3C*, M-*seco*-CTX3C methyl acetal*およびガンビエロールを使用した．このうち，アスタリスク（*）で示した成分は微量であり，秤量の精度が低いことから，定性分析用標準品とした.

3. マウス毒性試験（MBA）

試料10の筋肉について抽出物を調製しMBAに供した．MBAは食品衛生検査指針理化学編シガテラ[24]の試験法に準じて行い，抽出に用いる筋肉量は120 gとした．抽出物は1% Tween 60含有生理食塩水でエマルジョン化し，マウス（ddY系，オス，17～20 g，九動(株)）へ腹腔内投与後，24時間以内に死亡する最小投与量を1 MUとした．MBAの実施にあたっては，沖縄県衛生環境研究所動物実験委員会の承認を受け，その実施規定に従った.

4. LC-MS/MS

試料1～10の筋肉について試験液を調製しLC-MS/MSに供した．LC-MS/MSは既報[19]に準じて行った.

試験液の調製：各試料につき筋肉5 gを採肉し，アセトン15 mLでホモジナイズ，遠心分離（3,000 rpm, 10 min），上澄みの濃縮を2回繰り返した．濃縮液（1 mL以下）をジエチルエーテル5 mLにより2回再抽出した．エーテル層の濃縮残さを90%メタノール1.5 mLに溶解し，ヘキサン3 mLによる脱脂後，濃縮乾固し抽出物を得た．抽出物を酢酸エチル–メタノール（9 : 1）に溶解し，ジーエルサイエンス社製InertSep FL-PR（500 mg）に通液させ（4 mL），窒素気流下で濃縮乾固した．残さをアセトニトリル溶液としてInertSep PSA（200 mg）に負荷し（3 mL），つづいてメタノール3 mLにより溶出した．アセトニトリルおよびメタノールにより溶出した分析対象物を濃縮乾固後，それぞれメタノール1 mLに調製し，必要に応じ希釈して試験液とした.

分析装置：アジレント・テクノロジー社製のAgilent 1200 LCシステムおよびAgilent 6460トリプル四重極LC/MSシステムを用いた.

LC条件：分析対象物の重なりを最小にし，かつ短時間での分析を維持したまま，移動相の初期濃度，勾配，最終濃度の調整により，試料中マトリクスによる分析への影響を低減する条件を検討した．最終的に以下の条件を採用した．移動相には5 mmol/Lギ酸アンモニウムおよび0.1% (v/v) ギ酸を含む蒸留水（A）とメタノール（B）を用い，73%Bから90%Bまで11分間リニアグラジエントを行い4分間保持した．カラムはアジレント・テクノロジー社製のZorbax Eclipse Plus C18（2.1×50 mm, 1.8 μm）を40℃に維持し使用した．移動相の流速は0.4 mL/min，試験液の注入量は5 μLとした.

MS条件：イオン源にAgilent Jet Streamサーマルグラジエントフォーカシング技術を備えたエレクトロスプレーイオン化（ESI）法により，ダイナミックMRMモードで測定を行った．各パラメータは以下のとおり設定した．drying gas (300℃, 10 L/min), nebulizer gas (50 psi), sheath gas (400℃, 11 L/min), capillary voltage (5,000 V), nozzle voltage (1,000 V), fragmentor voltage (350 V), collision energy (CTXs: 40 eV, gambierol: 24 eV)．モニターイオンはプリカーサー／プロダクトイオン共にナトリウムイオン

付加分子を選択した．すなわち，$[M+Na]^+ > [M+Na]^+$ とし，溶出順に m/z 779.5（ガンビエロール），m/z 1,133.6（CTX1B），m/z 1,063.6（M-*seco*-CTX3C），m/z 1,079.6（2,3-dihydroxyCTX3C），m/z 1,061.6（51-hydroxyCTX3C），m/z 1,101.6（M-*seco*-CTX4A/B），m/z 1,117.6（52-*epi*-54-deoxyCTX1B，54-deoxyCTX1B），m/z 1,063.6（2-hydroxyCTX3C），m/z 1,077.6（M-*seco*-CTX3C methyl acetal），m/z 1,045.6（49-*epi*CTX3C，CTX3C），m/z 1,083.6（CTX4A，CTX4B）とした．得られたクロマトグラムのピーク積分値をCTXs標準品との比較により定量した．54-deoxyCTX1Bについては，そのエピ体と同等のイオン化効率とみなし，52-*epi*-54-deoxyCTX1Bの検量線を用いて定量した．標準品の検出下限（$S/N>3$）および定量下限（$S/N>10$）濃度は，CTX1Bと52-*epi*-54-deoxyCTX1Bはそれぞれ0.02および0.1 ng/mLであった．その他の標準品はそれぞれ0.05および0.2 ng/mLとした．

結　果

1. 魚種の同定

遺伝子解析により，試料10の16S rRNA後半領域の塩基配列577 bpはイッテンフエダイ *Lutjanus monostigma*（DQ784735）と一致した．一方で，試料3は前報でハマフエフキ *Lethrinus nebulosus* としていたが[20]，決定した塩基配列581 bp（AB793299）はハマフエフキ（AB793300）と約3%の変異が見られた．また，沖縄・奄美海域の代表的なシガテラ原因魚とも一致しなかった．

2. マウス毒性

試料10のマウス毒性は0.1 MU/gであった（Table 2）．

3. 毒 組 成

試料3～9の分析において，CTX1Bおよびガンビエロールの保持時間に重複するピークが検出されたが（痕跡値～0.06 ppb CTX1B相当および痕跡値～0.15 ppbガンビエロール相当），測定条件検討の結果，グラジエント条件の変更により対象成分と妨害ピークとの分離が可能となった．

CFPの原因食品であり，MBAで陽性であった試料1，2[20]および試料10のすべてからCTX1B類縁体が検出された．しかし，CTX3C類縁体やガンビエロールは検出されなかった（Fig. 2）．試料1はCTX1Bおよび54-deoxy-

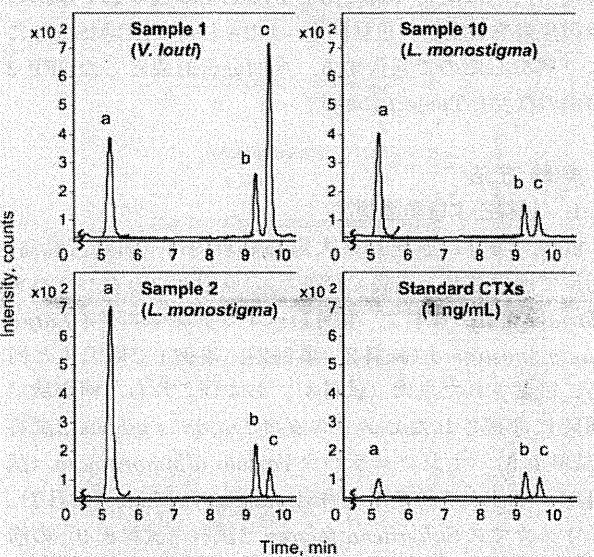

Fig. 2. Chromatograms of extracts from fish flesh implicated in CFP in Amami and Kakeroma Islands, and standard CTXs: CTX1B (a, 1 ng/mL), 52-*epi*-54-deoxyCTX1B (b, 1 ng/mL), and 54-deoxyCTX1B (c). The sample solutions used for injection were equivalent to 5 g flesh/mL (samples 1 and 10), and 1 g/mL (sample 2).

Table 2. Toxicity and contents of CTXs in fish flesh implicated in CFP and fish caught on the same occasion in coastal waters of Amami and Kakeroma Islands

Sample No.	Incident involved	Species	Toxicity[*1] (MU/g)	Toxin content[*2] (ng/g)							
				CTX1B	52-*epi*-54-Deoxy CTX1B	54-Deoxy CTX1B[*3]	CTX4A	CTX4B	M-*seco*-CTX4A/B	CTX3C type toxins[*4]	Gambierol
1	1	*V. louti*	0.2	1.12	0.32	1.48	ND	ND	ND	ND	ND
2	2	*L. monostigma*	0.8	8.78	1.18	0.90	ND	ND	ND	ND	ND
3	1	Unidentified[*5]	ND	0.13	0.04	0.04	ND	ND	ND	ND	ND
4	—	*L. gibbus*	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	—	*L. gibbus*	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	—	*V. albimarginata*	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	—	*A. rutilans*	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	—	*S. forsteri*	ND	ND	ND	ND	ND	ND	ND	ND	ND
9	—	*S. forsteri*	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	3	*L. monostigma*	0.1	1.11	0.16	0.15	ND	ND	ND	ND	ND

ND: not detected

[*1] Toxicity was determined by MBA (The MBA results of samples 1–9 were reported previously[20].)

[*2] Toxin contents were measured by LC-MS/MS.

[*3] 54-DeoxyCTX1B content in flesh was estimated by comparison with its 52-epimer as a quantifiable standard.

[*4] CTX3C, 49-*epi*CTX3C, 51-hydroxyCTX3C, 2,3-dihydroxyCTX3C, 2-hydroxyCTX3C, M-*seco*-CTX3C, and M-*seco*-CTX3C methyl acetal.

[*5] *Lethrinus* sp. can be identified by analysis of 16S rRNA gene on mtDNA (AB793299).

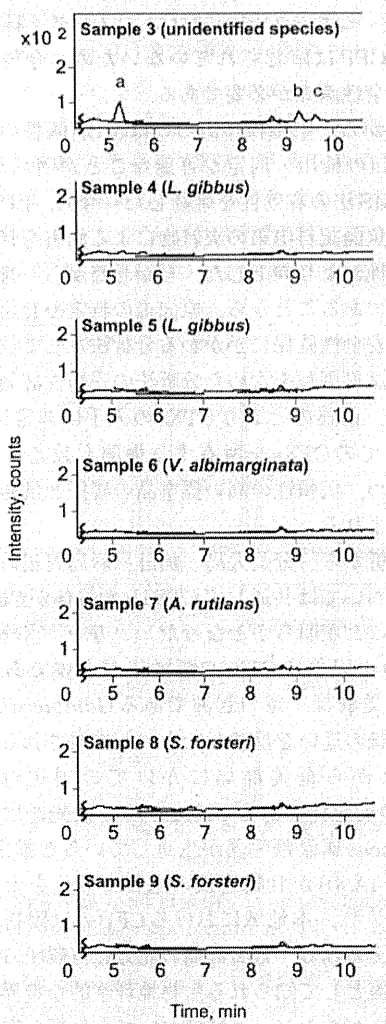

Fig. 3. Chromatograms of extracts from fish caught in coastal waters of Kakeroma Island. (a: CTX1B, b: 52-*epi*-54-deoxyCTX1B, c: 54-deoxyCTX1B)

The sample solutions used for injection were equivalent to 5 g flesh/mL.

CTX1Bの含量が高く（それぞれ1.12および1.48 ng/g），52-*epi*-54-deoxyCTX1Bは0.32 ng/gであった．試料2はCTX1Bを主要毒とし（8.78 ng/g），52-*epi*-54-deoxyCTX1Bおよび54-deoxyCTX1B含量はそれぞれ1.18および0.9 ng/gであった．試料10はCTX1Bを主要毒とし（1.11 ng/g），52-*epi*-54-deoxyCTX1Bおよび54-deoxyCTX1Bをそれぞれ0.16および0.15 ng/g含有した．

MBAで陰性（<0.025 MU/g）であった試料3～9のうち[20]，試料4～9については，すべての測定対象成分が検出下限値未満であった（Fig. 3）．一方，試料3からはCTX1B（0.13 ng/g），52-*epi*-54-deoxyCTX1B（0.04 ng/g）および54-deoxyCTX1B（0.04 ng/g）が検出された（Fig. 3）．

Table 2に各魚試料のマウス毒性およびLC-MS/MSによる各毒成分の定量結果を示す．

Table 3. Mouse toxicity obtained by MBA and calculated from LC-MS/MS results

Sample No.	MBA[*1] (MU/g)	LC-MS/MS[*2] (MU/g)	
		CTX1B	52-*epi*-54-DeoxyCTX1B
1	0.2–0.4	0.160	0.023
2	0.8–1.6	1.254	0.084
3	<0.025	0.018	0.003
10	0.1–0.2	0.158	0.012

[*1] MBA results are shown as the range of toxicity obtained with a dilution series of fish extracts.

[*2] The i.p. lethal dose levels in mice (7 ng for CTX1B and 14 ng for 52-*epi*-54-deoxyCTX1B)[23),25)] were used for conversion of the LC-MS/MS data to mouse lethality. The lethal dose for 54-deoxyCTX1B is unknown and so this compound was not included in the calculation.

考　　察

CFPの原因物質であるCTXsは，これまでに仏領ポリネシア産魚類および*G. toxicus*から20以上の類縁体が確認されており[23)]，基本骨格の違いからCTX1Bタイプと CTX3Cタイプに大別される（Fig. 1）．国内で発生するCFPの原因成分は，沖縄県で代表的なシガテラ原因魚がCTX1B類縁体のみであるのに対し，宮崎県沿岸で漁獲され食中毒の原因となった魚[17)]はCTX3C類縁体主体であり，また南鳥島沖で漁獲され食中毒の原因となった魚[15)]からはCTX1BおよびCTX3C両類縁体が検出され，国内でも地域によって毒組成が顕著に異なる傾向がある[19)]．今回，奄美大島および加計呂麻島で発生したCFPの原因成分は，いずれの試料もCTX1B類縁体であることが明らかとなった（Fig. 2）．CFPの原因となる魚種および毒組成は，沖縄県におけるCFP事例と類似していた．また，イッテンフエダイ（試料2, 10）は共通してCTX1Bが主要毒であるのに対し，バラハタ（試料1）では54-deoxyCTX1Bの含有率も高いことは（Table 2），沖縄産試料で確認された魚種特異的な毒組成[19)]とも一致する．

CTXsが検出された試料についてLC-MS/MS値からMUを算出した（Table 3）．MU換算に使用したマウス致死量は，CTX1Bを7 ng/MU，52-*epi*-54-deoxyCTX1Bを14 ng/MUとしたが[23),25)]，54-deoxyCTX1Bは比毒性が明確でないため除外した．また，MBAでは抽出物を2倍段階希釈により調製するため，決定した毒力はその値の2倍未満までの誤差範囲を持つこととした．これらを元にLC-MS/MS値とMBA結果を比較した（Fig. 4）．試料1以外はLC-MS/MSで測定した毒力の総和がMBA結果の範囲と一致した．試料1については，MBA結果に比してLC-MS/MS値が低かったが，換算に含めなかった54-deoxyCTX1B含量が相対的に高いため，その毒性が影響したものと考えられる．試料1および10はMBAによる毒力差があるが（それぞれ0.2および0.1 MU/g），LC-MS/MSで測定・換算した毒力はほぼ同程度である（Fig. 4）．一方で試料1の54-deoxyCTX1B含量は試料10の約10倍であり

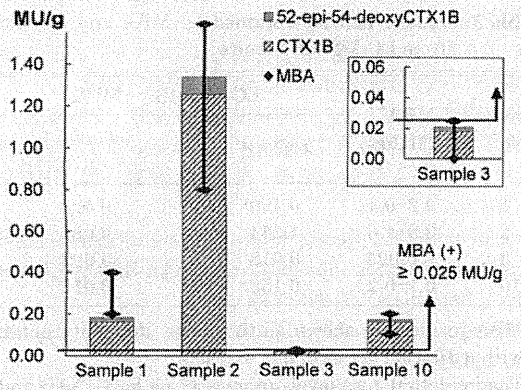

Fig. 4. Comparison of LC-MS/MS with MBA results for fish flesh implicated in CFP in Amami and Kakeroma Islands

The inset displays a magnified result for sample 3. The MBA results are shown as the range of toxicity. The line at 0.025 MU/g indicates the detection limit of MBA for ciguatoxins in fish.

(Table 2)，この毒組成差からも54-deoxyCTX1BのMBA結果への寄与が示唆される．

シガテラ毒のMBAにおいて0.025 MU/g以上の毒力を持つ試料は食用不適とされるが[24]，今回CFPの原因となった魚肉は，いずれもそれを大きく上回った（0.1～0.8 MU/g）．CTX1Bはシガトキシン類の中でも毒性が強く，太平洋地域におけるCFPの主要原因毒の1つであり[1]，本報の事例でも主要毒となっている（Fig. 4）．しかしながら，54-deoxyCTX1B含有率の高いバラハタやコクハンアラ[19]などの魚種については，同時に54-deoxyCTX1Bによる影響も考慮することが必要であろう．今回のLC-MS/MSおよびMBAで測定した毒力の比較（Fig. 4）から，54-deoxyCTX1Bのマウス致死量を推定すると，少なくとも90 ng/MUから最高9 ng/MUの値を取りうる．CTXsのマウス毒性については，Yasumoto[23]，杉山[25]が詳細に検討しており，前報[19]でも換算に使用した．これらの値とは多少異なる報告値もあるが[3,4]，使用するマウス系統の違いと微量試料の測定誤差によるものと考えられる．現在のところ，54-deoxyCTX1B標準品は天然試料から単離されたものが僅かに残るのみで，その使用が制限されるため，定量可能な標準品の確保と毒性の評価が急がれる．

事例1において原因魚（試料1）とともに摂食された試料3は，MBAにおいて陰性を示したため問題にされなかったが[20]，今回のLC-MS/MSによりCTXsを含有することが判明した（Fig. 3）．CTX1Bは10 MU（70 ng）以上の摂食によりヒトに中毒をもたらすとされるが[1,23]，試料3の主要毒であるCTX1B含量は試料1の1/9程度（0.018 MU/g）であることから（Table 3），今回のCFPへの寄与は少ないものであったと考えられる．しかしながら，MBAの検出境界にも近く（Fig. 4），摂食量によっては単独で食中毒を引き起こす可能性も否定できない．試料3は，遺伝子解析の結果からハマフエフキとは近縁種と推定されるが，これまで国内ではフエフキダイ科Lethrinidaeによる CFPは確認されていないため，今後詳細な魚種同定と安全性調査が必要である．

この事例から，中毒検体あるいはMBA陽性の検体でなくともCTXsの検出・同定が可能なことが明らかとなり，本LC-MS/MS法の有効性を確認した．今回，LC条件の検討により，魚肉試料由来の夾雑物による妨害を軽減し，分析上の特異性がより向上した．しかしながら，対象となる魚種が多様であることから，原因毒の特定や食用適否の判定など食の安全性確保にかかわる分析法として位置づけるためには，試料調製を含めた分析法の妥当性評価が必要である．また，前述のとおりCTXsの入手は非常に困難で，標準品としてのCTXsを所有する機関も数えるほどである．そのため，信頼性の高い標準品の確保と供給体制の確立が強く望まれる．

今回の分析から，奄美大島・加計呂麻島周辺および沖縄県の海域においては共通してCTX1B類縁体がCFPの原因物質であることが明らかとなった．一方で，宮崎県で発生したCFPの原因魚はCTX3C類縁体が主体である[19]．この毒組成の差異は，毒の起源である*Gambierdiscus*属渦鞭毛藻の系統の違いを反映したものと推察される．すなわち，沖縄県から奄美群島にかけての周辺海域では，CTX1B類の前駆体であるCTX4A/4Bを優先的に産生する*Gambierdiscus*属渦鞭毛藻が生息していると推定される．これらCTX4A/4Bが食物連鎖の過程で酸化を受けて毒性が増強され[1,23]，本地域におけるCFPの原因物質となっていると考えられる．奄美群島の北方に位置するトカラ列島には渡瀬線として知られる生物地理学的な境界線（トカラ構造海峡）があるが，海域においても魚類分布の変化が示されている[22]．また，同海域は黒潮が通過しており，その分断により*Gambierdiscus*属渦鞭毛藻の系統を分けていることが想定される．CTXs毒組成の差異と分布境界線との関係に興味が持たれる．

地球温暖化による海水温の上昇に伴い，CTXs産生者である*Gambierdiscus*属渦鞭毛藻の分布域拡大と温帯域でのCFP発生増加が世界的に懸念されている[3,9,18]．本稿でも示した標準品にかかわる問題が解決されれば，MBAに代わってLC-MS/MS法の普及が見込まれる．CFP原因食品の分析はもちろんのこと，広く食用魚の安全性調査や渦鞭毛藻のモニタリングが可能となり，食中毒防止への貢献が期待される．また現在，CTX3C群とCTX1B群に対する抗体が作製されており[26]，免疫学的測定法をはじめとする応用が期待されている．これらを評価・活用するうえで，対象物のCTXs組成を明らかにする必要があり，今回のLC-MS/MS分析で得た知見は，シガテラ原因魚の調査・検出に使用する抗体の適性を判断する重要な情報を提供するものである．すなわち，奄美群島から沖縄県にかけては抗CTX1B抗体の活用が期待できる．一方でそれ以外の地域から得られた分析試料は2検体に過ぎず，国内各地の試料について検討を継続しCTXs組成を明らかにしてい

くことが重要である.

謝　辞

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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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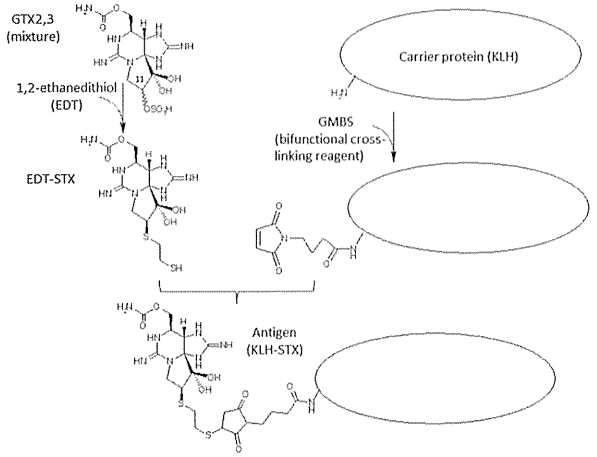

Figure 1. Preparation process and estimation structure of the KLH-STX antigen.

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).

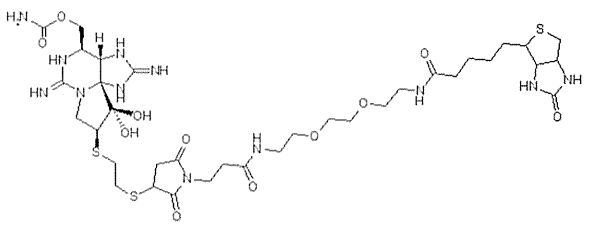

Figure 2. Estimation structure of biotin-STX, the competitive labeled antigen.

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

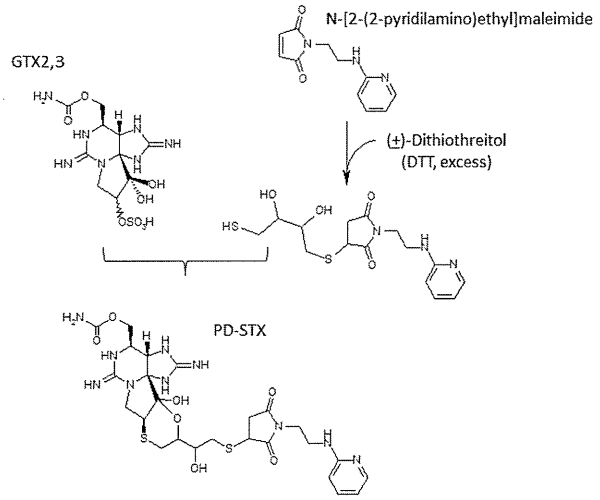

Figure 3. Preparation process and the estimation structure of PD-STX, the substitute standard of STX in ELISA.

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.

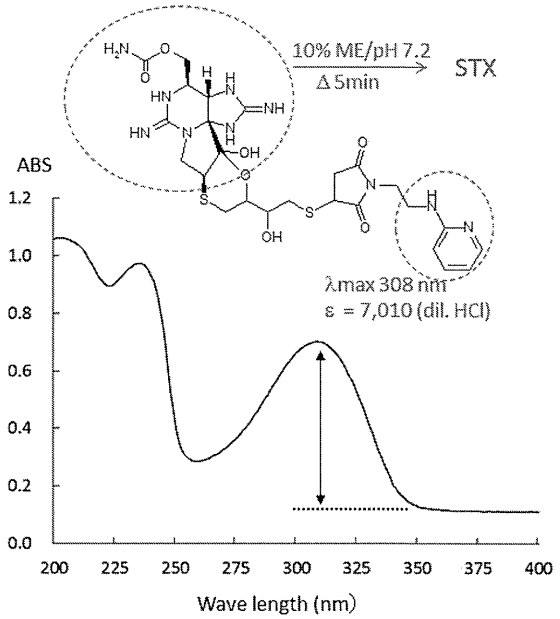

Figure 4. UV spectrum of PD-STX.

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

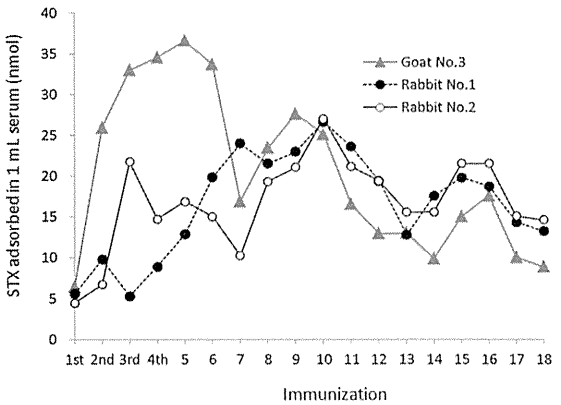

Figure 5. Enhancement of antibody activity in a goat and rabbits during immunization.

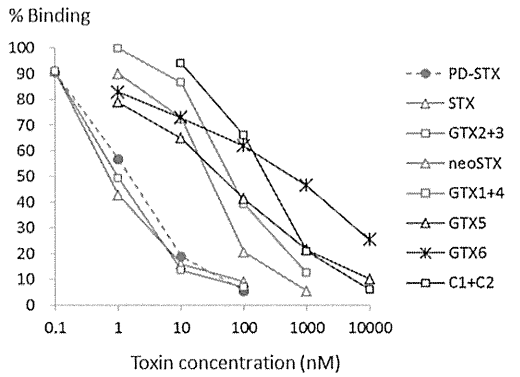

Figure 6. Comparison of the reactivity to the antibody of isolated toxins by ELISA. PD-STX, an artificial standard, is also analyzed.

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-

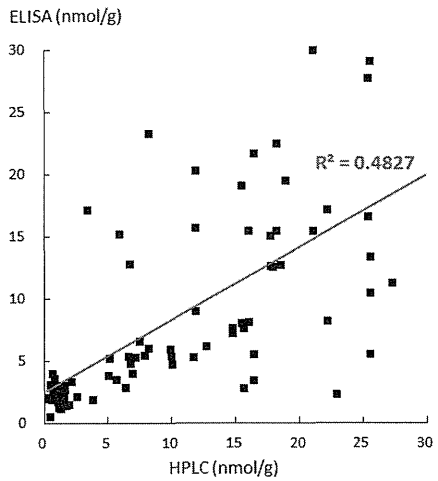

Figure 7. Comparison of the toxin concentration data obtained from ELISA with those from HPLC-fluorometric analysis. X-axis: crude extracts of shellfish were analyzed by a fluorometric HPLC system according to Oshima (16); Y-axis: crude extracts of shellfish were diluted with 0.1 M sodium phosphate (pH 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

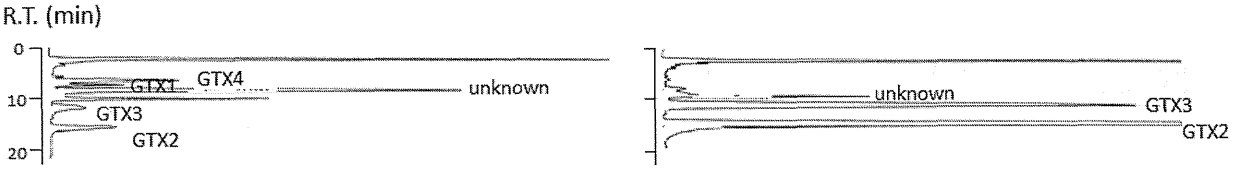

Figure 8. Chromatograms showing selective reduction of N1-OH of GTX1,4 to N1-H of GTX2,3 by ascorbic acid under presence of hemin as a catalyst. Left: before reduction. Right: after reduction. R.T. = retention time.

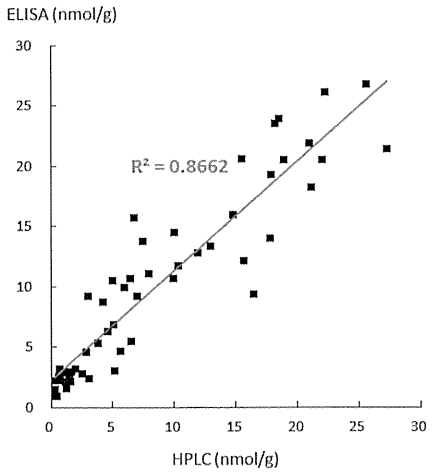

Figure 9. Comparison of the toxin concentration data of ELISA after transformation of toxin components by HCl treatment and selective reduction in the presence of hemin as a catalyst with those from HPLC-fluorometric analysis. X-axis: toxin level of crude extracts of shellfish analyzed by a fluorometric 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.

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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.

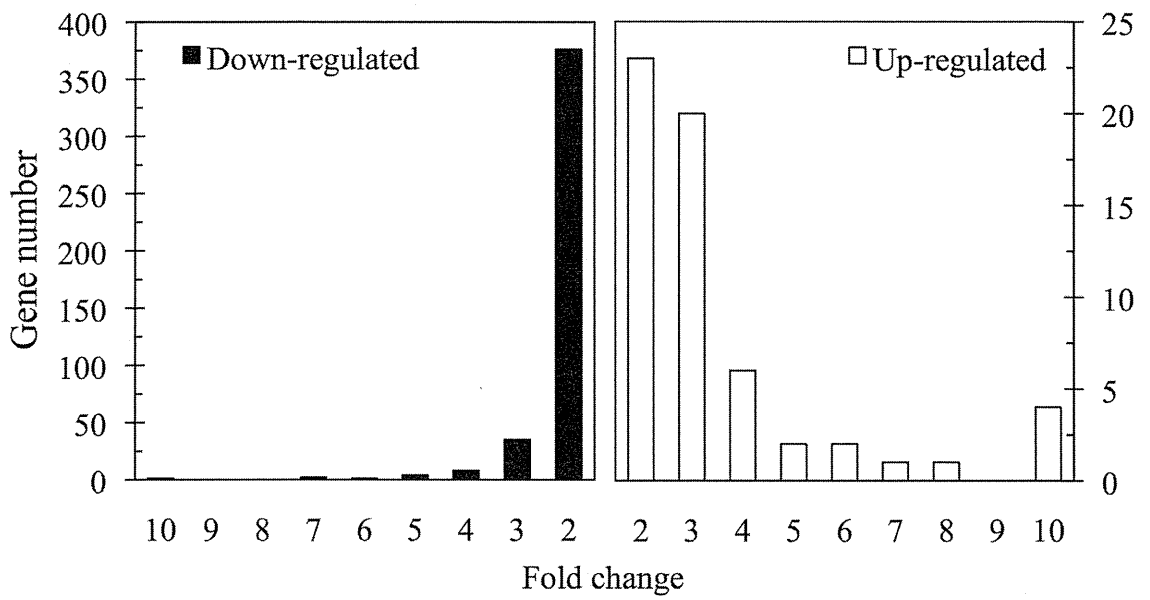

Figure 2. Functional classification of 59 genes significantly upregulated in the liver of *Takifugu rubripes* in the TTX-administered group.

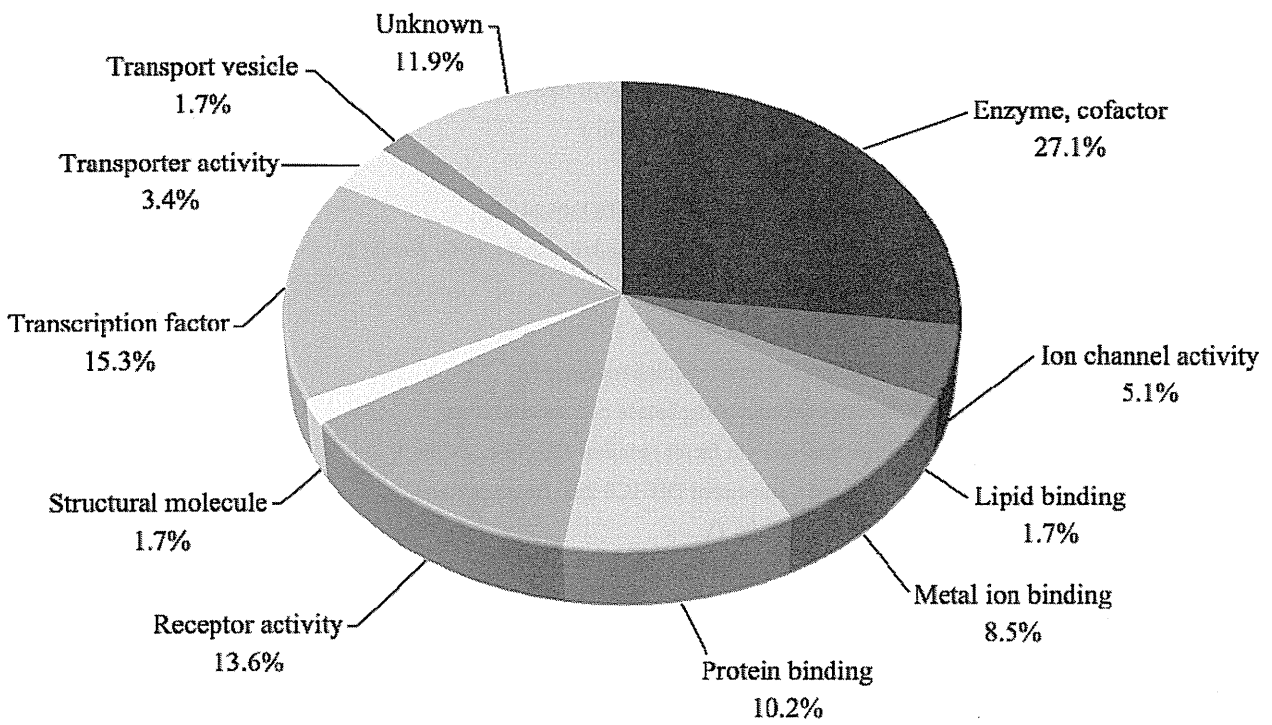

Table 2. List of genes upregulated in the liver of the TTX-administered pufferfish *Takifugu rubripes* group compared to those in the buffer-administered control group (FC [1] >3.0).

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).

In contrast, a total of 427 genes were found to be downregulated by TTX administration, as shown in Figure 1. The genes downregulated three-fold and more were extracted and are listed in Table 3. The highest downregulated gene in the TTX-administered group was elongation factor G2, and its FC value was −13.0. As shown in Figure 3, gene ontology classification of the downregulated genes showed that those involved in protein binding and enzyme and cofactors (metabolism) accounted for 20.8% and 13.1%, respectively, whereas those involved in the transcription factor, receptor activity and transporter activity accounted for 15.9, 10.1 and 6.6%, respectively.

To confirm the validity of the microarray data, real-time PCR was performed for the highest upregulated gene, chymotrypsin-like elastase family member 2A (Figure 4). There was a significant difference in the mRNA expression level between the TTX-administered (27.22 ± 4.18) and control group (1.00 ± 0.77) (p = 0.0019). This result is well correlated with the data of microarray analysis, FC 37.6 (Table 2).

Figure 3. Functional classification of 427 genes significantly downregulated in the liver of *Takifugu rubripes* in the TTX-administered group.