

PRACTICAL SANDWICH ELISA KIT FOR **CIGUATOXIN “CTX1B”**

Takeshi Sato¹, Mariko Yoshimura², Masako Hayakawa², Takeshi Tsumuraya³ and
Masahiro Hirama³

¹Cell Science Inc., Aoba-ku, Sendai 989-3212, Japan

²Wako Pure Chemical Industries, Ltd., Chuo-ku, Tokyo 103-0023, Japan

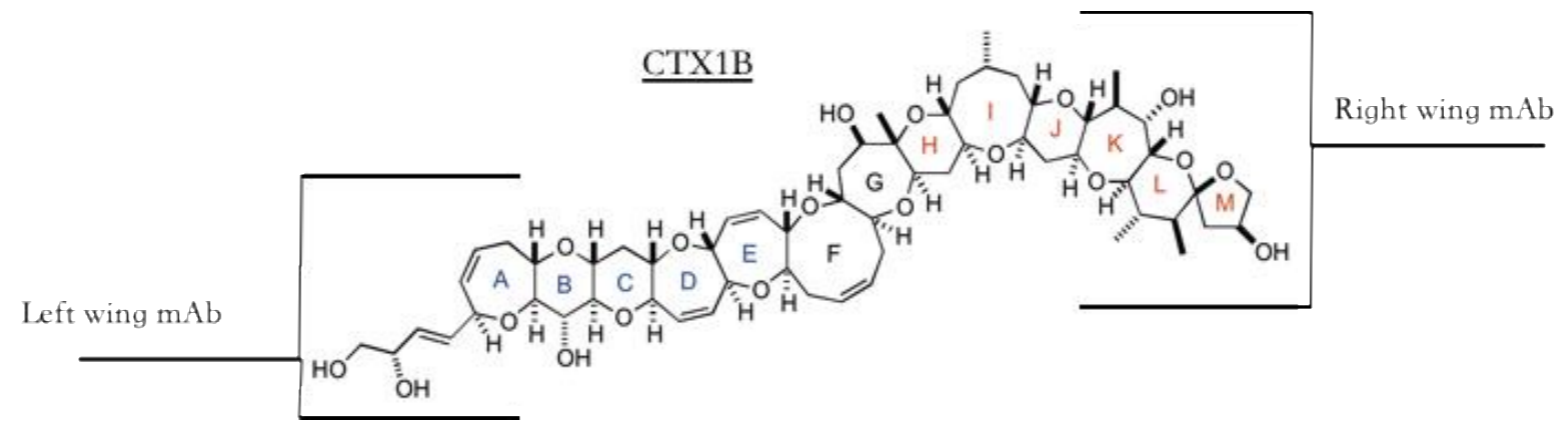
³Department of Biological Science, Graduate School of Science, Osaka Prefecture University,
Osaka 599-8531, Japan

Abbreviations

ELISA (Enzyme Linked Immuno-Sorbent Assay), CTX (Ciguatoxin), AP (Alkaline Phosphatase),
PNPP (Disodium p-Nitrophenyl Phosphate)

Introduction

Prof. Hirama and his coworkers of Tohoku University in Japan chemically synthesized four congeners of CTXs (CTX3C, CTX1B, 51-hydroxy-CTX3C, and 54-deoxy-CTX1B), which are mainly present in the Pacific Ocean. Subsequently, using synthetic hapten-KLH conjugates as antigens, Prof. Tsumuraya produced several kinds of monoclonal antibodies that strongly bind to the right side or the left side of CTXs molecule, and they successfully developed a highly sensitive immunochemical detection method.



Based on these researches, we have commercialized a new ELISA kit “CTX-ELISA 1B” to detect and quantify CTX1B, which is likely to be most abundant in the Pacific Ocean, at the detection range of 0.2 ~ 0.0005 ppb. Here we show the ELISA kit “CTX-ELISA 1B” and illustrate how to use it for an analysis of CTX1B in the flesh of a ciguatoxic fish.

Materials and Methods

“CTX-ELISA 1B” Kit



Contents of the Kit

- | | | |
|---|--|------|
| ① | Antibody coated 96 well plate “ CTX1B ” | 2 |
| ② | Wash buffer x20 conc. “ W-1 ” | 50ml |
| ③ | STD/Sample dilute “ D-1 ” | 50ml |
| ④ | Anti-CTX1B-AP dilute “ D-2 ” | 50ml |
| ⑤ | AP Substrate dilute “ R-1 ” | 50ml |
| ⑥ | CTX1B Standard DMSO solution | |
| ⑦ | AP-conjugated “ Anti-CTX1B-AP ” | |
| ⑧ | Plate cover sheet | 6 |

Table 1. AP-substrates usable in this kit and their detection condition

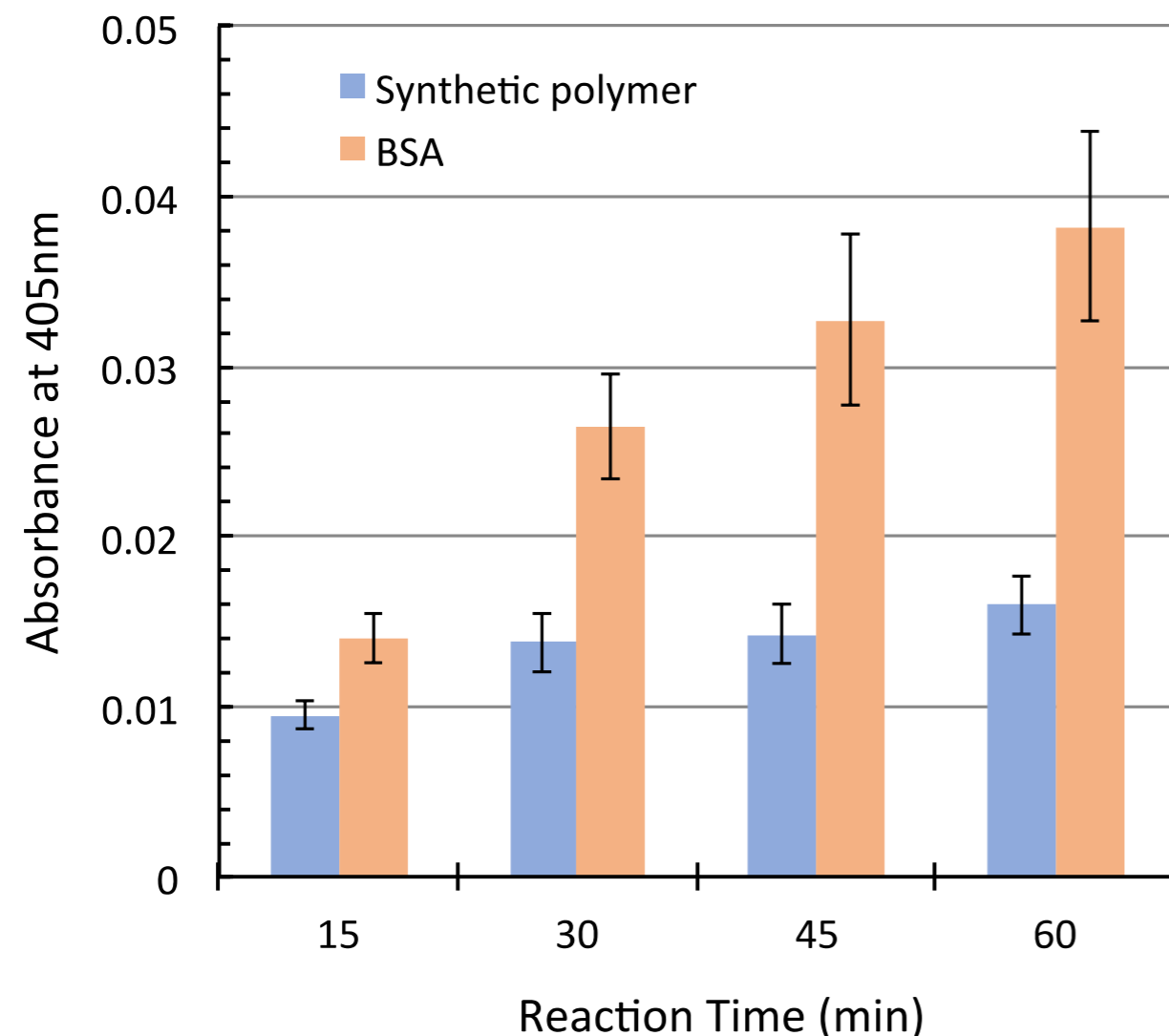
Detection	AP-Substrate	Substrate conc.	λ	Ex. λ	Em. λ
Colorimetry	PNPP (Wako Pure Chemical Ind. Inc., JAPAN)	10mg/12ml R-1	405nm		
Fluorometry	AtoPhos [□] AP Fluorescence Substrate (Promega Corporation, WI 53711-5399 USA)	Refer to instruction manual		440nm	575nm

Summary of assay procedure

- ① As described in the protocol of “CTX-ELISA 1B” Kit, prepare washing solution, test sample, CTX standard solution.
↓
- ② Add 100 μ L test sample and CTXs standard solution into each well.
↓ Incubate for 30 minutes at 37°C or at room temp.
- ③ Aspirate and wash 3 times with 200~300 μ L/well of wash buffer.
↓
- ④ Add 100 μ L Anti-CTXs-AP solution into each well.
↓ Incubate for 30 minutes at 37°C or at room temp.
- ⑤ Aspirate and wash 3 times with 200~300 μ L/well of wash buffer.
↓
- ⑥ Prepare AP-Substrate solution, and add 100~200 μ L/well.
↓ Incubate at 37°C.
- ⑦ Read Absorbance or Fluorescence Intensity within 30~45min.

Fig.1 Comparative test of blocking materials “Synthetic Polymer and Bovine Serum Albumin (BSA)” that suppress non-specific binding of CTX1B to the plastic surface.

- ① CTX1B solution of 250pg/ml was injected into a micro-test-plate pretreated with Synthetic Polymer or BSA, and incubated for 30minutes.
- ② After washing the plate, Anti-CTX1B-AP solution was added into each well, and incubated for 30 minutes.
- ③ After washing, added AP-substrate PNPP into each well to measure nonspecific binding of CTX1B.
- ④ Absorbance at 405nm was measured on each time.



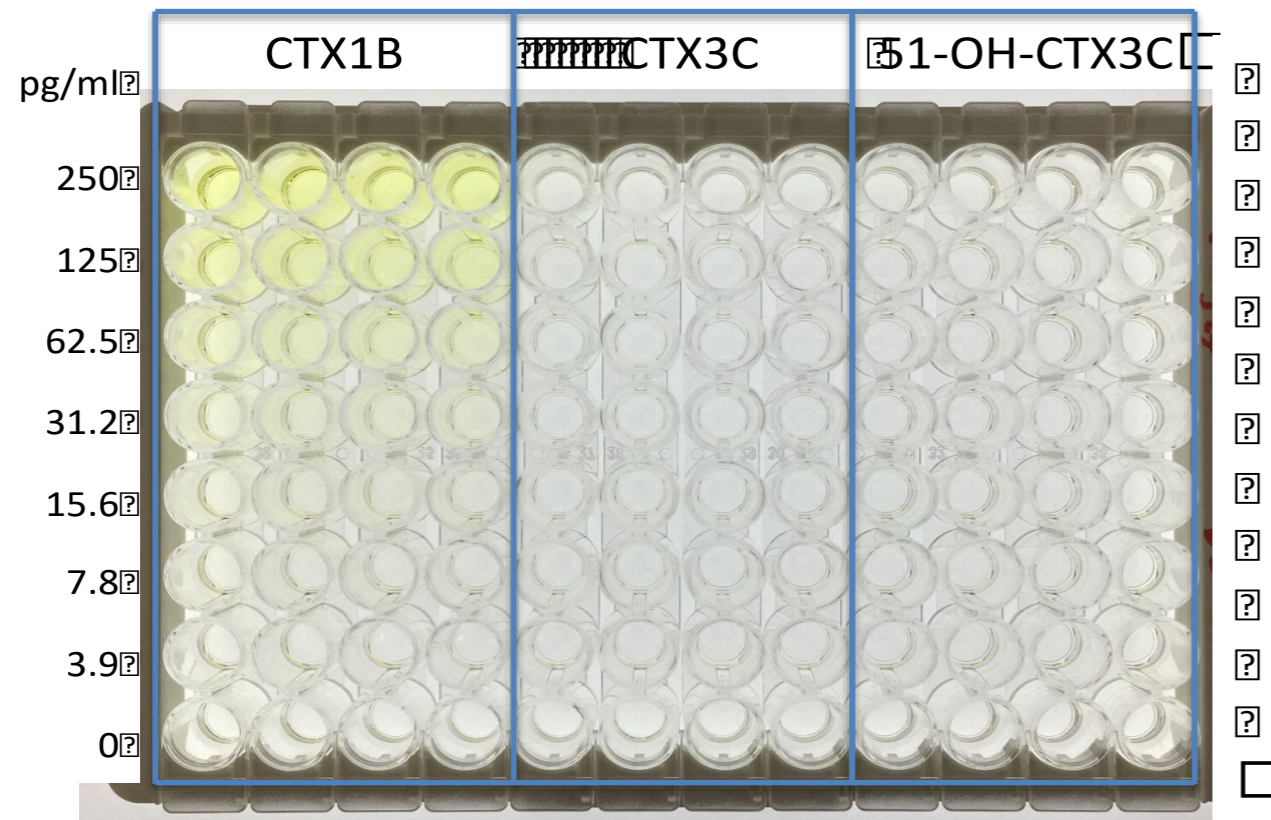
Results

1. Synthetic polymer strongly blocked non-specific binding of CTX1B and/or Anti-CTX1B-AP, and kept low absorbance for long time.
2. BSA that was used as a blocking reagent generally, did not adequately block the increase of background color.
3. The synthetic polymer effectively inhibited the nonspecific binding of Anti-CTX1B-AP and CTX1B to the plastic surface, and exerted excellent suppressing effect of background coloration. Synthetic polymer is extremely useful for improving the detection sensitivity.

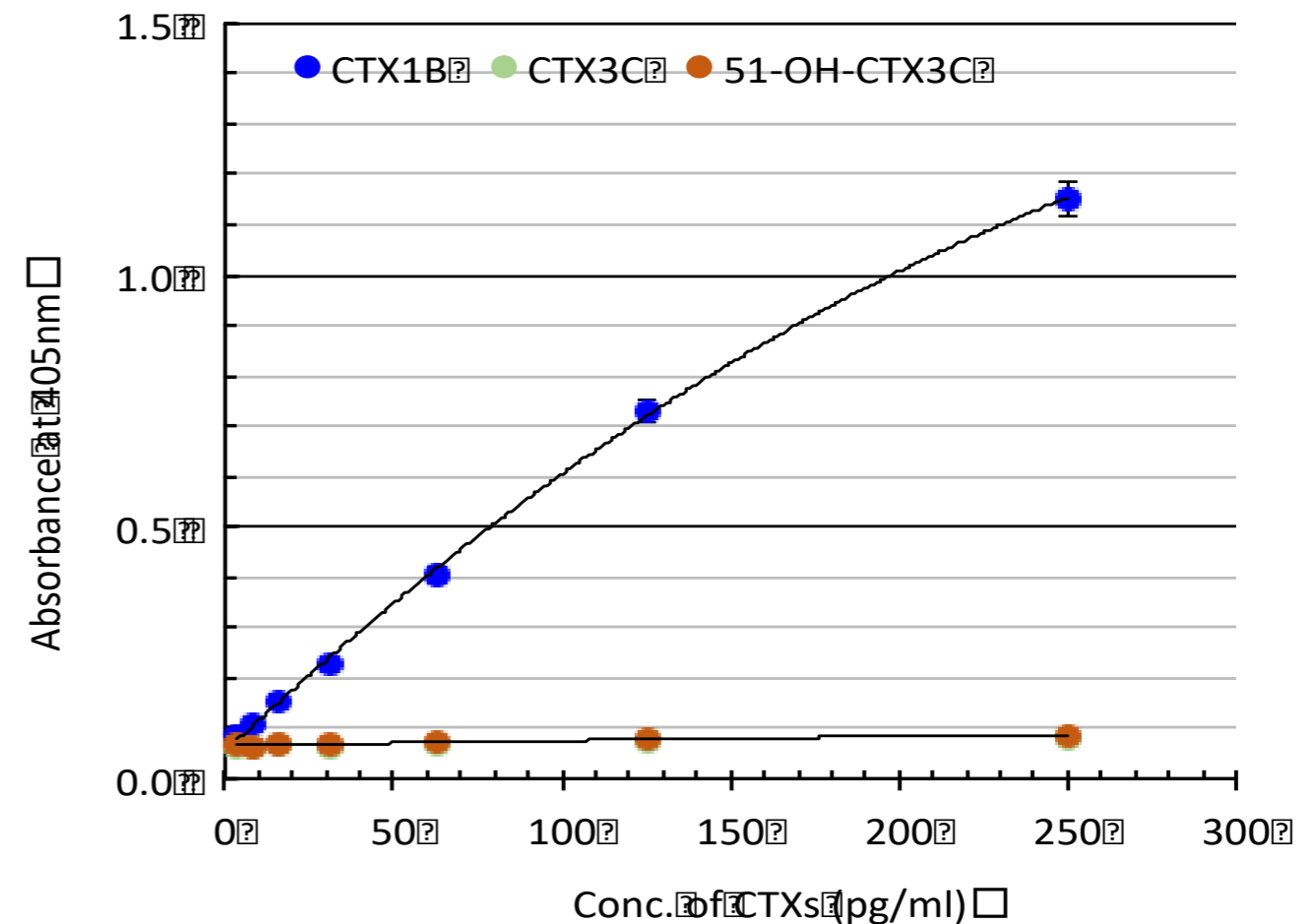
Fig.2 Specificity of the “CTX-ELISA 1B” Kit.

Three CTXs, “CTX1B, CTX3C and 51-OH-CTX3C” were separately assayed with “CTX-ELISA 1B” Kit, and the amount of CTXs were visualized using PNPP as substrate. Standard curve was prepared by measuring the absorbance at 405nm.

Photograph of micro test plate after 45min incubation



Standard curve of CTX1B, CTX3C and 51-OH-CTX3C



Result

“CTX-ELISA 1B” Kit can detect only CTX1B but not CTX3C and 51-OH-CTX3C.

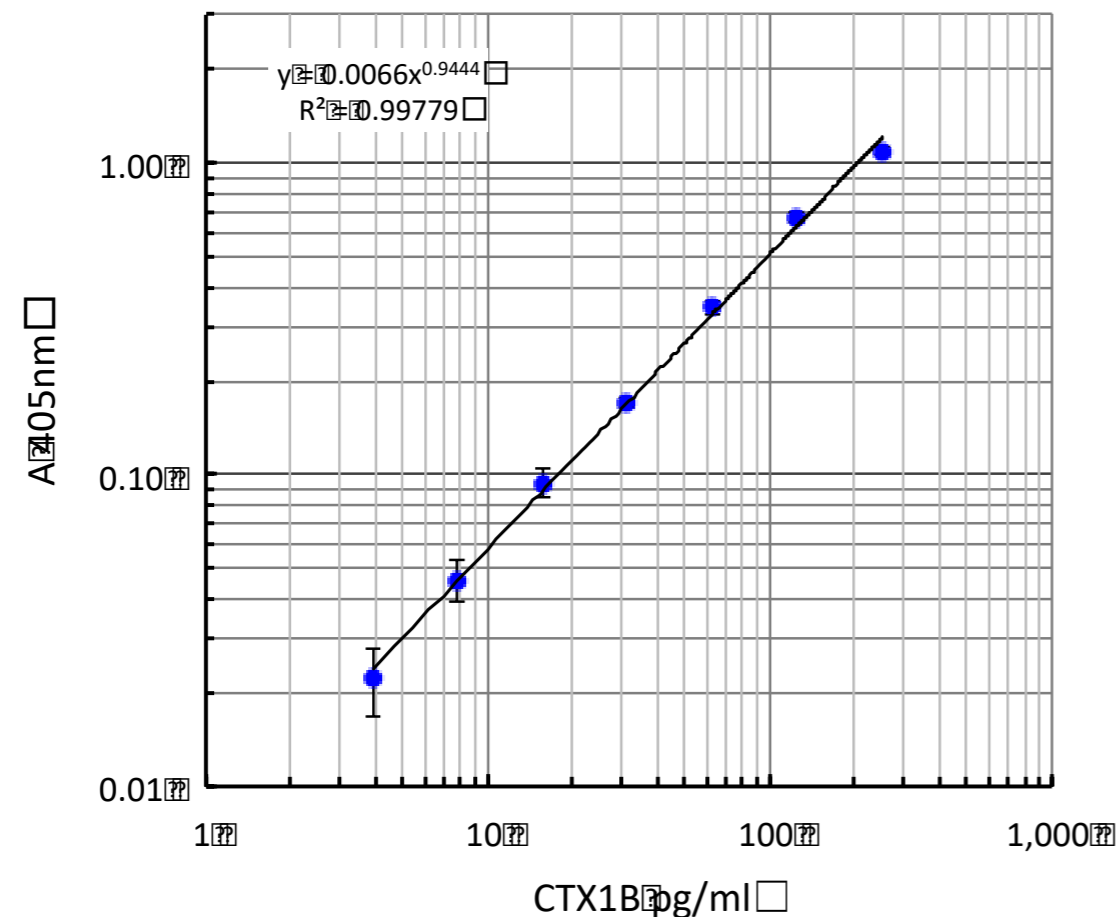
*** The "CTX-ELISA 1B" kit can specifically detect CTX1B.**

Fig.3 Comparative test of detection range for CTX1B by colorimetry and fluorometry.

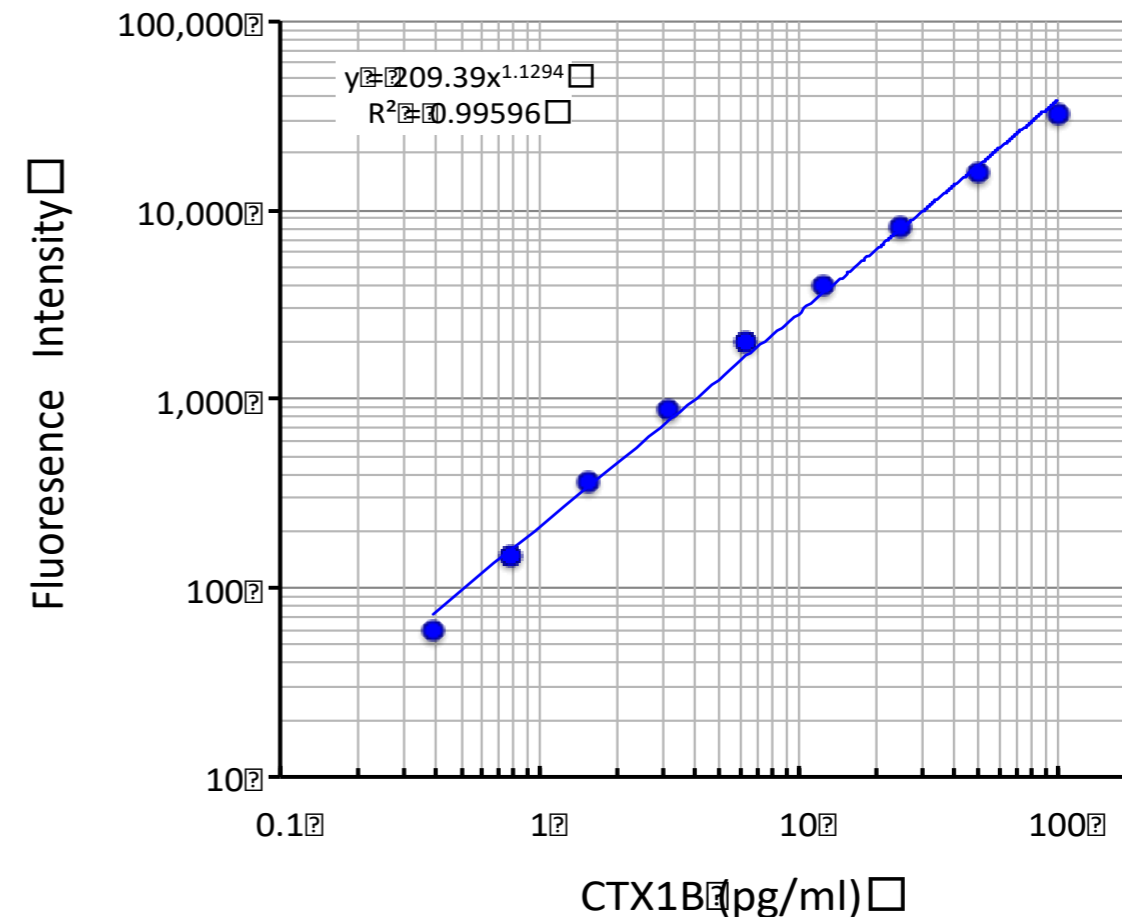
CTX1B of 0.1 ~ 250 pg/ml were measured by calorimetry and fluorometry.

PNPP and Atophos[□] AP substrate were used as AP-substrates for colorimetry and fluorometry, respectively, and compered detectable range between both procedures.

Colorimetry by PNPP



Fluorometry by AtoPhos[□] AP substrate



Result

- ① “CTX-ELISA 1B” Kit is usable for both of colorimetry and fluorometry.
- ② Detection limits by colorimetry and fluorometry are about 5 pg/ml (0.005ppb) and 0.5 pg/ml (0.0005ppb), respectively.
- ③ The fluorometry is 10 times more sensitive than the colorimetry, and sufficiently exceeds the FDA guidance level (0.01ppb).

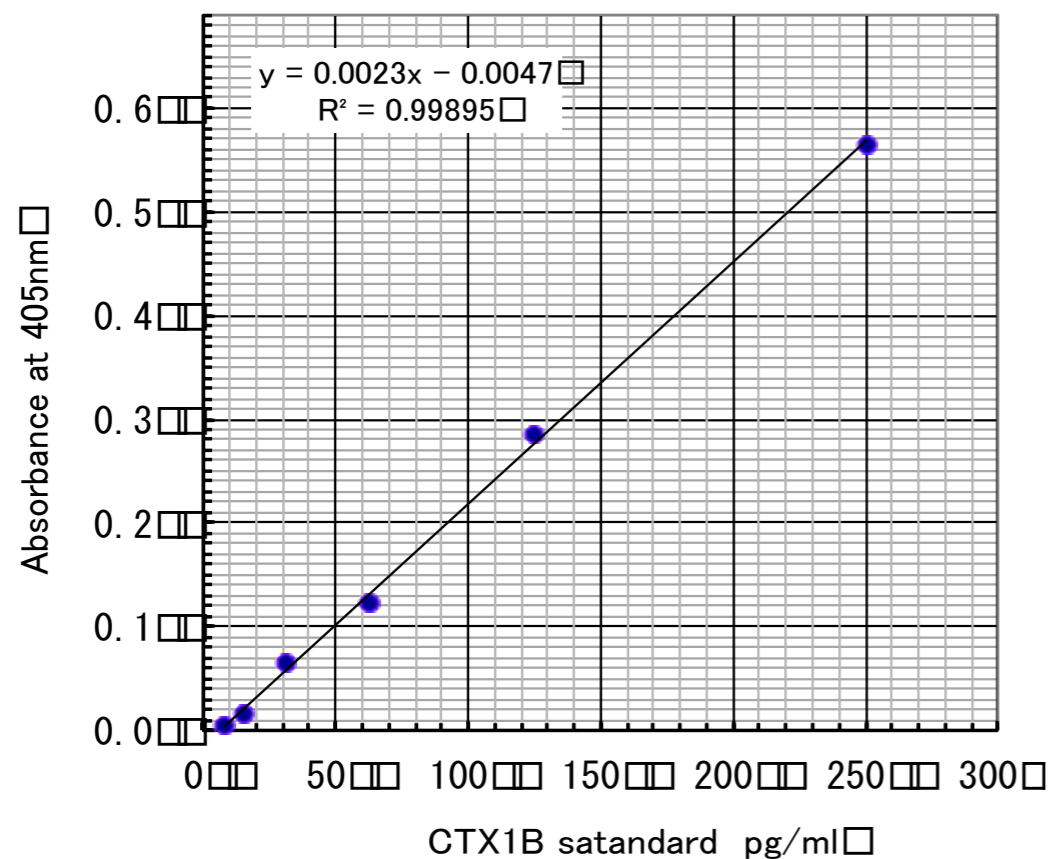
Fig.4 Detection of CTX1B containing in the ciguatoxic fish by “CTX-ELISA 1B”

Tentative procedure for preparing fish flesh extract.

- ① Put 5 g of fish flesh in a glass test tube, add 5 mL of methanol, and then mince it finely with ultra-sonic or glass homogenizer and so on.
- ② Centrifuge at 15,000 rpm for 10min, collect methanol layer into glass tube and then remove methanol/water by evaporation in vacuum.
- ③ Add 1 mL of DMSO and dissolve the residues by vortex for a few minutes.
- ④ Centrifuge and take the supernatant in a test tube as a test sample, and store in refrigerator until use.

Measurement of fish flesh extract

Calibration curve of CTX1B



Sample dilution 1:	Absorbance 405nm					CV%	CTX1B pg/ml	
	D1	D2	D3	Average	SD		Raw DATA	Calculated
10	0.365	0.428	0.279	0.357	0.075	20.9	157.4	1,574
20	0.216	0.294	0.249	0.253	0.039	15.5	112.0	2,241
40	0.148	0.127	0.131	0.135	0.011	8.2	60.9	2,435
80	0.061	0.059	0.065	0.062	0.003	5.0	29.4	2,349
Average conc.								2,342

Result

Approximately **2.34 ng/ml** of CTX1B was detected from the methanol extract of 5 g ciguatoxic fish flesh by **“CTX-ELISA 1B” Kit**. Therefore, the amount of CTX1B contained in the test fish was calculated to be about **468 pg/g**.

Conclusion

The newly developed ELISA kit “CTX-ELISA 1B” can detect CTX1B, which is likely to be most abundant in the Pacific Ocean, at the **wide range of 0.2~0.0005ppb**. This “CTX-ELISA 1B” is the first investigation tool that can specifically, quantitatively, and practically detect CTX1B. Since the detection sensitivity is much superior to the **FDA guidance level of 0.01ppb**, this kit will be useful not only for prevention of Ciguatera food poisoning (CFP) but also for the epidemiological and physiological studies.

Currently, we are also developing other CTX-ELISA kits, which can detect other CTXs. These new kits will be soon available from Wako Pure Chemical Industries, Ltd. of Japan.

Related papers

Ciguatera and its off-shoots-Chance encounters en route to a molecular structure. Scheuer, P. J.
Tetrahedron, **50**, 3 (1994).

Structures and configurations of ciguatoxin from moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. Murata, M., Legrand, A. M., Ishibashi, Y., Fukui, M., Yasumoto, T.
J. Am. Chem. Soc. **112**, 4380 (1990).

Marine toxins. Yasumoto, T., Murata, M. *Chem. Rev.* **93**, 1897 (1993).

Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. Yasumoto, T., Igarashi, T., Legrand, A.-M., Cruchet, P., Chinain, M., Fujita, T., Naoki, H.
J. Am. Chem. Soc. **122**, 4988 (2000).

The chemistry and biological function of natural marine toxins. Yasumoto, T.
Chem. Rec. **1**, 228 (2001).

Total synthesis of ciguatoxin CTX3C. Hirama, M., Oishi, T., Uehara, H., Inoue, M., Maruyama, M., Oguri, H., Satake, M.
Science, **294**, 1904 (2001).

Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. Oguri, H., Hirama, M., Tsumuraya, T., Fujii, I., Maruyama, M., Uehara, H., Nagumo, Y.
J. Am. Chem. Soc. **125**, 7608 (2003).

Total synthesis of ciguatoxin and 51-hydroxyCTX3C. Inoue, M., Miyazaki, K., Ishihara, Y., Tatami, A., Ohnuma, Y., Kawada, Y., Komano, K., Yamashita, S., Lee, N., Hirama, M.
J. Am. Chem. Soc. **128**, 9352 (2006).

Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins.
Tsumuraya, T., Fujii, I., Hirama, M.
Toxicon, **56**, 797 (2010).

Ciguatera incidence and fish toxicity in Okinawa, Japan. Oshiro, N., Yogi, K., Asato, S., Sasaki, T., Tamanaha, K., Hirama, M., Yasumoto, T., Inafuku, Y.
Toxicon, **56**, 656 (2010).

First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). Otero, P., Perez, S., Alfonso, A., Vale, C., Rodriguez, P., Gouveia, N. N., Gouveia, N., Delgado, J., Vale, P., Hirama, M., Ishihara, Y., Molgo, J., Botana, L. M.
Anal. Chem. **82**, 6032 (2010).

Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative alga from the Pacific. Yogi, K., Oshiro, N., Inafuku, Y., Hirama, M., Yasumoto, T.
Anal. Chem. **83**, 8886 (2011).

Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. Tsumuraya, T., Takeuchi, K., Yamashita, S., Fujii, I., Hirama, M.
Toxicon, **60**, 348 (2012).

Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: Sandwich ELISA detection of ciguatoxins. Tsumuraya, T., Fujii, I., Hirama, M.
J. ACAC. **97**, 373 (2014).

Practical route to the left wing of CTX1B and total syntheses of CTX1B and 54-deoxyCTX1B. Yamashita, S., Takeuchi, K., Koyama, T., Inoue, M., Hayashi, Y., Hirama, M.
Chem. Eur. J. **21**, 2621 (2015).

Highly Sensitive and Practical Fluorescent Sandwich ELISA for ^{[[L]]}_{SEP}Ciguatoxins. Tsumuraya, T., Sato, T., Hirama, M., Fujii, I., ^{[[L]]}_{SEP}
Anal. Chem. **90**, 7318 (2018).