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Original article

Haptenic properties of tetrodotoxin conjugated to carrier proteins by using dithiol reagents

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Introduction

Tetrodotoxin (TTX) has been detected as a toxic principle of various aquatic organisms such as puffer fish, goby, marine snails, horseshoe crab and xanthid crab. Traditionally, the toxicity of these organisms has been analyzed by mouse bioassay (MBA) [1]. However, MBA has negative aspects since it requires the use of laboratory animals. Various analytical methods for TTX such as those using HPLC-FD [2] offer alternatives to MBA. Among these, ELISA is the primary candidate as a simple and convenient method. Several research groups already have developed ELISA kits for TTX. Basically, monoclonal antibodies employed for these kits are raised against haptenic antigens in which a guanidino group of TTX is coupled with that of carrier proteins *via* formaldehyde. Toxic aquatic organisms often possess a significant amount of TTX analogues such as 11oxo-TTX and 4*epi*-TTX. These analogues seem to have insufficient affinity to monoclonal antibodies. Yotsu-Yamashita et al. [3] reported that 4,9-anhydro-tetrodotoxin (4,9*anh*-TTX), a derivative of TTX found in puffer fish, reacts with biological thiols such as cysteine (Cys) to form 4-S-Cys adduct of TTX. This finding enables us to design a new haptenic antigen. In this study, polyclonal antibodies were raised against TTX, based on newly designed antigens in which dithiol reagents were used as linkers between haptenic TTX and carrier proteins.

Material and methods

Preparation of antigens
A sulfhydryl moiety was introduced to TTX by a reaction between 4,9*anh*-TTX and dithiol compounds. Briefly, 4,9*anh*-TTX isolated from puffer fish was dissolved in dil. ammonium phosphate buffer (pH 8.0) containing excess amount of (±) dithiothreitol (DTT) or 1,2-ethanedithiol (EDT). These adducts (DTT-TTX and EDT-TTX) in the reaction mixtures were isolated by Bio-Gel P-2 column chromatography. Two types of new haptenic antigens were obtained from the mixture of a bifunctional coupling reagent (GMBS, Dojindo)-treated bovine serum albumin (BSA, Sigma, RIA grade) and DTT-TTX adduct, or GMBS-treated keyhole limpet hemocyanin (KLH, GBiosciences) and EDT-TTX adduct (Scheme 1).

Inoculation of antigens and analysis of titers
Antigens (BSA-DTT-TTX and KLH-EDT-TTX) each containing 0.3 mg were mixed with Freund's complete adjuvant and inoculated biweekly to rabbits. Periodically obtained antisera (100 μL) were mixed with an aliquot of TTX solution (1~20 μM), and treated with Nanosep 10K Omega (NMWL 10,000, Pall). The concentrations of TTX in the filtrates were analyzed by the HPLC-FD method [2] to estimate titer of the antisera for TTX.

Affinity for TTX analogues
After 7 months, 60 mL of antisera were obtained from a rabbit inoculated with KLH-EDT-TTX. Affinities for TTX and its analogues were examined as follows. Solutions each containing 100 μL of 4*epi*-TTX (10 μM), 4,9*anh*-TTX (10 μM), 11oxo-TTX (10 μM), and partially purified mixture of deoxy, dideoxy and trideoxy-TTX obtained from puffer fish ovary, were mixed separately with 100 μL of antisera and filtered through a ultrafilter device described above. The concentrations of respective analogues in the filtrates were determined by the HPLC-FD method [2] to estimate their affinity to the antisera.

Results

Titers of antisera raised against rabbits inoculated with BSA-DTT-TTX antigen fluctuated at relatively low level. In contrast, high titer of antisera was observed within 4 months for rabbits inoculated with KLH-EDT-TTX, though one rabbit died during inoculation (**Fig. 1**). One mL of the rabbit antisera after 14th inoculation of KLH-EDT-TTX antigen adsorbed 25 nmol of TTX, and 10 ~ 20 nmol for each of 4*epi*-TTX, 11oxo-TTX and partially purified

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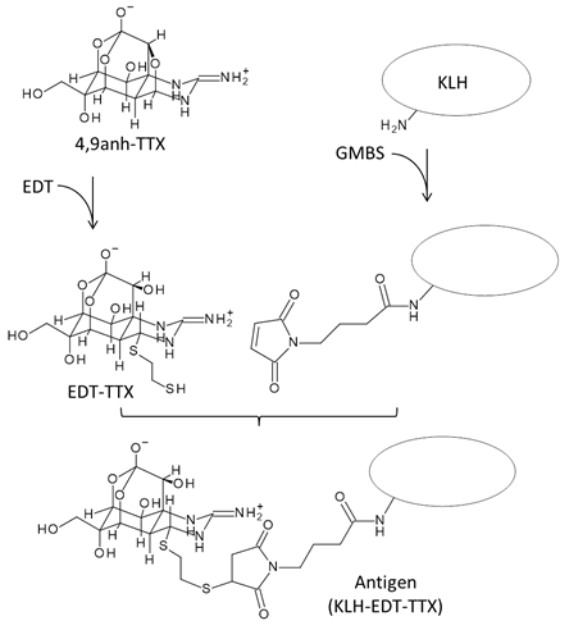
deoxy-TTXs. An ELISA kit based on these antisera is currently under construction.



Schme1 Preparation process and an estimated structure of the KLH-EDT-TTX antigen.

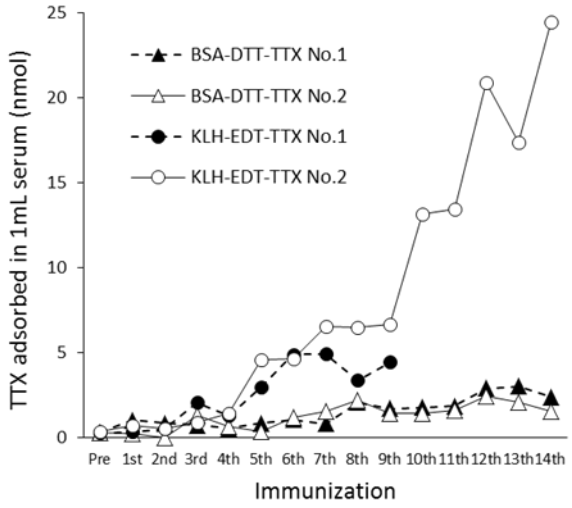


Fig. 1. Enhancement of antibody activity (titer) in rabbits during immunization. A rabbit (KLH-EDT-TTX No.1) died during immunization.

Discussion

Accumulation mechanism(s) of TTX in toxic aquatic organisms is not yet clear. It has been reported that some marine bacteria produce TTX, but the amount of TTX produced in bacterial culture is very low and cannot explain the high level of TTX found in toxic organisms such as puffer fish. Deoxy analogues such as 5,6,11-trideoxy-, 5,11-, 6,11-dideoxy, 5-deoxy and/or 11-deoxy-TTX have been found in various toxic organisms [4]. Some of these analogues may be produced by marine microorganisms and converted to TTX in toxic aquatic organisms including puffer fish. Polyclonal antibodies obtained in this study are expected as a new tool for the study to understand the mechanisms involved in toxin accumulation.

Acknowledgements

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References

1. Sato S, Kodama M (2015) Shokuhin Eisei Kensa Shishin, Rikagaku-hen (Standard Methods of Analysis in Food Safety Regulation), Japan Food Hygiene Association, Tokyo, pp. 813–820
2. Yotsu M, Endo A, Yasumoto T (1989) Agric Biol Chem 53: 893–895
3. Yotsu-Yamashita M, Goto A, Yasumoto T (2005) Chem Res Toxicol 18: 865–871
4. Yotsu-Yamashita M, Abe Y, Kudo Y, Ritson-Williams R, Paul VJ, Konoki K, Cho Y, Adachi M, Imazu T, Nishikawa T, Isobe M (2013) Mar Drugs 11: 2799–2813