論 文 の 内 容 の 要 旨

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論文題目　Studies on the detection methods for seafood noxious substances
（水産食品における有害物質の検出法に関する研究）

Food safety issue such as allergy and poisoning are always an important problem all around the world in the past decade. The consumers, governments and producers give increased attention to the safety and quality of food products in today's global marketplace, and detection technologies consequently become more important for ensuring food safety and security. The present study focuses on the detection of the food allergy of invertebrate major allergen, tropomyosin, and food poisoning of natural marine biotoxin, tetrodotoxin (TTX).

Food allergy is a dysfunction of the immune system against the ingested food or food additives. It is commonly mediated by immunoglobulin E (IgE) antibodies bound to mast cell, leading to release of histamine and other chemicals, which produces the symptoms such as urticaria, diarrhea, asthma and shock in severe in cases. Seafood allergy is common and major causes of food allergy in adult. During recent decades, seafood allergy is becoming a serious problem with increasing seafood consumption. Studies on food allergies demonstrated that tropomyosin is an invertebrate major allergen and shows cross-reaction among the given invertebrate species. Biotoxins are

substances synthesized by living organisms that are harmful to humans. They can be produced by many species of organisms such as bacteria, fungi, vertebrates, or marine microorganisms. The gastrointestinal tract and the nervous system of human can be affected by the biotoxins. One of popular biotoxin in Asia is TTX. TTX is a powerful neurotoxin found in several species, most notably puffer fish. In Japan, many puffer poisoning cases occur every year, resulting in numerous deaths.

Recent studies suggested that monoclonal antibodies (MAbs) are a useful tool in noxious substances detection and quantification. The present study is addressed to develop specific MAbs, and specific, rapid or sensitive immunoassays in order to detect and quantify invertebrate tropomyosin and TTX such as enzyme-linked immunosorbent assay (ELISA) and fluorescence resonance energy transfer (FRET) system.

This thesis is composed of four chapters. Mechanism of food allergy and TTX poisoning, common tests for assessment of food allergen and TTX are reviewed in Chapter 1.

In Chapter 2, the detection methods based on specific mouse MAbs against the sequences of IgE epitope shared by several shellfish tropomyosins were developed and characterized. The MAbs BE9, EB11 and DC3 raised against the sequences of T1 EKYKSISDELDQTFAEL and T2 KSISDELDQTFAEL recognized shellfish tropomyosins but also reacted with teleost proteins. The novel MAbs were then raised against the T3 sequence of SISDELDQTFAEL. A developed MAb, CE7B2, reacted to the crustacean, mollusks, arthropods and insects, but the reaction does not occur in vertebrate tropomyosin. Additionally, this MAb also recognized small fragments derived from tropomyosins in food products.

Subsequently, the MAb CE7B2 against invertebrate tropomyosin was applied to develop rapid or sensitive allergen detection methods by a sandwich ELISA and by fluorescence analysis using a FRET system for tropomyosin detection. Standard curves of the sandwich ELISA based on the MAb for quantification of crustaceans and mollusks were generated with serial purified tropomyosin dilutions (0.045-600 ng/ml). From these curves, detection limit were calculated as 0.09 ng/ml for kuruma prawn tropomyosin and 0.64 ng/ml for Japanese flying squid tropomyosin. The coefficient of variation (CV) analyses showed acceptable results of the intra- and inter-assay CVs, 1.5-5.1% and 1.2-4.2% in kuruma prawn tropomyosin standard curve and 0.8-3.9% and 0.6-3.2% in Japanese flying squid tropomyosin standard curve, suggesting that the sandwich ELISA assay is highly reproducible. The specificity of the sandwich ELISA

was checked using the tropomyosin-containing crude extracts from several processed food. The result suggests that the developed sandwich ELISA based on the MAb is useful for its application to the major food allergen tropomyosin of invertebrates.

Subsequently, a new allergen detection system, FRET system was developed in this study. The FITC-conjugated MAb CE7B2 was used as a fluorescent energy donor and TRITC-conjugated MAb 2A7H6 against shellfish tropomyosin was used as an acceptor. The fluorescence ratio was obtained in the presence of several concentrations of tropomyosin at the excitation of 470 nm and the emissions of 510-550 nm. The results showed gradual increases in the fluorescent intensity ratio from 510 nm to 550 nm with increasing tropomyosin concentration. It suggests that the FRET method with multi-antibodies would be useful to detect allergens with multi-epitopes without immobilization procedure. The system is more cost effective, as it required a shorter assay time and reduced investment in equipment than existing assay systems.

In Chapter 3, MAbs which produced against a novel derivative of TTX were prepared and characterized. The TTX was initially activated using *N*-[p-maleimidophenyl] isocyanate (PMPI) and subsequently conjugated to *S*-acetyl thioglycolic acid *N*-hydroxysuccinimide (SATA)-activated keyhole limpet hemocyanin (KLH) to immunization. To facilitate subsequent antisera and hybridoma evaluation, TTX was similarly conjugated to bovine serum albumin (BSA). Matrix-Assisted Laser Desorption Ionisation-Time Of Flight mass spectrometry (MALDI-TOF/MS) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed covalent attachment of toxins to BSA in the ratio of 3 mol per mol BSA for TTX. Although KLH-TTX conjugate could not be readily characterized prior to immunization, due to its large size (about 800 kDa), the approach of simultaneous synthesis and characterization of BSA-TTX conjugate by MALDI-TOF/MS confirmed that chemistry employed for protein modification was successful. Indeed, the subsequent mouse immunization with symptom of TTX-poisoning substantiated the validity of this strategy. Because the toxicity of TTX was retained in the obtained compound, it could be also used for elucidation of TTX biochemical production and accumulation mechanism.

MAbs against TTX were produced from the hybridoma cell lines, which were established by the fusion of P3-X63-Ag8.U1 (P3U1) myeloma cells with spleen cells isolated from BALB/c mouse immunized with the KLH-TTX conjugate. Five hybridoma clones were identified that secretes IgM, IgG1 and IgG2b MAbs against

TTX in twice fusions. However, the MAbs which produced in the first fusion had stronger cross-reaction to PMPI-SATA-activated BSA than TTX. Then the second fusion was carried out to prepare the specific MAbs against TTX. By using these MAbs, the indirect competitive ELISA showed the specificities of TTX were lower than BSA-TTX, but no cross-reaction to BSA, suggesting that the MAbs reacted with TTX-PMPI-SATA of BSA-TTX conjugate. In order to determine whether the MAbs react with free TTX, the fractions of MAbs co-incubated with TTX through IgG affinity column were analyzed by LC-TOF/MS, suggesting that the new synthesis procedure resulted in MAbs against free TTX. The MAbs obtained by the new synthesis might be used for develop TTX detection system.

In Chapter 4, the results obtained in this study and future perspectives are comprehensively discussed.

Food safety is a major worldwide problem, that detection methods for noxious substances are necessary to ensure our food security. In summary, the sandwich ELISA and FRET system based on MAb CE7B2 developed in the present study are very useful for a sensitive and rapid detection of invertebrate pan-allergen tropomyosin. The immunoassays are also more cost effective, as it required less sample preparation, a shorter assay time and reduced investment in equipment than either of the other assay systems. The new derivatization method with TTX could be not only for antibody generation, but also for elucidation of TTX biochemical production and accumulation mechanism. The developed MAbs against TTX would be used to develop detection system. The results presented in this thesis implicate that the developed MAbs and detection methods are could be useful to check and quality during food processing and to ensure the safety and security of food.