博士論文

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）

張　虹

Abstract

Food safety issues 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 cells, leading to release of histamine and other chemicals, which produces the symptoms such as urticaria, diarrhea, asthma and shock in severe 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 in 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 limits 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 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 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 electrospray ionization-time of flight mass spectrometry (ESI-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 developing 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, so 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 could be useful to check and quality during food processing and to ensure the safety and security of food.

List of abbreviations

BSA – Bovine serum albumin

CBB – Coomassie Brilliant Blue

CCD – Carbohydrate determinants

CHCA – α-Cyano-4-hydroxycinnamic acid

DBPCFC – Double-blind placebo-controlled food challenges

DDT – Dithiothreitol

DMF – Anhydrous dimethylformamide

DMSO – Dimethyl sulfoxide

DW – Distilled water

ELISA – Enzyme-linked immunosorbent assay

ESI-MS – Electrospray ionization mass spectrometry

ESI-TOF/MS – Electrospray ionization-time of flight/mass spectrometry

F/P – Fluorescein/protein

FABMS – Fast atom bombardment mass spectrometry

FITC – Fluorescein isothiocyanate

FRET – Fluorescence resonance energy transfer

GC-MS – Gas chromatography-mass spectrometry

GIT-BM – GIT medium containing 10% BM condimed H1

HAT – Hypoxanthine aminopterin thymidine

HPLC – High-performance liquid chromatography

HRP – Horseradish peroxidase

HAS –Haptasulfonic acid

HT – Hypoxanthine thymidine

IgA – Immunoglobulin A

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM – Immunoglobulin M

i.p. – intraperitoneal injection

KLH – Keyhole limpet hemocyanin

LC-MS – Liquid chromatography-mass spectrometry

MAb – Monoclonal antibody

MALDI-TOF/MS – Matrix-assisted laser desorption ionisation-time of flight/mass

MBS – Maleimidobenzoic acid-N-hydroxysuccinimide

NK – Natural killer

OPD – O-Phenylenediamine dihydrochloride

OVA – Ovalbumin

P3U1 – Myeloma P3-X63-Ag8.U1

PAb – Polyclonal antibody

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PEG – Polyethylene glycol

PMNs – Polymorphonuclear leukocytes

PMPI – *N*-[*p*-maleimidophenyl] isocyanate

PVDF – Polyvinylidene difluoride

QCM – Quartz crystal microbalance

RAST – Radioallergosorbent test

SATA – *S*-acetyl thioglycolic acid *N*-hydroxysuccinimide

SDS – Sodium dodecyl sulphate

SDS – PAGE–dodecylsulfate-polyacrylamide gel electrophoresis

SIM – Selected ion monitoring

SPR – Surface plasmon resonance

SPT – Skin prick test

TBS – Tris-Buffered-Saline

TBS-T – TBS containing Tween 20^{TM} at 0.5 % (v/v)

TCA – Trichloroacetic acid

TDA – Tetrodonic acid

TFA – Trifluoroacetic acid

TRITC – Tetramethylrhodamine isothiocyanate

TTX – Tetrodotoxin

Contents

Chapter 1 General introduction

Food safety issue such as allergy and poisoning are always important problems 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 (Gallart-Ayala and Moyano *et al.,* 2008), 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 now recognized as a worldwide problem of food safety. It is commonly mediated by immunoglobulin E (IgE) antibodies bound to mast cell, leading to release of histamine and other chemicals, which produce the symptoms such as urticaria, diarrhea, asthma and shock in severe in cases (Daul and Morgan 1993). Seafood allergy is common in many countries such as Europe, America and Australia, but seems to be more prevalent in Asian countries, since China and Japan are the first and second largest consumer of seafood (Lopata and O'Hehir *et al.,* 2010). The allergic reactions to shellfish are particularly very common in adults. The allergy of shellfish was almost three times more common in adults than in children in Japan, and five times in America (Hill and Hosking *et al.*, 1997; Sicherer and Munoz-Furlong *et al.,* 2004). Studies on food allergy demonstrated that tropomyosin is an invertebrate major allergen shows cross-reaction among the invertebrate species (Reese and Ayuso *et al.,* 1999).

Biotoxins are naturally occurring substances harmful to human produced by living organisms. They can be produced by many species of organisms such as bacteria, fungi, or vertebrates (Halstead 1959). The gastrointestinal tract and the nervous system of human can be affected by the biotoxins (Lewis and

Holmes 1993). Marine biotoxin have received particular attention in a part of food safety, following the damage caused to public health, water, and fish and shellfish cultures. One of popular marine biotoxin in Asia is TTX. TTX is a powerful neurotoxin found in several species, most notably in puffer fish. It has been reported that it is a stable toxin unaffected by cooking or freezing (Watters 1995). TTX poisoning often occurs in some countries such as Egypt, Korea, China and particularly Japan (Haque and Islam *et al.*, 2008). TTX poisoning in human is always due to ingestion of improperly handled toxic puffer fish. In Japan, many puffer poisoning cases occur every year, resulting in some deaths. In China and other countries of Asia, many food poisoning cases due to ingestion of wild puffer have occurred (Hwang and Noguchi 2007).

Food safety is a major worldwide problem, so that the detection of noxious substances technologies is necessary to ensure our food security. In this chapter, mechanism of food allergy and TTX poisoning, common tests for assessment of food allergens and TTX are introduced.

1.1 Basic introduction to food allergy and food allergen

Mechanism of IgE-mediated food allergy

Food allergy is now recognized as a worldwide problem, and like other allergy, it appears to be on the increase. Recent studies suggest that immunoglobulin E (IgE)-mediated food allergies affect 3.5 % to 4 % of Americans (Sicherer and Munoz-Furlong *et al.*, 2004). In addition, food allergy is the most common in childhood, affecting up to 8 % in infants (Kandyil and Davis 2009).

There are several different types of adverse reactions involving the immune system, which helps the body resist disease. In the case of food allergy, 'immediate hypersensitivity' is the most clearly understood. This reaction involves three primary components: food allergens, IgE, and mast cells and basophils (Sampson 2004). Food allergy can be divided into IgE-mediated and non-IgE-mediated reactions. Seafood allergy is commonly synonymous with IgE-mediated immediate-type I allergy (Daul and Morgan 1993). The symptoms with IgE-mediated reactions usually appear in 2 hours, except that some cases may appear after 4-6 hours (Murphy 2012). As shown in Fig. 1.1.1, when the allergenic foods ingested, IgE antibodies against allergens are produced by lymphocytes called B cell. During the circulating in the blood, the IgE antibodies are then bound to the IgE receptors (FcεRIs) on the surface of mast cells in the intestinal mucosa and trachea, or basophils in the blood. The individual becomes sensitization due to the bound IgE recognizing the same food allergen. The allergen binding to the IgE antibodies produces the cross-linking of the bound IgE-FcεRI complex with multivalent allergen, which causes the release of inflammatory mediators such as histamine, leukotrienes and prostaglandins responsible for the allergic symptoms (Pawankar 1999). The understanding of how food allergy represents an abrogation of normal oral tolerance is developing. Although any food can provoke a

reaction, relatively few foods are responsible for the vast majority of allergic reaction: in young children, milk, eggs, peanuts, soy, and wheat account for approximately 90 %; in adolescents and adults, peanuts, fish, shellfish and tree nuts account for approximately 85 % (Sampson 1999a). Diagnosis of food allergy includes observation case history, followed by laboratory studies, elimination diets, and challenges to ingest the food which may cause the allergy to confirm a diagnosis. Many food allergens have been characterized at a molecular level, which has increased the understanding of immunopathogenesis of food allergy. It can help develop new diagnostic and treatment methods. Currently, the most effective way to avoid recurrent of food allergy is keeping ingestion of food allergen out (Sicherer and Sampson 2006).

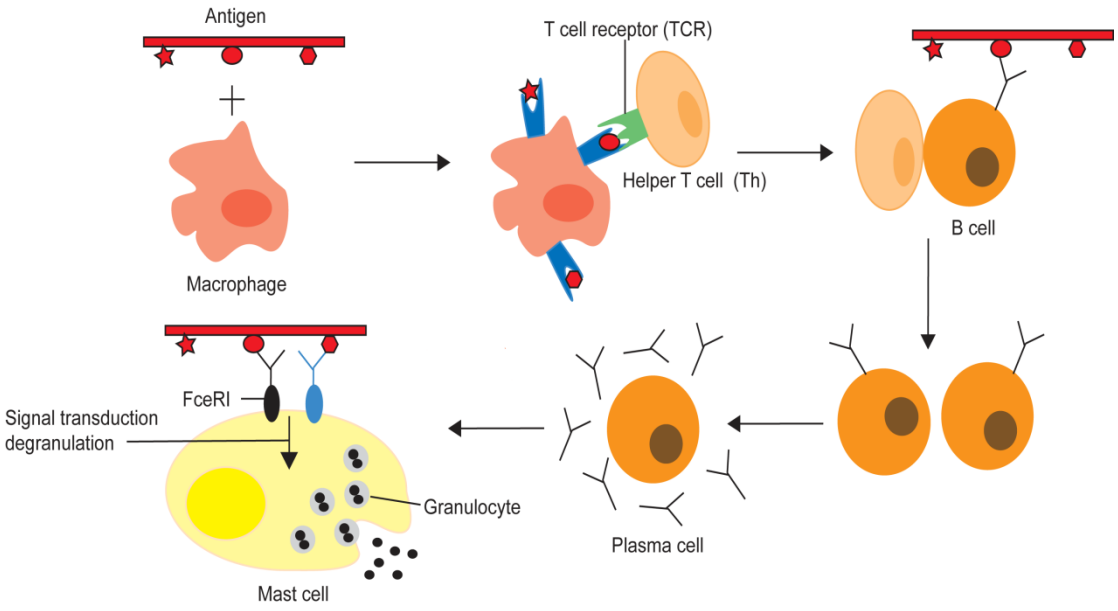


Fig. 1.1.1. General view for mechanism of IgE-mediated food allergy.

General introduction of food allergens

Although the human diet contains many different foods, only a small amount of these foods can induce food allergies. (Sampson 1999) Peanuts, fish, shellfish, and tree nuts are frequently listed in textbooks as the most relevant offenders in adult food allergy (Moneret-Vautrin and Morisset 2005). The allergic fraction of food is generally comprised of heat-stable, water-soluble glycoproteins ranging in size from 10 to 70 kDa. It is fairly resistant to industrial processing, heating, and cooking as well as to the digestive enzymes of the gut (Taylor and Hefle 2001).

The terminology 'food allergen' is ambiguous, because it has at least three interpretations: a food substance that reacts with IgE antibodies, induces allergic reactions, and induces allergic sensitization. Some food substances do all three of the above. However, some will do the first, but not do the second and third, whereas others will do the first and second, but not do the third one (Aalberse 1997). Therefore food allergens are food components that induce the production of IgE antibodies and are reactive to these antibodies to cause mediator release from mast cells and basophiles, resulting in immediate allergic reactions (Lehrer and Ayuso *et al.,* 2002). In order to induce immediate allergic responses, food allergens must possess the ability to stimulate immune responses and must be polyvalent molecules with at least two or more IgE antibody binding sites. Besides these essential properties, food allergens generally seem to share other several common properties.

Invertebrate major allergen tropomyosin

The major heat-stable allergen in shrimp was first described in 1981 (Hoffman and Day *et al.,* 1981) and later identified as tropomyosin (Shanti and Martin *et al.,* 1993). Tropomyosin belongs to a family of proteins associated with the thin filament in muscle, and microfilaments in many nonmuscle cells

(Smillie 1979). Allergic tropomyosins are found in invertebrates such as crustaceans, mollusks, arachnids, insects, whereas vertebrate tropomyosins are nonallergenic (Reese and Ayuso *et al.,* 1999). Studies of cross-reactivities among crustaceans, mollusks, cockroaches and dust mite have been established, and suggested tropomyosin as an important cross-sensitizing allergen (Reese and Ayuso *et al.,* 2012).

Invertebrate tropomyosin has been reported as a pan-allergen, meaning that a given invertebrate species can induce potent cross-reactive IgE antibodies that recognize tropomyosins of other species as well (Reese and Ayuso *et al.,* 1999; Lehrer and Ayuso *et al.,* 2002). The tropomyosin specific IgE antibodies cross react widely, since the similarity of invertebrate tropomyosins. Thus individuals with a shrimp allergy might also be allergic to other invertebrates (Shanti and Martin *et al.,* 1993). For example, IgE against tropomyosin from shrimp cross-reacts with tropomyosin from other crustaceans, molluscs (Leung and Chow *et al.,* 1996; Ishikawa and Nagashima *et al.,* 1999), and insects (Ayuso and Reese *et al.,* 2002; Boquete and Iraola *et al.,* 2011).

A number of studies focused on IgE binding sites of allergenic tropomyosins. Shanti et al. (1993) first identified two IgE-binding regions from shrimp *Penaeus indicus* by IgE reactivity of patients sera. Subsequently, a 36 kDa allergen, designated Pen a 1 isolated from brown shrimp (*Penaeus aztecus*) was identified as a muscle protein tropomyosin, broadly existing among crustaceans (Daul and Morgan 1993). Pen a 1 allergen contains five major IgE-binding regions, and eight IgE recognizing epitopes were identified in these five regions (Reese and Ayuso *et al.,* 2001). For the IgE-binding sites of mollusk tropomyosins, Ishikawa et al. (1998) proposed a 14-mer peptide as the IgE recognizing epitope on the oyster. However, it was revealed that the IgE-binding sites of gastropod reside in the carboxyl-terminal region (residues 245-284) of tropomyosin (Ishikawa and Ishida *et al.,* 1998).

Characterization of food products and their intermediates contribute to the future research. In order to ensure the standardization of the manufacturing process and food labelling be implement strictly, kinds of allergen should be analyzed and tested contained in foods through extensive, accurate and simple methods.(Besler and Steinhut *et al.,* 2001; Poms and Klein *et al.,* 2004). These will be introduced in the next section.

1.2 Common detection methods for food allergens

Currently, there are several technical possibilities for the detection of potential allergens in food products. The methods employed are either targeting the allergen itself or a marker that indicates the presence of the offending food. Immunological and molecular biological methods are commonly used as inexpensive, sensitive, and highly selective methods for the detection and quantification of a wide variety of noxious substances (Raybould and Bignami *et al.,* 1992). In the previous studies, the main molecular biological methods for detection of food allergen are polymerase chain reaction (PCR), and immunological methods: enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) biosensor technology and quartz crystal microbalance (QCM) immunosensor. ELISA is the most commonly used immunological method for the quantitative detection of food allergens. The principal advantages of these fully quantitative assays are their robustness and sensitivity. However, it is necessary to fix the antibody or antigen onto solid phase and the detection antibody must be labeled for measuring the enzyme reaction. SPR is produced on the basis of the evanescent wave phenomenon. A thin gold film is located between the prism and materials and the irradiation of light into materials at over critical angle produces evanescent fields. Surface plasmon energy resonance is occurred in proportional to the molecular density in the evanescent fields. QCM is based on the resonance frequency changing according to the mass when the material adhered to the surface of the electrode of the quartz vibrator. There were gradual increases in resonance frequency with the increasing material mass. In immunoassay, the antigens or antibodies were solid phased to the quartz vibrator, then the resonance frequency fall if the mass increased with the immune reaction. The fixation of antibody on quartz crystal is also necessary for QCM measurements of antibody-antigen interaction (Ansorena and Zuzuarregui *et al.,* 2011). Thus, although the ELISA method requires labeled reagent to detect

immunoreaction amounts and several experimental processes for immunoassay, SPR and QCM offer benefits to use non-labeled reagents for detection of molecular interaction (Steegborn and Skladal 1997; Bunde and Jarvi *et al.,* 1998). ELISA, SPR and QCM need the solid phase as described above, and the fluorescence resonance energy transfer (FRET) system is more cost effective, as it required a shorter assay time and reduced investment in equipment than existing assay systems.

1.3 Introduction of FRET system

Fluorescence resonance energy transfer (FRET) is a widely used method for monitoring interactions between or within biological macromolecules conjugated with suitable donor-acceptor pairs. FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein-protein interactions, protein–DNA interactions, and protein conformational changes (Jares-Erijman and Jovin 2003).

FRET is a mechanism describing energy transfer between two chromophores. A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through nonradiative dipole–dipole coupling. Measurements of FRET is frequently applied to determine if two fluorophores are within a certain distance of each other (Zheng 2006). For understanding functions on the molecular scale in biology, it is extremely important to reveal the information of intermolecular distances, reflecting interactions, distributions and supramolecular components. In addition, these informations are important when considering that many biological processes occur at the interface among molecules and supramolecular organizations. Because plenty of macromolecular systems are ideally suited for FRET application, FRET has been applied comprehensively including biotechnology and biophysics.

FRET

FRET phenomenon was first discovered in the early 20th century by Perrin, and in 1948, Forster formulated the principle of FRET, a phenomenon that occurs when two different chromospheres (donor and acceptor) with overlapping emission/absorption spectra are separated by a suitable orientation and a distance in the range 10–80 Å (Forster 1948). After the spatial proximity relationships of two

fluorescence-labelled sites in macromolecules was found, the use of FRET was established as a standard of spectroscopic measurement (Stryer 1978). Incidentally, in the early operating practice, fluorescent analogs of biomolecules and fluorescent reagents covalently or non-covalently attached to macromolecules were used as donors or acceptors of FRET. Intermolecular FRET occurs when the fluorescent energy donor and the acceptor are on different macromolecules. However, this form of FRET is more difficult to observe, because the stoichiometry of acceptors to donors can vary with transfection efficiencies, and also the donor and accepter host proteins may not be constitutively bound *in vivo*.

Donor florescence lifetimes in the absence and presence of acceptor molecules are often measured for the observation of FRET (Fig. 1.3.1). These lifetimes may originate from interacting and noninteracting molecules, which hamper quantitative interpretation of FRET data. Optimal conditions occur when all the donors are paired with an acceptor, as any unpaired protein adds noise to the signal. Additionally, if the distance or orientation between the pairs is unfavourable, FRET may not occur, even if the two proteins form a complex (Miyawaki and Tsien 2000). In this study, the FRET system was carried out for the detection of food allergen. The food allergy reaction on the surface of mast cell is modelled on this system based on two IgE epitope-specific MAbs.

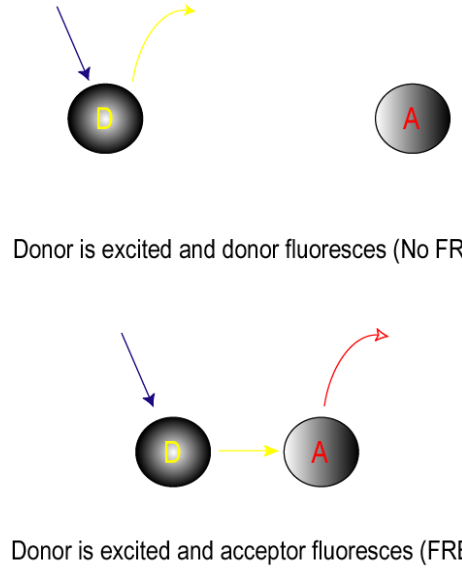


Fig. 1.3.1. Definition of fluorescence resonance energy transfer (FRET) and its relation to donor-acceptor proximity. D: donor. A: acceptor.

Application of FRET

FRET is increasingly occupying a centre stage in biological studies and in biotechnology. It has been applied in detection, quantitation, identification and characterization of structure and function of inorganic and organic compounds, and of biological processes. It is also successfully applied to the monitoring of biospecific reactions like immunoassays such as studies of antigen-antibody complexes and homogenous immunoassays (Oswald and Gruber *et al.*, 2001). However, at present there are no validated methods using the FRET system for the detection and quantification of food allergens.

Food allergy is commonly synonymous with type-I allergy as described in Section 2 in this chapter. As shown in Fig. 1.3.2A, it always occurs with the IgE antibodies binding to the IgE-binding sites (IgE epitopes) of the allergen, and then mediated by IgE antibodies bound to the IgE receptors (FcεRIs) of mast cell. The FcεRIs aggregate when the IgE antibodies are cross-linked by allergens with multi-epitopes, and then the FcεRIs spatially approach to each other. Subsequently, the mast cell degranulates and releases histamine, leukotrienes and other mediators (Daul and Morgan 1993). In this

study, two tropomyosin IgE epitope specific monoclonal antibodies were used as the allergen-specific IgE antibodies. In the presence of tropomyosin, the FRET would be observed when the two MAbs bind to the adjacent IgE epitope (Fig. 1.3.2B). The tropomyosin concentration can be determined by the intensity of fluorescence.

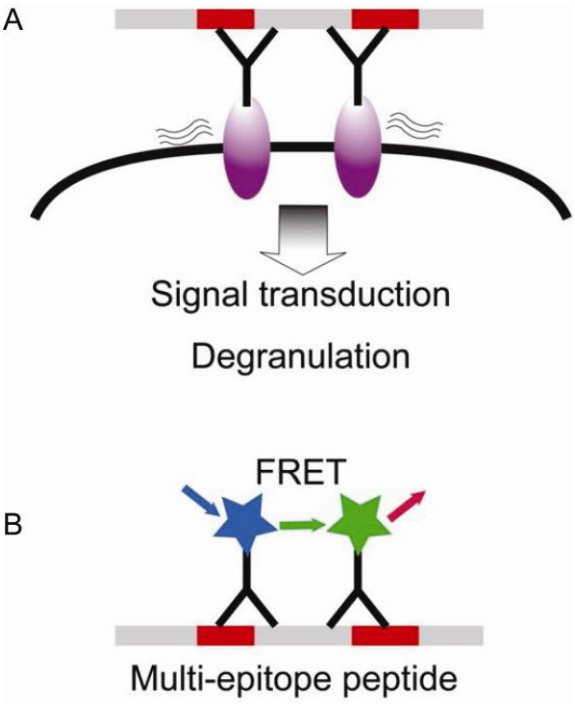


Fig. 1.3.2. Illustrations of IgE-mediated allergy (A) and FRET system in this study (B).

1.4 General introduction to TTX

Tetrodotoxin (TTX) has been received increasing interests due to its special properties. It is one of the most potent marine neurotoxins, which blocks the sodium channels of the excitable cell membrane of animals. Although TTX was originally isolated from puffer fish (Yokoo 1950), it has been found in widely divergent animal species, including puffer fish, gobies, frogs and shellfish (Tao and Wei *et al.*, 2010). It is a stable toxin unaffected by cooking or freezing (Watters 1995). TTX poisoning often occurs in some countries such as Egypt, Korea, China and Japan. TTX poisoning in human always due to ingestion of improperly handled toxic puffer fish. The toxicity of TTX is caused by it selectively blocking voltage-gated Na^+ channels on the surface of the nerve membranes causing blood vessels to relax, leading to a sudden drop in blood pressure. The main causes of death in TTX poisoning are paralysis and respiratory failure (Neagu and Micheli *et al.*, 2006).

Despite TTX has a long history and perfect knowledge of its toxicity and pharmacology, its pathway and biogenic origin are largely unknown. The unique structure of TTX lack of its detection and comparative biosynthesis studies have hindered the development of molecular tools for the purpose of pathway. Although a popular hypothesis proposes that a symbiotic or commensal bacterium living within those organisms are risible for TTX production, it is unclear how or why TTX occurs in such a diverse range of phylogenetically unrelated organisms.

Chemistry of TTX

TTX was isolated as a crystal by Yokoo in 1950 (Yokoo 1950), and chromatographically in the 1960s (Brown and Mosher 1963). Its structure was not confirmed until several groups elucidated it independent of each other in the middle 1960s (Tsuda and Tachikawa *et al.*, 1964; Woodward 1964;

Goto and Kishi *et al.*, 1965). Although toxicity and pharmacology of TTX are well known, much of the research regarding the biogenesis of TTX is still unclear. TTX is a low molecular weight hapten with no substructure to induce immunoreactions, which makes it necessary to couple TTX to a carrier molecule in order to produce antibodies against TTX. It has a very unique structure. TTX is containing a highly oxygenated carbon backbone which attached with a single guanidinium moiety (Fig. 1.4.1, in red). The carbon backbone of TTX consists of a 2,4-dioxaadamantane structure, decorated with four primary hydroxyl groups and one secondary hydroxyl group (Chau and Kalaitzis *et al.*, 2011). The molecular formula of TTX is $C_{11}H_{17}O_8N_3$. Pure TTX is insoluble in water and in most organic solvents such as methanol, ethanol and dimethylsufoxide. The acid dissociation constant *pKa* of TTX in water is 8.76 ± 0.01, it suggests that TTX dissolves in weak acid solution. Therefore, the citrate-treated TTX is always used as a commercial reagent for routine pharmacological studies. Since TTX is unstable at both high and low pH, it is best to keep solutions of the toxin at pH 5 with a suitable buffer. TTX can be stored in frozen for long periods without loss of toxicity (Moczydlowski 2013). Besides from TTX itself, its analogs are contained in puffer fish. TTX and analogues are displayed in Fig. 1.4.1.

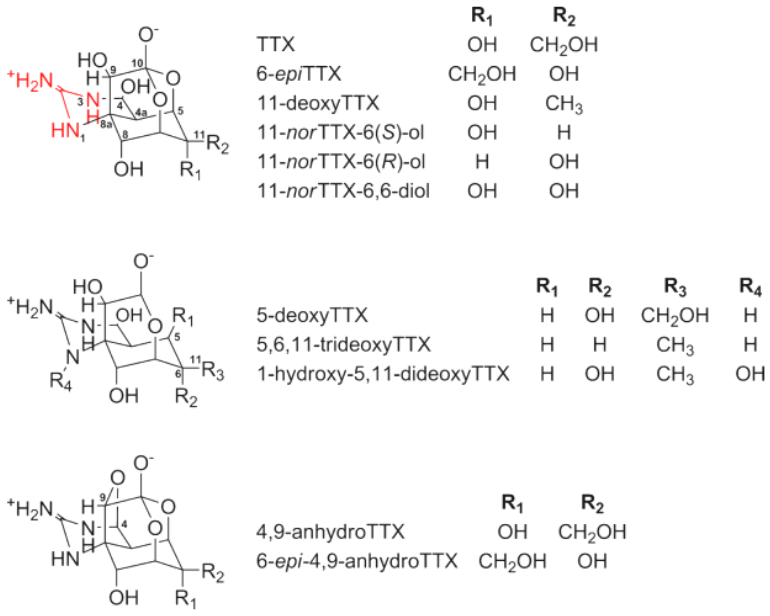


Fig. 1.4.1. TTX and analogues (Chau and Kalaitzis *et al.*, 2011).

Toxicity of TTX

TTX poisoning is a common poisoning along the coasts of Asia. Puffer poisoning has been reported in many Asian countries including Thailand, Malaysia, Bangladesh, Taiwan, China and particularly Japan (Haque and Islam *et al.,* 2008). TTX is present in high concentrations in the liver, ovaries, intestines and skin of puffer fish (Saoudi and Abdelmouleh *et al.,* 2010). TTX is known as one of the most toxic substances. It is 275 times more lethal than cyanide and 50 times more potent than strychnine or curate (Haque and Islam *et al.,* 2008). TTX is heat-stable and water-soluble, thereby boiling of puffer fish in water cannot destroy the toxin. In human, the MLD_{50} of TTX is approximately estimated to be 10,000 MU, equivalent to 2 mg (Noguchi and Ebesu 2001). Symptoms of poisoning generally start with perioral paraesthesia, followed by weakness of the limbs and progressive muscular paralysis, eventually causing death due to respiratory paralysis (Islam and Razzak *et al.,* 2011). There are no antidote has been developed and approved for human use.

TTX toxicological studies using a mouse bioassay standardized for TTX have been used to assess its level of toxicity, which is expressed as mouse units per mg TTX (MU/mg). Specific toxicity of TTX is 5000-8000 MU/mg where 1 MU is defined as amount of TTX that can kill a male ddY strain mouse with a body weight of 20 g in 30 min after intraperitoneal injection (i.p.). The material data safety sheet for TTX lists a LD_{50} for mice at 334 μg/g after oral application.

Pharmacology of TTX

The toxicity of TTX is caused by selectively blocking voltage-gated Na^+ channels on the surface of the nerve cell membranes causing blood vessels to relax, leading to a sudden drop in blood pressure (Neagu and Micheli *et al.,* 2006). TTX block voltage-gated Na^+ channels of nerve and muscle

membranes at nanomolar concentrations (Hille 1975). It is potent and potentially lethal marine toxins with great value to ion channel research. It allows separation of Na^+ currents from other ionic currents in native cells, since its specificity for Na^+ channels. Kao and other researchers (Kao 1986; Mosher 1986) have identified the toxicity active groups in TTX. These are the guanidinium group and the hydroxyls at C9 and C10 (Fig. 1.4.1). The guanidinium group is thought to form an ion-pair with an anionic site of the channel molecule, while the C9-OH and C10-OH form hydrogen bonds with other sites (Kao and Walker 1982). The C4 and C9 are joined by an oxygen bridge (Lipkind and Fozzard 1994). Therefore, the guanidinium group and oxygen group have very important places in TTX toxicity.

1.5 Common detection methods for TTX

TTX is one of the best known marine toxins that is usually isolated from puffer fish. The muscle that is the main edible portion is often toxic, resulting in paralytic poisoning and occasional death in humans. It is essential to monitor the TTX level in puffer fish tissue and select edible puffer fish species to ensure the safety of consumers, and the detection methods consequently become more important. Over the past decade, several biological and chemical qualitative and quantitative analysis methods have been developed. Of these methods, some are only used to identify TTX qualitatively, such as UV spectroscopy, IR spectrometry, gas chromatography-mass spectrometry (GC-MS), fast atom bombardment mass spectrometry (FABMS) and ^{1}H-NMR spectrometry (Noguchi and Mahmud 2001). Among the quantitative detection methods, the previously commonly used detection method is the mouse bioassay as described above. The other important methods are the cytotoxicity test, immunoassay, liquid chromatography-mass spectrometry (LC-MS), electrospray ionization-time of flight/mass spectrometry (ESI-TOF/MS) and high-performance liquid chromatography (HPLC). In this part, the common detection methods for TTX used in the present study are introduced.

Mouse bioassay

Mice have been commonly used for determination of several toxins. To know the concentration of TTX in a sample of interest, the mouse bioassay is usually used. It is also used to identify an unknown toxin extract in comparison with a TTX-specific dose-death time relationship curve. In the mouse bioassay, the unknown toxin extracted with 0.1 % acetic acid is prepared to a series of test solutions. Aliquots of each test solution are intraperitoneally injected into a group of mice. The unknown concentrations of toxin are determined from the drew dose-death time curve which used the known concentration of TTX (Hwang and Jeng 1991). Although the mouse has been commonly used for

determination of TTX, it has many disadvantages, sensitivity due to individual differences and humanitarian experiment to avoid using animal as possible.

High-performance liquid chromatography (HPLC)

HPLC method has been developed for both qualitative and qualitative analysis of TTX and improved constantly (Yasumoto and Nakamura *et al.*, 1982). The TTX in the samples is separated from other molecules in a buffer system and on a stationary phase with silica gel or ion exchange resin (Nagashima and Maruyama *et al.*, 1987; Yotsu and Endo *et al.*, 1989). A post column reaction is introduced that triggers the reaction of TTX to a C_9-quinazoline derivative (2-amino-6-hydroxymethyl-8-hydroxyquinazoline) (Fig. 1.5.1) (Goto and Kishi *et al.*, 1965). This reaction is commonly performed with 4 N NaOH from a separate pump in an oven set at about 110 ℃ and the reaction time of 2 min (Shoji and Yotsu-Yamashita *et al.*, 2001). In quantitative analysis of HPLC, the detection limit of the authentic TTX is about 0.03 μg (Yasumoto and Michishita 1985). But one of the disadvantages in this method is the varying fluorescent intensity among TTX and its cumbersome management.

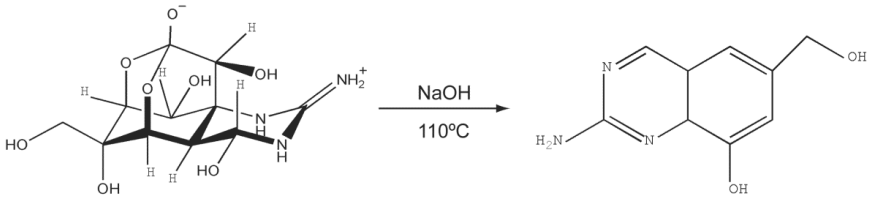


Fig. 1.5.1. Production of alkaline degradation of TTX to C_9 quinazoline derivative (Goto and Kishi *et al.*, 1965).

Electrospray ionization-time of flight/mass spectrometry (ESI-TOF/MS)

Although ESI-TOF/MS is not widely used in determination of TTX currently, it is a valuable technique. For TTX detection, the positive mode shows higher sensitivity than the negative mode

(Horie and Kobayashi *et al.,* 2002).

Matrix Assisted Laser Desorption Ionization -time of flight/mass (MALDI-TOF/MS)

This method is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules and large organic molecules, which tend to be fragile and fragment when ionized by more conventional ionization methods. Therefore the method is used for quick analysis both of low and high molecular materials.

UV spectroscopy

In this method, TTX is generally determined by irradiating a crude toxin with UV light. In the analysis, the UV spectrum of alkali-decomposed compounds of TTX strongly appears at around 276 nm (Tanu and Noguchi 1999).

Immunoassay

A variety of immunoassay methods for TTX detection have been developed and are still being improved such as ELISA, SPR, QCM and others, as described in the section of food allergen detection methods (Section. 1.2). In those immunoassay methods, the antibody played a most important part. Two of the latest immunoassay methods to detect and quantify TTX are the gold nanoparticle probe-based immunoassay and the fluidic force discrimination (FFD) assay. The gold nanoparticle probe-based immunoassay is a competitive immunoassay which uses gold nanoparticle-labeled monoclonal antibody against TTX and is performed on a cellulose-nitrate membrane (Zhou and Li *et al.,* 2010). The same style detection method is also used to detect food allergen. Major advantages of this assay are that it can be performed on location without need of any equipment, and this analysis takes only about 10 minutes at a reasonably sensitive detection limit. The FFD assay is also a competitive immunoassay which uses a sandwich format with microbeads (Yakes and Etheridge *et al.,*

2010). Since it renders extensive dilution series to find the concentration range, in which TTX from samples can be measured confidently, unnecessary.

Purpose of this study

Studies on food allergies demonstrated that tropomyosin is an invertebrate pan-allergen and shows cross-reactivities among the given invertebrate species (Reese and Ayuso *et al.,* 1999). Detection kits for shellfish tropomyosin are commercially available in Japan (Seiki and Oda *et al.,* 2007; Shibahara and Oka *et al.,* 2007). However, tropomyosin is relatively labile in enzymatic processing (Hoffman and Day *et al.,* 1981), the tropomyosin fragmented peptides containing IgE epitope sequences remain easily in processed food. Those detection kits with non-specific IgE epitope antibodies might fail to detect allergenic peptides in processed foods, such as fermented products and fish sauce. Emoto et al. (Emoto and Ishizaki *et al.,* 2009) demonstrated that an amino acid sequence around the C terminal is shared among shellfish tropomyosins and that the sequence is strongly recognized by sera of human individuals sensitive to shellfish. Immunological reaction-based techniques have been described to identify and quantify allergens in food including enzyme-linked immunosorbent assay (ELISA) (Jeoung and Reese *et al.,* 1997). Even though some monoclonal antibodies (MAbs) had been obtained against invertebrate tropomyosin (Barletta and Butteroni *et al.,* 2005; Lu and Ohshima *et al.,* 2007), it is necessary to develop an MAb recognizing the tropomyosin IgE epitopes shared by invertebrate species for rapid and highly sensitive detection.

For TTX detection, several immunoassay techniques have been developed so far. Watabe et al. reported that the MAb titer obtained from BALB/c mice immunized with TDA-bovine serum albumin (BSA) conjugated was low (Watabe and Sato *et al.,* 1989). Huot et al. prepared two MAbs against TTX. The capability for TTX was 50 ng/ml (Huot and Armstrong *et al.,* 1989). Kawatsu et al. developed a ELISA that can detect TTX at concentration range of 2-200 ng/ml (Kawatsu and Hamano *et al.,* 1997). Tao et al. reported a competitive ELISA for TTX detection with the limit of 5 ng/ml (Tao and Wei *et al.,*

2010). These investigators all used an immunogen prepared by treating a mixture of TTX and BSA, ovalbumin (OVA) or keyhole limpet hemocyanin (KLH) with an excess of formaldehyde (Fig. 1.1). As shown in Fig. 1.2, these resulting antibodies binding site is different from the binding site of biologically active site. It suggests that the conventional antibodies have low specificity to TTX toxicity active site.

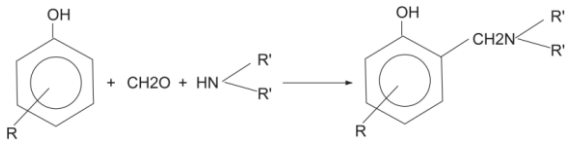


Fig. 1.1. The reaction formula of Mannich method (Lin and Speranza *et al.*, 1997).

Considering the merits that MAb is specific to particular antigen, and FRET system can rapid and sensitively detect the antibody-antigen interactions without any immobilization procedure. As the first goal of the present study is to prepare the MAbs specific to invertebrate tropomyosin and to develop rapid or sensitive immunoassays based on the MAbs for allergen detection and quantification by using sandwich ELISA and FRET systems.

In order to produce antibodies against TTX, it is necessary to couple TTX to a carrier protein since TTX is a low molecular weight of 319 hapten with no substructure to induce immunoreaction. Fig. 1.2 shows the location of the two conjugation chemistries on the TTX molecule. As described above, the guanidinium group and oxygen group have very important place in TTX toxicity. Generally, an indirect method is used to generate anti-TTX antibodies in earlier studies (Kawatsu and Hamano *et al.*, 1997; Zhou and Li *et al.*, 2009). These TTX-protein conjugate immunogens were all prepared using a modified formaldehyde method (Fig. 1.2B) (Huot and Armstrong *et al.*, 1989). Although this method can easily conjugate TTX with carrier proteins, the resulting monoclonal antibodies (MAbs) probably have low specificity to the toxicity active site (Fig. 1.2). The purpose of this study is preparing

antibodies with high specificity to toxicity active site of TTX (Fig. 1.2A). Using these MAbs, a useful immunoaffinity column chromatography could also been developed for isolation and identification of TTX. The MAbs can be used not only for detection the free TTX, but also for elucidation of TTX biochemical production and accumulation mechanism. Moreover the MAbs may be used as antidote to TTX.

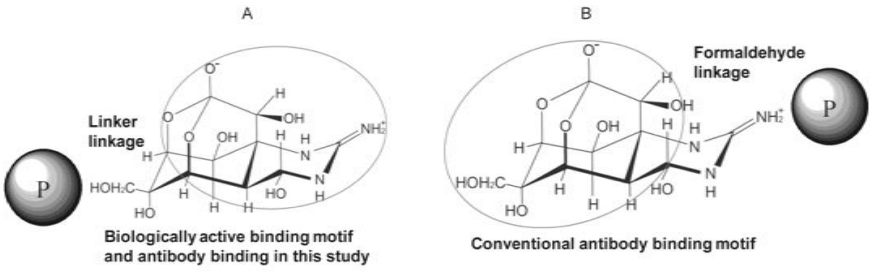


Fig. 1.2. Illustration of two conjugation sites for TTX. (A) Formaldehyde linkage was used in the production of the conventional antibody. (B) Linkers were used to conjugate TTX to carrier protein in this work which was biologically active to voltage-gated Na^+ channels. P: carrier protein.

Content of dissertation

This thesis composed of four chapters. Chapter 1 deals with general introduction.

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. Fistly, the MAbs BE9, EB11 and DC3 raised against the sequences of T1 EKYKSISDELDQTFAEL and T2 KSISDELDQTFAEL were obtained and characterized. Then MAb CE7B2 against the sequence of T3 SISDELDQTFAEL was established and characterized to be specific to the allergenic tropomyosins from different invertebrate species. Subsequently, a sandwich ELISA and a FRET system based on the MAb CE7B2 were developed and applied for detection of tropomyosin.

In Chapter 3, TTX was purified from the ovaries of natural puffer fish. The concentration of the purified TTX was analyzed by HPLC. A novel derivative of TTX for generation of antibodies was prepared and analyzed. The chemistry employed for protein modification was analyzed by MALDI-TOF/MS and SDS-PAGE. Subsequently, Five MAbs which produced against the novel derivative of TTX were prepared and characterized. The specificity of these MAbs were assayed by Screener blotter, competitive ELISA and ESI-TOF/MS.

The results obtained in the present study and future perspectives were comprehensively discussed in Chapter 4.

Some parts of this thesis have been already published as follows:

Hong Zhang, Ying Lu, Hideki Ushio, Kazuo Shiomi. 2014. Development of sandwich ELISA for detection and quantification of invertebrate major allergen tropomyosin by a monoclonal antibody. *Food Chemistry*, 150, 151-157.

Chapter 2 Development of detection and quantification methods based on monoclonal antibodies against an invertebrate major allergen tropomyosin

Shellfish are an increasingly important cause of IgE-mediated food allergy. One of the major shellfish allergen is trompomyosin. Tropomyosin belongs to a family of proteins associated with the thin filament in muscle, and microfilaments in many nonmuscle cells. Tropomyosin is soluble in water and in dilute salt solutions, the method of ammonium sulfate fraction is normally used for its extraction. Allergic tropomyosins are found in invertebrates such as crustaceans, mollusks, arachnids, insects, whereas vertebrate tropomyosins are nonallergenic (Reese and Ayuso *et al.*, 1999). Studies of cross-reactivities among crustaceans, mollusks, cockroaches and dust mite have been established, and suggested tropomyosin as an important cross-sensitizing major allergen (Reese and Ayuso et al., 2012).

Currently, there are several technical possibilities for the detection of potential allergens in food products. The methods employed are either targeting the allergen itself or a marker that indicates the presence of the offending food. Immunological and molecular biological methods are commonly used as inexpensive, sensitive, and highly selective methods for the detection and quantification of a wide variety of noxious substances (Raybould and Bignami et al., 1992). In the previous studies, the main molecular biological method is polymerase chain reaction (PCR), and immunological methods: enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) biosensor technology and quartz crystal microbalance (QCM) immunosensor. ELISA is probably the method that are used most commonly by the food industry and official food control agencies (van Hengel 2007). However, ELISA, SPR and QCM need to labeled regent or solid phase. For the purpose to develop rapid and/or

sensitive immunoassays based on the monoclonal antibodies for tropomyosin detection and quantification, in the present chapter, a sandwich ELISA and fluorescence resonance energy transfer (FRET) system were developed.

2.1 Preparation of tropomyosin-rich fractions and processed food extracts

2.1.1 Introduction

Crustaceans such as shrimp and crab are frequent causes of allergic reaction to foods. Several allergens were identified, but the muscle protein tropomyosin seems to be one major allergen (Reese and Ayuso *et al.,* 2012). Additionally, tropomyosins are important allergens in other crustaceans, mollusks and in other invertebrates such as house dust mite and cockroaches (Boquete and Iraola *et al.*, 2011). In this section, the tropomyosins were extracted and purified from 16 species of invertebrates and 9 kinds of processed food for characterize the developed monoclonal antibodies in later section.

2.1.2 Materials and Methods

Materials

The tropomyosin materials used in this study are listed in Table 1. Fresh specimens of kuruma prawn (*Marsupenaeus japonicus*), pink shrimp (*Pandalus borealis*), king crab (*Paralithodes camtschaticus*), swimming crab (*Portunus trituberculatus*), red snow crab (*Chionoecetes japonicus*), horsehair crab (*Erimacrus isenbeckii*), short-neck clam (*Raditapes philippinarum*), disk abalone (*Haliotis discus*), Sakhalin surf clam (*Pseudocardium sachalinense*), bloody cockle (*Anadara broughtonii*), Yezo scallop (*Patinopecten yessoensis*), whelk (*Neptunea polycostata*), Japanese flying squid (*Todarodes pacificus*), common octopus (*Octopus vulgaris*), sea cucumber (*Stichopus japonicus*), ascidian (*Halocynthia roretzi*), red sea bream (*Pagrus major*), American bullfrog (*Rana catesbeiana*), chicken (*Gallus gallus domesticus*) and cattle (*Bos primigenius*) were obtained from local markets. Dust mite (*Dermatophagoides pterouyssinus*) extract and American cockroach (*Periplaneta fluiginosa*) whole

body powder were obtained from Bio Stir (Tokyo, Japan).

The extracts from instant noodle soup mix of seafood style, Chinese-inspired bean-starch vermicelli soup mix (containing shellfish in materials), and Sichuan-inspired bean-starch vermicelli soup mix (showing no shellfish ingredients in the label on the package but a product through the same production line for shellfish-containing products), shrimp cracker, octopus cracker, shrimp powder, fish sauce, kimchi (showing no shellfish ingredient in the label on the package) and kimchi sauce were obtained from local supermarkets.

Table 1 Tropomyosin materials in this study

Phylum	*Class*	*Family*	*Species*	*Common name*
Arthropoda	**Malacostraca**	**Penaeidae**	***Marsupenaeus japonicus***	**Kuruma prawn**
			Pandalus borealis	**Pink shrimp**
		Lithodidae	***Paralithodes camtschaticus***	**King crab**
		Atelecyclidae	***Erimacrus isenbeckii***	**Horsehair crab**
		Oregoniidae	***Chionoecetes japonicus***	**Red snow crab**
		Portunidae	***Portunus trituberculatus***	**Swimming crab**
	Arachnida	**Pyroglyphidae**	***Dermatophagoides pterouyssinus***	**Dust mite**
	Insecta	**Blattidae**	***Periplaneta fluiginosa***	**American cockroach**
Mollusca	**Bivalvia**	**Veneridae**	***Raditapes philippinarum***	**Short-neck clam**
		Mactridae	***Pseudocardium sachalinense***	**Sakhalin surf clam**
		Arcidae	***Anadara broughtonii***	**Bloody cockle**
		Pectinidae	***Patinopecten yessoensis***	**Yezo scallop**
	Gastropoda	**Haliotidae**	***Haliotis discus***	**Disk abalone**
		Buccinidae	***Neptunea polycostata***	**Whelk**
	Cephalopoda	**Ommastrephidae**	***Todarodes pacificus***	**Japanese flying squid**
		Octopodidae	***Octopus vulgaris***	**Common octopus**
Echinodermata	**Holothuroidae**	**Holothuriidae**	***Stichopus japonicus***	**Sea cucumber**
Chordata	**Ascidiaca**	**Pyuridae**	***Halocynthia roretzi***	**Ascidian**
	Actinopterygii	**Sparidae**	***Pagrus major***	**Red sea bream**
	Amphibia	**Ranidae**	***Rana catesbeiana***	**American bullfrog**
	Aves	**Phasianidae**	***Gallus gallus domesticus***	**Chicken**
	Mammalia	**Bovidae**	***Bos primigenius***	**Cattle**

Preparation of tropomyosin-rich fractions and processed food extracts

The tropomyosin-rich acetone powder was prepared from each sample according to the method of Greaser and Gergely (Greaser and Gergely 1971) with slight modifications presented in Fig. 2.1.1. Fresh muscles of the samples were individually minced and extracted in a buffer A (1:5, w/v) containing 20 mM KCl, 1 mM $CaCl_2$ and 0.1 mM dithiothreitol (DDT) for 10min. After centrifuged at 10,000 g for 20 min at 4℃, the resulting precipitates were washed in a buffer A and centrifuged again. Then the obtained precipitates were washed four times with acetone and evaporated at room temperature overnight. Subsequently, the acetone powders of materials were extracted overnight in a buffer B (1:15, w/v) containing 1 M KCl, 25mM Tris (pH 8.0), 1 mM $KHCO_3$, 0.1 mM $CaCl_2$ and 0.1 mM DDT. After centrifugation at 15,000 g for 25 min, the supernatants were dissolved in 20 volumes of 50 mM phosphate buffer (pH 7.2), and subjected to salting-out (30-55% saturation of ammonium sulfate) to obtain myofibrillar proteins. In order to separate the heat-stable tropomyosin from the other myofibrillar proteins, the resulting tropomyosin solutions were kept at 100℃ for 30 min. (Hoffman and Day *et al.*, 1981). After centrifugation, the resulting supernatant was desalted through a PD-10 column (GE Healthcare Japan, Tokyo, Japan) and stored at -80℃ until use. On the other hand, the processed food materials were separately incubated in five volumes of 50 mM phosphate buffer (pH 7.2) overnight at 4℃. After centrifugation at 15,000 g for 25 min, the supernatant was desalted by the PD-10 column and stored at -80℃ until use.

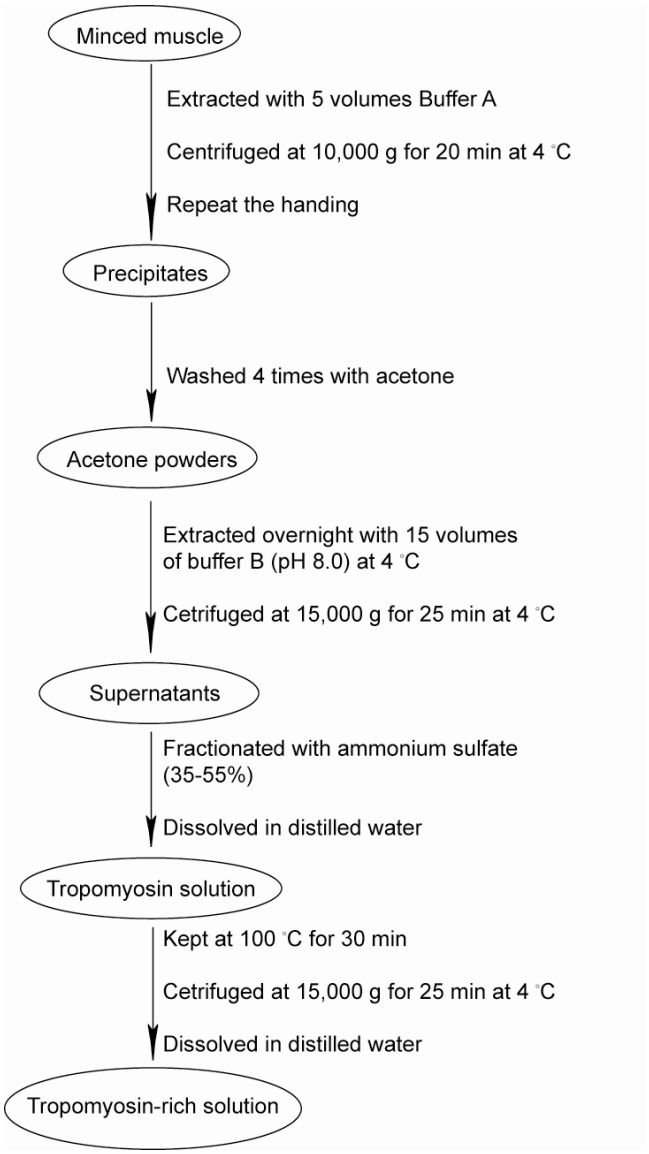


Fig. 2.1.1. Profile for preparation of tropomyosin antigens. Buffer A: 20 mM KCl, 1 mM CaCl2 and 0.1 mM DDT. Buffer B: 1 M KCl, 25mM Tris (pH 8.0), 1 mM KHCO3, 0.1 mM CaCl2 and 0.1 mM DDT. Arrows means the condition changes of the samples.

Protein quantification

Concentrations of obtained antigens were quantified by Bradford method (1976) using bovine serum albumin (BSA, Sigma-Aldrich Japan, Tokyo, Japan) as a standard protein.

Characterization of tropomyosin antigens by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 10% separating gel (Laemmli 1970). The proteins were stainined with Coomassie brilliant blue 250R (CBB). The short-neck clam tropomyosin, a 37 kDa protein in a molecular mass, was used as a tropomyosin marker protein. Tris-tricine SDS-PAGE was also performed with a separating gel (16.5% T, 3% C) and a stacking gel (4% T, 3% C) according to Schagger and von Jagow (Schagger and von Jagow 1987). In this case, the gel was stained with SYPRO Ruby protein gel stain (Bio-Rad Laboratories, Inc., Tokyo, Japan). Color Marker Ultra-low range (Sigma-Aldrich Japan, Tokyo, Japan) was used as molecular weight makers.

2.1.3 Results and Discussion

The presence of a heat-stable allergen in shrimp was first identified by Hoffman and Miller (Hoffman and Day *et al.,* 1981) with a molecular mass of 36.8 kDa. Further research demonstrated that the heat-stable allergen is actually tropomyosin, a myofibrillar protein composed of two identical subunits in molecular masses of 35-39 kDa, and was regarded as the major allergen of shrimps (Lopata and O'Hehir *et al.,* 2010). Study on shellfish allergy indicated that tropomyosin is a major cross-reactive allergy among crustaceans, mollusks and insects (Arruda 2005; Lopata and Lehrer 2009). In recent years, tropomyosin has been demonstrated to be a major allergen for various invertebrate with a molecular mass around 37 kDa, including crab (Leung and Chen *et al.,* 1998), squid (Miyazawa and Fukamachi *et al.,* 1996), octopus (Ishikawa and Suzuki *et al.,* 2001), dust mite (Boquete and Iraola *et al.,* 2011) and cockroach (Chu and Wong *et al.,* 2000).

In the SDS-PAGE CBB staining, the samples gave bands in the range of 34-39 kDa (Fig. 2.1.2). For dust mite and cockroach of whole body powder, the bands around 36 kDa were detected in the CBB staining gel. Two remarkable bands around 37 kDa of chicken and beef suggested that tropomyosin should be composed of two subunits (Lehman and Craig 2008). The observation that tropomyosin may be successfully extracted and purified from the fresh muscle samples.

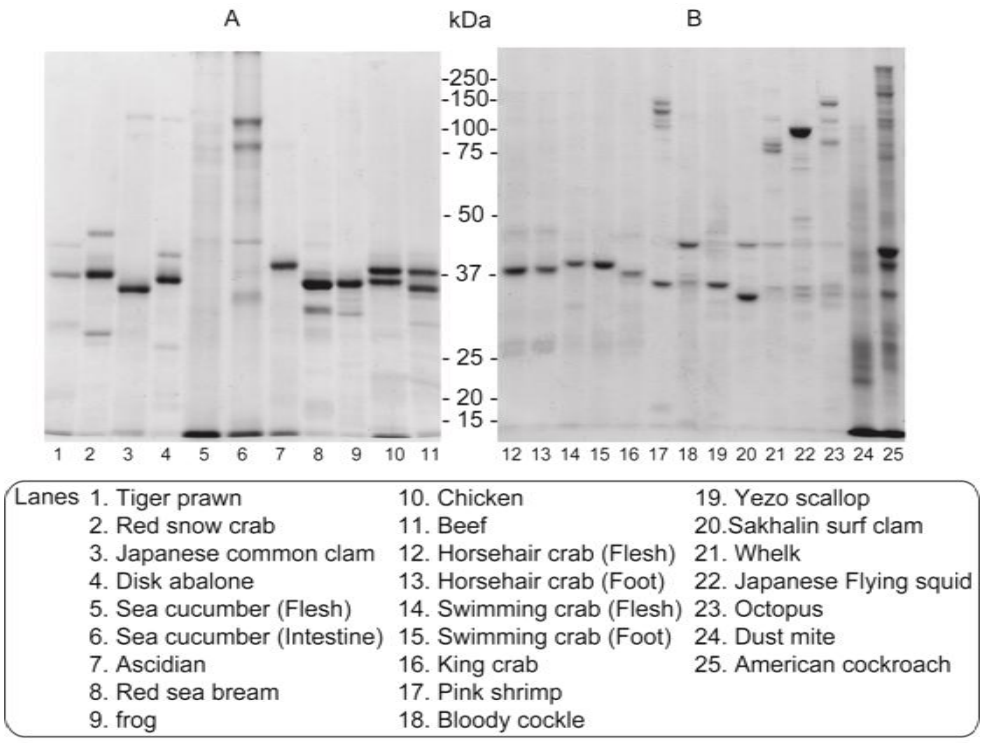


Fig. 2.1.2. Analysis of tropomyosin prepared (lane: 1-23) and extracted (lane: 24, 25) from various vertebrates and invertebrates and vertebrates by 10 % SDS-PAGE gels, stained with CBB. The protein concentrations of around 40 μg/ml were subjected to the SDS-PAGE. Each lane was around 200 ng for panel A and 100 ng for panel B.

Tris-Tricine SDS-PAGE was performed with short-neck clam tropomyosin as a reference molecular mass of 37 kDa (Fig. 2.1.3.). Color Marker Ultra-low Range (Sigma, Tokyo, Japan) was used as a molecular weight maker set. The samples gave the significant bands around molecular mass of 14 kDa,

20 kDa and 37 kDa in SYPRO Ruby gel stain (Fig. 2.1.3 A). On the other hand, CBB stain was performed in processed food extracts transferred PVDF to detect peptides (Fig. 2.1.3 B). The result showed the bands molecular mass below 1 kDa (Fig. 2.1.3 B, lanes 3-7, 9). The results suggest that the 37 kDa bands could be undegraded tropomyosin as compared with the reference protein (lane 1). In addition, tropomyosin is easily degraded by proteolytic enzymes (Rahman and Lopata *et al.*, 2010), many kinds of tropomyosin fragments could be contained with the molecular mass below 37 kDa. The successful extraction would be used to characterize the MAbs in later section.

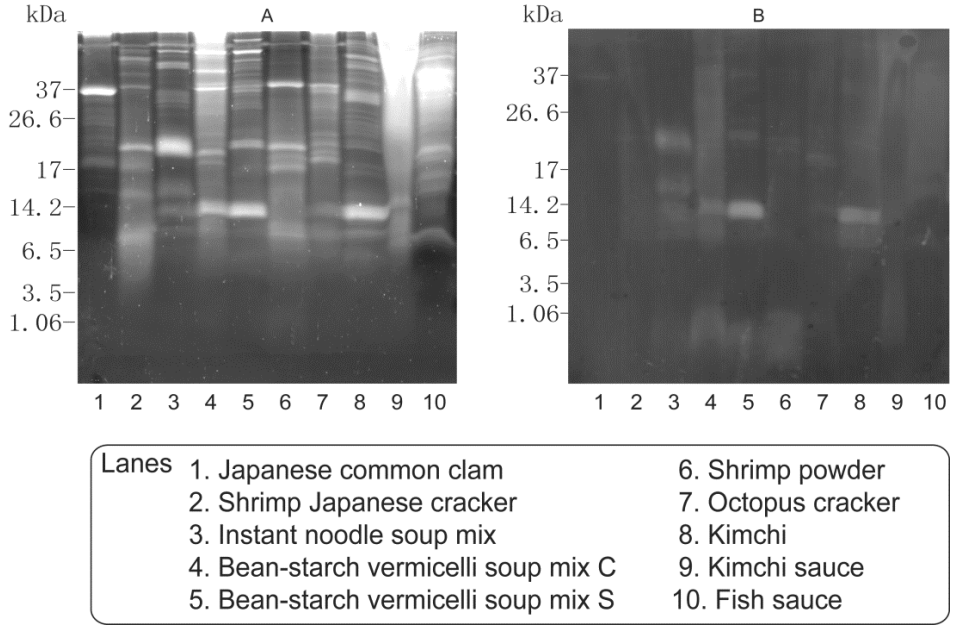


Fig. 2.1.3. Analysis of different processed foods (lane: 2-10) by Tris-Tricine SDS-PAGE. Proteins from processed foods were isolated by 10 % Tris-Tricine SDS-PAGE gels. The gel was stained with SYPRO Ruby protein gel stain (A). The proteins was blotted to PVDF membranes and stained with CBB (B). The materials of bean-starch vermicelli soup mix S contained no shellfish, but the same production line was used with bean-starch vermicelli soup mix C containing shellfish. C: Chinese-inspired. S: Sichuan style. Each lane was around 3 μg for panel A, B.

2.2 Preparation of monoclonal antibodies against invertebrate major allergen tropomyosin

2.2.1 Introduction

Monoclonal antibodies (MAbs) are useful tools for many molecular immunology investigations. In particular, when used in combination with techniques such as epitope mapping and molecular modeling, monoclonal antibodies enable the antigenic profiling and visualization of macromolecular surfaces. Monoclonal antibodies have become key components in clinical tests and detection techniques. The continuous culture of hybridoma cells that produce these antibodies offers the potential of an unlimited supply of reagent (Nelson and Reynolds *et al.,* 2000). In this section, the monoclonal antibodies (MAbs) against the sequences of IgE epitope shared by some shellfish tropomyosins were developed for tropomyosin detection.

2.2.2 Materials and methods

Synthetic peptide design

The previous studies demonstrated that a peptide sequence around C-terminal on the sequence of IgE epitope was shared by several shellfish tropomyosins (American lobster, brown shrimp, mantis prawn, krill, snow crab, octopus, Japanese abalone, scallop, clam, Japanese oyster). This sequence was strongly recognized by sera of human patients sensitive to shellfish tropomyosins (Emoto and Ishizaki *et al.,* 2009). The present peptides were designed based on the sequences of IgE epitope shared (Fig. 2.2.1) and synthesized through the Fmoc method (Atherton et al., 1979). The designed sequences in the

present study are T1: NH2-EKYKSISDELDQTFAELC-COOH, T2: NH2-KSISDELDQTFAELC-COOH, and T3: NH2-SISDELDQTFAELC-COOH (Fig. 2.2.1). In these sequences, the amino acid cluster with strong hydrophobicity was avoided and six very high homologous amino acid (ISDELD) residues were placed at center. The cysteine was added at C-terminal position for the following conjugation to carrier proteins. The designed peptides were conjugated with boving serum albumin (BSA) or keyhole limpet hemocyanin (KLH) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS, Thermo Fisher Scientific K.K., Yokohama, Japan) according to the manufacturer instructions. In this study, the KLH-conjugated peptides were used for immunization, and the BSA-conjugated peptides were used for hybridoma selection.

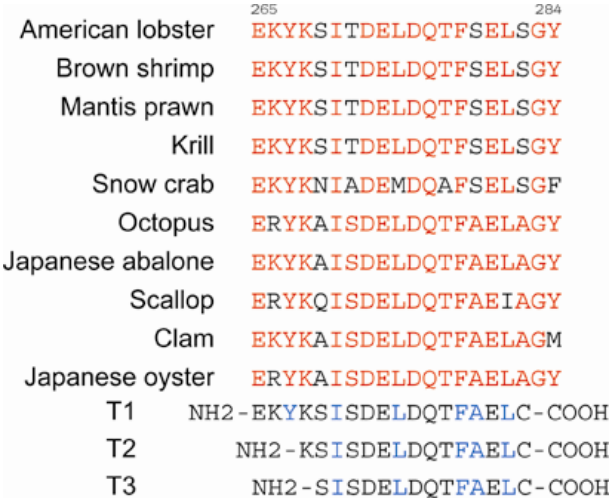


Fig. 2.2.1. The sequences of IgE epitope shared by several shellfish tropomyosins from GenBank database at regions 265-284. American lobster (fast tropomyosin soform, *Homarus americanus*, AAC48288), brown shrimp (*Farfantepenaeus aztecus*, AAZ76743), mantis prawn (Macrobrachium rosenbergii, ADC55380), krill (*Oratosquilla oratoria*, BAF95206), snow crab (slow-tonic isoform, *Chionoecetes opilio*, BAF47267), octopus (*Octopus vulgaris*, BAE54433), Japanese abalone (*Haliotis discus discus*, BAH10148), scallop (*Mizuhopecten yessoensis*, BAB17858), clam (*Venerupis philippinarum*, BAH10157), and Japanese oyster (*Crassostrea gigas*, BAH10152). The region colored red showed high homologous amino acids between different shellfish tropomyosin. The blue colored are hydrophobic residues.

Immunization

Three individuals of female BALB/c mice (6 weeks, Clea Japan, Tokyo, Japan) were intraperitoneally immunized respectively with an emulsion of 100 µl antigen solution containing 20 µg of each KLH-conjugated peptide and 100 µl TiterMax Gold (CytRx Cop., GA) on day 0 and the 14th day. The immunized BALB/c mice were given an intravenous injection the amount of 100 µl immunogen solution 3 days (25th day after the first immunization) before the following cell fusion procedure.

Cell fusion and selection

The cell fusion was carried out according to the method of Kohler and Milstein (Kohler and Milstein 1975) with slight modifications as described previously (Lu and Oshima *et al.,* 2004). The spleens were removed from immunized BALB/c mice 3days after the last boost and were fused with mouse myeloma cell line P3-X63-Ag8.U1 (P3U1) using polyethylene glycol (PEG) 1,500 (Roche Diagnostics Japan, Tokyo, Japan) and then were selected in a GIT medium (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) containing 10% BM-conditioned H1 (Roche Diagnostics Japan, Tokyo, Japan) and 1% hypoxanthine-aminopterin-thymidine (HAT) supplement (Roche Diagnostics Japan, Tokyo, Japan). Specific antibody screenings were performed by ELISA analysis. Positive hibridomas were cloned by limiting dilution method. The immunoglobulin isotypes and subclass of the new MAbs were determined using a mouse monoclonal antibody isotyping kit (Roche Diagnostics Japan).

MAb production and purification

Procedures for purification of MAb from cell culture and ascites fluid were illustrated in Fig. 2.2.2.

The IgG antibody fractions were prepared by ammonium sulfate precipitation, followed by the protein G column purification (GE Helthcare Japan, Tokyo, Japan). The concentrations of MAbs were quantified by UV spectrophotometry at 280 nm (IgG: A_{280}=1.4, UV-1700, Shimadzu Corporation, Kyoto, Japan). The resulting purified MAb powders were stored at -85 ℃.

ELISA analysis

The BSA-conjugated peptide or 0.2 μg/ml of tropomyosin protein samples as antigens (1: 10,000) were immobilized onto 96-well polystyrene plates (Corning Japan, Tokyo, Japan) at 100 μl/well of a 1 mg/ml concentration of protein in TBS for 1 h. After washing three times, each well was blocked with TBS containing 5% skim milk at 4 ℃ overnight, and then incubated with 100 μl of hybridoma culture supernatants at room temperature for 1 h. After washing, the plates were incubated for 1 h with 100 μl of horseradish peroxidase (HRP)-rabbit anti-mouse immunoglobulin G+A+M (H+L) antibody (1: 5,000 v/v, ZYMED Laboratories, Carlsbad, USA) in TBS containing 0.5% polyoxyethylene (20) sorbitan monolaurate (Wako, Tokyo, Japan) (TBST). Finally, 100 μl of freshly made substrate solution of Sigmafast OPD color former (Sigma-Aldrich Japan, Tokyo, Japan) was added and 1 M H_2SO_4 was used to stop the enzyme reaction. The absorbance was measured at 492 nm. TBS was used as a negative control.

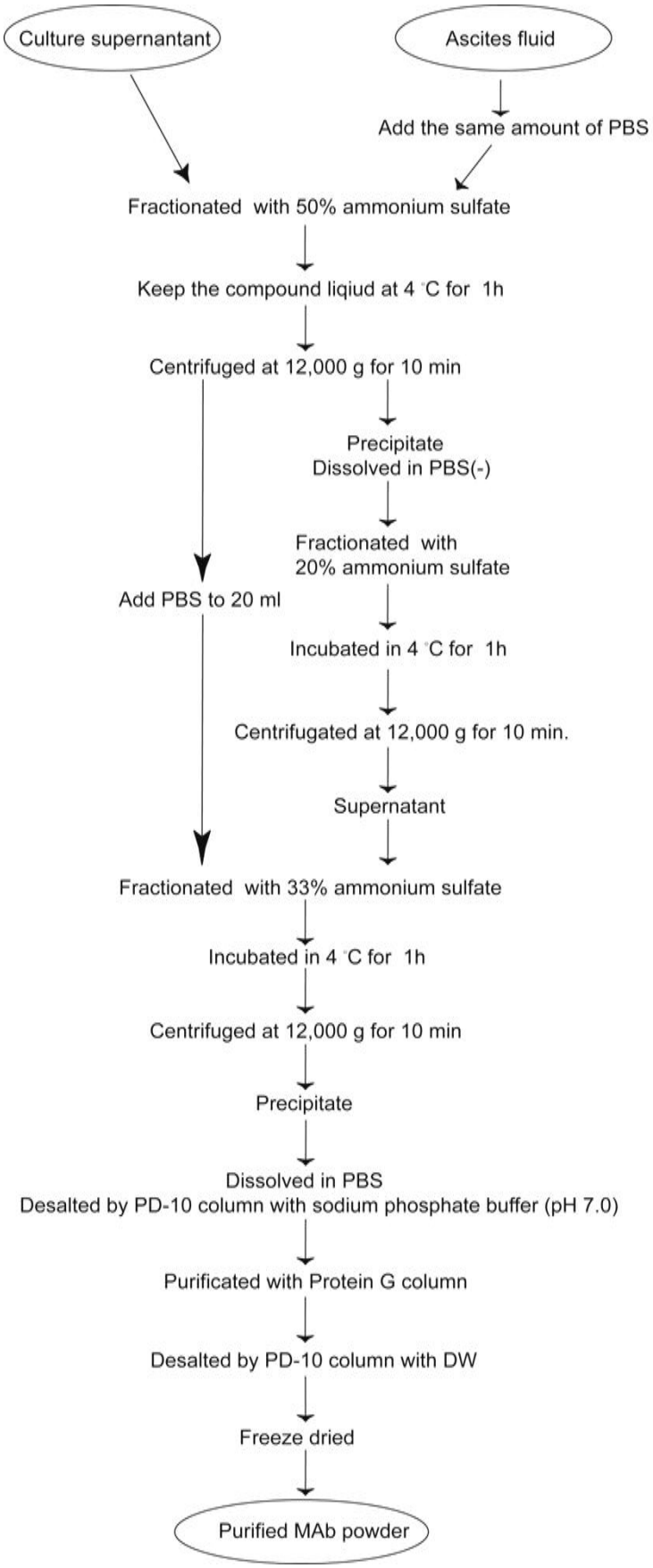


Fig. 2.2.2. Profile for purification of monoclonal antibody. Arrow means the experiment step.

Screener blotter analysis

Purified tiger prawn tropomyosin was electrophoresed on SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blocked with TBS containing 5% skim milk. After washing with TBST, the membrane was placed in a screener blotter (Screener Blotter Mini 12, Sanplatec, Osaka, Japan) apparatus. Hybridoma supernatants were applied to each lane and incubated at room temperature for 1 h with shaking. After washing in TBST, the membrane was incubated with goat anti-mouse IgG or IgM (H+L) Alexa Fluor 680. Finally, the reacted bands were visualized using the Odyssey (Fc Imaging System, MS Techonosystems, Tokyo, Japan).

2.2.3 Results and Discussion

Three days after the intravenous immunization, the mouse with the highest antibody titer against immunogen was splenectomized and the spleen cells were fused with P3U1 myeloma cells. Antibody levels in supernatants from the positive wells were detected by ELISA. Eventually, two hybridomas secreting the MAbs against KLH-T1 were selected through the limiting dilution method, designated as BE9 and EB11, respectively. Isotyping results indicated that the two MAbs were of subclass IgM with κ light chain (Table 2).

For the peptide KLH-T2, one positive MAb against peptide T2 was identified and designated as DC3; its isotype was identified as IgM with κ light chain (Table 2).

Most of the obtained hybridomas generated against the KLH-T3 immunogen showed positive response to BSA-T3 in the first ELISA screening. However, after the second screening by ELISA and Western blot analyses using a screener blotter, only the clone CE7B2 was found positive for crustacean

(Fig. 2.2.3) and molluscan tropomyosins (data not shown). The subtype of the MAb CE7B2 was identified as IgG 2a with κ light chain (Table 2).

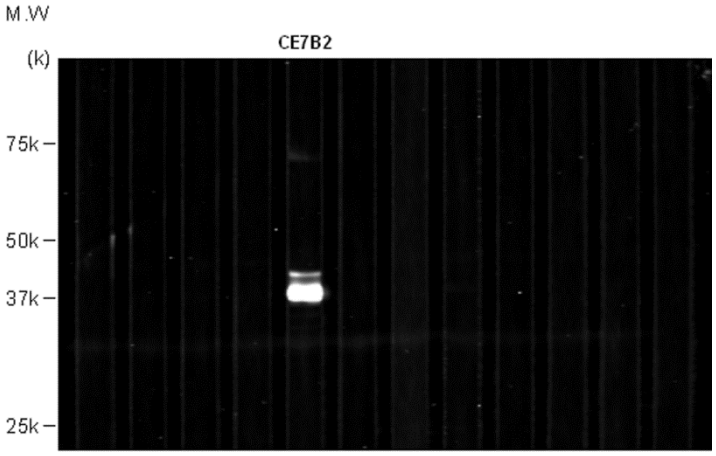


Fig. 2.2.3. Screener blotter analysis of BSA-T3 positive clones against purified tiger prawn tropomyosin.

Table 2. General survey of developed monoclonal antibodies

Monoclonal antibody	Immunogen	Subclass[a]	Light chain isotype[a]
BE9	KLH-T1	IgM	κ
EB11	KLH-T1	IgM	κ
DC3	KLH-T2	IgM	κ
CE7B2	KLH-T3	IgG2a	κ

a: Subclass and isotypes of the monoclonal antibodies were determined by a mouse monoclonal antibody isotyping kit.

2.3 Characterization of monoclonal antibodies against invertebrate major allergen tropomyosin

2.3.1 Introduction

In recent years, it has been reported several MAbs against shellfish or filarial tropomyosins for detecting invertebrate tropomyosin (Lu and Oshima *et al.*, 2004; Lu and Ohshima *et al.*, 2007; Sereda and Hartmann *et al.*, 2010). The obtained MAbs did not react to spread species invertebrate tropomyosin, since they were raised against only one type of intact invertebrate tropomyosin as an immunogen. The reported MAbs are presumably unable to detect specifically the common IgE epitopes of tropomyosin. Because the tropomyosin IgE epitope sequences are highly conserved among a wide range of invertebrate (Reese and Ayuso *et al.*, 1999), it is reported that some patients allergic to shrimp tropomyosin are also allergic to other crustacean, mollusks or non-edible insects tropomyosins (DeWitt and Mattsson *et al.*, 2004). These studies have indicated that the cross-reactivity among different allergens occurs because of shared similar or identical amino acid sequences of IgE-binding epitopes. In this section, the MAbs against the tropomyosin IgE epitope sequences were characterized.

2.3.2 Materials and methods

Electrophoresis and Western blot analysis

Because of the high sensitivity and low background, Western blot analysis is widely used as a tool for biological, clinical and biomedical researches (Roca-Sanjuan and Delcey *et al.*, 2011). In the routine clinical laboratory, chemiluminescence is commonly used for immunoassay (Kricka 2003). On

the other hand, in the fluorescence detection system, fluorescent antibodies were used with its high sensitivity. In this study, the chemiluminescent system was used to evaluate the allergenicity of tropomyosin by patient sera, and the fluorescent system was used to characterize and evaluate the developed MAbs.

The obtained MAbs were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Tris-tricine SDS-PAGE as described in the section 2.1.2. For Western blot analysis, the proteins separated by 10% SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P^{SQ}, Merk Japan, Tokyo, Japan). The membranes were blocked with Tris-buffered saline (TBS; Takara Bio Inc., Shiga, Japan) containing 5% skim milk or 15 mg/ml BSA at 4 ℃ overnight. After washing, the membranes were incubated with the supernatants from selected culture plate wells, purified MAbs solutions or patient sera for antibody characterization and allergenicity assessment. The Alexa Fluor 680 produced in goat anti-mouse IgM or IgG (1:10,000, Molecular Probes Lnc., USA) and goat horseradish peroxidase (HRP)-conjugated anti-human IgE were used as the secondary antibody. For the chemiluminescent assay, the specific reaction was detected with SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL., USA).

2.3.3 Result and Discussion

Muscle protein tropomyosin has been proved to be a major and common allergen in invertebrates (Reese and Ayuso *et al.*, 1999). It was reported that some MAbs against shellfish major allergen tropomyosin have been prepared (Lu and Oshima *et al.*, 2004; Lu and Ohshima *et al.*, 2007).

Alternatively, an invertebrate MAb NR1 against allergen filarial tropomyosin was characterized (Sereda and Hartmann *et al.,* 2010). But the MAbs cannot recognize all invertebrate tropomyosins. Lu and the colleagues demonstrated that the MAb 2A7H6 reacted to both of crustacean and mollusk tropomyosins, but not to octopus, clam, or others (Lu and Oshima *et al.,* 2004; Lu and Ohshima *et al.,* 2007) (Fig. 2.3.6). MAbs 5G5E1 and 1A3A7 reacted to crustacean tropomyosin species only (Fig. 2.3.6), whereas MAb NR1 was reported to specifically react to part of invertebrate tropomyosins but not to arthropods (Sereda and Hartmann *et al.,* 2010). It has been established that individuals sensitive to shrimp may also be allergic to other invertebrates such as crustacea, mollusk, house dust mite, cockroach and fruit fly, since the tropomyosin IgE epitope sequences are highly conserved in the invertebrates (Lopata and O'Hehir *et al.,* 2010). Therefore, it was important to prepare MAbs against the common IgE epitope sequences for detecting spread species invertebrate tropomyosin.

Characterization of the MAbs against peptide T1

In this study, Western blot assay was performed to evaluate the specific reaction of the obtained MAbs. The peptide T1 (NH2-EKYKSISDELDQTFAELC-COOH) was designed as described in Section 2.2 for recognition site of invertebrate tropomyosin. Cross-reactivity of the MAbs obtained against the peptide T1 was evaluated with various extracts from different species of invertebrates such as clam, abalone, ascidian, and sea bream. As shown in Fig. 2.3.1, the MAb BE9 not only reacts to the protein around 37 kDa of clam and abalone, but also reacts to vertebrate proteins around 37 kDa and 70 kDa of ascidian and red sea bream, respectively. On the other hand, the MAb EB11 weakly reacts to the proteins of clam and bream, however the reaction was much weaker than BE9, and its reactivity level was extremely low. The results suggest that the MAb BE9 and EB11 did not response to mollusks

tropomyosin with the molecular mass around 37 kDa, but strongly react to vertebrate proteins (ascidian and red sea bream). The molecular mass around 25 kDa could be the degradation products of tropomyosin, and the 70 kDa was presumably a dimer of the 37 kDa protein. In conclusion, the MAbs raised against the peptide T1 reacted to vertebrate proteins, and showed low specificity to invertebrate tropomyosin.

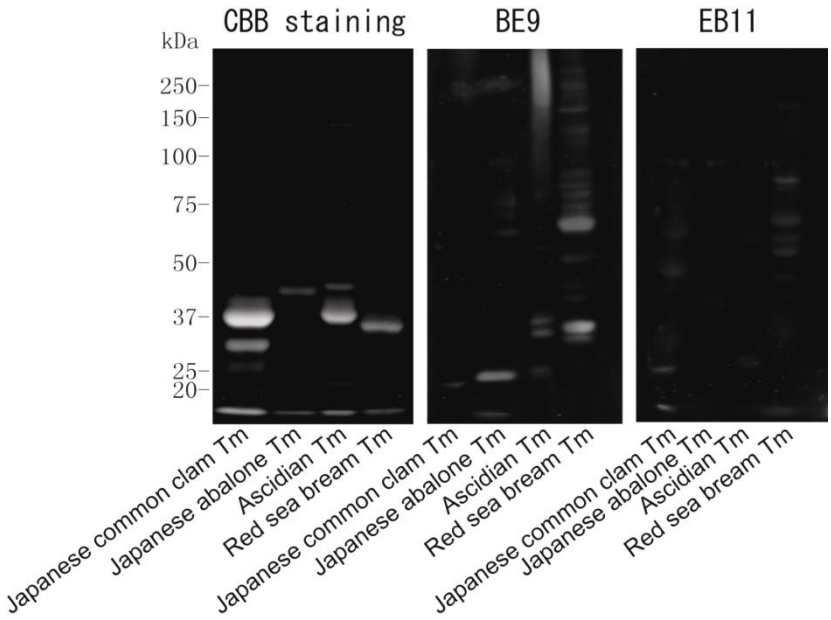


Fig. 2.3.1. Analysis of specificity of the MAbs generated against peptide T1 immunogen. Tropomyosins were extracted from Japanese common clam (*Ruditapes philippinarum*) and Japanese abalone (*Haliotis discus*) of invertebrate, ascidian (*Halocynthia roretzi*) and red sea bream (*Pagrus major*) of vertebrate. The proteins were separated by 10 % SDS-PAGE gel, and then stained by CBB (left panel). Proteins transferred PVDF membranes were analyzed by Western blotting. Purified MAb BE9 (middle panel) and EB11 (right panel) (1:5,000) were used as primary antibodies. Alexa Fluor 680 conjugated goat anti-mouse IgM (1:10,000) was used as a secondary antibody. The protein concentrations of each lane was around 10 μg. TM: tropomyosin.

Characterization of the MAb from peptide T2

The characterization of the MAbs generated against the peptide T2 (NH2-KSISDELDQTFAELC-COOH) as an immunogen is shown in Fig. 2.3.2. The cross-reactivity profiles of the MAbs were evaluated using extracts from various crustacean, mollusks and vertebrates (prawn, crab, clam, abalone, cucumber flesh and intestine, ascidian, and sea bream). In the studies on the reaction to tropomyosin extracts, the MAb DC3 reacted to the bands around 42 kDa, 37 kDa, 25 kDa, and low-molecular-weight substances. A serial of heat-stable tropomyosin with a molecular weight ranging from 34 to 38 kDa have been identified to be major allergen in different crustacean and mollusk species (Daul and Slattery *et al.,* 1994; Ishikawa and Ishida *et al.,* 1998; Leung and Chu 2001). Thus, the MAb DC3 obtained in the present study showed reactivity not only to prawn and crab of crustacean tropomyosin, but also to the clam and abalone of molluscan tropomyosin. But the MAb DC3 also reacted to the proteins around 37 kDa of red sea bream. These results indicated that the MAb DC3 was probably reactive to the common shellfish allergen tropomyosin and teleost myofibrillar proteins. In order to achieve a MAb specific against invertebrate tropomyosin, more studies was performed as follows.

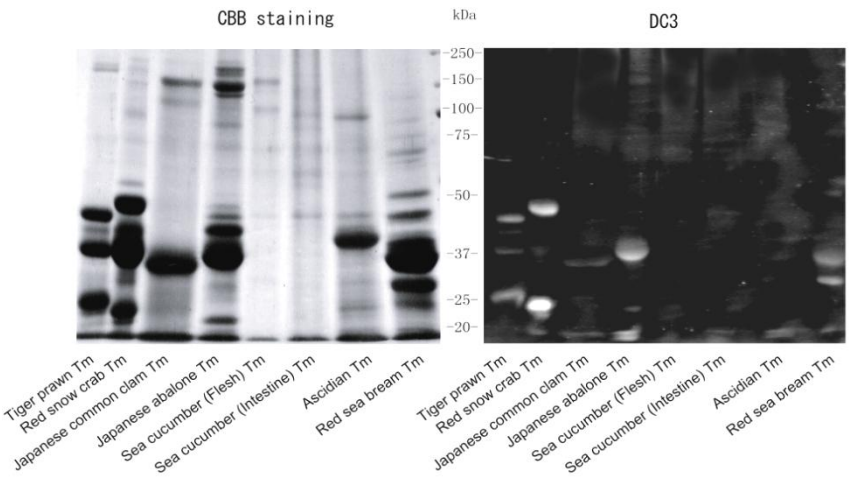


Fig. 2.3.2. Analysis of specificity of the MAb DC3 against peptide T2 immunogen. Tropomyosins were extracted from tiger prawn (*Penaeus monodon*), red snow crab (*Chionoecetes japonicus*), Japanese common clam (*Ruditapes philippinarum*), Japanese abalone (*Haliotis discus*) and sea cucumber flesh and intestine (*Stichopus japonicus*) of invertebrate, ascidian (*Halocynthia roretzi*) and red sea bream (*Pagrus major*) of vertebrate. The proteins were separated by 10 % SDS-PAGE gel, and then stained by CBB (left panel). Proteins blotted PVDF membranes were analyzed by Western blotting. Culture supernatant (1:1,000) from DC3 clones (right panel) was used as primary antibody. Alexa Fluor 680 conjugated goat anti-mouse IgM (1:10,000) was used as secondary antibody. The protein content of each lane was around 10 μg. TM: tropomyosin.

As shown in Fig. 2.3.1 and Fig. 2.3.2, all of the developed MAbs reacted to the teleost (red sea bream) protein with the molecular mass of nearly 37 kDa. These results raised a possibility that the designing peptides sequence having a high homology to sea bream tropomyosin. It was reported that a polyclonal antibody against carp tropomyosin reacted with proteins was degraded from tropomyosin by a myofibril-bound serine proteinase (Cao and Hara *et al.*, 1999). The research about tuna tropomyosins also showed two tropomyosin bands beta chain tropomyosin (37 kDa) and alpha chain tropomyosin (35 kDa) by SDS-PAGE analysis (Ochiai and Ozawa *et al.*, 2010). In this study, the bands around 37 kDa of red sea bream in Fig. 2.1.3 and Fig. 2.3.2 (CBB staining) were consistent with the other crustacean

and mollusks. Thus the protein with molecular mass of around 37 kDa for red sea bream would be tropomyosin. In addition, from the comparison of designed peptides with zebrafish alpha and beta chain of tropomyosin (Fig. 2.3.3), the identity is approximately 35%. The results indicated that the MAbs raised against peptides T1 (EKYKSISDELDQTFAEL) and T2 (KSISDELDQTFAEL) could cross-reacted with red sea bream tropomyosin partially due to the amino acid lysine (K).

```
                                265                 281
T1                              EKYKSISDELDQTFAEL
T2                                 KSISDELDQTFAEL
T3                                  SISDELDQTFAEL
Zebrafish alpha chain           L.Y.AI.E...HALNDM
Zebrafish beta chain            L.G.AL.E...LALNDM
                                 * *  * ***
```

Fig. 2.3.3. Comparison of designed peptides with zebrafish (*Danio rerio*) alpha (1, NP 571180) and beta (NP 001002119) chain tropomyosins. Points indicate the identities of redues with respect to the upper peptides. Asterisks indicate residues conserved in all the tropomyosins.

Characterization of the MAb from peptide T3

The peptide T3 (NH2-SISDELDQTFAELC-COOH) was newly designed on the basis of the discussion in Section 2.2. Cross-reactivity of the obtained MAbs was evaluated by different species tropomyosins. As shown in Fig. 2.3.4, the MAb CE7B2 was specifically reactive to the molecular mass around 37 kDa of invertebrate proteins except for American cockroach and sea cucumber, while this MAb did not recognized the vertebrate myofibrillar proteins. The MAb CE7B2 also recognized a band of around 75 kDa in bloody cockle and bands of smaller than 20 kDa in short-neck clam, dust mite and cockroach. On the other hand, the MAb weakly reacted with a protein band of 43 kDa of American cockroach. Because sea cucumber is easily autodigested by its proteases (Fu and Xue *et al.,* 2005), the tropomyosin extracted from the sea cucumber failed to be detected in SDS-PAGE and Western blot

analysis.

Previous studies reported that tropomyosins were allergic proteins with a molecular mass around 37 kDa in many invertebrate species such as shrimp (Ayuso and Lehrer *et al.*, 2002), abalone (Lopata and Zinn *et al.*, 1997), octopus (Ishikawa and Suzuki *et al.*, 2001), dust mite and cockroach (Ayuso and Reese *et al.*, 2002). As described above, several anti-tropomyopsin MAbs have been produced (Lu and Oshima *et al.*, 2004; Lu and Ohshima *et al.*, 2007; Sereda and Hartmann *et al.*, 2010), but they reacted to only one type of crustacean, molluscan and filarial tropomyosins. The MAbs were raised against intact tropomyosins as immunogens, suggesting that they may not detect common IgE epitopes specifically. Tropomyosin IgE epitope sequences are highly conserved in the invertebrates. It has been identified as the responsible allergen for cross-reactivity between seafood and insects (Boquete and Iraola *et al.*, 2011). In present study, the MAb CE7B2 against an IgE epitope shared by several shellfish tropomyosins was developed. This MAb showed reactivity not only to the allergenic proteins of crustacean tropomyosins, but also to the tropomyosins of mollusks and insects. The MAb CE7B2 would be probably reactive to the common invertebrate allergen tropomyosin. .

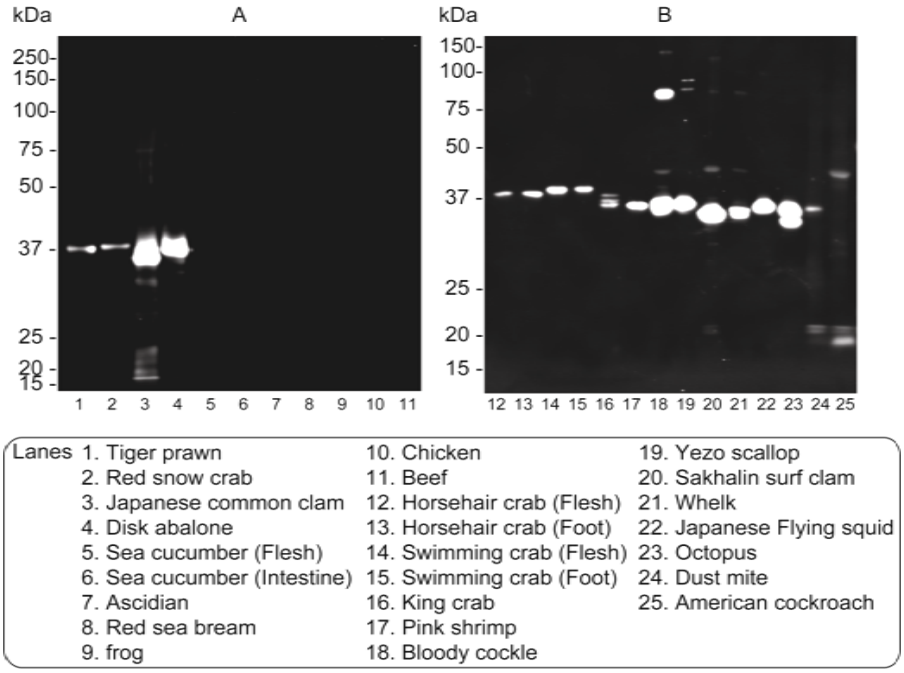


Fig. 2.3.4. Cross-reactivity of MAb CE7B2 to different crustaceans, mollusks, arachnids and vertebrates. Proteins were first separated by 10 % SDS-PAGE gels and then blotted to PVDF membranes. The MAb CE7B2 and Alexa Fluor 680 produced in goat anti-mouse IgG (1:10,000) were respectively used as primary and secondary antibody. The CBB stainings were shown in section 2.1 (Fig. 2.1.5). The protein concentrations of around 40μg/ml were subjected to the SDS-PAGE and each lane was around 200 ng for panel A and 100 ng for panel B.

As discussed above, the developed MAb CE7B2 against peptide T3 also reacted to a protein from cockroach with molecular mass around 43 kDa. A 43 kDa protein, tropomodulin contains similar amino acid sequence to tropomyosin was reported (Kostyukova and Hitchcock-DeGregori *et al.*, 2007). The protein is specifically associated with actin and tropomyosin at the thin filament pointed ends where it regulates thin filament length in the striated muscle (Leung and Chu 2001). In the present study, a region (230NISDEKLEQLFAAL243) of Drosophilidae tropomodulin was used instead of cockroach tropomodulin, since the amino acid sequences of American cockroach tropomodulin are not available in the GenBank. This region showed about 65% identity to the sequence of T3, suggesting that the

MAb CE7B2 raised against the peptide T3 might react to the insect tropomodulin which has not been reported as an allergen. To further determine whether tropomodulin is a novel allergen, the Western blot analysis with three shellfish tropomyosin-sensitive patient sera was carried out agaisnt different crustaceans, mollusks, and arachnids (Fig. 2.3.5). The results showed that the proteins detected by MAb CE7B2 also recognized by the sera. It proved once again that MAb CE7B2 should recognize invertebrate allergen tropomyosins. Additionally, the patient sera also reacted to a protein with molecular mass nearly 43 kDa from cockroach (Fig. 2.3.5A, B, C). It was proposed that the 43 kDa protein was also detected by MAb CE7B2, suggesting that tropomodulin with a molecular mass 43 kDa is probably a novel allergic protein, thereby further analysis should be carried out for its identification. This CE7B2 can be used in order to detect allergens in etiological foods instead of allergic patient sera.

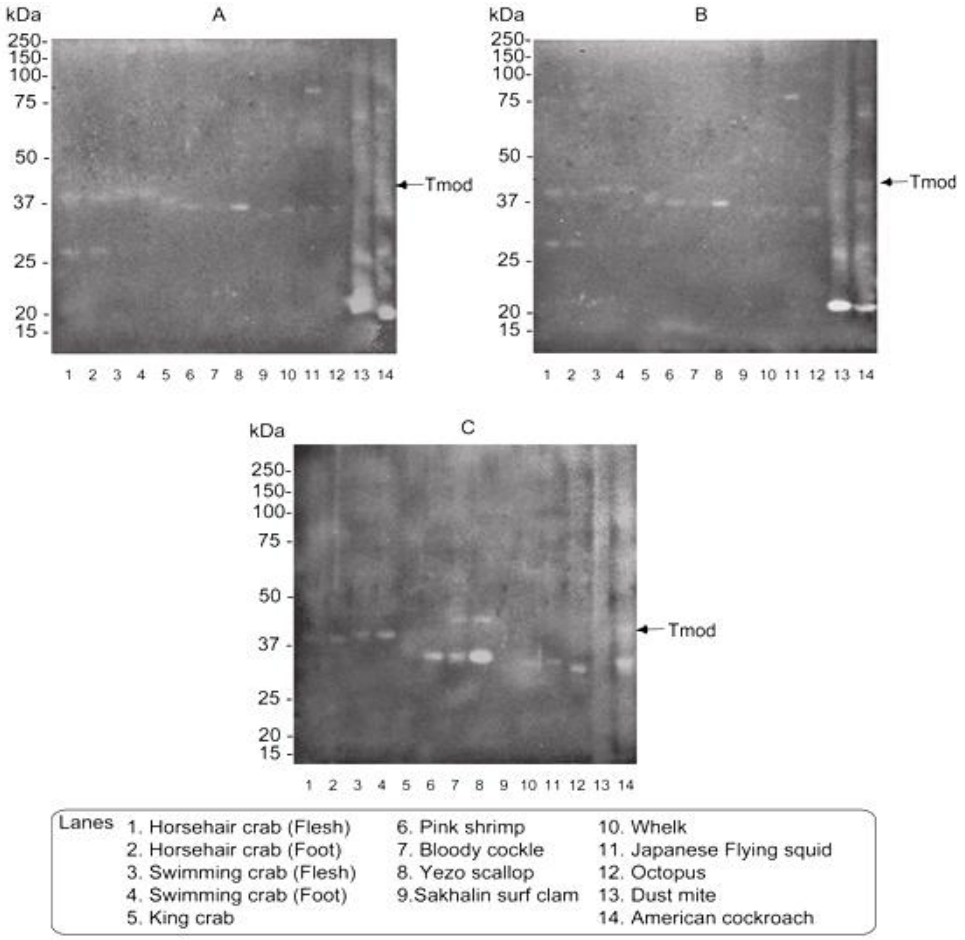


Fig. 2.3.5. Evaluation of the allergenic components in different crustaceans, mollusks, arachnids. The proteins were separated by 10 % SDS-PAGE gels and then blotted to PVDF membranes. Subsequently reacted to crustacean allergic patients sera (1:500) for allergenicity assessment assessment. Goat HRP-conjugated anti-human IgE (1:5,000) was used as secondary antibody. The protein concentrations of around 40μg/ml were subjected to the SDS-PAGE and the protein amount of each lane was around 100 ng. Tmod: tropomodulin.

It was reported that MAbs 2A7H6, 5G5E1, 1A3A7, AE9F9 (Lu and Oshima *et al.*, 2004; Lu and Ohshima *et al.*, 2007) and NR1 (Sereda and Hartmann *et al.*, 2010) were prepared as antibodies specifically reacting with tropomyosin. To confirm that the IgE epitope pattern of developed MAb CE7B2 is different from the reported MAbs, the MAbs 2A7H6, 5G5E1, 1A3A7, AE9F9 supplied from Dr. Lu was used in this work. As shown in Fig. 2.3.6, the MAb AE9F9 obtained against shellfish

tropomyosin was cross-reactive not only to the protein from crustaceans including crab and shrimp, but also to the proteins whelk of mollusk and chicken as reported (Lu and Oshima *et al.,* 2004). However, it did not react to scallop, clam squid, octopus, mite and cockroach, or slightly with cockle. The MAb 2A7H6 also reacted with shellfish, but not responded to cockle, scallop, clam, and mite. The MAb 5G5E1 reacted with only crustacean as reported by Lu *et al.* (Lu and Ohshima *et al.*, 2007), and the MAb 1A3A7 reacted with octopus and squid additionally. In contrast, the present MAb CE7B2 reacted to tropomyosins of the voluminous crustaceans, mollusks, arachnids, and insects (Fig. 2.3.4). Consequently, the MAb CE7B2 is useful for detection and quantification for invertebrate tropomyosins.

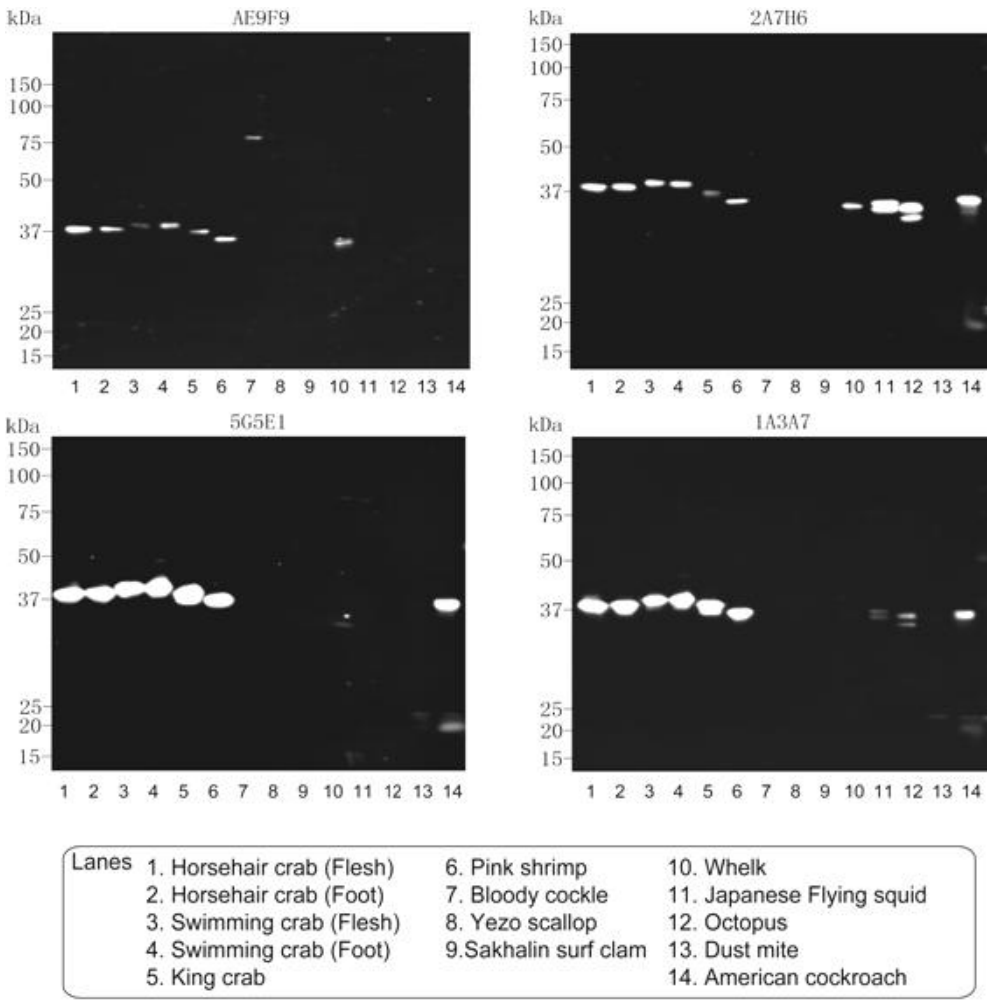


Fig. 2.3.6. Cross-reactivity of the MAbs AE9F9, 2A7H6, 5G5E1, and 1A3A7 (cells from Dr. Lu) to different crustaceans, mollusks, arachnids and vertebrates. Proteins were first separated by 10 % SDS-PAGE gels and then blotted to PVDF membranes. The culture supernatant from MAbs (1:1,000) and Alexa Fluor 680 conjugated goat anti-mouse IgG or IgM (1:10,000) respectively were used as primary and secondary antibody. The protein concentrations of around 40μg/ml were subjected to the SDS-PAGE and the protein amount in each lane was around 100 ng.

Tris-tricine SDS-PAGE was carried out with the short-neck clam tropomyosin used as a reference molecular mass of 37 kDa (Fig. 2.3.7). As shown in Fig. 2.3.7, the MAb CE7B2 reacted to the proteins around 37 kDa of shrimp cracker, shrimp powder and octopus cracker. The MAb reacted not only to the proteins of molecular mass around 17 kDa of clam; 14 kDa and 3.5 kDa of Chinese-inspired

bean-starch vermicelli soup mix, Sichuan-inspired bean-starch vermicelli soup mix (showing no shellfish ingredients in the label on the package but a product through the same production lines for shellfish-containing products) and kimchi sauce, but also to the peptides of a molecular mass below 1 kDa. The results suggest that the MAb CE7B2 could specifically not only detect intact tropomyosin but also recognize the fragmented peptides with an IgE epitope sequence of SISDELDQTFAEL in the processed foods. In conclusion, this CE7B2 is very useful for detecting the fragmented tropomyosin in product foods.

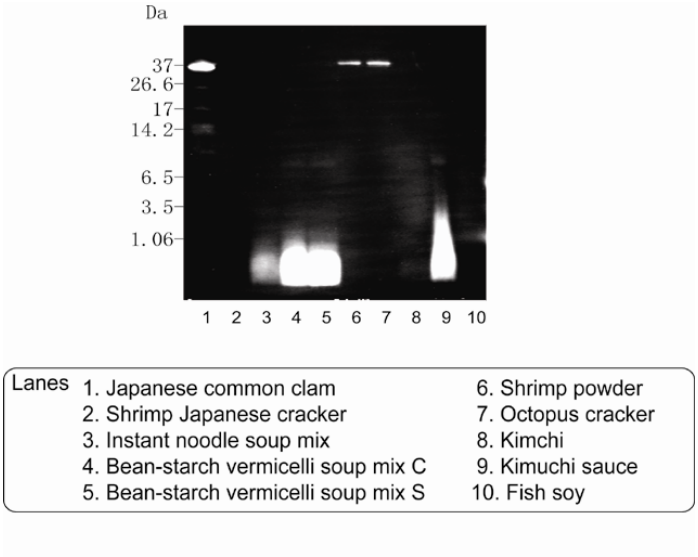


Fig. 2.3.7. Detection of intact and fragmented invertebrate tropomyosins in different processed foods using MAb CE7B2. The short-neck clam tropomyosin was used as a molecular mass reference (37 kDa). (A) Tris-tricine SDS-PAGE analysis of processed food extracts. The electrophoretic gel was stained with SYPRO Ruby protein gel stain. (B) Western blot analysis using MAb CE7B2 against invertebrate tropomyosin as a primary antibody and Alex Fluor 680 goat anti-mouse IgG (H+L) as a secondary antibody.

2.4 Applications of Monoclonal Antibodies against Invertebrate Major Allergen Tropomyosin

As shown in Section 2.3, the monoclonal antibody (MAb) CE7B2 against an invertebrate major allergen tropomyosin was prepared and characterized. The MAb CE7B2 showed reactivity not only to the seafood allergen such as crustaceans and mollusks, but also to the indoor allergen such as dust mite and cockroach. Immunoassays based on monoclonal antibody against allergen were also reported to measure allergens (Pollart and Smith *et al.,* 1991; Wu and Hsieh *et al.,* 1996) and enzyme-linked immunosorbent assay (ELISA) is the most commonly used immunological method for the quantitative detection of food allergens. The principal advantages of these fully quantitative assays are their robustness and sensitivity. In general, a sandwich ELISA is a good way for a quantitative assay of food allergens because non-specific protein bindings can be eliminated based on using of two specific antibodies possessing different binding sites. The antibodies may be monoclonal, detecting specifically a single peptide sequence in the target protein, or polyclonal, reacting to many epitopes in the target. Many studies have reported the sandwich ELISA based on MAbs or polyclonal antibodies (PAbs) for allergen detections such as shrimp and crustacean allergens (Jeoung and Reese *et al.,* 1997; Shibahara and Oka *et al.,* 2007). In addition to ELISA, fluorescence resonance energy transfer (FRET) is a widely used method for monitoring interactions between or within biological macromolecules conjugated with suitable donor-acceptor pairs. FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein-protein interactions, protein–DNA interactions, and protein conformational changes (Jares-Erijman and Jovin 2003). However, at present there are no validated methods using the FRET system for the detection and quantification of food allergens. In this section,

the sandwich ELISA and FRET system based on the MAb CE7B2 against the allergen tropomyosin were performed for allergen detection and quantification.

2.4.1 Application of Sandwich ELISA

2.4.1.1 Introduction

Immunoassays based on MAb against allergen were reported to identify and quantify allergens in food such as sandwich ELISA (Jeoung and Reese *et al.*, 1997). Sandwich ELISA is a good method for quantitative assay of allergen because of non-specific proteins can be eliminated using two types of antibody possessing different binding sites, for this reason the sandwich ELISA based on some MAbs against shellfish tropomyosin were developed (Lu and Ohshima *et al.*, 2007; Shibahara and Oka *et al.*, 2007). Food allergy commonly occurs with IgE antibodies binding to the IgE-binding sites (IgE epitopes) of the allergen. Consequently, using a MAb recognizing the tropomyosin IgE epitopes shared by invertebrate could raise the efficiency and sensitivity for the detection. The PAbs are usually used for ELISA, since its low cost. However, the PAbs easily show different antigen recognition abilities, even though they are raized against the same antigen. This point would affect the reliability of the detection methods. In comparison with PAbs, a MAb possesses a high level of selectivity for a single epitope and can be produced in unlimited amounts. Therefore, a sandwich ELISA based on a MAb was developed in this section.

2.4.1.2 Materials and methods

Sandwich ELISA

The microtitre wells of ELISA plate well were coated with 100 µl of the purified MAb at 2.5 µg/ml in 50 mM carbonate buffer (pH 9.6) at 4℃ overnight. After washing three times with TBST, each well was filled with 250 µl of blocking buffer (TBS containing 5% skim milk) and incubated at 37℃ for 1.5

h. After washing three times, 100 µl of samples were added to each well with different dilutions of the purified kuruma prawn tropomyosin, Japanese flying squid tropomyosin or production food extracted supernatants at 37 ℃ for 1 h. TBS was used as a negative control. After washing five times, 100 µl of a PAb raised in rabbits against king crab tropomyosin at 8 µg/ml was added to each well and incubated at 37 ℃ for 1 h. After washing in the same way as previous step, 100 µl of goat anti-rabbit immunoglobulin G+A+M (H+L) peroxidase conjugated antibody (Thermo Fisher Scientific K.K., Yokohama, Japan) at 1:10,000 dilution in TBST was added to each well and incubated at 37 ℃ for 1 h. The plate was washed again and then 100 µl of Sigmafast OPD color former solution (Sigma-Aldrich Japan, Tokyo, Japan) was added to each well and incubated in the dark for 30 min at room temperature. Reaction was terminated with 50 µl of 1 M H_2SO_4 and absorbance values at 492 nm were determined.

This developed sandwich ELISA would be performed to determine tropomyosin levels in different processed foodstuffs listed in Table 2. In this sandwich ELISA, the individual results were calculated as tropomyosin equivalents to the total ingredient weight. The equivalents were calculated using the standard curve for prawn tropomyosin, except that the equivalent for octopus cracker was calculated with squid standard curve (n=3).

Detection limit

The detection limit of the sandwich ELISA system for tropomyosin was calculated according to the equation of Miller and Miller (2005):

$$\mathrm{Ld} = \mathrm{a} + 3\left\{\sum(\mathrm{y_i} - \hat{\mathrm{y}}_\mathrm{i})^2/(n-2)\right\}^{1/2}$$

where Ld represents the value of detection limit concentration, a stands for the y-axis intercept deduced from line of regression of concentrations, $\mathrm{y_i}$ is the experimental values of every concentration, $\hat{y}_\mathrm{i}$

represents the individual values from line of regression of every concentration, and *n* is the number of samples.

Statistical method

The mean coefficients of variation (CV) were based on three performances on three different days for determination of the inter-assay and intra-assay precision.

2.4.1.3 Results and discussion

MAbs or PAbs are commonly used in sandwich ELISA systems. The MAb recognizes a single epitope, while PAb reacts with multi-epitopes in the target proteins. It is known that the quantitative capability of the sandwich ELISA is affected by false positives of PAbs, since PAbs may recognize structures similar in other related proteins. Consequently, the MAb was used as a capture antibody in order to detect the peptides fragmented by proteolysis but containing an IgE epitope sequence common in some invertebrates in the present sandwich ELISA assay.

In this study, a rabbit polyclonal antibody against king crab tropomyosin which was prepared previously was used in the developed sandwich ELISA. As shown in Fig. 2.4.1, the antibody reacted not only to the proteins from crustaceans, but also to the proteins from mollusks, and insects. In addition, it was reported that the antiserum did not react to the vertebrates (Lu and Oshima *et al.*, 2004). The antibody specificity to mollusks, arachnid and insect were more weakly than to crustaceans. The proteins around 37 kDa detected by the rabbit polyclonal antibody were also recognized by the MAb CE7B2, suggesting that the developed polyclonal antibody should recognize invertebrate tropomyosin and would be suitable for quantification of tropomyosin with the MAb CE7B2 in the sandwich ELISA.

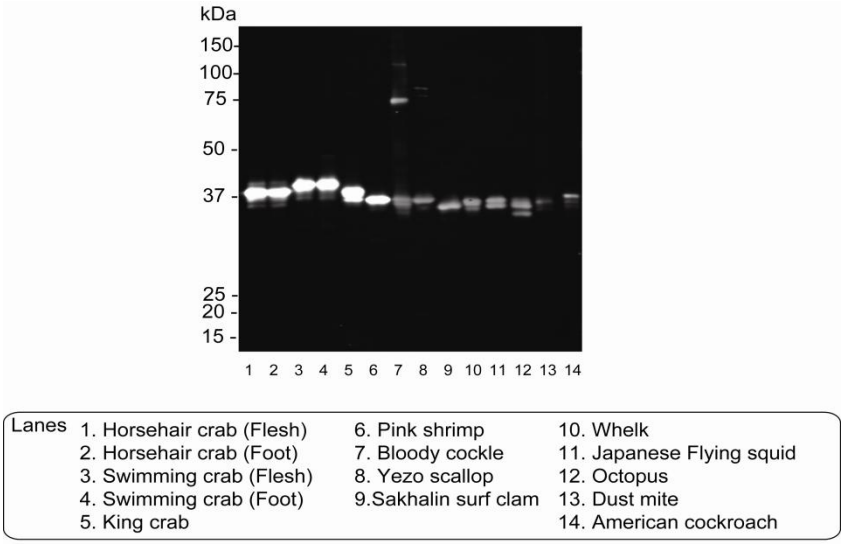


Fig. 2.4.1. Characterization of reactivity of rabbit antiserum (from Dr. Lu) against king crab (*Paralithodes camtschatica*) tropomyosin by Western blot assays. Proteins from different crustaceans, mollusks, arachnid and insect were isolated by a 10 % gel and blotted to a PVDF membrane. The rabbit antiserum (1:20,000) and Alexa Fluor 680 cojugated goat anti-rabbit IgG were used as primary and secondary antibody, respectively. The protein concentrations of around 40 μg/ml were subjected to the SDS-PAGE and each lane was around 100 ng for each panel.

Standard curves

The sandwich ELISA developed in the present study was used for detection and quantification of invertebrate tropomyosins by using the MAb CE7B2. The standard curves were performed with different dilutions from 0.045 to 600 ng/ml of kuruma prawn tropomyosin and 0.080 to 512 ng/ml of Japanese flying squid tropomyosin (Fig. 2.4.2). These standard curves can be applied to the quantification of crustacean and mollusk tropomyosins from several processed foods. The detection limit by this sandwich ELISA was 0.09 ng/ml for kuruma prawn and 0.64 ng/ml for Japanese flying squid tropomyosins, respectively.

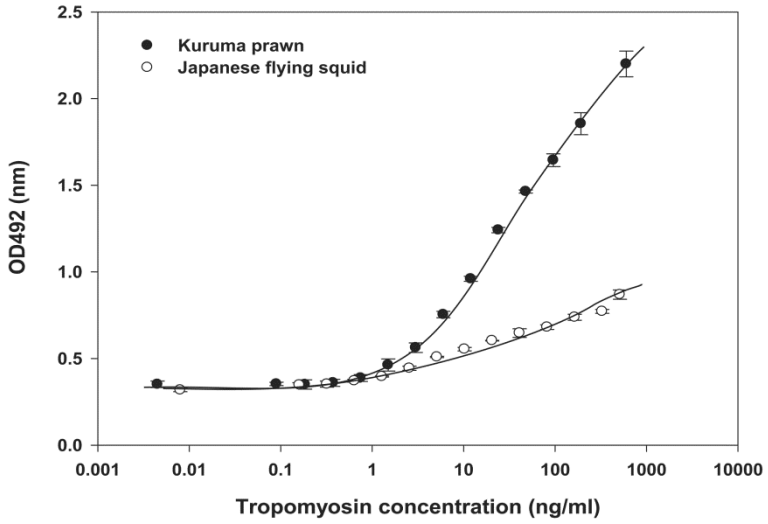


Fig. 2.4.2. Standard curves of sandwich ELISA with MAb CE7B2 for kuruma prawn (●) and Japanese flying squid (○) tropomyosins. The detection limits for kuruma prawn and Japanese flying squid tropomyosins were 0.09 and 0.64 ng/ml, respectively (n=3).

The intra- and inter-assay precision, expressed as the CV, were 1.5-5.1% and 1.2-4.2% in kuruma prawn tropomyosin, and 0.8-3.9% and 0.6-3.2% in Japanese flying squid tropomyosin (Table 2). The results suggest that the sandwich ELISA assay is highly reproducible.

Table 2 Coefficients of variation for intra- and inter-assay variations (n=3)

Kuruma prawn (ng/ml)	Intra-assay (% CV)	Inter-assay (% CV)
0.75	5.1	4.2
12	1.5	1.2
96	2.2	1.8
192	3.4	2.8
600	3.4	2.8
Japanese flying aquid (ng/ml)		
1.28	0.8	0.6
41	3.9	3.2
164	2.4	1.9
328	1.4	1.2
512	3.1	2.2

CVs were defined as the standard deviation divided by the mean and multiplied by 100.

Tropomyosin levels in processed foods

Tropomyosin concentrations of the crude extracts from different processed foods were determined by the developed sandwich ELISA standard curves. As shown in Table 3, the highest tropomyosin concentration was in shrimp powder reaching 392 μg/g and kimchi (showing no shellfish ingredient in the label on the package) having the least value of 10 μg/g. For the cup instant soup powders, the instant noodle soup mix of seafood style took a value of 239 μg/g, followed by 106 μg/g for Chinese-inspired bean-starch vermicelli soup mix and 118 μg/g for Sichuan-inspired bean-starch vermicelli soup mix (showing no shellfish ingredients in the label on the package but a product through the same production lines for shellfish-containing products). The levels of shrimp cracker and octopus cracker were 364 μg/g and 322 μg/g, respectively. The sauce prepared from the fish was reached a value of 22 μg/ml and from the kimchi of 113 μg/ml.

Table 3 Determination of tropomyosin from processed foods with the sandwich ELISA

Processed foods[a]	Tropomyosin/food[b] (μg/g)
Instant noodle soup mix of seafood style	239
Chinese-inspired bean-starch vermicelli soup mix	106
Sichuan-inspired bean-starch vermicelli soup mix[c]	118
Shrimp cracker	364
Octopus cracker	322
Shrimp powder	392
Kimchi[d]	10
	(μg/ml)
Fish sauce	22
Kimchi sauce	113

[a] All tropomyosins of processed foods, except for fish sauce and kimchi sauce, were extracted as described in Materials and Methods. For fish sauce and kimchi sauce, tropomyosins were extracted from liquid mixtures.

[b] Tropomyosin equivalents were calculated using the standard curve for prawn tropomyosin, except that the equivalent for octopus cracker was calculated with squid standard curve (n=3).

[c] Showing no shellfish ingredients but produced through the same production line for shellfish-containing products in the label on the package.

[d] Showing no shellfish ingredients in the label on the package.

In this study, a common immunological method, sandwich ELISA, based on the MAb CE7B2 against invertebrate tropomyosin for the quantitative detection of food and indoor allergens was developed. To determine tropomyosin in food, the sandwich ELISA method with the anti-tropomyosin antibodies have been recently developed as shellfish tropomyosin detection kits in Japan. One detection kits developed against purified tiger prawn tropomyosin, showed the detection limit of 0.4 ng/ml (Shibahara and Oka *et al.*, 2007), and one showed 0.71 ng/ml which raised against the similar tropomyosin. However, these detection kits without IgE epitope specificities might fail to detect allergenic peptides in processed foods. In comparison, the detection limit of the present sandwich ELISA is 0.09 ng/ml for kuruma prawn tropomyosin. This value is more sensitive than the previous systems. On the other hand, the detection limit of the present sandwich ELISA is 0.64 ng/ml for Japanese flying squid tropomyosin. This result confers the ability to detect mollusk and indoor pan-allergen tropomyosin. In particularly, compared to the previous studies, the present sandwich ELISA could detect both mollusk and dust mite samples. It is considered that the sandwich ELISA is a precise and reliable method to detect tropomyosin in processed food. Indeed, this study provided experimental evidence that this sandwich ELISA is applicable to various commercial processed foods as described above.

2.4.2 Development of FRET system

2.4.2.1 Introduction

FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein-protein interactions, protein–DNA interactions, and protein conformational changes (Jares-Erijman and Jovin 2003). The detection method for food allergen can be developed according to the property of FRET as described in Chapter 1. The FRET system detects the allergens only by mixing with the IgE epitope-specific antibodies without any immobilization procedure. However, at present there are no validated methods using the FRET system for the detection and quantification of food allergen. In this section, the FRET system was developed using fluorescein isothiocyanate (FITC)-conjugated MAb CE7B2 as a fluorescence energy donor and tetramethylrhodamine isothiocyanate (TRITC)-conjugated MAb 2A7H6 against shellfish tropomyosin as an acceptor. As described above, the MAb CE7B2 obtained in the present study showed specific reaction to an IgE epitope of invertebrate tropomyosin. In addition, the previous study demonstrated that the MAb 2A7H6 recognized one common epitope shared by crustacean and in part by mollusk species (Lu and Ohshima *et al.,* 2007). The FRET system with these two MAbs would be useful for detect allergens with multi-epitopes without any solid phase. In this part, the FRET system based on the two MAbs was developed for allergen detection.

2.4.2.2 Materials and methods

Preparation of FITC, TRITC-conjugated MAbs

The MAbs were purified by Protein-G affinity chromatography (HiTrap Protein G HP, GE

Heaithcare, Tokyo, Japan). FITC (Sigma, St. Louis, USA) was dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml and the MAb CE7B2 was dissolved in 0.1 M $NaHCO_3$ at 1.3 mg/ml. After 1 μl FITC solution was quietly added to the antibody solution, the mixture was incubated for 12 h at 4℃ in the dark. Then, the excess hydrolyzed FITC was removed by a PD-10 column (GE Healthcare, Tokyo, Japan) with PBS buffer. The conjugated fluorescein/protein (F/P) ratio was checked with the absorbance at 280 nm and 495 nm. On the other hand, the TRITC-conjugated MAb 2A7H6 was prepared procedure similar to FITC-conjugated MAb CE7B2. TRITC (Sigma, St. Louis, USA) was dissolved in DMSO at 1 mg/ml. The MAb 2A7H6 was dissolved in 0.1 M $NaHCO_3$ (1.3 mg/ml).

Determination of FRET system

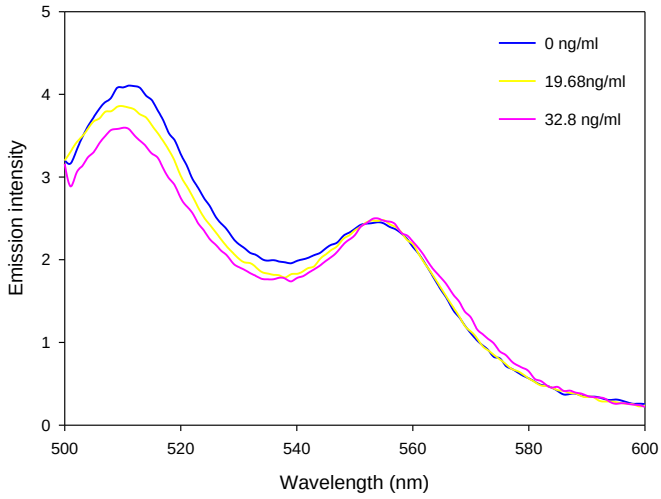
The fluorescence wavelengths were measured by fluorescence spectrophotometer (RF-1500, SHIMAZU CORPORATION, Kyoto, Japan). The fluorescent intensity was obtained at the ratio of the emission at 510 nm to 550 nm, and the excitation at 470 nm with several concentrations of tropomyosin. Bovine serum albumin (BSA) was used as a negative control.

2.4.2.3 Results and Discussion

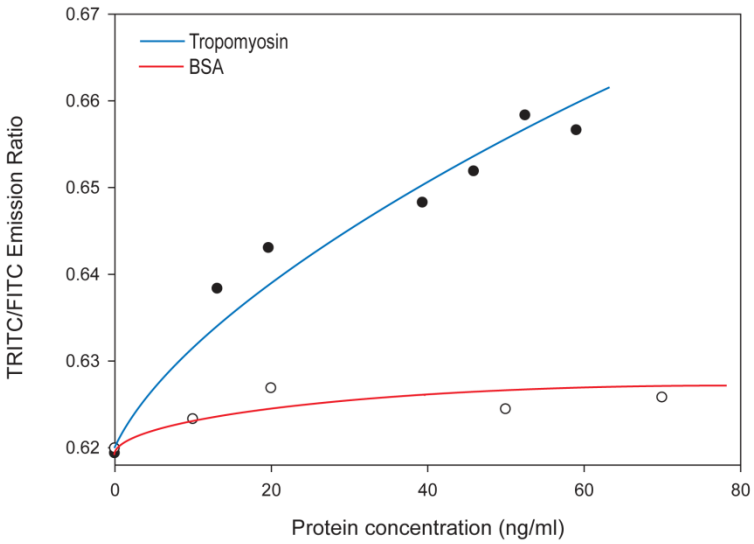
FRET is unique in generating fluorescence signals sensitive to molecular conformation, association, and separation in the 1–10 nm range. Intermolecular FRET occurs when the fluorescent energy donor and acceptor are on different macromolecules. Food and indoor allergies are commonly synonymous with type-I allergy. It always occurs with the IgE antibodies binding to the IgE-binding sites (IgE epitopes) of the allergen, and then mediated by IgE antibodies bound to the IgE receptors (FcεRIs) of mast cell. The FcεRIs aggregate when the IgE antibodies are cross-linked by allergens with

multi-epitopes, and then the FcεRIs spatially approach to each other. Subsequently, the mast cell degranulates and releases histamine, leukotrienes and other mediators (Daul and Morgan 1993). In this study, two tropomyosin IgE epitope specific MAbs, CE7B2 and 2A7H6, were used as the allergen-specific IgE antibodies. In the presence of tropomyosin, the FRET would be observed when the two MAbs bind to the adjacent IgE epitope. The tropomyosin concentration can be determined by the intensity of fluorescence.

As shown in Fig. 2.4.3A, the fluorescent intensity ratio of 555 nm to 510 nm gradual increased with increasing tropomyosin concentration. The donor FITC-conjugated MAb CE7B2 and acceptor TRITC-conjugated MAb 2A7H6 fluorescence was decayed and increased at the tropomyosin concentration of 0-2 molar, respectively. Consequently, the TRITC/FITC emission ratio increased. In order to evaluation of non-specific interactions with the multi-antibodies, the BSA was used instead of TM showing that addition of BSA was scarcely effective on this FRET system (Fig. 2.4.3B). The results suggest that the MAb CE7B2 and 2A7H6 should recognize the adjacent epitope and confer the energy transfer. In summary, the FRET method developed in the present study with multi-antibodies is useful to detect allergens with multi-epitopes without any immobilization procedure. Unfortunately, the present system has a very narrow dynamic range, probably because the orientations, *i.e.* the vectors of energy fields, of the donor and the acceptor are not fully optimized. But the system is more cost effective, as it required a shorter assay time and reduced investment in equipment than existing assay systems. The details for FRET systems should be further investigated.



A



B

Fig. 2.4.3. Fluorescence emission intensity ratio in FRET system. A, Emission spectra of 555 nm to 510 nm (excited at 470 nm). B, The florescence ratio was measured at the excitation of 470 nm (n=3). TM: tropomyosin.

Chapter 3 Development of detection and quantification methods based on monoclonal antibodies against tetrodotoxin

Tetrodotoxin (TTX) has been received increasing interests due to its special properties. It is one of the most potent marine neurotoxins, which blocks the sodium channels of the excitable cell membrane of animals. Although TTX was originally isolated from puffer fish (Yokoo 1950), it has been found in widely divergent animal species, including puffer fish, gobies, frogs and shellfish (Tao and Wei *et al.*, 2010). It is a stable toxin unaffected by cooking or freezing (Watters 1995). TTX poisoning often occurs in some countries such as Egypt, Korea, China and Japan. TTX poisoning in human always due to ingestion of improperly handled toxic puffer fish. The toxicity of TTX is caused by it selectively blocking voltage-gated Na^+ channels on the surface of the nerve membranes causing blood vessels to relax, leading to a sudden drop in blood pressure. The main causes of death in TTX poisoning are paralysis and respiratory failure (Neagu and Micheli *et al.*, 2006).

Most popular detection methods for TTX include the bioassay, chromatographic assay and immunoassay (Neagu and Micheli *et al.*, 2006). However, the disadvantages of the bioassay and chromatographic assay are the low sensitivity and the cumbersome management, respectively. The present study is focusing on the immunoassay because of its sensitivity, specificity, rapidity, simplicity, large-scale screening capability and low cost.

In order to producing antibodies against TTX, it is necessary to couple TTX to a carrier protein since TTX is a low molecular weight of 319 hapten with no substructure to induce immunoreaction. Fig. 3.1 shows the location of the two conjugation chemistries on the TTX molecule. As described in Chapter 1, the guanidinium group and oxygen group have very important place in TTX toxicity. Generally, an indirect method is used to generate anti-TTX antibodies in earlier studies (Kawatsu and Hamano *et al.*, 1997; Zhou and Li *et al.*, 2009). These TTX-protein conjugate immunogens were all prepared using a modified formaldehyde method (Fig. 3.1B) (Huot and Armstrong *et al.*, 1989). Although this method can easily conjugate TTX with carrier proteins, the resulting monoclonal antibodies (MAbs) probably have low specificity to free TTX since they do not bind the toxicity active

site (Fig. 3.1). For the purpose of preparing antibodies with high specificity to toxicity active site of TTX (Fig. 3.1A), in the present chapter, MAbs which were produced against a novel derivative of TTX were prepared and characterized.

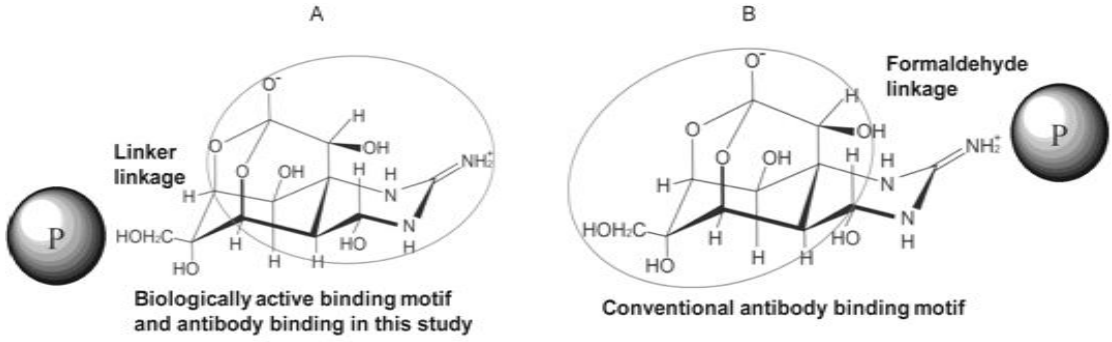


Fig. 3.1. Illustration of two conjugation sites for TTX. (A) Formaldehyde linkage was used in the production of the conventional antibody. (B) Linkers were used to conjugate TTX to carrier protein in this work which was biologically active to voltage-gated Na^+ channels. P: carrier protein.

3.1 Preparation of a novel derivative of tetrodotoxin

3.1.1 Introduction

Tetrodotoxin (TTX) has a very unique structure. It is containing a highly oxygenated carbon backbone which attached with a single guanidinium moiety. The carbon backbone of TTX consists of a 2,4-dioxaadamantane structure, decorated with four primary hydroxyl groups and one secondary hydroxyl group (Chau and Kalaitzis *et al.*, 2011). TTX is a low-molecular weight hapten with no substructure to induce immunoreactions (Zhou and Li *et al.*, 2009). Previous studies have used the modified formaldehyde method to prepare the TTX-protein conjugates for generation the MAbs against TTX. As described in introduction, this method could not obtain the antibodies specific to the toxicity active site of TTX. To directly address the goal, in this section, a novel derivative of TTX was prepared to generate MAbs against TTX.

3.1.2 Materials and Methods

Materials

Frozen puffer fish *T. rubripes* ovaries were obtained from Tokyo central wholesale market and transported to our laboratory.

TTX extraction and purification

Whole ovaries of *T. rubripes* were extracted with three volumes of 70% methanol (containing 5% acetic acid) and mixed for overnight with a tissue homogenizer. The extracts were centrifuged at 8,000 for 15 min. The supernatants were combined, concentrated to dryness by rotary evaporator and then

redissolved in 1% aqueous acetic acid. The aqueous extracts were then defatted with equal volume of chloroform. After removing the chloroform by centrifugation, the upper aqueous layer was filtered through an ultra filtration membrane (MWCO 1000 , Millipore, Bedford, MA, USA). Subsequently, the extracts were treated by active charcoal and purified by a column chromatography on a Bio-Rex 70 column (Bio-Rad Laboratories) twice by linear gradient elution with acetic acid (0-1N) as reported by Matsumoto et al. (2008).

HPLC-FLD

High-performance liquid chromatography (HPLC) coupled to a fluorescence detector (HPLC-FLD) was used as the method for detection and quantification which was modified from the method of Shoji *et al.* (Shoji and Yotsu-Yamashita *et al.,* 2001). As a mobile phase, a buffer system of 1% (v/v) acetonitrile and 10 mM ammonium formate were used containing haptasulfonic acid (HSA) at a concentration of 5 mM as ion-pair reagent (pH 5.0 with ammonium formate). 4 N NaOH was used as a reagent phase and degassed once for 5 min in an ultrasonic water bath before use. The stationary phase consisted of a Wakopak Navi C30-5 (Ø4.6 mm × 250 mm) reverse phase column (Tokyo, Japan).

The setup is schematically illustrated in Fig. 3.1.1. The mobile phase was degassed with a degassing unit (DG-2080-53, JASCO, Tokyo, Japan) and flow was controlled by a pump (PU-2080, JASCO, Tokyo, Japan) with a flow rate at 1.0 ml/min. Samples were injected in the injection coil and separated on the column resin at room temperature. Another pump (PU-1580, JASCO, Tokyo, Japan) was set at the same flow rate and transported 4 N NaOH as a reaction reagent, which was combined through a T-mixer with the mobile phase through after column separation. Subsequently, the reaction mixture was pumped through a plastic reaction coil (Ø0.5 mm × 20 m) in an oven (DOV-300A, AS ONE, Tokyo,

Japan) set at 105 ℃ to facilitate alkaline degradation of TTX and its analogs to detectable quinazoline derivatives, as described in the introduction. The calculated reaction time at 105 ℃ is about 2 min. After the reaction, the mixture was cooled down in a metal spiral embedded in ice water to decrease baseline noise levels in the detection signal and was detected with a fluorescence detector (FP-2020, JASCO, Tokyo, Japan) with excitation 365 nm and emission 510 nm. Finally, the signals were integrated on a PC running Chromato-Pro software system (Runtime Instruments, Japan).

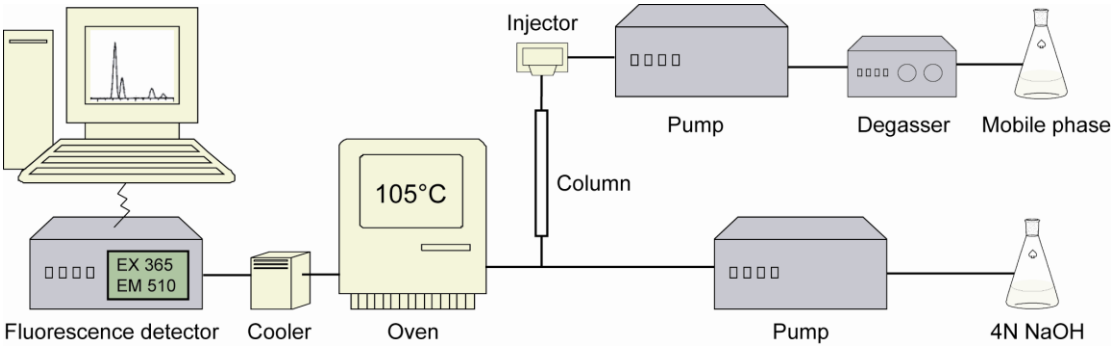


Fig. 3.1.1. HPLC-FLD setup.

TTX standard calibration curve and sample injection parameters

TTX standards were prepared from a dilution of commercial citrate-treated TTX (Sigma-Aldrich Japan, Tokyo, Japan). A dilution of 0.1 mg/ml in water was further diluted in HPLC mobile phase to concentration of 0.01 and 0.001 mg/ml. For confirmation of toxicity of toxic samples to be used, standard dilutions were applied with a microinjection syringe into the HPLC injector in different concentrations to obtain a calibration curve with four measurement points of TTX (10, 40, 80, 200 ng). The samples were measured in triplets and injected in volumes of 10-20µl, depending on preliminary one-point measurement results.

Preparation of PMPI-TTX conjugates

The TTX was activated through available secondary hydroxyl groups. The freeze-dried purified TTX was dissolved in anhydrous dimethylformamide (DMF) with minimum volume trifluoroacetic acid (TFA). The TTX solution reacted with *N*-[*p*-maleimidophenyl] isocyanate (PMPI) (Thermo Scientific, Tokyo, Japan) in DMF which represented 10-100 fold molar excess of PMPI over. After reacting overnight at room temperature, PMPI-activated TTX solution was added to the same volume of phosphate buffer (pH 7.3). The water-insoluble PMPI was removed by the filter (0.2 μm), and PMPI-TTX solution was collected for the following preparation of BSA-TTX conjugates. The most significant conjugation ratio between PMPI and TTX would be analyzed by an electrospray ionization mass spectrometry (ESI-MS) and a matrix-assisted laser desorption ionization-time of flight/mass (MALDI-TOF/MS) spectrometry. The conjugates of tetrodonic acid (TDA) and ethanol (EtOH) with PMPI were used as a control in the characterization of prepared TTX antibodies.

Preparation of BSA-TTX conjugates

S-acetyl thioglycolic acid *N*-hydroxysuccinimide (SATA) (Thermo Scientific, Tokyo, Japan) in DMF was added to 1.5 ml of BSA (100 molar fold with excess SATA) in 0.1 M phosphate, 0.15 M NaCl reaction buffer (pH 7.3). The solution was mixed gently, allowed to incubate for 30 min at room temperature and purified against the reaction buffer. The SATA-BSA was combined with deacetylation solution (0.5 M hydroxylamine, 0.025 M EDTA in reaction buffer, pH 7.3) and incubated for 2 h at room temperature before conjugation with PMPI-TTX. The extent of SATA incorporation was measured with cysteine hydrochloride monohydrate as a standard (Duncan 1983). The PMPI-TTX solution was added to the deacetylated BSA-SATA (0.1 M phosphate, 0.15 M NaCl and 0.001 M EDTA, pH 7.3). This combination of reactants represented a 20 fold molar excess of PMPI-TTX to BSA. After

the incubation overnight at 4 ℃, the conjugates were purified with a desalting column and analyzed by MALDI-TOF/MS. BSA-TDA and BSA-EtOH which prepared following a similar procedure to BSA-TTX were used as control in selection and characterization of prepared TTX antibodies. The chemical structures of the BSA conjugates were shown in Fig. 3.1.2.

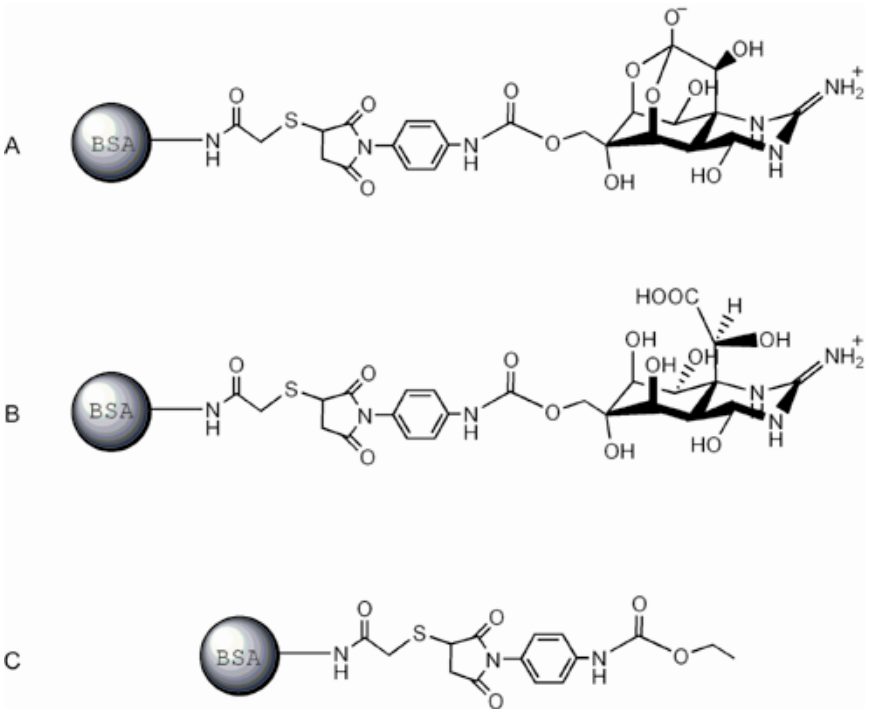


Fig. 3.1.2. The chemical structures of (A) BSA-TTX, (B) BSA-TDA, (C) BSA-EtOH.

ESI-MS analysis

The ESI-MS (Waters Alliance LC/MS system) analysis was performed at a spray voltage of +4.0 kV and the heated capillary temperature was maintained at 250 ℃. Nitrogen served both as the sheath gas at an operating pressure of 80 pis and as the auxiliary gas at a flow rate of approximately 50 μl/min. Three ions at *m/z* 214, 320 and 534 corresponding to the MH^+ ions of TTXs were detected in the selected ion monitoring (SIM) mode.

MALDI-TOF/MS analysis

The samples PMPI-TTX, BSA-TTX, BSA-TDA and BSA-EtOH were analyzed using a MALDI-TOF/MS (4800 plus, AB SCIEX, Tokyo, Japan). Each sample of 1 μl was mixed with 1 μl α-cyano-4-hydroxycinnamic acid (CHCA) matrix onto a mass spectrometry slide and crystallized at room temperature. Their mass spectra were analyzed under the acceleration voltage of 3.5 kV.

3.1.3 Results and Discussion

TTX analysis

A chromatogram of the TTX standard dilutions used for the TTX standard calibration curve is shown in Fig. 3.1.3. The TTX peak at a retention time (t_r) around 20 min is accompanied by two other prominent peaks, and clearly separated from each other. They were TTX analogs and 4-*epi*TTX (t_r=23 min) as reported by Tsuruda et al. (2002). The peak area became bigger with increasing amount of injected TTX. Depending on daily fluctuations of parameters such as room temperature or column condition, which is not unusual in HPLC analysis, although the exact results for the standard measurement varied a little, overall features remained the same. The TTX standard curves were linear in range of 10-200 ng TTX/injection, which was chosen to use in this work based on preliminary results by one-point analysis. The coefficient of determination was used on the verge of 0.99 in each calibration curve. The results of HPLC analysis of the purified TTX from puffer fish ovary sample were shown in Fig. 3.1.4. The chromatogram of the purified TTX showed an excellent peak separation and low noise levels. The retention time of the sample peak was the same as that of the TTX standard. The peaks occurring within several minutes of the TTX peak in the sample were most likely TTX

analogue (Nagashima and Maruyama *et al.*, 1987). The amount of the purified TTX is 3.1 mg (15,500 MU), and its purity is about 24 %.

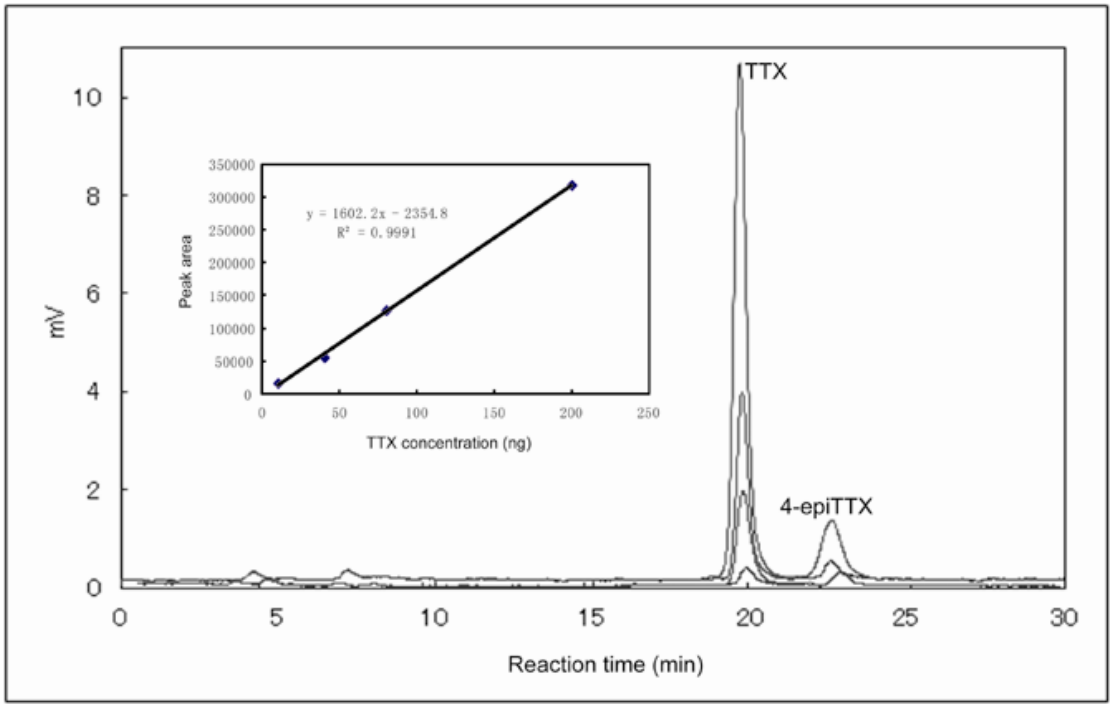


Fig. 3.1.3. HPLC analyses of TTX standard dilutions in different concentrations and calibration curve.

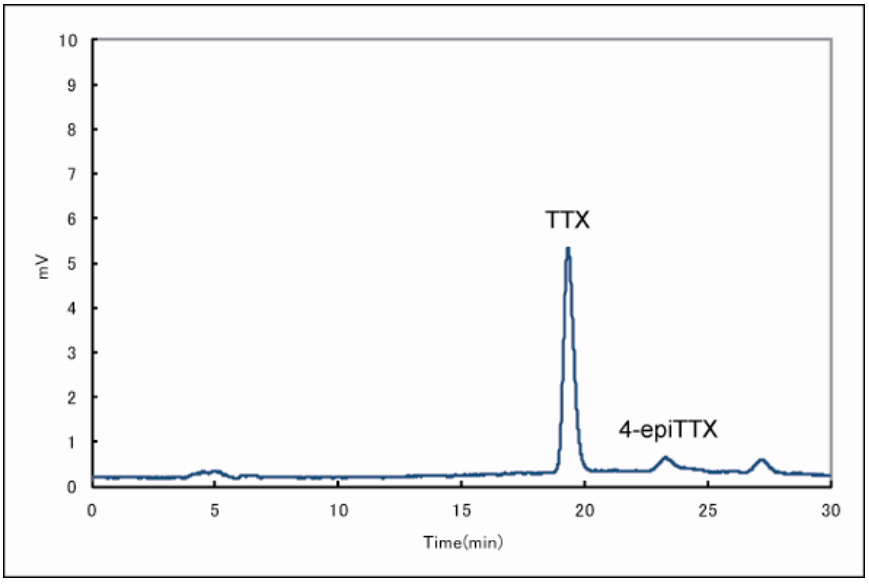


Fig. 3.1.4. HPLC chromatogram of purified TTX from puffer fish ovaries. TTX and its analogs have been identified using TTX standard. The TTX peak occurs at 19.3 min and peaks following within several minutes are TTX analogues.

ESI-MS analysis

The ion spectra of the molecular ions, MH^+, for PMPI in DMF with TFA of negative control (A), TTX (B) (*m/z* 320.41) and PMPI-TTX (C) (*m/z* 534.46) obtained by the ESI-MS are shown in Fig. 3.1.5. The spectrum of TTX was almost identical to that obtained by the ESI-MS of TTX reported by Shoji et al. (2001). The calculated molecular weight of PMPI-TTX should be around *m/z* 533, since the linker PMPI material data sheet shows its molecular weight is 214.18. As shown in Fig. 3.1.5B, the obtained PMPI-TTX spectra correspond approximately to the calculated spectrum. All spectra exhibited ions due to elimination of one or two water molecules. The results suggest that PMPI-TTX was successfully obtained in the reactions.

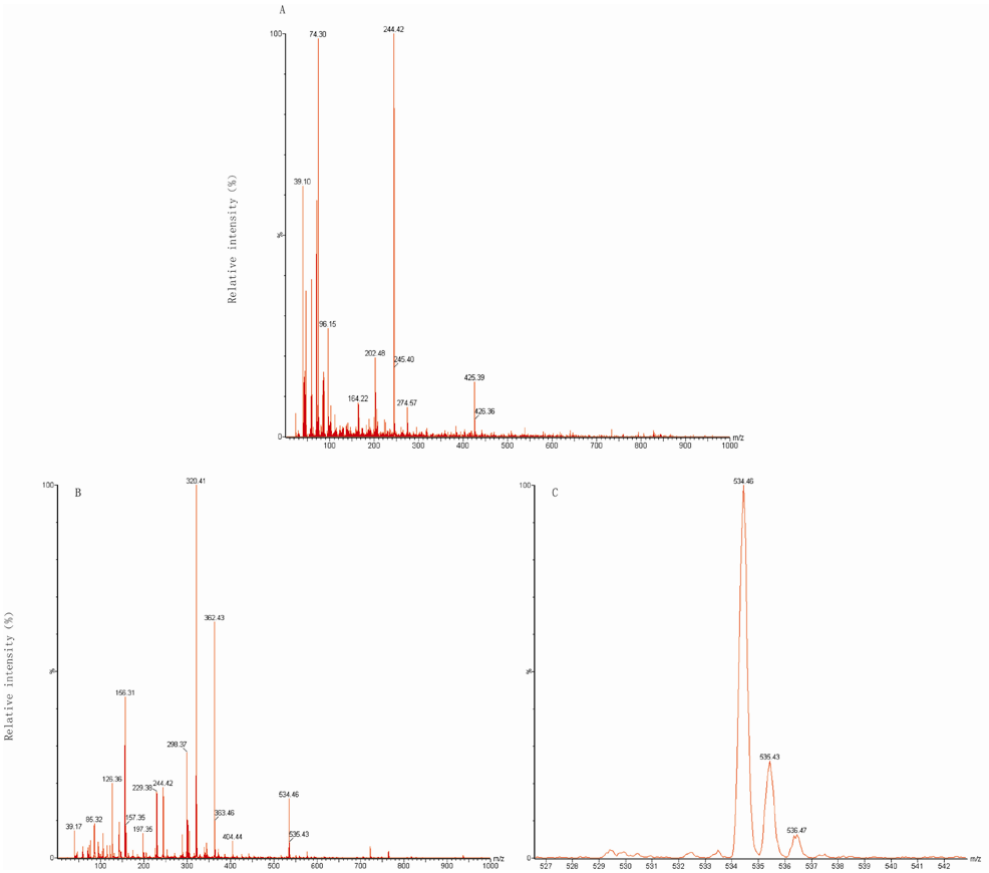


Fig. 3.1.5. The ion of MH^+ of PMPI in DMF with TFA of negative control (A), PMPI-TTX mixture (B) and PMPI-TTX conjugate (C). Each analog was injected into 50 µl/min of aqueous 100% CH_3CN.

MALDI-TOF/MS analysis

PMPI-TTX

The analytics of molecules with low molecular mass (below 800 Da) by MALDI-MS is not generally as established as the determination of larger biological molecules such as peptides or proteins (Persike and Zimmermann *et al.*, 2010). In thisanalysis, one problem is the intense background of matrix-ion signals. Although, significant interference is caused by the CHCA matrix ions (Fig. 3.1.6A), as a result of this accurate mass measurement and high resolution, the analyses were clearly separated from the interfering matrix background as shown in the inset (Fig. 3.1.6B-E). The main peak presence of TTX in results exhibited a molecular mass of 320+1 Da, this is consistent with the theoretical molecular weight of TTX ($C_{11}H_{17}N_3O_8$=319). Additionally, the conjugate PMPI-TTX shown *m/z* 534+1 was similar to the results in the ESI-MS analysis (Fig. 3.1.6C-D). Importantly, in comparison with the different ratios between PMPI and TTX, 20 molar fold excess PMPI to TTX showed the highest peak, suggesting that PMPI:TTX=20:1 is the most applicable in this study.

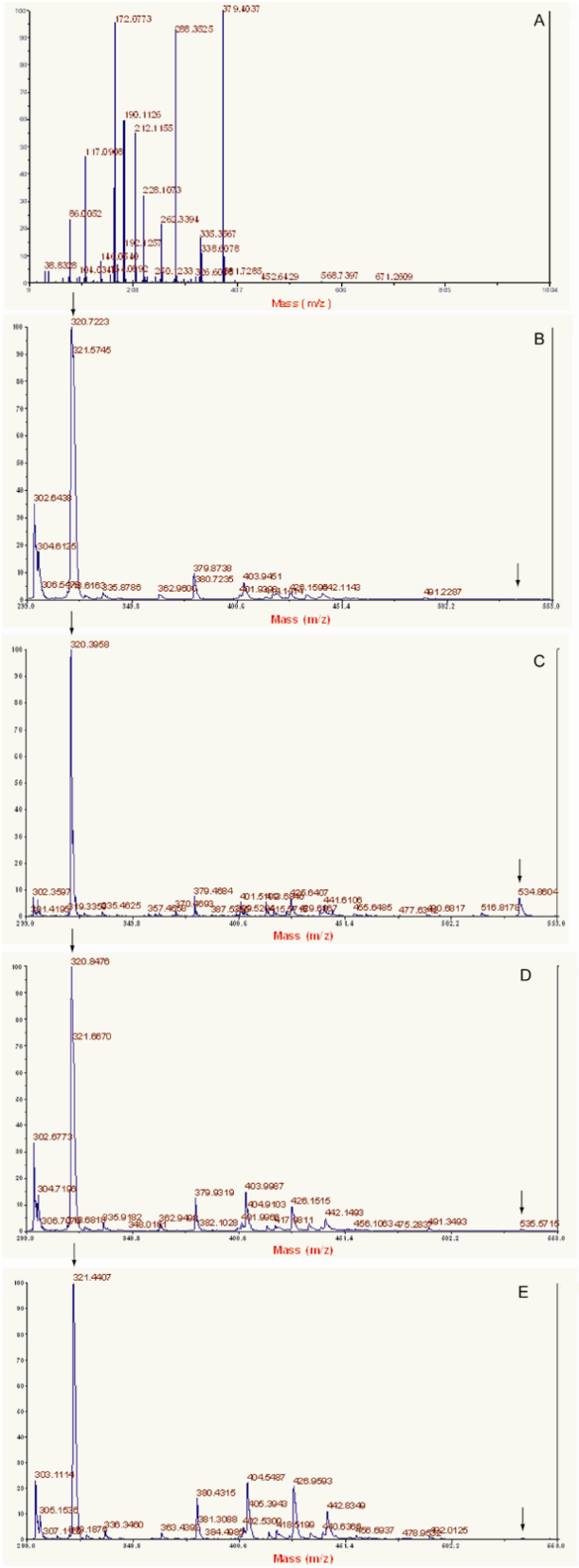


Fig. 3.1.6. MALDI-TOF/MS analysis of TTX and PMPI-TTX from different reaction ratios between PMPI and TTX, (A) 0; matrix CHCA (B) 10:1, (C) 20:1, (D) 50:1 and (E) 100:1. The arrows indicate the spectrum of TTX and PMPI-TTX.

BSA-TTX

The conjugates BSA-TTX, -TDA and -EtOH were analyzed by a MALDI-TOF/MS (Fig. 3.1.7). The hapten density can be determined by measuring the mass difference between the conjugated protein and the native protein. In comparison of the MALDI-TOF/MS results of native BSA with modified BSA, the signals for BSA-TTX and BSA -TDA indicated a hapten loading of only 3 mol TTX or TDA/mol BSA (Fig.3.1.7B-C). For the BSA-EtOH conjugate confirmed a hapten loading of 6 mol EtOH/mol BSA (Fig.3.1.7D). The results suggest that the chemistry (PMPI/SATA) employed for protein modification would be successful. Fox et al. have shown the maximum hapten loading of BSA to be 4 mol gliotoxin/mol BSA obtained by the similar procedure (Fox and Gray *et al.*, 2004). This is similar to the values for the hapten coupling to BSA in the present study, suggesting that the prepared BSA-TTX could be used to selection and characterization of obtained TTX antibodies.

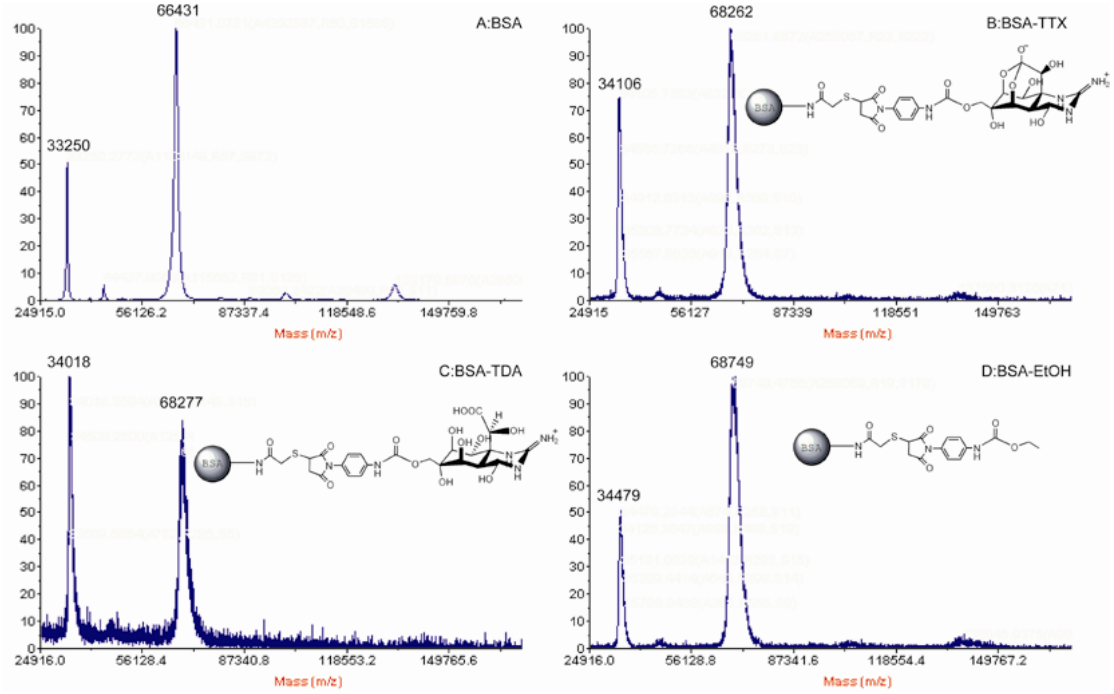


Fig. 3.1.7. MALDI-TOF/MS analysis of (A) BSA, (B) BSA-TTX, (C) BSA-TDA, and (D) BSA-EtOH conjugates.

3.2 Preparation of monoclonal antibodies against tetrodotoxin

3.2.1 Introduction

TTX, is a low molecular weight hapten with no substructure to induce immunoreactions, which makes it necessary to couple TTX to a carrier molecule in order to produce antibodies against TTX. Earlier studies have used a modified formaldehyde method to prepare TTX-protein conjugate immunogens. Although this method can easily conjugate TTX with carrier proteins, the resulting monoclonal antibodies (MAbs) probably have low specificity to free TTX since they do not bind the toxicity active site as described above. In order to improve this problem, MAbs specific to the TTX toxicity active site were produced by the novel derivative of TTX. .

3.2.2 Materials and Methods

Materials and animals

Frozen puffer fish *T. rubripes* ovaries were obtained from Tokyo central wholesale market in Japan.

Female BALB/c mice, 8 weeks old and 12 weeks old, used to immunize and thymectomy, respectively, were obtained from Sankyo Labo Service Corporation (Tokyo, Japan).

Preparation of artificial immunogen

3.5 mg S-acetyl thioglycolic acid N-hydroxysuccinimide (SATA) (Thermo Scientific, Yokohama, Japan) in anhydrous dimethylformamide (DMF) was added to 1 ml of in 0.1 M phosphate reaction buffer (pH 7.2) containing 0.15 M NaCl and 6.0 mg keyhole limpet hemocyanin (KLH) (Sigma-Aldrich Japan, Tokyo, Japan). The solution was mixed gently, allowed to incubate for 30 min at

room temperature, and applied on a NAP-10 column (GE Healthcare Japan, Tokyo, Japan). The resulting SATA-KLH solution was combined with a deacetylation solution (0.5 M hydroxylamine and 0.025 M EDTA in the reaction buffer, pH 7.2) and incubated for 2 h at room temperature before conjugation with PMPI-TTX as described in Section 3.1.2. The extent of SATA incorporation was measured with cysteine hydrochloride monohydrate as a standard. The PMPI-TTX solution was added to the deacetylated BSA-SATA solution (0.1 M phosphate, 0.15 M NaCl and 0.001 M EDTA, pH 7.3). After 2 h incubation at room temperature, the KLH-TTX conjugate was isolated through a desalting column and stored at -20 ℃.

Immunization procedures

The KLH-TTX was dissolved in PBS (-) and the resulting KLH-TTX solution was emulsified with an equal volume of a Freund's complete adjuvant (Difco laboratories Inc., Detroit, USA). Three individuals of 8 week old female BALB/c mice were given 600 μl of the KLH-TTX conjugate intraperitoneally (200 μl of KLH-TTX conjugate per mouse). Booster immunization was administered at one week intervals with the same amount of conjugate emulsified in a Freund' incomplete adjuvant (Difco laboratories Inc., Detroit, USA), and repeated three times. Blood samples were collected from the tails after each boost. The serum was diluted 1:100 with PBS and analyzed by a Western blot for antibody titers.

Cell fusion and cloning

The mouse with the highest polyclonal antibody titer was sacrificed for the following cell fusion procedure. The spleen was aseptically excised out and mashed with a medicine spoon through a

collector tissue sieve, producing a single-cell suspension which was combined with 1×10^7 P3-X63-Ag8.U1 (P3U1) myeloma cells. Cells were fused by incubating in the polyethylene glycol (PEG) 1500. After incubation for 5 min at a 37℃ bead bath, 5 ml of a GIT-BM-HAT medium were slowly added within 5 min. The cells were precipitated by a centrifugation at 1100 rpm and suspended in the medium, and then a 100 μl aliquot was poured into each well of Costar 96-well cell culture plates with mouse thymus cells for feeder cells. The colonies were fed every 7 days with a fresh GIT-BM-HAT medium. ELISA were used for the screening of hybridoma cells producing antibody against TTX approximately 7–14 days after fusion, in which the polystyrene microtiter plates for ELISA were coated with the BSA-TTX conjugate prepared in Section 3.1. BSA or BSA-EtOH was used as a negative control. The conjugates used for cell screening were different from the conjugates used for immunization in order to avoid reactions between the antibody and the carrier protein in the hybridoma selection. 300 wells were tested at the first screening. Each positive well was subcloned by the limiting dilution method into 96-well cell culture plates for two times until the 100% subcloning well was positive. Finally, the cloned cells were transferred into cell culture flasks for mass culture. The supernatant of positive hybridomas was collected for antibody characterizations.

MAb production and purification

The method of MAb purification from cell culture was performed as described in Section 2.2.2.

ELISA analysis

Two 96 well polystyrene plates (Corning Coaster, NY, USA) were coated with 100 μl/well of BSA-TTX and BSA or BSA-EtOH at several concentrations in TBS for 1 h at room temperature. Plates

were then washed three times with TBS containing 0.1% (v/v) Tween-20 (TBS-T). After blocking with 200 µl/well of 5% skim milk or 1% casein in TBS (Thermo Scientific, Tokyo, Japan) overnight at 4 ℃, the wells were washed three times with TBS-T. The anti-TTX antibodies obtained in the present study with 100 µl were added to each well, and plates were incubated at room temperature for 1 h. The wells were washed three times with TBS-T, then 100 µl/well of horseradish peroxidase-conjugated (HRP)-labeled rabbit antimouse IgG+A+M (H+L) conjugate, appropriately diluted with TBS-T was added. After incubation for 1 h at room temperature, the wells were washed five times with TBS-T. 100 µl of the substrate solution of o-phenylenediamine (OPD) (Sigma-Aldrich Japan, Tokyo, Japan) was finally added to each well. After developing the color for about 20 min, the reaction was terminated with 50 µl of 1 M H_2SO_4 and the absorbance values at 492 nm were read in a spectrophotometer.

3.2.2 Results and Discussion

In this study, the KLH was chosen as the carrier protein for immunization as theoretically it should contain a greater number of amino groups available for hapten coupling relative to BSA (Hermanson 1996). Due to the relatively large size (about 800 kDa), KLH conjugates could not be analyzed by MALDI-TOF/MS and the degree of hapten loading could not be determined by SDS-PAGE since limitations in resolution and the large protein size relative to that of the haptenylated form. Although KLH-toxin conjugate could not be readily characterized prior to immunization, 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, an apparent symptom of TTX-poisoning in the subsequent immunization of mouse substantiated the validity of this strategy. Because the toxicity of TTX was retained in the obtained high molecular weight compound, it could be

also used for discovery of the binding proteins of TTX in organisms.

In the 300 wells screened against BSA-TTX using the present purified TTX at the first trial, only about 6% wells had no reaction to the negative control (BSA) and, after cloning, two stable hybridoma cell lines, BD9G6 and CE4B11, producing antibodies against TTX were established.

In the second trial where commercially available TTX powder was used instead for the purified, 94% fusion efficiency was achieved at one week after fusion and 2288 hybrid clones were observed microscopically. Hybridoma supernatants were collected and used for the primary screening of hybrid clones by ELISA (Fig. 3.2.1). Finally, after the third cloning, clones CD2C4, DC10B8 and DC10G5 were obtained.

The subclass of these five clones were showed in Table 4. These MAbs had κ light chains.

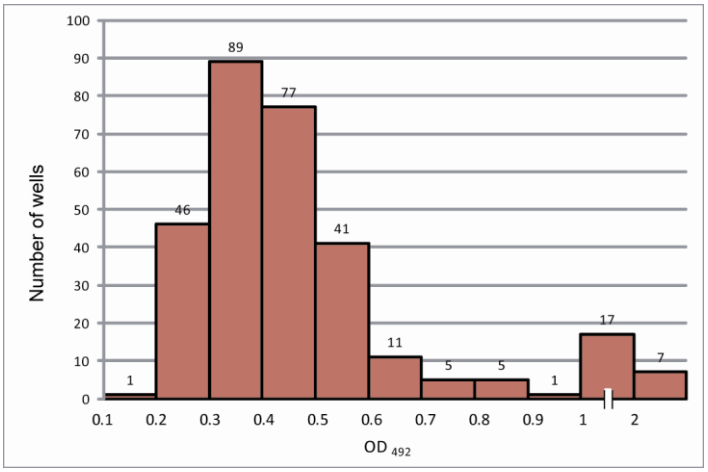


Fig. 3.2.1. Hybridoma cell line of anti-TTX antibodies were detected by ELISA.

Table 4. General survey of developed monoclonal antibodies

Monoclonal antibody	Immunogen	Subclass[a]	Light chain isotype[a]
BD9G6	KLH-TTX	IgG2b	κ
CE4B11	KLH-TTX	IgM	κ
CD2C4	KLH-TTX[b]	IgG1	κ
DC10B8	KLH-TTX[b]	IgG2b	κ
DC10G5	KLH-TTX[b]	IgG2b	κ

a: Subclass and isotypes of the monoclonal antibodies were determined by a mouse monoclonal antibody isotyping kit. b: The conjugated TTX was used commercial TTX powder.

3.3 Characterization of monoclonal antibodies against tetrodotoxin

3.3.1 Introduction

Fukiya and Matsumura developed a polyclonal rabbit anti-TTX antibody capable of passively protecting mice from lethal TTX challenge (Fukiya and Matsumura 1992). Others have reported monoclonal antibodies (MAbs) to tetrodonic acid (Watabe and Sato *et al.*, 1989) and to tetrodotoxin (Zhou and Li *et al.*, 2009; Tao and Wei *et al.*, 2010); however, none of these was prepared against the toxicity-active site of TTX. In this section, the obtained TTX-specific MAbs against toxicity active site of TTX were characterized.

3.3.2 Materials and Methods

Electrophoresis and Western blot analysis

The obtained MAbs were characterized by SDS-PAGE according to Leammli method as the description in section 2.1.2. For Western blot analysis, the proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-PSQ, Merk Japan, Tokyo, Japan). The membrane was blocked overnight with 1% casein in TBS (Thermo Scientific, Tokyo, Japan) overnight at 4 ℃. The membranes were incubated with culture supernatants from selected cell wells or purified MAbs. The Alexa Fluor 680-labeled goat anti-mouse IgM or IgG (1:10,000, Molecular Probes Lnc., USA) was used as the secondary antibody.

Screener blotter analysis

The antigens were electrophoresed on SDS-PAGE gel and transferred onto a PVDF membrane. The

membrane was incubated with TBS containing 1% casein. After washing with TBST, the membrane was placed in a screener blotter apparatus (Screener Blotter Mini 12, Sanplatec, Osaka, Japan). Hybridoma supernatants or purified MAb solutions were co-incubated with the commercial citrate-treated TTX or TTX-protein conjugates. Then the mixtures were applied to each lane and incubated at room temperature for 1 h with shaking. After washed in TBST, the membrane was incubated with goat anti-mouse IgG or IgM (H+L) Alexa Fluor 680. Finally, the reacted bands were visualized using the Odyssey Fc Imaging System (MS Techonosystems, Tokyo, Japan).

Competitive indirect ELISA

Each well was coated with 100 μl of BSA-TTX in TBS (1 μg/ml BSA-TTX) and incubated at room temperature for 1 h. The wells were washed three times with TBST. TBS containing 1 % casein used as the blocking buffer was added to each well, and the plate was incubated at 4 ℃ overnight. After washed three times, 50 μl TTX standards (0, 5, 25, 50, 100, 200, 500 ng) or BSA-TTX (0, 20, 40, 80, 160, 320 ng) were co-incubated in wells with 50 μl of anti-TTX MAb cell culture supernatants (1:500, 1:5,000) for 1 h at 37 ℃. After washed five times, the wells were incubated with 100 μl of rabbit anti-mouse IgG-HRP for 1 h in room temperature. After washed five times, the substrate solution was added to the plate (100 μl/well) and incubated at room temperature for 10 min. The color reaction was terminated with 50 μl of 1 N H_2SO_4 and the OD492nm was measured.

ESI-TOF/MS analysis

In order to confirm whether the obtained MAbs react with free TTX, TTX-MAb complexes were co-isolated with affinity chromatography and analyzed with mass spectrometry. The IgG fractions were

prepared from the hybridoma media by ammonium sulfate precipitation (30-50%) prior to the following steps. Each MAb and TTX mixture was incubated in a binding buffer (pH 7) containing 16 mM ammonium formate and loaded on a protein G column (GE Helthcare Japan, Tokyo, Japan). The column was washed with 10 ml of the binding buffer, followed by the elution with 5 ml of an elution buffer containing 0.1% formic acid. The eluted fractions were subjected to ultrafiltration with a 0.5 unit (Amicon Ultra-0.5 Centrifugal Filter, 50 kDa MWCO, Millipore, Bedford, MA, USA). After lyophilization, the samples were dissolved in 0.1% formic acid. The mass spectra were collected using an AB Sciex TripleTOF™ 5600 System (AB Sciex Japan, Tokyo) with the direct inlet flow rate of 1 μl/min. The declustering potential was set at 150 V and the collision energy was set at 47 V. Scan time was 1 min. The peak areas were calculated by the Peak View program (AB Sciex Japan). The binding buffer containing TTX alone was subjected the same procedures for a negative control. Commercial TTX citrate was also analyzed for a positive control.

3.3.3 Results and Discussion

TTX is one of the most lethal neurotoxins found in widely divergent animal species, including puffer fish, gobies, salamanders, frogs, octopus, shellfish and starfish (Miyazawa and Noguchi 2001). TTX was originally isolated from the puffer fish. It is a toxin stable to cooking or freezing (Watters 1995). Its intoxication in humans usually results from the ingestion of certain puffer fish species, in which high levels of TTX are found in the skin, gonads, liver and intestines. Raw puffer fish, commonly referred to as *fugu*, is regarded as a delicacy in China, Japan and several other countries. Therefore, it is necessary to develop a specific MAb for TTX detection.

Characterization of the MAbs against purified TTX-protein conjugates

In order to determine that the TTX-conjugate was recognized by the MAbs, Western blot analysis was performed (Fig. 3.3.1). Both MAbs CE4B11 and BD9G6 detected the conjugates with an apparent molecular mass around 70 kDa through MALDI-TOF/MS analysis (Section 3.1). On the other hand, the MAbs did not recognize BSA, suggesting that the MAbs could recognize the TTX-PMPI-SATA specifically.

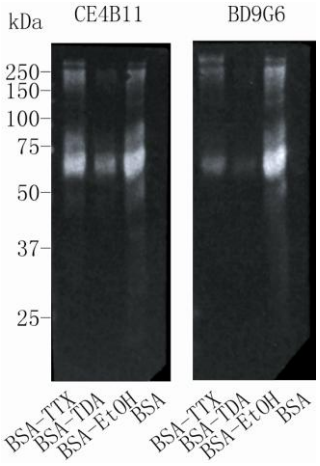


Fig. 3.3.1. Western blot analysis of BSA-TTX, BSA-TDA, BSA-EtOH and BSA using the developed MAb CE4B11 and BD9G6 cell culture supernatants.

To specify the specificity of the MAbs, Western blot analyses of the MAbs obtained in the present study under different TTX concentrations are shown in Fig. 3.3.2. Both of the MAb CEB11 and BD9G6 reacted to BSA-TTX. However, the results show no significant differences among the different TTX concentrations. The results suggest that the MAbs CE4B11 and BD9G6 have higher specificity to PMPI-SATA than TTX.

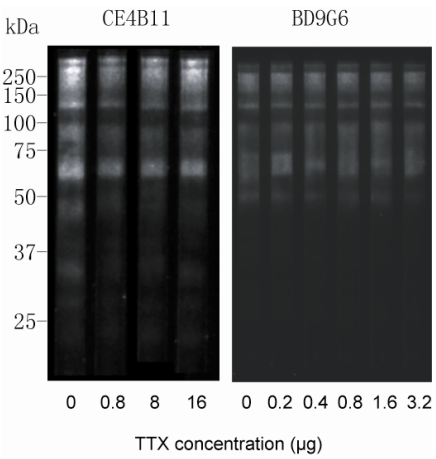


Fig. 3.3.2. Screener blotter analysis of specificity of the developed MAbs against KLH-TTX conjugate. BSA-TTX was prepared with the same method to KLH-TTX. The purified MAb CE4B11 (left panel, 1:300) and BD9G6 (right panel, 1:5,000) co-incubated with commercial TTX citrate were used as primary antibodies.

Characterization of the MAbs against commercial TTX-protein conjugates

All MAbs of the clone CD2 and DC10 were tested by ELISA with BSA-TTX and -EtOH (Fig. 3.3.3). The binding activities of the MAbs to BSA-TTX were extremely higher than to BSA-EtOH. Three of the MAbs (CD2C4, DC10G5, DC10B8) showed relatively higher ratio of BSA-TTX/-EtOH among them.

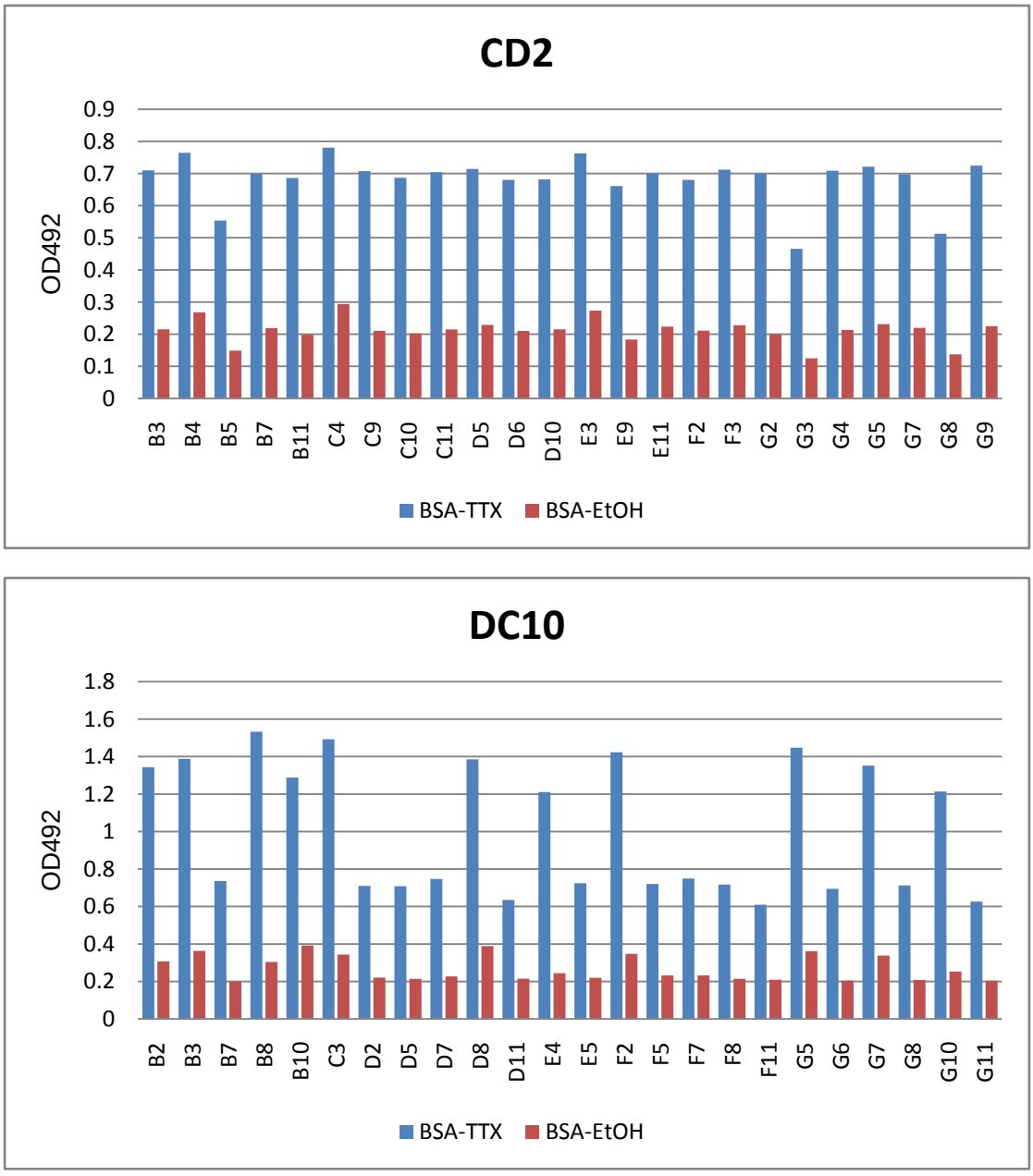


Fig. 3.3.3. ELISA analyses of MAbs against KLH-TTX. The first bar (■) represents BSA-TTX as a positive control, and the second bar (■) represents BSA-EtOH as a negative control. Each well of the ELISA plate was coated with 2 μg of conjugates in 100 μl of TBS.

To confirm the specificity of the MAbs, Western blot assay was performed using the conjugates, BSA-TTX, BSA-TDA and BSA-EtOH. The conjugate BSA-TTX showed a stronger reaction than the others, and the specific reaction bands were found around 70 kDa (Fig. 3.3.4).

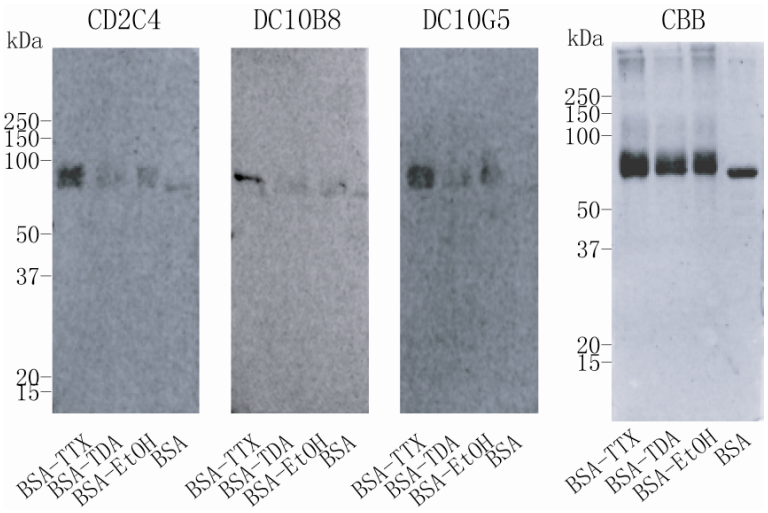


Fig. 3.3.4. Binding of the MAbs CD2C4, DC10B8 and DC10G5 to BSA-TTX, -TDA and EtOH. The protein concentration of each lane was around 1 μg.

The competitive ELISA was performed for the determination of the specificity to TTX of the obtained MAbs. As shown in Fig. 3.3.5 and Fig. 3.3.6, although the MAbs CD2C4, DC10B8 and DC10G5 are more sensitive to BSA-TTX than BSA-EtOH, there are no significant differences in the BSA-TTX/TTX competition assays. The results suggest that the MAbs CD2C4, DC10B8 and DC10G5 could react to TTX, but the specificity of MAbs against TTX were lower than BSA-TTX. In the ESI-TOF/MS analyses, the fractions co-isolated with the MAbs CD2C4 and DC10B8 gave the fragmented ion of 320.1 Da for TTX (Table 5, Fig. 3.3.7), where the corresponding peak areas and intensity were larger and higher than the negative control. These results suggest that the developed MAbs CD2C4 and DC10B8 would bind with free TTX.

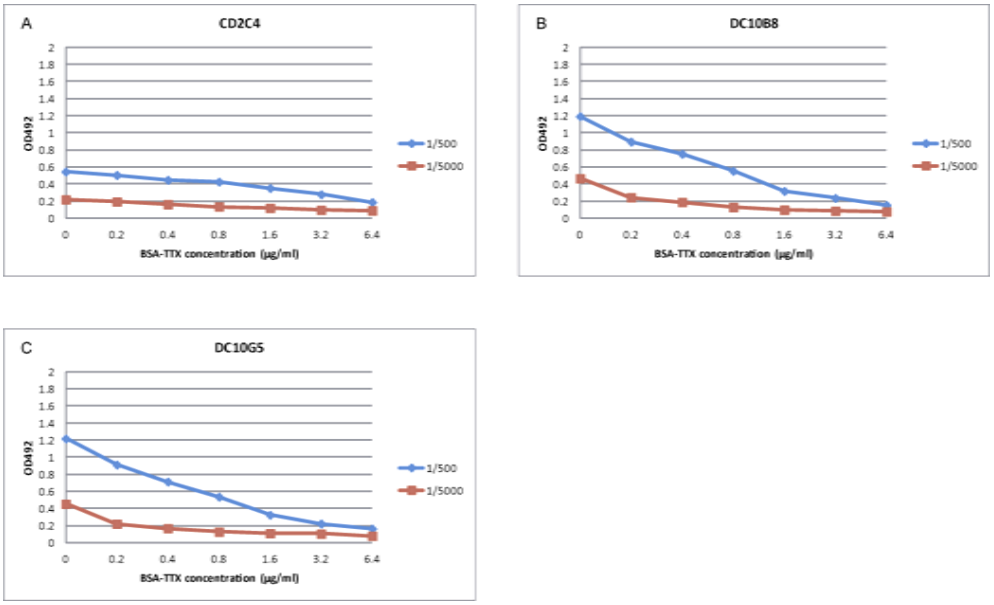


Fig. 3.3.5. Competitive indirect ELISA for determination of specificity between BSA-TTX and BSA-EtOH with the MAbs.

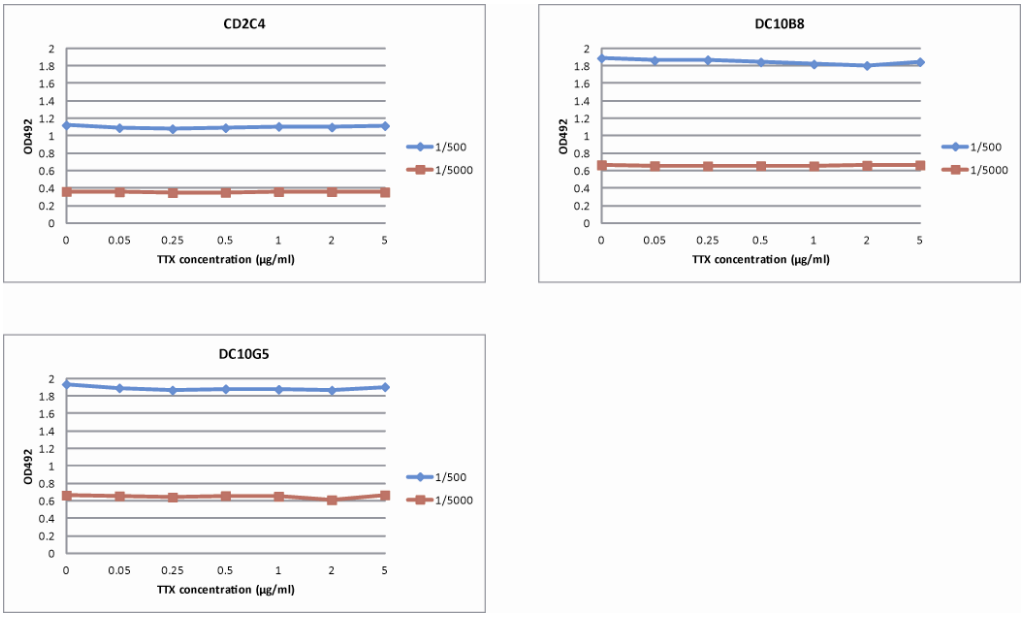


Fig. 3.3.6. Competitive indirect ELISA for determination of specificity between BSA-TTX and TTX with the MAbs.

Table 5 Product ion peak areas for observed the specific binding of the MAb DC10B8 and MAb CD2C4 against TTX

	m/z 162.06	*m/z* 302.09	*m/z* 320.1
0.3 mM TTX citrate	8529.816	6843.685	4723.552
Negative control	621.678	486.958	222.008
MAb DC10B8-TTX	827.120	833.517	462.506
MAb CD2C4-TTX	1080.362	1010.598	535.335

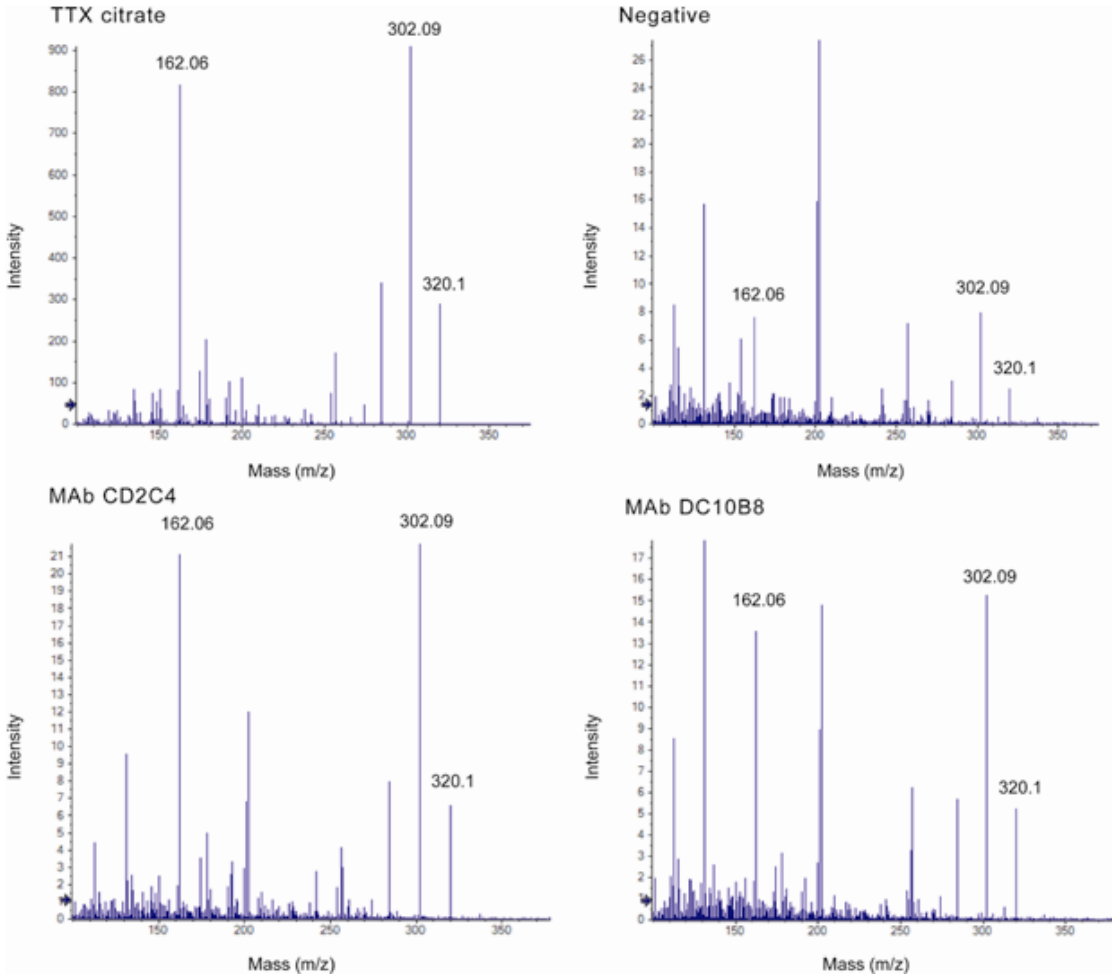


Fig. 3.3.7. TTX product ion mass spectrum of 162.06 Da and 302.09 Da ($M + H^+$).

Chapter 4 General Discussion

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.

Food is essential for our body maintenance, a major source of pleasure, and often intrinsic to our cultural identity. Food allergy is now recognized as a worldwide problem and appears to be on the increase. It is generally synonymous with type-I allergy reactions mediated by immunoglobulin E (IgE) antibodies bound to mast cell, leading to releases of histamine and other chemicals to the tissues, and causing the symptoms such as urticaria, diarrhea and asthma (Daul and Morgan 1993; Burks 2002). During recent decades, the number of patients suffering from food allergy has strikingly increased. Shellfish (crustaceans and mollusks) constitutes an important food source for human. Crustaceans such as shrimps and crabs are however the most frequent cause of food allergy in Japan. Mollusks such as shell and squid also exhibit over 20 % of seafood allergy. Shellfish have been thus known as a major cause of IgE mediated food allergy in adult (Crespo and Rodriguez 2003). Recent studies suggest that allergen-specific monoclonal antibodies (MAbs) are valuable tool in the allergen detection and quantification due to the property that MAb is specific to one particular antigenic structure (Pollart and Smith *et al.,* 1991; Wu and Hsieh *et al.,* 1996; Jeoung and Reese *et al.,* 1997). There are several technical principles for the detection of potential allergens in food products, which target either the allergen itself or a marker indicating the presence of the offending food. Immunological and molecular biological methods are commonly used as inexpensive, sensitive, and highly selective methods for the

detection and quantification of a wide variety of noxious substances (Raybould and Bignami *et al.*, 1992). In the previous studies, the main molecular biological method is polymerase chain reaction (PCR), and immunological methods: enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) biosensor technology and quartz crystal microbalance (QCM) immunosensor. ELISA, including a sandwich ELISA, has been commonly used in the detection and quantification of allergens, but the method needed the immobilization of antigens or antibodies on some solid phases. The recent development and application of optically sensing technologies such as a fluorescence resonance energy transfer (FRET) system, have allowed us to detect the allergen without immobilization procedure. This thesis was then focused to develop rapid or sensitive immunoassays based on the MAbs against major invertebrate allergens to detect and quantify by using a sandwich ELISA and FRET assays.

The major allergen of invertebrate species is a muscle protein called tropomyosin, which has been identified as a major and common protein in crustacean (Shanti and Martin *et al.*, 1993; Daul and Slattery *et al.*, 1994; Leung and Chen *et al.*, 1998), mollusks (Miyazawa and Fukamachi *et al.*, 1996; Chu and Wong *et al.*, 2000; Leung and Chu 2001), arachnids, and insects (Asturias and Gomez-Bayon *et al.*, 1999; Thomas and Smith *et al.*, 2002). Studies on the presence of a heat-stable allergen in shrimp have first identified by Hoffman and Miller (1981) with the molecular mass around 38 kDa. Since this reported, a serial of tropomyosins with a molecular mass ranging of 34-38 kDa have been identified as allergens in different invertebrate species (Shanti and Martin *et al.*, 1993; Leung and Chen *et al.*, 1998; Emoto and Ishizaki *et al.*, 2009). It was also demonstrated that this pan-allergen is a major cross-reactive allergen in invertebrate species (Reese and Ayuso *et al.*, 1999). That suggests that some allergenic structures in tropomyosins are shared by many invertebrate species. In the present study, several MAbs against the sequences of IgE epitope shared by several shellfish tropomyosins were

prepared and characterized. The MAbs BE9, EB11 and DC3 raised against the peptide sequences of T1 EKYKSISDELDQTFAEL and T2 KSISDELDQTFAEL recognized shellfish tropomyosins but also reacted with teleost proteins. The novel MAbs were then raised against the sequences of T3 SISDELDQTFAEL. The MAb CE7B2 obtained in the present study was specifically reactive to the molecular mass around 37 kDa of invertebrate proteins. As described above, tropomyosins were allergic proteins with a molecular mass around 37 kDa in many invertebrate species, suggesting that MAb CE7B2 reacted to the crustacean, mollusks, arthropods and insects tropomyosins, but not to vertebrate tropomyosin.

The Tris-tricine SDS-PAGE results in this study suggest that the MAb CE7B2 not only reacts to intact tropomyosin, but also recognizes small fragments derived from tropomyosins in food products. The MAb CE7B2 thus detects the allergenic tropomyosin from different processed foods. Several anti-tropomyopsin MAbs have been reported (Lu and Oshima *et al.,* 2004; Lu and Ohshima *et al.,* 2007; Sereda and Hartmann *et al.,* 2010), but they reacted to only one type of crustacean, molluscan and filarial tropomyosins. The MAbs were raised against intact tropomyosins as immunogens, suggesting that they may do not detect common IgE epitopes specifically. In general the MAb CE7B2 is not only valuable for detection of invertebrate tropomyosins, but also for detecting the fragmented tropomyosin in product foods.

The present study revealed that the MAb CE7B2 is specific to the invertebrate allergenic tropomyosin by comparison the binding reactivity of IgEs from crustacean allergic patient sera and that the 43 kDa protein was also detected by MAb CE7B2 and sera from shellfish-sensitive patients. This protein might be tropomodulin with a molecular mass around 43 kDa, widely expressed in various species (Fowler 1996). Tropomodulin is specifically associated with actin and tropomyosin at the thin

filament pointed ends where it regulates thin filament length in the striated muscle (Leung and Chu 2001). The results suggest that tropomodulin with a molecular mass 43 kDa is probably a novel allergic protein, thereby further analyses should be carried out for the identification and characterization.

In the present study, a common immunological method, sandwich ELISA, based on the MAb CE7B2 against invertebrate tropomyosin for the quantitative detection of food and indoor allergens was developed. To determine tropomyosin in food, the sandwich ELISA method with the anti-tropomyosin antibodies have been recently developed as shellfish tropomyosin detection kits in Japan. One commercial detection kit, developed against purified tiger prawn tropomyosin, showed the detection limit of 0.4 ng/ml (Shibahara and Oka *et al.,* 2007), and other showed 0.71 ng/ml which raised against the similar tropomyosin (Seiki and Oda *et al.,* 2007). However, these detection kits without IgE epitope specificities might fail to detect allergenic peptides in processed foods. In comparison, the detection limit of the present sandwich ELISA is 0.09 ng/ml for kuruma prawn tropomyosin. The present system is therefore more sensitive than the previous systems. On the other hand, the detection limit of the present sandwich ELISA is 0.64 ng/ml for Japanese flying squid tropomyosin. The coefficient of variation (CV) analyses results suggest that the sandwich ELISA assay is highly reproducible. The results confer the ability to detect mollusk and indoor pan-allergen tropomyosin. In particularly, compared to the previous studies, the present sandwich ELISA could detect both mollusk and dust mite samples. The developed sandwich ELISA based on the MAb is thus useful for its application to the major food allergen tropomyosin of invertebrates. It is considered that the sandwich ELISA is a precise and reliable method to detect tropomyosin in processed food. Indeed, this study provided experimental evidence that this sandwich ELISA is applicable to various commercial processed foods.

FRET is successfully used to quantify molecular dynamics, such as protein-protein interactions,

protein–DNA interactions, and protein conformational changes (Jares-Erijman and Jovin 2003). The advantage of that FRET system is the detection and quantification of the multi-epitope allergens without any immobilization procedure. However, at present there are no validated methods using the FRET system for the detection and quantification of food allergen. In this study, the FRET system was developed based on two tropomyosin IgE epitope specific MAbs, using the fluorescein isothiocyanate (FITC)-conjugated MAb CE7B2 as a fluorescence energy donor and the tetramethylrhodamine isothiocyanate (TRITC)-conjugated MAb 2A7H6 against shellfish tropomyosin as an acceptor were prepared. The fluorescence intensity ratio of the emissions of 510 nm to 555 nm at the excitation of 470 nm was obtained in the presence of several concentrations of tropomyosin. There were gradual increases in the fluorescent intensity ratio of 510 to 550 nm with increasing tropomyosin concentration. The result suggests that the FRET system with multi-antibodies would be useful to detect allergens with multi-epitopes. However, auto-fluorescent disturbed quantification of antigens in processed foods. The FRET system using a longer wavelength fluorophore-conjugated monoclonal antibody pairs would arrow us to detect and quantify tropomyosins and fragments with multiepitopes in processed foods without auto-fluorescent disturbance. On another front, as described in Chapter 1, the necessary condition of causing FRET is not only approach existence but also effective by the direction of donor and acceptor. In the present study, the fluorophores were conjugated to MAbs via amino residues, so that the molecular vectors of fluorophores are not fixed. There is a possibility that the energy transfer efficiency would increase if the fluorophores are rigidly connected with disulfide bond together with the amino residues of antibody.

Immunoassay based on monoclonal antibody and FRET system will be very useful for rapid and sensitive detection of allergen. As well, sandwich ELISA based on monoclonal and polyclonal

antibodies is applicable to quantification and detection allergens. The results presented in this thesis implicate that the antibodies specific to allergen will be a powerful tool for allergenicity assessment, allergen quantification and epitope mapping. As described above, the future technological development of FRET will permit it possible to develop more rapid, more highly sensitive detection and quantification of allergen and the MAb will be a helpful tool for the detection of allergen tropomyosin in a safe food consumption.

Marine biotoxins have received particular attention in a part of food safety. Biotoxins are synthetic substances harmful to human produced by living organisms. They can be produced by many species of organisms such as bacteria, fungi, vertebrates, or microorganisms (Halstead 1959). The gastrointestinal tract and the nervous system of human can be affected by the biotoxins (Lewis and Holmes 1993). One of popular biotoxin is tetrodotoxin (TTX). TTX is a powerful neurotoxin found in several species such as gobies, frogs and shellfish, and most notably puffer fish (Tao and Wei *et al.*, 2010). TTX poisoning often occurs in some countries such as Egypt, Korea, China and Japan. In Asian, it has been reported in many countries including Thailand, Malaysia, Bangladesh, Taiwan, China and particularly Japan (Haque and Islam *et al.*, 2008).

TTX is a stable toxin unaffected by cooking or freezing (Watters 1995). TTX poisoning in human always due to ingestion of improperly handled toxic puffer fish. The toxicity of TTX is caused by it selectively blocking voltage-sensitive Na^+ channels on the surface of the excitable cell membranes causing blood vessel smooth muscles to relax, leading to an impairment of blood circulation. The main causes of death in TTX poisoning are paralysis and respiratory failures (Neagu and Micheli *et al.*, 2006).

As described in general introduction, the guanidinium group and oxygen group have very important

place in TTX toxicity. Generally, an indirect method is used to generate anti-TTX antibodies in earlier studies (Kawatsu and Hamano *et al.,* 1997; Zhou and Li *et al.,* 2009). In order to develop antibodies against TTX, it is necessary to couple TTX to a carrier protein, since TTX is a hapten of a low molecular weight of 319 with no substructure to induce immunoreaction. These TTX-protein conjugate immunogens were all prepared using a modified formaldehyde method (Huot and Armstrong *et al.,* 1989). Although this method can easily conjugate TTX with carrier proteins, the resulting MAbs probably have low specificity to free TTX since they do not bind the toxicity active site. Considering for obtaining a highly specific anti-TTX MAb, the MAbs against a novel derivative of TTX were prepared and characterized. Although some proteins have been reported to be associated with toxification or accumulation of TTX in puffer fish, the biogenesis and toxification of TTX are still unclear (Yotsu-Yamashita and Sugimoto *et al.,* 2001; Yotsu-Yamashita and Okoshi *et al.,* 2013). Because the toxicity of TTX was retained, the novel derivative of TTX could be used for discovery of the TTX binding proteins which protect the toxicity active site, thereby could be used for elucidation of TTX biochemical production and accumulation mechanisms.

Fukiya and Matsumura developed a polyclonal rabbit anti-TTX antibody capable of passively protecting mice from lethal TTX dose (Fukiya and Matsumura 1992). Although others have reported MAbs to tetrodonic acid (Watabe and Sato *et al.,* 1989) and to tetrodotoxin (Zhou and Li *et al.,* 2009; Tao and Wei *et al.,* 2010), none of them was prepared against the toxicity-active site of TTX. In this study, the MAbs against the toxicity-active site of TTX were developed and five MAbs were obtained in twice fusions. However, the MAbs obtained in the first fusion had stronger cross-reaction to PMPI-SATA-activated BSA than TTX. This is probably because the purity of the purified TTX which was used to the immunogen is low. Then MAbs produced in the second fusion showed lower

specificity against TTX than BSA-TTX. The results suggest that the MAbs would probably react with a cross-linker moiety of PMPI-SATA in the BSA-TTX conjugate. The result of LC-TOF/MS, suggests that the developed MAbs CD2C4 and DC10B8 should bind with free TTX. The MAbs obtained by the new synthesis might be used for TTX detection and developing the detection system.

Although TTX was studied for a long time, the therapy for its poisoning has not been established. If TTX was ingested, a series of treatments should be done to keep the patient alive, emptying the stomach, including feeding the patient activated charcoal to bind the toxin, and taking standard life-support procdures, until the toxic effect is removed. Until now, no antidote has been developed or approved. The anti-TTX toxicity site MAbs obtained in the present study may be used for developing the TTX antidote. This article will be first carried out to the effective for reducing lethality in tests on mice.

Food safety is a major worldwide problem, and 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 equipment costs, compared to 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 and generate its antidote. The results presented in this thesis implicate that the developed MAbs and detection methods are could be useful to check the quality during food processing and to ensure the safety and security of food.

References

Aalberse, R. C. (1997). Food allergens. *Environmental Toxicology and Pharmacology.* 4(1-2): 55-60.

Ansorena, P., A. Zuzuarregui, E. Perez-Lorenzo, M. Mujika and S. Arana (2011). Comparative analysis of QCM and SPR techniques for the optimization of immobilization sequences. *Sensors and Actuators B-Chemical.* 155(2): 667-672.

Arruda, L. K. (2005). Cockroach allergens. *Current Allergy and Asthma Reports.* 5(5): 411-416.

Asturias, J. A., N. Gomez-Bayon, M. C. Arilla, A. Martinez, R. Palacios, F. Sanchez-Gascon and J. Martinez (1999). Molecular characterization of American cockroach tropomyosin (Periplaneta americana allergen 7), a cross-reactive allergen. *Journal of Immunology.* 162(7): 4342-4348.

Ayuso, R., S. B. Lehrer and G. Reese (2002). Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *International Archives of Allergy and Immunology.* 127(1): 27-37.

Ayuso, R., G. Reese, S. Leong-Kee, M. Plante and S. B. Lehrer (2002). Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. *International Archives of Allergy and Immunology.* 129(1): 38-48.

Barletta, B., C. Butteroni, E. M. R. Puggioni, P. Iacovacci, C. Afferni, R. Tinghino, R. Ariano, R. C. Panzani, C. Pini and G. Di Felice (2005). Immunological characterization of a recombinant tropomyosin from a new indoor source, *Lepisma saccharina*. *Clinical and Experimental Allergy.* 35(4): 483-489.

Besler, M., H. Steinhut and A. Paschke (2001). Stability of food allergens and allergenicity of processed foods. *Journal of Chromatography B.* 756(1-2): 207-228.

Boquete, M., V. Iraola, M. Morales, H. Pinto, F. Carballada, C. Carballas and J. Carnes (2011). Seafood hypersensitivity in mite sensitized individuals: is tropomyosin the only responsible allergen? *Annals of Allergy Asthma & Immunology.* 106(3): 223-229.

Brown, M. S. and H. S. Mosher (1963). Tarichatoxin: Isolation and Purification. *Science.* 140(356): 295-296.

Bunde, R. L., E. J. Jarvi and J. J. Rosentreter (1998). Piezoelectric quartz crystal biosensors. *Talanta.* 46(6): 1223-1236.

Burks, W. (2002). Current understanding of food allergy. *Genetically Engineered Foods Assessing Potential Allergenicity.* 964: 1-12.

Cao, M. J., K. Hara, K. Osatomi, K. Tachibana, T. Izumi and T. Ishihara (1999). Myofibril-Bound Serine Proteinase (MBP) and its Degradation of Myofibrillar Proteins. *Journal of Food Science.* 64(4): 644-647.

Chau, R., J. A. Kalaitzis and B. A. Neilan (2011). On the origins and biosynthesis of tetrodotoxin. *Aquatic Toxicology.* 104(1-2): 61-72.

Chu, K. H., S. H. Wong and P. S. C. Leung (2000). Tropomyosin is the major mollusk allergen: Reverse transcriptase polymerase chain reaction, expression and IgE reactivity. *Marine Biotechnology.* 2(5): 499-509.

Crespo, J. F. and J. Rodriguez (2003). Food allergy in adulthood. *Allergy & Clinical Immunology International - Journal of the World Allergy Organization.* 58: 98-113.

Daul, C. and J. Morgan (1993). Hypersensitivity reactions to cruscean and mollusks. *Clinical Reviews in Allergy & Immunology.* 11: 201-222.

Daul, C. B., M. Slattery, G. Reese and S. B. Lehrer (1994). Identification of the Major Brown Shrimp (Penaeus-Aztecus) Allergen as the Muscle Protein Tropomyosin. *International Archives of Allergy and Immunology.* 105(1): 49-55.

DeWitt, A. M., L. Mattsson, I. Lauer, G. Reese and J. Lidholm (2004). Recombinant tropomyosin from Penaeus aztecus (rPen a 1) for measurement of specific immunoglobulin E antibodies relevant in food allergy to crustaceans and other invertebrates. *Molecular Nutrition & Food Research.* 48(5): 370-379.

Duncan, P. A. (1983). Captive Behavior of the Short-Tailed Shrew (Blarina-Brevicauda). *Ohio Journal of Science.* 83(2): 4-4.

Emoto, A., S. Ishizaki and K. Shiomi (2009). Tropomyosins in gastropods and bivalves: Identification as major allergens and amino acid sequence features. *Food Chemistry.* 114(2): 634-641.

Forster, T. (1948). Intermolecular energy transfer and fluorescence. *Annalen der Physik (Leipzig).* 2: 55-75.

Fowler, V. M. (1996). Regulation of actin filament length in erythrocytes and striated muscle. *Current Opinion in Cell Biology.* 8(1): 86-96.

Fox, M., G. Gray, K. Kavanagh, C. Lewis and S. Doyle (2004). Detection of Aspergillus fumigatus mycotoxins: immunogen synthesis and immunoassay development. *Journal of Microbiological Methods.* 56(2): 221-230.

Fu, X. Y., C. H. Xue, B. C. Miao, Z. J. Li, W. G. Yang and D. F. Wang (2005). Study of a highly alkaline protease extracted from digestive tract of sea cucumber (Stichopus japonicus). *Food Research International.* 38(3): 323-329.

Fukiya, S. and K. Matsumura (1992). Active and Passive-Immunization for Tetrodotoxin in Mice. *Toxicon.* 30(12): 1631-1634.

Gallart-Ayala, H., E. Moyano and M. T. Galceran (2008). Liquid chromatography/tandem mass spectrometry (highly selective selected reaction monitoring) for the analysis of isopropylthioxanthone in packaged food. *Journal of Chromatography A.* 1208(1-2): 182-188.

Goto, T., Y. Kishi, Takahash.S and Y. Hirata (1965). Tetrodotoxin. *Tetrahedron.* 21(8): 2059-2088.

Greaser, M. L. and J. Gergely (1971). Reconstitution of troponin activity from three protein components. *Journal of Biological Chemistry.* 246(13): 4226-4233.

Halstead, B. W. (1959). Dangerous Marine Animals: That Bite, Sting, Shock, Or Are Non-Edible, Cornell Maritime Press.

Haque, M. A., Q. T. Islam and A. S. Ekram (2008). Puffer Fish Poisoning. *TAJ: Journal of Teachers Association.* 21(2): 199-202.

Hermanson, G. T. (1996). Bioconjugate techniques, Academic press.

Hill, D. J., C. S. Hosking, C. Y. Zhie, R. Leung, K. Baratwidjaja, Y. Iikura, N. Iyngkaran, A. Gonzalez-Andaya, L. B. Wah and K. H. Hsieh (1997). The frequency of food allergy in Australia and Asia. *Environmental Toxicology and Pharmacology.* 4(1-2): 101-110.

Hille, B. (1975). The receptor for tetrodotoxin and saxitoxin. A structural hypothesis. *Biophysical Journal.* 15(6): 615.

Hoffman, D. R., E. D. Day and J. S. Miller (1981). The major heat-stable allergen of shrimp. *Annals of Allergy.* 47(1): 17-22.

Horie, M., S. Kobayashi, N. Shimizu and H. Nakazawa (2002). Determination of tetrodotoxin in puffer-fish by liquid chromatography-electrospray ionization mass spectrometry. *Analyst.* 127(6): 755-759.

Huot, R. I., D. L. Armstrong and T. C. Chanh (1989). Protection against Nerve Toxicity by Monoclonal-Antibodies to the Sodium-Channel Blocker Tetrodotoxin. *Journal of Clinical Investigation.* 83(6): 1821-1826.

Hwang, D.-F. and S.-S. Jeng (1991). Bioassay of tetrodotoxin using ICR mouse strain. *J. Chin. Biochem. Soc.* 20: 80-86.

Hwang, D. F. and T. Noguchi (2007). Tetrodotoxin Poisoning. 52: 141-236.

Ishikawa, M., M. Ishida, K. Shimakura, Y. Nagashima and K. Shiomi (1998). Purification and IgE-binding epitopes of a major allergen in the gastropod Turbo cornutus. *Bioscience Biotechnology and Biochemistry.* 62(7): 1337-1343.

Ishikawa, M., Y. Nagashima and K. Shiomi (1999). Immunological comparison of shellfish allergens by competitive enzyme-linked immunosorbent assay. *Fisheries Science.* 65(4): 592-595.

Ishikawa, M., F. Suzuki, M. Ishida, Y. Nagashima and K. Shiomi (2001). Identification of tropomyosin as a major allergen in the octopus *Octopus vulgaris* and elucidation of its IgE-binding epitopes. *Fisheries Science.* 67(5): 934-942.

Islam, Q., M. Razzak, M. Islam, M. Bari, A. Basher, F. Chowdhury, A. Sayeduzzaman, H. Ahasan, M. Faiz and O. Arakawa (2011). Puffer fish poisoning in Bangladesh: clinical and toxicological results from large outbreaks in 2008. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 105(2): 74-80.

Jares-Erijman, E. A. and T. M. Jovin (2003). FRET imaging. *Nature Biotechnology.* 21(11): 1387-1395.

Jeoung, B. J., G. Reese, P. Hauck, J. B. Oliver, C. B. Daul and S. B. Lehrer (1997). Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA. *Journal of Allergy and Clinical Immunology.* 100(2): 229-234.

Kandyil, R. M. and C. M. Davis (2009). Shellfish allergy in children. *Pediatric Allergy and Immunology.* 20(5): 408-414.

Kao, C. Y. (1986). Structure-Activity Relations of Tetrodotoxin, Saxitoxin, and Analogs. *Annals of the New York Academy of Sciences.* 479: 52-67.

Kao, C. Y. and S. E. Walker (1982). Active Groups of Saxitoxin and Tetrodotoxin as Deduced from Actions of Saxitoxin Analogs on Frog-Muscle and Squid Axon. *Journal of Physiology-London.* 323(Feb): 619-637.

Kawatsu, K., Y. Hamano, T. Yoda, Y. Terano and T. Shibata (1997). Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin (vol 50, pg 133, 1997). *Japanese Journal of Medical Science & Biology.* 50(4-5): 208-208.

Kohler, G. and C. Milstein (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature.* 256: 495-497.

Kostyukova, A. S., S. E. Hitchcock-DeGregori and N. J. Greenfield (2007). Molecular basis of tropomyosin binding to tropomodulin, an actin-capping protein. *Journal of Molecular Biology.* 372(3): 608-618.

Kricka, L. J. (2003). Clinical applications of chemiluminescence. *Analytica Chimica Acta.* 500(1-2): 279-286.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227(5259): 680-685.

Lehman, W. and R. Craig (2008). Tropomyosin and the Steric Mechanism of Muscle Regulation. *Tropomyosin.* 644: 95-109.

Lehrer, S. B., R. Ayuso and G. Reese (2002). Current understanding of food allergens. *Genetically*

Engineered Foods Assessing Potential Allergenicity. 964: 69-85.

Leung, P. S. C., Y. C. Chen, M. E. Gershwin, H. Wong, H. S. Kwan and K. H. Chu (1998). Identification and molecular characterization of Charybdis feriatus tropomyosin, the major crab allergen. *Journal of Allergy and Clinical Immunology.* 102(5): 847-852.

Leung, P. S. C., W. K. Chow, S. Duffey, H. S. Kwan, M. E. Gershwin and K. H. Chu (1996). IgE reactivity against a cross-reactive allergen in crustacea and mollusca: Evidence fev tropomyosin as the common allergen. *Journal of Allergy and Clinical Immunology.* 98(5): 954-961.

Leung, P. S. C. and K. H. Chu (2001). cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster Crassostrea gigas. *Clinical and Experimental Allergy.* 31(8): 1287-1294.

Lewis, R. J. and M. J. Holmes (1993). Origin and transfer of toxins involved in ciguatera. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology.* 106(3): 615-628.

Lin, J. J., G. P. Speranza and H. G. Waddill (1997). Synthesis and reactivity of Mannich-derived polyoxyethylene amines as epoxy curing agents. *Journal of Applied Polymer Science.* 66(12): 2339-2346.

Lipkind, G. M. and H. A. Fozzard (1994). A structural model of the tetrodotoxin and saxitoxin binding site of the Na+ channel. *Biophysical Journal.* 66(1): 1-13.

Lopata, A. L. and S. B. Lehrer (2009). New insights into seafood allergy. *Current Opinion in Allergy and Clinical Immunology.* 9(3): 270-277.

Lopata, A. L., R. E. O'Hehir and S. B. Lehrer (2010). Shellfish allergy. *Clinical and Experimental Allergy.* 40(6): 850-858.

Lopata, A. L., C. Zinn and P. C. Potter (1997). Characteristics of hypersensitivity reactions and identification of a unique 49 kd IgE-binding protein (Hal-m-1) in abalone *(Haliotis midae)*. *Journal of Allergy and Clinical Immunology.* 100(5): 642-648.

Lu, Y., T. Ohshima, H. Ushio, Y. Hamada and K. Shiomi (2007). Immunological characteristics of monoclonal antibodies against shellfish major allergen tropomyosin. *Food Chemistry.* 100(3): 1093-1099.

Lu, Y., T. Oshima, H. Ushio and K. Shiomi (2004). Preparation and characterization of monoclonal antibody against abalone allergen tropomyosin. *Hybridoma and Hybridomics.* 23(6): 357-361.

Miyawaki, A. and R. Y. Tsien (2000). Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Applications of Chimeric Genes and Hybrid Proteins Pt B.* 327: 472-500.

Miyazawa, H., H. Fukamachi, Y. Inagaki, G. Reese, C. B. Daul, S. B. Lehrer, S. Inouye and M. Sakaguchi (1996). Identification of the first major allergen of a squid (Todarodes pacificus). *Journal of Allergy and Clinical Immunology.* 98(5): 948-953.

Miyazawa, K. and T. Noguchi (2001). Distribution and origin of tetrodotoxin. *Journal of Toxicology-Toxin Reviews.* 20(1): 11-33.

Moczydlowski, E. G. (2013). The molecular mystique of tetrodotoxin. *Toxicon.* 63: 165-183.

Moneret-Vautrin, D. A. and M. Morisset (2005). Adult food allergy. *Current Allergy and Asthma Reports.* 5(1): 80-85.

Mosher, H. S. (1986). The Chemistry of Tetrodotoxin. *Annals of the New York Academy of Sciences.* 479: 32-43.

Murphy, K. M. (2012). Janeway's Immunobiology 8th Ed, Garland Science.

Nagashima, Y., J. Maruyama, T. Noguchi and K. Hashimoto (1987). Analysis of paralytic shellfish poison and tetrodotoxin [in shellfish and fish] by ion-pairing high performance liquid chromatography. *Bulletin of the Japanese Society of Scientific Fisheries.* 53.

Nagashima, Y., J. Maruyama, T. Noguchi and K. Hashimoto (1987). Analysis of Paralytic Shellfish Poison and Tetrodotoxin by Ion-Pairing High-Performance Liquid-Chromatography. *Nippon Suisan Gakkaishi.* 53(5): 819-823.

Neagu, D., L. Micheli and G. Palleschi (2006). Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. *Analytical and Bioanalytical Chemistry.* 385(6): 1068-1074.

Nelson, P. N., G. M. Reynolds, E. E. Waldron, E. Ward, K. Giannopoulos and P. G. Murray (2000). Monoclonal antibodies. *Journal of Clinical Pathology-Molecular Pathology.* 53(3): 111-117.

Noguchi, T. and J. S. M. Ebesu (2001). Puffer poisoning: Epidemiology and treatment. *Journal of Toxicology-Toxin Reviews.* 20(1): 1-10.

Noguchi, T. and Y. Mahmud (2001). Current methodologies for detection of tetrodotoxin. *Journal of Toxicology-Toxin Reviews.* 20(1): 35-50.

Ochiai, Y., H. Ozawa, M. C. Huang and S. Watabe (2010). Characterization of two tropomyosin isoforms from the fast skeletal muscle of bluefin tuna Thunnus thynnus orientalis. *Archives of Biochemistry and Biophysics.* 502(2): 96-103.

Oswald, B., M. Gruber, M. Bohmer, F. Lehman, M. Probst and O. S. Wolfbeis (2001). Novel diode laser-compatible fluorophores and their application to single molecule detection, protein labeling and fluorescence resonance energy transfer immunoassay. *Photochemistry and Photobiology.* 74(2): 237-245.

Pawankar, R. (1999). Mast cell function modulating IgE-mediated allergy. *Allergology International.* 48(3): 171-182.

Persike, M., M. Zimmermann, J. Klein and M. Karas (2010). Quantitative determination of acetylcholine and choline in microdialysis samples by MALDI-TOF MS. *Analytical Chemistry.* 82(3): 922-929.

Pollart, S. M., T. F. Smith, E. C. Morris, L. E. Gelber, T. A. E. Plattsmills and M. D. Chapman (1991). Environmental Exposure to Cockroach Allergens - Analysis with Monoclonal Antibody-Based Enzyme Immunoassays. *Journal of Allergy and Clinical Immunology.* 87(2): 505-510.

Poms, R. E., C. L. Klein and E. Anklam (2004). Methods for allergen analysis in food: a review. *Food Additives and Contaminants.* 21(1): 1-31.

Rahman, A. M. A., A. L. Lopata, R. E. O'Hehir, J. J. Robinson, J. H. Banoub and R. J. Helleur (2010). Characterization and de novo sequencing of snow crab tropomyosin enzymatic peptides by both electrospary ionization and matrix-assisted laser desorption ionization QqToF tandem mass spectrometry. *Journal of Mass Spectrometry.* 45(4): 372-381.

Raybould, T. J. G., G. S. Bignami, L. K. Inouye, S. B. Simpson, J. B. Byrnes, P. G. Grothaus and D. C. Vann (1992). A Monoclonal Antibody-Based Immunoassay for Detecting Tetrodotoxin in Biological Samples. *Journal of Clinical Laboratory Analysis.* 6(2): 65-72.

Reese, G., R. Ayuso and S. B. Lehrer (1999). Tropomyosin: An invertebrate pan-allergen. *International Archives of Allergy and Immunology.* 119(4): 247-258.

Reese, G., R. Ayuso, S. Leong-Kee, M. Plante and S. Lehrer (2012). The IgE-binding regions of the major allergen Pen a 1: Multiple epitopes or intramolecular cross-reactivity? *International Archives of Allergy and Immunology.* 124(1-3): 103-106.

Reese, G., R. Ayuso, S. M. Leong-Kee, M. Plante and S. B. Lehrer (2001). The IgE-binding regions of the major allergen Pen a 1: Multiple epitopes or intramolecular cross-reactivity? *International Archives of Allergy and Immunology.* 124(1-3): 103-106.

Roca-Sanjuan, D., M. G. Delcey, I. Navizet, N. Ferre, Y. J. Liu and R. Lindh (2011). Chemiluminescence and Fluorescence States of a Small Model for Coelenteramide and Cypridina Oxyluciferin: A CASSCF/CASPT2 Study. *Journal of Chemical Theory and Computation.* 7(12): 4060-4069.

Sampson, H. A. (1999). Food allergy. Part 1: Immunopathogenesis and clinical disorders. *Journal of Allergy and Clinical Immunology.* 103(5): 717-728.

Sampson, H. A. (2004). Update on food allergy. *Journal of Allergy and Clinical Immunology.* 113(5): 805-819.

Saoudi, M., A. Abdelmouleh and A. El Feki (2010). Tetrodotoxin: a potent marine toxin. *Toxin Reviews.* 29(2): 60-70.

Schagger, H. and G. von Jagow (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry.* 166(2): 368-379.

Seiki, K., H. Oda, H. Yoshioka, S. Sakai, A. Urisu, H. Akiyama and Y. Ohno (2007). A reliable and sensitive immunoassay for the determination of crustacean protein in processed foods. *Journal of Agricultural and Food Chemistry.* 55(23): 9345-9350.

Sereda, M. J., S. Hartmann, D. W. Buttner, R. Volkmer, M. Hovestadt, N. Brattig and R. Lucius (2010). Characterization of the allergen filarial tropomyosin with an invertebrate specific monoclonal antibody. *Acta Tropica.* 116(1): 61-67.

Shanti, K. N., B. M. Martin, S. Nagpal, D. D. Metcalfe and P. V. S. Rao (1993). Identification of tropomyosin as the major shrimp allergen and characterization of Its IgE-binding epitopes. *Journal of Immunology.* 151(10): 5354-5363.

Shibahara, Y., M. Oka, K. Tominaga, T. Ii, M. Umeda, N. Uneo, A. Abe, E. Ohashi, H. Ushio and K. Shiomi (2007). Determination of crustacean allergen in food products by sandwich ELISA. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi.* 54(6): 280-286.

Shoji, Y., M. Yotsu-Yamashita, T. Miyazawa and T. Yasumoto (2001). Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: Liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Analytical Biochemistry.* 290(1): 10-17.

Sicherer, S. H., A. Munoz-Furlong and H. A. Sampson (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology.* 114(1): 159-165.

Sicherer, S. H. and H. A. Sampson (2006). 9. Food allergy. *Journal of Allergy and Clinical Immunology.* 117(2): 470-475.

Smillie, L. B. (1979). Structure and Functions of Tropomyosins from Muscle and Non-Muscle Sources. *Trends in Biochemical Sciences.* 4(7): 151-155.

Steegborn, C. and P. Skladal (1997). Construction and characterization of the direct piezoelectric immunosensor for atrazine operating in solution. *Biosensors & Bioelectronics.* 12(1): 19-27.

Stryer, L. (1978). Fluorescence Energy-Transfer as a Spectroscopic Ruler. *Annual Review of Biochemistry.* 47: 819-846.

Tanu, M. B. and T. Noguchi (1999). Tetrodotoxin as a toxic principle in the horseshoe crab

Carcinoscorpius rotundicauda collected from Bangladesh. *Journal of the Food Hygienic Society of Japan.* 40(6): 426-430.

Tao, J., W. J. Wei, L. Nan, L. H. Lei, H. C. Hui, G. X. Fen, L. Y. Jun, Z. Jing and J. Rong (2010). Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment.* 27(11): 1589-1597.

Taylor, S. L. and S. L. Hefle (2001). Ingredient and labeling issues associated with allergenic foods. *Allergy.* 56: 64-69.

Thomas, W. R., W. A. Smith, B. J. Hales, K. L. Mills and R. M. O'Brien (2002). Characterization and immunobiology of house dust mite allergens. *International Archives of Allergy and Immunology.* 129(1): 1-18.

Tsuda, K., R. Tachikawa, K. Sakai, C. Tamura, O. Amakasu, M. Kawamura and S. Ikuma (1964). On the structure of tetrodotoxin. *Chemical & Pharmaceutical Bulletin.* 12: 642-645.

van Hengel, A. J. (2007). Food allergen detection methods and the challenge to protect food-allergic consumers. *Analytical and Bioanalytical Chemistry.* 389(1): 111-118.

Watabe, S., Y. Sato, M. Nakaya, K. Hashimoto, A. Enomoto, S. Kaminogawa and K. Yamauchi (1989). Monoclonal-Antibody Raised against Tetrodonic Acid, a Derivative of Tetrodotoxin. *Toxicon.* 27(2): 265-268.

Watters, M. R. (1995). Organic Neurotoxins in Seafoods. *Clinical Neurology and Neurosurgery.* 97(2): 119-124.

Woodward, R. (1964). The structure of tetrodotoxin. *Pure Appl. Chem.* 9: 49-74.

Wu, C. H., M. J. Hsieh, J. H. Huang and S. F. Luo (1996). Identification of low molecular weight allergens of American cockroach and production of monoclonal antibodies. *Annals of Allergy Asthma & Immunology.* 76(2): 195-203.

Yakes, B. J., S. M. Etheridge, S. P. Mulvaney and C. R. Tamanaha (2010). Fluidic Force Discrimination Assays: A New Technology for Tetrodotoxin Detection. *Marine Drugs.* 8(3): 565-576.

Yasumoto, T. and T. Michishita (1985). Fluorometric determination of tetrodotoxin by high performance liquid chromatography. *Agricultural and Biological Chemistry.* 49(10): 3077-3080.

Yasumoto, T., M. Nakamura, Y. Oshima and J. Takahata (1982). Construction of a Continuous Tetrodotoxin Analyzer. *Bulletin of the Japanese Society of Scientific Fisheries.* 48(10): 1481-1483.

Yokoo, A. (1950). Study on chemical purification of tetrodotoxin (3)—purification of spheroidine. *J. Chem. Soc. Jpn.* 71(11): 590-592.

Yotsu-Yamashita, M., N. Okoshi, K. Watanabe, N. Araki, H. Yamaki, Y. Shoji and T. Terakawa (2013). Localization of pufferfish saxitoxin and tetrodotoxin binding protein (PSTBP) in the tissues of the pufferfish, *Takifugu pardalis*, analyzed by immunohistochemical staining. *Toxicon.*

Yotsu-Yamashita, M., A. Sugimoto, T. Terakawa, Y. Shoji, T. Miyazawa and T. Yasumoto (2001). Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, Fugu pardalis. *European Journal of Biochemistry.* 268(22): 5937-5946.

Yotsu, M., A. Endo and T. Yasumoto (1989). An Improved Tetrodotoxin Analyzer. *Agricultural and Biological Chemistry.* 53(3): 893-895.

Zheng, J. (2006). Spectroscopy-based quantitative fluorescence resonance energy transfer analysis. Ion Channels, Springer**:** 65-77.

Zhou, Y., Y. S. Li, S. Y. Lu, H. L. Ren, Z. H. Li, Y. Y. Zhang, F. G. Pan, W. S. Liu, J. H. Zhang and Z. S. Liu (2010). Gold nanoparticle probe-based immunoassay as a new tool for tetrodotoxin detection in puffer fish tissues. *Sensors and Actuators B-Chemical.* 146(1): 368-372.

Zhou, Y., Y. S. Li, F. G. Pan, Z. S. Liu and Z. Wang (2009). Identification of tetrodotoxin antigens and a monoclonal antibody. *Food Chemistry.* 112(3): 582-586.

Acknowledgment

During my studying in the Tokyo University of Marine Science and Technology, many people, my supervisor, my classmates, my husband and my parents, have offered me valuable help in my doctor's course.

First and foremost I would like to express my heartfelt gratitude towards my academic supervisor, Prof. Hideki Ushio, who, with his extraordinary patience and consistent encouragement, gave me great help by providing me with necessary materials, advice of great value and inspiration of new ideas. It is his suggestions that draw my attention to a number of deficiencies and make many things clear. Without his strong support, this thesis could not have been the present form. Moreover, he also gives me great help through all of my stay in Japan.

Secondly, I would like to express my heartfelt gratitude to Prof. Kazuo Shiomi for his important and constructive academic advice and for his subtle direction during my study. I would also wish to express my sincere gratitude to Dr. Gen Kaneko for his valuable suggestions and technical guidance for reading this dissertation and offering constructive comments.

I want to emphasize my special thanks towards Dr. Takuya Matsumoto and Yuji Nagashima, who I am truly indebted and thankful for. His guidance in this research was invaluable and by sharing his vast knowledge with me, I learn more than I ever hoped for.

I also want to thank all of the members of the Laboratory of Marine Biochemistry and Laboratory of Aquatic Molecular Biology and Biotechnology for their cooperation and friendly atmosphere.

Finally, in particular, I would like to express my gratitude to my family for their help and support. I am especially thankful for my husband Zhonghua Shen. Without his understanding and financial support it would be impossible for me to accomplish this research. His toleration and encouragement imparted me moving force over my study and life abroad.