

Preparation and Characterization of Monoclonal Antibody Against Abalone Allergen Tropomyosin

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ABSTRACT

Muscle protein tropomyosin is a major and common allergen of mollusks. In order to develop immunoassays based on monoclonal antibody (MAb) for allergen characterization, a MAb against Japanese abalone (*Haliotis discus*) was prepared and characterized in the present study. In comparison with the IgE reactivities of sera from crustacean allergic individuals, the selected MAb AE9F9 showed specific reaction to the abalone allergenic tropomyosin. The MAb AE9F9 reacted to the crustaceans including lobster, crab, and shrimp, but not to the mollusks other than abalone. It was surprising that the MAb AE9F9 was also reactive to the vertebrate chicken tropomyosin and smooth muscle tropomyosin of chicken gizzard. Comparison of amino acid sequences of tropomyosins among abalone, other mollusks, crustaceans, and chicken showed that two regions (90–105 and 147–165) have a high identity among abalone, crustaceans, and chicken, but are polymorphic among mollusks. In the two regions, substitutions at residues 99-Leu, 149-Lys, and 160-Arg of abalone tropomyosin are observed only in mollusks, suggesting that these residues might be important for determining the abalone tropomyosin structure recognized by the AE9F9 antibody. Because of the distinct binding site, the MAb AE9F9 might be useful for abalone allergen detection and epitope mapping of allergen tropomyosin.

INTRODUCTION

SHELLFISH, including crustaceans and mollusks, are important food sources throughout the world. However, shellfish hypersensitivity is among the most common and major causes of food allergy especially in adults, and shellfish allergy has been becoming a serious problem with the increasing rate of shellfish consumption.^(1,2) In general, the ingestion of shellfish would result in symptoms such as urticaria, angioedema, asthma, rhinitis, vomiting, and diarrhea and even shock in severe cases.⁽³⁾

Allergenic components of foods are commonly detected with *in vitro* tests such as enzyme linked immunosorbent assay (ELISA) and radioallergosorbent test (RAST) based on the specific IgE reactivity of patient serum. Because patient serum is comprised of polyclonal antibodies, it may result in false positive values for similar amounts of two allergens or false negative results for the minor allergen of the extract in the methods based on serological analysis.⁽⁴⁾ Consequently, it is necessary to develop other specific methods for allergen detection and quantification. In recent years, because of the high

specificity to one particular antigen, the allergen-specific monoclonal antibodies (MAbs) have been reported to be a useful tool in the field of allergen detection and quantification.^(5,6)

Studies on shellfish allergy showed that muscle protein tropomyosin is a major allergen in crustaceans and mollusks,^(7–12) and tropomyosin is believed to be an invertebrate pan-allergen.⁽¹³⁾ By the reactivity of IgE antibodies, it was shown that common antigenic structures are present in tropomyosins of crustaceans and mollusks.^(14–16) In order to develop immunoassays based on allergen-specific MAbs for mollusk allergen detection and quantification, monoclonal antibody against Japanese abalone (*Haliotis discus*) was prepared and characterized in the present study.

MATERIALS AND METHODS

Materials

Frozen specimens of Japanese abalone (*Haliotis discus*) and king crab (*Paralithodes camtschatica*), fresh Japanese oyster

(*Crassostrea gigas*), octopus (*Octopus vulgaris*), snow crab (*Chionoecetes opilio*), black tiger prawn (*Penaeus monodon*), Chinese prawn (*Penaeus chinensis*), common carp (*Cyprinus carpio*), sardine (*Sardinops melanosticta*), frog (*Rana catesbeiana*), chicken (*Gallus gallus domesticus*) leg muscle, and chicken gizzard were purchased from local suppliers. Patient sera were obtained from three individuals with histories of allergic reactions to both crustaceans and mollusks.

Preparation of immunogen

Edible portions of abalone were minced and mixed with 5 volumes (w/v) of phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). After an overnight extraction at 4°C, extracts were centrifuged at $15,220 \times g$ for 25 min; the supernatant was then precipitated by 30–55% sulfate ammonium to obtain the myofibrillar proteins. The precipitate was dissolved in PBS and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli's method.⁽¹⁷⁾ The proteins were separated by 10% separating gels and stained with coomassie brilliant blue 250R (CBB). Following SDS-PAGE, a gel strip containing the band around 38 k was cut from the CBB stained gel, and the protein was eluted from the gel strips by electrophoresis at 10 mA. The eluate was lyophilized, and then dissolved in PBS and stored at –85°C until use.

Preparation of cooked crustacean and mollusk samples

For crustacean samples, abdominal muscles of shrimp and leg muscles of crab were used. The adductor muscle of oyster and the tentacle muscle of octopus were used for mollusk samples. Because allergens are generally resistant to industrial processing, heating, and cooking, muscles from each species were first homogenized in distilled water (1:3, w/v) and then boiled for 10 min to simulate heat processing. After cooled down to 4°C, all samples were then centrifuged at $15,220 \times g$ for 25 min. The supernatants were separately stored at –20°C.

Preparation of vertebrate myofibrillar protein samples

Carp fast skeletal, sardine fast skeletal, frog fast skeletal, chicken fast skeletal, and chicken gizzard smooth myofibrillar protein samples were prepared by the method as described below. First, muscles were minced and homogenized with the 25 mM Tris-HCl (pH 8.0) buffer containing 20 mM KCl, 1 mM KHCO_3 , 0.1 mM CaCl_2 and 0.1 mM dithiothreitol (1:5, w/v) at 4°C. Following centrifugation at $5000 \times g$ for 15 min, the precipitates were extracted in the same buffer for 10 min and then recentrifuged. Extraction and centrifugation procedures were repeated three times to remove the soluble sarcoplasmic proteins. Finally, the suspensions were individually filtered with a layer of gauze in order to obtain small myofibrillar fragments. The filtrates were stored at –20°C until use.

Immunization and generation of monoclonal antibody

BALB/cA mice (female, 6 weeks, Crea, Tokyo, Japan) were immunized intraperitoneally with 100 μL of emulsions of im-

munogen (200 $\mu\text{g/mL}$) with complete and incomplete Freund's adjuvants on the 0 day and the 14th day, respectively. Twenty micrograms of the immunogen in PBS was intravenously injected on the 21st day. Three days after the intravenous injection, splenic lymphocytes were fused to murine myeloma cells (P3-X63-Ag8.U1) in the presence of polyethylene glycol (PEG) 1500 (Roche Diagnostics, Tokyo, Japan). The fused cells were first cultured in a GIT medium (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) containing 10% BM-conditioned H1 (Roche Diagnostics) and 1% hypoxanthine, aminopterin, thymidine supplement (HAT; Roche Diagnostics). The obtained hybridomas were screened by enzyme-linked immunosorbent assay as described below and cloned by the limiting dilution method.⁽¹⁸⁾ Isotypes of the obtained monoclonal antibodies were determined by a mouse monoclonal antibody isotyping kit (Roche Diagnostics).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was carried out to screen the hybridoma cell lines. The abalone myofibrillar protein sample obtained from the 30–55% sulfate ammonium suspension and the oyster sample from cooked water were used as antigens to select hybridomas. Briefly, 100 μL of antigen extracts were coated onto a flat-bottom microtiter plate (Costar®, Corning Inc., Tokyo, Japan) for 1 h at room temperature or overnight at 4°C. After washing four times with Tris-

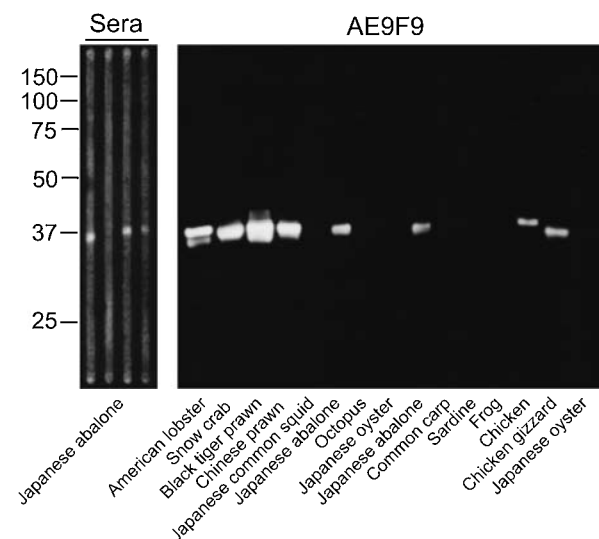


FIG. 1. Evaluation of the allergenic components of abalone extracts (left panel) and the cross-reactivity of MAb AE9F9 (right panel) to different crustaceans, mollusks, and vertebrates. The proteins were separated by precast 12.5% separating gels and transferred to polyvinylidene difluoride membranes. The sera (1:100) from crustacean allergic patients and culture supernatant (1:5) from AE9F9 clone were used as primary antibodies; and the HRP-conjugated anti-human IgE (1:3000) and anti-mouse IgM, IgG, IgA (H+L) (1:10000) were used as secondary antibodies, respectively. The proteins were visualized with the SuperSignal® West Pico chemiluminescent substrate and Polaroid film.

TABLE 1. COMPARISON OF TROPOMYOSIN SEQUENCES AT REGIONS 90-105 AND 147-165 AMONG MOLLUSKS, CRUSTACEANS, AND CHICKEN

Origin	Name	Amino acid residues						
		90	*	105	147	*	*	165
Mollusk	Hal d l	R-K-I-T-L-L-E-E-D-L-E-R-N-E-E-R			Q-L-K-E-A-K-Y-I-A-E-D-A-E-R-K-Y-D-E-A			
Abalone	Hal r l	-----	-----	-----	-----	-----	-----	-----
Abalone	Hal a l	-----	-----	-----	-----	-----	-----	-----
Mussel	Per v l	-----S-----	-----G-I-M-K-A-----	-----	-----T-----W-----E-D-K-----E-----	-----	-----	-----
Oyster	Cra g l	--R--Q-----	--M-----S-----	-----	--T-----L-----E-----K-----	-----	-----	-----
Scallop	Mim n l	--R-L-----	--Y-----S-----	-K	--E-T-----N-V-----T-----D-H-----F-----	-----	-----	-----
Crustacean								
Crab	Cha f l	--R--Q-----	-----S-----	-----	-----R-F-L-----E-D-----V	-----	-----	-----
Shrimp	Met e l	--R--Q-----	-----S-----	-----	-----R-F-L-----E-D-----V	-----	-----	-----
Lobster	Hom a l	--R--Q-----	-----S-----	-----	-----R-F-L-----E-D-----V	-----	-----	-----
Chicken	α -Tropomyosin	--R--Q---V---E---D---A-Q---	-----	-----	-----H-----E-D-----E-----V	-----	-----	-----

The above tropomyosin sequences are from GenBank database. The sequences at regions 90-105 and 147-165 of tropomyosins from three abalone species of *Haliotis diversicolor* (accession number AF216518), *Haliotis asinina* (AY320360), and *Haliotis rufescens* (X75218) were compared with the corresponding sequences from mussle *Perna viridis* (AF216519), oyster *Crassostrea gigas* (AF239173), scallop *Mimachiamys nobilis* (AF216520), crab *Charybdis furiatus* (AF061783), lobster *Homarus americanus* (AF034954), shrimp *Metapenaeus ensis* (U08008), and chicken *Gallus gallus* (α -tropomyosin, M32441).

buffered saline (TBS; Takara Bio Inc., Otsu, Shiga, Japan) containing 0.05% Tween 20 (TBS-T), the wells were then blocked with TBS containing 5% skim milk at ambient temperature for 1 h. One hundred microliters of the supernatants from cell lines were added and incubated at room temperature for 1 h. After washing four times with TBS-T, the wells were reacted with alkaline phosphatase conjugated goat anti-mouse IgM, IgG, IgA (H + L) secondary antibody (Cosmo Bio Co., Ltd., Tokyo, Japan) for 1 h. Finally, 100 μ L of freshly made substrate solution of *p*-nitrophenyl phosphate (Sigma-Aldrich Japan, Tokyo, Japan) was added for detection, and 50 μ L of 3 M NaOH was added to stop the enzyme reaction. The absorbance at 410 nm was measured on a densitometer (CS-9300 PC, Shimadzu Co., Kyoto, Japan) equipped with a multi-well apparatus for 96-well plates.

Chemiluminescence immunoassay

The chemiluminescence immunoassay was used to evaluate the allergenic components in abalone extract and to characterize the cross-reactivity of the MAb AE9F9. The cooked crustacean and mollusk water extracts and vertebrate myofibrillar protein samples were used for antibody characterization. Protein samples were first applied to SDS-PAGE using a precast 12.5% separating gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) by the Laemmli method.⁽¹⁷⁾ The separated proteins were then blotted onto a polyvinylidene difluoride membrane (Millipore Co., Japan, Tokyo, Japan) as described by Towbin et al.⁽¹⁹⁾ The membrane was blocked with TBS containing 20 mg/mL bovine serum albumin for 1.5 h for allergenic protein assay using patient sera or alternatively with TBS containing 5% skim milk for 1 h for antibody characterization. The membrane was then incubated for 1 h with optimum dilution of patient sera for allergenic protein assay or with culture supernatant from AE9F9 cell line for antibody characterization. The immunodetection was performed through incubation with rabbit horseradish peroxidase (HRP) labelled anti-human IgE or goat HRP-labelled anti-mouse IgM, IgG, IgA (H+L) secondary antibody for 1 h, followed by the detection with SuperSignal® West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The muscle protein tropomyosin has been proved to be a major and common allergen in mollusks.⁽²⁰⁾ In the present study, monoclonal antibodies against abalone were generated and characterized. For the hybridomas generated against abalone immunogen, about 40% wells (308 wells in total) showed positive responses to the abalone antigen in the first ELISA screening. It was reported that an allergenic protein around 38 k, supposed as tropomyosin, was present in oyster (*Crassostrea gigas*) and abalone (*Haliotis midae*).^(12,21) Although the oyster antigen was used to screen as well as the abalone antigen, it was difficult to retrieve the cell lines showing positive responses to both abalone and oyster antigens. This might be due to the polymorphism of the amino acid sequences in mollusk tropomyosins. Four hybridoma clones with positive responses

to abalone antigen were selected for the subsequent screening and cloning procedures. Among them, the MAb secreted by AE9F9 clone identified as an IgM subtype with κ light chain was further characterized by immunoblot assay.

The MAb AE9F9 was reactive to proteins with a molecular weight around 38 k from abalone and crustaceans (lobster, crab, and shrimp), and this 38 k protein was proven to be an allergenic component of abalone extract by the IgE reactivities of patient sera (Fig. 1). It was reported that an allergenic protein with a molecular weight of about 38 k was identified in South African abalone (*Haliotis midae*) and Australian abalone (*Haliotis rubra*);⁽²¹⁾ the 38 k allergenic protein has been proven to be tropomyosin and to be a common allergen in different crustaceans and mollusks.^(16,20,22) Consequently, we concluded that the MAb AE9F9 was specific to the abalone allergen tropomyosin and that this AE9F9 antibody would probably recognize the tropomyosin antigenic structures shared by abalone and crustaceans.

It was reported that shrimp allergen tropomyosin *Pen* a 1-specific MAbs and IgE of shrimp-allergic subjects did not bind to the vertebrate tropomyosins including beef, pork, rabbit, and chicken.⁽²³⁾ However, the MAb AE9F9 obtained in the present study reacted to tropomyosins of chicken and chicken gizzard, but not to those of other vertebrates (carp, sardine, and frog) and other mollusks (squid, oyster, and octopus) (Fig. 1). To assess whether the epitope shared by abalone, crustaceans, and chicken potentially exists, the amino acid sequences of tropomyosins from three abalone species (*Haliotis diversicolor*, *Haliotis asinina*, and *Haliotis rufescens*) were compared with those from other mollusks (mussel *Perna viridis*, oyster *Crassostrea gigas*, and scallop *Mimachiamys nobilis*), crustaceans (crab *Charybdis feriatus*, shrimp *Metapenaeus ensis*, and lobster *Homarus americanus*), and chicken *Gallus gallus* (α -tropomyosin) using the GenBank database. By comparing the amino acid sequences of these tropomyosins, two regions (90–105 and 147–165) with a high identity among abalone, crustaceans, and chicken, but polymorphism among mollusk species were found as listed in Table 1. The sequences at these two regions were observed with 100% identity among listed abalone species, suggesting that these two regions are monomorphic in abalone species. However, when they were compared to the corresponding sequences of other species, variations at residues 99-Leu, 149-Lys, and 160-Arg of abalone tropomyosins were observed only in mollusks (mussel, oyster, and scallop), while alteration at residue 153-Tyr was observed in all listed species. The residue 153-Phe previously reported to be critical for the allergenicity of shrimp *Pen* i 1⁽⁷⁾ is answered in crustacean allergen tropomyosins. Thus, the residue 153-Phe would be relatively important for the allergenic structure among crustaceans, and the three residues (99-Leu, 149-Lys, and 160-Arg) might play an important role in determining the abalone tropomyosin structure recognized by the AE9F9 antibody.

In the present study, amino acid sequences of *H. diversicolor*, *H. asinina*, and *H. rufescens* now available were used for the assessment of epitopes. Further studies are necessary to identify the epitope recognized by the MAb AE9F9 raised against *H. discus*. Because of its unique profiles, the MAb AE9F9 might be applied to abalone allergen detection or epitope mapping.

REFERENCES

- Hefle SL, Nordlee JA, and Taylor SL: Allergenic foods. *Crit Rev Food Sci* 1996;36:S69–S89.
- Crespo JF, and Rodriguez J: Food allergy in adulthood. *Allergy* 2003;58:98–113.
- Daul CB, Morgan JE, and Lehrer SB: Hypersensitivity reactions to crustacea and mollusks. *Clin Rev Allergy* 1993;11:201–222.
- Poulsen LK: In vivo and in vitro techniques to determine the biological activity of food allergens. *J Chromatogr B* 2001;756:41–55.
- Jeoung BJ, Reese G, Hauck P, Oliver JB, Daul CB, and Lehrer SB: Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA. *J Allergy Clin Immunol* 1997;100:229–234.
- Portnoy J, Brothers D, Pacheco F, Landuyt J, and Barnes C: Monoclonal antibody-based assay for Alt a 1, a major *Alternaria* allergen. *Ann Allergy Asthma Immunol* 1998;81:59–64.
- Shanti KN, Martin BM, Nagpal S, Metcalfe DD, and Subba Rao PV: Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* 1993;151:5354–5363.
- Daul CB, Slattery M, Reese G, and Lehrer SB: Identification of the major brown shrimp (*Penaeus aztecus*) as the muscle protein tropomyosin. *Int Arch Allergy Clin Immunol* 1994;105:49–55.
- Leung PSC, Chen YC, Mykles DL, Chow WK, Li CP, and Chu KH: Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Mol Mar Biol Biotechnol* 1998;7:12–20.
- Leung PSC, Chen YC, Gershwin ME, Wong SH, Kwan HS, and Chu KH: Identification and molecular characterization of *Charybdis feriatius* tropomyosin, the major crab allergen. *J Allergy Clin Immunol* 1998;102:847–852.
- Miyazawa H, Fukamachi H, Inagaki Y, Reese G, Daul CB, Lehrer SB, Inouye S, and Sakaguchi M: Identification of the first major allergen of a squid (*Todarodes pacificus*). *J Allergy Clin Immunol* 1996;98:948–953.
- Leung PSC, and Chu KH: cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster *Crassostrea gigas*. *Clin Exp Allergy* 2001;31:1287–1294.
- Reese G, Ayuso R, and Lehrer SB: Tropomyosin: an invertebrate pan-allergen. *Int Arch Allergy Immunol* 1999;119:247–258.
- Lehrer SB, and McCants ML: Reactivity of IgE antibodies with crustacea and oyster allergens: evidence for common antigenic structures. *J Allergy Clin Immunol* 1987;80:133–139.
- Halmepuro L, Salvaggio JE, and Lehrer SB: Crawfish and lobster allergens: identification and structural similarities with other crustacea. *Int Arch Allergy Appl Immun* 1987;84:165–172.
- Leung PSC, Chow WK, Duffey S, Kwan HS, Gershwin ME, and Chu KH: IgE reactivity against a cross-reactive allergen in crustacea and mollusca: evidence for tropomyosin as the common allergen. *J Allergy Clin Immunol* 1996;98:954–961.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- Köhler G, and Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495–497.
- Towbin H, Staehelin T, and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354.
- Chu KH, Wong SH, and Leung PSC: Tropomyosin is the major mollusk allergen: reverse transcriptase polymerase chain reaction, expression and IgE reactivity. *Mar Biotechnol* 2000;2:499–509.
- Lopata AL, Zinn C, and Potter PC: Characterization of hypersensitivity reactions and identification of a unique 49 kd IgE binding protein (Hal-m-1) in abalone (*Haliotis midae*). *J Allergy Clin Immunol* 1997;100:642–648.
- Leung PSC, and Chu KH: Molecular and immunological characterization of shellfish allergens. *Front Biosci* 1998;3:d306–312.
- Reese G, Tracey D, Daul CB, and Lehrer SB: IgE and monoclonal antibody reactivities to the major shrimp allergen Pen a 1 (tropomyosin) and vertebrate tropomyosins. *Adv Exp Med Biol* 1996;409:225–230.

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