

Allergen Analysis of Sea Urchin Roe Using Sera from Five Patients

Kenichi Tanaka^a Yasuto Kondo^a Chisato Inuo^b Yoichi Nakajima^a Ikuya Tsuge^b
Satoru Doi^c Shigeto Yanagihara^d Tetsushi Yoshikawa^b Atsuo Urisu^a

^aDepartment of Pediatrics, The Second Teaching Hospital, Fujita Health University, Nagoya, ^bDepartment of Pediatrics, School of Medicine, Fujita Health University, Toyoake, ^cHekinan Municipal Hospital, Hekinan, and ^dDepartment of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan

Key Words

Sea urchin · Allergen analysis · Major yolk protein · Egg cortical vesicle protein · Salmon roe · Cross reactivity

Abstract

Background: Sea urchin roe can cause anaphylactic reactions the first time they are consumed; therefore, careful clinical attention should be paid to their effects. However, no previous study has examined the allergens in sea urchin roe using sera from more than one patient. We attempted to identify sea urchin allergens using sera from 5 patients with sea urchin allergies. **Methods:** We enrolled 5 patients with relevant medical histories, positive results on a skin prick test and/or a food challenge test, and high levels of sea urchin-specific IgE in an enzyme-linked immunosorbent assay. We performed SDS-PAGE, immunoblotting, immunoblot inhibition, and N-terminal amino acid sequence detection. **Results:** Ten protein bands ranging from 18 to 170 kDa were detected in more than 2 patients' sera. In immunoblotting, the protein band for the 170-kDa major yolk protein was recognized by 4 of the 5 sera. Furthermore, the reaction between IgE and the protein band for egg cortical vesicle protein (18 kDa) was inhibited by the addition of salmon roe extract. **Conclusion:** Major yolk protein was confirmed to be

one of the main allergens in sea urchin roe. In addition, egg cortical vesicle protein (18 kDa) was demonstrated to be an important protein for cross-reactivity with salmon roe.

© 2014 S. Karger AG, Basel

Introduction

The incidence of marine-product allergies is considered to be 0.6% in children and 2.8% in adults [1]. While the overwhelming majority of cases are caused by shellfish including shrimps and crabs, there have been a few reports of allergies caused by other species.

Sea urchin allergies are quite rare. Two groups of researchers independently produced the first reports of patients with sea urchin allergies; i.e. Hickey [2] produced a case report about a patient who suffered an anaphylactic reaction after consuming sea urchin roe, and Rodriguez et al. [3] reported an analysis of the allergens of sea urchin roe which suggested that a 118-kDa protein might be a sea urchin allergen. Later, Yamasaki et al. [4] revealed that the 152-kDa major yolk protein (MYP) is the main allergen in sea urchins. All of the subjects in these studies developed anaphylactic reactions after eating sea urchin roe, which indicates the clinical significance of research into this top-

ic. However, none of these studies involved more than one subject. We collected sera from 5 patients with allergies to sea urchins and analyzed the IgE reactivity to sea urchin roe proteins.

Methods

Subjects

We diagnosed sea urchin allergy in a comprehensive manner, i.e. on the basis of: an obvious medical history, including clear symptoms that appeared within a few hours of the sea urchin roe being eaten; a positive skin prick test (SPT) result based on the sea urchin itself or its extract, and the detection of high levels of sea urchin-specific IgE via an enzyme-linked immunosorbent assay (ELISA). In one case, we received patient consent to perform an oral challenge test. We obtained approval for this study in advance from the research ethics committee of our university (10-216), and we obtained oral informed consent from all of the subjects.

Skin Prick Test

We conducted SPT on patients 2, 3, and 4 with a standard blood lancet [5] using normal saline as a negative control and 10 mg/ml of histamine as a positive control. The material used for the SPT in patients 2, 3, and 4 was protein extract, and in patients 1 and 5 it was fresh roe. With this test, we measured the diameter of all wheals that appeared 10–15 min after pricking. A positive result was defined as a wheal diameter that exceeded 50% of that of the positive control or measured ≥ 3 mm. The subjects stopped taking antihistamine agents 7 days before the SPT.

Oral Food Challenge Test

An allergist carefully performed an oral food challenge test via an open challenge. The material was fresh sea urchin roe purchased from a nearby store.

Sea Urchin Extract

We extracted raw proteins from the roe of *Strongylocentrotus nudus* by homogenizing the roe with 15 ml of 1 M KCL-phosphate-buffered saline (PBS) at pH 7.2, separated the supernatant from the resultant suspension by centrifugation, and then dialyzed it using dialysis tubing (molecular weight cut-off values: 6,000–8,000). The extract was then vacuum freeze-dried. The protein concentration of the extract was determined using a Pierce[®] bicinchoninic acid protein assay kit.

In addition, we prepared antigen extract from salmon roe to examine cross-reactivity according to the procedures outlined in a previous study by Kondo et al. [6] and vacuum freeze-dried it.

Enzyme-Linked Immunosorbent Assay

We dissolved the freeze-dried extract in PBS buffer (0.1 mg/ml) and then added 0.1 ml samples to each well of a Nunc-Immuno[™] Plate I (Nunc A/S, Roskilde, Denmark), which was then incubated for 1.5 h at room temperature. After discarding the extract solution, we added 0.15 ml of SuperBlock blocking buffer (Pierce, Rockford, Ill., USA) to each well and incubated the mixture overnight at 4°C. Then, we washed each well with 0.2 ml of 0.05% PBS-Tween and added 0.1 ml of the subject's serum diluted in Super-

Block blocking buffer at a ratio of 1:5 before incubating the plates overnight at room temperature. After washing the plates with PBS-Tween, we added 0.1 ml of goat anti-human IgE BIOT (1:1,000; Vector Laboratories, Inc., Burlingame, Calif., USA) to each well and incubated the plates for an hour at room temperature. Then, we washed the wells and added 0.1 ml of streptavidin-horseradish peroxidase (1:5,000; Southern Biotechnology Associates, Birmingham, Ala., USA) to each well and incubated them for an hour at room temperature. After washing the plates, we added 0.1 ml of 3,3',5,5'-tetramethylbenzidine (ICN Biomedicals, Aurora, Ohio, USA) to each well and then incubated the plates for 30 min under a light shield before the reactions were stopped by the addition of 0.1 ml of 1 N HCl to each well. The results were measured using LSPLATE manager 2001 (Wako, Osaka, Japan) and the optical density at 450 nm. The mean optical density value of 5 negative control sera was standard. The serum of a person with no food allergies was used as a negative control.

ELISA Inhibition

Before addition to the ELISA plate which was precoated with extract of sea urchin roe or salmon roe, sera were preincubated with solutions containing extracts (sea urchin roe or salmon roe) of 3 different concentrations (0.01, 0.1, and 1 mg/ml) as inhibitors at room temperature. The subsequent procedure was the same as that for ELISA described above.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

We carried out sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method [7]. After preparing 0.1 mg/ml of sea urchin roe extract solution from vacuum freeze-dried powder and centrifuging it, we electrophoresed the solution using 4–20% TRIS-Glycine Mini-Gel (Novex[®], Carlsbad, Calif., USA) under reducing conditions. We used 3-(N-morpholino) propanesulfonic acid buffer (MOPS) to separate middle-molecule substances and 2-(N-morpholino) ethanesulfonic acid buffer to separate low-molecular-weight substances. The electrophoretically fractionated proteins were then transferred to Immobilon-P membranes (Millipore, Bedford, Mass., USA) and blocked with SuperBlock blocking buffer, which contains a proprietary protein, i.e. the Kathon antimicrobial agent, in TRIS-buffered saline. Prior to the experiment, we prepared 15-fold diluted serum samples from the subjects and stored them at 4°C. We added these samples to the membrane and incubated them overnight. After washing the membranes, we added 2,000-fold-diluted biotin-labeled antihuman IgE antibodies to them and then incubated them for 3 h. We used BCIP/NBT phosphatase substrate (1-component) as a coloring agent to detect IgE.

Immunoblot Inhibition

In order to determine whether the IgE specifically bound to sea urchin roe allergens, we performed an immunoblot inhibition experiment using sea urchin roe extract as an inhibitor. First, we prepared pooled serum by mixing equal amounts of the sera from the 5 subjects. To produce a protein extract, we first diluted vacuum freeze-dried sea urchin roe powder in PBS to prepare a basic extract solution (3 mg/ml) and then further diluted the basic solution to create 4 solutions with different dilutions which contained 0, 3, 30, and 300 μ g of protein, respectively. We mixed each of these

Table 1. Clinical characteristics of patients with sea urchin roe allergy

Patient No.	Age/sex	First time SUR was consumed	Time to allergy after intake, min	Symptoms	Other food allergies
1	9/M	yes	~15	swelling of the lips, systemic urticaria, coughing spell, dyspnea	none
2	9/M	yes	~5	abdominal pain, soft feces, itching around the eyes	salmon roe
3	4/F	yes	~30	regional urticaria	salmon roe
4	27/F	no	~10	vomiting, systemic urticaria	none
5	22/M	no	~5	sore throat, nasal discharge, dyspnea	shrimp

SUR = Sea urchin roe.

solutions with the pooled serum, added them to polyvinylidene difluoride (PVDF) membranes containing sea urchin proteins, and incubated the membranes overnight. Membrane-bound IgE were detected in the same way as mentioned above.

At the same time, we examined whether there was any cross-reactivity between sea urchin and salmon roe antigens using immunoblot inhibition tests. We prepared pooled serum for subjects (cases 2 and 3) who were allergic to both sea urchin and salmon roe. We diluted the vacuum freeze-dried salmon roe extract powder in PBS to prepare a basic extract solution (1 mg/ml) and then further diluted the basic solution to create 3 solutions with different dilutions which contained 0, 3, and 30 µg of protein, respectively. Then, we performed immunoblot inhibition tests using the solutions as inhibitors.

Detection of N-Terminal Amino Acid Sequences

After immunoblotting, we colored the PVDF membranes with 0.1% amide black, cut out proteins whose molecular weights were similar to those of the proteins to which IgE bound, and then subjected their N-terminal amino-acid sequences to high-sensitivity detection via Edman degradation with Procise 494HT (ABI).

Results

The 5 subjects included 3 males and 2 females, and their mean age was 15 years (range 4–27). Four subjects exhibited more than one allergic symptom, and 3 of them developed anaphylactic reactions (table 1). Having acquired consent from subject 2, we conducted an oral challenge test using sea urchin roe and confirmed that they induced allergic symptoms. All subjects were SPT positive, and the specific IgE levels in sera measured by ELISA were high, except in patient 5 (table 2). Interestingly, 2 of the 5 subjects were also allergic to salmon roe (table 3). The ImmunoCAP assay detected salmon roe-specific IgE in both subjects, and the salmon roe induced allergic symptoms similar to those provoked by the sea urchin roe.

Table 2. Results of SPT and ELISA for sea urchin roe

Patient No.	SPT		Specific IgE (ELISA) ^d	
	wheel size	determination	patients	control average
1 ^a	18×10 mm	+	2.033	0.367
5 ^a	6×4 mm	+	0.394	0.339
2 ^b	3×2 mm ^c	3+	1.374	0.567
3 ^b	6×4 mm ^c	3+	2.539	0.565
4 ^b	4×3 mm ^c	3+	1.954	0.567

See Methods for details.

^a Prick-to-prick test with fresh sea urchin roe.

^b SPT with protein extract (3 mg/ml).

^c Positive control: histamine diphosphate (10 mg/ml); negative control: normal saline.

^d Determined via ELISA and the optical density at 450 nm.

Table 3. Allergic symptoms and IgE titers in 2 patients who were allergic both to sea urchin and salmon roes

Patient No.	Age/sex	Salmon roe		
		symptoms	specific IgE (UA/ml)	class
2	9/M	AP, D, V	51.0	5
3	4/F	U	0.9	2

AP = Abdominal pain; D = diarrhea; V = vomiting; U = urticarial.

Immunoblotting

While there were no shared bands to which the sera from all of the subjects reacted (table 4), sera from 2 or more subjects reacted with 10 bands, i.e. 170, 107, 90, 74, 47, 42, 36, 30, 20, and 18 kDa (fig. 1). Band 4, to which sera from most of the subjects reacted, was associated with a 170-kDa protein.

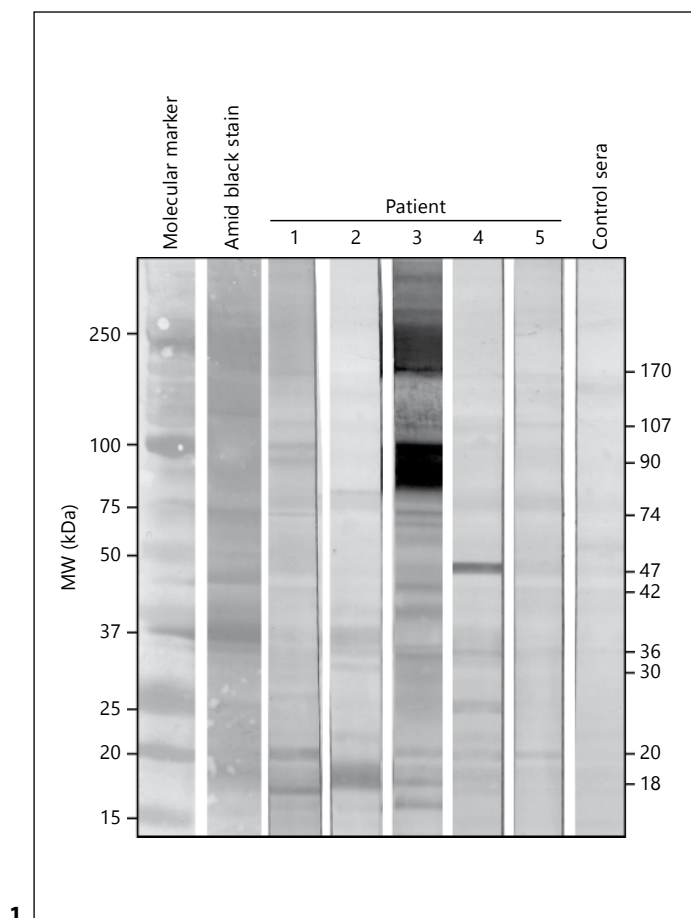


Fig. 1. Immunoblot of sea urchin roe using sera from 5 patients. MW = Molecular weight.

Fig. 2. Immunoblot inhibition of sea urchin roe. Dots indicate protein bands whose IgE binding was inhibited in a dose-dependent manner. Exes indicate protein bands whose IgE binding was not inhibited. MW = Molecular weight.

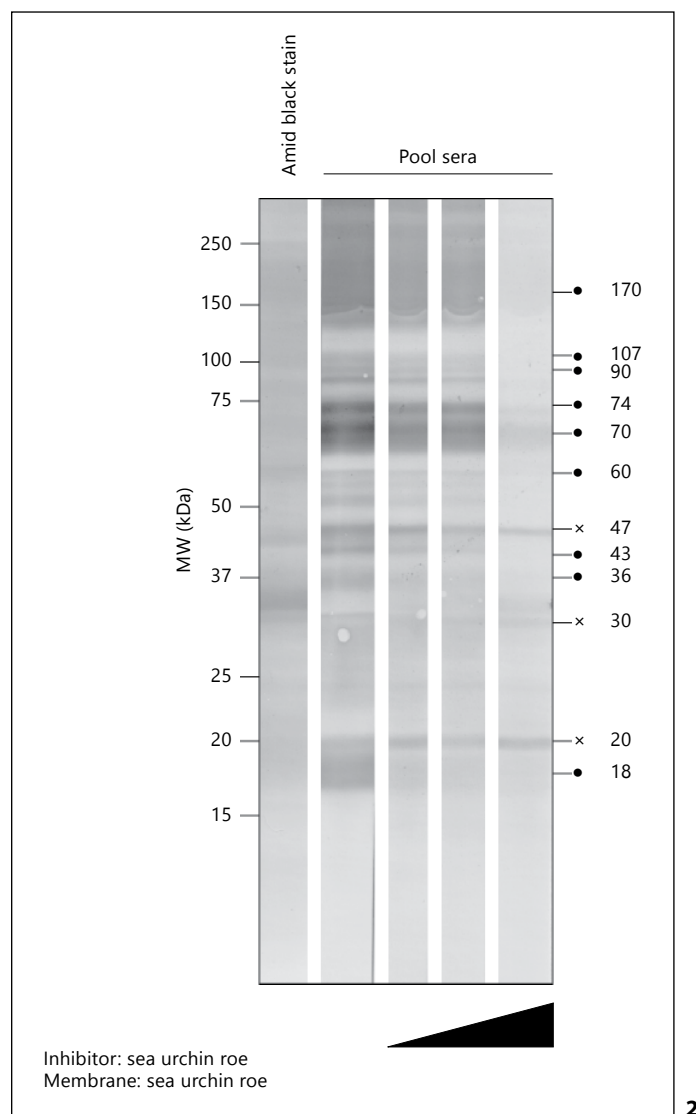


Table 4. IgE binding activity to sea urchin roe extract

MW (kDa)	Patient sera					Control serum pool	SUR inhibition
	1	2	3	4	5		
170	+		+	+	+		+
107	+		+	+	+		+
90	+	+					+
74	+		+				+
47			+	+	+	+	-
42	+		+				+
36		+		+			+
30		+		+			-
20	+		+	+	+	+	-
18		+	+				+

MW = Molecular weight; SUR = sea urchin roe.

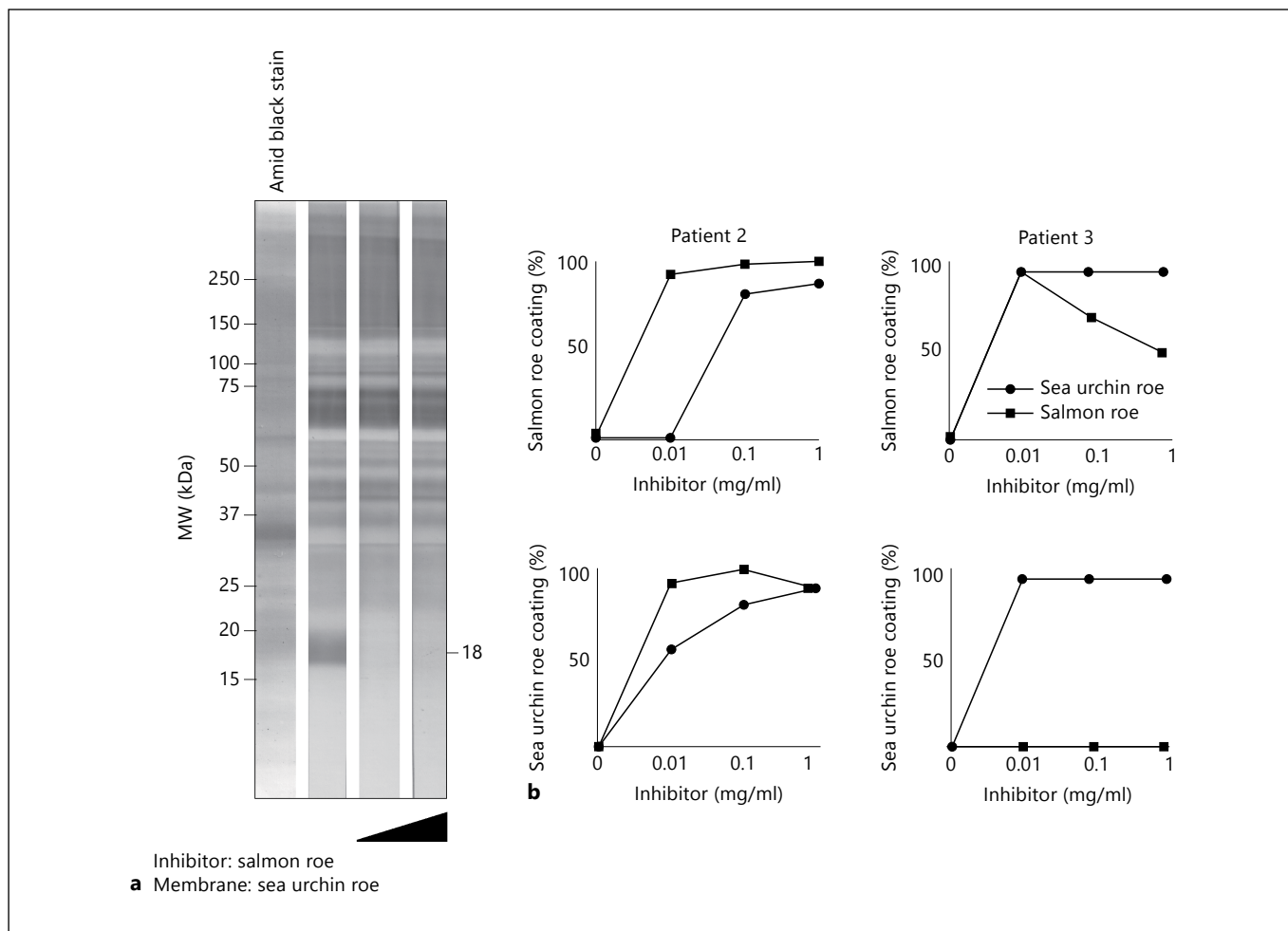


Fig. 3. Cross-reactivity between allergens in sea urchin and salmon roe. Immunoblot inhibition tests (**a**) and ELISA inhibition tests (**b**) were conducted using pooled serum from subjects who were allergic to both sea urchin and salmon roe. MW = Molecular weight.

Amino Acid Analyses

Among the above mentioned 10 bands, we set out to determine the N-terminal amino acid sequences of the 170-, 30-, 20-, and 18-kDa proteins. As for the 170-kDa band, 88.9% of its 18-base N-terminal amino-acid sequence was highly homologous to that of the MYP in *S. nudus*. In addition, the 20-base N-terminal amino-acid sequence of the 30-kDa protein exhibited 70% homology with that of the yolk granule protein Y in *S. purpuratus*, another sea urchin species. Furthermore, the 16-base N-terminal amino acid sequence of the 20-kDa protein was entirely consistent with that of the sperm-activating peptide of *S. purpuratus*. Lastly, the N-terminal amino acid sequence of the 18-kDa protein was also 100% identical to that of the 18-kDa egg cortical vesicle protein found in *S. purpuratus*.

Immunoblot Inhibition of Sea Urchin Roe

We conducted an immunoblot inhibition test to examine the specificity of the IgE that bound to sea urchin roe proteins. Among the 10 bands detected in the immunoblot test, the 47-, 30-, and 20-kDa bands did not disappear in the inhibition test in which the sea urchin protein was used as an inhibitor, which indicates that their reactions are nonspecific (fig. 2).

In the next step, we carried out another immunoblot inhibition test to see whether there was any cross-reactivity between the sea urchin and salmon roe allergens using sera from subjects who were allergic to both sea urchin and salmon roe, as well as salmon roe as an inhibitor. As a result, we found that the IgE that only bound to the 18-kDa sea urchin protein was inhibited by the addition of salmon roe antigen in a dose-dependent manner (fig. 3a).

ELISA Inhibition

ELISA inhibition indicated that each patient's primary allergen was different, i.e. salmon roe was the primary allergen for patient 2 while urchin roe was the primary allergen for patient 3 (fig. 3b).

Discussion

The prevalence of sea urchin allergies has not been clarified due to the low number of scientific or epidemiological studies that have investigated the condition. This is even true in Japan, which consumes more sea urchin produce than any other country in the world. However, previous reports have strongly suggested that sea urchin roe can cause anaphylactic reactions the first time they are consumed, and thus careful clinical attention should be paid to such allergies.

In this study, we performed allergen analyses using sera from 5 patients with allergies to sea urchins.

There were no bands that reacted with all 5 subjects' sera, but sera from 2 or more subjects reacted with 10 bands. We found that IgE bound to proteins other than MYP and the 118-kDa protein reported by Rodriguez et al. [3], which have been reported as sea urchin allergens. Among these, sera from 4 of the 5 sea urchin allergy sufferers reacted with 170-kDa MYP. MYP is a storage protein that belongs to the transferrin superfamily and plays a role in amino acid supply during gametogenesis and developmental processes [8]. It accounts for 80% of all proteins in mature gonads, which is the edible part of *Pseudocentrotus depressus* [8]. In summary, this indicates that MYP is the primary protein responsible for allergic reactions to sea urchin.

The 118-kDa protein which had previously been demonstrated to be a sea urchin antigen was not detected in the present study, probably because we extracted the allergens from raw sea urchin roe, while previous researchers heat-treated sea urchin roe during the extraction process.

Kondo et al. [6] previously reported that the 18-kDa egg cortical vesicle protein is responsible for cross-reactivity with salmon roe. However, this was not confirmed by the present study as immunoblotting revealed that IgE bound to the 18-kDa protein in 2 subjects who were allergic both to sea urchin roe and salmon roe. The results of ELISA inhibition suggested that the sensitizing agent is different in each patient with allergies both to sea urchin roe and salmon roe.

In Japan, salmon roe are known to cause allergies and can elicit anaphylactic reactions in small children [9]. Therefore, further research on the incidence of cross-reactivity between sea urchin and salmon roe is indispensable.

We conducted allergen analyses of sea urchin roe in 5 subjects, which showed that MYP is the primary protein responsible for sea urchin allergy because it tested positive most frequently in the immunoblotting test. In addition, egg cortical vesicle protein was demonstrated to be an important protein for cross-reactivity between sea urchin and salmon roe. Our findings need to be examined on a larger scale in the future.

Acknowledgements

We thank Miyuki Teshigawara for administrative assistance and support.

References

- 1 Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, Plaut M, Cooper SF, Fenton MJ, Arshad SH, Bahna SL, Beck LA, Byrd-Bredbenner C, Camargo CA Jr, Eichenfield L, Furuta GT, Hanifin JM, Jones C, Kraft M, Levy BD, Lieberman P, Luccioli S, McCall KM, Schneider LC, Simon RA, Teach SJ, Yawn BP, Schwaninger JM: Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-Sponsored Expert Panel. *J Allergy Clin Immunol* 2010; 126:S1–S58.
- 2 Hickey RW: Sea urchin roe (uni) anaphylaxis. *Ann Allergy Asthma Immunol* 2007;98:493–494.
- 3 Rodriguez V, Bartolome B, Armisen M, Vidal C: Food allergy to *Paracentrotus lividus* (sea urchin roe). *Ann Allergy Asthma Immunol* 2007;98:393–396.
- 4 Yamasaki A, Higaki H, Nakashima K, Osamu Y, Heinz KZ, Hitoshi T, Yuko C, Eishin M: Identification of a major yolk protein as an allergen in sea urchin roe. *Acta Derm Venereol* 2010;90:235–238.
- 5 Dreborg S: Skin testing: the safety of skin tests and the information obtained from using different methods and concentrations of allergen. *Allergy* 1993;48:473–475.
- 6 Kondo Y, Tanaka K, Inuo C, Tsuge I, Urisu A: A patient with salmon roe allergy showing taxonomy-unrelated cross-reactivity with sea urchin roe. *Ann Allergy Asthma Immunol* 2011;107:283–284.
- 7 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- 8 Unuma T, Yamamoto T, Akiyama T, Shirai-shi M, Ohta H: Quantitative changes in yolk protein and other components in the ovary and testis of the sea urchin *Pseudocentrotus depressus*. *J Exp Biol* 2003;206:365–372.
- 9 Akiyama H, Imai T, Ebisawa M: Japan food allergen labeling regulation – history and evaluation. *Adv Food Nutr Res* 2011;62:139–171.