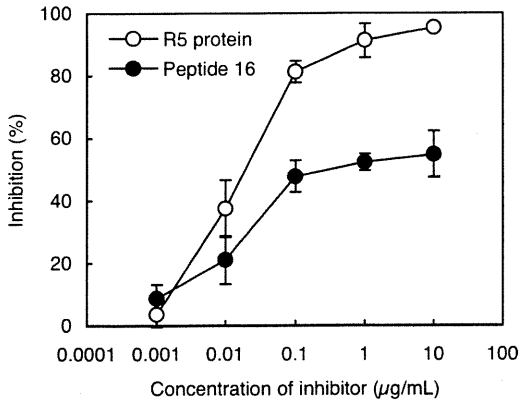

Fig. 4. Inhibition of the IgE reactivity to R5 protein by peptide 16 and R5 protein

Pooled serum (1 : 125 dilution) from patients 1–5 was preincubated with an equal volume of various concentrations of inhibitor (peptide 16 or R5 protein) and added to a plate coated with R5 protein (1 μg/mL). Data are ex- pressed as mean±SD (n=3).

minimal. It was thus concluded that the major IgE epitope of the rainbow trout collagen α2 chain is located in peptide 16 (region 941–960). As evaluated by inhibition ELISA using pooled serum from patients 1–5, peptide 16 accounted for more than 50% of the IgE reactivity to the R5 protein (Fig. 4).

IgE-binding ability of region 941–960 of collagen α2 chains

Fish collagen α2 chains have been completely sequenced for chum salmon *Oncorhynchus keta* (DDBJ/EMBL/GenBank accession number: AB075699), bastard halibut[16] and zebrafish,[14] as well as for rainbow trout[13]. As compared to the amino acid sequence of the rainbow trout collagen α2 chain, that of the chum salmon collagen α2 chain is completely conserved in region 941–960 (the most IgE-reactive region of the rainbow trout collagen α2 chain) and those of the bastard, halibut and zebrafish collagen α2 chains are highly conserved, with only one or two alterations (Fig. 5). On the other hand, the amino acid sequences of mammalian collagen α2 chains are homologous with one another in region 941–960 but are significantly different from those of fish collagen α2 chains. To examine whether region 941–960 is a common IgE epitope in fish collagen α2 chains, synthetic peptides Bh, Zf and Bv corresponding to region 941–960 of the bastard halibut, zebrafish and bovine collagen α2 chains, respectively, were analyzed for IgE-binding ability by fluorescence ELISA using sera from patients 1–7, in which significant reactivity to peptide 16 was observed. As shown in Fig. 6, no significant difference in reactivity with serum from patient 1, 2 or 3 was observed among peptides 16, Bh and Zf. However, the reactivity to peptides Bh and Zf was low in sera from patients 4–6 compared to that to peptide 16. No reactivity with serum from patient 7 was displayed by peptide Bh or Zf. In the case of the

	941								950								960
Rainbow trout	M	K	G	L	R	G	H	G	G	L	Q	G	M	P	G	P	N	G	P	S
Chum salmon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bastard halibut	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	S	-	-	-
Zebrafish	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-
Human	L	P	-	-	K	-	-	N	-	-	-	-	L	-	-	I	A	-	H	H
Bovine	L	P	-	-	K	-	-	N	-	-	-	-	L	-	-	L	A	-	H	H
Rabbit	L	P	-	I	K	-	-	N	-	-	-	-	L	-	-	L	A	-	Q	H
Mouse	L	P	-	-	K	-	Y	S	-	-	-	-	L	-	-	L	A	-	L	H

Fig. 5. Amino acid sequence alignment of the 941–960 region of collagen α2 chains from fish and mammals

DDBJ / EMBL / GenBank accession numbers: rainbow trout, AB052837; chum salmon, AB 075699; bastard halibut, AB196514; zebrafish, BC071278; human, BC042586; bovine, AB 008683; rabbit, D49399; mouse, AK132496.

peptide Bv, little reactivity with patients' sera was seen, except for patient 6. Patient 6 may be allergic to bovine collagen as well as fish collagen, although this was not confirmed. Nevertheless, in view of the fact that there are marked differences in amino acid sequence between peptides 16 and Bv, it seems unlikely that the patient 6 IgE reacting to peptide 16 can cross-react to peptide Bv.

Discussion

Collagen α chains, including α2 chain, are too large for direct analysis of their IgE epitopes by usual epitope mapping experiments with synthetic peptides. In this study, therefore, five overlapping proteins (R1–5) covering the entire sequence of the rainbow trout collagen α2 chain were expressed in *E. coli* and their IgE reactivity was examined by means of immunoblotting to identify major IgE-binding regions. This strategy was successful; the R5 protein was found to be the most IgE-reactive. Subsequent epitope mapping experiments, using synthetic overlapping peptides encompassing the sequence of the R5 protein, clearly demonstrated that the major IgE epitope of the rainbow trout collagen α2 chain is located in region 941–960 (MKGLRGHGGLQGMPGPNGPS). The major IgE epitope of the bovine collagen α2 chain has previously been reported to be included in region 485–494 (IPGEFGKPGP)[5]. Clearly, there is no overall sequence homology between the major IgE epitopes of the rainbow trout and bovine collagen α2 chains.

The chum salmon collagen α2 chain has the same amino acid sequence in region 941–960 as the rainbow trout collagen α2 chain (Fig. 5). In the case of the bastard halibut and zebrafish collagen α2 chains, one or two replacements (replacements of Gly by Pro at position 948 and Asn by Ser at position 957 for the bastard halibut collagen α2 chain and replacement of Gly by Pro at position 948 for the zebrafish collagen α2 chain) are recognized in region 941–960 compared to the amino acid sequence of the rainbow trout collagen α2 chain. However, the peptides Bh and Zf, corresponding to region 941–960 of the bastard halibut and zebrafish collagen α2 chains, respectively, were found to be reac-

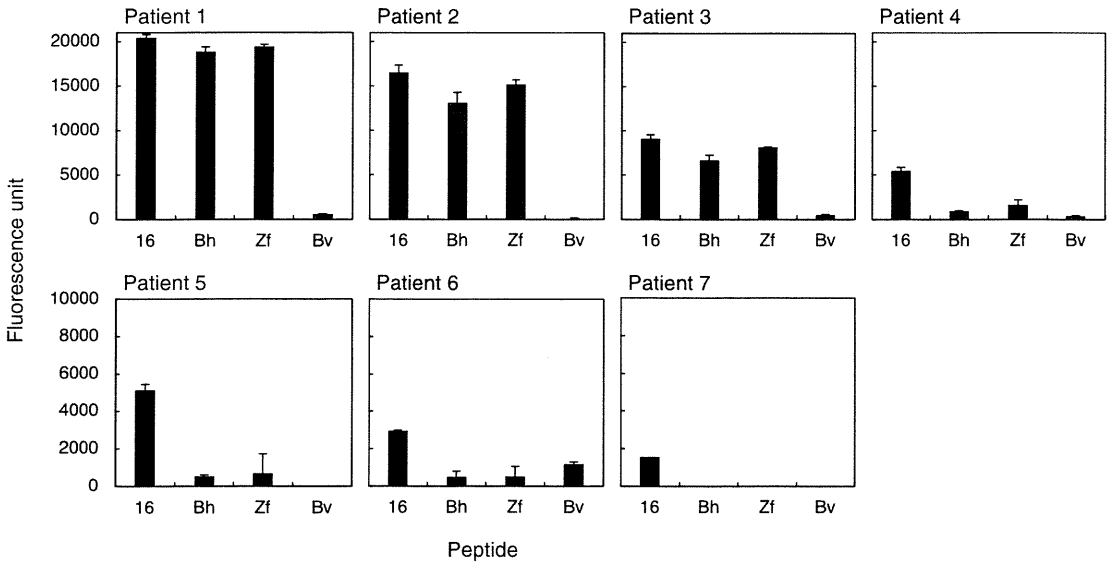

Fig. 6. IgE reactivity of the 941–960 region of fish and bovine collagen α2 chains analyzed by fluorescence ELISA
Data are expressed as mean±SD (n=3). Peptides: 16, rainbow trout; Bh, bastard halibut; Zf, zebrafish; Bv, bovine.

tive with patients' sera, although their IgE-binding ability was reduced in three sera and lost in one (Fig. 6). It is thus likely that region 941–960 is a major common IgE-binding epitope in fish collagen α2 chains. To confirm this, further amino acid sequence data on fish collagen α2 chains should be accumulated.

Bovine type I collagen is composed of two α1 chains and one α2 chain, like other mammalian type I collagens, and only the α2 chain has been demonstrated to be IgE-reactive[4]. The major IgE-binding epitope elucidated for the bovine collagen α2 chain is not found in collagen α1 chains from mammals, including bovine[5]. Differing from mammalian collagens, fish collagens take the form of either $(\alpha 1)_2\alpha 2$ or $\alpha 1\alpha 2\alpha 3$, depending on the fish species and tissues. Importantly, IgE cross-reactivity has been found among fish collagen α chains[11), 12)]. Nevertheless, the same sequence as that of the major IgE-binding epitope (region 941–960) determined for the rainbow trout α2 chain is not found in fish collagen α1 and α3 chains, including the rainbow trout collagen α1 and α3 chains. Further study on which residues in the major IgE epitope of the rainbow trout collagen α2 chain are crucial for IgE-binding would be helpful to understand the cross-reactivity among different α chains of fish collagens.

Finally, it should be noted that the major IgE epitope of the rainbow trout collagen α2 chain contains three Pro residues and one Lys residue. This is also the case with the major IgE epitope of the bovine collagen α2 chain. Pro residues are abundant in collagen, and many of them are hydroxylated by post-translational modification. Some Lys resides are also known to be hydroxylated. In the case of the major IgE epitope of the bovine collagen α2 chain, hydroxylation of Pro and Lys residues was suggested to be independent of the interaction with IgE. However, this has not been experimentally verified. Further study using analogous synthetic peptides with replacements of Pro and Lys residues by hydroxy-Pro and hydroxy-Lys residues, respectively, is needed to assess in more detail the IgE-binding ability of the major IgE epitopes determined for the rainbow trout and bovine collagen α2 chains.

Acknowledgements

The authors thank Drs. N. Shimojo and Y. Kohno, Department of Pediatrics, Chiba University, Dr. A. Urisu, Fujita Health University School of Medicine and Drs. H. Ohsuna and Z. Ikezawa, Department of Dermatology, Yokohama City University, for providing patients' sera. We also thank Miss Midori Suzuki for her technical assistance. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan.

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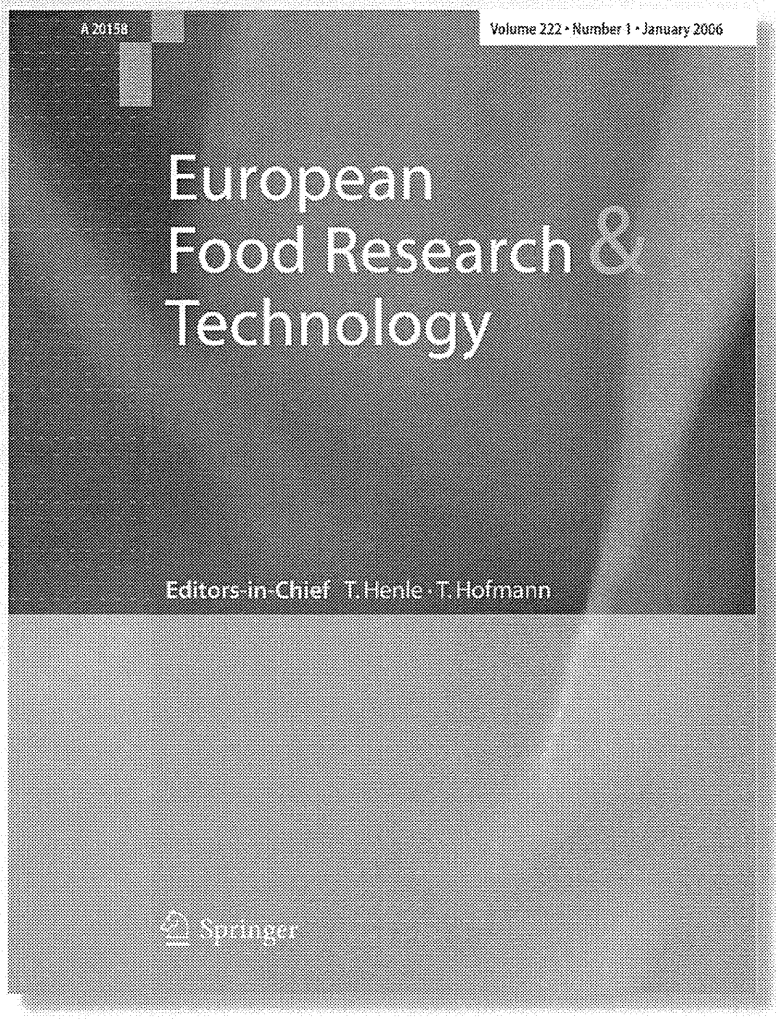
European Food Research and Technology
Zeitschrift für Lebensmittel-Untersuchung und -Forschung A

ISSN 1438-2377
Volume 233
Number 6

Eur Food Res Technol (2011)
233:991-997
DOI 10.1007/s00217-011-1602-x



Springer

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ORIGINAL PAPER

New extraction method suitable for immunoblotting analysis of fish allergens

Maki Kanamori · Hiroyuki Tanaka · Yuki Hamada · Yuji Nagashima · Kazuo Shiomi

Received: 10 August 2011 / Revised: 18 September 2011 / Accepted: 30 September 2011 / Published online: 13 October 2011

Abstract Immunoblotting is a simple method to analyze allergens in biological samples. In previous immunoblotting studies on fish allergens, however, collagen, an important allergen next to parvalbumin (major fish allergen), has not been detected in fish muscle extracts probably due to its unique chemical properties. This study was aimed to develop an extraction method suitable for immunoblotting analysis of fish allergens including collagen as well as parvalbumin. When various extracts from the Japanese eel white muscle were analyzed by SDS–PAGE, heating of the muscle homogenate at 80 °C for 20 min was found to be the most effective method to extract collagen as well as parvalbumin. The same extraction method was also effective for the other five species of fish analyzed (rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and Japanese flounder). Furthermore, parvalbumin and/or collagen were successfully identified as allergens in the six species of fish by immunoblotting using the heated extracts prepared by the method described above. It can be concluded that the extraction method (heating of the muscle homogenate at 80 °C for 20 min) developed in this study is useful not only for analyzing fish allergens by immunoblotting but also for preparing antigens for diagnosis of fish allergy by RAST (radioallergosorbent test).

M. Kanamori · H. Tanaka · Y. Nagashima · K. Shiomi (✉)
Department of Food Science and Technology,
Tokyo University of Marine Science and Technology,
Minato-ku, Tokyo 108-8477, Japan
e-mail: shiomi@kaiyodai.ac.jp

Y. Hamada
Laboratory of Fishery Nutritional Science, Faculty of Fisheries,
Nagasaki University, Nagasaki 852-8521, Japan

Keywords Allergen · Collagen · Extraction · Fish · Immunoblotting · Parvalbumin

Introduction

Food allergy mediated by immunoglobulin E (IgE) is frequently fatal due to anaphylactic shock, being a serious problem in industrialized countries. Fish is obviously among the most common causes of food allergy, especially in coastal countries including Japan. The major fish allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa, as demonstrated first with cod (*Gadus callarias*) [1, 2] and subsequently with a number of fish such as carp (*Cyprinus carpio*) [3, 4], salmon (*Salmo salar*) [5], mackerel (*Scomber japonicus*) [6], and tuna (*Thunnus obesus*) [7]. Besides parvalbumin, three classes of proteins, collagen [8, 9], aldehyde phosphate dehydrogenase [10], and transferrin [11] have also been identified as fish allergens, although not major ones. Aldehyde phosphate dehydrogenase was found as an allergen in codfish and transferrin as a cross-reactive allergen between tuna and marlin. However, these two classes of proteins have not yet been recognized as allergens in other species of fish, suggesting that they are allergens in limited species of fish. In contrast, our inhibition ELISA data revealed that collagen is a cross-reactive allergen among various species of fish [12]. Therefore, collagen is considered to be an important fish allergen next to parvalbumin.

An immunoblotting technique, which comprises separation of proteins by SDS–PAGE, transferring of proteins from a polyacrylamide gel to a membrane and immunological detection of targeting proteins on the membrane, has been widely used to identify allergens in crude extracts from biological samples. So far, fish allergens including

parvalbumin and/or other allergens (aldehyde phosphate dehydrogenase, transferrin, and unknown allergens) have also been effectively analyzed by a number of previous immunoblotting experiments [10, 11, 13–21]. However, it should be noted that immunoblotting is naturally helpless if targeting allergens are absent in the crude extract. As for parvalbumin, it is water-soluble and very stable to heating and hence can be easily extracted from the fish muscle under both non-heated and heated conditions. On the other hand, it may be not so easy to extract collagen from the fish muscle, due to its unique chemical properties. Firstly, native collagen, which is composed of three α-chains [in the form of $(\alpha 1)_2\alpha 2$ or $\alpha 1\alpha 2\alpha 3$] twisted together to form a triple helix [22], is insoluble in water at low temperatures, being usually absent in the non-heated extract from the fresh fish muscle. Secondly, native collagen is readily converted to a mixture of peptides (called gelatin) during heating of the fish muscle, as a result of both heat denaturation and digestion with muscle proteases, being hardly present in the heated extract. Thus, collagen as a fish allergen has so far been overlooked in previous immunoblotting experiments [10, 11, 13–21]. Recently, Weber et al. [23] prepared fish muscle extracts using urea solution (containing 8 M urea and 2% cyclohexylaminopropane) and detected collagen in the extracts by immunoblotting with polyclonal antibodies against fish collagen. It is, however, unknown whether their extraction method is suitable for analysis of fish allergens (both parvalbumin and collagen) by immunoblotting with patient sera.

As described above, collagen is not an ignorable fish allergen. To analyze fish allergens by immunoblotting, therefore, it is necessary to devise a sophisticated method to extract collagen without fragmentation, together with parvalbumin, from the fish muscle. We report here an extraction method suitable for immunoblotting analysis of fish allergens including collagen as well as parvalbumin.

Materials and methods

Fish samples

The following six species of fish were used in extraction experiments: Japanese eel (*Anguilla japonica*), rainbow trout (*Oncorhynchus mykiss*), Japanese horse mackerel (*Trachurus japonicus*), crimson sea bream (*Evynnis japonica*), Pacific mackerel (*Scomber japonicus*), and Japanese flounder (*Paralichthys olivaceus*). In the case of Japanese eel, live specimens were purchased at the Tokyo Central Wholesale Market, transported to our laboratory, and used for experiments immediately after killing. For the remaining five species, fresh specimens purchased at a local supermarket were immediately subjected to experiments.

Preparation of extracts

In this study, the Japanese eel white muscle was used as a model sample to prepare various extracts. The white muscle was collected from a specimen and well macerated with a mortar and a pestle. The macerate (4 g) was homogenized in three volumes of PBS (150 mM NaCl-10 mM phosphate buffer, pH 7.0) using an Ace Homogenizer AM10 (Nippon Seiki, Tokyo, Japan) under low temperature with ice. After centrifugation of the homogenate at 18,000×*g* and 4 °C for 20 min, the supernatant obtained was regarded as a non-heated extract. On the other hand, heated extracts were prepared as follows. The homogenate was transferred into a 50-mL polypropylene conical tube (30 × 115 mm; Nippon Becton–Dickinson, Tokyo, Japan), which was capped tightly and heated at 20, 40, 60, 80, or 100 °C in a water bath (about 2 L) for 20 min with shaking for initial 5 min. Then, the homogenate was cooled in an ice bath and centrifuged at 18,000×*g* and 4 °C for 20 min. The supernatant was used as a heated extract. In the case of the five species of fish other than Japanese eel, the heated extract was similarly prepared from the white muscle by heating of the homogenate at 80 °C for 20 min.

Purification of parvalbumin and collagen

Purification of parvalbumin from the white muscle of Pacific mackerel and bigeye tuna was achieved by gel filtration and reverse-phase HPLC, as reported previously [6, 7]. Collagen was purified from the white muscle of both species of fish by extraction with 500 mM acetic acid, followed by precipitation with 800 mM NaCl, according to the method of Miller and Rhodes [24].

SDS–PAGE

SDS–PAGE was carried out on a PhastSystem apparatus (GE Healthcare, Piscataway, NJ, USA) using ready-made polyacrylamide gels (PhastGel Gradient 8–25; GE Healthcare), as recommended by the manufacturer. Each sample was dissolved in 62.5 mM Tris–HCl buffer (pH 7.4) containing 2% SDS, 4 M urea, and 100 mM dithiothreitol, denatured by heating at 100 °C for 10 min and subjected to electrophoresis. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as references. After running, proteins were visualized by staining with Coomassie Brilliant Blue (CBB) R-250.

Human sera

Sera were obtained from eight fish-allergic patients (Table 1). These patients had been diagnosed to be allergic

Table 1 Characteristics of patients

Patient	Age	Gender	CAP-RAST class
1	27	Female	Sardine (3), salmon (4), cod (4), mackerel (3), horse mackerel (4), tuna (3)
2	4	Male	Mackerel (2)
3	3	Female	Mackerel (3), horse mackerel (4), tuna (3)
4	22	Female	Salmon (4)
5	19	Male	Sardine (2), cod (4), mackerel (3), horse mackerel (2), tuna (4)
6	18	Female	Horse mackerel (3), tuna (3), flounder (3)
7	4	Female	Salmon (4), cod (3)
8	3	Male	Tuna (3)

to fish at medical institutions (hospitals of Chiba University, Fujita Health University and Yokohama City University), based on the clinical histories of immediate allergic reactions after ingestion of fish and the CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) data. Written informed consent for the research use of serum was obtained from each patient.

ELISA

ELISA was performed to examine whether each patient serum reacts to bigeye tuna parvalbumin, bigeye tuna collagen, or both, as reported previously [12]. In brief, a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan) was coated with bigeye tuna parvalbumin or collagen solution (0.001–1.0 μg/mL) and incubated with patient serum (diluted 1:50), followed by horseradish peroxidase-conjugated goat antihuman IgE antibody (diluted 1:2,500; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Enzyme reaction was carried out using a substrate solution (0.1% *o*-phenylenediamine and 0.03% hydrogen peroxide), and the developed color was measured by absorbance at 490 nm. All ELISAs were performed in triplicate, and the data were expressed as the mean values.

Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as described elsewhere [20]. Briefly, the proteins separated by SDS–PAGE were electrotransferred from the polyacrylamide gel to a polyvinylidene difluoride membrane. Then, the membrane was incubated successively with patient serum (diluted 1:500) and horseradish peroxidase-conjugated goat antihuman IgE antibody (diluted 1:5,000). Antigen–antibody binding was detected using an ECL Plus Western Blotting Detection System (GE-Healthcare) and an ECL Mini Camera (GE-Healthcare), according to the manufacturer's instructions. For inhibition immunoblotting, patient serum (diluted 1:500) was preincubated with either Pacific mackerel parvalbumin (final concentration: 10 μg/mL) or Pacific mackerel collagen (final concentration: 10 μg/mL) at 37 °C for 2 h and used as a primary antibody. The subsequent procedure was the same as that for the immunoblotting described above.

Results

Analysis of fish muscle extracts by SDS–PAGE

When the non-heated and heated extracts from the white muscle of Japanese eel were analyzed by SDS–PAGE, a prominent 12 kDa protein band corresponding to parvalbumin was observed in all extracts (Fig. 1a). This is reasonably understood by the fact that parvalbumin is a thermostable sarcoplasmic (water-soluble) protein, being extractable with aqueous solvents regardless of heating temperatures. On the other hand, collagen α chain of about 120 kDa and β chain (dimer of α chain) of about 240 kDa were recognized in the extracts prepared by heating at 60, 80, and 100 °C but not in the other extracts. Based on the CBB-staining intensity, collagen concentration was assumed to be slightly higher in the 80 °C-heated extract than in the 60 °C-heated extract. In the case of the 100 °C-heated extract, both bands corresponding to collagen α and β chains were rather smear, suggesting that collagen was partly degraded. In the next step, heated extracts were prepared by heating at 80 °C for different times (10, 20, and 60 min) and similarly analyzed by SDS–PAGE. As shown in Fig. 1b, all the three kinds of extracts afforded bands of collagen α and β chains as well as that of parvalbumin. However, the collagen bands were considerably smeared in the 60 min-heated extract probably due to the gradual degradation of collagen during long heating.

The results described above allowed us to conclude that collagen as well as parvalbumin can be most effectively extracted from the Japanese eel white muscle with PBS by heating at 80 °C for 20 min. To evaluate whether this extraction method is commonly applicable to fish, the extracts from five species of fish (rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and

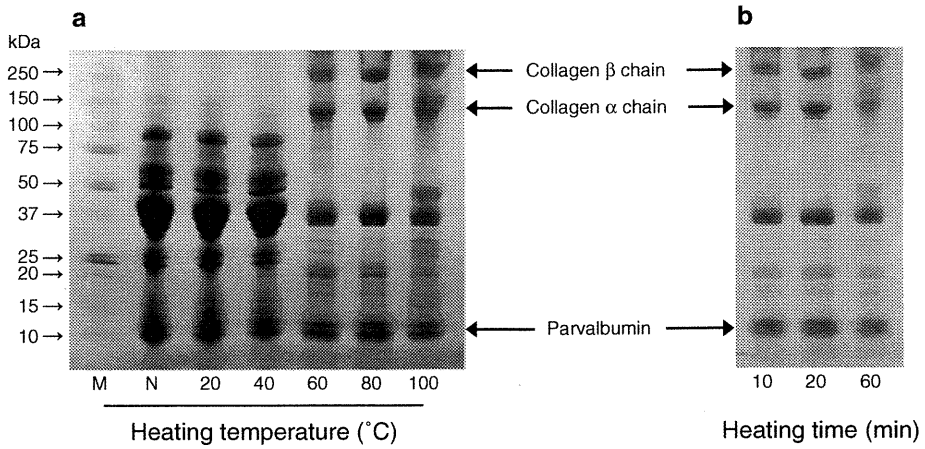

Fig. 1 SDS–PAGE of non-heated and heated extracts from the Japanese eel white muscle. **a** Extracts were prepared from the muscle by non-heating (labeled by N) or heating at different temperatures (20–100 °C) for 20 min. *Lane M*: molecular weight markers (Precision Plus Protein Standards). **b** Extracts were prepared from the muscle by heating at 80 °C for different times (10–60 min)

Japanese flounder) were similarly prepared by heating of the muscle homogenate at 80 °C for 20 min and analyzed by SDS–PAGE in comparison with the heated extract from the Japanese eel muscle. As shown in Fig. 2, both parvalbumin and collagen were certainly detected in the heated extracts from the five species of fish, as in that from Japanese eel. The observed CBB-staining intensity suggested no significant difference in collagen concentration at least among the six species of fish. In contrast, parvalbumin concentration markedly varied from fish to fish; the parvalbumin concentrations in rainbow trout and Pacific mackerel were much lower than those in the other four species of fish. It should be noted that a 14 kDa protein, together with parvalbumin (12 kDa), was clearly found only in crimson sea bream. We have already purified both 14 and 12 kDa proteins and identified them as isoforms of parvalbumin based on the determined partial amino acid sequences (unpublished data).

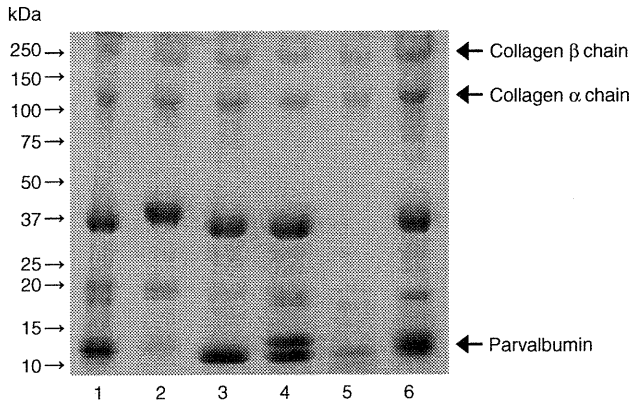

Fig. 2 SDS–PAGE of 80 °C-heated extracts from the white muscles of six species of fish. Fish samples: *lane 1*, Japanese eel; *lane 2*, rainbow trout; *lane 3*, Japanese horse mackerel; *lane 4*, crimson sea bream; *lane 5*, Pacific mackerel; and *lane 6*, Japanese flounder

Reactivity of patient sera to bigeye tuna parvalbumin and collagen

Prior to immunoblotting, eight patient sera were assessed for reactivity to bigeye tuna parvalbumin and collagen by ELISA. As a result, the sera were divided into the following three types: parvalbumin type (sera from patients 1–3) reacting only to parvalbumin, collagen type (sera from patients 4–6) reacting only to collagen, and parvalbumin/collagen type (sera from patients 7 and 8) reacting to both parvalbumin and collagen. In Fig. 3, the reactivities of the sera from patients 1, 4, and 7 to parvalbumin and collagen are shown as the typical examples of the parvalbumin, collagen, and parvalbumin/collagen types, respectively.

Analysis of fish muscle extracts by immunoblotting and inhibition immunoblotting

Allergens in the heated extracts from the six species of fish (Japanese eel, rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and Japanese flounder) were analyzed by immunoblotting using the eight patient sera. In the case of the parvalbumin-type sera (from patients 1–3), a 12 kDa blot corresponding to parvalbumin was observed in all the six species of fish although its intensity considerably varied among fish (Fig. 4). Similarly, the collagen-type sera (from patients 4–6) reacted to both collagen α and β chains of all species of fish and the parvalbumin/collagen-type sera (from patients 7 and 8) to parvalbumin, collagen α chain, and collagen β chain of all species of fish. Interestingly, the parvalbumin-type and parvalbumin/collagen-type sera reacted to the 12 kDa parvalbumin isoform in crimson sea bream but did not to another parvalbumin isoform of 14 kDa, conforming well to the fact that the latter isoform presents considerably weaker IgE reactivity than the former (unpublished data). Besides parvalbumin and collagen, 50–60 kDa proteins in rainbow trout, Japanese horse mackerel, and Japanese

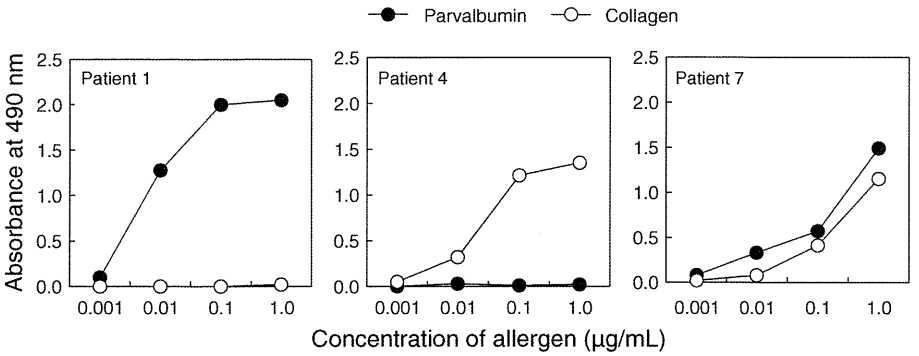

Fig. 3 ELISA analysis of reactivity of three patient sera to collagen and parvalbumin purified from bigeye tuna

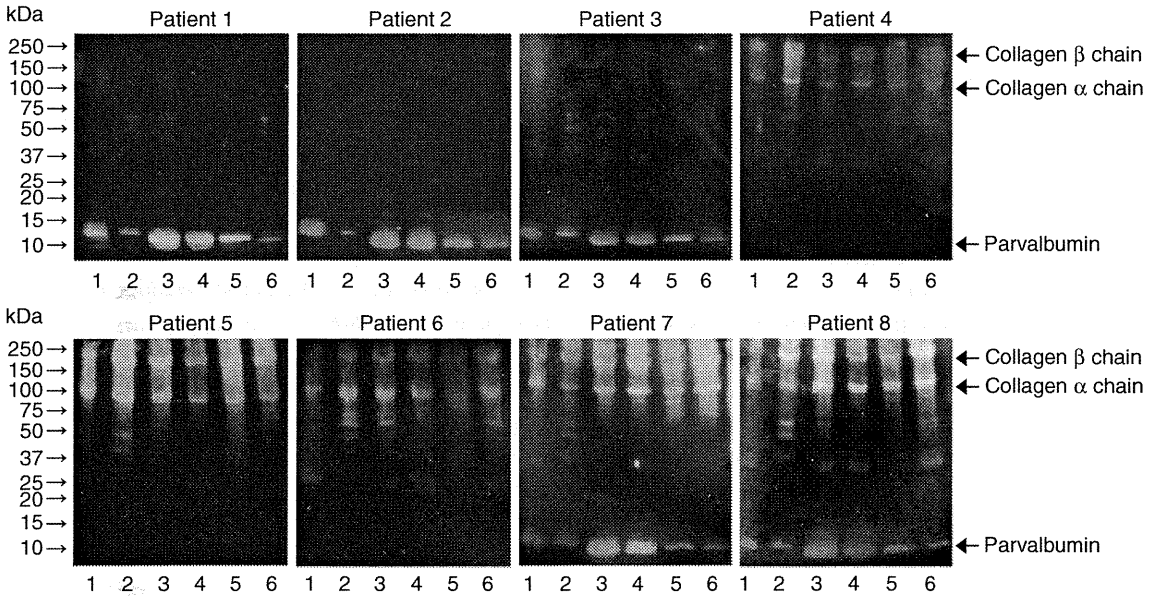

Fig. 4 Immunoblotting analysis of allergens reacting with eight patient sera in heated extracts from six species of fish. Fish samples: *lane 1*, Japanese eel; *lane 2*, rainbow trout; *lane 3*, Japanese horse mackerel; *lane 4*, crimson sea bream; *lane 5*, Pacific mackerel; and *lane 6*, Japanese flounder

flounder weakly reacted with certain sera (e.g., patient 6 serum) and 37 kDa proteins in Japanese eel and Japanese flounder with the patient 8 serum, indicating the presence of minor fish allergens to be identified in future.

When preincubated with Pacific mackerel parvalbumin, the patient 1 serum (parvalbumin type) showed no reactivity to any of the parvalbumins from the six species of fish (Fig. 5). Similarly, preincubation with Pacific mackerel collagen almost completely abolished the reactivity of the patient 4 serum (collagen type) to the collagen α and β chains of the six species of fish. In the case of the patient 7 serum (parvalbumin/collagen type), the reactivity to parvalbumins was completely inhibited by preincubation with Pacific mackerel parvalbumin. Although not completely, the reactivity of the patient 7 serum to collagens was greatly inhibited by preincubation with Pacific mackerel collagen.

Discussion

Collagen is insoluble in aqueous solvents at low temperatures and is readily converted to gelatin during heating. Due to these properties, collagen has been overlooked in previous immunoblotting studies [10, 11, 13–21], although it is an important fish allergen next to parvalbumin (major fish allergen). In this study, we found that collagen as well as parvalbumin can be most effectively extracted from Japanese eel by heating of the muscle homogenate at 80 °C for 20 min. The same extraction method was judged to be also effective for the other five species of fish (rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and Japanese flounder). Furthermore, when the heated extracts prepared from the six species of fish by the method described above were applied to immunoblotting analysis, collagen and/or parvalbumin were successfully

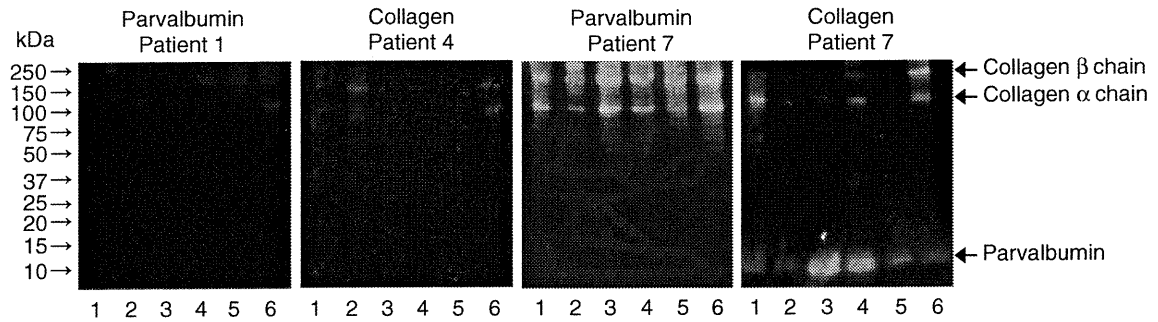

Fig. 5 Inhibition immunoblotting analysis of cross-reactivity among allergens in heated extracts from six species of fish. Fish samples: *lane 1*, Japanese eel; *lane 2*, rainbow trout; *lane 3*, Japanese horse mackerel; *lane 4*, crimson sea bream; *lane 5*, Pacific mackerel; and *lane 6*, Japanese flounder. Each patient serum was preincubated with the inhibitor (Pacific mackerel parvalbumin or collagen) indicated above the patient number and used as a primary antibody

identified as allergens in all species. Thus, it can be concluded that the extraction method (heating at 80 °C for 20 min) developed in this study is suitable for analyzing two important allergens (parvalbumin and collagen) in various species of fish by immunoblotting. However, it should be kept in mind that the extraction method from the fish muscle is useful to analyze thermostable allergens but is helpless for thermolabile ones.

With regard to the developed extraction method, two important points should be taken into account. Firstly, shaking of the muscle homogenate for initial 5 min during the heating process is essential for the effective extraction of collagen, because otherwise collagen bands (corresponding to both α and β chains) became smear on SDS–PAGE (data not shown) probably as a result of protease degradation. It is relevant to infer that the temperature of the muscle homogenate rapidly reaches the setting value (80 °C) through an efficient heat transfer by shaking, thereby preventing the degradation of collagen by muscle proteases (generally most active at 35–40 °C). Secondly, the fish muscle used for extraction is desirable to be as fresh as possible, since the native collagen (triple helix form) may be denatured into α-chains, which are susceptible to protease digestion, during storage of the fish muscle especially under unfavorable conditions. Indeed, our preliminary experiments showed that collagen bands were obscure on SDS–PAGE when the heated extract was prepared from the Japanese eel muscle previously kept overnight at room temperature.

It is particularly interesting to note that parvalbumin content differs among fish species as revealed by SDS–PAGE (Fig. 2); based on the CBB-staining intensity, parvalbumin content is significantly lower in rainbow trout and Pacific mackerel than in the other four species of fish. In consistence with this, immunoblotting experiments proved that the parvalbumin-recognizing sera (from patients 1, 2, 3, 7, and 8) weakly react to the parvalbumins from both rainbow trout and Pacific mackerel. Mackerels have previously been reported to be low in parvalbumin content and classified as low-allergenic fish species [19, 21]. This study newly showed that rainbow trout is also a low-allergenic fish species with low content of parvalbumin. In view of our findings, together with previous reports [19, 21], it can be assumed that there is a positive relationship between parvalbumin content and allergenicity of different fish species. Japanese flounder is an exception to this general assumption; although it has a large amount of parvalbumin as shown by SDS–PAGE, the IgE reactivity of its parvalbumin is weak (almost equivalent to the Pacific mackerel parvalbumin) as revealed by immunoblotting. Future molecular studies are needed to understand the weak IgE reactivity of the Japanese flounder parvalbumin.

For clinical diagnosis of allergic diseases, a RAST method (including a CAP-RAST method) is widely employed to determine antigens recognized by allergic subjects. Although commercial antigens (extracts from allergenic biological materials) are usually used for RAST, no careful attention is paid to whether or not they contain all allergens to be analyzed. If a commercial antigen does not contain an allergen recognized by a certain patient, its use in RAST leads to a wrong diagnosis that the patient is negative to the antigen. Since it is difficult to effectively extract collagen from the fish muscle, commercial fish antigens are likely to be devoid of collagen. In relation to this, it is worth mentioning that there are some fish-allergic patients who are judged to be negative to fish by RAST but manifest hypersensitive reactions after ingestion of fish. Such fish-allergic patients might recognize only collagen. To reduce false-negative results in the diagnosis of fish allergy by RAST, it is important to use fish antigens containing collagen. The extraction method developed for immunoblotting in this study is considered to be also applicable to the preparation of fish antigens for RAST.

In conclusion, an extraction method (heating at 80 °C for 20 min) suitable for immunoblotting analysis of fish allergens was developed in this study. Future

immunoblotting experiments using the heated extracts prepared by the established extraction method will identify both parvalbumin and collagen as allergens and also detect unknown allergens in a variety of fish species. Moreover, RAST using the heated extracts as fish antigens will improve the accuracy in diagnosis of fish allergy.

Acknowledgments The authors thank Drs. N. Shimojo and Y. Kohno, Department of Pediatrics, Chiba University, Dr. A. Urisu, Fujita Health University School of Medicine, and Drs. H. Ohsuna and Z. Ikezawa, Department of Dermatology, Yokohama City University, for providing patient sera. This study was partly supported by a Grant-in-Aid for Scientific Research (No. 19380120) from the Ministry of Education, Science, Sports, and Culture of Japan and a grant (H21-Food-General-004) from the Ministry of Health, Labour and Welfare of Japan.

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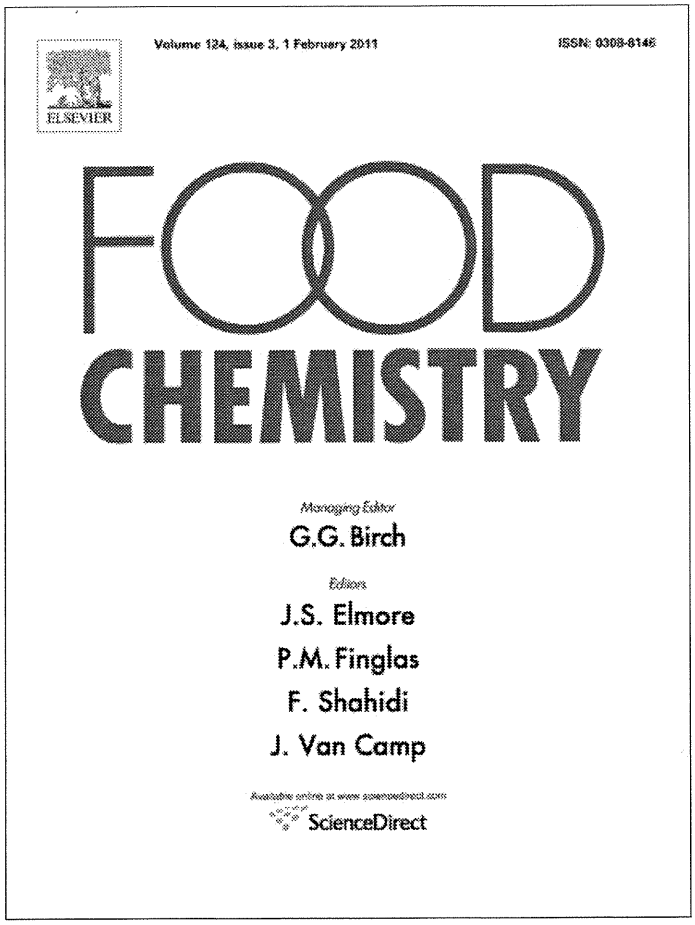

Food Chemistry 124 (2011) 921–926

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Paramyosin of the disc abalone *Haliotis discus discus*: Identification as a new allergen and cross-reactivity with tropomyosin

Midori Suzuki [a], Yukihiro Kobayashi [a,b], Yumiko Hiraki [a], Hiroki Nakata [a], Kazuo Shiomi [a,*]

[a] *Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan*
[b] *Physics and Chemistry Division, Kanagawa Prefectural Institute of Public Health, Chigasaki-shi, Kanagawa 253-0087, Japan*

ARTICLE INFO

Article history:
Received 7 April 2010
Received in revised form 23 May 2010
Accepted 8 July 2010

Keywords:
Allergen
Disc abalone
Cross-reactivity
Paramyosin
Tropomyosin

ABSTRACT

Tropomyosin, a 35–38 kDa myofibrillar protein, represents a major allergen in molluscs, as well as in crustaceans. Besides tropomyosin, a 100 kDa allergen was newly detected in the disc abalone *Haliotis discus discus*. The 100 kDa allergen was purified from the muscle of the disc abalone by salting-out and hydroxyapatite HPLC and identified as paramyosin based on the determined amino acid sequences of the peptide fragments produced by lysylendopeptidase digestion. Based on analysis by fluorescence ELISA, as many as 16 of the 18 patient sera tested, reacted to the disc abalone tropomyosin. The same patient sera also reacted to the disc abalone paramyosin, although rather less potently than to tropomyosin, suggesting that paramyosin is a major allergen. Immunoblotting data showed that IgE-reactive paramyosin is distributed in some species of molluscs other than the disc abalone. Interestingly, cross-reactivity between paramyosin and tropomyosin was demonstrated by inhibition immunoblotting and inhibition ELISA.

1. Introduction

Food allergy mediated by immunoglobulin E (IgE) is a public concern in industrialised countries. In sensitised subjects with high levels of specific IgE to a certain food, adverse reactions such as urticaria, asthma, diarrhoea and anaphylaxis are induced immediately after ingestion of the food; in severe cases, anaphylactic shock leads to even death. Of a number of allergenic foodstuffs, seafoods (fish, crustaceans and molluscs) are recognised as an important cause of food allergy especially in coastal countries, where their consumption is high. Previous studies showed that parvalbumin, a 12 kDa sarcoplasmic protein, is a major cross-reactive allergen in fish (Lehrer, Ayuso, & Reese, 2003; Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005; Wild & Lehrer, 2005). Besides parvalbumin, collagen has been additionally identified as a fish allergen although not major (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000). As for crustaceans and molluscs, tropomyosin, a 35–38 kDa myofibrillar protein, is a common major allergen (Chuo, Wong, & Leung, 2000; Emoto, Ishizaki, & Shiomi, 2009; Leung et al., 1996; Motoyama, Ishizaki, Nagashima, & Shiomi, 2006; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Reese, Ayuso, & Lehrer, 1999; Wild & Lehrer, 2005). IgE cross-reactivity of tropomyosin can be seen amongst crustaceans, amongst molluscs, between crustaceans and molluscs, and even between crustaceans and terrestrial arthropods, such as cockroaches and mites (Arlian, Morgan, Vyszenski-Moher, & Sharra, 2009; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002). Recently, arginine kinase (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007; Yu, Lin, Chiang, & Chow, 2003), a sarcoplasmic calcium-binding protein (Ayuso et al., 2009; Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008) and myosin light chain (Ayuso et al., 2008) have also been confirmed to be new allergens in crustaceans.

To prevent allergic accidents, labelling systems for processed food products containing allergenic food materials have recently been established in some countries. In Japan, 25 kinds of food materials are considered to be especially allergenic, and therefore are obligated or recommended to put labels on packages or bottles of processed food products containing them as raw materials. Abalone, together with other six kinds of seafoods (salmon, mackerel, shrimp, crab, squid and salmon roe), is amongst the 25 kinds of allergenic food materials. Molecular studies with two species of abalones, the Japanese abalone *Haliotis diversicolor* (Chuo et al., 2000), and the disc abalone *Haliotis discus discus* (Emoto et al., 2009), proved that their major allergen is tropomyosin, as in the other molluscs. During our study for allergens in disc abalone by immunoblotting, however, not only tropomyosin but also a 100 kDa allergen was found to be allergenic. This finding prompted us to purify and identify the 100 kDa allergen in the disc abalone. As a result, the 100 kDa allergen was demonstrated to be paramyosin by partial amino acid sequencing of the purified preparation. Subsequent ELISA experiments showed that paramyosin, as well as tropomyosin, is a major allergen in disc abalone. Interestingly,

* Corresponding author. Tel.: +81 3 5463 0601; fax: +81 3 5463 0669.
E-mail address: shiomi@kaiyodai.ac.jp (K. Shiomi).

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doi:10.1016/j.foodchem.2010.07.020

the IgE cross-reactivity between paramyosin and tropomyosin was also verified by inhibition experiments. We report here the identification of the 100 kDa allergen in the disc abalone, as paramyosin, and the cross-reactivity of paramyosin with tropomyosin.

2. Materials and methods

2.1. Animal samples

Live or fresh specimens of the disc abalone, turban shell *Turbo cornutus*, Mediterranean mussel *Mytilus galloprovincialis*, Yesso scallop *Patinopecten yessoensis*, Japanese flying squid *Todarodes pacificus*, and common octopus *Octopus vulgaris*, were purchased at the Tokyo Central Wholesale Market. Columellar muscle was collected from disc abalone, foot muscle from turban shell and Mediterranean mussel, adductor muscle from Yesso scallop, mantle muscle from Japanese flying squid, and leg muscle from common octopus. All muscle samples were stored at −20 °C until use.

2.2. Preparation of crude extracts

Each muscle sample was ground to powder in liquid nitrogen and homogenised with four volumes of 0.9 M NaCl–0.01 M phosphate buffer (pH 6.8). A part of the homogenate was centrifuged at 18,000*g* for 20 min, and the supernatant obtained was used as a non-heated extract. The remaining homogenate was heated in a boiling water bath for 10 min, similarly centrifuged, and the supernatant was regarded as a heated extract. Both non-heated and heated extracts were subjected to immunoblotting to examine whether the 100 kDa allergen under study is heat-stable or not.

2.3. Purification of tropomyosin

The disc abalone tropomyosin was purified from the muscle as reported in our previous paper (Motoyama et al., 2007). In brief, an acetone powder of myofibrillar proteins prepared from the muscle was extracted with 0.025 M Tris–HCl buffer (pH 8.0), containing 1 M KCl, 0.1 mM $CaCl_2$ and 0.1 mM dithiothreitol (DTT). The extract was subjected to salting-out with ammonium sulphate (30–60% saturation), followed by isoelectric precipitation (pH 4.6). Finally, small amounts of contaminants were removed by reverse-phase HPLC. The homogeneity of the final preparation was supported by SDS–PAGE.

2.4. Purification of a 100 kDa allergen

The muscle (5 g) of disc abalone was homogenised with 20 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.15 M NaCl and 0.5 mM cysteine. After centrifugation at 18,000*g* for 20 min, the supernatant obtained was regarded as a sarcoplasmic protein fraction. The residue was washed twice with the above solvent and shaken vigorously with 25 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl and 5 mM DTT at 4 °C, for 14 h. Then, $MgCl_2$ and ATP were added to the solution until a final concentration of 1 mM for each was reached, and shaken again for 1 h. After ultracentrifugation at 117,000*g* for 1 h, the supernatant obtained was used as a myofibrillar protein fraction. To purify the 100 kDa allergen, the myofibrillar protein fraction was first subjected to salting-out using ammonium sulphate. The precipitate at a 10–20% saturation of ammonium sulphate was dissolved in 10 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl, 5 mM DTT, and 0.01% thimerosal, and was applied to hydroxyapatite HPLC on a Bio-Scale CHT2-I column (0.7 × 5.2 cm; Bio-Rad Laboratories, Hercules, CA, USA). Elution was achieved with a linear gradient of 0.01–0.24 M phosphate buffer (pH 6.8) containing 0.9 M NaCl at a flow rate of 1 ml/min. The proteins were monitored at 280 nm with a UV detector and the eluate containing the 100 kDa allergen was collected.

2.5. Enzymatic digestion and isolation of the peptide fragments

The purified 100 kDa allergen (150 μg) was digested with 1.5 μg of lysylendopeptidase (Wako Pure Chemicals, Osaka, Japan) in 0.5 ml of 0.025 M Tris–HCl buffer (pH 8.5) containing 1 mM EDTA, 4 M urea and 0.02 M ethanolamine at 37 °C, for 18 h. To isolate the peptide fragments produced, the digest was applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh, Tokyo, Japan), which was eluted at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0–70% in 120 min) in 0.1% trifluoroacetic acid. Peptides were monitored at 220 nm with a UV detector.

2.6. Amino acid sequencing

Amino acid sequencing was performed by the automated Edman degradation method using a Procise 492HT protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.7. Human sera

Sera were obtained from 18 crustacean-allergic patients (patients 1–18) with clinical histories of immediate hypersensitivity reactions after ingestion of crustaceans. All patients were diagnosed to be allergic to crustaceans by the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) performed at hospitals; the determined CAP-RAST classes were 2–6 against shrimp, or both shrimp and crab. Patients 1, 2, and 12–14 were additionally shown to have CAP-RAST classes of 3–6 against molluscs. Written informed consent was obtained from each patient. In this study, sera from 19 healthy volunteers without adverse reactions after ingestion of any foods were used as controls. All sera were stored at −20 °C until use.

2.8. Fluorescence ELISA and inhibition ELISA

Fluorescence ELISA was performed as reported previously (Hamada et al., 2004). In brief, a polystyrene microtiter plate with 96 wells (Type H (black); Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of the 100 kDa allergen or the disc abalone tropomyosin solution (1 μg/ml) and reacted successively with the patient or control serum (diluted 1:200) and β-galactosidase-conjugated goat anti-human IgE antibody (0.25 μg/ml; American Qualex, San Clement, CA, USA). The enzyme reaction was carried out using as substrate a solution of 0.1 mg/ml 4-methylumbelliferyl-β-D-galactoside, and was stopped by addition of 0.1 M glycine-NaOH buffer (pH 10.3). Fluorescence units were measured with excitation and emission wavelengths at 367 and 453 nm, respectively. For inhibition ELISA, patient serum (diluted 1:100) was pre-incubated with an equal volume of inhibitor (100 kDa allergen or disc abalone tropomyosin) solution (0.002–20 μg/ml) at 37 °C for 2 h, and then used as a primary antibody. All ELISAs (including inhibition ELISAs) were performed in triplicate, and the data obtained were expressed in mean ± SD.

2.9. SDS–PAGE

SDS–PAGE was performed on a PhastSystem apparatus (GE-Healthcare, Buckinghamshire, UK) using ready-made gels (PhastGel Gradient 8-25; GE-Healthcare) and ready-made buffer strips (PhastGel SDS Buffer Strips; GE-Healthcare), according to the

manufacturer's instructions. Each sample was dissolved in 0.0625 M Tris–HCl buffer (pH 7.4) containing 2% SDS, 4 M urea and 0.1 M DTT, heated at 70 °C for 10 min, and subjected to electrophoresis. Precision plus protein standards (Bio-Rad Laboratories) were run as references, along with the samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

2.10. Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as reported previously (Kobayashi et al., 2006), using a pooled serum from 10 patients (patients 1–10) or 10 healthy volunteers. Briefly, the proteins of the crude non-heated and heated extracts, firstly separated by SDS–PAGE, were electrotransferred from the gel to a polyvynyliden difluoride membrane, which was reacted successively with patient or control serum (diluted 1:200) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:5000; Kirkegaard and Perry Laboratories, Gaitherburg, MD, USA). The blots were visualised using an ECL Plus Western Blotting Detection System (GE-Healthcare) and an ECL Mini Camera (GE-Healthcare), as recommended by the manufacturer. For inhibition IgE-immunoblotting, patient serum (diluted 1:100) was preincubated with an equal volume of inhibitor (100 kDa allergen or disc abalone tropomyosin) solution (20 μg/ml) at 37 °C for 2 h, and used as a primary antibody.

2.11. Protein determination

Protein was determined using a Protein Assay Kit (Bio-Rad Laboratories) based on the method of Bradford (1976). Bovine serum albumin was used as a standard protein.

3. Results

3.1. Purification and identification of a 100 kDa allergen in disc abalone

The 100 kDa allergen was mostly recovered in the myofibrillar protein fraction, suggesting that it is one of myofibrillar proteins. Its purification from the myofibrillar protein fraction was achieved by salting-out with ammonium sulphate (10–20% saturation), followed by hydroxyapatite HPLC on a Bio-Scale CHT2-I column. In hydroxyapatite HPLC, the 100 kDa allergen was eluted in a symmetrical peak at a retention time of 23.8 min (Fig. 1A). The 100 kDa allergen thus obtained was confirmed to be homogeneous, as analysed by SDS–PAGE (Fig. 1B).

Following digestion of the purified 100 kDa allergen with lysylendopeptidase, a number of peptide fragments was isolated by reverse-phase HPLC on a TSKgel ODS-120T column (data not shown). Of the isolated peptide fragments, four fragments (peptides 1–4) were randomly selected and sequenced. A database search by the BLAST algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990) revealed that the peptides 1–4 best fitted the 121–135, 147–158, 203–214 and 825–836 regions of Mediterranean mussel paramyosin (accession number AB016070 of the DDBJ/EMBL/GenBank databases), respectively (Fig. 2). The peptide 3 was completely identical with the 203–214 region of the Mediterranean mussel paramyosin, and the other peptides had only two to four alterations compared to the corresponding regions of Mediterranean mussel paramyosin. These results allowed us to conclude that the 100 kDa allergen was a paramyosin of the disc abalone.

3.2. Reactivity of patient sera to paramyosin and tropomyosin

Both paramyosin (100 kDa allergen) and tropomyosin purified from the disc abalone were evaluated for IgE reactivity by fluorescence ELISA using 18 sera from crustacean-allergic patients. Five patients (1, 2, and 12–14) had CAP-RAST classes of 3–6 against molluscs, whilst no information was available as to whether the other patients are allergic to molluscs. Nevertheless, positive reactivity to the disc abalone tropomyosin was found in as many as 16 patient sera (Fig. 3), conforming well to the current opinion that tropomyosin is a major cross-reactive allergen in crustaceans and molluscs (Chuo et al., 2000; Emoto et al., 2009; Leung et al., 1996; Motoyama et al., 2006, 2007; Reese et al., 1999; Wild & Lehrer, 2005). It was of particular interest that the same 16 patient sera also reacted to paramyosin although rather less potently than to tropomyosin. This demonstrated that paramyosin, like tropomyosin, is a major allergen of the disc abalone.

3.3. Distribution of allergenic paramyosin in molluscs

When analysed by SDS–PAGE, both 37 and 100 kDa proteins were recognised in the non-heated extracts from six species of molluscs (disc abalone, turban shell, Mediterranean mussel, Yes-

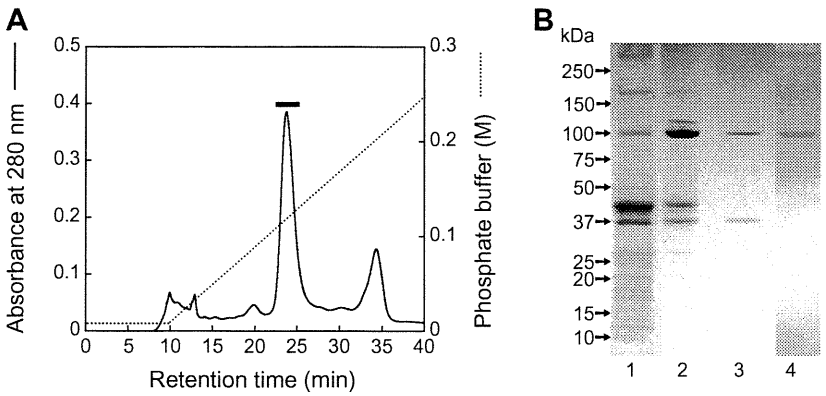

Fig. 1. Purification of a 100 kDa allergen in the disc abalone by hydroxyapatite HPLC (A) and analysis of its homogeneity by SDS–PAGE (B). (A) Sample, precipitate with a 10–20% saturation of ammonium sulphate from the myofibrillar protein fraction; column, Bio-Scale CHT2-I column (0.7 × 5.2 cm); elution, linear gradient of 0.01–0.25 M phosphate buffer (pH 6.8) containing 0.9 M NaCl; flow rate, 1 ml/min. A bar indicates the elution position of the 100 kDa allergen. (B) Samples: lane 1, sarcoplasmic protein fraction; lane 2, myofibrillar protein fraction; lane 3, precipitate with a 10–20% saturation of ammonium sulphate from the myofibrillar protein fraction; lane 4, purified 100 kDa allergen.

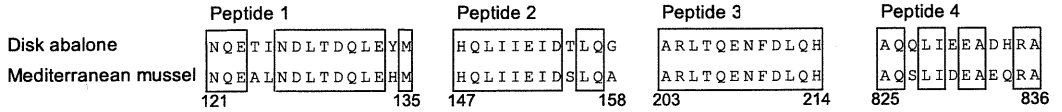

Fig. 2. Amino acid sequences of peptides 1–4 produced by digestion of the purified 100 kDa allergen with lysylendopeptidase, and the corresponding regions of Mediterranean mussel paramyosin. Identical residues are boxed. The accession number (DDBJ/EMBL/GenBank) for Mediterranean mussel paramyosin is AB016070.

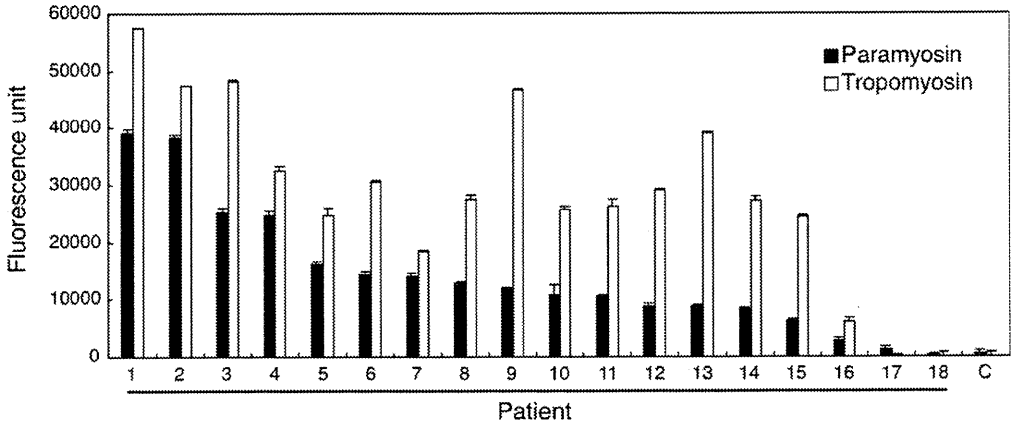

Fig. 3. Analysis of IgE reactivity of paramyosin and tropomyosin purified from the disc abalone by fluorescence ELISA using 18 patient sera. The control data (denoted by C) obtained with 19 healthy subjects were averaged and the values above mean + 2SD (fluorescence units of 1739 and 1466 for paramyosin and tropomyosin, respectively) were judged to be positive.

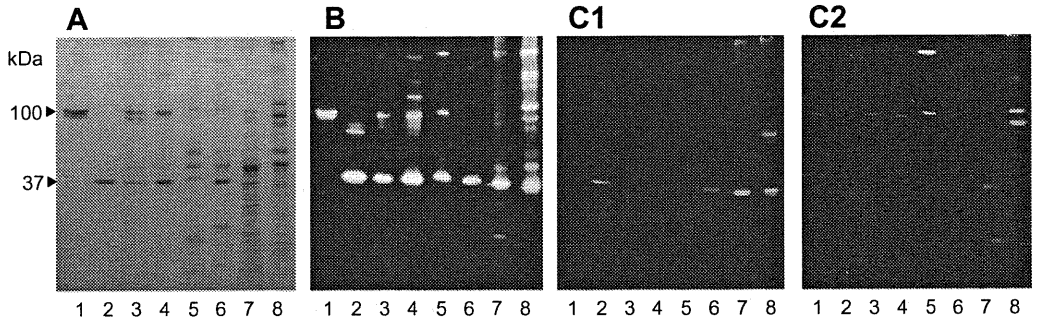

Fig. 4. Analysis of the non-heated extracts from six species of molluscs by SDS–PAGE (A), immunoblotting (B), and inhibition immunoblotting (C). Lanes: 1, disc abalone paramyosin; 2, disc abalone tropomyosin; 3, extract from disc abalone; 4, extract from turban shell; 5, extract from Mediterranean mussel; 6, extract from Yesso scallop; 7, extract from Japanese flying squid; 8, extract from common octopus. In immunoblotting, the patient serum (pooled from 10 patients; 1:200 dilution) was used as a primary antibody. In inhibition immunoblotting, the patient serum (1:100 dilution) was preincubated with an equal volume of inhibitor (disc abalone paramyosin and tropomyosin for C1 and C2, respectively) solution (20 μg/ml) prior to use as a primary antibody.

so scallop, Japanese flying squid and common octopus), although the staining intensity considerably differed from sample to sample (Fig. 4A). In immunoblotting, the control serum (pooled from 10 healthy volunteers) showed no reactivity to any proteins (data not shown) whilst the patient serum (pooled from patients 1–10) strongly reacted to the 37 kDa protein (corresponding to tropomyosin) in all the six species and weakly to the 100 kDa protein (corresponding to paramyosin) in at least four species (disc abalone, turban shell, Mediterranean mussel and common octopus) (Fig. 4B). In the case of the heated extracts, the 37 kDa protein (tropomyosin) was clearly observed in six species (Fig. 5A) and shown to be IgE-reactive as well (Fig. 5B). However, the 100 kDa protein (paramyosin) was not observed in any species, as analysed by either SDS–PAGE or immunoblotting. This suggested that paramyosin, unlike tropomyosin, becomes insoluble upon heat denaturation, being absent in the heated extracts.

3.4. IgE cross-reactivity of paramyosin with tropomyosin

In inhibition immunoblotting experiments using the disc abalone paramyosin as an inhibitor, the reactivity of the patient serum to molluscan paramyosins was completely lost, and that to molluscan tropomyosins was also lost or remarkably reduced (Fig. 4C1). On the other hand, the patient serum preincubated with the disc abalone tropomyosin showed no reactivity to both molluscan paramyosins and tropomyosins (Fig. 4C2). These results implied cross-reactivity, not only between molluscan paramyosins, but also between molluscan paramyosins and tropomyosins. Further evidence for the cross-reactivity between paramyosin and tropomyosin was obtained by the inhibition ELISA experiments. As shown in Fig. 6, the reactivity of the patient serum to either paramyosin or tropomyosin was inhibited in a dose-dependent manner by both paramyosin and tropomyosin, although much stronger inhibitory potencies were observed with tropomyosin.

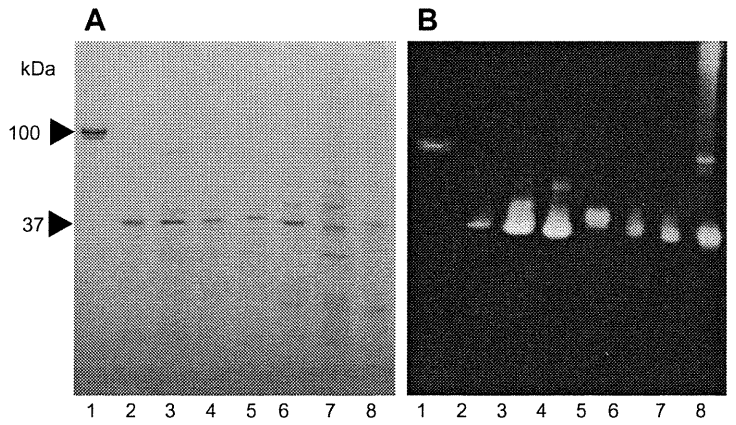

Fig. 5. Analysis of the heated extracts from six species of molluscs by SDS–PAGE (A) and immunoblotting (B) samples are the same as in Fig. 4.

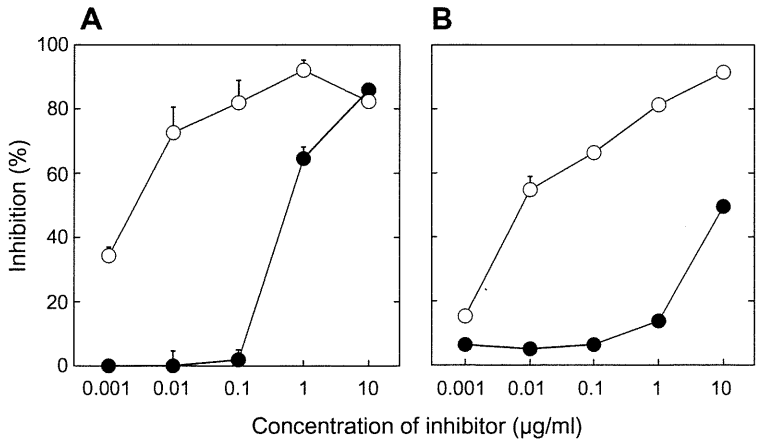

Fig. 6. Quantitative analysis of the IgE cross-reactivity between the disc abalone paramyosin and tropomyosin by inhibition ELISA. A microtiter plate was coated with 50 μl of paramyosin (A) or tropomyosin (B) solution (1 μg/ml). The patient serum (pooled from 10 patients; 1:100 dilution) was preincubated with an equal volume of the disc abalone paramyosin (●) or tropomyosin (○), and was used as a primary antibody.

4. Discussion

Tropomyosin, a 35–38 kDa myofibrillar protein, is a major allergen in molluscs as well as in crustaceans (Chuo et al., 2000; Emoto et al., 2009; Leung et al., 1996; Motoyama et al., 2006, 2007; Reese et al., 1999; Wild & Lehrer, 2005). In this study, however, a 100 kDa allergen differing from tropomyosin was purified from the muscle of the disc abalone *Haliotis discus discus* by salting-out and hydroxyapatite HPLC, and was clearly identified as paramyosin, one of the myofibrillar proteins, based on the determined partial amino acid sequence of the purified preparation. Immunoblotting and inhibition immunoblotting experiments suggested that paramyosin is not a specific allergen of the disc abalone but a cross-reactive allergen in some molluscs. Recently, arginine kinase (García-Orozco et al., 2007; Yu et al., 2003), sarcoplasmic calcium-binding protein (Ayuso et al., 2009; Shiomi et al., 2008) and myosin light chain (Ayuso et al., 2008) have been shown to be new allergens in crustaceans, whilst allergens other than tropomyosin have not been identified in molluscs. In this sense, paramyosin is the second allergen of molluscs, besides tropomyosin.

As described above, paramyosin is a cross-reactive allergen in some molluscs. Moreover, paramyosin is a major allergen in the disc abalone as demonstrated by fluorescence ELISA, in which as many as 16 of the 18 patient sera tested reacted to paramyosin, as well as to tropomyosin. This is presumably the case with other molluscan paramyosins. Despite these circumstances, paramyosin has long been overlooked in previous studies on molluscan allergens. In relation to this, it should be noted that heating procedures were included in preparing the extracts in previous studies; the extracts were prepared from the homogenates of boiled samples (Leung et al., 1996) or by heating the homogenates of raw samples (Emoto et al., 2009; Motoyama et al., 2006). Since tropomyosin is very thermostable, it can be detected in the extracts by SDS–PAGE and immunoblotting, or can be purified from the extracts by chromatography. In contrast, paramyosin is rather unstable to heating as evidenced in this study. This thermolabile property possibly accounts for the missing of paramyosin in previous studies on molluscan allergens.

Paramyosin is an invertebrate-specific protein that forms the core of the myosin-containing thick filaments (Watabe & Hartshorne, 1990). Prior to this study, paramyosin has already been established to be one of the allergens in some invertebrates, such as house dust mites (Tsai et al., 1999, 2005) and the fish parasite *Anisakis simplex* (Pérez-Pérez et al., 2000). It is thus possible that paramyosin, like tropomyosin, is a pan-allergen in invertebrates, although it is still unknown whether crustacean paramyosin is allergenic. However, paramyosin is obviously less IgE-reactive and also less thermostable than tropomyosin, suggesting that paramyosin contributes less to the adverse reactions induced after ingestion of molluscs, especially cooked molluscs, than tropomyosin.

The most significant finding of this study is that molluscan paramyosins are cross-reactive not only with one another but also with molluscan tropomyosins. The fact that the 16 patients recognising the disc abalone tropomyosin are all sensitive to the disc abalone paramyosin can be easily understood by the cross-reactivity between paramyosin and tropomyosin. So far, tropomyosins in various species of molluscs have been elucidated for their primary structures. In contrast, no information about the primary structures of molluscan paramyosins is available, except for the Mediterranean mussel paramyosin (AB016070 of the DDBJ/EMBL/GenBank databases). At present, therefore, it is impossible to explain at the molecular level the observed cross-reactivity between molluscan paramyosins and tropomyosins.

In conclusion, the present study demonstrated that paramyosin is the second allergen of molluscs. To clarify whether paramyosin is a pan-allergen in invertebrates, it is important to examine the allergenicity of crustacean paramyosins. Furthermore, the primary structures of various molluscan paramyosins should be elucidated for a molecular understanding of the cross-reactivity, not only amongst molluscan paramyosins, but also between molluscan paramyosins and tropomyosins.

Acknowledgments

The authors would like to thank Drs. A. Urisu and Y. Kondo, Fujita Health University School of Medicine, and Drs. H. Ohsuna and Z. Ikezawa, Department of Dermatology, Yokohama City University, for providing the patient sera. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and a grant from the Ministry of Health, Labour and Welfare of Japan.

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Food Chemistry 132 (2012) 835–840

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Purification, immunological properties and molecular cloning of two allergenic parvalbumins from the crimson sea bream, *Evynnis japonica*

Fang-Fei Guo, Hiroaki Kubota, Kazuo Shiomi *

Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan

ARTICLE INFO

Article history:
Received 3 April 2011
Received in revised form 16 October 2011
Accepted 9 November 2011
Available online 15 November 2011

Keywords:
Allergen
Crimson sea bream
Evynnis japonica
Parvalbumin

ABSTRACT

Parvalbumin, a calcium-binding sarcoplasmic protein of 12 kDa, represents a major allergen of fish. Previous immunoblotting experiments suggested the presence of a 14 kDa allergen, together with parvalbumin, in sea breams. In this study, the 14 kDa allergen (PA I) and parvalbumin (PA II) were purified from the white muscle of crimson sea bream, *Evynnis japonica*, by gel filtration and reverse-phase HPLC. Amino acid sequencing of lysylendopeptidase peptide fragments demonstrated that both PA I and PA II were isoforms of parvalbumin. As analysed by ELISA, PA I showed weaker reactivities with IgG (monoclonal or polyclonal antibody) and serum IgE in fish-allergic patients than did other fish parvalbumins, including PA II. The amino acid sequences of PA I and PA II were elucidated by cDNA cloning. PA I was found to have more specific amino acid residues at several positions compared to the other fish parvalbumins.

1. Introduction

Food allergy is a representative of type I allergies, mediated by immunoglobulin E (IgE) antibodies, and is a public concern in industrialised countries since even death may occur in severe cases. Among a number of allergenic foodstuffs, fish is recognised as an important cause of food allergy, especially in coastal countries, including Japan, where their consumption is high. The major fish allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa, as demonstrated with various species of fish, such as carp (Bugajska-Schretter et al., 1999; Swoboda et al., 2002), salmon (Lindstrøm, van Dô, Hordvik, Endresen, & Elsayed, 1996), cod (Elsayed & Aas, 1971a, 1971b; Elsayed & Bennich, 1975), horse mackerel (Shiomi, Hayashi, Ishikawa, Shimakura, & Nagashima, 1998), mackerel (Hamada et al., 2003) and tuna (Shiomi, Hamada, Sekiguchi, Shimakura, & Nagashima, 1999). Besides parvalbumin, three classes of proteins, namely collagen comprised of three 100 kDa α chains (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000), aldehyde phosphate dehydrogenase of 41 kDa (Das Dores et al., 2002) and transferrin of 94 kDa (Kondo et al., 2006), have so far been identified as fish allergens, although not major ones.

It should be noted that unidentified allergens, such as a 25 kDa allergen in swordfish (Kelso, Jones, & Yunginger, 1996) and a 63 kDa allergen in codfish (Mata et al., 1994), still exist in some species of fish. Our previous immunoblotting experiments have also revealed that the red sea bream, *Pagrus major*, contains a 14 kDa allergen, together with parvalbumin (Kobayashi et al., 2006). The 14 kDa allergen was considered to be distinguishable from parvalbumin, since it did not react with the monoclonal anti-frog muscle parvalbumin antibody. Interestingly, the 14 kDa allergen (having no reactivity with the monoclonal anti-parvalbumin antibody) was also found in the crimson sea bream, *Evynnis japonica*, in our preliminary experiments, suggesting that the 14 kDa allergen seems to be commonly contained in sea breams. For a better understanding of the sea bream allergy, this study was initiated to identify the 14 kDa allergen in the crimson sea bream. As a result, the 14 kDa allergen (named PA I) was demonstrated to be a parvalbumin isoform showing less IgG and IgE reactivities than the other 12 kDa parvalbumin (named PA II) of crimson sea bream and parvalbumins of horse mackerel, *Trachurus japonicas*, and Pacific mackerel, *Scomber japonicus*. We here report the purification, immunological properties and molecular cloning of two allergenic parvalbumin isoforms, PA I and II, from the crimson sea bream.

2. Materials and methods

2.1. Fish samples

Live specimens of crimson sea bream were purchased at the Tokyo Central Wholesale Market and fresh specimens of horse mackerel and Pacific mackerel at a local retail shop. White muscle samples collected from these fish were stored at −20 °C until used. For molecular cloning experiments, the white muscle obtained from a live specimen of crimson sea bream was immediately frozen in liquid nitrogen and stored at −80 °C until used.

* Corresponding author. Tel.: +81 3 5463 0601; fax: +81 3 5463 0669.
E-mail address: shiomi@kaiyodai.ac.jp (K. Shiomi).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.11.046