

Original article

Comparison of allergenicity and allergens between fish white and dark muscles

Background: Fish is one of the most frequent causes of immunoglobulin E (IgE)-mediated food allergy. Although the fish dark muscle is often ingested with the white muscle, no information about its allergenicity and allergens is available.

Methods: Heated extracts were prepared from both white and dark muscles of five species of fish and examined for reactivity with IgE in fish-allergic patients by enzyme-linked immunosorbent assay (ELISA) and for allergens by immunoblotting. Cloning of cDNAs encoding parvalbumins was performed by rapid amplification cDNA ends. Parvalbumin contents in both white and dark muscles were determined by ELISA using antiserum against mackerel parvalbumin.

Results: Patient sera were less reactive to the heated extract from the dark muscle than to that from the white muscle. A prominent IgE-reactive protein of 12 kDa, which was detected in both white and dark muscles, was identified as parvalbumin. Molecular cloning experiments revealed that the same parvalbumin molecule is contained in both white and dark muscles of either horse mackerel or Pacific mackerel. Parvalbumin contents were four to eight times lower in the dark muscle than in the white muscle.

Conclusions: The fish dark muscle is less allergenic than the white muscle, because the same allergen molecule (parvalbumin) is contained at much lower levels in the dark muscle than in the white muscle. Thus, the dark muscle is less implicated in fish allergy than the white muscle.

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Key words: allergenicity; dark muscle; fish; parvalbumin; white muscle.

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Accepted for publication 19 July 2005

Fish is an important food source rich in nutrients such as polyunsaturated fatty acids and lipid-soluble vitamins. However, it is obviously one of the most frequent causes of food allergy mediated by immunoglobulin E (IgE) antibodies, especially in coastal countries, such as Japan and Scandinavia, where the fish consumption is high. In fish-allergic patients, hypersensitivity reactions such as urticaria, asthma, vomiting are rapidly induced after ingestion of fish (1–3). Even fatal cases because of anaphylactic reactions have been reported (4, 5). The first identified fish allergen is parvalbumin (known as Gad c 1) of the cod *Gadus callarias* (6, 7). Parvalbumin is a calcium-binding sarcoplasmic protein with a molecular mass of about 12 kDa and is distributed universally in the white muscle of fish. In accordance with this, subsequent molecular studies with carp *Cyprinus carpio* (8), Atlantic salmon *Salmo salar* (9), Atlantic cod *G. morhua* (10) and Pacific mackerel *Scomber japonicus* (11), together with IgE-immunoblotting studies (2, 12–14), have demonstrated that parvalbumin represents the major and cross-reactive allergen in common with various species of fish.

Teleost fish have two types of muscle, white muscle (also called light muscle) and dark muscle (also called

red muscle). The white muscle is used for short bursts of swimming, while the dark muscle, which is located directly under the skin, is for continuous swimming. Accordingly, active fish such as tuna and skipjack have more developed dark muscle than bottom fish such as flounder and cod. Probably because the majority of the fish muscle is the white muscle even in active fish, all the previous studies on fish allergens have been performed using only the white muscle or that contaminated with a small amount of the dark muscle. However, the dark muscle of many species of fish is often ingested with the white muscle and hence is assumed to be implicated to some extent in hypersensitivity reactions caused by fish. Therefore, this study was initiated to clarify the allergenicity and allergens of the fish dark muscle for a better understanding of fish allergy. The results showed that the dark muscle was less allergenic than the white muscle and that the major allergen in the dark muscle was parvalbumin as in the white muscle. To obtain molecular evidence for the lower allergenicity of the dark muscle than the white muscle, primary structures and contents of parvalbumins in both white and dark muscles were also determined.

Materials and methods

Fish

Four species of active fish (sardine *Sardinops melanostictus*, yellowtail *Seriola quinqueradiata*, horse mackerel *Trachurus japonicus* and Pacific mackerel *Scomber japonicus*) and one species of bottom fish (red sea bream *Pagrus major*) were selected as samples, because these fish species are all widely eaten raw or cooked in Japan usually without removal of the dark muscle. Fresh specimens of each fish species were purchased at a local retail shop in Tokyo and immediately subjected to experiments or stored at -20°C until used. For cloning experiments, white and dark muscles were collected from a live specimen of either horse mackerel or Pacific mackerel, immediately frozen in liquid nitrogen and stored at -80°C until used.

Preparation of heated extracts

White and dark muscles of each fish specimen were separately homogenized with three volumes of 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. As this study was focused on parvalbumin, a heat-stable allergen, the homogenate was heated in a boiling water bath for 10 min. After centrifugation at 18 000 *g* for 20 min, the supernatant obtained was used as heated extract.

Purification of parvalbumin

Parvalbumins in the white muscles of horse mackerel and Pacific mackerel were individually purified by a combination of gel filtration and reverse-phase high-performance liquid chromatography (HPLC), as described elsewhere (11, 15).

Human sera

Sera were obtained from nine fish-allergic patients with documented clinical histories of immediate hypersensitivity reactions after ingestion of fish. Written informed consent was obtained from each patient. All the patient sera had been shown to elevate IgE specific for fish parvalbumin by our previous enzyme-linked immunosorbent assay (ELISA) experiments (16). In the present study, sera from nine healthy volunteers without adverse reactions after ingestion of any foods were pooled and used as a control. All sera were stored at -20°C until used.

ELISA

The ELISA was performed using a flat-bottomed polystyrene plate with 96-wells, as reported previously (17). To evaluate the allergenicity of the white and dark muscles, each heated extract of 1000-, 3000- or 10 000-fold dilution was coated on the plate and immunoreacted with patient serum (diluted 1 : 50), followed by peroxidase-conjugated goat antihuman IgE antibody (diluted 1 : 2500; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Enzyme reaction was carried out using substrate solution (0.1% *o*-phenylenediamine and 0.03% hydrogen peroxide in 0.05 M phosphate-citrate buffer, pH 5.0) and the developed colour was measured at 490 nm on a Kinetic Microplate Reader model 550 (Bio-Rad Laboratories, Hercules, CA, USA). For the quantification of parvalbumin in the white and dark muscles, each heated extract was diluted appropriately, coated on the plate and successively reacted with antiserum (diluted 1 : 5000; a gift from Mr H. Nakajima, Maruha Co., Tokyo, Japan) raised in rabbits against Pacific mackerel parvalbumin and peroxidase-conjugated goat

antirabbit IgG antibody (diluted 1 : 2500; Kirkegaard & Perry Laboratories). To make a calibration curve, various concentrations (0.001–10 $\mu\text{g/ml}$) of parvalbumin purified from horse mackerel or Pacific mackerel were subjected to ELISA. All ELISAs were performed in triplicate and the data were expressed in mean values.

The IgE-binding ability of parvalbumin, the target protein of this study, has been reported to be strongly reduced by treatment with chelating reagents leading to Ca^{2+} -depletion (18). In our preliminary ELISA experiments using purified Pacific mackerel parvalbumin, however, its IgE-binding ability did not depend on the presence of Ca^{2+} in the buffers used for dilution of patient sera and parvalbumin, suggesting that parvalbumin usually binds Ca^{2+} in the absence of chelating reagents. Moreover, heated extracts from fish muscles must be rich in Ca^{2+} . In the present study, therefore, Ca^{2+} was not added to the buffers used for dilution of patient sera, antiserum and sample solutions.

SDS-PAGE and immunoblotting

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem apparatus (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Ready-made gels (PhastGel gradient 8–25) and ready-made buffer strips (PhastGel SDS buffer strips) were purchased from Amersham Biosciences. Each sample was dissolved in 0.0625 M phosphate buffer (pH 7.5) containing 2% SDS, 3 M urea and 0.1 M dithiothreitol, heated at 100°C for 10 min and subjected to electrophoresis. Precision plus protein standards (Bio-Rad Laboratories) were run as a reference, along with samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

In immunoblotting, the proteins separated by SDS-PAGE were first electrotransferred from the gel to a polyvinylidene difluoride membrane using the PhastSystem apparatus equipped with a PhastTransfer (Amersham Biosciences), as described in the manufacturer's manual. Transfer of the proteins to the membrane was confirmed by staining with Ponceau S. The membrane was then washed with Tween-phosphate-buffered saline (PBS; Dulbecco's PBS, pH 7.4, containing 0.05% Tween 20) and blocked with 10% skimmed milk in Tween-PBS at 4°C overnight. After washing with Tween-PBS, the membrane was reacted successively with patient serum (diluted 1 : 500) at 37°C for 3 h and peroxidase-conjugated goat antihuman IgE antibody (diluted 1 : 10 000) at 37°C for 1 h. In the case of the detection of parvalbumin, the membrane was reacted with monoclonal antifrog muscle parvalbumin antibody (diluted 1 : 20 000; Sigma, St Louis, MO, USA) at 37°C for 1 h, followed by peroxidase-conjugated goat antimouse IgG antibody (diluted 1 : 20 000; Kirkegaard & Perry Laboratories) at 37°C for 1 h. Antigen-antibody binding was visualized using an ECL Plus Western blotting detection system (Amersham Biosciences) and an ECL mini camera (Amersham Biosciences), according to the manufacturer's instructions.

Cloning experiments

Total RNA was extracted from 2 g of each muscle sample with the Trizol reagent (Life Technologies, Rockville, MD, USA) and poly(A)⁺ mRNA was purified using an mRNA purification kit (Amersham Biosciences). A part of the purified mRNA was converted to cDNA, followed by ligation of API adapters, using a Marathon cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA, USA). The Marathon cDNA library thus constructed was applied to 3'- and 5'-rapid amplification cDNA ends (RACE). A forward primer (5'-TTGAGGAGGAGGAGCTGAAGCT-3') and

a reverse primer (5'-AGCTTCAGCTCCTCCTCTCAA-3'), which were designed based on the previously reported nucleotide sequence of the cDNA encoding the white muscle parvalbumin of Pacific mackerel (11), were used for 3'- and 5'-RACE, respectively, in combination with the API adapter primer. Amplification was performed using Ex Taq polymerase (Takara, Otsu, Japan) under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and 72°C for 7 min. The polymerase chain reaction (PCR) products were subcloned into the pT7Blue T-vector (Novagen, Darmstadt, Germany) and their nucleotide sequences were analysed using a PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and a PRISM 310 genetic analyzer (Applied Biosystems).

Results

Analysis of allergenicity by ELISA

A pooled serum from nine patients reacted in a dose-dependent manner to the heated extracts (1000-, 3000- and 10 000-fold dilutions) from the white and dark muscles of horse mackerel and Pacific mackerel (Fig. 1A). In both fish species, the reactivity to the dark muscle extract was significantly lower than that to the white muscle extract. Then, the heated extracts from the white and dark muscles of five species of fish were examined for IgE reactivity at a 1000-fold dilution, using three patient sera as well as the pooled serum. Regardless of the sera, essentially the same pattern of reactivity to the heated extracts was observed (Fig. 1B). First, the reactivity to the white muscle extract was high in horse mackerel and red sea bream and moderate in sardine and Pacific mackerel. Secondly, in common with these four species, the reactivity to the dark muscle extract was much lower than that to the white muscle extract. Thirdly, no

substantial reactivity was recognized with both white and dark muscle extracts of yellowtail.

Analysis of allergens by immunoblotting

Irrespective of the fish species and the muscle type, a 12 kDa protein was detected at the same position as the purified Pacific mackerel parvalbumin in the heated extract (Fig. 2A). This 12 kDa protein was reasonably identified as parvalbumin because it was reactive with the monoclonal antifrog muscle parvalbumin antibody (Fig. 2B). The pooled patient serum and three patient sera reacted almost specifically to the 12 kDa protein (parvalbumin), although no blots were observed in the dark muscle extract of yellowtail. It should be noted that the 12 kDa protein in the dark muscle was less stained with Coomassie Brilliant Blue and less reactive with both monoclonal antifrog muscle parvalbumin antibody and serum IgE than that in the white muscle, suggesting the lower content of parvalbumin in the dark muscle than in the white muscle.

Besides parvalbumin, another IgE-reactive protein of 14 kDa was found in both white and dark muscle extracts from red sea bream when analysed by the patient 3 serum (Fig. 2B). This protein did not react with the monoclonal antifrog muscle parvalbumin antibody. Analysis by SDS-PAGE showed that, among the five species of fish examined, only red sea bream contains the 14 kDa protein (Fig. 2A).

Primary structures of parvalbumins in white and dark muscles

The nucleotide sequences of the full-length cDNAs encoding parvalbumins contained in the white and dark

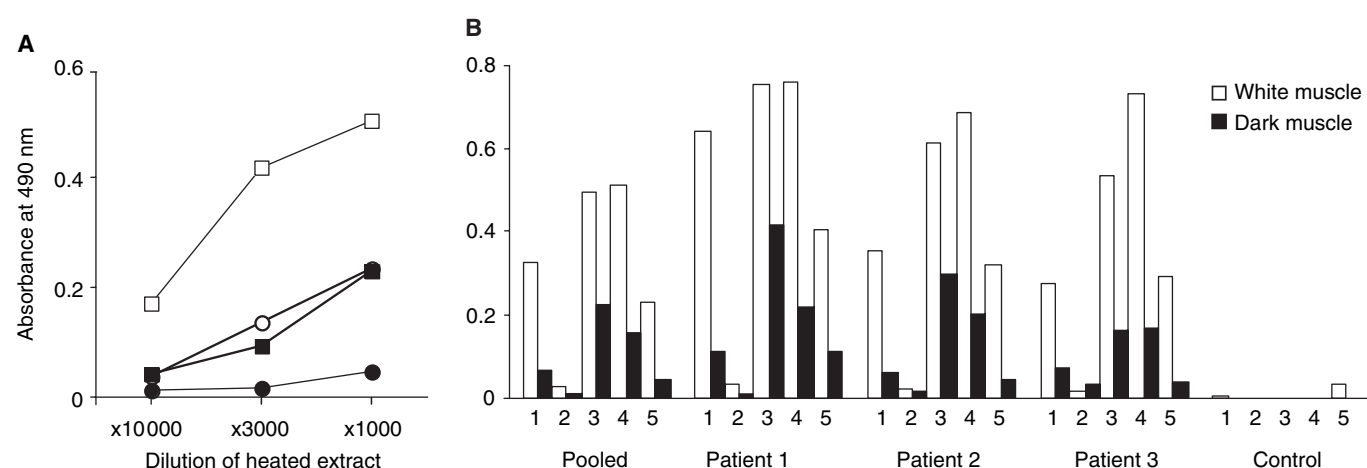


Figure 1. Analysis by enzyme-linked immunosorbent assay (ELISA) of the immunoglobulin E (IgE) reactivity of patient sera to the heated extracts from fish muscles. (A) IgE reactivity of the pooled patient serum to the heated extracts (1000-, 3000- and 10 000-fold dilutions) from horse mackerel white muscle (open square), horse mackerel dark muscle (closed square), Pacific mackerel white muscle (open circle) and Pacific mackerel dark muscle (closed circle). (B) IgE reactivity of the pooled patient serum and three patient sera to the heated extracts (1000-fold dilution) from white and dark muscles of sardine (1), yellowtail (2), horse mackerel (3), red sea bream (4) and Pacific mackerel (5).

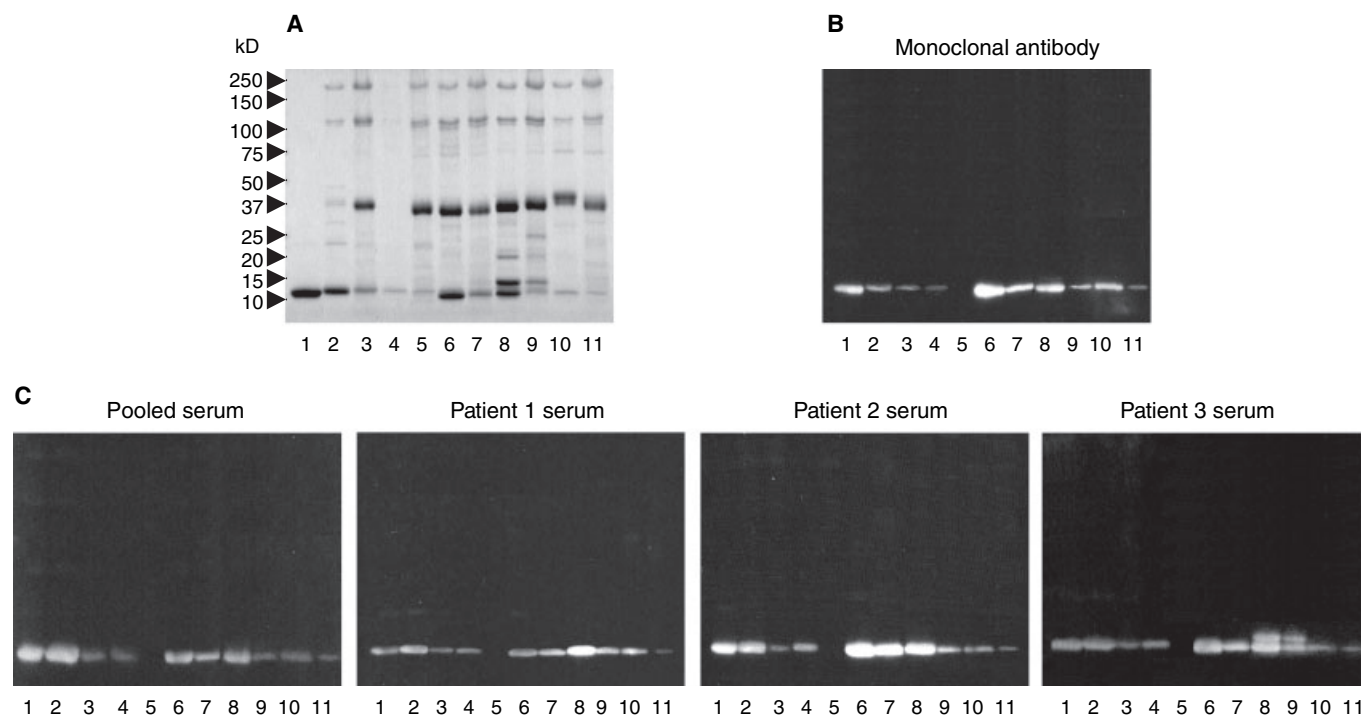


Figure 2. Analyses of the heated extracts from fish muscles by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; A), immunoblotting using monoclonal antifrog muscle parvalbumin antibody (B) and immunoglobulin E (IgE)-immunoblotting using the pooled patient serum and three patient sera (C). Lane 1, Pacific mackerel parvalbumin; 2, sardine white muscle; 3, sardine dark muscle; 4, yellowtail white muscle; 5, yellowtail dark muscle; 6, horse mackerel white muscle; 7, horse mackerel dark muscle; 8, red sea bream white muscle; 9, red sea bream dark muscle; 10, Pacific mackerel white muscle; 11, Pacific mackerel dark muscle.

muscles of horse mackerel and in the dark muscle of Pacific mackerel were elucidated by 3'- and 5'-RACE as aligned in Fig. 3 with that encoding parvalbumin in the white muscle of Pacific mackerel previously reported (11). In both fish species, the nucleotide sequence of the open reading frame encoding dark muscle parvalbumin is completely identical with that encoding white muscle parvalbumin. Only a slight difference is recognized between the cDNAs encoding parvalbumins in the white and dark muscles; when compared with the white muscle parvalbumin cDNA, the dark muscle parvalbumin cDNA of horse mackerel has five alterations in the 3'-untranslated region and that of Pacific mackerel has an insertion of 5 bp just before the poly(A) tail. It is thus apparent that the same parvalbumin molecule is expressed in both white and dark muscles of horse mackerel and Pacific mackerel. The horse mackerel parvalbumin showed 83% identity in amino acid sequence with the Pacific mackerel parvalbumin and was judged to be a member of β -type parvalbumins as previously discussed for the Pacific mackerel parvalbumin (11).

Contents of parvalbumin in white and dark muscles

Parvalbumin contents in both white and dark muscles of the five species of fish were determined by ELISA using

the antiserum raised in rabbits against the Pacific mackerel parvalbumin. In the present study, parvalbumins were purified from horse mackerel and Pacific mackerel and hence the calibration curves for the quantification of parvalbumin in these two species of fish could be separately made. As shown in Fig. 4, a little difference was observed between the two calibration curves, indicating that the antiserum used reacts to various fish parvalbumins with different potencies. In the case of sardine, yellowtail and red sea bream, for which purified parvalbumin preparations were unavailable, parvalbumin was determined using the calibration curve made for the Pacific mackerel parvalbumin. Although the parvalbumin contents determined for these three species of fish may slightly differ from the corrected values, it is considered that they accurately reflect the difference between the white and dark muscles, because the same parvalbumin molecule seems to be contained in both types of muscle of the three species of fish as established with horse mackerel and Pacific mackerel.

As summarized in Table 1, parvalbumin contents markedly varied among the fish species and also between the two types of muscle, being almost in parallel to the absorbances in ELISA reaction shown in Fig. 1. In the case of yellowtail, the dark muscle contained no detectable amounts of parvalbumin although only a small

A

White muscle	GAGACTTGTCTGTATCATCTGTACCCTACAAAGCAAAGACACCAAAGCTCACAATGGCCTTCAAAGGTGTACTGAATGATGCTGACGT	89
Dark muscle		88
	M A F K G V L N D A D V	12
White muscle	ACTGCAGCCCTGGACGGGTGCAAAAGTGCATTGACACACAAGGCATTCTTCAAAGCCTGCGGTCTGGCCGCCAAGTCCGCCGACGACAT	179
Dark muscle		178
	T A A L D G C K S A F D H K A F F K A C G L A A K S A D D I	42
White muscle	AAGAAGGCCTTCGCAATCATCGACCAGGACAAGAGCGGCTTCATTGAGGAGGATGAGCTGAAGCTGTTCTGCAGAACTTCTGCGCAGG	269
Dark muscle		268
	K K A F A I I D Q D K S G F I E E D E L K L F L Q N F C A G	72
White muscle	GCACGCGCACTGTCCGACGCCGAGACCAAGGCTTTCCTCAAGGCCGGTGACAGCGATGGTGACGGCAAGATTGGAGTTGATGAGTTCGC	359
Dark muscle		358
	A R A L S D A E T K A F L K A G D S D G D G K I G V D E F A	102
White muscle	GCCATGGTTAAGCATTAAACAGCAACTGACCAACAAC TGCTCTG ATGGAACAACAACAGCCACGTCGACCTCCCCCTTTTCA	447
Dark muscle	C.....T.....	447
	A M V K H *	107
White muscle	CTGAATATAAATAATTTTATACATTGCTTAAGAGACGTTTCCTCCTATGCAACACACTGTCCAAATGATGATTGTGAATGTCGCTC	537
Dark muscle		537
White muscle	GTTGTGTTTCATGTCTTAAATATGAATTTTGCTTACTGTAAATCTATGCACTTTCCAGG AACGAACGATATAAAAAGAAAAGAATAAA	626
Dark muscle	G.....	627
White muscle	ATTCTTTTGCTACGGG TAAAAAAAAAAAAAAAAAAAAA	664
Dark muscle	C.....	655

B

White muscle	CATCTGTACCCTACAAAGCAAAACATCAGAGATCACAATGGCCTTTGCAAGTGTACTGAAAGATGCTGA	71
Dark muscle	TCAAGTCTTGTCTGTAT.....	88
	M A F A S V L K D A E	11
White muscle	GTCACGCGCCCTGGATGGGTGCAAAGCCGCTGGTAGTTTCGACCACAAAAAGTTCTTCAAGGCATGCGGTCTGTCCGGCAAGTCCAC	161
Dark muscle		178
	V T A A L D G C K A A G S F D H K K F F K A C G L S G K S T	41
White muscle	GATGAAGTCAAGAAGGCTTTCGCCATCATTGACCAGGACAAGAGCGGCTTCATTGAGGAGGAGAGCTGAAGCTGTTCTTCGAGAACTT	251
Dark muscle		268
	D E V K K A F A I I D Q D K S G F I E E E E L K L F L Q N F	71
White muscle	AAGGCAGGTGCACGCGCACTCTCCGACGCCGAGACCAAGGCTTTCCTCAAGGCCGGTGACAGCGACGGTGATGGCAAGATCGGTATTGA	341
Dark muscle		358
	K A G A R A L S D A E T K A F L K A G D S D G D G K I G I D	101
White muscle	GAGTTTGAGCCATGATTAAGGGATAAAGAGGCTACTGACCAACACCCGCTCTGATGGAACAACAATGCCCGCTGACCTCCCCCTT	431
Dark muscle		448
	E F A A M I K G *	109
White muscle	TCATCTGAATATAAATAATTTTATACATTGCTTAAGAGACCTTTCCTCCTATGCAACACACTGTCTAAAATGATGATTGTGAATGT	521
Dark muscle		538
White muscle	GCTCCGTTGTGTTTCATGTCTTAAATATGAATTTTGCTTACTGTAAATCTATGCACTTTCAGGAACGAACGGTATAAAAAGAATAAAT	611
Dark muscle		628
White muscle	ATTCTTTTGCT AAAAAAAAAAAAAAAAAAAAAA	641
Dark muscle	ACGGCC.....AA	666

Figure 3. Nucleotide sequences of cDNAs encoding parvalbumins of horse mackerel (A) and Pacific mackerel (B). Deduced amino acid sequences are denoted below the nucleotide sequences. Dots in the nucleotide sequences for dark muscle parvalbumins represent the same nucleotides as those for white muscle parvalbumins. Stop codons are indicated by asterisks. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence database) are AB211364 for horse mackerel white muscle parvalbumin, AB211365 for horse mackerel dark muscle parvalbumin, AB091470 for Pacific mackerel white muscle parvalbumin and AB211366 for Pacific mackerel dark muscle parvalbumin.

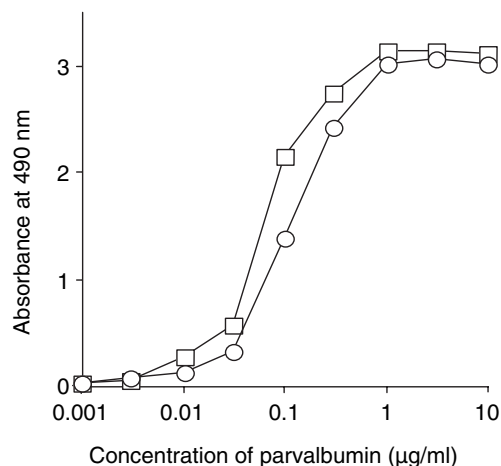


Figure 4. Calibration curves made using parvalbumins purified from horse mackerel (open square) and Pacific mackerel (open circle).

Table 1. Parvalbumin contents in the white and dark muscles of five species of fish

Fish	Number of specimens	Muscle	Parvalbumin content (µg/g)	
			Range	Mean ± SD
Sardine	4	White	114–187	149 ± 33
		Dark	21–50	38 ± 13
Yellowtail	3	White	12–25	20 ± 7
		Dark	ND	ND
Horse mackerel	6	White	1332–2066	1618 ± 312
		Dark	231–489	306 ± 108
Red sea bream	3	White	318–611	417 ± 168
		Dark	88–129	105 ± 21
Pacific mackerel	3	White	154–1376	686 ± 626
		Dark	18–130	80 ± 57

ND, not detected.

amount of parvalbumin was detected in the white muscle. As for the other four species of fish, parvalbumin contents were four to eight times lower in the dark muscle than in the white muscle, irrespective of the species.

Discussion

Parvalbumin represents a major and cross-reactive allergen in the fish white muscle. In the present study, therefore, patient sera containing IgE antibodies specific for fish white muscle parvalbumin were used to examine allergenicity and allergens of the dark muscle of five species of fish in comparison with those of the white muscle.

The IgE-immunoblotting experiments revealed that the major allergen in the dark muscle as well as in the white muscle is parvalbumin, except for the yellowtail dark

muscle for which no IgE-reactive bands were observed probably due to the scarcity of parvalbumin. However, analysis by SDS-PAGE strongly suggested that, in common with the five species of fish, the parvalbumin content in the dark muscle is significantly low compared with that in the white muscle. These results qualitatively supports the ELISA data that the dark muscle of the five species of fish is much less allergenic than the white muscle.

The significance of this study is in providing molecular evidence for the lower allergenicity of the dark muscle. Molecular cloning experiments clearly demonstrated that the same parvalbumin molecule is contained in both white and dark muscles of two species of fish, horse mackerel and Pacific mackerel. This is the first to report the primary structure of parvalbumin in the fish dark muscle. Because our finding is very likely to be applicable to all species of fish, the allergenicity of the two types of muscles largely depends on the parvalbumin content. Therefore, parvalbumin was then quantified for both white and dark muscles of the five species of fish by ELISA using the antiserum against Pacific mackerel parvalbumin. The results obtained showed that, regardless of the fish species, the parvalbumin content in the dark muscle is much lower than that in the white muscle. In addition, there is a good relationship in each muscle sample between the parvalbumin content and the IgE-binding potency of the heated extract. In preparing this paper, Lim et al. (19) has reported that parvalbumin is found in the white muscle of tuna *Thunnus tonggol* but absent in the dark muscle, conforming well to our study.

Two important findings were additionally obtained in this study. One finding is that yellowtail is extremely low in allergenicity. In ELISA, all the patient sera tested showed no substantial reactivity to the heated extracts from both white and dark muscles of yellowtail. In IgE-immunoblotting, the patient sera reacted very weakly to parvalbumin in the white muscle of yellowtail but not to that in the dark muscle. These results can be realized by the fact that yellowtail contains very low amounts and no detectable amounts of parvalbumin in the white and dark muscles, respectively. Although patients who have experienced allergic reactions to one species of fish are currently recommended to avoid all species of fish, some of them may be tolerable to yellowtail. Fish with very low allergenicity, such as yellowtail, will be discovered in future screenings with various species of fish. Another important finding is that a new allergen of 14 kDa is contained in both white and dark muscles of red sea bream, although it may be recognized by a limited number of fish-allergic patients. This allergen is not related to parvalbumin, because it does not react with the monoclonal antiparvalbumin antibody. Also, it is obviously distinguishable in molecular mass from collagen (16, 20, 21) and aldehyde phosphate dehydrogenase (22), which have been identified as minor allergens in fish. Purification of the 14 kDa allergen is under progress.

Based on the primary structures and contents of parvalbumin, this study proved at the molecular level that the fish dark muscle is less allergenic than the white muscle. The dark muscle is the minority of the whole muscle and is less frequently eaten than the white muscle. Taken together, we conclude that the dark muscle is much less implicated in fish allergy than the white muscle, being little worth consideration in future studies on fish allergens. Finally, it should be emphasized that a variety of fish species are eaten but only a part of them has been examined for allergenicity and allergens. Even this study with only five species of fish has established the low

allergenicity of yellowtail and the presence of a new 14 kDa allergen in red sea bream. Future study with many species of fish is needed to understand complicated features of fish allergy in more details.

Acknowledgments

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 Agriculture, Forestry and Fisheries of Japan.

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