

## Two Classes of Allergens, Parvalbumins and Higher Molecular Weight Substances, in Japanese Eel and Bigeye Tuna

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When assessed by ELISA using sera from five fish-sensitive subjects (1–5), the allergenicity of nine species of fish was found to vary not only among fish species but also among individual patients. In gel filtration on Sephadex G-75 of the crude extracts from Japanese eel and bigeye tuna, allergens were recognized in two fractions, *i.e.*, Fr. I, near the void volume and Fr. II, in the later fractions. Subject 3 and 4 sera reacted to Fr. I, subject 1 and 2 sera reacted to Fr. II, and subject 5 serum reacted both Frs. I and II. The four allergens (*Ang j* 1.01 and 1.02 from Japanese eel and *Thu o* 1.01 and 1.02 from bigeye tuna) for subjects 1 and 2 were isolated from Fr. II by reverse-phase HPLC on TSKgel ODS-120T. They were all identified as parvalbumins, based on their molecular weights, amino acid compositions, and positive reactions with a monoclonal anti-carp parvalbumin antibody. In conclusion, the major allergens in Japanese eel and bigeye tuna are parvalbumins and/or higher molecular weight substances, depending on patients.

**Key words:** allergen, fish, Japanese eel, bigeye tuna, parvalbumin

Food allergy mediated by IgE antibodies is a concern of public health in well-developed countries. Fish is obviously one of the most popular causes for food allergy, especially in Japan and some European countries (Norway, Sweden, Spain, etc.) where large quantities of fish are daily consumed. In fish-sensitive subjects, adverse reactions, such as urticaria, angioedema, asthma, and vomiting, are induced immediately after ingestion of fish. Even fatal and near-fatal anaphylactic reactions have been reported in severe cases.<sup>1,2)</sup>

It is well-known that the allergenicity of fish varies not only among fish species but also among fish-sensitive subjects.<sup>3–9)</sup> Despite this complicated feature of fish allergy, the major allergens in fish seem to be commonly parvalbumins belonging to the calcium-binding sarcoplasmic proteins in the ordinary muscle. In fact, parvalbumins have been clearly identified as the major allergens in three species of fish, codfish *Gadus callarias*,<sup>10,11)</sup> Atlantic salmon *Salmo salar*,<sup>12)</sup> and Japanese horse mackerel *Trachurus japonicus*.<sup>13)</sup> Some immunoblotting analyses have also suggested the major allergens in fish to be parvalbumins.<sup>5,6,8,9,14)</sup> On the other hand, minor allergens other than parvalbumins have been simultaneously detected in various species of fish by immunoblotting analyses.<sup>5,6,8,9,14)</sup> As one of the minor allergens in codfish, a 41 kDa protein has already been purified and characterized to some extent.<sup>15)</sup> In a rare case, a 25 kDa protein has been established to be the major allergen in swordfish.<sup>16)</sup> However, it should be pointed out that this allergen is recognized not by multiple fish-allergic subjects but by a peculiar subject allergic only to swordfish.

Most of the previous studies on fish allergenicity and

their allergens have been performed in Europe and USA. Although a variety of fish species are eaten raw or cooked in Japan, little study has been conducted about their allergenicity or allergens directed at Japanese fish-sensitive subjects. The sole conclusive information is that the major allergen for one patient isolated from the Japanese horse mackerel is parvalbumin.<sup>13)</sup> In this study, therefore, we first screened for allergenicity of nine species of fish, which are widely consumed in Japan, by enzyme-linked immunosorbent assay (ELISA) using sensitized sera from five subjects. Then, the crude extracts from two species of fish (Japanese eel and bigeye tuna) were applied to gel filtration in order to evaluate whether fish allergens are common to the five subjects. Finally, the major allergens for two of the five subjects were isolated from the two species of fish and examined for their physicochemical and immunological properties. We report here the screening data and evidence that the major allergens in Japanese eel and bigeye tuna are parvalbumins and/or higher molecular weight substances depending on the subjects.

### Materials and Methods

#### Fish

Fresh specimens of nine species of fish (Japanese eel *Anguilla japonica*, Pacific herring *Clupea pallasii*, chum salmon *Oncorhynchus keta*, walleye pollack *Theragra chalcogramma*, saury *Cololabis saira*, alfonsoin *Beryx splendens*, red sea bream *Pagrus major*, mackerel *Scomber japonicus*, and bigeye tuna *Thunnus obesus*) were purchased from a local market and stored at  $-20^{\circ}\text{C}$  until use.

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### Preparation of Crude Extract

The ordinary muscle of each fish was homogenized with 3 volumes of phosphate buffered saline (PBS; 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.0). After being heated at 100°C for 10 min, the homogenate was cooled to 5°C and centrifuged at  $18,000 \times g$  for 20 min. The supernatant was used as a crude extract.

### ELISA

ELISA was performed using a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan) as reported previously.<sup>17)</sup> In brief, each sample coated on the plate was reacted with sensitized or control serum (diluted 1:50), followed by peroxidase-conjugated goat anti-human IgE antibody (diluted 1:2,500; Kirkegaard & Perry Laboratories, Gaithersburg, USA). When monoclonal antibody against carp muscle parvalbumin (diluted 1:500,000; Sigma, St. Louis, USA) was used instead of the serum, peroxidase-conjugated goat anti-human IgE antibody was replaced by peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:50,000; The Binding Site, Birmingham, UK). Enzyme reaction was carried out using substrate solution containing 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.

### Sera

Sensitized sera were obtained from five subjects (subjects 1–5) who were aged 1–19 years. All subjects had a clinical history of immediate hypersensitivity reactions after ingestion of fish, except for subject 5 with a personal history. Subject 2 was also sensitive to egg, cow's milk, and crustaceans. Radioallergosorbent test (RAST), performed at hospitals using extracts from 1–4 species of fish, showed that subjects 1–4 had elevated serum IgE antibodies to fish. Serum from one subject (subject 6) without adverse reactions after ingestion of any foods served as a control. All sera were stored at  $-20^{\circ}\text{C}$  until use.

### Isolation Procedure

Allergens in Japanese eel and bigeye tuna were isolated as follows. The crude extract was subjected to a Sephadex G-75 column ( $2.5 \times 90$  cm; Pharmacia Biotech, Uppsala, Sweden), which was eluted with PBS at a flow rate of about 35 ml/h. Fractions of 10 ml were collected and measured for absorbance at 280 nm and allergenicity. Allergen-containing fractions were combined and applied to reverse-phase high performance liquid chromatography (HPLC) on a TSKgel ODS-120T column ( $0.46 \times 25$  cm; Tosoh, Tokyo, Japan). Elution was achieved at a flow rate of 1 ml/min by a gradient of acetonitrile (see Fig. 3 for details) in 0.1% trifluoroacetic acid. Proteins were monitored at 220 nm with a UV detector. The eluate corresponding to each allergen-containing peak was manually collected and lyophilized. The dried material was used as the isolated allergen.

### Protein Determination

Protein was determined by the method of Lowry *et al.*,<sup>18)</sup> using bovine serum albumin as a standard protein.

### Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem (Pharmacia Biotech, Uppsala, Sweden) according to the method described in the manufacturer's instructions. Ready-made gels (PhastGel High Density), ready-made buffer strips (PhastGel SDS Buffer Strips), and a molecular weight calibration kit (containing proteins or peptides with molecular weights ranging from 2,512 to 16,949) were purchased from Pharmacia Biotech. Prior to SDS-PAGE, samples were treated with 1% SDS in the presence or absence of 1% 2-mercaptoethanol at 100°C for 10 min. After running, the gel was stained with Coomassie Brilliant Blue R-250.

### Amino Acid Analysis

Each isolated allergen (about 30  $\mu\text{g}$ ) was hydrolyzed with 50  $\mu\text{l}$  of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in an evacuated tube at 115°C for 24 h. Following hydrolysis, the solution was neutralized with an equal volume of 3.5 M NaOH and diluted with 0.5 ml of 0.2 M citrate buffer (pH 2.2). A 0.5 ml-portion of the dilution was applied to an Atto MLC-703 amino acid analyzer.

## Results

### Allergenicity of Fish

In our previous paper concerning the Japanese horse mackerel allergens, no significant differences in ELISA were recognized between the heated extract and the non-heated extract.<sup>13)</sup> Thus, the allergenicity of nine species of fish was assessed by ELISA using the heated extracts.

The crude extracts from the nine species reacted more or less with almost all the sensitized sera from subjects 1–5 (Fig. 1), while they exhibited no positive reactions against the control serum from subject 6. On the whole, the crude extract from bigeye tuna reacted strongly with all the sensitized sera. Among the sensitized sera, those from subjects 1 and 2 showed relatively high reactivities against most of the crude extracts. However, there were slight differences in the reactivities between these two sera. For example, the subject 1 serum reacted more strongly with the extract from mackerel than the subject 2 serum, while the reactivities with the extracts from Japanese eel, chum salmon, and saury were higher in the latter than in the former. The sera from subjects 3 and 4 were comparable to each other as to the reactivities with the crude extracts. They were rather species-specific, reacting strongly with the extracts from two species (Japanese eel and bigeye tuna). As for the subject 5 serum, positive reactions, though not high, were equally recognized in all the crude extracts, except for that from mackerel.

### Isolation of Allergens from Japanese Eel and Bigeye Tuna

As described above, bigeye tuna was assumed to be strongly allergenic with all five subjects. In addition, subjects 3 and 4 were judged to be especially sensitive to Japanese eel as well as bigeye tuna. Considering these facts, we chose Japanese eel and bigeye tuna as samples and tried to isolate their allergens.

In gel filtration of the crude extract on Sephadex G-75,

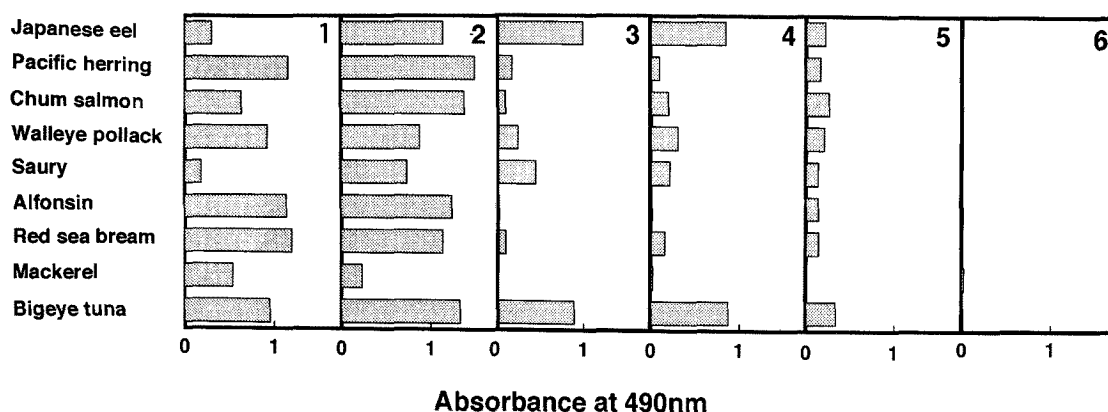


Fig. 1. Analysis by ELISA of the reactivity of the crude extracts from nine species of fish with six subject sera.

Each crude extract was diluted 1:500 and applied to ELISA. Subjects 1-5, patients; subject 6, control. Subject number is indicated at upper right corner of each figure.

the Japanese eel allergens were eluted at two different positions depending on the sensitized sera used in ELISA (Fig. 2A1 and A2). The major allergens for subjects 3 and 4 appeared near the void volume (Fr. I) and those for subjects 1 and 2 at around fraction 28 (Fr. II). Although the reactivities were considerably low, the major allergens for subject 5 were equally separated into both Frs. I and II. When analyzed by ELISA using the monoclonal anti-carp parvalbumin antibody, positive reactions were observed only in Fr. II (data not shown). Essentially the same results were also obtained when the crude extract from bigeye tuna was subjected to gel filtration (Fig. 2B1 and B2).

In this study, only the allergens contained in Fr. II were further purified by reverse-phase HPLC on TSKgel ODS-120T. As shown in Fig. 3A, the Japanese eel allergens, which were reactive with both subject 1 and 2 sera, were eluted in two peaks at retention times of 44 and 60 min. Thus, two allergens (named *Ang j* 1.01 and *Ang j* 1.02 in the order of elution on HPLC) were easily isolated from the Japanese eel muscle by only two chromatographic steps. In a typical run, 65 g of the muscle sample afforded 58 mg of *Ang j* 1.01 and 11 mg of *Ang j* 1.02. Similarly, two allergens (named *Thu o* 1.01 and *Thu o* 1.02) were also isolated from the bigeye tuna muscle (Fig. 3B). The yields of *Thu o* 1.01 and *Thu o* 1.02 from 320 g of the muscle sample were 14 and 8.3 mg, respectively.

#### Properties of the Isolated Allergens

On SDS-PAGE, the four isolated allergens migrated as a single band at almost the same position under both reduced and non-reduced conditions (Fig. 4). The molecular weights of the four allergens were estimated to be 10,000–11,000 in comparison with the mobilities of the reference proteins. As listed in Table 1, the amino acid compositions of the four allergens were comparable to one another; they were commonly rich in Asx, Ala, and Lys and lacked Met and Trp. *Ang j*s were characterized by the absence of His, whereas *Thu o*s by the absence of Tyr. *Ang j* 1.02 and *Thu o*s were further characterized by the absence of Pro. The determined molecular weights and amino acid compositions were closely similar to those of the known fish parvalbumins.<sup>13)</sup>

As shown in Fig. 5, the four allergens were capable of

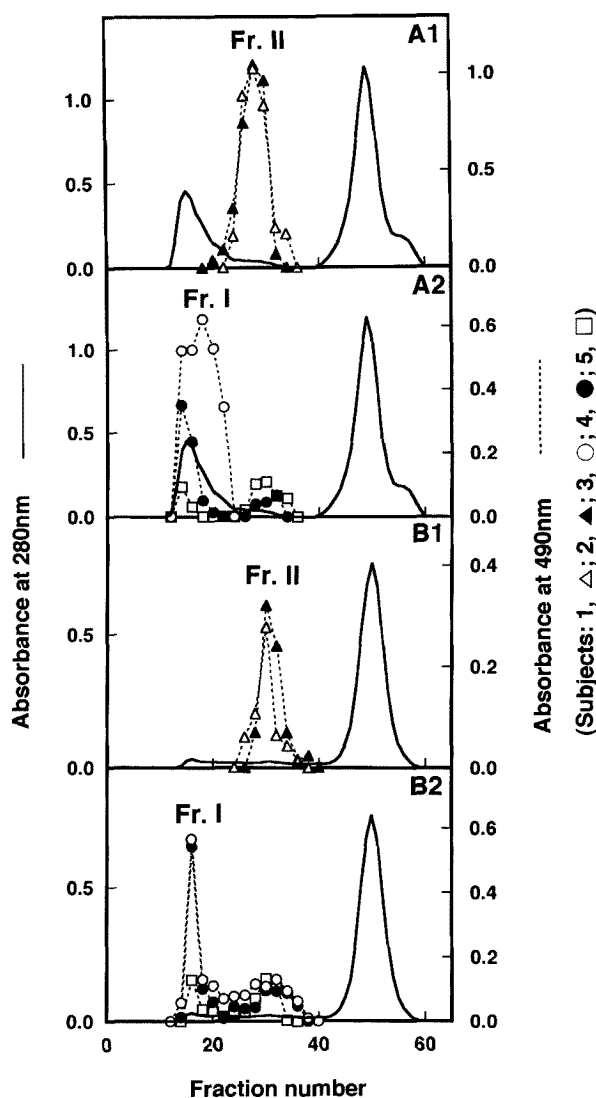


Fig. 2. Gel filtration on Sephadex G-75 of the crude extracts from Japanese eel (A1 and A2) and bigeye tuna (B1 and B2).

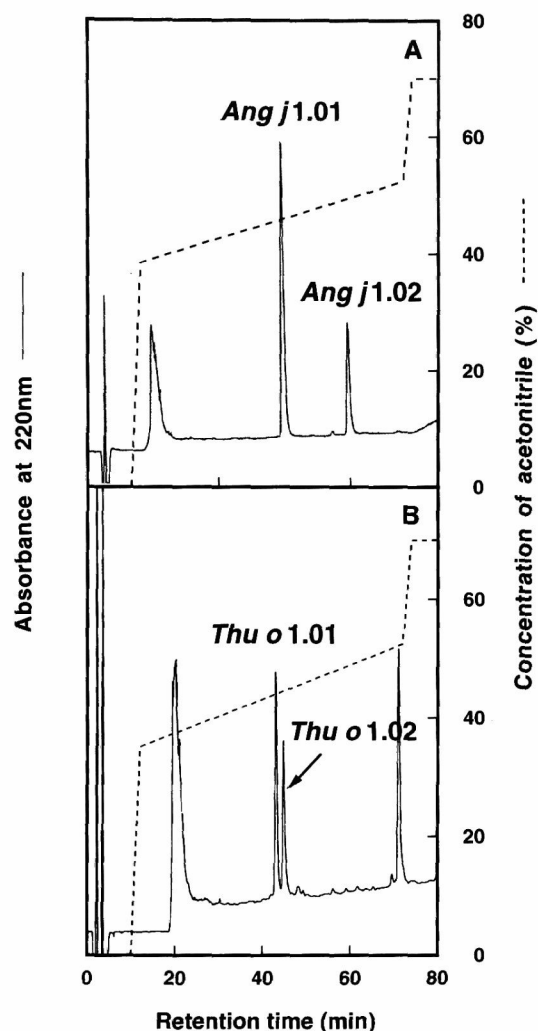


Fig. 3. Reverse-phase HPLC on TSKgel ODS-120T of the allergenic fraction (Fr. II) obtained by gel filtration.  
A, Japanese eel; B, bigeye tuna.

Table 1. Amino acid compositions of *Ang js* and *Thu os*

| Amino acid | Mol%              |                   |                   |                   |
|------------|-------------------|-------------------|-------------------|-------------------|
|            | <i>Ang j</i> 1.01 | <i>Ang j</i> 1.02 | <i>Thu o</i> 1.01 | <i>Thu o</i> 1.02 |
| Asx        | 16.5              | 13.6              | 14.2              | 13.9              |
| Thr        | 3.4               | 3.4               | 2.5               | 2.6               |
| Ser        | 5.0               | 7.2               | 4.8               | 5.0               |
| Glx        | 9.4               | 8.4               | 8.4               | 8.6               |
| Pro        | 1.3               | 0.0               | 0.0               | 0.0               |
| Gly        | 8.6               | 9.5               | 10.1              | 10.8              |
| Ala        | 11.9              | 17.9              | 17.6              | 17.3              |
| Val        | 3.7               | 5.5               | 3.0               | 3.3               |
| Cys(half)  | 0.2               | 0.0               | 1.3               | 0.6               |
| Met        | 0.0               | 0.0               | 0.0               | 0.0               |
| Ile        | 6.2               | 5.8               | 3.8               | 3.8               |
| Leu        | 7.9               | 8.2               | 8.2               | 8.3               |
| Tyr        | 1.0               | 1.1               | 0.0               | 0.0               |
| Phe        | 10.1              | 8.5               | 9.6               | 9.3               |
| His        | 0.0               | 0.0               | 2.7               | 2.8               |
| Trp        | 0.0               | 0.0               | 0.0               | 0.0               |
| Lys        | 13.7              | 9.4               | 13.1              | 12.9              |
| Arg        | 1.0               | 1.6               | 1.0               | 1.1               |

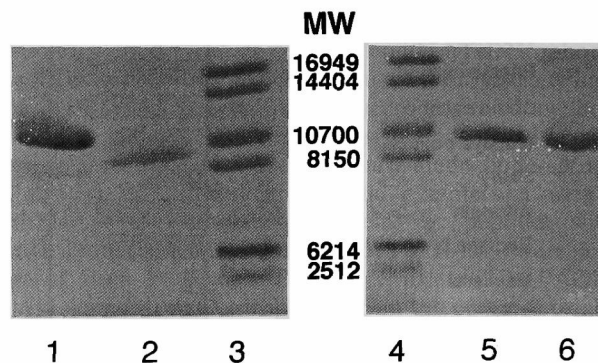


Fig. 4. SDS-PAGE of purified allergens.

Lanes: 1, *Ang j* 1.01; 2, *Ang j* 1.02; 3 and 4, standard proteins; 5, *Thu o* 1.01; 6, *Thu o* 1.02. Although this figure shows the migration patterns under reduced conditions, essentially the same results were obtained under non-reduced conditions.

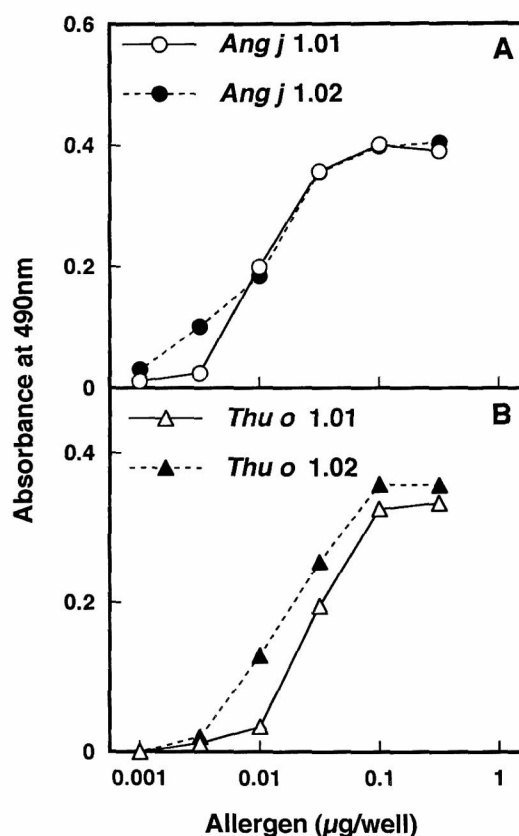


Fig. 5. Analysis by ELISA of the reactivity of *Ang js* (A) and *Thu os* (B) with monoclonal anti-carp parvalbumin antibody.

binding to the monoclonal anti-carp parvalbumin antibody, almost in the same magnitude, supporting that they are parvalbumins. The reactivities of patient sera with the four allergens are shown in Fig. 6. In accordance with the reactivity between Fr. II and each serum (see Fig. 2), the four allergens reacted significantly with both subject 1 and 2 sera, while the reactivities with the other sera were low or negligible. As to the subject 1 and 2 sera, the reactiv-

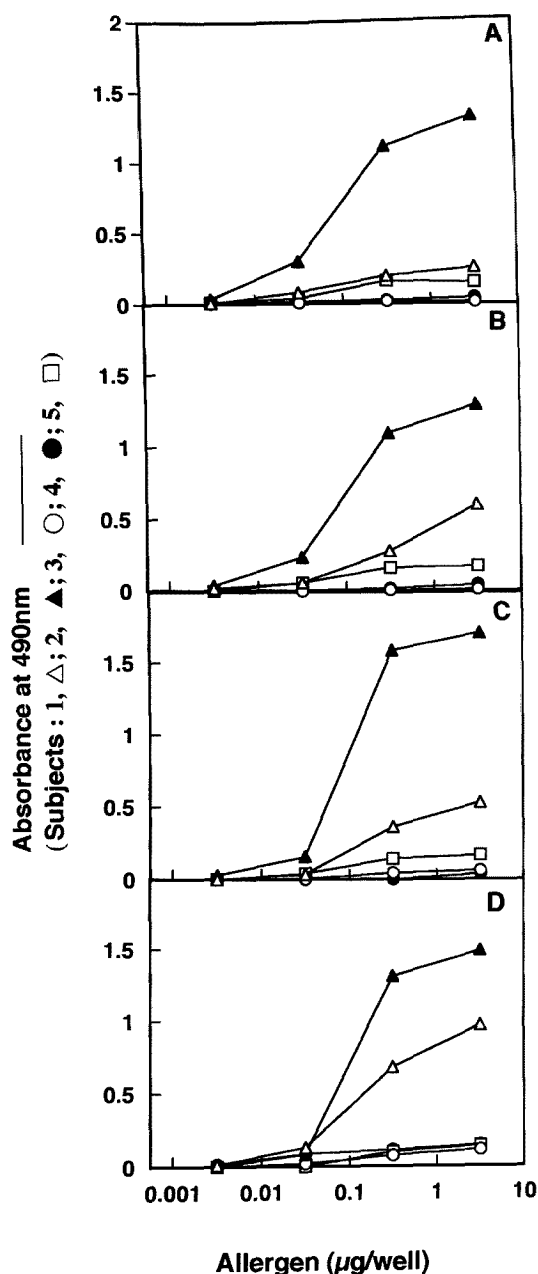


Fig. 6. Analysis by ELISA of the reactivity of *Ang j* 1.01 (A), *Ang j* 1.02 (B), *Thu o* 1.01 (C), and *Thu o* 1.02 (D) with the sera of five subjects.

ity with the latter was higher than that with the former, irrespective of the allergens. This conformed well to the fact that the crude extracts from Japanese eel and bigeye tuna react with the subject 2 serum more strongly than with the subject 1 serum (see Fig. 1). Although both *Ang j*s equivalently reacted with the subject 2 serum, they showed different reactivities against the subject 1 serum (Fig. 6A and B). Differences in the reactivities with either the subject 1 serum or the subject 2 serum were also recognized between *Thu os*. *Thu o* 1.01 reacted with the subject 1 serum more weakly than *Thu o* 1.02 and on the contrary the reactivities with the subject 2 serum were higher in the former than in

the latter (Fig. 6C and D).

## Discussion

Our screening data obtained by ELISA (Fig. 1) revealed that the allergenicity of fish depends not only on fish species but also on individual patients. Similar results have so far been obtained by several techniques such as skin tests and RAST-inhibition tests.<sup>3-9)</sup> Currently, patients who have experienced allergic reactions to one species of fish are recommended to avoid all species of fish. However, Bernhisel-Broadbent *et al.*<sup>5)</sup> have pointed out that this recommendation should be re-evaluated, since there are patients sensitive to certain species of fish but tolerant to other species.<sup>3-5)</sup> Indeed, our results suggest that subjects 3, 4, and 5 are substantially insensitive to mackerel.

Based on our screening data, the five fish-sensitive subjects can be classified into the following three groups: (1) subjects 1 and 2 with relatively high sensitivities to all the nine species of fish tested, (2) subjects 3 and 4 with especially high sensitivities to two species (Japanese eel and bigeye tuna), and (3) subject 5 with equal but low sensitivities to eight species. In accordance with this classification, the major allergens in Japanese eel and bigeye tuna vary among the three groups, as demonstrated by gel filtration on Sephadex G-75 (Fig. 2). Interestingly, despite the differences in allergens among the three groups, bigeye tuna appears to be highly allergenic with all the subjects. This fact suggests that bigeye tuna can be used at least in Japan as the most adequate fish sample in examining whether a person is fish-sensitive or not. It is also worth mentioning that several species of fish, such as Pacific herring, alfonsoin, and red sea bream, are much more allergenic to subjects 1 and 2 than to subjects 3 and 4. Hence, the co-use of these fish with bigeye tuna is assumed to be helpful not only from the diagnostic point of view but also in estimating which types of allergens (parvalbumins or larger substances, as discussed below) are major for a patient. Future study using many sera from Japanese patients and many species of fish consumed in Japan is needed to confirm this assumption.

The most common fish allergens are parvalbumins, as clearly demonstrated in three species of fish.<sup>10-13)</sup> This was the case with the four allergens for subjects 1 and 2, *Ang j* 1.01 and *Ang j* 1.02 isolated from Japanese eel and *Thu o* 1.01 and *Thu o* 1.02 from bigeye tuna. They are all unequivocally identified as parvalbumins, from the molecular weights, amino acid compositions, and positive reactions to the monoclonal anti-carp parvalbumin antibody. *Ang j* 1.01 and *Ang j* 1.02 or *Thu o* 1.01 and *Thu o* 1.02 are considered to be isomeric with each other.

Regardless of the fish species, the major allergens for subjects 1 and 2 are likely to be parvalbumins. In relation to this, we have previously isolated an allergen for subject 1 from Japanese horse mackerel and identified it as parvalbumin.<sup>13)</sup> It is, however, interesting to note that the observed differences in the reactivities with either the subject 1 serum or the subject 2 serum among the nine species of fish are caused not simply by the differences in parvalbumin contents but to some extent by those in the amino acid sequences of the IgE-binding epitopes of various parvalbumins. In fact, there must be some differences in the amino

acid sequences of the IgE-binding epitopes between *Ang j* 1.01 and *Ang j* 1.02 and between *Thu o* 1.01 and *Thu o* 1.02, since both *Ang js* react differently with the subject 1 serum and both *Thu os* with both subject 1 and 2 sera (Fig. 6). It is also noticeable that somewhat different spectra were seen in the reactions with the crude extracts from the nine species of fish between both subject 1 and 2 sera. This suggests that the epitopes of each parvalbumin recognized by serum IgE antibodies vary among individual patients. The IgE-binding epitopes of various fish parvalbumins should be elucidated using the sensitized sera from many patients, particularly in comparison with those previously reported for *Gad c* 1, the major allergen from codfish.<sup>19)</sup>

The most significant finding of this study is that the major allergens in Japanese eel and bigeye tuna for subjects 3 and 4 are distinguishable from parvalbumins. One of the major allergens for subject 5 is also not a parvalbumin. Though not purified, the detected unknown allergens have higher molecular weights than parvalbumins as judged by their behaviors in gel filtration. The monoclonal antibody against carp muscle parvalbumin did not react with the eluates in Fr. I, implying that the higher molecular weight allergens are neither aggregates of parvalbumins nor complexes of parvalbumin with some macromolecules. Previously, immunoblotting studies have shown the presence of allergens differing from parvalbumins in various species of fish<sup>5,6,8,9,14)</sup> and even the 41 kDa allergen has already been isolated from codfish.<sup>15)</sup> However, the allergens differing from parvalbumins are all minor. In a rare case, the 25 kDa protein has been detected as the major allergen in swordfish but this is allergenic only to a peculiar patient monospecific to swordfish.<sup>16)</sup> Thus, the present study is the first to report the occurrence of major allergens differing from parvalbumins against multiple fish-sensitive subjects.

In conclusion, this study shows that the major allergens in fish are parvalbumins for some patients but unknown higher molecular weight substances for other patients. As compared to the previous studies performed in Europe and USA, these circumstances may be peculiar to Japanese patients. In order to obtain more information about fish allergy, especially that in Japan, purification of the unknown allergens in Japanese eel and bigeye tuna is now under progress.

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