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Elucidation of a major IgE epitope of Pacific mackerel parvalbumin

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ABSTRACT

Ten overlapping peptides (20 amino acids in length with an offset of 10 amino acids) spanning the amino acid sequence of Pacific mackerel, *Scomber japonicus* parvalbumin (Sco j 1), were synthesized and evaluated for IgE-binding ability by fluorescence ELISA using fish-allergic patient sera. As a result, the region 21–40 was judged to contain a major IgE epitope of Sco j 1. However, this region was not necessarily assumed to be a major IgE epitope for the parvalbumins from seven other species of fish (sardine, Japanese eel, cod, horse mackerel, crimson sea bream, skipjack and flounder). Ala-scanning experiments revealed that eight amino acid residues (Ser-23, His-26, Lys-27, Lys-28, Lys-31, Cys-33, Leu-35 and Lys-38) are important for the IgE-binding of Sco j 1. Some of these residues are replaced by different residues in the parvalbumins of the other seven species of fish.

1. Introduction

Fish is rich in proteins and nutrients, such as vitamins and functional lipids (DHA and EPA), and hence is widely consumed as an important foodstuff, especially in coastal countries. However, fish is simultaneously well known as one of the most common causes of food allergy mediated by immunoglobulin E (IgE) antibodies. Immediately after ingestion of fish, sensitized individuals with high levels of fish-specific IgE develop allergic reactions, such as urticaria, diarrhea and anaphylactic shock; even fatal cases have been recorded (O'Neil, Helbling & Lehrer, 1993; Pascual, Esteban, & Crespo, 1992). Extensive studies with cod, *Gadus callarias*, first demonstrated that the major allergen (Gad c 1) is parvalbumin, a 12 kDa sarcoplasmic calcium-binding protein, probably implicated in muscle relaxation (Elsayed & Aas, 1971; Elsayed & Bennich, 1975). In the past decade, parvalbumin has been clearly identified as the major allergen in various species of fish, such as carp (Bugajska-Schretter et al., 1999; Swoboda et al., 2002), Atlantic salmon (Lindstrøm, van Dô, Hordvik, Endresen, & Elsayed, 1996), Pacific mackerel (Hamada et al., 2003) and bigeye tuna (Shiomi, Hamada, Sekiguchi, Shimakura, & Nagashima, 1999). Thus, parvalbumin is currently accepted as the major fish allergen, although some minor allergens, such as collagen (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000) have also been detected in fish.

The IgE cross-reactivity among fish parvalbumins has been established (Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005) but has not been fully understood at the molecular level. To give insight of the cross-reactivity among fish parvalbumins, it is essential to elucidate their IgE epitopes that are directly related to the hypersensitive reactions caused by the allergens. Moreover, elucidation of IgE epitopes of fish parvalbumins is a requisite, not only for the development of hypoallergenic fish products that can be eaten by fish-allergic patients, but also for the design of hypoallergenic parvalbumin molecules that will be used in future immunotherapy of fish allergy.

So far, studies on linear-type IgE epitopes of fish parvalbumins have been conducted only with Gad c 1; four regions have been proposed as IgE epitopes (Elsayed & Apold, 1983). However, these IgE epitope regions are not always conserved in other fish parvalbumins, implying that IgE epitopes of fish parvalbumins should be examined with each parvalbumin. On the other hand, the IgE reactivity of carp *Cyprinus carpio* parvalbumin (Cyp c 1; Swoboda et al., 2007) and Pacific mackerel *Scomber japonicus* parvalbumin (Sco j 1; Tomura, Ishizaki, Nagashima, & Shiomi, 2008) has been reported to be significantly reduced by depletion of Ca^{2+}, indicating the importance of conformational-type IgE epitopes for Cyp c 1 and Sco j 1. Nevertheless, linear-type IgE epitopes are not negligible for both parvalbumins, since their IgE reactivity is not completely lost by depletion of Ca^{2+}. In view of this, the present study was initiated to elucidate linear-type IgE epitopes of Sco j 1 by a mapping technique using overlapping peptides encompassing the amino acid sequence of Sco j 1. We report here the major IgE epitope of Sco j 1 and the amino acid residues crucial for the IgE-binding of Sco j 1.

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2. Materials and methods

2.1. Peptide synthesis

On the basis of the amino acid sequence of Sco j 1 (Hamada et al., 2003), 10 overlapping peptides (peptides A–J; Fig. 1), which were 20 amino acids in length (except for peptide J composed of 18 residues) with an offset of 10 amino acids, were chemically synthesized. Since peptide C (region 21–40) was found to contain a major IgE epitope, as described below in detail, peptides corresponding to peptide C were synthesized for the parvalbumins of seven species of fish (sardine *Sardinops melanostictus*, Japanese eel *Anguilla japonica*, cod *G. callarias*, horse mackerel *Trachurus japonicus*, crimson sea bream *Evynnis japonica*, skipjack *Katsuwonus pelamis* and flounder *Paralichthys olivaceus*) (Fig. 2). In this paper, these peptides are expressed in the combination of fish name and C (e.g. sardine-C). Furthermore, analogous peptides, in which the amino acid residues (except for two Ala residues at positions 21 and 32) of peptide C are individually replaced by Ala, were also synthesized. These analogous peptides are designated according to the original residue (in one letter), position number and A (e.g. G22A).

Peptide synthesis was performed on a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan), using 9-fluorenylmethyloxycarbonyl as an amino-protecting group and (benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate as a coupling reagent, as recommended by the manufacturer. After synthesis, each peptide was cleaved from the Fmoc resin (Shimadzu) with one of the cocktail solutions (Shimadzu) and precipitated with ether, according to the manufacturer's instructions. Purification of peptides was achieved by reverse-phase HPLC on a TSKgel ODS-120 T column (0.46 × 25 cm; Tosoh, Tokyo, Japan). The column was eluted with a linear gradient of acetonitrile (14–35% in 30 min) at a flow rate of 1 ml/min, with monitoring at 220 nm. To confirm the correct synthesis, each purified peptide was analyzed for its molecular weight by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry using a KOMPACT MALDI I instrument (Shimadzu). Insulin and sinapinic acid (matrix) were used to calibrate the instrument. Peptide was quantified by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

2.2. Human sera

Sera were obtained from 13 patients with a documented clinical history of immediate hypersensitive reactions, such as urticaria and diarrhea, after ingestion of fish. Written informed consent was obtained from each patient. Our preliminary ELISA experiments showed that all patients had elevated serum IgE to Sco j 1. Moreover, 11 of the 13 patients had been subjected to the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) at hospitals and recorded to have CAP-RAST classes of 2–4 against various species of fish, such as mackerel and tuna.

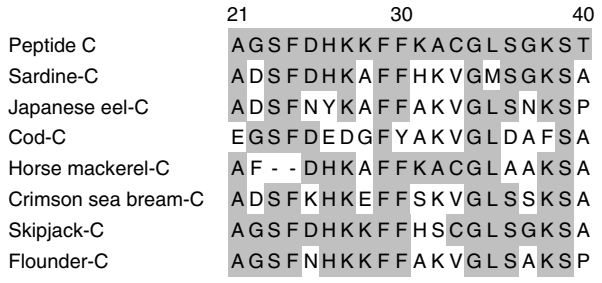

Fig. 2. Amino acid sequence comparison of the 21–40 region among parvalbumins from eight species of fish parvalbumins. Peptide C is the 21–40 region of Pacific mackerel parvalbumin (Sco j 1). Identical residues with peptide C are shaded. Note that horse mackerel-C is devoid of residues at positions 22 and 23 (indicated by dashes). Accession numbers for parvalbumins (UniprotKB/Swiss-Prot database for cod parvalbumin and DDBJ/EMBL/GenBank databases for the other parvalbumins): Pacific mackerel, AB091470; sardine, AB375262; Japanese eel, AB375263: cod, P02622; horse mackerel, AB211364; crimson sea bream, AB375264; skipjack, AB375265; flounder, AB375266.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Reactivity of each synthetic peptide with patient sera was assessed by fluorescence ELISA, essentially as reported previously (Hamada et al., 2004); the only exception was that a Nunc Immobilizer Amino plate for peptide (Nagel Nunc International, Rochester, NY, USA) was used for peptide coating. In brief, the plate was coated with 50 μl of peptide solution (10 μg/ml) and reacted successively with patient serum (diluted 1:250 or 1:500) and β-galactosidase-conjugated goat anti-human IgE antibody solution (0.25 μg/ml; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out using substrate solution (0.1 mg/ml 4-methylumbelliferyl-β-D-galactoside) and stopped by addition of 100 mM glycine-NaOH buffer (pH 10.3). Fluorescence intensity was measured on a SPECTRAmax GEMINI XS instrument (Molecular Devices, Sunnyvale, CA, USA) with excitation at 367 nm and emission at 453 nm. All ELISAs were performed in triplicate and the data obtained were expressed as means ± SD.

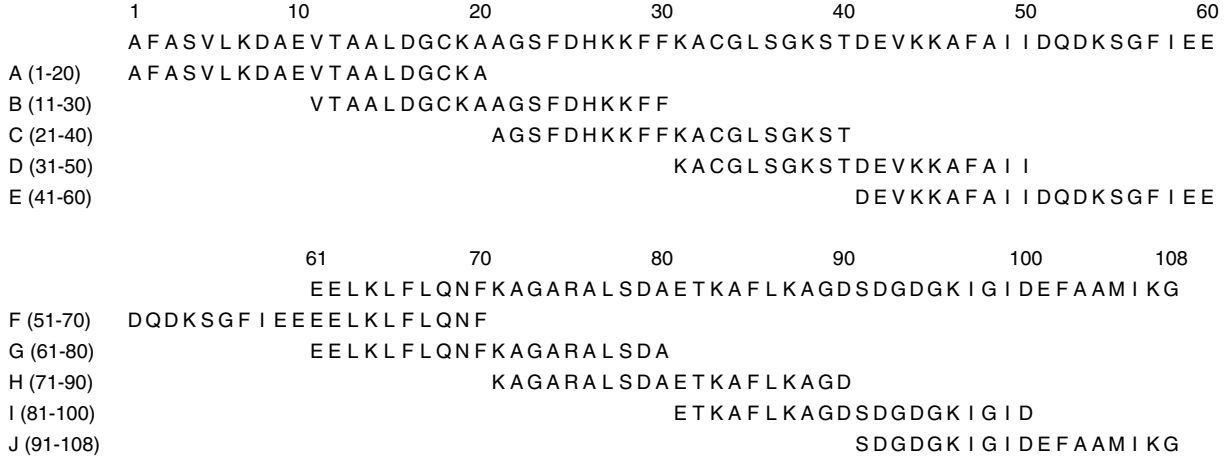

Fig. 1. Amino acid sequences of Sco j 1 and 10 synthetic overlapping peptides (A–J) spanning the amino acid sequence of Sco j 1. For each peptide, the region corresponding to Sco j 1 is indicated in a parenthesis.

3. Results

3.1. IgE reactivity of peptides A–J

Ten synthetic peptides (peptides A–J), spanning the entire amino acid sequence of Sco j 1, were assessed for IgE reactivity by fluorescence ELISA using 13 patients sera. As shown in Fig. 3, peptide C (region 21–40) obviously reacted with as many as 12 sera; only the reactivity with patient 13 serum was negligible. Although peptides D (region 31–50) and E (region 41–60) showed significant reactivity with two sera (from patients 4 and 7), their reactivity with the other patients sera was weak or negative. As for the remaining peptides, rather weak reactivity was observed in several sera. On the basis of these results, we concluded that the major IgE epitope of Sco j 1 is included in peptide C.

3.2. IgE reactivity of the region 21–40 of various fish parvalbumins

Although peptide C (region 21–40) contains a major IgE epitope of Sco j 1, as described above, diverse variations in amino acid sequence are recognized in the region 21–40 among fish parvalbumins (Fig. 2). In this study, therefore, synthetic peptides, which correspond to the region 21–40 of the parvalbumins from seven species of fish (sardine, Japanese eel, cod, horse mackerel, crimson sea bream, skipjack and flounder), were then evaluated for reactivity with 13 patients sera by fluorescence ELISA. As a result, five kinds of peptides (sardine-C, cod-C, horse mackerel-C, crimson sea bream-C and flounder-C) showed reactivity with only a few patients sera (Fig. 4). In the case of Japanese eel-C and skipjack-C, however, no substantial reactivity with any patient sera was observed. These results implied that the region 21–40 is not a common IgE epitope of fish parvalbumins but rather a specific IgE epitope of Sco j 1.

3.3. Ala-scanning experiments

To determine which amino acid residues in peptide C are crucial for IgE-binding, Ala-scanning experiments were performed. In these experiments, sera from patients 1–11, which were significantly reactive to peptide C (Fig. 3), were subjected to fluorescence ELISA. As shown in Fig. 5, IgE reactivity of each Ala-substituted peptide differed greatly from serum to serum. As compared to the IgE reactivity of peptide C, eight Ala-substituted peptides (S23A, H26A, K27A, K28A, K31A, C33A, L35A and K38A) showed more than 50% reduction in reactivity with at least six patients sera. In the case of four peptides (K27A, K28A, C33A and K38A), more than 50% reduction in IgE reactivity was recognized in at least nine patients sera and even complete loss of reactivity in several patients sera. These results suggested that four amino acid residues (Lys-27, Lys-28, Cys-33 and Lys-38) are crucial for the IgE-binding of Sco j 1 and that four residues (Ser-23, His-26, Lys-31and Leu-35) are also considerably important for IgE-binding. It is interesting to note that some Ala-substituted peptides showed higher reactivity with some patients sera than did peptide C. Particularly, peptide D25A reacted with as many as nine sera more highly than did peptide C.

4. Discussion

Four regions, 33–44 (VGLDAFSADELK), 49–64 (IADEDKEGFIEEDELK), 65–74 (LFLIAFAADL) and 88–96 (AGDSDGDGK), have been proposed as linear-type IgE epitopes of Gad c 1, based on the IgE-binding abilities of enzymatic peptide fragments and some synthetic peptides (Elsayed & Apold, 1983). This has been the sole available information on linear-type IgE epitopes of fish parvalbumins. Therefore, the present study with Sco j 1 is the second report on linear-type IgE epitopes of fish parvalbumins.

In this study, epitope mapping, using 10 overlapping peptides spanning the amino acid sequence of Sco j 1, demonstrated that the major IgE epitope of Sco j 1 is contained in peptide C (region 21–40). Peptide C partly overlaps with one (region 33–44) of the Gad c 1 IgE epitopes but has as many as five alterations in the overlapped region (33–40) compared to the amino acid sequence of Gad c 1. In the two Gad c 1 epitope regions (49–64 and 65–74), Sco j 1 has 4 and 5 alterations, respectively, probably accounting for the reason why peptides E (region 41–60), F (region 51–70) and G (region 61–80) were not evaluated to contain major IgE epitopes of Sco j 1. Although the remaining Gad c 1 IgE epitope (region 88–96) is completely conserved in Sco j 1, peptide I (region 81–100) showed IgE reactivity with only a limited number of

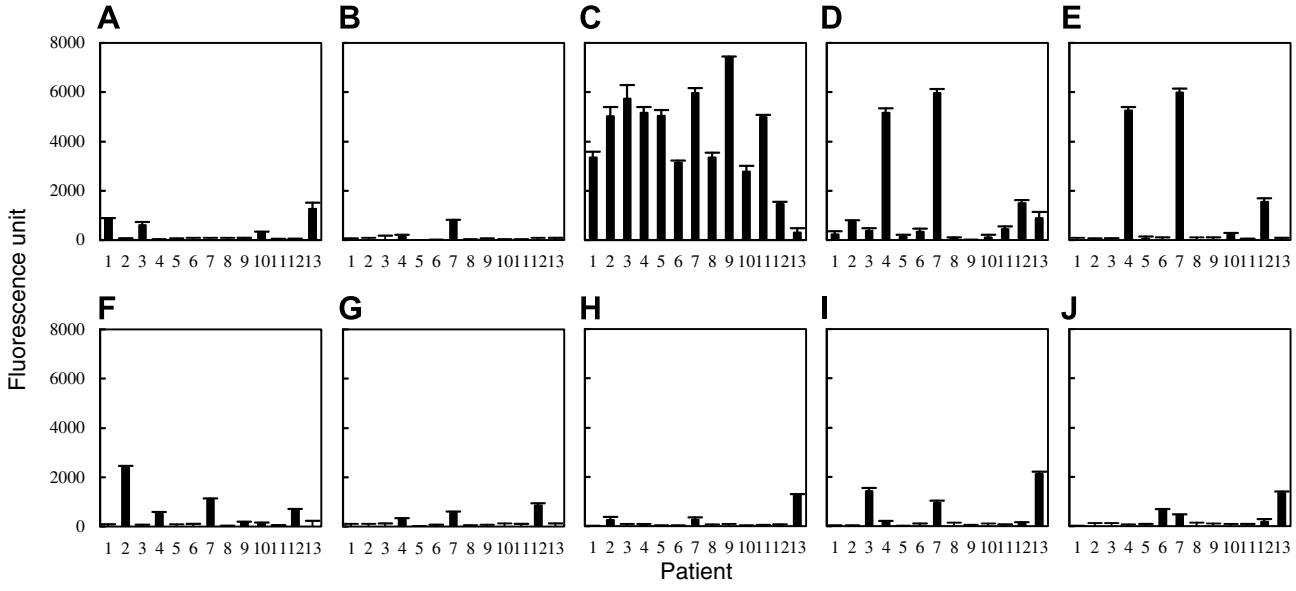

Fig. 3. IgE reactivity of 10 overlapping peptides spanning the sequence of Sco j 1. IgE reactivity was analyzed by fluorescence ELISA using sera from 13 fish-allergic patients. Each datum is expressed as mean ± SD (n = 3).

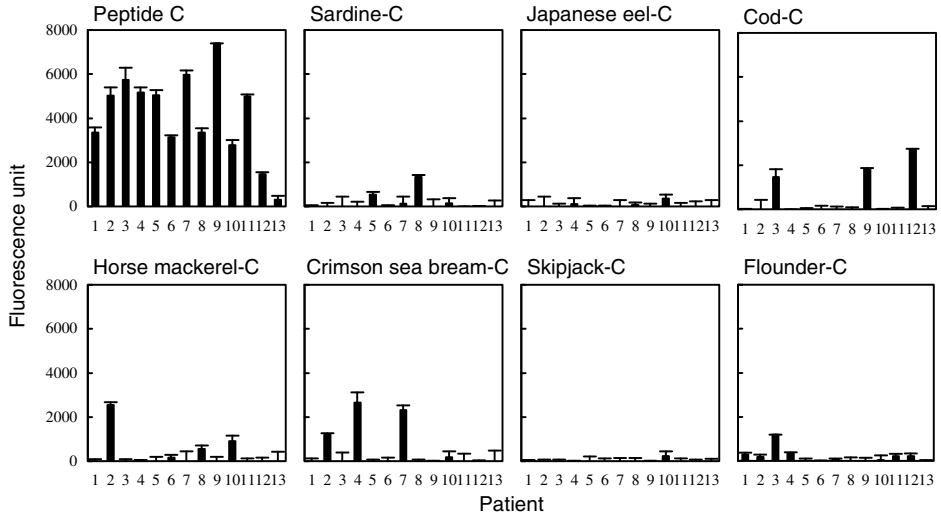

Fig. 4. IgE reactivity of peptides corresponding to the 21–40 region of various fish parvalbumins. IgE reactivity was analyzed by fluorescence ELISA using sera from 13 fish-allergic patients. Each datum is expressed as mean ± SD (n = 3).

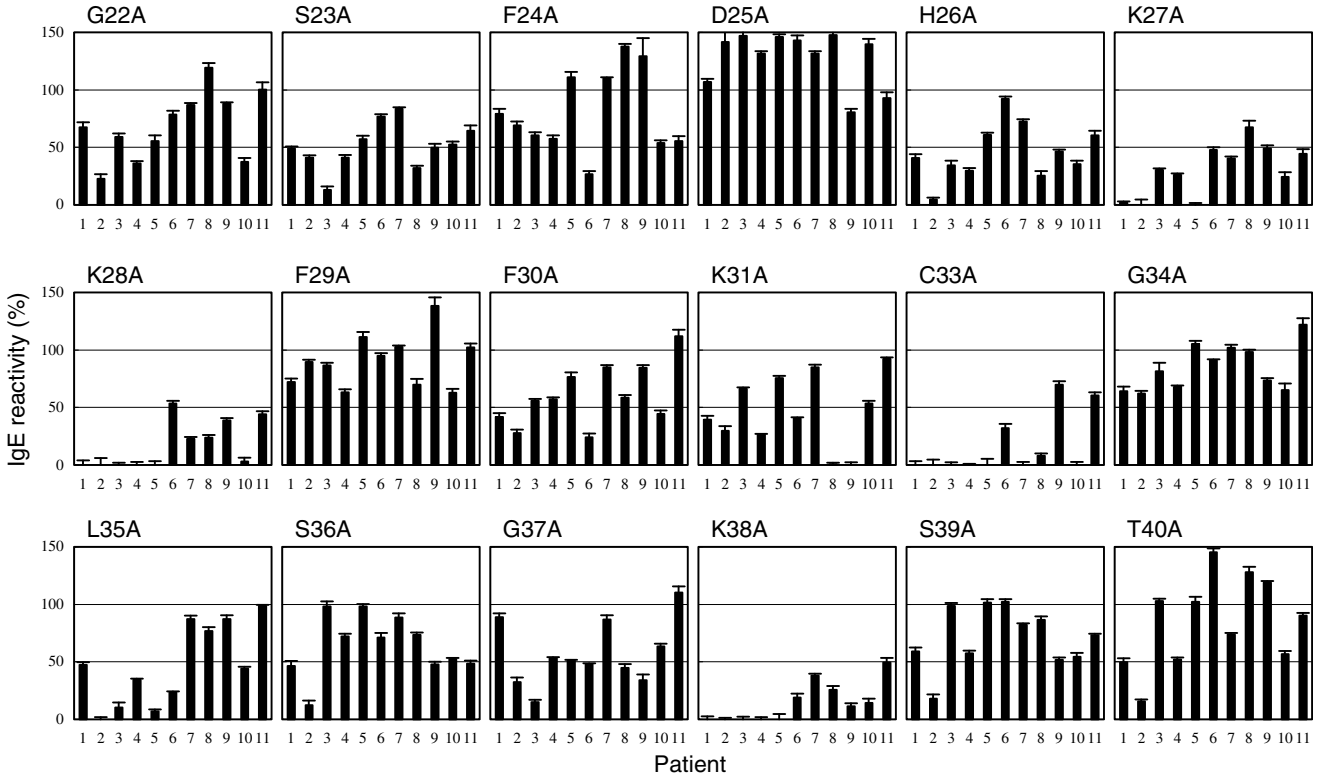

Fig. 5. IgE reactivity of peptide C-analogues with an Ala-substitution. IgE reactivity was analyzed by fluorescence ELISA using sera from 11 fish-allergic patients and expressed as % reactivity relative to peptide C. Each datum is expressed as mean ± SD (n = 3).

patients sera. This may be attributable to a racial difference, in view of the fact that IgE epitopes of Gad c 1 and Sco j 1 were examined using European and Japanese patients sera, respectively.

It should be noted that the region 21–40 is a major IgE epitope for Sco j 1 but not for the parvalbumins from seven species of fish. This can be mostly understood by the results of Ala-scanning experiments, which revealed the four crucial residues (Lys-27, Lys-28, Cys-33 and Lys-38) and four important residues (Ser-23, His-26, Lys-31 and Leu-35) for the IgE-binding of Sco j 1. Replacement of all the four crucial residues and two important residues by different residues is seen in cod-C (Fig. 2). In the case of sardine-C, Japanese eel-C, horse mackerel-C, crimson sea bream-C and flounder-C, one or two of the crucial residues and one or two of the important residues are replaced by different residues. These replacements seem to be the molecular basis for our finding that the region 21–40 of the parvalbumins from six species of fish does not contain a major IgE epitope. On the other hand, it is not easy to explain why skipjack-C showed no substantial reactivity with any patient sera, since it maintains all the four crucial residues. As compared to the amino acid sequence of peptide C, skipjack-C

has two alterations at positions 31 and 32. The replacement of the important residue Lys-31 by His is rather conservative but may be a little responsible for the reduction in IgE reactivity. Although the importance of Ala-32 could not be assessed by Ala-scanning experiments in this study, its replacement by a more hydrophilic residue (Ser) possibly contributes to the reduction in IgE reactivity. Future study using analogous peptides (K31H and A32S) will confirm how the two replacements are associated with the reduced IgE reactivity of skipjack-C.

It is worth mentioning that five of the eight amino acid residues crucial or important for the IgE-binding of Sco j 1 are basic amino acids (four Lys and one His). Furthermore, peptide D25A, in which the acidic residue (Asp) at position 25 is substituted by a neutral residue (Ala), showed higher reactivity with many patients sera than did the non-substituted peptide (equivalent to peptide C). Taken together, it seems to be reasonable to infer that the electrostatic interaction of positively charged residues of Sco j 1 with negatively charged residues of IgE is deeply implicated in the binding between Sco j 1 and IgE.

Conclusively, the present study showed that the region 21–40, identified as the major linear-type IgE epitope of Sco j 1, is rather specific to Sco j 1. This finding will facilitate future study on linear-type IgE epitopes of parvalbumins from various species of fish. In addition, this finding may conflict with the established IgE cross-reactivity among fish parvalbumins (van Dô et al., 2005). In relation to this, it is interesting to note that recent studies with Cyp c 1 and Sco j 1 suggest the importance of conformational-type IgE epitopes for fish parvalbumins (Swoboda et al., 2007; Tomura et al., 2008). It is therefore likely that the IgE cross-reactivity among fish parvalbumins largely depends on conformational-type IgE epitopes rather than linear-type IgE epitopes. For a better understanding of the IgE cross-reactivity among fish parvalbumins, further study is needed to elucidate both linear-type and conformational-type IgE epitopes of fish parvalbumins.

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