

Immunological Comparison of Shellfish Allergens by Competitive Enzyme-Linked Immunosorbent Assay

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Allergens from five species of shellfishes (oyster *Crassostrea gigas*, scallop *Patinopecten yessoensis*, short-neck clam *Tapes japonica*, turban shell *Turbo cornutus*, and whelk *Neptunea arthritica*) were immunologically compared by competitive ELISA inhibition analyses. The cross-reactivity was recognized between shellfish allergens and *Cra g 1*, the oyster allergen, suggesting that, irrespective of species, the major allergen in shellfishes is tropomyosin. When four synthesized overlapping peptides (P-1, LNRRIQLLEEDM; P-2, RIQLLEEDMERS; P-3, LLEEDMERSEER; P-4, EDMERSEERLQT), which comprehend a part of the sequence of K21d (IgE-binding epitope proposed for *Cra g 1*, IQ-LLEEDMERSEER), were subjected to competitive ELISA inhibition experiments, only P-3 inhibited the binding of *Cra g 1* to specific IgE antibodies in the sensitized serum. Comparison of the sequence of K21d with those of the four synthesized peptides revealed that both N- and C-terminal regions of K21d are essential for the binding to specific IgE antibodies. In addition, P-3 inhibited the binding of specific IgE antibodies to scallop and whelk allergens as well as oyster allergens, but substantially not to those from the other two species. It was thus concluded that shellfish allergens do not always have IgE-binding epitopes in the same regions, although they are all thought to be tropomyosins.

Key words: oyster, shellfish, allergen, competitive ELISA, tropomyosin, IgE-binding epitope, cross-reactivity, synthesized peptides

It is well-known that food-associated allergens in fish are parvalbumins,^{1–4)} while those in crustaceans are tropomyosins.^{5–8)} Recently, allergens in three species of mollusks, the squid *Todarodes pacificus*,⁹⁾ the oyster *Crassostrea gigas*,¹⁰⁾ and the turban shell *Turbo cornutus*,¹¹⁾ were shown to be tropomyosins as in crustaceans. However, IgE-binding epitopes of these molluscan tropomyosins are not necessarily common to each other. Our competitive ELISA (enzyme-linked immunosorbent assay) inhibition experiments, using enzymatic fragments of the allergens as inhibitors, showed that the oyster allergens (*Cra gs*) contain an IgE-binding epitope in the segment 92–105,^{12,13)} while the turban shell allergen (*Tur c 1*) in the C-terminal segment 256–284.¹¹⁾ It is also noted that neither the IgE-binding epitope for *Cra gs* nor that for *Tur c 1* are consistent with those (segments 50–66 and 153–161) previously proposed for the shrimp *Penaeus indicus* allergen (*Pen i 1*).⁶⁾

This study was initiated to obtain some immunological properties of shellfish allergens. For this purpose, the cross-reactivities of a sensitive human serum against shellfish antigens (*Cra g 1*, *Tur c 1*, and crude extracts of shellfish) were examined using a competitive ELISA inhibition technique. Furthermore, competitive ELISA inhibition analyses using four synthesized overlapping peptides as inhibitors were also performed in order to confirm the IgE-binding epitope of *Cra g 1* and to examine whether shellfish allergens have similar epitopic sites or not.

Materials and Methods

Shellfish

Three species of bivalves (oyster *Crassostrea gigas*, scallop *Patinopecten yessoensis*, and short-neck clam *Tapes japonica*) and two species of gastropods (turban shell *Turbo cornutus* and whelk *Neptunea arthritica*) were used in this study. All shellfish samples were purchased from a local market, shucked, and frozen at -20°C until use.

Extraction

The muscle part (*P. yessoensis*, *T. cornutus*, and *N. arthritica*) or the whole body (*C. gigas* and *T. japonica*) was homogenized with an equal volume of distilled water and centrifuged at $18,800 \times g$ for 20 min. The supernatant was heated in a boiling water bath for 10 min. After removal of insoluble matters by centrifugation, the resulting supernatant was used as crude extract.

Isolation of *Cra g 1* and *Tur c 1*

Both *Cra g 1* and *Tur c 1* were isolated as reported previously.^{10,11)} In brief, the crude extract was successively applied to gel filtration on Sephacryl S-300 (Pharmacia Biotech, Uppsala, Sweden), anion-exchange fast protein liquid chromatography on Mono Q HR 5/5 (Pharmacia Biotech), and reverse-phase high-performance liquid chromatography (HPLC) on TSKgel Phenyl-5PW RP (Tosoh, Tokyo, Japan).

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Peptide Synthesis

The four peptides of 12 residues overlapping each other by 3 residues (P-1, P-2, P-3, and P-4 corresponding to the segments 88–99, 91–102, 94–105, and 97–108 of *Cra g 1*, respectively; refer to Table 1 for their amino acid sequences) were prepared by Accord (Tokyo, Japan) using (9-fluorenylmethoxy)carbonyl group (Fmoc) as an amino-protecting group and benzotriazolylxytris(pyrrolidine) phosphonium hexafluorophosphate (PyBOP) as a coupling reagent. Since all the synthesized peptides were semi-purified products, they were further purified in our laboratory by reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh) with a linear gradient of acetonitrile (0–40% in 40 min) in 0.1% trifluoroacetic acid. Peptides were monitored by absorbance at 220 nm. Each isolated peptide was quantified by comparing the peak area with that of *Cra g 1* used as a standard.

Serum

Sensitized serum was obtained from a patient with immediate hypersensitivity reactions such as asthma and diarrhea after ingesting mollusks and crustaceans. The patient had been qualitatively diagnosed to be positive in specific IgE antibody reactivity to these seafoods by a radioallergosorbent test.

Competitive ELISA Inhibition Experiment

The cross-reactivity between *Cra g 1* and *Tur c 1* or between *Cra g 1* and allergens in each crude extract was assessed by the competitive ELISA method described before.¹²⁾ In brief, the inhibitor (*Cra g 1* or *Tur c 1*) solution (0.002–20 $\mu\text{g}/\text{ml}$) made in phosphate-buffered saline (pH 7.4, PBS) was pre-incubated with an equal volume of the sensitized serum (diluted 1:25 with 0.1% bovine serum albumin in PBS) at 37°C for one hour. Then 100 μl of the pre-incubation mixture was added to the microtiter plate (ELISA Plate H type with 96 wells; Sumitomo Bakelite, Tokyo, Japan) that had been previously coated with 100 μl of *Cra g 1* (1 $\mu\text{g}/\text{ml}$), *Tur c 1* (1 $\mu\text{g}/\text{ml}$) or each crude extract (diluted 1:1,000) in 0.05 M carbonate buffer (pH 9.5). The subsequent procedure was the same as in ELISA described earlier.¹⁰⁾

The ability of the four synthesized peptides to inhibit the binding of *Cra g 1* or allergens in each crude extract to IgE antibodies in the sensitized serum was also examined by competitive ELISA inhibition experiments. In this case, the microtiter plate was coated with 100 μl of *Cra g 1* (1 $\mu\text{g}/\text{ml}$) or each crude extract (diluted 1:1,000) and the synthesized peptides were used as inhibitors (2 $\mu\text{g}/\text{ml}$).

All competitive ELISA inhibition experiments were performed in triplicate and data were expressed in mean values. The IgE-binding ability of each inhibitor was evaluated by the decrease of absorbance at 490 nm.

Results and Discussion

Cross-reactivity of Shellfish Allergens

The cross-reactivity between *Cra g 1* and *Tur c 1* was demonstrated by competitive ELISA inhibition experiments. As shown in Fig. 1, pretreatment of the patient serum with *Cra g 1* or *Tur c 1* reduced the reactivity of both *Cra g 1* and *Tur c 1* to the specific IgE antibodies in the se-

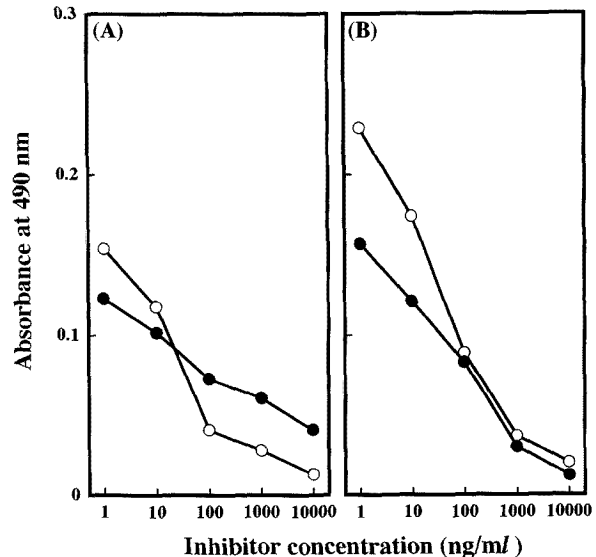


Fig. 1. Cross-reactivity between *Cra g 1* and *Tur c 1*.

Cra g 1 (○) or *Tur c 1* (●) was mixed with an equal volume of the sensitized serum (1:25 dilution). After incubation at 37°C for one hour, the mixture was added to the plate that had been previously coated with *Cra g 1* (A) or *Tur c 1* (B).

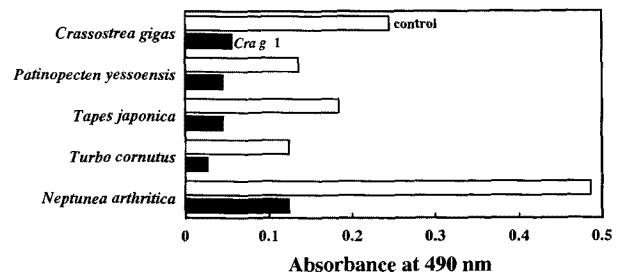


Fig. 2. Inhibitory ability of *Cra g 1* against the binding of allergens in the crude extracts of shellfishes to IgE antibodies in the sensitized serum.

Cra g 1 (2 $\mu\text{g}/\text{ml}$) was mixed with an equal volume of the sensitized serum (1:25 dilution). After incubation at 37°C for one hour, the mixture was added to the plate that had been previously coated with each crude extract of shellfish. Control represents the absorbance without the inhibitor (*Cra g 1*).

rum. These results suggest the occurrence of common epitopic sites in both *Cra g 1* and *Tur c 1*. In fact, one IgE-binding epitope (segment 256–284) proposed for *Tur c 1* is considered to be shared by *Cra g 1*, although that (segment 92–105) proposed for *Cra g 1* is not by *Tur c 1* due to replacements of several amino acid residues.¹¹⁾

The cross-reactivity among shellfish allergens was also examined by competitive ELISA inhibition analyses in which each crude extract was immobilized on the plate and *Cra g 1* was used as an inhibitor. As shown in Fig. 2, the absorbances of controls obtained without *Cra g 1* varied from species to species, indicating the difference in contents of allergens among shellfish. Nevertheless, the binding of crude extracts to the IgE antibodies in the patient serum was highly inhibited by the presence of *Cra g 1*, irrespective of species. Thus, it can be concluded that the

Table 1. Inhibition of the binding of *Cra g 1* to IgE antibodies in the sensitized serum by the synthesized peptides (P-1 to P-4)

Peptide	Amino acid sequence	Absorbance at 490 nm
P-1	⁸⁸ LNRRQLLEEDM ⁹⁹	0.722
P-2	⁹¹ RIQLLEEDMERS ¹⁰²	0.714
P-3	⁹⁴ LLEEDMERSEER ¹⁰⁵	0.587
P-4	⁹⁷ EDMERSEERLQT ¹⁰⁸	0.692
Control		0.742

Each inhibitor solution (2 µg/ml) was pre-incubated with an equal volume of the sensitized serum (diluted 1:25) at 37°C for one hour. The mixture was added to the plate that had been previously coated with *Cra g 1*. Control represents the absorbance without the inhibitor.

major allergen in shellfish is commonly tropomyosin having similar IgE-binding epitopes.

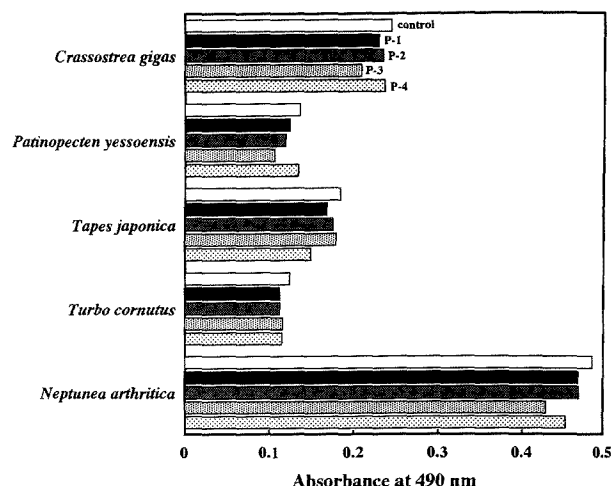
IgE-binding Epitope of *Cra g 1*

Four synthesized peptides (P-1 to P-4) were purified by reverse-phase HPLC (data not shown) and evaluated for their inhibitory ability against the binding of *Cra g 1* to IgE antibodies in the patient serum by competitive ELISA inhibition analyses. As summarized in Table 1, only peptide P-3 exhibited a high inhibitory ability. Comparison of the amino acid sequences of the synthesized peptides with that of K21d (IgE-binding epitope of *Cra g 1*, ⁹²IQLLEEDMERSEER¹⁰⁵) led us to assume that both *N*-terminal and *C*-terminal residues (⁹²IQLLE⁹⁶ and ¹⁰³EER¹⁰⁵) of K21d are more essential for the binding to the specific IgE antibodies than the interspaced segment ⁹⁷EDMERS¹⁰².

Although further competitive ELISA inhibition analyses using some synthesized peptides overlapping each other by one residue is needed to confirm the correct IgE-binding epitope of *Cra g 1*, our tentative results are comparable to those for *Gad c 1* (parvalbumin), the major allergen of the codfish *Gadus callarias*. In the case of *Gad c 1*, at least two of the three tetrapeptides (⁴¹DELK⁴⁴, ⁵¹DEDK⁵⁴, and ⁶¹DELK⁶⁴) interspaced by 6 amino acid residues are critical for a successful binding with the IgE antibodies but the interspaced segments are not.¹⁴⁾

Comparison of IgE-binding Epitopes of Shellfish Allergens

In order to compare the IgE-binding epitopes of shellfish allergens, the four synthesized peptides were subjected to competitive ELISA inhibition experiments and assessed for their inhibitory ability against the binding between crude extracts from various shellfish and IgE antibodies in the patient serum. As expected, only peptide P-3 exhibited a considerable decrease of absorbance with the crude extract from *C. gigas* (Fig. 3). Similar results were obtained with those from *P. yessoensis* and *N. arthritica*, indicating that the major allergen of these two species of shellfish has the epitopic sites with similar sequences to K21d of *Cra g 1*. In the case of the other two species (*T. japonica* and *T. cornutus*), however, no marked decrease of absorbance was caused by P-3. The result in *T. cornutus* coincides with our previous finding that the peptide fragment from *Tur c 1* corresponding to K21d of *Cra g 1* was not an IgE-binding epitope due to several substitutions of amino acid residues.¹¹⁾ The *T. japonica* allergen also appears to have significant substitutions of amino

**Fig. 3.** Inhibitory ability of the four synthesized peptides against the binding of allergens in the crude extracts of shellfishes to IgE antibodies in the sensitized serum.

ELISA conditions are as indicated in Fig. 2.

acid residues in the segment corresponding to K21d.

In conclusion, our results show that shellfish allergens are commonly tropomyosins but they do not always bear the same IgE-binding epitopes. At present, one IgE-binding epitope is known each for *Cra gs*^{12,13)} and *Tur c 1*.¹¹⁾ However, both *Cra gs* and *Tur c 1* require at least one more IgE-binding epitope to display allergenicity. In order to identify IgE-binding epitopes of shellfish allergens including *Cra gs* and *Tur c 1*, competitive ELISA inhibition experiments using various enzymatic fragments as inhibitors are needed. Also, various synthesized peptides containing a part of the possible IgE-binding epitopes should be subjected to competitive ELISA inhibition experiments to identify critical amino acid residues for the binding to specific IgE antibodies in sensitized sera. These future studies will give benefits to the development of hypoallergenic shellfish products without loss of physical, sensory, and nutritional functions by enzymatic hydrolysis as well as site-directed protein modification.

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