

Purification and IgE-Binding Epitopes of a Major Allergen in the Gastropod *Turbo cornutus*

Masaru ISHIKAWA, Masami ISHIDA, Kuniyoshi SHIMAKURA, Yuji NAGASHIMA, and Kazuo SHIOMI[†]

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

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The major allergen (*Tur c 1*) in the muscle of the gastropod, *Turbo cornutus*, was isolated by Sephacryl S-300, Mono Q HR 5/5 and TSKgel Phenyl-5PW RP column chromatography. ELISA showed *Tur c 1* to react strongly with sera from three individuals sensitive to both mollusks and crustaceans. SDS-PAGE showed *Tur c 1* to produce a major band corresponding to a molecular mass of 35 kDa under the reduced condition. Its amino acid composition was characterized by the abundance of Glx, followed by Leu, Ala and Lys in decreasing abundance, and the absence of Trp. In addition to these properties, the determined partial amino acid sequence identified *Tur c 1* to be a tropomyosin, as in the case of the known mollusk and crustacean allergens. However, the results of competitive ELISA inhibition experiments suggest that *Tur c 1* has an IgE-binding epitope in the C-terminal region which is dissimilar to those proposed for *Cra g 1* (the oyster *Crassostrea gigas* allergen) and *Pen i 1* (the shrimp *Penaeus indicus* allergen).

Key words: allergen; gastropod; IgE-binding epitope; tropomyosin

Introduction

Coastal countries such as Japan and the Scandinavian countries rank seafood as one of the main causes of the type I allergy reaction mediated by antigen-specific IgE antibodies in sensitized human sera. Two classes of proteins, parvalbumins and tropomyosins, have already been recognized as seafood allergens. As demonstrated for codfish *Gadus callarias*,^{1,2} Atlantic salmon *Salmo salar*³ and horse mackerel *Trachurus japonicus*,⁴ fish allergens are parvalbumins, while all the major allergens purified from three species of shrimp, *Penaeus indicus*,^{5,6} *P. aztecus*⁷ and *Metapenaeus ensis*,⁸ are not parvalbumins but tropomyosins. Similar to the case of shrimp, tropomyosins have recently been identified as the major allergens of two species of mollusks, oyster *Crassostrea gigas*^{9,10} and squid *Todarodes pacificus*.¹¹ It is interesting to note that the IgE-binding epitopes proposed for the *P. indicus* allergen (*Pen i 1*)⁶ and the *C. gigas* allergen (*Cra g 1*)¹⁰ differ from each other,

although both allergens are tropomyosins.

In order to confirm whether mollusk allergens would commonly be tropomyosins, we initiated this study on the gastropod, *Turbo cornutus*, which is taxonomically remote from both oyster and squid. Although the major allergen (designated as *Tur c 1* according to the International Union of Immunological Societies' nomenclature) purified from *T. cornutus* was found to be tropomyosin, as expected, its IgE-binding epitopes were distinguishable from those of *Cra g 1* and from those of *Pen i 1*. We describe here the purification of *Tur c 1* and its characterization, with special reference to the IgE-binding epitopes.

Materials and Methods

Shellfish. Live specimens of *T. cornutus* were purchased from a local market. They were shucked and dissected, and the muscle was used for the subsequent experiments.

Sera. Sensitized human sera were obtained from 4 subjects (subjects 1–4). Subjects 1–3 were intolerant to both mollusks and crustaceans, and subject 4 only to crustaceans. Control sera were obtained from 2 subjects (subjects 5 and 6) without adverse reactions after the ingestion of any seafood. These sera were stored at –20°C until use.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was carried out as outlined before.⁹ Briefly, the antigens immobilized on a microtiter plate (ELISA Plate H type with 96 wells; Sumitomo Bakelite, Tokyo, Japan) were immunoreacted with antigen-specific IgE antibodies in the sensitized sera (diluted 1:50) and then with the peroxidase-conjugated goat anti-human IgE antibody (diluted 1:2500; Kirkegaard & Perry Laboratories, MD, U.S.A.). The enzyme reaction was performed in 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.1% o-phenylenediamine and 0.03% H₂O₂.

The serum from subject 1 was used as a sensitized serum in competitive ELISA inhibition experiments. The serum was diluted (1:25) with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4)

[†] To whom correspondence should be addressed (Fax: +81-3-5463-0669, E-mail: shiomi@tokyo-u-fish.ac.jp).

Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; BSA, bovine serum albumin; CAM, S-carboxamidomethylated; TFA, trifluoroacetic acid; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; PBS, phosphate-buffered saline

and incubated at 37°C for 1 h with an equal volume of a sample solution (1 µg/ml) made in PBS. This solution was then added to a microtiter plate that had previously been coated with the isolated antigen (100 ng/well). The subsequent procedure was the same as that for ELISA. All ELISA runs were performed in triplicate, and the data are given as mean values.

Isolation procedure. The major allergen in the muscle of *T. cornutus* was isolated by the same procedure as that previously employed for *Cra* gs (the oyster *C. gigas* allergens).⁹⁾ In brief, the boiled aqueous extract from 23 g of the muscle was successively applied to gel filtration on a Sephacryl S-300 column (2.5 × 95 cm; Pharmacia Biotech, Uppsala, Sweden), anion-exchange fast protein liquid chromatography (FPLC) on a Mono Q HR 5/5 column (0.5 × 5 cm; Pharmacia Biotech) and reverse-phase high-performance liquid chromatography (HPLC) on a TSKgel Phenyl-5PW RP column (0.46 × 7.5 cm; Tosoh, Tokyo, Japan). Throughout the isolation procedure, the allergens were traced by ELISA, using the serum from subject 1 as the sensitized serum and that from subject 5 as the control.

Reduction and S-carboxamidomethylation. The isolated allergen (1 mg) was dissolved in 1 ml of 0.5 M Tris-HCl buffer (pH 8.5) containing 6 M guanidine-HCl and 2.5 mM EDTA, and then reduced with 2 mg of dithiothreitol (DTT) at room temperature for 2 h under nitrogen gas. Subsequently, 5 mg of iodoacetamide was added, and the reaction mixture was kept in the dark at room temperature for 30 min. The S-carboxamidomethylated (CAM) derivative produced was isolated by reverse-phase HPLC on a TSKgel Phenyl-5PW RP column (Tosoh). The column was eluted at a flow rate of 0.5 ml/min by a linear gradient of acetonitrile (0–70% in 100 min) in 0.1% trifluoroacetic acid (TFA). The derivatized allergen was monitored at 280 nm with a UV detector.

Preparation of the maleyl derivative. To the CAM-allergen (500 µg) dissolved in 10 ml of 0.1 M borate buffer (pH 9.0) was slowly added 1.5 ml of 1 M maleic anhydride in 1,4-dioxan. The reaction mixture was kept at room temperature for 10 min while stirring, and the pH value of the mixture was maintained at 9.0 by adding 0.1 M NaOH. The reactant was then dialyzed against dilute aq. NH₃ (pH 9.0) and lyophilized. To remove the side-reacted maleic anhydride from the hydroxyl group of Ser and Thr, the lyophilisate was dissolved in 0.8 M hydroxylamine (pH 9.0) and kept at 4°C for 18 h. After being dialyzed against dilute aq. NH₃ (pH 9.0) and lyophilized, the dried material was used as the maleyl-allergen.

Enzymatic digestion. The CAM-allergen (500 µg) was digested with 2 µg of lysylendopeptidase (E.C. 3.4.21.50; Wako Pure Chemicals, Osaka, Japan) in 1 ml of 0.1 M Tris-HCl buffer (pH 9.0) at 35°C for 6 h. The maleyl-allergen (500 µg) was hydrolyzed with 2 µg of TPCK-trypsin (E.C. 3.4.21.4; Worthington Biochemi-

cals, NJ, U.S.A.) in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM CaCl₂ at 37°C for 18 h. Maleic anhydride was unblocked from the tryptic digest by heating at 80°C for 45 min in 1 M formic acid. To separate the peptide fragments, each digest was applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh), which was eluted at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0–56% in 120 min) in 0.1% TFA. Peptides were monitored at 220 nm with a UV detector. Each isolated peptide was semi-quantified by comparing its peak area with that of the isolated allergen used as a standard.

Analytical methods. Protein was determined by the method of Lowry *et al.*,¹²⁾ using BSA as a standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a PhastSystem apparatus (Pharmacia Biotech), using ready-made slab gel (PhastGel Gradient 10–15) and ready-made buffer strips (PhastGel SDS Buffer Strips). Running, staining with Coomassie Brilliant Blue R-250 and destaining were carried out according to the manufacturer's instructions. Prior to SDS-PAGE, a sample was dissolved in 10 mM Tris-HCl buffer (pH 6.8) containing 1% SDS, 20% glycerin and 5% DTT, and then heated in a boiling water bath for 15 min. A low molecular weight calibration kit (Pharmacia Biotech) containing phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) was used as a reference.

For the amino acid analysis, the isolated allergen (45 µg) was hydrolyzed with 50 µl of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in an evacuated tube at 115°C for 24 h. After 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 0.45 ml portion of the diluate was applied to an Atto MLC-703 amino acid analyzer. The sequences of the peptide fragments were analyzed by the automated Edman degradation method with a gas-phase protein sequencer (LF-3400D TriCart with high sensitivity chemistry; Beckman, CA, U.S.A.).

Results

Isolation of Tur c 1

When the extract of *T. cornutus* was applied to a Sephacryl S-300 column, the major allergen detected by ELISA with the sensitized serum was eluted at around fraction 25 (Fig. 1). Anion-exchange FPLC on a Mono Q HR 5/5 column showed the major allergen as a large peak at a retention time of about 27 min (Fig. 2). The allergen-containing fraction was finally applied to reverse-phase HPLC on a TSKgel Phenyl-5PW RP column. As shown in Fig. 3, the allergen was eluted in the peak at a retention time of about 27 min. Thus, 1.8 mg of *Tur c 1* was obtained from 23 g of the starting material.

Properties of Tur c 1

It is apparent that *Tur c 1* was allergenic to subject 1, since the purification of *Tur c 1* was based on ELISA with the serum from subject 1. The allergenicity of *Tur c*

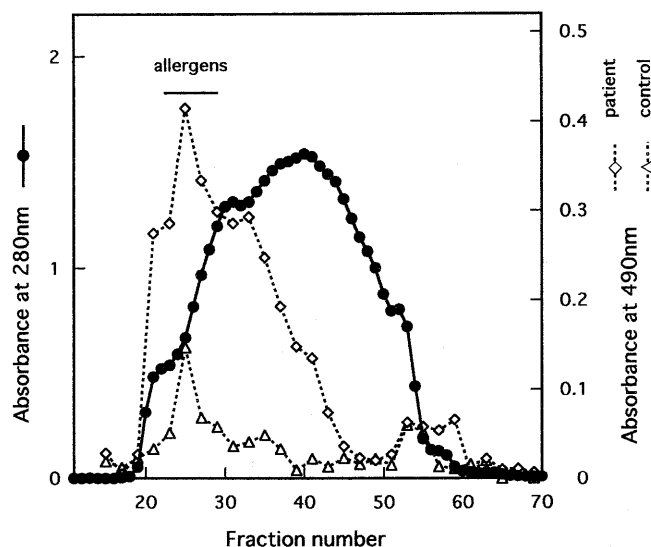


Fig. 1. Gel Filtration of the Crude Extract.

The boiled aqueous extract from the muscle of *T. cornutus* was loaded onto a Sephacryl S-300 column (2.5 × 95 cm) which was eluted with 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. Fractions of 10 ml were collected at a flow rate of 17 ml/h. The fractions indicated by a bar were pooled.

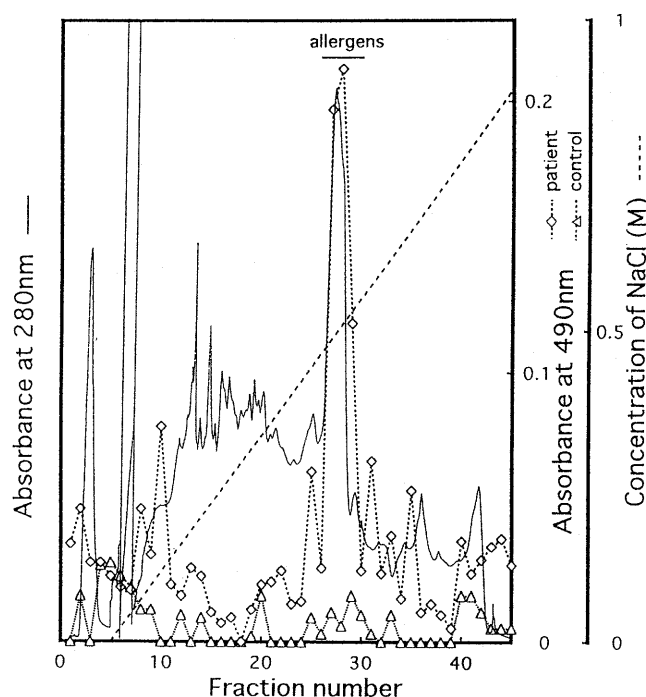


Fig. 2. Anion-exchange FPLC of the Allergen-containing Fraction Obtained by Gel Filtration.

The allergen-containing fraction obtained by gel filtration (see Fig. 1) was applied to a Mono Q HR 5/5 column (0.5 × 5 cm). The column was washed with 10 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient (0.0–1.0 M in 45 min) of NaCl in the buffer. Fractions of 1 ml were collected at a flow rate of 1 ml/min. The fractions indicated by a bar were pooled.

1 against the other patients (subjects 2 and 3 sensitive to both mollusks and crustaceans, and subject 4 sensitive only to crustaceans) and the controls (subjects 5 and 6)

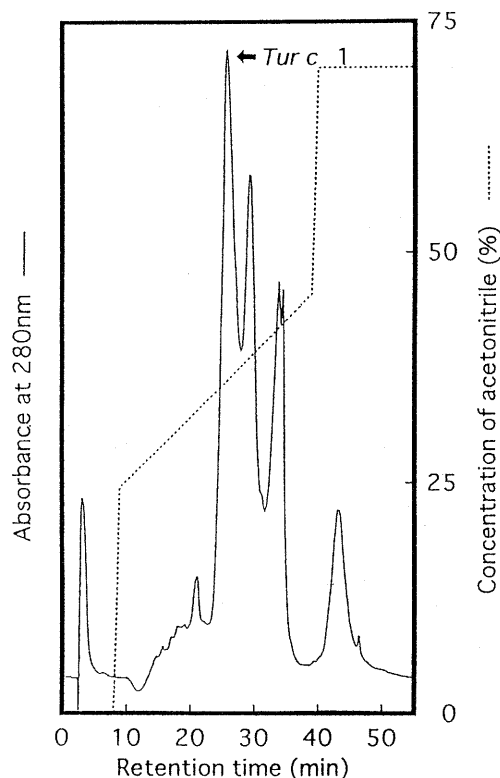


Fig. 3. Reverse-phase HPLC of the Allergen-containing Fraction Obtained by Anion-exchange FPLC.

The allergen-containing fraction obtained by anion-exchange FPLC (see Fig. 2) was chromatographed on a TSKgel Phenyl-5PW RP column (0.46 × 7.5 cm). Elution was performed with a gradient of acetonitrile in 0.1% TFA. The flow rate was maintained at 0.5 ml/min. *Tur c 1* was eluted in the peak at a retention time of 27 min, as indicated by the arrow.

was further assessed by ELISA, in which *Tur c 1* (100 ng/well) was reacted with each serum (diluted 1:50). As a result, *Tur c 1* strongly reacted with the sera from both subjects 2 and 3 to almost the same extent as that with the serum from subject 1, suggesting that *Tur c 1* was commonly allergenic to the 3 subjects. In contrast, the reaction with the serum from subject 4 was much weaker, and those with the control sera from subjects 5 and 6 were negligible.

SDS-PAGE performed under the reduced condition produced *Tur c 1* as a major band at the position corresponding to a molecular mass of 35 kDa (Fig. 4). The amino acid composition of *Tur c 1* is characterized by the abundance of Glx, followed by Leu, Ala and Lys in decreasing order of abundance, and the absence of Trp (Table). These chemical properties are very similar to those of tropomyosins from other sources.^{5,7,8,11)}

Partial amino acid sequence of *Tur c 1*

Twenty-four peptides (named K1-24 in their order of elution, Fig. 5) were isolated from the lyslendopeptidase digest of CAM-*Tur c 1* by reverse-phase HPLC, and 18 peptides (named MT1-18, Fig. 6) from the tryptic digest of maleyl-*Tur c 1*. Eight peptides (K7, 19, 20, 21, 23 and 24, and MT15 and 17) were selected for sequence analyses. The determined partial amino acid sequence

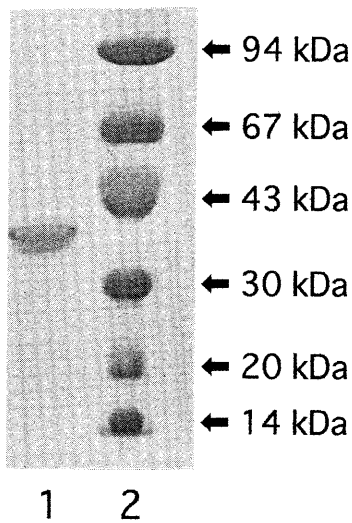


Fig. 4. SDS-PAGE of *Tur c 1*. Prior to electrophoresis, *Tur c 1* (lane 1) and standard proteins (lane 2) were treated with 1% SDS in the presence of 5% DTT. The protein bands were visualized with Coomassie Brilliant Blue R-250.

Table Amino Acid Composition of *Tur c 1*

Amino acid	Mol%
Asx	9.2
Thr	5.1
Ser	5.4
Glx	18.2
Pro	0.1
Gly	2.5
Ala	12.6
Val	4.0
Cys (half)	0.5
Met	1.8
Ile	4.0
Leu	14.5
Tyr	2.2
Phe	1.0
His	0.2
Trp	0.0
Lys	10.4
Arg	8.3

of *Tur c 1* is shown in Fig. 7, along with the partial amino acid sequences of tropomyosins from oyster *C. gigas* (*Cra g 1*)¹⁰ and shrimp *P. indicus* (*Pen i 1*)⁶ and the complete sequences of those from mussel *Mytilus edulis*, gastropod *Haliotis rufescens* and shrimp *M. ensis* (*Met e 1*).⁸ *Tur c 1* was obviously similar to the tropomyosins from other sources, having an especially close homology (95%) with that from *H. rufescens*. Thus, *Tur c 1* was identified as the tropomyosin of *T. cornutus*.

IgE-binding epitopes of Tur c 1

The ability of each peptide fragment to inhibit the binding of IgE antibodies in the sensitized serum to *Tur c 1* was assessed by competitive ELISA inhibition experiments. None of the 24 lysylendopeptidase peptides showed significant inhibitory ability (Fig. 8), suggesting

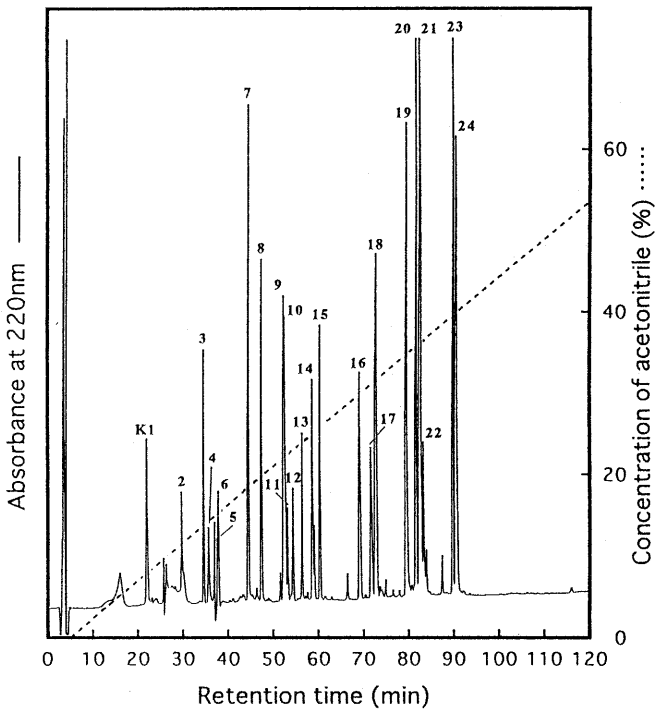


Fig. 5. Reverse-phase HPLC of the Peptides Produced by Digesting CAM-*Tur c 1* with Lysylendopeptidase. The lysylendopeptidase digest of CAM-*Tur c 1* was applied to a TSKgel ODS-120T column (0.46 × 25 cm). Elution was performed with a linear gradient of acetonitrile in 0.1% TFA. The flow rate was maintained at 1 ml/min. Twenty-four peptides (K1-24) were isolated.

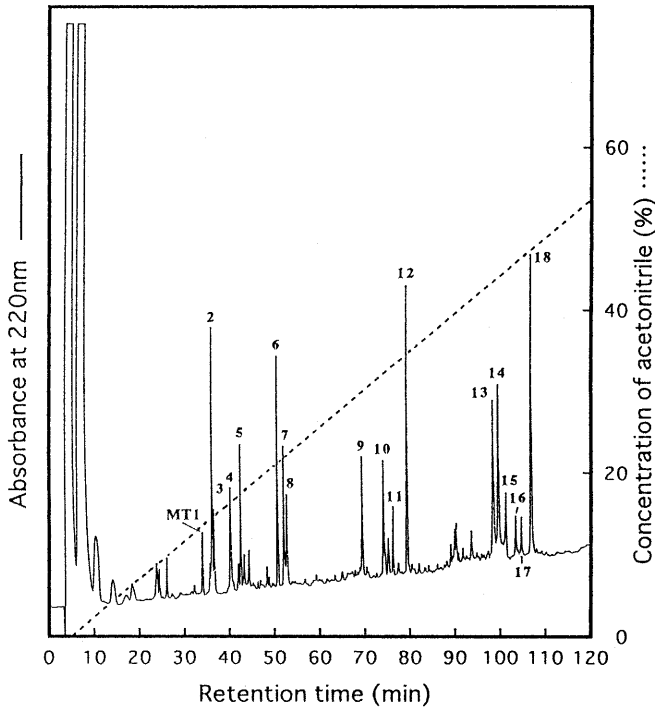
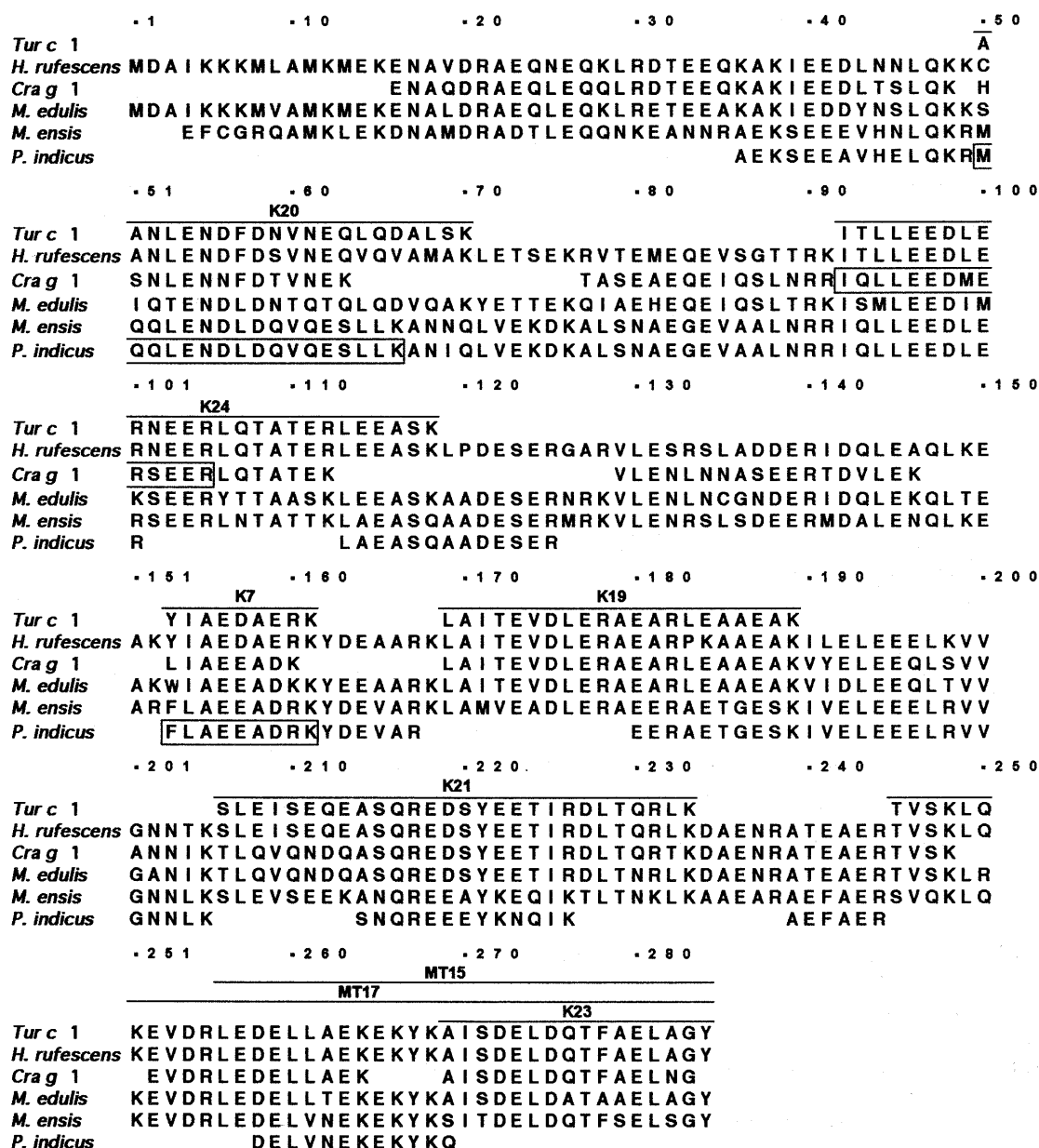


Fig. 6. Reverse-phase HPLC of the Peptides Produced by Digesting Maleyl-*Tur c 1* with Trypsin. The HPLC conditions were as in Fig. 5. Eighteen peptides (MT1-18) were isolated.



The determined partial amino acid sequence of *Tur c* 1 is aligned with the complete or partial amino acid sequences of tropomyosins from gastropod *Haliotis rufescens*, oyster *Crassostrea gigas* (*Cra g* 1), mussel *Mytilus edulis* and two species of shrimp, *Metapenaeus ensis* (*Met e* 1)⁶ and *Penaeus indicus* (*Pen i* 1).⁶ For the *H. rufescens* and *M. edulis* tropomyosins, putative amino acid sequences were translated from the nucleotide sequence databases (*H. rufescens*, submitted to EMBL under accession number X75218; *M. edulis*, submitted to EMBL, GenBank and DDBJ under accession number U40035). The denotations preceded with K and MT represent the peptides isolated from the lysylendopeptidase digest of CAM-*Tur c* 1 (see Fig. 5) and from the tryptic digest of maleyl-*Tur c* 1 (see Fig. 6), respectively. The sequences which are boxed represent the IgE-binding epitopes proposed for *Cra g* 1 and *Pen i* 1.

its retention time by reverse-phase HPLC.

Discussion

In this study, the major allergen (*Tur c 1*) in the muscle of the gastropod, *T. cornutus*, was isolated and demonstrated to be a tropomyosin. The determined partial amino acid sequence, together with the determined molecular mass and amino acid composition, provides evidence for *Tur c 1* being a tropomyosin. Tropomyosins are well known as shrimp allergens.⁶⁻⁸⁾ In addition,

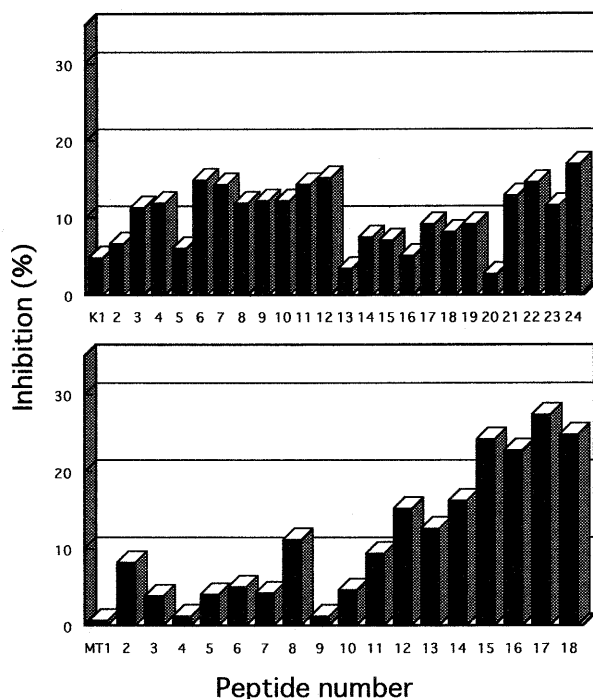


Fig. 8. Inhibition of the Binding of *Tur c 1* to IgE Antibodies in Sensitized Serum by the Lysylendopeptidase Peptides (K1-24) and Tryptic Peptides (MT1-18).

Each peptide solution (1 $\mu\text{g}/\text{ml}$ made in PBS) was mixed with an equal volume of the sensitized serum (1:25 dilution) from subject 1. After incubating at 37°C for 1 h, the mixture was added to the plate previously coated with *Tur c 1* (100 ng/well).

recent studies have established the major allergens of two mollusks (oyster^{9,10} and squid¹¹) to also be tropomyosins. Therefore, it is relevant to consider that tropomyosins are common allergens of mollusks as well as shrimps.

The results of competitive ELISA inhibition analyses reveal that one of the IgE-binding epitopes of *Tur c 1* is located in the C-terminal region. Both peptide MT15 (positions 256–284) and its N-terminal prolonged peptide MT17 (positions 245–284), like peptide MT18 that is supposed to be undigested maleyl-*Tur c 1*, showed a high inhibitory effect on the binding between IgE antibodies in the sensitized serum and *Tur c 1*. Importantly, peptide K23 (positions 269–284), which is the C-terminal fragment produced from CAM-*Tur c 1* by lysylendopeptidase digestion, was much less inhibitory. Moreover, we have previously reported that the two peptides obtained by lysylendopeptidase digestion of CAM-*Cra g 1*, one (positions 252–264) with the same sequence as *Tur c 1* and the other (positions 269–283) with only one alteration at position 282, were negative in competitive ELISA inhibition analyses.¹⁰ These results suggest that the 4 residues at positions 265–268 (or the 4 residues + several adjacent residues) are the critical amino acids for the binding of *Tur c 1* to specific IgE antibodies in sensitized human sera.

Of the tropomyosins identified as allergens in shrimps and mollusks, *Pen i 1* from shrimp *P. indicus*⁶ and *Cra g 1* from oyster *C. gigas*¹⁰ have been examined for their

IgE-binding epitopes. A comparison of the previous results with those obtained for *Tur c 1* leads to valuable information. One significant finding is that neither of the two IgE-binding epitopes (positions 50–66 and 153–161) proposed for *Pen i 1*⁶ were the IgE-binding epitopes for *Tur c 1* or *Cra g 1*. Although the two peptides K20 (positions 50–70) and K7 (positions 153–161), which include or completely correspond to the segments of the IgE-binding epitopes of *Pen i 1*, were obtained from lysylendopeptidase digestion of CAM-*Tur c 1*, they were not judged to contain IgE-binding epitopes based on competitive ELISA inhibition analyses. As compared to the sequence of *Pen i 1*, *Tur c 1* has many differences in both positions 50–66 (9 alterations) and 153–161 (4 alterations), accounting for the invalidity of K20 and K7 as IgE-binding epitopes. Such alterations are also apparent in the corresponding positions of *Cra g 1*. In contrast, the sequences corresponding to the IgE-binding epitopes of *Pen i 1* are completely preserved in *Met e 1* (the shrimp *M. ensis* allergen). Thus, the IgE-binding epitopes of shellfish tropomyosins differ from those of shrimp tropomyosins, although Lehrer and McCants¹³ have suggested the occurrence of common antigenic structures between shrimp and oyster allergens from the allergenic cross-reactivity of crude extracts.

Another significant finding is that each shellfish tropomyosin seems to have its own IgE-binding epitopes. Apart from the possibility that *Cra g 1*, like *Tur c 1*, may have one of the IgE-binding epitopes in the C-terminal region because of the high sequence homology between both allergens, *Cra g 1* has been reported to have an IgE-binding epitope in the segment ranging from positions 92 to 105.¹⁰ In this study, however, peptide K20 (positions 92–118) generated by the lysylendopeptidase digestion of CAM-*Tur c 1* did not inhibit the binding of *Tur c 1* to specific IgE antibodies in the sensitized serum. In the segment between positions 92 and 105, *Tur c 1* has three alterations (positions 93, 99 and 102) compared to *Cra g 1*. This fact indicates that at least one of the three residues (Gln-93, Met-99 and Ser-102) is essential for binding tropomyosins to specific IgE antibodies in sensitized sera.

In conclusion, *Tur c 1* has an IgE-binding epitope not in the regions containing the IgE-binding epitopes previously proposed for either *Pen i 1* or *Cra g 1* but in the C-terminal region. However, it should be kept in mind that results in competitive ELISA inhibition experiments may vary according to the serum used. Although the sera from subjects 2 and 3 were confirmed to strongly react with *Tur c 1*, it is still uncertain whether they would display the same reactivity with the peptide fragments produced by enzyme treatments as the serum from subject 1 that was used in the competitive ELISA inhibition experiments. In order to disclose more details about the IgE-binding epitopes of tropomyosins, competitive ELISA inhibition analyses should be conducted on many sensitized sera, enzymatic fragments of tropomyosins from various sources and synthesized peptides corresponding to the proposed IgE-binding epitopes.

Acknowledgments

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References

- 1) Elsayed, S. and Aas, K., Isolation of purified allergens (cod) by isoelectric focusing. *Int. Arch. Allergy Appl. Immunol.*, **40**, 428-438 (1971).
- 2) Elsayed, S. and Bennich, H., The primary structure of allergen M from cod. *Scand. J. Immunol.*, **4**, 203-208 (1975).
- 3) Lindstrøm, C. D-V., Van Dô, T., Hordvik, I., Endresen, C., and Elsayed, S., Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (*Salmo salar*). *Scand. J. Immunol.*, **44**, 335-344 (1996).
- 4) Shiomi, K., Hayashi, S., Ishikawa, M., Shimakura, K., and Nagashima, Y., Identification of parvalbumin as an allergen in horse mackerel muscle. *Fisheries Sci.*, **64**, 300-304 (1998).
- 5) Nagpal, S., Rajappa, L., Metcalfe, D. D., and Subba Rao, P. V., Isolation and characterization of heat-stable allergens from shrimp (*Penaeus indicus*). *J. Allergy Clin. Immunol.*, **83**, 26-36 (1989).
- 6) Shanti, K. N., Martin, B. M., Nagpal, S., Metcalfe, D. D., and Subba Rao, P. V., Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J. Immunol.*, **151**, 5354-5363 (1993).
- 7) Daul, C. B., Slaterry, M., Reese, G., and Lehrer, S. B., Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *Int. Arch. Allergy Immunol.*, **105**, 49-55 (1994).
- 8) Leung, P. S. C., Chu, K. H., Chow, W. K., Ansari, A., Bandea, C. I., Kwan, H. S., Nagy, S. M., and Gershwin, M. E., Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. *J. Allergy Clin. Immunol.*, **94**, 882-890 (1994).
- 9) Ishikawa, M., Shimakura, K., Nagashima, Y., and Shiomi, K., Isolation and properties of allergenic proteins in the oyster *Crassostrea gigas*. *Fisheries Sci.*, **63**, 610-614 (1997).
- 10) Ishikawa, M., Ishida, M., Shimakura, K., Nagashima, Y., and Shiomi, K., Tropomyosin, the major oyster *Crassostrea gigas* allergen and its IgE-binding epitopes. *J. Food Sci.*, **63**, 44-47 (1998).
- 11) Miyazawa, H., Fukamachi, H., Inagaki, Y., Reese, G., Daul, C. B., Lehrer, S. B., Inouye, S., and Sakaguchi, M., Identification of the first major allergen of a squid (*Todarodes pacificus*). *J. Allergy Clin. Immunol.*, **98**, 948-953 (1996).
- 12) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).
- 13) Lehrer, S. B. and McCants, M. L., Reactivity of IgE antibodies with crustacea and oyster allergens. Evidence for common antigenic structures. *J. Allergy Clin. Immunol.*, **80**, 133-139 (1987).