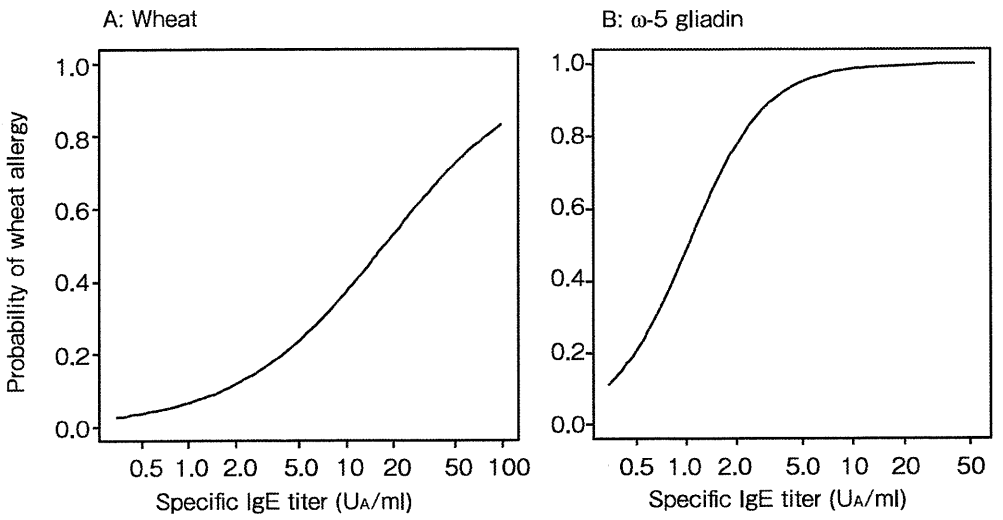

Fig. 3. Probability curves for wheat and ω-5 gliadin IgE
Fitted predicted probability curves for the diagnosis of wheat allergy at a given IgE titer to wheat (A) and ω-5 gliadin (B) are shown. These curves were created based on 59 wheat allergy patients (52 of whom were challenge positive), and 174 clinically evaluated non-wheat allergy subjects.

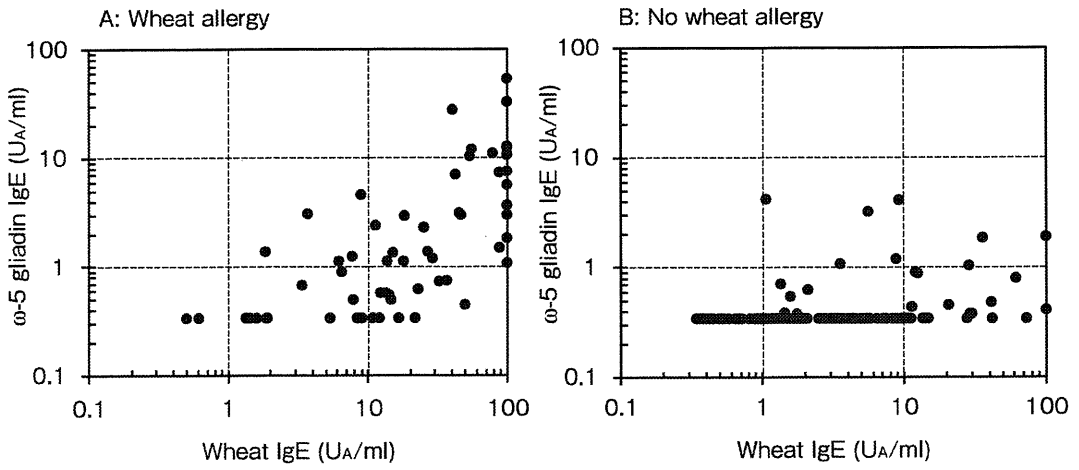

Fig. 4. Correlation between wheat and ω-5 gliadin IgE
Scatter diagrams of individual IgE titers to wheat and ω-5 gliadin in patients with wheat allergy (A) and no wheat allergy (B) are shown. Spearman's correlation coefficient was higher in patients with wheat allergy (A, $r=0.734$, $p<0.001$) than those with no wheat allergy (B, $r=0.357$, $p<0.001$).

考　察

本研究では，できるだけ臨床現場に即した条件下で，小麦アレルギーの診断に対する ω-5 グリアジン IgE の有用性を検討した．小麦アレルギー群は，採血後に負荷試験陽性，又はアナフィラキシー

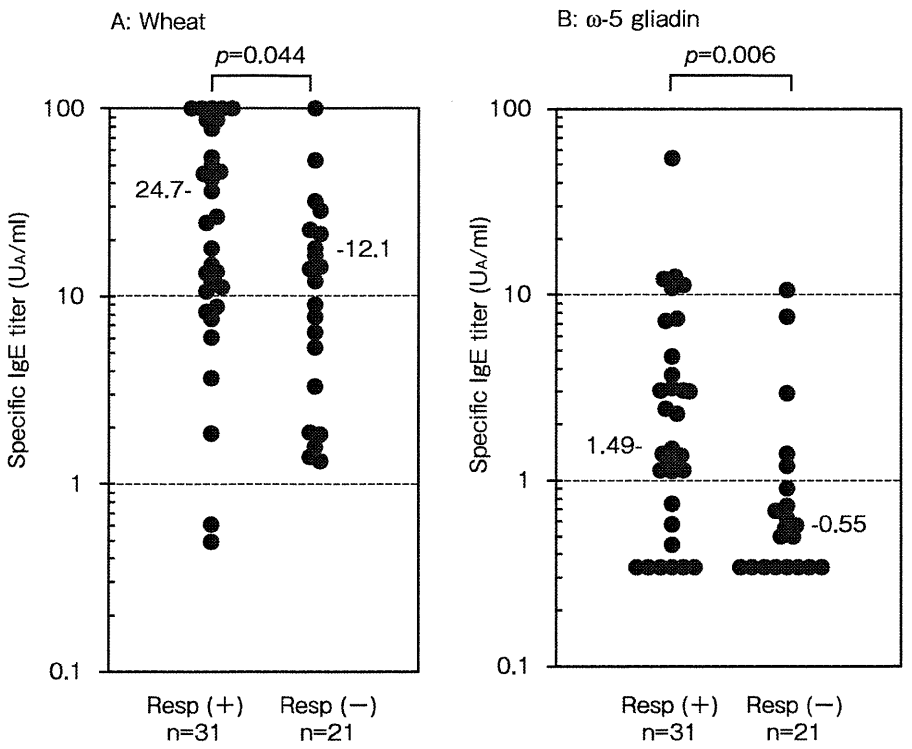

Fig. 5. Respiratory symptoms and IgE titers
Individual titers of IgE specific to wheat (A) and ω-5 gliadin (B) are shown in the challenge-positive patients, divided by the presence (n=31) or absence (n=21) of respiratory symptoms, designated as Resp (+) or Resp (−) in the figure. Significant difference in median IgE titer of wheat and ω-5 gliadin was detected Resp (+) versus Resp (−), using Mann-Whitney U test.

に近い誘発症状を繰り返す患者群である．非小麦アレルギー群は負荷試験陰性の結果も踏まえながら，日常的に小麦摂取が可能であるという問診を重視して判定した．対象者を負荷試験実施症例に限定しなかった理由は，主に次の3つである．

①負荷試験は耐性獲得の確認や，摂取後に何らかの症状の訴えがある症例に限定して実施することが多く，むしろ症例選択のバイアスを生じやすい．

②負荷陽性後にも，比較的短期間で自然に耐性が進む症例が存在する．

③負荷陰性の後も，何らかの症状を訴えて積極的に摂取を進めていない症例がしばしば存在する．

こうした情報を確認した上で，情報が曖昧な一部の症例を検討から削除することで，診断の正確さと症例選択のバイアスをできるだけ減らすことを期して評価を試みた．

その結果は，小麦感作症例を選択して報告した我々の既報[10]と基本的に一致しており，抗体価高値の場合に高い陽性的中率を示した．さらに既報では解析できなかった点として，0.35UA/mlをカットオフとすると診断特異度が87%であることを新たに示した．一方，小麦アレルギー患者の中にもω-5グリアジンIgE抗体陰性例が存在する，つまり診断感度には限界があることも再確認された．

これらの結果は，ω-5グリアジンが小麦の主要

なアレルゲンコンポーネントであることを証明する一方で，それ以外にも小麦アレルギーの症状に関与する重要なコンポーネントが存在することを示唆している．

小麦アレルゲンは多くのコンポーネントが報告されており，その多くは水溶性画分に存在する．古くはα-amylase/trypsin inhibitor[15]がBaker's asthmaの原因アレルゲンとされ，その他にもAcyl-CoA oxidase[16]，Peroxydase[17]，Triosephosphate isomerase[18]，Profilin[19]，Serine carboxypeptidase 2[20]，さらに最近でも新たにSerine proteinase inhibitor[21]，Lipid transfer protein[22]などが報告されている．また，グルテン構成タンパクでも，他のグリアジン画分や高分子量グルテニン[23]のアレルゲン性が報告されている．従って，ω-5グリアジンは主要なアレルゲンコンポーネントの一つではあるが，小麦アレルギーの全てを代表する成分ではない[24]．

小麦アレルギー患者の大部分は，臨床的にはコメやその他の穀物に交差反応を示さないことから，症状誘発に関与するアレルゲンコンポーネントやそのエピトープは，小麦特有のアレルゲン性を持つことが推測される[25]．一方小麦アレルギー患者の一部はライ麦，大麦に交差反応することが知られており，実際にライ麦のγ-70 secalin及びγ-35 secalin，大麦のγ-3 hordeinは小麦のω-5グリアジンと交差反応する[26]ことが知られている．

ω-5グリアジンIgEは，成人のWDEIAの主要なアレルゲンコンポーネントとして報告されてきた[7]．MatsuoらはWDEIA患者のIgE抗体が結合するエピトープ[23]を詳細に明らかにして，エピトープだけを組み合わせた合成ペプチドでもIgE抗体との反応性を有すること，小麦IgE抗体陰性患者においてもω-5グリアジンIgEが陽性となる者がおり，感度・特異度ともに小麦IgEより優れた臨床検査になる[9]ことを報告した．さらに，負荷試験において症状が誘発された患者の血清中からグリアジン抗原の検出[27]に成功し，運動やアスピリンによって抗原吸収が増加していることを証明した．

ω-5グリアジンが小児を中心とした即時型小麦アレルギーにおいても主要なアレルゲンであることは，Palosuoら[28]がELISAを使用したIgE抗体測定系で報告し，中でも小麦アナフィラキシーの症例[29]における診断的有用性が指摘された．一方Beyerら[30]は，アメリカ及びドイツの小麦アレルギー患者でω-5グリアジンIgEを検討し，診断的有用性に異議を述べる報告をした．しかしこの報告は症例数も少なく，負荷陽性者の中に小麦IgE抗体陰性例が多く含まれたり，負荷陽性・陰性群の間で小麦IgE抗体価の有意差を認めない母集団であるため，小麦アレルギーの臨床像が国や人種によって異なる可能性を否定できない．

我々は，小麦IgE陽性の小児を対象として小麦アレルギーの診断におけるω-5グリアジンIgEの有用性を検討し，抗体価がクラス3以上であればほぼ100%の診断特異性が得られること，抗体価が症状誘発閾値や誘発症状の重症度に関連することを報告した[10]．さらにTokudaら[31]は，ω-5グリアジンを用いたCD203cの発現増強を指標とする好塩基球刺激試験で，小麦アレルギーの診断上の有用性を報告している．

即時型小麦アレルギーの診断における特異的IgE抗体検査の評価として，Komataら[32]は小麦経口負荷試験に基づいた小麦IgEのprobability curveを報告し，特に1歳以上の症例では抗体価が100UA/mlでも陽性的中率は90%に至らないことを指摘した．今回我々が対象とした患者における小麦IgEのプロバビリティーカーブも，Komataらの報告と極めて近似した結果であった．その中でω-5グリアジンIgEは，小麦アレルギーの診断に対してクラス3で87%，クラス4以上の3人は全員小麦アレルギーと，我々の既報[10]と同様に高い特異度を確認した．

一方ω-5グリアジンIgEの診断感度は76.2%に留まり，ω-5グリアジンIgE陰性は小麦アレルギーを否定する根拠とはならない．同時に測定した小麦IgEは診断感度100%（小麦アレルギー群は全例陽性）であったことから，ω-5グリアジンIgEは単独で評価せず，必ず小麦IgEと併用して臨床診断に用いるべき検査と考えている．

小麦アレルギーの特徴の一つは，呼吸器症状の

誘発率が高いことである．今回の検討でも，呼吸器症状は負荷試験陽性者の61％に認められ，ω-5グリアジンIgE抗体価との関連も示唆された．呼吸器症状は，Baker's asthmaを対象とした多くの抗原分析が示すように，主として水溶性アレルゲンが関与している可能性がある．一方ω-5グリアジンは水溶性が低く，さらに摂取時には多くのタンパクが重合したグルテンとなっているため，消化酵素の働かない粘膜表面からは極めて吸収されにくいことが推測される．食物アレルゲンが呼吸器症状を誘発するメカニズムはほとんど解明されておらず，ω-5グリアジンが呼吸器症状にどのように関与するのかは今後の検討課題といえる．

Palosuoら[33]はin vitroの実験で，ω-5グリアジンがペプシンで分解された後，小腸のTissue transglutaminaseによって再重合し，WDEIA患者の特異的IgE抗体はそこにより強く結合することを報告している．この仮説はin vivoでは証明されていないが，グリアジンの示す複雑な抗原性を考察する上で興味深い．さらにグリアジンはセリアック病[34]の主要な抗原であること，患者血清からはグリアジン特異的IgA抗体と同時に，自己抗体としてTissue transglutaminase特異的IgA抗体が高率に検出されることからも，グリアジンは特異な免疫原性を持つことが示唆される．ω-5グリアジンは，グルタミン鎖の中にフェニルアラニンとプロリンが並んで構成されるIgEエピトープが全配列中に繰り返し存在する，極めてユニークなアレルゲン構造を示す．このアレルゲン構造と免疫応答との関わりを解明することは，今後の有益な研究課題になると思われる．

このように，同じ食物に対するアレルギーでも患者が認識するアレルゲンコンポーネントによって臨床像が異なる可能性がある．同様の解析は果物やナッツ類でも進んでおり，今後の食物アレルギー診療が発展する一つの方向性を示している[35]．

今回の対象者では，小麦IgE陰性の49名の中にω-5グリアジンIgE陽性者は見いだせず，全体として非小麦アレルギーの1例を除きω-5グリアジンIgEが小麦IgEを上回ることはなかった．成人のWDEIAで小麦IgE陰性者にω-5グリアジンIgE陽性患者が見いだされる[9]こととは，興味深い相違点である．今回の解析では，小麦アレルギー患者においては小麦IgEとω-5グリアジンIgEとの間に強い相関を認めたが，非小麦アレルギー患者においては弱い相関しか認めなかった．これは，小児の即時型小麦アレルギー患者は主としてω-5グリアジンに感作されているのに対して，非小麦アレルギー患者のIgE抗体はそれ以外のコンポーネントを多く認識していることを示唆している．

食物アレルギーの診断には，原因食品摂取時の誘発症状の確認が必須であり，IgE抗体価だけに基づく診断は必ずしも正確ではない．しかし，食物経口負荷試験は重篤な誘発症状を認めるリスクを伴い，高い陽性的中率を示す臨床検査は一般臨床において非常に有用である．

ω-5グリアジンIgEは，リコンビナントタンパクを用いた我が国初の食物アレルゲンコンポーネント特異的IgE抗体検査として，2010年10月から保険適応を取得した．今後，より多くのアレルゲンコンポーネント特異的IgE抗体が解析され，感度・特異度に優れた臨床検査が開発されることを期待したい．

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CLINICAL EVALUATION OF ω-5 GLIADIN-SPECIFIC IgE TEST

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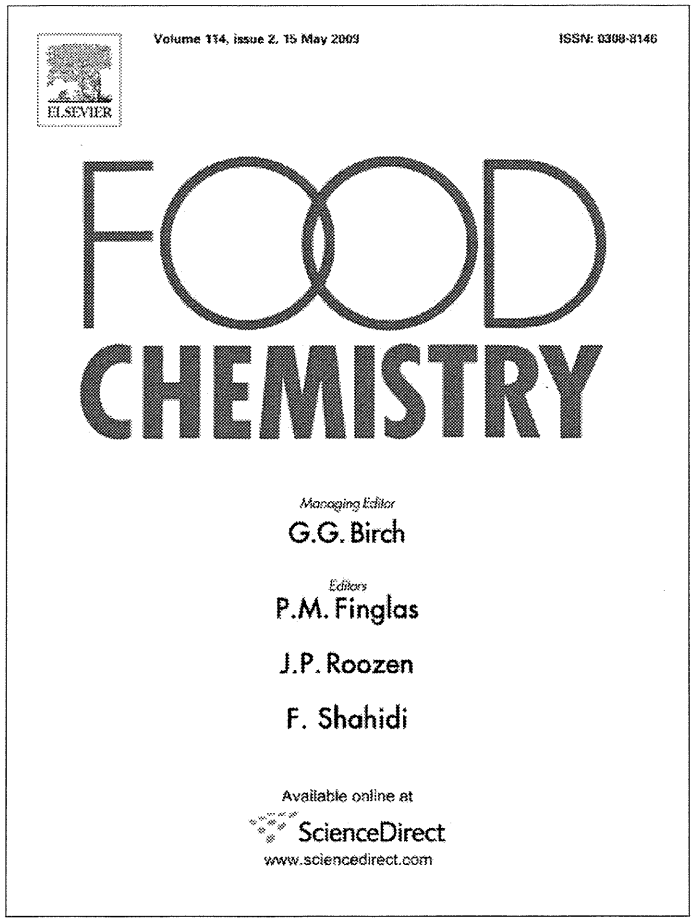
Objective: To reveal the clinical utility of an IgE test specific to ω-5 gliadin in the diagnosis of immediate-type wheat allergy.

Methods: We detected ω-5 gliadin-specific IgE in sera from all patients examined for wheat-specific IgE in our allergy clinic between January and October, 2008. The diagnostic value of the test was analyzed against the true diagnosis of wheat allergy based on oral wheat challenge or convincing clinical history. Subjects comprised 233 patients (median age, 3.6 years), 59 patients were diagnosed with wheat allergy, and 174 were judged to have no wheat allergy.

Results: The prevalence of being diagnosed as wheat allergy was 68% in the patients with CAP score 2 to ω-5 gliadin IgE (n = 31), and 87% in those with CAP score 3 (n = 15). All of the 3 patients with CAP score 4 or more were wheat allergic. However, 24% of patients with wheat allergy showed negative results (< 0.35UA/ml). According to the data, we proposed the probability curve of ω-5 gliadin-specific IgE.

Conclusions: IgE testing specific to ω-5 gliadin can offer a useful clinical marker for the diagnosis of immediate-type wheat allergy. But the data should always be evaluated with wheat-specific IgE titers, because of the low clinical sensitivity to detect the patients with wheat allergy.



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Tropomyosins in gastropods and bivalves: Identification as major allergens and amino acid sequence features

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ABSTRACT

Tropomyosin appears to be a major cross-reactive allergen of crustaceans and molluscs. In this study, four species of gastropods (disc abalone, turban shell, whelk and Middendorf's buccinum) and seven species of bivalves (bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam and short-neck clam) were confirmed to be allergenic by ELISA and their major allergen identified as tropomyosin by immunoblotting. Inhibition immunoblotting data showed the cross-reactivity of gastropod and bivalve tropomyosins with one another and also with cephalopod and crustacean tropomyosins. Then, amino acid sequences of tropomyosins from 10 species except for Middendorf's buccinum were elucidated by cDNA cloning. The known amino acid sequence data including our results reveal that molluscan tropomyosins share low sequence identities (about 60%) with crustacean tropomyosins and that they are highly homologous with one another within the same group (same family or same order) but not among the groups.

1. Introduction

Food allergy mediated by immunoglobulin E (IgE) antibodies is a public concern in industrialised countries. Sensitised subjects with high levels of IgE to a specific food can develop hypersensitive reactions, such as urticaria, asthma, diarrhoea and even anaphylaxis, immediately after ingestion of the food. In coastal countries, shellfish including crustaceans and molluscs are obviously among the most common causes of food allergy. The major allergen of crustaceans is tropomyosin, a 35–38 kDa myofibrillar protein involved in the muscle contraction, as demonstrated with the following various species: shrimps (Daul, Slattery, Reese, & Lehrer, 1994; Leung et al., 1994; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Shanti, Martin, Nagpal, Metcalfe, & Subba Rao, 1993), crayfishes (Leung et al., 1998a), lobsters (Leung et al., 1998a), crabs (Leung et al., 1998b; Motoyama et al., 2007), krills (Motoyama et al., in press; Nakano, Yoshimura, & Yamada, 2008), mantis shrimps (Motoyama et al., in press) and barnacles (Suma et al., 2007). Moreover, tropomyosin is considered to be a major allergen in molluscs primarily composed of cephalopods, gastropods and bivalves. Molecular evidence for this has been obtained with as many as nine species of cephalopods (cuttlefish, squid and octopus) (Ishikawa, Suzuki, Ishida, Nagashima, & Shiomi, 2001; Miyazawa et al., 1996; Motoyama, Ishizaki, Nagashima, & Shiomi, 2006). However, despite that gastropods and bivalves include a variety of groups, molecular studies have been limited to the following species: Japanese abalone *Haliotis diversicolor* (Chuo, Wong, & Leung, 2000), turban shell *Turbo cornutus* (Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998a), green mussel *Perna viridis* (Chuo et al., 2000), noble scallop *Chlamys nobilis* (Chuo et al., 2000), Japanese oyster *Crassostrea gigas* (Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998b; Ishikawa, Shimakura, Nagashima, & Shiomi, 1997) and constricted tagelus *Sinonovacula constricta* (Song, Li, Li, & Ran, in press).

IgE cross-reactivity is clinically and experimentally recognised among crustaceans, among molluscs and even between crustaceans and molluscs (Lehrer & McCants, 1987; Leung et al., 1996; Motoyama et al., 2006; Reese, Ayuso, & Lehrer,1999). Accumulated data on the primary structures of crustacean tropomyosins prove that they share extremely high sequence identities (mostly more than 90%) with one another (Motoyama et al., 2007), except for barnacle tropomyosin having considerably low identities (about 60%) with other crustacean tropomyosins (Suma et al., 2007). In accordance with this, the eight IgE epitopes proposed for brown shrimp *Penaeus aztecus* tropomyosin (Pen a 1) (Ayuso, Lehrer, & Reese, 2002b; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002a; Reese et al., 2005) are well conserved in other crustacean tropomyosins, being a molecular basis for the cross-reactivity among crustaceans. On the other hand, the cross-reactivity either among molluscs or between crustaceans and molluscs is not fully understood due to the shortage of information about the primary

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structures of molluscan tropomyosins, especially those of gastropod and bivalve tropomyosins. Amino acid sequences of gastropod and bivalve tropomyosins even including those with no evidence for allergenicity are known only for three species of abalones, three species of mussels, three species of scallops and one species of clam (refer to Table 2).

For a better understanding of the cross-reactivity either among molluscs or between crustaceans and molluscs, it is essential to elucidate amino acid sequences of allergenic tropomyosins from various species of gastropods and bivalves. In this study, therefore, four species of gastropods (disc abalone, turban shell, whelk and Middendorf's buccinum) and seven species of bivalves (bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam and short-neck clam), which are situated at various taxonomical positions (refer to Table 1), were selected as samples. We report here the identification of tropomyosins as the major allergens in these 11 species and the amino acid sequences of tropomyosins in 10 species (except for Middendorf's buccinum) determined by a cDNA cloning technique. The sequence features of molluscan tropomyosins are also discussed compared to those of crustacean tropomyosins.

2. Materials and methods

2.1. Animal samples

Live specimens of four species of gastropods (disc abalone *Haliotis discus discus*, turban shell *T. cornutus*, whelk *Neptunea polycostata* and Middendorf's buccinum *Buccinum middendorffi*), seven species of bivalves (bloody cockle *Scapharca broughtonii*, Japanese oyster *C. gigas*, Japanese cockle *Fulvia mutica*, surf clam *Pseudocardium sachalinensis*, horse clam *Tresus keenae*, razor clam *Solen strictus* and short-neck clam *Ruditapes philippinarum*) and American lobster *Homarus americanus* and fresh specimens of Japanese flying squid *Todarodes pacificus* were all purchased at the Tokyo Central Wholesale Market. The taxonomical positions of the gastropods and bivalves are summarised in Table 1. Columellar muscle was obtained from disc abalone, foot muscle from turban shell, whelk, Middendorf's buccinum, bloody cockle, Japanese cockle, surf clam and razor clam, adductor muscle from Japanese oyster, siphon (major edible muscle part) from horse clam, soft tissues from short-neck clam, abdominal muscle from American lobster and mantle muscle from Japanese flying squid. Muscle or soft tissue samples for extraction were stored at −20 °C until use and those for molecular cloning experiments were immediately immersed in liquid nitrogen and stored at −80 °C until use.

Table 1
Gastropods and bivalves used in this study.

Class	Order	Family	Species (common name)
Gastropoda	Vetigastropoda	Haliotidae	*Haliotis discus discus* (Japanese abalone)
		Turbinidae	*Turbo cornutus* (turban shell)
	Neogastropoda	Buccinidae	*Neptunea polycostata* (whelk)
			Buccinum middendorffi (Middendorf's buccinum)
Bivalvia	Arcoida	Arcidae	*Scapharca broughtonii* (bloody cockle)
	Ostreoida	Ostreidae	*Crassostrea gigas* (Japanese oyster)
	Veneroida	Cardiidae	*Fulvia mutica* (Japanese cockle)
		Mactridae	*Pseudocardium sachalinensis* (surf clam)
			Tresus keenae (horse clam)
		Solenidae	*Solen strictus* (razor clam)
		Veneridae	*Ruditapes philippinarum* (short-neck clam)

2.2. Preparation of heated extracts

Each muscle or soft tissue sample was homogenised with four volumes of 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). In view of the fact that tropomyosin, a target protein in this study, is thermostable, the homogenate was then heated in a boiling water bath for 10 min. After centrifugation at 18,000g for 15 min, the supernatant obtained was used as a heated extract. Protein concentrations of the heated extracts were quantified by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

2.3. Purification of tropomyosin

American lobster, Japanese flying squid and turban shell tropomyosins were purified from the abdominal, mantle and foot muscles, respectively, as reported previously (Motoyama et al., 2007). In brief, an acetone powder of myofibrillar proteins prepared from the muscle was extracted with 25 mM Tris–HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$ and 1 mM dithiothreitol. The extract was subjected to salting-out with ammonium sulfate (30–60% saturation), followed by isoelectrical precipitation (pH 4.6). Finally, small amounts of contaminants were removed by reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh, Tokyo, Japan). The homogeneity of the final preparation was supported by SDS–PAGE.

2.4. Human sera

Sera were donated from 10 crustacean-allergic patients (patients 1–10) with a history of hypersensitive reactions after ingestion of crustaceans. These patients had all been diagnosed to be allergic to crustaceans by the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) performed at hospitals; the determined CAP-RAST classes were 3–6 against shrimp and/or crab. Patients 5, 6, 8 and 9 had been additionally shown to have CAP-RAST classes of 3–6 against squid and octopus. In this study, sera from eight healthy volunteers without adverse reactions after ingestion of any foods were used as controls.

2.5. Fluorescence ELISA

Reactivity of the patient sera to each heated extract was evaluated by fluorescence ELISA as described elsewhere (Hamada et al., 2004). In brief, a polystyrene microtiter plate with 96 wells (Type H (black); Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of the heated extract (diluted 1:2000) and reacted successively with patient or control serum (diluted 1:500) and β-galactosidase-conjugated goat anti-human IgE antibody (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 the addition of 100 mM glycine-NaOH buffer (pH 10.3). Fluorescence intensity was measured on a SPECTRAmax GEMINI XS (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 in mean ± SD.

2.6. SDS–AGE

SDS–PAGE was performed on a PhastSystem apparatus (GE-Healthcare, Piscataway, NJ, USA) as described in the manufacturer's manual. Ready-made gels (PhastGel Gradient 8-25) and ready-made buffer strips (PhastGel SDS Buffer Strips) were purchased from GE-Healthcare. Each sample was dissolved in 62.5 mM phosphate buffer (pH 7.5) containing 2.5% SDS and 5% dithiothreitol, heated at 100 °C for 10 min and subjected to

electrophoresis. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were run as a reference, along with samples. After running, proteins were visualised by staining with Coomassie Brilliant Blue R-250.

2.7. Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as reported previously (Kobayashi et al., 2006). Briefly, the proteins separated by SDS–PAGE were electrotransferred from the gel to a nitrocellulose membrane, which was reacted successively with primary and secondary antibodies. In order to detect tropomyosin, an antiserum (diluted 1:30,000) raised in rabbits against king crab tropomyosin, which was kindly gifted from Dr. H. Ushio in our university, was used as a primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:20,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) as a secondary antibody. In IgE-immunoblotting, patient or control serum (diluted 1:500) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:10,000; Kirkegaard & Perry Laboratories) were used as primary and secondary antibodies, respectively. Blots were visualised using an ECL Plus Western Blotting Detection System (GE-Healthcare) and an ECL Mini Camera (GE-Healthcare), as recommended by the manufacturer. For inhibition IgE-immunoblotting, patient serum (diluted 1:250) was preincubated with an equal volume of American lobster, Japanese flying squid or turban shell tropomyosin solution (10 μg/ml) at 37 °C for 1 h and used as a primary antibody.

2.8. cDNA cloning of tropomyosins

The rapid amplification cDNA ends (RACE) method was employed for cDNA cloning of tropomyosins. Total RNA was extracted from 1.5 g of each muscle sample with 15 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For 3'RACE, first-stranded cDNA was synthesised from 5 μg of total RNA using the 3'RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's manual and used as a template. PCR was performed using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 2 min; and 72 °C for 7 min. In PCR, the following forward primers were used in combination of the abridged universal anchor primer (AUAP): 5′-CAGAGGTTGACCTTGAACGT-3′ (corresponding to the region 172–178 of the amino acid sequence) for disc abalone, 5′-GATGCCATCAAGAAGAAGATG-3′ (corresponding to the region 2–8) for turban shell and short-neck clam and 5′-GAGGCTGCCCGTAAACT(C/T)GC-3′ (corresponding to the region 164–170) for the other species. These primers were designed on the basis of the known cDNAs encoding molluscan tropomyosins (refer to Table 2 for the accession numbers of tropomyosins in the DDBJ/EMBL/GenBank databases). The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany) and at least three clones were analysed for nucleotide sequences using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and a PRISM 310 genetic analyser (Applied Biosystems). Based on the determined partial nucleotide sequence, 5′RACE was carried out using the 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's instructions. For each species, first-stranded cDNA was synthesised from 5 μg of total RNA using the gene-specific reverse primer and subjected as a template to PCR using another gene-specific reverse primer and the abridged anchor primer (AAP). In the case of disc abalone, nested-PCR was further performed. The PCR conditions in 5'RACE were the same as those in 3′RACE. All amplified products were subcloned and sequenced as described above.

3. Results and discussion

3.1. IgE reactivity of heated extracts from gastropods and bivalves

IgE reactivity of the heated extracts from gastropods and bivalves was analysed by fluorescence ELISA using five crustacean-allergic patient sera and a control serum pooled from eight healthy subjects. In comparison of the results with the control serum, each patient serum was judged to react with varied potencies to all of the heated extracts not only from American lobster but also from four species of gastropods and seven species of bivalves (Fig. 1). Prior to this study, the CAP-RAT determinations had demonstrated the occurrence of specific IgE against shrimp and/or crab in the five patient sera used and specific IgE against squid and octopus in the patient 5 serum. In the light of the established cross-reactivity among crustaceans (Motoyama et al., 2007; Reese et al., 1999), the reactivity of the five patient sera to the heated extract from American lobster is easily understood. Importantly, the results with the heated extracts from gastropods and bivalves show that at least the five patient sera recognise molluscs as well as crustaceans. This conforms well to the fact that tropomyosin is a cross-reactive allergen between crustaceans and molluscs as described below.

3.2. Identification of tropomyosins as major allergens in gastropods and bivalves

As analysed by SDS–PAGE, a 37 kDa protein, which is comparable in molecular mass to the turban shell tropomyosin used as a reference, was prominently detected in all of the heated extracts from four species of gastropods and seven species of bivalves (Fig. 2A). Irrespective of the species, the 37 kDa protein was judged to be tropomyosin from the positive reactivity with the antiserum against the king crab tropomyosin (Fig. 2B). In IgG-immunoblotting, minor blots above and/or below the 37 kDa protein (tropomyosin) were also observed in several species such as Japanese oyster and horse clam. It is unclear whether these blots are attributable to tropomyosin isoforms or other cross-reactive proteins. IgE-immunoblotting, using the pooled serum from 10 crustacean-allergic patients and individual six patient sera, showed that tropomyosins in all samples were IgE-reactive (Fig. 2C), although the reactivity of Japanese cockle tropomyosin with some patient sera was particularly low. In some species such as bloody cockle and Japanese oyster, IgE-reactive proteins of 100–150 kDa were also recognised. Nevertheless, IgE-immunoblotting data allowed us to conclude that tropomyosin is almost the sole IgE-reactive protein in all gastropods and bivalves examined. When four patient serum samples (pooled serum and three sera from patients 1–3) were individually preadsorbed with American lobster, Japanese flying squid or turban shell tropomyosin as an inhibitor, they completely lost their reactivity to tropomyosins from four species of gastropods and seven species of bivalves (only the results with the patient 1 serum are shown in Fig. 2D). These results qualitatively demonstrate the cross-reactivity among molluscan tropomyosins and also between crustacean and molluscan tropomyosins, as in previous studies (Lehrer & McCants, 1987; Leung et al., 1996; Motoyama et al., 2006; Reese et al., 1999).

3.3. Nucleotide sequences of cDNAs encoding gastropod and bivalve tropomyosins

In 3′RACE, no amplified products were obtained for Middendorf's buccinum. Attempts were made to amplify the cDNAs encoding the Middendorf's buccinum tropomyosin by RT-PCR using various primer sets but were unsuccessful. However, the

Table 2
Amino acid sequence identities (%) among molluscan and crustacean tropomyosins.

	Gastropods			Bivalves								Cephalopods	Crustaceans
	Order: Vetigastropoda		Order: Neogastropoda	Order: Arcoida	Order: Mytiloida	Order: Ostreoida		Order: Veneroida					
	Family: Haliotidae Abalones	Family: Turbinidae Turban shell	Family: Buccinidae Whelk	Family: Arcidae Bloody cockle	Family: Mytilidae Mussels	Family: Pectinidae Scallops	Family: Ostreidae Japanese oyster	Family: Cardiidae Japanese cockle	Family: Mactridae Surf clam Horse clam	Family: Solecurtidae Constricted tagelus	Family: Solenidae Razor clam	Family: Veneridae Short-neck clam		
Gastropods														
Abalones	96.1–99.6	94.0–97.2	75.4–77.8	77.5–79.6	71.5–75.4	68.3–81.0	76.0–78.5	73.6–75.4	72.5–75.7	70.8–73.2	73.9–76.1	74.6–76.8	77.8–82.7	59.2–63.7
Turban shell		100	77.1	80.3	74.3–74.6	70.8–80.6	77.8	75.4	74.6–76.4	73.2	76	76.7	79.9–81.3	60.9–63.4
Whelk			100	72.2	69.0–70.1	68.7–71.1	77.1	69.7	68.7–70.4	67.6	68.7	70.8	73.2–74.3	57.4–60.9
Bivalves														
Bloody cockle				100	75.4–75.7	70.4–73.2	79.2	72.9	72.9–73.6	70.1	73.2	73.9	71.8–73.2	57.7–60.2
Mussels					94.0–99.6	67.6–70.1	78.9–79.2	66.2–66.9	64.8–66.5	65.5–66.2	66.9–67.3	66.2–66.9	69.4–71.5	54.6–58.1
Scallops						84.4–91.9	72.2–73.9	66.2–70.4	66.5–71.5	65.1–67.6	66.9–68.7	68.0–71.5	69.7–74.6	56.7–60.0
Japanese oyster							100	72.5	72.9–74.3	69.7	72.9	74.3	75.0–76.0	59.9–62.3
Japanese cockle								100	85.9–88.7	83.8	85.6	86.6	70.1–70.8	57.0–58.5
Surf clam horse clam									94.4	87.7–88.4	92.3–92.6	92.3	71.8–72.9	55.3–57.4
Constricted tagelus										100	89.1	85.9	70.1–71.5	57.0–58.1
Razor clam											100	90.5	72.5–73.6	57.0–58.1
Short-neck clam												100	72.9–73.6	57.4–59.2
Cephalopods													91.2–99.6	61.3–64.4
Crustaceans														89.1–98.6

Gastropods: four species of abalones, tropical abalone *Haliotis asinina* (AY320360), Japanese abalone *Haliotis diversicolor* (AF216518), California red abalone *Haliotis rufescens* (X75218) and disc abalone *Haliotis discus discus*; turban shell *Turbo cornutus*; whelk *Neptunea polycostata*. Bivalves: bloody cockle *Scapharca broughtonii*; three species of mussels, blue mussel *Mytilus edulis* (U40035), Mediterranean mussel *Mytilus galloprovincialis* (AB000907) and green mussel *Perna viridis* (AF216519); three species of scallops, akazara scallop *Chlamys nipponensis* (AB021681), noble scallop *Chlamys nobilis* (AF216520) and Yesso scallop *Patinopecten yessoensis* (AB004636); Japanese oyster *Crassostrea gigas*; Japanese cockle *Fulvia mutica*; surf clam *Pseudocardium sachalinensis*; horse clam *Tresus keenae*; constricted tagelus *Sinonovacula constricta* (EU082209); razor clam *Solen strictus*; short-neck clam *Ruditapes philippinarum*. Cephalopods: golden cuttlefish *Sepia esculenta* (AB218913), big fin reef squid *Sepioteuthis lessoniana* (AB218914), Japanese flying squid *Todarodes pacificus*, neon flying squid *Onmastrephes bartrami* (AB218916) and common octopus *Octopus vulgaris* (AB218917). Crustaceans: brown shrimp *Penaeus aztecus*, pink shrimp *Pandalus eous* (AB270631), American lobster *Homarus americanus* (fast-type tropomyosin) (AF034954), American lobster (slow-twitch tropomyosin) (AF034953), American lobster (slow-tonic tropomyosin) (AY521627), snow crab *Chionoecetes opilio* (slow-tonic tropomyosin) (AB270634), horsehair crab *Erimacrus isenbekii* (slow-twitch tropomyosin) (AB270635), horsehair crab (slow-tonic tropomyosin) (AB270636), king crab *Paralithodes camtschaticus* (fast tropomyosin) (AB270632) and king crab (slow-tonic tropomyosins) (AB270633). Except for the species shown in Fig. 2, accession numbers (DDBJ/EMBL/GenBank nucleotide sequence database) are indicated in parentheses.

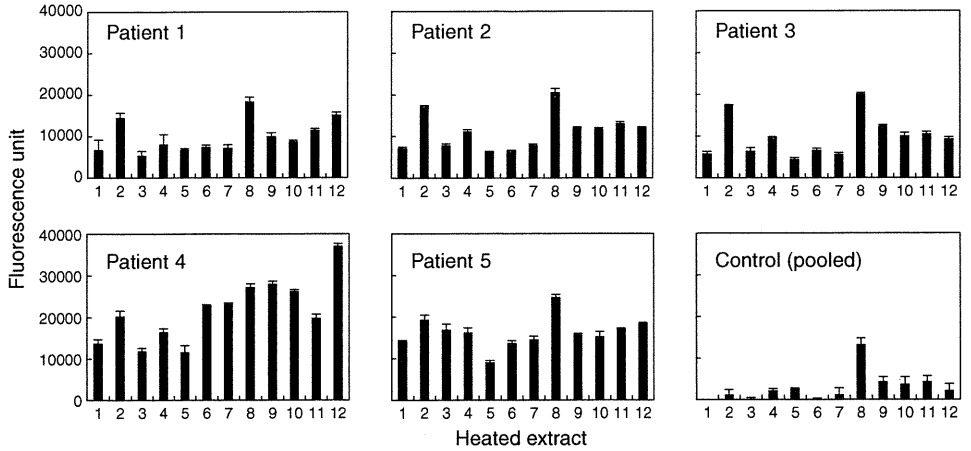

Fig. 1. Analysis by fluorescence ELISA of the IgE reactivity of patient sera to the heated extracts from four species of gastropods, seven species of bivalves and American lobster. Each heated extract (diluted 1:2000) was coated on the ELISA plate and reacted with patient or control serum (diluted 1:500). The control serum was pooled from eight healthy subjects. Heated extracts (protein concentration in mg/ml): 1, disc abalone (0.87); 2, turban shell (2.1); 3, whelk (3.8); 4, Middendorf's buccinum (2.1); 5, bloody cockle (5.5); 6, Japanese oyster (4.2); 7, Japanese cockle (2.8); 8, surf clam (9.3); 9, horse clam (6.2); 10, razor clam (4.6); 11, short-neck clam (7.5); 12, American lobster (3.6).

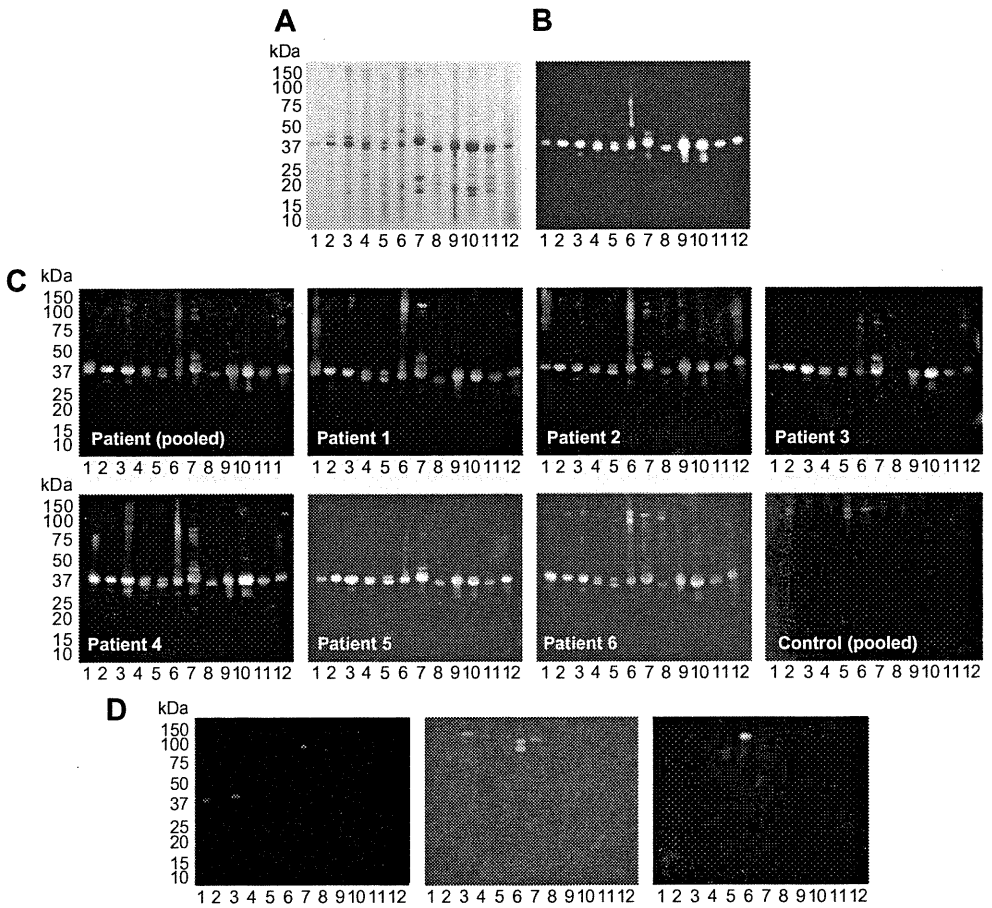

Fig. 2. Analysis of the heated extracts from four species of gastropods and seven species of bivalves by SDS–PAGE (A), IgG-immunoblotting (B), IgE-immunoblotting (C) and inhibition IgE-immunoblotting (D). Lanes: 1, tropomyosin purified from turban shell; 2, extract from disc abalone; 3, extract from turban shell; 4, extract from whelk; 5, extract from Middendorf's buccinum; 6, extract from bloody cockle; 7, extract from Japanese oyster; 8, extract from Japanese cockle; 9, extract from surf clam; 10, extract from horse clam; 11, extract from razor clam; 12, extract from short-neck clam. (A) Heated extracts (0.3 μl each) with the protein concentrations described in the legend for Fig. 1 were subjected to SDS–PAGE. (B) Antiserum against king crab tropomyosin was used to detect tropomyosins. (C) Patient sera (pooled serum from 10 patients and individual six patient sera) were used to detect IgE-binding proteins. (D) The patient 1 serum (diluted 1:250) was preincubated with an equal volume of inhibitor (left: American lobster tropomyosin, middle: Japanese flying squid tropomyosin, right: turban shell tropomyosin) solution (10 μg/ml) and used as a primary antibody.

full-length cDNAs encoding tropomyosins from the remaining 10 species could be cloned by both 3′ and 5′RACE. After subcloning of each PCR product into the pT7Blue-2 T-vector, at least three clones were analysed for nucleotide sequence. For each PCR product, there was no difference in nucleotide sequence among the clones analysed, suggesting that isoforms of the cloned tropomyosin, if present, are trace in the 10 species. The determined nucleotide sequences of the tropomyosin cDNAs, although not shown in this paper to save space, have been deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers: AB444939 for disc abalone (1223 bp), AB444940 for turban shell (1108 bp), AB444941 for whelk (1077 bp), AB444942 for bloody cockle (1079 bp), AB444943 for Japanese oyster (1110 bp), AB444944 for Japanese cockle (1119 bp), AB444945 for surf clam (1020 bp), AB444946 for horse clam (1081 bp), AB444947 for razor clam (1112 bp) and AB444948 for short-neck clam (1198 bp). All cDNAs commonly contain an open reading frame composed of 852 bp coding for 284 amino acid residues.

3.4. Amino acid sequences of gastropod and bivalve tropomyosins

The amino acid sequences of gastropod tropomyosins have so far been elucidated for three species of abalones (ear shell, Japanese abalone and California red abalone) and those of bivalve tropomyosins for three species of mussels (blue mussel, Mediterranean mussel and green mussel), three species of scallops (akazara scallop, noble scallop and Yezo scallop) and one species of clam (constricted tagelus) (refer to Table 2). In this study, the amino acid sequences of tropomyosins from three species of gastropods (disc abalone, turban shell and whelk) and seven species of bivalves

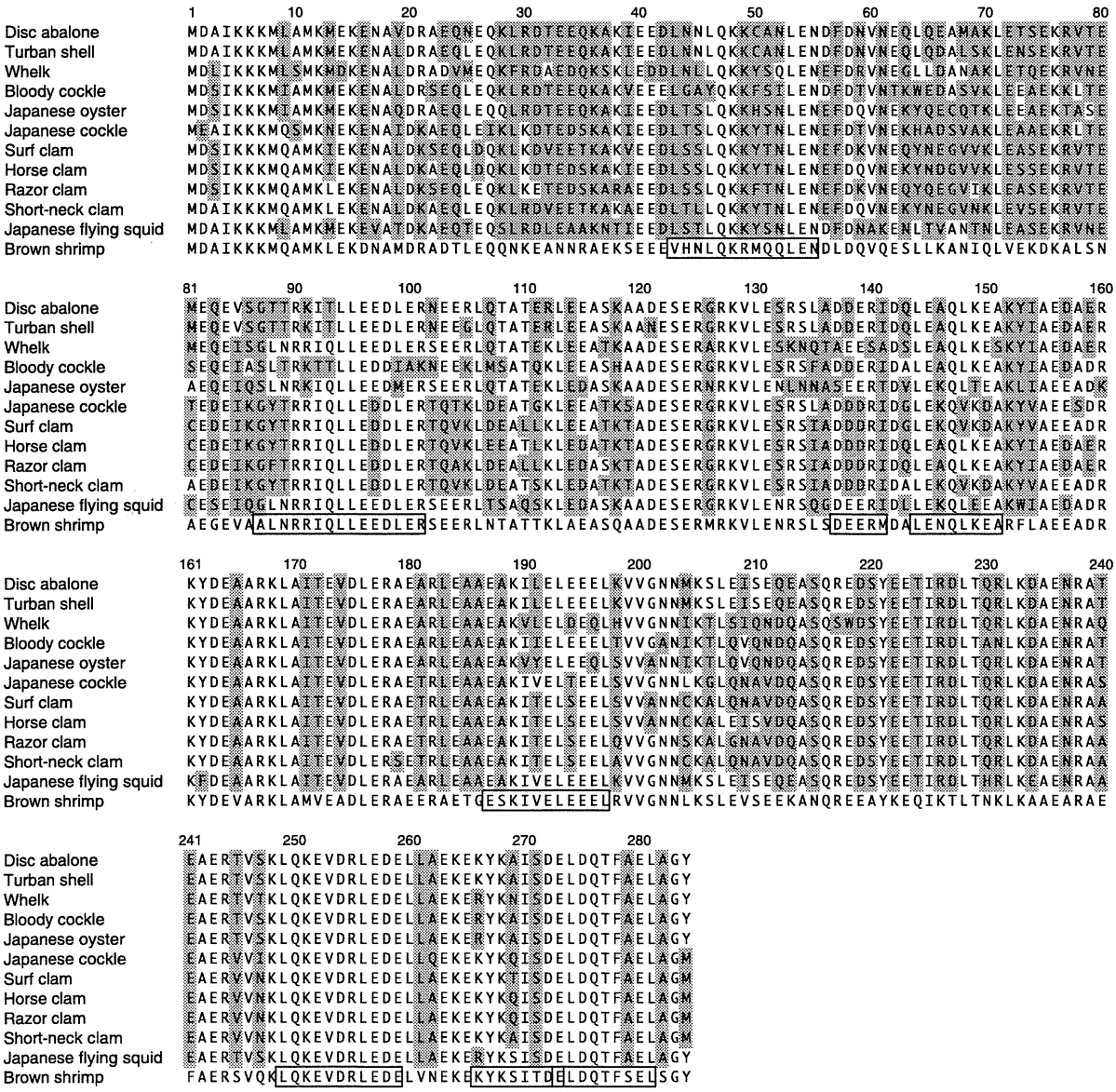

Fig. 3. Amino acid sequences of the gastropod and bivalve tropomyosins determined in this study and Japanese flying and brown shrimp tropomyosins as references. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): disc abalone *Haliotis discus discus*, AB444939; turban shell *Turbo cornutus*, AB444940; whelk *Neptunea polycostata*, AB444941; bloody cockle *Scapharca broughtonii*, AB444942; Japanese oyster *Crassostrea gigas*, AB444943; Japanese cockle *Fulvia mutica*, AB444944; surf clam *Pseudocardium sachalinensis*, AB444945; horse clam *Tresus keenae*, AB444946; razor clam *Solen strictus*, AB444947; short-neck clam *Ruditapes philippinarum*, AB444948; Japanese flying squid *Todarodes pacificus*, AB218915; brown shrimp *Penaeus aztecus*, DQ151457. The residues differing from brown shrimp tropomyosin are shaded. The IgE epitope regions proposed for brown shrimp tropomyosin are boxed.

(bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam and short-neck clam) were newly clarified. It is worth mentioning that, except for disc abalone, the gastropod and bivalve species used in this study are not only classified into diverse families and orders but also taxonomically discriminated from those used in the previous sequence studies. Thus, this study is of particular value in making it possible to understand the overall features of the amino acid sequences of tropomyosins from edible gastropods and bivalves.

The determined amino acid sequences of tropomyosins from the three species of gastropods and seven species of bivalves are aligned in Fig. 3, together with those of Japanese flying squid tropomyosin (a representative of cephalopod tropomyosins) and brown shrimp tropomyosin (a representative of crustacean tropomyosins). It is obvious that the amino acid sequences of gastropod and bivalve tropomyosins are significantly different from that of brown shrimp tropomyosin. As it is not easy to compare the amino acid sequences among molluscs only by the sequence alignment shown in Fig. 3, the sequence identities among molluscan and crustacean tropomyosins are summarised in Table 2, in which gastropods and bivalves are listed according to the family to which they belong. First, it should be noted that gastropod and bivalve tropomyosins share only 60% sequence identities with crustacean tropomyosins and considerably low identities (mostly 70–80%) with cephalopod tropomyosins. Another significant feature of the amino acid sequences of gastropod and bivalve tropomyosins is that they are highly homologous (mostly more than 90% identities) with one another within the same group (same family or same order) but not (mostly 70–80% identities) among the groups. Although further sequence data are needed, the gastropods and bivalves whose tropomyosins have already been sequenced can be divided into the following groups based on the sequence identities: two groups (members of the orders Vetigastropoda and Neogastropoda) for gastropods and five groups (members of the order Arcoida, the order Mytiloida, the families Pectinidae and Ostreidae of the order Ostreoida and the order Veneroida) for bivalves.

As described above, molluscan tropomyosins have low sequence identities with crustacean tropomyosins and are also distinguishable in sequence among the groups. Nevertheless, the cross-reactivity either among molluscan tropomyosins or between molluscan and crustacean tropomyosins was shown in this study as well as in previous studies (Lehrer & McCants, 1987; Leung et al., 1996; Motoyama et al., 2006; Reese et al., 1999). This can be realised to some extent in consideration of the eight IgE epitopes (regions 43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273 and 273–281) proposed for brown shrimp tropomyosin (Pen a 1) (Ayuso et al., 2002a, 2002b; Reese et al., 2005). As discussed in our previous paper (Motoyama et al., 2006), cephalopod tropomyosins have the same sequence in the region 249–259 as Pen a 1 and have only one replacement in the regions 88–101, 137–141, 187–197 and 273–281 compared to Pen a 1, accounting for the cross-reactivity between cephalopod and crustacean tropomyosins. In the case of gastropod and bivalve tropomyosins, the sequence of the region 249–259 is completely conserved as well. This partly supports the cross-reactivity either among molluscan tropomyosins or between molluscan and crustacean tropomyosins. However, gastropod and bivalve tropomyosins have extended replacements in the regions corresponding to the other Pen a 1 epitopes. Elucidation of IgE epitopes of gastropod and bivalve tropomyosins using synthetic peptides is needed to discuss in more detail their cross-reactivity with cephalopod and crustacean tropomyosins.

In conclusion, this study provided experimental evidence that a variety of gastropods and bivalves are allergenic and that their major allergen is tropomyosin cross-reacting with one another and also with cephalopod and crustacean tropomyosins. Importantly, our sequence data, together with the previous data, revealed that gastropods and bivalves contain tropomyosins with amino acid sequence features associated with their taxonomical positions. This might be a molecular basis for the fact that there are patients sensitive to some species of gastropods or bivalves but not to others. Therefore, future study on IgE epitopes of gastropod and bivalve tropomyosins should be performed in consideration of the taxonomical positions of species. Such study will greatly increase our molecular knowledge on the allergenicity of gastropod and bivalve tropomyosins.

Acknowledgements

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Original

A Major IgE Epitope of Rainbow Trout Collagen α2 Chain

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Bovine collagen is allergenic and its major IgE epitope has already been identified. Fish collagen is also allergenic but shows no IgE cross-reactivity with bovine collagen, implying that it has specific IgE epitopes. Therefore, this study was initiated to elucidate IgE epitopes of rainbow trout collagen α2 chain. Five overlapping proteins (R1–5; 221 or 225 amino acids long with an offset of 205 amino acids) covering the entire sequence of the rainbow trout collagen α2 chain were expressed in *Escherichia coli*. Immunoblotting experiments using 10 patients' sera reacting to fish collagen revealed that the major IgE epitope is included in the R5 protein (region 821–1,041). Then, 26 overlapping peptides (20 or 21 amino acids long with an offset of 8 amino acids) encompassing the sequence of the R5 protein were chemically synthesized and examined for IgE-binding ability by fluorescence ELISA. Region 941–960 was found to be most IgE-reactive. When evaluated by inhibition ELISA, this region accounted for more than 50% of the IgE reactivity to the R5 protein. Moreover, the same region was found to be IgE-reactive in bastard halibut and zebrafish collagen α2 chains, but not in bovine collagen α2 chain. Our results strongly suggest that region 941–960 is a major common IgE epitope of fish collagen α2 chains.

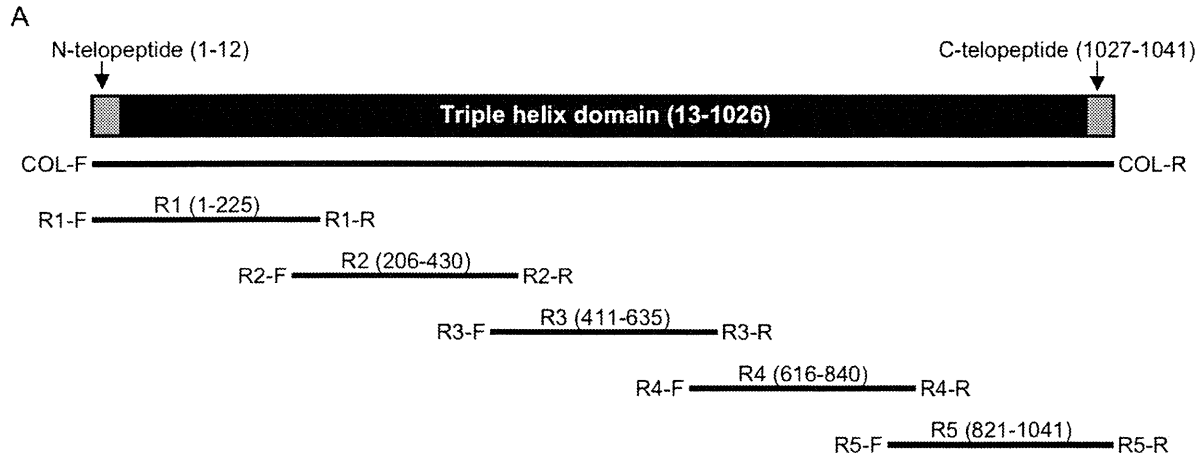
Key words: allergen; α2 chain; collagen; fish allergy; IgE epitope; rainbow trout

Introduction

Collagen is ubiquitously found in animals as a major protein in the skin, bone and intramuscular connective tissue of animals. It is composed of three α chains (in the form of $(\alpha 1)_2\alpha 2$ or $\alpha 1\alpha 2\alpha 3$) of about 110–120 kDa, which are twisted together to form a triple helix[1]. The denatured collagen (gelatin) from skin or bone of bovine and porcine is widely utilized as a raw material of jelly and as a supplement in cosmetics and foods. Because gelatin has long been believed to be nonimmunogenic and nonallergenic to humans, it has also been used as a stabilizer in vaccines, such as those for measles, mumps and rubella. However, immunoglobulin E (IgE)-mediated adverse reactions, including anaphylaxis, to the vaccines have been reported in some children and demonstrated to be mostly ascribable to gelatin in the vaccines[2), 3)]. Subsequently, of the α1 and α2 chains constituting bovine collagen, only the latter chain was found to be IgE-reactive[4)]. Moreover, the major IgE epitope of the bovine collagen α2 chain has been elucidated to reside in region 485–494[5)].

In fish, the major allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of about 12 kDa, as demonstrated with various species of fish, including cod[6)], carp[7)], Atlantic salmon[8)] and Pacific mackerel[9)]. Besides parvalbumin, collagen has also been identified as a fish allergen, although not a major one[10), 11)]. In contrast to bovine collagen, hetero α-chains of fish collagen all seem to be IgE-reactive[11), 12)]. Importantly, collagens from various species of fish are cross-reactive with one another, but not with those from mammals[10), 12)], implying that fish collagens have specific IgE epitopes distinct from those of mammalian collagens. To achieve a better understanding of the allergenicity of fish collagens, it is requisite to elucidate their specific IgE epitopes. Furthermore, information about IgE epitopes of fish collagens would be helpful to develop not only hypoallergenic processed fish products, but also hypoallergenic collagen molecules for immunotherapy of fish allergy.

All three α chains of collagen have been completely sequenced for only two species of fish, rainbow trout[13)] and zebrafish *Danio rerio*[14)]. Rainbow trout is an important edible fish that is widely consumed, while zebrafish is often used as a model fish in biology, but is not edible. Furthermore, the major IgE epitope has already been identified for the bovine collagen α2 chain[5)]. Here, we selected the rainbow trout *Oncorhynchus mykiss* collagen α2 chain as a target for analysis of IgE epitopes. First, overlapping recombinant proteins covering the entire sequence of the rainbow trout collagen α2 chain were examined for IgE-binding ability. Next, epitope mapping experiments were performed using synthetic overlapping peptides encompassing the sequence of the most IgE-reactive protein. Our results indicate that the



B

COL-F: 5'-CAGTTTGATGGAGGAAAGGG-3', COL-R: 5'-CAGTTTGATGGAGGAAAGGG-3'

R1-F: 5'-gaattcTCAGTTTGATGGAGGAAAGGGC-3', R1-R: 5'-gtcgacTTAGTTGTTACCAGGGTTACCAAC-3'

R2-F: 5'-gaattcAGAGCCTGGTATCAATGGAGCT-3', R2-R: 5'-gtcgacTTAACCACCCTTGCCAGCCTCACC-3'

R3-F: 5'-gaattcACCCGGTAACATCGGATTCCCC-3', R3-R: 5'-gtcgacTTACTCTCCACTGGGTCCACGAAG-3'

R4-F: 5'-gaattcGGGTGAGTCTGGCTCCTTCGGC-3', R4-R: 5'-gtcgacTTAAGTCATACCGGGCATGCCAAT-3'

R5-F: 5'-gaattcACCCGCTGGTGCCTCTGGACCC-3', R5-R: 5'-gtcgacTTAGGCTCTGTACTCATCGTAACC-3'

Fig. 1. Schematic presentation of the rainbow trout collagen α2 chain and five overlapping proteins (R1–5) covering the full sequence (A) and nucleotide sequences of the primers used to amplify the mature protein and R1–5 proteins (B)

(A) The regions corresponding to the N-telopeptide, triple helix domain, C-telopeptide and R1–5 proteins are indicated in parentheses. Forward and reverse primers are shown at the left and right sides, respectively, for each mature protein and R1–5 proteins. (B) The *Eco*RI and *Sal*I restriction sites are shown in small letters for forward and reverse primers, respectively.

major IgE epitope of the rainbow trout collagen α2 is included in the region 941–960. We also provide evidence that the same region is likely to be a common IgE epitope of fish collagen α2 chains.

Materials and Methods

Fish

A live specimen of rainbow trout was kindly supplied by Professor S. Sato of our university. The white muscle obtained from the specimen was immediately immersed in liquid nitrogen and kept at −80℃ until use.

Construction of a cDNA library

Total RNA was extracted from 2 g of the rainbow trout white muscle with TRIzol reagent (Life Technologies, Rockville, MD, USA) and poly(A)$^+$ mRNA was purified using an mRNA Purification Kit (GE Healthcare, Piscataway, NJ, USA). A Marathon cDNA library was constructed by conversion of a part of the purified mRNA to cDNA, followed by ligation of AP1 adapters, using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA).

Expression and purification of recombinant proteins

Five overlapping proteins, R1–5 (221 or 225 amino acids in length with an offset of 205 amino acids), which cover the entire sequence of the rainbow trout collagenα2 chain (Fig. 1A), were individually expressed in *Escherichia coli* as a glutathione-S-transferase (GST)-fusion form using the pGEX-6P-3 expression vector (GE Healthcare). First, a cDNA encoding the α2 chain[13] was amplified by PCR using the Marathon cDNA library as a template. Then, cDNAs of the R1–5 proteins, with addition of *Eco*RI and *Sal*I restriction sites at the 5′ and 3′ ends, respectively, were individually amplified by PCR using the α2 chain cDNA as a template. Nucleotide sequences of the primers used in PCR are summarized in Fig. 1B. Each PCR product and the expression vector were digested with *Eco*RI and *Sal*I and ligated using a DNA Ligation Kit (Takara, Otsu, Japan). *E. coli* JM109 was transformed with the ligated product and cultured overnight on LB agar containing 0.005% ampicillin at 37℃. A single colony was selected and grown in 500 mL of LB medium containing 0.005% ampicillin at 37℃ until the absorbance at 600 nm reached 0.6. Then, isopropyl-β-D-thiogalactoside (IPTG) was added to the culture to give a concentration of 1 mmol/L and incubation was continued for 3 hr. Bacteria were harvested by centrifugation and resuspended in 25 mL of 50 mmol/L Tris–HCl buffer (pH 7.5) containing 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride and 0.01% lysozyme. The bacterial suspension was sonicated and centrifuged,

and the GST-fusion protein recovered in the supernatant was purified by affinity chromatography on a GSTrap HP column (GE Healthcare) according to the manufacturer's instructions.

Human sera

Sera from 10 fish-allergic patients (patients 1–10) were used. This study was approved by the ethics committees of Chiba University, Fujita Health University and Yokohama City University. All patients had been diagnosed to be allergic to fish at hospitals, based on documented clinical histories of immediate hypersensitivity reactions after ingestion of fish and capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) classes of 2–4 against fish such as mackerel and tuna. In addition, their sera were all confirmed to contain specific IgE to fish collagen by ELISA, which was performed using either parvalbumin or collagen purified from bigeye tuna *Thunnus obesus* muscle as an antigen, as described in our previous paper[12]. Sera from two healthy volunteers without adverse reactions after ingestion of any foods were used as controls.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed on a ready-made gel (PhastGel Gradient 8–25; GE Healthcare) using a PhastSystem apparatus (GE Healthcare), as recommended by the manufacturer. Each sample was dissolved in 62.5 mmol/L phosphate buffer (pH 7.5) containing 2% SDS, 3 mol/L urea and 100 mmol/L dithiothreitol, heated at 100℃ for 10 min and subjected to electrophoresis. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were run as a reference, along with samples. After running, proteins were stained with Coomassie Brilliant Blue R-250.

Immunoblotting was carried out as reported previously[15]. In brief, the proteins separated by SDS-PAGE were electrotransferred to a polyvinylidene difluoride membrane, which was reacted successively with patients' serum (diluted 1 : 250) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1 : 5,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Antigen-antibody binding was visualized using an ECL Plus Western Blotting Detection System (GE Healthcare) and an ECL Mini Camera (GE Healthcare) according to the manufacturer's manual.

Peptide synthesis

The R5 protein (region 821–1041) was found to be most IgE-reactive, as described below. Therefore, 26 overlapping peptides (20 or 21 amino acids in length with an offset of 8 amino acids) covering the entire sequence of the R5 protein were chemically synthesized. Peptide 16 (region 941–960) was judged to be the most IgE-reactive as described below, and hence peptides corresponding to region 941–960 of bastard halibut *Paralichthys olivaceus*[16], zebrafish[14] and bovine[17] collagen α2 chains were additionally synthesized (named peptides Bh, Zf and Bv, respectively).

Peptide synthesis was performed on a PMMS-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, according to the manufacturer's instructions. After synthesis, each peptide was purified by reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan), which was eluted with a linear gradient of acetonitrile (49–63% in 40 min) at a flow rate of 1 mL/min. The molecular weight of each purified peptide was confirmed by MALDI-TOFMS using a KOMPACT MALDI I instrument (Shimadzu).

Enzyme-linked immunosorbent assay (ELISA)

IgE reactivity of synthetic peptides was assessed by fluorescence ELISA as reported previously[18]. In brief, a Nunc Immobilizer Amino plate for peptide (Nalge Nunc International, Rochester, NY, USA) was coated with 50 μL of peptide solution (10 μg/mL) and reacted successively with patients' serum (diluted 1 : 250) 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 mmol/L glycine–NaOH buffer (pH 10.3). Fluorescence intensity was measured on a SPECTRAmax GEMINI XS (Molecular Devices, Sunnyvale, CA, USA) with excitation at 367 nm and emission at 453 nm. Inhibition ELISA was also carried out to estimate what percentage of IgE reactivity to the R5 protein is accounted for by peptide 16. Pooled patients' serum (diluted 1 : 125) was incubated with an equal volume of inhibitor (peptide 16 or R5 protein) solution (0.002–20 μg/mL) at 37℃ for 1 hr and 50 μL of the mixture was then added to a microplate that had previously been coated with the R5 protein (1 μg/mL). The subsequent procedure was the same as in ELISA. All ELISAs (including inhibition ELISA) were performed in triplicate and the data obtained were expressed as mean±SD.

Results

IgE-binding ability of recombinant R1–5 proteins

Five proteins (R1–5) covering the entire sequence of the rainbow trout collagen α2 chain were individually expressed in *E. coli* as GST-fusions. In each expression experiment, a 45–48 kDa band was prominently observed in both soluble and insoluble fractions from IPTG-induced bacteria as analyzed by SDS-PAGE (data not shown). Based on the molecular masses of GST (about 26 kDa) and R1–5 proteins (about 20 kDa), these bands were attributed to the GST-fusion proteins. Following affinity chromatography on a GSTrap HP column, each GST-fusion protein was obtained in electrophoretically pure state from the soluble fraction (Fig. 2A).

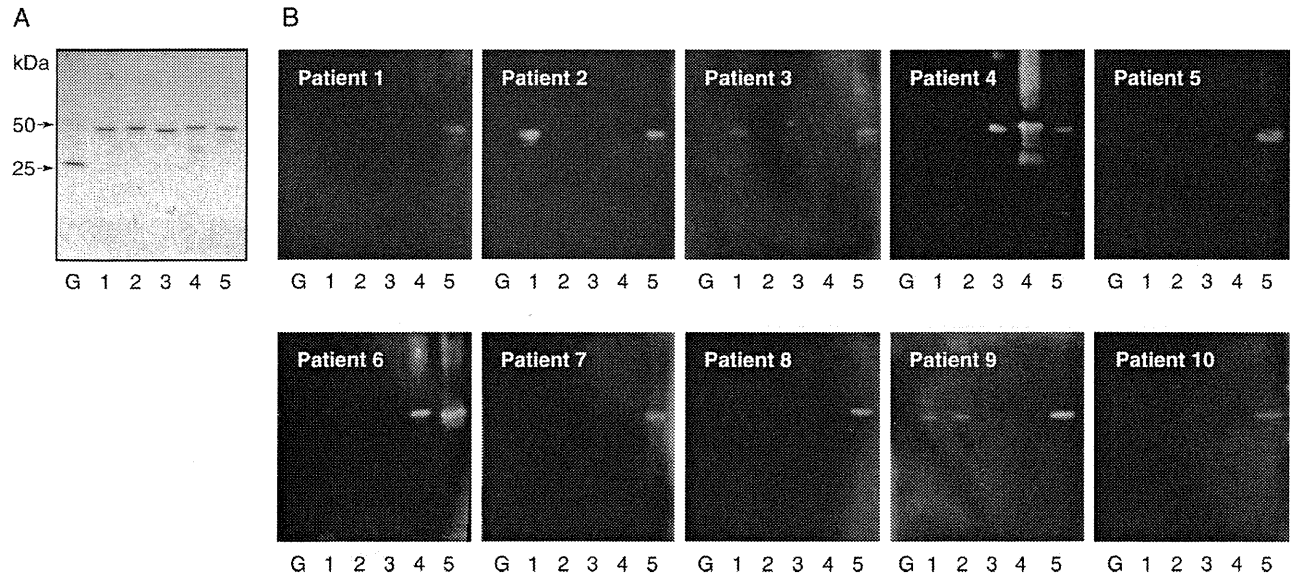


Fig. 2. Analysis of GST and recombinant R1–5 proteins by SDS-PAGE (A) and immunoblotting (B)
Lanes: G, GST; 1, GST-fusion R1; 2, GST-fusion R2; 3, GST-fusion R3; 4, GST-fusion R4; 5, GST-fusion R5.

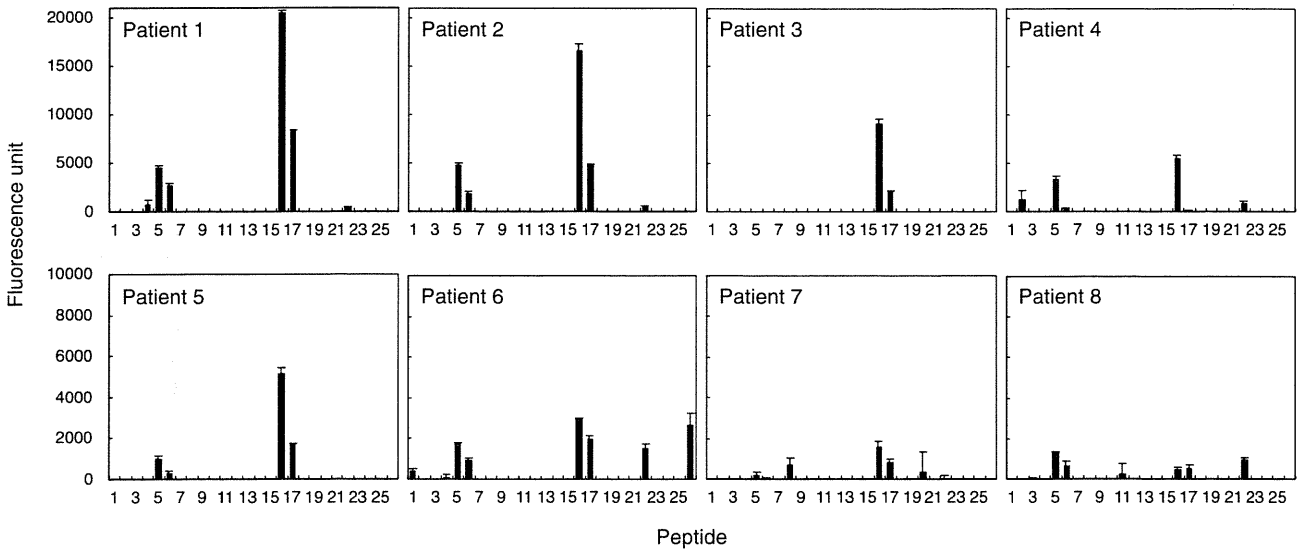


Fig. 3. IgE reactivity of peptides 1–26 analyzed by fluorescence ELISA
Data are expressed as mean±SD (n=3).

When analyzed by immunoblotting, sera of 10 patients reacted to at least one of the five GST-fusion proteins (Fig. 2B), while two control sera showed no reactivity to any of them (data not shown). IgE reactivity to GST was not recognized in any of the patients' sera, supporting the view that the reactivity between the patients' sera and the GST-fusion proteins is attributable to the R1–5 portions of the fusion proteins. Sera from patients 2, 3, 4, 6 and 9 reacted not only to the R5 protein, but also to one or two of the R1–4 proteins; for example, serum of patient 2 also reacted to the R1 protein and serum of patient 4 to the R3 and 4 proteins. The remaining five patients' sera showed positive reactivity only to the R5 protein. These results indicate that the major IgE epitopes of the rainbow trout collagen α2 chain are included in the C-terminal R 5 protein (region 821–1,041).

IgE-binding ability of synthetic peptides 1–26

To evaluate further the major IgE epitopes, 26 overlapping peptides covering the entire sequence of the R5 protein were synthesized and examined for IgE-binding ability by fluorescence ELISA. Since insufficient serum was available from patients 9 and 10, only the remaining eight sera were subjected to fluorescence ELISA. Each serum reacted to several peptides (Fig. 3), although control sera reacted to none of the peptides (data not shown). Among the 26 peptides, peptides 5, 16 and 17 were recognized at high frequencies by the patients' sera. Seven sera showed the highest reactivity to peptide 16; the only exception was serum from patient 8 serum, in which the reactivity to peptide 16 was