

Purification and molecular cloning of a major allergen from *Anisakis simplex*[☆]

Kuniyoshi Shimakura^{a,*}, Hironori Miura^a, Kaori Ikeda^a, Shoichiro Ishizaki^a,
Yuji Nagashima^a, Toshihiro Shirai^b, Shiro Kasuya^c, Kazuo Shiomi^a

^a Department of Food Science and Technology, Faculty of Marine Science, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan

^b Department of Internal Medicine, Fujinomiya City General Hospital, Fujinomiya 418-0076, Japan

^c Faculty of Regional Studies, Gifu University, Gifu 501-1112, Japan

Received 6 March 2003; received in revised form 1 May 2003; accepted 14 January 2004

Abstract

A heat-stable allergen with a molecular weight of 21 k was purified from larvae of the nematode *Anisakis simplex* by gel filtration, anion-exchange FPLC and reverse-phase HPLC. When analyzed by immunoblotting and ELISA, seven of eight patient sera reacted to the 21 k allergen, demonstrating that this protein is a major allergen of *A. simplex*. A full-length cDNA encoding the 21 k allergen was cloned by a combination of 3'RACE and screening of an expression library with DIG-labeled DNA probes. The precursor of the 21 k allergen was judged to be composed of a signal peptide (23 residues) and a mature protein (171 residues). As compared to the N-terminal amino acid sequence (up to the 17th residue) of Ani s 1 previously identified as the major allergen, the 21 k allergen has only one replacement, suggesting that the 21 k allergen belongs to the same protein family of Ani s 1. Although the 21 k allergen was found to have 30–40% sequence identity with Kunitz-type trypsin inhibitor domain containing hypothetical proteins of *Caenorhabditis elegans*, it lacked inhibitory activity against trypsin. The 21 k allergen was successfully expressed in *Escherichia coli* as a GST-fusion protein showing reactivity with IgE in patient sera. © 2004 Elsevier B.V. All rights reserved.

Keywords: Allergen; *Anisakis simplex*; Molecular cloning; Purification

1. Introduction

The nematode *Anisakis simplex*, in the third larval stage, parasitizes fish, cephalopods and crustaceans. When infected raw or undercooked fish is ingested by humans, the parasite occasionally penetrates the gastrointestinal mucosa, causing a disease known as anisakiasis [1]. Typical symptoms of anisakiasis are abdominal pain, nausea and vomiting. Besides these gastrointestinal symptoms, IgE-mediated allergic reactions, such as urticaria, angioedema and anaphylaxis,

are induced in some patients sensitized by previous contact with *A. simplex* [2–6]. Diagnosis of *A. simplex* allergy has been performed by laboratory assays based on the measurement of specific IgE using crude parasite extracts [2,5,7–10]. However, these assays seem to lead to false-positive results, since high levels of specific IgE are often detected even in asymptomatic subjects [7,10]. The use of purified or recombinant allergens will make the diagnosis of *A. simplex* allergy much more reliable. Along this line, three classes of proteins, a 24 k protein [11], a 21 k protein homologous to nematode troponins [12] and paramyosin [13], have recently been purified or cloned one after another and identified as major or minor allergens of *A. simplex*. Both 24 k and 21 k allergens were named Ani s 1 by different authors. To avoid confusion, the 24 k allergen is hereafter referred to Ani s 1 and the 21 k allergen to the troponin-like allergen.

In Japan, fish consumption is very high and the habit of eating raw seafood prevails. In reflection of these circumstances, incidents of anisakiasis occur much more frequently in Japan than in other countries. According to Murata [14],

Abbreviations: DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

[☆] Note: Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession number AB100095.

* Corresponding author. Tel.: +81-3-5463-0607; fax: +81-3-5463-0669.

E-mail address: kunisan@s.kaiyodai.ac.jp (K. Shimakura).

as many as 32,300 cases of anisakiasis are recorded in Japan from 1960 to 1997, accounting for more than 90% of the cases reported in the world. Although not surveyed in detail, allergy due to *A. simplex* is also considered to be prevalent in Japan. It is, however, unknown whether the known *A. simplex* allergens described above are also responsible for allergic reactions in Japanese patients. This prompted us to initiate a study on *A. simplex* allergens against Japanese patients. As a result, a heat-stable 21 k protein that is closely similar to but not identical with Ani s 1 was identified as the major allergen. The present paper deals with the purification and molecular cloning of the 21 k allergen.

2. Materials and methods

2.1. Parasite sample

Cysts of *A. simplex* were collected from the surface of hepatopancreata of the walleye pollack (*Theragra chalcogramma*) captured at Raus, Hokkaido, and digested with pepsin according to the method of Bier et al. [15]. The third stage larvae thus obtained were washed with Young's Ringer solution for seawater fish and stored at -20°C until used. Some of the larvae were stored at -80°C for molecular cloning experiments.

2.2. Human sera

Sera were obtained from eight patients (patients 1–8) with a history of hypersensitivity reactions, such as urticaria and anaphylaxis, after ingestion of raw or cooked fish. The patients were all diagnosed to be allergic to *A. simplex* but not to fish, based on the determined CAP-RAST classes (4–6 against *A. simplex* and 0 against fish). In this study, sera from two healthy subjects without an episode of any hypersensitivity reactions were used as controls. All sera were stored at -20°C until used.

2.3. Preparation of crude extract

The larvae of *A. simplex* were well macerated in a mortar with four volumes of 0.01 M phosphate buffer (pH 7.2) containing 0.15 M NaCl and centrifuged at $20,000 \times g$ at 0°C for 30 min. The supernatant was used as non-heated extract. A part of the non-heated extract was heated in a boiling water bath for 10 min. After removal of insoluble materials by ultracentrifugation at $100,000 \times g$ at 0°C for 30 min, the supernatant obtained was used as heated extract.

2.4. Purification procedure

Throughout the following purification procedure, allergens were followed by ELISA using patient 1 serum. The heated extract from *A. simplex* was first loaded onto a Sephacryl S-300 column (2.5 cm $\times$ 90 cm; Amersham

Pharmacia Biotech, Buckinghamshire, UK), which was eluted with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0) at a flow rate of about 35 ml/h. Fractions of 10 ml were collected and measured for absorbance at 280 nm and allergenic activity. Allergen-containing fractions were combined, dialyzed against distilled water and lyophilized. The dried products were dissolved in 5 ml of 0.01 M Gly–NaOH buffer (pH 9.5) and subjected to FPLC on a Mono Q HR 5/5 column (0.5 cm $\times$ 5 cm; Amersham Pharmacia Biotech). The column was washed with the same Gly–NaOH buffer and then eluted at a flow rate of 1 ml/min with a linear gradient of NaCl (0–0.7 M in 35 min) in the buffer. Proteins were monitored at 280 nm with a UV detector and fractions of 1 ml were collected for the assay of allergenic activity. Allergenic fractions were pooled and finally applied to reverse-phase HPLC on a TSKgel Phenyl-5PW RP column (0.46 cm $\times$ 7.5 cm; Tosoh, Tokyo, Japan), which was eluted at a flow rate of 0.5 ml/min with a linear gradient of acetonitrile (21–70% in 80 min) in 0.1% trifluoroacetic acid. Proteins were monitored at 220 nm with a UV detector. The eluate containing an allergen was manually collected, lyophilized and used for subsequent experiments.

2.5. ELISA

ELISA was performed using a polystyrene microtiter plate with 96-wells (ELISA plate H Type; Sumitomo Bakelite, Tokyo, Japan) essentially by the same method as described elsewhere [16]. In brief, the antigen coated on the plate was reacted successively with patient or control serum (diluted 1:50) and peroxidase-conjugated goat anti-human IgE antibody (diluted 1:2500; Kirkegaard & Perry Laboratories, Gaithersburg, MD). Then the enzyme reaction was carried out using substrate solution (0.1% *o*-phenylenediamine dihydrochloride and 0.03% H_2O_2 in 0.05 M phosphate-citrate buffer, pH 5.0) and the developed color was read at 490 nm on a Kinetic Microplate Reader model 550 (Bio-Rad Laboratories, Hercules, CA). All ELISAs were performed in triplicate and the data were expressed in mean values.

2.6. Protein determination

Protein was determined by the method of Lowry et al. [17], using bovine serum albumin as a standard.

2.7. SDS–PAGE and immunoblotting

SDS–PAGE was performed on a PhastSystem (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Ready-made gels (PhastGel Gradient 10–15) and ready-made buffer strips (PhastGel SDS Buffer Strips) were purchased from Amersham Pharmacia Biotech. Prior to loading, each sample was dissolved in 2% SDS–0.01 M Tris–HCl buffer (pH 6.8) with or without 2%

2-mercaptoethanol and heated at 100 °C for 15 min. After running, the gel was stained with Coomassie Brilliant Blue R-250. A low molecular weight calibration kit (Amersham Pharmacia Biotech) was used as a reference.

In immunoblotting, the proteins separated by SDS–PAGE were first electrotransferred to a nitrocellulose membrane using the PhastSystem equipped with a PhastTransfer (Amersham Pharmacia Biotech) as described in the manufacturer's manual. The membrane was blocked with blocking solution (Dulbecco's phosphate buffer, pH 7.2, containing 2.5% skimmed milk and 0.05% Tween 20) at 37 °C overnight. Then, the membrane was reacted successively with patient 1 serum against the purified allergen, patients 1 and 5 sera against the expressed allergen (diluted 1:25) at 37 °C for 3 h and peroxidase-conjugated goat anti-human IgE antibody (diluted 1:1000) at 37 °C for 3 h or with goat anti-GST IgG (diluted 1:2500; Amersham Pharmacia Biotech) and peroxidase-conjugated donkey anti-goat IgG antibody (diluted 1:10,000; Rockland, Gilbertsville, PA). Antigen–antibody binding was visualized by incubating the membrane with substrate solution (0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.05% H₂O₂ in 0.05 M citrate buffer, pH 5.0) at room temperature for 20 min.

2.8. Amino acid sequence analysis

Amino acid sequencing was performed by the automated Edman degradation method with a gas-phase protein sequencer (LF-3400D TriCart with high sensitivity chemistry; Beckman Coulter, Fullerton, CA).

2.9. Construction of cDNA library

Total RNA was extracted from 2 g of *A. simplex* larvae with the TRIzol reagent (Life Technologies, Rockville, MD) and poly(A)⁺ mRNA was purified by affinity chromatography on oligo(dT)-cellulose using an mRNA purification kit (Amersham Pharmacia Biotech). A part of the purified mRNA was converted to cDNA, followed by ligation of AP1 adapters using a Marathon cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA). In this paper, the library thus constructed is called "Marathon cDNA library". A part of the purified mRNA was also used to construct an expression cDNA library. In this case, synthesis of cDNA and ligation of the double-stranded cDNA to *Not* I/*Sal* I ends of λ ZipLox were carried out using the SuperScriptTM Lambda System (Life Technologies) and packaging in λ phase using the Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA).

2.10. Cloning of cDNA encoding the purified allergen

The Marathon cDNA library was applied to 3'RACE analysis using an AP1 reverse primer and degenerate forward primer (5'-MGIACIGARTGYCARYTICC-3') which

was designed according to the determined N-terminal amino acid sequence of the purified allergen. The amplified product was analyzed for DNA sequence using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosciences, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosciences). cDNA library was infected to *Escherichia coli* Y1090 and plated on LB agar. After incubation at 42 °C for 3.5 h plaques formed were blotted onto a nitrocellulose membrane previously soaked in 10 mM isopropyl-1-thio- β -galactopyranoside (IPTG). Positive plaques were detected with DIG-labeled DNA probes using a DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany). The DIG-labeled DNA probes were prepared using the DNA product (606 bp) that was amplified from the Marathon cDNA library by PCR with gene specific primers. Following infection of the λ ZipLox clones into *E. coli* DH 10B, the plasmid DNA was purified from the selected colonies of *E. coli* grown in LB medium using a Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories) and subjected to DNA sequencing.

2.11. Expression of the purified allergen

The open reading frame corresponding to the mature 21 k allergen, with addition of recognition sites of *Bam*H I and *Eco*R I to the upstream and the downstream, respectively, was amplified by PCR and ligated to the pGEX-6p-3 vector (Amersham Biosciences, Piscataway, NJ). Following introduction of the ligation product into *E. coli* JM 109, transformed cells were grown overnight at 37 °C in 5 ml of LB medium containing 0.005% ampicillin. Then 50 μ l of the culture was inoculated into 5 ml of the same medium and incubated with stirring at 37 °C until the absorbance of the medium at 600 nm was increased to 0.5. To induce protein expression, the culture was added with 1 mM IPTG and further incubated at 37 °C for 3 h. Bacteria harvested by centrifugation were suspended in 150 μ l of 0.01 M phosphate buffer (pH 7.0) containing 1 mM EDTA and sonicated. After centrifugation, both supernatant (soluble fraction) and precipitate (insoluble fraction) were subjected to SDS–PAGE and immunoblotting.

3. Results

3.1. IgE reactivity of sera

Analyses by ELISA showed that all patient sera reacted to both non-heated and heated extracts from *A. simplex* (Fig. 1) but two control sera did not. Interestingly, five patient sera (from patients 1, 3, 6–8) had almost the same potency in IgE reactivity against both non-heated and heated extracts, while the remaining three patient sera (from patients 2, 4 and 5) were about 40, 30 and 55% less reactive, respectively, to the heated extract than to the non-heated extract. These results strongly suggest that the major allergen of *A. simplex*

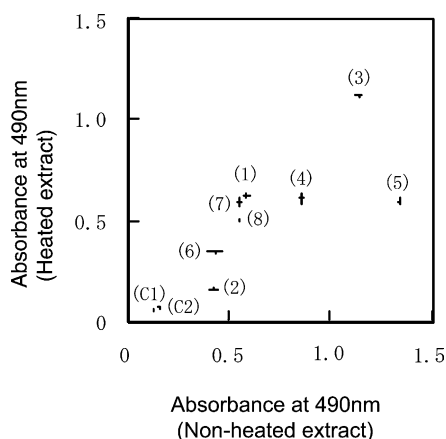


Fig. 1. IgE reactivity of eight patient sera (1–8) and two control sera (C1 and C2) to the non-heated and heated extracts from *A. simplex*. Each extract was diluted 1:2500 and subjected to ELISA.

is thermostable, although thermolabile allergens recognized by some patients coexist. Therefore, this study was focused on thermostable allergens and their purification was carried out using the heated extract.

3.2. Purification and properties of allergens

When the heated extract from *A. simplex* was chromatographed on Sephacryl S-300, allergens appeared at around fraction 36 (Fig. 2A). In anion-exchange FPLC on Mono Q, allergens were adsorbed on the column at pH 9.5 and eluted with a linear gradient of NaCl (Fig. 2B). Finally, an allergen was purified by reverse-phase HPLC on TSKgel Phenyl-5PW RP; the allergen was eluted in a single peak with a retention time of 33 min (Fig. 2C). In a typical run, 200 μ g of the purified allergen was obtained from 90 g of *A. simplex* larvae. The purified allergen migrated as a single band of 21 k in SDS-PAGE under both reduced (Fig. 3A) and non-reduced (data not shown) conditions. In this paper, the purified allergen is called “21 k allergen”.

Immunoblotting proved that the 21 k allergen is reactive with serum IgE in seven patients except for patient 7 (typical results with patient 1 serum are shown in Fig. 3B). The eight patient sera were further evaluated for their IgE reactivity to the 21 k allergen by ELISA (Fig. 4). In accordance with the results in immunoblotting, seven patient sera (from patients 1 to 6 and 8) reacted to the 21 k allergen with varied potencies, while no reactivity was observed in patient 7 serum similar to two control sera.

Analysis by a protein sequencer identified N-terminal 26 amino acid residues of the 21 k allergen as follows: DRTECQLPLDKGTPCTQEGGVKPSVA. The determined N-terminal sequence was closely similar to that of Ani s 1 [11]. As compared to the N-terminal sequence up to the 17th residue reported for Ani s 1, the 21 k allergen had only one alteration at position 14 (Pro for the 21 k allergen and Gln for Ani s 1).

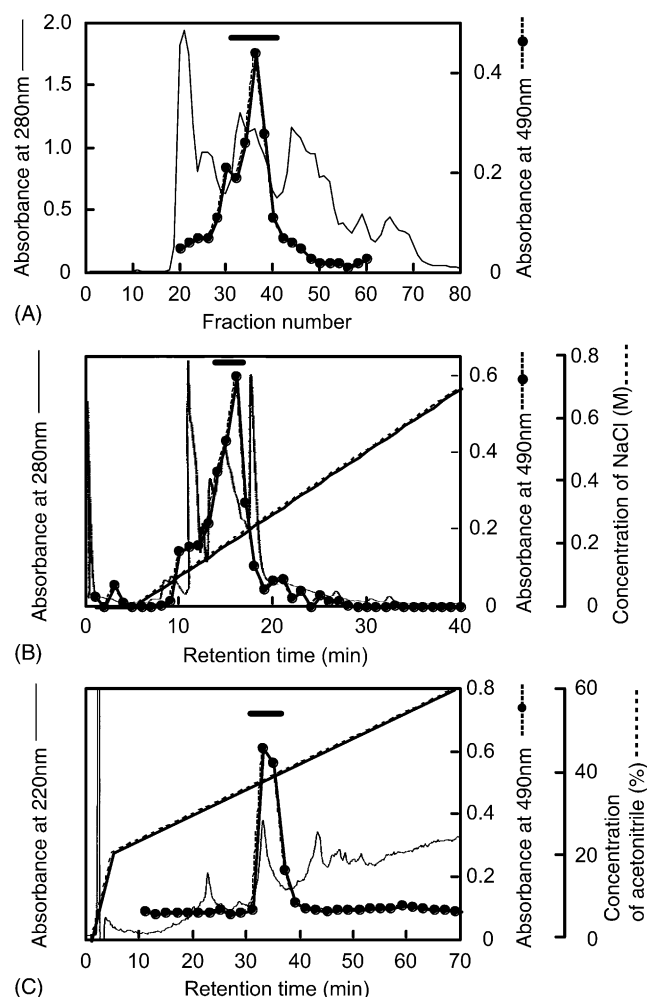


Fig. 2. Purification of a major allergen from *A. simplex* by gel filtration on Sephacryl S-300 (A), anion-exchange FPLC on Mono Q (B) and reverse-phase HPLC on TSKgel Phenyl-5PW RP (C). A crossbar in each panel represents pooled fractions.

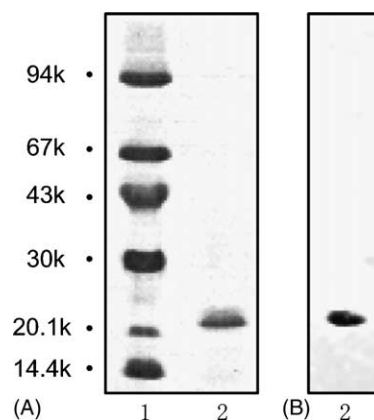


Fig. 3. SDS-PAGE (A) and immunoblotting (B) of the purified allergen. Sample: lane 1, reference proteins; lane 2, purified allergen.

3.3. Molecular cloning of the purified allergen

The 3'-end nucleotide sequence of the 21 k allergen cDNA was elucidated by the 3'RACE method applied to

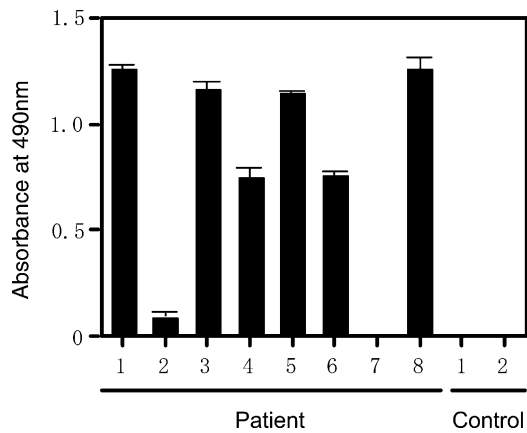


Fig. 4. IgE reactivity of eight patient and two control sera to the 21 k allergen. The protein concentration of the 21 k allergen was adjusted to 1 µg/ml and subjected to ELISA.

the Marathon cDNA library. However, no DNAs were amplified by the 5'RACE method, although PCRs were tried using various reverse primers combined with the AP1 forward primer. Therefore, the cDNA encoding the 21 k allergen was cloned following the screening of the expression cDNA library with DIG-labeled DNA probes. The full length cDNA thus obtained has 1008 bp, comprising 30 bp of 5' untranslated sequence, 585 bp of an open reading frame and 393 bp of 3' untranslated sequence (GenBank accession number AB100095). The precursor protein of the 21 k allergen was composed of 194

amino acid residues and the segment 24–194 was judged to be a mature protein from the determined N-terminal amino acid sequence (Fig. 5). Analysis by the SignalP (<http://www.cbs.dtu.dk/services/SignalP-2.0>) suggested the segment 1–23 to be a signal peptide. The calculated molecular weight (18,869) of the mature protein having 171 amino acid residues was rather lower than the value (21 k) estimated by SDS–PAGE, suggesting that the 21 k allergen is partly glycosylated. A homology search using the NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) revealed that the 21 k allergen has 30–40% identity with Kunitz-type trypsin inhibitor domain containing hypothetical proteins of *Caenorhabditis elegans*. Similar to the hypothetical proteins of *C. elegans*, the 21 k allergen also has a domain of Kunitz-type protease inhibitors {FX₃GCX₆(F or Y)X₅C} at positions 62–80 (Fig. 5). We preliminarily detected trypsin inhibitory activity in the heated extract from *A. simplex*. In gel filtration on Sephacryl S-300, however, the activity was eluted later than the 21 k allergen, being ascribable to the serine protease inhibitors comprising 78 amino acid residues previously reported [18]. It was, therefore, assumed that the 21 k allergen is devoid of trypsin inhibitory activity, although not confirmed with the purified preparation due to scarcity of available sample.

3.4. Expression of the mature 21 k allergen in *E. coli*

In this study we tried to express the 21 k allergen in *E. coli* as a GST fusion protein. Based on the molecular weights

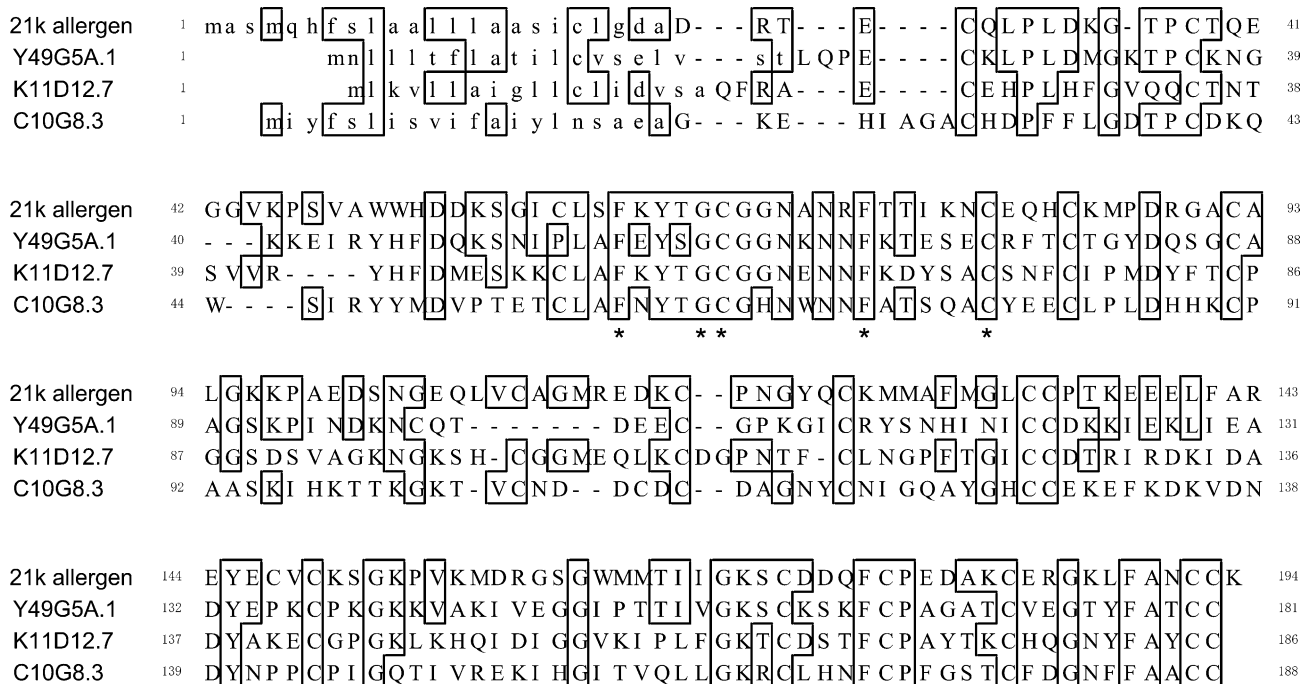


Fig. 5. Alignment of the amino acid sequence of the 21 k allergen with those of three Kunitz-type trypsin inhibitor domain containing hypothetical proteins (Y49G5A.1, GenBank AAD14760.1; K11D12.7, GenBank AAK95883.1; C10G8.3, GenBank AAD31051.1) from *Caenorhabditis elegans*. Amino acid residues identical with the 21 k allergen are boxed. Dashes represent gaps. A domain of Kunitz-type protease inhibitors is indicated by asterisks below the sequence of the protein C10G8.3. Signal peptides predicted by the SignalP (<http://www.cbs.dtu.dk/services/SignalP-2.0>) are shown in lower-case letters.

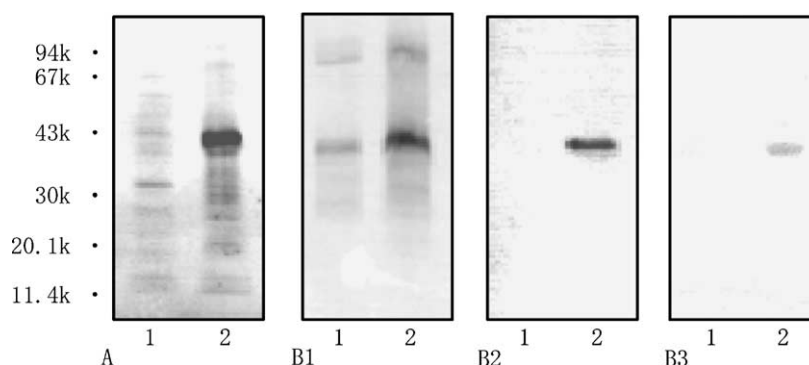


Fig. 6. SDS-PAGE (A) and immunoblotting (B) of soluble and insoluble fractions prepared from *E. coli* expressing the 21 k allergen fused with GST. Sample: lane 1, soluble fraction; lane 2, insoluble fraction. Primary antibody used for immunoblotting: B1, anti-GST IgG; B2, patient 1 serum; B3, patient 5 serum.

of the mature 21 k allergen (18.9 k) and GST (25 k), that of the GST fusion protein should be 44 k. When analyzed by SDS-PAGE, a prominent protein band was observed at the position corresponding to a molecular weight of 44 k only in the insoluble fraction from *E. coli* used for expression (Fig. 6A). This protein band was shown to react with anti-GST IgG (Fig. 6B1) and IgE from patient 1 (Fig. 6B2) and 5 sera (Fig. 6B3) by immunoblotting. In addition, the reactivity with patients 1 and 5 sera were inhibited by pre-absorption of the serum with the 21 k allergen (data not shown). However, the fusion protein was recovered only in the insoluble fraction from *E. coli*, implying its formation of inclusion body. In this study, therefore, the recombinant 21 k allergen could not be obtained in a pure state.

4. Discussion

In this study, a heat-stable 21 k allergen was purified from *A. simplex* larvae and its primary structure elucidated by a molecular cloning technique. Based on the fact that seven of the eight Japanese patients having high levels of IgE specific for *A. simplex* recognize the 21 k allergen, we presume that this allergen is a major allergen of *A. simplex* against Japanese sufferers.

The determined amino acid sequence reveals that the 21 k allergen is closely similar to Ani s 1 recently purified as a major allergen by Moneo et al. [11], although it is distinct from the other known *A. simplex* allergens (troponin-like allergen [12] and paramyosin [13]). Only the N-terminal 17 amino acid residues are known for Ani s 1. However, there is only one alteration in the N-terminal region between the 21 k allergen and Ani s 1; at position 14, the former has Pro and the latter Gln. This fact strongly suggests that the 21 k allergen belongs to the same protein family as Ani s 1, being an isoform of Ani s 1. In relation to this, it should be noted that the *A. simplex* larvae used in this study are from the hosts captured near Hokkaido, Japan, while those used by Moneo et al. are supposed to be from the hosts captured near

Europe. Since the 21 k allergen and Ani s 1 are purified from *A. simplex* specimens inhabiting different sea areas, it is not strange that they are genetically different from each other to a slight extent. Presumably, the 21 k allergen is recognized by European patients and conversely Ani s 1 by Japanese patients.

It is likely that *A. simplex* allergy is mostly associated with antigens secreted from invaded live larvae. Of the known allergens, Ani s 1 is obviously a secretory allergen because of its limited distribution in the secretory gland of the parasite [11]. Judging from the close similarity to Ani s 1, the 21 k allergen is also considered to be localized in the secretory gland and act as a secretory allergen. However, several authors [3,8,19] have described that cooking as well as freezing, during which parasitized larvae become extinct, cannot prevent *A. simplex* allergy, suggesting that allergic reactions are caused by not only live larvae but also dead larvae. In our study, this is the case with patient 1 who had an episode of allergic reactions after ingestion of not raw but grilled fish. In the light of these circumstances, it should be noted that the 21 k allergen is heat-stable as to allergenicity. The occurrence of heat-stable allergens such as the 21 k allergen seems to be a molecular basis for the inefficiency of cooking of fish against *A. simplex* allergy. We thus assume that the 21 k allergen is not only associated with allergy by live larvae but also can behave as if it were a food allergen when dead larvae are ingested with fish.

The cDNA encoding the 21 k allergen was cloned by a combination of 3'RACE using the Marathon cDNA library and screening of the expression cDNA library with DIG-labeled DNA probes. Previously, both troponin-like allergen [12] and paramyosin [13] were identified as *A. simplex* allergens by molecular cloning studies, in which patient sera with high levels of specific IgG for *A. simplex* were used to screen an expression cDNA library. In these studies, however, no cDNA clones encoding Ani s 1 or our 21 k allergen were detected. Similarly, we could not detect any positive clones encoding the 21 k allergen by the screening of our expression cDNA library with patient sera. In view

of these results, it is likely that a sufficient number of cDNA clones encoding the 21 k allergen occur in the expression library but their expression is largely inhibited due to unknown factor or the 21 k allergen or its precursor is cleaved with proteases after expression.

The 21 k allergen was successfully expressed in *E. coli* as a GST fusion protein, although the recombinant allergen could not be obtained in a pure state. Immunoblotting and inhibition immunoblotting data confirmed that the fusion protein has an ability to bind IgE, similar to the purified 21 k allergen. It is, therefore, likely that carbohydrate moieties assumed to be contained in the purified 21 k allergen are not essential for the allergenicity. In addition, the glycosylation does not appear to contribute to the heat stability of the 21 k allergen, since the fusion protein was demonstrated to be allergenic by immunoblotting following the heat treatment.

It is worth mentioning that minor allergens other than the 21 k allergen are detectable in *A. simplex*. Three sera from patients 2, 4 and 5 are less reactive to the heated extract than to the non-heated extract, suggesting the occurrence of heat-labile allergens (Fig. 1). In addition, one serum from patient 7 shows no IgE reactivity to the 21 k allergen (Fig. 4), leading us to assume that IgE from patient 7 recognizes heat-stable allergens differing from the 21 k allergen. The detected unknown heat-labile and heat-stable allergens may be analogous to either one of the troponin-like allergen [12] and paramyosin [13].

In conclusion, we have purified and cloned a heat-stable 21 k allergen from *A. simplex* and demonstrated it to be an isoform of Ani s 1. The 21 k allergen is a major allergen of *A. simplex* and appears to be deeply responsible for allergic reactions caused by both live and dead larvae. In addition to the availability of its cDNA, future acquisition of its recombinant preparation will facilitate acute diagnosis and desirable immunotherapy of *A. simplex* allergy.

Acknowledgements

We wish to thank Dr. Masami Ishida, Tokyo University of Marine Science and Technology, for analyzing the N-terminal amino acid sequence of the 21 k allergen. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

- [1] Sakanari JA, McKerrow JH. Anisakiasis. Clin Microb Rev 1989;2: 278–84.
- [2] Audicana MT, Fernández de Corres L, Muñoz D, Fernández E, Navarro JA, del Pozo MD. Recurrent anaphylaxis caused by *Anisakis simplex* parasitizing fish. J Allergy Clin Immunol 1995;96:558–60.
- [3] Fernández de Corres L, Audicana M, del Pozo MD, et al. *Anisakis simplex* induces not only anisakiasis. Report on 28 cases of allergy caused by this nematode. J Invest Allergol Clin Immunol 1996;6:315–9.
- [4] Moreno-Ancillo A, Caballero MT, Cabañas R, et al. Allergic reactions to *Anisakis simplex* parasitizing seafood. Ann Allergy Asthma Immunol 1997;79:246–50.
- [5] del Pozo MD, Audicana M, Diez JM, et al. *Anisakis simplex*, a relevant etiologic factor in acute urticaria. Allergy 1997;52:576–9.
- [6] Montoro A, Perteguer MJ, Cuéllar C, Chivato T. Recidivous acute urticaria caused by *Anisakis simplex*. Allergy 1997;52:985–91.
- [7] Gutierrez-Ramos R, Tsuji M. Detection of antibodies to *Anisakis simplex* larvae by enzyme-linked immunosorbent assay and immunoelectrophoresis using crude or purified allergens. J Helminthol 1994;68:305–9.
- [8] del Pozo MD, Moneo I, Audicana MT, et al. Laboratory determinations in *Anisakis simplex* allergy. J Allergy Clin Immunol 1996;97:977–84.
- [9] García M, Moneo I, Audicana MT, et al. The use of immunoblotting as a diagnostic tool in *Anisakis simplex* allergy. J Allergy Clin Immunol 1997;99:497–501.
- [10] Pascual CY, Crespo JF, San Martín S. Cross-reactivity between IgE-binding proteins from *Anisakis*, German cockroach, and chironomids. Allergy 1997;52:514–20.
- [11] Moneo I, Caballero ML, Gómez F, Ortega E, Alonso MJ. Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. J Allergy Clin Immunol 2000;106:177–82.
- [12] Arrieta I, del Barrio M, Vidarte L, et al. Molecular cloning and characterization of an IgE-reactive protein from *Anisakis simplex*: Ani s 1. Mol Biochem Parasitol 2000;107:263–8.
- [13] Pérez-Pérez J, Fernández-Caldas E, Marañón F, et al. Molecular cloning of paramyosin, a new allergen of *Anisakis simplex*. Int Arch Allergy Immunol 2000;123:120–9.
- [14] Murata I. Anisakiasis. Shonika Rinsho 1999;52:607–10.
- [15] Bier JW, Jackson GJ, Adams AM, Rude RA. Parasitic animals in foods. FDA bacteriological analytical manual. 8th ed. Revision A. Gaithersburg. AOAC International; 1998. 19.01-9.
- [16] Ishikawa M, Shimakura K, Nagashima Y, Shiomi K. Isolation and properties of allergenic proteins in the oyster *Crassostrea gigas*. Fish Sci 1997;63:610–4.
- [17] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
- [18] Lu CC, Nguyen T, Morris S, Hill D, Sakanari JA. *Anisakis simplex*: mutational bursts in the reactive site centers of serine protease inhibitors from an ascarid nematode. Exp Parasitol 1998;89:257–61.
- [19] Audicana L, Audicana MT, Fernández de Corres L, Kennedy MW. Cooking and freezing may not protect against allergenic reactions to ingested *Anisakis simplex* antigens in humans. Vet Rec 1997;140:235.