

Purification and cDNA cloning of a new heat-stable allergen from *Anisakis simplex*[☆]

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Received 2 May 2007; received in revised form 25 June 2007; accepted 25 June 2007

Available online 30 June 2007

Abstract

The nematode *Anisakis simplex* is a representative parasite for marine animals and occasionally causes not only anisakiasis but also allergic reactions in sensitized subjects. Besides the known allergens, a number of unidentified allergens have been suggested to still exist in *A. simplex*. In this study, a new heat-stable allergen of 15 kDa (named Ani s 8) was purified from the third stage larvae of *A. simplex* by gel filtration on Sephacryl S-300, anion-exchange HPLC on Mono Q and reverse-phase HPLC on TSKgel Phenyl-5PW RP. Analysis by fluorescence ELISA showed that 7 of 28 *Anisakis*-allergic patients had elevated serum levels of IgE to Ani s 8. On the basis of the determined partial amino acid sequence, the complete sequence of Ani s 8 (composed of 150 amino acid residues) was elucidated by cDNA cloning, in which as many as 32 homologs of the cDNA encoding 10 isoforms of Ani s 8 were detected. Ani s 8 shares amino acid sequence homology (up to 36%) with several members of the SXP/RAL-2 protein family, including Ani s 5 (15 kDa) previously identified as an *A. simplex* allergen. Inhibition ELISA data demonstrated the IgE cross-reactivity between Ani s 8 and Ani s 5.

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Keywords: Allergen; Ani s 8; *Anisakis simplex*; cDNA cloning; Nematode

1. Introduction

The nematode *Anisakis simplex* is a representative parasite for marine animals, especially for fish. Following ingestion of raw or undercooked fish infected with third stage larvae of *A. simplex*, humans occasionally suffer from gastrointestinal symptoms, such as abdominal pain, nausea, vomiting and diarrhea, known as anisakiasis [1]. In addition to anisakiasis, even IgE-mediated allergic reactions, such as urticaria, angioedema and

anaphylaxis, are also induced in individuals who have previously been sensitized with *A. simplex* [2–6].

To date, the following seven kinds of proteins have been identified as *A. simplex* allergens and well characterized: a secretory protein of 21 kDa (Ani s 1) [7,8], paramyosin of 100 kDa (Ani s 2) [9], tropomyosin of 41 kDa (Ani s 3) [10,11], a cysteine protease inhibitor of 9 kDa (Ani s 4) [12,13], a 15 kDa protein homologous with the SXP/RAL-2 family proteins (Ani s 5) [14], a protease inhibitor of 7 kDa (Ani s 6) [14] and a troponin-like protein of 21 kDa [15]. Recently, Ani s 7 of 139 kDa was added as a new allergen to the list of allergens provided by Allergen Nomenclature Sub-Committee (<http://www.allergen.org/Allergen.aspx>), although its properties have not been reported. Besides the allergens described above, a number of unidentified allergens seem to still exist in *A. simplex* as suggested by either immunoblot analysis or crossed immunoelectrophoresis [16–18].

It has previously been reported that cooking of fish as well as freezing of fish, to which parasitized larvae are intolerable, cannot always prevent *A. simplex* allergy [2–4,19–21], suggesting

Abbreviations: CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; IgE, immunoglobulin E; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

[☆] Note: Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession numbers AB300625–AB300656.

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the involvement of heat-stable allergens in some incidents of *A. simplex* allergy. Moreover, Armentia et al. [22] have recently reported that ingestion of chicken meat contaminated with *A. simplex* allergens, which are possibly derived from fishmeal used for feeding chicken, induces hypersensitive reactions in *A. simplex*-allergic patients. Heat-stable allergens in *A. simplex* must be also responsible for this case, since manufacturing of fishmeal involves a long and severe heating process. Although Ani s 1 and Ani s 4 are heat-stable among the known *A. simplex* allergens, other unknown heat-stable allergens are likely to occur. This study was hence initiated to obtain more information about heat-stable allergens possibly implicated in *A. simplex* allergy. We report here the purification of a new heat-stable allergen of 15 kDa (named Ani s 8) from *A. simplex* and its primary structure elucidation by a cDNA cloning technique.

2. Materials and methods

2.1. Parasite

Third stage larvae of *A. simplex* with cysts were collected from the surface of hepatopancreas of walleye pollack (*Theragra chalcogramma*). After the cysts were digested with pepsin according to the method of Bier et al. [23], the larvae were washed with 0.9% NaCl solution and stored at -20°C until use.

2.2. Preparation of crude extract

The larvae were extracted twice with two volumes of 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl and the extract was used as a non-heated extract. A part of the non-heated extract was heated in a boiling water bath for 10 min. After ultracentrifugation at $1,00,000 \times g$ for 60 min, the supernatant obtained was used as a heated extract. The protein concentrations of the non-heated and heated extracts were determined to be 13.0 and 6.4 mg/ml, respectively, by the method of Lowry et al. [24] using bovine serum albumin as a standard.

2.3. Human sera

Sera were obtained from 28 patients with clinical histories of allergic reactions, such as urticaria and anaphylaxis, after eating raw or cooked fish. These patients were diagnosed to be allergic to *A. simplex* but not to fish at hospital, based on the determined CAP-RAST classes (2–6 against *A. simplex* and 0 against fish). In this study, sera from 17 healthy subjects having no specific IgE against *A. simplex* were used as controls. All sera were stored at -20° until use.

2.4. ELISA

Reactivity of patient sera to the crude extract or the purified allergen was assessed by fluorescence ELISA as reported previously [25]. In brief, a polystyrene microtiter plate with 96 wells

(Type H (black); Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of the crude extract (diluted 1:600) or the purified allergen solution (1 $\mu\text{g}/\text{ml}$) and reacted successively with patient or control serum (diluted 1:200) and β -galactosidase-conjugated goat anti-human IgE antibody (0.25 $\mu\text{g}/\text{ml}$; American Qualex, San Clement, CA, USA). Enzyme reaction was performed using 0.1 mg/ml 4-methylumbelliferyl- β -D-galactoside and arrested by addition of 100 mM glycine–NaOH buffer (pH 10.3). Fluorescence intensity was measured at 367 nm (excitation) and 453 nm (emission). During chromatographic steps, allergens were detected by normal ELISA as reported previously [26]. Briefly, a polystyrene microtiter plate with 96 wells (Type H; Sumitomo Bakelite) was coated with 50 μl of sample solution and reacted with patient serum (diluted 1:100), followed by peroxidase-conjugated goat anti-human IgE antibody (0.4 $\mu\text{g}/\text{ml}$; Kirkedgaard & Perry Laboratories, Gaithersburg, MD, USA). Enzyme reaction was carried out using substrate solution containing 0.1% *o*-phenylenediamine and 0.03% H_2O_2 and arrested by addition of 1 M sulfuric acid. The developed color was measured by absorbance at 490 nm. All ELISAs were performed in triplicate and the data were expressed in mean $\pm$ S.D.

2.5. Inhibition ELISA

As described below in detail, Ani s 8 was found to be homologous with Ani s 5, a 15 kDa protein identified as an *A. simplex* allergen in our previous paper [14]. Therefore, antigenic cross-reactivity of Ani s 8 with Ani s 5 was examined by inhibition ELISA. For Ani s 5, a recombinant preparation was expressed in *Escherichia coli* as reported previously [14] and used. Patient serum (diluted 1:100) was incubated with an equal volume of Ani s 8 or Ani s 5 solution (0.02–20000 ng/ml) at 37°C for 2 h and a 50 μl portion of this solution was added to the plate which had previously been coated with Ani s 8 (50 ng/well). The subsequent procedure was the same as adopted for fluorescence ELISA.

2.6. SDS-PAGE

SDS-PAGE was performed using a PhastSystem apparatus (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Each sample was dissolved in 62.5 mM Tris–HCl buffer (pH 6.8) containing 2% SDS or 2% SDS, 6 M urea and 100 mM dithiothreitol, heated at 70°C for 10 min and run on a PhastGel Gradient 8–25 gel (GE Healthcare Bio-Sciences). Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as references. Proteins were detected by staining with Coomassie Brilliant Blue R-250.

2.7. Purification procedure

The heated extract from *A. simplex* larvae was applied to gel filtration on a Sephacryl S-300 HR column (2.5 cm $\times$ 90 cm; GE Healthcare Bio-Sciences), which was eluted with 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 20 ml/h. Fractions of 10 ml were collected, measured

for absorbance at 280 nm and assayed for IgE reactivity by normal ELISA. Allergen-containing fractions were pooled, dialyzed against distilled water and lyophilized. The dried products were dissolved in 10 mM Tris–HCl buffer (pH 8.0) and subjected to anion-exchange HPLC on a Mono Q HR 5/5 column (0.5 cm × 5 cm; GE Healthcare Bio-Sciences). After being washed with the same Tris–HCl buffer to remove unadsorbed proteins, the column was eluted at a flow rate of 1 ml/min with a linear gradient of NaCl (0–300 mM in 45 min) in the buffer, with monitoring at 280 nm using a UV detector. The eluate containing allergens was collected and then applied to reverse-phase HPLC on a TSKgel Phenyl-5PW RP column (0.46 cm × 7.5 cm; Tosoh, Tokyo, Japan). Elution of the column was achieved by a linear gradient of acetonitrile (14–70% in 60 min) in 0.1% TFA at a flow rate of 1 ml/min. Proteins were monitored at 220 nm with a UV detector. The eluate corresponding to an allergen peak was lyophilized and dissolved in desired solvents for subsequent experiments.

2.8. Enzymatic digestion and isolation of peptide fragments

The purified allergen (50 µg) was digested with 0.5 µg of endoprotease Glu-C (Roche, Mannheim, Germany) in 100 µl of 100 mM ammonium carbonate buffer (pH 7.8) containing 2 mM EDTA at 25 °C for 6 h. To isolate peptide fragments produced, the digest was applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 cm × 25 cm; Tosoh), which was eluted with a linear gradient of acetonitrile (0–42% in 120 min) in 0.1% TFA at a flow rate of 1 ml/min. Peptides were monitored at 220 nm with a UV detector.

2.9. Amino acid sequencing

Amino acid sequencing was performed by the Edman degradation method with an automatic gas-phase protein sequencer

(LF-3400D TriCart with high sensitivity chemistry; Beckman Coulter, Fullerton, CA, USA).

2.10. Cloning experiments

The cDNA phage library used was the same as constructed in our previous study [14]. To amplify a 5'-end fragment of the allergen cDNA, the cDNA library was subjected to PCR using a forward vector primer (5'-AGCGGATAACAATTTTCACACAGG-3') and a reverse degenerate primer (5'-TCNGCYTTYTGNGCRTCNGG-3') designed based on the determined internal amino acid sequence. Amplification was performed using HotMaster Taq polymerase (Eppendorf, Tokyo, Japan) under the following conditions: 94 °C for 2 min and 35 cycles of 94 °C for 20 s, 50 °C for 10 s and 70 °C for 50 s. PCR products were ligated into the pT7Blue-2 T-vector (Takara, Otsu, Japan) using a DNA Ligation Kit (Takara). *E. coli* JM109 was transformed with the ligation products and cultured on LB agar containing 0.01% ampicillin, 0.01% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 2 mM isopropyl-β-D-thiogalactoside. After incubation at 37 °C overnight, each white colony was cultured in LB medium containing 0.005% ampicillin at 37 °C overnight. The plasmid DNA was purified from a bacterial suspension using a Quantum Prep Plasmid Mini Prep Kit (Bio-Rad Laboratories) and sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). To determine the remaining sequence of the allergen cDNA, PCR was further performed using a gene specific forward primer (5'-CGCGTCTTTAACTTAGCTC-3'), which was designed based on the 5'-end sequence, and a reverse vector primer (5'-GTTTTCCCAGTCACGACGT-3') under the same conditions as described above. Amplified products were similarly subcloned into the pT7Blue-2 T-vector and sequenced.

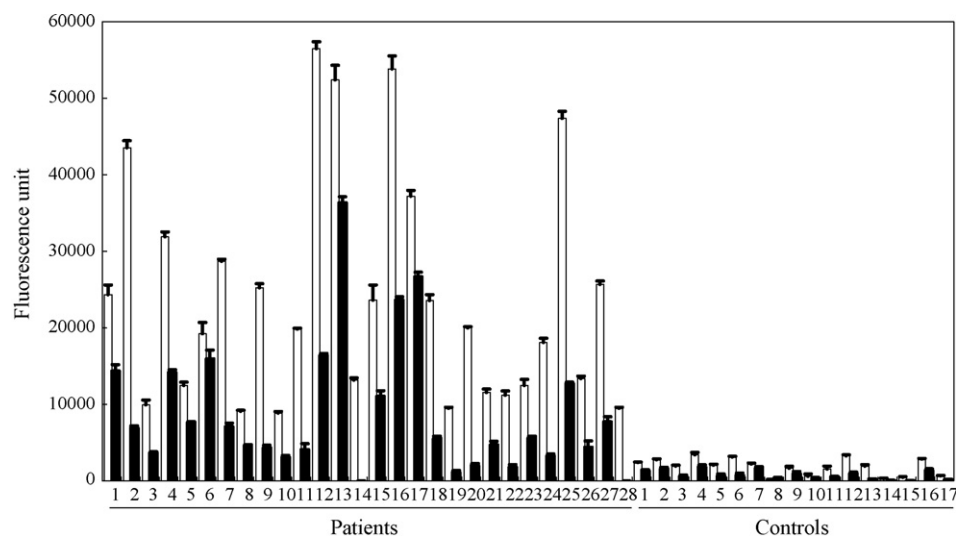


Fig. 1. Analysis of IgE reactivities of the extracts from *Anisakis simplex* larvae by fluorescence ELISA using 28 patient and 17 control sera. Open and closed bars represent the non-heated and heated extracts, respectively. Each datum is expressed in mean ± S.D. ($n = 3$). The value above the control mean + 2S.D. (equivalent to fluorescence unit 4008 for the non-heated extract and 1904 for the heated extract) was judged to be positive.

3. Results

3.1. Reactivity of patient sera to the crude extracts

Analysis by fluorescence ELISA showed that all 28 patient sera reacted to the non-heated extract from *A. simplex* with varied potencies (Fig. 1). In addition, as many as 24 sera (except for four sera from patients 14, 19, 22 and 28) were positive to the heated extract as well. The heated extract maintained 10–83% IgE reactivity of the non-heated extract depending on the patient sera.

3.2. Purification of allergens

Throughout the purification procedure, the patient 13 serum was used to detect allergens by ELISA, not only because it exhibited the highest reactivity to the heated extract (Fig. 1) but also because its available volume was sufficient at our hand. It should be also noted that the patient 13 serum strongly reacts to a 15 kDa protein differing from Ani s 5 in the non-heated extract [14]. In our preliminary immunoblotting experiments, the 15 kDa protein was found to be almost the sole allergen in the heated-extract recognized by patient 13. Thus, the use of the patient 13 serum for ELISA seemed to make it easy to purify the 15 kDa allergen.

In gel filtration on Sephacryl S-300 HR, allergens contained in the heated extract were eluted between fractions 40 and 42 (Fig. 2A). When the allergenic fraction obtained by gel filtration was subjected to anion-exchange HPLC on Mono Q HR 5/5, allergens appeared at retention times of 29–32 min (Fig. 2B). In the final step of purification by reverse-phase HPLC on TSKgel Phenyl-5PW RP, allergens were separated into three fractions (Frs. A–C in Fig. 2C). In either Fr. A or Fr. B, however, no obvious protein peak was observed. In accordance with this, no protein bands were detected in both Frs. A and B as analyzed by SDS-PAGE (data not shown). On the other hand, Fr. C afforded a single band of 15 kDa in SDS-PAGE under both reduced and non-reduced conditions (Fig. 3). In a typical run, 2 mg of the 15 kDa allergen (Ani s 8) was obtained from 70 g of *A. simplex* larvae. When analyzed by fluorescence ELISA, 7 of 28 patient sera, including the patient 13 serum used in purification, were found to react to Ani s 8 (Fig. 4).

3.3. Primary structure of Ani s 8

Analysis of Ani s 8 by a protein sequencer yielded no N-terminal amino acid residue, suggesting the N-terminal blocking of the allergen. Therefore, Ani s 8 was digested with endoprotease Glu-C to obtain information about its internal amino acid sequence. Of the peptide fragments isolated from the digest by reverse-phase HPLC, two (named peptides 1 and 2 in the order of elution) were sequenced as follows: HKKLVAALPPDAQKADAE for peptide 1 and LTA-IADDASLTAAKHDKIVQTFE for peptide 2.

On the basis of the amino acid sequence of peptide 1, a degenerate reverse primer (corresponding to PDAQKAD) could be designed to amplify the 5'-end region of the Ani s 8 cDNA by

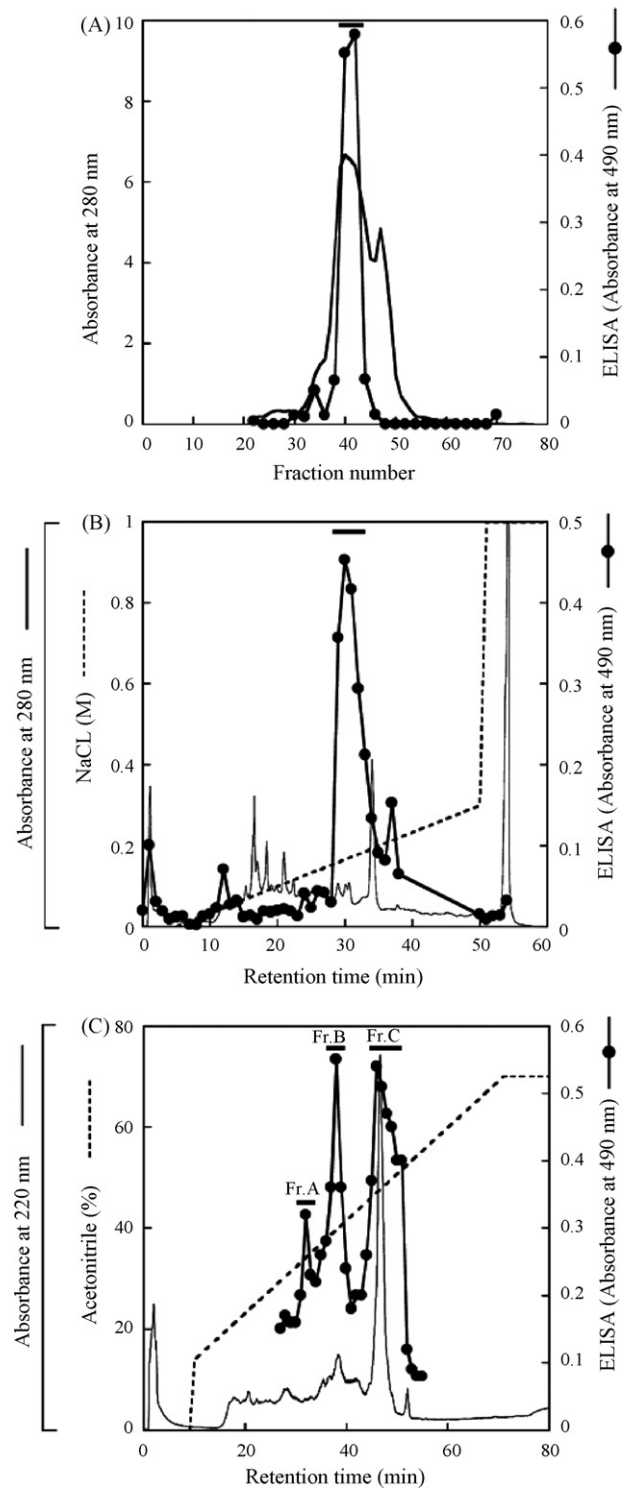


Fig. 2. Purification of Ani s 8 from *Anisakis simplex* larvae by gel filtration on Sephacryl S-300 (A), anion-exchange FPLC on Mono Q (B) and reverse-phase HPLC on TSKgel Phenyl-5PW RP (C). A bar in each panel represents the pooled fractions.

PCR. Following subcloning of the amplified products into the pT7Blue-2 T-vector, six clones were analyzed for nucleotide sequence. Although there were several alterations in the determined nucleotide sequences among the six clones, the 5'-end sequence up to the 22nd position was completely conserved. In

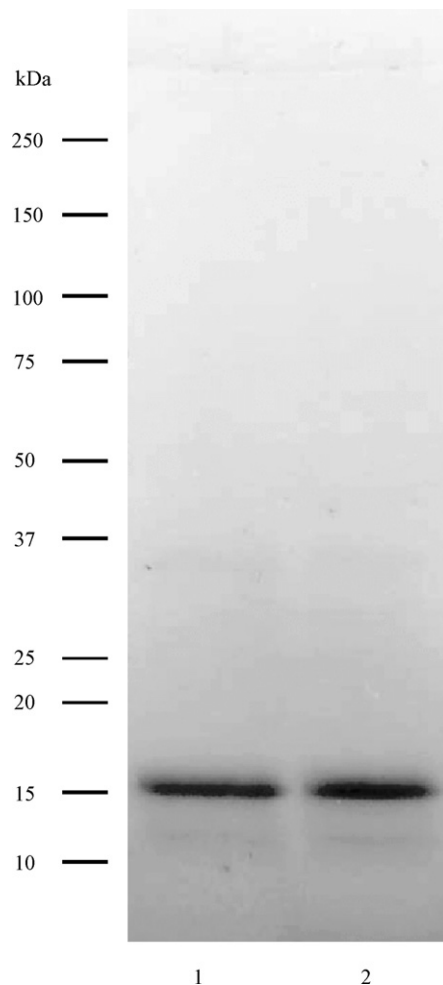


Fig. 3. SDS-PAGE of Ani s 8 under reduced (lane 1) and non-reduced (lane 2) conditions.

the next step, therefore, the Ani s 8 cDNA was amplified by PCR using the specific forward primer (corresponding to positions 1–20) and the reverse vector primer. Nucleotide sequence analysis of 37 clones revealed the presence of as many as 32 kinds of the Ani s 8 cDNA (747–973 bp; DDBJ/EMBL/GenBank accession numbers AB300625–AB300656), the open reading frames of which were commonly composed of 450 bp encoding 150 amino acids.

As shown in Fig. 5, 10 different amino acid sequences were deduced for the precursor protein of Ani s 8 from the determined nucleotide sequences, indicating the occurrence of at least 10 isoforms of the allergen. The isoform 1 was encoded by 23 kinds of cDNA, while the isoforms 2–10 having one or two alterations compared to the isoform 1 were individually encoded by 9 kinds of cDNA, suggesting that the isoform 1 is the major form of Ani s 8. Peptides 1 and 2 are recognized at positions 90–107 and 108–131 of all isoforms, respectively, except for that isoforms 7 and 9 have only one mutation at positions 111 and 108, respectively. Irrespective of the isoforms, the N-terminal segment (up to the 16th residue) was predicted to be a signal peptide by the SignalP analysis [27], implying that the mature Ani s 8 corresponds to the segment 17–150 (134 residues). The calculated molecular mass of the mature Ani s 8 is 14447–14517 Da, which is comparable to the value (15 kDa) estimated by SDS-PAGE.

A homology search by the BLAST program [28] revealed that Ani s 8 exhibits 30–36% sequence identities with some members of the SXP/RAL-2 protein family from various species of nematodes, such as the AS16 and AS14 proteins from *Ascaris suum*, the RAL-2 homolog from *Acanthocheilonema viteae*, the immunodominant hypodermal antigen from *Onchocerca volvulus* and the antigen from *Brugia malayi* (Fig. 6). The DUF148 domain (accession number PF02520), which is commonly contained in the SXP/RAL-2 protein family, is also recognized at positions 25–147 of Ani s 8, as analyzed by the Pfam program [29]. It should be noted that Ani s 5 (152 amino acid residues)

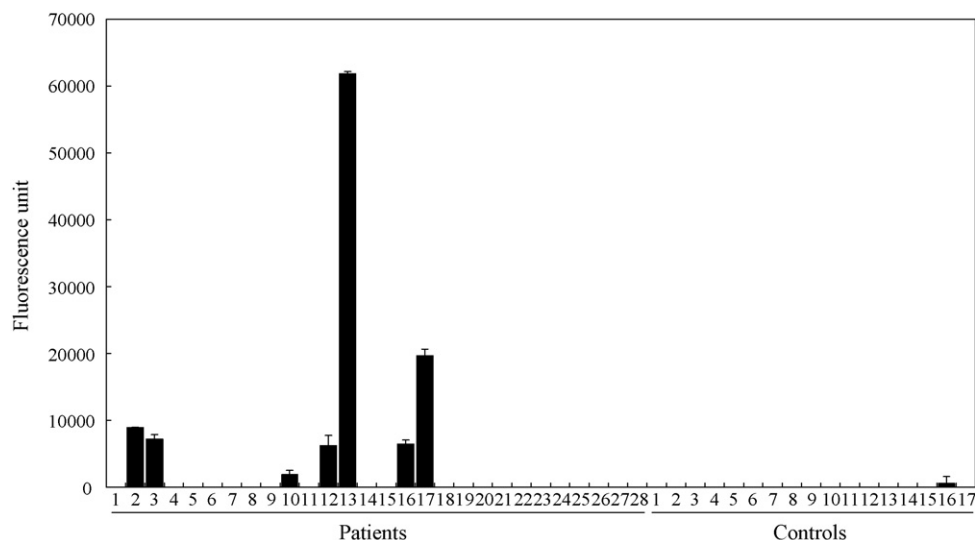


Fig. 4. Analysis of IgE reactivity of Ani s 8 by fluorescence ELISA using 28 patient and 17 control sera. Each datum is expressed in mean $\pm$ S.D. ($n = 3$). The value above the control mean + 2S.D. (equivalent to fluorescence unit 290) was judged to be positive.

Isoform 1	MKVLFFVAAVVAVSLAQDQGPPPLPKFLDGADQATKDAFAALAQTTFKDDTDKQVEDAVQQFVNDHPAIKDAYEAE	[75]
Isoform 2	.R.....	[75]
Isoform 3	D.....	[75]
Isoform 4	P.....	[75]
Isoform 5	Y.....	[75]
Isoform 6	R.....	[75]
Isoform 7	A.....	[75]
Isoform 8	N.....	[75]
Isoform 9	IIFV.I.VIAF..GPQG.	[75]
Isoform 10		[75]

	Peptide 1	Peptide 2	
Isoform 1	KKEVLAAQQAEEEEHKKLVAAALPPDAQKADAELTAIADDASLTAAKHDKIVQTFESLPPAVKEELNKNLQQGQS		[150]
Isoform 2			[150]
Isoform 3	T.....		[150]
Isoform 4			[150]
Isoform 5			[150]
Isoform 6			[150]
Isoform 7	T.....		[150]
Isoform 8			[150]
Isoform 9	S.....		[150]
Isoform 10	D.....		[150]

Fig. 5. Deduced amino acid sequences of 10 isoforms of Ani s 8. Dots represent the residues identical with the sequence of the isoform 1.

Ani s 8	MKVLFFVAAVVAVSLAQDQGPP	LPKFLDGADQATKDAFAALAQT	[46]
Ani s 5	..T.IVA.LFCTIGM.LADDT..	P.P..A..P.DVVK..FE.LKKD	[46]
AS16	..Y.IT.TCLFVLA.VEG.T.SR	V.P..V..PESAVKD.FE.IKKD	[46]
AS14	...IIFV.I.VIAF..GPQG.	.P..V..PANVVAE.KQIITGA	[44]
RAL-2	NSARAARVSC.FAQQ.Q.Q.QLQQQQQ	FPV.P..I..SPKVISE.QQ.INS	[52]
IHA	..FVILLTIGLL.VA.IP.RRQQQQQQQQQQRDEREI	P..E..PPSVI.E.YN.LK.D	[60]
BMA	EFRSVFGEE.QFMI	.P..L..PESAVREMKE.F.KY	[36]

Ani s 8	KDDTDKQVEDAVQQFVN DHPAIKDAYEAEKKEVLAAQQAEEEEHKKLVAAALPPDAQKAD	[105]
Ani s 5	ETK..PEI.KDLDAW.DTLGGDY.AKF.TF...MK.KEAELAKA.EEA..KMT.E.K...	[106]
AS16	EEK..PEI.ADIDA..AKLGADYTNKFK.F.A.LK.HEAEY.KA.AAAI.KFS.A.KE..	[106]
AS14	P.K..AEIDRDIENW.ARQG.K..TEFNKF.TQMQQGKAR..AA.RASI.KFS.A.KA..	[104]
RAL-2	RQR..REM.Q..ESWIAQQSQK.QVSFQKF.QQA.N.L.Q..AQ.QASA.K.S.E.RRV.	[112]
IHA	ENK..Q.T.AD.EA.I.RLGGSY.VRFTQFME..KK.RADY.RI.QQA..RFS.A.KD..	[120]
BMA	ANKP.S.L.A..EEW..GKGGM..EK.SQF.ANMQLMHEKTALLR.AMAEN.S.E.K...	[96]

Ani s 8	AELTAIADDASLTAAKHDKIVQTFESLPPAVKEELNKNLQQGQS	[150]
Ani s 5	...SK..E.D..NGIQ.AQ..QAIYKT..QS..D..E.GIGPAVPQ	[152]
AS16	.K....E..K.NGIQ.RQ..KE.M....KE.RD..E.AIAG.A	[150]
AS14	.Q.....NPN.KGRE.QQ..TGLLQ...A..QA.FQ.EM.G	[154]
RAL-2	.Q.SS...KSV...PMQ.QEQ.DMIISK..EN..Q..QRAI.G	[162]
IHA	.RMS...SPH..TRQ.SQQ.QAIMD..SES.RR.IINALSPQE	[164]
BMA	VD.SN.GK.KT.SEQQ.RE.FKEYLRN.S.V.RN..	[132]

Fig. 6. Amino acid sequence alignment of Ani s 8 (isoform 1) with six members of the SXP/RAL-2 protein family. DDBJ/EMBL/GenBank accession numbers: Ani s 8, AB300625–AB300647; Ani 5, AB274998; AS16 (AS16 protein from *Ascaris suum*), AB089179; AS14 (AS14 protein from *A. suum*), AB057441; RAL-2 (RAL-2 homolog from *Acanthocheilonema viteae*), AF000609; IHA (immunodominant hypodermal antigen from *Onchocerca volvulus*), U00693; BMA (antigen from *Brugia malayi*), M95550. The DUF148 domain is shaded. Dots represent the residues identical with the sequence of Ani s 8.

previously identified as an *A. simplex* allergen is also homologous to the SXP/RAL-2 protein family; the sequence identity between Ani a 5 and Ani s 8 is 35%.

3.4. IgE cross-reactivity of Ani s 8 with Ani s 5

IgE cross-reactivity of Ani s 8 with Ani s 5 was evaluated by inhibition ELISA using five patient sera reacting to Ani s 8. As shown in Fig. 7, the reactivity of four patient sera to Ani s 8 was inhibited in a dose-dependent manner by Ani s 8 and more strongly by Ani s 5, demonstrating the cross-reactivity between both allergens. The only exception was observed with patient 13 serum; the reactivity of this serum to Ani s 8 was inhibited by Ani s 8 but not by Ani s 5.

4. Discussion

Most *Anisakis*-allergic patients recognize heat-stable allergens as evidenced by ELISA using the non-heated and heated extracts from the third stage larvae of *A. simplex* (Fig. 1). This suggests that heat-stable allergens seem to be implicated in *A. simplex* allergy, although future study is needed to assess their clinical relevance in detail. Of the eight known *A. simplex* allergens, Ani s 1 and Ani s 4 have been shown to be heat-stable. In this study, the third heat-stable allergen of 15 kDa (Ani s 8) was purified from the third stage larvae of *A. simplex* and confirmed to be specific to 25% (7 of 28 patients) of *A. simplex*-allergic patients (Fig. 4). The primary structure determined by cDNA cloning revealed that Ani s 8 is a member of the SXP/RAL-

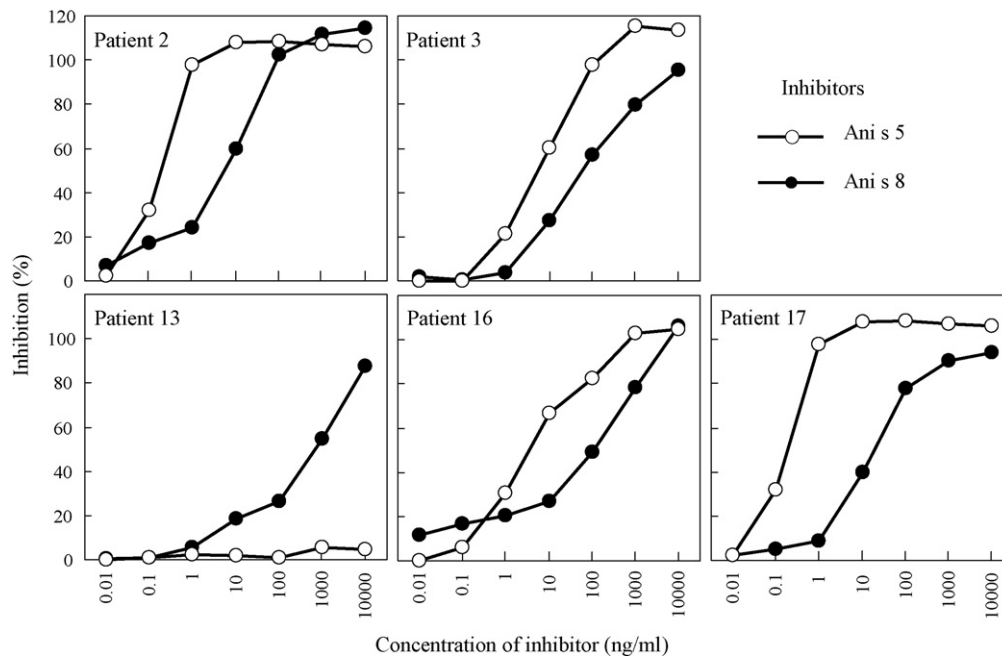


Fig. 7. Inhibitory activity of Ani s 5 and Ani s 8 against the reactivity of five patient sera to Ani s 8 determined by inhibition ELISA. Patient serum (diluted 1:100) was incubated with an equal volume of Ani s 5 or Ani s 8 solution (0.02–20000 ng/ml) at 37 °C for 2 h and a 50 µl portion of this solution was added to the plate which had previously been coated with Ani s 8 (50 ng/well).

2 protein family from various species of nematodes, similar to Ani s 5 (15 kDa) previously identified as an *A. simplex* allergen [14]. Inhibition ELISA data established the cross-reactivity between Ani s 8 and Ani s 5 (Fig. 7). Caballero and Moneo [30] described the occurrence of a heat and pepsin-resistant 14 kDa allergen detected by 25% of *Anisakis*-allergic patients. In view of the comparable molecular mass and seroprevalence, the heat and pepsin-resistant 14 kDa allergen might be identical with Ani s 5 and/or Ani s 8.

Interestingly, at least 32 homologs of the Ani s 8 cDNA, 23 encoding the isoform 1 and 9 individually encoding the isoforms 2–10, were found in cloning experiments. These cDNA homologs might be paralogs resulted from gene multiplication or mRNA splicing. Moreover, it should be noted that the cDNA phage library used in this study was constructed from about two hundreds of *A. simplex* larvae. Therefore, the observed variations of the Ani s 8 cDNA is possibly attributable to the polymorphism derived from individual larvae.

Some proteins of the SXP/RAL-2 family, such as the AS16 protein from *A. suum* (a gastrointestinal parasite distributing in both human and swine) [31] and the SXP/RAL-2 protein from *Meloidogyne incognita* (a major parasite of plant) [32], have been demonstrated or suggested to be secreted to the outside of the parasite. As discussed previously for Ani s 5 [14], it is likely that Ani s 8 is also secreted into the human gastrointestinal tract from the third stage larvae of *A. simplex* infecting fresh or undercooked seafood and that the secreted Ani s 8 stimulates human immune systems leading to the production of IgE antibodies against Ani s 8.

Our previous study showed that seven sera (from patients 2, 3, 10, 13, 16, 17 and 24) reacted to Ani s 5 [14], while seven sera (from patients 2, 3, 10, 12, 13, 16 and 17) were found to react

to Ani s 8 in this study (Fig. 4). Apparently, patients 12 and 24 specifically recognize Ani s 8 and Ani s 5, respectively. When five of the six sera reacting to both Ani s 5 and Ani s 8 were used in inhibition ELISA experiments, the cross-reactivity between both allergens was observed with four sera (from patients 2, 3, 16 and 17) (Fig. 7). In the case of the patient 13 serum, however, no cross-reactivity between both allergens was recognized, suggesting that the patient 13 serum has different IgE recognizing the peculiar regions of Ani s 5 and Ani s 8. Although Ani s 5 is recognized by patient 13, its purification was not achieved in this study. This may be due to the low concentration of Ani s 5 in *A. simplex* or to the weaker reactivity of the patient 13 serum to Ani s 5 than to Ani s 8.

In conclusion, a new heat-stable 15 kDa allergen (Ani s 8) was purified from *A. simplex* and identified as a member of the SXP/RAL-2 protein family. Since *A. simplex* allergens greatly vary from patient to patient [17], even minor allergens such as Ani s 8 should not be underestimated in *A. simplex* allergy. Further characterization of unidentified allergens (probably minor allergens) is needed for a better understanding of *A. simplex* allergy and also for the future accurate diagnosis and therapy of *A. simplex* allergy.

Acknowledgments

We are grateful to Dr. T. Shirai, Department of Internal Medicine, Fujinomiya City Central Hospital, and Dr. S. Kasuya, Faculty of Regional Studies, Gifu University, for providing sera from *A. simplex*-allergic patients. 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 and a grant from the Ministry of Health, Labour and Welfare of Japan.

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