



## Identification of novel three allergens from *Anisakis simplex* by chemiluminescent immunoscreening of an expression cDNA library

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### ARTICLE INFO

#### Article history:

Received 12 September 2010

Received in revised form 9 January 2011

Accepted 17 January 2011

Available online 22 January 2011

#### Keywords:

Allergen

Ani s 11

Ani s 12

*Anisakis simplex*

Immunoscreening

### ABSTRACT

*Anisakis simplex* is a representative nematode parasitizing marine organisms, such as fish and squids, and causes not only anisakiasis but also IgE-mediated allergy. Although 10 kinds of proteins have so far been identified as *A. simplex* allergens, many unknown allergens are considered to still exist. In this study, a chemiluminescent immunoscreening method with higher sensitivity than the conventional method was developed and used to isolate IgE-positive clones from an expression cDNA library of *A. simplex*. As a result, three kinds of proteins, Ani s 11 (307 amino acid residues), Ani s 11-like protein (160 residues) and Ani s 12 (295 residues), together with three known allergens (Ani s 5, 6 and 9), were found to be IgE reactive. Furthermore, ELISA data showed that both recombinant Ani s 11 and 12 expressed in *Escherichia coli* are recognized by about half of *Anisakis*-allergic patients. Ani s 11 and Ani s 11-like protein are characterized by having six and five types of short repetitive sequences (5–16 amino acid residues), respectively. Both proteins share as high as 78% sequence identity with each other and also about 45% identity with Ani s 10, which includes two types of short repetitive sequences. On the other hand, Ani s 12 is also structurally unique in that it has five tandem repeats of a CX<sub>13–25</sub>CX<sub>9</sub>CX<sub>7,8</sub>CX<sub>6</sub> sequence, similar to Ani s 7 having 19 repeats of a CX<sub>17–25</sub>CX<sub>9–22</sub>CX<sub>8</sub>CX<sub>6</sub> sequence. The repetitive structures are assumed to be involved in the IgE-binding of the three new allergens.

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### 1. Introduction

*Anisakis simplex* is a representative nematode parasitizing marine animals such as fish and squids. When the third stage larvae of *A. simplex* are ingested orally via infected fish, they occasionally penetrate to the human gastrointestinal mucosa, causing a disease called anisakiasis; manifestations of anisakiasis are nausea, diarrhea, abdominal pain, vomiting and so on [1]. In addition, some individuals previously sensitized by *A. simplex* have elevated levels of immunoglobulin E (IgE) to this nematode and develop IgE-mediated gastroallergic anisakiasis ranging from urticaria to life-threatening anaphylactic shock [2–6].

**Abbreviations:** BSA, bovine serum albumin; CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; IgE, immunoglobulin E; IPTG, isopropyl-1-thio-β-galactoside; GST, glutathione-S-transferase; PBST, Dulbecco's phosphate buffered saline containing 0.05% Tween 20; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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To date, the following 10 kinds of proteins have been identified as *A. simplex* allergens: Ani s 1 (secretory gland protein) [7,8], Ani s 2 (paramyosin) [9], Ani s 3 (tropomyosin) [10], Ani s 4 (cysteine protease inhibitor) [11,12], Ani s 5 (SXP/RAL-2 family protein) [13], Ani s 6 (serine protease inhibitor) [13], Ani s 7 (novel protein with repetitive sequences) [14], Ani s 8 (SXP/RAL-2 family protein) [15], Ani s 9 (SXP/RAL-2 family protein) [16] and a troponin C-like protein [17]. Recently, Ani s 10 was added as a new allergen to the DDBJ/EMBL/GenBank databases (accession number GU187358), although its properties have not been clarified. Moreover, Arlian et al. [18] reported that at least 18 different allergens exist in *A. simplex*, suggesting the occurrence of a considerable number of unidentified allergens.

Immunoscreening of an expression cDNA library using patient sera is an effective technique to identify allergens. Indeed, four *A. simplex* allergens (Ani s 2 [9], Ani s 5 [13], Ani s 6 [13] and troponin C-like protein [17]) have been found by this technique. Since immunoscreening provides cDNAs encoding allergens, it has two additional advantages. First, primary structures of allergens are easily deduced by sequencing of the cDNAs without purification of allergens. Second, acquisition of the allergen cDNAs makes it possible to produce not only recombinant allergens for diagnosis but also even recombinant hypoallergenic proteins for future allergen-specific immunotherapy.

However, the conventional immunoscreening method, which is based on the detection of antigen–antibody complexes by an enzymatic color reaction, requires a great deal of patient sera due to low sensitivity and often results in a high ratio of false positive clones. In this study, therefore, a chemiluminescent immunoscreening method with higher sensitivity than the conventional one was newly developed. Application of the new method to the expression cDNA library of *A. simplex* suggested three kinds of proteins to be new allergens of the nematode. In addition, two of the three proteins were expressed in *Escherichia coli* and their recombinants were confirmed to be IgE reactive by enzyme-linked immunosorbent assay (ELISA) using *Anisakis*-allergic patient sera. In this paper, these two allergens are called Ani s 11 and Ani s 12 according to the nomenclature of the Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies [19]. The names have already been assigned by the Sub-Committee. We report here the identification of new allergens in *A. simplex* by the developed chemiluminescent immunoscreening method.

## 2. Materials and methods

### 2.1. Human sera

Sera were obtained from 30 patients with clinical symptoms, such as urticaria, angioedema and anaphylaxis, after ingestion of raw or undercooked fish. These patients were all diagnosed to be allergic not to fish but to *A. simplex* at hospitals, based on the determined capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) classes (2–6 against *A. simplex* and 0 against fish). In this study, sera from 16 healthy volunteers were used as controls. This study using human sera was approved by the Ethics Board of the Fujinomiya City Central Hospital and the Ethics Committee of Gifu University.

### 2.2. Immunoscreening

The phage ( $\lambda$ ZipLox) expression cDNA library of *A. simplex* used in this study was the same as previously constructed from the third stage larvae that were collected from the walleye pollack (*Theragra chalcogramma*) captured at Raus, Hokkaido [8]. Immunoscreening of the cDNA library was performed using the patient 18 serum because of the sufficient volume available. Moreover, our preliminary immunoblotting experiments showed that the patient 18 serum gives different IgE responses to the *A. simplex* crude extract from the patient 13 serum used in our previous immunoscreening [13], suggesting that the use of the patient 18 serum in immunoscreening can lead to the finding of new allergens.

We employed two kinds of immunoscreening methods as shown in Fig. 1. One method (called conventional immunoscreening method) was the same as described in our previous paper [13]. In brief, *E. coli* Y1090 (ZL) infected with the phage cDNA library was plated on an LB agar plate and incubated. Plaques formed by bacteriophages were transferred onto a nitrocellulose membrane previously soaked in 10 mM isopropyl-1-thio- $\beta$ -galactoside (IPTG). After blocking with 3% bovine serum albumin (BSA) in Dulbecco's phosphate buffered saline containing 0.05% Tween 20 (PBST), the membrane was reacted successively with the patient serum (diluted 1:20) and peroxidase-conjugated goat anti-human IgE antibody (1  $\mu$ g/mL; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Enzyme reaction was performed using substrate solution containing 0.03% 4-chloro-1-naphthol, 10 mM imidazole and 0.017% H<sub>2</sub>O<sub>2</sub>. Clones corresponding to the positive signals were individually picked up and subjected to subcloning.

The other method (called chemiluminescent immunoscreening method) was developed in this study. Culturing of *E. coli* infected with the cDNA library on LB agar, transferring of plaques onto a nitrocellulose membrane and washing of the membrane with PBST were performed in

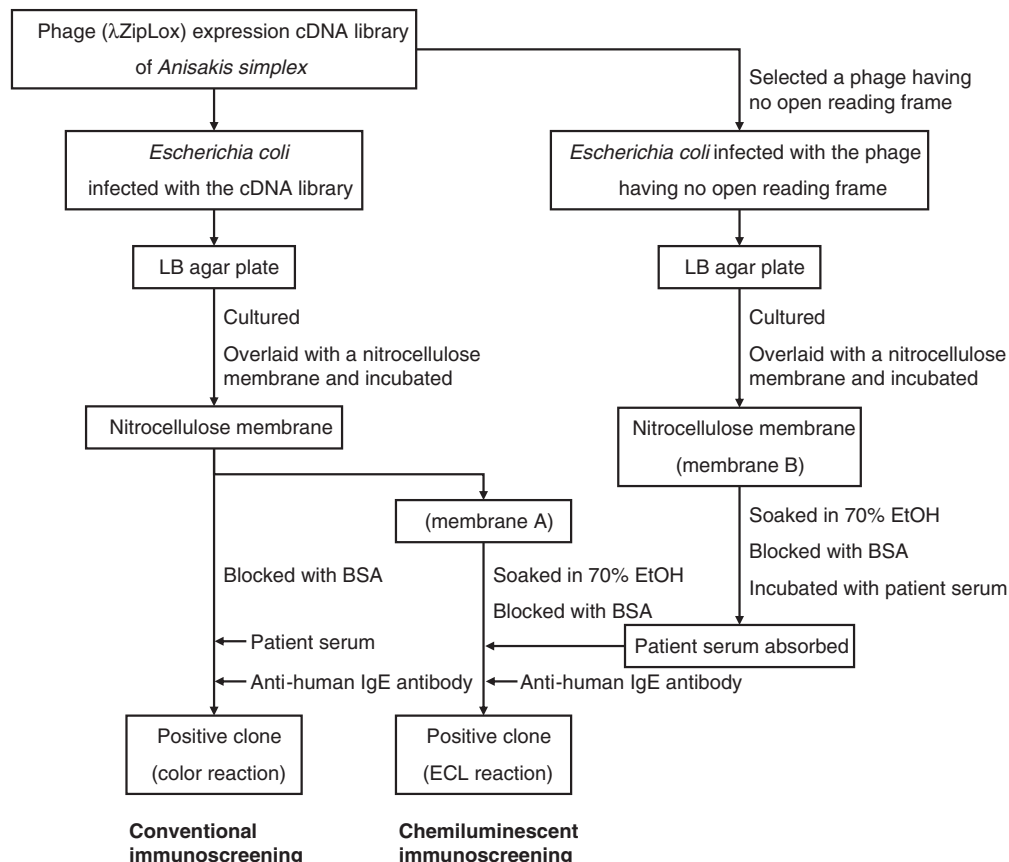


Fig. 1. Schematic diagram of the immunoscreening methods applied to the expression cDNA library of *Anisakis simplex*.

the same way as adopted for the conventional immunoscreening method. The membrane (called membrane A) was then soaked in 70% ethanol for 10 s to sterilize *E. coli* and inactivate bacterial peroxidase and washed with PBST. After being blocked with 3% BSA in PBST at 4 °C overnight, membrane A was washed again with PBST. Aside from membrane A, another membrane (called membrane B) was similarly prepared using an LB agar plate including *E. coli* infected with the phage that had only a part of the 3'-untranslated region (no open reading frame) and hence was isolated as a false-positive clone in conventional immunoscreening. Membrane B was immersed in the patient serum (diluted 1:200) at 37 °C for 4 h; during this procedure, nonspecific antibodies in the serum were expected to be absorbed by the membrane. Then membrane A was reacted with the retrieved serum at 37 °C for 4 h, washed with PBST and reacted with peroxidase-conjugated goat anti-human IgE antibody (0.2 µg/mL) at 37 °C for 1 h. After washing with PBST, antigen-antibody binding was visualized using an ECL Plus Western Blotting System (GE-Healthcare Biosciences, Piscataway, NJ, USA). Positive clones were individually picked up and subjected to subcloning.

### 2.3. Subcloning and DNA sequencing

*E. coli* DH10B was infected with each positive λZipLox clone and plated on LB agar containing 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 0.01% ampicillin and 2 mM IPTG. After being incubated at 37 °C overnight, each white colony was picked up and cultured in LB medium containing 0.005% ampicillin at 37 °C overnight. The plasmid DNA was extracted from the bacterial suspension using a Quantum Prep Plasmid Mini Prep Kit (Bio-Rad Laboratories, Hercules, CA, USA) 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).

### 2.4. Expression and purification of recombinant Ani s 11 (rAni s 11)

Ani s 11 was expressed in *E. coli* as a glutathione-S-transferase (GST)-fusion protein using a pGEX-6P-3 vector (GE-Healthcare Biosciences). A cDNA encoding mature Ani s 11 (refer to Fig. 2) was amplified by polymerase chain reaction (PCR) using the isolated clone as a template. The following primers were used: a forward primer

with *Bam*H I restriction site (underlined) 5'-TATGGATCCAATCCTCCTCGTTCC-3' and a reverse primer with *Not* I restriction site (underlined) 5'-ATGCGGCGCGCTTACAACGAATTCTCCTCTTTC-3'. Amplification was performed using HotMaster Taq DNA Polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min and 35 cycles of 94 °C for 20 s, 55 °C for 10 s and 70 °C for 50 s. The PCR product and the pGEX-6P-3 vector were digested with *Bam*H I and *Not* I and ligated with each other using a DNA Ligation Kit (Takara, Otsu, Japan). *E. coli* JM109 was transformed with the ligated product and cultured on an LB agar plate containing 0.005% ampicillin at 37 °C overnight. A single colony was picked up and cultured in 500 mL of LB medium containing 0.005% ampicillin at 37 °C until the absorbance at 600 nm reached 0.7. The medium was then added with IPTG at a final concentration of 1 mM and further incubated with shaking for 3 h. Bacteria were harvested by centrifugation, suspended in 25 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride and sonicated well. After centrifugation, the GST-fusion Ani s 11 in the supernatant was isolated by affinity chromatography using a Glutathione Sepharose 4B column (GE-Healthcare Biosciences) according to the manufacturer's manual. The isolated protein was digested with 100 units of PreScission Protease (GE-Healthcare Biosciences) at 4 °C for 18 h. Following application of the digest to the same affinity column, GST-free rAni s 11 was obtained in the flow-through fraction. The Ani s 11 fraction was then subjected to reverse-phase high performance liquid chromatography (HPLC) on a TSKgel Phenyl-5PW RP column (0.46 × 7.5 cm; Tosoh, Tokyo, Japan), which was eluted by a linear gradient of acetonitrile (0–42% in 30 min) in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. Proteins were monitored at 220 nm with a UV detector. The eluate corresponding to rAni s 11 was manually collected and lyophilized.

### 2.5. Expression and purification of recombinant Ani s 12 (rAni s 12)

rAni s 12 was expressed in *E. coli* as a histidine (His)-tagged protein using a pQE-30 Xa vector (Qiagen, Hilden, Germany). A cDNA corresponding to mature Ani s 12 (refer to Fig. 3) was amplified by PCR using the isolated clone as a template and the following primers: a forward primer with *Bam*H I restriction site (underlined) 5'-TGGATCCGAACGAGAGGAATTGCG-3' and a reverse primer with

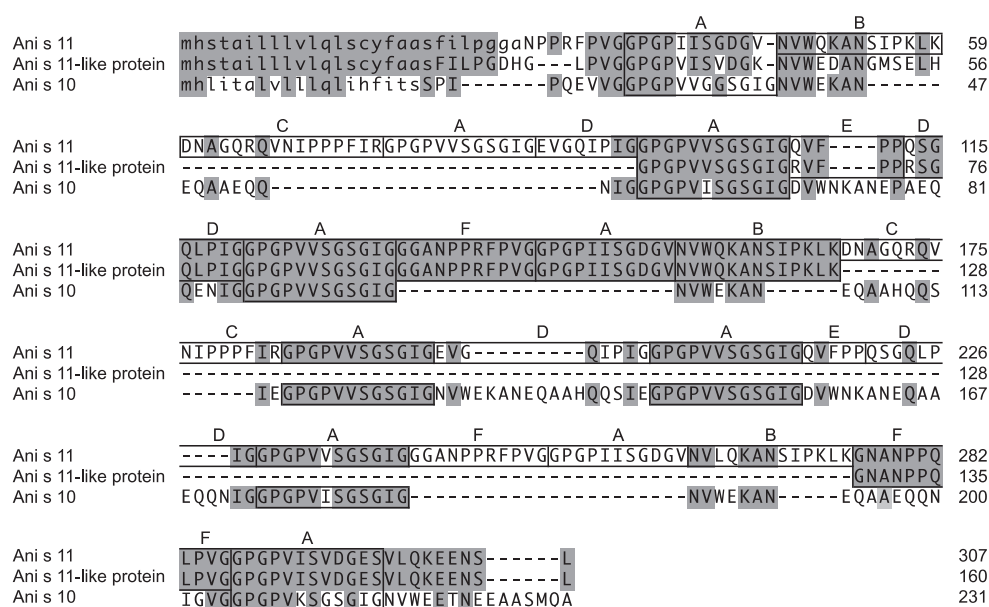


**Fig. 2.** Amino acid sequences of Ani s 11, Ani s 11-like protein and Ani s 10. Putative signal peptides are shown in lowercase letters. Dashes represent gaps. Amino acid residues identical to those of Ani s 11 are shaded. Amino acid numbers are shown at the right. Repetitive sequences are boxed and their types (A–F) are denoted above the sequence of Ani s 11. DDBJ/EMBL/GenBank accession numbers: Ani s 11, AB555754; Ani s 11-like protein, AB555755; and Ani s 10, GU187358.

*Sal* I restriction site (underlined) 5'-TATGTCGACTCAGAGGCCTTTCGC-3'. The DNA polymerase used and the conditions of PCR amplification were the same as in the case of Ani s 11. The PCR product and the expression vector were digested with *Bam*HI and *Sal* I and ligated with each other as described above. Subsequent expression procedures, including transformation of *E. coli* JM109 with the ligated product, culturing of the transformants on an LB agar plate, growing of the isolated transformant in LB medium, induction of protein expression by IPTG and harvesting of bacteria, were the same as adopted for Ani s 11 (refer to Section 2.4). The bacterial pellet thus obtained was suspended in 25 mL of 20 mM phosphate buffer (pH 7.4) containing 500 mM NaCl and 1 mM phenylmethylsulfonyl fluoride, digested with 0.1 mg/mL lysozyme at 4 °C for 1 h and sonicated. After centrifugation, the precipitate was dissolved by sonication in 25 mL of 20 mM phosphate buffer (pH 7.4) containing 500 mM NaCl, 10 mM imidazole, 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol. The His-tagged Ani s 12 was purified in the presence of 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol by affinity chromatography using a HisTrap Chelating HP column (GE-Healthcare Biosciences) as recommended by the manufacturer. Then, the His-tagged Ani s 12 fraction was dialyzed five times against 50 volumes of 400 mM L-arginine-HCl buffer (pH 8.0) for refolding and centrifuged at 20,630 g. Refolded His-tagged Ani s 12 in the supernatant was finally purified by reverse-phase HPLC on a TSKgel Phenyl-5PW RP column as in the case of Ani s 11.

## 2.6. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

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

## 2.7. Protein determination

Protein concentrations of the recombinant allergens were estimated by the method of Gill and von Hippel [20], using an extinction coefficient ( $E_{M}^{280}$ ) of 11,380 M<sup>-1</sup> cm<sup>-1</sup> for rAni s 11 and 35,640 M<sup>-1</sup> cm<sup>-1</sup> for rAni s 12, calculated from the number of tyrosine, tryptophan and cysteine residues.

## 2.8. Fluorescence ELISA

Fluorescence ELISA was performed as reported previously [21]. Each recombinant allergen was coated on a 96-well Type H (black) microtiter plate (Sumitomo Bakelite, Tokyo, Japan) and reacted successively with patient serum (diluted 1:200) and  $\beta$ -galactosidase-conjugated goat anti-human IgE antibody (0.25  $\mu$ g/mL; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out using 4-methylumbelliferyl- $\beta$ -D-galactoside as a substrate and fluorescence units were determined with excitation and emission wavelengths at 367 and 453 nm, respectively.

IgE reactivity of the His-tag portion (28 amino acid residues) corresponding to the N-terminus of rAni s 12 was also evaluated by fluorescence ELISA using a Nunc Immobilizer Amino plate for peptide (Nalge Nunc International, Rochester, NY, USA). The His-tag peptide (MRGSHHHHHHSGSGSGIEGRPYNGTGS) was synthesized with a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan) by the Fmoc/PyBOP strategy according to the manufacturer's instructions and purified by reverse-phase HPLC as described elsewhere [22].

## 3. Results

### 3.1. Positive clones obtained by immunoscreening

The results obtained by immunoscreening of the expression cDNA library using the patient 18 serum are summarized in Table 1. When approximately  $9.7 \times 10^5$  phages were subjected to the conventional immunoscreening method, 11 IgE-positive clones were isolated. DNA sequencing revealed that 1 clone codes for Ani s 5 and 2 clones separately for two kinds of proteins (Ani s 11 and Ani s 11-like protein) differing from the known *A. simplex* allergens, while the remaining 8 clones (73%) are false positive because of having no open reading frame. On the other hand, chemiluminescent immunoscreening of approximately  $5.0 \times 10^4$  phages yielded as many as 38 IgE-reactive clones, of which only 3 clones (8%) were judged to be false positive. Of the 35 clones except for the false positive clones, 23, 2 and 5 clones coded for Ani s 5 (including some Ani s 5 variants; data not shown), Ani s 6 and Ani s 9, respectively, and the remaining 5 clones for three kinds of proteins (Ani s 11, Ani s 11-like protein and Ani s 12) distinguishable from the known *A. simplex* allergens. Taken together, our immunoscreening data nominated three kinds of proteins (Ani s 11, Ani s 11-like protein and Ani s 12) as candidates of new *A. simplex* allergens.

### 3.2. Amino acid sequences of Ani s 11, Ani s 11-like protein and Ani s 12

Nucleotide sequence data of the Ani s 11 cDNA (1437 bp), Ani s 11-like protein cDNA (985 bp) and Ani s 12 (1129 bp) have been deposited in the DDBJ/EMBL/GenBank databases (refer to Figs. 2 and 3 for accession numbers). Amino acid sequences of Ani s 11 (307 residues), Ani s 11-like protein (160 residues) and Ani s 12 (295 residues) were deduced from the nucleotide sequences of the open reading frames (921 bp for Ani s 11, 480 bp for Ani s 11-like protein and 885 bp for Ani s 12).

The deduced amino acid sequences of Ani s 11 and Ani s 11-like protein are shown in Fig. 2. A high sequence identity (78%) is observed between both proteins, although the chain length is remarkably different from each other. The SignalP analysis [23] indicated that the segments 28–307 and 21–160 are mature portions of Ani s 11 and Ani s 11-like protein, respectively. Interestingly, short sequences comprising 6–15 amino acid residues, which are classified into six types (types A–F) based on the amino acid sequence similarity, are repeatedly recognized in the orders of ABCADAEDAFABCADAEDAFABFA for mature Ani s 11 and AAEDAFABFA for mature Ani s 11-like protein. A search by the BLAST algorithm [24] showed that Ani s 10 (231 residues) is homologous to Ani s 11 and Ani s 11-like protein; it shares 49 and 43%

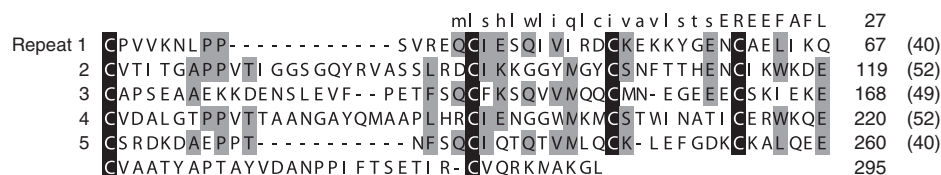


**Fig. 3.** Amino acid sequence of Ani s 12 with alignment of five repeats (repeats 1–5) of the CX<sub>13–25</sub>CX<sub>9</sub>CX<sub>7,8</sub>CX<sub>6</sub> motif. A putative signal peptide is shown in lowercase letters. Dashes represent gaps. Consensus amino acid residues in the five repeats are shaded. Amino acid numbers are shown at the right. The number in each parenthesis after the amino acid number indicates the length of each repeat. DDBJ/EMBL/GenBank accession number for Ani s 12: AB555757.

**Table 1**

IgE-positive clones obtained by immunoscreening of an *Anisakis simplex* cDNA library using the patient 18 serum.

| Type of IgE-positive clones |                       | No. of IgE-positive clones |                                  |
|-----------------------------|-----------------------|----------------------------|----------------------------------|
|                             |                       | Normal immunoscreening     | Chemiluminescent immunoscreening |
| Known allergen              | Ani s 5               | 1                          | 23                               |
|                             | Ani s 6               | 0                          | 2                                |
|                             | Ani s 9               | 0                          | 5                                |
| New allergen                | Ani s 11              | 1                          | 2                                |
|                             | Ani s 11-like protein | 1                          | 2                                |
|                             | Ani s 12              | 0                          | 1                                |
| False positive clone        |                       | 8                          | 3                                |
| Total                       |                       | 11                         | 38                               |

Number of phages subjected to immunoscreening: approximately  $9.7 \times 10^5$  for normal immunoscreening and approximately  $5 \times 10^4$  for chemiluminescent immunoscreening.

sequence identities with Ani s 11 and Ani s 11-like protein. In the case of Ani s 10, seven repeats of the type A sequence are observed (Fig. 2). Furthermore, three repeats (positions 41–57, 99–115 and 186–202) of another type of sequence composed of 17 residues, the N-terminal region (up to the 7th residues) of which is highly homologous to that (NVWQKAN or NVLQKAN) of the type B sequence, alternate with the type A sequence in Ani s 10.

The deduced amino acid sequence of Ani s 12 is shown in Fig. 3. The mature Ani s 12 was predicted to correspond to the segment 20–295 (276 residues) by the SignalP analysis. Ani s 12 is structurally characterized by having five tandem repeats of a novel CX<sub>13–25</sub>CX<sub>9</sub>CX<sub>7,8</sub>CX<sub>6</sub> motif composed of 40–52 amino acid residues. Although all repeats commonly have four Cys residues at regular intervals, no appreciable sequence similarities are observed among the repeats. At least 19 tandem repeats of a similar motif (CX<sub>17–25</sub>CX<sub>9–22</sub>CX<sub>8</sub>CX<sub>6</sub>) have already been reported for Ani s 7 (composed of at least 1096 amino acid residues) [14]. There is no prominent sequence homology between the motifs of Ani s 7 and 12.

### 3.3. Expression and purification of rAni s 11 and rAni s 12

Ani s 11 was successfully expressed in *E. coli* as a GST-fusion protein. As analyzed by SDS-PAGE, GST-fusion Ani s 11 (approximately 55 kDa) was observed as a soluble protein in the lysate of IPTG-induced *E. coli* (lane 1 in Fig. 4A). Purification of GST-fusion Ani s 11 was achieved by affinity chromatography on a Glutathione Sepharose 4B column (lane 3 in Fig. 4A). After treatment with PreScission Protease, GST-free rAni s 11

was passed through the same affinity column and purified by reverse-phase HPLC on a TSKgel Phenyl-5PW RP column. On SDS-PAGE, the purified rAni s 11 preparation migrated as a single band with approximately 30 kDa (lane 4 in Fig. 4A). In a typical run, the yield of rAni s 11 was 9.5 mg/L of the bacterial culture.

His-tagged rAni s 12 expressed in *E. coli* was detected as an inclusion body in the IPTG-induced bacteria (lane 2 in Fig. 4B). After affinity chromatography on a HisTrap chelating HP column, followed by protein refolding and reverse-phase HPLC, His-tagged rAni s 12 was obtained in a pure state (lane 3 in Fig. 4B). Typically, 10.0 mg of His-tagged rAni s 12 was obtained from 1 L of the bacterial culture.

### 3.4. IgE reactivity of rAni s 11 and rAni s 12

Both rAni s 11 and rAni s 12 were evaluated for IgE reactivity by fluorescence ELISA using 30 patient sera. As shown in Fig. 5, 14 sera (from patients 1, 4, 6–10, 13, 16, 18, 19, 25, 29 and 30) reacted to rAni s 11 and 17 sera (from patients 1, 2, 4, 8–11, 13–19, 25, 29 and 30) to rAni s 12, with remarkably varied potencies. Obviously, the patient 18 serum, which was used in the immunoscreening assay, exhibited high reactivities to both rAni s 11 and rAni s 12. In this study, Ani s 11 was expressed as a GST-fusion protein but the final recombinant preparation was free of GST. On the other hand, rAni s 12 was finally obtained as a His-tagged protein. However, none of the patient sera recognizing rAni s 12 reacted to the synthesized His-tag peptide (data not shown), implying that the His-tag portion is not involved in the IgE binding of rAni s 12.

## 4. Discussion

Immunoscreening of an expression cDNA library with patient sera has been used to identify Ani s 2 [9], Ani s 5 [13], Ani s 6 [13] and troponin C-like protein [17] as *A. simplex* allergens. However, the previous immunoscreening method (corresponding to the conventional immunoscreening method in this study), in which positive clones transferred from the LB agar plate to the membrane have been detected by an enzymatic color reaction, has two drawbacks, low sensitivity of detection and high frequency of false positive results. In this study, the low sensitivity of detection was greatly improved by replacement of the color reaction to the chemiluminescent reaction. Based on the dilutions (1:20 in the conventional immunoscreening method and 1:200 in the chemiluminescent immunoscreening method) of the patient 18 serum needed for immunoscreening, the chemiluminescent method was judged to be 10 times higher in sensitivity than the conventional method. On the other hand, another drawback, high frequency of false positive results, was improved by ethanol treatment of the transferred membrane for sterilization of *E. coli* and inactivation of endogenous peroxidase and also by pretreatment of the patient serum with a membrane for removal of non-specific antibodies. As a result of these treatments, it became much easier to distinguish between positive and negative clones in the chemiluminescent immunoscreening method, remarkably decreasing false positive results. Indeed, as many as 8 (73%) of the 11 IgE-positive clones isolated from the cDNA library were false positive in the conventional immunoscreening method, while false positive clones were only 3 (8%) of the 38 IgE-positive clones isolated by the chemiluminescent immunoscreening method. We assume that the established chemiluminescent immunoscreening method is useful for identification of not only *A. simplex* allergens but also those from various animals and plants.

In this study, the developed method was successfully applied to immunoscreening of the expression cDNA library with the patient 18 serum; besides the three known allergens (Ani s 5, 6 and 9), three proteins (Ani s 11, Ani s 11-like protein and Ani s 12) were suggested to be new allergens of *A. simplex*. Ani s 11 and Ani s 11-like protein were also detected by the conventional immunoscreening method but

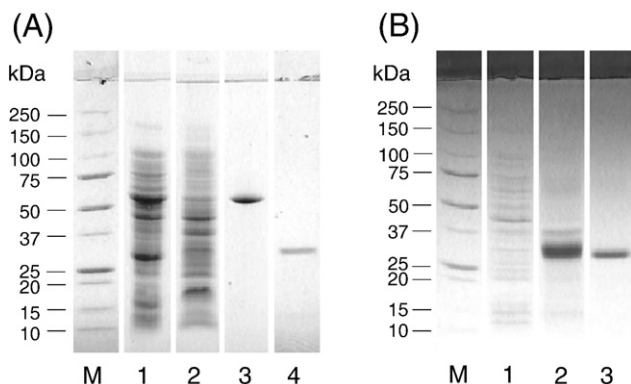


**Fig. 4.** SDS-PAGE of recombinant Ani s 11 (A) and 12 (B). (A) Lanes: M, molecular weight marker proteins; 1, soluble fraction from IPTG-induced *E. coli*; 2, insoluble fraction from IPTG-induced *E. coli*; 3, purified GST-fusion Ani s 11; and 4, purified GST-free Ani s 11. (B) Lanes: M, molecular weight marker proteins; 1, soluble fraction from IPTG-induced *E. coli*; 2, insoluble fraction from IPTG-induced *E. coli*; and 3, purified His-tagged Ani s 12.

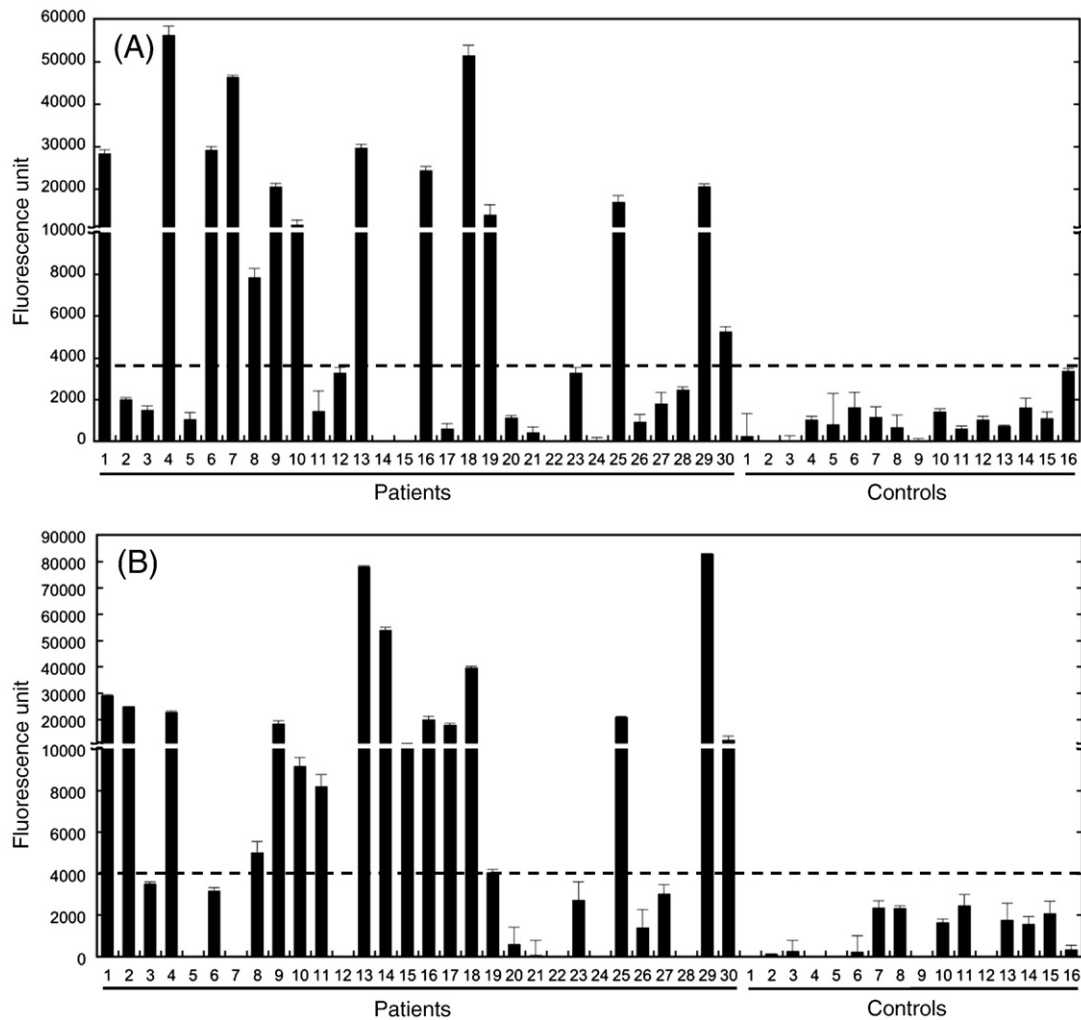


**Fig. 5.** Analysis of the IgE reactivity of rAni s 11 (A) and rAni s 12 (B) by fluorescence ELISA using 30 patient sera. Data are expressed as mean  $\pm$  SD ( $n=3$ ). Control data obtained with 16 healthy subjects were averaged and the mean  $\pm$  3SD (equivalent to fluorescence units 3511 for Ani s 11 and 4028 for Ani s 12) is represented by the horizontal dotted line in each figure. The values above this line were judged to be positive.

Ani s 12 was not. Both Ani s 11 and Ani s 12 were expressed in *E. coli* and their recombinants were confirmed to be IgE reactive by fluorescence ELISA using 30 patient sera. This allowed us to conclude that both Ani s 11 and Ani s 12 are certainly new *A. simplex* allergens. It is relevant to consider that Ani s 11-like protein is also a new allergen since its primary structure is closely similar to Ani s 11.

Of the 10 *A. simplex* allergens so far identified, only three allergens (Ani s 1, 2 and 7) are known as major allergens recognized by more than 50% of *Anisakis*-allergic patients. However, *A. simplex* allergens greatly vary from patient to patient and hence even minor allergens should not be underestimated in *A. simplex* allergy. In view of these circumstances, it is interesting to note that Ani s 11 and 12 may be major allergens since they are recognized by about half of *Anisakis*-allergic patients. Therefore, the recombinant preparations of Ani s 11 and 12 could be useful tools in future diagnosis of *A. simplex* allergy.

Ani s 11 and Ani s 11-like protein are structurally unique in that they have five or six types of short repetitive sequences comprising 6–15 amino acids. In relation to this, it is worth mentioning that various parasites have antigenic proteins with short repetitive sequences (e.g., those from the malaria parasite *Plasmodium falciparum* [25–27]), which usually induce a strong humoral immune response in the infected host. Furthermore, the parasite antigenic proteins with tandem repeats often localize on the body surface, presumably for protection against hosts' immune responses, and tend

to be readily recognized by hosts [28]. Similarly, both Ani s 11 and Ani s 11-like protein might be surface antigens.

Another new allergen, Ani s 12, also has a tandem repeat structure but its repetitive sequence CX<sub>13–25</sub>CX<sub>9</sub>CX<sub>7,8</sub>CX<sub>6</sub> comprising 40–52 amino acid residues is much longer than those of Ani s 11 and Ani s 11-like protein. As many as 19 repeats of a closely similar motif CX<sub>17–25</sub>CX<sub>9–22</sub>CX<sub>8</sub>CX<sub>6</sub> can be seen in Ani s 7 [14]. Furthermore, a protein with seven repeats of a motif CX<sub>15–26</sub>CX<sub>9–13</sub>CX<sub>8</sub>CX<sub>6</sub> has been cloned from *A. simplex* by immunoscreening using UA3 (monoclonal antibody recognizing two *A. simplex* glycoproteins of 139 and 154 kDa), although its detailed structure and properties have not been reported [14]. Such repetitive unique motifs including four Cys residues at regular intervals may be widely found in *A. simplex* allergenic proteins, although not recognized in any proteins from organisms other than *A. simplex*. Although similar tandem motifs are contained in both Ani s 7 and 12, they have no significant homology with each other, suggesting that Ani s 12 has specific IgE epitopes differing from the major IgE epitope proposed for Ani s 7.

In conclusion, we have identified Ani s 11, Ani s 11-like protein and Ani s 12 as new allergens of *A. simplex* by the chemiluminescent immunoscreening method developed in this study. Importantly, the three allergens are structurally unique; Ani s 11 and Ani s 11-like protein have five or six types of short repetitive sequences and Ani s 12 has a novel tandem motif with four Cys residues. For a better understanding of

*A. simplex* allergy, future study is needed to elucidate IgE epitopes of the three allergens having unique repetitive sequences.

## Acknowledgements

The authors 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 human sera. 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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