Molecular Cloning and Expression of a New Major Allergen, Ani s 14, from Anisakis simplex

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The nematode Anisakis simplex is a representative parasite infecting marine animals. When third stage larvae of A. simplex infecting fish and squid are ingested by humans, individuals previously sensitized by this parasite may experience IgE-mediated allergic reactions. So far, as many as 13 kinds of proteins (Ani s 1–13) have been identified as A. simplex allergens but several more unknown allergens are suggested to exist. In this study, therefore, chemiluminescent immunoscreening of an expression cDNA library constructed from the third stage larvae was conducted to identify a new allergen. As a result, an IgE-positive clone coding for a 23.5 kDa protein (named Ani s 14) composed of 217 amino acid residues was isolated. The regions 4–147 and 34–123 of Ani s 14 share identity with the region 2–91 of Ani s 7 and 32% identity with the region 2–91 of Ani s 12, respectively. Recombinant Ani s 14 was successfully expressed in Escherichia coli as a His-tagged protein and shown to be IgE reactive to 14 (54%) of 26 sera from Anisakis-allergic patients. In conclusion, Ani s 14 is a new major allergen of A. simplex that is specific to Anisakis-allergic patients.

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Key words: Anisakis simplex; allergen; expression; molecular cloning

Introduction

The nematode Anisakis simplex is a representative parasite infecting marine animals. Third stage larvae of A. simplex infect various fish and squid. When humans ingest fish or squid infected with the third stage larvae of A. simplex, the larvae may penetrate the gastrointestinal wall and cause adverse reactions, such as gastric or abdominal pains, nausea and vomiting, which are called anisakiasis1,2. It should be noted that A. simplex causes not only food poisoning, but also immunoglobulin E (IgE)-mediated allergy. Some individuals previously sensitized by the larvae have elevated IgE specific for A. simplex and develop IgE-mediated gastroallergic anisakiasis, including urticaria, angioedema and anaphylaxis3–6. Diagnosis of A. simplex allergy is currently performed based on IgE reactivity to a somatic crude extract of A. simplex using CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) and skin tests. However, subclinical subjects are often misdiagnosed to be false positive, because the crude extract of A. simplex contain some proteins that react nonspecifically with IgE5,6. To avoid misdiagnosis, it is desirable to use purified or recombinant allergens specific for Anisakis-allergic patients in the diagnostic tests. For this purpose, detailed information on A. simplex allergens is needed.

So far, the following 13 kinds of proteins have been identified as A. simplex allergens: Ani s 1 (Kunitz-type serine protease inhibitor)7,8, Ani s 2 (paramyosin)9,10, Ani s 3 (tropomyosin)11, Ani s 4 (cysteine protease inhibitor)12,13, Ani s 5 (SXP/RAL-2 family protein)14, Ani s 6 (serine protease inhibitor)15, Ani s 7 (novel protein with repetitive sequences)16, Ani s 8 (SXP/RAL-2 family protein)17, Ani s 9 (SXP/RAL-2 family protein)18, Ani s 10 (novel proteins with repetitive sequences)19, Ani s 11 (novel proteins with repetitive sequences)20, Ani s 12 (novel proteins with repetitive sequences)21 and Ani s 13 (hemoglobin)22. However, Arlian et al.22 reported that at least 18 different allergens exist in A. simplex. Therefore, the present study was initiated to identify a new A. simplex allergen by applying the chemiluminescent immunoscreening method to the expression cDNA library of A. simplex. The chemiluminescent immunoscreening method was recently developed and shown offer be much higher sensitivity and much lower frequency of false-positive results than the conventional immunoscreening method based on an enzymatic color reaction23.

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deed, the developed immunoscreening method has already been used to identify two allergens (Ani s 11 and 12)\textsuperscript{30}. In this study, using the same immunoscreening method, another protein was identified as a new allergen. Furthermore, the protein was expressed in Escherichia coli and the recombinant protein was confirmed to be IgE-reactive by enzyme-linked immunosorbent assay (ELISA) using Anisakis-allergic patients’ sera. The new allergen was named Ani s 14 according to the nomenclature of the Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies\textsuperscript{30}. The name Ani s 14 has already been accepted by the Sub-Committee. We report here the identification of a new allergen (Ani s 14) in A. simplex by the chemiluminescent immunoscreening method.

**Materials and Methods**

**Human sera**

Sera were obtained from 26 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 CAP-RAST classes (2–6 against A. simplex and 0 against fish). In this study, sera from 13 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. All experiments using human sera were performed in compliance with the Ethical Guidelines of Tokyo University of Marine Science and Technology ( Permit Number: 26–002).

**Immunoscreening**

The phage (λZipLox) expression cDNA library of A. simplex used in this study was the same as previously constructed from third stage larvae that were collected from walleye pollack (Theragra chalcogramma) captured at Raus, Hokkaido\textsuperscript{23}. The immunoscreening method employed was the same as described in our previous paper\textsuperscript{30}. 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 incubated in patient 15 serum. Plaques formed by bacteriophages were inoculated. Bacteria were incubated on a LB agar plate containing ampicillin. IPTG was added to the medium and the incubation was continued. Bacteria were harvested by centrifugation, suspended in phosphate buffer, digested with lysozyme and sonicated. After centrifugation, the precipitate (containing His-tagged rAni s 14) was dissolved by sonication in phosphate buffer containing 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol. The His-tagged rAni s 14 was purified by nickel-chelate affinity chromatography on a His-Trap Chelating HP column (GE-Healthcare Biosciences, Piscataway, NJ, USA). Positive clones were individually picked up and subjected to subcloning.

**Subcloning and DNA sequencing**

Subcloning and DNA sequencing were performed as described previously\textsuperscript{20}. E. coli DH10B was infected with a positive λZipLox clone and plated on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside, ampicillin and IPTG. The plate was incubated, and each white colony was picked up and cultured in LB medium containing ampicillin. The plasmid DNA was extracted from the bacterial suspension and sequenced using a DNA sequencer.

**Analysis of amino acid sequence of Ani s 14**

The amino acid sequence of Ani s 14 deduced from the nucleotide sequence was analyzed by the SignalP 4.1 server\textsuperscript{20} for signal peptide, the SMART server\textsuperscript{30} for domains and the Pfam server\textsuperscript{20} for motifs. Homologous proteins were searched through the DDBJ/EMBL/GenBank databases by the BLAST algorithm on the National Center for Biotechnology Information server\textsuperscript{20}.

**Expression and purification of recombinant Ani s 14**

rAni s 14 was expressed in E. coli as a histidine (His)-tagged protein using a pQE-30 Xa vector (Qiagen, Hilden, Germany) as described in our previous paper\textsuperscript{20}. An open reading frame of Ani s 14 was amplified by polymerase chain reaction (PCR) using the isolated clone as a template and the following primers: a forward primer with Stu I restriction site (underlined) 5′-AAT AGG CCT ATG CAA GTG AAG TGT ATC GC-3′ and a reverse primer with Hind III restriction site (underlined) 5′-TATA AGG CCT TCA CGT CTG ATC CAC GGT C-3′. Amplification was performed using HotMaster Taq DNA Polymerase (Eppendorf, Hamburg, Germany). The PCR product and the expression vector were digested with Stul and Hind III and ligated with each other. E. coli JM109 was transformed with the ligated product and cultured on an LB agar plate containing ampicillin. A single colony was picked up and cultured in LB medium containing ampicillin. IPTG was added to the medium and the incubation was continued. Bacteria were harvested by centrifugation, suspended in phosphate buffer, digested with lysozyme and sonicated. After centrifugation, the precipitate (containing His-tagged rAni s 14) was dissolved by sonication in phosphate buffer containing 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol. The His-tagged rAni s 14 was purified by nickel-chelate affinity chromatography on a His-Trap Chelating HP column (GE-Healthcare Biosciences) and refolded by dialysis against 400 mM L-arginine–HCl buffer. Protein concentration of rAni s 14 was quantified using Bio-Rad Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).
**Results and Discussion**

IgE-reactive clones obtained by chemiluminescent immunoscreening

Chemiluminescent immunoscreening of the expression cDNA library of *A. simplex* was performed using the patient 15 serum (the same as the patient 18 serum used in our previous study) as it was available in sufficient quantity. DNA sequencing showed that about 60% of the IgE-reactive clones coded for Ani s 5 and the rest for Ani s 6, 9, 11, 11-like or 12, in accordance with the previous study. However, one clone coding for a new allergen (Ani s 14) was eventually found.

Amino acid sequence of Ani s 14

Nucleotide sequence data of the Ani s 14 cDNA (1,259 bp) have been deposited in the DDBJ/EMBL/GenBank databases (accession number: LC027371). The start codon coding for methionine and stop codon are located at positions 23–25 and 674–676 bp, respectively. Thus, the amino acid sequence of Ani s 14 (23.5 kDa protein comprising 217 residues) was deduced from the nucleotide sequence of the open reading frame (651 bp), as shown in Fig. 1. No signal peptide was found in the deduced amino acid sequence as analyzed by the SignalP 4.1 server. The sequence was characterized by having two homologous regions at positions 5–44 and 54–97. These regions are composed of a common structure of CX₉CX₂₆CX₁₂,₂₆C and share 36% sequence identity with each other. It should be noted that tandem repeats of similar structures having four Cys residues at regular intervals have been recognized in two *A. simplex* allergens as follows: 19 repeats of CX₉CX₂₆CX₁₂,CX₉ in Ani s 7 (16), and five repeats of CX₉CX₂₆CX₁₂,CX₉ in Ani s 12 (29). However, there is no sequence homology among the repeats of Ani s 7, 12 and 14. In addition to the two homologous regions, three kinds of redundant sequences, Cys-Ile-Ala among the repeats of Ani s 7, 12 and 14. In addition to the two homologous regions, three kinds of redundant sequences, Cys-Ile-Ala among the repeats of Ani s 7, 12 and 14. In addition to the two homologous regions, three kinds of redundant sequences, Cys-Ile-Ala among the repeats of Ani s 7, 12 and 14. In addition to the two homologous regions, three kinds of redundant sequences, Cys-Ile-Ala among the repeats of Ani s 7, 12 and 14. In addition to the two homologous regions, three kinds of redundant sequences, Cys-Ile-Ala among the repeats of Ani s 7, 12 and 14.

![Fig. 1. Amino acid sequence of Ani s 14 deduced from the nucleotide sequence](image-url)

DDBJ/EMBL/GenBank accession number is LC027371. Amino acid numbers are shown at the left and right sides. Two homologous regions are boxed and identical residues in both regions are shaded. Three kinds of redundant sequences are indicated by open circles, closed circles and asterisks under the amino acid residues.
A New Anisakis simplex Allergen, Ani s 14

Expression and IgE reactivity of rAni s 14

Ani s 14 was successfully expressed in *E. coli* as a His-tagged protein. As analyzed by SDS-PAGE, a dominant protein band of 27 kDa was detected in the insoluble fraction from IPTG-induced *E. coli* (lane I in Fig. 3). The calculated molecular mass (26,398 Da) of His-tagged rAni s 14 was close to 27 kDa. Moreover, in our separate small-scale culture experiment, the 27 kDa protein band did not appear in the insoluble fraction from bacteria not induced by IPTG (data not shown). Accordingly, the 27 kDa protein band in the insoluble fraction was ascribable to His-tagged rAni s 14. After nickel-chelate affinity chromatography, followed by refolding, His-tagged rAni s 14 was obtained from the insoluble fraction of the IPTG-induced bacteria in a pure state (lane P in Fig. 3). In a typical run, 1 L of the bacterial culture yielded 8 mg of His-tagged rAni s 14.

rAni s 14 was evaluated for IgE reactivity by fluorescence ELISA using sera from 26 patients. As shown in Fig. 4, 14 sera (54%) including the patient 15 serum used for immunoscreening reacted to rAni s 14. In this study, rAni s 14 was finally obtained as a His-tagged protein. However, none of the 14 patients’ sera recognized Ani s 7 and Ani s 12. Thus, the functions of Ani s 14 are also unclear.

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ing rAni s 14 reacted to the synthesized His-tag peptide (data not shown), implying that the His-tag portion is not involved in the IgE binding of His-tagged rAni s 14. Of the 13 A. simplex allergens so far identified, five (Ani s 1, 2, 7, 12 and 13) are major allergens recognized by more than 50% of Anisakis-allergic patients. Ani s 14 is the sixth major A. simplex allergen. However, Ani s 14, together with Ani s 12, exhibits a lower rate of reactivity with patients’ sera than Ani s 1, 2, 7 and 13, which are recognized by about 80% of patients or more.

In this study, we have identified Ani s 14 as a new major allergen of A. simplex by means of the chemiluminescent immunoscreening method. Recombinant Ani s 14 was verified to be IgE reactive, and hence could be useful as a diagnostic tool for A. simplex allergy. Although Ani s 14 is partly homologous in amino acid sequence with Ani s 7 and 12, it is structurally unique and does not belong to any known protein families. Elucidation of the IgE epitopes of this unique allergen will increase our knowledge of A. simplex allergy. Finally, it should be emphasized that the recently developed chemiluminescent immunoscreening method was again found to be useful in identifying new allergens of A. simplex in this study. Future studies with this immunoscreening method using sera from other patients should identify further unknown allergens of A. simplex.

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