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Short communication

Ani s 10, a new *Anisakis simplex* allergen: Cloning and heterologous expression ☆

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ABSTRACT

Anisakiasis is a human disease caused by accidental ingestion of larval nematodes belonging to the *Anisakidae* family. Anisakiasis is often associated with a strong allergic response. Diagnosis of *A. simplex* allergy is currently carried out by test based on the IgE reactivity to a complete extract of L3 *Anisakis* larvae although the specificity of these diagnostic tests is poor. Improving the specificity of the diagnostic test is possible using purified recombinant allergens. A new *Anisakis* allergen, named Ani s 10, was detected by immunoscreening an expression cDNA library constructed from L3 *Anisakis simplex larvae.* The new allergen was overproduced in *Escherichia coli*; it is a protein of 212 amino acids and it was localized as a 22 kDa protein band in an ethanol fractionated extract from the parasite. Ani s 10 has no homology with any other described protein, and its sequence is composed by seven almost identical repetitions of 29 amino acids each. A total of 30 of 77 *Anisakis* allergic patients (39%) were positive both to rAni s 10 and natural Ani s 10 by immunoblotting. The new allergen could be useful in a component-resolved diagnosis system for *Anisakis* allergy.

1. Introduction

Anisakiasis is a human disease caused by the accidental ingestion of larval nematodes belonging to the family *Anisakidae*. Humans acquire the infection by eating raw or undercooked seafood. Anisakiasis is a serious zoonotic disease, and there has been a dramatic increase in its reported prevalence throughout the world, in the last two decades [1]. The onset of infection may present acute epigastric pain, sometimes accompanied by nausea and vomiting. The other common manifestation of human anisakiasis is an IgE-mediated reaction in sensitized individuals. These allergic symtoms can include urticaria, angioedema and anaphylaxis few hours after ingestion of infected fish [2].

Diagnosis of *Anisakis* allergy is currently carried out by tests based on the IgE reactivity to a complete extract of L3 *Anisakis* larvae. However, there are healthy people with positive results in these tests, which means that these tests are not specific enough [3]. The best alternative to improve specificity in the diagnostic tests is employing purified recombinant *Anisakis* allergens with the same immunological properties as their natural counterparts.

On the other hand, the diagnosis in allergy is changing to systems that use microchips containing isolated recombinant allergens (termed component-resolved diagnosis) [4]. These systems allow us to know the profile of allergen recognition of each patient. For this purpose, identification and molecular cloning of *A. simplex* allergens are requisite.

We report here the cDNA cloning expression and purification of a new *Anisakis* allergen in *Escherichia coli*. This new allergen has been designated Ani s 10 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

2. Materials and methods

We constructed the cDNA library starting from 150 mg of L3 *Anisakis* larvae. mRNA was isolated from L3 using Dynabeads mRNA direct kit (Invitrogen Dynal AS, Oslo, Norway), following the manufacturer's instructions (standard protocol). A polymerase chain reaction PCR-based cDNA library was subsequently prepared according to the instructions for the SMART PCR cDNA library construction Kit (protocol PT3000-1, Version PR7Y2399, Clontech. Palo Alto, CA, USA). The amplified cDNA was ligated into the Sfi I-digested Lambda TriplEx2 vector supplied in the library construction kit (Clontech). The ligation reaction was packed using Gigapack Gold III packaging extract from Stratagene/Biocrest (Cedar Creek, TN, USA) following the supplier's recommended protocol. The library was plated by infecting log-phase *E. coli* XL1-Blue (Clontech). The L3 cDNA library was estimated to contain 0.75×10^6 plaque forming units (final titer after amplification 0.27×10^{10} pfu ml^{-1}). Upon amplification this cDNA library was immunoscreened with an *Anisakis* allergic patient's serum.

☆ The nucleotide sequence of Ani s 10 cDNA has been deposited in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number GU187358. The new allergen has been submitted to the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee.

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The immunoscreening of cDNA library was performed according to the protocol included in the instructions for SMART PCR cDNA library Construction Kit (protocol PT3003-1, Version PR7Y2399). Specific IgE immunoblotting was performed with patient's serum diluted 1:10, as described [5]. Reacting plaques were rescreened until a homogeneous population of immunopositive recombinant phages was obtained.

In order to DNA sequence the insert contained in the immunopositive phages, phage insert from each purified plaque was directly PCR amplified with the primers: sense 5′-TCCGAGATCTGGACGAGC-3′ and antisense 5′-TAATACGACTCACTATAGGG-3′. The product of this PCR was employed as template for the DNA sequencing reaction, performed with the following primers: sense 5′-AGCGCGCCATTGTGTTGG-3′ or antisense 5′-CCGCATGCATAAGCTTGC-3′. The BLAST network server of the National Center for Biotechnology Information [6] was used to analyze the nucleotide sequences and deduced amino acid sequences. Analysis of the signal sequence was performed using SignalP V 3.0 at the Center of Biological Sequence Analysis (http://www.cbs.dtu.dk/services/SignalP/index.html).

The secondary structure prediction was performed at NPS@ web server [7] with the following secondary structure prediction methods: DSC, HNNC, MLRC, PHD and PREDATOR.

The coding region of Ani s 10, was PCR amplified using the following primers: sense 5′-GACGACGACAAGATGTCACCTATCCCA-CAAGAAG-3′ and antisense 5′- GAGGAGAAGCCCGGTCAAGCTTG-CATGGAGGCA-3′. The primers employed included the appropriate sequence (underlined) for directional cloning into the plasmid expression vector pET46 EK/LIC (Novagen Merck KGaA, Darmstadt, Germany). This plasmid produces a protein with a hexa-histidine N-terminal tag. The resultant plasmid was transferred into *E. coli* KRX (Promega, Madison WI, USA). The transformed bacteria were grown at 37 °C in LB medium supplemented with ampicillin (50 μg/ml) and the protein expression was induced overnight at 25 °C with rhamnose 0.1% and 1 mM IPTG. The culture was centrifuged and the pellet was resuspended in lysis buffer (BugBuster Reagent, Novagen).

Recombinant Ani s 10, expressed as a 6-His tagged protein, was purified from the soluble protein fraction using the His Spin Trap kit and the His Buffer kit (GE Healtcare Bio-Sciences AB. Uppsala, Sweden), following manufacturer's specifications. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules CA, USA).

The *Anisakis* protein extract enriched in natural Ani s 10 was prepared by means of an ethanol fractionation procedure. *Anisakis* L3 larvae were extracted from muscle of *Micromesistius poutassou*. The parasites were identified as *A. simplex* by morphological analysis. Larvae (1.5 g) were mixed with 5 ml of PBS and ground with a mortar and pestle. The mixture was incubated for 30 min at room temperature and centrifuged at 4000×*g* for 15 min. The supernatant ("crude extract", 5 ml) was mixed with 5 ml of ethanol, incubated for 30 min at room temperature and centrifuged at 4000×*g* for 15 min. The supernatant was mixed again with ethanol to increase the ethanol concentration to 66%, incubated for 30 min at room temperature and centrifuged at 4000×*g* for 15 min. The supernatant was mixed again with ethanol to increase the ethanol concentration to 90%, incubated for 30 min at room temperature and centrifuged at 4000×*g* for 15 min. The supernatant was discarded and the pellet was resuspended in 500 μl of PBS ("fraction 60–90%"). The fraction 66–90% was enriched in natural Ani s 10 [8].

Sera from 77 patients allergic to *Anisakis* were studied to find out the frequency of recognition of the new allergen. The diagnosis was made on the basis of a convincing clinical history and confirmed by *Anisakis*-specific IgE antibody assessments by CAP FEIA (Phadia, Uppsala, Sweden). Levels of *Anisakis*-specific IgE antibodies ranged from 0.70 kU/l to >100 kU/l (Table 1). Sera from 5 patients allergic to several pollens with negative CAP to *Anisakis* were tested as negative controls.

Table 1
Clinical data, specific IgE levels and sensitization to Ani s 10 of the thirty selected patients with allergy to *Anisakis simplex*. The numeration of the patients is the same as in Fig. 3.

Patient	Age/sex	Symptoms	Specific IgE(KU/l)	Ani s 10
1	49/F	Dyspnea/urticaria	>100	+
2	56/M	Urticaria/vomit/dyspnea	>100	+
3	49/F	Anaphilaxia	>100	+
5	50/F	Urticaria/epigastralgia	>100	+
9	57/F	Anaphilaxia/urticaria	26.1	+
12	22/F	Angioedema	2.07	+
13	44/M	Epigastralgia	2.84	+
14	63/F	Urticaria	10	+
15	53/F	Urticaria	>100	+
16	30/F	Urticaria/epigastralgia	37.3	+
18	29/M	Urticaria/angioedema	15.1	+
20	46/F	Anaphilaxia	19.3	+
22	68/F	Vomit/nausea/fever	>100	+
25	49/M	Urticaria	7	+
30	61/F	Urticaria/dyspnea	>100	+
33	62/F	Urticaria/diarrhea/angioedema/nausea	51.2	+
36	39/M	Abdominal pain/diarrhea	8.3	+
39	44/M	Vomit/nausea/diarrhea	3.5	+
41	63/F	Anaphilaxia	24.4	+
42	67/M	Epigastralgia/urticaria	27.6	+
52	40/F	Vomit/diarrhea	20.7	+
54	80/F	Urticaria	91.8	+
59	60/M	Abdominal pain/vomit/pruritus	17.9	+
60	61/M	Abdominal pain/vomit	16.2	+
63	67/F	Pruritus	43.1	+
64	68/F	Pruritus	40.4	+
66	45/M	Anaphilaxia/urticaria	>100	+
67	80/M	Urticaria	91.8	+
69	43/M	Anaphilaxia/urticaria	>100	+
74	60/F	Anaphilaxia	50.7	+

M, male; F, female; Negative result for IgE<0.35 kU/l.

3. Results and discussion

The immunoscreening of the cDNA library was performed by IgE-immunoblotting with a patient's serum that recognized *Anisakis* allergens not described yet. This serum recognized proteins with an apparent molecular weight different from the allergens previously described, when an Inmmunoblotting with L3 *Anisakis* larvae extracts prepared by means of an ethanol fractionation procedure was performed (see Material and methods section).

Patient's serum detected four clones, of which two contained the sequence of Ani s 10. However, the entire cDNA sequence of Ani s 10 was only present in one clone.

The cDNA encoding Ani s 10 comprised a 696 bp open reading frame coding for a protein of 231 amino acids (Fig. 1A). The first 19 amino acids corresponded to a signal peptide. Removal of the signal peptide resulted in a mature protein with 212 amino acids, a calculated molecular mass of 21,126.7 Da and a theoretical isoelectric point of 4.16.

Ani s 10 sequence is composed by seven amino acid repeats of 29 amino acids. Each of these repeats has theoretical cleavage sites for trypsin (K, lysine in positions 45, 74,103, 132, 161, 190, and 210) and pepsin (W, tryptophan in positions 43, 72, 101, 130, 159, 188, and 219) (Fig. 1B). There are two cockroach allergens, Bla g 1 from *Blattella germanica* and Per a 1 from *Periplaneta Americana,* that have tandem repeats of 100 amino acid residues long. These allergens are secreted in the cockroach digestive tract and cleaved by trypsin-like enzymes before being excreted [9]. Bla g 1 and Per a 1 allergens do not have homology with Ani s 10, but it has 7 putative cleavage points for pepsin and trypsin in the middle of the repetitions. It could be possible that Ani s 10 was cleaved in 7 small active peptides at the digestive tract as happens with Bla g 1.

The secondary structure prediction shows that each repetition contains a short region with extended strand structure, followed by