



Short communication

Cloning and expression of a biologically active *Anisakis simplex* allergen Ani s 1 in the yeast *Pichia pastoris*

Rosa Rodriguez-Perez*, Maria Luisa Caballero, Miguel Gonzalez-Munoz,
Ana Rodriguez-Mahillo, Ignacio Moneo

Servicio de Inmunología, Hospital Carlos III, C/Sinesio Delgado 10, 28029 Madrid, Spain

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Anisakis simplex is a nematode parasite that can infect humans when raw or undercooked seafood is eaten. The onset of infection may present acute epigastric pain, sometimes accompanied by nausea and vomiting [1]. The other common manifestation of human anisakiasis is an allergic reaction in sensitised individuals. These allergic symptoms can include urticaria, angioedema [2] and anaphylaxis few hours after ingestion of infected fish [3].

Nowadays, available commercial diagnosis systems to detect specific IgE use crude parasite extracts. These crude extracts can present cross-reactions with other parasites. On the other hand, the presence of healthy people with specific IgE against allergens from the crude extract of the parasite makes difficult the serologic diagnosis. To overcome this problem, one way is to use the most important and specific allergens of *Anisakis*, purified or recombinant.

Ani s 1 was described as a major allergen from the *A. simplex* parasite [4]. In a previous work it has been found that 85% of patients with *Anisakis* allergy have specific IgE to Ani s 1 [5]. For that reason, specific IgE determination to Ani s 1 is considered a useful tool for the diagnosis of *Anisakis* allergy [5].

The purification of natural Ani s 1 is a hard task and the low yield obtained makes impossible its use as a routine diagnosis reagent [4]. The best alternative is to use a recombinant Ani s 1 protein with the same immunological properties as its native counterpart.

Ani s 1 was cloned and expressed as a GST-fusion protein in *Escherichia coli*, but the protein was recovered only in insoluble form [6]. This means that the incorrect folding of the protein could affect their properties and characteristics, and its use as a diagnostic tool was discarded.

One way to overcome the protein expression problems in bacteria is to employ an eukaryotic system like the yeast *Pichia pastoris*. This approach has solved problems of correct folding and expression in other cloned allergens [7,8].

The aim of this work has been to achieve a recombinant Ani s 1 allergen with equivalent immunological properties than the native form.

We designed specific oligonucleotide primers for PCR amplification from the mature form of Ani s 1, without leader peptide. The primers were based on the known cDNA sequence of Ani s 1 (accession number AB100095). The sense primer 5'-ctcgagaaaagagagGATAGAACGGAATG-3' contains an XhoI restriction site (underlined), the sequence coding for the pre-prosequence of the *Sacharomyces cerevisiae* α -factor present in plasmid pPIC9, and the N-terminal end of mature Ani s 1 (capital letter). The antisense primer 5'-gaattcTTATTTACAACAATTTGCAAATA-3' includes the C-terminal end of Ani s 1, a stop codon and EcoRI restriction site (underlined). The PCR product was purified and cloned directly in the plasmid pGem included in the pGem TEasy vector cloning kit (Promega, Madison, WI, USA). The recombinant plasmid was used to transform TOP10 *E. coli* cells.

Isolation and sequencing of the obtained cDNA revealed a 513 bp open reading frame coding for a 171 amino acid polypeptide. The complete amino acid sequence deduced of the whole Ani s 1 molecule presented only a single change at position 162

Abbreviations: RP-HPLC, reverse phase high performance liquid chromatography; MS, mass spectrometry; rAni s1, recombinant Ani s 1; nAni s 1, natural Ani s 1

* Corresponding author. Tel.: +34 914532656; fax: +34 913150034.

E-mail address: mrosa_ro@yahoo.es (R. Rodriguez-Perez).

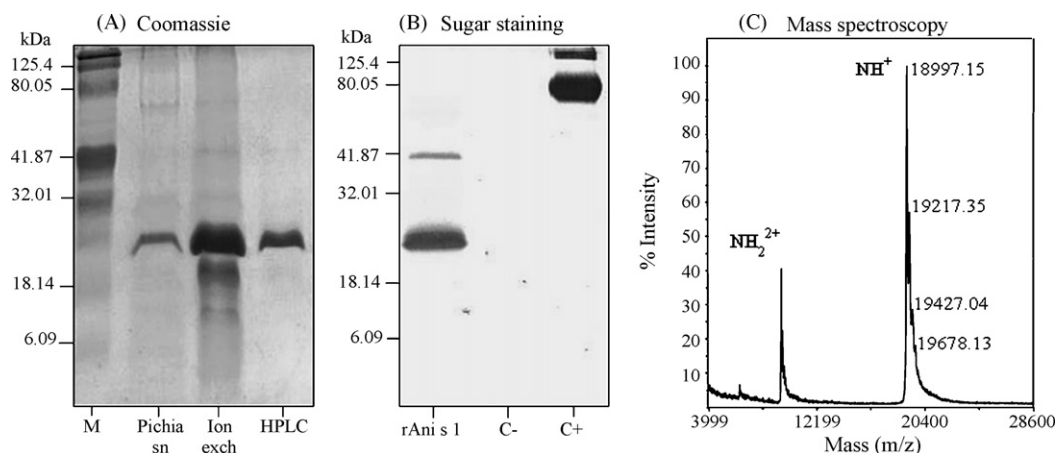


Fig. 1. Expression and purification of recombinant Ani s 1. (A) Coomassie blue staining after SDS-PAGE of the purification steps; M: molecular mass markers; *Pichia sn*: supernatant of *Pichia* culture. The culture medium obtained after induction was dialysed against distilled water, and freeze-dried; Ion exch: the *Pichia* medium was chromatographed on a High S cartridge (Bio-Rad, Hercules, CA, USA) cationic interchange column in a 50 mM acetate buffer, pH 5.0. The fraction containing rAni s 1 was eluted from the column with 50 mM acetate buffer, pH 6.0, dialysed and freeze-dried; HPLC: the ion exchange fraction was separated by RP-HPLC on a Hypersil 300-C4 column (250 mm × 46 mm; particle size 5 µm; Teknokroma, Barcelona, Spain). Elution was performed with a linear gradient of acetonitrile (20–60% in 60 min; 1 ml/min) in 0.1% trifluoroacetic acid. (B) Sugar staining of purified protein transferred to membrane after SDS-PAGE. The carbohydrate detection was performed using the DIG Glycan Detection Kit (Roche Diagnostics GmbH, Penzberg, Germany). Non-glycosylated protein creatinase (C–) and glycosylated protein transferring (C+) were used as controls. (C) Mass spectroscopy of recombinant Ani s 1. The analysis was performed on a Voyager DETM STR mass spectrometer (PerSeptive Biosystems, Framingham, MA).

(R → Q), when compared with the deduced amino acid sequence of Ani s 1 previously described [6].

The pGem plasmid containing Ani s 1 sequence was digested with XhoI/EcoRI (Fermentas, Ontario, Canada). The DNA fragment containing Ani s 1 was subcloned into the same sites of the plasmid pPIC9 (kindly supplied by Dr. Gabriel Salcedo from ETSIA, Madrid) in frame with the sequence coding for the pre-prosequence of the *S. cerevisiae* α-factor present in plasmid pPIC9, downstream of the AOX1 promoter. This sequence contains the signal peptidase cleavage that allows the secretion of the recombinant protein. The action of this peptidase leaves a glutamic residue at the N-terminal end of the mature recombinant protein.

The pPIC9/Ani s 1 was linearized with Sac I (New England Biolabs, Beverly, USA) restriction enzyme, and the purified fragment was used to electroporate GS115 strain of *P. pastoris* (kindly supplied by Dr. Gabriel Salcedo from ETSIA of Madrid) following the Invitrogen manual recommendations (Invitrogen Corporation, De Schelp, the Netherlands). Transformed cells were grown on minimal glucose plates at 30 °C during 3–4 days, and screening for positive clones was performed by patching the colonies in replica plating on minimal glucose versus minimal methanol plates. To produce recombinant Ani s 1 (rAni s 1), selected (His⁺, Mut⁺) strains were cultured in buffered glycerol complex medium for 24 h at 30 °C. The cells were then collected by centrifugation, and expression of the recombinant protein was induced by resuspension in buffered minimal methanol medium. Methanol induction of *P. pastoris* transformed cells resulted in the extracellular secretion of rAni s 1. After induction, the maximum secretion was achieved 4 days later.

Purification of recombinant Ani s 1 was carried out by a two steps chromatographic procedure. The dialysed extracellular medium of the culture was first fractionated by cation-exchange.

The fraction containing rAni s 1 was dialysed, freeze-dried and then separated by RP-HPLC. The different steps of purification were analyzed by SDS-PAGE (Fig. 1A). The final output was 800 µg of pure protein from 1 l of *P. pastoris* culture.

The carbohydrate detection of the rAni s 1 revealed that it is glycosylated (Fig. 1B). The lack of any N-glycosylation site implies that an O-glycosylation should be responsible for the carbohydrate detection.

Mass spectroscopy of the protein (Fig. 1C) revealed a main peak of 18,997 Da which is in agreement with the calculated molecular weight (18997.8 Da). There are small peaks with molecular weights of 19217.35; 19427.04 and 19678.13 that correspond to glycosylated forms with one or more mannoses (159 Da).

N-terminal sequencing of the purified rAni s 1 was performed automatically using a 477 gas-phase sequencer (Applied Biosystems, Foster City, California). The obtained sequence was the expected sequence EDRTEXQLP, which confirmed the purity and the correct processing of the protein (the X residue corresponds with a C in the native protein, and C residues are not detected by this technique).

Both natural Ani s 1 and recombinant Ani s 1 displayed similar IgE-binding capacity when a pool of sera from *Anisakis* allergic patients was used for specific IgE immunodetection (data not shown).

To determine the allergenic equivalence between natural Ani s 1 and recombinant Ani s 1, an immunoblotting inhibition was performed (Fig. 2B). A full IgE-binding inhibition was obtained using rAni s 1 as inhibitor against nAni s 1 present in the enriched extract. The same was true when the enriched extract of nAni s 1 was tested as inhibitor against rAni s 1.

A total of 30 patients with positive CAP to *Anisakis* and convincing history of *Anisakis* allergy were tested using natural Ani

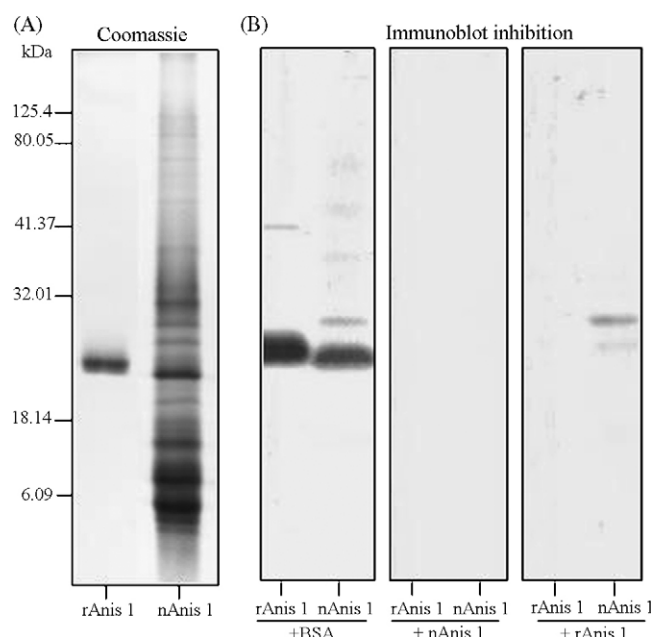


Fig. 2. Immunological properties. (A) Coomassie blue staining after SDS-PAGE of rAni s 1 (1 μ g) and enriched extract in nAni s 1 (10 μ g). To obtain the enriched extract of Ani s 1, a PBS extract of L3 larvae, was mixed with the same amount of absolute ethanol (50%, v/v) and left for 30 min at 4 °C. Thereafter the mixture was centrifuged at 4000 $\times$ g for 15 min, and the pellet was discarded. The supernatant was mixed again with the same amount of absolute ethanol (66% ethanol concentration). After 30 min at 4 °C, the mixture was centrifuged at 4000 $\times$ g for 15 min, and the pellet obtained was resuspended in 0.5 mL of PBS [4]. (B) Immunoblot inhibition assays. + BSA, with bovine serum albumin, negative control (30 μ g). + nAni s 1, enriched extract (5 μ g). + rAni s 1, recombinant Ani s 1 (5 μ g). The nitrocellulose membranes were incubated with a pool of sera from *Anisakis* allergic patients diluted 1:20 and pre-incubated for 2 h at room temperature with each inhibitor.

s 1 and recombinant Ani s 1 by immunoblot. Of those patients, 28 recognized Ani s 1 (93% of the patients), and all of them recognized both, the natural and the recombinant form (data not shown). The most important finding was that the different sera recognized with the same intensity the natural and the recombinant allergens. This fact demonstrated that the recombinant Ani s 1 can replace the use of the natural allergen in diagnostic procedures.

The biologic activity of rAni s 1 was analyzed measuring basophil activation by flow cytometry. Blood of 10 patients was used, 5 of them were IgE negative for both nAni s 1 and rAni s 1, and the other 5 were IgE positive for nAni s 1 and rAni s 1 by immunoblot and CAP. To analyze basophil activation, cells were stained with 20 μ L CD63 (IgG1)-FITC/CD123(IgG1)-PE/HLA-DR(IgG2a)-PerCP reagent mixture (BD Biosciences) and acquired in a FACScan flow cytometer (BD Biosciences). The basophil population was gated using side scatter (SSC) and CD123. Then, gated cells were displayed in a CD123/HLA-DR dot plot to eliminate HLA-DR+ cells. Subsequently, CD63 expression was detected in the CD123+ HLA-DR– basophils. In the CD63/CD123 dot plots, the quadrants were set with the negative control of the test, in order to identify activated basophils by high CD63 expression. Table 1 summarizes the results obtained with the allergen concentrations that produced maximal basophil

Table 1

Basophil activation test with rAni s 1 of positive patients for Ani s 1 and negative patients for Ani s 1

Patient	CAP <i>Anisakis</i> (kUA/l)	Immunoblot Ani s 1		% Basophil activation
		Natural	Recombinant	
Positive				
1	16.00	+	+	91.30 ^a
2	23.50	+	+	53.28
3	3.80	+	+	52.07
4	>100	+	+	70.51
5	41.80	+	+	97.61
Negative				
6	1.16	—	—	2.84
7	<0.35	—	—	1.56
8	<0.35	—	—	3.11
9	<0.35	—	—	1.54
10	<0.35	—	—	1.01

^a The percentage of basophil activation is the highest of the three amounts of rAni s 1 tested (1.5, 0.15 and 0.015 μ g).

activation. All patients positive for Ani s 1 (natural and recombinant) by immunoblot had positive basophil activation. None of the control subjects negative for Ani s 1 by immunoblot was positive for basophil activation test at any of the concentrations assayed, although patient 6 had a low positive CAP value for *Anisakis*.

The expression of the recombinant proteins in the yeast *P. pastoris* as an extracellular non-fusion proteins, allows the avoidance of solubility problems that sometimes appear when *E. coli* are used. In this work, the recombinant allergen and the native allergen have similar IgE-binding capacity, as shown by immunodetection (see Table 1) and by immunoblot inhibition assay (Fig. 2). Besides, the recombinant form has in vivo activation capacity as it has been demonstrated by basophil activation test. These data indicate that the recombinant protein is properly expressed and the IgE epitopes are well conserved. But in SDS-PAGE, the apparent molecular mass of recombinant and natural Ani s 1 is different, the apparent molecular weight under non-reducing conditions for natural Ani s 1 is 21 kDa, but our recombinant Ani s 1 seems to have an increased molecular weight. We know that recombinant Ani s 1 is *O*-glycosylated and it has one N-terminal amino acid more than the natural protein. It is possible that these two factors explain this discrepancy. On the other hand, the MALDI analysis shows the expected molecular weight and the N-amino terminal sequence was the expected.

In conclusion, recombinant Ani s 1 here described and natural Ani s 1 have similar allergenic properties. This recombinant allergen could be considered as a tool for clinical diagnosis and it is at present used in our laboratory for this purpose. Even more, it could be considered as an alternative in the future for its use in immunotherapy in patients with allergy to *Anisakis* as described for other recombinant allergens [9,10].

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