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Short communication

Identification and allergenic characterisation of a new isoform of the *A. simplex* allergen Ani s 4

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ABSTRACT

Anisakis simplex hypersensitivity is a growing disease in developed countries. A positive diagnosis usually leads to the dietary recommendation of avoiding fish and seafood consumption. The protein Ani s 4 is a clinically relevant allergen due to its heat and pepsin resistant properties and its importance in the anaphylaxis process. The attempt of cloning Ani s 4 has led to the identification and characterisation of a new isoform that differs only in one amino acid with the previously published. This isoform was produced as an His tagged recombinant protein and its allergenic properties were tested by IgE immunoblot and by a flow cytometry basophil activation test. The results were compared to the allergenic properties of the isoform previously described. Both isoforms of Ani s 4 showed different capacities to bind IgE from sensitised patients and different potencies in the basophil activation test.

1. Introduction

A positive diagnosis of *Anisakis simplex* hypersensitivity is usually considered if a patient has a compatible clinical history and positive skin prick test and/or a positive specific anti-*Anisakis* IgE value. A compatible clinical history includes symptoms such as abdominal pain, vomiting, nausea, urticaria, angioedema, breathing difficulty or anaphylaxis within the first 12 h after eating fish.

Parasite crude extracts are used in the allergy diagnosis to measure the quantity of anti-*Anisakis* IgE and to perform the skin prick tests. This kind of extract induces in both cases a high rate of false positives among healthy population [1], due to the high homology of some of the *A. simplex* allergens with proteins found in other parasites, insects, dust mites, crustaceans, etc. [2–4]. IgE immunoblotting using crude and semi-purified *Anisakis* extracts [5,6], ELISA using *O*-deglycosilated allergens [1] or the determination of *Anisakis*-induced basophil activation by flow cytometry [7] have been postulated as more specific diagnosis methods.

To avoid false positives, the use of recombinant proteins is widely used in the diagnosis of several illnesses. Regarding the parasitoses, different serological tests using recombinant proteins have been developed for taeniasis, filariasis, leishmaniasis, malaria, etc. [8–11].

In order to design a diagnosis test based in the use of recombinant proteins for the *A. simplex* sensitivity, several allergens of this nematode have been cloned and characterised [12,13]. Among them, Ani s 4 was described as a minor allergen, resistant to heat and pepsin, frequently recognised by people suffering anaphylaxis due to *A. simplex* [14]. When its amino end was sequenced, it was found that the second and the third amino acids of the mature protein could vary between M/T and L/P respectively. The isoform starting with the sequence GML was recently cloned and characterised [13].

The aims of this study were, first, to produce and characterise other Ani s 4 isoforms as His tagged recombinant proteins, and second, to determine if there were differences in the allergic properties between the isoforms.

2. Material and methods

2.1. Patients

A patient was considered to be sensitised to *Anisakis simplex* (pAS) when he showed a compatible clinical history (acute urticaria, abdominal pain, vomiting, angioedema and/or anaphylaxis within the first 12 h of eating fish) confirmed by a positive *A. simplex* specific IgE value by FEIA-CAP and a positive recognition of an *A. simplex* allergens of a parasite crude extract by immunoblotting.

Sensitivity to Ani s 4 (pAS/pA4) was considered if an *A. simplex* sensitised patient recognised the natural allergen Ani s 4 by

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IgE immunoblotting. If a patient did not recognise Ani s 4, he was considered pAS/nA4.

Samples from non-allergic individuals (nAS) were included in all the assays as controls.

2.2. Production of recombinant Ani s 4

A. simplex total RNA was isolated and mRNA was reverse transcribed using a polyT primer. Ani s 4 specific cDNA was PCR amplified using the primer pair P1 (5′-GAGGAGAAGCCCGGTTTTT-3′) and P2 (5′-GATTCATAAATATGCAATCCAG-3′). The cDNA obtained was sequenced by the DNA Automatic Sequence Service (CIB, CSIC, Madrid, Spain) using an adaptation of the Sanger method (Sanger 1975). The sequences obtained were aligned using Clustalw multiple alignment software (www.ebi.ac.uk/Tools/clustalw2/index.html). This alignment showed that two Ani s 4 mRNA were produced by *A. simplex*. These two mRNA were translated to proteins, which showed that one of them coincided with the Ani s 4 sequence previously published (GenBank accesion number CAK50389) while the other one differed in the amino acid in the position 25 of the complete protein (the third amino acid of the mature protein without the signal peptide), where a proline was found instead of the leucine previously described. In this article, the recombinant Leu^3Ani s 4 will be named rAni s 4-L, while the recombinant Pro^3Ani s 4 will be named rAni s 4-P.

The two recombinant Ani s 4 were produced as described [13] by transforming the bacteria strain *Escherichia coli* BL21 Star (DE3) One Shot (Invitrogen, Carlsbad, CA, USA) with the DNA sequence of their mature form (without the signal peptide) cloned into the pET 46 EK/LIC plasmid (Novagen, Merck, Darmstadt, Germany). The PCR primers used to amplify the Ani s 4 mature were P3 (5′-GGAATGCYAGGCGGATCGTC-3′), P4 (5′-GACGACGACAAGATAGGAATGCTAG-3′) and P5 (5′-GACGACGACAAGATAGGAATGCCAG-3′). P4 was used to amplify rAni s 4-L and P5 was used to amplify rAni s 4-P.

The recombinant Ani s 4 proteins, expressed as 6x His tagged proteins, were purified from the soluble protein fraction using His*Bind Resin (Novagen) and concentrated. Imidazole was then eliminated by filtration using Microsep 3 K Cartridge (Filtron Technology Corporation, Northborough, MA, USA).

The recombinant protein concentration was determined using Bradford Reagent (Fluka, Sigma–Aldrich Inc., St. Louis. MO, USA), using BSA (bovine serum albumin) as the standard. A_{450} was read on a microplate reader 340 ATC (SLT Labinstruments, Salzburg, Austria).

2.3. IgE immunoblotting

The proteins (100 μg) were applied to 16% acrylamide–Tris–Tricine buffered SDS-PAGEs without lanes [13]. After the electrophoresis, the proteins were transferred to nitrocellulose membranes by diffusion [15]. After blocking (3% Nonidet P-40 in PBS for 30 min), membranes were placed on a Mini-Protean II Multiscreen (Bio-Rad, Hercules, CA, USA) and subsequent incubations with individual human sera, mouse anti-human IgE monoclonal antibody (Ingenasa, Madrid, Spain) and alkaline-phosphatase labelled goat anti-mouse IgG (Biosource International, CA, USA) were performed as described. The immunoblots were revealed for 30 min with BCIP-NBT (5-bromo-4-choro-3-indolyl phosphate-nitro blue tetrazolium).

Thirteen pAS/pA4 and seven pAS/nA4 patients' sera were used to test their recognition of the two rAni s 4 isoforms. To compare the recognition intensity of rAni s 4-L and rAni s 4-P by each patient, the immunoblotting bands were densitometred and analysed with the Quantity One software (BioRad, Hercules, CA, USA)

To compare the epitopes of rAni s 4-L and rAni s 4-P, an immunoblotting inhibition assay was performed. The protocol was followed as described above, except that a mixture of pAS/pA4 patients' sera was pre-incubated with 1, 0.2, 0.04, 0.008 or 0 μg of rAni s 4 for 2 h. The inhibition was observed as the attenuation of the recognition intensity and measured by densitometry of the immunoblot bands using the Quantity One software. An inhibition with an irrelevant recombinant protein was also performed in the same conditions as assay control.

2.4. Detection of rAni s 4 induced basophil activation

After checking if the patients' basophils were responders to mouse anti-human-IgE monoclonal antibody, patients samples were incubated as described [7,13], first with 1 μg/mL of an *A. simplex* crude extract to test if they were *A. simplex*-sensitised, and then with different concentrations of the recombinant allergens to study the allergens capability of specifically activate basophils.

We used the following allergen concentrations: 1 μg/mL, 0.1 μg/mL and 0.01 μg/mL of rAni s 4-L and rAni s 4-P. A positive activation was determined by flow cytometry measuring the increment of the CD63 expression by basophils when incubated with the allergens.

2.5. Statistical analysis

The statistics analysis was performed using SPSS 13.0 software (Chicago, IL, USA). The quantitative variables were described with median and range and they were correlated using the non-parametric Spearman's rho.

3. Results

3.1. Production of rAni s 4

We analysed 30 sequences of the Ani s 4 cDNAs obtained by PCR using the specific primer pair P1 and P2. The translation of all of them produced identical proteins except for one amino acid. The amino acid in position 25 of the complete proteins, including the signal peptide (the third amino acid of the mature protein), was a leucine in 50% of the sequences, while the other 50% had a proline.

The two recombinant proteins, rAni s 4-L and rAni s 4-P, were produced in a bacterial system, purified by affinity chromatography (Fig. 1A) and their recognition by patients IgE *in vitro* and *ex vivo* was studied.

We could not confirm in this assay the existence of any Ani s 4 isoform with a Thr instead of a Met in the second position of the mature protein.

3.2. Comparison of the allergenicity by IgE immunoblotting

Twenty pAS (13 pAS/pA4 and 7 pAS/nA4) patients' sera were used in the immunoblotting assay to compare the intensity of their recognition of each recombinant Ani s 4. The results of that rAni s 4-P was recognised by the same patients that r-Ani s 4L (Fig. 1B). The immunoblotting bands were densitometred and the recognition intensity was compared for each patient. Ani s 4-P recognition was more intense in 70% of the A4+ patients studied, although the intensities of recognition of both proteins showed a high correlation ($r = 0.940$, $p < 0.001$).

The immunoblotting assay showed different inhibition rates by the two recombinant proteins (Fig. 1C). The soluble rAni s 4-L was able to almost completely inhibit the binding of the patients IgE to both immobilised recombinant allergens, while soluble rAni s