ORIGINAL ARTICLE

Expression of a Recombinant Protein Immunochemically Equivalent to the Major *Anisakis simplex* allergen Ani s 1

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Abstract

Background: Anisakis simplex is a nematode which can parasitize humans, producing anisakiasis and can induce immunoglobulin-(Ig)-E–mediated allergic symptoms. Parasite recombinant proteins, such as the major allergen Ani s 1, may be useful tools to avoid misdiagnosis of *A simplex* allergy due to cross-reactivity when whole parasite extracts are used.
Objective: To obtain Ani s 1 allergen as a recombinant protein with IgE-binding properties similar to its natural counterpart.
Methods: Ani s 1-encoding cDNA was amplified by polymerase chain reaction and cloned. The allergen was expressed in *Escherichia coli* as a nonfusion protein. Natural and recombinant Ani s 1 were investigated by means of Western blotting, enzyme allergosorbent test, enzyme-linked immunosorbent assay (ELISA), and ELISA inhibition using sera from 53 patients with *A simplex* allergy.
Results: Residues of the amino acid sequence of the encoded protein were 99.4% identical to the reported one. Purified rAni s 1 was obtained with a yield of 2 mg/L of culture while the yield of the natural counterpart was only 50 µ/g of larvae. rAni s 1 reactivity was not significantly different from that of the natural allergen; the correlation was excellent ($\rho = 0.92$, $P < .001$). ELISA-inhibition experiments showed that the dose-response inhibition curve obtained with rAni s 1 overlapped with that of nAni s 1. In an enzyme allergosorbent analysis, 86.8% of the *A simplex*-allergic patient sera reacted to rAni s 1.
Conclusion: Recombinant Ani s 1 is immunochemically equivalent to its natural counterpart and therefore might be useful for the in vitro diagnosis of anisakiasis and *A simplex*-mediated allergy.

Key words: Nematode. Recombinant allergen. Biologically active allergen Diagnostic tool. Major allergen.

Resumen

Antecedentes: Anisakis simplex es un nemátodo que puede parasitar a humanos produciendo anisakiasis e inducir síntomas alérgicos mediados por inmunoglobulina (Ig)E. Las proteínas recombinantes del parásito, como el alérgeno mayoritario Ani s 1, pueden ser unas herramientas útiles en el diagnóstico de alergia a *A simplex* para evitar los errores, debidos fundamentalmente a fenómenos de reactividad cruzada, que se observan cuando se utilizan extractos completos del parásito.
Objetivo: Disponer de una forma recombinante del alérgeno Ani s 1 que conserve las mismas propiedades de unión a anticuerpos IgE que su equivalente natural.
Métodos: Se amplificó, mediante la reacción en cadena de la polimerasa, y se clonó el ADNc que codifica para Ani s 1 y su expresión se realizó en *E coli* como una proteína no fusionada. Se estudiaron mediante inmunodetección, enzimoalergoadsorción (ELISA) e inhibición de ELISA las formas natural y recombinante de Ani s 1 utilizando suero de 53 pacientes con alergia a *A simplex*.
Resultados: La secuencia de aminoácidos de la proteína codificada tenía un 99,4% de residuos idénticos a los de la secuencia descrita. El rendimiento obtenido con el alérgeno recombinante Ani s 1 purificado fue de 2 mg/L de cultivo mientras que el rendimiento para el alérgeno natural fue sólo de 50 µg/g de larvas. La reactividad humoral de rAni s 1 no fue significativamente diferente de la obtenida con el alérgeno natural obteniéndose una correlación excelente ($\rho = 0,92$, $P < 0,001$). La curva dosis-respuesta de rAni s 1, obtenida a partir de experimentos de inhibición de ELISA, solapaba con la obtenida con nAni s 1. Según los ensayos de enzimoalergoadsorción un 86,8% de los pacientes alérgicos a *A simplex* reaccionaban también con rAni s 1.
Conclusión: La forma recombinante de Ani s 1 es inmunoquímicamente equivalente a su homólogo natural y por ello podría ser utilizada en el diagnóstico *in vitro* de la anisakiasis y la alergia a *A simplex*.

Palabras clave: *Nemátodo. Alérgeno recombinante. Alérgeno activo biológicamente. Herramienta diagnóstica. Alérgeno mayoritario.*

* The first 2 authors contributed equally to the study.

Introduction

Anisakis simplex is a nematode which parasitizes fish and cephalopods. Humans become accidental hosts by eating raw or undercooked infested fish or seafood, leading to a gastrointestinal disease known as anisakiasis. *A simplex* can also cause immunoglobulin (Ig) E-mediated allergic reactions such as urticaria and anaphylaxis in some sensitized patients [1]. The diagnosis of *A simplex* allergy is based on the measurement of serum specific IgE using crude parasite extracts, but this approach is hampered by the frequent presence of false-positive results probably due to cross-reactions [2-4].

Nine allergens have been isolated from *A simplex* (www.allergen.org; www.allergome.org). Ani s 1 is a major allergen without cross-reactions with other allergens [5,6]. The presence of Ani s 1-specific IgE, with a sensitivity of 86% and specificity of 90%, has been postulated to be a more specific marker for *A simplex* hypersensitivity and anisakiasis than IgE detection against the crude extract [1,7]. If purified *A simplex* allergens are to make diagnosis more reliable, well-defined recombinant allergens will become necessary for the standardization of diagnostic methods. Furthermore, they would offer the possibility of component-resolved diagnosis by means of in vivo and in vitro procedures [8] since these molecules could be produced with suitable purity and batch consistency. The aim of the present study was to obtain the major allergen Ani s 1 as a recombinant protein with IgE-binding properties similar to its natural counterpart.

Methods

Serum Samples

Serum samples were obtained from 53 patients with *A simplex* allergy recruited at the Allergy Service of the General Hospital of Segovia, Spain. All patients had experienced several allergic reactions (urticaria/angioedema, abdominal pain, or anaphylaxis) after eating marinated or cooked fish. Patients had a positive skin test to *A simplex* extract, a positive specific IgE determination (class ≥3) to *A simplex* extract by enzyme immunoassay (ImmunoCAP, Phadia, Uppsala, Sweden), and negative reactivity to several fish extracts. Sera from patients with allergy to common aeroallergens and without food allergy were used as controls.

Amplification of the Gene Encoding Ani s 1 by Polymerase Chain Reaction

Poly A+-enriched RNA was isolated from 100 mg of *A simplex* by using the Quick Prep MicroRNA Purification Kit (GE-Healthcare, Uppsala, Sweden). 1 μg mRNA was reverse transcribed with the First-Strand cDNA Synthesis Kit (GE-Healthcare). The Ani s 1 coding region was amplified from first-strand cDNA by the use of primers designed according to a previously reported sequence [9]. The primers were 5´-CGGGATCCGATAGAACGGAATGTCAG-3´ (sense) and 5´-CGGAATTCTTATTTACAACAATTTGCAAA-3´ (anti-sense). *Bam*HI and *Eco*RI restriction sites were underlined. Polymerase chain reaction (PCR) product was isolated and cloned into pGEM-T vector (Promega, Madison, Wisconsin, USA) and the nucleotide sequence of the DNA insert was determined. Ani s 1-encoding cDNA was cloned into the expression vector pKN172 [10] as a *Bam*HI-*Eco*RI fragment and used to transform competent *Escherichia coli* BL21(DE3) cells.

Expression and Purification of rAni s 1

For expression, cells were grown to an optical density (OD) 600 of 0.6 in Luria broth-containing 200 μg/mL of ampicillin, and after addition of 0.6 mM isopropyl-thio-ß-galactoside incubation was continued for another 3 hours. The bacterial pellet was treated with lysozyme followed by sonication. rAni s 1 was obtained as inclusion bodies which were solubilized with 6 M guanidine hydrochloride. After buffer exchange to 6 M urea, expressed protein was diluted to 1 mg/mL and refolded successively against 3, 1.5, 0.75, 0.38, and 0.19 M urea, each for 90 minutes followed by oxidative refolding in the presence of cysteine-cystine (5 mM:1 mM) in 50 mM Tris pH 8.0 overnight at 4ºC. The monomeric molecule was separated from aggregates by means of gel permeation chromatography (Superdex S200 column, ÄKTA prime system [GE-Healthcare]) with a final yield of 2 mg/L culture. Purified recombinant protein was used to obtain polyclonal antibodies by immunizing New Zealand rabbits with 5 boosts of 200 μg of rAni s 1 every 2 weeks.

Allergen Extracts and Purified Natural Allergens

A simplex L3 larvae, extracted from the viscera and body cavity of blue whiting (*Micromesistus poutassou*) were supplied by the Department of Microbiology and Parasitology of the University of Santiago de Compostela, Spain. The larvae were washed in phosphate buffered saline (PBS), snap-frozen in liquid nitrogen and ground in a mortar. Afterwards, proteins were extracted by magnetic stirring (4 hours at 4°C) in saline solution, clarified by centrifugation, dialyzed, and lyophilized. In order to purify nAni s 1, an immunosorbent column was prepared by coupling rabbit anti-Ani s 1 polyclonal antibodies to CNBr-activated Sepharose 4B (6 mg of antibodies per milliliter of gel) as recommended by the manufacturer (GE-Healthcare). Subsequently, *A simplex* extract was passed through the column and after extensive washing with PBS, bound protein was eluted with 100 mM citrate buffer, pH 2.7. Fractions were collected in tubes containing neutralizing buffer (1 M Tris-HCl, pH 8.0), pooled, and concentrated by ultrafiltration using an Amicon Ultra-4 device with a 5 kDa cutoff (Millipore Co, Bedford, Massachusetts, USA). Further purification was performed by chromatography onto a Superdex S75 column in the Smart system (GE-Healthcare). The protein content was determined by the Bradford method.

Analytical Methods

Specific IgE levels to *A simplex* extract and Ani s 1 purified allergens were evaluated by enzyme allergosorbent test (EAST) following the manufacturer's instructions for specific IgE

enzyme immunoassay (Hytec, Hycor Biomedical, Kassel, Germany). After preliminary testing, optimal concentrations of *A simplex* extract and Ani s 1 (at 1.8 and 0.1 mg/mL, respectively) were coupled to CNBr-activated paper discs [11]. For ELISA, microwell plates (Greiner, Frickenhausen, Germany) were coated overnight at room temperature with 100 ng of Ani s 1 (100 μL of 1 μg/mL) in 0.1 M bicarbonate buffer, pH 9.6, and blocked with 1% bovine serum albumin and 0.05% Tween 20 in PBS. Afterwards, sera from *A simplex*-allergic patients and control individuals (dilution 1:2) were added and incubated for 1 hour at 37°C, and detection was carried out as previously described [12]. Levels higher than 3 times the conjugate control were considered positive. For ELISA-inhibition assays, the pool of sera from *A simplex*-allergic patients was preincubated at 4°C overnight with solutions containing various concentrations of natural and recombinant Ani s 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [13] in 12.5% polyacrylamide gels. Proteins were stained with Coomassie blue or transferred electrophoretically onto polyvinylene difluoride membranes and blocked for 1 hour at room temperature with 5% skimmed milk and 0.1% Tween 20 in PBS. Membranes were incubated overnight at 4°C with the pool of sera (diluted 1:5) followed by incubation with anti-human IgE conjugated to horseradish peroxidase (Southern Biotech, Birmingham, Alabama, USA). Reacting-bands were detected by chemiluminescence as recommended by the manufacturer (ECL-Plus, GE-Healthcare).

Statistical Analysis

Statistical data analysis was performed by using the Wilcoxon signed rank test and calculating the Spearman correlation coefficient (ρ) with the SPSS version 11.0 package for Windows (SPSS Inc, Chicago, Illinois, USA). A *P* value of less than .05 was considered significant.

Results

Expression and Purification of rAni s 1

cDNA coding for Ani s 1 was obtained by reverse transcription-PCR from mRNA of *A simplex* larvae with primers flanking the mature protein designed from the nucleotide sequence reported previously [9]. The amino acid sequence of the encoded protein contained 99.4% identical amino acids to the reported one, except for the replacement Asn185→Lys (numbering of the precursor protein), due to a G→A substitution in the nucleotide sequence. rAni s 1 expressed in *E coli* was purified from inclusion bodies solubilized with 6 M guanidine hydrochloride. The expressed protein was refolded by dilution and progressive step dialysis followed by oxidative refolding in the presence of cysteine-cystine. The monomeric molecule was separated from aggregates (dimeric, trimeric, and polymeric forms) by means of gel permeation chromatography with a final yield of 2 mg/L culture. The recombinant allergen showed a molecular mass migration in SDS-PAGE slightly higher than the natural molecule (Figure 1A).

Purification of nAni s 1

Natural Ani s 1 was purified from *A simplex* L3 larvae extract using immunoaffinity chromatography with anti-rAni s 1 polyclonal antibodies, followed by final size exclusion chromatography. The purified protein migrated as a single band of 21 kDa under reduced (Figure 1A) and nonreduced (data not shown) conditions. nAni s 1 was present in small amounts in the crude larvae extract (less than 0.2% of the

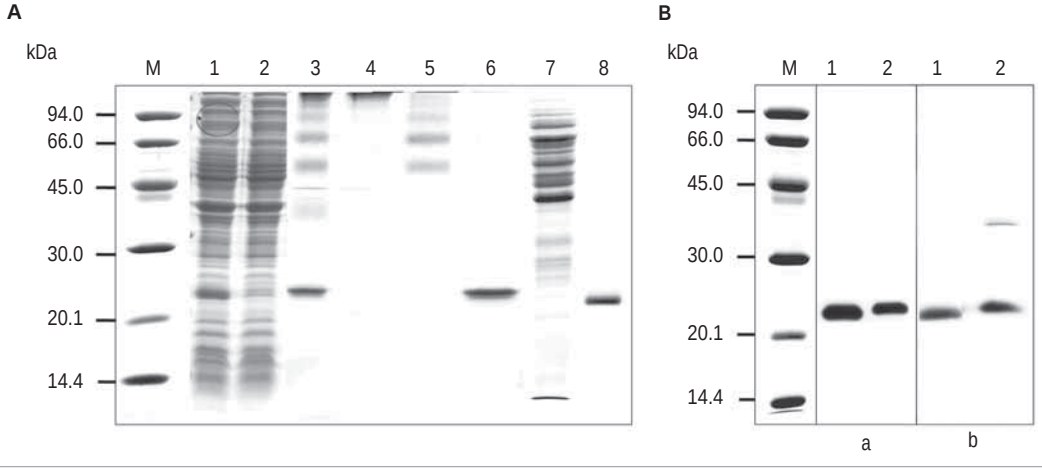

Figure 1. Electrophoretic analysis of Ani s 1. (A) Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions from Ani s 1 purification: lane M, molecular mass markers; lane 1, bacterial expression; lane 2, soluble fraction; lane 3, inclusion bodies fraction; lanes 4 and 5, polymeric and oligomeric fractions, respectively, of rAni s 1 after permeability chromatography; lane 6, purified monomeric rAni s 1; lane 7, whole extract of *A simplex* extract; lane 8, purified nAni s 1. (B) Immunoblots of nAni s 1 (lane 1) and rAni s 1 (lane 2) incubated with rabbit antiserum against Ani s 1 (a) or a pool of sera from *Anisakis*-species-allergic patients (b).

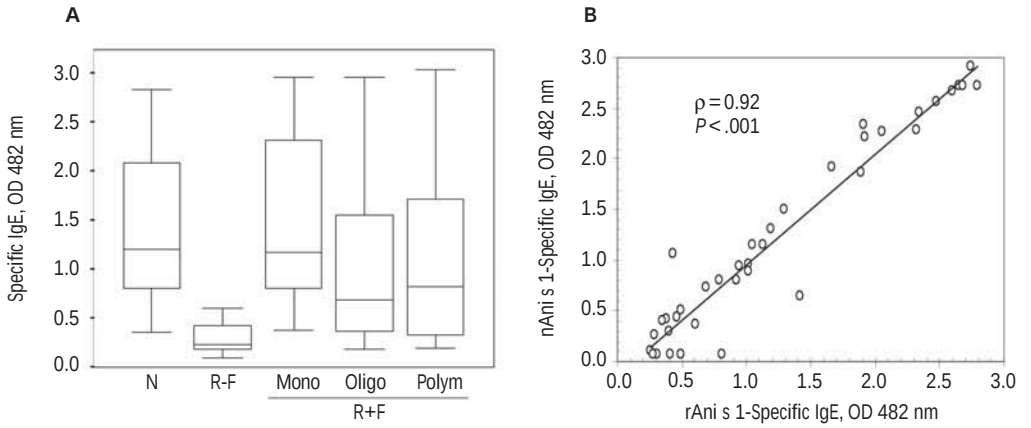

Figure 2. (A) Box plots of the mean of duplicate measurements of specific immunoglobulin E (IgE) from Ani s 1-reactive sera to purified nAni s 1 (N), unfolded rAni s 1. R-F indicates recombinant without refolding; Mono, monomeric; Oligo, di- and trimeric; Polym, polymeric forms of correctly folded rAni s 1 (recombinant with refolding, R+F). (B) Enzyme-linked immunosorbent assay with natural or recombinant Ani s 1 in the solid phase, incubated with individual sera from Ani s 1-reactive sera. OD indicates optical density; ρ is the Spearman correlation coefficient.

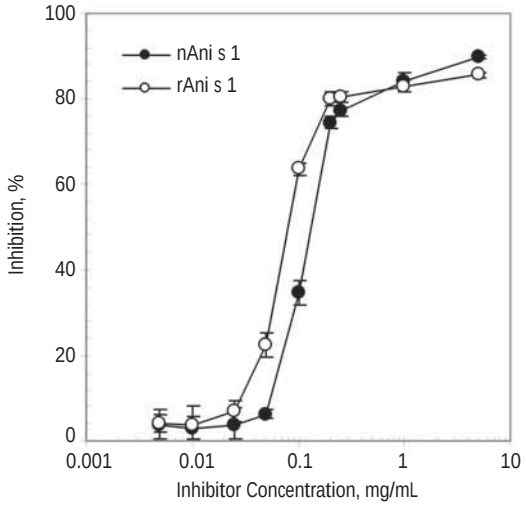

Figure 3. The binding of specific immunoglobulin E from a pool of sera from *A simplex*-allergic patients to nAni s 1 was inhibited by the addition of rAni s 1. An inhibition curve with nAni s 1 was used for comparison. Samples were analyzed in triplicate. Error bars indicate the SD of the measurements

extract protein as measured by a specific ELISA for Ani s 1 based on monoclonal antibodies) [14]. The final yield after purification was 50 μg/g of larvae.

Immunochemical Properties

Immunoblotting experiments with rabbit polyclonal antisera and a pool of sera from *A simplex*-allergic patients showed that both natural and monomeric recombinant Ani s 1 reacted similarly (Figure 1B). Preliminary ELISA experiments showed that rAni s 1, before the oxidative refolding, gave lower IgE-binding levels than those obtained for nAni s 1, resulting in 55% false negative results (data not shown). After oxidative refolding, monomeric rAni s 1 reactivity to 53 sera from *A simplex*-allergic patients was not significantly different from that of the natural allergen (Figure 2A). In contrast, IgE-reactivities of oligomeric (dimers and trimers) and polymeric aggregates were significantly lower than that of the natural counterpart ($P < .001$). IgE-binding activity of the monomeric recombinant form gave an excellent correlation ($\rho = 0.92$, $P < .001$) with that of the natural allergen (Figure 2B). Using individual sera from *A simplex*-allergic patients, EAST experiments showed that 46 out of 53 sera (86.8%) reacted to rAni s 1 while only 4 reactions were observed among 40 control sera. Furthermore, when the IgE-binding capacity of rAni s 1 was analyzed by means of ELISA inhibition with the same pool of sera from *A simplex*-allergic patients, the dose-response inhibition curve obtained with monomeric rAni s 1 overlapped with that of nAni s 1, reaching up to 90% inhibition of IgE-binding (Figure 3) and demonstrating that IgE-binding epitopes are preserved in rAni s 1.

Discussion

In recent years, *A simplex* has been recognized as an important cause of disease in man both as an active pathogen and as a food-borne allergen source [1]. However, serodiagnosis of both diseases is hindered by the unacceptable proportion of false-positive results when whole parasite extract is used [2,3]. Allergenic cross-reactivity could be responsible for this effect since it has been described that *A simplex* share IgE-binding components with other nematodes, but also with dust mites, chironomids, crustaceans, and cockroaches [1].

In this study, EAST showed that 86.8% of sera from

A simplex-allergic patients have specific IgE against Ani s 1. That result is similar to the reactivity prevalence of 85% previously detected by immunoblotting with raw extracts [5,7]. Both results confirm the designation of Ani s 1 as a major allergen. Determination by immunoblotting of specific IgE directed to Ani s 1 has been reported as a useful diagnostic method due to its high sensitivity and specificity (86% and 90%, respectively) [7,15,16]. Nevertheless, the use of well-defined and pure allergen for in vivo and in vitro techniques will overcome some of the problems found in the diagnosis of sensitized patients. Unfortunately, the purification of natural Ani s 1 is laborious and expensive since larvae must be extracted manually from parasitized fish and the protein is present in a very small amount in the *A simplex* extract [14]. Cloning of Ani s 1 cDNA did not facilitate the availability of recombinant allergen since the recovery of *E coli*-expressed protein occurs in the insoluble fraction [9] and poor correlation was found between the IgE-binding properties of inclusion bodies-solubilized rAni s 1 and those of natural allergen (Figure 2A). That the efficient production of immunochemically equivalent recombinant allergens is a bottleneck in the use of recombinant proteins for diagnosis has been reported for other allergens, such as Der p 1, Phl p 1, or Pla a 2 [17-19]. Very recently, the expression of a biologically active Ani s 1 has been reported using the *Pichia pastoris* expression system, although this method is time-consuming and a lower yield (0.8 mg/L) was obtained [20]. We show here that the oxidative refolding of an *E coli*-expressed rAni s 1 followed by chromatographic separation of monomeric molecules was essential for IgE recognition as was demonstrated by means of immunoblotting, ELISA and ELISA-inhibition experiments. The monomeric rAni s 1 obtained was immunochemically equivalent to the natural counterpart and therefore it could be useful for the in vitro diagnosis of anisakiasis and *A simplex*-mediated allergy.

In conclusion, we report the production in *E coli* of rAni s 1 in a soluble and correctly folded form with most of its immunochemical features identical to those of its natural counterpart. The results described here may have clinical applications in the future although additional purified allergens should be studied in order to find the right cocktail of recombinant allergens needed for an accurate molecular diagnosis of *A simplex* sensitization.

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