

Cloning and characterisation of the *Anisakis simplex* allergen Ani s 4 as a cysteine-protease inhibitor [☆]

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Abstract

Anisakis simplex is a nematode that can parasitise humans who eat raw or undercooked fish containing live L3s. Larvae invading the gastrointestinal mucosa excrete/secret proteins implicated in the pathogenesis of anisakiasis that can induce IgE mediated symptoms. Misdiagnosis of anisakiasis, due to cross-reactivity, makes it necessary to develop new diagnostic tools. Recombinant allergens have proved to be useful for diagnosis of other parasitoses. Among the *Anisakis* allergens, Ani s 4 was considered to be a good potential diagnostic protein because of its heat resistance and its importance in the clinical history of sensitised patients. Therefore, the objective of this study was to clone and characterise the cDNA encoding this allergen. The Ani s 4 mRNA sequence was obtained using a PCR-based strategy. The Ani s 4 amino acid sequence contained the characteristic domains of cystatins. Mature recombinant Ani s 4 was expressed in a bacterial system as a His-tagged soluble protein. The recombinant Ani s 4 inhibited the cleavage of a peptide substrate by papain with a K_i value of 20.6 nM. Immunoblotting, ELISA, a commercial fluorescence-enzyme-immunoassay and a basophil activation test were used to study the allergenic properties of rAni s 4, demonstrating that the recombinant allergen contained the same IgE epitopes as the native Ani s 4, and that it was a biologically active allergen since it activated basophils from patients with allergy to *A. simplex* in a specific concentration-dependent manner. Ani s 4 was localised by immunohistochemical methods, using a polyclonal anti-Ani s 4 anti-serum, in both the secretory gland and the basal layer of the cuticle of *A. simplex* L3. In conclusion, we believe that Ani s 4 is the first nematode cystatin that is a human allergen. The resulting rAni s 4 retains all allergenic properties of the natural allergen, and can therefore be used in immunodiagnosis of human anisakiasis.

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1. Introduction

Anisakis simplex is a nematode of the Anisakidae family that parasitises humans who eat raw or undercooked fish that contain live L3s. The L3 can invade the gastrointestinal

mucosa releasing excretory/secretory (E/S) proteins involved in the pathogenesis of anisakiasis (Matthews, 1982; Hotez et al., 1994; Dziekonska-Rynko et al., 2004.). This tissue-penetrating parasite can induce IgE-mediated reactions with several clinical manifestations ranging from urticaria or angioedema to anaphylaxis (Kasuya et al., 1990; Audicana et al., 1995; Gonzalez-Quijada et al., 2005). In addition, allergic occupational symptoms induced by *A. simplex* antigens, such as urticaria, conjunctivitis and asthma (Armentia et al., 1998; Scala et al., 2001), and sub-clinical sensitisation among healthy populations (Garcia

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et al., 1997; Caballero and Moneo, 2003) have also been reported. Current diagnostic methods, such as a skin prick test or fluorescence-enzyme-immunoassay (FEIA) use parasite crude extracts consisting of multiple antigens, some of which cross-react with other parasites (Fernandez-Caldas et al., 1998; Iglesias et al., 1996; Lozano et al., 2004; Perteguer et al., 2003) that can cause false positives in patients infected with other parasites (Lorenzo et al., 2000; Caballero and Moneo, 2001). Approaches to decrease the rate of false positives included the use of purified (Moneo et al., 2005; Rodero et al., 2004, 2005) or deglycosylated antigens (Lorenzo et al., 2000) of the parasite in ELISA, immunoblotting and skin prick tests. Another tool proposed for *A. simplex* allergy diagnosis was E/S proteins (Baeza et al., 2004). The availability and characterisation of recombinant proteins have improved the specificity of the diagnostic procedures for some parasite infections (Rodríguez-Perez et al., 2003; Baskar et al., 2004). These reagents defined the involvement of these proteins in the parasite life cycle and pathogenesis (Ford et al., 2005), and their use in specific immunotherapy was suggested by some authors (Ghosh et al., 2006).

Four cDNAs encoding *A. simplex* allergens have been cloned to date. Ani s 1, a 24 kDa major allergen showing certain homology with Kunitz-type serine protease inhibitors, was expressed in bacteria (Shimakura et al., 2004), although the recombinant protein did not show the same IgE-binding properties as the natural allergen. There is a 21 kDa minor allergen, also named Ani s 1, that it is a homologue to *Onchocerca volvulus* and *Caenorhabditis elegans* troponin C and it is recognised by the 20% of sensitised patients (Arrieta et al., 2000). Ani s 2 is a paramyosin and a major allergen with immunologic cross-reactivity with paramyosin from other nematodes, trematodes and arthropods, due to high sequence homology (Perez-Perez et al., 2000). Ani s 3, a tropomyosin (Asturias et al., 2000a,b), seems to be an unimportant protein for *A. simplex* allergy because it is only recognised by patients having specific IgE against *A. simplex* allergens due to cross-reactivity with proteins from other sources, such as house dust mites or insects. Finally, we purified an allergen from *A. simplex*, Ani s 4, that appears in the E/S protein fraction of the parasite (Moneo et al., 2005). It was a highly heat-resistant protein and it was suggested that glycosylation was not involved in its allergenicity. We observed that after heating a crude parasite extract, some patients did not lose the ability to respond to this protein, suggesting that this protein must be clinically relevant for them. Ani s 4 was recognised by 27% of sensitised patients and it was observed that anaphylactic episodes were more frequent among those patients who recognised this allergen than those who did not recognise it (Moneo et al., 2005). The thermal stability of Ani s 4 is shared by other parasite antigens, such as ABA-1, a non-glycosylated lipid-binding polyprotein allergen of *Ascaris* (Xia et al., 2000). Therefore, Ani s 4 was considered a good candidate to be cloned and expressed, in order to study this protein in depth and to test its potential use as a diagnostic tool.

Here we describe the cloning and expression of Ani s 4, a member of the cystatin family, and show that Ani s 4 is expressed in the secretory gland and the cuticle of *A. simplex* L3.

2. Materials and methods

2.1. Patients' sera

IgE-mediated hypersensitivity to *A. simplex* (As+ patients) was considered when the following criteria were met: compatible clinical history, *A. simplex*-positive specific IgE values and negative for specific IgE against fish. Compatible clinical history included anaphylaxis, abdominal pain, vomiting, acute urticaria and/or angioedema within 12 h of eating fish. Sensitivity to Ani s 4 (As+ A4+ patients) was considered when this allergen was recognised by the patients' IgE by immunoblotting. Control samples (As-) from healthy people with no clinical history of allergy and atopic individuals with no *A. simplex* hypersensitivity were included in the assays.

2.2. Ani s 4 mRNA sequencing

Total RNA was isolated by TRIzol extraction (Gibco, Invitrogen, Carlsbad, CA, USA) from 180 mg of *A. simplex* L3 extracted from the muscle of hake (*Merluccius merluccius*). Two micrograms of total RNA was reverse transcribed to cDNA using an oligo d(T)₂₅ primer and Omniscript RT Kit (Qiagen). PCRs were performed in a Px2 Thermal Cycler (Thermo Electron Corporation, Foster City, CA, USA) using *Taq* DNA Polymerase Core kit (Qiagen). The PCR primers (Table 1) were designed considering the back-translation of the Ani s 4 N-terminal amino acid sequence (Moneo et al., 2005) and its comparison with protein sequences from databases available at NCBI (<http://www.ncbi.nlm.nih.gov>) and with nematode expressed sequence tag (EST) databases from the Nematode EST Sequencing Project (<http://www.nematode.net>). Two PCR strategies were performed to amplify Ani s 4 mRNA. First, PCR using the primers AS4-S6 and AS4-AS5 was performed under the following conditions: initial denaturation: 95 °C for 5 min; amplification: 94 °C for 1 min, 50 °C for 2 min, 72 °C for 1 min, 35 cycles; final elongation step: 72 °C for 1 min. As no single band was obtained, PCR products were reamplified using the primers AS4-S5 and AS4-AS4 under the same conditions. Second, hotstart touchdown nested PCRs were performed using the primers AS4-S6 and AS4-AS6 for amplification, and the primers AS4-S5 and AS4-AS6 for reamplification, under the following conditions: initial denaturation: 95 °C for 5 min; amplification: 94 °C for 30 s, 60 °C, decreasing 1 °C in each cycle until 50 °C, for 30 s, 72 °C for 30 s, 40 cycles; final elongation step: 72 °C for 1 min. All PCR products were purified using QIAquick Gel Extraction kit (Qiagen), cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and expanded in *Escherichia coli*

Table 1
Nucleotide sequences of primers used for PCR amplification of Ani s 4

Name	Nucleotide position	Primer sequence ^{a,b}
AS4-S5	113–135	5'-GAYGTBAAYGAYCCRGARATHAA-3'
AS4-S6	85–102	5'-GGAATGCCCKGGTGGYWSC-3'
AS4-AS3	–	5'-GAGGAGAAGCCCGGTTTT-3'
AS4-AS4	313–332	5'-GAAGTTCTCCCARGGCTTCT-3'
AS4-AS5	329–354	5'-CRTTTATGCAGMTTTCATYCTTTGA-3'
AS4-AS6	237–256	5'-TTTGTACCAGCGACTACCTG-3'
AS4-D1	85–104	5'-GGAAYGCGYAGGCGGATCGTC-3'
AS4-D3	85–94	5'-GACGACGACAAGATAGGAAYGCGYAG-3'
AS4-3R	137–159	5'-GAGCTCGCTGGTAAGTCTATCGC-3'
AS4-5R	187–310	5'-CCAAATGGTAACGGTACACAGGTC-3'
AS4-E	9–30	5'-GATTCATAAATATGCAATCCAG-3'

Anisakis simplex nucleotide positions for primers AS4-AS4, AS4-AS5 and AS4-AS6 are only indicative.

^a R = A/G, Y = C/T, M = A/C, K = G/T, S = C/G, W = A/T, B = C/G/T, H = A/C/T, N = A/C/G/T.

^b Nucleotides in italics are non-specific for Ani s 4.

OneShot TOP 10 (Invitrogen) or *E. coli* NovaBlue (Novagen, Merck KGaA, Darmstadt, Germany). Bacterial plasmid DNA was obtained using a QIAprep Spin Miniprep Kit (Qiagen) and it was sequenced by the DNA Automatic Sequence Service (Centro de Investigaciones Biológicas, Centro Superior de Investigaciones Científicas Madrid, Spain). The DNA sequences obtained were aligned using Clustalw multiple alignment software. This alignment allowed us to design specific primers (AS4-3R and AS4-5R; Table 1) to be used in rapid amplification of cDNA 3'- and 5'-ends (RACE) PCR in order to obtain the complete sequence of Ani s 4 mRNA, using a BD Smart RACE cDNA Amplification Kit (BD Biosciences, Erembodegum, Belgium). PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced as described above. The complete open reading frame (ORF) of Ani s 4 mRNA was obtained by PCR using the primer pair AS4-E (designed from the sequences obtained in RACE PCRs) and AS4-AS3 (Table 1).

2.3. *rAni s 4* production and purification

Anisakis simplex mRNA was reverse transcribed using the d(T)₂₅ primer as described above. Mature Ani s 4 cDNA sequence was obtained by PCR using specific primers to amplify the product of the RT. KOD HiFi DNA Polymerase (Novagen) was used in a PCR using the primers AS4-D1 (Table 1) and AS4-AS3 under the following conditions: initial denaturation: 95 °C for 5 min; amplification: 94 °C for 30 s, 72 °C, decreasing 1 °C in each cycle until 58 °C, for 30 s, 72 °C for 30 s, 45 cycles; elongation: 72 °C for 1 min. To include the appropriate sequence previous to the mature Ani s 4 sequence for directional cloning into a pET46 EK/LIC (Novagen) vector, the PCR product (1 µl) was re-amplified using the primers AS-D3 (Table 1) and AS4-AS3 and the PCR conditions: initial denaturation: 95 °C for 5 min; amplification: 94 °C for 30 s, 68 °C (decreasing 1 °C in each cycle until 58 °C) for 30 s, 72 °C for 30 s, 40 cycles; final elongation: 72 °C for 1 min. The PCR product was purified from an agarose

gel band as described and cloned into a pET46 vector. *Escherichia coli* BL21 Star (DE3) One Shot (Invitrogen) were transformed with the recombinant plasmid. An overnight culture was diluted 1:20, grown at 37 °C to an OD₆₀₀ of 0.6 and induced with 0.75 mM isopropyl thiogalactoside (IPTG) for 1 h at 37 °C. The culture was centrifuged and the pellet was resuspended in BugBuster Reagent (Novagen). Recombinant Ani s 4, expressed as a 6×His tagged protein, was purified from the soluble protein fraction using His*Bind Resin (Novagen) following manufacturer's specifications, except that the elution buffer was 100 mM imidazole. The purified recombinant protein was concentrated and imidazole was eliminated by filtration using a Microsep 3K Cartridge (Filtron Technology Corporation, Northborough, MA, USA).

2.4. *rAni s 4* modelling

Modelling of the *rAni s 4* theoretical structure was performed with the First Approach Mode of Swiss-Model software (www.swissmodel.expasy.org) using the Protein Data Bank (PDB) template 1EQK (cystatin from *Oryza sativa*).

2.5. *nAni s 4* purification

Anisakis simplex L3s (1.5 g) extracted from hake muscle (*M. merluccius*) 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 4,000g 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 4,000g 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 4,000g for 15 min. The supernatant was discarded and the pellet was resuspended in 500 µl of PBS. Native Ani s 4 was purified from this resuspended pellet by reversed-phase HPLC as described (Moneo et al., 2000).

2.6. Protein concentration determination

The protein concentration was determined using Bradford Reagent (Fluka, Sigma–Aldrich Inc., St. Louis, Missouri, USA), using BSA as the standard. A_{450} was read on a microplate reader.

2.7. Functional characterisation of rAni s 4

A papain solution (50 mM $(\text{PO}_4)^{3-}$, 10 mM EDTA 5 mM Cys, 1 U/ml papain, pH 6.7) was activated for 30 min at 37 °C and pre-incubated with 1, 0.5, 0.25, 0.125 µg/ml (final concentrations) of rAni s 4, or 1 µg/ml of a non-allergenic *A. simplex* recombinant protein expressed in *E. coli*, for 20 min at room temperature. This non-allergenic recombinant protein was a misfolded serine protease inhibitor from *A. simplex* that did not show any activity. Papain activity was assayed using Ac-Phe-Gly-pNA (Calbiochem, EMD Biosciences, Merck, Darmstadt, Germany) (1–0.125 mM; final volume 200 µl). A_{405} was measured each minute for 20 min. K_i values were determined from double reciprocal plotting of the data (Dixon et al., 1979) expressing the velocity of the reaction as $\Delta A_{405} \text{ min}^{-1} \text{ ml}^{-1}$.

2.8. SDS–PAGE and immunoblotting

Different fractions of protein, with or without 350 mM β-mercaptoethanol treatment, were applied to a 16% polyacrylamide gel as described (Moneo et al., 2000). For purified protein, 16% acrylamide–Tris–Tricine buffered SDS–PAGEs were used as described (Schagger and Von Jagow, 1987). For immunoblotting assays, proteins were transferred to nitrocellulose membranes by diffusion (Moneo et al., 1995). Then, membranes were blocked (3% Nonidet P-40 in PBS for 30 min) and subsequent incubations with pools of human sera, mouse anti-human IgE mAb (Ingenasa, Madrid, Spain) and alkaline-phosphatase labelled goat anti-mouse IgG (Biosource International, CA, USA) were performed. BCIP–NBT (5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium) was the substrate used for revealing the immunoblots. For specific IgE detection in individual human samples, the antigen was loaded on a 16% polyacrylamide gel without lanes and the electrophoresis and the transfer were carried out as previously described except that after blocking, membranes were placed on a Mini-Protean II Multiscreen (Bio-Rad) and incubated with individual human sera (Moneo et al., 1995). For immunoblotting inhibition assays, the same experiment was performed, except that human sera was pre-incubated with 0, 1, 0.2, 0.04 or 0.008 µg protein for 2 h. The inhibition was measured by densitometry using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.9. Immunocapture ELISA

One hundred micrograms of rAni s 4 was conjugated with 0.8 mg m-periodate treated peroxidase as described

(Cuevas et al., 1983). Microtiter plate wells were coated with 1 µg of mouse anti-human IgE mAb (Ingenasa) and blocked with PBS, 0.05% Tween 20, 1% BSA for 1 h at room temperature. Then, subsequent incubations were performed with individual human sera (eight from As+ patients and 2 As– controls) for 18 h at 4 °C, peroxidase-conjugated-rAni s 4 (0, 0.5, 1, 2 and 4 µg) for 1 h at room temperature and finally with 2 mg/mL *o*-phenylenediamine in 0.1 M citrate–phosphate buffer, pH 5, 0.03% H_2O_2 for 90 min at room temperature. Reaction was stopped by adding 100 µl of 2 N H_2SO_4 and A_{492} was read in a microplate reader.

2.10. Detection of specific anti-Ani s 4 IgE

One milligram of rAni s 4 was incubated with 100 µg of biotin *N*-hydroxysuccinimide ester for 2 h and biotin-conjugated rAni s 4 was purified in a Sephadex G25 column. Specific IgE against rAni s 4 was measured by FEIA (ImmunoCAP System, Pharmacia, Uppsala, Sweden). ImmunoCAPs coated with streptavidin were incubated for 30 min at room temperature with 50 µl of biotin-conjugated rAni s 4 (69 µg/ml). Then, the immunoCAPs were hand loaded into a UniCAP 100® (Pharmacia) and the usual procedure was followed as indicated by the manufacturer.

2.11. Detection of rAni s 4-induced basophil activation

Flow cytometry was the method chosen to test if rAni s 4 was capable of activating basophils of *A. simplex*-sensitised patients. The assay was performed as described (Gonzalez-Muñoz et al., 2005). Proteins used for basophil activation consisted of an *A. simplex* crude extract (1.5 µg/ml) and purified rAni s 4 (1, 0.1 and 0.01 µg/ml). The same non-allergenic *A. simplex* recombinant protein used in the rAni s 4 enzymatic assay was used as a control (1 µg/ml). Nineteen patients were tested, 12 of them were As+A4– and seven were As+A4+. Ten As– samples, from six healthy and four atopic individuals, were included as controls.

2.12. Immunohistochemistry

Parasitised livers from hakes (*M. merluccius*) were dissected, cut into small fragments (5 × 5 mm), fixed in 10% buffered formalin for 24 h and immersed in paraffin. Four-micrometer paraffin sections were stained with H&E for histological study. Two paraffin blocks, which contained several cross-sections of larvae of *A. simplex*, including all parasite structures and organs, were selected for immunohistochemistry. Four-micrometer sections, placed on positively charged slides, were incubated with rabbit polyclonal antisera raised against rAni s 4 and diluted 1/8,000 in Dako Real antibody diluent (Dako, Glostrup, Denmark). The immunocomplexes formed were detected using a commercial detection system (Dako Real

Detection kit, Dako), according to the manufacturer's instructions. The kit is based on the labelled streptavidin–biotin method and is composed of biotinylated goat anti-mouse and anti-rabbit immunoglobulins, streptavidin conjugated to horseradish peroxidase and diaminobenzidine chromogen. All incubations were carried out on a Dako TechMate Horizon autostainer (Dako). Hematoxylin was used for counter staining. Controls included tissue sections treated with pre-immune rabbit serum as the primary antibody.

3. Results

3.1. Ani s 4 belongs to the cysteine-protease inhibitor family

Total RNA isolated from *A. simplex* L3 was reverse transcribed and amplified by PCR using degenerate primers in two different strategies. All the products obtained were cloned into a pGEM-T Easy vector and sequenced. Four products, of 250 and 100 bp, were compatible with the known Ani s 4 amino terminal amino acid sequence (Moneo et al., 2005). The 3' and 5' ends of Ani s 4 cDNA were obtained by RACE PCRs using specific primers that were designed according to the sequences obtained in the PCR rounds. The complete cDNA sequence of Ani s 4 was also obtained by PCR.

The Ani s 4 amino acid sequence (PDB Accession No. CAK50389) was deduced from the Ani s 4 mRNA nucleotide sequence (GenBank Accession No. AM279414) (Fig. 1). The ORF of Ani s 4 defines a protein of 115 amino acid residues with an estimated molecular weight of

12.7 kDa and a calculated isoelectric point of 5.56. No predicted glycosylation sites were found. Amino acids 1–22 are not found in mature Ani s 4 protein and they constitute a predicted signal peptide (<http://www.cbs.dtu.dk/services/SignalP>). This information is compatible with the appearance of nAni s 4 (sequence starting at Gly 23) among the E/S antigens of *A. simplex*. The Ani s 4 complete amino acid sequence was compared with protein databases, showing some similarities with family 2 cystatins (Fig. 1). The alignment of Ani s 4 with other cystatins showed an identity of 36% with *O. sativa* cystatin, 27% with *O. volvulus* CPI-1, 25% and 24% with *Brugia malayi* CPI-1 and CPI-2, respectively, and 18% with human cystatin C. Ani s 4 possesses two of the three characteristic domains of cystatins. It has a Gly close to the amino terminal end of the mature protein and a QVVAG domain (amino acids 70–74) that constitutes the first hairpin loop, but it lacks the second characteristic hairpin loop PW. In this place, Ani s 4 contains KW (amino acids 90–91). Ani s 4 also possesses two cysteine residues (Cys 92 and Cys 112) that likely form a disulfide bond, found in other members of family 2 cystatins (Fig. 1). Ani s 4 is smaller than other family 2 cystatin members; it has 28 amino acid residues less than *C. elegans* cystatin C (GenBank Accession No. CAC33822) or 46 less than *B. malayi* CPI-2. The sequence deleted in Ani s 4 with respect to the *C. elegans* and *B. malayi* cystatin sequences corresponds to a central region between the functional domains QxVxG and PW and to a sequence previous to the functional domain GG (Fig. 1).

The molecular structure of rAni s 4, modelled on the X-Ray structure from *O. sativa* cystatin, includes a five turn

Ani s 4	-----MQSRIVVAVVVFCCIILMQTE-----AGMLGGSSDQVDNDPE	37
CAC33822	-----MKAILVFALIAIS--IISVNA-----GMMTGGSVEQDASQKE	35
AAD51085	-----MFFPIVWLSVLLIISKSEFAR-----EIRLSRATY-NEDDEE	35
AAD51087	-----MFYLIWFSAFATISKSEFAR-----EIRLGKLTNDNEDDEE	36
AAD51086	MMSTMSIKEGLLVILLSLFLDFTTALHREIPHME SKGQMQRGQVLLGGWQERSPED	60
AAL30830	-----MS SDGG-----FVLGGVEFVGNENDLH	22
CAA36497	--MAGPLRAPLILLAL LAVALAVS PAAGSSP-----GKPPRLVGGPMDASVEEEG	48
Ani s 4	<u>IKELAGKSI</u> AKISAMINDGKP--HELKVVSAKKQV VAGEKYTLEILV-----	83
CAC33822	YSDKAWKAVKGINDQASNNGPYIYAPIKVTKASTQV VAGISTKLEVLVGESNCKKGLQA	95
AAD51085	IQEVAEKAMEQVNDQTRYRNL--YKLVRVISAQTQV VAGIKYYLTILAAPTTCRKG---A	90
AAD51087	IQEVAEKAMIQVNEKMKYDNL--YKLVKVINARTQV VAGMKYYLTILTAPTTCRK---N	90
AAD51086	I L E L L P S V L T K V N Q Q S N D E Y H -- L M P I K L L K V S S Q V V A G V K Y K M E V Q V A R S E C K K S -- A S	116
AAL30830	LVDLARFAVTEHNKKAN----SLLEFEKLVSVKQV VAGTLYYFTIEVK-----	67
CAA36497	VRRALDFAVGEYNKASNDMYH--SRALQVVRARKQIVAGVNYFLDVELGRITCTCTKT--QP	104
Ani s 4	-----KGDGHQDLCTVTIWQKKWENFEFVKVLKCDHQ-----	115
CAC33822	HEITSSNCQIKDGGSRALYQVTIWKEKPWENFEQFTVEKIRDVTADEQF	143
AAD51085	VGMNEMKCTIDSSKPTKQFKIEVWSAPWQNTFKVTLT-----	127
AAD51087	SGMS PANCAIDHSKPKKKVILEVWSAPWQNI FKVTMA-----	127
AAD51086	EQVNLKTCKKLEGHFDQVMTLEVWEKPWEDFLQVNI LETKVLSSV---	161
AAL30830	-----EGDAKKLYEAKVWEKPWMDFKELQEFKPVDAASANA--	102
CAA36497	NLDNCFPHDQPHLKRKAFCSFQIYAVPWQGTMTLSKSTCQDA-----	146

Fig. 1. Comparison of Ani s 4 amino acid (PDB Accession No. CAK50389) sequence with other family 2 cystatin sequences. Ani s 4 from *A. simplex* was compared with cystatins from *O. sativa*, *B. malayi* CPI-1, *B. malayi* CPI-2, *O. volvulus* CPI-1, *O. volvulus* CPI-2 and *Homo sapiens* cystatin C (GenBank Accession Nos. AAL30830, AAD51085, AAD51086, AAD51087, CAC33822, CAA36497, respectively). Gaps (–) were included for optimizing the alignment. Amino acids conserved in five of the proteins are in bold. The identities of the N-terminal amino acid sequences of recombinant and natural Ani s 4 are underlined. The cleavage site of Ani s 4 signal peptide is located between Ala 21–Gly 22. No putative glycosylation sites were found for Ani s 4 sequence.

α -helix and a five stranded antiparallel β -pleated sheet. This three dimensional structure brings together the functional cystatin domains (Fig. 2).

3.2. The rAni s 4 produced by *E. coli* retains the allergenic characteristics of nAni s 4

rAni s 4 was obtained mostly as a soluble protein from bacteria after induction with IPTG for 30 min. The rAni s 4 production kinetics showed maximum production after 1 h of induction and there were no significant differences in the rAni s 4 amounts recovered from the bacterial extracts during the following 3 h (data not shown). Purification through affinity chromatography, using a Ni^{2+} resin, recovered the protein mainly as a monomer, as can be seen by SDS-PAGE and immunoblot (Fig. 3). β -Mercaptoethanol treatment converted all the protein to its monomeric form without losing allergenicity (Fig. 3).

With the aim of comparing the IgE binding capacity of rAni s 4 with that of nAni s 4, both proteins were tested by immunoblot with 15 As+ patients' sera. Five As- patients' sera were tested as controls. We observed that all the sera that recognised nAni s 4 also recognised rAni s 4 (Fig. 4). To compare the IgE binding characteristics of natural and recombinant Ani s 4, immunoblot inhibition studies were performed. Competition for IgE binding to immobilised natural allergen by soluble rAni s 4 was visualised as an attenuation of IgE binding to immobilised protein

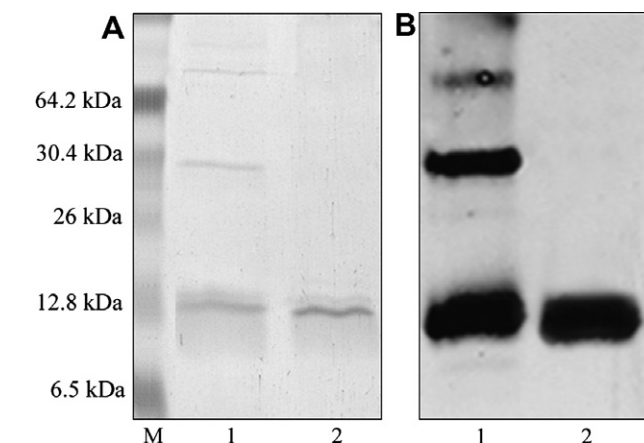


Fig. 3. Purified rAni s 4 treatment with β -mercaptoethanol. (A) SDS-PAGE. (B) Immunoblotting revealed with a pool of As+ patients' sera. Five micrograms of purified rAni s 4 without treatment (lane 1) or treated with 350 mM β -mercaptoethanol (lane 2). Lane M, molecular weight markers.

after pre-incubation of patient sera with soluble rAni s 4. The same experiment was performed with immobilised rAni s 4. Both assays showed concentration-dependent inhibitions (>95%) of the soluble protein used in pre-incubations of patient sera (Fig. 4). No inhibition of the IgE binding was observed when the sera were pre-incubated with an irrelevant recombinant protein.

IgE binding capacity of the recombinant protein was assessed with eight sera from *A. simplex* allergic patients.

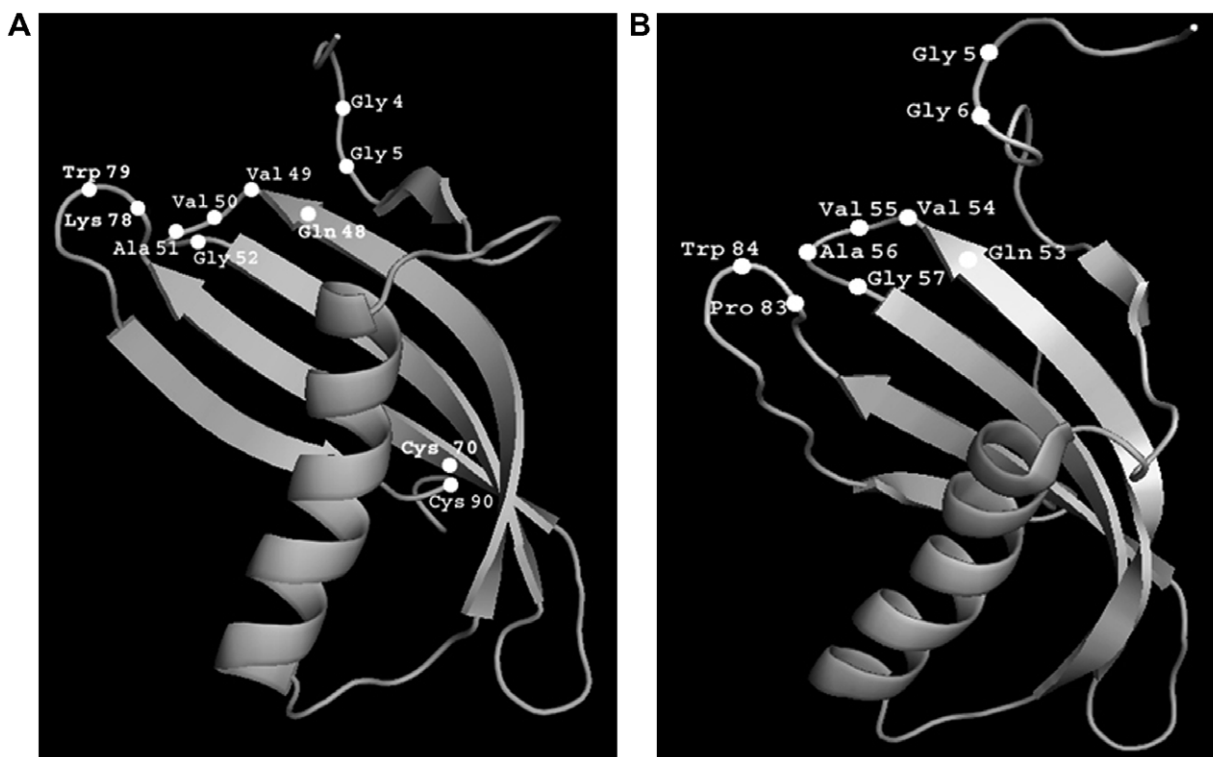


Fig. 2. Ani s 4 (A) and oryzacystatin (B) three-dimensional structures. Ani s 4 was modelled on the crystal structure of rice (*O. sativa*) cystatin. Ribbon representation of Ani s 4 shows a five turn α -helix and five stranded antiparallel β -pleated sheet. The amino acids of the three functional domains of cystatins are highlighted in both structures and the two cysteines, conserved in the Ani s 4 sequence but not in the rice cystatin sequence, are also indicated.

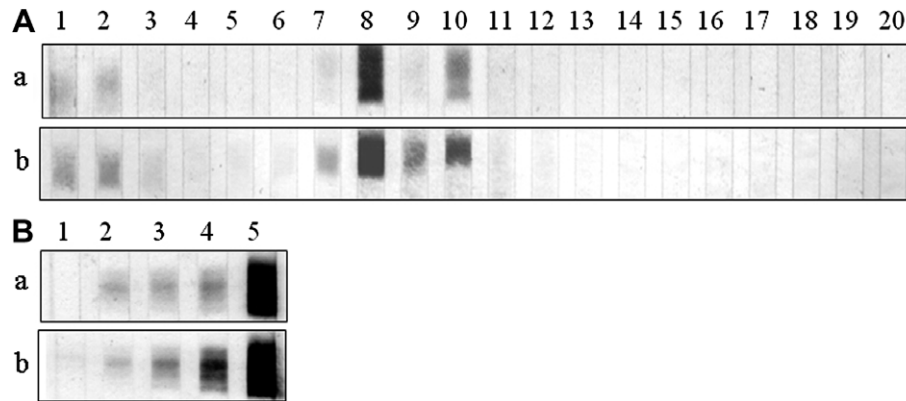


Fig. 4. Specific IgE recognition of Ani s 4 by sensitised patients. (A) Native (a) and recombinant (b) Ani s 4 recognition by individual patients IgE. Lanes 1–15: As+ patients. Lanes 16–20: As- patients. (B) Inhibition immunoblotting of immobilised natural (a) and recombinant (b) Ani s 4 by soluble rAni s 4 (lane 1, 1 µg; lane 2, 0.2 µg; lane 3, 0.04 µg; lane 4, 0.008 µg; lane 5, 0 µg).

Six of the eight As+ patients were also A4+, as shown by immunoblot, while the other two had not recognised rAni s 4 (Table 2). All patients who recognised rAni s 4 by immunoblot had positive ELISA and FEIA values, except subject 3, showing a FEIA value <0.35 kUA/L and a weak recognition of rAni s 4 by immunoblot. Comparison of the specific IgE anti-Ani s 4 values obtained by ELISA and FEIA showed a good correlation by Spearman's rho non-parametric test (correlation coefficient = 0.806; $P = 0.005$).

3.3. rAni s 4 inhibits the activity of papain

In order to check if rAni s 4 possessed cystatin activity, as suggested by its amino acid sequence, 0.2 U of the cysteine protease, papain, were inhibited with different concentrations of rAni s 4 and its activity as a protease was assessed using a colorimetric substrate. A_{405} data were analysed and it was shown that rAni s 4 did indeed inhibit papain from cleaving its substrate according to the double reciprocal plots (data not shown) with a K_i value of 20.6 nM. No inhibition of the protease activity was observed when pre-incubating the papain solution with an irrelevant recombinant protein.

3.4. rAni s 4 activates basophils from *A. simplex*-sensitised patients

In order to check if rAni s 4 was a biologically active allergen, whole blood samples from 19 patients (seven As+A4+ and 12 As+A4- by immunoblot) were challenged with the recombinant protein and the basophil specific activation was measured by flow cytometry. Ten controls were also tested.

The 12 As+A4- patients showed a basal activation control (without allergen) of 1.5% (0.4–4.9%, median and range) while the value obtained with the positive controls (anti-IgE stimulation) was 69.3% (18.1–94.5%). The percentage of activated basophils for these patients was

50.1% (2.7–93.4%) after incubation with *A. simplex* crude extract and 1.1% (0.0–10.6%) using 1 µg/ml rAni s 4.

The negative control for the seven As+A4+ patients was 1.7% (0.0–5.8%) and the positive control was 91.0% (25.8–94.9%). The percentage of basophil activation for these patients was 88.4% (24.1–97.5%) with *A. simplex* crude extract and 91.7% (61.3–98.6%) using rAni s 4 extract. The rAni s 4 percentage of basophil activation in As+A4+ patients was dependent on the protein concentration used in stimulation (Fig. 5). The activated basophil percentage in three of the As+A4+ patients, when stimulated with an irrelevant recombinant protein, was 1.2% (0.9–1.5%).

The 10 healthy and atopic controls (As-) showed percentages of basophil activation of 1.2% (0.0–2.4%) for the negative control and 85.4% (9.3–97.0%) for the positive control, while they showed percentages of 1.4% (0.0–2.4%) when stimulated with *A. simplex* crude extract and 0.8% (0.0–3.9%) using rAni s 4.

3.5. An antiserum raised against rAni s 4 localises nAni s 4 in the E/S gland and the cuticle of *A. simplex*

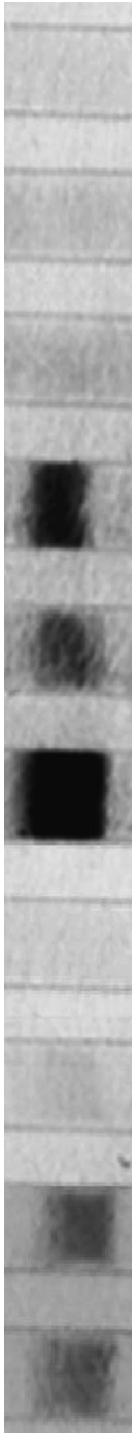
Parasite sections incubated with anti-rAni s 4 polyclonal antibody showed clear staining of the excretory gland (Fig. 6A). Intense labelling was also seen in the basal layer of the cuticle in some larvae (Fig. 6C). No staining of the excretory gland or any other structure of the parasite was found in control sections (Fig. 6B and D).

4. Discussion

Human anisakiasis is characterised by an IgE-mediated immune response to somatic and E/S proteins from the parasite. Laboratory diagnosis is usually based on the detection of IgE antibodies which bind to proteins in crude parasite extract. Due to immunological cross-reactivity between nematode proteins, detection of specific IgE against parasite E/S products has been proposed as a more

Table 2
Comparison between results obtained with *A. simplex* allergic patient sera tested by different assays

Subject No. Type	1 (As+A4+)	2 (As+A4+)	3 (As+A4+)	4 (As+A4+)	5 (As+A4+)	6 (As+A4+)	7 (As+A4+)	8 (As+A4+)	9 (As+)	10 (As+)
Specific IgE anti- <i>A. simplex</i> (kU/L)	14.1	54.4	19.0	5.58	>100	2.47	41.7	89.4	<0.35	<0.35
Specific IgE anti-Ani s 4 (A ₄₉₂)	0.746	0.944	0.148	0.089	0.671	0.125	0.516	0.091	0.083	0.092
Specific IgE anti-Ani s 4 (kU/L)	0.92	2.37	<0.35	<0.35	0.97	<0.35	0.84	<0.35	<0.35	<0.35
Specific IgE binding by immunoblotting										



Specific IgE anti-*A. simplex* was measured by fluorescence enzyme immunoassay (FEIA) CAP. Specific IgE anti-Ani s 4 was measured by ELISA, by FEIA CAP and by immunoblotting. A FEIA CAP value <0.35 kU/L was considered as a negative result. Subjects 9 and 10 were healthy controls. Sensitised patients that recognised Ani s 4 are noted as AS+A4+. Sensitised patients that did not recognise Ani s 4 are noted as AS+A4-. Healthy controls are noted as AS-.

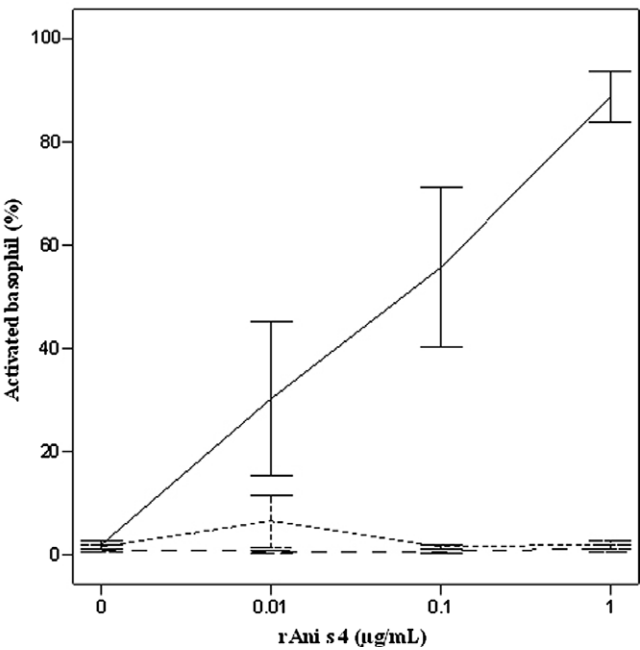


Fig. 5. rAni s 4-induced basophil activation. Basophil activation of As+A4+ patients (continuous line) was dependent on the rAni s 4 concentration used to stimulate whole blood basophils, while basophils of As+A4- patients (spotted line) and non-sensitised donors (discontinuous line) were not activated in the presence of the recombinant allergen. Results are expressed as percentages of CD63⁺ basophils above basal values (means ± SEM).

accurate diagnostic method for *Anisakis* allergy (Baeza et al., 2004). A component of the E/S products of the parasite, Ani s 4, was described as a heat-resistant 9 kDa allergen that was recognised by IgE in 27% of As+ patients (Moneo et al., 2005). We concluded that Ani s 4 was an important allergen and sought to clone and characterise the recombinant protein as a potential diagnostic tool.

The cDNA sequence data agreed with the sequence analysis of the amino terminus of the mature native protein (Moneo et al., 2005), implying that Ani s 4 is synthesised as a precursor protein with a signal peptide that is eliminated before secretion. The amino acid sequence of Ani s 4 showed that this protein belongs to the cystatin family, due to the presence of a consensus motif (QxVxG) (Abrahamson, M., Alvarez-Fernandez, M., Nathanson, C.M., 2003. Cystatins. Biochem. Soc. Symp. 70, 179–199) and the conserved Gly near the N-terminal amino acid. The contribution of both motifs to enzyme inhibition has been demonstrated in human cystatin C (Hall et al., 1993; Bjork et al., 1995) and chicken egg white cystatin (Auerswald et al., 1995). But the presence of this Gly residue is not a general requirement for the inhibitory activity, as described for the nematode Bm-CPI-1 cystatin (Gregory et al., 1997). Another highly conserved motif of cystatins is the Pro and Trp next to the C-terminal amino acid. This motif is located in the second hairpin loop and its contribution to enzyme binding has been reported (Auerswald et al., 1995). The Ani s 4 sequence showed a substitution of Pro for Lys in this region. A replacement of the Pro has also

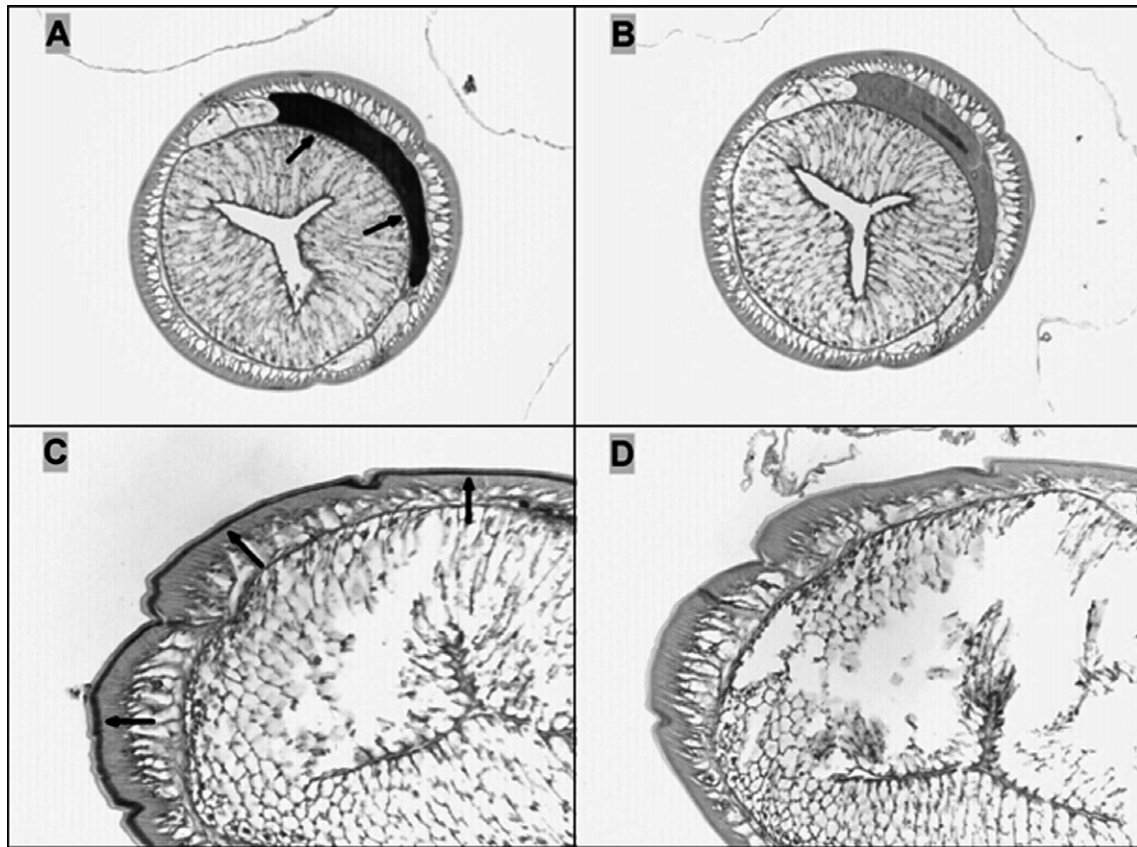


Fig. 6. Immunolocalisation of Ani s 4 in sections of *A. simplex* L3. Polyclonal anti-rAni s 4 antibodies strongly labelled the excretory gland (arrows, A) and, in some larvae, the basal layer of the cuticle (arrows, C). No staining was observed in sections incubated with normal rabbit serum (B and D).

been reported in *Anisakis viteae* cystatin (Hartmann et al., 1997). Since both *A. viteae* and *A. simplex* cystatins are functional protease inhibitors, the presence of the Pro in the second hairpin loop does not appear to be essential for activity. Like other nematode cystatins (Maizels et al., 2001), Ani s 4 has two cysteine residues near the C-terminal amino acid, in contrast to the mammalian and avian family 2 cystatins, that contain four cysteine residues (Abrahamson, 1994). This feature makes it difficult to include nematode cystatins in any of the three described families (Abrahamson, 1994) although their characteristics locate them closer to cystatin family 2 (Maizels et al., 2001).

Immunohistochemistry showed Ani s 4 is located in both the excretory gland and underneath the cuticle in the L3 anatomy. Its presence in excretory glands is consistent with Ani s 4 detection as a parasite excretory product (Moneo et al., 2005). Another allergenic protein from the parasite is the major allergen Ani s 1, also located in the excretory gland (Moneo et al., 2000). Among cystatins, onchocystatin is detected in the hypodermis and the basal layer of the cuticles of *O. volvulus* L3 and is believed to be involved in larval moulting (Lustigman et al., 1992) since it co-localises with a cysteine protease required for the moulting (Lustigman et al., 1996). Because Ani s 4 has two locations, it appears to have two action sites, and one can speculate that it would be involved in moulting

of the *A. simplex* L3 and in inhibiting cysteine proteases in the surrounding environment. Another possible explanation is that there might be two different proteins sharing epitopes with different biological functions.

To obtain a more reliable diagnosis of *A. simplex* allergy and to decrease the frequency of false positives caused by cross-reactivity, several purified or recombinant parasite proteins have previously been used (Moneo et al., 2000; Arrieta et al., 2000; Perez-Perez et al., 2000). Results obtained in this study demonstrated that rAni s 4 retained the allergenic characteristics of nAni s 4. Characterisation of rAni s 4 as an allergen was demonstrated by its IgE-binding capacity assessed by immunoblot, ELISA and FEIA, and by its ability to activate basophils. Our studies with patient sera showed that the recombinant protein contained the same IgE epitopes as nAni s 4. The absence of glycosylation sites predicted for the mature Ani s 4 sequence and the expression of the recombinant protein in a bacterial system support previous data which suggested that no carbohydrate epitopes were involved in its IgE binding capacity (Moneo et al., 2005).

We previously reported the detection of basophil activation by flow cytometry as a valuable approach for the diagnosis of *Anisakis* allergy (Gonzalez-Muñoz et al., 2005). We sought to determine if rAni s 4 was able to induce specific basophil activation and found rAni s 4 to be a

biologically active allergen as demonstrated by its capacity to activate basophils in a concentration-dependent manner. The finding that rAni s 4 distinguished between control subjects versus sensitised patients and A4– versus A4+ patients, both in serologic and cellular assays, supports its use as a diagnostic reagent for anisakiasis.

Several nematode cystatins, mainly of filarial-origin, have been described, including Bm-CPI-1 and 2 from *B. malayi* (Gregory et al., 1997; Manoury et al., 2001), Ov-CPI-2 from *O. volvulus* (Lustigman et al., 1992) and Av-CPI-1 from *A. viteae* (Hartmann et al., 1997). No cystatins have been described from *Ascaris suum*, *Ascaris lumbricoides* or *Toxocara canis*, nematodes more closely related to *A. simplex* than filariae. The allergenic potential of nematode cystatins has only been reported for *A. viteae* cystatin, characterised as a potent allergen in a rodent model (Hartmann et al., 2003). However, there are two cystatins identified as allergenic for humans: the cat allergen Fel d 3 (Ichikawa et al., 2001) and the kiwifruit phytocystatin (Bublin et al., 2004).

Several proteases from various sources have been identified as allergens (Donnelly et al., 2006) and the significance of their activity in provoking IgE responses has been reported (Schulz et al., 1995; Hewitt et al., 1995; Donnelly et al., 2006). The allergenicity demonstrated for the above mentioned cystatins and the *A. simplex* cystatin suggests that protease inhibitors can also be relevant allergens. Whether their inhibitory activity is involved in the allergenicity of cystatins has to be studied.

In conclusion, we identified Ani s 4 as the first cystatin in the *Nematoda* class that causes allergy in humans and obtained a recombinant counterpart that retains all allergenic properties of the natural allergen, suggesting its usefulness as a diagnostic tool in *A. simplex* allergy.

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