An antibody-based ELISA for quantification of Ani s 1, a major allergen from *Anisakis simplex*

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SUMMARY

Anisakis simplex is a nematode parasite that can infect humans who have eaten raw or undercooked seafood. Larvae invading the gastrointestinal mucosa excrete/secrete proteins that are implicated in the pathogenesis of anisakiasis and can induce IgE-mediated symptoms. Since Ani s 1 is a potent secreted allergen with important clinical relevance, its measurement could assess the quality of allergenic products used in diagnosis/immunotherapy of *Anisakis* allergy and track the presence of *A. simplex* parasites in fish foodstuffs. An antibody-based ELISA for quantification of Ani s 1 has been developed based on monoclonal antibody 4F2 as capture antibody and biotin-labelled polyclonal antibodies against Ani s 1 as detection reagent. The dose-response standard curves, obtained with natural and recombinant antigens, ranged from 4 to 2000 ng/ml and were identical and parallel to that of the *A. simplex* extract. The linear portion of the dose-response curve with nAni s 1 was between 15 and 250 ng/ml with inter-assay and intra-assays coefficients of variation less than 20% and 10%, respectively. The assay was specific since there was no cross-reaction with other extracts (except *Ascaris* extracts) and was highly sensitive (detection limit of 1·8 ng/ml), being able to detect Ani s 1 in fish extracts from codfish and monkfish.

Key words: *Anisakis simplex*, *Anisakis* allergy, Ani s 1, quantification, monoclonal antibody, immunoassay, food allergy, recombinant antigen, parasite detection.

INTRODUCTION

Anisakis simplex is a nematode parasite that, in the larval stage, can parasitize many different kinds of fish or cephalopods, being marine mammals the final hosts, in which the adult stage is reached. Man is an accidental host who acquires the larvae by eating raw or undercooked fish. The parasitization of human subjects by the living larvae produces a disease known as anisakiasis giving rise to digestive symptomatology. Allergy to *A. simplex* has been reported as allergic type I hypersensitivity reaction (urticaria-angioedema, anaphylaxis, and occupational conjunctivitis and asthma) following ingestion or handling of the infected fish (Audicana *et al.* 1995; Del Pozo *et al.* 1997; Montoro *et al.* 1997; Añibarro and Seoane, 1998; Armentia *et al.* 1998; Pulido *et al.* 2000; Scala *et al.* 2001).

Nine allergens have been cloned and identified as major or minor allergens from *A. simplex* (www.allergen.org; www.allergome.org). Ani s 1, a 21 kDa allergen produced in the excretory gland (Gómez-Aguado *et al.* 2003), is a good antigen for the diagnosis of hypersensitivity and intestinal anisakiasis because it does not have cross-reaction with other allergens (Moneo *et al.* 2000; Caballero and Moneo, 2002; Valls *et al.* 2003). It has been demonstrated that detection of IgE specifically directed against Ani s 1 has a high accuracy in the diagnosis of anisakiasis (sensitivity, 86%; specificity, 90%) (Caballero and Moneo, 2002). Due to the importance of this allergen, Ani s 1 concentration would have to be measured in the extracts used for the clinical diagnosis and immunotherapy in order to guarantee the quality of allergenic products. Monoclonal antibody (mAb)-based assays allow quantification of specific allergen levels affording the sensitivity, specificity and reproducibility required (van Ree, 2004). The aim of this work was to develop, for the first time, a 2-site sandwich ELISA to determine Ani s 1 concentration in *A. simplex* extracts and provide an alternative procedure to detect *Anisakis* parasitization in fish foodstuff useful for seafood safety assessments.

MATERIALS AND METHODS

Anisakis simplex *extracts*

A. simplex larvae were supplied by the Santiago de Compostela University (Parasitology Laboratory, Pharmacy Faculty). The parasites were washed in

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PBS and harvested by centrifugation at 4000 ***g*** for 10 min. Then, they were snap-frozen in liquid nitrogen and ground in a mortar. The proteins were extracted by magnetic stirring (4 h at 4 °C) in saline solution. The extract was clarified by centrifugation for 15 min at 3300 ***g***. The supernatant was stored and the pellet was resuspended, extracted and centrifuged under the same conditions. Both supernatants were filtered through glass fibre pre-filters (Millipore Corp., Bedford, MA, USA) and dialysed with an 8000 Da cut-off. Protein content was determined by the Bradford method (Bradford, 1976).

Purification of natural and recombinant Ani s 1

rAni s 1 expressed in *E. coli* was purified from inclusion bodies solubilized with 6 M guanidine hydrochloride as previously described (Ibarrola *et al*. 2008). Briefly, the allergen was refolded by dilution and progressive step dialysis followed by oxidative refolding in the presence of cysteine-cystine (5 mM : 1 mM) in 50 mM Tris pH 8·0 overnight at 4 °C. The monomer was separated from aggregates by means of size exclusion chromatography onto a Sephadex 200 column in an AKTA Prime System (GE-Healthcare, Buckinghamshire, UK). Natural Ani s 1 was purified from *A. simplex* L3 extract using an immuno-affinity column made with the obtained anti-recombinant Ani s 1 polyclonal antibodies, followed by a final size exclusion chromatography step onto a Superdex S75 column (GE-Healthcare) as previously described (Ibarrola *et al*. 2008).

Production and purification of Ani s 1-specific antibodies

Polyclonal antibodies were obtained by immunizing a New Zealand rabbit with 5 boosts of 200 μg of rAni s 1 emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). Serum samples collected 10 days after the last injection were tested and stored at −80 °C. For monoclonal antibody production, BALB/c mice were immunized intraperitoneally with 20 μg of Ani s 1 emulsified in complete Freund's adjuvant. In the next boosts the allergen was emulsified in incomplete Freund's adjuvant (Difco), and finally an injection without adjuvant was given 3 days before the fusion. Detailed procedures for the fusion and hybridoma maintenance have been previously described (Arilla *et al*. 1997). Positive hybridomas were identified by ELISA and subcloned by limiting dilution. Isotype detection was done by IsoStrip kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Antibodies were purified from rabbit anti-Ani s 1 serum or from hybridoma culture supernatants by affinity chromatography with a Hitrap Protein G (GE-Healthcare). The purified rabbit antibodies were labelled with biotin using the ECL protein biotinylation module™ (GE-Healthcare).

Quantification of Ani 1 by a 2-site sandwich ELISA

Microtitre 96-well plates (Greiner, Frickenhausen, Germany) were coated with 100 μl of mAb 4F2 at 5 μg/ml in phosphate-buffered saline (PBS) overnight at room temperature. After blocking with 200 μl of PBS containing 1% BSA and 0·05% Tween 20 (PBS-B-T), the plates were incubated with a dilution series of natural or recombinant Ani s 1 ranging from 0·004 to 2 μg/ml or *A. simplex* extracts from 3 to 400 μg/ml and then biotinylated anti-Ani s 1 polyclonal antibodies were added at 0·25 μg/ml. Finally, bound antibodies were detected by incubation with streptavidin-horseradish peroxidase at 0·25 μg/ml (Sigma Chemical Co., St Louis, MO, USA). All the incubations were performed with 100 μl/well in PBS-B-T for 1 h at 37 °C and with 3 washes of 200 μl/well of PBS containing 0·05% Tween 20 between successive steps. Wells were incubated for 30 min at room temperature in the dark with 200 μl of freshly prepared enzyme substrate *o*-phenylenediamine (Sigma), and the colour reaction was stopped by adding 50 μl of 3 M H_2SO_4. The optical density was measured at 492 nm. Wells without coated mAb, labelled antiserum, or Ani s 1 were included as controls to determine the background for the assay. The Ani s 1 concentration in the crude extracts was determined by interpolating the optical densities of the extracts in the linear portion of the standard curve.

Detection limit, inter-assay and intra-assay variations, and recovery experiments were performed as previously described (Asturias *et al*. 1999). In the recovery experiments, several extracts of fish (hake), nematodes (*Ascaris lumbricoides*), crustaceans (shrimp and prawn), and cephalopods (octopus, squid, and cuttlefish) at 1 mg/ml were spiked in the wells of the standard curve and the obtained results were compared to the expected results according inter-assay and intra-assay variations.

Electrophoresis and immunoblotting

Proteins (20 μg of crude extract and 2 μg of purified molecules) were analysed by SDS-PAGE under reducing conditions (Laemmli, 1970) and stained with Coomassie Brilliant Blue R-250. For immunoblotting experiments, proteins (5 μg of crude extract and 50 ng of purified molecules), after SDS-PAGE, were electrophoretically transferred onto polyvinylidene difluoride membranes (Hybond™-P, GE-Healthcare) which were blocked for 1 h at room temperature with 5% skimmed milk, 0·1% Tween 20 in PBS. Then, the blots were incubated with the mAb 4F2 (0·5 μg/ml) or rabbit anti-Ani s 1 serum, diluted 1 : 500 000, overnight at 4 °C. After

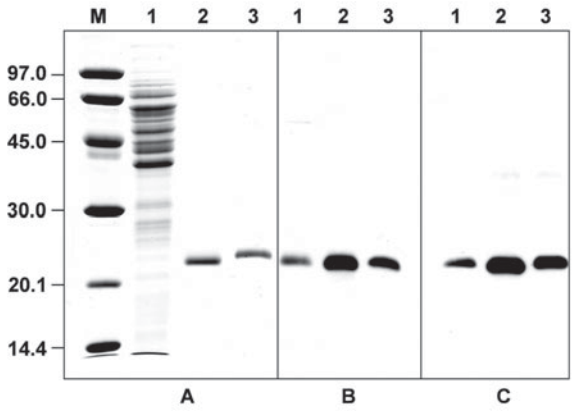

Fig. 1. Coomassie-stained SDS-PAGE (A) and Western blot immunostained with mAb 5D4 (B) or with rabbit anti-Ani s 1 serum (C) of *Anisakis simplex* extract (lane 1), nAni s 1 (lane 2), and rAni s 1 (lane 3).

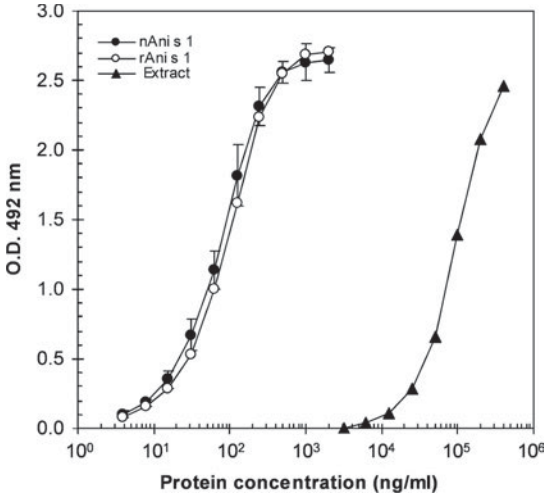

Fig. 2. Dose-response curves in 2-site sandwich ELISA of nAni s 1, rAni s 1, and of *Anisakis simplex* extract.

washing, the membranes were incubated for 1 h at room temperature with anti-mouse IgG antibodies (Sigma) or anti-rabbit immunoglobulins (Dako, Copenhagen, Denmark) linked to horseradish peroxidase (1 : 10 000). After further washes, they were exposed to ECL plus Western Blotting Detection System (GE-Healthcare) for 5 min before being revealed with Hyperfilm^{TM} ECL (GE-Healthcare).

RESULTS

After fusion of spleen cells from BALB/c mice immunized with rAni s 1 and Sp2/0 myeloma cells, hybridomas secreting anti-Ani s 1 antibodies were selected by ELISA. The mAb secreted by 4F2 hybridoma (IgG1 subclass, κ ligh chains) was chosen because it was able to capture Ani s 1 in solution when biotin-labelled polyclonal antibodies detected the formed complexes. Antibody specificities were checked by immunoblotting experiments with *A. simplex* extracts, showing that both 4F2 mAb and polyclonal anti-serum reacted with a single 21 kDa band in extracts, and also recognized natural and recombinant allergen under reducing conditions (Fig. 1). Immunoblotting experiments using several raw extracts from different origins including an extract of *Ascaris lumbricoides* gave no positive reaction even after loading 10 times more protein (data not shown). Similarly, immunoblots of raw extract and purified proteins from *Anisakis* incubated with an irrelevant mAb (anti-Lol p 1) (Arilla *et al.* 2001) gave negative results.

Several assays were performed to determine the minimal antibody concentrations that gave rise to a dose-response curve with the highest sensitivity. These concentrations were 5 μg/ml for 4F2 mAb and 250 ng/ml for biotin-labelled polyclonal antibodies and the curve was established from 4 to 2000 ng/ml. It revealed a sigmoid shape when plotted on a semi-logarithmic scale, being linear in the range from 15 to 250 ng/ml with a coefficient of regression of 0·993 (Fig. 2) and a detection limit of 1·8 ng/ml. The intra-assay coefficients of variation were 6, 10, 8, 5 and 6% at the concentrations of 15·6, 31·2, 62·5, 125 and 250 ng/ml respectively while the inter-assay CVs were 18, 16, 12, 12, and 6% at the same concentrations. The practical working range extended from 8 to 2000 ng/ml because at these concentrations the CVs were <20. The assay was applied to rAni s 1 and the resulting standard curve was statistically similar to that obtained with the natural allergen (Fig. 2). These results, and the $99{\cdot}7 \pm 11{\cdot}5$% recovery obtained, supported the validity of the assay.

The specificity of the 2-site sandwich ELISA was tested at a protein concentration of 200 μg/ml with extracts from different origins: pollen (*Lolium perenne*), mould (*Alternaria alternata*), mite (*Dermatophagoides pteronyssinus*), insects (*Pseudococcus*, *Tenebrio molitor*, *Thaumetopoea pytiocampa*), annelids (*Lumbricus terrestris*, *Glycera dibranchiata*, *Paranereis acrata*, *Nereis diversicolor*, *Marphysa sanguinea*, *Sipunculus nudus*) and nematode (*Ascaris lumbricoides*). There was no significant reaction except in the case of *Ascaris* extract where the assay detected 0·04 mg of an Ani s 1-like protein per gramme of extract. Nevertheless, spiking experiments using 1 mg/ml of *A. lumbricoides* extract showed that this extract had no significant interference with the assay since the obtained values were within the variability range of the expected values (data not shown).

Ani s 1 content was calculated in 7 different *A. simplex* extracts by interpolation in the linear portion of the standard curve and ranged between 0·2 and 0·4 mg per gramme of extract with an average of 0·3 and a variation coefficient of 25%. The

developed ELISA was applied to track the presence of Ani s 1 in some commercial fish extracts intended for allergy diagnostic uses such as seabream, monkfish, sardine, anchovy, sole, hake, and codfish. Positive results were obtained with raw and boiled extracts of monkfish (2 and 10 ppm, respectively) and codfish (5 and 33 ppm, respectively). Several extracts from organisms involved in the life-cycle of *Anisakis* larvae were also tested in spiking experiments to assess the specificity of the assay. None of the extracts from crustaceans (shrimp and prawn), and cephalopods (octopus, squid, and cuttlefish) had significant interference with the described assay.

DISCUSSION

The quantification in mass units of major allergens has become an important goal for the standardization of allergen products intended for clinical use. ELISA has the advantage of high sensitivity, reproducibility and reliability, without drawbacks of earlier immunoassays (e.g. radioactivity). Due to those characteristics ELISA is becoming the standard among the allergenic protein quantification techniques. In this study, the combination of 4F2 mAb as capture and biotin-labelled anti-Ani s 1 polyclonal antibody as tracer were applied in a 2-site sandwich ELISA to quantify Ani s 1. Both monoclonal and polyclonal antibodies were highly specific as the immunoblotting analysis of the complete extract demonstrated. This combination was chosen since it yielded the Ani s 1 standard curve with the highest sensitivity (1·8 ng/ml) to be applied to the routine measurement of the Ani s 1 content in production batches of extracts and prick-test solutions. A recombinant Ani s 1 (rAni s 1) with equivalent immunological properties to the natural counterpart has been expressed in *Escherichia coli* (Ibarrola *et al*. 2008). Purified nAni s 1 showed a dose-response curve parallel to that of crude *A. simplex* extracts, suggesting that the assay was measuring the same molecule in the different preparations and that the allergen had not been altered during the purification process. The assay reproducibility was proven by the low intra- and inter-assay CV, to be less than 10% and 20%, respectively. The purification of natural Ani s 1 is laborious and expensive since larvae should be extracted manually from parasitized fishes and the low yield of purified protein obtained (50 μg/g of larvae in this work) makes impossible its use as a routine reagent (Moneo *et al*. 2000). Therefore, rAni s 1 was used as a standard in the developed assay and the resulting curve was statistically similar to that obtained with the natural allergen. Therefore, the recombinant Ani s 1 could become a reference material for a specific Ani s 1 assay because it has several advantages over its natural counterpart. Recombinant Ani s 1 is accurately quantified and can be produced in quantities large enough to develop stability studies and a suitable lyophilization strategy.

The assay did not give any significant reaction with several extracts from different origins. The unique exception was in the case of *A. lumbricoides* extract where the assay detected 0·1 mg of an Ani s 1-like protein per gramme of extract. Cross-reactivity among ascarids and excretory-secretory antigens from *A. simplex* had been previously detected but no identification of the antigens involved had been reported (Kennedy *et al*. 1988; Lozano *et al*. 2004). Nevertheless, this aspect needs more studies since no reaction in immunoblot has been found between the Ani s 1 antiserum or the 4F2 mAb and the ascarid extract, both under reducing and non-reducing conditions.

Allergy to *A. simplex* has been reported following ingestion or handling of the infected fish (Audicana *et al*. 1995; Del Pozo *et al*. 1997; Montoro *et al*. 1997; Añibarro and Seoane, 1998; Armentia *et al*. 1998; Pulido *et al*. 2000; Scala *et al*. 2001). Current diagnosis of *A. simplex* allergy is based on anamnesis, detection of *A. simplex*-specific IgE antibodies and cutaneous tests with positive reactivity against *A. simplex* extracts but negative reactivity to fish extracts (Lorenzo *et al*. 2000; Baeza *et al*. 2004). Therefore, the presence of Ani s 1 traces in commercial fish extracts used for allergy diagnostics (monkfish and codfish extracts), detected by the developed ELISA, gives warning of possible misdiagnosis of fish allergies due to positive results when cutaneous or serum tests have been performed with fish extracts made from *Anisakis* larvae-parasitized raw material. Furthermore, a recent article has described the putative presence of *Anisakis* proteins in chicken meat due to the high proportion in chicken feed of fishmeal which might possibly be contaminated by this nematode (Armentia *et al*. 2006).

The amount of Ani s 1 found in a single larva is about 244 ng and, taking into account the detection limit of the assay, it could detect as low as 25 larvae per 100 g of fish. Similar infection levels have been found in heavily-infected fish described in a New Zealand survey (Wharton *et al*. 1999). Although a higher degree of sensitivity should be reached in order to detect mild and low infection levels, the developed ELISA can constitute a first step for improving current methods for detection of nematodes in fish, especially having taken into account the reported low detection efficiency of candling, the commonly recommended inspection method (Levsen *et al*. 2005).

In conclusion, we have described for the first time a 2-site sandwich ELISA, based on a mAb and a specific rabbit antiserum, to determine Ani s 1 concentrations in *A. simplex* extracts. The described assay is a specific and sensitive method that allows standardization of *A. simplex* extracts intended for

clinical use and could help in the detection of *A. simplex* parasitization in marketable fish foodstuffs.

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