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Isolation of a heat-resistant allergen from the fish parasite *Anisakis simplex*

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Abstract The thermal stability of allergenic peptides from the fish parasite *Anisakis simplex* has not been fully elucidated. This is of special relevance for physicians who should clearly indicate if sensitized patients should avoid ingestion of raw fish only or whether well-cooked fish should also be avoided, if allergenic peptides derived from the parasite remain immunologically detectable. An allergen was purified after heating a crude parasite extract for 30 min. The allergen was further purified by an ethanol fractionation procedure followed by a reversed-phase HPLC. The N-terminal amino acid sequence was obtained. This allergen was detected by 27% of sensitized subjects. The N-terminal amino acid sequence of the 9 kDa allergen showed no similarities to other known proteins. A minor low molecular weight allergen from *A. simplex* is highly resistant to heating and it could therefore have significant clinical relevance.

Keywords Allergen · *Anisakis* · IgE · Parasite · Nematode

Introduction

Eating raw or undercooked fish can lead to hypersensitivity reactions due to allergy to the fish parasite *Anisakis simplex*. The acute symptoms experienced by sensitized subjects, known as gastroallergic anisakiasis (Daschner et al. 2000) are due to infection by live larvae during their mucosal invasion (Moneo and Caballero 2002), and are probably the result of the release to the circulation of secretory products, such as the major

allergen Ani s 1, described by us some time ago (Moneo et al. 2000).

Knowing whether allergic reactions are the result of an active parasite infection is at present a central point for understanding this disease, as important dietetic recommendations are currently empirical. Some authors described sensitized patients tolerating the ingestion of frozen larvae (Sastre et al. 2000; Trujillo et al. 2002; Garcia et al. 2001; Alonso-Gomez et al. 2004). They therefore state that these subjects can eat well-cooked or frozen fish, even if they contain parasites. On the contrary, the presence of heat-resistant allergens has been described by other authors, implying that parasite-allergic patients could present symptoms after eating fish containing allergens derived from dead larvae (Audicana et al. 2002; Falcao et al. 2002). In our experience some, though not all, sensitized patients reported symptoms after eating well-cooked fish, even after ingestion of canned fish. We therefore studied the effect of pepsin and/or heat and found that a heat-resistant low molecular weight allergen was detected by 22.5% of the sensitized patients (Caballero and Moneo 2004).

This paper describes the isolation and characterization of this heat-resistant allergen from *Anisakis simplex*.

Materials and methods

Sera

Sera from 30 patients with clinical symptoms after ingesting raw fish who had specific IgE levels higher than 3.5 kU/L, as measured by CAP (Pharmacia, Uppsala, Sweden), were used for the study. A serum pool was used for IgE detection during purification.

Antigen

Larvae were manually extracted from livers of *Micromesistius poutassou* obtained from local markets. One gram of fresh larvae was mixed with 10 mL of PBS, ground in a

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mortar and centrifuged at 1,000 *g* and the pellet discarded. The protein content of this supernatant was 2 mg/mL, as measured by the Bradford method (Bradford 1976) using BSA as a standard. Excretory/secretory allergens were obtained by incubating larvae at 37°C at low pH as previously described (Moneo and Caballero 2002).

Antigen purification procedure

1. Heat treatment: the crude extract was incubated for 30 min in a boiling water bath. Thereafter, the extract was centrifuged at 4,500 *g* for 15 min and the pellet discarded.
2. Ethanol fractionation: the above described supernatant was mixed with the same volume of absolute ethanol and left for 30 min at room temperature. The mixture was then centrifuged at 4,000 *g* and the pellet discarded. The supernatant was mixed again with ethanol to raise the final alcohol concentration to 66%. After 30 min, the mixture was centrifuged at 4,000 *g* and the supernatant discarded. The pellet was resuspended in a 1/10th of the original volume in distilled water and considered 10× concentrated.
3. Reversed-phase HPLC: This 10× concentrated parasite extract (0.25 mL) was applied to a 7.8 × 200 mm C-4 Nucleosil column (Vydac, Hesperia, California, USA) and eluted using a linear (0–100% in 60 min) acetonitrile gradient in 0.1% TFA at a flow rate of 1 mL/min. Absorbance was monitored at 220 nm and the collected fractions were studied by SDS-PAGE and IgE-immunoblotting.

Antigen treatments

The heated crude supernatant extract was treated as follows:

- Reduction: 25 µL of the heated crude supernatant extract were mixed with the same volume of sample buffer with 1% 2-ME and boiled for 5 min.
- Periodate oxidation: 25 µL of the heated crude supernatant extract were mixed with 5 µL of the oxidant solution (30 mM sodium m-periodate, 2 M sodium acetate, pH 4.7) and incubated for 30 min at 4°C in the dark. Thereafter, the same volume of sample buffer was added to the reaction mixture.
- Pepsin digestion: the heated crude supernatant extract (25 µL) was mixed with the same volume of 1 wt/vol percentage of pepsin A (sigma, St. Louis, MO) in 50 mM HCl and incubated for 1 h at 37°C. The same volume of sample buffer was added after this incubation.

N-terminal amino acid sequence analysis

The HPLC fraction with the highest recognition on the IgE immunoblot was separated in a 16% acrylamide gel

and transferred to a Sequiblot PVDF (Bio-Rad Lab, Hercules, CA) membrane by diffusion for 18 h. After washing, the membrane was stained with Coomassie blue for 5 min and destained with 50% ethanol. The protein band was excised and submitted for N-terminal amino acid analysis. N-terminal amino acid sequence analysis was carried out in a Perkin Elmer/Applied Biosystems Procise 494 microsequencer running in pulse liquid mode.

SDS-PAGE and immunoblotting

The different fractions or column peaks obtained were applied in individual wells of a 16% polyacrylamide gel and the SDS-PAGE was performed at 150 V under standard conditions (Moneo et al. 2000). After running, nitrocellulose sheets were placed on both sides of the gel and transfer was performed by diffusion for 18 h as previously described (Moneo et al. 1995). After washes and blocking for 30 min in 3% NP-40 in PBS, the sera or the serum pool were incubated for 18 h while shaking. Thereafter, the membranes were washed and then incubated with 10 mL of a 1/1,000 dilution of a mouse monoclonal anti-IgE antiserum for 3 h (Ingenasa, Madrid, Spain). After new washes, the membranes were incubated again with 10 mL of a 1/2,500 dilution of an alkaline-phosphatase labeled goat anti-mouse IgG (Biosource Int, Camarillo, CA, USA) antiserum for 1 h. Finally, the membranes were washed and the substrate (BCIP-NBT) was added for 30 min (Moneo et al. 2000).

For specific IgE detection in several human samples, the antigen was loaded on a 16% acrylamide gel without lanes and the electrophoresis and transfer were carried out as described above. After blocking, the membranes were placed on a Mini-Protean II Multiscreen (Bio-Rad Labs). The sera were placed in the individual lanes (0.6 mL of a 1:5 dilution) and incubated overnight while shaking. The following day, the membranes were taken out of the device and the rest of the assay was performed as described.

Results

Heating a crude parasite extract led to a significant loss of protein and to a reduction of detected allergens by the different sera. Figure 1 shows that this drastic treatment did not destroy the recognition of some IgE-binding proteins and that a low molecular weight allergen was highly resistant to heat and could therefore be potentially relevant for sensitized patients.

The parasite extract was subjected to a differential ethanol fractionation procedure in order to purify the allergen. The low molecular weight allergen was not present in the resulting pellet, obtained when the sample was incubated in a final 50% v/v ethanol concentration. The allergen was found in the pellet obtained from the 50–66% ethanol fraction. A further increase of the

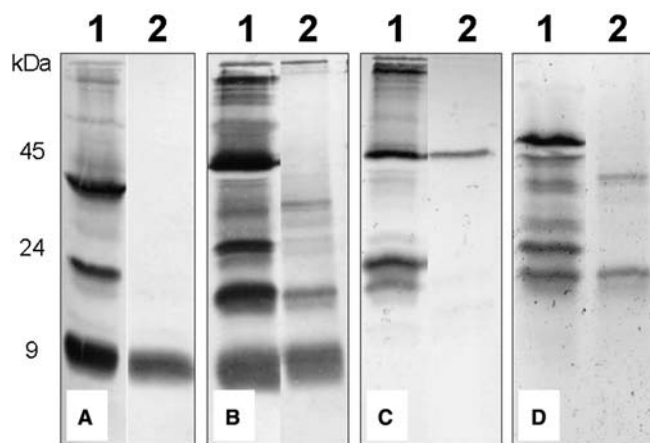


Fig. 1 Results on four representative cases of the effect induced by heating for 30 min in a boiling water bath of a crude parasite extract. Lane 1 IgE immunoblotting using the crude extract. Lane 2 IgE immunoblotting using the heated extract

alcohol concentration to 90% did not precipitate more allergen, indicating that the 50–66% fraction, which produced the smallest pellet, contained the majority of the allergen (Fig. 2). This fraction was then purified further by reversed phase HPLC. This method allowed the recovery of an allergen with an estimated molecular weight of 9 kDa according to its mobility in the SDS-PAGE. The fraction with the highest IgE-binding properties was transferred to a membrane and the N-terminal amino acid sequence obtained was GMLGGSSDVDVNDPEIK. At positions two and three, we always found an alternative amino acid, M/T and L/P, which suggests a genetic polymorphism at this region. No similarities to known proteins were found and the allergen was registered in the Swiss-Prot Protein Knowledgebase and assigned accession number P83885.

The presence of specific IgE antibodies to this allergen was studied in the sera of 30 patients with clear

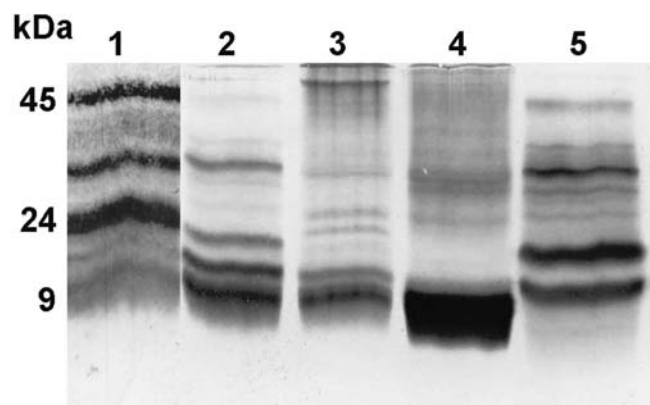


Fig. 2 Effect on immunoblotting using a positive serum pool of the initial allergen purification steps. Lane 1 crude extract, lane 2 after heating for 30 min, lane 3 pellet obtained after 50% ethanol fractionation, lane 4 pellet obtained after 50–66% ethanol (10× concentrated), lane 5 pellet obtained after 66–90% ethanol fractionation (10×)

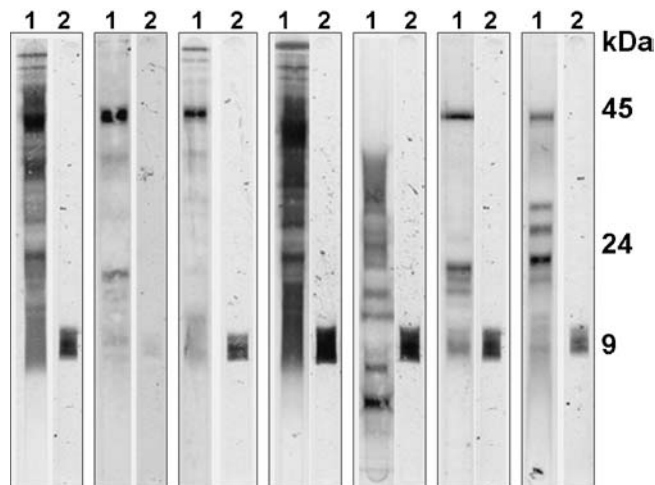


Fig. 3 IgE immunoblot of several sera reactive to Ani s 4. Lane 1 crude extract, lane 2 IgE immunoblot using purified Ani s 4

clinical symptoms of anisakiasis and antibodies were found in eight of them. Therefore this protein was considered a minor parasite allergen by the Allergen Nomenclature Sub-Committee of the IUIS and registered as Ani s 4. Figure 3 shows that the presence of antibodies to Ani s 4 did not correlate with a larger amount of antibodies to other antigens of the crude extract, or with a special pattern of antigenic recognition because Ani s 4 detection occurred in sera with different reactivities to the crude extract. The group of patients sensitized to this allergen had significantly more anaphylactic episodes ($P < 0.001$, two tailed Fisher's exact test) and were attended more often in Emergency Units ($P < 0.001$) than subjects having specific IgE to other parasite allergens.

The allergen was treated in several ways in order to exclude the possibility that specific IgE to Ani s 4 was directed to a carbohydrate moiety. Pepsin induced a complete loss of recognition, 2-ME a partial loss but the allergen was insensitive to m-periodate. These results suggested that the epitope/s were not sugar residues (Fig. 4).

The allergen was found in excretory/secretory products of the larvae (Moneo and Caballero 2002), as shown in Fig. 5, strongly suggesting this protein could be stored in the excretory gland of the parasite, as was demonstrated for Ani s 1 (Moneo et al. 2000; Gomez-Aguado et al. 2003).

Discussion

This paper describes the isolation and partial characterization of a heat-resistant allergen. After heating a crude extract for 30 min, specific antibodies to this protein were found in 27% of the sensitized patients. This low frequency of detection could be attributed to an MHC restriction of the immune response as

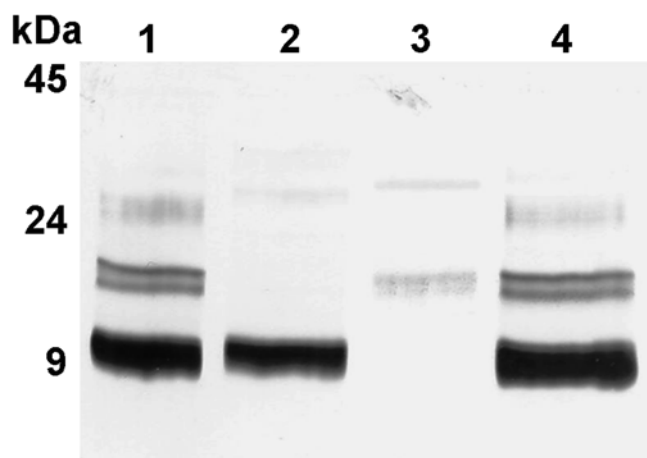


Fig. 4 Effect of different treatments on the 50–66% fractionation to measure their influence on Ani s 4 recognition. Lane 1 no treatment, lane 2 after incubation with 2-ME, lane 3 after pepsin digestion, lane 4 treatment with m-periodate

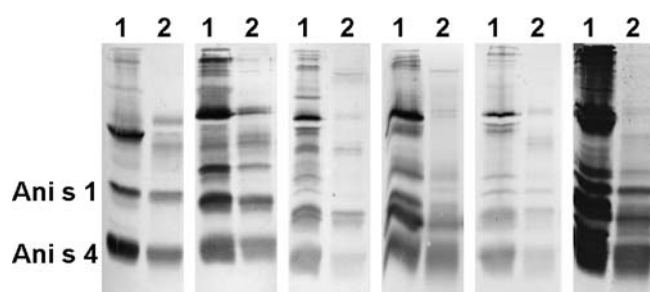


Fig. 5 Detection by IgE-immunoblot of Ani s 4 in excretory/secretory products by different positive sera. Lane 1 IgE-immunoblot using a crude parasite extract, lane 2 the same blot using excretory/secretory allergens

described years ago after experimental infection of mice with *Ascaris suum* (Tomlinson et al. 1989). According to the Allergen Nomenclature Subcommittee of the IUIS, this protein should be considered a minor allergen, based on the definition that major allergens are only those IgE-binding proteins that are detected by more than 50% of the sera (Aalberse 2000). However, this classification has often been considered unacceptable and many authors believe that frequency of recognition is not equivalent to clinical relevance (Aalberse 2000). Perhaps Ani s 4 could serve as a model for this hypothesis. After heating a crude parasite extract, some patients only detect this allergen, a fact that leads one to suspect that this protein must be highly relevant, at least for these patients. The finding that subjects sensitized to Ani s 4 have more severe symptoms than patients who did not recognize this allergen further supports the clinical relevance of this allergen.

The N-terminal amino acid analysis did not reveal any similarity to known proteins and therefore no information about the biological role of this protein was obtained. However, patients having antibodies to Ani s 4

detect the allergen in excretory/secretory products suggesting that this allergen is contained in the excretory gland of the larvae, as demonstrated by our study of Ani s 1 with immunohistochemistry (Moneo et al. 2000; Gomez-Aguado et al. 2003). If this is the case, proteins contained in the excretory gland of this parasite should be considered highly relevant. We could not perform this type of study due to the small amount of protein obtained that hindered the production of an antiserum to be used as a tracer for the immunohistochemical localization of the allergen.

Our study suggested that the thermal resistance of the allergen was due to intrinsic properties of the allergen and not to the fact that the epitopes were carbohydrates. Thermal stability of parasite allergens has been previously described for ABA-1, a non-glycosylated allergen of *Ascaris* (Xia et al. 2000; McDermott et al. 2001). These proteins have been found in *Ascaris suum*, *Ascaris lumbricoides*, *Toxocara canis* and *Anisakis* (Yahiro et al. 1998; Christie et al. 1990; Kennedy et al. 1988), but the N-terminal amino acid sequence demonstrated that Ani s 4 did not belong to this family of allergens. Resistance to m-periodate or sensitivity to pepsin are only indirect proofs of the composition of the epitope/s. A bacteria-derived recombinant allergen lacking any carbohydrate and retaining the allergenicity of the native molecule is probably the best proof of this hypothesis. We are developing this kind of reagent that, which could also be highly useful when applied to clinical diagnosis. With the help of this recombinant allergen, we could also measure the concentration of this allergen in different foods such as in canned fish, because we suspect that the amount of allergen present in some cans should be high enough as to induce symptoms referred by some patients sensitized to Ani s 4.

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