

Isolation of Ani s 5, an excretory–secretory and highly heat-resistant allergen useful for the diagnosis of *Anisakis* larvae sensitization

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Abstract Allergens Ani s 1 and Ani s 4 have demonstrated their utility for the diagnosis of the sensitization to larvae of the genus *Anisakis*. The aim was to determine the number of patients with compatible clinical history, who did not recognize Ani s 1 and Ani s 4, and characterize the allergens responsible for their sensitization. Eighty-four patients were studied by CAP and immunoglobulin E (IgE) immunoblotting. The 12% of the patients recognized allergens different from Ani s 1 and Ani s 4, being half sensitized to a heat-resistant 15-kDa allergen, which was isolated by ethanol fractionation, followed by a hydroxyapatite chromatography and a reversed-phase high-performance liquid chromatography and identified by its amino terminal sequence as Ani s 5. A total of 41 of the 84 patients studied (49%) showed specific IgE to Ani s 5 that was detected among the excretory–secretory products and immunohistochemically located at the excretory gland, ventriculus, and the luminal surface of the intestinal epithelium of the larvae.

Introduction

Anisakis larvae produce severe hypersensitivity reactions when larvae stages or their products are ingested in raw or undercooked fish. However, some sensitized patients reported symptoms after eating well-cooked or canned fish. The parasite heat-resistant allergens Ani s 1, Ani s 4, and Ani s 8 could be responsible for these manifestations (Caballero and Moneo 2004; Moneo et al. 2005; Kobayashi et al. 2007b). Ani s 1 and Ani s 4 have demonstrated their utility for the diagnosis (Caballero and Moneo 2002; Moneo et al. 2007). Thus, as conclusion of a study performed with 64 patients referred for suspected *Anisakis* larvae sensitization, we found that 95% of the sensitivity of the diagnosis by immunoglobulin E (IgE) immunoblotting could be achieved using only these two allergens (Moneo et al. 2007). However, the existence of patients with compatible clinical history, some of them with reactions after eating well-cooked or canned fish, that do not recognize Ani s 1 and Ani s 4 makes necessary the search of the allergens responsible for their sensitization to complete the battery of reagents for the diagnosis.

Material and methods

Eighty-four patients with clinical symptoms after consumption of fish were studied by CAP (Phadia) and an IgE multiblot using different antigenic extracts (Moneo et al. 2007). About 500 *Anisakis* larvae were ground in 10 ml of phosphate-buffered saline and centrifuged for 15 min at 4,500×g. The supernatant (crude extract) was heated for 30 min in a boiling water bath and centrifuged. An ethanol fractionation was performed with the new supernatant obtained. Crude and boiled extracts, and differential ethanol

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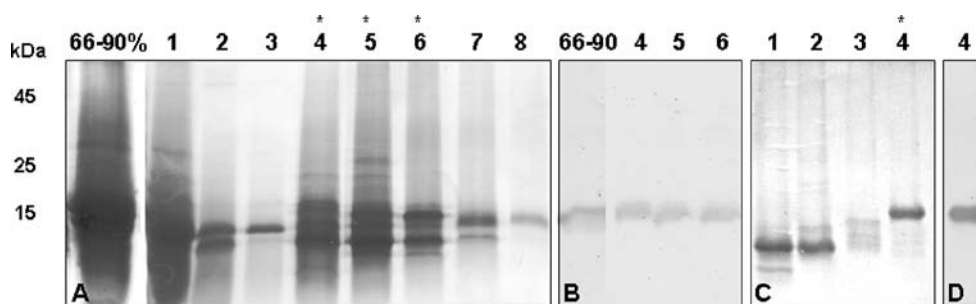


Fig. 1 Isolation of Ani s 5 by first: hydroxyapatite chromatography of the 66–90% ethanol fraction; **a** SDS-PAGE of the eluted fractions and **b** IgE immunoblotting corresponding with the positive fractions

fractions 50–66% and 66–90%, were analyzed by 16% acrylamide–Tris glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and IgE immunoblotting (Moneo et al. 2000).

To isolate allergens from the 66–90% ethanol fraction, 300 μ l were loaded onto an Econo-Pac CHT-II hydroxyapatite column (Bio-Rad) and eluted with a linear gradient of Na_2HPO_4 (5 mM–0.5 M). The chromatography was performed at a neutral pH (7.4) and the eluted fractions were dialyzed, lyophilized, and analyzed by SDS-PAGE and IgE immunoblotting. An eluted fraction (100 μ l) was loaded on a Hypersil 300-C4 column (250 $\times$ 46 mm; particle size 5 μ m; Teknokroma, Barcelona, Spain) and eluted using a linear gradient of acetonitrile (10–90% in 1 h) in 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min. Elution was monitored at 280 nm, and fractions were collected and analyzed in a 16% acrylamide–Tris tricine SDS-PAGE (Schägger and von Jagow 1987) and IgE immunoblotting. The high-performance liquid chromatography (HPLC) fraction recognized by the patients was electrotransferred onto a polyvinylidene fluoride membrane (Sequiblot, Bio-

Rad) and stained with Coomassie blue (Jorgensen et al. 2004). The protein band was excised and submitted for N-terminal amino acid analysis in a Perkin Elmer–Applied Biosystems Procise 494 microsequencer in pulse liquid mode at the Proteomic Service in Centro de Investigaciones Biológicas (Madrid, Spain).

The excretory–secretory (ES) products were obtained by incubating 1.2 g of live *Anisakis* larvae in 10 ml of 50 mM HCl for 18 h at 37°C. The larvae were discarded and supernatant was adjusted to pH 7.2, dialyzed against distilled water, freeze-dried, and resuspended in 300 μ l of distilled water. ES products collected were analyzed by SDS-PAGE and IgE immunoblotting.

Sections (4 μ m) of formalin-fixed paraffin-embedded *Anisakis* larvae were placed on positively charged slides and incubated overnight at room temperature, with a 1/3 diluted serum from a patient who only recognized Ani s 5 in the parasite extract before boiling. The serum from a nonsensitized subject (negative CAP value) was used as control. Specific IgE was detected with a mouse antihuman IgE monoclonal antibody (Ingenasa, Madrid, Spain) diluted

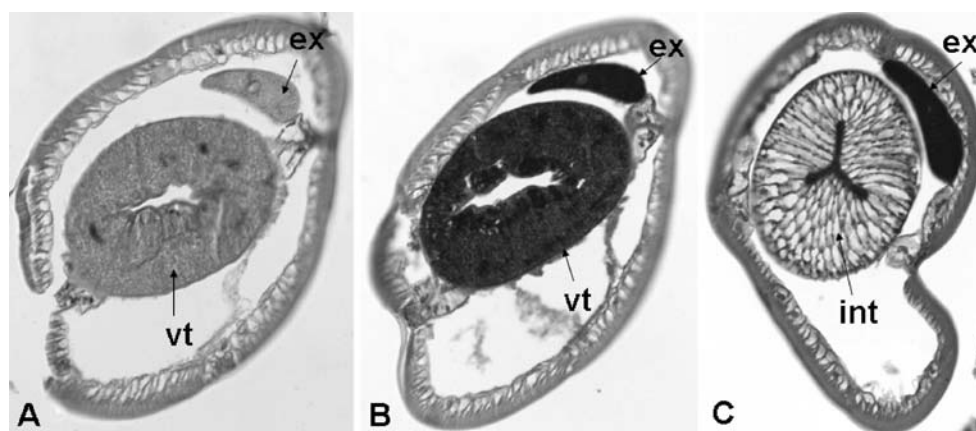


Fig. 2 Immunohistochemical localization of Ani s 5 in *Anisakis* larvae sections incubated with the serum from a patient who only recognized Ani s 5 among the proteins of the parasite extract before boiling. **a** Negative control, **b** positive reaction in excretory gland and ventric-

ulus, **c** positive reaction in excretory gland and luminal epithelium of the intestine. Abbreviations: *ex*, excretory gland; *int*, intestine; *vt*, ventriculus (original magnification $\times 20$)

Table 1 Results obtained in studying *Anisakis*-sensitized patients by IgE immunoblotting ($n=84$)

	Positive patients, n (%)
Ani s 1	56 (67)
Ani s 5	41 (49)
Ani s 4	25 (30)
Negative to both Ani s 1 and Ani s 4	10 (12)

1:100 and a biotinylated goat antimouse immunoglobulin with a labeled streptavidin–biotin peroxidase system LSAB-2 HRP (Dako Real Detection kit, Dako). The images of the immunohistochemical localization were obtained with a photomicroscope Leica DM 5000B and digital camera Leica DC 300.

Results and discussion

Ten of the 84 patients studied (12%) recognized allergens different from Ani s 1 and Ani s 4. Five of these ten patients (50%), who had previously reported symptoms after the ingestion of cooked and/or canned fish, detected a 15-kDa allergen contained both in the heated parasite extract and in the 66–90% ethanol fraction. This fraction was subjected to a hydroxyapatite chromatography to purify the allergen, which was detected in three of the eluted fractions by the serum from a sensitized patient (Fig. 1a, b). The fifth eluted fraction was subjected to a reversed-phase HPLC and the 15-kDa allergen was only detected in the fourth fraction, eluted at 62% acetonitrile concentration (Fig. 1c, d). The N-terminal amino acid sequence of the protein isolated was: DDPPPPFLAGAPQ, corresponding with Ani s 5, an allergen recently cloned and expressed, belonging to the SXP/RAL-2 protein family (Kobayashi et al. 2007a). This group has also cloned another heat-stable 15-kDa allergen, named Ani s 8, that shares two amino acid motifs with Ani s 5 and other members of the SXP/RAL-2 protein family (Kobayashi et al. 2007b). To our knowledge, nothing is known about the function of these proteins, but several members of the family have been described as immunologically active proteins.

We described the *Anisakis* allergens Ani s 1 and Ani s 4 as excretory–secretory proteins (Moneo et al. 2000; Caballero and Moneo 2004). In the present study, Ani s 5 has been also detected among the ES products released by larvae, and it has been immunohistochemically located in the excretory gland, ventriculus, and luminal surface of the intestinal epithelium of the parasite (Fig. 2). Some proteins of the SXP/RAL2 family like As16, homologous to Ani s 5, are secreted outside the parasite among the excretory–secretory products and localized in the hypodermis and

cuticle, as well as the epithelium of the intestine in the adult parasite (Tsuiji et al. 2001).

Kobayashi et al (2007a) defined Ani s 5 as an important allergen for some *Anisakis* allergic patients, seven of 28 reacted to recombinant Ani s 5, concluding that this protein could be useful for the diagnosis. Our work supports these conclusions showing that 49% (41 of the 84) of the patients studied detected native Ani s 5. Therefore, in our study, Ani s 5 was the second more frequently recognized allergen among sensitized patients (Table 1). Furthermore, the fact that 50% of the patients negative to Ani s 1 and Ani s 4 are sensitized to this excretory–secretory and heat-resistant allergen suggests that this protein should be considered as a new tool for the diagnosis of the *Anisakis* larvae sensitization.

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The experiments comply with the current laws of the country in which they have been performed.

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