

María Luisa Caballero · Ignacio Moneo

Several allergens from *Anisakis simplex* are highly resistant to heat and pepsin treatments

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Abstract Ingestion of raw or undercooked fish can lead to infection with *Anisakis simplex*. Sensitized patients show specific IgE to proteins from this parasite. The aim of this study was to assess the frequency of specific IgE recognition directed to heat and/or pepsin-resistant allergens from *A. simplex* among sensitized patients. Twenty-seven patients with positive specific IgE and immunoblotting with a crude parasite extract were included in the study. Specific IgE detection against allergens resistant to boiling for 30 min and/or a pepsin digestion of an *A. simplex* extract was performed by immunoblotting. A total of 81% of the patients showed specific IgE to pepsin-resistant allergens and 67% had specific IgE to heat-resistant allergens. Thirty percent of patients recognized allergens after both treatments, one being the allergen detected by 75% of the patients of this group. Heat- and/or pepsin-resistant allergens from *A. simplex* could explain reactions and symptoms after the ingestion of well-cooked or canned fish.

Introduction

Anisakis simplex is a nematode parasite frequently found in a great diversity of fish and cephalopod species commonly consumed by humans. The ingestion of raw or undercooked fish can lead to infection with *A. simplex* larvae. This causes not only symptoms directly associated with the gastrointestinal infection, but also IgE-mediated hypersensitivity reactions, sometimes of great severity (Ishikura et al. 1993; Audicana et al. 1997).

A recent study performed by the Spanish Society of Allergology and Clinical Immunology concluded that sensitization to this parasite was found in up to 60% of

patients suffering from acute urticaria in regions where raw fish is consumed, but not in other parts of the country where well-cooked fish is eaten (Moneo et al. 2000a). Although cooking or freezing kills the parasite, it does not destroy its allergenic capacity (Kennedy et al. 1988). It has been found that at least some patients recognize heat-treated *Anisakis* allergens (Audicana et al. 1997).

We studied several sensitized patients who showed symptoms after the ingestion of well-cooked or even canned fish. The influence of pepsin in the allergenicity of the *A. simplex* protein has not been studied previously. The aim of this work was to assess the frequency of specific IgE recognition directed to heat- and/or pepsin-resistant allergens from *A. simplex* among sensitized patients.

Materials and methods

Patients

A total of 27 patients with specific IgE to *A. simplex* determined by specific IgE (Pharmacia Diagnostics, Uppsala, Sweden) and positive immunoblotting with a parasite crude extract were included in the study.

Antigens

A. simplex extract was prepared from larvae obtained from blue whiting, *Micromesistius poutassou*. About 300 larvae were ground in a mortar with 10 ml PBS. The mixture was centrifuged for 15 min at 4,500 g, and the pellet discarded. The supernatant was separated into four tubes; one of them was stored at –20°C without any treatment, as a crude extract. For pepsin digestion, the other tube was incubated at 37°C for 3 h in 50 mmol/l HCl in the presence of 1 mg/ml porcine pepsin (Sigma, St. Louis, Mo.). To test resistance to heat, a tube was incubated for 30 min in boiling water. Finally, the

M. L. Caballero (✉) · I. Moneo
Department of Immunology, Hospital Carlos III,
Sinesio Delgado 10, 28029 Madrid, Spain
E-mail: mlcsoto@hotmail.com
Tel.: +34-91-4532656
Fax: +34-91-7336614

fourth tube with the crude extract was boiled and digested with pepsin under the same conditions described above. After these treatments, the tubes were centrifuged and supernatants were assayed to study protein content and allergenicity.

Excretory/secretory antigens released by *A. simplex* larvae were obtained following the procedure described in a previous work (Moneo et al. 2002).

SDS-PAGE and immunoblotting

SDS-PAGE was carried out in 16% polyacrylamide minigels and stained with Coomassie brilliant blue (Bio-Rad Lab, Hercules, Calif.). Immunoblotting was performed after an overnight transfer to nitrocellulose using a diffusion method followed by incubation for 30 min in 3% Nonidet P-40. Nitrocellulose was incubated with a pool of sera from sensitized patients to study the influence of the treatments. Bound IgE was detected using a monoclonal anti-IgE (Ingenasa, Madrid, Spain) at a 1/1,000 dilution for 3 h and an alkaline-phosphatase-labeled goat antimouse (Biosource, Camarillo, Calif.) at a 1/4,000 dilution for 1 h, as previously described (Moneo et al. 2000b). To study the individual responses of the patients to crude and treated extracts, 0.1 ml of each was electrophoresed in a minigel without lanes. After transfer and incubation in Nonidet P-40, the membranes were placed in a miniblotter (Bio Rad) and incubated with 0.1 ml of each serum diluted 1/6 in incubation buffer (Moneo et al. 2000b).

Results

Figure 1 shows the protein patterns obtained when an *A. simplex* extract was subjected to boiling for 30 min and/or to pepsin digestion. Several of the proteins that remain in solution after the treatments bound IgE as shown by the immunoblotting carried out with a pool of positive sera (Fig. 1B). Allergens with molecular weights between 9 and 26 kDa were found after boiling for

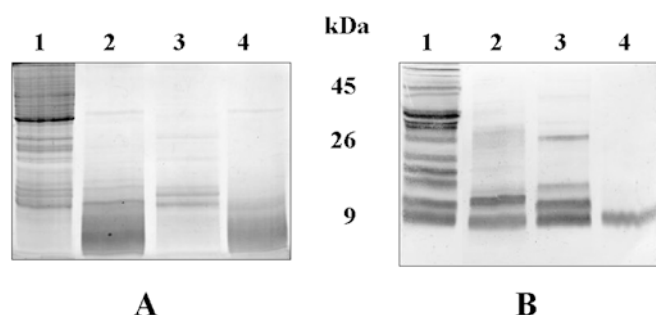


Fig. 1A, B Heating and/or pepsin treatment of an *Anisakis simplex* extract. **A** Coomassie blue staining and, **B** specific IgE-immunoblot using a pool of positive sera. *Lane 1* Crude extract without any treatment, *lane 2* after pepsin digestion, *lane 3* after boiling for 30 min, and *lane 4* after boiling followed by pepsin treatment

30 min, one being the 9-kDa allergen which was also resistant to pepsin digestion.

Two representative patients were studied with the *A. simplex* extract. Their treatments are shown in Fig. 2. Patient A had suffered from acute anisakiasis and patient B had non-ulcer dyspepsia. Even though the two patients had positive specific IgE and immunoblotting, the protein patterns obtained were different, but both had specific IgE directed to heat and/or pepsin-resistant allergens.

Figure 3 shows the frequencies of specific IgE recognition directed to the treatment-resistant allergens. Some 81% of the sensitized patients (22/27) showed specific IgE to pepsin-resistant allergens, and 67% (18/27) had specific IgE to heat-resistant allergens. However, only 30% of patients (8/27) recognized allergens, with estimated molecular weights of about 26, 14 and 9 kDa, after both treatments. The most frequently detected was

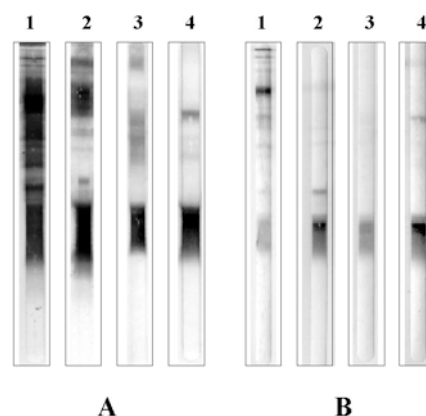


Fig. 2 Specific IgE detection patterns of sera from: **A** a patient who had suffered an acute anisakiasis, and **B** a patient without allergic episodes suffering non-ulcer dyspepsia and abdominal pain after fish ingestion. *Lane 1* Crude extract without any treatment, *lane 2* after pepsin digestion, *lane 3* after boiling for 30 min, and *lane 4* after boiling followed by pepsin treatment

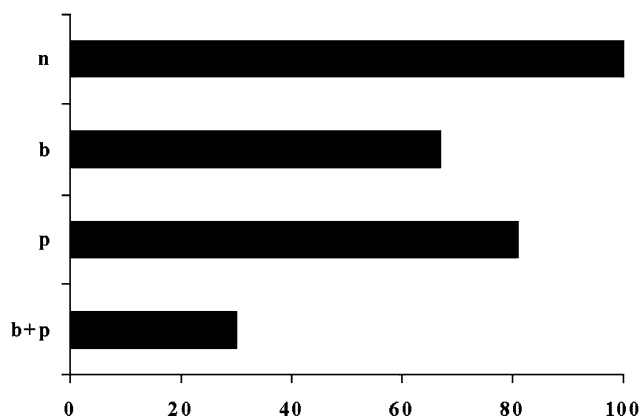


Fig. 3 The frequency of specific IgE recognition directed to treatment-resistant allergens: *n* native without any treatment, *b* boiling for 30 min, *p* pepsin digestion, and *b+p* boiling for 30 min followed by pepsin digestion

the 9-kDa allergen detected by 75% of these patients (Fig. 4).

The distribution of clinical features among the patients with specific IgE to the 9-kDa allergen is shown in Fig. 5. Of the patients, 33% had had allergic but no digestive symptoms after fish ingestion. Fifty percent of patients without allergic episodes suffered gastrointestinal symptoms with abdominal pain and/or vomiting, and 17% of patients had both types of symptoms.

To test if the 9-kDa allergen was a somatic or excretory protein, selected patients were studied by blotting against secretory allergens. As shown in Fig. 6, the patients studied recognized Ani s1 and three, who detected the 9 kDa heat-and-pepsin-resistant allergen, also recognized an excretory/secretory allergen of the same molecular weight present among the antigens released by larvae. This suggests that this 9-kDa allergen is a secretory protein.

Discussion

Several studies on dietary recommendations for patients sensitized to *A. simplex* have recently been published, but at present clear guidelines are not available.

Many allergists recommend the avoidance of fish for all patients with a positive skin test or specific IgE result

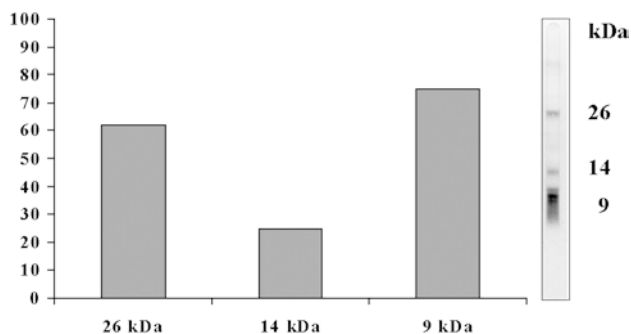


Fig. 4 The frequency of detection of the three most relevant heat- and pepsin-resistant allergens among patients with specific IgE to these allergens

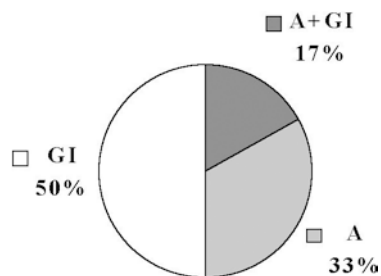


Fig. 5 The distribution of clinical features among patients with specific IgE to heat- and pepsin-resistant allergens. *A* Patients with allergic symptoms after fish ingestion (urticaria and/or angioedema). *GI* Patients without allergic episodes but with gastrointestinal symptoms: abdominal pain and/or vomiting, within 48 h after fish ingestion. *A+GI* Patients with both allergy and gastrointestinal symptoms

to *A. simplex*. However, one group demonstrated that patients with gastroallergic anisakiasis can tolerate deep-frozen seafood, in which the parasites are dead and non-infective (Alonso et al. 1999). Another study reported that patients who had suffered severe anaphylactic reactions after the ingestion of fish can eat frozen fish (Garcia et al. 2001). Other authors (Trujillo et al. 2002) evaluated the utility of different types of diets on preventing further reactions in patients allergic to *A. simplex* and also concluded that they can eat fish frozen at -20°C for 48 h without the risk of a severe allergic reaction. The general belief is that cooking at 70°C or freezing to -20°C for 72 h are the best preventive measures (Bouree et al. 1995).

However, these measures do not destroy the allergenic capacity of *A. simplex* for susceptible patients. As shown above, some sensitized patients have specific IgE directed to proteins from the parasite resistant to boiling for 30 min and to pepsin digestion. These highly resistant allergens could explain the reactions and symptoms of non-ulcer dyspepsia frequently found by us in some *A. simplex* sensitized patients after the ingestion of well cooked or canned fish. In particular, a 9-kDa allergen was detected by 75% percent of patients sensitized to heat-and-pepsin-resistant allergens. Patients who detected this 9-kDa allergen had symptoms such as abdominal pain, pyrosis, nausea and vomiting. Moreover, they had been using antacids or proton pump inhibitors and had required gastroscopy. These symptoms have not been attributed to an *A. simplex* hypersensitivity until now.

Food allergic patients can show severe anaphylactic reactions after the ingestion of raw food, but they can tolerate the ingestion of the same food once cooked (Sanchez-Monge et al. 2000). This is due to the disappearance of the antigenicity by cooking. Proteins resistant to gastrointestinal digestion are especially prone to elicit IgE-mediated immediate-type hypersensitivity reactions, among which anaphylactic shock is the most severe (Astwood et al. 1996). For this reason, digestion assays using pepsin at low pH or trypsin in alkaline conditions have become a standard procedure for

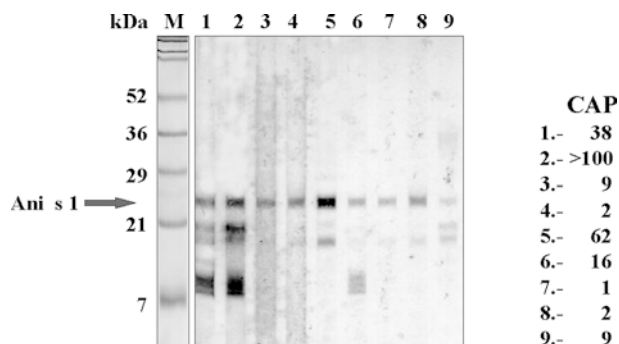


Fig. 6 Specific IgE detection to excretory/secretory allergens from *A. simplex* among selected patients. Lane M Protein standards. Lanes 4 and 8 correspond to a patient monosensitized to Ani s1 used as marker

examining the allergenic properties of food proteins (Astwood et al. 1996; Jensen-Jarolim et al. 1999).

In conclusion, pepsin and heat resistant allergens seem to have a high clinical relevance due to their stability against both treatments.

In a previous study (Moneo et al. 2000b), we found that Ani s 1, an excretory gland protein with a molecular weight close to 25 kDa, had a high clinical relevance and can be considered as a useful tool for the in vitro diagnosis of *A. simplex* hypersensitivity and intestinal anisakiasis (Caballero and Moneo 2002). In this sense, the 9-kDa allergen, also found among the antigens released by larvae, could be used in the future as a marker to evaluate the risk of reactions in sensitized patients after the ingestion well cooked or canned fish. We are therefore trying to isolate and characterize this allergen in order to test its clinical relevance in depth.

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