

Research Note

Allergenic Properties and Cuticle Microstructure of *Anisakis simplex* L3 after Freezing and Pepsin Digestion

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ABSTRACT

This article examines the viability of and the alterations to the larval cuticle and the pattern of the antigens released when live or frozen *Anisakis simplex* larvae were treated with acid and pepsin. The results showed that freezing did not greatly alter the larva body. If ruptures were observed, the antigen release to the incubation media was not enhanced, and most of the antigenic content was retained inside the bodies of the larvae. The immunoblotting assay demonstrated that most of the antigens released, including the allergen Ani s 4, were resistant to pepsin. Freezing killed the larvae, but their survival was not compromised by acid treatment or pepsin digestion when kept chilled. All these findings support recommendations about freezing fish for consumption raw or undercooked to prevent human infection by *A. simplex* larvae. However, our data show that the antigenicity of the larvae is preserved after freezing and may explain why some sensitized patients develop symptoms after ingestion of infested frozen fish.

The presence of *Anisakis simplex* and other Anisakidae nematodes has dramatically increased throughout the world in the last 2 decades in several fishing areas, infecting most widely consumed commercially valuable saltwater fish and cephalopod species (1, 2, 11, 18). The fishing industry and public health authorities have long been aware of the problems caused by the infestation of fish by parasites, as some of them affect both the commercial value of catches and consumer health. Among the parasites that infect fish, species of the Anisakidae family are important because of the human illnesses associated with the ingestion of fish or cephalopods parasitized with the live third-stage larvae (L3).

The immunoglobulin E (IgE) from sensitized patients recognizes somatic allergens from *A. simplex* and also proteins secreted/excreted by the parasite under pH conditions similar to those found in the stomach (15). In fact, pepsin and acid pH are potent stimuli for *A. simplex* to secrete/excrete proteins and even to molt from the L3 to the adult stage (12, 13). Many *A. simplex* allergens are resistant to heat and pepsin treatments (10). Of these, Ani s 4 has been described as the secreted/excreted allergen recognized most frequently by *A. simplex*-sensitized patients suffering anaphylaxis (16, 19).

Freezing fish for consumption raw or with only mild treatments, under time and temperature conditions consid-

ered sufficient to kill the larvae, is mandatory in many countries. The conditions vary from freezing at not more than -20°C in all parts of the product for at least 24 h, as recommended by the European Union (3, 5), to freezing at -20°C for at least 7 days or -35°C for 15 h, as recommended by the U.S. Food and Drug Administration (4).

The aim of this study was to evaluate the allergenic and antigenic capacities and the release of antigens from the larvae to the medium when live and frozen *A. simplex* larvae are treated with pepsin and acid.

MATERIALS AND METHODS

Sample preparation. Live *A. simplex* larvae in the third stage (L3) were obtained from viscera and ovaries of heavily parasitized fresh hake (*Merluccius merluccius*) caught in the Northwest Atlantic fishing ground (FAO area 27) and the Bay of Biscay. Approximately 100 g of larvae, with ovary or viscera tissue attached, was sent to the laboratory at the Instituto del Frío (CSIC) and immediately stored at $5 \pm 1^{\circ}\text{C}$ (chilled larvae) for further preparation. An aliquot of the larvae and the extraneous tissue was frozen in individual test tubes at $-20 \pm 1^{\circ}\text{C}$. The final temperature was reached in 30 min, and the larvae were stored at -20°C for at least 48 h (frozen larvae). Frozen larvae were thawed at room temperature (<30 min) before application of each treatment. The chilled and frozen storage temperatures chosen were the average temperature in domestic refrigerators and the highest temperature recommended for killing the larvae, respectively.

Treatment of the larvae. Chilled and frozen *A. simplex* larvae (0.5 g in each individual assay) were treated with acid (0.3

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M HCl, pH 1 [harsh conditions] or 30 mM NaCl, 0.06 M HCl, pH 1.8 [mild conditions]) and with acid and pepsin (10 mg of pepsin per ml; proteolytic activity, 1:10,000 NF [U.S. National Formulary], equivalent to 2,000 International Pharmaceutical Federation [FIP]-U/g; Panreac, Castellar del Vallès, Spain), under harsh conditions (17) or 3.3 mg of pepsin per ml under mild conditions (6) at a larva-solution ratio of 1:2 (wt/vol). Pepsin treatments under harsh conditions were selected to mimic the method used in the fish industry to isolate larvae from fish flesh, and the treatments under mild conditions were to mimic the gastric digestion conditions. Capped tubes were incubated in a water bath ($37 \pm 0.5^\circ\text{C}$, 4 h) with continuous shaking (250 rpm). After treatment, the larvae were separated from the incubation liquid with a sieve, and the pH of the filtrated medium was adjusted to ≥ 6 to halt the pepsin reaction. Assay controls were larvae treated with phosphate-buffered saline (PBS).

Study of the larval viability. Spontaneous and stimulated movements of the larvae before and after treatments were visually observed and recorded by a video camera (Sony Handycam DCR-Sr70) immediately after treatment and at intervals over a space of 2 h. When no movement was recorded, the larva was considered dead. A bluish fluorescence of the chilled and frozen larvae before and after incubation was examined in a dark chamber by exposing them to a UV light source (366 nm) 15 cm above the parasites. The intensity of the fluorescent emission was rated in arbitrary units as maximum, medium, slight, or no fluorescence.

SEM. For scanning electron microscopy (SEM) observation, control and treated larvae were immediately fixed as described previously (20).

Immunoblotting. Two hundred milligrams of each sample of *A. simplex* larvae was mixed with 2 ml of PBS, ground with a potter, and sonicated (30 s; output power, 11 W) and centrifuged (10 min, $16,000 \times g$, 20°C) to discard the insoluble debris. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting assays were performed as described previously (19).

The work was performed at least twice with different *A. simplex* batches.

RESULTS AND DISCUSSION

Larval viability. Spontaneous movements were detected in all chilled larvae before and after all treatments; larvae were more active when treated under harsh conditions. No movement was detected in the frozen larvae. The highest fluorescence intensities were observed in the frozen larvae. The chilled (live) larvae treated under harsh conditions emitted slight or intermediate fluorescence when treated in acid or pepsin, respectively. No fluorescence was observed in the chilled larvae treated under mild conditions.

SEM. The cuticle of the untreated chilled and frozen larvae presented continuous wide transverse striations (annular furrows) with a smooth fibrillar surface (Fig. 1A). In the PBS controls, the surface of the frozen larvae presented deep invaginations (Fig. 1B) and disrupted zones with emission of internal material (not shown), which was not observed in the chilled larvae. No changes were observed in the live larvae treated with acid or pepsin under mild conditions, whereas the frozen larvae showed smooth zones along with deep invaginations (Fig. 1C). The larvae treated with acid under harsh conditions presented an undulate appearance with marked annular furrows, more evident in the

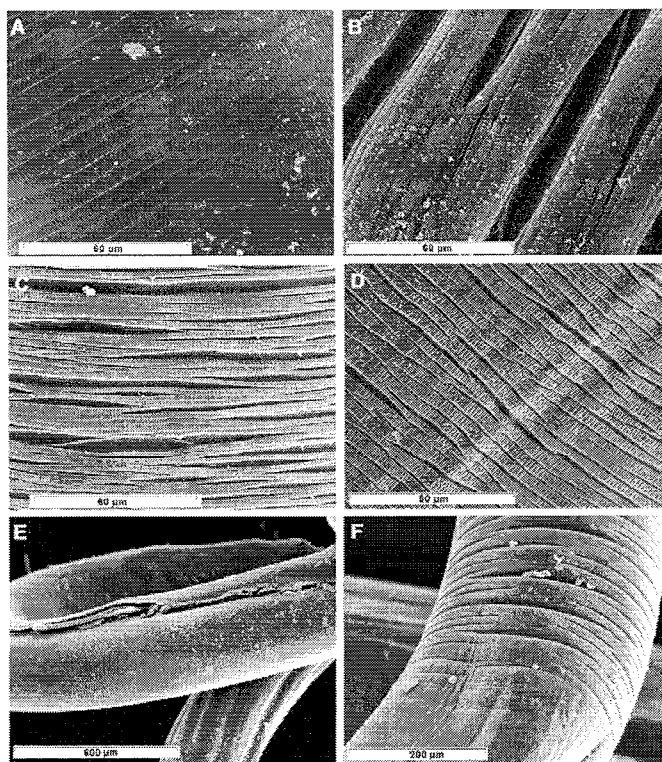


FIGURE 1. SEM of the cuticle of *A. simplex* L3 larvae. (A) Chilled (4°C) control, not treated; (B) frozen (-20°C) larvae treated with PBS; (C) frozen larvae, acid treated under mild conditions; (D) frozen larvae, acid treated under harsh conditions; (E) chilled larvae, acid and pepsin treated under harsh conditions; (F) frozen larvae, acid and pepsin treated under harsh conditions.

frozen larvae (Fig. 1D). Longitudinal breaks in the cuticle with emission of material from the interior were clearly appreciable in the chilled larvae treated with pepsin in harsh conditions (Fig. 1E); the frozen larvae showed marked invaginations together with smooth areas, although no fractures in the cuticle were detected (Fig. 1F).

Immunoblotting. The antigenic and allergenic activities of the *A. simplex* extracts and the proteins released to the incubation media were studied by IgG immunoblotting using an anti-Ani s 4 polyclonal rabbit antiserum and by IgE immunoblotting, using pooled human sera. The rabbit-IgG immunoblotting assay showed the presence of Ani s 4 (9 kDa) in all the whole-larva body extracts and all the incubation media filtrates. The human-IgE immunoblotting assay (Fig. 2) demonstrated that the human sera were able to recognize several allergenic proteins, in a range from 6.5 to more than 90 kDa, in all the larva extracts and the incubation media filtrates. The addition of pepsin to the incubation media yielded a profile of detected proteins different from the profile obtained under acid conditions.

This work was designed to evaluate the effects of chilling and freezing on the viability, cuticle structure, and antigen/allergen release by *A. simplex* larvae. The results showed that incubation with acid and pepsin for 4 h did not kill the larvae, which presented active movements, even when the HCl or the pepsin concentrations were higher than those reported for the gastric juice and longitudinal break-

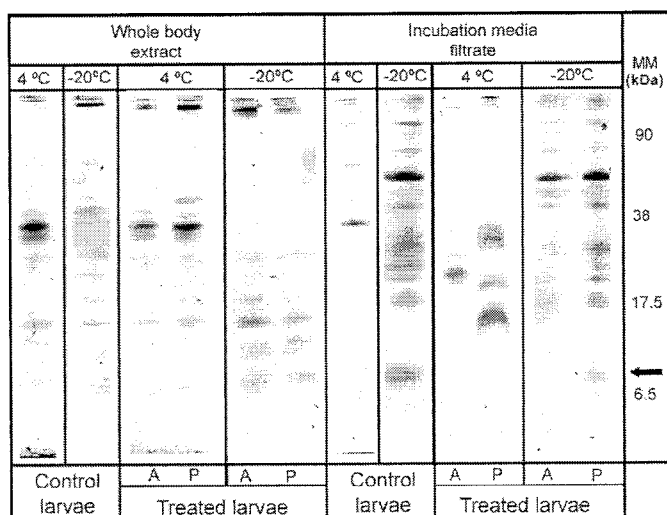


FIGURE 2. IgE immunoblotting of the chilled (4°C) and frozen (-20°C) larvae, untreated (control larvae) or treated in acid media, without (A) or with (P) pepsin, revealed with a pool of sera from *A. simplex*-sensitized patients. The estimated molecular masses (MM) are indicated in kilodaltons. An arrow marks the position of the allergen Ani s 4.

ages in the cuticle were observed by SEM. On the other hand, freezing at -20°C for 48 h killed the larvae and produced increased fluorescence emission. Freezing the fish muscle enhances emission of fluorescence of the larvae, and this is used to determine the prevalence and intensity of infection of fish by L3 larvae (9). This phenomenon has been associated with the death of the anisakid larvae (14). In this study, we found that live larvae were slightly fluorescent when treated with acid at a high concentration, and this effect was enhanced when pepsin was added to the medium. This indicated that fluorescence did not discriminate between live and dead larvae and suggested that it might be related to the stress produced in the larvae by the treatment.

SEM micrographs revealed that acid did not cause big changes in the cuticle or the body shape of live or dead larvae. On the other hand, the digestion of the live larvae with pepsin under harsh conditions caused ruptures along their bodies, although the larvae remained alive, resembling the beginning of molting. The frozen larvae incubated in PBS presented fractures in their bodies and changes in the microstructure of their cuticle. Changes in their cuticles were also observed when frozen larvae were treated with acid and pepsin under mild and harsh conditions, suggesting that freezing might alter the permeability of the *Anisakis* cuticle, as observed in a previous work (20).

The release of Ani s 4 and other allergens by the *Anisakis* larvae during the different treatments was not directly related to changes in the surface of the larvae. Ani s 4 has been described as a heat- and pepsin-resistant allergen frequently recognized by patients suffering from anaphylaxis due to *A. simplex* (16, 19). However, our findings indicate that this allergen is resistant to pepsin even at harsher acid and pepsin concentrations than those found in the human stomach. The IgE immunoblotting assay demonstrated the presence of several other allergens in both the larval bodies

and the incubation media. It was not the purpose of this article to identify and characterize these allergens, but the results of this assay revealed a significant amount of allergens, resistant to very low pH and high pepsin concentrations, that are able to bind the human IgE.

The results of this study demonstrate that during the different treatments *Anisakis* larvae retained several allergens resistant to pepsin and acid inside their bodies and release them into the incubation media, even when the larvae were dead. The presence of *A. simplex* allergens in the fish muscle, with their allergic capacity intact after digestion, could provoke symptoms in previously sensitized patients. In fact, some authors have reported clinical symptoms in sensitized individuals following the ingestion of frozen or canned fish presumably containing dead larvae or parasite antigens (7, 8). This may explain why some sensitized patients develop symptoms after ingestion of infested frozen fish. Further studies are necessary to determine the clinical relevance of these findings and may help to design personalized diets adjusted to the characteristics of the allergens recognized by each patient, and also to find further uses for heavily parasitized fish.

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