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Anisakis Simplex Antigens in Fresh and Frozen-thawed Muscle of Anchovies in Vinegar

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Marinated fish treatment using low pH to enlarge the storage life of fish as in anchovies in vinegar, does not kill *Anisakis simplex* larvae infesting fish muscle. To kill the larvae it is compulsory in many countries to freeze fish intended to be marinated raw, which prevents the consumer to be infested with the live larvae. However, it is not known if *A. simplex* antigens are released to the media after freezing and vinegar processing, which may cause allergic reaction to *A. simplex* sensitized consumers. Anchovy fillets were artificially infested with *A. simplex* L3, treated with a vinegar solution and chilled stored for 10 days. Infested frozen-thawed fillets were treated and stored in the same conditions. Viability of the larvae, SEM, immunoblotting and immunohistochemistry were performed on the treated fillets before and after pepsin treatment. Viability of the larvae was detected only in the chilled fillets; however, *A. simplex* antigens were detected in the chilled and in the frozen-thawed fillets even after pepsin treatment. This suggests that the consumption of anchovies in vinegar may be a potential hazard when ingested by sensitized consumers, even if freezing kills the larvae.

Key Words: *Anisakis simplex*, antigens, anchovies, vinegar, food hygiene

INTRODUCTION

Several food-processing treatments use low pH to increase the storage life of fish products to avoid or slow the growth of spoilage microorganisms. Marinated fish prepared under different recipes is very popular in many countries and often the fish preserved in these conditions is considered as a delicatessen (anchovies in vinegar, ceviche, lomi-lomi, etc.). However, these treatments are not sufficient to kill some parasites infesting fish. Among fish parasites, *Anisakis* sp. is considered a major hazard due to the fact that it can cause in the consumers infestation by the live larvae and allergy. In general when fish is infested with *Anisakis simplex* larvae (L3), treatments based on the decrease of pH do not kill the larvae during the storing period in the

industrial and household conditions used to prepare the fish in vinegar or other common processes of fish based in lowering the pH. Treatments based on the action of acetic acid at strong concentration followed by washing to reduce the acetic acid concentration to levels acceptable to consumers have been proposed to ensure the rapid death of *Anisakis* larvae (Sánchez-Monsalvez et al., 2005).

To prevent the infestation of the consumer by the L3 larvae it is compulsory in many countries to freeze the fish that is intended to be consumed raw or processed by mild treatments that do not kill the parasite. The freezing and storing conditions vary from a temperature of not more than -20°C in all parts of the product for at least 24 h recommended in the European Union (1991, 2004) to freeze at -20°C for at least 7 days or -35°C for 15 h recommended for the FDA (2001). The sensitization to *A. simplex* has been found to be associated with the ingestion of raw fish, suggesting that an active infestation with live L3 larvae is necessary for IgE synthesis (Moneo et al., 2000) although a development of allergic asthma has been found to occur in mouse models after nasal sensitization with *Anisakis pegreffii* antigens (Kirstein et al., 2007). Nevertheless, although freezing the fish at -20°C for a time ≥ 24 h

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avoid the infestation of the consumer with the live larvae, this procedure may not be adequate to avoid allergy in individuals previously sensitized to *A. simplex* allergens. Allergy to *Anisakis* has a high prevalence in Spain (Pichler, 1999) and has been associated to the consumption of a very popular fish preparation as it is anchovies in vinegar in which the anchovies are marinated in a solution of vinegar and salt for several hours.

In a previous work we have detected changes in the cuticle of *A. simplex* larvae after freezing and food processing treatments, which may lead to a different permeability of the cuticle in the frozen larvae (Tejada et al., 2006a, b). Moreover, *Anisakis* antigens have been detected in fish muscle parasitized by live *A. simplex* L3 (Solas et al., 2008). It has been reported that live *A. simplex* L3 larvae release allergens even during a short incubation in diluted acid (Moneo and Caballero, 2002) which suggests that an increase of allergen release may also be produced in parasitized fish treated at low pH. In addition, the live larvae release allergens when incubated in conditions that mimic the gastric juice, some of them pepsin resistant, which can explain the acute allergic symptoms that appear in some patients after eating parasitized fish (Caballero and Moneo, 2004).

The aim of this work is to ascertain if antigens from *A. simplex* live and dead (killed by freezing) larvae are secreted/excreted or released to the media or to the fish muscle in the pH and salt conditions employed to produce anchovies in vinegar and if they are detected after pepsin treatment.

MATERIALS AND METHODS

Samples

Larvae

Live *A. simplex* larvae in the third stage (L3) were obtained from heavily parasitized fresh hake (*Merluccius merluccius*) ovaries at the central fish market in Madrid, Spain (Mercamadrid). The hakes were caught in the Northwest Atlantic fishing ground. Approximately 100 g of larvae including ovary tissue were sent to the laboratories at the Instituto del Frio (IF) and immediately stored at $5 \pm 1^\circ\text{C}$ until used (maximum period of storage 72 h).

Anchovies

Twelve kilograms of anchovies (*Engraulis encrasicolus*) were transported from Mercamadrid to the laboratory and immediately treated. The fish was caught in the Atlantic waters of Morocco. The temperature of the fish on arrival to the laboratory was 3°C . The mean length and weight of the fish was 13.8 ± 0.5 cm and 29.3 ± 3.2 g.

The anchovies were headed, gutted, filleted, and washed with ice water to remove blood.

Methods

Sample preparation

To standardize the conditions of the study, units of 50 ± 0.5 g of fillets were placed in plastic containers in 2 layers, artificially infested with 12 live *A. simplex* larvae placed between both layers and stored at $5 \pm 1^\circ\text{C}$ for 24 h to let the migration of the larvae into the fish muscle. After that, part of the fish was frozen at $-20 \pm 1^\circ\text{C}$ and stored for 5 days. The frozen samples were thawed overnight at $12 \pm 1^\circ\text{C}$ before further treatment. Chilled and frozen-thawed anchovy fillets were treated in the containers with 90 mL of a vinegar solution (white wine vinegar, water, and salt (50:50:6, v:v:w) and stored in a cold room set at $5 \pm 1^\circ\text{C}$ for 24 h. The conditions utilized are usual for preparing 'anchovies in vinegar'. After that the liquid was drained and the anchovies were stored up to 12 days in the cold room at $5 \pm 1^\circ\text{C}$. Control lots for the chilled vinegar treated samples (no *A. simplex* added) and for the *A. simplex* infested chilled fish (no vinegar treated) were also prepared and stored in the same conditions. Four containers with 50 ± 0.5 g of fillets were prepared for each treatment and storage period studied. Chilled and frozen storage temperatures were chosen as the average temperature in home refrigerators and the highest temperature recommended for killing the *A. simplex* larvae, respectively. All the lots were studied after 24 h of vinegar treatment and at intervals during storage. For immunoblotting studies chilled and frozen-thawed larvae were treated with vinegar (rate 1:2 larvae:vinegar solution) and chilled stored until measured.

Proximate analyses and pH

Moisture and ash (AOAC, 1995), crude fat (Knudsen et al., 1985) and crude protein (LECO FP-2000 nitrogen/protein determinator (LECO Corp., St Joseph, MI, USA) using a nitrogen to protein conversion factor = 6.25) were measured in the fish muscle. The results of the proximate analyses are expressed as gram per kilogram of muscle and are the mean value of three determinations. The protein in the retentates and filtrates of the samples after pepsin treatment was measured and expressed as percent of protein to total protein in the sample. The pH was determined at room temperature using 10 g of chopped muscle in 100 g distilled water. Measurements were carried out with a glass electrode (pH-electrode blue line Elektrolyt L300; Schott Instruments, Mainz, Germany).

Pepsin treatment

Twenty-five grams (± 1 g) of the chilled and frozen-thawed anchovies in all lots were treated with a pepsin solution (0.3M HCl, 10 mg/mL pepsin (Proteolytic Activity 1:10,000 NF (2000 FIP—U/g) Panreac, Spain) at 37°C ($\pm 0.5^\circ\text{C}$) at a rate of 1:2 (w:v) for 6 h 45 min in a water bath with continuous shaking. The final time was set when fresh (0 days storage) no infested anchovy muscle was digested. Previously we had checked that the larvae remained alive after this treatment. The digested protein was measured by LECO after passing through a sieve (2 mm mesh). Isolated chilled and frozen-thawed vinegar treated larvae were separated from the vinegar solution and subjected to pepsin treatment (rate 1:2 larvae:pepsin solution, w:v) in the time and temperature conditions described. After pepsin treatment the pH was adjusted at ≥ 6 to stop pepsin reaction.

Larvae viability

Movement

The movement of the larvae was visually observed and recorded by a video camera (Sony Handycam DCR-Sr70). When the movement of the larvae was not observed immediately, 3-min videos were taken at intervals within 2 h to assure that no movement was produced.

Fluorescence

The emission of a bluish fluorescence of the chilled and frozen larvae, was examined by exposing the fillets to UV light (366 nm) and observing the fluorescence emission in a dark chamber, holding the UV light ~ 15 cm above the fillets. Pictures at visible and UV light were systematically taken with a Nikon camera (Digital Camera Nikon Coolpix 5000). The intensity of the fluorescent emission was rated in arbitrary units as maximum, intermediate, slight and no fluorescent emission.

Immunoblotting

For immunological studies, isolated chilled and frozen *A. simplex* larvae treated with the vinegar solution up to 12 days were pepsin treated as stated, and mixed with 2 mL of PBS and then they were ground with a potter, sonicated (30 s, 11 watts output power, Microson ultrasonic cell disruptor. Misonix, Farmingdale, NY, USA) and centrifuged (10 min, 16,000 g, 20°C) to discard the insoluble debris. Soluble proteins were diluted 1/50 in electrophoresis sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% Glycerol, 0.042% Bromophenol Blue) and applied to a 16% Tris-tricine polyacrylamide gel in order to separate them according to their

molecular weights. Vinegar fractions obtained after the removal of the larvae were made up to 2 mL and centrifuged (10 min, 16,000 $\times$ g, 20°C). Supernatants were diluted 1/2 in electrophoresis sample buffer before being applied to the same tris-tricine polyacrylamide gels. After electrophoresis, proteins were stained with Coomassie or transferred to nitrocellulose membranes for immunoblotting (Hitchcock, 1950; Kasuya and Koga, 1992). Briefly, for the rabbit IgG immunoblotting assay, the membranes were blocked (3% Nonidet P-40 in PBS for 30 min) and subsequent incubations with rabbit anti-Ani s 4 and anti-*A. simplex* crude extract antisera (1/10,000 dilution), obtained as described (Kasuya and Koga, 1992), and alkaline-phosphatase labeled goat anti-rabbit IgG (1/5000 dilution) (Biosource International, CA, USA) were performed. BCIP-NBT (5-Bromo-4-Choro-3-Indolyl Phosphate-Nitro Blue Tetrazolium) was the substrate used for revealing the immunoblots.

Scanning Electron Microscopy

For Scanning Electron Microscopy (SEM) observation, small blocks of parasitized tissue (1.5-cm-sided cubes) were cut in all lots. The samples were immediately fixed with a mixture (1:1, v:v) of paraformaldehyde (4%) and glutaraldehyde (0.2%) in 0.1 M phosphate buffer (pH 7.2) and stored at $5 \pm 1^\circ\text{C}$ for at least 4 h. The fixed samples were postfixed with OsO_4 , washed, dehydrated in increasing concentrations of acetone, critical point dried, sputter coated with gold-palladium in a metallizer (Balzer SCD 040, Liechtenstein) and scanned by SEM (Jeol JSM 6400, Akishima, Tokyo, Japan) at 20 kV. A large number of micrographs were taken in SEM in order to select the most representative ones.

Immunohistochemistry

Antigen source

Crude antigen extract was obtained after crushing in a mortar manually collected larvae (Garcia et al., 1997). Recombinant Ani s 4 was produced in a bacterial system as previously reported (Rodriguez-Mahillo et al., 2007). Thereafter, a rabbit anti-serum to Ani s 4 and an anti-serum to a crude extract were obtained to detect antigens and Ani s 4 in parasitized fish muscle.

IHC study

Chilled and frozen vinegar treated muscle after 8 days storage before and after pepsin treatment were subjected to Immunohistochemistry (IHC) observation. Small pieces of parasitized tissue were fixed in 0.1% glutaraldehyde in phosphate buffer saline for 1 h at 4°C. After washing in PBS, samples were dehydrated in successive ethanols with progressive lowering of temperature

to -30°C and finally embedded in Lowicryl K4M (PolyScience Inc. Washington, USA) at -30°C (PLT technique). K4M resin was polymerized under UV light for 24 h at -30°C and then under UV for 48 h at room temperature. Low temperature dehydration embedding and polymerization was performed on automatic freeze substitution system (AFS, Leica, Vienna, Austria). Ultrathin sections (70 nm) were obtained with an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) and collected on 100-mesh formvar coated nickel grids. Grids carrying ultrathin sections were sequentially floated in 0.1M glycine in PBS and 5% bovine serum albumin (BSA) in PBS. Then, grids were incubated overnight at 4°C with rabbit anti-Ani s 4 or anti-crude extract antisera diluted 1:200 with PBS containing 1% BSA. After washing with PBS, the sections were incubated with a secondary antibody, goat anti-rabbit IgG (80 ng/mL) conjugated to 15 nm gold particles (British Biocell, Cardiff, UK) diluted 1:200 in PBS for 1 h at room temperature. After PBS and water washings, sections were stained with 2% uranyl acetate (20 min) and Reynold's lead citrate (3 min) and examined in a Jeol 1010 electron microscope. Controls were performed excluding the primary antibody. Simultaneously, negative controls were processed with non-immune rabbit serum instead of primary anti-Ani s 4 and anti-crude extract antibody.

RESULTS AND DISCUSSION

The proximate analyses of the anchovy muscle were: moisture: $777.7 \pm 3.2 \text{ g kg}^{-1}$; crude protein; $203.3 \pm 4.2 \text{ g kg}^{-1}$; fat: $22.7 \pm 4.1 \text{ g kg}^{-1}$ and ash: $18.1 \pm 1.6 \text{ g kg}^{-1}$. The pH of the samples treated with the vinegar solution the initial pH was around 4.1 and remained with small changes during the storage (Figure 1). In the infested lots the pH of the frozen-thawed fish was significantly lower than the chilled fish at the end of the storage period. In the lot infested with *A. simplex*, no vinegar added, the pH rose during storage at the same values than in the fish muscle.

Viability

Movement of the larvae was detected in the unfrozen lots during all the storage period. One live larva was observed after 10 days in the anchovy fillets treated with vinegar where no larvae were added, which indicates that the muscle was naturally infested. This differs with the results found in anchovy muscle parasitized with *Anisakis pegreffii* larvae (Marrone et al., 2007). No movement of the larvae was detected in the parasitized frozen-thawed lot.

A maximum emission of fluorescence by the larvae was appreciated in the frozen-thawed lot during all the storage period; nevertheless the intensity of the emission decreased at an intermediate fluorescence at the end of the storage period. A slight fluorescent emission was observed in some live larvae, no vinegar treated, at one day storage. It is accepted that emission of fluorescence of the *A. simplex* L3 larvae improves by freezing and thawing of the samples (Anonymous, 1995) and is reported by some authors as an indicator of the death of the larvae (Karl et al., 1995).

The larvae remained live in all the unfrozen lots in the vinegar treated muscle after 10 days storage and after pepsin digestion, which supports the recommendation to freeze the fish intended to be consumed raw or subjected to mild treatments, as vinegar, in order to avoid the

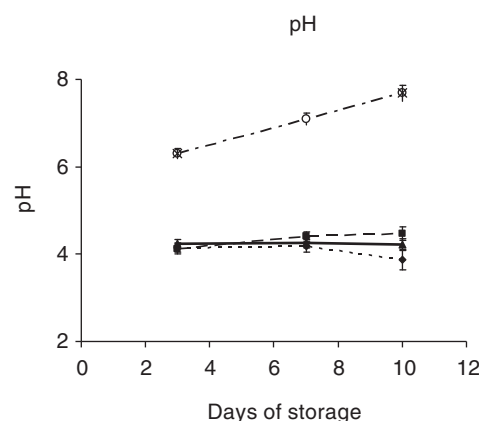


Figure 1. pH of the muscle during chilled storage at $5 \pm 1^{\circ}\text{C}$. AF: anchovy fillets; V: vinegar solution; As: *A. simplex*; Fr-T: frozen-thawed. (▲) AF + V, (■) AF + As + V, (◆) AF + As + Fr-T + V, (○) AF + As, (◇) AF.

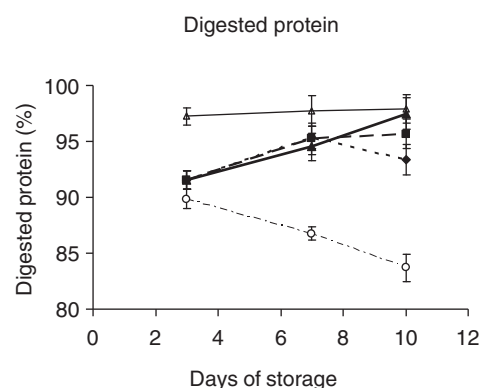


Figure 2. Percent of crude protein (Nx6.25) digested by pepsin solution. (▲) AF + V, (■) AF + As + V, (◆) AF + As + Fr-T + V, (○) AF + As, (◇) AF.

infestation of the consumer with the live larvae and also to prevent initial sensitization to *A. simplex*.

Protein in the filtrates after pepsin treatment

Most of the anchovy muscle was digested in the conditions employed (Figure 2). In the vinegar treated samples the percentage of protein in the filtrates remained above 91% during all the studied period whereas in the infested lot without vinegar processing, the protein digested with the pepsin treatment was lower than in the vinegar treated

lots, and decreased at longer storage time. This may be due to an increase of production or release of protease inhibitors to the muscle by the live larvae in the untreated lot during storage, which may decrease the action of pepsin on the fish muscle. Pepsin inhibitors have been identified in several nematodes, among them *Ascaris lumbricoides* (Ng et al., 1999) and there is direct and indirect evidence of the production of pepsin inhibitors by *A. simplex* (Morris and Sakanari, 1994; Dzik, 2006; Knox, 2007). The release of pepsin inhibitors or their activities seem to decrease in acidic media.

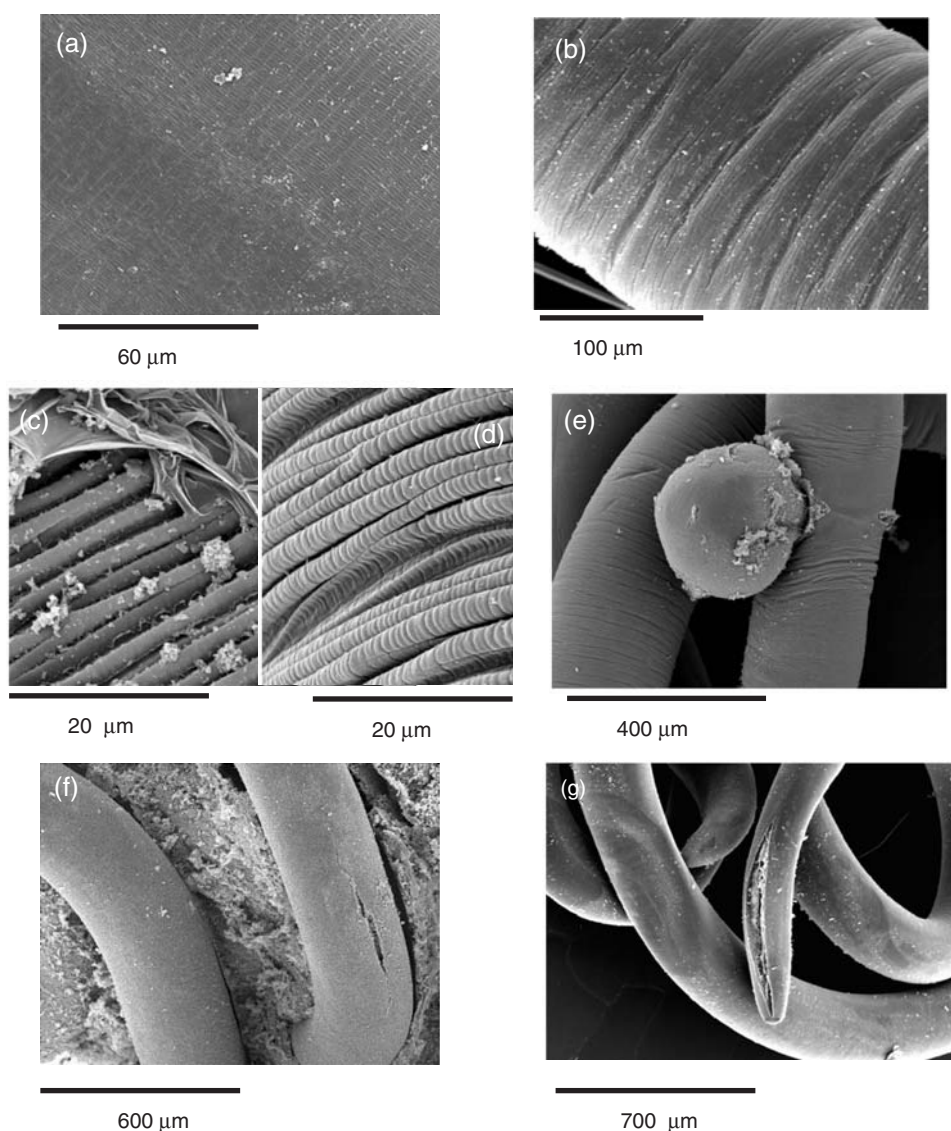


Figure 3. SEM of *A. simplex* larvae in the vinegar treated anchovies. Chilled: Appearance of the cuticle: without (a) and with (b) vinegar treatment. Cuticle of the vinegar treated larvae at 1 day of storage (c). Cuticle of the vinegar and pepsin treated larvae after 5 day of storage (d). Breakages in the cuticle in the vinegar treated larvae with emission of the interior in larvae chilled stored for 10 days (e). Frozen-thawed: Ruptures in the surface observed in individuals at 1 day storage (f); Appearance of the vinegar treated larvae after pepsin treatment (g). Storage at $5 \pm 1^\circ\text{C}$.

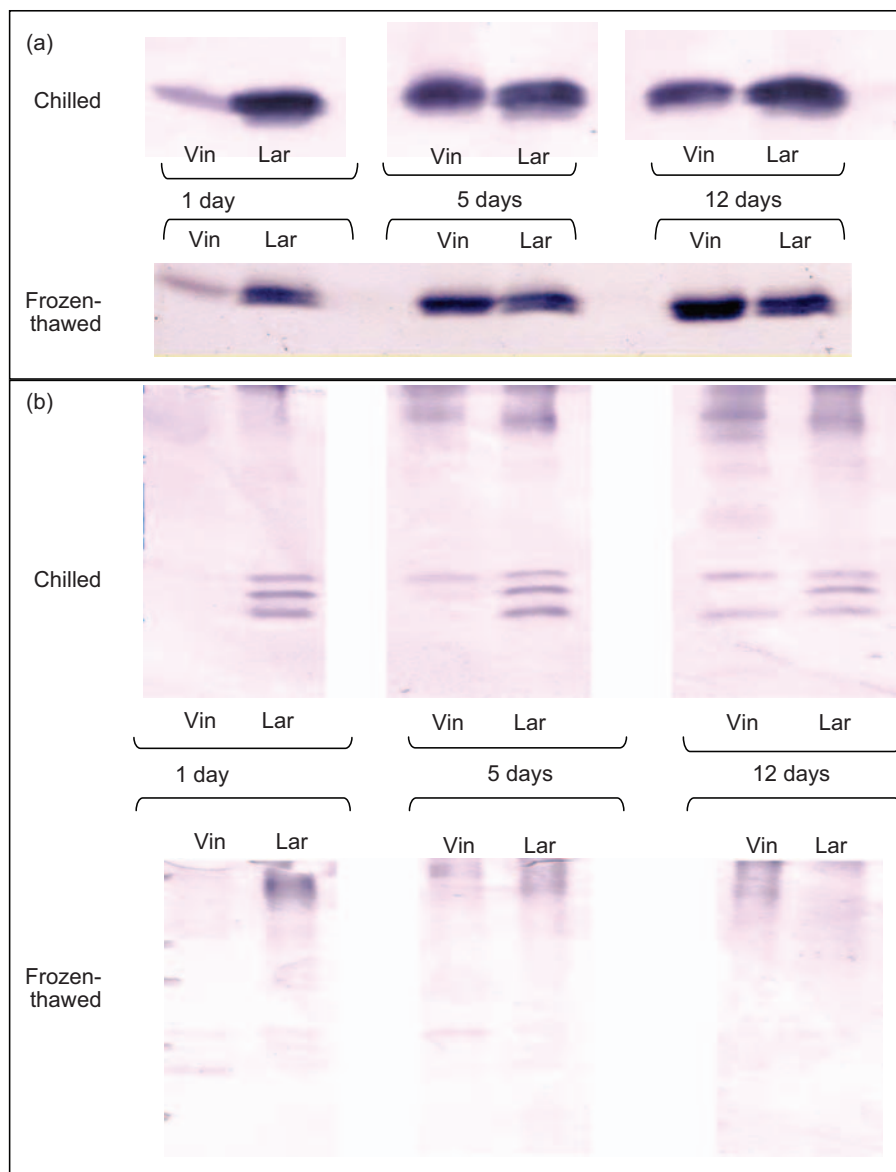


Figure 4. Proteins of the vinegar media and the vinegar treated whole body *A. simplex* extracts after pepsin treatment of the chilled and frozen-thawed larvae. Incubations with rabbit anti-Ani s 4 (a) and anti-*A. simplex* crude extract antisera (b) (1/10,000 dilution). Vinegar media (Vin); larvae after pepsin treatment (Lar).

SEM

The smooth appearance observed in the surface of the chilled untreated larvae (Figure 3a) changed to one with marked grooves in the vinegar treated lots (Figure 3b) where a fibrous appearance of the cuticle was observed along the storage (Figure 3c) and was maintained after pepsin treatment (Figure 3d). Ruptures in the surface of the parasite with emission of material from the interior of some of the vinegar treated larvae were found mainly at the end of the

storage period (Figure 3e). In the frozen-thawed vinegar treated lot, the dead larvae presented a round shape in most of the cases although some longitudinal breakages were observed in some larvae (Figure 3f). After pepsin treatment the frozen larvae presented ruptures and dehydrated areas (Figure 3g).

Immunoblotting

Ani s 4 was detected in the larvae and in the vinegar fraction of the chilled and frozen-thawed larvae during

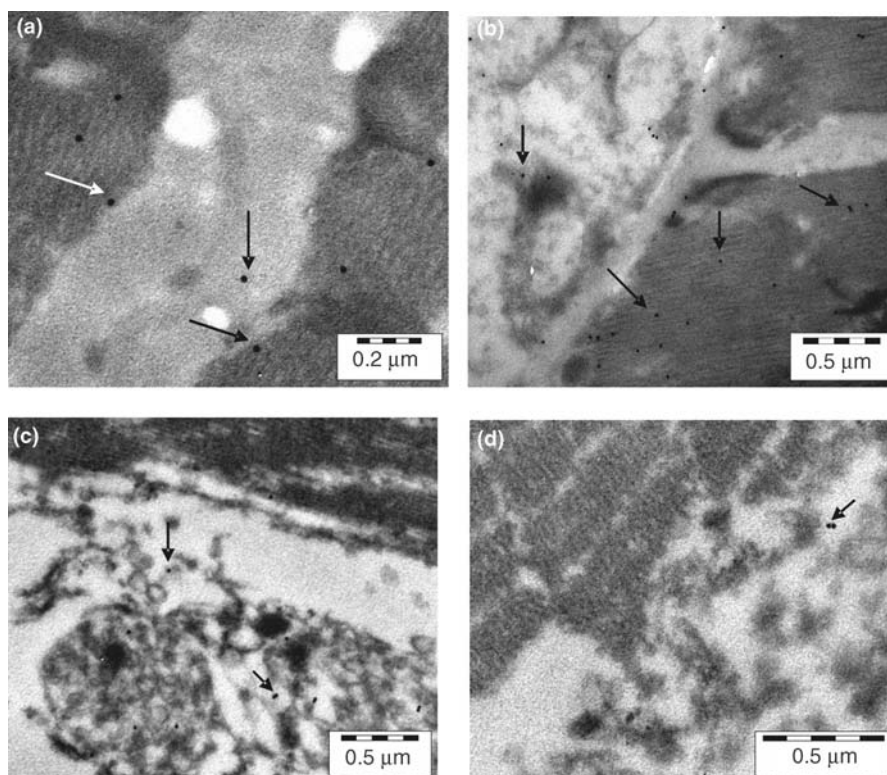


Figure 5. Immunolocalization of Ani s 4 in frozen-thawed *A. simplex* L3 treated with a vinegar solution before (a) and after (b) pepsin treatment. Immunolocalization of Ani s 4 (c) and *A. simplex* anti crude antigens (d) in the contact area larvae-anchovy muscle of chilled (c) and frozen-thawed (d) muscle treated with vinegar. Micrographs obtained from samples at 8 days of storage at 5 ± 1 °C. The 15 nm gold particles conjugated with the secondary antibody appear in the micrograph as dark circles, like those pointed out by arrows.

all the period studied (Figure 4a). Anti-*A. simplex* crude extract antisera (Figure 4b) was detected in the chilled lots in the vinegar and pepsin treated larvae. However, in the frozen ones no clear bands appear in the vinegar and in the pepsin treated larvae.

Immunohistochemistry

Ani s 4 was detected in the interior of the larvae even after freezing-thawing, vinegar and pepsin treatments (Figure 5a, b). Ani s 4 was observed in the vinegar treated muscle in areas in contact with the larvae (Figure 5c) and in areas where the sarcomer's structure was preserved (Figure 6a) even after the harsh pepsin treatment given to the infested fish muscle (Figure 6b). Ani s 4 was also detected in the infested muscle frozen-thawed and vinegar treated before (Figure 6c) and after pepsin treatment (Figure 6d).

A. simplex crude antigens were immunolocalized in areas in contact with the larvae (Figure 5d) and in the anchovy muscle in all conditions studied (Figure 7). The apparent difference with the immunoblotting

studies in the frozen-thawed samples may be due to differences in both protocols. In the immunoblotting assay only soluble proteins obtained from the larvae extracts (diluted 1/50 in electrophoresis sample buffer) or vinegar supernatants (made up to 2 mL and diluted 1/2 in electrophoresis sample buffer) were applied to the gels. It is well known that many proteins precipitate in acidic media or aggregate in frozen storage (Tejada et al., 1996), becoming unextractable in the assayed immunoblotting conditions.

The structure of the sarcomers after vinegar treatment presented a denser appearance in the frozen-thawed anchovy muscle than in the unfrozen one, which was much more evident in the muscle recovered after pepsin treatment, where the digested unfrozen muscle after 8 days of storage (Figure 6b and 7b) was more damaged than the frozen-thawed one (Figure 6d and 7d). This suggest that some changes were caused by freezing and frozen storage, which modify the appearance of the infested muscle when treated with vinegar, even in the short period (5 days) that the anchovy fillets remained frozen.

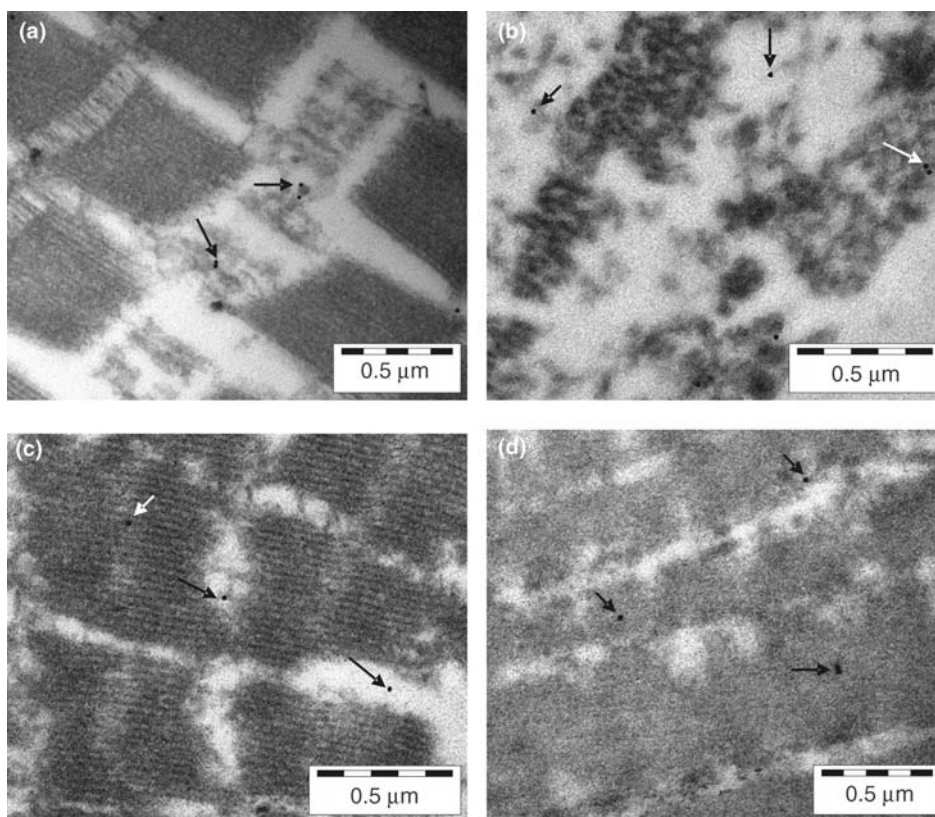


Figure 6. Immunolocalization of Ani s 4 in sarcomers of anchovy muscle artificially infested with live *A. simplex* L3. Chilled, vinegar solution treated: Sarcomers before (a) and after (b) pepsin treatment. Frozen-thawed, vinegar solution treated: Sarcomers before (c) and after (d) pepsin treatment. Micrographs obtained from samples after 8 days of storage at 5 ± 1 °C. The 15 nm gold particles conjugated with the secondary antibody appear in the micrograph as dark circles, like those pointed out by arrows.

As stated, *A. simplex* antigens were detected in the infested anchovy muscle by the specific anti Ani s 4 and *A. simplex* anti crude extracts in all the conditions studied. In the chilled samples, the presence of antigens in the muscle could be due to excretion/secretion by the live larvae. An increase of excretion/secretion or release of allergens by the live larvae in acidic media at 37 °C in conditions that mimic the gastric juice has been previously reported (Moneo and Caballero, 2002). Nevertheless the live larvae presented breakages in the surface, mainly at the end of the storage period, which probably increased the release of allergens to the muscle, as Ani s 4 is also located in the basal lamina of the cuticle (Rodríguez-Mahillo et al., 2007). In the frozen-thawed lots, the presence of antigens in the muscle may be due to the secretion/excretion by the live larvae in the 24 h elapsed before freezing or to the release of antigens by the dead larvae. Changes in the permeability of the cuticle of the frozen larvae observed by Environmental SEM, and release of antigens by isolated frozen larvae have been previously reported (Tejada et al., 2006a, b). The detection of *A. simplex* antigens in the muscle in all the studied conditions confirm the

resistance to acid and pepsin action even at stronger conditions than the ones that mimic the gastric juice previously reported in isolated larvae (Caballero and Moneo, 2004).

A. simplex larvae remained alive after 10 days of vinegar treatment and after pepsin digestion. This supports the recommendation given by the sanitary authorities to freeze the fish intended to be consumed raw or subjected to mild treatments in order to avoid the infestation of the consumer with the live larvae and also to prevent initial sensitization to *A. simplex* in healthy consumers. However, Ani s 4 was detected in the vinegar fraction and both, Ani s 4 and *A. simplex* antigens were detected in fish muscle and isolated larvae even when the infested muscle or the larvae had been previously frozen to kill the L3 larvae and after pepsin treatment. The results obtained suggest that the consumption of anchovies in vinegar infested with *A. simplex* may be a potential hazard when consumed by sensitized individuals, even if the larvae are killed by freezing. Nevertheless, we cannot estimate at present if the detected amount support an allergic reaction against *A. simplex* allergens in patients previously sensitized against the parasite.

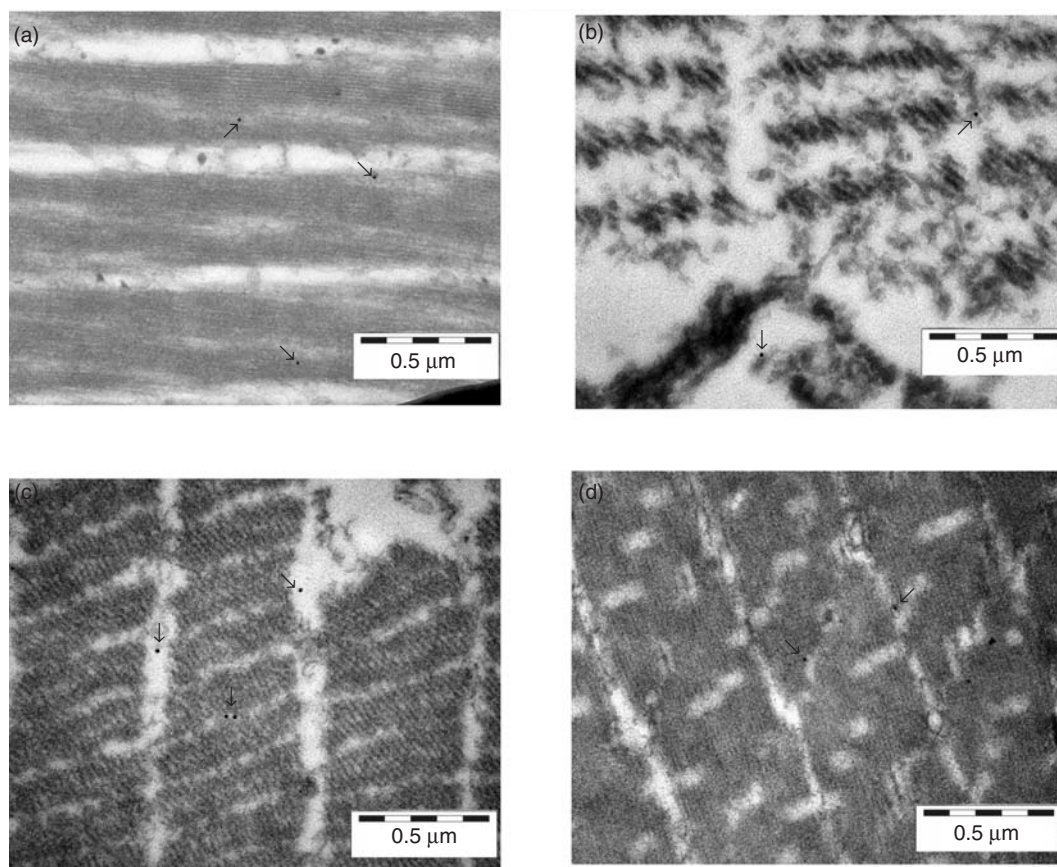


Figure 7. Immunolocalization of *A. simplex* anti crude antigens in sarcomers of anchovy muscle artificially infested with live *A. simplex* L3. Chilled, vinegar solution treated: Sarcomers before (a) and after (b) pepsin treatment. Frozen-thawed, vinegar solution treated: Sarcomers before (c) and after (d) pepsin treatment. Micrographs obtained from samples after 8 days of storage at $5 \pm 1^\circ\text{C}$. The 15 nm gold particles conjugated with the secondary antibody appear in the micrograph as dark circles.

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REFERENCES

- Anonymous (1995). Isolation and identification of Anisakid roundworm larvae in fish. Bureau of Microbial Hazards. Food Directorate. Laboratory Procedure ExFLP-1. Health Canada. Health products and food branch, Ottawa. Available from: http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume5/opflp_02-eng.php (accessed February 25, 2009).
- AOAC (1995). *Official Methods of Analysis*, 16th edn, Arlington, Va: Association of Official Analytical Chemists.
- Caballero M.L. and Moneo I. (2004). Several allergens from *Anisakis simplex* are highly resistant to heat and pepsin treatments. *Parasitology Research* **93**: 248–251.
- Dzik J.M. (2006). Molecules released by helminth parasites involved in host colonization. *Acta Biochimica Polonica* **53**(1): 33–64.
- European Union (1991). Council Directive 91/493/EEC laying down the health conditions for the production and the placing on the market of fishery products. OJ L 268 24.9.1991.
- European Union (2004). Corrigendum to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L 139 Bull 30.4.2004.
- FDA (2001). Processing parameters needed to control pathogens in cold smoked fish. In: *Potential Hazards in Cold-smoked Fish: Parasites*, Rockville, MD: US Food and Drug Administration, Center for Food Safety and Applied Nutrition.
- García M., Moneo I., Audicana M.T., del Pozo M.D., Muñoz D., Fernández E., Díez J., Etxenagusia M.A. and Ansotegui I.J. (1997). The use of IgE immunoblotting as a diagnostic tool in *Anisakis simplex* allergy. *Journal of Allergy and Clinical Immunology* **99**(4): 497–501.
- Hitchcock D.J. (1950). Parasitological study on the Eskimos in the Bethel area of Alaska. *Journal of Parasitology* **36**: 232–234.
- Karl H., Roepstorff A., Huss H.H. and Bloemsma B. (1995). Survival of *Anisakis* larvae in marinated herring fillets. *International Journal of Food Science and Technology* **29**: 661–670.
- Kasuya S. and Koga K. (1992). Significance of detection of specific IgE in *Anisakis*-related diseases. *Alerugi* **41**: 106–110.
- Kirstein F., Nieuwenhuizen N.E., Worsnell G., Brombacher F., Mattiucci S. and Lopata A.L. (2007). Different routes of sensitization to *Anisakis pegreffii* in a mouse model of allergic asthma. *Parassitologia* **49**(2): 222.
- Knox D.P. (2007). Proteinase inhibitors and helminth parasite infection. *Parasite Immunology* **29**: 57–71.
- Knudsen L., Reimers K., Berner L. and Jensen N.C. (1985). A modification of Bligh and Dyer's oil extraction method reducing

- chloroform vapour outlet. In: *15th Western European Fish Technologists' Association (WEFTA) Meeting*, Hamburg, Germany.
- Marrone R., Damiano S., Panzardi M., Palma G., Mattiucci S. and Anastasio A. (2007). Occurrence of *Anisakis pegreffii* larvae in anchovies (*Engraulis encrasicolus*) caught off Naples Gulf (Italy). *Parassitologia* **49**(2): 225.
- Moneo I., Caballero M.L. and Jimenez S. (2000). Inmunodetección de IgE específica (immunoblotting) en el estudio de prevalencia de sensibilización a *Anisakis simplex* en España. *Alergología e Inmunología Clínica* **15**: 255–261.
- Moneo I. and Caballero M.L. (2002). *Anisakis simplex* larvae release allergens during a short incubation in diluted acid which can be used for clinical diagnosis. *Alergología e Inmunología Clínica* **17**: 201–207.
- Morris S.R. and Sakanari J.A. (1994). Characterization of the serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis simplex*. *Journal of Biological Chemistry* **269**: 27650–27656.
- Ng K.S., Petersen J.F.W., Cherney M.M., Garen C., James M.N.G., Zalatoris J.L., Rao-Naik C., Dunn B.M., Martzen M.R. and Peanasky R.J. (1999). Structure determination of *Ascaris* pepsin inhibitor-3 by MAD phasing. Available from: <http://www.aps.anl.gov/News/Reports/1999/ngk1.pdf> (accessed August 10, 2007).
- Pichler W.J. (1999). *Anisakiasis*: immunity, allergy or both? Lessons on the natural role of immunoglobulin E from a nematode infestation. *Clinical and Experimental Allergy* **29**: 1161–1163.
- Rodriguez-Mahillo A.I., Gonzalez-Muñoz M., Gomez-Aguado F., Rodriguez-Perez R., Corcuera M.T., Caballero M.L. and Moneo I. (2007). Cloning and characterisation of the *Anisakis simplex* allergen Ani s 4 as a cysteine-protease inhibitor. *International Journal for Parasitology* **37**(8–9): 907–917.
- Sánchez-Monsalvez I., De Armas-Serra C., Martínez J., Dorado M., Sánchez A. and Rodríguez-Caabeiro F. (2005). A new procedure for marinating fresh anchovies and ensuring the rapid destruction of *Anisakis* Larvae. *Journal of Food Protection* **68**(5): 1066–1072.
- Solas M.T., García M.L., Rodriguez-Mahillo A.I., González-Munoz A.I., De las Heras C. and Tejada M. (2008). *Anisakis* antigens detected in fish muscle infested with *Anisakis simplex* L3. *Journal of Food Protection* **71**(6): 1273–1276.
- Tejada M., Careche M., Torrejon P., Del Mazo M.L., Solas M.T., García M.L. and Barba C. (1996). Protein extracts and aggregates forming in minced cod (*Gadus morhua*) during frozen storage. *Journal of Agricultural and Food Chemistry* **44**: 3308–3314.
- Tejada M., Solas M.T., Navas A. and Mendizábal A. (2006a). Effect of freezing and different heat treatments on *Anisakis* larvae: preliminary study, In: Luten J.B., Jacobsen C., Bekaert K., Sæbø A. and Oehlenschläger J. (eds), *Seafood Research from Fish to Dish, Quality, Safety and Processing of Wild and Farmed Fish*, Wageningen: Academic Publishers, pp. 309–316.
- Tejada M., Solas M.T., Navas A. and Mendizábal A. (2006b). Scanning electron microscopy of *Anisakis* larvae following different treatments. *Journal of Food Protection* **69**(6): 1379–1387.