Research Article

Received: 20 February 2009 Revised: 29 May 2009 Accepted: 12 June 2009 Published online in Wiley Interscience: 21 August 2009

(www.interscience.wiley.com) DOI 10.1002/jsfa.3712

Effect of high hydrostatic pressure on mortality and allergenicity of *Anisakis simplex* L3 and on muscle properties of infested hake

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Abstract

BACKGROUND: High pressure (HP) ranging from 100 to 350 MPa (1–15 min) was applied to *Anisakis simplex* larvae and parasitised hake (*Merluccius merluccius*) muscle. The aim of the study was to kill the larvae to prevent human anisakidosis, to evaluate the effect on *A. simplex* allergens and to minimally alter fish muscle quality.

RESULTS: The larvae were killed at pressures ≥200 MPa and times ≥1 min, producing alterations in the larva body and ruptures in the cuticle when observed by scanning electron microscopy. Nevertheless, Ani s 4 and *A. simplex* crude antigens were recognised by immunoblotting and immunohistochemistry at all HPs assayed. Small changes in colour and texture were observed in fish muscle under all pressure/time conditions. Major changes were observed visually at 300 MPa, where the muscle appeared as slightly cooked. Apparent viscosity of muscle homogenates decreased significantly at longer times or higher applied pressure. No changes were detected at 200 MPa in the electrophoretic pattern of proteins treated with or without β-mercaptoethanol, suggesting that disulfide bonds were not formed.

CONCLUSION: Application of HP at 200 MPa for up to 5 min would kill *A. simplex* larvae, avoiding infestation of the consumer and causing small changes in the hake muscle perceived sensorially. However, HP-treated *A. simplex*-parasitised fish would still be a potential hazard for consumers allergic to the larvae.

Keywords: *Anisakis simplex*; allergen; fish muscle; high pressure; hake; SEM; IHC

INTRODUCTION

Infestation of marine fish and cephalopods with *Anisakis* sp. has increased worldwide in the last few decades, affecting most commercially valuable species and many fishing grounds and causing consumer health problems related to infestation with live larvae (anisakidosis) when fish are consumed raw or undercooked. The growing importance of this problem has been highlighted recently.[1–3]

The location of the larvae may be gastric, intestinal or ectopic. When the larvae penetrate the mucosa, they may cause gastrointestinal symptoms of varying intensity, such as acute abdominal pain, bowel obstruction, vomiting and diarrhoea. Owing to the diversity of symptoms, anisakidosis has often been misdiagnosed.[4] The development of allergy to *Anisakis simplex* has also been associated with the ingestion of raw or undercooked fish, with symptoms such as angioedema, urticaria and even anaphylaxis being manifested in the consumer, thus suggesting that active infestation with live L3 larvae is necessary for immunoglobulin E (IgE) synthesis.[3,5] However, there is increasing evidence that patients sensitised to *A. simplex* show allergic symptoms after consuming products that have been properly processed to kill larvae.[6–8] This may be due to the presence of parasite allergens that are stable to treatments able to cause the death of larvae.[8,9] The impact of *A. simplex* on human health is described in a recent publication based on reports published by the European Community's Rapid Alert System for Food and Feed, which states that *A. simplex* is responsible for 33% of specific biological hazard alerts.[10]

Methods to kill the larvae causing anisakidosis, such as freezing, cooking and microwave treatment of infested fish, have been studied intensively over the last few decades.[11–17] However, cooking and microwave treatment of fish muscle completely change the 'raw' appearance of the muscle, preventing utilisation of the flesh in specific cases where a raw appearance is necessary; even freezing may confer to the fish flesh some texture and flavour characteristics detectable by consumers.

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High hydrostatic pressure (HP) has been used for treating food to extend its shelf life and has been reported to kill *A. simplex* larvae, although the main uses of HP in food have been to inhibit endogenous enzymes and inactivate micro-organisms.[18–22] The pressure required to kill *A. simplex* larvae is generally much lower than that used for destroying micro-organisms. Pressures of 200 MPa for 10 min and 207 MPa for 3 min were reported to kill 100% isolated *A. simplex* larvae[19] and larvae in fish muscle[18] respectively. Regardless of its effect on the larvae, HP may cause some sensory and functional changes in the fish muscle, perceived as changes in texture, colour and lipid oxidation, which will differ according to the pressure/time conditions applied.[18,21,22] HP treatment of fish muscle causes denaturation of some proteins, leading to modification of some inter- and intra-protein bonds, which has been used by the fish industry to improve the properties of surimi gels.[23–26] Such changes could also occur in allergenic proteins, thus modifying their allergenic potential. It is reported that some *A. simplex* allergens, Ani s 4 among others, are resistant to heat, acid and pepsin even if excreted or released by the larvae to the surrounding muscle.[27,28] However, no data have been found related to the effect of HP on *A. simplex* allergenic potential.

Therefore the objective of the present work was to study the effect of HP on hake (*Merluccius merluccius*) muscle infested with *A. simplex*, with the aim of killing the larvae while producing minimum changes in fish muscle properties, and evaluate the resistance of *A. simplex* antigens under different pressure/time conditions.

EXPERIMENTAL

Materials

Anisakis simplex larvae

Live *A. simplex* larvae in the third stage (L3) were obtained from heavily parasitised hake (*M. merluccius*) ovaries and viscera at the central fish market (MERCAMADRID) in Madrid, Spain from hake caught in the Northwest Atlantic fishing grounds. For each experiment a new batch of larvae was used. Approximately 100 g of larvae with ovary or viscera tissue attached were sent each time to the laboratories at the Instituto del Frío and immediately stored at $5 \pm 1\ ^{\circ}C$.

Hake

Hake were obtained from MERCAMADRID in November 2007. Wild hake were selected because of their high economic value and high prevalence and intensity of infestation by *Anisakis* sp. L3. The length and weight of the fish were 49.7 ± 5.43 cm and 1.15 ± 0.26 kg respectively. Nine fish were used in the experiment. The fish were beheaded, gutted and cut into steaks (1 cm thick). The temperature during handling of the samples was $\leq 5\ ^{\circ}C$.

HP treatment

HP was exerted on the samples by means of an HP prototype (GEC Alsthom ACB, Nantes, France) consisting of an HP chamber (2.35 L capacity) filled with water as pressure-transmitting medium. Thermocouples placed inside Pasteur pipettes filled with water or inserted in the fish muscle were used to fix the working conditions and to measure sample temperatures. An ethanol/water mix in an internal cooling circuit of the HP chamber was used as thermoregulating fluid. Working pressures (100, 200, 300 and 350 MPa) were reached within 1–2 min and were released within 1–5 s after treatment. Compression was accompanied by an increase in temperature in the range from 2 to 12 °C for the pressures exerted at 100, 200 and 300 MPa and from 14 to 24 °C for the pressure applied at 350 MPa. Expansion during pressure release was accompanied by a temperature decrease symmetrical to the temperature increase during compression.

HP on isolated larvae

HP was applied to isolated *A. simplex* larvae in order to establish the minimum pressure/time conditions required to achieve 100% mortality. For that purpose, groups of five live *A. simplex* larvae stored at $5 \pm 1\ ^{\circ}C$ were placed inside plastic Pasteur pipettes (Deltalab SL, Barcelona, Spain), which were filled with distilled water (~5 mL) and sealed to avoid the formation of air bubbles. For each treatment, three pipettes were placed inside a plastic bag, which was vacuum sealed before HP treatment. Pressures applied were 100, 200, 300 and 350 MPa for 1 min, two cycles of 1 min separated by 5 min (1 + 1), 2 min and two cycles of 2 min separated by 5 min (2 + 2). Pressure at 200 MPa for 5 and 15 min was also applied for specific studies.

HP on muscle infested with *A. simplex* larvae

Studies on parasitised hake muscle were conducted under selected pressure/time conditions in order to determine the influence of HP on mortality and antigen release by the larvae as well as the influence of the pressure treatment on hake flesh. In order to standardise the infestation, sandwiches of hake steaks were parasitised by placing 12 live *A. simplex* larvae on the surface of one hake steak (1 cm thick, 8 cm long, 6 cm wide), which was then covered with another steak. All sandwiches were vacuum sealed in heat-resistant bags (WIPAK/GRYSPEERT Model PAE 110 K FP, oxygen permeability 30 mL m^{-2} day^{-1} measured at 23 °C and 75% relative humidity; ILPRA Systems España, SL, Mataró, Spain) and stored at $5 \pm 1\ ^{\circ}C$ for 24 h to allow migration of the larvae inside the muscle.[28,29] HP was applied to the samples at 200 MPa for 1, 2, 2 + 2 and 5 min and at 300 MPa for 1 min. Untreated sandwiches were used as controls. Parallel studies were performed on isolated larvae in order to check possible differences from batch to batch.

Methods

Larvae

Spontaneous and stimulated movements of the larvae, either isolated or transferred from the fish sandwiches to distilled water, were observed visually after treatments and recorded by video camera (Handycam DCR-Sr70, Sony, Osaka, Japan) at intervals over a period of 2 h. When no movement of a larva was recorded, it was considered dead. Emission of a bluish fluorescence of the larvae before and after treatment was examined in a dark chamber by exposure to a UV light source (366 nm) positioned 15 cm above the parasites. Emission of fluorescence was observed immediately after treatment and following storage for 24 h at 5 °C. The intensity of the fluorescent emission was rated in arbitrary units as maximum, medium, slight or no fluorescence.

Muscle

All analyses were performed after removing the larvae from the fish muscle following HP treatments.

Proximate analyses

Moisture, ash, crude protein (LECO FP-2000 nitrogen/protein determinator (LECO Corp., St Joseph, MI, USA), using a nitrogen-to-protein conversion factor of 6.25) and crude fat contents of

hake muscle samples were measured and expressed in g kg^{-1} muscle.[30,31]

Colour

The colour of HP-treated and untreated raw samples was measured in a HunterLab model D25-9 colorimeter (D45/21) (Hunter Associates Laboratory Inc., Reston, VA, USA) using a CIELab scale (L^* (lightness: 100, white; 0, black), a^* ($-a^*$, greenness; $+a^*$, redness) and b^* ($-b^*$, blueness; $+b^*$, yellowness).[32]

Shear resistance

Control and HP-treated muscle samples (23 ± 0.5 g) were packed into cylindrical stainless steel containers (30 mm height, 30 mm diameter), which were hermetically sealed with screw-fitting tops and bottoms and kept at 5 ± 1 °C for 18 h. The samples were then cut into two slices (1.5 mm height). Each determination was performed on two slices in a TA-XT2 texture analyser (Stable Micro Systems Ltd, Godalming, UK) fitted with a Kramer shear cell at a maximum force of 25 kN and a speed of 2 mm s^{-1}. The maximum load was measured and expressed in N g^{-1}.

Apparent viscosity of muscle homogenates

Apparent viscosity was determined in 1 : 4 (w/v) homogenates of hake muscle in 50 g L^{-1} NaCl (pH 7) using a Brookfield Model LVTD rotary viscometer (Brookfield Engineering Labs, Stoughton, MA, USA).[33] Results were expressed in cP. The amount of protein in muscle homogenates was measured in a LECO FP-2000 nitrogen/protein determinator (LECO Corp.) to check if differences in apparent viscosity were due to the amount of protein in the homogenates.

Polyacrylamide gel electrophoresis

Protein fractions of muscle homogenates were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Phastsystem horizontal apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) using 12.5% polyacrylamide gels. Samples treated with SDS (25 g L^{-1} SDS and 0.02 g L^{-1} bromophenol blue) or SDS + β-mercaptoethanol (β-ME, 50 mL L^{-1}) were heated for 5 min in a boiling water bath. Electrophoresis conditions were 4 mA per gel, 250 V and 3 W. Protein bands were stained with Coomassie brilliant blue (Pharmacia LKB Biotechnology). The molecular masses of the main component proteins in the samples were estimated by comparing their mobility with that of a standard (High-Range Rainbow Molecular Weight Markers, Amersham Pharmacia Biotech UK Limited, Amersham Place, UK) containing myosin (220 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa).

Electron microscopy

For scanning electron microscopy (SEM) observation, larvae and small blocks of parasitised tissue (1.5 cm-sided cubes) were cut from all lots, immediately fixed with a 1 : 1 (v/v) mixture of paraformaldehyde (40 g L^{-1}) and glutaraldehyde (2 mL L^{-1}) in 0.1 mol L^{-1} phosphate buffer (pH 7.2) and stored at 5 ± 1 °C for at least 4 h. The fixed samples were post-fixed with OsO_4, washed, dehydrated in increasing concentrations of acetone, critical point dried, sputter coated with gold/palladium in a metalliser (SCD 040, OC Oerlikon Balzers Ltd., Principality Liechtenstein) and scanned by SEM (Jeol JSM 6400, Akishima, Tokyo, Japan) at 20 kV. A large number of micrographs were taken of larvae subjected to all HP treatments in order to select the most representative ones.

For immunohistochemistry (IHC) observation a rabbit anti-Ani s 4 antiserum was used to evaluate the presence of Ani s 4 in parasitised fish muscle. Anti-*A. simplex* crude extract and anti-Ani s 4 antiserum used for immunohistochemical and immunoblotting assays were generated by immunisation of rabbits with 1 mg of parasite crude extract and 0.7 µg of recombinant Ani s 4[27] respectively (Sigma-Aldrich, Haverhill, Suffolk, UK). Small pieces of control and selected samples of parasitised HP-treated muscle tissue were treated as described previously.[28] Controls were performed excluding the primary antibody. Simultaneously, samples processed with pre-immune rabbit serum instead of primary anti-Ani s 4 were considered as controls.

Immunoblotting

The antigenic activities of the *A. simplex* extracts were studied by immunoblotting using rabbit anti-*A. simplex* crude extract and anti-Ani s 4 antiserum. HP-treated (200 MPa for 5 and 15 min) *A. simplex* larvae before and after pepsin treatment (0.3 mol L^{-1} HCl, 10 mg mL^{-1} pepsin (proteolytic activity 1 : 10 000 U.S. National Formulary (NF) (2000 International Pharmaceutical Federation (FIP)- U g^{-1}); Panreac, Castellar del Vallés, Spain), larvae/solution ratio 1 : 2 (w/v), incubated with continuous shaking at 37 ± 0.5 °C for 4 h) were homogenised with 2 mL of phosphate-buffered saline (PBS), ground with a potter, sonicated (30 s, 11 W output power) and centrifuged (16 000 × *g*, 10 min, 20 °C) to remove insoluble debris. Samples were subjected to electrophoresis and transferred to nitrocellulose membranes. The membranes were then incubated with rabbit antiserum and subsequently with alkaline phosphatase-labelled goat anti-rabbit IgG (Biosource International, Camarillo, CA, USA). Finally, immunoblots were revealed with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) as substrate.

Sensory evaluation

In order to assess the action of HP on fish muscle, sensory evaluation was carried out by a panel of nine trained assessors on non-infested hake samples treated at 200 MPa for times proved to be efficient to kill the isolated larvae without producing visual changes in appearance of the raw fish. The fish were filleted and skinned by hand, checked to be free of *Anisakis* and then packed in heat-resistant bags (see details above). The samples were high pressured at 200 MPa for 1, 2, 2 + 2 and 5 min and then cooked for 10 min at 91 °C in a saturated steam oven (Rational Combi-Master CM6, Grossküchentechnik GmbH, Landsberg Lech, Germany). HP-untreated samples were used as controls. Panel members were asked to evaluate visual aspect, succulence, firmness, elasticity, juiciness, flavour, odour and colour using structured scales.

Statistical analysis

Results for colour, shear resistance, apparent viscosity and protein content of the muscle homogenates were subjected to one-way analysis of variance (ANOVA) and Tukey's honestly significant difference test using the SPSS Version 9.0 program (SPSS, Chicago, IL, USA). The level of significance was set at $P < 0.05$.

RESULTS

Isolated larvae

Spontaneous movements were evident in the larvae after HP treatment at 100 MPa under all time conditions applied; therefore

this pressure was discarded for further studies on infested fish muscle. At higher pressure, no movements of the larvae were observed under any pressure/time conditions assayed (Table 1). No additional effect on mortality was observed when the pressure was applied in two cycles.

Emission of fluorescence by the larvae was clearly visible at all pressures applied, except at 200 MPa for 1 + 1 min and 350 MPa for 1 min where a small percentage of larvae (7%) did not emit fluorescence (Table 1).

Muscle parasitised with *A. simplex* larvae

The pressures applied to the sandwiches were 200 MPa for 1, 2, 2 + 2 and 5 min and 300 MPa for 1 min, since these conditions proved to be efficient to kill the isolated larvae.

Larvae

As stated, the larvae were transferred after treatment from the fish sandwiches to distilled water to observe movement and emission of fluorescence. No movements of the larvae were detected under any of the conditions employed. Fluorescent emission of varying intensity was observed under all pressure conditions assayed (Table 1).

Hake muscle

The proximate composition of the hake muscle was as follows: moisture, 799.4 ± 1.5 g kg^{-1}; crude protein, 195.5 ± 0.9 g kg^{-1}; crude fat, 10.8 ± 3.7 g kg^{-1}; ash, 12.9 ± 0.4 g kg^{-1}.

Table 1. Movement and fluorescent emission of isolated *Anisakis simplex* larvae and larvae in hake muscle after HP treatment

Larvae	Pressure (MPa)	Time (min)	Movement (%)	Fluorescence (%)[a]			
				–	*	**	***
Isolated	100	1	100	0	53	47	0
		1 + 1	100	0	33	53	13
		2	100	0	50	50	0
		2 + 2	100	0	20	53	27
	200	1	0	0	0	100	0
		1 + 1	0	7	0	13	80
		2	0	0	0	73	27
		2 + 2	0	0	0	40	60
	300	1	0	0	33	53	13
		1 + 1	0	0	50	50	0
		2	0	0	20	53	27
		2 + 2	0	0	0	100	0
	350	1	0	7	0	13	80
		1 + 1	0	0	0	73	27
		2	0	0	0	40	60
		2 + 2	0	0	33	53	13
In muscle	200	1	0	0	25	50	25
		2	0	0	47	35	18
		2 + 2	0	0	44	39	17
		5	0	0	67	17	17
	300	1	0	0	43	50	7

[a] Asterisks indicate intensity of fluorescent emission in arbitrary units: *** maximum intensity; ** medium intensity; * slight intensity; – , no fluorescent emission.

Colour

The colour of the fish muscle after treatment is shown in Table 2. Significantly higher values of L^* were observed at 200 MPa applied in two cycles of 2 min (2 + 2 min) and at 300 MPa applied for 1 min, with the highest L^* being found under the latter conditions. At 200 MPa, no significant changes in a^* were found, whereas a decrease in b^* was detected for all times studied, but there were no significant differences among lots due to the time of application of HP. However, in lots treated at 300 MPa, a significant increase in a^* was measured but no significant change in b^*.

The visual appearance of the muscle treated at 300 MPa for 1 min was partly white and opaque, resembling a slightly cooked fish muscle, which was not observed when 200 MPa was applied even for the longest time.

Shear resistance

The shear resistance of the fish muscle increased significantly in all HP-treated lots, but there were no significant differences among lots due to the pressure/time conditions (Table 3).

Apparent viscosity

The apparent viscosity of the muscle homogenates decreased significantly in all HP-treated samples. Lower viscosity values were achieved at higher pressure or longer time applied, with the lowest value being reached at 300 MPa for 1 min (Table 3). The protein content of the homogenates did not differ between control samples and samples treated at 200 MPa for all times assayed (Table 3); however, the protein content was significantly lower in samples treated at 300 MPa.

Electrophoretic pattern

The protein bands obtained in the homogenates by SDS-PAGE after pressure treatment of hake muscle did not change with different pressure/time conditions or β-ME treatment of the extracts, presenting in both cases a pattern as shown in Fig. 1.

Electron microscopy

Deep longitudinal invaginations in the larvae were observed by SEM after treatment at 200 MPa for 1 min (Fig. 2(a)). The larva cuticle appeared disrupted after 5 min at 200 MPa (Fig. 2(b)). After 15 min at 200 MPa, large zones of the cuticle showed breaks longitudinal to the body axis resembling a blast of the cuticle (Fig. 2(c)). Fractures in the cuticle were also observed when the larvae were treated for short times at higher pressures (Figs 2(d) and 2(e)).

Table 2. Colour of HP-treated hake muscle

Treatment	L^*	a^*	b^*
Control	51.96 ± 1.61a	0.71 ± 0.79a	−1.75 ± 0.89a
200 MPa, 1 min	52.37 ± 1.32a	0.73 ± 0.51a	−3.79 ± 0.94b
200 MPa, 2 min	53.38 ± 2.32a	−0.26 ± 0.66a	−2.88 ± 1.77ab
200 MPa, 2 + 2 min	57.88 ± 3.38b	−0.18 ± 0.72a	−3.49 ± 1.01b
200 MPa, 5 min	52.82 ± 2.28a	0.06 ± 1.05a	−3.69 ± 0.60b
300 MPa, 1 min	63.75 ± 1.44c	2.36 ± 1.07b	−0.14 ± 1.11a

Different letters within a column indicate significant differences between treatments ($P < 0.05$).

Table 3. Shear resistance, apparent viscosity and protein content of muscle homogenates

Treatment	Shear resistance (N g^{-1})	Apparent viscosity (cP)	Protein content (g kg^{-1})
Control	1.13 ± 0.11a	10 579 ± 146a	39.0 ± 0.5a
200 MPa, 1 min	2.16 ± 0.20 b	8457 ± 2674b	39.2 ± 2.6a
200 MPa, 2 min	1.80 ± 0.25b	4324 ± 710c	38.8 ± 1.1a
200 MPa, 2 + 2 min	2.02 ± 0.22b	1729 ± 134d	40.2 ± 0.7a
200 MPa, 5 min	1.88 ± 0.17b	1253 ± 59d	37.4 ± 0.2a
300 MPa, 1 min	1.67 ± 0.21b	117 ± 16e	28.6 ± 0.7b

Different letters within a column indicate significant differences between treatments ($P < 0.05$).

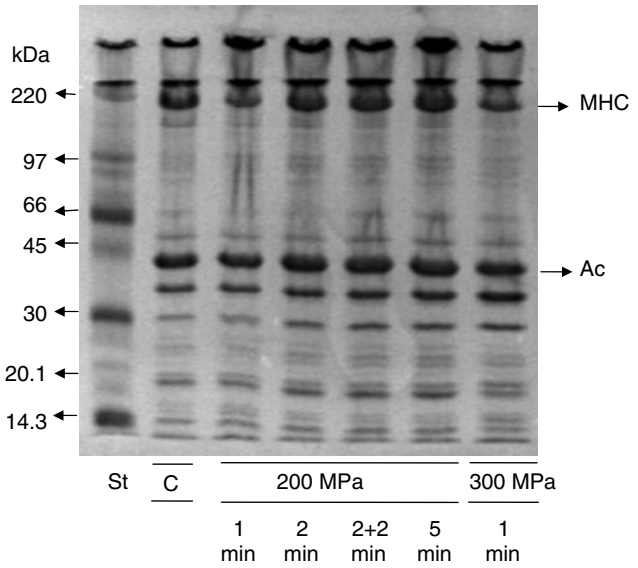

Figure 1. SDS-PAGE (12.5%) of hake muscle homogenates. Samples were treated for electrophoresis without β-ME. Abbreviations: St, standard; C, control (no HP treatment); MHC, myosin heavy chain; Ac, actin.

Immunolocalisation of Ani s 4 was observed by IHC in the hake muscle after HP treatment (Fig. 2(f)).

Immunoblotting

The immunoblotting assay (Fig. 3) showed the presence of antigenic proteins in *A. simplex* after treatment at 200 MPa for 5 and 15 min. Ani s 4 and *A. simplex* crude antigens were detected in the larvae even after pepsin treatment.

Sensory evaluation

Treatment of the hake muscle by HP caused perceptible differences between control and HP-treated samples that were recognised by all assessors. However, no clear differences among HP-treated samples were detected by most panellists. HP-treated samples were mainly described as having poorer odour, succulence and flavour and higher firmness and elasticity. Nevertheless, the changes in texture were not perceived by some assessors as a negative factor, and none of the samples was rejected.

DISCUSSION AND CONCLUSION

This work was designed to produce the death of *A. simplex* infesting fish muscle to avoid anisakidosis in consumers, while causing minor changes in the fish flesh, by applying HP and to evaluate the effect of the selected conditions on *A. simplex* allergens. In order to study HP treatments in a comparative way, the work was performed on isolated larvae and on fish muscle steaks parasitised in the laboratory with the same number of live larvae, storing the muscle for a time to let the larvae migrate inside the fish muscle, following a previous procedure.[28,29] Our results indicated that *A. simplex* larvae, either isolated or in artificially infested muscle, were killed by HP in less time or at lower pressure than reported previously.[18,19] However, treatment of hake muscle by HP caused perceptible sensory differences between control and treated samples.

Emission of fluorescence by the larvae is used to detect *A. simplex* L3 in fish muscle and has been associated with the death of larvae in frozen muscle[34] and of HP-treated larvae.[19] Nevertheless, we have observed previously that fluorescent emission by dead larvae depends on the conditions of storage and processing.[17] In the present study we found that all larvae subjected to a pressure of 100 MPa maintained spontaneous movements (remained live) and emitted fluorescence at higher intensity when the pressure was applied in two cycles. This supports our previous results in which we considered that emission of fluorescence did not discriminate between live and dead larvae and might be related to the stress produced in the larvae by the treatment.[7]

Changes in colour of the fish muscle were detected under all pressure/time conditions. However, visual changes in appearance of the fish muscle were observed only at 300 MPa. Increase in L^* and cooked appearance of the flesh in fish species have been reported by other authors using different pressure/time conditions, generally longer times than those applied in the present study, and have been related to the denaturation of myofibrillar and sarcoplasmic proteins.[22,35–38]

A modification of texture detected as an increase in shear strength and perceived sensorially as higher firmness and elasticity was found in all HP-treated lots. An increase in shear resistance and hardness has been reported previously in HP-treated fish muscle, depending on the species and pressure/time conditions.[18,21,39]

The main changes were measured as a decrease in apparent viscosity of muscle homogenates at longer time or higher pressure applied. The decline in apparent viscosity is considered an early indication of changes in myofibrillar protein and has been related to the decrease in extractability at longer frozen storage times, to a lower myosin heavy chain/actin (MHC/Ac) ratio in salt solutions and to changes in the protein conformation and/or formation of intermolecular bonds with formation of aggregates, which alter the number and size of particles, their axial relationship and their water-binding capacity.[40–42] Likewise, unfolding, aggregation, precipitation and gel formation have been detected in isolated tilapia myosin when subjected to increasing pressure ranging from 50 to 200 MPa.[43] In the present study, no changes were detected in the amount of protein in the homogenates of muscle treated at 200 MPa. This suggests that at this pressure a different conformation of the proteins was induced that lowered their capacity to bind water. However, when 300 MPa was applied, a significantly lower amount of protein in the homogenates was measured, which implies that, at this pressure, protein aggregates of higher molecular weight were formed that were retained by the mesh in the procedure used to measure apparent viscosity in muscle homogenates.[33] This behaviour may be related to the

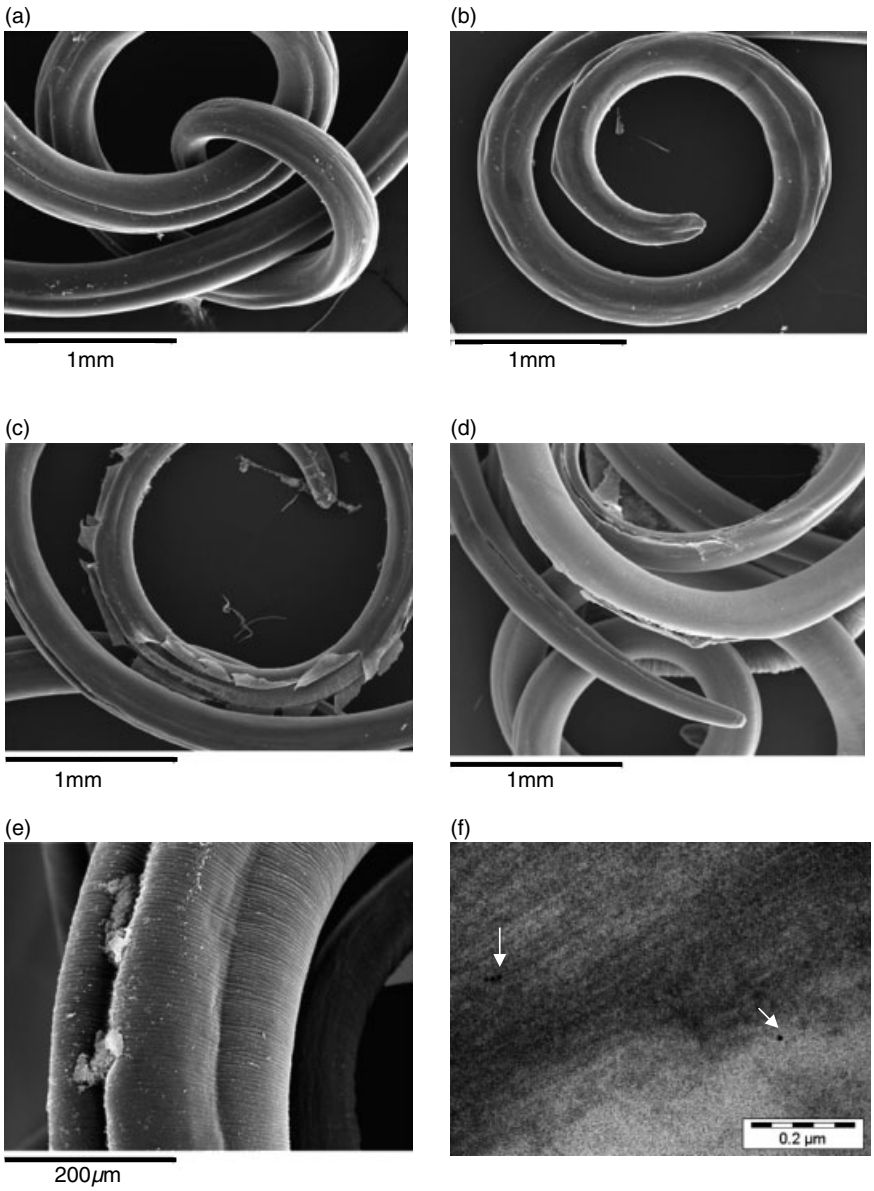

Figure 2. SEM images of HP-treated *Anisakis simplex*: (a) 200 MPa for 1 min; (b) 200 MPa for 5 min; (c) 200 MPa for 15 min; (d) 300 MPa for 2 + 2 min; (e) 350 MPa for 1 min. (f) Immunolocalisation of *A. simplex* allergen Ani s 4 in hake muscle. The 15 nm gold particles conjugated with the secondary antibody appear as dark circles such as those indicated by arrows.

visual changes observed in the muscle treated at this pressure and to the decrease in apparent viscosity observed in this lot. The aggregation of protein in this lot was not due to an increase in temperature as a result of HP treatment, as the fish muscle temperature ranged from 2 to 12 °C during treatment.

The electrophoretic pattern of the proteins in extracts treated with or without β-ME did not differ under any HP condition studied. In the lots treated at 200 MPa, the results suggest that changes in viscosity in hake muscle were mainly due to a different conformation of proteins that lowered their water-binding capacity and therefore made them less viscous and in which disulfide bonds were not involved. In the lot treated at 300 MPa, only the filtered homogenates were studied and they maintained the same pattern as those obtained at 200 MPa. The data obtained at 200 MPa differ from the results obtained in cod muscle, where disulfide bond formation was found at this pressure applied for a longer time (20 min).[21] Differences in the type of bonds involved in protein aggregation in cod and hake muscle during frozen storage have been reported previously.[42]

Nematodes are round in cross-section, with a cuticle described as extremely tough, elastic and flexible.[44] However, HP at $\geq$200 MPa produced changes in the cuticle and larva body, losing the round appearance of the untreated larvae[17] and showing breakages in the cuticle, more evident when applied for longer time or at higher pressure. Nevertheless, Ani s 4 and *A. simplex* crude antigens in the larvae or released to the muscle maintained their allergenic properties, which makes infested fish treated by HP potentially dangerous to consumers previously sensitised to *A. simplex* larvae.

Taking into account that different batches of larvae may present different resistance to HP, as observed in their resistance to heat,[45] for fish intended to be consumed raw or undercooked it would be

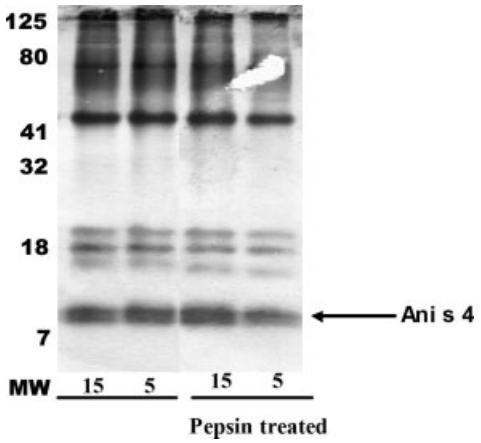

Figure 3. Immunoblotting, revealed with anti-*Anisakis simplex* crude extract and anti-Ani s 4 antiserum, of *A. simplex* larvae treated at 200 MPa for 5 and 15 min and analysed before and after pepsin treatment (0.3 mol L^{-1} HCl, 10 mg mL^{-1} pepsin, larvae/solution ratio 1 : 2 (w/v), incubated at 37 ± 0.5 °C for 4 h). Estimated molecular weights are indicated.

safe to apply 200 MPa for the maximum time that assure the death of larvae without producing visual changes in the fish flesh.

ACKNOWLEDGEMENTS

This work has been financed by the Spanish project Plan Nacional de I+D+i AGL2005-05699-C02-01/02 ALI, and CSIC projects: PIE 2004 7 0E 160 CSIC and PIE 2004 7 0E 340. Sanja Vidacek carried out her work on a grant provided by the Spanish Ministry of Foreign Affairs/Spanish Agency of International Cooperation (MAE-AECI Scholarship). Thanks are due to Mr Angel Mendizábal, Public Health Institute, Madrid City Council, Madrid, Spain for supplying *A. simplex* larvae and to Dr Antonio Molina from the Instituto del Frío (CSIC) for his help in fixing the HP parameters.

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