Food Science and Technology International OnlineFirst, published on September 4, 2009 as doi:10.1177/1082013209342914

Quality of Fish Muscle Infested with *Anisakis simplex*

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Marine fish species infested with Anisakidae larvae are reported in many fishing grounds. Besides the problems related to consumer's health when the live larvae are ingested (anisakidosis) or to allergic problems caused by the larvae, the products excreted/secreted or released by the larvae are recognized as proteases, protease inhibitors or antioxidants, which can cause changes in fish muscle during storage. To study this problem, live larvae or a homogenate of larvae in water were added in controlled conditions to gelatin agar gels and minced fish muscle and stored at 20 or 5 °C for 7 days. A high activity in the gelatin agar gels was observed, whereas the inclusion of larvae or larvae homogenates in the muscle did not produce detrimental changes in texture, viscosity, color, or oxidation of fish muscle. The electrophoretic pattern of the extracted natural actomyosin presented fewer changes during storage in the larvae and larvae homogenate added lots than in the control lots. Nevertheless in naturally infested fish different parasite-host mechanisms might be involved. Parasitized fish has to be processed with treatments which kill the larvae to avoid consumers' anisakidosis. However, as some of the *Anisakis* allergens are very stable to heat, patients allergic to *Anisakis* sp. have to be informed before ingesting parasitized fish or fish products.

Key Words: *Anisakis* sp. larvae, excretion/secretion products, fish quality, food hygiene

INTRODUCTION

Marine fish species infested with L3 larvae of *Anisakis* sp. represent nowadays a problem for the fish industry. *Anisakis* sp. are geographically located in many fishing grounds and reports on infestation of a range of fish species are rising in the last years (Wharton et al., 1999; Mladineo, 2001; Quijada et al., 2005; Valero et al., 2006). According to Abollo et al. (2001) the third stage larvae of *Anisakis simplex* have been recorded world-wide in approximately 200 fish species and in 25 cephalopod species, some of them with a high economical value.

Most of the larvae are reported to be found in the internal viscera, mesentery, and peritoneum of the fish and in a less amount in musculature (Smith, 1984; Wharton et al., 1999; Valero et al., 2006). In the latter case a high percent of the larvae are found in the belly flaps. The prevalence and number of larvae in the musculature appears to be dependent on fish species (Abollo et al., 2001) and have been connected with *post-mortem* migration of the larvae from viscera cavity (Smith, 1984); however other studies did not confirm this relation (Wharton et al., 1999; Mladineo, 2001).

The ingestion of fish parasitized with the live larva is also a potential human health hazard as may cause in the consumer an infestation (anisakidosis) and as a source of food-borne allergens causing allergy when ingested. To date, 9 different *A. simplex* allergen types have been identified (Kobayashi et al., 2007; Rodriguez-Perez et al., 2008). Studies concerning proteins related to *Anisakis* have been mostly addressed to characterization of *Anisakis* allergens (Moneo et al., 2005; Kobayashi et al., 2007; Rodriguez et al., 2008; Rodriguez-Perez et al., 2008), identification of pathogenesis-associated enzymes (Nguyen et al., 1999; Podolska and Napierska, 2006), and, to a lesser extent, identification of metabolism-associated proteins (Dávila et al., 2006). Serine, metalloproteinases, cathepsin D-like aspartic proteinase and cathepsin L-like cysteine proteinase, serine and cysteine proteinase inhibitors, antioxidative enzymes and nucleotide enzymes have been discovered so far, some of them being secreted by the larvae to the environment (Sakanari and McKerrow, 1989; Nguyen et al., 1999; Dzik, 2006; Rodriguez-Mahillo et al., 2007; Yu et al., 2007). The synthesis and release of these enzymes by the larvae may also affect the fish muscle and modify the quality during its storage and commercialization; however this effect has not been studied up to now. Therefore, the aim of this work was to determine if the presence of live or dead *Anisakis* larvae in the fish muscle have influence on the fish quality during storage, due to the possible action of

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Received 13 March 2008; revised 19 June 2008.

Food Sci Tech Int 2009;0(00):0001–0008

Los Angeles, London, New Delhi and Singapore
ISSN: 1082-0132
DOI: 10.1177/1082013209342914

the enzymes synthesized/released by the larvae to the surrounding tissues.

MATERIALS AND METHODS

Material

Anisakis simplex

Live *A. simplex* larvae (L3) were obtained from heavily parasitized hake (*Merluccius merluccius*) ovaries and viscera at the central fish market in Madrid (Mercamadrid, Spain). For every experiment a new batch of larvae was used. The hake samples were caught in the Northwest Atlantic fishing grounds. Approximately 100 g of larvae, with ovary or viscera tissue attached, were sent to the laboratories and immediately stored at 5 ± 1 °C (chilled larvae) for further preparation.

Gelatin Agar

A preliminary work, to determine the proteolytic activity of live or dead (homogenates) *A. simplex* larvae, was performed on an inert medium, gelatin agar (DEV-gelatine agar, *Sigma*) with the following composition: agar (15.0 g/L), gelatin (10.0 g/L), meat extract (10.0 g/L), meat peptone (10.0 g/L), sodium chloride (5.0 g/L); final pH 7.3 ± 0.2) – stored at different temperatures. For the live larvae, 5 *A. simplex* L3 larvae were placed on gelatin agar in Petri dishes and stored at 20 ± 2 °C up to 5 days. The larvae were placed in two locations: on top of the gelatin agar and in double-layered gelatin agar. Homogenates from live (chilled) or frozen (-20 ± 2 °C up to 7 days) – thawed larvae were obtained by homogenizing larvae with cold distilled water for 2 min (Braun MR6550 Hand Blender, position 3) at a concentration 0.5 larvae per milliliter of distilled water. One milliliter of homogenate was homogeneously spread on the upper surface of gelatin agar cylinders (3 cm diameter, 1.5 cm height) and stored in Petri dishes at 5 ± 1 °C up to 8 days. A preliminary study for fixing the larvae concentration was performed. The appearance of the gelatin agar was monitored and recorded every 24 h.

The experiment was performed in triplicate for every condition examined [live *A. simplex* (surface and double layer) and dead (homogenized – chilled and frozen)].

Fish Muscle

Farmed gilthead seabream (*Sparus aurata*) muscle was used to study the effect of L3 *A. simplex* in the quality of fish muscle. In the natural environment the Sparidae family may present infestation, however the muscle of the farmed individuals are nearly free of *A. simplex*. Hake (*M. merluccius* L.) muscle was used for comparative studies in proteins. Prevalence and infestation in hake is high in the natural environment and may present a different response muscle-parasite than that of gilthead seabream.

Gilthead seabreams (10–12 kg in each batch) were purchased from Mercamadrid in May and June 2007. The mean length and weight of the fish were 21.2 cm and 0.42 kg (May batch) and 21.8 cm and 0.44 kg (June batch), respectively. Hake was obtained from Mercamadrid in March 2007. The length and weight of the fish was 56 cm and 1.36 kg, respectively. The hypaxial muscle was naturally infested with *A. simplex* larvae, therefore, only the axial muscle was used for the experiment.

The fish was delivered in refrigerated boxes to the laboratory and immediately headed, gutted, filleted, and chopped. Live larvae and larvae homogenates were added to hake and gilthead seabream muscle, respectively. Larvae homogenates were used to standardize the conditions in order to reduce in each batch differences in activity of individual larvae as reported by Rodriguez Mahillo et al. (2007).

To study the effect of live larvae 15 g of chopped hake muscle were placed into stainless steel containers (30 mm height by 30 mm diameter); five live *A. simplex* larvae were placed on the top and covered with additional 15 g of chopped muscle. Larvae homogenates were obtained by homogenizing live larvae with cold distilled water at a concentration of 5 larvae per milliliter of water as described. One milliliter of homogenate was mixed with 30 g of chopped gilthead seabream muscle and placed in stainless steel containers. The containers were hermetically sealed with screw-fitting tops and stored up to 7 days at 5 ± 1 °C. Lots were named incubated when *A. simplex* larvae (live or homogenates) were added and stored as described.

Control samples were performed mixing 1 mL of distilled water with 30 g of chopped muscle. At least three containers were prepared for every condition and analyses performed.

Methods

Proximate Composition and pH

Moisture, ash (AOAC, 1995), crude protein in LECO FP-2000 nitrogen/protein determinator (LECO Corp., St Joseph, MI, USA; using a nitrogen to protein conversion factor = 6.25) and crude fat (Smedes, 1999) were measured. The results were expressed in gram per kilogram of muscle. The pH was determined at room temperature using 10 g of thawed chopped dorsal muscle in 100 mL distilled water. Measurements were carried out with a glass electrode (pH-electrode blue line Elektrolyt L300; Schott Instruments, Mainz, Germany).

Apparent Viscosity

Apparent viscosity was determined in a homogenate of muscle in 5% NaCl, pH 7 (1 : 4) (w : v) (Borderías et al., 1985) using a Rotary Viscometer Brookfield Mod LVTD (Brookfield Engineering Labs, Stoughton, Mass., USA). Results were expressed in centipoise (cP).

Shear Resistance

The stainless steel containers were heated at 91 °C for 10 min in a saturated steam oven (Rational Combi-Master CM6), cooled for 30 min in ice water then kept refrigerated until measured (≤2 h). Each cylinder was cut into two slices (1.5 mm height) and measured in an Instron Universal Testing Machine, model 4501 (Instron Engineering, Canton, Mass., USA) fitted with a Kramer shear cell (Kramer et al., 1951), at a maximum force of 25 kN and a speed of 2 mm/s. The results were analyzed using the Instron Series IX software (Automated Materials Testing System V.5). The maximum load was measured and expressed as Newton per gram sample (N/g sample).

Extraction of Protein

Extraction of proteins was performed in the incubated (larvae homogenates or live larvae added) and control gilthead seabream and hake muscle.

Supernatants

The fish muscle was homogenized with 5 volumes of phosphate buffer pH 7.5 (3.38 mM potassium dihydrogen phosphate/15.5 mM disodium hydrogen phosphate), centrifuged at 5000 × *g* for 15 min (0–5 °C) (RC 5B refrigerated centrifuge, Sorvall Instruments, DuPont, Wilmington, Del., USA) and the precipitate was washed twice following the same process as before. The supernatants (S) obtained in the three successive washes in phosphate buffer were collected, made up to a fixed volume (80 mL) with phosphate buffer and saved for SDS-electrophoresis. The protein in the supernatants was determined by the LECO FP-2000 nitrogen/protein determinator and expressed as mg S/g of muscle.

Natural Actomyosin Extraction

The resulting precipitate was homogenized in an Omnimixer with 3 volumes of 0.8 M NaCl pH 7.5 (3.38 mM potassium dihydrogen phosphate/15.5 mM disodium hydrogen phosphate) for 3 min at 6000 rpm, in an ice-water bath. The homogenate was transferred to a beaker and the homogenizer vase was rinsed with 2 volumes of the 0.8 M NaCl solution and added to the previous protein extract. After standing for 2 h in an ice-water bath, the protein extract was centrifuged for 20 min at 5000 *g* (0–5 °C). The supernatant was diluted with 10 volumes of cold water (0–2 °C) and left to stand in ice water for ~20 min until the protein precipitated. The top layer was siphoned off and the rest, containing the protein suspended in water, was centrifuged for 15 min at 5000 × *g* (0–5 °C). Then 3 M NaCl (50 mM Tris maleate pH 7.0) was added to the precipitate to bring the salt concentration up to 0.6 M NaCl. The mixture was dialysed against 0.6 M NaCl (50 mM Tris maleate pH 7.0) overnight in a refrigeration chamber. Protein concentration in the supernatants was determined by the LECO FP-2000 nitrogen–protein determinator. Results were expressed as milligram NAM per gram of muscle.

Polyacrylamide Gel Electrophoresis

The extracted fractions (S and NAM) were analyzed by SDS-PAGE in a Phastsystem horizontal apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) using 12.5% polyacrylamide gels. Samples were treated with 2% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue, heated for 5 min in a boiling water bath. Aliquots (1 μL) were applied to the electrophoresis gel. For NAM, the protein concentration was adjusted to 5 mg/mL. Electrophoresis conditions were 4 mA/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 Biosciences): 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 Lysozime 14.3 kDa.

Color

Color measurements in raw samples were performed using a CIELab scale ($L^*a^*b^*$) (Young and Whittle, 1985). Measurements were performed in a HunterLab model D25-9 colorimeter (D45/21; Hunter Associates Laboratory Inc., Reston, VA, USA)

TBARS

The thiobarbituric acid reactive substances, TBARS (Vyncke, 1970) were determined in the June batch after observing visual changes in color in the May lot, to check if these changes were related to lipid oxidation. A standard curve was prepared with 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St Louis, MO, USA). Results are expressed as μmol of malonaldehyde 100/g of muscle (μmol MA 100/g muscle). All determinations were performed at least in triplicate.

The significance of the variables studied was assessed by an *F*-test according to the SPSS 9.0 program (Chicago, Il, USA). The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Gelatin Agar

High activity of the live larvae in gelatin agar gels was observed after 1 day of storage at 20 ± 2 °C for both, the larvae placed on the gel surface and between 2 layers of gelatin agar (Figure 1(a) and (b)). Larvae homogenates also showed a strong activity during storage at 5 ± 1 °C, nevertheless the first visible activity was evident after 6 days of storage (Figure 1(c)). The activity of the homogenates was described as strong (homogenates from

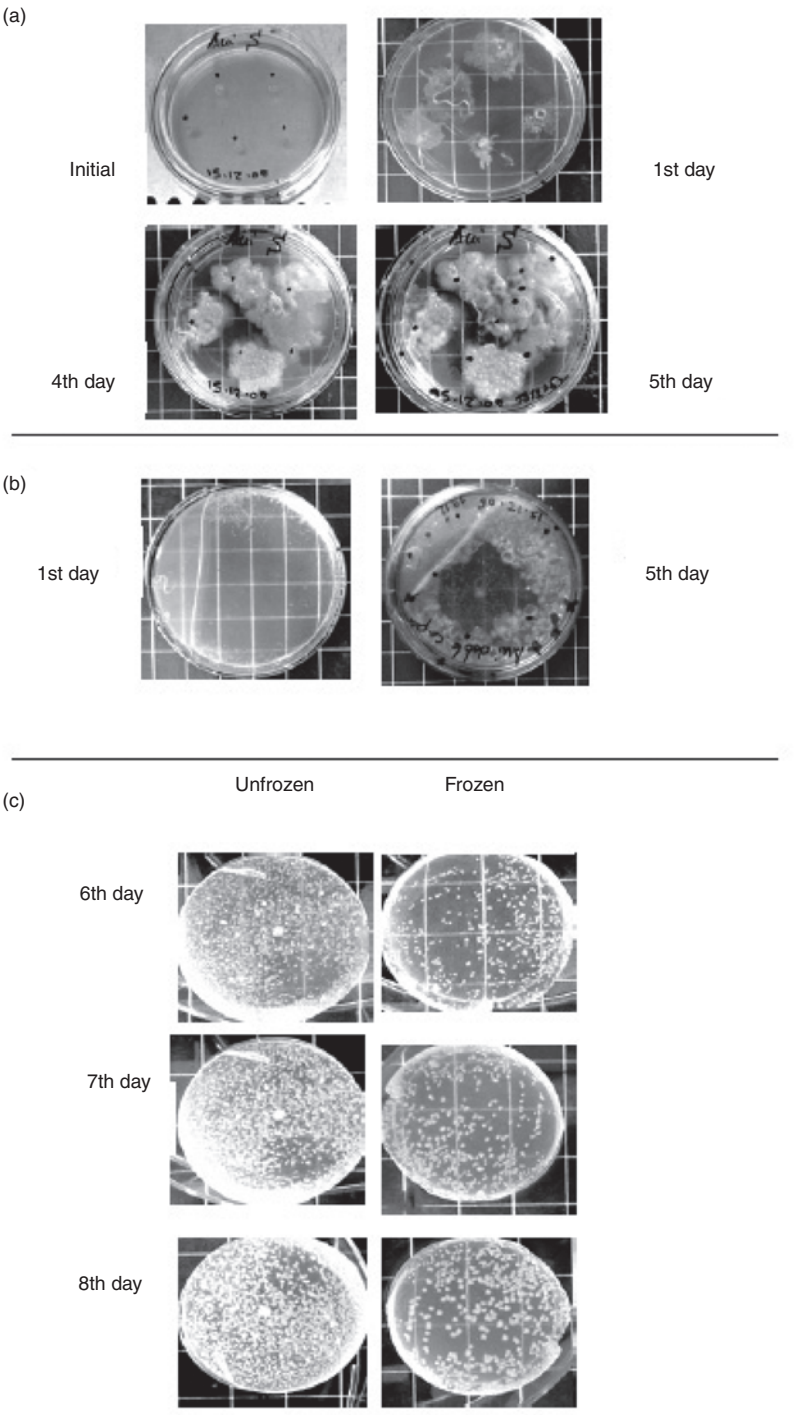

Figure 1. Gelatin agar gels incubated with (a): *A. simplex* L3 larvae placed on top of the gel and stored at 20 ± 2 °C up to 5 days. (b): *A. simplex* L3 larvae placed in double layered gelatin agar gels and stored at 20 ± 2 °C for 5 days. (c): *A. simplex* L3 homogenates (0.5 larvae/mL distilled water per gel) stored at 5 ± 1 °C up to 8 days: UNFROZEN = Live *A. simplex* larvae chill stored; FROZEN = *A. simplex* larvae frozen (-20 ± 2 °C for 7 days) – thawed.

chilled larvae) or moderate (homogenates from frozen larvae). With prolonged storage, the observed activity was increasing for both conditions of larvae (live and homogenates) and temperature (20 ± 2 °C and 5 ± 1 °C). The experiment was stopped when the control gels became visibly contaminated (after 5 days at 20 ± 2 °C and after 8 days at 5 ± 1 °C).

The results evidenced a high proteolytic activity of *A. simplex* on gelatin agar, even when the larvae had been killed previously. However, the relationship between the larvae and fish muscle tissue was expected to be more complex as a consequence of different enzymes present in the muscle tissue and synthesized or released by the larvae.

Fish Muscle

Proximate Composition

The proximate composition of the hake and gilthead seabream muscles is presented in Table 1.

The pH of the control gilthead seabream samples did not changed significantly during the storage period (6.49 ± 0.03 and 6.51 ± 0.07 for initial and at 7 days storage, respectively). This has been previously observed for this species and storage time (Tejada and Huidobro, 2002). After 7 days of storage the pH of the incubated sample was significantly higher than the initial pH (6.68 ± 0.13).

Apparent Viscosity

The apparent viscosity of gilthead seabream in the May batch was significantly lower than in the June batch (Table 2). After 7 days of storage, a decrease was observed in the control lots in both batches, nevertheless this decrease was only significant in the May batch. The viscosity of the incubated lots remained stable during the storage period. A decrease in viscosity has been related to a lower amount of MHC extracted at different storage times, although other parameters that affect the viscous behavior of protein solutions or dispersions, such as changes in the conformation and arrangement of intermolecular bonds which modify the number and size of the particles, their axial relationship, and their water binding capacity are also involved (Del Mazo et al., 1999)

Shear Resistance

The shear resistance of the cooked muscle was significantly lower in the May batch during all the storage period (Table 2). Differences between control and incubated lots were only significant in the May batch after 7 days storage. These results would be in accordance with the lack of activity detected on gelatin agar gels stored at the same temperature up to 6 days.

Extracted Proteins

The amount of protein extracted in the supernatants in hake, decreased in both the control and the incubated lot as iced storage progressed. No significant differences were observed in gilthead seabream (Table 3).

The amount of extracted NAM in the initial controls for gilthead seabream were in the range of the ones

Table 1. Composition of hake and gilthead seabream.

Fish species	Moisture	Crude protein (g/kg muscle)	Crude fat (g/kg muscle)	Ash
Hake	797 ± 1.2	199 ± 3.0	3.0 ± 0.3	10.2 ± 0.2
Gilthead seabream				
May	766 ± 4.6	216 ± 9.6	19.8 ± 2.0	14.3 ± 0.2
June	743 ± 6.6	223 ± 4.0	27.5 ± 7.8	13.8 ± 0.1

Table 2. Apparent viscosity and shear resistance of gilthead seabream.

Batch	Lot	Days	Apparent viscosity (cP)	Shear resistance (N/g)
May	C	1	$5696 \pm 263^{x/a}$	$1.28 \pm 0.40^{x/a}$
		5	$5698 \pm 105^{x/a}$	$1.18 \pm 0.19^{x/a}$
		7	$4936 \pm 113^{x/b}$	$1.17 \pm 0.09^{x/a}$
	I	1	$5326 \pm 179^{x/a}$	$1.19 \pm 0.17^{x/a}$
		5	$5083 \pm 133^{y/a}$	$1.11 \pm 0.13^{x/a}$
		7	$5219 \pm 119^{y/a}$	$1.49 \pm 0.04^{y/b}$
June	C	1	6141 ± 715^{a}	1.56 ± 0.24^{a}
		5	$6277 \pm 397^{x/a}$	$1.72 \pm 0.36^{x/a}$
		7	$5935 \pm 566^{x/a}$	$2.40 \pm 0.28^{x/b}$
	I	5	$6243 \pm 383^{x/a}$	$2.12 \pm 0.37^{x/a}$
		7	$6398 \pm 353^{x/a}$	$2.24 \pm 0.74^{x/a}$

Different letters (x, y) within a column of a batch indicate significant differences between the control (C) and incubated (I) lots after 1, 5, or 7 days of storage ($p < 0.05$). Different letters (a, b) within a column of a batch indicate significant difference between the days of storage of the same lot ($p < 0.05$).

Table 3. Extracted proteins from hake and gilthead seabream.

Fish species	Lot	Days	mg supernant/g muscle	mg NAM/g muscle
Hake	C	1	$75 \pm 1^{x/a}$	$80 \pm 2^{x/a}$
		7	$60 \pm 5^{x/b}$	$74 \pm 6^{x/a}$
	I	1	$82 \pm 3^{x/a}$	$77 \pm 8^{x/a}$
		7	$58 \pm 1^{x/b}$	$75 \pm 1^{x/a}$
Gilthead seabream	C	1	66 ± 2^{a}	71 ± 3^{a}
		7	$71 \pm 2^{x/a}$	$65 \pm 3^{x/a}$
	I	7	69 ± 0^{x}	71 ± 13^{x}

Different letters (x, y) within a column of a fish species indicate significant differences between the control (C) and incubated (I) lots after 1 or 7 days of storage ($p < 0.05$). Different letters (a, b) within a column of a fish species indicate significant difference between the days of storage of the same lot ($p < 0.05$).

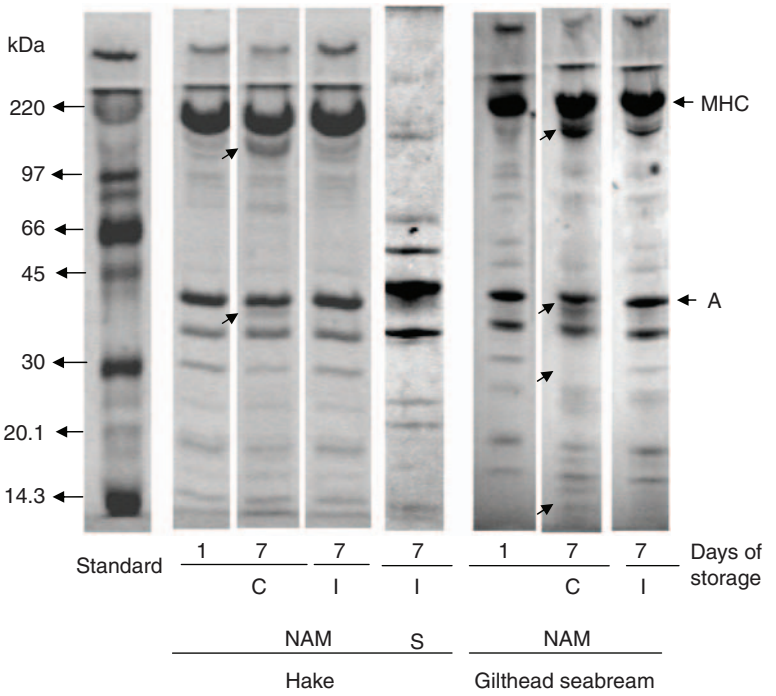

Figure 2. Electrophoretic pattern of natural actomyosin (NAM) and supernatants (S) extracted from hake and gilthead seabream muscle after 1 and 7 days of chilled storage (5 ± 1 °C). MHC = myosin heavy chain; A = actin; C = control; I = incubated. Main changes observed in the bands after 7 days storage are marked with small arrows.

obtained previously for this species; however the values of NAM obtained for hake were lower than the ones previously reported (Del Mazo et al., 1999; Tejada et al., 2003). No significant differences were found in amount of extracted NAM during storage for both lots (control and incubated) and fish species (hake or gilthead seabream).

Polyacrylamide Gel Electrophoresis

The electrophoretic profile of NAM after 7 days storage presented differences between the control and the incubated lots (Figure 2). In hake, clear bands were observed in the control lot at MW between 220 and 97 and at lower MW than actin, whereas the pattern of the incubated lot after 7 days storage did not present differences with the control at 1 day storage. A similar pattern was observed in NAM extracted from gilthead seabream muscle, where changes in the control were also observed at kilodalton <30 after 7 days storage.

This suggests that the proteolytic activity of the larvae had less influence than the action of protease inhibitors from the live larvae or larvae homogenates. The small changes detected in NAM may be in accordance with differences in apparent viscosity observed between incubated and control lots after one week chilled storage, as parameters other than amount of protein are involved in the viscous behavior of protein homogenates.

The number and intensity of bands of the extracted supernatants in hake and gilthead seabream were not modified in the control and the incubated lots after 7 days of storage presenting an electrophoretic profile as shown in Figure 2 (S).

Color

Visual differences in color between the control and incubated lots were observed in the May batch after 7 days of storage where color in the incubated lots was more similar to the initial one. $L^*a^*b^*$ was determined at this point and measured as higher a^* value and lower b^* value of the incubated lot at the end of the storage period (Table 4). In the June batch differences were only found in a^* values between the control and incubated lots at 7 days storage, with higher values also found in the incubated lot where no differences were observed with the initial control. Nevertheless visual differences between lots were not clearly appreciated in this batch.

TBARS

The incubated lot presented significant lower values during storage than the control lot (Table 5), which increased with storage time at a rate found previously for this species (Tejada and Huidobro, 2002).

The lots including larvae homogenates presented less changes in visual color, slower increase of TBARS, and

Table 4. Color (*L* a* b**) values of gilthead seabream.

Batch	Lot	Days	*L**	*a**	*b**
May	C	7	78.0 ± 0.5^{x}	0.1 ± 0.2^{x}	9.5 ± 0.1^{x}
	I	7	77.9 ± 0.2^{x}	1.1 ± 0.2^{y}	8.2 ± 0.1^{y}
June	C	1	82.4 ± 2.0^{a}	-1.2 ± 0.0^{a}	12.1 ± 0.7^{a}
		5	$86.2 \pm 1.9^{x/ab}$	$-1.3 \pm 0.3^{x/a}$	$10.4 \pm 1.4^{x/ab}$
		7	$87.5 \pm 2.5^{x/b}$	$-1.8 \pm 1.1^{x/b}$	$9.7 \pm 1.3^{x/b}$
	I	5	$83.1 \pm 6.0^{x/a}$	$-1.1 \pm 0.2^{x/a}$	$9.8 \pm 0.6^{x/a}$
		7	$87.5 \pm 1.2^{x/a}$	$-1.4 \pm 0.3^{y/a}$	$10.5 \pm 0.1^{x/a}$

Different letters (x, y) within a column of a batch indicate significant differences between the control (C) and incubated (I) lots after 5 or 7 days of storage ($p < 0.05$). Different letters (a, b) within a column of the June batch indicate significant difference between the days of storage of the same lot ($p < 0.05$).

Table 5. TBARS- Gilthead seabream.

Batch	Lot	Days	μmol MA/100 g muscle
June	C	1	0.26 ± 0.04^{a}
		5	$0.46 \pm 0.08^{x/b}$
		7	$0.57 \pm 0.14^{x/b}$
	I	5	$0.34 \pm 0.03^{y/a}$
		7	$0.38 \pm 0.04^{y/a}$

Different letters (x, y) within a column of the June batch indicate significant differences between the control (C) and incubated (I) lots after 5 or 7 days of storage ($p < 0.05$). Different letters (a, b) within a column of a batch indicate significant difference between the days of storage of the same lot ($p < 0.05$).

lesser changes in *a** and *b** during chilled storage, probably due to the presence of antioxidative enzymes existing in the parasite described by Dzik (2006) and Yu et al. (2007).

The results showed that the inclusion of a homogenate of *A. simplex* had no detrimental effect on quality of fish muscle even when a high ratio larvae : muscle was used. Although high activity of the larvae homogenates was observed in gelatin agar gels, the incubated muscle presented fewer changes than the control lots during storage. Differences in the results obtained between batches could be the consequence of batch-to-batch differences in gilthead seabream and in *A. simplex* (Tejada et al., 2006; Rodriguez Mahillo et al., 2007). Nevertheless it is important to point out that most of this work has been conducted in fish that was not naturally infested with the larvae, with the aim of studying the changes in the muscle in controlled conditions due to the difficulty of standardize the naturally infested fish. However, if the fish is heavily infested and/or the larvae are present in the muscle in the live fish, different parasite-host mechanisms might be involved, since the immune system of the fish would be still active and one of the roles of the *A. simplex* enzymes is fight against the host immune system. Therefore, further studies should be conducted in order to determine properties of muscle tissue of fish naturally infested with *A. simplex* larvae.

Thermal or other processing conditions of fish infested with *A. simplex* safe for allergic individuals have not been established so far. Killing the larvae by the methods that prevent anisakiasis (cooking, freezing, etc.) may not prevent allergy in consumers that have already developed allergy to *A. simplex,* as some of the *A. simplex* allergens are reported to be heat stable (Ani s 1, Ani s 4, and Ani s 8; Moneo et al., 2005; Kobayashi et al., 2007) and some allergens including Ani s 4 maintain its allergenic capacity although the fish infested with *A. simplex* had been previously frozen (Tejada et al., 2007). Therefore, patients who have developed allergy to *Anisakis* sp. have to be informed before ingesting parasitized fish or fish products.

ACKNOWLEDGMENTS

This work has been financed by the Spanish projects AGL2005-05699-C02-01 ALI, PIE 2004 7 0E 160, and PIE 2004 7 0E 340 CSIC. Dr 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 Angel Mendizábal, Public Health Institute, Madrid City Council, for supplying *A. simplex* larvae.

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