Journal of Food Protection, Vol. 78, No. 4, 2015, Pages 767–771
doi:10.4315/0362-028X.JFP-14-441

Effect of Allyl Isothiocyanate against *Anisakis* Larvae during the Anchovy Marinating Process

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MS 14-441: Received 11 September 2014/Accepted 7 December 2014

ABSTRACT

Allyl isothiocyanate (AITC), is a natural compound found in plants belonging to the family Cruciferae and has strong antimicrobial activity and a biocidal activity against plants parasites. Anisakidosis is a zoonotic disease caused by the ingestion of larval nematodes in raw, almost raw, and marinated and/or salted seafood dishes. The aim of this work was to evaluate the effect of AITC against *Anisakis* larvae and to study its potential use during the marinating process. The effects of AITC against *Anisakis* larvae were tested in three experiment: in vitro with three liquid media, in semisolid media with a homogenate of anchovy muscle, and in a simulation of two kinds of anchovy fillets marinating processes. For all tests, the concentrations of AITC were 0, 0.01, 0.05, and 0.1%. Significant activity of AITC against *Anisakis* larvae was observed in liquid media, whereas in the semisolid media, AITC was effective only at higher concentrations. In anchovy fillets, prior treatment in phosphate buffer solution (1.5% NaCl, pH 6.8) with 0.1% AITC and then marination under standard conditions resulted in a high level of larval inactivation. AITC is a good candidate for further investigation as a biocidal agent against *Anisakis* larvae during the industrial marinating process.

Human anisakidosis is caused by parasites of the Anisakidae and Raphidascaridae families. *Anisakis, Pseudoterranova, Contracaecum,* and *Hysterothylacium* are all human health threats *(15)*. Anisakidosis is caused by either an infection following ingestion of viable parasites or an allergic (hypersensitivity) reaction to parasite antigens *(4, 11)*. The species most commonly associated with human infection is *Anisakis simplex,* followed by *Pseudoterranova decipiens (11)*. Human anisakidosis is associated with the consumption of raw or almost raw seafood products; several fish and cephalopod species are intermediate hosts in which a very large number of larvae can accumulate *(18)*. Of the approximately 20,000 anisakidosis cases reported up to 2010 by the European Food Safety Authority (EFSA) worldwide, more than 90% have occurred in Japan (where approximately 2,000 cases are diagnosed annually), with most of the rest from Spain, the Netherlands, and Germany *(11)*. For these reasons, European Commission Regulation No 853/2004 *(9)* for "fishery products consumed raw or almost raw" and "marinated and/or salted fishery products, if the processing is insufficient to destroy nematode larvae" established a freezing treatment at −20°C for 24 h to inactivate *Anisakis* larvae. Because freezing can affect the sensorial characteristics of marinated or slightly salted fishery products, several alternative methods have been evaluated to obtain an equivalent effect. Several studies *(1, 3, 13, 29)* have been carried out to assess the effect of salting and/or marinating technologies on the larval inactivation rate and the reduction of pathogenicity, but parasite responses have been highly variable. This variability could be due to the stage of larval development or the encystment of larvae on the serous membranes of fish, which are related to the modality of fish infection, the environmental conditions at the fishing site, the species of fish, the morphological and immune conditions of gastrointestinal tract of the parasitized fish *(26)*, and the redox potential of the fish coelomic cavity *(25)*. Results of a recent investigation *(14)* indicated that the wide variability of *Anisakis* larval responses to marinating and thermal treatments could be related to larval size. In the last decade, some innovative techniques have been introduced for inactivation of anisakid larvae in fish products, although methods such as irradiation *(31)* and high hydrostatic pressure *(8, 22)* can have a negative effect on the sensorial parameters of the product. Sanchez-Monsalvez et al. *(30)* proposed a new marinating technique based on 5 days at 4°C in a marinade containing 12% salt and 6% acetic acid followed by posttreatment washes to reduce the acetic acid concentrations to levels acceptable to consumers. Under these conditions, the death of all parasites present in the anchovy fillets was observed after 13 days at 4°C. Of the various chemical additives, only hydrogen peroxide has been recognized for its effect against anisakid larvae *(2)*, although its use it is not allowed in the European Community. Recently, an interesting debate has arisen about the use of the allyl isothiocyanate (AITC) as a

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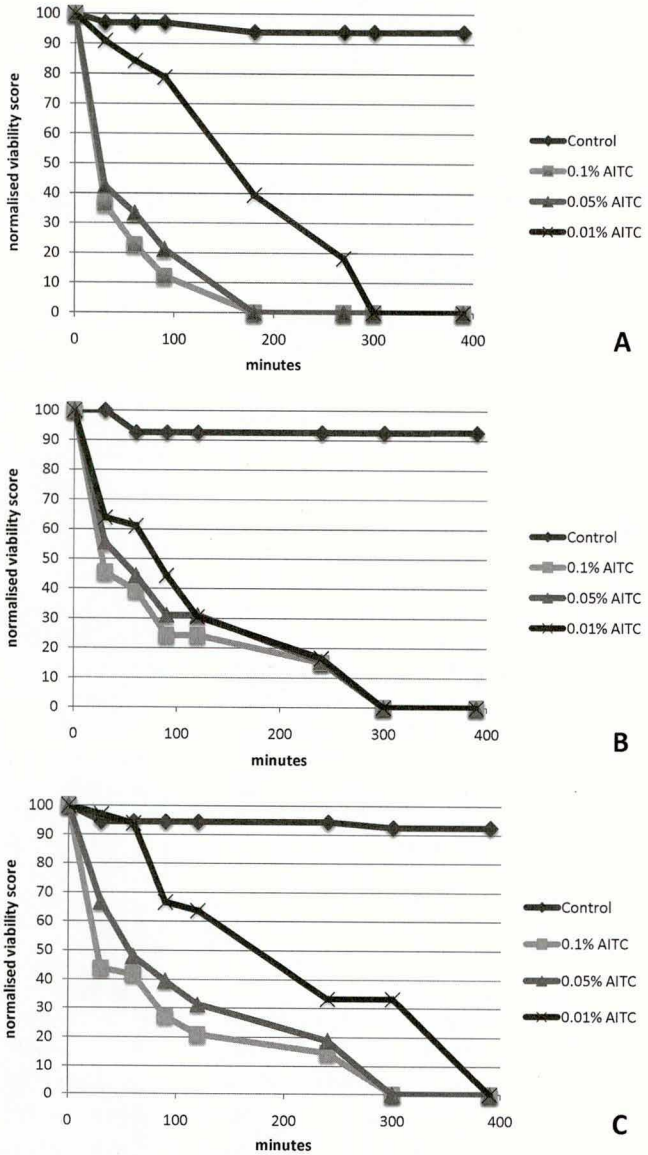

FIGURE 1. *Experiment 1: in vitro test. Inactivation rate (IR) (%) of* Anisakis *larvae in (A) strong marinade, (B) mild marinade, and (C) physiological saline with 0, 0.01, 0.05, and 0.1% AITC at 20°C.*

food preservative. AITC is one of many natural antimicrobials found in the seeds, stems, leaves, and roots of cruciferous plants *(21, 24)*, including horseradish, black and brown mustard, cabbage, Brussels sprouts, broccoli, cauliflower, kohlrabi, kale, turnip, rutabaga, watercress, wasabi, radish, and papaya *(6, 7, 20)*. AITC from natural sources is permitted for use as a food preservative in Japan and as a generally recognized as safe flavoring agent in the United States *(7, 19)*. The essences and extracts of horseradish and mustard are listed as flavoring preparations in Canada. The EFSA *(12)* provided a scientific opinion on the safety of AITC and noted that the mean daily total exposure to AITC from all sources including natural occurrence in food, use as a flavoring, and application as an antispoilage agent results in an intake two- to fourfold greater than the acceptable daily intake in children and up to an eightfold greater intake in adult consumers. Starting in 2012, the use of AITC was authorized in European countries as a flavoring substance by Commission Regulation No 872/2012 *(10)*.

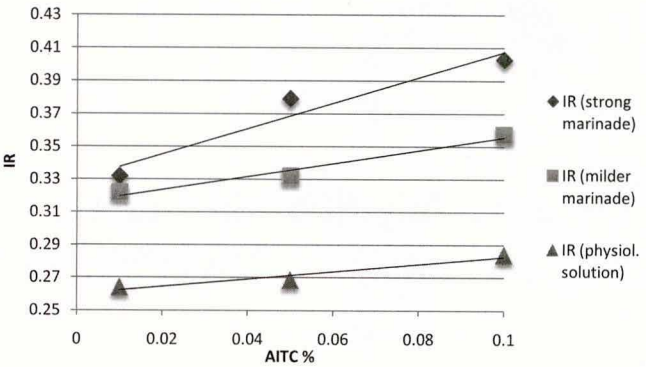

FIGURE 2. *Experiment 1: in vitro test. Relation between IR, AITC concentration, and marinade solution.*

Although the food preservative activity of AITC has been widely studied with regard to the antimicrobial effect on pathogens and spoilage agents *(7, 19–21, 27)*, no studies on the potential effect of AITC against food parasites have been conducted. However, isothiocyanates often have activity against a variety of soil crop pests, including nematodes *(5, 32)*. In light of this information, the aim of this work was to evaluate the effect of AITC on *Anisakis* larvae and to study the use of AITC during the anchovy marinating process.

MATERIALS AND METHODS

Experimental plan. The present study was carried out with three experiments. In the first experiment, in vitro tests were conducted in which *Anisakis* larvae were exposed to three AITC (Sigma Aldrich, Milan, Italy) concentrations in three liquid media to obtain a preliminary evaluation of AITC effectiveness. In the second experiment, semisolid media were created with homogenate of anchovy muscle and three AITC concentrations to evaluate the potential effect of fish muscle proteins on the activity of AITC against *Anisakis* larvae. The third experiment simulated two kinds of anchovy fillet marinating processes with different AITC concentrations. Each experiment were conducted with at least 50 *Anisakis* larvae collected from 12 specimens of *Lepidopus caudatus* within 8 h of harvest. Larvae were evaluated microscopically for viability and to confirm their identification as *Anisakis* genus type I (which includes *A. simplex* sensu stricto, *A. pegreffi, A. simplex* C, *A. ziphidarum, A. typical,* and *A. nascettii) (23)*. At each fixed time interval during the experiments, larval viability was microscopically determined according to the criteria of Hirasa and Takemasa *(17)* based on a scoring scheme of 3 (viable), 2 (reduction of mobility), 1 (mobility only after stimulation), and 0 (death). Larvae were considered dead when no mobility in saline solution (0.9% NaCl) was observed under a stereoscopic microscope. The normalized mean viability score was then used to assess the inactivation rate (IR; percent viability reduction in 1 min under fixed treatment conditions) according to the method of Giarratana and others *(14)*.

Experiment 1: in vitro. In vitro tests were conducted in the following liquid media: (i) a 1:1 (vol/vol) solution of distilled water and vinegar (6% acetic acid), 3% NaCl, and 1% citric acid (strong marinade) to reproduce the marinating solution used by several producers *(16)*; (ii) a 3:1 (vol/vol) solution of distilled water and vinegar (6% acetic acid), 1.5% NaCl, and 0.5% citric acid to reproduce a milder marinating solution (mild marinade); and (iii) a physiological saline solution (0.9% NaCl).

TABLE 1. *Larval inactivation rate (IR) during in vitro test*

AITC (%)	IR (%)[a]		
	Strong marinade	Mild marinade	Physiological saline
0	0.0220	0.0504	0.0239
0.01	0.3317	0.3218	0.2639
0.05	0.3790	0.3318	0.2683
0.1	0.4029	0.3572	0.2840

[a] Percent viability reduction in 1 min under fixed treatment conditions according to the method of Giarratana et al. *(14)*.

Each liquid medium was used to obtain solutions with the three AITC concentrations: 0.1, 0.05, and 0.01%; a fourth solution was prepared without AITC as a control. *Anisakis* larvae were introduced into 20 ml of each solution, maintained at 20°C, and checked for viability at 0, 30, 60, 90, 180, 270, 300, and 390 min.

Experiment 2: semisolid media. For this experiment, 40 g of muscle from 10 specimens of *Engraulis encrasicolus* was homogenized with 100 ml of a 1:1 (vol/vol) solution of distilled water and vinegar (6% acetic acid), 3% NaCl, and 1% citric acid (strong marinade, medium A). Another homogenate was obtained with the same amount of physiological solution (medium B). These homogenates were used to produce four semisolid media for each diluent, with 0.1, 0.05, 0.01, and 0% (as the control) AITC. *Anisakis* larvae were introduced into 20 ml of each solution, maintained at 20°C, and checked for viability at 0, 90, 150, 270, and 420 min.

Experiment 3: experimental marinating processes. To reproduce a marinating process, a 1:1 (vol/vol) solution of distilled water and vinegar (6% acetic acid), 3% NaCl, and 1% citric acid was prepared. The marinade was used for the preparation of three solutions of AITC (0.1, 0.05, and 0.01%), and a fourth aliquot of marinade without AITC was used as a control. Ten fillets of anchovy were marinated in 100 ml of each solution. For each test, the fillets were pressed into 15 blocks (approximately 2 by 1 by 0.4 cm, height by width by depth), and a notch (3 to 4 mm) was incised into each block to contain one larva. Each notch was closed with a commercial solution of cyanoacrylamide (Loctite, Milan, Italy). Fish blocks were marinated for 24 h at 10°C, and larval viability was monitored at 0, 3, 7, 19, and 24 h. This treatment protocol also was applied to 30 whole fillets (10 for each AITC concentration) to assess the sensory characteristics with regard to mustard and sourish odors and flavors. In another treatment, 15 anchovy muscle blocks prepared as described above were immersed in phosphate buffer (pH 6.8) with 1.5% NaCl and 0.1% AITC and in the same solution without AITC as a control. Fish blocks containing *Anisakis* larvae and blocks of 30 whole anchovy fillets (for the sensory analysis) were maintained in these buffers for 30 h at 4°C, rinsed in tap water for 3 h (water flow, 4.8 liters/min), and then marinated for 24 h at 4°C in the strong marinade solution (1:1 distilled water and vinegar, 3% NaCl, and 1% citric acid). Larval viability was monitored at 0, 6, 12, 18, 24, 30, 33, 49, and 57 h, and sensory analysis was conducted after rinsing (at 33 h) and at the end of the marinating treatment (at 57 h).

RESULTS

Experiment 1: in vitro. In vitro tests revealed significant activity of AITC against *Anisakis* larvae; complete inactivation was observed after 180 to 300 min (5 h) in the marinating solutions with all AITC concentrations (Fig. 1A and 1B) and after 300 to 390 min in the physiological solutions (Fig. 1C). Figure 2 and Table 1 show the close relationship between IR and AITC concentration and between IR and the strength of the marinade.

Experiment 2: semisolid media. In both semisolid media with 0.1% AITC, 100% inactivation was achieved after 100 min of treatment. With 0.05% AITC, complete inactivation was observed for the homogenate in only the strong marinating solution (Fig. 3A); complete larval inactivation was not obtained in the semisolid medium with physiological saline (Fig. 3B). With 0.01% AITC, in both kinds of homogenates a small percentage of larvae were not inactivated.

Experiment 3: experimental marinating processes. The use of AITC as part of the industrial marinating process did not result in satisfactory inactivation of *Anisakis* larvae despite the high concentration of AITC (0.1%) (Fig. 4A). This concentration of AITC also conferred a strong mustard flavor and odor to the fillets. However, pretreatment in phosphate buffer (pH 6.8) and 1.5% NaCl, then 0.1% AITC, followed by rinsing in tap water resulted in greater larval inactivation (Fig. 4B) and the absence of the mustard flavor and odor; further marinating treatment resulted in complete larval inactivation. All of the devitalized *Anisakis*

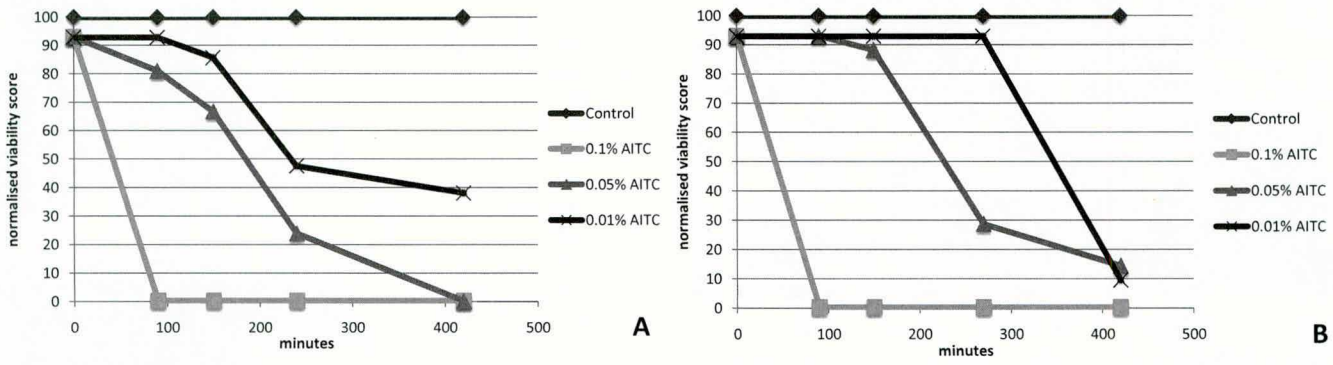

FIGURE 3. *Experiment 2: semisolid media test. Inactivation rate (IR) (%) of* Anisakis *larvae in 40 g of* Engraulis encrasicolus *muscle homogenized with 100 ml of (A) strong marinade or (B) physiological saline with 0, 0.01, 0.05, and 0.1% AITC at 20°C.*

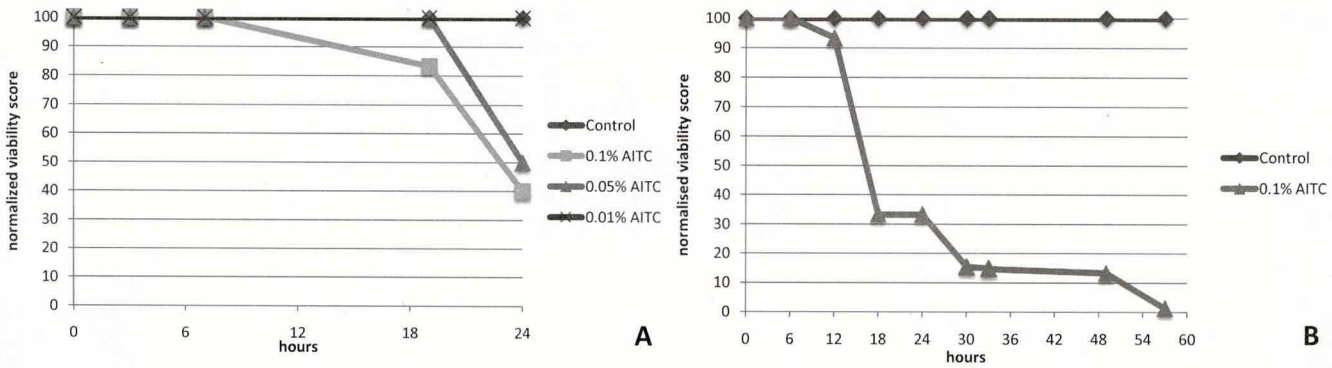

FIGURE 4. *Experiment 3: experimental marinating processes. Inactivation rate (IR) (%) of* Anisakis *larvae in* Engraulis encrasicolus *fillets in (A) strong marinade with 0, 0.01, 0.05, and 0.1% AITC for 24 h at 10°C and (B) phosphate buffer solution (1.5% NaCl, pH 6.8) with 0 and 0.1% AITC for 24 h at 4°C, then rinsed in tap water for 3 h (time reported under solid line), and marinated with a normal strong marinade.*

larvae viewed under the stereomicroscope sustained damage of the digestive tract (Fig. 5).

DISCUSSION

This work is the first structured study concerning the effect of AITC on *Anisakis* larvae. The results revealed larvicidal activity that appears proportional to the AITC concentration. The larvicidal activity probably is related to the damage found in the parasite digestive tract (Fig. 5). However, no specific studies on the mechanisms of action of AITC against parasites have been reported. In bacteria, AITC produces cellular damage and structural changes of the cellular membrane and causes the leakage of ions and other cell contents *(21)*.

The pH of the liquid medium seems to play a key role in structural changes and decomposition of AITC, which influences the effectiveness of AITC against *Anisakis* larvae *(28)*. These results indicated that pH did not affect the activity of AITC against *Anisakis* larvae in vitro or in semisolid media but probably interferes with AITC solubility in fish muscle, as indicated by the marinating experiments. Low pH slows the diffusion of AITC through the fish muscle, but pretreatment in phosphate buffer allowed efficient diffusion of AITC into the fish muscle. The subsequent removal of the AITC by rinsing in tap water was effective for removing the marked mustard flavor associated with AITC.

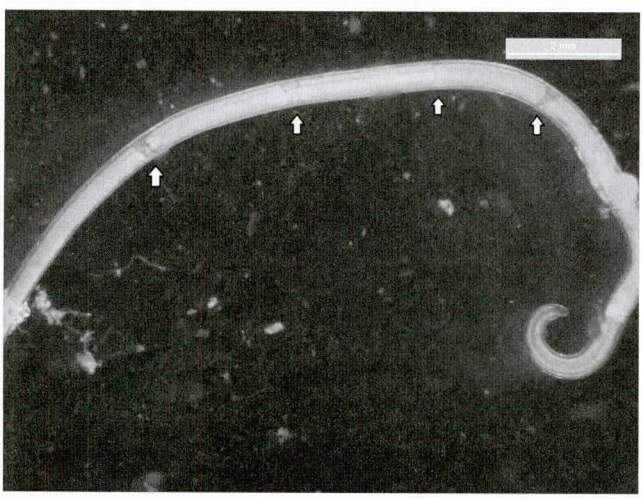

FIGURE 5. *Inactivated (score 0)* Anisakis *larvae under a stereomicroscope. Arrows indicate damage of the larval digestive tract observed after the experimental marinating process (experiment 3B).*

The use of natural compounds as preservative in foods is receiving increased attention because of the relatively safety, wide acceptance by consumers, and potential functional and technological uses of these compounds. Studies have revealed several biochemical and physiological properties of AITC, such as antimicrobial, antiinflammatory, and anticarcinogenic properties *(7, 20, 21)*. The results obtained in the present study support the use of AITC as part of the anchovy marinating process to inactivate *Anisakis* larvae. The effectiveness of AITC against *Anisakis* larvae demonstrated in these experiments justifies further investigations to evaluate the potential use of AITC for treatment of human anisakidosis.

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