

Identification of Autoclave-Resistant *Anisakis simplex* Allergens

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ABSTRACT

Anisakis simplex is a fish parasite able to induce allergic reactions in humans infected when eating raw or undercooked fish parasitized with viable third-stage larvae. Some authors claim that exposure to nonviable *Anisakis* material can result in allergic symptoms in previously sensitized patients, indicating that parasite allergens are resistant to the thermal treatments of usual cooking procedures. Furthermore, some patients report symptoms after eating canned fish. The aim of this work was the analysis of parasite allergen stability in heating to 121°C in an autoclave to simulate the thermal process applied to canned fish. Third-stage larvae were subjected to autoclaving for 20, 40, and 80 min, and parasite crude extracts were analyzed by electrophoresis, immunoblotting, and a flow-cytometric basophil activation test. Allergens resistant to autoclaving were separated by reversed-phase high-performance liquid chromatography and identified by ion trap mass spectrometry. Protein analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that autoclaving considerably reduced the number and intensity of identifiable protein bands in a time-dependent manner. Several allergens were detected by immunoblotting with a pool of *A. simplex* allergic patients' sera after autoclaving. Allergens of 9 and 14 kDa resistant to autoclaving were identified as Ani s 4 and Ani s 1 allergens, respectively. Functional analysis showed that allergens retain their capacity to activate basophils even after autoclaving for 80 min. In conclusion, some relevant *A. simplex* allergens retain their capacity to bind immunoglobulin E and activate basophils after being subjected to autoclaving, which is a method equivalent to that used in industrial canning processes.

Anisakis simplex is a fish parasite able to induce allergic reactions in humans that have been infected when eating raw or undercooked fish parasitized with viable third-stage larvae (L3) of *A. simplex* (4). After infection, humans can become sensitized, and allergic symptoms can appear in subsequent exposures to the parasite. Some authors consider hypersensitivity reactions only occur after sensitized subjects are exposed to viable L3 (5, 20), while others conclude that exposure to parasite allergens can trigger adverse reactions even when L3 are killed by freezing and/or cooking the fish (2, 14, 23). The possibility of inducing allergic reactions by nonviable *Anisakis* material indicates that parasite allergens are resistant to thermal treatments applied for killing larvae. It has been reported that several *A. simplex* antigens and allergens are heat stable, as observed when the parasite is subjected to different time-temperature conditions (7, 25). Heat stability of the parasite proteins when cooking parasitized hake has been also shown (15). Other evidence that epitopes of *A. simplex* allergens are preserved after thermal treatments are provided by specific immune cellular responses. Extracts from cooked parasitized fish and boiled *A. simplex* L3 are able to induce activation of basophils and T cells from allergic patients (8, 15).

Characterization of *A. simplex* proteins has allowed the description of several heat-resistant allergens: the major allergen, Ani s 1 (11, 21); cystatin Ani s 4 (16); and allergens belonging to the SXP/Ral family, Ani s 5 (9), Ani s 8 (10), and Ani s 9 (18). The presence of these thermostable allergens can account for the occurrence of hypersensitivity reactions to nonviable *A. simplex* material contaminating fishery products.

The aim of this work was the analysis of parasite allergen stability in heating to 121°C in an autoclave to simulate the thermal process applied to canned fish (26). Allergen identification was performed by mass spectrometry, and preservation of allergenic properties was studied by immunoblotting and a flow cytometric basophil activation test.

MATERIALS AND METHODS

Material preparation, processing, and preparation of parasite extracts. Live *A. simplex* in L3 were obtained from heavily parasitized hake (*Merluccius merluccius*) ovaries and viscera at the central fish market (Mercamadrid) in Madrid, Spain, from fish caught in the northwest Atlantic fishing grounds. L3 were extracted from fish tissue, washed in phosphate-buffered protein (PBS), and immediately frozen at –20°C until use. L3 were autoclaved in a vertical bench top autoclave (Selecta, model Mediclave, Barcelona, Spain) at 121°C, 105 kPa for 20, 40, and 80 min. The number of L3 for each temperature was 30 in 30 ml of

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PBS. Then, untreated and autoclaved L3 were ground in a Potter-ELV homogenizer (Corning Incorporated Life Sciences, Tewksbury, MA) and sonicated at 18 W for 5 s. Extracts were obtained after centrifugation at $16,000 \times g$ at 4°C for 10 min. Autoclaving and preparation of larvae extracts were carried out in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

Patient samples. Samples were obtained from eight patients with allergic and/or gastrointestinal symptoms within 12 h after ingestion of fish. All of them showed specific immunoglobulin (IgE) to *A. simplex* >0.5 kU_A/liter and undetectable IgE to fish (ImmunoCAP; ThermoScientific, Waltham, MA). Samples from five healthy subjects were used as controls. This study was approved by the Ethics Committee of the Hospital Carlos III (Madrid, Spain), and all subjects included were asked to sign an informed consent form.

Electrophoresis and immunoblotting. Extracts from untreated and heat-treated L3 were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 4 to 20% Tris-glycine gel (Novex, Life Technologies, Grand Island, NY) and stained with Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA). For immunoblotting, proteins were transferred to nitrocellulose membranes by applying a constant current of 1.3 A for 7 min in a Trans-Blot Turbo Instrument (Bio-Rad). Membranes were blocked with PBS, 0.05% Tween 20, and 1% bovine serum albumin for 1 h at room temperature and then incubated with either a rabbit polyclonal anti-*A. simplex* crude extract (1/10,000) or a pool of *A. simplex* allergic patients' sera (1/5). Then, membranes were incubated with the corresponding secondary antisera, according to previously published methods (16).

Basophil activation test. Venous blood samples were drawn from five *A. simplex* allergic patients with positive anti-*A. simplex* IgE values. One hundred microliters of whole blood was incubated for 15 min at 37°C with 20 µl of stimulation buffer containing interleukin 3 (5 ng/ml; Pharmingen/BD Biosciences, Erembodegem, Belgium) and protein extracts of untreated or autoclaved *A. simplex* L3. Ten microliters of undiluted and 1/5 and 1/25 protein extracts were assayed. Negative and positive controls were included by incubating the blood without allergen or 10 µl anti-IgE (10 mg/ml; Pharmingen), respectively. Basophil activation was stopped by adding 10 µl of EDTA (20 mM; Sigma-Aldrich) at room temperature for 5 min. To analyze basophil activation, cells, in the dark and on ice, were stained with 20 µl of CD63–fluorescein isothiocyanate/CD123–phycoerythrin/human leukocyte antigen-DR–Peridinin–chlorophyll protein reagent mixture (BD Biosciences) for 20 min. Then, samples were incubated with 2 ml of FACS Lysing solution (BD Biosciences) for 15 min at room temperature. After centrifugation ($300 \times g$, 5 min) and washing with PBS, cells were resuspended with 300 µl of PBS and analyzed on a FACS sort flow cytometer (BD Biosciences). The percentage of activated basophils was calculated by subtracting spontaneous CD63 expression (negative control) from the maximum percentage value obtained from the concentration curves of protein extracts.

Reversed-phase high-performance liquid chromatography. A 50 to 90% ethanol fraction (0.7 ml) of protein extract from L3 after autoclaving at 121°C for 40 min was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a BioBasic18 column (C18, 25 by 0.46 cm; 5-µm particle size; ThermoScientific). Sample injection and elution was performed with an Agilent 1200 Series liquid chromatograph (Agilent Technologies, Inc., Santa Clara, CA). Elution was carried

out with a linear gradient of acetonitrile (ACN) in 0.1% (vol/vol) trifluoroacetic acid (0 to 5% for 10 min and 5 to 100% for 60 min, at a flow rate of 1 ml/min). Each fraction was analyzed by SDS-PAGE and tested for IgE binding by immunoblot using a pool of allergic patient sera.

Ion trap mass spectrometry. Mass spectrometry was performed at the Proteomics and Genomics Facility, Centro de Investigaciones Biológicas (Madrid, Spain). Protein bands from RP-HPLC fractions, detected by human sera and visualized with Coomassie R-250, were excised from gel and destained in 50 mM ammonium bicarbonate/50% ACN, dehydrated with ACN, and dried. Gel pieces were rehydrated with 12.5 ng/liter trypsin in 50 mM ammonium bicarbonate and incubated overnight at 30°C. Peptides were extracted at 37°C using ACN 100% and then 0.5% trifluoroacetic acid, dried, cleaned using ZipTip (Millipore, Billerica, MA), and reconstituted in 5 µl of 0.1% formic acid–2% ACN. Peptides were analyzed by a nanoflow HPLC (ThermoScientific) coupled on line to a Linear Trap Quadrupole (LTQ) Orbitrap Velos (ThermoScientific) mass spectrometer via a nanoelectrospray ion source (ThermoScientific). Briefly, peptides were loaded onto a C18-A1 ASY-Column 2-cm precolumn (Thermo Scientific) and then eluted onto a Biosphere C18 column (C18, inner diameter, 15 cm by 75 µm, 3-µm particle size; Nano Separations, Nieuwkoop, The Netherlands) and separated with a linear gradient from 2 to 99% ACN in 0.1% formic acid at a flow rate of 250 nl/min. Mass spectra were acquired on the LTQ Orbitrap Velos in the positive ion mode. Full-scan mass spectrometry spectra (*m/z* 300 to 1,700) were acquired in the Orbitrap with a target value of 1,000,000 at a resolution of 60,000, and the 15 most intense ions were selected for collision-induced dissociation fragmentation in the LTQ, with a target value of 10,000 and normalized collision energy of 35%. Mass spectra *.raw files were searched against the UniProtKB database using SEQUEST and MASCOT (version 2.3, Matrix Science, Boston, MA) search engines through Proteome Discoverer (version 1.3.0.339, ThermoScientific). Search parameters included a maximum of two missed cleavages allowed, carbamidomethylation of cysteines as a fixed modification, and oxidation of methionine as variable modifications. Precursor and fragment mass tolerance were set to 10 ppm and 0.8 Da, respectively. Identified peptides were filtered by false discovery rate of 0.01 using a peptide validator.

Statistics. Quantitative variables are described by median and range.

RESULTS

The effect of autoclaving on parasite proteins was analyzed by SDS-PAGE. The main effect observed was that autoclaving considerably reduced the number and intensity of identifiable protein bands. There was no difference in protein profile between autoclaving for 20 and 40 min, while a high reduction in intensity of protein bands was observed after 80 min (Fig. 1). A protein band of 12 kDa was clearly identifiable even at 80 min of autoclaving, and diffuse protein bands at about 7 to 16 kDa were also visible, suggesting that protein degradation occurred during processing. Antigen stability to autoclaving was tested with specific rabbit polyclonal anti-*A. simplex* crude extract antiserum. Most proteins detected in the untreated L3 became undetectable after autoclaving, and the antigen bands were mainly located in the range of 6 to 16 kDa.

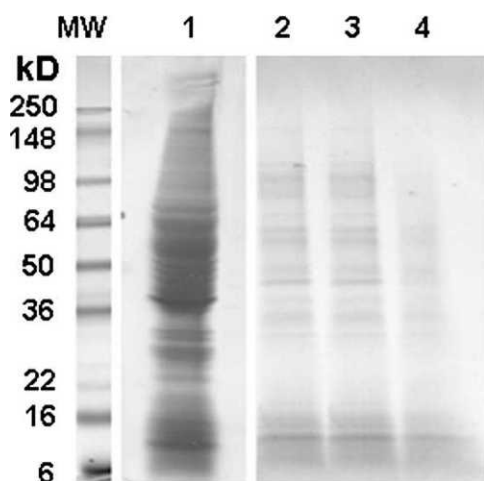


FIGURE 1. Effect of autoclaving on *A. simplex* proteins. Crude extracts of untreated (lane 1) and treated L3 for 20, 40, and 80 min at 121 °C (lanes 2, 3, and 4, respectively) were analyzed by SDS-PAGE. MW, molecular weight standards.

Autoclaving for 80 min had a greater effect on antigen recognition as observed in the SDS-PAGE analysis, and only one protein band at 7 kDa was detected (Fig. 2A).

Regarding allergenic epitopes, autoclaving reduced the allergens detected by a pool of human sera as compared with the allergen profile found in untreated L3 (Fig. 2B). Allergens of 250, 35, and 9 kDa as well as a wide band of 12 to 15 kDa were identified after 40 min of autoclaving. The low-molecular-weight allergens were highly heat resistant because they were still detected after 80 min.

To characterize the heat-resistant low-molecular-weight allergens detected after autoclaving, a 50 to 90% ethanol fraction of the protein extract after 40 min of autoclaving was subjected to RP-HPLC. Several proteins were detected in the eluted fractions by SDS-PAGE (Fig. 3A). In specific IgE-immunoblotting assay with human pooled sera, several

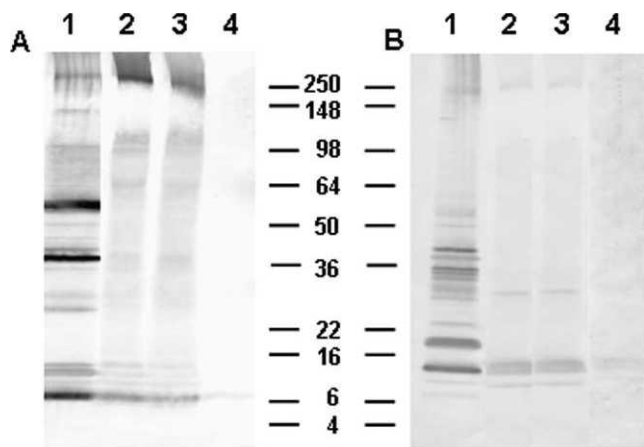


FIGURE 2. Effect of autoclaving on *A. simplex* antigens and allergens. Crude extracts of untreated (lane 1) and treated L3 for 20, 40, and 80 min at 121 °C (lanes 2, 3, and 4, respectively) were analyzed by immunoblotting. Antigen stability was analyzed with an antiparasite crude extract antiserum (A). Allergenicity was determined by IgE-immunoblotting with a pool of *A. simplex* allergic patients' sera (B). MW, molecular weight standards.

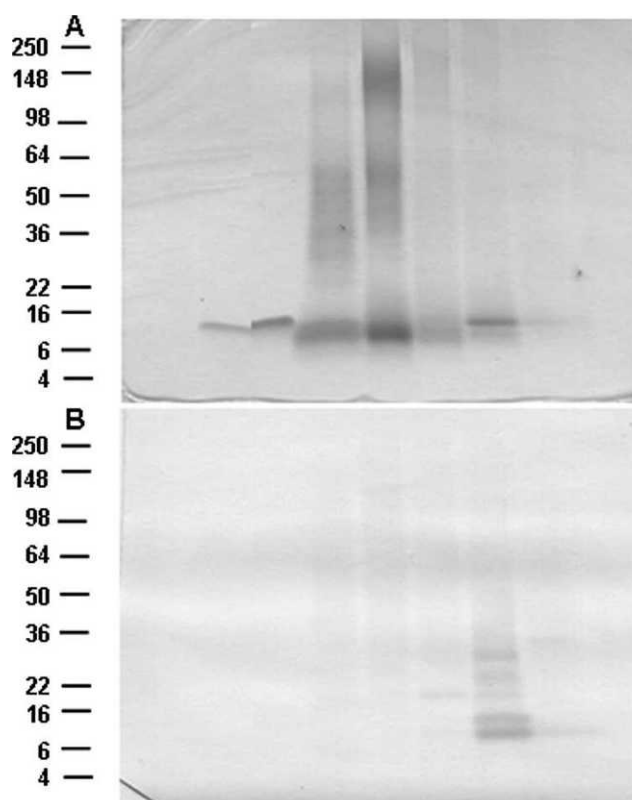


FIGURE 3. RP-HPLC of a 50 to 90% ethanol fraction of the L3 protein extract after 40 min of autoclaving. Consecutive RP-HPLC fractions were resolved by electrophoresis. Eluted proteins (A) and allergens (B) were analyzed by SDS-PAGE and IgE-immunoblotting, respectively. (A) and (B) are aligned with the same RP-HPLC fractions. MW, molecular weight standards.

allergen bands were also identified (Fig. 3B). Among them, only two proteins of 14- and 9-kDa bands were visible in SDS-PAGE and were then identified by mass spectrometry. The 14-kDa protein was identified as Anis 1, a main *A. simplex* allergen, and the 9-kDa protein was Anis 4, a heat- and pepsin-resistant allergen. Figure 4 shows the peptides identified for Anis 1 and Anis 4.

To determine if parasite allergens retained their allergenicity after autoclaving, a basophil activation test was performed with five samples from *A. simplex*-sensitized patients. The median of spontaneous (4%, range 0.6 to 9.4%) and anti-IgE induced CD63 expression (82%, 76 to 98%) in patients ($n = 5$) were similar to those found in healthy controls ($n = 5$), that is, 3%, 1 to 8% and 80%, 79 to 97% for spontaneous and anti-IgE induced CD63 expression, respectively. No basophil activation with extracts from untreated (2%, 1 to 6%) or autoclaved L3 (3%, 2 to 7%) was found in healthy controls. Basophil activation was observed when patient samples were incubated with extracts obtained from L3 subjected to autoclaving for 40 and 80 min (Table 1). The results of basophil activation test were variable because both an autoclaving time-dependent decrease and increase have been found. Apparently there was not any relationship between the patients' anti-*A. simplex* IgE values and the percentage of activated basophils (Table 1).

FIGURE 4. A. *simplex* allergens analyzed by ion trap mass spectrometry. Peptides identified of Ani s 1 (accession no. Q7Z1K3) and Ani s 4 (accession no. Q14QT4) are underlined.

Ani s 1
 MASMQHFSLA ALLLAASICL GDADRTECQL PLDKGTPCTQ EGGVKPSVAW WHDDKSGICL
SFKYTGCGGN ANRFTTIKNC EQHCKMPDRG ACALGKKPAE DSNGEQLVCA GMREDKCPNG
 YQCKMMAFMG LCCPTKEEEL FAREYEGVCK SGKPVKMDRG SGWMMTILGK SCDDQFCPED
AKCERGLFA NCCK

Ani s 4
 MQSRIVVAVV VFCCIILMQT EAGMLGGSSD VDVNDPEIKE LAGKSIAKIS AMINDGKPHE
LVKVVSAAKQ VVAGDKYTL ILVKDGDHOD LCTVTIWQKK WENFEEVKVL KCDHQ

DISCUSSION

Our results showed that the autoclaving process caused profound changes in the SDS-PAGE protein profile and *A. simplex* allergen recognition by specific IgE. However, some allergenicity is preserved because the capacity of binding IgE and activating basophils is retained by the protein extract from larvae after autoclaving even for 80 min. Clinical interest in *A. simplex* allergens' thermostability comes from descriptions of patients reporting allergic symptoms after eating cooked fish (2, 3, 13) or canned fish (1, 3, 13, 14, 23).

Previous reports described the effect of different thermal procedures aimed to kill L3 on the parasite antigens showing that several parasite proteins retained their antigenic characteristics (7, 17, 24, 25). Our immunoblotting analysis allowed identifying low-molecular-weight allergens resistant to temperature higher than those previously reported and similar to canning processes. Ani s 1 was identified as one of the allergens resistant to autoclaving. This protein was the first allergen identified and was characterized as a major allergen recognized by more than 80% of *Anisakis* allergic patients (6, 11, 21). According to our IgE immunoblotting assays, Ani s 1 is detected with a lower molecular weight than expected. We cannot explain the change in its molecular weight, although the action of certain endogenous proteases could not be fully excluded, despite that the autoclaving and protein extraction processes were carried out in the presence of protease inhibitors. Ani s 4, the other identified allergen detected in 27% of allergic patients, was the first nematode protease inhibitor (cystatin) described as an allergen and has been previously shown to be heat stable (boiling for 30 min) and resistant to pepsin digestion (7, 12, 16). Its resistance to autoclaving along with pepsin resistance suggests that Ani s 4 could be clinically relevant in cases in which Ani s 4-sensitized patients are exposed following consumption of processed parasite-contaminated fishery products.

With regard to Ani s 1, pepsin digestion stability has not been analyzed so far. Further studies on pepsin stability would strengthen our results.

Taking into account that IgE binding, as shown by IgE immunoblotting, is not sufficient to predict that an allergen is able to trigger the allergic cascade (19, 22), a basophil activation test with processed L3 extracts was performed. Functional analysis showed that *A. simplex* allergens were able to induce basophil activation after autoclaving. Despite a marked decrease in protein content observed after treatment for 80 min (Fig. 1), the residual parasite allergens can induce CD63 upregulation in the patients tested. The capacity to induce basophil activation seemed to be patient dependent because decreased, increased, or unchanged basophil activation was observed after heat treatment. Similar findings have been reported with blanched or roasted peanuts (19). On the other hand, patients' anti-*A. simplex* IgE levels were not predictable for the magnitude of basophil activation. Although basophil activation tests do not provide information on cellular immune response in the gut, it allows the study of potential allergenicity of *Anisakis* allergens after food processing, because it mimics the in vivo pathway leading to symptoms.

In conclusion, autoclaving is a thermal process that considerably decreases the potential allergenicity of *A. simplex*. However, some relevant allergens retain their capacity to bind IgE and activate basophils after being subjected to this heat treatment, which is equivalent to that used in thermal industrial processes. Our findings are relevant for nonfish allergic subjects who are allergic to *Anisakis* and present symptoms after eating fishery products contaminated with parasite allergens. Although exposure to functionally active allergens after eating parasitized and thermal processed fish can occur, studies on gastroduodenal digestion stability are needed to demonstrate the relevance of allergens that remain allergenic after treatments that simulate a canning process.

TABLE 1. Specific anti-*Anisakis* IgE and percentage of activated basophils with protein extracts of untreated and autoclaved *A. simplex* L3

Patient	Untreated <i>Anisakis</i> L3	Autoclaving, 121°C		Specific IgE (kU _A /liter)
		40 min	80 min	
1	94	93	62	28.8
2	74	30	2	0.60
3	80	67	47	0.71
4	71	78	81	0.74
5	53	73	78	7.33

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