Characterisation of potential novel allergens in the fish parasite

Anisakis simplex

Christiane Kruse Fæste a,*, Karen R. Jonscher b, Maaike M.W.B. Dooper a, Wolfgang Egge-Jacobsen c, Anders Moen c, Alvaro Daschner d, Eliann Egaas a, and Uwe Christians b

a Norwegian Veterinary Institute, Oslo, Norway
b University of Colorado Denver, Aurora, CO, USA
c University of Oslo, Oslo, Norway
d Instituto de Investigación Sanitaria-Hospital Universitario de La Princesa, Madrid, Spain

Abstract

The parasitic nematode Anisakis simplex occurs in fish stocks in temperate seas. A. simplex contamination of fish products is unsavoury and a health concern considering human infection with live larvae (anisakiasis) and allergic reactions to anisakid proteins in seafood. Protein extracts of A. simplex produce complex band patterns in gel electrophoresis and IgE-immunostaining. In the present study potential allergens have been characterised using sera from A. simplex-sensitised patients and proteome data obtained by mass spectrometry. A. simplex proteins were homologous to allergens in other nematodes, insects, and shellfish indicating cross-reactivity. Characteristic marker peptides for relevant A. simplex proteins were described.

Keywords

Allergenicity; Anisakis simplex; Cross-reactivity; Immunoblotting; Mass spectrometry; Proteomic analysis

1. Introduction

Anisakis simplex (herring or whale worm) is the only known fishery product-contaminating parasite eliciting clinical allergic responses [1]. In gastro-allergic anisakiasis allergic symptoms can arise as secondary immune response after a previous infestation by live larvae [2]. There is, however, an on-going discussion regarding whether primary sensitisation by antigens from dead larvae can also occur [3–6]. Four clinical allergic manifestations, i.e. gastric, intestinal, ectopic, and systemic, have been associated with A. simplex, and...
responses might depend on the route of sensitisation [7]. More than 90% of the anisakiasis cases resulted from infection with a single larva [2]. The seafood-transmitted zoonotic disease is caused by the accidental ingestion of third-stage larvae lying encapsulated in the edible tissues of infected fish that is eaten raw or under-cooked [8]. Allergic incidents can, however, also be elicited by hidden allergens in processed fish and products thereof [9], by *A. simplex* protein transmission through the food chain [7], and by occupational exposure to aerosols [4,10]. Recently, the European Food Safety Authority has concluded that *A. simplex* larvae have a considerable allergic potential, emphasising the need for routine testing of fishery products [6].

The occurrence of anisakid nematodes has been reported from all major oceans and seas [11]. The *A. simplex* life cycle is complex involving planktonic crustaceans, fish and marine mammals. In fish, the larvae are mainly situated in the visceral cavity; however, a minor proportion may migrate deeply into the fillets [12]. In recent years, an increasing number of anisakiasis cases have been observed [13], and this development has been connected to the increase of marine mammal populations, a more globalised cuisine, faster cooking practices (e.g. microwaving), the trend to avoid overcooked food for vitamin preservation, and a generally higher consumption of fish for health reasons [1]. Over 90% of the anisakiasis cases world-wide are reported from Japan, and most others occur in Spain, Italy, the USA (Hawaii), the Netherlands, and Germany in regions, where traditionally raw or undercooked fish dishes such as sushi and sashimi, pickled anchovies, lomi-lomi, and salted herring are consumed [5].

In Norway, a country with proportionally high per-capita fish consumption, the number of anisakiasis incidents is low, possibly because mainly cooked or fried fish products are eaten [14]. The demographic IgE sensitisation to *A. simplex* proteins is less than 2%, a relatively low value as compared to about 12% in Japan and Spain. Immunoblot analyses using crude *A. simplex* extracts have shown very heterogeneous and individually different IgE compositions between patients and populations [15–17], and genetic predisposition is thought to be a possible cause for the observed differences in disease susceptibility [18]. The prevalence of anti- *A. simplex* IgE in a population may also result from subclinical and undiagnosed anisakiasis, cross-reactivity with other nematodes or insects due to homologous allergens, or cross-reactions with carbohydrate- and phosphorylcholine-groups on post-translationally modified proteins [19].

Since positive IgE values are not a reliable marker for allergic reactivity, the discrimination between symptomatic and asymptomatic individuals by other than serodiagnostic analyses is important for the determination of *A. simplex* allergy [15]. Established methods include skin prick testing (SPT), basophil activation (BAT) measurement, and oral challenge. However, even the outcome of double-blind placebo-controlled food challenges (DBPCFC) is influenced by patient recruitment and choice of the *A. simplex* challenge material [5,20]. Whereas some studies reported that sensitised patients tolerated deep-frozen or well-cooked fish with anisakid larvae, others have described patients getting allergic symptoms from heat-processed contaminated fish.
In addition to the great variability in responsiveness between individuals, differences may also result from the complexity of the *A. simplex* proteome and specific protein characteristics. Several anisakid allergens have been shown to be relatively resistant to digestion or heat treatment and may even renature under cooling [20,21]. Furthermore, allergenicity appeared to be allocated to sequential epitopes and independent from glycosylation [22].

Three different groups of potential allergenic proteins originate from *A. simplex*. Excretory/secretory (ES) proteins are expressed by the larvae in high amounts during host infestation, somatic proteins are constituents of the larvae body, and cuticular proteins on the larvae’s surface serve as protection from digestion [1]. Together with the different routes of sensitisation (ingestion, inhalation, mucosal, or cutaneous contact) this diverse immunogenic composition is likely a major cause for the development of differential clinical responses.

*A. simplex* protein extracts produce complex band patterns in gel electrophoresis [23,24]. A number of proteins have been recognised as allergens and are registered in the Aller-gome database [25]. Among these are known proteins such as secreted proteinase inhibitors (Ani s1, Ani s 4) and somatic paramyosin (Ani s 2) and tropomyosin (Ani s 3), but also a number of un-characterised proteins, whose functions have not yet been established (Ani s 7, Ani s 10–12, Ani s 24) (Table 1).

The complex binding pattern of *A. simplex* proteins observed in IgE immunoblots suggests that the description of allergens is incomplete. Indeed, several new allergens have been detected by using high-resolution protein purification methods and immunoscreening of protein-expressing cDNA libraries or phage display systems constructed from *A. simplex* larvae [26].

In the present study a different approach using mass spectrometry-based proteomic analysis was attempted to characterise *A. simplex* proteins and to identify potential novel allergens.

### 2. Materials and methods

#### 2.1. Patients

Sera from two different patient populations were obtained including 14 Norwegian and 13 Spanish patients with IgE against *A. simplex* and positive skin prick tests (Table 2). The Norwegian patients were originally recruited by newspaper advertisements for a study on shellfish allergy; however, they were also tested for cross-reactivity to *A. simplex* and mite. Skin prick testing (SPT) was performed with total PBS extract of *A. simplex* 3rd stage larvae retrieved from contaminated Blue Withing (*Micromesistius poutassou*) caught in the Norwegian Sea. Positive responders were studied further using a basophile activation test, ImmunoCap™ (Phadia, Uppsala, Sweden) analyses, and immunoblotting. Specific IgE levels to *A. simplex* (p4, *Anisakis* spp.), shrimp (f24, *Pandalus borealis*, *Penaeus monodon*, *Metapenaeopsis barbata*, *Metapenaeus joyneri*), and mite (d1, *Dermatophagoides pteronyssinus*) were measured. The sera were stored in conformity with Norwegian law in a registered diagnostic bio-bank.
The Spanish patients were admitted to clinical treatment either because of anisakiasis or allergy to *A. simplex* proteins. Skin prick tests were performed with *A. simplex* antigen (Lab IPI, Madrid, Spain), and SPT responses were considered positive when they had a mean diameter of at least 3 mm × 3 mm. Histamine (1%) and isotonic saline solution (0.9% NaCl) were the positive and negative controls, respectively. Measurements of total and specific IgE were performed using ImmunoCap™. The studies were approved by the study centre’s institutional review board and all patients gave their written informed consent.

2.2. *A. simplex* protein extracts

The protein was extracted from 3rd stage *A. simplex* larvae that were freed from host tissue as described earlier [24]. Proteins were extracted with phosphate-buffered saline (PBS) (pH 7.4) for 1 h at room temperature. Total protein contents were determined using the Lowry Protein Assay (BioRad Laboratories, Hercules, CA). Aliquots were stored at −20 °C until use.

2.3. Gel electrophoresis and immunoblot

The NuPage Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein samples by SDS-PAGE, in accordance with the manufacturer’s instructions as previously described [24]. Samples contained 10 μg and 30 μg *A. simplex* protein for the immunoblotting and mass spectrometry experiments, respectively. Proteins were either stained with SimplyBlue™ Safe Stain (Invitrogen) and used for in-gel digestion and MS experiments, or transferred electrophoretically onto nitrocellulose membrane (Bio-Rad) in an XCell II Blot Module (Invitrogen) and used for immunostaining.

Immunoblots were developed as described before using Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.6) as washing buffer and TBS-T containing 3% BSA as blocking and assay buffer [27]. After incubating at 4°C overnight with 1:20 diluted patient sera the blots were washed (3 × 15 min) and incubated subsequently with rabbit anti-human IgE antibody (1:1000; Dako, Glostrup, Denmark) and HRP-conjugated goat anti-rabbit antibody (1:5000; Zymed, San Francisco, CA) for 2h each with intermediate washing. After washing (3 × 10 min), the membrane was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Zymed) until bands of satisfactory intensity appeared (2–10 min). All washing and incubation steps were performed under gentle shaking at RT.

2.4. GelPro Analyzer® image analysis

Immunoblots were scanned and processed using GelPro Analyzer® Version 6.3 (MediaCybernetics, Bethesda, MD). IgE-binding signal intensities were determined by applying Standard Optical Density Fitting (second order polynomial) correlating the number of pixels measured to the optical density (OD). The relative protein amount in an individual band was approximated in proportion to the protein quantity loaded in each lane (10 μg). All lanes were processed individually so that potential lane-to-lane intensity differences were compensated.
2.5. Sample preparation for MS experiments

Protein bands of interest were excised from the SDS-page gels, destained, alkylated, digested and extracted as described previously [27]. Briefly, the gel slices were destained with acetonitrile/50 mM NH₄HCO₃ (50/50) at room temperature (RT), dried, reduced with dithiothreitol (Sigma Chemicals, St. Louis, MD) at 56 °C, alkylated with iodoacetamide at RT in the dark, washed and dried. Proteins were digested in gel with 0.1 μg/ml trypsin (Trypsin Gold mass spectrometry grade, Promega, Madison, WI) at 37 °C overnight. Tryptic peptides were extracted from the gel, acidified with formic acid, and analysed by mass spectrometry.

2.6. Protein identification by nanoLC/quadrupole ion trap MS/MS

Tryptic *A. simplex* peptides were analysed by reversed-phase nano-liquid chromatography electrospray quadrupole-iontrap mass spectrometry (nanoLC ESI-MS/MS) using an Agilent 1100 HPLC-system equipped with a nanopump coupled to an Agilent LC/MSD Trap XCT Plus (Agilent Technologies, Palo Alto, CA) mass spectrometer. Peptides were loaded onto a Zorbax C18 column (75 μm ID × 10 cm, 300 Å porosity, 5 μm particles) (Agilent Technologies) for 2 min using a micro-well plate autosampler and a capillary pump delivering a flow of 5 μl/min without split. Peptides were eluted by a gradient of solvent A (0.1% formic acid) and solvent B (90% acetonitrile, 0.1% formic acid) at a flow rate of 300nl/min. The gradient was ramped from 3% to 8% B in 1 min, from 8% to 45% B in 85 min, and finally to 90% B in 5 min, until the mobile phase was returned to the initial conditions after 10 min. Spray was established using 8 μm ID emitters (New Objective, Woburn, MA) and a capillary voltage of 1600 V. Spectra were collected over 350–1800 m/z. Three fragmentation spectra were collected for the three most abundant m/z values. Subsequently, those m/z were excluded from analysis for 1 min and the next three most abundant m/z values were selected for fragmentation to enable analysis of lower abundance peptide ions.

The Spectrum Mill database search algorithm (Agilent Technologies, Santa Clara, CA) was used to search the NCBI nr and UniProt databases, employing the taxonomy filter for nematodes. Parameters used for the search included the monoisotopic mass, a peptide mass tolerance of 1.2 Da and a fragment ion mass tolerance of 0.6 Da. Furthermore, tryptic peptides were only allowed two missed cleavages, and carbamidomethylation of cysteine was chosen as a fixed modification. Post-translational modifications (glycosylations and/or phosphorylations) as possible variable peptide modifications were not included in the search parameters. Database matches were validated by reverse database scoring using SpectrumMill software. Proteins with SpectrumMill scores above 13, peptide scores above 10 and scored percent intensity (SPI) of 70% were used as a cutoff for initial “hit” validation. Additionally, search result using MASCOT were included when protein scores were above the significance threshold (p<0.05) and peptide expectation values below 10⁻⁵.

2.7. High resolution proteomics by nanoLC-ESI-orbitrap-MSMS

The tryptic *A. simplex* peptides were further analysed using high-resolution reversed-phase nano-liquid chromatographic ESI-Orbitrap-MSMS. The system consisted of two Agilent 1200 HPLC binary pumps (nano and capillary) with autosampler, column heater and
integrated switching valve (Agilent, Waldbronn, Germany) coupled to a nanoelectrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide solutions (4μL) were extracted on 5-mm × 0.3-mm Zorbax 300 SB-C18 5 μm columns (Agilent) by washing with 97% 0.1% formic acid/3% acetonitrile at a flow rate of 4μL/min provided by the capillary pump. After 7 min, the integrated switching valve was activated, and peptides were eluted onto a 150-mm × 0.075-mm C18, 3-μm resin column (GlycproSIL C18-80 Å, Glycpromass, Stove, Germany). Chromatographic separation was achieved using an acetonitrile/water (0.1% formic acid) binary gradient from 5% to 55% acetonitrile in 70 min and a flow rate of 0.2 μL min\(^{-1}\) provided by the nanoflow pump.

Mass spectra were acquired in the positive ion mode applying a data-dependent automatic switch between survey scan and tandem mass spectra (MS/MS) acquisition. Peptide samples were analysed with a high-energy collisional dissociation (HCD) fragmentation method, acquiring one Orbitrap survey scan in the mass range of \(m/z\) 300–2000 followed by MS/MS of the three most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap was 1,000,000 for survey scans at a resolution of 30,000 for \(m/z\) 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Collision-induced fragmentation was performed with a target value of 5000 ions. The ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180 s.

Mass spectrometric data were first analysed by generating msf-files from raw MS and MS/MS spectra using the Proteome Discoverer 1.0 software (Thermo Fisher Scientific). Database searches were performed by using the NCBI-database applying the taxonomy filter for nematodes. Both the SEQUEST search engine (La Jolla CA, USA) involving the criteria enzyme name (trypsin), missed cleavage sites (2), precursor mass tolerance (10 ppm), fragment mass tolerance (0.6 Da), fixed modifications (carbamidomethyl), variable modification (oxidation), and the MASCOT search engine (Matrix Science Inc., Boston, MA) with the criteria enzyme name (trypsin), fixed modifications (carbamidomethyl), variable modifications (oxidation), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (±7 ppm), fragment mass tolerance (±0.6 Da), and maximum missed cleavages (1) were used. Proteins were considered as significant hits if the XCorr was higher than 1.5 (SEQUEST) or if the score was higher than 30 (MASCOT).

3. Results

3.1. Norwegian patients included in the study

The 14 Norwegian patients (Table 2) had serous total IgE levels ranging from 51 to 4569 kU/L and specific IgE levels of classes 0–4 (<0.35–8.5 kU/L) for *Anisakis* ssp, classes 0–5 (<0.35–56.3 kU/L) for shrimp, and classes 1–5 (0.6–58.5 kU/L) for mite. Skin prick testing (SPT) with total *A. simplex* extract resulted in strong reactions in four patients, medium reactions in two patients and a slight reaction in one patient whereas there was no reaction in seven patients. Reactivity in SPT and specific IgE serum levels appeared not to be directly correlated. Patient N14 had no measurable anti-*Anisakis* IgE, but experienced one of the strongest reactions in SPT. Patient N7 had class 3 anti-*Anisakis* IgE, but was negative in SPT, and N10 had both high anti-*Anisakis* IgE and a strong SPT reaction.
All patients were additionally sensitised to house dust mite, and several were also sensitised to shrimp. Patients N3, N4, N10, and N11 had IgE classes of 2–5 for all three allergens, and strong SPT reactions. N1 and N14 were sensitised to mite and not to *Anisakis*, however, they were positive in SPT. Four patients, N5, N6, N8, and N9, had no anti-*Anisakis* IgE, and were negative in SPT, but were included in the study because of their elevated anti-mite IgE levels with the aim to study potential cross-reactivity.

3.2. Spanish patients included in the study

The 13 Spanish patients (Table 2) could be divided into two subgroups: Patients S1–S7 were allergic to *A. simplex*, whereas patients S8–S13 had been diagnosed with gastro-allergic (GA) anisakiasis. All patients were positive in SPT with *A. simplex* and had at least class 1 serum IgE against *Anisakis* ssp. proteins, although in average the GA-patients had higher levels. S13 had the highest anti-*Anisakis* IgE serum level of all patients included in this study. Patients S1–S7 were also sensitised to shrimp, and partly also to mite, whereas S8–S13 had little or none IgE to shrimp and mite.

3.3. Determination of allergenic *A. simplex* proteins using patient sera

Sera from 14 Norwegian and 13 Spanish patients (Table 2) were used to detect allergenic *A. simplex* proteins using immunoblot. The individual sera bound to multiple protein bands, creating patient-specific binding patterns. The signal intensities of 39 individual protein bands ranging from 5 to 200 kDa were determined using image analysing software (Fig. 1a). The respective molecular weights of the signal bands (presented with a decimal for better differentiation) were determined by the software in relation to the pre-stained molecular weight marker. For each band, the approximated relative protein amounts were summed up for all patients (Fig. 1b). *A. simplex* proteins ranging from 25 to 80 kDa showed particularly strong IgE-binding. Several proteins were detected by all sera, and IgE-binding to the band at 55.5 kDa was the strongest, followed by bands at 37.7 and 73.3 kDa. Second to this triplet were four protein bands at 40.8, 47.5, 63.7, and 68.0 kDa, followed by a group of eight bands at 25.3, 34.4, 35.9, 39.3, 43.2, 50.3, 58.6, and 204.6 kDa. In contrast, some proteins were recognised with considerable strength only by a few patient sera such as 18.2 kDa by N1, N4, N7, S4, and S6 and 13.7 kDa by N12, S11, S12, and S13. Only S11 bound to a protein band at 5.5 kDa.

Evaluating the two patient groups separately (figures not shown) showed very similar binding patterns for the Norwegian and Spanish sera. There were, however, slight differences for five protein bands. Whereas the combined Norwegian sera appeared to bind stronger to the protein at 68.0 kDa, the Spanish sera showed stronger binding to bands at 13.7, 25.3, 47.5, and 63.7 kDa.

3.4. Characterisation of *A. simplex* proteins using MS-based proteomics

Total protein extract from *A. simplex* was separated by one-dimensional gradient electrophoresis under denaturing conditions (Fig. 2). At least 22 protein bands in the range from 3 to 200 kDa were visible after gel staining, forming a multi-band pattern. Protein bands at about 40, 48, 56 and 73 kDa were particularly intense, but many other bands in the upper molecular range were also clearly visible.
Tryptic peptides of 16 *A. simplex* protein bands (Fig. 2) were analysed with LC/MSMS. The resulting peptide masses, patterns and sequences were compared to the database entries for nematodes. *A. simplex* proteins were identified by peptide homologies to known nematode proteins (Table 3). The numbers of detected matching peptides varied from only 1 to 56, resulting in uncertainties for proteins with low hit rates. However, among the 10 proteins described by one peptide (Table 3) were two known *A. simplex* proteins.

103 *A. simplex* proteins were characterised in this study, of which 94 had not been described before. Currently, both the Universal Protein Knowledgebase (UniProtKB) and the NCBI database contain data for 44 unique *A. simplex* proteins, some of them listed with many protein species and fragments so that the total numbers of entries are 153 and 536, respectively.

The proteins characterised in the present study (Tables 3 and S1) included many structural proteins and locomotoric muscle proteins. Furthermore, proteins associated with transcription or translation processes, the cellular energy supply, or the nuclear DNA repair system, and protein synthesis-associated proteins such as ribosomal subunits, translation initiation factors, and elongation factors were identified. In addition, regulatory proteins as well as transport-related proteins were discovered in the *A. simplex* extract, but catabolic enzymes accounted for the biggest part of the characterised proteins (Table 3). Many of the enzymes achieving the best Mascot scores were involved in sugar metabolism processes (glycogenolysis, citric acid cycle, glycolysis, pyruvate dehydrogenase, pentose phosphate pathway). Several key enzymes of other metabolic pathways could also be characterised (Table 3) such as enzymes involved in amino acid metabolism. Finally, proteins associated with detoxification reactions as well as invertebrate haemoglobin were found.

### 3.5. Allocation of *A. simplex* proteins to allergen families

A considerable number of the detected *A. simplex* proteins could be classified into 33 allergen families (Table 4) as defined in the AllFam database ([http://www.meduniwien.ac.at/allergens/allfam](http://www.meduniwien.ac.at/allergens/allfam)) [28]. The classification is only made with respect to specific peptide sequence motifs and domains and is without prejudice to the actual allergenicity of the respective proteins. Nevertheless, each AllFam class contains known allergens originating from different species. Many of the 16 analysed gel bands (Fig. 2) contained potentially allergenic proteins.

Several known allergens from *A. simplex* (Table 1) were detected in the present analysis, including paramyosin (Ani s 2) containing myosin tail (AF100), tropomyosin (Ani s 3) (AF054), Ani s troponin including an EF-hand domain (AF007), SXP/RAL-2 protein Ani s 8 (AF137), and fructose 1,6-bisphosphatase Ani s FBPP (without AllFam number). However, the majority of the newly characterised *A. simplex* proteins, which could be allocated to an allergen family, had not been described before (Table 4).
3.6. Comparison of potential *A. simplex* allergens to known nematode, insect, and shellfish allergens

Focussing on AllFam families containing homologous allergenic proteins from potentially cross-reacting species such as other nematodes, insects, or shellfish, 17 allergen candidates from *A. simplex* have been identified in this study (Table 5).

Myosin-4 contains the well-conserved EF-hand motif of structural proteins, and some of the major allergens of animal origin belong to this protein class. Myosin light chain from German cockroach is an inhalant allergen [29], myosin heavy chain from biting midge is allergenic by bite [30], and European white shrimp myosin is a known food allergen [31]. Carbonic anhydrase is a major antigen in human body louse and the sanyak plant [32]. Lipid transport proteins including apolipoporphin, and vitellogenin have been determined as allergens in German cockroach and three species of house dust mites [33]. Calponin-like protein, belonging to the EB1 family, is as a major allergen in pig roundworm [34]. Myophilin, from the same protein family, is recognised as a muscle-specific antigen in dog tapeworm [35]. Alpha-amylase has homologues in yellow fever mosquito, mites, and midge, which all have been shown to elicit allergenic reactions by sting, bite, or inhalation [33].

Heat shock protein 70 (HSP70) is a known allergenic inhalant in house dust mite [36], storage mite, biting midge, black fly, and cockroach [29]. Endochitinase, a glycoside hydrolase from the chitinase class III group, was detected a 70 kDa. The weight of the nematode aglycone from *A. suum* is 40 kDa but chitinases are generally highly glycosylated. The enzyme has been identified as a major mite allergen for cats, dogs, and humans [33,37]. Disulphide isomerase contains a thioredoxin domain. Thioredoxins (TRX) with IgE-binding potential have been determined in Indian meal moth and white shrimp. Tubulin α has been found to cause asthma and allergy after inhalation of dust containing fodder mite or dust mite. Enolase is a major cross-reacting allergen in plants, fungi, and fish, and has also been recognised as an allergen in cockroach [29]. Arginine kinase (ATP-guanido phosphotransferase) is an important cross-reactive pan-allergen in invertebrates. So far, IgE-binding arginine kinases have been identified in 11 insect and shellfish species, including cockroaches, mite, moth, shrimps, crabs, and lobster. Haemoglobin is a known allergen in mites. Fructose-bisphosphate aldolase I (FPA) has not been assigned an AllFam-number yet, although it has shown allergenicity in cockroach and biting midge [30]. The 60S ribosomal protein (AF070) is allergenic in horses by sting or bite from midge [38]. Moreover, the protein is an inhalant or ingestion allergen in many fungi and some plants. Triosephosphate isomerase (TPI) has been characterised as an allergen in several invertebrates and plants. The enzyme can elicit allergic reactions in humans by inhalation of dust containing cockroach debris [29], by midge bite or sting [30], or by ingestion of crayfish and shrimp. Glutathione-S-transferase (GST) has been identified as an important allergen in German and American cockroaches [39,40]. The enzyme is also termed Group 8 mite allergen [33]. Furthermore, GST has shown allergenic potential in several parasitic nematodes.

3.7. Combination of immunoblot and proteomics data

Several *A. simplex* proteins of particular interest emerged when the immunoblot analysis and the proteomics data were considered together. The alignment of blot and gel gave an
indication of which proteins could be responsible for IgE-binding. Several strong signals (Fig. 1a and b) coincided with known *A. simplex* allergens (Table 1) or proteins that are known allergens in other invertebrate species (Table 5). Due to this approximation, the high-molecular weight proteins myosin-4, apolipoprotein, and carbonic anhydrase might have caused binding at about 200 kDa. In the range from 82 to 185 kDa, the combined total IgE-binding was rather low, although individual patients reacted strongly to some proteins (Fig. 1b). The proteomics data suggested the presence of calponin-like protein and paramyosin (*Anis 2*) at this molecular weight. Comparably, it could be assumed that the immunoblot signal at 85 kDa might be associated with the presence of heat shock protein 90 or aconitate hydratase (Table 4) and that the signal triplet at 73, 68, and 64 kDa was associated with α-amylase, heat shock protein 60, and endochitinase (Table 5). The strongest observed IgE-binding signal for all patients at 56 kDa might be correlated to disulphide isomerase and myophilin (Table 5). The tubulins α and β could be associated to the signal duplet at 59 and 50 kDa and enolase could have produced the signal at 48 kDa. The four signals from 43 to 38 kDa are potentially related to the presence of arginine kinase, haemoglobin, fructose-1,6-bisphosphate aldolase 1, and 60S acidic ribosomal protein in the same molecular range on the gel. The IgE-binding at about 35 kDa could result from tropomyosin (*Anis 3*) (Table 1). Triosephosphate isomerase or glutathione-S-transferase was candidates to have caused the relatively strong signal at 25 kDa. Troponin C (*Anis troponin*) and the SXP/RAL-2 proteins (*Anis 5, Anis 8, Anis 9*) were potentially responsible for some of the scattered immunoblot signals at lower molecular weights (Fig. 1a).

### 3.8. Marker peptides of potential novel *A. simplex* allergens

Unique allergen peptides could be suitable for the screening of potentially contaminated food products. Several of the newly discovered allergen candidates from *A. simplex* were therefore studied in more detail using high-resolution MS/MS for the identification of possible biomarkers. Considering the qualities of the individual data, peptides from eleven *A. simplex* proteins were selected (Table S2).

Enolase (Fig. S1a) showed the best results under the chosen measurement conditions. Comparison to the *A. simplex* enolase protein sequence in the UniProt database revealed no mismatches in the 30 peptides that had been determined by MS/MS-analysis. Enolase was the only of the potential novel allergens, for which *A. simplex* sequence information was available in the NCBI and UniProt protein databases. One in many species highly conserved enolase peptide and two *A. simplex*-specific peptides were identified as good marker peptide candidates (Table S1, Fig. S1b and c). Additionally, an *A. simplex*-specific peptide was found in myosin-4 (Fig. S2), with identity in 13 or 14 of the 15 amino acids, respectively, to homologous myosin-4 in the nematodes *A. suum* and *Caenorhabditis elegans*. The marker peptides detected in α-amylase, HSP70, disulphide isomerase, tubulin α, arginine kinase, 60S acidic ribosomal protein, triosephosphate isomerase, and glutathione-S-transferase are all highly conserved in nematodes (Table S2, Fig. S2). In contrast, nematode haemoglobin is less homologous. The proteins from cod worm (*Pseudoterranova decipiens*), pig roundworm (*Ascaris suum*), and canine roundworm (*Toxocara canis*) share only 60 to 65% homology. However, a C-terminal peptide of *A. simplex* haemoglobin had 100% sequence
identity to the homologous peptide in the closely related *Anisakis peregraeffi* (Table S2, Fig. S2).

### 4. Discussion

Allergy to *A. simplex* and gastro-allergic anisakiasis caused by contaminated fishery products have been recognised as a food safety concern [6]. At the same time, there is a lack of data regarding the allergenic potential of anisakid proteins. The *A. simplex* genome is not completely identified and only a relatively small number of *A. simplex* proteins have been entered into protein databases to date. However, the observed complex patterns in immunoblots using patient sera have led to the entry of 21 *A. simplex* proteins into the Allergome database [25] reflecting the ambiguous situation. The respective importance of the listed allergens is under discussion because a high frequency of false-positive results in immunoblots with sera from sensitised patients without clinical manifestations and even in healthy control individuals has been observed [1,14,16,41]. Additionally, the allergenic potentials of several of the listed *A. simplex* proteins designated as allergens, including Ani s 5, Ani s 6, Ani s 7, Ani s 10, Ani s 11, and Ani s 12, have been determined only by immunoscreening of an expression cDNA or phage display library with serum from a single patient [26].

In order to characterise the allergenic potential of *A. simplex* proteins further we have performed immunostaining with sera from sensitised Spanish and Norwegian patients and proteomic analysis.

#### 4.1. Sensitisation to *A. simplex* proteins in the patient groups

Compared to the high incidence of infestation of wild-caught marine edible fish by *A. simplex* larvae, cases of anisakiasis and clinically manifested allergy to anisakid proteins are surprisingly uncommon. The occurrence of anisakiasis is directly connected to special dietary habits such as the consumption of raw and undercooked fish in certain geographical regions. In contrast the ratio of anti-*A. simplex* IgE seropositive persons in a population is considerably higher, but again, sensitisation appears to be geographically dependent [1].

There is apparently a connection between the frequency of anisakid infections and the rate of sensitisation in specific populations. It has been suggested that infection with parasitic worms may modulate the immune reactivity of the host [42]. The nematode presumably blocks the mechanisms that trigger allergic incidents resulting in an overall systemic anti-allergic effect although the nematode allergens stimulate the generation of specific IgE. In this context, invertebrate tropomyosin, a conserved muscle protein present in high amounts in all nematodes, is regarded as a promising candidate for a vaccine against allergy to nematodes [42]. On the other hand it has been argued that acute or sporadic forms of parasitism, such as gastro-allergic anisakiasis, are associated with an elevated risk of allergy [43,44].

The IgE-immunoblots performed in our study showed little variability between Norwegian and Spanish patients. Reactivity in SPT and specific IgE serum levels did not appear to be directly correlated, and IgE classes were not recognisably connected to binding patterns and
intensities. The observed binding patterns showed more inter-individual than inter-group differences, as previously noted [45]. Three regions on the immunoblots, depicting IgE-binding to proteins with molecular weights of 80–150 kDa, 30–40 kDa, and <20 kDa, demonstrated the most diversity between patients.

The greatest difference with regard to the two patient groups was the consistent co-sensitisation to mite in the Norwegian patients. More than 90% of the study subjects had anti-mite IgE of class 2 or more, whereas the Spanish patients were not, or to a lesser degree, sensitised to mite. This difference might explain the observed small variations in immunoblot binding intensities to five protein bands when comparing the combined results of each group. Although the results could only be considered as indicative due to the uncertainties connected to some variances in the background noise of the blots and the image processing performed, they allowed the observation of some trends: The Norwegian sera appeared to bind notably stronger to a protein band at 68 kDa, which according to the proteomic analysis could contain *A. simplex* heat shock protein 70, a known mite allergen [36]. Furthermore, α-amylase, another insect allergen, was presumably recognised at 73 kDa. The potential importance of cross-reactivity of anti-insect IgE with anisakid proteins was further confirmed by the results for the four patients who were sensitised to mite but not to *A. simplex*. Cross-sensitisation and allergenic cross-reactivity to mite had also been observed in Norwegians with anti-*A. simplex* IgE in previous studies [14,46].

The Spanish sera bound rather intensely to a protein band at 14 kDa, tentatively characterised as an SXP/RAL2-protein (Ani s 8). Excretory/secretory (ES) proteins such as Ani s 8 are up-regulated following host infection. Not surprisingly, the sera of the anisakiasis patients S10–S13 showed the strongest binding to this allergen. In total somatic extracts from *A. simplex* larvae, as used in the present study, the secretory proteins originate from the excretory glands and are generally underrepresented [23]. Consequently, we found only a few ES proteins by proteomic analysis. Three other signals that appeared to be preferentially represented in the immunoblot from the Spanish sera were provisionally aligned with triosephosphate isomerase (25 kDa), enolase (48 kDa), and endochitinase (64 kDa). A 48 kDa protein, presumably enolase, was also well recognised in another study involving Spanish patients [16,45].

In general, the IgE-binding patterns to *A. simplex* proteins found in the present experiment were comparable to those in previous published studies describing complex patterns with multiple bands in the range from 14 to 190 kDa [15,16,20,23,47,48]. Sera of anisakiasis patients bound preferentially to ES allergens and their carbohydrated forms [17,47], with dominant bands at 14, 56, and 72 kDa [41]. In somatic extracts, the strongest binding occurred at 43, 48, and 56 kDa [23,45], which could be related to the allergen candidates arginine kinase, enolase, disulfide isomerase, and myophilin determined in the present study. Based on our results, the designated pan-allergens paramyosin (Ani s 2) and tropomyosin (Ani s 3) were of lesser importance confirming previous findings that have questioned the clinical relevance of these anisakid proteins [43].
4.2. Analysis of the A. simplex proteome

The proteomic analysis of the somatic A. simplex extract resulted in the identification of numerous proteins by comparison to homologous peptides from database-listed nematode proteins. Since entries for nematodes of the Anisakidae family (including A. simplex and Pseudoterranova decipiens) are scarce, we used data of the phylogenetically closely related Ascaris suum of the Ascaridae family as the best fit. Both families belong to the same Ascaridoidea superfamily and Ascaridida order \[49\], whereas the “model” nematode Caenorhabditis elegans, the genome of which has been totally sequenced, belongs to the Rhabditida order and is more distantly related.

Many of the characterised A. simplex proteins were enzymes involved in carbohydrate metabolism. Parasitic nematodes use glucose from the host environment at a high rate, and glycogen has been shown to be their main endogenous carbohydrate \[50\]. Structural and muscle proteins were likewise present in considerable abundance in the somatic extract. They account for an essential part of the nematode’s total body weight and are easily detected. Generally, when using a mass spectrometry-based proteomics approach without targeted enrichment, the more abundant proteins in an organism achieve the highest sequence coverage and best peptide Mascot scores, as observed here.

4.3. Novel A. simplex allergen candidates

Recently, the determination of new allergens has preferably been based on methods of molecular allergology such as the immunoscreening of cDNA libraries rather than the previously applied immunoblots \[26\]. However, this approach is somewhat limited in that allergen detection is based on a single patient serum, and protein expression in culture is not necessarily the same as in a live organism. In contrast, immunoblot analysis uses representative protein extracts and often multiple patient sera, but allergen detection is restricted to molecular weight comparisons or confirmation by monoclonal antibodies.

In the present study we combined the advantages of IgE-based immunoscreening with mass spectrometry-based proteomics, allowing for the direct identification of protein bands of interest and description of a number of new allergen candidates extracted from A. simplex. Somatic protein extracts contain a large variety of proteins and the sensitivity and reactivity of patients are highly variable as multi-band immunoblot patterns confirm \[16,48\]. Nevertheless, proteins have to be fairly abundant to elicit IgE-sensitisation and are therefore well-suited for MS-based strategies, so that both techniques are mutually supportive.

Considering the observed cross-reactivity among ecdysozoan species \[46\] we were particularly interested in potentially allergenic A. simplex proteins that were homologous to known allergens in related phyla such as other nematodes, insects, or shellfish. According to our findings, myosin, heat shock protein 70, α-amylase, disulphide isomerase, myophilin, enolase, arginine kinase, haemoglobin, fructose-1,6-biphosphate aldolase 1, 60S acidic ribosomal protein, triosephosphate isomerase, and glutathione-S-transferase are all candidates for causing insect-nematode cross-allergies.
4.4. Marker peptides for A. simplex

Peptide sequence analyses of selected A. simplex proteins resulted in the determination of a number of marker peptides for the specific detection of this nematode, underlining the great potential of MS-based proteomic analysis. This technique may allow the differentiation between homologous proteins from related nematode species and could therefore be used for the classification of food and feed contaminants. In a next step, it will be advantageous to verify our findings by using fractionated or recombinant proteins for the immunoblotting experiments or to perform two-dimensional electrophoresis, and to study the allergenicity of selected allergen candidates in more detail.

In conclusion, A. simplex is known for its diversity of antigens that are responsible for the development of differential clinical responses [1]. By comparing serum analyses and proteome data we have characterised a number of potential novel allergens of this fish parasite. They will facilitate further studies on the mechanisms leading to A. simplex sensitisation and on the risk characterisation with regard to its allergic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Arne Levensen from the National Institute of Nutrition and Seafood in Bergen, Norway, for the sourcing and characterisation of Anisakis simplex 3rd stage larvae. We greatly appreciate Marianne Werner from the Norwegian Veterinary Institute for preparing the protein extracts. Furthermore, we are very grateful to Agnieszka A. Kendrick from the University of Colorado at Denver for technical assistance with the ESI-IT-MSMS and to Christian Köhler from the University of Oslo for help with the protein database searching.

Additional funding for the activities at the University of Colorado was received by NIH/NIDDK P30 DK48520.

Funding: This study was funded by the Orkla Foundation, Norway.

References

1. Audicana MT, Kennedy MW. Anisakis simplex: from obscure infectious worm to inducer of immune hypersensitivity. Clin Microbiol Rev. 2008; 21:360–79. [PubMed: 18400801]
2. Daschner A, Alonso-Gómez A, Cabañas R, Suarez-de-Parga JM, López-Serrano MC. Gastroallergic anisakiasis: borderline between food allergy and parasitic disease-clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by Anisakis simplex. J Allergy Clin Immunol. 2000; 105:176–81. [PubMed: 10629469]
3. Audicana MT, Fernández de Corres L, Muñoz D, Fernández E, Navarro JA, del Pozo MD. Recurrent anaphylaxis caused by Anisakis simplex parasitizing fish. J Allergy Clin Immunol. 1995; 96:558–60. [PubMed: 7560669]
4. Nieuwenhuizen N, Lopata AL, Jeebhay MF, Herbert DR, Robins TG, Brombacher F. Exposure to the fish parasite Anisakis causes allergic airway hyperreactivity and dermatitis. J Allergy Clin Immunol. 2006; 117:1098–105. [PubMed: 16675338]
5. Pravettoni V, Primavesi L, Piantanida M. Anisakis simplex: current knowledge. Eur Ann Allergy Clin Immunol. 2012; 44:150–6. [PubMed: 23092000]
6. EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on risk assessment of parasites in fishery products. EFSA J. 2010; 8:1543–634.
7. Armentia A, Martin-Gil FJ, Pascual C, Martín-Esteban M, Callejo A, Martínez C. Anisakis simplex allergy after eating chicken meat. J Investig Allergol Clin Immunol. 2006; 16:258–63.
8. Deardorff TL, Kayes SG, Fukumura T. Human anisakiasis is transmitted by marine food products. Hawaii Med J. 1991; 50:9–16. [PubMed: 2022472]

9. Añíbarro B, Seoane FJ, Múgica MV. Involvement of hidden allergens in food allergic reactions. J Investig Allergol Clin Immunol. 2007; 17:168–72.

10. Mazzucco W, Lacca G, Cusimano R, Provenzani A, Costa A, Di Noto AM, et al. Prevalence of sensitisation to Anisakis simplex among professionally exposed populations in Sicily. Arch Environ Occup Health. 2012; 67:91–7. [PubMed: 22524649]

11. Sakanari JA, McKerrow JH. Anisakiasis. Clin Microbiol Rev. 1989; 2:278–84. [PubMed: 2670191]

12. Levsen A, Lunestad BT. Anisakis simplex third stage larvae in Norwegian spring spawning herring (Clupea harengus L.), with emphasis on larval distribution in the flesh. Vet Parasitol. 2010; 171:247–53. [PubMed: 20413223]

13. Klimpel, S.; Palm, HW. Anisakid nematode (Ascaridoidea) life cycles and distribution: increasing potential in the time of climate change?. In: Melhorn, H., editor. Progress in parasitology. 1st. Berlin, Heidelberg, Germany: Springer-Verlag; 2011. p. 201–22.

14. Lin AH, Florvaag E, Van Do T, Johansson SG, Levsen A, Vaali K. IgE sensitisation to the fish parasite Anisakis simplex in a Norwegian population: a pilot study. Scand J Immunol. 2012; 75:431–5. [PubMed: 22420531]

15. Del Pozo MD, Moneo I, de Corres LF, Audicana MT, Muñoz D, Fernandez E, et al. Laboratory determinations in Anisakis simplex allergy. J Allergy Clin Immunol. 1996; 97:977–84. [PubMed: 8655894]

16. García M, Moneo I, Audicana MT, del Pozo MD, Muñoz D, Fernández E, et al. The use of IgE immunoblotting as a diagnostic tool in Anisakis simplex allergy. J Allergy Clin Immunol. 1997; 99:497–501. [PubMed: 9111494]

17. Hwang YK, Kim JS, Lee JB, Song TJ, Joo KW, Lee JS, et al. Human anisakiasis: diversity in antibody response profiles to the changing antigens in larval excretions/secretions. Parasite Immunol. 2003; 25:1–7. [PubMed: 12753432]

18. Sánchez-Velasco P, Mendizábal L, Antón EM, Ocejo-Vinyals G, Jerez J, Leyva-Cobián F. Association of hypersensitivity to the nematode Anisakis simplex with HLA class II DRB1*1502-DQB1*0601 haplotype. Hum Immunol. 2000; 61:314–9. [PubMed: 10689122]

19. Lorenzo S, Romarís F, Iglesias R, Audicana MT, Alonso JM, Leiro J, et al. O-glycans as a source of cross-reactivity in determinations of human serum antibodies to Anisakis simplex antigens. Clin Exp Allergy. 2000; 30:551–9. [PubMed: 15895253]

20. Caballero ML, Moneo I, González-Muñoz M, Rodríguez-Mahillo AI, Rodríguez-Perez R, Silva A. Isolation of a heat-resistant allergen from the fish parasite Anisakis simplex. Parasitol Res. 2005; 96:285–9. [PubMed: 15895253]

21. Caballero ML, Moneo I. Several allergens from Anisakis simplex are highly resistant to heat and pepsin treatments. Parasitol Res. 2004; 93:248–51. [PubMed: 15138891]

22. Moneo I, Audicana MT, Alday E, Curiel G, del Pozo MD, García M. Periodate treatment of Anisakis simplex allergens. Allergy. 1997; 52:565–9. [PubMed: 9201369]

23. Moneo I, Caballero ML, Gómez F, Ortega E, Alonso MJ. Isolation and characterization of a major allergen from the fish parasite Anisakis simplex. J Allergy Clin Immunol. 2000; 106:177–82. [PubMed: 10887322]

24. Werner MT, Faeste CK, Levsen A, Egaas E. A quantitative sandwich ELISA for the detection of Anisakis simplex in seafood. Eur Food Res Technol. 2011; 232:157–66.

25. Mari A, Rasi C, Palazzò P, Scala E. Allergen databases: current status and perspectives. Curr Allergy Asthma Rep. 2009; 9:376–83. [PubMed: 19671381]

26. López I, Pardo MA. A phage display system for the identification of novel Anisakis simplex antigens. J Immunol Methods. 2011; 373:247–51. [PubMed: 21893063]

27. Faeste CK, Christians U, Egaas E, Jonscher KR. Characterization of potential allergens in fenugreek (Trigonella foenum-graecum) using patient sera and MS-based proteomic analysis. J Proteomecs. 2010; 73:1321–33. [PubMed: 20219717]

28. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol. 2008; 21:847–52. [PubMed: 18395549]
29. Chuang JG, Su SN, Chiang BL, Lee HJ, Chow LP. Proteome mining for novel IgE-binding proteins from the German cockroach (Blattella germanica) and allergen profiling of patients. Proteomics. 2010; 10:3854–67. [PubMed: 20960453]

30. Chen YH, Lee MF, Lan JL, Chen CS, Wang HL, Hwang GY, et al. Hypersensitivity to Fociopomia taiwana (biting midge): clinical analysis and identification of major For t 1, For t 2 and For t 3 allergens. Allergy. 2005; 60:1518–23. [PubMed: 16266384]

31. Ayuso R, Grishina G, Bardina L, Carrillo T, Blanco C, Ibáñez MD, et al. Myosin light chain is a novel shrimp allergen, Lit v 3. J Allergy Clin Immunol. 2008; 122:795–802. [PubMed: 18760458]

32. Hur GY, Park HJ, Kim HA, Ye YM, Park HS. Identification of Dioscorea batatas (sanyak) allergen as an inhalant and oral allergen. J Korean Med Sci. 2008; 23:72–6. [PubMed: 18303202]

33. Thomas WR, Smith WA, Hales BJ, Mills KL, O’Brien RM. Characterization and immunobiology of house dust mite allergens. Int Arch Allergy Immunol. 2002; 129:1–18. [PubMed: 12372994]

34. Wang J, Czech B, Crunk A, Wallace A, Mitreva M, Hannon GJ, et al. Deep small RNA sequencing from the nematode Ascaris reveals conservation, functional diversification, and novel developmental profiles. Genome Res. 2011; 21:1462–77. [PubMed: 21685128]

35. Virginio VG, Hernández A, Rott MB, Monteiro KM, Zandonai AF, Nieto A, et al. A set of recombinant antigens from Echinococcus granulosus with potential for use in the immunodiagnosis of human cystic hydatid disease. Clin Exp Immunol. 2003; 132:309–15. [PubMed: 12699422]

36. Aki T, Fujikawa A, Wada T, Jyo T, Shigeta S, Murooka Y, et al. Cloning and expression of cDNA coding for a new allergen from the house dust mite, Dermatophagoides farinae: homology with human heat shock cognate proteins in the heat shock protein 70 family. J Biochem. 1994; 115:435–40. [PubMed: 8056755]

37. O’Neil SE, Heinrich TK, Hales BJ, Hazall LA, Holt DC, Fischer K, et al. The chitinase allergens Der p 15 and Der p 18 from Dermatophagoides pteronyssinus. Clin Exp Allergy. 2006; 36:831–9. [PubMed: 16776685]

38. Althaus H, Müller N, Busato A, Mellor PS, Torsteinsdottir S, Martí E. Cloning and sequencing of a cDNA expressing a ribosomal P0 peptide from Culicoides nubeculosus Diptera). Vet Immunol Immunopathol. 2004; 99:99–111. [PubMed: 15113658]

39. Arruda LK, Vailes LD, Ferriani VP, Santos AB, Pomés A, Chapman MD. Cockroach allergens and asthma. J Allergy Clin Immunol. 2001; 107:419–28. [PubMed: 11240940]

40. Jeong KY, Lee H, Shin KH, Yi MH, Jeong KJ, Hong CS, et al. Sequence polymorphisms of major German cockroach allergens Bla g 1, Bla g 2, Bla g 4, and Bla g 5. Int Arch Allergy Immunol. 2008; 145:1–8. [PubMed: 17703949]

41. Del Rey Moreno A, Valero A, Mayorga C, Gómez B, Torres MJ, Hernández J, et al. Sensitisation to Anisakis simplex s.l. in a healthy population. Acta Trop. 2006; 97:265–9. [PubMed: 16438926]

42. Sereda MJ, Hartmann S, Lucius R. Helminths and allergy: the example of tropomyosin. Trends Parasitol. 2008; 24:272–8. [PubMed: 18450511]

43. Daschner A, Cuéllar C, Rodero M. The Anisakis allergy debate: does an evolutionary approach help? Trends Parasitol. 2012; 28:9–15. [PubMed: 22079162]

44. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. Science. 2002; 296:490–4. [PubMed: 11964470]

45. Baeza ML, Rodriguez A, Matheu V, Rubio M, Tornero P, de Barrio M, et al. Characterization of allergens secreted by Anisakis simplex parasite: clinical relevance in comparison with somatic allergens. Clin Exp Allergy. 2004; 34:296–302. [PubMed: 14987311]

46. Johansson E, Aponno M, Lundberg M, van Hage-Hamsten M. Allergenic cross-reactivity between the nematode Anisakis simplex and the dust mites Acarus siro, Lepidoglypus destructor, Tyrophagus putrescentiae, and Dermatophagoides pteronyssinus. Allergy. 2001; 56:660–6. [PubMed: 11421925]

47. Akao N, Ohyama T, Kondo K. Immunoblot analysis of serum IgG, IgA and IgE responses against larval excretory-secretory antigens of Anisakis simplex in patients with gastric anisakiasis. J Helminthol. 1990; 64:310–8. [PubMed: 2283472]

48. Rodero M, Cuéllar C, Chivato T, Mateos JM, Laguna R. Western blot antibody determination in sera from patients diagnosed with Anisakis sensitisation with different antigenic fractions of
Anisakis simplex purified by affinity chromatography. J Helminthol. 2007; 81:307–10. [PubMed: 17974043]

49. Kim KH, Eom KS, Park JK. The complete mitochondrial genome of Anisakis simplex (Ascaridida: Nematoda) and phylogenetic implications. Int J Parasitol. 2006; 36:319–28. [PubMed: 16442542]

50. Mansour, TE. The pharmacology and biochemistry of parasitic helminths. In: Garattini, S.; Shore, PA., editors. Advances in pharmacology. 3rd. New York, USA: Academic Press; 1964. p. 129-65.

Abbreviations

- **A. simplex**  Anisakis simplex
- **m/z**  mass-to-charge ratio
- **NanoLC ESI-MS/MS**  nano-liquid chromatography electrospray ionisation tandem mass spectrometry
- **OD**  optical density
Fig. 1.
(a) IgE-immunoblot with total *A. simplex* extract using sera from 14 Norwegian (N1–N14) and 13 Spanish (S1–S13) patients with sensitivity to *A. simplex* (see Table 2). Relevant lanes were extracted from the scanned individual patient immunoblots and presented as a composite gel. M: SeeBluePlus2 molecular weight marker; relative protein molecular weights (kDa) are indicated on the right. 
(b) Bar Chart showing summarised binding intensities of 27 patients for 22 IgE-binding signals on immunoblot (Fig. 1a). Protein molecular weights (kDa) as determined by GelPro Analyzer® are given on the ordinate, protein amounts (ng), calculated as described in Section 2, are on the abscissa. The contributions of individual patients to the combined results are marked by different patterns as shown in the legend.
Fig. 2.
Coomassie-stained SDS-PAGE of total *A. simplex* protein extract. Gel bands (No. 1–16) were excised, digested with trypsin, and subjected to mass spectrometric analysis. SeeBlue Plus2 molecular weight marker; relative protein sizes (kDa) are given on the left side of the gel.
Table 1

*A. simplex* allergens described in literature (retrieved from the Allergome database ([www.allergome.org](http://www.allergome.org)) [25].

| Allergen       | Accession no. | Protein name                      | MW (kDa) | Protein family AllFam/Pfam     |
|----------------|---------------|-----------------------------------|----------|-------------------------------|
| Ani s 1.0101   | Q7Z1K3        | Animal Kunitz serine protease inhibitor | 21.2     | AF003/PF00014                 |
| Ani s 2.0101   | Q9NJA9        | Paramyosin                        | 100      | AF100/PF01576                 |
| Ani s 3.0101/Ani s 3.0102 | Q9NAS5G4XTD3 | Tropomyosin                      | 33.3 33.2 | AF054/PF00261                 |
| Ani s 4.0101   | P83885        | Cystatin                          |          | Fragment[^a]                 |
| Ani s 5.0101   | A1IKL2        | SXP/RAL-2 proteins                | 16.6     | AF137/PF02520                 |
| Ani s 6.0101   | A1IKL3        | Serine protease inhibitor         | 9.7      | AF027/PF01826 & PF08742       |
| Ani s 7.0101   | A9XBJ8        | Unknown                           | 119      | Unknown                       |
| Ani s 8.0101   | A7M606        | SXP/RAL-2 proteins                | 16.1     | AF137/PF02520                 |
| Ani s 9.0101   | B2XCP1        | SXP/RAL-2 proteins                | 15.5     | AF137/PF02520                 |
| Ani s 10.0101  | D2K835        | Unknown                           | 23.3     | Unknown                       |
| Ani s 11.0101  | E9RFF3        | Unknown                           | 30.0     | Unknown                       |
| Ani s 12.0101  | E9RFF6        | Unknown                           | 32.9     | Unknown                       |
| Ani s 24 kDa   | G1FMP3        | Unknown                           | 23.5     | Unknown                       |
| Ani s CCOS3    | Q1X6K9        | Cytochrome C oxidase subunit 3    | 29.0     | ~/PF00510                     |
| Ani s cytochrome B | Q1X6L0    | Cytochrome B                      | 42.2     | ~/PF00032                     |
| Ani s FBPP     |              | Fructose 1,6-bisphosphatase       | ~40      | ~/PF00316[^b]                 |
| Ani s NADHDS4L | Q1X6K2        | NADH dehydrogenase subunit 4L     | 9.2      | ~/PF00420                     |
| Ani s NARaS    |              | Nicotinic acetylcholine receptor alpha subunit | ~60 | ~/PF02931[^b]                 |
| Ani s PEPB     |              | Phosphatidylethanolamine-binding protein | ~24 | ~/PF01161[^b]                 |
| Ani s troponin | Q9U3U5        | Troponin C                        | 18.5     | AF007/PF00036 & PF01036       |

[^a] Cystatin in *Caenorhabditis elegans* 15.6 kDa.

[^b] By comparison with *Ascaris suum* proteins.
Patient groups from Norway and Spain. Results of total serum IgE analysis and analysis of individual IgE to *Anisakis* spp., shrimp (*Pandalus borealis*, etc.), and house dust mite (*Dermatophagoides pteronyssimus*). Several patients were positive in skin prick testing with *A. simplex* extract.

| ID  | Age | Sex | Total IgE (kU/l) | A. simplex | Shrimp | Mite | SPTb |
|-----|-----|-----|------------------|------------|--------|------|------|
|     |     |     | Classa IgE (kU/l) | Classa IgE (kU/l) | Classa IgE (kU/l) |     |      |
| Norwegian group |     |     |      |            |        |      |      |
| N1  | 38  | F   | 51    | 0 <0.35   | 0 <0.35 | 1    | 0.6  | +    |
| N2  | 37  | F   | 780   | 2 1.5     | 3 6.3   | 2    | 2.2  | ++   |
| N3  | 33  | M   | 124   | 2 3.5     | 4 24.5  | 3    | 4.4  | +++  |
| N4  | 49  | M   | 71    | 2 0.8     | 3 5.5   | 2    | 2.3  | +++  |
| N5  | 43  | M   | 268   | 0 <0.35   | 0 <0.35 | 3    | 7.7  | −    |
| N6  | 55  | M   | 2308  | 0 <0.35   | 3 10.7  | 3    | 12.9 | −    |
| N7  | 21  | M   | 3287  | 3 3.6     | 3 12.1  | 3    | 8.2  | −    |
| N8  | 59  | M   | 610   | 0 <0.35   | 1 0.6   | 3    | 7.0  | −    |
| N9  | 65  | M   | 491   | 0 <0.35   | 2 2.5   | 2    | 1.9  | −    |
| N10 | 27  | M   | 328   | 3 8.5     | 4 43.1  | 4    | 39.9 | +++  |
| N11 | 37  | M   | 242   | 3 5.6     | 5 56.3  | 5    | 58.5 | ++   |
| N12 | 45  | F   | 4569  | 2 1.0     | 2 1.6   | 4    | 39.0 | −    |
| N13 | 50  | M   | 1237  | 1 0.4     | 2 2.6   | 2    | 1.5  | −    |
| N14 | 42  | F   | 467   | 0 <0.35   | 3 4.2   | 3    | 3.7  | +++  |
| Spanish group |     |     |      |            |        |      |      |
| S1  | 37  | F   | 106   | 2 2.4     | 3 8.0   | 3    | 8.0  | pos. |
| S2  | 32  | F   | 271   | 3 14.7    | 5 70.0  | 3    | 10.5 | pos. |
| S3  | 33  | F   | 92    | 2 2.8     | 2 0.8   | 0    | <0.35| pos. |
| S4  | 58  | F   | 57    | 1 0.8     | 2 1.6   | 2    | 1.2  | pos. |
| S5  | 24  | F   | 27    | 1 0.5     | 2 2.4   | ≥1   | ≥0.35| pos. |
| S6  | 40  | F   | 229   | 3 9.4     | 2 2.5   | n.m. | n.m. | pos. |
| S7  | 48  | F   | 199   | 2 0.9     | 2 2.9   | ≥1   | ≥0.35| pos. |
| S8c | 41  | M   | 434   | 3 7.2     | 2 1.3   | ≥1   | ≥0.35| pos. |
| S9c | 39  | M   | 87    | 3 8.0     | 2      |      |      |      |
| ID  | Age | Sex | Total IgE (kU/l) | A. simplex | Shrimp | Mite | SPT<sup>b</sup> |
|-----|-----|-----|-----------------|------------|--------|------|--------------|
|     |     |     | Class<sup>a</sup> | IgE (kU/l) | Class<sup>a</sup> | IgE (kU/l) | Class<sup>a</sup> | IgE (kU/l) |
| S10 | 49  | F   | 3               | 12.3       | 1      | 0.4  | 1            | 0.4        | pos.      |
| S11 | 65  | M   | 3               | 10.2       | 0      | <0.35 | n.m.        | n.m.       | pos.      |
| S12 | 68  | M   | 3               | 14.2       | 0      | <0.35 | n.m.        | n.m.       | pos.      |
| S13 | 60  | M   | 4               | 18.1       | 0      | <0.35 | n.m.        | n.m.       | pos.      |

<sup>a</sup>IgE classes by ImmunoCap according to IgE levels (kU/L): class 0: <0.35; class 1: 0.35–0.7; class 2: 0.8–3.5; class 3: 3.6–17.5; class 4: 17.6–50; class 5: 51–100; class 6: >100.

<sup>b</sup>Skin prick testing: −: no reaction; +: <20% of positive control; ++: <50%; +++: ≤80%; pos.: positive; n.m.: not measured.

<sup>c</sup>Patients with gastro-allergic anisakiasis.
### Table 3

*Anisakis simplex* proteins identified by proteomic analysis using ESI-Iontrap or ESI-Orbitrap mass spectrometry and database search (UniProt).

| No. | Protein name                                    | Protein family | Access. no. | MW (kDa) \(^{a}\) | Peptide match \(^{b}\) | Mascot score | Sequence coverage (%) \(^{c}\) |
|-----|-----------------------------------------------|----------------|-------------|-------------------|------------------------|--------------|---------------------------------|
| 1   | Myosin-4                                      | AF007/PF00063 & PF0273 | F1KQ88      | 200               | 45                     | 1323         | 27 (Asc s)                      |
|     | Filamin-A                                     | –/PF00630       | F1KPN0      | 181               | 3                      | 146          | 3 (Asc s)                       |
|     | Apolipoporin                                  | AF092/PF01347 & PF09172 | F1KPM2      | 119               | 3                      | 44           | 1 (Asc s)                       |
|     | Carbonic anhydrase                            | AF139/PF00194  | E0VS50      | 1                 | 1                      | 44           | 1 (Ped h)                       |
| 2   | RAS GTPase-activating protein                 | –/PF03836       | F1KR99      | 170               | 3                      | 89           | 2 (Asc s)                       |
|     | Clathrin heavy chain                          | –/PF00637       | F1KQ49      | 2                 | 2                      | 36           | 1 (Asc s)                       |
|     | ATP-dependent RNA-helicase                    | –/PF00270 & PF04408 | Q7QCW2      | 23                | 2                      | 32           | 20 (Ano g)                      |
|     | Coiled-coiled protein                         | –/PF03915       | Q7PQ25      | 13                | 1                      | 32           | 17 (Ano g)                      |
| 3   | Pyruvate carboxylase 1                        | –/PF02786 & PF02436 | F1KRV7      | 100               | 10                     | 84           | 8 (Asc s)                       |
|     | Kinesin light chain                           | –/PF09311 & PF00515 | Q05090      | 18                | 3                      | 33           | 25 (Str p)                      |
|     | Calponin-like protein                         | AF164/PF00307 & PF00402 | F1KPY3      | 56                | 6                      | 34           | 26 (Asc s)                      |
| 4   | Elongation factor 2                           | –/PF00009 & PF00679 | F1KWZ4      | 91                | 25                     | 772          | 33 (Asc s)                      |
|     | α-Actinin                                     | AF164/PF00307 & PF00435 | F1KR95      | 21                | 21                     | 486          | 25 (Asc s)                      |
|     | Glycogen phosphorylase                        | –/PF00343       | F1KS3       | 17                | 17                     | 413          | 20 (Asc s)                      |
|     | Tetrahydrofolate synthase                     | –/PF01268       | F1KRW4      | 8                 | 8                      | 342          | 11 (Asc s)                      |
|     | Transitional endoplasmic reticulum ATPase 1   | –/PF02359 & PF02933 | F1LCZ2      | 8                 | 8                      | 376          | 26 (Asc s)                      |
|     | 10-Formyltetrahydrofolate dehydrogenase       | AF040/PF00551 & PF00171 | F1KT06      | 14                | 14                     | 279          | 13 (Asc s)                      |
|     | Paramyosin (Ani s 2)                          | AF100/PF01576   | Q9NJA9      | 12                | 12                     | 251          | 17 (Asc s)                      |
|     | Nuclease-domain containing protein 1          | –/PF00565 & PF00657 | F1KT77      | 8                 | 8                      | 195          | 10 (Asc s)                      |
|     | 26S proteasome subunit 2                      | –/PF01851       | F1KV05      | 5                 | 5                      | 164          | 5 (Asc s)                       |
|     | Aminopeptidase                                | –/PF01433 & PF11838 | F1KUM7      | 6                 | 6                      | 138          | 9 (Asc s)                       |
|     | Glycine dehydrogenase                         | –/PF02347       | F1KTS9      | 4                 | 4                      | 72           | 5 (Asc s)                       |
| 5   | Proponyl-CoA carboxylase α                    | –/PF00289 & PF00364 | F1KUZ6      | 83                | 83                     | 1123         | 30 (Asc s)                      |
|     | Methylmalonyl-CoA mutase                      | –/PF01642       | F1KWB3      | 20                | 20                     | 836          | 35 (Asc s)                      |
|     | Heat shock protein 90                         | AF042/PF00183   | C1KG49      | 20                | 20                     | 495          | 29 (Asc s)                      |
|     | 6-Phosphofructokinase                         | –/PF00365       | F1KSL6      | 8                 | 8                      | 326          | 13 (Asc s)                      |
|     | Aconitate hydratase                           | AF186/PF00330 & PF00694 | F1KYA7      | 7                 | 7                      | 268          | 10 (Asc s)                      |
| 6   | Heat shock protein 70                         | AF002/PF00012   | A8Q5Z6      | 70                | 70                     | 946          | 35 (Bra m)                      |
| No. | Protein name                              | Protein family | Access. no. | MW (kDa) | Peptide match | Mascot score | Sequence coverage (%) |
|-----|-------------------------------------------|----------------|-------------|----------|---------------|--------------|-----------------------|
| 1   | Phosphoenolpyruvate carboxykinase         | --/PF00821     | Q05893      | 21       | 617           | 35 (Asc s)   |                       |
| 2   | 1,4-α-Glucan-branching enzyme (α-amylase) | AF033/PF00128 & PF02806 | F1KTZ0 | 9         | 283           | 15 (Asc s)   |                       |
| 3   | Transketolase-1                           | --/PF00456     | A8WUX5      | 6         | 281           | 8 (Cae b)    |                       |
| 4   | Moesin                                    | --/PF00769 & PF03979 | F1KX42 | 9         | 193           | 20 (Asc s)   |                       |
| 5   | Glycogen synthase                         | --/PF05693     | F1KYL5      | 9         | 147           | 14 (Asc s)   |                       |
| 6   | ATP synthase subunit A                    | AF048/ PF00006 | F1KW99      | 8         | 131           | 13 (Asc s)   |                       |
| 7   | Intermediate filament protein B           | AF008/ PF00038 | P23731      | 2         | 114           | 4 (Asc s)    |                       |
| 8   | Succinate dehydrogenase                   | --/PF00890 & PF02910 | Q8WSR3 | 3         | 64            | 6 (Asc s)    |                       |
| 9   | Endochitinase                             | AF077/ PF00704 | F1LF6       | 1         | 42            | 3 (Asc s)    |                       |
| 10  | Glutamate dehydrogenase                   | --/PF00208 & PF02812 | F1LID2 | 56        | 780           | 23 (Asc s)   |                       |
| 11  | Glucose-6-P-isomerase                     | --/PF00032     | F1KUW6      | 9         | 570           | 17 (Asc s)   |                       |
| 12  | Heat shock protein 60                     | --/PF00118     | F1KV8       | 13        | 287           | 20 (Asc s)   |                       |
| 13  | Phosphoglucomutase-1                      | --/PF02878 & PF00408 | F1LY5  | 8         | 230           | 15 (Asc s)   |                       |
| 14  | Disulphide isomerase                      | AF023/ PF00085 | B2REF9      | 7         | 145           | 17 (Asc s)   |                       |
| 15  | Protein phosphatase PP2A                  | --/PF02985     | F1KVC0      | 4         | 83            | 8 (Asc s)    |                       |
| 16  | Plastin-2                                 | --/PF00307     | F1KY7       | 2         | 63            | 3 (Asc s)    |                       |
| 17  | Myofilin                                  | AF164/ PF00307 | Q24799      | 1         | 59            | 9 (Ech g)    |                       |
| 18  | Translation initiation factor 3           | --/PF10255     | F1KUY0      | 2         | 55            | 2 (Asc s)    |                       |
| 19  | Calrecticulin                             | AF055/ PF00262 | Q0V774      | 2         | 52            | 7 (Hel p)    |                       |
| 20  | Aldehyde dehydrogenase                    | AF040/ PF00171 | F1KZ18      | 1         | 37            | 1 (Asc s)    |                       |
| 21  | Tubulin α                                 | AF025/ PF00091 & PF03953 | F1L649 | 52        | 695           | 43 (Asc s)   |                       |
| 22  | Tubulin β                                 | AF025/ PF00091 & PF03953 | F1L7U3 | 15        | 649           | 43 (Asc s)   |                       |
| 23  | ATP-synthase subunit B                    | AF048/ PF00006 | F1L06      | 7         | 311           | 15 (Asc s)   |                       |
| 24  | Dihydrolipoyl dehydrogenase              | --/PF00070 & PF02852 | F1L686 | 7         | 263           | 22 (Asc s)   |                       |
| 25  | Propionyl-CoA carboxylase β               | --/PF01039     | F1L4Y2      | 6         | 255           | 14 (Asc s)   |                       |
| 26  | UTP-G-1-P-uridylyltransferase             | --/PF01704     | F1KYX7      | 9         | 240           | 21 (Asc s)   |                       |
| 27  | Fumarase                                  | --/PF00206 & PF00415 | E1FR7T | 6         | 228           | 21 (Lot b)   |                       |
| 28  | Cytosolic dipeptidase                     | --/PF01546 & PF07687 | F1L670 | 5         | 184           | 15 (Asc s)   |                       |
| 29  | 6-Phosphogluconate dehydrogenase         | --/PF00393 & PF03446 | F1L0H1 | 6         | 147           | 15 (Asc s)   |                       |
| 30  | 26S protease subunit 4                    | --/PF00004     | F1L7Q7      | 4         | 108           | 9 (Asc s)    |                       |
| 31  | Importin α                                | --/PF00514     | F1L5L6      | 2         | 43            | 5 (Asc s)    |                       |
| No. | Protein name                                             | Protein family AllFam/Pfam | Access. no. | MW (kDa) | Peptide match | Mascot score | Sequence coverage (%) |
|-----|---------------------------------------------------------|----------------------------|-------------|----------|---------------|--------------|-----------------------|
| 9   | Glycine/serine hydroxymethyltransferase                | –/PF00464                  | B7PG87      | 1        | 1             | 42           | 1 (Ixo s)             |
|     | Enolase                                                | AF031/PF00113 & PF03952    | Q8MU59      | 49       | 20            | 18           | 2 (Ixo s)             |
|     | Rab GDP dissociation inhibitor α                       | –/PF00996                  | F1KV11      | 12       | 12            | 596          | 40 (Ani s)            |
|     | Elongation factor 1 α                                  | AF011/PF00009              | Q9U600      | 10       | 10            | 534          | 36 (Ani s)            |
|     | Phosphoglycerate kinase                                | AF145/PF00162              | F1L2P3      | 10       | 10            | 235          | 25 (Asc s)            |
|     | Adenosylhomocysteine                                   | –/PF05221 & PF00670        | E1FVC1      | 4        | 1             | 201          | 11 (Lol l)            |
|     | Hexokinase                                             | –/PF00349 & PF03727        | F1KVA2      | 5        | 5             | 174          | 17 (Asc s)            |
|     | Isocitrate dehydrogenase                               | –/PF00180                  | F1L8D7      | 5        | 4             | 157          | 13 (Asc s)            |
|     | Initiation factor 4A                                   | –/PF00270 & PF00271        | F1KY60      | 8        | 8             | 131          | 18 (Asc s)            |
|     | 4-Hydroxybutyrate-CoA transferase                      | –/PF02550                  | F1KWR3      | 1        | 1             | 36           | 4 (Asc s)             |
|     | CAMP-dependent protein kinase regulatory subunit       | –/PF00027 & PF02197        | F1LA32      | 2        | 2             | 63           | 9 (Asc s)             |
|     | Serpin serine proteinase inhibitor                     | AF018/PF00079              | F4MST7      | 5        | 5             | 45           | 12 (Ani s)            |
|     | Imidazolone propionase                                 | –/PF13147                  | F1LM6       | 2        | 2             | 43           | 4 (Asc s)             |
|     | Nucleosome assembly protein                            | –/PF00956                  | Q9U602      | 1        | 1             | 36           | 8 (Ani s)             |
| 10  | Actin                                                  | –/PF00022                  | Q25010      | 42       | 20            | 521          | 58 (Hel a)            |
|     | Pyruvate dehydrogenase                                 | –/PF00676                  | P26268      | 9        | 9             | 44           | 30 (Asc s)            |
| 11  | Glyceraldehyde-3-P-dehydrogenase                       | AF184/PF00044 & PF02800    | P48812      | 40       | 12            | 424          | 37 (Bru m)            |
|     | Tropomyosin (Ani s 3)                                  | AF054/PF00261              | Q9NAS5      | 11       | 11            | 299          | 37 (Ani s)            |
|     | Fructose-1,6-bisphosphatase (Ani s FBPP)               | –/PF00316                  | E3MY15      | 3        | 3             | 233          | 15 (Cae t)            |
|     | Fructose-bisphosphate aldolase 1                       | –/PF00274                  | A8PJ35      | 2        | 2             | 230          | 15 (Bru m)            |
|     | Haemoglobin                                            | AF009/PF00042              | P26914      | 3        | 3             | 149          | 5 (Pse d)             |
|     | Malate dehydrogenase                                   | AF014/PF00056 & PF02866    | F1L7C0      | 3        | 3             | 102          | 5 (Asc s)             |
|     | Arginase kinase                                        | AF049/PF00217 & PF02807    | E1GB10      | 3        | 3             | 53           | 10 (Lot h)            |
|     | 60S acidic ribosomal protein                           | AF070/PF00428 & PF00466    | A8PFQ5      | 1        | 1             | 42           | 3 (Bru m)             |
|     | Antigenic IgI-domain                                   | –/PF07679                  | Q8MY16      | 7        | 7             | 34           | 20 (Asc s)            |
| 12  | 14-3-3 Protein                                         | –/PF00244                  | F1KWX6      | 34       | 13            | 880          | 42 (Asc s)            |
|     | Proteasome subunit α 1                                 | AF149/PF00227              | F1LM2       | 7        | 7             | 226          | 33 (Asc s)            |
|     | 40S ribosomal protein S3                               | AF185/PF00189 & PF07650    | F1L5X2      | 7        | 7             | 200          | 34 (Asc s)            |
|     | 60S ribosomal protein S5                               | –/PF00861 & PF14204        | F1L6Y6      | 2        | 2             | 111          | 23 (Bru m)            |
|     | Glucosamine-6-phosphate deaminase                      | –/PF01182                  | F1KD94      | 2        | 2             | 92           | 10 (Sack)             |
|     | ADP/ATP translocase                                    | –/PF00153                  | F1LB38      | 5        | 5             | 71           | 15 (Asc s)            |
| No. | Protein name                          | Protein family AllFam/Pfam | Access. no. | MW (kDa)a | Peptide matchb | Mascot score | Sequence coverage (%f) |
|-----|--------------------------------------|---------------------------|-------------|----------|---------------|-------------|------------------------|
| 3   | 3-Oxoacyl-reductase                   | AF028/PF00106             | O17915      | 2        | 64            |             | 9 (Asc s)              |
| 13  | GTP-binding nuclear protein ran-1    | -/PF00071                 | F1KXW6      | 3        | 48            |             | 14 (Cae e)             |
| 14  | Triosephosphate isomerase             | AF032/PF00121             | P91919      | 4        | 166           | 20 (Cal p)   |                        |
| 15  | Thioredoxin peroxidase 2              | AF167/PF0417&PF00578      | Q17172      | 4        | 107           | 29 (Bru m)   |                        |
| 15  | 60S ribosomal protein L11             | -/PF00281 & PF00673       | Q94793      | 4        | 74            |             |                        |
| 15  | 40S ribosomal protein S7              | -/PF01251                 | P33514      | 5        | 62            |             | 32 (Ano g)             |
| 15  | Glutathione-S-transferase             | AF010/PF00043 & PF02798   | P46436      | 3        | 50            |             | 15 (Asc s)             |
| 15  | SXP/RAL-2 protein (Ani s 8)           | AF137/PF02520             | A7MQ66      | 15       | 40            |             | 28 (Ani s)             |
| 16  | Calmodulin                           | AF007/PF00036 & PF01036   | O16305      | 1        | (20)          |             | 11 (Cae e)             |
| 16  | Troponin-like protein (Ani s troponin)| AF007/PF00036 & PF01036   | Q9U3U5      | 1        | (10)          |             | 14 (Ani s)             |
| 16  | Histone 4                            | -/PF00047                 | Q6WV72      | 12       | 14            | 38          | 83 (Myt t)             |

aProtein band number and molecular weight from SDS-PAGE gel (Fig. 1).

bProteins characterised by only one peptide match are uncertain.

cSequence coverages as found to homologous protein species in other ecdysozan species including Anisakis simplex (Ani s), Anopheles gambiæ (Ano g), Ascaris suum (Asc s), Brugia malayi (Bru m), Caenorhabditis briggsae (Cae b), Caenorhabditis elegans (Cae e), Caenorhabditis remanei (Cae r), Culex pipiens (Cal p), Echinococcus granulosus (Ech g), Helicoverpa armigera (Hel a), Heligmosomoides polygyrus (Hel p), Ixodes scapularis (Iso s), Loa loa (Lol l), Mytilus trossulus (Myt t), Pediculus humanus corporis (Ped h), Pseudoterranova decipiens (Pse d), Saccoglossus kowalevski (Sac k), Strongylocentrotus purpuratus (Str p), Toxocara canis (Tox c).
Table 4
Classification of detected *A. simplex* proteins to known allergen families.

| AllFam | Protein family                        | No.\(^a\) | Taxonomy                        | *A. simplex*\(^b\)                                                                 |
|--------|--------------------------------------|------------|---------------------------------|-----------------------------------------------------------------------------------|
| AF002  | Heat shock protein (HSP) 70          | 6          | Fungi, plants, animals          | HSP70                                                                             |
| AF007  | EF hand domain allergens             | 63         | Plants, animals                 | Myosin-4, Ani s troponin, calmodulin                                               |
| AF008  | Intermediate filament protein        | 1          | Animal                          | Intermediate filament protein B                                                   |
| AF009  | Globin                              | 11         | Animals                         | Haemoglobin                                                                      |
| AF010  | Glutathione S-transferase            | 8          | Fungi, plants, animals          | Glutathione S-transferase                                                         |
| AF011  | Eukaryotic elongation factor I       | 1          | Fungus                          | Elongation factor 1α                                                              |
| AF014  | Lactate/malate dehydrogenase        | 3          | Fungi, plants                   | Malate dehydrogenase                                                             |
| AF018  | Serpin serine protease inhibitor    | 4          | Plants, animals                 | Serine protease inhibitor                                                         |
| AF023  | Thioredoxin                         | 11         | Fungi, plants, animals          | Disulphide isomerase                                                             |
| AF025  | Tubulin/FtsZ family                 | 2          | Animals                         | Tubulin α, tubulin β                                                             |
| AF028  | Short-chain dehydrogenase           | 3          | Fungi                           | 3-Oxoacyl-reductase                                                               |
| AF031  | Enolase                             | 12         | Fungi, plants, animals          | Enolase                                                                          |
| AF032  | Triosephosphate isomerase           | 4          | Plants, animals                 | Triosephosphate isomerase                                                        |
| AF033  | Alpha-amylase                       | 10         | Bacteria, fungi, plants, animals | α-Amylase                                                                        |
| AF040  | Aldehyde dehydrogenase              | 3          | Fungi                           | 10-Formyltetrahydro-folate dehydrogenase, aldehyde dehydrogenase                  |
| AF042  | Heat shock protein (HSP) 90         | 2          | Fungi, plants                   | HSP90                                                                            |
| AF048  | ATP synthase                        | 1          | Animal                          | ATP synthase subunit A, ATP synthase subunit B                                   |
| AF049  | ATP-guanido phototransferase        | 11         | Animals                         | Arginine kinase                                                                  |
| AF054  | Troponymosin                        | 47         | Animals                         | Troponymosin                                                                     |
| AF055  | Calrecticulin family                | 1          | Fungus                          | Calrecticulin                                                                    |
| AF070  | 60S acidic ribosomal protein        | 11         | Fungi, plants, animals          | 60S acidic ribosomal protein                                                      |
| AF077  | Glycoside hydrolase family          | 9          | Plants, animals                 | Endochitinase                                                                    |
| AF092  | Lipoprotein                         | 6          | Animals                         | Apolipopophorin                                                                  |
| AF100  | Myosin tail                         | 5          | Animals                         | Paramyosin (Ani s 2)                                                            |
| AF137  | SXP/RAL-2 family                   | 3          | Animals                         | SXP/RAL-2 protein (Ani s 8)                                                       |
| AF139  | Eukaryotic-type carbonic anhydrase  | 1          | Plant                           | Carbonic anhydrase                                                               |
| AF145  | Phosphoglycerate kinase             | 1          | Fungus                          | Phosphoglycerate kinase                                                           |
| AF149  | Proteasome subunit                 | 1          | Plant                           | Proteasome subunit α 1                                                           |
| AF164  | EB1 family                          | 1          | Animal                          | Calponin-like protein, α-actinin, myophilin                                      |
| AF167  | Peroxiredoxin                      | 1          | Plant                           | Thioredoxin peroxidase 2                                                          |
| AF184  | Glyceraldehyde 3-phosphate dehydrogenase | 2    | Fungus, animal                 | Glyceraldehyde 3-phosphate dehydrogenase                                         |
| AF185  | Ribosomal protein S3               | 1          | Fungus                          | 40S ribosomal protein S3                                                          |
| AF186  | Acomitase                          | 1          | Fungus                          | Acomitase                                                                        |

\(^a\) Number of known allergens in this AllFam protein family [28].

\(^b\) Extracted from Table 3.
### Table 5

Potential novel allergens in *A. simplex* and homologues in nematodes, insects, and crustaceans.

| Protein                      | MW\(^a\) (kDa) | AllFam\(^b\) | Homologous allergens\(^c\)                                                                 |
|------------------------------|----------------|--------------|--------------------------------------------------------------------------------------------|
| Apolipopophorin              | 353.6          | AF092        | Cockroach (Blag vitellogenin) Mite (Der p 14, Der f 14, Eur m 14)                             |
| Calponin-like protein        | 256.1          | AF164        | Roundworm (Asc s calponin)                                                                   |
| Carbonic anhydrase           | 233.7          | AF139        | Louise (Ped h carbonic anhydrase)                                                             |
| Myosin-4                     | 218.6          | AF007        | Cockroach (Blag 8) Midge (For t myosin) Shrimp (Lit v 3)                                      |
| α-Amylase                    | 84.4           | AF033        | Mite (Blo t 4, Der p 4, Eur m 4, Tyr p 4) Midge (Cul n 8)                                     |
| Heat shock protein 70        | 70.5           | AF002        | Mite (Der f HSP, Blo t HSP) Cockroach (Blag HSP) Midge (Cul n HSP) Fly (Sim vi 70 kDa)         |
| Myophilin                    | 21.2           | AF164        | tapeworm (Ech g myophilin)                                                                   |
| Disulphide isomerase         | 55.6           | AF023        | Moth (Pio z 2) Shrimp (Lit v TRX)                                                             |
| Tubulin αβ                   | 55.6/51.3      | AF025        | Mite (Lep d alpha tubulin, Tyr p alpha tubulin)                                              |
| Enolase                      | 47.7           | AF031        | Cockroach (Blag enolase)                                                                      |
| Myoophorin                   | 40.0           | AF077        | Mite (Der f 15, Der f 18, Der p 15, Der p 18, Blo t 15)                                       |
| Haemoglobin                  | 39.5           | AF009        | Midge (Chi k 1, Pol n 1, Chi t 1-9)                                                           |
| Fructose-1,6-bisphosphate aldolase 1 | 39.5 |                   | Cockroach (Blag FPA) Midge (For t FPA)                                                        |
| 60S acidic ribosomal protein | 34.9           | AF070        | Midge (Cul n 1)                                                                              |
| Triosephosphate isomerase    | 26.3           | AF032        | Cockroach (Blag TPI) Midge (Per t TPI)                                                        |
| Glutathione-S-transferase    | 23.6           | AF010        | Cockroach (Blag 5, Per a 5) Mite (Aca s 8, Ale o 8 Blo t 8, Der f 8, Der p 8, Gly d 8, Lep d 8, Sui m 8, Tyr p 8, Sar s GST) Nematode (Asc s GST, Bru m GST, Loa lo GST, Onc v GST, Sch j GST, Wuc ba GST) |

\(^a\)Molecular weight according to peptide sequence (UniProt database).

\(^b\)AllFam [28].

\(^c\)Homologous allergens in other ecdysozoan species: *Aedes aegypti* (Aed a), *Aeluroglyphus ovatus* (Ale o), *Archaepotamoeba sihiriensis* (Arc s), *Ascaris suum* (Asc s), *Blatella germanica* (Bla g), *Blomia tropicalis* (Blo t), *Brugia malayi* (Bru m), *Chironomus kieni* (Chi k), *Chionoecetes opillo* (Chi o), *Chironomus thummi thumani* (Chi t), *Cragon crongan* (Cra c), *Culicoides nubeculosus* (Cul n), *Dermatophagoides farinae* (Der f), *Dermatophagoides pteronyssinus* (Der p), *Echinococcus granulosus* (Ech g), *Euroglyphus maynei* (Eur m), *Forcipomyia taiwana* (For t), *Glycyphagus domesticus* (Gly d), *Homarus gammarus* (Hom g), *Lepidoglyphus destructor* (Lep d), *Litopenaeus vannamei* (Lit v), *Loa loa* (Lol lo), *Metapenaeus ensis* (Met e), *Onchocerca volvulus* (Onc v), *Pediculus humanus corporis* (Ped h), *Penaeus monodon* (Pen m), *Periplaneta americana* (Per a), *Phodius interpunctella* (Plo i), *Polypedilum nubiferum* (Pol n), *Sarcopes scabiei* (Sar s), *Schistosoma japonicum* (Sch j), *Scylla serrata* (Sey s), *Simulium vittata* (Sim vi), *Suidsasia medanensis* (Sui m), *Typhochlaena putrescentiae* (Tyr p), *Wuchereria bancrofti* (Wuc ba).