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Characterisation of potential novel allergens in the fish parasite *Anisakis simplex*

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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.

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

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.

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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 Allergome 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*, *Metapenaus 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,

Table 1 – *A. simplex* allergens described in literature (retrieved from the Allergome database (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 3.0101Ani s 3.0102	Q9NAS5G4XTD3	Tropomyosin	33.3 33.2	AF054/PF00261
Ani s 4.0101	P83885	Cystatin	Fragment[a]	AF005/PF00031
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.

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 2 h 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 NH_4HCO_3 (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.

Table 2 – 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		SPT[b]
				Class[a]	IgE (kU/l)	Class[a]	IgE (kU/l)	Class[a]	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.
S8[c]	41	M	434	3	7.2	2	2.3	≥1	≥0.35	pos.
S9[c]	39	M	87	3	8.0	2	1.3	≥1	≥0.35	pos.
S10[c]	49	F	441	3	12.3	1	0.4	1	0.4	pos.
S11[c]	65	M	141	3	10.2	0	<0.35	n.m.	n.m.	pos.
S12[c]	68	M	75	3	14.2	0	<0.35	n.m.	n.m.	pos.
S13[c]	60	M	166	4	18.1	0	<0.35	n.m.	n.m.	pos.

[a] 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.
[b] Skin prick testing: –: no reaction; +: <20% of positive control; ++: <50%; +++: ≤80%; pos.: positive; n.m.: not measured.
[c] Patients with gastro-allergic anisakiasis.

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 300 nl/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 NCBInr 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 (4 μL) 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 4 μL/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 again *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

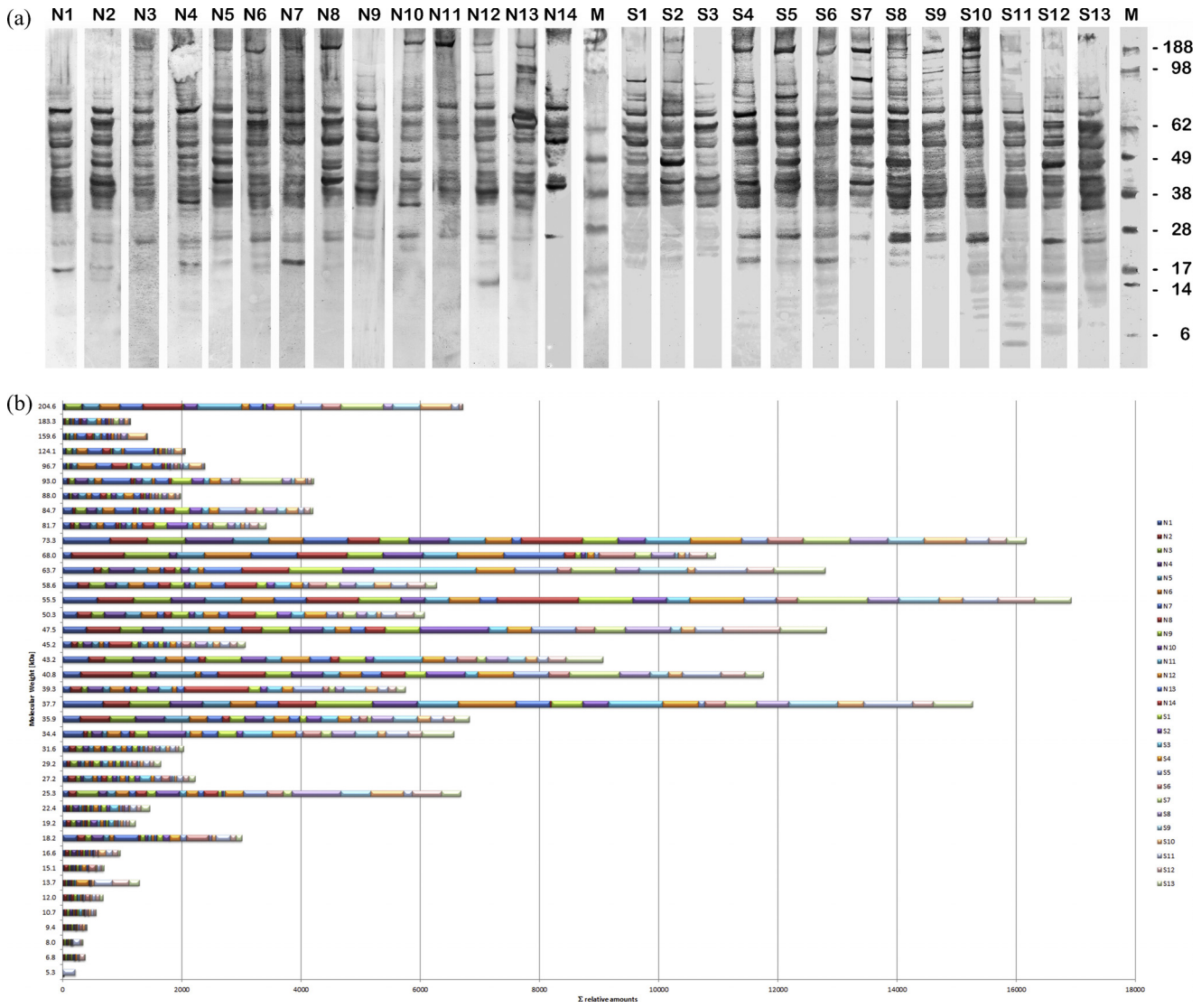

Fig. 1 – (a) IgE-immunoblot with total *A. simplex* extract using sera from14 Norwegian (N1–N14) and 13 Spanish (S1–S13) patients with sensitivity to *A. simplex* (s. Table 2). Relevant lanes were extracted from the scanned individual patient immunoblots and presented as a composite gel. M: SeeBluePlus2 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.

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 multiband 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

Table 3 – *Anisakis simplex* proteins identified by proteomic analysis using ESI-Iontrap or ESI-Orbitrap mass spectrometry and database search (UniProt).

No.[a]	Protein name	Protein family AllFam/Pfam	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		6	146	3 (*Asc s*)
	Apolipophorin	AF092/PF01347 & PF09172	F1KPM2		3	45	1 (*Asc s*)
	Carbonic anhydrase	AF139/PF00194	E0VS50		1	44	<1 (*Ped h*)
2	RAS GTPase-activating protein	–/PF03836	F1KR99	170	3	89	2 (*Asc s*)
	Clathrin heavy chain	–/PF00637	F1KQ49		2	36	1 (*Asc s*)
	ATP-dependent RNA-helicase	–/PF00270 & PF04408	Q7QCW2		23	32	20 (*Ano g*)
	Coiled-coiled protein	–/PF03915	Q7PQ25		13	32	17 (*Ano g*)
3	Pyruvate carboxylase 1	–/PF02786 & PF02436	F1KRV7	100	10	84	8 (*Asc s*)
	Kinesin light chain	–/PF09311 & PF00515	Q05090		18	33	25 (*Str p*)
	Calponin-like protein	AF164/PF00307 & PF00402	F1KPY3		56	34	26 (*Asc s*)
4	Elongation factor 2	–/PF00009 & PF00679	F1KWZ4	91	25	772	33(*Asc s*)
	α-Actinin	AF164/PF00307 & PF00435	F1KR95		21	486	25 (*Asc s*)
	Glycogen phosphorylase	–/PF00343	F1KSK3		17	413	20 (*Asc s*)
	Tetrahydrofolate synthase	–/PF01268	F1KRW4		8	342	11 (*Asc s*)
	Transitional endoplasmic reticulum ATPase 1	–/PF02359 & PF2933	F1LCZ2		8	376	26 (*Asc s*)
	10-Formyltetrahydrofolate dehydrogenase	AF040/PF00551 & PF00171	F1KT06		14	279	13 (*Asc s*)
	Paramyosin (Ani s 2)	AF100/PF01576	Q9NJA9		12	251	17 (*Ani s*)
	Nuclease-domain containing protein 1	–/PF00565 & PF00567	F1KT77		8	195	10 (*Asc s*)
	26S proteasome subunit 2	–/PF01851	F1KV05		5	164	5 (*Asc s*)
	Aminopeptidase	–/PF01433 & PF11838	F1KUM7		6	138	9 (*Asc s*)
	Glycine dehydrogenase	–/PF02347	F1KTS9		4	72	5 (*Asc s*)
5	Propionyl-CoA carboxylase α	–/PF00289 & PF00364	F1KUZ6	83	20	1123	30 (*Asc s*)
	Methylmalonyl-CoA mutase	–/PF01642	F1KWB3		20	836	35 (*Asc s*)
	Heat shock protein 90	AF042/PF00183	C1KG49		20	495	29 (*Asc s*)
	6-Phosphofructokinase	–/PF00365	F1KSL6		8	326	13 (*Asc s*)
	Aconitate hydratase	AF186/PF00330 & PF00694	F1KYA7		7	268	10 (*Asc s*)
6	Heat shock protein 70	AF002/PF00012	A8Q5Z6	70	21	946	35 (*Bru m*)
	Phosphoenolpyruvate carboxykinase	–/PF00821	Q05893		21	617	35 (*Asc s*)
	1,4-α-Glucan-branching enzyme (α-amylase)	AF033/PF00128 & PF02806	F1KTZ0		9	283	15 (*Asc s*)
	Transketolase-1	–/PF00456	A8WUX5		6	281	8 (*Cae b*)
	Moesin	–/PF00769 &PF03979	F1KX42		9	193	20 (*Asc s*)
	Glycogen synthase	–/PF05693	F1KYL5		9	147	14 (*Asc s*)
	ATP synthase subunit A	AF048/PF00006	F1KW99		8	131	13 (*Asc s*)
	Intermediate filament protein B	AF008/PF00038	P23731		2	114	4 (*Asc s*)
	Succinate dehydrogenase	–/PF00890 & PF02910	Q8WSR3		3	64	6 (*Asc s*)
	Endochitinase	AF077/PF00704	F1L1F6		1	42	3 (*Asc s*)
7	Glutamate dehydrogenase	–/PF00208 & PF02812	F1L1D2	56	11	780	23 (*Asc s*)
	Glucose-6-P-isomerase	–/PF00342	F1KUW6		9	570	17 (*Asc s*)
	Heat shock protein 60	–/PF00118	F1KVK8		13	287	20 (*Asc s*)
	Phosphoglucomutase-1	–/PF02878 & PF00408	F1L0Y5		8	230	15 (*Asc s*)
	Disulphide isomerase	AF023/PF00085	B2REF9		7	145	17 (*Asc s*)
	Protein phosphatase PP2A	–/PF02985	F1KVC0		4	83	8 (*Asc s*)
	Plastin-2	–/PF00307	F1KY27		2	63	3 (*Asc s*)
	Myophilin	AF164/PF00307	Q24799		1	59	9 (*Ech g*)
	Translation initiation factor 3	–/PF10255	F1KUY0		2	55	2 (*Asc s*)
	Calrecticulin	AF055/PF00262	Q0VJ74		2	52	7 (*Hel p*)
	Aldehyde dehydrogenase	AF040/PF00171	F1KZ18		1	37	1 (*Asc s*)

Table 3 – (*Continued*)

No.[a]	Protein name	Protein family AllFam/Pfam	Access. no.	MW (kDa)[a]	Peptide match[b]	Mascot score	Sequence coverage (%)[c]
8	Tubulin α	AF025/PF00091 & PF03953	F1L649	52	15	695	43 (*Asc s*)
	Tubulin β	AF025/PF00091 & PF03953	F1L7U3		15	649	43 (*Asc s*)
	ATP-synthase subunit B	AF048/PF00006	F1L006		7	311	15 (*Asc s*)
	Dihydrolipoyl dehydrogenase	–/PF00070 & PF02852	F1L686		7	263	22 (*Asc s*)
	Propionyl-CoA carboxylase β	–/PF01039	F1L4Y2		6	255	14 (*Asc s*)
	UTP-G-1-P-uridylyltransferase	–/PF01704	F1KYX7		9	240	21 (*Asc s*)
	Fumarase	–/PF00206 & PF10415	E1FRT7		6	228	21 (*Lol l*)
	Cytosolic dipeptidase	–/PF01546 & PF07687	F1L670		5	184	15 (*Asc s*)
	6-Phosphogluconate dehydrogenase	–/PF00393 &PF03446	F1L0I1		6	147	15 (*Asc s*)
	26S protease subunit 4	–/PF00004	F1L7Q7		4	108	9 (*Asc s*)
	Importin α	–/PF00514	F1L5L6		2	43	5 (*Asc s*)
	Glycine/serine hydroxymethyltransferase	–/PF00464	B7PG87		1	42	1 (*Ixo s*)
9	Enolase	AF031/PF00113 & PF03952	Q8MU59	49	30	4392	80 (*Ani s*)
	Rab GDP dissociation inhibitor α	–/PF00996	F1KV11		12	596	40 (*Ani s*)
	Elongation factor 1 α	AF011/PF00009	Q9U600		10	534	36 (*Ani s*)
	Phosphoglycerate kinase	AF145/PF00162	F1L2P3		10	235	25 (*Asc s*)
	Adenosylhomocysteinase	–/PF05221 & PF00670	E1FVC1		4	201	11 (*Lol l*)
	Hexokinase	–/PF00349 & PF03727	F1KVA2		5	174	17 (*Asc s*)
	Isocitrate dehydrogenase	–/PF00180	F1L8D7		5	157	13 (*Asc s*)
	Initiation factor 4A	–/PF00270 & PF00271	F1KY60		8	131	18 (*Asc s*)
	4-Hydroxybutyrate-CoA transferase	–/PF02550	F1KWR3		1	83	4 (*Asc s*)
	CAMP-dependent protein kinase regulatory subunit	–/PF00027 & PF02197	F1LA32		2	63	9 (*Asc s*)
	Serpin serine proteinase inhibitor	AF018/PF00079	F4MST7		5	45	12 (*Ani s*)
	Imidazolone propionase	–/PF13147	F1L4M6		2	43	4 (*Asc s*)
	Nucleosome assembly protein	–/PF000956	Q9U602		1	36	8 (*Ani s*)
10	Actin	–/PF00022	Q25010	42	20	521	58 (*Hel a*)
	Pyruvate dehydrogenase	–/PF00676	P26268		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	299	37 (*Ani s*)
	Fructose-1,6-bisphosphatase (Ani s FBPP)	–/PF00316	E3MYI5		3	233	15 (*Cae r*)
	Fructose-bisphosphate aldolase 1	–/PF00274	A8P3E5		3	230	15 (*Bru m*)
	Haemoglobin	AF009/PF00042	P26914		3	149	5 (*Pse d*)
	Malate dehydrogenase	AF014/PF00056 & PF02866	F1L7C0		3	102	5 (*Asc s*)
	Arginine kinase	AF049/PF00217 & PF02807	E1GBI0		3	53	10 (*Lol l*)
	60S acidic ribosomal protein	AF070/PF00428 & PF00466	A8PQF5		1	42	3 (*Bru m*)
	Antigenic IgI-domain	–/PF07679	Q8MY16		7	34	20 (*Asc s*)
12	14-3-3 Protein	–/PF00244	F1KXW6	34	13	880	42 (*Asc s*)
	Proteasome subunit α 1	AF149/PF00227	F1L3M2		7	226	33 (*Asc s*)
	40S ribosomal protein S3	AF185/PF00189 & PF07650	F1L5X2		7	200	34 (*Asc s*)
	60S ribosomal protein L5	–/PF00861 & PF14204	F1L6Y6		2	111	23 (*Bru m*)
	Glucosamine-6-phosphate deaminase	–/PF01182	F1KZ94		2	92	10 (*Sac k*)
	ADP/ATP translocase	–/PF00153	F1LB38		5	71	15 (*Asc s*)
	3-Oxoacyl-reductase	AF028/PF00106	O17915		2	64	9 (*Asc s*)
	GTP-binding nuclear protein ran-1	–/PF00071	F1KXW6		3	48	14 (*Cae e*)

Table 3 – (*Continued*)

No.[a]	Protein name	Protein family AllFam/Pfam	Access. no.	MW (kDa)[a]	Peptide match[b]	Mascot score	Sequence coverage (%)[c]
13	Triosephosphate isomerase	AF032/PF00121	P91919	27	4	166	20 (*Cul p*)
14	Thioredoxin peroxidase 2	AF167/PF10417&PF00578	Q17172	22	4	107	29 (*Bru m*)
	60S ribosomal protein L11	–/PF00281 & PF00673	Q94793		4	74	30 (*Tox c*)
	40S ribosomal protein S7	–/PF01251	P33514		8	62	32 (*Ano g*)
	Glutathione-S-transferase	AF010/PF00043 & PF02798	P46436		5	50	15 (*Asc s*)
15	SXP/RAL-2 protein (Ani s 8)	AF137/PF02520	A7M6Q6	15	4	40	28 (*Ani s*)
	Calmodulin	AF007/PF00036 & PF01036	O16305		1	(20)	11 (*Cae e*)
	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*)

[a] Protein band number and molecular weight from SDS-PAGE gel (Fig. 1).
[b] Proteins characterised by only one peptide match are uncertain.
[c] Sequence coverages as found to homologous protein species in other ecdysozoan species including *Anisakis simplex* (Ani s), *Anopheles gambiae* (Ano g), *Ascaris suum* (Asc s), *Brugia malayi* (Bru m), *Caenorhabditis briggsae* (Cae b), *Caenorhabditis elegans* (Cae e), *Caenorhabditis remanei* (Cae r), *Culex pipiens* (Cul p), *Echinococcus granulosus* (Ech g), *Helicoverpa armigera* (Hel a), *Heligmosomoides polygyrus* (Hel p), *Ixodes scapularis* (Ixo 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).

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) [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 apolipophorin, 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

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 1	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	Thioreduxin	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, aldehydedehydrogenase
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 phosphotransferase	11	Animals	Arginine kinase
AF054	Tropomyosin	47	Animals	Tropomyosin
AF055	Calreticulin family	1	Fungus	Calrecticulin
AF070	60S acidic ribosomal protein	11	Fungi, plants, animals	60S acidic ribosomal protein
AF077	Glycoside hydrolase family 18	9	Plants, animals	Endochitinase
AF092	Lipoprotein	6	Animals	Apolipophorin
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	Aconitase	1	Fungus	Aconitase

[a] Number of known allergens in this AllFam protein family [28].
[b] Extracted from Table 3.

antigen in dog tapeworm [35]. Alpha-amylase has homologues in yellowfever 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

Table 5 – Potential novel allergens in *A. simplex* and homologues in nematodes, insects, and crustaceans.

Protein	MW[a] (kDa)	AllFam[b]	Homologous allergens[c]
Apolipophorin	353.6	AF092	Cockroach (Bla g 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	Louse (Ped h carbonic anhydrase)
Myosin-4	218.6	AF007	Cockroach (Bla g 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) Mosquito (Aed a 4) Midge (Cul n 8)
Heat shock protein 70	70.5	AF002	Mite (Der f HSP, Blo t HSP) Cockroach (Bla g HSP) Midge (Cul n HSP) Fly (Sim vi 70 kDa)
Myophilin	21.2	AF164	tapeworm (Ech g myophilin)
Disulphide isomerase	55.6	AF023	Moth (Plo i 2) Shrimp (Lit v TRX)
Tubulin α/β	55.6/51.3	AF025	Mite (Lep d alpha tubulin, Tyr p alpha tubulin)
Enolase	47.7	AF031	Cockroach (Bla g enolase)
Arginine kinase	41.8	AF049	Cockroach (Bla g 9, Per a 9) Mite (Der p 20) Moth (Plo i 1) Shrimp (Lit v 2, Cra c 2, Met e 2, Pen m 2) Crab (Chi o 2, Scy s 2) Lobster (Hom g 2)
Endochitinase	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 (Bla g FPA) Midge (For t FPA)
60S acidic ribosomal protein	34.9	AF070	Midge (Cul n 1)
Triosephosphate isomerase	26.3	AF032	Cockroach (Bla g TPI) Midge (For t TPI) Crayfish (Arc s 8) Shrimp (Cra c 8)
Glutathione-S-transferase	23.6	AF010	Cockroach (Bla g 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 aeqypti* (Aed a), *Aleuroglyphus ovatus* (Ale o), *Archaeopotamobius sibiriensis* (Arc s), *Ascaris suum* (Asc s), *Blatella germanica* (Bla g), *Blomia tropicalis* (Blo t), *Brugia malayi* (Bru m), *Chironomus kiiensis* (Chi k), *Chionoecetes opillo* (Chi o), *Chironomus thummi thummi* (Chi t), *Crangon crangon* (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 mondon* (Pen m), *Periplaneta americana* (Per a), *Plodia interpunctella* (Plo i), Polypedilum *nubiferum* (Pol n), *Sarcoptes scabiei* (Sar s), *Schistosoma japonicum* (Sch j), *Scylla serrata* (Scy s), *Simulia vittata* (Sim vi), *Suidasia medanensis* (Sui m), *Tyrophagus putrescentiae* (Tyr p), *Wuchereria bancrofti* (Wuc ba).

is a known allergen in midges. 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

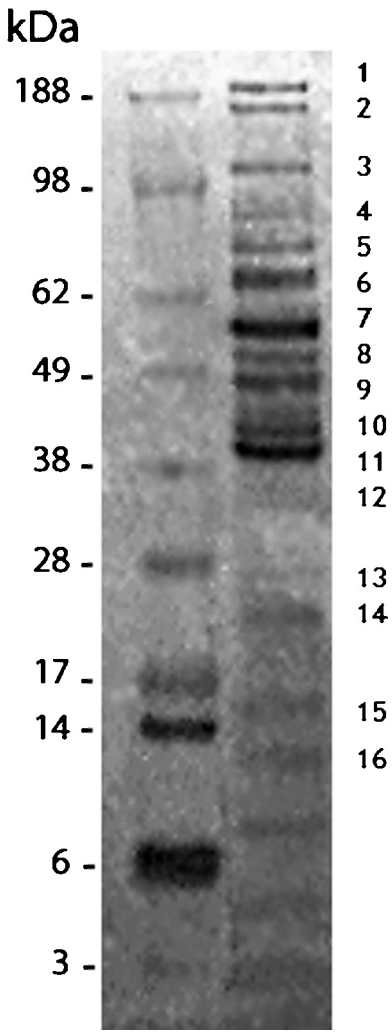

Fig. 2 – Coomassie-stained SDS-PAGE of total *A. simplex* protein extract. Gel bands (No. 1–16) were excised, digested with trypsin, and subjected to mass spectrometric analysis. SeeBlue Plus2 molecular weight marker; relative protein sizes (kDa) are given on the left side of the gel.

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 (Ani s 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 (Ani s 3) (Table 1). Triosephosphate isomerase or gluthatione-S-transferase was candidates to have caused the relatively strong signal at 25 kDa. Troponin C (Ani s troponin) and the SXP/RAL-2 proteins (Ani s 5, Ani s 8, Ani s 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 haemoglobins are 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 peregreffi* (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 isomerise, 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.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.06.006.

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