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PROTEOMIC PROFILING REVEALS NEW INSIGHTS INTO THE ALLERGOMES OF *ANISAKIS SIMPLEX*, *PSEUDOTERRANOVA DECIPIENS,* AND *CONTRACAECUM OSCULATUM*

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KEY WORDS

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Putative Allergen

ABSTRACT

Anisakis simplex, *Pseudoterranova decipiens,* and *Contracaecum osculatum* third-stage larvae (L3) are fish-borne nematodes that can cause human anisakidosis. Although *A. simplex* is a known source of allergens, knowledge about the allergic potential of *P. decipiens* and *C. osculatum* is limited. Therefore, we performed comparative proteomic profiling of *A. simplex*, *P. decipiens,* and *C. osculatum* L3 larvae using liquid chromatography–tandem mass spectrometry. In total, 645, 397, and 261 proteins were detected in *A. simplex*, *P. decipiens*, and *C. osculatum* L3 larvae, respectively. Western blot analysis confirmed the cross-reactivity of anti-*A. simplex* immunoglobulin (Ig)G antibodies with protein extracts from *P. decipiens* and *C. osculatum* L3 larvae. The identified proteins of the Anisakidae proteomes were characterized by label-free quantification and functional analysis, and proteins involved in many essential biological mechanisms, such as parasite survival, were identified. In the proteome of *A. simplex* 14, the following allergens were identified: Ani s 1, Ani s 2 (2 isomers), Ani s 3 (2 isomers), Ani s 4, Ani s 8, Ani s 9, Ani s 10, Ani s 11-like, Ani s 13, Ani s fructose 1,6-bisphosphatase, Ani s phosphatidylethanolamine-binding protein (PEPB), and Thu a 3.0101. The following 8 allergens were detected in *P. decipiens*: Ani s 2, Ani s 3 (2 isomers), Ani s 5, Ani s 8, Ani s 9, Ani s PEPB, and Ani s troponin. In *C. osculatum* 4, the following allergens were identified: Ani s 2, Ani s 5, Ani s 13, and Asc l 3. Furthermore, 28 probable allergens were predicted in *A. simplex* and *P. decipiens*, whereas in *C. osculatum*, 25 possible allergens were identified. Among the putative allergens, heat shock proteins were most frequently detected, followed by paramyosin, peptidyl-prolyl cis-trans isomerase, enolase, and tropomyosin. We provide a new proteomic data set that could be beneficial for the discovery of biomarkers or drug target candidates. Furthermore, our findings showed that in addition to *A. simplex*, *P. decipiens* and *C. osculatum* should also be considered as potential sources of allergens that could lead to IgE-mediated hypersensitivity.

Anisakidae nematodes are one of the most important food-borne parasites. Live third-stage larvae (L3) of anisakids consumed with fish or seafood dishes can cause anisakidosis. Each year, approximately 2,000 new cases of anisakidosis are found in Japan; in South Korea, 200; in Europe, 500; and in the United States, 70 (Arizono et al., 2012; Lim et al., 2015). The main etiologic agents of the disease are 2 species: *Anisakis simplex* and *Anisakis pegreffii*. *Pseudoterranova* spp. and *Contracaecum* spp. human infections have been detected less frequently. In the course of anisakidosis, live L3 larvae cause organ damage at the location of the larvae, predominantly in the gastrointestinal tract. The L3 larvae can penetrate the wall of the alimentary tract and migrate to the internal organs. In addition, allergens of *A. simplex* and *A. pegreffii* L3 larvae may cause allergic reactions in the form of urticaria, angioedema, and even life-threatening anaphylactic shock (Choi et al., 2009). Furthermore, employees of the fish processing industry have had episodes of allergic asthma (Scala et al., 2001) and conjunctivitis (Añíbarro and Seoane, 1998) induced by *Anisakis* allergens. Nephrotic syndrome (Meseguer et al., 2007) and arthritis (Cuende et al., 1998) associated with *Anisakis* allergy have also been reported. Because of the thermostability of many *Anisakis* allergens, immunoglobulin (Ig)E-mediated hypersensitivity reactions can also occur after the ingestion of highly processed fish products containing *Anisakis* allergens (Bao et al., 2015).

Many studies on various aspects of Anisakidae nematodes have been performed, and among them, investigations of the allergic properties of *Anisakis* are particularly important. Nonetheless, knowledge about the allergic potential of *Pseudoterranova* and *Contracaecum* is very limited. Because of the phylogenetic closeness of anisakids, we suppose that *Pseudoterranova decipiens* and *Contracaecum osculatum* could contain allergens similar to *A. simplex*. Moreover, for the majority of human *Anisakis* allergy cases, the pathogenic agent was not confirmed by genetic or morphological tests. Consequently, although other nematodes from the Anisakidae family may cause allergic reactions, these cases are attributed to *Anisakis*.

A powerful tool allowing large-scale identification and expression analysis of proteins as well as many allergens is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Additionally, proteomic studies enable the identification of protein modifications not present in the deoxyribonucleic acid sequence and allow accurate determination of protein abundance (Zhang et al., 2014), in contrast to genomic or transcriptomic analyses, which are also used for high-throughput allergen detection (Gygi et al., 1999; Cho, 2007). To date, only 2 proteomic investigations have been conducted to identify the allergens of *Anisakis* nematodes (Arcos et al., 2014; Fæste et al., 2014). Moreover, new allergens and potential allergens of *Anisakis* spp. are still being discovered, and many allergens of *Anisakis* spp. have yet to be discovered.

Therefore, we aimed to provide more insight into Anisakidae proteomes by comparative investigation of *A. simplex*, *P. decipiens,* and *C. osculatum* L3 larvae using LC-MS/MS technology. Furthermore, we carried out a functional analysis of the identified proteins to further understand anisakid biology and pathogenicity. Above all, special emphasis was placed on the identification of allergens of Anisakidae parasites, and here we reported novel data concerning the allergens and predicted allergens of *A. simplex*, *P. decipiens,* and *C. osculatum*.

MATERIALS AND METHODS

Anisakidae L3 larvae collection and identification

Anisakis simplex, *P. decipiens*, and *C. osculatum* L3 larvae were collected from marine fish and identified as previously described (Kochanowski et al., 2019). In brief, anisakid larvae were manually purified from fish tissue and extensively rinsed with sterile 0.01 M phosphate-buffered saline solution (PBS), pH 7.4. Anisakidae parasites were then identified by using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) (Zhu et al., 1998). The results of the PCR-RFLP assay were validated by Sanger sequencing of PCR products before the enzymatic digestion step. Sequences were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession nos. MF967307.1, MF988284.1 and MF980981.1.

Protein extraction from anisakid L3 larvae

Extracts from whole nematodes were obtained as described previously with minor modifications (Kochanowski et al., 2019). Briefly, approximately 0.5 g of *A. simplex*, *C. osculatum*, and *P. decipiens* L3 larvae were initially homogenized with 3 ml of sterile PBS by grinding with a mortar and pestle followed by further disintegration using a high-speed homogenizer. Afterward, to increase the protein extraction yield, parasite homogenates were sonicated in ice (10-μm amplitude for 30 sec) and incubated for 1 hr at 4 C. Finally, the lysates were clarified by centrifugation (20,000 *g* for 30 min at 4 C). Protein concentration was determined by measuring the absorbance at 280 nm using an ultraviolet–visible spectrophotometer (Implen, München, Germany) and adjusted to 4 mg/ml. Three independent biological replicates of each parasite extract were performed. Protein extracts were kept at –80 C for further analysis.

Generation of rabbit anti-*A. simplex* antisera

Rabbits were immunized by intramuscular injection of 2.0 mg of *A. simplex* extract mixed with Freund's complete adjuvant (Sigma, St. Louis, Missouri) according to our previously described protocol (Kochanowski et al., 2019). Preimmune serum was collected before immunization and used as a negative control.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB) analysis

Whole parasite extracts were analyzed by 4–20% gradient SDS-PAGE (BioRad, Hercules, California) under reducing conditions (Laemmli, 1970), and gels were stained with colloidal Coomassie G-250 (PageBlue, Thermo Fisher Scientific, Rockford, Illinois). For WB analysis, parasite extracts from the 3 species were first separated by 4–20% gradient SDS-PAGE in duplicate and then transferred onto a 0.45-μm nitrocellulose membrane (BioRad, München, Germany). After the blocking step, the membrane was cut; 3 strips loaded with different Anisakidae species extracts were incubated with rabbit anti-*A. simplex* hyperimmune serum diluted 1:400; another 3 strips were incubated with preimmune rabbit serum. The anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma) was used as a secondary antibody. SDS-PAGE and WB profiles were analyzed with GelAnalyzer software (ver. 2010a; http://www.gelanalyzer.com/).

Sample processing and LC-MS/MS analysis

Protein solutions were subjected to the standard procedure of tryptic digestion, during which proteins were reduced with 0.5 M (5 mM final concentration [f.c.]) tris (2-carboxyethyl) phosphine for 1 hr at 60 C; the cysteine residues were subsequently alkylated with 200 mM (10 mM f.c.) methyl methanethiosulfonate for 10 min at room temperature and finally cleaved overnight with 10 μl of 0.1 μg/μl trypsin at 37 C. A total of 20 μl of the resulting peptide mixtures was loaded onto a reversed-phase RP-18 precolumn (Waters, Milford, Massachusetts) using 0.1% (v/v) formic acid (FA) in water as a mobile phase and then transferred to a nano-high-performance liquid chromatography (nano-HPLC) RP-18 column (internal diameter 75 μm [Waters]) using a linear acetonitrile gradient of 0–35% (v/v) over 160 min in the presence of 0.1% (v/v) FA at a flow rate of 250 nl/min. The nano-HPLC column outlet was coupled directly to the ion source of a Q Exactive mass spectrometer (Thermo Electron Corp., San Jose, California) working in the regime of data-dependent MS to MS/MS switch with higher-energy collisional dissociation (HCD)-type peptide fragmentation. A blank run ensuring the absence of cross-contamination from previous samples preceded each analysis.

Data analysis

Database search and protein identification: Raw LC-MS/MS data were processed and queried using MaxQuant software with the integrated Andromeda search engine ver. 1.6.1.0 (http://www.coxdocs.org) (Tyanova et al., 2016) against the *A. simplex* reference proteome (20,786 sequences; proteome ID: UP000036680) obtained from Universal Protein Resource (UniProt) (http://www.uniprot.org/) and searched against the reverse sequences from the same database as generated by MaxQuant along with 248 sequences of common contaminants. The search parameters were set as follows: trypsin was specified as the cleavage enzyme, and the maximum number of missed cleavages was set to 1. The allowed precursor mass deviation for the initial search used for mass recalibration was 20 parts per million (ppm). In the main Andromeda search, the precursor and isotope match tolerances were set to 10 ppm and 5 ppm, respectively. Fixed modification searches including oxidation of methionine and beta-methylthiolation of cysteine were performed as variable modifications. The false discovery rate (FDR) for proteins, peptides, and modification sites was fixed at 0.01. The minimum peptide length was set to 7, and requantification was enabled.

Additionally, mass spectrometric data were preprocessed with Mascot Distiller software ver. 2.6 (Matrix Science, London, U.K.; http://www.matrixscience.com/distiller.html) and analyzed with the Mascot search engine server ver. 2.5 (Matrix Science; http://www.matrixscience.com/server.html) against the nonredundant National Center for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov) with a taxonomy filter for Metazoa (13,299,211 sequences in June 2017). To reduce mass errors, the peptide and fragment mass tolerance settings were established separately for individual LC-MS/MS runs after a measured mass recalibration, resulting in values of 5 ppm for the parent and 0.01 Da for fragment ions in HCD MS/MS mode. Peptide sequences were searched using trypsin specificity allowing 1 missed cleavage; the ion type was set as monoisotopic, and protein mass was set as unrestricted. Beta-methylthiolation of cysteine was used as a fixed modification, whereas oxidation of methionine was set as a variable modification. Proteins with Mascot scores $\geq$50 were considered significant ($P < 0.05$).

Scaffold software ver. 4.8.7 (Proteome Software Inc., Portland, Oregon; http://www.proteomesoftware.com/) (Searle, 2010) was used to merge search results from search engines, validate the peptide and protein identifications, and estimate the relative protein abundance. Peptide identifications were accepted at $\geq$95.0% probability with the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003) were accepted if they were established with $\geq$99.0% probability and if at least 1 peptide was identified in all 3 biological replicates. Proteins that contained similar peptides and that could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. Label-free quantification of the protein amounts was conducted using total spectral count (Liu et al., 2004) and visualized in Morpheus (https://software.broadinstitute.org/morpheus/).

In silico proteome maps: In silico proteome maps (virtual 2-dimensional [2D] protein gel electrophoresis) of *A. simplex*, *P. decipiens*, and *C. osculatum* were generated using JVirGel software ver. 2.0 (http://www.jvirgel.de/index.html) (Hiller et al., 2006). The same software was applied to calculate the theoretical isoelectric point (pI) and molecular weight (Mw) of detected proteins.

Protein annotation: Homology searches, InterPro motifs searches, Gene Ontology (GO) annotation into 3 categories (molecular function, biological process, and cellular component), Clusters of Orthologous Group annotations, annex augmentation, enzyme search, and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed using Blast2GO Pro software ver. 5.1.13 (https://www.blast2go.com/) (Conesa et al., 2005). Annotations obtained from the different databases were merged via Blast2GO. All of these analyses were run with default settings and thresholds.

Identification of putative allergens: Identified proteins were evaluated for putative allergenicity by web servers using different bioinformatics approaches and databases: (1) searching against The Food Allergy Research and Resource Program AllergenOnline.org database ver. 18B (http://www.allergenonline.com/) (2,089 sequences, March 2018) using full-length FASTA alignment (e-value cut-off: 1e-05; 70% identity match); (2) scanning with the AllerTOP web server ver. 2.0 (http://www.ddg-pharmfac.net/AllerTOP/method.html) (Dimitrov et al., 2013); (3) searching in the PREAL web server (http://www.gmobl.sjtu.edu.cn/PREAL/index.php) (Wang et al., 2013) at a probability threshold of 0.7. Only predicted allergens identified with at least 2 different servers were accepted.

RESULTS

SDS-PAGE and WB analysis of extracts of anisakid larvae

The parasite extracts were separated by SDS-PAGE, and the displayed protein profiles are shown in Figure 1a. SDS-PAGE band patterns of *A. simplex* and *P. decipiens* extracts were similar and characterized by a large number of bands at a wide range of molecular weights (16–246 kDa), whereas the profile of the *C. osculatum* extract was slightly different with a smaller number of major bands.

In WB, the anti-*A. simplex* rabbit IgG antibodies reacted with extracts from all 3 anisakids (Fig. 1b). All Anisakidae extracts had a quite similar reaction profile with a multiband pattern; however, the WB profiles of the *C. osculatum* and *P. decipiens* extracts were characterized by a reduced number or intensity of bands compared with that of the *A. simplex* extract.

Identification of proteins

In *A. simplex*, 645 proteins corresponding to 2,417 total unique peptides were detected. A total of 397 proteins of *P. decipiens* based on 1,111 unique peptides and 261 proteins of *C. osculatum* based on 621 unique peptides was detected. Furthermore, 423, 165, and 152 proteins were unique to the *A. simplex*, *P. decipiens*, and *C. osculatum* proteomes, respectively (see Fig. 2a). Most of the predicted Anisakidae proteins were phylogenetically related to *A. simplex*, *Toxocara canis*, and *Ascaris suum*. Identification was conducted with high confidence since the protein and peptide FDR in all cases was $\leq$0.2%.

Virtual 2D gels showing the identified protein distributions according to theoretical Mw and pI values are presented in Figure 2d. Proteins from all 3 nematodes had an approximate Mw in the range of 2–800 kDa and pI in the range of 3–15. Nevertheless, the majority of proteins had a Mw in a narrower range of 15–100 kDa and pI in the range of 4–8.

Among all detected proteins, 51 were identified in 3 nematodes, and they are listed in Table I. Sequences of heat shock proteins (HSPs), spindle- and centrosome-associated proteins, and fructose bisphosphate aldolase were the most frequently detected shared proteins.

Relative quantification

Spectral counting analyses of MS/MS data were used to calculate the relative abundances of proteins in proteomes. This

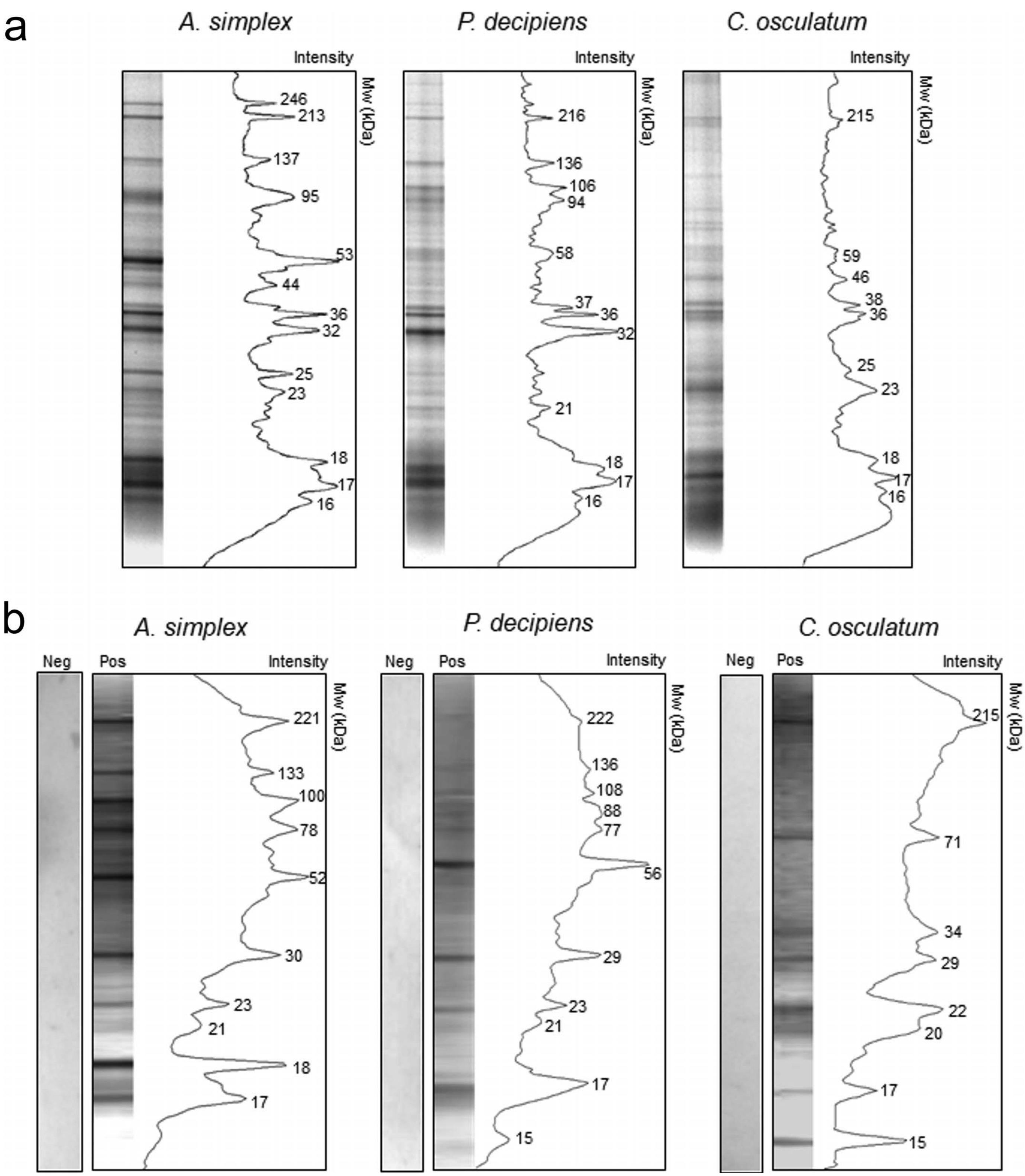

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of extracts prepared from *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* L3 larvae (**a**). Western blot analysis of Anisakidae extracts immunoreacted with rabbit anti-*A. simplex* immunoglobulin G antibodies; densitometric analysis and molecular weight (Mw) quantification (kDa) were performed by GelAnalyzer software (**b**).

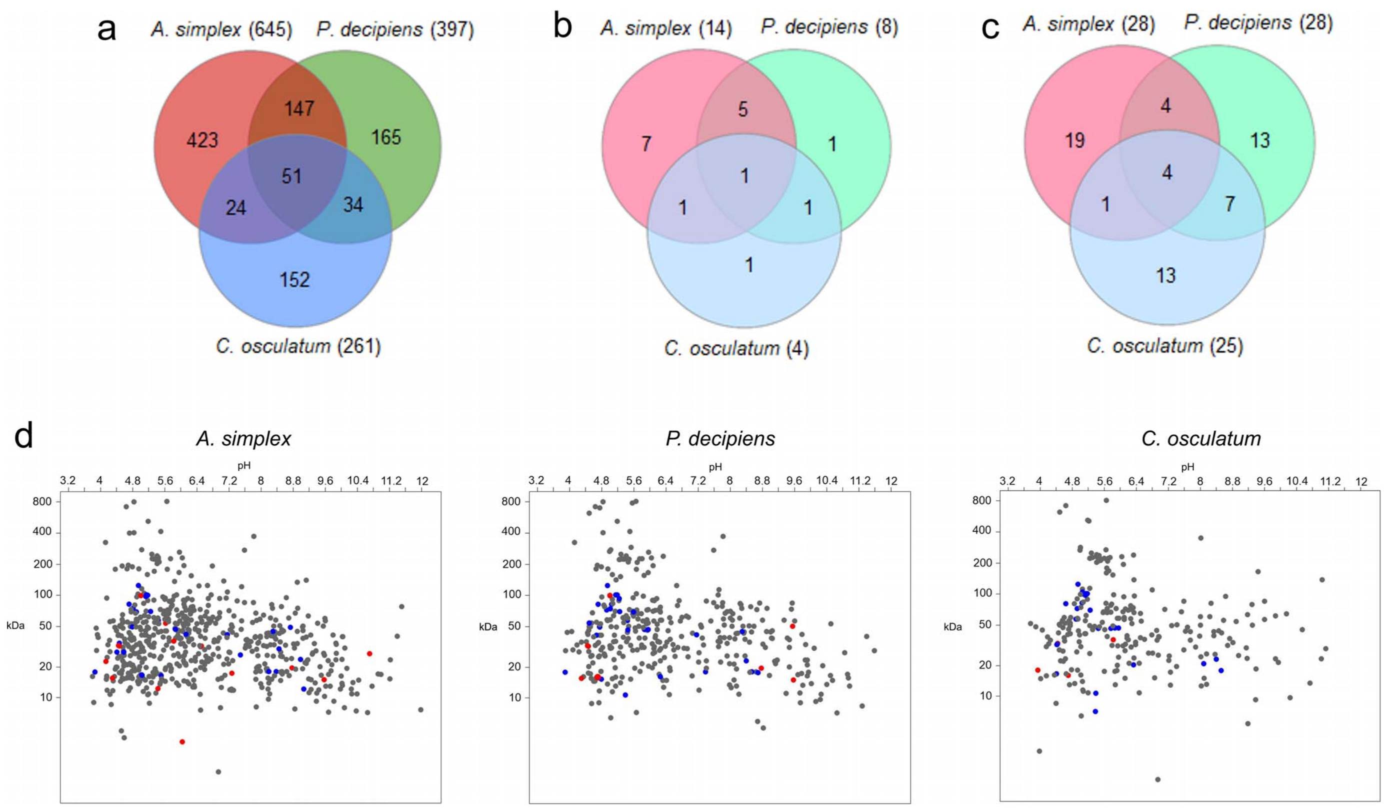

Figure 2. Venn diagrams of all identified proteins (**a**), allergens (**b**), and putative allergens (**c**) from *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum*. Distribution of detected anisakid proteins based on the molecular weight (Mw) and isoelectric point (pI) values; detected allergens are displayed in red; predicted allergens are in blue (**d**). Color version available online.

parameter is not an accurate measurement of protein concentration; nevertheless, it allows an approximate level to be estimated. The top 50 most abundant proteins of the 3 nematodes are displayed in the heat map (Fig. 3). The most highly expressed proteins of *A. simplex* L3 larvae were SXP/RAL-2 family protein isoform 1 (allergen Ani s 8) and enolase. Hemoglobin and disorganized muscle protein 1 were the most abundant in the *P. decipiens* proteome, whereas enolase proteins were the most abundant in the *C. osculatum* proteome. Moreover, many of the top 50 most abundant proteins were allergens or putative allergens.

GO annotation of identified proteins

GO functional analysis was performed using Blast2GO software to assign functions and processes to the identified anisakid larvae proteins. From the analysis, GO terms were assigned for 527 proteins of *A. simplex*. For *P. decipiens* and *C. osculatum*, 297 and 205 proteins were assigned, respectively. For *A. simplex* proteins, a total of 2,442 annotations was assigned. For *P. decipiens* and *C. osculatum* proteomes, in total 1,171 and 871 annotations were assigned, respectively. Nevertheless, the structures of GO terms among the 3 proteomes were quite similar.

The most common biological process categories of Anisakidae proteins were organic substance metabolic process (15%; GO:0071704), primary metabolic process (14–15%; GO:0044238), cellular metabolic process (14%; GO:0044237), and nitrogen compound metabolic process (12–14%; GO:0006807), followed by small-molecule metabolic process (8–9%; GO:0044281) and biosynthesis process (7–8%; GO:0009058).

Concerning the GO analysis for molecular function, anisakid proteins were mainly involved in ion binding (15–17%; GO:0043167), organic cyclic compound binding (12–13%; GO:0097159), heterocyclic compound binding (12–13%; GO:1901363), small molecule binding (11–12%; GO:0036094), carbohydrate derivative binding (8–9%; GO:0097367), hydrolase activity (7–10%; GO:0016787), drug binding (7–8%; GO:0008144), and protein binding (6–9%; GO:0005515).

In terms of cellular components, the major groups were intracellular (22%; GO:0005622), intracellular part (22%; GO:0044424), intracellular organelle (15–17%; GO:0043229), nonmembrane bounded organelle (11–12%; GO:0043228), and intracellular organelle part (11%; GO:0044446). More details regarding GO analysis are shown in Figure 4 and Suppl. File S1.

Identification of enzymes and metabolic pathways

Enzymes were assigned according to Enzyme Commission (EC) numbers and KEGG pathway analysis to better understand the function of the detected proteins. Potential enzymes of anisakids were mapped on the basis of the chemical reaction they catalyze. Enzyme classification revealed that hydrolases were the largest group in 3 proteomes (45–82 sequences; EC 3). Less abundant enzymes were oxidoreductases (11–48 sequences; EC 1), lyases

Table I. Shared proteins identified in *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* L3 larvae.

No.	Accesion no.*	Protein	Organism	Mw§	pI§
1	A0A0M3K9Z6‡	Uncharacterized protein	*Anisakis simplex*	41.92	4.75
2	A0A0M3KC30‡	Uncharacterized protein	*Anisakis simplex*	81.64	4.85
3	A0A0M3K916‡	Uncharacterized protein	*Anisakis simplex*	77.55	6.26
4	A0A0M3K6M6‡	Uncharacterized protein	*Anisakis simplex*	36.61	6.21
5	A0A0M3KBE3‡	Uncharacterized protein	*Anisakis simplex*	48.35	5.86
6	A0A0M3J6C9‡	Uncharacterized protein	*Anisakis simplex*	26.75	8.16
7	A0A0M3J2S6‡	Uncharacterized protein	*Anisakis simplex*	54.66	4.55
8	A0A158PN23‡	Twitchin	*Anisakis simplex*	807.33	5.65
9	A0A0G2YNB9‡	Tubulin beta chain	*Anisakis simplex*	50.31	4.48
10	A0A0M3JYT8‡	Proteasome subunit alpha type	*Anisakis simplex*	28.52	6.12
11	A0A158PP35‡	Paramyosin	*Anisakis simplex*	101.67	5.15
12	A0A0M3JD61‡	Methylmalonyl-CoA epimerase, mitochondrial	*Anisakis simplex*	13.07	8.02
13	A0A0M3KBA5‡	Malate dehydrogenase	*Anisakis simplex*	34.24	8.82
14	A0A0M3JUZ4‡	Hemicentin-1	*Anisakis simplex*	517.51	5.19
15	A0A0M3JM12‡	Heat shock 70 kDa protein F, mitochondrial	*Anisakis simplex*	11.99	5.06
16	A0A0M3K4H2‡	Glutamate dehydrogenase, mitochondrial	*Anisakis simplex*	60.92	8.24
17	A0A0M3K444‡	Fructose-bisphosphate aldolase	*Anisakis simplex*	39.57	7.98
18	A0A0M3JYW9‡	Fructose-bisphosphate aldolase	*Anisakis simplex*	43.91	8.34
19	A0A0M3K0Q9‡	Chaperonin homolog Hsp-60, mitochondrial	*Anisakis simplex*	50.67	4.74
20	A0A158PMX4‡	Adenylate kinase isoenzyme 1	*Anisakis simplex*	24.04	9.16
21	CAD43170.1†	Enolase	*Anisakis simplex*	47.47	5.87
22	AJB44650.1†	Actin	*Anisakis simplex*	41.81	5.12
23	AIU38247.1†	Heat shock protein 70	*Anisakis pegreffii*	70.65	5.25
24	AIT71762.1†	Glyceraldehyde 4 phosphate dehydrogenase	*Anisakis simplex*	36.16	7.75
25	AHY24647.1†	Glycogen phosphorylase	*Anisakis simplex*	99.46	5.84
26	KHN80459.1†	Myosin-3	*Toxocara canis*	226.24	5.29
27	KHN81411.1†	Heat shock 70 kDa protein F, mitochondrial	*Toxocara canis*	71.46	6.25
28	KHN88357.1†	Spectrin alpha chain	*Toxocara canis*	277.34	5.03
29	KHN76765.1†	Putative leucine-rich repeat-containing protein	*Toxocara canis*	714.33	4.64
30	NP_001156363.1†	Trypsinogen precursor	*Sus scrofa*	25.88	6.95
31	XP_015307599.1†	Predicted: Low-quality protein: actinlike	*Macaca fascicularis*	22.7	4.58
32	KHN76191.1†	Propionyl-CoA carboxylase alpha chain, mitochondrial	*Toxocara canis*	81.89	8.26
33	KHN74012.1†	Intermediate filament protein ifa-1	*Toxocara canis*	66.61	5.57
34	A0A0M3K1Y0‡	Uncharacterized protein	*Anisakis simplex*	195.00	6.07
35	KHN77077.1†	Fructose-bisphosphate aldolase 1	*Toxocara canis*	50.01	7.29
36	KHN75145.1†	Propionyl-CoA carboxylase beta chain, mitochondrial	*Toxocara canis*	58.46	6.26
37	KHN75937.1†	Dihydrolipoyl dehydrogenase, mitochondrial	*Toxocara canis*	57.06	7.52
38	KHN72825.1†	Spindle- and centromere-associated protein	*Toxocara canis*	251.21	5.28
39	XP_019640346.1†	Predicted: uncharacterized protein LOC109482101	*Branchiostoma belcheri*	84.67	5.07
40	KHN88332.1†	Chaperonin -like protein Hsp-60, mitochondrial	*Toxocara canis*	85.76	7.59
41	A0A0M3K1Z5‡	Uncharacterized protein	*Anisakis simplex*	211.29	5.46
42	KHN75599.1†	Phosphoenolpyruvate carboxykinase	*Toxocara canis*	72.02	6.13
43	KHN85055.1†	Spindle- and centromere-associated protein	*Toxocara canis*	241.51	5.41
44	KHN76782.1†	Adenylate kinase isoenzyme 1	*Toxocara canis*	57.14	9.6
45	KHN78603.1†	Spindle- and centromere-associated protein	*Toxocara canis*	227.48	5.75
46	KHN78570.1†	Protein disulfide-isomerase A3	*Toxocara canis*	55.32	6.52
47	KHN73857.1†	Intermediate filament protein A, partial	*Toxocara canis*	73.27	5.68
48	CRZ24364.1†	BMA-MYO-5	*Brugia malayi*	231.81	6.04
49	KHN72220.1†	Hsc70-interacting protein	*Toxocara canis*	26.23	4.53
50	A0A0M3K5C5‡	Myosin-3	*Anisakis simplex*	125.56	4.95
51	A0A0M3K659‡	Uncharacterized protein	*Anisakis simplex*	178.93	5.67

* Accession numbers were obtained from National Center for Biotechnology Information nonredundant protein database (†) or UniProtKB (‡).
§ Molecular weights (Mw) and isoelectric points (pI) were calculated using JVirGel software.

Figure 3. Heat map of the top 50 most abundant proteins of *Anisakis simplex*, *Pseudoterranova decipiens,* and *Contracaecum osculatum* determined by label-free quantification mass spectrometry. The average total spectrum count of proteins identified in all 3 replicates was log2-transformed and visualized using Morpheus. Accession numbers of proteins were obtained from the National Center for Biotechnology Information nonredundant protein database (*) or UniProtKB (†). Color version available online.

(14–27 sequences; EC 4), transferases (16–31 sequences; EC 2), and isomerases (11–30 sequences; EC 5). Ligases (EC 6) were the smallest group of enzymes in *A. simplex* (10 sequences) and *P. decipiens* (1 sequence), whereas ligases were not detected in *C. osculatum*. The identified Anisakidae enzymes are listed in Table II.

The predicted enzymes of *A. simplex*, *P. decipiens*, and *C. osculatum* were assigned to 70, 43, and 32 KEGG pathways, respectively. The most represented pathways of 3 proteomes were biosynthesis of antibiotics (17–73 sequences; 17–37 enzymes), purine metabolism (12–63 sequences; 5 enzymes), glycolysis/gluconeogenesis (9–41 sequences; 9–14 enzymes), citrate cycle (6–32 sequences; 6–13 enzymes), pyruvate metabolism (5–26 sequences; 5–7 enzymes), carbon fixation in photosynthetic organisms (4–21 sequences; 4–9 enzymes), carbon fixation pathways in prokaryotes (4–18 sequences; 4–12 enzymes), pentose phosphate pathway (4–17 sequences; 4–8 enzymes), fructose and mannose metabolism (3–15 sequences; 3–7 enzymes), and methane metabolism (3–18 sequences; 3–8 enzymes). The complete list of predicted pathways with the number of identified protein sequences and enzymes is shown in a heat map (Fig. 5). Suppl. File S1 shows the details of enzyme identification and KEGG pathway mapping.

Anisakid allergome

Allergen identification: In the proteome of *A. simplex* 14, the following allergens were identified: Ani s 1, Ani s 2 (2 isomers), Ani s 3 (2 isomers), Ani s 4, Ani s 8, Ani s 9, Ani s 10, Ani s 11-like, Ani s 13, Ani s fructose 1,6-bisphosphatase, Ani s phosphatidylethanolamine-binding protein (PEPB), and Thu a 3.0101. Eight allergens were detected in *P. decipiens*: Ani s 2, Ani s 3 (2 isomers), Ani s 5, Ani s 8, Ani s 9, Ani s PEPB, and Ani s troponin. In *C. osculatum* 4, the following allergens were predicted: Ani s 2, Ani s 5, Ani s 13, and Asc l 3. Moreover, the following allergens were among the top 50 most abundant Anisakidae proteins: Ani s 2, Ani s 3, Ani s 8, and Ani s 13 (see Fig. 3). All allergens identified in the tested anisakids are presented in Table III and Figure 2b.

Predicted allergen identification: To increase the sensitivity and accuracy of allergen prediction, we have combined the results of 3 commonly used bioinformatics tools: AllergenOnline, AllerTop, and PREAL. The analysis predicted 28 probable allergens

Figure 4. Gene Ontology (GO) pie chart of *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* L3 larvae proteins assigned using Blast2GO. The identified GO terms at level 3 with the corresponding number of identified proteins and percentage distribution of sequences among GO categories are presented. Color version available online.

of *A. simplex* and the same number of putative allergens of *P. decipiens*, whereas in *C. osculatum*, 25 possible allergens were detected. In total, 61 unique candidate allergens were identified in the 3 Anisakidae parasites (see Fig. 2c; Table IV). Among them, sequences of HSPs were most frequently detected, followed by paramyosin, peptidyl-prolyl cis-trans isomerase, enolase, and tropomyosin. Furthermore, putative allergens such as tropomyosin, paramyosin, myosin-3, enolase, HSPs, and *Caenorhabditis briggsae* CBR-CYN-3 protein were the most abundant Anisakidae proteins (see Fig. 3). The 3 anisakids shared 4 putative allergens: hsp70, myosin-3, enolase, and paramyosin.

DISCUSSION

Here, we performed LC-MS/MS profiling of L3 larvae of *A. simplex*, *P. decipiens,* and *Contracaecum osculatum*. To date, few studies have explored the proteomes of *A. simplex* and *P. decipiens* (Arcos et al., 2014; Fæste et al., 2014; Carrera et al., 2016; Stryiński et al., 2019), whereas the proteome of *C. osculatum* was examined for the first time in the present survey. We predicted a higher number of *A. simplex* proteins (n = 645) compared with *P. decipiens* (n = 397) and *C. osculatum* (n = 261) proteins probably because the protein database for *Anisakis* is much larger because, in contrast to *Pseudoterranova* and *Contracaecum*, the whole genome of *Anisakis* was sequenced. In a previous study, 98 proteins of *Anisakis* spp. were identified using 2D gel-based matrix-assisted laser desorption–time-of-flight (TOF)/TOF mass spectrometry (Arcos et al., 2014), whereas 103 proteins of *A. simplex* were detected using a SDS-PAGE gel-based LC-MS/MS approach (Fæste et al., 2014). In the present study, more than 6 times the number of *A. simplex* proteins were predicted compared with the previously mentioned investigations; this result could have been caused primarily by 3 factors: (1) the available database of the whole *A. simplex* proteome; (2) we used a gel-free approach, which is considered to be more sensitive (Abdallah et al., 2012); (3) 2 databases of proteins and 2 search engines were used to increase the sensitivity of LC-MS/MS detection. Nonetheless, Carrera et al. (2016) identified 1,231 and 1,276 proteins in *A. simplex* and *P. decipiens* L3, respectively, using a gel-free approach. Moreover, Stryiński et al. (2019) reported 1,872 proteins of *A. simplex* L3. Carrera et al. (2016) and Stryiński et al. (2019) probably predicted a larger number of proteins because they used a LC-MS/MS system with better resolution and different search engines.

HSPs were found most frequently among all detected anisakid proteins. HSPs are also abundant in the proteomes of many nematodes, and these proteins are essential for parasite survival, particularly during temperature stress (Chen et al., 2014; da Silva et al., 2018). The protective activity of HSPs can be one of the main mechanisms of resistance of Anisakidae larvae to low and high temperatures, which are used to kill larvae in fish products (e.g., cold smoking, freezing in domestic freezers). Furthermore,

Table II. Enzyme identification of *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* L3 larvae.

	Sequences count		
Enzyme	*A. simplex*	*P. decipiens*	*C. osculatum*
EC 1. Oxidoreductases	48	15	11
EC 1.1. Acting on the CH–OH group of donors	9	5	2
EC 1.2. Acting on the aldehyde or oxo group of donors	7	3	2
EC 1.3. Acting on the CH–CH group of donors	5	3	1
EC 1.4. Acting on the CH–NH(2) group of donors	3	—*	—
EC 1.5. Acting on the CH–NH group of donors	6	1	—
EC 1.6. Acting on NADH† or NADPH	2	—	—
EC 1.8. Acting on a sulfur group of donors	5	—	—
EC 1.9. Acting on a heme group of donors	1	—	—
EC 1.11. Acting on a peroxide as acceptor	7	2	5
EC 1.12. Acting on hydrogen as donors	1	—	—
EC 1.15. Acting on superoxide as acceptor	2	1	1
EC 2. Transferases	31	16	18
EC 2.1. Transferring one-carbon groups	4	2	—
EC 2.2. Transferring aldehyde or ketonic groups	2	1	3
EC 2.3. Acyltransferases	4	2	1
EC 2.4. Glycosyltransferases	6	3	4
EC 2.5. Transferring alkyl or aryl groups, other than methyl groups	3	—	1
EC 2.6. Transferring nitrogenous groups	2	—	—
EC 2.7. Transferring phosphorus-containing groups	9	8	9
EC 2.10. Transferring molybdenum- or tungsten-containing groups	1	—	—
EC 3. Hydrolases	82	55	45
EC 3.1. Acting on ester bonds	4	1	1
EC 3.2. Glycosylases	3	2	-
EC 3.3. Acting on ether bonds	2	—	1
EC 3.4. Acting on peptide bonds (peptidases)	26	11	7
EC 3.5. Acting on carbon–nitrogen bonds, other than peptide bonds	2	—	—
EC 3.6. Acting on acid anhydrides	45	41	36
EC 4. Lyases	26	27	14
EC 4.1. Carbon–carbon lyases	20	19	5
EC 4.2. Carbon–oxygen lyases	6	8	9
EC 5. Isomerases	30	17	11
EC 5.1. Racemases and epimerases	1	—	—
EC 5.2. Cis-trans-isomerases	10	5	3
EC 5.3. Intramolecular oxidoreductases	13	9	6
EC 5.4. Intramolecular transferases	6	3	2
EC 6. Ligases	10	1	—
EC 6.1. Forming carbon–oxygen bonds	2	—	—
EC 6.2. Forming carbon-–ulfur bonds	2	—	—
EC 6.3. Forming carbon–nitrogen bonds	5	1	—
EC 6.4. Forming carbon–carbon bonds	1	—	—

*— = Not detected.
† NADH = nicotinamide adenine dinucleotide, reduced form; NADPH = NAD phosphate, reduced form.

many HSPs are known as allergens (Aki et al., 1994; Chiung et al., 2000; Movérare et al., 2000).

The functional analysis conducted in this study allowed the characterization of the proteins and proteomes of Anisakidae nematodes. We found that the main GO categories, biological process, molecular functions, and cellular component, were very similar for the 3 anisakids. KEGG analysis revealed that carbohydrate metabolism pathways were the major group among all identified pathways. Certainly, carbohydrate metabolism pathways are essential for Anisakidae larvae since carbohydrates are probably the main source of energy. Moreover, it was reported that trehalose, which is one of the major carbohydrates of *A. simplex*, could play a protective role in the stress response (Łopieńska-Biernat et al., 2006, 2007). Surprisingly, a high abundance of proteins putatively involved in the biosynthesis pathway of antibiotics was found. Nevertheless, antibiotic biosynthesis pathways were also reported in studies of the transcriptome (Llorens et al., 2018) and proteome (Stryiński et al., 2019) of *Anisakis* spp. L3 larvae. Furthermore, we found that hydrolases were the most common enzyme class of *A. simplex*, *P. decipiens*, and *C. osculatum* L3 larvae. Indeed, the hydrolytic activity of *Anisakis* spp. and *Contracaecum* spp. larvae were previously reported (Łopieńska-Biernat et al., 2004; Zółtowska et al., 2007). Hydrolases could play a relevant role in anisakidosis pathogenesis by allowing penetration of host tissue (Hotez et al., 1994; Bahlool et al., 2013).

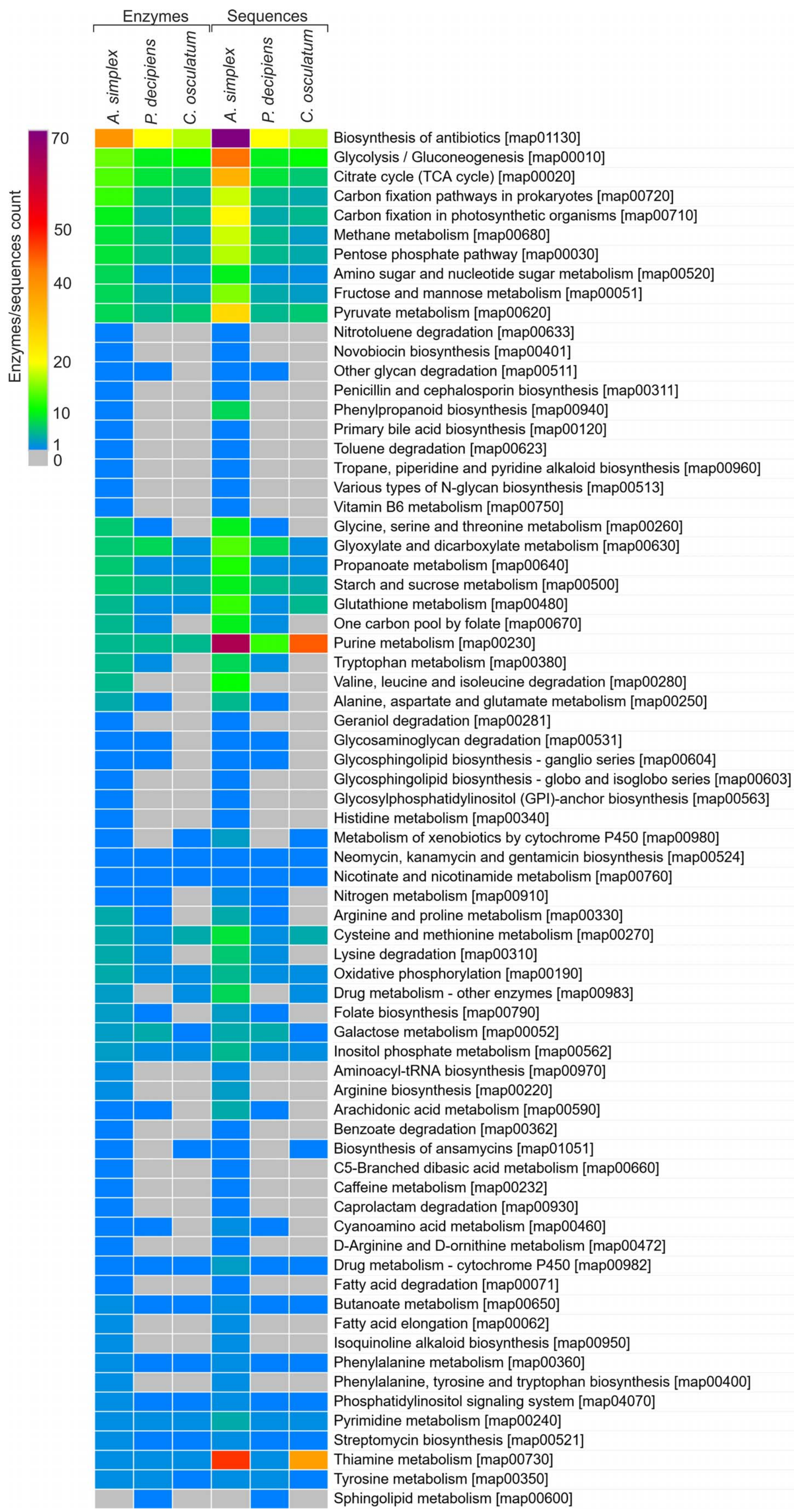

Figure 5. Heat map of predicted metabolic pathways of *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* with the number of corresponding protein sequences and enzymes. Color version available online.

Table III. Allergens detected in *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* L3 larvae.

No.	Accession no.*	Protein	Allergen	Organism	Mw§	pI§
A. simplex, *P. decipiens*, *C. osculatum*						
1	Q9NJA9.1†	Paramyosin	Ani s 2	*Anisakis simplex*	100.45	5
A. simplex, *P. decipiens*						
2	ABV55106.1†	SXP/RAL-2 family protein	Ani s 9	*Anisakis simplex*	15.49	9.57
3	A0A0M3J1J1‡	Phosphatidylethanolamine-binding protein homolog	Ani s PEPB	*Anisakis simplex*	20.08	8.78
4	AEQ28167.1†	Tropomyosin	Ani s 3	*Anisakis simplex*	32.92	4.47
5	BAF75681.1†	SXP/RAL-2 family protein 2 isoform 1	Ani s 8	*Anisakis simplex*	16.13	4.29
6	Q9NAS5.1†	Tropomyosin	Ani s 3	*Anisakis simplex*	33.2	4.45
A. simplex, *C. osculatum*						
7	AFY98826.1†	Hemoglobin, partial	Ani s 13	*Anisakis pegreffii*	36.66	5.82
P. decipiens, *C. osculatum*						
8	BAF43534.1†	SXP/RAL-2 family protein	Ani s 5	*Anisakis simplex*	16.59	4.7
A. simplex						
9	CAK50389.1†	Cysteine protease inhibitor	Ani s 4	*Anisakis simplex*	12.73	5.43
10	P86979.1†	Fructose-bisphosphate aldolase A	Thu a 3.0101	*Thunnus albacares*	3.91	6.04
11	AAF75225.1†	Paramyosin isoform, partial	Ani s 2	*Anisakis simplex*	54.37	5.63
12	BAJ78222.1†	Unknown	Ani s 11-like	*Anisakis simplex*	27.7	10.71
13	ACZ95445.1†	Unknown	Ani s 10	*Anisakis simplex*	23.28	4.13
14	AGC60032.1†	Kunitz-type serine protease inhibitor	Ani s 1	*Anisakis pegreffii*	17.91	7.27
15	A0A0M3JWX2‡	Fructose-1,6-bisphosphatase	Ani s FBPP	*Anisakis simplex*	32.78	6.52
P. decipiens						
16	CAB58171.1†	Troponin-like protein	Ani s troponin	*Anisakis simplex*	18.52	3.94
C. osculatum						
17	ACN32322.1†	Tropomyosin	Asc l 3	*Ascaris lumbricoides*	33.55	4.44

* Accession numbers were obtained from the National Center for Biotechnology Information nonredundant protein (†) database or UniProtKB (‡).
§ Molecular weights (Mw) and isoelectric points (pI) were calculated using JVirGel software.

To date, 22 allergens of *A. simplex* have been identified (see File S1), and 14 of them (Ani s 1 to Ani s 14) were approved by the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Subcommittee. As mentioned before, little is known about the allergenic potential of *P. decipiens* and *C. osculatum*. Human IgE-mediated responses to *P. decipiens* and *C. osculatum* have not been tested thus far. However, it was found that infection with *P. decipiens* can induce an allergic reaction in mice (Ludovisi et al., 2017). Furthermore, allergens of *A. simplex*, such as Ani s 2, Ani s 8, and Ani s 9, were reported in *P. decipiens* L3 larvae (Carrera et al., 2016). Data about allergens in *C. osculatum* larvae are not available. However, in a previous study, we found that excretory–secretory and crude antigens from *A. simplex*, *P. decipiens*, and *C. osculatum* L3 larvae also react with IgG antibodies from the sera of anisakidosis patients (Kochanowski et al., 2019). Similarly, in the present study, we confirmed by Western blot the cross-reactivity of rabbit anti-*A. simplex* IgG antibodies with protein extracts from *P. decipiens* and *C. osculatum* L3 larvae.

In this survey, allergomes of Anisakidae L3 larvae were investigated. We found 14, 8, and 4 allergens in *A. simplex*, *P. decipiens,* and *C. osculatum*, respectively (see Table III). Among the identified proteins, the major allergen, Ani s 2 (Carballeda-Sangiao et al., 2016), was shared among the 3 Anisakidae nematodes. It is known that major allergens cause the IgE response in more than 50% of sanitized patients. Moreover, the following major allergens were identified in anisakid larvae: Ani s 1, Ani s 11-like, and Ani s 13 (Carballeda-Sangiao et al., 2016). Ani s 2 and Ani s 3 detected in anisakids are considered panallergens with high similarity to related muscle proteins of invertebrates (Daschner et al., 2012; Quiazon et al., 2013). Therefore, Ani s 2 and Ani s 3 could be responsible for cross-reactions with proteins of other nematodes, crustaceans, or insects and could lead to IgE-dependent hypersensitivity in allergic patients (Guarneri et al., 2007). It is worth mentioning that the detected allergens Ani s 1, Ani s 4, Ani s 5, Ani 8, Ani s 9, Ani s 10, and Ani s 11-like are resistant to high temperatures (Moneo et al., 2005; Baird et al., 2014; Carballeda-Sangiao et al., 2016). This creates a risk of allergic reactions in sensitized humans even after highly processed food contaminated with Anisakidae allergens are ingested.

Since many Anisakidae allergens have yet to be discovered, we investigated the proteomes of nematodes to identify potential allergens. Computational web servers were used to predict allergens on the basis of the similarity of sequence and physicochemical properties between known allergens and possible allergens. In total, 61 candidate allergens were found in 3 anisakid species. Possible allergens of *P. decipiens* and *C. osculatum* were identified for the first time, and potential allergens of *A. simplex* were detected previously in the transcriptome (Baird et al., 2016; Llorens et al., 2018) and proteome (Arcos et al., 2014; Fæste et al., 2014). The largest allergome database for *Anisakis* spp. was provided by Llorens et al. (2018) (AnisakisDB; http://anisakis.mncn.csic.es/public/allergome), and it contains 937 consensus

Table IV. Predicted allergens detected in *Anisakis simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum* L3 larvae. Homologous allergens identified by AllergenOnline and AllerTop are also presented.

No.	Accession no.*	Protein	Organism	Mw§	pI§
A. simplex, *P. decipiens*, *C. osculatum*					
1	AIU38247.1†	Heat shock protein 70	*Anisakis pegreffii*	70.65	5.25
2	A0A0M3K5C5‡	Myosin-3	*Anisakis simplex*	125.56	4.95
3	CAD43170.1†	Enolase	*Anisakis simplex*	47.47	5.87
4	A0A158PP35‡	Paramyosin	*Anisakis simplex*	101.67	5.15
A. simplex, *P. decipiens*					
5	AIU38242.1†	Heat shock protein 90	*Anisakis pegreffii*	82.99	4.71
6	A0A0M3J3H0‡	Uncharacterized protein	*Anisakis simplex*	57.85	5.44
7	A0A0M3K8L6‡	Uncharacterized protein	*Anisakis simplex*	42.33	7.16
8	APG31415.1†	Arginine kinase, partial	*Anisakis simplex*	45.37	8.3
A. simplex, *C. osculatum*					
9	P13392.2†	Paramyosin	*Dirofilaria immitis*	98	5.13
P. decipiens, C. osculatum					
10	XP_015089903.1†	Predicted: peptidyl-prolyl cis-trans isomerase 3	*Vicugna pacos*	11.09	5.39
11	ADH95417.1†	Enolase	*Steinernema carpocapsae*	47.17	5.45
12	KHN76390.1†	Heat shock 70 kDa protein A	*Toxocara canis*	101.56	5.17
13	ERG82498.1†	Heat shock 70 kDa protein c	*Ascaris suum*	72.77	4.93
14	ADQ00605.1†	Enolase	*Ascaris suum*	47.43	5.96
15	KHN76385.1†	Heat shock 70 kDa protein 1A/1B	*Toxocara canis*	82.92	5.05
16	AJH66211.1†	Sigma class glutathione S-transferase	*Baylisascaris schroederi*	23.68	8.4
A. simplex					
17	A0A0M3J6G4‡	Peptidyl-prolyl cis-trans isomerase	*Anisakis simplex*	12.61	9.07
18	PDM67365.1†	Unc-15	*Pristionchus pacificus*	100.77	5.18
19	CDJ92091.1†	Tropomyosin domain containing protein	*Haemonchus contortus*	35.03	4.47
20	CDJ82690.1†	Peptidyl-prolyl cis-trans isomerase domain containing protein	*Haemonchus contortus*	18.53	8.38
21	ACT55267.1†	EF-hand family protein	*Onchocerca volvulus*	18.49	3.86
22	XP_003340820.1†	PREDICTED: tropomyosin alpha-4 chain isoform X4	*Monodelphis domestica*	28.61	4.41
23	BAF80467.1†	HSP70 protein	*Poecilia reticulata*	69.82	4.89
24	ARD05107.1†	Putative heat shock 70 kDa protein, partial	*Onchocerca boehmi*	17.18	5.03
25	KIH65495.1†	ATP:guanido phosphotransferase, catalytic domain protein, partial	*Ancylostoma duodenale*	49.67	8.74
26	ALR73973.1†	Putative heat shock 70 kDa protein, partial	*Acanthocheilonema odendhali*	17.3	5.02
27	KHN80686.1†	Tubulin alpha-3 chain	*Toxocara canis*	50	4.79
28	XP_007542787.1†	Predicted: tropomyosin alpha-4 chainlike isoform X1	*Poecilia formosa*	28.66	4.58
29	ACT35690.1†	Cathepsin L-like cysteine proteinase	*Ditylenchus destructor*	42.4	6.13
30	KHN80348.1†	Peptidyl-prolyl cis-trans isomerase 3	*Toxocara canis*	8.2	18.56
31	A0A0M3KGA6‡	Probable arginine kinase	*Anisakis simplex*	16.99	5.51
32	A0A0M3K5H6‡	78 kDa glucose-regulated protein	*Anisakis simplex*	75.4	4.81
33	KHN89119.1†	Peptidyl-prolyl cis-trans isomerase 7	*Toxocara canis*	24.36	8.99
34	AEB31319.1†	Hypothetical protein, partial	*Epinephelus bruneus*	26.99	7.49
35	AMB66820.1†	70 kDa heat shock protein C, partial	*Euphausia crystallorophias*	30.89	8.45
P. decipiens					
36	ERG81087.1†	Tropomyosin	*Ascaris suum*	41.84	4.67
37	ACX47902.1†	Cyclophilin	*Gnathostoma spinigerum*	18.58	8.54
38	XP_003746930.1†	Predicted: heat shock 70 kDa protein cognate 4-like	*Galendromus occidentalis*	69.59	5.6
39	KHN87109.1†	Heat shock 70 kDa protein C	*Toxocara canis*	74.69	5.01
40	KHN83332.1†	Superoxide dismutase (Cu-Zn)	*Toxocara canis*	16.76	6.26
41	KRX57761.1†	Paramyosin, partial	*Trichinella* sp. T9	93.97	5.23

Table IV. Extended.

Homologous allergen					
AllergenOnline			AllerTop		
Accession no.	Protein	Organism	Accession no.	Protein	Organism
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	P40918‡	Heat shock 70 kDa protein	*Davidiella tassiana*
—‖	—	—	Q6Y2F9‡	HDM allergen	*Dermatophagoides pteronyssinus*
ACH70931.1†	Enolase 3-2	*Salmo salar*	-	-	-
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
-	-	-	-	-	-
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	P40918‡	Heat shock 70 kDa protein	*Davidiella tassiana*
-	-	-	Q6Y2F9‡	HDM allergen	*Dermatophagoides pteronyssinus*
-	-	-	ACD50950.1†	Der p 20 allergen	*Dermatophagoides pteronyssinus*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
AAP35065.1†	Der f Mal f 6 allergen	*Dermatophagoides farinae*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
ACH70931.1†	Enolase 3-2	*Salmo salar*	—	—	—
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	—	—	—
ABF18258.1†	Heat shock cognate 70	*Aedes aegypti*	—	—	—
ACH70931.1†	Enolase 3-2	*Salmo salar*	—	—	—
AIO08848.1†	Der f 28 allergen	*Dermatophagoides farinae*	P40918‡	Heat shock 70 kDa protein	*Davidiella tassiana*
P46436.3†	Glutathione *S*-transferase 1	*Ascaris suum*	P46436‡	Glutathione *S*-transferase 1	*Ascaris suum*
AEY79726.1†	Cyclophilin	*Daucus carota*	P22011‡	Peptidyl-prolyl cis-trans isomerase	*Candida albicans*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
ACN32322.1†	Tropomyosin	*Ascaris lumbricoides*	P15846‡	Tropomyosin, muscle	*Trichostrongylus colubriformis*
AEY79726.1†	Cyclophilin	*Daucus carota*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
CAB58171.1†	Troponin-like protein	*Anisakis simplex*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
AFV53352.1†	Tropomyosin	*Oreochromis mossambicus*	P67936‡	Tropomyosin alpha-4 chain	*Homo sapiens*
ABF18258.1†	Heat shock cognate 70	*Aedes aegypti*	CAC14168.1†	Putative luminal binding protein	*Corylus avellana*
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	CAC14168.1†	Putative luminal binding protein	*Corylus avellana*
-	—	—	P14208‡	Arginine kinase	*Homarus gammarus*
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	Q92260.1†	Heat shock 70 kDa protein	*Penicillium citrinum*
AIO08861.1†	Der f 33 allergen	*Dermatophagoides farinae*	Q8WQ47.2†	Tubulin alpha chain	*Lepidoglyphus destructor*
-	—	—	AAF72534.1†	Tropomyosin	*Blattella germanica*
-	—	—	AAB09252.1†	34 kDa maturing seed vacuolar thiol protease precursor	*Glycine max*
CAA59468.1†	Cyclophilin	*Catharanthus roseus*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
—	—	—	ACD50950.1†	Der p 20 allergen	*Dermatophagoides pteronyssinus*
ABF18258.1†	Heat shock cognate 70	*Aedes aegypti*	CAC14168.1†	Putative luminal binding protein	*Corylus avellana*
—	—	—	Q9X721‡	Collagenase ColG	*Clostridium histolyticum*
—	—	—	AAQ24544.1†	Blo t 6 allergen	*Blomia tropicalis*
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	AAQ24544†	Blo t 6 allergen	*Blomia tropicalis*
-	—	—	Q27249‡	Tropomyosin isoforms c/e	*Caenorhabditis elegans*
CAA59468.1†	Cyclophilin	*Catharanthus roseus*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	—	—	—
ABF18258.1†	Heat shock cognate 70	*Aedes aegypti*	—	—	—
—	—	—	CAI43283.4†	Mala s 12 allergen precursor	*Malassezia sympodialis*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*

Table IV. Continued.

No.	Accession no.*	Protein	Organism	Mw§	pI§
42	ALR74014.1†	Putative heat shock 70 kDa protein, partial	*Onchocerca skrjabini*	15.78	4.79
43	KHN83883.1†	Troponin C, isoform 2	*Toxocara canis*	18.51	3.89
44	AAY27745.1†	Alpha tubulin	*Onchocerca volvulus*	50.04	4.75
45	KHN76665.1†	Paramyosin	*Toxocara canis*	101.42	5.19
46	AID21512.1†	Putative enolase protein, partial	*Senilites tristanicola*	18.66	7.39
47	KKA70091.1†	Thioredoxin	*Pristionchus pacificus*	26.99	7.49
48	AFK10904.1†	Peptidyl-prolyl cis-trans isomerase	*Callorhinchus milii*	18.24	8.69
C. osculatum					
49	ERG86750.1†	Paramyosin	*Ascaris suum*	105.58	5.06
50	BAJ11924.1†	Tropomyosin	*Thunnus thynnus*	32.76	4.42
51	ERG80132.1†	Peptidyl-prolyl cis-trans isomerase 3	*Ascaris suum*	21.54	8.09
52	XP_015236319.1†	Predicted: keratin, type II cytoskeletal 8-like	*Cyprinodon variegatus*	57.53	4.9
53	EJD75137.1†	Tropomyosin	*Loa loa*	33.2	4.43
54	XP_002638373.1†	*Caenorhabditis briggsae* CBR-CYN-3 protein	*Caenorhabditis briggsae*	18.45	8.52
55	A0A0M3K0I5‡	Paramyosin	*Anisakis simplex*	100.55	5.06
56	ACM24799.1†	Heat shock protein 90	*Steinernema feltiae*	81.2	4.64
57	ABC86903.1†	Paramyosin	*Ancylostoma caninum*	101.08	5.17
58	BAN20388.1†	Enolase	*Riptortus pedestris*	46.92	5.77
59	KRX11527.1†	Triosephosphate isomerase, cytosolic, partial	*Trichinella nelsoni*	7.45	5.38
60	AID21495.1†	Putative enolase protein, partial	*Anisomeria bistriata*	20.89	6.33
61	A0A0M3KA05‡	SXP/RAL-2 family protein 2 isoform 1	*Anisakis simplex*	17.10	4.41

* Accession numbers were obtained from National Center for Biotechnology Information nonredundant protein database (†) or UniProtKB (‡).
§ Molecular weights (Mw) and isoelectric points (pI) were calculated using JVirGel software.
|| — = Not detected.

transcripts from 121 different allergens. The putative allergens predicted in our study are generally consistent with the AnisakisDB allergome database (Llorens et al., 2018) and the majority of possible allergens identified by us are in this database. Putative anisakid allergens, such as sequences of HSPs, paramyosin, peptidyl-prolyl cis-trans isomerase, enolase, and tropomysin, were highly represented in our investigation and are also abundant in previously mentioned studies (Fæste et al., 2014; Baird et al., 2016; Llorens et al., 2018). Similarly, less common in this study, possible Anisakidae allergens such as alpha-tubulin, arginine kinase, cathepsin L-like cysteine proteinase, myosin-3, sigma class glutathione *S*-transferase, superoxide dismutase (Cu-Zn), SXP/RAL-2 family protein 2 isoform 1, thioredoxin, triosephosphate isomerase, troponin C isoform 2, and tubulin alpha-3 chain were also previously identified as putative allergens on the basis of the proteome (Fæste et al., 2014) or transcriptome (Baird et al., 2016; Llorens et al., 2018) of *A. simplex*. It should be noted, however, that the allergenicity of possible allergens should be confirmed by immunological investigations in future studies.

In summary, our study provides novel scientific insight from proteomic investigations of *A. simplex*, *P. decipiens,* and *C. osculatum* L3 larvae. LC-MS/MS analysis supported by the bioinformatics approach allowed the identification and functional analysis of anisakid proteins. For the first time, the proteomes of *P. decipiens* and *C. osculatum* L3 larvae were deeply investigated. We anticipate that our data set will be beneficial for the discovery of new Anisakidae biomarkers and drugs against anisakids. More important, anisakid allergomes were investigated to detect allergens and possible allergens. On the basis of these results we can also conclude that in addition to *A. simplex*, *P. decipiens* and *C. osculatum* should be considered potential sources of allergens that could lead to IgE-mediated hypersensitivity.

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Animal experiments were conducted in accordance with protocols approved by the Local Ethical Commission for Animal Experimentation in Lublin (license no. 66/2012).

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Table IV. Continued, extended.

Homologous allergen					
AllergenOnline			AllerTop		
Accession no.	Protein	Organism	Accession no.	Protein	Organism
AOD75395.1†	Heat shock-like protein	*Tyrophagus putrescentiae*	—	—	—
CAB58171.1†	Troponin-like protein	*Anisakis simplex*	Q9U3U5‡	Troponin-like protein	*Anisakis simplex*
AIO08861.1†	Der f 33 allergen	*Dermatophagoides farinae*	Q8WQ47.2†	Tubulin alpha chain	*Lepidoglyphus destructor*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
ACH70931.1†	Enolase 3-2	*Salmo salar*	—	—	—
—	—	—	ACO34814.1†	Sal k 3 pollen allergen, partial	*Kali turgidum*
AAP35065.1†	Der f Mal f 6 allergen	*Dermatophagoides farinae*	P22011‡	Peptidyl-prolyl cis-trans isomerase	*Candida albicans*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
AFV53352.1†	Tropomyosin	*Oreochromis mossambicus*	P09493‡	Tropomyosin alpha-1 chain	*Homo sapiens*
AEY79726.1†	Cyclophilin	*Daucus carota*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
—	—	—	P02538.3†	Keratin, type II cytoskeletal 6A	*Homo sapiens*
ACN32322.1†	Tropomyosin	*Ascaris lumbricoides*	ACN32322.1†	Tropomyosin	*Ascaris lumbricoides*
AEY79726.1†	Cyclophilin	*Daucus carota*	CAA59468.1†	Cyclophilin	*Catharanthus roseus*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
—	—	—	Q71EE1‡	Heat-shock protein	*Hevea brasiliensis*
Q9NJA9.1†	Paramyosin (Ani s 2)	*Anisakis simplex*	AAF72796.1†	Paramyosin	*Anisakis simplex*
CBL79146.1†	Enolase	*Salmo salar*	—	—	—
CAC14917.1†	Triosephosphat-isomerase	*Triticum aestivum*	Q9FS79‡	Triosephosphat-isomerase	*Triticum aestivum*
ACH70931.1†	Enolase 3-2	*Salmo salar*	—	—	—
BAF75681.1†	SXP/RAL-2 family protein 2 isoform 1	*Anisakis simplex*	BAF75710.1†	SXP/RAL-2 family protein 2 isoform 8	*Anisakis simplex*

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