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In-Depth Proteomic and Glycomic Analysis of the Adult-Stage *Cooperia oncophora* Excretome/Secretome

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Abstract

Cooperia oncophora is one of the most common intestinal parasitic nematodes in cattle worldwide. To date, *C. oncophora* infections are treated using broad-spectrum anthelmintics. However, during the past decade, reports of anthelmintic resistance in this parasite species have

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The authors declare no competing financial interest.

Supporting Information

Supplementary Figure 1. One-dimensional gel electrophoretic profile of the HMW fraction isolated from the *C. oncophora* ES proteome by SEC. Supplementary Figure 2. MALDI-TOF MS analysis of the N-glycans released from the ES proteome. Supporting Information File 1. Adult-stage *Cooperia oncophora* transcriptome database. Supporting Information File 2. Detailed documentation regarding MS and MS/MS identifications of the spots and bands observed in Figure 1A, B. Supplementary Table 1. Oligonucleotides used in RT-PCR experiments. Supplementary Table 2. List of isotigs identified (after NemaBLAST search at <http://www.nematode.net>) and employed to generate the newly deposited 10 and 5 consensus sequence variants corresponding to the LMW1 (top panel) and LMW2 antigens (lower panel), respectively (Figure 2). Supplementary Table 3. Glycopeptide variants detected in the tryptic digest of the single-domain ASP, analyzed by MS/MS. Different glycoforms, in line with the MMW ES N-glycan profile, were detected with H5N2 and H6N2 glycoforms giving rise to the highest ion intensities. This material is available free of charge via the Internet at <http://pubs.acs.org>.

emerged worldwide, necessitating new avenues for its control, possibly through vaccination. In this frame, we analyzed the adult-stage *C. oncophora* excretome/secretome (ES), covering both the protein and glycan components, since this fraction constitutes the primary interface between parasite and host and may hold potential vaccine candidates. Two-dimensional gel electrophoretic separation of the ES material enabled the MALDI-TOF mass spectrometry (MS)-directed identification of 12 distinct proteins, grouped in three separate molecular weight fractions: (i) a high molecular weight fraction consisting of a double-domain activation-associated secreted protein (ASP), (ii) a midmolecular weight fraction predominantly containing a single-domain ASP, a thioredoxin peroxidase and innexin, and (iii) a low molecular weight protein pool essentially holding two distinct low molecular weight antigens. Further MS-driven glycan analysis mapped a variety of *N*-glycans to the midmolecular weight single-domain ASP, with Man₆GlcNAc₂ oligomannosyl glycans as the major species. The predominance of the nonglycosylated double-domain ASP in the high-molecular weight fraction renders it ideal for advancement toward vaccine trials and development.

Keywords

Cooperia oncophora; excretome/secretome; helminth proteomics; glycomics; sequence analysis

INTRODUCTION

Although being regarded as a mild pathogen, the helminth *Cooperia oncophora* is one of the most common intestinal parasitic nematodes in cattle in temperate climate regions worldwide¹ and as such carries a substantial economical footprint as it holds a profound share in production losses.^{2,3} A previous study demonstrated that helminth infections increase United States beef production costs by nearly \$190 per head according to 2005 market prices.⁴ Anthelmintics have long been the method of choice to treat and prevent parasite infections in a reasonably efficient and low-cost manner. However, during the past decade, a large number of reports on the development of anthelmintic resistance have surfaced from all over the world, in particular for *Cooperia* spp.^{5–9} Therefore, alternative routes in controlling helminth infections need to be explored, one of which may involve the development and administration of prophylactic vaccines.

The most promising and interesting helminth protein fraction to scout for potential vaccine antigens is probably its excretome/secretome (ES), as it constitutes the primary interface between host and worm. The ES fraction essentially consists of proteins and other compounds secreted or released from the worm, likely playing a role in helminth survival, propagation, host infection, and evasion of host immunological responses.^{10,11} To date, comprehensive ES proteome surveys have been conducted for the sheep liver fluke *Fasciola hepatica*,^{12,13} the ruminant gastrointestinal parasite *Hemonchus contortus*,¹⁴ the blood flukes *Schistosoma mansoni*¹⁵ and *S. japonicum*,¹⁶ the sheep and goat parasitic nematode *Teladorsagia circumcincta*,¹⁷ the 3larial parasite *Brugia malayi*,^{18,19} the murine parasite *Heligmosomoides polygyrus*,²⁰ the rat parasite *Strongyloides ratti*,²¹ and the hookworm *Ancylostoma caninum*.²² For *C. oncophora*, apart from a previously described family of low molecular weight antigens,^{23,24} much less is known about the composition of the ES material in terms of proteins and protein-linked glycans.

In this study, we aimed to explore the complete excretome/secretome of adult *C. oncophora* worms by means of two-dimensional (2D) gel electrophoresis combined with mass spectrometry. In terms of protein identification, the mass spectrometric data were BLAST-searched against a transcriptome data set produced from eight different life-stages of *C. oncophora*, more specifically, the egg, L1, L2, L3-sheathed, L3-exsheathed, L4, adult

female, and adult male stages. Furthermore, the transcriptome data set was employed in obtaining the full-length coding sequences of the ES proteins and in gaining insights in the existence of possible sequence isoforms. Additionally, glycan components of the excretome/secretome were identified by mass spectrometric analysis of released glycans and tryptic glycopeptides.

EXPERIMENTAL SECTION

Preparation of Adult *Cooperia oncophora* Excretory and Secretory Products

Calves were infected with an in-house *C. oncophora* strain according to De Graef et al.²⁵ Adult-stage *C. oncophora* were collected from the large intestine 21 days postinfection. The worms were subsequently placed on a modified Baermann apparatus filled with physiological water at 37 °C. Worms migrating to the bottom of the funnel were then collected and washed five times in physiological water at 37 °C. In a next step, the helminths were transferred and cultured for three consecutive days in RPMI medium (Gibco, Invitrogen; Carlsbad, CA, USA) at 37 °C. The medium was refreshed on a daily basis and stored at –80 °C. After this three-day culturing period, all –80 °C stored media, containing the ES protein fraction, were pooled and passed through a 0.22 µm filter and simultaneously concentrated and dialyzed to PBS at 4 °C using an Amicon ultrafiltration unit and Ultracel Regenerated Cellulose ultrafiltration discs (both Millipore; Billerica, MA, USA). The protein concentration of the obtained ES sample was determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Size Exclusion Chromatography

Size exclusion chromatographic (SEC) fractionation of *C. oncophora* total ES material was carried out using a self-packed Superdex 200 16/70 column (GE Healthcare Bio-Sciences AB; Uppsala, Sweden). Maximum volumes of 0.5 mL of a concentrated ES protein solution were injected and eluted in PBS at a flow rate of 1 mL/min. Protein elution was monitored by absorbance measurements at 280, 254, and 214 nm, and when suitable 1.0 mL fractions were collected (ÄKTA Explorer, GE Healthcare Bio-Sciences AB; Uppsala, Sweden). PBS column equilibration and washing steps of at least two column volumes were carried out prior to and after each experiment, respectively.

One- and Two-Dimensional Electrophoresis and Tryptic Digestion of ES Proteins

Denaturing one-dimensional (1D) electrophoresis (SDS-PAGE) was carried out according to Laemmli,²⁶ including and omitting β-mercaptoethanol in reducing and nonreducing SDS-PAGE, respectively.

For two-dimensional gel electrophoresis (2D-PAGE), protein samples were precipitated by adding 5 vol of ice-cold acetone to 1 vol of ES material. This was vortexed briefly and incubated at –20 °C for 1 h, after which the protein pellet was recovered by centrifugation at 2000g for 5 min. The supernatant was discarded, and the pellet was allowed to air-dry, after which it was resolubilized in 8 M urea, 2 M thiourea, 2% w/v CHAPS, 20 mM DTT, 0.2% v/v carrier ampholytes (pH 3–10; GE Healthcare Bio-Sciences AB; Uppsala, Sweden). This was left at room temperature for 1 h, followed by centrifugation at 5000g for 5 min to remove any insoluble protein still present. Sufficient volumes of the supernatant (exact amounts in micrograms are provided in the figure legends) were applied to 7 cm, pH 3–10, Immobiline DryStrip IEF strips (GE Healthcare Bio-Sciences AB; Uppsala, Sweden). Covered with mineral oil, this was left overnight at room temperature for rehydration of the strip and uptake of the protein sample. Isoelectric focusing was carried out at room temperature using an Ettan IPGphor3 isoelectric focusing instrument (GE Healthcare Bio-Sciences AB; Uppsala, Sweden) with an initial 3 h focusing period at 300 V, followed by a

five hour linear gradient from 300 to 3500 V and a final 18 h long time-span at 3500 V, yielding a total voltage load of approximately 73 kVh. Proteins resolved in the first dimension strips were reduced and alkylated prior to second-dimensional electrophoresis by incubating the strips for 15 min at room temperature in a 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2% w/v DTT solution followed by another 15 min in an identical solution containing 2.5% w/v iodoacetamide instead of the DTT. The second-dimensional electrophoresis (SDS-PAGE), with the IEF strip embedded in the stacking gel, was carried out as described for One-Dimensional Gel Electrophoresis.

SimplyBlue SafeStain (Invitrogen; Carlsbad, CA, USA) was used to visualize the proteins according to the manufacturer's instructions. Pore size of the gels, provided as a percentage of total acrylamide-bisacrylamide monomer concentration, is indicated in the corresponding figure legends.

Prior to tryptic digestion, protein bands and/or spots of interest were excised from the gel, washed twice for 20 min at 30 °C using a mixture of 50% acetonitrile (ACN)-200 mM ammonium bicarbonate, and then air-dried. Sequencing grade trypsin (Promega; Madison, WI, USA) was added to a final amount of 0.1 μ g, and the trypsin-gel slice mixture was then kept on ice for 45 min, after which 50 mM ammonium bicarbonate was added until the gel slices were completely submerged. Digestion was performed by overnight incubation at 37 °C. Peptides were extracted by adding 60% ACN-0.1% formic acid twice to the gel spots. The extraction buffer was evaporated in a Speedvac apparatus with the remaining peptides being redissolved in 8 μ L of 0.1% formic acid.

Mass Spectrometric Analysis of Proteins

Tryptic peptides were spotted on a stainless steel matrix-assisted laser desorption ionization (MALDI) target plate and covered with α -cyano-4-hydroxycinnamic acid matrix (7 mg/mL in 50% ACN, 0.1% trifluoroacetic acid, 1 mM ammonium citrate) in a 1:1 ratio. After spotting, samples were allowed to dry by ambient air. Prior to each set of analyses, instrument calibration was carried out using a mixture of angiotensin I, Glu-fibrino-peptide B, ACTH (1-17), and ACTH (18-39) (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's instructions. Protein identification was obtained on a MALDI-tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) system (model 4800 proteomic analyzer; Applied Biosystems; Foster City, CA, USA) in positive ion MS mode. This mass spectrometer uses a 200-Hz frequency tripled Nd:YAG laser operating at a wavelength of 355 nm and was used in reflectron mode (acceleration voltage 20 kV; reflectron voltage 15.6 kV). For MS data acquisition, a total of 1000 shots were collected (50 subspectra accumulated from 20 laser shots each). The result-dependent MS/MS experiments were performed using the job-wide interpretation method. In this strategy, full mass spectra were collected, and individual molecular ions were chosen for fragmentation from the spots in which their peak intensity was the highest. The spot-to-spot precursor selection was set to 200 ppm, and the S/N ratio of the parent ion was at least 200. MS/MS analysis was performed from the eight highest peaks, from the highest to the least intense peak. All MS/MS data were acquired in the 1 keV MS/MS mode using air as the collision gas (1.2×10^{-7} Torr). A total of 4000 shots (50 subspectra accumulated from 80 laser shots each) were acquired, and the timed-ion-selector window was set to resolution 400 (fwhm). The 4800 Proteomics Analyzer was externally calibrated prior to analysis using fragment ion of Glu-fibrinopeptide, following the manufacturer's instructions.

Protein Identification

For peptide mass fingerprint analysis, the obtained spectra were searched against the *Cooperia oncophora* transcriptome database, downloaded from <http://www.nematode.net>

and consisting of 31,774 amino acid sequences, supplemented with existing NCBI depositions for *C. oncophora*, using the GPS Explorer V2 software platform (Applied Biosystems; Foster City, CA, USA) which makes use of the Mascot search engine 2.3 (Matrix Science Inc.; Boston, MA, USA). The spectra, of which the 50 most intense ions were loaded, were searched using a 200-ppm peptide mass and a 0.8-Da MS/MS tolerance, with carbamidomethylation (Cys) and methionine-oxidation as variable modification parameters and with a maximum tolerance of two missed cleavage events during trypsin digestion of the protein. The significance threshold was set at $p < 0.5$ (ion score = 58).

Sequence Analysis

Following their MS identification, the obtained sequences were subjected to BLASTP analysis²⁷ employing a nonredundant protein sequence database to assign an identification based on sequence identity. Where available, full-length amino acid sequences were deposited at the GenBank database (www.ncbi.nlm.nih.gov/) with their accession numbers provided in Table 1.

The double-domain ASP sequence was determined as follows: two degenerate primers (forward, ATGYAACAGKAYTGGG-TGAGG and reverse, ATACACATGGAYAAAYTGTGTGCT, with Y and K representing T/C and G/T, respectively) were designed based on conserved terminal regions found in the *Cooperia punctata* double-domain ASP sequence (GenBank accession number: AAK35199.1) and isotig sequences from the *C. oncophora* transcriptome database (<http://www.nematode.net>). Polymerase chain reaction (PCR) using the *GoTaq* enzyme (Promega; Madison, WI, USA) was carried out by mixing 1 μ L of adult-stage *C. oncophora* cDNA with 1 μ M of both forward and reverse primers and one unit of *GoTaq* in reaction buffer containing 0.2 mM of each dNTP and 1.2 mM MgCl₂. PCR experiments were carried out on a Mastercycler Ep instrument (Eppendorf; Hauppauge, NY, USA) where 2 min at a denaturing temperature of 95 °C were followed by 35 cycles of denaturing (30 s at 95 °C), annealing (30 s at 58 °C) and elongation (DNA synthesis for 90 s at 72 °C). Afterward, a final elongation step at 72 °C for 5 min was included, after which the PCR mixtures were kept at 10 °C. The obtained PCR products were subsequently ligated in pGEM-T Easy (Promega; Madison, WI, USA) according to the manufacturer's instructions, after which DH5 α competent cells (Invitrogen; Carlsbad, CA, USA) were transformed with these vector constructs as described by the manufacturer and plated on X-gal (5-bromo-4-chloro-indolyl- β -D-galactopyranoside) containing medium. Five white colonies were picked and analyzed by mixing 15 μ L of clone suspension (in water) with identical amounts of the reagents mentioned above. PCR conditions were also duplicated from the above-mentioned, be it that only 32 cycles of denaturing, annealing, and elongation were carried out. The obtained PCR fragments were sequenced (Genetic Service Unit, Ghent University Hospital; Ghent, Belgium), followed by matching of the obtained data to the previously recorded MS and MS/MS spectra. The corresponding double-domain ASP sequence was also deposited at the GenBank database (Table 1).

In Silico Protein Characterization

Following full-length amino acid sequence assessment of the identified proteins, molecular weight, and isoelectric point values were determined using the corresponding ExPASy calculation tools (http://web.expasy.org/compute_pi/), whereas N-terminal signal sequences were predicted using SignalP 3.0,²⁸ or, when lacking, nonclassical secretory proteins were assessed using SecretomeP 2.0.²⁹ Regarding SignalP 3.0 predictions, identifications were considered positive when both neural network and hidden Markov model algorithms offered corroborative estimations. Nonclassical secreted proteins were predicted to yield a neural network score exceeding the normal threshold of 0.5 but not to contain a signal peptide.

RNA Extraction and Reverse-Transcriptase PCR

C. oncophora eggs, L1, L2, L3, L4, and adult male and female worms were collected and RNA was extracted as previously described.³⁰ For each of the genes of interest, unique internal primers were designed using the online Primer3 software (version 0.0.4; <http://frodo.wi.mit.edu/primer3/>), which are listed in Supplementary Table 1, Supporting Information. The SuperScript One-Step RT-PCR with Platinum *Taq* system (Invitrogen; Carlsbad, CA, USA) was applied. PCR mixtures typically consisted of 200 ng of RNA, 0.8 μ M of both forward and reverse primers and one unit of RT/Platinum *Taq* Mix in reaction buffer containing 0.2 mM of each dNTP and 1.2 mM MgCl₂. PCR experiments were carried out on a Mastercycler Ep instrument (Eppendorf; Hauppauge, NY, USA) with program settings as follows: after an initial 30 min at 50 °C a denaturing temperature of 94 °C is maintained for 2 min, followed by 45 cycles of denaturing (15 s at 94 °C), annealing (30 s at 56–63 °C), and elongation (DNA synthesis for 12 s at 72 °C). Afterward, a final elongation step at 72 °C for 10 min was included, after which the PCR mixtures were kept at 10 °C. *C. oncophora* GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was included as an internal standard.

Release, Purification, and Derivatization of *N*- and *O*-Glycan Moieties

Total ES material as well as separate size exclusion chromatography-obtained ES fractions, i.e., HMW, MMW, and LMW fractions, were subjected to serial treatment with PNGase F and PNGase A to obtain released *N*-glycans. To this end, lyophilized proteins were redissolved in PBS with 1.3% SDS and 0.1% β -mercaptoethanol, and incubated at 95 °C for 10 min, followed by addition of 1.3% NP-40. The samples were then incubated with trypsin-coupled Sepharose for 16 h at 37 °C while shaking. Beads were spun down, the supernatant was transferred to a fresh tube, and PNGase F was added, followed by incubation for 24 h at 37 °C while shaking. The mixture was then applied to a C₁₈ RP cartridge (500 mg; JT Baker, Philipsburg, NJ, USA), and the flow-through and wash fractions (2 mL 10% acetonitrile (ACN) and 4 mL of water, respectively) were subsequently applied to carbon cartridges (150 mg Carbograph; Grace, Deerfield, IL, USA). After a wash with 6 mL of water, glycans were eluted with 3 mL of 25% ACN and 3 mL of 50% ACN containing 0.1% TFA. The (glyco)-peptides that remained on the C₁₈ RP cartridge were eluted by applying 5 mL of 30% ACN/0.1% TFA and 5 mL of 60% ACN/0.1% TFA, and these combined eluates were subsequently vacuum-dried. The (glyco)-peptides were dissolved in sodium acetate (NaAc) pH 4.5 and, after addition of PNGase A (Roche Diagnostics GmbH, Mannheim, Germany), incubation for 24 h at 37 °C. The mixture was applied to a C₁₈ RP cartridge and a carbon cartridge as outlined above. The purified PNGase F- and PNGase A-released *N*-glycans in the carbon cartridge eluates were each subjected to labeling with 2-aminobenzoic acid (2-AA) as described previously.³¹ Briefly, released *N*-glycans were taken up in 50 μ L of water and mixed with 25 μ L of a freshly prepared solution of 2-AA (48 mg/mL in DMSO containing 15% glacial acetic acid). Subsequently, 25 μ L of freshly prepared solution of 2-picoline-borane (1 M in DMSO) were added, followed by 5 min of shaking and incubation at 65 °C for 2 h. Free label and reducing agent were removed from the samples using Carbograph cartridges conditioned with 2 mL of 80% ACN in water, followed by equilibration with 3 mL of water. Samples were mixed with 300 μ L of water and were loaded on the cartridges. After being washed with 4 mL of water, oligosaccharides were eluted using 1 mL of 50% ACN containing 0.1% TFA. For cleanup, labeled glycan moieties were loaded on Biogel P-10 (BioRad, Veenendaal, The Netherlands) in 75% ACN and after washing with 80% ACN eluted with water. In parallel, to obtain *O*-glycan alditols, ES samples were treated with 0.1 M NaOH/1 M NaBH₄ at 40 °C for 24 h. Samples were then neutralized on ice using 4 M acetic acid, and boric acid was removed by repeated evaporation and addition of 1% acetic acid in MeOH. Released *O*-glycans were purified using C₁₈ and carbon cartridges as described above. Permethylation was carried out as

described previously.³² Briefly, dried *O*-glycans were dissolved in DMSO, after which NaOH (spatula-tip with powder) was added, and the sample was left at room temperature for 10 min while shaking regularly. Then, 100 μ L of iodomethane was added, followed by 10 min of regular shaking and subsequently the addition of 400 μ L of dichloromethane and 500 μ L of water. Shaking by inversion, removal of the aqueous layer, and addition of fresh water were repeated five times before the remaining organic layer was dried under a flow of nitrogen.

Excretome/Secretome Glycan Analysis by Mass Spectrometry

Employing the assembled amino acid sequences of the identified proteins, *in silico* *N*-glycosylation analyses were performed using NetNGlyc 1.0 Server (Technical University of Denmark; <http://www.cbs.dtu.dk/services/NetNGlyc/>). Experimental glycan analyses were carried out as follows: 2-AA-labeled *N*-glycan and permethylated *O*-glycan pools were analyzed with an Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonics; Bremen, Germany) operating in the negative-ion (*N*-glycans) or positive-ion (*O*-glycan) reflectron mode using DHB (Bruker Daltonics) as a matrix. A sample of MMW *N*-glycans was incubated with Jack bean α -mannosidase (Sigma) in NaAc pH 4.5 for 16 h at 37 °C and analyzed by MALDI-TOF MS after application to Zip-Tip C₁₈ and direct elution onto the target plate with a solution of 20 mg/mL DHB in 30% ACN. MALDI-TOF MS spectra were annotated in terms of monosaccharide composition (F_xH_yN_z) applying the Glyco-Peakfinder tool (www.glyco-peakfinder.org), followed by manual interpretation in-line with the mannosidase treatment results, using Bruker Daltonics FlexAnalysis software (Bruker Daltonics; Bremen, Germany). For glycopeptide analysis, a sample of the tryptic glycopeptide/peptide mixture extracted from the gel slice was applied to a reverse-phase column (PepMap, 3 μ m, 75 μ m·100 mm; Dionex/LC Packings, Amsterdam, The Netherlands) using an Ultimate 3000 nano-LC system (Dionex/LC Packings). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water) at a flow rate of 200 nL/min. After injection of the sample, elution conditions were switched to 10% solvent B (95% acetonitrile, 0.1% formic acid) followed by a gradient to 60% B in 45 min and a subsequent isocratic elution with a duration of 10 min. The LC column was coupled to an Esquire HCT-Ultra ESI-ion trap-MS (Bruker-Daltonics, Bremen, Germany) equipped with an online nanospray source operating in the positive-ion mode. For electrospray (1100–1250 V), electropolished stainless steel LC/MS emitters (150 μ m OD, 30 μ m ID) from Proxeon A/S (Odense, Denmark) were used. The solvent was evaporated at 175 °C employing a nitrogen stream of 7 L/min. Ions from *m/z* 500 to *m/z* 1800 were registered in the MS mode. When operated in the auto MS/MS mode, registering ions from *m/z* 140 to 2200, each MS scan was followed by the acquisition of MS/MS spectra of up to three of the most abundant ions in the MS spectrum. Selected MS/MS spectra were interpreted manually using Bruker Daltonics DataAnalysis software (Bruker Daltonics; Bremen, Germany).

RESULTS

The Adult-Stage *Cooperia oncophora* Excretome/Secretome

Two-dimensional gel electrophoretic separation of the adult-stage *C. oncophora* ES protein fraction revealed 25 clearly visible protein spots, grouped in three clusters termed HMW, MMW, and LMW representing the high, medium, and low molecular weight protein pools to which they respectively belong, as shown in Figure 1A. All spots were excised and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)-directed identification, resulting in the successful annotation of 22 spots. As summarized in Table 1, the HMW fraction mainly consisted of a double-domain activation-associated secreted protein (ASP),³³ displaying some heterogeneity in terms of isoelectric point (pI) values, hence the observed train of spots 1–4 in Figure 1A. The MMW

fraction contained three distinct proteins, more specifically innexin, thioredoxin peroxidase, and a single-domain ASP.³³ Finally, in the LMW protein fraction, despite displaying a multitude of protein spots, five proteins were identified with as of yet unknown functions (Table 1). Given the high degree of complexity in terms of 2D-PAGE spot number and distribution (Figure 1A), these proteins are expected to bear extensive amino acid sequence variation and/or chemical modifications, thereby yielding a considerable number of protein isoforms both in terms of molecular mass and pI values.

In parallel to 2D electrophoretic analysis, the adult *C. oncophora* ES proteome was fractionated by size exclusion chromatography in its three constituting fractions (HMW, MMW, and LMW) which were subsequently analyzed through 1D gel electrophoresis in order to visualize and identify low-abundance proteins, which may have been undetectable or masked in 2D-PAGE, and to obtain sufficient material for further glycan analyses. As shown in Figure 1B and summarized in Table 1, the analysis demonstrated that, besides the double-domain ASP, the HMW fraction additionally contained very low amounts of a ‘hypothetical protein’, which curiously was observed only under reducing conditions. Two additional proteins were identified in the MMW fraction, more specifically aldose reductase and a ‘parasitic stage specific protein 2’, the latter with no further functional information currently available. In contrast to the HMW and MMW fractions, no additional proteins were identified from the LMW fraction.

Oligomeric Species in the Adult-Stage ES Proteome

Whereas the reducing conditions applied in 2D-PAGE hampered its observation, intriguingly, when comparing reducing to nonreducing 1D-GE migration profiles, traces of disulfide bridge-based oligomeric species were shown to be present in the HMW protein fraction (Figure 1B, band 1). As no peptides other than those originating from double-domain ASP were noted upon MALDI-MS analysis and lower percentage non-reducing PAGE of this oligomeric species demonstrated its approximately 140-kDa molecular weight (Supplementary Figure 1, Supporting Information), it can be concluded that the double-domain ASP is able to form homodimeric structures.

Determination and *in Silico* Characterization of Full-Length Protein Sequences

Of the 12 identified proteins present in the adult-stage *C. oncophora* excretome/secretome, three were found to have their full-length sequences already documented and available in the GenBank database (Table 1), more particularly 14-kDa ES antigen, ES antigen 1, and ES antigen 2 with accession numbers AAD09212.1, CAC38986.1, and AAD09213.1, respectively. Apart from the double-domain ASP, the HMW hypothetical protein and innexin, the available *C. oncophora* transcriptome database, combined with MALDI-MS based identification, allowed six more *quasi* full-length protein sequences to be determined (single-domain ASP, thioredoxin peroxidase, aldose reductase, parasitic stage-specific protein 2, and two as of yet unknown proteins) (Table 1), which were deposited at the GenBank sequence database. On the basis of homologues identified in other species, the sequences obtained for the HMW hypothetical protein (isotig24792; highest homology to the *Caenorhabditis remanei* hypothetical protein; GenBank accession number XP_003114672) and innexin (isotig14500; highest homology to the *C. remanei* innexin; GenBank accession number XP_003096841.1) are expected to still lack 733 and 160 amino acids at their N-termini, respectively. Finally, for the double-domain ASP, we were able to clone its *quasi* full-length sequence by using a degenerate PCR approach. On the basis of its closest homologue in *Cooperia punctata* (AAK35199.1), we expect it to lack roughly 31 and 20 residues at the N- and C-termini, respectively, including the signal peptide.

As an initial *in silico* characterization, software-driven signal sequence prediction, combined with *N*-linked glycosylation consensus site searches, revealed only the single-domain ASP and the 14-kDa ES protein as potentially carrying asparagine-linked sugar moieties (Table 1). Notably, whereas the occurrence of a signal peptide could not be elucidated for the double-domain ASP, due to the lacking of an N-terminal amino acid stretch, our analyses did show the presence of two *N*-glycan acceptor sites (Asn-Xxx-Ser/Thr) in its sequence. For all identified proteins, further *in silico* analysis of the available full-length sequences yielded molecular weight values corresponding well to those observed in-gel, as demonstrated upon comparison of the values listed in Table 1 to Figure 1A, B.

Sequence Polymorphisms in *C. oncophora* Low-Molecular Weight Antigens

Given the observation that 12 out of the 18 protein spots observed in the LMW region all corresponded to either one of three previously described proteins (Figure 1A and Table 1), more specifically 14-kDa ES antigen, ES antigen 1 and ES antigen 2, we sought to unravel this apparent redundancy by thorough sequence analysis. First, upon aligning the three aforementioned protein sequences, we noted that the ES antigen 1 (CAC38986.1) and the 14-kDa ES protein (AAD09212.1) show 76% sequence identity, disregarding the C-terminal 18 amino acid stretch present in the 14-kDa ES protein (Figure 2A). From here on, we will collectively refer to these proteins as “LMW1 antigens”. In contrast, ES antigen 2 (AAD09213.1) shares only 35% sequence identity with LMW1 and will thus be referred to as “LMW2 antigen”. In order to investigate the existence of different isoforms or allelic variants of these proteins, an online available *C. oncophora* transcriptome data set (<http://www.nematode.net>) was BLAST searched using the sequences of LMW1 (both CAC38986.1 and AAD09212.1) and LMW2 antigens (AAD09213.1), yielding 36 and 15 homologous isotig hits, respectively (Supplementary Table 2, Supporting Information), from which, after eliminating all redundant and overlapping sequences, a total of 10 and 5 different proteins were identified with significant homology to LMW1 and LMW2 antigens, respectively. An alignment of these sequence variants is shown in Figure 2, whereas their GenBank accession numbers are included in Supplementary Table 2, Supporting Information.

Transcription Pattern of the Excretory-Secretory Proteins

The results of the gene specific RT-PCRs performed on RNA collected from seven different life stages are shown in Figure 3. Apart from the hypothetical protein identified in the HMW fraction, transcripts were detected for all other identified adult *C. oncophora* ES proteins. For the double-domain ASP, aldose reductase, innexin, and thioredoxin peroxidase, transcript was detected in all life stages, whereas for the single-domain ASP, parasitic stage specific protein and the LMW1 and LMW2 antigens this was the case in all stages apart from the eggs. Finally, transcription for the unknown proteins 1 and 2 was demonstrated exclusively in the L4 and adult stages.

Glycan Analysis of the *Cooperia oncophora* ES Proteome

Overall ES glycosylation was studied by MALDI-TOF MS analysis upon release of the *N*- and *O*-linked glycan moieties. Whereas a clear set of PNGase F-released glycans was detected (Supplementary Figure 2, Supporting Information), no signals could be detected in the PNGase A-specific release (data not shown), thereby indicating that no significant amounts of *N*-glycans with α 1–3-linked fucose modifications of the asparagine-linked *N*-acetylglucosamine (GlcNAc) are present in the excretome/secretome of adult *C. oncophora*. To obtain more information on the presence of glycan moieties on specific proteins in the *C. oncophora* ES fraction, the *N*- and *O*-glycans, released from the LMW, MMW and HMW protein clusters, were analyzed by MALDI-TOF MS. The major *N*-glycan signals observed

in the total ES pool were found to be present in the MMW *N*-glycan spectrum (Figure 4A), whereas no *N*-glycans could be detected in the LMW and HMW fractions. No *O*-glycan derived signals could be detected in the three ES fractions.

Using the monoisotopic mass of each of the *N*-glycan peaks observed, monosaccharide compositions were assigned and putative structures deduced. Major signals detected in the spectrum of MMW *N*-glycans that also appeared in the total ES *N*-glycan spectrum were derived from glycan moieties with the compositions F1H3N2, H5N2, H6N2 and H7N2 at m/z values 1176.4, 1354.4, 1516.5, and 1678.5 $[M - H]^-$, respectively (F, fucose, Fuc; H, hexose, Hex; *N,N*-acetylhexosamine, HexNAc) (Figure 4A). These compositions are indicative for a paucimannosidic, core-fucosylated glycan (F1H3N2) and a series of oligomannose glycans $\text{Man}_{5-7}\text{GlcNAc}_2$ (Man, mannose). A sample of the MMW *N*-glycans was incubated with α -mannosidase, leading to a spectrum containing detectable signals only at m/z 706.3 $[M - H]^-$ and 852.3 $[M - H]^-$ for H1N2 ($\text{Man}_1\text{GlcNAc}_2$) and F1H1N2 ($\text{Fuc}_1\text{Man}_1\text{GlcNAc}_2$) (data not shown), thereby confirming the presence of the mannose extensions in the untreated sample.

Prompted by the presence of a putative *N*-glycosylation acceptor site in the single-domain ASP, the most abundant protein in the MMW protein cluster (Figure 1B and Table 1), the tryptic glycopeptides derived from the corresponding single-domain ASP gel slice were subjected to nanoliquid chromatography (LC) MS/MS analysis. The obtained data were interrogated for the presence of glycosylated peptides by searching for MS/MS spectra containing the common glycan fragment ions H1N1 (m/z 366.1 $[M + H]^+$) and H2N1 (m/z 528.2 $[M + H]^+$). Interestingly, a series of parent ions was detected that could be assigned to the H5N2, H6N2, and H7N2 glycoforms of the single-domain ASP tryptic peptide WNCTLEAK (1021.5 $[M + H]^+$, with cysteine as carbamidomethyl derivative) based on (i) the overall mass of the glycopeptide, and (ii) the collision induced dissociation fragment spectra showing the fragmentation patterns of the glycosidic linkages in each peptide glycoform. An MS/MS spectrum of the major glycopeptide WNCTLEAK-Hex₆HexNAc₂ is provided in Figure 4B (with an overview of detected and fragmented glycopeptide moieties derived from the same gel slice shown in Supplementary Table 3, Supporting Information). From the parent ion m/z 1201.1 $[M + 2H]^{2+}$ a clear series of $[M + H]^+$ fragment ions indicated the loss of six Hex residues and one HexNAc, leaving the corresponding peptide-fragment ion with a single GlcNAc residue linked to the asparagine residue at m/z 612.8. Similar, but less intense, spectra were obtained for the H5N2 and H7N2 glycoforms of the same peptide, indicating that the $\text{Man}_{5-7}\text{GlcNAc}_2$ *N*-glycans released from MMW proteins are present on a single glycosylation site of the *C. oncophora* single-domain ASP.

DISCUSSION

The combined MALDI-MS-obtained identifications from both 2D gel electrophoretic and size exclusion chromatographic fractionation of the adult-stage *C. oncophora* excretome/secretome yielded 12 different proteins distributed over 3 distinct molecular weight clusters. Although such a number of proteins may appear as modest when compared to the ES fractions of other helminths such as *Ascaris suum*,³⁴ *Schistosoma japonicum*,¹⁶ and *Hemonchus contortus*,¹⁴ often revealing up to a hundred or more different proteins in their excretome/secretome, the protein profile observed in the current study was in accordance to previously published data.²⁴ In addition, higher-sensitivity gel analyses (silver staining) did not reveal the presence of any additional proteins when compared to those observed on Coomassie-stained gels (data not shown). Whereas the reason for such quantitative differences in the number of ES proteins between species is still unclear, it may reflect differences in specific host-worm interfaces.

Among the proteins identified we observed two different activation-associated secreted proteins (ASP) from the *C. oncophora* excretome/secretome. ASPs are found in the ES fraction of numerous parasitic nematodes (reviewed in ref 35) and constitute a Strongylida-specific subgroup of the CAP protein superfamily (also termed sperm-cell glycoprotein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) proteins; Pfam PF00188), which displays an extreme diversity both in occurrence, covering prokaryotes and eukaryotes, and function, having been shown to be involved in processes as diverse as reproduction, cancer and immune regulation.³⁶ To date, however, the true biological function of ASPs in nematodes still remains enigmatic, despite substantial research efforts^{37–41} where ASPs have been suggested to play a key role in the transition from the free-living to the parasitic phase and in immune evasion, emphasizing their importance at the host-parasite interface. However, in our hands transcripts of both the single- and double-domain ASPs were found at all stages from egg to adult, thereby hinting toward a broader functionality than initially considered.

ASPs have been found in three configurations: (i) as double domain ASPs, composed of two distinct but related CAP domains, (ii) as C-type single domain ASPs, and (iii) as N-type single domain ASPs, the second and the latter bearing the highest homology to the C- and N-terminus of the double domain ASPs, respectively.⁴² Our study yielded a double-domain ASP as part of the HMW fraction, and a single-domain ASP, belonging to the MMW fraction. Whereas the double-domain ASP bears two consensus acceptor sites for N-linked glycosylation, it was revealed not to carry any such moieties. Furthermore, upon 2D-PAGE analysis, a train of four distinct spots was observed, all of which correspond to this double-domain ASP, possibly reflecting certain amino acids carrying chemical modifications and/or some level of sequence diversity. Curiously, whereas unnoticed upon 2D–GE analysis, the 1D gel migration pattern for the double-domain ASP showed a doublet both under reducing and nonreducing conditions. Since it does not carry any sugar moieties, this observation may be attributed to partial processing of a signal peptide. Perhaps the most surprising observation regarding this double-domain ASP was a small portion of it migrating as a dimer, exclusively upon nonreducing gel electrophoresis, thereby implying it to be disulfide bridge-based. Whereas single-domain ASPs are known to dimerize in solution,³⁷ to our knowledge this is the first indication of a double-domain ASP to behave similarly. Even though only a small fraction dimerizes, the question remains whether its dimeric structure would affect protein stability and/or have functional consequences, for instance resulting in the completion or restoration of an active enzymatic site and/or enabling the proper orientation of domains to allow for putative substrate binding and conversion.

In contrast to its double-domain counterpart, the single-domain ASP, as found in the MMW fraction, displayed no signs of detectable sequence diversity or oligomerization. Interestingly, the single-domain ASP was shown to carry an N-linked glycan moiety at Asn93, thereby corroborating *in silico* predictions in terms of signal sequence and N-glycosylation consensus sites. Although its general impact on protein structure and immunogenicity is still to be conclusively determined, the glycan group was found to localize N-terminally to α -helix 2 in a classic ASP secondary structural organization,³⁹ which is the same region where one of two N-linked glycan structures have previously been found on an *Ostertagia ostertagi* single-domain ASP.^{39,43} Whereas structural and functional information on ASP-borne sugar moieties is still scarce, the fact that (i) glycans are found in the same regions of *C. oncophora* and *O. ostertagi* ASPs, and (ii) these glycans both contain terminal mannoses, suggests that they may govern and/or support binding by certain lectin receptors on, e.g., antigen presenting cells. Given these resemblances, the question remains whether these would also be observed in ASPs from other helminths, and if so, what the underlying rationale behind it may be.

Analogous to the double-domain ASP, upon 1D gel electrophoretic analysis of the single-domain ASP a doublet of bands was observed with the lower molecular weight band much less abundant when compared to the double-domain ASP situation where both bands of the doublet were *quasi* equally intense. Possibly, the lower single-domain ASP band represents aberrant signal sequence processing or the protein itself, devoid of its Asn93-linked glycan moiety.

The proteomic analysis also revealed a low molecular weight spot cluster mainly consisting of several variants of two low-molecular weight antigens, which have previously been employed in a diagnostic assay²⁴ but whose true function(s) remain unclear. Apart from the homologues identified in the closely related parasite *Cooperia punctata*,⁴⁴ so far this protein family has not been identified in any other nematode species. Why these proteins display such extensive diversity, as seen in their 2D-PAGE spot profile, remains unclear, although it has been hypothesized that this may create antigenic diversity and/or a set of redundant proteins, which may be eliminated through the host immune response without serious detrimental effects on parasite viability.⁴⁴

Additionally, our *C. oncophora* proteome analyses revealed several proteins that have previously also been found in the excretory/secretory material of other nematode species. Aldose reductase and thioredoxin peroxidase are two proteins that are known to be involved in detoxification mechanisms, the first also encountered in parasites such as *Giardia lamblia* and converting the mutagenic and toxic methylglyoxal, a byproduct of glycolysis, into acetol,⁴⁵ and the latter found in a wide array of parasites^{46–48} and acting as an antioxidant which eliminates reactive oxygen species generated during oxygen metabolism, oxidative processes and host immune responses.⁴⁹ The importance of these proteins may further be highlighted by the fact that transcripts were consistently observed in all *C. oncophora* life-stages.

Another protein that was found to be abundantly transcribed from egg to adult stages in *C. oncophora* and identified in the adult ES fraction was innexin (Pfam: PF00876), an invertebrate equivalent to the connexin family of molecules. These are gap-junction proteins that, upon oligomerization, form intercellular channels through which ions and small molecules may pass, thereby allowing intercellular communication.⁵⁰ Furthermore, innexins have been attributed a role in locomotion, with some of them also being involved in modulating the response to the anthelmintic ivermectin.^{51–53}

In conclusion, this study provides the first in-depth analysis of the adult-stage *C. oncophora* excretome/secretome. On the basis of the obtained insights, we propose the HMW-predominant and nonglycosylated double-domain ASP for advancement toward further vaccination trials and large-scale recombinant production, both cornerstones in vaccine discovery and development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2-AA	2-aminobenzoic acid
ACN	acetonitrile
ASP	activation-associated secreted protein
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DEPC	diethylpyrocarbonate
DHB	2,5-dihydroxybenzoic acid
DTT	dithiotreitol
ES	excretome/secretome
ESI	electrospray ionization
EST	expressed sequence tag
Fuc	fucose
GlcNAc	<i>N</i> -acetylglucosamine
Hex	hexose
HexNAc	<i>N</i> -acetylhexosamine
HMW	high molecular weight
LC	liquid chromatography
LMW	low molecular weight
MALDI	matrix-assisted laser desorption ionization
Man	mannose
MMW	medium molecular weight
MS	mass spectrometry
PBS	phosphate buffered saline
PMF	peptide mass fingerprint
RP	reversed phase
SEC	size-exclusion chromatography
TFA	trifluoro acetic acid
TOF	time-of-flight

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Figure 1.

Representative 2D electrophoresis profile of the *Cooperia oncophora* ES proteome (A) and size exclusion chromatographic elution profile of total adult *C. oncophora* ES material paralleled by the corresponding 1D denaturing and nondenaturing gel electrophoretic migration patterns (B). (A) Proteins (150 μ g) were separated across a linear pH range from 3 to 10 in the first dimension, followed by SDS-PAGE analysis on a 13.5% gel in the second dimension. The total 2D-electrophoresis experiment was performed in triplicate. Gels were Coomassie Blue G stained and spots of interest were excised and subjected to MALDI mass spectrometric identification. The gel was divided into three major parts, i.e., HMW, MMW, and LMW representing the high, medium, and low molecular weight regions observed on the gel, respectively. In total, 25 unique spots were excised, the MS identifications of which can be found in Table 1. (B) For each SEC experiment, 1 mg of total ES protein was loaded on the column. Upon elution the peaks were collected and analyzed for protein content by reducing (+) and nonreducing (–) SDS-PAGE. The gel was of a 12% total acrylamide–bisacrylamide monomer concentration, loaded with 10 μ g of protein per lane and SimplyBlue stained. Indicated bands (1–12) were excised and identified by MALDI mass spectrometry (Table 1).

Figure 2. ClustalW alignment of NemaBLAST-generated (<http://www.nematode.net>) amino acid sequences (isotigs) corresponding to LMW1 (A) and LMW2 antigens (B), both part of the LMW fraction of the adult-stage *C. oncophora* ES proteome. GenBank accession numbers and sequences of the previously deposited proteins, on which the homology-based MS-directed identification was based, are given on top of the panels. After the *C. oncophora* database was queried, all resulting isotigs were aligned, followed by elimination of redundant sequences and taking into account single amino acid sequence diversity (Supplementary Table 2). The remaining sequences, i.e., variants of one protein, were deposited in GenBank. In panel A, upon alignment of the protein sequences, it is clear that CAC38986.1 and AAD09212.1 represent homologues of each other. Whereas the AAD09212.1 GenBank deposition lacks its signal sequence, it does carry a C-terminal amino acid stretch, which was not found in CAC38986.1. For both LMW1 and LMW2 antigens, our approach allowed us to extend the previously deposited sequences until full-length.

Figure 3.

Monitoring the transcript levels of the 2D-PAGE identified proteins in different *Cooperia oncophora* life stages. Reverse-transcriptase PCR experiments were conducted on RNA from egg, L1, L2, L3, L4, and adult (male and female) stages of *Cooperia oncophora* using the primers listed in Supplementary Table 1, Supporting Information. The constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GADPH) was included as an internal control.

Figure 4.

MALDI-TOF MS analysis of released *N*-glycans from the ES MMW subfraction and LC-MS/MS spectrum of the major *N*-glycopeptide glycoform found in the single-domain ASP. (A) *N*-Glycans were released from ES MMW material using PNGase F and labeled with anthranilic acid. The major glycans detected in the *N*-glycan pool are oligomannosyl *N*-glycans carrying 5–7 Man residues, as confirmed by α -mannosidase digestion. The branch location of the outer Man residues is not precisely known. (B) Tryptic (glyco)peptides derived from the single-domain ASP containing gel slice were subjected to LC-MS/MS analysis. Glycopeptide fragmentation spectra were selected for further analysis by monitoring glycan marker ions at m/z 366 and 528. Only glycosylation variants of the ASP peptide containing *N*-glycosylation site Asn93 were detected (Supplementary Table 3, Supporting Information). The major glycoform found is shown, carrying the Man₆GlcNAc₂ glycan that also corresponds to the major structure detected in the overall MMW ES glycan profile (panel A). (Green) circle, Man; (blue) square, GlcNAc; (red) triangle, Fuc.

Table 1

MS-Directed Identification and Annotation of the Adult-Stage *Cooperia oncophora* Excretome/Secretome^a

homology-based ID ^b	GenBank accession no. ^c	spots in Figure 1A	bands in Figure 1B	ions matched ^d			M _r /pI ^g (kDa)(-)
				PMF	MS/MS	secre. pred. ^e /N-glyc (#) ^f	
double-domain ASP	<i>HF586918</i>	1-4	1-3	21	5 (17%)	4 (17%)	SP/Y (2)
hypothetical protein	<i>HAAAM01000001</i>		4	21	2 (9%)	1 (8%)	
aldose reductase	<i>HAAAM010000003</i>		5	37	22 (50%)	4 (16%)	N/Y (2)
single-domain ASP	<i>HAAAM010000002</i>	5	6, 7	15	1 (12%)	1 (12%)	SP/Y (1)
inexin	<i>HAAAM010000004</i>	6		35	11 (16%)	1 (4%)	
thioredoxin peroxidase	<i>HAAAM010000005</i>	7		24	8 (38%)	2 (13%)	N/N
paras. stage spec. prot.	<i>HAAAM010000006</i>		8	18	11 (65%)	0 (0%)	NC/N
14 kDa ES protein	<i>AAD09212.1</i>	10, 24	11	15	4 (24%)	1 (11%)	NC/Y (1)
ES antigen 2 protein	<i>AAD09213.1</i>	11, 12, 19		53	18 (52%)	2 (12%)	NC/N
ES antigen 1	<i>CAC38986.1</i>	13-16, 18, 20, 21	10, 12	16	11 (35%)	3 (19%)	SP/N
unknown protein 1	<i>HAAAM010000007</i>	8	9	11	3 (32%)	2 (32%)	SP/N
unknown protein 2	<i>HAAAM010000008</i>	9, 23		16	5 (32%)	2 (13%)	SP/N

^aThe top, middle, and lower panels of the table reflect the HMW, MMW, and LMW ES fractions, respectively.

^bBased on BLASTP-search.

^cAccession numbers in italic represent new GenBank deposited protein sequences.

^dBoth on peptide mass fingerprint (PMF) and MS-MS levels the sequence coverage is given as a percentage (%) between brackets. Values for the best MS-identification are provided, for values per protein spot/band, see Supplementary File 2, Supporting Information.

^ePrediction of protein secretion using the full-length protein sequences as determined in this study or when available at GenBank. SP: presence of a signal peptide as determined by SignalP 3.0; NC: nonclassical secretory protein as determined by SecretomeP 2.0; N: not secreted.

^fThe presence of possible N-glycosylation sites as determined by NetNGlyc 1.0 analysis using the protein sequences as determined in this study (Y: yes; N: no) (number of consensus sites found in the amino acid sequence is given between brackets).

^gM_r and pI values were determined using the ExPASy molecular weight and isoelectric point calculation tool. Values corresponding to full-length proteins are provided. Cleavage of possible signal peptide was not taken into account.