



Isolation and Proteomic Analysis of Rhoptry-Enriched Fractions from *Cryptosporidium parvum*

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Abstract

Background: Rhoptries are unique secretory/excretory organelles that are found exclusively in the Apicomplexa, and their contents are discharged at the time of invasion and are critical in the establishment of productive infection. Several rhoptry proteins have been identified in *Toxoplasma gondii*, *Plasmodium falciparum* and *Neospora caninum* and have been linked not only with the parasites' adhesion and invasion processes but also with their intracellular pathways. To date, only one *Cryptosporidium parvum* rhoptry protein candidate related to TgRON1 of *T. gondii* and PfASP of *P. falciparum* has been reported.

Methods: Subcellular fractionation of sporozoites was performed to obtain highly purified organelles. One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by liquid chromatography coupled with mass spectrometry was applied for fraction analysis, and 22 potential novel rhoptry proteins were detected by protein domain analysis using online softwares.

Results: Twenty-two potential novel rhoptry proteins were detected. A protein with *T. gondii* and *N. caninum* rhoptry protein homologs and some proteins with domains similar to that of *T. gondii* rhoptry proteins were identified.

Conclusion: These novel candidate proteins may be considered targets for researching the invasion pathway of *C. parvum* and the pathogenic mechanisms of rhoptry proteins. The present work provides a starting point towards the elucidation of the repertoire and function of *C. parvum* rhoptry proteins.

Keywords: *Cryptosporidium parvum*, Rhoptry, Liquid chromatography, Mass spectrometry, Protein domain analysis

Introduction

The apicomplexan parasite *Cryptosporidium parvum* is a waterborne enteric protozoan with significant veterinary importance (1). Cryptosporidiosis in the immunocompetent host is a self-limiting diarrheal illness, but in immunosuppressed individuals such as those with human immunodeficiency virus (HIV) infection, the disease can be serious and life threatening (1, 2). In addition to humans, *C. parvum* has been reported as a common serious pri-

mary cause of diarrheal outbreaks in farm animals, especially newborn ruminants such as cows that result in significant economic losses (3). To date, due to the lack of effective drugs and approved vaccines, the prevention and treatment of cryptosporidiosis remains a big problem (4).

Most apicomplexan parasites, such as *Toxoplasma* spp., *Cryptosporidium* spp., *Plasmodium* spp., *Babesia* spp., and *Eimeria* spp., have the capacity to invade

and replicate within the cells of their vertebrate hosts (5). All of these genera possess three kinds of secretory vesicles consisting of the apical organelles (rhoptries, micronemes and dense granules), which secrete substances that enable parasites to adhere selectively to and invade host cells; once within a host, they cause further modifications and eventually escape (6). Rhoptries are perhaps the most unusual organelles found within apicomplexan parasites (7, 8). They are the largest of the apical organelles, and their name alludes to their club-like shape that is visible in the apicomplexan parasites (6).

The number of rhoptries differs among apicomplexan parasites. Each zoite has two rhoptries in *Plasmodium* merozoites and sporozoites, *Babesia caballi* has three (9), *Theileria parva* merozoites have six (10), and *T. gondii* has eight or more in every sporozoite. However, sporozoites of *C. parvum* reportedly have only one (11). Rhoptry proteins have been linked with both the adhesion and invasion processes of the parasites and with intracellular pathways such as the delivery of signals to the host cell nucleus that is achieved by crossing the parasitophorous vacuole membrane (PVM) (12) or through evacuoles (13). Furthermore, some rhoptry proteins were shown to be important virulence factors (14). Although *C. parvum* contains rhoptry, few rhoptry proteins in *C. parvum* have been reported to date.

In the present study, subcellular fractionation was coupled with proteomic techniques to identify novel rhoptry components in *C. parvum*.

Materials and Methods

Oocyst preparation and sporozoite isolation

The *C. parvum* Iowa isolate used in this study was purchased from Waterborne™ Inc. and maintained by passage in newborn *Cryptosporidium*-free Holstein bull calves (15). Oocysts were isolated from calf feces using sucrose density gradient centrifugation (16) and 0.5% (wt/vol) hypochlorite treated immediately at 4°C for 10 min. Oocysts excystation was conducted in 0.75% (wt/vol) Na-taurocholate (Sigma, Hercules, CA, USA) at 37°C for 2 h until it exceeded 90% (15).

Isolation of rhoptry organelles

A total of 1×10^9 sporozoites were collected by centrifugation at $1,300 \times g$ for 10 min at 4°C. The subsequent purification steps were performed as previously described (17). The sporozoites were washed once in phosphate-buffered saline (PBS) and in R buffer (250 mM sucrose, 10 mM MOPS, pH 7.2, 2 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid [EDTA], complete protease inhibitor mixture), respectively. The pellet was resuspended in R buffer (5×10^8 sporozoites/ml) and then disrupted by passage through a French® Type Pressure Cell Disrupter (STANSTED FLUID POWER Ltd.) as previously described (18). After being centrifuged at $1,300 \times g$ for 20 min, the supernatant was transferred to a new microcentrifuge tube.

The organelle pellet was created by centrifugation at $25,000 \times g$ for 25 min and resuspended in R buffer containing 30% (wt/vol) Percoll. The mixture was centrifuged at $61,500 \times g$ for 25 min and a brownish band was collected. The band was diluted in R buffer and pelleted again at $100,000 \times g$ for 90 min to remove the Percoll. The mixture (after Percoll removal and R buffer dilution) was overlaid with steps of sucrose (36%, 39%, 42%, 45%, 48%, and 60%) in S buffer (10 mM MOPS, 2 mM dithiothreitol, 1 mM EDTA, and complete protease inhibitor mixture) and centrifuged at $150,000 \times g$ for 18 h. Fractions in each gradient were collected, diluted in R buffer, and centrifuged at $100,000 \times g$ for 90 min. These fractions were stored in R buffer at -80°C.

Electron microscopy detection

Pooled gradient fractions were respectively fixed in 2.5% glutaraldehyde, 4% sucrose, and 0.05 M phosphate buffer (pH 7.4) for 2 h, washed three times with ice-cold PBS, and centrifuged at 14,000 rpm for 10 min. Each sample was processed for electron microscopy using standard methods (19).

Protein extraction

Total protein was extracted using a ReadyPrep™ Protein Extraction Kit (Total Protein) (BIO-RAD, USA) according to the manufacturer's instructions. The extraction process could solubilize many

types of cell proteins, including both soluble and membrane fractions. Briefly, the purified rhoptry fraction was added to 1 ml of sample buffer (7 M urea, 2 M thiourea, 1% [wt/vol] ASB-14 detergent, 40 mM Tris base, and 0.001% bromophenol blue) in a 2-ml microcentrifuge tube and sonicated on ice for 30-sec bursts (typically 3–4 times). The mixture was centrifuged at $16,000 \times g$ for 30 min at 20°C. We removed and transferred the supernatant to a clean tube. Unused extract was stored in aliquots at -80 °C.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1-D SDS-PAGE)

Rhoptry proteins were separated in 15% SDS-PAGE and visualized using Coomassie Blue staining. The entire protein gel profile was excised from the gel and maintained in the new tube with MilliQ water until the protein was digested.

Liquid chromatography coupled with mass spectrometry (LC/MS-MS) and bioinformatics analysis

The entire 1-D SDS-PAGE gel profile was hydrolyzed to cleave the peptides and then analyzed using LC/MS-MS as described below.

First, the sample was minced and washed twice with MilliQ water, decolorized twice with 50% MeOH for 30 min and subsequently washed with MilliQ water, 50% acetonitrile and 100% acetonitrile. After the sample was reduced with 10 mM DTT for 1 h at 55 °C, alkylated 55 mM iodoacetamide was added for 45 min in the dark and the sample was washed again with MilliQ water, 50% acetonitrile and 100% acetonitrile. Finally, the sample was dried in vacuo for 30 min.

Second, the dried sample was digested with 3 µl of 12.5 ng/µl trypsin (Promega V5280) overnight at 37°C and centrifuged, and the supernatant was acidified using 1% formic acid. Digested peptides were dissolved using 25 mM NH_4HCO_3 (0.1% formic acid and 2% acetonitrile) were analyzed using LC/MS-MS on an LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany). The peptides were separated on a BioBasic C18 PicoFit column (100 µm, 10 cm long, 3 µm

resin; Michrom Bioresources, Auburn, CA, USA) at a flow rate of 300 nl/min. Water and acetonitrile added with 0.1% formic acid were used as solvents A and B, respectively. The gradient was started at 5% solvent B and increased to 35% solvent B for 120 min. Peptide ions were analyzed in data-dependent MS experiments and detected in a survey scan from 400 to 1700 amu (3 scans) followed by five data-dependent MS/MS scans (5 scans each; isolation width, 3 amu; 35% normalized collision energy; dynamic exclusion for 3 min). Bioworks 3.2 software was used to process the data and convert it to an MGF file.

The search was performed using the MASCOT in the NCBI, CryptoDB v5.0, and EupathDB v2.16 databases. Protein functions were predicted using SMART, TMpred and SignalP 4.0 software.

Results

Isolation of rhoptry organelles

The *Cryptosporidium* oocysts with >90% viability were used to excystate and disrupte. The disrupted sporozoite composition was subjected to sucrose gradient floatation using a gradient of 36–60% sucrose. Fractions of each gradient (36%, 39%, 42%, 45%, 48%, and 60% sucrose) were processed for electron microscopy, and those that were enriched with rhoptry proteins were identified. The only fraction between 36% and 39% sucrose was enriched with rhoptries as indicated by its black tadpole-like appearance (Fig. 1).

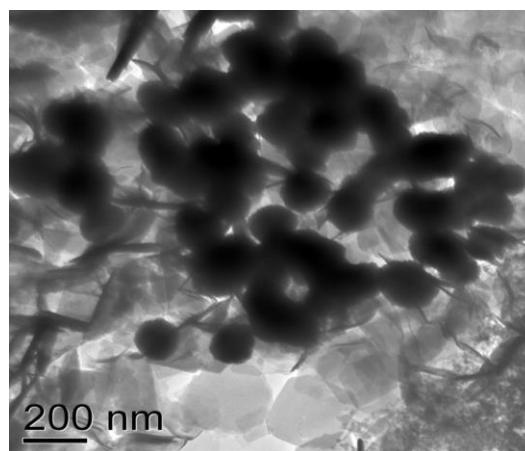


Fig.1: Electron microscopy of sucrose graction of rhoptry fraction

Other sucrose concentrations did not find any similar structures. The crystalline objects in the background may be the leaving sugars. Based on these results, pooled gradient fractions of *C. parvum* were used for the subsequent studies.

Proteomic identification of rhoptry organelles using gel LC/MS-MS

Different protein compositions were observed among the subcellular fractions in 1-D SDS-PAGE (Fig. 2). The molecular weights of some of the proteins were >260 kDa (A), while the others were 25–260 kDa (B). These two sections were excised and identified using LC/MS-MS. A total of 22 proteins (four from part A and 18 from part B) were identified, including kinases, secret proteins, large proteins, membrane protein, ATPase, peptidase, dikinase, transmembrane protein, and hypothetical proteins (Table 1). Among these proteins, a serine threonine (Ser/Thr) protein kinase of *C. parvum* (cgd7_590) possessed 40% amino acid sequence identity with *T. gondii* ME49 ROP17 (TgROP17) and 34% identity with *N. caninum* ROP20 (NcROP20).

Protein domain analysis by software online

Most hypothetical *C. parvum* rhoptry proteins have N-terminal signal peptides, C- or N-terminal transmembrane domains, and several low complexity regions. The cgd7_4820 protein has a predicted GPI anchor signal. The cgd7_590 and cgd3_3230 proteins contain Ser/Thr kinase domain, which usually results in a functional change of the target protein by changing enzyme activity, cellular location, or the association with other proteins. The cgd7_590 protein is located on chromosome VII and contains the signature Ser/Thr kinase domains such as TgROP16, TgROP17, and TgROP18. The putative RON protein (cgd3_1540) identified in *C. parvum*, which is located on chromosome III, owned two signal peptides in the N-terminal, two internal repeats, and several low complexities such as TgRON 4. The cgd6_3870 protein was located on chromosome VI and had 12 transmembrane domains, similar to TgROP14. The cgd3_590 protein had DUF900, a hydrolase-like domain. Despite the presence of N-terminal signal peptides and C-terminal transmembrane domains, the cgd2_450 protein also had a serine proline-rich domain.

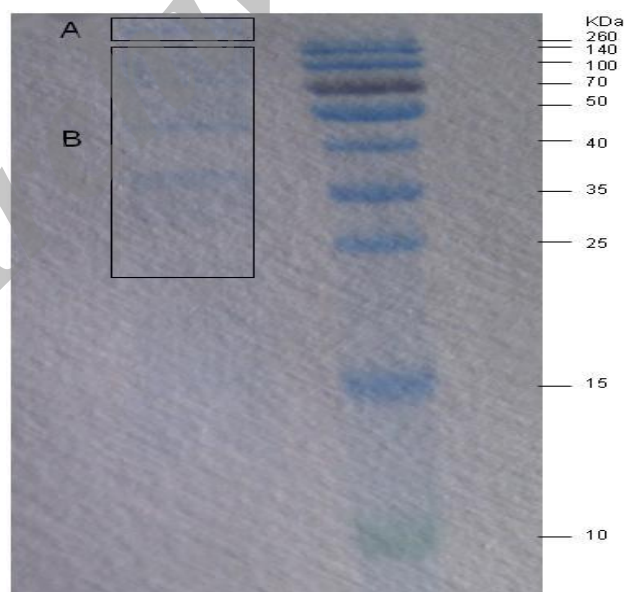


Fig.2: 1-D SDS-PAGE resolution of the rhoptry fraction

A : The proteins with molecular weight more than 260 kDa;

B : The proteins with molecular weight between 25 kDa to 260 kDa

Table.1: Identification of rhopty proteins by LC/MS-MS

Protein name	Genome localization	Predicted Molecular Weight (kDa)	Score *	No. matched peptides **	Amino acid sequence of peptides ***	Accession No.	Blast homology (score /Identities /Positives)
Ser/thr protein kinase	Chr VII	26.66	106	8	ENAIKYDLYCIGMI-MI-FIEMWTSALK/NLSGQR/LAADPAWSAGK	cgd7_590	TGME49_058580 Toxoplasma_gondii_ME49 Rhopty kinase family protein ROP17 (83/40%/66%); NCLIV_028170 Neospora_caninumRhopty kinase family protein ROP20, putative,(83/34%/48%)
Large protein with a SPRY domain and HECT domain	Chr I	538.22	806	22	DSVIDTKSSFLDYNEI-EK/HYESR/KSPELIDDLFVK/NVNQLIELI NAITK/NVLYNIPDTEIYSLK/TPNNDSPS NNVNHNLNILHDTYSNHDIIK FDEHLNNTNIPLQFKYNSLEI-EHPCK/SLFFFLELSLITNSSTYIPNDNK/A IIFHGGG-NFGDLYSHHHELR/TLDGMAFLSR	cgd1_1920	
Secreted protein with signal peptide, fringe-like glycosyltransferase domain and a WcaK like glycosyltransferase domain	Chr VI	95.52	538	16	FDEHLNNTNIPLQFKYNSLEI-EHPCK/SLFFFLELSLITNSSTYIPNDNK/A IIFHGGG-NFGDLYSHHHELR/TLDGMAFLSR	cgd6_1450	
Large protein with signal peptide. cysteine-rich, threonine-rich, possible mucin	Chr III	184.65	551	19	ELSIIPFEDVEDHIK/CTNSNDK/GWPIG-MEAL-NGNWDLNR/ALSSITYTYTK/EMPIPFPTAS LPLIGQLAANAFPMIK	cgd3_1540	
Ser/thr protein kinase	Chr III	178.11	279	10	NLIIDEKTQNEINCNER/NPQIEYRLTQI-INIIK/SGNPLVLCNNILK/LLNFLQK	cgd3_3230	
Signal peptide plus GPI anchored membrane protein with N-terminal multiple cysteines	Chr VII	45.22	235	10	TKETMTMSR/LFGLTPEEQCK/EDEDK-DEAEK	cgd7_4820	
Vacuolar proton translocatingATpase with 7 transmembrane regions near C-terminus	Chr IV	104.81	356	6	GDGILFAPLNHMR/NINLIK/VVSVI-YFQGATTSVYDKISR/FTSFTNSSK	cgd4_1470	
WD repeat protein	Chr V	127.45	306	9	IHIQESQQDISDK/EENCLELSK/LIHSSA WMNNNIVIFGGEDHIVR	cgd5_2540	
WD repeat protein	Chr IV	63.01	260	11	YLISSAHDGTIKLWNSLNGK/YENLEIP-ENIYSLFGRK	cgd4_950	
Secreted insulinase like peptidase, signal peptide	Chr III	146.83	127	12	LSDILQEIS-SPT(2)/VFMVYDK/SIPTQNTQNSLK	cgd3_4280	
R1 like alpha-glucan water dikinase	Chr II	192.83	148	8	HSNSMKLI-WEK/INFLYR/LLAQSAETASLTLISK	cgd2_2340	
3 transmembrane region protein	Chr VIII	43.85	168	3	MAK-NLWLFQSQEIMESNAAK/RFLGFCVNGIK	cgd8_4880	
Very large secreted protein, signal peptide	Chr VII	374.84	484	15	LYLNSSQIEGLEIIPK/LYLIPMQK/VNELF DK/GDLDFDLGCIR/INLTVITPGK	cgd7_4340	
Hypothetical protein with signal peptide and one or more transmembrane domain regions	Chr II	135.07	406	12	LNY-NILLNSILENSR/LVQSSK/NYSINMANEEF K/INFFANNFIFELNDLK	cgd2_1710	
Hypothetical protein with signal peptide and possible transmembrane region at C-terminus	Chr V	56.88	245	8	VEIIDREISLD-GQMSLR/PIISELFDMLLENI-EAKR/PIQFSSFNIVTSK	cgd5_4280	
Extracellular protein with a signal peptide, FN3 domain and a predicted transmembrane region	Chr IV	284.24	731	18	MKLSNLIIPGR/FFDYEYDLSR/LSD-FLYVTFDNNIR/LDWKIFYNFGNPLDLEI FEK/IINTFSISYLESDTIEQVEEEEWQVK/ELNGNNEEVSISR	cgd4_640	

Table 1: Cond...

Multiprotein bridging factor type 1 like transcriptional co-activator	Chr III	177.24	111	6	LAMPNAARLDQDTGDYR/QNIPQNAAK	cgd3_3750
Nuclear VCP like protein with 2 AAA ATPase domains	Chr V	77.24	206	10	VVNQLLTELDT- GVGER/LTALYSK/IYVPLPNEMGR	cgd5_2010
Hypothetical integral membrane protein with 12 transmembrane domains	Chr VI	63.49	411	11	LCWVVFIFCLSLFGMLDIITSLM- NNYR/VNCLTDGHLK/FALTLHGIIIGFFLF VTPCIFTYDMDNLVAK	cgd6_3870
Signal peptide, serine proline rich, possible low mw mucin glycoprotein locus of 6 genes	Chr II	19.34	72	3	DFVSVVGLNTENRNSVELGNK/GSIEIGN PTQPK	cgd2_450
Hypothetical protein with signal peptide and one or more transmembrane domain regions	Chr II	135.07	480	13	LNYNILLNSILENSR/VFSIIHILISLVFTA- TIFIPLILIESEK/ISQHSLK/KQLMGIFR	cgd2_1710
Hypothetical protein with 4 transmembrane domains, possible unusual phyletic distribution	Chr III	134.07	299	7	MESNELNSLSIDIP- SGNEQNLK/TSSEGMKGGYSSDTK/GFPL CHIYPAPSIGMNR	cgd3_590

*: MASCOT MS protein score, the probability score was <0.05.

***: Number of peptide masses values matched.

***: Amino acid sequence identified by MS-MS.

Discussion

Rhoptries are unique secretory/excretory organelles that are found exclusively in Apicomplexa, and their contents are discharged at the time of invasion and are critical to the establishment of a productive infection. Rhoptries were first isolated from *Eimeria nieschilzi* (20) and have since been isolated from several Apicomplexa including *P. falciparum*, *T. gondii*, *Babesia* spp., *Eimeria* spp., and *N. caninum*. Bradley et al. reported 38 novel proteins identified by MS of a purified rhopty fraction from *T. gondii* (17). Marugán-Hernández et al. identified an extensive number of potential rhopty proteins in *N. caninum* using proteomic approaches (21). Sanderson et al. assessed the potential array of rhopty-related proteins detected in the *C. parvum* sporozoite proteome by performing homologous BLAST searches using protein sequences and detected 12 putative sporozoite rhopty proteins (22). However, the functions of these proteins were not studied here. To date, only one *C. parvum* putative rhopty protein, CpPRP1, has been reported, and it is the only *C. parvum* protein that shows significant homology to the known rhopty neck proteins (23). The reason for the low primary sequence conserva-

tion between *C. parvum* and other apicomplexan parasites may be that the parasites are able to adapt to distinct biological niches, i.e., nucleated cells for *T. gondii* and *N. caninum*, erythrocytes/hepatocytes for *P. falciparum*, and enterocytes for *C. parvum* (23). Therefore, it is difficult to use homology comparisons to obtain novel *C. parvum* rhopty proteins. In this study, the subcellular fractionation technique was used to gain enriched rhopty organelle fractions prior to the use of 1-D SDS-PAGE and LC/MS-MS.

Most proteins examined here from *C. parvum* rhopty-enriched fractions possessed an N-terminal signal peptide that was recognized by SignalP. However, the internal transmembrane domains were not recognized by TMpred in all proteins. Additionally, many proteins contained several stretches of low complexity regions, which were predicted to form α -helical regions within the *T. gondii* rhopty proteins. These characteristics of the 22 novel predicted proteins we gained here were similar in overall structure to those of the *T. gondii* rhopty proteins.

These novel proteins identified by LC/MS-MS could be categorized into two classes. In the first class, only one novel protein (cgd7_590) identified in *C. parvum* had *T. gondii* and *N. caninum* homologs. The sequences of the *C. parvum* cgd7_590 protein shared 40% and 34% identity with TgROP17 and NcROP20, respectively. The cgd7_590 protein was located on chromosome VII and contained the signature Ser/Thr kinase domain just like TgROP16, TgROP17, and TgROP18. There are few studies on TgROP17, but it shared high structural similarity with TgROP16 and TgROP18 of the TgROP2 superfamily (24, 25). With regard to domain structures, TgROP17 and TgROP18 contained a conserved catalytic triad and showed excellent conservation of other key residues (26). Moreover, TgROP17 showed stronger conservation within the ATP-binding clefts like TgROP16 and TgROP18, which were related to kinase activity (27). As we all know, TgROP16 and TgROP18 are the main virulence factors with kinase activity (14, 28). TgROP18 remains at the PVM (29); however, TgROP16 is translocated into the host cell nucleus (14). It is highly possible that TgROP17 will work as a functional kinase for regulating the virulence of *T. gondii* from this point. On the other hand, the cgd3_3230 protein contained an Ser/Thr kinase domain and seemed to be a functional kinase as well.

In the other class, some proteins with domains similar to that of *T. gondii* rhoptry proteins were identified. One putative RON protein (cgd3_1540) identified in *C. parvum* located on chromosome III contained two signal peptides in the N-terminal, two internal repeats, and several low complexities like TgRON 4, a secreted rhoptry neck protein of *T. gondii* that migrates at the moving junction in association with TgAMA1 during invasion (17). The TgRON4 protein also contains two amino acid repeats that may have particular importance to the function of their trafficking to the rhoptries. The cgd6_3870 protein was located on chromosome VI and had 12 transmembrane domains, similar to TgROP14. TgROP14 lacks an apparent signal peptide but contains multiple predicted transmembrane do-

main, a feature common to other *Toxoplasma* proteins that have been demonstrated to enter the secretory pathway in the absence of a signal peptide (30). At the same time, the cgd3_590 protein contained DUF900, a hydrolase-like domain. In addition to the signal peptide and transmembrane region, the cgd2_450 protein was the owner of a serine- and proline-rich domain and a possible low molecular weight mucin glycoprotein locus of six genes. These functional structures above were similar to those of other known rhoptry proteins, but their detailed functions remain unknown.

Prior to this research, only one *C. parvum* rhoptry protein candidate (cgd8_2530) related to TgRON1 of *T. gondii* and PfASP of *P. falciparum* had been reported. However, this protein was not detected in the present study. Naturally, several peptides from *C. parvum* rhoptry were not identified due to fewer total proteins or their low abundance. The identification of several non-rhoptry proteins proved that rhoptry-enriched fractions possibly contain other proteins. In *T. gondii* (31), *E. tenella* (32) and *N. caninum* (21), different degrees of contamination (such as dense granules) (6) were also observed in the secretory organelle-enriched fractions.

In this study, 22 novel proteins were identified by LC/MS-MS from purified rhoptry fractions of *C. parvum*. The molecular weights of the 18 proteins from part B were between 25–260 kDa, while those from part A were >260 kDa. Due to protein abundance and other reasons, some proteins were not detected. However, the present work provided a starting point for the study of the repertoire and function of *C. parvum* rhoptry proteins. Nonetheless, further studies must be conducted to confirm their characteristics, localization within rhoptries, synergistic effect with other proteins, and functional role in the *C. parvum* life cycle.

Conclusion

Twenty-two potential novel rhoptry proteins were detected from *Cryptosporidium parvum* rhoptry-enriched fractions by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis

followed by liquid chromatography coupled with mass spectrometry analysis. These novel candidate proteins may be considered targets for researching the invasion pathway of *C. parvum* and the pathogenic mechanisms of rhoptry proteins.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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