Pakistan J. Zool., vol. 52(2), pp 649-658, 2020. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20190707110736

Proteomic Analysis by Shotgun LC-MS/MS of Cyst Wall Protein Components From the Protozoan Parasite *Cryptocaryon irritans*, the Causative Agent of White Spot Disease in Marine Fish

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Article Information
Received 07 June 2019
Revised 12 July 2019
Accepted 19 July 2019
Available online 27 January 2020

Authors' Contribution
HW and FY conceived and designed the study. HW, SP, SS, KW performed experiments, analyzed the sequencing data and interpreted the data. HW wrote the manuscript. FY helped in interpreted the data and wrote the paper.

Key words
***Cryptocaryon irritans*, Protozoan parasite, Cyst wall, Proteomics, LC-MS/MS.**

ABSTRACT

To illustrate the protein composition of the *Cryptocaryon irritans* cyst wall, a shotgun-liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was applied and all identified proteins were categorised using a bioinformatics approach. The results showed that 3,472 proteins were identified with a theoretical molecular weight of 11.53–610.90 kDa and an isoelectric point of 4.13–11.77. A total of 63.07% or 50.09% proteins were annotated by Kyoto Encyclopaedia of Genes and Genomes (KEGG) or Gene Ontology (GO) terms, respectively. These results highlight that mass material is transported and catabolised and growth- and death-related proteins are highly expressed, including a number of vesicle-mediated transport, cytoskeletal and extracellular secretion-related proteins. These results provide a platform to study tomont cell characteristics and provide a further understanding of cyst wall formation and mechanisms.

INTRODUCTION

Cryptocaryon irritans, a parasitic ciliate that penetrates the epithelium of the gills, skin, and fins of marine fish, causes acute mortality in cultured fish. The tomont of *C. irritans* is an important stage for cell division and proliferation. Therefore, it is necessary to cut off the life cycle before the parasites proliferate and reinfect the host.

The cyst wall is the cell's 'armour', which prevents a large number of drugs from penetrating the cell, which may otherwise cause damage to the protoplasm (Dan, 2006; Zahid et al., 2018). Ultrastructural observations show that the thickness of the *C. irritans* cyst wall is about 4 μm, including a multi-layered structure composed of three regions with different densities but with similar forms (Ma *et al.*, 2017). The chemical composition of the *C. irritans* cyst wall, however, is not known. The chemical composition of the protozoan cyst wall is mainly protein and polysaccharides (Calvo *et al.*, 2003). Furthermore, as the structural and regulatory proteins of the cyst wall of parasitic protozoa are often used as targets for screening and designing new anti-parasite drugs (Jarroll and Sener, 2003; Aguilar-Díaz *et al.*, 2011), identifying the *C. irritans* cyst wall proteins will be helpful to screen and develop drugs that inhibit cyst wall formation.

Shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a technique used to determine proteins in complex systems. This method increases the probability that a protein of low abundance is identified. This technique has been widely used in many fields of life science (Aggarwal *et al.*, 2006), especially proteomics, including for large-scale screening of different proteins in *Pseudourostyla cristata* (Gao *et al.*, 2015). In the present study, shotgun- LC-MS/MS and bioinformatics analysis techniques were used to identify the protein components of the *C. irritans* cyst wall.

MATERIALS AND METHODS

Cryptocaryon irritans cyst wall

C. irritans were isolated from naturally infected *Larimichthys crocea*, and the same species of fish with an

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0030-9923/2020/0002-0649 $ 9.00/0

average body mass of 100 g were used as animal models to establish the passage system (Yin *et al.*, 2018). A large number of tomonts adhered to the bottom of the aquaria (1062-L, Φ_{bottom}=130 cm ×H=80 cm) four days after the infection. The fish were transferred to another clean aquarium, and residual tomonts were collected by carefully discarding the debris. The tomonts were incubated in a one liter beaker. The theronts were released about -four days later, and the empty cyst wall was harvested under a microscope. Approximately 1.5×10^4 empty cyst walls were gathered and combined in a 1.5 ml centrifuge tube. The empty cyst walls were washed with three times in fresh seawater and clean empty cyst walls (Fig. 1) were stored at −80°C before the proteomic analysis.

Seawater salinity and temperature for aquaculture were 29%–31% and 28±1°C, respectively.

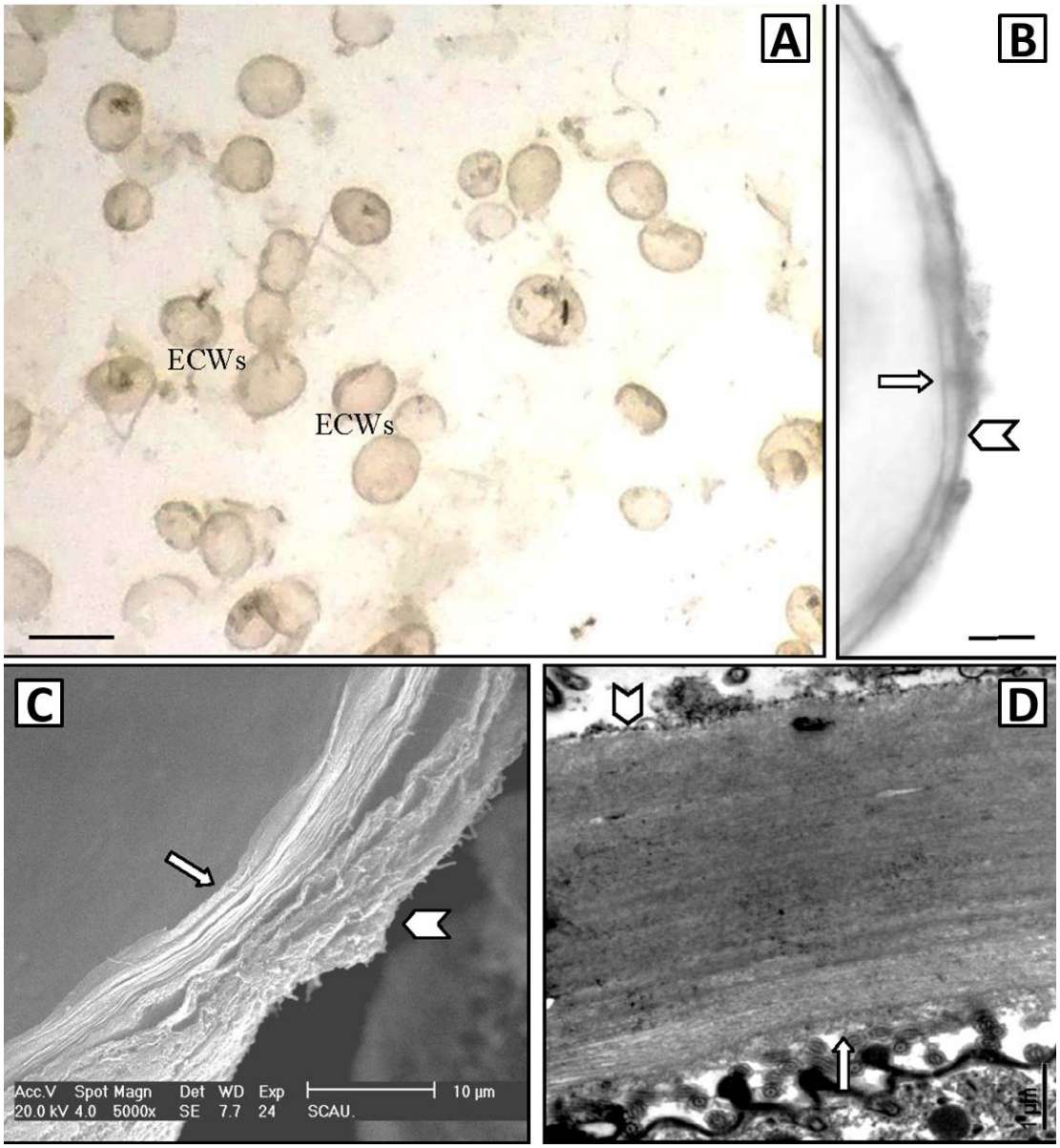

Fig. 1. Cyst wall of *Cryptocaryon irritans* viewed by an optical microscope (A, B), SEM (C), and TEM (D). A, a holistic view of empty cyst walls; B, partial view of empty cyst walls; C, cyst wall stripped off in SEM sample preparation, showing the multi-layer structure of the thick cyst wall; D, sectional view of the cyst wall. ECWs represent empty cyst wall. Arrows indicate the inner surface of cyst wall, and Arrowheads indicate the outer surface of the cyst wall. Scale bars = 500 µm (A), 10 µm (B), 10 µm (C), and 1 µm (D).

Protein extraction

The frozen clean empty cyst walls were transferred to tubes and ground with SDT lysis buffer (pH 8.5, 4% SDS, 100 mM Tris-HCl and 100 mM DL-dithiothreitol (DTT) using the MP FastPrep-24 5G homogeniser (MP Biomedical, Santa Ana, CA, USA). The homogenised cyst wall samples were incubated in a buffer of six M urea containing 2% SDS and 5 % β-mercaptoethanol at 100°C for 10 min to extract the cyst wall proteins. Subsequently, the cyst wall proteins were concentrated using an Ultrafree-0.5 centrifugal filter device with a high-flux Biomax-100 membrane (Millipore, Bedford, MA, USA). First, 500 µl of solubilised protein sample was added to the tube and centrifuged at 14,000×g for 30 min; the column was inserted into a new tube and centrifuged at 1,000×g for two min. Finally, 20 µl (20 mg/ml) of concentrated protein was obtained (Wang *et al.*, 2017). The protein content in the supernatant was quantified by the Bradford method (Bradford, 1976). Aliquots were stored at −80°C until use.

Cyst wall protein enzymolysis

The filter-aided proteome preparation method was used for enzymolysis of the cyst wall proteins. Total-protein samples (200 µg) diluted in 100 mM DTT solution to a concentration of 100 mg/mL were boiled for five min. The samples were left to cool to 25°C and were then loaded into a 10 kDa ultrafiltration tube (Sartorius, Goettingen Germany), followed by adding 200 µL UA buffer (8 M urea and 150 mM Tris-HCl pH 8.0) to the tube and centrifugation at 14,000×g for 15 min, followed by a wash in UA buffer. Then, 100 µL iodoacetamide (100 mM IAA in UA buffer) was added to block reduced cysteine residues, and the samples were incubated for 30 min in the dark. The filters were washed three times with 100 µL UA buffer and then twice with 100 µL 25 mM NH_4HCO_3 buffer. Finally, the protein suspensions were digested with 4 µg trypsin (Promega, Madison, WI, USA) in 40 µL of 25 mM NH_4HCO_3 buffer overnight at 37°C, and the resulting peptides were collected as the filtrate. The peptides in each sample were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL; Sigma, St. Louis, MO, USA), concentrated by vacuum centrifugation, and reconstituted in 40 µL of 0.1% (v/v) formic acid. Finally, the concentration of the resulting peptide was analysed at OD280, and aliquots of the samples were freeze-dried for the proteomic analysis.

LC-MS/MS analysis

Peptide mixtures from the above extraction were separated by reverse phase-high-performance liquid chromatography (RP-HPLC) followed by tandem MS analysis. RP-HPLC was carried out using a surveyor EASY-nLC system (Thermo Finnigan, San Jose, CA, USA). The fractions were thawed and dissolved in buffer A (0.1% v/v formic acid in Milli-Q water). The column was equilibrated for 20 min with 95% (v/v) buffer A. The peptide mixtures were separated using a Thermo Scientific EASY column (2 cm × 100 µm 5 µm-C18) and a Thermo Scientific EASY column (75 µm × 100 mm 3 µm-C18) at a flow rate of 300 nL/min. The peptides were separated with solvent B [acetonitrile with 0.1% (v/v) formic acid] using a segmented gradient from 4–9% (v/v) in one min, from 9–50% in 54 min, from 50–90% (v/v) in one min and then at 90% (v/v) for 20 min.

A Q-Exactive (Thermo Finnigan) mass spectrometer was set to perform data acquisition in the positive ion mode, with a selected mass range of 300–1,800 mass/charge (m/z). Resolving power for the Q-Exactive was set to 70,000 for the MS scan and 17,500 for the MS/MS scan at m/z 200. MS/MS data were collected using the top 20 most abundant precursor ions with charge ≥ 2 as determined from the MS scan. The maximum ion injection times for the survey scan and the MS/MS scans were 50 and 60 ms, respectively, and the automatic gain control target values for both scan modes were set to 3×10^6. These parameters were selected with an isolation window of 2 m/z and fragmented by higher-energy collisional dissociation with normalised collision energy of 27 eV. Dynamic exclusion for selected precursor ions was set to 60 s and the under ratio on the Q-Exactive was 0.1%.

Mass spectrometry data analysis and functional annotation of the proteins

Raw files were searched using MASCOT 2.2 software for database retrieval against the protein database (uniprot_Ciliophora_169050_20171127.Fasta, http://www.uniprot.org). The following search parameters were set: trypsin as the enzyme; missed cleavage sites were set to 2; fixed modification was carbamidomethyl (C); dynamic modification of setting oxidation (M). Blast2Go (https://www.blast2go.com/) software was used to screen the differentially expressed proteins in the Gene Ontology (GO) functional annotations. The expression levels of the proteins were annotated using a KO analysis (http://www.genome.jp/kegg/tool/map_pathway2.html). Kyoto Encyclopaedia of Genes and Genomes automatic annotation server (KASS) was used for the default parameters to perform the pathway annotation.

RESULTS AND DISCUSSION

Cyst wall proteins tandem mass spectrometric analysis

C. irritans cyst wall proteins were excised, digested and analysed by shotgun LC-MS/MS. The *C. irritans* cyst

wall proteins were identified by searching the Ciliophora database at the NCBI; 3,472 proteins were identified with mascot scores ≥ 20, of which 1,194 proteins had a unique peptide value ≥ 2 (Supplementary Table I). All 3,472 proteins were distributed in a range of molecular weights of 5.72–998.30 kDa and isoelectric points of 4.13–11.77, according to the spectrometric analysis (Figs. 2, 3; Supplementary Table I).

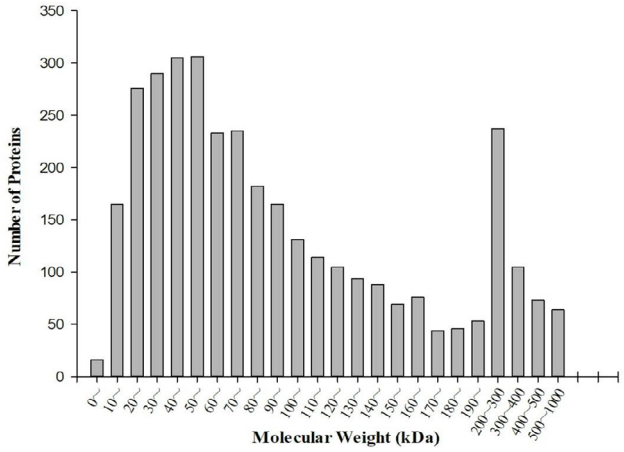

Fig. 2. Distributions of molecular mass (MW) for cyst wall of *Cryptocaryon irritans* by the shotgun LC-MS/MS approach.

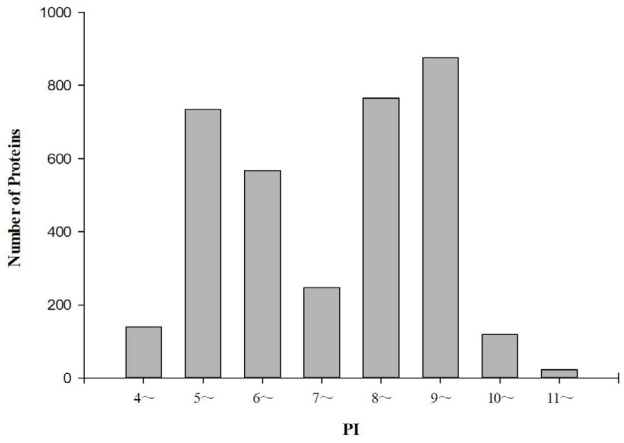

Fig. 3. Distributions of isoelectric point (pI) for cyst wall of *Cryptocaryon irritans* by the shotgun LC-MS/MS approach.

GO and KEGG categories of C. irritans cyst wall proteins

In this study, a total of 1,739 proteins (50.09 %) (Table I) were assigned predicted GO terms (Fig. 4, Supplementary Table II). These terms were summarized into 2167 sub-categories under three GO terms corresponding to the biological process (BP) category (1248), cellular component (CC) category (299), and molecular function (MF) category (620). In the BP category, the top 20 sub-categories were above 35 %, which proteins most enriched, included cellular process, several metabolic process, organism process, macromolecule modification, localization, and phosphorylation. In the MF category, catalytic activity, cyclic compound binding, ion binding, small molecular binding, nucleotide binding, and hydrolase activity were most enriched sub-categories, were above 52% of this part. In the CC category, the cell, memebrane, intracellular, organelle, cytoplasm, macromolecular complex, and cytoskeleton were the most enriched parts, were above 65%.

Table I.- Annotation of proteins of proteomic profiles of *Cryptocaryon irritans* cyst wall.

Database	Number of annotated proteins	Percentage of annotated proteins in protein group
KO	2,499	71.98%
GO	1,739	50.09%
KEGG	2,190	63.07%
Protein group (Total)	3,472	100.00%

The proteins were also categorized using the KEGG database to identify the biological pathways in *C. irritans* cyst wall proteins. A total of 2,190 proteins (63.07 %) (Table I) were further annotated by KEGG and classified into six categories with 47 subclasses (290 known KEGG pathways) (Fig. 5).

Studies on the components of the cyst wall have deepened. As early as the 1960s, researchers used cytochemical labelling to suggest that the *Entamoeba* cyst wall is composed of a carbohydrate-protein complex (McConnachie, 1969). In 1980, biochemical analysis, fluorescence labelling, ultrastructural observations and X-ray diffraction were combined to confirm that chitin is the main component of the *E. invadens* cyst wall (Arroyo-Begovich *et al.*, 1980). In recent years, studies have demonstrated that the cyst wall of protozoa is mainly composed of proteins and polysaccharides (Jarroll and Sener, 2003), and the protein components of the cyst walls of a variety of protozoa have been identified. For example, SDS-PAGE has been used to isolate small molecular proteins (Rios *et al.*, 2012); MS analysis of purified *E. invadens* cyst walls revealed chitinase and two lectins (Samuelson and Robbins, 2011); eight SDS-PAGE strips were analysed by MALDI-TOF MS, and 42 proteins were found in the *E. encysticus* cyst wall (Wang *et al.*, 2017). In the present study, 3,472 proteins were identified by LC–MS/MS.

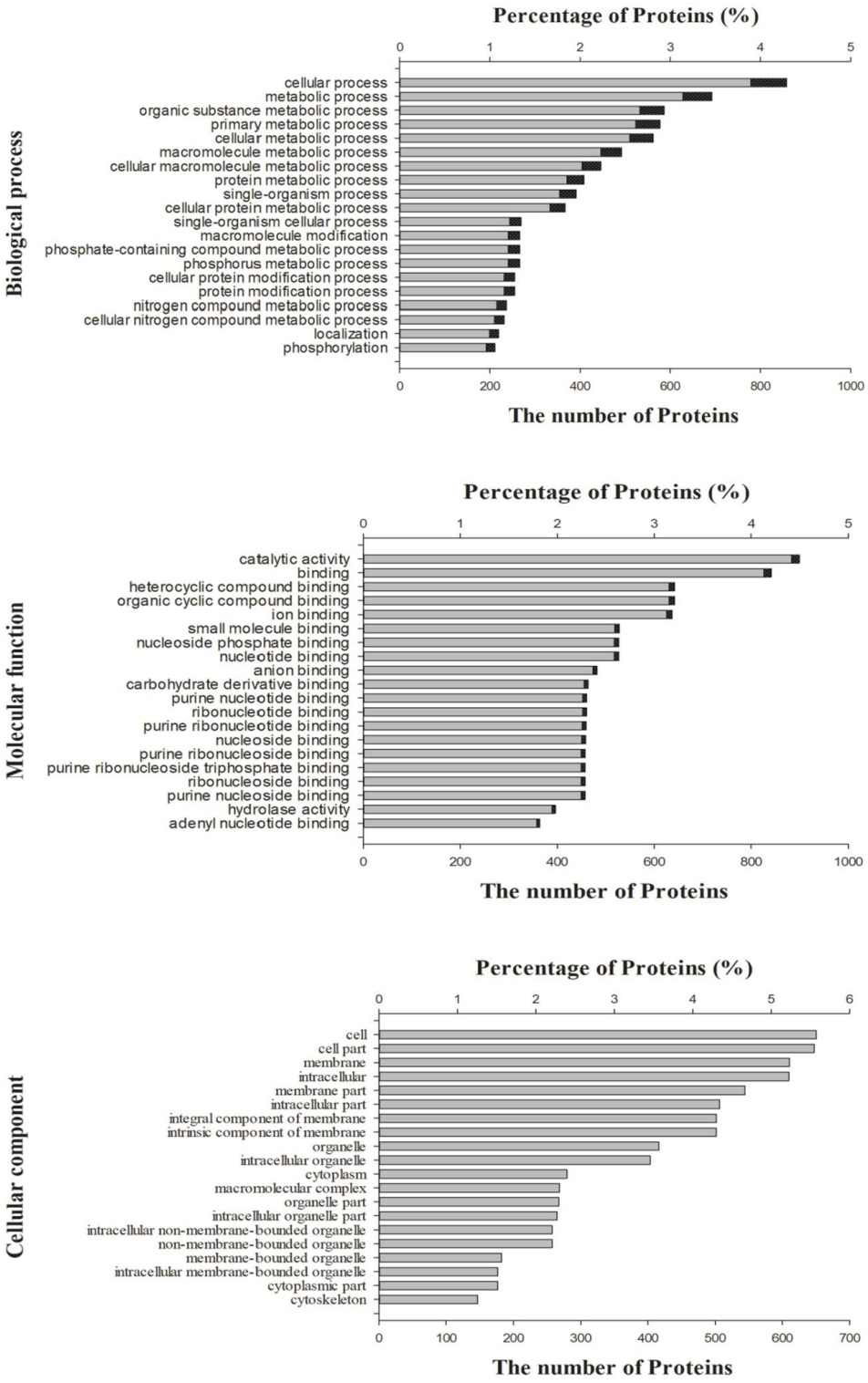

Fig. 4. GO categories of *Cryptocaryon irritans* cyst wall proteins. These terms were summarized into 2167 sub-categories under 3 GO terms. Bar charts represented the top 20 sub-categories of the biological process (BP) category, cellular component (CC) category, and molecular function (MF) category, respectively. Grey bars showed the number of proteins, the black bars indicated the percentage of proteins.

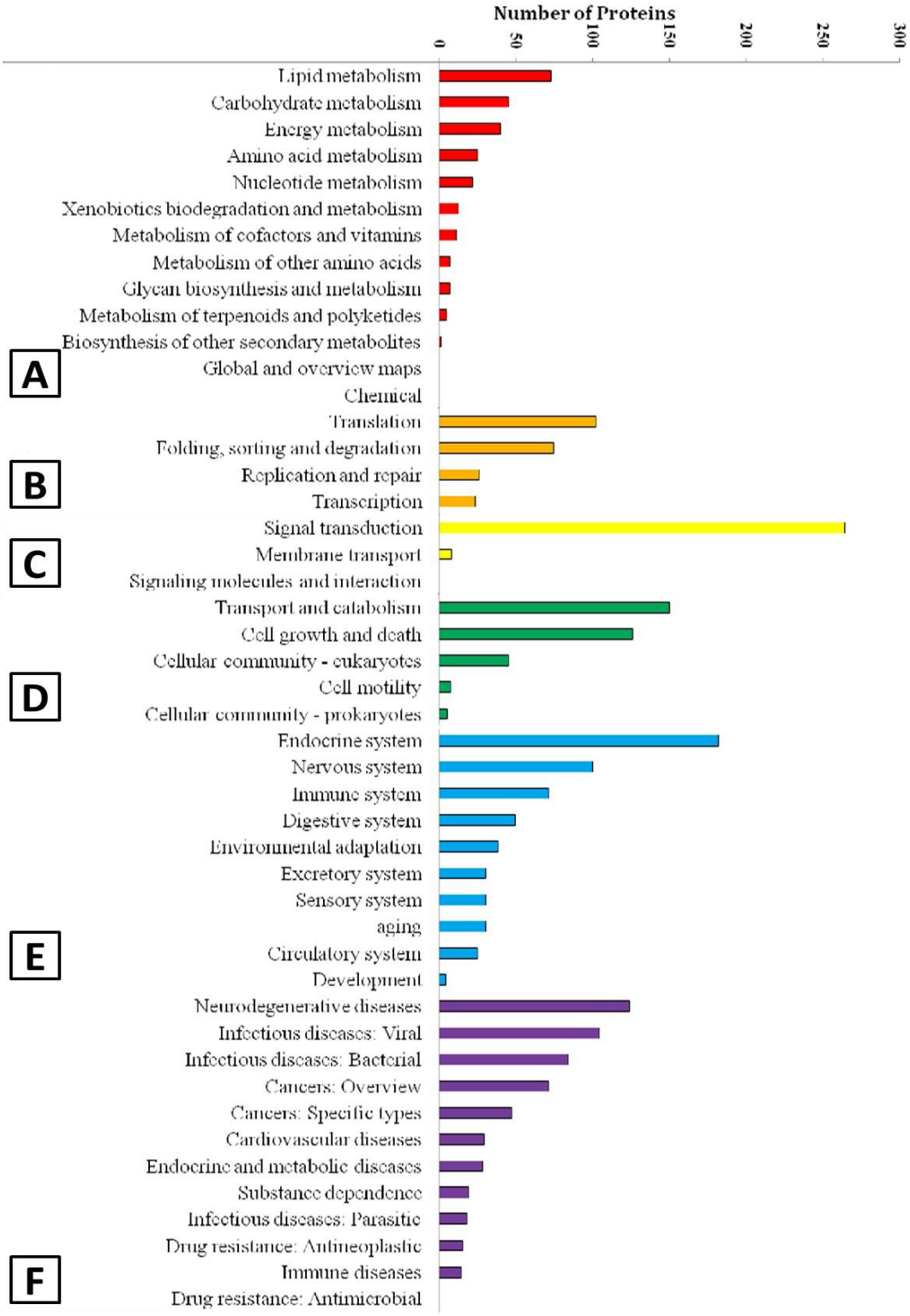

Fig. 5. KEGG categories of *Cryptocaryon irritans* cyst wall proteins. A total of 2,190 proteins were annotated by KEGG and classified into 6 categories with 47 subclasses. The upper cases represent the title of 6 categories. A, metabolism; B, genetic information processing; C, environmental information processing; D, cellular processes; E, organismal systems; F, human diseases.

Table II.- The top 1% confident proteins in turn following unique peptide count.

	Reference	Unique pep count
1	**Agglutination/immobilization antigen** OS=*Cryptocaryon irritans* PE=2 SV=1	22
2	**Agglutination/immobilization antigen** OS=*Cryptocaryon irritans* PE=2 SV=1	20
3	**Surface antigen** OS=*Cryptocaryon irritans* PE=2 SV=1	19
4	**Immobilization antigen** (Fragment) OS=*Cryptocaryon irritans* PE=2 SV=1	17
5	**Agglutination/immobilization antigen** OS=*Cryptocaryon irritans* GN=iAg PE=4 SV=1	14
6	**ADP/ATP carrier** OS=*Cryptocaryon irritans* PE=2 SV=1	14
7	**Tubulin beta chain** OS=*Tetrahymena thermophila* PE=1 SV=1	13
8	**Agglutination/immobilization antigen** (Fragment) OS=*Cryptocaryon irritans* PE=2 SV=1	12
9	**Tubulin beta chain** OS=*Tetrahymena thermophila* GN=BTU1 PE=1 SV=1	12
10	**Tubulin beta chain** OS=*Euplotes focardii* PE=3 SV=1	11
11	**Immobilization antigen** (Fragment) OS=*Cryptocaryon irritans* PE=2 SV=1	10
12	**Tubulin beta chain** (Fragment) OS=*Sorogena stoianovitchae* GN=sbt PE=2 SV=1	10
13	**Tubulin beta chain** OS=*Euplotes crassus* PE=3 SV=1	10
14	**Actin** (Fragment) OS=*Metopus es* PE=3 SV=1	10
15	**Actin** (Fragment) OS=*Heterometopus palaeformis* PE=3 SV=1	10
16	**Actin** (Fragment) OS=*Metopus es* PE=3 SV=1	10
17	**Agglutination/immobilization antigen isoform 1** OS=*Cryptocaryon irritans* PE=2 SV=1	9
18	**Agglutination/immobilization antigen isoform 3** (Fragment) OS=*Cryptocaryon irritans* PE=2 SV=1	9
19	**Agglutination/immobilization antigen isoform 5** (Fragment) OS=*Cryptocaryon irritans* PE=2 SV=1	9
20	**Uncharacterized protein** OS=*Paramecium tetraurelia* GN=GSPATT00035237001 PE=4 SV=1	9
21	**Tubulin beta chain** (Fragment) OS=*Chilodonella uncinata* GN=btub PE=3 SV=1	9
22	**Tubulin beta chain** (Fragment) OS=*Colpoda* sp. PE=3 SV=1	9
23	**Tubulin beta chain** OS=*Stylonychia lemnae* GN=TUBB1 PE=3 SV=1	9
24	**Tubulin beta chain** OS=*Gastrostyla steinii* PE=3 SV=1	9
25	**ATP synthase subunit alpha** OS=*Stentor coeruleus* GN=SteCoe_10017 PE=3 SV=1	9
26	**ATP synthase subunit alpha** OS=*Paramecium tetraurelia* GN=GSPATT00039376001 PE=3 SV=1	9
27	**Tubulin alpha chain** OS=*Tetrahymena thermophila* PE=1 SV=1	9
28	**Tubulin alpha chain** (Fragment) OS=*Frontonia* sp. PE=3 SV=1	9
29	**Tubulin alpha chain** OS=*Paramecium tetraurelia* GN=alphaPT4 PE=3 SV=1	9
30	**ATP synthase subunit beta** OS=*Stylonychia lemnae* GN=Contig4879.g5215 PE=3 SV=1	9
31	**Tubulin beta chain** OS=*Stentor coeruleus* GN=BTU01 PE=3 SV=1	8
32	**Tubulin beta chain** OS=*Stentor coeruleus* GN=BTU05 PE=3 SV=1	8
33	**Tubulin beta chain** (Fragment) OS=*Myrionecta rubra* PE=2 SV=1	8
34	**Tubulin beta chain** (Fragment) OS=*Nyctotherus ovalis* PE=3 SV=1	8
35	**Tubulin beta chain** (Fragment) OS=*Chilodonella uncinata* GN=Btub PE=3 SV=1	8

If the unique peptide count was considered a reference indicator for the confidence of correctly identifying the proteins, the top 1% (35 in 3,472) of proteins were antigens, ATP/ADP transfer/creation-related proteins, and cytoskeleton-related proteins (tubulins and actin) (Table II). In addition, the top 100 proteins also included glyceraldehyde-3-phosphate dehydrogenase, heat shock proteins, ubiquitin, elongation factor (EF) 1-α, p-loop containing nucleoside triphosphate hydrolase, histone H4, calmodulin 1, fat domain-containing protein, transitional endoplasmic reticulum ATPase, histone variant H3.5 and preprotein translocase subunit-like protein (Supplementary Table I). It proved that the cytoskeleton-related proteins comprise the major components and protect against adverse environmental conditions in the resting cyst wall of *E. encysticus* (Wang *et al.*, 2017). Agglutination/immobilisation and surface antigens have been identified in all stages of the *C. irritans* life cycle (Huang *et al.*, 2012; Lin *et al.*, 2013). Fish immunised with *C. irritans* tomonts with a cyst wall produce specific antibody (Bai *et al.*, 2008), suggesting that antigens are present in the cyst wall. Heat shock proteins are important housekeeping genes and are involved in encystation of parasitic protozoa when they encounter a hostile environment (Wang *et al.*, 2012). ADP glycosylation factor-ARFs in *Pseudvurostyla cristata* cells are related to intracellular transport and

extracellular polymerisation of cyst wall materials (Gao *et al.*, 2015). The cyst wall protein-EF-Tu of *C. cucullus* is related to the formation of a fibrous or network-like structure of the scale layer of its cyst wall (Funadani *et al.*, 2016). Feeding RNAi to silence the EF-1α gene suppresses the formation of resting cysts in *C. cucullus* (Sogame *et al.*, 2016).

Structural and regulatory proteins of the cyst wall are often targets for screening and designing new drugs (Jarroll and Sener, 2003). For example, the formation of a cyst wall requires the synthesis of a large number of proteins, and puromycin is a protein synthesis inhibitor; a 50 μg mL^{-1} dose can cause failure of the *C. cucullus* cyst wall to form (Funadani *et al.*, 2016). β-1,3-glucan is a component of the oocyst wall of *Toxoplasma* and *Eimeria*, and biosynthesis of glucans is regulated by glucan-hydrolase/synthase. This enzyme is also a target of echinocandins, which inhibit formation of the cyst wall by interfering with glucan-hydrolase/synthase activity (Bushkin *et al.*, 2012). Synthesis of *E. invadens* cysts relies on chitin synthase and chitinase, while inhibitors (poly D, nikkomycin and allosamidin) of these enzymes lead to failed cyst formation (Ghosh *et al.*, 1999). Protein kinases, as an important drug target, are also widely regarded in the control of parasitic protozoa, and different protein kinases inhibitors (staurosporine, chelerythrine chloride and Calphostin C) exhibit an inhibitory effect on encystation (Samanta *et al.*, 2018). Human Glrac has been considered a target for designing anticancer drugs (Aznar and Lacal, 2001). Because Glrac regulates the trafficking and secretion of cyst wall proteins in the protozoa *Giardia*, it has also been used as a potential target to develop new anti-parasite drugs (Krtková *et al.*, 2016). In this study, eight nuclear transport-related proteins (40S ribosomal protein SA, GTP-binding nuclear protein, transmembrane proteins, N-acetyltransferase B complex non-catalytic subunit, GTP-binding nuclear protein ran and nucleoporin nup155) were found in the *C. irritans* cyst wall. Our previous study confirmed that the nuclear transporter inhibitor leptomycin B effectively induces *C. irritans* theronts and tomontsdeath (Yin *et al.*, 2016). In addition, a number of cyst wall formation-related proteins, particularly some proteins peculiar to the tomonts of the *C. irritans*, were identified (Supplementary Table II) (Mai *et al.*, 2015). These proteins will provide a basis for researchers to develop anti-*C. irritans* drugs.

Table III.- Vesicle-mediated transport-related proteins.

	Reference	MW (kDa)	PI
1	**Dynein heavy chain 1, axonemal protein** OS=*Tetrahymena thermophila* (strain SB210) GN=TTHERM_00558640 PE=4 SV=2	487.70	5.79
2	**WD40-repeat-containing domain** OS=*Pseudocohnilembus persalinus* GN=PPERSA_00474 PE=4 SV=1	222.44	8.9
3	**Zinc-binding dehydrogenase family oxidoreductase** OS=*Tetrahymena thermophila* (strain SB210) GN=TTHERM_00384890 PE=4 SV=2	201.13	6.07
4	**Intraflagellar transport protein, putative** OS=*Ichthyophthirius multifiliis* (strain G5) GN=IMG5_148350 PE=4 SV=1	198.15	6.55
5	**Clathrin heavy chain** OS=*Ichthyophthirius multifiliis* (strain G5) GN=IMG5_187840 PE=3 SV=1	198.04	5.97
6	**Uncharacterized protein** OS=*Paramecium tetraurelia* GN=GSPATT00020489001 PE=4 SV=1	133.07	7.81
7	**Transmembrane protein, putative** OS=*Tetrahymena thermophila* (strain SB210) GN=TTHERM_00923100 PE=4 SV=3	113.30	8.2
8	**Adaptin amine-terminal region family protein** OS=*Tetrahymena thermophila* (strain SB210) GN=TTHERM_00155580 PE=4 SV=3	113.00	5.73
9	**Phospholipase** OS=*Paramecium tetraurelia* GN=GSPATT00019109001 PE=3 SV=1	112.86	8.67
10	**Coatomer subunit gamma** OS=*Tetrahymena thermophila* (strain SB210) GN=TTHERM_00444320 PE=3 SV=1	105.39	5.47
11	**Adaptin amine-terminal region family protein** OS=*Tetrahymena thermophila* (strain SB210) GN=TTHERM_00287890 PE=4 SV=2	100.45	5.8
12	**Coatomer subunit beta'** OS=*Stentor coeruleus* GN=SteCoe_76 PE=3 SV=1	97.81	5.08
13	**Coatomer subunit beta** OS=*Pseudocohnilembus persalinus* GN=PPERSA_03040 PE=4 SV=1	97.32	5.92
14	**Uncharacterized protein** OS=*Pseudocohnilembus persalinus* GN=PPERSA_05656 PE=4 SV=1	91.97	5.3
15	**Phospholipid-transporting ATPase** OS=*Ichthyophthirius multifiliis* (strain G5) GN=IMG5_078220 PE=3 SV=1	91.56	8.21
16	**Transport protein** OS=*Stylonychia lemnae* GN=Contig1948.g2110 PE=4 SV=1	86.28	5.74
17	**Sec1-like protein** OS=*Pseudocohnilembus persalinus* GN=PPERSA_07320 PE=3 SV=1	69.11	6.2
18	**Uncharacterized protein** OS=*Stentor coeruleus* GN=SteCoe_14443 PE=3 SV=1	62.67	5.33
19	**Uncharacterized protein** OS=*Paramecium tetraurelia* GN=GSPATT00026361001 PE=3 SV=1	50.81	8.54
20	**Mu1 adaptin** OS=*Oxytricha trifallax* GN=OXYTRI_04462 PE=3 SV=1	50.28	8.65
21	**Uncharacterized protein** OS=*Paramecium tetraurelia* GN=GSPATT00015008001 PE=4 SV=1	48.82	6.45
22	**Uncharacterized protein** OS=*Stentor coeruleus* GN=SteCoe_5626 PE=4 SV=1	23.96	9.1

ACKNOWLEDGEMENTS

The authors gratefully acknowledge funding support from the Natural Science Foundation of Shanghai (Grant No. 17ZR1439600), the Natural Science Foundation of Zhejiang Province (Grant No. Y18C190005), and the K.C. Wong Magna Fund in Ningbo University.

Supplementary material

There is supplementary material (Tables I and II) associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190707110736

Statement of conflict of interest

The authors declare no conflict of interest.

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