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Identification of cyst wall proteins of the hypotrich ciliate *Euplotes encysticus* using a proteomics approach

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Euplotes encysticus **is a species of Hypotrich ciliates, which form cyst wall by secreting the special substances on encounter of adverse environment. It has critical significance to study the component and mechanism underlying resting cyst, during resisting unfavorable conditions in dormancy induction. The present study was aimed to investigate the effects of cyst wall proteins of *Euplotes encysticus* by using biochemical methods. Therefore, protein extracts were separated by SDS-PAGE, identified and analyzed by MALDI-TOF MS and Bioinformatics tools. We detected 42 cyst wall proteins, 26 were functional proteins and 16 proteins consist of unknown function; which is consistent with cyst wall specificity. These results partially revealed the components of resting cyst wall formed after the cells differentiation of *Euplotes encysticus*. In addition, our data suggested that the function of cyst wall proteins are more likely involved in the mechanical protection, signal transduction, material transport, protein degradation and energy metabolism to survival, with potentially importance implications in the molecular mechanism of eukaryocyte dormancy under stress condition.**

Keywords: *Euplotes encysticus*, encystment, cyst wall proteins, mass spectrum identification, fluorescence taxtoid

Introduction

As is well known, ciliate protozoa have a special physiological status to form the resting cyst when they are encounter adverse environment. A series of physiological and morphological changes occur during the stress condition such as: starvation, sharp temperature changes and high-density. These changes include drastic cytoplasmic dehydration, cells shrinking and turning around, forming cyst wall. However, excystment will occur when the external environmental conditions become suitable (Liu *et al.*, 2009; Verni and Rosati, 2011). This survival strategy is basically associated with the dormant mechanism, which is an important part of ciliates system biology and developmental biology. Study on ciliates dormant phenomenon is also one of the most important aspects of researching the eukaryotic cell mechanisms of functional regulation and resistance to adversity.

Notably, the characteristics of the encystment are sharply changed the cell morphological structure. Meanwhile, alternation in the cell morphological structure is inevitably accompanied by the rapid change in the proteins expression. Therefore, one of the tasks for revealing the encystment mechanism is identification of the encystment-related proteins. In our previously study, we focused on screening and identifying the differential expression proteins in the cytoplasm and nucleus of the cyst comparing with the vegetative cell. Namely, pointed out the encystment-related proteins, targeting the cytoplasm and nucleus of the cyst (Chen *et al.*, 2014).

It is believed that encystment play an important role in the formation of spherical and protected cyst wall (Grisvard *et al.*, 2008). The emergence of cyst wall is mainly hallmark of cyst formation. The cyst wall pursuing as the barrier and/or door for the resting cysts; in contrast to the barrier, protecting cell against adverse environmental factors, maintaining substances exchanges between resting cysts and their surroundings. So far, most of the work on the resting cyst are mainly descriptive and lack of biological role and interaction; nevertheless, some structural and morphological associations are good studies and characterized on the microscopic and submicroscopic level. Unfortunately, information on the biochemical composition of resting cyst wall is very scarce and most studies focused on the parasitic protozoa cyst wall (Aguilar-Díaz *et al.*, 2011; Samuelson and Robbins, 2011). Although, the cyst wall composition of some free-living protozoa have been reported, such as *Opisthonecta henneguyi* (Calvo *et al.*, 2003), *Histriculus cavicola* (Calvo and Miguel, 1996), *Laurentia acuminate* (Gutierrez *et al.*, 1983). These studies showed that major components of cyst wall are proteins, carbohydrates and glycoproteins. Certainly, researchers mainly focus on the carbohydrate, while unclear investigation of cyst wall proteins has been evaluated. To date, little is known about the exact protein components of free-living protozoa cyst wall. Moreover, parasitic protozoa form the cyst wall to avoid digested by the host, whereas free-living ciliates form cyst wall to cope the adverse environmental conditions. Therefore, the cyst wall protein components and functions of these two species of protozoa are significantly different during encystation. In addition, the protein events behind the cyst wall formation of free-living ciliates

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are little known. Here, in this study, cyst wall proteins were extracted from the *Euplotes encysticus* using biochemical methods and separated via SDS-PAGE, identified by MALDI-TOF MS and Bioinformatics technology. Additionally, one-dimensional cyst wall protein maps and proteins were identified. Overall, our study establishes platform for the investigation of cyst wall proteins function in *Euplotes encysticus* and other related organisms, which sheds new light on the basic information of resting mechanism of ciliate and eukaryote organisms. To the best of our knowledge, this is the first proteomic analysis of the cyst wall proteins from free-living ciliates.

Materials and Methods

Culture and encystment induction

Hypotrichous ciliates *Euplotes encysticus* was used as the materials because of its easy culture and induction qualities, which was kind gifted by Prof. Fukang Gu of East China Normal University. Culture and encystment induction of the ciliates was performed as previously reported (Chen *et al.*, 2014). Briefly, the ciliates were cultured in 10 cm dishes with filtered and sterilized water from the pond (Zi zhuyuan in East China Normal University) and fed with cultured *Chilomonas paramecium*. The dishes were incubated at 25°C in the incubator. To spur the formation of cysts, vegetative cells were cultured to a high density, stop feeding and accompanied by low temperature. The cyst formation was established after the 10 days of starvation. Thus, protein extraction and all subsequent investigations were carried out as described below.

Separation and purification of cyst wall proteins

After the formation of cysts, approximately 5×10^6 cysts were transferred to Eppendorf tube (1.5 ml) and centrifuged at 1,000 × g for 5 min at 4°C, then removed the supernatant and resuspended harvest cells in encystation buffer. Repeat twice to ensure that impurities attached to cyst wall were removed. Cyst wall proteins were extracted as described by Izquierdo *et al.* with slight modifications. Briefly, the harvested cells were mixed with 0.5 ml encystation buffer (10 mM Tris-HCl, pH 6.8) and were subjected to intermittently sonication (20 Kc/s, 50 W) for 10 min until the entire population of cyst had been ruptured (Izquierdo *et al.*, 2000). The lysate was centrifuged at 5,800 × *g* for 3 min to separate cyst wall from the cystic cytosol. The pellets were washed with encystation buffer for several times. Then isolated cyst walls were incubated in buffer of 6 M urea containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol at 100°C for 10 min to extract 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). Firstly, 500 µl solubilized protein samples were added to the tube and centrifuged at 14,000 × *g* for 30 min and then the column was inserted into a new tube, centrifuged at 1,000 × *g* for 2 min. Finally, 20 µl (20 mg/ml) concentrated proteins were obtained. The protein sample was stored at -20°C for future use.

SDS-PAGE and MALDI-TOF-MS analysis of the cyst wall proteins

Prior to electrophoresis, protein sample was diluted 10 µg/µl with 10 mM Tris-HCl (pH 6.8), the sample was mixed with an equal volume of 2 × electrophoresis loading buffer (10 mM Tris-HCl, 1% SDS, 20% v/v glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8) and heated at 100°C for 3 min, and cooled down to room temperature. Subsequently, 20 µl sample containing 100 µg cyst wall proteins was applied and separated by SDS-PAGE on 12.5% acrylamide separating gel and 5% acrylamide stacking gels at 80 V for 0.5 h and then 120 V for 2 h (Fig. 1). The prestained protein marker was run in parallel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue (CBB) R-250 (Sigma) for 1 h, and then bleached was eluated (75 ml acetic acid, 50 ml methanol, 875 ml ddH_2O) overnight. After visualization by Coomassie staining, the apparent molecular weight of cyst wall proteins was determined using protein molecular weight markers. Then the gel lanes were cut and subjected to in-gel tryptic digestion as described previously (Shevchenko *et al.*, 1996).

After in-gel digested, the gel lanes were analyzed by combined peptide mass fingerprinting (PMF) and MS/MS as described previously (Yue *et al.*, 2008) with some modifications. Briefly, trypsin digested extracted peptides were subsequently mixed with MALDI matrix (5 mg/ml CHCA diluted in 0.1% TFA/50% ACN) and spotted on to the 192-well stainless steel MALDI target plates. MS measurements were carried out on the ABI 4,800 Proteomics Analyzer with delayed ion extraction (Applied Biosystems). The MS spectra were recorded in the positive reflector mode in a mass range from 800 to 4,000 using a 200-Hz ND: YAG laser operating at 355 nm. For one main MS spectrum 25 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. For MS calibration, autolysis peaks of trypsin ([M + H] + 842.5100 and 2211.1046) were used as internal calibrates, and 10 of the most intense ion signals were selected as precursors for MS/MS acquisition, excluding the trypsin autolysis peaks and the matrix ion signals. In MS/MS mode, 50 sub-spectra with 50 shots per sub-spec-

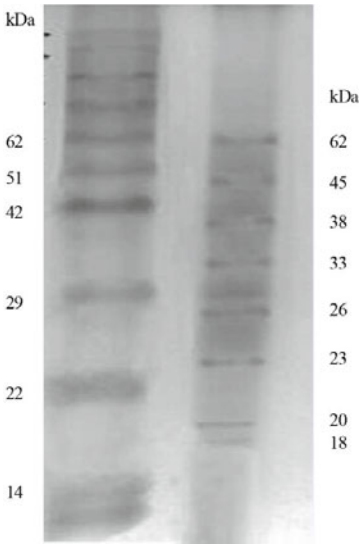

Fig. 1. SDS-PAGE pattern of Cyst wall proteins (CWPs) of *E. encysticus*. The SDS-PAGE image shown was representative of three independent experiments. The sample was separated by 12.5% resolving gel and the gel was stained with Coomassie Brilliant Blue R-250.The left lane is protein marker with molecular weight from 10.5 kDa to 175 kDa and eight gel bands were separated from CWPs.

trum were accumulated for one main MS spectrum using a random search pattern. Using the individual PMF spectra, peptides exceeding a signal-to-noise ratio of 20 that passed through a mass exclusion filter were submitted to fragmentation analysis. The 10 most and 10 least intense ions per MALDI spot, with signal-to-noise ratios > 50 were selected for subsequent MS/MS analysis in the 2-kV mode. Whereas, air served as the collision gas. The data was calibrated using the ABI 4700 Calibration Mixture (Applied Biosystems).

FLUTAX fluorescent labeling method for revealing microtubular cytoskeleton components of the resting cyst wall and the vegetative cell

As our previous reported method (Chen *et al.*, 2014) with slight modification, about 30 μl of the resting cysts and the vegetative cells were added on a clean glass slide respectively. While, the excess water was removed. Then defined amount of saponin (0.05%) was permeated drop wise into the resting cysts or the vegetative cells for 30 sec. After washing with PHEM, the resting cysts or vegetative cells were fixed with 4% paraformaldehyde (PFA) for 1 min and washed once with PHEM. The resting cysts or vegetative cells were stained with 1 μM FLUTAX (Invitrogen) for 6 min in dark, subsequently rinsed with 0.01 M PBS (pH 7.2) for three times. Afterward, the stained resting cysts or the vegetative cells were examined and taken photos by Olympus BX51 fluorescence microscope (excitation wavelength = 492 nm, emission wavelength = 520 nm).

Mass spectrometry data analysis

The obtained MS data were analyzed, searched and identified with GPS 3.6 (Applied Biosystems) and Mascot 2.1 software (http://www.matrixscience.com/). Namely, the obtained data of peptide mass fingerprinting and MS/MS spectrum were comprehensive analyzed with GPS 3.6 and Mascot 2.1 software. The corresponding proteins were searched in NCBI and Uniprot databank. NCBI was searched for the counterpart of MS/MS spectra using GPS-Explorer Software 3.6 with MASCOT as the database search engine, with the following parameter settings: the mass accuracy was 100 ppm, one missed trypsin cleavage was allowed, carbamido methylation was set as a fixed modification, the oxidation of methionine was allowed as a variable modification, and the MS/MS fragment tolerance was set to 0.4 kDa. A GPS Explorer protein confidence index ≥ 95% was used for further manual validation.

Results

Cyst wall proteins electrophoresis

We performed three independent experiments for biochemistry extracts, electrophoresis separation and mass spectrum identification of cyst wall proteins of *Euplotes encysticus*. All the samples of the cyst wall proteins were separated by SDS-PAGE electrophoresis. Representative electrophoresis spectrum of the cyst wall proteins was showed in Fig. 1. The electrophoresis spectrum presented 8 protein bands with a molecular weight of 18, 20, 23, 26, 33, 38, 45, and 62 kDa according to the protein marker. Subsequently electrophoretic gel bands were in-gel digested, following by tandem mass spectrometric analysis.

Table 1. **The cyst wall proteins of *E. encysticus* identified by MALDI-TOF-MS**

Band	Protein name	Accession No.	Protein MW	Protein PI	Pepcounts	Protein score	Protein score C. I.%
~ 62 kDa	Aminoglycoside phosphotransferase [*Oxytrichatrifallax*]	gi|403374432	83981.2	5.72	16	77	99.654
	Protein kinase domain containing protein [*Tetrahymena thermophila* SB210]	gi|89303410	74431.8	9.28	13	63	97.424
	Caspase [*Marsupenaeusjaponicus*]	gi|296933458	59370.6	5.44	15	84	90.809
	Hypothetical protein [*Paramecium tetraurelia* strain d4-2]	gi|145528319	74540.9	5.75	13	59	80.54
	Tetratricopeptide repeat protein 26 [*Oxytrichatrifallax*]	gi|403344643	81979	7.95	13	50	0
~ 45 kDa	β-Tubulin [*Moneuplotescrassus*]	gi|290685	50349.1	4.75	4	72	99.997
	Unnamed protein product [*Paramecium tetraurelia*]	gi|124422913	49937	4.82	4	72	99.002
	Hypothetical protein [*Paramecium tetraurelia* strain d4-2]	gi|145491103	43782.1	8.5	12	66	95.542
	Hypothetical protein IMG5_172430 [*Ichthyophthiriusmultifiliis*]	gi|340501834	42768.8	5.78	11	65	95.112
	PX domain containing protein [*Tetrahymena thermophila* SB210]	gi|146142861	45466.1	8.35	12	56	87.09
~ 38 kDa	α-Tubulin, partial [*Euplotespetzi*]	gi|399146149	41141.5	6.44	8	45	98.365
~ 33 kDa	Hypothetical protein IMG5_081230 [*Ichthyophthirius multifiliis*]	gi|340506324	31483.5	9.25	11	60	82.253
~ 26 kDa	Ubiquitin [*Tetrahymenapyriformis*]	gi|578550	25661.8	6.85	7	47	4.294
~ 20 kDa	Hypothetical protein [*Moneuplotesminuta*]	gi|260076095	21269.4	10.25	6	33	72.867
~ 18 kDa	NADH dehydrogenase subunit 10 [*Moneuplotesminuta*]	gi|260076082	18948.5	9.08	6	38	91.993

Table 1. Continued

Band	Protein name	Accession No.	Protein MW	Protein PI	Pepcounts	Protein score	Protein score C. I.%
> 90 kDa	Hypothetical protein CPAR2_404140 [*Candida parapsilosis*]	gi|354547876	98531.9	6.37	19	98	99.634
	Tubulin-tyrosine ligase family protein [*Oxytrichatrifallax*]	gi|403359260	160677.6	9.33	21	68	99.619
	Dynein heavy chain [*Oxytrichatrifallax*]	gi|403337372	509984.9	5.76	41	75	99.439
	Hypothetical protein [*Paramecium tetraurelia* strain d4-2]	gi|145489349	111730.9	6.82	18	68	97.606
	Hypothetical protein OXYTRI_24007 [*Oxytrichatrifallax*]	gi|403358365	283370.2	9.14	31	68	97.314
	Viral A-type inclusion protein repeat containing protein [*Tetrahymenathermophila* SB210]	gi|89304620	311258.3	6.01	27	62	96.444
	NFX1-type zinc finger-containing protein 1 [*Oxytrichatrifallax*]	gi|403353380	212597.4	8.55	20	57	95.201
	Hypothetical protein OXYTRI_14239 [*Oxytrichatrifallax*]	gi|403333074	185593.4	5.57	18	55	92.217
	microtubule-actin crosslinking factor 1 [*Musmusculus*]	gi|123244271	836664.6	5.29	48	84	91.808
	Hypothetical protein Mpop_4982 [*Methylobacterium populi* BJ001]	gi|188584168	281832.1	4.93	25	83	89.922
	Hypothetical protein Pmar_PMAR028244 [*Perkinsus marinus* ATCC 50983]	gi|294892798	427330.5	4.77	36	83	89.201
	Putative tape measure tail fibre protein [*Yersinia phage* phiR1-37]	gi|358356566	271266.8	9.81	35	83	87.884
	Cation channel family protein [*Tetrahymenathermophila* SB210]	gi|89303351	156645.4	8.78	19	55	82.584
	Hypothetical protein [*Paramecium tetraurelia* strain d4-2]	gi|145479115	96711.9	5.26	17	59	78.166
	Hypothetical protein OXYTRI_07201 [*Oxytrichatrifallax*]	gi|403344906	258014.4	5.2	27	58	75.502
	Putative ABC transporter [*Oxytrichatrifallax*]	gi|403344281	173406.2	5.86	19	58	71.872
	Hypothetical protein TTHERM_00393070 [*Tetrahymena thermophila*]	gi|118357267	186809.6	5.01	27	52	71.097
	Hypothetical protein OXYTRI_08843 [*Oxytrichatrifallax*]	gi|403342191	458047.9	6.7	34	58	69.86
	Dynein heavy chain family protein [*Tetrahymena thermophila*]	gi|118378437	496299.6	6.12	31	51	61.011
	RHS repeat family protein [*Tetrahymenathermophila*]	gi|118350474	774668.7	8.87	37	51	59.174
	ATPases of the AAA+ class [*Oxytrichatrifallax*]	gi|403335402	90957.6	5.27	12	46	30.631
	IP3 receptor calcium ion channel protein [*Tetrahymena thermophila* SB210]	gi|89284243	388136.8	5.73	26	49	29.052
	Cullin family protein [*Tetrahymenathermophila*]	gi|118359062	93775.3	8.87	14	45	0
	Cytoplasmic dynein heavy chain 2 protein [*Tetrahymena thermophila]*	gi|54660021	490256.6	6.32	31	46	0
	Spc97 / Spc98 family protein [*Tetrahymenathermophila* SB210]	gi|146143647	108337	6.56	13	46	0
	Ubiquitin carboxyl-terminal hydrolase family protein [*Tetrahymenathermophila* SB210]	gi|89304797	425714.2	5.31	25	48	0
	Adenylate cyclase, family 3 (some proteins containing HAMP domain) [*Oxytrichatrifallax*]	gi|403374931	141860.2	9.52	14	43	0

Tandem mass spectrometric analysis of eight bands

After in-gel digested, these eight gel bands were identified by 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX), and the identified results were shown in Table 1. As we know, the criteria of a successful protein identification are protein score C.I.% > 95% and protein score > 50–55. Due to lack of whole gene sequence of *Euplotes encysticus* and limitation of protozoan protein databases, the identified proteins whose protein score C.I.% < 95%, even uncharacterized proteins with lower protein score C.I. and protein score > 40 might consider to be successfully identified.

According to the criteria, a total of 42 cyst wall proteins of *Euplotes encysticus* were identified by searching the related protein databases. Among 42 proteins, 26 were identified proteins listed in Table 1. on the basis of their molecular weights from high to low except that those proteins whose molecular weights > 90 kDa were listed at the end. Nevertheless, there is no identified protein met the criteria in 23 kDa

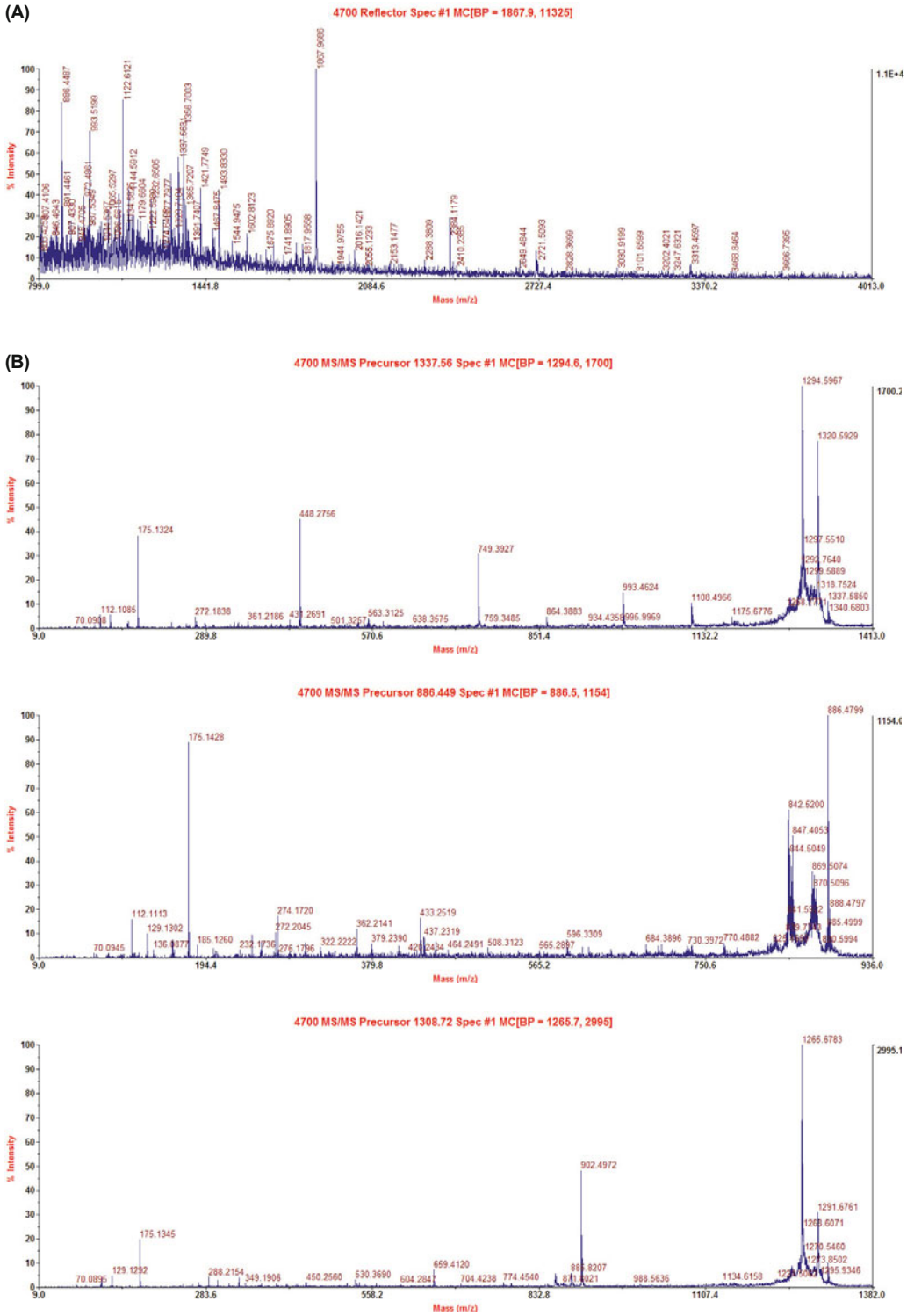

Fig. 2. MALDI-TOF mass spectra of the 33 kDa proteins from the wall cyst. (A) Represent peptide mass fingerprinting of the 33 kDa protein. (B) Represents part of MS/MS spectra of the 33 kDa protein.

gel band, which might be mainly because of the current restriction of relevant protein databases.

These eight gel bands identified by 4800 Plus MALDI TOF/TOF analyzer provided many peptide mass fingerprinting and MS/MS spectra. Here, we only look at 33 kDa protein as a representative, the peptide mass fingerprinting and part of MS/MS spectra of this protein was shown (Fig. 2). These spectra provided us with more detailed information of analysis and identification of specific proteins in the cyst wall.

Bioinformatics analysis of identified cyst wall proteins

In order to provide a comprehensive understanding of the proteins identified in this article, we queried against the UniProtKB/Swiss-Prot protein databases and Kyoto Encyclo-

Table 2. Functional categories of the identified cyst wall proteins of *E. encysticus*

Functional categories	Numbers of proteins
Cell wall anchor protein	4
Cytoskeleton proteinss	8
Energy metabolism	3
Transport and catabolism	2
Processing, folding, sorting and degradation	5
Signal transduction	2
Membrane transport	2
Unknown function	16

pedia of Genes and Genomes (KEGG) website (http://www.genome.jp/kegg/kegg2.html). The resultant proteins were classified into several aspects including cell wall anchor proteins, cytoskeleton proteins, energy metabolism, transport and catabolism, degradation, signal transduction, membrane transport. The results of functional categories of identified proteins were shown in Table 2.

According to the functional categories in Table 2, cytoskeleton-associated protein including α-tubulin and β-tubulin accounted for a significant proportion of the identified proteins, whose percentage was approximately 19.0%. The 9.52% of the 42 proteins were cell wall anchoring proteins, including PX domain containing protein, tape tail fiber protein, IP3 receptor calcium ion channel proteins, adenylate cyclase. Proteins involved in processing, folding, sorting and degradation occupied 11.9%, which contained cullin family protein, ubiquitin, ubiquitin carboxyl-terminal hydrolase family protein. Proteins related to energy metabolism engaged 7.14%, which integrated aminoglycoside phosphotransferase, NADH dehydrogenase subunit 10 and ATPases of the AAA+ class. Proteins related to transport and catabolism, membrane transport and signal transduction had the same percentage 4.76. These proteins may be important in the regulation and transportation of cyst wall substances concerned in the process of cyst wall production, and their functions will be discussed below. However, a large proportion of 42 proteins were unknown function, which might also play important roles in the regulation of cyst wall formation. The proportion of each functional category was shown in Fig. 3.

FLUTAX fluorescent labeling method for revealing microtubular cytoskeleton components of the resting cyst wall and vegetative cell

Fluorescence taxtoid (FLUTAX) is a specific fluorescent dye for staining cytoskeleton. Fig. 4A indicated the vegetative cell. Fig. 4B interestingly, signify the ellipse vegetative cell just transformed into the spherical cyst in very early stage of the encystment, when the volume had not yet shrunk. The cyst surface generated many rows spots with FLUTAX fluoresce,

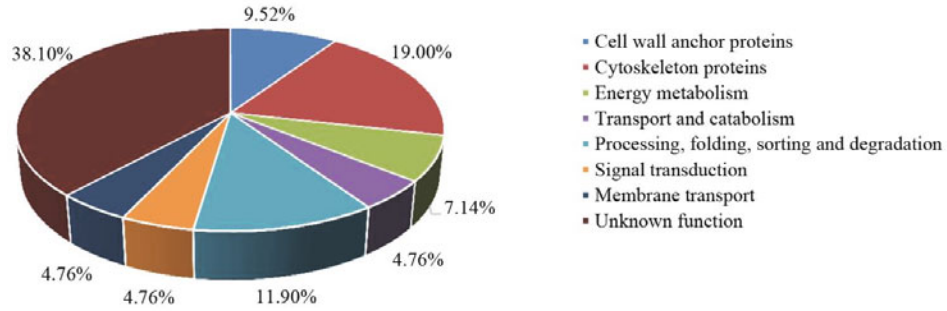

Fig. 3 The ratio of each functional category of identified cyst wall proteins. The identified proteins were divided into eight categories including Cell wall anchor proteins, Cytoskeleton proteins, Energy metabolism, Transport and catabolism, Processing, folding, sorting and degradation, Signal transduction, Membrane transport and Unknown function proteins. In this pie chart, uncharacterized the protein accounted for a large proportion followed by cytoskeletal proteins; 11.9% proteins were involved in processing, folding, sorting and degradation; Cell wall anchor proteins occupied 9.52% and energy metabolism proteins occupied 7.14%. And the remaining three categories accounted for the same ratio 4.76%.

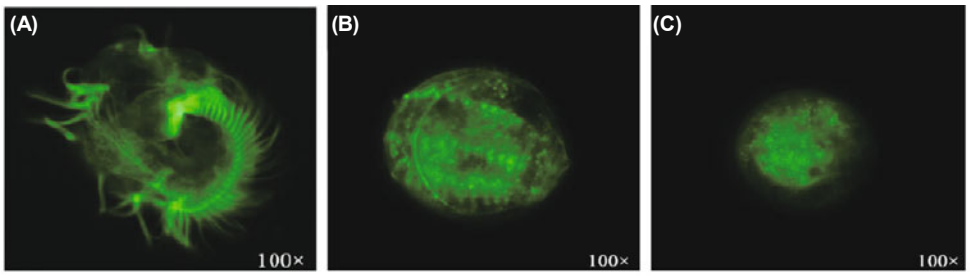

Fig. 4. Representative fluoresce microscopic image of the resting cyst for *Euplote sencysticus*. (A) The vegetative cell. (B) The ellipse vegetative cell just transformed into the spherical cyst. The cyst surface generated many rows spots with FLUTAX fluoresce, suggesting theses spots with microtubular cytoskeleton components because FLUTAX was a specific fluorescent dye for staining cystoskeleton. But the volume had not yet shrunk. (C) The volume of mature cyst had sharply shrunk due to cytoplasm shrinking and so on. The resting cyst wall presented many spots with FLUTAX fluoresce, which indicated cyst wall with microtubular cytoskeleton components.

suggesting that the cyst wall contained microtubular cytoskeleton components. Fig. 4C indicated that the volume of the cyst had sharply shrunk in very late stage of the encystment due to cytoplasm shrinking, macronuclear chromatin condensation, etc. The cyst wall appeared many spots with FLUTAX fluoresce, which showed cyst wall contained microtubular cytoskeleton components. These results and the identified results of above cytoskeleton proteins, confirmed that cytoskeleton proteins presented in the resting cyst wall.

Discussion

In our experiment, the sample of the cyst wall proteins separated by SDS-PAGE electrophoresis presented 8 protein bands with a molecular weight of 18, 20, 23, 26, 33, 38, 45, and 62 kDa. In the cyst wall of the other protozoa similar molecular weight proteins were reported. For example, three proteins (Mol.wt. 22, 26 and 39 kDa) were observed by SDS-PAGE electrophoresis in cyst wall of *Giardia lamblia* (Luján *et al*., 1995; Boone *et al*., 1999). Four proteins (Mol.wt. 18, 20, 26, and 44 kDa) were detected in cyst wall of hypotrichous ciliate *Paraurostyla* sp. (Rios *et al*., 1989, 1992). One protein (Mol.wt. 61 kDa) was also detected in cyst wall of ciliate *Colpoda inflate* (Martingonzalez *et al*., 1991).

Subsequently the electrophoretic gel bands were in-gel digested, following by MALDI-TOF MS analysis. So far, MALDI-TOF-MS seemed to be nearly a synonym for protein analysis, this technique is thought to be a useful tool in protein analysis because of its fast, simple and convenient performance (Ekström *et al*., 2001; Sparbier *et al*., 2006). In comparison with SDS-PAGE, which cannot ensure the protein separation completely, MALDI-TOF-MS has a higher sensitivity. Therefore, it is not surprising that we can get several proteins from one gel band.

Using MALDI-TOF MS, 42 proteins of the cyst wall were identified in this study. To further understand the functions of these identified proteins, we examined the protein databases on UniProtKB/Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) website. In terms of composition ratio of cyst wall proteins, differences were existed among the different functional proteins. Function-unknown proteins attained a large portion (38.1%), due to the limitation of protozoan protein databases. The function-known proteins were categorized into several aspects: cell wall anchoring, cytoskeleton, energy metabolism, transport and catabolism, signal transduction, membrane-associated transportation, processing, sorting and degradation. Accordingly, the majority of identified proteins might demonstrate their importance and contribution in encystment process.

Considerable research has proven that most of the compositions of ciliate resting cyst walls consist of carbohydrates and proteins (Gutiérrez *et al*., 1990, 1998). In this study, we found that cyst wall proteins supported some structural and functional proteins through Bioinformatics analysis. As is known, the most important function of protozoan cyst wall, as well as the bacteria cell wall, acted to be maintains the cell shape. Therefore, it obviously needs the involvement of cytoskeleton proteins, such as tubulin and actin. These structural proteins existed in cyst wall of *Euplotes encysticus* also retain the function to protection against adverse environmental conditions of resting cyst. Therefore, cysts of *Euplotes encysticus* are more resistant against stimuli than vegetative cells most likely because of their specific wall. On the other hand, the movement of ciliate mainly depends on its cilia, which is mainly composed of cytoskeleton proteins like tubulin. Nonetheless, the cells stay in resting status when cyst emerges, the ciliary organelles have not disappeared completely (Janisch, 2000; Tian *et al*., 2002). The cyst of *Euplotes encysticus* belongs to non-kinetosome-resorbing cysts (Gu, 1995); so far, the structural proteins partially retained during the phase of cyst formation. Fluorescent spots in Fig. 4, at least in part, showed this non-resorbing-kinetosome. Our FLUTAX fluorescent labeling experiment further proved that the cyst wall contained microtubular cytoskeleton components.

During ciliate encystment, one of the most remarkable changes is drastic decrease in cellular volume, which is partially depend on the loss of intracellular water and degradation of cytoskeleton system. The reduction of the cell proteins also reveals enhancement of protein degradation activity in the process of the encystment. In our study, ubiquitin, ubiquitin carboxyl-terminal hydrolase family protein and cullin family protein were identified. As is known, ubiquitin-proteasome pathway (UPP) is an important signaling pathway to selectively degrade intracellular proteins (Jung *et al*., 2009; Chen *et al*., 2013). This identified result demonstrated that protein degradation was involved in resting cyst formation, which is consistent with previous reports (Honts and Williams, 2003; Yue-Hua, 2004). This evidence leads us to believe that most of non-essential proteins degraded during encystment and only essential proteins are left to maintain life activities of resting cyst. Nevertheless, resting cysts of protozoa is one of the cryptobiotic forms (Gutiérrez *et al*., 2001), as well as the conditions become favorable, they will rapidly excyst to proliferate. We speculate that ubiquitin, ubiquitin carboxyl-terminal hydrolase family protein and cullin family protein might play an important role in the process of excystment. They might also involve in the degradation of cyst wall proteins to make a gap of cyst wall, which is beneficial for ciliates to get out of the resting cyst. In addition, ubiquitin system also has a number of important non-proteolytic functions, including intracellular signal transduction, transcriptional regulation, membrane transport and protein kinase activation (Mukhopadhyay and Riezman, 2007; Chen *et al*., 2012). Therefore, these ubiquitin related proteins not only involve in proteins degradation but also likely participate in the other activities of the encystment and excystment.

The emergence of cyst wall is a main feature of cyst formation. As already described by Gutierrez (Gutiérrez *et al*., 2001), the synthesis and intracellular transport of the cyst wall components and the assembly of the extracellular cyst wall polymers are involved in cyst wall production. All these processes are closely associated with material exchange and transport. It had been reported that there was material exchange channel between pellicle and granular layer in resting cyst of *paraurostyla weissei* (Fu, 1999). Resent findings also demonstrated that there were several channel proteins including cation channel family protein, IP3 receptor calcium ion channel protein and transport proteins like ABC trans-

porter in cyst wall. These proteins have the potential to sense environmental cues. Energy related proteins including NADH dehydrogenase subunit 10 and AAA+ type ATPase were also identified. Noteworthy, the life activities of ciliates merely tend to be enervated but not quitted during the process of vegetative cell transforming into resting cyst. Resting cyst still experiences some processes, such as intracellular digestion, energy utilization, material exchange between cyst and environment through the cyst wall. These functional proteins mentioned above prompted that resting cyst of *Euplotes encysticus* needs to keep a low level of material and energy exchange with the outside world to maintain the basic living although the cell physiological metabolism level is low during encystment.

Apart from being related to cytoskeleton, material transportation and degradation, the cyst wall also involved signal transduction. As the cyst cells can excyst back to vegetative cell when environment getting favorable (Calvo *et al.*, 2003), this depends on signal transduction of wall proteins. A number of the identified cyst wall proteins including protein kinase, NFX1-type zinc finger-containing protein, RHS Repeat family protein, cation channel family protein, IP3 receptor calcium ion channel receptor protein, adenylate cyclase have signal transduction function. We infer that these proteins play important roles in the cyst wall signal transduction. In addition to, some enzymes were discovered in the cyst wall, including protein kinase, caspase, NADH dehydrogenase, adenylate cyclase. Functions of these enzymes involve in a wide range roles including protein phosphorylation, regulating protein synthesis and cell differentiation, signal transduction, stress response and oxidative phosphorylation. So these enzymes play many potential roles in protecting cyst surviving under stresses.

Despite the above-mentioned functions of these cyst wall proteins, expressed much higher functions as expected, in comparison with our previous report. Our results validated that cyst wall not only play role in mechanical protection, but also have many other functions including signal transduction, material transport, proteins degradation and energy metabolism. Indeed, the cyst wall contains abundant number of proteins which play vital role in the multi-function. Besides, it also characterized that the resting cyst could survive in adverse environment.

Notably, proteins with unknown function among the identified cyst wall proteins were those which were usually not similar to any known protein. However, their expressions might be associated with the stress tolerance and encystment. Therefore, these proteins are likely novel wall proteins and may be the best candidates for sources of resisting adverse environmental stress in future studies.

In conclusion, the cyst wall protein components are complicated with many structural and functional proteins. These functional proteins involve in cyst wall structural formation, signal transduction, material transportation and protein degradation. The cyst wall composed of many functional proteins can protect and coordinate cell dormancy against adverse environment, this composition mechanism is significant for resting cyst survival. It is reported that cyst wall emergence the symbol of vegetative cell turning to resting cyst, so we infer that cyst sensing outside changes is likely through cyst wall proteins transiting signals. This study also suggests that we can further study on resting mechanism through analysis the differences between cyst wall proteins before and after cell differentiation.

This study identified at scale cyst wall proteins of *Euplotes encysticus* by MALDI -TOF MS technology and revealed many cyst wall proteins. Revealing these cyst wall proteins and their function will help clarify interaction relationship between eukaryocyte and environment, as well as insight into molecular mechanism of resisting adverse circumstance. Our data also partially explained why *Euplotes encysticus* could survive under adverse environment.

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Conflicts of Interest

Competing financial interests: The authors declare no competing financial interests.

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