Korean J Biol Sci 6: 341-347, 2002

Changes in Profiles of Major Proteins in Encysting *Acanthamoeba castellanii*

Joon Tae Park, Young Eui Jeong, and Tae In Ahn*

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

Key Words:
Cellular differentiation
Ubiquitin
Encystment
Acanthamoeba
Protein profile

The life cycle of *Acanthamoeba* is comprised of two distinct stages, trophozoite and cyst. During periods of stress, trophozoites undergo cellular differentiation into cyst. In order to understand the cellular differentiation, we followed changes in profiles of major proteins by 2D-PAGE and ubiquitinated proteins by immunoblotting with anti-ubiquitin (Ub) monoclonal antibody (mAb) as a probe. We observed 51 proteins present in trophozoite were lost with the encystment. We found that 43 proteins within 24 h, and 8 proteins in 96 h of encystment. Among them, 17 proteins were stained with anti-Ub mAb. In cysts, 16 proteins including 2 anti-Ub mAb-reactive proteins were newly synthesized. Four proteins were newly detected in 24 h-cyst and disappeared in 96 h-cyst, one protein was synthesized in 24-96 h-cyst and disappeared in 168 h-cyst, and 11 proteins appeared upon encystment and were present in all cyst stages. We identified a cyst specific 33 kDa protein as subtilisin-like serine proteinase by N-terminal sequencing. Identification of these proteins lost and newly synthesized with encystment would improve our understanding of cysting protozoan parasites.

Acanthamoeba can cause sight-threatening keratitis, life threatening granulomatous amoebic encephalitis, pneumonitis and dermatitis. They are ubiquitous in nature and are found in diverse habitats such as soil, water and dust (Marciano-Cabral et al., 2000). They are relatively large in size, grow rapid in axenic culture, and exhibit unicellular differentiation.

The life cycle of *Acanthamoeba* is comprised of two distinct stages, trophozoite and cyst. During periods of stress, trophozoites of *Acanthamoeba* undergo a cellular differentiation process (termed encystment). Traditionally, encystment has been seen as a strategy for overcoming adverse conditions such as dehydration, extremes of temperature, lack of available food, and changes in salinity, nutrients or pH (Corliss et al., 1984). The cysts of *Acanthamoeba* are formed from trophozoites when starvation, desiccation, or other adverse conditions prevail (Neff et al., 1964) and can reverse to trophozoites when the conditions are favorable (Martieze, 1985). The initiation of encystment induces a reduction of metabolism so that cell synthesizes a chemically and structurally complex cell wall. Wall synthesis is accompanied by a decrease in cytoplasmic mass and a gradual dehydration of the organism (Bowers et al., 1969). Studies on the chemical composition of cysts have shown that the formation of cyst wall involves the production of cellulose and protein, which are components of the wall structure that are not found in trophozoite (Neff et al., 1969). These changes induce cyst to be more resistant to biocide than trophozoite. Thus, *Acanthamoeba* could be an attractive model of cellular differentiation. However, only one protein that displays cyst specific expression has been identified (Hirukawa et al., 1998).

In eukaryotic cells, the turnover of intracellular proteins is carried out by two distinct systems - the lysosomal and Ub-dependent proteolysis systems. The Ub-dependent proteolysis system is essential for maintaining the differentiation of protozoan parasites (Gonzalez et al., 1999). It is the major non-lysosomal pathway of proteolysis and accounts for the degradation of most short-lived, misfolded or stress-damaged proteins. When cultured cells are subjected to a sudden increase in temperature, a burst of degradation of normally long-lived proteins is observed. The increase in proteolysis coincides with reduction in free and histone-conjugated Ub and an increase in multi-Ub-protein conjugates (Parsell et al., 1993). However, substrates for ubiquitination in stressed cells have not yet been well characterized. Characterization of various target proteins in the ubiquitination pathway is an important task in the study of stress physiology.

In this study, we followed changes in profiles of major proteins in encysting amoebae to establish the basics in the study of cellular differentiation and stress physiology of *Acanthamoeba*. In order to detect masses of proteins, we applied 2D-PAGE and immunoblotting

* To whom correspondence should be addressed.
Tel: 82-2-880-7771 Fax: 82-2-880-7771
E-mail: ahntaein@snu.ac.kr

using anti-Ub mAb.

Materials and Methods

Cell culture

Trophozoites: *Acanthamoeba castellanii* (Castellanii strain, ATCC No, 50374) used in this study was a gift from Dr. D. I. Chung (Kyungpook National University, Korea). Trophozoites were grown axenically in PYG medium consisted of 2% proteose peptone, 0.1% yeast extract, 1.8% glucose, 0.4 M $MgSO_4 \cdot 7H_2O$, 0.1 M sodium citrate, 0.005 M $Fe(NH_4)_2(SO_4)_2$, 0.25 M Na_2HPO_4 and 0.25 M KH_2PO_4 (pH 6.5) (Stratford et al, 1978). Trophozoites from stationary phase(3.5×10^6 cells/mL) of culture were inoculated into PYG medium (7.4×10^5 cells/mL) and grown at 30℃ in a shaking incubator agitated at 120 rpm/min.

Encystment: Trophozoites in stationary phase were collected aseptically by centrifugation at 800×g for 2 min, washed twice in encystment medium (EM) containing 95 mM NaCl, 5 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$ and 20 mM Tris-HCl, pH 9.0 (Bowers et al., 1969), inoculated into EM (3.5×10^6 cells/mL), and incubated at 30℃ with agitation for encystment.

Density of cells in suspension was determined by direct counting with a hemacytometer and averaged from four counts (Freshney, 1994).

Protein sample preparation

Trophozoites and cysts were collected and washed twice with PBS by centrifugation at 800×g for 2 min. Trophozoites were resuspended in 2 volumes of lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1 mM EDTA) and homogenized by sonication. Subsequently, the homogenate was centrifuged for 15 min at 16,800×g and the supernatant was collected. The cyst pellet was resuspended in 2 volumes of lysis buffer, and transferred to 1.5 mL tubes. Cyst suspensions were mixed with cold glass beads (Sigma, 0.425-0.60 mm diameter) and the tubes were vigorously agitated with vortex mixer for 10 sec each time over a 20 min period. The extent of homogenization was determined by phase-contrast microscopy. Then, the supernatant was collected by the same procedure for trophozoites. To prepare a concentrated protein sample, proteins in the supernatant were precipitated with 10% TCA on ice for 30 min and collected by centrifugation for 5 min at 10,000×g. Residual TCA was removed by two washes with acetone and proteins were dissolved in urea sample buffer (9.5 M urea, 4% Chaps, and 100 mM DTT). Protein concentration was estimated according to a modified Bradford method (Ramagli et al., 1985).

Two-dimensional electrophoresis of proteins

Isoelectric focusing (IEF) was performed according to the method of O'Farrell (1975) with a slight modification. IEF gels were made in glass tubing (155×3 mm inner diameter). The gel mixture (10 mL) contained 9.0 M urea, 4% acrylamide, 2% NP-40, and 2% ampholines (1.6% pH 5-7, 0.4% pH 3.5-10). For analytical purpose, 800 μg proteins were loaded in each tube. Electrophoresis was performed at 200 V for 1 h, 400 V for 1 h, 600 V for 15 h, then 800 V for 1 h. After IEF, the gels were equilibrated in equilibration buffer (0.06 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 100 mM DTT) for 1 h and immediately loaded on the second dimensional discontinuous SDS gel as described by Laemmli (1970). Electrophoresis was carried out at 30 mA/gel for 5.5 h. The gel was stained with Coomassie Brilliant Blue R-250.

Immunoblotting

After electrophoresis, proteins in gels were transferred electrophoretically onto nitrocellulose (NC) membrane (PROTRAN BA85; Schleicher & Schuell). Then, the NC membrane was immunostained with anti-Ub mAb (Santa-Cruz Biotech. Inc., diluted 1 : 1,000 in PBS) and subsequently with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, diluted 1 : 10,000 in PBS). Bound antibodies were visualized using a chemiluminescence reagent (Amersham International).

Image acquisition and analysis

The stained 2D-gels were photographed by using Camedia Digital Camera C-3030 (Olympus Co. Ltd, Tokyo, Japan). Data analysis was performed with Melanie II 2-D polyacrylamide gel electrophoresis software (release 2.2, Bio-Rad). Reference points (landmark) were marked on image to align and match.

N-terminal amino acid sequencing and database search

The cyst specific proteins resolved in 2D-PAGE were electrophoretically transferred onto polyvinylidene difluoride membrane (WESTRAN; Schleicher & Schuell). The membrane was stained with Coomassie Brilliant Blue R-250, and was then submitted to the Korea Basic Science Institute Seoul Branch (Seoul, Korea) for amino acid sequencing. The automated micro sequencing was done using a Precise Protein Sequencing System (Applied Biosystems, Cambridge, UK) with Edman degradation procedure. The determined amino acid sequence was compared with other sequences in Databases using FASTA program.

Results and Discussion

Morphological changes and cysting kinetics

The trophozoite of *A. castellanii* is irregular in shape and has numerous acanthopodia and spongiosome contractile vacuole system (Fig. 1A, T). Logarithmic

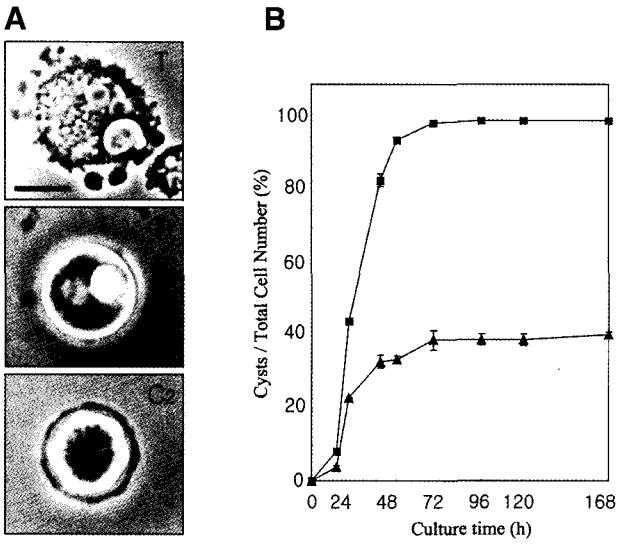

Fig. 1. Morphological changes (A) and encysting kinetics (B) of *Acanthamoeba castellanii* in nutrient-free medium. In nutrient-free media, trophozoites (T) differentiated to ectocyst (C_1) with ectocyst wall, and matured cyst (C_2) showing walls meet at several places forming star-like shape. The bar in (T) represents 10 μm for all pictures. In B, trophozoites were inoculated into nutrient-free media at a density of 3.5×10^6 cells/mL to induce encystment. Data of total cysts (■) and matured cysts (▲) represent average ±S.D. fom four different experiments.

growth phase of trophozoites in PYG media lasts 120 h until ~2.5×10^6 cells/mL in density. In nutrient-free EM, the amoebae maintained their typical trophozoite appearance for the first 8-12 h of encystment. After 14 h of encystment, the cell began to round and ectocyst wall appeared (Fig. 1A, C_1). The number of cysts began to increase dramatically after 24 h and trophozoites disappeared after 48 h (Fig. 1B). After 16 h of encystment, the endocyst wall began to appear. The matured cyst was characterized by a double-walled envelope, an outer ectocyst and inner endocyst. The two walls meet at several places forming a star-like or angular shape (Fig. 1A, C_2). We considered those cysts with double-walled envelope as matured cysts. However, the proportion of matured cysts did not increase with incubation time after 48 h. It was observed that total maximum percentage of matured cysts did not reach 50% up to 168 h after induction. It was impossible to separate matured cysts from less matured ectocyst by differential centrifugation or two phase-polymer systems applied. Durations of encystment were comprised of cyst wall synthesis and cyst maturation stages, which were 20-24 h and 2-7 d in nutrient-free media, respectively (Bowers et al., 1969). In this study, we subdivided the cysts into 24 h, 96 h and 168 h-cyst for protein analysis.

Changes in protein profiles during encystment

In comparisons of BCB stained 2D-gels (Fig. 2) and immunoblots of corresponding gels using anti-Ub mAb as probe (Fig. 3), we could detect 51 proteins that were present in trophozoites disappeared and 16 proteins newly appeared with encystment (Table 1). Massive turnover of 51 proteins could be ascribed to a decrease in cellular contents of protein to be used as a source of precursors in cell wall synthesis or as a source of energy for the cells that have no external food source (Neff et al., 1969). Among them, 43 proteins (No. 1-33 PDDD and U1-U10 PDDD in Table 1) disappeared within 24 h of encystment, and 8 proteins (No. 34 PPDD and U11-U17 PPDD) were present until 24 h-cyst and disappeared thereafter.

Those 33 PDDD proteins were mostly acidic in pI-range (pI 4.72-6.25) except for 3 proteins (No. 21; pI 7.00, 27; pI 6.58, and 28; pI 6.62). Actin was one of the PDDD type proteins (No. 20). Actin is one of the most abundant and major protein constituents in *A. castellanii* (7.7% of total protein) (Jantzen, 1981). It is a contractile apparatus in free-living *A. castellanii*, but is not necessary for non-motile cysts. As encystment proceeded, actins decreased entirely (Fig. 4A). On the other hand, the pI range of anti-Ub mAb reactive U1-U10 PDDD proteins and U11-U17 PPDD proteins was rather basic (pI 5.85-7.51) except for one protein (SU1 NSSD).

Of the 16 newly synthesized proteins, 4 proteins (S1-S4 NSDD) appeared in 24 h-cyst and disappeared in 96 h- and 168 h-cyst, one protein (SU1 NSSD) was detected in 24 h-, 96 h-cyst and disappeared in 168 h-cyst (Fig. 4B), and 11 proteins (S5-S14 and SU2 NSSS) appeared upon encystment and were present in all cyst stages. On the other hand, a cyst specific SU1 NSSD protein (21 kDa, pI 5.20) was one of the major proteins in 24 h- and 96 h-cyst, but completely lost in 168 h-cyst. The ubiquitinated SU1 protein was similar to the cyst specific CSP21 in molecular mass and behavior (Hirukawa et al., 1998). However, its pI was more acidic than CSP21 (pI 9.06). Immunoblot analysis using anti-CSP21 antibody demonstrated that the protein was detectable after 12 h encystment and its expression was restricted at an early stage of encystment (Hirukawa et al., 1998). Among the 11 NSSS proteins, the anti-Ub mAb reactive SU2 protein (33 kDa, pI 6.3) was not detected in trophozoites, but was the most major protein in 24 h-, 96 h-, and 168 h-cyst (Fig. 4C). On the basis of N-terminal sequences (Table 2), the protein had a high similarity with a subtilisin-like serine proteinase of *Acanthamoeba healyi* (Hong et al., 2000).

When *Acanthamoeba* are induced to encystment, massive turnover of organelles and cellular membrane system take place (Griffiths et al., 1969). This rapid autophagy is also the essential prerequisite for establishment of new architecture of the cyst (Bowers et al., 1969). We assumed that 34 proteins (PDDD and PPDD type) not reacting with anti-Ub mAb were degraded by the lysosomal system. Under the condition of cellular stress (e.g., starvation), cytoplasmic

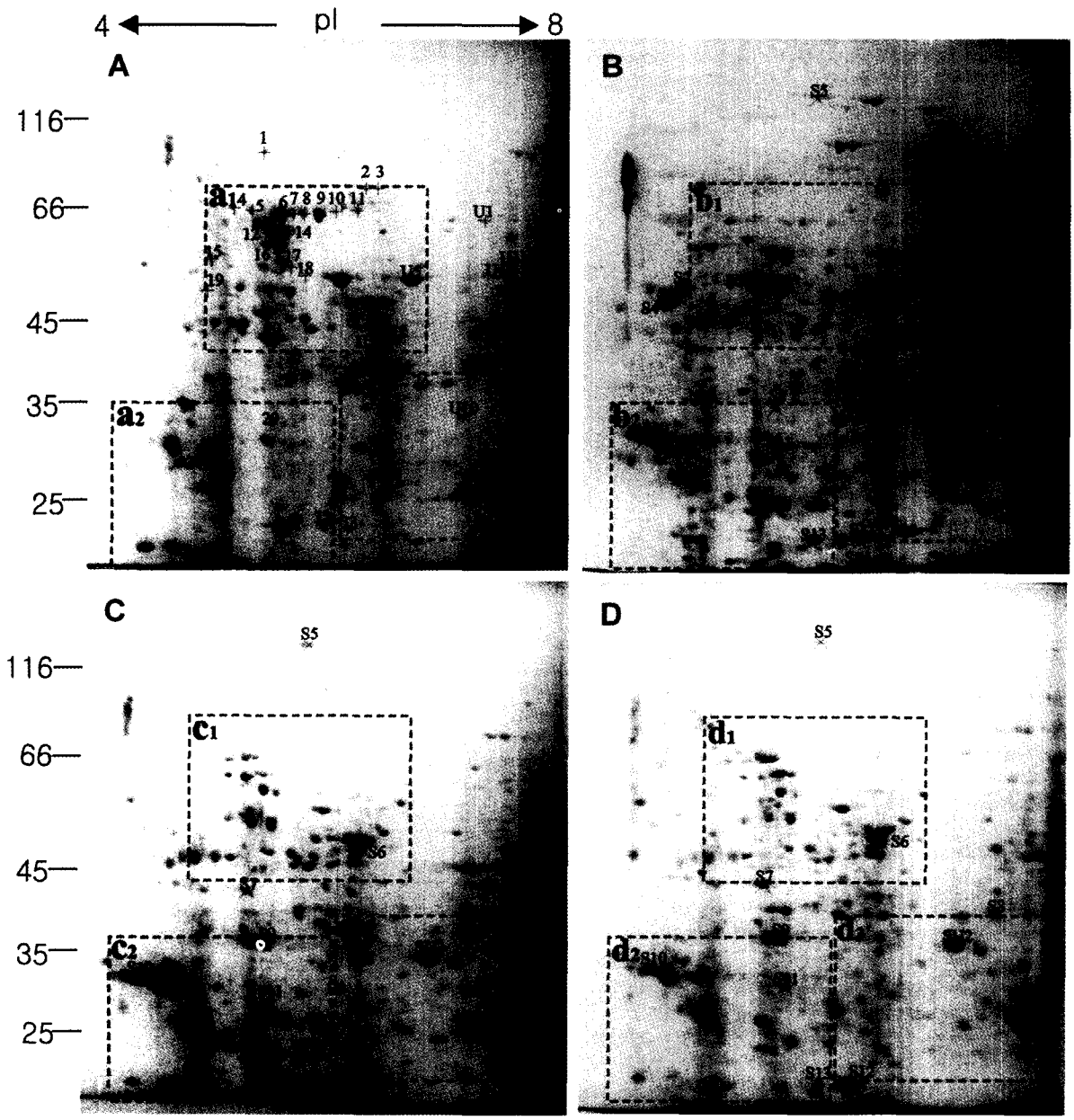

Fig. 2. Comparisons of 2D protein profiles during encystment (10% gel). (A) Trophozoite, (B) 24 h-cyst, (C) 96 h-cyst, (D) 168 h-cyst. Selected proteins in rectangles (a_1-a_3, b_1-b_3, c_1-c_3, d_1-d_3) are compared in Fig. 4. Symbols in gel pictures: +; present in trophozoites and disappeared in cysts, △; present in trophozoites and 24 h-cyst, and lost thereafter, O; newly synthesized in 24 h-cyst and lost thereafter, □; newly synthesized in 24 h and 96 h-cyst, and lost in 168 h-cyst ×; newly synthesized in 24-h cyst and persisted in all encystment stage.

proteins are delivered to lysosome and the processes of autophagy are observed (Selgen et al., 1992). By using electron microscope, lysosome has been identified as a prominent component of the cytoplasm of cyst. In the vacuole of lysosome, dense contents of mitochondria, lipid droplet and glycogen are found (Bowers et al., 1969). We observed that 17 proteins reacting with anti-Ub mAb were lost with encystment. Since they were reactive with anti-Ub mAb, most of them could be ubiquitinated proteins undergoing Ub-dependent degradation. The Ub-dependent proteolysis system is essential for maintaining differentiation of protozoan parasites (Gonzalez et al., 1999). The proteins degraded by the Ub-dependent proteolysis system are soluble cytosolic proteins, integral membrane proteins and nucleus proteins (Jensen et al., 1995). Until recently, substrates for ubiquitination in stressed cells have not yet been well characterized. In our results, anti-Ub mAb reactive proteins were rather basic than non-reactive proteins. This observation might have

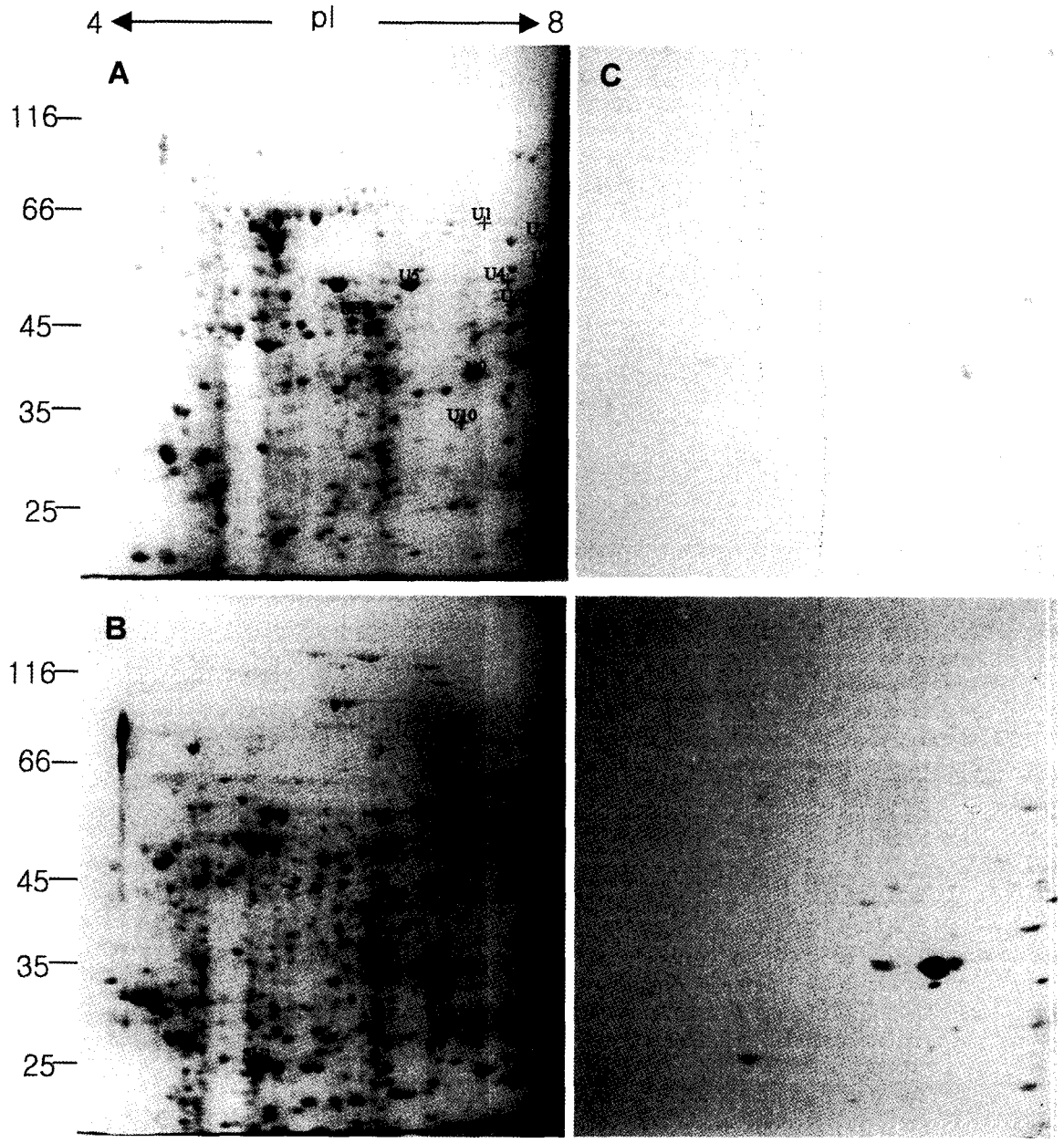

Fig. 3. Proteins of trophozoite and 24 h-cyst stained with BCB (A, B) and with mAb against Ub mAb (C, D). Symbols are the same as in Fig. 2.

some implication in stress cell physiology.

During encystment, synthesis of the cyst-specific proteins begins shortly after induction. These changes are ascribed to the synthesis of cyst-specific components such as acid insoluble proteins and celluloses. The acid insoluble protein-containing ectocyst wall is first synthesized and the cellulose-containing endocyst wall is synthesized within the ectocyst wall. These changes induce cysts to be more resistant to biocide than trophozoites. Since their roles as human pathogens causing keratitis and encephalitis have become recognized, finding these cyst-specific proteins is very important. In this study we detected 16 cyst-specific proteins including two anti-Ub mAb reactive proteins. Among them, four NSDD type proteins (S1-S4) were 24 h-cyst specific. Autolysosomes appeared upon encystment and were present by the time the ectocyst forms a complete layer around the cell (Bowers et al., 1969). Thus, the transiently existing S1-S4 proteins could be the constituents of transient organelles such as autolysosomes.

Those newly synthesized cyst specific NSSS type

Table 1. Summary of proteins changed with encystment

Spot no.	M.W.	pI	Level changes*	Spot no.	M.W.	pI	Level changes*
1	105.4	5.30		U1	64.2	6.51	
2	76.0	5.92		U2	63.0	7.39	
3	76.0	6.00		U3	59.6	7.51	
4	66.2	5.01		U4	58.0	6.60	
5	65.0	5.21		U5	54.0	6.20	PDDD
6	64.2	5.42		U6	48.2	6.93	
7	64.3	5.48		U7	48.2	7.11	
8	64.3	5.52		U8	46.0	5.85	
9	64.2	5.60		U9	38.2	6.48	
10	65.0	5.70		U10	33.0	6.40	
11	65.2	5.85		34	30.0	4.72	
12	63.0	5.37		U11	59.5	6.93	
13	63.5	5.40		U12	41.6	6.08	
14	64.0	5.46		U13	40.8	5.93	PPDD
15	59.7	4.90		U14	37.2	6.70	
16	60.0	5.40		U15	33.2	6.03	
17	59.5	5.47	PDDD	U16	32.1	7.30	
18	58.0	5.52		U17	28.8	7.11	
19	53.8	4.85		S1	53.9	6.40	
20	41.6	5.37		S2	50.0	4.78	NSDD
21	40.0	7.00		S3	48.2	4.72	
22	39.0	5.48		S4	47.0	4.65	
23	38.2	6.03		SU1	21.0	5.20	NSSD
24	38.0	5.90		S5	137.5	5.56	
25	37.5	6.14		S6	47.0	5.92	
26	37.0	6.25		S7	41.8	5.19	
27	35.4	6.58		S8	37.2	6.48	
28	35.0	6.62		S9	34.0	5.35	
29	32.1	5.37		S10	30.0	4.43	NSSS
30	28.2	6.05		S11	28.8	5.40	
31	25.2	5.74		S12	14.4	5.80	
32	16.0	5.65		S13	13.0	5.51	
33	15.0	5.80		S14	8.0	5.70	
				SU2	33.0	6.30	

Spot numbers of proteins correspond to those shown in Fig. 2.
Level changes of the proteins in trophozoite, 24 h, 96 h, and 168 h-cyst are shown in one letter code, N: not present, P: present, D: disappeared, and S: newly synthesized.

proteins (S5-S14 and SU2) could involve in the formation of cyst wall components. The ectocyst walls are composed of acid insoluble proteins (Weisman, 1976). However, the constituents of ectocyst wall or late developing endocyst wall have not been characterized. We identified SU2 protein as a subtilisin-like serine proteinase. Proteinase is known to play an important role in cellular differentiation. Treatment of proteinase inhibitor, lactacystin, delayed encystment of *Entamoeba invadens* (Gonzalez et al., 1999).

In this study, we detected 67 proteins of interest in cellular differentiation of *Acanthamoeba*. Among them, 51 proteins were degraded or lost and 16 proteins were newly synthesized with encystment. The 17 proteins of the lost 51 proteins and two proteins of newly synthesized proteins were reactive with anti-Ub mAb. These proteins will be good markers in the study of cellular differentiation of *Acanthamoeba* and stress physiology of cells. Identification and characterization of these proteins in follow-up studies will enable us to find molecular targets in cellular differentiation of encysting protozoans and developing biocides to human-threatening *Acanthamoeba*. Recently we cloned two cDNAs in *Acanthamoeba* and characterized one as a novel protein kinase whose expression decreased with encystment and the other as a novel Ub-like protein whose expression increased with encystment (unpublished result).

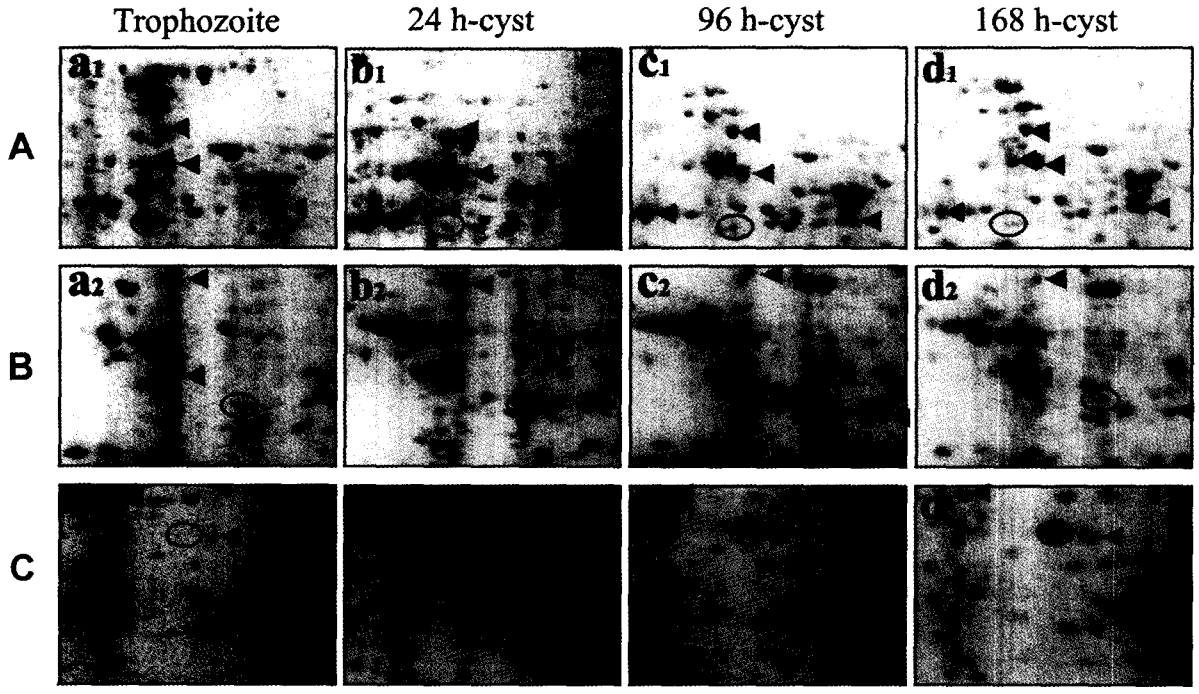

Fig. 4. Detailed comparisons of proteins in selected areas of two- dimensional gels shown in Fig. 2. Circled proteins in A; actin (Spot no. 20), B; 21 kDa protein (SU1), and C; 33 kDa protein (SU2). Arrowheads indicate reference spots (land mark) to align the gels.

Table 2. Comparisons of N-terminal sequences of the cyst specific SU2 protein with subtilisin-like serine proteinase of *A. healyi*

Organisms	Sequences	M.W.(kDa)	pI	Sources
33 kDa protein (Spot no. SU2)	NXQRQDSAIXNLQRINTH	33	6.3	This study
Acanthamoeba healyi. Subtilisin-like serine proteinase	118 NCLSQSGAIWNLQRINAQ 135 * *..** ******::	33	6.3	Hong et al., 2000

Acknowledgement

This study was supported by a grant from Seoul National University. J. T. Park and Y. E. Jeong were supported by BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

References

Bowers B and Korn ED (1969) The fine structure of *Acanthamoeba castellanii* (Neff strain) II encystment. *J Cell Biol* 41: 786-805.

Corliss JO and Esser SC (1984) Comments on the role of the cysts in the life cycle and survival of free-living protozoa. *Trans Am Microsc Soc* 93: 578-593.

Freshney RI (1994) Culture of Animal Cells. Wiley-Liss, New York.

Griffiths AJ and Hughes DE (1969) The physiology of encystment of *Hartmannella castellanii. J Protozool* 16: 93-99.

Gonzalez J, Bai G, Frevert U, Corey EJ, and Eichinger D (1999) Proteasome-dependent cyst formation and stage-specific Ub mRNA accumulation in *Entamoeba invadens. Eur J Biochem* 264: 897-904.

Hirukawa Y, Nakato H, Izumi S, Tsuruhara T, and Tomino S (1998) Structure and expression of a cyst specific protein of *Acanthamoeba castellanii. Biochim Biophys Acta* 1398: 47-56.

Hong YC, Kong HH, Ock MS, Kim IS, and Chung DI (2000) Isolation and characterization of a cDNA encoding a subtilisin-like serine proteinase (ahSUB) from *Acanthamoeba healyi. Mol Biochem Parasitol* 111: 441-446.

Jantzen H (1981) Control of actin synthesis during the development of *Acanthamoeba castellanii. Dev Biol* 82: 113-126.

Jensen TJ, Loo MA, Pind S, Williams DB, Goldberg AL, and Riordan JR (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* 83: 129-135.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* 227: 680-685.

Marciano-Cabral F, Puffenbarger R, and Cabral GA (2000) The increasing importance of *Acanthamoeba* infections. *J Euk Microbiol* 47: 29-36.

Martieze AJ (1985) Free-living amoebas. In: Ration B (ed), Natural History, Prevention, Diagnosis, Pathology and Treatment of Disease, CRC Press, pp 5-42.

Neff RJ, Ray SA, Benton WF, and Wilborn M (1964) Induction of synchronous encystment (differentiation) in *Acanthamoeba sp.* In: Prescott DM (ed), Methods in Cell Physiology I, Academic Press Inc, New York, pp 55.

Neff RJ and Neff RH (1969) The biochemistry of amoebic encystment. *Symp Soc Exp Biol* 23: 51-81.

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007-4021.

Parsell DA and Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Ann Rev Genet* 27: 437-496.

Ramagli LS and Rodriguez LV (1985) Quantitation of microgram amounts of protein in two dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6: 559-563.

Selgen PO and Bohley P (1992) Autophagy and other vacuolar protein degradation mechanisms. *Experientia* 48: 158-172.

Stratford MP and Griffiths AJ (1978) Variations in the properties and morphology of cysts of *Acanthamoeba castellanii. J Gen Microbiol* 108: 33-37.

Weisman RA (1976) Differentiation in *Acanthamoeba castellanii. Ann Rev Microbiol* 30: 189-219.

[Received October 10, 2002; accepted November 13, 2002]