

Antifreeze Water-Rich Dormant Cysts of the Terrestrial Ciliate *Colpoda cucullus* Nag-1 at -65°C : Possible Involvement of Ultra-Antifreeze Polysaccharides

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Abstract. We found that the water-rich (osmolality below 0.052 Osm/l) wet resting cysts of the soil ciliate *Colpoda cucullus* Nag-1 were tolerant to extremely low temperature (-65°C). When cell fluid obtained from the resting cysts was cooled at -65°C , small particles of ice crystals did not grow into large ice crystals. At -65°C , the cysts shrank due to an outflow of water, because a vapor pressure difference was produced between the cell interior and freezing surrounding medium. The osmolality of these shrunk cells was estimated 0.55 Osm/l, and the freezing point depression of the shrunk cell fluid was estimated to be 1.02°C . Hence, the antifreeze ability of wet cysts at -65°C can not be explained by freezing point depression due to elevation of cytoplasmic osmolality.

The cytoplasm of resting cysts was vividly stained red with periodic acid-Schiff (PAS) and stained purple with toluidine blue. On the other hand, the excystment-induced cysts were not stained with PAS, and exhibited a loss of the antifreeze activity. PAS staining of SDS-PAGE gel obtained from encysting *Colpoda* cells showed that a large amount of PAS-positive macromolecules accumulated as the encystment stage progressed. These results suggest that antifreeze polysaccharides may be involved in the antifreeze activity of *C. cucullus* Nag-1 dormant forms.

Keywords: water-rich resting cyst, antifreeze, polysaccharides, osmolality.

INTRODUCTION

In dormant forms (resting cysts of protists and endospores of bacteria) and vegetative forms of microorganisms, tolerance to harsh environments including desiccation (Corliss and Esser 1974), UV rays (Nicholson and Galeano 2003, Lonnen *et al.* 2014, Matsuoka *et al.* 2017, Yamane *et al.* 2020) and freezing (Müller *et al.* 2010, Anderson 2016, Cramm *et al.* 2019) is an adaptive strategy for survival in terrestrial environments. To survive on the soil surface where puddles appear temporarily and dry out rapidly, soil unicellular eukaryotes such as the ciliate *Colpoda* promptly transform into resting cysts when they detect approaching desiccation. They excyst and proliferate rapidly when favorable aquatic environments are recovered.

Dry resting cysts of *Colpoda cucullus* are known to be tolerant to a temperature of -180°C (Taylor and Strickland 1936). *Colpoda cucullus* Nag-1 wet cysts are reported to be tolerant to a temperature of -30°C (Maeda *et al.* 2005). Such antifreeze activity of *Colpoda* cysts is not surprising, because it is generally thought that even wet resting cysts are dehydrated, and thus they do not produce large ice crystals. In the present study, however, we found that wet resting cysts of *C. cucullus* Nag-1 were not dehydrated, and such water-rich resting cysts were also tolerant to a temperature of -65°C . In the present paper, we propose that possible antifreeze polysaccharides might be responsible for the antifreeze activity of *Colpoda* resting cysts.

Encystment of *C. cucullus* Nag-1 can be induced by increasing the cell density in the Ca^{2+} -containing surrounding medium (Matsuoka *et al.* 2009). On the other hand, excystment is induced by the addition of hay infusion (Haagen-Smit and Thimann 1938), wheat leaves infusion (Watoh *et al.* 2003, Tsutsumi *et al.* 2004), chlorophyllin (Tsutsumi *et al.* 2004), and certain peptides (Akematsu and Matsuoka 2007).

In encystment-induced *C. cucullus* Nag-1 cells, mucus is first expelled into the extracellular space, followed by extrusion of small sticky globules called lepidosomes (Foissner *et al.* 2011). The lepidosomes are trapped in a mucus layer, forming a mucus/lepidosome layer (Funatani *et al.* 2010, Funadani *et al.* 2016). At 2–4 h after encystment induction, the cells become rounded and then surrounded by a single rigid layer (ectocyst layer), followed by the formation of several endocyst layers between the ectocyst layer and plasma membrane (Funatani *et al.* 2010). The formation of

a cyst wall (consisting of a mucus/lepidosome layer, an ectocyst layer and endocyst layers, in order from outside to inside) is accompanied by the digestion of vegetative cell structures such as cilia (Funatani *et al.* 2010), and the halting of mitochondrial activity (Funatani *et al.* 2010, Sogame *et al.* 2014) to finally complete the dormant form within several days.

MATERIALS AND METHODS

Colpoda cucullus Nag-1 (Funadani *et al.* 2016) (18S ribosomal RNA gene: GenBank Accession No. AB918716) was cultured in a 0.05% (w/v) dried wheat leaves infusion. Resting cyst formation (encystment) was induced by suspending 2-day cultured vegetative cells in an encystment-inducing medium containing 1 mM Tris-HCl (pH 7.2) and 0.1 mM CaCl_2 at a high cell density ($> 30,000$ cells/ml). Suspensions (300 μl each) of encystment-induced cells were dispensed onto watch glasses, and then placed under humid conditions at room temperature. Watch glasses with suspensions of encysting cells at various stages were then placed in a freezer at -65°C , and kept for more than 24 h. The frozen samples were thawed at room temperature, the medium in the cyst-adhered watch glass was discarded, and a fresh 0.05% wheat leaves infusion was poured to induce excystment. Cysts were randomly chosen, and the number of vacant cysts from which vegetative cells had emerged was counted at 24 h after the start of excystment induction.

For periodic acid-Schiff (PAS) staining of cells, vegetative and encysting cells of *C. cucullus* Nag-1 were dried on a slide glass or watch glass, and stained using a PAS Stain Kit (Muto Pure Chemicals, Tokyo) following the manufacturer's protocol. For toluidine-blue staining of thick sections of resting cysts, the resting cysts were fixed for 6 h in a prefixative [6% glutaraldehyde, 100 mM cacodylate buffer (pH 7.2) and 4 mM sucrose], and then postfixated for 1 week in a postfixative [1% OsO_4 , 100 mM cacodylate buffer (pH 7.2) and 2 mM sucrose]. The postfixated samples were washed with pure water, dehydrated through a graded ethanol series, and finally suspended in acetone. The samples were embedded in Spurr's resin. The thick sections were stained with 0.1% toluidine blue with warming.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially according to Laemmli's method (Laemmli 1970). The *C. cucullus* Nag-1 cells were solubilized in the SDS-sample buffer [30 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% glycerol], and then boiled for 3 min. A sample containing approximately 50 μg proteins in each lane, corresponding to about 5,000 cells, was electrophoresed on a 12.5% gel at 150 V.

For PAS staining of gels, SDS-PAGE gels were incubated for 30 min in 12.5% (v/v) trichloroacetic acid (Wako Pure Chemicals, Osaka, Japan), rinsed in pure water, and then kept for 50 min in a periodic acid solution containing 3% (v/v) acetic acid and 1.08% (w/v) orthoperiodic acid (Wako Pure Chemicals). The treated gels were rinsed six times in pure water for 10 min each, and incubated for 50 min in Schiff's reagent (Wako Pure Chemicals) in the dark. Thereafter, the gels were kept for 30 min in 0.5% (w/v) sodium

bisulfite (Wako Pure Chemicals) solution, and finally soaked in pure water overnight. In this case, the pure water was changed several times. In order to detect protein bands, the PAS-stained gels were stained with 0.2% Coomassie brilliant blue (CBB) R250 dissolved in a solution containing 45% (v/v) methanol and 10% (v/v) glacial acetic acid, and then destained in a 27% (v/v) methanol, 9% (v/v) glacial acetic acid solution.

For preparation of the cell fluid contained in mature cysts, vegetative cells of *C. cucullus* Nag-1 were induced to encyst in a Petri dish and maintained for more than 2 weeks. The cyst-adhered Petri dishes were washed with pure water, and then the cysts were scraped off the dish with a cutter blade and collected by centrifugation (8,000 × g for 10 min). A 20 µl pellet was homogenized using an As One Model 226A microhomogenizer (As One, Osaka, Japan) in a microfuge tube or Teflon homogenizer, then centrifuged (8,000 × g for 10 min) to obtain 10 µl supernatant.

RESULTS AND DISCUSSION

We first examined the tolerance of *C. cucullus* Nag-1 wet resting cysts in various encysting stages to cooling at -65°C (Fig. 1A-1, closed circles). The freeze tolerance of encysting cells was acquired within 2 days after encystment induction. When the resting cysts were cooled at -65°C , they were expected to shrink due to an outflow of water across the plasma membrane, because a vapor pressure difference was produced between the cell interior and freezing surrounding medium. In some resting cysts that had been frozen and thawed, the cell body remained shrunk and was detached from the cyst wall (Fig. 1A-1, inset photograph), indicating that the membrane selective permeability may be destroyed. Such shrunk cysts failed to excyst (data not shown).

When cell fluid obtained from the mature cysts was cooled at -65°C , small ice particles did not grow into large ice crystals (Fig. 1A-2, lower photograph) in contrast to the production of large ice crystals of pure water (Fig. 1A-2, upper photograph). This may be the reason why the cell structure was not destroyed.

When mature resting cysts were placed in a hypertonic surrounding medium (encystment-inducing medium containing 1 M sucrose), they shrank immediately (Fig. 1B-1). The steady-state diameter of cysts kept in sucrose solutions was plotted against sucrose concentrations (Fig. 1B-2). Judging from Fig. 1B-2, the osmolality of the cytosol of mature resting cysts was estimated to be below 0.052 Osm/l [sum of the osmolality of 0.05 mol/l sucrose (0.05 Osm/l), 1 mM Tris-HCl (0.0019 Osm/l), and 0.1 mM CaCl₂ (0.0003 Osm/l)].

As mentioned above, when the mature resting cyst sample was cooled and the surrounding medium began

to freeze, the cysts were shrunk due to an outflow of water. After the cysts were frozen and thawed, some cysts whose selective membrane permeability was destroyed did not return to their original size (see Fig. 1A-1, inset 'b'). The irreversibly shrunk (killed) cells may reflect the cell size when the surrounding medium is frozen. The mean size of the shrunk cells was 23 µm (75% of normal size) (n = 30 cells). Judging from the cell size shown in Fig. 1B-2, the osmolality of the cytosol of shrunk cells was estimated to be 0.55 Osm/l.

Freezing point depression (ΔT) can be estimated by the following formula:

$$\Delta T = K_f m$$

Here, ' K_f ' and ' m ' represent the freezing point of the medium (K_f of water: 1.85) and mass molarity, respectively. If the cytosolic osmolality of resting cysts is 0.55 Osm/l (intracellular mass molarity: \approx 0.55 mol/kg), the freezing point depression (ΔT) can be estimated as 1.02°C . This estimation means that resting cysts may be frozen at -1.02°C . Consequently, failure of *C. cucullus* Nag-1 cysts to freeze -65°C may not be explained merely by a freezing point depression resulting from an elevation in the osmolality of the cytosol.

When the *C. cucullus* Nag-1 resting cysts that had undergone extreme shrinking in 1 M sucrose solution were returned to the medium without sucrose, their size was regained in all cells (n = 15 cells), indicating that the destruction of membrane permeability in some frozen and thawed cysts was not due to dehydration of the cell body. The freezing-mediated destruction of the plasma membrane function may be attributable to mechanical injury by ice crystals produced outside the cells.

C. cucullus Nag-1 encysting cells at different stages were subjected to PAS staining (Fig. 2A). The results showed that vegetative cells were not stained red at all (Fig. 2A, a). On the other hand, encysting cells became vividly stained at 3 h after encystment was induced (Fig. 2A, b–d). The thick sections of mature resting cysts were also stained purple with toluidine-blue (Fig. 2A, f). However, the cells were not stained with PAS at 10 min after onset of excystment induction (Fig. 2A, e), and this absence of staining was accompanied by a disappearance of tolerance to -65°C (Fig. 2B). This strongly suggests that antifreeze polysaccharides may be involved in the tolerance of resting cysts to extremely low temperatures. When cell fluid obtained from the mature cysts was cooled at -65°C , the small ice particles still did not grow into large ice

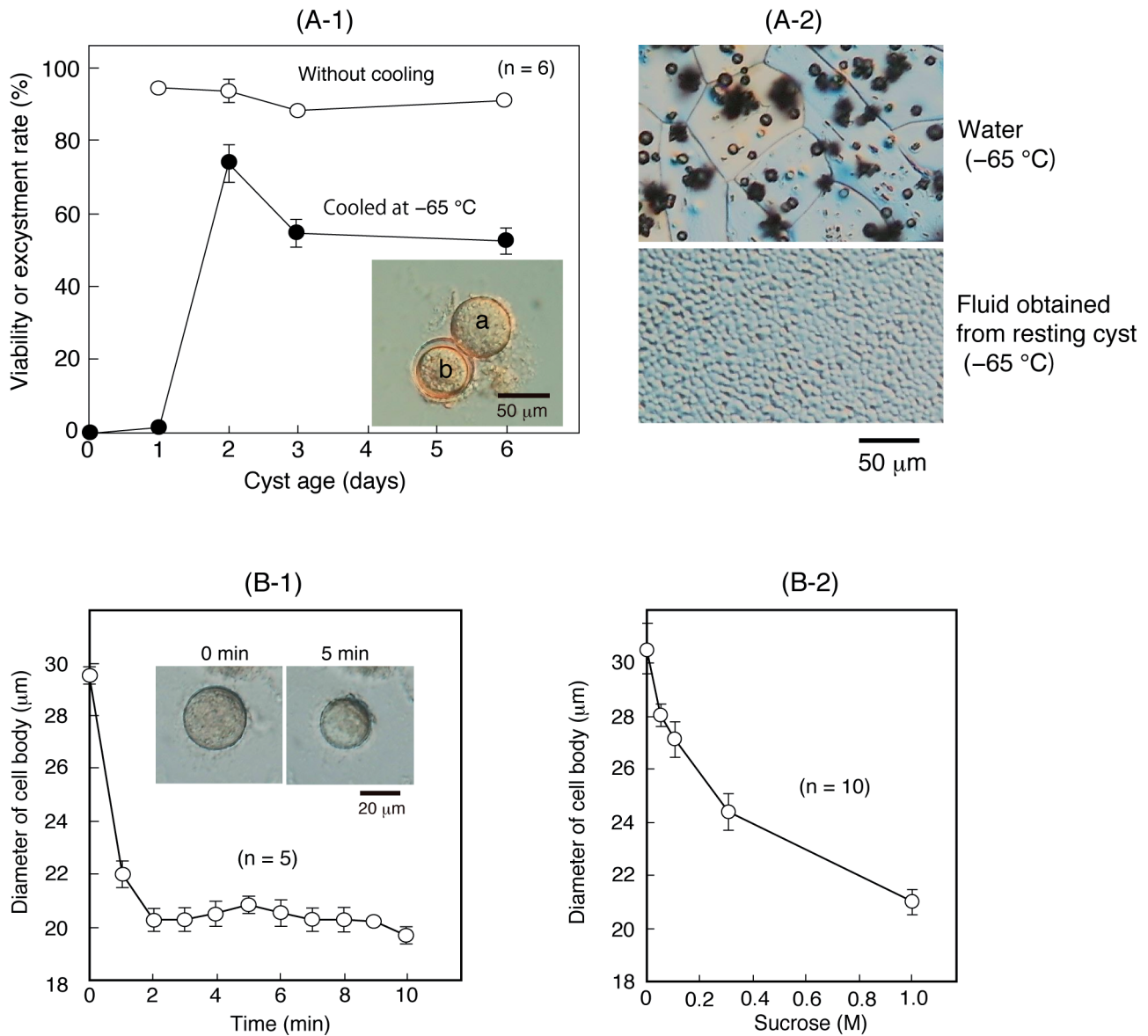


Fig. 1. Tolerance and antifreeze activity of wet resting cysts of *C. cucullus* Nag-1 in response to cooling (-65°C) and their osmolality. (A-1) Tolerance of encysting cells at various encystment stages. The abscissa indicates the cyst age of encysting cells. The ordinate indicates the excystment rates (%) of resting cysts (aged 1 day or more) or viability (%) of vegetative cells. Closed and open circles show the excystment rate or viability of the cells cooled at -65°C for 24 h or more and those without cooling, respectively. The viability (%) of frozen vegetative cells is expressed as a percentage of the total number of tested cells (> 50 cells). The rate of excystment was expressed as a percentage of the total number of observed cells (50 cells). Points and attached bars correspond to the means of 6 measurements and the standard errors (SE), respectively. In each set of experiments (i.e., the cooling group and control group experiments), the same lot samples were used. (A-1, inset photograph) 1-week-old cysts cooled at -65°C for 24 h, and thawed at room temperature. 'a': a living cyst, 'b': a cyst whose cell body shrank and detached from the cyst wall (killed cysts). (A-2) Inhibition of ice crystal growth in cell fluid obtained from *C. cucullus* Nag-1 resting cysts (2 or more weeks old). Upper and lower photomicrographs are pure water and cell fluid obtained from *Colpoda* cysts cooled at -65°C for 30 min. (B-1) Changes in cell size of 2-week-old cysts after transfer from encystment-inducing medium without sucrose to medium containing 1 M sucrose. One run of measurement was done in the same cells. Points and attached bars correspond to the mean diameter obtained from 5 cells and the SE, respectively. (B-1, inset photograph) A 1-week-old resting cyst kept in encystment-inducing medium (left), and the same cyst transferred and kept for 5 min in the encystment-inducing medium containing 1 M sucrose (right). (B-2) Cell size of 2-week-old resting cysts immersed for 10 min in encystment-inducing medium containing various concentrations of sucrose. One run of measurement (0, 0.05, 0.1, 0.3, 1 M sucrose) was done in the same cell. Points and attached bars correspond to the mean diameter obtained from 10 cells and the SE, respectively.

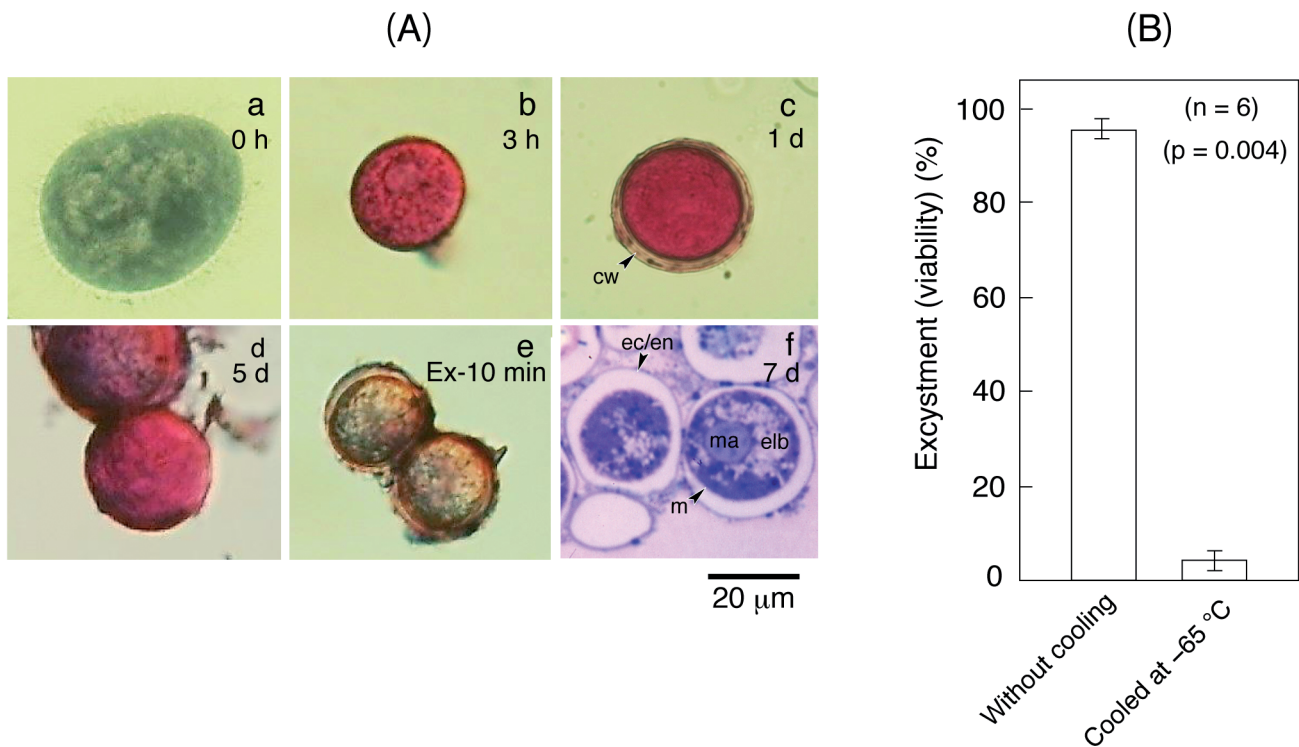


Fig. 2. Detection of polysaccharides in *C. cucullus* Nag-1 encysting cells. (A) A PAS-stained vegetative cell (a), a 3-h-old immature cyst (b), a 1-day-old immature cyst (c), an almost mature cyst (5 days old) (d) and a toluidine-blue stained thick section of a 1-week-old mature cyst (f). (e) PAS-stained 1-week-old mature cysts at 10 min after onset of excystment induction. cw: cyst wall, ec/en: an ectocyst layer lined with endocyst layers, ma: macronucleus, m: plasma membrane, elb: electron-lucent body (probably stored carbohydrate granules). (B) Tolerance of excystment-induced cells (1-week-old cysts) to a temperature of -65°C . Excystment was induced for 10 min, and then the cells were cooled at -65°C for 24 h and then thawed at room temperature. The excystment rate (%) was measured at 24 h after thawing, and was expressed as a percentage of the randomly chosen total number of cells (50 cells). Columns and attached bars correspond to the means of 6 measurements and the standard errors (SE), respectively. In each set of experiment (i.e., the cooling group and control group experiments), the same lot of samples was used. There was significant difference between the columns ($p < 0.01$, Mann-Whitney test).

crystals (Fig. 1A-2). Presumably, the antifreeze polysaccharides accumulated in *Colpoda* resting cysts suppressed the growth of ice crystals by binding to the small ice crystals, as has been observed in antifreeze proteins (Rahman et al. 2019).

SDS-PAGE and PAS staining were carried out for detection of glycoproteins and polysaccharides prior to CBB staining (Fig. 3, left). In the PAS-stained gels, a broad smear band at around 55 kDa, and a faint sharp band at around 45 kDa were observed (Fig. 3, left). The smear band around 55 kDa increased as encystment progressed, but the 45 kDa band hardly changed (Fig. 3, left). PAS-positive macromolecules accumulated at the top of lanes that might contain polysaccharides increased as the cyst formation progressed (Fig. 3, left, arrow). Analysis of the gel stained with CBB and PAS (Fig. 3, right) showed that the PAS-stained smear

band at around 55 kDa was not a glycoprotein, because the corresponding CBB-stained smear band was not detected.

The encysting cells became PAS-stained at 3 h after onset of encystment induction (Fig. 2A). The PAS-stained SDS-PAGE gel also showed that the presumed polysaccharides increased at 3 h after encystment induction (Fig. 3, left). On the other hand, antifreeze activity was not observed until 2 days after encystment induction (Fig. 1A-1). The precursor materials for endocyst layers which have not solidified yet contain toluidine blue-stained substances, suggesting that the endocyst layers may contain polysaccharides (Funatani et al. 2010). Therefore, the precursor of endocyst layers synthesized in the cytoplasm may be PAS-stained, although solidified layers of endocyst were not stained with PAS [Fig. 2A, c; cyst wall (cw)].

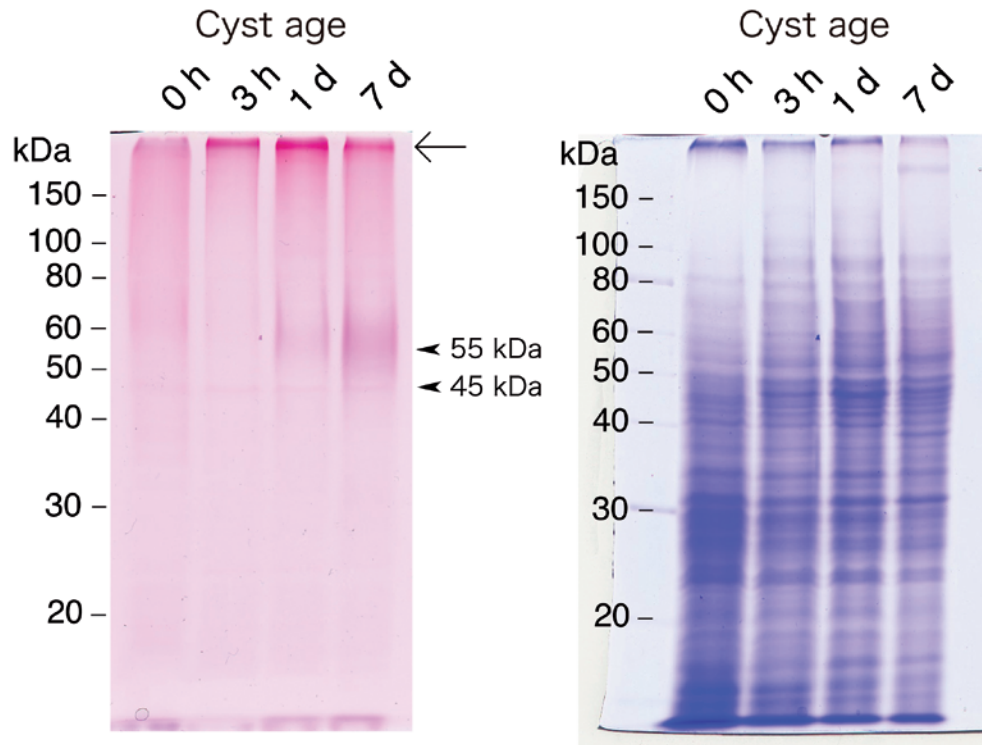


Fig. 3. PAS-stained SDS-PAGE gel (left) analyzing total proteins in encystment-induced cells, and CBB-staining of PAS-stained gel (right).

On the other hand, the PAS-stained components accumulated in the cytoplasm of mature cysts (Fig. 2A, d) may be polysaccharides for antifreeze, not for endocyst formation, because the formation of endocyst layers was completed by this encystment stage.

Antifreeze molecules such as antifreeze proteins, polysaccharides such as xylomannan, and glycolipids have been found in various organisms that exhibit freezing tolerance (Walters *et al.* 2009, Duman 2015, Kawahara *et al.* 2016, Kim *et al.* 2017). The freezing point of antifreeze proteins and xylomannan drops by at most 3–4°C (Walters *et al.* 2009, Kim *et al.* 2017). Therefore, the antifreeze activity of *C. cucullus* Nag-1 resting cysts at –65°C is difficult to explain by the already-known antifreeze molecules alone. A combination of antifreeze proteins and glycerol may cause a further drop in the freezing point, as reported in cryotolerant terrestrial arthropods (Duman 2001). It is possible that the antifreeze activity of *C. cucullus* Nag-1 is accomplished by a combination of multiple cryoprotectants.

In a future work, it will be necessary to isolate the presumed *Colpoda* antifreeze polysaccharides in order to determine their molecular structure and the freezing temperature.

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