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Suppression effects of nutrients on Ca^{2+}-induced encystment of *Colpoda cucullus*

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Summary

'Starvation' is known to be a common factor for encystment induction among a number of protozoans. This may involve that the encystment is suppressed by nutrient molecules contained in a surrounding medium or by incorporated ones. In the present study, what kinds of the nutrients suppress Ca^{2+}-mediated encystment of *Colpoda cucullus* was examined. Among the tested substances (sugars, vitamins, protein, peptides, amino acids), only a bovine serum albumin (BSA) and its digested products (peptides) markedly suppressed encystment, but free amino acids did not show a marked encystment-suppression effect. The suppression of encystment by BSA was not affected when the endocytic internalization of BSA was blocked. The results suggest that the suppression of Ca^{2+}-mediated encystment by BSA may not be correlated with certain receptors on the cell surface stimulated by some partial conformations of the peptides or proteins.

Key words: *Colpoda*, encystment suppression, endocytosis, nutrients, starvation

Introduction

Resting cyst formation (encystment) of terrestrial ciliate *Colpoda* is promptly induced by an increase in external Ca^{2+} concentration (Yamaoka et al., 2004) or overpopulation of *Colpoda* vegetative cells (Strickland, 1940; Maeda et al., 2005). The Ca^{2+}-induced encystment of *Colpoda* is, on the other hand, suppressed by components released from bacteria (Yamasaki et al. 2004) and components contained in plant leaves (Tsutsumi et al. 2004). In *C. cucullus*, however, encystment is induced slowly even when the vegetative cells are transferred into water in which neither encystment-inducing element nor encystment-suppressing one is contained (our observation). In this case, encystment induction might be responsible for starvation which is believed to be a common factor for encystment induction among many protozoans (Corliss and Esser, 1974). The encystment induction by the starvation might be correlated with a cancellation of the encystment suppression due to a stop of supply of encystment-suppressing molecules such as nutrients to the surrounding medium or cell interior. In the present study, the nutrient molecules which can suppress Ca^{2+}-mediated encystment of *C. cucullus* were examined. Among the tested substances (sugars, vitamins, protein, peptides, amino acids), only a protein (bovine serum albumin - BSA) and its digested products (peptides) markedly suppressed

encystment. The present paper will discuss whether these molecules stimulate certain receptors on the cell surface or affect intracellular signaling pathway leading to encystment induction.

Material and methods

Colpoda cucullus was cultured in an infusion of dried cereal leaves (0.05-0.1 %) inoculated with bacteria (*Enterobacter aerogenes*) at 23° C in the dark. Bacteria were cultured on agar plates containing 1.5% agar, 0.5% polypeptone, 1% meat extract and 0.5% NaCl. Encystment was induced by suspending the vegetative cells cultured for 1.5-3 days in a standard saline solution containing 1 mM $CaCl_2$, 1 mM KCl and 5 mM Tris-HCl (pH 7.2); among these salts, Ca^{2+} is a crucial element for encystment induction (Yamaoka et al., 2004). Prior to assays for encystment induction, vegetative cells were quickly rinsed 3 times in standard saline solution containing each test molecule by transferring the cells into fresh solutions using a thin glass pipette, and 50-100 cells were finally suspended in 1.5 ml of each test solution. Bacterial density above 104 cell/ml in the surrounding medium influences salt-induced encystment (Yamasaki et al., 2004). Therefore, bacteria must be eliminated from the test solution. By transferring the *Colpoda* cells into fresh medium 3 times, the number of bacteria could be reduced to about 10 cells/ml (Yamasaki et al., 2004). If the bacteria proliferate twice per 1.2 hr under the most conducive conditions (Yamasaki et al. 2004), their density is still not expected not to exceed 2×10^3 cells/ml in 8 hr.

The rate of encystment was expressed as the percentage of the total number of tested cells (50-100 cells), which was determined at 8 hr after the vegetative cells were transferred into each solution.

The images for fluorescence micrographs were acquired by a digital camera (Nikon, Coolpix 4500) equipped with a microscope (Olympus, BX50). In this case, the cells were fixed with about 3.7 % paraformaldehyde. In order to digest bovine serum albumin (BSA) with trypsin, BSA and trypsin were dissolved at the ratio of 10 : 1 (w/w), respectively, in a medium containing 2% 2-mercaptoethanol and 10 mM NH_4HCO_3 and incubated for 48 hr at 37 °C. After the digestion, the sample was centrifuged (8,000 g, 10 min) and the supernatant was decanted to dialyze in distilled water through a 1,000-Da cutoff membrane (Float-A-Lyzer, Spectrum Laboratories, Inc.) for 3 days on ice. For analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the dialyzed sample was mixed at a ratio of 1 : 1 (vol/vol) with a solution containing 2% SDS, 60 mM Tris-HCl (pH 6.8), 2% 2-mercaptoethanol and 20% glycerol, and then boiled for 3 min. Gels were run at 150 V, stained for 1 hr with 0.2% Coomassie brilliant blue R250 in a 45% (vol/vol) methanol, 10% glacial acetic acid solution, and destained in a 10% (vol/vol) methnanol, 10% (vol/vol) glacial acetic acid solution. For standard proteins, a molecular standards kit (Bio-Rad) was employed.

D-Glucose, D (-)-mannitol and riboflavin were purchased from Nacalai Tesque (Japan), trypsin, BSA, dextran and dextran-FITC from Wako (Japan), BSA-FITC from Sigma, nicotinic acid from Aldrich, and amino acids from Wako (Japan). All chemicals were dissolved in a standard saline solution.

Results and Discussion

As shown in Figs 1A-a and 1B-b, the addition of 1mg/ml of BSA or 1 mg/ml of BSA conjugated with fluorescein isothiocyanate (BSA-FITC) in the surrounding medium evoked prominent suppression of Ca^{2+}-induced encystment in *C. cucullus.* In this case, the encystment rates in the presence of 1 mg/ml BSA or 1 mg/ml BSA-FITC (Fig. 1A-a) were significantly different from the rate in the medium without BSA (open triangle) ($p < 0.05$, Mann-Whitney test). By the addition of peptides obtained by tryptic digestion of 2 mg/ml BSA (Fig. 1B-a), the rate of Ca^{2+}-induced encystment was prominently decreased, although there is no significant difference by comparison with control ('None') ($p = 0.05$, Mann-Whitney test). Other nutrient molecules (dextran, dextran-FITC, glucose, mannitol, ribo-flavin, nicotinic acid, free amino acids) did not showed a marked encystment-supression effect (Figs 1A-b, c, d; Fig. 1C). At a concentration of 1 mg/ml, riboflavin did not dissolve completely; in this medium, no encystment suppression effect was observed (Data not shown). However, encystment induction by lower concentrations of Ca^{2+} (eg. 0.1 mM Ca^{2+}) or overpopulation was somewhat suppressed by the addition of glucose (in preparation). The present encystment induction by 1 mM Ca^{2+} seems to be strong, so that it may not be influenced by the addition of glucose.

When the vegetative cells were suspended in standard saline solution containing BSA-FITC (1 mg/ml), fluorescence became visible in small-sized vacuoles at 30 min after incubation; subsequently,

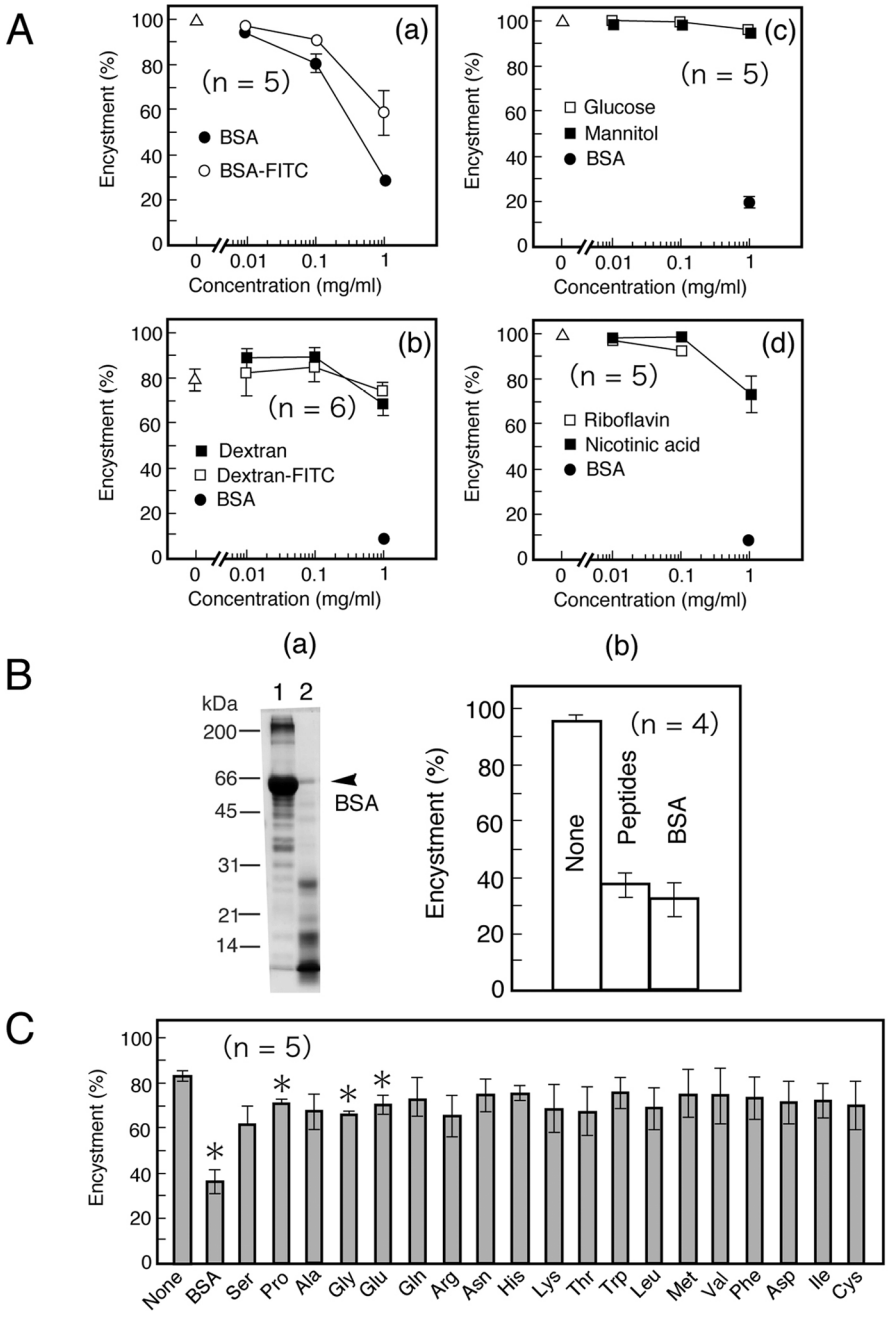

Fig. 1. Suppression effects of the addition of nutrients on Ca2+-induced encystment of Colpoda cucullus. 1A: (a) - BSA (closed circles) and BSA-FITC (open circles); (b) - dextran (closed squares) and dextran-FITC (open squares); (c) - D-glucose (open squares) and D (-)-mannitol (closed squares); (d) - riboflavin (open squares) and nicotinic acids (closed squares); closed circles in (a)-(d) - 1 mg/ml of BSA; open triangles (a)-(d) - standard saline solution (1 mM CaCl2, 1 mM KCl, 5 mM Tris-HCl, pH 7.2) without any nutrients. 1B: (a) - SDS-PAGE of the products obtained by tryptic digestion of BSA. Lane 1, BSA; Lane 2, products obtained by tryptic digestion. The arrowhead indicates a band of BSA; (b) - effect of the addition of peptides produced by tryptic digestion of BSA on Ca2+-induced encystment. None, standard saline solutions without any other components; peptides, products obtained by tryptic digestion of 2 mg/ml BSA; BSA, 2 mg/ml BSA. 1C: effect of the addition of free amino acids (1 mg/ml each) on the Ca2+-induced encystment. The 'amino acid mixture' (labeled 'Mix') contained amino acids (Ser, Pro, Ala, Gly, Glu, Gln, Arg, Asn, His, Lys, Thr, Trp, Leu, Met, Val, Phe, Asp, Ile, Cys; 1 mg/ml each). Columns (points) and attached bars correspond to the means of 4-6 identical measurements (n = 4-6) and SE, respectively. Asterisks (*) in figures indicate significantly different from each other ($p < 0.05$, Mann-Whitney test).

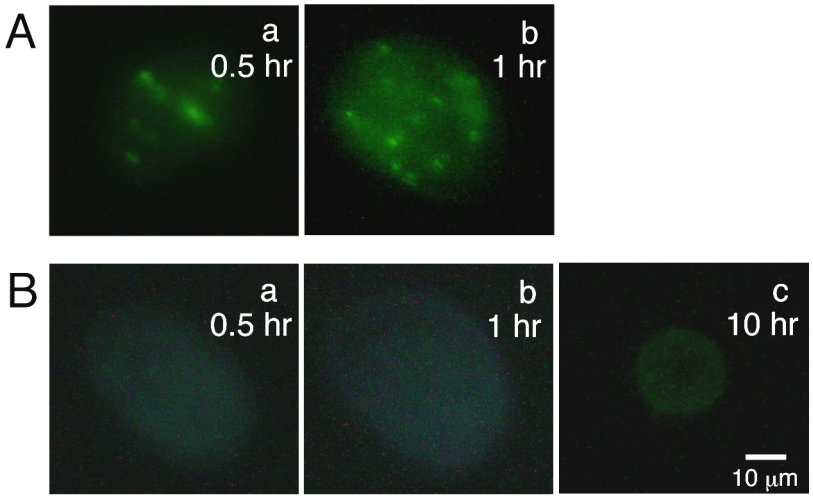

Fig. 2. Fluorescence micrographs of cells fed BSA-FITC (1 mg/ml) for 30 min (A, a) and 1 hr (A, b), and cells fed dextran-FITC (1 mg/ml) for 30 min (B, a), 1 hr (B, b) and 10 hr (B, c).

the number of vacuoles with fluorescence gradually increased (Fig. 2A). Such vacuoles are probably endosomal compartments or early food vacuoles produced by a fusion of endocytic vesicles. On the other hand, no intracellular fluorescence was detected when the vegetative cells were incubated in standard saline solution containing only dextran-FITC (Fig. 2B), which is known to be internalized by fluid-phase endocytosis (Klein and Satre, 1986). These results indicate that only the endocytically internalized molecule (BSA) has encystment-suppressing activity. The question then arises: is the endocytic process and/or nutrition via endocytosis correlated with the suppression of encystment? It has been reported that receptor-mediated endocytosis is inhibited in hypertonic medium (Daukas and Zigmond, 1985; Ramoino et al., 2001). The present study also reconfirmed that the formation of vesicles containing BSA-FITC was inhibited in hypertonic solution containing 0.2 M 4 sucrose (Fig. 3A). Despite endocytosis is blocked, BSA or BSA-FITC tended to be suppressed Ca^{2+}-mediated encystment (Fig. 3B). The encystment was reduced in the hypertonic medium without BSA or BSA-FITC (column labeled 'Sucrose'). This may be attributed to stress caused by exposure to an extreme hypertonic medium. Judging from these results, it is likely that neither the endocytic process nor nutrition via endocytosis is correlated with the suppression of encystment induction. Instead, encystment suppression may be mediated by certain receptors on the cell surface that are activated by proteins such as BSA. The question then arises, what kinds of molecular structures of BSA are recognized by these presumed receptors? Addition of a single amino acid (Ser, Pro, Ala, Gly, Glu, Gln, Arg, Asn, His, Lys, Thr, Trp, Leu, Met, Val, Phe, Asp, Ile, Cys) into external medium showed no prominent suppression of encystment (Fig.1C). Presumably, partial conformation of proteins is responsible for the suppression of encystment induction. A marked encystment suppression by the addition of an amino acid mixture (1 mg/ml each) may be attributed to the stress of higher concentration of the external medium, because in this medium, some of the cells were killed or their cell shape disordered

The Ca^{2+}-induced encystment of *Colpoda* is suppressed by components contained in cereal infusion (Tsutsumi et al. 2004). Peptides and proteins bearing structures responsible for the suppression of encystment are possibly contained in cereal infusion, and the encystment induction by "starvation" may be due to consumption of such molecules in the surrounding medium. If so, in 2-3 day-old culture, in which a number of cells began to encyst, the amount of these molecules must have been reduced. In fact, the absorbance in UV range of 3-day-old exhausted culture medium was extremely reduced (data not shown). And the addition of BSA or peptides into the cell culture (1.5-2 day-old) maintained cell growth for a while (data not shown).

References

Corliss J.O. and Esser S.C. 1974. Comments on the role of the cyst in the life cycle and survival

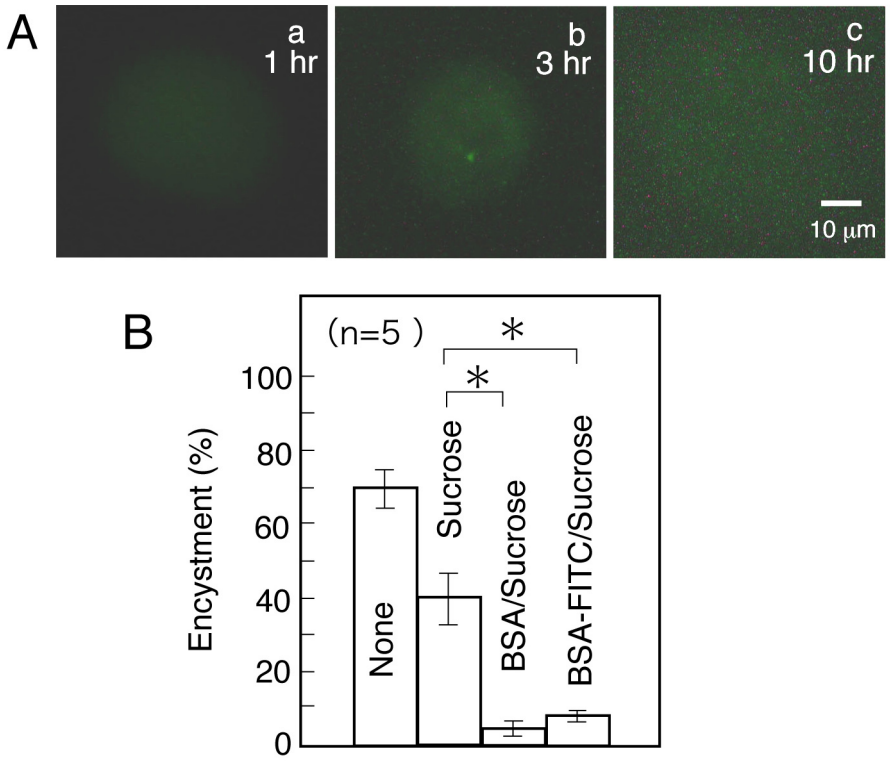

Fig. 3. Effects of hypertonic medium (standard saline solution containing 0.2 M sucrose) on uptake of 1 mg/ml BSA-FITC (A) and on the suppression of Ca2+-induced encystment by BSA or BSA-FITC (B). Cells incubated in the medium containing BSA-FITC for 1hr (A, a), 3 hr (A, b) and 10 hr (A, c). None, standard saline solution without any other component; Sucrose, that containing 0.2 M sucrose; BSA/Sucrose, that containing 1 mg/ml BSA and 0.2 M sucrose; BSA-FITC/Sucrose, that containing 1 mg/ml BSA/FITC and 0.2 M sucrose.

of free-living protozoa. Trans. Amer. Micros. Soc. 93, 578-593.

Daukas G. and Zigmond S.H. 1985 Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. J. Cell Biol. 101, 1673-1679.

Klein G. and Satre M. 1986. Kinetics of fluid-phase pinocytosis in *Dictyostelium discoideum* amoebae. Biochem. Biophys. Res. Commun. 138, 1146-1152.

Maeda H., Akematsu T., Fukui R. and Matsuoka T. 2005 Studies on the resting cyst of ciliated protozoan *Colpoda cucullus*: Resistance to temperature and additional inducing factors for en-or excystment. J. Protozool. Res. 15, 7-1.

Ramoino P., Fronte P., Beltrame F., Robello M. and Diaspro A. 2001. Fluid phase and receptor-mediated endocytosis in *Paramecium primaurelia* by fluorescence confocal laser scanning microscopy. Eur. Biophys. J. 30, 305-312.

Strickland A. G. R. 1940. The effect of concentration of *Colpoda duodenaria* on the time required for encystment in food-free medium. Physiol. Zool. 13, 356-365.

Tsutsumi S., Watoh T., Kumamoto K., Kotsuki H. and Matsuoka T. 2004. Effects of porphyrins on encystment and excystment in ciliated protozoan *Colpoda* sp. Jpn. J. Protozool. 3, 119-126.

Yamaoka M., Watoh T. and Matsuoka T. 2004. Effects of salt concentration and bacteria on encystment induction in ciliated protozoan *Colpoda* sp. Acta Protozool. 43, 93-98.

Yamasaki C., Kida A., Akematsu T. and Matsuoka T. 2004. Effect of components released from bacteria on encystment in ciliated protozoan *Colpoda* sp. Jpn. J. Protozool. 37, 111-117.

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