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Entamoeba invadens: cysteine protease inhibitors block excystation and metacystic development

Asao Makioka[a,*], Masahiro Kumagai[a], Seiki Kobayashi[b], Tsutomu Takeuchi[b]

[a] *Department of Tropical Medicine, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan*
[b] *Department of Tropical Medicine and Parasitology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*

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Abstract

We examined the effects of six cysteine protease inhibitors on the excystation and metacystic development of *Entamoeba invadens*. Excystation, which was assessed by counting the number of metacystic amoebae after the induction of excystation, was inhibited by the cysteine protease inhibitors Z-Phe-Ala-DMK and E-64d in a concentration-dependent manner during incubation compared to the controls. Neither inhibitor had a significant effect on cyst viability; thus, their inhibitory effects were not due to the toxic effect on cysts. Metacystic development, when determined by the number of nuclei in amoeba, was also inhibited by these protease inhibitors, because the percentage of 4-nucleate amoebae was higher than in the controls on Day 3 of incubation. Although other cysteine protease inhibitors, Z-Phe-Phe-DMK, E-64, ALLM, and cathepsin inhibitor III, had a weak or little effect on the excystation, they inhibited cysteine protease activity in the lysates of *E. invadens* cysts. Broad bands with gelatinase activity of metacystic amoebae, as well as cysts and trophozoites, were detected in the gelatin substrate gel electrophores and were inhibited by Z-Phe-Ala-DMK. There was a difference in the protease composition between cysts and trophozoites, and the protease composition of metacystic amoebae changed from cyst-type to trophozoite-type during development. These results strongly suggest that cysteine proteases contribute to the excystation and metacystic development of *E. invadens*, which leads to successful infection.

Index Descriptors and Abbreviations: Entamoeba invadens; Protozoa; Cysteine protease; Excystation; Metacystic development; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

1. Introduction

Cysteine proteases are not only important virulence factors, but also play a role in the growth and differentiation in many protozoan parasites (McKerrow, 1989; Rosenthal, 1999; Sajid and McKerrow, 2002). This is also true of *Entamoeba histolytica*, because there is accumulating evidence for the potential role of cysteine proteases in the pathogenesis of invasive amebiasis as well as in the growth of the parasites (reviewed by Que and Reed, 2000). In *Entamoeba invadens*, which has been used as a model of encystation and excystation of *E. histolytica*, specific cysteine protease inhibitors significantly reduced the efficiency of encystation, although the effect of inhibition was secondary through decreased trophozoite multiplication (Sharma et al., 1996). However, no studies on the role of cysteine proteases in the excystation and metacystic development of *Entamoeba* have so far been reported.

Excystation and metacystic development are necessary for *Entamoeba* infection, and their processes have been described for *E. histolytica* (Cleveland and Sanders, 1930; Dobell, 1928). Since *E. histolytica* does not encyst

* Corresponding author. Fax: +3 3431 4459.
E-mail address: makioka@jikei.ac.jp (A. Makioka).

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efficiently in axenic culture, *E. invadens*, a reptilian parasite, has been commonly accepted as a model for the study of encystation and excystation (Eichinger, 1997; López-Romero and Villagómez-Castro, 1993). Excystation is the process through which the whole organism escapes from the cyst through a minute perforation in the cyst wall. Metacystic development is the process in which a hatched metacystic amoeba with four nuclei divides to produce eight amoebulae, which grow to become trophozoites (Cleveland and Sanders, 1930; Dobell, 1928; Geiman and Ratcliffe, 1936). The transfer of *E. invadens* cysts in an encystation medium to a growth medium induces in vitro excystation (Garcia-Zapien et al., 1995; Makioka et al., 2002; McConnachie, 1955; Rengpien and Bailey, 1975). In this study, we examined the effect of cysteine protease inhibitors on the excystation and metacystic development of *E. invadens*. Here, we report that cysteine proteases contribute to these processes of *E. invadens*.

2. Materials and methods

Trophozoites of the IP-1 strain of *E. invadens* were cultured in an axenic growth medium, BI-S-33 (Diamond et al., 1978), at 26 °C. To obtain cysts, trophozoites (5×10^5 cells/ml) were transferred to an encystation medium called 47% LG (LG is BI without glucose; Sanchez et al., 1994). After three days of incubation, the percentage of encystation reached 80% on average. The cells were harvested and treated with 0.05% sarkosyl (Sigma Chemical, St. Louis, MO) to destroy the trophozoites (Sanchez et al., 1994). The remaining cysts were washed with phosphate-buffered saline, counted, and then suspended in a growth medium. The viability of the cysts was determined by trypan blue dye exclusion, and the number of nuclei per cyst was determined after staining with modified Kohn's stain (Kumagai et al., 2001). Cyst preparation included 30% dead or denatured cysts and 70% viable cysts, where 4-nucleate cysts are 30% and 1- to 3-nucleate cysts are 70%. For the experiments on the excystation and metacystic development of *E. invadens*, duplicate cultures of 5×10^5 cysts/ml were incubated with inhibitors for three days. Metacystic amoebae were counted in a hemocytometer on Days 1 and 3, and their viability was determined by trypan blue dye exclusion. Viable metacystic amoebae and cysts were clearly distinguished as light yellow and light blue in color, respectively. The former was also identified by positive motility. The cysteine protease inhibitors used in this study, purchased from Calbiochem (San Diego, CA), are listed in Table 1. These inhibitors were previously used in cultures of human fibroblasts infected with *Toxoplasma gondii* to examine their effect on the intracellular development of parasites (Shaw et al., 2002). All of the inhibitors were dissolved in dimethyl sulfoxide (DMSO).

Table 1
Cysteine protease inhibitors used in the present study

Inhibitor	Specificity
Cysteine protease inhibitors	
Z-Phe-Ala-DMK	Cysteine proteases
Z-Phe-Phe-DMK	Cysteine proteases
E-64	Cysteine proteases
E-64d	Cysteine proteases
Calpain inhibitor 2 (ALLM)	Ca^{2+}-dependent cysteine proteases
Cathepsin (cysteine) protease inhibitor	
Cathepsin inhibitor III	Cathepsin proteases

Abbreviations: Z-Phe-Ala-DMK, Z-Phe-Ala-diazomethylketone; Z-Phe-Phe-DMK, Z-Phe-Phe-diazomethylketone; E-64d, (2S, 3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethylester; E-64, *N*-[*N*-(L-3*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine; calpain inhibitor 2, *N*-acetyl-leu-leu-methioninal (ALLM); and cathepsin inhibitor III, Z-Phe-Gly-NHO-Bz-*p*OMe.

The control cultures received the same volume of DMSO.

Metacystic development was determined by the number of nuclei per amoeba. The cells were harvested on Days 1 and 3 in cultures with or without inhibitors and stained with modified Kohn's stain. The number of nuclei per amoeba was determined by the double-counting of least 100 amoebae.

For the assay of cysteine protease activity, cysts (2×10^7/ml) were harvested, washed, and subjected to three freeze–thaw cycles in a phosphate-buffered saline. After centrifugation, the supernatants were obtained as lysates. Protease activity was quantified by the cleavage of synthetic peptide substrate Z-Arg-Arg-AMC (benzyloxycarbonyl-arginine-arginine-4-amino-7-methyl-coumarin; Sigma) as previously described (Keene et al., 1986), and recorded as the initial velocity of the cleavage of the fluorescent 4-amino-7-methylcoumarin group/5 μl lysate. The lysates were preincubated for 15 min at room temperature with 10 and 50 μM each of the cysteine protease inhibitors as described above.

Protease gel activity was assessed by gelatin substrate gel electrophoresis as previously described (Keene et al., 1986). The cysts and trophozoites were solubilized using a Laemmli sample buffer (Laemmli, 1970) without a reducing agent, and the supernatants after centrifugation were used. To obtain metacystic amoebae with 4-nuclei and 1-nucleus separately, the cysts were transferred to the growth medium with or without 10 μg/ml aphidicolin (Sigma). The cultures with aphidicolin on Day 1 contained a higher percentage of 4-nucleate amoebae, while the cultures without the drug on Day 3 included that of 1-nucleate amoebae (Makioka et al., 2003). Metacystic amoebae in both cultures were lysed by treatment with a small volume of 0.05% sarkosyl, which had no effect on the cysts. The supernatants after centrifugation were then treated with the Laemmli sample buffer. In certain experiments, trophozoites were treated similarly as for metacystic amoebae. SDS–PAGE was conducted in non-

reducing conditions on 10% gels that had been copolymerized with 0.1% gelatin: equivalent to 5×10^4 loaded per lane. After electrophoresis, the gels was washed for 1 h in 2.5% Triton X-100 to remove SDS, rinsed twice in distilled water, and incubated in 100 mM Tris–HCl (pH 7.4) buffer containing 5 mM EDTA and 2 mM DTT with or without 1 mM Z-Phe-Ala-DMK for 12–18 h at 37 °C. After staining with Coomassie blue and several cycles of destaining, the gelatinase activity was detected as clear bands on the Coomassie blue-stained background of the control gels. All of the experiments of this study were performed at least three times and similar results were obtained. Therefore, the data presented in the results are representative.

3. Results

The effect of cysteine protease inhibitors on the number of metacystic amoebae of *E. invadens* after the transfer of cysts to a growth medium is shown in Fig. 1. Among the inhibitors tested, two cysteine protease inhibitors, Z-Phe-Ala-DMK and E-64d, were effective. For this reason, only the results of these inhibitors are shown. The number of metacystic amoebae in cultures with 10 μM Z-Phe-Ala-DMK during incubation was comparable to the controls, whereas it significantly decreased in cultures with 50 and 100 μM Z-Phe-Ala-DMK compared to the controls. Similarly, metacystic amoebae decreased in number during incubation in cultures with more than 50 μM E-64d. The effects of cysteine protease inhibitors on cyst viability are shown in Fig. 2. The number of viable cysts in the control cultures decreased during incubation. It is considered that most immature cysts contained in culture degenerate or die during incubation. The number of viable cysts in cultures containing 50 and 100 μM Z-Phe-Ala-DMK or E-64d during incubation was comparable to or greater than that of the controls, except for 100 μM E-64d on Day 3.

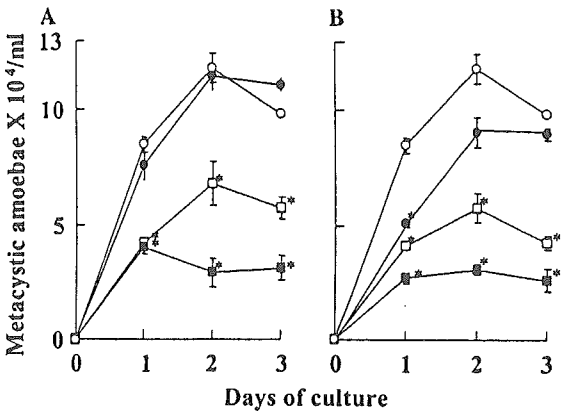

Fig. 1. Effect of cysteine protease inhibitors on the number of metacystic amoebae of *Entamoeba invadens*. Cysts were transferred to a growth medium containing various concentrations of cysteine protease inhibitors Z-Phe-Ala-DMK (A) and E-64d (B). The mean numbers ± SE of metacystic amoebae for the duplicate cultures are plotted (each asterisk indicates $P < 0.05$). Concentrations of 0, 10, 50, and 100 μM are indicated by the white circles, black circles, white squares, and black squares, respectively.

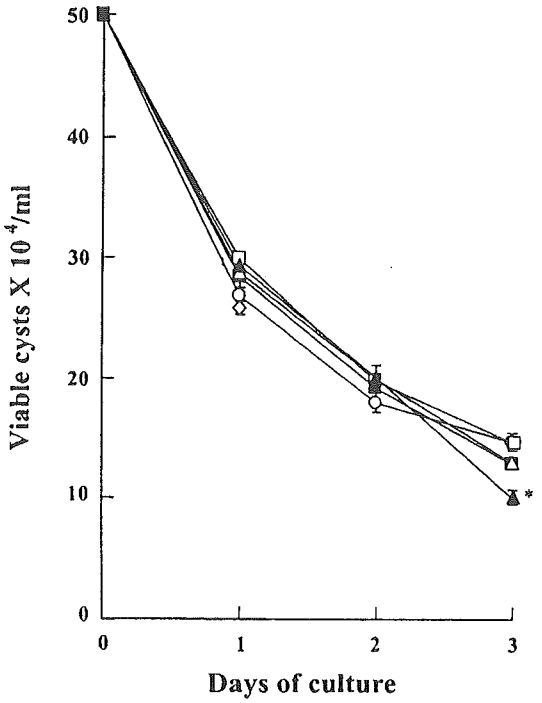

Fig. 2. Effect of cysteine protease inhibitors on the cyst viability of *E. invadens* in the growth medium. The experimental conditions were the same as those for Fig. 1. The mean numbers ± SE of viable cysts for the duplicate cultures are plotted (each asterisk indicates $P < 0.05$). Control (white circles), 50 μM Z-Phe-Ala-DMK (white squares), 100 μM Z-Phe-Ala-DMK (black squares), 50 μM E-64d (white triangles), and 100 μM E-64d (black triangles).

The effects of cysteine protease inhibitors on metacystic development were examined by counting the number of nuclei per cell. As shown in Fig. 3, 9% of the metacystic amoebae were 4-nucleate on Day 1 of incubation in the control cultures, whereas 29 and 34% of the amoebae were in cultures with 100 μM each of Z-Phe-Ala-DMK and E-64d, respectively. The percentage of 4-nucleate amoebae in the control cultures then decreased to 3% on Day 3, following the increased percentages of 1- to 3-nucleate amoebae. In contrast, the percentage of 4-nucleate amoebae in cultures with Z-Phe-Ala-DMK and E-64d was still 19 and 27% on Day 3, respectively, suggesting the inhibition of metacystic development due to these inhibitors.

Since there was a difference in the inhibitory effect on excystation among the cysteine protease inhibitors used, we examined the effects of these inhibitors on cysteine protease activity in cyst lysates. As shown in Fig. 4,

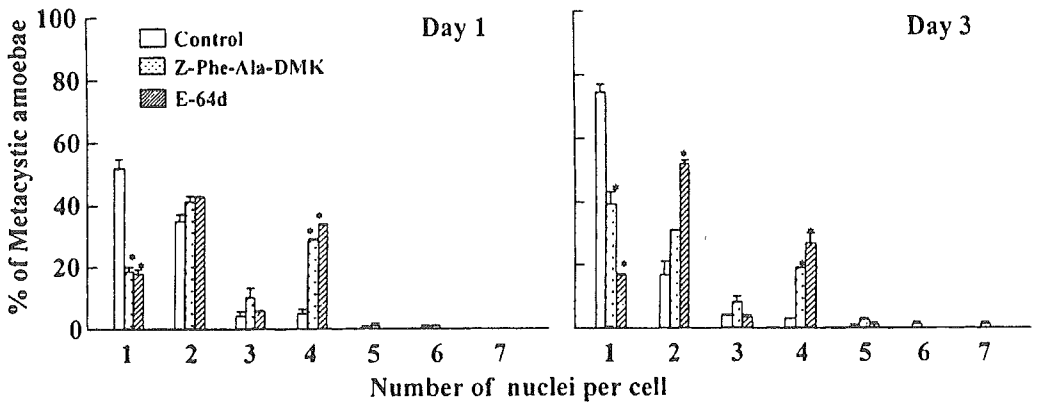

Fig. 3. The effect of cysteine protease inhibitors on the metacystic development of *E. invadens*. The cysts were transferred to a growth medium with or without 100 μM of Z-Phe-Ala-DMK or E-64d. The numbers of nuclei per metacystic amoeba stained with modified Kohn on Days 1 and 3 of incubation were counted, and the percentage of amoebae in each class (1- to 7-nucleate) was determined (each asterisk indicates $P < 0.05$).

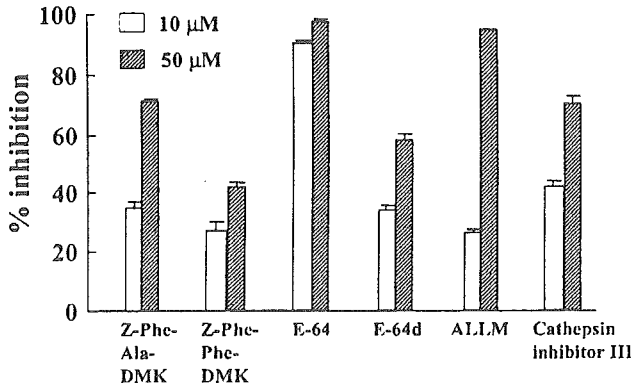

Fig. 4. The effect of cysteine protease inhibitors on cysteine protease activity in the lysates of *E. invadens* cysts. The lysates of *E. invadens* cysts (2×10^7/ml) were incubated with 10 or 50 μM each of the six inhibitors shown in Table 1. The percentages ± SE of inhibition against the control are plotted.

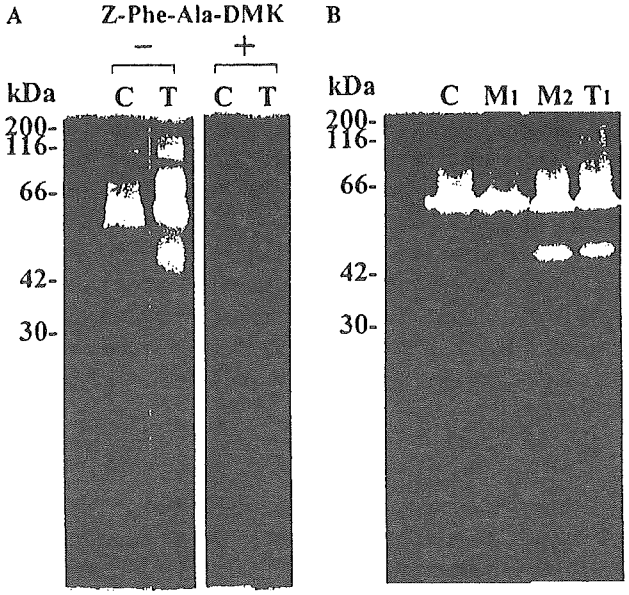

Fig. 5. Gelatin substrate SDS–PAGE of the lysates of *E. invadens* cysts, metacystic amoebae, and trophozoites. (A) Cysts (C); tophozoites (T). Following the removal of SDS, the gels were incubated in buffer alone (–) or with 1 mM Z-Phe-Ala-DMK (+). (B) M1 and M2 were metacystic amoebae from cultures with aphidicolin on Day 1, and those from cultures without the drug on Day 3, respectively. T1 was trophozoites treated similarly as those for metacystic amoebae.

cysteine protease activity in cyst lysates against synthetic peptide substrate Z-Arg-Arg-AMC was inhibited by all of the inhibitors, although there was a difference in their potency.

As shown in Fig. 5A, gelatin substrate SDS–PAGE indicated a major band of 56 kDa, broad bands of 58–66 kDa and 44–54 kDa, and a minor band of 43 kDa in cyst lysates (C). The 56 kDa band and these broad bands detected in cysts were also seen in trophozoite lysates (T). Additional broad bands of 29–41 kDa were also detected in the trophozoite lysates, suggesting a qualitative difference between these two forms. Most of these bands disappeared in the presence of Z-Phe-Ala-DMK. Newly hatched metacystic amoebae with four nuclei (M1) showed a band pattern similar to that of cysts, while more developed metacystic amoebae with one nucleus (M2) showed a band pattern similar to that of trophozoites (T1) (Fig. 5B).

4. Discussion

These results strongly suggest the participation of cysteine proteases in the excystation and metacystic development of *E. invadens*. As cyst viability was not affected by the two cysteine protease inhibitors, Z-Phe-Ala-DMK and E-64d, reduced excystation cannot be due to their toxic effect on cysts. Since other cysteine protease inhibitors used in the present study inhibited cysteine protease activity in cyst lysates, their failure to block

excystation must be due to their lower cell permeability. This is true for E-64, which had a strong inhibitory potency on the cysteine protease activity in cyst lysates. On the other hand, its inhibitory effect on excystation was much lower than E-64d, which is a membrane-permeable synthetic analog of E-64. The process of excystation includes the loosening and separation of amoeba from the cyst wall; the amoeba begins to move about within the cyst. The amoeba then flows back and forth through a small pore in the cyst wall and escapes from the cyst. Thus, cyst wall destruction is necessary for minute perforation of the cyst wall. Since the *Entamoeba* cyst wall contains a mix of protein and chitin (Arroyo-Begovich and Carbez-Trejo, 1982; Frisardi et al., 2000), it is conceivable that both protease and chitinase are essential for cyst wall destruction in the excystation process. The walls of *E. invadens* cysts are electron dense and have a uniform thickness of ~100 nm when observed by electron microscopy (Frisardi et al., 2000). Electron-dense materials were also present in the secretory vesicles and along the plasma membrane. Furthermore, the formation of a crescent-shaped space between the plasma membrane and the cyst wall was observed, and, frequently, some electron-dense bodies projected towards this newly formed space (Chavez-Munguia et al., 2003). Metacysts that endocytose the cyst wall residues were also observed. These observations suggest that secretory vesicles, including proteases and chitinase, are sent in close apposition to the plasma membrane. These enzymes are then secreted into the space between the plasma membrane and the cyst wall to destroy the cyst wall.

The hatched 4-nucleate metacystic amoeba grows rapidly and divides to form eight amoebulae. The results indicate that cysteine proteases are also involved in this metacystic development because the percentage of 4-nucleate amoebae was higher than in the controls on Day 3 of incubation. The results indicate the difference in the band pattern of protease activity between cysts and trophozoites, also changing the band pattern form from cyst-type to trophozoite-type during metacystic development. This is related to our previous results that suggest change in the expression of proteins during metacystic development (Makioka et al., 2003).

Regarding other proteases, we have previously demonstrated that lactacystin, a specific inhibitor of proteasome, had little effect on the excystation and metacystic development of *E. invadens*, suggesting the little contribution of proteasome to these processes (Makioka et al., 2002), although lactacystin inhibited the encystation in vitro of *E. invadens* (Gonzalez et al., 1999; Makioka et al., 2002).

It has recently been demonstrated that *E. histolytica* contains 20 cysteine protease (CP) genes, of which only a small subset is expressed during in vitro cultivation (Bruchhaus et al., 2003). Therefore, it is likely that at least some of these enzymes are required to infect the human host and/or complete the parasite life cycle (Bruchhaus et al., 2003). The gene that encodes CP5 is missing in the closely related but non-pathogenic *E. dispar*, suggesting the potential role of CP5 in the host tissue destruction of *E. histolytica*. Since cyst wall destruction is necessary for excystation by both amoebae, it appears that CP5 is not responsible for cyst wall destruction in either *E. histolytica* or *E. dispar*, or that other CP isoforms are used for it in *E. dispar*. Regarding CP genes in *E. invadens*, it has recently been demonstrated that among the 20 CP genes of *E. histolytica*, 14 homologous genes are found in this parasite (Wang et al., 2003).

Future study will focus on the identification and characterization of CP isoforms responsible for the excystation and metacystic development of *Entamoeba*, which will lead to a more accurate understanding of these processes and also to the identification of targets for vaccination and chemotherapy to inhibit *Entamoeba* infection.

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GROWTH-PROMOTING EFFECT ON IRON-SULFUR PROTEINS ON AXENIC CULTURES OF *ENTAMOEBA DISPAR*

KHALIFA S.A.M.*, IMAI E.*, KOBAYASHI S.*, HAGHIGHI A.**, HAYAKAWA E.* & TAKEUCHI T.*

Summary:

A growth-promoting factor (GPF) that promotes the growth of *Entamoeba dispar* under axenic culture conditions was found in fractions of mitochondria (Mt), hydrogenosomes (Hg) and chloroplasts (Cp) obtained from cells of six different protozoan, mammalian and plant species. We were able to extract the GPF from the Cp-rich leaf cells of a plant (spiderwort: *Commelina communis* L.) in an acetone-soluble fraction as a complex of chlorophyll with low molecular weight proteins (molecular weight (MW) approximately 4,600). We also found that pre-treatment with 0.6 % complexes of 2-mercaptoethanol (2ME) complexes of chlorophyll-a with iron-sulphur (Fe-S) proteins (e.g., ferredoxins (Fd) from spinach and *Clostridium pasteurianum*) and noncomplex rubredoxin (Rd) from *C. pasteurianum* have a growth-promoting effect on *E. dispar*. These findings suggest that *E. dispar* may lack a sufficient quantity of some essential components of Fe-S proteins, such as Fe-S center.

KEY WORDS : growth, mitochondria, hydrogenosomes, chloroplasts, iron-sulphur proteins, *Entamoeba dispar*.

Résumé : LES EFFETS D'ACCÉLÉRATION DE CROISSANCE DES PROTÉINES FER SOUFRE DANS LA CULTURE AXENIQUE D'*ENTAMOEBA DISPAR*

*Des facteurs d'accélération de croissance (Growth promoting factor: GPF) favorisant le développement d'*Entamoeba dispar *ont été détectés dans la composition de la mitochondrie, de l'hydrogénosome et du chloroplaste isolés à partir de six sortes de cellules issues de protozoaires, mammifères et plantes. De plus, les GPF de cellules de mésophile contenant une grande quantité de chloroplaste végétal (*Commelina communis *L.) ont pu être extraits comme une substance composée de protéines de faible masse moléculaire (≈ 4600) et de chloroplastes dans la composition acétone-soluble. À partir de ces résultats, nous avons préalablement pu découvrir des effets d'accélération de croissance dans le corps composé de protéines fer-soufre (épinard et ferrédoxine de* Clostridium pasteurianum*) et de chlorophylle-a traité au 2-mercaptoéthanol 0,6 % et du corps simple rubrédoxine de* C. pasteurianum*. Ces observations ont suggéré qu'une composante essentielle formant la protéine fer-soufre d'*E. dispar *(comme noyau fer-soufre) semblait être insuffisante.*

MOTS CLÉS : *Croissance, entamoeba histolytica, hydrogénosome, chloroplaste, protéines fer-soufre, Entamoeba dispar.*

INTRODUCTION

Entamoeba dispar grows well under xenic and monoxenic culture conditions along with enteric bacteria such as *Escherichia coli* or anaerobic bacteria such as *Fusobacterium symbiosum* (*Clostridium symbiosum* ATCC 14940) (Robinson, 1968; Diamond, 1982; Vargas *et al.*, 1990). However, even after the introduction of the axenic yeast extract-iron serum (YI-S) medium for *E. dispar* (Diamond *et al.*, 1995; Clark, 1995) the axenic cultivation of *E. dispar* remains difficult. Compared with *E. dispar*, pathogenic *E. histolytica*, which is closely related to *E. dispar*, easily adapts to the axenic culture medium (TYI-S-33) (Diamond *et al.*, 1978). Additionally, *Entamoeba histolytica* is capable of invading the mucosa of the large intestine. We have also developed a new yeast extract-iron-gluconic acid-dihydroxyacetone-serum medium (YIGADHA-S) (Kobayashi *et al.*, 2005) based on the YI-S medium and on the results of an investigation on bacterial metabolic products, and have succeeded in culturing five strains under axenic conditions. However, despite using the YIGADHA-S culture system, the axenic growth of four of five strains of *E. dispar* was found to be very poor, with the exception of one primate-derived strain (CYNO 09: TPC) isolated from a cynomolgus monkey. A further search for useful growth promoting factors (GPFs) revealed that autoclaved (121°C, 15 minutes) bacteria and more than 20 types of protozoan, mammalian and plant cells containing mitochondria (Mt), hydrogenosomes (Hg) and chloroplasts (Cp) have a significant growth-promoting effect on *E. dispar*. However, the degree of these growth-promoting effects differs among the GPFs. In the present study, we were able to extract GPF from the Cp-rich leaf cells of a plant (spiderwort: *Commelina communis* L.) in an acetone-soluble fraction as a chlorophyll complex with low mole-

* Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan.

** Department of Medical Parasitology and Mycology, School of Medicine Shaheed University of Medical Sciences, Evven, Teheran 19395, Iran.

Correspondence: Seiki Kobayashi, PhD., Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.

Tel.: +81-3-5363-3761 – Fax: +81-3-3353-5958

E-mail: skobaya@sc.itc.keio.ac.jp

cular weight proteins. Significant amounts of iron (Fe), sulphur (S) and molybdenum (Mo) atoms were found in the protein-chlorophyll complex fraction along with the magnesium (Mg) atom of chlorophyll.

Based on these findings, we hypothesized that some common components of Fe-S proteins present in Mt, Hg, Cp and bacteria support the growth of *E. dispar*. This is because in *E. dispar*, an essential redox Fe-S protein [ferredoxin (Fd)] is expected to be involved in energy metabolism, such as oxidative decarboxylation of pyruvate to acetyl-coenzyme A (acetyl-CoA), which is similar to that found in *E. histolytica* (McLaughlin & Aley, 1985).

MATERIALS AND METHODS

E. DISPAR ISOLATE

In this study, one human-derived strain of axenically grown E. dispar (AS-16-IR) (Kobayashi *et al.*, 2005) was used to determine the growth promoting effect of Fe-S proteins.

AXENIC CULTIVATION OF *E. DISPAR*

The YIGADHA-S medium (Kobayashi *et al.*, 2005) containing 15 % heat-inactivated bovine serum was used as the axenic cultivation medium for *E. dispar*.

DONOR CELLS OF MT, HG, CP AND MITOSOMES

The Mt donors are as follows: protozoan parasites: i) *Crithidia fasciculata* (ReF-1, PRR strain, ATCC 50083); ii) *Trypanosoma cruzi* (Tulahuen strain); iii) *Leishmania major* (MHOM/SU/73/5-ASKH strain, ATCC 50155); and iv) *Acanthamoeba castellanii* (ATCC 30011). v) The vertebrate cells used as Mt donors were from the mouse lymphoblast cell line (P388D1, ATCC CCL-46). The Hg donors are as follows: protozoan parasites: vi) *Trichomonas vaginalis* (KO-11 strain) (Qi *et al.*, 1995) and vii) *Trichomonas foetus* (Okamoto *et al.*, 1998). viii) Leaves of the spiderwort (*Commelina communis* L.) were used as the Cp donors. Consisting amitochondrial protozoan parasite mitosomes that are mitochondrial remnant organelles (Tovar *et al.*, 2003) was used as the mitosome donor, ix) *Giardia intestinalis* (syn. *lamblia*), (Portland-1 strain, ATCC 30888). The bacterium used was *Pseudomonas aeruginosa* (PA:KEIO strain) (Kobayashi *et al.*, 1998).

PREPARATION OF CELLS

C. fasciculata and *T. cruzi* were axenically cultured in liver infusion tryptose (LIT) medium (Gutteridge *et al.*, 1969) supplemented with 10 % foetal bovine serum (FBS). After cultivation for three and seven days respectively, 2×10^7 cells of each parasite were harvested by centrifugation (650 g × 8 minutes). The cells of each parasite were washed three times by centrifugation with 10 mM phosphate-buffered saline (PBS) (pH 7.4), and each pellet was suspended in approximately eight times its volume of 50 mM Tris-HCl (pH 7.4). *L. major* was axenically cultured in Schneider's medium (Gibco™) supplemented with 15 % FBS at 26° C for four days and harvested by centrifugation (650 g × 8 minutes). *A. castellanii* was axenically cultured in peptone-yeast glucose (PYG) medium (Rowbotham, 1983; ATCC media formulations No. 712) for four days and harvested by centrifugation (650 g × 4 minutes). The P388D1 cell line was cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Taito-ku, Tokyo, Japan) supplemented with 10 % FBS for four days and harvested by centrifugation (125 g × 4 minutes). *T. vaginalis* and *T. foetus* were axenically cultured for three days in BI-S-33 medium in which the peptone components of TYI-S-33 medium (Diamond *et al.*, 1978) are substituted by Biosate (BBL, Becton Dickinson Co., Cockeysville, Maryland, USA), and harvested by centrifugation (275 g × 4 minutes). The Cp-rich fraction from the wild spiderwort (*C. communis* L.) was isolated. The leaves of wild spiderwort were picked from the private field of Keio University (Tokyo) during the flower season from June to August (2000-2003). Fresh green spiderwort leaves were stripped from the stems, washed with tap water and then with distilled water. The leaves were ground with serum-free RPMI 1640 medium (RPMI) in an earthenware mortar by using a wooden pestle. The resulting leaf cell suspension was filtered through a double thickness gauze to remove the residue. The cell suspension was then washed three times with RPMI by centrifugation (440 g × 10 minutes). *P. aeruginosa* was cultured in Biosate-Iron broth (Diamond *et al.*, 1978) for three days and harvested by centrifugation (650 g × 4 minutes). *G. intestinalis* was axenically cultured in modified BI-S-33 medium (Keister, 1983) for three days and harvested by centrifugation (275 g × 4 minutes).

PREPARATION OF MT, HG AND CP FRACTIONS

Mt, Hg and Cp rich fractions were prepared as described by Hogeboom (1955), Opperdoes *et al.* (1984) and Gorham (1955), respectively. Since the methodology for mitosome isolation has not been established, intact cells of *G. intestinalis* were tested for their growth-promoting effect without preparing the mitosome rich fraction. After washing the fractions with RPMI once by centrifugation (440 g × 10 minutes), each fraction was suspended in 2 ml of RPMI. In order to test for their growth-promoting effect under axenic culture conditions, the fractions were sterilized by autoclaving (121° C, 15 minutes), and 0.2 ml of each suspension was added into the YIGADHA-S medium (5 ml). The

medium was then inoculated with a 0.8 ml suspension of *E. dispar* (AS16IR strain = AS 16 IR) (final density: 200-2,700 amoebae/ml).

NuPAGE® Novex 4-12 % Bis-Tris gradient peptide gel electrophoresis and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The lyophilized cell fraction of spiderwort was extracted with 20 %, 40 %, 80 % and 100 % acetone solutions (20 mg/ml) for 10 minutes at 4° C. After centrifugation (17,800 g × 10 minutes, 4° C) of each acetone extract, the supernatant was collected. The supernatants that were extracted with 80 % and 100 % acetone solutions were diluted with distilled water to obtain an acetone concentration of 50 %. Each supernatant was then concentrated by evaporation by using a SpeedVac system (SVC 100; Savant Instruments, Inc., Farmingdale, New York, USA) until evaporated to dryness. The molecular weight of the proteins in each extract was determined by NuPAGE® Novex 4-12 % Bis-Tris gradient peptide (Invitrogen Corp., Carlsbad, California, USA) gel electrophoresis.

The bands of complexes of low molecular weight protein with chlorophyll that were detected in the supernatants of 80 % and 100 % acetone extracts were isolated by preparative SDS-PAGE on a 12 % gel in a Mini Prep Cell (Bio-Rad Laboratories, Inc., Hercules, California, USA) electrophoresis unit. SDS-PAGE was performed according to the procedure described by Laemmli (1970). For NuPAGE® (Novex 4-12 % gradient peptide gel = Novex 4-12 %) each dried and concentrated supernatant of the acetone extract was redissolved in the sample buffer with 50 mM dithiothreitol and heated at 70° C for 10 minutes to reduce the disulfide bonds. For SDS-PAGE (12 % gel) each supernatant was redissolved in the sample buffer with 1 % (v/v) 2-mercaptoethanol (2ME) and boiled for four minutes. Following NuPAGE®, the gel was stained with 2D-silverstain II (Daiichi Pure Chemicals Co. Ltd., Chuo-ku, Tokyo, Japan).

Assay of the acetone extracts of spiderwort leaf cells for a GPF

One millilitre supernatants (obtained after centrifugation; 17,800 g × 10 minutes, 4° C) of the 20 %, 40 %, 80 % and 100 % acetone extracts of the lyophilized cell fraction of spiderwort (20 mg/ml) was dialyzed three times against 200-250 volumes of distilled water for 18 hours by using a Spectra/Por® 3 (3500 MWCO) dialysis membrane for 18 h; the volume of each extract was adjusted to 2.4 ml. Each extract was sterilized by filtration (Sartorius membrane filter; 0.2-mm pore size), and 0.2 ml of each extract was added to the YIGADHA-S medium to test the growth-promoting effect of the extract on *E. dispar* (AS 16 IR).

Analysis using a scanning electron microscopy (SEM)/energy dispersive X-ray analyser (EDX)-integrated analysis system

Following the preparative SDS-PAGE, the complexes of chlorophyll with low molecular weight proteins in the 80 % and 100 % acetone-soluble fractions were dialyzed in the same manner as described above. After lyophilization, the dialyzed complexes were examined using an SEM and EDX-integrated analysis system SEM-EDX III Type N/H (Hitachi Science Systems, Ltd., Hitachinaka, Ibaragi, Japan) in the Nihonbashi laboratory, Hitachi High-Technologies Co., Tokyo, Japan.

Preparation of chlorophyll-a complexes with purified iron-sulphur proteins

Purified Fd from spinach (1 mg/ml; Sigma F-3013) and *C. pasteurianum* (1 mg/ml; Sigma F-7629) and purified rubredoxin (Rd) from *C. pasteurianum* (15.7 mg/ml; Sigma R-2512) were dissolved in 0.6 % 2ME/H2O (20 mg of Fd or 31.4 mg of Rd/4 ml of 0.6 % 2ME), and the Fd and Rd solutions were dialyzed in the same manner as described above. Purified chlorophyll-a (Sigma C-5753) was dissolved in acetone (25 mg/25 ml acetone), and after adding 4 ml of distilled water, the solution was dialyzed in the same manner as described above. The Fd and Rd solutions were mixed with dialyzed chlorophyll-a and incubated for 10 minutes at 25° C. The solutions of chlorophyll-a complexes were then sterilized by filtration, and 0.2 ml of each solution was added to the YIGADHA-S medium to test their growth-promoting effect on *E. dispar* AS 16 IR.

Statistical analysis of growth-promoting effect on *E. dispar*

All experiments for testing growth-promoting effect were repeated at least twice. The data of each experiment were subjected to one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post hoc test; the levels of statistical significance were taken as $p < 0.05$ and $p < 0.01$.

RESULTS

The Mt, Hg and Cp isolated from eight different types of cells were tested to determine whether they promoted the growth of axenically grown *E. dispar* AS 16 IR. The intact cells of *G. intestinalis* and *P. aeruginosa* were also tested for the same. A growth-promoting effect was apparently observed for every fraction except for the fraction of intact cells of *G. intestinalis*. Apparently, the intact cells of *E. histolytica* (HM-1: IMSS clone 6 strain) containing mitosomes (Leon-Avila & Tovar, 2004) also did not produce a

growth-promoting effect (data not shown). Although the growth-promoting effect of the two fractions of Mt from *T. cruzi* and the Hg fraction from *T. vaginalis* retained the statistically significant difference by Dunnett's test ($p < 0.05$), the growth-promoting effect of the other six fractions containing Mt, Hg and Cp was significantly different ($p < 0.01$ or $p < 0.05$). In particular, the effect of the Cp fraction from spiderwort exceeded that of the intact cells fraction of *P. aeruginosa* as shown in Figure 1. Thus, it was concluded that some of the Mt, Hg and Cp fractions, which were obtained under appropriate conditions as well as the intact bacterial cells contain a GPF.

Acetone extraction was used to obtain a water-soluble GPF from Cp-rich leaf cells of some plant species (e.g., spiderwort, cherry and morning glory), which have sufficiently strong leaf-cell membranes that can withstand the leaf crushing and cell isolation process. Figure 2 shows the results obtained on testing the 0 %, 20 %, 40 %, 80 % and 100 % acetone-soluble fractions from lyophilized Cp-rich leaf cells of spiderwort for a growth-promoting effect. The results showed that the 20 % and 80 % acetone-soluble fractions had a stronger growth-promoting effect than the other acetone-soluble fractions (0 %, 40 % and 100 %). The growth-promoting effects of the 20 % and 80 % acetone-soluble fractions were statistically significant at $p < 0.01$ (Dunnett's test); however, the effects of the 0 %, 40 % and 100 % acetone-soluble fractions were retained at $p < 0.05$. The silverstain NuPAGE® analysis of these acetone-soluble fractions yielded a distinct dense protein band (MW 4600) that was common to the 20 %, 40 %, 80 % and 100 % acetone-soluble fractions. The intensity of the bands of the 20 % and 80 % acetone-soluble fractions were stronger than the bands of the other acetone-soluble fractions (40 % and 100 %) (Fig. 3). These results indicated that the efficacy of the growth-promoting effect was correlated with the intensity of the low molecular weight protein band (Figs 2, 3). The band of the complex of the chlorophyll with low molecular weight proteins (green colour) present in both the 80 % and 100 % acetone-soluble fractions was obtained by SDS-preparative electrophoresis; both the fractions showed a growth-promoting effect on *E. dispar* AS 16 IR (data not shown). The result showed that the band of the complex of chlorophyll with low molecular weight proteins, which was present in the acetone-soluble fractions, contained a GPF. The reduction in the intensity of the low molecular weight protein bands obtained by using the 40 % acetone-soluble fraction was considered to be caused by the incomplete solubility of chlorophyll. Some protein parts are believed to be precipitated together with the insoluble chlorophyll when extraction is performed with an intermediate concen-

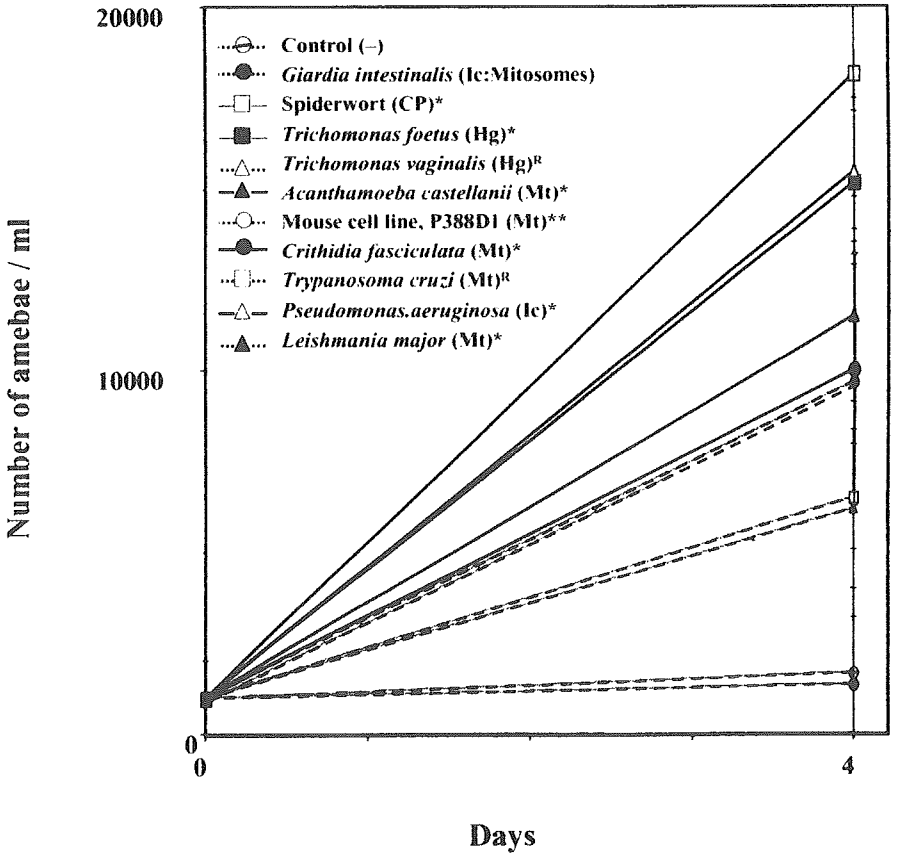

Fig. 1. – The effect of intact cells (Ic) and the fractions of mitochondria (Mt), chloroplasts (Cp) and hydrogenosomes (Hg) from ten types of cells, including bacterial, mammalian, plant and protozoan cells, on the growth of *Entamoeba dispar*.

The cellular components were sterilized by autoclaving at 121° C for 15 minutes. The growth kinetics of the *E. dispar* AS 16 IR strain in the YIGADHA-S medium are shown (mean numbers of amoebae in duplicate cultures are plotted). *, **: the mean of the growth-kinetic level was significantly higher than that of the control (*$p < 0.01$ and **$p < 0.05$ by Dunnett's test). R: the significant difference was retained by Dunnett's test ($p < 0.05$).

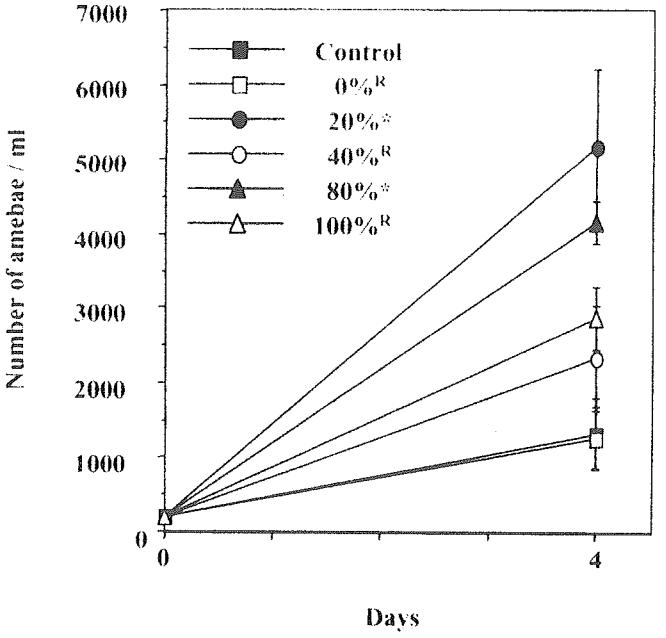

Fig. 2. Effect of soluble fractions of spiderwort Cp-rich leaf cells extracted with five different concentrations of acetone (0 %, 20 %, 40 %, 80 % and 100 %) on the growth of *E. dispar*.
The growth kinetics of the *E. dispar* AS 16 IR strain in the YIGADHA S medium are shown (mean numbers of amoebae in duplicate cultures are plotted). *: the mean of the growth-kinetic level was significantly higher than that of the control ($^*p < 0.01$ by Dunnett's test). R: the significant difference was retained by Dunnett's test ($p < 0.05$).

tration of acetone such as 40 %. As compared to an 80 % acetone concentration, the 100 % concentration of acetone was considered to be slightly severe for the extraction of the low molecular weight proteins without decreasing the activity of a GPF. As a result, the intensity of the low molecular weight protein bands obtained by using the 40 % and 100 % acetone-soluble fraction was believed to be reduced.

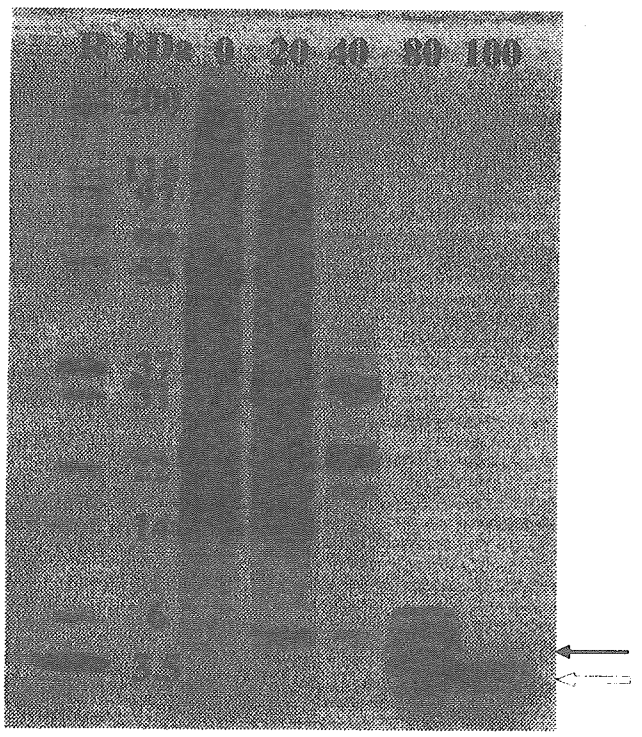

Fig. 3. NuPAGE® (Novex 4-12 %) of five acetone soluble fractions of spiderwort Cp-rich leaf cells extracted with different concentrations of acetone (0 %, 20 %, 40 %, 80 % and 100 %).
Molecular mass markers (M) are shown on the left. ⟵ : acetone soluble low molecular weight protein bands were isolated from the 20 %, 40 %, 80 % and 100 % acetone-soluble fractions. ⟸ : broad green band of chlorophyll.

The SEM/EDX-integrated analysis system revealed that the low molecular weight protein band contained Fe, S and Mo atoms along with the Mg atom of chlorophyll (Figs 4, 5). We believed that Fd present in Cp is a type of Fe-S protein containing Fe and S atoms. In order to confirm the growth-promoting effect of Fd, we examined the effects of purified spinach Fd (MW 12,000), *C. pasteurianum* Fd (MW 6,000) and purified *C. pasteurianum* Rd (MW 19,000) on the growth of *E. dispar* AS 16 IR. The analysis revealed that 0.6 % 2ME-treated *C. pasteurianum* Rd and each of the water-soluble chlorophyll-a complexes with 0.6 % 2ME-treated spinach Fd and *C. pasteurianum* Fd have a growth-promoting effect on *E. dispar* AS 16 IR with a statistically significant difference ($p < 0.01$) (Fig. 6). The non-complexed Fd from spinach and from *C. pasteurianum* treated with 0.6 % 2ME also had a slight growth-promoting effect when compared with the growth kinetics of the control with or without chlorophyll-a; however, these growth-promoting effects were retained ($p < 0.05$).

These results suggest that the GPF in the acetone-soluble fraction of spiderwort Cp-rich leaf cells is the complex of the chlorophyll-a with a component of an Fe-S protein, such as an inorganic Fe-S centre, released by the breakage of disulfide bonds due to treatment with 2ME. The Fe-S redox proteins are commonly found in enteric bacteria (e.g., Fd), mitochondria (e.g., Fe-S proteins in complex I and II), chloroplasts (e.g., Fd) and hydrogenosomes (e.g., Fd); however, there are diffe-

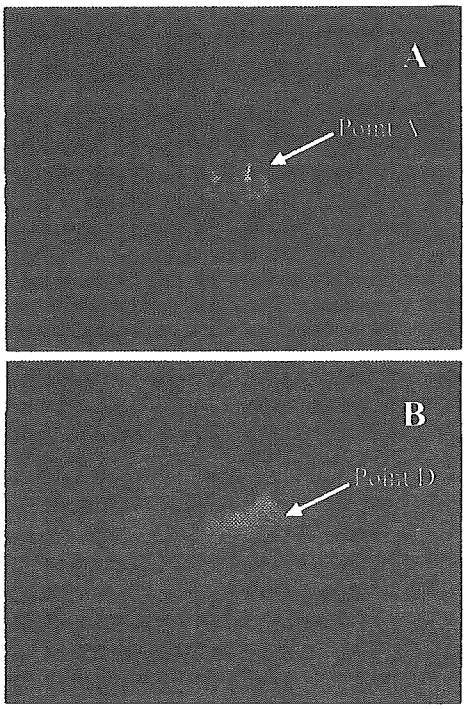

Fig. 4. – Scanning electron microscope (SEM) images of crystals observed in complexes of chlorophyll with low molecular weight proteins present in the 80 % (A) and 100 % (B) acetone-soluble fractions.
By using an SEM-energy dispersive X-ray (EDX) analyzer, Fe atoms were detected at point A (A) and S and Mo atoms were detected at point D (B).

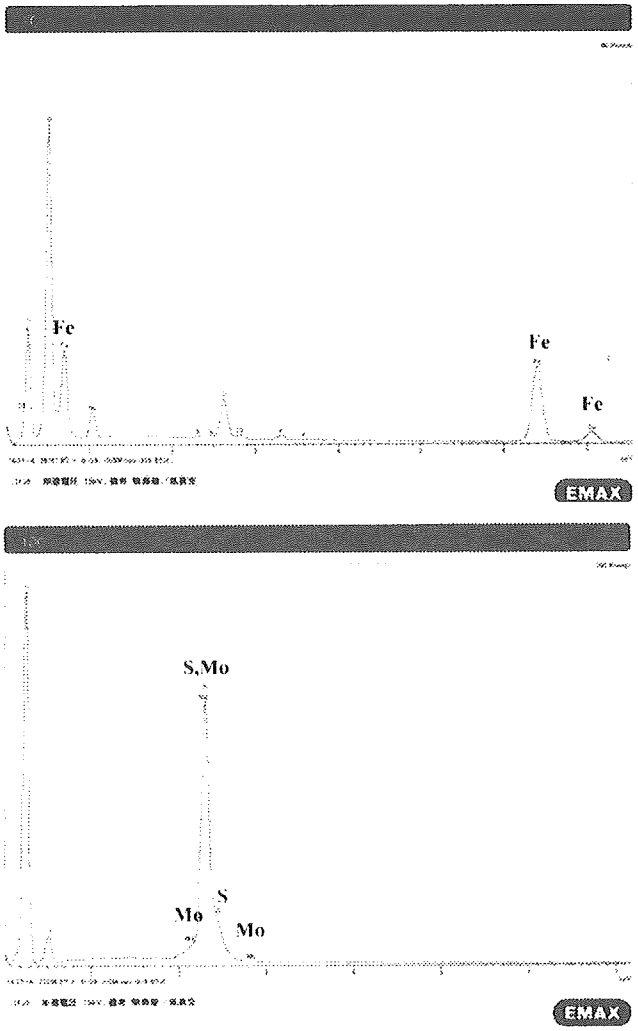

Fig. 5. – Specific peaks of the Fe atom at point A (Fig. 3) and S and Mo atoms at point D (Fig. 3) detected in the complexes of chlorophyll with low molecular weight proteins in the 80 % (A) and 100 % (B) acetone-soluble fractions by using an SEM-EDX analyzer.

rences in the chemical structure of their Fe-S centers. In *E. dispar*, Fd is considered as an essential redox protein involved in energy metabolism, similar to that in *E. histolytica*, although neither species contains Mt. These findings suggest that *E. dispar* grown under axenic culture conditions may lack a sufficient quantity of some essential component of the Fe-S proteins (e.g., Fe-S centre).
The growth-promoting effect of the GPF was tested on three other strains of *E. dispar* (SAW 1734R clone AR, AS 2 IR and CYNO 16:TPC), (Koyabashi *et al.*, 2005); it was confirmed that the GPF produced the same effect on their growth (data not shown).

DISCUSSION

Previously, we designed the YIGADHA-S medium (Kobayashi *et al.*, 2005) for axenic cultivation of *E. dispar*. It contains dihydroxyacetone (DHA) that has a significant growth-promoting effect on *E. dispar*. DHA is a ketotriose and functions as a sugar source for *E. dispar*; therefore, it is considered to be directly metabolized to DHA phosphate, which is an intermediary metabolite in the Embden-Meyerhof-Parnas glycolytic pathway. However, the growth of *E. dispar* was poor despite the presence of DHA.
In the present study, a GPF was detected in the autoclaved Mt, Hg and Cp fractions and in intact bacteria and was demonstrated to show a growth-promoting effect on *E. dispar*. However, the efficacy of each GPF from these organelles varied under different conditions. Prior to the present study, detection of a GPF from trophozoites of *E. histolytica* (HM-1:IMSS clone 6 strain) was attempted. Since *E. histolytica* is genetically closely related to *E. dispar*, it contains an abundance of Fe-S proteins, such as Fd, which are only stable under anaerobic conditions, and it contains mitochondrial remnant mitosomes (Leon-Avila & Tovar, 2004). However, neither the intact cells nor any extract from the cells could promote the growth of *E. dispar* AS 16 IR (data not shown). One reason for the failure to extract a GPF from *E. histolytica* trophozoites may be the fragility of

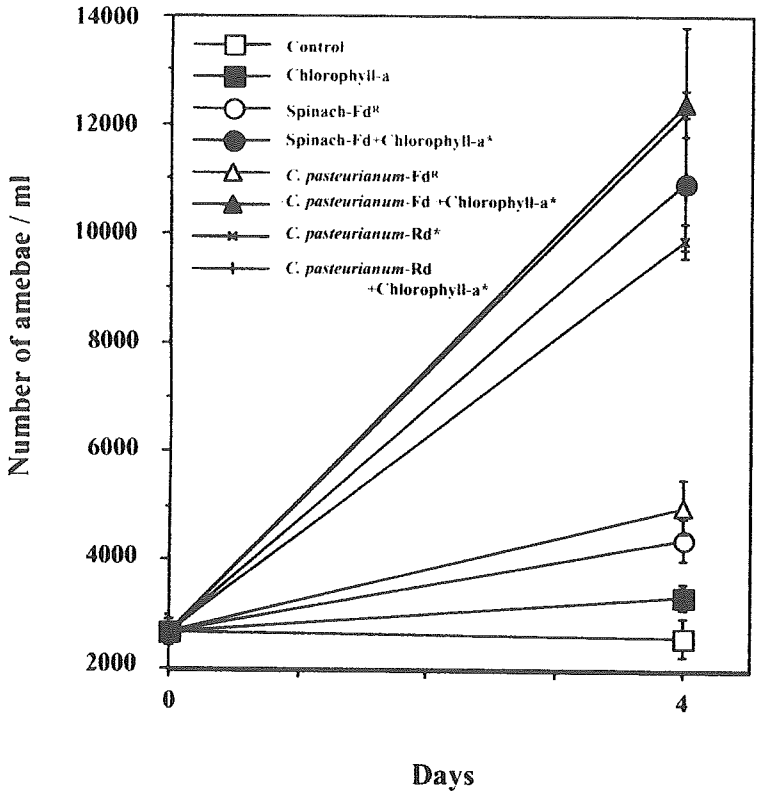

Fig. 6. - Effect of chlorophyll-a (—■—), 0.6 % 2ME-treated spinach ferredoxin (Fd) (—○—), *Clostridium pasteurianum* Fd (—△—) and *C. pasteurianum* rubredoxin (Rd) (—×—), and complexes of chlorophyll a with spinach Fd (—●—), *C. pasteurianum* Fd (—▲—) and *C. pasteurianum* Rd (—•—) on the growth of *E. dispar*, and control (—⊟—).
The growth kinetics of the *E. dispar* AS 16 IR strain in the YIGADHA-S medium are shown (mean numbers of amoebae in duplicate cultures are plotted). *: the mean of the growth-kinetic level was significantly higher than that of the control (*$p < 0.01$ by Dunnett's test). R: the significant difference was retained by Dunnett's test ($p < 0.05$).

the cell membrane and organelles (e.g.,mitosomes) similar to that of the spinach leaf cells. The GPF was assumed to be composed of a heat-stable substance and some other common simple inorganic substances. It was feasible to isolate the GPF from acetone-soluble fractions of Cp-rich leaf cells as bands of low molecular weight proteins complexed with chlorophyll containing Fe, S and Mo atoms.

The results of this study indicate that the protein bands may be derived from Fe-S (e.g., Fd) or Mo-Fe-S proteins [e.g., molybdoferredoxin, which is a common component of nitrogenase (present in rhizobium species)]. It appears that the GPF requires to form a complex with chlorophyll because the growth-promoting effects of purified Fd from spinach, *C. pasteurianum* and *E. histolytica* strain HM-1: IMSS clone 6 (the Fd consists of 4Fe-4S centre) on *E. dispar* AS 16 IR were not remarkable (data not shown). We found that the complex of chlorophyll-a with 0.6 % 2ME-treated Fd containing a 2Fe-2S centre from spinach and that containing 4Fe-4S centre from *C. pasteurianum* have a growth-promoting effect on *E. dispar* AS 16 IR. This effect was also observed with 0.6 % 2ME-treated Rd, which has the most simple Fe-S centre (1 Fe and 4 cysteine-residues without inorganic S), without chlorophyll-a. It is not clearly understood why complex formation must occur between chlorophyll-a and the components (Fe-S centres and cysteine residues) of bacterial and plant Fd to produce a growth-promoting effect is not clearly understood. However, it is considered that the complex of chlorophyll-a with the Fd component (MW greater than 100,000 by gel permeation chromatography) may be efficiently taken up by amoeba cells as a compact mass by a process, such as pinocytosis, and the complex may be degraded until an appropriate size of the Fe-S compound is obtained. We speculate that the Fe-S compounds are utilized as components of some essential Fe-S proteins such as amoebic Fd, which is an essential redox protein involved in oxidative decarboxylation of pyruvate to acetyl CoA in *E. dispar*; this is similar to that reported in *E. histolytica* (McLaughlin & Aley, 1985). It is reported that the amitochondrial protozoan parasite *E. histolytica*, which is closely related to *E. dispar*, contains numerous fermentation enzymes that are similar to bacterial enzymes, including the pyruvate Fd oxidoreductase, Fd and alcohol dehydrogenase E, derived from concomitant enteric bacterial DNA by horizontal transfer (Rosenthal *et al.*, 1997).

We succeeded in developing a more stable axenic cultivation system by adding the complex of chlorophyll with a component of Fe-S protein (e.g., Fd from spiderwort) treated with 2ME to the YIGADHA-S medium. We achieved an improved axenic cultivation system *for E. dispar* by using cellular components of protozoan, mammalian and plant cells as well as intact bacteria. However, we recognize that it is difficult to develop an axenic cultivation system for *E. dispar* that is at par

with the well-established cultivation system of *E. histolytica* for use in biological studies such as biochemical and immunological studies, etc.

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AXENIC CULTIVATION OF *ENTAMOEBA DISPAR* IN NEWLY DESIGNED YEAST EXTRACT–IRON–GLUCONIC ACID–DIHYRDOXYACETONE–SERUM MEDIUM

Seiki Kobayashi, Eiko Imai, Ali Haghighi*, Shaden A. Khalifa, Hiroshi Tachibana†, and Tsutomu Takeuchi

Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. *e-mail: skobaya@sc.itc.keio.ac.jp*

ABSTRACT: Yeast extract–iron–gluconic acid–dihyrdoxyacetone-serum medium that allows axenic cultivation of *Entamoeba dispar* was designed based on casein-free yeast extract-iron-serum (YI-S) medium, and the usefulness of the medium was assessed. The main differences from YI-S medium are replacement of glucose by gluconic acid, addition of dihydroxyacetone and D-galacturonic acid monohydrate, and sterilization by filtration. This medium promoted the axenic growth of 5 strains of *E. dispar* (2 strains of nonhuman primate isolates and 3 strains of human isolates). In addition, to clarify the biological basis for the growth of *E. dispar* in this medium, analyses of relevant enzymes on the glycolytic pathway of the amoebae as well as of the protozoans that are the best culture supplement for amoebae are being performed.

After axenic cultivation of *Entamoeba dispar* (strain: SAW760RRcloneAR) was reported (Clark, 1995) in casein-free yeast extract–iron–serum (YI-S) medium (Diamond et al., 1995) supplemented with gastric mucin, the efficacy of this axenic culture system was assessed for other *E. dispar* strains. However, despite its utility, the only *E. dispar* strain established as an axenic amoeba in YI-S medium thus far is SAW760RR clone A.

On the other hand, when a monoxenic culture system for *E. dispar* in biosate–cysteine–starch–iron–serum (BCSI-S) medium with *Pseudomonas aeruginosa* was developed (Kobayashi et al., 1998), a significant growth-promoting effect of dihydroxyacetone (DHA) was observed. DHA is a ketotriose and as a sugar source for *E. dispar* is thought to be directly metabolized to DHA phosphate (DHAP), which is an intermediary metabolite in the Embden–Meyerhof–Parnas glycolytic pathway.

Another significant result was that during characterization of the glycolytic pathway of *Crithidia fasciculata*, an effective culture associate of *E. dispar* in BI-S-33 medium (Diamond et al., 1978), approximately 35 times as much glucose-6-phosphate dehydrogenase (G6PDH) activity as that of *Trypanosoma cruzi* (Tulahuen strain) was detected. *Trypanosoma cruzi* can be used as a growth-promoting supplement for monoxenic culture of *E. histolytica*. However, it does not promote the growth of *E. dispar*, whereas *C. fasciculata* does (S. Kobayashi, unpubl.). G6PDH activity has not been detected in axenically grown *E. histolytica* (Reeves, 1972) or *E. dispar* (S. Kobayashi, unpubl.). G6PDH is an essential enzyme not only in the pentose-phosphate pathway but also in the Entner–Doudoroff pathway, which starts with G6P. G6PDH catalyzes the transformation of G6P to 6-phosphogluconate (6PG) via 6-phosphogluconolactone, and we focused attention on the glycolytic pathway after 6PG in *E. dispar* and examined the reactions after 6PG. Both the pentose-phosphate and Entner–Doudoroff glycolytic pathways can branch from 6PG. The activity of 6PG dehydratase (EC 4.2.1.12.) (Gottschalk and Bender, 1982; Nguyen and Schiller, 1989), which is one of the enzymes in the Entner–Doudoroff pathway, was detected in the lysates of both *E. histolytica* (HM-1:IMSS clone 6) and *E. dispar* (CYNO 16:TPC), and its activity in *E. dispar* was 2.87 times greater than in *E. histolytica*. By contrast, no 6PG dehydrogenase activity in the pentose-phosphate pathway was detected in either lysate (S. Kobayashi, unpubl.). On the other hand, DHA, which has a growth-promoting effect, is thought to be metabolized to G3P. through DHAP, which is converted to glyceraldehyde-3-phosphate (G3P) by triose phosphate isomerase (EC 5.3.1.1.). Thus, if viable *C. fasciculata* actually supplies a growth-promoting substance like DHA, DHA may be preferable to viable *C. fasciculata* as a culture ingredient. On the basis of this information, we hypothesized the existence of some failure in the upstream portion of the glycolytic pathway in *E. dispar*, before DHAP or G3P production from glucose in YI-S medium. We, therefore, attempted to design an axenic culture medium for *E. dispar* by modifying the ingredients of YI-S medium, and we tested its usefulness.

MATERIALS AND METHODS

Reagents

All chemicals used in this study were of the highest quality commercially available unless otherwise stated.

Entamoeba dispar isolates

Five strains of *E. dispar* were subjected to a trial of axenic cultivation in this study. Two strains (CYNO 09:TPC and CYNO 16:TPC) from nonhuman primates were isolated in Japan (Kobayashi et al., 1998) in Robinson's medium (Robinson, 1968), and 2 strains (AS 2 IR and AS 16 IR) were isolated from humans in Iran. An *E. dispar* strain, SAW 1734R clone AR (SAW1734RclAR), was used as the reference strain. The xenic *E. dispar* strains were transferred to monoxenic culture medium with viable *C. fasciculata* and antibiotics (as in the classic approach Diamond used for *E. histolytica*, Diamond, 1983) in BI-S-33 medium. The *C. fasciculata* was then replaced with antibiotic-sensitive *P. aeruginosa* in BCSI-S medium because *P. aeruginosa* is removed easily by antibiotics and antibiotic-resistant *C. fasciculata* usually survive in test medium for axenic culture even after 2–3 subcultures, and it promotes the growth of *E. dispar*.

Yeast–iron–gluconic acid–dihydroxyacetone–serum medium

Yeast–iron–gluconic acid–dihydroxyacetone (YIGADHA) broth was prepared by first dissolving the following ingredients in 880 ml of water distilled and purified with a Milli-Q column (Millipore Co., Billerica, Massachusetts): K_2HPO_4, 1 g; KH_2PO_4, 0.6 g; NaCl, 2.0 g; yeast extract (BBL, Becton Dickinson Co., Cockeysville, Maryland), 30 g; gluconic acid, 5.0 g; DHA (Sigma Chemical Co., St. Louis, Missouri), 1.0 g; D-galacturonic acid monohydrate, 0.2 g; L-cysteine hydrochloride, 1.0 g; ascorbic acid, 0.2 g; and ferric ammonium citrate (brown), 22.8 mg.

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* Present address: Department of Medical Parasitology and Mycology, School of Medicine Shaheed Beheshti University of Medical Sciences, Eveen, Teheran 19395, Iran.

† Present address: Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan.

The pH was adjusted to 6.5 with 1 N NaOH, and the solution was sterilized by filtration (Sartorius membrane filter; 0.2-μm pore size). An 88 ml volume of YIGADHA broth was aseptically dispensed into a 100-ml, screw-capped glass bottle and stored at −30 C.

To complete the YIGADHA-serum (YIGADHA-S) medium, 2 ml of vitamin mixture #18 prepared according to the instructions for PDM-805 medium (Diamond and Cunnick, 1991), and 16 ml of heat-inactivated bovine serum was aseptically added to 88 ml of the YIGADHA broth. A 5-ml volume of the complete medium was then dispensed into 13- × 100-mm, screw-capped borosilicate glass culture tubes (Asahi Techno Glass Co., Chuo-ku, Tokyo, Japan).

Establishment of the culture of *Entamoeba dispar* with sterilized *Crithidia fasciculata*

After removing *P. aeruginosa* by washing and addition of antibiotics (polymyxin B, 130 units/ml), trophozoites of the 5 strains of *E. dispar* were transferred to YIGADHA-S medium with sterilized *C. fasciculata* (2–3 million/ml, ReF-1:PRR, ATCC no. 50083) by fixation with 10% (v/v) formalin as described in the literature (Clark, 1995). All 5 strains of *E. dispar* adapted to the culture at 35.5 C in the YIGADHA-S medium with the associate within 2 wk after 3–4 whole-medium changes by centrifugation (275 *g*, 4 min) and eventually started to grow continuously. When the same growth-promoting effect was later found with autoclaved *C. fasciculata* in Hanks' solution as with the formalin-fixed *C. fasciculata*, we switched the method of sterilizing *C. fasciculata* to autoclaving (121 C, 15 min) because of its simplicity.

When the amoebae were subcultured, 0.6–0.8 ml of the amoeba suspension (2.5 × 10^4 to 5 × 10^4/ml) was usually transferred to the fresh medium, after the cultures were chilled for 5 min in an ice-water bath. They were then inverted several times to disperse the amoebae. Subcultures were successfully prepared at intervals of 3 and 4 days.

Growth kinetics of the amoebae

At 24-hr intervals for 96 hr, the number of amoebae in 5 μl of the homogeneous amoeba suspension diluted with known volume of YIGADHA-S medium were counted under a microscope after chilling the culture tubes for 5 min in an ice-water bath. The data were then converted to number per milliliter.

Zymodeme analysis and polymerase chain reaction

Zymodeme analysis (Sargeaunt, 1988) and polymerase chain reaction (PCR) analysis (Tachibana et al., 1991; Cheng et al., 1993) of the amoeba isolates were performed to characterize the amoebae grown in the axenic culture.

RESULTS

YIGADHA-S medium was designed, and the culture associates tested could be removed with no loss of *E. dispar* viability as a result of searching for the improved basic medium and condition for axenic cultivation of *E. dispar*, based on the casein-free YI-S medium.

YI-S medium was therefore modified as follows. Glucose was replaced by GA, DHA and D-galacturonic acid monohydrate were added because of the significant growth-promoting effects under the axenic culture conditions, and the medium was sterilized by filtration and adjusted to pH 6.5 with NaOH.

As a result of these modifications, trophozoites of *E. dispar* started to grow continuously in the absence of viable *C. fasciculata* or *P. aeruginosa* for the first time, although supplementation with the formalin-fixed organisms was still necessary for growth.

The growth-promoting ability of *C. fasciculata* was also maintained in this YIGADHA-S culture system even after it was autoclaved (121 C, 15 min) in Hanks' solution. There was no difference between the growth-promoting effect of autoclaved *C. fasciculata* and of formalin-fixed organisms. Because the procedure was easier, the amoebae were maintained in the medium with autoclaved *C. fasciculata*. The growth kinetics of the 5 strains of *E. dispar* in this culture system are shown in Figure 1.

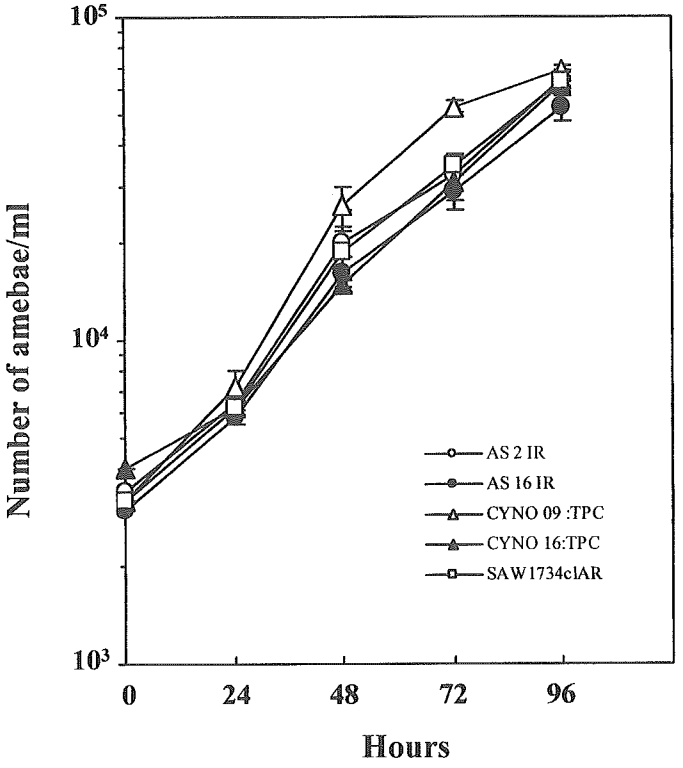

FIGURE 1. Growth kinetics of 5 strains of *Entamoeba dispar* in the 4-yr passaged cultures in YIGADHA-S medium supplemented with autoclaved *Crithidia fasciculata*. Mean numbers of amoebae in duplicate cultures are plotted.

After 3 mo of maintenance in this culture system, 1 strain of *E. dispar* (CYNO 16:TPC) started to grow without any supplement, e.g., without autoclaved *C. fasciculata*. However, addition of 6PG (Sigma P-7877, 67 μg/ml), an intermediary metabolite in the Entner–Doudoroff pathway (Fig. 3), was necessary to maintain this strain without the autoclaved organisms.

After culturing with autoclaved *C. fasciculata* for 1 yr and 4 yr, respectively, other *E. dispar* strains started to grow axenically in the YIGADHA-S medium without the addition of any culture associates. The growth kinetics of 5 strains of axenically grown *E. dispar* are shown in Figure 2. The clear differences in growth kinetics between monoxenic culture (Fig. 1) and axenic culture (Fig. 2), except for the CYNO 09:TPC strain, suggest the existence of some other growth-promoting factor in autoclaved *C. fasciculata*.

The CYNO 16:TPC and CYNO 09:TPC strains were usually subcultured by transferring 1 ml of amoeba suspension to fresh YIGADHA-S medium, and the AS 16 IR, AS 2 IR, and SAW1734RclAR strains were subcultured by transferring 1 ml of amoeba suspension concentrated to an adequate density for subculture (3 × 10^4 to 6 × 10^4 amoebae/ml) by centrifugation (275 *g*, for 4 min).

The zymodemes of the 5 strains grown in both the monoxenic and axenic YIGADHA-S media were all judged to be type I. All 10 DNA samples of the 5 strains used in the PCR

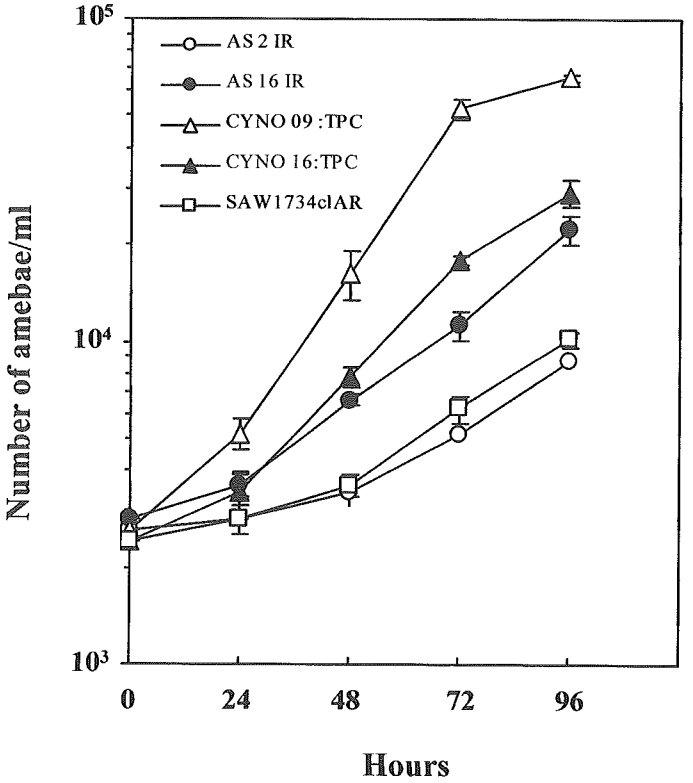

FIGURE 2. Growth kinetics of 5 strains of axenically grown *Entamoeba dispar* in 2-yr (CYNO 09: TPC), 3-yr (CYNO 16:TPC), and 2-mo (AS 2 IR, AS 16 IR, and SAW 1734RclAR) passaged cultures in YIGADHA-S axenic medium. Mean numbers of amoebae in duplicate cultures are plotted.

analyses described above were amplified with *E. dispar*–specific primers alone; the *E. histolytica*–specific primers did not elicit any DNA amplification. These findings are summarized in Table I.

DISCUSSION

We have previously reported a monoxenic culture system for *E. dispar* in BCSI-S medium in which glucose was replaced with starch and to which sterilized *C. fasciculata* were added after heat treatment at 56 C for 30 min followed by incubation in 1% hydrogen peroxide at 4 C for 24 hr (Kobayashi et al., 1998). The sterilized parasite appeared to be metabolically inactive as judged by the nuclear magnetic resonance spectra. Although the growth-promoting effect of *C. fasciculata* was partially lost as a result of this sterilization step, the minimum essential effect persisted, which made it possible to establish a long-term monoxenic culture of *E. dispar* with metabolically inactive *C. fasciculata*. In this study, we succeeded in producing an axenic culture system of *E. dispar* by using YIGADHA-medium designed on the basis of YI-S medium from the monoxenic culture of the amoeba with sterilized *C. fasciculata*.

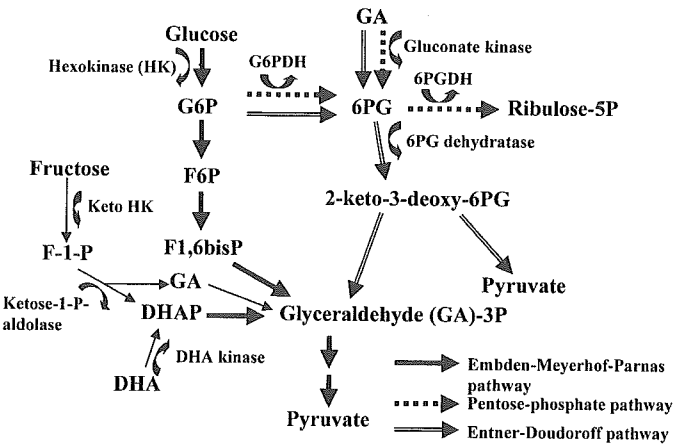

FIGURE 3. Metabolic map of 3 glycolytic pathways.

During attempts to improve the culture system, it was found that by replacing some kinds of sugar from starch, the growth of *E. dispar* was promoted significantly in the BCSI-S monoxenic culture system. First, a marginal growth-promoting effect of fructose was found, although its effect was insufficient for axenic cultivation of *E. dispar*, and a more useful compound, DHA, was used. The concentration (0.1%) of DHA required for the growth of *E. dispar* in YIGADHA-S medium was very critical: DHA concentrations below 0.05% and greater than 0.15% failed to effectively promote the growth of *E. dispar*. However, despite the clear effect of DHA and fructose on the growth of *E. dispar*, the results of our biochemical assays for phosphorylation of DHA by DHA kinase, ketohexokinase, and ketose-1-phosphate aldolase, the enzymes involved in the conversion of fructose or DHA to DHAP (Fig. 3), showed no significant activity of these enzymes in the crude lysates of axenic *E. dispar* (CYNO 16:TPC) or *E. histolytica* (HM-1:IMSS clone

TABLE I. Zymodeme and PCR analyses of 5 strains of *Entamoeba dispar*.

Strain	Xenic (date)	Zymodeme*		PCR†	
		Monoxenic (date)	Axenic (date)	Monoxenic	Axenic
SAW 1734RclAR	Z-III‡ (March 1985)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
AS 2 IR	Z-I (June 1998)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
AS 16 IR	Z-I (June 1998)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
CYNO 09:TPC	Z-I (April 1992)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
CYNO 16:TPC	Z-III (April 1992)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*

* Zymodemes type I and III are classified as *E. dispar*.
† PCR analysis using 2 sets of oligonucleotide primers each (p11 plus p12 and p13 plus p14, respectively) for amplification of the DNAs of *E. histolytica* and *E. dispar*. The zymodeme and PCR analyses were performed around the same time.
‡ Data cited from Mirelman et al. (1986).

6) (data not shown). The mechanism of growth-promoting effect of DHA and fructose is not yet understood.

Pimenta et al. (2002) demonstrated an interesting morphological characteristic of *E. dispar* by electron microscopy: some concomitant bacteria cells were free and viable in the cytoplasm of *E. dispar*, without being surrounded by a distinct phagosome membrane. This suggests that some bacterial species can survive in the cytoplasm and exist in a symbiotic relationship with *E. dispar*. If the Entner–Doudoroff pathway does indeed function in glycolysis in *E. dispar*, as reported in *E. histolytica* under xenic conditions (Hilker and White, 1959), the reactions upstream in the Embden-Meyerhof-Parnas pathway (Fig. 3) including a reaction regulating the transformation of fructose-6-phosphate to fructose-1,6-bisphosphate or vice versa, usually catalyzed by 6-phosphofructokinase (6-PFK) and fructose-bisphosphatase (EC 3.1.3.11.) and affecting the both the glycolysis and glycogenesis pathways, may not function well in *E. dispar*. The regulatory reactions in *E. histolytica* are well known to be regulated by a single unique enzyme (ppi-dependent 6-PFK; EC 2.7.1.90.), and the reaction is reversible and has no apparent regulatory function (Reeves et al., 1974, 1976).

The clear growth-promoting effect of autoclaved *C. fasciculata* (or *P. aeruginosa*) indicated that they contain as yet unidentified heat-stable growth-promoting substances for *E. dispar*. If the substances can be identified, YIGADHA-S medium will be improved. Further analyses of the axenically grown *E. dispar* will enable us to further elucidate the biological properties of *E. dispar* that differ from those of pathogenic *E. histolytica*.

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