Establishment of an abalone digestive gland cell line secreting various glycosidases in protein-free culture

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Abstract

A cell line designated as ADG was established from an abalone digestive gland using ERDF medium supplemented with 8% fetal bovine serum (FBS), 8% abalone hemolymph, and high concentrations of NaCl, KCl, MgCl₂, MgSO₄, and CaCl₂. ADG cells proliferated better in protein-free medium than in FBS-supplemented medium. Among 9 kinds of media examined, ERDF medium was shown to be optimal for cell growth. ADG cells secreted 13 different kinds of glycosidases in protein-free medium: α -L-fucosidase, β -L-fucosidase, α -D-galactosidase, β -D-galactosidase, N-acetyl- α -D-galactosaminidase, N-acetyl- β -D-galactosaminidase, α -D-glucosidase, β -D-glucosidase, N-acetyl- α -D-glucosaminidase, N-acetyl- β -D-glucosaminidase, α -D-mannosidase, β -D-mannosidase, β -D-xylosidase, and 1-3 xylanase. When ADG cells were cultured in Grace's insect cell medium, the activity of some secreted glycosidases increased 25-fold to 65-fold per cell as compared with control cells cultured in ERDF medium.

Abbreviations: ADG – abalone digestive gland; ERDF – enriched RDF; FBS – fetal bovine serum; L-15 – Leibovitz's L-15 media; DME – Dulbecco's modified Eagle medium; F-12 – nutrient mixture (Ham); LDF – L-15; DME: F-12 = 10 : 7 : 3; MEM – minimum essential medium; RPMI – RPMI medium 1640; 199 – media 199; GIC – Grace's insect cell medium; pNP – *p*-nitrophenol.

Introduction

Algae, especially seaweed such as laver and tangle have been valuable as food materials in Japan since old days. Seaweed has been also widely utilized as forage for cattle or cultured fishes, medical drugs, cosmetics, food additives, industrial materials, fuel and so on. Seaweed is rich in proteins, minerals, vitamins and other nutrients. However, polysaccharides which make up their cell walls hinder their digestion, resulting in lowering the value of seaweed as nutritional foods. Preparation of protoplasts of seaweed is not only indispensable for cell fusion for breeding of valuable seaweed or for gene introduction in marine technolo-

gy (Rochaix, 1982; Juras, 1994), but also would be useful to increase the value of seaweed as food materials and forages because of their increased digestion efficiencies.

Polysaccharide-degrading enzymes derived from bacteria or digestive gland enzymes from sea mollusks such as abalone and aplysia which eat seaweed have been used for preparation of protoplasts of seaweed (Kawashima and Tokuda, 1990; Ishikawa and Saga, 1990; Nakanishi and Saga, 1990). The enzymes derived from bacteria degrades can be obtained in large scale, their activities are not so strong and limited to the cell walls of seaweed. The digestive gland cells derived from abalone and aplysia secrete various glycosidas-

es which degrade various polysaccharides in the cell walls of seaweed. Actually, the marked abalone acetone powder is used for preparation of protoplast of seaweed's polysaccharides. However, these enzymes have not been purified and characterized thoroughly.

Establishment of digestion gland cell lines of sea mollusks which secrete various glycosidases will be useful to produce various glycosidases in a large quantity to digest polysaccharides of seaweed as well as to clarify the cell physiology of sea mollusks. Few cell lines derived from sea mollusks have been established so far. Here we first established a cell line derived from an abalone digestive gland, which rapidly grows and secretes various kinds of glycosidases in protein-free medium. Although the possibility exists that the cells we describe in this report are parasitic organisms derived from the digestive gland rather than true abalone cells, we do not think this is the case for the following reasons. The size of the cells is considerably larger than most yeasts, and the cells do not show the fluidity of protoplasm or shape of protists. Cells similar in morphology to the cells we have cultured can be identified in the digestive gland of the abalone. We expect in the future to deal with this issue further by electron microscopy.

Materials and methods

Preparation of abalone hemolymph

The abalone hemolymph was extracted by cutting the muscle tissue with a scalpel, the resulting liquid was centrifuged at 3,000 rpm for 20 min, sterilized with 0.22 μ m membrane filter (MILLIPORE), and finally stored at -20°C until used for cell culture.

Establishment of an abalone digestive gland cell line

The abalone digestive gland (about 40 g) was removed from an abalone (about 350 g) with a scalpel and scissors after the abalone's metabolism was slowed in ice cold seawater disinfected with 70% ethyl alcohol. A portion of the digestive gland was minced with a scalpel, suspended in ERDF medium (enriched RDF), and centrifuged at 800 rpm for 90 s. The process of suspension and centrifugation were repeated at least five times to completely separate the desired cells from other digestive gland components. Pellets containing the cells were resuspended in ERDF medium including 5 kinds of high concentrations of salts (designated

as salts-ERDF, Table 1) supplemented with 8% fetal bovine serum (FBS, Whittaker Bioproducts) and 8% abalone hemolymph, placed in 24-well microplates, and incubated at 23°C in air. Isolated cells were observed with a phase contrast microscope everyday and subcultured every 3 to 4 days. An established abalone digestive gland cell line was designated, ADG cell line.

The addition of salts to the medium

To determine basal salt concentrations in salts-ERDF medium, we tried to simulate conditions of not only sea water, but also optimal culture condition for *Aplysia* neurons (Schacher, 1983; Schacher, 1985) and Kuruma Prawn (*Penaeus japonicus*) midgut gland cell. We used salts-ERDF medium with 340 mM NaCl, 10 mM KCl, 21 mM MgCl_2 , 18 mM MgSO_4 , and 8 mM CaCl_2 for the primary culture of abalone digestive gland cells and subcultured for about one month, as shown Table 1.

Effect of FBS on cell growth

ADG cells were suspended in salts-ERDF medium (Table 1) with the following amounts of FBS; 0%, 1%, 2%, 4%, 7%, 10% and 15%. Cells were seeded in 6-well tissue culture plates (3 ml culture medium/well) at a density of 1.0×10^5 cells/ml, and incubated at 23°C in air. Cell number was determined daily using a cell counter.

Effect of different culture media on cell growth

In protein-free culture, ADG cells were suspended in the following media with the appropriate salts (Table 2); ERDF (enriched RDF; KYOKUTO Pharmacy Industrial Co., Japan), LDF (L-15 : DME : F-12 = 10 : 7 : 3), L-15 (Leibovitz's L-15 media; GIBCO), DME (Dulbecco's modified Eagle medium; NISSUI Pharmaceutical Co., Japan), F-12 (nutrient mixture (Ham); GIBCO), MEM (Minimum essential media; NISSUI Pharmaceutical Co., Japan), RPMI 1640 (RPMI medium 1640; ICN), 199 (MEDIUM 199; ICN), and GIC (Grace's insect cell medium; GIBCO), and then cells were seeded in 6-well plates (3 ml culture medium/well) at a density of 1.0×10^5 cells/ml, and incubated at 23°C in air.

Table 1. Salt concentration in media for Aplysia and Prawn cells, sea water, ERDF medium, and salts-ERDF medium for ADG cell growth

Salt	Aplysia	Prawn	Sea water (mM)	ERDF	Salts-ERDF
NaCl	400	320	520	110	340
KCl	10	10	9.4	5	10
MgCl ₂	27	16	53	0.0	21
MgSO ₄	27	19	22	0.52	18
CaCl ₂	11	7.2	6.8	0.72	7.7

Table 2. Salt concentrations in different culture media

Salt	ERDF	LDF	L-15	DME	F-12 (mM)	MEM	RPMI	199	GIC
NaCl	260	260	260	260	260	260	260	260	16
KCl	10	10	10	10	10	10	10	10	55
MgCl ₂	21	21	21	21	21	21	21	21	21
MgSO ₄	18	18	18	18	18	18	18	18	18
CaCl ₂	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	7.7

Effect of salts on cell growth

To estimate the effect of varying concentrations of the previously mentioned salts (Table 1) in protein-free medium, ADG cells were suspended in ERDF medium with following concentrations of salt; A(NaCl): 110 mM, 170 mM, 260 mM, 340 mM, and 510 mM; B (KCl): 5.0 mM, 5.5 mM, 6.0 mM, 10 mM, 16 mM, and 37 mM; C (MgCl₂): 0 mM, 4.0 mM, 8.0 mM, 39 mM, 78 mM, and 155 mM, D (MgSO₄): 0.4 mM, 4.0 mM, 8.0 mM, 39 mM, 77 mM, and 154 mM, and E (CaCl₂): 0.7 mM, 1.4 mM, 2.2 mM, 7.7 mM, 15 mM, and 29 mM. Also other 4 kinds of salt-concentrations in each experiments (A, B, C, D, and E) were 370 mM NaCl, 10 mM KCl, 39 mM MgCl₂, 0.4 mM MgSO₄, and 0.7 mM CaCl₂, respectively, but other salt-concentrations in D (MgSO₄) were 0 mM for examining the effect of both MgCl₂ and MgSO₄. ADG cells seeded in 6-well plates (3 ml culture medium/well) at a density of 1.0×10^5 cells/ml, and incubated at 23 °C in air.

Examination of secreted glycosidases

The following substrates to determine enzymatic activities were purchased from SIGMA Co.; p-nitrophenyl (pNP)- β -D-fucopyranoside, pNP- α -L-fucopyranoside, pNP- β -L-fucopyranoside, pNP- α -D-galactopyranoside, pNP- β -D-galactopyranoside,

pNP-N-acetyl- α -D-galactosaminide, pNP-N-acetyl- β -D-galactosaminide, pNP- α -D-glucopyranoside, pNP- β -D-glucopyranoside, pNP-N-acetyl- α -D-glucosaminide, pNP-N-acetyl- β -D-glucosaminide, pNP- α -D-mannopyranoside, pNP- β -D-mannopyranoside, and pNP- β -D-xylopyranoside. 1-3 Xylan (prepared from *Pophyra yezoensis*) (Iriki, 1960), porphyran (prepared from *Porphyra yezoensis*) (Su & Hassid, 1964), mannan (WAKO Pure Chemical industries Co., Japan), κ -carrageenan (WAKO Pure Chemical industries Co., Japan), and sodium alginate (WAKO Pure Chemical industries Co., Japan) were also used for experiments. Spent medium after cultivation for one week was used to measure glycosidase activities. Glycosidase activity was measured by using two methods, the *p*-nitrophenol and Somogyi-Nelson assays (Somogyi, 1952).

When using the *p*-nitrophenol assay, enzyme reaction mixture contained the following components in total volume of 40 ml: 20 ml of the above prepared medium, and 20 ml of 0.16 mmoles substrate in H₂O. After incubation at 37 °C for 100 min, the reaction was terminated with 460 ml of 0.2 M borate buffer (pH 10). The incubated mixtures were centrifuged at 12,000 rpm for 15 s to remove the insoluble components. The liberated nitrophenol was quantitatively determined at its absorption maximum (400 nm) with a spectrophotometer. One unit of enzyme was defined as the amount of enzyme to hydrolyze 1 mmole of the substrate. The specific activity of enzyme was expressed

as units per spent medium (ml).

The Somogyi-Nelson assay was performed according to Somogyi *et al.* (1952). Culture medium was centrifuged at 3,000 rpm for 15 min to remove the cells and the insoluble components, and dialyzed against 10 mM sodium acetate buffer (pH 5.0) overnight to remove reducing sugars in medium. Enzyme reaction mixtures contained the following components; 20 ml of above prepared medium, and 20 ml of 1% substrate in H₂O. After incubation at 37 °C for 180 min, enzyme activity was measured with the Somogyi-Nelson assay. As with the *p*-nitrophenol assay, one unit of enzyme was defined as the amount of enzyme to hydrolyze 1 mmole of the substrate per min.

Results

Establishment of an ADG cell line

The ADG cell line was established in salts-ERDF medium supplemented with 8% FBS, 8% abalone hemolymph and increasing concentrations of NaCl, KCl, MgCl₂, MgSO₄ and CaCl₂. In the primary culture of the abalone digestive gland cells, various types of cells such as nerve cells, fibroblasts, and leukocytes were observed. Most of these cells could not persist in subculture, but only round and weakly adhesive cells were rapidly proliferated. The round cells could proliferate in both the adhesive and floating states without changing the morphology during the long-term culture (Figure 1). The cells rapidly proliferated with the doubling time of 12 h to 13 h, and reached confluency at a high-density of 1.0×10^7 cells/ml when the cells were seeded at a density of 1.0×10^5 cells/ml and cultivated for 5 days in a 90 mm plastic dish. The round cells were cloned by the limiting dilution method. The cloned cell line designated as ADG showed both the similar morphology and proliferative ability as the cell population before cloning, suggesting that a single type of cells proliferated and were selected in the medium used. The ADG cell line obtained could actively proliferate and secrete various types of glycosidases for more than 13 months.

Protein-free culture of the ADG cell line

When plated, ADG cells did not proliferate in protein-free salts-ERDF medium for the first 24 h, then quickly proliferated maintaining a spherical morphology and reached confluency at a higher density than in FBS

Figure 1. Photograph of an abalone digestive gland (ADG) cell line ($\times 200$).

Figure 2. Effect of the various FBS concentrations on the growth of abalone digestive gland cells. Abalone digestive gland cells were suspended in salts-ERDF medium supplemented various concentrations of FBS and seeded in 6-well plastic plates at a cell number of 1.0×10^5 cells/ml. Cell number was determined daily with a cell counter as well as in the following figures. ●—●, 0% FBS; ○—○, 1% FBS; ■—■, 2% FBS; □—□, 4% FBS; ▲—▲, 7% FBS; △—△, 10% FBS; —, 15% FBS.

supplemented medium (Figure 2). Only a little number of cells were observed to be lysed.

In media supplemented with 1, 2, and 4% FBS, the cell proliferated more rapidly than in protein-free medium, however, in low concentrations of 1% FBS many lysed cells were observed in culture medium. One fourth to one fifth of total cells perished on the sixth day. When serum concentrations were increased to 7%, 10%, and 15%, the cell growth rates decreased or stopped. The morphology of the cells in higher serum concentrations changed to spherical to oval and

Figure 3. Effect of the various media on the growth of ADG cells. Abalone digestive gland cells were suspended in 9 different kinds of culture media with salts and seeded in 6-well plastic plates at a cell number of 1.0×10^5 cells/ml. ●—●, ERDF; ○—○, LDF; ■—■, L-15; □—□, DME; ▲—▲, F-12; △—△, MEM; +, RPMI; —, 199; —, GIC.

died. These results indicate that FBS contains both growth factors and toxic substances for ADG cells.

The optimal medium for growth of the ADG cell line in protein-free culture

As shown in Figure 3, ADG cells proliferated to the highest density in protein-free ERDF medium with salts among nine basal media examined. LDF and L-15 media also supported the proliferation of ADG cells well, although the cell density reached were lower than ERDF medium. Cell damages were observed when cultured in F-12, MEM, RPMI 1640 and DME media, suggesting that these media were not suitable for the ADG cell line. Many of ADG cells cultured in GIC medium became large but not damaged. GIC medium was not suitable for the growth of ADG cells but suitable for secretion of various glycosidases, as well be described later.

The optimal salts concentrations for the ADG cell line in protein-free culture

The optimum NaCl, MgCl₂ and MgSO₄ concentrations in protein-free ERDF medium for the growth of ADG cells were around 260 mM to 430 mM, 4 mM to 78 mM and 4 mM to 30 mM, respectively (Figure 4). When ADG cells were cultured in ERDF medium contain-

ing 430 mM NaCl, 4 mM MgCl₂, and 8 mM MgSO₄, the cell viability was high at a confluent state. The addition of KCl to the ERDF medium exhibited no effect on the cell growth. CaCl₂ caused cell damages, mostly lysis in concentrations of more than 7.7 mM, suggesting that CaCl₂ was toxic at higher concentrations for ADG cells. Varying concentrations of salts did not remarkably change the ability of ADG cells to secrete glycosidases (data not shown).

Glycosidases secreted from the ADG cell line in protein-free culture

ADG cells secreted the following 9 kinds of glycosidases in protein-free salts-ERDF medium; α -L-fucosidase, α -D-galactosidase, N-acetyl- β -D-galactosaminidase, α -D-glucosidase, N-acetyl- β -D-glucosaminidase, α -D-mannosidase, β -D-mannosidase, β -D-xylosidase (Table 3), in addition to 1-3 xylanase. The ability of ADG cells to secrete these glycosidases was also confirmed in other media (Table 3). In particular, GIC medium was suitable for the secretion of various glycosidases. In comparison of the specific enzyme activities per cell in GIC medium with those in ERDF medium, ADG cells cultured in GIC medium exhibited about a 65-fold higher enzyme activity for α -D-mannosidase and about 25-fold higher activities for the following glycosidases; α -D-galactosidase, N-acetyl- β -D-galactosaminidase, and N-acetyl- β -D-glucosaminidase. These results demonstrated that ERDF and GIC media were more suitable for cell growth or production of glycosidases, respectively. ADG cells secreted a small amount of 1-3 xylanase but not other enzymes which degrade porphyrin, mannan, κ -carrageenan, and sodium alginate (data not shown).

Discussion

This paper describes the first establishment of an abalone digestive gland cell line. ADG cells were established by subculturing of abalone digestive gland cells in ERDF medium supplemented with 8% FBS, abalone hemolymph, and increased concentrations of 5 kinds of salts. We note that FBS may include both cell growth factors and cell toxic factors for the ADG cell line. ADG cells initially proliferated in 8% FBS supplemented ERDF medium with salts, but higher concentrations of FBS inhibited the cell growth and induced cell death. The fact that ADG cells were suc-

Table 3. Glycosidases activities secreted into the supernatant of abalone digestive gland cells cultured in different media

Glycosidase	ERDF	LDF	L-15	DME	F-12	MEM	RPIM	199	GIC
	(μ units/ml)								
β-D-fucosidase	—	—	—	—	—	—	—	—	—
α-L-fucosidase	—	0.06	0.06	—	—	—	—	0.09	0.21
β-L-fucosidase	0.10	0.10	0.14	0.09	0.10	0.10	0.09	—	0.06
α-D-galactosidase	3.55	5.25	0.06	—	—	—	—	0.09	10.0
N-acetyl-α-D-galactosaminidase	—	0.12	0.09	—	—	—	—	—	0.27
β-D-galactosidase	—	—	—	—	—	—	—	—	0.19
N-acetyl-β-D-galactosaminidase	6.20	11.5	6.15	2.02	0.63	0.69	1.39	1.75	15.3
α-D-glucosidase	0.09	—	0.09	0.12	0.08	0.06	—	0.10	0.16
N-acetyl-α-D-glucosaminidase	—	—	—	—	—	—	—	—	0.08
β-D-glucosidase	—	—	—	—	—	—	—	0.06	0.08
N-acetyl-β-D-glucosaminidase	4.55	8.70	3.55	1.36	0.31	0.57	0.92	1.09	15.1
α-D-mannosidase	0.27	0.27	0.23	0.37	0.27	0.26	0.21	0.43	2.14
β-D-mannosidase	0.21	0.21	0.24	0.19	0.19	0.16	0.13	0.15	0.40
β-D-xylosidase	0.21	0.23	0.13	0.10	0.10	0.13	0.10	0.10	0.37

— not detectable

Figure 4. Effect of 5 kinds of salts on the growth of ADG cells. ADG cells were suspended in ERDF medium with various concentrations of salts, which were described in the section of Materials and methods, seeded in 6-well plastic plates at a cell number of 1.0×10^5 cells/ml. The cell densities on fifth day were shown. ■, salt concentrations in salts-ERDF medium used for primary culture; □, salt concentrations in original ERDF medium.

cessfully established in ERDF medium supplemented with 8% FBS and abalone hemolymph suggests that abalone hemolymph may contain some factors which neutralize the action of the toxic substances in FBS. ADG cells could proliferate to a high-density without any observable damage in protein-free culture. These results lead to a conclusion that FBS is not necessary for ADG cell culture. ADG cells grown in protein-free medium are not adherent because of no supplying adhesive factors such as fibronectin from FBS.

NaCl, MgCl₂, and MgSO₄ promoted cell growth but CaCl₂ caused strong cell damages at concentrations higher than 7.7 mM. It is likely that NaCl, MgCl₂, MgSO₄, and CaCl₂ may affect the cell growth signals via the change of the osmotic pressure.

There was no cell growth during the first 24 h before starting rapid cell growth when ADG cells were plated on low cell densities in fresh protein-free medium. This suggests that ADG cells may secrete some autocrine growth factors to assist their own growth. No detectable change in the morphology and proliferative ability of ADG cells was observed during the cultivation in protein-free ERDF medium containing 430 mM NaCl, 4 mM MgCl₂ and 8 mM MgSO₄ for more than 14 months, suggesting that this protein-free medium was optimum for the cultivation of this cell line. ADG cells grown in protein-free medium to a high density produced clumps like the bunch of grapes, which were also observed in original digestive gland tissue of abalone.

ERDF medium and GIC medium are most suitable for cell growth and secreting glycosidases, respectively. When comparing ERDF medium with GIC medium, GIC medium contains the following components which ERDF does not have; α -ketoglutaric acid, fumaric acid, malic acid, succinic acid, fructose, sucrose, and several kinds of amino acids. When ADG cells were cultured in GIC medium, the number of large spherical cells was increased, suggesting that these components in GIC medium may stimulate the differentiation of ADG cells to high productive cells or accelerate the production and secretion of glycosidases in ADG cells.

Only 1,3-xylanase among polysaccharide degrading enzymes was secreted from ADG cells. It remains to be examined if other polysaccharide-degrading enzymes can be induced or not. Glycosidases secreted from ADG cells may be useful to clarify the characteristics of invertebrate glycosidases. Purification of some glycosidases secreted from ADG cells are now undergoing and will be published elsewhere.

ADG cells can rapidly proliferate to a very high density (1.0×10^7 cells/ml in a dish) with a very short doubling time of 12–13 h in a non adherent state in protein-free medium. These cells can grow at low temperature like 23 °C without a CO₂ incubator. ADG cells may become a powerful vehicle for mass-production of recombinant proteins like insect cells.

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