Purification and characterization of α -D-galactosidase produced by ADG cell line established from abalone digestive gland

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Received 24 February 1999; accepted 9 February 2000

Key words: abalone digestive gland cell, protein-free culture, α -D-galactosidase

Abstract

ADG cell line was established from an abalone digestive gland and previously characterized. ADG cells have the potential to grow in protein-free culture and secrete 13 types of glycosidases. In this article, we determined the origin of ADG cell line, using electron microscopy, and purified a glycosidase secreted by these cells. The electron microscopic analysis showed that ADG cell line contains several nuclei, which suggests that they may be derived from protist cells. Moreover, α -D-galactosidase that hydrolyzes *p*-nitrophenyl galactopyranoside was purified 130-fold from the spent culture medium of ADG cells. The molecular weight of the enzyme, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration analysis, was shown to be 43 and 42 kDa, respectively, and it appeared to consist of a single polypeptide chain. The purified enzyme preparation was practically free from other glycosidases secreted from the cells. Catalytic activity was optimal at pH 5.5 and at a temperature of 37 °C. The enzyme was also the most stable at pH 5.5.

Abbreviations: ADG, abalone digestive gland; ERDF, enrich RDF; M.W., molecular weight; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

Polysaccharide-hydrolyzing enzymes derived from bacteria or mollusks such as abalone and aplysia have been used for the preparation of seaweed protoplasts (Rochaix and Dillewijn, 1982; Kawashima and Tokuda, 1990; Ishikawa and Saga, 1990; Nakanishi and Saga, 1990; Kuras and Wollman, 1994). The enzymes obtained from sea mollusks can digest various polysaccharides in the cell walls of seaweed. Actually, the marked abalone acetone powder is widely used for the preparation of seaweed protoplast. However, these enzymes have not been completely purified or characterized. Also, seaweed is rich in protein, minerals, and vitamins, and has been widely used as food, medical materials, cosmetics, food additives, or indus-

trial materials. The value of seaweed can be raised by digestion of these cell walls.

Establishment of digestive gland cell lines from sea mollusks which secrete various glycosidases will be useful, not only to produce these enzymes in a large quantity to digest seaweed polysaccharides, but also to clarify the cell physiology of sea mollusks. A few cell lines derived from sea mollusks have been established so far. We previously described the establishment of ADG cell line isolated from sea mollusks, which rapidly grows and secretes various types of glycosidases in protein-free medium (Kusumoto et al., 1997). Although the possibility exists that these cells are parasitic organisms derived from the digestive gland rather than true abalone cells, the morphology was different from yeasts, bacteria, or molds. In this article, we estimated the origins of ADG cell line by using

Figure 1. Morphology of an ADG cell. (A) scanning electron microscopy; (B) transmission electron microscopy. Marks used were N, nuclei; M, mitochondria; ER, endoplasmic reticulum; G, Golgi apparatus; S, secretory vesicle.

electron microscopy. Moreover, we tried to purify and characterize α -D-galactosidase secreted by ADG cells that hydrolyzes *p*-nitorophenyl galactopyranoside.

Materials and methods

Materials

ERDF medium was purchased from KYOKUTO Pharmaceutical Industrial Co., Ltd. Econo-Pac 10DG column and silver stain kit were purchased from BIO-RAD Laboratories. Molecular weight standards, catalase, aldolase, ovalbumin, and chymotrypsinogen A were from Pharmacia Biotech. DEAE-Toyopearl

Figure 2. DEAE-Toyopearl 650M chromatography of α -D-galactosidase. The enzyme was eluted with a linear gradient between 20 to 150 mM sodium phosphate buffer, pH 7.5. Glycosidase activity of the each fractions was measured as described under the Materials and Methods section. The used mark was O, α -D-galactosidase activity.

Figure 3. Toyopearl HW55 F chromatography of α -D-galactosidase. The enzyme solution from step 4 (3.66 mg per ml of protein) was applied to the column, which had been previously equilibrated with 200 mM sodium phosphate buffer, pH 6.9. The column was developed with the same buffer at a flow rate of 1 ml per min. Glycosidase activity of each fraction was measured as described under the Materials and Methods section. The used mark was O, α -D-galactosidase activity. The heavy line shows the absorbance at 280 nm.

650M column and Toyopearl HW55 F column were from TOSOH Co. A substrate, *p*-nitorophenyl galactopyranoside was from Sigma Chemical Co. All other reagents were from Wako Pure Chemical Industries.

Electron microscopy

Ultrastructural analysis was performed by transmission electron microscopy (H-7000 model, Hitachi,

Figure 4. Polyacrylamide (12%) gel electrophoretic analysis of α -D-galactosidase. The gels were fixed in 40% methanol/10% acetic acid and stained using the silver stain kit. For details, see the Materials and Methods section. Lane 1, M.W. standards. Lane 2, α -D-galactosidase (M.W. = 43 kDa).

Ltd.) and scanning electron microscopy (S-2100B model, Hitachi, Ltd.).

Enzyme assay

Activity of the α -D-galactosidase was determined by using *p*-nitrophenyl galactopyranoside at 37 °C. One unit of the enzymes was defined as the amount of enzymes, which hydrolyze 1 μ mol of *p*-nitrophenyl galactopyranoside per min.

Molecular weight determination

Toyopearl HW55 F column was operated as described in step 4. Catalase (M.W. 232 kDa), aldolase (M.W. 158 kDa), ovalbumin (M.W. 43 kDa), and chymotrypsinogen A (M.W. 24 kDa) were used for calibration. The molecular weight was also determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were fixed in 40% methanol/10% acetic acid and stained using the silver stain kit.

Results

Ultrastructure of ADG cells

We previously reported that ADG cells were significantly different in the morphology from yeasts, bacteria, or molds, and were similar to cells observed in the abalone digestive gland. Scanning electron microscopy of ADG cells showed that the surface of the cells is uneven and not flat (Figure 1A). Using transmission electron microscopy, several nuclei, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, and secretory vesicle were observed in ADG cells (Figure 1B). An electron micrograph of ADG cells showed that the cells contains several nuclei, indicating that they are not derived from true abalone cells but rather from unicellular organisms. These organisms may be parasitic protist cells living in the abalone digestive gland. We suggest that ADG cell line may be from the family of the zoosporic fungi, which comprise a polyphyletic grouping of four classes, the *Plasmodiophoromycetes*, *Oomycetes*, *Hyphochytriomycetes* and *Chytridiomycetes*. Although ADG cells had not tinsel-like flagellum like the zoosporic fungi, *Mastigomycotina* (Sparrow, 1973; Barr, 1981, 1988; Awaji, 1989), the morphology was similar in several nuclei or Golgi apparatus.

Figure 5A–C. Catalytic properties of α -D-galactosidase. (A) Optimal pH on the enzyme activity of α -D-galactosidase. (B) Stable pH on the enzyme activity of α -D-galactosidase. Buffer used was 10 mM citric acid-10 mM sodium phosphate buffer, pH 3.0 to 8.5. The enzyme reaction was carried out at 37 °C for 30 min. (C) Optimal temperature on the enzyme activity of α -D-galactosidase. The enzyme solution was incubated in 1.0 mM citric acid-10 mM sodium phosphate buffer, pH 5.5, for 30 min at various temperatures from 0 to 80 °C. The used mark was O, α -D-galactosidase.

Moreover, the results that several large secretory vesicles and developed Golgi apparatus were observed, suggest that ADG cells actively secretes various types of glycosidases, probably to digest seaweeds in the abalone gland. Several Golgi apparatuses were observed around one of nucleus, which suggest that this one will be used for gene expression while the other nuclei are used to procreate.

α -D-galactosidase purification

Step 1: Cell culture – ADG cell line was derived from an abalone digestive gland and secreted the various types of glycosidases in protein-free medium: fucosidase, galactosidase, N-acetyl-galactosaminidase, glucosidase, N-acetyl-glucosaminidase, mannosidase, xylosidase, and xylanase. For enzyme production, ADG cells were cultured in protein-free ERDF medium containing 260 mM NaCl, 10 mM KCl, 4 mM MgCl₂ and 8 mM MgSO₄ at 23 °C for 1 week. About 2 l of culture medium were obtained and the cells were removed by centrifugation at 3000 rpm for 15 min. The clear supernatant contained 0.1 mg per ml of protein (4.26 unit per liter of α -D-galactosidase). All remaining steps were conducted at 4 °C.

Step 2: Ammonium sulfate precipitation – The supernatant from step 1 was brought to 90% saturation by the addition of 657 g of solid ammonium sulfate per 1 l of the solution. The mixture was stirred for 3 h at 4 °C and centrifuged at 10 000 rpm for 30 min. The precipitate was dissolved in a minimal volume of 20 mM sodium phosphate (NaH₂PO₄) buffer, pH 7.5, and desalted by passage through an Econo-Pac IODG column, which was equilibrated with 20 mM sodium phosphate buffer (pH 7.5).

Step 3: DEAE-Toyopearl 650M chromatography – The desalted extracts were pooled (20 ml) and applied on a DEAE-Toyopearl 650M column (4 × 80 cm) at a flow rate of 2 ml per min. The column, equilibrated previously with 20 mM sodium phosphate buffer, pH 7.5, was again washed with this buffer at 2 ml per min until the unabsorbed protein was eluted. Elution was continued with a linear gradient between 20 to 150 mM sodium phosphate buffer, pH 7.5 (Figure 2). Fractions of 85 ml were collected. The α -D-galactosidase containing fractions (24 to 38) were pooled and brought to 90% saturation by ammonium sulfate. The precipitate obtained from centrifugation

at 10 000 rpm for 30 min was dissolved in 1 ml of 200 mM sodium phosphate buffer (pH 6.9).

Step 4: Toyopearl HW55 F chromatography – The enzyme solution from step 3 (3.66 mg per ml of protein) was applied to a Toyopearl HW55 F column (4 × 80 cm) which had been equilibrated previously with 200 mM sodium phosphate buffer, pH 6.9 (Figure 3). The column was developed with the same buffer at a flow rate of 1 ml per min. Fractions of the eluted enzymes were collected and used for characterizations.

As shown in Table 1, α -D-galactosidase was purified 130-fold from the culture medium with a 5.2% recovery of activity. The final peak from the Toyopearl HW55 F column was perfectly homogeneous consisting of a single polypeptide chain as judged by SDS-PAGE (Figure 4). The molecular weight estimated by its mobility on the SDS-PAGE was 43 kDa, and by the elution volume in molecular sieve chromatography on the Toyopearl HW55 F column was about 42 kDa (data not shown). When the purified enzyme was concentrated to 0.08 mg per ml, at least 70% of the activity remained after 1 month at -20°C . The enzyme preparation was free from the following glycosidases acting on *p*-nitrophenyl galactopyranoside: α - or β -fucosidase, β -galactosidase, N-acetyl- α - or β -galactosaminidase, α - or β -glucosidase, N-acetyl- α - or β -glucosaminidase, mannosidase, and β -xylosidase.

Catalytic properties

Optimal pH: Figure 5 shows the rate of enzyme activities at various pH in 10 mM citric acid-10 mM sodium phosphate buffer (pH 3.0–8.5). The enzyme reaction was carried out at 37°C for 30 min.

The highest enzyme activity was observed at pH 5.5 (Figure 5A). The enzyme solution was incubated in the buffer ranging from pH 3.0 to 8.5 at 4°C for 24 h. The most stable pH was 5.5, similar to optimal pH for activity (Figure 5B). **Optimal temperature:** The enzyme solution was incubated in the buffer (pH 5.5) for 30 min at various temperature (from 0 to 80°C) to examine the effect of temperature on the enzyme activities. α -D-galactosidase exhibited the highest activity at 37°C in the buffer (Figure 5C).

Discussion

α -D-galactosidase has been purified from a variety of prokaryotic and eucaryotic species, such as *Coffea canephora* (Haibach, 1991), *Colocasia esculenta* (Chien, 1991), *Phaseolus vulgaris* (Dhar, 1994), or *Aspergillus tamaraii* (Civas, 1984). Most of α -D-galactosidase derived from these species has similar low molecular weight (about 35 to 55 kDa). In this article, we first purified α -D-galactosidase from the spent medium of cultured ADG cells, protist, which may be the family of zoosporic fungi. The α -D-galactosidase derived from ADG cells had a similar molecular weight (about 43 kDa) as those derived from the species described previously. Moreover, the optimal pH of α -D-galactosidase from ADG cells was 5.5, which was also similar to that of α -D-galactosidase derived from other species (pH 5.0 to 6.0). These results suggested that α -D-galactosidase from ADG cells have similar characteristics as that from the other species. ADG cells can rapidly grow with a doubling time of 12 to 13 h in protein-free medium at a low temperature of 23°C without a CO_2 incubator. ADG cells can grow and secrete glycosidases in larger scale of batch culture (about 3 l) (data not shown). Therefore, ADG cells may become a powerful and stable vehicle for the mass-production of glycosidases.

Acknowledgements

We are grateful to Dr. David W. Barnes, ATCC, for valuable discussion on the morphology of ADG cell line.

References

- Awaji M, Machii A, Nakamura HK and Wada KT (1989) Characterization of a zoosporic fungus appearing in primary cultures of the kuruma prawn midgut gland. *Bull Natl Res Inst Aquaculture* 15: 19–27.
- Barr DJ (1981) The phylogenetic and taxonomic implications of flagellar rootlet morphology among zoosporic fungi. *Biosystems* 14: 359–370.
- Barr DJ (1988) How modern systematics relates to the rumen fungi. *Biosystems* 21: 351–356.
- Chien SF and Lin-Chu M (1991) The conversion of group B red blood cells into group O by an α -D-galactosidase from taro (*Colocasia esculenta*). *Carbohydr Res* 217: 191–200.
- Civas A, Eberhard R, Le Dizet P and Petek F (1984) Glycosidases induced in *Aspergillus tamaraii*. secreted α -D-galactosidase and β -D-mannanase. *Biochem J* 219: 857–863.

- Dhar M, Mitra M, Hata J, Butnariu O and Smith D (1994) Purification and characterization of *Phaseolus vulgaris* α -D-galactosidase isozymes. *Biochem Mol Biol Int* 34: 1055–1062.
- Haibach F, Hata J, Mitra M, Dhar M, Harmata M, Sun P and Smith D (1991) Purification and characterization of a *Coffea canephora* α -D-galactosidase isozymes. *Biochem Biophys Res Commun* 181: 1564–1571.
- Ishikawa Y and Saga N (1990) Study on the breeding of marine algae applying tissue culture technique. *Soshiki Baiyou* 16: 173–177.
- Kawashima Y and Tokuda H (1990) Protoplast isolation from several macroalgae and cell fusion. *Soshiki Baiyou* 16: 157–161.
- Kuras R and Wollman FA (1994) The assembly of cytochrome b6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*. *EMBO J* 13: 1019–1027.
- Kusumoto K, Shirahata S, Katakura Y, Murakami H and Kamei Y (1997) Establishment of an abalone digestive gland cell line secreting various glycosidases in protein-free culture. *Cytotechnology* 24: 169–175.
- Nakanishi K and Saga N (1990) Production of useful compounds in marine algae cell culture. *Soshiki Baiyou* 16: 177–180.
- Rochaix J and Dillewijn IV (1982) Transformation of the green alga *Chlamydomonas reinhardtii* with yeast DNA. *Nature* 296: 70–72.