Isolation and Identification of a Porphyrin-degrading Bacterium

Takahiko Aoki,*¹ Toshiyoshi Araki,*¹
and Manabu Kitamikado*²

(Received November 27, 1989)

A screening was undertaken to acquire bacteria with porphyrin-degrading enzyme productivity. Several strains were obtained from the sea environment in Fukuoka Prefecture. Among them, the strain AP-2 from an alga showed the highest productivity of the enzyme. The organism needed the extract from *P. yezoensis* as an inducer to produce porphyrin-degrading enzyme, and released a large amount of the enzyme into the culture fluid, when incubated with shaking at 25°C for 5 days in the following liquid medium; 0.5% peptone, 0.1% yeast extract, 3% NaCl in the extract from *P. yezoensis*, pH 7.4. The enzyme exhibited a high activity at pH 6.5. The enzyme seems to be an β -agarase which cleaves not only porphyrin but also agar to give various sizes of neoagarooligosaccharides.

According to criteria of the Bergey's Manual of Systematic Bacteriology, Vol. 1, the bacterium was assigned to the genus *Vibrio*, but not listed species name for this organism.

Porphyrin-degrading enzyme is a useful tool for the structural analysis of porphyrin and also an essential enzyme to prepare protoplasts from algae containing porphyrin in their cell wall, *Bangia* and *Porphyra*.¹⁾

As enzyme capable of degrading porphyrin have been reported β -agarases (EC 3.2.1.81) from *Cytophaga* sp.²⁾ and *Pseudomonas atlantica*³⁾ which hydrolyzed agar with a structural component a similar to porphyrin. However, these enzymes degraded only a defined part of porphyrin structure and exhibited low hydrolyzing activity on porphyrin compared with agar.

To the purpose of preparation of protoplasts from *Porphyra*, a popular food stuff in Japan, we tried to search bacteria which produce enzymes having higher activity against porphyrin than these reported β -agarases. This paper describes isolation, culture condition, and identification of bacterium which produces a large amount of porphyrin-degrading enzyme exhibiting a high activity, in nearly neutral and alkaline conditions.

Materials and Methods

Substrates

Porphyrin was prepared by the method of Su and Hassid⁴⁾ from a red alga *Porphyra yezoensis* collected from Ariake Bay in Fukuoka Prefecture.⁵⁾

The extract from *P. yezoensis* was prepared as follows; five grams of dry leaf powder of *P. yezoensis* was suspended in 600 ml of hot water in a 2,000 ml flask and heated in a boiling water bath for 3 h. The mixture was centrifuged at 6,000 r.p.m. for 20 min and the supernatant was used as the extract of *P. yezoensis*. Neoagarosaccharides (neoagarobiose to neoagarooctaose) was prepared by the method of Groleau and Yaphe⁶⁾ from hydrolysis products of the agar with β -agarase from *P. atlantica* (Sigma Chemical Co.). Agar (Agar Noble) and κ -carrageenan were purchased from Difco Lab. and Wako Pure Chem. Ind., respectively.

Media used for Screening of Bacteria

The medium for the bacteria of marine origin was composed of 0.5% peptone (Daigo Eiyokagaku Co.), 0.1% yeast extract (Daigo Eiyokagaku Co.), and 3% NaCl, pH 7.6-7.8. For those of freshwater and land soil origins, we used a nutrient broth (Difco Lab.), pH 7.0-7.2. These media were used after adding 0.2% porphyrin and solidifying with 1.5% agar (Difco Lab.), as required.

Sources of Porphyrin-degrading Bacteria

Seventy-two samples, collected during 1985 in Fukuoka Prefecture, were examined. Those of

*¹ Department of Chemistry of Fishery Resources, Faculty of Bioresources, Mie University, Tsu, Mie 514, Japan (青木恭彦, 荒木利芳: 三重大学生物資源学部).

*² Department of Fisheries, Faculty of Agriculture, Kyushu University, Higashi, Fukuoka 812, Japan (北御門学: 九州大学農学部).

marine origin included: 4 from coastal sea water, 18 from the bottom mud of coastal sea, 19 from algae (green, brown, and red), 5 from gills and 6 from intestinal contents of marine fish. Those from land and fresh-water origins included: 10 from the soil, 4 from river water, and 6 from pond water.

Method of Screening for Porphyrin-degrading Bacteria

Before screening, each solid sample was blended with a small volume of either sterilized 3% NaCl solution (for samples of marine origin) or 0.9% NaCl solution (for those of land and fresh-water origins). A small quantity of the sample was streaked on the surface of agar medium in a petri dish, and incubated aerobically at 25°C for 4–7 days.

As porphyrin-degrading bacteria were presumed to be able to hydrolyze agar, colonies capable of liquefying agar were isolated. The isolated bacteria were inoculated into 5 ml of liquid medium containing 0.2% porphyrin. After incubation with shaking at 25°C for 4 days, a volume of 0.5 ml of each culture grown was added to 2 ml of acid albumin (3.26 g of sodium acetate, 4.56 ml of glacial acetic acid, and 1.0 g of bovine albumin fraction V were dissolved in 1 liter of water, pH adjusted to 3.72 to 3.78 with 1:1 concentration of HCl).⁷⁾ Acid albumin combined with porphyrin so that it forms turbidity in the mixture, but not when porphyrin is degraded by enzyme. The porphyrin-degrading bacteria selected by the acid albumin test were inoculated on agar slant medium containing 0.2% porphyrin, stored in a refrigerator, and transferred monthly in homologous agar media to maintain viability.

Assay of Porphyrin-degrading Enzyme

A 0.5 ml of enzyme solution was added to 1 ml of 100 mM sodium acetate buffer, pH 6.0, containing 3.0% of porphyrin and 2 mM CaCl_2 . After incubation at 37°C for an appropriate period, the reaction was halted by heating the mixture in a boiling water bath for 5 min. The reducing sugar produced was then determined by Somogyi-Nelson's method⁸⁾ and expressed as D-galactose. One unit of the enzyme was defined as the activity which liberated 1 μM of galactose per min, under the above condition.

Preparation of Enzyme solution from Isolates

Cells taken from agar slant culture of each isolate were transferred to 20 ml of sterile liquid

medium containing 0.2% porphyrin in a 100 ml flask. The preparation was then incubated with shaking by a reciprocal shaker (8 cm amplitude, 90 strokes/min) at 25°C for 4 days. Each culture was centrifuged, and the enzyme activity of the supernatant obtained was determined. For the determination of optimal pH and thin layer chromatography (TLC) of hydrolysis products, salting-out preparation (75% saturation with solid ammonium sulfate) of the supernatant was used as enzyme solution.

Cultural Conditions for Enzyme Production of Strain AP-2

The following conditions were examined: initial pH of culture medium; addition of porphyrin, agar, κ -carrageenan, and the extract from *P. yezoensis* to medium as an inducer of porphyrin-degrading enzyme; culture temperature and period; with or without shaking by a reciprocal shaker.

Detection of Hydrolysis Products of Porphyrin with the Enzyme from Strain AP-2

TLC was carried out by an ascending method using TLC-plastic sheet silica gel 60 (Merck & Co. Inc.) with a solvent system of n-butanol-acetic acid-water (2:1:1, v/v), and the saccharides developed were visualized by spraying the plate with a naphthoresorcinol reagent.⁹⁾

Decomposing Ratio of Substrate with the Enzyme from Strain AP-2

Decomposing ratio of porphyrin and agar was determined as follows; to 2 ml of substrate solution [1.0 mg in 100 mM sodium acetate buffer, pH 6.0, containing 2 mM CaCl_2] was added 0.5 ml of enzyme solution (10 units in the same buffer). After incubation at 38°C for 12 h, the mixture was dialyzed for 12 h (Spectra/Por tubing, size 3) against 100 ml of water, and total carbohydrate was determined for inner and outer solutions by phenol- H_2SO_4 method of Dubois *et al.*¹⁰⁾

Identification Method for Strain AP-2

Identification of the strain AP-2 was made according to the criteria described in Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984).¹¹⁾ Motility, morphology, and gram-staining characteristics were determined by light microscopy. The test of ability to utilize different organic compounds was used by BM (basal medium). The oxidase test was measured by the method of Kovacs.¹²⁾ The mol% G+C of the DNA range was measured by the method of Marmur.¹³⁾

Other identification methods were used, as required.¹⁴⁾

Results and Discussion

Screening of Porphyran-degrading Bacteria

As the result of screening, bacteria capable of degrading porphyran were obtained one isolate from the bottom mud of the coastal sea, and four from algae. An isolate from an alga exhibited the highest enzyme productivity among them, that is, its enzyme activity was 178 m units per 1 ml of culture fluid after 4-day incubation, and the enzymes from the other bacteria were in range of 70 to 157 m units per ml. Therefore, the organism was termed strain AP-2 and used for the following experiments.

Cultural Condition for the Production of Porphyran-degrading Enzyme on Strain AP-2

The strain AP-2 couldn't produce the enzyme without an inducer. The extract from *P. yezoensis*

was an excellent inducer as well as porphyran, and better than agar and carrageenan (Fig. 1). When grown in the liquid medium at initial pH 7.4 and temperature of 25°C with shaking, the organism produced a large amount of the enzyme. The enzyme activity of the culture fluid reached the highest level after 5-day incubation (Fig. 2).

Effects of pH and Porphyran Concentration on the Enzyme Activity

The optimal pH of enzyme was determined with M/10 sodium acetate (pH 3.2–6.0), and M/15 phosphate (pH 6.0–8.0), M/20 Tris-HCl (pH 7.5–9.0) buffers in the assay system. The maximal activity of the enzyme was pH 6.5 (Fig. 3).

As shown in Fig. 4, the enzyme activity was considerably affected by porphyran concentration in the reaction mixture. To obtain the maximal reaction was needed 3% porphyran of final concentration.

Fig. 1. Productivity of porphyran-degrading enzyme of strain AP-2 grown on different carbohydrates.

As culture medium was used liquid medium containing 0.2% of different carbohydrate except the extract from *P. yezoensis*.

Fig. 2. Effect of incubation times on porphyran-degrading enzyme production of strain AP-2.

Fig. 3. pH-Activity curve of porphyran-degrading enzyme. ○, sodium acetate buffer (pH 3.2–6.0); ×, phosphate buffer (pH 6.0–8.0); △, Tris-HCl buffer (pH 7.5–9.0).

Fig. 4. Influence of the substrate concentration on the enzyme activity.

Fig. 5. Thin layer chromatogram of hydrolyzates of porphyran with the enzyme from strain AP-2. S, neoagarobiose to neoagarooctaose (DP 2, 4, 6, 8; degree of polymerization); Gal, galactose; 15 min, 30 min, 3 h; incubation time.

Thin Layer Chromatography of Hydrolytic Products

A volume of 2 ml of the salting-out enzyme (ca. 2.5 units) from the culture fluid of strain AP-2 was added to 12 ml of 2 mM sodium acetate buffer, pH 6.0, containing 3% porphyran and 2 mM CaCl_2 , and the mixture was incubated at 37°C. At intervals, a part of the reaction mixture was withdrawn and spotted on TLC-plate. Porphyran was cleaved and formed various neoagarooligosaccharides and other oligosaccharides (Fig. 5).

The enzyme of strain AP-2 hydrolyzed not only porphyran but also agar, and the decomposing ratio was 52% and 84%, respectively. While, using β -agarase from *P. atlantica*, the decomposing ratio on them was 23% and 75%, respectively.^{8,15)} This result showed the strain AP-2 produced an β -agarase.

Identification of Strain AP-2

The morphological and biochemical characteristics of strain AP-2 are shown in Table 1. The organism was a gram-negative rod, facultative anaerobic, oxidase positive and sensitive to 2,4-diamino-6,7-diisopropyl pteridine, and formed acid but no gas from glucose. It was assigned to the genus *Vibrio*, according to the criteria of

Table 1. Morphological, cultural, and biochemical characteristics of isolate AP-2

Form	rod
Size	0.8 to 1.2 by 1.8 to 2.2 μm
Gram strain	Negative
Motility	+
Flagellation in liquid media	Single polar
3-12 polar flagella	
Aerobic growth	+
Unanaerobic growth	+
O-F test	Fermentation
Catalase	+
Vibriostatic agent sensitivity	+
Swarming on solid complex media	-
PHB-accumulation	-
Pigmentation	
Yellow-orange	-
Blue-black	-
Red	-
Arginine dihydrolase	-
Oxidase	+
Reduction of NO_3^- to NO_2^-	+
Luminescence	-
Gas from D-glucose	-
Indol production	-
Production of acetoin and/or diacetyl	-
Methyl red test	+
Thornley arginine	-
Na^+ required for growth	+
Requirement for organic growth factors	-
Growth at:	
4°C	-
30°C	+
35°C	+
40°C	-
Production of:	
Amylase	+
Gelatinase	+
Lipase	+
Alginase	-
Chitinase	+
Utilization of:	
D-Xylose	-
L-Arabinose	-
D-Mannose	+
D-Galactose	-
Sucrose	+
Trehalose	+
Cellobiose	+
Lactose	-
Salicin	+
Citrate	+
D-Mannitol	+
D-Sorbitol	+
meso-Inositol	+
Ethanol	-
L- α -Alanine	-
L-Serine	-
L-Leucine	-
L-Histidine	-
L-Proline	+
L-Tyrosine	+
D-Gluconate	+
D-Glucuronate	-
Mol% G+C of DNA	46

Bergey's Manual of Systematic Bacteriology. The strain AP-2 closely resembled *Vibrio harveyi* in the Manual on biochemical characteristics, but the strain differed in the ability to utilize some organic compounds (e.g. D-glucuronate and L-serine). Therefore, the species of the strain has not been decided.

When the thalli of *P. yezoensis* (ca. 100 mg) were dipped into the 5 ml of MES buffer solution containing 1 unit each of the porphyran-degrading enzyme, β -1,4-mannanase, and β -1,3-xylanase, and 0.7 M mannitol, and incubated at 22°C for 90 min with shaking, a large number of protoplasts (10^5 – 10^6) were formed.¹⁸⁾

The porphyran-degrading enzyme from strain AP-2 was a useful enzyme to prepare protoplasts from *P. yezoensis*. Purification and characterization of the enzyme from strain AP-2 will be published on another occasion.

References

- 1) E. Percival and R. H. McDowell: Chemistry and Enzymology of Marine Algal Polysaccharides, Academic Press, London, 1967, pp. 134–137.
- 2) M. Duckworth and J. R. Turvey: *Biochem. J.*, **113**, 687–692 (1969).
- 3) L. M. Morrice, M. W. McLean, F. B. Williamson, and W. F. Long: *Eur. J. Biochem.*, **135**, 553–558 (1983).
- 4) J. C. Su and W. Z. Hassid: *Biochemistry*, **1**, 468–474 (1962).
- 5) T. Aoki, T. Araki, and M. Kitamikado: *Nippon Suisan Gakkaishi*, **54**, 277–281 (1988).
- 6) D. Groleau and W. Yaphe: *Can. J. Microbiol.*, **23**, 672–679 (1977).
- 7) M. B. Mathews: in "Methods in Enzymology" (ed. by E. F. Neufeld and V. Ginsburg) Vol. 8, Academic Press Inc., New York, 1966, pp. 654–662.
- 8) M. Somogyi: *J. Biol. Chem.*, **195**, 19–23 (1952).
- 9) M. Duckworth and W. Yaphe: *J. Chromatogr.*, **49**, 482–487 (1970).
- 10) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith: *Anal. Chem.*, **28**, 350–356 (1956).
- 11) P. Baumann, A. L. Furniss, and J. V. Lee: in "Bergey's Manual of Systematic Bacteriology" (ed. by N. R. Krieg and J. G. Holt) Vol. 1, Williams & Wilkins, Baltimore, 1984, pp. 518–538.
- 12) N. Kovacs: *Nature*, **178**, 703 (1956).
- 13) J. Marmur: *J. Mol. Biol.*, **3**, 208–218 (1961).
- 14) R. Sakazaki and A. Balows: in "The Prokaryotes" (ed. by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel), Springer-Verlag, New York, 1981, pp. 1272–1301.
- 15) L. M. Morrice, M. W. McLean, W. F. Long, and F. B. Williamson: *Eur. J. Biochem.*, **137**, 149–154 (1983).
- 16) T. Araki, T. Aoki, and M. Kitamikado: *Nippon Suisan Gakkaishi*, **53**, 1623–1627 (1987).