Production of Fucoidan-Degrading Enzymes, Fucoidanase, and Fucoidan Sulfatase by *Vibrio* sp. N-5

Shin-ichi Furukawa,*¹ Tatsuo Fujikawa,*¹ Daizo Koga,*²
and Akio Ide*²

(Received February 7, 1992)

When the fucoidan-utilizing bacterium strain N-5, identified as genus *Vibrio*, was grown on a synthetic medium containing fucoidan, both fucoidanase and fucoidan sulfatase were detected in the cells. The latter enzyme was produced with a time lag, while fucoidanase appeared rapidly. Both enzymes were induced with the addition of fucoidan as a carbon source. Both enzymes showed approximately the same properties for activity and stability as a function of pH and temperature. Intracellular fucoidanase was found in the cells grown on the medium containing 0.3% fucoidan hydrolyzed gagome- and Mozuku-fucoidans, whereas fucoidan sulfatase acted on Gagome-, Mozuku-, and Futomozuku-fucoidans to release sulfate ions.

Fucoidan is a sulfated polysaccharide. Although it has been isolated from various species of brown algae, its chemical structure, biological and physiological functions have not yet been unraveled.

Some fucoidan-degrading bacteria such as *Pseudomonas atlantica*, *P. carrageenovora*,¹⁾ and *Vibrio* sp.²⁾ have been isolated, and their production of fucoidan-hydrolyzing glycosidases have been reported. However, there are no reports on bacteria that are capable of producing sulfatase acting on fucoidan.

In our previous paper,³⁾ five strains of fucoidan-utilizing bacteria were isolated from sea sands and their growing conditions were characterized. We also reported that the N-5 strain produces a large amount of fucoidan-degrading enzymes, fucoidanase, and fucoidan sulfatase.

In this paper, we describe the production and properties of fucoidanase and fucoidan sulfatase by strain N-5.

Materials and Methods

Microorganism, Culture Medium, and Cultivation

The fucoidan-utilizing bacterium strain N-5 isolated from sea sands was used for the production of fucoidanase and fucoidan sulfatase. The bacterium was grown at 25°C on a fucoidan agar medium consisting of 2.0 g NH_4NO_3 , 1.0 g

Gagome-fucoidan, and 1.8 g agar in 1 l of sterilized natural seawater, and was then stored at 4°C.

A basal medium consisting of 2.0 g NH_4NO_3 , 2.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g Na_2HPO_4 , 25.0 g NaCl, and 1.0 g fucoidan in 1 l of distilled water was used for culture of the bacterium. A broth medium consisting of 15 g dry bouillon (Nissui Pharmaceutical Co., Ltd) in 1 l of distilled water and fucoidan seawater (FS) medium were used for the production of fucoidanase and fucoidan sulfatase.

The FS medium was prepared by adding $6 \times 10^{-6}\text{M}$ of thiamine and 3.0 g of fucoidan to the basal medium. The pH was adjusted to 8.0 with a concentrated Na_2CO_3 solution. The bacterium was cultivated at 25°C in 200 ml of FS medium or broth medium with shaking (8 cm amplitude, 90 strokes/min) for 2 days. The cells were harvested and then resuspended in 5-fold volumes of 100 mM sodium acetate buffer (pH 6.0) and disrupted with 20 kHz of ultrasonication for 6 min at 0°C. The suspension was centrifuged at $12,000 \times g$ at 4°C for 15 min. The resulting supernatant was diluted to a certain volume. The diluted supernatant was referred to as enzyme solution. Bacterial growth was monitored spectrophotometrically at 660 nm or by weighing the harvested cells.

*¹ Laboratory of Nutrition Chemistry, Home-economics, Hijiya Women's Junior College, Higashi, Hiroshima 732, Japan (古川真一, 富士川龍郎: 比治山女子短期大学家政科).

*² Laboratory of Biochemistry, Faculty of Agriculture, Yamaguchi University, Yoshida, Yamaguchi 753, Japan (古賀大三, 井手明雄: 山口大学農学部).

Preparation of Fucoidan

Fucoidan was prepared from Gagome *Kjelmaniella crassifolia*, Mozuku *Nemacystus decipiens*, and Futomozuhu *Tinocladia crassa*, by the method of Fujikawa.⁴⁾ The crude fucoidan extracted with hot water from algal thalli was treated with cetylpyridinium chloride (CPC) at pH 2. The mixture was centrifuged, and the precipitate obtained was washed several times with distilled water. The precipitate was dissolved in a KCl solution (final concentration of about 2 M). The solution was mixed with 2 volumes of 99% ethanol. The resulting precipitate was washed successively with 99% ethanol, 80% ethanol, and 95% acetone to remove free CPC and KCl, and then dried in air. The resulting fucoidan was treated with barium acetate to remove insoluble barium conjugating materials. This fucoidan preparation was used as the carbon source for the cultivation of a bacterium or enzyme substrate.

Preparations of Fucoidanase and Fucoidan Sulfatase

The bacterium was cultivated at 25°C for 3 days in 250 ml of FS medium with shaking. Cells were harvested by centrifugation and washed twice with 2% NaCl solution. For preparation of a crude enzyme solution, 8 g wet weight of bacterial cells were suspended in 80 ml of 100 mM sodium acetate buffer (pH 6.0). Cells were disrupted by 20 kHz of ultrasonication for 6 min at 0°C. The disrupted cell suspension was centrifuged, and the supernatant was used as a crude enzyme solution.

Enzyme Assay

Fucoidanase activity was measured by a colorimetric analysis of reducing sugar released from fucoidan.^{5,6)} A mixture consisting of 1.0 ml of substrate solution (0.1% Gagome-fucoidan dissolved in 100 mM sodium acetate buffer, pH 6.0) and 1.0 ml of enzyme solution was incubated at 38°C for 24 h. The reducing sugar released was measured by the Somogyi-Nelson method.⁷⁾ Enzyme activity was expressed as the amount of released L-fucose ($\mu\text{g/ml}$ reaction mixture). Fucoidan sulfatase activity was measured by nephelometry toward BaSO_4 . A mixture consisting of 1.0 ml of substrate solution (0.1% Gagome-fucoidan dissolved in 100 ml of sodium acetate buffer, pH 6.0) and 1.0 ml of enzyme solution was incubated at 38°C for 15 h, and then 25% trichloroacetic acid (0.5 ml) was added to ter-

minate the reaction and to remove proteins. Sulfate ions released were monitored spectrophotometrically at 660 nm according to the method described in the previous reports.^{5,6)} The enzyme activity was expressed as the amount of sulfate ($\mu\text{g/ml}$ reaction mixture). The amount of total protein was determined by the method of Lowry *et al.*⁸⁾ with bovine serum albumin as standard.

Results

Identification of Fucoidan-Utilizing Bacterium Strain N-5

The morphological properties were studied by the method of Shimizu.⁹⁾ The shape of the bacterium was composed of short rods ($0.8\text{--}1.0 \times 1.0\text{--}1.8 \mu\text{m}$). The results of diagnostic tests for this strain indicated that the bacterium was Gram-negative, motile, positive for nitrate reduction and H_2S production, negative for gas formation from D-glucose, and positive for catalase and oxidase productions. The bacterium grew at 6% NaCl under anaerobic conditions. The optimal growth temperature was 20 to 25°C. On the basis of these observations, this bacterium was characterized as genus *Vibrio*.¹⁰⁾

Determination of Culture Medium for Production of Fucoidanase and Fucoidan Sulfatase

The effects of nitrogen sources, vitamins, and fucoidan concentration on bacterial growth in the basal medium were investigated. Of several nitrogen sources and vitamins tested, ammonium nitrate and thiamine were found to promote bacterial growth (Tables 1 and 2). As shown in Table 3, the optimum concentration of fucoidan was 0.3% for both bacterial growth and enzyme production. The bacterial growth and produc-

Table 1. Effects of nitrogen sources on the bacterial growth

Additions*	Cell growth (OD ₆₆₀)
None	0.20
NH_4NO_3	0.22
NH_4Cl	0.20
$(\text{NH}_4)_2\text{SO}_4$	0.18
NaNO_3	0.17
NaNO_2	0.01
Urea	0.15

* Concentrations: 0.2%.

The bacterium was cultivated at 25°C for 3 days in 200 ml of the basal medium with shaking.

Table 2. Effects of vitamins on the bacterial growth

Additions*	Cell growth (OD ₈₈₀)
None	0.21
Thiamine HCl	0.30
Pantothenate	0.22
Riboflavin	0.25
Folic acid	0.23
Ascorbic acid	0.21

* Concentrations: 6×10^{-8} M.

The bacterium was cultivated at 25°C for 3 days in 200 ml of the basal medium with shaking.

Table 3. Effect of Gagome-fucoidan on the growth and the production of fucoidanase and fucoidan sulfatase by *Vibrio* sp. N-5

Fucoidan concentration (%)	Bacterial growth (OD ₈₈₀)	Activities of fucoidan-degrading enzymes	
		Fcdnase* ¹	Fcd sulfatase* ²
0.1	0.25	15.0	0.18
0.3	0.53	24.4	0.30
0.5	0.50	23.7	0.31

The bacterium was cultivated at 25°C for 3 days in the basal medium with shaking. *¹ Fcdnase, fucoidanase; *² Fcd sulfatase, fucoidan sulfatase.

Table 4. The productions of fucoidanase and fucoidan sulfatase by *Vibrio* sp. N-5 in the basal medium and in FS medium

Medium	Cell yield (mg/ml)	Productions of fucoidan-degrading enzymes	
		Fcdnase* ¹	Fcd sulfatase* ²
Basal	0.17	11.0	0.10
FS	2.10	168.3	1.31

The bacterium was cultivated at 25°C for 48 h.

*¹ Fcdnase, fucoidanase; *² Fcd sulfatase, fucoidan sulfatase.

Fig. 1. Growth of *Vibrio* sp. N-5 and production of fucoidanase and fucoidan sulfatase in a synthetic medium containing fucoidan.

---, The pH of the culture supernatant; ○, bacterial growth; △, fucoidanase activity in the cell extract; □, fucoidan sulfatase activity in the cell extract; ▲, fucoidanase activity in the culture medium; and ■, fucoidan sulfatase activity in the culture medium.

tions of fucoidanase and fucoidan sulfatase were also investigated in the basal medium and FS medium. Table 4 shows that FS medium enhanced the productivity of bacterial cells and both enzymes 12 to 13-fold more than those of the basal medium.

Production of Fucoidanase and Fucoidan Sulfatase by *Vibrio* sp. N-5

To examine the time-courses of the production of fucoidanase and fucoidan sulfatase, the bac-

Fig. 2. Effects of Gagome-fucoidan on the production of fucoidanase and fucoidan sulfatase of *Vibrio* sp. N-5 in the broth medium.

Addition of 1.5% fucoidan (50 ml) is shown by an arrow.

Closed symbols, 1.5% fucoidan was added; open symbols, no fucoidan was added. ● and ○, bacterial growth; ▲ and △, fucoidanase activity in the cell extract; ■ and □, fucoidan sulfatase activity in the cell extract; and ■, fucoidan sulfatase activity in the culture medium.

terium was cultivated at 25°C for 7–10 days in a FS medium or broth medium. As shown in Fig. 1, *Vibrio* sp. N-5 produced fucoidanase with the growth time, whereas the production of fucoidan sulfatase was delayed to some extent. The activities of neither enzyme was detected in the culture medium. The productivity of fucoidanase and fucoidan sulfatase by the bacterium reached their maximum on the 3rd and 4th day of cultivation, respectively. On the other hand, as shown in Fig. 2, the bacterium produced a small amount of fucoidanase in the cells when grown on broth medium, but fucoidan sulfatase was not observed. However, the addition of 1.5% fucoidan to the same medium at the 2nd day of the cultivation immediately triggered the fucoidanase activity. It should be noted that the fucoidan sulfatase activity appeared gradually in both the cells and the culture medium after the addition of fucoidan.

Properties of Fucoidanase and Fucoidan Sulfatase

(1) Effects of pH and Temperature: The pH-activity profiles of fucoidanase and fucoidan sulfatase are shown in Fig. 3A. The optimum pHs were 6.0 and 7.5 for fucoidanase and fucoidan sulfatase, respectively. The optimum temperatures for both enzymes were around 40°C. (Fig. 3B).

(2) Effects of pH and Temperature on Enzyme Stability: As shown in Fig. 4A, fucoidanase was stable between pH 5 and 8, while fucoidan sulfatase was stable between pH 5 and 9. As shown in Fig. 4B, less than 50% of fucoidan sulfatase activity was restored with heating at 70°C, while fucoidanase was completely inactivated over 70°C.

(3) Substrate Specificities: Substrate specificities of both enzymes were investigated using three kinds of fucoidans, Gagome-, Mozuku (sulfated fucan)- and Futomozuku-fucoidans (sulfated galactofucan) as substrates. As shown in Table 5, the fucoidanase hydrolyzed Gagome- and Mozuku-fucoidans, but did not act on Futo-

Fig. 3. Effects of pH and temperature on the activities of fucoidanase and fucoidan sulfatase.

A) Reaction mixtures were incubated at 38°C for 15 h. The pH was adjusted with the following buffers; 100 mM McIlvaine buffer (pH 3.0–8.0), 100 mM Tris-HCl buffer (pH 8.0–9.0), and 100 mM glycine-NaOH buffer (pH 9.0–10.0).

B) The reactions were carried out at pH 6.0 for 15 h. ○, Fucoidanase activity; ●, fucoidan sulfatase activity.

Fig. 4. Effects of pH and temperature on the stabilities of fucoidanase and fucoidan sulfatase.

A) The enzyme solution was pre-incubated at various pHs for 2 h. The buffers used were the same as in Fig. 3-A. Residual activities were measured in 100 mM sodium acetate buffer (pH 6.0), at 38°C for 15 h, and expressed as the percent of the activity at pH 6.0.

B) The enzymes were pre-incubated at various temperatures for 15 min in 100 mM sodium acetate buffer (pH 6.0). Residual activities were measured in the same buffer at 38°C for 15 h, and expressed as the percent of the activity at 4°C.

○, Fucoidanase activity; ●, fucoidan sulfatase activity.

Table 5. Substrate specificities of fucoidanase and fucoidan sulfatase

Substrate (fucoidan)	Reaction time (h)	Activities of fucoidan-degrading enzymes	
		Fcdnase* ¹	Fcd sulfatase* ²
Gagome	24	15.26	0.34
Mozuku	24	16.36	0.34
Futomozuku	30	—* ³	0.14

A reaction mixture consisting of 1.0 ml of enzyme solution and 1.0 ml of 0.1% fucoidan solution was incubated at 38°C. *¹ Fcdnase, fucoidanase; *² Fcd sulfatase, fucoidan sulfatase; *³ —, not detected.

mozuku-fucoidan. The fucoidan sulfatase acted on the former two substrates, whereas less activity was observed with Futomozuku-fucoidan.

Discussion

The fucoidan-utilizing bacterium strain N-5 isolated from sea sands was identified as genus *Vibrio*. *Vibrio* sp. N-5 grew on the synthetic medium containing fucoidan (FS medium) (Fig. 1), and on the broth medium in the presence or absence of fucoidan (Fig. 2). When grown in the fucoidan-containing media, *Vibrio* sp. N-5 produced both fucoidanase and fucoidan sulfatase (Fig. 1). This suggests that both enzymes are induced by fucoidan. This speculation is strongly supported by the observation that the bacterium produced both enzymes in the cells after addition of fucoidan to the broth medium (Fig. 2). However, the induction response was distinct for each species. Fucoidan sulfatase was induced with a lag time, whereas fucoidanase was induced rapidly. Fucoidan sulfatase might be induced by products degraded from fucoidan by fucoidanase.

Fucoidanase and fucoidan sulfatase produced in FS medium showed similar behavior in enzyme activities and stability to pH and temperature (Figs. 3 and 4). However, both enzymes had different properties in substrate specificity against three kinds of fucoidan. Fucoidanase acted on Gagome- and Mozuku-fucoidans, but not to Futomozuku-fucoidan (Table 5). This suggests that the action of fucoidanase produced in the cells of *Vibrio* sp. N-5 is specific to sulfated fucan. Fucoidan sulfatase acted on Gagome-, Mozuku-, and Futomozuku-fucoidan to release sulfate ions. This suggests that fucoidan sulfatase might have a broad substrate specificity or might be heterogeneous enzymes.

Among fucoidan-degrading bacteria strain, *Vibrio* sp. No. 6-2-9 was reported to produce a glycosidase capable of hydrolyzing fucoidan.²⁾

However, it was uncertain whether bacteria produce sulfatase acting on fucoidan or not.²⁾ In this study, we indicate clearly that fucoidan sulfatase is produced together with fucoidanase in *Vibrio* sp. N-5. These fucoidan-degrading enzymes, fucoidanase, and fucoidan sulfatase, may be useful for the analysis of polysaccharide structure and for the potential application of fucoidan.

Acknowledgment

We wish to express our thanks to Professor Y. Takahashi, Shimonoseki University of Fisheries, for technical assistance in the identification of this bacterium, and for his valuable suggestions.

References

- 1) W. Yaphe and K. Morgan: Enzymic hydrolysis of fucoidin by *Pseudomonas atlantica* and *Pseudomonas carrageenovora*. *Nature*, **183**, 761-762 (1959).
- 2) T. Morinaga, T. Araki, M. Ito, and M. Kitamikado: A search for fucoidan degrading bacteria in coastal sea environments of Japan. *Nippon Suisan Gakkaishi*, **47**, 621-625 (1981).
- 3) S. Furukawa and T. Fujiwara: Growth and fucoidan sulfatase production in fucoidan-utilizing bacteria from sea sand. *Nippon Nogekagaku Kaishi*, **58**, 1123-1126 (1984).
- 4) T. Fujikawa: Purification method of fucoidan with barium salt. *Bull. Hijiya Wom. Jun. Coll.*, No. **24**, 151-158 (1990).
- 5) T. Fujikawa, K. Koyabu, and M. Wada: Enzymes in hepatopancreas of abalone active on fucoidan (1), crude enzyme and unabsorbed fraction on CM-cellulose. *Nippon Nogekagaku Kaishi*, **53**, 87-95 (1979).
- 6) T. Fujikawa, K. Koyabu, and M. Wada: Enzymes in hepatopancreas of abalone, active on fucoidan (2), enzyme fraction adsorbed on CM-cellulose. *Nippon Nogekagaku Kaishi*, **53**, 197-202 (1979).
- 7) M. Somogyi: Notes on sugar determination. *J. Biol. Chem.*, **95**, 19-23 (1952).
- 8) O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and J. R. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).
- 9) U. Shimizu: Taxonomy and Ecology of Marine Bacteria in "Marine Microbiology" (ed. by N. Taga), Tokyo Univ. Press., 1974 (in Japanese), pp. 45-51.
- 10) J. M. Shewan and M. Veron: Bergey's manual of determinative bacteriology, 8th ed., The Williams and Wilkins Co., Baltimore, 1974, pp. 340-345.