海洋性細菌No.9-12が生産する抗腫瘍性粘質物について

誌名	日本水産學會誌
ISSN	00215392
著者	奥谷, 康一
巻/号	43巻3号
掲載ページ	p. 323-328
発行年月	1977年3月

Bulletin of the Japanese Society of Scientific Fisheries 43 (3) 323-328 (1977)

A Viscous Antitumor Substance Obtained from a Marine Bacterium No. 9-12*1

Koichi OKUTANI*2

(Received August 16, 1976)

A marine bacterium No. 9-12, isolated from sea water, produced an extracellular viscous substance in sea water medium.

The viscous substance was precipitated from the fermentation broth with acetone, extracted with M/100 EDTA and lyophilized after dialysis against water. The substance thus obtained exhibited antitumor activity for mouse sarcoma-180 (ascites type). Upon intraperitoneal injection of 1 mg/Kg/day ×5 and 5 mg/Kg/day ×5 of this substance, the growth of mouse ascites carcinoma was inhibited 84.4% and 87.2%, respectively.

Chemical analysis showed that the substance contained 25.8 % C, 4.2% H, 6.1% N, and 27.3% ash.

The homogeneity of the crude extract was determined by column chromatography on Sephadex G-75, Sephadex G-200 and Sepharose 6B. The data suggested that the polysaccharide was separated from the protein moiety and that the polysaccharide was heterogenous.

Extracellular viscous substance produced by microorganisms are usually composed of the polysaccharides.[1-4] The polysaccharide preparations from many origin are known to be exhibit interesting biological activities such as an antitumor activity.[5-9] As antitumor polysaccharides produced by microorganisms[5,7,9] many reports are also available on the lipopolysaccharide of bacteria and the cell wall polysaccharide of the yeast. The antitumor activity of the plant polysaccharides including these microbial polysaccharides are thought to be host mediated and expected to be a new way for cancer immunochemotherapy. Studies on the polysaccharide produced by marine bacteria are carried out by the present author and in the previous paper[10,11] the author reported the antitumor polysaccharide produced by a marine *Vibrio* sp. However, the knowledge on polysaccharide produced by marine bacteria is not enough at present.

A marine bacterium, isolated from the sea water by the present author, produced a viscous substance in the sea water media and the substance was precipitated with acetone.

In the present paper, (1) the author mentioned about the method for recovering these viscous substances from the culture fluid, and about the homogeneity and antitumor activity of the substance, and (2) the author also pointed out that the viscous substance contained the polysaccharides.

---

*1 Contribution No. 5 from the Aji Marine Biological Station, Faculty of Agriculture, Kagawa University (香川大学農学部，庵治臨海実習施設業績，第5号).

*2 Faculty of Agriculture, Kagawa University, Kagawa 761-07 Japan (香川大学農学部).

Materials and methods

Bacterial strain A marine bacterium No. 9–12 was used. This bacterium was isolated from the sea water at Seto Inland Sea by modified ZoBell's 2216E media.

Isolation procedures for the viscous substance Five hundred milliliters of sea water media containing 5 per cent peptone and 1 per cent yeast extract in 1 liter of conical flask were autoclaved at 120°C for 20 minutes. The bacterium No. 9–12 which was incubated in 5 m*l* of the same media at 25°C for 2–3 days was inoculated in these 500 m*l* media and incubated statically at 25°C for 5–7 days. At the end of the incubation, the clear supernatant solution was obtained by filtration of culture media through filter paper (Toyo No. 5B) with Hyflo-super-cel.

The supernatant was added with equal volumes of acetone and mixed in the cold. After one night in the cold the precipitates were collected on filter paper (Toyo No. 5B) with Hyflo-super-cel. The precipitates were then extracted with M/100 EDTA. This EDTA solution was carried out to dialysis (Visking tube) against deionized water.

The dialysate was then filtered (Whatman GF/C) and freeze-dried. The pale brown powder was obtained as the preparation of the viscous substance. These isolation procedures were summarized in Fig. 1.

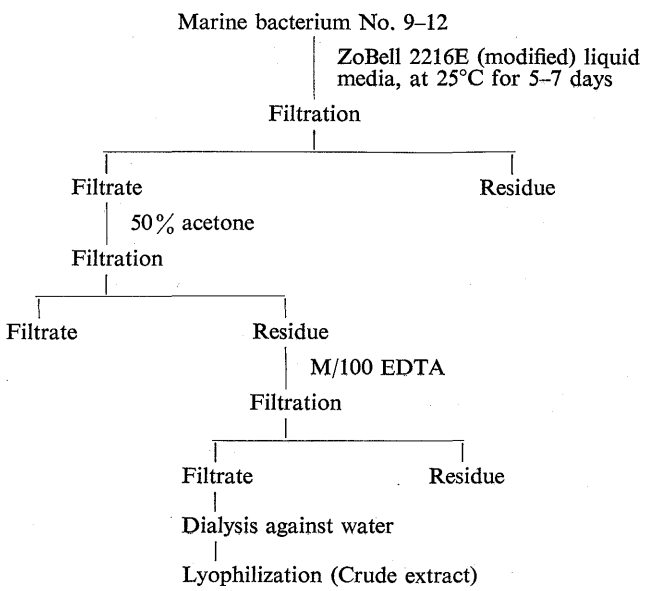

Fig. 1. Isolation procedures for an antitumor polysaccharide preparation from marine bacterium No. 9–12.

Chemical analysis The determinations of C, H, N, and ash of the sample were carried out by using Perkin-Elmer CHN corder (model 240).

Gel filtration Sephadex G–75, Sephadex G–200 and Sepharose 6B column were used.

Void volume (V_0) and total volume (V_t) of the column were determined by using Blue dextran-2000 and urocanic acid respectively.

Antitumor activity Ascites types of sarcoma-180 (S–180A), Ehrlich carcinoma (EAC) and solid type of Nakahara-Fukuoka-sarcoma (NFS) were used in the present experiments. Female mice (ddY) weighing about 20 g were used for the antitumor assay. S–180A and EAC were injected intraperitoneally, and NFS was injected subcutaneously. Samples were intraperitoneally injected dialy for 5 days after 24 hours of the tumor plantation.

Results and discussion

Bacterial culture and the isolation of the viscous substance A marine bacterium No. 9–12 grew well on sea water media and the culture fluids became viscous for 2–3 days' incubation. This viscosity reached at maximum in 5–7 days' incubationat 25°C.

As it was difficult to remove the cell from the culture fluid by usual centrifugation, the culture fluid was filtered with Hyflo-super-cel. Not only acetone but also ethanol and methanol precipitated this viscous substance from the culture filtrate. About three hundred milligrams of the crude substance were obtained from 1 liter of the culture fluid.

The crude extract was positive against phenol-sulfuric acid and Cu-FOLIN reactions.

Chemical analysis Chemical analysis of the substance were indicated as follows: C=25.8%, H=4.2%, N=6.1% and ash=27.3%.

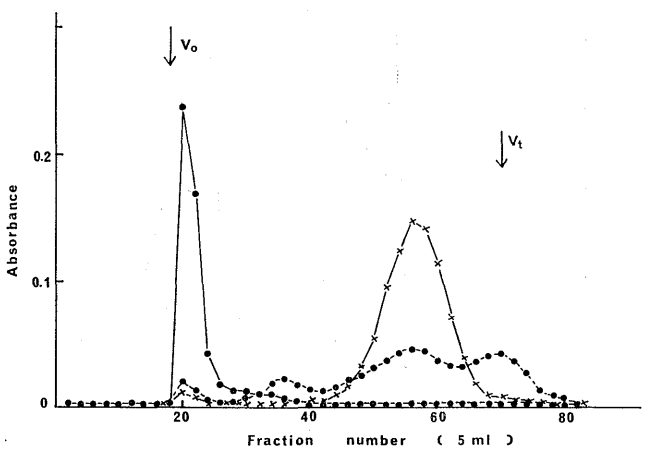

Fig. 2. Sephadex G–75 column chromatography of the crude extract

The crude extract (22.5 mg) was dissolved in 3 ml of 0.5M–NaCl solution. After filtration (Whatman GF/C), the solution was applied to the column (2.2×95.5 cm) and fractionated with 0.5M–NaCl at a flow rate of 1 ml/min. Each 5 ml fractions was collected and analyzed.

Sugar was determined by the method of phenol-sulfuric acid (—●—) at 490 mμ. Protein was determined by the absorbance at 280 mμ (---●---), and by the method of LOWRY *et al.* at 640 mμ (—×—).

Void volume (V_0) and total volume (V_t) of the column were indicated by arrows.

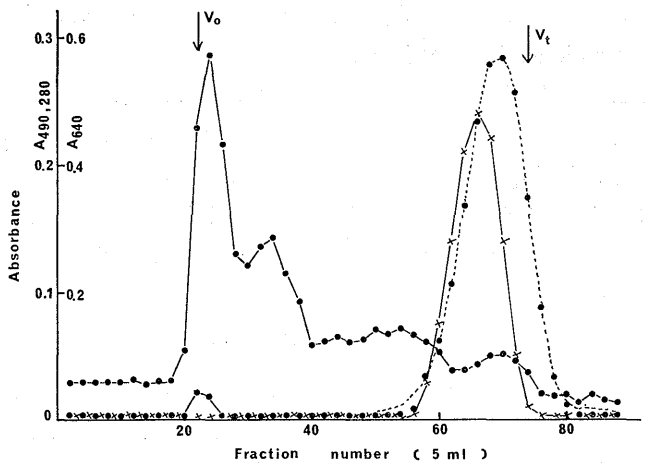

Fig. 3. Sephadex G–200 column chromatography of the crude extract.
The crude extract (50.5 mg) was dissolved in 4 ml of 0.5M–NaCl solution. After filtration (Whatman GF/C), the solution was applied to the column (2.3×92.4 cm) and fractionated with 0.5M–NaCl at a flow rate of 10 ml/hr.
Refer to the foot-note in Fig. 2 for further detail.

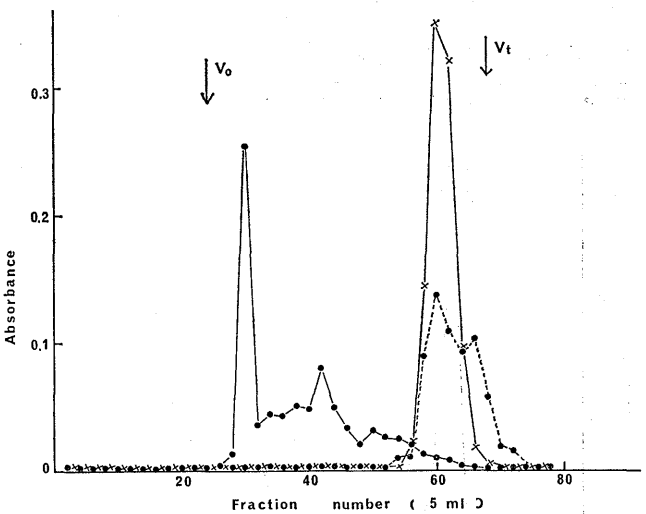

Fig. 4. Sepharose 6B column chromatography of the crude extract.
The crude extract (20.7 mg) was dissolved in 5 ml of 0.5 M–NaCl solution. After filtration (Whatman GF/C), the solution was applied to the column (2.5×82 cm) and fractionated with 0.5 M–NaCl at a flow rate of 0.6 ml/min.
Refer to the foot-note in Fig. 2 for further detail.

Gel filtration of the viscous substance The elution pattern of the column chromatography on Sephadex G–75 was shown in Fig. 2. Fig. 2 shows that the polysaccharide was eluted at V_0 and separated from the protein moiety. This result also shows that the protein moiety was relatively small molecule.

The elution pattern of the column chromatography on Sephadex G-200 was shown in Fig. 3. As shown in Fig. 3, one fraction of the polysaccharide was eluted at V_0 and other fractions of the polysaccharide were eluted at the elution volume between V_0 and V_t.

The elution pattern of the column chromatography on Sepharose 6B was shown in Fig. 4. As shown in Fig. 4, several fractions of the polysaccharide were observed in the elution volume between V_0 and V_t. The above mentioned results suggested that the crude extract was composed of the polysaccharides of wide varieties of the molecular weight and the protein moiety.

Antitumor activity The criteria for evaluation of activity are shown in Table 1. The antitumor activity of the crude extract was shown in Table 2. As shown in Table 2, the crude extract inhibited the growth of tumor cells. In the intraperitoneal injection of 1 mg/Kg/day $\times 5$ and 5 mg/Kg/day $\times 5$ of this substance the growth of mouse S-180A was inhibited at the ratio of 84.4% and 87.2% respectively. Only 25.7% inhibition was found in NFS. On the contrary, the growth of the tumor cells of EAC was rather stimulated by this substance.

The spectrum on the antitumor activity seemed to be narrow from the results obtained in the present experiments.

Table 1. Criteria for the evaluation of activity

Tumors	Activity			
	−	+	++	+++
S-180A, EAC (T/C%)	100–66	65–41	40–11	10–0
NFS (T/C%)	100–71	70–51	50–21	20–0

The tumor growth (T/C%): $\frac{\text{The mean value of treated group}}{\text{That of control}} \times 100$

Table 2. Antitumor activity of a polysaccharide preparation from marine bacterium No. 9–12.

Tumors	Drug	Dose (mg/Kg× days)	Route	Number of mice	Mean±SE.[1]	Tumor growth[2] (T/C%)	Activity
S-180A	Sample	1.0×5	ip	7	0.17±0.02	15.6	++
		5.0×5	ip	7	0.14±0.05	12.8	++
	Control	×5	ip	10	1.09±0.17	*	*
EAC	Sample	1.0×5	ip	7	1.37±0.23	138.4	—
		5.0×5	ip	7	1.87±0.16	188.9	—
	Control	×5	ip	10	0.99±0.10	*	*
NFS	Sample	5.0×5	ip	10	1.02±0.21	145.7	—
		10.0×5	ip	10	0.52±0.31	74.3	——
	Control	×5	ip	10	0.70±0.18	*	*

1) S-180A and EAC: Total packed cell volume. NFS: Tumor weight.

2) The tumor growth T/C%: $\frac{\text{The mean value of treated group}}{\text{That of control}} \times 100$

A more detail understanding of the antitumor activity awaits the purification of the substance. Work on the purification is in progress.

The bacterium No. 9–12 is thought to be a genus *Pseudomonas* but the bacteriological characteristics will be shown in the future paper.

References

1) K. Fukagawa, H. Yamaguchi, D. Yonezawa, and S. Murao: *Agr. Biol. Chem.*, **38**, 29–35 (1974).
2) E. Ninomiya, T. Kizaki, and K. Hanada: *Nippon Nogeikagaku Kaishi*; **42**, 178–184 (1968).
3) S. Omata, T. Sakai, Y. Ichihara, and M. Yoshioka: *J. Ferment. Technol.*, **53**, 786–792 (1975).
4) S. Murao, N. Morita, and Y. Takahara: *J. Ferment. Technol.*, **51**, 653–660 (1973).
5) H. Hojo, M. Uchiyama, and S. Suzuki: *Yakugaku Zasshi*, **93**, 947–950 (1973).
6) G. Chihara, Y. Maeda, J. Hamuro, T. Sasaki, and F. Fukuoka: *Nature*, **222**, 687–688 (1969).
7) N. Kumano, K. Kurita, and S. Oka: *Gann*, **64**, 529–533 (1973).
8) Y. Maeda, K. Ishimura, and G. Chihara: *Protein, nucleic qcid and Enzyme*: **21**, 425–435 (1976).
9) Y. Nishikawa: *Pharmacia*, **8**, 543–548 (1974).
10) K. Okutani: *Tech. Bull. Fac. Agr. Kagawa Univ.*, **26**, 75–78 (1974).
11) K. Okutani: This Bull., **42**, 367–370 (1976).