MARINACTAN, ANTITUMOR POLYSACCHARIDE PRODUCED BY MARINE BACTERIA

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Extracellular polysaccharides of marine bacteria were screened for their antitumor activity against sarcoma-180 solid tumor in mice.

An active polysaccharide was purified and named marinactan. The producing microorganism has a typical marine bacterial nature requiring sea water for growth and was identified as *Flavobacterium uliginosum*. Marinactan is a novel heteroglycan consisting of glucose, mannose and fucose in a ratio of approximately 7 : 2 : 1. Marinactan, 10~50 mg/kg daily for 10 days i.p., produced 70~90% inhibition of the growth of solid sarcoma 180. Complete regression of the tumor was observed in some treated mice. Its administrations before and after tumor transplantation showed almost the same inhibitory effect. Marinactan prolonged markedly the survival period of mice bearing ascites sarcoma 180.

Microorganisms of marine origin[1~3] as well as marine organisms[4,5] produce varieties of interesting biologically active compounds which have never been found in products of terrestrial organisms. It has been known that some marine organisms produce polymeric materials such as agar or other slimy materials. Agar-liquefying microorganisms are found frequently in the marine environment, but rarely in terrestrial areas. These facts suggest that the marine environment may be a significant source of organisms which synthesize and degrade such polymeric materials as polysaccharides. We were therefore stimulated to investigate bioactive polysaccharides produced by marine bacteria.

As the result of our screening 1,083 marine isolates, 167 isolates were found to produce significant amounts of extracellular polymeric materials which were easily precipitated by the addition of alcohol to fermented broths after separation of the cells. These precipitates were dissolved in saline and injected intraperitoneally into mice bearing sarcoma 180 solid tumors. Six of 100 precipitates exhibited remarkable suppressive or completely regressive effects on the tumor. The producers were unique in their requirement of sea water for growth. One of the above producers was identified as *Flavobacterium uliginosum* and the antitumor principle was purified and named marinactan.

In this paper, we report identification of the producing organism, fermentation and isolation processes, and the antitumor activity of marinactan.

Isolation of Marine Bacteria and Screening for Antitumor Polysaccharide

Marine bacteria were isolated from sea water, sea mud and sea weed by incubation and colony formation on an agar medium containing 0.6% glucose, 0.5% polypeptone and 0.1% yeast extract in artificial sea water (Jamarin S, Jamarin Laboratory, Osaka). The collected sea water was filtered using a membrane filter. Sea mud of particles of sea water on the filter was suspended in Jamarin S, stirred

vigorously and then let stand for several minutes. Sea weed was cut into small pieces with scissors and suspended in Jamarin S. The suspension was gently homogenized with a teflon-glass homogenizer and then let stand for several minutes. The upper part of these suspensions was diluted with Jamarin S. The diluted suspension of the samples were plated onto the agar medium and incubated at 27°C for 2~3 days.

Isolated colonies picked from the above cultured agar medium were cultured in 100 ml of liquid medium consisting of the same ingradients as above at 27°C for 2~3 days in 500 ml flasks on a reciprocal shaker (7-cm amplitude, 120 rpm) The cultured broths were centrifuged and 1.5 volumes of ethanol was added to the resulting supernatant to precipitate extracellular polysaccharide materials. A significant amount of polysaccharide was noted in 167 strains out of 1,083 isolates. The polysaccharide materials thus obtained were dissolved in saline and each aqueous preparation was administered intraperitoneally to ICR mice daily in doses of 20 and 100 mg/kg for 10 days starting at the day following the subcutaneous inoculation of 3×10^6 sarcoma 180 cells. At the 5th week after the tumor cell inoculation, the tumor inhibitory activity was determined by measuring the tumor weight in 5 mice. Remarkable antitumor activity was observed in 6 out of 100 samples tested. One active principle of strain MP-55 was purified and named marinactan.

Marinactan-producing Organism

The bacterial strain MP-55 was isolated from the homogenates of a sea weed (*Ishige foliacea* like) collected at Odawa Bay, Kanagawa Prefecture, Japan.

The taxonomical characteristics of strain MP-55 are described in Tables 1~3. On the basis of the morphological, cultural and physiological characteristics, strain MP-55 was determined to belong to the genus Flavobacterium using BERGEY's Manual of Determinative Bacteriology, 8th edition as reference. A comparative study of the strain and known species most similar to it was made. The strain was most identical to *F. uliginosum* in respect to: color tone of colony, no growth at 37°C, no high salt resistance, liquefaction of gelatin, no growth on a medium without sea water, reduction of nitrates, and production of acids from glucose, sucrose and maltose. On the other hand, they were different from each other with respect to production of acid from lactose and a somewhat different DNA-GC content. Since no key has been recognized in respect to the above differences for speciation within genus Flavobacterium, we concluded that the strain MP-55 is not a new species, but belongs to *F. uliginosum* ZoBell and Upham 1944.

Table 1. Morphological characteristics of the strain MP-55.

Form and size of the cell	Rod, 0.2 to 0.3 μm× 0.6 to 0.9 μm
Pleomorphism	Non-pleomorphic
Motility	Non-motile
Sporogenicity	Non-sporulating
Gram staining	Negative
Acid-fastness	Negative

Table 2. Cultural characteristics of the strain MP-55.

Bouillon agar plate culture	No growth
Marine agar 2216 medium (Difco) plate culture	Medium growth, circular, flat to semilenticular, entire, smooth, glistening, translucent, mucoid, and yellow to yellow orange
Bouillon agar slant culture	No growth
Marine agar 2216 slant culture	Moderate growth, membraneous, thread-like, glistening, and yellow to orange
Bouillon liquid culture	No growth
Marine 2216 medium liquid culture	Slightly turbid, and no membrane is formed on the surface
Bouillon gelatin stab culture	No changes
Marine 2216 medium gelatin stab culture	Liquefaction
Litmus milk	No changes

Table 3. Physiological characteristics of the strain MP-55.

Reduction of nitrates	Positive
Denitrification	Negative
Methyl-red test	Negative
Voges-Proskauer test	Negative
Production of indole	Negative
Production of hydrogen sulfide	Negative
Utilization of citric acid	Negative on a Koser medium and negative on a Christensen medium
Utilization of inorganic nitrogen source	Utilize nitrates and ammonium salts
Production of dye	No formation of water-soluble dye
Urease	Negative
Oxidase	Positive
Catalase	Positive
Growth ranges: Temperature	Good growth at 8 to 35°C
Optimum temperature	24 to 30°C; and no growth at 37°C
pH	6 to 9
Attitude toward oxygen	Aerobic
O-F test (according to the Hugh and Leifson Method)	No acid is formed
Acid formation from carbohydrates (basic culture composition: 0.5% peptone, 2% NaCl, 1% $MgCl_2$, 0.2% $CaCl_2$, 0.1% KCl and 0.02% Bromocresol purple)	Positive without gas formation; D-glucose, D-xylose, maltose, sucrose, cellobiose and rhamnose. Negative without gas formation; L-arabinose, D-mannose, D-fructose, D-galactose, lactose, trehalose, D-sorbitol, D-mannitol, inositol, glycerin, starch, raffinose and D-ribose
Salt tolerance	Growth on Marine 2216 medium with addition of 2% NaCl, but no growth with addition of 4% NaCl
Nutrient requirement	No growth occurs unless sea water is added
DNA-GC content	36.3% (according to the Tm method)

Fermentation and Isolation of Marinactan

The medium employed for fermentation of marinactan was composed of same ingradients which were described above. The pH of the medium was adjusted to 7.2 before sterilization. Strain MP-55 was incubated at 27°C for 24 hours in 100 ml medium containing in a 500-ml flask on a reciprocal shaker (7-cm amplitude, 120 rpm). Four ml portions of the growth in the above broth was transferred to 500-ml flasks containing 100 ml of the medium and shaken as described previously. The production of polysaccharides generally reached a maximum after 2~3 days.

Fermentation studies were also carried out in a tank fermentor. A 12-liter portion of the seed culture of strain MP-55 in a 20-liter jar fermentor was inoculated to 300-liters of the medium in a 500-liter tank fermentor which was operated at 27°C with aeration of 150 liters/minute and agitation of 300 rpm. The broth pH decreased slightly at the beginning of cultivation and then increased gradually to reach pH 7.5~8.3. The peak production of polysaccharides was obtained after 18~22 hours' cultivation.

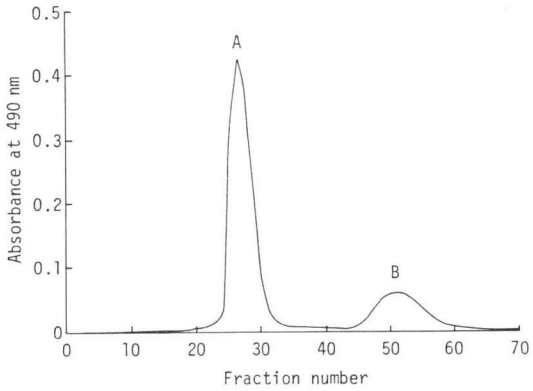
Harvested broth was centrifuged at 7,000×g for 15 minutes. To the supernatant, an equal volume of ethanol was added with vigorous stirring. The precipitate thus obtained was collected by centrifugation and extracted with hot water using 1/10~1/15 the broth volume, followed by centrifugal separation of the insoluble portion. The extraction procedure was repeated using half the volume of hot water of

the first extraction on the insoluble fraction. The combined supernatant thus obtained was dialyzed overnight against running water and then, 1.5 volumes of ethanol was added to the dialyzed liquid. The precipitate was dried under reduced pressure to give 400~500 mg of crude preparation (preparation I) from one liter of centrifuged broth.

In order to remove protein impurities contained in preparation I, 1/3 volume of chloroform and 1/30 volume of *n*-butanol were added to a 0.2% aqueous solution of preparation I. The mixture was vigorously stirred for 30 minutes and subjected to centrifugal separation at 3,000 rpm for 15 minutes. The above procedure was repeated several times to finally yield partially purified preparation II.

Fig. 1. Chromatography of partially purified marinactan,

Preparation II (2 mg) was dissolved in 1 ml of 0.5 M NaCl and applied to a Sepharose CL-4B column (2×85 cm). The column was eluted with 0.5 M NaCl and fractions (3 ml) were collected at a flow rate of 7 ml/hour. Total sugar was determined by phenol-sulfuric acid method at 490 nm.



Preparation II was chromatographed on a column of Sepharose CL-4B to give an elution pattern consisting of two peaks (A & B) of polysaccharide fractions, as shown in Fig. 1. The peak A portion showed antitumor activity, while the peak B portion contained mannan and did not show any antitumor activity. The A portion was dialyzed and precipitated by adding 1.5 volumes of ethanol. The resulting precipitate was dried under reduced pressure to give a purified preparation III of marinactan. This preparation (50 mg/kg/day) produced 79% inhibition of the growth of solid sarcoma 180.

Physicochemical Properties

Marinactan (preparation III) was soluble in water but insoluble in most organic solvents such as dimethylsulfoxide, ethanol, chloroform and ether, and behaved as a neutral substance. The color reaction was positive with phenol-sulfuric acid, anthrone and orcinol-sulfuric acid, but negative with ninhydrin. It is hygroscopic and the water content varied depending on the humidity. One example of elemental analysis of marinactan was: C 37.43%, H 5.61%, ash 5.53%, and water content 16.3%. The sugar content (calculated as glucose) determined by the phenol-sulfuric acid method was 75~84% and protein content determined by LOWRY method[6] was 0~0.4%. The ultraviolet absorption spectrum showed no absorption maximum. The infrared absorption spectrum (KBr tablet) is shown in Fig. 2. The optical rotation was $[\alpha]_{589}^{22}$ +69° (*c* 0.13, H_2O).

When gel filtration of marinactan was carried out on a column of Sepharose CL-4B, only one peak was observed as shown in Fig. 3 with indication of a molecular weight of greater than 1,000,000.

Density gradient centrifugation with CsCl was performed as shown in Fig. 4. It exhibited a single and almost symmetrical peak which was calculated to be approximately 1.65 g/cm^3.

The sugar composition of marinactan (preparation III) was studied as follows. It was treated with 5% HCl-methanol at 90°C for 20 hours. The reaction mixture was neutralized by adding silver carbonate and the resulting insoluble material was removed. The solution was dried under reduced pressure and trimethylsilylated for gas chromatography. The results of gas chromatographic analysis using standard samples as references showed that marinactan was composed of glucose, mannose and fucose

Fig. 2. Infrared spectrum of marinactan (KBr).

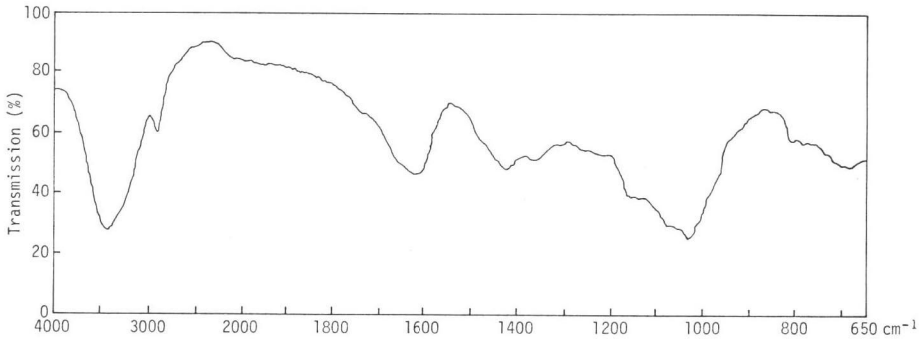


Fig. 3. Elution pattern of marinactan on Sepharose CL-4B chromatography.

Preparation III (2 mg) was dissolved in 1 ml of 0.5 N NaCl, applied to the column (1.6×80 cm) and fractionated with 0.5 N NaCl at a flow rate of 7 ml/hour.

Total sugar was determined by phenol-sulfuric acid method at 490 nm.

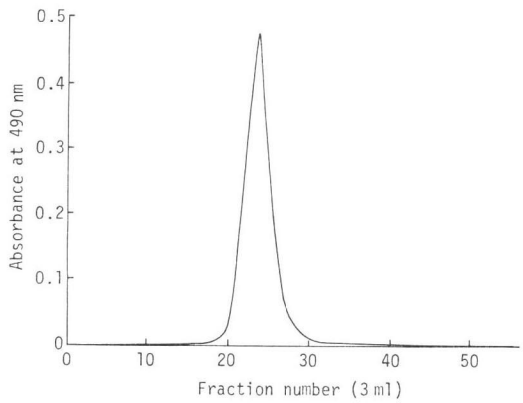
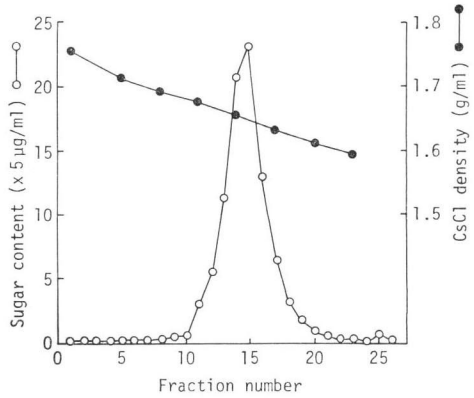


Fig. 4. CsCl density gradient centrifugation of marinactan.

268 μg of marinactan was centrifuged in 6.4 ml of 53.5% (w/w) CsCl in 6 mM tris-HCl buffer pH 8.0 at 120,000×g, 20°C for 70 hours. The sugar content of fractions in the centrifuge tube was analyzed by the phenol-sulfuric acid method.



in the following ratio (approximately 7: 2: 1): glucose 69.5±0.9%, mannose 19.6±1.4%, fucose 10.9±0.9%.

Further details of the physiological properties and structural studies of marinactan will be reported elsewhere.

Antitumor Activity

The marinactan preparations obtained by the procedure described above were examined for their antitumor activity against sarcoma 180 solid tumor in mice. As shown in Table 4, the intraperitoneal administration of each preparation showed remarkable antitumor activity. The activity of purified preparation III was almost the same as the activity of partially purified preparation II which consists of about 80% marinactan and a small amount of inactive B as shown in Fig. 1.

The prophylactic effect of marinactan against solid sarcoma 180 and the life prolongation effect on mice bearing ascites sarcoma 180 were examined using preparation II. As shown in Fig. 5, daily administration of marinactan for 10 days starting 10 days before the tumor transplantation inhibited growth of

Table 4. Antitumor activity of polysaccharide preparations from *F. uliginosum* MP-55.

Marinactan	Dose (mg/kg×day)	Number of mice	Inhibition ratio (%)	Complete regression
Preparation I	10×10	5	90	0 / 3
	20×10	7	86	4 / 7
	50×10	7	86	1 / 7
Preparation II	10×10	7	79	3 / 7
	20×10	7	90	3 / 7
	50×10	7	77	3 / 7
Preparation III	50×10	7	79	1 / 7

ICR/CRJ mice were transplanted s.c. with 3×10^6 cells of sarcoma 180. Samples were administered i.p. once a day from the next day after the tumor transplantation. Tumor weight was estimated at 5th week.

Fig. 5. The effect of marinactan on sarcoma 180 solid tumor in mice.

ICR/CRJ mice were inoculated s.c. with sarcoma 180 tumor (3×10^6 cells). Marinactan (preparation II) of 20 mg/kg daily was started to inject i.p. at 10 days before tumor transplantation (□) and at the next day after tumor transplantation (○). Tumor size was expressed by multiplication of the largest diameter and the smallest diameter of the tumor.

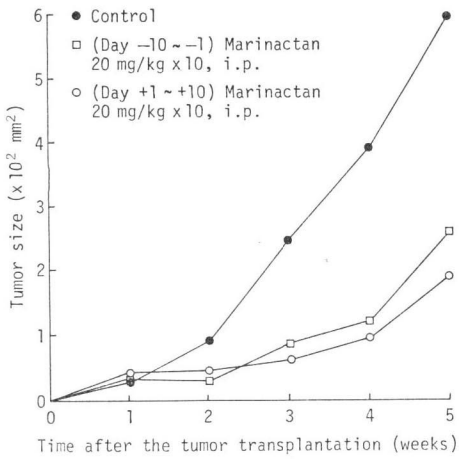
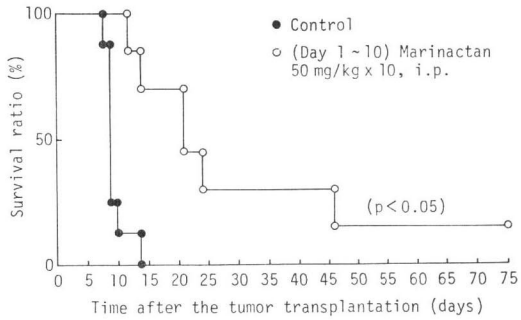


Fig. 6. Life prolongation effect of marinactan on sarcoma 180 ascites tumor bearing mice.

ICR/CRJ mice were inoculated i.p. with sarcoma 180 tumor (1×10^6 cells). Marinactan (preparation II) of 50 mg/kg, daily was started to inject i.p. at the next day after tumor transplantation.



the solid tumor to the same extent as administration starting the day after tumor transplantation. The survival period of mice bearing sarcoma 180 ascites tumor was markedly prolonged by administration of preparation II as shown in Fig. 6.

Discussion

Marine bacteria produced extracellular polysaccharides as expected, and some of the polysaccharides had significant antitumor activity.

F. uliginosum MP-55 which produced marinactan required sea water for its growth, suggesting that it was native in the marine environment.

Marinactan has been obtained as a water-soluble white powder showing a single peak by gel filtration with Sepharose CL-4B or in a density gradient centrifugation with CsCl. There have not been previously reported microbial polysaccharides composed of glucose, mannose and fucose and therefore, marinactan is a novel heteroglycan.

There have been many reports in recent years of antitumor polysaccharides obtained from microorganisms. Lentinan[7], schizophyllan[8], PS-K[9] and KS-2[10] which are obtained from basidiomycetes consist of glucose except for mannose in KS-2. A polysaccharide of yeast[11] is composed of mannose

and TC-13[12], obtained from actinomycetes, consists of glucose and mannose. Except for schizophyllan, they all are intracellular polysaccharides and are obtained by extraction of the microbial cell mass. On the other hand, the antitumor polysaccharides of *Alcaligenes faecalis* var. *myxogenes*[13] and *Serratia marcescens*[14] are known to be produced extracellularly, as well as schizophyllan. The polysaccharide of Alcaligenes is composed only of glucose and is insoluble in water. The polysaccharide of Serratia is composed of mannose only.

Marinactan shows a marked antitumor effect against sarcoma 180 in both solid and ascites form. The administration of marinactan before as well as after tumor transplantation resulted in antitumor activity. Marinactan (100 μg/ml) showed no inhibition of growth of tumor cells *in vitro*. Thus, marinactan should be a biological response modifier. Marinactan has low acute toxicity; 100 mg/kg intraperitoneally or subcutaneously does not cause death of mice. The effects of marinactan on the mouse immunological system and its effect on bacterial infection in mice will be reported in a forthcoming paper.

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