Antitumor Activity of Protein-Polysaccharides Produced from *Vibrio anguillarum*

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ABSTRACT—**The antitumor activity of protein-polysaccharide produced by a strain, *Vibrio anguillarum*, No. 17 isolated from sea water was investigated.**

The extracellular protein-polysaccharide used in this experiment was obtained through the cultivation of *Vibrio anguillarum* No. 17 at 25°C for 5-7 days in the sea water medium containing 0.5% peptone and 0.1% yeast extract.

The compositional monosaccharides of protein-polysaccharide were xylose, mannose, galactose, glucose and fructose in order and its major amino acids were glutamic acid, serine and aspartic acid.

The antitumor activity of the protein-polysaccharide at a dose of 0.5mg/kg/day or 5mg/kg/day against Sarcoma-180 in mice were 20.9% and 43.9%, respectively.

Keywords □ Antitumor activity, Protein-polysaccharide, *Vibrio anguillarum*

Recently, investigations into the chemistry and biological activity of marine products have been intensively studied in the world. From the great varieties of marine organisms, it might be expected that new chemical compounds with useful biological activity could be isolated from them, some of those compounds have been tested in various biological screening system from which many compounds found to possess interesting biological activities such as antitumor,[1-5] antibiotics,[6-9] cardiovascula[10-12] insecticides[2-4] activities, etc.

Particalarly marine organisms have proved to be good sources for antitumor compound which containes polysaccharides. The polysaccharides also has been isolated from marine microorganism.[1, 4-7, 13-15]

Although the number of known as microorganism antitumor compounds has increased dramatically in the formation on the specific mechanisms by which they inhibit tumor.

In this papers, we were prepared to polysaccharides from marine *Vibrio* sp. and extracts was tested for antitumor activity against sarcoma-180 implanted in ICR strain mice.

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MATERIALS AND METHODS

Bacterial strain—A marine bacterium, strain, No. 17, were isolated from sea water at kwang An beach by modified Zobell's media and then identified by the method of Bain and Shewan[16], Buchanan *et al.*[17] and Hayes, *et al.*.[19]

For identification of *Vibrio* sp., we were compared with *Vibrio anguillarum*.

Animal and tumors—Female mice of ICR strain and sarcoma 180 was also supplied by the college of pharmacy, Seoul National University.

Extraction a protein-polysaccharides—Those procedure were performed by the method of Oku-

tani.[15] The *Vibrio anguillarum* No. 17 which was incubated in 5m*l* of the sea water media containing 5.0% peptone, 1.0% yeast extract at 25 °C for 2-3days was inoculated in these 500m*l* media and incubated statically at 25 °C for 5-7days. After incubation, the clear supernatant was obtained by filteration of culture media through filter paper(Toyo No. 5B) with Hyfor-super-cel.

The supernatant was added with equal volumes of acetone and mixed at 4 °C for one night. After extraction, precipitates were collected at 4 °C on the filter paper(Toyo No. 5B) with Hyfol-super-cel. The precipitates were then extracted with 1/100M-EDTA. This EDTA solution was carried out to dialysis (Vasking tube) against deionized water. The dialysate was then filtered(whatman GF/C) and dried by lysophilization.

Inhibition ratio against Sarcoma 180—Sarcoma-180 cells (1.0×10^7/m*l*) obtained from ascites fluid were subcutaneouly inoculated into the right flank of ICR female mice of 25 ± 1g. A protein-polysaccharide fraction (0.5mg/kg or 5mg/kg) was injected intratumorally for 10 consecutive days starting 24hrs after tumor transplantation. Physiological saline was used as the control.

Antitumor activity of a protein-polysaccharides sample was evaluated by suppressive effect of tumor size during the observation of 26 days and also by tumor weight at the end of the experiment. All the surivors were sacrificed on the 26 days or the experiment and solid tumors were excised and weighted. Inhibition(I.R.) was calculated as an index of antitumor activity according to the following formula

$$\text{I.R. (\%)} = \text{Cw-Tw} / \text{Cw} \times 100$$

Where in Cw is average tumor weight of the control group and Tw is that of the treated group.

Polysaccharides analysis—The polysaccharide content was determined by anthrone test the monosaccharide content of a protein-polysaccharide fraction was determined according to the methods of Mitrucka[19] with some modification. The dried sample(20mg) in the ampoule was mixed with 3.0% HCl-MeOH. The ampoule was sealed after replacement with nitrogen gas and then heated at 85 °C during 20hr for methanolysis. The hydrolyzate was neutralysed with $AgCO_3$ and filtered(Toyo No. 5B). The filterate was evaporated with rotary evaporator and then the sample was dried on P_2O_5 in vacuo. The dried sample was mixed with 1m*l* of TMS reagent(The supernatant of the mixture of pyridine 10m*l*, hexamethyldisilazand 4m*l* and trimethylchlorosilane 2m*l*) and then was heated at 70-80 °C for 3min. The supernatant of the reaction mixture was subjected to the gas-chromatography(Table 1). Several monosaccharides of the extract were identified by comparison with retention times and pick areas with those of standard monosaccharides.

Table 1. Conditions of gas chromatography.

Model	Shimadzu RPR-G1	
Column	3% OV-1	130 °C-180 °C
Temperature	column	rate ; 3 °C/min
	injection	250 °C
Flow rate	air	0.6kg/cm^2
	hydrogen	0.8kg/cm^2
	nitrogen	50m*l*/min
Sensitivity	attenuate	3
	range	2
Detector	FID	

Amino acid analysis—Amino acids compositions carried out according to method of Spackmann *et al.*.[20] After the hydrolysis of the protein with 6N-HCl, amino acid were analyzed with amino acid analyzer model JLC-6AH No. 310.

RESULTS AND DISCUSSION

Morphological and physiological characteristics—Among the 8 colonies isolated from sea water were identified to the *Vibrio anguillarum* No. 17 according to their morphological and physiological characteristics. But the test of them couldn't be identified. The morphological and cultural characteristics of strain, No. 17 was shown in Table 2. The cell had shown slightly curved, short rods or straight and motility of a

Table 2. Morphological and cultural characteristics of strain No. 17.

Morphology of sell
Slightly curved, short rods or straight, 1.0-3.5 m.
Motile with a single polar flagellum.
Asporogeneous and gram negative.
Growth on agar
Abundant growth, flat, smooth, entire, opague.
Pigment not produced.
Growth on liquid media
Uniformly turbid, pellicle. Pigment not produced.

Culture were incubated in sea water containing 0.5% peptone, 0.5% yeast extract.

single polar flagellum. Gram negative of these strains were grown well onto agar medium at 25-30 °C and pigment were not produced onto agar slants, liquid media. Physiological and characteristic of strain, No. 17 was shown in Table 3. This strain were also hydrolyzed both of starch, chitin and gelatin, and reduction, glycerol and mannitol were assimilated 1.0% carbohydrate and also ultilized glutamate and arginine by the strains, No. 17. The strain, No. 17 was compared with *Vibrio anguillarum*. On the basis of the morphological, cultural and physiological characteristics, the strain, No. 17 indicated that it belongs to the *Vibrio anguillarum*.

Chemical properties of protein-polysaccharide fraction(PPF)—To determine the compositions of monosaccharide and amino acids, quantitative analysis of polysaccharide were carried out with above described method. The polysaccharide was 8.13%. The monosaccharides of were found to xylose, mannose, galactose, glucose and fructose was 61.39, 14.51, 11.53, 9.30% and 3.27%, respectively (Table 4). There have been many reports in recent years of antitumors polysaccharides obtained from microorganism.

Letinan[20], schizophyllan[22], PS-K[23] and KS-2[14]. Which are obtained from basidiomycetes consist of glucose except for mannose in KS-2. A polysaccharide of yeast[24] is composed of mannose and Tc-13, obtained from actinomycetes, consist of glucose and mannose.

Table 3. Physiological characteristics of strain No. 17 which compared with *Vibrio anguillarum*.

	strain No. 17	Vibrio anguillarum
Hydrolysis of starch	+	+
Hydrolysis of chitin	+	+
Hydrolysis of gelatin	+	-
Reduction of nitrite	+	+
Production of indole	+	+
Production of catalase	+	+
Production of hydrogen sulfide	-	-
Optimum temperature for growth	25-27 °C	25-27 °C
Gas from D-glucose	-	-
Utilization of:		
Sucrose	+	+
Maltose	+	+
Lactose	-	-
Dextrin	+	+
Cellulose	-	-
Glucose	+	+
Galactose	-	-
Furactose	+	+
Mannose	+	+
Arabinose	+	+
Xylose	-	-
Raffinose	-	-
Melibiose	-	+
Glycerol	+	+
Mannitol	+	+
Sorbitol	+	+
Glutamate	+	+
Arginine	+	-
Ornithine	-	-
Sodium citrate	+	+
ONPG hydrolysis	+	+

Culture were incubated in 0.5% peptone sea water containing BCP as a pH indicator and 1.0% of the carbohydrate, using Durham tubes for the detection of gas.

Okutani[12] reported that the polysaccharide of *Vibrio* sp. contained 11.2% carbohydrate as mannose. These results indicated that polysaccharide of *Vibrio anguillarum* No. 17 were composed of

Table 4. Polysaccharide and monosaccharide contents of *Vibrio anguillarum* No. 17.

Polysaccharide (%)	8.13
Monosaccharide(%)	
Glucose	9.30
Galactose	11.53
Mannose	14.51
Xylose	61.39
Fructose	3.27

widely varying monosaccharide.

On the other hand, *Vibrio anguillarum* No. 17 was composed of 15 amino acids which contained glutamic acid, serine, aspartic acid and isoleucine as major amino acids(Table 5). The ratio of the acidic amino acids was high, whereas that of the basic amino acids was low and sulfur containing amino acid was cysteine.

Antitumor test of PPF—The antitumor activity of the PPF was recognized in vivo by the suppression of the tumor growth against Sarcoma 180 in ICR mice. In the intraperitional injections of 0.5 mg/kg/day and 5mg/kg/day of this substance were inhibited at the ratio of 20.9% and 43.9%, respectively(Table 6).

The prolongation of life span of the PPF treated group indicated that PPF exerted a high antitumor effect and a low toxidity. All of the mice survived at the end of the observation periods. However, all of the treated mice showed since ill symptoms. Okutani[12] was indicated that polysaccharide of *Vibrio* sp. cured tumor completely in 33% of mice at 100mg/kg.

Table 6. Antitumor activities of *Vibrio anguillarum* No. 17.

	Average tumor weight(g)	Inhibition ratio(%)	Complete regression
Control (saline)	4.81 ± 1.25	—	6/0
0.5mg/kg	3.98 ± 1.67	20.9	6/0
5mg/kg	2.70 ± 1.12	43.9	6/0

Table 5. Amino acid composition of *Vibrio anguillarum* No. 17.

Amino acid	Content(%)
Asp	11.40
Thr	4.80
Ser	11.46
Glu	31.45
Gly	2.89
Ala	3.71
Cys	2.34
Val	4.42
Met	3.24
Ile	6.35
Leu	4.17
Phe	5.23
Lys	2.35
His	0.73
Pro	5.48

Viscous antitumor substance from a marine bacterium was inhibited ranging from 84.4% to 87.2% against growth of mouse ascites carcinoma[15].

It was considered that antitumor effects of PPF were similar to those of indicated antitumor components of some reports[1,25].

In conclusion, polysaccharide is a heteroglycan consisting of glucose, mannose, galactose, fructose and xylose, and major amino acid were glutamic acid, serine and aspartic acid. The polysaccharide of *Vibrio anguillarum*, No. 17 shows a antitumor activity effect against Sarcoma-180 in ascite form.

We couldn't discussed about amino acid compositions, because data of amino acid compositions in PPF was not found in another marine microorganism.

국문요약

해수로부터 분리 확인한 비브리오균에서 생산하는 단백 다당체의 항종양 활성에 대하여 조사하였다.

비브리오균을 0.5%-peptone와, 0.5% yeast extract가 함유된 해수를 기질로 배양하여 단백 다당체를 얻었다. 단백 다당

체의 단당류의 함량은 xylose, mannose, galactose, glucose 및 fructose의 순서이고, 주요 아미노산은 glutamic acid, serine 및 aspartic acid였다.

단백 다당체의 항종양 활성을 Sarcoma-180에 대하여 0.5mg/kg/day 및 5mg/kg/day을 쥐에 주사했던 바 20.9% 및 43.9%의 억제 효과가 있었다.

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