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Isolation and Antitumor Activity of a New Rhamnose Polysaccharide, Clostrhamnan from *Clostridium saccharoperbutylacetonicum*

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A new rhamnose polysaccharide was isolated from the hot water-extract of *Clostridium saccharoperbutylacetonicum* cells. This wall-associated polysaccharide, Clostrhamnan purified by affinity chromatography with Con A-Sepharose and gel filtration with Sephadex G-75, contained chemically similar two components, except for molecular weight (16,000 and 15,000). Both of components were consisted of 95 % rhamnose and 5 % glucose, and immunologically showed antitumor activity on sarcoma-180.

INTRODUCTION

Several fungal polysaccharides were reported as antitumor substance with an immunological potentiation activity. Particularly PS-K and Lentinan were studied in details (Chihara and Maeda, 1969; Chihara et *al*, 1970; Hirase *et al, 1976;* Abe *et al, 1978).* We reported previously extracellular polysaccharide produced by *Coriolus* sp. No. 20 and hot water-extract from C. *saccharoperbutylacetonicum* showed antitumor activity (Hayashida and Watanabe, 1983 a, b). We obtained a rhamnose-polymer fraction with antitumor activity on sarcoma-180 from the hot water-extract of *C. saccharoperbutylacetonicum*. This paper describes on the isolation and purification of rhamnose polysaccharide and its antitumor activity.

MATERIALS AND METHODS

Materials.

Water soluble fraction of hot water-extract of C. *saccharoperbutylacetonicum* was used for the preparation of polysaccharide. C. *saccharoperbutylacetonicum* was cultured in a modified TYA medium (Hayashida and Watanabe, 1983b) at 30°C for about 60 hr, and the harvested cells were collected and washed 3 times with cold deionized water before extraction. The washed cells were added to 750ml of deionized waler containing 1.5 ml of 40 % formaldehyde solution, and stirred for 30 min at 70°C. The hot water-extract was precipitated with 85 % ethanol, and the precipitate was dialyzed against running tap water and deionized water successively, and finally lyophilized. Water soluble fraction was prepared by centrifuging the mixure of 1 g hot water-extract and

100 ml deionized water.

Purification of polysaccharide.

The water soluble fraction of hot water-extract was dissolved in 0.05 M phosphate buffer of pH **7.0** and applied to a DEAE-Sephadex A-50 column (2.4 x35 cm) equilibrated with the same buffer. This column was washed with 300ml of the buffer, and the unadsorbed part was concentrated, then placed on a Sephadex G-50 column (2.8x90 cm). Elution was performed with deionized water and the elute was collected in 5ml fraction. The 30th to 60th fractions were combined and lyophilized (F-l). Lyophilized F-l was dissolved in 0.15 M phosphate buffered saline (PBS), pH 6.8, containing 0.1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$. The solution was applied to a column (3.0×25cm) of Con A-Sepharose previously equilibrated with 0.15 M PBS, and eluted with the same buffer (Ohtani et al, 1980). Unadsorbed part was concentrated and applied to a column (2.8 x90 cm) of Sephadex G-75. Elution was performed with deionized water. The purified preparation thus obtained was lyophilized. Detection of carbohydrates was generally carried out according to anthrone-sulfuric acid method (Koehler, 1952) and phenol-sulfuric acid method (Dubois, 1956).

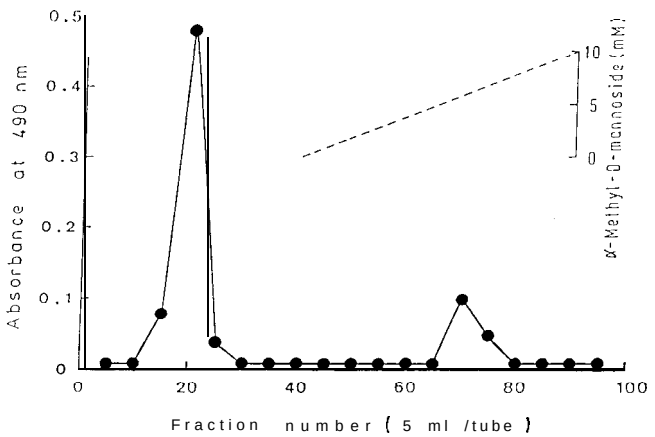


Fig. 1. Elution profile of Con A-Sepharose column (3.0 × 25 cm) chromatography of the F-l.
●, Absorbance at 490 nm. Elution was performed with 0.15 M phosphate buffered saline containing 0.1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$. Each fraction was 5 ml. Unadsorbed part was collected from 10th to 25th fractions and used for further study.

General methods.

Gas-liquid chromatography (GLC) was performed with a Hitachi 163 gas chromatograph fitted with a flame ionization detector, using a stainless steel column of 10 % silicone SE-52 on a 80-100 mesh Chromosorb W-AW support (3 mm x2 m) programmed from 125°C to 250°C (2°C per min). For GLC analysis, the complete hydrolysis of polysaccharide was performed by heating at 100°C

with 2N trifluoroacetic acid for 6 hr in a sealed tube, and neutral sugars were converted into the corresponding trimethylsilyl derivatives by Sweeley's method (Sweeley et al, 1963). Tentative identification of trimethylsilyl derivatives of neutral sugars were made by comparing their absolute and relative retention time to those of the authentic sugar derivatives. Quantification of all peaks were accomplished by measurement with integrator and convertion to weight percentage using a standard curve between peak ratio (sugar/internal standard) and weight ratio (sugar/internal standard).

Sedimentation analysis was performed with a Spinco model E ultracentrifuge at a rotor speed 51,310rpm at 20°C. Concentration of polysaccharide was 1.0% in deionized water.

Optical rotation was measured with a Union Giken PM-101 Polarimeter at 20°C. Concentration of polysaccharide was 2.0 % in deionized water.

Determination of molecular weight.

The average molecular weight of polysaccharide was determined by high-performance liquid chromatography (HPLC) in a column of Toyo Soda TSK G-5000PW (7.5 ID×60) with 0.1 M tris buffer, pH 8.0, as the carrier at room temperature, under a flow rate 0. 9 ml/min. Purified pullulan fractions having definite molecular weight were used as standards. Polysaccharides were detected with Shodex RI SE-11 refractive-index indicator.

Antitumor bioassay.

Antitumor bioassay was made by using ddY male mice of 6 to 8 weeks old. Implantation of sarcoma-180 was performed to intraperitoneally inject in 0.2 ml doses after dilution to 5 x 10^6 cells of sarcoma-180 per ml with PBS (−) (phosphate buffered saline without Ca^{2+} and Mg^{2+}) by measuring the cell population on Burker-Turk haemacytometer. The sample was dissolved in PBS (−) (5 mg/ml) and injected intraperitoneally 10 times after tumor implantation for 10 days in 0.2 ml doses. As control, only PBS (−) was injected under the same conditions. Complete regression was measured as the ratio of survivors over 40 days after tumor implantation against test mice. Survival days were estimated as the mean survival days and standard deviation of died mice.

RESULTS AND DISCUSSION

Isolation of rhamnose polysaccharide.

Water soluble fraction of hot water-extract was applied to a DEAE-Sephadex A-50 column chromatography, as the first step. The polysaccharide fraction that was not adsorbed on this column was collected and desalted by a Sephadex G-50. The 30th to 60th fractions with absorbance at 620 nm by anthrone-sulfuric acid method were combined and lyophilized (F-l) for further study. Purification of the polysaccharide was performed by two steps of chromatography using columns of Con A-Sepharose and Sephadex G-75. The Con A-Sepharose affinity chromatography afforded two parts that were unadsorbed part and adsorbed part eluted with about 5 mM methyl-α-D-man-

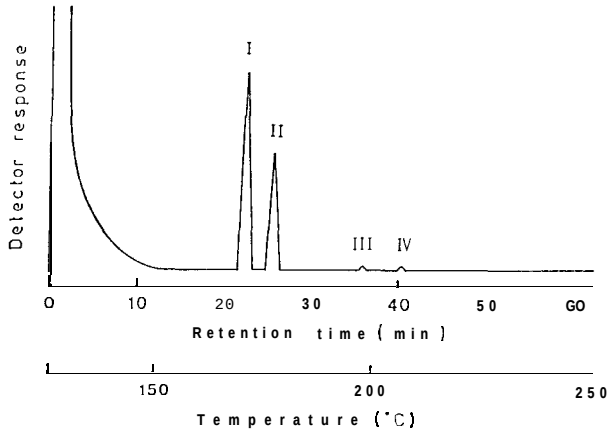


Fig. *2.* Analysis of sugar composition of *the* unadsorbed part to Con A-Sepharose column by GLC.
I, α-rhamnose; II, β-rhamnose; III, α-glucose; IV,)-glucose

noside; the elution profile is shown in Fig. 1. On gas chromatograph analysis, the adsorbed part contained only D-gulcose and the unadsorbed part contained L-rhamnose and extremely small amount of n-glucose (Fig. 2). From the convertion of the peak area integrated to the weight percent, the unadsorbed part composed of 95 % rhamnose and 5 % glucose on a average.

Properties of rhamnose polysaccharide.

The extremely rhamnose-rich part was further fractionated using a column of Sephadex G-75. The gel filtration revealed that the rhamnose polysaccharide consisted of two components with higher (F-l-L, $[\alpha]_D = +32.5$) and lower (F-l-S, $[\alpha]_D = +28.0$) molecular weights; the elution profile is shown in Fig. 3. Each one gave a single and symmetric peak on the sedimentation analysis as shown in Fig. 4, so they were found to be homogeneous in molecular weight. The sedimentation constants of F-1-L and F-1-S were 2.20S and 1.85S in convertion to water solution at 20°C. The purified rhamnose polysaccharide was soluble in cold water. Optical rotation was positive for the β-configuration. From the elution time of pullulans by HPLC, the molecular weights of F-1-L and F-1-S were 16,000 and 15,000, respectively (Fig. 5). F-1-L and F-1-S were quite similar except in molecular weight.

Kochetkov and Malysheva (1980) chemically obtained the regular polysaccharide α-(1→3)-L-rhamnanbypoly condensation of L-rhamnose. On the other hand, rhamnose-containing polysaccharides were isolated and purified from several bacterial cell walls (Prakobphol *et al,* 1980; Prakobphol and Linzer,1980; Neal and Wilkinson, 1979; Pazur *et al,* 1978). However, there is no previous report on the isolation of such a rhamnose-rich (95 %) polysaccharide from natural sources. The new polysaccharide isolated from *C. saccharoperbutylacetonicum* cells was designated as Clostrhamnan.

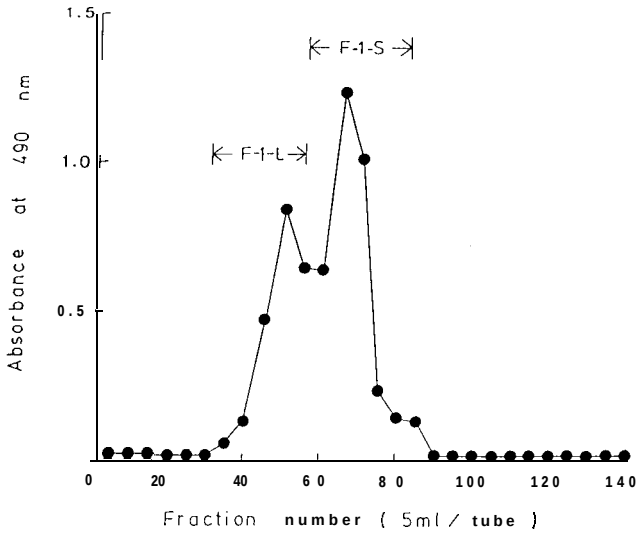
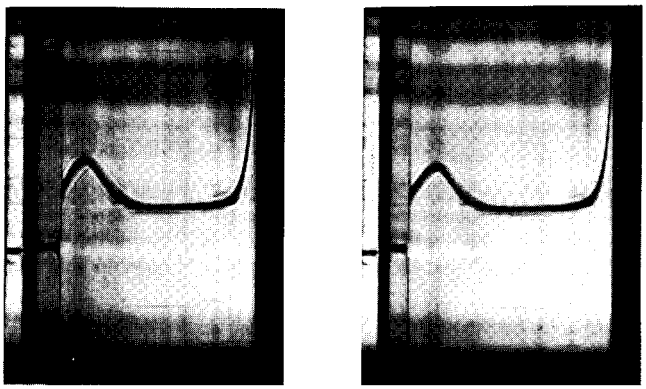


Fig. 3. Elution profile of Sephadex G-75 column (2.8×90 cm) chromatography of the eluate from the **Con** A-Sepharose column.
●, Absorbance at 490 nm. Each fraction was 5 ml.



(A) (B)

Fig. 4. Sedimentation diagrams of purified Clostrhamnan F-1-L (A) and F-l-S (B). Measurement was made at a concentration of 10 mg/ml of polysaccharides in deionized water. The pictures were taken at 105 min (A) and 144 min (B) after attaining top speed (56,100 rpm) at 20°C, using a Spinco model E ultracentrifuge (sedimented left to right).

Antitumor activity of Clostrhamnan.

Effect of Clostrhamnan on sarcoma-180 is shown in Fig. 6. All control mice died within about 15 days after tumor implantation. When lmg of this polysaccharide was administered 10 times, 3 out of 10 mice did not get cancer and survived over 40 days. In this case, the other died mice lived for 29

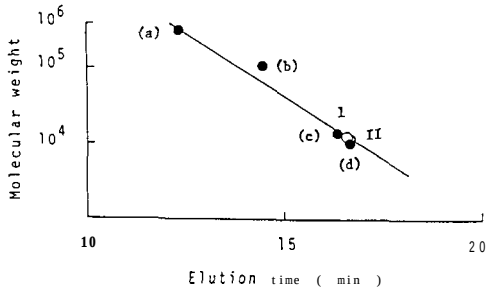


Fig. 5. Determination of the molecular weight of Clostrhamnan F-l-L and F-l-S by HPLC.
I, F-1-L; II, F-l-S; (a) Pullulan MW 800,000; (b) MW 100,000; (c) MW 20,000; (d) MW 10,000.

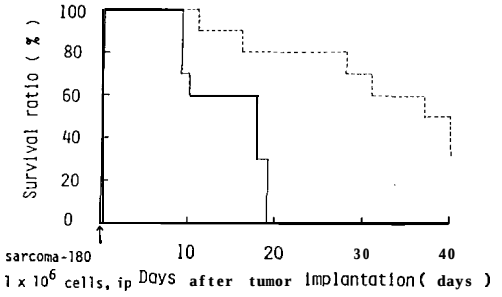


Fig. 6. Effect of Clostrhamnan on sarcoma-180.
·····, Clostrhamnan 1 mg×10;——, control
ddY mice were given intraperitoneal injection of 50 mg/kg/day of sample in PBS (−). Only PBS (−) was injected as control.

days after tumor implantation in average. Accordingly, effect of delayed death was found in these died mice. It is known that PS-K and Lentinan inhibit the tumor growth to augment the defence of the host against the tumor (Kaibara et ***al, 1977;*** Abe ***et al, 1978;*** Chihara and Maeda, 1969; Maeda et al, 1973). Both materials showed high antitumor activity to solid-type tumor, but low to ascite-type tumor. Clostrhamnan showed antitumor activity to ascite-type tumor without any toxicity. Further details of chemical structure and antitumor activity of Clostrhamnan will be described in the following papers.

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