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Effects of Antitumor Polysaccharides from *Albizza julibrissin* on Immune Function

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Abstract □ Polysaccharide fractions were prepared from *Albizzia julibrissin* by different extraction schedules. The fractions obtained were designated as PS-I and PS-II, respectively. Further purification of PS-I by Sephadex G-200 column chromatography gave two subfractions, Alju A and Alju B. Each fraction showed marked antitumor activity against sarcoma 180 solid form but not ascite form. PS-I and PS-II increased delayed hypersensitivity in normal and tumor bearing mice. PS-I treatment led to moderate restoration of the suppressed antibody production in tumor bearing mice.

Keywords □ Polysaccharide, *Albizzia julibrissin*, Antitumor activity, Hemolytic plaque assay, Delayed hypersensitivity test.

In recent years, although the medicine has continuously developed, cancer hazard remains still one of our most pressing health problems. The events that lead to the development of cancer, and the factors that control it, are poorly understood.

Furthermore, conventional treatment programs for tumor patients such as surgical method, radiation therapy and chemotherapy have some limitations in effects. Therefore, in addition to these established methods, development of a substance which enhances host defense mechanism in the human body has been one of the most important means of cancer treatment.[1]

In this aspect, the polysaccharides with antitumor activity, such as lentinan[2], picibanil (OK-432)[3], PS-K[4], TC-13[5]etc., have been found in a variety of natural sources. In these points of view, we have reexamined a number of folk remedies that have been used for treatment of malignant tumor patients in Korea for a long time and observed that some plants polysaccharides have antitumor activities against sarcoma 180 solid tumor. [6,7,8] Among them, we carried out a more detailed study on the polysaccharide from *Albizzia julibrissin*. In this study, we isolated active principles of *Albizzia julibrissin* and pruified them with gel filtration using Sephadex G-200.

In order to elucidate the action mechanism of the polysaccharides, we investigated the effects of the polysaccharides on hemolytic plaque forming cells against sheep erythrocytes and delayed type hypersensitivty in mice.

EXPERIMENTAL METHODS

Materials

Albizziae Cortex was commercially obtained from Kyung-Dong Herbdrug market in Seoul, Korea.

ICR and BALB/c mice were obtained from the Experimental Animal Breeding Center of

Seoul National University. Sarcoma-180 was maintained by serial i.p. passage in ICR mice.

Preparation of Polysaccharides

Polysaccharide fractions were obtained from *A. julibrissin* according to the method of Caldes *et al.*[9] with slight modification.

In this case, alkali extraction (PS-I) method was the same as before[7,8] but hot water fraction (PS-II) was extracted with distilled water for 10 hrs in a 80~90°C water bath instead of 0.5M NaOH overnight.

Purification of crude polysaccharide

Sephadex G-200 medium gel was prepared and packed onto a column with 4cm (ID) × 90cm(L).[10] The hot water fraction (200mg) of *A. julibrissin* polysaccharide was dissolved in 15 ml of distilled water and insoluble material was removed by filtration and the filtrate was applied onto the top of the column bed. The elution rate was 6ml/hr and hydrostatic pressure was maintained 10cm H_2O by using Mariotte flask. The eluent was collected in a glass tube every 30 miniutes, and polysaccharide contents of every odd-numbered fractions were determined by anthron test.

The anthron test positive fractions were concentrated, dialyzed against distilled water for 3 days and lyophilyzed. These fractions were devided into two groups (35~65, 90~110) and designated as Alju A and Alju B, respectively.

Antitumor test

Soild tumor growth inhibition test was carried out by the same way as that reported on the antitumor activity of lentinan.[2] Male ICR mice of about 20g were implanted s.c. with 1×10^6 cells of sarcoma 180 into the left groin 24 hrs before the start of sample administration. Samples were administered once a day for 10 days by i.p. injection with each dose.

On the 21st day after tumor implantation, the mice were sacrificed and the solid tumors were excised and inhibition ratios were calculated from their weights.

Hemolytic plaque assay

Male BALB/c mice weighing about 20g were administered i.p. with samples at a dose of 20mg/kg daily for seven consecutive days. In case of tumor bearing mice, sarcoma 180 cells (1×10^6cell/mouse) were implanted into the left groin 24 hrs before sample injection. Three-days after the last sample administration, the mice were immunized by i.p. injection with 8×10^7 SRBC.

Four days later, the mice were sacrificed and hemolytic plaque assay was accomplished according to Cunningham's modified method.[11]

Briefly, spleen cell suspensions of each mouse were mixed together with 12.5% SRBC in BSS and guinea pig complement (Denka Seiken Co.) and 50 μl reaction mixtures were pipetted into Cunningham chambers by capillary action. Each sample was assayed duplicatedly. Following pipetting of reaction mixture, the chambers were incubated for 1 hr in a humidified CO_2 incubator (Flow Lab. 220) gassed with 5% CO_2 and 95% air at 37°C. And then, the number of plaques formed by the antibody producing cells were counted.

Delayed type hypersensitivity (DTH)

Footpad test was performed as a model for delayed type hypersensitivity. In this study, effects on DTH were examined in normal and tumor bearing mice.

1) *DTH test in normal mice*

Heat aggregated bovine serum albumin (AB SA) was prepared according to the method of Richard G.T. *et al.*[12] Male ICR mice about 20g were injected i.p. with samples at a dose of 20mg/kg daily for 7 consecutive days. Three days after the last sample administration, mice

were sensitized by injecting 40 μg of antigen emulsified in 25 μl of Complete Freund's Adjuvant (CFA, Wako Chem Co.) s.c into left side of the tail base. (As a negative control, saline alone was injected instead of ABSA). Seven days later, DTH was elicited by challenging the animals in the left footpad with 30 μl of a 2% suspension of ABSA in saline. The thickness of footpad was measured with a micrometer (Mitutoyo MFC Co.) just before and 24hr after injection of challenging antigen into footpads and increase in footpad thickness was calculated.

2) *DTH test in tumor bearing mice*

Antigen fraction was prepared according to the modified method of Abe et al.[13] In this study, sarcoma 180 cells were used and protein concentration was adjusted to 500 μg/0.03ml in Hanks' solution. Male ICR mice about 20g were implanted s.c with 1×10^6 cells/mouse of sarcoma 180 into the left groin 24hrs before the start of sample administration and samples were administered as the same way in normal mice. DTH test was performed at the 8th day after the last sample injection. In all experiments, 0.03ml of the antigen fraction of tumor cells was injected into the left footpad of treated and control mice, and the increase of footpad thickness was calculated as the same method in normal mice.

Stastistical Analysis

Values of each group were expressed as the means±standard errors. T-value was calculated by Student's t-test and p-value was determined using an exchange table.

RESULTS AND DISCUSSION

The antitumor activity of polysaccharide was recognized by the suppression of the solid tumor growth. In the mice treated with polysaccharides, the average tumor weights were 2.106±0.575g in PS-I, 2.572±0.586g in PS-II, which were significantly lower than that of control mice (7.227±0.381g) ($p<0.01$) and there was no significant difference in antitumor activity between PS-I and PS-II (Table I). Since PS-II was the polysaccharide extracted with hot water, the "active" polysaccaride from *Albizziae Cortex* is heat stable. AljuA, larger molecular fraction from PS-II, showed stronger antitumor activity than Alju B. In the previous study[7] we observed that total polysaccharde fraction from *Albizzia julibrissin* showed strong antitumor activity against sarcoma 180 solid form but not against ascite form. We found also in this study, that polysaccharide extracted with hot water didn't prolong the life span (data are not shown). This is the quite similar response pattern to Lentinan, a polysaccharide from *Lentinus edodes* which completely regress sarcoma 180 solid form, and Forco C, polysaccharide from *Forsythia corea* which has marked antitumor activity.[8]

Table I. Antitumor activities of *A. julibrissin* polysaccharides against sarcoma 180 solid tumor

Sample	Dose (mg/kg×days)	Tumor wt. (g/mouse)	I.R.	p-value
Control	—	7.227±0.381	—	—
PS-I[1]	20×10	2.016±0.575	72.1	$p<0.01$
PS-II[2]	20×10	2.572±0.586	64.4	$p<0.01$
Control	—	4.727±0.533	—	—
Alju A	10×10	1.588±0.389	66.4	$p<0.01$
Alju B	10×10	3.196±0.542	32.4	$p<0.01$

1) PS-I=Polysaccharide I: Alkali extraction
2) PS-II=Polysaccharide II: Hot water extraction

We used delayed hypersensitivity reaction to measure the antitumor cellular immunity. The DTH reaction was measured as increase in footpad thickness after injection of sonicated sarcoma 180 cells as antigen in tumor bearing

Table II. The effects of *A. julibrissin* polysaccharide on delayed type hypersensitivity

Sample	sensitizing Ag	challenging Ag	Increase in footpad thickness(mm) (mean±S.E.)	p-value
Normal mice				
Negative control	—	ABSA	0.41±0.042	
Positive control	BSA	ABSA	0.54±0.025	
PS-II	BSA	ABSA	0.78±0.074	$p<0.05$
Tumor mice				
Control	—	Tumor homogenate	0.09±0.039	
PS-I	—		0.51±0.067	$p<0.01$
PS-II	—		0.39±0.036	$p<0.01$

BSA: Bovine serum albumin ABSA: Heat Aggregated BSA
PS-I: Polysaccharide I: Alkali extraction PS-II: Polysaccharide II: Hot water extraction

mice and heat aggregated bovine serum albumin in normal mice. The effects of polysaccharides on DTH in normal and tumor bearing mice are shown in Table II. As shown in the table, the swelling produced by intradermal injection of challenging antigen into ICR mice showed significant difference between control and treated group. The high increase of footpad swelling in treated groups in normal and tumor bearing mice indicates that *A. julibrissin* polysaccharide accelerated delayed hypersensitivity.

The plaque assay was carried out to show whether polysaccharide from *A. julibrissin* would increase the number of antibody proudcing cells in the spleen against sheep erythrocytes. The experiment was performed in 4 groups of mice; non treated control (group NC), mice given PS-II (group NT), mice grafted with sarcoma 180 and given PS-II (group TT), and mice grafted sarcoma 180 and not treated (group TC). As shown in Table III, the results obtained in tumor bearing mice were different from those in normal mice. In normal mice, the PFC number of treated group was lower than that of control mice (NC), but, in tumor bearing mice, the PFC number of treated group (TT) increased although lower than that in normal mice. Because of the complexity of PFCs-affecting factors it is difficult to say distinctly, but the increased PFC number of treated group exhibited the possibility that *A. julibrissin* PS somewhat restored the supressed antibody production in tumor

Table III. Hemolytic plaque forming cells (PFCs) in spleen of BALB/c mice immunized with sheep red blood cells (8×10^7/mouse)

	No. of mice	Spleen cell count ($\times10^6$) (mean±S.E.)	PFC/10^6 spleen cells (mean±S.E.)	PFC/spleen ($\times10^4$) (mean±S.E.)
NC	6	251.3±19.29	400.6±59.9	10.4±2.00
NT	5	246.5±18.55	88.4±33.6	2.50±1.00
TC	6	265.2±14.24	64.0±33.9	1.68±0.88
TT	5	336.4±17.40	125.0±41.7	4.48±1.63

NC: Nontreated control NT: Mice given polysaccharide
TC: Mice grafted with sarcoma 180 TT: Mice grafted with sarcoma 180 and given polysaccharide

bearing mice. In lentinan treated mice,[14] tumor inoculated s.c continued to grow 2 weeks, as in controls, and then started to regress. Even in untreated tumor bearing mice, 2-3 weeks after tumor inoculation, humoral reaction starts to increase and cellular immunity is depressed. In case of lentinan treatment, antitumor IgG activity slightly increased in the serum or restored and strengthened the T-DHR just before tumor regression and maintained it at high level but passive transfer of antitumor antibody did not cause regression of tumors at 2-3 weeks after tumor inoculation.[15]

The factors above mentioned may offer at least one of antitumor action mechanism of *A. julibrissin* polysaccharide. Namely the T-DHR potentiation by *A. julibrissin* polysaccharide may have an important role in its antitumor action. This idea is supported by the previous finding that T-DHR was correlated with the antitumor acti*in vitro* many systems.[16] In addition to above results, *A. julibrissin* polysaccharide markedly increased Arthus reaction and did not show direct cytocidal effect against sarcoma 180 cells in vitro. (data are not shown).

Put together above results, *A. julibrissin* polysaccharide showed a marked antitumor activity against sarcoma 180 solid form, but had no direct cytotoxicity against the tumor cells. And this polysaccharide also increased delayed hypersensitivity in normal and tumor bearing mice.

As far as the results reported here are concerned, there is no evidence that *A.julibrisssin* polysaccharide accelerates all humoral immune parameters but at least one of the antitumor mechanism may be related to activated cell mediated immunity.

Further studies on the mechanism of host defense against transplantable tumor, various parameters, such as macrophage mediated cytolysis[17,18,19,20], the function of T-lymphocyte[21], and natural killer cell activity, are under experiment.

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