Immunostimulating Polysaccharides from *Panax notoginseng*[1]

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Purpose. The main purpose of this study is to prepare and characterize polysaccharides from *Panax notoginseng,* investigate their effects on immune system *in vitro* in order to find new immunostimulants for the prevention and supporting treatment of infection and immunodeficiency related diseases.

Methods. Polysaccharides were extracted with aqueous solution, separated with column chromatography. Their anticomplementary activities were investigated by using human serum and antibody-sensitized sheep red blood cells. Interferon-γ and tumor necrosis factor inductive activities were studied by using isolated mouse spleen lymphocytes and peritoneal macrophages, respectively.

Results. Four polysaccharides, homogeneous in gel-filtration chromatography, were prepared and designated PF3111, PF3112, PBGA11, and PBGA12. Component sugar analysis revealed that they are heteroglycans with MWs ranging from 37 kD to 760 kD, composed of glucose, galactose, arabinose, mannose, and xylose in different molar ratios. Fraction PBGA12 has the most anticomplementary activity which is mediated through both alternative and classical pathways. All the polysaccharides except PBGA11 induced the production of interferon-γ in the presence of concanavalin A. They induced the production of significant amount of TNF-α in cell cultures.

Conclusions. The polysaccharides from *P. notoginseng* have immunostimulating activities *in vitro.*

KEY WORDS: *Panax notoginseng;* polysaccharides; interferon-γ; anticomplementary activity; TNF-α.

INTRODUCTION

Immunotherapy is currently receiving great attention as supporting treatment modalities in the management of cancer and AIDS patients whose immune function is compromised. Polysaccharides as immunostimulating agents from various sources including higher plants, have aroused great interest in recent years (1). Many of these polysaccharides have profound effects on the immune system, and are relatively nontoxic. Lentinan, a polysaccharide from *Letinus edodes,* has been successfully used in the treatment of various types of cancer (2). New sources of these compounds are constantly being discovered, and the demands for immunostimulants for the treatments of various diseases associated with compromised immune function are high. *Panax notoginseng* is a well-known traditional Chinese medicinal herb used as a hemostatic, cardiotonic, and antiinfective agent, and has been found to enhance the immune system and possess anti-tumor effect (3). Extensive studies have been conducted with ginsenosides from this herb. The effects of polysaccharides from this herb on immune system remain to be investigated. The present paper describes the preparation of polysaccharides from *Panax notoginseng,* and the *in vitro* investigation of their effects on the immune system.

EXPERIMENTAL

Materials

A dry powder made from the root of *Panax notoginseng* (Native & Animal Products Factory, Kunming, Yunnan, China) was used. Goat anti-human complement C3 antiserum were purchased from Sigma®. IgM-sensitized sheep red blood cells were obtained from DiaMedix. TNF-α and interferon-γ ELISA Kits were purchased from Genzyme Diagnostics. Male BDF1 mice (8 ~ 10 weeks old) were provided by Charles River Laboratories.

Preparation of Polysaccharides

Polysaccharides from *P. notoginseng* were prepared according to the Scheme shown in Fig. 1. H_2SO_4-Phenol method (4) was used to detect polysaccharide in column chromatography.

Determination of Molecular Weight

Gel filtration on a 2 × 40 cm column of Sepharose 4B-200 was used to determine the average molecular weights of the polysaccharides using water as the eluent and phenol-concentrated H_2SO_4 as detecting agent. A calibration curve was constructed by using dextrans with known molecular weights (39, 73.5, 500, and 2000 kD). The elution volumes were taken in 0.5-ml fractions. Then the average molecular weight of each polysaccharide fraction was determined by using the calibration curve.

Identification of Component Monosugars

The component sugars were analyzed with a modified HPLC method of Muramoto et al. (5). Two ml of polysaccharide solution (2 mg/ml) was mixed with 2.5 ml of 4 M trifluoroacetic acid and heated at 100°C for eight hrs, dried under vacuum, and redissolved in 5 ml of water. Five μl of the hydrolysates was mixed with 40 μl of DABS-hydrazide (dimethylaminoazobenzen-4′-sulfonyl hydrazide) solution (prepared according to Anderson's method (6)) in a test tube. The tube was sealed and heated at 50°C for 2 hrs, and centrifuged. The supernatant was kept at 4°C prior to HPLC analysis. Monosugar derivatives were analyzed on a Gilson-712 HPLC system with a reversed phase Xorbax ODS column (4.6 × 150 cm, particle size 5 μm), and detected at 485 nm. The column was preequilibrated with running solvent (30% acetone in 0.08 M aqueous acetic acid) for 30 min. before 2 to 10 μl of sample solution was injected. After each run, the column was washed with washing buffer (80% acetone in 0.08 M aqueous acetic acid) for 30 min. Authentic monosugars, glucose, galactose, mannose, arabinose, xylose, rhamnose, fucose, fructose, galacturonic acid, and glucuronic acid were also derivatized with the same method.

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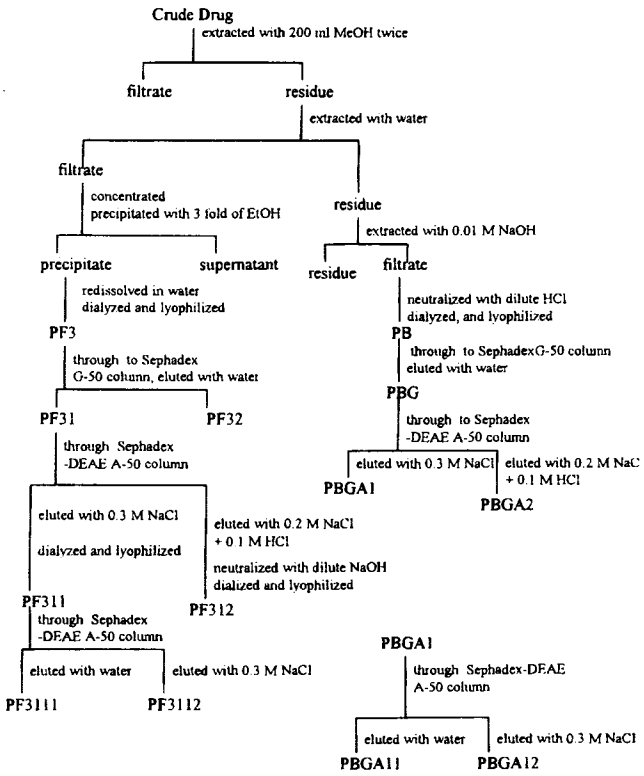

Fig. 1. The scheme for the preparation of polysaccharides from *P. notoginseng.*

The system was calibrated with zero to 20 μM of derivatized glucose. The peak heights of HPLC spectra were measured, and the molar ratio of monosugar composition of each polysaccharide was calculated against authentic monosugars.

Determination of Anticomplementary Activity

The anticomplementary activity was determined according to the method of Gonda et al (7). Various dilutions of polysaccharides solution in H_2O were incubated with 50 μl normal human serum (NHS) obtained from a healthy adult and 50 μl GVB^{2+} (Gelatin-veronal-buffered (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+}). The residual hemolytic activity was determined by using antibody-sensitized sheep red blood cells. NHS incubated with PBS without polysaccharides provided as negative controls. The anticomplementary activity was calculated as the following:

$$\text{anticomplementary activity} = \left(\frac{TCH_{50t} - TCH_{50c}}{TCH_{50t}}\right) x100\%$$

TCH_{50}: The volume of NHS needed to lyse 50% of sheep red blood cells;
TCH_{50t}: for polysaccharide treated serum;
TCH_{50c}: for negative control (without polysaccharide treatment).

The anticomplementary activity through alternative pathway was determined as the following: Fifty μl of various concentrations of polysaccharide solution was incubated with 50 μl of NHS and 50 μl of gelatin-veronal-buffered saline (pH 7.4) containing 10 mM EGTA and 2 mM $MgCl_2$ (EGTA-GVB) at 37°C for 30 min., and mixed with 350 μl of EGTA-GVB. The residual hemolytic activity was determined as described above. NHS was incubated with PBS and EGTA-GVB to provide a control.

The identification of C3 protein activation was carried out by using crossed immunoelectrophoresis. NHS (50 μl) was incubated with 50 μl of the polysaccharide solution (500 μg/ml) with both GVB^{++} and EGTA-GVB buffers for 30 min at 37°C. The serum was then subjected to crossed immunoelectrophoresis to locate the C3 cleavage products. Shortly after the first run (barbital buffer, pH 8.6; ionic strength, 0.025; with 1% of agarose, gel thickness is 2 mm, 1 mA/cm for 6 hr), the second run was carried out on a gel plate (2.0 mm) containing 3% of goat anti-human C3 serum at a potential gradient of 2 mA/cm for 24 hrs. After the electrophoresis, the plate was fixed and stained with 0.8% coomassie brilliant blue G methanol solution, and washed with aqueous methanol solution containing 10% acetic acid.

Determination of Interferon Inductive Activity

Male BDF1 mice (8–10 weeks old) were injected intraperitoneally (i.p.) with 3 ml of 3% thioglycollate medium to induce macrophages, and killed by cervical dislocation 4 days later. Mouse peritoneal macrophages were collected by lavage with cold phosphate buffered saline (PBS). The macrophages were washed with cold PBS, and resuspended in RPMI 1640 medium (10^6 cells/ml). Mouse spleens were removed, washed with Hank's balanced salt solution (HBSS). Sterile spleen cell suspension was prepared, washed with HBSS, and resuspended in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. RBCs were removed by using Histo-Paque® at a spin speed of 400 × g for 30 min.. Spleen lymphocytes were collected, washed with HBSS, and resuspended in RPMI 1640 medium containing 10% FCS (10^6 cells/ml).

The interferon induction experiment was carried out in 24-well flat-bottomed cell culture plates. A mixture of spleen lymphocytes and peritoneal macrophage suspensions (9:1) was used. For the interferon induction, we have three experimental groups. 1) To each well, 0.9 ml of spleen lymphocyte suspension was added. Polysaccharides were dissolved in RPMI 1640 medium, diluted to 3 different concentrations (5000, 2500, 1250 μg/ml), and added to cultured spleen lymphocytes (0.1 ml/well). The cells were cultured at 37° C with 5% CO_2 for 24, 48, 72 hrs, respectively. Supernatants were collected by centrifugation, and frozen at −75°C until interferon-γ determination by Enzyme-Linked Immunosorbent Assay (ELISA) method. Cell viability was checked by trypan blue exclusion method. 2). The procedure is the same as above except Con-A (10 μg/well) was added to the cell culture. 3). Cells were incubated with interferon α/β (30 IU/well) for 6 hrs, washed with HBSS for three times, and resuspended in RPMI 1640 medium containing 10% FCS. Then cells were incubated with different concentrations of polysaccharides. Cell cultures with medium without polysaccharides were negative controls.

Determination of TNF-α Inductive Activity

Mouse peritoneal macrophages (10^6 cells/ml) were cultured with different concentration of polysaccharides at 37°C in 5% CO_2 for 24 hrs. Supernatants were collected and stored

Table 1. Anticomplementary Activities of Polysaccharides

polysaccharide	Anticomplementary activity (inhibition%) 1000 μg/ml	500 μg/ml	250 μg/ml	125 μg/ml
PF3111	14.9 ± 1.3	11.9 ± 0.9	11.4 ± 0.9	8.3 ± 0.8
PF3112	38.7 ± 1.0[a,b]	31.3 ± 0.8[a,b]	25.6 ± 0.3[a]	18.8 ± 0.1
PBGA11	7.2 ± 0.8	7.5 ± 0.8	4.1 ± 1.0	1.9 ± 0.5
PBGA12	95.5 ± 0.2[a,b]	86.1 ± 2.2[a,b]	80.2 ± 0.3[a,b]	74.5 ± 0.3[ab]
	(38.0 ± 0.0)[c]	(32.6 ± 0.6)	(29.6 ± 0.0)	(23.5 ± 1.0)
LPS	27.4 ± 0.2	22.0 ± 0.0	18.4 ± 0.6	18.3 ± 0.4
Lentinan	21.8 ± 1.6	21.8 ± 0.6	24.2 ± 0.9	23.8 ± 3.4

[a] Statistically significant ($P < 0.05$) as compared with LPS.
[b] Statistically significant ($P < 0.05$) as compared with Lentinan.
[c] Values in parenthesis are the activity through alternative pathway.

at −75°C until assay for TNF-α by ELISA method. Cells cultured with added same amount of medium without polysaccharides served as negative controls.

Statistics

Significance ($P < 0.05$) was determined by Student's T-test. Quantitative structure-activity relationship (QSAR) analysis was implemented with BMDP program.

RESULTS

The yields of polysaccharide fractions PF3111, PF3112, PBGA11 and PBGA12 are 3.0, 2.2, 1.4, and 0.1%, respectively. They are homogeneous on gel-filtration column chromatography (only one peak detected) with molecular weights of 685, 37, 45, and 760 kD, respectively. Surprisingly, all the polysaccharides are composed of galactose, glucose, mannose, arabinose, and xylose with molar ratios of 3.5:10.8:3.5:1.0:2.3, 2.9:5.3:2.8:1.0:2.1, 3.1:4.2:5.3:1.0:3.2, and 2.5:7.2:4.3:1.0:8.1 for PF3111, PF3112, PBGA11 and PBGA12, respectively.

The anticomplementary activities of the polysaccharides tested are shown in Table 1. PF3112 and PBGA12 have statistically significantly higher anticomplementary activity as compared with lipopolysaccharide(LPS) and lentinan at 95% level. PBGA12 has the highest anticomplementary activity. The anticomplementary activity through the alternative pathway of PBGA12 is significantly reduced when Ca is chelated, but not totally abolished. It appears that the anticomplementary activity of the polysaccharide is mediated through both classical and alternative pathways. This is also verified by crossed immunoelectrophoresis. PBGA12 did split C3 protein into two peaks, the native C3 protein peak and the C3 cleavage product peak indicted by arrows as shown in Fig. 2. Similar result was obtained for PF3112. From these figures, one also can see that when polysaccharides were incubated with Mg^{2+}-EGTA-GVB, the ratio of the height of the cleavaged peak to the native protein peak is significantly reduced. This means that polysaccharides activated C3 through both classical and alternative pathways.

It was found that the polysaccharides could not induce significant amount of interferon-γ as compared with the blank control (Table 2). Pretreatment of spleen lymphocytes with mouse interferon α/β did not enhance the production of interferon-γ. However, the addition of Con-A (10 μg/ml), a T-cell mitogen, could significantly enhance the production of interferon-γ compared with either the blank control or the Con-A control at 95% significance level. Interferon-γ production induced by PBGA12 reached the highest level in the cell culture at 48 hrs, followed by a decrease in interferon-γ concentration in the supernatant. No significant amount of interferon activity

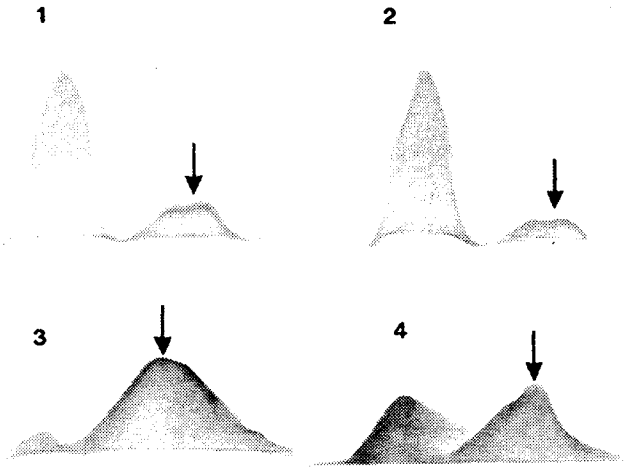

Fig. 2. The crossed immunoelectrophoresis pattern of NHS incubated with (1) PBS and GVB^{2+}; (2) PBS and Mg^{2+}-EGTA-GVB; (3) PBGA12 and GVB^{2+}; (4) PBGA12 and Mg^{2+}-EGTA-GVB. The C3 cleavage product peaks are indicated with arrows.

Table 2. Interferon-γ Induction by Polysaccharides (200 μg/ml)

Compound	Interferon-γ concentration (mean ± SD)(pg/ml)[a] Treatment A	Treatment B	Treatment C
PBGA11	0	1083.0 ± 86.7	266.2 ± 83.7
PBGA12	401.4 ± 66.1	1387.1 ± 97.0[b]	942.0 ± 4.4[b]
PF3111	554.1 ± 39.7	1531.0 ± 94.0[b]	413.1 ± 19.1
PF3112	361.7 ± 44.1	808.3 ± 52.9	0
LPS	1282.8 ± 116.1[b]	2650.4 ± 35.3[b]	1085.9 ± 69.0[b]
Control	419.0 ± 89.6	706.9 ± 33.8	335.9 ± 40.7

[a] Treatment A. Compound only; Treatment B. Compound + Con-A; Treatment C. Compound + interferon (α/3) pretreatment.
[b] Significant ($P < 0.05$) as compared with controls.

Table 3. TNF-α Induction in Mouse Peritoneal Macrophages by Polysaccharides

Compound	TNF-α concentration (mean ± SD)(pg/ml)		
	500 μg/ml	200 μ/ml	20 μ/ml
PBGA11	11041 ± 159[a]	3559 ± 18	2770 ± 22
PBGA12	13018 ± 159[a]	11694 ± 106[a]	10035 ± 177[a]
PF3111	10441 ± 653[a]	8235 ± 529[a]	3206 ± 124
PF3112	9418 ± 53[a]	7459 ± 71[a]	3665 ± 53
LPS	13812 ± 459[a]		
Control	3471 ± 35		

[a] Statistically significant ($P < 0.05$) as compared to the control.

in the supernatant could be detected by using cytopathic effect-inhibition method with mouse interferon α/β as the standard (8). Therefore, the induced interferon is mainly interferon-γ.

All the polysaccharides induced significant amount of TNF-α in the cell cultures at concentration of 500 μg/ml (see Table 3). Lipopolysaccharide from *E. coli* has the highest TNF-α inductive activity. PBGA12 was the most active fraction. This activity followed a dose-dependent manner.

The Log(molar activity), which is defined as log (activity/molar concentration), and the Log MW of polysaccharides from *P. notoginseng* and *V. yedoensis* (9) are listed in Table 4. The correlations of log(molar activity) for anticomplementary, interferon-γ inductive, and TNF-α inductive activities are shown in Eq. [1], Eq. [2], and Eq. [3], respectively. From the QSAR analysis, it seems that the molecular weight of a polysaccharide may be a major factor in affecting its mitogenicity.

anticomplementary activity:

$$\text{Log (molar activity)} = 1.28\ \text{Log MW} + 0.07$$
$$(n=10,\ r=0.94,\ s=0.33,\ F_{1,8} = 62.41) \quad (1)$$

Interferon-γ induction:

$$\text{Log (molar activity)} = 1.10\ \text{Log MW} + 3.42$$
$$(n=10,\ r=0.97,\ s=0.20,\ F_{1,8}=92.18) \quad (2)$$

Table 4. Molecular Weights and Molar Activities of Polysaccharides

PS	log MW	Log(molar activity)[a]		
		anti-complementary	IFN-γ induction	TNF-α induction
PF3111	5.84	7.01	9.72	10.16
PF3112	4.57	6.16	8.17	8.84
PBGA11	4.65	5.51	8.39	9.00
PBGA12	5.88	7.86	9.72	10.30
YF41[b]	6.02	7.88	10.13	10.39
YF42[b]	4.57	5.75	8.46	8.74
YF3111[b]	5.84	7.66	9.90	10.00
YF3112[b]	4.57	6.23	8.76	9.03
YF3121[b]	5.84	7.65	10.04	10.21
YF3122[b]	4.65	6.20	8.69	9.09

[a] Anticomplementary activity at 1000 μg/ml; IFN-γ induction at 200 μg/ml with Con-A; TNF-α induction at 500 μg/ml.
[b] Polysaccharides from *V. yedoensis*.

TNF-α induction:

$$\text{Log (molar activity)} = 1.00\ \text{Log MW} + 4.34$$
$$(n=10,\ r=0.99,\ s=0.11,\ F_{1,8} = 358.6) \quad (3)$$

DISCUSSIONS

It has been shown that most polysaccharides from higher plants are heteroglycans with high molecular weights (1). A polysaccharide, Sanchinan-A composed of galactose and arabinose with a molecular weight of 1500 kD, has been previously prepared from *Panax notoginseng* with acid hydrolysis and enzymatic hydrolysis by Ohtani et al. (10). Their product is different from our polysaccharides obtained in terms of monosugar composition and molecular weight. Their differences are most likely due to different procedures used.

Complement system plays an important role in fighting against various infections and tumors. It has been shown that polysaccharides with higher anticomplementary effects have higher antitumor activities. Hashimoto et al. (11) have found that the pretreatment with mouse interferon α/β could enhance endotoxin-induced interferon production by rabbit tissue cells. This is different from our results. Con-A could significantly enhance the production INF-γ, which has also been shown in other experiments (12). The interferon induction experiment indicates that these polysaccharides alone may not be able to activate T cells, but may enhance the production of interferon-γ by activated T-cells. Interferon α/β is mainly secreted by activated macrophages and other somatic cells, while interferon-γ is primarily produced by activated T cells. In our cell culture, the majority of cells are lymphocytes, namely T cells and B cells. INF-γ is important in immune defense against various viral infections and in the regulation of cell-mediated immune response. TNF-α has profound effects in immune system. It has tumor cytotoxic, antiviral, and anti-parasitic activities. TNF-α also can cause inflammation and endotoxic shock. This may be responsible for some possible side effects of polysaccharides.

In the biological studies, concentrations of polysaccharides were expressed on a μg/ml basis. The higher the molecular weight of the polysaccharide, the fewer the molecules will be contained on a μg/ml basis. Therefore, it is more meaningful to convert the concentrations to a molar basis as is usually done in quantitative structure-activity relationship (QSAR) analysis (2,13). From the QSAR analysis, it can been seen that molecular weight is very important in determining the mitogenicity of polysaccharides. This phenomenon has also been found in the QSAR analysis of antitumor activities of polysaccharides (2). These results reveal that specific binding sequence may exist in polysaccharide molecules. The bigger the molecule, the more the binding sequences will be contained in the molecule. A more thorough QSAR analysis in terms of the secondary and tertiary structures of polysaccharides with their biological activities can be obtained only with complete structural determination.

CONCLUSION

Four distinct homogeneous polysaccharide fractions have been prepared from dried *P. notoginseng*. PBGA12 has a strong anticomplementary activity. PF3111 and PBGA12 significantly induced the production of interferon-γ in the presence of Con-

A. At concentrations from nanomolar to low micromolar range, all the polysaccharides can significantly induce the production of TNF-α by thioglycollate-induced mouse peritoneal macrophages. It is apparent that the biological activities of polysaccharides are highly correlated with their molecular weights, which suggests that the molecular weight is a very important parameter in determining the mitogenicity of a biologically active polysaccharide. Those active polysaccharides can stimulate the complement system, enhance the production of INF-γ and TNF-α production. Through further investigation, they can be potent immunstimulating agents which can be used to treat immune deficiency related diseases.

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