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Original article

Extraction, purification and macrophage stimulatory activity of polysaccharide isolated from leaves of *Perilla frutescens* BRITTON var. *crispa*

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Summary. The crude polysaccharide portion of *Perilla frutescens* BRITTON var. *crispa* (PFB-1) was prepared by following steps, hot water extraction (PFB-0), decolorization with methanol reflux and ethanol precipitation. The PFB-1 was separated by anion-exchange column chromatography (fraction: PFB-1-0). Purified PFB-1-0-ii was obtained from gel permeation column chromatography purification steps of PFB-1-0. The PFB-1-0-ii was shown to be one polysaccharide with molecular mass of 10 kDa (kilodalton). PFB-1-0-ii consists of mannose, galactose and glucose as major sugars with 82.8% carbohydrate. Next, this study investigated the effects of PFB-0, PFB-1, PFB-1-0, and PFB-1-0-ii on macrophage stimulatory activity in primary macrophages. PFB-0, PFB-1, PFB-1-0 and PFB-1-0-ii increased macrophage cellular lysosomal enzyme activities. Among the samples tested, PFB-1-0-ii exhibited the highest macrophage lysosomal enzyme activities. The macrophage-stimulating activity was significantly enhanced during the purification step. The results support the utility of purified polysaccharide from Perilla as immunostimulatory activity agent.

Key words: immunostimulatory activity, macrophage, *Perilla frutescens* BRITTON var. *crispa,* polysaccharide, purification

Introduction

Labiatae, the common name of *Perilla frutescens* BRITTON var. *crispa* (Perilla) is one of the most common herbs in many Asian countries such as Korea, China and Japan (1). Perilla leaves have been used as diuretic, sedative, anti-dote and anti-febrile in Japanese traditional medicine. In addition, they frequently accompany seafood as the leaves are believed to prevent food poisoning and to protect the digestive tract from inflammatory diseases. To elucidate these effects, many other extracts and ethanol extracts of Perilla leaves has been investigated, and contain perillaldehyde, a sedative and an anti-fungal agent. Several studies on the pharmacological activities of the herb in relation to sedation, indigestion, and food poisoning have been reported Makino et al. (2). Traditional usage of the herb has led to the expectation that the leaves possess inhibitory effect against food allergies. For example, several anti-allergies effects of the leaves have been reported Makino et al. (3). Recently, the polysaccharides from herbs have been reported to contain immune enhancing effects such as augmentation of macrophage function. Several polysaccharides, including βglucan and chitin, have been shown to induce anti-tumorial activity *via* macrophage activation in the host (4). Similar to other polysaccharides, polysaccharide from Perilla may show immunostimulatory

activity by macrophage activation. However, the immunostimulatory activity by macrophage activation of Perilla and the composition of the pure PFB have not been investigated. Accordingly, we analysed chemical properties, homogeneity and molecular weight of pure PFB after extraction and purification and evaluated the macrophage stimulatory activity of purified PFB.

Materials and Methods

Materials and chemicals

Leaves of PFB, which were cultivated and dried at Hongcheon, Kangwon-do in South Korea, were purchased from commercially *Korea Kyung-Dong Market* (Seoul, South Korea). DEAE-Toyopearl 650 M, Sephadex G-100 were obtained from Pfizer Inc. (New York, NY). Rosewell Park Memorial Institute (RPMI-1640) medium, Triton X-100 and *p*-nitrophenyl phosphate were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Penicillin and streptomycin were obtained from Flow Laboratories (Irvine, Scotland). All other chemicals and solvents were of analytical grade.

Purification procedure

Leaves of PFB (1 kg) were decocted with water (20 liter) at 100˚C to half volume and filtered with Whatman No. 4 filter paper. The filtrate was then lyophilized to obtain hot-water extract (PFB-0; yield = 16.0%), and PFB-0 was refluxed with methanol (5 liter) for 1 h (5 times) and centrifuged to remove methanol-soluble fraction (PFB-M; yield = 0.4%). The methanol-insolubles were precipitated in 80% ethanol, resulting in ethanol-solubles (PFB-E; yield = 4.9%) and precipitate (PFB-1; yield = 9.8%). The PFB-1 (500 mg) was passed through ion-exchange column chromatography using a DEAE-Toyopearl 650 M (Sigma-Aldrich Co. LLC., St. Louis, MO) column (4.0 i.d. × 30 cm), obtaining eight subfractions (PFB-1-0 ~ 7). Eight fractions were collected, dialyzed against tap water and lyophilized. After absorption of the sample solution into the column bed, the column was washed with water to obtain neutral sugar portion, and resultantly obtained one sugar fraction (PFB-1-0). And then the absorbed acidic polysaccharides were eluated by stepwise gradient NaCl solution (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 2.0 mol/L), and resultingly divided into seven sugar fractions (PFB-1-1, -2, -3, -4, -5, -6, and -7). The lyophilisate PFB-1-0 (40 mg) was applied to gel permeation column chromatography using a Sephadex G-100 column (2.5 i.d. × 94 cm) equilibrated with 0.2 mol/L NaCl. The polysaccharide was eluated with 0.2 mol/L NaCl at a flow rate of 0.2 ml/min and each fraction was concentrated, desalted and lyophilized for macrophage-stimulating activity. Two fractions, PFB-1-0-i and –ii, were obtained by this column (Fig. 1) and chemical composition of two fractions were analyzed contents of carbohydrate (5), uronic acid (6) and proteins (7) (Table 1). Homogeneity of the purified polysaccharide (PFB-1-0-ii) was confirmed again by high perfomance liquid chromatography (HPLC, HP Agilent series 1690, Waldbronn, Germany) equipped with refractive index detector and GPC typed column (TSK-gel G5000PW and TSKgel G3000PW, 3.75 i.d. × 60 cm). Ten micro- liter of each polysaccharide solution (1 mg/ml) was analyzed by using an isocratic mobile phase, 0.2 mol/L NaCl at a flow rate of 0.25 ml/min. Standard dextrans (T-2000, T-500, T-70, T-40, glucose) were also analyzed in the same condition.

Gas liquid chromatography (GLC)

The component sugars of polysaccharide were analyzed by GLC method of Canonica & Pisano (8). Polysaccharides were hydrolyzed using trifluoroacetic acid, and after reduction, the neutral sugars and uronic acids

Table 1 . Chemical composition and yield of PFB-1-0 subfractions separated by Sephadex G-100 gel permeation column chromatography

	PFB-1-0-i	PFB-1-0-ii
(%)		
Yield	66.5 ± 4.2	74.9 ± 5.2
Carbohydrate[a]	43.2 ± 2.5	82.8 ± 4.8
Uronic acid[b]	0.8 ± 0.1	0.9 ± 0.1
Proteinc	0.8 ± 0.1	1.5 ± 0.1

[a]Carbohydrate content : phenol–sulfuric acid method (as Gal).
[b]Uronic acid content: m–hydroxybiphenyl method (as GalA).
cProtein content: Lowry method (as bovine serum albumin).

were converted to the corresponding alditol acetates. Flame ionization detector/gas chromatography (Young Lin Instrument Co., Ltd., M600D, Gyeonggi-do, South Korea) analysis was conducted on a SP-2380 (30 m × 0.25 mm, 0.2 μm) capillary column at 250°C. Nitrogen gas was used as carrier gas at a flow rate of 0.5 ml/min. Molecular ratio of sugars was calculated from peak areas and molecular weights of the converted sugars.

Isolation and culture of macrophages from male ICR mice

For the preparation of macrophage monolayers, specific pathogen-free male ICR mice (5-6 weeks old; Daehan-Biolink Co, Chungcheongbuk-do, South Korea) were intraperitoneally injected with 1 ml of 3% thioglycolate (TG) medium. After 3 days, peritoneal exudates cells (PEC) were collected, and seeded into a 96-well microplate (1.0 x 10^5 cells/well) in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin (PEST), in humid atmosphere of 5% (v/v) CO_2 and 95% (v/v) air at 37°C. The growth medium was replaced to sample solution (1, 10 and 100 μg/ml) dissolved in the medium, and it was maintained for 24 h under the same condition. Animal care and handling were performed according to protocols approved by the Animal Experimentation and Ethics Committee of Korea University (Seoul, South Korea).

Measurement of macrophage cellular lysosomal enzyme activity

Macrophage cellular lysosomal enzyme activity was measured according to the procedure of Suzuki et al. (9). Primary macrophage monolayers in a 96-well microplate (1.0 ´ 10^5 cells/well) were solubilized by the addition of 25.0 μl of 0.1% Triton X-100. One hundred fifty microliters of 10.0 mmol/L *p*-nitrophenyl phosphate was added as a substrate for acid phosphatase. Then, 50.0 μl of 0.1 mol/L citrate buffer (pH 5.0) were added to the well. After incubation for 1 hour at 37°C, 50.0 μl of 0.2 mol/L borate buffer (pH 9.8) was added to the reaction mixture, and the absorbance at 405 nm was photometrically measured by using a microplate reader (Model 3550-UV, Bio-Rad, Hercules, CA). The lysosomal enzyme activity was calculated as follows: Lysosomal enzyme activity (Relative activity; %) = T/C ´ 100 where C is the absorbance of a saline as control, and T is that of a test sample at 405 nm.

Statistical analysis

Data were expressed as means ± SD and average values from three to eight values per experiment. Each experiment was performed a minimum of three times. Analysis of variance (ANOVA) was conducted, and Duncan's multiple range tests were used to determine the significance of differences between groups. The level of statistical significance was set to $P < 0.05$.

Results and Discussion

Polysaccharide was isolated and purified from leaves of Perilla by hot water extraction, decolorization with methanol reflux, ethanol precipitation, ion-exchange column chromatography and gel permeation column chromatography (Fig. 1) (10). The seven sugar fractions (PFB-1-0, PFB-1-1, PFB-1-2, PFB-1-3, PFB-1-4, PFB-1-5, PFB-1-6, PFB-1-7) were obtained by ion-exchange column chromatography (Fig. 1). PFB-1-0 contained high carbohydrate (Fig. 2). Thus, the PFB-1-0 fraction further separated by gel permeation column chromatography and two fractions, PFB-1-0-i and PFB-1-0-ii, were obtained by this column (Fig. 1 and Fig. 4). The PFB-1-0-i and PFB-1-0-ii was mostly carbohydrate (Fig. 4). Chemical composition of the purified polysaccharide (PFB-1-0-i and PFB-1-0-ii) was determined as shown in Table 1. The PFB-1-ii was composed of carbohydrate (82.8%), uronic acid (0.9%) and protein (1.5%). Homogeneity of the purified polysaccharide (PFB-1-0-ii) was confirmed by HPLC and PFB-1-0-ii showed a single peak on the HPLC chromatogram (Fig. 3A). Carbohydrate in PFB-1-0-i was 43.2% (Table 1). The hot water extract of PFB before purification consisted of 43.2% total carbohydrate, 5.8% uronic acid and 13.8% protein. During the methanol reflux and ethanol precipitation, protein content markedly declined to 8.9%, indicating that denaturated protein was eliminated (PFB-1). The finally purified polysaccharide (PFB-1-0-ii) showed the molecular mass of 10 kilodalton (kDa) (Fig. 3B). The polysaccharides ranged in size of 1,000 kDa being relatively high molecular weight (MW) compared with other known acidic immune-stimulating polysaccharides. Polysaccharides from *Lithosperum euchromum* is known to have the MW more than 500 kDa and it

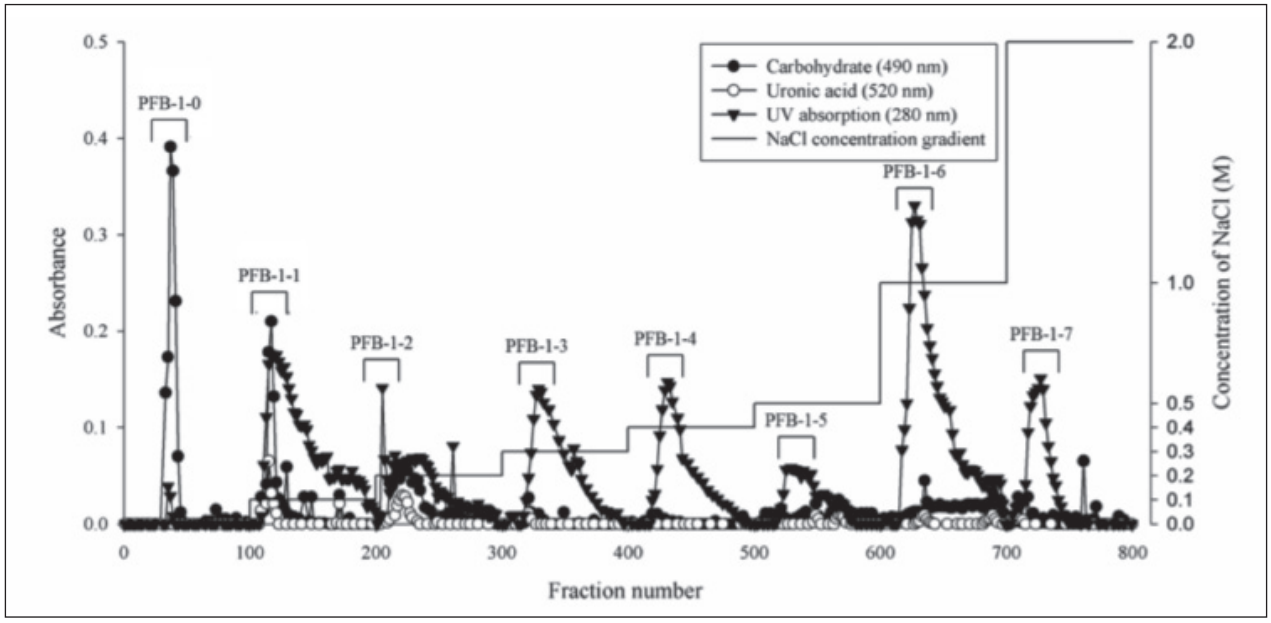

Figure 1. Ion-exchange column chromatogram of PFB-1 on the column of DEAE-Toyopearl 650 M. The column was eluted first with distilled water until sugar was no longer detected and then eluted in stepwise gradient (0.1 mol/L ~ 2.0 mol/L NaCl).

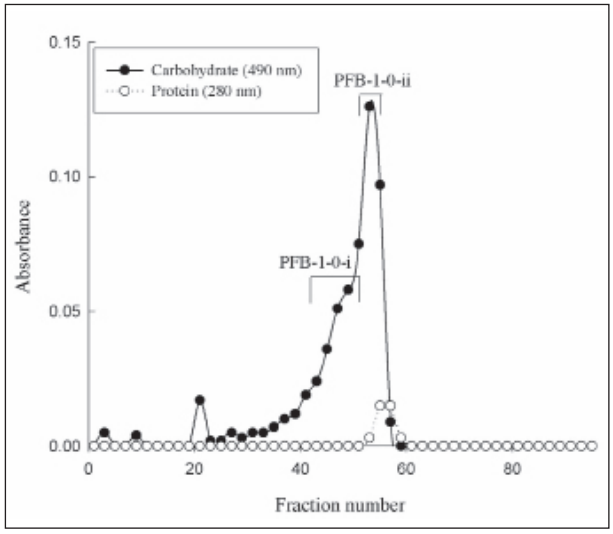

Figure 2. IGel permeation column chromatogram of PFB-1-0 on Sephadex G-100. Further purification of PFB-1-0 by gel permeation column chromatography on Sephadex G-100. Fraction PFB-1-0 was applied to a column (2.5 i.d. X 94 cm) and eluted with 0.2 mol/L NaCl.

showed immune-stimulating activities (11) but most immune-stimulating polysaccharides such as polysaccharides from *Bupleurum falcaltum* (12) and polysaccharides from *Panax ginseng* (13) have the size less than 10 kDa. Na et al. (14) reported that water-soluble polysaccharide isolated from *Capsosiphon fulvescens* have immunostimulating activity and the apparent average molecular weight of the purified polysaccharide was estimated to be approximately 385 kDa by size-exclusion HPLC.

The sugar composition of active fractions (PFB-1, PFB-1-0, PFB-1-0-ii) obtained from purification step on PFB was shown in Table 2. The sugar composition of the purified PFB-1-0-ii was determined to be arabinose (9.6%), xylose (0.2%), mannose (39.8%), galactose (14.5%) and glucose (45.6%) in molar percentage, based on the GLC analysis (Table 2). The monosaccharide composition of PFB-1 and PFB-1-0 was determined to be arabinose, xylose, mannose, galactose and glucose and major sugar of PFB-1, PFB-1-0 and PFB-1-0-ii were mannose, galactose and glucose (Table 2). The pharmacologically active polysaccharides are reported to contain arabinose and galactose as the component sugars of rhamnogalacturonam (12). As the purification process went on, arabinose, xylose and galactose decreased to minor portion, while mannose and glucose mainly occupied the purified polysaccharides. Moreover, in the beginning stage of purification, the percentage of glucose was much higher than that of mannose, but in the final stage, the ratio of glucose was raised clearly.

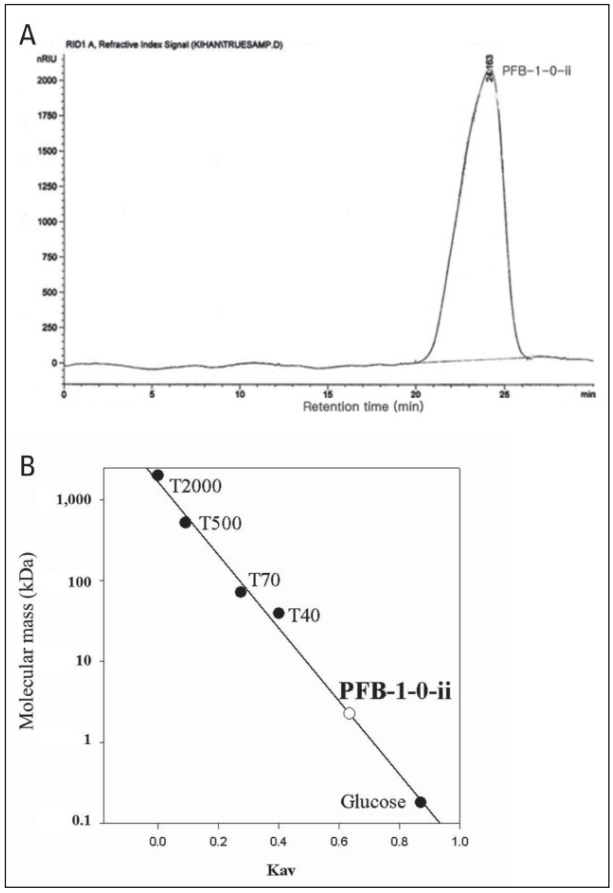

Figure 3. HPLC profile of purified fraction PFB-1-0-ii. HPLC was performed on a HP Agilent series 1690 instrument equipped with continuous two columns (TSKgel G5000PW and TSKgel G3000PW) and developed with 0.2 mol/L NaCl. Refractive index detector was used.

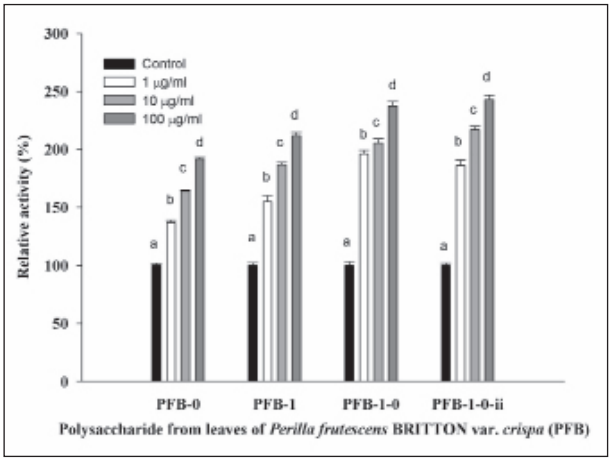

Figure 4. Macrophage lysosomal phosphatase activities of purified fractions obtained during purification process. The lysosomal enzyme activity was calculated as the percentage of the absorbance of a sample to a control (saline) at 405 nm. The values shown are means ± SD (n = 3) and means with different letters differ significantly from the same sample ($P < 0.05$), as determined by Duncan's multiple range test.

Macrophage stimulating activities of water and various solvents extracts such as ethanol, methanol, butanol, acetone, 0.8% HCl, 0.95% NaCl, 0.1 mol/L NaOH, and 2.5 mol/L NaOH containing 8% urea were tested. When macrophage stimulatory activities (relative activity, %) at three concentration of the samples (1, 10, 100 μg/ml) were tested, water extract showed higher macrophage stimulatory activities than other extracts at tested all concentrations (Data not shown). Therefore, water extract was selected for further investigations on macrophage cellular lysosomal enzyme activities of fractions obtained during purification process. The two crude fractions, PFB-0 and PFB-1, showed higher macrophage cellular lysosomal enzyme activity of more than 190% of relative value at 100 μg/ml PFB-0 or PFB-1 treated primary macrophage (Fig. 4). PFB-1-0 isolated from PFB-1 by ion-exchange column chromatography was more effective macrophage stimulators than PFB-0 and PFB-1. PFB-1 and PFB-1-0 exhibited 214% and 240% macrophage stimulatory relative activity in 100 μg/ml PFB-1 or PFB-1-0 treated primary macrophage. Continuously, PFB-1-0 was divided into two subfractions (PFB-1-0-i and PFB-1-0-ii) by gel permeation column chromatography. PFB-1-0-ii, high molecular portion, showed higher macrophage stimulating activ-

Table 2 .Sugar composition of active fractions obtained from purification step on *Perilla frutescens* BRITTON var. *crispa* (Unit: mol %)

Sugar composition*	PFB-1	PFB-1-0	PFB-1-0-ii
Rhamnose	-	-	-
Fucose	-	-	-
Arabinose	10.3 ± 0.6	8.9 ± 1.1	9.6 ± 1.0
Xylose	1.3 ± 0.1	0.9 ± 0.1	0.2 ± 0.0
Mannose	20.3 ± 3.5	25.9 ± 2.6	39.8 ± 3.8
Galactose	17.6 ± 2.1	16.8 ± 1.6	14.5 ± 1.3
Glucose	18.9 ± 1.7	24.9 ± 2.3	45.6 ± 4.1

*Alditol acetate derivatives prepared from the acid-hydrolysates of polysaccharides were analyzed by gas liquid chromatography.

ities than that of PFB-1-i (Data not shown). Among the samples tested, PFB-1-0-ii exhibited the highest macrophage cellular lysosomal enzyme activities. PFB-1-0-ii at a concentration of 100 μg/ml increased the macrophage cellular lysosomal enzyme activities by a 245% (relative activity), compared to untreated control (100%). The macrophage cellular lysosomal enzyme activities of PFB-0, PFB-1, PFB-1-0 and PFB-1-0-ii were increased in a dose-dependent manner. The macrophage cellular lysosomal enzyme activities of samples at all concentration were found to be in the following order: PFB-1-0-ii > PFB-1-0 > PFB-1 > PFB-0. The macrophage-stimulating activity was significantly enhanced during the purification step (Fig. 4). These results are practically consistent with reports of other investigators (12, 15). Many algal polysaccharides have been reported for their immunostimulatory activities *in vitro* and *in vivo* as they modify the activity of macrophages (14, 16-18). Purified polysaccharide isolated from *Capsosiphon fulvescens*, a green alga, can modulate the immune function of macrophages (14).

In conclusions, PFB-1 and PFB-1-0 were fractionated and purified, and the last purified homogeneous macrophage-stimulating fraction PFB-1-0-ii showed the highest lysosomal enzyme activity. The polysaccharide portion, the high molecular mass fraction, showed potent macrophage-stimulating activity. In addition, PFB-1-0-ii consists of mannose, galactose and glucose as major sugars. The molecular weight of the last purified polysaccharide PFB-1-0-ii was estimated approximately 10 kDa. PFB-1-0-ii may therefore be interesting compounds for the development of novel immunostimulatory activity drugs and functional food. However, further investigations characterizing PFB-1-0-ii are need. In addition, further animal and human studies are needed to determine whether dietary feeding of PFB-1-0-ii can increase immunostimulatory activity as observed in the present in vitro study. We also need mechanistic studies on PFB-1-0-ii.

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