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Isolation and Immunomodulating Activity of an Extracellular Polysaccharide Produced by *Bacillus* sp. PS-12

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A bacterial strain producing highly viscous extracellular polysaccharide was isolated from soil. Through morphological, physiological and chemotaxonomical studies, it was identified as a *Bacillus* sp. and named *Bacillus* sp. PS-12. The extracellular polysaccharide, named PS-12 was purified by ethanol precipitation, cetylpyridinium chloride (CPC) precipitation and gel permeation chromatography. The purified polysaccharide was found to consist of glucose, mannose, galactose, and fucose, with a molar ratio of approximately 7:3.2:2:1, respectively. PS-12 was investigated for its immunostimulating activity on murine macrophage RAW264.7 cells using an ELISA assay. PS-12 stimulated the production of TNF-α to a level 50 times greater than the control and also induced IL-6 secretion in a dose-dependent manner. The cytotoxicity on RAW264.7 cells by PS-12 was relatively low with 10% cytotoxicity at 2 μg/ml. These results indicate that PS-12 is less cytotoxic to immune cells and possess immunomodulating activity in which it can produce cytokines including TNF-α and IL-6 from macrophages.

Key words : *Bacillus* sp., extracellular polysaccharide, immunomoulating activity

Introduction

Many microorganisms have an ability to synthesize extracellular polysaccharides and excrete them out of the cell either as soluble or insoluble polymers. Many kinds of extracellular polysaccharides produced by microorganisms have been discovered and developed for commercial application [1,9,23]. These polysaccharides produced by bacteria have characteristic rheological and physiological properties which are different from those of natural gums and synthetic polymers. They are biodegradable and generally not harmful to the environment. Therefore, microbial polysaccharides have recently attracted much attention as a subject for research. Some of these polysaccharides have found many applications in various areas of industry such as oil recovery, foods, detergents, textiles, adhesives, cosmetology, pharmacology, paints, concretes, and wastewater treatments [5,29]. Biological functions of microbial polysaccharides led many investigators to apply these polysaccharides to human and veterinary healthcare, and thus production and applications of polysaccharides as therapeutic agents have been increasingly important topics of intensive researches.

Macrophages play an important role in regulating innate immunity as well as adaptive immune responses by production of cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ), and various types of chemokines such as RANTES, MCP-1, MIP-1α, TARC etc [14,24]. Splenocytes, mainly composed of immune-related cells such as T cells, B cells dendritic cells and macrophages, regulate humoral and cellular immune responses against foreign antigens, like bacteria and viruses, and endogenous antigens, like tumors. The immunomodulating activity by splenocytes is partly associated with the production of cytokines and chemokines [13]. TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. IL-6 also considered an important mediator of acute inflammatory responses. Moreover, IL-6 functions as a differentiation and growth factor of hematopoietic precursor cells, B cells, T cells, keratinocytes, neuronal cells, osteoclasts, and endothelial cells [2,4].

Several polysaccharides isolated from natural sources, bacteria and some fungi have immunomodulating activities. Numerous studies have shown that (1→3)-β-D glucans isolated from mushrooms enhance the functional status of macrophages and neutrophils [34] and modify immunosuppression [6]. Capsular polysaccharides isolated from *Klebsiella pneumoniae* serotype 1 (K1) and 3 (K3) could induce TNF-α in mice [8]. Peptidoglycan (PG), a polymer present in the cell wall

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of almost all bacterial species, possesses various immunomodulating activities [12]. PG is a mitogen [10,16] and a polyclonal activator [11] of B cells and induces macrophages to secrete various cytokines, including IL-1, IL-6, and TNF-α [20].

In the course of our studies on screening and industrial application of microbial polysaccharides, we isolated a bacterial strain from soil producing highly viscous polysaccharides. This polysaccharide was purified, characterized, and examined for its immunomodulating activity in murine macrophage, RAW264.7 cells.

Materials and Methods

Microorganism and culture medium

Exopolysaccharide-producing bacteria were isolated from many kinds of soil samples. The composition of the screening medium was as follows : 40 g glucose, 1.0 g NH_4NO_3, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot 4H_2O$, 0.05 g NaCl, and 0.1 g yeast extract in 1 l distilled water. The initial pH of the medium was adjusted to 7.0 with 1.0 N NaOH. The culture was deriver from a single colony and grown for 36 h on a rotary shaker at 30°C.

Measurement of apparent viscosity

The apparent viscosity of cell-free solution was measured by Brookfield Digital rheometer (model DV-Ⅲ, USA) equipped with a much-sample adapter (spindle SC4-34) and small-sample adapter (spindle SC4-18).

Identification of strain PS-12

The isolated strain PS-12 was identified from its morphological and physiological properties, according to Bergey's Mannual of Systemetic Bacteriology [28]. The chemotaxonomical characteristics of the strain were examined by the procedures of Komagata and Suzuki [22], Miller [26], and Tamaoka and Komagatas [32].

Preparation of extracellular polysaccharide (EPS) hydrolysate

Purified EPS was dissolved in 20-ml of distilled, deionized water (ddH_2O, 0.2%, w/v) and then hydrolyzed at 121°C for 1 hr after adding an equal volume of 4.0 M trifluoroacetic acid (TFA). Insoluble materials were removed by centrifugation at 35,000× *g* for 30 min and the supernatant was filtered through a 0.2-μm pore-size filter. The filtrate was dried on Speed Vac and redissolved in ddH_2O. To completely remove residual TFA, this procedure was repeated 3-4 times.

Analysis of sugar components

Complete hydrolysis of the PS-12 was carried out with 4.0 M TFA at 121°C for 1 hr. After hydrolysis, the solution was neutralized with 1.0 N NaOH and lyopphilized. Sugar constituents in the TFA-hydrolyzate were analyzed by HPLC. Monosaccharide composition of PS-12 was analyzed by applying the acid hydrolysate to an HPLC system equipped with Microspherogel carbohydrate column (6.5×300 mm, Beckman, U.S.A.) and Refractive Index Detector (RID). Carbohydrates were eluted with water at a flow rate of 0.5 ml/min.

Cell culture

The RAW264.7 cells (ATCC TIB-71) were maintained in RPMI1640 (Gibco, USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (Gibco, USA). The cells were grown at 37°C in a humidified 5% CO_2 incubator.

MTT cytotoxicity assay

RAW264.7 cells (5×10^4 cells/well) were plated on a 96-well microplate in RPMI1640 media. PS-12 and lipopolysaccharide (LPS) were dissolved in 0.1% dimethylsulfoxide (DMSO) in serum free media. Different concentrations of the PS-12 were prepared with serial dilution in triplicate. 0.1% DMSO in serum free media was used as a control. The toxicity profiles of PS-12 were assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay as described by Mosmann [27]. After 24 hr of incubation, MTT (5 mg/ml) was added to each well and the plates incubated for a further 4 hr and the media removed. DMSO was later added into each well to solubilize the formazan crystals. The absorbance was read at 595 nm using a Microplate reader (Molecular Devices, USA).

Assay for TNF-α and IL-6 release

TNF-α and IL-6 release were tested on the murine RAW264.7 cell line, using an ELISA assay (Quantikine Immunoassay Mouse TNF-α, Quantikine Immunoassay Mouse IL-6, R&D systems, USA). RAW264.7 cells (5×10^4

cells/well) were seeded on a 96-well microplate in RPMI1640 media. Cells were incubated for 24 hr in the presence of various concentrations of PS-12 with LPS (with or without test compounds). Treated LPS was *Escherichia coli* EH100 endotoxin (Sigma). Supernatants were harvested and cytokine release was measured by optical density following the manufacturer's instructions.

Results and Discussion

Screening and identification of the EPS-producing bacteria

For the screening of EPS-producing bacteria, more than 200 bacterial strains which excreted mucous material on the agar plate of the screening medium were isolated from soils. A mucoid collony on the agar plate presumed to be an EPS producer was cultured in a 250-ml Erlenmeyer flask 50 ml of the culture medium. For selection, materials produced from each isolate were tested for viscosity and pH stability. Among the materials tested, mucoid from the strain PS-12 showed the highest viscosity and relative stability in a wide range of pHs (pH 4~12). From these results, strain PS-12 was selected to be the most suitable candidate as a practical EPS producer.

The morphological and biochemical characteristics of strain PS-12 are shown in Table 1. After incubation for 3 day at 30°C on glucose-nutrient agar medium, colonies were circular, convex, and milky-white. Strain PS-12 was rod shaped, gram-positive, and formed spores. This strain grew in the medium with 7% (w/v) NaCl, at temperature of 25°C and 40°C but did not grow at 65°C. The isolated strain was able to liquefy gelatin and to form indol. The strain showed a positive reaction in Voges-Proskauer and catalase test, and produced acid from glucose, arabinose, mannitol and xylose. Thus it was considered to belong to *Bacillus* sp. as reported by Gorden *et al.* [15].

To elucidate the relationship between strain PS-12 and the Genus *Bacillus* in detail, its chemotaxonomical characteristics were examined. The peptioglycan of the strain had the meso-type of diaminopimelic acid in the *Bacillus* sp. [28]. The main fatty acids were branched-chain fatty acids, such as 13-methyl tetradecanoic acid (iso-15 : 0) and 12-methylteradecanoic acid (anteiso-15 : 0); this finding is in agreement with that of Suzuki and komagata [30]. Quinone composition and G+C molar ratio of strain PS-12 wered menaquinone-7 (MK-7) and 45.20%, respectively.

Table 1. Characteristics of strain PS-12

Characteristics	Results
Morphological	
Gram staining	+
Shape	rod
Width of rod	0.9~1.1 μm
Length of rod	3.2~3.7 μm
Spore	+
Motility	+
Acid fast	—
Cultural	
Colony color	milky-white
Growth in air	+
Growth anaerobically	−
Growth at 25°C	+
Growth at 40°C	+
Growth at 65°C	−
Growth at pH 5.7	−
Growth at 7% NaCl	+
Physiological	
Catalase	+
Oxidase	+
O/F (Oxidation/Fermentation)	F
Carbohydrates, acid from	
glucose	+
arabinose	+
sucrose	+
xylose	+
Hydrolysis of	
gelatin	+
starch	+
casein	+
Voges-Proskauer reaction	−
Nitrate reduction	+
Indole reduction	+
Urease	−
Chemical	
DAP (Diaminopimelic acid)	*meso* type
Fatty acid	bracnched C_{15}
Quinone	MK-7 (menaquinone)
G+C molar ratio	45.20%

When the keys to genera listed in Bergey's Manual [28] were traced on the basis of the these results, strain PS-12 was identified as *Bacillus* species, and designated as *Bacillus* sp. PS-12.

Production of EPS

In the flask, the optimum medium for the production of EPS was as follows: 30 g glucose, 0.7 g NH_4NO_3, 2.0 g K_2HPO_4, 0.1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot 4H_2O$, 0.07 g NaCl, in 1 l distilled water. The initial pH was 8.0 and the culture temperature was 30°C. In the optimized

medium, the EPS was produced in a yield 2 fold higher than in the basal medium.

In the jar-fermentor culture, the culture broth become progressively viscous with the production of EPS. The EPS production began in an early exponential phase of growth and continued until it reached the stationary phase of growth. It was apparent that EPS production by *Bacillus* PS-12 was definitely growth-dependent. The cell concentration increased gradually with the extension of cultivation time and reached the maximum value of 3.27 g-DCW/l after 70 hr of cultivation. The concentration of EPS increased in proportion to the increasing cell concentration and reached the maximum level of 13.5 g/l after 65 hr of cultivation. This result indicates that the concentration of EPS from *Bacillus* sp. PS-12 is much higher than that of other polysaccharide-producing microorganism [3,31]. The viscosity of the culture broth at that time was 86,200 centipoise, and then, decreased gradually with prolonged cultivation. Because the concentration of EPS remained constant, the decrease of the viscosity in later stages of cultivation might suggest that structural changes occurred during this stage.

Purification of EPS

In order to remove the bacterial cells, the culture broth (5 l) of *Bacillus* sp. PS-12 was diluted with ten vol. of distilled water. Most of the bacterial cells were removed by centrifugation at 9,000× *g* for 30 min. The cell-free culture broth was concentrated to 2.1 l. The concentrated supernatant (2.1 l) was precipitated crude EPS was dried with a vacuum evaporator (2.8 g), and redissolved in distilled water. Then, 10% CPC solution was added until no more precipitate was formed. The insoluble EPS-CPC complex was collected by centrifugation, and redissolved in a 10% sodium chloride solution. After dialysis against distilled water, the EPS was precipitated by the addition of two vol. of ethanol and dissolved in distilled water. The EPS obtained was dialyzed against distilled water and lyophilized. Purified EPS solution (0.1%) was loaded onto a Sephacryl S-500 column (4.3×62 cm) and eluted with distilled water. Fractions of 5 ml each were collected and examined by the phenol-sulfuric acid method [7]. Fractions containing EPS were combined and lyophilized. The final preparation was named PS-12.

Chemical composition of PS-12

The sugar constituents in the TFA-hydrolyaste were glucose, mannose, galactose, and fucose in an approximate molar ratio of 7:3.2:2:1. According to an elemental analysis, the constituent elements were 39.3% carbon, 6.7% hydrogen, 42.5% oxygen, and 0.6% nitrogen. The phenol-sulfuric acid method [7] showed that the polysaccharide contained 83.2% (w/w) of total sugar. In the hydrolysate of PS-12, 4.15% acetic acid, 5.62% pyruvic acid, 30.32% uronic acid and 5.13% protein were detected (Table 2). The protein might be bound to the polysaccharide by electrostatic interaction between protein and negatively charged groups of the polysaccharide.

Table 2. Chemical components of PS-12

Chemical components	Contents (w/w, %)	Elemental analysis	Contents (w/w, %)
Total sugar	82.20	Carbon	39.3
Protein	5.13	Hydrogen	6.7
Acetic acid	4.15	Oxygen	42.5
Pyruvic acid	5.62	Nitrogen	0.6
Uronic acid	30.32	Sulfur	0.2

Cytotoxicity of PS-12

The direct cytotoxicity of PS-12 and LPS were tested against the macrophage-like cell line, RAW264.7. The cells were treated with the various concentration of PS-12 (0-10 μg/ml). When the concentration of PS-12 was 2 μg/ml, cell viability was approximately 90%. Therefore maximum concentration of PS-12 was determined to 2 μg/ml (Fig. 1A). Then 2 μg/ml of PS-12 and different concentration of LPS (0-5 μg/ml) were treated to RAW264.7. Nearly 0.1 μg/ml of LPS was no influence of cell cytotoxicity (Fig. 1B). From this analysis, treatment with 0.1 μg/ml of LPS and maximum 2 μg/ml of PS-12 were not found to have any appreciable effect on cell viability.

Macrophage activation by PS-12

Macrophages are the first line of defense among innate immune responses against microbial infection [19]. RAW264.7 cells are known to produce cytokines in response to the addition of LPS, and this system is used to detect the modulating activities of compounds on cytokine production. The defense mechanism of macrophages against pathogens includes proinflammatory cytokines such as TNF-α, IL-1, IL-6, and others [25]. PS-12 stimulated the production of TNF-α to a level 50 times greater than the normal (Fig. 2A). Also PS-12 induced IL-6 secretion from RAW264.7 cells in a dose-dependent manner (Fig. 2B).

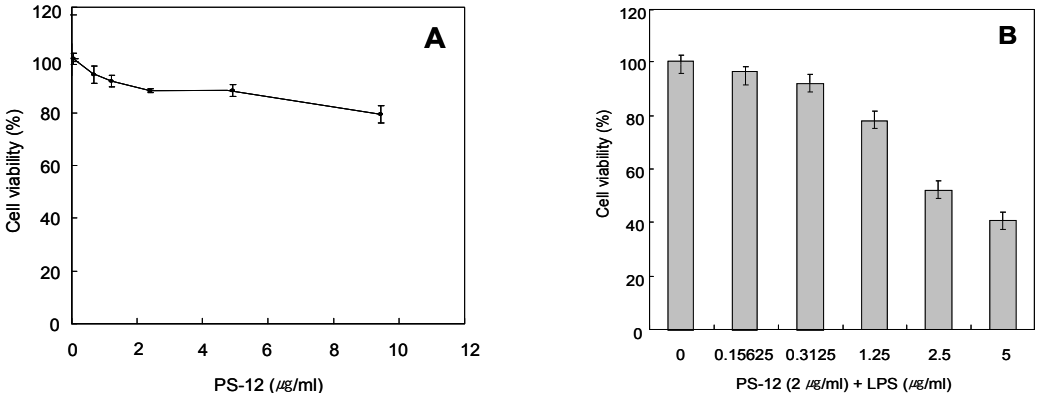

Fig. 1. Cell cytotoxicity of polysaccharides extracted from PS-12. Cells were incubated for 24 hr in the presence of PS-12 (A) and 2 μg/ml of PS-12 with various concentrations of LPS (B)

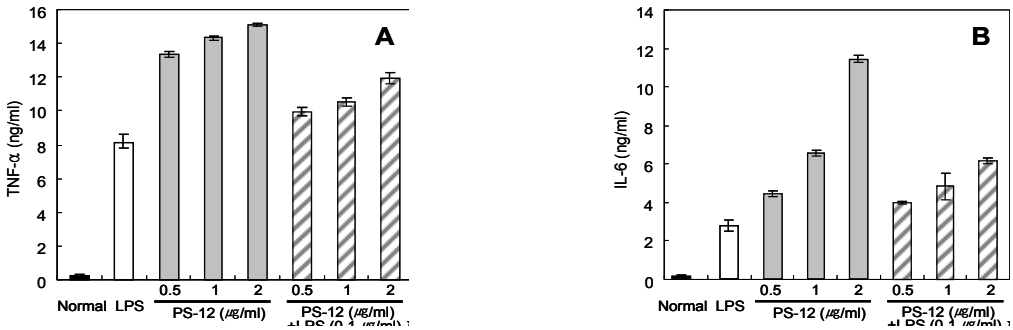

Fig. 2. Immunomodulating effects of purified polysaccharides extracted from PS-12. RAW264.7 cells were co-incubated with the indicated doses of PS-12 for 24 hr. As a control, commercial LPS (0.1 μg/ml) was used. The level of TNF-α (A) and IL-6 (B) in the supernatants of the cultures was determined by ELISA kit. The cells were treated with the mixture of LPS (0.1 μg/ml) and different concentration (0.5, 1, 2 μg/ml) of PS-12.

These results suggest that PS-12 may have beneficial effects on immunostimulation.

On the other hand, when the cells were co-treated with PS-12 and LPS, the TNF-α secretion was slightly decreased and, especially, IL-6 level was markedly reduced (Fig. 2). Several LPS receptors have been identified in different macrophages [18,33]. The most thoroughly studied receptor is CD14, which has been identified in several types of macrophages and macrophage cell lines, including the mouse peritoneal macrophage cell line, RAW264.7 [17,21]. Thus, the significant reduction in IL-6 induction by the co-treatment of PS-12 and LPS suggests that CD 14 may be the possible receptor for our PS-12 on RAW264.7 cells.

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초록 : *Bacillus* sp. PS-12가 생산하는 extracellular polysaccharide의 분리 및 immunomodulating activity

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토양에서 분리한 세포외 다당류 생산균주 PS-12는 형태학적, 생리학적, 화학적 분석에 의하여 *Bacillus* sp.에 속하는 균주로 동정되었으며, 분리균주 PS-12는 *Bacillus* sp. PS-12로 명명하였다. *Bacillus* sp. PS-12가 생산하는 세포외 다당류는 에탄올 침전, cetylpyridinium chloride (CPC) 침전과 gel permeation chromatography를 이용하여 정제하였으며, 정제된 다당류 PS-12의 당조성은 glucose, mannose, galactose와 fucose가 7:3.2:2:1의 몰비로 구성되어있었다. *Bacillus* sp. PS-12로부터 분리된 다당류 PS-12를 이용하여 면역증강효과를 확인하였다. TNF-α 및 IL-6 측정은 RAW264.7 대식세포주를 사용하였으며 cytokine정량을 위하여 ELISA kit를 이용하였다. RAW264.7 세포주에 대한 PS-12의 세포독성을 확인하기 위하여 세포독성이 10% 미만을 나타내는 농도인 2 μg/ml을 PS-12의 최대농도로 측정하였다. PS-12는 2 μg/ml에서 TNF-α를 정상세포보다 50배 이상 높은 수치로 생산하였다. 또한 IL-6의 생산을 농도 의존적으로 증가시켰다. 이러한 결과로부터 PS-12가 면역세포에 대해 세포독성을 거의 나타내지 않는 농도에서 대식세포로부터 TNF-α와 IL-6의 cytokine 생산을 함으로써 면역증강효과를 나타낸다는 것을 확인하였다.