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# In Vivo Anti-influenza Virus Activity of an Immunomodulatory Acidic Polysaccharide Isolated from *Cordyceps militaris* Grown on Germinated Soybeans

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An acidic polysaccharide (APS) was isolated from the extract of *Cordyceps militaris* grown on germinated soybeans. Analyses of sugar composition indicated that APS consisted of D-galactose, L-arabinose, D-xylose, L-rhamnose, and D-galacturonic acid. On the basis of the result of methylation analysis, APS was considered to be mainly composed of Ara*f*-(1→, →5)-Ara*f*-(1→, →4)-Gal*p*-(1→ and →4)-GalA*p*-(1→ residues. When the polysaccharide was intranasally administered, it decreased virus titers in the bronchoalveolar lavage fluid and the lung of mice infected with influenza A virus and increased survival rate. Furthermore, APS increased TNF-α and IFN-γ levels in mice when compared with those of untreated mice. APS enhanced nitric oxide (NO) production and induced iNOS mRNA and protein expressions in RAW 264.7 murine macrophage cells. The induction of mRNA expression of cytokines including IL-1β, IL-6, IL-10, and TNF-α was also observed. These results demonstrated that APS might have beneficial therapeutic effects on influenza A virus infection at least in part by modulation of the immune function of macrophages.

**KEYWORDS: Anti-influenza virus activity; polysaccharide; *Cordyceps militaris*; soybean; macrophage**

## INTRODUCTION

Recently, there have been increasing risks of emerging viral infectious diseases such as avian influenza virus. Influenza is a highly contagious, acute respiratory disease that affects all age groups. A major problem is that influenza shows high genetic variability, resulting in the rapid emergence of antiviral drug resistant pathogens. The two medicinal approaches available for the control and prevention of influenza are the use of vaccines and the use of antiviral agents (*1*). Although vaccines are effective methodology for the prevention of infection, their efficacy of protection from a possible new pandemic influenza virus strain strongly depends on the vaccine quality. From another perspective, it is suggested that the stimulation of host innate immune response might provide prophylactic protection against infectious diseases. One advantage of this strategy is to prevent infection not only by the specified virus but also by other infectious organisms. To accomplish this, it is thought that food ingredients might be favorable resources employed to stimulate the host immune response. So far, many functional foods and ingredients have been studied for their effects on the immune system (*2*). Among food ingredients, some non-nutritional polysaccharides (dietary fibers) are known to possess immunomodulatory effects. The function of polysaccharides has attracted much attention in the biomedical area because of their broad spectrum of therapeutic properties and their relatively low toxicity (*3*, *4*). Therefore, the discovery of novel polysaccharides from edible natural resources would be important in the development of therapeutic agents for infectious diseases.

Various fungi have been traditionally used in many countries for health maintenance as well as prevention and treatment of various diseases. *Cordyceps militaris* is a representative insect-borne fungus that has been folklorically used. Various biological activities such as antifibrotic, anti-inflammatory, and antinociceptive activities have been reported (*5*, *6*). However, naturally occurring *C. militaris* is not easily available for food in large amounts because of its high production cost. Recently, however, Park et al. developed a method of growing *C. militaris* on germinated soybeans known to be rich in nutrients and biologically active compounds such as isoflavones (*7*, *8*). Thus, *C. militaris* grown on germinated soybeans might be a promising source for nutraceutical applications.

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In this paper, we describe the isolation of an acidic polysaccharide (APS) from *C. militaris* grown on germinated soybeans and the evaluation of its usefulness as an anti-influenza agent. The information of this study is valuable for the application of polysaccharides to initiate host immune function.

## MATERIALS AND METHODS

**Materials.** Eagle's minimal essential medium (MEM) and Dulbecco's MEM (DMEM) were obtained from Nissui Pharmaceutical (Tokyo, Japan). DEAE 650 M and $GMPW_{XL}$ columns were purchased from Tosoh (Tokyo, Japan). Sepharose 6B and Sephacryl S-500 HR were from GE Healthcare (Piscataway, NJ). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Extraction and Fractionation.** Mycelium of *C. militaris* was inoculated on germinated soybeans and cultured at 20–25 °C for 50 days. The cultured material (200 g) was ground and extracted with boiling $H_2O$ for 1 h under reflux. The obtained extract was filtered, and the residue was extracted in the same manner. After extraction three times, the combined extract was concentrated in vacuo and lyophilized to give a brown powder (C, 56.2 g). C was dissolved in $H_2O$ and centrifuged at 9000 rpm for 15 min to remove the insoluble portion. The soluble portion was dialyzed with a seamless cellulose tube (MWCO: 14,000, Wako Pure Chemicals) against $H_2O$, and the nondialysate was lyophilized to give CH (yield = 39%). CH dissolved in $H_2O$ was applied to a Toyopearl DEAE 650 M anion exchange column (5 i.d. × 15 cm; Tosoh Corp.), which was successively eluted with $H_2O$, 0.5 M NaCl, 1.0 M NaCl, 2.0 M NaCl, 4.0 M NaCl, and 0.2 M NaOH. The yields of the eluates were 12% (CH-1), 58% (CH-2), 2.2% (CH-3), 1.2% (CH-4), 2.2% (CH-5), and 4.9% (CH-6), respectively. CH-2 was applied to a Toyopearl DEAE 650 M column (5 i.d. × 10 cm), which was eluted with a linear gradient system prepared with $H_2O$ and 2 M NaCl. Fractions of 10 mL were collected and monitored by the phenol−$H_2SO_4$ method (*9*) and UV absorbance at 260 nm. CH-2A (0.8%), CH-2B (0.6%), CH-2C (46%), and CH-2D (23%) were obtained on the basis of their elution profiles. CH-2C was applied to a Sepharose 6B gel filtration (4.4 i.d. × 90 cm; GE Healthcare UK Ltd., Buckinghamshire, U.K.) and eluted with 0.01 M citrate buffer containing 0.1 M NaCl. Fractions of 15 mL were collected and monitored by the phenol−$H_2SO_4$ method and UV absorbance at 260 nm to give two fractions, CH-2C-1 (42.9%) and CH-2C-2 (28.6%). CH-2C-1 was subjected to a Sephacryl S-500 HR gel filtration (2.2 i.d. × 95 cm; GE Healthcare UK Ltd.), which was eluted with 0.01 M citrate buffer containing 0.1 M NaCl. Fractions of 5 mL were collected and monitored by the phenol−$H_2SO_4$ method and UV absorbance at 260 nm (yield = 65.5%). The fraction containing carbohydrate was rechromatographed on Sepharose S-500 HR to give a polysaccharide (APS).

**Cellulose Acetate Membrane Electrophoresis.** APS was applied to a cellulose acetate membrane (Separax; Jokoh Co. Ltd., Tokyo, Japan) in 0.1 M pyridine/0.47 M formic acid buffer (pH 3) and run at 1 mA/cm. The membrane was stained with 0.25% toluidine blue.

**Estimation of Apparent Molecular Weight.** The apparent molecular weight of the polysaccharide was estimated by HPLC analysis. The sample was applied on TSK $GMPW_{XL}$ gel filtration columns (7.6 × 300 mm × 2; Tosoh Corp.) and eluted with 0.1 M NaCl at 0.5 mL/min. Commercially available pullulans (Shodex P-52; Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers.

**Chemical Analyses of Polysaccharide.** Uronic acid content was determined by the *m*-hydroxydiphenyl method (*10*). Protein content was determined using a Bio-Rad protein assay kit. Sugar composition was determined as follows: APS (1 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. After removal of TFA under $N_2$ gas, the hydrolysates were converted to alditol acetates, which were analyzed by GC using an SP-2330 column (30 m × 0.32 mm i.d.; Supelco Inc., Bellefonte, PA) with the oven temperature of 200–240 °C (4 °C/min). Absolute configuration of monosaccharides was analyzed as 2-butyl-glycoside (*12*). The polysaccharide was methylated according to the Hakomori method (*11*). The methylated polysaccharide was hydrolyzed with 2 M TFA at 120 °C for 1 h, reduced with $NaBH_4$, and acetylated (*13*). The partially methylated alditol acetates were analyzed on GC using an SP-2330 fused silica capillary column and GC-MS using a DB-1MS fused silica capillary column. Identification of partially methylated alditol acetates was carried out on the basis of retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and its mass fragmentation patterns (*13*).

**Animals, Cells, and Virus.** All animals were obtained from Japan SLC, Shizuoka, Japan. All animal experiments were conducted in accordance with the animal experimentation guidelines of the University of Toyama. No observable side effects such as diarrhea due to drug administration were detected throughout the experiments. RAW 264.7 and MDCK cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and MEM supplemented with 5% FBS, respectively. Influenza A virus (NWS strain, H1N1) was grown on MDCK cells.

**In Vitro Antiviral Activity and Cytotoxicity.** For cell growth inhibition studies, MDCK cells were cultured for 72 h in the presence of increasing amounts of polysaccharide. Viable cell yield was determined by the trypan blue exclusion test. The inhibition data were plotted as dose–response curves, from which the 50% cell growth inhibitory concentration ($CC_{50}$) was obtained. Cell monolayers in 48-well plates were infected with virus at 0.2 plaque-forming unit (PFU) per cell at room temperature. After 1 h of viral infection, the monolayers were washed three times with phosphate-buffered saline (PBS) and incubated in maintenance medium (MEM plus 2% FBS) at 37 °C. Samples were added during infection and throughout the incubation thereafter or immediately after virus infection. Virus yields were determined by plaque assay after 2 days of incubation. The antiviral activity was expressed as the 50% replication inhibitory concentration ($IC_{50}$), which was the sample concentration that reduced plaque numbers by 50% in the treated cultures as compared with no drug control.

**Animal Experimental Design.** Female BALB/c mice (5 weeks old) were inoculated intranasally with 50 μL of viral suspension ($2 \times 10^5$ PFU/mouse). CH (2.5 mg/0.1 mL in PBS) was orally administered two times a day (every 12 h) from 7 days before virus inoculation to 3 days postinoculation. Control mice were treated orally with 0.1 mL of vehicle ($H_2O$) alone. APS was intranasally administered to anesthetized mice at a dose of 0.1 mg/15 μL per mouse 10 min before and after 2, 24, and 48 h of virus inoculation. Control mice were treated intranasally with 15 μL of vehicle ($H_2O$) alone. Mice ($n$ = 10–11) were observed for 28 days to monitor body weight change and mortality. In other groups of mice, blood, bronchoalveolar lavage fluid (BALF), and the lung were individually collected at 3 and 5 days after virus inoculation ($n$ = 5–6 each). Blood samples were centrifuged at 3000 rpm for 10 min, and sera were stored at −20 °C. Lung samples were sonicated for 10 s after the addition of 1 μL of PBS/mg of lung tissue and centrifuged at 10000 rpm for 30 min to separate the supernatants, which were then stored at −80 °C. BALFs were prepared by four washes with 0.8 mL of ice-cold PBS via a tracheal cannula and centrifuged at 1500 rpm for 10 min to obtain the supernatants, which were then stored at −80 °C.

**Cytokine Assays.** TNF-α and IFN-γ in blood, BALF, and lung homogenates were assayed by the ELISA technique. Each sample was diluted 10-fold with PBS, and then 50 μL of the dilution was incubated in a 96-well formatted flexible plate at 37 °C for 1 h. After three washings with PBS, uncoupled binding sites in the wells were blocked with 10% skim milk in PBS. After three washings with PBS containing 0.05% Tween 20, the plate was incubated at 37 °C for 1 h in the presence of rabbit antimouse/rat TNF-α or rabbit antimouse IFN-γ antibodies (BioSource International Inc., Camarillo, CA). The plate was then developed using peroxidase-labeled goat antirabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and peroxidase substrate.

**NO Production.** Accumulated nitrite, which is a stable oxidized products of NO, in the culture media of RAW 264.7 cells was measured using a colorimetric assay based on the Griess reaction (*14*). Briefly, the cells ($2.0 \times 10^5$) were seeded in a 96-well plate and incubated in the absence or presence of sample at 37 °C for 20 h. The culture supernatants were reacted with Griess reagent at room temperature for 10 min, and then nitrite concentration was determined by measuring the absorbance at 550 nm. The standard curve was obtained using the known concentrations of sodium nitrite.

**RT-PCR.** RAW 264.7 cells ($2.4 \times 10^5$) were incubated in the absence or presence of sample at 37 °C for 8 h. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was reverse transcribed from total RNA using a Superscript III system (Invitrogen, Carlsbad, CA). PCR amplification was carried out using Taq DNA polymerase (TaKaRa, Japan) and specific primers. The primers were as follows: iNOS, 5′-ACCTACTTCCTGGACATTACGACCC-3′ and 5′-AAGGAGCAATGCCCGTACCAGGCC-3′; IL-1$\beta$, 5′-GCAACTGTTCCTGAACTCAA-3′ and 5′-CTCGGAGCCTGTAGTGCAG-3′; IL-6, 5′-TTCCTCTCTGCAAGAGACT-3′ and 5′-TGTATCTC TCTGAAGGACT-3′; IL-10, 5′-TACCTGGTAGAAGTGATGCC-3′ and 5′-CATCATGTATGCTTCTATGC-3′; IFN-α, 5′-CTACTGGCCAA CCTGCTCTC-3′ and 5′-CTACTGGCCAACCTGCTCTC-3′; TNF-α, 5′-ATGAGCACAGAAAGCATGATC-3′ and 5′-TACAGGCTTGT CACTCGAATT-3′; $\beta$-actin, 5′-TGGAATCCTGTGGCATCCATGA AAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. $\beta$-Actin was used as internal standard. PCR was performed in an iCycler (Bio-Rad, Hercules, CA). The thermal cycle was as follows: 60 s of denaturation at 94 °C, 60 s of annealing at 53 °C (IL-6, 30 cycles), 55 °C (TNF-α, 25 cycles), 56 °C (IL-10, 30 cycles), 59 °C (IFN-α, 27 cycles), and 60 °C (IL-1$\beta$, iNOS, and $\beta$-Actin, 25 cycles), respectively, and 90 s of extension at 72 °C. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

**Western Blotting.** RAW 264.7 cells ($2.0 \times 10^6$) were plated on 35 mm dishes and incubated for 12 h with or without sample. The cells were washed three times with PBS and lysed with RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) followed by centrifugation. The supernatants were subjected to 12% SDS-PAGE and transferred onto a PVDF membrane. The membrane was washed with Tris-buffered saline (TBS) and blocked with 5% skim milk in TBS. After incubation with antibodies against iNOS or $\beta$-actin, the membranes were incubated with appropriate HRP-conjugated antibodies. The bands were developed by using a Chemiluminescence Luminol Reagent (Santa Cruz) according to the manufacturer's instructions.

**Statistical Analysis.** The data are presented as the mean ± SD. The differences between groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparisons was made by using Dunnett's multiple-comparison test. A comparison between the two groups was made by using Student's *t* test.

## RESULTS AND DISCUSSION

In the preliminary study for the evaluation of the usefulness of *C. militaris* grown on soybeans in the treatment of influenza infection, the hot water extract (CH) was applied to mice infected with influenza A virus. Reduced virus titers in lung tissues [$(74.4 \pm 22) \times 10^4$ pfu/100 mg] and BALF [$(58 \pm 47) \times 10^4$ pfu/100 $\mu$L] were observed at 3 days postinfection when compared with those of untreated mice [$(209 \pm 59) \times 10^4$ pfu/100 mg and $(237 \pm 132) \times 10^4$ pfu/100 $\mu$L for the lung and BALF, respectively). These results prompted us to isolate an active component in CH. CH was applied to a DEAE 650 M anion exchange chromatography and fractionated into six fractions. Among the fractions, CH-2 eluted with 0.5 M NaCl, which was the most abundant fraction, was subjected to anion exchange column chromatography on DEAE 650 M with linear gradient elution. The most abundant fraction (CH-2C) was collected and separated by gel filtration on Sepharose 6B and Sephacryl S-500. Analytical HPLC showed that the obtained fraction was eluted as a single and symmetrical peak on the chromatogram, and the fraction was detected as a single band on the cellulose acetate membrane electrophoresis (**Figure 1**). Thus, the fraction was a homogeneous polysaccharide on the basis of molecular weight and charge distribution. Its apparent molecular mass was estimated to be $5.76 \times 10^5$ Da by HPLC analysis. A Bradford assay revealed that the polysaccharide, APS, did not contain a protein portion because no color was developed. Uronic acid was detected by using the *m*-hydroxydiphenyl method (7%), and the IR spectrum also supported the presence of uronic acid because the C═O stretching absorption (1740 $cm^{-1}$) was observed. From the analysis of neutral sugar composition by the conversion of the hydrolysates into alditol acetates and (−)-2-butyl glycosides, APS was found to consist of D-galactose (58.3%), L-arabinose (27.8%), D-xylose (7.5%), and L-rhamnose (6.4%). The uronic acid present in the molecule was revealed to be D-galacturonic acid. Methylation analysis revealed that APS was considered to be mainly composed of Ara*f*-(1→, →5)-Ara*f*-(1→, →4)-Gal*p*-(1→ and →4)-GalA*p*-(1→ residues (**Table 1**). These results indicated that APS might be an arabinogalactan-type polysaccharide.

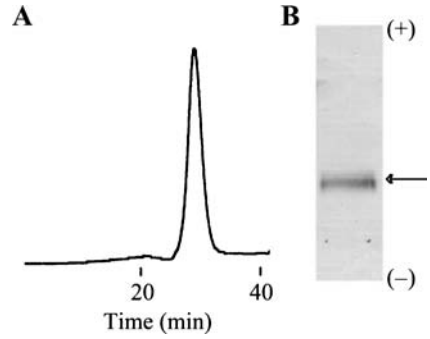


**Figure 1.** HPLC and cellulose acetate membrane electrophoresis of APS: (**A**) HPLC analysis was performed with a TSK-gel $GMPW_{XL}$ column (7.6 × 300 mm × 2) at 0.6 mL/min of 0.1 M NaCl (the peak was monitored by RI detector). (**B**) Electrophoresis was performed on a cellulose acetate membrane in pyridine−formic acid buffer (pH 3.0). Polysaccharide (the arrow) was stained with 0.5% toluidine blue.

**Table 1.** Result of Methylation Analysis of APS

| deduced linkage | methylated sugar | mol % |
|---|---|---|
| rhamnosyl | | |
| Rha*p*-(1→ | 2,3,4-$Me_3$-Rha[a] | 1.1 |
| →2)-Rha*p*-(1→ | 3,4-$Me_2$-Rha | 2.1 |
| →2,4)-Rha*p*-(1→ | 3-Me-Rha | 3.7 |
| arabinosyl | | |
| Ara*f*-(1→ | 2,3,5-$Me_3$-Ara | 6.1 |
| →5)-Ara*f*-(1→ | 2,3-$Me_2$-Ara | 8.9 |
| →3,5)-Ara*f*-(1→ | 2-Me-Ara | 2.5 |
| →2,5)-Ara*f*-(1→ | 3-Me-Ara | 0.7 |
| xylosyl | | |
| →3)-Xyl*p*-(1→ | 2,4-$Me_2$-Xyl | 1.9 |
| galactosyl | | |
| Gal*p*-(1→ | 2,3,4,6-$Me_4$-Gal | 3.7 |
| →4)-Gal*p*-(1→ | 2,3,6-$Me_3$-Gal | 51.4 |
| →3)-Gal*p*-(1→ | 2,4,6-$Me_3$-Gal | 0.8 |
| →3,4)-Gal*p*-(1→ | 2,6-$Me_2$-Gal | 4.5 |
| →2,4)-Gal*p*-(1→ | 3,6-$Me_2$-Gal | 2.9 |
| galacturonosyl | | |
| →4)-GalA*p*-(1→ | 2,3-$Me_2$-Gal | 9.8 |

[a] 2,3,4-$Me_3$-Rha was 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol.

The efficacy of intranasally administered APS against influenza virus infection was evaluated on the basis of the weight loss and survival rate (**Figure 2** and **Table 2**). APS (0.1 mg) was given intranasally four times, that is, at 10 min before infection and at 2, 24, and 48 h postinfection. During these experiments, no nasal exudates were observed by administration of APS. Control mice treated with vehicle showed high mortality (70%) with marked reduction of body weight. In mice treated with APS, although the loss of body weight was also found at a similar extent to that shown by control mice, the mortality (18%) was significantly lower than that of the vehicle group. These results suggested that intranasal APS would protect mice from lethal influenza infection.

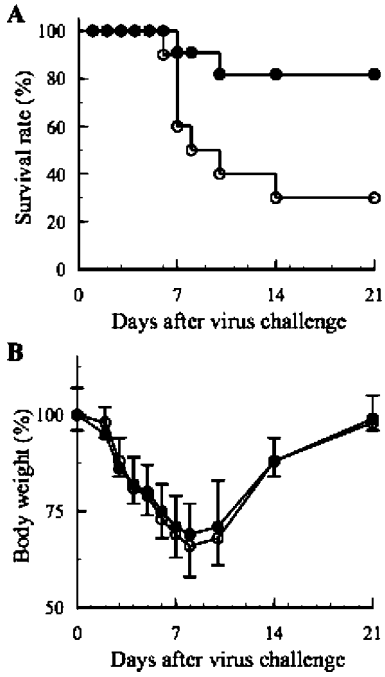


**Figure 2.** Effects of intranasal treatment of APS on survival rate (**A**) and body weight loss (**B**) in mice infected with influenza A virus (NWS strain, H1N1). The numbers of female BALB/c mice in the vehicle group (○, 3 survived) and the APS group (●, 9 survived) were 10 and 11, respectively. APS (0.1 mg/15 $\mu$L/mouse) was intranasally administered under anesthesia. After 10 min, influenza virus ($2 \times 10^5$ PFU/50 $\mu$L/mouse) was inoculated intranasally. After 2, 24, and 48 h postinfection, mice were anesthetized and intranasally administered the same dose of the sample. Control mice were treated intranasally with 15 $\mu$L of vehicle ($H_2O$) alone.

**Table 2.** Effect of APS on the Mortality of Mice Infected with Influenza A Virus

| | | | day of death | |
|---|---|---|---|---|
| treatment | no. of mice surviving/total | mortality (%) | mean ± SD | median |
| $H_2O$ | 3/10 | 70 | 7.7 ± 1.4 | 7.0 |
| APS | 9/11 | 18 | 8.5 ± 2.1 | 8.5 |

To examine whether or not APS could suppress the virus loads in mice, virus titers in the lung and BALF were determined at 3 and 5 days of virus inoculation. As shown in **Figure 3**, APS significantly decreased virus titers of both the lung and BALF when compared with control group. APS, however, showed no inhibitory effect on in vitro influenza virus replication (data not shown). These results suggested that APS did not show direct interaction with virus particles. Therefore, the protective effect of APS observed in mouse model was suggested to be involved in host mediated mechanism(s) such as the stimulating effect of host immune function. Thus, in the next experiments, the effects of APS were determined on the levels of TNF-α and IFN-$\gamma$, the cytokines having antiviral activities, in the blood, BALF, and lung samples of virus-infected mice (**Figure 4**). At 3 days postinfection, there was no difference in cytokine levels between vehicle and APS groups (**Figure 4A−C**). At 5 days postinfection, however, both TNF-α and IFN-$\gamma$ were increased in the blood and lung homogenate samples from APS-treated mice (**Figure 4D**,**F**), whereas less increase was observed in BALF (**Figure 4E**). Therefore, the protective effect of APS on lethal infection of influenza virus might be due at least in part to the enhancement of TNF-α and IFN-$\gamma$ production.

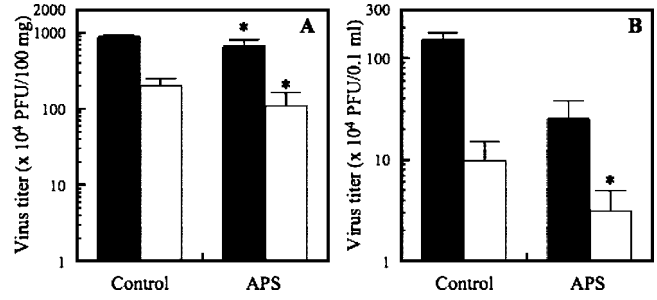


**Figure 3.** Effect of APS on the virus replication in the mice infected with influenza virus. BALB/c mice ($n$ = 5–6) were treated as described in **Figure 2**. Virus titers in the lung (**A**) and bronchoalveolar lavage fluid (BALF) (**B**) were determined at 3 (solid bar) and 5 days (open bar) after virus infection. Data are shown as mean ± SD. Asterisk indicates statistically significant differences as compared with vehicle controls: ∗, $p < 0.05$.

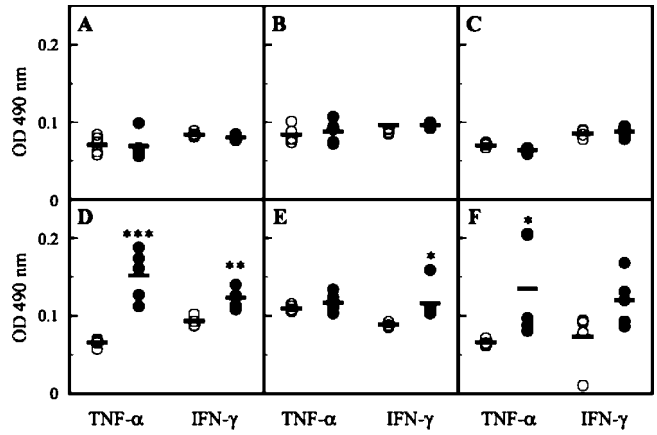


**Figure 4.** Effects of APS on TNF-α and IFN-$\gamma$ levels in mice infected with influenza virus. BALB/c mice ($n$ = 5–6) were treated as described in **Figure 2**. Blood (**A**, **D**), BALF (**B**, **E**), and lung samples (**C**, **F**) were collected from mice at 3 (**A**−**C**) and 5 days (**D**−**F**) after virus infection and subjected to the measurement of the cytokine levels by ELISA. Open and solid circles indicate vehicle- and APS-treated mice, respectively. Asterisks indicate statistically significant differences as compared with vehicle controls: ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$.

Although host defense mechanisms are complex, it is known that macrophages are key participants in the innate immune system's ability to respond to the invasion of pathogenic organisms. Thus, we investigated the effects of APS on the activation of macrophage (**Figure 5**). RAW 264.7 murine macrophage cells were incubated with APS for 20 h, and NO concentrations in the culture supernatants were measured by the Griess reaction. As shown in **Figure 5A**, APS showed a stimulatory effect on NO production in a dose-dependent manner. Unlike lipopolysaccharide (LPS), APS caused no significant difference in NO production between the treatment and without treatment with polymyxin B (data not shown). We further examined whether or not APS-induced NO production of macrophages was catalyzed by iNOS. When APS was administrated to cell culture at 100 $\mu$g/mL, expression levels of iNOS mRNA and protein were increased 3.4- and 3.2-fold in comparison with untreated control, respectively, by densitometric analyses. The results showed that APS induced iNOS mRNA expression (**Figure 5B**) and, hence, increased iNOS protein levels in RAW 264.7 cells (**Figure 5C**).

As many immunomodulatory compounds are known to modulate cytokine production, we analyzed the immunomodulatory properties of APS by detection of cytokine mRNA by

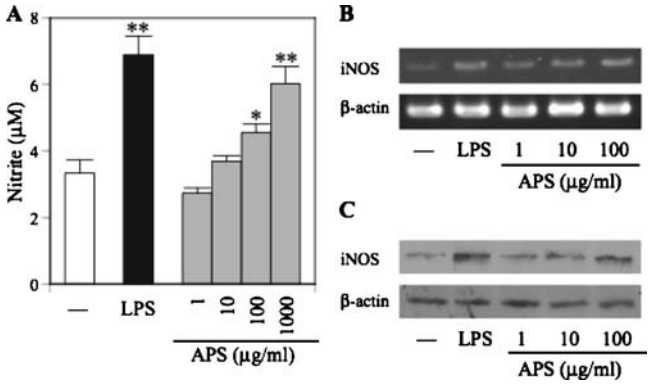


**Figure 5.** Effects of APS on NO production and iNOS expression in RAW 264.7 cells. (**A**) The cells were incubated with different concentrations of APS for 20 h. NO production was determined by measuring the accumulation of nitrite in the culture medium. No sample control (−) and lipopolysaccharide (LPS) (0.1 $\mu$g/mL) were also assayed. Data are mean ± SD of triplicate cultures, and asterisks indicate statistical difference compared with controls: ∗, $p < 0.01$; ∗∗, $p < 0.001$. (**B**) The cells were incubated with different concentrations of APS for 8 h. iNOS mRNA expression was detected by RT-PCR. (**C**) The cells were incubated with different concentrations of APS for 12 h. iNOS protein was detected by Western blotting.

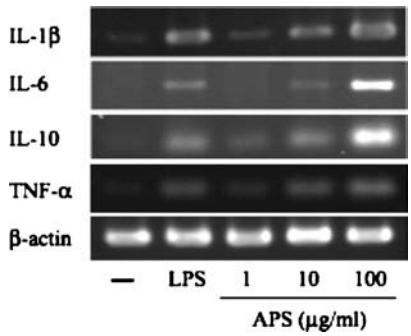


**Figure 6.** Effect of APS on cytokine mRNA expression in RAW 264.7 cells. RAW 264.7 cells were cultured in the absence or presence of LPS (0.1 $\mu$g/mL) or different concentrations of APS for 8 h. Cytokine mRNA expression was detected by RT-PCR.

RT-PCR. Treatment of RAW 264.7 cells with APS resulted in an increased mRNA expression of cytokines including IL-1$\beta$, IL-6, IL-10, and TNF-α in a dose-dependent manner (**Figure 6**). IFN-α mRNA expression, however, was not induced in APS-treated RAW 264.7 cells (data not shown).

Influenza A virus enters the host through the upper respiratory tract and encounters pulmonary phagocytic cells including alveolar macrophages, as an early line of defense. In many cases, macrophages are the first barrier of the defense system responding rapidly and creating an environment for a subsequent immune process (*15*). This study clearly demonstrated that intranasal administration of APS protected mice from lethal infection with influenza A virus. In vivo virus production in APS-treated mice was suppressed, whereas the polysaccharide exerted no in vitro inhibitory effect on influenza A virus replication. These observations suggested that host-mediated factors, especially immune systems, could contribute to the protective effect of APS on influenza A virus infection. Indeed, the levels of cytokines including TNF-α and IFN-$\gamma$ were increased in APS-treated mice. These cytokines are well-known to possess an antiviral effect against influenza virus infection. In particular, TNF-α has been reported to exert an anti-influenza virus effect, which is greater than that of $\gamma$- or α-interferon (*16*). Thus, the effect of APS on the stimulation of TNF-α and IFN-$\gamma$ production could contribute to its anti-influenza virus activity in animals. Ichinose et al. reported that prophylactic intranasal administration of chitin microparticles prevented pathogenesis of H1N1 and H5N1 influenza viruses (*17*). The polysaccharide-based particles also showed a protective effect on lethal infection with influenza virus and was suggested to stimulate an immunological reaction. Therefore, it was thought by analogy that intranasal administration of APS, another class of polysaccharides, could stimulate an immunological function, leading to the reduction of viral replication in vivo.

To elucidate the effect of APS on immune components, we studied its immunomodulating effect on the murine macrophage cell line, RAW 264.7. As a result, APS stimulated NO production through the induction of iNOS mRNA and the increment of iNOS protein level. NO has been shown to be a principal effector molecule produced by macrophages for cytotoxic activity and can be used as an index of macrophage activation (*18*). In addition, APS was shown to stimulate the induction of mRNAs of several cytokines including IL-1$\beta$, IL-6, IL-10, and TNF-α. Therefore, APS might enhance the production of both inflammatory (IL-1$\beta$, IL-6, and TNF-α) and anti-inflammatory (IL-10) cytokines. IL-1$\beta$, IL-6, and TNF-α are often implicated as key mediators produced by macrophages in response to infection and inflammatory stimuli. The release of pro-inflammatory cytokines is essential for host survival from infection and is also required for the repair of tissue injury. On the other hand, it has been suggested that IL-10 might act in a negative feedback mechanism to prevent potential detrimental effects from excessive macrophage activation during inflammation (*19*). It is known that the balance between pro-inflammatory and anti-inflammatory cytokines is crucial to the host's response to infection.

Structural analyses indicated that APS was an arabinogalactan-type polysaccharide. So far, many arabinogalactans have been isolated from various plant resources, and some of them showed immunomodulatory effects (*4*). Larch wood arabinogalactan has been suggested to be an activator of lymphocytes and macrophages, and its properties might contribute to immunoprevention of cancer (*20*). It has been reported that an arabinogalactan from *Juniperus scopolorum* also showed a similar cytokine production pattern in murine macrophage cell lines (*21*). Although their structural features for expressing immunomodulatory effects are still unknown, it was mentioned that high molecular mass arabinogalactans (200 and 680 kDa) showed potent immunomodulatory effects. As the apparent molecular mass of APS was estimated to be 576 kDa, its molecular mass might be sufficient to contribute to its immunomodulatory effect. Frequent contamination of LPS is always a matter of concern for tested compounds, because LPS is a strong activator of macrophages. In the present study, however, the macrophage activation by APS was not due to LPS contamination because it was not suppressed by polymyxin B treatment.

In summary, we have demonstrated that APS is a potent immunostimulating polysaccharide isolated from cultured *C. militaris* on germinated soybeans. The polysaccharide has in vivo anti-influenza virus A activity and stimulates NO production and cytokine mRNA expression in a murine macrophage cell line, RAW 264.7. From these results, APS isolated from cultured *C. militaris* on germinated soybeans is suggested to activate macrophage function and might have potential as a functional food source.

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