

## Immunomodulating Activity of Crude Polysaccharide from *Inonotus obliquus* Sclerotia by Fractionation including MeOH Reflux

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### Abstract

To obtain the immunomodulating polysaccharide from chaga mushroom (*Inonotus obliquus* sclerotia, IO), crude polysaccharide fractions (IO-M-CP and IO-CP, respectively) prepared from hot-water extract (IO-W) of *I. obliquus* by EtOH precipitation after MeOH reflux or not. After IO-W was re-dissolved in water followed by EtOH addition in the case without MeOH reflux, EtOH mixture was fractionated into EtOH-soluble (IO-E) and crude polysaccharide (IO-CP). In the meanwhile, MeOH-soluble fraction (IO-M) was separated from IO-W after MeOH reflux. The residue was dissolved in water and was added by EtOH, and then EtOH mixture was also fractionation into EtOH-soluble (IO-M-E) and crude polysaccharide (IO-M-CP). As a result of the macrophage stimulating activity of these fractions, IO-CP and IO-M-CP showed significantly increased cell proliferation and cytokines production than IO-W. Particularly, IO-M-CP promotes the production of IL-12 more than IO-CP. In the splenocytes proliferating activity and intestinal immune system modulating activity through Peyer's patch, both of 2 crude polysaccharide fractions were significantly promoted in cell proliferation and cytokines production than IO-W, and IO-M-CP was more potent than IO-CP in IL-2 production from splenocytes and GM-CSF production (10 µg/mL) in Peyer's patch cells. In addition, immunomodulating polysaccharide fractions (IO-M-CP and IO-CP) prepared from IO-W by EtOH precipitation with or without EtOH reflux showed no significant difference in the chemical composition and component sugar. These results suggested that MeOH reflux might exclude low-molecular weight materials from IO-W and consequently increase the immunomodulating activity of IO-M-CP. Therefore, it was confirmed that immunomodulation of polysaccharide prepared from hot-water extract of chaga mushroom was enhanced by fractionation including MeOH reflux and EtOH precipitation.

Key words: *Inonotus obliquus* Sclerotia, MeOH reflux, crude polysaccharide, immunomodulating activity, component sugar

### Introduction

A mushroom is the fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. Mushrooms have long been attracting a great deal of interest in many areas of foods and biopharmaceutical industries (Chang ST 1999). Edible and medicinal mushrooms have an established usage history in the human diet and traditional therapies, and the pharmacological importance of the mushrooms is very high in the Far East as a traditional medicine in treating various

diseases (Wasser SP 2002). For the mushroom, it has been ascribed that a number of biologically active and immunostimulatory ingredients (such as polysaccharides, glycoproteins and proteoglycans) stimulates various immune system responses in the host and inhibits tumor growth in preliminary research (Kim et al. 2006; Zhang et al. 2007; Lemieszek & Rzeski 2012). Especially, the polysaccharides isolated from sclerotia and submerged culture mycelia have been used as an immune-boosting agent, which is an ideal biological response modifier (BRM) that induces cellular and humoral immunities (Wasser SP 2002;

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Sánchez C 2004).

*Inonotus obliquus* is a black parasitic fungus that grows on the living trunks of mature birch and has been used as a folk remedy in North-East Asian countries, such as Korea, Japan, China, and in Russia for five hundred years (Xu & Zhu 2011). This dark, hard and cracked fungus, which often appears like burnt charcoal, is called the sclerotium (Campbell & Davidson 1938). *I. obliquus* is considered a medicinal mushroom in Russian and Eastern European folk medicine since the sixteenth centuries, and it has been reported by the antitumor, anti-oxidative, and hyperglycemic activities (Mizuno et al, 1999; Cui et al. 2005; Hu et al, 2009; Huang et al. 2012). For medicinal use, an extraction process is strongly needed to make at least some of the bioactive component bioavailable. Scientific studies and researches are generally focused on highly concentrated extracts, and traditional Russian usage is based on a form of hot-water extraction. In previous study, the extraction conditions of *I. obliquus* sclerotia are affected the physiological activity (Baek et al. 2012). Meanwhile, because the bioactivities of polysaccharide are affected significantly by structural features and molecular weights, the isolation of a specific polysaccharide is essential for the studies of physiological activity of polysaccharides. Xu et al. (2014) suggested the possibility of separating fractions of different structures from the starting mixture using appropriate alcohol concentration. The stepwise ethanol-precipitation procedure is more selective with respect to pectin structural features and surface properties than the one-step ethanolic precipitation (Guo et al. 2016). Du et al. (2013) also reported on chemical analysis and antioxidant activity of polysaccharides fractionated by using different concentrations of alcohol precipitation from *I. obliquus* sclerotia. However, up to now, few detailed investigation has been reported on the polysaccharide from the *I. obliquus* sclerotia fractionated by MeOH reflux and EtOH precipitation on the immunostimulatory activity and its chemical characteristics.

To prepare crude polysaccharide with enhanced immunomodulating activity, therefore, hot-water extract (IO-W) from *I. obliquus* sclerotia was fractionated with MeOH reflux or non-reflux, followed by EtOH precipitation and fractionated into IO-CP or IO-M-CP, respectively. These crude polysaccharides were also compared with IO-W or each other on immunomodulating activity and chemical property to investigate the effect of MeOH reflux treatment.

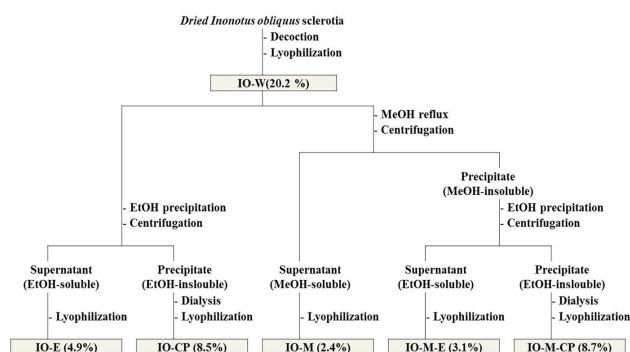
## Materials and Methods

### 1. Extraction and solvent fractionation of *Inonotus obliquus* sclerotia

*Inonotus obliquus* sclerotia were obtained from Functional Polysaccharide Research Institute Ltd. (Seoul, Korea). Hot-water extraction and solvent fractionation from *I. obliquus* sclerotia is shown in Fig 1. The wild sclerotia were grinded with commercial blender into fine powder (60 mesh), and the powder was extracted with 20 volumes of water by decoction (over 90°C, thrice). After centrifugation (10,000 × g, 4°C, 30 min) to remove insoluble material, the aqueous supernatant was lyophilized to obtain hot-water extract from *I. obliquus* (IO-W, 20.2% yield of raw material). IO-W was refluxed with 5 volumes of absolute MeOH at 50°C (thrice), and then MeOH-soluble supernatant was lyophilized to yield MeOH-soluble fraction (IO-M, 2.4%). The residue was re-dissolved with small amounts of water, and then 95% EtOH was added slowly to 80% final content of EtOH and stirred at 4°C for 12 h. After centrifugation, the precipitate was dialyzed using a Spectra/Por 2 membrane (molecular weight cutoff, MWCO 12,000-14,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Ethanol-soluble supernatant and non-dialyzable portion of EtOH-precipitates were lyophilized to yield EtOH-soluble and crude polysaccharide fraction (IO-M-E and IO-M-CP, 3.1 and 8.7%), respectively. In the meanwhile, MeOH-untreated IO-E (4.9%) and IO-CP (8.5%) were also fractionated from IO-W of *I. obliquus* by aforementioned procedure except MeOH reflux (Fig. 1).

### 2. Analytical methods

The contents of total carbohydrate, uronic acid, and protein were measured by the phenol-sulfuric acid method (Dubois et al. 1956), *m*-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen 1973), and Bradford method (Bradford MM 1976) with protein



**Fig. 1. Preparation of crude polysaccharide from *Inonotus obliquus* sclerotia by fractionation including MeOH reflux.**

assay dye (Bio-Rad laboratories, Hercules, CA, USA), respectively, using galactose (Gal), galacturonic acid (GalA), and bovine serum albumin (BSA) as the respective standards. Monosaccharide compositions of the polysaccharide were analyzed by the alditol acetate derivatives method (Jones & Albersheim 1972) with a slight modification using GC on an ACME-6100 GC system (Young-Lin Co., Gyeonggi-do, Korea) equipped with a SP-2380 capillary column (0.2  $\mu\text{m}$  film, 0.25 mm i.d  $\times$  30 m; Supelco, Bellefonte, PA, USA). The analysis was performed with the following temperature schedule: 60°C $\rightarrow$ 220°C (30°C/min), 220°C for 8 min, 220°C $\rightarrow$ 250°C (8°C/min), 250°C for 15 min. The respective molar ratio of monosaccharides was determined from the peak areas and response factors on a flame ionization detector (FID).

### 3. Mice and cell culture

Pathogen-free, 6-week old female Balb/c and C3H/He mice were purchased from Orient Bio (Gyeonggi-do, Korea) and acclimated to their housing environment for 1 week prior to the experiments. The animal room conditions were controlled on a 12 h light/dark cycle, 24 $\pm$ 1°C temperature, and 55% humidity. A pellet diet and water were supplied *ad libitum*. Animal experiments were approved and performed in accordance with Guidelines for Care and Use by the Animal Research Committee of Korea University (KUIACUC-2015-184). Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and fungizone (amphotericin B) were obtained from Gibco BRL Co. (Grand Island, NY, USA). Conventional water-soluble tetrazolium assay kit, Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan).

### 4. Macrophage stimulatory activity

Macrophage stimulatory activity was estimated by the procedure of Suzuki et al. (1990) with slight modification. Balb/c mice were injected intraperitoneally with 1 mL of sterile 5% thioglycollate medium to induce peritoneal macrophage. After 72-96 h, macrophage cells were obtained from peritoneal cavity of the mice by washing twice with 5 mL of cold RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/mL of penicillin and 100  $\mu\text{g}/\text{mL}$  of streptomycin. An aliquot (200  $\mu\text{L}$ ) of the cell suspension ( $1 \times 10^6$  cells/mL) was seeded onto a 96-well cell culture plate (Corning Costar Corp., Cambridge, MA, USA) and pre-incubated for 2 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. After non-adherent cells were removed by washing

with PBS, the adherent cells were treated with sample and incubated for 24 h. The cell proliferation of macrophage was measured by using WST (water-soluble tetrazolium salt)-based colorimetric assay (Ishiyama et al. 1996). Briefly, 20  $\mu\text{L}$  of CCK-8 solution was added into the cultured cells, and then cell counts were evaluated according to the manufacturer's instructions. Macrophage proliferating activity of sample was expressed as a percentage of the negative control treated with saline alone. In addition, 100  $\mu\text{L}$  of culture supernatant was individually collected for the quantification of macrophage-induced mediators, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-12. Levels of TNF- $\alpha$  (eBioscience Inc., San Diego, CA, USA) and IL-12 (BD Biosciences Co., San Diego, CA, USA) in the culture supernatant were measured using a respective ELISA set with the manufacturer's instructions.

### 5. Splenocyte stimulatory activity

A 180  $\mu\text{L}$  of spleen lymphocytes ( $5 \times 10^6$  cells/mL) in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/mL of penicillin and 100  $\mu\text{g}/\text{mL}$  of streptomycin was isolated from Balb/c mice, and then co-cultured with 20  $\mu\text{L}$  of sample in a 96-well cell culture plate for 48 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. Twenty  $\mu\text{L}$  of CCK-8 solution was added into the cultured cells, and then splenocytes counts were evaluated according to the manufacturer's instructions. Spleen lymphocyte proliferating activity of sample was expressed as a percentage of negative control. In addition, 100  $\mu\text{L}$  of culture supernatant was individually collected for the quantification of IL-2 and granulocyte macrophage-colony stimulating factor (GM-CSF) (BD Biosciences Co.). Levels of these cytokines in the culture supernatant were measured using a respective ELISA set with the manufacturer's instructions.

### 6. Intestinal immune system modulating activity through Peyer's patch

The activity was estimated by the procedure of Yu et al. (1998) with slight modification. After Peyer's patches of C3H/He mice were carefully dissected out from the wall of the small intestine, these organs were placed in ice cold RPMI-1640 medium containing 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 10% heat-inactivated FBS (FBS-RPMI). To obtain a single cell suspension, Peyer's patches were homogenized by tapping them gently with a rubber rod over a 100 mesh sterile stainless steel sieve and filtered through a 100  $\mu\text{m}$  cell strainer (BD

Falcon, Franklin Lakes, NJ, USA), and then suspended at a density of  $2 \times 10^6$  cells/mL in FBS-RPMI. For the experiment, an aliquot (180  $\mu$ L) of the cell suspension was cultured with 20  $\mu$ L of sample in a 96-well cell culture plate for 5 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. The resulting supernatant (50  $\mu$ L) was further incubated with a 100  $\mu$ L of bone marrow cell suspension ( $2.5 \times 10^5$  cells/mL) from C3H/He mice for 6 days in the same incubator. The cells were incubated for 4 h in 20  $\mu$ L of CCK-8 solution, and then cell counts were evaluated according to the manufacturer's instructions. Intestinal immune system modulating activity through Peyer's patch was compared to negative control (bone marrow cells cultured with the supernatant of Peyer's patch cell suspension incubated in only saline). In addition, 100  $\mu$ L of culture supernatant was collected for the quantification of GM-CSF. Levels of GM-CSF in the culture supernatant were measured using an ELISA set with the manufacturer's instructions.

## 7. Statistical analysis

All data are presented as mean $\pm$ standard deviation (S.D.). All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA). Differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range tests, and were considered significant at  $p < 0.05$ .

## Results

### 1. Fractionation including MeOH reflux of hot-water extracts from *Inonotus obliquus* sclerotia

As shown in Fig. 1, there was 20.2% yield of hot-water extract (IO-W) from *I. obliquus* sclerotia, whereas MeOH-soluble fraction (IO-M) isolated from IO-W by MeOH reflux was only 2.4%. Methanol-insoluble residue of IO-W was re-dissolved by DIW and fractionated into two fractions by EtOH precipitation; IO-M-E (3.1%) was recovered in EtOH-soluble supernatant and IO-M-CP (8.7%) was recovered in EtOH-precipitate that is believed to be a crude polysaccharide with relatively high-molecular weight. In the meanwhile, IO-E and IO-CP (4.9 and 8.5%) were also separated from IO-W by EtOH precipitation without MeOH reflux to compare the chemical properties and immunological activity. The yields of two crude polysaccharide fractions (IO-M-CP by MeOH reflux and IO-CP without MeOH reflux) were similar (8.7 and 8.5%), but the yield of IO-M-E (3.1%) isolated from

IO-M-CP was lower than IO-E (4.9%) from IO-CP. These results suggest that MeOH reflux result in separation of hydrophilic low-molecular weight substances from IO-W and finally increased isolation of crude polysaccharide from IO-W.

Ethanol precipitation is one of the most widely used methods for preparing natural polysaccharides, in which ethanol concentration significantly affects the yield of total sugar, is usually set at 70-80% (Xu et al. 2014). Only in the last few years, polysaccharides have been fractionated by stepwise ethanol precipitation, replacing almost all of the purification protocols that include at least one such step (Teleman et al. 2000). However, up to now, few detailed investigation has been reported on the polysaccharide fractionated by MeOH reflux and EtOH precipitation on the immunostimulatory activity and yield of crude polysaccharide from the *I. obliquus* sclerotia. Therefore, MeOH reflux and EtOH precipitation has proposed useful processes to prepare of crude polysaccharide.

### 2. Chemical component and monosaccharide composition

As we all know, it is important to know the monosaccharide composition and molar ratio of polysaccharides, which contribute to their bioactivity (Lv et al. 2009). The properties of two crude polysaccharide fractions (IO-CP and IO-M-CP), including total sugar, uronic acid and protein content as well as monosaccharide composition were summarized and shown in Table 1. Crude

**Table 1. Chemical properties and sugar compositions of crude polysaccharides separated from IO-W by fractionation including MeOH reflux**

		IO-E	IO-CP	IO-M-E	IO-M-CP
Chemical properties (%) <sup>1)</sup>	Neutral sugar	21.7 $\pm$ 2.2	75.0 $\pm$ 3.9	14.5 $\pm$ 1.7	83.5 $\pm$ 3.7
	Uronic acid	N.D. <sup>3)</sup>	16.3 $\pm$ 2.1	N.D.	7.4 $\pm$ 2.3
	Protein	1.5 $\pm$ 0.4	8.7 $\pm$ 0.2	1.1 $\pm$ 0.1	9.1 $\pm$ 0.1
Component neutral sugar (Mole%) <sup>2)</sup>	Rhamnose	7.0	11.6	9.0	11.5
	Fucose	N.D.	1.6	N.D.	2.1
	Arabinose	22.1	6.3	22.4	6.8
	Xylose	18.6	20.5	16.6	22.4
	Mannose	10.2	7.9	8.0	8.2
	Galactose	5.2	12.6	4.8	13.6
	Glucose	36.8	23.2	39.2	27.9

<sup>1)</sup> Percentage (%) in each dry material

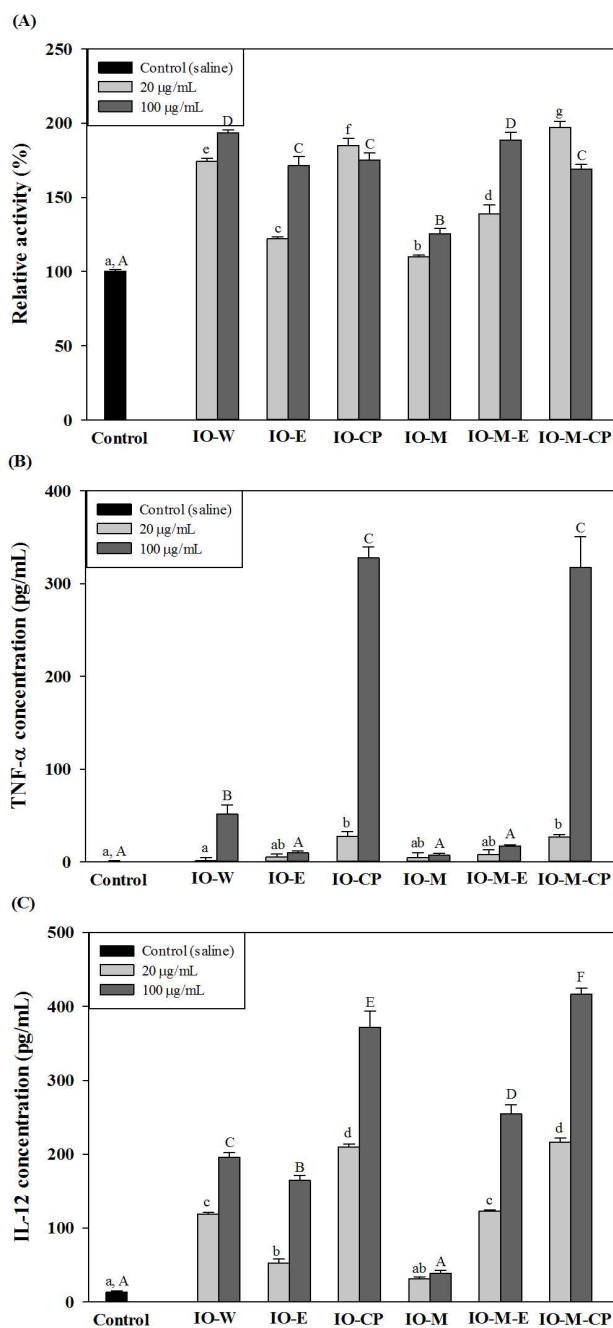
<sup>2)</sup> Mole% was calculated from the detected total carbohydrate.

<sup>3)</sup> N.D.: not detected.

polysaccharide (IO-M-CP) prepared by MeOH reflux and EtOH precipitation from IO-W consisted mainly of neutral sugar (83.5%) in addition to relatively small amounts of uronic acid (7.4%) and protein (9.1%). The component sugar analysis indicated that IO-M-CP contained rhamnose, xylose, galactose and glucose-rich polysaccharide (molar ratio; 0.41:0.80:0.49:1.00), such as hemicellulosic polysaccharides of xyloglycan. This result is similar to the partially purified polysaccharide, xylogalactoglucon, possessing antitumor and hypoglycemic activities from *I. obliquus* sclerotia and mycelia (Mizuno et al. 1999). On the other hand, IO-CP from IO-W without MeOH reflux contained less neutral sugar (75.0%) and more uronic acid (16.3%) than those of IO-M-CP. However, monosaccharide composition of IO-CP was very similar to that of IO-M-CP (rhamnose, xylose, galactose and glucose; molar ratio of 0.50:0.88:0.54:1.00, Table 1). In the meanwhile, IO-M-E and IO-E were also shown closely similar compositions and percentages (arabinose, xylose and glucose) in component sugar analysis (Table 1). From the sugar analysis that the kinds of sugars contained in the crude polysaccharides and EtOH fractions were different, it was predicted that crude polysaccharide was composed mainly of polysaccharides whereas that of EtOH fractions were composed of the non-polysaccharide fraction, such as monosaccharide or oligosaccharide. These results suggested that MeOH reflux can contribute to the separation of non-polysaccharide materials from hot-water extract of *I. obliquus* sclerotia.

### 3. Macrophage stimulatory activity of crude polysaccharide fractions

Macrophage can initiate innate immune recognition and phagocytosis of pathogens, thereby secreting inflammatory mediators such as TNF- $\alpha$ , IL-6, IL-12, and nitric oxide. Furthermore, modulation of these systems can significantly affect both humoral and cellular immune responses (Beutler 2004). When the peritoneal macrophages isolated from Balb/c mice were stimulated with crude polysaccharides, IO-M-CP enhanced the significant proliferation (1.97-fold against saline control) compared to those of IO-W (1.74-fold) or IO-CP (1.85-fold) at a dose of 20  $\mu\text{g}/\text{mL}$  (Fig. 2A). However, macrophage stimulating activity in IO-M-CP and IO-CP was lower rather at higher sample concentration. IO-M-E (1.88-fold) was also shown the significantly high activity at 100  $\mu\text{g}/\text{mL}$  but other fractions including MeOH-soluble fraction (IO-M) showed only a negligible effect. Next, the effects of crude polysaccharides on the production of macrophage-

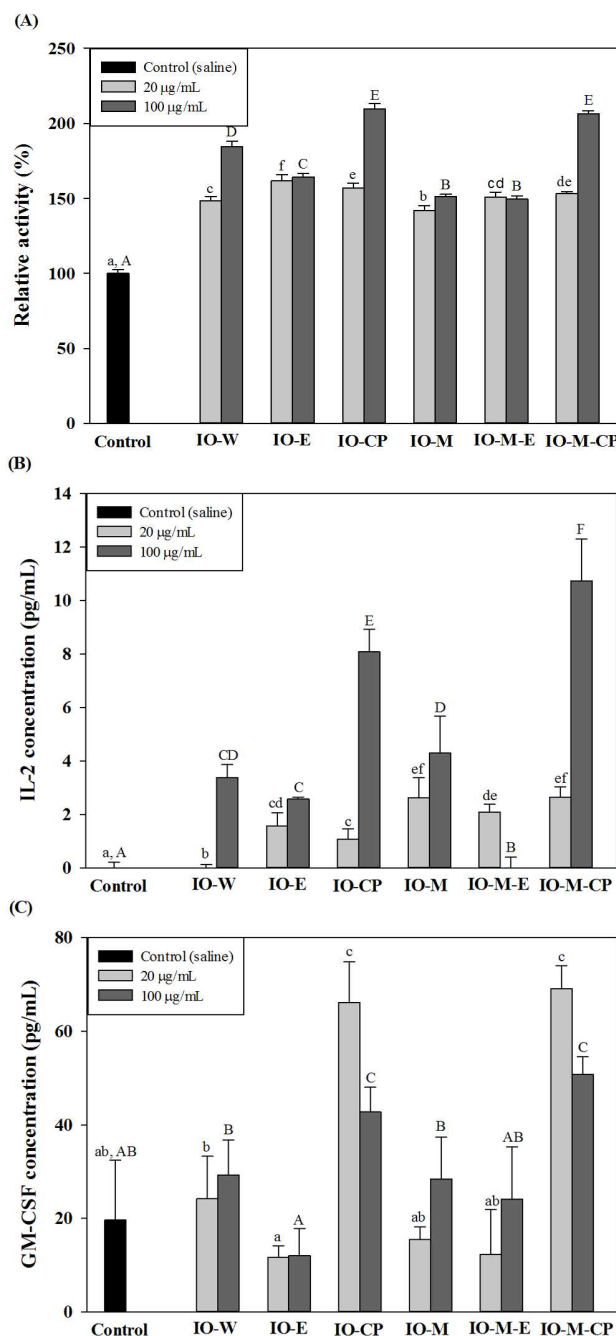


**Fig. 2. Macrophage stimulating activity of crude polysaccharide on (A) macrophage proliferation and production of macrophage-induced cytokine, (B) TNF- $\alpha$  and (C) IL-12.** The macrophage proliferating activity was determined by CCK-8 kit, and the levels of TNF- $\alpha$  and IL-12 in the culture supernatant were quantified by ELISA. The difference between lower and upper case letters on the bar means that there is a significant difference ( $p < 0.05$ ) among all tested samples at 20  $\mu\text{g}/\text{mL}$  and 100  $\mu\text{g}/\text{mL}$  by Duncan's multiple range test.

induced cytokines were also quantified. The concentration of TNF- $\alpha$  was significantly high at 100  $\mu\text{g/mL}$  of only IO-CP (6.39-fold against IO-W) and IO-M-CP (6.19-fold) but it was not significant between each other of crude polysaccharide (Fig. 2B). However, IO-M-CP significantly stimulated the IL-12 production (416.09 pg/mL) from peritoneal macrophage compared to IO-W (195.61 pg/mL) or IO-CP (371.85 pg/mL) at the concentration of 100  $\mu\text{g/mL}$  (Fig. 2C). These results showed that crude polysaccharides stimulated macrophage activity more than hot-water extract regardless of MeOH reflux. And then, although there was no significant difference in the activity of crude polysaccharides, IO-M-CP prepared by fractionation including MeOH reflux stimulated significantly higher production of IL-12 than IO-CP.

#### 4. Splenocyte stimulatory activity of crude polysaccharide fractions

To assess the stimulatory activity of the polysaccharide fraction on lymphocyte, we investigated the proliferating activity of splenocyte from Balb/c mice. The present result indicated that IO-CP (2.10-fold against saline control) and IO-M-CP (2.06-fold) significantly increased mitogenic activity at 100  $\mu\text{g/mL}$  of sample concentration than IO-W (1.85-fold) or other solvent fractions (1.50-1.64-fold). However, there was no significant difference between crude polysaccharides. To investigate the effects of crude polysaccharides on the production of lymphocyte-induced cytokines, IL-2 and GM-CSF were quantified in the culture supernatant of splenocyte. As shown in Fig. 3B, IL-2 concentration was significantly higher through stimulation with IO-M-CP (10.74 pg/mL) than other fractions including IO-CP (8.08 pg/mL) at a dose of 100  $\mu\text{g/mL}$ . Of various cytokines produced by lymphocytes, IL-2 is a pleiotropic cytokine produced primarily by CD4<sup>+</sup> T cells that drives T cell growth, augments natural killer (NK) cytolytic activity, induces the differentiation of regulatory T cells, and mediates activation-induced cell death (Liao et al. 2011). Although there was no significant difference between each other, IO-M-CP and IO-CP enhanced also production of GM-CSF (69.03 and 66.14 pg/mL) compared with IO-W (24.16 pg/mL) or other solvent fractions (11.72-15.50 pg/mL) at 20  $\mu\text{g/mL}$  (Fig. 3C). However, GM-CSF production was exhibited lower effect at a high sample concentration in both IO-M-CP and IO-CP. GM-CSF is an important hematopoietic growth factor and immune modulator which is produced by a variety of cell types including T cells, macrophages, endothelial cells and fibroblasts upon receiving immune stimuli (Shi et al. 2006). Taken together,

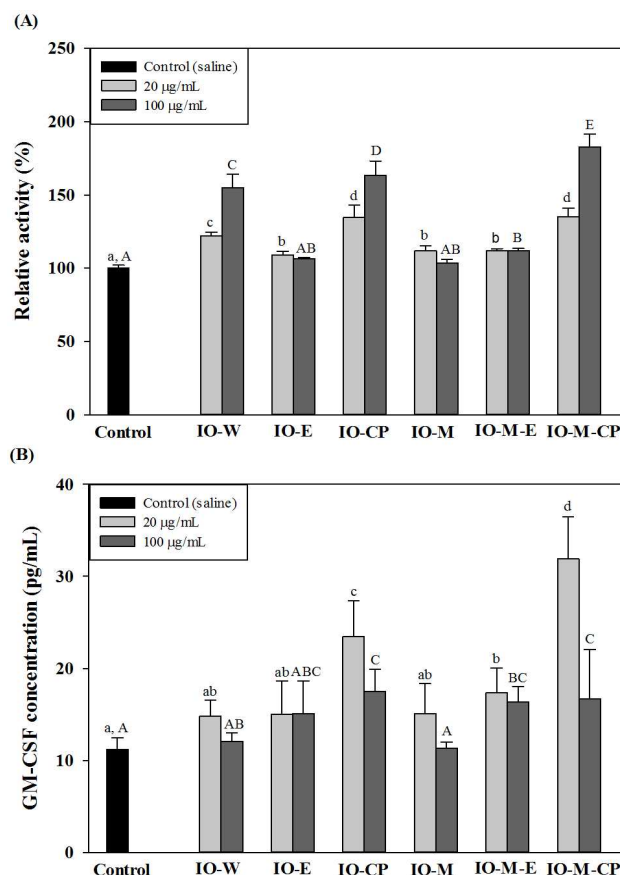


**Fig. 3. Splenocyte stimulatory activity of crude polysaccharide on (A) splenocytes proliferation and production of splenocyte-induced cytokine, (B) IL-2 and (C) GM-CSF.** The splenocyte proliferating activity was determined by CCK-8 assay, and the levels of IL-2 and GM-CSF in the culture supernatant were quantified by ELISA. The difference between lower and upper case letters on the bar means that there is a significant difference ( $p < 0.05$ ) among all tested samples at 20  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$  by Duncan's multiple range test.

IO-M-CP showed the splenocyte-stimulatory activity among other fractions including IO-CP, suggesting that MeOH reflux-including fractionation of *I. obliquus* sclerotia is useful method for a significant enhancement of lymphocyte-stimulatory activity.

### 5. Intestinal immune system modulating activity of crude polysaccharide fractions

Lymphocytes in the intestinal mucosa such as Peyer's patches first interact with antigen and further differentiate and mature in the germinal center of the lymphoid follicles. They also rapidly eliminate from the mucosa and migrate through the mesenteric lymph nodes to reach systemic circulation. Therefore, the intestinal immune system including Peyer's patches not only contributes to the defense system of the mucosa but also regulates systemic inflammation, resulting in suppression of allergic reactions and autoimmune diseases (James & Zeitz, 1994). In this study, the effect of crude polysaccharide on the intestinal immune system modulating activity through Peyer's patch was investigated as bone marrow cell proliferation due to the presence of various cytokines in the culture supernatant of Peyer's patch cells stimulated with samples for 5 days. At low concentration of 20  $\mu\text{g}/\text{mL}$ , IO-M-CP showed no more proliferation of bone marrow cells than IO-CP, but it further stimulated bone marrow cell proliferation more than IO-CP at a higher concentration (100  $\mu\text{g}/\text{mL}$ ). In terms of bone marrow cell proliferation, IO-M-CP (1.83-fold than saline control) had the most potent proliferating activity among all fractions tested including IO-CP (1.64-fold) at the dose of 100  $\mu\text{g}/\text{mL}$ . In the meanwhile, IO-M-CP showed the potent enhancement of GM-CSF production in the Peyer's patch cell-culture supernatant (31.91  $\text{pg}/\text{mL}$ ), and it exhibited significantly higher production than other fractions including IO-CP (14.98  $\text{pg}/\text{mL}$ ) at a low dose. GM-CSF is an important hematopoietic growth factor and immune modulator that has profound effects on the functional activities of various circulating leukocytes and is produced locally because it acts in a paracrine manner to recruit circulating neutrophils, monocytes and lymphocytes to enhance their functions in host defense (Shi et al. 2006). It was well known that Peyer's patches are mainly composed of B cells and T cells, which are known to be a source of CSFs including GM-CSF, and various cytokines (Lee et al. 2015). These results suggested that IO-M-CP can contribute to bone marrow cell proliferation resulting from Peyer's patch stimulation followed by GM-CSF production. Therefore, hot-water extract from *I. obliquus* by fractionation including MeOH reflux seems to play



**Fig. 4. Intestinal immune system modulating activity of crude polysaccharide on (A) bone marrow cell proliferation through Peyer's patch and (B) production of GM-CSF from Peyer's patch cell.** Peyer's patch cells from C3H/He mice were treated with polysaccharide fractions for 5 days. The culture supernatant was further incubated with bone marrow cells without polysaccharide fractions for 6 days. The bone marrow cell proliferation through Peyer's patch was determined by CCK-8 assay, and the level of GM-CSF in the culture supernatant of Peyer's patch cells was quantified by ELISA. The difference between lower and upper case letters on the bar means that there is a significant difference ( $p < 0.05$ ) among all tested samples at 20  $\mu\text{g}/\text{mL}$  and 100  $\mu\text{g}/\text{mL}$  by Duncan's multiple range test.

an important role in preparation crude polysaccharide with enhanced immunomodulating activity from chaga mushroom.

## Discussion

Traditionally, the extracts of *I. obliquus* sclerotia can be divided into three parts, i.e. MeOH-soluble materials that

composed mainly with triterpenoids and steroids, aqueous EtOH-soluble substances that consisted of phenolic compounds as main antioxidants, and water-soluble polysaccharides or proteoglycans prepared by EtOH precipitation after hot-water extraction that consisted mainly of  $\beta$ -glucan or mannan (Chen et al. 2010; Cui et al. 2005). Cui et al. (2005) reported that 80% ethanol-soluble fractions (Fc and Fd) isolated from hot-water extract of *I. obliquus*, which contains polyphenolic components, showed strong anti-oxidant. We previously reported that extraction conditions affect the physiological activity of *I. obliquus*, and immunostimulating polysaccharide fractionated from *I. obliquus* by decoction is composed mainly of neutral sugars (Baek et al. 2012). The purpose of this study is to focus on the isolation and chemical property of solvent fractions from *I. obliquus* sclerotia, and explored their immunostimulatory activities for seeking new natural functional ingredients used in food and pharmaceutical industry. For the goal, the crude polysaccharide from hot-water extract of *I. obliquus* sclerotia was isolated by MeOH reflux and EtOH precipitation, and its immunostimulatory activity was investigated. Among the fractions, IO-M-CP which is polysaccharide prepared by fractionation including MeOH reflux was composed mainly of neutral sugar such as rhamnose, xylose, galactose and glucose, speculating hemicellulosic polysaccharide xyloglycan. Interestingly, IO-CP which is crude polysaccharide prepared without MeOH reflux showed closely similar to that of IO-M-CP in monosaccharide composition. These result indicated that low molecular-weight materials such as polyphenols and sterols, rather than neutral polysaccharide were isolated from hot-water extract of *I. obliquus* sclerotia by MeOH reflux. IO-M-CP possesses the most potent activity macrophage stimulating, mitogenic, and intestinal immune system modulating activity through Peyer's patch than those of other fractions including IO-CP. Conclusively, MeOH reflux-including fractionation of *I. obliquus* sclerotia is useful method for a significant enhancement of immunostimulatory activity.

Although the precise structure is yet unknown, based on the results presented above and the knowledge that cytokines such as TNF- $\alpha$ , IL-12, IL-2, and GM-CSF, known to act on the mainly of macrophage and T cells, we conclude that IO-M-CP may stimulate both innate and adaptive immunity, providing the body with defense against disease and cancer. In conclusion, the crude polysaccharide isolated from sclerotia of *I. obliquus* by using MeOH reflux and EtOH precipitation stimulates the various immune systems and could be beneficial to human health

and possible of using as a functional food.

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