J. Appl. Glycosci., **71**, 47−54 (2024)
doi:10.5458/jag.jag.JAG-2023_0012

Regular Paper

Polysaccharides from a Fermented Beverage Induce Nitric Oxide and Cytokines in Murine Macrophage Cell Line

(Received October 11, 2023; Accepted February 27, 2024)

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Abstract: Super Ohtaka®, a fermented beverage of plant extracts, is prepared from approximately 50 kinds of fruits and vegetables. Natural fermentation is mainly performed by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp.). Four water-soluble polysaccharide fractions were obtained from Super Ohtaka® by dialysis, ion exchange chromatography, and gel filtration chromatography; these fractions were designated as OEP1-1, OEP1-2, OEP2, and OEP3. OEP1-1 is a polysaccharide composed solely of glucose. The other fractions contained polysaccharides composed of glucose, galactose, mannose, and a small amount of arabinose. OEP2 and OEP3 contained phosphorus, which was not detected in OEP1-1 and OEP1-2. Furthermore, the immunomodulatory activity of the polysaccharides was investigated in murine macrophage cell lines. OEP2 and OEP3 significantly induced nitric oxide (NO) secretion by macrophages in a dose-dependent manner (concentration range of 4 to 100 μg/mL). When the concentration of OEP3 was 100 μg/mL, NO production was almost identical to lipopolysaccharide (LPS; 10 ng/mL) used as a positive control. Notably, OEP3 induced NO secretion more strongly than OEP2. This trend was also observed for TNF-α, IL-1β, IL-6, and IL-12 p40 secretion. Overall, our *in vitro* studies on polysaccharides isolated from Super Ohtaka® suggest that the fermented beverage stimulates macrophages and activates the immune system.

Key words: fermented beverage, polysaccharides, immunomodulatory activity, murine macrophage cell line

INTRODUCTION

The naturally fermented drinks called "kôso", a mixture of various vegetables and saccharides, are garnering a great deal of attention, and there are also reports on the transition of microorganisms during fermentation [1]. One of these unique beverages is "Super Ohtaka®". Super Ohtaka® (fermented beverage of plant extracts) is produced by fermentation of an extract from 50 kinds of fruits and vegetables [2]. The extract is obtained using sucrose-osmotic pressure in a cedar barrel for seven days and is fermented by lactic acid bacteria and yeast for 180 days. The fermented beverage displayed scavenging activity against the radical 1,1-diphenyl-2-picrylhydrazyl *in vitro*, and its oral administration significantly reduced ethanol-induced damage of the gastric mucosa in rats [2]. Moreover, Ogawa *et al*. reported the remarkable healing effect of fermented beverages (Super Ohtaka®) on the sarcoma transplanted rats [3]. On the other hand, there are some reports on the immunostimulatory action of yeast and black yeast-derived β-glucan [4, 5]; as mentioned above, Super Ohtaka® was fermented with yeast and lactic acid bacteria. For this reason, the β-glucan of Super Ohtaka® was measured. We confirmed the presence of β-glucan (150−766 ng/mL), which is thought to be derived from the yeast cell wall [6].

High-performance anion exchange chromatography (HPAEC) showed that this beverage contains high levels of saccharides, estimated to be between 550 and 590 g/L, mainly glucose and fructose, and a small amount of undetermined oligosaccharides. We have previously examined the preparation of saccharides of fructopyranoside series as Super Ohtaka®, such as β-D-fructo pyranosyl-(2→6)-D-glucopyranose [7], β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose [8], β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose [8], β-D-fructopyranosyl-(2→6)-D-fructo furanose [9], β-D-fructopyranosyl-(2→1)-D-fructopyranose [9], β-D-fructopyranosyl-(2→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside [9], and β-D-fructopyranosyl-(2→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside [9]. The characteristics of β-D-fructopyranosyl-(2→6)-D-glucopyranose are non-cariogenicity, low digestibility, and unfavorable bacteria that produce mutagenic substances do not use the saccharide [10]. In addition, we have isolated and identified

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Abbreviations: ABEE, *p*-aminobenzoic acid ethyl ester; Da, dalton; ELISA, enzyme-linked immunosorbent sorbent assay; EPS, extracellular polysaccharide; FBS, fetal bovine serum; HPAEC, high-performance anion-exchange chromatography; HPLC, highperformance liquid chromatography; IL, interleukin; LPS, lipopolysaccharide; NEAA, non-essential amino acids; NK cell, natural killer cell; NMR, nuclear magnetic resonance; NO, nitric oxide; PBS, phosphatebuffered saline; TNF, tumor necrosis factor.

novel non-reducing trisaccharides, such as 1^F-β-glucosylsucrose and 1^F-β-galactosylsucrose from the beverages [11]. Two oligosaccharides containing an α-fructofuranoside linkage were also detected in this beverage [12]. Furthermore, those novel saccharides were confirmed to be produced by fermentation [7, 9, 11, 12].

Notably, polysaccharides isolated from botanical sources are considered biological response modifiers for their antioxidant, anticancer, and immune-modulation activities [13−15]. The main structural characteristics of polysaccharides include molecular mass, saccharide composition, glycosidic bonds, degree of branching, and triple helix conformation [16, 17]. Several bioactive polysaccharides have been isolated from botanical sources such as mushrooms, algae, lichens, and higher plants and many are relatively non-toxic with no significant side effects [16, 18, 19]. Recently, polysaccharides have attracted a great deal of attention; many studies have demonstrated that botanical polysaccharides have the potential to activate cells involved in innate immunity [16, 18, 19]. The extracellular polysaccharides (EPS) of bacteria such as lactic acid bacteria are also known to stimulate immune cells such as macrophages, and there are some reports on their isolation and functionality [20−22].

In this study, we isolated polysaccharides and other high-molecular-weight compounds and obtained water-soluble polysaccharide fractions from the fermented beverage Super Ohtaka®. Partial characterization of these fractions was also performed. Further, the immunostimulatory and immunoregulatory activities of these fractions were investigated in murine macrophage cell lines.

MATERIALS AND METHODS

Materials. Dextran standards (12, 50, 150, 410, and 670 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The murine macrophage RAW 264 cell lines (ECACC 85062803) and J774.1 cell lines were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Eagle's medium, penicillin-streptomycin, and phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Lipopolysaccharide from *Escherichia coli* 0111: B4 (LPS) was purchased from Sigma-Aldrich. NO_2/NO_3 assay kit-C 2 (Colorimetric) was purchased from DOJINDO Laboratories Co., Ltd. (Kumamoto, Japan). Mouse TNF-α, IL-1β, and IL-12 p40 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcom Co., Ltd. (Cambridge, UK). The Mouse IL-6 ELISA kit was purchased from Proteintech Co., Ltd. (Rosemont, IL, USA). All the other chemicals and reagents were of analytical grade.

Preparation of fermented beverage of plant extracts (Super Ohtaka®). To prepare the "fermented beverage of plant extracts" (Super Ohtaka®; Ohtakakohso Co., Ltd., Otaru, Japan), 50 kinds of fruits and vegetables were used, as previously reported [2, 23]. The 50 fruits and vegetables were cut, sliced, or diced into small pieces, mixed, and put in cedar barrels. Then, an equivalent weight of sucrose was added to the samples, mixed well to allow high sample sucrose, and the barrels were left for one week at room temperature. The juice exudates were separated from the solids without compression and used for fermentation. The fermented beverage was obtained by incubating the extract at 37 ˚C in the dark by natural fermentation using yeast (mainly *Zygosaccharomyces* spp., *etc*.) and lactic acid bacteria (mainly *Leuconostoc* spp., etc.). After 7 days, the fermented beverage was subjected to additional maturation and aging to obtain a slightly sticky, brown liquid.

Preparation of crude polysaccharides from Super Ohtaka®. Super Ohtaka® (1 L) was filtered using a Vivaflow 200 (Membrane: 10,000 MWCO, Sartorius, Göttingen, Germany). Diafiltration samples containing non-permeable components of the ultrafiltration membrane were collected, concentrated, and lyophilized. The lyophilizate was suspended in distilled water and dialyzed against distilled water using a dialysis tube for 5 days. The dialysate was then suspended in distilled water and centrifuged. The supernatant was filtered through a 0.45 μm filter and lyophilized to form a water-soluble fraction (crude polysaccharides) used in subsequent experiments.

Separation and purification of crude polysaccharides. DEAE-Sepharose Fast Flow (Sigma-Aldrich) column chromatography was used for fractionation. The crude polysaccharides (50 mg) were dissolved in 0.02 M Tris-HCl buffer (pH 8.6, 2 mL), loaded onto a column (1 × 15 cm), and eluted in a stepwise gradient with 50 mL of 0.02 M Tris-HCl buffer (pH 8.6) containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 M NaCl, respectively, at a flow rate of 0.3 mL/min. Each fraction was analyzed using the phenol-sulfuric acid method [24]. The polysaccharide fractions were collected and dialyzed against distilled water using dialysis tubes. Subsequently, these fractions were purified repeatedly using a Toyopearl HW-65s (1.7 × 94 cm) column (Tosoh Co., Tokyo, Japan) at room temperature and eluted with distilled water at a flow rate of 9 mL/h. Relevant fractions were collected, pooled, and concentrated by freeze-drying. Polysaccharide fractions were passed through a Sep-Pak® Light Silica cartridge (Waters Co., Milford, MA, USA). The total carbohydrate content of the polysaccharide fractions was determined using the phenol-sulfuric acid method and anthrone method [25] with glucose as the standard. Protein content was determined using the method described by Lowry [26] with BSA as the standard.

Determination of molecular masses of the polysaccharides. Estimation of molecular masses of the polysaccharide fractions was done based on the elution volume with Toyopearl HW-65s (1.7 × 94 cm) and Toyopearl HW-55s column (1.7 × 94 cm). Elution was performed using distilled water at a flow rate of 9 mL/h at room temperature. The molecular mass was calibrated using standard dextran (12, 50, 150, 410, and 670 kDa; 5 mg/0.5 mL).

Determination of chemical composition. The monosaccharide composition was determined using a Dionex Bio LC series system (Sunnyvale, CA, USA) equipped with an HPLC carbohydrate column (Carbo Pack PA1, inert styrene divinyl benzene polymer, Sunnyvale, CA, USA), and pulsed amperometric detection (PAD) [27−29]. Glucuronic acid and galacturonic acid were measured by HPLC with the *p*-aminobenzoic acid ethyl ester (ABEE)-conversion method [30, 31]. Twenty μL of the polysaccharides solution (4 mg/mL) was mixed in 20 μL, 8 M trifluoroacetic acid and hydrolyzed by heating at 100 ˚C for 3 h. The trifluoroacetic acid was removed using a centrifugal evaporator. The residue was diluted appropriately with distilled water and used for measurements.

The phosphorus content of the polysaccharides was

determined by the Fiske-Subbarow method [32] using KH_2PO_4 as a standard. The sample was decomposed using 5 M sulfuric acid at 160 ˚C for 4.5 h. A small amount of 30 % H_2O_2 was added during decomposition. Finally, the solution was neutralized with NaOH and used for the experiments.

Cell culture and immunomodulatory activity assay. The macrophage cell line RAW 264 was cultured in modified Eagle's medium (MEM) supplemented with 10 % fetal bovine serum (FBS), 0.1 mM non-essential amino acids (NEAA: Gibco), 100 µg/mL streptomycin, and 100 units/mL penicillin at 37 ˚C in a 5 % CO_2 humidified atmosphere cultivator. J774.1 cells were cultured in RPMI1640 medium with 10 % FBS, and the other culture conditions were the same as those used for RAW 264 cells (NEAA was not added). The cells were plated at 1×10^5 cells/well in 24-well plates and incubated with various concentrations of each polysaccharide sample for 24 h. Supernatants were collected by centrifugation and used for NO and cytokine assays. LPS dissolved in PBS was used as a positive control and adjusted to a final concentration of 10 ng/mL. The concentrations of NO, TNF-α, IL-1β, IL-6, and IL-12 p40 in the supernatants were determined using NO-detecting kit and mouse TNF-α, IL-1β, IL-6, and IL-12 p40 ELISA kits, respectively, according to the manufacturer's instructions. J774.1 cells were used to measure IL-12 because RAW cells do not produce IL-12 in response to LPS [33]. Other cytokines were measured in RAW 264 cells.

Cell viability assay. The macrophages were cultivated in a medium supplemented with various concentrations of polysaccharides. Ten µL of Cell Counting Kit-8 was added (DOJINDO) to 200 µL of the culture medium and incubated at 37 ˚C for 1 h. The absorption was measured at 450 nm, and cell viability was calculated as per the manufacturer's protocol.

Statistical analysis. Data are expressed as mean ± standard deviation (SD) values with triplicates. The group mean was compared using a one-way analysis of variance and Tukey's tests. The statistical difference was considered significant at $P < 0.05$. The analysis was performed using SPSS Version 27.

RESULTS AND DISCUSSION

Isolation of polysaccharides from Super Ohtaka®. Approximately 0.2 g crude polysaccharides were obtained from Super Ohtaka® (1 L) using Vivaflow 200 and dialysis tubes. The freeze-dried powder was brown. A portion (50 mg) of the lyophilizate was separated by ion exchange chromatography using a DEAE-Sepharose Fast Flow column, and three peaks were observed (Fig. 1A). Polysaccharide peaks were observed in the non-adsorbed fraction (peak 1), and the 0.1 M (peak 2) and 0.2 M NaCl (peak 3) eluted fractions and were fractionated into neutral polysaccharide and two kinds of acidic polysaccharides. Each fraction was further purified by gel filtration chromatography using a Toyopearl HW-65s column. As shown in Fig. 1B, two peaks were observed, mainly in the non-adsorbed fraction (peak 1). These were designated as OEP1-1 (Fraction No. 40−52) and OEP1-2 (Fraction No. 73−90). The 0.1 M and 0.2 M NaCl elution fractions were purified by removing the region with UV absorption (Figs. 1C and 1D) and designated as OEP2 (Fraction No. 42−52) and OEP3 (Fraction No. 42−56), respectively. Purified OEP1-1 (9.0 mg), OEP1-2 (7.4 mg), OEP2 (25.5 mg), and OEP3 (11.5 mg) were obtained as white powders (Table 1). The carbohydrate and protein contents of each fraction are presented in Table 1. The

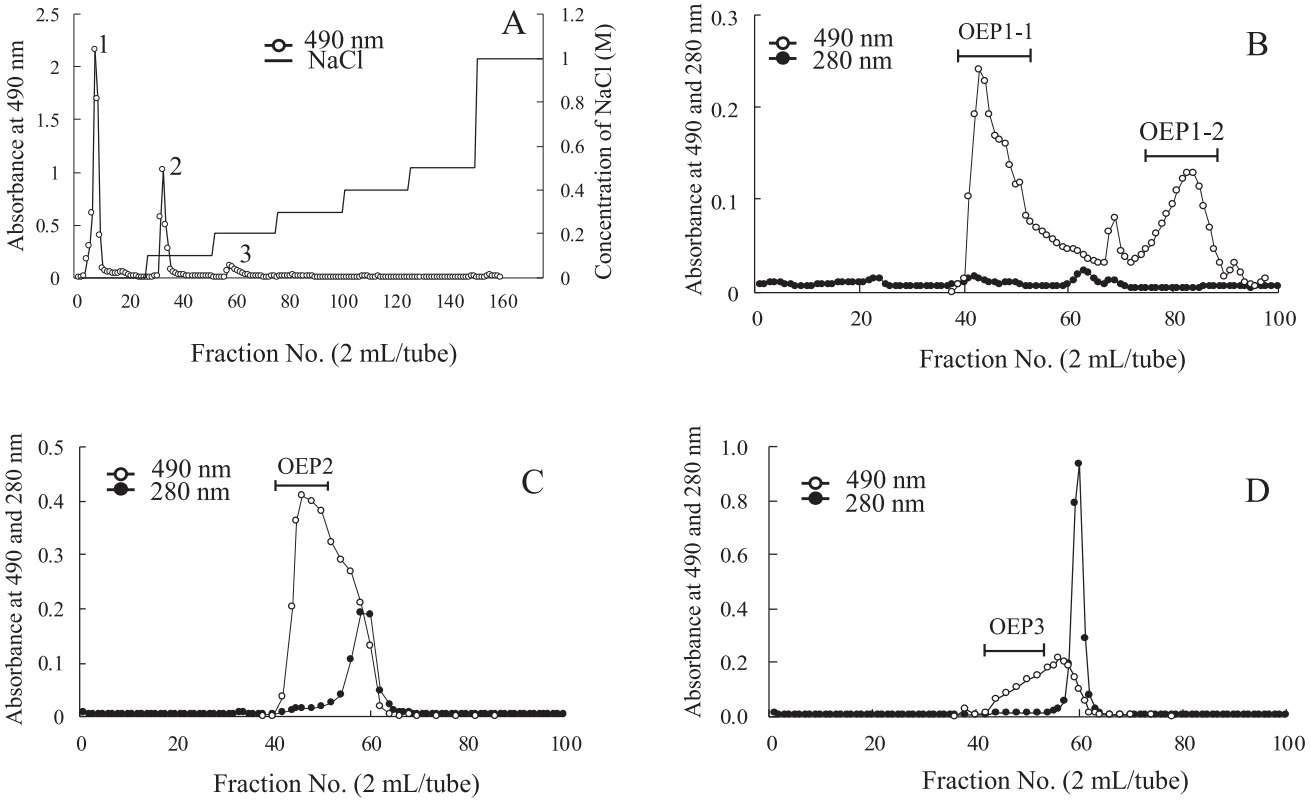

Fig. 1. DEAE-Sepharose Fast Flow column chromatogram (A) and Toyopearl HW-65s column chromatogram (B, C, and D) of the fermented beverage of plant extracts (Super Ohtaka®).

carbohydrate contents of the polysaccharide fractions OEP1-1, OEP1-2, OEP2, and OEP3 were 74.4, 66.3, 86.4, and 68.5 %, respectively, as estimated using the phenol-sulfuric acid method. A small amount of protein was detected in the isolated polysaccharides.

Molecular mass distribution. The molecular mass distributions of OEP1-1, OEP1-2, OEP2, and OEP3 were determined by gel filtration chromatography using Toyopearl HW-65s and -55s columns, as shown in Fig. 2. Based on the calibration curve obtained from the analysis of dextran molecular mass standards, the average molecular masses of the isolated fractions were determined. OEP1-1 and OEP2 eluted earlier than the 670 kDa standard dextran; therefore, their molecular masses could not be calculated. The average molecular masses of OEP1-2 and OEP3 were 19 and 320 kDa, respectively (Figs. 2A and 2B).

Chemical composition of the polysaccharides. The monosaccharide compositions of the isolated fractions were analyzed using the HPAEC and ABEE methods, and the results are presented in Table 2. OEP1-1 is a polysaccharide composed solely of glucose. The other fractions were polysaccharides composed of glucose, galactose, and mannose; a small amount of arabinose was also observed. Fructose, glucuronic acid, and galacturonic acid were not detected in any polysaccharides. As shown in Table 2, all four polysaccharides had different monosaccharide compositions. Furthermore, OEP2 and OEP3 were shown to be polysaccharides containing 0.7 ± 0.03 and 5.1 ± 0.63 % phosphorus, respectively. The purity of these polysaccharide was confirmed by NMR. The correlation between phosphorus and saccharide residue was confirmed by ^{1}H-NMR. The correlation between phosphorus and saccharide residue was also confirmed by 2D-NMR (P/H HMBC: data not shown). The EPS produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1, which is known to have immunomodulatory activity, is reported to have a phosphorus content of 0.01 % (wt/wt) [34]. Although it is unknown whether OEP2 and OEP3 are EPS, they had a high phosphorus content compared to the EPS of *L. bulgaricus* OLL1073R-1. The reason for this is currently unknown, so we would like to investigate whether it is produced by fermentation microorganisms or increases due to maturation. In addition, phosphorous was not detected in OEP1-1 and OEP1-2. OEP1-1 was analyzed by NMR, and branching of α1→6 was determined by HMBC analysis (data not shown). *Leuconostoc* spp. is known to produce glucans composed of α1→6 linkages [35]. As mentioned above, the presence of *Leuconostoc* spp. has been confirmed in the liquid during fermentation of Super Ohtaka®. Thus, OEP1-1 may have been produced by *Leuconostoc* spp. The main saccharide constituents of OEP2 were mannose and galactose, whereas those of OEP3 were mainly glucose. The galactose-to-mannose ratio of OEP2 is

Table 1. Chemical composition of OEP1-1, OEP1-2, OEP2, and OEP3.

Sample name	Carbohydrate (%)		Protein (%)	Content /L of Super Ohtaka®
	(Anthrone method)*	(Phenol-sulfuric acid)*	(Lowry method)	(mg)
OEP1-1	51.2 ± 1.1	74.4 ± 5.1	3.3	9.0 ± 0.8
OEP1-2	37.8 ± 3.2	66.3 ± 0.9	1.4	7.4 ± 0.9
OEP2	41.3 ± 1.4	86.4 ± 3.2	8.4	25.5 ± 2.3
OEP3	54.4 ± 3.6	68.5 ± 4.1	7.4	11.5 ± 0.5

*Glucose standard.

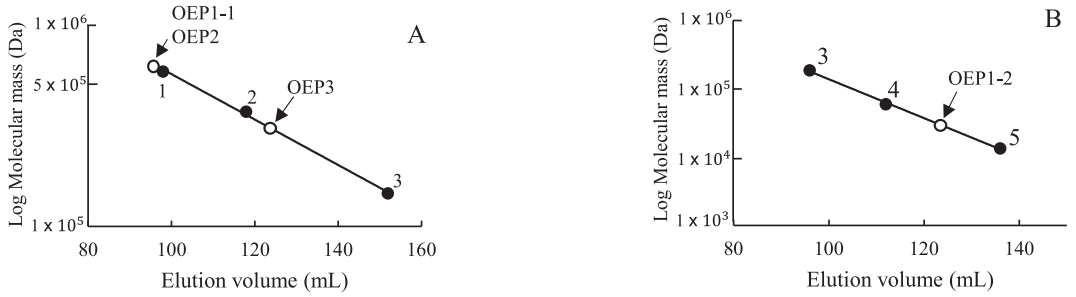

Fig. 2. Calibration curve for determining the molecular mass of polysaccharides of the fermented beverage of plant extracts (Super Ohtaka®) based on the elution volume and the molecular mass of standard dextran series.
(A) Toyopearl HW-65s, (B) Toyopearl HW-55s. ●: Dextran standard (**1**, 670 kDa; **2**, 410 kDa; **3**,150 kDa; **4**, 50 kDa; **5**,12 kDa).

Table 2. Molecular mass, monosaccharide composition, and substitution ratio of the isolated polysaccharides.

Sample name	Molecular mass		Ratio	Molar ratio							Substitution ratio
	(kDa)	Color	(%)	Glc	Gal	Man	Ara	Fru	GlcA	GalA	Phosphorus
OEP1-1	>670	White	17.8	1	–	–	–	–	–	–	–
OEP1-2	19	White	15.6	1	1.6	1.6	0.1	–	–	–	–
OEP2	>670	White	45.7	1	2.6	6.4	0.5	–	–	–	0.7 ± 0.03 %
OEP3	320	White	20.9	1	0.2	0.4	Tr	–	–	–	5.1 ± 0.63 %

Tr: Trace.

similar to that of legume-derived galactomannans. Legumes (green gram, black gram, and clover) were used as raw materials for Super Ohtaka® [2] but in small quantities (total 2−3 %). Glucose and arabinose were also detected in OEP2, suggesting that they were not legume-derived galactomannans. There are several reports on the EPS of lactic acid bacteria, and it has been reported that polysaccharides in EPS produced by *Lactobacillus plantarum* No. 14 strain were separated by an ion-exchange column [20]. In this report, several EPSs were separated and named H-NPS, L-NPS, W-APS, and S-APS. The monosaccharide compositions of W-APS (Glc: Gal: Man = 1.00:0.41:6.74) and S-APS (Glc: Gal: Man = 1.00:0.20:0.08) reported here were relatively similar to those of OEP2 and OEP3. Because *Lactiplantibacillus plantarum* subsp. *plantarum* (formerly *Lactobacillus plantarum*) was isolated during the fermentation of Super Ohtaka®, these polysaccharides may have been produced by *L. plantarum.* However, W-APS and S-APS contain carboxyl and sulfate groups, respectively, and have been reported to contain no phosphorus. Furthermore, these polysaccharides differ in molecular mass.

It is still unclear whether these polysaccharides are derived from raw plants or during the fermentation by microorganisms, and structural analyses have not yet progressed. However, from the 7th day of fermentation to the 180th day, the four types of polysaccharides isolated this time increased by about 1.5 to 2 times (data not shown). Based on this, we speculate that the production of these polysaccharides from fermentation, and we would like to isolate fermentation microorganisms and investigate the production of polysaccharides.

Immunomodulatory activities. The immunomodulatory activity was evaluated by measuring NO, TNF-α, IL-1β, and IL-6 secretions from RAW 264 cells and IL-12 p40 secretions from J774.1 cells. OEP1-1 and OEP1-2 were used as unfractionated mixtures on Toyopearl HW-65s, named OEP1, for

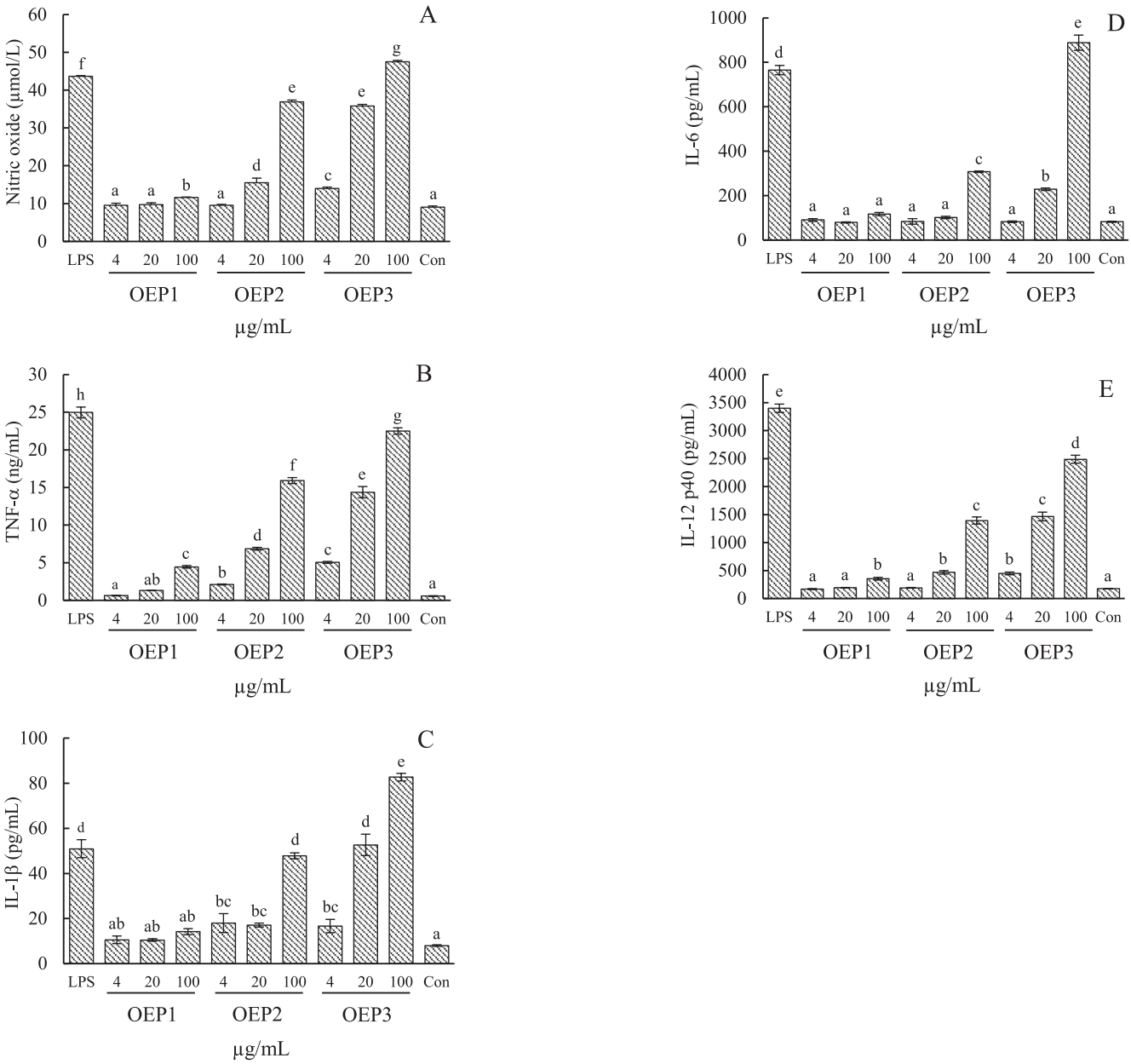

Fig. 3. Effect of the isolated polysaccharides on the production of NO (A), TNF-α (B), IL-1β (C), IL-6 (D), and IL-12 p40 (E).

Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. The height and bar of each column represents the means ± SD (n = 3); those not sharing a common superscript letter are significantly different at $p < 0.05$. Con: Control.

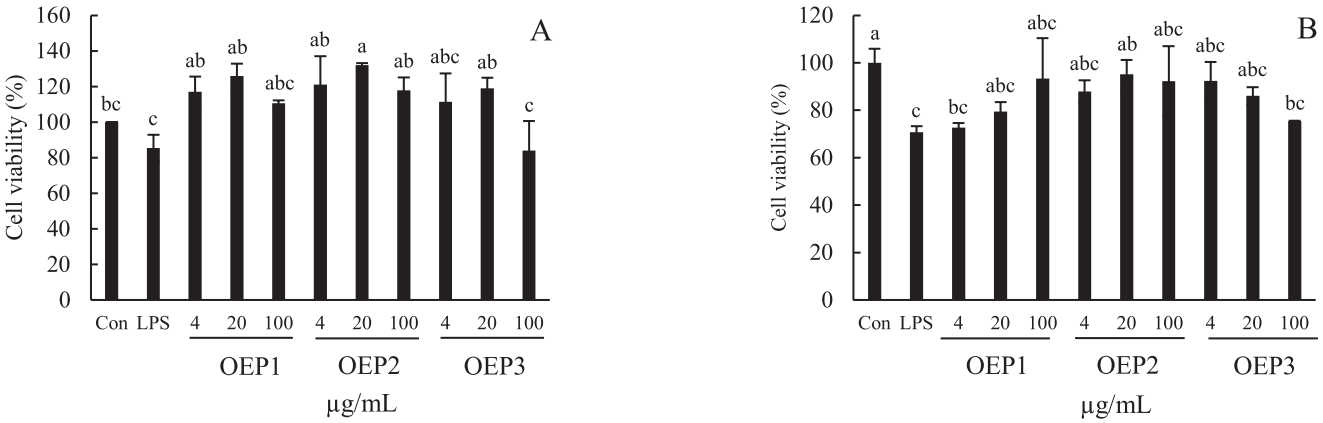

Fig. 4. Toxicity of the isolated polysaccharide fractions at different concentrations assayed by cell viability.

Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. The height and bar of each column represents the means ± SD (n = 3); those not sharing a common superscript letter are significantly different at $p < 0.05$. (A) RAW 264 cells, (B) J774.1 cells. Con: Control.

testing. Cytokines (IL-1β, IL-6, and TNF-α) released from activated macrophages play important roles in pro-inflammatory responses. IL-12 is a typical example of an LPS mediator that is a pro-inflammatory cytokine and functions in both innate and adaptive immune systems [36]. This cytokine takes part in the production of INF-γ by NK cells and T cells [37], induction of Th1 responses [38], and enhancement of resistance to intracellular infections [39, 40]. The effects of different concentrations of OEP1, OEP2, and OEP3 on NO, TNF-α, IL-1β, IL-6, and IL-12 p40 production are shown in Fig. 3. A slight increase in NO secretion from macrophages was observed in cultures supplemented with OEP1. However, OEP2 and OEP3 significantly increased NO secretion dose-dependently (Fig. 3A). This trend was also observed in TNF-α, IL-1β, and IL-12 p40 secretions (Figs. 3B, 3C, and 3E). However, IL-6 levels were significantly increased following OEP3 treatment (Fig. 3D).

Among the polysaccharides isolated from Super Ohtaka®, only those in which phosphorus was detected stimulated macrophages, and the results reflected the phosphorus content. There are several reports on the immunostimulatory action of EPS against lactic acid bacteria. H-APS, a high molecular mass fraction of acidic EPS produced by *L. bulgaricus* OLL1073R-1 has been reported to stimulate mouse splenocytes and significantly increase interferon-γ production. It has also been reported that oral administration of H-APS enhances the activity of natural killer (NK) cells [34], and that the phosphate groups of polysaccharides are important for this activity [41]. In addition, human trials using yogurt made with this bacterium suggest that it enhances resistance to viruses [42].

Cell viability. The effects of various polysaccharide fractions derived from Super Ohtaka® on the viability of mouse macrophages are shown in Fig. 4A (RAW 264 cells) and 4B (J774.1 cells). For the RAW 264 cells shown in Fig. 4A, except when OEP3 was added at a concentration of 100 μg/mL (84.0 ± 16.7 %), although it showed some variation, the viability was over 100 % compared to the control. Therefore, samples other than OEP3 likely had a low influence on NO and cytokine production in RAW 264 cells. For the J774.1 cells in Fig. 4B, 4 μg/mL concentration of OEP1 (72.7 ± 1.98 %) and LPS had similar viability (70.7 ± 2.65 %) but did not induce IL-12 p40. Therefore, we speculated that the addition of OEP1 at a concentration of 4 μg/mL would have little influence on the release of cytokines due to macrophage cell death. But, OEP1 did not exhibit dose-dependent cytotoxicity. This may be because OEP1 was a mixture of OEP1-1 and OEP1-2. Moreover, OEP1 was a fraction that was not adsorbed by ion exchange chromatography, so it was not sufficiently separated, and there may have been a small amount of contaminants. When OEP2 was used, the viability was approximately 90 % at all concentrations. OEP3 had a viability of 92 ± 8.09 and 86 ± 3.72 % when added at 4 and 20 μg/mL, respectively, and showed IL-12 p40 induction even at these concentrations. Since the viability was 75 ± 0.22 % when 100 μg/mL was added, there may have been a cytotoxic influence on cytokine production.

In this study, we isolated for the first time several polysaccharides from Super Ohtaka® that are different from β-glucan. The isolated polysaccharides stimulated immune cells to produce NO and cytokines, which correlated with the phosphorus content of the polysaccharides. Stimulation with these polysaccharides produces IL-12 p40 from macrophages, which is expected to activate NK and T cells and contribute to the anticancer effects. It would be interesting to study the role of these polysaccharides in intestinal immunity. We intend to explore further the relationship between the structures and functions of these polysaccharides.

CONFLICTS OF INTERESTS

Hideki Okada, Akira Yamamori, Naoki Kawazoe, and Michimasa Hirata are employees of Ohtakakohso Co., Ltd.

ACKNOWLEDGMENTS

We thank Drs. E. Fukushi and Y. Takata (Hokkaido University, Sapporo) for NMR spectral measurements.

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