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Isolation and Characterization of Intestinal Immune System Modulating and Anticancer Active Fractions from the Herbal Prescriptions

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Abstract The prescriptions (DB-1 – DB-5) were prepared with the active herbal medicines, *Acanthopanax senticosus*, *Glycyrrhiza uralensis*, *Polygonatum odoratum*, and *Cichorium intybus*. The most active crude polysaccharide fraction (DB-2-3), which was isolated through the fractionation of hot-water extract from DB-2, was significantly reduced by periodate oxidation (52.7 and 63.7%) on intestinal immune system modulating and anticancer activity. When DB-2-3 was further fractionated by column chromatographies, DB-2-3IIc-2 showed the most potent activities. In addition, DB-2-3IIc stimulated the proliferation of bone marrow cells via Peyer's patch in dose-dependent pattern by oral administration. The metastasis of colon 26-M3.1 lung carcinoma had significantly inhibited in mice fed DB-2-3IIc at 1 mg/mouse (43.8%). DB-2-3IIc-2 mainly contained uronic acid (46.1%) and 42.5% of neutral sugar with a small amount of protein (7.6%), and component sugar analysis also showed that DB-2-3IIc-2 was composed Ara, Gal, and GalA (molar ratio; 0.50:0.63:1.00). It may be suggested that activities of DB-2-3IIc-2 are resulted from pectic polysaccharides containing a polygalacturonan moiety with side chain of neutral sugars, such as Ara and Gal.

Keywords: herbal prescription, intestinal immune system modulating activity, anticancer activity, pectic polysaccharide

Introduction

Traditional herbal medicines, which consist of several kinds of component herbs, generally take orally as the crude extracts which are obtained by the decoction of the mixture of several herbs (prescription). Crude extract contains not only low molecular weight substances such as alkaloids, saponins, and flavonoids (1), but also high-molecular weight substances such as proteins, tannins, and polysaccharides (2,3). Although biologically active substances with low molecular weight in herbal medicines have been studied well, these substances are restricted by usage as food materials owing to the organic solvent extraction resulted in safety and food processing problem, which was caused by addition with a large amount of inactive macromolecules. From these viewpoints, further progress in the functional food application of bioactive high molecular weight materials, such as polysaccharides and proteoglycan, is expected.

In our previous patent (4), hot-water extracts from *Acanthopanax senticosus*, *Glycyrrhiza uralensis*, *Cichorium intybus*, and *Polygonatum odoratum* showed strong intestinal immune system modulating and anticancer activities. Although hot water extracts from *A. senticosus* and *G uralensis* enhanced as intensive as biological response modifier, *C. intybus* and *P. odoratum* also exhibited higher intestinal immune system modulating and anticancer activity than those of the control and the other herbal medicines (4). Four kinds of these herbal medicines are known to be prophylactic to various diseases such as allergic condition (5), inflammation (6), and cancer (7), and various high molecular weight compounds such as polysaccharides and proteoglycan (8,9) from each herb are related to diverse biological activities. However, there are a few reports about the active constituents (polysaccharides) and biological activity from the prescription of these active herbal medicines.

In the present paper, therefore, the major active principles for intestinal immune system modulating and anticancer activities of a new herbal prescription were isolated and identified to develop the polysaccharide materials of functional food, which include the active polysaccharides in great quantities and have higher biological activities.

Materials and Methods

Materials *Acanthopanax senticosus* (AS), *Glycyrrhiza uralensis* (GU), *Polygonatum odoratum* (PO), and *Cichorium intybus* (CI), were obtained from the big mart (Cheongju, Korea), considering classes of plants, a place of production and the condition of breeding. Voucher specimens of these plants and extracts were deposited at Division of Food and Biotechnology, Chungju National University (Herbarium No. 001-004 and 011-014, Jeungpyong, Korea). Diethyl aminoethyl (DEAE)-Sepharose CL-6B and Sepharose CL-6B were obtained from Pharmacia Biotech (Uppsala, Sweden), and RPMI-1640 medium, Hank's balanced salt solution (HBSS), Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS), and thioglycollate were purchased from Gibco (Grand Island, NY, USA). Penicillin, streptomycin, and amphotericin B were obtained from Flow Laboratories (Irvine, Scotland), and Alamar Blue™ from Alamar BioSciences Inc. (Sacramento, CA, USA).

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Preparation of hot-water extracts from mixtures Four kinds of herbal medicines having immunological and anticancer activities, such as AS, GU, PO, and CI, were mixed by a particular % ratio considered each activity (4). Each prescription (DB-1 – DB-5) of dried herbal medicines (50 g) was homogenized by Ultra-Turrax® T-50 at 5,000 rpm for 20 min (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). After decoction with water (400 mL) to half volume followed filtration with metal mesh, the residues were re-extracted by the same procedure (3 times). The extracts were centrifuged to remove insoluble material, and lyophilized to obtain hot-water extracts from mixtures.

Fractionation of hot water extract from DB-2 The most active prescription (500 g), DB-2, which consisted of ratio (%) with AS:GU:PO:CI=35:35:15:15 was decocted with water (10 L) to half volume and the residual material was re-extracted by the same procedure. The extracts were combined and centrifuged to remove insoluble material. The supernatant was lyophilized to give hot-water extract (DB-2-0; yield 31.3%). DB-2-0 was refluxed with MeOH (5 L) for 1 hr (5 times) and centrifuged to obtain MeOH-soluble (DB-2-1; yield 4.9%) and MeOH-insoluble fractions. The MeOH-insoluble fraction was dissolved in water and then added 4 volumes of EtOH. The supernatant (DB-2-2; yield 1.1%) was separated by centrifugation and the resulting precipitate was dialyzed against distilled water. After the insoluble materials were removed by centrifugation, the non-dialyzable portion was lyophilized to obtain crude polysaccharide fraction (DB-2-3; yield 14.1%).

Chemical and enzymatic treatments of DB-2-3 Periodate oxidation was performed as described previously (10). Briefly, DB-2-3 was stirred in 50 mM acetate buffer (pH 4.5) containing 25 mM $NaIO_4$ at 4°C for 96 hr in the dark. The oxidized products were reduced with $NaBH_4$ and dialyzed to obtain the oxidized-products. Chlorite treatment was performed according to the procedure of Oka *et al.* (11). DB-2-3 was dissolved in 4% acetic acid and added $NaClO_2$. The solution was stirred at 70°C until the color had changed, and neutralized with 3 N NaOH in an ice bath. The reaction products were dialyzed and lyophilized to obtain the chlorite-treated products. In addition, DB-2-3 was digested with Actinase E (Kaken-Kagaku Co., Ltd., Tokyo, Japan) in 50 mM Tris-HCl buffer (pH 7.9) containing 10 mM $CaCl_2$ at 37°C for 48 hr. After the reaction was terminated by neutralization with 0.1 N HCl, the mixtures were dialyzed and lyophilized to obtain the digested products (10).

Ion exchange and gel filtration chromatography of DB-2-3 DB-2-3 was applied to a column (4.0×40 cm) of DEAE-Sepharose CL-6B (Cl^- form) equilibrated with deionized water (DIW). The column was eluted with DIW to obtain an unadsorbed fraction (DB-2-3I; yield 3.3%). The adsorbed fractions were eluted by stepwise gradient elution with 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 2.0 M NaCl. Seven adsorbed fractions were obtained as lyophilizates after dialysis (yields: DB-2-3IIa, 4.2%; 3IIb, 12.7%; 3IIc, 15.4%; 3IId, 5.5%; 3IIe, 3.5%; 3IIf, 3.7%; 3IIg, 2.3%). DB-2-3IIc having the significant immunological and anticancer activity was further fractionated by Sepharose CL-6B (3.0×90 cm, 0.2 M NaCl), and the most active polysaccharide, DB-2-3IIc-2 (yield, 2.8%), was obtained in the intermediate volume.

General methods Total carbohydrate, uronic acid, and protein were determined by phenol-sulfuric acid (12), *m*-hydrxydiphenyl (13), and Bradford's method (14) by using Gal, GalA, and BSA as the respective standards. Component sugars of polysaccharides were analyzed as alditol acetates after hydrolysis of polysaccharides with 2 M trifluoroacetic acid (TFA) for 1.5 hr at 121°C (15) and analyzed by gas liquid chromatography (GLC) as described previously (16). GLC was performed on an HP-6890 Series II gas chromatography (Hewlett-Packard, Palo Alto, CA, USA) equipped with a SP-2380 capillary column (0.2 μm film, 0.25 mm i.d.×30 m, Supelco); the temperature program was: 60°C for 1 min; 60°C→220°C (30°C/min), 220°C for 8 min; 220°C→250°C (8 °C/min); 250°C for 15 min. The molar ratios were calculated from the peak areas and response factors using the flame-ionization detector (FID).

Mice and cell culture ICR and specific pathogen-free female BALB/c or C3H/He mice (5-7 weeks old) were purchased from Daihan-Biolink Co. (Chungbuk, Korea). The mice were housed and maintained at 24±1°C with constant humidity (55%). They had access to commercial chew pellet diet (Samyang Co., Ltd., Incheon, Korea), and water was freely available. Colon 26-M3.1 lung carcinoma was maintained in EMEM supplemented with 7.5% FBS, Na-pyruvate, non-essential amino acid, and L-glutamine.

Intestinal immune system modulating activity through Peyer's patch One-hundred-eighty μL of Peyer's patch cell suspension (2×10^6 cells/mL in RPMI 1640-FBS) prepared from the small intestine of untreated C3H/He mice or C3H/He orally administered DB-2-3IIc for 7 days (0.5, 1.0, and 2.0 g/kg/day) was cultured with 20 μL sample or without sample in a 96-well flat bottom microtiter plate for 5 days at 37°C. The culture supernatant (50 μL) was incubated with bone marrow cell suspension (2.5×10^5 cells/mL in RPMI 1640-FBS) from untreated C3H/He mice for 6 days (17). After 20 μL of Alamar Blue™ solution was added to each well, the cells were continuously cultured for 5-24 hr (18). The fluorescence intensity was measured to count the cell numbers using Fluoroskan II (Labsystems, Helsinki, Finland) at an excitation wavelength of 544 nm and emission wavelength of 590 nm during the cultivation. The intestinal immune system modulating activity was expressed as the stimulation of bone marrow cell growth compared with that of the control, in which Peyer's patch cells were incubated or administered with saline instead of the test sample.

Assays of anticancer activities Experimental lung metastasis of colon 26-M3.1 cells was assessed by *i.v.* inoculation of tumor cell lines into syngeneic BALB/c mice (19). Mice administered with the indicated dose (1 mg/mouse) of samples by the oral administration were then inoculated *i.v.* with colon 26-M3.1 (2.7×10^4/mouse) cells 1, 3, and 5 day after treatment. The mice were dissected 14 days after tumor inoculation and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted under

a dissecting microscope. For NK cell-mediated tumor cytotoxicity, 2 Balb/c mice/group were intravenously administered their respective extract (100 μg/mouse), and their splenocytes were harvested after 1 day. Single splenocytes cell suspensions (100 μL/well) were added to ^{51}Cr-labelled Yac-1 cells (1×10^4 cells/100 μL/well) to obtain effector (splenocytes)-to-target (Yac-1) cell ratios (E/T ratio) of 25:1 and 100:1 in U-bottomed 96-well plates. After incubation for 6 hr at 37°C, the plates were centrifuged for 10 min at 900×g. The supernatant (100 μL) of each well was absorbed onto a cotton swab and monitored for radioactivity using a gamma counter (20). The % cytotoxicity generated by the NK cells was calculated from the radioactivity (count/min).

Statistical analysis The differences between the control (only saline without sample) and the treatment in the experiments were tested for statistical significance by the Student's *t*-test. A value of $p<0.05$ was considered to show that the test sample had statistically significant immunostimulating and anticancer activities.

Results and Discussion

Preparation of the active herbal prescription In order to develop new herbal prescription as food materials with health-promoting effect, we prepared hot water extracts of various mixtures by ratio compounded the activity of individual herbal medicine from *A. senticosus* (AS), *G. uralensis* (GU), *P. odoratum* (PO), and *C. intybus* (CI), which showed the potent intestinal immune system modulating and anticancer activity (4). When the intestinal immune system modulating activity and NK cell-mediated cytotoxicity against Yac-1 of hot-water extracts from prescriptions (DB-1 – DB-5) were compared, hot-water extract from DB-2 (% ratio; AS:GU:PO:CI=35:35:15:15) showed the more potent activity than those of individual herbal medicine or other mixtures: hot-water extract from only AS had 1.76-fold (100 μg/mL) of the control whereas hot-water extract from DB-2 showed 1.63 (10 μg/mL) – 2.15-fold (100 μg/mL) on intestinal immune system modulating activity (Fig. 1A); DB-2 also showed the most potent NK cell cytotoxicity (3.53 – 6.35-fold at 25:1 and 100:1 of E/T ratio, 100 μg/mouse, respectively), but AS had the intermediate activity (5.85-fold of 100:1, Fig. 1B). Hot-water extract from DB-1 prescription (AS:GU:PO:CI=30:30:20:20), which was mixed by small weights of AS and GU compared to DB-2, showed much lower the activity than DB-2 (1.76-fold at 100 μg/mL; 5.47-fold at 100:1 of E/T ratio) as active as one from only AS, whereas hot-water extracts from DB-3 (AS:GU:PO:CI=40:30:10:20), DB-4 (AS:GU:PO:CI=40:35:10:15), and DB-5 (AS:GU:PO:CI=40:40:10:10) by large weight of AS and GU as the main active components had the same activity of DB-2 (Fig 1A and 1B), suggesting no effect on the activity of mixture even if AS and GU added the larger weight than DB-2.

The chemical compositions of a prescription are very complicated. After combining with other herbs to form a prescription, the action of each of the original herbs will be changed. The essential change is in the chemical compositions, i.e. the formation of new substances (21), which are the material bases for functional effects. Although the biological activity of the herbal prescription also nearly showed the sum activity of each herb by the these reactions for decoction in generally, hot-water extract of DB-2 showed the higher intestinal immune system modulating and anticancer activity than the other mixture of AS. Therefore, it was suggested that a new herbal prescription of DB-2 contained some healthful chemical ingredients, and it could provide beneficial biological activities

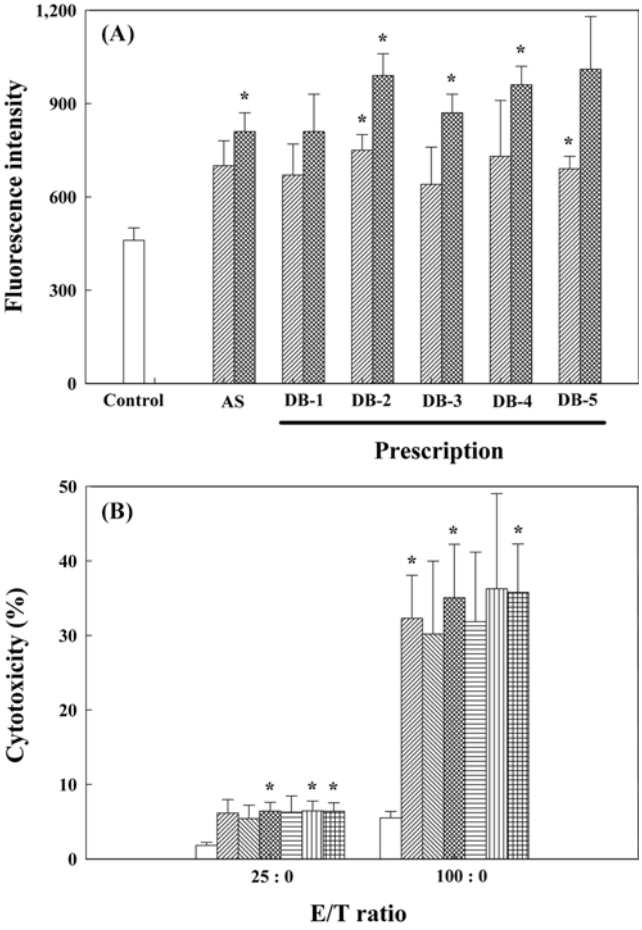

Fig. 1. Effects of hot-water extracts from 5 prescriptions on (A) intestinal immune system modulating activity and (B) NK cell-mediated tumor cytotoxicity. AS, hot-water extract from *A. senticosus*. Prescription, DB-1 – DB-5 mixtures with AS, GU, PO, and CI. E/T ratio was effector (splenocytes)-to-target (Yac-1), and sample 100 μg/mouse *i.v.* Mean±SD (n=4); *Significance between control and sample at $p<0.05$. (A) ▨, sample (10 μg/mL); ▩, sample (100 μg/mL), (B) □, control; ▨, AS; ▧, DB-1; ▩, DB-2; ▤, DB-3; ▥, DB-4; ▦, DB-5.

Isolation and characterization of the biological active polysaccharides from hot-water extract of DB-2 After the fractionation of hot-water extract of DB-2 (DB-2-0), only DB-2-3 (crude polysaccharide fraction) showed the most intestinal immune system modulating and NK cell-mediated cytotoxicity against Yac-1, but other subfractions showed no activity even at a higher concentration or E/T ratio (Fig. 2A and 2B). In order to examine which moiety in DB-2-3 contributed to expression of the activity, effect of chlorite treatment, periodate oxidation and pronase

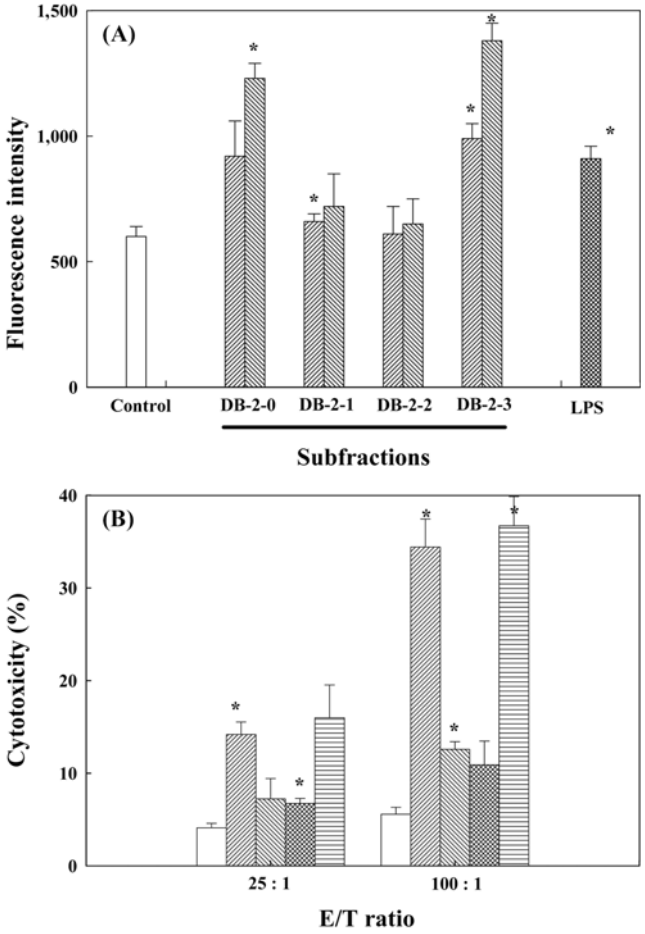

Fig. 2. Effects of subfractions from DB-2-0 on (A) intestinal immune system modulating activity and (B) NK cell-mediated tumor cytotoxicity. Subfractions: DB-2-0, hot-water extract of DB-2; DB-2-1, MeOH-soluble fraction; DB-2-2, EtOH-soluble fraction; DB-2-3; crude polysaccharide fraction. LPS: lipopolysaccharide, positive control (10 μg/mL). (A) ▨, sample (10 μg/mL), ▩, sample (100 μg/mL); (B) □, control; ▨, DB-2-0; ▧, DB-2-1; ▩, DB-2-2; ▤, DB-2-3.

Table 1. Effects of chemical and enzymatic treatments of DB-2-3 on immunological and anticancer activity

Fraction	Fluorescence intensity[1)]	Cytotoxicity[2)]
Control	530±40	4.50±0.55
DB-2-3	1,080±60*[3)]	27.68±2.63*
Periodate-oxidized DB-2-3	790±50*	12.91±1.13*
Chlorite-treated DB-2-3	960±170	24.91±2.69*
Pronase-digested DB-2-3-3	920±60*	20.57±3.57

[1)]The final concentration of sample was 100 μg/mL.
[2)]The final amount of sample was 100 μg/mouse at 100:1 of E/T ratio.
[3)]Mean±SD (n=4); *Significant difference between control and sample at $p<0.05$.

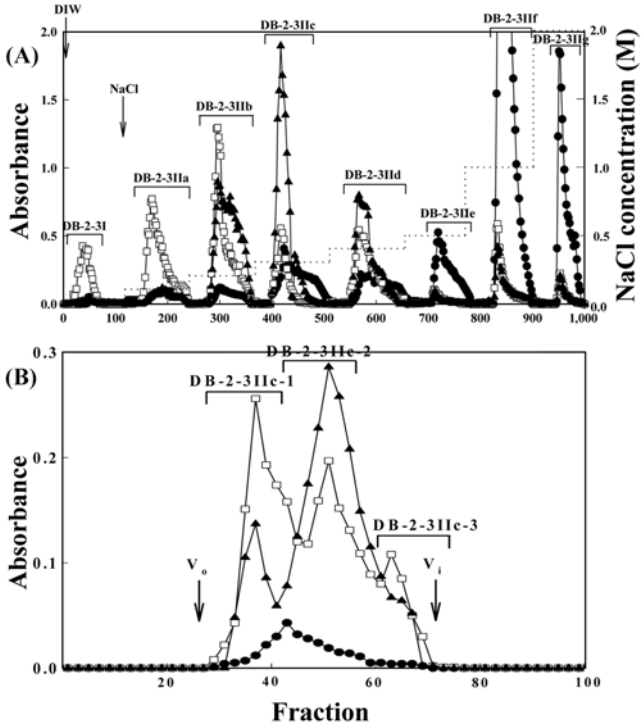

Fig. 3. Elution patterns of (A) DB-2-3 on DEAE-Sepharose CL-6B (Cl^- form), and (B) DB-2-3IIc on Sepharose CL-6B. ●, UV-absorbance (280 nm); □, carbohydrate (490 nm); ▲, uronic acid (520 nm).

digestion were tested on the activity of DB-2-3. As shown in Table 1, $NaIO_4$ oxidation significantly reduced the activity of DB-2-3 (52.7% reduction at 100 μg/mL, and 63.5% reduction at 100:1 of E/T ratio, respectively) whereas $NaClO_2$ and pronase treatments did not affect activities, suggesting that the polysaccharide moiety in DB-2-3 contributed to expression of the activity. From these results, we found that macromolecules such as polysaccharides, rather than low molecular weight substances, are the potent immunological and anticancer compounds of DB-2 mixture.

When DB-2-3 was further fractionated by anion-exchange chromatography on DEAE-Sepharose CL-6B (Cl^- form), 1 unadsorbed (DB-2-3I) and 7 adsorbed fractions (DB-2-3IIa – 3IIg) were obtained (Fig. 3A). When the intestinal immune system modulating activities of the subfractions were compared, DB-2-3IIc showed the most potent activity (2.23-fold of the control at 100 μg/mL) (Table 2). DB-2-3IIb and 3IId had intermediate activity (1.84- and 1.66-fold) whereas other fractions expressed weak activity only (Table 2). Among these subfractions, DB-2-3IIc also showed the most significant and potent NK cell-mediated cytotoxicity (6.32-fold at 100:1 of E/T ratio, 100 μg/mouse), and the order of the cytotoxicity seemed to be DB-2-3IIb (5.87-fold)>DB-2-3IId (5.23-fold) (Table 2). Since DB-2-3IIc mainly contained carbohydrate and its yield was relatively higher than the others, the active substances were identified from DB-2-3IIc in the present study. When DB-2-3IIc was fractionated by gel filtration on Sepharose CL-6B, 3 subfractions (DB-2-3IIc-1 – 3) were obtained (Fig. 3B). DB-2-3IIc-2 showed the most potent intestinal immune system modulating activity (2.14-fold at 100 μg/mL) and NK cell-mediated cytotoxicity (6.25-fold at 100:1 of E/T ratio, 100 μg/mouse), but DB-2-3IIc-1 and 3IIc-3 had the weaker activities than DB-2-3IIc-2 (Table 3).

The immunological and anticancer active fraction, DB-

Table 2. Intestinal immune system modulating and anticancer activities of subfractions from DB-2-3 on DEAE-Sepharose CL-6B

Subfraction	Fluorescence intensity[1)]	Cytotoxicity[2)]
Control	440±30	5.24±0.79
DB-2-3	920±50*[3)]	32.85±4.45*
DB-2-3I	520±30*	8.23±1.16*
DB-2-3IIa	660±100	11.52±2.19
DB-2-3IIb	810±100	30.76±4.34*
DB-2-3IIc	980±50*	33.12±4.17*
DB-2-3IId	730±40*	27.41±5.64
DB-2-3IIe	610±60	20.69±2.74*
DB-2-3IIf	540±80	12.31±2.38
DB-2-3IIg	500±30*	11.11±2.46

[1)]The final concentration of sample was 100 μg/mL.
[2)]The final amount of sample was 100 μg/mouse at 100:1 of E/T ratio.
[3)]Mean±SD (n=4); *Significant difference between control and sample at $p<0.05$.

Table 3. Immunological and anticancer activity of subfractions from DB-2-3IIc on Sepharose CL-6B

Subfraction	Fluorescence intensity[1)]	Cytotoxicity[2)]
Control	420±20	6.28±0.82
DB-2-3IIc	910±40*[3)]	39.38±4.36*
DB-2-3IIc-1	740±60	34.16±4.02*
DB-2-3IIc-2	900±30*	39.25±4.12*
DB-2-3IIc-3	510±20*	11.22±2.19

[1)]The final concentration of sample was 100 μg/mL.
[2)]The final amount of sample was 100 μg/mouse at 100:1 of E/T ratio.
[3)]Mean±SD (n=4); *Significant difference between control and sample at $p<0.05$.

2-3IIc-2, mainly contained uronic acid (46.1%) in addition to a considerable amount of neutral sugar (42.5%) and a small amount of protein 7.6% (Table 4), but DB-2-3IIc-1, which had intermediate activity, comprised of about 20.8% uronic acid, 69.3% neutral sugar, and 2.3% protein. Component sugar analysis showed that DB-2-3IIc and its identified fraction DB-2-3IIc-2 consisted mainly of GalA, Gal, and Ara (molar ratio of 1.00:0.63:0.50 and 1.00:0.58:0.59) (Table 4). In the present study, although further detailed characterization of DB-2-3IIc-2 was not performed, the active fraction was considered to comprise mainly pectic-arabinogalactan polysaccharides, which contained a galacturonan region [poly- or oligomerized α-(1→4]-GalA] and a 'ramified region' (rhamnogalacturonan core with side chains, such as arabinogalactan) because DB-2-3IIc-2 contained much of GalA, Ara, Gal, and Rha (molar ratio; 1.0:0.59:0.58:0.20). Aspinall (22) and Clarke *et al*. (23) classified plant arabinogalactans and arabinogalactan-proteins into type I, type II, and another type according to the structure of arabinogalactan portion. Type I arabinogalactans are arabino-4-galactan which have a (1→4)-β-D-galactan backbone with arabinosyl oligosaccharide side chains. Type II arabinogalactans are arabino-3,6-galactans which

Table 4. Physicochemical properties of subfractions obtained from DB-2-3IIc on Sepharose CL-6B

	DB-2-3IIc	DB-2-3IIc-1	DB-2-3IIc-2
Content[1)] (%)			
Carbohydrtae	54.7	69.3	42.5
Uronic acid	40.5	20.8	46.1
Protein	6.1	2.3	7.6
Component sugar (mol%)			
Arabinose	16.1	20.1	19.7
Xylose	5.1	10.4	3.9
Rhamnose	4.6	2.5	6.6
Fucose	2.0	1.5	1.7
Mannose	4.6	6.2	4.4
Galactose	20.2	22.4	19.2
Glucose	9.3	17.7	5.7
Galacturonic acid	31.9	16.2	33.2
Glucuronic acid	6.2	3.0	5.6

[1)]Analyzed by: carbohydrate content, phenol-sulfuric acid method (as galactose); uronic acid content, m-hydroxydiphenyl method (as galacturonic acid); protein content, Bradford method (as BSA).

comprise a highly branched (1→3)-β-D-galactan backbone possessing (1→6)-β-D-galactosyl and/or arabinosyl side chains attached at position 6 to some of the galactosyl residues in the backbone. Other arabinogalactan types are polysaccharides with arabinogalactan side chains such as a pectic arabinogalactan from *Angelica acutiloba* Kitagawa (24). Since DB-2-3IIc-2 did not reacted with the β-glucosyl-Yariv antigen (data not shown), which can recognize type II arabinogalactan (23), it was suggested to be not an arabino-3,6-galactan but pectic polysaccharide with side chain of neutral sugars. However, studies on the structure and structure-activity relationship of the active polysaccharides, DB-2-3IIc-2, must await further study.

Intestinal immune system modulating and anti-metastatic activities by oral administration of DB-2-3IIc The active fraction, DB-2-3IIc, identified from hot-water extract of DB-2 mixture on DEAE-Sepharose CL-6B was orally administered to investigate the intestinal immune system modulation via Peyer's patch and anti-metastasis using colon 26-M3.1 lung carcinoma model in mice. The *ex vivo* effect of DB-2-3IIc on how Peyer's patch cells mediate the hematopoietic responses of bone marrow cell was investigated Peyer's patch cells were obtained from C3H/He mice that had been administered with DB-2-3IIc for 7 days at 0.5, 1.0, and 2.0 g/kg doses, and the culture supernatant was added to the culture of bone marrow cells that had been isolated from untreated mice. When the intestinal immune system modulating activity through Peyer's patch of orally administered DB-2-3IIc was compared, 2.0 g/kg showed the most potent activity (1.57-fold of the saline control), whereas other doses group had intermediate activity (Fig. 4). This result suggested that oral administration of 2.0 g/kg of DB-2-3IIc enhanced the stimulatory responses of bone marrow cell proliferation through Peyer's patch. Intestinal immune system, including Peyer's patches, not only contributes to the defense system of the mucosa but also regulates systemic

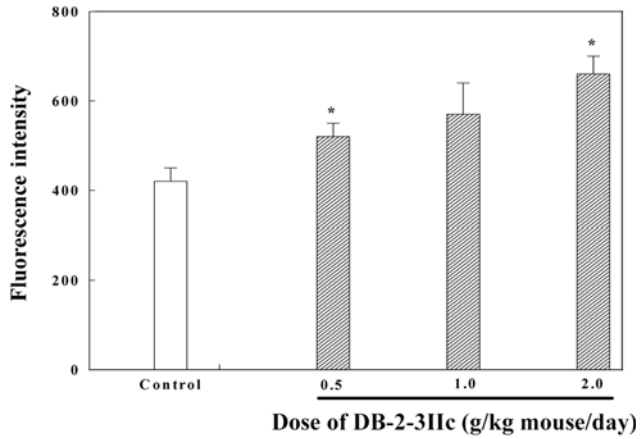

Fig. 4. Effect of orally administered DB-2-3IIc on intestinal immune system modulating activity. Data were expressed as mean±SD of quadruplicate cultures. □, Control (only saline was administered); ▨, oral administered doses of DB-2-3IIc.

Table 5. Effects of orally administered DB-2-3IIc on anti-metastatic activity

Orally administered fraction[1)]	Number of lung metastasis of colon 26-M3.1	
	Mean±SD[2)] (inhibition %)	Colony range
Tumor control[3)]	144±25 (100.0)	118-175
DB-2-3	100±16 (30.6)*[4)]	80-121
DB-2-3IIb	133±35 (7.6)	98-171
DB-2-3IIc	81±11 (43.8)*	70-89

[1)]Final amount of sample was 1 mg/moue.
[2)]Inhibition (%)=[(No. of lung metastasis of tumor control−No. of lung metastasis of sample)/No. of lung metastasis of tumor control] ×100.
[3)]Tumor-beared one without sample administration.
[4)]*Significant difference between the tumor control and sample at $p<0.05$ ($n=4$).

inflammation, resulting in the suppression of allergic reactions and autoimmune diseases (25). In several studies, hot-water extracts from rhizomes of *Atractylodes lancea* (2) and roots of licorice (26) have been shown to have a modulating effect on the intestinal immune system. Since these herbal medicines, including DB-2-3IIc from hot-water extract of DB-2 mixture, are orally administered, there is a possibility that these natural sources express their clinical effects through the intestinal immune system. In our previous study (4), it was observed that the systemic administration of hot-water extracts from individual AS and GU significantly inhibited tumor metastasis produced by colon 26-M3.1 carcinoma cells, and this effect was due to the activation of innate immune system in mice such as macrophage and NK cells. In addition, hot-water extracts of these herbal medicines could augment the natural immunity against tumor metastasis produced by colon 26-M3.1 carcinoma (4). In the present study, the oral administration of DB-2-3 and DB-2-3IIc fractionated from DB-2 mixture of herbal medicines including AS and GU dramatically inhibited the lung metastasis produced by colon 26-M3.1 tumors in a dose dependent manner (Table 5). Oral administration of DB-2-3 and DB-2-3IIc (1 mg/mouse) 1, 3, and 5 days before tumor inoculation similarly inhibited lung metastasis of colon 26-M3.1 cells (30.6 and 43.8% inhibition, respectively) even if the cytotoxicity via oral administration had lower than one of *i.v.* administration, whereas DB-2-3IIb was not significantly. It was also well documented that oral administration of BRMs alone or with antigens could induce the enhancement of host defense system non-specifically (27) as well as elicit specific and protective immune response to foreigners (28,29). Indeed, our previous study showed that the oral administration of glycoprotein fractionated from *A. senticosus* significantly inhibited experimental lung metastasis by colon 26-M3.1 cells (8), and its anti-metastatic activity was due to the activation of immune effector cells such as macrophage and NK cells in mice (8,30). Consequently, these results suggested that DB-2-3 and DB-2-3IIc was able to induce a prophylactic effect against lung metastasis induced by colon 26-M3.1 tumor cells, and these fractions are one of the components related with this anti-tumor activity.

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