

Anti-metastatic effect of polysaccharide isolated from *Colocasia esculenta* is exerted through immunostimulation

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Abstract. In the present study, an edible corm of the plant *Colocasia esculenta*, commonly known as Taro was extracted with cold water (4°C). Finally, 10.44 g (1.04%) of the crude polysaccharide (Taro-0) was obtained from Taro. The purified active compound (Taro-4-I) was isolated using DEAE-Sepharose FF and Sephadex G-100. The anti-complementary activity of Taro-4-I (57.3±4.5%) was similar to that of polysaccharide K (used as the positive control). The molecular weight of Taro-4-I was 200 kDa and it was a polysaccharide composed of 64.4% neutral sugars and 35.6% uronic acid. Taro-4-I activated the complement system through the classical and alternative pathways. The treatment of peritoneal macrophages with Taro-4-I significantly increased the production of interleukin (IL)-6 and tumor necrosis factor- α (TNF- α) in a dose-dependent manner. However, IL-12 production showed maximal activity at 56 μ g/ml and subsequently decreased. Splenocytes obtained from mice which were administered Taro-4-I intravenously showed a higher toxicity to Yac-1 cells compared to those obtained from untreated mice in a effector-to-target (E/T) ratio-dependent manner. The group treated with 50 μ g/ml Taro-4-I showed a significantly increased toxicity to Yac-1 cells compared to the group treated with 500 μ g/ml Taro-4-I. The administration of Taro-4-I significantly inhibited the lung metastasis of B16BL6 melanoma cells. However, the group treated with 50 μ g/mouse Taro-4-I had a significantly lower number of tumors compared to the group injected with 500 μ g/mouse Taro-4-I.

Introduction

It is well known that the majority of cancer-related deaths are not due to the primary tumor itself but to the dissemination of

tumor cells to secondary sites by a series of events collectively known as the metastatic cascade (1). Cancer metastasis, the spread of cancer cells from the primary neoplasm to distant sites and their growth there, is the major cause of mortality in patients with various types of cancer (2). Tumor invasion and metastasis involve multiple processes and various cytophysiological changes, including changed adhesion capability between cells and the extracellular matrix and damaged intercellular interactions (3). Most cancer treatments such as surgery, radiation therapy, or chemotherapy, usually attack not only cancer cells but normal cells as well, causing harmful side-effects (4). In recent years, a number of botanical compounds isolated from food or natural herbal medicines have been found to inhibit proliferation, induce apoptosis, suppress angiogenesis, retard metastasis and enhance chemotherapy, exhibiting anti-cancer potential *in vitro* and *in vivo* (5). While they possess anti-cancer properties, toxicity to normal tissues is rare (6).

Colocasia esculenta Linn. (*C. esculenta*) (family, Araceae) is an annual herbaceous plant with a long history of use in traditional medicine worldwide, particularly in tropical and subtropical regions. The edible corm of *C. esculenta* is commonly termed Taro, it is found throughout India and is also cultivated worldwide (7). Taro has been known since ancient times for its curative properties and has been utilized for the treatment of various ailments such as asthma, arthritis, diarrhea, internal hemorrhaging, as well as neurological and skin disorders (8). Brown *et al* (9) reported that the soluble extracts of a starchy paste made from Taro showed anti-proliferative activity against the rat YIT colon cancer cell line and activated the lymphocytes from splenocytes. However, they only used the soluble extracts of the starchy paste, without knowing the identity of the active compound. Therefore, the purpose of this study was to identify the active compound with immunostimulating activity in the edible corm of *C. esculenta* and elucidate the mechanisms by which it stimulates the immune system.

Materials and methods

Plant material. The edible corm of *C. esculenta* (Taro), which was cultivated in Gyeongbuk, Korea in 2009, was purchased from a commercial market. A voucher specimen was deposited at the Graduate School of Food Biotechnology, Kyonggi University, Gyeonggi, Korea.

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Isolation and purification of the polysaccharides from Taro. Taro (1 kg) was sliced and mixed with three volumes of distilled water and stirred at 4°C overnight. After centrifugation at 6,000 rpm for 30 min, the supernatant was precipitated with four volumes of ethanol, dialyzed and lyophilized. Finally, 10.4 g (1.04%) of crude polysaccharides were obtained. The crude polysaccharides were purified by ion exchange chromatography on a DEAE-Sephacel FF (Cl⁻ form) column (GE Healthcare, Uppsala, Sweden) with a stepwise gradient of NaCl (0, 0.05-2 M NaCl). Each fraction was collected, dialyzed against tap water and freeze-dried. The active fraction, Taro-4, was eluted with 0.2 M NaCl and was further purified by size-exclusion chromatography on a Sephadex G-100 column (GE Healthcare) using 50 mM ammonium formate buffer (pH 5.5). High-performance size-exclusion chromatography (HPSEC) of Taro-4-I was performed on an high-performance liquid chromatography (HPLC)-9500 instrument (Young-Lin Co., Gyeonggi, Korea) equipped with a Superdex 75 GL column (GE Healthcare). A total of 10 μ l of each polysaccharide solution were analyzed using an isocratic mobile phase (50 mM ammonium formate buffer, pH 5.5) at a flow rate of 0.5 ml/min at room temperature. The molecular weights of the purified polysaccharides were estimated from a calibration curve constructed with standard pullulans (P-800, 400, 200, 100, 50, 20, 10 and 5; Showa Denko Co., Ltd., Tokyo, Japan).

General analytical methods. Total carbohydrate, uronic acid and protein were determined using phenol-H₂SO₄ (10), m-hydroxydiphenyl (11) and the Bradford method (12) with a protein assay kit, using galactose, galacturonic acid and bovine serum albumin as the respective standards. The sugar composition of the polysaccharide samples was determined by gas chromatography (GC) analysis of their alditol acetates. The samples were then hydrolyzed with 2 M trifluoroacetic acid for 1.5 h at 121°C, converted into the corresponding alditol acetates (13) and analyzed by GC at 60°C for 1 min, 60-220°C (30°C/min), 220°C for 12 min, 220-250°C (8°C/min), and 250°C for 15 min, using a GC (GC 6000 series; Young-Lin Co.) equipped with an SP-2380 (Supelco, Bellefonte, PA, USA) capillary column. The molar ratios were calculated from the peak areas and response factors using a flame ionization detector.

Anti-complementary activity assay. Anti-complementary activity was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by residual complement (14). Normal human serum (NHS) was obtained from volunteer adults. A total of 50 μ l aliquots of exopolysaccharide of various concentrations (100, 500 and 1,000 μ g/ml) were mixed with equal volumes of NHS and gelatin veronal-buffered saline (GVB²⁺, pH 7.4) containing 500 mM Mg²⁺ and 150 mM Ca²⁺, respectively. The mixtures were pre-incubated at 37°C for 30 min and the residual total hemolytic complement (TCH₅₀) was determined using IgM hemolysin-sensitized sheep erythrocytes (EA cells, 1x10⁸ cells/ml). The NHS was incubated with water and GVB²⁺ to provide a control. The anti-complementary activity of the isolated polysaccharides is expressed as the percent inhibition of the control TCH₅₀ polysaccharide K (PSK) (15) from

Coriolus versicolor. $TCH_{50} (\%) = TCH_{50} (\text{control}) - TCH_{50} (\text{treated with sample}) / TCH_{50} (\text{control})$.

Immunoelectrophoresis. Alternative activation of the C3 protein was examined using standard one- and two-dimensional immunoelectrophoresis methods. NHS was incubated with Taro-4-I and an equal volume of one of the following three solutions: i) GVB²⁺, ii) 10 mM ethylene-glycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) solution containing 2 mM MgCl₂ in GVB²⁺ (Mg²⁺-EGTA-GVB), or iii) 10 mM EDTA solution in GVB²⁺ (EDTA-GVB). The incubations were carried out at 37°C for 30 min. The serum was then subjected to crossed immunoelectrophoresis to observe the C3 cleavage products (16). Shortly after the first run in barbital buffer (pH 8.6; ionic strength, 0.025 with 1% agarose), the second run was performed on a gel plate (layer thickness, 1.5 mm) containing 0.5% anti-human C3 serum (Sigma Chemical Co, St. Louis, MO, USA) which recognizes both C3a and C3b, at a potential gradient of 15 mA/plate for 15 h. After electrophoresis, the plate was fixed and stained with 0.2% bromophenol blue in MeOH:water:acetic acid (5:4:1) (17).

Animals. Specific pathogen-free (SPF), 6-week-old female BALB/c mice were purchased from G-Bio Animal, Inc. (Seoul, Korea). The mice were maintained in a clean rack in an SPF room at Kyonggi University. Water and a diet of pellets were supplied *ad libitum*. All animal experiments were carried out according to the instructions of the Ethics Committee for Use of Experimental Animals at Kyonggi University (2011-003).

Macrophage proliferation and cytokine production. Peritoneal macrophages were harvested from thioglycollate-treated 6-week-old BALB/c mice as described previously (18). The cells (1x10⁶/well) were suspended in complete RPMI-1640 medium and plated in 96-well culture plates. After 2 h of incubation in a 5% humidified CO₂ incubator, non-adherent cells were removed by washing with PBS and the adherent macrophages were incubated with the indicated doses of Taro-4-I for 24 h. Macrophage proliferation was assayed using the Cell Counting kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA) (19) and the concentrations of various cytokines in the medium were determined by enzyme-linked immunosorbent assay kits (Becton-Dickinson and Co., Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Natural killer (NK)-mediated cytotoxicity assay. Yac-1 is a Moloney murine leukemia virus-induced lymphoma that lacks the expression of MHC-I and is sensitive to lysis by NK cells (20). Therefore, NK-mediated cytotoxicity was determined in Yac-1 and primary cultured splenocytes from sample-treated animals (21). Briefly, three BALB/c mice/group were administered Taro-4-I intravenously (i.v.) (5, 50 and 500 μ g/mouse) and their splenocytes were harvested three days after treatment. Single-cell suspensions of splenocytes were added to the Yac-1 cells (1x10⁵ cells/ml) to obtain effector-to-target (E/T) cell ratios of 100:1, 50:1 and 25:1 in U-bottomed nine-well plates, after which the cultures were incubated for 6 h. Following incubation, the culture supernatants (100 μ l/well) were mixed with lactate dehydrogenase (LDH) solution (Promega Co., Madison, WI, USA) and the absorbance value of each well

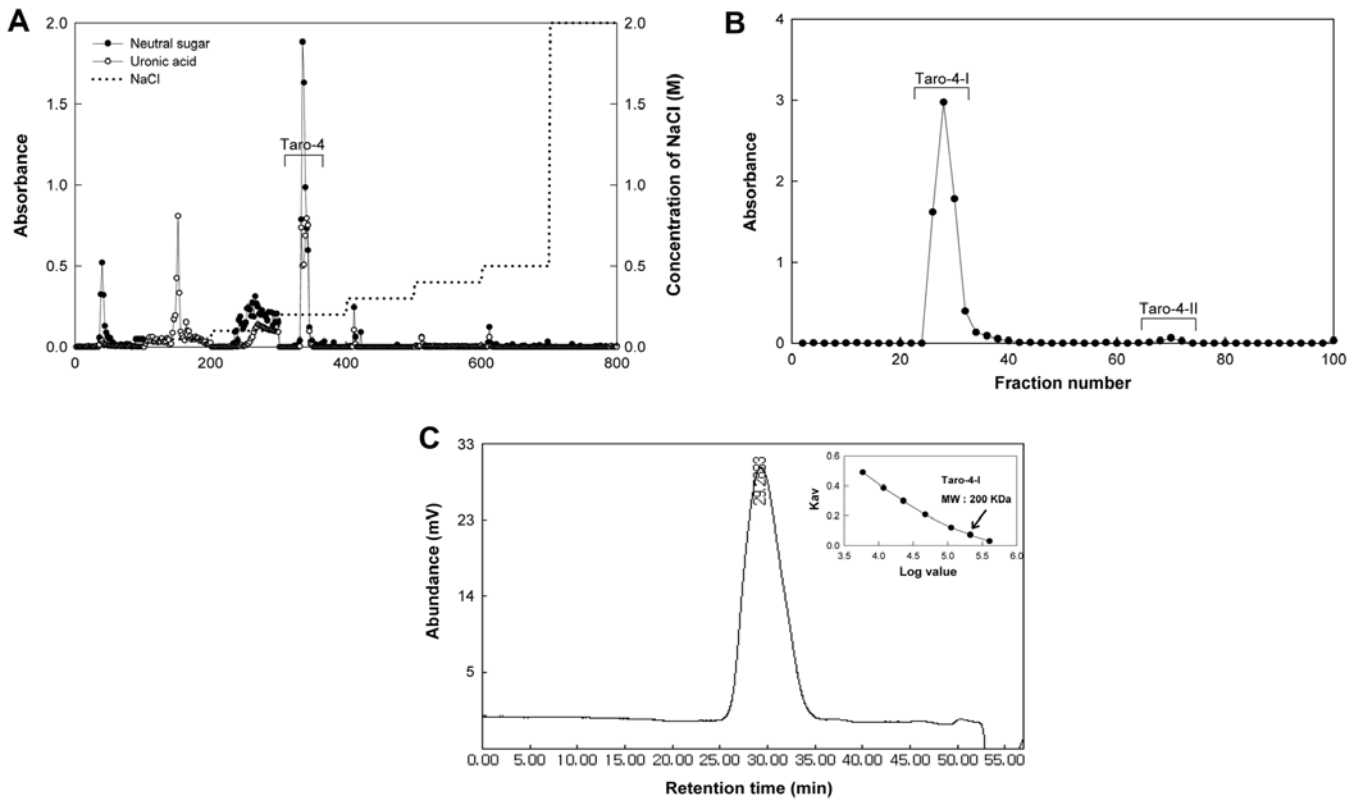


Figure 1. Purification steps of Taro-4-I from the edible corm of *Colocasia esculenta*. (A) Anion-exchange chromatography on DEAE-Sepharose FF (Cl⁻ form) column with stepwise gradient of NaCl (0, 0.05-2 M NaCl). (B) Size exclusion chromatography on Sephadex G-100 or elution pattern of Taro-4 on Sephadex G-100. Taro-4 was subjected on a Sephadex G-100 column (2.5x90 cm) and eluted with 50 mM ammonium formate buffer (pH 5.5) at a flow rate of 0.2 ml/min. (C) HPLC profile and molecular weight determination (in box) of Taro-4-I. HPLC equipped with Asahi-Pak GS-520 + GS-320 + GS-220 linked columns.

was measured at 490 nm. The percentage of NK cellular cytotoxicity was calculated using the following formula: cytotoxicity (%) = [(experimental release-spontaneous release)/(maximum release-spontaneous release)] x100.

Anti-metastatic activity in vivo. Experimental lung metastasis was assessed by *i.v.* inoculation of B16BL6 melanoma cells (2.7×10^4 cell/mouse) into syngeneic BALB/c mice (22). Treatment with various Taro-4-I doses was carried out two days prior to or one day after *i.v.* inoculation with B16BL6 melanoma cells. The mice were sacrificed 14 days following tumor inoculation and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted under a dissecting microscope.

Statistical analysis. 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 a one-way Analysis of variance (ANOVA) and Duncan's multiple range test. All data are presented as the means \pm standard deviation (SD).

Results

Purification of an active compound from *C. esculenta* with anti-complementary activity. The edible corm of *C. esculenta* was extracted with cold water (4°C) and 10.44 g (1.04%) of the crude polysaccharide (Taro-0) was obtained. The polysaccharide was then applied to column chromatography using

DEAE-Sepharose FF. Anti-complementary activities of subfractions from DEAE-Sepharose FF were detected in the following order: fraction eluted with 0.4 M NaCl (Taro-6) and fraction eluted with 0.3 M NaCl (Taro-5) > fraction eluted with 0.5 M NaCl (Taro-7) > fraction eluted with 0.2 M NaCl (Taro-4) > fraction eluted with 1.0 M NaCl (Taro-3). The yield of Taro-4 (30.5%) was higher than that of Taro-6 (3.1%), Taro-5 (3.9%) and Taro-7 (0.1%). Therefore, we selected the Taro-4 fraction for the following purification step. When Taro-4 was applied to a Sephadex G-100 column, it was divided into two subfractions, Taro-4-I and Taro-4-II (Fig. 1B) and the Taro-4-I subfraction was found to exhibit higher anti-complementary activity, as shown in Table I. Taro-4-I showed a single peak on HPLC, indicating that this fraction was highly purified (Fig. 1C).

Characterization of purified compound having anti-complementary activity. The anti-complementary activity of Taro-4-I (57.3 \pm 4.5%) was similar to that of PSK, which was used as the positive control (Table I). The molecular weight of Taro-4-I was 200 kDa (Fig. 1C). Taro-4-I was a polysaccharide composed of 64.4% neutral sugars and 35.6% uronic acid. Taro-4-I mainly comprised of galactose (38.9 mole %), mannose (19.2 mole %) and glucose (4.2 mole %) in the neutral sugar portion (Table II).

Activation mode of the complement system. The most important effector of the complement system, C3, is present in the human

Table I. Purification procedure and yield of each fraction.

Purification step	Recovery	Yield (%)	Anti-complementary activity ITCH ₅₀ (%) ^a
<i>Colocasia esculenta</i>	1.0 kg		
Cold water extract	10.4 g	100.0	41.3±0.8 ^d
DEAE Sepharose FF			
0.00 M NaCl	637 mg	6.1	30.8±3.4 ^e
0.05 M NaCl	1,936 mg	18.5	34.0±1.1 ^e
0.10 M NaCl	1,617 mg	15.5	43.1±1.0 ^d
0.2 M NaCl (Taro-4)	3,188 mg	30.5	55.8±3.1 ^c
0.3 M NaCl	410 mg	3.9	65.1±1.3 ^a
0.4 M NaCl	319 mg	3.1	66.4±0.4 ^a
0.5 M NaCl	Trace	0.1	61.7±1.6 ^b
2 M NaCl	273 mg	2.6	34.1±2.0 ^e
Sephadex G-100			
Taro-4-I	2,060 mg	19.7	57.3±4.5 ^c
Taro-4-II	Trace		

The purification process is described in detail in Materials and methods. Anti-complementary activity (1,000 µg/ml) was measured by the complement fixation test based on complement consumption and the degree of red blood cell lysis by the residual complement protein. ^aInhibition of TCH₅₀ (%) = [(TCH₅₀ of control - TCH₅₀ of sample)/TCH₅₀ of control] x 100. Superscripted lower case letters indicate significant differences between groups (P<0.05) as shown by Duncan's multiple range tests.

Table II. Chemical properties of the purified compound (Taro-4-I) from *Colocasia esculenta*.

Composition/component	(%)
Chemical composition ^a	
Neutral sugar	64.4
Uronic acid	35.6
Protein	0.0
Sugar component ^b	(Mole %) ^c
Rhamnose	0.1
Fucose	0.0
Arabinose	1.7
Xylose	0.3
Mannose	19.2
Galactose	38.9
Glucose	4.2
Galacturonic acid + glucuronic acid	35.3

^aBased on dry material; ^bsugar components were analyzed using alditol acetates by GC; ^cmole % calculated from the detected total carbohydrate.

plasma in large quantities (800-1,800 µg/ml) and it is converted to C3a and C3b by cleavage, which is the major reaction in complement activation (23). Both Mg²⁺ and Ca²⁺ are required to activate the classical pathway, but only Mg²⁺ is required to activate the alternative pathway. Taro-4-I was used in different buffer systems to evaluate the complement activation pathway.

Under the GVB²⁺ experimental condition containing Mg²⁺ and Ca²⁺ ions, Taro-4-I cleaved C3 and exhibited a clear second precipitin line (C3a and C3b protein) (Fig. 2Aa). Additionally, under the GVB²⁺ experimental condition containing Mg²⁺, Taro-4-I exhibited a clear second precipitin line. Under the GVB²⁺ experimental condition containing Mg²⁺ and Ca²⁺, the detected anti-complementary activity was ~50.5±4.5% at 1,000 µg/ml (Fig. 2B), which was the outcome of participation in both complement-activated pathways leading to cellular lysis. Furthermore, when anti-complementary activity was determined under the Ca²⁺-depleted experimental condition (GVB²⁺ containing Mg²⁺) (Fig. 2Ab), which only acts on the alternative pathway, Taro-4-I cleaved C3 and exhibited a clear second precipitin line and the detected anti-complementary activity was ~29.0±2.0% of the activity at 1,000 µg/ml (Fig. 2B). These results indicated that the mode of complement activation by Taro-4-I was via not only the classical, but also the alternative pathway, although to a lesser extent.

Effect of Taro-4-I on macrophage activation. We examined the Taro-4-I toxicity on primary cultured peritoneal macrophages by incubating the cells with doses up to 500 µg/ml. Taro-4-I at the maximum dose did not affect cell viability compared to the control (Fig. 3A). Subsequently, the effect of Taro-4-I on various cytokines, such as interleukin (IL)-6, IL-12 and tumor necrosis factor (TNF)-α, was assessed by incubating peritoneal macrophages with doses up to 500 µg/ml. The treatment of peritoneal macrophages with Taro-4-I significantly increased the production of IL-6 (Fig. 3B) and TNF-α (Fig. 3D) in a dose-dependent manner. The production of IL-12 showed maximal activity at 56 µg/ml, after which it declined (Fig. 3C).

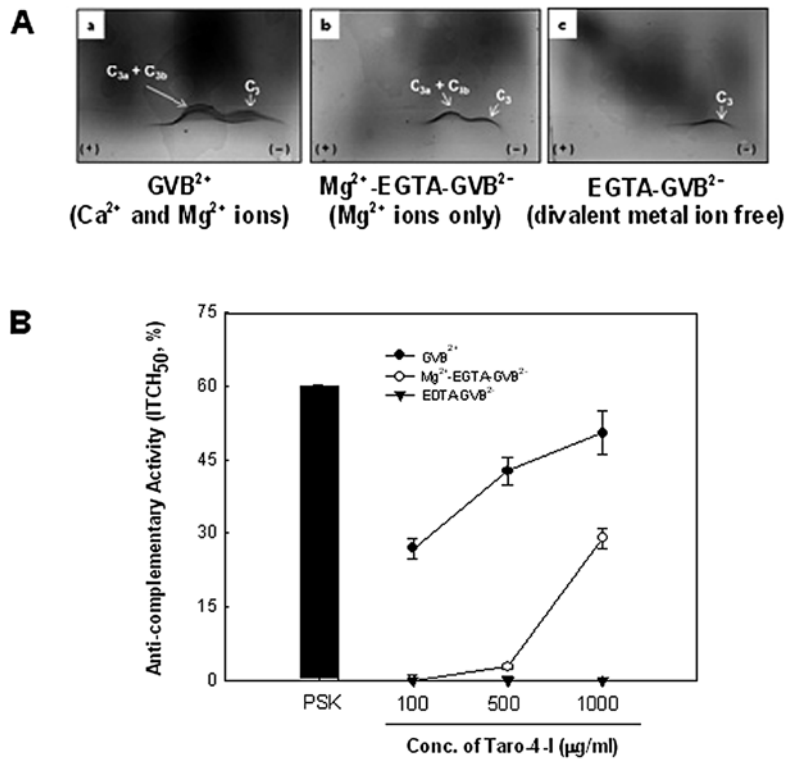


Figure 2. Alternative C3 fragmentation by Taro-4-I from the corms of *Colocasia esculenta*. (A) Image of immunoelectrophoresis with goat anti-human C3 antibody. (B) Anti-complementary activity with/without Ca²⁺ and Mg²⁺. Immunoelectrophoresis was carried out as described in detail in Materials and methods. Anti-complementary activity was presented as the inhibition of 50% total complement hemolysis by Meyer's method. Polysaccharide K (PSK), a known immunoactive polysaccharide from *Coriolus versicolor*, was used as the positive control. Values are presented as the means ± SD (n=3). Conc., concentration.

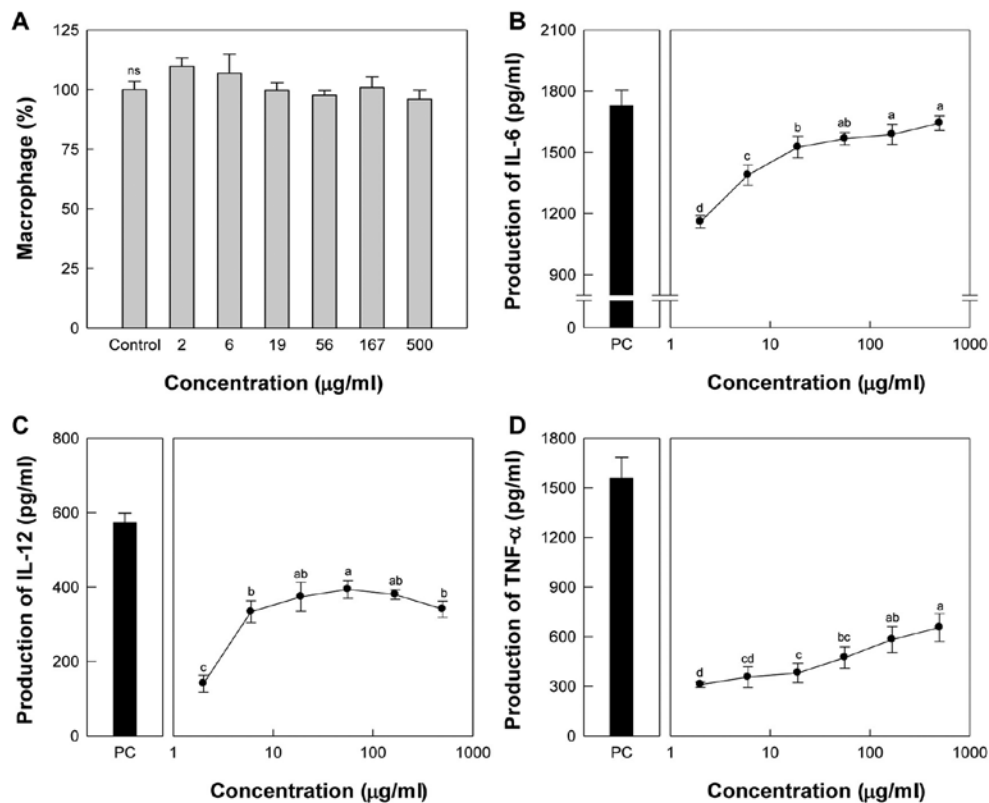


Figure 3. (A) Cytotoxic effect and production of cytokines including (B) IL-6, (C) IL-12 and (D) TNF-α by Taro-4-I in murine peritoneal macrophages. Peritoneal macrophages (1x10⁶/well) were treated with various concentrations of Taro-4-I in 96-well plates for 24 h. Cytotoxicity was determined using the Cell Counting kit-8 (CCK-8) and the concentrations of various cytokines in the medium were determined by ELISA kits. A total of 5 µg/ml lipopolysaccharide (LPS) was used as the PC (positive control). Closed symbols (●) with lower case letters (a-d) indicate significant differences between groups (P<0.05) as shown by Duncan's multiple range tests. ns, not significant.

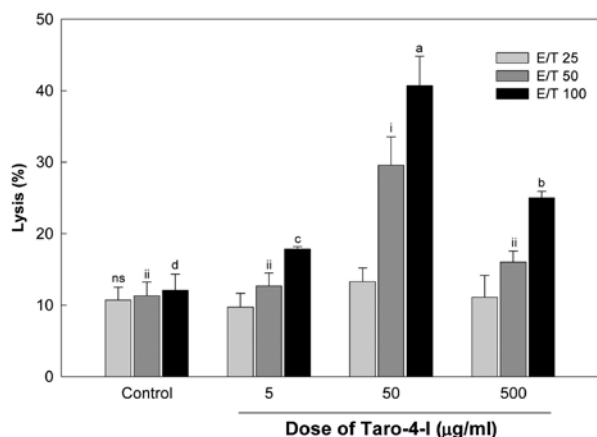


Figure 4. Effect of Taro-4-I on the activation of NK cells. NK-mediated cytotoxicity was determined in Yac-1 and primary cultured splenocytes from sample-treated animals. Following incubation, the culture supernatants were assayed with lactate dehydrogenase (LDH) content. Bars with lower case letters (a-d or i-ii) indicate significant differences ($P < 0.05$) as shown by Duncan's multiple range tests. ns, not significant.

Effect of Taro-4-I on NK cell activity. The effect of Taro-4-I on NK cell activity was estimated by the cytotoxic activity against Yac-1 cells, a NK-sensitive mouse lymphoma cell line,

using an LDH release assay. Splenocytes obtained from mice administered with Taro-4-I showed a higher toxicity to Yac-1 cells compared to those obtained from untreated mice in a E/T ratio-dependent manner (Fig. 4). The group treated with 50 µg/ml Taro-4-I showed a significantly higher toxicity to Yac-1 cells than the group treated with 500 µg/ml Taro-4-I.

Inhibitory effect of Taro-4-I on lung metastasis. We examined the effect of Taro-4-I on the experimental lung metastasis produced by B16BL6 melanoma cells. The administration of Taro-4-I significantly inhibited the lung metastasis of B16BL6 melanoma cells (Fig. 5). However, the group treated with 50 µg/mouse Taro-4-I had a significantly lower number of tumors than the group treated with 500 µg/mouse Taro-4-I.

Discussion

The innate immune system is phylogenetically older than the acquired (adaptive) or specific immune system and provides rapid but incomplete host defense until the slower, more definitive, acquired immune response develops (24). The complement system is an essential component of innate immunity and also plays an important role in modulating adaptive immunity (25). The complement system is a key component of the innate immune system, playing a central role in host defense against pathogens or cancer (26).

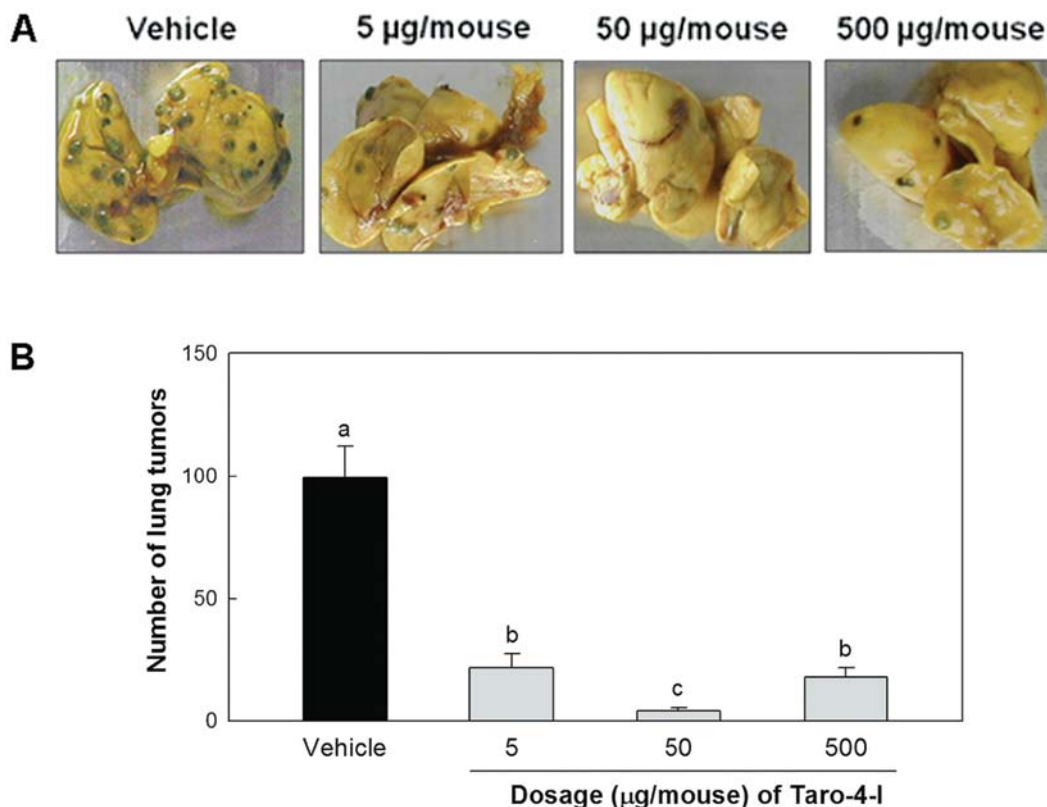


Figure 5. Anti-metastatic effect of Taro-4-I isolated from the corms of *Colocasia esculenta* on lung metastasis produced by i.v. inoculation of B16BL6 melanoma cells. (A) Scanned images of mouse lungs produced by i.v. inoculation of B16BL6 melanoma cells. (B) Five BALB/c mice per group were inoculated i.v. with 4×10^4 B16BL6 melanoma cells and injected i.v. with the indicated doses of Taro-4-I suspended in PBS two days prior to tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for the evaluation of tumors. Bars with lower case letters (a-d) indicate significant differences ($P < 0.05$) as shown by Duncan's multiple range tests.

Research of food-derived bioactive components for cancer prevention and cancer therapy is expanding due to the relatively low or undetected toxicity (27) and better bioavailability of these components. Over the last three decades, polysaccharides isolated from botanical sources (mushrooms, algae, lichens and higher plants) have also attracted a great deal of attention in the biomedical arena, due to their broad-spectrum therapeutic properties (28) and relatively low toxicity. The most promising biopharmacological activities of these biopolymers are their immunomodulatory and anti-cancer effects (29). Three anti-tumor mushroom polysaccharides, lentinan, schizophyllan and protein-bound PSK (Krestin), isolated from *Lentinus edodes*, *Schizophyllum commune* and *Coriolus versicolor*, respectively, have become major commercial items in Japan (29). Although the mechanism of their antitumor action is not yet completely understood, these polysaccharides and polysaccharide-protein complexes have been shown to enhance cell-mediated immune responses *in vivo* and *in vitro* and act as biological response modifiers (BRMs). BRMs are considered a useful tool in tumor growth suppression and inhibition of metastasis.

In this study, we identified and purified a potentially novel therapeutic compound (Taro-4-I), derived from Taro. This compound is a polysaccharide containing 35.6% uronic acid and is mainly comprised of galactose (38.9 mole%), mannose (19.2 mole%) and glucose (4.2 mole%) with a molecular weight of 200 kDa (Fig. 1C). The molecular weights of most immunostimulating polysaccharides are in the range of 6-1,000 kDa (30) and Taro-4-I falls within this range.

Anti-complementary activity is measured by the complement fixation test (14) and is expressed as the percentage inhibition of the control TCH₅₀ PSK (15) from *Coriolus versicolor* as a positive control. The antitumor activity of PSK has been evaluated in Japan for the prevention of esophageal, gastric and lung cancers in humans with promising results and is even sold as a drug (29). The polysaccharide has been found to be well-tolerated and compatible with chemotherapy and radiation therapy. However, the mechanism of action of PSK is not yet completely understood. Animals administered PSK have shown increased neutrophil levels with concomitant toxicity of target cells and a marked decrease in size and number of metastatic lung foci (31). Torisu *et al* (32) evaluated the clinical efficacy and the mechanism of action of PSK using a randomized double-blind trial in 111 patients who underwent surgery for colorectal cancer. They reported that the survival rate of patients was significantly higher ($P < 0.05$) in the PSK group than in the control group and the polymorphonuclear leukocytes from PSK-treated patients showed remarkable enhancement in their activities, such as random and/or chemotactic locomotion and phagocytic activity, when compared with those in the control group. In this study, the anti-complementary activity of Taro-4-I ($57.3 \pm 4.5\%$) was found to be similar to that of PSK ($60.0 \pm 0.0\%$) (Table I). Taro-4-I activated the complement system via the classical and alternative pathways (Fig. 2). The complement system plays an important role in host defense, inflammation and allergic reactions and is activated via the classical and alternative pathways. The classical pathway is activated by an immune complex containing IgM and IgG antibodies, the acute phase protein, C-reactive protein and RNA tumor viruses. The alternative pathway does not require antibodies and is directly activated by polysac-

charides, certain immunoglobulins, viruses, fungi, bacteria, certain animal cells and parasites.

Macrophages are ancient and phylogenetically conserved cells found in all multicellular organisms and they, together with neutrophils, represent the first line of host defense after the epithelial barrier. Macrophages participate both in non-specific defense (innate immunity) and in the initiation of specific defense mechanisms (adaptive immunity) in vertebrate animals. The production of IL-12 in a co-incubation system of peritoneal macrophages with Taro-4-I showed maximal activity at $56 \mu\text{g/ml}$ (Fig. 3C). The group treated with $50 \mu\text{g/ml}$ Taro-4-I showed a significantly higher toxicity to Yac-1 cells, a NK-sensitive mouse lymphoma cell line, compared to the group treated with $500 \mu\text{g/ml}$ Taro-4-I (Fig. 4). The group treated with $50 \mu\text{g/mouse}$ Taro-4-I had a significantly lower number of tumors compared to the group treated with $500 \mu\text{g/mouse}$ Taro-4-I (Fig. 5). IL-12 is produced mainly by macrophages and is a NK cell stimulatory factor. NK cell cytotoxicity may represent a way to eliminate overstimulated macrophages. NK cells are lymphocytes of the innate immune system that are involved in early defense against both allogeneic (non-self) cells and autologous cells undergoing various forms of stress, such as infection (with viruses, bacteria, or parasites) and malignant transformation (33). *In vitro* studies using cells from humans and several other mammalian species, as well as *in vivo* studies using mice and rats, have suggested that tumor cells are recognized as NK cell targets (34). It is thus speculated that Taro-4-I stimulates the complementary system and induces the secretion of various cytokines, such as IL-6, IL-12 and TNF- α from macrophages. The NK cells activated by IL-12 inhibit tumor metastasis.

The anti-cancer activity of the polysaccharides from Taro has previously been reported by Brown *et al* (9). However, this is the first study to demonstrate the anti-metastatic activity of the active compound isolated from Taro. Our data provide a scientific foundation for the anti-cancer and anti-metastatic activity of Taro-4-I, demonstrating that it exerts its effects through immunostimulation. The group treated with $50 \mu\text{g/mouse}$ Taro-4-I showed remarkable preventive activity ($96.2 \pm 1.3\%$).

Based on our data, the administration of $\sim 162.5 \text{ mg/day}$ of Taro-4-I or 824.85 mg/day of the cold water extract would be expected to have an anti-metastatic effect in humans (65 kg body weight) as calculated by the FDA dose calculator program. However, the anti-metastatic activity of Taro-4-I showed a bell-shaped profile. Its molecular weight is approximately 200 kDa . It is therefore questionable whether the whole structure is required for its anti-metastatic effects. Therefore, in future studies, we aim to further explore the optimal range for clinical trials and identify the essential structure.

Acknowledgements

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