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Purification, Characterization and Biological Activities of a Garlic Oligosaccharide

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Abstract : A novel oligosaccharide was purified from garlic (*Allium sativum* L.) bulbs via hot water extraction, ammonium sulfate precipitation, gel filtration and ion exchange chromatography. The molecular weight of the oligosaccharide was determined to be 1800. A nuclear magnetic resonance (NMR) study showed that ten fructose molecules were connected by β 1-2 linkage to a terminal glucose. The oligosaccharide had cytotoxic activities against human malignant lymphoma cells (U937) and colon adenocarcinoma cells (WiDr) in vitro. Furthermore, this oligosaccharide significantly suppressed the growth of murine colon adenocarcinoma cells (colon 26) in vivo. The oligosaccharide also stimulated interferon- γ production by human peripheral blood lymphocyte in vitro, indicating that it may activate the immunological pathways and suppress the growth of tumors in vivo.

Key words : garlic bulb, oligosaccharide, NMR, cytotoxic effect, immunological activity.

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Introduction

Garlic (*Allium sativum* L) bulbs are composed of 26~30% (w/w) carbohydrates, which are the main ingredients aside from moisture. Garlic contains the polysaccharides fructan and galactan, and oligosaccharide scorodose [1]. An epidemiological research has indicated that garlic is an effective cancer-preventive food [2]. Organosulfur compounds in the garlic bulbs have been thought to be the main components as anti-tumor substances. For example, diallylsulfide and diallyldisulfide were shown to exert an anti-tumor activity [3]. Diallyltrisulfide had the same effect [4]. Moreover, allicin (diallyl thiosulfinate) was shown to be toxic in various mammalian cells in a dose-dependent manner in vitro [5]. Ajoene had an anti-tumor activity not only in vitro but also in vivo [6].

We have long been interested in a purified garlic lectin protein and reported its anti-tumor activity [7]. During the process of garlic lectin purification, we also prepared a garlic oligosaccharide. We report here the anti-growth effect of garlic oligosaccharide on tumor cells both in vitro and in vivo, and an immunological activity as well.

Materials and Methods

Purification of garlic oligosaccharide

We mixed 164 g of fresh garlic bulbs grown in Japan with 164 ml of distilled water. They were then homogenized for 2 min at room temperature and heated for 30 min at 85°C. The infusion was filtrated through gauze to remove insoluble materials. The crude extract was adjusted to 80% saturation with (NH₄)₂SO₄ maintained at 4°C for 30 min, and centrifuged at 8000 rpm for 10 min. The precipitate was resuspended in 30 ml of 0.02 M acetate buffer (pH5.0) and centrifuged at 8000 rpm for 10 min. The supernatant was applied to a Sephadex G-75 column (4.5×65 cm), equilibrated with the 0.02 M acetate buffer (pH5.0), and eluted with the same buffer. Aliquots of 15 ml were collected, and the protein content in each fraction was measured by optical density at 280 nm. To measure the sugar content of each fraction, 50 µl was extracted and colored by the phenol sulfuric acid method and subsequently measured at 490 nm. Three peaks were detected at 280 nm; the second peak was the highest and contained sugar and it also contained lectin protein. The aliquots were concentrated by ultrafiltration using the AMICON YM10 membrane to 10 ml and then applied to a DEAE-Toyopearl column (3.2×33 cm), equilibrated with the 0.02 M phosphate buffer (pH6.0) and eluted with the same buffer by NaCl linear gradient (0—0.25 M). The sugar fraction, the first peak, was eluted and then pooled. Thereafter it was dialyzed against water and freeze-dried.

Measurement of molecular weight

The molecular weight of the oligosaccharide was measured by gel filtration chromatography (GFC) using a Bio-Gel-P2 column (2.0×64.1 cm: Bio-Rad Laboratories). Glucose (MW=180.16), sucrose (MW=342.34), raffinose (MW=522.46), stachyose (MW=666.58) and maltohexase (MW=990.87) were employed as standard reference compounds, and distilled water was used as an eluent.

Structural analysis

The matrix assisted laser-desorption ionization-time of flight (MALDI-TOF) mass spectrum was obtained with BIFLEX III (Bruker) using 2, dihydroxybenzoic acid as a matrix. ^1H NMR and ^{13}C NMR spectra were measured on a Varian UNITY INOVA 500 spectrometer at 500 MHz and 125 MHz, respectively.

Chemical shifts are given by relation to that of internal TSP-d4 ($\text{Me}_3\text{CD}_2\text{CD}_2\text{CO}_2\text{Na}$) in D_2O . Various techniques were examined, such as distortion enhancement by polarization transfer (DEPT), double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), rotating frame overhauser enhancement spectroscopy (ROESY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC).

Cell culture

Human histiocytic lymphoma cells (U937) and human colon adenocarcinoma cells (WiDr) were offered by the Health Science Research Bank at Japan Health Foundation. Murine colon adenocarcinoma cells (Colon-26) were kindly supplied by Dr. T. Obata at Kanazawa University Cancer Research Institute. U937, WiDr and Colon-26 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ of streptomycin and 50 $\mu\text{g}/\text{ml}$ of gentamicin sulfate in a humidified atmosphere of 5% CO_2 and 95% air at 37°C . At subconfluence, WiDr cells were harvested by treating with 0.25% trypsin and 0.02% EDTA.

Cell viability analysis (in vitro experiment)

U937 (1×10^4 cells) and WiDr (5×10^3 cells) cells were cultured in flat-bottomed microtiter culture plat wells with or without several concentrations of garlic oligosaccharide in RPMI 1640 medium containing 10% FBS at 37°C for the indicated days. U937 cells were harvested every day and viable cells were counted with a hemocytometer after staining with trypan blue [8]. Viable WiDr cells were determined after adding cell counting kit-8 solution (Dojin Chemical Co. Ltd., Kumamoto, Japan) to each well of a microtiter plate containing the cells. The cells were incubated in a CO_2 incubator for 3 h, and then the absorbance of each well was measured by a microplate reader at 450 nm.

Animals

Male BALB/cN Sea mice were obtained from Kyudo, Nakatsu, and maintained on the basal diet CE-II (CLEA Japan Inc., Tokyo, Japan) until use in the present experiment at age 8 weeks.

Anti-tumor analysis in vivo experiment

BALB/cN Sea mice (body weight : 23–24 g) were inoculated subcutaneously with 1×10^5 cells of murine colon adenocarcinoma (Colon 26) in 0.2 ml of phosphate-buffered saline (PBS) on day 0. The mice were given oral administration of oligosaccharide at a dose of 0.5 mg and 10 mg in 0.5 ml of PBS every other day from day 0. Control mice received only a solvent (PBS). Each group consisted of 6 mice. The anti-tumor activity of oligosaccharide was evaluated by

measuring tumor growth on days 19 and 22 after tumor inoculation and expressed as the ratio of tumor size of the oligosaccharide-treated mice to that of the control mice. Tumor size (mm^3) was measured with calipers and calculated using the following formula: Tumor size (mm^3) = [longest diameter] $\times$ [shortest diameter] $\times$ [shortest diameter].

All experimental procedures were in accordance with the Kyushu Institute of Technology Animal Care and Use Regulations.

Assay of cytokine production stimulating activity of human peripheral blood lymphocytes (PBL)

PBLs were obtained from 6 healthy male humans via the centrifugation method. An assay of cytokine production stimulating activity was performed in a 96-well culture plate. Human PBLs were inoculated at 1×10^6 cells /ml in an RPMI 1640 medium containing 0, 0.1 and 5 mg/ml of oligosaccharide. Following cultivation in a CO_2 incubator at 37°C , interferon- γ concentrations in the culture medium were measured by the ELISA method. The study on this assay using blood samples was approved by the Ethical Committee of the SRL Inc., Tokyo, Japan.

Results and Discussion

Structure elucidation

GFC analysis suggested that the molecular weight of the oligosaccharide was ca. 1800 (Fig.1). This was supported by MALDI-TOF mass spectrum, as shown in Fig. 2. The typical-162 (fragment) peaks, which are accompanied by +18 (water molecule) peaks, indicated the existence of a regularly connected C6-monosaccharide unit resembling fructose. Actually, the NMR spectra (DEPT, HSQC, HMBC, and DQF-COSY) suggested that a β 1-2 fructan sequence with a glucose terminus would form the bulk of the oligosaccharide (Fig.3). As can be seen from Table 1, the major peaks appeared in the ^1H and ^{13}C NMR spectra of the oligosaccharide and corresponded with those of commercial inulin (n =ca. 20~30).

In vitro studies

Figure 4a shows the dose-dependent effects of the garlic oligosaccharide on the growth of U937 and WiDr cells. After a 96 h treatment at a concentration of 5 mg/ml, the viabilities of the U937 and WiDr cells were 31 and 20%, respectively. The time-dependent effects of garlic oligosaccharide on the growth of U937 and WiDr cells are shown in Fig. 4b. It had quite a stronger cytotoxic effect on the WiDr cells by the 24h and 48h treatments compared to that of the U937 cells. We previously reported that a garlic lectin, which is purified with an oligosaccharide at the step of gel chromatography, exhibited strong anti-growth effects on these tumor cells [7], indicating that our purified products may be the major cancer-preventive components in the garlic bulbs besides the organosulfur compounds [3 – 5].

In vivo studies

The effect of oligosaccharide on the growth of subcutaneously implanted Colon-26 cells in

Fig. 1. Measurement of molecular weight.

The molecular weight of the oligosaccharide was measured by gel filtration chromatography (GFC) using a Bio-Gel-P2 column. ●: 1. Glucose(MW=180.16), 2. sucrose (MW=342.34), 3. raffinose (MW=522.46), 4. stachyose (MW=666.58) and 5. maltohexase (MW=990.87). ○: oligosaccharide.

Fig. 2. MALDI-TOF mass spectrum of the garlic oligosaccharide.

The matrix assisted laser-desorption/ionization-time of flight (MALDI-TOF) mass spectrum was obtained with BIFLEX III (Bruker) using 2, dihydroxybenzoic acid as a matrix. ^1H NMR and ^{13}C NMR spectra were measured on a Varian UNITY INOVA 500 spectrometer at 500 and 125 MHz, respectively.

Fig. 3. Speculated structure of the oligosaccharide obtained from garlic.

G: glucose, F: fructose ($n=9$), Ft: fructose terminal.

Fig. 4. Dose-dependent effects (a) and time-dependent effects (b) of the garlic oligosaccharide on cell viability. For the dose-dependent effects, cells were treated with different concentrations of oligosaccharide for 96 h and cell viabilities were determined. For the time-dependent effects, cells were treated with the oligosaccharide (5 mg/ml) for 24, 48, 72 and 96 h and cell viabilities were determined ●: U937, ■: WiDr. The cell viability is represented as percent of that of untreated control cells. All data are average $\pm$ S.E. from hexa wells.

Table 1. Comparison of the NMR data of the oligosaccharide and inulin

	¹³ C (δ)/ppm		¹ H (δ)/ppm	
G1	94.9	(94.9)	5.37	(5.36)
G2	73.8	(73.6)	a	(3.46)
G3	74.3,	75.2 (74.2, 75.0)		—
G4	71.8	(71.6)	a	(3.40)
G5	74.3,	75.2 (74.8, 75.0)		—
G6	a	(62.5)		—
F1	62.0~	64.5 (63.3)	3.60~3.90	(3.60~3.85)
F2	105.0~	107.0 (105.6)		—
F3	79.0~	82.0 (79.3)	4.10~4.20	(4.18)
F4	77.0~	79.0 (76.6)	4.00~4.10	(4.02)
F5	82.0~	85.0 (83.4)	3.80~3.90	(3.80)
F6	64.5~	66.0 (64.5)	3.55~3.95	(3.60~3.85)
Ft1	a	(62.8)		—
Ft2	a	(106.1)		—
Ft3	a	(79.1)		—
Ft4	a	(76.2)		—
Ft5	a	(83.4)		—
Ft6	a	(64.5)		—

In parentheses are shown the chemical of authentic inulin

^a Not determined due to peak overlapping

— : No signal

Table 2. Inhibitory effect of the oligosaccharide on the growth of Colon-26 cells

Dose (mg/mouse)	Tumor size (mm ³) mean±S.E.	Inhibition ratio (%)	Body weight (g)	Number of survivors/ Number tested
On the 19th day				
0	226.2 ± 85.7	0	27.3	6/6
0.5	36.4 ± 10.4	84	26.8	6/6
10	44.4 ± 14.6	80	29.0	6/6
On the 22nd day				
0	307.9 ± 102.5	0	27.5	6/6
0.5	32.3 ± 6.2*	90	27.0	6/6
10	55.6 ± 16.6*	82	29.6	6/6

Student's *t* test was used for the statistical analysis of differences*: Significantly different from control mice at *P*<0.05

Table 3. Interferon- γ production from human peripheral lymphocytes stimulated with the garlic oligosaccharide

Oligosaccharide (mg/ml)	Interferon- γ (IU/ml)
0.0	0.21 ± 0.03
0.1	$0.27 \pm 0.02^*$
5.0	$2.13 \pm 0.68^*$

Student's *t* test was used for the statistical analysis of differences

*: Significantly different from control mice at $P < 0.05$

BALB/cN Sea mice is shown in Table 2. Oral administration of the oligosaccharide resulted in a high inhibitory effect on the growth of Colon-26 cells. Table 2 shows that more than 80% of tumor growth inhibitions were observed on the 19th and 22nd days, after oral administrations of oligosaccharide at 0.5 and 10 mg /mouse/every other day. We observed a dose-dependent production of interferon- γ in human peripheral lymphocytes treated with the garlic oligosaccharide, as shown in Table 3. We also measured the stimulatory effect of the garlic oligosaccharide on the production of interferon- γ in mouse spleen cells (data not shown). Taken together, the oligosaccharide probably binds to cancer cells and inhibits the growth of the cells; furthermore, it may activate the immunological pathways and suppress the growth of tumors in vivo. The above results suggest that the oligosaccharide will provide us with effectiveness in its clinical application.

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ニンニクオリゴ糖の精製とその生理的活性

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要 旨：我々はニンニク鱗茎より糖質を精製した。この化合物は10 個のフルクトースと β 1-2 で1 個のグルコースに結合する分子量1800 のオリゴ糖であり、植物イヌリンと類似した構造を持つものであった。このオリゴ糖はヒトのリンパ球性ガン細胞U937 および大腸ガン細胞WiDrの増殖を抑制した。さらにマウスに経口的投与をした場合において、マウスに移植したColon26 ガン細胞の増殖を抑制することがわかった。またヒト末梢血リンパ球に対してインターフェロン γ の産生を増大させることから、ニンニクオリゴ糖はガン細胞に直接結合してガン細胞の増殖を阻害する働きがあると同時に、in vivoの系において免疫的活性を増大させてガン細胞の増殖を抑制する可能性があることが示唆された。

キーワード：ニンニク鱗茎, オリゴ糖, 核磁気共鳴, 細胞毒性, 免疫的活性。

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