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Effects of *Microbacterium laevaniformans* Levans Molecular Weight on Cytotoxicity

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Abstract Levans produced from *Microbacterium laevaniformans* were isolated, characterized, and fractionated by molecular weight. TLC, HPLC, and GC-MS analyses of the exo-polysaccharide showed that it was a fructan-type polymer and was composed of (2,6)- and (2,1)-glycosidic linkages. ^{13}C-NMR analysis proved that the polysaccharide was mainly a β-(2,6)-linked levan-type polysaccharide. To investigate the cytotoxicity of the acetone-precipitated levan fractions such as M1, M2, and M3, HepG2, P388D1, U937, SNU-1, and SNUC2A cell lines were screened. Among the cell lines tested, the cytotoxicity of M1-M3 fractions were detected from only SNU-1 and HepG2 cells at the dosage level of 100-800 μg/ml. The M2 fraction (M_r 80,000) at 400 μg/ml had the greatest cell growth inhibition (84.6%) on SNU-1, while the M1 (M_r 50,000) at 800 μg/ml showed the greatest (46.32%) on HepG2. To obtain more uniform M_r fractions of levan, the levan was further fractionated from S1 (M_r 1,000,000) to S5 (M_r 10,000) using gel permeation chromatography. Again, the S1-S5 fractions had strong cytotoxicity on SNU-1 and HepG2 cell lines. The greatest inhibition effects of S4 (M_r 80,000) on SNU-1 and S5 (M_r 10,000) on HepG2 were shown to be 49.5% and 73.0%, respectively. The cytotoxicity of the levan fractions was more effective on SNU-1 than on HepG2. Although the relationship between the Mw and the cytotoxicity was not clear, smaller M_r fractions of levan showed greater growth inhibition effect on the cancer cell lines in general. Therefore, it was indicated that a specific Mw class of levan is responsible for the effective cytotoxicity.

Key words: Levan, cytotoxicity, *Microbacterium laevaniformans*, molecular weight

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Levan, primarily found as a microbial product, is a β-(2,6)-linked fructose polymer with β-(2,1)-linked branched side chains, although it was also found in various plants [7, 9]. Microbial levans are produced from sucrose-based substrates by the action of levansucrase from a variety of microorganisms [6, 13, 14, 19, 22].

Many plant- or bacterial-derived polysaccharides possess multiple biological activities such as immunostimulating, prebiotic, antitumor, and anti-inflammatory activities [1, 2, 12, 17, 23]. Levan and fructan, which are fructose-based biopolymers, are also categorized as bioactive polysaccharides. Previously, *Aerobacter* levan was shown to have antitumor and immunostimulating activities [12, 18]. It was recently reported, as well, that levans produced by four different *Zymomonas mobilis* strains displayed antitumor activity, and the antitumor activity was somewhat related to the specific molecular weight [3, 4]. In addition to the role of levan in biological activity, the high viscosity and high molecular weight of levan enable its use as a thickener or stabilizer in the food, pharmaceutical, and cosmetic industries [11, 16]. Levan is also a promising raw material for fructose production.

The differences in composition of sugar, type of glycosidic linkage, and branching degree of levan influence the secondary and tertiary structures of the single chain and their macromolecular assembly, determining the biological and physical properties of the levan. Therefore, it is important to establish a structure-function relationship of levan polysaccharide for food and drug industrial applications. In this study, the structure of *Microbacterium* levan is characterized and levan is fractionated by molecular weight using acetone-precipitation or GPC separation. Using these fractionated levan samples, the MTT assay was pursued to find out the relationship between molecular weight and cytotoxicity of the levan polysaccharide.

MATERIALS AND METHODS

Materials

MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.). Kisegel 60 F_{254} TLC plate was purchased from Merck Co. (Darmstadt, Germany). Other chemicals (Sigma Chemical Co., St. Louis, MO, U.S.A.) were reagent grade and used without further purification.

Production of Levans

Levan was produced from the culture of *Microbacterium laevaniformans* KCTC 9732. The organism was grown on a defined medium, which consisted of sucrose, 1.0%; peptone, 1.0%; yeast extract, 0.3%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%. The pH of the medium was adjusted to 7.0-7.2. The bacterial cell culture was fermented for levan production in a shaking incubator for 48 h at 37°C with a seed culture grown in stationary flasks for 24 h. Cells were discarded by centrifugation at 5,500 ×*g* for 20 min. Extracellular levan in the supernatant was precipitated with an equal volume of acetone and stored at 4°C overnight. The precipitates were retrieved by centrifugation at 5,500 ×*g* for 20 min, and the final levan samples were freeze-dried.

Characterization of Levan from *Microbacterium laevaniformans*

Levan (0.04 g) was hydrolyzed using 0.5 M H_2SO_4 (15 ml) at 90°C for 3 h, and neutralized with 4 N NaOH. The resulting acid hydrolyzates were used for TLC (thin layer chromatography) and HPLC (high performance liquid chromatography) analyses. For the TLC analysis, 10 µl of the hydrolyzed sample was spotted on a silica gel plate (DC-Alufolien Kieselgel 60 F254, Merck & Co. Inc., Darmdstadt, Germany), and a mixture of methyl chloride: methanol (7:3, v/v) was used as a developing solvent. Para-anisaldehyde sulfuric acid solution was sprayed on the plate, and color was developed at 110°C for identifying sugars in the levan samples. The sample was subjected to HPLC analysis using a Waters Series (Pump 515, RI Detector 486, and Integrator 746, Waters Co., U.S.A.). The samples were eluted, at a flow rate of 1 ml/min, on a Zorbax NH_2 column (4.6×150 mm, DuPont Instruments, U.S.A.) with deionized water. The column temperature was maintained at 25°C, and the sample size was 10 µl. Methylation of the levan samples was achieved by following the procedure of Hakomori [8]. Ten milligrams of the sample was dissolved in 1 ml of DMSO, and then the resulting solution was reacted with 1.0 ml of methylsulfinyl cabanion at 25°C for 3 h. After 1 ml of methyl iodide (CH_3I) was added to the reaction solution and kept at 25°C overnight, the methylated levan sample was extracted with chloroform three times, then was recovered using petroleum ether. The reductive cleavage of the methylated samples was conducted by the Rolf and Gray method [18], and the resulting samples were treated with acetic anhydride (50 µl) for 1 h. Methylated alditol acetate was dissolved in dichloromethane and extracted with water several times, and then the organic layer was subjected to GC-MS analysis. The reduced derivatives were separated using a DB-5MS capillary column (0.25 mm i.d.×30 m) equipped in a Hewlett Packard 6890N Network GC system. The column temperature increased from 90 to 150°C at 2°C/min with 5-min hold at the initial temperature. Nitrogen flow was 1 ml/min with a split injection (1,000:1). The fragmented ions were identified with a 5973 mass selective detector. ^{13}C-NMR analysis of the levan samples dissolved in D_2O was performed with a 300 MHz NMR spectrometer (Unity INOVA, Varian Co., Palo Alto, CA, U.S.A.).

Fractionation of Levan by Molecular Weight

The levan in the culture supernatant was fractionated sequentially by precipitating it with different final concentrations of acetone. Seventy-five, 66, and 50 percent of acetone-precipitates were named M1, M2, and M3, respectively. The precipitates were collected and freeze-dried. GPC (Gel permeation chromatography) analysis was performed to determine average molecular weights (M_r) of the fractionated levan samples as described below.

To obtain more uniform molecular weight of levan, the levan was precipitated with 3 vol ethanol. The levan was reprecipitated twice from water with ethanol and dialyzed for 72 h. The GPC separation of levan on a column (1.6× 75 cm) of Sepharose-4B (Sigma Chemical Co., U.S.A.) was performed with distilled water as an eluent. Fractions were collected, and analyzed with phenol-sulfuric acid reagent, which were finally divided into five different Mw fractions from S1 to S5. The calibration curve was obtained to determine relative molecular weight (M_r) of the levan fractions using pullulan standards (Mw: 788,000, 404,000, 112,000, 47,300 dalton).

Maintenance of Cell Lines

The cytotoxicities of the levans from *Microbacterium laevaniformans* were investigated on a mouse monocyte cancer cell line (P388D1), a human monocyte cancer cell line (U937), a human colorector cancer cell line (SNUC2A), a human liver cell line (HepG2), and a human stomach cancer cell line (SNU-1). The cancer cell lines were purchased from Korea Cell Lines Bank, and were stored at -70°C. The cell lines were subcultured in RPMI-1640 medium with 10% (v/v) fetal bovine serum, and 100 units/ml penicillin/streptomycin, and incubated in a 5% CO_2 incubator at 37°C.

MTT Assay

The MTT assay for evaluating cytotoxicity was performed following the procedure of Carmichael *et al.* [5]. The cells

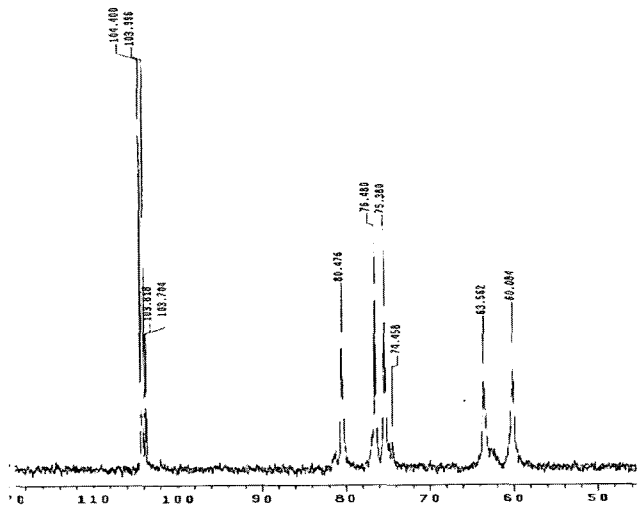

Fig. 1. ^{13}C-NMR spectrum of levan from *Microbacterium laevaniformans*.

(1×10^5 cells/well; 100 µl) were plated on 96-well tissue culture plates and treated with 100 µl of the levan samples. The treated cell cultures were incubated in a 5% CO_2 incubator at 37°C for 72 h. Once 20 µl of MTT stock solution (5 mg/ml) was added to the incubated culture, the plate was wrapped with aluminum foil and incubated for another 4 h in the same conditions. The medium in the culture was removed by centrifugation at 2,000 ×*g*, and the converted dye from the cells was solubilized with 200 µl of dimethylsulfoxide (DMSO). The absorbance of the released dye was determined at 540 nm. The dosage level of levan on the selected cell lines was in the range of 100–800 µg/ml. The cytotoxicity was determined by the degree of cell proliferation when the cancer cell specimen was treated with the levan solution. The percent of growth inhibitory effect was evaluated by the following equation:

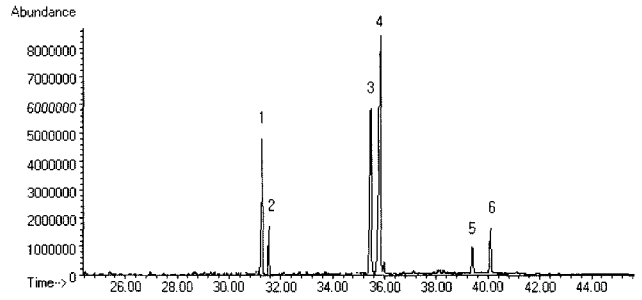

Fig. 2. Gas chromatogram of the partially *O*-methylated anhydroalditol acetates derived from reductive cleavage of levan from *Microbacterium laevaniformans*.

. 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol; 2. 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-glucitol; 3. 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-mannitol; 4. 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol; 5. 1,6-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol; 6. 1,6-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-glucitol.

Table 1. Fructosyl linkage composition of inulinase-modified M-levans.

Linkage type	Relative proportion (%)
β(2→6)-linked fructose	70.2
Branch points (at C1, C2, and C6)	12.9
Terminal groups (at 1- or 2-position)	17.0

$$\text{Growth inhibitory effect (\%)} = (DAbs_{control} - DAbs_{sample})/DAbs_{control} \times 100$$

Statistics

Statistical analysis was performed with ANOVA in SPSS (Statistical Package for the Science) program. Significance of difference at the level of $p<0.05$ was estimated by Duncan's multiple range test.

RESULTS AND DISCUSSION

Identification of Levan from *Microbacterium laevaniformans*

The structure of exopolysaccharide produced from *Microbacterium laevaniformans* was characterized using various analytical techniques. TLC and HPLC analyses of the acid hydrolyzates showed that the exopolysaccharide was fructan, which was constituted with only fructose (data not shown). ^{13}C-NMR analysis proved that the exopolysaccharide had a peak pattern of levan with β-anomeric form, which was identified by the chemical shifts of individual carbon atoms in the furanose ring (Fig. 1). The positions of six main peaks at C2 (104.4), C5 (80.5), C3 (76.5), C4 (75.4), C6 (63.6), and C1 (60.1 ppm) were almost identical with the peak positions for levan previously reported by Simms *et al.* [20] and Han and Clarke [10]. Again, GC-MS analysis clearly showed that the polysaccharide was a fructose polymer (Fig. 2). In addition, GC-MS analysis showed that 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-mannitol and 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol were the most abundant products. It indicates that

Table 2. MTT assay of differentially acetone-precipitated levans on the cytotoxicity of SNU-1 (Human stomach carcinoma) cells.

Dosage level (µg/ml)	Growth inhibition effect (%)		
	M1	M2	M3
100	$^{A1)}$43.91±6.38$^{a2)}$*	^{A}41.37±8.77^{a}	^{A}33.87±8.01^{a}
200	^{A}32.70±5.08^{a}	^{A}45.36±9.86^{a}	^{B}69.97±3.72^{b}
400	^{A}42.47±7.87^{a}	^{B}84.56±5.58^{b}	^{B}70.65±8.47^{b}
800	^{B}42.49±9.92^{a}	^{A}66.54±9.69^{b}	^{A}59.67±8.92^{b}

*Mean±S.D., n=3.

$^{1)A,B}$Values with different superscripts within the same row are significantly different among samples at $\alpha=0.05$ level by Duncan's multiple range test.

$^{2)a,b}$Values with different superscripts within the same column are significantly different among samples at $\alpha=0.05$ level by Duncan's multiple range test.

Table 3. MTT assay of differentially acetone-precipitated levans on the cytotoxicity of HepG2 (Human hepatocellular carcinoma) cells.

Dosage level (μg/ml)	Growth inhibition effect (%)		
	M1	M2	M3
100	$^{A1)}$44.91±1.94$^{a2)}$*	^{B}36.70±0.60^{b}	^{C}2.67±1.17^{c}
200	^{A}41.67±1.26^{a}	^{B}21.49±1.44^{a}	^{C}12.76±0.91^{a}
400	^{A}42.48±2.48^{a}	^{B}24.04±8.37^{a}	^{B}22.70±0.65^{a}
800	^{A}46.32±3.86^{a}	^{B}33.60±3.15^{b}	^{B}36.56±1.27^{a}

*Mean±S.D., n=3.

$^{1)A-C}$Values with different superscripts within the same row are significantly different among samples at α=0.05 level by Duncan's multiple range test.

$^{2)a-c}$Values with different superscripts within the same column are significantly different among samples at α=0.05 level by Duncan's multiple range test.

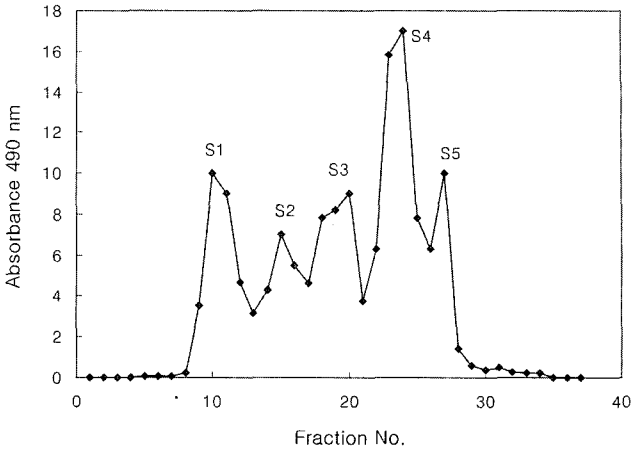

Fig. 3. Elution profile of levan polysaccharide from *Microbacterium laevaniformans* by Sepharose 4B-gel chromatography.

the main backbone chain of levan consisted of β-(2,6)-linkage. The peaks of 1,6-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol and 1,6-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-glucitol were derived from the main chain fructose having β-(2,1)-linked side chain. The ion fragmentation pattern of acid hydrolyzate of this polymer was identical with that of fructose (data not shown). Therefore, it was confirmed that the exopolysaccharide was a fructose polymer consisting of 70.2% of β-(2,6)-linked main chains and 12.9% of β-(2,1)-linked branches, when the individual peaks were quantified (Table 1).

MTT Assay of Acetone-Precipitated Levan Fractions

The average molecular weights of the acetone-precipitated levans, such as M1, M2, and M3, were 50,000, 80,000, and 200,000, respectively, when judged by GPC analysis. To evaluate the cytotoxicity of differentially acetone-precipitated levans, several cancer cell lines, such as HepG2, P388D1, U937, SNU-1, and SNUC2A, were screened. Among the cell lines tested, the growth of two cell lines, SNU-1 and HepG2, were strongly inhibited by the fractionated levan samples.

As indicated in Table 2, the cytotoxicity of the acetone-fractionated levans on the SNU-1 was in the range of 32.74-84.50% at the dosage levels of 100-800 μg/ml. The M2 fraction (MW 80,000) showed the greatest cytotoxicity (84.56%) at a concentration of 400 μg/ml. Then, the activity of M2 decreased at the higher level (800 μg/ml). In the case of HepG2, M1 (MW 50,000) treatment had the greatest (46.32%) inhibition effect at 800 μg/ml (Table 3). Overall, the smallest Mw fraction, M1, seemed to show the greatest activity at all dosage levels. The cytotoxicity of M2 decreased as the dosage level increased, while that of M3 increased along with increasing levan concentration. In most cases, however, cytotoxicity of the acetone-fractionated levans showed less than 50% on the HepG2 cell line. These results indicated that a specific class of molecular weight might be responsible for effective cytotoxicity.

MTT Assay of GPC-Fractionated Levans

To obtain more uniform molecular weight fractions of levan, the original levan samples were further divided into five fractions using Sepharose-4B gel chromatography. The relative molecular weight, M_r, of the GPC-fractionated levans were 1,000,000 (S1), 300,000 (S2), 100,000 (S3), 50,000 (S4), and 10,000 (S5) as shown in the elution profile (Fig. 3).

The cytotoxicity of S1-S5 were evaluated using cancer cell lines HepG2, P388D1, U937, SNU-1, and SNUC2A, and the strong growth inhibition effect was detected in the SNU-1 and HepG2 cell lines.

Table 4. MTT assay of single molecular weights on the cytotoxicity of SNU-1 (Human stomach carcinoma) cells.

Dosage level (μg/ml)	Growth inhibition effect (%)				
	S1	S2	S3	S4	S5
100	$^{C1)}$15.37±3.23$^{b2)}$*	^{A}48.46±3.70^{b}	^{A}37.47±2.85^{b}	^{A}49.44±0.99^{a}	^{B}21.17±3.09^{c}
200	^{C}15.35±2.92^{b}	^{A}63.18±1.49^{a}	^{B}50.88±2.88^{a}	^{B}51.40±1.89^{a}	^{B}51.37±0.68^{b}
400	^{D}18.37±1.63^{b}	^{B}64.09±5.23^{a}	^{AB}62.72±1.44^{a}	^{A}73.44±4.47^{a}	^{C}49.45±9.67^{b}
800	^{B}29.82±8.57^{a}	^{A}56.73±0.00^{b}	^{A}55.61±0.20^{a}	^{B}31.70±7.48^{b}	^{A}58.04±2.01^{a}

**Mean±S.D., n=3.

$^{1)A-D}$Values with different superscripts within the same row are significantly different among samples at α=0.05 level by Duncan's multiple range test.

$^{2)a-d}$Values with different superscripts within the same column are significantly different among samples at α=0.05 level by Duncan's multiple range test.

Table 5. MTT assay of single molecular weights on the cytotoxicity of HepG2 (Human hepatocellular carcinoma) cells.

Dosage level (μg/ml)	Growth inhibition effect (%)				
	S1	S2	S3	S4	S5
100	$^{C1)}3.41\pm0.14^{c2)}$*	$^{A}22.03\pm0.74^{c}$	$^{B}13.25\pm0.00^{c}$	$^{C}22.56\pm0.74^{b}$	$^{C}25.42\pm2.84^{c}$
200	$^{B}30.47\pm0.33^{b}$	$^{B}32.98\pm0.82^{b}$	$^{C}23.93\pm1.19^{b}$	$^{C}26.79\pm3.59^{b}$	$^{A}42.76\pm3.44^{b}$
400	$^{B}39.43\pm0.21^{a}$	$^{C}34.83\pm2.54^{b}$	$^{D}29.43\pm1.35^{a}$	$^{C}34.46\pm1.56^{a}$	$^{A}41.60\pm2.84^{b}$
800	$^{B}38.96\pm0.29^{a}$	$^{A}45.78\pm0.36^{a}$	$^{C}30.86\pm0.82^{a}$	$^{B}38.59\pm8.00^{a}$	$^{A}49.40\pm1.91^{a}$

*Mean±S.D., n=3.

1) $^{A-D}$Values with different superscripts within the same row are significantly different among samples at α=0.05 level by Duncan's multiple range test.

2) $^{a-d}$Values with different superscripts within the same column are significantly different among samples at α=0.05 level by Duncan's multiple range test.

The GPC-fractionated levans tested against SNU-1 did not show any clear relationship between the molecular weight and the cytotoxicity (Table 4). As the dosage level of levan fractions increased up to 400 μg/ml, the cell growth inhibition effect increased in general. When the dose increased to 800 μg/ml, however, the cytotoxicity of S2, S3, and S4 fractions tended to decrease. Among all the tested fractions, the S4 fraction showed the greatest inhibition activity (73.4%) at 400 μg/ml, while the S1 fractions showed the lowest cytotoxicity at each dosage level.

When the levan fractions were applied to the HepG2 cell line, the inhibition effect appeared to increase with increasing dose (Table 5). For instance, the effect of cell growth inhibition rapidly increased as the dosage level of S1 fraction changed from 100 to 200 μg/ml and further increased up to 400 μg/ml, then the effect kept at a consistent level starting at 400 μg/ml. In other fractions, S2-S5, the level-off effect existed either in a lower or in a similar dosage level. Although the S5 fraction (the smallest M_r fraction) was shown to have the greatest activity at all dosage levels, the molecular weight seemed not to be related to the degree of cytotoxicity. The cytotoxicity against HepG2 showed less than 50% in all test groups, as expected from the result of acetone-precipitated levan samples, which means that the levan is less effective on HepG2 than on SNU1. Previously, Calazans *et al.* [4] classified the levans of *Zymomonas mobils* by the viscosity-average molecular weight and the antitumor activities were evaluated against Sarcoma 180. They reported that the greatest antitumor activity was found in a levan with Mw 456,900 (72%), and the inhibition effects were not found to have relationship between the Mw and the dosage level. Therefore, it was concluded from the present study that there were a specific molecular weight class and dosage level of levan for displaying effective cytotoxicity.

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