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Characterization of Anti-complementary Polysaccharide from *Teucrium viscidum var. miquelianum*

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Abstract

Water-soluble crude polysaccharide (TM-1) prepared from the leaves of *Teucrium viscidum var. miquelianum* was fractionated into three polysaccharide fractions, TM-2, TM-3 and TM-4 by the addition of cetyltrimethylammonium bromide. The major polysaccharide fraction, TM-2, consisted of glucose, galactose, rhamnose, mannose, glucuronic acid and galacturonic acid, and all fractions contained galactose and glucose as the major neutral sugars. TM-4 showed the highest anti-complementary activity. When TM-4 was futher fractionated by anion exchange chromatography, TM4- II a and TM4- II b showed the most potent anti-complementary activity. TM4- II a was composed mainly of galactose, arabinose and glucose in the molar ratios of 2.13 : 0.94 : 1.00 respectively, and contained a small amount of galacturonic acid and glucuronic acid.

Key words: anti-complementary, polysaccharide, *Teucrium viscidum var. miquelianum*

INTRODUCTION

Teucrium viscidum var. miquelianum is given orally to patients for the treatment of several diseases such as convulsions, coughs, and pneumonia in traditional medicine. It relies upon the use of crude extracts of herbal drugs to bring about a general improvement in the homeostasis. Recently, several immunomodulatory activities have been observed in the high molecular weight fractions of the hot water extracts from the herbs but less observed in the low molecular weight fractions. Some studies have reported that the polysaccharides of the high molecular weight fraction possessed interferon-inducing activity (1), antineoplastic activity (2), lymphocyte mitogenic activity (3), anti-complementary activity (4-7), and anti-inflammatory activity (8). The complement system consists of over 20 serum proteins including nine complement components (C1,C2,C3,C4,C5,C6,C7,C8 and C9) and their regulators. It is important in initiating inflammation, and its activation might result in opsonization, activation of leukocytes, mast cell degranulation or lysis of target cells by the endproduct C5b-9 of the cascade. Several studies have reported on the anti-complementary activities : the root of *Angelica acutiloba* (4,9) contains six kinds of anti-complementary polysaccharides (two kinds of arabinogalactans and four kind of pectic polysaccharides), the leaf of *Artemisia princeps* contains two kinds of anti-complementary acidic heteroglycans (10), the grain of *Coix lachryma-jobi var.* ma-yuen contains two kinds of anti-complementary acidic heteroglycans (11). The present paper describes the chemical characterization of the major anti-complementary polysaccharides from the leaves of *Teucrium viscidum var. miquelianum.*

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MATERIALS AND METHODS

Materials

Teucrium viscidum var. miquelianum was purchased for experimental use at Pusan-Jin market in Pusan, Korea. DEAE-Sepharose Fast Flow were obtained from Sigma Co., Ltd.

Isolation of water-soluble polysaccharide

The dried leaves of *Teucrium viscidum var. miquelianum* (600 g) were decocted with hot water. The yield of the extract (TM-0) was approximately 12.6% of the dry weight of the *Teucrium viscidum var. miquelianum.* The lyophilised extract was refluxed with methanol (3 L) for 2h, and then the MeOH insoluble precipitate was dissolved in distilled water. The addition of ethanol (5 volumes) gave a precipitate that was collected by centrifugation and redissolved in distilled water. The distilled water soluble fraction was dialysed against running distilled water through Visking cellophane tubing, and the crude polysaccharide (TM-1) was obtained as the lyophilisate of the internal solution (yield, 7.9% of the dried *Teucrium viscidum var. miquelianum*).

Fractionation of crude polysaccharide

Crude polysaccharide (TM-1) was fractionated into three fractions as follows. TM-1 (15.8 g) was dissolved in distilled water (790 mL) and treated with an equal volume of 8% solution of cetyltrimethylammonium bromide (cetavlon) by the method of Yamada et al. (12). After standing at 20°C

for 20h., the precipitate was collected by centrifugation and redissolved in 10% NaCl. Potassium acetate and 2 volumes of ethanol were added to the solution, and the resulting precipitate was redissolved in distilled water, followed by dialysis against running distilled water. The acidic polysaccharide fraction (TM-2) was then obtained as the lyophilisate of the non-dialysble fraction (yied, 32.7% from TM-1). 1% H_3BO_3 (790 mL) was added to the supernatant. The solution was stirred and the pH was adjusted to 8.8 by the addition of 2M NaOH. The resulting precipitate was acidified with 2% acetic acid and dissolved in 10% NaCl. Three volumes of ethanol were added to the solution together with potassium acetate, and the resulting precipitate was dissolved in distilled water and then dialysed. The non-dialysable fraction (TM-3) was obtained as the lyophilisate (yield, 16.8% from TM-1). The final supernatant of the cetavlon fraction was acidified to pH 4.4 with acetic acid, and 3 volumes of ethanol were added to the solution together with potassium acetate. The precipitate was dissolved in distilled water, then dialysed, and TM-4 was obtained as the lyophilisate of the non-dialysable portion (yield, 26.3% from TM-1).

Ion-exchange chromatography

TM-4 was applied to a DEAE-Sepharose Fast Flow column (3.2×45 cm) with chloride as counter-ion. The column was first eluted with water at 1 mL/min, followed by NaCl gradient (0～1 M). The carbohydrate profile was determined using the phenol-sulpuric acid.

General method

The total carbohydrate and uronic acid contents were determined by the phenol-sulfuric acid method (13) and the *m*-hydroxydiphenyl method (14) respectively, using glucose and galacturonic acid as the respective standards. The amount of protein was assayed by the method of Lowry et al. (15) with bovine serum albumin as the standard.

Methanolysis and GC

The polysaccharide samples (100 μg) and standards (100 nmol) together with myo-inositol were dried in a vacuum over P_2O_5 for 24 h, and then subjected to methanolysis with methanolic HCl for 16 h at 70°C (16). The samples were dried with nitrogen at room temperature, 2-methyl-2-propanol was added and the samples were dried again. Prior to trimethylsilylation the samples were dried in a vacuum over P_2O_5 for 4 h. The samples were subjected to gas chromatographic analysis on a Varian 3400 instrument and a flame ionization detector, a split-spilitlless injector. The column was DB-1 fused silica capillary column (30 m×0.32 mm i.d) with film thickness 0.25μm. Helium was used as carrier gas at a flow rate of 3.0 mL/min. Both injection-and detector temperature were 280°C. The column temperature was initially 140°C (15 min), then an increase of 15°C/min to 170°C (1 min), followed by 10°C/min to 250°C (6 min) and then 15°C/min to 290°C (10 min).

Pronase digestion of the crude polysaccharide

TM-1 (200 mg) was dissolved in 50 mL of 50 mM Tris-HCl, pH 7.9, containing 10 mM $CaCl_2$, and then 50 mg of pronase was added. The reaction mixture was incubated at 37°C for 48 h with a small amount of toluene. The reaction was terminated by boiling for 5min. The mixture was then dialysed against distilled water for 2 days, and the non-dialysable portion was lyophilised to obtain the TM-1 pronase digest.

Periodate oxidation of the crude polysaccharide

TM-1 (50 mg) was dissolved in 30 mL of 50 mM acetate buffer, pH 4.5, and then 50 mM $NaIO_4$ (10 mL) was added. The reaction mixture was incubated at 4°C in the dark for 3 days. Ethylene glycol (5 mL) was added to destroy the excess periodate, and the mixture was dialysed against distilled water for 2 days. The non-dialysable solution was concentrate while being continuously stirred for 12 h at room temperature. After the neutralisation of the reaction mixture with acetic acid, the H_3BO_3 in the sample was removed by the repeated addition and evaporation of methanol. Finally, the oxidised TM-1 was obtained as the lyophilisate after dialysis.

Anti-complementary activity

Gelatin-veronal-buffered saline (pH 7.2) contained 500 μM Mg^{++} and 150 μM Ca^{++} (GVB^{++}) was prepared as previously described (9) and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of polysaccharide fractions in distilled water, polysaccharides (50 μL) were incubated with 50 μL of NHS and 50 μL of GVB^{++}. The mixtures were incubated at 37°C for 30 min and the residual total haemolytic complement (TCH_{50}) was determined by a method using IgM haemolysin-sensitized sheep erythrocytes (EA) at 1108 cell/mL (17). NHS was incubated with distilled water and GVB^{++} to provide a control. The anti-complementary activity of the polysaccharide fractions was expressed as the percent inhibition from TCH_{50} of the control.

Statistical analysis

All data were subjected to an analysis of variance and tested for significant differences by Duncans multiple range test.

RESULTS AND DISCUSSION

Isolation of water-soluble polysaccharide

The leaves of *Teucrium viscidum var. miquelianum* showed the most potent anti-complementary activity among tested herbs in the screening procedures. The leaves and stems of *Teucrium viscidum var. miquelianum* were extracted with hot water, and their activities and yields were examined. The chemical analysis and the yield of the extract from each portion of *Teucrium viscidum var. miquelianum* are shown in Table 1. The yield of extract from the leaves was

Table 1. Chemical properties and yields of hot water extracts from the leaves and stems of *Teucrium viscidum var. miquelianum* (%)

	Leaves	Stems
Total sugar	54.2	36.6
Total uronic acid	15.1	8.5
Total protein	13.1	6.7
Yield	12.6	7.2

12.6% and it is higher than that of the stems (7.2%). The contents of total sugar, uronic acid and protein of the hot water extract from leaves were 54.2, 15.1 and 13.1%, respectively. The anti-complementary activity of the extract from the leaves of *Teucrium viscidum var. miquelianum* showed a more potent activity than that of the extract from the stems (Fig. 1). At 100 μg/mL concentration, the leaves showed a high activity of more than 62%, while the stems hardly showed any activity. The sample showed an overall increase in activity depending on the concentration increase. Therefore, the leaves of *Teucrium viscidum var. miquelianum* was used for the experimental material (TM-0). The yield of TM-1 was 7.9% from dried *Teucrium viscidum var. miquelianum*. TM-1 showed potent anti-complementary activity, but methanol (TM-M) and ethanol soluble materials (TM-E) obtained during the extraction process of the crude polysaccharide showed much lower anti-complementary activity than that of TM-1 (Fig. 2). The anti-complementary activity of the deproteinised polysaccharide did not change significantly contrasting with that of TM-1, but the activity decreased by the periodate oxidation of the polysaccharide (Fig. 3). These results indicate that the carbohydrate moiety contribute to the anti-complementary activity. It has been reported that several immunomodulating activities were found in the crude polysaccharide fractions of the higher plants (9-12).

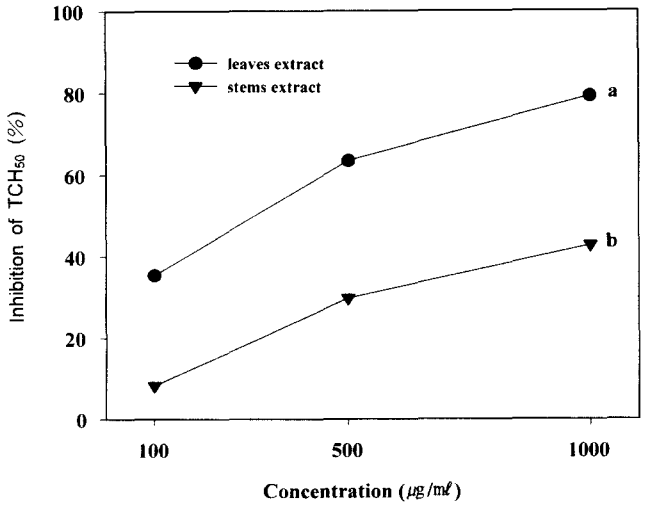

Fig. 1. Anti-complementary activity of hot water extracts from the leaves and stems of *Teucrium viscidum var. miquelianum*. Value are means of three replicates and those with different letters are significantly different at $p<0.01$.

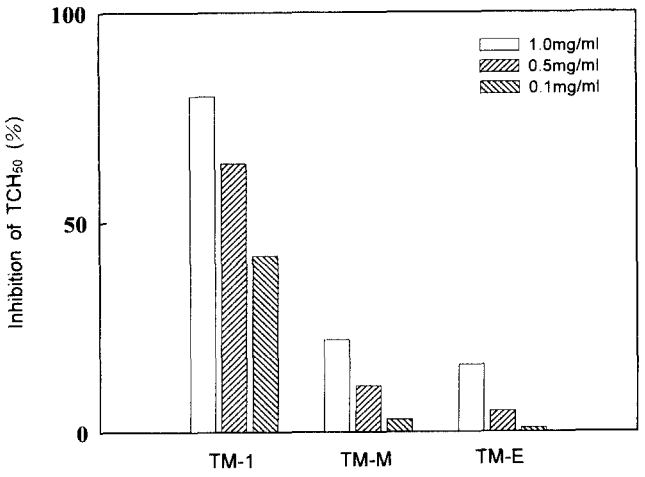

Fig. 2. Anti-complementary activites of several extracts obtained from the leaves of *Teucrium viscidum var. miquelianum*. TM-1: Ethanol precipitate, TM-M: Methanol soluble component, TM-1: Ethanol soluble component.

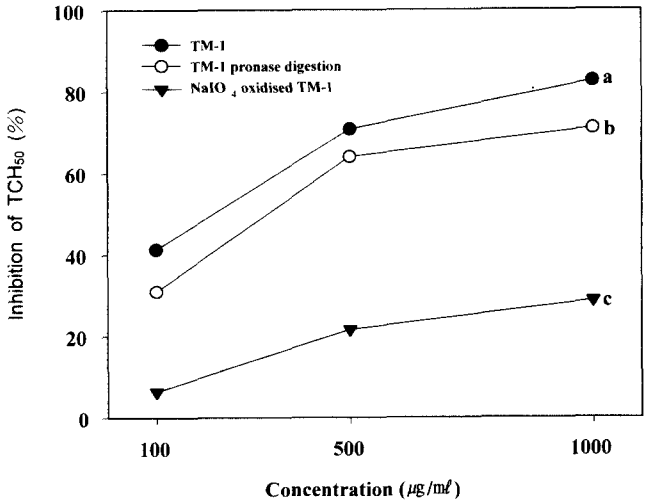

Fig. 3. Lability of polysaccharide anti-complementary activities to periodate and pronase treatment. Value are means of three replicates and those with different letters are significantly different at $p<0.01$.

Fraction of crude polysaccharide by cethyltrimethylammoniumbromide

Crude polysaccharide (TM-1) was fractionated into three polysaccharide fractions by the addition of cetavlon. The chemical properties of TM-1, TM-2, TM-3 and TM-4 are summarized in Table 2. TM-1 contained glucose, galactose and galacturonic acid as major component sugars. The major polysaccharide fraction, TM-2, contained glucose, galactose, and rhamnose as the major neutral sugar, and a large amount of uronic acid which was estimated to be galacturonic acid. Whereas TM-4 contained rhamnose, galactose and glucose as the major neutral sugars, and its uronic acid content was the lowest among the polysaccharide fractions.

The anti-complementary activities of these cetavlon-fractions are shown in Fig. 4. The anti-complementary ac-

Table 2. Chemical properties and yields of polysaccharide fractions on cetavlon treatment of TM-1

	TM-1	TM-2	TM-3	TM-4
Total sugar (%)	66.7	73.2	81.1	76.8
Total uronic acid (%)	24.2	37.9	24.6	18.4
Total protein (%)	12.4	14.8	11.4	13.7
Yield (%)	7.9	2.6	1.3	2.1
(Component sugar)		(Molar ratios)		
Rhamnose	0.69	0.66	0.60	0.64
Arabinose	0.31	-[1)]	0.37	0.26
Xylose	0.12	-	0.16	0.03
Mannose	0.39	0.57	0.51	0.42
Galactose	0.61	0.65	0.94	0.64
Glucose	1	1	1	1
Glucuronic acid	0.42	0.55	0.49	0.28
Galacturonic acid	0.53	1.42	0.64	0.37

[1)]Not detected.

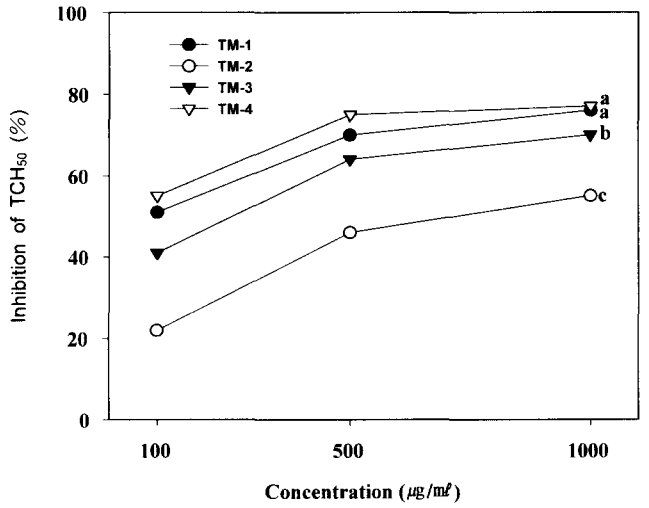

Fig. 4. Anti-complementary activities of the polysaccharide fractions obtained by cetavlon treatement of TM-1. Value are means of three replicates and those with different letters are significantly different at $p<0.01$.

tivity was the hightest in TM-4, followed by TM-1, TM-3 and TM-2 (Fig. 4), which was proportional to the decease in their uronic acid content. Yamada et al. (4). have reported that neutral polysaccharide (arabinogalactan) from *Angelica acutiloba* showed the hightest anti-complementary activity among cetavlon-fractions. However, *Angelica acutiloba* root also contains another pectic polysaccharide fraction which has moderate anti-complementary activity and contains a large proportion of galacturonic acid together with small amounts of neutral sugars (18,19). Thus, further studies of acidic polysaccharide fraction (TM-2) are now in progress.

Fractionation of TM-4 on DEAE-sepharose chromatography

Since TM-4 showed a high anti-complementary activity, it was further fractionated on the column of DEAE-sepharose Fast Flow (Cl^-) into unabsorbed fraction (TM4-I) and absorbed fractions (TM4-IIa, TM4-IIb, TM4-IIc and TM4-IId) by the elution with a linear gradient of 1 M NaCl (Fig. 5). The chemical properties of these sub-fractions are summarised in Table 3. TM4-I consisted mainly of arabinose, galactose and glucose in molar proportions of 0.20 : 0.29 : 1.00, respectively and contained a small amount of uronic acid. TM4-IIa was composed mainly of galactose, arabinose and glucose in the molar ratios of 2.13 : 0.94 : 1.00, respectively. TM4-IIc showed the highest hexose content among the other sub-fractions, and glucose and galactose were found to be a major component sugars. The fraction with the poorest yield, TM4-IIb, showed glucose, galactose and arabinose as major component sugars. The fraction TM4-IId consisted mainly of arabinose, rhamnose, xylose, glucose and galactose, and contained significant amounts of uronic acid. Most of the uronic acid of TM4-IId was identified as galacturonic acid.

Fig. 6 shows the anti-complementary activity after the incubation of different concentrations of the polysaccharide fractions with NHS. Among sub-fractions of TM-4, TM4-IIa

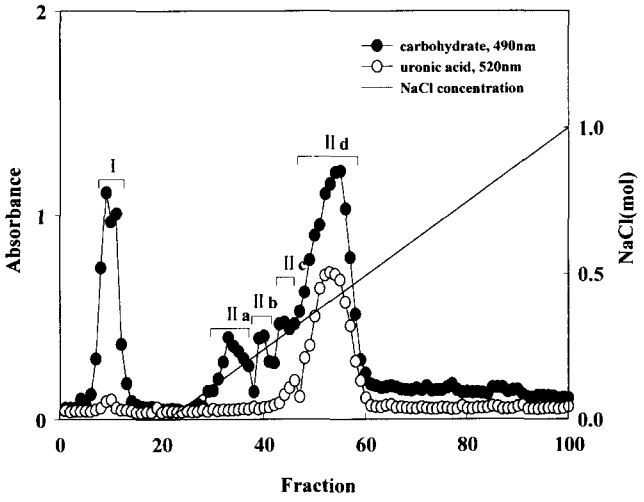

Fig. 5. DEAE-Sepharose Fast Flow (Cl^--form) chromatography of TM-4. TM-4 was dissolved in distilled water. Linear gradient elution was carried out with NaCl.

Table 3. Chemical properties of TM-4 subfractions

	TM4-I	TM4-IIa	TM4-IIb	TM4-IIc	TM4-IId
Total sugar (%)	76.1	87.2	86.5	91.8	65.3
Total uronic acid (%)	1.4	3.8	4.6	12.0	38.9
Total protein (%)	3.7	12.1	14.2	17.5	23.7
Yield (%)[1)]	23.7	7.5	3.7	4.3	32.8
(Component sugar)			(Molar ratio)		
Rhamnose	0.03	0.48	0.62	0.67	1.70
Arabinose	0.20	0.94	1.60	0.69	1.96
Xylose	0.03	0.36	0.57	0.50	0.86
Mannose	0.07	0.69	0.88	0.86	0.60
Galactose	0.29	2.13	2.92	1.95	2.05
Glucose	1	1	1	1	1
Glucuronic acid	trace[2)]	0.28	0.38	0.41	1.82
Glacturonic acid	0.01	0.35	0.43	0.44	4.15

[1)]yields from TM-4.
[2)]below 0.01 molar ratio.

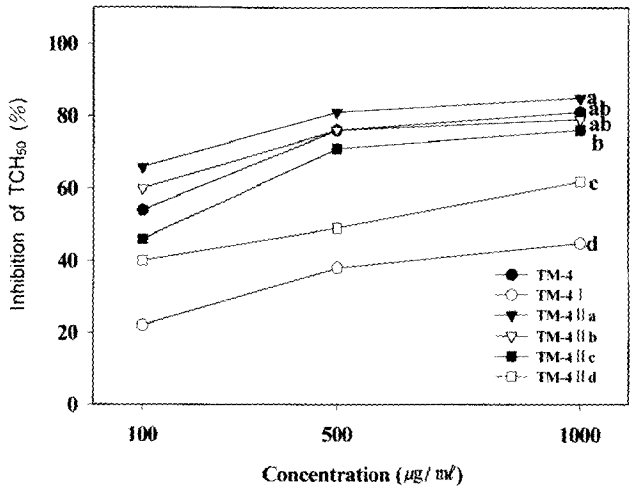

Fig. 6. Anti-complementary activites of TM-4 subfractions from ion chromatography. Values are means of three replicates and those with different letters are significantly different at $p<0.01$.

and TM4-IIb showed a high anti-complementary activity. In particular, when 1000 μg/mL of TM4-IIa was incubated with an equal volume of NHS, about 85% of the TCH_{50} was reduced.

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