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Structural Features and Anti-Complement Activity of an Acidic Polysaccharide from *Forsythia suspensa*

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Abstract

Background: The complement system is an important host defence mechanism against the invasion of foreign materials. However, excessive or uncontrolled activation of the complement system might lead to severe complement-mediated disorders which are harmful to human bodies. There are still no desirable therapeutic compounds available on the market for complement inhibition. In recent years, researches have been focused on natural polysaccharides to modulate of the immune system because of their immunomodulatory activities and safety. In this study, Fs-8-ba2, a homogeneous acidic polysaccharide (MW ca. 7.2 kDa) was isolated from *Forsythia suspensa* and its anti-complementary effect was investigated.

Method: The major primary structural features of Fs-8-ba2 were elucidated using HPGPC, IR, absolute configuration, component analysis and methylation analysis, periodate oxidation, carboxyl reduction partial acid hydrolysis, and NMR spectroscopy, etc. To identify the anti-complementary activity of Fs-8-ba2, the hemolytic assays through the classical pathway (CP) and the alternative pathway (AP) of complement system *in vitro* were taken.

Results: The backbone of Fs-8-ba2 was composed of 7 homogalacturonan (HG) and 2 rhamnogalacturonan (RG-I) moieties with the side chains attached at O-4 of 1,2,4-linked α-L-Rha in the RG-I moieties. The anti-complement assay showed that the native acidic polysaccharide (Fs-8-ba2) possessed stronger inhibitory effect on the complement activation than the carboxyl-reduced polysaccharide (Fs-8-ba2re) through the classical (IC50: 0.311 ± 0.020 mg/mL *vs.* 3.292 ± 0.032 mg/mL) and alternative pathways (IC50: 0.218 ± 0.015 mg/mL *vs.* no activity). It indicated that the anti-complement effect of pectic polysaccharide was related to GalA content (carboxyl group). Preliminary mechanism studies by using complement-depleted sera indicated that Fs-8-ba2 selectively interacted with C1q, C1r, C1s, C2, C3 and C9, but not C4 and C5.

Conclusion: These results suggested that Fs-8-ba2, an acidic polysaccharide, could be of potential benefits in the treatment of the complement-mediated diseases.

Keywords: *Forsythia suspensa*; Acidic polysaccharide; Carboxyl group; Rhamnogalacturonan; Anti-complement

Introduction

The complement system can be activated by three separate pathways including the classical pathway (CP), alternative pathway (AP) and lectin pathway (LP) to mobilize defence mechanisms against the invasion of foreign materials such as bacteria and viruses [1]. All three pathways comprise different components and are activated in different fashions but use a common terminal pathway. The CP is activated when C1q interacts with its ligands such as immune-complexes (ICs), the LP is activated when MBL or ficolins binds to certain carbohydrates and the AP can be activated by spontaneous C3 hydrolysis or via properdin, thereby generating C3 (H_2O) which can bind factor B (fB). Activation of each of these pathways generates C3-convertases resulting in activation of the common terminal pathway and generating several effector processes, such as chemotaxis by C3a and C5a, opsonization by C3b, and lysis by the membrane attack complex [2,3]. Excessive or uncontrolled activation of the complement system might lead to severe complement-mediated disorders, such as Alzheimer's disease, Pick's disease and Myasthenia gravis (MG) [4-6]. A large number of anti-complement therapeutic agents have been developed and some have been found to be beneficial in reducing tissue injury in a number of animal models of severe complement-dependent inflammation.

Numerous natural or semi-synthetic polyanions, such as complestatin, derivatized dextrans, chondroitin sulfate, dextran sulfate and heparin have been found to inhibit complement activation. However, many of these compounds are toxic and most of them require relatively high concentrations for inhibiting complement activation both *in vitro* and *in vivo* [7]. For example, heparin is recognized as one of the best characterized molecule that shows profound anti-complement activity. However, at the concentration sufficient to inhibit the complement system, heparin will demonstrate undesirable anti-coagulant side effect, which limits its clinical application in the treatment of anti-complementary diseases. In this

work we have extended the search for a natural polysaccharide without sulfate group, which can be used to treat complement-mediated diseases. *Forsythia suspensa* is the dried fruit of *Forsythia suspensa* (Thunb.) Vahl. Its active flavonoids, alkaloids, lignans, glycosides and other chemical constituents [8] have been proved to possess urease inhibitory activity, against lipid peroxidation [9], anti-inflammatory activity [10], antioxidant and antibacterial activity [11]. However, no polysaccharide with anti-complement activity from *Forsythia suspensa* has yet been reported. We report here the isolation, purification and characterization of a homogeneous polysaccharide with anti-complement activity and its mechanism.

Materials and Method

Materials

The dried medicinal materials of *Forsythia suspensa* were collected in Shanxi province, China, in May 2011, and authenticated by Prof. L.H. Wu at Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai. A voucher specimen (0213001) was deposited at the Herbarium of the Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai. CMC was purchased from Sigma-Aldrich (St Louis, MO, USA). TFA, $NaBH_4$ and NaCl were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). All other reagents were analytical grade.

Sheep erythrocytes were collected in Alsevers' solution. Anti-sheep erythrocyte antibody was from Zhengzhou Bestgene biotechnological Co., Ltd. NHS was obtained from healthy adult donors. Heparin (sodium salt, 150 IU/mg, BR) was from Sinopharm Chemical Reagent Co. Anti-C1q, Human (Goat); Anti-C1r, Human (Goat), Anti-C1s, Human (Goat); Anti-C2, Human (Goat); Anti-C4, Human (Goat) and Anti-C5, Human (Goat) were from Biorbyt Ltd (Cambridge, UK). Anti-C9, Human (Goat) were from Abcam Plc. Anti-C3, Human (Goat) was from Merck Millipore (Darmstadt, Germany).

Buffers: Veronal buffer saline (VBS) containing 0.1% gelatin (GVB), GVB containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+} (GVB^{2+}), and GVB containing 2.5 mM Mg^{2+} and 8 mM EGTA (Mg^{2+}-EGTA-GVB) were prepared as described previously [12].

General methods

All evaporations were carried out at below 60°C under reduced pressure. IR spectrum (Nujol) was recorded on a Perkin-Elmer 591B spectrophotometer. Specific rotation was detected by a Perkin-Elmer 241M digital polarimeter at 20°C. The ^1H-, ^{13}C- and 2D-NMR spectra were recorded on a Bruker 400 NMR spectrometer. All chemical shifts were reported relative to acetone. GC-MS was analyzed with a Thermo TRACE DSQ apparatus equipped with a TR-5ms column (length: 60 m × 0.25 mm, thickness of liquid phase: 0.25 μm).

Extraction and isolation of Fs-8-ba2

The hemolytic assay on the classical pathway was used as biological activity guide in the purification of the anti-complement compounds from the *Forsythia suspensa*.

The dried medicinal materials of *Forsythia suspensa* (5 kg) were extracted with quintuple 95% EtOH by refluxing process for 3 h at 60°C. The residue was dried and then extracted with boiled water (50 L) for three times. The extract was concentrated by evaporation, and was poured into three volumes of 95% EtOH under vigorous stirring. This mixture was kept at room temperature overnight, and then centrifuged. After volatilizing the EtOH, the precipitate was re-dissolved in water, and then lyophilized. The crude polysaccharide preparation thus obtained a brownish powder named Fsp.

Fsp (8 g) was dissolved in water (150 mL), and the insoluble substance was removed by centrifugation. The supernatant was applied to a DEAE-cellulose column (Cl^-, 50 cm × 5 cm), and eluted first with water, and then with a 0-2 M gradient of NaCl solution (0.1 M, 0.2 M, and 2.0 M NaCl), leading to the isolation of four subfractions: Fs-1, Fs-2, Fs-3 and Fs-8. Of those, fraction Fs-8 showed the relatively high activity. After dialysis and lyophilized, Fs-8 was obtained from the 2 M NaCl eluent, in a yield of 400 mg (5%).

Fs-8 was purified on tandemly linked Superdex 75 and Superdex 30 columns (100 cm × 2.6 cm) with 0.2 M NaCl as solvent and eluent, with the refractometric detection, giving Fs-8-ba2. The complete extraction and isolation process was illustrated in Figure 1.

Homogeneity and molecular size

The homogeneity and molecular weight of Fs-8-ba2 were determined by HPGP [13,14] and GPC software respectively, on a linked column of KS-804 and KS-802 columns, eluting with 0.2 M NaCl at a flow rate of 0.8 mL/min. The column was pre-calibrated by standard dextran (P-5, P-10, P-20, P-50, P-100, P-200, P-400, P-800, Shodex Co.). The sample was prepared as a 0.2 M NaCl solution, and 20 μL of the solution was analyzed each time.

Determination of sugar composition

The native sample (2 mg) was treated with 2 mL 2 M TFA at 120°C for 2 h. After cooling to room temperature, the hydrolysate was divided into two parts. One part was evaporated to dryness with the addition of methanol. Then the residue was reduced by $NaBH_4$ overnight and acetylated with Ac_2O [15]. The resulting alditol acetates were detected by GC-MS. The other portion was analyzed by TLC [16] using glucuronic acid and galacturonic acid as references. The uronic acid content was estimated by the m-hydroxydiphenyl method [17].

Carboxyl reduction

Carboxyl reduction was carried out as described by Taylor and Conrad [18]. The native polysaccharide (50 mg) was dissolved in distilled water (20 mL) and then 150 mg of CMC was added. As the reaction proceeded, the reaction mixture was maintained at pH 4.75 by automatic titration with 0.1 M HCl for at least 3 h. After hydrogen ion uptake had ceased, 2 M $NaBH_4$ was added slowly. The mixture was maintained at pH 7.0 by automatic titration with 4 M HCl. A total of 25 mL of the $NaBH_4$ solution was required for the reduction, which was completed in 60 min. The reduction process was repeated three times until the signal at 172.2 and 176.1 ppm in the 13C-NMR spectrum disappeared.

Methylation analysis

Fs-8-ba2re (5 mg) was methylated for three times according to the method described by Needs and Selvendran [19]. After complete methylation as shown by IR spectrum, the permethylated polysaccharide was hydrolyzed in 2 M TFA at 120°C for 2 h. After $NaBH_4$ reduction and acetylation, the partially methylated alditol acetates were analyzed by GC-MS.

Partial acid hydrolysis

Fs-8-ba2re (50 mg) was hydrolyzed with 0.1 M TFA at 100°C for 2 h, and further condensed at reduced pressure to remove TFA. After evaporation to remove TFA, the hydrolyzate was re-dissolved in water. Then the solution was chromatographed on Sephadex G-10. The main polysaccharide (named Fs-8-ba2rem) was collected, and freezed-dried.

Anti-complement activity

Anti-complement activity through the classical pathway: Assays were based on the haemolysis of erythrocytes by the membrane generated after complement activation. Based on the method described by Zhang and Chen [20], EAs were prepared by incubation of sheep erythrocytes 2% (v/v) with the same volume of rabbit anti-sheep erythrocyte antibody in GVB2+. Our polysaccharides were dissolved in GVB2+, and so is heparin used as positive control. Then the sample solutions were diluted into various concentrations for assay. The 1:3.5 diluted serum of normal human was chosen to give sub-maximal lysis in the absence of complement inhibitors. Various dilutions of tested samples (100 μL) were pre-incubated with 100 μL NHS and 200 μL GVB2+ at 37°C for 10 min. Then, 200 μL EAs were added and the mixture was incubated at 37°C for 30 min. The different assay controls were incubated in the same conditions: (1) all blanks, 200 μL EAs in 400 μL GVB2+; (2) 100% lysis, 200 μL EAs in 400 μL water; (3) sample control, 100 μL dilution of each sample in 500 μL GVB2+; (4) complement control: 100 μL NHS (1:3.5, v/v) and 200 μL EA in 300 μL GVB2+. The reaction mixture was centrifuged immediately. Optical density of the supernatant was measured at 405 nm with a UV-VIS spectrophotometer (Power Wave XS, BioTek Instrument, Winooski, USA). The percentage inhibition was calculated by subtracting sample control absorbance from each value to obtain corrected absorbance. Anti-complement activity through the classical pathway was determined as a mean of triplicate per concentration and expressed as the 50% inhibitory concentration value (CP50 value) from complement-dependent haemolysis of the control.

Anti-complement activity through the alternative pathway: According to the method of Klerx et al. [12] each sample was dissolved in Mg^{2+}-EGTA-GVB, and various dilutions of which were made. After pre-incubation of dilutions of each sample (150 μL) with 1:3.5 diluted NHS (150 μL) at 37°C for 10 min, 200 μL rabbit erythrocytes (ERs 0.5%, v/v) were added. Following a second incubation step at 37°C for 30 min, cell lysis was determined as described in the section anti-complement activity through the classical pathway. Controls for all blanks, 100% lysis and sample control were included. Anti-complement activity through the alternative pathway was determined as a mean of triplicate per concentration and expressed as the 50% inhibitory concentration value (AP50 value) from complement-dependent haemolysis of the control.

Preparation of complement-depleted serum with complement antibody: Tests to prepare complement-depleted serum were conducted according to the method described by Zhu et al. [21]. Various dilutions of each antiserum were incubated with the same volume of NHS (1:3.5) at 37°C for 15 min. After centrifugation, the supernatant (200 μL) was incubated with 200 μL EAs and 200 μL GVB2+ at 37°C for 30 min as previously said. Then cell lysis was measured as described in the anti-complement activity through the classical pathway section. The antiserum dilution against the NHS hemolytic capacity was determined. The optimal dilutions of complements (1:8 for C1q, 1:4 for C1r, 1:3 for C1s, 1:1 for C2, 1:32 for C3, 1:1 for C4, 1:6 for C5, 1:32 for C9) were incubated with the same volume of NHS (1:3.5) at 37°C for 15 min following by centrifugation. The supernatant was stored as C-depleted sera in aliquot at -80°C before used in hemolytic assays.

Interaction with individual complement components: Tested compounds were dissolved in GVB2+, and diluted to the required concentration which were just sufficient to completely inhibit the hemolysis of 1:10 diluted NHS through classical pathway. 200 μL EAs and 200 μL individual C-depleted sera of C1q, C1r, C1s, C2, C3, C4, C5 or C9 were added to 200 μL sample-treated NHS, and the mixture was incubated at 37°C for 30 min. After centrifugation and measurement of the optical density of the supernatant, the percentage of hemolysis was calculated. For the assay of individual depleted serum group, C-depleted sera were directly incubated with EAs under the same condition, and the hemolytic activity was calculated. The controls: (1) all blanks, 200 μL EAs in 400 μL GVB2+; (2) 100% lysis: 200 μL EAs in 400 μL water; (3) complement control: 100 μL NHS (1:3.5, v/v) and 200 μL EAs in 300 μL GVB2+; (4) sample control: 100 μL dilution of sample in 500 μL GVB2+, were prepared under the same condition.

Results

Sugar composition analysis of Fs-8-ba2

Fs-8-ba2 was prepared from Fs-8 with a yield of 1.47% as shown in Figure 1. The HPGPC profile (Figure 2) showed a symmetrical peak, indicating that Fs-8-ba2 was a homogenous polysaccharide with a molecular weight around 7.2 kDa based on comparison with dextran of known molecular weights. The specific rotation [α]D was +16.0 (c 0.5, H_2O). For the composition analysis, a high portion (37.5%) of uronic acid was detected by the method of uronic content determination, and then identified as GalA by TLC. To confirm the ratio of monosaccharides, the native polysaccharide was reduced with NaBH4 three times and the reduced polysaccharide Fs-8-ba2re was revealed to consist of Rha, Glu and Gal (derived from GalA and native Gal), in the molar ratio of 1.25: 1.00: 2.60, respectively. A combination of sugar composition analysis of carboxyl-reduced Fs-8-ba2re and the uronic content revealed that Fs-8-ba2 was composed of Rha, Glu, Gal and GalA in the molar of 1.25: 1.00: 0.78: 1.82, respectively. The Rha/GalA ratio was 0.69, which fell within the RG-I range of 0.05-1.0 [22]. Therefore, Fs-8-ba2 is likely a RG-I type pectin containing a backbone of a repeating unit [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→] [23].

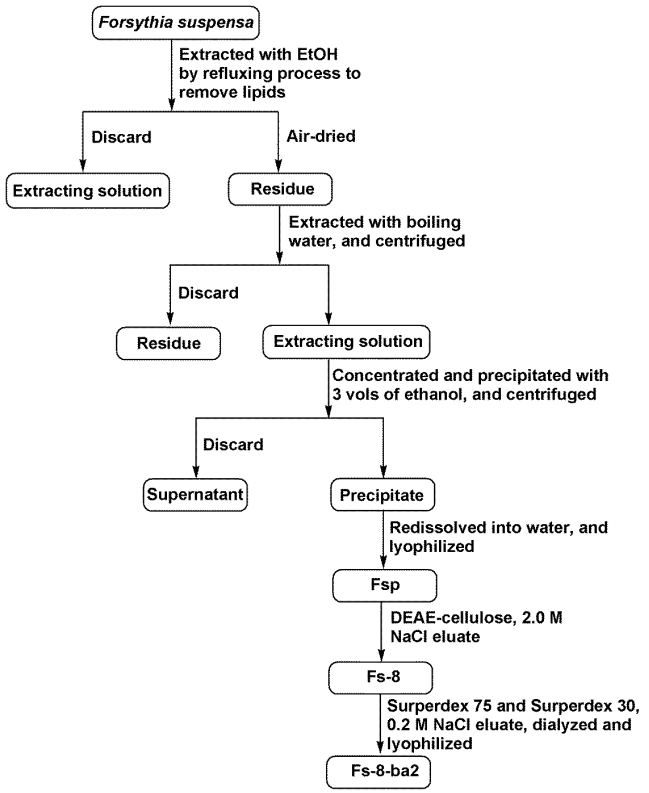

Figure 1: Scheme of isolation of polysaccharide Fs-8-ba2 from *Forsythia suspensa.*

The dried medicinal materials of *Forsythia suspensa* were extracted with EtOH by reflux for 3 h at 60°C to remove lipids. The residue was extracted with boiled water; the aqueous extract was precipitated with EtOH concentration to 75%, giving Fsp. After successive separation by DEAE-cellulose column, Superdex 75 and Superdex 30 gel permeation chromatographic steps, it afforded the carbohydrate fraction Fs-8-ba2.

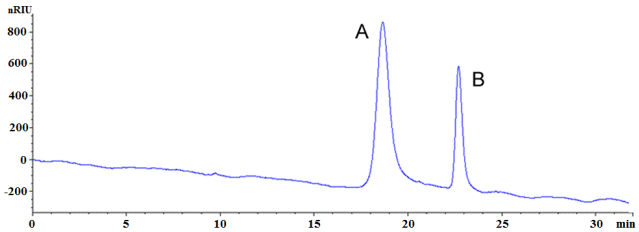

Figure 2: HPGPC of Fs-8-ba2.

The sample was analyzed by a linked column of KS-804 and KS-802 columns, and eluted with 0.2 M NaCl at a flow rate of 0.8 mL/min. Peak A was a single symmetrical peak of Fs-8-ba2 and Peak B was the peak of the solvent.

Methylation analysis

The carboxyl-reduced derivative Fs-8-ba2re was methylated three times and then subjected to acid hydrolysis, reduction and acetylation to yield partially methylated alditol acetates (PMAAs). The PMAAs were analyzed by GC-MS, and the results were shown in Table 1. Methylation analysis indicated that Fs-8-ba2re contained a large proportion of 1,4-linked Gal (42.7%). According to the sugar analysis, a portion of Gal from Fs-8-ba2re was derived from GalA, indicating that the native polysaccharide contained 1,4-linked GalA. The native polysaccharide also contained 1-Rha, 1,2-Rha, 1,2,4-linked Rha, 1,6-Glc together with other neutral residues. The identification of 1,4-linked GalA, 1,2- linked Rha and 1,2,4-linked Rha residues also suggested the existence of RG-I in the polysaccharide [24,25].

NMR (Nuclear magnetic resonance) spectroscopy

In the 1H-NMR spectrum of the polysaccharide, the signal at 3.7 ppm was derived from methyl groups binding to carboxyl group of D-GalpA. Signals at 1.14 and 1.20 ppm derived from methyl groups of L-Rhap were assigned to the O-2 linked Rha and to the O-2, 4 linked Rha, respectively [25]. Other signals (Table 2) were assigned according to the 1H-1H COSY spectrum (Figure 1S).

From the 13C-NMR spectrum (Figure 2S) of the native polysaccharide, the chemical shifts in the area of anomeric carbon atoms suggested two α- linkages for the L-Rhap residues (98.9 ppm) and the D-GalpA residues (100.5 ppm) [26]. The signal at 17.7 ppm was assigned to C-6 of the L-Rhap residues, and the signals at 54.1 ppm and 172.2 ppm suggested methyl groups binding to carboxyl groups of D-GalpA [27]. The substitution at O-4 of GalA was ascertained by the strong signal at 79.3 ppm, assigned in reference to literature data [28]. Other signals (Table 2) were assigned according to the 1H-13C HSQC spectrum (Figure 3S).

In the NOESY spectrum of the native polysaccharide (Figure 4S), a cross peak between H-1 and H-2 in D-GalA was observed, which indicated that the polysaccharide contained α-D-GalA residues. A cross peak between H-1 and H-2 in Rha indicated that the Rha was α-L-Rha. Inspection of the NOESY spectrum showed the GalA H-1 track NOE connectivity with Rha H-2 in agreement with a GalA (1→2) Rha linkage and the Rha H-1 track NOE connectivity with GalA H-4 in agreement with a Rha (1→4) GalA. And a cross peak between H-1 and H-4 in D-GalA indicated that the polysaccharide contained HG moiety which was composing of α-(1→4)-linked D-GalA residues.

Methylated sugar	Molar Ratio	Linkage
2,3,4-Me3-Rha	4.6	1-Rha
3,4- Me2-Rha	7.1	1,2-Rha
3-Me-Rha	2.8	1,2,4-Rha
2,3,4,6-Me4-Glc	3.1	1-Glc
2,3,6-Me3-Glc	5.7	1,4-Glc
2,3,4-Me3-Glc	18.8	1,6-Glc
2,3-Me2-Glc	0.8	1,4,6-Glc
2,3,4,6-Me4-Gal	7.1	1-Gal
2,3,6-Me3-Gal	42.7	1,4-Gal

3,6-Me2-Gal	1.6	1,2,4-Gal
2,6-Me2-Gal	4	1,3,4-Gal
2,3-Me2-Gal	4.6	1,4,6-Gal

Table 1: Methylation analyses of Fs-8-ba2re.

Residues	C1(H1)	C2(H2)	C3(H3)	C4(H4)	C5(H5)	C6(H6)	OCH 3
1-Rha	98.9 (4.92)	72.7 (3.52)	74.7 (3.64)	73.0 (3.52)	70.6 (3.67)	17.7 (1.19)	
1,2-Rha	98.9 (4.92)	77.6 (4.07)	72.6 (3.46)	73.0 (3.52)	70.6 (3.67)	17.9 (1.14)	
1,2,4-Rha	98.9 (4.92)	77.6 (4.07)	72.6 (3.46)	82.9 (4.14)	70.6 (3.67)	17.9 (1.20)	
1,4-GalA(OMe)	100.5 (5.04)	69.3 (3.69)	69.9 (3.93)	79.3 (4.37)	72.4 (4.71)	172.2	54.1 (3.70)
1,4-GalA	100.5 (5.04)	69.3 (3.69)	69.9 (3.93)	79.3 (4.37)	71.9 (5.04)	176.1 17.7	

Table 2: 13C-NMR and 1H-NMR (in parentheses) of Fs-8-ba2 (ppm, r.t, D2O).

Partial acid hydrolysis

The carboxyl-reduced polysaccharide was methylated twice after partial acid hydrolysis and methylated sugars were converted to partially methylated alditol acetates. As a result of methylation analysis, Fs-8-ba2rem contained 1,4-linked Gal and 1,2-linked Rha in the molar ratio of 4.6: 1.0. It was thus concluded that the backbone of Fs-8-ba2 was composed of 7 HG moieties and 2 RG-I moieties. And the branches were attached to O-4 of Rha of the RG-I moieties. From the results, the schematic structural model of the backbone of Fs-8-ba2 was tentatively proposed as in Figure 3.

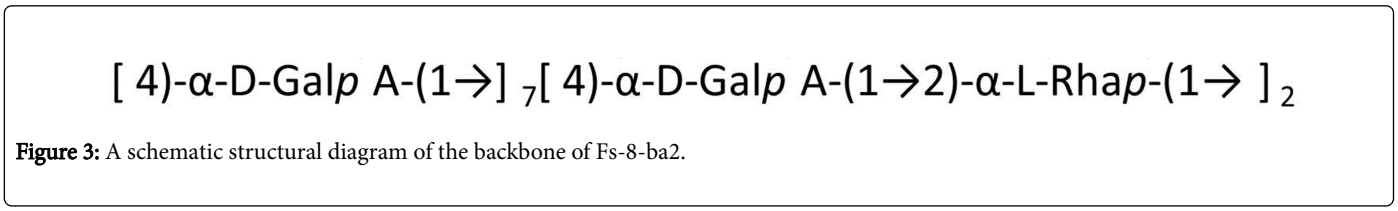

Figure 3: A schematic structural diagram of the backbone of Fs-8-ba2.

Anti-complement activity

The effect of the homogeneous polysaccharide on activation of human complement through the classical pathway was examined in 1:3.5 diluted NHS with heparin as a positive control. In the classical pathway, the percentage of activation that 1:3.5 diluted NHS occurred was 98.11 ± 1.03% in the complement control. As shown in Figure 4A, the concentrations that expressed as the 50% inhibitory concentration from complement-dependent haemolysis of the control were 0.311 ± 0.020, 3.292 ± 0.032, 0.045 ± 0.002 mg/mL for Fs-8-ba2, Fs-8-ba2re and heparin, respectively. The results indicated that the anti-complement activity of Fs-8-ba2 was largely reduced by the carboxyl-reduced product, suggesting carboxyl groups played an important role in the properties of pectin [29].

In the alternative pathway, the percentage of activation for the sample 1:3.5 diluted NHS was 96.14 ± 3.08% in the complement control group. The anti-complement activities of Fs-8-ba2, Fs-8-ba2re and heparin on the alternative pathway were shown in Figure 4B. Both Fs-8-ba2 and heparin blocked hemolysis of ERs in a dose-dependent manner, but Fs-8-ba2re showed negligible activity. In the AP50 assay, the concentrations that resulted in 50% inhibition of hemolysis were 0.218 ± 0.015 mg/mL for Fs-8-ba2 and 0.165 ± 0.034 mg/mL for heparin. Therefore, Fs-8-ba2 is a potent polysaccharide in inhibiting complement system activation through the alternative pathway.

The effects of Fs-8-ba2 on individual complement components were investigated in the system with C-depleted reagents and a limited amount of human serum. The capacities of various depleted sera for restoring the hemolytic capacity of Fs-8-ba2-treated serum were also examined. Under these conditions, the complement component under investigation is the limiting factor in the component-mediated hemolysis assay. Thus, the failure of restoring the hemolysis could be attributed to the interaction between the tested sample and the corresponding complement component.

As shown in Figure 5, the percentage induced hemolysis by the 1:3.5 NHS solution through the classical pathway was 98.11 ± 1.03% in the complement control group. Fs-8-ba2 at a concentration of 1.00 mg/mL exhibited a considerable inhibitory effect on this hemolysis (4.78 ± 2.08%). Further studies indicated that Fs-8-ba2 selectively interacted with C1q, C1r, C1s, C2, C3 and C9, but not with C4 and C5. Therefore, Fs-8-ba2 inhibited complement activation by blocking the target complements C1q, C1r, C1s, C2, C3 and C9.

Fs-8-ba2 possessed a good inhibitory effect on the complement activation through both the classical and alternative pathways (IC50: 0.311 ± 0.020 mg/mL and 0.218 ± 0.015 mg/mL, respectively), which exhibited similar inhibitory effect with the homogeneous polysaccharide (D3-S1) from the roots of Bupleurum smithii (IC50: 0.34 ± 0.02 mg/mL and 0.081 ± 0.003 mg/mL, respectively) [30]. Sulfated polysaccharides demonstrated a stronger inhibitory effect than Fs-8-ba2, for example, heparin in this work. However, it is regrettable that it demonstrated undesirable anti-coagulant side effect as the existence of sulfated groups, which limits its clinical application in the treatment of anti-complementary diseases [31].

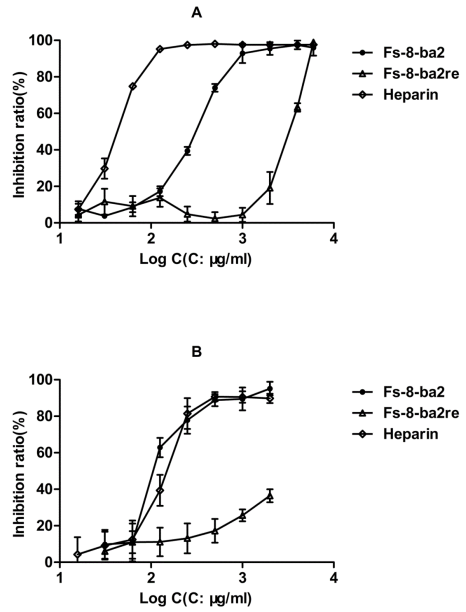

Figure 4: A showed the inhibition of classical pathway-mediated hemolysis of EAs in 1:3.5 diluted NHS in the presence of increasing amounts of Fs-8-ba2 and Fs-8-ba2re.

Figure 4A showed the inhibition of classical pathway-mediated hemolysis of EAs in 1:3.5 diluted NHS in the presence of increasing amounts of Fs-8-ba2 and Fs-8-ba2re. Results are expressed as percent inhibition of hemolysis. Heparin was used as reference. Data were expressed as mean ± SD (n=3). Figure 4B showed the inhibition of alternative pathway-mediated lysis of ERs in 1:3.5 diluted NHS in the presence of increasing amounts of Fs-8-ba2 and Fs-8-ba2re. Heparin was used as positive control. Results are expressed as percent inhibition of hemolysis. Data were expressed as mean ± SD (n=3).

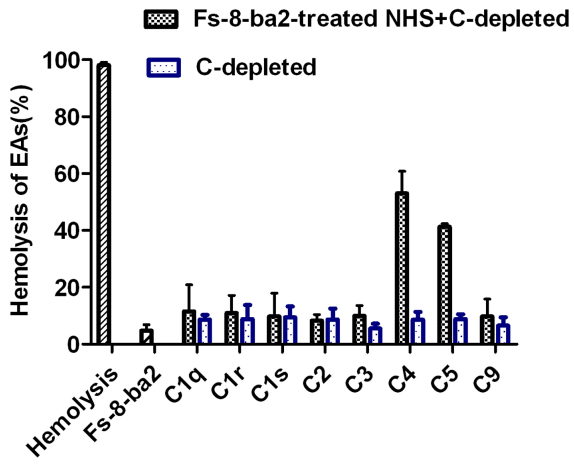

Figure 5: Hemolytic assays for identification the targets of Fs-8-ba2 utilizing C-depleted serum.

Fs-8-ba2-treated serum was mixed with various complement-depleted sera and the capacity of these complement-depleted sera to restore hemolytic capacity in the classical pathway was estimated by adding sheep antibody-sensitized erythrocytes. Results are expressed as hemolysis percentages. Data were expressed as mean ± SD (n=3). Abbreviation: Cont., complement control group.

Discussion

Previous study reported by Haruki Yamada showed that seventeen water-soluble polysaccharides obtained from various plants were tested for anti-complement activities, considerable activity was observed for most of these polysaccharides, and interestingly these active polysaccharides commonly contained [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→] residues as the main chain [32]. It is thus indicated that the presence of a backbone of a repeating unit [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→] in polysaccharide might be very important to anti-complement effect of the polysaccharides. In our study, we reported that Fs-8-ba2, a RG-I type acidic polysaccharide containing a backbone of a repeating unit [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→] with the side chains (1,4-Glc and 1,6-Glc) attached at O-4 of 1,2,4-linked α-L-Rha in the RG-I moieties, possessed stronger inhibitory effect on the complement activation than the carboxyl-reduced polysaccharide (Fs-8-ba2re) through the classical (IC50: 0.311 ± 0.020 mg/mL *vs.* 3.292 ± 0.032mg/mL) and alternative pathways (IC50: 0.218 ± 0.015 mg/mL *vs.* no activity). Our results provided more clues to show that the presence of a backbone of a repeating unit [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→] in polysaccharide is very important to anti-complement effect of the polysaccharides. More interestingly, the anti-complement activity of Fs-8-ba2 was largely reduced by the carboxyl-reduced product, it is suggested carboxyl groups played an important role in the anti-complement activity of Fs-8-ba2. What's more, it was reported that the rhamnosyl residues of RG-I can be substituted at O-4 with neutral sugars side chains; these side chains are mainly composed of galactosyl and/or arabinosyl residues [33]. However, the side chains of Fs-8-ba2 mainly consist of 1,4-Glc and 1,6-Glc, which is uncommon in natural products. It was revealed that Fs-8-ba2 was a type RG-I with some novel structural features.

Heparin is a highly sulfated copolymer of uronic acid and glucosamine. Sulfate groups of heparin had been reported to function as a crucial factor for the activities of anticoagulation and anti-complement activation [30]. In our study, Fs-8-ba2, an acidic polysaccharide, was isolated and purified to homogeneity from *Forsythia suspensa*. IR and NMR assay revealed the absence of sulfated group in the polysaccharide, and suggested that Fs-8-ba2 may take a different mechanism during complement inhibition. The anti-complement activity of heparin could be attributed to its typical chelating-like structure in which abundant negatively charged sulfate groups are present [34]. Because of the lack of sulfate groups in Fs-8-ba2, the other mechanisms are likely involved in Fs-8-ba2 mediated-complement inhibition. The data of C-depleted sera hemolysis indicated that Fs-8-ba2 might selectively interact with C1q, C1r, C1s, C2, C3 and C9, but not with C4 and C5. Therefore, Fs-8-ba2 inhibited complement activation by blocking the target complements C1q, C1r, C1s, C2, C3 and C9, which is different from heparin with blocking 13 kinds of target complements. Moreover, as the lack of abundant negatively charged sulfate groups in heparin and sulfated homogalacturonans, Fs-8-ba2 demonstrated lower inhibitory effect than heparin and sulfated homogalacturonans. However, it also showed less undesirable anti-coagulant side effect than heparin and sulfated homogalacturonans as the absence of sulfated groups, which

might improve its clinical application in the treatment of anti-complementary diseases.

Conclusion

In our study, a homogeneous acidic polysaccharide (Fs-8-ba2), with the backbone of 7 homogalacturonan (HG) and 2 rhamnogalacturonan (RG-I) moieties, and the side chains attached at O-4 of 1,2,4-linked α-L-Rha in the RG-I moieties, was isolated from *Forsythia suspensa*. The anti-complement assay showed that Fs-8-ba2 possessed strong inhibitory effect on the complement activation through the classical and alternative pathways. The data of complement-depleted sera indicated that Fs-8-ba2 selectively interacted with C1q, C1r, C1s, C2, C3 and C9, but not C4 and C5. These results suggested that Fs-8-ba2 could be considered as a promising candidate of an anti-complement agent in the treatment of many diseases with characteristics of excessive activation of the complement system, such as system lupus erythematosus, rheumatoid arthritis, and acute respiratory distress syndrome.

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Author's Contribution

Songshan Shi and Hui Lian contributed equally to this work. Shunchun Wang and Songshan Shi conceived, designed the experiments, and contributed reagents, materials and analysis platforms; Hui Lian, Chao Zhu, Huijun Wang, Ruimin Liu, and S.W. Annie Bligh analyzed the data, interpreted the results, prepared figures, wrote the manuscript, and revised the article; Songshan Shi, Hui Lian, and Chao Zhu carried out the laboratory experiments.

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