Homogalacturonans from Preinfused Green Tea: Structural Characterization and Anticomplementary Activity of Their Sulfated Derivatives

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Ⓢ *Supporting Information*

ABSTRACT: Two homogeneous water-soluble polysaccharides (TPSR4-2B and TPSR4-2C) were obtained from preinfused green tea. Their average molecular weights were estimated to be 41 kDa and 28 kDa, respectively. A combination of composition, methylation, and configuration analysis, as well as NMR spectroscopy, indicated that both TPSR4-2B and TPSR4-2C were poly-(1−4)-α-D-galactopyranosyluronic acid in which 30.5 ± 0.3% and 28.3 ± 0.5%, respectively, of uronic acid existed as methyl ester. Two sulfated derivatives (Sul-R4-2B and Sul-R4-2C) from TPSR4-2B and TPSR4-2C were prepared after sulfation with a 2:1 chlorosulfonic acid−pyridine ratio. The anticomplementary assay showed that Sul-R4-2B and Sul-R4-2C demonstrated a stronger inhibitory effect on the complement activation through the classic pathway, compared to that of heparin. Preliminary mechanism studies by using complement component depleted-sera indicated that both Sul-R4-2B and Sul-R4-2C selectively interact with C1q, C1r, C1s, C2, C5, and C9 but not with C3 and C4. The relationship between DS and the anticomplementary activity of sulfated derivatives of homogalacturonans showed that low sulfated derivatives of homogalacturonans also exhibited potent anticomplementary effect, which might greatly reduce the side effects related to heparin and oversulfated chondroitin sulfate, such as anticoagulant activity and allergic-type reaction. These results suggested that sulfated derivatives of homogalacturonans might be promising drug candidates for therapeutic complement inhibition.

KEYWORDS: *Camellia sinensis L., green tea residue, homogalacturonan, sulfated derivative, anticomplement*

■ INTRODUCTION

The complement system can be activated by different cascades of signaling pathways including the classical pathway (CP), alternative pathway (AP), and the mannan binding lectin pathway (MBL). Although it is of great importance in host immune defense against infection through the clearance of antigen−antibody complexes from the bloodstream, its excessive activation might result in various diseases such as system lupus erythematosus (SLE), rheumatoid arthritis (RA), and acute respiratory distress syndrome (ARDS), etc.[1,2] Up to now, many naturally occurring agents have been reported to have anticomplementary effects, and they offer the prospect of inexpensive and nontoxic strategies for therapeutic inhibition of the complement.[2] The relationship between chemical characteristics and the anticomplementary activity of natural and synthetic sulfated polysaccharides indicated that sulfated polysaccharides could be a prospect for inexpensive and nontoxic strategies for therapeutic inhibition of a complement.[3,4] For instance, fucan, a sulfated polysaccharide extracted from brown seaweed, was reported to have certain resistance to excessive complement activity with a moderate adverse effect.[3]

Green tea, collected from the plant *Camellia sinensis* L., is one of the most widely consumed beverages around the world. It is increasingly recognized as a health drink in China and Japan, with many of its bioactive components already characterized.[5] For example, polyphenols from green tea are well known to have antioxidative,[6] antibacterial,[7] and immunomodulatory effects.[8,9] In recent years, polysaccharides from green tea were also reported to be bioactive, with demonstrated hypoglycemic benefit,[10−13] antioxidant potential,[14,15] antiadhesion of pathogenic bacteria,[16] and enhancement of immune activity.[13,17,18] However, most interest focused on polyphenols. The spent leaf of infused green tea, which might be rich in polysaccharides, was commonly discarded. There is no report on the polysaccharides contained in preinfused green tea. With numerous reported studies on the biological activity of polysaccharides in green tea, it is valuable to study the polysaccharides in spent green tea and investigate the potential of making use of such discarded resources.

In our studies, green tea samples after polyphenol extraction are investigated. Two homogalacturonans were extracted from

preinfused samples, and their structural features are characterized using chemical and spectral methods. The sulfated derivatives of the extracted homogalacturonans were prepared, and their anticomplement activities through the classic pathway were tested.

■ MATERIALS AND METHODS

Materials. Dried spent leaf of green tea after countercurrent extraction at 80 °C was provided by Shanghai Novanat Bioresources Co., Ltd. DEAE-cellulose was purchased from Amersham Biosciences. Sephacryl S-300 HR (100 × 2.6 cm) was obtained from GE Healthcare Bio-Sciences. 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimidemetho-p-toluenesulfonate (CMC), pectin methylesterase, and trifluoroacetic acid (TFA) were purchased from Sigma. All other reagents were of analytical grade.

General Methods. IR spectra were measured with a Perkin-Elmer 2000 FTIR spectrometer as KBr pellets. High performance gel permeation chromatography (HPGPC) was performed with an Agilent 1100 instrument fitted with GPC software, using KS-805 and KS-804 connections in series (Shodex CO.). Gas chromatography (GC) was performed with a Thermo TRACE GC apparatus equipped with a DB-624 column (length, 30 m × 0.32 mm; thickness of liquid phase, 1.8 μm) for the determination of O-methyl and O-acetyl groups. GC-MS was analyzed with a Thermo TRACE DSQ apparatus equipped with a TR-5 column (length, 60 m × 0.25 mm; thickness of liquid phase, 0.25 μm).

Isolation and Purification of Polysaccharides. TPSR4-2B and TPSR4-2C were isolated according to the scheme shown in Figure 1. Briefly, dried samples of preinfused green tea (1.948 kg) were extracted with boiling water twice for 2 h, and the solid−liquid ratios were 1:15 and 1:10, respectively. After centrifugation, 95% EtOH was added until the EtOH concentration reached 40%, rendering crude polysaccharide TPSR4 (46.6 g, yield 2.39%). The supernatant left was further supplemented with 95% EtOH to obtain 70% of EtOH aqueous solution, and the precipitate was designated as the crude polysaccharide TPSR7 (25.3 g, yield 1.30%).

TPSR4 was fractionated on a column of DEAE-cellulose (50 cm ×5 cm, Cl⁻ form), and eluted stepwise with distilled water and 0.1, 0.2, and 0.5 M NaCl solutions. The fraction eluted with 0.2 M NaCl (TPSR4-2, yield 28.7% from TPSR4) was further fractionated on a column of Sephacryl S-300 HR (100 cm × 2.6 cm) and eluted with water to give two major fractions TPSR4-2B (yield 16.9% from TPSR4-2) and TPSR4-2C (yield 35.8% from TPSR4-2).

Homogeneity and Molecular Weight. Measurements were performed by HPGPC on two columns of KS-805 and KS-804 in series.[19] The column was calibrated by standard pullulans with known molecular weight (P-5, P-10, P-20, P-50, P-100, P-200, P-400, and P-800; Shodex CO.). The column temperature was kept at 40.0 ± 0.1 °C. NaCl at 0.2 M was used as eluant, and the flow rate was kept at 0.8 mL/min. All samples were finally prepared as 2 mg/mL solutions, and a 20 μL aliquot was injected for each run.

Monosaccharide Composition Analysis. The acidic polysaccharide was hydrolyzed with 2 M TFA at 121 °C for 2 h. After evaporation to completely remove TFA, the residue was dissolved in distilled water (0.1 mL) and analyzed on a PEI-cellulose plate (E. Merck), developed with 5:5:1:3 (v/v) EtOAc−pyridine−AcOH−water. The plate was visualized by spraying O-phthalic acid reagent and heated to 100 °C for 5 min.[20] The remaining hydrolysate was reduced with NaBH₄ at room temperature for 3 h. After neutralization with AcOH and evaporation to dryness, the residue was acetylated with Ac₂O for 1 h at 100 °C. The resulting alditol acetates were subjected to GC-MS analysis. Reduction of carboxyl groups was carried out with CMC-NaBH₄ three times as described.[21,22] The reduced polysaccharides (R4-2Bre and R4-2Cre) were hydrolyzed and converted into alditol acetates as described above. The configurations of reduced polysaccharides (R4-2Bre and R4-2Cre) were determined by comparing with standard D-galactose and L-galactose using the double hydrolysis/reductive amination method of Cases Cerezo, and Stortz.[23]
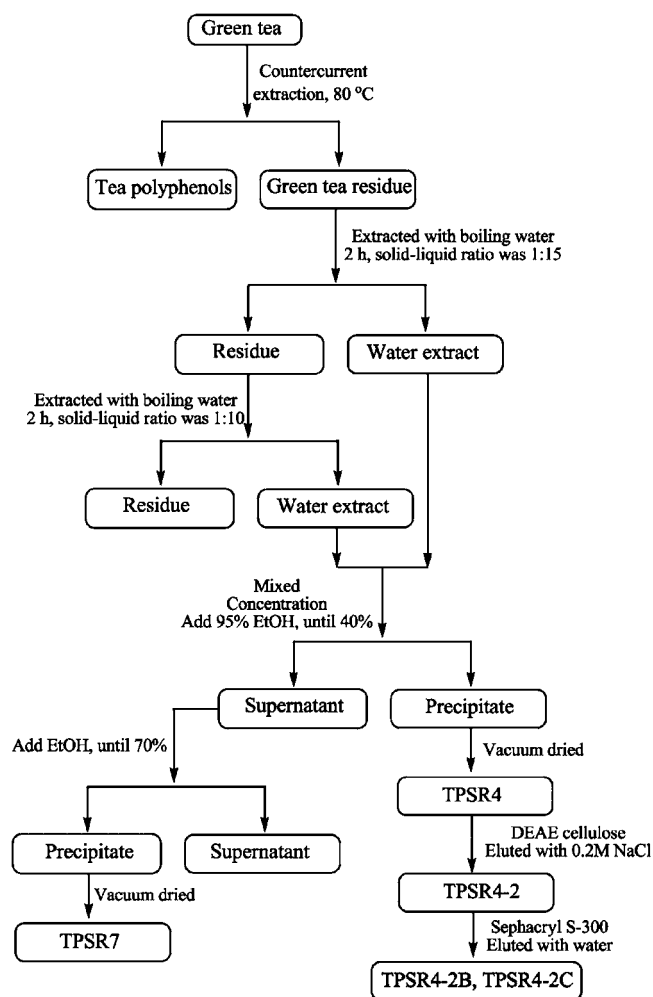

Figure 1. Scheme of isolation of polysaccharides TPSR4-2B and TPSR4-2C from green tea. The dried green tea was extracted at 80 °C with the method of countercurrent extraction to give tea polyphenols, whereas the remaining residue was collected to give the green tea residue. The green tea residue was extracted with boiling water; the aqueous extract was precipitated with EtOH concentration to 40% and 70%, giving TPSR4 and TPSR7, respectively. After successive separation by DEAE-cellulose anion-exchange and Sephacryl S-300 HR gel permeation chromatographic steps, it afforded the carbohydrate fractions TPSR4-2B and TPSR4-2C.

Determination of Esterification Degree (O-Methyl and O-Acetyl Groups). Internal calibration curves for quantification of methanol and acetic acid were prepared by adding the standards in the concentration range between 20 and 100 μg/mL for methanol and acetic acid, and isopropanol as the internal standard. The saponification of the polysaccharide occurred by the addition of 0.8 mL of 2 M NaOH at 25 °C, and after 1 h, the reaction was terminated by the addition of 0.8 mL of 2 M HCl, and pH was adjusted to 2.0, as described by Nunes et al.[24] The GC oven temperature for methanol was set at 65 °C (held for 8 min) and for acetic acid at 100 °C (held for 8 min). The flow rate of the carrier gas (N₂) was set at 2 mL/min.

Methylation Analysis. The reduced polysaccharides (R4-2Bre and R4-2Cre, 5 mg) were methylated three times with methyl iodide and powdered sodium hydroxide in dimethyl sulfoxide as described by Needs and Selvendran.[25] Samples R4-2Bre and R4-2Cre were dried overnight in vacuo (P₂O₅) at room temperature, then dissolved in 4 Å molecular sieve-dried DMSO (1.5 mL), and methylated using Need's method. The permethylated polysaccharide was hydrolyzed and converted into partially acetylated partially methylated alditol acetates and analyzed by GC-MS.[26]
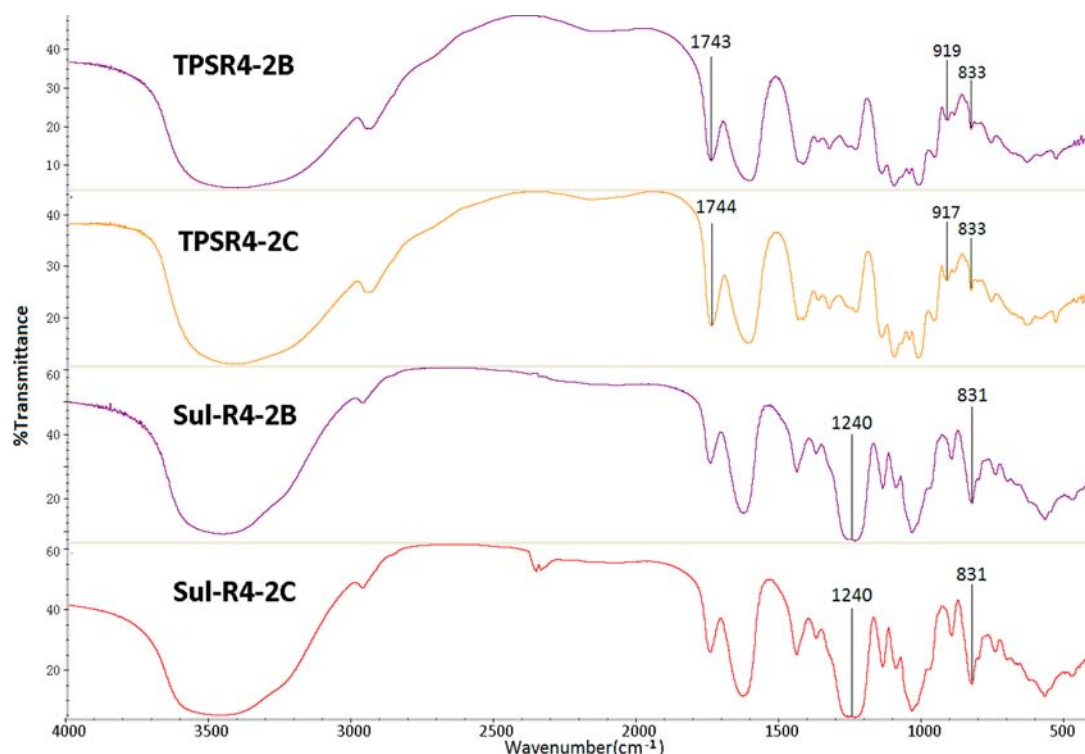
Figure 2. IR spectra of TPSR4-2B and TPSR4-2C, and their sulfated derivatives Sul-R4-2B and Sul-R4-2C. For TPSR4-2B and TPSR4-2C, the IR spectra showed an absorption of the C=O in uronic acid at 1743 cm^{-1} and 1744 cm^{-1}, respectively. The absorption at 833 cm^{-1} was characteristic of α configuration, and 919 and 917 cm^{-1} were characteristic of pyranose. For Sul-R4-2B and Sul-R4-2C, the IR spectra of both showed a C−O−S absorption at 831 cm^{-1} and a S=O vibration at 1240 cm^{-1}, indicating that both were successfully sulfated.

Preparation of Sulfated Polysaccharide. Sample TPSR4-2B and TPSR4-2C were suspended in dry formamide and kept at room temperature for 15 min followed by the addition of 2:1 chlorosulfonic acid and pyridine (2 mL). The mixture was maintained at 0 °C for 2 h and then kept at room temperature for 3 h. After the reaction, the mixture was adjusted to pH 7−8 by 5 M NaOH solution, dialyzed first against saturated NaHCO$_3$ overnight and then against deionized water extensively. The retentates were lyophilized to give sulfated derivatives (Sul-R4-2B and Sul-R4-2C). Furthermore, Sul-R4-2B-1-1 and Sul-R4-2B-6h were the sulfated derivatives from TPSR4-2B when the ratio of chlorosulfonic acid and pyridine changed from 2:1 to 1:1, and the reaction time at room temperature from 3 h to 4 h. The methyl ester of TPSR4-2B was removed by pectinesterase (P5400, Sigma) giving TPSR4-2B-Me, and Sul-R4-2B-Me was the sulfated derivative from TPSR4-2B-Me. The degree of substitution (DS) was measured by the BaCl$_2$ gelatin method.[27,28] DS was given by the following formula (% W is weight percent sulfate content, 96 is the molecular weight of SO$_4^{2-}$, 176 and 80 are the molecular weight per galacturonic acid unit and per sulfate group unit in sulfated derivatives of homogalacturonans, respectively):

$$DS = \frac{176 \times \% W}{96 - 80 \times \% W}$$

NMR Spectra. Native polysaccharides and sulfated polysaccharides (50 mg) were deuterium-exchanged three times and dissolved in 0.4 mL of D$_2$O (99.8%) for ^{13}C NMR, and 30 mg was dissolved in 0.5 mL of D$_2$O for ^{1}H NMR and 2D NMR. ^{1}H NMR, ^{13}C NMR, and 2D NMR were measured with a Bruker Avance III 400 spectrometer with a dual probe at 25 °C. The chemical shifts of ^{13}C NMR and ^{1}H NMR are expressed in ppm using acetone as the internal standard at 31.50 ppm for ^{13}C NMR and HDO at 4.70 ppm for ^{1}H NMR. All of the experiments were recorded, and data were processed using standard Bruker software; for ^{1}H NMR, typical parameters were relaxation delay time (D1) = 1.0 s, spectral width (SW) = 20.5 ppm, acquisition time (AQ) = 3.985 s, and transmitter frequency offset (Q1P) = 6.175 ppm.

For ^{13}C NMR, D1 = 2.0 s, SW = 238 ppm, AQ = 1.363 s, and Q1P = 105 ppm.

The 2D ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) was obtained using a Bruker standard experiment hsqcetgpsisp. Typical parameters were coupling constant, JCH = 145 Hz, D1 = 2.0 s, SW (1H) = 10 ppm, 2 k data points, AQ = 0.256 s, SW (13C) = 238 ppm, 256 data points, and AQ = 0.00534 s. The data were processed using shifted sine-bell squared functions in both dimensions; the size of the real spectrum was set to 1k for both axes before Fourier transformation.

The 2D ^{1}H−^{1}H correlated (COSY) spectra were obtained using a Bruker standard experiment cosygpmfsw. Typical parameters were D1 = 2.0 s, AQ = 0.192 s, SW = 13.3 ppm, O1P = 4.0 ppm, and 2 k data points. The data were processed using shifted sine-bell squared functions, and the size of the real spectrum was set to 1 k for both axes before Fourier transformation.

Anticomplementary Activity through the Classical Pathway. According to the modified method described by Mayer,[29] sensitized erythrocytes (EAs) were prepared by the incubation of sheep erythrocytes (4.0 × 10^8 cells/mL) with equal volumes of rabbit antisheep erythrocyte antibody in barbital buffered saline (BBS). Samples and heparin (used as positive control) were dissolved in BBS. Normal human serum (NHS) was used as the complement source. Diluted NHS was chosen to give submaximal lysis in the absence of complement inhibitors. Various dilutions of BBS-dissolved samples (100 μL) were preincubated with 100 μL of NHS and 200 μL of BBS at 37 °C for 10 min. Then 100 μL of EA was added, and the mixture was incubated at 37 °C for 30 min. A range of different assay controls were incubated in the same conditions: (1) vehicle control, 100 μL of EAs in 500 μL of BBS; (2) 100% lysis, 100 μL of EAs in water (500 μL); and (3) sample control, 100 μL dilution of each sample in 500 μL of BBS. The reaction mixture was centrifuged immediately. Optical density of the supernatant was measured at 405 nm with a spectrophotometer (Wellscan MK3, Lab systems Dragon). Corrected absorbance was obtained by subtracting sample control absorbance from corresponding samples, and the percent inhibition was calculated
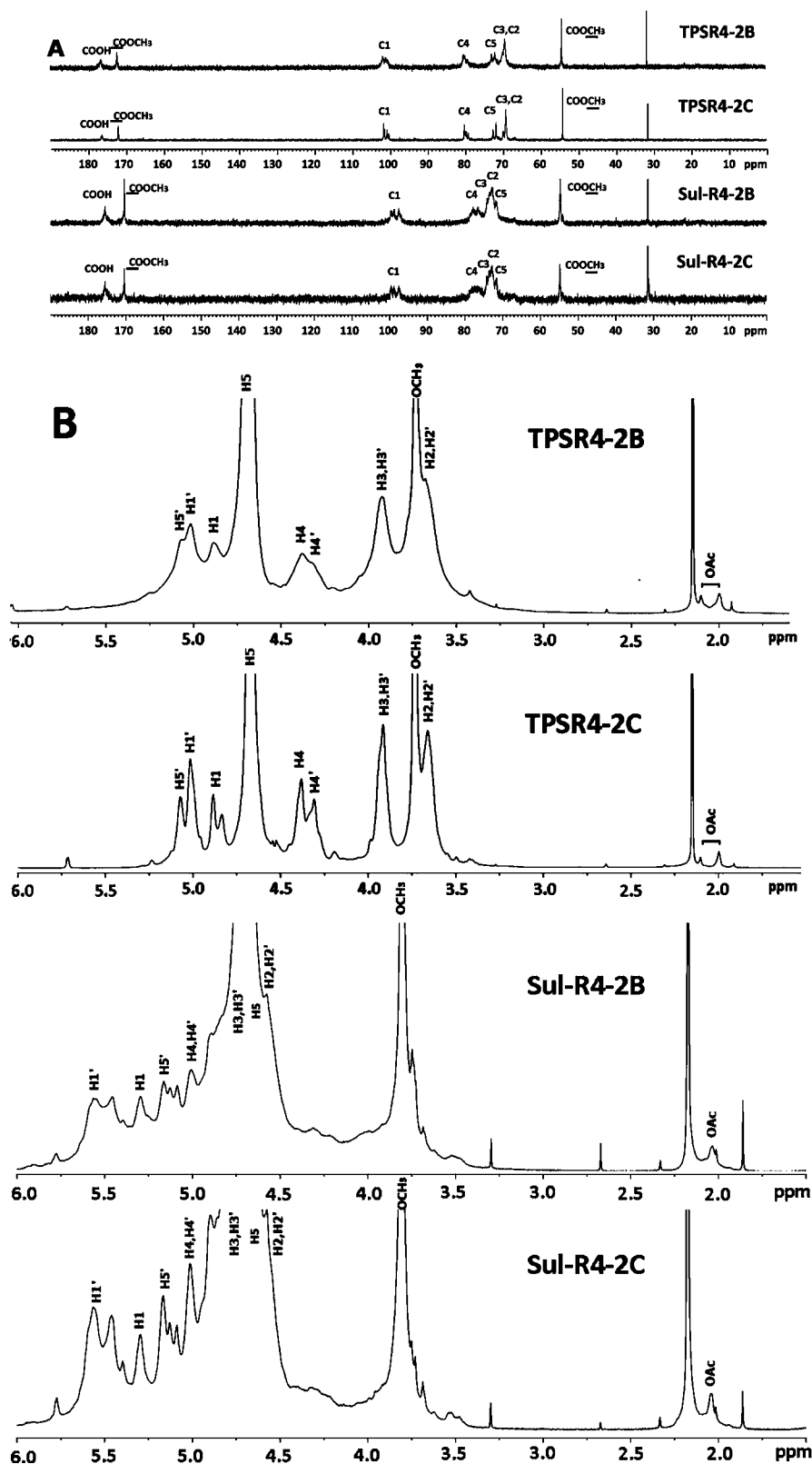
Figure 3. ^{13}C NMR and ^{1}H NMR spectra of TPSR4-2B, TPSR4-2C and their sulfated derivatives Sul-R4-2B and Sul-R4-2C obtained at 25 °C in D$_2$O. In ^{1}H NMR spectra, H was 1,4-α- GalpA or its sulfated derivative, and H′ was 1,4-α- GalpA(OMe) or its sulfated derivative. (A) For ^{13}C NMR of TPSR4-2B and TPSR4-2C, the low-field signal at 176.2−176.3 ppm corresponded to C-6 of (1−4)-linked GalpA. The signals at 172.0−172.2 ppm and 54.1 ppm indicated that some of the GalpA residues existed as the methyl ester. The anomeric signals at 100.7−101.6 ppm were assigned to C-1 of (1−4)-linked α-GalpA. The signal at 71.8−72.7 ppm was attributed to C-5 of (1−4)-linked GalpA. For ^{13}C NMR of Sul-R4-2B and Sul-R4-2C, the chemical shift of C-2 and C-3 of GalpA was downfield shifted about 4 ppm from 69.2 and 69.9 ppm to 72.8 and 73.5 ppm, indicating that the sulfonyl substitution was exclusively at C-2 and C-3 on 1, 4-linked GalpA. Meanwhile, the presence of sulfate groups at C-2 made the signal of the anomeric carbon shifted upfield from 100.7−101.6 ppm to 97.3−99.5 ppm; the signal at C-4 highfield shifted from 79.5−80.2 ppm to 76.9 ppm. The

Figure 3. continued

signals at 71.6 and 69.4 ppm were assigned to C-5 of nonmethyl and methyl esterified Sul-R4-2B and Sul-R4-2C. (B) For ^{1}H NMR of TPSR4-2B and TPSR4-2C, the signal at 3.78−3.79 ppm showed the characteristics of methyl protons in methyl esterified (1−4)-linked Gal$p$A. The weak signals at 2.02 and 2.12 ppm suggested that both of them contained a very small amount of O-acetyl groups in C-2 and C3. The other signals were also assigned, and the Arabic numerals refer to atoms in sugar residues. For ^{1}H NMR of Sul-R4-2B and Sul-R4-2C, the chemical shifts of H-2 and H-3 of Gal$p$A were downfield shifted about 0.9 ppm from 3.66 and 3.91 ppm to 4.59 and 4.85 ppm after TPSR4-2B and TPSR4-2C were sulfated. With the presence of sulfate groups at C-2 and C-3, the anomeric proton shifts downfield about 0.5 ppm from 4.88−5.04 ppm to 5.30−5.56 ppm, and the signals at H-4 shift from 4.31−4.38 ppm to 5.1 ppm. The signals at 4.81 and 5.09 ppm were assigned to H-5 of nonmethyl and methyl esterified Sul-R4-2B and Sul-R4-2C.

with the 100% lysis control as reference. Inhibition of lysis (%) = 100 − 100 × (OD$_{sample}$ − OD$_{sample\ control}$)/OD$_{100\%\ lysis}$. The concentration of sample that resulted in 50% hemolytic inhibition was defined as the CH$_{50}$ value toward the classical pathway.[30]

Preparation of Complement-Depleted Serum with Complementary Antibody. The experiment was conducted according to the method of Zhou et al.[31] 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 of EA and 200 μL of BBS at 37 °C for 30 min as previously described. Then cell lysis was measured as described in the anticomplementary activity through the classical pathway section. The antiserum dilution against the NHS hemolytic capacity was determined. The optimal dilutions of the complements (1:8 for C1q, 1:4 for C1r, 1:3 for C1r, 1:1 for C4, and 1:6 for C5) were incubated with the same volume of NHS (1:3.5) at 37 °C for 15 min followed by centrifugation. The supernatants were stored as C-depleted sera in aliquot at −80 °C before use in hemolytic assays.

Interaction with Individual Complement Components. Tested compounds were dissolved in BBS, diluted to the required concentration, and adjusted to the sufficient level to completely inhibit the hemolysis of 1:10 diluted NHS through the classical pathway. Two hundred microliters of EA and 200 μL of individual complement-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 EA under the same condition, and the hemolytic activities were calculated. The controls, (1) all blanks, 200 μL of EA in 400 μL of BBS; (2) 100% lysis, 200 μL EA in 400 μL water; (3) complement control, 100 μL of NHS (1:3.5, v/v) and 200 μL of EA in 300 μL of BBS; and (4) sample control, 100 μL of dilution of sample in 500 μL of BBS were prepared under the same conditions.

■ RESULTS AND DISCUSSION

Isolation of TPSR4-2B and TPSR4-2C. As described in Figure 1, the water-soluble polysaccharides were extracted from dried spent leaves of green tea. Through fractional precipitation with 40% and 70% of ethanol, TPSR4 (yield, 2.39%) and TPSR7 (yield, 1.30%) were obtained. TPSR4 was further purified by anion-exchange chromatography and size-exclusion chromatography to obtain TPSR4-2B (yield, 16.9%) and TPSR4-2C (yield, 35.8%). Both TPSR4-2B and TPSR4-2C were homogeneous, as they were eluted as a single symmetrical peak, corresponding to molecular weights of 41 kDa and 28 kDa, respectively. Both TPSR4-2B and TPSR4-2C are acidic polysaccharides since the IR spectra of TPSR4-2B (Figure 2) showed an absorption of the C=O in uronic acid at 1743 cm^{-1}. The absorptions at 3424, 2953, and 1422 cm^{-1} were attributed to the stretching from O−H, C−H, and carboxyl C−O groups, and those at 1145, 1103, and 1017 cm^{-1} to various in-plane C−O vibrations; similarly, TPSR4-2C (Figure 2) showed an absorption of the C=O in uronic acid at 1744 cm^{-1}. The absorptions at 3405, 2942, and 1421 cm^{-1} were attributed to the stretching from O−H, C−H, and carboxyl C−O groups, and those at 1146, 1103, and 1017 cm^{-1} to various in-plane C−O vibrations.[32] The bands at 833 cm^{-1} were characteristic of α configuration, and the absorption at 919 and 917 cm^{-1} was characteristic of the pyranose of TPSR4-2B and TPSR4-2C.[33]

Monosaccharide Composition Analysis and Determination of Esterification Degree. Complete hydrolysis followed by TLC showed that both TPSR4-2B and TPSR4-2C contained mainly uronic acid. This was confirmed by the titration of uronic acid using 3-phenylphenol (m-hydroxybiphenyl).[34,35] TPSR4-2B and TPSR4-2C were found to contain 99.0% and 86.1% of uronic acid, respectively, when D-galacturonic acid was used as the standard.

TPSR4-2B and TPSR4-2C were reduced three times with sodium borohydride by Taylor's method[22] to give carboxyl-reduced polysaccharides, R4-2Bre and R4-2Cre. IR spectra of R4-2Bre and R4-2Cre showed the disappearance of C=O absorption at 1743(1744) cm^{-1}. Complete hydrolysis of R4-2Bre and R4-2Cre followed by TLC analysis also indicated that they did not contain uronic acid. After the hydrolysate was converted into the corresponding alditol acetates and analyzed by GC-MS, R4-2Bre and R4-2Cre were found to only contain galactose resulting from galacturonic acid that existed in TPSR4-2B and TPSR4-2C. The configuration of galactose in R4-2Bre and R4-2Cre (Supporting Information, Figure 1) were assigned as the D configuration by comparing with standard D-galactose and L-galactose using the GC-MS method developed by Cases et al.,[23] indicating D-galacturonic acid as the major uronic acid in TPSR4-2B and TPSR4-2C.

As determined, 30.5 ± 0.3% and 28.3 ± 0.5% of carboxylic groups of galacturonic acid residues in TPSR4-2B and TPSR4-2C existed as the methyl ester, respectively. The degrees of O-acetylation of the TPSR4-2B and TPSR4-2C were determined as 1.27 ± 0.07% and 0.92 ± 0.08%. It might be possible that galacturonic acid residues in TPSR4-2B and TPSR4-2C are 2- and/or 3-O-acetylated, which is commonly observed in the homogalacturonan regions of pectic polysaccharides.[36]

Linkage Analysis of TPSR4-2B and TPSR4-2C. R4-2Bre and R4-2Cre were methylated three times to give completely methylated polysaccharides. GC-MS analysis indicated that both R4-2Bre and R4-2Cre were (1−4)-linked linear galactans, inferring that the native TPSR4-2B and TPSR4-2C were (1−4)-linked linear homogalacturonans.

NMR Analysis of TPSR4-2B and TPSR4-2C. The signals of TPSR4-2B and TPSR4-2C in 1D ^{1}H, ^{13}C NMR, and 2D NMR (^{1}H−^{13}C HSQC and ^{1}H−^{1}H-COSY) spectra were assigned as completely as possible based on the chemical shifts reported in the literature. The ^{13}C NMR spectrum of TPSR4-2B and TPSR4-2C (Figure 3A) showed that the signals at 176.2−176.3 ppm corresponded to C-6 of nonmethyl esterified (1−4)-linked Gal$p$A. The signals at 172.0−172.2 ppm and 54.1

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Table 1. ^{13}C NMR and ^{1}H NMR Chemical Shifts (ppm) for TPSR4-2B, TPSR4-2C, and Their Sulfated Derivatives Sul-R4-2B and Sul-R4-2C

residues	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6	−OCH$_3$
→4)-α-GalpA-(−1→	101.6	69.2	69.9	80.0	72.7	176.3	
(TPSR4-2B, A)	4.88	3.68	3.92	4.38	4.69		
→4)-α-GalpA6Me-(−1→	100.7	69.2	69.9	79.5	71.8	172.0	54.1
(TPSR4-2B, A′)	5.02	3.68	3.92	4.32	5.07		3.78
→4)-α-GalpA-(−1→	101.3	69.2	69.9	80.2	72.7	176.2	
(TPSR4-2C, A)	4.91	3.66	3.91	4.38	4.69		
→4)-α-GalpA6Me-(−1→	100.7	69.2	69.9	79.6	71.8	172.2	54.1
(TPSR4-2C, A′)	5.04	3.66	3.91	4.31	5.07		3.79
→4)-α-GalpA2/3Sul-(−1→	99.5	72.8	73.5	76.9	71.6	175.5	
(Sul-R4-2B/Sul-R4-2C, B)	5.30	4.59	4.85	5.01	4.81		
→4)-α-GalpA6Me2/3Sul-(−1→	97.3	72.8	73.5	76.9	69.4	171.4	54.7
(Sul-R4-2B/Sul-R4-2C, B′)	5.56	4.59	4.85	5.01	5.09		3.81

ppm indicated that partial GalpA residues existed as the methyl ester in both TPSR4-2B and TPSR4-2C. The anomeric signals at 101.3−101.6 and 100.7 ppm were assigned to C-1 of nonmethyl and methyl esterified (1−4)-linked GalpA. The signals at 72.7 and 71.8 ppm were attributed to C-5 of nonmethyl and methyl esterified (1−4)-linked GalpA, and the signals at 80.0−80.2 and 79.5−79.6 ppm to C-4 of nonmethyl and methyl esterified (1−4)-linked GalpA. The signals at 69.2 and 69.9 ppm were assigned to C-2 and C-3 of both nonmethyl and methyl esterified (1−4)-linked GalpA.[4,20,37]

In the ^{1}H NMR of TPSR4-2B and TPSR4-2C (Figure 3B), the signal at 3.78−3.79 ppm showed the characteristics of protons in methyl esterified (1−4)-linked GalpA. The intense anomeric proton signals at 4.88−4.91 and 5.02−5.04 ppm corresponded to the nonmethyl and methyl esterified (1−4)-linked GalpA, which also indicated that GalpA residues possessed α configuration.[38] The signals at 4.69 and 5.07 ppm were attributed to H-5 of nonmethyl and methyl esterified (1−4)-linked α-GalpA and the signals at 4.38 and 4.31−4.32 ppm to H-4 of nonmethyl and methyl esterified (1−4)-linked α-GalpA. The signals at 3.66−3.68 and 3.91−3.92 ppm were assigned to H-2 and H-3 of both nonmethyl and methyl esterified (1−4)-linked α-GalpA.[38,39] In addition, the weak signals at 2.02 and 2.12 ppm suggested that both TPSR4-2B and TPSR4-2C contained a very small amount of O-acetyl groups in C-2 and C-3.[40]

In the case of nonmethyl esterified (1−4)-linked α-GalpA, the cross-peaks 4.88/101.6, 3.68/69.2, 3.92/69.9, 4.38/80.0, and 4.69/72.7 ppm in the HSQC spectrum of TPSR4-2B corresponded to H-1/C-1, H-2/C-2, H-3/C-3, H-4/C-4, and H-5/C-5.[41,42] In the HSQC spectrum of TPSR4-2C (Supporting Information, Figure 2), 4.91/101.3, 3.66/69.2, 3.91/69.9, 4.38/80.2, and 4.69/72.7 ppm corresponded to H-1/C-1, H-2/C-2, H-3/C-3, H-4/C-4, and H-5/C-5, respectively. In the ^{1}H−^{1}H COSY (data not shown), the cross-peaks 4.88/3.68, 3.68/3.92, and 3.92/4.38 ppm were detected in TPSR4-2B, and the cross-peaks 4.91/3.66, 3.66/3.91, and 3.91/4.38 ppm were detected in TPSR4-2C, which corresponded to H-1/H-2, H-2/H-3, and H-3/H-4, respectively.

As for the methyl esterified (1−4)-linked α-GalpA, the cross-peaks 5.02/100.7, 3.68/69.2, 3.92/69.9, 4.32/79.5, and 5.07/71.8 ppm in the HSQC spectrum of TPSR4-2B corresponded to H-1/C-1, H-2/C-2, H-3/C-3, H-4/C-4, and H-5/C-5, and the signal at 3.78/54.1 ppm corresponded to methoxyl group.[41,42] In the HSQC spectrum of TPSR4-2C (Supporting Information, Figure 2), 5.04/100.7, 3.66/69.2, 3.91/69.9, 4.31/

79.6, and 5.07/71.8 ppm corresponded to H-1/C-1, H-2/C-2, H-3/C-3, H-4/C-4, and H-5/C-5, respectively, and the signal at 3.79/54.1 ppm corresponded to the methoxyl group. In the ^{1}H−^{1}H COSY (data not shown), the cross-peaks 5.02/3.68, 3.68/3.92, and 3.92/4.32 ppm were detected in TPSR4-2B, and the cross-peaks 5.04/3.66, 3.66/3.91, and 3.91/4.31 ppm were detected in TPSR4-2C, which corresponded to H-1/H-2, H-2/H-3, and H-3/H-4, respectively. The complete ^{1}H and ^{13}C NMR signal assignments for TPSR4-2B and TPSR4-2C are summarized in Table 1.

Preparation and Characterization of Sulfated Derivatives of TPSR4-2B and TPSR4-2C. Two sulfated derivatives, designated as Sul-R4-2B and Sul-R4-2C, were prepared from TPSR4-2B and TPSR4-2C, respectively, by the sulfation reagent. The IR spectra of both (Figure 2) showed a C−O−S absorption at 831 cm^{-1} and a S=O vibration at 1240 cm^{-1}, indicating that Sul-R4-2B and Sul-R4-2C were successfully sulfated.[19] Interestingly, molecular weights of both Sul-R4-2B and Sul-R4-2C were reduced (Table 2), which might be due to

Table 2. Molecular Weight (MW), Degree of Substitution (DS), and CH$_{50}$ of TPSR4-2B, TPSR4-2C, and Their Sulfated Derivatives^{a}

samples	molecular weight (Da)	DS^{b}	CH$_{50}$ (μg/mL)
TPSR4-2B	4.1×10^4	0	>2000
TPSR4-2C	2.8×10^4	0	>2000
TPSR4-2B-Me	4.0×10^4	0	>2000
Sul-R4-2B	2.6×10^4	1.35	5.4 ± 0.6
Sul-R4-2C	1.2×10^4	1.42	5.2 ± 0.4
Sul-R4-2B-1-1	3.1×10^4	0.48	6.1 ± 0.6
Sul-R4-2B-6h	2.5×10^4	1.69	8.1 ± 0.1
Sul-R4-2B-Me	2.5×10^4	1.12	5.6 ± 0.3

^{a}The methyl ester of TPSR4-2B was removed giving TPSR4-2B-Me; Sul-R4-2B-Me was the sulfated derivative from TPSR4-2B-Me; Sul-R4-2B-1-1 and Sul-R4-2B-6h were the sulfated derivatives from TPSR4-2B when the ratio of chlorosulfonic acid and pyridine was changed from 2:1 to 1:1, and the reaction time at room temperature from 3 h to 4 h, respectively. The results showed that the sulfated derivative of low DS (0.48) exhibited similar anti-complementary activity with sulfated derivatives of high DS (1.12, 1.35, and 1.42) and even higher anti-complementary activity than the sulfated derivative of the highest DS (1.69). ^{b}DS is calculated as 176 × % W/ (96 - 80 × % W); % W is weight percent sulfate content, 96 is the molecular weight of SO$_4^{2-}$, 176 and 80 are the molecular weight per galacturonic acid unit and per sulfate group unit in sulfated derivatives of homogalacturonans. CH$_{50}$ of heparin was 8.9 ± 0.3 μg/mL.
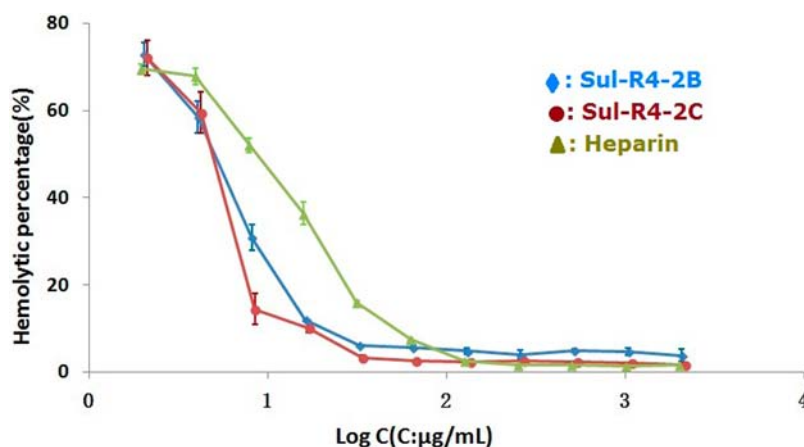
Figure 4. Inhibition of classical pathway-mediated hemolysis of Sul-R4-2B, Sul-R4-2C, and heparin. Inhibition of classical pathway-mediated hemolysis of EAs in 1:10 diluted NHS in the presence of increasing amounts of Sul-R4-2B (♦) and Sul-R4-2C (●). Heparin (▲) was used as the reference. Results are expressed as hemolytic percentage. The concentrations that resulted in 50% inhibition (CH_{50}) were 5.4 ± 0.6 μg/mL, 5.2 ± 0.4 μg/mL, and 8.9 ± 0.3 μg/mL for Sul-R4-2B, Sul-R4-2C, and heparin, respectively. The 100% hemolysis corresponds to the hemolytic activity of HPS in the absence of Sul-R4-2B and Sul-R4-2C. At a concentration of 16.1 μg/mL, 16.8 μg/mL, and 62.5 μg/mL, Sul-R4-2B, Sul-R4-2C, and heparin, respectively, almost abolished all of the hemolytic activity. On the contrary, at the concentration of 2000 μg/mL, TPSR4-2B and TPSR4-2C did not exhibit inhibitory activity of the classical pathway. Data are the mean from 4 determinations ± SEM.

the partial acid hydrolysis or to the high negative-charge density condition for sulfation.[43] The degrees of substitution (DS) of Sul-R4-2B and Sul-R4-2C were 1.353 and 1.420 (Table 2) respectively, based on Chaidedgumjorn et al.'s method.[27] According to the chemical shifts in ^{13}C NMR spectra described by others,[4,44] sulfate groups were probably substituted at the C-2 and C-3 of Gal$p$A residues. As shown in Figure 3A, the chemical shifts of C-2 and C-3 of Gal$p$A were downfield shifted about 4 ppm from 69.2 and 69.9 ppm to 72.8 and 73.5 ppm after TPSR4-2B and TPSR4-2C were sulfated. Meanwhile, the presence of sulfate groups at C-2 and C-3 made the signals of the anomeric carbon shift from 100.7−101.6 ppm to 97.3−99.5 ppm and the signals at C-4 shift from 79.5−80.2 ppm to 76.9 ppm. The signals at 71.6 and 69.4 ppm in the spectra of Sul-R4-2B and Sul-R4-2C were assigned to C-5 of nonmethyl esterified Sul-R4-2B and Sul-R4-2C (B) and methyl esterified Sul-R4-2B and Sul-R4-2C (B′). In the ^{1}H NMR of Sul-R4-2B and Sul-R4-2C (Figure 3B), the chemical shifts of H-2 and H-3 of Gal$p$A were downfield shifted about 0.9 ppm from 3.66 and 3.91 ppm to 4.59 and 4.85 ppm after TPSR4-2B and TPSR4-2C were sulfated. Because of the presence of sulfate groups at C-2 and C-3, the anomeric proton downfield shifted about 0.5 ppm from 4.88−5.04 to 5.30−5.56 ppm, and the signals at H-4 shifted from 4.31−4.38 ppm to 5.01 ppm. The signals at 4.81 and 5.09 ppm in the spectra of Sul-R4-2B and Sul-R4-2C were assigned to H-5 of B and B′, respectively. In the case of nonmethyl esterified Sul-R4-2B and Sul-R4-2C, the cross-peaks of HSQC spectra (Supporting Information, Figure 2) 5.30/99.5, 4.59/72.8, 4.85/73.5, 5.01/76.9, and 4.81/71.6 ppm corresponded to H-1/C-1, H-2/C-2, H-3/C-3, H-4/C-4, and H-5/C-5 of B; for methyl esterified Sul-R4-2B and Sul-R4-2C, the cross-peaks of HSQC spectra (Supporting Information, Figure 2) 5.56/97.3, 4.59/72.8, 4.85/73.5, 5.01/76.9, and 5.09/69.4 ppm corresponded to H-1/C-1, H-2/C-2, H-3/C-3, H-4/C-4, and H-5/C-5 of B′; the signal at 3.81/54.7 ppm corresponded to the methoxyl group of B′. The complete ^{1}H and ^{13}C NMR signal assignments for Sul-R4-2B and Sul-R4-2C are shown in Table 1.

Inhibition of Sul-R4-2B and Sul-R4-2C against the Classical Pathway of the Complement System. The effect

of native galacturonans and their sulfated derivatives on the activation of a human complement system through the classical pathway were examined in 1:10 diluted NHS, and heparin was used as reference.[29] In the control group, the percentage of complementary activation was 98.75 ± 1.32%. As shown in Figure 4, the concentrations that resulted in 50% inhibition (CH_{50}) were 5.4 ± 0.6 μg/mL, 5.2 ± 0.4 μg/mL, and 8.9 ± 0.3 μg/mL for Sul-R4-2B, Sul-R4-2C, and heparin, respectively. Sul-R4-2B and Sul-R4-2C were more potent than heparin in inhibiting the activation of the classical pathway. In contrast, the native galacturonans (TPSR4-2B and TPSR4-2C) did not exhibit an anticomplementary effect (Table 2). At a concentration of 16.1 μg/mL, Sul-R4-2B almost abolished all of the hemolytic activity of NHS (percent inhibition, 88.99 ± 0.40%). Similarly, at a concentration of 16.8 μg/mL, Sul-R4-2C almost abolished all of the hemolytic activity of NHS (percent inhibition, 89.74 ± 2.34%). However, at a concentration of 15.6 μg/mL, the percent inhibition of heparin was 63.54 ± 2.59%. Up to a concentration of 62.5 μg/mL, heparin almost abolished all of the hemolytic activity of NHS (percent inhibition, 92.46 ± 0.37%). Thus, both Sul-R4-2B and Sul-R4-2C exhibited inhibitory effects on complement activation through the classic pathway stronger than that of heparin.

Identification of the Target Individual Complement of Sul-R4-2B and Sul-R4-2C in the Complement Activation Cascade. The effects of Sul-R4-2B and Sul-R4-2C on individual complement components were investigated in the system with complement-depleted reagents and a limited amount of human serum. The capacities of various depleted sera to restore the hemolytic capacity of Sul-R4-2B or Sul-R4-2C-treated serum were examined. Under these conditions, the complement component under investigation is the limiting factor in the component-mediated hemolysis assay. Thus, the failure in restoring the hemolysis could be attributed to the interaction between the tested sample and corresponding complement component.

As shown in Figure 5, the percentage of 1:3.5 NHS induced hemolysis through the classical pathway was 97.12 ± 1.15% in the complement control group. Sul-R4-2B and Sul-R4-2C at a concentration of 16.1 μg/mL and 16.8 μg/mL exhibited a
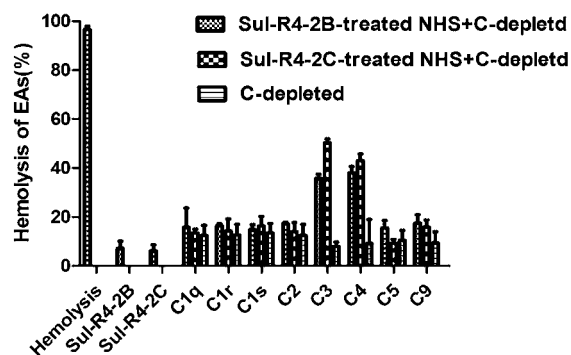
Figure 5. Hemolytic assays for individual components utilizing C-depleted serum. Sul-R4-2B or Sul-R4-2C treated serum was mixed with various depleted sera, and the capacity of these depleted sera to restore the classical pathway hemolytic capacity was estimated by adding sheep antibody-sensitized erythrocytes. The results indicated that both of the Sul-R4-2B and Sul-R4-2C selectively interact with C1q, C1r, C1s, C2, C5, and C9 but not with C3 and C4. Results are expressed as hemolysis percentages. Data are the means from 3 determinations ± SD.

strong inhibitory effect on this hemolysis, and their hemolysis percentage was 8.16 ± 1.68% and 6.81 ± 1.02%, respectively. Further studies indicated that both Sul-R4-2B and Sul-R4-2C selectively interacts with C1q, C1r, C1s, C2, C5, and C9 but not with C3 and C4. Therefore, Sul-R4-2B and Sul-R4-2C inhibit complement activation by blocking the target complements C1q, C1r, C1s, C2, C5, and C9 used in this assay.

Discussion. In our studies, two distinct homogalacturonans (TPSR4-2B and TPSR4-2C) were first isolated from spent leaves of green tea after polyphenol extraction. The chemical and spectral characterization indicated they were linear (1−4)-α-D-galacturonans with molecular weights of 41 kDa and 28 kDa. The methyl-esterification degrees of substitution of TPSR4-2B and TPSR4-2C were determined to be 30.5 ± 0.3% and 28.3 ± 0.5%, while the degrees of substitution of O-acetylation were 1.27 ± 0.07% and 0.92 ± 0.08%, respectively. The sulfated derivatives at C-2 and/or C-3 of GalpA of TPSR4-2B and TPSR4-2C, designated as Sul-R4-2B (DS,1.35) and Sul-R4-2C (DS, 1.42), were obtained. Both sulfated derivatives demonstrated a stronger inhibitory effect (CH_{50}, 5.4 ± 0.6 μg/mL for Sul-R4-2B; CH_{50}, 5.2 ± 0.4 μg/mL for Sul-R4-2C) than that of heparin (CH_{50}, 8.9 ± 0.3 μg/mL) on the activation of the complement system through the classic pathway. The data of C-depleted sera hemolysis indicated that both of them selectively block C1q, C1r, C1s, C2, C5, and C9. In contrast, native TPSR4-2B and TPSR4-2C had no such effects. Our native acid polysaccharides (TPSR4-2B and TPSR4-2C) and the native acid polysaccharides with demethylation, which exhibited high negative charge (TPSR4-2B-Me), did not show high activity on the activation of the complement system through the classic pathway; however, their sulfated derivatives showed a more potent effect than heparin (Table 2). This indicates that sulfation of homogalacturonans played a crucial role in anticomplement activity and is consistent with the concept that sulfated groups are necessary, although not sufficient for anticomplementary effects of polysaccharides.[3]

It was reported earlier that fully sulfated chondroitin sulfate, where all of the hydroxyl groups of both the uronic acid and galactosamine residues were sulfated, might cause acute, rapid onset of significant side effects indicative of an allergic-type reaction.[45] It is crucial to investigate the relationship between the DS and anticomplementary activity of sulfated derivatives of galacturonans. Here, sulfated derivatives of TPSR4-2B with different DS were prepared as shown in Table 2. The results showed that native homogalacturonans (DS = 0) did not exhibit an anticomplementary effect but that sulfated derivatives of homogalacturonans (DS from 0.48 to 1.69) exhibited a more potent effect than heparin (CH_{50} = 8.9 ± 0.3 μg/mL) in inhibiting the activation of the classical pathway. In Table 2, the CH_{50} result of Sul-R4-2B-6h (DS = 1.69, MW = 2.5 × 10⁴ Da, and CH_{50} = 8.1 ± 0.1 μg/mL) was statistically significantly higher than the other four results, which indicated that Sul-R4-2B-6h exhibited lower anticomplementary activity than the other four sulfated derivatives; the four CH_{50} results (Sul-R4-2B, Sul-R4-2C, Sul-R4-2B-1-1, and Sul-R4-2B-Me) were not significantly different between each other; even the CH_{50} results of Sul-R4-2C (DS = 1.42, MW = 1.2 × 10⁴ Da, and CH_{50} = 5.2 ± 0.4 μg/mL) and Sul-R4-2B-1-1 (DS = 0.48, MW = 3.1 × 10⁴ Da, and CH_{50} = 6.1 ± 0.6 μg/mL) did not show significantly different data. Taking into account only three sulfated derivatives with similar MW (2.5−2.6 × 10⁴), derivatives with DS of 1.12 and 1.35 had statistically similar CH_{50} results 5.6 ± 0.3 and 5.4 ± 0.6 with similar anticomplementary activity; however, the derivative with the much higher DS, 1.69, had higher CH_{50} results, 8.1 ± 0.1, with a little bit lower anticomplementary activity. The other two sulfated derivatives (Sul-R4-2C and Sul-R4-2B-1-1) with MWs of 1.2 × 10⁴ and 3.1 × 10⁴ Da and greatly dissimilar DS values of 1.42 and 0.48, respectively, had CH_{50} results statistically similar to the results for the low DS at constant MW derivatives (Sul-R4-2B and Sul-R4-2B-Me). It implied that sulfated derivatives of homogalacturonans might block complements equally when DS and MW were in a certain range (DS from 0.48 to 1.42 and MW from 1.2 × 10⁴ to 3.1 × 10⁴ Da); however, the derivative with much higher DS (1.69) blocked fewer complements. It might account for site-specific sulfation and site-specific DS acetyl, or there might even be two thresholds in sulfate DS values (from 0 to 2) in combination with MW that lead stably blocked complements to exhibit high anticomplementary activity; the derivatives with too low or too high DS did not block or blocked fewer complements. Because of the limited nature of our data, more work was needed to study in depth the relationship among DS, MW, site-specific sulfation, site-specific DS acetyl, and anticomplementary activity.

Interestingly, our results inferred that sulfated derivatives of low DS (0.48) exhibited similar anticomplementary activities with sulfated derivatives of high DS (1.12, 1.35, and 1.42); with even higher anticomplementary activity than the sulfated derivative of highest DS (1.69) in Table 2, the sulfated derivative of higher DS sulfate did not lead to higher anticomplementary activity. This was different from the anticoagulant activity of sulfated derivatives of homogalacturonans which depend on the level of sulfate substitution.[44] It can be inferred that low sulfated derivatives of homogalacturonans also exhibited potent anticomplementary effects, which may greatly reduce the side effects, such as anticoagulant activity and allergic-type reaction. It can be also inferred that the sulfated derivatives of homogalacturonans from the spent leaves of green tea can be considered as a promising candidate of an anticomplement 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Configuration of R4-2Bre and R4-2Cre and ^{1}H/^{13}C HSQC spectra of TPSR4-2B, TPSR4-2C, and their sulfated derivatives Sul-R4-2B and Sul-R4-2C. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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