Chemistry of Natural Compounds, Vol. 47, No. 1, March, 2011 [Russian original No. 1, January–February, 2011]

POLYSACCHARIDES FROM Fabaceae. V. α-GLUCAN FROM *Sophora flavescens* ROOTS

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UDC 582.738:677.46

Water-soluble polysaccharides from Sophora flavescens *(Fabaceae) roots were studied. The dominant polymer S_fP-1-1/2 of molecular weight 85 kDa was isolated using ion-exchange and gel chromatography. The structure of the isolated compound was α-(1→4)-glucan partially substituted at the C-6 position by single glucopyranose units. A pharmacological study showed that S_fP-1-1/2 exhibited under azathioprine suppression pronounced immunostimulating activity.*

Keywords: *Sophora flavescens*, Fabaceae, α-4,6-glucan, immunostimulating activity.

Sophora flavescens Soland. is a herbaceous perennial of the family Fabaceae that is indigenous to the Russian steppes and woody-steppe communities of Zabaikal, Khabarovsk, and Primoriya Territories and Irkutsk and Amur Oblasts. Roots of *S. flavescens* have been used in Tibetan medicine under the name *sle tres* as an antipyretic agent and for nerve diseases, malaria, and acute and chronic infections [1]. Studies of the chemical composition of *S. flavescens* roots identified chalcones [2], flavonoids [3], isoflavones [4], pterocarpanes [5], coumarins [6], phenylpropanoids [7], alkaloids [8], and triterpenes [9]. The composition and structure of polysaccharides from this plant have not been reported. Our goal was to study the composition and structure of water-soluble polysaccharides (WSPS) from *S. flavescens* roots.

Polysaccharides from *S. flavescens* roots were isolated from raw material that was treated preliminarily with several organic solvents (hexane, $CHCl_3$, EtOAc, acetone) and EtOH (80%). Three free carbohydrates (saccharose, fructose, and glucose) in a 63:37:1 ratio were detected in the EtOH extract. Their content in the raw material was 4.42, 2.61, and 0.07%, respectively.

The WSPS were extracted from *S. flavescens* by hot water with subsequent precipitation of the aqueous extract by EtOH, demineralization, and deproteinization of the resulting polysaccharide complex to afford a WSPS total fraction (S_fP) in 3.24% yield of the raw material mass. Solutions of S_fP had a positive specific rotation (+155.7°) and reacted with I_2. The carbohydrate content in S_fP was 95.4% with up to 3.82% uronic acids. The ash and protein contents were less than 0.5%. Total hydrolysis of S_fP produced glucose as the dominant monosaccharide (92.7 mol%) with galactose, arabinose, and rhamnose in a 9:6:1 ratio and traces of mannose and xylose (Table 1).

S_fP was fractionated using chromatography over DEAE-cellulose to produce six fractions (Table 1). The neutral I_2-positive fraction S_fP-1 that was eluted by water was the dominant one (88% of S_fP mass) and consisted of only glucose. Fractions S_fP-2 and S_fP-3 were eluted by NaCl solutions (0.1 and 0.3 M, respectively), were also neutral, reacted with I_2, and contained small quantities of galactose and arabinose in addition to glucose. Subsequent elution of the DEAE-cellulose by NaCl solutions (0.5 and 1 M) and NaOH (0.1 M) produced fractions with uronic acids amounting to 12.4–47.3 mol%. Thus, the WSPS complex from *S. flavescens* roots was a heterogeneous mixture of neutral and acidic components with the neutral fractions dominating.

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TABLE 1. Properties of Fractions S_fP and S_fP-1–S_fP-6

Fraction	Yield, %	$[\alpha]_D$, °[c]	Reaction with I_2	Monosaccharide composition, mol%						
				Ara	Gal	Glc	Man	Rha	Xyl	GalUA
S_fP	3.24[a]	+ 155.7	+	1.2	1.8	92.7	Tr.[d]	0.2	Tr.	4.0
S_fP-1	88.0[b]	+ 146.1	+			99.9				
S_fP-2	0.7[b]	n.d.	+		4.6	95.3				
S_fP-3	0.5[b]	n.d.	+	3.0	3.7	93.2				
S_fP-4	3.0[b]	+ 152.4	+	10.2	14.7	58.9	Tr.[d]	2.8	0.9	12.4
S_fP-5	0.4[b]	n.d.	–	15.4	19.7	32.2	0.3	3.3	1.2	27.8
S_fP-6	5.3[b]	+ 184.6	–	16.8	21.3	7.2	2.1	4.2	1.0	47.3

[a]Of raw material mass; [b]of S_fP mass; [c]c = 0.5, NaOH (1%); n.d., not determined; [d] <0.05 mol%; Tr.: traces.

TABLE 2. Properties of Fractions S_fP-1 and S_fP-1-1–S_fP-1-4

Fraction	Yield, % of S_fP-1 mass	$[\alpha]_D$, °[a]	λ_{max}, nm[b]	B_V[c]		MW, kDa (relative content, %)
				610 nm	680 nm	
S_fP-1	–	+ 146.1	582	0.170	0.144	–
S_fP-1-1	66.9	+ 154.0	610	0.316	0.256	120 (5), 85 (83), 43 (12)
S_fP-1-2	11.9	+ 142.7	555	0.108	0.078	85 (53), 52 (20), 43 (27)
S_fP-1-3	9.1	+ 110.5	554	0.056	0.046	85 (9), 52 (18), 43 (30), 11 (43)
S_fP-1-4	4.4	+ 69.9	555	0.024	0.022	43 (11), 11 (89)

[a]c = 0.5, NaOH (1%); [b]absorption maximum of complex with I_2; [c]"blue" value.

Next, fractionation of S_fP-1 by stepwise precipitation by EtOH produced four subfractions that typically had a positive specific rotation and positive reaction with I_2 (Table 2). The dominant subfraction was S_fP-1-1 (66.9% of S_fP-1 mass) that was precipitated by EtOH (36%). The absorption maximum of the I_2 complex for S_fP-1-1 was 610 nm, which is similar to that for a slightly branched amylose [10]. The high "blue" values (0.256 at 680 nm and 0.316 at 610 nm) for S_fP-1-1 also suggested that the polymer was slightly branched [11]. The "blue" values were much lower for subfractions precipitated at higher EtOH concentrations. The absorption maxima of the complexes with I_2 shifted to 554–555 nm. This indicated that their structures were more branched. All isolated fractions were heterogeneous and contained an array of components with molecular weights (MWs) in the range 11–120 kDa.

Three components S_fP-1-1/1, S_fP-1-1/2, and S_fP-1-1/3 with MW 120, 85, and 43 kDa, respectively, were detected in S_fP-1-1. The dominant polymer S_fP-1-1/2 was isolated by preparative gel chromatography. Glucan S_fP-1-1/2 (MW 85 kDa) typically gave a positive reaction with I_2 (λ_{max} 612 nm) and had high specific rotation (+157.4°) and "blue" values (0.281 at 680 nm and 0.364 at 610 nm). The IR spectrum of S_fP-1-1/2 showed bands indicative of an α-bond (850 cm^{-1}) and pyranose rings (1021, 1050, 1146). The IR spectrum was similar to those of α-(1→4)-glucans [12].

The polysaccharide was acetylated and treated subsequently with CrO_3 in order to determine the configuration of the glucose in S_fP-1-1/2. The oxidation product was hydrolyzed. Analysis of the products found glucose, which confirmed that it had the *a*-configuration.

Periodate oxidation of S_fP-1-1/2 consumed 1.12 mol IO_4^- per anhydro unit and released 0.11 mol HCOOH per anhydro unit. Smith degradation of the periodate oxidation product detected in the hydrolysate glycerin and erythrite (GC/MS) in a 1:8.55 ratio. Cleavage of this nature indicated that the structure of S_fP-1-1/2 included both (1→4)- and (1→6)-bonds.

Hydrolysis of the permethylate of S_fP-1-1/2 and analysis of the cleavage products as the aldonitrile acetates (GC/MS) detected 1,5-diacetyl-2,3,4,6-tetra-*O*-Me-Glc*p*, 1,4,5-triacetyl-2,3,6-tri-*O*-Me-Glc*p*, and 1,4,5,6-tetraacetyl-2,3-di-*O*-Me-Glc*p* in a 1.09:8.27:1 ratio. According to the results, S_fP-1-1/2 contained in the main chain (1→4)-bonded glycopyranose units substituted 10.7% at the C-6 position by single glucopyranose units.

TABLE 3. ^{13}C NMR Data of Glucan S_fP-1-1/2

Unit	^{13}C chemical shifts, ppm					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Glc*p*-1→	100.21	72.42	74.97	69.59	71.78	60.09
→4-α-Glc*p*-1→	100.21	72.42	74.97	78.15	71.78	60.09
→4,6-α-Glc*p*-1→	98.56	72.30	74.71	78.15	72.14	65.44

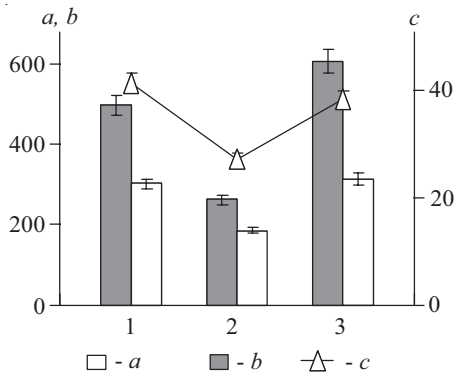

Fig. 1. Effect of glucan S_fP-1-1/2 on antibody formation (*a*, absolute number of antibody-forming cells in spleen × 100; *b*, number of antibody-forming cells per 10^6 spleenocytes) and extent of delayed-type hypersensitivity reaction (*c*, %). Experimental groups: water (untreated) (1), azathioprine (50 mg/kg) (2), azathioprine (50 mg/kg) + S_fP-1-1/2 (50 mg/kg) (3).

Partial hydrolysis of S_fP-1-1/2 produced in the hydrolysate four compounds SO-1–SO-4. Compounds SO-1, SO-2, and SO-3 were isolated and identified using chromatographic mobility, physicochemical properties, and methylation results as glucose, maltose, and maltotriose, respectively. The chromatographic mobility of SO-4 agreed with that of maltotetraose.

Enzymatic cleavage of S_fP-1-1/2 by α-amylase gave hydrolysis products, chromatography (HPTLC) of which detected glucose, maltose, maltotriose, maltotetraose, and several components that were probably polymerized maltose homologs.

The ^{13}C NMR spectrum of the studied glucan exhibited resonances that were assigned to single glucopyranose side chains and unsubstituted and substituted glucopyranose units of the main polymer chain (Table 3).

The α-configuration of the glucose units was confirmed by the position of the C-1 resonance at weak field (98.56 and 100.21 ppm). The C-4 resonance was shifted (+7.75 ppm) compared with that of free α-glucopyranose for the glucopyranose units of the main chain (substituted and unsubstituted). This was possible if this atom was involved in forming (1→4)-bonds [13]. A shift of +5.35 ppm was also found for C-6 of substituted glucose in the main chain and was explained by branching at this position. The magnitude of this shift (65.44 ppm) indicated that this atom was glycosylated by an α-substituent [14]. This was confirmed by a shift of the C-5 resonance of substituted glycopyranose units in the main chain by +0.36 ppm compared with that of the unsubstituted units that was due to the α-effect of the C-6 substituent.

A study of the effect of S_fP-1-1/2 on antibody-formation processes found that the glucan restored the humoral immune response index under azathioprine immunosuppression. Administration of azathioprine reduced both the absolute number of antibody-forming cells (AFC) and the number of AFC per 10^6 spleenocytes by 47.4 and 38.8%, respectively, compared with those of the untreated group (Fig. 1). Administration of S_fP-1-1/2 during immunosuppression increased reliably the number of AFC both in absolute values and calculated per 10^6 spleenocytes. The first index exceeded the level of azathioprine suppression by 2.31 times; the second, by 1.69 times. A study of the effect of S_fP-1-1/2 on cell-mediated reaction of delayed-type hypersensitivity (DTH) found that the tested compound restored the index of this reaction (IR DTH) under azathioprine immuosuppression. Administration of azathioprine reduced the IR DTH by 34.7% compared with the same index for the untreated group. Administering S_fP-1-1/2 with immunosuppression increased the IR DTH by 42% compared with the control. Thus, glucan S_fP-1-1/2 was capable of ameliorating the suppressive activity of azathioprine on the indices of the humoral and cellular immune response.

We found that WSPS from *S. flavescens* roots were a heterogeneous complex. The dominant polymer was slightly branched glucan S_fP-1-1/2 that contained in the main chain α-(1→4)-bonded glucopyranose substituted at C-6 by single α-glucopyranose units. Glucans of similar structure were isolated earlier from roots of other members of the Fabaceae family, e.g., *Astragalus mongolicus* [15], *Hedysarum polybotrys* [12], and *Sophora subprostrata* [16].

EXPERIMENTAL

Roots of *S. flavescens* were collected in Zabaikal Territory (Tsokto-Khangil; 17 Sept. 2006; 50°54′20″ N, 114°37′34″ E). The species was assigned by Cand. D. V. Sandanov (IGEB, SB, RAS). The raw material was stored in the Herbarium of the Department of Biologically Active Compounds, IGEB, SB, RAS (No. Fb/r-82/4-01/0906).

Gel chromatography was performed over Sephadex G-100 (Pharmacia); ion-exchange chromatography, cation-exchanger KU-2-8 (Reakhim) and anion-exchangers ARA-5pT40 (Reakhim) and DEAE-cellulose (Reanal); HPTLC, Sorbfil PTSKh-AF-V silica-gel plates (Imid Ltd.); PC, FN-16 chromatography paper (Filtrak). The solvent systems were PrOH:$CHCl_3$:H_2O (1, 7:4:1) (double development to heights of 3.5 and 7 cm); EtOAc:AcOH:MeOH:H_2O (2, 12:3:3:2), and *i*-PrOH:H_2O (3, 8:2). Compounds were detected using *p*-hydroxydiphenylphosphate (1%). Spectrophotometric studies were carried out on a UV-Vis-mini spectrophotometer (Shimadzu). Optical rotation was determined on a SM-3 polarimeter (Zagorsk Optico-Mechanical Plant). IR spectra were recorded on a Spectrum 100 IR-Fourier spectrometer (Perkin–Elmer) as films on KRS-5 plates in the range 4,000–650 cm^{-1}. GC/MS was performed in a 5973 N instrument (Agilent Technologies) with a 6890 N mass-selective detector (Agilent Technologies), diffusion pump, PH-Innowax capillary column (30 m/250 μm/0.50 μm). ^{13}C NMR spectra were recorded from DMSO-d_6 solutions (1%) on a VXR 500S NMR spectrometer (Varian) at operating frequency 125.7 MHz. Dialysis was carried out in dialysis tubes with 1 kDa exclusion limit (Sigma). Quantitative analysis of gel-chromatograms used the Leonardo 1.01 program (Nauka Plyus). The carbohydrate contents were determined using the phenol–H_2SO_4 method [17]; uronic acids, reaction with 3,5-dimethylphenol [18]; protein, by the Bradford method [19]; ash content, gravimetrically after ashing; "blue" value (B_v), by the Morrison–Laignelet method [20]. We used standard samples of maltose (CAS No. 6363-53-7), maltotriose (CAS No. 1109-28-0), and maltotetraose (CAS No. 34612-38-9) (all Acros Organics); protease from *Streptomyces griseus* (EC 3.4.24.31, Fluka, CAS No. 9036-06-0, 6 U/mg); α-amylase from *Aspergillus oryzae* (EC 3.2.1.1, Sigma, CAS No. 9001-19-8, 150-250 U/mg); and azathioprine (Sigma).

Isolation of WSPS from *S. flavescens*. Ground *S. flavescens* roots (300 g) were extracted in a Soxhlet apparatus successively by hexane, $CHCl_3$, EtOAc, and acetone. The solid raw material was dried and extracted by EtOH (80%, 1:12, 5 × 1.5 L) and then H_2O (1:15) on a boiling-water bath for 2 h (3×). The aqueous extracts were separated by centrifugation (6,000 g, 20 min) and combined. The WSPS were precipitated by EtOH (95%, 1:4). The precipitate was centrifuged (6,000 g, 20 min), washed with EtOH (95%), and dried. The WSPS fraction was demineralized over cation-exchanger KU-2-8 (H^+-form, 3 × 40 cm, H_2O eluent) and deproteinized by protease [21] to afford fraction S_fP (9.72 g, 3.24% of raw material mass).

S_fP, $[\alpha]_D$ +155.7° (*c* 0.5, 1% NaOH). Glc, 92.7 mol%; Gal:Ara:Rha, 9:6:1; Man, Xyl, traces; uronic acids, 4 mol%. IR spectrum (ν, cm^{-1}): 610, 660, 743, 765, 834, 851, 883, 916, 956, 1019, 1049, 1075, 1101, 1143, 1223, 1333, 1696, 1732, 2930, 3398.

Total Hydrolysis. The compound (20 mg) was dissolved in TFA (5 mL, 2 M) and heated at 120°C for 2 h. The hydrolysate was concentrated in vacuo in the presence of MeOH and analyzed by HPTLC (system 1) and GC/MS (as methyl esters).

Fractionation of S_fP over DEAE-cellulose. A weighed portion of S_fP (9 g) was dissolved in H_2O (400 mL), placed on a column of DEAE-cellulose (OH^--form, 3 × 60 cm), and eluted successively by H_2O, NaCl solutions (0.1–1.0 M), and NaOH (0.1 M). The effluents were neutralized by HCl (2 M), dialyzed, and precipitated by acetone (1:4). Six fractions were obtained: S_fP-1 (H_2O, 7.92 g), S_fP-2 (0.1 M NaCl, 0.062 g), S_fP-3 (0.3 M NaCl, 0.046 g), S_fP-4 (0.5 M NaCl, 0.27 g), S_fP-5 (1.0 M NaCl, 0.038 g), and S_fP-6 (0.1 M NaOH, 0.48 g).

Fractionation of S_fP-1 by Stepwise Precipitation by EtOH. A weighed portion of S_fP-1 (7 g) was dissolved in H_2O (300 mL) and treated in portions with EtOH (95%). The resulting precipitates were centrifuged and dried to produce four fractions: S_fP-1-1 (36% EtOH, 4.68 g), S_fP-1-2 (48%, 0.83 g), S_fP-1-3 (67%, 0.64 g), S_fP-1-4 (88%, 0.31 g).

Isolation of S_fP-1-1/2. A weighed portion of S_fP-1-1 (3 g) was dissolved in NaCl solution (0.3%, 100 mL), placed on a column of Sephadex G-100 (3 × 60 cm), and eluted by NaCl solution (0.3%). The course of the elution was monitored using phenol–H_2SO_4. Fractions containing the dominant component were combined, dialyzed, and precipitated by acetone (1:3) to afford S_fP-1-1/2 (1.83 g).

S_fP-1-1/2. $[\alpha]_D$ +157.4° (*c* 0.5, 1% NaOH). MW 85 kDa. UV spectrum of the complex with I_2 (λ_{max}, nm): 612. B_V (λ): 0.364 (610 nm), 0.281 (680 nm). IR spectrum (ν, cm^{-1}): 640, 765, 834, 850, 894, 916, 964, 1021, 1050, 1102, 1146, 1279, 1330, 1370, 1423, 2945, 3391.

Oxidation by CrO_3 of preliminarily acetylated S_fP-1-1/2 was performed as before [22]. The oxidation product was hydrolyzed and analyzed by GC/MS. Periodate oxidation and Smith degradation were carried out by the literature methods [23].

Methylation of S_fP-1-1/2 was carried out using methyliodide by the literature method [24]; formolysis and hydrolysis of the permethylate, as before [25]. The solid methylated carbohydrates were treated with $NaBH_4$, acetylated by Ac_2O, and analyzed by GC/MS [26].

Partial hydrolysis of S_fP-1-1/2. A weighed portion of S_fP-1-1/2 (380 mg) was dissolved in H_2O (50 mL), treated with H_2SO_4 (10 mL, 5%), and heated at 100°C for 8 h. The hydrolysate was neutralized by anion-exchanger ARA-5pT40 (CO_3^{2-}-form), concentrated, and analyzed by HPTLC (system 2). Hydrolysis products were separated by preparative PC (system 3) to afford three compounds, SO-1, SO-2, and SO-3, that were identified as glucopyranose [R_f 0.48, mp 156°C, $[\alpha]_D$ +54.2° (*c* 0.5, H_2O)], *O*-α-glucopyranosyl-(1→4)-glucopyranose [maltose; R_f 0.38, mp 131°C, $[\alpha]_D$ +138.4° (*c* 1.0, H_2O); 2,3,4,6-tetra-*O*-Me-Glc*p*:2,3,6-tri-*O*-Me-Glc*p*, 1:1], and *O*-α-glucopyranosyl-(1→4)-*O*-α-glucopyranosyl-(1→4)-glucopyranose [maltotriose; R_f 0.31, mp 132°C, $[\alpha]_D$ +161.4° (*c* 0.3, H_2O); 2,3,4,6-tetra-*O*-Me-Glc*p*:2,3,6-tri-*O*-Me-Glc*p*, 1:2], respectively. A minor component SO-4 was identified using chromatographic mobility as *O*-α-glucopyranosyl-(1→4)-*O*-α-glucopyranosyl-(1→4)-*O*-α-glucopyranosyl-(1→4)-glucopyranose (maltotetraose; R_f 0.23).

Enzymatic Hydrolysis of S_fP-1-1/2. A weighed portion of S_fP-1-1/2 (30 mg) was dissolved in phosphate buffer (20 mL, 20 mM Na_2HPO_4 containing 6.7 mM NaCl, pH 6.9), treated with α-amylase solution (20 mL) in double distilled water, heated in a thermostat at 37°C for 24 h, heated at 100°C for 15 min, and centrifuged at 6,000 g. The supernatant was concentrated to dryness. The dry solid was dissolved in H_2O (200 μL) and analyzed by HPTLC (system 2).

Immunostimulating Activity of S_fP-1-1/2. Experiments were performed on male CBA mice (18-20 g). The activity of the test compound was studied in untreated animals and animals under immunosuppression induced by azathioprine administered perorally at a dose of 50 mg/kg once per day for five days. An aqueous solution of S_fP-1-1/2 was administered perorally to the azathioprine test group at a dose of 50 mg/kg once per day for 14 days. The control group received water according to an analogous schedule. The condition of humoral immunity was estimated from the number of AFC using the Cunningham method [27]. Mice were immunized i.p. by sheep erythrocytes (SE) at a dose of 2×10^8 cells/mouse. The immune response was estimated from the number of AFC in spleen and per 10^6 cells with nuclei on the fifth day after immunization. The condition of the cellular immune response was estimated from the delayed-type hypersensitivity (DTH) reaction using the standard local DTH method. Mice were sensitized by i.p. administration of a suspension of SE (0.1%) in physiological saline. The decisive antigen dose (50 μL, 50% SE suspension) was administered on the fourth day of hind-paw plantar fasciitis. Physiological saline of the same volume was injected into the contralateral paw. The DTH reaction was estimated after 24 h from the mass difference of the treated and control paws.

ACKNOWLEDGMENT

We thank Cand. D. V. Sandanov of the IGEB, SB, RAS (Ulan-Ude) for supplying *S. flavescens* raw material. The work was supported financially by a Lavrent′ev Award for Young Scientists, SB, RAS.

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