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# Structural characterization of the water-extractable polysaccharides from *Sophora subprostrata* roots

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**Abstract**

The crude polysaccharide SSa, obtained from the roots of *Sophora subprostrata* by boiling-water extraction and ethanol precipitation, was fractionated by anion-exchange and gel permeation chromatography, giving four polysaccharide fractions termed SSa1, SSa2, SSa3, and SSa4. Their structural features were investigated by sugar analysis, methylation analysis, partial hydrolysis, and NMR spectroscopy. SSa1 and SSa2 were both characterized as highly branched amylopectins with small average unit chain length of 8–10 except that SSa2 was contaminated with small amount of arabinogalactan. SSa3 was mainly an arabinogalactan, attached to a rhamnogalacturonan core. SSa4 was a rhamnogalacturonan I (RG-I) pectic polysaccharide having a backbone consisting of 1,4-linked α-D-galacturonic acid and 1,2-linked α-L-rhamnose, with various branches attached to O-4 of L-rhamnose.


*Keywords:* *Sophora subprostrata*; Leguminosae; Roots; Polysaccharide

## 1. Introduction

*Sophora subprostrata* Chun et T Chen is a traditional Chinese medicinal plant (Leguminosae), distributed chiefly in South China. Its roots are used in traditional Chinese medicine as an antipyretic, antidote, or analgesic (Chen & Jiang, 1994). It has been known to contain flavonoids, alkaloids, saponins, phenols, and lignins, etc. The antitumor activity of *S. subprostrata* crude preparation has been reported, and theorized to be the result of cytotoxicity of its alkaloids (Sakamoto et al., 1992; Zheng, Dong, & She, 1997). In order to understand the role of polysaccharide fractions in its pharmacological activities, we isolated several polysaccharides from its water and alkaline extracts respectively. A xyloglucan and a heteroxylan from the alkaline extract have been reported previously (Dong, Ding, & Fang, 1998; Dong, Ding, Yang, & Fang, 1999). In this paper we report on the structural features and properties of four polysaccharide fractions isolated from water extract.

## 2. Experimental

### 2.1. Materials

The roots of *S. subprostrata* were purchased commercially as crude drug, and the original plant was collected from *Zhejiang* Province of China. The crude drug was identified by comparison with the authenticated species. The α-amylase (E.C.3.2.1.1) and amyloglucosidase (E.C. 3.2.1.3) were Sigma products.

### 2.2. General methods

All evaporations were carried out at below 40 °C under reduced pressure. Optical rotation was recorded with a WZZ-1S polarimeter (Shanghai Physical Optics Instrument Co.). Thin layer chromatography (TLC) was performed on precoated Pei-cellulose plate (10 cm × 5 cm, Merck) and developed with EtOAc: Pyridine: HOAc: $H_2O$ (5:5:1:3, v/v), and visualized by spraying with *o*-phthalic acid reagent (1.6 g of *o*-phthalic acid and 0.9 ml of aniline were dissolved in 100 ml of water-saturated *n*-butanol.) and heating at 105 °C for 5 min. Gas liquid chromatography (GLC) was performed on a DP-1701 capillary column (30 m × 0.225 mm) with a flame ionization detector (FID).

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The column temperature was 210 °C for composition analysis. GLC–MS was performed on a Varian VISTA 64 instrument equipped with SCOT DB-1701 column (25 m × 0.256 mm i.d.), and detected by FID (detector temperature: 250 °C). The column temperature was kept at 200 °C for 13 min and then increased to 250 °C in a rate of 8 °C/min. The homogeneity and molecular weight of polysaccharides were evaluated and determined by high performance gel permeation chromatography (HPGPC) method on a Bio-Rad HPLC pump equipped with a Shodex sugar KS-805 column (Showa Denkko), and detected refractometrically (Showa Denkko, SE-51). The column was calibrated with T-series dextrans of known molecular weights (Mrs) (Dextran T-2000, T-500, T-110, T-70, T-40, T0-20). Neutral carbohydrate was determined with sulfuric acid–phenol method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), with D-glucose as standard; uronic acid determined by *m*-hydroxyl diphenyl method (Blumencrantz & Asboe-Hansen, 1973), with D-glucuronic acid as standard; protein determined by Lowry method (Bensadoun & Weinstein, 1976), with bovine serum albumin as standard.

### 2.3. Isolation and purification of polysaccharides

The *S. subprostrata* crude drug (5 kg) was refluxed with ethanol for 16 h to remove lipids. After filtration, the residue was dried by air at room temperature, and then extracted with boiling water (20 l) thrice (8 h for each). The extracts were combined, concentrated and dialyzed against running water. The nondialysate was concentrated to 8 l, centrifuged, and the supernatant was precipitated with three volumes of ethanol. The precipitate was collected by centrifugation, washed successively with ethanol and acetone, then dried at 50 °C at reduced pressure, giving SSa (126 g) as a grayish powder.

A portion of this preparation (30 g) was dissolved in water (300 ml), the insoluble residue removed by centrifugation. The supernatant was applied in five runs to a DEAE-Sephadex A-25 column ($Cl^-$, 42 cm × 4.5 cm), eluted first with water and then with 0–2 M gradient NaCl. The eluate was monitored polarimetrically. SSa-A-1 (7.0 g) was obtained from the water eluate, and SSa-B-1, SSa-B-2, SSa-B-3 were obtained successively from NaCl eluate.

SSa-A-1 was purified by gel permeation chromatography on a column of Sephadex G-200 (2.6 cm × 90 cm). SSa-B-1 was purified by repeating anion-exchange chromatography on a column of DEAE-Sephadex A-25 and then on a column of Sephadex G-200 (2.6 cm × 90 cm). SSa-B-2 was purified on a column of Sephacryl S-300 (2.6 cm × 90 cm). SSa-B-3 was refractionated on a column of DEAE-Sephadex A-25, and then purified on a column of Sephadex G-200 (2.6 cm × 90 cm), to which a column of Sephacryl S-300 (2.6 cm × 90 cm) was tandemly linked. All gel permeation chromatography was performed with 0.2 M NaCl as the eluent, monitored with phenol–$H_2SO_4$ method, and refractionation on the DEAE-Sephadex A-25 column (2.6 cm × 60 cm) was performed as described above.

### 2.4. Sugar analyses

The polysaccharide sample (3 mg) was hydrolyzed in 2 ml of 2 M trifluoroacetic acid (TFA) at 110 °C for 1.5 h. TFA was removed by evaporation at reduced pressure with the addition of methanol. A small portion of the residue was subjected to TLC analysis, and the remaining portion was transformed into the corresponding alditol acetates, and analyzed by GLC (Dong et al., 1998). If a polysaccharide contains uronic acid according to TLC, another part of it was carboxyl-reduced with $NaBH_4$ (Taylor & Conrad, 1972), and then hydrolyzed and analyzed by GLC as described above. The sugar composition was calculated by comparing the results based on native and carboxyl-reduced polysaccharides.

### 2.5. Methylation analysis

The vacuum dried polysaccharide (5 mg) was methylated with the method of powdered sodium hydroxide in dimethyl sulfoxide (Needs & Sevendran, 1993). Methyl iodide (0.3 ml) was added dropwise as the methylating agent. The methylated polysaccharide was recovered by dialysis against distilled water and freeze-drying. The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in IR (Nujol). The permethylated polysaccharide was hydrolyzed by treatment with 90% formic acid (100 °C, 3 h), and then 2 M TFA (100 °C, 4 h). The partially methylated sugars were reduced with $NaBH_4$ (25 mg) at room temperature for 3 h, and acetylated with acetyl anhydride (2 ml) at 100 °C for 1 h. The partially methylated alditol acetates were analyzed by GLC–MS.

### 2.6. $^{13}C$ and $^{1}H$ NMR spectra

45 mg of the polysaccharide sample was dissolved in $D_2O$ (0.5 ml), freeze-dried, and redissolved in $D_2O$ (0.5 ml). The $^{13}C$ NMR and $^{1}H$ NMR spectra were measured with 5 mm tubes at room temperature with Brucker AM-400 NMR spectrometer. All the chemical shifts were in relative to $Me_4Si$.

### 2.7. Enzymic hydrolysis of SSa2

Five milligrams of SSa2 dissolved in distilled water (1 ml) was added with 50 μl of amylase preparation, containing 5 mg/ml of α-amylase and 5 mg/ml of amyloglucosidase. This mixture was kept at 50 °C for 8 h, then heated at 100 °C for 15 min, and centrifuged. The supernatant was applied to a column of Sephadex G-10 (1.6 cm × 90 cm), eluted with $H_2O$, and detected with $H_2SO_4$–phenol method. Two fractions were obtained, the high-MW fraction termed SSa2-EP1 (3 mg), and the low-MW fraction termed SSa2-EP2 (1 mg). SSa2-EP2 was analyzed by TLC. SSa2-EP1 was dialyzed against distilled water and subject to composition analysis. The nondialyzate

was hydrolyzed with 2 M TFA (110 °C, 1.5 h), and then analyzed by TLC, and GLC as the alditol acetates.

### 2.8. Mild hydrolysis of SSa4

SSa4 (40 mg) was dissolved in 0.01 M TFA (10 ml), and heated at 100 °C for 1 h. After TFA was removed by evaporation, the residue was dissolved in water and dialyzed against distilled water (500 ml × 3). The dialyzable fraction was concentrated, applied to a column of Sephadex G-10 (90 cm × 1.6 cm) with $H_2O$ as the eluent, detected refractometrically. Each pooled fraction was concentrated and its sugar composition was analyzed by TLC and GLC as the alditol acetates after hydrolysis. The nondialysate was also subjected to sugar analysis.

## 3. Results and discussion

### 3.1. Isolation and purification of polysaccharides

The crude polysaccharide SSa obtained from the hot-water extract of *S. subprostrata* roots contained 75.0% of neutral carbohydrate and 12.0% of protein. After fractionation on a DEAE-Sephadex A-25 anion-exchange column, SSa-A-1 (7.0 g) was obtained from the water eluate, and SSa-B-1 (0.46 g), SSa-B-2 (0.65 g), SSa-B-3 (1.24 g) were obtained successively from NaCl eluate. These fractions were refractionated on a DEAE-Sephadex A-25 column or purified by gel permeation chromatography on columns of Sephadex G-200 or Sephacryl S-300, giving SSa1, SSa2, SSa3, and SSa4, in corresponding to the previous SSa-A-1, SSa-B-1, SSa-B-2, and SSa-B-3, respectively. SSa1 through to SSa4 each showed only one symmetrical peak on HPGPC.

Mrs, specific rotations, and sugar compositions of these polysaccharides were determined and given in Table 1.

Table 1
Properties and sugar compositions of polysaccharides of *Sophora subprostrata*

| Fractions | SSa1 | SSa2 | SSa3 | SSa4 |
|---|---|---|---|---|
| $[\alpha]_D$ | +180° | +37.5° | −56.7° | −36.4° |
| Mr | $9.1 \times 10^3$ | $1.3 \times 10^5$ | $6.6 \times 10^5$ | $4.2 \times 10^5$ |
| Carbohydrate (wt%) | 100 | 100 | 93.8 | 49.3 |
| Protein (wt%) | 0 | 0 | 0 | 11.0 |
| Uronic acid (wt%)[a] | 0 | 2.0 | 6.2 | 35.6 |
| Sugar composition (mol%) | | | | |
| Rha | | | 11.0 | 16.7 |
| Ara | | 7.4 | 60.0 | 21.3 |
| Xyl | | | | 6.5 |
| Man | | | | 2.9 |
| Gal | | 4.8 | 23.9 | 18.5 |
| Glc | 100 | 87.8 | | 4.1 |
| GalA[b] | 0 | | 6.0 | 29.8 |

[a] Determined by *m*-hydroxyl diphenyl method.
[b] Calculated according to the GLC results based on native and carboxyl-reduced polysaccharides.

### 3.2. Structural characterization of SSa1

SSa1 contains only D-glucose and it is negative to $I_2$–KI reagent. The high proportion of 2,3,6-tri-*O*-methyl-glucose in methylation analysis indicated a 1,4-linked glucosyl backbone (Table 2), and the presence of 2,3,4,6-tetra-*O*-methyl-glucose and 2,3-di-*O*-methyl-glucose suggested that SSa1 had side chains at some O-6. The average unit chain length (CL) was 10, judging by the methylation analysis. The IR absorption at 845 $cm^{-1}$ indicated an α anomeric configuration to the glucosyl residues, in agreement with its large positive specific rotation. All these results suggested that SSa1 was a highly branched amylopectin which was negative to iodine probably due to its short average unit CL. Most amylopectins were reported to have CLs of 18–28 (Aspinal, 1985).

### 3.3. Structural characterization of SSa2

Composition analysis showed that SSa2 contains glucose, galactose, arabinose, and trace of uronic acid, with the molar ratio as shown in Table 1. SSa2 gave a weak pink color to $I_2$–KI reagent at the concentration of more than 1 mg/ml. Methylation analysis revealed that it consisted mainly of terminal, 1,4- and 1,4,6-linked glucose, terminal arabinose, 1,3-, and 1,3,6-linked galactose. Since terminal, 1,4- and 1,4,6-linked glucosyl residues are usual constituents of amylopectin, and the other units are common in type II arabinogalactan (arabino-3,6-galactan), SSa2 was probably a highly branched amylopectin contaminated with arabinogalactan. The average unit CL was 9, according to the result of methylation analysis. Although SSa2 has a small average CL, it showed weak color reaction to iodine, while SSa1 did not. This is probably because the high-Mr SSa2 has a chain profile that involves a small proportion of long side chains. In the anomeric region of its $^1H$ NMR spectrum (Fig. 1), three closely neighbored strong signals (δ5.41, 5.38, and 5.36 ppm) could be assigned to H-1 of α-glucose. The other signals at δ5.24, 4.97 ppm were attributed to terminal and 1,5-linked α-L-arabinose. The weak signal at δ4.57 ppm arose from α-D-galactose.

After treating SSa2 with amylase and amyloglucosidase, the dialyzable fraction, designated SSb2-EP2, was analyzed by TLC. Large amount of D-glucose was identified, indicating a 1,4-linked α-D-glucan backbone. The non-dialyzable fraction EP1 was composed mainly of arabinose and galactose as shown by GLC, indicating that the arabinogalactan was not covalently attached to glucose as side chains. It was thus concluded that SSa2 was a mixture of amylopectin and arabino-3, 6-galactan, which were co-eluted on Sephadex G-200 column and on HPGPC, probably due to their similar molecular size or complex formation via intermolecular force.

Table 2
GLC–MS results of methylation analyses of polysaccharides SSa1 through to SSa4

| Methyl positions | Linkages | Molar ratios | | | | | |
|---|---|---|---|---|---|---|---|
| | | SSa1 | SSa2 | SSa3 | SSa3R[a] | SSa4 | SSa4R[a] |
| 2,3,5-$Me_3$–Ara | T- | | 3.8 | 31.7 | 26.7 | 15.9 | 14.6 |
| 2,3-$Me_2$–Ara | 1,5- | | 2.2 | 22.9 | 22.6 | 9.3 | 9.1 |
| 2-Me–Ara | 1,3,5- | | | | | 8.3 | 6.9 |
| 3-Me–Ara | 1,2,5- | | | 10.5 | 12.3 | | |
| 3,4-$Me_2$–Rha | 1,2- | | | 2.9 | 5.0 | 7.0 | 10.7 |
| 3-Me–Rha | 1,2,4- | | | 5.3 | 5.0 | | 9.9 |
| 2,3,4,6-$Me_4$–Gal | T- | | | | | 7.0 | 8.7 |
| 2,4,6-$Me_3$–Gal | 1,3- | | | 5.1 | 4.8 | | 1.5 |
| 2,3,6-$Me_3$–Gal | 1,4- | | | | 7.9 | 6.5 | 25.0 |
| 2,3,4-$Me_3$–Gal | 1,6- | | | 5.4 | 4.2 | 7.7 | 6.2 |
| 2,4-$Me_2$–Gal | 1,3,6- | | 2.6 | 16.2 | 11.8 | 4.3 | 7.4 |
| 2,3,4,6-$Me_4$–Glc | T- | 10.2 | 10.3 | | | | |
| 2,3,6-$Me_3$–Glc | 1,4- | 76.4 | 69.9 | | | | |
| 2,3,4-$Me_3$–Glc | 1,6- | 1.5 | | | | | |
| 2,3-$Me_2$–Glc | 1,4,6- | 9.3 | 11.2 | | | | |

[a] Carboxyl-reduced polysaccharides.

### 3.4. Structural characterization of SSa3

Compositional analysis revealed that SSa3 was composed mainly of L-arabinose, D-galactose, and small amount of L-rhamnose and D-galacturonic acid. Methylation analysis on the carboxyl-reduced SSa3 showed that arabinosyl residues were positioned at nonreducing terminal, 1,2,5- and 1,5-linked, and that galactosyl residues were

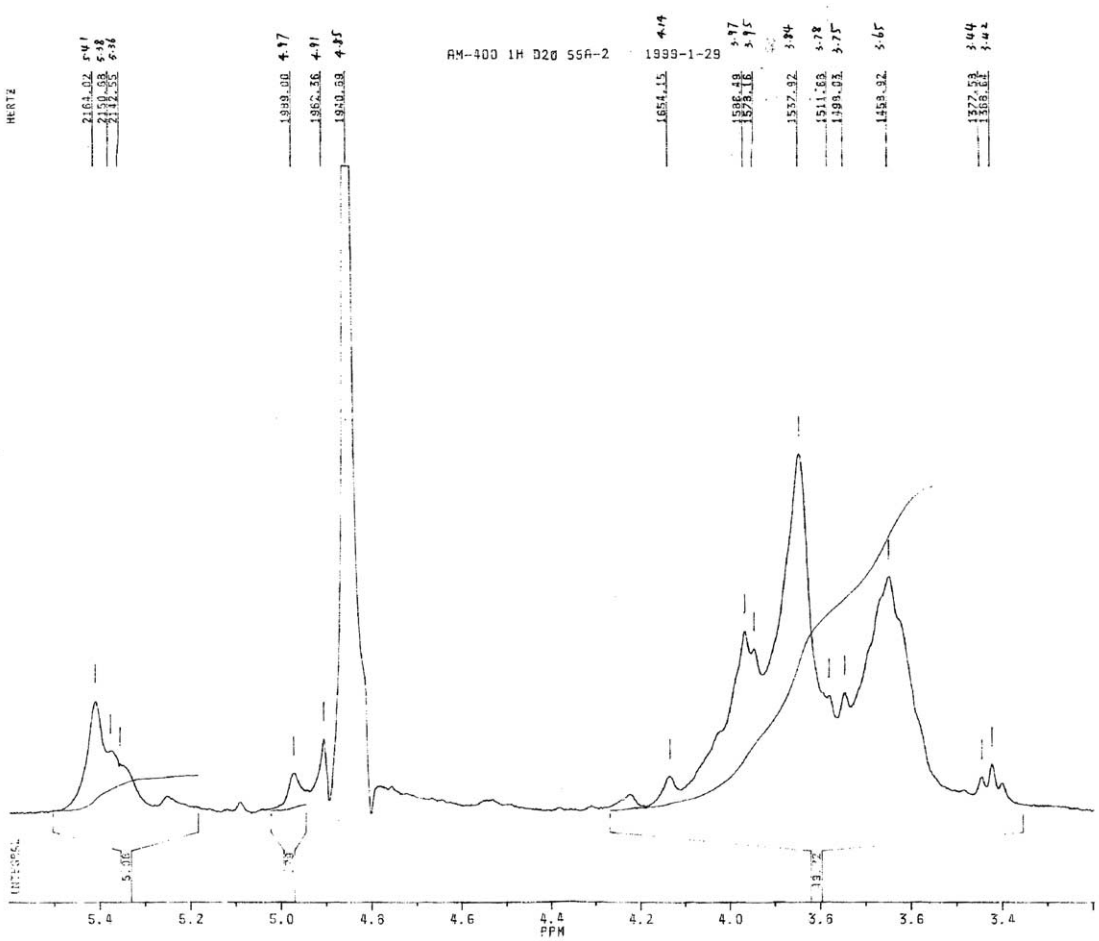


Fig. 1. $^1$H NMR spectrum of SSa2.

mainly 1,3-, 1,6-, and 1,3,6-linked. Rhamnosyl residues were 1,2- and 1,2,4-linked, as was commonly reported in rhamnogalacturonan I (RG-I) (Thomas, Darvill, & Albersheim, 1989). According to methylation analysis, the native SSa3 did not contain 1,4-linked galactosyl residues, so the 1,4-linked galactose in SSa3R should result from carboxyl-reduced galacturonosyl residues, which were usually 1,4-linked in RG-I. In the $^{13}C$ NMR spectrum (Fig. 2), the four signals at $\delta$111.26, 109.54, 109.00, and 108.56 ppm should be assigned to α-L-arabinofuranose according to their strength and literature value (Saulnier, Brillouet, Moutounet, du Penhot, & Michon, 1992). Other signals at $\delta$105.28, 104.76, 100.50 ppm could be assigned to 4-linked α-D-galactose, 6-linked α-D-galactose, and α-L-rhamnose, respectively. All these data indicate that SSa3 probably contains a rhamnogalacturonan core, to which different side chains, consisting of arabinose and galactose, are attached (Kiyohara et al., 1994).

### 3.5. Structural characterization of SSa4

As the fraction eluted at high NaCl concentration in anion-exchange chromatography, SSa4 was subject to further purification with ion-exchange chromatography and gel permeation chromatography. The SSa4 thus obtained contained neutral carbohydrate (49.3%), uronic acid (35.6%), and protein (11.0%). Sugar analysis showed that it was a typical RG-I pectic fraction (Table 1). Methylation analysis on native SSa4 (Table 2) identified 1,6-, 1,4-, and 1,3,6-linked galactosyl as the major components, and arabinose positioned at nonreducing terminal or 1,5-linked. The proportion of rhamnose in methylation analysis of SSa4 was much lower than that in sugar analysis, probably resulting from α-elimination and oxidation under the alkaline condition of repetitive methylation (Yang & Montgomery, 2001). The result on carboxyl-reduced SSa4 (SSa4R) was in approximate accordance with that of sugar analysis in rhamnose proportion (Table 2). In its $^{13}C$ NMR spectrum (Fig. 3), two strong signals at $\delta$100.58, and 99.50 ppm could be assigned to α-L-rhamnose and α-D-galacturonic acid respectively; the two weak signals at $\delta$109.50 and 105.70 ppm were attributable to α-L-arabinofuranose and α-D-galactopyranose, respectively. The substitution at O-4 of galacturonic acid was ascertained by the strong signal at 79.41 ppm, assigned in reference to literature value (De Pinto et al., 1996). C2 signals for 1,2- and 1,2,4-linked rhamnose were at $\delta$78.61 and 78.13 ppm, respectively. Other characteristic signals were $\delta$176.59 ppm (C6 of galacturonic acid) and 18.68 ppm (C6 of rhamnose).

On mild acid hydrolysis, the major dialyzable fraction contained arabinose and galactose, in the ratio of 30:1,

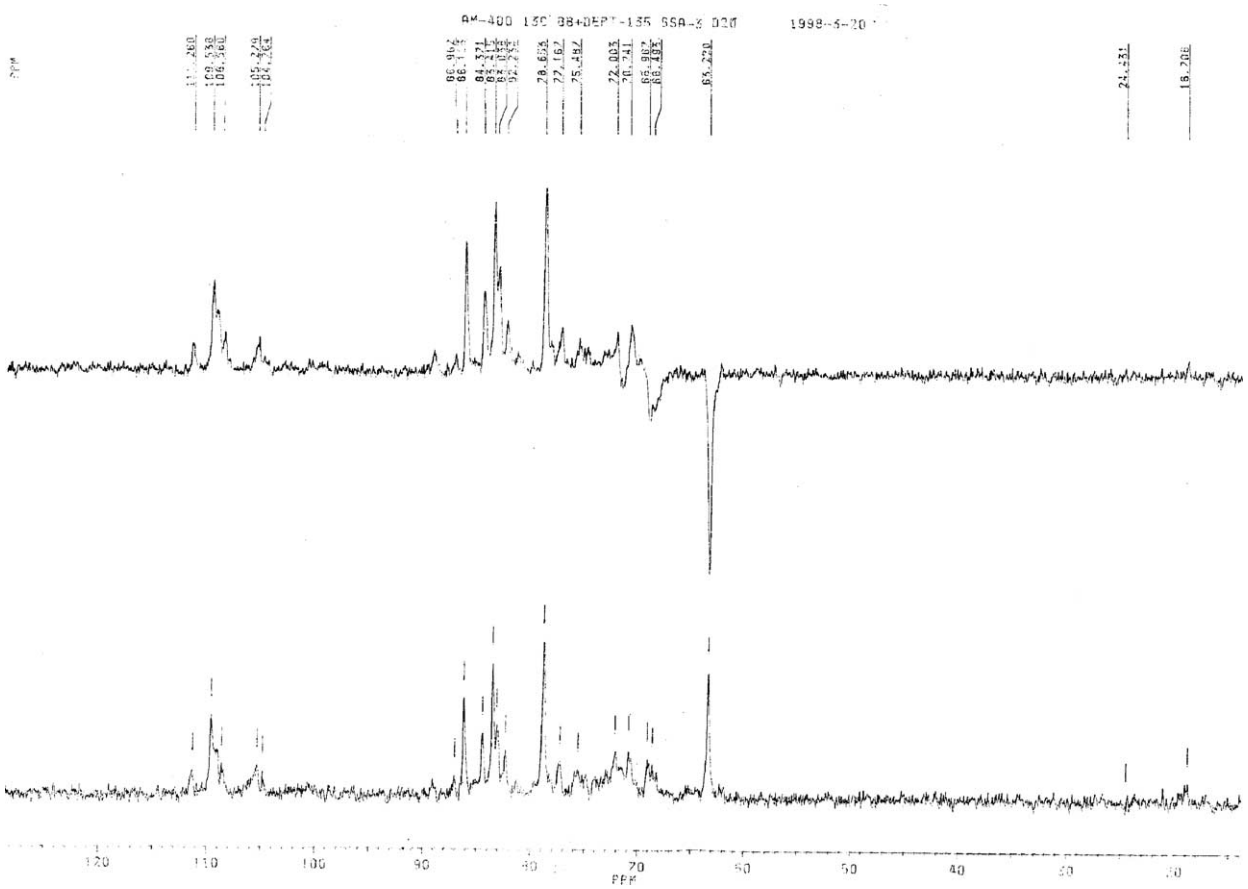


Fig. 2. $^{13}C$ NMR spectrum of native SSa3.

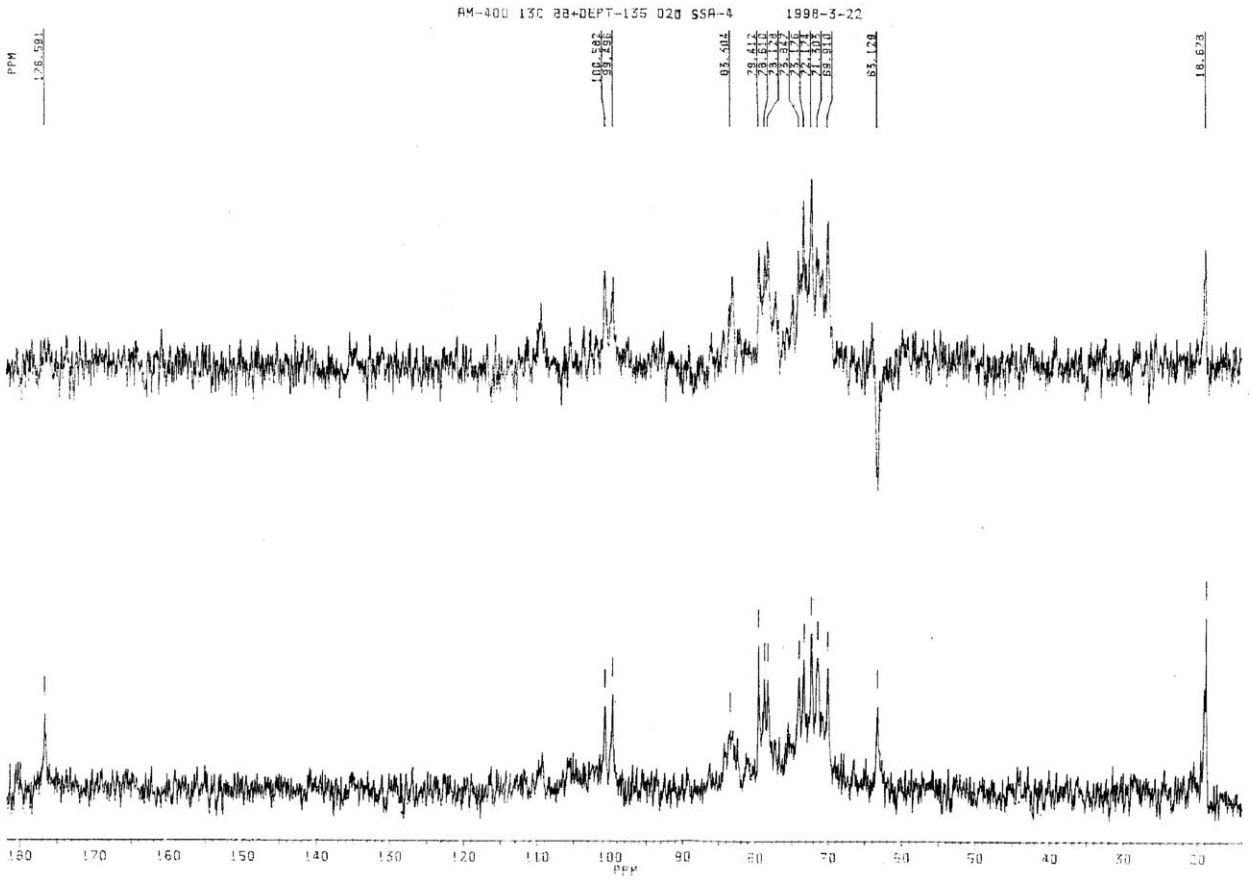


Fig. 3. $^{13}C$ NMR spectrum of native SSa4.

indicating that they were present at outer chains. The nondialyzable fraction was a modified polysaccharide composed mainly of galacturonosyl and rhamnosyl residues. It was thus concluded that SSa4 is a RG-I polysaccharide, having a backbone composed of 1,4-linked galacturonic acid and 1,2-linked rhamnose, with branches attached to O-4 of some rhamnose. The branches probably contain arabinogalactan, arabinan and galactan, and the fine structure remained for further investigation. The small amount of xylose, mannose, and glucose indicated the contamination of hemicellulosic polysaccharides.

## 4. Conclusions

According to the results above, it was concluded that the water extract of *S. subprostrata* contained several types of water extractable polysaccharides, predominantly water-soluble amylopectins (SSa1 and SSa2) and pectins (SSa3 and SSa4). Starch should be the major reserve polysaccharide of *S. subprostrata* roots, due to the high content of SSa1. Although both are pectic polysaccharides, SSa3 is mainly arabinogalactans attached to small amount of rhamnogalacturonan core, while SSa4 a typical rhamnogalacturonan I.

Many plant polysaccharides with immunological activities have been reported (Shimizu, Tomoda, Kanari, & Gonda, 1991; Wang, Zhao, Li, Li, & Li, 1996; Yamaoka, Kawakita, Kaneko, & Nomoto, 1995), most of which are arabinogalactans and rhamnogalacturonans. Preliminary immunopharmacological tests showed that SSa3, SSa4 in vitro were effective in increasing the weight of spleen, and the serum IgG level, and SSa1 and SSa2 were noneffective. Further studies are necessary to relate such activities with the pharmacological effect of *S. subprostrata* roots.

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