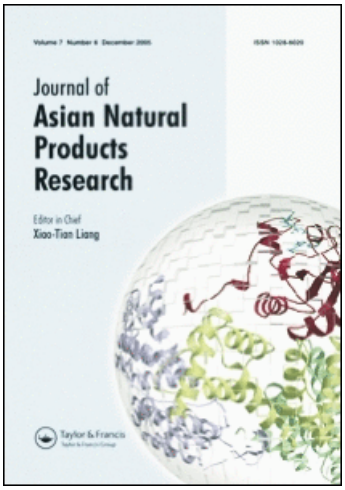

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Structural features of a pectic polysaccharide from mulberry leaves

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A pectic polysaccharide SDA was obtained from the boiling water extract of mulberry leaves. It consisted of rhamnose, arabinose, xylose, glucose, galactose, and galacturonic acid units in the molar ratio of 5:4:1:2:6:38. Its molecular weight was determined to be 1.5×10^4 by high-performance gel filtration chromatography. A combination of linkage analysis, partial acid hydrolysis, ESIMS, ^{1}H-NMR, and ^{13}C-NMR spectral analyses revealed its structural features. It was found that SDA possessed an α-(1 → 4)-galacturonan backbone with some insertions of (1 → 2) Rha residues, with the side chains attached to the O-3 or O-2 position of GalA residues, or the O-4 position of 1,2-linked Rha residues. The side chains contained branched (1 → 5) linked arabinan, branched (1 → 3) linked rhamman, linear (1 → 4) linked xylan, linear (1 → 4) linked glucan, and linear (1 → 2) linked galactan. SDA was a new acidic polysaccharide isolated from mulberry leaves for the first time.

Keywords: mulberry leaves; polysaccharide; structure; pectin

1. Introduction

Mulberry leaves have long been used in traditional Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine, and lower blood pressure. Up to now, a great of diversity of bioactive components with low molecular weights including steroids, alkaloids, and flavones from mulberry leaves have been elaborated [1,2]. Several reports described sugar composition and molecular weights of polysaccharides isolated from mulberry leaves [3,5]. However, there was no structural study on the polysaccharides from mulberry leaves. The aim of this work was to study in detail the chemical composition and structural features of a new pectic polysaccharide obtained from mulberry leaves.

2. Results and discussion

The hot water extract of the dried mulberry leaves was precipitated with four volumes of anhydrous ethanol to give the crude polysaccharide S. The aqueous solution of S was further decolored by resin AB-8, which was then concentrated and precipitated with four volumes of anhydrous ethanol to obtain SD. SD was successively subjected to DEAE-cellulose chromatography and Sephacryl S-200 gel filtration chromatography, which resulted in the isolation of a polysaccharide SDA. SDA showed a symmetrical narrow peak on HPGFC, as shown in Figure 1. Its molecular weight was determined to be 1.5×10[4] in reference to standard dextrans.

Quantitative determination of neutral sugars by GC-MS showed that SDA consisted of rhamnose (Rha), arabinose (Ara), xylose (Xyl), glucose (Glc), and galactose (Gal) in the molar ratio of 5:4:1:2:6. It was also shown to contain 70% uronic acid by the sulfuric acid–carbazole method. The presence of galacturonic acid (GalA) was established by the increase of Gal

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content in carboxyl-reduced SDA (SDA-R). A combination of sugar composition analysis of native SDA and SDA-R revealed that SDA contained Rha, Ara, Xyl, Glc, Gal, and GalA in the molar ratio of 5:4:1:2:6:38.

The linkage analysis of the carboxyl-reduced polysaccharide, SDA-R, was performed with the sodium hydroxide–iodomethane (NaOH-MeI) procedure. The results were given in Table 1, column b. As shown, the total content of Gal residues was increased comparing with the result of the component analysis. The 1,4-linked and 1,3,4-linked Gal residues could possibly originate from the GalA residues, since 1,4-linked GalA and 1,3,4-linked GalA were commonly found in pectic substances of the plant kingdom. The results of methylation analysis of native SDA confirmed that 1,4-linked, 1,2,4-linked, and 1,3,4-linked Gal residues were derived from GalA residues (Table 1, column a), although direct methylation analysis of acidic polysaccharide could only lead to qualitative information because of β-elimination reaction. Due to the dominant feature of SDA consisting mainly of a linear chain of 1,4-linked GalA units, it was a pectic polysaccharide. A small portion of rhamnose residues were most likely incorporated in the main chain of SDA due to the presence of 1,2-linked Rha residues, which were normally inserted in the galacturonan backbone giving rise to a rhamnogalacturonan backbone, a typical feature of pectic polysaccharide.[6] Besides the 1,2-, 1,2,4-linked Rha residues, rhamnose was also present as 1,3-linked and 1,3.4-linked residues, which were rare in pectic polysaccharides. Ara formed a $1 \rightarrow 5$ linked arabinan with branches at the O-3 position of Ara residues.

SDA was partially hydrolyzed under mild acidic condition (0.01 mol/l TFA, 100°C, 1 h) and then dialyzed, giving SDA-1 (nondialysate) and SDA-G (dialysate). SDA-G was repeatedly fractionated on columns of Sephadex G-25 and Sephadex G-10. Three carbohydrate fractions, H-1, H-2, and H-3, were obtained.

SDA-1, with a molecular weight 1.2×10^4, was eluted as a single symmetrical peak on HPGFC. It was composed of Rha, Ara, Xyl, Glc, Gal, and GalA in the molar ratio of 6:9:10:4:15:380, based on the composition analysis of SDA-1 and the carboxyl-reduced SDA-1 (SDA-1R). Compared with the sugar composition of SDA, the content of Rha, Ara, and Gal was remarkably decreased.

In comparison with that of SDA-R, the results of methylation analysis of SDA-1R (Table 1, column c) indicated that the content of 1,4-linked Gal and 1,2-linked Rha had increased and the molar ratios of 1,2,4-linked, 1,3,4-linked Gal residues and 1,2,4-linked Rha had decreased, followed by the release of the side chains including 1,5-linked, 1,3,5-linked Ara residues, terminal, 1,3-linked, 1,3,4-linked Rha residues, and 1,2-linked Gal residues. The results suggested that the side chains were attached to the backbone at O-3 or O-2 of 1,4-linked Gal residues, or O-4 of 1,2-linked Rha residues.

H-1 consisted of Rha, Ara, Xyl, Glc, Gal, and GalA, of which GalA was the major component. The results suggested that H-1 had a structure similar to SDA except for a difference in the molar ratio of neutral and acidic components, which possibly originated from the backbone.

H-2 showed a broad peak on HPGFC, indicating it was a mixture composed of several oligosaccharides. After complete hydrolysis, GC-MS analysis indicated the presence of Rha, Ara, and Xyl in the molar ratio of 8:5:1. The results suggested that H-2 was a mixture of neutral oligosaccharides present as side chains in native SDA released during the mild acidic hydrolysis.

H-3 consisted of Rha and Xyl. The ESIMS of H-3 showed three pseudomolecular ions at *m/z* 323, 337 and, 351, corresponding to $[\text{Xyl-Xyl} + H_2O + \text{Na}]^+$, $[\text{Xyl-Rha} + H_2O + \text{Na}]^+$, and $[\text{Rha-Rha} + H_2O + \text{Na}]^+$. According to the methylation results, Xyl-Xyl could be identified as Xyl-$(1 \rightarrow 4)$-Xyl. The xylan side chains, either attached to the O-3, O-2 positions of GalA residues, or attached to the O-4 position of 1,2-linked Rha residues, thus Xyl-Rha could be identified as Xyl-$(1 \rightarrow 4)$-Rha.

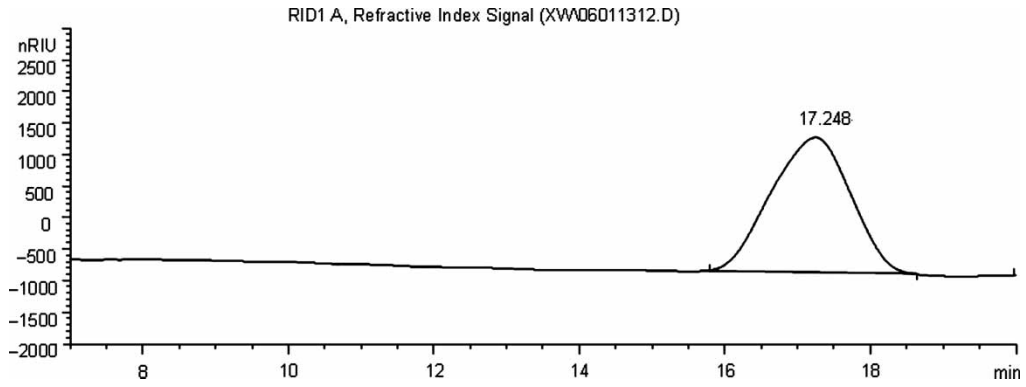

Figure 1. HPGFC chromatogram of SDA.

For further investigation on the structure of native SDA, SDA-1 was hydrolyzed with 0.25 mol/l TFA 100°C for 2 h and then dialyzed, giving SDA-2 (nondialysate). SDA-2, with a molecular mass of 9.8×10^3, was eluted as a single peak on HPGFC. It contained almost 100% GalA. The results of the linkage analysis of the carboxyl-reduced SDA-2 (Table 1, column d) suggested that the 1,4-linked Gal residues originating from GalA were almost the only detectable linkage, except trace 1,2-linked Rha, which further indicated that SDA possessed an α-(1 → 4)-galacturonan backbone.

H-4 consisted of GalA and rhamnose. The pseudomolecular ion *m/z* at 339 and 369 attributed to $[Rha\text{-}GalA\text{-}H]^-$, $[GalA\text{-}GalA\text{-}H]^-$ in ESIMS. Thus, SDA possessed the structure sequence GalA → GalA and Rha → GalA (or GalA → Rha). These results indicated that the main chain of SDA contained the alternating sequence of GalA and Rha units, besides the predominant sequential GalA → GalA.

The chemical structure of SDA was also studied by ^{1}H-NMR and ^{13}C-NMR spectroscopy. The ^{13}C-NMR spectrum for SDA was shown in Figure 2. Predominant signals at δ 99.0, 77.2 were assigned to C-1 and C-4 of 1,4-linked GalA residues, respectively. In the low field, the signals for the resonances of carbonyl groups (carboxyl and ester carbonyls) at δ 175.4 and 171.0 corresponded to the C-6 of unesterified (COOH) and methyl esterified ($COOCH_3$) of GalA units, respectively. The signal at δ 53.0 represented methyl carbons of the methyl ester ($COOCH_3$) of GalA units. In the high field, the signals at δ 17.4 and 20.3 corresponded to C-6 of Rha residues and methyl carbons of acetyl groups, respectively. However, no anomeric carbon signals of other glycosyl residues were detected in the ^{13}C-NMR spectrum, probably due to the presence of rhamnose, Ara, etc. in SDA as minor components together with the lower sensitivity of ^{13}C-NMR.

In the ^{1}H-NMR spectrum (Figure 3) of SDA, anomeric protons at δ 5.08, 5.02, 4.98 were all of α-GalA residues. Signals at δ 1.20 and 2.00 related to C-6 in rhammose residues and acetyl protons, respectively.

The signals from GalA unit, which was the principal sugar of SDA-2, were dominating in the ^{13}C-NMR spectrum (Figure 4). The carbon signal at δ 172.5 was attributed to C-6 of the carboxyl. The resonances at δ 99.9, 77.6, 71.5, 68.0, and 67.8 were assigned to C-1, C-4, C-5, C-3, and C-2 of the repeating unit of (1 → 4)-α-GalA → , respectively, in reference to previous work [7]. In addition, after two partial acid hydrolysis, signals from carboxylic acid methyl esters disappeared. The NMR and methylation results were in good agreement and demonstrated that SDA possessed an α-(1 → 4)-galacturonan backbone.

Table 1. Linkage analysis of SDA (a), SDA-R (b), SDA-1R (c) and SDA-2R (d).

Methylated sugars	Molar ratio				Linkages
	a	*b*	*c*	*d*	
2,3,4-Me_3-Rha	0.9	0.8	n.d.	n.d.	Terminal
3,4-Me_2-Rha	n.d.	0.9	0.3	Trace	1,2-
2,4-Me_2-Rha	1.3	1.5	n.d.	n.d.	1,3-
2-Me-Rha	0.7	0.9	n.d.	n.d.	1,3,4-
3-Me-Rha	n.d.	0.4	trace	n.d.	1,2,4-
2,3,5-Me_3-Ara	1.8	1.3	0.4	n.d.	Terminal
2,3-Me-Ara	0.6	0.4	n.d.	n.d.	1,5-
2-Me-Ara	1.5	1.4	n.d.	n.d.	1,3,5-
2,3-Me_2-Xyl	1.0	1.0	0.5	n.d.	1,4-
2,3,4,6-Me_4-Glc	0.3	0.2	0.1	n.d.	Terminal
2,3,6-Me_3-Glc	0.9	1.1	0.2	n.d.	1,4-
2,3,4,6-Me_4-Gal	2.1	3.5	0.7	n.d.	Terminal
2,3,6-Me_3-Gal	n.d.	19.6	21.1	1.0	1,4-
3,4,6-Me_3-Gal	Trace	2.1	n.d.	n.d.	1,2-
2,6-Me_2-Gal	n.d.	8.9	3.5	n.d.	1,3,4-
3,6-Me_2-Gal	n.d.	4.1	0.9	n.d.	1,2,4-

n.d.: not detected.

The pectic polysaccharides reported so far commonly consisted of smooth regions with blocks of homogalacturonan and hairy regions with blocks of rhamnogalacturonan carrying side chains [8]. From the above results, it could be concluded that SDA had different structural features that the neutral side chains were attached to the acidic backbone by the O-3, O-2 of GalA residues or O-4 of Rha residues. These structural features of SDA resembled those of the pectic polysaccharide DL-2A isolated from the leaves of *Diospyros kaki* [7]. In contrast, the unusual structural features were that SDA contained a small but significant proportion of 1,3-, 1,3,4-linked Rha residues as the side chains since 1,2-, 1,2,4-linked Rha residues were commonly found as part of the backbone in pectic substances from the plant kingdom. This new finding expanded the knowledge on the fine structures of pectic substances in plant kingdom.

3. Experimental

3.1 General experimental procedures

Thin layer chromatography (TLC) was performed on precoated cellulose plate (10 cm × 5 cm) 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. IR spectrum was recorded on a Nicolet Magna IR550 spectrometer. GC-MS was performed on an Agilent 6890/5975 instrument with a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm). The homogeneity and molecular weight of polysaccharide were evaluated and determined by high-performance gel filtration chromatography (HPGFC) on an Agilent 1100 HPLC instrument equipped with TSK G4000 PW column, and detected refractometrically. The column was calibrated with standard dextrans T-1 ($M_w = 1270$), T-2 ($M_w = 5220$), T-3 ($M_w = 11{,}600$), and T-4 ($M_w = 48{,}600$) and eluted with 0.1 mol/l $NaNO_3$ (0.5 ml/min) at 35°C. Neutral carbohydrate was determined with sulfuric acid–phenol method, with D-glucose as standard; uronic acid determined by sulfuric acid–carbazole method, with D-glucuronic acid as standard. The ^{1}H-NMR and ^{13}C-NMR spectra were measured at room temperature on Bruker AM 400 spectrometer;

60 mg of the polysaccharide sample was dissolved in D_2O (0.5 ml). All chemical shifts were reported relative to Me_4Si. Reduction of carboxyl groups was carried out with CMC-$NaBH_4$ for three times according to the method of Taylor and Conrad [9]. ESIMS was recorded with Agilent 1100 LC/MSD SL system.

3.2 Plant material

Dried mulberry leaves were collected in Chongming District of Shanghai in China. DEAE-cellulose, Sephacryl S-200, Sephadex G-25, and Sephadex G-10 were purchased from Amersham Bioscience (Uppsala, Sweden). Standard dextrans were from Fluka Co. (Steinheim, Germany). 1-Cyclo-hexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMC) and trifluoroacetic acid (TFA) were Sigma-Aldrich products (Steinheim, Germany). All the other reagents were of analytical grade as available (Shanghai, China).

3.3 Separation and purification of native SDA

The dried mulberry leaves (1 kg), previously refluxed with anhydrous ethanol to remove fats and waxes, were extracted three times with the boiling water (25 l for 1 h per time). The extract was concentrated, precipitated with four volumes of anhydrous ethanol, and washed by acetone and vacuum-dried at 40°C to yield the crude polysaccharide S (100 g). The 4% aqueous solution of S (2.5 l) was further decolored by Resin AB-8 column (Φ5.0 × 40 cm), and concentrated and precipitated with four volumes of anhydrous ethanol to obtain SD (66 g). A portion of SD (5.0 g) was fractionated on DEAE-cellulose column (Cl^- type, Φ5.0 × 50 cm), eluted first with water, and then with 0.1, 0.2, 0.3 mol/L NaCl successively. SD_{H_2O} (85 mg) was obtained from the water eluate, $SD_{0.1}$ (370 mg), $SD_{0.2}$ (950 mg), $SD_{0.3}$ (52 mg), were obtained from NaCl eluate, respectively. The major fraction $SD_{0.2}$ was further purified on Sephacryl S-200 column (Φ1.6 × 80 cm) to give the homogenous polysaccharide SDA (780 mg).

3.4 Sugar analysis

The polysaccharide sample (3 mg) was hydrolyzed in 2 ml of 2 mol/l TFA at 120°C for 2 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 GC-MS. The column temperature was isothermal at 180°C for 2 min, followed by a 5°C/min gradient up to 250°C. If a polysaccharide contained uronic acid according to TLC, another part of it was carboxyl-reduced with $NaBH_4$ and hydrolyzed and analyzed by GC-MS as described above. The sugar composition was calculated by comparing the results based on native and carboxyl-reduced polysaccharides.

3.5 Methylation analysis

The vacuum-dried polysaccharide (8 mg) was methylated with the method of powered sodium hydroxide in dimethylsulfoxide. Methyl iodide (0.6 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 spectrum. The permethylated polysaccharide was hydrolyzed with 2 mol/l TFA (120°C, 2 h). The partially methylated sugars were reduced with $NaBH_4$ (25 mg) at room temperature for 3 h, and acetylated with acetyl anhydride (3 ml) at 100°C for 1 h. The partially methylated alditol acetates were analyzed by GC-MS. The column temperature was isothermal at 120°C for 2 min, followed by a 5°C/min gradient up to 250°C [10].

3.6 Two steps partial acid hydrolysis

SDA (1 g) was treated with 0.01 mol/l TFA at 100°C for 1 h. After TFA was removed by evaporation, the residue was dissolved with a small amount of water and dialyzed (cutoff 1000 Da) against distilled water

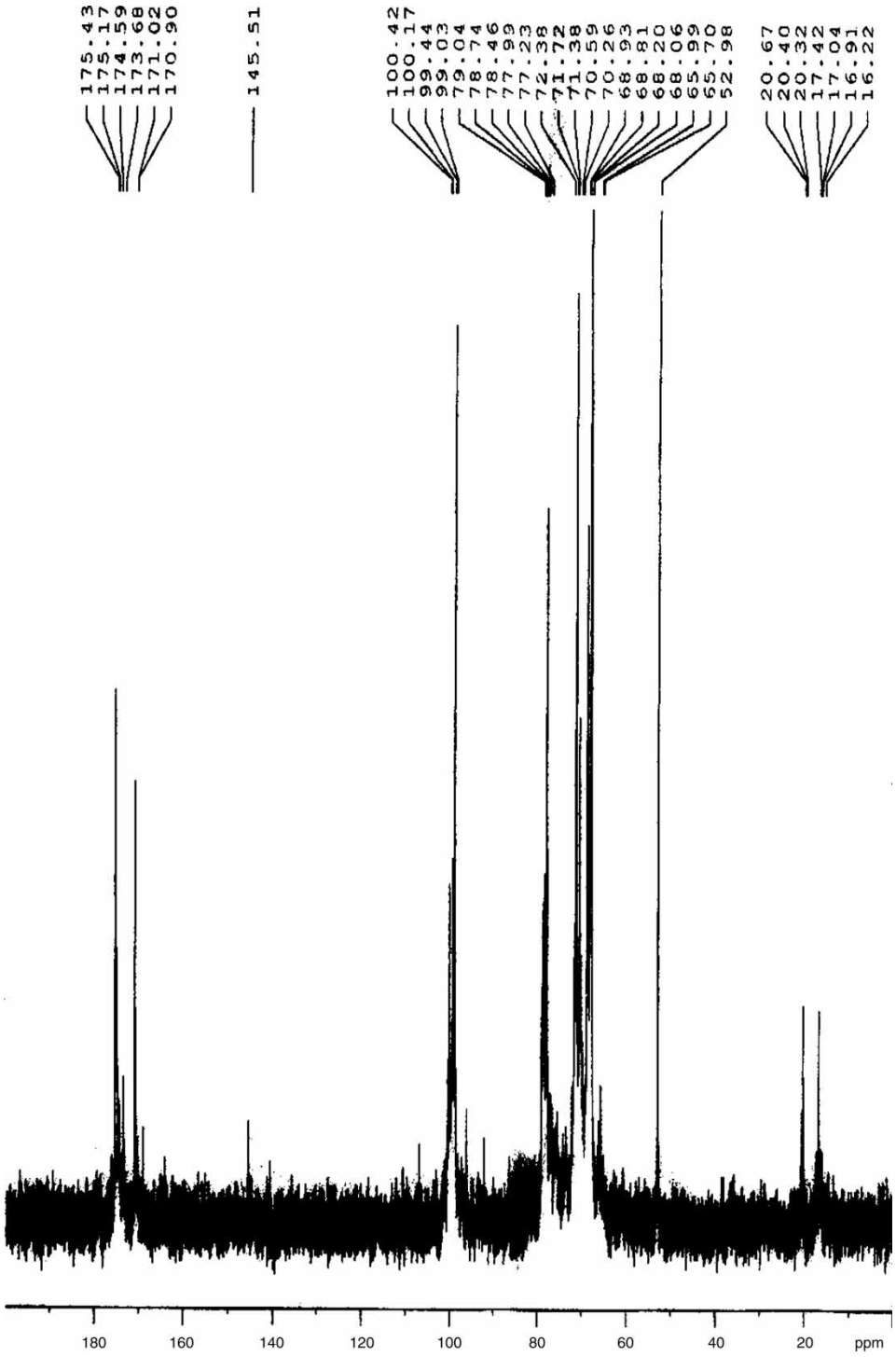

Figure 2. ^{13}C-NMR spectrum of SDA.

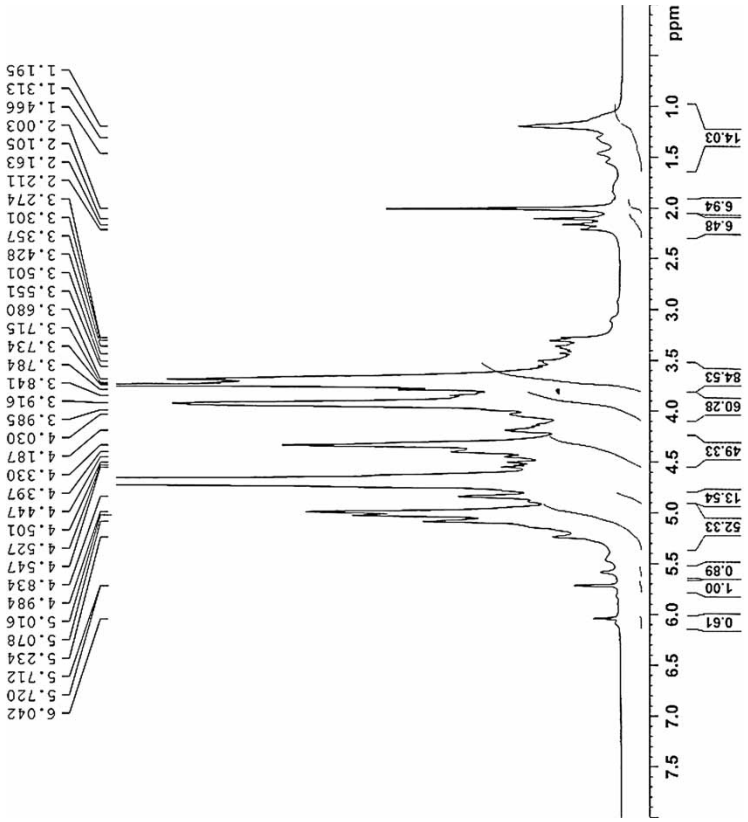

Figure 3. ^{1}H-NMR spectrum of SDA.

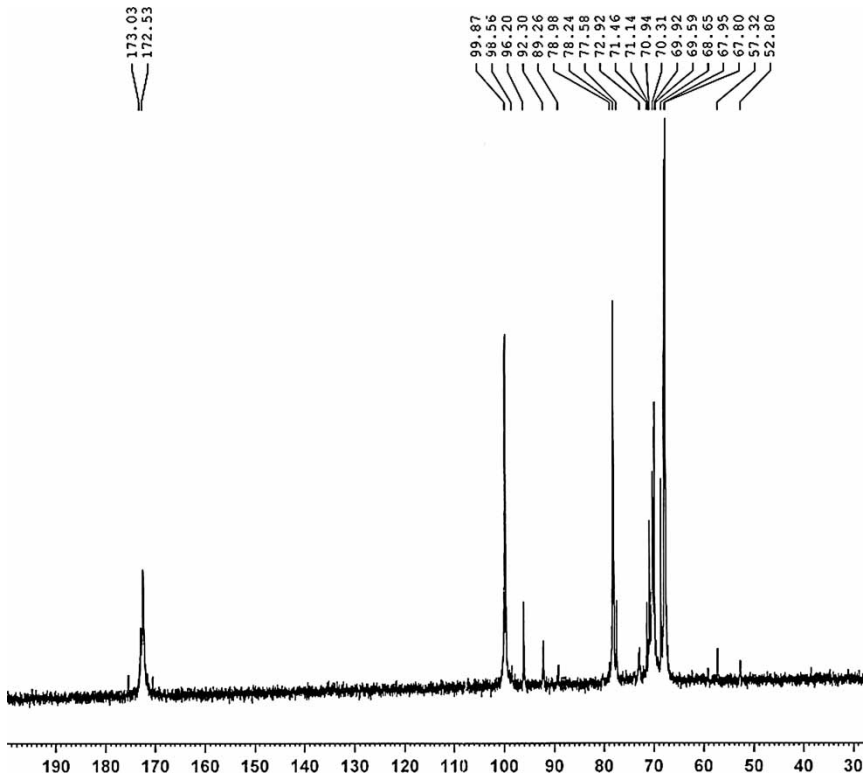

Figure 4. ^{13}C-NMR spectrum of SDA-2.

(3 × 1000 ml). The dialyzable fraction was concentrated and then applied to columns of Sephadex G-25 and Sephadex G-10 (Φ1.6 × 90 cm) with H_2O as the eluent to obtain the three fractions H-1, H-2, H-3. The nondialysate named SDA-1 (yield 82% from SDA) was further hydrolyzed with 0.25 mol/l TFA at 100°C for 2 h and the mixture was treated as described above. The retentate was concentrated, lyophilized, and SDA-2 was obtained (yield 80% from SDA-1). The dialyzable fraction was concentrated and then applied to a column of Sephadex G-10 (Φ1.6 × 90 cm) to obtain fraction H-4.

References

[1] W.L. Han, L. Liu, X.Q. Zhang, W.C. Ye, Y.L. Pan, and X.S. Yao, *China J. Chin. Mater. Med.* **32**, 695 (2007).

[2] L. Liu, Z.Q. Yin, X.T. Zhang, Y.L. Pan, and W.C. Ye, *Sci. Sericulture* **31**, 331 (2005).

[3] Y.W. Chen and Z. Xue, *J. China-Japan Friendship Hosp.* **4**, 210 (1990).

[4] Y.Z. Ou, J. Chen, and Y.H. Li, *Food Sci.* **26**, 181 (2005).

[5] C.Y. Jun, T. Wu, Q. Shen, W.M. Zhang, C.M. Lu, and G.R. Wu, *Food Sci.* **28**, 43 (2007).

[6] S. Hokputsa, W. Gerddit, S. Pongsamart, K. Inngjerdingen, T. Heinze, A. Koschella, S.E. Harding, and B.S. Paulsen, *Carbohydr. Polym.* **56**, 471 (2004).

[7] J.Y. Duan, Y. Zheng, Q. Dong, and J.N. Fang *Phytochemistry* **65**, 609 (2004).

[8] J.A. De Vries, F.M. Rombouts, A.G.J. Voragen, and W. Pilnik, *Carbohydr. Polym.* **2**, 25 (1982).

[9] R.L. Taylor and H.E. Conrad, *Biochemistry* **11**, 1385 (1972).

[10] P.W. Needs and R.R. Sevendran, *Carbohydr. Res.* **245**, 1 (1993).