

Lei SHI, Kaoshan CHEN, Qun DONG, Jinian FANG, Kan DING

Separation, purification and structure elucidation of a polysaccharide from root of *Cudrania tricuspidata*

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Abstract A water-soluble polysaccharide (CPS-0) was obtained from the root of *Cudrania tricuspidata* (Carr.) Bur., by hot water extraction (70°C), deproteinization using enzymolysis and Sevag method, precipitation with ethanol, and fractionation through DEAE-Sephadex A-50 chromatography. The purity of CPS-0 was determined by HPLC and the structure was elucidated by monosaccharide composition analysis, methylation analysis, GC, GC-MS, NMR spectral (¹H-NMR, ¹³C-NMR, HMQC), UV, IR, and elemental analysis. The CPS-0 was found to contain glucose residues only. The average repeating unit is a decasaccharide having a backbone consisting of 1,4-linked α -D-glucopyranosyl residues to which the side chain consisting of terminal and 1,4-linked α -D-glucopyranosyl residues was attached at position 6 of the branching residues.

Keywords root of *Cudrania tricuspidata* (Carr.) Bur., polysaccharide, separation and purification, structural characterization

Cudrania tricuspidata, a plant of the family of Moraceae, is a deciduous shrub or tree widely distributed in the eastern, south central and southwestern part of China to the southern part of Hebei Province. The root, stem, leaf and fruit of the plant are used as traditional Chinese medicines [1]. Aside from its use as treatment for gonorrhoea, rheumatism, jaundice, boils, scabies, bruising and dysmenorrhoea, its roots were also applied for the treatment of esophagus, stomach, and digestive tract carcinoma [2,3]. Therefore, the roots of *Cudrania tricuspidata* are very valuable for developing anti-tumor drugs. Thus

Translated from *Chemical Journal of Chinese Universities*, 2007, 28(6): 1088–1091 [译自: 高等学校化学学报]

Lei SHI, Kaoshan CHEN
School of Life Science, Shandong University, Jinan 250100, China

Lei SHI, Qun DONG, Jinian FANG, Kan DING (✉)
Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
E-mail: kding@mail.shcnc.ac.cn

far, the compounds isolated and identified from *Cudrania tricuspidata* are mainly xanthenes and flavones as well as some alkaloids, lignins, coumarins, etc. [4–10]. However, to our knowledge, there are no reports of polysaccharides from *Cudrania tricuspidata*. Recently three polysaccharides from the root of this plant were isolated and purified. *In vitro* bioactivity assay showed that they were immunopotentiators [11]. Here we reported physicochemical properties and structure of another polysaccharide, designated as CPS-0, isolated from the root of *Cudrania tricuspidata*.

1 Experimental

1.1 Materials, reagents and apparatus

The roots of *Cudrania tricuspidata* were purchased from a herb distributing center in Bozhou city in Anhui Province, China. They were identified by Professor Xu Yutai of the School of Life Sciences, Shandong University. T-series Dextran and DEAE-Sephadex A-50 were from obtained Pharmacia. Silica gel plates 60GF₂₅₄ were purchased from Merck. The standard monosaccharide and BSA used were Sigma products. DMSO was from Fluka. All other reagents used were domestic analytical reagents.

The instruments used were: Cintra 5 UV-Visible spectrophotometer (GBC, Australia), Modulyo D-230 vacuum freeze dryer (Thermo Savant, USA), SORVALL high speed refrigerated centrifuge (Kendro, USA), NEXUS 470 FTIR infrared spectrometer (Nicolet, USA), AVANCE-600 superconductive NMR spectrometer (Bruker, Switzerland), Waters 515 HPGPC equipped with a Waters 2410 differential detector (Waters, USA), VarioEL III elemental analyzer (Elementar, Germany), GC-14B gas chromatograph (Shimadzu, equipped with a 3% OV-225/AW-DMCS-Chromosorb W column (2.5 m × 3 mm) and an FID detector), GCT GC-MS (Waters, USA; equipped with an Agilent HP6890 gas chromatograph), Büchi 461 rotatory evaporator (Büchi, Switzerland).

1.2 Methods

1.2.1 Extraction, isolation, and purification

The air-dried and crushed roots (1 kg) were extracted with 8 volumes of water at 70°C three times (2 h each time). The supernatant was centrifuged and passed through a D3520 column to remove flavones, etc. The eluent was concentrated to one-third of its original volume. After centrifugation, the supernatant was precipitated with 4 volumes of 95% ethanol and placed in the refrigerator overnight. The precipitate was washed with dehydrated ethanol, acetone, and diethyl ether and dried under infrared light. The precipitate was then redissolved in H₂O and subjected to enzymolysis and the Sevag method for 5 times to remove proteins [12]. The resulting solution was dialyzed and lyophilized to achieve a yellow product (CPS). CPS was dissolved in distilled H₂O, centrifuged and passed through a DEAE-Sephadex A-50 column. The eluent used was H₂O. The eluent was detected using the phenol-H₂SO₄ method. Fractions exhibiting a single peak were collected, pooled, dialyzed, concentrated and lyophilized to give a polysaccharide, CPS-0.

1.2.2 Homogeneity and molecular weight

High-performance gel permeation chromatography (HPGPC) was carried out with a Waters 515 pump equipped with a Waters Ultrahydrogel[®] 1000 column and a Waters 2410 RI detector [13]. The column was calibrated with standard T-series Dextran (T-2000, T-110, T-70, T-40, and T-20) with 2.5×10^{-3} mol/L (pH = 6.0) phosphate buffer as the mobile phase at a flow rate of 0.7 mL/min. All samples were prepared as 1 mg/mL solutions and 20 μ L of solution was analyzed in each run. The molecular weight of the polysaccharide was estimated by comparing the result with the standard curve. The data were processed using the Waters GPC Millennium³² software.

1.2.3 Monosaccharide analysis [14]

CPS-0 (4 mg) was hydrolyzed using 2 mol/L TFA in a sealed tube at 110°C for 2 h followed by evaporation *in vacuo* to dryness with methanol. The residue was redissolved in H₂O (0.2 mL) and 20 μ L of the solution was used for TLC analysis with a developing reagent (ethyl acetate:pyridine:glacial acetic acid:water in a 5:5:1:3 ratio). The other portion was reduced with NaBH₄ (25 mg) at room temperature for 3 h then acetylated with Ac₂O (2 mL) at 100°C for 1 h. The resulting alditol acetate was examined by GLC.

1.2.4 Methylation analysis

CPS-0 (5 mg) was methylated four times using the modified Ciucanu method [15]. The permethylated CPS-0 was

depolymerized with 90% formic acid (100°C, 4 h) followed by hydrolysis with 2 M TFA (100°C, 6 h). The hydrolysate was converted into partially methylated alditol acetate and analyzed by GC-MS with a Shimadzu QP Class-5000 instrument.

1.2.5 Characterization

The IR spectrum of CPS-0 (2.0 mg) was obtained with a KBr pellet in the range of 4000–400 cm⁻¹. ¹H and ¹³C NMR spectra were measured using a Bruker AM-400 NMR instrument equipped with a dual probe in the FT mode at 20°C. CPS-0 (50 mg) was dissolved in D₂O at a concentration of 50 mg/0.5 mL. Chemical shifts are referred to the residual signal of HOD at $\delta = 4.70$ for ¹H-NMR spectrum and the external standard tetramethylsilane for ¹³C-NMR spectra.

2 Results and discussion

2.1 Physicochemical properties of CPS-0

CPS-0 obtained from the root of *Cudrania tricuspidata* is a white floccule. It has no smell and is hygroscopic in air. CPS-0 is soluble in water and insoluble in diethyl ether, chloroform, acetone, *n*-butanol, ethanol, etc. It is also stable when heated. The specific rotation $[\alpha]_D$ of CPS-0 was +179.2 (*c* 0.60, H₂O). The I₂-KI assay (CPS-0:1 mg/mL) was carried out. The result are shown in Table 1. The color of the reaction of I₂-CPS-0 was light red, different from the typical starch – I₂ complex which is blue.

Table 1 Characteristics of complex of I₂-CPS-0

	color	λ_{\max}/nm	absorbance
soluble starch	blue purple	582.8	1.640
CPS-0	light red	497.8	0.191

2.2 Elemental analysis, homogeneity and molecular weight

The carbon (40.54%) and H (6.597%) content were estimated by elemental analysis. Results show that CPS-0 contains no N or S. This result proved that CPS-0 contains no protein. The molar ratio of C:H:O was 1:2:1. On HPGPC, CPS-0 showed a symmetrical peak, indicating a homogenous fraction. The average molecular weight was estimated to be 5.9×10^3 .

2.3 Structural elucidation

After complete hydrolysis with TFA, TLC analysis showed that CPS-0 contains no uronic acid. GLC analysis indicated that it is composed exclusively of glucose.

Table 2 GC-MS data for methylation analysis of CPS-0

methylated sugars	molar ratio	MS data of main fragments/ <i>m/z</i>	linkage types
2,3,4,6-Tetra-O-Me-Glc	1.0	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	Glc (1→
2,3,6-Tri-O-Me-Glc	8.0	43, 45, 87, 99, 101, 117, 161, 233	→4) Glc (1→
2,3-Di-O-Me-Glc	1.0	43, 85, 99, 101, 117, 127, 159, 161, 201, 261	→4,6) Glc (1→

The IR spectrum (not shown) indicated that CPS-0 possesses characteristic absorption peaks for polysaccharides. The strong peak at 3315.53 cm^{-1} is the absorption of the O–H bond and the peak at 2921.22 cm^{-1} is the absorption of C–H bond. The peak at 1636 cm^{-1} is the absorption of hydrated water in CPS-0 [14]. The peaks at $1200\text{--}1000\text{ cm}^{-1}$ are the absorption of C–O bond. The absorption at 851.70 cm^{-1} indicated that CPS-0 has α -glucopyranosidic linkage which was further supported by its high $[\alpha]_D$ value of $+179.2$.

After methylating for four times using the modified Ciucanu method, the permethylated CPS-0 was depolymerized and converted into partially methylated alditol acetate. GC-MS analysis showed three types of linkages, corresponding to T-Glc_p (terminal), 1, 4-linked Glc_p and 1, 4, 6-linked Glc_p, respectively, approximately in the molar ratio of 1:8:1. The data indicate an O-6-branched (1→4)- α -D-glucan structure of CPS-0 (Table 2). The backbone therefore was deduced to be composed of 1, 4-linked glucose residues with a branch on every 10 residues.

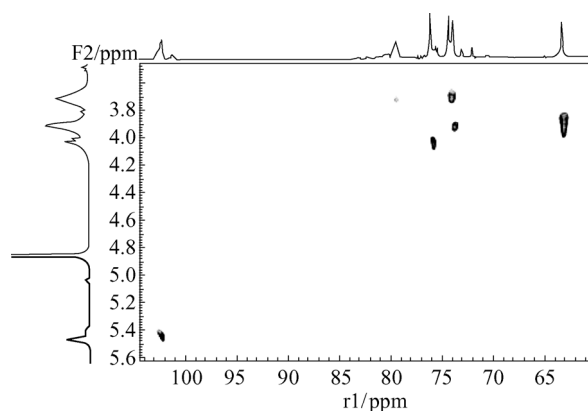
The ^{13}C -NMR spectrum contained two anomeric signals at $\delta = 102.09$ and 101.12 , indicating an α -anomeric configuration for glucopyranosyl units. The signal at $\delta = 102.09$ and 101.12 arose from the anomeric atoms of 1, 4-linked Glc_p and 1, 4, 6-linked Glc_p, respectively. The signal at $\delta = 62.96$ was assigned to C-6 of (1→4)- α -D-glucosyl residues, as it was supported by the corresponding reversed peak in the DEPT spectrum (not shown). Other ^{13}C -NMR signals were tentatively assigned and the assignments are shown in Table 3 [16,17].

Table 3 ^{13}C -NMR signal assignments for CPS-0

residues	C1	C2	C3	C4	C5	C6
1-Glc	102.09	74.23	75.38	71.82	73.68	62.96
1, 4-Glc	102.09	74.23	75.38	79.20	72.87	62.96
1, 4, 6-Glc	101.12	74.04	75.38	79.20	72.87	–

In HMQC spectrum of CPS-0 (Fig. 1), the proton signal at $\delta = 5.46$ had a cross with the anomeric carbon signal at $\delta = 102.09$, while the proton signal at $\delta = 5.04$ had a cross with the anomeric carbon signal at $\delta = 101.12$. The result further confirmed that the signal at $\delta = 102.09$ was assigned to C-1 of the 1, 4-linked glucose residue and the signal at $\delta = 101.12$ arose from C-1 of the 1,4,6-linked glucose residue.

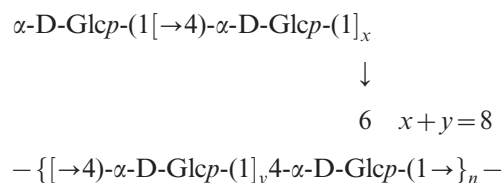
In the ^1H spectrum of CPS-0, the proton signals at $\delta = 5.46$ and 5.04 indicate an α -anomeric configuration for the glucopyranosyl units. The strong signal at $\delta = 5.46$

**Fig. 1** HMQC spectrum of CPS-0

is from the anomeric hydrogen signal of 1,4-linked glucose residue, while the weak signal at $\delta = 5.04$ might arise from the anomeric hydrogen signal of 1,4,6-linked glucose residue which further supported the above conclusion.

A large proportion of glucans obtained from plants are α -D-glucans having a backbone consisting of 1,4-linked glucose residues. This sort of α -D-glucans also include starch. According to degree of branching, starch is classified between amylose and amylopectin. In the I_2 -KI assay, the complex of I_2 -amylose is strong blue, while the complex of I_2 -amylopectin is purple. The complex of I_2 -CPS-0 was light red, which suggested that CPS-0 is similar to highly branched amylopectin. The average repeating chain length of CPS-0 was ten. Generally, the complex of I_2 -starch shows blue when the average chain length of starch is more than thirty-six [18]. The results show that the complex of I_2 -CPS-0 was light red and the color strength of the complex was also weak. We deduced the reason was that CPS-0 is highly branched and branches affect the formation of the helix structure which is essential for the I_2 binding.

Our conclusion is that CPS-0 contains only glucose residues. The average repeating unit is a deca-saccharide which has a backbone consisting of 1, 4-linked α -D-glucopyranosyl residues to which the side chain consisting of terminal and 1, 4-linked α -D-glucopyranosyl residues is attached at position O-6 of the branching residues, as shown below:



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