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Isolation and structural characterization of the water-extractable polysaccharides from *Cassia obtusifolia* seeds

Mingsheng Shang, Xiaoman Zhang, Qun Dong*, Jian Yao, Qin Liu, Kan Ding**

Glycochemistry and Glycobiology Lab, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China

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ABSTRACT

The seed of *Cassia obtusifolia* is a food or herbal medicine used for improving eyesight, treating constipation and other disorders, and polysaccharides have been implicated in these pharmacological activities. The endosperm of the seeds, *Cassia* gum, is a commercial thickening or gelling agent, composed mainly of galactomannans. However, the whole seeds of *C. obtusifolia*, rather than the endosperm, are used in folk medicine or food, which might contain more complex constituents of polysaccharides. In this study, the whole seeds of *C. obtusifolia* were extracted with boiling water, and from the water extract, three homogeneous fractions were isolated, designated CFAA-1, CFAA-3, and CFBB2, respectively, after treatment with Fehling solution followed by anion-exchange and gel permeation chromatography. Using chemical and spectroscopic methods, CFAA-1, and CFAA-3 were elucidated to be both branched galactomannans with different molecular weights, consisting of 1,4-linked β-D-mannopyranosyl backbone with single-unit α-D-galactopyranosyl branches attached to O-6 of mannose, while CFBB2 was shown to be a linear (1 → 4)-α-polygalacturonic acid.

1. Introduction

Cassia obtusifolia L. is an annual herb (family: Leguminosae), whose dried whole seeds are used in China as a drink or a folk medicine. The seeds are reported to have the effects of improving eyesight, alleviating constipation, and lowering hypertension and hyperlipidemia (Hao, Sang, & Zhao, 2001). In recent years, there are many studies on its chemical constituents and pharmacological activities, particularly on the anthraquinones and their neuroprotective effects (Drever et al., 2008; Ju et al., 2010; Kim et al., 2011; Li, Xiao, Li, Zhang, & Pang, 2009; Wu et al., 2011). The endosperm flour of *C. obtusifolia* or *Cassia tora* seeds, after removing the husk and germ, is *Cassia* gum, which is used widely as thickener or gelling agent. It was reported that the gum contains more than 75% of galactomannans (Hallagan, La Du, Pariza, Putnam, & Borzelleca, 1997). Wu and Abbott (2005) reported that gum content and monosaccharide composition varied with particle size after fine grinding and sieving. The galactomannan can be chemically modified for hair conditioning (Staudigel et al., 2007). However, the galactomannan from the endosperm probably could not account for all the polysaccharides in *C. obtusifolia* seeds used in a traditional Chinese medicine since the whole seeds rather than the husk-removed ones are used. Therefore, it is possible that the polysaccharides other than galactomannan are responsible for the pharmacological effects of the seeds. In order to get a full understanding of the types and structural features of the polysaccharides in *C. obtusifolia* seeds, we isolated and characterized the water-extractable polysaccharides from the whole seeds of *C. obtusifolia*.

2. Experimental

2.1. Material

The dried whole seeds of *C. obtusifolia* were purchased as unprocessed crude drug from Shanghai Xuhui TCM Slices Co. Ltd., and the original plants were collected from Anhui Province. DEAE-cellulose 32 was purchased from Whatman International Ltd. (Bradstone, England), Sephacryl S-300 HR from Amersham Biosciences AB (Uppsala, Sweden), and Bio-Gel P-2 from Bio-Rad Laboratories, Inc. (Hercules, USA). L-Arabinose, D-galactose, D-mannose, D-galacturonic acid, sodium borohydride, iodomethane, and N-cyclohexyl-N′-(2-morpholino-ethyl)-carbodiimidemethyl-*p*-toluene-sulfonate (CMC) were obtained from Fluka Chemie GmbH (Buchs, Switzland). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO) and PEI-cellulose precoated TLC plate were obtained from Merck KGaA (Damstardt, Germany). Dialysis tubes (Membra-cei™, MWCO 3500) were purchased from Sino-American Biotechnology Co. (SABC, Shanghai). Other reagents are

* Corresponding author. Tel.: +86 21 50806600x3203; fax: +86 21 50806928.
** Corresponding author. Tel: +86 21 50806928; fax: +86 21 50806928.
E-mail addresses: qdong@mail.shcnc.ac.cn (Q. Dong), kding@mail.shcnc.ac.cn (K. Ding).

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all analytical grade from Shanghai Chemical Reagent Company unless claimed otherwise.

2.2. General methods

IR spectra were determined with a Perkin-Elmer 591B spectrophotometer as KBr pellets. Optical rotations were determined with a Perkin-Elmer 241M digital polarimeter. NMR spectra were recorded on a Varian Mercury 400 NMR spectrometer. DEPT experiments were carried out using a polarization transfer pulse of 135°. Gas chromatography (GC) was performed with a Shimazu-14B apparatus equipped with a 3% OV-225/AW-DMCS-Chromosorb W column (2.5 m × 3 mm), with N_2 as the carrier gas at a flow rate of 25 ml/min. The column temperature was kept at 210 °C for sugar composition analysis and at 190 °C for methylation analysis. GC–MS was performed on a Finnigan Trace/DSQ instrument, equipped with an HP-5 capillary column. The initial temperature was 120 °C, increased to 250 °C at the gradient of 1.5 °C/min, and kept for 5 min. The neutral carbohydrate and uronic acid were determined with phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and *m*-hydroxyl diphenyl method (Blumencrantz & Asboe-Hansen, 1973), using D-glucose and D-glucuronic acid as the standards with appropriate correction by response factors, respectively. Protein was determined with Lowry method (Bensadoun & Weinstein, 1976).

2.3. Isolation and purification of polysaccharides

The dried seeds of *C. obtusifolia* (2.0 kg) were defatted twice by soaking in 95% ethanol (10 l) for 5 days at room temperature (25 °C). After filtration, the residue was dried at ambient atmosphere, and then extracted 5 times with boiling water, each for 5 h. The extracts were concentrated, and dialyzed against running water for 2 days. The retentate was centrifuged, and to the supernatant were added 3 volumes of 95% ethanol under vigorous stirring. After standing overnight at 4 °C, the precipitate was obtained by centrifugation, washed successively with absolute ethanol and acetone, and dried in vacuum, to give the water-extracted crude polysaccharide COA (152 g, 7.6%).

Treatment with Fehling solution was performed as described (Jones, 1965). Briefly, to the solution of COA (2 g in 100 ml H_2O), Fehling solution was added dropwise with rigorous stirring until no more precipitate formed. The suspension was stirred at room temperature for 4 h, and then centrifuged. The precipitate was washed with deionized water, then macerated for 0.5 h with 5% (v/v) hydrochloric acid in ethanol and filtrated on a sintered glass funnel, washed with ethanol until the filtrate was free of chloride ion. The pellet was dried in vacuum to give CFA (0.66 g, yield 33%). The supernatant was neutralized with HOAc, dialyzed, concentrated, and precipitated with 3 volumes of ethanol, to give CFB (0.68 g, yield 34%).

CFA (10.1 g) was fractionated by anion-exchange chromatography on a DEAE-cellulose column (5 cm × 60 cm, Cl^- form), which was eluted stepwise with water, 0.2, 0.4, and 0.8 M NaCl, and monitored with phenol-sulfuric acid method, to give 3 fractions, designated CFAA (6.03 g, 59.8%), CFAB1 (112 mg, 1.1%), and CFAB2 (78 mg, 0.8%) from water, 0.2, and 0.4 M NaCl elution, respectively. No carbohydrate was detected in 0.8 M NaCl eluate. CFAA (1.06 g) was purified on a Sephacryl S-300 column, equilibrated and eluted with 0.2 M NaCl, giving CFAA-1 (166 mg, 15.7%), CFAA-2 (153 mg, 14.4%), and CFAA-3 (168 mg, 15.9%). CFB (5 g) was also fractionated into three fractions by using the DEAE-cellulose column as described above, designated as CFBA (397 mg, 7.9%), CFBB1 (276 mg, 5.5%), and CFBB2 (890 mg, 17.8%).

2.4. Homogeneity and molecular weight

The homogeneity and molecular weight of polysaccharides were estimated by HPGPC method (Alsop & Vlachoogiannis, 1982; Wei & Fang, 1989). The HPLC instrument was equipped with series-connected Ultrahydrogel™ 2000 and Ultrahydrogel™ 500 columns, eluted with 0.045 M phosphate buffer at a flow rate of 0.5 ml/min. The column was calibrated by molecular weight-known standard Dextrans (T-700, 580, 300, 110, 80, 70, 40, 9.3 and 4, Pharmacia). The column temperature was kept at 30.0 ± 0.1 °C. All samples were prepared as 0.2% (w/v) solutions, and 20 μl of solution was analyzed in each run.

2.5. Intrinsic viscosity [η] and viscosity average molecular weight (Mv)

The intrinsic viscosity was measured at 25 °C by using an Ubbelohde capillary viscometer. The polysaccharide was dissolved in distilled water in an original concentration of 2 mg/ml. The kinetic energy correction was negligible. [η] was estimated using the Huggins and Kraemer equations by extrapolation to infinite dilution as follows:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c$$

$$\ln \eta\frac{r}{c} = [\eta] - k''[\eta]^2 c$$

where η_{sp}/c is the reduced viscosity; $\ln \eta r/c$, the inherent viscosity; c, sample concentration; k' and k'', constants for a given polymer under given condition in a given solvent.

Viscosity average molecular weights, Mv, were calculated using the established Mark–Houwink relationship for galactomannans, taking into account the ratios of M/G in galactomannans (Cerqueira et al., 2009).

$$[\eta] = 11.55 \times 10^{-6}[(1-\alpha) \times \mathrm{Mv}]^{0.98}$$

where $\alpha = 1/[(\mathrm{M/G}) + 1]$ and [η] is expressed in dl/g.

2.6. Glycosyl composition analysis (Blakeney, Harris, Henry, & Stone, 1983)

The polysaccharide sample (2 mg) was hydrolyzed with 2 M TFA (2 ml) at 110 °C for 2 h in a sealed test tube. TFA was removed under reduced pressure by repeated evaporation with methanol. An aliquot of the hydrolysate was analyzed by TLC on a Pei-cellulose plate, developed with EtOAc–pyridine–HOAc–H_2O (5:5:1:3, v/v). The other part was dissolved in 2 ml of H_2O, and reduced with 25 mg of $NaBH_4$ at room temperature for 2 h. The boric acid was removed by repeated evaporation under reduced pressure with methanol after neutralization with acetic acid. After acetylation with acetic anhydride, the alditol acetates was extracted with chloroform and analyzed by GC.

For uronic acid-containing polysaccharides, the carboxyl reduction of uronic acid was performed according to Conrad procedure (Taylor & Conrad, 1972) before acid hydrolysis and derivatization.

2.7. Methylation analysis

The polysaccharides were methylated thrice with the modified method of Ciucanu as described by Needs and Selvendran (1993). The permethylated polysaccharide was depolymerized with 90% formic acid at 100 °C for 4 h, followed by hydrolysis with 2 M TFA at 100 °C for 4 h. The hydrolyzate was then converted into the partially methylated alditol acetates and analyzed by GC–MS (Sweet, Shaprio, & Albersheim, 1975).