Carbohydrate Research 393 (2014) 43–50

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structure and activities of a novel heteroxylan from *Cassia obtusifolia* seeds and its sulfated derivative

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ARTICLE INFO

Article history:
Received 17 February 2014
Received in revised form 3 April 2014
Accepted 21 April 2014
Available online 30 April 2014

Keywords:
Cassia obtusifolia
Structure
Glucuronoxylan
Anti-angiogenesis
Anti-tumor

ABSTRACT

COB1B1S2 was isolated from an alkaline extract of *Cassia obtusifolia* seeds, and purified by anion-exchange and gel permeation chromatography. It contains arabinose, xylose, and glucuronic acid, in the molar ratio of 5:81:14, with an apparent molecular weight estimated to be 70.4 kDa. Elucidated by using chemical and spectroscopic methods, COB1B1S2 was shown to have a backbone consisting of 1,4-linked β-D-Xyl*p*, with one single-unit terminal α-D-Glc*p*A or α-L-Ara*f* substituted at O-2 for nearly every five 1,4-linked Xyl*p*. COB1B1S2 is structurally different from typical glucuronoxylans by its absence of methylation at O-4 of GlcA. The native COB1B1S2 showed no significant inhibition on the tube formation of human microvascular endothelial cells (HMEC) and on the growth of liver and colon cancer cells. On the contrary, COB1B1S2-Sul, prepared as the sulfated derivative of COB1B1S2, exhibited a significant inhibition on tube formation of HMEC in a dose-dependent manner, and on the growth of Bel7402 liver cancer cells. These results indicated that the introduction of sulfate groups significantly enhanced the biological activity of glucuronoxylan.

1. Introduction

Semen Cassiae is the dry ripe seed of *Cassia obtusifolia* (Leguminosae). This herb is distributed mainly in China, Korea, India, and West tropical regions.[1] As a traditional Chinese herbal medicine, *Semen Cassiae* was first recorded in Shennong's Classic of Materia Medica in China, and used widely as a drink or a folk medicine. In India, it was also consumed as roasted tea or as an ingredient for coffee.

The whole seed of *Cassia obtusifolia* is widely used for treatment of headache, ophthalmic diseases, constipation, hypertension as well as hyperlipidemia. In recent years, more and more reports have elucidated the relationship between its chemical constituents and pharmacological activities. For example, it was reported that the methanolic extract of *Semen Cassiae* contains an appreciable content of total free phenolic compounds and displayed a significant inhibition of α-amylase and α-glucosidase as well as a free radical-scavenging effect against 2,2-diphenyl-1-picrylhydrazyl (DPPH).[1] A water-soluble polysaccharide extracted from *Cassia obtusifolia* exhibited an inhibition on α-amylase and pancreatic lipase activity and rendered an increase in protease activity.[2] In addition, it can bind with bile acids and reduce the amount of cholesterol. Previously, we have reported the galactomannan and homogalacturonan isolated from boiling-water extract of *Cassia obtusifolia*.[3] The galactomannan consisted of 1,4-linked β-D-mannopyranosyl backbone with branches of a single unit α-D-galactopyranosyl attached to O-6 of mannose, and the homogalacturonan was a linear 1,4-linked α-D-galacturonic acid.

Heteroxylans are distributed widely in various higher plants, whether in woods or grass, as the major hemicellulosic polysaccharides that constitute the major components of plant cell wall and extracellular matrix, along with pectin and cellulose. Most heteroxylans contain a characteristic 1,4-linked β-D-xylopyranosyl backbone, with single-unit arabinose and/or 4-O-Me-D-glucuronic acid (MeGlcA) residues attached to O-2/O-3 of backbone xylose, and thus are categorized into arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. In glucuronoxylan, the 4-OH of glucuronic acid is mostly methylated,[4–6] and the MeGlcA/Xyl molar ratios can vary from 1:5 to 1:20, depending on species, source of tissue, and period of harvest. Many attempts have been made to modify xylans to obtain the bioactive derivatives or functional materials. The cationic xylans not only improved the strength properties of bleached hardwood kraft pulp in the application of papermaking but also showed antimicrobial activity against some Gram-negative and Gram-positive bacteria. The trimethylammonium-2-hydroxypropyl xylan was prepared as beater additive. Sulfated xylan from beechwood glucuronoxylan was studied as an anticoagulant for nearly thirty years in

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http://dx.doi.org/10.1016/j.carres.2014.04.016
0008-6215/

Europe.[7] Xylan sulfates have also been studied for their distinct antiviral, anti-cancer, anti-metastatic and anti-inflammatory activities.[7] Xylans from *Scinaia hatei* after sulfation exhibit a strong anti-HSV bioactivity but no direct inactivating effect on virions in a virucidal assay.[8] Marshall et al.[9] found that the xylan sulfate was an effective inhibitor of heparin-binding growth factors in vitro and inhibited the development and progression of tumors in nude mice. However, up to now, no research has been reported on xylans and their sulfated derivatives with respect to their anti-angiogenic activity and inhibition on liver cancer cells.

In this study, we isolated and characterized a glucuronoxylan with novel structural features from alkali-extract of *Cassia obtusifolia* seeds. In addition, we examined the anti-angiogenesis and anti-tumor activities of the glucuronoxylan and its sulfated derivative.

2. Experimental

2.1. Materials

The crude drug of *Cassia obtusifolia* seeds was purchased from Shanghai Xuhui TCM Slices Co. Ltd. Diethylaminoethyl– (DEAE–) cellulose 32, Sephacryl S-300 HR were purchased from the same manufacturers as described previously.[3] T-series Dextrans were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) was purchased from Tokyo Chemical Industry Co. Ltd. Sodium borohydride, iodomethane, and trifluoroacetic acid (TFA) were purchased from Sinopharm Chemical Reagent Co. Ltd. All reagents were of analytical grade unless claimed otherwise.

2.2. Isolation of polysaccharide COB1B1S2 from *Cassia obtusifolia* seeds

The dried whole seeds of *Cassia obtusifolia* (2 kg) were treated with 95% ethanol (10 L) twice for 5 days at room temperature. After filtration, the ethanol-insoluble residue was extracted 5 times with boiling water, 5 h for each. The residue was collected by filtration and treated twice with 5% sodium hydroxide (20 L) for 3 h under intermittent stirring at 4 °C. After filtration, the alkaline extract was neutralized with dilute hydrochloric acid, then concentrated and dialyzed against running water for 2 days. The retentate was centrifuged and to the supernatant were added 3 volumes of 95% ethanol with vigorous stirring. After standing overnight, the precipitate was collected by centrifugation, washed successively with absolute ethanol and acetone, and dried in vacuum, to give the crude alkali-extracted polysaccharide (COB1, 23.0 g).

COB1 (22.3 g) was fractionated on a DEAE-cellulose column (50 cm × 5 cm, Cl^- form), eluted stepwise with water, 0.2, 0.4 M aqueous sodium chloride and monitored by phenol–sulfuric acid method. The fraction eluted with 0.2 M NaCl was collected as the major component, and designated as COB1B1 (7.6 g, 34% of COB1). COB1B1 was further purified by gel permeation chromatography on a Sephacryl S-300 column (100 cm × 2.6 cm), equilibrated, and eluted with 0.2 M NaCl, and fractionated into three fractions. The major fraction was collected and designated as COB1B1S2 (yield 40.9% of COB1B1). The other two fractions were designated as COB1B1S1 (yield 6.2%) and COB1B1S3 (yield 13.4%), respectively.

2.3. Homogeneity and molecular weight determination

Homogeneity and molecular weight were measured by high performance gel permeation chromatography (HPGPC) on an Agilent 1260 HPLC system equipped with series-connected Ultrahydrogel 2000 and 500 columns,[10] with 0.1 M $NaNO_3$ used as the mobile phase at a flow rate of 0.5 mL/min. All samples were prepared as 0.2% (w/v) in mobile phase, and 20 μL of solution was injected in each run. The eluate was monitored with an RI and a UV detector, and the column temperature was kept at 25 °C.

2.4. Monosaccharide composition determination

The monosaccharide composition of polysaccharides was determined by gas liquid chromatography (GC) as alditol acetates. Briefly, 2 mg of polysaccharide was hydrolyzed with 2 M TFA at 110 °C for 4 h. The monosaccharides were converted into alditol acetates as described,[11] and analyzed by GC. For quantification of uronic acid, COB1B1S2 was first carboxyl-reduced as described by Taylor & Conrad,[12] and then hydrolyzed, derivatized, and analyzed as described above.

2.5. Methylation analysis

Due to its poor solubility in DMSO, the polysaccharide (10 mg) was first methylated with the modified Haworth's method.[13] The polysaccharide was dissolved with 0.5 mL of H_2O and then 0.5 mL of 30% (w/v) sodium hydroxide was added. 0.5 mL dimethyl sulfate was added dropwise into the mixture and stirred at room temperature for 3 h. The mixture was dialyzed and the retentate was lyophilized. It was further methylated thrice with modified method of Ciucanu.[14] The permethylated polysaccharide was hydrolyzed and converted into the partially methylated alditol acetates (PMAA) and analyzed by GC–MS. GC–MS analysis was carried out on a Shimadzu QP2010 plus GC–MS,[15] equipped with a DB-5 ms capillary column using a temperature programing of 140 °C (3 min)–250 °C (40 min) at 2 °C/min. Helium was used as the carrier gas.

2.6. Infrared spectroscopy (IR) and optical rotation

The IR spectra were measured on a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, USA). Native polysaccharide (2 mg) was ground with KBr and pressed into pellets, then scanned from 4000 to 600 cm^{-1}. Methylated polysaccharide was measured according to the Nujol method. Specific rotation was determined with an Autopol VI instrument (Rudolph Research Analytical) at 25 °C at a wavelength of 589 nm.

2.7. NMR analysis

The ^1H NMR, ^{13}C NMR, COSY, hetero-nuclear single quantum coherence (HSQC), and hetero-nuclear multiple bond correlation (HMBC) spectra were recorded at 25 °C on a Bruker AVANCE III NMR spectrometer operating at 500 MHz. The polysaccharides (30 mg) were deuterium-exchanged and dissolved in 0.5 mL of D_2O (99.8% D). The chemical shifts were calibrated by reference to HOD as the internal standard at 4.85 ppm in ^1H NMR and to acetone as the internal standard at 31.50 ppm in ^{13}C NMR.

2.8. Reduction of uronic acid[12]

COB1B1S2 (20 mg) was dissolved in 10 mL H_2O. CMC was added and the pH was kept at 4.75 with 0.01 M HCl. After 2 h, 2 M aqueous sodium borohydride (15 mL) was added slowly to the mixture, and 4 M HCl was added to keep pH at 7.0 at room temperature. The mixture was stirred for 1 h, and then dialyzed against distilled water. The retentate was lyophilized. The procedure above was repeated thrice, and the resulting carboxyl-reduced polysaccharide designated COB1B1S2-R was determined for the uronic acid