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# Characterization of arabinogalactans from *Larix principis-rupprechtii* and their effects on NO production by macrophages

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ABSTRACT

Arabinogalactans are a source of dietary fiber with health benefits. In this work, two arabinogalactans assigned as AGW and AGS were isolated from *Larix principis-rupprechtii*, and characterized by gel permeation chromatography (GPC), monosaccharide analysis, methylation analysis and NMR spectroscopy analysis. The average molecular weights of AGW and AGS were $1.53 \times 10^4$ and $1.84 \times 10^4$ Da, respectively. Methylation analysis and NMR spectra suggested that AGW and AGS have a 1,3-linked Gal*p* backbone, branched at C-6 with 1,6-linked Gal*p* side residues. The Ara residues were substituted at C-6 of 1,6-linked Gal*p* consisting of α-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→6)-β-D-Gal*p*-(1→ and β-L-Ara*p*-(1→6)-β-D-Gal*p*-(1→. Significantly, AGS (0.74%) was shown to contain 25 times more uronic acid than AGW (0.03%), which demonstrated a polyelectrolyte effect. Application of these two polysaccharides to macrophage RAW 264.7 cells was shown to increase nitric oxide (NO) production. These results provide a basis for studying the relationship between the structure and biological activity of arabinogalactans.

## 1. Introduction

Macromolecular and biological polysaccharides have begun to receive wide attention as benefactors to human health, due to their ability to function across several biological activities. Examples of such activities include polysaccharides serving as antioxidants, anti-virulents, antineoplastics and even immune system enhancers (Chen et al., 2017; Xie et al., 2017; Zou et al., 2017). Previous research upon the beneficial biological activities of polysaccharides found that the key variables to polysaccharide functionality with regards to human health are monosaccharide constitution, molecular weight, types of glycosidic linkages, extent of intramolecular branching, and macromolecular conformation (Bohn & Bemiller, 1995; Sletmoen & Stokke, 2008). The complicated relationships between the aforementioned molecular complexities and human health benefits have led to a significant uptick in research investigating this topic.

*Larix principis-rupprechtii* is a unique softwood species which is native to China, growing mainly in Hebei and Shanxi Provinces (Di, Li, Wang, & Wang, 2014). *L. principis-rupprechtii* hemicellulose contains a particularly unique polysaccharide: a highly water-soluble arabinogalactan (AG) that features a high degree of intramolecular branching. Similar AG hemicelluloses can be found in other species of the genus *Larix* (larch). AG from *L. occidentalis*, *L. dahurica*, and *L. laricina* have been the subject of previous studies, and each work concluded in agreements regarding the unique structural properties of AG from *Larix*. It was found that AG features 1,3-linked β-D-galactopyranosidic units which serve as a molecular backbone with branches at C6 hydroxyl position to either single D-Gal*p* units or to extended oligosaccharide like structures consisting of Gal*p*, Ara*p*, Ara*f*, Rha*p* and Glc*p*A (Churms, Merrifield, & Stephen, 1978; Goellner, Utermoehlen, Kramer, & Classen, 2011; Ponder & Richards, 1997a; Ponder, 1998, 1997b, 1997c; Prescott, Groman, & Gulyas, 1997). It is generally believed that the corresponding molar ratios of Gal: Ara in larch AG is about 6:1, and both of comprise more than 98% of the total carbohydrate content of AG. (Côté, Day, Simson, & Timell, 1966; Goellner et al., 2011; Willför & Holmbom, 2004). A trace amount of glucuronic acid moieties have also been identified in the AG, but further understanding of how it is connected is not yet known, due to a lack of elucidative analytical techniques (Odonmaiig, Ebringerova, Machova, & Alfijldi, 1994; Ponder & Richards, 1997a). The significance of uronic acid groups in AG lie in the dramatic effect which a few uronic acid groups have on chromatographic behavior of isolated AG, where differences in separation were observed around system pH and uronic acid pKa (~3) (Ponder & Richards, 1997a). The molecular weight distribution of larch

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arabinogalactans is reported to be very broad, with molecular weight from 3 to 100 kDa having been reported with high polydispersities. (Churms et al., 1978; Eremeeva & Bykova, 1992; Prescott, Enriquez, Jung, Menz, & Groman, 1995, 1997; Teratani, Kato, Kai, & Yamashita, 1987).

AG extracted from different larch species have been shown to possess diverse biological properties, including immunological activity (Currier, Lejtenyi, & Miller, 2003; Groman & Gou, 1997; Kelly, 1999; Kim, Waters, & Burkholder, 2002), antitumor (Beuth, Ko, Schirrmacher, Uhlenbruck, & Pulverer, 1988), fecal microbial population regulators (Grieshop, Flickinger, & Jr, 2002; Robinson, Feirtag, & Slavin, 2001), antiviral effects (Enriquez, Chu, Josephson, & Tennant, 1995), and ocular benefits (Burgalassi et al., 2007). AG from *Larix laricina* has even been demonstrated to play a unique role in reducing the incidence of the common cold (Riede, Grube, & Gruenwald, 2013). Finally, AG from larch has properties that make it suitable as a carrier for delivering diagnostic or therapeutic agents to hepatocytes via the asialoglycoprotein receptor (Groman, Enriquez, Jung, & Josephson, 1994), and as protecting agent for maintaining precious metal nanoparticles in colloidal suspension (Mucalo, Bullen, Manley-Harris, & Mcintire, 2002).

Macrophages are an important class of immune cells. It has demonstrated that some polysaccharides have unique patterns of influence upon the immune system (Wang et al., 2017; Xu, Chen, Zhang, & Ashida, 2012). Nitrous oxide (NO) is an important cytotoxic mediator contributing to both the antitumor and antimicrobial activity of macrophages (Bogdan, 2001). Among signaling qualities, NO affects cellular decisions of life and death either by turning on apoptotic pathways or by shutting them off (Brüne, 2003). It has been reported that macrophage-derived NO can kill or reduce replication of infectious agents and cause cytostasis or kill tumor cells (Degroote & Fang, 1999; Pervin, Singh, & Chaudhuri, 2001; Xie, Dong, & Fidler, 1996). However, there is less research on larch arabinogalactans.

In this study, we isolated two different fractions of AG from a bulk source of AGs extracted from *L. principis-rupprechtii*. Molecular structures within each AG fraction were investigated using a variety of analytical techniques, such as high performance anion-exchange chromatography (HPAEC), methylation analysis, and a range of NMR analyses (1D $^{1}H$, $^{13}C$, 2D COSY, TOCSY, HSQC, HMBC and NOESY). The analyzed AGs have been characterized in terms of physicochemical properties, monosaccharide constitution, glycosidic bond types and macromolecular conformations. To relate this structural data to biological function, the effect of characterized AG upon NO production from macrophage RAW 264.7 cells was studied. The cumulative goal of this research is to contribute to the scientific discourse regarding the relationship between structure and function of AGs with respect to human health.

## 2. Materials and methods

### 2.1. Materials and reagents

Wood chips from *L. principis-rupprechtii* certified by plant taxonomist were supplied by Beijing Forest Bureau, Beijing, China. Prior to experimentation, the chips were crushed to 20–80 mesh wood powder. DEAE-Sepharose Fast Flow and Superdex 75 pre grade gel filtration media were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Deuterium oxide ($D_2O$), dextrans of different molecular weights and monosaccharide standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from Gibco. Nitric Oxide (NO) assay kits were obtained from Beyotime (Shanghai, China). All other employed reagents were of analytical grade.

### 2.2. Extraction and purification of polysaccharides

Wood powder from *L. principis-rupprechtii* (300 g) was extracted twice with 3 L of distilled water for 2 h at 60 °C to isolate AG. After extraction, both solid and liquid were combined and centrifuged to obtain the AG-containing supernatant. To recover AG from the liquid phase, precipitation was induced by adding three volumes of 95% ethanol by holding at 4 °C overnight. After time, the precipitate was separated by centrifuging at 9600 g for 10 min and then collected. The recovered precipitate was then re-dissolved in distilled water, and the precipitation protocol was repeated twice more. After the conclusion of third cycle, the precipitate was freeze-dried to obtain crude AG.

Crude AG was redissolved in deionized water and purified with a DEAE-Sepharose Fast Flow column (XK1.6 × 15 cm, GE-healthcare). The column was eluted with deionized water, 0.05, 0.1, 0.2, 0.3 and 0.5 M NaCl. Resultant eluents were monitored by the phenol-sulfuric acid colorimetric assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1955). The eluted samples was collected and further purified on a Superdex 75 pre grade column (Tricon 1 × 60 cm) using deionized water as eluent, collecting main fraction, respectively. Finally, two purified fractions named AGW (Water elution fraction) and AGS (NaCl elution fraction) were obtained.

### 2.3. Elementary analysis

The elemental composition of AG was determined by measuring the atomic contents of carbon, hydrogen and nitrogen in AGW and AGS with Thermo Scientific Flash 2000. For analysis, ~1.5 mg of AG was weighed into a tin capsule. A blank value of an empty tin capsule was recorded under the same conditions to establish a baseline value.

### 2.4. Molecular weight determination

The average molecular weights and polydispersity of both AGW and AGS were determined by GPC on Agilent 1260 system (Agilent, USA) equipped with Ultrahydrogel 250 and Ultrahydrogel 2000 columns in series (7.8 × 300 mm, Waters Corp, USA). Column temperature was kept at 50 °C. The sample solution (2 g/L) was eluted with 0.05 M $NaNO_3$ flowing at the rate of 0.6 mL/min. The average molecular weights were calculated based upon retention time results of different dextrans standards.

### 2.5. Monosaccharide composition analysis

To assay the monosaccharide constitution of various AG preparations, ~10 mg of the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h. Excess TFA was removed by mixing in methanol and forcing co-evaporation under vacuum. Resultant monosaccharides from hydrolysate were analyzed by a high performance anion-exchange chromatography system (Dionex ICS-5000, USA) equipped with a CarboPac™ PA10 column (2 × 250 mm) and a pulsed amperometric detector. The elution program consisted of an initial isocratic elution in 37 mM NaOH from 0 to 20 min, followed 200 mM $CH_3COONa$ from 20 to 35 min, and finally equili-brated in 37 mM NaOH from 35 to 50 min.

### 2.6. Methylation analysis

Methylation of AGW and AGS was conducted according to the method of previous reports (Hakomori, 1964). Methylated polysaccharides were dialyzed with distilled water for 24 h and next extracted by chloroform. The chloroform layer was concentrated and dried. The methylated products were hydrolyzed with 2 mol/L TFA at 105 °C for 6 h. The hydrolyzate was reduced with sodium borodeuteride (10 mg) for 4 h at room temperature, following by an acetylation step utilizing acetic anhydride (0.5 mL) and pyridine (0.5 mL) for 2 h at 100 °C. Methylated alditol acetates were finally analyzed by a Thermo Trace ISQ GC–MS system (Thermo Fisher Scientific, USA). The initial column temperature was set at 80 °C (held for 2 min), and programmed