Fraction and chemical analysis of antioxidant active polysaccharide isolated from flue-cured tobacco leaves

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ABSTRACT

Background: The metabolic compounds from tobacco were investigated to posses various biological activities, such as antioxidant, antimicrobial and neuroprotective activities. **Materials and Methods:** Polysaccharides were isolated from the ultrasound-assisted extraction of flue-cured tobacco with hot water. The purified polysaccharides were analyzed by GC-MS, FT-IR, and TGA (Thermogravimetric analysis). The antioxidant activities of purified polysaccharides were evaluated in vitro. **Results:** Two polysaccharides (Fr-I and Fr-II) were isolated and purified. The analysis of monosaccharide composition in the polysaccharide by GC/MS revealed that Fr-I and Fr-II were heteropolysaccharides. Fr-I consisted of D-mannose and galactose, ribose and arabinose, while Fr-II was mainly composed of glucose, allose, galactose and mannose. Thermo gravimetric analysis (TGA) indicated that the degradation temperature (Td) of the Fr-I (241 °C) was higher than that of Fr-II (220 °C). Both samples showed inhibitory effects on OH (hydroxyl) and DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) radical in a concentration-dependent manner. Comparing Fr-I with Fr-II, the latter has a strong scavenging ability. **Conclusion:** Both polysaccharide fractions showed significant antioxidant effects. Various factors influenced the antioxidant activity of polysaccharides.

Key words: Antioxidant activity, flue-cured tobacco leaves, fraction, polysaccharide

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INTRODUCTION

In recent years, research has indicated that polysaccharides extracted from higher plants have a certain antioxidant activity towards free radicals.[1,2] Moreover, most plant-derived polysaccharides are relatively non-toxic and do not cause severe side effects. Thus, plant polysaccharides represent ideal candidates as food additives and medicines.[3,4]

Flue-cured tobacco (*Nicotiana tabacum* L) is one of the most important commercial types of tobacco for production in the world. The metabolic compounds from tobacco were investigated to posses various biological activities, such as antioxidant, antimicrobial and neuroprotective activities.[5,6] To the best of our knowledge, water-soluble polysaccharides from flue-cured tobacco leaves have been little investigated.

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In recent years, ultrasound-assisted extraction (UAE) has been employed for preparing polysaccharides from plant materials efficiently, mainly attributed to its mechanical effects, which greatly facilitate mass transfer between immiscible phases through a super agitation.[7] In the present work, with the method of UAE, the polysaccharide was obtained from the leaves of flue-cured tobacco by water as extracting solvent and ethanol as precipitating agent. Then, two polysaccharide fractions were purified by Sepharose CL-6B column chromatography. Both purified polysaccharides were analyzed by GC-MS, FT-IR, and TGA (Thermogravimetric analysis). Finally, their associated antioxidant activities on hydroxyl and DPPH radicals were evaluated *in vitro*.

MATERIALS AND METHODS

Materials and chemicals

The flue-cured tobacco leaves (Yunyan 87) were kindly donated from the RandD center of Shanghai Tobacco (Group) Co., Ltd. (Shanghai, China). The tobacco leaves were dried at 45°C and ground into powder prior to the experiments. The standard monosaccharides including rhamnose, fucose, arabinase, xylose, mannose,

galactose, glucose, glucuronic acid and galacturonic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Sepharose CL-6B were obtained from Sigma (St. Louis, MO, USA). All other reagents/chemicals used were of analytical grade.

Extraction, separation and purification of the polysaccharides

The dried tissue powder (1g) was extracted with distilled water in a JY92-2D Ultrasonic cell grinder (Ningbo Scientz Biotechnology Co., LTD, China) with the following conditions: extraction power of 500 W, extraction temperature of 60°C, extraction time of 8 min and ratio of water to raw material of 25:1 (mL g^{-1}). The solution was concentrated in vacuo to 30% of original volume and precipitated with four volumes of absolute ethanol at 4°C overnight in a refrigerator to precipitate the polysaccharides. The precipitate was separated by centrifugation (10,000rpm for 15 min) and repeatedly washed sequentially with smaller amounts of ethanol, acetone and ether then dialyzed and lyophilized in vacuum, yielding the water-extractable crude polysaccharides.

The crude polysaccharide above was re-dissolved in 0.2 M NaCl buffer, then applied to a Sepharose CL-6B column (2.4 cm × 100 cm) and eluted with the same buffer at a flow rate of 0.6 mL min^{-1}. Fractions (5.0 mL/tube) were collected and combined according to the total carbohydrate content determined by the phenol–sulfuric acid method at 490nm.[8] Protein concentration was determined according to the Bradford method at 280nm.[9] The two fractions obtained were concentrated and dialyzed, then concentrated and precipitated with a 4-fold volume of 95% ethanol, dialyzed and freeze-dried to obtain the polysaccharide.

Physicochemical properties analysis

The purified polysaccharides were ground with dry KBr powder (spectroscopic grade) and then pressed into a 1mm pellet for FT-IR measurement on a FT-IR infrared spectrometer (Bruker Tensor 27) in the frequency range 4000- 550 cm^{-1}.[10]

Monosaccharide composition was measured according to the following procedure: the polysaccharide (5mg) was first dissolved into 2mL of 2M trifluoroacetic acid (TFA), then the solution was heated at 120°C for 2h. The internal standard sugars were prepared and subjected to GC/MS analysis separately in the same way. The alditol acetates of polysaccharide fraction were analyzed by GC/MS (Varian Co., Model: Star 3600 CX, Lexington, MA, USA) fitted with a fused silica capillary column (Na form, 30 m × 0.25 mm, Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector. The quantity of fractions was determined from the peak area using response factors.[11]

Thermogravimetric analysis was done with a TA Q4000IR TGA apparatus using 15mg polysaccharide fraction of the test material. The TGA curve plots the TGA signal, converted to percent weight change on the *Y*-axis against the reference material temperature on the *X*-axis.[12]

Antioxidant activity assays

For the evaluation of antioxidant activity of polysaccharide extracted from tobacco leaves, DPPH Radical scavenging activity and OH radical scavenging activity were determined according to the methods of Eloff, *et al.*, and Wang *et al.*, respectively.[13,14] The radical-scavenging activities of polysaccharides, expressed as percentage inhibition of OH and DPPH, were calculated according to the formula: Radical scavenging rate (%) = $(A_{sample}-A_{blank})/(A_{control}-A_{blank})\times 100$ Where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, control (without polysaccharide) and blank (without H_2O_2, or DPPH), respectively. In both assays, the polysaccharide samples were pre-dissolved in water and tested at various concentrations in parallel with vitamin C (Vc) as an antioxidant reference (positive control).

Statistical analysis

All the data are shown in means ± S.D. (n = 3). Statistical analysis was carried out with three groups using one-way analysis of variance (ANOVA using SPSS (version 11.0, Chicago, IL).). The values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Fractions eluted with 0.2M NaCl revealed two higher metachromatic activities, named Fr-I and Fr-II respectively. The elution curve for polysaccharide fraction was shown in Figure 1. Although further detection by electrophoresis is

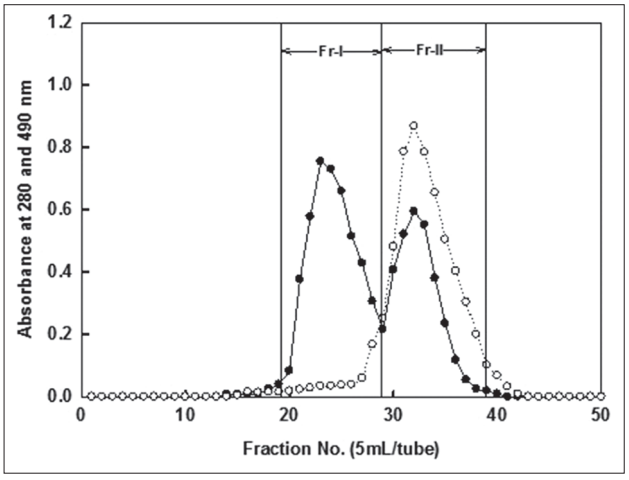

Figure 1: TGA thermogram of polysaccharide fractions (Fr-I and Fr-II) from flue-cured tobacco leaves

necessary, it might be revealed that Fr-II was a glycoprotein. The two polysaccharide fractions were respectively, pooled, dialyzed and lyophilized.

The IR spectrum could be seen in Figure 2. Two samples (Fr-I and Fr-II) were analyzed with FT-IR for detecting functional groups. Infrared spectra of both samples showed a broad stretching intense characteristic peak at approximately the region of 3285 cm^{-1} could be assigned to the stretching vibrations of hydrogen-bonded OH groups. The weak absorption bands at about 2928 cm^{-1} were attributed to C-H stretching.[15] Both samples exhibited a relatively strong absorption band appeared at 1585.3-1603.0 cm^{-1}, which was assigned to the C = O asymmetric stretching vibrations of the carboxylate ($–COO^-$) groups.[15] The stretching vibration peaks at around 1032.1-1039.7 cm^{-1} suggested the presences of C-O-H link bond position.[16]

GC-MS traces of the polysaccharide hydrolysates showed the monosaccharide components of the samples compared with standard monosaccharides. The monosaccharide composition of Fr-I and Fr-II are illustrated in Table 1. The result indicated that Fr-I was mainly composed of mannose (34.21%), galactose (18.05%), ribose (14.72%) and arabinose (10.04%), while Fr-II was mainly composed of glucose (25.76%), allose (17.99%), galactose (11.37%) and mannose (10.62%).

Thermal stability is one important physicochemical property for applications of polysaccharide.[12] The TGA analysis of purified Fr-I and Fr-II was carried out dynamically (weight loss versus temperature) and the experimental results are presented in Figure 3. According to the TGA curve, the degradation temperature (Td) of Fr-I and Fr-II was determined to be at 241°C, and 220°C, respectively. Furthermore, the weight of each fraction was dramatically lost around 300°C and continued gradually to decrease and the final residue was 9.14% for Fr-I and 24.97% for Fr-II. The results indicated that both fractions have high thermal stability. This data suggested that polysaccharide isolated can be used for functionalization and many other chemical modifications.

The antioxidant activities of two fractions from the flue-cured tobacco leaves were evaluated by two established *in vitro* systems, including hydroxyl radical and DPPH radicals. In this experiment, scavenging rates of Fr-I and Fr-II on DPPH free radical were investigated. As showed in Figure 4a, both EPS fractions demonstrated concentration-dependent antiradical activity by reducing the stable radical DPPH. The scavenging activities of Fr-II were stronger than those of Fr-I, and finally reached to 90% at the concentration of 2.0 mg mL^{-1}. The results of OH radical scavenging activities of two polysaccharide fractions are shown in Figure 4b. The results showed that the OH radical scavenging activities enhanced corresponding to

Table 1: Carbohydrate composition in the purified polysaccharide fractions (Fr-I and Fr-II) extracted from the flue-cured tobacco leaves

Carbohydrate composition (%)	Fr-I	Fr-II
D-deoxyribose	3.03	1.68
D-arabinose	10.04	6.63
L-rhamnose	5.34	7.30
D-ribose	14.72	6.95
D-(−)-lyxose	1.85	0.97
D-xylose	3.14	4.96
D-deoxyglucose	nd	4.29
D-(+)-tarot pyranose	4.99	1.49
D-(+)-galactose	18.05	11.37
D-glucose	2.00	25.76
D-(+)-mannose	34.21	10.62
D-allose	nd	17.99
D-glucuronic acid	2.63	nd

nd: Not detected

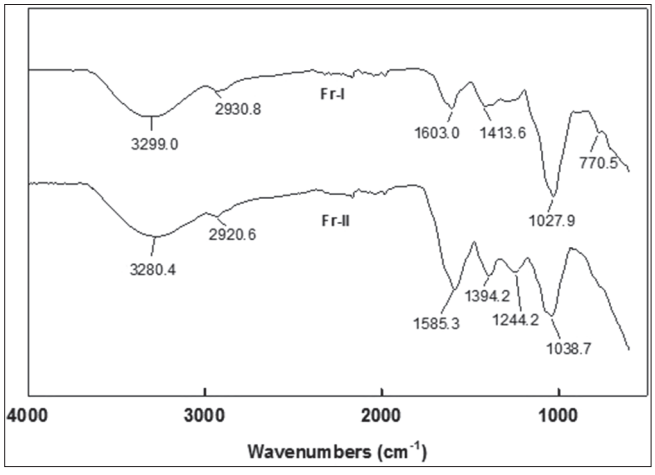

Figure 2: The FT-IR spectra of the polysaccharide fractions (Fr-I and Fr-II) from flue-cured tobacco leaves

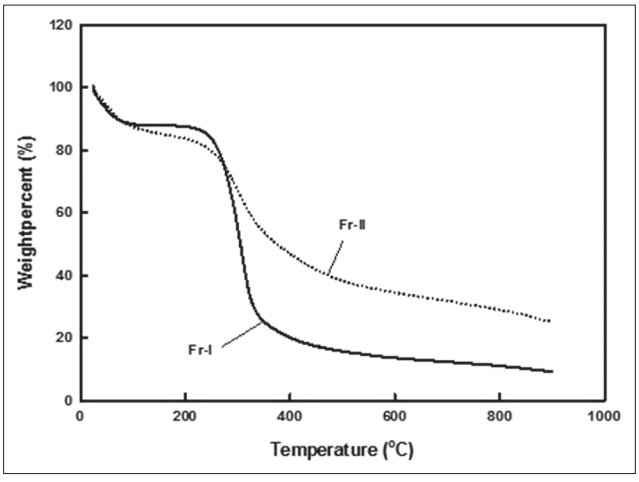

Figure 3: TGA thermogram of polysaccharide fractions (Fr-I and Fr-II) from flue-cured tobacco leaves

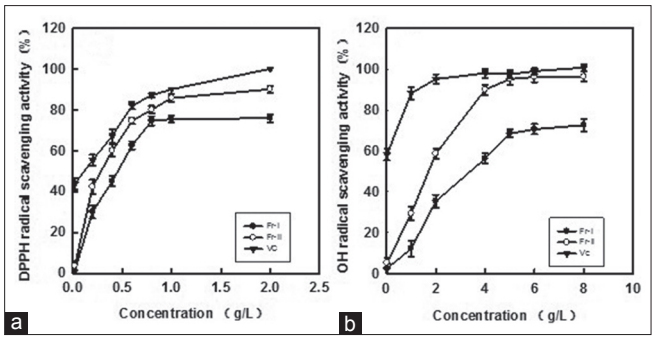

Figure 4: Antioxidant activity of polysaccharide fractions (Fr-I and Fr-II) from flue-cured tobacco leaves. The results represent mean ± S.D. (n = 3). DPPH (a) and OH (b) radical scavenging activity of polysaccharide., Fr-I (●), Fr-II (○) and Vitamin C (▼)

the increase with the concentration (0.02-8 mg/mL^{-1}). The ability of Fr-II was stronger than that of Fr-I at every concentration point and the OH radical scavenging rate at 5 mg mL^{-1} concentration was 95.21% (close to scavenging activity of vitamin C). In this experiment, both polysaccharide fractions showed significant antioxidant effects. The Fr-II has stronger antioxidant activities than Fr-I, which could be attributed to their monosaccharide compositions.

In conclusion, two homogeneous polysaccharides were isolated, and their physicochemical properties and antioxidant activities were determined. Various factors influenced the antioxidant activity of polysaccharides. Thus, further research is needed to explore the complete structure, conformation and mechanism of antioxidant activity.

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