Antioxidant Activity Potential of Virginia (Flue-Cured) Tobacco Flower Polysaccharide Fractions Obtained by Ultrasound-Assisted Extraction

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Ultrasound-assisted extraction was employed to extract polysaccharide from Virginia (flue-cured) tobacco flowers. The orthogonal matrix method (L₉(3)⁹) was used to determine the optimal extraction conditions as to ultrasound power, extraction time, ratio of solvent to solid, and extraction temperature at 300 W, 4 min, 35 (mL/g), and 70 °C respectively. The crude extract was successively purified by chromatography, yielding two major polysaccharide fractions, termed Fr-I and Fr-II. Both fractions are heteropolysaccharides, mainly containing glucose, mannose, and allose with an α-configuration. Thermo gravimetric analysis (TGA) indicated that the degradation temperatures (Td) of Fr-I and Fr-II were 185 °C and 190 °C respectively. The preliminary antioxidant activity test in vitro showed both fractions could potentialize the scavenging effect on hydroxyl and DPPH radicals in a dose-dependent manner. In conclusion, the two polysaccharides may be useful as naturally potential antioxidant agents for application in food and medicinal fields.

Key words: extraction; optimization; polysaccharide; antioxidant activity; tobacco flower

Polysaccharides are generally present in the cell walls of plants. They can have a thickening, stabilizing, or jelling effect that maintains plant texture. In recent years, bioactive polysaccharides of low cytotoxicity extracted from plants have emerged as important bioactive materials.1,2) The polysaccharides extracted from higher plants are widely used in the food, pharmaceutical, and cosmetic industries as gelling agents, thickeners, and stabilizing agents.3) Tobacco metabolites have been found to pose various biological activities in several pharmacological areas, including central nervous system (CNS), obesity disease and other diseases.4–6) To the best of our knowledge, although the characteristics and bioactivities of polysaccharides for several plant flowers are known, polysaccharides from tobacco flowers have been little investigated.7,8)

Various novel techniques for the extraction of bioactive substances from plants have been developed recently. Ultrasound-assisted extraction (UAE) has the advantage of accelerating the extraction process, causing less change to the structural and molecular properties of plant materials.9) For these reasons, it is widely used today to assist in the extraction of polysaccharides from plant materials.10)

In this present study, four parameters, ultrasound power, extraction time, ratio of solvent to solid and extraction temperature, were optimized by the orthogonal matrix method. Crude polysaccharide was purified by Sepharose CL-6B column chromatography. The chemical composition and structural features of the purified polysaccharide were measured and its thermal stability was confirmed. Finally, the antioxidant properties of flower polysaccharide are reported here for the first time. This report introduces flue-cured tobacco flower polysaccharide as a possible valuable source of the unique antioxidant properties.

Materials and Methods

Materials and chemicals. Flue-cured tobacco flowers were kindly provided by the Xuchang Tobacco Planting Area (Henan, China). The tobacco flowers were dried at 45 °C and sieved with a 40-mesh sieve (pore size, 0.42 mm). Standard monosaccharides were purchased from Sigma–Aldrich (St. Louis, MO), and Sepharose CL-6B was also from Sigma. All other chemicals were of analytical reagent grade.

Ultrasound-assisted extraction of polysaccharide. The procedure for UAE was as developed by Wu et al., with some modifications.11) One g of tobacco flower powder (TFP) was extracted with distilled water in a JY92-2D Ultrasonic cell grinder (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). Extraction was carried out at a variety of ultrasound power levels (200–400 W) at various temperatures (50–70 °C), ratios of solvent to solid (25–35; v/w), and extraction times (2–6 min). Debris fragments of the polysaccharide extracts were removed by centrifugation. After filtration to remove the debris fragments, the filtrate was concentrated with a rotary evaporator and precipitated with four volumes of absolute ethanol for 48 h at 4 °C. The precipitates were washed 3 times with 50, 75, and 100% ethanol and filtered in order to remove mono- and disaccharides. The resulting precipitates were collected by centrifugation (10,000 r/min, 15 min) and redissolved in distilled water. The ethanol precipitate of the crude polysaccharide was deproteinized with Sevag reagent (1:4 n-butanol/chloroform, v/v). After removal of free proteins and Sevag reagent by centrifugation, the aqueous phase was dialyzed against deionized water and dried by vacuum freeze-drying to obtain the crude polysaccharide (CPS). The percentage polysaccharide extraction yield (%) was calculated as follows:

\[ \text{Extraction yields (%, w/w)} = \frac{W_{\text{CPS}}}{W_{\text{TFP}}} \times 100 \]  

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Table 1. Experimental Factors and Their Levels for Orthogonal
Projects*  

<table>
<thead>
<tr>
<th>Level</th>
<th>Power (A) W</th>
<th>Time (B) min</th>
<th>Ratio of solvent to solid (C)</th>
<th>Temperature (D) °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>2</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>4</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>6</td>
<td>35</td>
<td>70</td>
</tr>
</tbody>
</table>

* A, B, C, and D represent factors for extraction. 1, 2, and 3 represent concentration levels of a factor.

where W_{CS} was defined as the weight of CPS and W_{TTP} as the weight of TFP used.

Optimization of polysaccharide extraction. An orthogonal L_{0}(3^4) test design was applied to optimize the extraction conditions for polysaccharide from flue-cured tobacco flowers. As seen in Table 1, the extraction experiment was carried out with four factors at three levels based on preliminary single-experiment results. The extraction yield (%) of polysaccharide was chosen as the response.

Purification of crude polysaccharide. Crude polysaccharide was redissolved in 0.2 M NaCl buffer, loaded onto 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. Fractions of 5.0 mL/tube were collected by means of a fraction collector. The protein concentration was determined by the Bradford method with bovine serum albumin as standard. The total carbohydrate content of the polysaccharide was measured by the phenol-sulfuric acid method, using glucose as standard. The total sugar content of the polysaccharide was determined by the phenol-sulfuric acid method with glucose as standard. The protein moiety in the polysaccharide was monitored by measuring the absorbance at 280 nm, and the carbohydrate moiety was monitored at 490 nm. The peaks with the highest polysaccharide content were collected, dialyzed, and lyophilized to obtain purified polysaccharide for further analysis.

Monosaccharide composition analysis. To identify and quantify monosaccharide, the polysaccharide fraction (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 110°C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol, reduced with NaBH₄ for 30 min at 20°C and acetylated with acetic anhydride and pyridine at 100°C for 20 min. Internal standard sugars were prepared and subjected to GC/MS analysis separately in the same way. The alditol acetates of the polysaccharide fraction were analyzed by GC/MS (Star 3600, Varian, Lexington, MA) fitted with a fused silica capillary column (Na form, 30 m × 0.25 mm, Supelco, Bellefonte, PA) and a flame ionization detector.

Fourier transform-infrared (FT-IR) spectroscopy. FT-IR spectroscopy (Bruker Tensor 27, Bruker, Karlsruhe, Germany) was analyzed with the KBr disc for detecting functional groups. The purified copy (Bruker Tensor 27, Bruker, Karlsruhe, Germany) was analyzed by the KBr disc for detecting functional groups. The purified copy (Bruker Tensor 27, Bruker, Karlsruhe, Germany) was analyzed by the KBr disc for detecting functional groups. The purified copy (Bruker Tensor 27, Bruker, Karlsruhe, Germany) was analyzed by the KBr disc for detecting functional groups.

Thermo gravimetric analysis. Thermogravimetric analysis of the polysaccharide was done by TA Q5000IR TGA apparatus (TA Instruments, New Castle, DE) with a 15-mg 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.

Antioxidant activity assays. To evaluate the antioxidant activity of the polysaccharide extracted from tobacco flowers, DPPH radical scavenging activity and OH radical scavenging activity were determined by the methods of Eloff et al. and Wang et al. respectively. In both assays, the polysaccharide samples were predissolved in water and tested at various concentrations in parallel with vitamin C (Vc) as antioxidant reference (positive control).

Statistical analysis. All statistical analyses were performed in triplicate, and data were expressed as mean ± SD. Statistical significance was determined by Student’s t-test. Experimental results from the experimental design were statistically subjected to analysis of variance (ANOVA) by SPSS (version 11.0, Chicago, IL). Probability values of <0.05 and <0.01 were regarded as statistically significant and highly significant respectively.

Results and Discussion

Optimization of extraction conditions by the orthogonal matrix method

The orthogonal matrix L_{0}(3^4) method was used to optimize the process of ultrasound-assisted extraction and to investigate the relationships between variables of the extraction factors. The experimental conditions for each project are listed in Table 2, and the experimental results are also included, in the last column of this table. By the orthogonal method, the effect of those extraction factors on extraction yield was calculated, and the results are shown in Table 2. According to the magnitude order of R (Max Diff), the order of effect of all factors on extraction yield was determined: extraction temperature > ratio of water to raw material > extraction power > extraction time. This result indicates that the effect of extraction temperature was more important than the other factors. To obtain the optimization levels for each of the factors, the optimum extraction conditions were A_{2}B_{2}C_{3}D_{1}, that is, extraction power 300 W, extraction time 4 min, ratio of water to raw material 35:1 (mL/g), and extraction temperature 70°C. Through a confirmatory test, a 3.76% extraction yield was obtained under the above optimum conditions. This indicates that the selected conditions were truly the most suitable.

Isolation, purification, and analysis of polysaccharide

In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, two fractions (designated Fr-I and Fr-II) of polysaccharide were co-eluted, as shown in Fig. 1. Although further detection is necessary by electrophoresis, it might be that both fractions were glycoproteins. The details of the monosaccharide compositions of the carbohydrate in the two polysaccharide fractions by the trifluoroacetic acid hydrolysis and the GC-MS analysis method are illustrated in Table 3. The results indicate that both fractions were heteropolysaccharides consisting mainly of mannose, allose, and glucose. However, the main difference was that Fr-II was an acidic polysaccharide composed of galacturonic acid and glucuronic acid, while Fr-I was a neutral polysaccharide.

FT-IR spectroscopy of the polysaccharides

FT-IR is an effective analytical instrument for acquiring covalent bonding information and detecting functional groups. Typical IR spectra for two polysaccharide fractions are presented in Fig. 2. All samples exhibited a characteristic broad intense stretching peak at approximately the region of 3,270 cm⁻¹ for the carbohydrate ring, and a weak C–H band at about 2,928 cm⁻¹. A characteristic absorption band appeared at 1,600 cm⁻¹, and was assigned to the stretching vibration of the carboxyl group (C=O) of the polysaccharide. The band at 1,550 cm⁻¹ of Fr-I corresponds to a stretching or N–H deformation of amine II. The prominent absorption bands at 1,071–1,074 cm⁻¹ sug-
ggested the presence of a pyranose ring. Characteristic absorption at 882 cm$^{-1}$ was found in the IR spectrum, indicated an $\alpha$-dominating configuration in pyranose form of the sugar.

**Thermal analysis of the polysaccharides**

The increasing importance of polysaccharides has motivated scientists to study their physicochemical properties to find property-based applications. For most of these applications, thermal stability is important. Thermal stability can be determined by TGA. This involves measuring a sample’s change in mass with variations in temperature, and it is a very useful technique for analyzing samples that either gain or lose mass during heating. TGA analysis of the purified polysaccharide fractions was carried out dynamically (weight loss versus temperature). The experimental results are presented in Fig. 3. On TGA, both fractions exhibited endothermic weight losses of 8–18% in the 100–190 °C range, due to a loss of trapped water. The degradation temperatures (Td) of Fr-I and Fr-II were determined to be 185 °C and 190 °C respectively. This suggests that the material should not be submitted to the temperature Td in order not to compromise the physical integrity of the material evaluated. The Tds of both fractions were lower than hemicellulose (220–315 °C) and cellulose at (315–400 °C), due to their different chemical compositions. Furthermore, the weight of each fraction was dramatically lower at about 240 °C and continued gradually to decrease, and the final residue was 19.91% for Fr-I and 28.55% for Fr-II. Thus, it appears that two fractions from flue-cured tobacco flowers possessed high thermal stability. Furthermore, Fr-I and Fr-II showed similar behavior as to degradation, probably due to their similar chemical compositions.

**Antioxidant property analysis of the polysaccharides**

The *in vitro* antioxidant capacities of the two fractions from flue-cured tobacco flowers were evaluated by different biochemical methods, including hydroxyl and DPPH radical scavenging assay. The hydroxyl radical can induce significant damage to adjacent biomolecules. Hence removing hydroxyl radicals is important for antioxidant defense in living cell systems. The results for the hydroxyl radical scavenging activities of the two polysaccharide fractions with Vc as positive control are shown in Fig. 4A. They indicated that the two fractions

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**Table 2. Application and Analysis of L$\sigma$(3$^\alpha$) Orthogonal Projects to Polysaccharide Extraction from Flue-Cured Tobacco Flowers**

<table>
<thead>
<tr>
<th>Run</th>
<th>A: Power (W)</th>
<th>B: Time (min)</th>
<th>C: Ratio of solvent to solid</th>
<th>D: Temperature (°C)</th>
<th>Extraction yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1(200)</td>
<td>1(2)</td>
<td>1(25)</td>
<td>1(50)</td>
<td>3.310 ± 0.043</td>
</tr>
<tr>
<td>2</td>
<td>1(200)</td>
<td>2(4)</td>
<td>2(30)</td>
<td>2(60)</td>
<td>3.618 ± 0.037</td>
</tr>
<tr>
<td>3</td>
<td>1(200)</td>
<td>3(6)</td>
<td>3(35)</td>
<td>3(70)</td>
<td>3.741 ± 0.032</td>
</tr>
<tr>
<td>4</td>
<td>2(300)</td>
<td>1(2)</td>
<td>2(30)</td>
<td>3(70)</td>
<td>3.710 ± 0.050</td>
</tr>
<tr>
<td>5</td>
<td>2(300)</td>
<td>2(4)</td>
<td>3(35)</td>
<td>1(50)</td>
<td>3.556 ± 0.035</td>
</tr>
<tr>
<td>6</td>
<td>2(300)</td>
<td>3(6)</td>
<td>1(25)</td>
<td>2(60)</td>
<td>3.417 ± 0.028</td>
</tr>
<tr>
<td>7</td>
<td>3(400)</td>
<td>1(2)</td>
<td>3(35)</td>
<td>2(60)</td>
<td>3.372 ± 0.068</td>
</tr>
<tr>
<td>8</td>
<td>3(400)</td>
<td>2(4)</td>
<td>1(25)</td>
<td>3(70)</td>
<td>3.367 ± 0.022</td>
</tr>
<tr>
<td>9</td>
<td>3(400)</td>
<td>3(6)</td>
<td>2(30)</td>
<td>1(50)</td>
<td>3.318 ± 0.071</td>
</tr>
</tbody>
</table>

$k_i$ = (Σ extraction yield at $A_i$)/3
$k_i$ = max[$k_i$] − min[$k_i$]

**Table 3. Carbohydrate Compositions of Purified Polysaccharide Fractions (Fr-I and Fr-II) Extracted from Flue-Cured Tobacco Flowers**

<table>
<thead>
<tr>
<th>Carbohydrate composition (%)</th>
<th>Fr-I</th>
<th>Fr-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>4.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Ribose</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.8</td>
<td>6</td>
</tr>
<tr>
<td>Talose</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>12.3</td>
<td>9.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.6</td>
<td>17.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>25.3</td>
<td>22.8</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Allose</td>
<td>24.7</td>
<td>21</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>0</td>
<td>9.1</td>
</tr>
</tbody>
</table>

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**Fig. 1. Elution Profiles of Pectic Polysaccharide on Sepharose CL-6B Chromatography.**

Eluates were analyzed by measuring the absorbance at 490 nm for carbohydrates (●) and the absorbance at 280 nm for proteins (○).
exhibited scavenging activities towards hydroxyl radicals in a concentration-dependent manner. The potency of Fr-I was stronger than that of Fr-II at every concentration point, and the OH radical scavenging rate at 4 mg/mL was 91.2% (close to the scavenging activity of vitamin C). The DPPH radical is widely used to evaluate the free-radical scavenging ability of natural compounds.24 In this experiment, the scavenging rates for Fr-I and Fr-II on the DPPH free radical are showed in Fig. 4B. The results indicate that both polysaccharide fractions showed significant antioxidant effects, and that the polysaccharide fractions increased radical scavenging activity in a concentration-dependent manner. The scavenging activities of Fr-I were stronger than those of Fr-II, and finally reached 94.5% at a concentration of 2.0 mg/mL. This suggests that the composition of the non-dominated monosaccharide composition of polysaccharide might affect their antioxidant activity. The results indicate that both fractions are potential free-radical scavengers, and that their activities against radicals were closely associated with their polysaccharide compositions. The antioxidant activity of polysaccharides, might be attributed to their hydroxyl groups and other functional groups, such as –COOH, C=O, and –O–, which can donate electrons, reducing the radicals to a more stable form or reacting with free radicals to terminate the radical chain reaction.25)

In conclusion, the orthogonal matrix method was applied to optimize ultrasound-assisted extraction parameters for polysaccharides, from flue-cured tobacco flowers. The highest yield of crude polysaccharide, 3.76% (w/w), was obtained under optimal conditions: ultrasound power 300 W, extraction time 4 min, ratio of water to raw material of 35:1 (mL/g), and extraction temperature 70°C. Two fractions of polysaccharides were obtained through purification of crude polysaccharide by Sepharose CL-6B column chromatography. Characterization of both fractions was done by FT-IR spectroscopy, GC, and TGA. Both polysaccharide fractions showed strong antioxidant activity. In order to understand the structure-function relationship better, further work on the molecular features and bioactivities of carbohydrate and protein moieties in both fractions is in progress in our laboratory.

References