Cellulase-Assisted Extraction, Characterization, and Bioactivity against Rheumatoid Arthritis of Astragalus Polysaccharides

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This study investigated the effect of cellulase on the isolation of crude Astragalus polysaccharide (APS), analyzed the monosaccharide component of deproteinized APS, detected the molecular weights of purified APS, and examined the biological activities and the preliminary mechanism against rheumatoid arthritis (RA). Compared with water extraction method, cellulase-assisted extraction increased the yield of crude APS to 154% and polysaccharide contents to 121%. Crude APS was then purified by ethanol precipitation, Sevag deproteinization, and high-performance liquid chromatography (HPLC) analysis; monosaccharide contents of APS were different after cellulase-assisted method, especially galacturonic acid content which significantly increased. DEAE-52 cellulose column chromatography isolated three polysaccharide fractions, including a neutral polysaccharide (APS-water) and two acidic polysaccharides (APS-NaCl1 and APS-NaCl2). Using high-performance gel permeation chromatography (HPGPC), the molecular weights of APS-water, APS-NaCl1, and APS-NaCl2 were identified as 67.7 kDa, 234.1 kDa, and 189.4 kDa, respectively. Then their therapeutic effects and possible mechanism against RA were explored using type II collagen-induced arthritis (CIA) rat model. APS could significantly reduce paw swelling, serum concentration of IL-1β and TNF-α, and the expression levels of NF-κB-p65 and IκBα in synovial membranes in CIA rats. Our study indicated that cellulase significantly increases the yield and polysaccharide contents of crude APS, improves the product quality, and preserves the biological features against RA in CIA rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, and systemic disease. The pathological features of RA are joint synovitis and pannus formation [1]. Currently, there is no known cure; the treatment aim is to prevent RA progression by keeping away from joint inflammation, destruction, and function loss [2]. Inflammation is the basis of RA development; therefore, it is an important target for RA therapy [3]. It has been proven that clinical agents targeting inflammatory mediators or various cytokines, such as antibody drugs (adalimumab and anakinra) which interfere with the progression of inflammation [4] and glucocorticoid drugs which inhibit cellular and humoral immunity [5], could prevent RA. Chemical drug treatment, alone or in combination, although can take significant effect in short term, is easy to illness relapses and side effects on patients. Biological agents, although work highly effective with low side effects, are very expensive [6]. Thus, there are still a lot of limitations in the clinical application of drugs against RA inflammation. It is necessary to continuously research and develop anti-inflammation, high-efficiency, low-toxicity, and moderate-priced drugs against RA.

Treatment of RA using Chinese herbal remedies has a long history [7]. In recent years, effective herbal medicine...
extraction, such as tripterygium polyglycoside (TG), has shown good anti-inflammatory effects in the treatment of both RA animal models and patients [7]. It has been reported [8] that Astragalus polysaccharides (APS) inhibited cell growth and proinflammatory responses in IL-1β-stimulated fibroblast-like synoviocytes (FLSs). And APS is safe without any distinct toxicity and side effects [9], thus can be used for a long time. However, APS is mainly extracted using water extraction method, which has several disadvantages, such as low active ingredients (polysaccharide content), especially the yield (only about 2.5%) of crude APS [10], and high cost. All of these limit the development and utilization of APS. Enzyme engineering technology is a bioengineering technology used in recent years for the extraction of active constituents from natural plants [11]. Appropriate enzyme can decompose the plant tissues more gently and release the active ingredients faster, therefore increasing the extraction efficiency. The cell wall of Astragalus membranaceus is majorly composed of cellulose and is also the main barrier in releasing macromolecules, such as intracellular polysaccharides. The use of cellulase can hydrolyze cell walls and facilitate the dissolution of intracellular components [12].

In this study, cellulase was used to assist extraction of crude APS from Astragalus membranaceus, aimed at improving APS extraction efficiency, shortening the extraction time, preserving the biological activity of APS, and maximizing the utilization of APS. Then the crude APS was further purified and identified, and the effect and preliminarily mechanism of purified APS on treating RA in rat models were investigated.

2. Materials and Methods

2.1. Materials. Raw roots of Astragalus membranaceus (Fisch.) Bge. were obtained from Tongrentang Group Corporation (Beijing, China).

2.2. Extraction of Crude APS. The roots were dried and pulverized then were soaked in 80% ethanol overnight. After filtering with gauze, the residue was soaked with pure water at a mass ratio of 1:8 and was boiled for 2 h. When the temperature was lowered to 50°C, pH was adjusted to 5.5 with 0.5% sulfuric acid, and then cellulase (SDG-2425, Sunson, Beijing, China) was added to the final enzyme activity at 60 U per gram of raw materials. The residue was stirred for 90 min at 50°C and then was boiled for 1 h to inactive cellulase. After filtering with gauze again, the residue was soaked with pure water at a mass ratio of 1:5, then the cellulase hydrolysis process was repeated. The filtrates from two extraction processes were combined and concentrated by rotary evaporation, followed by removing impurity through stirring and soaking in 95% ethanol for 24 h. The precipitate was centrifuged, dried, and weighed, to be named crude APS. The crude APS extraction yield was calculated by the following:

\[
\text{Crude APS extraction yield} = \left( \frac{\text{Weight of crude APS}}{\text{Weight of raw materials used}} \right) \times 100\%.
\]  

2.3. Determination of Polysaccharide Contents. The polysaccharide contents of crude APS were determined by phenol-sulfuric acid method. Crude APS was dissolved in deionized water, then 2.0 ml of each dissolved sample was mixed with 1.0 ml of 5% phenol, followed by shaking well. And then 5.0 ml of sulfuric acid was added and shaken vigorously at room temperature for 5 min. After boiling for 15 min and then cooling for 30 min, absorption at 490 nm was recorded on the basis of deionized water as blank control. Polysaccharide concentration was calculated according to the glucose standard curve. Then polysaccharide content was calculated by formula (2), and APS extraction yield was calculated by formula (3):

\[
\text{APS content} = \left( \frac{\text{Weight of polysaccharide tested}}{\text{Weight of crude APS used}} \right) \times 100\%.
\]

\[
\text{APS extraction yield} = \text{APS content} \times \text{Crude APS extraction yield}.
\]

2.4. Monosaccharide Composition Analyses. The protein in crude APS was removed with solvent of chloroform: n-butanol (3:1), and the supernatant was collected by centrifugation then was dried and weighed [13]. 10 mg APS was first hydrolyzed with 1 ml sulfuric acid solution (2 mol/l) at 110°C for 6 h. After precipitation and neutralization by barium sulfate, the sample was centrifuged and the supernatant was retained. Then HPLC was applied for monosaccharide composition analyses of vacuum dried APS. 1-Phenyl-3-methyl-5-pyrazolone (PMP) derivatives of various monosaccharide standards (Sigma, MO, USA) and monosaccharide from APSs were prepared by the reported method [14]. Then a Shimadzu LC-20A HPLC system (Kyoto, Japan) was used to analyze the monosaccharide composition of the APS according to the literature methods [15].

2.5. Isolation and Purification of APS. After purification by Sepag method and vacuum drying, APS was dissolved in deionized water to 0.2 g/ml and filtered using syringe filter (0.45 μm pore). 10 ml APS filtrate was loaded on a DEAE-52 cellulose column (2.4 × 70 cm, 4057-200, Whatman, Buckinghamshire, UK) that had been equilibrated with deionized water. The column was subsequently eluted with 400 ml deionized water, followed by 800 ml 0.5 M NaCl aqueous solution with a flow rate of 1 ml per min. The eluate was collected into each tube (10 ml per tube), respectively. Polysaccharide content in each tube was measured through phenol-sulfuric acid method at 490 nm. The eluates of each major fraction were pooled, dialyzed, concentrated, and lyophilized. The three combined eluted fractions were denoted as APS-water, APS-NaCl1, and APS-NaCl2.

2.6. Molecular Weight Determination. High-performance gel permeation chromatography (HPGPC) was used to evaluate molecular weights and homogeneity of the three polysaccharide fractions from DEAE-52 cellulose column [16]. APS-water, APS-NaCl1, or APS-NaCl2 was dissolved in deionized water to 2 mg/ml and filtered through syringe filter (0.45 μm
pore). 20 μl filtrate was applied to a TSKgel G-3000PWXL liquid chromatography column which was eluted with deionized water with a flow rate of 0.6 ml/min. A series of different molecular weight of dextran standards (1.0-20.0 kDa, Sigma, MO, USA) were applied for calibration. The standard curve was obtained through plotting the logarithm of the known molecular weight of each standard against the corresponding retention time. Afterwards, the molecular weight was determined from the previously constructed standard curve according to the retention time of each sample.

2.1. Western Blotting. The mean values of volume were calculated and plotted using a plethysmometer (520-IITC, Thermo, MA, USA). The mean values of volume were measured to indicate the hind paw swelling once a week at day 0, after CFA injection, and before and after treatments. Paw volume was measured to indicate the hind paw swelling using a plethysmometer (520-IITC, Thermo, MA, USA). The mean values of volume were calculated and plotted at each time point.

2.9. ELISA. 2 h after administration on day 28, all rats were sacrificed. Blood was collected from the femoral artery and centrifuged for 15 min at 4000 rpm at 4°C. The serum (supernatant) was collected and kept at -80°C. Cytokine concentrations (TNF-α and IL-1β) were quantified through ELISA kits (Enzo, Ramssdonksveer, Netherlands) according to the manufacturer’s procedure. Optical density (OD) values were recorded at 450 nm in a microplate reader. Concentrations were calculated according to the standard curve.

2.10. Western Blotting. After all rats were anesthetized and sacrificed on the last day of experiment, the synovial membranes were collected together in each group and immediately stored at -80°C. For western blotting, each tissue was lysed to extract total protein by using RIPA buffer (Sigma, MO, USA). Then the protein concentration for each sample was determined by BCA kit (Thermo, MA, USA) and loaded SDS by boiling for 5 min with loading buffer (Boston Biochem, MA, USA). The SDS-loaded protein was separated by SDS-PAGE according to procedures in Bio-Rad system (CA, USA), followed by transferring to PVDF membrane (Merck Millipore, MA, USA). The membranes were blocked with 5% goat serum (in TBST), then incubated in refrigerator overnight with the following primary antibodies, anti-NF-κB-p65 monoclonal (Cell Signaling Technology, 1:800, MA, USA) and anti-IκBα monoclonal (Cell Signaling Technology, 1:1000, MA, USA). Signals were further detected using appropriate IRDye 800CW-conjugated secondary antibodies at room temperature for 1 h. The complex was visualized with imaging systems (Li-Cor Biosciences, USA). GAPDH on the same membrane was used as a loading control. All western blotting analyses were performed in triplicate.

2.11. Data Analysis. All statistical analyses of this study were run with IBM SPSS 19.0. We performed one-way analysis of variance (ANOVA) to determine significant differences, followed by LSD-Q test for comparison between groups. Results were presented as means ± standard deviation. P values less than 5% reflected statistical significance.

3. Results and Discussions

Traditional Chinese medicine (TCM) has a long history of usage in treating RA [18]. Recently, a variety of TCM drugs and methods with good curative effect and few side effects have emerged. These can reduce the side effects in therapy with NSAIDs (nonsteroidal anti-inflammatory drugs) and glucocorticoids, showing the characteristics and advantages of TCM in treating RA [18]. Fangji Huangqi decoction is a common prescription for RA treatment, and its therapeutic effect has been clinically proven [19]. Astragalus membranaceus is the major component of Fangji Huangqi decoction. Pharmacological studies have shown that APS, as the main ingredient of Astragalus membranaceus, has various therapeutic functions, such as immune regulation [20, 21], anti-inflammation [7, 22], antioxidation [23], antivirus [24, 25], antibacterial [26], lowering blood sugar [27], and protecting the liver [28] and kidney [29]. Furthermore, it has been reported that APS could prevent RA progression [8, 30]. However, as a macromolecule, the extraction yield and purity of APS are greatly limited [10]. Thus, a variety of technologies are used to assist the water extraction process to elevate the yield and purity of APS. Cellulase can accelerate the release of APS from plant cells; more importantly, it also preserves the structural integrality and bioactivity of APS without destruction because cellulase swells the cell wall with high specificity [12]. This present study was to investigate the assisted effect of cellulase on crude APS extraction. Furthermore, the crude APS was purified and the physicochemical properties of purified APS were determined, and
3.1. Cellulase Increased the Extraction Efficiency of Crude APS from *Astragalus membranaceus*. As shown in Table 1, crude APS yield increased from 2.45%±0.32% by water extraction method to 3.78%±0.43% with cellulase-assisted extraction method, increasing 54%. Polysaccharide content in crude APS increased from 25.71%±1.82% by water extraction method to 31.24%±2.71% with cellulase treatment. Thus, extraction yield of APS was increased to 187% by water extraction method, from 0.63%±0.11% to 1.18%±0.25%. Our results indicated that the yield and polysaccharide content of crude APS were greatly improved by cellulase-assisted extraction method, compared to conventional method. Chen et al. [11] studied the effect of various enzymes on the yield of APS; similar to our results, they found that cellulase could improve APS extraction yield nearly to 1.5-fold. The function of cellulase is to decompose cellulose [12], which is the main structural component of the cell wall. It helps to increase the release of polysaccharides from the cytoplasm, thereby ultimately increasing yield and polysaccharide content. In our cellulase-assisted extraction procedure, repeated water extraction and cellulase treatment after boiling are two key steps. On the one hand, softening of raw material by boiling contributes to the full functionality of the cellulase. On the other hand, a large amount of saccharides obtained by initial enzymatic hydrolysis might exert inhibitory feedback on cellulase function. It has been reported that sucrose and glucose in the extract have a negative effect on cellulase activity [31]. Therefore, the secondary enzymatic treatment to the filter residue after filtration is necessary, and it helps to improve the efficiency of cellulase in the second extraction and fully extract the remaining polysaccharides.

Our study showed that the isolation procedure with cellulase-assisted treatment is simple and feasible and can greatly increase APS yield, indicating that cellulase treatment is an effective process in utilizing *Astragalus membranaceus*. However, there is still a need for identifying the biological activity and effectiveness of APS obtained in this process.

3.2. Purification of APS, Monosaccharide Composition, and Molecular Weight Analyses. Crude APS was isolated by hot water extraction, cellulase hydrolysis, and ethanol precipitation. After purification by Sevag method, vacuum drying, decomposition, and HPLC analysis, APS was identified with the composition of glucose, galactose, arabinose, mannose, and galacturonic acid (Table 2), and it showed the homogeneity of monosaccharide types of APSs extracted by different methods. However, the extraction method significantly influenced the monosaccharide contents (%). The contents of galacturonic acid, mannose, and arabinose of APS from cellulase-assisted extraction were 2.88%, 2.08%, and 12.23%, respectively, which were significantly higher than those of APS from water extraction (0.97%, 1.24%, and 6.56%). The result was similar to a study by Shang et al. [32] and suggested a better antioxidant activity and potential bioactivity of APS extracted from cellulase-assisted method [33].

Then after DEAE-52 cellulose ion-exchange column chromatography, three purified polysaccharide fractions were obtained (Figure 1). Anionic DEAE-52 cellulose chromatography column was selected because polysaccharide was negatively charged. Tube 11-22, tube 51-72, and tube 82-94 were combined, respectively, and then dialyzed and

### Table 1: Yield and content of *Astragalus* polysaccharides extracted by different methods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Crude APS extraction yield (%)</th>
<th>APS content (%)</th>
<th>APS extraction yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extraction</td>
<td>2.45 ± 0.32</td>
<td>25.71 ± 1.82</td>
<td>0.63 ± 0.11</td>
</tr>
<tr>
<td>Cellulase-assisted extraction</td>
<td>3.78 ± 0.43*</td>
<td>31.24 ± 2.71*</td>
<td>1.18 ± 0.25*</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>0.013</td>
<td>0.043</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation (*n* = 3). Significant effect compared to result by water extraction method: *P* < 0.05.

### Table 2: Monosaccharide composition of *Astragalus* polysaccharides extracted by different methods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (%)</th>
<th>Galactose (%)</th>
<th>Arabinose (%)</th>
<th>Mannose (%)</th>
<th>Galacturonic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extraction</td>
<td>65.11 ± 0.02</td>
<td>26.12 ± 0.81</td>
<td>6.56 ± 0.11</td>
<td>1.24 ± 0.07</td>
<td>0.97 ± 0.12</td>
</tr>
<tr>
<td>Cellulase-assisted extraction</td>
<td>47.32±0.03***</td>
<td>35.49±0.08****</td>
<td>12.23±0.18***</td>
<td>2.08±0.25**</td>
<td>2.88±0.08***</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.005</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation (*n* = 3). Significant effect compared to result by water extraction method: **P < 0.01; ***P < 0.001.

![Figure 1: Elution curve of polysaccharide fractions from crude APS by DEAE-52 cellulose column chromatography.](image-url)
lyophilized to obtain the fractions, APS-water, APS-NaCl1, or APS-NaCl2. APS-water is a neutral polysaccharide fraction which does not bind to the column and could be eluted by deionized water. APS-NaCl1 and APS-NaCl2 are acidic polysaccharides which bind to the column and could be eluted by 0.5 M NaCl solution.

Molecular weights of purified polysaccharide fractions were determined by HPGPC. As shown in Figure 2, three polysaccharide fractions exhibited a single peak, which indicated that the fractions were homogeneous. Based on the calibration curve of dextran standards, the average molecular weights of APS-water, APS-NaCl1, and APS-NaCl2 were 67.7 kDa (Figure 2(a)), 234.1 kDa (Figure 2(b)), and 189.4 kDa (Figure 2(c)), respectively. Different from our results, Yan et al. [34] reported that the molecular weight distribution of APS is extensive. One possible reason is that APS obtained by Yan et al. [34] may not be purified. Notably, consistent with Yan et al.’s result [34] that the molecular weights of most APS (57.6%) were more than 150 kDa, in our study, molecular weights of APS-NaCl1 and APS-NaCl2 were more than 150 kDa and constituted the main ingredients of purified APS.

3.3. Relief of RA Symptoms In Vivo by APS. In order to verify the biological activity of APS obtained by cellulase hydrolysis, the anti-RA effect of purified APS in CIA rats was investigated. There are two classical rodent models of RA, adjuvant arthritis (AA) rats and CIA rats. In previous reports, the anti-RA effect of APS was detected in AA rats [30]. However, compared with AA rat, CIA rat has more obvious and serious synovial hyperplasia, cartilage destruction, and other secondary lesions. Moreover, in CIA rats, RA duration is much longer, and signs are similar to human RA. Thus, the CIA rat model is more ideal for the study of RA [35]. Rat foot volume was measured by displacement method and significantly increased in CIA rats from day 7 to day 14 after the primary injection (Figure 3). Foot volumes in CIA rats were notably larger
Figure 4: Serum levels of proinflammatory cytokines after 2 weeks of drug administration in type II collagen-induced arthritis (CIA) rats and vehicle rats. (a) Serum IL-1β levels. (b) TNF-α levels. Data represent the mean ± SD of 6 rats per group. Significant effect compared to the CIA group: *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5: Western blotting analysis of the effects of APS administration on protein expression of NF-κB-p65 and IκBα, as well as GAPDH in synovial membrane homogenates from rats in each group. (a) Representative western blots are shown. (b) Ratios of optical density are calculated on NF-κB-p65 to GAPDH and IκBα to GAPDH. Data represent the mean ± SD of 6 rats per group. Significant effect compared to the CIA group: *P < 0.05, **P < 0.01, ***P < 0.001.
than those in vehicle rats on day 14 (P < 0.05), indicating the successful CIA rat models. After 2 weeks of administration, the hind paw volumes of rats in the CIA group continued to increase, while the volumes in the APS group or the TG group decreased notably (P < 0.05), compared to those in the CIA group. Although volume was slightly smaller in the TG group from day 21 to day 28, there was no statistical difference in the hind paw volumes in the TG group versus the APS group.

3.4. The Anti-inflammatory Activity on CIA Rats In Vivo by APS. Inflammation is the basic and core step in RA progression. Cytokines are involved in the regulation of various inflammatory responses and play a key role in each stage of the RA pathological process [36]. As shown in Figure 3, APS had an acceptable effect in reducing RA symptoms in CIA rats. And it was speculated that APS might decrease inflammation by reducing serum cytokines levels. Proinflammatory cytokines, TNF-α and IL-1β, are pleiotropic molecules which play central roles in swelling, pannus formation and perpetuation, and so on [37]. After 2 weeks of administration, rat serum IL-1β (Figure 4(a)) and TNF-α (Figure 4(b)) were significantly lower in the APS group than in the CIA group (P < 0.05), indicating that APS contributed to the inhibition of serum cytokines and alleviated inflammation. Interestingly, this result was consistent with previous studies [8]. Meng et al. [8] reported that APS could in vitro decrease TNF-α secreted in RA fibroblast-like synoviocytes. Jiang et al. [30] also verified that APS could in vivo diminish serum TNF-α and IL-1β in AA rats.

3.5. Inhibitory Effect to NF-κB Activation in CIA Rats by APS. NF-κB signaling pathway is considered as the main "switch" in secreting proinflammatory cytokines and can activate and regulate a variety of cytokines, including TNF-α and IL-1β [38]. In Figure 5, compared with the CIA group, APS and TG significantly decreased NF-κB-p65 expression (P < 0.05) and significantly increased IκBα expression (P < 0.05). In physical status, NF-κB binds to its inhibitory protein, IκB, to form a complex, which is stabilized in the cytoplasm and cannot function as a transcription factor. In pathological status, NF-κB releases from the complex because IκB is phosphorylated by IKK kinase and phosphorylated IκB is subsequently ubiquitinated and degraded by proteasome [39]. The dissociative NF-κB then travels into the cell nucleus, binds promoter sequences, and activates transcription of various genes to lead to transcriptional expression of downstream inflammatory molecules [40]. Numerous studies have shown that NF-κB exerts harmful and crucial effect in the positive feedback mechanism in RA inflammation. NF-κB not only upregulates the transcriptional levels and serum concentrations of IL-1β, TNF-α, and IL-6 but also can be activated by increased IL-1β and TNF-α through intracellular cascade responses. The positive feedback contributes to maintain and accelerate the inflammatory response of RA and leads to structural damage of the bone and cartilage ultimately [41, 42]. In this study, APS administration in CIA rats reversed the expression levels of NF-κB-p65 and IκBα, thereby blocking a harmful feedback and cycle (increased proinflammatory cytokines → activated NF-κB signaling → induced release and increasing of proinflammatory cytokines).

4. Conclusion

In summary, our results indicate that cellulase can significantly increase the APS yield and polysaccharide content, improve the product quality, and preserve the biological features in reducing RA symptoms, cytokine secretion, and NF-κB activation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

No conflicts of interest were declared by the authors.

References


