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Comparative study of the extracting polysaccharides from Traditional Chinese Medicine *Astragali Radix* in the field of new biological cellulase-assisted enzymes technology

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Traditional Chinese Medicine *Astragali Radix* (AR) that is included in the edible herbal list made by the Ministry of Health of China possesses multiple immunomodulatory effects. This study had shown that *Astragalus* polysaccharide (APS) among ingredients that play the significant role in immune regulation is the most active one. Biological enzymes, as innovative biological technology in the field of new energy, such as cellulase-assisted enzyme, were used in preparing the APS from *Astragali Radix* in this study. The conventional water reflux extraction was employed as the control group. Using the content of total polysaccharides as the evaluation index, anthrone-sulfuric acid method was applied to determine polysaccharide content. With ratio of raw material to water 1:30 g/mL，the polysaccharide content of the traditional process could be up to 4.82% when time was 120 min and temperature was 90°C. Cellulase-assisted enzymatic extraction conditions of polysaccharide was optimized by single factor experiments. The optimal extraction conditions were as follows: dosage of cellulase, enzymolysis temperature 55°C, and pH 5.0. Under the optimal extraction conditions, the content of polysaccharide was 1.56%. Although, the content of cellulase-assisted method used to extract polysaccharide was a litter lower，but the macromolecules in AR were extracted quickly and transformed into small molecules with molecular weight less than 10 kDa on the basis of simulating the temperature and enzyme-like environment, with which cellulase enzymes are more easily to break down plant cell walls and could be absorbed effectively by the digestive tract in the human body. Therefor it provides theoretical reference for further development APSs.

Keywords: *Astragalus* polysaccharides, cellulase-assisted enzymes extraction, water reflux extraction, comparative study

INTRODUCTION

Astragali Radix (AR), also well-known as Huangqi in China, it is derived from the dried root of perennial legume *Astragalus* membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao and A. membranaceus (Fisch.) Bge. [1]. AR is one of the essential and commonly used Traditional Chinese Medicine (CHM) to tonify Qi， its medicinal history has been recorded for more than 2000 years. Moreover, AR has been included in the list of national drug and food homology in 2018. To date, over 30 polysaccharides had been found in AR. Most of them are heteropolysaccharides. The bioactivities of polysaccharides are related to their structures, glycosidic bonds, monosaccharide compositions, ratio of monosaccharides, molecular weights, and so on. The immunoregulatory effects of *Astragalus* polysaccharide (APS) were mainly related to their structures α-(1 → 4)-D-glucan. Generally, the molecular weights of the heteropolysaccharides are in the range of 8.7 kDa to 4.8×10^3 kDa，the molecular weights range with good water solubility were from 10 to 1.0×10^3 kDa. The activities were the strongest when the molecular weights were between 10 kDa and 50 kDa [2-3]. Studies have shown that APS has significant immunomodulatory effects on antitumor and antiinfection [4]. APS-Ⅱ are the most immune active parts of *Astragalus* polysaccharides (APSs), and their molecular weights are about 10 kDa. In the monosaccharide compositions of the immune active APSs, Glu is the most abundant, then followed by Ara and Gal. The molecular weights of APSs for injection are mainly concentrated in 10 kDa approximately, indicating that the molecular weights of 10 kDa were the main parts of their efficacy [5]. It was preliminarily speculated that AHPS (*Astragalus* heteropolysaccharides, about 11 kDa) was mainly transported and absorbed by small intestinal cells by everted gut sac method in rats. Preparation processing, especially the methods for herbal extraction, would bring different pharmacological effect. Currently, the most common polysaccharide extraction method is hot-water extraction and alcohol sedimentation. In recent years, various innovative green extraction techniques, such as ultrasonic assisted extraction, semi-bionic extraction [6-7] and enzyme assisted extraction are in practice for isolation of bioactive polysaccharides. Enzyme assisted extraction technology offers many advantages, such as higher

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extraction yield, lower energy requirements and suitable for the organism to absorb owing to the imitative biological environment, compared to conventional extraction methods. The effects of biological enzymatic extraction of polysaccharides are also superior to the other methods in terms of biological activities such as antioxidant and anticancer effects. It may enhance the activities of polysaccharides by keeping their relative molecular weights at a suitable low level [8-10].

The conventional water reflux extraction was employed as the control group. Anthrone-sulfuric acid method was applied to determine the content of total polysaccharides (in terms of Glu) [11]. This research aims to describe a comparative study of the extracting polysaccharides from AR between the new biological cellulase-assisted enzymes technology and conventional extraction method. With total polysaccharides content as index, single factor experiments were employed to optimize parameters of the influencing factors such as the enzyme amount, enzyme treated temperature and the range of pH value.

EXPERIMENTS

Plant materials, reagents

AR slices (5 years, wild or imitation wild) were purchased from Datong of Shanxi and certified by professor Y. Bai from Shanxi Medical University as *Astragalus* membranaceus (Fisch) Bge. Var. momgholicus (Bge). Hsiao. Cellulase (activity of 30 units/mg, food grade) was acquired from Zhejiang Yinuo Biotechnology Co., Ltd (Zhejiang, China). D (+)-glucose (080M00143V, >98%) was obtained from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). All the other reagents used were of analytical grade.

Apparatus

A pulverizer (Model LG-200, Ruian Baixin Medicine Machine and Instrument Factory, China) was used to pulverize AR slices. An ultrasonic apparatus (Model KQ3200E, Kunshan Ultrasonic Instrument Co. Ltd, China) was used to degrease AR power. A water bath (Model HH-2, Shanghai Guohua Electric Appliance Co. Ltd, China) was applied to polysaccharides extraction. An electronic balance with 0.0001 g readability (Model TP-213, Beijing Danfu Instrument Co. Ltd., China) was used for weighing AR degreased power. An oven (Model DHG-9035A, Shanghai Qixin Scientific Instrument Co., Ltd, China) was used to dry polysaccharides from AR. A low-speed centrifuge (Model 800, Shanghai Surgical Instrument Factory, China) was used for separating polysaccharides. An ultraviolet-visible spectrophotometer (Model 1200, Shanghai Meipuda Instrument Co. Ltd, China) was used for quantifying AR polysaccharides.

Pre-treatment of AR slices

The slices were pulverized and passed through a 50-mesh sieve. Then, 100 g powder was ultrasoniced by 500 mL of 60~90% petroleum ether at normal temperature (about 25°C) for 40 min and filtration. The filtrate was discarded, the solvent in the filter residue evaporates to obtain AR degreased powder.

Extraction of APS with cellulase

AR degreased powder (5.0 g) was placed into a flask and added with distilled water obtain a material-to-water ratio of 1:30 (g/mL). The mixture was heated in a water bath for 120 min at designated dosage of enzyme, pH and temperature. After enzymolysis process, the extract was rapidly heated for 10 min in boiling water bath, cooled, filtered. The filtrate was concentrated and precipitated in three times the volumes of 95% ethanol, then let it stand for about 15 h. The polysaccharide precipitate was collected by centrifugation at 3000 r/min for 25 min, washed with anhydrous ethanol. The precipitate was dried in an oven at 60°C to get the crude enzymatic extraction polysaccharide (APS-E).

Optimization of cellulase-assisted enzymatic extraction conditions

A single factor experimental design was used to determine optimal extraction conditions including dosage of cellulase (ranging from 0.5 to 2.0%), pH value (ranging from 4.0 to 7.0, pH was adjusted to the desired value with 1 mol/L HCl and 1 mol/L NaOH), and temperature (ranging from 35 to 65°C) [12-14]. One factor was changed, while other factors kept constant in each experiment. The contents of total polysaccharides as the evaluation index, the optimal extraction conditions were determined.

Extraction of APS with water reflux as the control group

The water reflux extraction was carried out using the same material-to-water ratio 1:30 (g/mL) and the same time of extraction (120 min). AR degreased powder (5.0 g) was added into a flask

with 150 mL of water，the mixture was refluxed for 120 min at 90°C, and then filtered. The filtrate was concentrated and then submitted to the same steps that were taken in the cellulase extraction to get the crude water extraction polysaccharide (APS-W) *as the control group*.

Content determination for total polysaccharides with UV-vis spectrophotometry

Total polysaccharides were quantitatively analyzed by anthrone-sulfuric acid method using glucose as the standard monosaccharide.

1. Drawing of glucose standard curves

12.5 mg of glucose standard monosaccharide was weighed precisely, transferred quantitatively into a 50 mL volumetric flask after dissolution with distilled water to prepare the corresponding standard store solution (250 μg/mL), then stored in a refrigerator at 4°C. When in use, the solutions with mass concentration of 12.5, 15,17.5, 20, 22.5, and 25 μg/mL glucose standard solution were prepared, and the mixed liquor was shaken well, then 5.0 mL of 0.2% anthrone-sulfuric acid solution (100 mL of 80% concentrated sulphuric acid was added to 0.2 g of anthrone, it was prepared when it was needed) was added, respectively. After the mixed liquor was heated for 5 min in a boiling water bath，it cooled down at room temperature. The absorbance values (OD) of these solutions were measured by UV-vis spectrophotometer at the maximum absorption wavelength for making standard curves with corresponding gradient concentration.

2. Methodological verification of the precision

In order to verify the precision of the method, the OD values of the glucose standard solution (15 μg/mL) were measured six times under the maximum absorption wavelength, and the relative standard deviation (RSD) (%) value of these six measured OD were calculated.

3. Methodological verification of the stability and the repeatability

In order to verify the stability of the method, the test sample solution (APS-E, 18 μg/mL) was determined every 10 min and lasted for 50 min under the maximum absorption wavelength, and their OD values were used to calculate the RSD (%) as the stability. Furthermore, the RSD (%) of the other six portions same concentration solution were also tested for the repeatability of the method.

4. Methodological verification of the accuracy

The accuracy of the method was appraised by recovery experiments. The nine samples (APS-E, 18 μg/mL, 1 mL) of known concentrations were divided into three groups, and then each group was added with different amounts of glucose standard (7.5, 15, 23 μg), respectively. Nine portions solutions were diluted with distilled water and the OD values were measured under the maximum absorption wavelength, to calculate the recovery rate according to Eq. (1):

The recovery rate (%) = (the measured quantity after added standard substance – the known quantity of sample) × 100% / the mass of added standard substance (1)

5. Content determination for total polysaccharides from AR

Polysaccharides of APS-E and APS-W were formulated into a test solution of 18 μg/mL with distilled water, respectively. The OD values of samples were determined at the wavelength of 612 nm to calculate total polysaccharides content (%) according to standard curve. The content was calculated as shown in the Eq. (2):

$$\text{polysaccharide concent (\%)} = (C \times D) / W \times 100\% \quad (2)$$

where:

C - glucose concentration in the sample, μg/mL;

D - the dilution volume, mL;

W - mass of AR, μg.

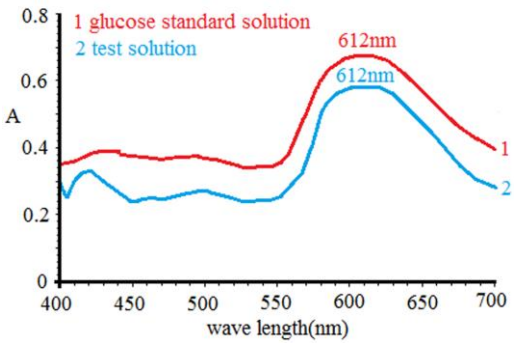

Fig.1. Results of wavelength scanning for glucose standard solution and test solution

RESULTS AND DISCUSSIONS

The content determination for total polysaccharides

1. Drawing of glucose standard curves

The content of total polysaccharides was determined by UV-vis methods. UV wavelength scanning for standard solution and test solution

were showed in Fig.1. With wavelength scanning from 400 to 700 nm, the maximum absorption of standard sample and test sample at 612 nm. Using UV method, the linear regression equation of glucose was equal to $Y = 0.0289\ X - 0.0068$ (Y was OD value, X was glucose concentration), of which $r = 0.9995$, range of linearity was 12.5~25 μg/mL.

2. Results of methodological verification

According to the results of UV method evaluation, the RSD (%) of the precision, stability and repeatability of the experiments is 0.2%, 1.9% and 1.0%, respectively. They were all with in 2%. The results of recovery rate were shown in Tab.1.

Table 1. The results of recovery rates

Mass of sample	Mass of glucose	Measured amount	Recovery rate	Average recovery rate	RSD
μg	μg	μg	%	%	%
15.5	7.8	23.2	98.7		
15.5	7.7	23.3	101.3		
15.5	7.8	23.2	98.7		
15.5	15.5	30.7	98.1		
15.5	15.5	30.7	98.1	99.2	1.3
15.5	15.5	30.6	97.4		
15.5	23.2	38.8	100.4		
15.5	23.1	38.7	100.4		
15.5	23.2	38.7	100.0		

From recovery rates data, the recovery rates of glucose were within the limits (95.0~105.0%). All of these methodological verification could met the requirements of quantitative analysis.

Single factor experiments analysis

The optimal conditions for enzymatic hydrolysis of factors were obtained by single factor test in the preliminary experiments.

1. Effect of dosage of cellulase on content of polysaccharides

In order to study the effect of different dosage of cellulase on the content of APS, the extraction was performed at different dosage of cellulase (0.5%, 1.0%, 1.5% and 2.0%), keeping the pH value, raw material to water ratio, temperatures and time constant at pH 5.0，1:30 g/mL, 120 min, and 55°C, respectively. As shown in the Fig.2a, with the increasing dosage of cellulase, the content of polysaccharides growed to top and decreased afterward. Generally, high dosage of cellulase promotes the diffusion of polysaccharides from the cells. When the amount of enzyme reaches a certain value, such as 1.5%, the reaction with the substrate will reach a saturated state, and if excessive enzyme is added at this time, the dissolution of polysaccharides will be blocked. Therefore, 1.5% was chosen as the optimum dosage of cellulase.

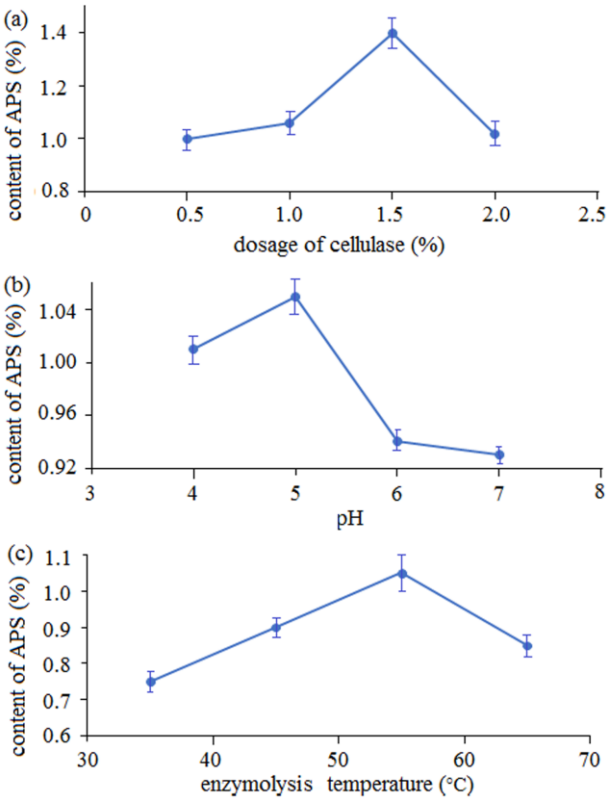

Fig.2. Results of single factors experiments (a) dosage of cellulase (b) pH value (c) enzymolysis temperature

2. Effect of pH value on content of polysaccharides

As shown in Fig.2b, the effect of pH value on content of polysaccharides was investigated. The pH value was changed from 4.0 to 7.0, while the other extraction conditions were: dosage of cellulase 1.5%, raw material to water ratio 1:30 g/mL, time 120 min, and temperature 55°C. The content increased with pH until it was up to 5.0 and then began to decrease. So, 5.0 was chosen as the optimum pH value.

3. Effect of temperature on content of polysaccharides

Extraction was performed at different temperatures (35, 45, 55, and 65°C). The dosage of cellulase was 1.5%, the raw material to water ratio was 1:30 g/mL, the time was 120 min and the pH was 5.0. Generally, high temperature promotes the diffusion of polysaccharides from the cells, which

indicates that lower temperature might prevent the degradation of polysaccharide and assure its bioactivities. As shown in the Fig.2c, at first the content increased, and when 55°C, it subsequently decreased with increasing temperature. Hence, 55°C was deemed as the optimal temperature.

According to the single factor experiments, the extraction conditions were optimized as follows: dosage of cellulase, 1.5%; temperature, 55°C and pH value, 5.0.

The content determination for total polysaccharides of AR

The total polysaccharides content of AR samples obtained with cellulase-assisted treatment under the optimized extraction conditions (dosage of cellulase, 1.5%; temperature, 55°C and pH value, 5.0) and in control group were 4.82% (n=3) and 1.56% (n=3), respectively. This results suggest that although, the content of cellulase-assisted method used to extract polysaccharide was a litter lower by far，but the macromolecules in AR were extracted quickly and transformed into small molecules with molecular weight less than 10 kDa on the basis of simulating the temperature and enzyme-like environment, with which cellulase enzymes are more easily to break down plant cell walls and could be absorbed effectively by the digestive tract in the human body. Therefor it provides theoretical reference for further development APSs.

CONCLUSIONS

The macromolecules in AR were extracted quickly and transformed into small molecules, because cellulose-assisted method extracted polysaccharides reduce the molecular weight of polysaccharides, break the long-chain structure of the original polysaccharides, thus releasing more active units, and do not destroy the polymerization structure of the active units, so that the relative molecular weight of the polysaccharide is kept at a suitable low level, for example, less than 10 kDa, on the basis of simulating the temperature and enzyme-like environment, which could be absorbed effectively by the digestive tract in the human body [15]. So, the cellulase-assisted method provides reference value for the extraction of bioactive polysaccharides by multiple enzyme extraction or the combination on other extraction technology with enzyme extraction technology, also provides theoretical basis for later pharmacological and pharmacokinetic studies.

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ABBREVIATIONS

AR- Astragali Radix;
CHM - Chinese Medicine;
APS -Astragalus polysaccharide;
AHPS-Astragalus heteropolysaccharides;
APS-E- enzymatic extraction polysaccharide;
APS-W- water extraction polysaccharide*;*
UV-vis- ultra violet-visible;
OD -absorbance values;
RSD- relative standard deviation;
Da - dalton;
Glu - glucose;
Ara - arabinose;
Gal - galactose.

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