Characterization and Evaluation of the Pro-Coagulant and Immunomodulatory Activities of Polysaccharides from Bletilla striata

Wanchen Zhai, Enwei Wei, Rui Li, Tianyi Ji, Yueyao Jiang, Xiaoxiao Wang, Yiyng Liu, Zhiying Ding,* and Hongli Zhou*

1. INTRODUCTION

Bletilla striata (Family Orchidaceae) is a perennial herb that is mainly distributed in China, including Guangxi, Guizhou, Yunnan, and Sichuan provinces. The Chinese Pharmacopoeia (2020 edition) states that B. striata stanches bleeding, reduces inflammation, promotes tissue regeneration, and relieves hematemesis, hemoptysis, and detumescence. Several components, such as triterpenoids, saponins, steroidals, flavonoids, polysaccharides, and polyphenols, have been isolated from B. striata. Polysaccharides from B. striata (BSPs) are one of the major bioactivity-contributing components due to their various beneficial activities, including wound healing, anti-inflammation, anti-angiogenesis, anti-oxidation, anti-hepatic, and immunomodulatory properties. Inspired by a research indicating that purified BSP possesses hemostatic effects, we aimed to fractionate promising polysaccharides with the highest pro-coagulant activity.

The BSPs extracted by hot water are composed of mannose and glucose with a molar ratio of 3:1 and can be regarded as glucomannan polymers with a molecular size of 135 kDa.11,12 Although these polysaccharides have been preliminarily identified by infrared (IR) and nuclear magnetic resonance spectroscopies, with regard to detailed structural information, further characterization is still pending. The X-ray photoelectron spectroscopy (XPS) analysis has received increasing attention for chemical composition evaluation and quantitative analysis of polysaccharides.13 By using XPS analysis, Wang et al.14 confirmed that –SO₃ groups (S⁺⁺, high binding energy of 168.7 eV) are abundant in Artemisia sphaerocephala polysaccharides; a similar observation was reported in another work.15 Thus, in the present work, XPS analysis was used to obtain the fine structural information of BSP for the first time.

The concept of "immunothrombosis" has been proposed, wherein the immune system uses clotting factors to cause the coagulation of hemolymph and protect them against invading microorganisms.16,17 Peng et al. found that BSPF2 significantly induced the proliferation of spleen cells from Balb/C mice and exhibited immunological activity.9 In this regard, the present study was designed to explore the immunomodulatory activity of BSP in vitro by using RAW 264.7 cells. Herein, BSP was extracted by hot water and isolated using DEAE-52 cellulose chromatography. Physicochemical characteristics were determined through ultraviolet-visible (UV-vis) spectroscopy, IR spectroscopy, XPS analysis, and high-performance liquid chromatography (HPLC). In addition, assays of biological activities including in vitro pro-coagulant...
and immunomodulatory function were conducted. The obtained results would provide scientific basis for comprehensive utilization of BSP.

2. MATERIALS AND METHODS

2.1. Plants and Chemicals. *B. striata* was obtained from Yunnan Province (east longitude: 97°31′–106°11′; north latitude: 21°8′–29°15′, China) in January 2019 and authenticated by Prof. Guangshu Wang, School of Pharmaceutical Sciences, Jilin University, Changchun, China.

Standard sugars including mannose (Man), ribose (Rib), rhamnose (Rha), glucuronic acid (Glu A), galacturonic acid (Gal A), glucose (Glu), galactose (Gal), and arabinose (Ara) were purchased from Sino-pharm Chemical Reagent Co., Ltd. (Shanghai, China). T-series dextran standards including T-10, T-40, T-70, T-100, and T-500 kDa were obtained from the National Institutes of Food and Drug Control (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and lipopolysaccharide (LPS) were acquired from Sigma-Aldrich (St Louis, USA). The nitric oxide (NO) assay kit was supplied by Meta-Flam (Shanghai, China). Trypsin, penicillin, and streptomycin were provided by Sciencell Technology Co. (Jiangsu, China). Yunnan Baiyao and normal saline were acquired from a local hospital (Jilin, China).

2.2. Extraction of Polysaccharides. Fresh tubers of *B. striata* were washed and cut into slices, which were extracted based on a previous study with the following conditions: an extraction temperature of 70 °C, extraction time of 1 h, and liquid-to-solid ratio of 30:1 (mL/g). Two extraction solutions were merged, filtered with gauze, and concentrated by rotary evaporation under reduced pressure. The solutions were precipitated twice with four volumes of 95% ethanol at 4 °C overnight and lyophilized to obtain crude BSP.

2.3. Purification and Isolation of Polysaccharides. Crude BSP powder was dissolved in deionized water and deproteinized with Sevag solution (chloroform and n-butanol at a ratio of 4:1). Protein content was calculated using Bradford assay. After removal of the Sevag reagent, the purified BSP solution was dialyzed (MD10, Viskase, Darien, IL, USA) in distilled water for 72 h to remove small molecular impurities. The solution in the dialysis bag was concentrated, dried, and subjected to a DEAE-S2 cellulose column (2.6 cm × 30 cm). The polysaccharides were eluted stepwise with a gradient concentration of NaCl solution (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 M) at a flow rate of 1.0 mL/min (10 mL/tube) and sequentially named as BSP-1, BSP-2, BSP-3, BSP-4, and BSP-5 (0.4–0.7 M NaCl). BSP-5 was shelfed owing to its low content. The eluting fractions were dialyzed, concentrated, and lyophilized. The separated BSPs were stored. Total polysaccharide content was assayed by the phenol sulfuric acid method at 490 nm. Uronic acid content was determined by meta-hydroxydiphenyl assay at 520 nm.

2.4. Physicochemical Properties of Polysaccharides. 2.4.1. Sulfate Group Identification. The sulfate group (–SO_3H) content was detected by the BaCl_2–gelatin turbidity method with slight modifications. In brief, 2 g of BaCl_2 was dissolved in a 0.3% gelatin solution prepared in hot water (60–70 °C). About 5 mg of each BSP-1–BSP-4 was sealed with 4 mL of HCl (1 M) and hydrolyzed at 105 °C for 12 h. The hydrolysate was dried under a nitrogen atmosphere and dissolved in 1 mL of water, then 1 mL of HCl (1 M) and 0.5 mL of barium chloride–gelatin (5 mg/mL) were added. The mixture was fully shocked and then incubated for 20 min at 25 °C. In standard curve determination, 0.2 mL of the polysaccharide solution was added to measure sulfur content (S%). Degree of substitution (DS) was calculated using the following equation:

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%}$$

2.4.2. UV–Vis and IR Spectrum Analysis. The UV–vis spectra of BSP-1–BSP-4 solutions (1 mg/mL) were recorded by a UV–vis spectrophotometer (LS5, INESA Analytical Instrument Co., Ltd., Shanghai, China) from 200 to 800 nm. The IR spectra of BSP-1–BSP-4 fractions were recorded using KBr compressed into tablets (1:100) through an FTIR-650 Fourier transform infrared spectrophotometer (Gangdong Sci. & Tech. Development Co., Ltd., Tianjin, China) within the range of 4000–400 cm⁻¹. Second-derivative IR spectra obtained by the OMNIC 8.0 Savitzky–Golay derivative were used to distinguish overlapping peaks and obtain high-resolution peaks.

2.4.3. X-ray Photoelectron Spectroscopy Analysis. XPS analysis was carried out on a bioemulsifer film deposited on a glass slide by using a PHI5600 photoelectron spectrometer (Physical Electronics, Eden Prairie, MN, USA) to reveal the chemical states of the elements in BSP-1–BSP-4 fractions. The emitted photoelectrons were detected by a hemispherical analyzer set at an angle of 45°. The total acquisition time was 2 min and 0.3 s. Core-level spectroscopy with a constant pass energy mode of 20 eV was equipped with an energy step size of 0.05 eV. Data analysis was performed by XPS PEAK 4.1 software.

2.4.4. Molecular Weight Analysis. HPLC (Elite P230IHHPLC, Elite Analytical Instruments Co., Ltd., Dalian, China) with a gel chromatographic column was used to determine the molecular weights (Mw) of BSP-1–BSP-4 fractions as previously reported. The chromatographic conditions were as follows: a column temperature of 50 °C, flow rate of 1.0 mL/min, RID temperature of 35 °C, and total run time of 30 min. The dextran standards were applied to prepare a calibration curve for determining the molecular weight of the samples.

2.4.5. Monosaccharide Composition Analysis. Based on a previous report, 20 mg of the samples were hydrolyzed to monosaccharides by adding 2 M TFA (2 mL) into a reactor and kept at 110 °C for 5 h. The hydrolytic liquid was evaporated thoroughly, and the obtained powder sample was re-dissolved in methanol. This process was repeated three times to remove any residual TFA. The hydrolyzed samples (0.2 mL) and monosaccharide standards dissolved in water were mixed with 0.3 mol/L NaOH solution (0.2 mL). The mixture was added with 0.5 M PMP (0.2 mL) dissolved in methanol solution and heated in a water bath for 1 h at 70 °C. The solution was cooled and added with 0.3 M hydrochloric
acid solution (0.2 mL) to neutralize. The solution was extracted using 1 mL of chloroform and swirled for 30 s. The solution was centrifuged, and the chloroform layer was discarded. The process was repeated three times. The final supernatant was filtered using a 0.22 μm membrane. In brief, 10 μL of the samples after derivatization were injected into Ultimate 3000 HPLC (Thermo, USA) coupled with a Supersil ODS2 column (5 μm, 4.6 mm × 250 mm) and an Ultimate 3000 diode array detector (DAD, Thermo) to determine the monosaccharide composition of BSP-1–BSP-4 fractions. The chromatographic conditions were as follows: mobile phase consisting of PBS (pH = 6.8) and acetonitrile (82: 18, v/v), a flow rate of 0.8 mL/min, column temperature of 30 °C, detector wavelength of 245 nm, and run time of 85 min. The monosaccharide composition was confirmed by comparing the retention time of the standard sugars. The area normalization method was used to calculate the molar ratio.

2.5. In Vitro Coagulant Activity Analysis. All samples were prepared at concentrations of 50, 100, 200, 400, and 800 μg/mL and dissolved in normal saline (0.9%) for subsequent analysis. APTT, PT, TT, and FIB contents were determined using a previously described method with some modifications. For determination of APTT, 100 μL of the APTT reagent, 100 μL of plasma, and 100 μL of the sample solution were mixed and incubated for 3 min at 37 °C. The solution was added with 100 μL of calcium chloride (37 °C) and analyzed by a coagulometer (InDaM, Shanghai, China). For determination of PT or TT, the samples and plasma (100 μL) were mixed and incubated for 1 min at 37 °C. Then, the solution was added with a PT reagent (200 μL) or pre-warmed TT reagent (100 μL), respectively. Clotting time was recorded.

Fibrinogen was detected in accordance with the kit’s instruction. In brief, 100 μL of plasma and 100 μL of the sample were added with a dilute solution (0.9 mL), and then the mixture was incubated for 30 s at 37 °C. Clotting time was recorded after mixing the FIB reagent (50 μL) and the above solution (0.1 mL). FIB content was calculated using a standard curve.

Normal saline served as the blank control. Yunnan Baiyao, a well-known Chinese medicine that has been used for hemostasis in China for approximately 100 years, was used as the positive control.31,32

2.6. In Vitro Immunomodulatory Activity Assay. 2.6.1. Cell Culture. RAW 264.7 cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). After recovery, cells in the humidified 5% CO2 incubator (at 37 °C) were cultured in DMEM containing 10% fetal bovine serum and 1% each of penicillin and streptomycin. Cell passages were performed with 0.25% trypsin–EDTA when cells reached a density of 80–90%.

2.6.2. Cell Viability Analysis. MTT assay was used to assess the effect of BSP-1–BSP-4 fractions on the RAW 264.7 cells viability.33 The sample powder was dissolved in DMEM and diluted to 25, 50, 100, 200, 400, and 800 μg/mL. After ultrasonic dissolution, the sample solution was stored at −20 °C. The cells (100 μL) were plated into 96-well plates (1 × 104 cells/well) for 12 h and treated with 25, 50, 100, 200, 400, and 800 μg/mL BSP-1–BSP-4 for 24 h. The blank control group was added with DMEM without BSP, and the positive group was added with lipopolysaccharide (LPS, 25 μg/mL). The cells were added with 15 μL of MTT (5 mg/mL) in a 5% CO2 incubator at 37 °C for another 4 h. The supernatant was discarded, and formazan was dissolved in 150 μL of DMSO.

The mixture was shaken for 10 min to ensure complete dissolution of the purple crystals. Optical density was recorded at 490 nm by using a spectrophotometric plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The absorbance of the blank control (0 μg/mL) was considered as 100%. Five independent wells for each concentration were analyzed.

2.6.3. Nitric Oxide and Phagocytosis Analysis. RAW 264.7 cells were plated into 96-well plates as described in Section 2.6.2. The supernatant was collected to determine NO production by using a NO assay kit. The supernatant was mixed with an equal volume of Griess Reagent I (1% sulfanilamide) and Griess Reagent II (0.1% N-1-naphthylethylene diamine dihydrochloride in 5% phosphoric acid) at room temperature for 10 min. Absorbance at 540 nm was recorded by a spectrophotometric plate reader, and NO production was calculated by comparison with NaNO2 standards.

Neutral red phagocytosis assay was conducted to evaluate the phagocytic capacity of RAW 264.7 cells.34 After removal of the supernatant, the non-adherent cells were removed by washing twice with PBS. Each well was then added with 100 μL of neutral red solution and incubated for 1 h. The solution was discarded, and the cells were washed twice with PBS to remove residual neutral red solution. The cells were broken down by lysate solution (ethanol and 1.0 mol/L acetic acid in a 1:1 ratio). The plates were kept overnight at room temperature. Absorbance at 490 nm was recorded using a spectrophotometric plate reader.

2.7. Statistical Analysis. GraphPad Prism 6.01 software (LaJolla, CA, USA) was used for analysis. One-way analysis of variance (ANOVA) and Bonferroni multiple comparisons tests were used for intergroup comparisons. A value of p < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Extraction, Purification, and Isolation of BSP. The yield of BSP using hot water reached 45.55%. After purification procedures including ethanol sedimentation, deproteination, and dialysis, the purified BSP was separated on anion exchange DEAE-S2 cellulose and eluted with distilled water (BSP-1), 0.1 M NaCl (BSP-2), 0.2 M NaCl (BSP-3), and 0.3 M NaCl (BSP-4) to obtain four fractions with the ratio of 3:10:3:2 (Figure 1). The contents of polysaccharides in the above-
mentioned fractions reached 93.3, 93.5, 96.23, and 90.21%, respectively (Table 1). Meanwhile, a small amount of protein was detected, and BSP-2 had the highest content of uronic acids among the fractions (Table 1).

3.2. Sulfate Group Identification. The results of the BaCl$_2$–gelatin turbidity method showed that BSP-1 and BSP-2 became turbid under a black background compared with the blank control group. The turbidity degrees of BSP-3 and BSP-4 were higher than those of BSP-1 and BSP-2. These observations suggested that BSP-1–BSP-4 fractions contained sulfate and that BSP-3 and BSP-4 had higher sulfate content than BSP-1 and BSP-2. The DS of BSP-1–BSP-4 decreased in the following order: BSP-4 (1.70) > BSP-3 (1.59) > BSP-1 (0.29) > BSP-2 (0.03). Overall, these results demonstrated that BSP belonged to natural polysaccharides with a sulfate radical.

3.3. Analysis of UV–Vis and IR. Figure 2 shows weak absorption peaks at 260 and 280 nm, indicating that the BSP-1–BSP-4 fractions contained a small amount of nucleic acids and proteins. These results are consistent with the quantitative analysis in Table 1.

The IR analysis indicated that all polysaccharides exhibited similar absorption peaks in Figure 3a. In particular, the characteristic absorption peaks were as follows: the absorption peak near 3414 cm$^{-1}$ corresponding to the O–H stretching vibration, the peak at 2925 cm$^{-1}$ corresponding to the C–H stretching vibration in $\text{–CH}_2$, the peak at 1643 cm$^{-1}$ corresponding to the asymmetric stretching vibration of COO$^-$, the peak at 1421 cm$^{-1}$ that was considered as the $\text{–OH}$ bending vibration, and the peak at approximately 1066 cm$^{-1}$ that was due to the C–O–C symmetric stretching vibration of carbohydrates. These results demonstrated that all the BSP fractions possessed the typical structural characteristics of polysaccharides.

Second-derivative IR spectroscopy is a method to distinguish overlapping peaks and obtain high-resolution peaks; this method was used to compare differences in peaks especially within 2000–500 cm$^{-1}$ to explore the fine structures of BSP-1–BSP-4. In Figure 3b, the peak at 1730 cm$^{-1}$ was assigned to the C=O of the carbonyl group in ester, and BSP-2 showed the highest absorption; the peak at 1150 cm$^{-1}$ was

![Figure 2. UV–vis spectra of BSP-1, BSP-2, BSP-3, and BSP-4.](https://dx.doi.org/10.1021/acsomega.0c05171)

![Figure 3. (a) IR spectra and (b) second-derivative IR spectra of BSP-1, BSP-2, BSP-3, and BSP-4.](https://dx.doi.org/10.1021/acsomega.0c05171)

![Figure 4. Wide-survey XPS spectra of BSP-1, BSP-2, BSP-3, and BSP-4.](https://dx.doi.org/10.1021/acsomega.0c05171)

Table 1. General Components in BSP-1–BSP-4 Fractions

| Polysaccharide | Uronic acids (%) | Proteins (%) | Total carbohydrates (%) |
|----------------|------------------|--------------|-------------------------|
| BSP-1          | 2.47 ± 0.15      | 1.05 ± 0.19  | 93.3 ± 3.75             |
| BSP-2          | 3.54 ± 0.19      | 0.46 ± 0.13  | 93.5 ± 5.15             |
| BSP-3          | 1.10 ± 0.7       | 0.67 ± 0.12  | 96.23 ± 4.18            |
| BSP-4          | 2.37 ± 0.31      | 1.32 ± 0.46  | 90.21 ± 6.16            |
assigned to the bending vibrations of C−O;40 the peak at 879 cm$^{-1}$ was related to β-glucosidic bonds.41 The most important peak of BSP fractions at 1252 cm$^{-1}$ could be attributed to the asymmetric stretching vibration of S=O, and the peak at 816 cm$^{-1}$ represented the symmetrical vibration of C−O−S that was associated to a C−O−SO$_3$ group; BSP-4 exhibited the strongest absorption.42,43

### 3.4. Analysis of XPS

XPS analysis was conducted to determine the presence of the valence state of different elements of BSP-1−BSP-4. Figure 4 shows the survey spectra of four elemental components assigned to 168.5 (S), 283.4 (C), 400.1 (N), and 532.1 eV (O);44 in particular, C 1s and O 1s had strong binding energy and were the main components of the polysaccharides. Furthermore, small amounts of nitrogen and sulfur were detected.

**Table 2. Monosaccharide Composition (Molar Ratio %) of BSP-1−BSP-4**

| monosaccharide composition | sample |
|----------------------------|--------|
|                           | BSP-1  | BSP-2 | BSP-3 | BSP-4 |
| Man                       | 22.34  | 52.32 | 3.20  | 10.35 |
| Rib                       | 0.24   | —     | 1.20  | 1.18  |
| Rha                       | 0.23   | —     | 1.00  | 1.00  |
| GluA                      | 0.89   | —     | —     | —     |
| Glu                       | —      | 2.44  | 0.87  | 1.21  |
| Gal                       | —      | 31.26 | 8.77  | 6.12  |
| GalA                      | 1.00   | 1.00  | 1.00  | —     |
| Xyl                       | 1.28   | —     | —     | 1.06  |
| Ara                       | —      | —     | —     | 0.62  |
| Fuc                       | 0.56   | —     | —     | —     |

“−” means not present.

**Table 3. Molecular Weights of BSP-1−BSP-4**

| name   | BSP-1  | BSP-2  | BSP-3  | BSP-4  |
|--------|--------|--------|--------|--------|
| Mw (Da) | 761,123| 843,940| 950,302| 715,462|

**“Mw means average molecular weight**
In addition to content analysis, the curve fitting of C 1s, O 1s, N 1s, and S 2p is shown in Figure 5a. The C 1s peak could be divided into three positions: the peaks at 284.5 eV were assigned to non-functionalized carbon (C–C and C–H);45 the peaks at 286.4 eV were due to C–O, C–N, or C–S bonds;46 and the peaks centered at the binding energy of 287.7 were attributed to O=C=O or H–N=C=O.15 Changes in the intensity ratio of carbon contributions are found in Figure 5a. BSP-1, BSP-3, and BSP-4 had a strong intensity at 284.5 eV, and BSP-2 and BSP-4 had a strong intensity at 286.4 eV compared with the other peaks.

The O 1s spectra are shown in Figure 5b and could be decomposed into two positions at 531.5 and 532.6 eV. The peak at 531.4 eV was associated with O double bonded to C in carboxylic acid, ester, or amide, while the O 1s peak at 532.6 eV arose from the alcohol, hemiacetal, or acetal group.14,47,48 These findings indicated that the contents of C=O and C=O bonds were relatively high in BSP-2, consistent with the results of glucuronic acid in Table 1 and ester carbonyl group at 1730 cm⁻¹ in the IR analysis. BSP-2 and BSP-4 had a strong intensity at 286.4 eV compared with the other peaks.

Nitrogen and sulfur were also detected in the samples (Figure 5c,d). The N 1s peak was divided into two positions at 313.5 and 326.6 eV. The peak at 313.4 eV was associated with O double bonded to C in carboxylic acid, ester, or amide, while the O 1s peak at 326.6 eV arose from the alcohol, hemiacetal, or acetal group.14,47,48 These findings indicated that the contents of C=O and C=O bonds were relatively high in BSP-2, consistent with the results of glucuronic acid in Table 1 and ester carbonyl group at 1730 cm⁻¹ in the IR analysis. BSP-2 and BSP-4 had a strong intensity at 286.4 and 326.6 eV, which might be related to the acetyl oxygen group.10,48

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Nitrogen and sulfur were also detected in the samples (Figure 5c,d). The N 1s peak was divided into two parts, at 400.2 and 399.1 eV. The peak at 399.1 eV was related to the NH₂ or NH group,49 and the peak at 400.1 eV was attributed to NH–C=O bonds.50 The weak N 1s peak might be due to the protein residuals or glycoprotein complex. In addition to C, O, and N, S was the first element found in the BSP fractions. With regard to the S 2p peak, the peak at the binding energy 168.5 eV was associated with SO₃⁻ bonds that possessed negatively charged groups.49 BSP-3 and BSP-4 had stronger intensity at 168.5 eV than BSP-1 and BSP-2, suggesting that they possessed higher sulfate content. These results were consistent with the turbidity experiment in Section 3.2.

3.5. Analysis of Monosaccharide Composition. Retention time was compared between the samples and the standard mixture to determine the monosaccharide composition (Table 2). BSP-1–BSP-4 had higher proportions of mannose and glucose. In particular, BSP-1 and BSP-4 contained more types of monosaccharides than BSP-2 and BSP-3. BSP-2 had the highest proportion of GalA among the fractions.

3.6. Analysis of Molecular Weight. Table 3 shows the molecular weight of three fractions determined based on the calibration curve. The average Mw of BSP-4 was 7.15 × 10⁵ Da, which was the lowest among the fractions.51

3.7. Analysis of In Vitro Coagulant Activity Assay. The coagulant activities of BSP-1–BSP-4 were assessed by measuring APTT, PT, TT, and FIB. The effect of polysaccharides on APTT was first examined. As shown in Figure 6a, BSP-1 (0.2–0.8 mg/mL), BSP-2 (0.4–0.8 mg/mL), BSP-3 (0.2–0.8 mg/mL), and BSP-4 (0.2–0.8 mg/mL) exhibited significant pro-coagulant activity (p < 0.05); in
particular, 0.8 mg/mL BSP-4 had better pro-coagulant activity that had been found to exceed Yunnan Baiyao. Hence, BSP fractions could activate the intrinsic pathway of coagulation.52

The effect of BSP fractions on PT was then determined. Figure 6b shows that BSP-1 (0.2−0.8 mg/mL), BSP-2 (0.4−0.8 mg/mL), BSP-3 (0.4−0.8 mg/mL), and BSP-4 (0.4−0.8 mg/mL) significantly shortened the PT than normal saline (p < 0.05). Hence, BSP fractions possessed high pro-coagulant activity by converting prothrombin to thrombin in a short time via the extrinsic coagulation pathway.53

Active thrombin can convert soluble fibrinogen into insoluble fibrin, leading to clotting.54 The acceleration of this process can shorten the clotting time. TT is considered an indicator for common coagulant pathways. In this regard, BSP-1 (0.2−0.8 mg/mL), BSP-2 (0.4−0.8 mg/mL), BSP-3 (0.4−0.8 mg/mL), and BSP-4 (0.2−0.8 mg/mL) reduced the TT compared with normal saline (p < 0.05). Hence, BSP fractions possessed high pro-coagulant activity by converting prothrombin to thrombin in a short time via the extrinsic coagulation pathway.55

Overall, BSP is more likely to be a promising coagulant by regulating intrinsic, extrinsic, and common coagulant pathways.

In general, the body’s own coagulation process can transform blood into insoluble fibrin, which is mainly considered to be involved in primary hemostasis. When no hemostatic agent is available to implement hemostasis timely, especially in the battlefield, operating rooms, and emergency rooms, many deaths will occur due to uncontrollable bleeding.56 Thus, an ideal hemostatic material or agent should be developed. Thus far, a wide variety of polysaccharides have been investigated and applied as hemostatic agents because of their advantages including low price, minimal side effects, and biodegradable properties.57−59 A previous study reported that BSP hydrogels exhibited pro-coagulant activity.11 Moreover, most sulfated polysaccharides play an important role in the coagulation pathway via the mechanism that the negatively charged groups of polymeric sulfates could bind to the

Figure 7. Effect of BSP-1−BSP-4 on (A) cell viability, (B) pinocytic capability, and (C) secretion levels of NO of RAW 264.7 cells. The group without BSP was used as the blank control group, and the group treated with LPS (25 μg/mL) was used as the positive control. *p < 0.05 versus the blank control group.
Macrophage activation was also determined using neutral increased the uptake rate compared with the blank control (mannose receptors and trigger pinocytosis). This fact explains are composed of fucose or mannose residues could attach to BSP in a future study. We will focus on the structure and mechanism of the extracted its lowest uronic acid content and highest molecular weight. BSP was extracted by hot water, and four polysaccharides were isolated and obtained using DEAE-52 cellulose. BSP fractions belonged to natural polysaccharides with a sulfate radical, as isolated and obtained using DEAE-52 cellulose. BSP fractions which might be ascribed to its higher sulfate content and lower density increases upon the incorporation of carboxyl groups. BSP-2 (50, 100, and 200 μg/mL), BSP-3 (100 μg/mL), and BSP-4 (50–800 μg/mL) significantly increased the amount of NO production (p < 0.05) compared with the blank control. The BSP-3 group had no enhancing effect on NO production (p > 0.05). Similar to the results of pinocytic activity, 100 μg/mL BSP-2 showed the best effect on increasing NO production among the fractions.

In addition to mannose residues, uronic acids are associated with immunomodulatory activities of polysaccharides. BSP-2 with an appropriate molecular weight and high uronic acid content might exhibit higher immunomodulatory activity than other BSP fractions, similar to previous reports. BSP-3 showed inapparent immunomodulatory activity possibly due to its lowest uronic acid content and highest molecular weight. We will focus on the structure and mechanism of the extracted BSP in a future study.

4. CONCLUSIONS
BSP was extracted by hot water, and four polysaccharides were isolated and obtained using DEAE-52 cellulose. BSP fractions belonged to natural polysaccharides with a sulfate radical, as confirmed by the results of second-derivative IR spectroscopy and XPS analysis. The results of the in vitro coagulant assay revealed that BSP fractions had stronger pro-coagulant activity compared with normal saline. Finally, BSP fractions were observed to possess significant immunomodulatory activity by enhancing pinocytic capacity and NO production. Overall, BSP can be used as natural pro-coagulant and immunomodulatory agents in pharmaceutical and nutraceutical industries.

AUTHOR INFORMATION

Corresponding Authors
Zhiying Ding – School of Pharmaceutical Sciences, Jilin University, Changchun 130012, PR China; Phone: +86 13843180286; Email: dzy@jlu.edu.cn; Fax: +86 13843180286

Hongli Zhou – Jilin Engineering Research Center for Agricultural Resources and Comprehensive Utilization, Jilin Institute of Chemical Technology, Jilin 132022, PR China; orcid.org/0000-0002-5561-2587; Phone: +86 432 62185246; Email: zhouhongli@jlct.edu.cn; Fax: +86 432 62185246

Authors
Wanchen Zhai – School of Pharmaceutical Sciences, Jilin University, Changchun 130012, PR China
Enwei Wei – Bethune Institute of Epigenetic Medicine, The First Hospital, Jilin University, Changchun 130012, PR China
Rui Li – Department of Pharmacy, China-Japan Union Hospital of Jilin University, Changshun 130012, PR China
Tianyi Ji – School of Pharmaceutical Sciences, Jilin University, Changshun 130012, PR China
Yuecao Jiang – School of Pharmaceutical Sciences, Jilin University, Changshun 130012, PR China
Xiaoxiao Wang – Jilin Engineering Research Center for Agricultural Resources and Comprehensive Utilization, Jilin Institute of Chemical Technology, Jilin 132022, PR China
Yiyang Liu – Jilin Engineering Research Center for Agricultural Resources and Comprehensive Utilization, Jilin Institute of Chemical Technology, Jilin 132022, PR China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05171

Notes
The authors declare no competing financial interest.

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