Structural characterization and immunomodulatory activity of a water soluble polysaccharide isolated from *Botrychium ternatum*

Xiaoyong Zhao^a, Jinying Li^b, Yaqin Liu^a, Datong Wu^a, Pengfei Cai^a, Yuanjiang Pan^{a,*}

Affiliations:

^a Department of Chemistry, Zhejiang University, Hangzhou 310027, China

^b College of Life Science, Zhejiang University, Hangzhou 310058, China

***Corresponding author.** Tel.: +86 571 87951629; fax: +86 571 87951629.

E-mail: panyuanjiang@zju.edu.cn (Y. Pan).

Abstract

As a folk medicine, *Botrychium ternatum* has been used for thousands of years in China. In the present work, a water soluble polysaccharide BTp1 was extracted and purified from *B. ternatum*. Based on the MALDI matrix 3-aminoquinoline- α -cyano-4-hydroxycinnamic acid, the molecular weight of BTp1 was determined to be 11638 Da directly. Monosaccharide analysis showed that BTp1 was composed of arabinose (Ara). Combining enzymatic hydrolysis and subsequent MALDI-TOF analysis, a linear backbone of BTp1, consisted of (1 $\rightarrow$ 5)-linked α -L-Araf, was inferred quickly. Then according to NMR experiments, the whole structure of BTp1 was established. The repeating unit of BTp1 was deduced as a linear backbone with branches at O-2, O-3 and its neighboring O-2 positions

terminated with (1→)-linked α -L-Araf, respectively. The immunomodulatory assay exhibited that BTp1 could significantly enhance the viability and promote the release of NO in RAW 264.7 cells, suggesting that BTp1 could be a potential immunomodulatory agent in pharmacological fields.

Keywords: *Botrychium ternatum*; Polysaccharide; MALDI-TOF-MS analysis; NMR spectroscopy; Immunomodulatory activity

Chemical compounds studied in this article:

Arabinose (PubChem CID: 5460291); Hydroxylamine hydrochloride (PubChem CID: 443297); 3-Aminoquinoline (PubChem CID: 11375); α -Cyano-4-hydroxycinnamic acid (PubChem CID: 5328791); 2,5-Dihydroxybenzoic acid (PubChem CID: 3469) NO (PubChem CID: 145068); Lipopolysaccharide (PubChem CID: 11970143)

1. Introduction

Polysaccharides exist as one of the mainly bioactive compounds in the medicinal plants, which have attracted a great deal of attention in the pharmacological area (Li, Wu, Lv, & Zhao, 2013). Among them, the water-soluble polysaccharides are most significant owing to their non-cytotoxic properties and to a variety of bioactivities, such as immunomodulatory (Lin, Liao, & Ren, 2016; Yu et al., 2015), anti-cancer (Chien, Yen, Tseng, & Mau, 2015; Xie, Zou, & Li, 2015), and anti-diabetic activities (Jin, Zhao, Huang, & Shang, 2014; Wang, Zhao, Yang, Wang, & Kuang, 2016). The physico-chemical properties that have been related with such activity are molecular weight (M_w), the monosaccharide composition, glycosidic-linkage and branching characteristics (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015). Therefore, the structure analysis is very important to better understand the chemical structure-bioactivity relationship of polysaccharides.

Several methods have been introduced to analyze structure of polysaccharides, include methylation analysis, MALDI-TOF-MS, NMR, and so on (Hu, Cheong, Zhao, & Li, 2013). The features of glycosidic linkages could be analyzed by methylation analysis (Ciucanu, & Kerek, 1984). However, it is difficult to achieve permethylation product, especially for the high M_w polysaccharides, which may lead incorrect conclusion. MALDI-MS has proven to be an effective tool for the analysis of polysaccharides. It could be employed to obtain the average molar masses (López-García, García, Vilarinho, & Rodríguez, 2016). However, up to now, using MALDI-MS for direct analysis of polysaccharides with high M_w (>10,000 Da) has

been hindered by the low ionization efficiency of these compounds (Hsu et al., 2007). And MALDI-MS have also been used to detect hydrolysate from polysaccharides to assist the structure illumination (Ding et al., 2015, 2016). NMR analysis is a powerful method for the structural analysis of polysaccharides (Cheng & Neiss, 2012). It is a reliable and nondestructive technique, which can provide detailed structural information for polysaccharides.

Botrychium ternatum, belonging to the family of Ophiolossaceaus, is a traditional Chinese medicine. It has been prescribed to treat asthma and whooping cough and been considered to have anti-toxic effects *in vivo*. The major components of *B. ternatum* are flavonoids and polysaccharides (Qi, 2012). A few researchers have focused on the flavonoids in *B. ternatum* (Warashina, Umehara, & Miyase, 2012; Yuan et al., 2013). Totally 33 small molecules have been identified in methanol extract (Warashina et al., 2012) and 70% ethanol extract of *B. ternatum* has been selected to treat allergic asthmatic in mouse (Yuan et al., 2013). However, to the best of our knowledge, although preparations of *B. ternatum* have already been used clinically, the understanding of chemical composition in this herb, especially for the polysaccharides, is still limited.

Herein, a homogeneous polysaccharide BTp1 was extracted and purified from *B. ternatum*. Precise *M_w* of BTp1 was determined by MALDI-MS directly. In combination with enzymatic hydrolysis, the backbone of BTp1 was deduced quickly. The NMR spectrometry made the whole structural establishment of BTp1 available. Moreover, the immunomodulatory activity of BTp1 was investigated *in vitro* using

RAW 264.7 macrophage cells. These results would provide valuable information about the structure and immunomodulatory bioactivity of the *B. ternatum* polysaccharide.

2. Materials and methods

2.1. Materials and reagents

The whole herb of *B. ternatum* were purchased from a local pharmacy in Hangzhou, China. Arabinose (Ara), rhamnose (Rha), fucose (Fuc), mannose (Man), glucose (Glu), galactose (Gal), T-series dextrans, lipopolysaccharide (LPS), 2,5-dihydroxybenzoic acid (DHB), 3-aminoquinoline (3AQ) and α -cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (MO, USA). DEAE-Sepharose fast-flow and Sephadex G-100 were obtained from GE Healthcare Life Science (Piscataway, NJ, USA). *Endo*-arabinanase (EC 3.2.1.99) was purchased from Megazyme (Wicklow, Ireland). Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), phosphate-buffered saline (PBS, pH 7.4), streptomycin, and penicillin were obtained from Hyclone Co. (UT, USA). Nitric oxide (NO) kit and Cell Counting Kit-8 (CCK-8) were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Deionized water was purified using a Milli-Q system (Milford, MA, USA). All other chemicals and reagents were analytical grade.

2.2. Extraction and purification

The process of extraction and purification of the crude polysaccharide was

carried out according to previous method (Hu, Kong, Yang, & Pan, 2011) in our lab. In brief, dried sample was ground into a fine powder. After defatted with ethanol, the mixtures (1 kg) were dried at room temperature. Then the mixtures were boiled with deionized water for 3 h and filtered. The combined filtrates were concentrated to dryness under reduced pressure. The residue was resuspended in deionized water (800 mL) and washed successively with petroleum ether, EtOAc and finally n-BuOH. To precipitate the polysaccharides, four volumes of 95% ethanol were gradually added to the remaining aqueous. After 12 h at 4 °C, the pellets was collected by centrifugation, and washed with acetone and 95% ethanol three times. Protein was removed using Sevage reagent (Sevag, Lackman, & Smolens, 1938). The deproteinated solution was decolorized by passing through a macroreticular anion-exchange resin column (D315). This resulting solution was dialyzed in 3500 Da molecular weight cut-off (MWCO) tubing at 37 °C for 48 h, and the solution within the dialysis membrane was freeze-dried to yield crude polysaccharide (30 g, 3%).

The decolorized fraction (400 mg) was dissolved in deionized water (2 mL), and centrifuged. The supernatant was then applied to a column of DEAE-Sephadex fast-flow (2.6×40 cm) eluted successively with 0.1 M, 0.15 M, and 0.2 M NaCl (300 mL each). The content of polysaccharide was detected by phenol-H₂SO₄ spectrophotometric method (Dubois, Gilles, Hamilton, & Rebers, 1956). According to the elution profile, main part (0.1 M) was dialyzed against deionized water in 3500 Da MWCO tubing as mentioned above. The residue within membrane was then concentrated, lyophilized, and further chromatographed on a Sephadex G-100

gel-filtration column (2.6×40 cm) using deionized water as eluent. Appropriate fractions were combined, dialyzed and lyophilized to yield BTP1 (200 mg).

2.3. Purity, molecular weight and monosaccharide composition

The purity of BTP1 was determined by high-performance gel permeation chromatography (HPGPC) carried out on a Waters 515 instruments (Milford, MA) fitted with a Waters 2410 refractive index detector and a TSK G4000-SWXL (Tosoh Biosep, made in Japan) size exclusion analytical column (7.8×300 mm). The mobile phase was deionized water and the sample concentration was 2 mg mL⁻¹. T-series dextran standards, which have definite molecular masses ranging from 10 to 500 kDa, were used to calibrate the HPGPC system. During the experiment process, the column was kept at 40 °C. Data obtained by the system were collected and analyzed using the Waters Millennium 32 software package.

Monosaccharide composition was measured according to method (Yang, Prasad, & Jiang, 2016), with modifications. Briefly, BTP1 was hydrolysed by 2.0 M trifluoroacetic acid at 100 °C for 2 h, dried by vacuum rotary evaporation. Hydroxylamine hydrochloride and pyridine were added to the hydrolysates or monosaccharide standard mixture, and reacted at 100 °C for 30 min. After acetic anhydride was added, the reaction was carried out at 100 °C for 30 min. The acetylated derivatives were loaded onto gas chromatography (Thermo Fisher Scientific) fitted with a HP-5 capillary column and a flame ionization detector. A gradient temperature program (150-300 °C, 2 min initial hold, 15 °C min⁻¹ ramp rate, and hold until the end of run) was used. The ionization potential was 70 eV, and the temperature of the ion

source was 200 °C.

2.4. Structure analysis of BTp1

2.4.1. FT-IR spectroscopy

Infrared (IR) spectrometry of BTp1 was performed in the 4000-400 cm^{-1} region on a Beckman Acculab-10-FTIR spectrometer. Samples was pressed into tablets with KBr and subjected to 64 scans at 4 cm^{-1} resolution in reference to air to obtain an average spectrum.

2.4.2. MALDI-TOF-MS analysis

MALDI-TOF-MS analyses were performed using a Bruker UltrafleXtreme™ mass spectrometer equipped with a modified Nd: YAG laser (355 nm, 2000 Hz). The mixture of matrix CHCA and peptides standard (Peptide Calibration Standard II, Bruker Daltonics, Bremen, Germany) were used for calibration of spectra. Linear mode was used to detect BTp1 directly, while reflectron mode was used to analyse hydrolysate of BTp1 treated by *endo*-arabinanase. Data were collected and analyzed by Bruker flexControl 3.4 (Germany).

2.4.2.1. Matrix preparation

DHB matrix solution was prepared by dissolving 15.4 mg of DHB into 1 mL 50% ACN. 3AQ-CHCA was prepared according to previous method (Kaneshiro, Fukuyama, Iwamoto, Sekiya, & Tanaka, 2011), with modification. 1 M of 3AQ was prepared with 50% ACN as solvent. Then CHCA was dissolved in the obtained 3AQ (1M) with a final concentration of 0.1 M. The resulting mixtures was vortexed for 5 min to get the 3AQ-CHCA ionic liquid matrix.

2.4.2.2. Enzymatic digestion

Enzymatic digestion of BTP1 was carried out according to (Lin, Wu, Xie, Zhao, & Li, 2015), with modification. In brief, BTP1 solution (2 mg mL^{-1}) was mixed with *endo*-arabinanase (the final concentration was 5 U mL^{-1}) in a total volume of 1 mL and digested overnight (12 h) at 40°C . The mixtures were then heated at 90°C for 20 min to denature the enzymes. After centrifugation (12000 rpm for 10 min), the supernatant was obtained and stored at 4°C for subsequent MALDI analysis.

2.4.3. Nuclear magnetic resonance (NMR) spectroscopy

BTP1 was dissolved in $0.5 \text{ mL D}_2\text{O}$ to a final concentration of 10% (w/v). NMR spectra were obtained at 25°C on an Agilent 600 MHz instrument. The measured spectroscopy included ^1H , ^{13}C , DEPT-135, $^1\text{H}/^1\text{H}$ correlation spectroscopy (COSY), $^1\text{H}/^1\text{H}$ nuclear overhauser effect spectroscopy (NOESY), $^1\text{H}/^1\text{H}$ total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC). ^1H NMR spectrum was recorded by suppressing the HOD signal (δ 4.74) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegthart, 1992). Both ^1H and ^{13}C NMR were decoupled. Acetone (δ 30.89) was used as internal standard to calibrate the ^{13}C chemical shift. Chemical shifts were expressed in ppm.

2.5. Cell culture and immunomodulatory activity assay

2.5.1. Cell culture

RAW 264.7 macrophage cells were purchased from American Type Culture Collection (Rockville, Md., USA). Cells were cultured in DMEM supplemented with

10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂.

2.5.2. Cell cytotoxicity assay

Cell cytotoxicity assays were performed using CCK-8, following the manufacturer's instructions. Briefly, cells were seeded in 96-well plates (5×10^3 well⁻¹). After 24 h of incubation at 37 °C, cells were allowed to grow in medium alone (blank control), in medium with serial concentrations (62.5, 250 and 1000 µg mL⁻¹) of BTp1 and LPS (50 µg mL⁻¹). After 48 h, CCK-8 (10 µL) was added to each well, and the cells were incubated for 2 h. The absorbance of each well was measured at 450 nm and results were expressed as ratio of absorbance values between treatment and blank control.

2.5.3. Nitric oxide assay

RAW 264.7 cells (5×10^4 well⁻¹) were seeded in 96-well microplates overnight. The culture medium was refreshed and cells were incubated with serial concentrations (62.5, 250 and 1000 µg mL⁻¹) of BTp1 and LPS (50 µg mL⁻¹) as the positive control, respectively. An equal volume of culture medium was used as the blank control. The supernatants were collected after incubation for 24 h. The concentrations of NO in the supernatants were determined using NO-detecting kit according to the manufacturer's instructions at 540 nm.

2.5.4. Statistical analysis

Experiments were carried out independently in triplicates and the data were expressed as mean ± SEM values. All data were analyzed using one-way analysis of

variance statistically. Significant differences were set at $p < 0.05$. All statistical analysis was performed through statistical software (SPSS, Version 17.0).

3. Results and discussion

3.1. Isolation and purification

The water-soluble polysaccharides were extracted from *B. ternatum* by hot-water extraction strategy. After filtrates were concentrated, the aqueous solution was defatted with ethanol. The resulting fraction was deproteinated using Sevage reagent and decolorized using a weakly basic anion-exchange styrene macroreticular resin, consecutively. Then the obtained part was separated through DEAE-Sepharose fast-flow. It gave three fractions, which were eluted sequentially by 0.1 M, 0.15 M and 0.2 M NaCl (Fig. 1). The fraction eluted by 0.1 M NaCl had a higher yield and had a good water solubility. It has been collected according to result of phenol-H₂SO₄ method and then further purified by Sephadex G-100 gel-filtration chromatography column. Combining ion exchange chromatography with gel chromatography has been proved to be an efficient strategy for polysaccharides purification (He, Liang, Zhang, & Pan, 2014; Hu et al., 2011). Finally, main peak has been collected and lyophilized to obtain BTp1.

Fig.1. Stepwise elution of crude BTp1 on a DEAE-Sepharose fast-flow column

3.2. Molecular weight and monosaccharide composition

The HPGPC profile (Fig. 2A) showed a symmetrically single peak, indicating that BTp1 was a homogeneous polysaccharide. According to the calibration curve, the average *M_w* of BTp1 was about 12 kDa. BTp1, estimated to be 98% carbohydrate by spectrophotometric analysis (data not shown), appeared as a white powder and had no absorption at 280 or 260 nm in the UV spectrum, suggesting the absence of protein and nucleic acid.

MALDI continues to be a major technique for the analysis of carbohydrates (Harvey, 2015). In this study, BTp1 has also been analyzed by MALDI (Fig. 2B) using 3AQ-CHCA as matrix in linear and positive-ion mode directly. The uniform and Gaussian distribution of peaks (different degree of polymerisation) confirmed the homogeneous property of BTp1 and average *M_w* detected was 11787.5 Da with an index of polydispersity value of 1.028. The peak-to-peak mass difference of 132 Da was observed between neighboring units, corresponding to the the elimination of one Ara unit. And peaks could be assigned to the mass of the singly charged $[M+3AQ+Na]^+$, calculated as $132.05n+126.06+18.01+22.99$, where *n* is the number of Ara units. According to 11787.5 Da, BTp1 was composed of about 88 Ara units and average *M_w* was 11638 Da.

Fig.2. The HPGPC profile (A), MALDI-TOF-MS spectrum (B) of BTp1 and gas chromatography of six monosaccharide standard mixture (C), BTp1 sample after derivatization (D). In figure C and D, the numbers of 1, 2, 3, 4, 5 and 6 represent Rha, Ara, Fuc, Man, Glu and Gal, respectively..

The monosaccharide composition of BTp1 was determined by acid hydrolysis, aldonitrile acetate derivatization and gas chromatography analysis (Fig. 2C & D). Based on retention time compared with monosaccharide standard mixture and electron impact profiles, BTp1 was composed of Ara monomers. Determination of the absolute configurations of the sugars present in BTp1 was based on the GC-MS analysis of their acetylated (+)-2-butyl glycosides (Gerwig, Kamarling, & Vliegenthart, 1978), which indicated the L-configuration of the constructive sugars.

3.3. Structure prediction of BTp1

3.3.1. FT-IR spectroscopy

The IR spectrum of BTp1 (Fig. 3) showed bands at 3430, 2929, 1637, 1384, 1316, 1221, 1080, 1040, 860, and 804 cm^{-1} . The broad and intense IR band at 3430 cm^{-1} was assigned to OH stretching vibration, and the band at 2929 cm^{-1} arose from C-H stretching vibration. The band in region of 1637 cm^{-1} was due to the absorbed water. The peak at around 1384 cm^{-1} was ascribed to the OH bending vibration. The bands at 1316 and 1221 cm^{-1} were attributed to the bending vibration of C-H. The bands at 1200-1000 cm^{-1} were due to the stretch vibration of C-O-C and C-O-H. It was observed that BTp1 was free of protein as confirmed from the absence of bands corresponding to amide I ($\sim 1654 \text{ cm}^{-1}$) and amide II ($\sim 1538 \text{ cm}^{-1}$) vibrations (Synytsya et al., 2009). And the absence of absorption peak at around 1730 cm^{-1} confirmed the absence of uronic acid (Siu, Xu, Chen, & Wu, 2016).

Fig.3. FT-IR spectrum of BTp1

3.3.2. MALDI-TOF-MS analysis of enzymatic hydrolysate

MALDI-TOF-MS spectrometry is a convenient tool for the structural analysis of

polysaccharide (Barrientos, Clerigo, & Paano, 2016; Ding et al., 2015, 2016), and it has been used to probe the major connectivity of BTp1 in the present study. *Endo*-arabinanase is an enzyme that catalyses 1,5- α -L-arabinan specifically. The properties of *endo*-arabinanase was shown in Table S1 & S2. After treated by this enzyme, the *Mw* of polysaccharide suffered from a sharp decrease in comparison with that of the original BTp1. As shown in Fig. 4A & B, a series of ions were detected with strong intensity in the mass spectrum using 3AQ-CHCA and DHB matrices, respectively. The distinct mass peaks were corresponding to the mass number of $[M+3AQ+Na]^+$ and $[M+Na]^+$ in Fig. 4A and Fig. 4B. The data above indicated that the backbone of BTp1 was composed of (1 $\rightarrow$ 5)-linked α -L-Araf.

Fig.4. MALDI-TOF spectrum of enzymatic hydrolysates from BTp1 using 3AQ-CHCA (A) and DHB (B) as matrices.

3.3.3. NMR spectroscopy

The 600 MHz ^1H NMR spectrum (Fig. 5A) of the polysaccharide shows five anomeric proton signals at δ 5.10, 5.05, 5.03, 5.00, 4.93 with the ratio of peak areas at approximate 1:1:2:1:2. The, the signals at δ 5.03 and 4.93 corresponded to two residues respectively while the signals at δ 5.10, 5.05, and 5.00 indicated the presence of only one residue. The sugar residues were described to be A-E according to their decreasing anomeric proton chemical shifts. In the 150 MHz ^{13}C NMR spectrum (Fig. 5B) and DEPT-135 NMR spectrum (Fig. 5C), four anomeric signals at δ 108.95, 109.41, 109.73, and 110.08 were present in a ratio of nearly 2:2:1:2. Signal at δ 108.95 was assigned to anomeric carbons of A and B residues, whereas signals at δ 109.41, 109.73, and 110.08 were assigned for anomeric carbons of C, D, and E residues, respectively. All the ^1H and ^{13}C signals (Table 1) were assigned, in combination with literatures data (Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Mandal et al., 2011; Xia, Liang, Yang, Wang, & Kuang, 2015), using COSY (Fig. 5D), NOESY (Fig. 5E), $^1\text{H}/^1\text{H}$ TOCSY (Fig. 5F), HSQC (Fig. 5G), and HMBC (Fig. 5H) experiments. The assigning principles have been used in this study. Firstly, anomeric proton signals were assigned according to anomeric carbon signals in HSQC (Fig. 5G) spectrum. Then the other proton signals of the residues have been assigned on the basis of COSY (Fig. 5D) and $^1\text{H}/^1\text{H}$ TOCSY (Fig. 5F) spectrum. Finally, assignment of all carbon signals has been carried out in accordance with HSQC (Fig. 5G). The NOESY (Fig. 5E) and HMBC (Fig. 5H) have been used for the confirmation of all the NMR signals and also for determination of residues sequence.

Table 1 Assignments of ^1H and ^{13}C NMR spectra for BTp1

Residues	Linkage		1	2	3	4	5
A	1,2,3,5- α -L-Araf	C	108.95	87.43	82.64	83.35	68.57
		H	5.10	4.16	4.15	4.10	3.80-3.78; 3.70-3.68
B	1,2,5- α -L-Araf	C	108.95	89.46	77.73	83.44	68.95
		H	5.05	4.01	4.01	4.16	3.75-3.73; 3.67-3.63
C	1- α -L-Araf	C	109.41	83.88	79.07	86.57	63.61
		H	5.03	3.97	3.81	3.92	3.69-3.65; 3.58-3.55
D	1- α -L-Araf	C	109.73	83.9	79.10	86.63	63.66
		H	5.00	3.97	3.81	3.88	3.69-3.65; 3.58-3.55
E	1, 5- α -L-Araf	C	110.08	83.85	79.35	84.80	69.56
		H	4.93	3.97	3.86	4.05	3.75-3.73; 3.63-3.61

Fig.5. NMR spectra of BTp1 in D_2O . (A) ^1H spectra (B) ^{13}C spectra (C) DEPT-135 spectra (D) COSY spectra (E) NOESY spectra (F) $^1\text{H}/^1\text{H}$ TOCSY spectra (G) HSQC spectra (H) HMBC spectra.

For furanose residue attended in glycosidic linkage, the chemical shifts of anomeric carbon present at δ 105–110 (Yang et al., 2016). The very high anomeric carbon chemical shifts (δ 108.95-110.08, A-E) in the present study indicated that all these residues were present as furanose. In addition, coupling constants ($J_{\text{H1-H2}}$) of α -anomeric protons differ remarkably from that of β -oriented anomer for arabinosyl

moiety (Dourado et al., 2006; Mandal et al., 2011; Xia et al., 2015). They can be used for the identification of the anomeric configuration of the corresponding residues. In this study, the J_{H1-H2} of the anomeric protons in all of the arabinosyl residues were observed as a broad singlet, revealing that they were present as an α -anomer.

Residue A has an anomeric carbon chemical shift at δ 108.95 with an anomeric proton signal at δ 5.10. The downfield shifts of C-2, C-3, and C-5 (Fig. 5B & C, Table 1) compared with the standard methyl arabinofuranosides (Agrawal, 1992) illustrated that the residue A was 1,2,3,5-linked arabinofuranose. The anomeric carbon chemical shift of residue A was confirmed by the cross-peaks (AH1, BH5a), (AH1, BH5b) in NOESY (Fig. 5E), and (AC1, BH5a), (AC1, BH5b) in HMBC experiment (Fig. 5H).

Residue B has an anomeric carbon chemical shift at δ 108.95 with an anomeric proton signal at δ 5.05. The downfield shifts of C-2 and C-5 (Fig. 5B & C, Table 1) with respect to the standard methyl arabinofuranosides (Agrawal, 1992) indicated that the residue B was 1,2,5-linked arabinofuranose. The anomeric carbon chemical shift of residue B was confirmed by the cross-peaks (BH1, EH5a), (BH1, EH5b) in NOESY (Fig. 5E), and (BC1, EH5a), (BC1, EH5b) in HMBC experiment (Fig. 5H).

The anomeric proton signals at δ 5.03 and 5.00, and anomeric carbon chemical shifts at δ 109.41 and 109.73 could be assigned for C and D, respectively. The carbon chemical shifts of residue C and D corresponded nearly to the value of standard methyl arabinofuranosides. The downfield shifts of C-1 (Fig. 5B & C, Table 1) with respect to the standard arabinofuranosides (Voelter & Breitmaier, 1986) indicated that the residue C and D were terminal-arabinofuranosyl moieties (1 $\rightarrow$). The anomeric

carbon chemical shift of residue C and D were confirmed by the cross-peaks (CH1, AH2), (CH1, BH2), (DH1, AH3) in NOESY (Fig. 5E), and (CC1, AH2), (CC1, BH2), (DC1, AH3) in HMBC experiment (Fig. 5H).

Residue E has an anomeric carbon chemical shift at δ 110.08 with an anomeric proton signal at δ 4.93. The downfield shift of C-5 (Fig. 5B & C, Table 1) with respect to the standard methyl arabinofuranosides (Agrawal, 1992) indicated that the residue E was 1,5-linked arabinofuranosyl moiety. The anomeric carbon chemical shift of residue E was confirmed by the cross-peaks (EH1, AH5), (EH1, EH5) in NOESY (Fig. 5E), and (EC1, AH5), (EC1, EH5) in HMBC experiment (Fig. 5H).

Based on the results of monosaccharide composition, FT-IR spectroscopy, MALDI-TOF-MS and 1D and 2D NMR spectroscopy, the following sequences of BTp1 were hypothesized as shown in Fig. 6.

Fig. 6. The chemical structure of BTp1

3.4. Immunomodulatory activity of BTp1

Macrophages play indispensable roles in the innate and adaptive immune response to pathogens and in-tissue homeostasis (Hao et al., 2012). Nitric oxide (NO) is one of the cytokines released by stimulating agents and was directly involved in defending against pathogen invasion (Commins, Borish, & Steinke, 2010; Lee et al., 2013).

Therefore, the effects of BTp1 on the production of NO released from the RAW 264.7 cells was investigated. Fig. 7A shows the effects of BTp1 on the viability of cells. The results showed that BTp1 at a series concentration (62.5-1000 $\mu\text{g mL}^{-1}$) had no cytotoxic effects, and could stimulate the proliferation of RAW 264.7 cells ($p < 0.05$). In addition, compared with blank control, the release of NO was significantly promoted by BTp1 in a dose-dependent manner (Fig. 7B). When the polysaccharide dosage reached 1000 $\mu\text{g mL}^{-1}$, the production of NO was even higher than that of group treated by LPS (positive control). All results above suggested that RAW 264.7 can be activated by BTp1 in the tested concentration range. And detailed bioactivities and the structure-bioactivity relationship of the purified polysaccharide BTp1 will be further investigated.

Fig. 7. Effects of BTp1 on cell viability (A) and production of NO (B) of RAW 264.7 *in vitro*. * and ** represent $p < 0.05$ and $p < 0.01$ compared with blank control, respectively.

4. Conclusion

In summary, a water-soluble polysaccharide BTp1 was isolated and purified from *B. ternatum* in the present study. Using 3AQ-CHCA as MALDI matrix, precise M_w of BTp1 was determined to be 11638 Da. On the basis of monosaccharide analysis,

MALDI-TOF and NMR study, the structure of BTP1 was putatively identified. The backbone was constructed by (1→5)-Araf and the branch chains were (1→)-linked α -L-Araf at O-2, O-3 and adjacent O-2 positions. The immunomodulatory assay showed that BTP1 could significantly enhance the viability of RAW 264.7 cells, and promote the release of NO. These results suggested that *B. ternatum* polysaccharide might be a good candidate for the development of new immunomodulatory functional food supplement.

Acknowledgement

Financial support from the National Science Foundation of China (Grant No. 21327010 and No. 21372199) are gratefully acknowledged.

References

- Agrawal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, 31, 3307-3330.
- Barrientos, R. C., Clerigo, M. M., & Paano, A. M. C. (2016). Extraction, isolation and MALDI-QTOF MS/MS analysis of β -D-Glucan from the fruiting bodies of *Daedalea quercina*. *International Journal of Biological Macromolecules*, 93, 226-234.
- Cheng, H. N., & Neiss, T. G. (2012). Solution NMR spectroscopy of food polysaccharides. *Polymer Reviews*, 52, 81-114.
- Chien, R. C., Yen, M. T., Tseng, Y. H., & Mau, J. L. (2015). Chemical characteristics

- and anti-proliferation activities of *Ganoderma tsugae* polysaccharides. *Carbohydrate Polymers*, 128, 90-98.
- Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, 131, 209-217.
- Commings, S. P., Borish, L., & Steinke, J. W. (2010). Immunologic messenger molecules: Cytokines, interferons, and chemokines. *Journal of Allergy and Clinical Immunology*, 125, S53-S72.
- Ding, H. H., Cui, S. W., Goff, H. D., Chen, J., Wang, Q., & Han, N. F. (2015). Arabinan-rich rhamnogalacturonan-I from flaxseed kernel cell wall. *Food Hydrocolloids*, 47, 158-167.
- Ding, H. H., Cui, S. W., Goff, H. D., Chen, J., Guo, Q., & Wang, Q. (2016). Xyloglucans from flaxseed kernel cell wall: Structural and conformational characterisation. *Carbohydrate Polymers*, 151, 538-545.
- Dourado, F., Cardoso, S. M., Silva, A. M., Gama, F. M., & Coimbra, M. A. (2006). NMR structural elucidation of the arabinan from *Prunus dulcis* immunobiological active pectic polysaccharides. *Carbohydrate Polymers*, 66, 27-33.
- DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350-356.
- Ferreira, S. S., Passos, C. P., Madureira, P., Vilanova, M., & Coimbra, M. A. (2015). Structure-function relationships of immunostimulatory polysaccharides: A

review. *Carbohydrate Polymers*, 132, 378-396.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. (1978). Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary GLC. *Carbohydrate Research*, 62, 349-357.

Hao, N. B., Lü, M. H., Fan, Y. H., Cao, Y. L., Zhang, Z. R., & Yang, S. M. (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clinical and Developmental Immunology*, 2012.

Hård, K., Van zadelhoff, G., Moonen, P., Kamerling, J. P., & Vliegenthart, J. F. (1992). The Asn- linked carbohydrate chains of human Tamm- Horsfall glycoprotein of one male. *The FEBS Journal*, 209, 895-915.

Harvey, D. J. (2015). Analysis of carbohydrates and glycoconjugates by matrix- assisted laser desorption/ionization mass spectrometry: An update for 2011–2012. *Mass Spectrometry Reviews*, 9999, 1-168

He, Z., Liang, F., Zhang, Y., & Pan, Y. (2014). Water-soluble polysaccharides from finger citron fruits (*Citrus medica* L. var. *sarcodactylis*). *Carbohydrate Research*, 388, 100-104.

Hsu, N. Y., Yang, W. B., Wong, C. H., Lee, Y. C., Lee, R. T., Wang, Y. S., et al. (2007). Matrix-assisted laser desorption/ionization mass spectrometry of polysaccharides with 2', 4', 6'-trihydroxyacetophenone as matrix. *Rapid Communications in Mass Spectrometry*, 21, 2137-2146.

Hu, C., Kong, Q., Yang, D., & Pan, Y. (2011). Isolation and structural characterization of a novel galactomannan from *Eremurus anisopterus* (Ker. et Kir) Regel

- roots. *Carbohydrate Polymers*, 84, 402-406.
- Hu, D. J., Cheong, K. L., Zhao, J., & Li, S. P. (2013). Chromatography in characterization of polysaccharides from medicinal plants and fungi. *Journal of Separation Science*, 36, 1-19.
- Jin, M., Zhao, K., Huang, Q., & Shang, P. (2014). Structural features and biological activities of the polysaccharides from *Astragalus membranaceus*. *International Journal of Biological Macromolecules*, 64, 257-266.
- Kaneshiro, K., Fukuyama, Y., Iwamoto, S., Sekiya, S., & Tanaka, K. (2011). Highly sensitive MALDI analyses of glycans by a new aminoquinoline-labeling method using 3-aminoquinoline- α -cyano-4-hydroxycinnamic acid liquid matrix. *Analytical Chemistry*, 83, 3663-3667.
- Lee, J. S., Synytsya, A., Kim, H. B., Choi, D. J., Lee, S., Lee, J., et al. (2013). Purification, characterization and immunomodulating activity of a pectic polysaccharide isolated from Korean mulberry fruit Oddi (*Morus alba* L.). *International Immunopharmacology*, 17, 858-866.
- Li, S. P., Wu, D. T., Lv, G. P., & Zhao, J. (2013). Carbohydrates analysis in herbal glycomics. *TrAC Trends in Analytical Chemistry*, 52, 155-169.
- Lin, P. C., Wu, D. T., Xie, J., Zhao, J., & Li, S. P. (2015). Characterization and comparison of bioactive polysaccharides from the tubers of *Gymnadenia conopsea*. *Food Hydrocolloids*, 43, 199-206.
- Lin, Z., Liao, W., & Ren, J. (2016). Physicochemical characterization of a polysaccharide fraction from *Platycladus orientalis* (L.) franco and its

- macrophage immunomodulatory and anti-hepatitis B virus activities. *Journal of Agricultural and Food Chemistry*, 64, 5813-5823.
- López-García, M., García, M. S. D., Vilariño, J. M. L., & Rodríguez, M. V. G. (2016). MALDI-TOF to compare polysaccharide profiles from commercial health supplements of different mushroom species. *Food Chemistry*, 199, 597-604.
- Mandal, S., Patra, S., Dey, B., Bhunia, S. K., Maity, K. K., & Islam, S. S. (2011). Structural analysis of an arabinan isolated from alkaline extract of the endosperm of seeds of *Caesalpinia bonduc* (Nata Karanja). *Carbohydrate Polymers*, 84, 471-476.
- Qi, J. H. (2012). A summary of recent studies on *Botrychium* Sw. *Journal of Xi'an University of Arts & Science (Natural Science Edition)*, 2, 014.
- Sevag, M. G., Lackman, D. B., & Smolens, J. (1938). The isolation of the components of streptococcal nucleoproteins in serologically active form. *Journal of Biological Chemistry*, 124, 425-36.
- Siu, K. C., Xu, L., Chen, X., & Wu, J. Y. (2016). Molecular properties and antioxidant activities of polysaccharides isolated from alkaline extract of wild *Armillaria ostoyae* mushrooms. *Carbohydrate Polymers*, 137, 739-746.
- Synytsya, A., Míčková, K., Synytsya, A., Jablonský, I., Spěváček, J., Erban, V., et al. (2009). Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers*, 76, 548-556.
- Voelter, W., & Breitmaier, E. (1986). *Carbon-13 NMR spectroscopy: High-resolution*

methods and applications in organic chemistry and biochemistry. John Wiley & Sons.

Wang, P. C., Zhao, S., Yang, B. Y., Wang, Q. H., & Kuang, H. X. (2016). Anti-diabetic polysaccharides from natural sources: A review. *Carbohydrate Polymers*, *148*, 86-97.

Warashina, T., Umehara, K., & Miyase, T. (2012). Flavonoid glycosides from *Botrychium ternatum*. *Chemical and Pharmaceutical Bulletin*, *60*, 1561-1573.

Xia, Y. G., Liang, J., Yang, B. Y., Wang, Q. H., & Kuang, H. X. (2015). Structural studies of an arabinan from the stems of *Ephedra sinica* by methylation analysis and 1D and 2D NMR spectroscopy. *Carbohydrate Polymers*, *121*, 449-456.

Xie, X., Zou, G., & Li, C. (2015). Antitumor and immunomodulatory activities of a water-soluble polysaccharide from *Chaenomeles speciosa*. *Carbohydrate Polymers*, *132*, 323-329.

Yang, B., Prasad, K. N., & Jiang, Y. (2016). Structure identification of a polysaccharide purified from litchi (*Litchi chinensis* Sonn.) pulp. *Carbohydrate Polymers*, *137*, 570-575.

Yu, Q., Nie, S. P., Wang, J. Q., Huang, D. F., Li, W. J., & Xie, M. Y. (2015). Signaling pathway involved in the immunomodulatory effect of *Ganoderma atrum* polysaccharide in spleen lymphocytes. *Journal of Agricultural and Food Chemistry*, *63*, 2734-2740.

Yuan, Y., Yang, B., Ye, Z., Zhang, M., Yang, X., Xin, C., et al. (2013). *Sceptridium ternatum* extract exerts antiasthmatic effects by regulating Th1/Th2 balance and

the expression levels of leukotriene receptors in a mouse asthma model. *Journal of Ethnopharmacology*, 149, 701-706.