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Water-soluble polysaccharide from *Bupleurum chinense* DC: Isolation, structural features and antioxidant activity

Liwei Sun *, Kai Feng, Rui Jiang, Jiaqing Chen, Yu Zhao, Rui Ma, Haibin Tong *

Life Science Research Center, Beihua University, Jilin 132013, China

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ABSTRACT

The water-soluble polysaccharide (BCPS-1) was isolated from *Bupleurum chinense* DC. BCPS-1 (M_w = 29 kDa) was composed of Ara; Gal; Glc with a molar ratio of 2.1:2.5:1. According to FT-IR, partial acid hydrolysis, periodate oxidation and Smith degradation, methylation and GC-MS analysis, the results indicate BCPS-1 had a backbone of (1→5)-linked Ara, (1→4)-linked Gal and (1→3)-linked Gal residues with occasionally branches at O-6. The branches were composed of (1→4)-linked Glc, and terminated with Gal residues. The *in vitro* antioxidant activity evaluated by DPPH radical scavenging method showed that BCPS-1 had a significant antioxidant effect in a concentration-dependent manner.

1. Introduction

Free radicals are by-products of the cellular metabolism and transition-metal ions, and they seem to play an important role in causing biomacromolecule damage *in vivo* (Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). Increasing oxidative stress and disorders in energy metabolisms may lead to mutations and eventually to many severe diseases. Antioxidants may play an essential role in protecting our bodies from various oxidative damages which are linked to diabetes, cancer, cardiovascular disease and neurodegenerative diseases, including Parkinson's and Alzheimer's disease (Lin & Beal, 2003). Hence, the search for new sources of natural antioxidants are currently of major interest of scientists.

Bupleurum chinense DC, belongs to the plant family *Bupleurum* spp., is a well-known Traditional Chinese Medicine (TCM) that has been used for more than thousand years, distributed mainly in Hebei, Liaoning, Jilin, Heilongjiang and Neimeng province of China (Li, Song, Li, Chen, & Bi, 2005). It customarily called "Bei chaihu" in China. First mentioned in the Treatise on Cold Induced Febrile Disease (Shang Han Lun) as a primary ingredient of an ancient Chinese medicinal formula known as *Xiao Chai Hu Tang* from the 1st century BC (Yen, Lin, Chuang, & Liu, 1991), *B. chinense* is one of China's "harmony" herbs, balancing different organs and energies within the body, and it is also used as a tonic herb because of its ability to strengthen the action of the digestive tract, improve liver and circulatory system function, and relieve liver tension (Kuang, Sun, Yang, Xia, & Feng, 2009; Zhu, Liang, Han, & Dong, 2009).

In recent years, increasing researches have been carried out on *B. chinense*, and the results show that the saikosaponin, flavonoid, essential oil and fatty acid possessed several proven pharmacologic activities, including hepato-protective, mild sedative, antipyretic, analgesic, anti-tussive, immunomodulatory effect, anti-fibrotic effect, inhibit growth of liver cancer cells and promotion of liver regeneration (Chang et al., 2007; Sun, 2006; Yin, Pan, Chen, & Hua, 2008; Zhang, Zhou, & Wang, 2007). Therefore, in Japan, Korea and China, it has been widely prescribed to outpatients for treating chronic liver diseases.

Nowadays, lots of researches have tended to focus on the activities of saikosaponin. There is relatively little information pertaining to isolation, purification, and activity determination of the water-soluble polysaccharide from *B. chinense*, especially detailed studies of the structure. Since structure and functions are intimately related, an in-depth study of the structure of the polysaccharides would be of interest. Therefore, we specifically focused on elucidation the isolation and structural characterization of water-soluble polysaccharide from *B. chinense* and antioxidant effects *in vitro*, ultimately, assessing the putative mechanism underlying the antioxidant effects *in vitro*.

2. Experimental

2.1. Materials and chemicals

The roots of *B. chinense* were purchased from local medicine market in Jilin City, and identified according to the identification standard of Northeast Plant Retrieval List of China.

* Corresponding authors. Tel.: +86 432 4608354; fax: +86 432 4608350 (H. Tong).
E-mail addresses: sunliwei1970@yahoo.com (L. Sun), tonghaibin@gmail.com (H. Tong).

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DEAE Sepharose Fast Flow and Sephacryl S-200 were purchased from Amersham (Sweden). T-series dextrans, 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA) and standard sugars were obtained from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was purchased from Fluka. All other chemical reagents were analytical grade.

2.2. Isolation and purification of polysaccharide

The powdered roots of *B. chinense* (500 g) were extracted with 80% (v/v) ethanol for 24 h. After filtered, the residues were dried and extracted with distilled water at 80 °C for three times. The whole extract was filtered and centrifuged. The supernatant was concentrated by evaporation under reduced pressure and treated with three volumes of ethanol for precipitation at 4 °C overnight. The precipitation was obtained by centrifugation, and dried under reduced pressure. The sample was dissolved in distilled water, then frozen at −20 °C, thawed at room temperature and centrifuged to remove insoluble materials. The supernatant was deproteinated by a combination of proteinase and Sevag method. Finally the supernatant was lyophilized to give crude *B. chinense* polysaccharides (CBCP).

The crude polysaccharides (CBCP) was redissolved in distilled water and loaded onto DEAE Sepharose Fast Flow column (3 × 30 cm), using ÄKTA explore 100 FPLC purification system (Amersham Biosciences division of GE Healthcare) equipped with a P-900 series pump, Monitor UV-900, Monitor pH/C-900, Fraction collector Frac-950 and Auto-sampler A-900, eluted first with distilled water and then with stepwise gradient of NaCl solution (0.1, 0.3, 0.5, and 1 M) at a flow rate of 5 ml/min. Four sharp peaks were detected. All fractions was collected, dialyzed, lyophilized, and the neutral fraction eluted with distilled water was further fractioned on a Sephacryl S-200 column (2.6 × 100 cm), eluted with 0.15 M NaCl at a flow rate of 1 ml/min. One main fraction was collected, dialyzed and lyophilized to obtain purified polysaccharide, named as BCPS-1.

2.3. Homogeneity and molecular weight

The homogeneity and molecular weight of BCPS-1 was evaluated and determined by HPGPC. The sample solution (10 μl of 0.5%) was applied to Shimadzu HPLC system equipped with a TSK-GEL G3000 PWXL column (7.8 × 300 mm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.6 ml/min and detected by a RID-10A refractive index detector. The columns were calibrated with T-series dextran (T-200, T-70, T-40, T-20, and T-10) and glucose (M_w = 180, Sigma) as standards. The molecular weight of BCPS-1 was estimated by reference to the calibration curve made above.

2.4. Measurement of monosaccharide composition, carbohydrate and protein contents

Gas chromatography (GC) was used for identification and quantification of the monosaccharide compositions. BCPS-1 was hydrolyzed with 2 M TFA (2 ml) at 120 °C for 2 h. The hydrolyzed product was converted into the alditol acetates as described by Lehrfeld (1985) and analyzed by GC. GC was performed on a Varian 3400 instrument (Hewlett–Packard Component, USA) equipped with DM-2330 capillary column (30 m × 0.32 mm × 0.2 μm) and flame-ionization detector (FID).

Total carbohydrate content of the polysaccharide was determined by phenol-sulfuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as the standard. In addition, protein in the polysaccharide was quantified according to the Bradford's method (1976) using bovine serum albumin (BSA) as the standard.

2.5. Spectroscopic methods

Ultraviolet–visible spectra of BCPS-1 was recorded with a Varian Cary-100 Ultraviolet Visible Spectrophotometer (Varian Inc., Victoria, Australia). Fourier transform infrared spectra of BCPS-1 was recorded with a Nicolet 5700 FT-IR spectrometer (Nicolet Instrument, Thermo Company, Madison, USA) in the range 4000–400 cm^{-1}. The material was incorporated in KBr disks.

2.6. Partial acid hydrolysis

Partial acid hydrolysis of BCPS-1 (80 mg) was performed according to the method described by Tong, Liang, and Wang (2008) using 0.5 M TFA (3 ml) at 90 °C for 3 h. The hydrolyzed BCPS-1 was centrifuged to remove the precipitation (BCPS-1a), and the supernatant was dialyzed against distilled water for 24 h. After dialysis, the fraction removed was collected and named as BCPS-1d, and then ethanol was added to the solution in dialysis sack, and the precipitation and supernatant designated as BCPS-1b and BCPS-1c, respectively. All fractions were dried for GC analysis as mentioned above.

2.7. Periodate oxidation and Smith degradation

Periodate oxidation and Smith degradation of polysaccharide was carried out as described by Tong et al. (2009). Briefly, BCPS-1 (25 mg) dissolved in 12.5 ml of distilled water was mixed with 12.5 ml of 30 mM $NaIO_4$, and the mixture was kept in dark at 4 °C for 72 h. Ethylene glycol (2 ml) was added to terminate the reaction. About 2 ml periodate-oxdized product was used to calculate the yield of formic acid by 0.00397 M sodium hydroxide, and the rest was extensively dialyzed for 24 h. The content inside the dialysis sack was concentrated and reduced with $NaBH_4$, neutralized with 50% acetic acid, and then dialyzed. One-third of solution mentioned above was freeze-dried and fully hydrolyzed for GC analysis, others were added to the same volume of 1 M sulfuric acid, kept for 40 h at 25 °C, neutralized to pH 6.0 with $BaSO_4$, and filtered for analysis of Smith degradation. The filtrate was dialyzed, and the content out of sack was lyophilized for GC analysis; the content inside was concentrated to a small volume, and added 5 vols. of ethanol (12 ml), the supernatant and precipitate were also dried for GC analysis after centrifugation.

2.8. Methylation analysis

BCPS-1 (20 mg) was methylated twice according to the method described by Ciucanu and Kerek (1984). The methylated products were extracted by $CHCl_3$, and showed no absorption peak in the region 3600–3300 cm^{-1} in the IR spectrum analysis, which indicated methylated products were methylated completely. The product was hydrolyzed with formic acid and 2 M TFA, and excess acid was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with $NaBH_4$ for 24 h, and acetylated with acetic anhydride-pyridine (1:1) at 100 °C for 2 h. The alditol acetates of the methylated sugars were analyzed by GC-MS.

2.9. DPPH radical scavenging activity of CBCP and BCPS-1

The antioxidant activity of polysaccharides was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical test according to the method

described by Yen, Yang, and Mau (2008) with some modifications. Samples (CBCP and BCPS-1) were dissolved in distilled water at 0 (control), 0.05, 0.1, 0.2, 0.4, 1, 2, 4, and 8 mg/ml. One milliliter test samples were thoroughly mixed with 2 ml of freshly prepared DPPH (0.1 mM) in 50% ethanol. After shaking vigorously, the mixture was incubated at 25 °C for 30 min in the dark, and then the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Ascorbic acid was used as a positive control. The experiment was carried out in triplicate and averaged. The scavenging activity of DPPH radicals was calculated as:

(1 - absorbance of sample/absorbance of control) × 100%.

3. Results and discussion

3.1. Isolation, purification and structural analysis of polysaccharide BCPS-1

In this study, the crude polysaccharide was obtained as a water-soluble dust-coloured powder from *B. chinense* by hot water extraction. The total yield of crude water-soluble polysaccharides was 6.5% of the dried material. After the freeze–thaw process and deproteination by a combination of proteinase treatment and the Sevag method, the crude polysaccharide CBCP was purified by ÄKTA explore 100 FPLC systems, and the neutral main fraction (BCPS-1) was separated by DEAE Sepharose Fast Flow and Sephacryl S-200 columns. BCPS-1 was collected for further analysis of structure and antioxidant activity.

BCPS-1 had a negative response to the Bradford's method and no absorption was detected by the UV spectrum at 280 and 260 nm indicating the absence of protein and nucleic acid. BCPS-1 could be eluted as a single peak and its average molecular weight was estimated as 29 kDa by HPGPC according to the calibration curve with standard dextran and glucose. The HPGPC profile also demonstrated that BCPS-1 might be a homogeneous polysaccharide on the basis of its molecular weight and polarity. The total carbohydrate content of BCPS-1 was 97.5% determined by phenol–sulfuric acid method. GC analysis showed BCPS-1 was composed of three kinds of monosaccharides, namely arabinose, galactose, and glucose with a molar ratio of 2.1:2.5:1. IR spectrum of BCPS-1 (Fig. 1) revealed a typical major broad stretching peak at 3411 cm^{-1} for the hydroxyl group, and a weak band at 2919 cm^{-1} showed C–H stretching vibration. The broad band at 1610 cm^{-1} was due to the bound water. The band at 842 cm^{-1} and 877 cm^{-1} indicated α- and β-configurations of the sugar units simultaneously existing in the polysaccharide.

After partial acid hydrolysis of BCPS-1, four fractions BCPS-1a (precipitation after hydrolysis), BCPS-1b (precipitation in the dialysis sack), BCPS-1c (supernatant in the dialysis sack), and BCPS-1d (fraction out of dialysis sack) were obtained, and all the fractions were subjected to GC analysis. The results of GC analysis shown in Table 1 indicated that Ara and Gal in BCPS-1a and BCPS-1b could be the backbone structure of BCPS-1, and Glc and Gal in BCPS-1c and BCPS-1d could be the branched structure of BCPS-1.

BCPS-1 was oxidized with 0.015 M $NaIO_4$ at RT in the dark for 3 days. The results from periodate oxidation showed that 0.896 mmol periodate was consumed and 0.153 mmol formic acid was produced per sugar residue, indicating the existence of small amount of monosaccharides which are 1→ linked or (1→6)-linked. The amount of consumed periodate was more than twice the yield of generated formic acid, indicating the existence of large amounts or 1→4 or (1→2)-linked sugar residue.

The periodate-oxidized products were fully hydrolyzed and analyzed by GC analysis (Table 1). The existence of large amounts of Gal revealed that lots of Gal residues was (1→3)-linked, (1→2,3)-linked, (1→2,4)-linked, (1→3,4)-linked, (1→3,6)-linked or (1→2,3,4)-linked that can not be oxidized. No Ara and Glc were observed, and large amount of glycerol and erythritol were obtained, demonstrating that Ara and Glc were all linkages which can be oxidized by periodate.

GC analysis for Smith degradation shown in Table 1 indicated there was no precipitation in the sack, demonstrated that the backbone of BCPS-1 should be oxidized completely by HIO_4. Hence, it can be concluded that the linkages of backbone are (1→), (1→2), (1→6), (1→2, 6), (1→4) and (1→4, 6) that can be oxidized producing glycerin and erythritol detected out of sack.

The fully methylated BCPS-1 was hydrolyzed with acid, converted into alditol acetates, and analyzed by GC–MS (Table 2). The results showed the presence of five peaks, including 2,3-Me_2-Ara, 2,3,4,6-Me_4-Gal, 2,3,6-Me_3-Glc, 2,3,6-Me_3-Gal and 2,4-Me_2-Gal in molar ratios of 3.8:2.1:2.2:1:1.9. This showed a good correlation between terminal and branched residues, and these molar ratios agree with the overall monosaccharide composition described above.

The results from analysis of GC-MS, which were consistent with the results from partial acid hydrolysis, periodate oxidation and Smith degradation, indicated that 2,3-Me_2-Ara (1,5-linked Ara), 2,3,6-Me_3-Gal (1,4-linked Gal) and 2,4-Me_2-Gal (1,3,6-linked Gal) were major components of the backbone structure, part of Gal

Table 1
GC analysis result of partial acid hydrolysis, periodate-oxidized products and Smith degradation of BCPS-1.

Fractions	Molar ratios				
	Glycerol	Erythritol	Arabinose	Galactose	Glucose
Partial acid hydrolysis					
BCPS-1a[a]			2.8	2.4	
BCPS-1b[b]			0.8	1	
BCPS-1c[c]			Trace	1	0.9
BCPS-1d[d]				1	0.8
Periodate-oxidized products					
Full acid hydrolysis	5.2	3.4		2.4	
Smith degradation					
Out of sack	4.5	2.6		2.1	
Supernatant in sack					
Precipitation in sack					

[a] BCPS-1a, precipitation after hydrolysis.
[b] BCPS-1b, precipitation in the sack.
[c] BCPS-1c, supernatant in the sack.
[d] BCPS-1d, fraction out of sack.

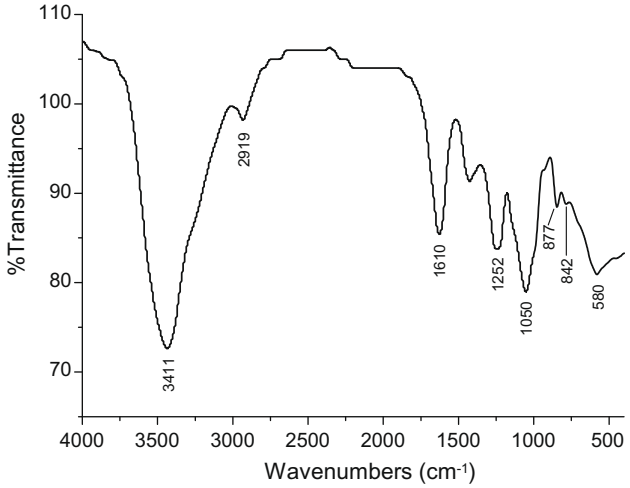

Fig. 1. IR profile of BCPS-1.

Table 2
GC-MS analysis of methylated BCPS-1.

Methylated sugars (as alditol acetates)[a]	Retention time (min)	Mass fragments (*m/z*)	Molar ratio	Type of linkage
2,3-Me_2-Ara	13.04	43,87,101,117,129,189	3.8	1,5-linked Ara
2,3,4,6-Me_4-Gal	13.76	43,45,71,87,101,117,129,145,161,205	2.1	Terminal Gal
2,3,6-Me_3-Glc	15.14	43,45,87,99,101,113,117,233	2.2	1,4-linked Glc
2,3,6-Me_3-Gal	16.01	43,45,87,99,101,113,117,233	1	1,4-linked Gal
2,4-Me_2-Gal	18.43	43,87,117,129,189	1.9	1,3,6-linked Gal

[a] 2,3,4,6-Me4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactose, etc.

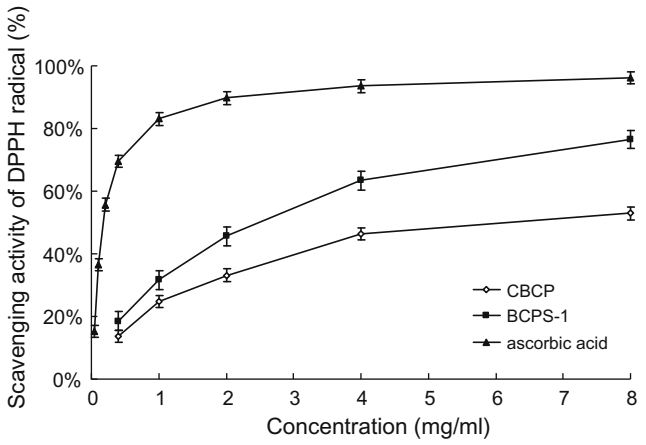

Fig. 2. Scavenging ability of CBCP and BCPS-1 against DPPH radical. Results were presented as means ± SD ($n = 3$).

and all Glc were distributed in branches, and residues of branches terminated with Gal.

3.2. Assay for antioxidant activity of CBCP and BCPS-1

Fig. 2 demonstrated DPPH scavenging activity caused by different concentrations of CBCP and BCPS-1. The DPPH radical scavenging activity of CBCP and BCPS-1 reached 52.9% and 76.5% at 8 mg/ml, respectively. The EC_{50} values of CBCP and BCPS-1 for DPPH radicals were 6.4 mg/ml and 3.8 mg/ml, respectively. Scavenging activity of BCPS-1 was significantly higher ($P < 0.05$) than that of CBCP at the concentration rang of 2–8 mg/mL. The scavenging activity increased steadily at the concentration rang of 0.05–8 mg/ml for CBCP and BCPS-1, while the scavenging reached a maximum plateau from 0.05 to 2 mg/ml for ascorbic acid, which indicated that the scavenging activity of CBCP and BCPS-1 against DPPH radical was less than that of ascorbic acid.

4. Conclusion

This study demonstrated that BCPS-1 isolated from *Bupleurum chinense* DC, which is a heteropolysaccharide consisting of Ara; Gal; Glc with a molar ratio of 2.1:2.5:1, had a backbone of (1→5)-linked Ara, (1→4)-linked Gal and (1→3)-linked Gal residues which occasionally branches at O-6. The branches were composed of (1→4)-linked Glc, and terminated Gal residues. The results of DPPH radical scavenging activity show that the purified polysaccharide (BCPS-1) had a significant antioxidant activity in a concentration-dependent manner. The DPPH radical scavenging activity of BCPS-1 reached 76.5% at 8 mg/ml. The study suggested BCPS-1 could potentially be used as natural antioxidants. The correlation between different extraction technique and antioxidant activity of *B. chinense* polysaccharide will be further investigated in future work.

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