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Structural characterization and anti-inflammatory activity of two water-soluble polysaccharides from *Bellamya purificata*

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ABSTRACT

Two water-soluble polysaccharides of *Bellamya purificata* named BPS-1and BPS-2 were isolated from the foot muscle of *B. purificata*, purified by anion-exchange, gel-permeation chromatography and their anti-inflammatory activity was tested. They were homogenous with a molecular weight of 7.2×10^6 Da and 8.3×10^6 Da by HPGPC, respectively. BPS-1 was composed of glucose, fucose, arabiinose and xylose with a molar ratio of 99:2:1:1, it has a backbone of α-(1 → 4)-linked glucose residues, with branches at C-6 consisting of terminal and α-(1 → 3)-linked glucose residues; BPS-2 was composed of glucose, fucose, arabinose, galactose, xylsoe and mannose with a molar ratio of 218:5:3:2:1:1, and it has a backbone of α-(1 → 4)-linked glucose residues, with branches at C-3 and C-6 consisting of terminal and α-(1 → 3)-linked glucose residues. Anti-inflammatory activity experiments showed that BPS-1 and BPS-2 possessed potent inhibiting functions on mouse ear edema induced by xylene, with the inhibition rate of 57.56% and 56.46% at dose 1 mg/mL, respectively.

1. Introduction

Polysaccharides and polysaccharide–protein complexes have been found as common polymers in shellfish and received considerable attention in recent years because of their anti-inflammatory, antitumor and immunoregulatory activities (Dai, Zhang, Zhang, & Wang, 2009; He et al., 2007; Liu et al., 2008, 2007).

Bellamya purificata belongs to Mollusca phylum, Gastropoda class, Prosobranchia subclass and Viviparidae family, which is a species of mud snail found in fresh water in China (Xia, Wang, & Xu, 2007). It is distributed in the Yangtze River drainage area and most of the east-China provinces with a yearly output of 10 million tonnes. *B. purificata* is a popular aquatic food with nutritional and pharmacological activity in China. It has been used as traditional Chinese medicine to treat dysentery, hemorrhoids, jaundice and eczema.

The foot muscle of *B. purificata* contains abundant protein, polysaccharides, and the like. It is valuable to exploit them. Recently research has tended to focus on the nutritional value of *B. purificata*, there is no reports about purification, and activity of its polysaccharides, especially on its structure. This paper reports the isolation, purification, structure and anti-inflammatory activity of two water-soluble polysaccharides from the foot muscle of *B. purificata*.

2. Materials and methods

2.1. Materials

B. purificata was purchased from local market in Hangzhou, China. DEAE-Sephacel and Sepharose 6B were purchased from Amersham Biosciences, CA, USA; trypsin, papain and dextrans were from Sigma Chemical Co., St. Louis, MO, USA; aqueous solutions were prepared with ultrapure water made by a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used in this paper were analytical grade.

2.2. Isolation and purification of polysaccharides

The fresh foot muscle of *B. purificata* (50 g) was defated for 4 h with acetone. The extract was discarded and the residue was further extracted with 1400 mL water at 75 °C for 5.2 h. The extract was filtered and centrifuged at 3500 rpm for 10 min at room temperature. The supernatant was concentrated in a rotary evaporator under reduced pressure at 50 °C and precipitated by the addition of ethanol in 1:3 ratios (v/v) at 4 °C. After overnight precipitation, the sample was centrifuged at 8000 rpm for 10 min at 4 °C. The precipitate was dissolved in 500 mL of distilled water and deprotein by a combination of proteinase and sevag method (isoamyl alcohol and chloroform in 1:4 ratio) (Staub, 1965). The resulting aqueous

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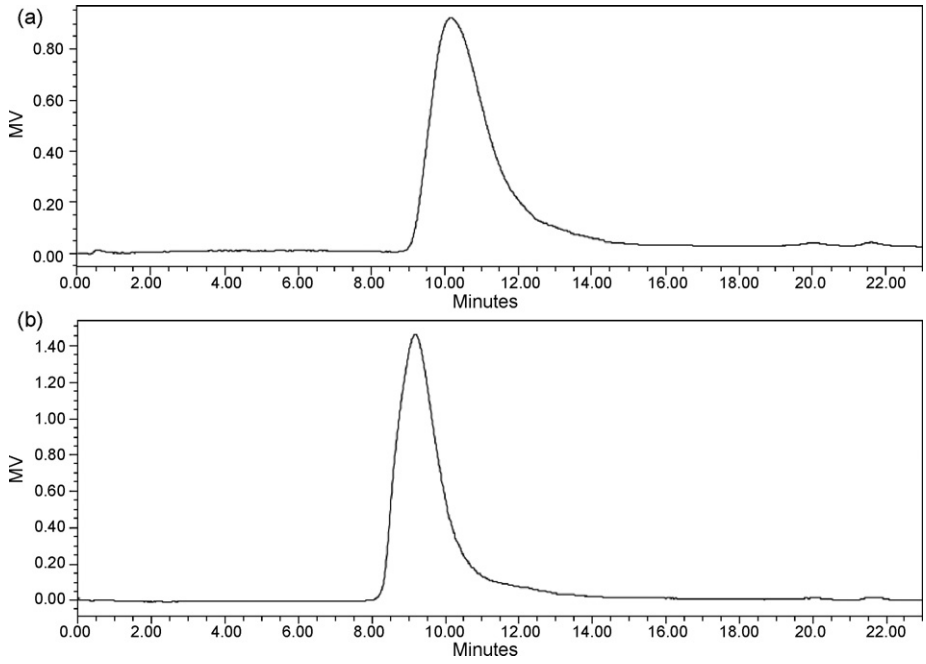

Fig. 1. HPGPC profile of BPS-1 (a) and BPS-2 (b).

fraction was extensively dialyzed against double-distilled water for 2 days and precipitated by addition of 3 vol. of anhydrous EtOH. After centrifugation, the precipitate was washed with anhydrous EtOH and then freeze dried to yield the crude polysaccharide (CBPS, 1.00 g).

The crude polysaccharide was dissolved in distilled water, applied to a DEAE-Sephacel column (1.6 cm × 40 cm) and eluted with distilled water, followed by 0.1 M NaCl and 0.5 M NaCl, respectively. Each fraction of 5 mL was collected at a flow rate of 30 mL/h and monitored by the phenol-sulfuric acid method at 490 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The collected fractions were further chromatographed on a Sepharose 6B (1.6 cm × 120 cm) eluted with distilled water, fractions were collected and lyophilized to give white power polysaccharides of *B. purificata* named BPS-1 and BPS-2, respectively, which were subjected to the subsequent analyzes.

2.3. Homogeneity and molecular weight

The homogeneity and molecular weight of BPS-1 and BPS-2 were determined on a Waters HPLC system (Allances 2695, Waters, USA) equipped with a Waters Ultrahydrogel linear column (7.8 mm × 300 mm) and a Waters 2410 differential refractometer. A sample solution (10 μL of 0.1%) was injected in each run, with ultrapure water as the mobile phase at 0.5 mL/min. The HPLC system was precalibrated with T-series Dextran standards (T-10, T-40, T-70, T-500 and T-2000).

2.4. Monosaccharide analysis

BPS-1 and BPS-2 (10 mg), dissolved in 2 M trifluoroacetic acid (TFA, 2 mL), was hydrolyzed at 120 °C for 3 h. The hydrolyzate was repeatedly co-concentrated with methanol, followed by successive reduction with hydroxylamine hydrochloride and acetylation with acetic anhydride–pyridine (1:1, v/v; 2 mL) at 90 °C for 1 h. The resulting aldononitrile acetates were subjected to GLC analysis on a HP 6890 GC (Hewlett-Packard, USA) instrument equipped with a hydrogen flame ionization detector, using a DB-23 column (60 m × 0.25 mm × 0.25 μm) at a temperature program of 180 °C (1 min) followed by 5 °C/min to 210 °C (10 min) and 1 °C/min to 230 °C (1 min), and then 5 °C/min to 260 °C (5 min). Highpurity-nitrogen was used as the carrier gas at a flow rate of 1.5 mL/min. The injector and detector temperature were 270 °C and 300 °C, respectively.

2.5. Partial hydrolysis with acid

BPS-1 and BPS-2 (20 mg) were hydrolyzed with 0.2 M TFA, kept at 100 °C for 2 h, centrifuged, dialyzed the supernatant with distilled water for 48 h, and then the supernatant in the dialysis tube were dried, and then GC analysis was carried out as described in Section 2.4.

2.6. Methylation analysis

The BPS-1 and BPS-2 (100 mg) were methylated three times according to Hakomori (1964). The pre-methylated product was depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 120 °C for 3 h. The partially methylated residues were reduced and acetylated; the resulting products were analyzed by GC–MS. Complete methylation was confirmed by the disappearance of the OH^- band (3200–3700 cm^{-1}) in the IR spectrum. GC–MS was done on a TRACE DSQ II GC/MS chromatography (Thermoelectron Company, USA) fitted with a fused silica capillary column (30 m × 0.25 mm × 0.25 μm) of TR-35 MS. The column oven was first cooled to 80 °C for 3 min, and raised to 200 °C at 15 °C/min and maintained for 1 min, and then raised to 260 °C at 10 °C/min for 5 min. The nitrogen flow rate was 1.0 mL/min and the ion-source temperature was 250 °C.

Table 1
The monosaccharide and backbone composition of BPS-1, BPS-2.

Sample	Composition	Molar ratio
BPS-1	Glc, Fuc, Xyl, Ara	99:2:1:1
BPS-2	Glc, Fuc, Ara, Gal, Xyl, Man	218:5:3:2:1:1
BPS-1-H (partial acid hydrolysis of BPS-1)	Glc, Fuc, Xyl	42:5:1
BPS-2-H (partial acid hydrolysis of BPS-2)	Glc, Fuc, Man,	133:2:1

Table 2
GC–MS date of aldononitrile acetate derivatives from the methylated product of BPS-1 and BPS-2.

Acetates of sugar[a]	R_t (min)	Molar ratio	Mass fragments (m/z)	Linkage
BPS-1				
2,3,4,6-Me_4-Glu (Residue-A)	14.73	6	45,59,71,101,113,129,145,161,205	Terminal
2,4,6-Me_3-Glu (Residue-B)	14.95	1	43, 87, 101, 127, 145, 161	→3)Glup(→1
2,3,6-Me_3-Glu (Residue-C)	16.82	32	43, 55, 87, 99, 113, 129, 147, 173, 233, 244	→4)Glup(→1
2,3-Me_2-Glu (Residue-D)	18.56	4	43, 56, 85, 99, 127, 141, 159, 201, 261	→4,6)Glup(→1
BPS-2				
2,3,4,6-Me_4-Glu (Residue-A)	14.73	14	45,59,71,101,113,129,145,161,205	Terminal
2,4,6-Me_3-Glu (Residue-B)	14.95	3	43, 87, 101, 127, 145, 161	→3)Glup(→1
2,3,6-Me_3-Glu (Residue-C)	16.80	88	43, 55, 87, 99, 113, 129, 147, 173, 233, 244	→4)Glup(→1
2,6-Me_2-Glu (Residue-E)	17.41	1	43, 87, 115, 129, 184, 211, 244, 286	→3,4)Glup(→1
2,3-Me_2-Glu (Residue-D)	18.56	11	43, 56, 85, 99, 127, 141, 159, 201, 261	→4,6)Glup(→1

[a] 2,3,4,6-Me_4-Glu = 1,5-di-o-acetl-2,3,4,6-tetra-o-methyl-glucose, etc.

2.7. Periodate oxidation-smith degradation

For analytical purpose, 25 mg of the polysaccharides (BPS-1 and BPS-2) were dissolved in 25 mL of 18 mM $NaIO_4$. The solution was kept in the dark at 4 °C; 0.1 mL aliquots were withdrawn at 24 h intervals, diluted to 25 mL with distilled water and read in a spectrophotometer at 223 nm (Deters, Dauer, Schnetz, Fartasch, & Hensel, 2001). Ethylene glycol (2 mL) was added at the end of the reaction which continued for 3 days. The solution of the periodate product (2 mL) was sampled to calculate the yield of formic acid by titration with 0.01 M sodium hydroxide, and the rest was dialyzed against distilled water for 24 h. The content inside was concentrated and reduced with sodium borohydride (100 mg), and the mixture was left for 20 h at room temperature, neutralized to pH 6.0 with 1 M acetic acid, dialyzed as described above, and hydrolyzed with 2.0 M TFA at 120 °C for 3 h after lyophilized. The hydrolyzate was finally converted into aldononitrile acetates which were analyzed by GC and GC–MS (procedure and conditions described in Sections 2.4 and 2.6).

2.8. UV, IR and NMR analysis

UV–vis absorption spectra were recorded with a Shimadzu UV-2550 spectrophotometer between 190 and 400 nm. The FT-IR spectra (KBr pellets) were recorded on Nicolet is10 FT-IR in a range of 400–4000 cm^{-1}. For NMR measurements, BPS-1 and BPS-2 were dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium (Dueñas-Chasco et al., 1997) by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharides (30 mg) were put in a 5-mm NMR tube and dissolved in 0.5 mL 99.96% D_2O. Spectra were recorded with a Bruker AV-500 spectrometer. The ^1H and ^{13}C NMR spectra were recorded at 50 °C. Acetone was used as an internal standard (δ 31.12 ppm) for the ^{13}C spectrum. The ^1H NMR spectra were recorded fixing the HOD signal at δ 4.68 ppm at 50 °C.

2.9. Measurement of anti-inflammatory activity

Female ICR mice (grade II, 8 weeks old) weighing 26.87 ± 0.27were purchased from Zhejiang Experimental Animal Center (Hangzhou, China). They were housed in an acryl fiber cage in a controlled room (temperature 22 ± 3 °C) and were maintained on a 12 h light/dark cycle. Food and tap water were freely available.

The edema was induced by solution of BPS-1 or BPS-2 in xylene at concentrations of 0 (control), 0.05, 0.5, 1.0 mg/mL, respectively. Each solution in volume of 0.02 mL was smeared to each of inner and outer surfaces of right ear, the left ear remained untreated. After 4 h, the animals were sacrificed, and each ear was perforated with a metal punch to provide a 9-mm diameter disc. Edema was assessed by subtracting the weight of untreated ear disc from the weight of treated ear disc, thus determining the inhibition ratio of edema (Xu, Bian, & Chen, 2002).

$$\text{Inhibition (\%)} = \left(\frac{\text{control thickness increased} - \text{sample thickness increased}}{\text{control thickness increased}}\right) \times 100\% \quad (1)$$

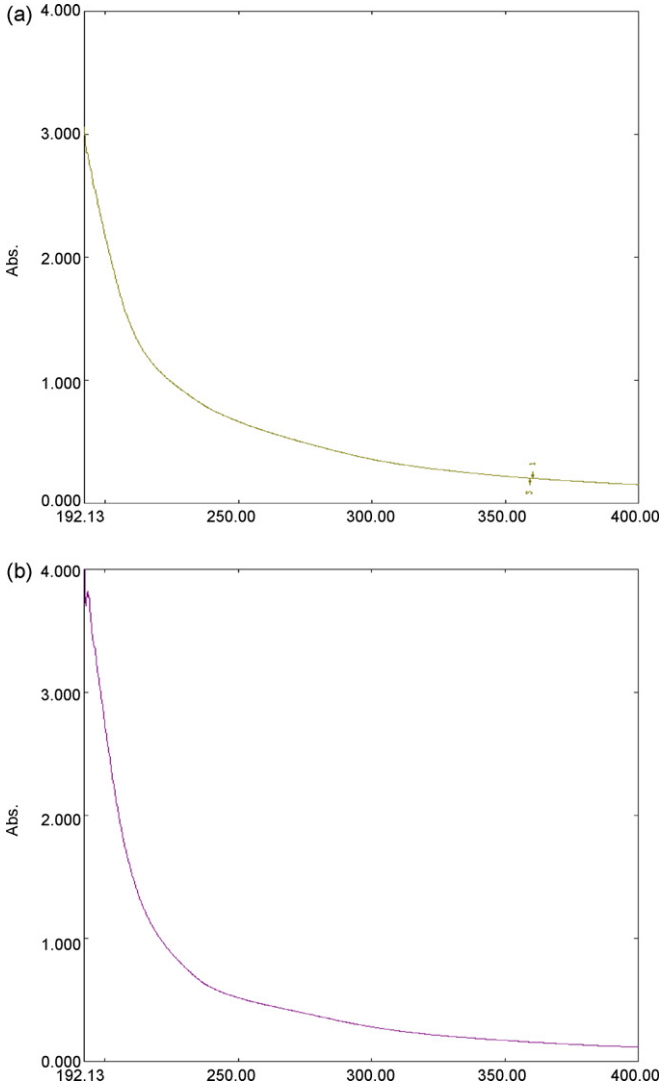

Fig. 2. UV spectrum of BPS-1 (a) and BPS-2 (b).

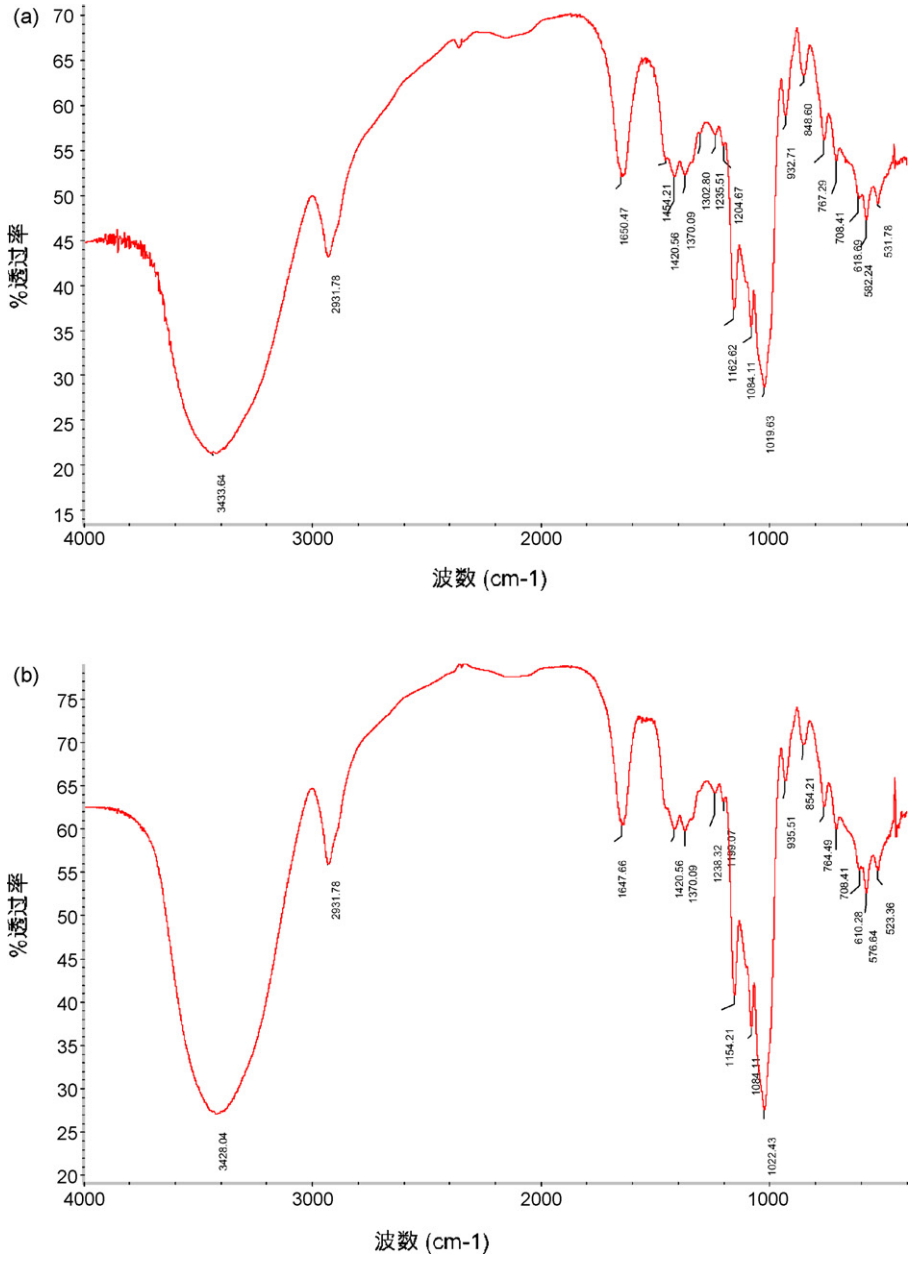

Fig. 3. IR spectrum of BPS-1 (a) and BPS-2 (b).

2.10. Statistical analysis

The data were presented as mean ± SEM. Statistical analyses were performed using Student's *t*-test and one-way analysis of variance. All computations were done by employing the statistical software (SPSS, version 11.5). Significant difference between two groups was defined as $p < 0.05$.

3. Results and discussion

3.1. Isolation, purification of polysaccharides

The crude polysaccharide was extracted with hot water from the foot muscle of *B. purificata*. This fraction was separated and sequentially purified through DEAE-Sephacel and Sepharose-6B gel-permeation chromatography, leading to the isolation of two water-soluble purified polysaccharides BPS-1 and BPS-2, with their yield being 1.25% and 0.46% of the fresh material, respectively. The fractionation procedure was monitored by carbohydrate content detected with the phenol-sulfuric acid assay.

BPS-1 and BPS-2 appeared as a white powder. Their homogeneity and average molecular weight analyzed by high-performance gel-permeation chromatography (HPGPC). The calibration curve was linear, equation $y = -0.3254x + 10.166$, with a correlation coefficient of $R^2 = 0.9875$. The HPGPC profile (Fig. 1) showed a single and symmetrically sharp peak, indicating that BPS-1 and BPS-2 was a homogeneous polysaccharide, with average molecular weight of 7.2×10^6 Da and 8.3×10^6 Da, respectively. Total carbohydrate content of BPS-1 and BPS-2 was determined to be 99.14% and 97.06% using phenol-sulfuric acid method, respectively.

3.2. Monosaccharide and backbone composition of polysaccharides

Monosaccharide constitution analysis by GC, aldononitrile acetates derivatives of BPS-1 and BPS-2 hydrolyzate showed that

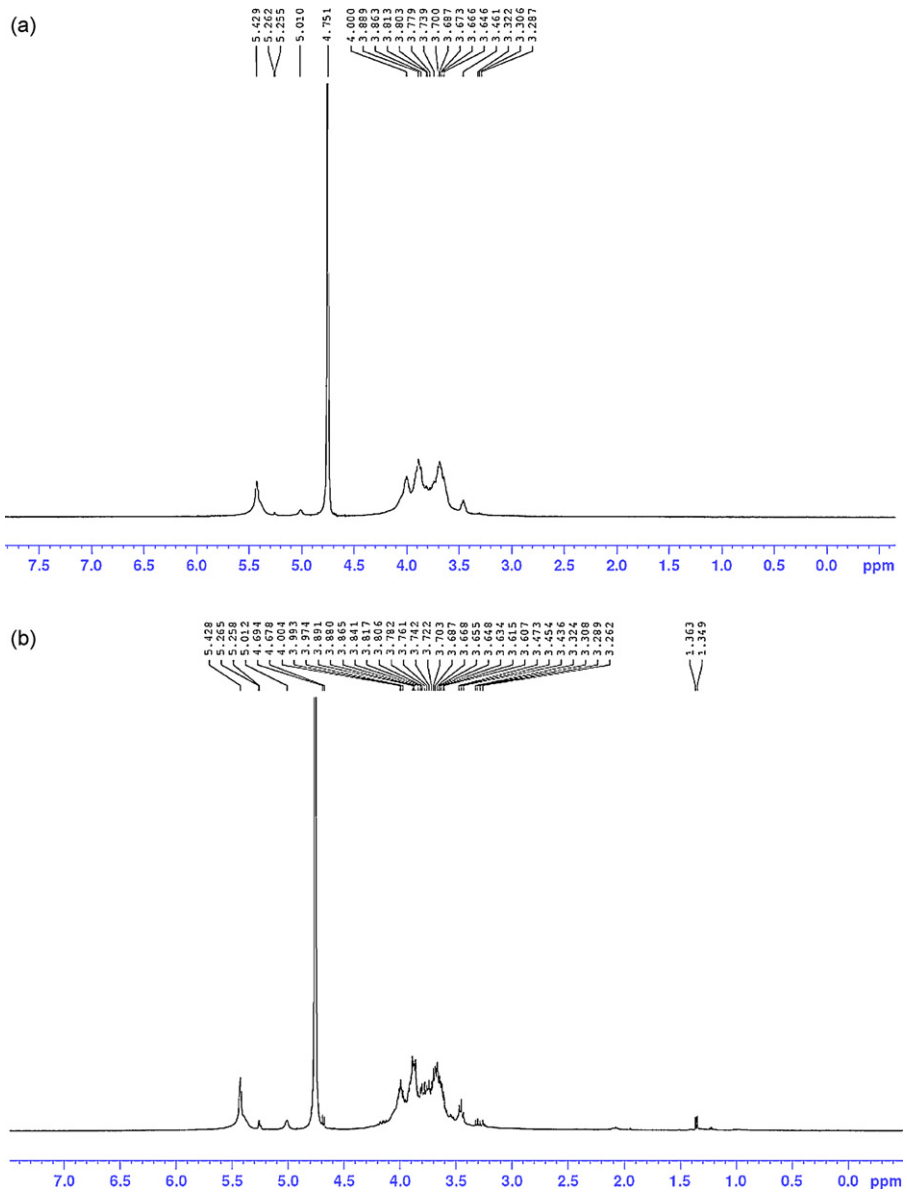

Fig. 4. ^{1}H NMR spectrum of BPS-1 (a) and BPS-2 (b).

BPS-1 was composed of D-glucose, D-fucose, D-xylose, D-arabinose in the molar ratio of 99:2:1:1; BPS-2 was composed of D-glucose, D-fucose, D-arabinose, D-galactose, D-xylose and D-mannose with the molar ratio of 218:15:3: 2:1:1 (Table 1).

The backbone of polysaccharides ascertains by partial acid hydrolysis and GC analysis. After partial acid hydrolysis of BPS-1 and BPS-2, BPS-1-H and BPS-2-H (supernatant in the dialysis sack) were obtained. BPS-1-H and BPS-2-H were subjected to GC. The results indicated that glucose, with a little fucose and xylose could be on the backbone of BPS-1, glucose and arabinose could be on the branch of BPS-1; glucose, with a little fucose and mannose could be on the backbone of BPS-2, and glucose, arabinose, xylose and galactose could be on the branch of BPS-2.

3.3. The backbone structure and linkage of polysaccharides

BPS-1 and BPS-2 were oxidized with 0.018 M sodium metaperiodate ($NaIO_4$) at RT in the dark for 3 days. A total of 1.05 mol $NaIO_4$ was consumed per mole of sugar residues for BPS-1, and 0.92 mol $NaIO_4$ was consumed per mole of sugar residues for BPS-2, based on the average molar mass (180) of a glycosyl residue. The productions of formic acid were 0.14 mol and 0.11 mol, respectively. It was thus deduced that the non-reducing terminal residues or (1 → 6)-linked glycosyl bonds amounted to 14% for BPS-1, with (1 → 2)-/(1 → 4)-/(1 → 4,6)-linked and (1 → 3)-/(1 → 3,4)-linked glycosyl bonds amounting to 77% and 9%,

Table 3
Anti-inflammatory activity of BPS-1 and BPS-2 on mouse ear edema induced by xylene ($x \pm s$, $n = 5$).

Substance	Dose (mg/mL)	Edema (mg)	Inhibition (%)
Control	–	5.42 ± 0.70	–
BPS-1	1	2.30 ± 0.84*	57.56
	0.5	3.26 ± 0.67*	39.85
	0.05	4.66 ± 0.59	14.02
BPS-2	1	2.36 ± 0.68*	56.46
	0.5	3.14 ± 0.69*	42.07
	0.05	4.82 ± 0.68	11.07

* Significant (Student's t-test $p < 0.05$).

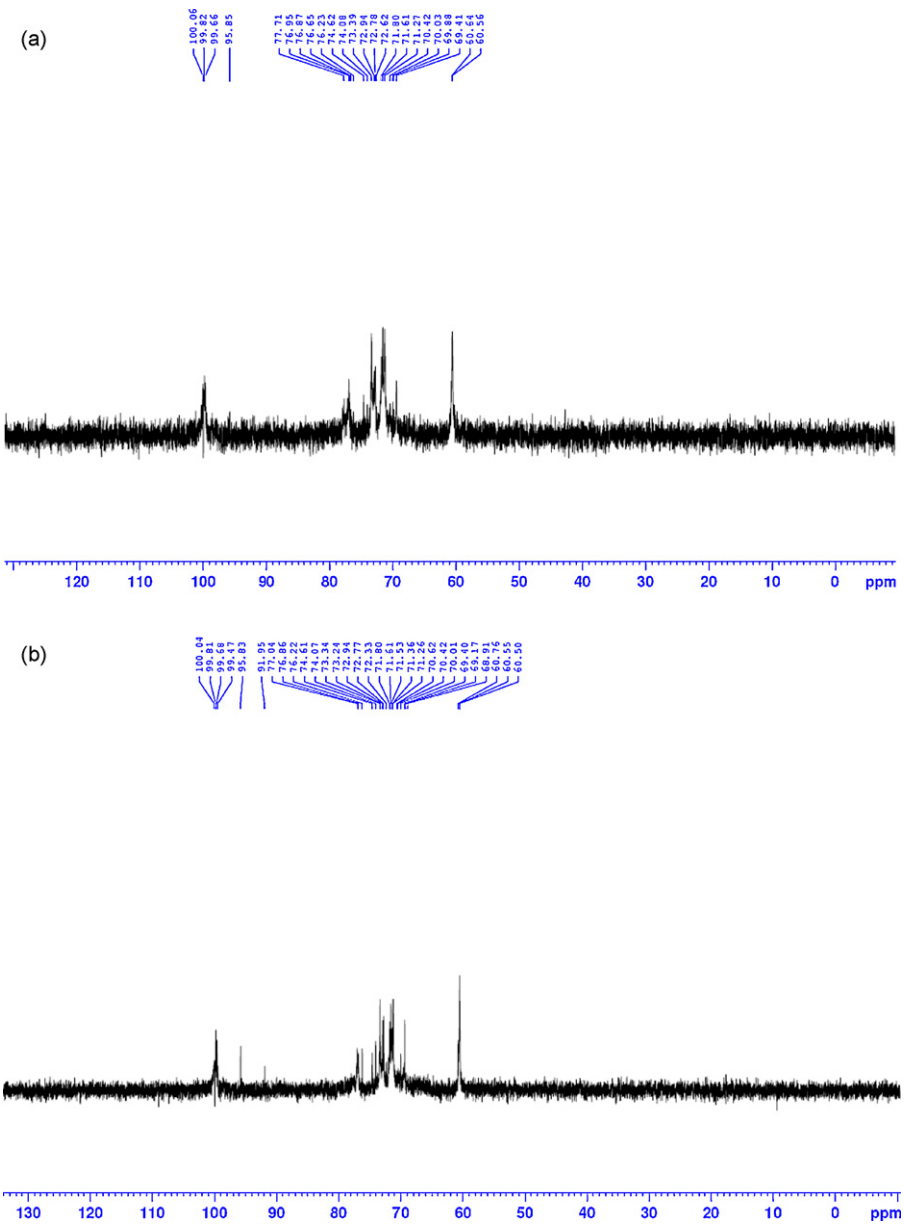

Fig. 5. ^{1}C NMR spectrum of BPS-1 (a) and BPS-2 (b).

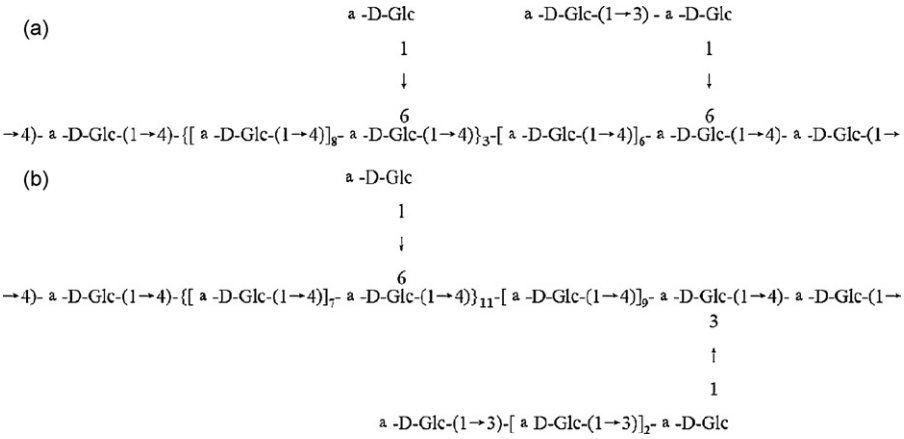

Fig. 6. The structure of BPS-1 (a) and BPS-2 (b) isolated from *Bellamya purificata*.

respectively. The non-reducing terminal residues or (1 → 6)-linked glycosyl bonds of BPS-2 amounted to 11%, with (1 → 2)-/(1 → 4)-/(1 → 4,6)-linked and (1 → 3)-/(1 → 3,4)-linked glycosyl bonds amounting to 70% and 19%, respectively.

The oxidized products were reduced and hydrolyzed. GC–MS and GC analysis identified the presence of erythritol, glucose, fucose and glycerol in the oxidized products of BPS-1 and BPS-2 with the molar ratio of 84:2:0.4:13 and 81:3:0.5:16, respectively. The presence of glucose and fucose indicating a part of glucose and fucose are (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, namely linkages that cannot be oxidized. Arabinose and xylsoe were absent, then it should be inferred that arabinose and xylsoe were all linkages that can be oxidized, namely (1 → 2)-linked or (1 → 4)-linked. The products of glycerol and erythritol suggested the existence of (1 → 6)/(1 → 2)-linked glycosyl residues and (1 → 4)-linked glycosyl in BPS-1and BPS-2, respectively.

The fully methylated products were hydrolyzed with acid, converted into aldononitrile acetates, and analyzed by GC–MS (Table 2). The results indicated that the backbone of BPS-1 chain are mainly (1 → 4)-linked-α-D-glucosyl (Residue-A) and (1 → 4,6)-linked-α-D-glucosyl (Residue-B). The two side chains attached to the C-6 position of Residue-B contained (1 → 3)-linked-α-D-glucosyl (Residue-C) and single non-reducing terminal (1 →)-linked-α-D-glucosyl (Residue-D) groups. According to the peak areas, four types of residues are in the ratio of 32:4:1:6. The backbone of BPS-2 chain are mainly (1 → 4)-linked-α-D-glucosyl (Residue-A), (1 → 4,6)-linked-α-D-glucosyl (Residue-B) and (1 → 3,4)-linked-α-D-glucosyl (Residue-C). The BPS-2 chains have two branches, one side chains attached to the C-6 position of Residue-B contained single non-reducing terminal (1 →)-linked-α-D-glucosyl (Residue-E) groups and the other side chains attached to the C-3 position of Residue-C contained (1 → 3)-linked-α-D-glucosyl (Residue-D). According to the peak areas, five types of residues are in the ratio of 88:11:1:3:14.

3.4. The UV, IR and NMR of polysaccharides

The data on UV, IR and NMR analysis of BPS-1and BPS-2 were showed in Figs. 2–5. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum (Fig. 2), indicating the absence of protein and nucleic acid. On the IR spectrum (Fig. 3), the bands in the region of 3433.64 cm^{-1} and 3428.04 $cm^{-1}$are due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2931.78 cm^{-1} and 2931.75 $cm^{-1}$are due to C–H stretching vibration, and the bands in the region of 1650.47 cm^{-1} and 1647.66 cm^{-1} are due to associated water. Moreover, the characteristic absorptions at 848.60 cm^{-1} and 854.21 cm^{-1} in the IR spectra indicated α-configurations existing in BPS-1and BPS-2.

In the anomeric region of the ^{1}H NMR spectrum of BPS-1 (Fig. 4a), four signals occurred at 5.429, 5.255, 5.262, and 5.010 ppm, which were assigned as Residue-A, Residue-B, Residue-C and Residue-D, respectively. Accordingly in the anomeric region of the ^{13}C NMR spectrum (Fig. 5a), four carbon resonances appeared at 99.82, 100.06, 99.66 and 95.85 ppm. All the results confirmed the presence of four sugar residues and their configurations: Residue-A, Residue-B, Residue-C and Residue-D are form of α-configuration, consistent with GC and FT-IR data. In the high magnetic field, the 60.56 ppm signal should come from C-6 resonance of Residue-B. In the anomeric region of the ^{1}H NMR spectrum of BPS-2 (Fig. 4b), four signals occurred at 5.428, 5.258, 5.265, and 5.012 ppm, which were assigned as Residue-A, Residue-B, Residue-D and Residue-E, respectively. And accordingly in the anomeric region of the ^{13}C NMR spectrum (Fig. 5b), five carbon resonances appeared at 99.81, 100.04, 99.47, 99.68 and 95.83 ppm. All the results confirmed the presence of five sugar residues and their configurations: Residue-A, Residue-B, Residue-C, Residue-D and Residue-E are form of α-configuration, consistent with GC and FT-IR data. In the high magnetic field, the 60.55 ppm signal should come from C-6 resonance of Residue-B. All the NMR chemical shifts were compared with the literature values (Cui et al., 2007; Dong, Yao, Yang, & Fang, 2002; Ishurd et al., 2004; Lu, Cheng, Lin, & Chang, 2010; Pang et al., 2007; Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005; Sun et al., 2008).

On the basis of above-mentioned results, it can be concluded the suggested repeat unit of BPS-1and BPS-2 (Fig. 6).

3.5. Anti-inflammatory activity in vitro

The difference in weight between the two plugs was taken as a measure of oedematous response, and reached the maximum at 30 min followed by a decline after embrocating xylene.

Topical anti-inflammatory activity of BPS-1 and BPS-2 was evaluated as inhibition of the xylene-induced ear edema in mice. This method has certain advantages for natural product testing and has a good predictive value for screening anti-inflammatory agents (Jacobs, Culver, Langdom, O'Brien, & White, 1985). Topical application of xylene induced cutaneous inflammation at the ears of mice and caused significant increase in ear plug weight of the right ear when compared to the left ear. The effect of BPS-1 and BPS-2 on ear edema in mice is shown in Table 3. When BPS-1 and BPS-2 were applied at dose of 0.05, 0.5, and 1.0 mg/mL, it provided inhibitory effect on ear edema formation in a dose-dependent manner. Treatment of BPS-1 and BPS-2 at 1.0 mg/mL gave 57.56% and 56.46% inhibition in ear plug weight, respectively, indicating that BPS-1 and BPS-2 contains anti-inflammatory activity. The result was in accordance with the previous reports that several glucan-type polysaccharides were shown to possess anti-inflammatory activity (Czarnecki & Grzybek, 1995).

4. Conclusions

In summary, it was concluded that the water extract of the foot muscle of *B. purificata* contained two predominant water extractable polysaccharides (BPS-1 and BPS-2). They were α-configuration, and theirs repeating unit of structure were proposed as described above. According to the anti-inflammatory activity test vitro, BPS-1 and BPS-2 could significantly provide inhibitory effect on xylene-induced ear edema formation in a dose-dependent manner. The further studies are needed to elucidate the structure, function, their relationships and mechanisms responsible for its anti-inflammatory activity.

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