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Morphological and structural characterization of a polysaccharide from *Gynostemma pentaphyllum* Makino and its anti-exercise fatigue activity

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ABSTRACT

The crude polysaccharide was obtained from *Gynostemma pentaphyllum* Makino by water extraction followed by ethanol precipitation. The polysaccharide was successively purified by chromatography on DEAE-52 and SephadexG-150 column, and three polysaccharide fractions were obtained and termed GPP1-a, GPP2-b, and GPP3-a, respectively. The administration with GPP1-a markedly prolonged exhaustive exercise time of the mice. Structural features of GPP1-a were investigated by a combination of instrumental and chemical analyses, including atomic force microscope (AFM), scanning electron microscope (SEM), partial acid hydrolysis, periodate oxidation, Smith degradation, methylation analysis, gas chromatography–mass spectrometry (GC–MS) analysis and NMR spectroscopy. The results indicate that GPP1-a has a backbone of (1 → 4)-linked α-D-Glucose residues, which occasionally branches at O-6. The branches are mainly composed of (1 → 6)-linked α-D-Glucose, (1 → 3)-linked β-D-Galactose and (1 → 6)-linked α-D-Galactose residues, and terminated with β-D-Galactose residues and β-L-Arabinose residues.

1. Introduction

It is well accepted that carbohydrates serve as structural components and energy source in the cell. Their highly complex structure allows very specific interactions, so that these bio-molecules are involved in a variety of molecular recognition processes in intercellular communication and signal transductions such as cell adhesion, differentiation, development, regulation, etc. (Varki, 1993; Wang, Luo, & Ena, 2007).

As we all have known the chemical structure of polysaccharides is the base of their bioactivities, for example, polysaccharides from the different strains of *Poria cocos* mycelia showed differences *in vivo* and *in vitro* anti-tumor activities, depending on their monosaccharide composition, molecular mass, and chain conformation (Jin et al., 2003). A more recent study has shown that the different fractions from the crude polysaccharide have distinct biological activities (Tiffany, Yi, Anne, & Cheng, 2007). Therefore, it is valuable that the isolation and characterization of the fractions with strong biological activities from crude polysaccharide is beneficial to their usage in the prevention and treatment of related-diseases, such as tumour.

It has been found that the structure of polysaccharide is intimately related to its functions. This notion is further supported by the study indicating that the majority of polysaccharides from mushroom with anti-tumor activity have β (1 → 3)-D-glucan structure in the main chain (Akira, Mariko, Takuma, Motohiro, & Hideki, 1981). Glucan with three helix structure and molecular weight of more than 90,000 may enhance immune functions (Ohno, Miura, Miura, Adachi, & Yadomae, 2001), indicating that the molecular morphology of polysaccharide is closely associated with its functions. Therefore, the structural identification of polysaccharide is necessary for a better understanding of the relationship between structure and activity.

Gynostemma pentaphyllum Makino is a kind of perennial herbs climbing shrub of *G. pentaphyllum* genus of gourd family, and it mainly grows in oriental countries (Hu, Chen, & Xie, 1996). Numerous studies on *G. pentaphyllum* Makino have illustrated a variety of biological activities with minimal pharmacological toxicity (Attawish et al., 2004), including anti-gastric ulcer (Rujjanawate, Kanjanapothi, & Amornlerdpison, 2004), anti-tumour (Li, Jiao, & Lau, 1993), enhancing immune functions (Zhou, Ye, & Ren, 2006) and cardioprotection (Circosta, Pasquale, & Occhiuto, 2005). To date, its biological activities are mainly attributed to gypenosides. However, recent studies have suggested that the polysaccharide components of *G. pentaphyllum* Makino also exhibit significant bioactivities, including anti-aging (Luo & Wang, 2005), anti-oxidant stress (Wang & Luo, 2007) and improving immune competence (Qian, Wang, & Tang, 1999). Moreover, the crude

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polysaccharide from *G. pentaphyllum* Makino enhances exercise capacity in mice (Fu, 2000). These results clearly demonstrated that polysaccharide is also an active component of *G. pentaphyllum* Makino. However, whether polysaccharide consists of several distinct fractions remains elusive. More importantly, the structural and morphological information of the fractions of polysaccharide with the strongest biological activities molecular is lacking.

The objective of this study is to isolate the anti-exercise fatigue fraction of polysaccharide from *G. pentaphyllum* Makino, and to further characterize its structure and morphology.

2. Materials and methods

2.1. Materials

Gynostemma pentaphyllum Makino was purchased from Shaanxi Province (China). The columns of DEAE-52 cellulose, SephadexG-150 and Sephadex G-200 were from Pharmacia (Sweden). Standard dextran was from Sigma. All other reagents were of the highest available quality in China.

2.2. Isolation and purification of polysaccharides

After the extraction in 80% ethanol at 50 °C for 2 h twice, *G. pentaphyllum* Makino (500 g) was dried and extracted with distilled water at 80 °C for 2 h twice (Solid–liquid ratio is 1:15). After each extraction, the polymers were separated from residues by filtration, and extracts were combined, concentrated and removed free protein layer by the use of method of Sevage, and then concentrated and dialyzed against running water for 48 h. The above extract was subjected to the precipitation with 4-fold volumes of ethanol. The *G. pentaphyllum* polysaccharide (GPP) was collected by centrifugation, washed successively with ethanol, acetone, and ether, and then freeze-dried.

DEAE-52 cellulose column chromatography was used for the fractionation of this preparation. The sample GPP (600 mg) was dissolved in 10 mL distilled water and centrifuged. The supernatant was injected into a column (2.5 × 60 cm) of DEAE-52 cellulose equilibrated with distilled water. After loading with sample, the column was eluted with 300 mL of distilled water at rate of 0.8 mL/min, followed by the stepwise elution using 300 mL of NaCl solution (from 0 to 1 mol/L) at rate of 0.6 mL/min, and then sediment was collected with automatic fraction collector and detected by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and each eluting peak was recorded, dialyzed against tap water and distilled water for 48 h, and then purified by gel-filtration chromatography on a column of Sephadex G-150 (2.5 × 80 cm). The sample was dissolved in the minimal volume of 0.1 mol/L NaCl solution and added to the column, and then eluted with 0.1 mol/L NaCl solution. The main fraction is collected as described above.

2.3. Purification determination and general chemical properties of the polysaccharides

The purification of the polysaccharides was conducted by a Sephadex G-200 column chromatography and methods of optical rotation (Wang, Liang, & Luo, 2004). The sample was dissolved in 0.1 mol/L NaCl solution, centrifuged, and then the supernatant was applied to a Sephadex G-200 column (1 × 80 cm), which was eluted with 0.1 mol/L NaCl solution at a rate of 0.2 mL/min. Polysaccharides were detected by the phenol-sulfuric acid method. Elution curve was drawn by the tube number as abscissa and absorbance as vertical coordinate. In addition, the sample was dissolved in distilled water, and its optical rotation was measured with a WZZ-2SS polarimeter (Xi'an Instrument Co., Xi'an, China) at 20 °C.

The molecular weight was calculated by the calibration curve obtained by using various standard dextran (Wang, Liang, & Zhang, 2001). Gas chromatography (GC) was used for the identification and quantification of monosaccharide composition (Wang & Luo, 2007). The sample was carried out by the trimethylsilylation reagent. GC was then performed on a 6890 N instrument (Agilent Technologies Co., USA) equipped with capillary column (30 m × 0.32 mm × 0.25 μm). The operation was performed by the following conditions: H_2: 20 mL/min; air: 200 mL/min; N_2: 20 mL/min; injection temperature: 250 °C; detector temperature: 250 °C; the column temperature was kept at 150 °C for 2 min, increased to 190 °C at rate of 7 °C/min and held for 2 min at 190 °C, and then increased to 260 °C at rate of 7 °C/min and held at 260 °C for 2 min.

The infrared (IR) spectrum of the sample was determined by the KBr-disk method with a Fourier transform Infrared Spectrometer (Brucher Corp., Germany) in the range of 400 to 4000 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004). The UV spectrogram of samples was measured from 190 to 600 nm of wave length with a Lambd 950 instrument (Perkin–Elmer Corp., USA).

2.4. Measurement of exhaustive exercise time in mice

Kunming male mice, 2-month-old, were randomly divided into 13 groups, 10 mice per group. The main fractions of polysaccharide collected in the present study were dissolved in a small amount of water, respectively. Mice were orally given polysaccharide solution for a week, and the doses were 50, 100, and 150 mg/kg/d, respectively. Mice in the control group were given the equal volume of water. A week later, mice were swam with wire of 5% body weight tied their tails in the pool (length: 65 cm, width: 50 cm, depth: 50 cm) filled with 30 cm depth of water at 30 to 35 °C. Mice were regarded as exhaustion when they were underwater for 8 s, and their swimming time was immediately recorded.

2.5. Observation of molecular morphology of polysaccharide

Polysaccharide powder was placed on the sample stage, and covered with electric layer (10 nm in thickness) using the vacuum evaporator. Molecular morphology of sample was observed by a Quanta 200 instrument (Philips–FEI Company, Holland).

The sample was dissolved in deionized water at 10 μg/mL, and the solution was kept either at room temperature for 2 h or at 45 °C for 2 h. The solution (5 μL) was dropped on the surface of mica schist and dried at room temperature. Molecular morphology of sample was observed and taken a photo under tapping mode using a SPM-9500J3 instrument (Shimadzu Corp., Kyoto, Japan).

2.6. Structural analysis of polysaccharide

2.6.1. Partial hydrolysis with acid assay

Polysaccharide was hydrolyzed in 0.1 mol/L trifluoroacetic acid at 80 °C for 14 h, and then centrifuged. The precipitate was dried, and then analyzed by using GC. The supernatant was dialyzed with distilled water for 48 h using the dialysis bag, and the solution out of the bag was dried for GC analysis. The solution in the bag was further precipitated in ethanol, and the precipitate and supernatant were dried, and then GC analysis was carried out, respectively.

2.6.2. Periodate oxidation-Smith degradation

The sample (20 mg) was dissolved in 12.5 mL of distilled water and 12.5 mL of $NaIO_4$ (30 mmol/L) were subsequently added. The solution was kept in the dark at room temperature, and 0.4 mL aliquots were withdrawn at 4–8 h intervals, diluted

to 100 mL with distilled water and read in a spectrophotometer at 223 nm. Glycol (2 mL) was used to cease periodate oxidation. The solution of periodate product (2 mL) was titrated to calculate the yield of formic acid by 0.01 mol/L NaOH, and the rest was extensively dialyzed against tap water and distilled water for 48 h, respectively. The residue inside was concentrated and reduced with sodium borohydride (40 mg), and the mixture was placed at room temperature for 24 h, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, and concentrated to a volume of 10 mL. One-third of solution described above was freeze-dried and analyzed with GC. The rest was added to the same volume of 1 mol/L sulfuric acid, kept at 25 °C for 40 h, neutralized to pH 6.0 with barium carbonate, and filtered. The filtrate was dialyzed as described above, and the content out of bag was desiccated for GC analysis; the content inside was precipitated with ethanol, and the supernatant and precipitate were also dried for GC analysis.

2.6.3. Methylation analysis

Methylation analysis was performed by the method of Hakomori (1964). The sample was dissolved in 6 mL of dimethylsulfoxide (DMSO), removed air with nitrogen, stirred for 15 h at 25 °C. Sodium hydroxide–DMSO solution was then added (6 mL) and blended well. The mixture was methylated with 3.6 mL of methanol iodide and reacted for 7 min exactly.

The product was dialyzed against tap water and distilled water for 24 h, respectively, and desiccated, and the product was retreated twice as described above. The solution was concentrated to 10 mL, treated thrice with chloroform, fully shocked for 30 min to extract the methylated polysaccharide. The methylated polysaccharide was dried with sodium sulfate for 24 h, filtered and evaporated to 1 mL. The methylated polysaccharide was dried and its IR spectrum was measured. Absorption peak of hydroxyl was not identified at 3400 cm^{-1}, indicating that polysaccharide was completely methylated.

The methylated polysaccharide was hydrolyzed in 1 mL of 85% formic acid at 100 °C for 4 h under nitrogen conditions. Then, the excess formic acid was removed by methanol, and the hydrolyzed polysaccharide was dried under vacuum conditions, and further hydrolyzed in 2 mL of 2 mol/L trifluoroacetic acid for 6 h at 100 °C. The mixture was neutralized to pH 6.5 using ethanol, and the distilled water was used to remove ethanol. The hydrolyzed product was reduced with sodium borohydride for 24 h, and then acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. After the removal of acetic anhydride using ethanol, the product was dried under vacuum conditions, and then dissolved in chloroform. The resultant was subjected to gas chromatography–mass spectrometry (GC–MS) analysis with a GCMS–QP2010 instrument (Shimadzu Corp., Kyoto, Japan). The methylated sugar linkages were identified on the basis of relative retention time and fragmentation pattern (Needs & Selvendran, 1993). The molar ratios of each sugar were calibrated using the peak areas and response factor of the flame-ionisation detector in GC.

2.6.4. Nuclear magnetic resonance (NMR) spectroscopy

The sample was diluted in D_2O. ^{1}H and ^{13}C spectra were recorded at 333 K on an AVANCF300MHZ instrument (Brucher Corp., Switzerland) (Habibi, Heyraud, Mahrouz, & Vignon, 2004; Shi, Chen, Dong, Fang, & Ding, 2007).

2.7. Statistical analysis

Data were presented as means ± SD. Student's *t*-test was used to assess significance of difference. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. The extraction and purification of polysaccharide

GPP was isolated from the hot-water extract of *G. pentaphyllum* Makino with a yield of 11.44%, and fractionated on DEAE-52 cellulose column. Three main fractions were then obtained, and termed GPP1 from the water elute fraction, GPP2 and GPP3 from the NaCl elute fractions, respectively (Fig. 1a). Three fractions were further purified by gel chromatography on Sephadex G-150 column, and obtained three main fractions, named GPP1-a, GPP2-b, and GPP3-a, respectively (Fig. 1b).

The purification of GPP1-a, GPP2-b, and GPP3-a was identified on both Sephadex G-200 column and WZZ–2SS automatic optical polarimeter at room temperature. The results showed only one symmetrical peak on gel-filtration chromatography and the same optical rotation in different low concentrations of ethanol, clearly demonstrating that they all are single composition. The general chemical properties of GPP1-a, GPP2-b, and GPP3-a were determined and shown in Table 1.

3.2. The effects of GPP and its three main fractions on exhaustive exercise time in mice

To explore the bioactivities of GPP and its three main fractions, mice were orally given either GPP or its three main fractions for 1 week. Mice were then swum and the exhaustive exercise time was measured. As shown in Fig. 2, GPP and GPP1-a significantly prolonged exhaustive exercise time in mice in dose-dependent manner, and exhaustive exercise time in GPP1-a group was longer than that in GPP group at doses of 100 and 150 mg/kg/d ($P < 0.05$). However, GPP2-b and GPP3-a failed to prolong exhaustive exercise time in mice. These results indicate that GPP1-a is the major active fraction of GPP.

The underlying mechanisms by which GPP1-a prolongs exhaustive exercise time in mice may be associated with the role of GPP1-

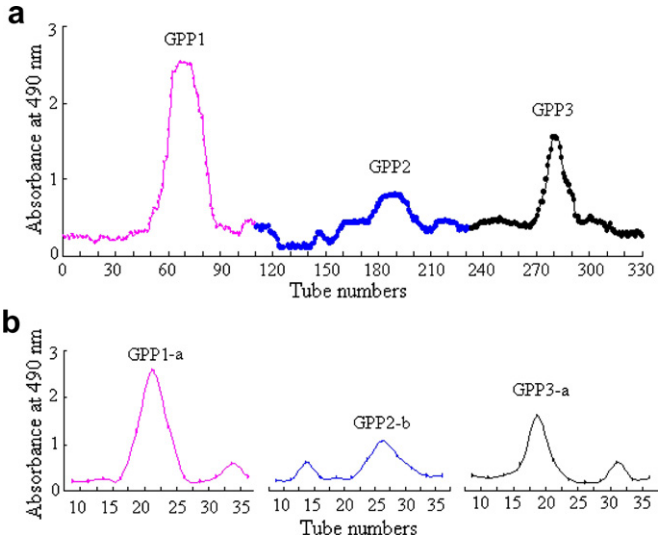

Fig. 1. DEAE-52 cellulose and SephadexG-150 column chromatography of GPP. (a) Polysaccharide (GPP) from *Gynostemma pentaphyllum* Makino was dissolved in distilled water, centrifuged, and the supernatant was injected into a column of DEAE-52 cellulose equilibrated with distilled water. After loading with sample, the column was eluted with distilled water, followed by NaCl solution. Three components were obtained, and termed GPP1, GPP2, and GPP3, respectively. (b) Following crude isolation, GPP1, GPP2, and GPP3 were purified using SephadexG-150 column chromatography, respectively, and the main fractions, GPP1-a, GPP2-b, and GPP3-a, were obtained from GPP1, GPP2, and GPP3, respectively.

Table 1
General chemical properties of GPP1-a, GPP-b, and GPP3-a

Samples	GPP1-a	GPP2-b	GPP3-a
Yield (%, w/w)[a]	21.59	3.65	11.1
Total carbohydrate (%, w/w)	99.6	98.2	99.4
Average molecular weight (kD)	89.2	197.5	253.6
Sugar components (mol %)			
Arabinose	0.18	0.38	Nd[b]
Ribose	Nd	0.64	1.62
Fructose	Nd	Nd	0.54
Xylose	Nd	0.97	Nd
Galactose	0.72	1.26	0.49
Glucose	1.00	1.00	1.00
Main typical	α[c]	β[d]	α
Nucleic acid and protein	Nd	Nd	Nd

[a] Based on GPP.
[b] Nd: Not detected.
[c] α: The IR spectra has absorption at 844±8 cm^{-1}.
[d] β: The IR spectra has absorption at 897±8 cm^{-1}.

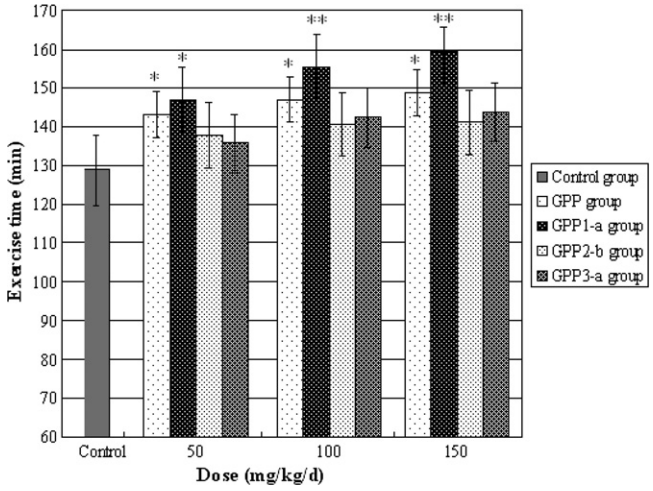

Fig. 2. Effects of GPP, GPP1-a, GPP2-b, and GPP3-a on exhaustive exercise time in mice. Mice were orally given GPP, GPP1-a, GPP2-b, or GPP3-a at doses of 50, 100, and 150 mg/kg/d for 1 week, respectively. Mice were then swam with a load of 5% body weight tied their tails. The exhaustive exercise time was recorded. Values are expressed as means ± SD of ten, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

a in scavenging reactive oxygen species (ROS) excessively produced in exhaustive exercise. This hypothesis is supported by the studies showing that GPP1-a may scavenge ROS *in vivo* and *in vitro* experiments. Considerable evidence has indicated that the role of polysaccharide in scavenging ROS is likely associated with hydrogen and hydroxyl of polysaccharide chain (Schuchmann & Sonntag, 1978), and their locations in polysaccharide chain also significantly affect the role of polysaccharide in scavenging ROS (Kardosova & Machova, 2006). In addition, our experiments under way clearly indicate that GPP1-a may significantly increase the levels of glycogen in skeletal muscle and liver (unpublished results, 2007), in part, responsible for the GPP1-a-induced increase in exhaustive exercise time, because increased glycogen levels in skeletal muscle and liver may enhance exercise performance in experimental animals (Niu, Wu, Yu, & Wang, in press). However, the mechanisms by which GPP1-a increases glycogen levels warrant further investigations.

3.3. Molecular morphology of GPP1-a

Having demonstrated that the major active ingredient of GPP was GPP1-a, the structural characterization of GPP1-a was further investigated by SEM and AFM. The sample surface scanning images were obtained by SEM, and surface information of images was analyzed by the image software. As shown in Fig. 3, GPP1-a consisted mainly of randomly distributed individual spherical particles under 2000× conditions. Moreover, these spherical particles were made of smaller spherical particles with the diameter of 500–1000 nm.

The surface topography of GPP1-a was accurately observed by AFM as described previously (Cai, Li, & Lu, 1999; Ma, Bai, Sun, & Liu, 2000). The photos of GPP1-a taken by AFM were shown in Fig. 4. The results showed that there were many clusters with different sizes at room temperature (Fig. 4a). The formation of these clusters may be attributable to the aggregation of one or more polysaccharide chains (Dragnevski & Donald, 2008). On the clusters, there were many small branches (Fig. 4c) with less than 3.98 nm in height, indicating that GPP1-a was a multi-branched structure. When GPP1-a was heated at 45 °C for 2 h, these clusters were markedly expanded, and the highness of the branches was decreased to less than 3.65 nm (Fig. 4b and d). These changes in the size of the clusters and the highness of the branches were major attributable to the heat-induced aggregation of GPP1-a molecules.

3.4. Structural characterization of GPP1-a

3.4.1. Partial hydrolysis with acid and periodate oxidation analysis-smith degradation products of GPP1-a

GPP1-a was decomposed by partial acid hydrolysis, and its products were subjected to GC analysis. The results showed that

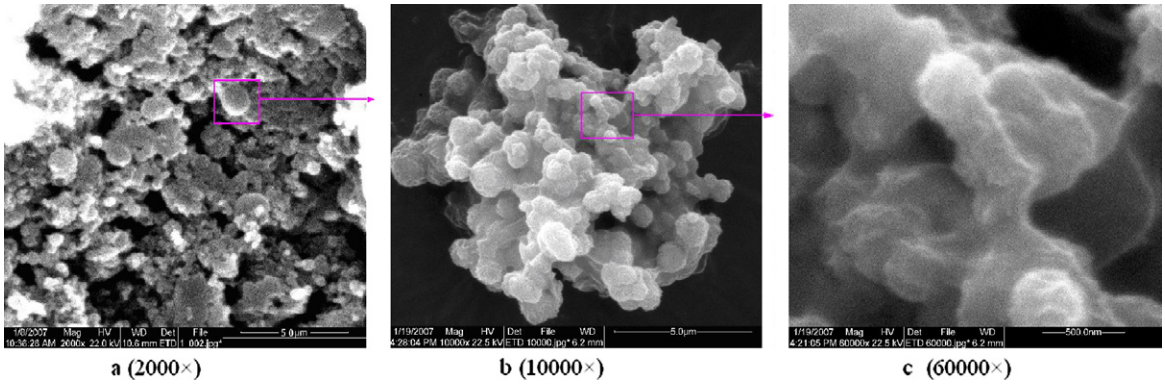

Fig. 3. SEM images of GPP1-a. The molecular morphology of GPP1-a was directly observed using scanning electron microscope (SEM). (a) The morphology of GPP1-a at 2000×. GPP1-a was composed of many individual spherical particles connected with each other. (b) The morphology of GPP1-a at 10,000×. The particles were composed of more little spherical particles. (c) The morphology of GPP1-a at 60,000×. The diameter of those particles was 500–1000 nm.

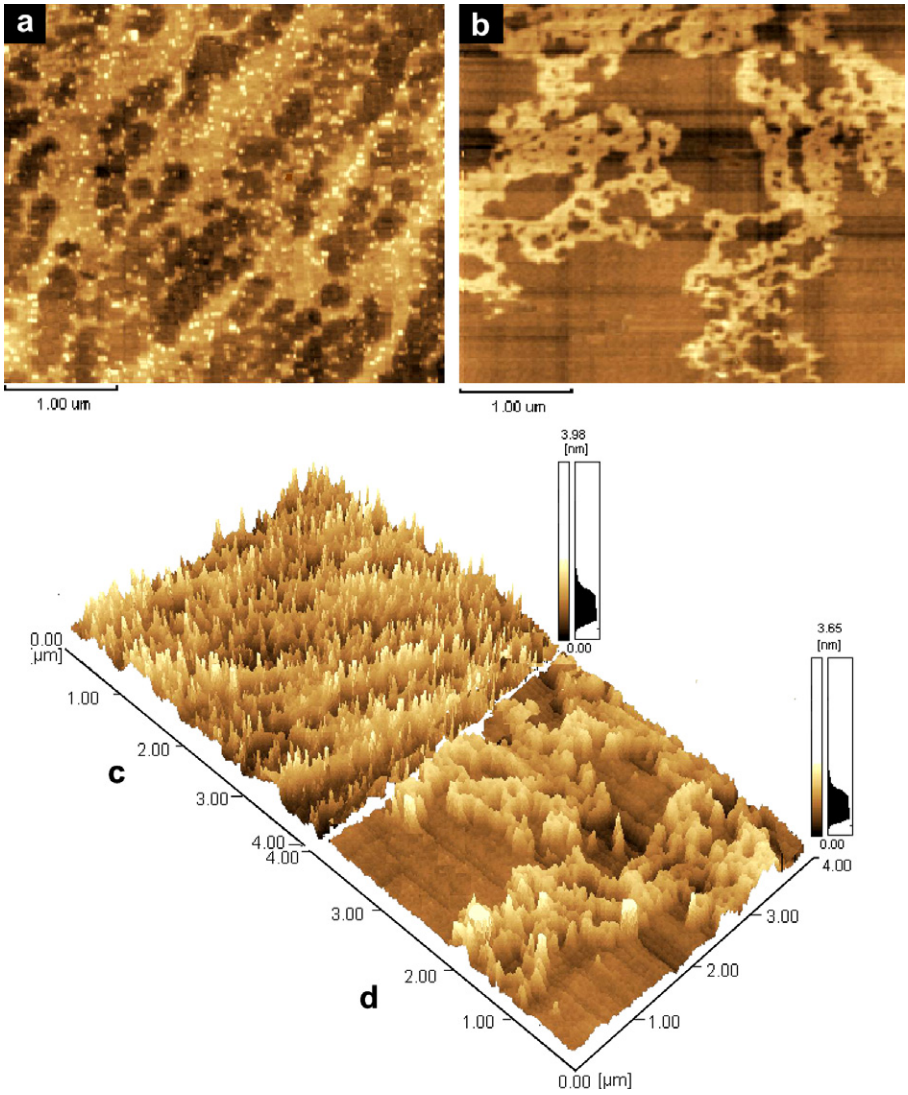

Fig. 4. AFM images of GPP1-a. GPP1-a was dissolved in deionized water at the concentration of 10 μg/mL. The solution was kept either at room temperature for 2 h or at 45 °C for 2 h, and molecular morphology of sample was observed using a SPM-9500J3 instrument (Shimadzu Corp., Kyoto, Japan). (a) Two-dimensional map of GPP1-a at room temperature for 2 h. (b) Two-dimensional map of GPP1-a at 45 °C for 2 h. (c) Three-dimensional map of GPP1-a at room temperature for 2 h. (d) Three-dimensional map of GPP1-a at 45 °C for 2 h.

the precipitations, the components of backbone structure of GPP1-a, from both partial acid hydrolysis and the dialysis by the dialysis bag were composed mainly of Glucose (Glu) with little amount of Galactose (Gal). Moreover, a number of Gal and part of Glu (Molar ratio 0.35:1) were present in the supernatant in the dialysis bag, indicating that Gal and Glu could be present in the branch chains of GPP1-a. Arabinose (Ara) was present out of dialysis bag, indicating its existence in the terminal position of branch chains.

Results from 9 d of periodate oxidation of GPP1-a showed that the ratio of the consumption of HIO_4 (1.25 mol/mol sugar residue) to the production of formic acid (0.35 mol/mol sugar residue) was more than 2, indicating the existence of monosaccharide with either 1 → linked or 1 → 6 linked. These two linkages accounted for 55.4% of total linkages.

The periodate-oxidized products were further hydrolyzed and examined by GC. The presence of Gal indicated that a part of link-

Table 2
GC-MS results of methylation analyses of GPP1-a

Retention time (min)	Methylated sugar	Mass fragments (*m/z*)	Molar ratio[a]	Linkage type
12.98	2,3,5-Me_3-Ara	43,45,71,87,101,117,129,161	0.671	1-linked Ara
13.08	2,3,4,6-Me_4-Gal	43,59,71,87,101,117,129,145,161,205	0.342	1-linked Gal
13.92	2,4,6-Me_3-Gal	43,45,87,101,117,129,161	0.668	1,3-linked Gal
14.18	2,3,6-Me_3-Glu	43,58, 87,99,101,113,117,129,131,161,233	2.053	1,4-linked Glc
14.79	2,3,4-Me_3-Glu	43,87,99,101,107,129,161,189	0.673	1,6-linked Glc
15.05	2,3,4-Me_3-Gal	43,87,99,101,107,129,161,189	1.326	1,6-linked Gal
15.64	2,3-Me_2-Glu	43,85,87,99,101,117,127, 161,201,261	1.000	1,4,6-linked Glc

[a] Calculated from the ratio of peak areas of GC.

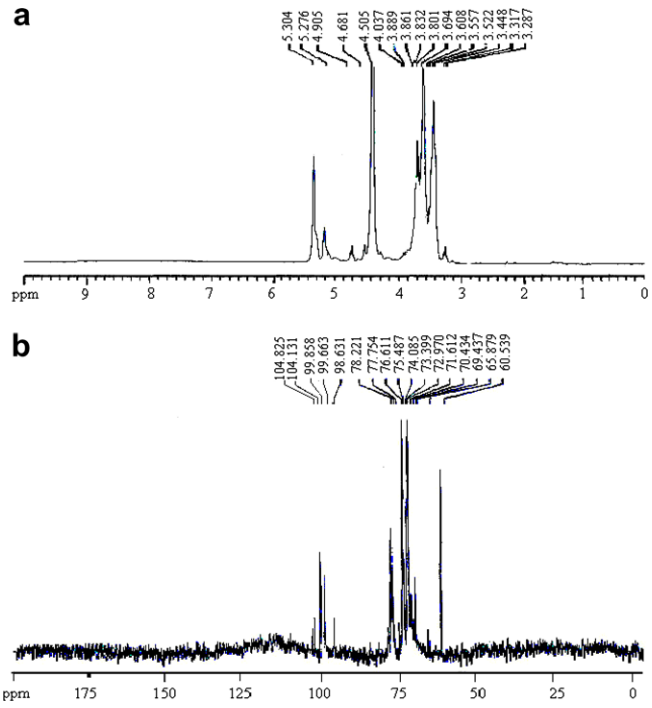

Fig. 5. NMR spectra of GPP1-a in D_2O at 60 °C. (a) ^1H NMR spectrum. (b) ^{13}C NMR spectrum.

ages were (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 through Gal, and these linkages were not able to be oxidized. Glu and Ara were not measured, likely indicating that Glu and Ara constituted all linkages, including 1 → linked, (1 → 6)-linked, (1 → 2)-linked, (1 → 2,6)-linked, (1 → 4)-linked or (1 → 4,6)-linked, which were able to be oxidized.

Results of Smith-degradation analysis showed that the precipitate was not observed in dialysis bag, indicating that the backbone of GPP1-a was completely oxidized by HIO_4. The linkages of backbone were 1 → 4 or 1 → 4,6 linked that could be oxidized to produce erythritol out of dialysis bag. The linkages of branch chains were 1 → , 1 → 6, 1 → 2, or 1 → 2,6 linked that could be oxidized to produce glycerin out of dialysis bag.

3.4.2. Methylation of GPP1-a

GC profile of methylated GPP1-a on GC–MS showed the presence of seven main components, and their methylation analysis was showed in Table 2. According to the results from partial acid hydrolysis and periodate oxidation-Smith degradation, these components were 2,3,5-Me_3-Ara, 2,3,4,6-Me_4-Gal, 2,4,6-Me_3-Gal, 2,3,6-Me_3-Glu, 2,3,4-Me_3-Glu, 2,3,4-Me_3-Gal, and 2,3-Me_2-Glu in molar ratios of 0.671:0.342:0.668:2.053:0.673:1.326:1.00.

Results of partial acid hydrolysis and methylation linkage analysis of GPP1-a indicated that 2,3,6-Me_3-Glu was a major component of the backbone structure, and the branch residue was (1 → 4,6)-linked Glu, which accounted theoretically for 32.7% of the backbone structure, namely on average one branch point for each three residues of backbone. Residues of branch structure were (1 → 3)-linked Gal, (1 → 6)-linked Glu and (1 → 6)-linked Gal, and their comparative quantities were able to be confirmed by molar ratios.

3.4.3. NMR spectra of GPP1-a

The ^1H and ^{13}C NMR spectra of GPP1-a were shown in Fig. 5. Based on periodate oxidation, methylation analysis and on the data available in the literature (Cozzolino et al., 2006; Elisabeth, Johannes, & Johannes, 2001; Zhang, 1999), H-1 signals were assigned to the (1 → 4)-linked α-glucopyranose (Glu*p*) residues (δ 5.304), the (1 → 6)-linked α-galactopyranose (Gal*p*) residues (δ 5.276), (1 → 4,6)-linked α-Glu*p* residues (δ 4.905) and (1 → 6)-linked β-Gal*p* residues (δ 4.681), respectively (Fig. 5a). In the ^{13}C NMR spectrum of GPP1-a (Fig. 5b), five main signals were detected in the anomeric carbon region of 98–105 ppm, namely δ 98.631, 99.663, 99.858, 104.131, and 104.825. Based on the data available in the literatures (Gulin, Kussak, Jansson, & Widmalm, 2001; Sims & Newman, 2006; Ciucanu & Kerek, 1984; Xu, Chen, & Zhang, 2004; Zhang, 1999), in the anomeric carbon region, signal at δ 98.631 was attributed to C-1 of (1 → 6)-linked α-Glu*p*, δ 99.663 to C-1 of (1 → 6)-linked α-Gal*p*, δ 99.858 to C-1 of (1 → 4)-linked α-Glu*p*, δ 104.131 to C-1 of (1 → 3)-linked β-Gal*p* and δ 104.825 to the terminal β-L-Ara*f*, respectively.

On the basis of partial acid hydrolysis, periodate oxidation, Smith degradation, methylation analysis, GC–MS analysis and NMR spectroscopy, the backbone of GPP1-a was composed of (1 → 4)-α-linked Glu, and branches were attached to O-6 of Glu each three (1 → 4)-α-linked Glu. These branches were mainly composed of (1 → 6)-linked α-D-Glu, (1 → 3)-linked β-D-Gal and (1 → 6)-linked α-D-Gal residues. Also, methylation analysis of GPP1-a revealed the terminal Gal residues and terminal Ara residues with molar ratios of 1:2, and the terminal Gal residues should be attached to the (1 → 3)-linked β-Gal. These results indicate that GPP1-a is composed of a repeating unit with the possible structure shown in Fig. 6.

4. Conclusion

The present study demonstrates for the first time that GPP1-a and GPP may significantly prolong exhaustive exercise time in mice, and the role of GPP1-a excels that of GPP in prolonging exhaustive exercise time, suggesting that GPP1-a is a major active component in GPP. The backbone of GPP1-a is (1 → 4)-linked Glu with three kinds of branches. GPP1-a is a polymer composed of individual spherical particles characterized by clusters with a number of branches. Moreover, these clusters and branches may be changed by heating. Taken together, these results improve our understanding of the relationship between polysaccharide structure and bioactivities.

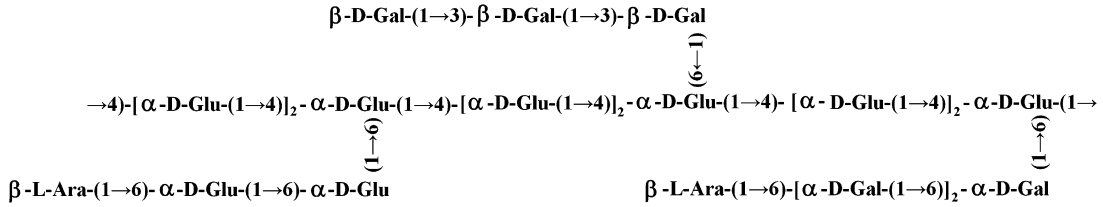

Fig. 6. Possible structure of GPP1-a.

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