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Structure analysis and anti-fatigue activity of a polysaccharide from *Lepidium meyenii* Walp

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**ABSTRACT**

A polysaccharide was obtained from *Lepidium meyenii* Walp by hot water extraction and purification by Millipore (100 kD) and Sephadex G-200. The content of polysaccharide was examined to be 89.9% with phenol-sulfuric acid method. Its average molecular weight was estimated to be $2.213 \times 10^6$ Da by High Performance Gel Permeation Chromatography (HPGPC). Monosaccharide analysis showed that the polysaccharide was composed of arabinose, mannose, glucose and galactose with the molar ratio of 2.134: 1: 2.78: 2.82. After Smith degradation, methylation, infrared spectroscopy and NMR, the primary structure of the polysaccharide was identified. The backbone of the polysaccharide was composed of $\beta-D-Galp-(1\rightarrow4)-\alpha-D-Galp-(1\rightarrow$ and $\beta-D-Galp-(1\rightarrow4)-\alpha-D-Galp-(1\rightarrow$), while the branches were comprised of $\beta-D-Araf-(1\rightarrow6)-\beta-D-Glup-(1\rightarrow$), $\alpha-D-Manp-(1\rightarrow3,6)-\alpha-D-Galp-(1\rightarrow$), $\beta-D-Glup-(1\rightarrow$, and $\alpha-D-GluP-(1\rightarrow$. The anti-fatigue effect of the polysaccharide was evaluated using exhaustive swimming test and biochemical indexes. The results indicated the polysaccharide has anti-fatigue effect.

**KEYWORD**

*Lepidium meyenii* Walp; polysaccharide; construction; anti-fatigue

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1. Introduction

Recently, polysaccharides have attracted a great deal of attention from medicine and food industry as a functional ingredient for medical application and food additives (Perez et al. 2015). Polysaccharides are an important component of living organisms and widely found in higher plant cell wall and microbial cell walls (Chen et al. 2012; Zhao et al. 2012). Besides, they have numerous biological activities such as antitumor, anti-radiation, antiviral, anti-inflammatory, anti-fatigue, anti-aging, hypoglycemic, hypolipidemic and immune regulation (Bai et al. 2012; Sun et al. 2012; Wang et al. 2012; Ktari et al. 2017). The various bioactivities of polysaccharides were due to structural and physical features such as molecular weight, sequence of linkages, monosaccharide composition and morphology (Xu et al. 2016).

In recent years, many biological activities compounds of Lepidium genus have been found in a large number of studies, such as the hypoglycemic and antioxidant activities of garden cress (Lepidium sativum L.) seed on alloxan-induced diabetic male rats (Attia et al. 2017); and the production of a new muclilage compound in Lepidium sativum callus by optimising in vitro growth conditions (Golkar et al. 2018). Lepidium meyenii Walp (named as Maca), an annual herbaceous plant of the cruciferous family, grows in Peru of the Andes (3500–4500 m altitude). Maca is traditionally used as an herb to enhance energy, sexual function and fertility. Maca has a good fame with ‘Peruvian ginseng’ and ‘South American ginseng’ in local folk (Zha et al. 2014; Li et al. 2017; Zhang et al. 2017). Without any side effects, Maca, as a main raw material of various health products, has raised the attention of scientists. Li’s research results showed that the Lepidium meyenii Walp (Maca) has two fractions of polysaccharides (MPS-1 and MPS-2), the MPS-1 was composed of xylose, arabinose, galactose and glucose. The IR spectrum indicated that only α-pyranose existed in MPS-1, and both α-pyranose and β-pyranose existed in MPS-2 (Li et al. 2017). However, there is few reports on the structure identification of Maca polysaccharides, which greatly limited their practical applications (Chaplin and Kennedy 1986; Lee et al. 2016; Ling et al. 2017; Zhang et al. 2017). So, the aim of this study is to elucidate the structure and conformation of a novel polysaccharide from Maca and to reveal the structure-function relationship of Maca polysaccharide by testing their anti-fatigue activity.

2. Results and discussion

2.1. Isolation and purification

The water-soluble polysaccharides were extracted from Lepidium meyenii Walp. The starch and proteins were further removed from the crude polysaccharides. Maca polysaccharide displayed no absorbance at 280 nm and a negative response to the iodine reagents, indicating that polysaccharide was free of protein and starch. After being analyzed by HPGPC (Figure S1), Maca polysaccharides contained three fractions ‘a’, ‘b’ and ‘c’. Polysaccharides were purified by Millipore (100 kD) and Sephadex G-200(Figure S2). There was a single symmetrical narrow peak in Figure S3It was named as M-PL. The sugar content of M-PL was 89.9% using phenol-sulfuric acid method, and the extraction rate of M-PL was 1.76%. The specific rotation of M-PL was [α]_D^25° = +130°. According to the molecular weight method, the peak time of the HPGPC of M-PL was 8.91 min, the molecular weight of M-PL was about 2.213 × 10^6 Da based on the calibration with standard dextrans derived from linear regression.
(log$M_w = -0.3177t + 9.1724, R^2 = 0.9949$). Uronic acid content of M-PL is negative. Therefore, it was well proved that the M-PL is a neutral polysaccharide.

### 2.2. Monosaccharide identification of M-PL

GC-MS chromatogram of standard monosaccharide, as shown in Figure S4, M-PL was composed of the arabinose, mannose, glucose and galactose in a molar ratio of 2.134: 1: 2.78: 2.82. Sigida et al. showed that the Lepidium meyenii Walp was consisted of rhamnose, arabinose, glucose, and galactose (Sigida et al. 2013). (Li et al. 2017) verified that the polysaccharide of *Lepidium meyenii* Walp was composed of xylose, arabinose, galactose and glucose with the mole ratio 1:1.7:3.3:30.5. It suggested that the monosaccharide composition difference of this polysaccharide could be affected by various factors such as the origin, species and extraction parts of raw material.

### 2.3. FT-IR spectrum analysis of M-PL

M-PL was assayed by the FT-IR and the result was shown in Figure S5. A broadly-stretched intense peak at 3416.08 and 1332.79 cm$^{-1}$ was attributed to the O–H stretching vibration for the characteristic absorptions of polysaccharides (Zhang, Ming et al. 2010), and the signals at 2928.08 and 1239.67 cm$^{-1}$ were the result of the stretching vibration of C–H bond, the sharp peak near 1332.79 cm$^{-1}$ indicated O–H bending vibration (Mitić et al. 2009). The stronger characteristic peak occurring between 1740.03 and 1620.25 cm$^{-1}$ were derived from the ester aldehyde (–C=O) groups (Wang et al. 2011). In addition, the band at 1620.25 cm$^{-1}$ could be also attributed to the ring stretching of glucose (Fang et al. 2006). The characteristic absorption of 1144.70 cm$^{-1}$ was attributed to the C–O stretching vibration. The characteristic absorptions peak at 1097.41, 1048.35, and 1020.24 cm$^{-1}$ suggested the presence of pyranoside. Moreover, the absorption peak at 881.82 and 919.05 cm$^{-1}$ indicated the presence of the $\alpha$-glycosidic linkage and $\beta$-glycosidic linkage (Zhang, He et al. 2010), respectively. In addition, the absorption peak at 881.82 cm$^{-1}$ could also indicate the presence of galactopyranose and mannopyranose units (Du et al. 2016; Ustyuzhanina et al. 2016).

### 2.4. Periodate oxidation and Smith degradation

The periodate oxidation experiment showed that 0.175 mol of NaIO$_4$ was consumed and 0.0219 mmol of formic acid was produced per mole sugar residue for M-PL. Previous studies indicated (Zha et al. 2007) that the production of formic acid is lower than the consumption of sodium periodate, which demonstrated that there were $1\rightarrow3$, $1\rightarrow4$, (or $1\rightarrow2$), $1\rightarrow6$ glycosidic linkages or molecular terminal groups. The consumption of NaIO$_4$ exceeded a double of that of HCOOH, indicating the existing of $1\rightarrow2$ or $1\rightarrow2,6$ or $1\rightarrow4$ or $1\rightarrow4,6$ linked glycosidic bonds in M-PL. These could infer that the non-reducing terminal residues or $1\rightarrow6$ linked glycosidic bonds were existed in M-PL.

The periodate-oxidized product of polysaccharide was hydrolyzed and was detected with GC-MS (Figure S6), so that the glycosidic linkage of the polysaccharide could be deduced. The predominant presence of erythritol revealed that the linkages of backbone were $1\rightarrow4$ and $1\rightarrow4,6$ glycosidic linkages that could be oxidised to produce erythritol (Wang et al. 2011). A small number of glycerol revealed that the linkages of backbone were $1\rightarrow2$ or $1\rightarrow6$ or $1\rightarrow2,6$.
glycosidic linkages that could be oxidised to produce glycerol. Galactose demonstrated that there were \(1\rightarrow3\) glycosidic linkages. So, it could be inferred that the non-reducing terminal residues or \(1\rightarrow6\) linked glycosidic bonds were existed in M-PL. The GC–MS chromatogram of M-PL after periodic acid oxidation and Smith degradation was presented in Figure S6. According to the presence of glycerin, it was deduced that the linkages of main chain could be \(1\rightarrow2\) or \(1\rightarrow6\) or \(1\rightarrow2,6\) glycosidic linkages. The presence of erythritol indicated that the \(1\rightarrow4\) or \(1\rightarrow4,6\) glycosidic linkages were present in the backbone of M-PL. Galactose and mannose were produced in the Smith degradation of M-PL, which meant that some glycosyls were not oxidised in periodic acid oxidation. The presence of galactose and mannose indicated that the \(1\rightarrow3\) or \(1\rightarrow3,6\) galactose and mannose glycosidic linkages were present in side chain of M-PL. The results showed that the molecular molar ratio of erythritol: glucose: mannose: galactose was 3.331: 16.95: 1: 3.095, indicating that there were \(1\rightarrow4\), \(1\rightarrow3\), \(1\rightarrow6\) (or \(1\rightarrow3,6\)) glycosidic linkages in the M-PL.

2.5. **Methylation analysis**

Methylation is an important means to analyze polysaccharide linkages. After the methylation treatment, Figure S7 showed that the IR spectrum was significantly enhanced by the infrared absorption around 2900 cm\(^{-1}\), and the absence of the OH band at 3500 cm\(^{-1}\) absorption significantly decreased, which indicated that the polysaccharide was completely methylated. The fully methylated samples were analyzed by GC-MS (Liu et al. 2017). GC–MS results (Table 1) of PMAAs (Figure 3(c)) derived from M-PL gave six peaks. These results indicated that M-PL polysaccharide was consisted of 2,3,6-tri-O-methyl-galactose, 2,3,4-di-O-methyl-glucose, 2,3,4-tri-O-methyl-arabinose, 2,4-tri-O-methyl-mannose, 2,3,4,6-tetra-O-methyl-glucose, 2,4,6-tri-O-methyl-galactose. As the PMAAs (Figure S8) shown, the presence of 2,3,6-tri-O-methyl-Galactose and the relative molar ratio: 30.3% was in agreement with monosaccharide analysis and the Smith degradation. The results indicated that there was \((1\rightarrow4)\)-Galactose linkage in the main chain. In addition, 2,4,6-tri-O-methyl-Galactose which originated from the \((1\rightarrow3)\)-Galactose residues was also detected. Its relative molar was 6.1% according to Figure S8. The presence of \((1\rightarrow6)\)-linked glucose units was demonstrated by the presence of amounts of 2,3,4-di-O-methyl-glucose (24.2%). In addition, terminal glucopyranose was also observed in small amounts (9.1%). According to the PMAAs, the presence of 2,3-tri-O-methyl-arabinose indicated \((1\rightarrow5)\)-linked Araf units was detected, and the hydroxyl groups at C-5 of arabinose have been replaced. Manose residues at O-3 and O-6 position were presented as 2,4-tri-O-methyl-manno with a portion of more than 12.3%. This was in accordance to former studies (Rana et al. 2009; Wang et al. 2011). Methylation and GC–MS analysis were performed to reveal the integrated structure of Maca polysaccharide, (Tang et al. 2017) research showed that the content of 1,3-galp (26.97%) was the most abundant, and then 1,3-GlcP (22.58%), and 1,3-Man (5.59%), It suggested that the main chain of Maca polysaccharide with the repeating unit for 1,3-Galp, 1,3-Glup, and 1,3-Manp with ratio: 5:4:1. In Maca polysaccharide, arabinose (10.74%) residues also existed in the O-3 linked form, 3,4,6-galp (A), and 3,4,6-GlcP as terminal residues for sugar chain. Nevertheless, the results (Zha et al. 2018) showed that the major alditol acetate derivatives from the methylated products of *Lepidium meyenii* polysaccharides only had Araf and Glcp alditol acetate derivatives and 2,3,5-Me3-Araf, 2,3,4,6-Me4-GlcP, 2,4,6-Me3-GlcP, 2,3,6-Me3-GlcP, 2,3,4-Me3-GlcP, and 2,3-Me2-GlcP accounted for 6.31, 13.25, 7.09, 53.66, 6.24 and 13.45%, respectively. The
presence of 1,4,6-linked-Glc p indicated that *Lepidium meyenii* polysaccharide contained branched structure. However, the study suggested that the (1→4)-Galp (30.3%) was the main chain, (1→6)-Glc p (24.2%), (1→5)-Araf (18.4%), (1→3)-Galp residues (6.1%), and (1→3,6)-Manp residues (12.3%) acted as the branch chains, Glc p (9.1%) was the terminal residues for sugar chain.

### 2.6. NMR spectroscopy analysis of M-PL

The $^1$H NMR spectrum of polysaccharide was mainly used to figure out the glycosidic bond configuration. The residue configuration could be deduced according to its coupling constant. In general, the coupling constant of the anomeric protons of α-configuration pyranoside chemistry shift around at 4.9–5.8 HZ, and that of anomeric protons of β-configuration pyranoside around at 4.4–4.9 HZ (Zhang et al. 2006; Liu et al. 2017). The $^1$H NMR spectrum of M-PL showed (Figure S9) that the range of heterotopic hydrogen residue was between 4.5 and 5.4 ppm. The proton resonance signal at $\delta$ 4.78 ppm corresponds to the peak of residual water. According to the results of literature, the $\delta$ value at 5.171 ppm corresponds to $\alpha$-D-Glu p-(1→ heterotopic hydrogen chemical shift (Yang et al. 2016); the $\delta$ 4.895–4.902 (coupling constant $J = 2.8$ Hz) corresponds to $\alpha$-D-Galp-(1→ heterotopic hydrogen resonance displacement (Dobruchowska et al. 2008); In the $^1$H NMR spectrum of M-PL, the signal at $\delta$ 5.102–5.074 with a coupling constant 11.2 Hz indicated the existence of β-configuration →6)-D-Glu p-(1→ residue (Capek et al. 2010); the $\delta$ 5.329–5.319 ppm (coupling constant $J = 4$ Hz) corresponded to →3)-α-D-Galp-(1→ heterotopic hydrogen signal (Kokoulin et al. 2016). The peak at $\delta$ 5.007 ppm was attributed to the terminal proton signal of →3,6)-α-D-Manp-(1→ (Bock and Thersen 1982). The peak at $\delta$ 4.544–4.564 ppm (coupling constant $J = 8$ Hz) corresponded to →4)-β-D-Galp-(1→ heterotopic hydrogen chemical shift (He et al. 2016); the peak at $\delta$ 5.136–5.146 ppm corresponded to →5)-β-D-Araf-(1→ heterotopic hydrogen chemical shift (Chi et al. 2015). The literature showed that the signal of the anomeric carbon is generally between 90 and 110 ppm in the $^{13}$C NMR spectrum (Fontana et al. 2015). In general, the anomeric carbon resonance chemical shift of the α-configuration pyranoside is around 90–103 ppm and that of the β-configuration pyranoside anomeric carbon is around 103–106 ppm (Nep et al. 2016). The related literature showed that $\delta$ 20 ppm is the characteristic chemical shift of methyl carbon on the acetyl substituent, around $\delta$ 170 ppm was the resonance peak of carbonyl resonance. No such resonance absorption peak was observed in the spectrum (Figure S10), which indicated that M-PL was neutral sugars without acetyl substitutent (Zhang et al. 2017). The resonant signals of the sugar residues C-1 to C-6 were concentrated in the range of 60–110 ppm, among which there were seven low-field resonance peaks in the range of 92.13 to 107.522, the $\delta$ 95.946/4.895–4.902, $\delta$ 106.96/4.544–4.564, $\delta$ 107.522/5.102–5.074, $\delta$ 97.779/5.329–5.319, $\delta$ 92.129/5.007, $\delta$ 97.759/5.171, and $\delta$ 107.491/5.136–5.146 ppm, which were designated →4)-α-D-Galp-(1→, →4)-β-D-Galp-(1→, →6)-β-D-Glu p-(1→, →3)-α-D-Galp-(1→, →3,6)-α-D-Manp-(1→, →α-D-Glu p-(1→, and →5)-β-D-Araf-(1→ heterotopic carbon resonance displacement. All the chemical shifts are summarised in Table.2. The chemical shift of C-4 usually ranged from 69 to 77 ppm. Also it may also existed in the down-field of $\delta$ C 77–85 ppm when the hydrogen atoms were substituted; There was same situation for $\delta$ C-6 and $\delta$ C-3 with a usual range of $\delta$ C 60–64 ppm, $\delta$ C65–75 ppm, and $\delta$ C 64–70 ppm, 75–85 ppm with substitution, respectively (Perepelov et al. 2005; Fontana et al. 2015). According to the monosaccharide composition, Smith degradation, methylation and NMR spectroscopy, the molecular composition of M-PL was predicted as shown in Figure S11.
2.7. *Simulation gastric juice and intestinal juice experiment in vitro*

Amylase, a major protein component of human saliva in the mouth, has been reported to initiate the breakdown of α-(1→4) linkages in starch or other carbohydrates (van Ruth and Roozen 2000). Therefore, the digestion of CM-PL by simulation gastric and intestinal juice was carried out in this work. It can be seen from the Figure S12 that there was no change in the content of Maca polysaccharide in the simulated gastric juice (Figure S12-(A)) and intestinal juice (Figure S12-(B)) experiment *in vitro* compared with CM-PL sample (Figure S1). There was no change for molecular weight of CM-PL in both retention time and response value after digestion, and no monosaccharide was detected after digestion, which can be inferred that the Maca polysaccharides could not be decomposed into monosaccharide in the body. Chen et al. (2018) revealed that there was no change in molecular weight, monosaccharide and reducing sugars in polysaccharides from Fuzhuan brick tea after saliva, simulated gastric and small intestinal digestion, indicating that Fuzhuan brick tea polysaccharide could pass through the digestive system without being broken down and reach the large intestine safely. In fact, most of natural non-starch plant carbohydrates can not be digested by human digestive tract (Asano et al. 2003).

2.8. *Biochemical indexes of mice after exercise*

From Table S3-(A), it was found that there was no significant difference in the body weight of the mouse after 45 days of continuous administration (*p* > 0.05). It indicated that M-PL had no significant effect on the body weight of mouse.

The ability to improve exercise endurance is the most powerful manifestation of the anti-fatigue activity of drugs. Meanwhile, the length of swimming time could reflect the fatigue degree of animal. As we could see from Table S3-(B), the swimming time of the low dose group (0.01 g/(kg·d)), middle dose group (0.03 g/(kg·d)), and high dose group (0.05 g/(kg·d)) was significantly longer than that of the blank control group (*P* < 0.01), which confirmed that Maca polysaccharide can significantly prolong the swimming time of mouse. This effect was most obviously observed in the high dose group. The result was in accordance with most previous researches which also verified that the polysaccharides have anti-fatigue activity. Zhang showed (Zhang et al.) that the roots of *Morinda officinalis* polysaccharides have anti-fatigue activity by mice weight-loaded swimming model. Table S3-(C) showed that after continuous administration of Maca Polysaccharide 45 d, compared with the normal control group, the high (0.05 g/(kg·d)) dose group could significantly reduce the serum urea nitrogen content after swimming (*p* < 0.001), and this effect was not obviously observed in low dose group. As the previous study indicated that *Radix Rehmanniae Preparata* polysaccharide enhanced the swimming capacity of mice by decreasing the accumulation of serum urea nitrogen, by delaying the accumulation of lactic acid and by improving the energy storage (Tan et al. 2012); When the body fails to utilise energy from carbohydrate and fat, in that condition energy is derived from protein and amino acid catabolism. SUN is outcome of protein and amino acid catabolism. Consequently, the excess production of SUN will reflect the protein decomposition which will attenuate the muscle contraction and induces fatigue. SUN level will significantly increased when body is poorly adapted for exercise tolerance (You et al. 2011).
The endurance and workload capacity of the body depends on the level of energy source including glycogen storage. The consumption rate of HG is a main factor for extreme fatigue. Rapid consumption of hepatic glycogen results in hypoglycemia during intense exercise, which diminishes nervous system (Jung et al. 2007). The content of liver glycogen and muscle glycogen in mouse significantly increased after swimming in the high dose group (0.05 g/(kg·d)) \((p < 0.001)\), however, this effect was not obvious in low dose group (0.01 g/(kg·d)). This was in consistence with a similar study of Chi (Chi et al.), which reported that Ziyang green tea polysaccharides could recover muscle glycogen level in mouse. Zhang et al. (2009) demonstrated that the anti-fatigue activity of polysaccharides may be linked to the progress in the metabolic control of exercise and the stimulation of energy metabolism.

Lactate dehydrogenase is known as an accurate indicator of muscle activity. Lactate dehydrogenase was very small in the blood and a rise in serum level suggests that muscle damage has occurred, or is occurring (Huang et al. 2011). The LDH level of the M-PL (high dose group) significantly highest than that of the control group. It implied that supplementation of M-PL could affect the LDH activity. The content of some other chemicals for mouse after swimming such as blood lactic acid, lactate dehydrogenase significantly decreased \((p < 0.001)\) in the high dose group (0.05 g/(kg·d)). The results were also in accordance with the study of (Zhao et al. 2017), which showed that corn silk polysaccharide could significantly prolong the swimming time, decrease BUN and LA levels, increase LDH activities, and increase the contents of HG in the corn silk polysaccharide-rich treated mice.

### 3. Experimental section

All experimental procedures are described in the supplementary materials.

### 4. Conclusion

The polysaccharide from Maca with average molecular weight of 2,213 × 106 Da is a branched neutral polysaccharide. The total sugar content was 89.9%. The results of monosaccharide analysis showed that the molar ratio of arabinose, mannose, glucose and galactose was 2.134: 1: 2.78: 2.82. After Smith degradation, methylation, infrared spectroscopy and NMR analysis, the primary structure of the polysaccharide was identified. The backbone of the polysaccharide was composed of \(\rightarrow4\)-\(\beta\)-D-Galp-\((1\rightarrow\) and \(\rightarrow4\)-\(\alpha\)-D-Galp-\((1\rightarrow\), while the branches were comprised of \(\rightarrow6\)-\(\beta\)-D-Glup-\((1\rightarrow\), \(\rightarrow5\)-\(\beta\)-D-Araf-\((1\rightarrow\), \(\rightarrow3,6\)-\(\alpha\)-D-Manp-\((1\rightarrow\), \(\rightarrow3\)-\(\alpha\)-D -Galp-\((1\rightarrow\), \(\alpha\)-D-Glup-\((1\rightarrow\). Anti-fatigue experiment showed that M-PL enhanced the swim capacity of mice by decreasing the accumulation of SUN, and Lactate dehydrogenase delaying the accumulation of lactic acid, and by improving the energy storage Liver glycogen, muscle glycogen. Our study provides a detailed structural description of M-PL and anti-fatigue activity. This would allow one to extend its application as a therapeutic agent as well as functional food.

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