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Structure characterization, modification through carboxymethylation and sulfation, and *in vitro* antioxidant and hypoglycemic activities of a polysaccharide from *Lachnum sp.*

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ABSTRACT

An exopolysaccharide (LEP) was isolated from the fermented broth of *Lachnum* YM240. The structure of LEP was analyzed by fourier transform infrared spectrometry (FT-IR), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC–MS), and 1D and 2D-nuclear magnetic resonance spectroscopy (NMR). The carboxymethylated and sulfated derivatives of LEP was difined as CLEP and SLEP which with molecular weight (*Mw*) of 3.64×10^4 and 2.17×10^4 g/mol respectively. The yield of CLEP and SLEP were 60.27 ± 3.04% and 42.88 ± 2.16%, with degree of substitution (*DS*) values of 0.36 and 0.14 respectively. The result of antioxidant activity and hypoglycemic activity showed that CLEP with higher (*DS*) and (*Mw*) exhibited stronger antioxidant abilities on scavenging DPPH radical and hydroxyl radical compared with SLEP and LEP, with EC_{50} value of 1.05 and 0.80 mg/mL respectively. The reducing power of CLEP (0.533) and SLEP (0.428) was greater than LEP (0.353). In addition, CLEP and SLEP showed higher alpha-glucosidase inhibitory and alpha-amylase inhibition activities and exhibited significant inhibitory effects against the diffusion of glucose compared to LEP. Furthermore, CLEP and SLEP showed more significant effects than LEP at the same dosage, indicating that carboxymethylation and sulfation modifications were effective ways to enhance antioxidant and hypoglycemic activities of the polysaccharide.

1. Introduction

Polysaccharides are considered as important bioactive biological macromolecules products, which have been widely studied in the biochemical and medical area due to their bioactivities. Therefore, increasing attention has been placed on extraction and characterization of new bioactive polysaccharides which may be applied as functional food and medicine [1]. In recent decades, pharmacological studies have shown that fungal polysaccharides possesses various biological activities including anti-oxidation, anti-radiation, hypolipidemic and hypoglycemic, anti-fatigue and anti-tumor, which are also research relevant subjects on functional factors of drugs and healthcare food [2]. *Lachnum* polysaccharides are a polysaccharide acquired from fungal which obtained from fermentation show potential bioactivites in *vivo* such as hypoglycemic, antioxidant, anti-aging, antitumor, anti-fatigue, immunostimulatory, and so on [3]. Studies have shown that tissue antioxidant status may play an important role in the etiology of diabetes [4]. Oxidative stress may constitute a focal point for multiple therapeutic interventions and for therapeutic synergy [5]. Nutritional factors including antioxidants have great influence in the management of diabetes mellitus (DM) and its complications [6]. An imbalance between oxidative stress and antioxidative defense mechanisms in diabetics can result in cell and tissue damage and accelerate diabetic complications. So administration of appropriate antioxidants could prevent or retard diabetic complications to some extent. Many extracts from plant and fungi have shown significant antioxidant effect which can be applied to the treatment of many kinds of diseases [7].

Previous studies have demonstrated that DM exhibits enhanced oxidative stress and highly reactive oxygen species production in pancreatic islets due to persistent and chronic hyperglycemia, thereby depletes the activity of the antioxidative defense system, and thus promotes free radical generation [8]. Oxygen free radicals have been suggested to be a contributory factor in complications of DM.

Chemical modification such as sulfation, carboxymethylation, phosphorylation, selenylation and so on are considered to be an effective means to introduce functional groups and improve the bioactivity of polysaccharides. At the same time, the activity of polysaccharide depends on several structural parameters such as the *Mw*, *DS*, the

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substitution position, type of sugar, and glycosidic branching [9]. Polysaccharides have significant efficacy, low incidence of side effects, low cost and relative safety, while synthetic anti-diabetic drugs can produce serious side effects, as hypoglycemic coma and disturbances of the liver and kidneys. It has been demonstrated that chemical sulfation and carboxymethylationof water-insoluble polysaccharide is awide methods to modified the natural polysaccharides which could change the chainconformation, resulting in the improvement of their biological activities [10]. In this context, the purpose of this study was evaluate in *vitro* antioxidant and hypoglycemic activities of a polysaccharide from *Lachnum* YM240 and its derivatives, aming to provide scientific evidence for development of a potential natural oral hypoglycemic agent or functional food.

2. Materials and methods

2.1. Materials and reagents

The fruiting bodies of *Lachnum* YM240 collected from Yunnan Province (China) were separated and preserved in Microbial Resource and Application Laboratory of Hefei University of Technology. DEAE-Cellulose-52 and Sephadex G-100 were purchased from Whatman Co. (Maid-stone, Kent, UK) and Pharmacia Co. (Sweden), respectively. SO_3-pyridine complex (≥92%) was purchased from TCI Development Co. (Ltd. Japan). Standards of monosaccharides were purchased from Aladdin Chemistry Co. (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), α-amylase, α-glucosidase, ferrous chloride and ascorbic acid were purchased from Sigma Chemicals Co. (St Louis, USA). Glucose assay kits were purchased from Rong-sheng Biotech Co. (Shanghai, LTD, China). All other chemicals and solvents used in the study were of analytical reagent grade and were obtained from Sinopharm Chemical Reagent Co. (Ltd. Shanghai, China).

2.2. Extraction and purification of LEP

Extraction and purification of LEP were carried out according to the previously reported method with minor modification [11]. The crude extracellular polysaccharide from *Lachnum* YM240 fermenting liquor were obtained by the sequential process of ethanol precipitation, H_2O_2 decolorization, Sevage deproteination, salting out and dialysis. The retained fraction was vacuum-dried to obtain crude polysaccharide. Crude polysaccharide was sequentially purified by DEAE-cellulose-52 column chromatography (2.6 × 40 cm), eluted with different concentration of NaCl in water (0, 0.3 and 0.5 M NaCl). Then the 0.3 M NaCl eluted fractions were collected and further purified by Sephadex G-100 chromatographic column (1.6 × 100 cm), eluted with distilled water at 0.3 mL/min. The collected fractions were pooled, freeze–dried, and designated as LEP.

2.3. Structural characterization of LEP

2.3.1. Homogeneity and Mw of LEP

The homogeneity and average molecular weight of LEP were determined by HPLC (1260 Infinity, Agilent Technologies). The conditions of HPLC were as follows: capillarycolumn was Ultrahydrogel™ 2000 (7.8 mm × 300 mm) and Ultrahydrogel™ 500 (7.8 mm × 300 mm) with 2489 UV/ Visibledetector. Sample solution was injected in each run, with double distilled water as the mobile phase at a flow rate of 0.5 mL/min. The temperature of the column was set at 35 °C and the response time of the 1260 refractive index detector was fixed at 4 s. The purity was examined by the shape and the distribution of the peak(s) and the *Mw* was calculated according to the calibration curve established by the standard dextran. Standard T-2000, T-500, T-110, T-40 and T-10 dextrans were used as molecular markers.

2.3.2. Monosaccharide composition analysis of LEP

LEP was hydrolyzed with 5 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 4 h. Excess TFA was removed by co-distillation with 5 mL of methanol and the procedure was repeated for six times after the hydrolysis was completed. After reduction with 40 mg $NaBH_4$, hydrolyzed sample was treated with 5 mL acetic anhydride and 3 mL pyridine at 100 °C for 1 h. Afterward, the acetylated derivative component of the totally hydrolyzed LEP were detected by a GC–MS analyzer. The chromatographic data was acquired using the NIST Mass Spectral Database (NIST11) with NIST MS search program v.2.0. The conditions of GC–MS were as follows: capillary column was HP-5 (30 m × 0.25 mm × 0.25 μm); column temperature was initially set at 50 °C, increased to 250 °C with 10 °C/min; injector temperature was 260 °C; helium flow rate was 1 mL/min; ion source was EI, 70 eV; molecular weight range was 35–650 amu s^{-1}.

2.3.3. Methylation analysis of LEP

The step of methylation was determined according tothe method of Ping *et al.* (2017) with some modification [12]. LEP (40 mg) was depolymerized in carbonylamines, dissolved in 2 mL DMSO (≥99.5%) in hydrolysis tube under nitrogen, treated with ultrasound for 20 min at low temperature till LEP dissolved and then treated with 50 mg NaOH powder. Then the tube was sealed with N_2 and submitted to ultrasonic vibrated at the temperature of 4 °C for 60 min. Iodomethane (1 mL) was added dropwise, and the tube was sealed with N_2 and ultrasonic vibrated at the temperature of 4 °C for 60 min. The reaction solution was mixed with 2 mL distilled water to terminate the reaction, and the reactants were lyophilized. The completeness of methylation was confirmed by disappearance of the hydroxyl absorption in IR spectrum at 3400 cm^{-1}. Finally, LEP was first reduced to its corresponding carboxyl-group reduced product, and hydrolyzed, deoxidized, acetylated then analyzed with GC—MS as described above.

2.3.4. FT-IR and NMR analysis

Infrared spectra (IR) were recorded on a Nicolet 67 FT-IR (Spectrum One, PerkinElmer Co., USA) spectrometer. LEP was ground with potassium bromide powder and pressed into a pellet for spectrometric measurement in the frequency range of 4000–400 cm^{-1}. ^1H NMR, ^{13}C NMR, polarization transfer spectroscopy (DEPT), ^1H–^1H correlated spectroscopy (COSY), ^1H–^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC) and ^1H–^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) experiments were recorded at 30 °C using a Bruker AV-500. LEP was dissolved in 1 mL D_2O followed by centrifugation and freeze–dried. The process was repeated three times, and the final sample was dissolved in 1.0 mL D_2O.

2.4. Chemical modification of LEP

2.4.1. Carboxymethylation reaction

The carboxymethylation of LEP was achieved by mixing 300 mg LEP with 12.5 mL isopropanol, and the mixture vigorously stirred for 15 min at room temperature. Then 5 mL of 20% NaOH was added drop wise. After being stirred at room temperature for 3 h, the carboxymethylation agent (a mixture of 2.63 g chloroacetic acid, 5 mL of 20% NaOH and 12.5 mL isopropanol) was added under stirring. The reaction was maintained at 60 °C for 4 h. After the solution was cooled to room temperature, the pH value of the solution was adjusted to 7 with 0.5 M HCl [13]. The product was dialyzed against flowing tape water for 12 h and then deionized water for 48 h. The non-dialyzable phase was concentrated and precipitated with 95% (v/v) ethanol at 4 °C for 12 h, then washed sequentially with ethanol, acetone and ether. The sample was freeze-dried to obtain the carboxymethylated derivative and coded as CLEP.

The *DS* values for CLEP was assigned as the average number of substituent groups on each sugar residue. The values can be calculated from elemental composition in the polysaccharides (*C*%) on the basis of