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Biochemical analysis and hypoglycemic activity of a polysaccharide isolated from the fruit of *Lycium barbarum* L

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ABSTRACT

Purification, characterization and hypoglycemic activities of polysaccharide from *Lycium barbarum* L. were investigated in this study. A water soluble polysaccharide (LBP) was obtained with ultrafiltration membranes separation, which was further purified by chromatography of DEAE cellulose column and Sephadex G-150 to get LBP3a and LBP3b. The high performance permeation chromatography (HPGPC) analysis showed that the average molecular weight (Mw) of LBP3b was 4.92 kDa. Monosaccharide composition analysis revealed that the LBP3b was comprised of mannose, rhamnose, glucose, galactose and xylose with a molar ratio of 5.52:5.11:28.06:1.00:1.70. The preliminary structure features of LBP3b were investigated by UV, FT-IR, NMR and SEM. *In vitro* cell experiments showed that LBP3b had significantly inhibited the absorption of glucose in a dose-dependent manner. The study showed that LBP3b had potential use as an anti-diabetic agent.

1. Introduction

Diabetes mellitus (DM), which has become a major health problem around the world, is a chronically metabolic disorder with abnormally high levels of blood glucose (so called hyperglycemia), which is caused by the deficiency in insulin secretion and/or the decreased response of the organs to insulin [1]. The worldwide incidence of DM including types 1 and 2 is dramatically increasing and is estimated to be more than 400 million by 2030 [2]. Many oral hypoglycemic agents, such as biguanides and sulfonylureas, are available for the treatment of diabetes, but these agents are chemically synthetic and deficient in multiple dosage regimen, high-cost, adverse side effects and toxicity [3,4]. To explore and discover novel safer and more effective substitutes is important and significant. The traditionally edible and medicinal resources have become the focus of investigation for hypoglycemic activity [5,6].

Lycium barbarum L., belongs to the *Solanaceous* family, also called wolfberry or *goji* berry, is a famous traditional Chinese herbal medicine which has been used for more than 2300 years [7]. And nowadays the fruit from *L. barbarum* L. has been widely used as a popular functional food [8], with a large variety of beneficial effects, such as reducing blood glucose and serum lipids, nourishing eyes, kidneys and liver, anti-radiation, immunity improvement, anti-aging, anticancer, anti-fatigue, enhancing hemopoiesis and male infertility [9–12]. Several types of components including carotenoids, amino acids, trace minerals, vitamins, fatty acids, polysaccharides and betaine identified in the small dried fruits of boxthorn were reported to have such biological and health-related activities [13,14].

Among these chemical constituents of *L. barbarum* fruits, the most well researched components are a group of water-soluble glycoconjugates (*L. barbarum* polysaccharides or LBP), which are estimated to comprise 5–8% of the dried fruits. Many studies on pharmacology and photochemistry have demonstrated that LBP was one of the major active ingredients responsible for above biological activities [15–17]. However, because of the complexity structures of LBP, using different methods of extraction and purification will get some LBP fractions with different components, structures and functions [8,15,18]. The structure and function of every LBP factions have never been comprehensive and in-depth discussion.

In this study, the crude LBP was extracted from *L. barbarum* fruits by the method of ultrafiltration membranes separation, purified the crude LBP by methods of DEAE ion-exchange cellulose and Sephadex gel filtration successively, pre column derivatization high performance liquid chromatography was employed to identify the monosaccharide composition, and then the structure of LBP subfraction (LBP3b) were characterized by UV, FT-IR, NMR and SEM.

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In addition, *in vitro* cell experiment was carried out to evaluate the hypoglycemic effect of LBP3b.

2. Materials and methods

2.1. Materials and chemicals

The *L. barbarum* fruits were purchased in the market of Ningxia Hui Autonomous Region, China. The plant material was dried at ambient temperature and stored in a dry place prior to use. DEAE-cellulose, Sephadex G-150, glucose, galactose, arabinose, rhamnose, mannose, xylose, and trifluoroacetic acid (TFA) were purchased from Sigma. All the other chemicals and solvents used were analytical grade.

2.2. Preparation of crude polysaccharides

Polysaccharides were prepared by the method of Yin and Dang [19]. The dried fruit of *L. barbarum* fruits was powdered with a blender and the ground samples were immersed into a given volume of hot water at 60 °C. Under certain conditions, the mixed extractions were ultrafiltered by organic membrane separated molecular weight from 300 to 50 kDa (Obtained from Suntar Membrane Technology Co., Ltd., Xiamen, China). After ultrafiltration, the filtration liquid was refluxed three times to remove lipids with chloroform: methanol solvent (2:1) (v/v). After filtering the residues were air-dried and then refluxed again with 80% ethanol. The combined filtrate was precipitated using 95% ethanol, 100% ethanol and acetone in turn. After filtering and centrifuging, the precipitate was collected and vacuum-dried, giving crude polysaccharides (glycoconjugates) named crude LBP (CLBP).

2.3. Separation and purification of CLBP

The CLBP was deproteinized three times with Sevag reagent [20] after which the resulting polysaccharide solution was lyophilized to give a crude product. The crude product was dissolved and subjected to a DEAE cellulose column (OH^-, 2.6 cm × 90 cm) [21], and eluted at a flow rate of 30 ml/h with distilled water and 0.05–0.5 mol/l NaCl, the eluent was collected by automatic fraction collector and monitored by UV absorption at 280 nm and phenol sulfuric acid assay at 490 nm, and four homogenous sub-fractions, designated as LBP1, LBP2, LBP3 and LBP4 were obtained. As the content is highest, the sub-fraction LBP3 was further purified using Sephadex G-150 columns (2.5 cm × 60 cm). After centrifuging, concentrating and freeze-drying, LBP3b was used for subsequent experiments.

The total sugar content of LBP3b was measured to be 96.53% *via* the phenol–sulfuric acid method [22] using D-glucose as standard sample.

2.4. Analysis of monosaccharide composition and molecular weight determination

LBP3b sample was hydrolyzed with trifluoroacetic (TFA) and derivated by 1-phenyl-3-methyl-5-pyrazolone (PMP) [23]. The monosaccharide composition of LBP3b was carried out by reversed-phase liquid chromatography (Agilent 1260, VWD detector, USA) on a ZORBAX Eclipse $XDBC_{18}$ column (250 mm × 4.6 mm, Agilent, USA) with a mobile phase composed of 0.1 mol/l PBS (pH 6.7) and acetonitrile in the ratio of 83:17, the flow rate of mobile phase was 1 ml/min, the temperature was kept at 30 °C and the injection volume was 10 μl, The detection was carried out at 250 nm. D-Mannose, L-rhamnose, D-glucose, D-galactose, D-xylose, D-arabinose were used as references.

The average molecular weight of LBP3b was determined by high performance gel permeation chromatography (HPGPC). The sample solution was applied to Agilent High Performance Liquid Chromatography (HPLC) equipped with Shodex OHpak SB-802HQ and Shodex OHpak SB-805 HQ columns (8.0 mm × 300 mm, Showa Denko, Japan), eluted with 0.1 mol/l phosphate buffer, pH 5.5, containing 0.2 mol/l NaCl solution at a flow rate of 0.8 ml/min and detected by a Agilent 1260 Refractive Index Detector. The columns were calibrated with Dextran P-82 series standard of known molecular weight (805 000, 393 000, 210 000, 48 800, 21 700, 10 000, 6000, 180 Da). The molecular weight of LBP3b was estimated by reference to the calibration curve made above.

2.5. UV and FT-IR analysis

The homogeneous LBP3b was dissolved and diluted to a proper concentration, and scanned from 200 to 500 nm with the UV spectrophotometer (CARY 50 UV-vis, Agilent, USA). The FT-IR of LBP3b was determined using a Fourier transform infrared spectrophotometer (NEXUS 870, USA) equipped with OMNIC software. The LBP3b samples were respectively ground with KBr powder and then pressed into pellets for transformation infrared spectra measurement in a frequency range of 4000–400 cm^{-1} [24,25].

2.6. NMR analysis

The polysaccharide was exchanged three times with deuterium in DMSO by lyophilizing. ^1H and ^{13}C NMR spectra were recorded on a NMR spectrometer (Brucker AVANCE III) at 600 MHz.

2.7. Scanning electron microscope

The polysaccharides were coated with gold and examined with a scanning electron microscope system (Hitachi S-3400N, Japan) under high vacuum condition at an accelerating voltage of 5 kV, as well as image magnifications from 500× to 10 000×

2.8. Effect of LBP3b on glucose absorption in Caco-2 cell culture model

Cell culture: Caco-2 cells were obtained from the Cell Bank of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were grown in 25 cm^2 plastic flasks. They were cultured in DMEM medium with 10% fetal bovine serum at 37 °C, 5% CO_2 and 85–90% relative humidity, the medium was changed every other day. Exponentially growing cells were used for experiments. The cells were seeded in the Transwell polycarbonate membrane on the culture plate according to a certain amount of inoculation of cell concentration each bore (Corninig, 3413). Medium was changed every other day during the first week, afterward, once a day, trained for 19–21 days. Monolayer integrity was evaluated by transepithelial electrical resistance values (TEER) measured with a Millicell-ERS (Millipore Corp.). The TEER of single cell layer ≥300 Ω cm^2. It said that the growth of cells were close film, which could be used as *in vitro* model for intestinal absorption.

Glucose uptake assay: the culture medium of above Caco-2 monolayer cell model was removed. Cells were washed twice with 2 ml HBSS (pH 7.2) and balanced 20 min in CO_2 incubator at second washing times. According to the experimental group, took moderate glucose and LBP3b, soluted in HBSS, filtrated and sterilized, heated to 37 °C before use. MTT assays were to test the toxicity of LBP3b to Caco-2 cells. Mixed the glucose and (or) LBP3b with HBSS, joined 0.1 ml to AP side, added 0.7 ml HBSS to BL side as incoming fluid, and placed them in the incubator. During this period, draw the liquid of AP side to detect the excess glucose. By the amount of lessons, put an equivalent amount of liquid into the AP side again