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Structural characterization of the polysaccharide moiety of an aqueous glycopeptide from mannatide

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ABSTRACT

A homogeneous glycopeptide with a molecular weight of 1.61×10^5 Da, termed as MT2-A, was isolated from crude mannatide by DEAE-52 cellulose column and Sephacryl S-300 gel column. The polysaccharide moiety of MT2-A was mainly composed of mannose and trace amount of glucose. Besides, MT2-A contained 16 kinds of amino acids and the total amino acid content was 4.41%. The detailed chemical structure of MT2-A was elucidated using methylation analysis, Fourier transform infrared spectroscopy (FT-IR), partial acid hydrolysis, selective acetolysis and 1D/2D nuclear magnetic resonance (NMR) spectroscopy. Based on the experimental results, it was concluded that the polysaccharide moiety of MT2-A had a backbone of (1 → 6)-α-D-mannopyranose residues, which highly branched at O-2 position of (1 → 2,6)-α-D-mannopyranose residues. The side chains were mainly composed of (1→)-α-D-mannopyranose, (1 → 2)-α-D-mannopyranose and (1 → 3)-α-D-mannopyranose residues. The length of the side chains could not be longer than four mannose residues.

1. Introduction

Mannatide (Polyactin A) was a new type of immunoenhancer, which was developed for the first time in China. It was isolated from the fermentation broth of cultured buccal α-hemolytic Streptococcus 33# [1]. Clinical observation suggested that Mannatide had remarkable immunological activity [2–5]. It had been widely used to treat old age disease, recurrent respiratory tract infection, aplastic anemia and adjuvant therapy of tumors [6,7]. In recent years, the effects of mannatide on increasing mitogenic index of lymphocytes, stimulating cytotoxic effects of natural killer (NK) cells and macrophages, and up-regulating the gene expression and secretion of cytokines and chemokines are well studied [8]. Therefore, it was very important to investigate the relationship between the structure and biological activity of mannatide. Unfortunately, most current reports on mannatide focused only on its pharmacological activity and therapeutic effects. Zou and Xu [1] reported a Chuan-lian polysaccharide isolated from Duo kang jia su (Mannatide) and its primary structure was studied through Smith degradation, methylation analysis and 1D NMR. The results indicated that the Chuan-lian polysaccharide was α-mannan binding protein and the polysaccharide chains include (1→)-linked, (1 → 2)-linked, (1 → 3)-linked and (1 → 2,6)-linked mannose residues. However, to our knowledge, there is no other report focusing on the mannatide's detailed structure. In our study, a homogeneous glycopeptide MT2-A was isolated from mannatide by DEAE-52 cellulose column and Sephacryl S-300 gel column. The detailed structure of the polysaccharide moiety of MT2-A was elucidated using methylation analysis, partial acid hydrolysis, selective acetolysis, FT-IR, 1D NMR (^{1}H NMR, ^{13}C NMR) and 2D NMR including ^{1}H—^{1}H correlated spectroscopy (^{1}H—^{1}HCOSY), total correlation spectroscopy (TOCSY), nuclear overhauser effect spectroscopy (NOESY), heteronuclear singular quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC).

2. Materials and methods

2.1. Materials

Mannatide was obtained from Chengdu Leer Pharmaceutical Co., Ltd. (Chengdu, Sichuan China). DEAE-cellulose was purchased from Whatman Corp. (Maidstone, UK). Sephacryl S-300 gel and dialysis bags (molecular weight cut off 3500) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dextran T-series standards (M_w: 12, 50, 270, 670 kDa) were from Fluka Corp.

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(Washington, USA). Monosaccharides (D-glucose, L-rhamnose, D-mannose, D-galactose, D-xylose, D-galacturonic acid, L-arabinose) were bought from Shanghai Yuanju Bioscience Technology Limited Company. The other chemicals were analytical grade and made in China.

2.2. Isolation and purification of MT2-A

Crude mannatide (300 mg) was fractionated on DEAE-cellulose column (5.0 cm × 26 cm) eluting with distilled water and 0.1 M NaCl gradually at a flow rate of 3 mL/min. The eluent was monitored by the phenol–sulfuric acid method [9]. Fractions containing polysaccharides were collected, concentrated, dialyzed and lyophilized to give MT1 (50 mg) and MT2 (200 mg), respectively. The major fraction MT2 (75 mg) was further purified on Sephacryl S-300 gel column (1.6 cm × 80 cm) at a flow rate of 15 mL/h to give the homogeneous glycopeptide MT2-A (50 mg).

2.3. Homogeneity and molecular weight of MT2-A

High performance gel filtration chromatography (HPGFC) technique [10] was used to determine the homogeneity and molecular weight. The HPGFC instrument was equipped with an Agilent 1100 HPLC system matched with a TSK PW_{XL} G4000 gel filtration column, an Agilent G1362A refractive index detector and an Agilent G1315B diode array detector. The sample was eluted with 0.7% Na_2SO_4 at a flow rate of 0.6 mL/min. Data was analyzed by Agilent GPC Software. The molecular weight was calculated based on calibration curve obtained by Dextran T-series standards (M_w: 12, 50, 270, 670 kDa).

2.4. Monosaccharide composition of MT2-A

The monosaccharide composition of MT2-A was determined by the PMP-labeling procedure [11]. MT2-A (5 mg) was hydrolyzed with 2 M TFA (trifluoroacetic acid) at 120 °C for 2 h. Excess acid was removed by evaporating at reduced pressure with the addition of methanol for five times after completing the hydrolysis. Dry hydrolysis product was labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP) through the addition of 2 mL 0.6 M NaOH and 1 mL of 0.5 M PMP methanol solution. The mixture was reacted at 70 °C for 100 min. Later, the reaction product was neutralized with 1 mL of 0.3 M HCl solution and extracted with 20 mL chloroform three times. The aqueous phase was finally filtered through 0.45 μm nylon membrane (Shanghai, China) for HPLC analysis. 20 μL of the resulting solution was injected into the Aligent Eclipse XDB-C_{18} column (250 mm × 4.6 mm). The sample was eluted with a mixture of acetonitrile and (pH 6.8) 0.1 M phosphate buffer (20:80) [12] at a flow rate of 1.0 mL/min and detected at 250 nm. Standard monosaccharides were determined by the same procedure.

2.5. Amino acid analysis of MT2-A

MT2-A (10 mg) was dissolved with 6 M HCl and hydrolyzed at 110 °C for 24 h. The amino acid constituents were separated on an ion exchange column (Biochrom, 200 mm × 4.6 mm × 8 μm) and analyzed on an Amino Acid Analyzer [13] (Biochrom, UK). Spectrometer was 570 nm (VIS1) and 440 nm (VIS2). Preliminary calibration of the column was conducted by Amino Acids Mixture Standard Solution.

2.6. Methylation analysis

Methylation analysis was performed according to the method of Needs and Selvendran [14] with minor modifications. The sample (20 mg) was dissolved in anhydrous DMSO (4 mL), stirred constantly for 5 min in a waterless condition. Then NaOH (100 mg) grinded under infrared lamp was quickly added into the sample solution. The mixture was treated by ultrasonic bath for 30 min to obtain a clear solution. After that iodomethane (1.0 mL) was added into the solution slowly in an ice bath. The mixture reacted for 30 min with constant stirring at room temperature. Then the reaction was terminated with the addition of water, evaporated to remove excess iodomethane, dialyzed with flowing water for 48 h, and freeze-dried. Completely methylated polysaccharide was obtained by repeating the above procedure for three times. Complete methylation was verified by the disappearance of hydroxyl absorption in the ATR-IR spectrum. The methylated product (4 mg) was completely hydrolyzed at 120 °C for 2 h, reduced with $NaBH_4$ (100 mg) at room temperature for 2 h and acetylated with acetic anhydride (3 mL) at 100 °C for 1 h. The partially methylated alditol acetate was extracted by chloroform for three times and the chloroform layer was evaporated to dryness and dissolved again in 0.5 mL chloroform. 1 μL of the above filtered solution was injected automatically in split mode to GC–MS system (Agilent 6890/5975 GC–MS System, USA) with the HP-5 column (30 m × 0.25 mm × 0.25 μm). The split ratio was 5:1. The oven temperature was initially set at 120 °C for 2 min, programmed from 120 °C to 250 °C at the speed of 5 °C/min and held at 250 °C for 10 min.

2.7. Partial acid hydrolysis

MT2-A (200 mg) was hydrolyzed in 0.2 M TFA (100 mL) and 1.0 M TFA (100 mL) at 100 °C for 8 h, respectively. Excess acid was removed by evaporation at reduced pressure with the addition of methanol for five times after completing the hydrolysis. The sample was dialyzed with distilled water for 48 h. Fractions in the dialysis bags named MT2-A-0.2 and MT2-A-1.0 were prepared for methylation analysis.

2.8. IR spectroscopy analysis

MT2-A (1–2 mg) was mixed with KBr powder and pressed into tablets, which was measured on Nicolet Magna-IR 550 spectrometer.

2.9. NMR spectroscopy analysis

70 mg of MT2-A and MT2-A-0.2 was, respectively, deuterium exchanged three times with 99.98% D_2O by freeze-drying. The ^1H NMR and ^{13}C NMR spectra were given at 23 °C on a Varian VNMRS-600. ^1H—^1H COSY, TOCSY, NOESY, HSQC and HMBC experiments were conducted by the standard programs. TMS was used as external standard for the ^{13}C NMR spectrum, and D_2O was used as internal standard for ^1H NMR spectrum [15].

2.10. Selective acetolysis of MT2-A

MT2-A (100 mg), anhydrous pyridine (10 mL) and acetic anhydride (10 mL) were reacted in a sealed round-bottom flask at 100 °C for 24 h. Excess pyridine was removed by evaporation with the addition of methylbenzene for five times. Afterwards 10 mL acetic acid, 10 mL acetic anhydride and 1 mL concentrated sulphuric acid were added, respectively, and then reacted at 40 °C for 12 h. The reaction was terminated with addition of anhydrous pyridine (40 mL). Excess pyridine was removed by evaporating. Afterwards, the product was extracted with 10 mL chloroform and 6 mL water under shaking, and separated by centrifuging, gathering the chloroform layer. Water was substituted by 6 mL 1 M HCl and 6 mL 1 M $NaHCO_3$ successively, and the mixture was treated following the same steps. The obtained chloroform layer was dried