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Purification and characterization of a novel glycoprotein from *Streptomyces* sp. ZX01

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ABSTRACT

A novel glycoprotein GP-1 with antiviral activity against plant virus was isolated from the fermentation broth of the actinomycete *Streptomyces* sp. ZX01. MALDI-TOF-MS proved that molecular weight of GP-1 approximately was 8.5 kDa. GP-1 was a heat-sensitive glycoprotein with decreasing antiviral activity after treated from 80 °C to 100 °C for 30 min. GP-1 contained 40.23% carbohydrate with N-linked and O-linked glycan. FT-IR and NMR spectra proved that GP-1 contained protein and carbohydrate portions with α-D-(1,6)-glucose residues. Circular dichroism revealed that GP-1 was a glycoprotein with a large unordered content. Moreover, protein sequencing was predicted by using MALDI-TOF-MS and Mascot search. These results suggested that glycoprotein GP-1 could be used as a novel natural antiviral agent in agricultural industry.

1. Introduction

Glycoprotein is a kind of glycoconjugate, which is linked to proteins or peptides [1]. Generally, glycoproteins have different and excellent bioactivity in various fields, and it has different structure and bioactivity mechanism compared with its polysaccharide. Although widely distributed in eukaryote, glycoproteins appear to be relatively fewer in prokaryotic organisms. In the last two to three decades, the glycoproteins have been reported in some prokaryotic organisms [2], an oligosaccharyltransferase (PglB) in *Campylobacter jejuni* was the first general glycosylation system in bacteria [3]. The bacterial glycoproteins also produced by Actinomycetes, a class of high G + C content and Gram-positive bacteria, such as, *Mycobacterium* sp., *Corynebacterium* sp. and *Streptomyces* sp. People pay more attention to *Mycobacterium* in recent years [4,5], because many secreted antigens of glycoproteins were found in *Mycobacterium* sp. [5–9].

Streptomyces is a famous prokaryotic gram-positive bacterium in pharmaceuticals industry. The majority antibiotics were produced by *Streptomyces*. It also could produce other useful macromolecules, such as, polysaccharide [10,11] and protein [12]. However, there were only a small amount of reports about glycoproteins from *Streptomyces*, such as glucanase and xylanases from *S. lividans* [13–15], PstS from *S. coelicolor* [16].

Streptomyces sp. ZX01 was isolated from forest soil around the Kanas Lake in China (in the submissions). As an unknown species of *Streptomyces*, lots of works about its metabolites were underway. Our research showed the metabolites of ZX01 had powerful antiviral activity against plant virus, especially *Tobacco mosaic virus* (TMV). Meanwhile, many features of glycoprotein appeared in preliminary study. So, it is very necessary to isolate and structurally elucidate the glycoproteins from the metabolites of ZX01 strain.

In the present paper, we report the purification and structural characteristics of glycoprotein GP-1 isolated from *Streptomyces* sp. ZX01 as well as the assay of thermostability and anti-TMV activity of GP-1.

2. Materials and methods

2.1. Strain and culture conditions

The strain of *Streptomyces* sp. ZX01 was isolated from forest soil around the Kanas Lake in Xinjiang Province of China. The stock culture was maintained on Gause's No.1 medium slant at 4 °C, and

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sub-cultured every two month. The strain was grown at 28 °C in a 70 L mechanical stirred fermentor (Green Bio-engineering Co., Ltd., Zhenjiang, China) with working volume of 50 L with the fermentation medium (millet 1%, glucose 1%, peptone 0.3%, $CaCO_3$ 0.2%, NaCl 0.25%). The fermentation broth was filtered and processed spray-drying.

2.2. Purification of glycoprotein

The powder of fermentation broth (entire culture, including medium and metabolites) was dissolve in water, then extracted with ethyl acetate and *n*-butanol. The water phase was applied to a column (8 cm × 60 cm) with macroporous resin (Diaion 101, nonpolar styrene-divinyl polymer). The column was eluted sequentially with water, 20%, 40%, 60% methyl alcohol/water (v/v). The 40% MeOH eluents was concentrated in vacuum, then, proceed to alcohol precipitation at 4 °C. The precipitates were purified by DEAE-cellulose (DE-52) column (2 cm × 50 cm) eluted with 0.1, 0.3 and 0.5 M NaCl at pH 7.0 (every fraction tube 5 mL). The saccharide content of the eluate was determined spectrophotometrically at 490 nm using the method of phenol–sulfuric acid [17], the protein content was determined by coomassie brilliant blue method at 595 nm. Two factions F4-1 and F4-2 were collected, dialyzed and freeze-dried. F4-1 was then applied to a Sephadex G-75 gel-filtration column (1.2 cm × 100 cm), and eluted with deionized water at a flow rate of 0.3 mL/min. Two peaks GP-1 and GP-2 were collected and lyophilized for antiviral activity assessment and structural analysis.

2.3. Assay of antiviral activity

The antiviral activity against TMV of glycoprotein fractions was determined as follows. First, a light layer of 500-mesh carborundum was scattered on the leaf surface of *Nicotiana glutinosa* L. Second, each sample was mixed with an equal volume of TMV solution (10 μg/mL final concentration) and was used immediately to mechanically inoculate the left half-leaves, and the right halves were treated with a solution of 10 μg/mL TMV containing the same solvent as a control. Finally, a little of sterile water was sprayed on the tobacco plant [18]. Each treatment was performed five times. The number of necrosis spots was recorded after 3–4 days. The inhibition rate was calculated according to the formula:

$$\text{Inhibition rate}\,(\%) = \frac{1-T}{C} \times 100$$

where T is the mean of necrosis spots on the half-leaf of treatment and C is the mean of spots on the half-leaf of control [19].

2.4. Thermostability of glycoprotein

Glycoprotein GP-1 (1 mg/mL) was treated at 40, 60, 80, 100 °C for 30 min, and then, comparing the antiviral activity and circular dichroism (CD) spectra by spectropolarimeter (Jasco, Japan) at 25 °C in the region of 190–250 nm.

2.5. Molecular mass of glycoprotein

The molecular mass of GP-1 was estimated by SDS-PAGE with 5% stacking gel and 20% separating gel, the gel was stained with Coomassie Brilliant Blue (CBB) [20]. To determine whether GP-1 was a glycoprotein or not, the gel also was stained with Periodic Acid-Schiff (PAS) [21]. Sample and marker loading amount both was 10 μg. The BSA (Albumin from bovine serum) was a negative control.

The molecular weight of GP-1 was also determined by using an autoflex III smartbeam MALDI-TOF-MS (Bruker, Germany) in linear and positive ion mode. The matrix compound was DHB (2,5-dihydroxy benzoic acid). GP-1 was dissolved in 0.1% TFA solution, and mixed with matrix solution (1:1, v/v). The protein calibration standards I (Brucker Daltonics, USA) was used to mass calibrate before the analysis of a sample.

2.6. Monosaccharide composition and amino acid composition

Carbohydrate content of GP-1 was determined by the phenol-sulfuric acid method [17] using glucose as the standard. Monosaccharide composition was analyzed by gas chromatography (GC). GP-1 (5 mg) was absolutely hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 mL) for 4 h at 120 °C in ampule. The residual TFA was removed with methanol by rotary vacuum evaporator. The hydrolyzed monosaccharides were converted to alditol acetate derivatives then analyzed on an Agilent 6820 GC system (Agilent, USA) with capillary column (30 m × 0.32 mm). The operation conditions were reported in the previous study [22]. Protein content was determined by method of Coomassie brilliant blue using Bovine serum albumin (BSA) as the standard. Amino acid composition was analyzed by HPLC. GP-1 (5 mg) was dissolved in 5 mL 6 M HCl and hydrolyzed at 110 °C for 24 h in ampule. After neutralization using NaOH, Agilent HPLC 1260 (Agilent, USA) with Agilent ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm) was performed to analyze the amino acid composition. Flowing phase: (A) 0.05 mol/L sodium acetate (pH 6.8); (B) 50% (v/v) acetonitrile/deionized water. Gradient procedure: 0–15–25–35 min (30–55–100–30%, B). Flow rate: 1 mL/min; temperature of column: 30 °C; wavelength: 360 nm.

2.7. Conformation analysis

The types of glycosidic bond were analyzed by UV spectrum and enzymolysis. UV Spectroscopic analysis of GP-1 was performed by using UV spectrophotometer (LabTech, China) ranging from 200 nm to 400 nm to determined O-linked glycan. GP-1 was dissolved in 0.2 M NaOH for 30 min followed by UV analysis. GP-1 without sodium hydroxide treatment was used as the control. Molecular mass analysis of GP-1 treated with glycosidase PNGase F was proceed by using MALDI-TOF-MS. The circular dichroism (CD) spectrum of GP-1 (dissolve in distilled water) was obtained by spectropolarimeter (Jasco, Japan) at 25 °C in the region of 190–250 nm. CDPRO software with three matching programs (CONTIN, SELCON3 and CDSSTR) and a reference protein package consisting 48 kinds of protein were used to analysis the second structure percentages. FT-IR spectrum of GP-1 was recorded with FT-IR spectrometer (Jasco, Japan) in the range of 400–4000 cm^{-1} using the potassium bromide (KBr) disk method. The ^1H and ^{13}C NMR spectra of GP-1 were generated with a Bruker AVANCE III 600 MHz spectrometer (Germany). Samples were dissolved in D_2O and mainly examined at room temperature.

2.8. In-gel tryptic digestion

The band of GP-1 in SDS-PAGE was excised, then rinsed two times with deionized water and destained with 200 μL destaining solution (25 mM NH_4HCO_3, 50%CH_3CN). The gel slices were dehydrated by 200 μL acetonitrile, and dried completely in a Speedvac evaporator. The dried gel pieces were incubated with 10 mM dithiothreitol at 56 °C for 1 h. Cooling and dried gel pieces then incubated with 55 mM Iodoacetamide in dark for 45 min. The gels were rinsed, in sequence, with 25 mM NH_4HCO_3, 50% CH_3CN and CH_3CN, then dried completely in Speedvac evaporator [23]. The dried gel pieces were reswollen in 2 μL of 25 mM ammonium bicarbonate containing 10 ng/μL trypsin. After solvent had infiltrated into gels, 10 μL