Structural Investigation of an Antibacterial Polysaccharide from *Streptomyces virginia* H03

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The chemical structure of a polysaccharide from the broth of cultured *Streptomyces virginia* H03 was investigated. There might be 1→2 and 1→4 and no 1→6 glycosidic linkages in the polysaccharide according to periodate oxidation and Smith degradation. Four fragments including 2,3,4,6-Me_4-Man, 2,3,6-Me_3-Gal, 2,4,6-Me_3-Glc, and 3,6-Me_2-Man were found in the methylated polysaccharide. Furthermore, the polysaccharide has a β-Glc(1→4)-α-Man(1→4)-α-Gal(1→3)-linked backbone and a branch at the C-2 position of (1→2)-linked mannose residues as determined by the method of nuclear magnetic resonance (NMR) spectroscopy. The assumed structure of the polysaccharide is

$$
\begin{array}{l}
[\rightarrow 3)\text{-}\beta\text{-Glc}(1\rightarrow 4)\text{-}\alpha\text{-Man}(1\rightarrow 4)\text{-}\alpha\text{-Gal}(1\rightarrow]_n. \\
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\qquad\qquad\qquad\qquad\quad\;\; \uparrow \\
\qquad\qquad\qquad\quad \alpha\text{-Man}(1
\end{array}
$$

Additionally, the polysaccharide has a wide antibiogram and was found to be most effective against *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli*.

Key words: Polysaccharide, *Streptomyces virginia* H03, Antibacterial Activity

Introduction

There are very few reports on antibacterial polysaccharides produced by *Streptomyces*, which are Gram-positive bacteria and famous for producing many kinds of bioactive secondary metabolites including antibiotics, antifungal, antiviral, anticancer, and immunosuppressant agents, insecticides and herbicides (Williams *et al.,*1983). In our laboratory, over 1000 strains, mostly unusual genera of actinomycetes, were isolated and purified from all kinds of soil and water. *Streptomyces virginia* H03 was found to produce a novel polysaccharide which was made up of mannose (Man), glucose (Glc), and galactose (Gal) in a 2:1:1 proportion (He *et al.*, 2008). The structure of the antibacterial polysaccharide was determined by periodate oxidation, Smith degradation, permethylation, and nuclear magnetic resonance (NMR). Furthermore, the antibiograms and minimal bactericidal concentrations (MBCs) were assayed to investigate the antibacterial activity of the polysaccharide.

Material and Methods

Materials

Streptomyces virginia H03 was obtained from soil of Dabieshan, China. It was identified and conserved in China Center for Type Culture Collection (CCTCC), and its serial number is M 207049. A novel antibacterial polysaccharide was isolated and purified from the broth of *Streptomyces virginia* H03 by the method reported by He *et al.* (2008). Six strains of food spoilage and poisoning microorganisms including the bacteria *Staphylococcus aureus* (*S. aureus* ATCC 25923), *Bacillus subtilis* (*B. subtilis* ATCC 6633), *Listeria monocytogenes* (*L. monocytogenes* ATCC 43251), and *Escherichia coli* (*E. coli* ATCC 25922), and the yeasts *Zygosaccharomyces bailii* (*Z. bailii* ATCC 36947) and *Candida utilis* (*C. utilis* ATCC 9256) were obtained from the College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China.

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Periodate oxidation and Smith degradation

The methods of periodate oxidation and Smith degradation were developed by Fleury and Lange (1933). A suspension of 50 mg polysaccharide dissolved in 50 ml 15 mM $NaIO_4$ was kept in the dark at room temperature. At 6-h intervals, 0.1-ml aliquots were withdrawn to determine the periodate consumption. The excess of periodate was reduced with ethylene glycol, and the liberated formic acid was titrated with 5 mM NaOH. After the oxidized product was dialyzed against distilled water for 3 d, the residue in the dialysis tube was filtered and re-dissolved in distilled water. Then, it was reduced with 0.2 g $NaBH_4$ for 20 h at 20 °C, and the excess of sodium borohydride was decomposed by addition of 10% acetic acid. After the reaction mixture was dialyzed against distilled water, a white powder of the polysaccharide polyalcohol was obtained. Subsequently, 10 mg polysaccharide polyalcohol were hydrolyzed into the sugars and alcohols with 1 M trifluoroacetic acid at 100 °C for 6 h in sealed tubes, then the mixture was evaporated to dryness. Finally, the sugars and alcohols were converted into their alditol acetates (Osborne *et al.*, 1999) and subjected to gas chromatography. Galactose, fucose, mannose, glycerol, erythritol, and glycolaldehyde were used as controls.

Methylation analysis

80 mg of polysaccharide were methylated three times based on the method of Hakomoris (1964). The reaction mixture was dialyzed against distilled water, the fraction in the dialysis tube was filtered, re-dissolved in $CHCl_3$, and then the solution was evaporated to dryness. The methylated polysaccharide was hydrolyzed with 1 M trifluoroacetic acid for 6 h at 100 °C until the mixture showed no absorption for free hydroxys group in its infrared spectrum. The sugars were obtained after the mixture was evaporated to dryness. Finally, the sugars were converted into their alditol acetates (Osborne *et al.*, 1999) for GC-MS analysis.

NMR spectroscopy

NMR spectroscopy was used to determine the chemical shifts of the glycosyl residues of the polysaccharide. The polysaccharide (30 mg) was dissolved in 1 ml D_2O, and ^{13}C NMR spectra were recorded with a Bruker DRX Avance 600 MHz spectrometer for 33920 scans at 30 °C.

Antibacterial activity of the polysaccharide

Firstly, six strains of familiar pathogenic or food spoilage microorganisms including bacteria, such as *B. subtilis*, *S. aureus*, *L. monocytogenes*, and *E. coli*, and yeasts, such as *Z. bailii* and *C. utilis*, were selected for testing the antibiograms of the polysaccharide by the method of filter disc diffusion plate with slight modifications. Each strain with the inoculum density of 10^6 colony forming units (CFU) per ml was inoculated on the medium. Then a filter with the purified polysaccharide solution (300 μg ml^{-1}) was placed in the middle of the medium. After the medium was incubated for 48 h at 30 °C, the inhibition zones were measured. Nisin, an antimicrobial peptide produced by *Lactococcus lactis*, was widely used in the food industry as a natural antimicrobial (De Vos *et al.*, 1995; Cheigh *et al.*, 2002). It was considered as a control in the present study.

Secondly, the minimal bactericidal concentrations (MBCs) of the polysaccharide were determined as described by Hacek *et al.* (1999) with slight modifications. Two-fold serial dilutions of the polysaccharide (2028, 1024, 512, 256, 128, 64, 32, 16, 8, and 4 μg ml^{-1}) were prepared, 100 μl polysaccharide solutions with each concentration were pipetted into tubes, and 100 μl freshly grown bacteria were added into the tubes with a density of 10^6 CFU per ml. A series of tube dilutions were incubated on a rotary shaker with a speed of 160 rotations per minute (rpm) for 48 h at 30 °C. On the following day, aliquots of each dilution were transferred on agar plates and incubated. The number of colonies was evaluated and the initial concentrations retrospectively calculated. The MBC was the lowest concentration of the polysaccharide that prevented visible growth on the subculture plate. Nisin was used as a control.

Results and Discussion

Analysis of periodate oxidation and Smith degradation

No formic acid was found indicating no 1→6 glycosidic linkages (Liu *et al.*, 2007; Luo and Fang, 2008). After the polysaccharide was hydrolyzed, glycerol, glyceraldehyde, erythritol, and glycolaldehyde were detected in the periodate-oxidized products by gas chromatography which showed that there might be 1→2 and 1→4 glycosidic linkages (Feng *et al.*, 2008; Wu *et al.*, 2009). Ad-

Table I. Methylation analysis of the polysaccharide.

Methylated sugar[a] (as alditol acetate)	Mode of linkage	Molar ratio
2,3,4,6-Me_4-Man	Man(1→	0.94
2,3,6-Me_3-Gal	→4)Gal(1→	0.92
2,4,6-Me_3-Glc	→3)Glc(1→	0.98
3,6-Me_2-Man	→4)Man(1→ and a branch at C-2	1.1

[a] 2,3,4,6-Me_4-Man, 2,3,4,6-tetra-*O*-methyl-mannose; 2,3,6-Me_3-Gal, 2,3,6-tri-*O*-methyl-galactose; 2,4,6-Me_3-Glc, 2,4,6-tri-*O*-methyl-glucose; 3,6-Me_2-Man, 3,6-di-*O*-methyl-mannose.

ditionally, the content of erythritol was about two times that of glycerol suggesting that the amount of 1→4 glycosidic linkages was two times that of 1→2 glycosidic linkages.

Analysis of methylation

After the fully methylated product of the polysaccharide was hydrolyzed with acid, it was converted into alditol acetates, which were analyzed by GC-MS (Table I). Four peaks appeared, including 2,3,4,6-Me_4-Man, 2,3,6-Me_3-Gal, 2,4,6-Me_3-Glc, and 3,6-Me_2-Man, in a molar ratio of 0.94:0.92:0.98:1.1. In detail, the peaks of 2,3,4,6-Me_4-Man, 2,3,6-Me_3-Gal, and 2,4,6-Me_3-Glc indicated glycosidic linkages of Man(1→, →4)Gal(1→, and →3)Glc(1→, respectively. Additionally, the peak of 3,6-Me_2-Man showed a →4) Man(1→-linked backbone and a branch at the C-2 atom.

Analysis of the NMR spectrum

The 600-MHz ^{13}C NMR spectrum of the polysaccharide is shown in Fig. 1, and the chemical shifts of the glycosyl residues of the polysaccharide are listed in Table II. Anomeric carbon (C-1) signals of glycosides were observed at 100 – 104 ppm, and C-2, C-3, C-4, C-5, and C-6 signals of glycosides were found from 60 – 80 ppm

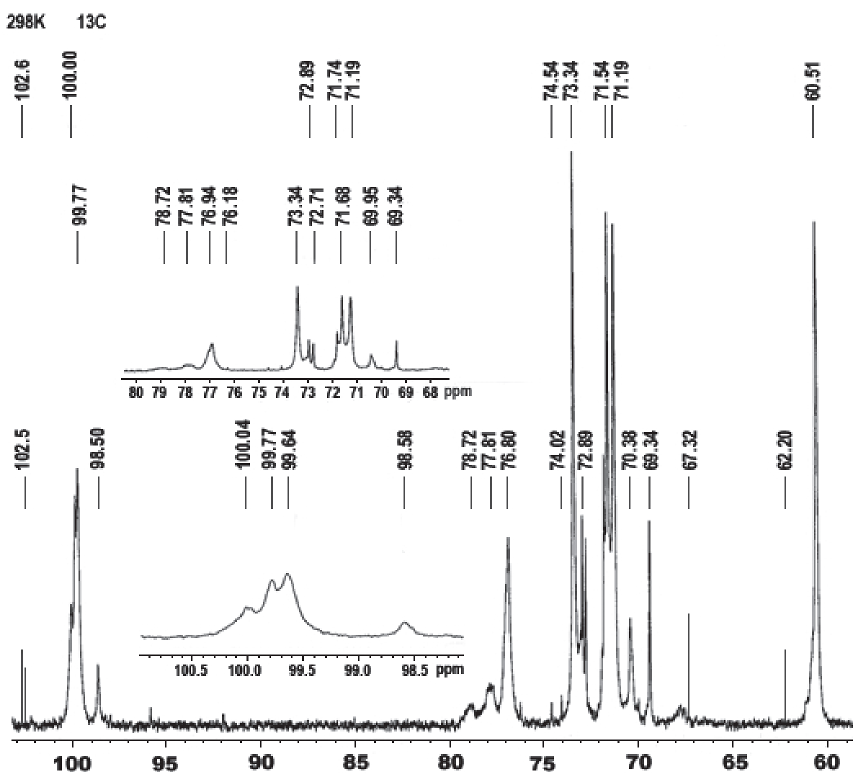

Fig. 1. 600-MHz ^{13}C NMR spectrum of the polysaccharide.

Table II. Chemical shifts of the glycosyl residues of the polysaccharide in ^{13}C NMR spectra (δ in ppm).

Residue	C-1	C-2	C-3	C-4	C-5	C-6
β-Glc(1→4)	102.6	74.0	74.5	78.7	76.8	60.5
α-Man(1→4)	102.5	71.7	71.6	67.3	74.5	62.2
→4)-α-Gal(1→	100.0	70.4	69.3	76.9	70.4	62.2
→3)-β-Glc(1→	102.5	69.3	69.3	78.7	69.3	60.5
α-Man(1→2)	102.5	74.5	72.9	76.9	77.81	60.5
→2)-α-Man(1→	99.6	79.1	71.2	67.3	73.3	62.2

Table III. Antibiograms and MBCs of the polysaccharide and nisin.

Microorganism	Diameter of inhibition zone [mm]		MBC [μg ml^{-1}]	
	Polysaccharide	Nisin	Polysaccharide	Nisin
B. subtilis	38.5 ± 0.3	36.5 ± 0.3	32	64
S. aureus	37.4 ± 0.5	34.8 ± 0.3	32	64
L. monocytogenes	36.8 ± 0.2	34.6 ± 0.4	32	–
E. coli	40.4 ± 0.4	–	16	–
Z. bailii	28.5 ± 0.3	–	128	–
C. utilis	29.1 ± 0.2	–	128	–

(Ishurd *et al.*, 2004; Omaira *et al.*, 2005; Kawagishi *et al.*, 1990). The anomeric carbon sign of both α- and β-configurations were detected at 100 and 104 ppm, respectively (Pramanik *et al.*, 2005; Cui *et al.*, 2007). Chemical shifts of glycosyl residues such as β-Glc(1→4), α-Man(1→4), →4)-α-Gal (1→, α-Man(1→2), and →3)-β-Glc(1→ were also observed from Fig. 1. Based on the results mentioned above, the conclusion could be drawn that the polysaccharide is a hetero-polysaccharide, which has a β-Glc(1→4)-α-Man(1→4)-α-Gal(1→3)-linked backbone with a branch at the C-2 position of (1→2)-linked mannose residues. According to the results of periodate oxidation, Smith degradation, permethylation, and NMR spectroscopy, the structure of the polysaccharide might be as follows:

[→3)-β-Glc(1→4)-α-Man(1→4)-α-Gal(1→]$_n$.
2
↑
α-Man(1

Antibiogram and MBCs of the polysaccharide

The result of the antibacterial activity test of the purified polysaccharide *in vitro* is shown in Table III. Of all the microorganisms tested, the purified polysaccharide was found to be the most effective against the bacteria *B. subtilis*, *S. aureus*, *L. monocytogenes*, and *E. coli* with inhibition zones of 38.5, 37.4, 36.8, and 40.4 mm, respectively, followed by the yeasts, with inhibition zones of 28.5 and 29.1 mm, respectively, for *Z. bailii* and *C. utilis*. It could not only inhibit the growth of Gram-positive and Gram-negative bacteria but also of yeasts. However, nisin could only inhibit the growth of Gram-positive bacteria. Therefore, the conclusion could be drawn that the antibiogram of the polysaccharide is wider than that of nisin which is the only natural, nonpoisonous and effective antimicrobial accepted in the world.

The MBCs of the polysaccharide against *B. subtilis*, *S. aureus*, *L. monocytogenes*, *E. coli*, *Z. bailii*, and *C. utilis* were 32, 32, 32, 16, 128, 128 μg ml^{-1}, respectively (Table III). However, the MBC of nisin was higher than that of the polysaccharide at the same conditions, which was consistent with the results of inhibition zones. Therefore, the results that the antibacterial activities of the polysaccharide were stronger than that of nisin suggested that the polysaccharide might be used as a potential antimicrobial in food.

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