JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. 7(Spl. Edn.), p. 711-717

# Structural Characterization and in vitro Antiviral Activity of Oligo-glucomannan Sulfate Prepared from Amorphophallus konjac

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(Received: 27 September 2013; accepted: 04 November 2013)

**Oligo-glucomannan (OGM) could be obtained through enzymolysis from KGM, a water-soluble polysaccharide isolated from Konjac flour. The sulfated derivative of OGM (OGMS) was prepared by chlorosulfonic acid method with formamide as a dehydration-condensation agent. OGMS was one kind of low molecular weight polysaccharide sulfate which had different weight-average molecular mass (Mw) ranging from 1.8 to 2.0 KDa and different degree of substitution (DS) ranging from 1.25 to 1.73. FT-IR and $^{13}C$ NMR spectra indicated that the sulfated groups had been introduced at C-2 0C-6 and C-3 positions of OGMS. OGMS had significant inhibitory effect on the growth of HepG2 and Hela cells in vitro at concentration higher than 0.2mg/mL. On the other hand, OGMS was a potent inhibitor of viruses HBV, IAV and $CBV_3$, with $IC_{50}$ values determined in vitro of 8.3, 4.2 and 3.1$\mu$ÀgmL$^{-1}$, respectively due to its sulfated groups and low molecular weight. The results showed that konjac oligo-glucomannan sulfate could be developed as a new type of medicine which had potential antiviral activity.**

**Key words:** Sulfated derivative, Oligo-glucomannan, Cytotoxicity, Antiviral

Polysaccharides from plants are safe, biocompatible and biodegradable substances which have many kinds of biological activities such as enhancing immunity, antioxidation and antitumor[5-7].

Since the inhibitory activity of a sulfated polysaccharide against the human immuno-deficiency virus (HIV) was first reported in 1987, sulfated polysaccharides had become the focus of considerable research[6]. They were a kind of polysaccharides with sulfated groups replacing their hydroxyl groups, including natural sulfated polysaccharides extracted from plants or derivatives synthesized from natural polysaccharides[12, 13]. Sulfated polysaccharides could not only enhance the water solubility but also change the chain conformation, resulting in antiviral and anticoagulant activities[2, 10, 11] which depended on their structural parameters, including the degree of substitution (DS)[8], the weight-average molecular mass (Mw), the position of sulfate, and glycosidic branching[9].

Konjac was a plant of the genus Amorphophallus using as an immuno regulation and healthcare food for a long time which was native to warm subtropical and tropical eastern Asia, from Japan and China south to Indonesia. After harvesting the mature konjac tubers, the tubers were washed, sliced, dried and milled by Raymond mill. After that the mixed power was separated to removing the konjac 'dancing powder', so the konjac flour could be obtained[1]. It

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contained significant amounts of konjac glucomannan (KGM) ranging from 51.3 to 96.9% .KGM, a primary polysaccharide component of konjac flour, was a kind of multifunctional natural polymer consisting of ²-D-mannose and ²-D-glucose residues (molar ratio 1.6:1) linked by β-1, 4-glycosidic bonds which was the main effective ingredient with weak biological activities, such as enhancing immunity, anti-oxidation and anti-tumor[2-4].

In the present study, Oligo-glucomannan sulfate (OGMS) was prepared from Oligo-glucomannan (OGM) which was obtained through enzymolysis. To introduce the sulfate group to OGM, chlorosulfonic acid method was adopted with formamide which was added as a dehydration-condensation agent in the reaction system to increase the reaction rate and hence decreasing the depolymerization of polysaccharides. The cytotoxicity effects of OGMS on host cells and antiviral activity on viruses were discussed in vitro to prove that sulfated modification could improve its antiviral activities.

## MATERIAL AND METHODS

### Materials and reagents

OGMS had been introduced into the experimental field of chemical engineering and technology College, Wu Han University of Science and Technology, WuHan, and a voucher specimen was deposited in Biological engineering institute, Hubei University of technology, WuHan. The preparation of OGMS had been described previously [8]. In short, OGMS was sulfated by Formamide chlorosulfonic acid and extracted by ethanol precipitation which was purified by gel and ion exchange chromatography. After dialysis and freeze-drying, pure OGMS was obtained.

### Cellulose acetate electrophoresis (CAE)

The method was adapted from that described by Stanley et al. Samples (10 mg $mL^{-1}$, 8 µL) were applied to cellulose acetate strips (SepraphorIII) and electrophoresis was conducted in a Gelman semi-micro bath containing $ZnSO_4$ buffer (0.2 M, pH 5.1) for 60 min at 6 mA, 100 V. Strips were stained with 1% Alcian Blue and destained with 5% aq. HOAc containing 10% EtOH. Hyaluronic acid and chondroitin sulfate were used as standard charged polysaccharides.

### Sulfate and sugar content

Total sugar content was analyzed by the phenol sulfuric acid method using D-galactose as standard. The Sulfur contents (S %) of the sulfated derivatives were determined by an elemental analyzer (Vario EL, Elementar Co., Germany), and DS, which referred to the average number of sulfated residues on each monosaccharide residue was calculated:

$$DS=(162\times S\%)/(32-102\times S\%)$$

The molecular weight of OGMS was determined by Gel Chromatography (GPC) on a TSK 3000sw column eluted with 0.7% HAc-NaAc buffer solution at a flow rate of 1.0 mL/min at 35°C. Elution was monitored by a refractive index detector.

### Sulfate group analysis

FT-IR spectra was recorded on a Nexus FT-IR spectrophotometer (Thermo Nicolet Corporation, USA). $^{13}C$ NMR spectra of 40 mg/ml solution in $D_2O$ was recorded at 40°C with a Bruker 600 MHz spectrometer (Germany), and the chemical shifts were expressed in ppm relative to the resonance of internal standard 2,2-dimethyl- 2-silapentane -5-sulfonate (DSS).

### Cell Growth inhibition assay

The HepG2 and Hela cell were provided by the virus research Institute, Science Academe of China and maintained with RPMI 1640 medium containing 10% fetal bovine serum and 100 ng/ml, each of penicillin and streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$.

The inhibition effects of OGMS on HepG2 and Hela cell were evaluated in vitro using MTT assay. Cells ($5\times10^5$ cells/well) were incubated for 12 h before adding the compounds. Cells were exposed and were treated with varied concentration of OGMS for 48 h at 37°C. After drug exposure, the culture medium was removed and 100 µl of MTT reagent (diluted in culture medium, 0.5 mg/ml) was added. Following incubation for 4 h, the MTT/medium was removed and DMSO (100 µl) was added to dissolve the formazan crystals. Absorbance of the colored solution was measured on a micro plate photometer using a test wavelength of 490nm. Results were evaluated by comparing the absorbance of the wells containing compound treated cells with the absorbance of wells containing 0.1%DMSO alone (solvent control). Conventionally, cell viability was determined for

each assay including blank wells that did not contain cells. All experiments were performed in triplicate. To validate the induction of apoptosis by identifying morphological features, phase-contrast microscopy was used to show the dose-dependent manner in cell viability in treatment of cells with OGMS.

The inhibition rate (IR) was calculated according to the formula below:

$$\text{Growth inhibition rate}(\%) = \frac{1\text{-}A_{\text{drug-blank}}}{-A_{\text{control-blank}}} \times 100$$

**Antiviral assay**

Cells were inoculated into 96-well micro titer trays ($2 \times 10^5$cells per well) and the plates were incubated at 35°C in 5% $CO_2$ until the cells were confluent. Four uninfected cell control wells, four virus-infected control wells and eight serial two-fold dilutions of each test material in MEM in quadruplicate rows were used for each virus. After removal of the growth medium, MEM was added to the cell control wells (100 $\mu$L) and the virus-infected control wells (50 $\mu$L). The test solutions (50 $\mu$L) were added to the remaining 32 wells. Dilutions of the virus were prepared in MEM for the inoculum [multiplicity of infection (MOI) =0.05] and aliquots (50 $\mu$L) were added to all except the cell control wells. All plates were incubated at 35°C in 5%$CO_2$ for 48 h. The plates were then examined micro scopically, the cytopathic effect (CPE) was scored and the cells were fixed. Following fixation, each well was washed four times with 300 $\mu$L of wash solution (PBS containing 0.2% BSA and 0.05% Tween 20). The $IC_{50}$ value corresponds to the lowest concentration reducing the plaques by 50% or more.

## RESULTS AND DISCUSSIONS

**Component analysis of OGMS**

Cellulose acetate electrophoresis of OGMS (Fig. 1) showed one mobile blue band, which appears to indicate that OGMS component was homogeneous.

The molecular masses of OGMS with different DS were investigated (Table 1). It showed that the molecular masses of OGMS increased from 1.8 to 2.0 kDa, and DS increased from 1.25 to 1.73. It implied that hydroxyl groups were substituted efficiently by sulfated groups on the polysaccharide.

After sulfated modification, the molecular mass of polysaccharide had not been decreased obviously. It might be the reason of adding N, N-dicyclohexyl- -carbodiimide (DCC) in synthesis reaction, and making the reaction achieve dynamic equilibrium fast, which decreased the depolymerization of polysaccharide. DCC had a

**Table 1.** Component analysis of OGMS

| OGMS (sample) | Time(h) | M w (kDa) | Elemental analysis (%) C | H | S | DS |
|---|---|---|---|---|---|---|
| OGMS-1 | 2 | 1.80 | 20.53 | 3.92 | 13.04 | 1.25 |
| OGMS-2 | 2 | 1.85 | 19.86 | 3.88 | 13.82 | 1.38 |
| OGMS-3 | 2 | 1.95 | 19.23 | 3.66 | 14.88 | 1.55 |
| OGMS-4 | 2 | 2.00 | 18.91 | 3.72 | 15.91 | 1.73 |

**Table 2.** Growth inhibition of OGM and OGMS against HepG20 Hela cells in vitro

| Group | Result | $C^c$(μg/ ml) 200 | 400 | 600 | 800 | 1000 |
|---|---|---|---|---|---|---|
| OGM | $IR^a$(%) | -16.3±0.4 | -10.7±1.5 | -8.2±2.4 | -6.3±1.6 | -4.4±0.9 |
| OGMS | $IR^a$(%) | 30.1±3.5 | 36.0±2.3 | 45.7±1.8 | 55.7±2.6 | 61.4±2.4 |
| OGM | $IR^b$(%) | -5.1±0.9 | -2.5±0.6 | 7.7±1.9 | 9.6±1.5 | 13.4±2.2 |
| OGMS | $IR^b$(%) | 22.5±2.7 | 31.1±3.2 | 35.2±2.9 | 38.6±2.1 | 41.6±1.2 |

$IR^a$, growth inhibition rate against HepG2 cell; $IR^b$, growth inhibition rate against Hela cell; $C^c$, concentration of OGM and OGMS

**Table 3.** Antiviral activity ($IC_{50}$) of OGMS

| | HBVμgmL$^{-1}$ | IAV μgmL$^{-1}$ | $CBV_3$ μgmL$^{-1}$ |
|---|---|---|---|
| OGMS | 8.3 | 4.2 | 3.1 |
| Virazole | 7.5 | 3.8 | 3.0 |

molecular masse about 472 Da which worked as a dehydration agent to afford dicyclohexylurea in the reaction.

**Sulfate group analysis of OGMS**

The FT-IR spectra of the native OGM and OGMS were shown in Fig. 2. Compared with OGM, two characteristic absorption bands appeared in the FT-IR spectra of OGMS, one at near 1148 cm$^{-1}$ describing an asymmetrical S=O stretching vibration and the other at near 834 cm$^{-1}$ representing a symmetrical C–O–S vibration. They indicated that OGMS was successfully sulfated.

Sulfated position on the polysaccharide was usually determined by $^{13}C$ NMR spectrum. The $^{13}C$ NMR spectra for the native OGM and OGMS were shown in Fig. 3. The $^{13}C$ NMR spectrum of the native OGM exhibited six signals around 96.4, 78.2, 74.9, 73.1, 70.9, and 60.8 ppm, attributed to C-1, C-3, C-5, C-2, C-4, and C-6, respectively. Compared with it, there were several new signals caused by sulfated groups in OGMS. The peak at 66.8 was assigned to the signal of C-6s; the peak at 72.7 was assigned to the signal of C-2s. The peak at 61.6 was weakened, which indicated that C-6 had been substituted by the sulfated group, but C-2 had been partially substituted. Because their strong peaks existed in the NMR spectra, the peak at 72.7 for C-2s was much weaker than that of C-6s at 66.8. We could conclude that the C-6 position was more active than the C-2 position due to the steric hindrance. Furthermore, a new peak appeared at 93.9, and the native C-1 peak at 96.4 became weaker in NMR spectra of OGMS. It was known that the signal of C-1 splitted if hydroxyl group on C-2 was

**Fig. 1.** Cellulose acetate electrophoresis of OGMS

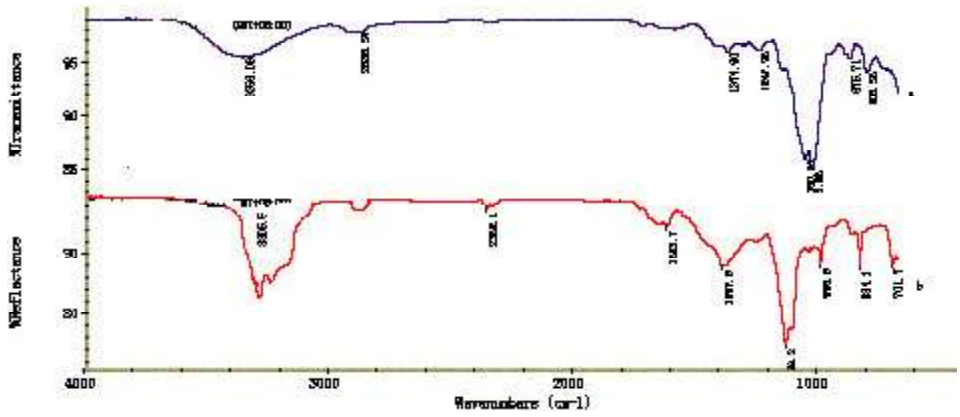



**Fig. 2.** FT-IR spectra of OGM (a) OGMS (b)

functionalized. It could be explained by the fact that C-2 had been substituted, which could influence the adjacent C-1 to split into two peaks. New peaks at 65–75 ppm meant sulfation of other positions occurred besides C-6 and C-2.

**Growth inhibition of OGMS against host cells**

In the present study, the growth inhibitory effects of OGM and OGMS against HepG20Hela cells in vitro were first examined in Table 2.OGM was determined to have no cytotoxicity against HepG2 cells , but OGMS showed cytotoxicity. In the concentration range from 200 µg/ ml to 1,000 µg/ ml, OGMS inhibited significantly the growth of HepG2 cells. Especially, at the concentration of 1,000 µg/ ml, the inhibition activity of OGMS was the highest with an inhibition

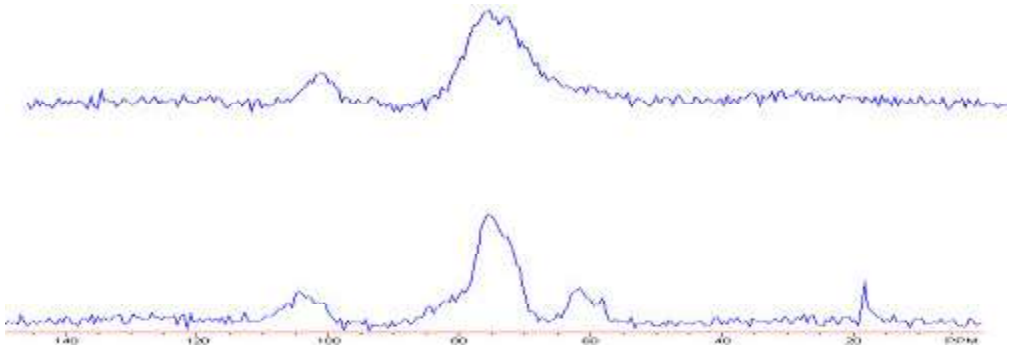
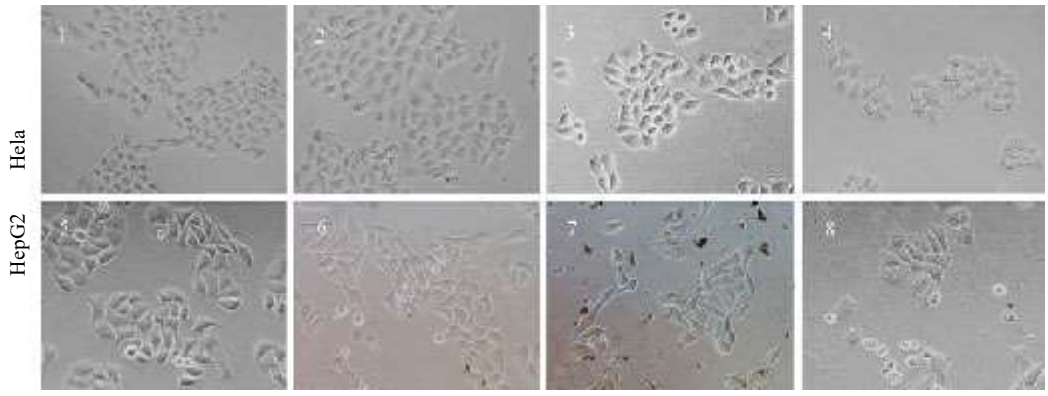


**Fig. 3.** $^{13}C$ NMR spectrum of OGM (a)0OGMS (b) (600MHz)




HepG2 and Hela cells were seeded into tissue culture flasks and treated with increasing concentrations of OGMS. 1-4 was HepG2 cells: (1) Control, (2)–(4) Concentration of OGMS was 200, 600 and 1,000 µg/ml, respectively. 5–8 were Hela cells: (5) Control, (6)–(8) Concentration of OGMS was 200, 600 and 1,000 µg/ml, respectively. All cells were photographed after 48 h drug treatment.

**Fig. 4.** Morphological characteristics of HepG2 and Hela cells treated with OGMS

ratio of 61.4%±2.4%. This suggested that sulfated modification could improve cytotoxicity of OGM on tumor cells.

In the meantime, OGM exhibited lower inhibition activity against Hela cells, but OGMS exhibited much stronger inhibition ratios against the growth of Hela cells. In the concentration range from 200 µg/ ml to 1,000 µg/ ml, OGMS inhibited significantly the growth of Hela cells. Especially, at the concentration of 1,000 ìg/ ml, the inhibition activity of OGMS was the highest with an inhibition ratio of 41.6%±1.2%. It indicated that the sulfated groups could contribute to direct cytotoxicity of OGM on tumor cells in vitro.

Figure 4 showed that HepG2 and Hela cells treated with OGMS were accompanied by

morphological changes. Two days after treatment with OGMS, cell numbers decreased visibly with OGMS concentration increasing from 200 µg/ ml to 1,000 µg/ ml. Moreover, the distinctive morphological features of cells included detachment and cell shrinkage. After cells were killed, cytoplasm came out. We believed that the death of HepG2 and Hela cells induced by OGMS were very obvious in vitro.

**Antiviral activity of OGMS**

The antiviral activities of OGMS and virazole (control group) were the same, within experimental error, against all three viruses (Table 3). From the table 3, we could conclude that OGMS was a potent inhibitor of viruses HBV0IAV and $CBV_3$, with $IC_{50}$ values determined in vitro of 8.3, 4.2 and 3.1µgmL$^{-1}$, respectively, which was close to virazole. On the other hand, OGMS had much lower cytotoxicity than virazole at the same concentration.

The potency of OGMS against the HBV virus was comparable to that found by Thompson and Drager, ($IC_{50}$ 2.2 µgmL$^{-1}$), but the potency against the IAV virus was greater than that found by these authors ($IC_{50}$ 6.1 µgmL$^{-1}$). The reason for the discrepancy between the two viruses was unclear.

## CONCLUSIONS

Sulfated derivatives of OGM from Konjac were prepared by chlorosulfonic acid method using DCC as a dehydration condensation agent. FT-IR spectra and $^{13}C$ NMR spectra indicated that the sulfation reaction had actually occurred. Depending on the reaction conditions, sulfated derivatives showed different DS ranging from 1.25 to 1.73, and different Mw ranging from 1.8 to 2.0 KDa. It implied that hydroxyl groups were substituted efficiently by sulfated groups on the polysaccharide with a little degradation.

Sulfated derivatives exhibited obvious inhibition effects on HepG2 and Hela cells in vitro, compared with OGM. It was determined that the sulfation of the native OGM could improve its cytotoxicity on tumor cells. OGMS was a potent inhibitor of viruses HBV0IAV and $CBV_3$, with $IC_{50}$ values determined in vitro of 8.3, 4.2 and 3.1µgmL$^{-1}$, respectively, which was close to virazole. We think sulfated derivatives of OGM will have an assistant role with antiviral agent in the treatment of virus in the future.

## ACKNOWLEDGMENTS

This study was supported by research grants from the international cooperation between Poland and China, and from HuBei Province National Science Fund. We thank Chen XinWenÿLiang ChangYong, Fu YouRong and Dr Yao XiangJie for their help.

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