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Chemically sulfated natural galactomannans with specific antiviral and anticoagulant activities

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ABSTRACT

Naturally occurring galactomannans were sulfated to give sulfated galactomannans with degrees of substitution of 0.7–1.4 per sugar unit and molecular weights of $\overline{M}_n = 0.6 \times 10^4$–$2.4 \times 10^4$. Sulfated galactomannans were found to have specific biological activities in vitro such as anticoagulant, anti-HIV and anti-Dengue virus activities. The biological activities were compared with those of standard dextran and curdlan sulfates, which are polysaccharides with potent antiviral activity and low cytotoxicity. It was found that sulfated galactomannans had moderate to high anticoagulant activity, 13.4–36.6 unit/mg, compared to that of dextran and curdlan sulfates, 22.7 and 10.0 unit/mg, and high anti-HIV and anti-Dengue virus activities, 0.04–0.8 μg/mL and 0.2–1.1 μg/mL, compared to those curdlan sulfates, 0.1 μg/mL, respectively. The cytotoxicity on MT-4 and LCC-MK2 cells was low. Surface plasmon resonance (SPR) of sulfated galactomannans revealed strong interaction with poly-L-lysine as a model compound of virus proteins, and suggested that the specific biological activities might originate in the electrostatic interaction of negatively charged sulfate groups of sulfated galactomannans and positively charged amino groups of surface proteins of viruses. These results suggest that sulfated galactomannans effectively prevented the infection of cells by viruses and the degree of substitution and molecular weights played important roles in the biological activities.

1. Introduction

Naturally occurring galactomannans are important polysaccharides in the food industries [1] because they interact with other polysaccharides such as xanthan, carrageenan, and agarose to form highly viscous gels [2]. Recently, we reported the structural analysis of four galactomannans with different proportions of galactose and mannose residues from fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust bean gum (LG) produced by seeds of leguminous plants. High resolution proton, carbon, and 2D NMR measurements of the galactomannans indicated that the structure of galactomannans had a (1 → 4)-β-D-mannopyranosidic main chain with (1 → 6)-α-D-galactopyranosidic side chains [3].

On the other hand, the biological activities of sulfated polysaccharides have been attracting attention, and especially their antiviral and anticoagulant activities are well known [4]. In addition, it is important for development of biomedical materials to elucidate the relationship between the structure and biological activities of polysaccharides. Since sulfated polysaccharides were found to have anti-influenza virus activity by Geber in 1958 [5], many investigations of the antiviral activities have been reported. In 1985, Nakashima et al. found anti-HIV activity of natural sulfated polysaccharides extracted from sea alga [6,7]. We have investigated the synthesis and biological activities of sulfated polysaccharides obtained by sulfation of both synthetic and naturally occurring polysaccharides. Synthetic polysaccharides with stereoregularity were prepared by ring-opening polymerization of anhydro sugar derivatives. Recently, we reported the synthesis and biological activities of sulfated galactomannans by ring-opening polymerization and copolymerization of a new dis-

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accharide monomer, benzylated 4-*O*-galactopyranose-branched 1, 6-anhydro mannose with a monosaccharide monomer, benzylated 1, 6-anhydro mannose. The synthetic galactomannans obtained had a 1, 6-α-D-mannopyranosidic main chain with a 4-*O*-β-D- galactopyranose branch in a sugar unit of the main chain, which is the different structure from natural galactomannans. Several reports have appeared on the biological activities of sulfated galactomannans [4]. The anticoagulant activity of sulfated galactomannans was tested by the inhibition of the coagulant factors IIa and Xa [8], APTT time [9], and plasma clot time [10]. High antioxidant activity, EC_{50} = 3.99–7.99 μg/mL, was also reported and compared with that of natural galactomannans without sulfation. The degree of substitution and low molecular weights were found to promote the antioxidant activity [11]. In addition, antiviral activities against herpes simplex [11], yellow fever [12], Dengue [13], and simian rotaviruses [14] were reported. However, there are no reports on the relationship between structure and biological activities.

In this paper, we report the synthesis of sulfated natural galactomannans with potent anti-HIV and anti-Dengue virus activities. We also describe the results of anticoagulant activities, which were examined by using bovine blood plasma according to the United States Pharmacopoeia. The biological mechanism was investigated by surface plasmon resonance (SPR) using poly-L-lysine as a model compound of virus surface proteins. The structure of sulfated galactomannans was determined by high resolution NMR and IR spectroscopies.

2. Materials and methods

2.1. Materials

Naturally occurring galactomannans, fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust bean gum (LG), were purchased from Air Green Co., Ltd. or Sigma-Aldrich, Japan, respectively. The proportions of mannose and galactose were 1.0 and 1.20 for FG, 1.0 and 0.61 for GG, 1.0 and 0.35 for TG, and 1.0 and 0.30 for LG, respectively. The galactomannans were treated with 5% H_2SO_4 solution for 2 h at 60 °C to give low molecular weight galactomannans according to our previously described method [3]. Poly-L-lysine with the molecular weight of 1000–5000 Da was supplied from Sigma-Aldrich, Japan. Piperidine-*N*-sulfonic acid was synthesized from piperidine and sulfonic acid by the reported method [14]. Sulfur trioxide pyridine (SO_3-pyridine) complex was purchased from Sigma-Aldrich, Japan.

2.2. Sulfation of natural galactomannans

A typical procedure for sulfation is as follows. Galactomannan from FG (0.25 g, 1.5 mmol) with $\overline{M}_n = 16.3 \times 10^4$ was added in anhydrous DMSO (30 ml) under N_2 atmosphere, and then piperidine-*N*-sulfonic acid (0.75 g, 4.6 mmol) was added. The mixture was stirred for 120 min at 100 °C and then cooled to room temperature. The mixture was dialyzed for 24 h against deionized water after neutralizing with 5% NaOH solution. The dialysate was freeze-dried to give a sulfated galactomannan in 0.24 g yield. The specific rotation was $[\alpha]_D^{25} = +49^\circ$ (H_2O, c1%), and the molecular weight was $\overline{M}_n = 0.9 \times 10^4$. Elemental analysis: C, 24.1%; H, 3.5%; S, 14.3%.

2.3. Biological activities

Anticoagulant activity of sulfated galactomannans in vitro was measured by using bovine plasma according to the modified method of the US Pharmacopeia [15], and the activity was calculated by comparison with that of standard dextran sulfate (H-39), 22.7 units/mg. The anti-HIV activity in vitro was assayed by the MTT method according to the reported method [16]. MT-4 cells, which are HIV sensitive, the HIV-$1_{HTLV\text{-}IIIB}$ strain, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) were used. The anti-HIV activity was evaluated by the EC_{50}, which is the concentration that inhibited HIV infection of MT-4 cells by 50% [16]. The cytotoxicity was measured as the concentration of sulfated galactomannans at which 50% of the cells survived [16]. The anti-Dengue virus activity in vitro was determined by using LLC-MK2 cells, a rhesus monkey kidney cell strain, and serotype 2 Dengue virus. The anti-Dengue virus activity was evaluated by the same procedure of our recent paper [17] and the MTT method was conducted to calculate the activity (EC_{50}) and cytotoxicity (CC_{50}) of sulfated galactomannans [17].

2.4. Measurements

The 400 MHz ^1H and 100 MHz ^{13}C NMR spectra were recorded using a JEOL ECM-400 spectrometer in D_2O at 50 °C with 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS) as an internal standard. FT-IR spectra were measured using a Perkin Elmer Spectrum One spectrometer. Optical rotation was measured in H_2O at 25 °C using a JASCO DIP-140 digital polarimeter. Molecular weights of galactomannans and sulfated galactomannans were determined by an aqueous phase GPC equipped with Tosoh TSK-gel columns (7.6 mm × 600 mm) of $G2500PW_{XL}$, $G3000PW_{XL}$, and $G4000PW_{XL}$ eluted with 66.7 mM of phosphate buffer solution (pH 6.86) at 40 °C. A standard pullulan (Shodex standard P-82, Showa Denko Co., Japan) was used as a reference. A Biacore X100 system (GE Healthcare UK, Ltd.) was used to measure surface plasmon resonance (SPR) at 25 °C. The measurements were carried out according to the company-supplied method using a CM5 sensor chip. The apparent kinetic constants, association-(k_a), dissociation-(k_d) rate, and dissociation (K_D) constants, were calculated by the company-provided software.

3. Results and discussion

3.1. Sulfation of natural galactomannans

Galactomannans are naturally occurring polysaccharides produced by plant seeds; they have a (1 → 4)-β-D-mannopyranosidic main chain with (1 → 6)-α-D-galactopyranosidic branches in proportions dependent on the kind of seed. In this study, four galactomannans from fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust bean gum (LG) were used to investigate anticoagulant and antiviral activities after sulfation. The solubility of galactomannans is low because galactomannans have molecular weights higher than 100×10^4 Da. Therefore, before sulfation, acid hydrolysis was carried out to decrease the molecular weights according to the previously reported procedure [3]. The structure of hydrolyzed galactomannans was determined to be the same as that before hydrolysis without molecular weights by measuring specific rotation and high resolution NMR [3]. Sulfation was performed by using piperidine-*N*-sulfonic acid (PSA) or SO_3-pyridine complex as a sulfating reagent, as shown in Scheme 1. Table 1 shows the results of sulfation. Galactomannans were sulfated by PSA in DMSO at temperatures higher than 85 °C to give sulfated galactomannans with relatively low molecular weights ranging from $\overline{M}_n = 0.5 \times 10^4$ to 2.3×10^4. When SO_3-pyridine complex in DMF was used for the sulfation at temperatures below 85 °C, sulfated galactomannans had relatively higher molecular weights between $\overline{M}_n = 0.9 \times 10^4$ and 2.4×10^4. The molecular weights of sulfated galactomannans were also dependent on those before sulfation. The degree of substitution (DS) was between 0.7 and 1.4 in one sugar residue, and the