Doctoral Thesis

Development of Antiviral Biomaterials by Naturally Occurring Polysaccharides

September, 2011

Tegshi Muschin

Kitami Institute of Technology, Japan

Contents

Acknowledgements ..... **V**

Chapter 1. General introduction **1**

1.1. HIV / AIDS 1

1.2. HIV replication mechanisms 2

1.3. Anti-HIV agents 4

1.4. Influenza 4

1.5. Polysaccharides in the virus treatment research 7

1.6. Summary of this thesis 10

Reference 12

Chapter 2. Synthesis and specific influenza A virus-adsorptive functionality of alkyl curdlan sulfate-coated membrane filter **15**

ABSTRACT 15

2.1. Introduction 16

2.2. Experimental Section 19

2.2.1. Materials 19

2.2.2. Measurements 19

2.2.3. Alkyl curdlan sulfate 20

2.2.4. Anti-HIV activity of alkyl curdlan sulfate 21

2.2.5. Alkyl curdlan sulfate-coated membrane filter 22
2.2.6. Hemagglutination inhibition assay (HA) of alkyl curdlan sulfate-coated membrane filter 22
2.3. Results and Discussion 23
2.3.2. Synthesis and anti-HIV activity of alkyl curdlan sulfate 24
2.3.3. Structure of alkyl curdlan sulfate 27
2.3.4. Preparation of alkyl curdlan sulfate-coated membrane filter 31
2.3.5. Influenza hemagglutination inhibition assay (HA) of alkyl curdlan-sulfate coated membrane filter 37
2.4. Conclusion 41
References and Notes 42

Chapter 3. Structural Analysis of Galactomannans by NMR Spectroscopy 45

Abstract 45
3.1. Introduction 46
3.2. Experimental 48
3.2.1. Galactomannans 48
3.2.2. Hydrolysis of galactomannans by dilute H_2SO_4 aqueous solution 49
3.2.3. Measurements 49
3.3. Results and Discussion 51

3.3.1. Acid degradation of galactomannans and structural identity 51
3.3.2. Assignment of proton and carbon signals by DQF-COSY and HSQC NMR measurements 59
3.3.3. Structural analysis of galactomannans by HMBC and ROESY NMR measurements 65
References 72

Chapter 4. Sulfation of galactomannans and their biological activities 75

ABSTRACT 75
4.1. Introduction 76
4.2. Experimental Section 78
4.2.1. Materials 78
4.2.2. Measurements 78
4.2.3. Sulfation 79
4.2.4. Biological activities 81
4.3. Results and Discussion 82
4.3.1. Sulfation of Galactonannans 82
4.3.2. Anti-HIV activity and Blood anticoagulant activity 85
References and Notes 89

Acknowledgment

I dedicated with best respect and particularly indebted to my supervior, professor Dr. Takashi Yoshida of Kitami Institute of Technology, Japan. The works of my doctoral course in graduate school was achieved under his wonderful direction. I am grateful to him and professor Dr. Gereltu Borjihan of Inner Mongolia University, China, award me a valuable opportunity to came in Japan begin my researches in doctoral course, it take to me fresh career.

I am grateful to professor Dr. Nakashima Hideki and Taisei Kanamoto of St. Marianna University school of Medicine, Japan, for their help with biological activities experiment. I also thank all of the members of Yoshida laboratory for supports and assistance during my doctoral course.

I Thank HEIWA NAKAJIMA Foundation and Japan Student Services Organization (JASSO) for economical support, it is important to my stable and concentrated research.

Finally, I would like to thank my wife Surina for supports during the doctoral researches and patience to fight together in doctoral curse.

Chapter 1

General introduction

1.1. HIV / AIDS

The acquired immunodeficiency syndrome (AIDS) is a disease caused by retrovirus, human immunodeficiency virus (HIV) (**Figure 1**), HIV attacks the body`s immune system, infection leads to progressive immunologic deterioration, AIDS is the advanced stage, when the immune system becomes irreparably damaged, and opportunistic infections and cancers.

The first cases of AIDS were reported in the United States in 1981, amongst male homosexuals in Los Angeles and New York.[1] Two years later, Gallo et al. discovered that a novel retrovirus may have been infecting AIDS patients,[2,3] and after, be named human immunodeficiency virus (HIV). HIV has two major types of HIV-1 and HIV-2, HIV-1 is by far the most widespread worldwide, HIV-2 is found mainly in West Africa. As of December 2006, the World Health Organization estimated that 40 million adults and children were infected with HIV; 25 million of those infected resided in Africa and >1 million in North America. What is more sad, 3 million deaths occurred globally in 2006 as a result of HIV and AIDS.[4]

1.2. HIV replication mechanisms[4-6]

HIV enters macrophages and $CD4^+$ T cells by the adsorption of glycoproteins on its surface to receptors on the target cell followed by fusion of the viral envelope with the cell membrane and the RNA genome and viral proteins are released into the cell. Reverse transcription occurs to form a double-stranded DNA copy of the genome, which translocates and integrates into the host genome. Viral protein expression occurs and precursors are protealytically cleaved by the viral protease (and host proteases) to form functional proteins. These new proteins are packaged into a new virion, which is subsequently released to infect a new host cell (**Figure 2**).

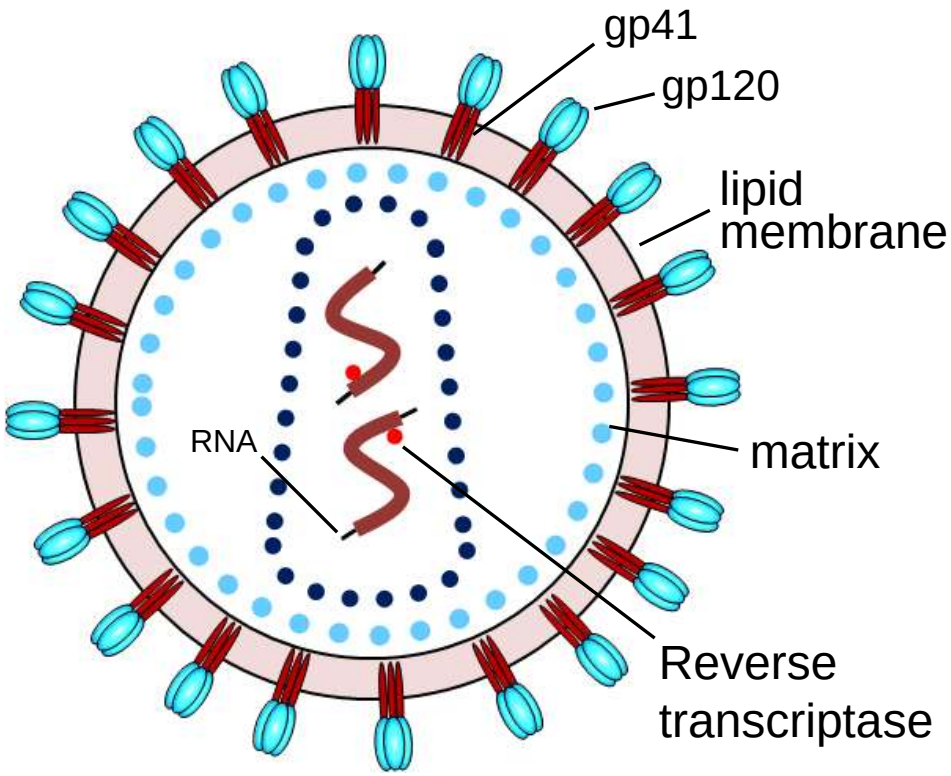

Figure 1. Structure of HIV

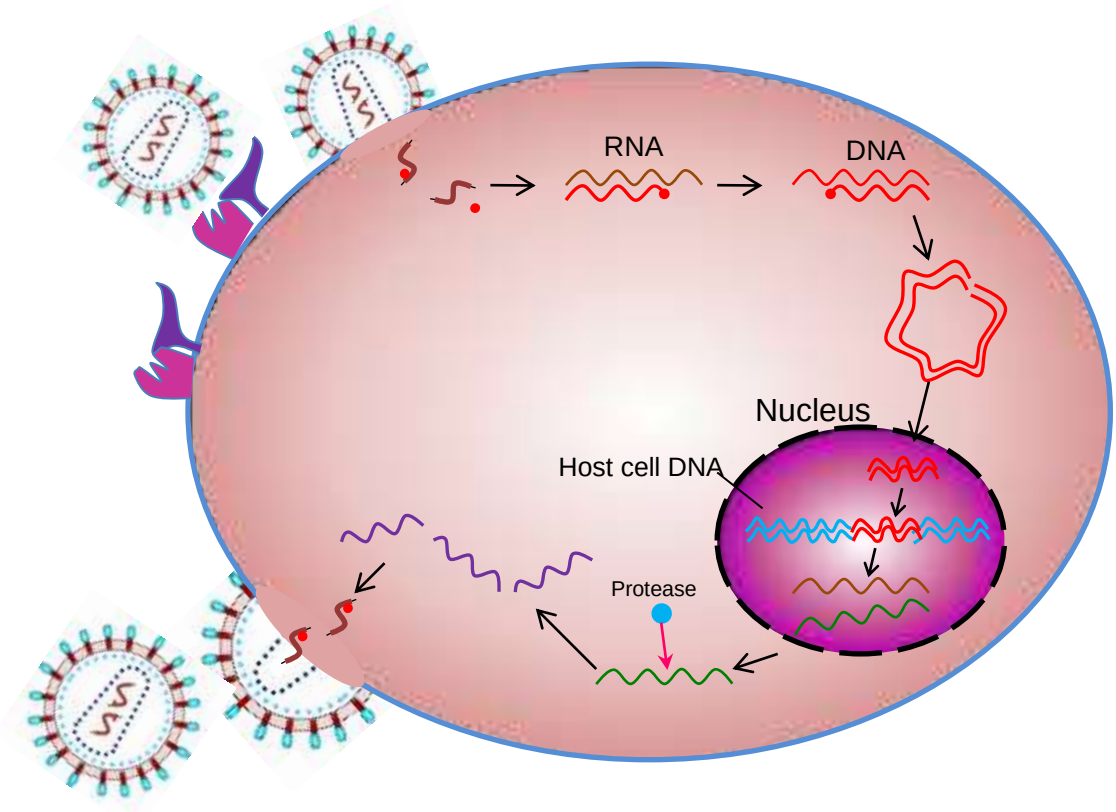

Figure 2. HIV replication cycle

1.3. Anti-HIV agents

Since AIDS discovered and pandemic in the worldwide, nowadays near third decade, under the researchers around the world have worked hard, more than 20 antiretroviral drugs have been approved by regulatory authorities for the treatments for the treatment of HIV infection. Available drugs belong to 6 different classes: nucleoside (nucleotide) reverse transcriptase (RT) inhibitors (NtRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs) and integrase inhibitor, which are targeted at viral enzymes; a fusion inhibitor, which prevents the fusion of the virus envelope with the host-cell membrane; and a CCR5 inhibitor, which blocks the interaction of the virus with one of its receptors on the host cell.[7,8] However, some of these drugs are minimally used, because of their serious side effects, commercial burden, or inconvenient administration schedules. On the other hand, sulfated polysaccharides focus attention upon due to their excellent and promising antiviral activities and low cytotoxicity.

1.4. Influenza

Influenza virus is an acute infectious disease caused by a member of the orthomyxovirus family, have three types as influenza A, B and much lesser extent, C virus. Influenza A and B viruses carry two surface enveloped glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA). HA is the key protein for cell entry and the release of virus genome into the cytoplasm of host cell,[9] virus

attachment to the cells mediated by the interaction of HA with the sialic acid on the host cell surface, after that endocytosis and fusion of the viral envelope with the cell membrane, then uncoating of the viral particles within the endosomes following penetration of H^+ ions, through the M2 matrix ion channel, into the interior of the virions, and transcription of the viral (-)RNA genome to messenger (+)RNA, through the polymerase complex (PA, PB1, and PB2), translation of mRNA to viral proteins, then packaging and budding of progeny virus particles from the cell surface resulting in the release of these progeny virions, and allowing further spread of the virus infection(**Figure 3**).[10-12]

Drugs, such as oseltamivir (Tamiflu) is neuraminidase inhibiter, and amantadine drugs is preventing infection by block M2 ion channel.

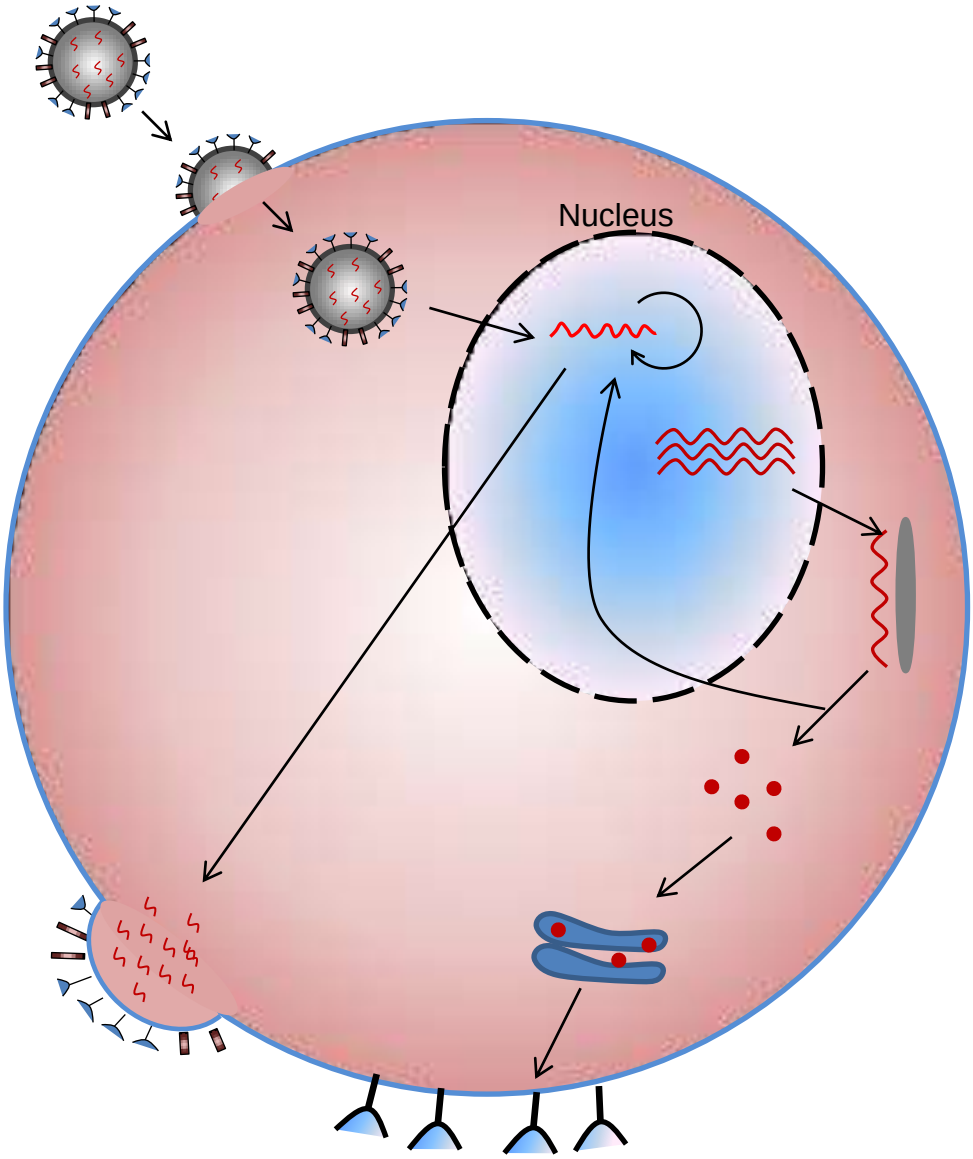

Figure 3. Influenza virus replication cycle

1.5. Polysaccharides in the virus treatment research

In last several decades, naturally occurring polysaccharides and oligosaccharides are getting a high attention because of their specific biological activities.

The first report on the natural polysaccharides having anti-virus activity in 1958, by Paul Gerber,[13] inhibited effect for infection of influenza B virus or mumps virus, which was the polysaccharide extracted from seaweeds, composition is mainly a linear polysaccharide of D - galactose units linked 1→3, with a small number of sulfate groups.[14] Inhibitory effects of polysaccharide on the herpes simplex virus (HSV) were also reported in 1964.[15,16] Sulfated polysaccharides have been recognized as having potent *in vitro* anti-HIV activity since the late 1980s. Dextran sulfate (**Figure 4a**) and heparin were investigated by Baba M. et al. for their antiviral effect on the human immunodeficiency virus (HIV) *in vitro*[17], In 1987. And at the same year, H. Nakashima et al. reported that a sea algal extract from *Schizymenia pacifica* , which was a sulfated polysaccharide, consist of galactose (73%), 3,6-anhydrogalactose (0.65%), and sulfonate (20%), inhibited the reverse transcriptase (RT) of avian myeloblastosis virus and HIV *in vitro*.[18]

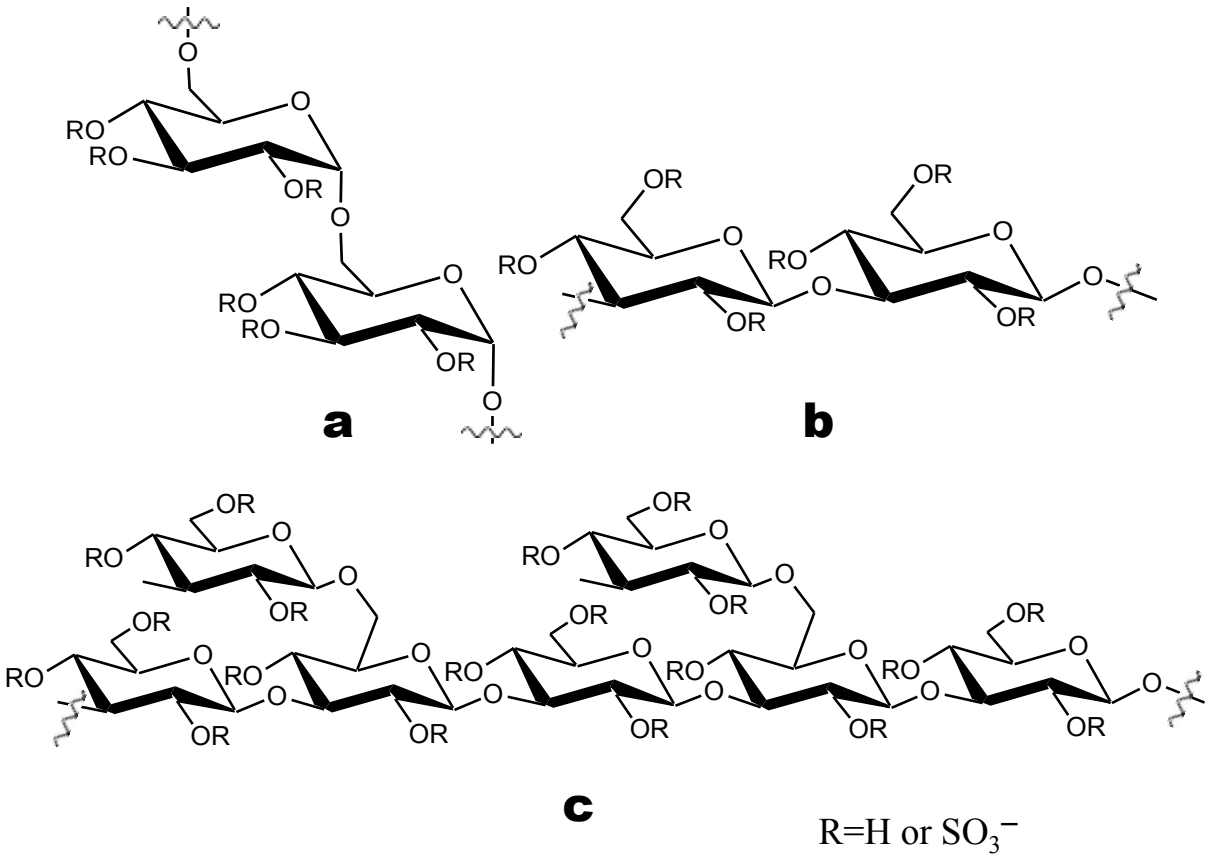

Figure 4. Structure of dextran sulfate (**a**), curdlan sulfate (**b**), and lentinan sulfate (**c**).

Also, having a large number of natural occurring polysaccharides reported to have biological activities of anti-coagulant and anti-virus. However, natural polysaccharides have complex structure due to their heterogeneity including many kinds of monosaccharide unit, it is difficult to elucidate the relationship between structures and biological activities. Therefore, we have presented many papers on the synthesis of polysaccharides having defined structures by the ring-opening polymerization of anhydrosugar derivatives and examined the structure and biological activity,[19,20] It was found that synthetic sulfated polysaccharides from D-ribose, D-xylose, and D-glucose monomers completely inhibited the infection of MT-4 cells by HIV at the concentration of 3.3 μg/ml and had low cytotoxicity.[21] In the beginning of the development of anti-HIV polysaccharides, lentinan,it is a naturally occurring polysaccharide in mushroom, from *L.edodes Sing.* and with a branched (1→3)-β-D-glucopyranosidic structure and immunomodulating activity, was sulfated to prepare sulfated lentinan (**Figure 4c**), which also had potent anti-HIV activity at concentrations as low as 3.3μg/ml.[22] Especially, in later, we was revealed that curdlan sulfate (**Figure 4b**), which was sulfated derivative of curdlan, a naturally occurring polysaccharide isolated from two types of bacteria, *Agrobacterium biobar* and *Agrobacterium radiobactor*, with a linear 1, 3-β-linked glucopyranosidic structure, when it was with a high sulfur content of 14.4% also completely inhibited the infection of MT-4 cells by HIV at concentrations as low as 3.3 μg/ml.[23]

1.6 Summary of this thesis

In this work, To develop a biomaterial with an influenza virus-adsorptive functionality, an alkyl curdlan sulfate was prepared by ionic interaction between a positively charged didodecyldimethyl ammonium bromide and a negatively charged sulfate group of curdlan sulfate, which has potent anti-HIV activity, and then coated on a membrane filter with a 1μm pore size by hydrophobic interaction with the long alkyl groups in the curdlan sulfate. However, It was found that the membrane filter (3 sheets) coated with 1.6 mg of alkyl curdlan sulfate selectively removed influenza A viruses.

Secondly, the structure of naturally occurring galactomannans was characterized by high resolution NMR spectroscopy involving two-dimensional (2D) NMR measurements of the field gradient DQF-COSY, HMQC, HMBC, and ROESY experiments. Appearance of galactose side chain GH1-O-GC4 and GH1-O-MC6 correlation signals in HMBC spectrum and the coupling constant $J_{GH1,\ 2}$ = 3.4 Hz suggested that at least two galactose residues were glycosylated by a α-linkage from the C6 position of main chain mannose. This result was supported by the NOE correlation (ROESY) signals. Main chain mannose should have β-linkage because of the appearance of MH1-O-MH4 NOE correlation signal between two mannose residues and decrease of specific rotations with decreasing proportion of galactose side chains.

Finally, The specific biological activities such as blood anticoagulant and anti-HIV activities of the galactomannans after sulfation, with different proportions of galactose (G) and mannose (M), from fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust been gum (LG), were investigated. These sulfated galactomannans exhibited potent anti-HIV activity as high as that of dextran sulfate and curdlan sulfate, represented by the 50% protecting concentration (EC_{50}) in the range of 1.14-2.86 μg/ml. In addition, the sulfated galactomannans had high blood anticoagulant activity in the range of 29.4-71.0 unit/mg, which values were higher than that of standard dextran sulfate (22.7 unit/mg) and curdlan sulfate (10 unit/mg). It was presumed that sulfated branched polysaccharides were interacted strongly with surface glycoproteins of HIV and blood anticoagulant proteins, because of their flexible branched structures.

Reference

[1] Centers for Disease Control. Pneumocystis pneumonia—Los Angeles. *MMWR Morb. Mortal. Wkly. Rep.* 1981, *30*, 250–252

[2] Gallo, R. C.; Sarin, P. S.; Gelmann, E. P.; Robert-Guroff, M.; Richardson, E.; Kalyanaraman, V. S.; Mann, D.; Sidhu, G. D.; Stahl, R. E.; Zolla-Pazner, S.; Leibowitch, J.; and Popovic, M. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* 1983, *220*, 865–867

[3] Barre-Sinoussi, F.; Chermann, J. C.; Rey, F.; Nugeyre, M. T.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Vezinet- Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983, ***220***, 868–871

[4] Basavapathruni, A; Anderson, KS. Reverse transcription of the HIV-1 pandemic. *The FASEB Journal* 2007, *21*, 3795–3808

[5] Chan, D; Kim, P. HIV entry and its inhibition. *Cell* 1998, *93*, 681–684.

[6] Wyatt, R.; Sodroski, J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 1998, *280*, 1884–1888.

[7] Marie-Pierre de Béthune. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989–2009). *Antiviral Research*, 2010, *85*, 75-90.

[8] De Clercq, E. Anti-HIV drugs: 25 compounds approved within 25

years after the discovery of HIV. *Int. J. Antimicrob. Agents* 2009, *33*, 307–320.

[9] Korte, T.; Ludwig, K.; Huang, Q.; Rachakonda, PS.; Herrmann, A. Conformational change of influenza virus hemagglutinin is sensitive to ionic concentration. *Eur. Biophys. J.* 2007, *36*, 327–335.

[10] Bouvier, NM.; Palese, P. The biology of influenza viruses. *Vaccine* 2008, *26*, 49–53

[11] Lagoja, IM.; De Clercq E. Anti-influenza virus agents: synthesis and mode of action. *Med. Res. Rev.* 2008, *28*, 1-38.

[12] Palese P. Influenza: old and new threats. *Nat. Med.* 2004, ***10***, S82-87.

[13] Gerber, P.; Dutcher, JD.; Adams, EV.; Sherman, JH. Protective effect of seaweed extract for chicken embryos infected with influenza B or rnurnps virus. *Proc. Soc. Erp. Biol. Med.* 1958, *99*, 590-593.

[14] T. Mori, *Seaweed Polysaccharide,* Adv. Carbohydr. Chem. 1953, *8*, 315-350.

[15] Nahmias, AJ.; Kibrick, S.; Bernfeld, P. Effect of synthetic and biological polyanions on herpes simplex virus. *Proc. SOC . Exptl. Biol. Med.* 1964, *115*, 993-996.

[16] TAKEMOTO, KK.; P. FABISCH. Inhibition of herpes virus by natural and synthetic acid polysaccharides. *Proc. Soc. Exptl. Biol. Med.* 1964, *116*, 140-144.

[17] Ito M.; Baba M.; Sato A.; Pauwels R.; De Clercq E.; Shigeta S. Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) in vitro. *Antiviral*

Res.1987, *7*, 361-367.

[18] Nakashima, H.; Kido, Y.; Kobayashi, N.; Motoki, Y.; Neushul, M.; Yamamoto, N.; Purification and characterization of an avian myeloblastosis and human immunodeficiency virus reverse transcriptase inhibitor, sulfated polysaccharides extracted from sea algae. Antimicrob. Agents Chemother. 1987, *31*, 1524-1528.

[19] Yoshida, T. Synthesis of polysaccharides having specific biological activities. *Prog. Polym. Sci.* 2001, *26*, 379–441.

[20] Uryu, T. Artificial polysaccharides and their biological activities, *Prog. Polym. Sci.* 1993, *18*, 717-761

[21] Yoshida, T. Synthetic and natural polysaccharides having specific biological activities; in Polysaccharides, Structural diversity and functional versatility 2nd ed.; Dumitriu, S., Ed.; Marcel Dekker, New York; 2005.

[22] Yoshida, O.; Nakashima, H.; Yoshida, T.; Kaneko, Y.; Yamamoto, I.; Matsuzaki, K.; Uryu, T.; Yamamoto, N. Sulfation of the immunomodulating polysaccharide lentinan: A novel strategy for antiviral to human immunodeficiency virus (HIV); Biochem Pharmacol 1988, *37*, 2887 - 2891.

[23] Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Yasuda, N.; Mimura, T.;Yoshida, O.; Yamamoto, N. Synthesis and structural analysis of curdlan sulfate with potent anti-AIDS virus activity; Macromolecules 1990, *23*, 3717 - 3722.

Chapter 2

Synthesis and specific influenza A virus-adsorptive functionality of alkyl curdlan sulfate-coated membrane filter

---

ABSTRACT.

To develop a biomaterial with an influenza virus-adsorptive functionality, an alkyl curdlan sulfate was prepared by ionic interaction between a positively charged didodecyldimethyl ammonium bromide and a negatively charged sulfate group of curdlan sulfate, which has potent anti-HIV activity, and then coated on a membrane filter with a 1μm pore size by hydrophobic interaction with the long alkyl groups in the curdlan sulfate. The alkyl curdlan sulfate with the degree of alkylation (DOA) of 0.03 (1 didodecyldimethyl group/12 sugar residues with 36 hydroxyl or sulfate groups) showed potent anti-HIV activity in a 50% effective concentration (EC_{50}) as low as 0.87 μg/ml (standard curdlan sulfate EC_{50} = 0.3 μg/ml), and the activity decreased with increasing DOA. A DOA higher than 0.1 (1 didodecyldimethyl group/three sugar residues with nine hydroxyl or sulfate groups) gave no anti-HIV activity. Although both curdlan sulfate and alkyl curdlan sulfates did not inhibited infection of MDCK cells by influenza viruses, the alkyl curdlan sulfate-coated

membrane filter was found to have a specific adsorptive functionality for influenza A virus in vitro. When 1.6 mg of the alkyl curdlan sulfate with the DOA of 0.03 was coated on a membrane filter (13 mmØ, pore size: 1μm), three stacked alkyl curdlan sulfate-coated membrane filters dramatically decreased hemagglutination to 1/4 - 1/32. However, the membrane filter did not effectively remove on influenza B viruses, and a membrane filter without alkyl curdlan sulfate was not effective against influenza viruses. These results can therefore be presumed to demonstrate that the alkyl curdlan sulfate-coated membrane filter removed influenza A viruses by adsorption between the negatively charged sulfate groups and the positively charged envelope protein of the virus.

KEYWORDS: curdlan sulfate; membrane filter; adsorptive functionality; influenza A virus

2.1. Introduction

Sulfated polysaccharides have specific biological activities such as blood anticoagulant and anti-HIV activities.[1] We have studied on the synthesis of stereoregular polysaccharides by the ring-opening polymerization of anhydro sugar monomers to elucidate the relationship between the structures of polysaccharides and their biological activities.[2] It was found that synthetic sulfated polysaccharides from D-ribose, D-xylose, and D-glucose monomers completely inhibited the infection of MT-4 cells by HIV at the concentration of 3.3 μg/ml and had low

cytotoxicity.[3] In the beginning of the development of anti-HIV polysaccharides, a naturally occurring polysaccharide, lentinan with a branched (1→3)- β-D-glucopyranosidic structure and immunomodulating activity, was sulfated to prepare sulfated lentinan, which also had potent anti-HIV activity at concentrations as low as 3.3 μg/ml.[4] Later, it was revealed that curdlan sulfate, which was prepared by sulfation of the naturally occurring polysaccharide curdlan with a linear 1, 3-β-linked glucopyranosidic structure, also completely inhibited the infection of MT-4 cells by HIV at concentrations as low as 3.3 μg/ml.[5] In general, sulfated polysaccharides like heparin have high blood anticoagulant activity, [6] making them unsuitable for AIDS treatment. However, curdlan sulfate has low blood anticoagulant activity (10 unit/mg) and low cytotoxicity.

To elucidate action mechanism of the anti-HIV activity in curdlan sulfate, Uryu et al synthesized the oligopeptide in the envelope of HIV, glycoprotein (gp) 120, which is the helical region consisting of residues no. 506 to no. 518 at the C terminus Observations of the interaction of the oligopeptide and curdlan sulfate by NMR spectroscopy[7, 8] revealed chemical shifts and broadening of the oligopeptide and curdlan sulfate signals. Kozbor and coworkers investigated the role of the V2, V3, and CD-4 binding domains of gp120 on HIV, and indicated that the V3 domain appeared to determine the extent of the interaction of curdlan sulfate with the V2 and CD-4 regions in the gp120.[9] They considered that the electrostatic interaction of curdlan sulfate and the V3 domain occurred

because basic amino acid residues such as lysine and arginine existed in the V3 domain of gp120.

In 1958, Gerber showed the inhibitory effects of agar, which contained sulfated polysaccharides, from seaweeds in chicken embryos on infection with influenza B virus.[10] They also reported that the polysaccharides had no effect on the multiplication of influenza A virus. Recently, Santos reported the inhibitory effect of dextran sulfate on Madin-Darby canine kidney (MDCK [a]) cells infected with the Influenza virus (A/Puerto Rico (H1N1) strain).[11] Although high molecular weight dextran sulfate (500kDa) was inhibited the virus fusion at a concentration as low as 5 μg/ml at pH 5.0, low molecular weight dextran sulfate (5kDa) had no effects. Several reports on the purification and concentration of influenza viruses have been published. Sulfated cellulose membranes were used to purify the influenza virus from the MDCK cell culture supernatant for vaccine production.[12] Ten stacked sheets of sulfated cellulose membrane exhibited influenza virus adsorption capacities of 13 μg HA/cm^2 at a loading velocity of 15 ml/min. The Influenza A virus in the cell culture solution was isolated effectively by size-exclusion and anion-exchange chromato- gramphic purification, and proteins and host cell DNA were also removed.[13, 14]

The purpose of this work was to synthesize an alkyl curdlan sulfate-coated membrane filter with influenza virus-adsorptive functionality. The alkyl curdlan sulfate was prepared by ionic interaction of

didodecyldimethyl ammonium bromide and curdlan sulfate, which has with potent anti-HIV activity. In addition, the preparation of alkyl curdlan sulfate-coated membrane filters for the development of the biomaterials and the ability of the membrane filters to remove influenza viruses are described. The structure of alkyl curdlan sulfate was determined by high resolution NMR and FT-IR measurements. The surface of the alkyl curdlan sulfate- coated membrane filter was observed by SEM and attenuated total reflection infrared spectroscopy (ATR/FT-IR) measurements.

2.2. Experimental Section

2.2.1. Materials

Curdlan sulfate with the molecular weight of $\overline{M}_n$ = 15.9 X 10^4 and S content of 12.87% was used. It has anti-HIV activity at the EC_{50} of 0.3 μg/ml. Commercially available didodecyldimethylammonium bromide (DDAB) was used. Nitrocellulose membrane filters with the pore size of 1 μm and diameter of 13 mm were purchased from Toyo Roshi Kaisha, Ltd. Influenza viruses, A/Puerto Rico (H1N1), A/Brisbane (H1N1), A/Yamagata(H1N1), B/Florida(H1N1), and B/Hong Kong (H1N1), were purchased from Denka Seiken Co, Ltd.

2.2.2. Measurements

The 400 MHz ^{1}H and 100 MHz ^{13}C NMR spectra were obtained using a JEOL ECM-400 spectrometer in D_2O at 40°C with 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS) as an internal standard. The specific rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at 25°C in a water-jacketed 10 ml quartz cell. The attenuated total reflection infrared (ATR/FT-IR) spectrum was recorded using a Perkin Elmer Spectrum One FT-IR spectrometer. Molecular weights of polymers were determined by an aqueous phase GPC equipped with TOSOH TSK-gel columns (7.6 mm X 600 mm X 3) of G2500PW_{XL}, G3000PW_{XL}, and G4000PW_{XL} eluted with 66.7 mM phosphate buffer solution using standard pullulan (Shodex standard P-82) as a reference at 40 °C. The UV (UV-8020) and RI (RI-8021) detectors were used. Scanning electron microscopy (SEM) was performed using a JEOL JSM-5800 at accelerated voltage of 20 keV. The weight of membrane filters was measured using a Mettler Toledo XS3DU microbalance. The degree of alkylation was calculated from the ^{1}H NMR by comparison with the intensities of the terminal methyl group and H1 proton due to the didodecyl group and glucose residue of curdlan sulfate, respectively.

2.2.3. Alkyl curdlan sulfate

Curdlan sulfate was dissolved in 20 ml of ion exchange water, and then didodecyldimethylammonium bromide (DDAB, 0.05g) in 10 ml of methanol was added dropwise at room temperature. The mixture was

stirred for 30min. After dialyzation against deionized water for 24 h, the residual water in the dialyzed tube was filtered and then freeze-dried to give an alkyl curdlan sulfate with the degree of alkylation (DOA) of 0.03 (1 didodecyldimethyl group/12 sugar residues with 36 hydroxyl or sulfate groups) at a yield of 0.50 g. The DOA was calculated from the ^{1}H NMR spectroscopy by comparisons of the intensities of the methyl signal and H1 proton due to the didodecyl group and curdlan sulfate, respectively.

2.2.4. Anti-HIV activity of alkyl curdlan sulfate[15]

The anti-HIV activity of alkyl curdlan sulfate was assayed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium) method. Human T4-positive cells carrying human T-lymphotropic virus type 1 (3.0 X 10^4/well, MOI: 0.01) (MT-4 cells) were infected with HIV-1$_{HTLV-IIIB}$ at the multiplicity of 0.01, and HIV-1 and mock-infected MT-4 cells were incubated in the presence of various concentrations of alkyl curdlan sulfate for 5 days at 37°C in a CO_2 incubator. The viability of both HIV-1- and mock-infected cells was assayed spectrophotometrically by the reduction of MTT. The anti-HIV activity was represented by the EC_{50}, which is defined as the concentration of the test compound that decreased the HIV-induced cytopathic effect 50%. The CC_{50} was determined as the concentration of the test compound that was cytotoxic on 50% of MT-4 cells.

2.2.5. Alkyl curdlan sulfate-coated membrane filter

The alkyl curdlan sulfate-coated membrane filter was prepared as follows. Before preparation, the weight of the nitrocellulose membrane filter was measured precisely by a Metter microbalance instrument. The average weight of 10 filters was used. A 1% aqueous solution (2 ml) of alkyl curdlan sulfate with a DOA of 0.03 was passed slowly through the nitrocellulose membrane filter in the holder by a syringe and dried overnight under vacuum below 40 °C to give an alkyl curdlan sulfate-coated membrane. The amount of fixed curdlan sulfate on the membrane filter was determined by weighting to be around 1.61 mg. The results are shown in Table 2.

2.2.6. Hemagglutination inhibition assay (HA) of alkyl curdlan sulfate-coated membrane filter[16]

A typical procedure for the assay is as follows. The two-fold dilution method was used. Three of the alkyl curdlan sulfate coated membrane filters were put on a 24-well plate, and then three defined dilution of virus stocks with various concentrations of A/Yamagata virus in phosphate-buffered saline (PBS, 500 µl) were added to the plate. The plate was shaken constantly for 30 sec by a plate mixer. The plate was allowed to stand for 5 min at room temperature, and 50 µl was removed from each

fraction; the solution (50 µl) was added to a solution of chicken erythrocytes in PBS, and then HA was performed to determine whether the erythrocytes were precipitated or not. The basic HA value of A/Yamagata used here was 128. The results are shown in Table 3.

2.3. Results and Discussion

2.3.1. Anti-influenza virus activity of curdlan sulfate

Among the sulfated polysaccharides we have synthesized so far, a curdlan sulfate prepared by sulfation of a naturally-occurring polysaccharide, curdlan, with a linear 1, 3-β glucopyranosidic structure was found to have potent anti-HIV activity and low cytotoxicity. A phase I/II clinical test of curdlan sulfate has been carried out in the United States.[17] Like HIV, influenza virus has an envelope protein; thus, we selected curdlan sulfate as a sulfated polysaccharide to investigate the anti-influenza virus activity using the hemagglutination inhibition assay published by WHO.[18] Influenza A virus/Puerto Rico (H1N1) was inoculated into MDCK cells in the presence or absence of curdlan sulfate (225 and 45 µg/ml) and then the mixture was incubated with chicken erythrocytes in 96-well plates. Tamiflu (225 or 45µg/ml), which is a specific remedy for influenza, was used as a reference agent. After 1h, agglutination of the erythrocytes was observed in the wells containing Tamiflu, revealing that Tamiflue prevented the infection of the MDCK

cells by the influenza virus. However, curdlan sulfate did not effectively prevent infection by the influenza virus because no agglutination of the erythrocytes occurred. Neither did other sulfated and acidic polysaccharides, dextran sulfate, heparin, and sodium hyaluronate, coagulate the erythrocytes. These results suggest that sulfated polysaccharides have no anti-influenza virus activity.

As described in the introduction section, because sulfated polysaccharides have preventive effects against HIV infection, we expected sulfated polysaccharides to have an influenza virus-adsorptive functionality due to the electrostatic interaction because influenza virus also has the envelope protein hemagglutinin.

2.3.2. Synthesis and anti-HIV activity of alkyl curdlan sulfate

For development of influenza virus-adsorptive biomaterials, we prepared an alkyl curdlan sulfate-coated membrane filter, and the adsorptive functionality was examined. The synthetic scheme is shown in Scheme 1. Curdlan sulfate with $\overline{M}_n$ = 15.9 x 10^4 and S = 12.87%

Scheme 1. Synthesis of alkyl curdlan sulfate. Didodecyldimethyl group was introduced into curdlan sulfate (S = 12.87%, $\overline{M}_n$ = 15.9 x 10^4) by ionic interaction.

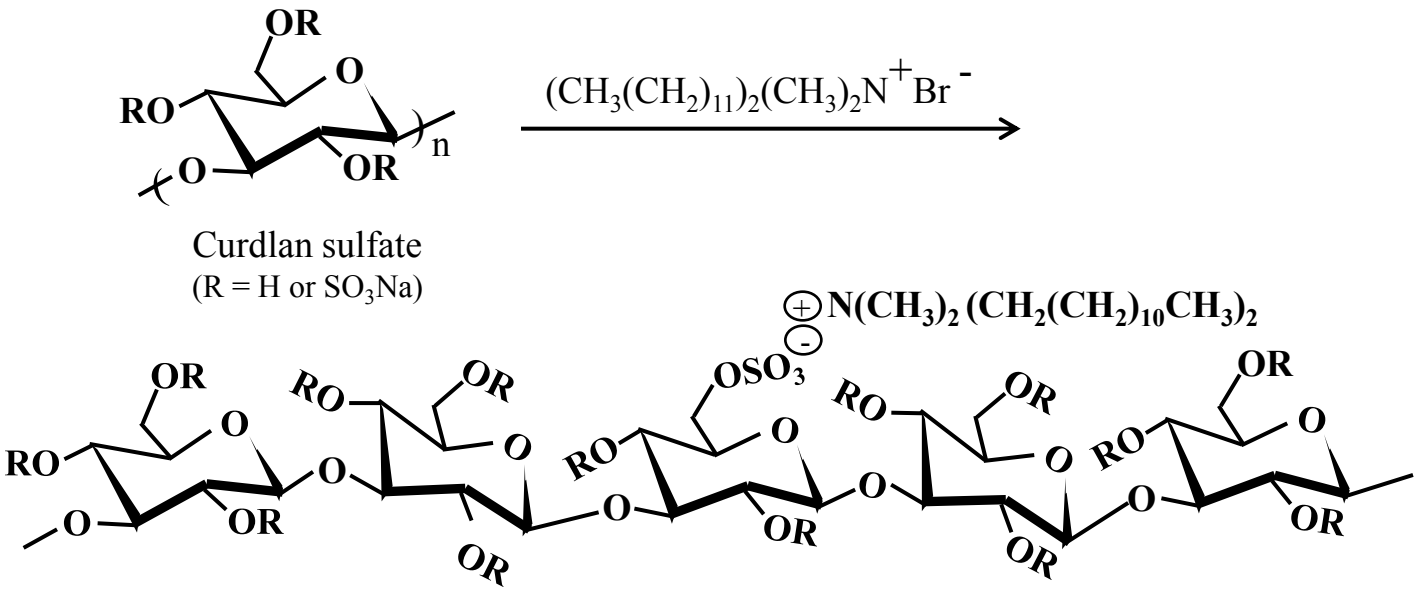

Table 1. Preparation of alkyl curdlan sulfate and their anti-HIV activity

			Alkyl curdlan sulfate		
No	CS[a]	DDAB[a]	Yield	Degree of alkylation	Anti-HIV activity[b]
g	g	g		(Alkyl group/Sugar unit)	EC_{50}, μg/ml
1	0.5	0.05	0.50	0.03 (1/12)	0.87
2	0.5	0.1	0.52	0.06 (1/6)	1.44
3	0.5	0.2	0.57	0.1 (1/3)	>1000
4	0.5	0.5	0.82	0.5 (3/2)	>1000

[a]CS: Curdlan sulfate with S content of 12.87%. DDAB: Didodecyldimethylammonium bromide. [b]Anti-HIV activity was determined by the comparison with that of standard curdlan sulfate with the EC_{50} = 0.3 μg/ml.

in water was added to the methanol solution of didodecyldimethyl ammonium bromide (DDAB) to give alkyl curdlan sulfates with a variety of different proportions of didodecyldimethyl groups, which are listed in Table 1. When 0.05g (0.11 mmol) of DDAB and 0.5g of curdlan sulfate were used, an alkyl curdlan sulfate with the DOA of 0.03 was obtained (No.1). The DOA increased with increases in the amount of DDAB. A large amount of DDAB (0.5 g) gave an alkyl curdlan sulfate with a large proportion of didodecyldimethyl groups (No. 4).

The anti-HIV activity of the alkyl curdlan sulfates obtained was evaluated, and the results are shown in Table 1. The alkyl curdlan sulfate with the DOA of 0.03 was found to have potent anti-HIV activity at concentrations as low as the EC_{50} = 0.87 μg/ml. However, the activity decreased with an increase in DOA. The alkyl curdlan sulfate with a DOA more than 0.1 (1 didodecyldimethyl group/3 sugar residues with 9 hydroxyl or sulfate groups) had scarcely any anti-HIV activity, and the EC_{50} = 1000 μg/ml. Therefore, we used the alkyl curdlan sulfate with the DOA of 0.03 for this work.

2.3.3. Structure of alkyl curdlan sulfate

The structure of alkyl curdlan sulfate was analyzed by a combination of 1D and 2D NMR measurements including H-H COSY and HMQC experiments. Figure 1 shows the ^1H NMR spectrum of alkyl curdlan

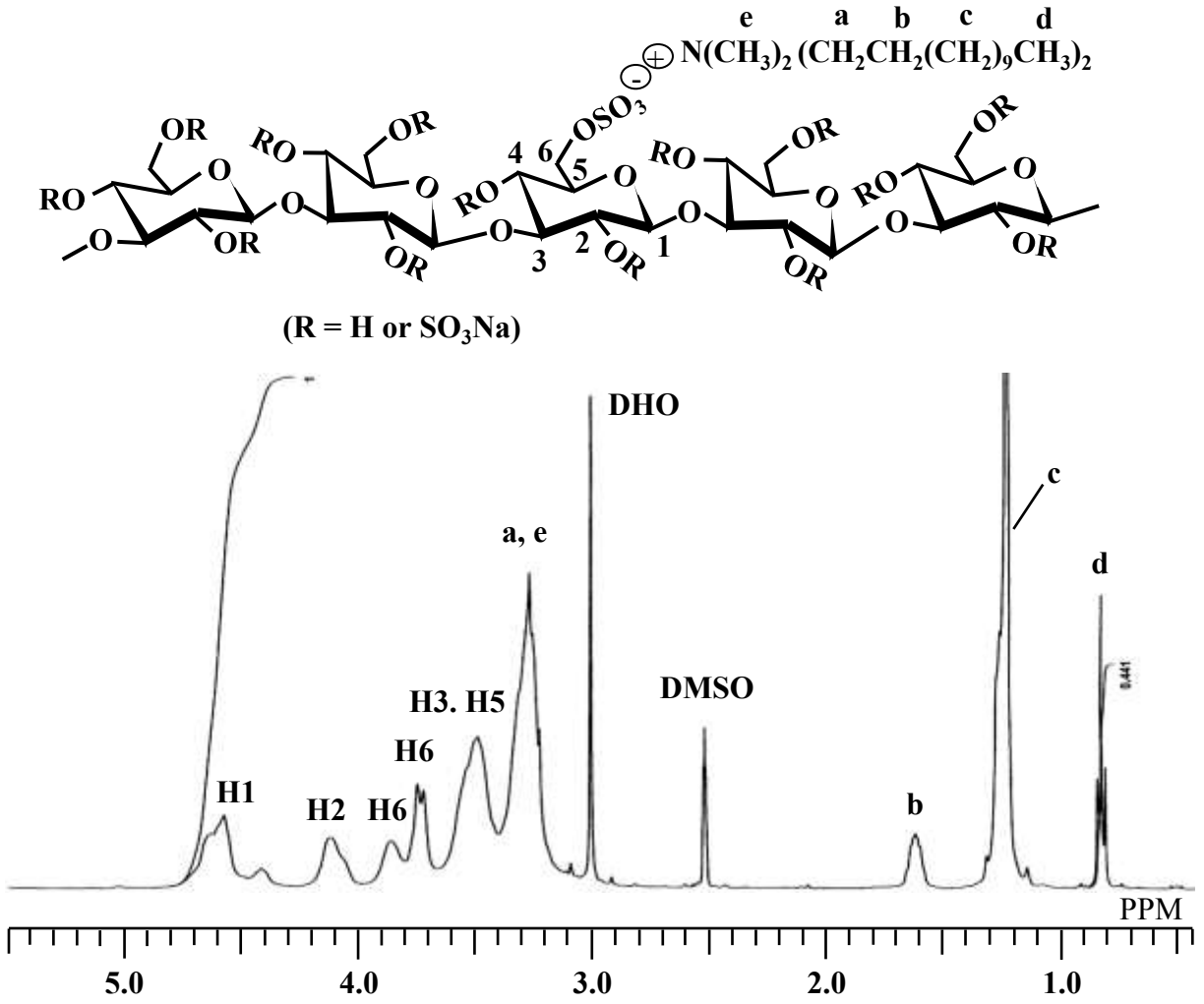

Figure 1. 400 MHz ^{1}H NMR spectrum of alkyl curdlan sulfate with DOA of 0.03 (1 didodecyldimethyl group/12 sugar residues with 36 hydroxyl or sulfate groups) (DMSO-d_6, 40 ℃).

sulfate with the DOA of 0.03. The two methyl signals (d) at the terminal of the didodecyl group appeared at 0.8 ppm as a triplet signal. The two methylene (a) and methyl (e) signals shifted downfield around 3.25 ppm as a broad peak because these groups were connected directly to the cationic ammonium N atom. The H1 proton of the glucose residue in curdlan sulfate appeared independently at 4.6 ppm. Therefore, the DOA was calculated by the peak intensities of the H1 proton at 4.6 ppm and methyl signal (d) at 0.8 ppm.

Figure 2 shows the ^{13}C NMR spectra of the original curdlan sulfate (A) and alkyl curdlan sulfates (B-D) with DOA of 0.03, 0.06, and 0.1,

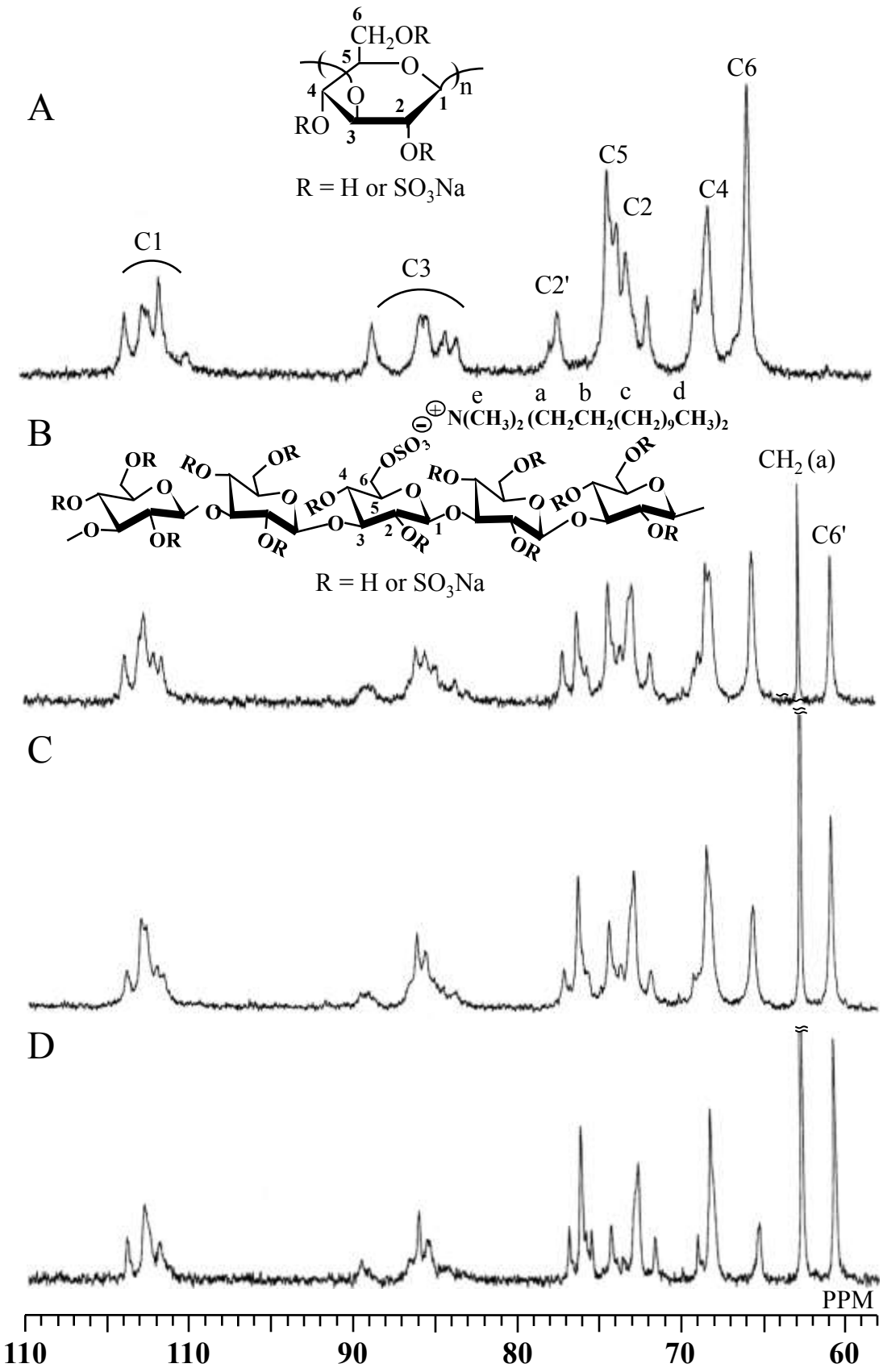

Figure 2. 100 MHz ^{13}C NMR spectra of curdlan sulfate (A) and alkyl curdlan sulfates (B~C) with the degree of alkylation of 0.03, 0.06, and 0.1 per glucose residue, respectively, (DMSO-d_6 at 40 ℃).

respectively. With the increasing proportion of the didodecyldimethyl group, the intensity of the C6 signal at 66 ppm (2A) decreased gradually and a new signal appeared at 60.5 ppm due to the C6 carbon substituted in the didodecyldimethyl group (2B - 2D). The intensities of the C6 signal at 60.5 ppm and methylene (a) signal in the didodecyl group at 62.5 ppm increased with the increasing proportion of the didodecyl group, but the other signals did not shift as much, suggesting that didodecyldimethyl groups were mainly introduced at the C6 sulfate groups of curdlan sulfate by ionic interaction.

2.3.4. Preparation of alkyl curdlan sulfate-coated membrane filter

An alkyl curdlan sulfate-coated membrane filter was prepared by passing a 1% aqueous alkyl curdlan sulfate solution with the DOA of 0.03 through commercially available nitrocellulose membrane filters (13 mmØ) with the pore size of 1 μm by means of a syringe. Table 2 presents the results for the preparation of the alkyl curdlan sulfate-coated membrane

Table 2. Preparation of alkyl curdlan sulfate coated membrane filter[a]

C[b] wt%	Weight of membrane filter[c] Before mg	Weight of membrane filter[c] After mg	Increased weight[d] mg
0.0	5.16±0.00	5.03±0.02	-0.13±0.02
0.5	5.22±0.00	5.72±0.08	0.50±0.08
1.0	5.24±0.00	6.85±0.16	1.61±0.16

[a] Commercially available nitrocellulose membrane filter with pore size of 1 μm and diameter 13 mm and alkyl curdlan sulfate with the DOA of 0.03 were used. Two ml solution of alkyl curdlan sulfate in deionized water was passed through the filter. [b] Concentration of alkyl curdlan sulfate in deionized water. [c] Average weight of 10 membrane filters after drying overnight under vacuum at 40 °C. [d] Weight of alkyl curdlan sulfate on the membrane filter.

filter. When 0.5% and 1% solution of the alkyl curdlan sulfate were passed through the filter, the weights of the filter were increased to 0.50 and 1.61 mg, respectively, after drying, indicating that alkyl curdlan sulfate was fixed on the surface by hydrophobic interaction between the long alkyl groups and the surface of the membrane filter. The alkyl curdlan sulfate was not removed from the filter by washing with water because no alkyl curdlan sulfate was detected in the concentrated wash water by HPLC with high sensitive UV and RI detectors.

Figure 3 shows the ATR/FT-IR spectra of nitrocellulose membrane filter (A) before and (B) after coating with the alkyl curdlan sulfate with

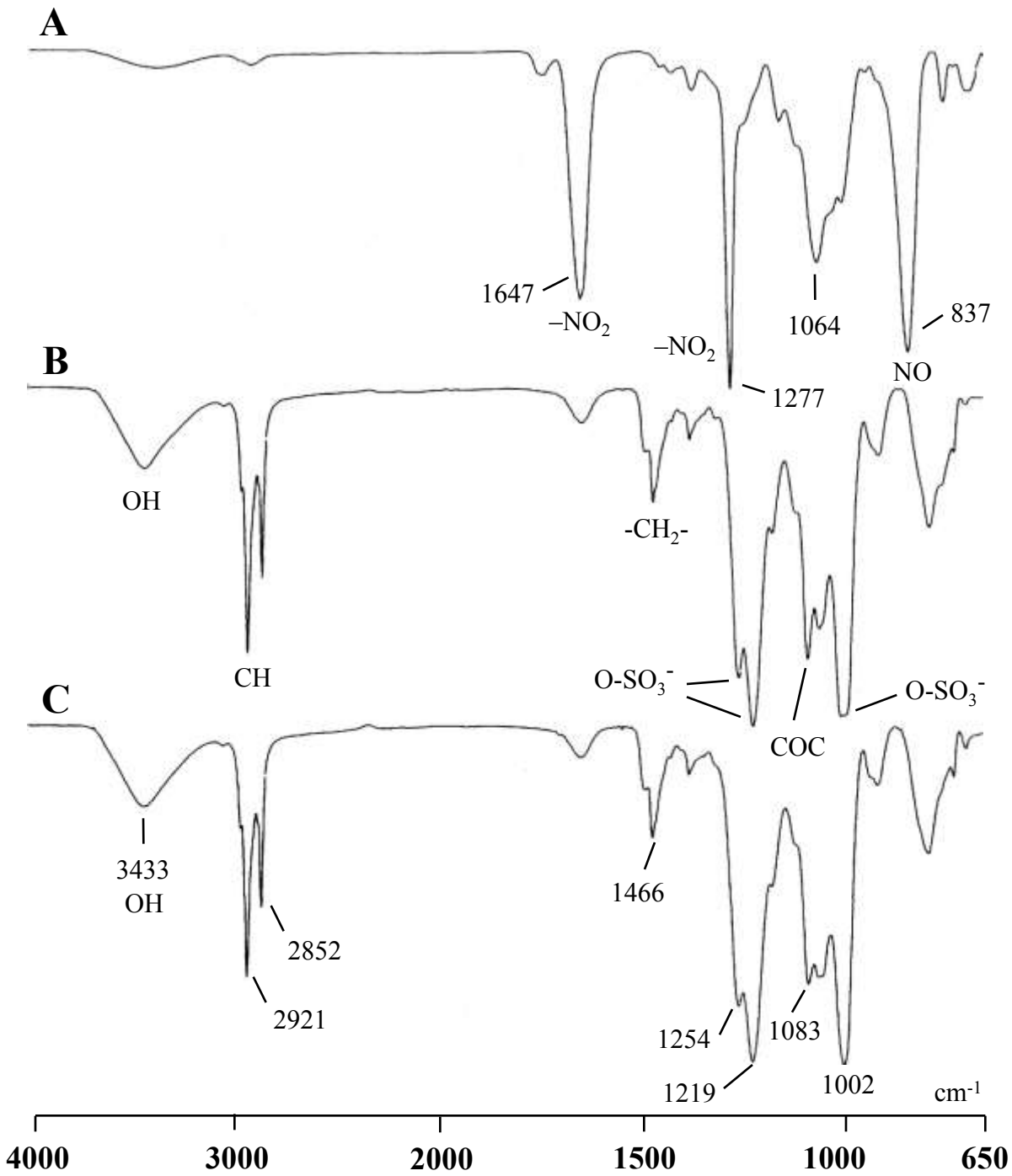

Figure 3. ATR/FT-IR spectra of (A) commercially available nitrocellulose membrane filter, (B) alkyl curdlan sulfate (DOA of 0.03) coated membrane filter, and (C) alkyl curdlan sulfate with DOA of 0.03, respectively.

the DOA of 0.03. In the spectrum in Figure 3 (A), the nitrocellulose membrane filter has characteristic absorptions at 1647, 1277, and 837 cm^{-1} ascribed to -NO_2, -NO_2, and N=O stretching vibrations, respectively. After coating (Figure 3B), these characteristic absorptions disappeared and a broad absorption at 3433 cm^{-1} due to OH stretching and large peaks at 1254, 1219, and 1002 cm^{-1} due to –OSO_3^- stretching vibrations of sulfate groups appeared. In addition, the spectrum in Figure 3B was similar to that of the alkyl curdlan sulfate (Figure 3C). These results indicate that the alkyl curdlan sulfate successfully coated the membrane filter due to the hydrophobic interaction of the long alkyl groups. The SEM observations of the surface of the alkyl curdlan sulfate-coated membrane filter are shown in Figure 4, in which photos A and B are a commercially available

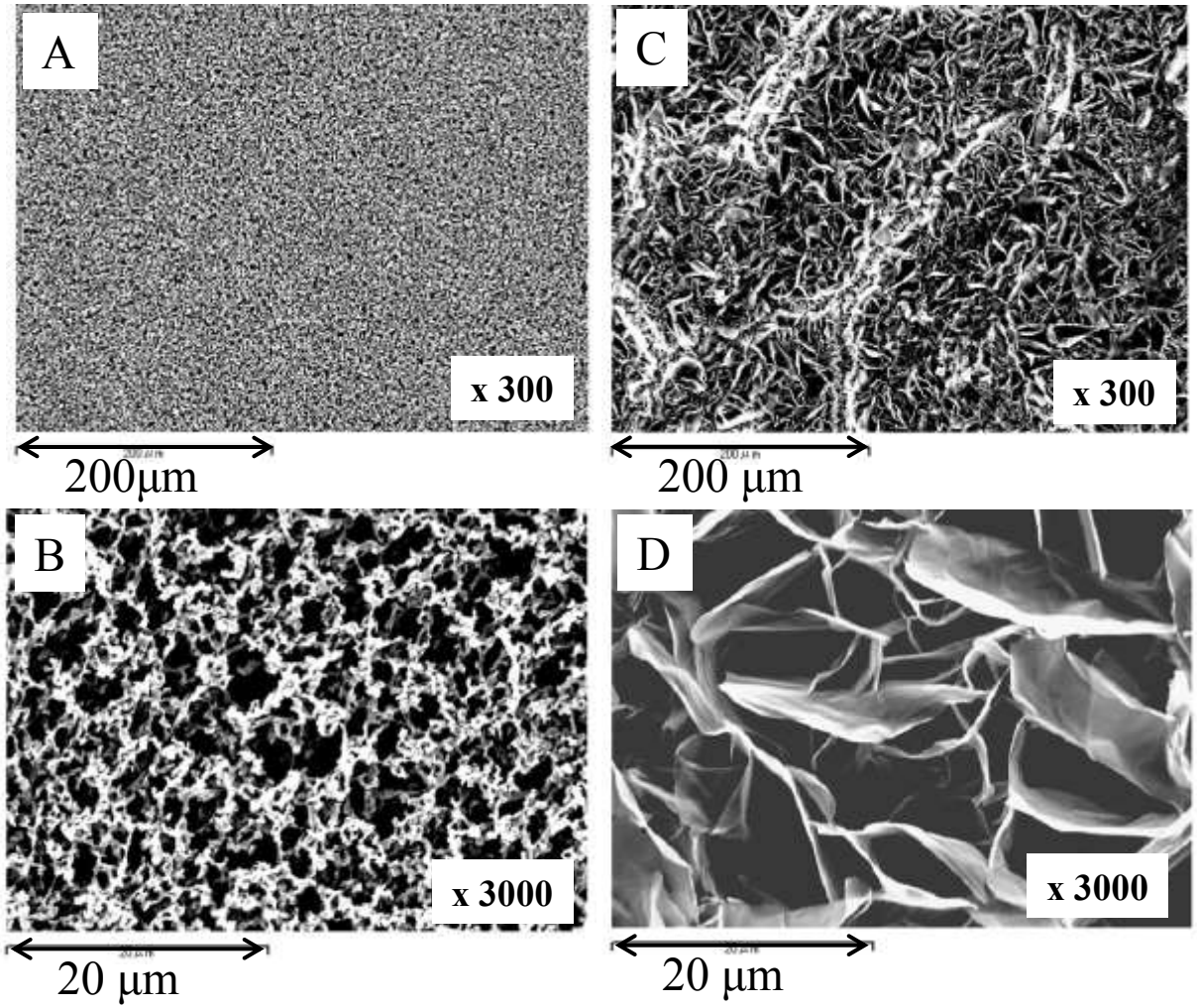

Figure 4. Typical SEM images of the surfaces of (A) and (B) commercially available nitrocellulose membrane filter (13 mm diameter) with pore size of 1 μm and of (C) and (D) alkyl curdlan sulfate coated membrane filter with the magnifications of 300 (A) and (C), and of 3000 (B) and (D), respectively. Alkyl curdlan sulfate with DOA of 0.03 was used.

nitrocellulose membrane filter and photos C and D are the alkyl curdlan sulfate-coated membrane filter. The SEM photos are taken at low (x 300) and high (x 3000) magnifications, respectively. After coating with the alkyl curdlan sulfate, micropores with a diameter around 10 μm were observed. Both SEM observations and ATR/FT-IR measurements demonstrated that the membrane filter was successfully coated by the alkyl curdlan sulfate.

2.3.5. Influenza hemagglutination inhibition assay (HA) of alkyl curdlan-sulfate coated membrane filter

The HA assay is one method to determine the level of influenza virus present in a sample.[18] Two-fold dilutions of virus solutions were prepared. The alkyl curdlan sulfate-coated membrane filter (1 or 3 sheets) was then placed in the wells. After 5 min, each solution (50 μl) was removed from the 24-well plate and transferred to another 24-well plastic plate, to which a specific amount of chicken erythrocytes was added. The results of HA was presented in Table 3. Influenza virus has an envelope glycoprotein, hemagglutinin, that binds to erythrocytes to form a lattice. If the influenza

Table 3. HA assay of alkyl curdlan sulfate coated membrane filter

No	Filter[b] sheet	ACS[c] mg	Dilution: Influenza virus A/Brisbane	A/Yamagata	A/Uruguay	A/PelutoRico	B/Florida	B/HongKong
1[a]	--	--	256	128	128	64	128	256
2	1	--	128	64	--	64	128	--
3	3	--	128	64	64	32	64	256
4	1	0.5	256	--	--	--	128	--
5	3	0.5	128	--	--	--	32	256
6	1	1.6	256	64	--	64	128	--
7	3	1.6	<64	<4	<16	<16	64	128
			(1/4)[d]	(1/32)[d]	(1/8)[d]	(1/4)[d]	(1/2)[d]	(1/2)[d]

[a] Base HA value of each virus. [b] The membrane filters coated with 0.5 mg and 1.6 mg of alkyl curdlan sulfate were used. [c] ACS: Alkyl curdlan sulfate on a sheet. [d] The ratio of decreasing HA value (No. 7) compared with the base value of each virus (No. 1).

virus is absent, a precipitate of erythrocytes appeared on the bottom of the well. The HA titer of the A or B type viruses was 128 or 256, which was the base value of this assay (No. 1). In Nos. 2 - 5, the viruses were not adsorbed very much on the membrane filters (1 or 3 sheets) without or with a small amount of alkyl curdlan sulfate (0.5 mg). In No. 6, one membrane filter coated with 1.6 mg of alkyl curdlan sulfate was not effective in decreasing the amount of A/Brisbane and B/Florida viruses. When three stacks of filters were used (No. 7), the HA value on the A type viruses decreased to 1/4 - 1/32. On the other hand, the membrane filter did not remove B type B/Florida and B/Hong Kong viruses very well. Suzuki and coworkers reported that bovine brain sulfatides or galactosylceramide (glycolipid) - coated nonwoven fabrics which was prepared in the emulsion of ethylene - vinyl acetate copolymer reduced the HA from 512 to 16 or 32, suggesting that the glycolipids on the nonwovens interacted specifically with henagglutinine on the surface protein of influenza viruses.[19] On the other hand, curdlan sulfate-coated filter had the similar inhibitory activity, however, the synthesis of curdlan sulfate and preparation of the filter without any organic solvents are easily and simple.

Although the diameter of viruses is 0.1 - 0.4 μm (100 - 400 nm) in general, the alkyl curdlan sulfate-coated membrane filter prepared here had larger pores (1 μm), as shown in Figure 4, than the diameter of the viruses, suggesting that the alkyl curdlan sulfate-coated membrane filter

adsorbed the influenza A viruses by electrostatic interaction between negatively charged sulfate groups and the positively charged envelope protein, hemagglutinin, on the viruses. For the B type influenza viruses, the alkyl curdlan sulfate-coated membrane filter did not work effectively. According to the influenza research database,[18] the envelope protein, hemagglutinin, of A type viruses has a larger amount of the basic amino acid residues with a positive charge compared to those of B type viruses. Therefore, we assumed that the alkyl curdlan sulfate-coated membrane filter selectively removed A type viruses. As well-known, sialic acids in cell surface glycoprotein play an important role in the carbohydrate - protein recognitions leading to infection of influenza viruses to host cells. Nishida and Kobayashi reported that *p*-nitrophenyl *N*-acetyl-6-*O*-sulfo-β-D-glucosamine (*p*NP GlcNHAc) had potent neuramidase inhibitory activity and showed both of the sulfate and NHAc groups were necessary for the inhibitory activity, indicating that the neuramidase recognized the absolute structure of *p*NP GlcNHAc as a competitor of *N*-acetylneuraminic acid.[20] In addition, Kovensky reviewed the carbohydrate - protein interactions mediated by highly specific protein sequences or by electrostatic interactions.[21] Therefore, we considered that curdlan sulfate might recognize specifically influenza viruses through the specific interaction with surface glycoproteins of the influenza viruses. The detailed adsorptive mechanism is under further investigation.

2.4. Conclusion

A long alkyl chain from didodecyldimethylammonium bromide was introduced into curdlan sulfate ($\overline{M}_n$ = 15.9 x 10^4 and S content of 12.87%) to give alkyl curdlan sulfates with various DOA due to the ionic interaction between the negative sulfate group and positive ammonium salt with a didodecyldimethyl group. The alkyl curdlan sulfate with the DOA of 0.03 had potent anti-HIV activity at concentrations as low as EC_{50} = 0.87 μg/ml. It was coated on a nitrocellulose membrane filter due to the hydrophobic interaction between the long alkyl groups and the surface of the filter to give an alkyl curdlan sulfate-coated membrane filter, which has pores with a diameter around 10 μm. It was found that the membrane filter (3 sheets) coated with 1.6 mg of alkyl curdlan sulfate selectively removed influenza A viruses. However, the membrane filter did not work effectively on influenza B viruses. The removal mechanism was assumed to due to the adsorption of influenza A viruses on the alkyl curdlan sulfate-coated membrane filter by the electrostatic interaction between negatively charged sulfate groups and a positively charged envelope protein, hemagglutinin, of the viruses. The elucidation of the adsorptive mechanism containing the specific interactions of curdlan sulfate and influenza viruses is now being investigated.

References and Notes

[1] Paulsen, B. S., *Bioactive Carbohydrate Polymers*; Ed.: Kluwer Academic Publishers: Dordrecht, 2000.

[2] Yoshida, T. Synthesis of polysaccharides having specific biological activities. *Prog. Polym. Sci*. 2001, *26*, 379 - 441.

[3] Yoshida, T. Synthetic and natural polysaccharides having specific biological activities. In *Polysaccharides, Structural diversity and functional versatility*, 2nd ed.; Dumitriu, S., Ed.; Marcel Dekker: New York, 2005. 839-851

[4] Yoshida, O.; Nakashima, H.; Yoshida, T.; Kaneko, Y.; Yamamoto, I.; Matsuzaki, K.; Uryu, T.; Yamamoto, N. Sulfation of the immunomodulating polysaccharide lentinan: A novel strategy for antiviral to human immunodeficiency virus (HIV). *Biochem. Pharmacol*. 1988, *37*, 2887 - 2891.

[5] Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Yasuda, N.; Mimura, T.; Yoshida, O.; Yamamoto, N. Synthesis and structural analysis of curdlan sulfate with potent anti-AIDS virus activity. *Macromolecules* 1990, *23*, 3717 - 3722.

[6] Lindahl, U.; Backstrom, G.; Thunberg, L. The antithrombin-binding sequence in heparin. *J. Biol. Chem*. 1983, *258*, 9826 - 9830.

[7] Jeon, K. J.; Katsuraya, K.; Kaneko, Y.; Mimura, T.; Uryu. T. Studies on interaction of sulfated polysaccharides as an AIDS drug by NMR. *Macromolecules* 1997, *30*, 1997 - 2001.

[8] Jeon, K. J.; Katsuraya, K.; Inazawa, K.; Kaneko, Y.; Mimura, T.; Uryu. T. NMR spectroscopy detection of interactions between a HIV protein sequence and a highly anti-HIV active curdlan sulfate. *J. Am. Chem. Soc*. 2000, *122*, 12536 - 12541.

[9] Jagodzinski, P. P.; Wustner, J.; Kmieciak, D.; Wasik, T. J.; Fertala, A.; Sieron, A. L.; Takahashi, M.; Tsiji, T.; Mimura, T.; Fung, M. S.; Gorny, M. K.; Kloczewiak, M.; Kaneko Y.; Koznor, D. Role of the V2, V3, and CD4-binding domains of gp120 in curdlan sulfonate neutralization sensitivity of HIV-1 during infection of T lymphocytes. *Virology* 1996, *226*, 217 – 227.

[10] Gerber, P.; Dutcher, J. D.; Adams, E. V.; Sherman, J. H. Protective effect of seaweed extracts for chicken embryos infected with influenza B or mumps virus. *Proc. Soc. Exp. Biol. Med*. 1958, *99*, 590 - 593.

[11] Ramalho-Santos, J.; Lina, M. C. Fusion and infection of influenza and sendai viruses as modulated by dextran sulfate: A comparative study. *Biosci. Rep*. 2001, *21*, 294 - 304.

[12] Opitz, L.; Lehmann, S.; Reichl, U.; Wolff, M. W. Sulfated membrane adsorbers for economic pseudo-affinity capture of influenza virus particles. *Biotech.Bioeng*. 2009, *103*, 1144 - 1154.

[13] Kalbfuss, B.; Wolff, M.; Morenweiser, R.; Reichl. U. Purification of cell culture-derived human influenza A virus by size-exclusion and anion-exchange chromatography. *Biotech. Bioeng*. 2006, *96*, 932 - 944.

[14] Kalbfuss, B.; Wolff, M.; Geisler, L.; Tappe, A.; Wickramasinghe, R.; Thom, V.; Reichl. U. Direct capture of influenza A virus from cell culture supernatant with Sartobind anion-exchange membrane adsorbers. *J. Membr. Sci*. 2007, *299*, 251 - 260.

[15] Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn. P.; Desmyter, J.; DeCercq, E. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* 1988, *20*, 309 – 321.

[16] World health organization. 2002., E. Identification of influenza isolates by hemagglutination inhibition. In *WHO manual on animal influenza diagnosis and surveillance*, http://dosei.who.int/

[17] Gordon, M.; Deeks, S.; Marzo, C.; Goodgame, J.; Guralnik, M.; Lang, W.; Mimura, T.; Pearce, D.; Kaneko, Y. Curdlan sulfate in a 21-day intravenous tolerance study in human immunodeficiency virus (HIV) and cytomegalovirus (CMV) infected patients. *J. Med*. 1997, *28*, 108 - 128.

[18] *Influenza Research Database*: http://www.fludb.org/

Chapter 3

Structural Analysis of Galactomannans by NMR Spectroscopy

Abstract

The structure of naturally occurring galactomannans was characterized by high resolution NMR spectroscopy involving two-dimensional (2D) NMR measurements of the field gradient DQF-COSY, HMQC, HMBC, and ROESY experiments. Four galactomannans with different proportions of galactose (G) and mannose (M), from fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust bean gum (LG), wereinvestigated. Because these galactomannans had very high molecular weights, hydrolysis by dilute H_2SO_4 was carried out to give the corresponding low molecular weight galactomannans, the structural identities of which were established by comparison of the specific rotations, shape of the GPC profiles, and NMR spectra with those of higher molecular weight galactomannans. The correlation signals GH1 - GC4, - GC5, and - MC6 in HMBC and GH1 - GH6 in ROESY spectra of FG showed that more than two galactopyranose units with the 1→4 linkage were connected at C6 of the mannopyranose main chain. The coupling constant ($J_{H1,\ 2}$) of galactose was 3.4Hz, indicating that galactose has an α-linkage. The main chain mannose was found to connect through the 1→4 linkage,

because of the appearance of the correlation signals MH1 - MC4, and MC1 - MH4 in the HMBC spectrum due to the long-range correlation signals between two neighboring mannopyranose residues through the M4 - O - M1 bond. Although the main chain mannose $J_{H1, 2}$ was not observed, probably because of the high molecular weight, the specific rotation of LG with a higher proportion of mannose was low, $[\alpha]_D^{25}$ = +10.8°, compared with that of FG with a lower proportion of mannose, $[\alpha]_D^{25}$ = +90.5°, suggesting that the mannose in the main chain had a β-linkage. These results suggest that the galactomannans comprise a (1→4)-β-mannopyranosidic main chain connected with more than two (1→4)-α-galactopyranosidic side chains, in addition to the single galactopyranose side chain, at C6 of the mannopyranose main chain.

3.1. Introduction

Galactomannans are naturally occurring branched polysaccharides consisting of a mannopyranose main chain with galactopyranose side chains that are distributed widely in the seeds of the leguminous plants. Galactomannans are used as stabilizing, thickening, binding, and gelling agents in the food industry.[1] The proportions of galactose and mannose in the galactomannans are dependent on the source. The fundamental structure reported in the literature was mainly a (1→4)-β-D-mannopyranosidic main chain with various proportions of single (1→4)- or (1→6)-α-D-galactopyranosyl side chains, which was determined by methylation analysis.[2] In general, naturally occurring galactomannans are difficult to dissolve in water because they have

high molecular weights of more than 100 x 10^4 and high viscosity in aqueous solution. In addition, galactomannans show lyotropic and thermotropic liquid crystallinities at a certain concentrations in water;[3, 4] thus, they are one of the important naturally occurring groups of branched polysaccharides.

We have reported the synthesis of branched polysaccharides by the ring-opening polymerization of anhydro disaccharide and trisaccharide monomers and have investigated the relationship between the structure and biological activities, such as the blood anticoagulation and anti-HIV activities of sulfated polysaccharides after sulfation of the synthetic and natural polysaccharides.[5, 6] For example, a new oligosaccharide-branched polysaccharide, 3-*O*-(β-D-lactosyl)-(1→5)-α-D-ribofuranan, was obtained for the first time by the ring-opening polymerization of a trisaccharide monomer, 1, 4-anhydro-2-*O*- benzyl- 3-*O*-(2', 3', 6', 2", 3", 4", 6"-hepta-*O*-benzyl-β-D-lactosyl)-α-D-ribopyranose, using BF_3OEt_2 as a catalyst into perbenzylated 3-*O*-(β-D- lactosyl)-α-D- ribofuranan and subsequent removal of the protective benzyl groups.[7] The structure was identified by high-resolution NMR measurements. Similarly, the polysaccharides in lacquer tree sap are acidic branched polysaccharides with specific biological activities such as promoting blood coagulation and antitumor activities [8] and have complex structures.[9, 10] We carried out structural analysis of Asian lacquer polysaccharides from Vietnam, Myanmar, Cambodia, Taiwan, and Japan in comparison with a Chinese lacquer polysaccharide, revealing that the structures of polysaccharides

in China and Japan, Taiwan and Vietnam, Myanmar and Cambodia, were similar to each other, and the polysaccharides in Myanmar and Cambodia had larger amounts of L-arabinose and L-rhamnose than those in other Asian lacquer polysaccharides.[11]

After sulfation, branched polysaccharides are expected to have potent and specific anti-HIV, anti-influenza virus, and blood anticoagulant activities.[12, 13] Therefore, determination of the structure without destruction of the molecules of galactomannans is quite important not only to know the structural details but also to reveal the relationship between the structures and their biological activities. Several kinds of galactomannans with various proportions of galactose branches depending upon the botanical source are known. However, to our knowledge, there are few reports on the precise structural analysis by high resolution NMR spectroscopy because they have poor solubility due to their high molecular weights. In this paper, we report for the first time structural comparisons and determinations of galactomannans with different proportions of galactose and mannose residues obtained from different sources by NMR spectroscopy including 2D measurements after acid hydrolysis to decrease the molecular weight.

3.2. Experimental

3.2.1 Galactomannans

Four kinds of galactomannans with different proportions of

galactose and mannose were used. Fenugreek gum (FG) from the seeds of *Trigonella foenum-graecum* was purchased from Air Green Co., Ltd., Japan, guar gum (GG) from the seeds of *Cyamopsis tetragonolobus* and locust bean gum (LG) from the seeds of *Ceratonia siliqua* were obtained from Sigma-Aldrich, Japan, and tara gum (TG) from the seeds of *Caesalpinia spinosa* was provided by Iwate Chemical Co., Japan, respectively.

3.2.2 Hydrolysis of galactomannans by dilute H_2SO_4 aqueous solution

A typical procedure for the hydrolysis of galactomannans was as follows. The galactomannan from FG (0.5 g) was added to 20% H_2SO_4 aqueous solution and stirred for 30 min at 50 °C. After cooling in a water bath, the reaction mixture was neutralized by saturated $NaHCO_3$ solution, and then dialyzed against deionized water overnight. The dialyzate was filtered to remove the water-insoluble precipitate and then freeze-dried to give 0.37 g of a low molecular weight galactomannan. The specific rotation was $[\alpha]_D^{25}$ = +90.5° (H_2O, c 1) and the molecular weight was $\overline{M}_n$ = 2.7 x 10^4.

3.2.3 Measurements

The ^1H NMR and ^{13}C NMR spectra were recorded with a JEOL ECM-400 or with a JEOL α-500 spectrometer at 400 MHz and 100 MHz or 500 MHz and 125 MHz, respectively, for solutions in D_2O at 50 °C

with 3-(trimethylsilyl)-1- propanesulfonic acid sodium salt (DSS) as an internal standard or in DMSO-d_6 at 60 °C. The assignment of the proton and carbon signals was carried out by a combination of the double–quantum filtered correlation spectroscopy (DQF-COSY) and heteronuclear single–quantum correlation (HSQC) 2D NMR measurements. The DQF-COSY was carried out with a 1024 x 256 data matrix, and four transients were acquired for each t1 value. The spectral width was 1870.58 Hz in both dimensions and the pulse delay was 1.5 s. The HSQC was carried out with a 4096 x 512 data matrix, and four transients were acquired for each t1 value. The proton and carbon spectral widths were 1870.87 Hz and 8544.65 Hz, respectively. The heteronuclear multiple–bond correlation (HMBC) measurement was carried out with a 4096 x 512 data matrix. The proton and carbon spectral width were 1870.87 Hz and 10000.52 Hz, respectively. The evolution time for the HMBC experiments was set to 62.5 ms. The 2D rotating–frame Overhauser effect spectroscopy (ROESY) experiment was recorded with a 4096 x 512 data matrix, with a 200 ms mixing time. The spectral width was 1869.76 Hz in both dimensions.

Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. The molecular weight of polymers was determined by an aqueous phase GPC (column; Tosoh TSK-gel G2500PW_{XL}, G3000PW_{XL}, and G4000PW_{XL}, 7.6 mm x 300 mm x 3 eluted with 66.7 mM of phosphate buffer, pH = 6.86) with a Tosoh RI detector using pullulan as a standard. Optical rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at

25 °C in a water-jacketed 10 ml quartz cell.

3.3. Results and Discussion

3.3.1 Acid degradation of galactomannans and structural identity

Galactomannans were extracted from the leguminous plants and included fenugreek gum (FG) with the approximate proportions of galactose and mannose of 1.0:1.04~1.20 [14 - 16], guar gum (GG) with 1.0:1.54~1.80 [17 - 19], tara gum (TG) with 1.0:2.50 [17], and locust bean gum (LG) with 1.0:3.0~3.75 [17, 20], respectively. Because the molecular weights of the galactomannans were very high, more than 100 x 10^4, the galactomannans were difficult to dissolve in water and molecular weights were difficult to measure by GPC. Some studies on structural analysis of galactomannans have been reported, but there are few reports on precise structural determinations by high resolution NMR spectroscopies using 1D and 2D NMR. Therefore, in this work, the galactomannans were hydrolyzed in a dilute aqueous H_2SO_4 solution to give low molecular weight galactomannans, structural identities of which were established by the specific rotations, shape of the GPC profiles, and NMR

Table 1. Hydrolysis of galactomannans by dilute sulfuric acid[a]

Galactomannan[b]			H_2SO_4	Time	Yield	$\overline{M}_n$[c]	$\overline{M}_w/\overline{M}_n$	$[\alpha]_D^{25}$[d]
	Mole ratio of							
	G	M	%	min	g	x10^4		deg
FG			5	45	0.47	30.0	2.0	+89.0
			10	30	0.38	16.3	1.6	+87.2
	1.0	0.83	20	30	0.37	2.7	2.2	+90.5
GG			5	60	0.46	16.0	1.8	+56.3
			10	30	0.35	12.1	1.9	+58.9
	1.0	1.64	20	30	0.29	1.4	2.2	+58.9
TG			5	60	0.47	7.3	2.0	+21.9
			10	60	0.37	4.4	2.2	+21.2
			15	30	0.37	1.7	2.5	+20.7
	1.0	2.88	20	30	0.38	1.3	2.2	+20.0
LG			5	45	0.46	20.4	1.6	+12.8
			10	30	0.44	8.5	1.9	+10.3
			15	30	0.34	2.6	2.2	+11.5
	1.0	3.33	20	30	0.30	0.9	2.2	+10.8

(a) Galactomannan, 0.5g; sulfuric acid, 50ml; temp., 50°C.
(b) The mole ratio of galactose (G) and mannose (M) was determined by the integral values of the H1 signals. FG: Fenugreek gum; GG: guar gum; TG: tara gum; LG: locust bean gum.
(c) Calculated by GPC using pullulan standards.
(d) Measured in H_2O at 25 °C (c 1).

measurement, respectively. Table 1 shows the results of the hydrolysis. When FG was hydrolyzed with 5% H_2SO_4 for 45 min at 50°C, the molecular weight decreased to $\overline{M}_n$ = 30.0 x 10^4 and the specific rotation was $[\alpha]_D^{25}$ = +89.0°. The molecular weight was further decreased to $\overline{M}_n$ = 2.7 x 10^4 by increasing the concentration of H_2SO_4 to 20%, and the specific rotation was almost the same, $[\alpha]_D^{25}$ = +90.5°. The other galactomannans shown in Table 1 were hydrolyzed in the same manner to give low molecular weight galactomannans. There was little difference in the specific rotations between high and low molecular weights. Figure 1 exhibits the GPC profiles after hydrolysis, in which (A) is the original FG and its hydrolyzates with the number-average molecular weights ($\overline{M}_n$) of (B) 30.0 x 10^4, (C) 16.3 x 10^4, and (D) 2.7 x 10^4, respectively. The molecular weight of the original FG was difficult to measure by GPC because substances with molecular weights higher than 100 x 10^4 are difficult to dissolve in water, as shown in Figure 1A. Hydrolysis decreased the molecular weight of FG, but the shapes of

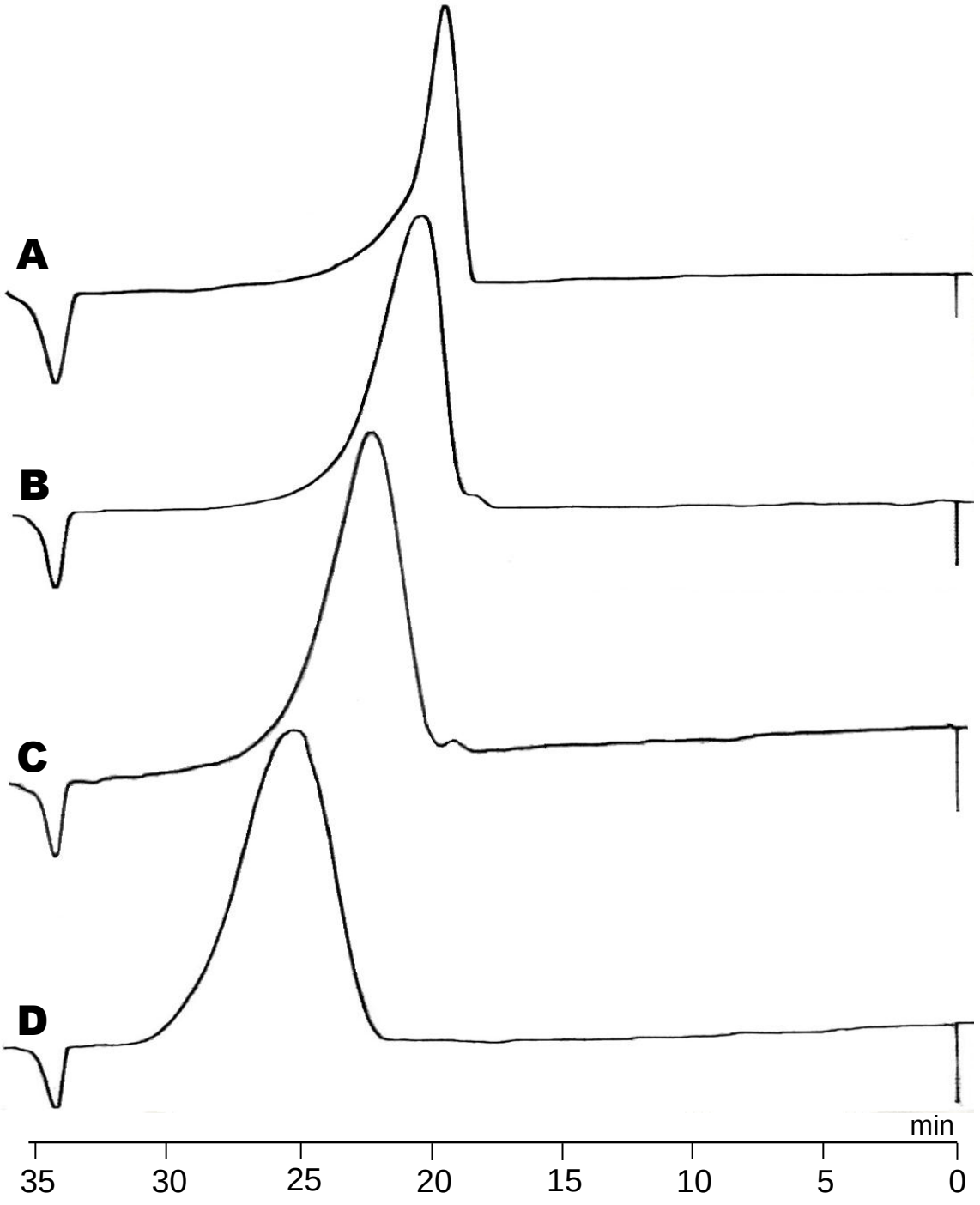

Figure 1. Aqueous GPC profiles of galactomannan from fenugreek gum. (A) Original galactomannan with the molecular weight of $\overline{M}n$ > 100 x 10^4, (B) with $\overline{M}n$ = 30.0 x 10^4 and $[\alpha]_D^{25}$ = +89.0° (H_2O, c 1), (C) $\overline{M}n$ = 16.3 x 10^4 and $[\alpha]_D^{25}$ = +87.2° (H_2O, c 1), and (D) $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5° (H_2O, c 1).

Figure 1

the GPC profiles were unchanged, and the molecular weight distribution are almost the same in Figures 1B-1D. After hydrolysis, the FGs with the lower molecular weights were easily dissolved in water. Figure 2 represents the ^{13}C NMR spectra of FGs with different molecular weights, in which all signals were assigned by the correlation signals of the DQF-COSY and HSQC spectra, are represented in Figure 5-7. The original FG in Figure 2A had low peak resolutions and gave a noisy spectrum in the highly viscous solution due to its high molecular weight. The peak resolutions were found to increase with decreasing molecular weight, and the intensity of each signal was not changed much by the decrease in molecular weights. Taking into account the specific rotations, shapes of the GPC profiles and NMR spectra, the structural identity was retained after hydrolysis.

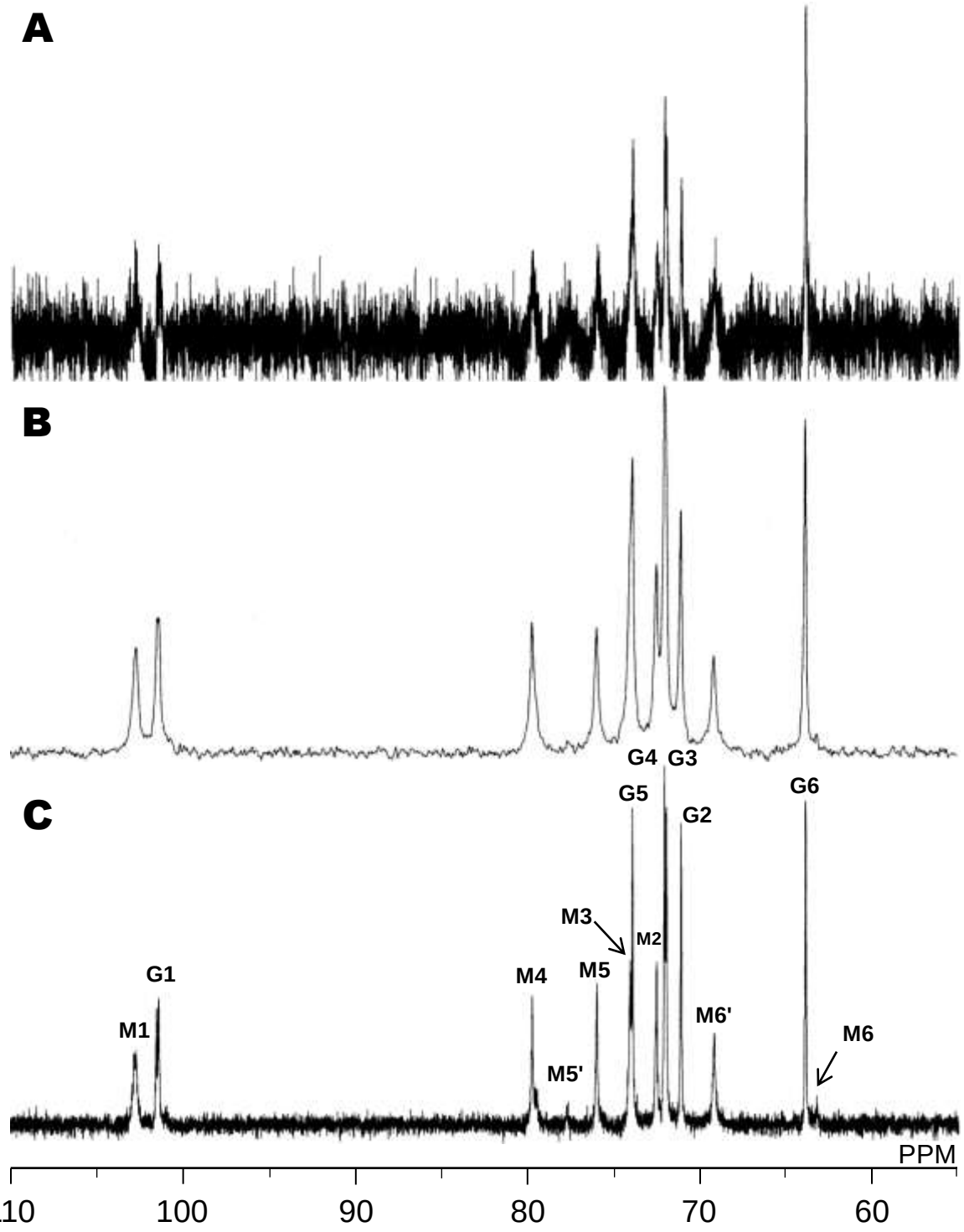

Figure 2. 125MHz ^{13}C NMR spectra of galactomannans from rom fenugreek gum. (A) Original galactomannan with $\overline{M}_n$ = 100 x 10^4, (B) with $\overline{M}_n$ = 16.3 x 10^4 and $[\alpha]_D^{25}$ = +89.0° (H_2O, c 1), (C) $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5° (H_2O, c 1). The signals were assigned by both DQF-COSY and HSQC spectra in D_2O at 50°C.

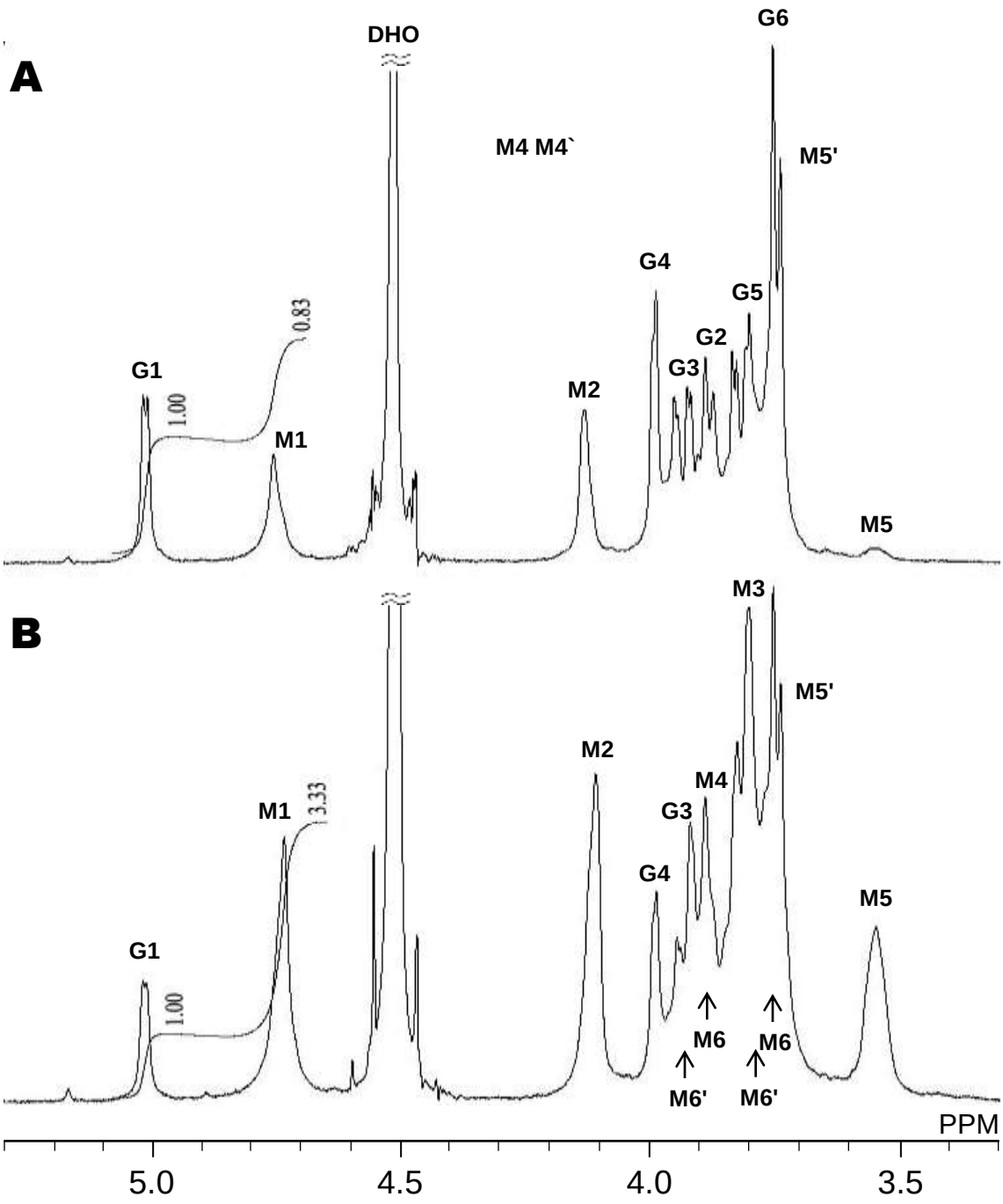

Figure 3. 400MHz ^{1}H NMR spectra of galactomannans (A) with $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5° (H_2O, c 1) from fenugreek gum and (B) with $\overline{M}_n$ = 0.9 x 10^4 and $[\alpha]_D^{25}$ = +20.0° (H_2O, c 1) from locust bean gum in D_2O at 50 °C. ': Mannose or galactose attached sugar signal.

Figure 3 shows the ^{1}H NMR spectrum of galactomannans with molecular weights of $\overline{M}n = 2.7 \times 10^4$ from FG and $\overline{M}n = 0.9 \times 10^4$ from LG, respectively. The galactomannans were found to have different proportions of galactose and mannose residues by the integrated ratios of the H1 protons as exhibited in Table 1. Using the coupling constant $J_{H1,\ 2}$ of galactose in the side chain, $J_{H1,\ 2} < 3.4$ Hz, the galactose residues were found to be glycosylated by an α-linkage. The integrated ratio of the H1 signals of the ^{1}H NMR spectra showed that the low molecular weight FG consisted of galactose and mannose in the proportions of 1.0 and 0.83, GG in 1.0 and 1.64, TG in 1.0 and 2.88, and LG in 1.0 and 3.33, respectively, proportions that were almost the same as reported previously. The ^{13}C NMR spectra of galactomannans with low molecular weights are shown in Figure 4. The carbon signals of the galactomannans appeared at the same chemical shift points, and only the signal intensities were different, suggesting that the galactomannans used here had the same structure, and the only proportions of galactose and mannose residues were different. The intensity of the signal at 63 ppm increased with increasing proportions of mannose, and the signals at 63 and 69 ppm were assigned to the C6 mannose by the DQF-COSY and HSQC spectra as described in the next section. The signal at 63 ppm was assigned to C6 without an attached galactose side chain. The signal at 69 ppm, with almost the same intensity in Figures 4A to 4D, was due to the C6 with an attached galactose side chain.

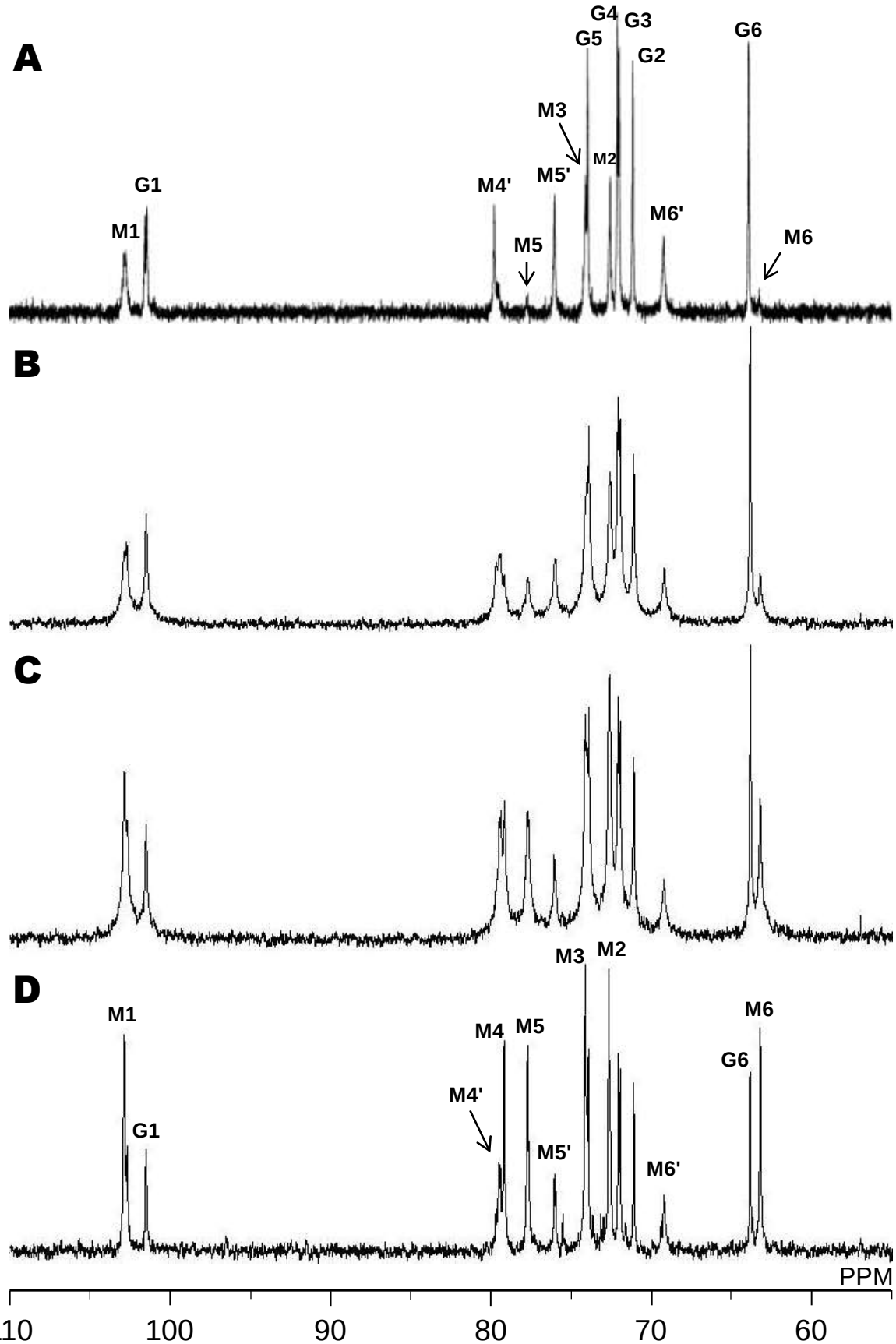

Figure 4. 125MHz and 100MHz ^{13}C NMR spectra of galactomannans (A) with $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5° (H_2O, c 1) from fenugreek gum, (B) with $\overline{M}_n$ = 1.4 x 10^4 and $[\alpha]_D^{25}$ = +58.9° (H_2O, c 1) from guar gum, (C) with $\overline{M}_n$ = 1.3 x 10^4 and = +20.0° (H_2O, c 1) from tara gum, and (D) with $\overline{M}_n$ = 0.9 x 10^4 and $[\alpha]_D^{25}$ = +10.8° (H_2O, c 1) from locust bean gum, respectively, in D_2O at 50°C. ': Mannose or galactose attached sugar signal.

3.3.2 Assignment of proton and carbon signals by DQF-COSY and HSQC NMR measurements.

Low molecular weight galactomannans were measured by the DQF-COSY and HSQC spectra in D_2O to assign the signals. Figure 5-7 exhibits the 2D spectra of galactomannan from fenugreek gum and locust bean gum. In the DQF-COSY spectrum of galactomannan from fenugreek gum (Figure 5), the H1 signals due to galactose (GH1) and mannose (MH1) residues appeared at 5.02 and 4.74ppm, respectively. The correlation signal of the GH1 - GH2 was observed to assign the GH2 signal to 3.84 ppm. The GH3 signal was exhibited at 3.93 and 3.95 ppm assigned by correlation signals of GH2 - GH3. GH4 and GH5 signals were determined in a similar manner, as exhibited in Figure 5. The GH6 signal was assigned by the correlation of the GH6 and GC6 signals in the HSQC spectrum (Figure 7). In the DQF-COSY spectrum of galactomannan from locust bean gum (Figure 6), Although the MH2 and MH3 signals were found to appear at 4.12 and 3.82 ppm, respectively, by the correlation of signals of MH1-MH2 and MH2-MH3, other mannose proton signals were determined in a similar manner, as exhibited in Figure 6. In the HSQC spectrum shown in Figure 7, the carbon signals were assigned from the corresponding proton signals. The MC6 at 63 and 69 ppm were separated into two correlation signals, respectively, of the corresponding two MH6 and MH6' protons at 3.96 and 3.78 ppm and 3.91 and 3.75 ppm, respectively, due to the presence and absence of attached galactose side chains, as mentioned in 3.3.1

section above. The MC4 and MC5 signals were correlated to the MH4 and MH5 signals at 3.86 and 3.75 ppm in the DQF-COSY and HSQC spectra of FG, respectively. Although the galactomannan from FG gave complex ^{1}H and ^{13}C spectra, all signals were assigned by the decreasing molecular weight. The proton and carbon signals of other galactomannans with low molecular weights were also assigned on the basis of the FG data and by the measurements of the 2D NMR spectra.

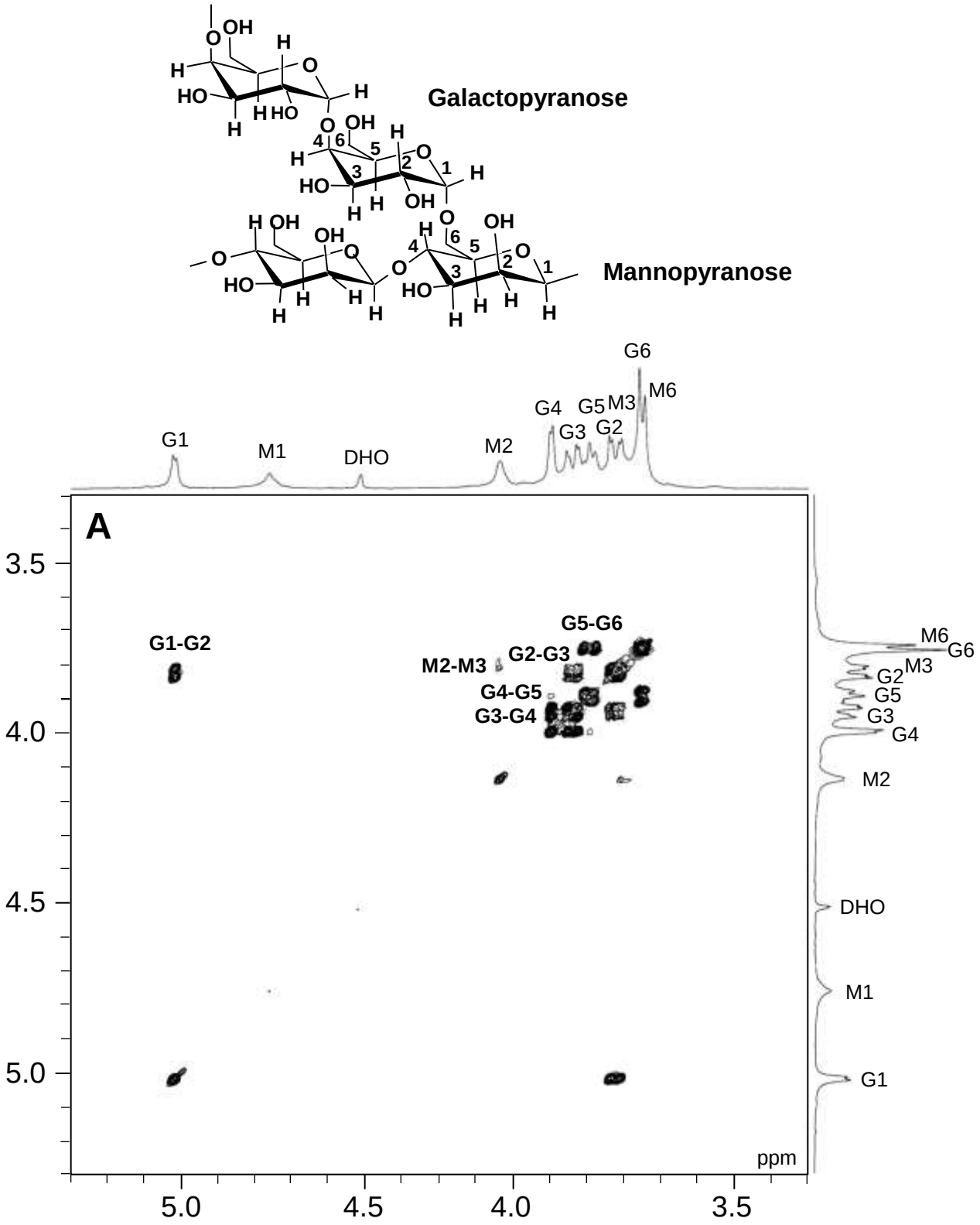

Figure 5. DQF-COSY spectra of low molecular weight galactomannan with $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5°(H_2O, c 1) from fenugreek gum in D_2O at 50°C. M: mannose, G: galactose.

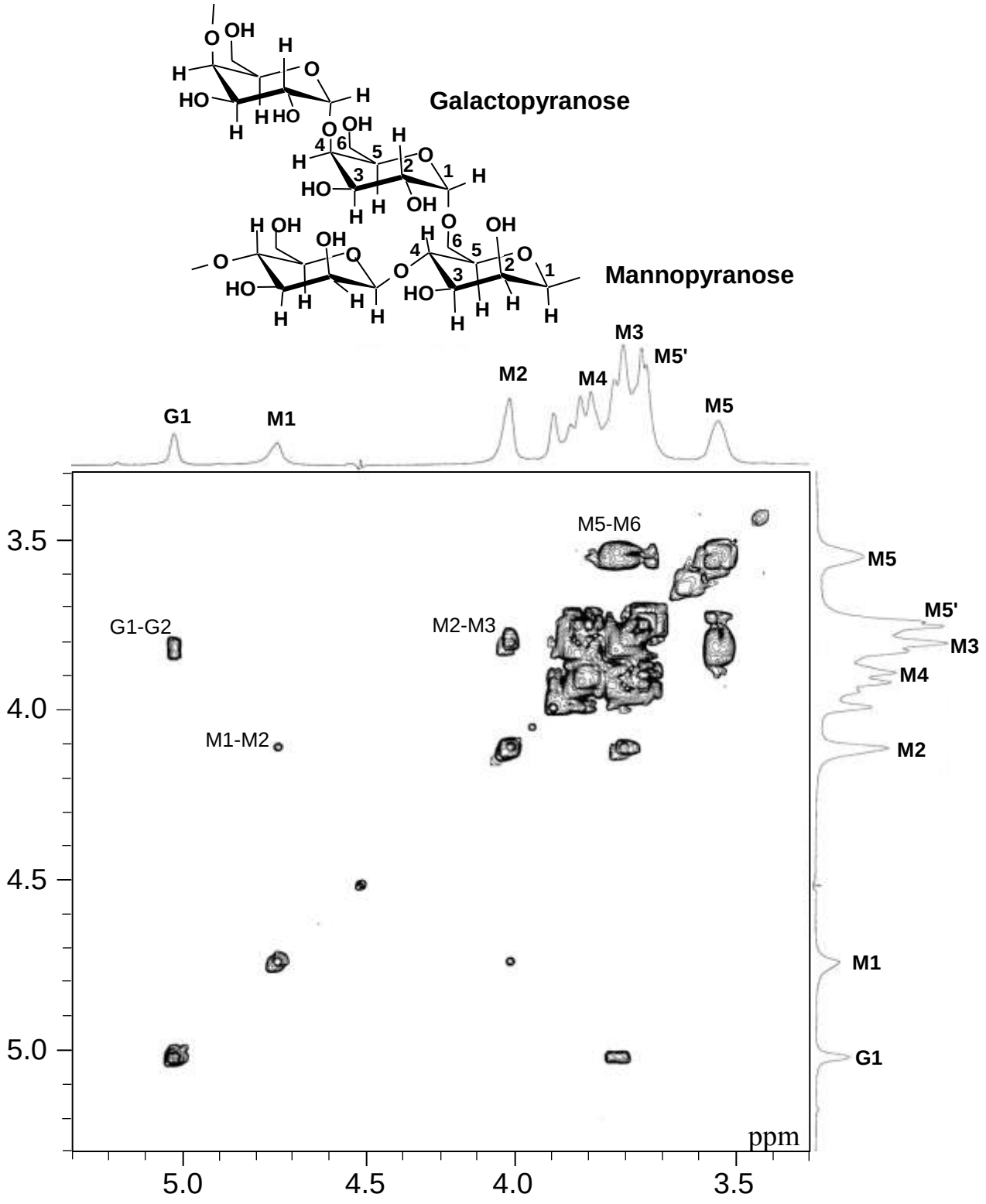

Figure 6. DQF-COSY spectra of low molecular weight galactomannan with $\overline{M}_n$ = 0.9 x 10^4 and $[\alpha]_D^{25}$ = +10.8°(H_2O, c 1) from locust bean gum in D_2O at 50°C. M: mannose, G: galactose. ': Mannose or galactose attached sugar signal.

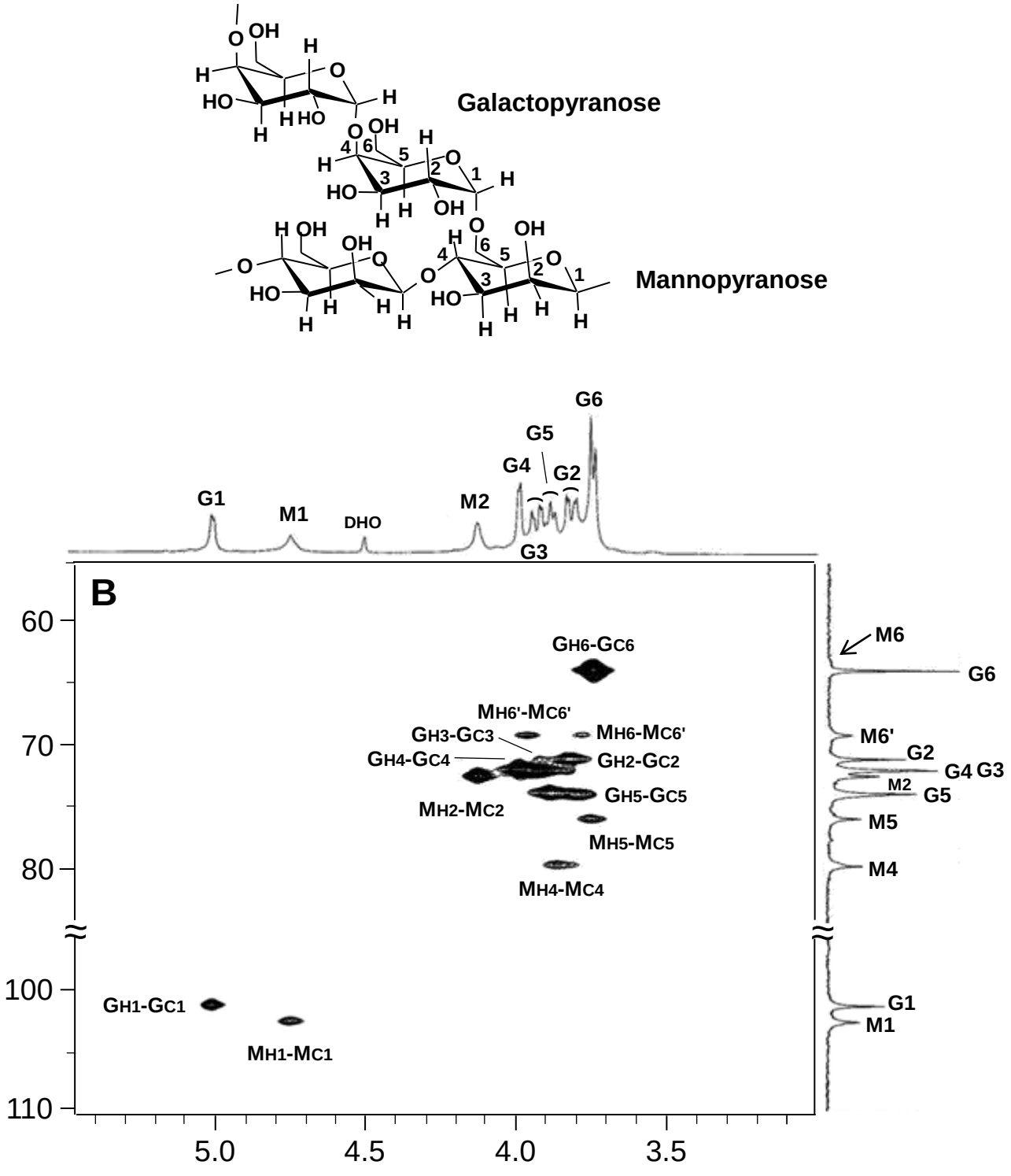

Figure 7. HSQC spectra of low molecular weight galactomannan with $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5 °(H_2O, c 1) from fenugreek gum in D_2O at 50 °C. ': Mannose or galactose attached sugar signal.

3.3.3 Structural analysis of galactomannans by HMBC and ROESY NMR measurements.

Because most proton and carbon signals of NMR spectra were assigned by the DQF-COSY and HSQC measurements, the whole structure of the galactomannans was elucidated by the HMBC and ROESY spectra. HMBC measurement is suitable for the determination of multiple bond connectivity and ROESY for the analysis of protons that locate near each other by use of NOE. Figure 8 gives the HMBC spectrum of galactomannan ($\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$= +90.5° (H_2O, c 1)) from FG. The correlation signals between GH1 - GC5 through the G1 - O - G5 oxygen and GH1 - GC3 and GC1-GH5 in the one galactopyranose residue, and the GH1 - GC4 due to the two neighboring galactopyranose residues through the G1 - O - G4 oxygen appeared successively on the GH1 and GC1 tracks, respectively. The appearance of the correlation signals between two galactose residues revealed that more than two galactose residues were glycosylated from the main chain mannose were glycosylated in addition to the single galactopyranose side chains. In addition, the long-range correlation signals between GH1 and MC6 through the G1 - O - M6 oxygen appeared on the GH1 track, suggesting that galactose branches were attached at the C6 carbon of the main chain mannose.

The correlation signals between MH1 - MC4, and MC1 - MH4 on the MH1 and MC1 tracks appeared clearly, respectively, due to the long-ranged correlation signals through the M4 - O - M1 oxygen

between two neighboring mannopyranose residues, indicating that mannose has a 1→4-linked pyranoside structure. In addition, the specific rotations of galactomannans decreased with increasing proportions of mannose residues, as demonstrated in Table 1, revealing that mannopyranose residues were glycosylated by the β-linkage in the main chain.

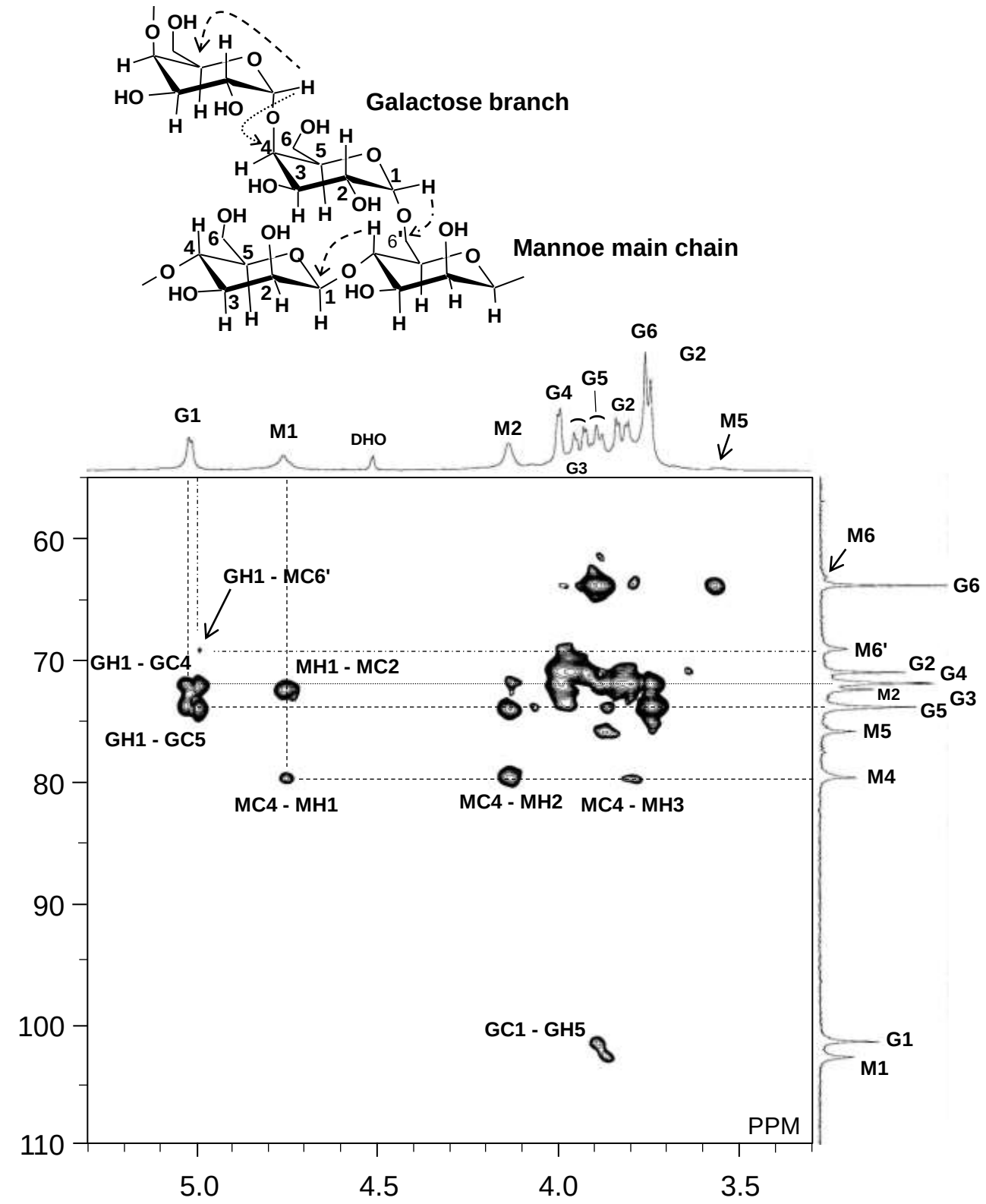

Figure 8. HMBC spectrum of low molecular weight galactomannan with $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5°(H_2O, c 1) from fenugreek gum in D_2O at 50°C. ': Mannose or galactose attached sugar signal.

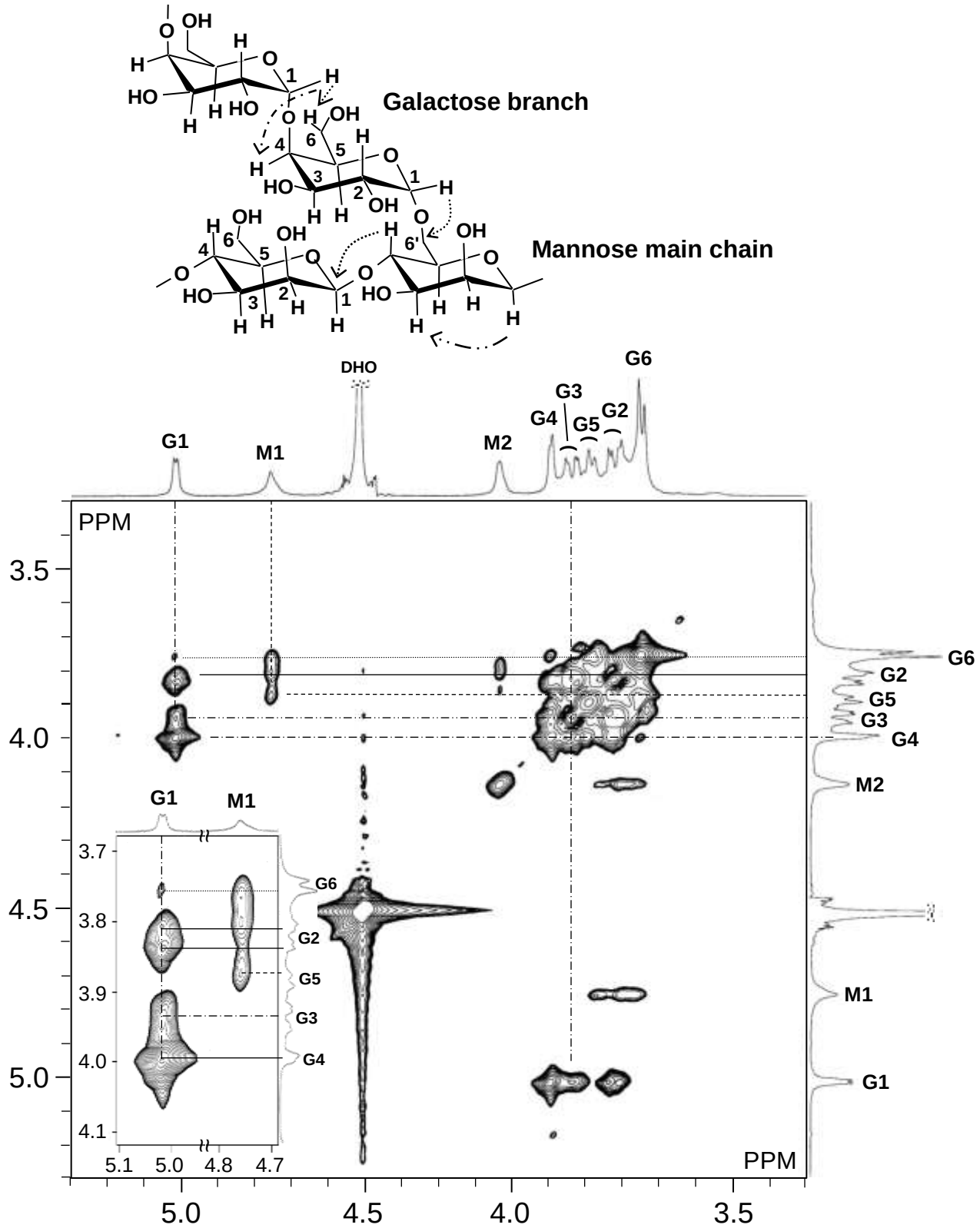

Figure 9. ROESY spectrum of low molecular weight galactomannan with $\overline{M}_n$ = 2.7 x 10^4 and $[\alpha]_D^{25}$ = +90.5°(H_2O, c 1) from fenugreek gum in D_2O at 50°C. ': Mannose or galactose attached sugar signal.

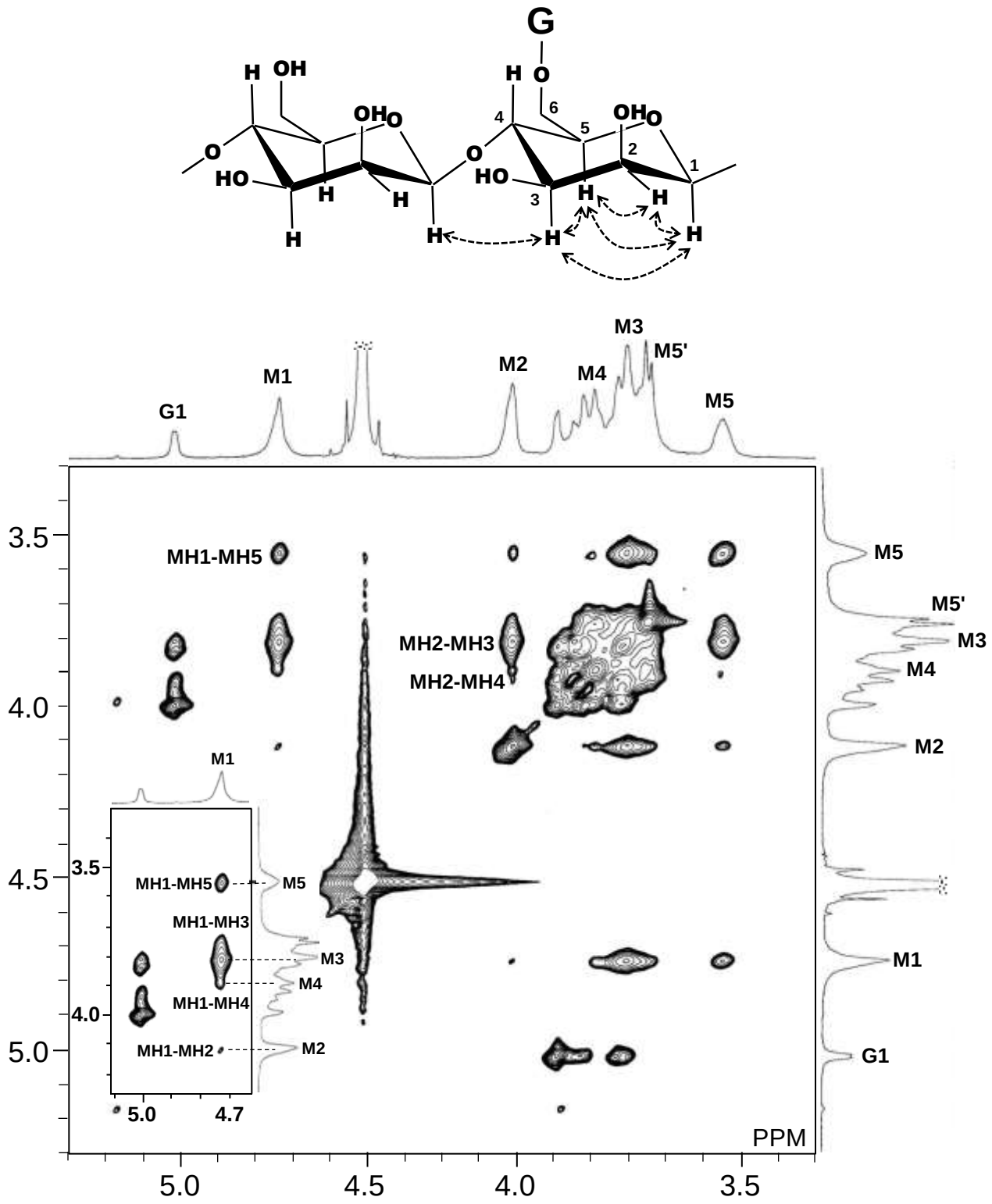

Figure 10. ROESY spectrum of low molecular weight galactomannans with $\overline{M}_n$ = 0.9 x 10^4 and $[\alpha]_D^{25}$ = +10.8° (H_2O, c1) from locust bean gum in D_2O at 50 °C. **'**: Mannose or galactose attached sugar signal.

Further structural analysis of galactomannans was carried out by ROESY, and the spectra are represented in Figures 9 (FG) and 10 (LG), in which the proportions of galactose and mannose in FG were 1.0 and 0.83 and LG were 1.0 and 3.33, respectively, according to the results of the ^{1}H NMR spectra as shown in Figure 3. In Figure 9, the NOE correlation signals between GH1 - GH2, - GH3, - GH4, and - GH6 of FG appeared clearly on the GH1 track; however, the correlation signal due to GH1 - GH5 was not observed because of the distance between the two protons was long, according to the pyranose structure. On the other hand, correlation signal between GH1 - GH6 protons due to the neighboring two galactose residues appeared, suggesting that these two protons were located closely. From the HMBC and ROESY spectra of FG galactomannan, galactose was attached at the C6 of the main chain mannopyranose with the β-linkage. In the ROESY spectrum (Figure 10) of LG, which has a large proportion of mannose residues, NOE correlation signals between MH1 - MH2, - MH3, and -MH5, and MH2 - MH3 and - MH5, and MH5 - HM6' in the one mannose residue, and between MH1 - MH4 between the neighboring two mannose residues were observed. However, NOE correlation signals between MH1 - MH6 did not appear clearly because the two protons were spatially very distant. These results of the 2D NMR also suggest that the mannopyranose in the main chain was glycosylated by the (1→4)-β-linkage.

In conclusions, the NMR signals of galactomannans with different proportions of galactose and mannose residues from several seeds were

assigned by the DQF-COSY and HSQC 2D NMR measurements. The linkages and whole structures of the galactomannans were revealed by the combination of HMBC and ROESY spectroscopies using the long-range and NOE correlations. Appearance of galactose side chain GH1-O-GC4 and GH1-O-MC6 correlation signals in HMBC spectrum and the coupling constant $J_{GH1, 2}$ = 3.4 Hz suggested that at least two galactose residues were glycosylated by a α-linkage from the C6 position of main chain mannose. This result was supported by the NOE correlation (ROESY) signals. Main chain mannose should have β-linkage because of the appearance of MH1-O-MH4 NOE correlation signal between two mannose residues and decrease of specific rotations with decreasing proportion of galactose side chains. After sulfation of the galactomannans, the relationship between the branched structure and biological activities, such as antiviral and blood anticoagulant activities, is under investigation.

References

[1] Srivastava,M., & Kapoor, V. P. Seed galactomannans: an overview. *Chem. Biodiversity*, 2005, *2*, 295 - 317.

[2] McDougall, G. J., Morrison, I. M., Stewart, D., & Hillman, J. R. Plant cell walls as dietary fibre: range, structure, processing and function. *J. Sci. Food Agric*., 1996, *70*, 133 - 150.

[3] Hatakeyama, T., Naoi, S., & Hatakeyama, H. Liquid crystallization of glassy guar gum with water. *Termochim. Acta*., 2004, *416*, 121 - 127.

[4] Tanaka, R., Hatakeyama, T., Hatakeyama, H. Formation of locust bean gum hydrogel by freezing -thawing. *Polym. Internal*., 2004, *45*, 118 - 126.

[5] Yoshida, T. Synthesis of polysaccharides having specific biological activities. *Prog. Polym. Sci*., 2001, *26*, 379 - 441.

[6] Yoshida, T. Synthetic and natural polysaccharides having specific biological activities, in Polysaccharides: Structural diversity and functional versatility, 2nd Ed., S. Dumitriu, Ed., Marcel Dekker, Inc, New York, 2005.

[7] Han, S., Kanematsu, Y., Hattori, K., Nakashima, H., Yoshida, T. Ring-opening polymerization of benzylated 1, 6-anhydro-β-D-lactose and specific biological activities of sulfated (1→6)-α-D-lactopyranans, *J. Polym. Sci. Part A: Polym. Chem*., 2009, *47*, 913 - 924.

[8] Lu, R. Hattori, K. Yoshida, T. Nakashima, H. Premanathan, M.

Aragaki, R. Mimura, T. Kaneko, Y. Yamamoto, N. Miyakoshi, T. Uryu, T. Specific biological activities of Chinese lacqure polysaccharides. *Carbohydr. Polym.*, 2000, *43*, 47 - 54.

[9] Lu, R. Hattori, K. Yoshida, T. Yang, J. Du, Y. Miyakoshi, T. Uryu, T. Structural analysis of polysaccharide in Chinese lacquer by high resolution NMR spectroscopy. *Sen'i Gakkaishi*, 1999a, *55*, 47 - 56.

[10] Lu, R. Yoshida, T. Uryu, T. Viscosity behaviors of Chinese urushi polysaccharide in aqueous solutions. *Sen'i Gakkaishi*, 1999b, *55*, 402 - 407.

[11] Lu, R. Yoshida, T. Specific Structure and Molecular Weight Changing of Asian Lacquer Polysaccharides. *Carbohydr. Polym.*, 2003, *54*, 419 - 424.

[12] Yoshida, T. Yasuda, Y. Uryu, T. Nakashima, H. Yamamoto, N. Mimura, T. Kaneko, Y. Synthesis and in vitro inhibitory effect of L-glycosyl- branched curdlan sulfates on AIDS virus infection. *Macromolecules*, 1994, *27*, 6271 - 6276.

[13] Tegshi, M., Han, S., Kanamoto, T., Nakashima, H., Yoshida, T. Synthesis and specific influenza A virus-adsorptive functionality of alkyl curdlan sulfate- coated membrane filter", *J. Polym. Sci. Part A: Polym. Chem.*, 2011, *49*, 3241 – 3247.

[14] Andrews, P. Hough, L. Jones, J. K. N. Mannose-containing polysaccharides. Part II. The galactomannan of fenugreek seed (Trigonella fœnum-græcum). *J. Chem. Soc.*, 1952, 2744 - 2750.

[15] Daoud, K. M. The reserve polysaccharide of the seeds of fenugreek: its digestibility and its fate during germination. *Biochem*

J., 1932, *26*, 255 - 263.

[16] Ramesh, H. P., Yamaki, K., Ono, H. Tsushida, T. Two-dimensional NMR spectroscopic studies of fenugreek (Trigonella foenum-graecum L.) galactomannan without chemical fragmentation. *Carbohydr. Polym.*, 2001, *45*, 69 - 77.

[17] Anderson, E. Endosperm mucilages of legumes. *Ind. Eng. Chem.*, 1949, *41*, 2887 – 2890.

[18] Dea, I. C. M., Morrison, A. Chemistry and interactions of seed galactomannans. *Adv. Carbohydr. Chem. Biochem.*, 1975, *31*, 241 – 312.

[19] Stéphane, J., Debon, J., Tester, R. F. In vitro binding of calcium, iron and zinc by non-starch polysaccharides. *Food Chem.*, 2001, *73*, 401 - 410.

[20] Vieira, M. C., Gil, A.M. A solid state NMR study of locust bean gum galactomannan and Konjac glucomannan gels. *Carbohydr. Polym.*, 2005, *60*, 439 - 448.

Chapter 4

Sulfation of galactomannans and their biological activities

ABSTRACT.

The specific biological activities such as blood anticoagulant and anti-HIV activities of the sulfated galactomannans with different proportions of galactose (G) and mannose (M), from fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust been gum (LG), were investigated. The sulfation of the galactomannans was carried out by piperidine-N-sulfonic acid or chlorosulfonic acid and piperidine-N-sulfonic acid to give sulfated galactomannans with degrees of sulfation of 0.53 to 0.84. These sulfated galactomannans exhibited potent anti-HIV activity as high as that of dextran sulfate and curdlan sulfate, represented by the 50% protecting concentration (EC_{50}) in the range of 1.14-2.86 μg/ml. In addition, the sulfated galactomannans had high blood anticoagulant activity in the range of 29.4-71.0 unit/mg, which values were higher than that of standard dextran sulfate (22.7 unit/mg) and curdlan sulfate (10 unit/mg). The reason of why sulfated galactomannans had both high activities of anti-HIV and blood anticoagulant activities were presumed that galactomannans are branched polysaccharides, that is, sulfated branched polysaccharides

were interacted strongly with surface glycoproteins of HIV and blood anticoagulant proteins, because of their flexible branched structures.

4.1. Introduction

In the third chapter, we elucidated the structure of the galactomannans by using of 1D and 2D NMR measurements,[1] suggesting that galactopyranose residues were glyarylated with 1,4-α linkage ($J_{H1,2}$= 3.4 Hz) and were branched from the C6 position of main chain mannose. Main chain mannose had β-linkage because of the appearance of the NOE correlation signal due to MH1-O-MH4 linkages between two mannopyranose residues.

In our laboratory, we have reported many papers on the sulfation of synthetic and naturally occurring polysaccharides and then examined the relationship between the structure and biological activities of sulfated polysaccharides such as blood anticoagulant and anti-HIV activities.[2, 3]

The biological activities were assumed that negative charged sulfate groups in sulfated polysaccharides electrostatic interacted with positive charged portions of the surface proteins. For example, heparin, a naturally occurring sulfated polysaccharide has potent anticoagulant activity by the electrostatic interaction between negatively charged pentasaccharide portions in heparin and positively charged portions of antithrombin III. The ionic interaction is induced the conformation changes of antithrombin III and then activated antithrmbin III is bound

with thrombin.[4, 5]

Therefore, heparin is widely used to inhibit blood coagulation in clinic, through accelerating the inactivation of serine protease of the coagulation cascade by interacting with antithrombin.[6] Heparin is a linear polysaccharide, however, has complex structure consisting of uronic acid (1→4)-D-glucosamine repeating disaccharide subunits.[7] Although heparin and some other naturally occurring polysaccharides have potent anticoagulant activity, it is difficult to elucidate the relationship between their structures and biological activities, because of their complex structures.

In our recent works, branched polysaccharides after sulfation had strongly biological activities than those of linear sulfated polysaccharides and we reported the relationship between branched structures and biological activities such as anti-HIV, blood anticoagulant, and anti-tumor activities, Galactomannans are naturally occurring polysaccharides and abundant in nature. Therefore, we carried out the sulfation of galactomannans both to examine the biological activities and to elucidate the relationship between the branched structures and biological activities.

To our knowledge, there are few reports on the relationship between the precise structures and biological activities, because of their poor solubility and high molecular weights. In this chapter, we describe the sulfation of galactomannans with different proportions of galactose and mannose residues obtained from different sources, and their anti-HIV and blood anticoagulant activities were examined. Also we

investigate the relationship between the branched structure and biological activities of sulfated galactomannans.

4.2. Experimental Section

4.2.1. Materials

Four kinds of galactomannans with different proportions of galactose and mannose were used. Fenugreek gum (FG) from the seeds of *Trigonella foenum-graecum* was purchased from Air Green Co., Ltd., Japan; guar gum (GG) from the seeds of *Cyamopsis tetragonolobus* and locust bean gum (LG) from the seeds of *Ceratonia siliqua* were obtained from Sigma-Aldrich, Japan; and tara gum (TG) from the seeds of *Caesalpinia spinosa* was provided by Iwate Chemical Co., Japan, respectively.

4.2.2. Measurements

The 400 MHz ^{1}H and 100 MHz ^{13}C NMR spectra were obtained 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. IR spectra were recorded by a Shimadzu FT-IR 8300 spectrometer. Optical rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at 25°C in a water-jacketed 10 ml quartz cell. Molecular weights of polymers were determined by an aqueous phase gel permeation chromatography (GPC) equipped with TOSOH TSK-gel columns (7.6 mm X 600 mm X 3) of G2500PW$_{XL}$,

G3000PW$_{XL}$, and G4000PW$_{XL}$ eluted with 66.7 mM of phosphate buffer solution (pH 6.86) using standard pullulan (Shodex standard P-82; showa Denko Co., Japan) as a reference at 40 °C. RI (RI-8021) detectors were used.

4.2.3. Sulfation

Galactomannan was sulfated by the as following two method: 1. Sulfate two time continuously by the used of chlorosulfonic acid in pyridine (CSA method), and piperidine –N-sulfonic acid in the DMSO (PSA method), 2. Treatment the galactomannan by sulfuric acid to decrease molecular weight, and then sulfate by used of PSA method.

CSA method

Galactomannan (1.0 g, 6.2 mmol) was added to chlorosulfonic acid (2.2 g, 18.6 mmol) in the dry pyridine (100 ml) and the mixture stirred for 24 h at 100 °C, after cooled with the ice bath, neutralized by the saturated $NaHCO_3$ solution, and then acetone was added until the acetone layer apeared. The lower aqueous layer was washed three times with acetone, with subsequently dialysis against deionied water overnight. The dialyzate was concentrated and freeze-dried to give sulfated fenugreek gum (SFG-c), yield 1.16 g.

PSA method for further sulfate

SFG-c (0.5g) was dissolved in the dry DMSO (50 ml) and then

piperidine-N-sulfonic acid (1.5 g, 9.1 mmol) was added to the solution. The mixture was stirred for 2 h at 100 °C, after cooled with in ice bath, neutralized by the saturated $NaHCO_3$ solution (40 ml) and then acetone was added until the precipitate appeared. The precipitate was collected by the centrifugation, then washed three times with acetone, redissolved in water (100 ml), and dialyzed against deionized water overnight. The dializate was concentrated to approximately 30 ml and freeze-dried to give two time sulfated fenugreek gum (SFG-c-p), yield 0.47 g, $[\alpha]_D^{25}$=+41.7° (H_2O, c 1%), $\overline{M}_n$=0.9 X 10^4. Elem anal. Found: C, 21.62; H, 3.34; S, 9.43.

Treatment of galactomannan[1]

Galactomannan`s fenugreek gum of 0.5g was added in 10% sulfuric acid solution (50 ml) and stirred for 30 min at 50 °C, after cooling with water bath, the reaction mixture was neutralized by saturated $NaHCO_3$ solution, and dialyzed against deionized water overnight. The dialyzate was after filtered to removing water insoluble precipitate, and freeze-dried to give low molecular weight fenugreek gum: yield 0.38 g; $[\alpha]_D^{25}$=+87.2° (H_2O, c 1%); $\overline{M}_n$= 16.3 X 10^4.

Sulfation of low molecular weight galactomannan

Low molecular weight fenugreek gum (0.25 g, 1.5 mmol) was dissolved in dry DMSO (25 ml) and piperidine-N-sulfonic acid (0.74 g, 4.5 mmol) was added to the solution. The mixture was stirred for 2 h at 100 °C. Sulfated low molecular weight fenugreek gum was obtained after the

same treatment as previously described: yield 0.24g, $[\alpha]_D^{25}$=+48.8° (H2O, c 1%), $\overline{M}_n$=0.9 X 10^4. Elem anal. Found: C, 24.14; H, 3.52; S, 7.54.

4.2.4. Biological activities

Anti-HIV Activity[8]

The anti-HIV activity was determined by the protection from HIV-induced cytopathic effects *in vivo*.MT-4 cell was infected with HIV-$1_{HTLV-\ IIIB}$ strain at the multiplicity of infection of 0.01. HIV infected and uninfected MT-4 cells (3.0 x 10^4 cells/mL) were co-cultured in the presence of various concentrations of sulfated polysaccharides for 5 days at 37 °C in CO_2 incubator. The viability of both HIV and mock-infected cells was assayed photometrically by the MTT method in which color change by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue formazan product is proportional to the number of viable cells. The concentration of sulfated polysaccharides for 50% of inhibition of virus infection in 5-day HIV-infected MT-4 cell culture was defined as EC_{50} and for 50% cytotoxicity in 5-day MT-4 cell culture was designated as CC_{50}.

Blood Anticoagulant Activity[9]

The anticoagulant activity testing of sulfated galactomannans was measured by using bovine plasma according to modification of the US pharmacopeia and was calculated in comparison with the activity of

standard destran sulfate (H-39), 22.7 units/mg

4.3 Results and Discussion

4.3.1 Sulfation of Galactonannans

Purchased natural galactomannans were difficult to be sulfated, because of the poor solubility in water or other organic solvents, higher viscosity and gelling behavior.

However, we understood in previous study that it was became more easily to be analyzed after decreasing the molecular weight by the acid hydrolysis. Therefore, we sulfate galactomannans after acid hydrolysis to decrease the molecular weight, the sulfation was carried out with piperidine-N-sulfonic acid (PSA) in DMSO. On the other hand, we have tried to carry out sulfation two times with chlorosulfonic acid (CSA) in pyridine and PSA in DMSO.

As shown in **Table 1**, sulfated galactomannans having degrees of sulfation of 0.53 to 0.84 were obtained. Molecular weight were approximately 1 x 10^4 except one of SGG was 14.6 x 10^4, and STG was 3.9 x 10^4. Specific rotations were decreased greatly after sulfation, such as FG being around +90°,

Table 1. Sulfation of galactomannans with piperidine-N-sulfonic acid(PSA)[a]

No.	GM	Sulfating regent g (mol per suger unit)	Temp. °C	Time h	yield g	$\overline{M}_n$[b] X 10^4	$[\alpha]_D^{25}$ [c] (°)	elemental analysis (%) C	H	S	DS
1	FG	0.74 (3)	100	2	0.24	0.9	+48.8	24.1	3.5	9.7	0.71
2	FG	1.5 (>3)	100	2	0.47	0.9	+41.7	21.6	3.3	9.2	0.66
3	GG	0.74 (3)	100	2	0.41	1.8	+35.1	21.1	3.1	8.2	0.57
4	GG	0.74 (3)	85	1	0.36	14.6	+35.7	24.1	3.7	7.8	0.53
5	TG	0.74 (3)	85	1	0.40	3.9	+14.0	23.0	3.5	8.5	0.59
6	LG	0.74 (3)	100	2	0.33	1.2	+6.4	20.5	3.1	9.9	0.73
7	LG	1.5 (>3)	100	2	0.49	0.8	+4.4	20.8	3.2	10.8	0.84

[a] No. 1 and 3 ~ 6, 0.25 g of galactomannan was used; No. 2 and 7, 0.5 g of sulfated galactomannan (sulfation with chlorosulfonic acid in pyridine at 100 °C, 24h).

[b] Determined by GPC.

[c] Measured in H_2O (c 1%).

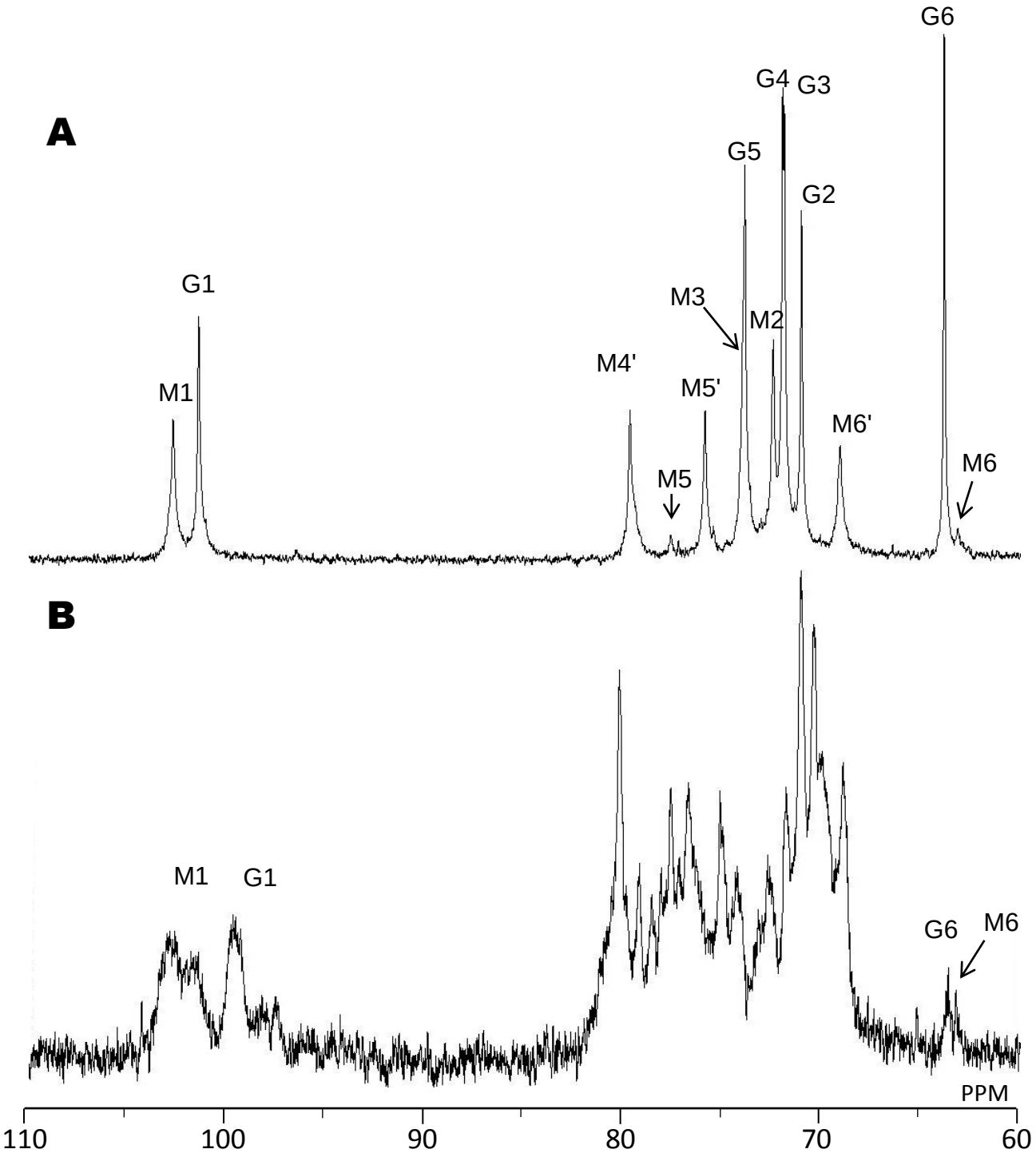

Figure 1. 100MHz ^{13}C NMR spectra of galactomannans from fenugreek gum. (A) Fenugreek gum, (B) Sulfation fenugreek gum.

and by the sulfation specific rotation became to +48.8 and +41.7.

According to the 13C NMR spectrum of sulfated galactomannan, as shown in **Figure 1**, comparison with Figure 1A, we found that the signal of C6 carbon shifted downfield from 63.7 ppm, suggested that the sulfate group was introduced at the C6 hydroxyl group. It was difficult to clear out the shifting of C2 and C3 carbon because of the signal between 67 ppm to 83 ppm is so complex after sulfation. However, it must be a little proportion of sulfate groups was introduced at C2 and C3 of both of mannopyranoside and galactopyranoside.

4.3.2 Anti-HIV activity and Blood anticoagulant activity

Since we obtained sulfated galactomannans with the well-defind structure and molecular weights, specific biological activities such as anti-HIV and blood anticoagulant activities were examined to elucidate the structure-activity relationship of the sulfated galactomannans. The results of the biological activities are summarized in Table 2. Anti-HIV activity was evaluated by the 50% inhibitory concentration (EC_{50}) of sulfated polymers on the infection of HIV to MT-4 cell. All of the sulfated galactomannans shown potent anti-HIV activity as high as curdlan sulfate (0.61 μg/ml) and dextran sulfate (2.36 μg/ml). Comparing the anti-HIV activities of the different ratio of Gal/Man, we found there was no potent difference in 4 kinds of galactomannans of FG, GG, TG, and LG.

The cytotoxicity of the sulfated galactomannans was low, since the CC_{50}, 50% cytotoxic concentration of sulfated polymers on MT-4 cell, was more than 100 µg/ml.

Table 2. Biological activity of sulfated galactomannans

No.	SGM	M_n X 10^4	$[\alpha]_D^{25}$ deg	DS	EC_{50} [a] ug/ml	CC_{50} [b] ug/m l	AA [c] unit/mg
1	SFG	0.9	+48.8	0.71	1.65	> 100	13.4
2	SFG	0.9	+41.7	0.66	1.14	> 100	29.4
3	SGG	1.8	+35.1	0.57	1.42	> 100	50.5
4	SGG	14.6	+35.7	0.53	1.31	> 100	71.0
5	STG	3.9	+14.0	0.59	2.86	> 100	48.1
6	SLG	1.2	+6.4	0.73	2.29	> 100	47.6
7	SLG	0.8	+4.4	0.84	1.24	> 100	42.2
8	DS [d]	0.85	+92.1	2.1	2.36	= 139.2	22.7
9	CS [d]	15.9	-0.3	0.72	0.61	= 555.5	10
10	AZT (uM)				0.033	= 246.7	
11	ddC (uM)				4.52	= 2461.3	

[a] 50% of effective concentration.

[b] 50% of cytotoxic concentration.

[c] Anticoagulant activity; dextran sulfate H-039, 22.7 unit/mg.

[d] DS = Dextran sulfate; CS = Curdlan sulfate.

Another important biological activity, is blood anticoagulant activity. Table 2 shows the results of the blood anticoagulant activity of sulfated galactomannans. The anticoagulant activity measurement was performed at 37 °C using bovine plasma according to the US Pharmacopeia and compared with that of the commercial dextran sulfate H-039 (22.7 unit/mg) as a reference. Sulfated galactomannans had high anticoagulant activity of 42.2 unit/mg ~ 71.0 unit/mg, excepte SFG. It showed more potent active than linear polysaccharides of curdlan sulfate (10 unit/mg) and dextran sulfate (22.7 unit/mg).

Previous papers had been reported the branched sulfated polysaccharides had more potent anticoagulant activity than that of linear sulfated polysaccharides.[10, 11] Yoshida et al. had reported in many papers about synthesized branched polysacharides such as sulfated ribopyranan having 23% of glucose branch also showed anticoagulant activities of 47 unit/mg, it is higher than no branched (1→ 4)-β-ribopyranan (29 unit/mg).[12] In this research, the sulfated galactomannans reveal more potent blood anticoagulant activity than that of linear polysaccharides of dextran sulfate and curdlan sulfate et al.. It suggested that sulfate branched polysaccharides more strongly interacting with the glycoprotein, due to their flexible branched structure.

In conclusion, four kinds of galactomannans with different proportions of galactose (G) and mannose (M), from fenugreek gum (FG), guar gum (GG), tara gum (TG), and locust been gum (LG), were sulfated, and their specific biological activities such as anti-HIV and

blood anticoagulant activities were investigated, then to elucidate the relationship between the activities and the structure. It was found that the anti-HIV activities of the sulfated galactomannans were as high as that curdlan sulfate and dextran sulfate. Blood anticoagulant activities of sulfated galactomannans were potent higher than that of the linear polysaccharides of curdlan sulfate and dextran sulfate. Detailed investigations on the relationship between the activities and the structure of polymers are in progress.

References and Notes

[1] Muschin, T., Yoshida, T. Structural Analysis of Galactomannans by NMR Spectroscopy, 2011, in press.

[2] Yoshida, T. Synthesis of polysaccharides having specific biological activities. *Prog. Polym. Sci*., 2001, *26*, 379 - 441.

[3] Yoshida, T. Synthetic and natural polysaccharides having specific biological activities, in Polysaccharides: Structural diversity and functional versatility, 2nd Ed., S. Dumitriu, Ed., Marcel Dekker, Inc, New York, 2005.

[4] Lindahl, U.; Backstrom, G.; Thunberg, L. The antithrombin–binding sequence in heparin; *J. Biol. Chem*. 1983, *258*, 9826.

[5] Villanueva, G. B. Prediction of the secondary structure of antithrombin III and the location of heparin–binding site; *J. Biol. Chem*. 1984, *259*, 2531.

[6] I. Björk and U. Lindahl, Mechanism of the anticoagulant action of heparin; *Mol. Cell. Biochem.*, 1982, *48*, 161-182.

[7] D. L. Rabenstein; Heparin and heparan sulfate: structure and function; *Nat. Prod. Rep*., 2002, *19*, 312-331

[8] Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn. P.; Desmyter, J.; DeCercq, E. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds; *J. Virol. Methods* 1988, *20*, 309 – 321.

[9] U. S. Pharmacopoeia National Formulary, USP XXI 1985, pp. 480 – 483.

[10] S. Alban and G. Franz; Partial synthetic glucan sulfates as potential new antithrombotics: A review. *Biomacromolecules*, 2001 *2*, 354–361

[11] S. Alban, A. Schauerte, G. Franz; Anticoagulant sulfated polysaccharides: Part I. Synthesis and structure–activity relationships of new pullulan sulfates; 2002, *47*, 267-276.

[12] Yoshida, T. Synthetic and natural polysaccharides having specific biological activities, in Polysaccharides: Structural diversity and functional versatility, 2nd Ed., S. Dumitriu, Ed., Marcel Dekker, Inc, New York; 2005.