Doctoral Thesis

STRUCTURE AND ANTIVIRAL ACTIVITY OF WATER-SOLUBLE POLYSACCHARIDES IN ULAAN GOYO *(CYNOMORIUM SONGARICUM* RUPR.) PLANT

(鎖陽植物中の糖鎖構造と抗ウイルス性)

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A thesis Submitted to the Graduate School of Engineering in Partial Fulfillment of the Requirements for the Degree of

Doctor of Engineering

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September, 2015

Preface

The plant, *Cynomorium songaricum* Rupr. is used as a traditional medicine in China and Mongolia. In the present study, the new three water-soluble polysaccharides isolated from *C. songaricum* Rupr. were purified by successive Sephadex G-100, G75, G-50 and DEAE cellulose column chromatographies and then characterized by high resolution NMR and IR spectroscopies.

The molecular weights of the three polysaccharides were determined by an aqueous GPC to be $\overline{M}_n$=3.7 x 10^4 (CSP-1), 2.9 x 10^4 (CSP-2), and 1.0 x 10^4 (CSP-3), respectively. In addition, it was found that the polysaccharide with the larger molecular weights was an acidic polysaccharide (CSP-1 and CSP-3). It was found that the iodine-starch reaction of both CSP-1 and CSP-2 polysaccharides were negative and the methylation analysis gave 2, 4, 6-tri-*O*-methyl alditol acetate as a main product with 2, 3, 6-tri-*O*-methyl alditol as a small branch. NMR and IR measurements and sugar analysis revealed that both polysaccharides (CSP-1 and CSP-2) had a (1→3)-α-D-glucopyranosidic main chain with a small number of branches.

CSP-3 polysaccharide was negative in iodine-starch reaction and methylation analysis, sugar analysis, NMR and IR measurements gave 2, 3, 6-tri-*O*-methyl galactose and 2, 5-di-*O*-methyl-D-arabinose main chain with branches those are 2, 3, 4-tri-*O*-methyl arabinose and 2, 4, 6-tri-*O*-methyl rhamnose.

After sulfation, the sulfated CSP-1 and CSP-2 polysaccharides were found to have a potent inhibitory effect on HIV infection of MT-4 cells at a 50% effective concentration of EC_{50} = 0.3−0.4 μg/ml, which concentrations have almost the same high activity as standard dextran and curdlan sulfates, EC_{50} = 0.35 and 0.14 μg/ml, respectively. The 50% cytotoxic concentration was low, CC_{50}>1000 μg/ml.

In order to know the interaction between the sulfated polysaccharides (SCSPM, SCSP-1 and SCSP-2) and poly-L-lysine as a model protein compound was investigated by a surface plasmon resonance to reveal the anti-HIV mechanism. Before sulfation, *C. songaricum* Rupr. polysaccharides had no interaction with poly-L-lysine. After sulfation, sulfated *C. songaricum* Rupr. polysaccharides showed strong interaction. The apparent kinetic constants, association-rate (k_a), dissociation-rate (k_d), and association (K_D) constants, k_a = 9.8-40 x 10^4 1/Ms, k_d = 1.7-2.9 x 10^{-4} 1/s, and K_D = 0.4-2.9 x 10^{-9} M were obtained. These values were the same orders as those of the standard dextran sulfate with potent anti-HIV activity, suggesting that the sulfated *C. songaricum* Rupr. polysaccharides were strongly bound to poly-L-lysine and that the interaction was stable for a long time, probably due to the electrostatic interaction of the negatively-charged sulfated groups of the polysaccharides and positively-charged amino groups of poly-L-lysine.

For more investigation of the interaction between sulfated polysaccharides and poly-L-lysine, we measured particle size and zeta potential of CSP-M, CSP-1, CSP-2, sulfated CSP-M, CSP-1 and CSP-2 polysaccharides by Photal ELSZ-1000 analyzer. The particle size of sulfated

CSP-M polysaccharide was increased from 71.9±9.5 to 77.1±26.1 nm, when poly-L-lysine (40.7±6.9 nm) was added. SCSP-1 and SCSP-2 were also successfully increased from 17.2±6.5 to 154.7±11.2 nm and from 78.1±27.9 to 97.7±12.8 nm respectively. Also zeta potential (ζ) of sulfated *C.songaricum* polysaccharides (-25.96; -26.8; and -23.07 mV) were being close to zero (-18.36; -8.87; and -8.62 mV), when poly-L-lysine (ζ=0.41 mV) was added.

These results suggest that the sulfated polysaccharides gave strong interaction to poly-L-lysine and the interaction should be the reason why sulfated polysaccharides (SCSP-M, SCSP-1 and SCSP-2) had strong anti-HIV activity.

In this work, we report the new three polysaccharides extracted from of *C. songaricum* Rupr. Structural analyses of the polysaccharides were carried out by methylation analysis, high resolution NMR and IR spectroscopies and by comparison with the structure of starch. In addition, after sulfation, the polysaccharides were found to have strong anti-HIV activity.

Keywords: *Cynomorium songaricum* Rupr.; water-soluble polysaccharide; structure; NMR; Anti-HIV activity; SPR.

Contents

Chapter 1.

General Information

1.1. *Cynomorium Songaricum* Rupr. plant

Kingdom: *Plantae*
Phylum: *Tracheophyta*
Class: *Magnoliopsida*
Series: *Cynomoriales*
Family: *Cynomoriaceae*
Genus: *Cynomorium*
Species: *Cynomorium songaricum* Rupr.

Figure 1. *Cynomorium Songaricum* Rupr. [39].

Cynomorium songaricum Rupr. (Fig 1.) is an annual herb plant that is distributed widely in northwest Inner Mongolia of China and southern Mongolia. Other common name of this plant is red thumb, Chinese cynomorium, or desert thumb.

In Mongolia, the stem of *Cynomorium songaricum* Rupr. is called Ulaan goyo and also called Soyang in China, is parasitic on the roots of salt-tolerant plants, mainly species of genera Nitraria, but is also known to parasitize the roots of other plants (*Atriplex* so on). It is mainly produced in Gobi-Altai province of Mongolia and Inner Mongolia, Gansu, Qinghai and Xinjiang of China.

The chemical composition reported from *C.songaricum* include triterpenes [1] [2] [3] [4] [5], fructosides [6], flavanoids, condensed tannins [7], lignan glucopyranosides, alkaloids [8], and acidic heteropolysaccharides [8]. The triterpenes and tannins of *C.songaricum* were also reported to have inhibitory activity against HIV protease [9].

For Chinese and Mongolian traditional medicine, the stem of *C.songaricum* is used to improve immunity and kidney function and also treat various types of disease [10], facilitating catharsis [10], and improving lumbar weakness [6].

The pharmacological properties, which has been demonstrated various constituents of *C.songaricum*, such as catechin, binding tannides, polysaccharides, etc [13]. The *C.songaricum* gave not only showing anti-hypoxia or anti-anoxia effects [4] [5], anti HIV activity [7], immunity improvement [14], physical endurance enhancement [15], and anti-oxidation [16], but also having many actions such as anti-dementia [17], antibrain aging [18], anti-epilepsy [19], anti-stress [20], and anti-fatigue [21].

1.2 Plant polysaccharides

In general, polysaccharides are important to several biological function in organism. For example, starch is converted to energy and stored in liver and muscles. The cellulose is major component of structure in plant.

In Asian traditional medicine, plants are generally used to treat various types of illnesses, including wounds, both external and internal since ancient times. The many plants can be found as part of traditional medicine on all over the world. For example, in the Western countries, plants are still in use and called “Traditional remedies” [31]. In medicinal plants, polysaccharides have more several pharmacological activities than another high molecular weight components of medicinal plants [32].

Plant polysaccharides have been subject of studies for a long time, the main reason for focusing on their physic and chemical properties and their application. The last 30 years there is an increasing interest in polysaccharides from various plant sources.

Polysaccharides appear in many different forms and in different parts of plants. There are two types of polysaccharides that consist of one type of monosaccharide, is called a homo-polysaccharide, and composed of two and up to approximately ten different types, some of which may be in repeating units, is called a hetero-polysaccharide: they can be linear or branched and be substituted with different types of organic groups like methyl and acetyl groups [31].

Biological activity of plant polysaccharides have been tested in various systems. The isolation, purification, and structural elucidation of medicinal plant polysaccharides may have important implications in the treatment of various other diseases [33].

1.2.1 *Cynomorium Songaricum* polysaccharides

Several reports on the isolation and structure of polysaccharides from the plant have been published. However, no complete structural analysis has been reported [24] [25] [26] [9]. A water-soluble polysaccharide from the plant was purified by Ultrahydrogel 500 chromatography to give a heteropolysaccharide consisting of arabinose, glucose, and galactose [25].

Sugar analysis of the polysaccharide performed by GC-MS indicated that the polysaccharide was composed of rhamnose (3.70%), arabinose (15.15%), mannose (13.58%), glucose (44.26%), and galactose (23.30%) [27].

1.3 Biological activities

1.3.1 Biological activity of *Cynomorium songaricum* polysaccharides

In Mongolia and China, the root and stem of the plant have been used as a traditional medicine to improve immunity and kidney function and to treat constipation by relaxing the bowels. There are several reports on the

constituents and biological activity of the plant. Zhao et al found that components of the stem extracted with chloroform and n-butyl alcohol strongly activated dopamine and norepinephrine uptake by D8/N cells and decreased γ-aminobutyric acid and serotonin uptake by G/S6 cells, respectively. The active constituents have not been identified yet [37].

The triterpene, ursolic acid and its hydrogen malonate, extracted by CH_2Cl_2 and MeOH from the plant were reported to have potent inhibitory activity against HIV-protease at a 50% inhibitory concentration (IC_{50}) of 6-8 μM, respectively. In addition, water-extracted flavanols also potently inhibited HIV-1 protease activity [33]. The selenide polysaccharide was synthesized by selenylation of *C. songaricum* Rupr. polysaccharides and the structure characterized by UV, IR, and Raman spectra [26]. The selenide polysaccharides significantly decreased blood glucose levels and increased serum insulin levels in streptozotocin-induced diabetic rats [25], however, antitumor activity was not shown [24] [26].

1.3.2 HIV / AIDS

The Human Immunodeficiency Virus (HIV) is a retrovirus that causes the acquired immunodeficiency syndrome (AIDS). But structure is different from other retroviruses. It is roughly spherical with diameter of about 120 nm. HIV targets the immune system ($CD4^+$T cells) and defense systems against infections and some types of cancer [34] [35]. Immune function is typically measured by CD4 cell count. HIV is grouped in two types named

HIV-1 (global) and HIV-2 (West Africa). HIV-2 is muss less pathogenic than HIV-1 and is restricted in its worldwide distribution.

Many researchers are attempting to prevent and treat HIV/AIDS in the world.

1.3.3 Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) was first demonstrated by Otto in 1968, but was not made commercially available for interaction applications under the fall of almost two decades ago by Biocare (GE healthcare). SPR biosensors can be used for sensing purposes, for example it's a main tools for biomolecular interaction analysis.

In this study, we examined interaction of poly-L-lysine (model of glycoprotein) and isolated polysaccharides of *Cynomorium songaricum* Rupr. (CSP-M, CSP-1 and CSP-2) by a Biocare X100 (GE healthcare) and a CM5 sensor chip.

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Chapter 2.

Sugar analysis and Isolation of *Cynomorium Songaricum* Rupr. polysaccharides

2.1 Abstract

The plant, *Cynomorium songaricum* Rupr. is used as a traditional medicine in China and Mongolia. In the present study, new three water-soluble polysaccharides isolated from *C. songaricum* Rupr. were purified by successive Sephadex G-100., G-75, G-50 and DEAE Cellulose column chromatographies and then characterized by high resolution NMR and IR spectroscopies. The molecular weights of the two polysaccharides were determined by an aqueous GPC to be $\overline{M}_n$=3.7 x 10^4, 2.9 x 10^4 and 1.0 x 10^4, respectively. In addition, it was found that the polysaccharide with the larger molecular weight was an acidic polysaccharide.

Keywords: *C.songaricum*, column chromatography, sugar analysis, GPC.

2.2. Introduction

Cynomorium Songaricum Rupr., called Suo Yang in China and Ulaan goyo in Mongolia, is an annual herb plant that is distributed widely in norwest Inner Mongolia in China and southern Mongolia.

The structural characterization of a Chinese lacquer polysaccharide, *Rhus vernicifera*, performed by NMR spectroscopy including 2D NMR measurements, indicated that the lacquer polysaccharide had a 1, 3-β-linked D-galactopyranosidic main chain with complex side chains consisting of D-galactose, 4-*O*-methyl-D-glucuronic acid, D-glucuronic acid, L-arabinose, and L-rhamnose [1].

In addition, the lacquer polysaccharide had two molecular weight fractions, $\overline{M}_n = 10 \times 10^4$ and $\overline{M}_n = 3.0 \times 10^4$. The two fractions were isolated by a Sephadex G-100 column chromatography and then treated with dilute alkaline solution to decrease the molecular weights, suggesting that the lacquer polysaccharides have an associated structure with several low molecular weight polysaccharides of $\overline{M}_n = 1.4 \times 10^4$ [2].

2.3. Experimental

2.3.1 *Materials*

The plant material, *Cynomorium songaricum* Rupr. was obtained in Hohhot, Inner Mongolia, China. After washing with water and then drying, the dried *C.songaricum* Rupr was homogenized to a fine powder and kept in a desiccator at room temperature.

The monosaccharide analysis was carried out with a Honenpak C18 column (4.6 mmϕ x 75 mm) eluted with 7% acetonitrile/0.2 M potassium borate buffer (pH 8.9) solution by a fluorescence detector at 305 nm and 360 nm.

Reagents

A 4-aminobenzoic acid ethyl ester (ABEE) labeling kit and standard monosaccharide mixture-11 were purchased from J-Oil Mils Co., Ltd., Tokyo, Japan, for the sugar analysis. Other reagents were obtained from companies and used without further purification.

2.3.2 Measurements

Specific rotation of 0.5 wt% of polysaccharide samples in H_2O solution was recorded by a JASCO DIP-140 digital polarimeter at 25°C. Infrared spectra were measured by a Perkin Elmer Spectrum One FT-IR spectrometer. An aqueous phase GPC (Hitachi Co., Tokyo, Japan) was used for

determination of the molecular weights of polysaccharide at 40°C by TOSOH TSK-gel columns (7.6 mm x 300 mm x 3) of G2500PW$_{XL}$, G3000PW$_{XL}$, and G4000PW$_{XL}$, eluted with 66.7 mmol phosphate buffer, pH=6.68) for water-soluble polysaccharides using pullulan as a reference.

In this study, the monosaccharide components of the CSP-1, CSP-2, CSP-3 and CSP-M were identified with 4-aminobenzoic acid ethyl ester (ABEE) according to the instruction manual (J-Oil Mills Co., Ltd., Tokyo, Japan) for the GlyScope sugar labeling Kit ABEE labeling kit.

2.3.3 Purification and Isolation of crude polysaccharides

Extraction of crude polysaccharides

The powdered *C.songaricum* Rupr (200g) was treated with 500 ml of 98% ethanol preheated to 50^{o}C for 3 hours twice. It was then filtered through filter paper, after which the insoluble material was treated three times with boiling water (1:5 w/v) for 3 hours, 3 times. After filtration, the combined filtrate was concentrated to 100 ml by a rotary evaporator under reduced pressure and then added cold 95% ethanol (v/v) was added at 4^{o}C to produce precipitates for overnight, which were collected by centrifugation for 20 min (5000 rpm) and washed several times with ethanol. The precipitate obtained was dissolved in ion-exchanged water and dialyzed (MWCO: 14000 Dalton) for 48 hours to obtain 2.5 g of crude polysaccharide (CSP-M) after vacuum freeze-drying.

Purification and Isolation of polysaccharides

The crude polysaccharides were subjected to a Sephadex G75 column and eluted with the phosphate buffer at the pH of 6.86. The fractions containing polysaccharides were automatically collected by a fraction collector. The two combined solutions were dialyzed (MWCO: 14000 Dalton) with deionized water, respectively, and then freeze-dried to obtain white powders, CSP-1 (53.3 mg), CSP-2 (15.1 mg) and CSP-3 (20 mg).

The two white powders were further purified by Sephadex G50 column chromatography to give two pure polysaccharides, respectively. An aqueous phase gel permeation chromatography (GPC) was used for determination of the molecular weights of polysaccharide at 40^{o}C by TOSOH TSK-gel columns (7.6 mm x 300 mm x 3) of G2500PW$_{XL}$, G3000PW$_{XL,}$ and G4000PW$_{XL}$, eluted with 66.7mmol/l phosphate buffer, pH= 6.86) for water-soluble polysaccharides with IR detector using TOSOH pullulan standards having molecular weights of 0.59 x 10^{4}, 1.18 x 10^{4}, 2.28 x 10^{4}, 4.73 x 10^{4}, 11.2 x 10^{4}, 21.2 x 10^{4}, 40.4 x 10^{4}, and 78.8 x 10^{4}. The elution rate and pressure of the phosphate buffer eluent were 0.8ml/min and 48 kgf/cm^{2}, respectively.

Specific rotation of 0.5 wt% of polysaccharide samples in H_2O solution was recorded by a JASCO DIP-140 digital polarimeter at 25°C in a water-jacketed 10 ml quartz cell.

2.3.4 Reduction of methyl glucuronate in polysaccharide CSP-1

The polysaccharide was reduced according to a carboxyl reduction method of Taylor and Conrad [3], followed by either monosaccharide analysis or by monosaccharide linkage analysis (methylation). In this method, carboxylic group are first reduced with sodium borodeuteride ($NaBD_4$) to generate 6,6'-dideuteriosugars, which can be distinguished from neutral sugars by GC-MS by the presence of fragment ions with increased masses (M^++2) [4].

The CSP-1 polysaccharide (40 mg) was dissolved in deuterium oxide (D_2O) (10 ml) and then 2 M $NaBD_4$ solution in D_2O (25 ml) was added dropwise. pH of the solution was kept at 7 by 4 N HCl solution. The mixture was stirred for 1 h at room temperature and acidified with 4 N HCl to pH 4. The solution was dialyzed against deionized water for 48 h and then freeze-dried to give 37 mg of a reduced CSP-1 polysaccharide. Further evaporate three times with 2-3 ml of 5% acetic acid in methanol and three times with 2-3 ml of methanol to remove boric acid.

In IR and ^{13}C NMR spectra, the absorption due to the carboxylic acid disappeared.

2.3.5 Sugar analysis

The monosaccharide components of CSP-M, CSP-1, CSP-2 and CSP-3 were analyzed by the 4-aminobenzoic acid ethyl ester (ABEE) method according to the company's instructions (J-Oil Mils Co., Ltd.) by using an ABEE labeling kit [5]. A typical procedure is as follows: the CSP-1 polysaccharide solution (10 µl) (1 mg of dried CSP-1 polysaccharide dissolved in 100 µl of H_2O) was hydrolyzed with 4 M trifluoroacetic acid (TFA) (10 µl) at 100°C for 3 h. After the solution was evaporated to dryness, *N*-acetylation was carried out with acetic anhydride (10 µl) in pyridine-methanol (1:9 v/v) solution (40 µl) for 30 min at room temperature. The solution was evaporated under reduced pressure to dryness and then H_2O (10 µl), and the ABEE solution (40 µl) were added. The mixture was stirred for 1 h at 80°C to introduce ABEE into aldehydes at the C1 position of hydrolyzed monosaccharides by a reductive amination. The solution was cooled to room temperature, and then H_2O (200 µl) and chloroform (200 µl) were added and stirred. The aqueous phase was collected and then filtered to give an aqueous solution of ABEE-labeled monosaccharides, which were analyzed by a Honenpak C18 column on a HPLC instrument to obtain D-glucose, D-galactose, D-mannose, L-arabinose, and D-ribose in the proportions of 83.6%, 2.4%, 2.9%, 3.6%, 3.5%, and 2.0%, respectively. The other polysaccharides, CSP-M, 2 and CSP-3, were analyzed by the same procedure.

2.4 Result and discussion

Extraction and purification of polysaccharides from C. songaricum Rupr.

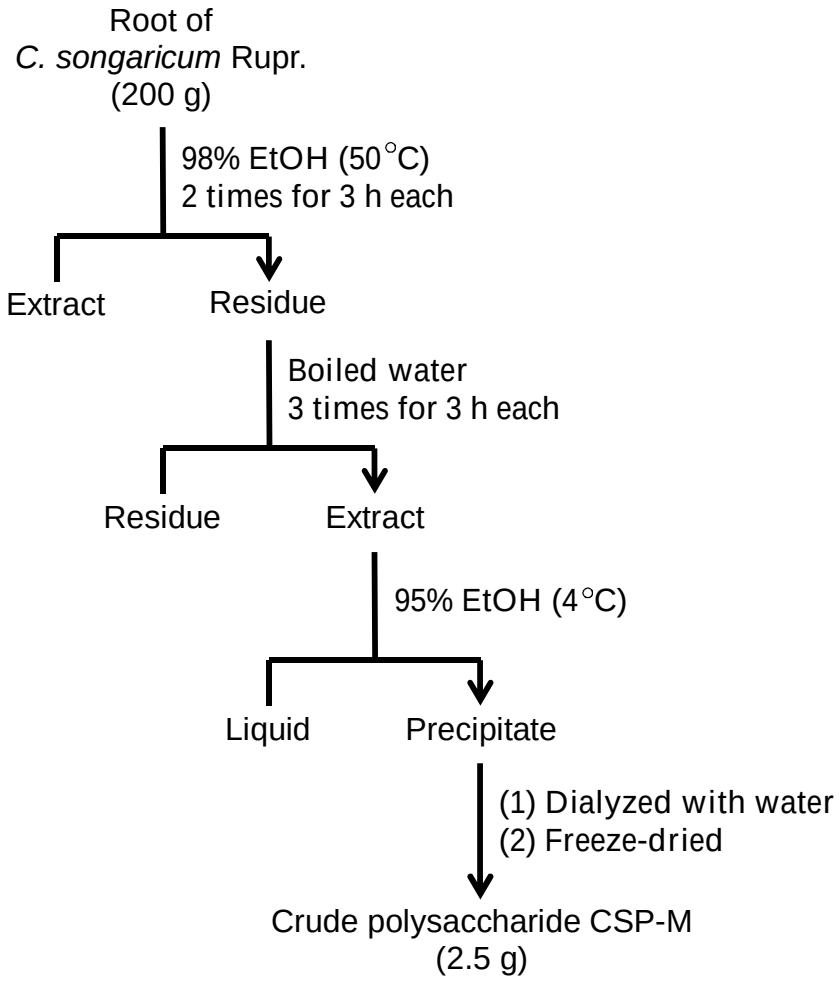

Figure 2. Extraction of water-soluble polysaccharides from the root of *C. songaricum* Rupr., a plant used as a traditional medicine in China and Mongolia.

Figure 2 shows the extraction scheme of the polysaccharides from *C. songaricum* Rupr. After removal of oily constituents in the dried and

powdered *C. songaricum* Rupr. (200 g) by extraction with ethanol two times, the residue was treated with boiling water to give a water-soluble extract in 2.5 g yield after precipitation with a cold ethanol and then freeze-drying of the precipitate dissolved in water. We found that the water-soluble extraction had three molecular-weight fractions measured as the aqueous GPC. The three fractions were isolated by the Sephadex G75 column and subsequent G50 chromatography to give three pure CSP-1, CSP-2 and CSP-3 fractions.

Figure 3 shows the GPC profiles of the water-soluble extract CSP-M (3A) and the three purified fractions, CSP-1 (3B), CSP-2 (3C) and CSP-3 (3D), respectively. The molecular weights of the three fractions were $\overline{M}_n$ = 3.7 x 10^4, $\overline{M}_n$ = 1.0 x 10^4 and $\overline{M}_n$ = 2.9 x 10^4 for CSP-1,CSP-2 and CSP-3, as shown in Figures 4B, 4C and 4D respectively. The specific rotation of the CSP-1 and CSP-2 fractions were positive and large, +148.6° and +172.5°, respectively, suggesting that the polysaccharides have an α-glycosidic linkage. But CSP-3 fraction was negative - 87.7°

Structure of polysaccharides

Infrared spectra were measured by a Perkin Elmer Spectrum One FT-IR spectrometer (PerkinElmer Co., Tokyo, Japan).

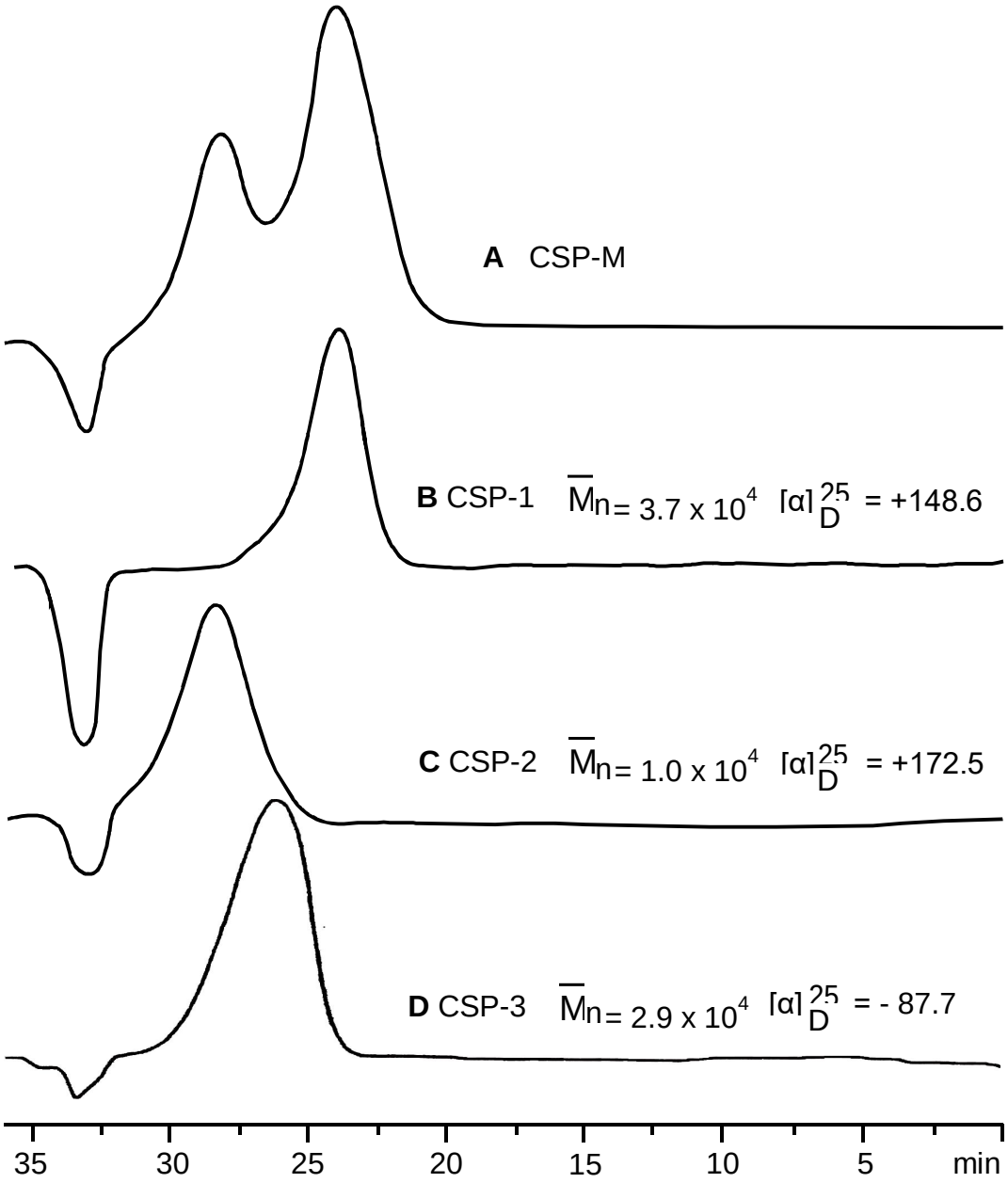

Figure 3. Aqueous GPC profiles of *C.songaricum* Rupr. polysaccharides after extraction and isolation by Sephadex column chromatography. (A) Crude polysaccharides CSP-M after extraction, (B), (C) and (D) isolated polysaccharides.

FTIR analysis

The structures of the two polysaccharides CSP-1, CSP-2 and CSP-3 were analyzed by IR and NMR spectroscopies. Figure 4 exhibits the IR spectra of the water-soluble extractions.

CSP-M (4A), CSP-1 (4B), CSP-2 (4C) and CSP-3 (4D), respectively, in which the spectra were similar to each other. The characteristic large signals at 3398 cm^{-1} and 1050 cm^{-1} due to OH and C-O stretching vibrations of the sugar residues appeared in the spectra.

In the spectrum 4B for CSP-1, it was found that the absorption at 1740 cm^{-1} appeared due to a stretching vibration of carboxylate, suggesting that the CSP-1 fraction consists of glucuronate residues. On the other hand, no absorption at 1740 cm^{-1} appeared in Figure 4C, indicating that the CSP-2 fraction had no acidic sugar residues.

The IR results suggest that the purified CSP-1, CSP-2 and CSP-3 are polysaccharides. In the spectrum of CSP-M, the absorption at 1050 cm^{-1} was relatively weaker than that of the CSP-1 and CSP-2, suggesting that polysaccharides in the CSP-M fraction are fewer in quantity.

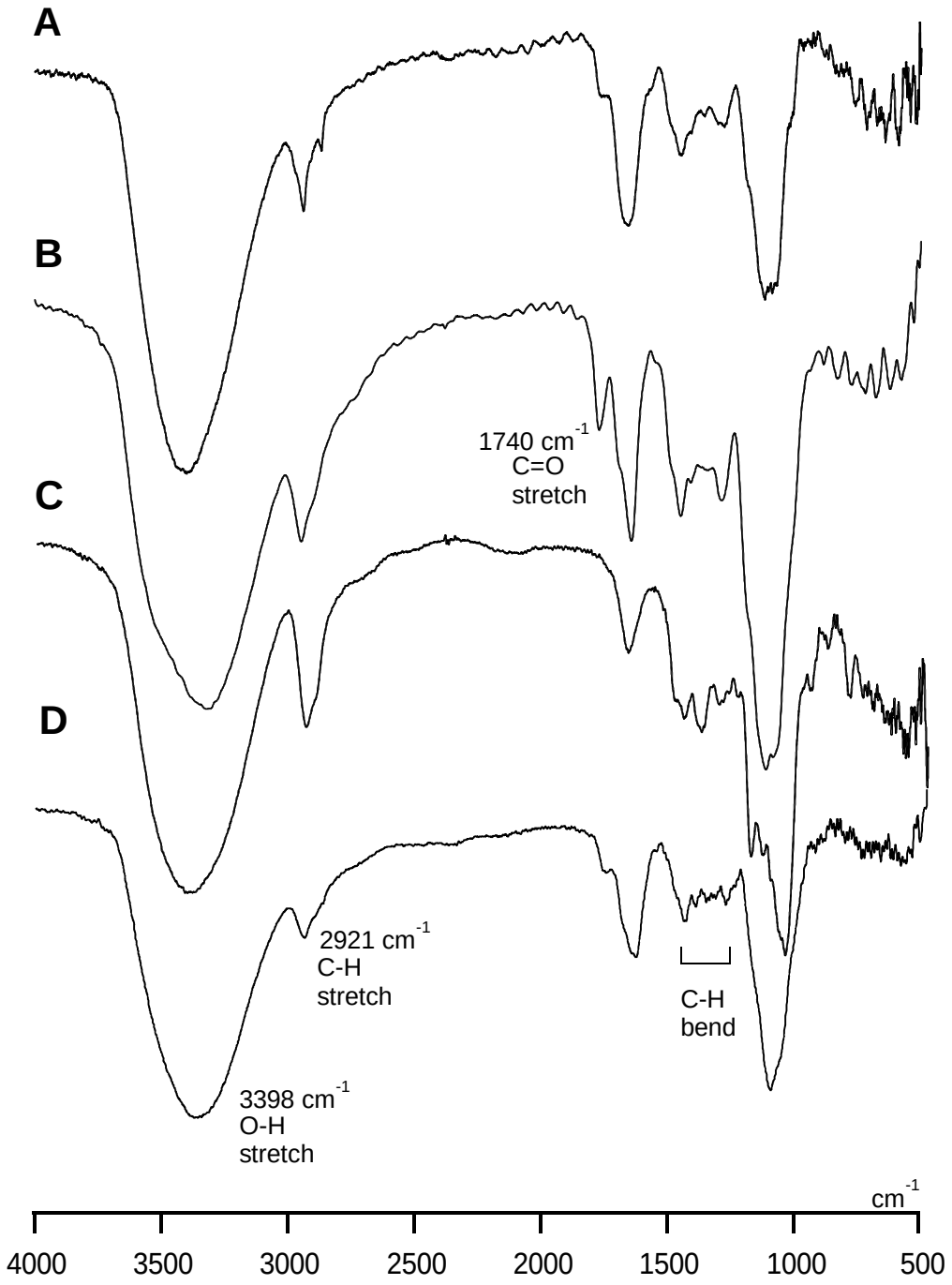

Figure 4. FT-IR spectra of *C.songaricum* Rupr. polysaccharides (KBr method). (A) CSP-M, (B) CSP-1, (C) CSP-2.and (D) CSP-3.

Sugar analysis of CS polysaccharides by HPLC using for ABEE labeling Kit

Sugar analysis of the CSP-M, CSP-1, CSP-2, and CSP-3 polysaccharides was carried out by degradation with 2N TFA according to the company's instructions [5]. Before hydrolysis with 2N TFA, CSP-1 was directly reduced by $NaBD_4$ into deuterated glucose because the CSP-1 polysaccharide had methyl glucuronate revealed by the measurements of IR and NMR as mentioned below.

Figure 5 shows the HPLC profiles of monosaccharide residues and the profile of the monosaccharide standards is also presented in Figure 5D. In Figure 5B and 5C, D-glucose was found to be a main residue and small amounts of D-galactose, D-mannose, and L-arabinose were detected.

Table 1. Sugar composition

	Retention time		*CSP-1 Conc. (%)*	*CSP-2 Conc. (%)*	*CSP-3 Conc. (%)*	*CSP-M Conc. (%)*
Gal	10.43	D-Galactose	2.4	1.7	35.3	26.3
Man	13.20	D-Mannose	2.9	3.6	-	5.2
Glc	15.28	D-Glucose	83.6	84.8	5.8	11.7
Ara	16.36	L-Arabinose	4.6	3.5	43.6	43.8
Rib	18.37	D-Ribose	2.0	2.0	-	1.0
Rha	28.40	L-Rhamnose	-	-	8.5	-

Methyl D-glucuronate was not detected by HPLC, because the carboxyl methyl group at the C6 position due to methyl glucuronate in the CSP-1 polysaccharide was reduced by $NaBD_4$ into deuterated glucose. Also no fucose and rhamunose with methyl group were detected in CSP-1 and CSP-2 polysaccharides. The results of the sugar analysis are shown in Table 1. Taking into account the integration values of the peaks, the proportions of D-glucose were calculated to be 83.6% and 84.4% in the CSP-1 and CSP-2 polysaccharides, respectively.

As shown in Table 1, the CSP-1 and CSP-2 fractions contained larger amounts of D-glucose. But galactose and arabinose were major sugar components in the CSP-3 and CSP-M.

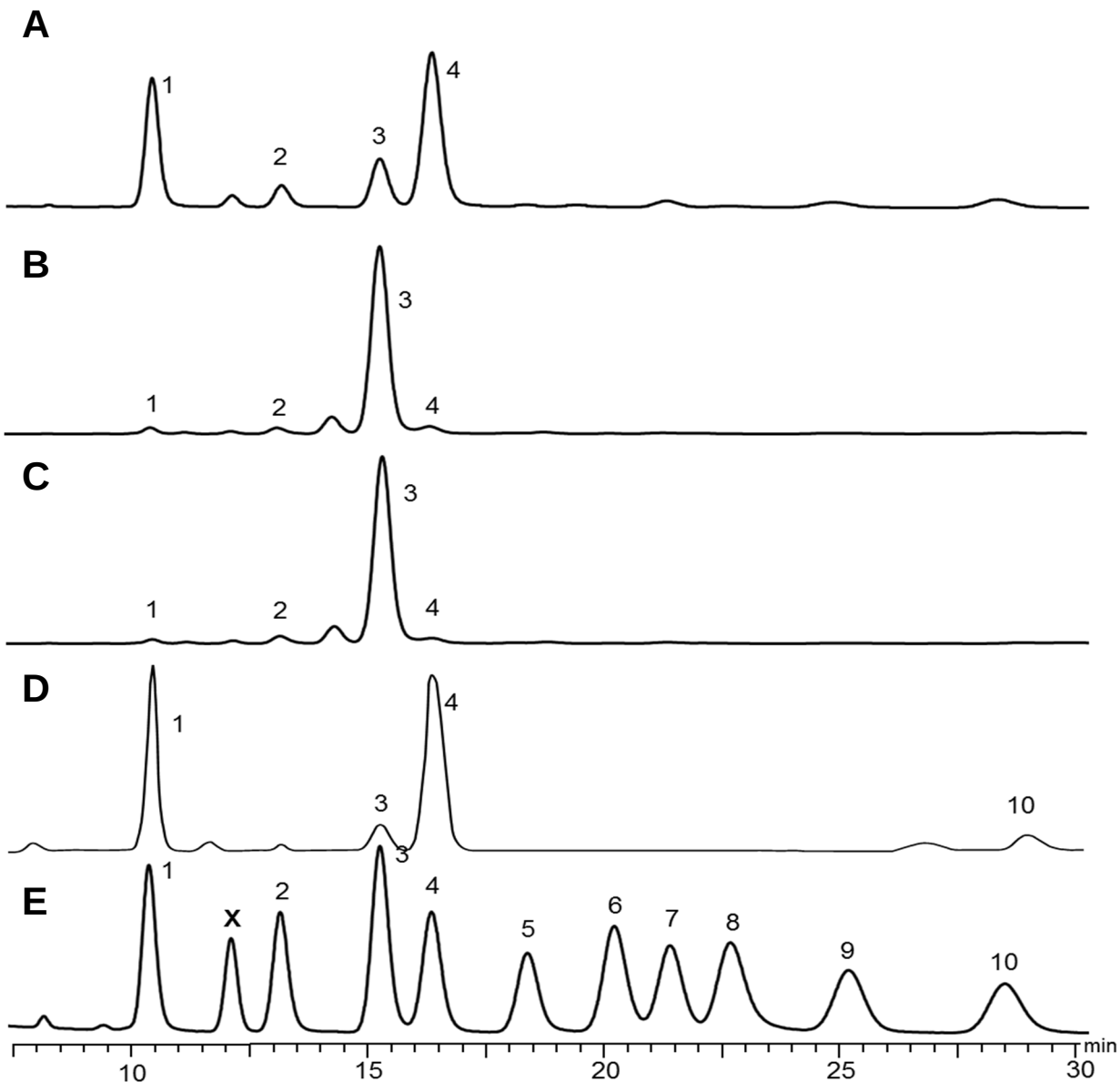

Figure 5. HPLC profiles of CSP-M (A), CSP-1 (B), CSP-2 (C), CSP-3 (D) monosaccharide residues after hydrolysis (2N TFA) of *C.songaricum* Rupr. polysaccharide, and monosaccharide standards (D). Peaks: 1. D-galactose, 2. D-mannose, 3. D-glucose, 4. L-arabinose, 5. D-ribose, 6. *N*-acetyl-D-mannosamine, 7. D-xylose, 8. *N*-acetyl-D-glucosamine. HPLC condition: column: Honenpak C18. Solvent A: 0.2M potassium borate buffer / acetonitrile (93 / 7), solvent B: 0.02% TFA / acetonitrile (50 / 50). Flow rate: 1 mL / min.

2.5 Conclusion

In this chapter we found that the water-soluble extraction had three molecular-weight fractions measured as the aqueous GPC. The CSP-1 and CSP-2 fractions were almost the same structure by IR, monosaccharide analysis and specific rotations. The molecular weights of the three fractions were $\overline{M}_n$ = 3.7 x 10^4, $\overline{M}_n$ = 1.0 x 10^4 and $\overline{M}_n$ = 2.9 x 10^4 for CSP-1, CSP-2 and CSP-3, respectively.

The specific rotation of the CSP-1 and CSP-2 fractions were positive and large, +148.6° and +172.5°, respectively, suggesting that the polysaccharides have an α-glycosidic linkage. But CSP-3 fraction was negative - 87.7°.

In sugar analysis of CSP-1 and CSP-2 fractions, D-glucose (83.6 % and 84.8 %) was found to be a main residue and small amount of D-galactose, D-mannose and L-arabinose. CSP-3 fraction containing residues of D-galactose (35.3 %), L-arabinose (43.6 %) and L-rhamnose (8.5 %).

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Chapter 3.

Structure analysis of *Cynomorium Songaricum* Rupr. polysaccharides by NMR spectroscopy and Methylation analysis

3.1 Abstract

Structural characterization of the *Cynomorium Songaricum* polysaccharides were performed by means of high resolution NMR spectroscopy (600 MHz) involving two dimensional NMR measurements such as field gradient COSY, TOCSY, HMQC, and HMBC experiments. Most of complicated proton and carbon absorptions were assigned. It was identified that the CSP-1 and CSP-2 polysaccharides had a 1, 3-linked D-glucopyranosidic main chain with a small number of branches. The coupling constants ($J_{1,2}$) of anomeric protons in the COSY spectrum suggested that the glucopyranose residue in the main chain (4.96 ppm) had α-linkage, and the D-glucopyranose (5.14 ppm) in the side-chain had α-linkage.

The proportions of the component sugar units were calculated by the intensity of the anomeric carbon signals and sugar analysis. CSP-1 was found that the polysaccharide with the larger molecular weight was an

acidic polysaccharide. It was found that the iodine-starch reaction of both isolated polysaccharides was negative and the methylation analysis gave 2, 4, 6-tri-*O*-methyl alditol acetate as a main product.

1D, 2D NMR measurement and methylation analysis revealed the presence of the following units:

→4)-α-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→3)-α-Ara*f*-(1→3)-α-D-Glc*p*-(1→

Methylation analysis of CSP-3 (2.9×10^4) polysaccharide gave to have a 2, 3, 6-tri-*O*-methyl galactose and 2, 5-di-*O*-methyl-D-arabinose main chain with branches those are 2, 3, 4-tri-*O*-methyl arabinose and 2, 4, 6-tri-*O*-methyl rhamnose.

Keywords: NMR spectroscopy, methylation analysis, linkage analysis.

3.2 Introduction

*Cynomorium songaricu*m is distributed widely in Mongolia and China. The chemical composition reported from *C.songaricum* include triterpenes, fructosides, flavanoids, condensed tannins, lignan glucopyranosides, alkaloids, and acidic heteropolysaccharides. The triterpenes and tannins of *C.songaricum* were also reported to have inhibitory activity against HIV protease.

The structural characterization of a Chinese lacquer polysaccharide, *Rhus vernicifera*, performed by NMR spectroscopy including 2D NMR measurements, indicated that the lacquer polysaccharide had a 1, 3-β-linked D-galactopyranosidic main chain with complex side chains consisting of D-galactose, 4-*O*-methyl-D-glucuronic acid, D-glucuronic acid, L-arabinose, and L-rhamnose [2].

Several reports on the isolation and structure of polysaccharides from the *C.songaricum* plant have been published. The structure analysis of *C.songaricum* polysaccharide reported to have →3)-α-ara*f*-(1→3)-α-d-glc*p*-(1→4)-α-d-Gal*p*A6Me-(1→ structure [3]. However, no complete structural analysis has been reported.

The structure analysis of polysaccharides requires specialized techniques and methods. For example, NMR and methylation analysis. NMR spectroscopy has become the most powerful chemical technique for establishing polysaccharides structure. The glycosidic linkage positions of sugar units in a polysaccharides is established by methylation analysis.

3.3 Experimental

3.3.1 Materials

The ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded by a JEOL ECX-600 spectrometer (Jeol Co., Tokyo, Japan) in D_2O

as a solvent at 40°C. The 2D ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC spectra were measured on the JEOL-supplied programs.

Methylation analysis was carried out by the Hakomori method [1] using sodium hydride (NaH) and methyl iodine (CH_3I). The structure of the alditol acetate was analyzed by a JEOL JMS-T100 GCV GC-MS spectrometer (Jeol Co., Tokyo, Japan).

3.3.2 Measurement

NMR spectroscopy

The isolated and fractionated *Cynomorium songaricum* polysaccharide samples (50 mg) were prepared for NMR measurements. All NMR spectra were recorded at 40°C in D_2O. Sodium 4, 4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as an internal standard at δ 0 ppm for ^{1}H and δ 0.015 ppm for ^{13}C spectra.

Methylation analysis

The methylation analysis was carried out according to the method of Hakomori [1]. The typical procedure is as follows. The CSP-1 and CSP-3 polysaccharides (10 mg) was dissolve in dry DMSO solution (10 ml) of 2M methyl sulfinylmethyl sodium under N_2 atmosphere. After stirring for 10 h at 30°C, methyl iodine (5 ml) was added to the mixture, which mixture was further stirred for 3 h at 30°C. The methylated polysaccharide was obtained

after dialyzing with deionized water overnight, extraction with chloroform, and evaporation. The methylation procedure was repeated three times until the OH signal of IR spectrum on the methylated polysaccharide disappeared.

The methylated polysaccharide was hydrolyzed with 2N trifluoroacetic acid at 110°C for 16 h. The hydrolyzed product was reduced with $NaBH_4$ and then acetylated with acetic anhydride (10 ml) and dry pyridine (10 ml) solution at 100°C to give alditol acetate.

3.4 Result and Discussion

3.4.1 Assignment of proton and carbon signals by COSY and HMQC 2D NMR measurements

The ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded by a JEOL ECX-600 spectrometer (Jeol Co., Tokyo, Japan) in D_2O as a solvent at 40°C. The 2D H-H COSY, HMQC, TOCSY and HMBC spectra were measured on the JEOL supplied programs. The COSY (Figure 8, 10) was carried out with 1024 x 1024 data matrix and pulse delay was 1.5 s.

Figure 7 shows the ^{13}C NMR spectra of the water-soluble extracts CSP-M (7A), CSP-1 (7B), CSP-2 (7C), and, CSP-3 (7D) in D_2O, respectively. After purification by the Sephadex column chromatography, the ^{13}C NMR signals of the CSP-1 and CSP-2 fractions (Figures 7B and 7C) became simple. Carbonyl and methyl carbon signals at 184 ppm and 26 ppm were found to appear in the CSP-1 spectrum (Figure 7B), assuming that the CSP-1

polysaccharide had carboxyl methyl groups at the C6 position, probably due to methyl glucuronate residues.

However, the proportion of methyl glucuronate in the polysaccharide was small according to the intensity of the signals.

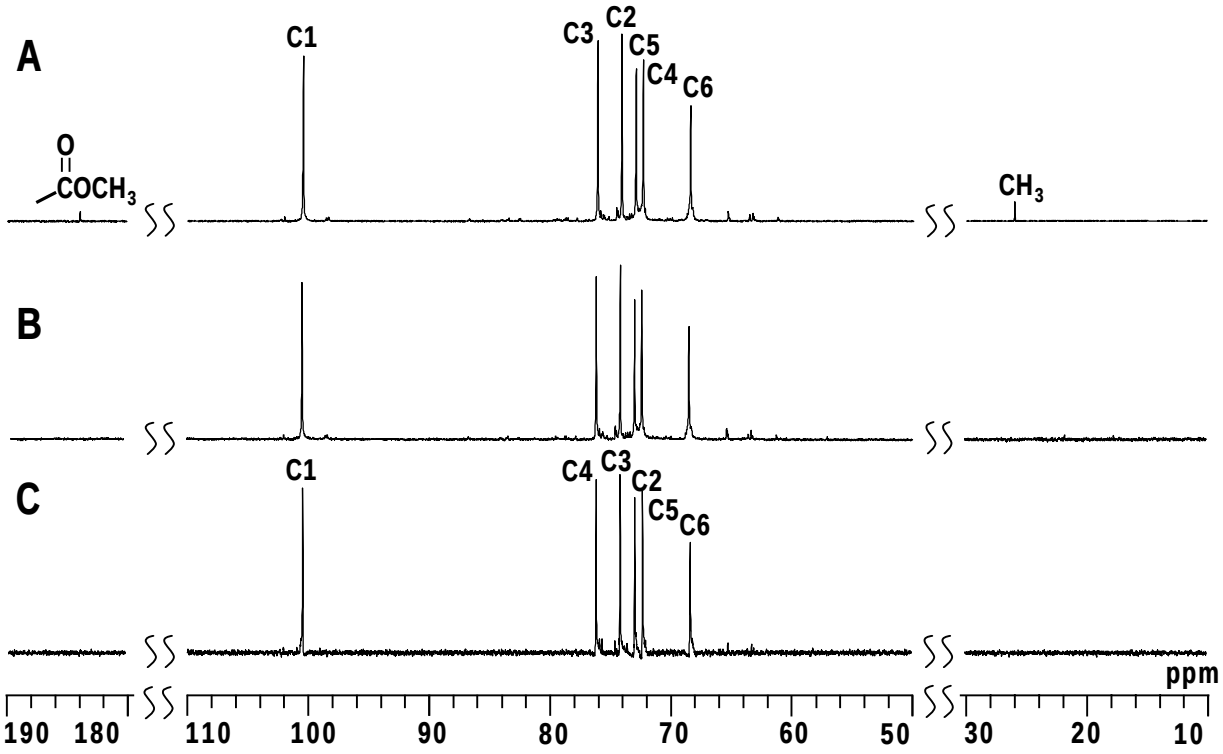


Figure 6. 600 MHz ^{13}C NMR spectra of (A) CSP-1 (B) CSP-2 and (C) Starch with $\overline{M}n$ = 3.0 x 10^4 and $[\alpha]_D^{25}$ +152.1 (c 1, H_2O). DSS (0.015 ppm) was used as an internal standard. Signals were assigned by both COSY and HMQC measurements in D_2O at 40° C.

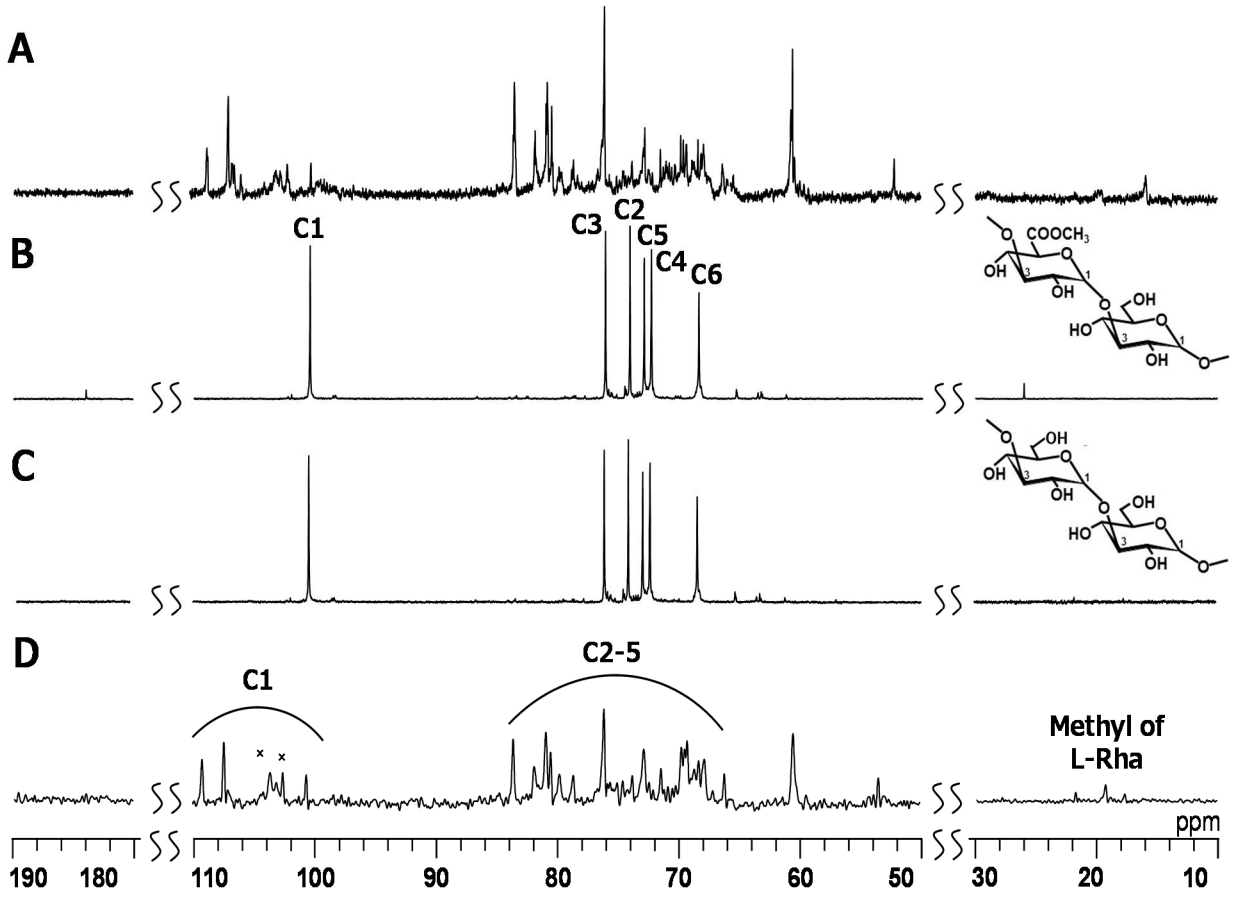


Figure 7. 600 MHz ^{13}C NMR spectra of (A) CSP-M (B) CSP-1 and (C) CSP-2 and (D) CSP-3. DSS (0.015 ppm) was used as an internal standard. Signals were assigned by both COSY and HMQC measurements in D_2O at 40° C.

The CSP-1 and CSP-2 polysaccharides had six main signals due to glucose residues, respectively, which signals appeared at the same positions in Figures 7B and 7C.

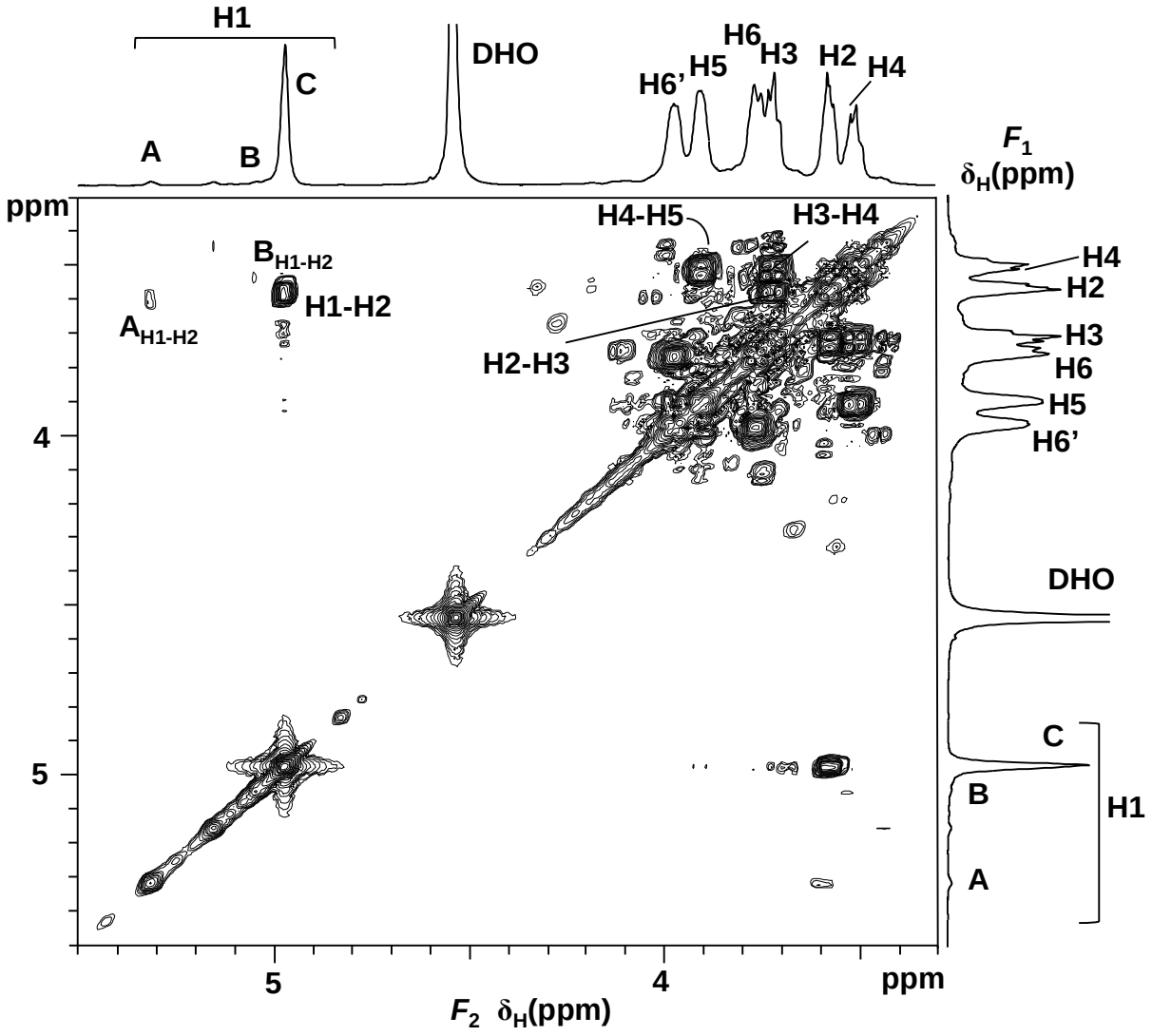


Figure 8. ^{1}H-^{1}H COSY spectra of CSP-1 and CSP-2 (D_2O as solvent, 50°C).

After purification by the DEAE cellulose and G75 sephadex column chromatography, the ^{13}C NMR signals of the CSP-3 fraction (Figures 7D) is roughly same to CSP-M fraction. In the proton spectrum, doublet signal appeared at 1.25 ppm, which was correlated to the carbon signals at 19.3

ppm in HMQC spectrum (Figure 11). These result suggested that the C6 methyl group of L-rhamnose could be assigned at 1.25 ppm and 19.3 ppm for proton and carbon signals, respectively.

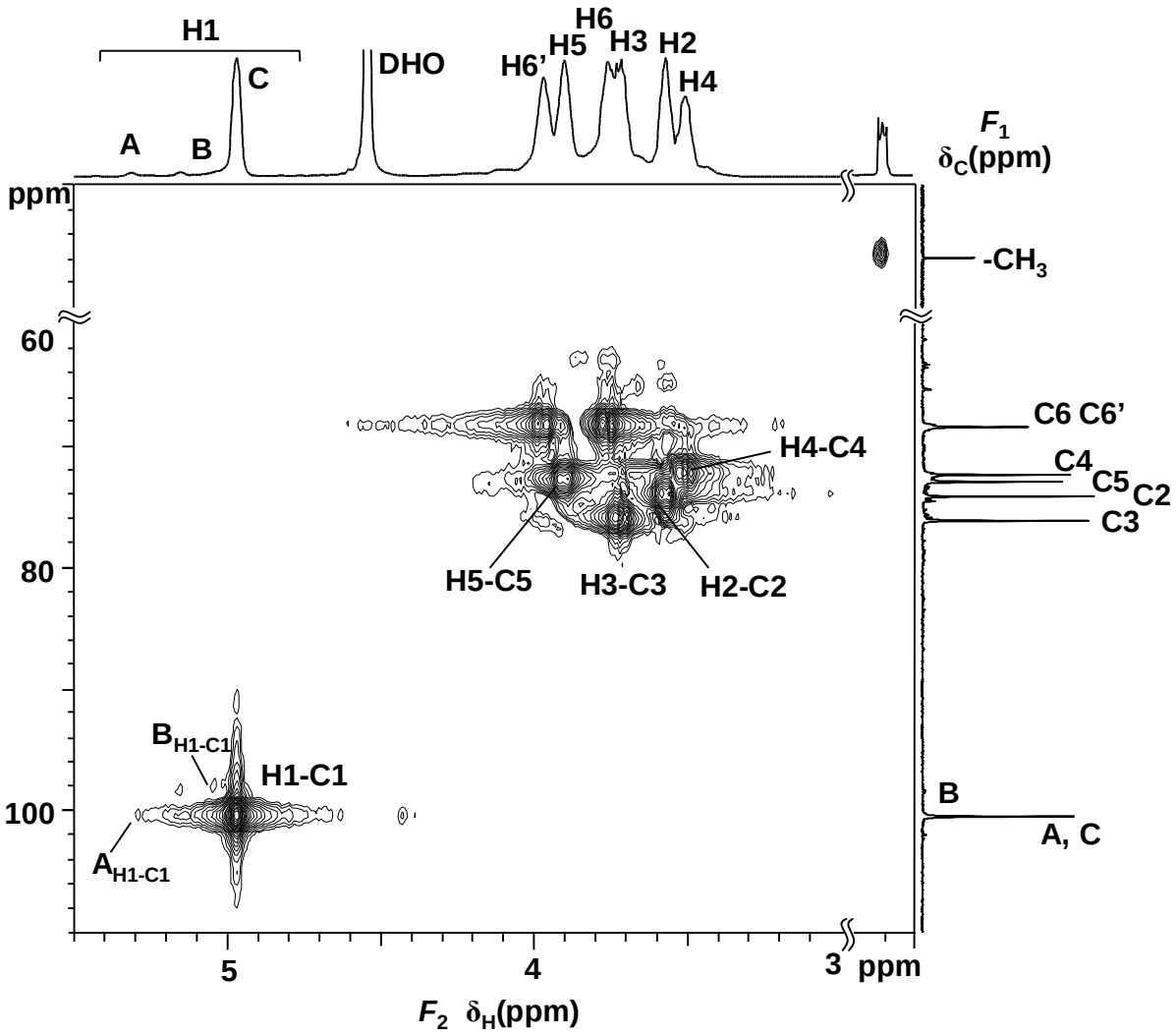


Figure 9. ^{1}H-^{13}C HMQC spectra of CSP-1 and CSP-2 (D_2O as solvent, 50°C).

The signals were assigned by the combined 2D NMR of ^{1}H-^{1}H COSY and HMQC measurements (Figure 8, 9, 10, 11). The C1 signal appeared at 101 ppm as a singlet peak and the specific rotations were large positive values, as represented in Table 3. The results of NMR and specific rotation indicated that the CSP-1 and CSP-2 polysaccharides were composed of α-glucopyranosidic linkages.

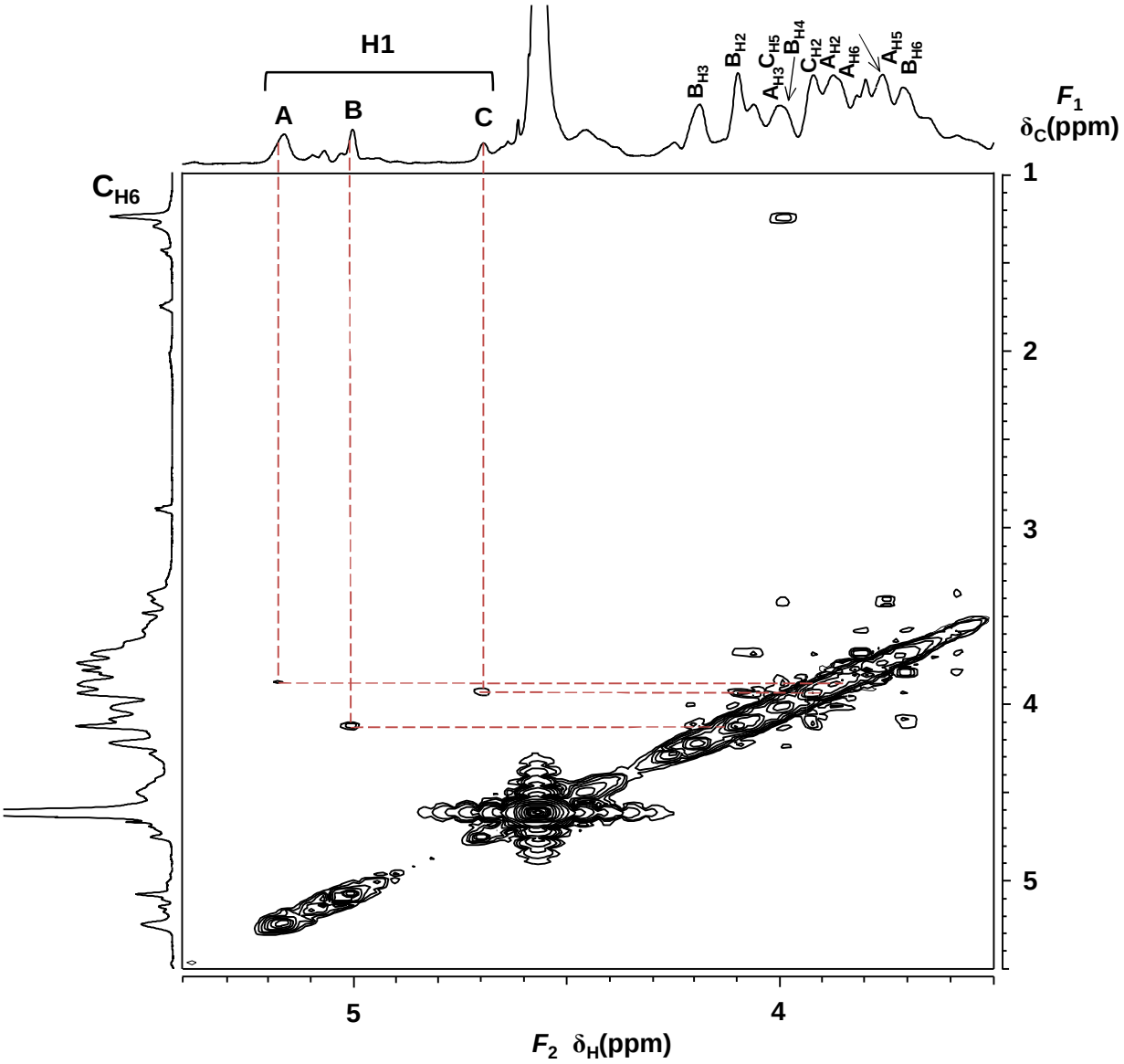


Figure 10. ^{1}H-^{1}H COSY spectra of CSP-3 (D_2O as solvent, 50°C).

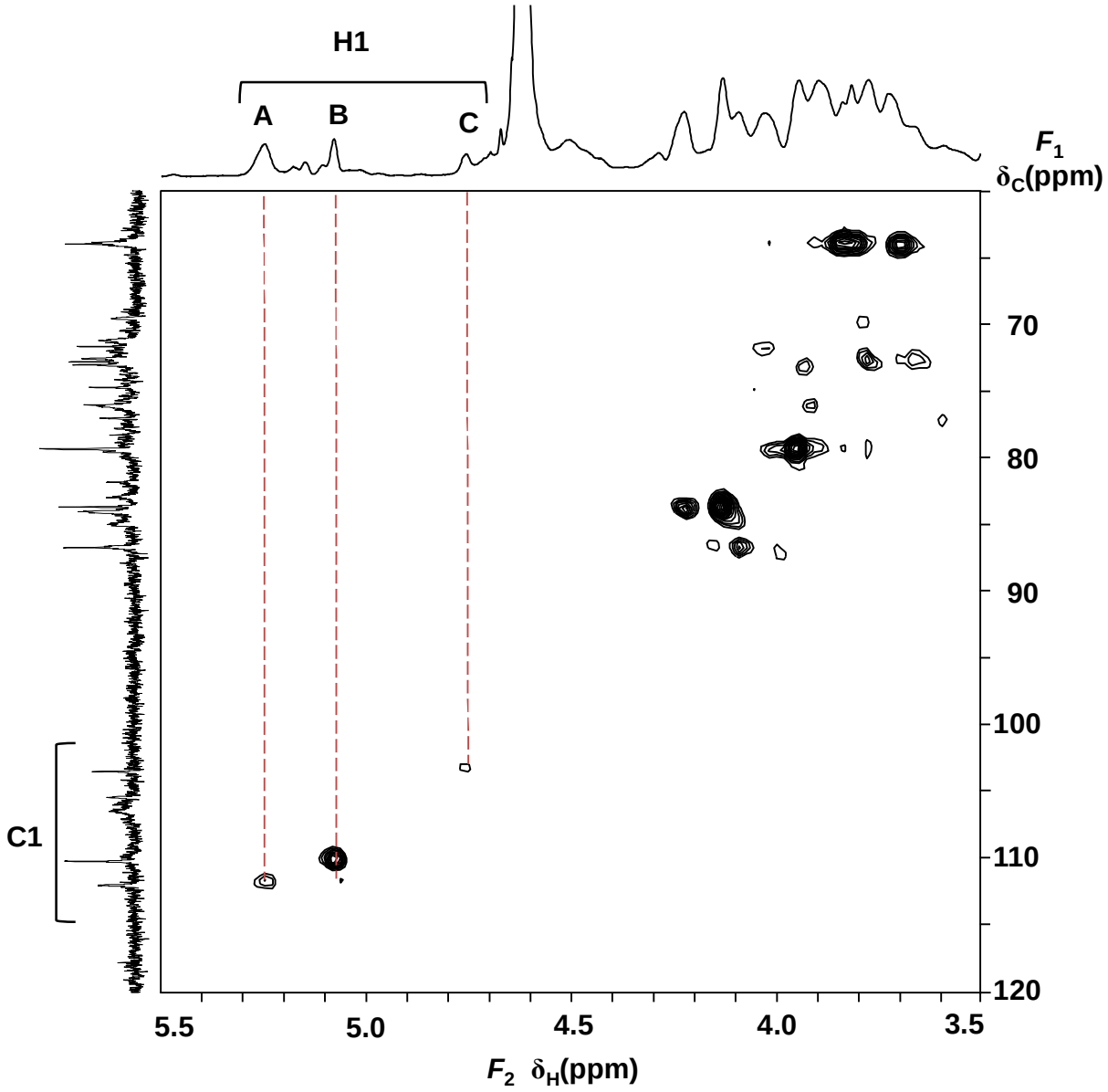


Figure 11. ^{1}H-^{13}C HMQC spectra of CSP-3 (D_2O as solvent, 50°C).

Therefore, we assumed that the CSP-1 and CSP-2 polysaccharides had a structure similar to starch. Figure 6C shows the ^{13}C NMR spectrum of starch with $\overline{M}_n$ = 3.0 x 10^4 and $[\alpha]_D^{25}$=+152.1 (c 1, H_2O). The signals appeared the

same positions as those of the CSP-1 and CSP-2 polysaccharides in Figures 6A and 6B, respectively. However, the assignment as shown in Figure 6C by using ^{1}H-^{1}H COSY and HMQC spectra gave completely difference from those in Figures 6A and 6B for the CSP-1 and CSP-2 polysaccharides. From the methylation analysis, the CSP-1 and CSP-2 polysaccharides gave only 2, 4, 6-tri-*O*-methyl alditol acetate as a main signal and the iodine-starch reaction of the CSP-1 and CSP-2 polysaccharides was negative. In addition, the specific rotation had positive and large values, $[\alpha]_D^{25}$ +148.6 and +172.5, respectively. These results indicated that the CSP-1 and CSP-2 polysaccharides had (1→3)-α-D-glucopyranosidic structure. Several small signals also appeared in the spectra of the CSP-1 and CSP-2 polysaccharides.

The C1 signal of CSP-3 fraction appeared at 103.4 ppm, 110.1 ppm and 111.9 ppm as singlet peak (Figure 7D). The specific rotation was -87.7. After further methylation analysis, the precise structure of the polysaccharides will be analyzed.

3.4.2 Structural analysis of CS polysaccharides by HMBC 2D NMR measurement and methylation analysis

In this paper, carbon signals were identified both HMQC and HMBC spectra. HMBC measurement is suitable for the determination of multiple bond connectivity. Figure 12 and 13 gives the HMBC spectrum of CSP-1 ($\overline{M}_n$=3.7 x 10^4), CSP-2 ($\overline{M}_n$=1.0 x 10^4) and CSP-3 ($\overline{M}_n$=2.9 x 10^4) polysaccharides from *Cynomorium songaricum* Rupr. In the HMBC spectra of CSP-1, the

correlation signals between BH1-CC4 and CH1-CC3 in the glucopyranoside residues. The correlation signal of CH1-O-CC5 oxygen is also glucopyranoside residue.

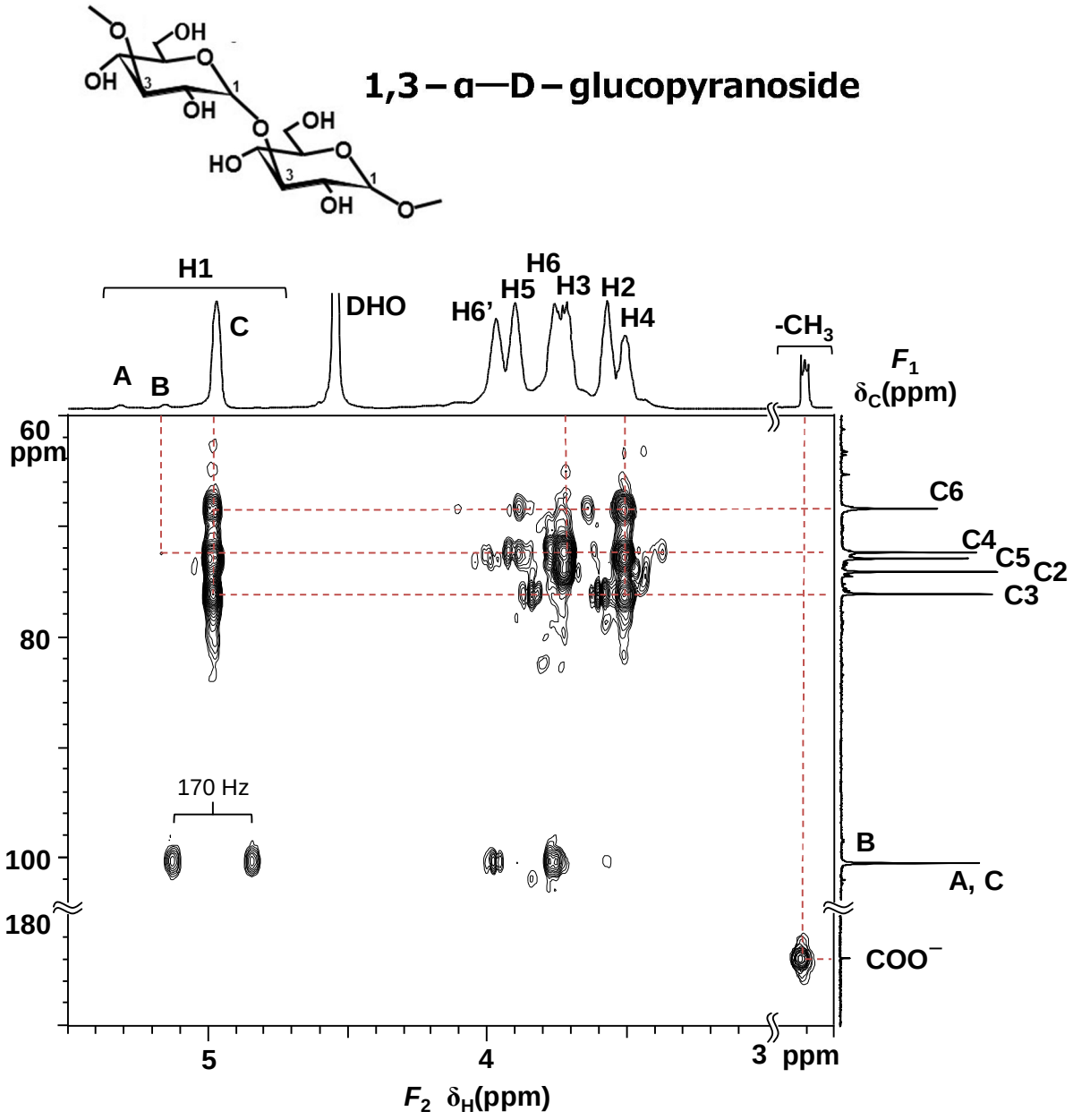


Figure 12. ^{1}H-^{13}C HMBC spectra of CSP-1 (D_2O as solvent, 50°C).

The methylation analysis of the *C.songaricum* polysaccharides (CSP-1, 2 and 3) were carried out using methyl iodine (CH_3I) and sodium hydride (NaH) in DMSO and according to the method of Hakomori [1]. After hydrolysis (TFA), reduction ($NaBD_4$), and acetylation (pyridine/acetic anhydride), the partially methylated alditol acetates were analyzed by the JEOL JMS-T100 GCV GC-MS spectrometer. After methylation analysis, the individual peaks of the PMAA and fragmentation patterns were identified

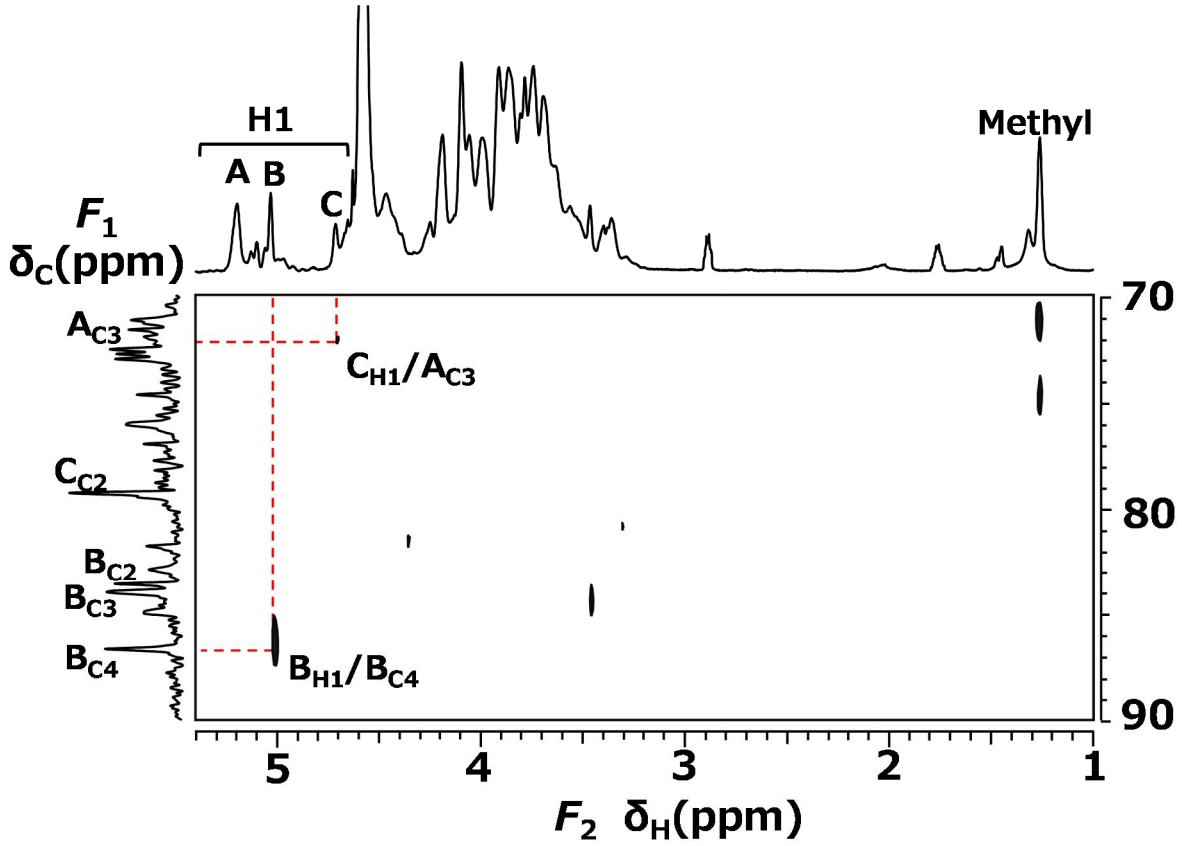


Figure 13. ^{1}H-^{13}C HMBC spectra of CSP-3 (D_2O as solvent, 50°C).

by their retention time (Figure 14) in GC and by comparison with literature mass spectra patterns.

Based on this analysis of PMAA, the linkage patterns of CSP-1 is shown in Figure 15. The data from the methylation analysis and NMR analysis suggested that CSP-1 is mainly composed of a Glc*p* backbone linked 1→3. The trace amounts of linkage of 1→4-Glc*p* could only be assigned to incorporate randomly in the side chain.

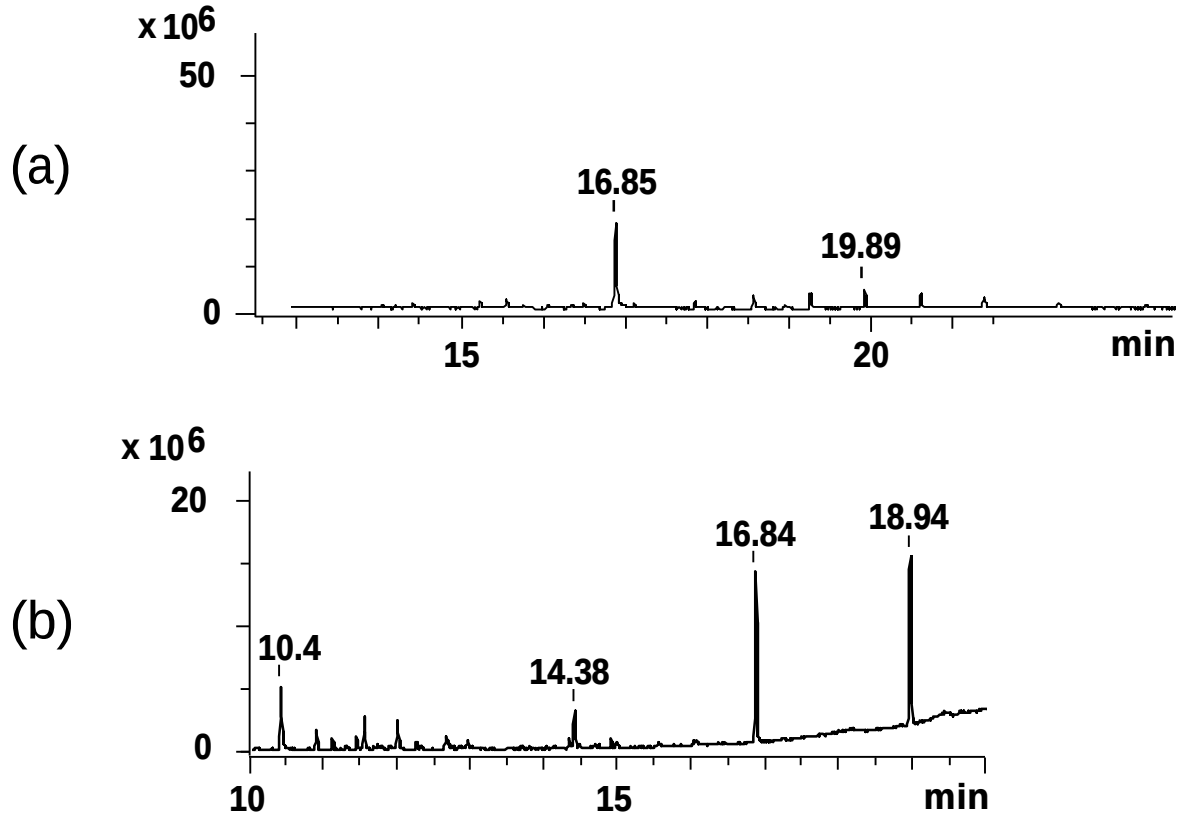
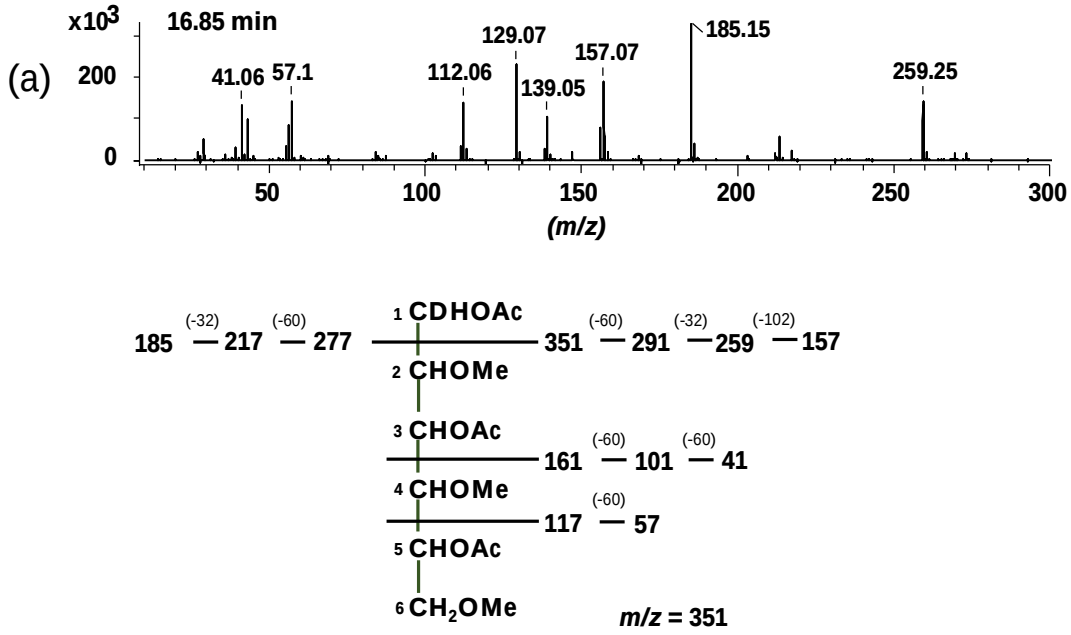


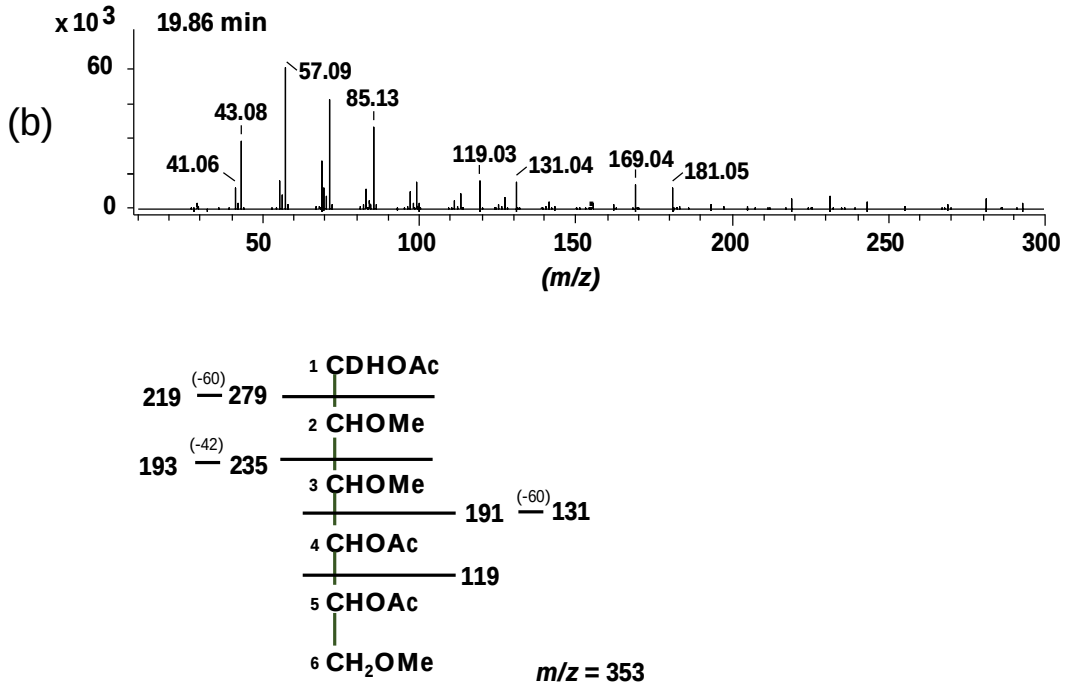
Figure 14. Total ion current (TIC) chromatogram of GS-MS. (a) CSP-1 (b) CSP-3 polysaccharide.

The result of methylation analysis (Figure 16) suggested CSP-3 is mainly composed of a 1→4-linked Gal*p*, 1→3-linked Gal*p*, 1→3-linked Ara*f*, and 1→4-linked Glc*p* backbone with terminal-6-deoxy-L-Rha*p* as the side chain.

The result of 1D and 2D NMR spectroscopy analysis confirms the conclusions drawn from methylation analysis and provides us with more details of the *C.songaricum* polysaccharides structure.



1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol



1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol

Figure 15. EI-MS spectrums of CSP-1 alditol-acetate. (a) 16.85 min and (b) 19.89 min spectrum of TIC and primary and secondary fragmentation of the ions on EI-MS spectrum.

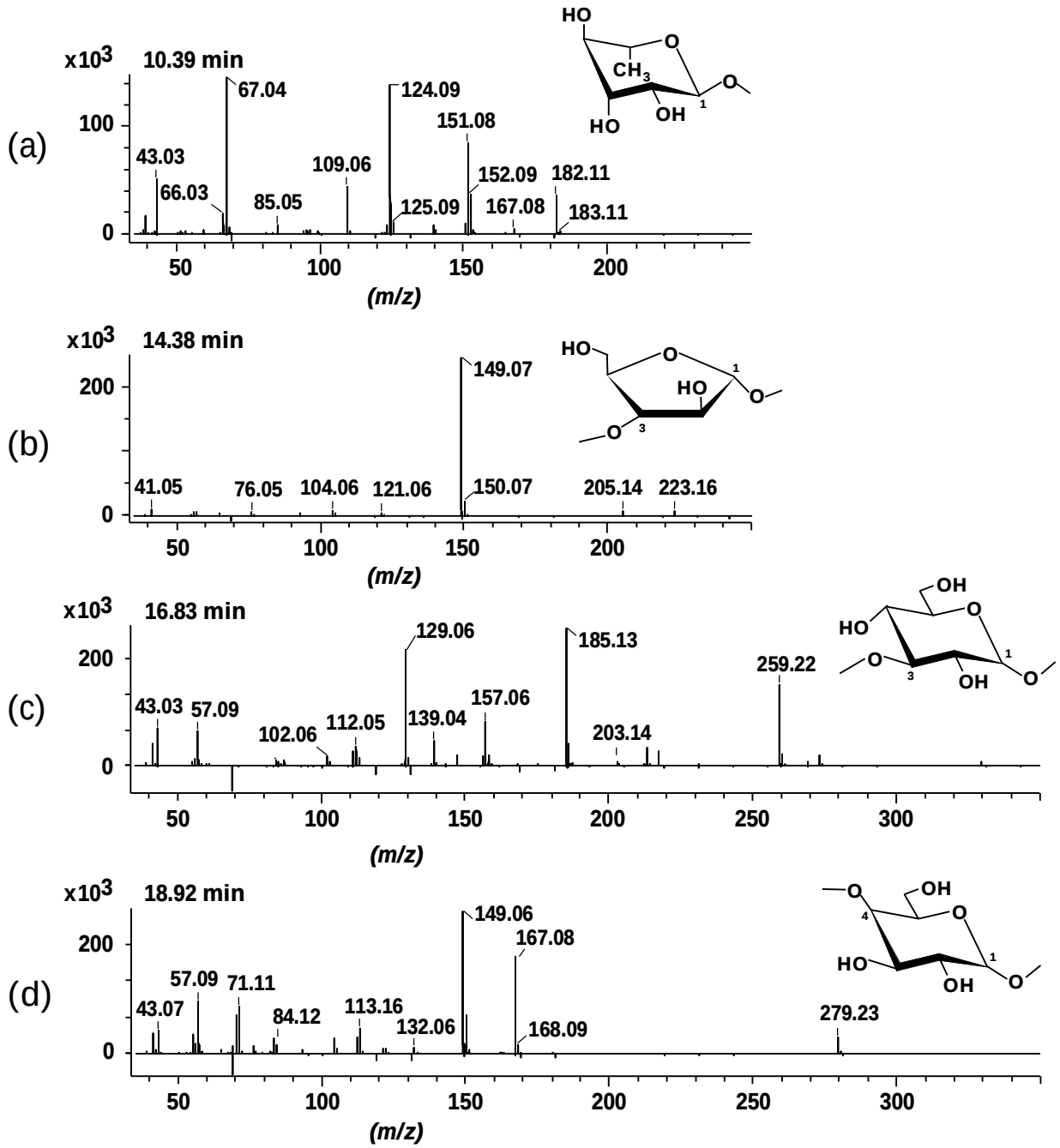


Figure 16. EI-MS spectrums of CSP-3 alditol-acetate. (a) 10.39 min (b) 14.38 min (c) 16.83 min and (d) 18.92 min spectrum of TIC and primary and secondary fragmentation of the ions on EI-MS spectrum.

The HMBC spectrum (Figure 13) indicated that the H-1 signals of 1,3-linked L-ara*f* correlate with C-3 of 1,4-linked-D-Glc*p* residue. It was also observed that H-1 of 1, 4-linked-D-Gal*p* correlate with C-4 of 1,3-linked-D-Gal*p* residue. Thus we combine the information from sugar analysis, NMR and methylation analysis, a complete assignment of all the linkage patterns can be identified as shown in following units:

→4)-α-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→3)-α-Ara*f*-(1→3)-α-D-Glc*p*-(1→

+

t-6-deoxy-L-Rha*p*

3.5 Conclusion

It was concluded that the three new polysaccharides with glucose as a main component were isolated from *C. songaricum* Rupr. a plant used as a traditional medicine in Mongolia and Inner Mongolia. One (CSP-1) of the two polysaccharides contained a small amount of methyl glucuronate.

The structure was analyzed by high resolution NMR, sugar analysis, methylation analysis and IR spectroscopies, indicating that the CSP-1 polysaccharide had a linear 1→3-linked-α-D-glucopyranoside main chain with small amount of 1→4-linked-α-D-glucopyranoside side chains. The trace amount of methyl glucuronic acid could only be assigned to incorporate randomly in the backbone. Several small signals also appeared in the spectra of the CSP-1 and CSP-2 polysaccharides. CSP-3 polysaccharide had a 1→4-linked Gal*p*, 1→3-linked Gal*p*, 1→3-linked Ara*f*, and 1→4-linked Glc*p* backbone with terminal-6-deoxy-L-Rha*p* as the side chain.

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Chapter 4.

Interaction of *C.Songaricum* polysaccharides with poly-L-lysine and their anti-HIV activity

4.1 Abstract

In this chapter, the *C. songaricum* Rupr. polysaccharides, CSP-M, CSP-1, and CSP-2, were sulfated with piperidine *N*-sulfated acid in DMSO at 80 °C to give sulfated polysaccharides, SCSP-M, SCSP-1, and SCSP-2 with degrees of sulfation (DS) of 1.3, 1.8, and 1.4 (maximum: 3.0), respectively.

After sulfation, the sulfated *C. songaricum* Rupr. polysaccharides were found to have a potent inhibitory effect on HIV infection of MT-4 cells at a 50% effective concentration of 0.3-0.4 μg/ml, a concentration that has almost the same high activity as standard dextran and curdlan sulfates, EC_{50} = 0.35 and 0.14 μg/ml, respectively. The 50% cytotoxic concentration was low, CC_{50}>1000 μg/ml. In addition, the interaction between the sulfated polysaccharides and poly-L-lysine as a model protein compound was investigated by a surface plasmon resonance to reveal the anti-HIV mechanism.

Keywords: *Cynomorium songaricum*; anti-HIV activity; SPR; DSL

4.2 Introduction

Cynimorium songaricum Rupr., called Suo Yang in China and Ulaan Goyo in Mongolia, is an annual herb plant that is distributed widely in northwest Inner Mongolia in China and southern Mongolia.

We found that sulfated polysaccharides, which were prepared by sulfation of synthetic and natural occurring polysaccharides, had potent anti-HIV activity and the relationship between structure of polysaccharides and biological activity has been investigated [1]. In addition, we have investigated the structural analysis of naturally occurring lacquer polysaccharides to reveal the relationship between the structure and biological activities such as blood anticoagulant, antitumor, and anti-HIV activities [2]. The structural characterization of a Chinese lacquer polysaccharide, *Rhus vernicifera*, performed by NMR spectroscopy including 2D NMR measurements, indicated that the lacquer polysaccharide had a 1, 3-β-linked D-galactopyranosidic main chain with complex side chains consisting of D-galactose, 4-*O*-methyl-D-glucuronic acid, D-glucuronic acid, L-arabinose, and L-rhamnose [3, 4].

After sulfation, sulfated lacquer polysaccharides had high anti-HIV activity as evaluated by the 50% effective concentration, EC_{50} = 0.3 μg/ml, and low blood anticoagulant activity, 10 units/mg, compared with those of the standard dextran sulfate (around 0.3 μg/ml and 20 units/mg), respectively [4].

4.3 Experimental

4.3.1 Materials

The SPR spectrum was measured on a Biacore X100 (Biacore Co., Tokyo, Japan) instrument at 25°C using a CM5 sensor chip. Commercially available poly-L-lysine (Sigma-Aldrich, Co.) with the molecular weight of 1000-5000 was immobilized on the CM5 sensor chip according to the instruction manual. A HBS-EP+10x (GE Healthcare Japan, Co.) running buffer (including 0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 30mM EDTA, and 0.5 v/v% polyoxyethylene (20) sorbitan monolaurate surfactant (surfactant P20)) was diluted ten times with Milli-Q water.

4.3.2 Measurement

Piperidine N-Sulfonic acid

Piperidine N-Sulfonic acid according to a Nagasawa & Yoshidome method [8] with slight modification A mixture of 75.2 g (87.2 ml) of piperidine and 300 ml of chloroform ($CHCl_3$) was cooled at -5-0^oC, and it was added to 100 ml of $CHCl_3$ solution containing 17.2 g (9.8 ml) of chlorosulfonic acid (HSO_3Cl). The reaction temperature of the mixture was controlled not to exceed 0^o and the mixture was stirred for 3 hours. After the distillation of $CHCl_3$ from the mixture, the residue was dissolved in 200 ml

of 10% Sodium carbonate (Na_2CO_3), extracted with ethyl ether for 3 times to remove piperidine. The water layer was separated an adjusted to pH 1 with Diaion SK1B, then added with 10% barium acetate solution to remove inorganic sulfate. After the removal of precipitates, the clear solution was concentrated and crystallized from hot water to afford long needles.

Sulfation by Piperidine N-Sulfonic acid

The typical procedure for sulfation is as follows: the CSP-1 polysaccharide (0.2 g) was dissolved in dry DMSO (25 ml) and then piperidine *N*-sulfonic acid (1.2 g) was added. The mixture was stirred for 2 h at 80°C. After cooling to room temperature, the solution was neutralized with 10% NaOH aqueous solution and then dialyzed against deionized water for 48 h. The dialysate was concentrated to 30 ml and then freeze-dried to give the sulfated SCSP-1 polysaccharide (0.3 g).

Anti-HIV activity

The assay of anti-HIV activity for sulfated *C. songaricum* Rupr. polysaccharides was carried out by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium) method in our laboratory using MT-4 cell that is a HIV-sensitive cell line and HIV-$1_{HTLV-IIIB}$ virus according to the method of DeCercq [5]. The various concentration of test substance and HIV-infected MT-4 cells (3.0×10^4/well, MOI: 0.01) are immediately added in 96 wells

microtiter plate. In order to know cytotoxicity characterization of the samples against MT-4 cells, cultured virus infected MT-4 cells with various concentration of sample. After culturing at 37°C for five days in the CO2 incubator, we measured the number of viable cells according to the MTT method.

The anti-HIV activity was evaluated by the 50% effective concentration (EC_{50}) of sulfated polysaccharides and the cytotoxicity at 50% cytotoxic concentration (CC_{50}) compared with standard dextran and curdlan sulfates, which have $\overline{M}_n$ = 0.9 x 10^4 and 7.9 x 10^4, S contents = 18.4 and 14.1, EC_{50} = 0.35 and 0.14 μg/ml, and CC_{50} >1000 μg/ml, respectively.

SPR measurement

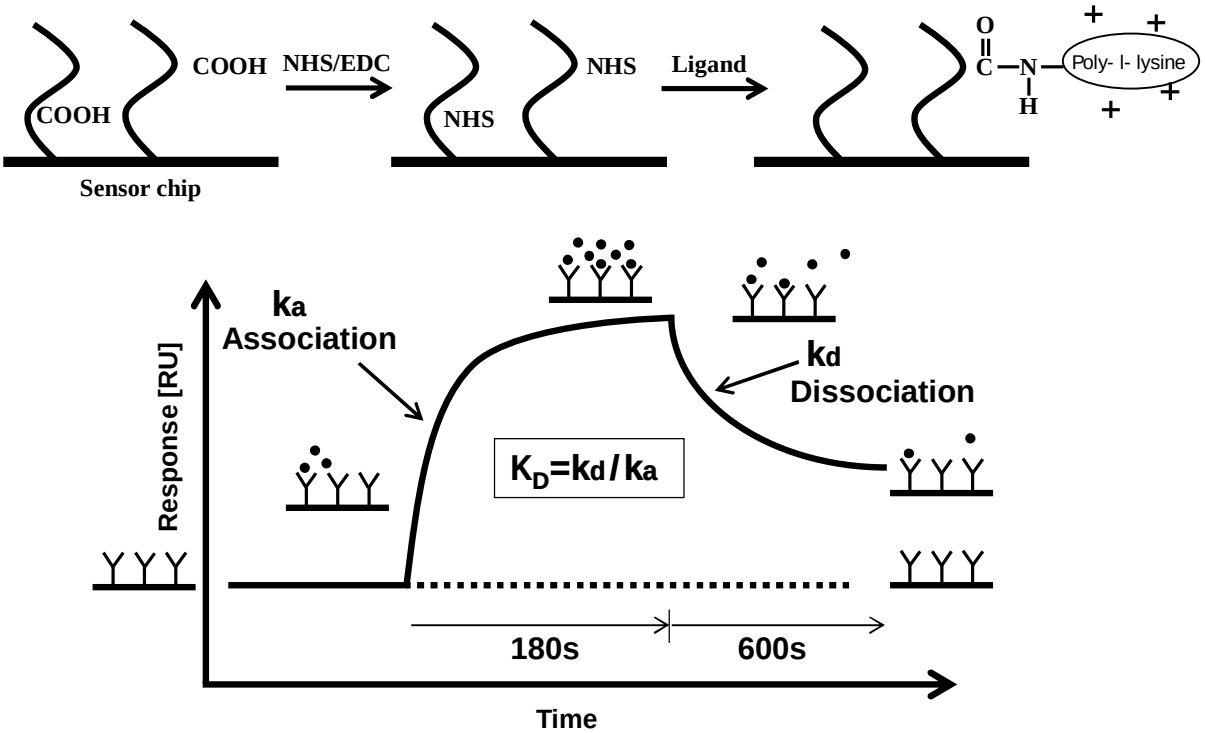

Figure 17. SPR sensorgram

Poly-L-lysine in 10 mM sodium acetate buffer (pH 5.5) was immobilized on the CM5 sensor chip using an amine coupling kit according to the Biacore protocols. The value for the immobilized poly-L-lysine was 2000 response [4] units (RU). SCSP-1, 2 and SCSP-M polysaccharides (500 μg/ml) with different molecular weights were injected poly-L-lysine immobilized sensor chip, and then the HBS-EP+ running buffer solution was injected to determine the association and dissociation rate constants, as shown in Figure 20. The initial concentration of sulfated polysaccharides with high interaction was 5 μg/ml.

Particle size and Zeta potential measurement

The dynamic light scattering (DLS) and zeta (ζ)potential were performed at 25◦C on an Otsuka Electronics ELSZ-1000ZS zeta potential and particle size analyzer in phosphate buffer solution (pH 7.4) at the concentrations of 1mg/mL of sulfated polysaccharides and poly-l-lysine, respectively, and the data were analyzed by the maker provided software.

4.4 Result and Discussion

4.4.1 Sulfation of polysaccharides

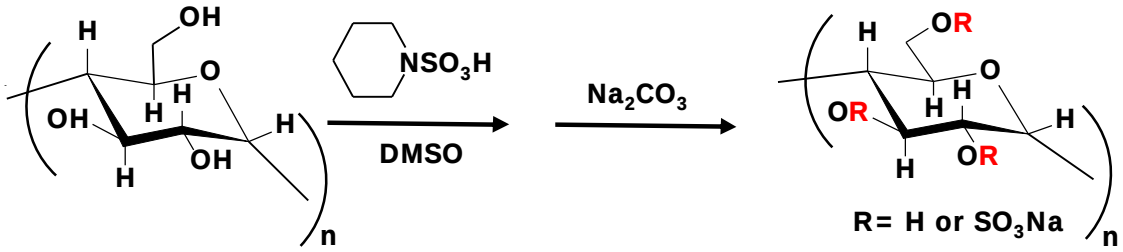

Figure 18. Sulfation of polysaccharide

The *C. songaricum* Rupr. polysaccharides, CSP-M, CSP-1, and CSP-2, were sulfated with piperidine *N*-sulfated acid in DMSO at 80°C to give sulfated polysaccharides, SCSP-M, SCSP-1, and SCSP-2 with degrees of sulfation (DS) of 0.7, 1.8, and 1.4 (maximum: 3.0), respectively.

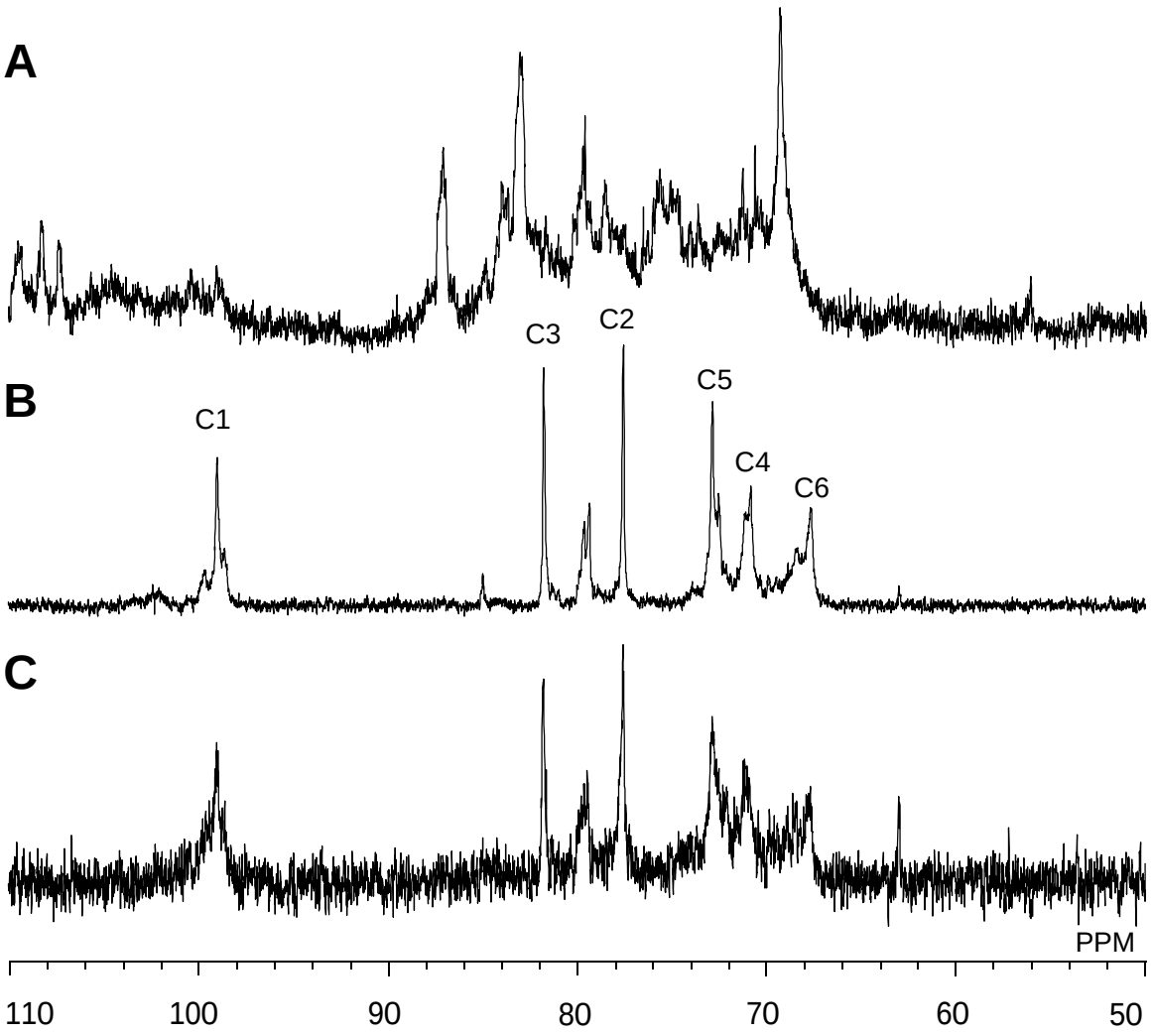

Figure 19. 600 MHz ^{13}C NMR spectra of Sulfated (A) CSP-M (B) CSP-1 and (C) CSP-2 in D_2O. DSS (0.015 ppm) was used as an internal standard at 50°C.

Figure 19 shows the ^{13}C NMR spectra of sulfated *C. songaricum* Rupr. polysaccharides, SCSP-M (19A), SCSP-1 (19B), and SCSP-2 (19C) in D_2O at 40°C, respectively. After sulfation, the signals became complex and broadened. The C1 signals were shifted to higher magnetic field at 95 ppm, because of the introduction of sulfate groups into hydroxyl groups of the polysaccharides. The results of sulfation are summarized in Table 3. The

molecular weight of the sulfated polysaccharides was more than $\overline{M}_n$ = 10 x 10^3.

4.4.2 Anti-HIV activity

The prevention of HIV infection of MT-4 cells in the presence of sulfated *C. songaricum* Rupr. polysaccharides at several concentrations was examined by MTT assays and then evaluated by the 50% effective concentration (EC_{50}) [5], which was compared to that of standard dextran and curdlan sulfates with potent anti-HIV activity at 0.35 and 0.14 μg/ml. Although dextran sulfate had $\overline{M}_n$ = 0.9 x 10^3 that is almost the same molecular weight of SCSP-M, SCSP-1, and SCSP-2, curdlan sulfate had higher molecular weight ($\overline{M}_n$ = 7.9 x 10^3) than that of these three polysaccharides.

Before sulfation, the *C. songaricum* Rupr. polysaccharides, CSP-M, CSP-1, and CSP-2, had no anti-HIV activity. After sulfation, the sulfated *C. songaricum* Rupr. polysaccharides, SCSP-M, SCSP-1, and SCSP-2, were found to have potent anti-HIV activity at the EC_{50} = 0.3-0.4 μg/ml, which was a potency as high as that of the standard dextran sulfate (EC_{50} = 0.35 μg/ml). In general, from our results, polysaccharides with high molecular weights more than 7000 Da and with high DS more than 1.0 gave higher anti-HIV activity [6, 7]. Therefore, the three sulfated *C. songaricum* Rupr. polysaccharides, had potent anti-HIV activity. However, because the CSP-M fraction was the mixture, further purification is performed.

The cytotoxicity of the 50% cytotoxic concentration was low; that is, the MT-4 cells survived under concentrations higher than 1000 μg/ml of sulfated *C. songaricum* Rupr. polysaccharides. These results indicate that the sulfated *C. songaricum* Rupr. polysaccharides worked effectively for the inhibition of HIV infection into MT-4 cells with low cytotoxicity.

It was assumed that the anti-HIV activity of sulfated polysaccharides was originated by the electrostatic interaction between negatively charged sulfated polysaccharides and positively charged surface proteins of HIV [1].

4.4.3 Particle size and Zeta potential of CS polysaccharides

Additionally, the interaction between sulfated polysaccharides and poly-L-lysine, we measured particle size and zeta potential of CSP-M, CSP-1, CSP-2, sulfated CSP-M, CSP-1 and CSP-2 polysaccharides by Analyzer of Photal ELSZ-1000 to elucidate their connection. The particle size of sulfated CSP-M polysaccharide was increased from 71.9±9.5 to 77.1±26.1 nm, when poly-L-lysine (40.7±6.9 nm) was added. SCSP-1 and SCSP-2 were also successfully increased from 17.2±6.5 to 154.7±11.2 nm and from 78.1±27.9 to 97.7±12.8 nm respectively. Also zeta potential (ζ) of sulfated *C.songaricum* polysaccharides (-25.96; -26.8; and -23.07 mV) were being close to zero (-18.36; -8.87; and -8.62 mV), when poly-L-lysine (ζ=0.41 mV) is added (Table 2).

Table 2. The result of particle size and zeta potential

Sample	$\overline{M}_n$ [a] x10[4]	DS	Poly-l-lysine: Absent Particle size (nm)	Poly-l-lysine: Present Particle size (nm)	Absent ζ (mV)	Present ζ (mV)
CSP-M	0.6	-	26.8 ± 30.2	79.0 ± 25.4	-18.84	-5.88
CSP-1	3.7	-	15.3 ± 13.0	130.8 ± 68.0	-9.98	-6.45
CSP-2	1.0	-	23.6 ± 51.6	63.3 ± 23	-13.99	-12.69
SCSP-M	1.0	1.3	71.9 ± 9.5	77.1 ± 26.1	-25.96	-18.36
SCSP-1	1.9	1.8	17.2 ± 6.5	154.7 ± 11.2	-26.8	-8.87
SCSP-2	1.4	1.4	78.1 ± 27.9	97.7 ± 12.8	-23.07	-8.62
Dextran Sulfate	0.6		18.2 ± 14.8	32.7± 106.6	-9.37	-5.31
Dextran Sulfate	0.9				-10.81	-2.62

Particle size and Zeta potential were analyzed by Zeta-potential and Particle size Analyzer of Photal ELSZ-1000.

(Poly-l-lysine MW:2000-4000; ζ =0.41 mV; DSL=40.7±6.9 nm).

4.4.4 Interaction of *C.songaricum* polysaccharides with poly-L- lysine

Poly-L-lysine with positively charged amino groups was used as a model compound of HIV surface proteins and interaction of sulfated *C. songaricum* Rupr. polysaccharides with poly-L-lysine was estimated by

surface plasmon resonance (SPR), as shown in Table 3. Before sulfation, *C. songaricum* Rupr. polysaccharides had no interaction with poly-L-lysine.

After sulfation, sulfated *C. songaricum* Rupr. polysaccharides showed strong interaction. The apparent kinetic constants, association-rate (k_a), dissociation-rate (k_d), and association (K_D) constants, k_a = 9.8-40 x 10^4 1 / Ms, k_d = 1.7-2.9 x 10^{-4} 1 / s, and K_D = 0.4-2.9 x 10^{-9} M were obtained. These values were the same orders as those of the standard dextran sulfate with potent anti-HIV activity, suggesting that the sulfated *C. songaricum* Rupr. polysaccharides were strongly bound to poly-L-lysine and that the interaction was stable for a long time, probably due to the electrostatic interaction of the negatively-charged sulfated groups of the polysaccharides and positively-charged amino groups of poly-L-lysine.

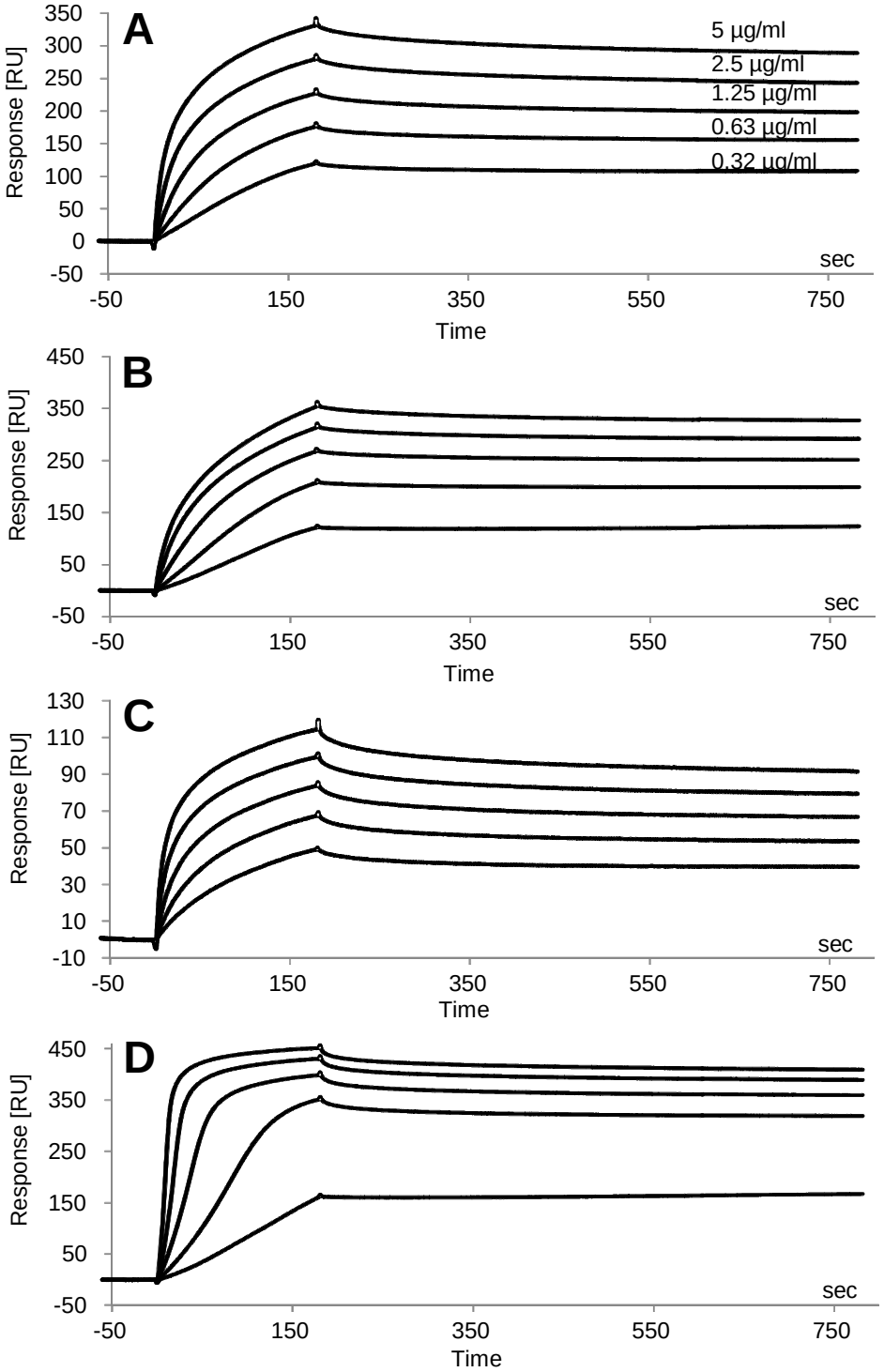

Figure 20. Binding curves of *Cynomorium songaricum* polysaccharides with poly-L-lysine. Binding of (A) sulfated CSP-M, (B) sulfated CSP-1, (C) sulfated CSP-2 and (D) dextran sulfate. Concentration of polysaccharides were 5.0, 2.5, 1.25, 0.62, and 0.31 µg/ml.

Table 3. Anti-HIV activity and kinetic constants of sulfated C*. songaricum* Rupr. polysaccharides

Sample	$\overline{M}_n$ [a]	$[\alpha]_D^{25}$	Elemental analysis (%)					EC_{50} [b]	CC_{50} [c]	Kinetic constant[d]		
			C	H	N	S	DS[e]			k_a	k_d	K_D
	x10^4	deg						µg/ml	µg/ml	x10^4 1/Ms	x10^{-4} 1/s	x10^{-9} M
CSP-M	0.6	+5.72	42.9	6.15	0.93	-	-	>1000	>442			
CSP-1	3.7	+148.6	40	5.58	0.07	-	-	>1000	>1000			
CSP-2	1.0	+172.5	39.1	5.31	0.07	-	-	>1000	>1000			
SCSP-M	1.0	+4.56	22.4	3.39	0.32	13.8	0.7	0.3	>1000	9.8	2.8	2.9
SCSP-1	1.9	+102.1	18.7	2.49	0.13	14.7	1.8	0.4	>1000	40	1.7	0.4
SCSP-2	1.4	+135.7	19.6	3.48	0.31	12.4	1.4	0.3	>1000	17.9	2.9	1.7
Dextran sulfate	0.9					18.4		0.35	>1000	171	2.4	0.1
Curdlan sulfate	7.9					14.1		0.14	>1000			
AZT (uM)								0.014	196			
ddC (uM)								0.59	1014			

a) Determined by GPC.
b) 50% Effective concentration
c) 50% Cytotoxic concentration
d) Degree of sulfation
e) k_a: Association-rate, k_d: dissociation-rate constants, and K_D: dissociation constant, = k_d / k_a

4.5 Conclusion

In this study, three new polysaccharides were isolated from *C. songaricum* Rupr. a plant used as a traditional medicine. But only CSP-1,2 and CSP-M polysaccharides were used for anti-HIV activity and SPR measurement. After sulfation, sulfated *C. songaricum* Rupr. polysaccharides (SCSP-1, SCSP-2 and SCSP-M) were found to have anti-HIV activity as potent as that of the standard dextran sulfate. From the SPR measurement, the activity was assumed to originate from the electrostatic interaction of sulfate groups of the sulfated *C. songaricum* Rupr. polysaccharides and amino groups of poly-L-lysine used as a model compound of proteins.

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polymerization of a 1,4-anhydroribose derivative,"*Carbohydr. Res.* vol.282, pp. 113-123, 1996.

Chapter 5. Conclusion

In this study, three new polysaccharides with glucose, arabinose and galactose as a main component were isolated from *C. songaricum* Rupr. a plant used as a traditional medicine in Mongolia and Inner Mongolia of China by using Sephadex size exclusive chromatography. The molecular weights of three polysaccharides were determined by an aqueous GPC to be $\overline{M}_n$=3.7 x 10^4, 2.9 x 10^4, and 1.0 x 10^4, respectively.

One (CSP-1) of the three polysaccharides contained a small amount of methyl glucuronate. It was found that the iodine-starch reaction of both isolated polysaccharides was negative and the methylation analysis gave 2, 4, 6-tri-*O*-methyl alditol acetate as a main product. The structure was analyzed by high resolution NMR and IR spectroscopies, indicating that the polysaccharide had a linear (1→3)-α-D-glucopyranosidic with methyl glucuronate structure. It was found that both of CSP-1 and CSP-2 polysaccharide show the same structure in ^{13}C NMR spectroscopy. But CSP-3 polysaccharide, which has 1→4-linked Gal*p*, 1→3-linked Gal*p*, 1→3-linked Ara*f*, and 1→4-linked Glc*p* backbone with terminal-6-deoxy-L-Rha*p* as the side chain. Biological activity such as anti-HIV activity of CSP-3 polysaccharide that had been not studied in this study.

After sulfation, sulfated *C. songaricum* Rupr. polysaccharides were found to have anti-HIV activity (0.3-0.4 μg/ml) as potent as that of the standard dextran sulfate. The 50% cytotoxic concentration was low, CC_{50}>1000 μg/ml.

Additionally, from the SPR measurement, the activity was assumed to originate from the electrostatic interaction of sulfate groups of the sulfated *C. songaricum* Rupr. polysaccharides and amino groups of poly-L-lysine used as a model compound of proteins. Before sulfation, *C. songaricum* Rupr. polysaccharides had no interaction with poly-L-lysine. After sulfation, sulfated *C. songaricum* Rupr. polysaccharides showed significantly high interactions. The apparent kinetic constants, association-rate (k_a), dissociation-rate (k_d), and association (K_D) constants, k_a = 9.8-40 x 10^4 1 / Ms, k_d = 1.7-2.9 x 10^{-4} 1 / s, and K_D = 0.4-2.9 x 10^{-9} M were obtained. These results suggest that the minus charge of sulfate groups, compared to minus charge of carboxyl groups give stronger attraction to plus charge of amino groups, and the interaction should be the reason why sulfated *C.songaricum* polysaccharides had potent high anti-HIV activity.

In the further study, elucidate the relationship between the biological activity and structure of the CSP-3 polysaccharides.

Acknowledgement

Firstly, I would like to thanks my supervisor professor Dr. Takashi Yoshida of Kitami Institute of Technology, Japan, who is supporting and providing me with an excellent atmosphere for doing research. I would never have been able to finish my study without his excellent guidance and advice.

I would like to thank professor Dr. Sukhdolgor Jigmed of National University of Mongolia, award me a valuable opportunity to came in Japan begin my researches in doctoral course.

I would also thanks assistant professor Dr. Masashi Komata and Dr. Han Suqin, for all they taught in the laboratory.

Thank my friends Dr. Budragchaa, Dr. Oyunjargal, senior Dr. Tegshi, Dr. Surina, Dr. Yuting, Dr. Shiming and his wife, and other worker and students in the research laboratory of Prof. Yoshida for helped me a lot during my doctorate research.

I also wish to thank YONEYAMA Scholarship Foundation of Rotary Club and Kitami-West Rotary Club for economical support.

I would like to thank my husband Odkhuu B. and my lovely son Odjargal O. They were always supporting me from Mongolia during my doctorate course.

This research was partly supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science 2012-2014 (No. 24550129).