Doctoral Thesis

ELUCIDATION OF INTERACTION MECHANISM BETWEEN LACQUER POLYSACCHARIDES AND PROTEINS

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Elucidation of interaction mechanism between lacquer polysaccharides and proteins
（漆糖鎖とペプチド間の相互作用メカニズムの解明）

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Preface

Lacquer polysaccharides, which exist in the sap of Asian lacquer tree, are highly branched acidic polysaccharides with 1,3-β-galactan backbone and glucouronic acid terminals. Lacquer polysaccharides were reported to be antitumor active and blood coagulation promoting effective, which specific activities might be caused from electrostatic attraction between negative-charged carboxyl groups from the uronic acid terminal of polysaccharides, and positive-charged amino groups from target proteins. By use of surface plasmon resonance (SPR), the interaction dynamics between the polysaccharides and proteins can be quantitatively evaluated.

Lacquer polysaccharides are usually found with two molecular weight fractions, 10 $x10^4$ and 3.0 $x10^4$Dalton in the proportion of about 25% and 75% respectively. In the previous study, the single fraction of $10x10^4$ was reported to be degraded into 3.0 $x10^4$ after the sap was collected. The two pure fractions were first isolated in this study by using

Sephadex size exclusive chromatography. It was found that both of the two fractions show the same structure by ^{1}H and ^{13}C NMR characterization. It was also found that addition of ferric or ferrous ion can lead to a gelation of lacquer polysaccharides with lower molecular weights in aqueous solution, suggesting that the degradation may cause by breakage of metal-carboxyl association linkages. Appearance at 1612 cm^{-1} due to COO^{-} ion of the IR spectra and alkaline degradation of 3.0 $x10^{4}$ fraction into $1.4x10^{4}$, which halved the molecular weight, could also be a proof of existence of metal-carboxyl association.

In the dynamic study of proteins-polysaccharides interaction by SPR, poly-L-lysine as an all-positive charged polypeptide model was used to be evaluated the activity of lacquer polysaccharides and their derivatives. It was found that the interaction increased with increasing molecular weight of lacquer polysaccharides. In addition, the sulfated derivatives showed significantly high interactions with dissociation-rate constant of k_d = 1.74 $x10^{-4}$ [1/s], association-rate constant of k_a = $4.31x10^{4}$ [1/Ms] and dissociation constant of K_D=$4.0x10^{4-9}$ [M] respectively, while original lacquer polysaccharides gave only k_d = 3.54 $x10^{-4}$

[1/s], k_a = 4.99 x10^2 [1/Ms] and K_D = 7.10 x10^{-7} [M]. These result suggest that the sulfate groups, compared to carboxyl groups give stronger attraction to amino groups, and the interaction should be the reason why sulfated lacquer polysaccharides had potent anti-HIV acctivity.

To evaluate the affection of sulfation to anti-HIV activities, lacquer polysaccharides were sulfated 1, 2 and 3 times respectively, by piperidine-*N*-sulfate to gives sulfated lacquer polysaccharides with different degree of sulfations. According to the amino acid sequence of V3 loop, C-terminus, and CD4-binding domain of HIV surface glycoprotein gp120 were synthesised and immobilized to the sensor chip separately. The C-terminus gave the highest response that increased with increasing the degree of sulfation.

While the association-rate constant was different from the molecular weights, degree of sulfation, and binding ligands, the dissociation-rate constant of lacquer polysaccharide derivatives was not affective so much and gave rather low values. This implies high stability of the interaction and sulfated polysaccharides were expected for

long-term effectives and were possible candidates as an anti-HIV medicine in the future.

Key words: lacquer, polysaccharides, surface plasmon resonance, HIV, oligopeptide, biosensor

Contents

Chapter 1

General Introduction

1.1 Introduction to lacquer tree and lacquer

1.1.1 Lacquer tree

Chinese and Japanese lacquers are derived from *Toxicodendron vernicifluum* (also *Rhus verniciflua, T. verniciferum, R. vernicifera)*, a deciduous tree of the family *Anacardiaceae* that is native to central and western China and widely cultivated by Chinese (see **Table 1.1**). The tree was introduced into Japan at a very early time and grows well in Japan. The trees are cultivated and tapped for their toxic sap, which is used as a highly durable lacquer to make Chinese, Japanese, and Korean lacquer wares.

Table 1.1 Scientific classification of Japanese lacquer

Kingdom	*Plantae*	Plants
Subkingdom	*Tracheobionta*	Vascular plants
Superdivision	*Spermatophyta*	Seed plants
Division	*Magnoliophyta*	Flowering plants
Class	*Magnoliopsida*	Dicotyledons
Subclass	*Rosidae*	
Order	*Sapindales*	
Family	*Anacardiaceae*	Sumac family
Genus	*Toxicodendron Mill.*	Poison oak
Species	*Toxicodendron vernicifluum (Stokes) F. A. Barkley*	Chinese lacquer

Tapping usually commences when the tree grows up to 30 cm in diameter Midsummer is the best season to tap lacquer sap because lacquer sap is too fluid in spring and too thick in autumn. Lacquer sap is secreted by the laticiferous canals, which distribute in phloem of almost all parts of the plant, including roots, stems, leaves, and immature fruits, without seeds. Direct contact with the plant, exposure to smoke or fumes from a burning plant or even contact with pets or animals that have touched the plant can cause severe allergic dermatitis in some individuals. Even plant specimens 100 or more years old

can cause problems (Langenheim, J.H. 2003; Mills & White 1994).

Lacquer in China is divided into four categories: Maoba lacquer, Chengkou lacquer, southwest lacquer, and northwest lacquer. The Maoba lacquer, which produced in Hubei province of China, is famous for high urushiol content, thickness, high viscosity, and brightness and well drying. The sample used in this study is mainly produced from lacquer tree cultivated in Maoba, Hubei province of China.

1.1.2 Lacquer

For several centuries, natural lacquer was widely used as a coating material in Asia, which is called Qi in Chinese or Urushi in Japanese, was of high value of artistic level and used only by novel family because of its mysterious power of beauty (Ragueb, B V., 1976; Neill, J. P. O., 1992).

Archaeologists have found Chinese lacquer ware used to coat small objects in the early Late Stone Age (4000 B.C.). The techniques had reached an advanced level by the Shang dynasty (1600-1100 B.C.). The art reached its highest level of development during the Ming dynasty A.D. 1368-1644), however, the methods kept secret (Mills & White 1994).

However, lacquer ware that was used to be the only coating material, nowadays, is almost replace by modern synthetic chemical material in our daily life, by significantly lower price and mass industrial production. However, natural lacquer is of great importance both in artistic and modern medical area.(Lu & Yoshida 2000)

Lacquer sap is usually neutral and contains 60-65% of urushiol, 20-30% of water, 3-5% of polysaccharides, and 1-3% of laccase (Lu & Yoshida 1999)

1.2 Polysaccharides

Polysaccharides are used to be vital important to human being as a kinds of common energy supply like starch and basic clothing materials like cotton, and nowadays become increasingly important as kinds of abundant sources of wide variety products in almost every area of our daily life.

In general, plants were used as a traditional medicine, in both western and eastern countries, to treat various kinds of illnesses and wounds, external and internal, shown by the modern science. Plants also contain polysaccharides that are exhibited several kinds of biological activities, including antitumour, antioxidant, antiulcer, immunomodulation, immunostimulation, and coagulation promotion(Yamada et al., 1990; Sakurai, Yamada et al., 1998; Guo, Yamada et al., 2000; Bao et al., 2002 Duan et al., 2003 Sonoda et al., 1998; Chun et al.,2002). Most of these polysaccharides could be separated into four kinds of categories according to compositions and structures:

arabinogalactan type I, arabinogalactan type II, and rhamnogalacturonan type I, and rhamnogalacturonan type II (Klemm, 2003).

1.2.1 Arabinogalactans

Occurrence of Arabinogalactans is widely throughout the plant kingdom Arabinogalactans that occur in two structural variations, type I and type II. Arabinogalactans may act as structural component of cell wall or a soluble component in plants. Type I gives a leaner structure of a 1, 4-galactose backbone with 1, 5-arabinose short side chains, and is commonly found in pectin from citrus, apple, and potato. Type II is more widely present in nature and has a 1, 3-galactan backbone heavily substituted at 6 position of a sugar unit of mono- and oligosaccharides.

Type I is found to have variable degrees in the cell wall and is composed of a β -1, 4 linked galactan backbone with side chains of arabinans. Type II have back bone of a β-1, 3

linkage with a side chain of 1, 6 linked sugar units, often found of uronic acid ends. Type II polysaccharides form precipitate by addition of a reagent called Yarive reagent. This type of arabinogalactan is frequently found bound to rhamnogalacturonan type I, and also found bound to rhamnose unit of pectin at the position 4 of the galactose unit.

In the most cases, type II arabinogalactans are found in plants as an associated complex with the substantial amount of protein. Even it has not always been proved that the linkage of protein to carbohydrates is covalent or not, the alkaline treatment is usual method of isolation and purification procedure. The alkaline treatment also cause a the diminishing proportion of nitrogenous component, which could be seen as non-bonded proteins or peptides has been removed. (Aspinall, 1983 p 126)

1.2.2 Lacquer polysaccharides

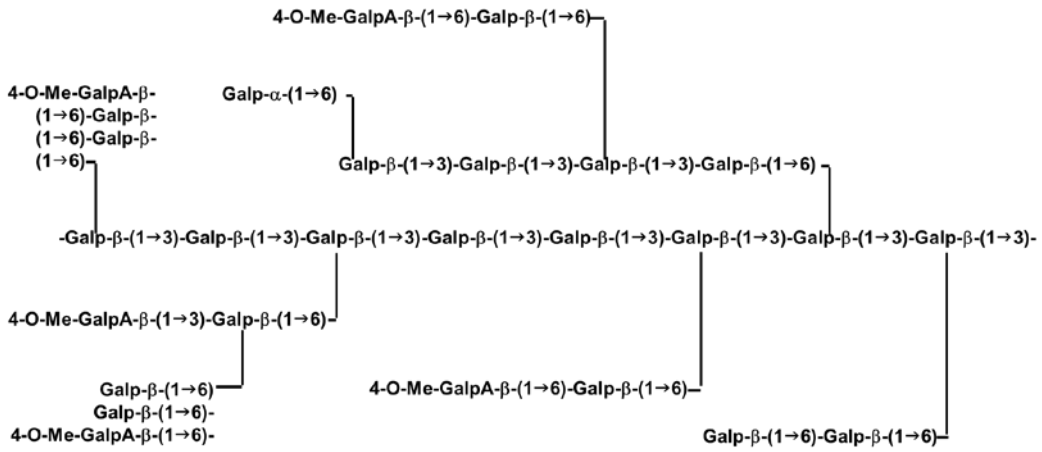

Figure 1.1 Partial structure of lacquer polysaccharides

The structure of lacquer polysaccharides in the sap of lacquer tree was characterized by a sugar analysis (Oda, Ishida & Honnda, 1962), methylation (Oshima & Kumanotani, 1984), and NMR analyses (Lu et al., 1999), indicating that lacquer polysaccharides had a 1, 3 -β-galactopyranosidic main chain having complex branches with 4-*O*-methyl glucuronic acid in the terminal. The monosaccharide components were d-galactose, 4-*O*-methyl-D-glucuronic acid, L-arabinose, and L-rhamnose. Biological activities such as blood coagulant-promoting and antitumor activities were observed (Lu et al., 2000).

1, 3-β-galactan backbone and glucuronic acid terminals are the same as the typical Type II arabinogalactans. Lacquer polysaccharides were reported to be antitumor active and blood coagulation promoting effective, which specific activities might be caused from electrostatic attraction between negative-charged carboxyl groups of the uronic acid terminal of polysaccharides, and positive-charged amino groups of target proteins.

Lacquer polysaccharides are usually found with two molecular weight fractions, 10×10^4 and 3.0×10^4 Dalton in the proportion of about 25% and 75% respectively. In the previous study, the single fraction of 10×10^4 was reported to be degraded into 3.0×10^4 after the sap was collected. The two pure fractions were first isolated in this study by using Sephadex size exclusive chromatography. It was found that both of the two fractions show the same structure by ^{1}H and ^{13}C NMR characterizations. It was also found that addition of ferric or ferrous ion can lead to a gelation of lacquer polysaccharides with lower molecular weights in aqueous solution, suggesting that the degradation may

cause by a breakage of metal-carboxyl association linkages. Appearance at 1612 cm-1 due to COO- ion of the IR spectra and alkaline degradation of 3.0 x 10^4 fraction into 1.4x10^4, which halved the molecular weight, could also be a proof of existence of metal-carboxyl association.

1.2.3 Association of polysaccharides

Associated structure of polysaccharides occurs in many kinds of polysaccharides, especially in those with uronic acid side chains, such as pectin, and alginic. Alginic acid was associated by a ferrous ion and then formed a gelation. Acidic polysaccharides should take associate structures with many kinds of multivalent metal ions.

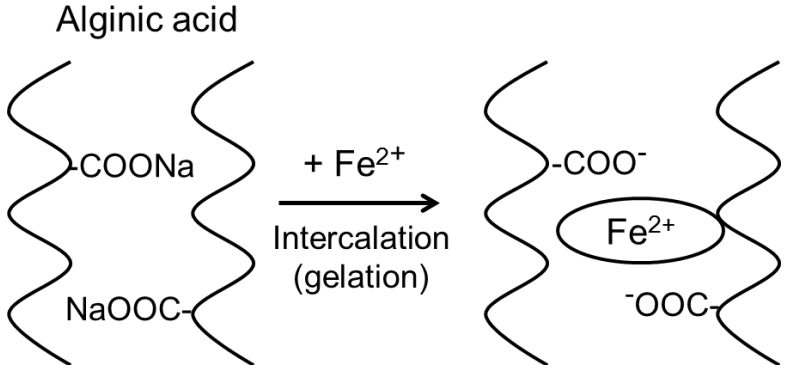

Figure 1.2 Association structure of alginic acid (Aspinall 2003)

On the other hand, metal bridged association are widely occurred in some natural algae. 1:3 or 1:2 complex was found in golden algae. The metal ions that connected to polysaccharides might potentially affect to become the key fact of the biological activities and the chemical behaviours (Aspinall, 2003).

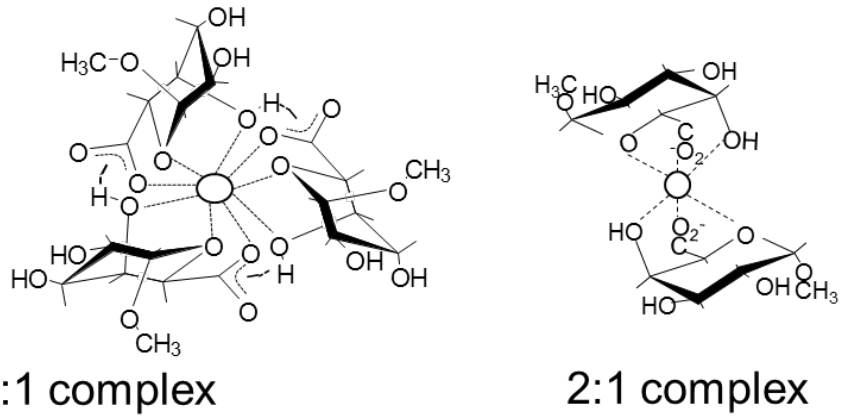

Figure.1.3 Associated structure of polysaccharides from golden algae (Aspinall 2003)

1.3 Biological activities

Plants as important sources of traditional medicine are usually contained of many kinds of polysaccharides that may have potent special biological effects such as

antitumour, anti-virus, and immune system modulating activities.

1.3.1 Biological activities of lacquer polysaccharides

Since branched 1, 3-β glucans have potent anti-tumour activity, lacquer polysaccharides were expected to have antitumour activity. Therefore, antitumour assay was carried out by using Sarcoma 180 tumour in rat. After 35 days of transplantation, the weights of tumour in rat without any polysaccharides increased to 20g. When lentinan (1 mg/kg), a potent antitumour polysaccharide, provided to rats by an intraperitonal injection (i.p.), tumour of rats disappeared after 35 days of transplantation. For the natural lacquer polysaccharide, it was found that the weights of tumour decreased to 10, 11, and 13 g by 50 and 5 mg/kg by p.o., and 1 mg/kg by i.p., respectively.. These results indicate that the 1, 3-β structure of lacquer polysaccharide might play an important role in the antitumour (see **Figure1.4**, Lu, & Yoshida, 1999).

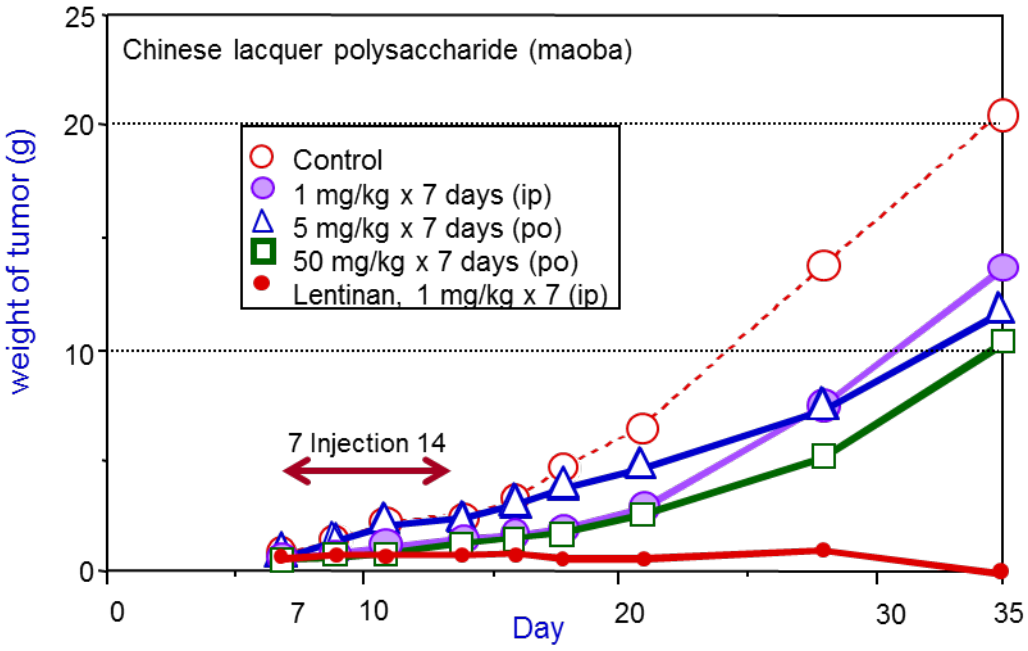

Figure 1.4 Antitumous activities of lacquer polysaccharides

As shown in the **Figure 1.5,** the lacquer polysaccharide was revealed to promote the blood coagulation more than 1 min on the blank (4 min and 25 s). After sulfation, the lacquer polysaccharides showed weak anticoagulant activity, which increased slightly with increasing degree of sulfation.

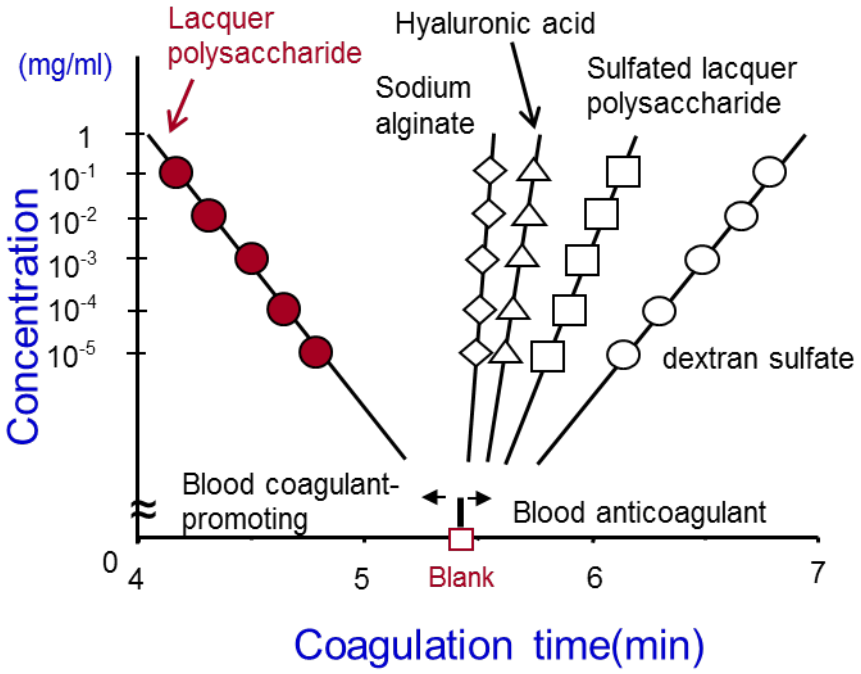

Figure 1.5 Blood coagulation affect to blood coagulation of polysaccharides

However, the sulfated lacquer polysaccharides provided low anticoagulant activity, probably because of competition with anticoagulant activity originated from sulfate groups and activation of coagulant factors by the branched structure.

1.3.2 Surface plasmon resonance

Biomolecular interactions in an important factor for virtual every biological phenomenon such as ligand-receptor interactions, signal transduction, regulation of gene expression, etc. These interaction are all controlled by specific recognition of interacting partners. In the broad spectrum of technologies for interaction analysis, label-free biomolecular interaction analysis has special place. Surface plasmon resonance (SPR) has become very important with a yearly impressive increase in number of publications. The SPR method is a label-free technique, only one of the interacting partners has to be immobilized on a surface of sensor chip. The SPR is one of the few techniques that generate equilibrium data and kinetic data.

Talking about the history ,the first SPR phenomenon were observated at the beginning of twentieth century, by Wood, who detected an anomalous diffraction pattern of light and dark hands when visible polarized light reflects on metal grating. And in late 1960s Otto and Kretschmann

made an optical excitation of surface plasmons possible. In their setting light falls through a glass prism under condition of total reflection and onto a mental film evaporated on the glass. This is the basic configuration for an SPR sensor. In the 1980s Liedberg et al. first realized that SPR-based sensors can be used to study binding, as the SPR signal is sensitive to a change in refractive index. The refractive index is influenced by the accumulation of mass near the metal surface. The configuration for SPR sensors developed by Kretschmann and Otto opened the way for development of commercial SPR instrumentation, initially by Pharmacia Biosensor in Sweden, a predecessor of what now is GE-Biacore. Next to Biacore instruments, various other types of SPR instrumentation are available.

The SPR sensorgram of a single circle of the among the several circles in the multi-circle method is shown in **Figure1.6**. The multi-circle method is common method that we use to determine association rate constant k_a, dissociation rate constant k_d, and dissociate constant K_D. The fitting model is used usually by 1:1 Langmuir model,

and the χ2 is one of the ways to determine whether the fitting went normally. A lower value of χ2 is preferable.

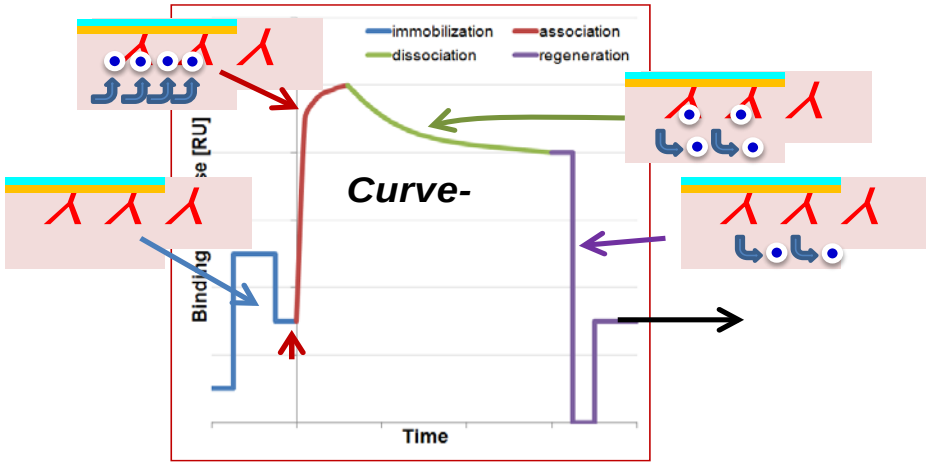

Figure.1.6 SPR sensorgram

To prepare the surface of sensor chip, there are two major methods, EDC/NHS assays, and biotin assay are often used for the immobilization of peptides.

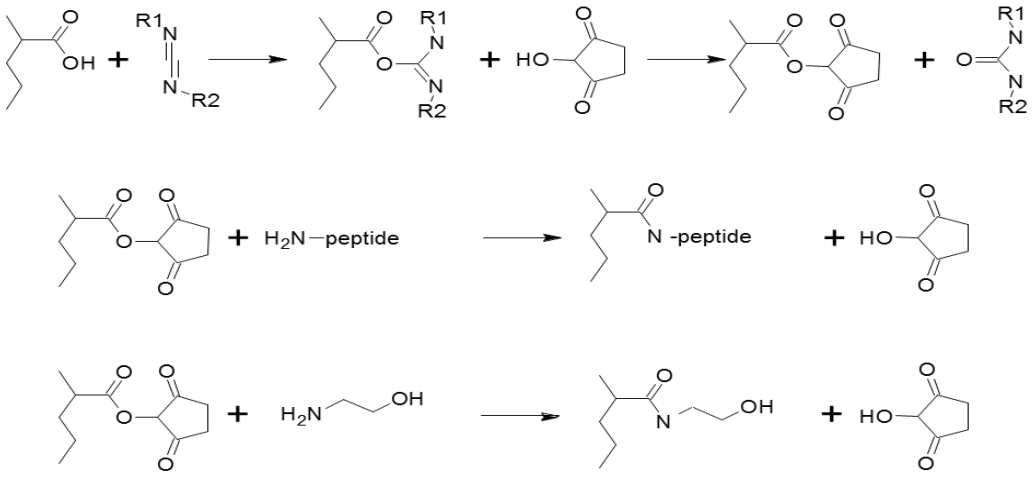

Figure 1.7 EDC/NHS assay of peptide immobilization

Peptides with one end connected to biotin, can be easy immobilized on SA sensor chip on which streptavidin are pre-immobilized. EDC/NHS assay, shown in **Figure 1.7**, is used to immobilize peptides to CM sensor chip, on which dextran with carboxyl group are pre-immobilized.

1.4 Reference

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Chapter2

Structure and molecular weight of lacquer polysaccharides

2.1 Abstract

The partial structure of lacquer polysaccharide was studied in the previous research, highly branched 1, 3-β-galactan backbone and 1, 6 side chains with glucuronic terminals. Crude lacquer polysaccharide is a mixture of two fractions with molecular weight 10 x 10^4 and 3.0 x 10^4 respectively. The crude lacquer polysaccharide was hydrolysed at 60℃ in aqueous solution for from 1 to 30 hours, and found out that from 1 to 4 hours, only the fraction with molecular weight 10 x 10^4 was degraded, shown in the GPC chart is the vanish of one of the peaks,

and give a high yield up to 96%. However the hydrolysis for longer duration only gave a single peak with molecular weight decreases gradually. On the other hand, even smaller fraction of polysaccharide obtained by means of alkaline treatment, molecular weight about 1.4×10^4. Addition of ferric and ferrous ion caused the gelation of lacquer polysaccharide, and the elemental analysis result of crude polysaccharide shows that trace amount of Fe and Cu was found. It is possible that lacquer polysaccharide form a triple cluster via metal ion bridge, which cause vanish larger fraction, and made alkaline degradation occur.

2.2 Introduction

Normally, the Chinese lacquer polysaccharide was reported to have two molecular weight fractions around 3.0 x104 and 9.0 x104 in the proportion of roughly 25:75 mol%. The ratio of constitutional sugar residues was the same as the two fractions (Lu et al., 1999; Oshima & Kumanotani, 1984). However, the polysaccharide with single fraction (9.0 x104) was found in the lacquer sap collected in Aizu,

Fukushima prefecture, Japan, degradation of larger fraction was observed by the normal storage before the addition of acetone (see **Figure 2.1**, Lu & Yoshida., 2003).

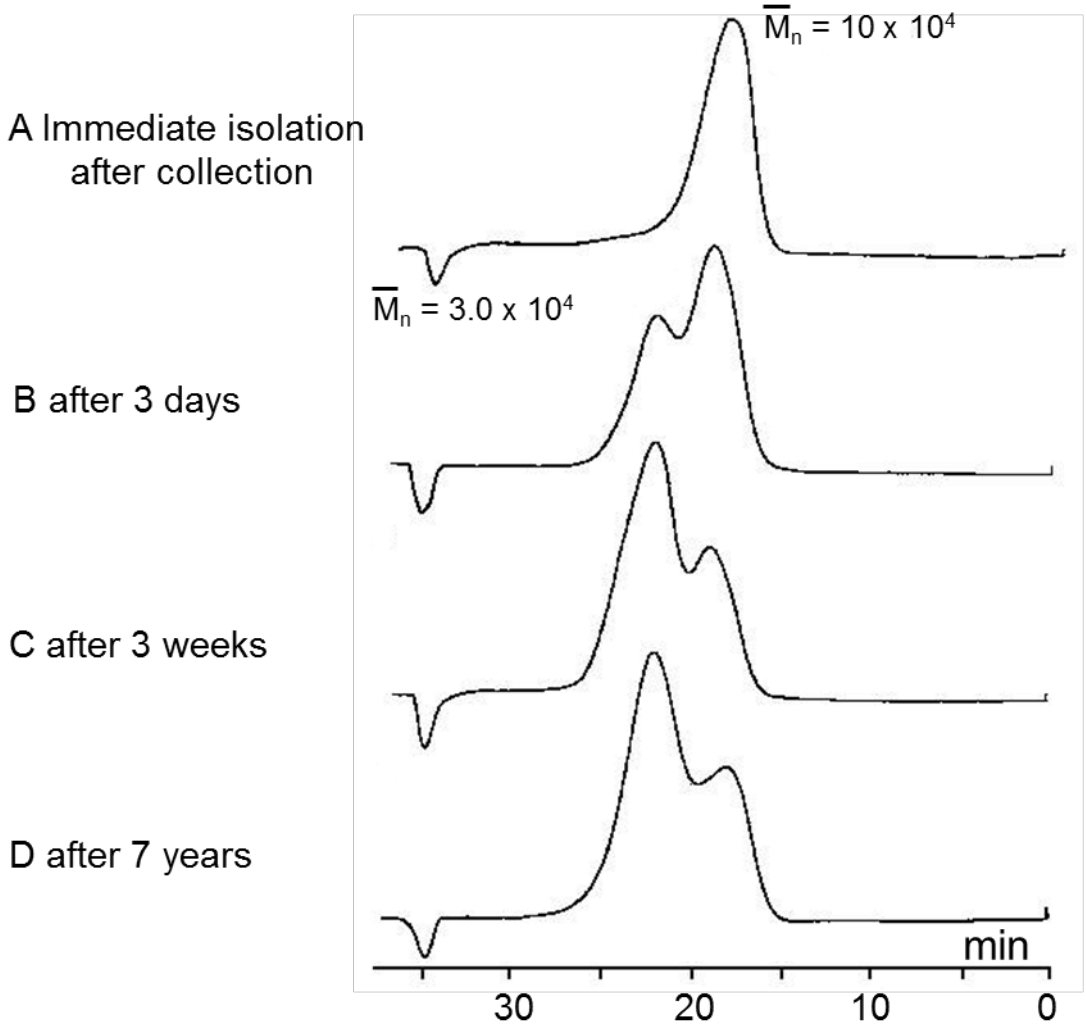


Figure 2.1 Molecular weight changes of lacquer polysaccharides after collection lacquer sap

The structure of lacquer polysaccharides in the sap of lacquer tree was characterized by sugar (Oda, Ishida &

Honnda, 1962), methylation (Oshima & Kumanotani, 1984), and NMR analyses (Lu et al., 1999), indicating a 1,3-galactopyranosidic main chain having complex branches with 4-O-methyl glucuronic acid in the terminal. The monosaccharide components were d-galactose, 4-O-methyl-d-glucuronic acid, l-arabinose, and l-rhamnose. Biological activities such as blood coagulant-promoting and antitumor activities were observed (Lu et al., 2000).

Association structure has been reported in many forms of polysaccharides, especially those with uronic acid terminals, like alginic acid (Klemm, 2010), and Polysaccharides from Golden Algae (Aspinall, 1983), with help of multivalent metal ion such as Ferrous and Eu.

.

In this Chapter, acid hydrolysis, alkaline treatment, addition of metal ion, and X-ray fluorescence spectroscopy was carried out to study the detailed structure of lacquer polysaccharides.

2.3 Experimental

2.3.1 Materials

The lacquer polysaccharides was isolated from acetone powder made from the lacquer sap produced in Maoba, Hubei China. Poly-L-lysine with a molecular weight of 1000-5000 was purchased from Sigma-Aldrich, Co. A CM5 sensor chip, an amine coupling kit, HBS-EP+ 10x buffer (including 0.1M 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 30mM EDTA, and 0.5 v/v% polyoxyethylene (20) sorbitan monolaurate surfactant (surfactant P20)), and 50 mM NaOH solution were supplied by GE Healthcare Japan, Co. Ltd. The HBS-EP+ 10x buffer was diluted 10 times with Milli-Q water.

2.3.2 Measurement

^{13}C NMR spectra were recorded on a JEOL JNM ECX-400 or JEOL ECX-600 spectrometer at 100 MHz or 150 MHz at 40 ℃ in D_2O or DMSO-d_6 solvents, respectively. Chemical shifts are expressed as ppm downfield from 4, 4'-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard.

Molecular weights of polysaccharides were determined at 40℃ by an aqueous phase GPC column (Tosoh TSK-gel G2500PWXL, G3000PWXL, and G4000PWXL, 7.6 mmtimes300 mmtimes3 eluted with 66.7 mmol phosphate buffer, pH=6.68) with a Tosoh RI detector using pullulan standards.

Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. Specific rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at 25℃ in a water-

jacketed 10 ml quartz cell. The surface plasmon resonance (SPR) spectrum was taken on a Biacore X100 instrument at 25℃ using a CM5 sensor chip. Elemental analysis was carried out by S8 Tiger X-ray fluorescence spectrometer, from Brucker.

2.3.3 Separation of two fractions and alkaline treatment

Separation of two fractions is carried out by Sephadex G 100 size exclusive chromatography. Which will be detailed discussed in **Chapter 3**. Alkaline treatment was carried out in aqueous solution. Lacquer polysaccharide fraction with with molecular weight 3.0×10^4 (200 mg) was dissolved in 5% NaOH solution (20 ml) and the mixture was stirred for 12 h at 60℃. Followed by dialysis and freeze-drying Sephadex G-50 column chromatography, polysaccharides with molecular weight 1.4x104 was obtained gave a 85% yield.

2.3.4 Interaction to metal ions

Ferric Chloride was solved by water and added drop-wisely into the solution of lacquer polysaccharides. By stirred and duration for several minutes, the gel like light brown precipitate formed. By centrifuge and water-washed then froze-dried, the precipitate was collected. IR spectra was determined.

2.3.5 Elemental analysis

Lacquer polysaccharides was finely grounded and put into a 3 mm high, with 12 mm diameter PVC tube, and then pressed into a tablet used for X-ray fluorescence determination. Rh source and room temperature.

2.3.6 Acidic hydrolysis of lacquer polysaccharides

Every 50mg of crude lacquer polysaccharide was solved in deionized water, kept at 60℃ for 1, 2, 3, 4, 15, and 30 h, respectively. After been cooled to room temperature, and neutralized with sodium carbonate, each sample was dialysis against deionized water for 48h, and freeze dried to give an almost white products.

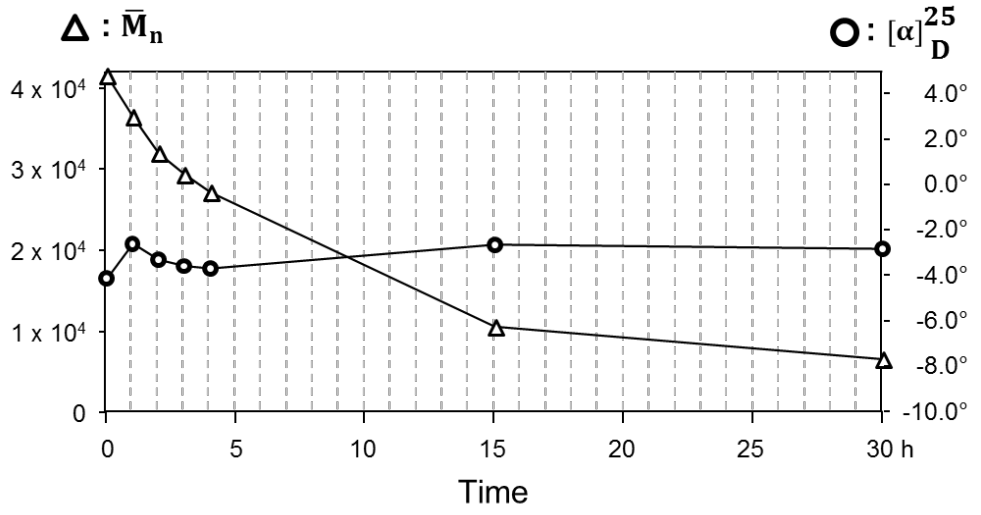


Figure 2.2 result of changes of Molecular weight and special rotation by acidic hydrolysis

2.4 Result and discussion

Highly branched acidic polysaccharides with 1, 3-β-galactan backbone and glucouronic acid terminals, lacquer polysaccharides are usually found with two molecular weight fractions, $10x10^4$ and 3.0×10^4 Dalton in the proportion of about 25% and 75% respectively. The single fraction of 10 x 104 was reported to be degraded into 3.0x104 after the sap was collected. While treated with diluted sulphuric acid at 60℃ for about 4 hours long, the larger fraction with molecular weight 10x104 hydrolysed into 3.0x104, gave a high yield of 97%. Which suggest that the two fraction act different while encounter to acid, may resulted from the structural different. Since two pure fractions were first isolated by using Sephadex size exclusive chromatography, It was found that both of the two fractions show the same structure by ^1H and ^{13}C NMR characterization. That means two fractions possess some repeating unit in both fraction. Which is lead to a consideration that, the larger fraction is built from three unique unit of smaller fraction.

It was also found that addition of ferric or ferrous ion can lead to a gelation of lacquer polysaccharides with lower molecular weights in aqueous solution, suggesting that the degradation may cause by breakage of metal-carboxyl association linkages. Appearance at 1612 cm-1 due to COO- ion of the IR spectra and alkaline degradation of 3.0x104 fraction into 1.4x104, which halved the molecular weight, could also be a proof of existence of metal-carboxyl association

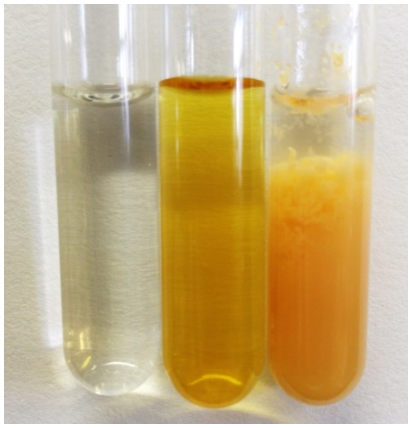


Figure 2.3 gelation of lacquer polysaccharides by addition of Ferrous ion

The result of alkaline treatment, that fraction with molecular weight of $3.0x10^4$ degraded into $1.4x10^4$, just one half of its size, also suggesting the repeating unit and

association structure. while most of the carbohydrate do no reaction with alkaline, the result of degradation may cause by sodium ion, who replaced the bridging metal and destroyed the association structure.

On the XRF spectra shown below, we can find out absorption of potassium, sodium which are introduced by the phosphate buffer we used, and trace amount of iron and copper was also found. which might be the metal ion which functions the association bridge between two acidic polysaccharides.

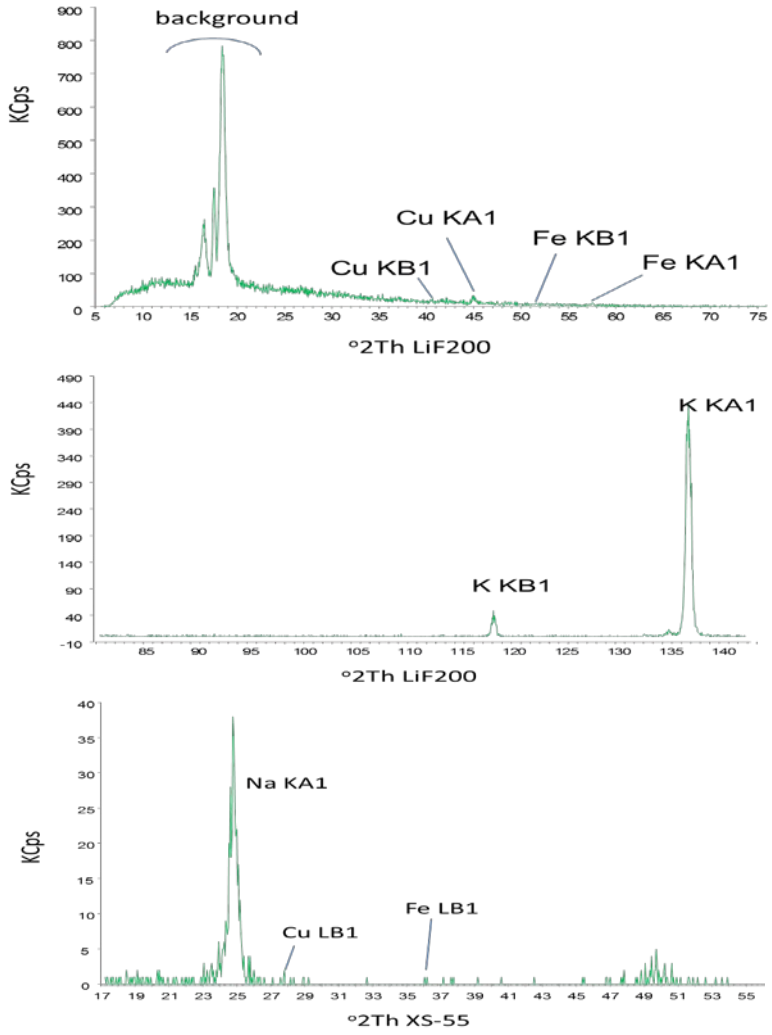


Figure 2.4 Result of elemental analysis by X-ray fluorescence

All the result proving that, lacquer polysaccharides could form a bridging structure and the association do exist in the lacquer polysaccharides. However the detailed mechanism was not clear enough, such as what kind of connection and why it stopped in the lacquer sap when certain proportion reached.

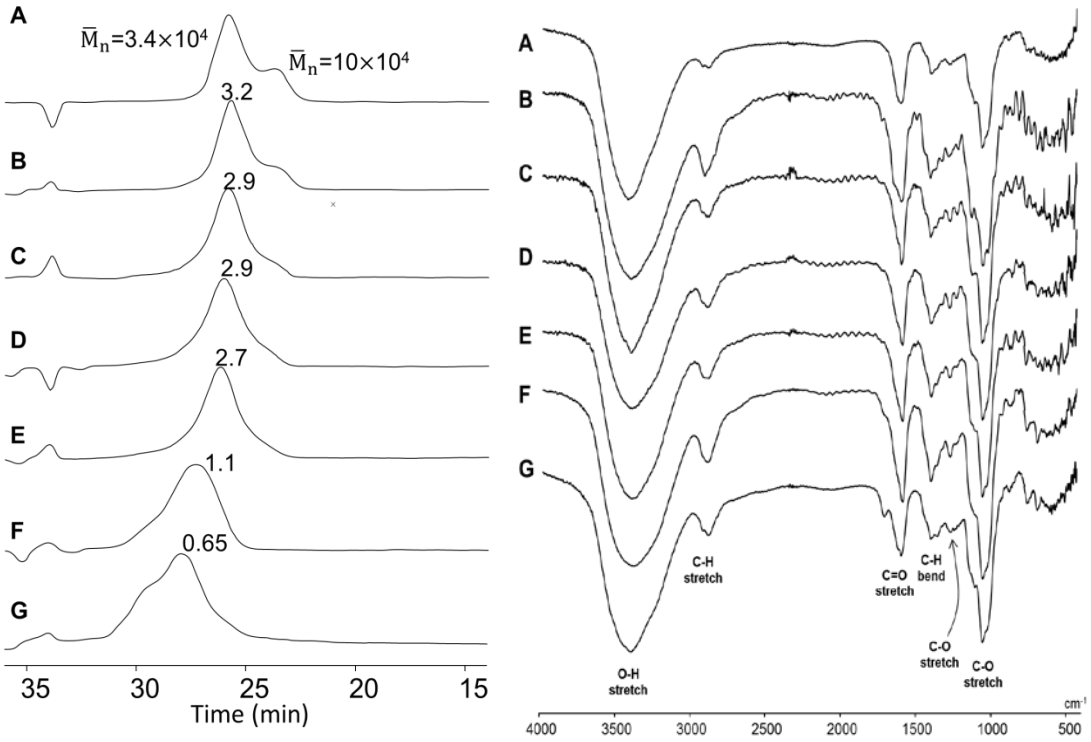


Figure 2.5 GPC profile (left) and FTIR spectra of lacquer polysaccharides, (A). Crude lacquer polysaccharide, (B)~(G) acidic hydrolysed lacquer polysaccharides, by 1, 2, 3, 4, 15, and 30 hours.

By determine the interaction of acidic hydrolysed lacquer polysaccharides, it is found that the interaction to Poly- L-lysine increased from 0 to 4 hours and then decreased from 4 to 30 hours. Which may consider that the lacquer polysaccharides was activated by diluted sulphuric

acid while the larger fraction degraded completely at 4 hours reaction

.

2.5 Conclusion

In this chapter, we mainly discussed the association of lacquer polysaccharides the structure of lacquer polysaccharides, which had not been well studied, was discussed. The association structure, which cause the special form of degradation of larger fraction of lacquer polysaccharide into one third molecular weight, including natural degradation in the lacquer sap by exposure to air and acidic hydrolysis by diluted sulphuric acid, was well discussed.

Two pure fractions were first isolated in this study by using Sephadex size exclusive chromatography. It was found that both of the two fractions show the same structure by ^{1}H and ^{13}C NMR characterization. It was also found that addition of ferric or ferrous ion can lead to a

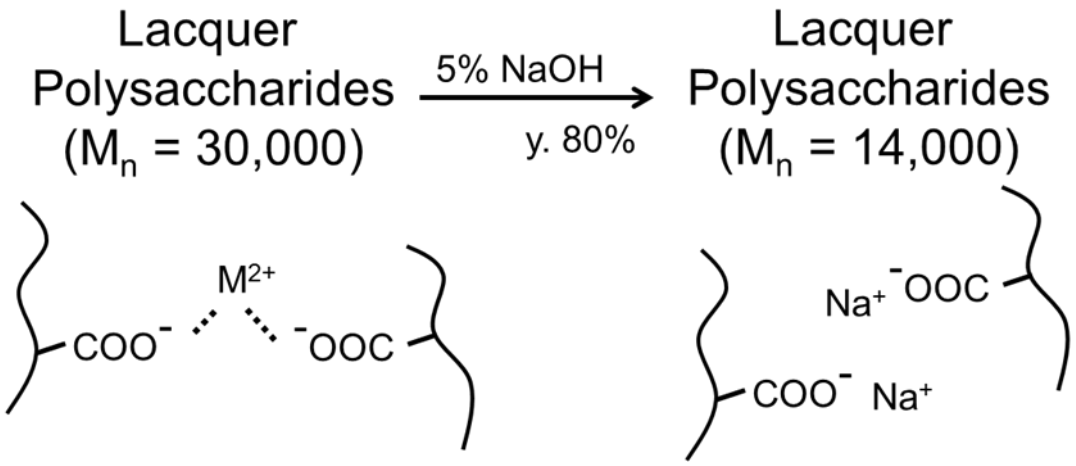
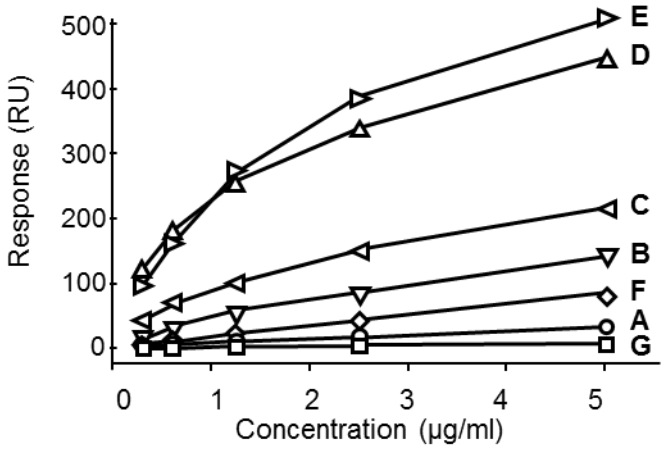


Figure 2.6 Alkaline treatment of lacquer polysaccharides

gelation of lacquer polysaccharides with lower molecular weights in aqueous solution, suggesting that the



degradation may cause by breakage of metal-carboxyl association linkages. Appearance at 1612 cm^{-1} due to COO^- ion of the IR spectra and alkaline degradation of 3.0×10^4

fraction into 1.4 x 10^4, which halved the molecular weight, could also be a proof of existence of metal-carboxyl association.

What interesting is that, lacquer polysaccharides by 4 hour acidic hydrolysis give a high affinity to polylysine, like sulfated polysaccharide. The mechanism is still uncovered, and might be studied in the future.

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Chapter 3

Separation of lacquer polysaccharides and interaction with poly-L-lysine

3.1 Abstract

A naturally occurring acidic lacquer polysaccharide with glucuronic acid at the terminals of the complex branches has specific biological activities including promotion of blood coagulation and antitumor activities. The polysaccharide has two molecular weight fractions $\overline{M}_n$ = 10 x 10^4 and $\overline{M}_n$ = 3.0 x 10^4.

In the present work, two pure fractions were isolated for the first time by Sephadex G-100 column chromatography. Then, each fraction was treated with diluted alkaline solution to decrease the molecular weights to $\overline{M}_n$ = 3.0 x 10^4 and $\overline{M}_n$ = 1.4 x 10^4, respectively. The NMR and IR spectra and specific rotations of the fractionated and original lacquer polysaccharides were almost identical, suggesting that the lacquer polysaccharides are an associated structure with several low molecular weight polysaccharides of $\overline{M}_n$ = 1.4 x 10^4. Interactions between each lacquer polysaccharide and poly-L-lysine, a model compound of proteins and peptides with positively-charged amino groups, were investigated by surface plasmon resonance (SPR) to elucidate the biological mechanism. The apparent dissociation-rate (k_d), association-rate (k_a), and dissociation constant (K_D) obtained by SPR indicate that the lacquer polysaccharides had weaker interactions with poly-L-lysine than sulfated polysaccharides and that the interaction depended on the molecular weight. These SPR results suggest that the specific biological activities of lacquer polysaccharides originate from electrostatic interaction.

3.2 Introduction

Naturally occurring lacquer (*urushi*) is the only natural product polymerized by the enzyme laccase; it has been used as a coating material for several thousand years in Japan and Asian countries (Lu et al., 2013). The lacquer sap contains mainly urushiol (a general term for 3-alkenylcatechols), polysaccharides, and the enzyme laccase.

Fundamental and applied studies on natural lacquer have focused on the polymerization mechanism of urushiol, properties of the coating films, functionality of lacquer sap, and development of new lacquer paints. Kumanotani investigated the chemistry (*urushi*) for use in the future as a coating material (Kumanotani, 1995). The polymerization mechanism of urushiols was elucidated by the electron spin resonance (ESR) detection of a semiquinone radical and by the structure of the separated urushiol dimers (Oshima et al., 1985). Natural lacquer requires a long time for complete polymerization; in general, it takes more than one month under humidity at 70% and a temperature around 25°C. Miyakoshi et al. have developed hybrid lacquers that

polymerize quickly at humidity below 50% and temperature below 20°C by adding amine-functionalized organic silicone reagents to natural lacquer (Nagase et al., 2004). As a coating material, the new natural lacquer with a precious metal colloid was found to give a beautiful and durable surface. The Au and Ag colloids produce lacquer films with homogeneous red and yellow colors, respectively (Lu et al., 2006).

However, only limited studies on the other components of lacquer sap, lacquer polysaccharides, have been reported. Oshima and Kumanotani revealed the structure of lacquer polysaccharides by gel permeation chromatography (GPC), sugar analysis, methylation analysis, and Smith degradation, which suggest that the lacquer polysaccharide is a mixture of two fractions with the molecular weights of $\overline{M}_n$ = 2.3 x 10^4 and 6.7 x 10^4 and that it has a (1→3)-β-D-galactopyranosidic main chain with complex branches having glucuronic acid at the terminals (Oshima & Kumanotani, 1984). We also reported the structural analysis of the lacquer polysaccharides by high resolution nuclear magnetic resonance (NMR)

measurements including 2D NMR spectroscopies, in which complex signals were assigned and the monosaccharide components were revealed to be D-galactose, 4-*O*-methyl-D-glucuronic acid, L-arabinose, and L-rhamnose (Lu et al., 1999). Furthermore, GPC studies of the time-course of the degradation of Japanese lacquer polysaccharides in the sap suggest that the molecular weight of polysaccharides in the lacquer sap was originally $\overline{M}_n$ = 6.7 x 10^4 with a narrow molecular weight distribution, and that then the lacquer polysaccharides separated gradually into two molecular weights after collection of the sap (Lu & Yoshida, 2003).

Several biological activities of lacquer polysaccharides in vivo were reported, including the prevention of leukopenia induced by cyclophosphamide in mice and stimulating the growth of leucocytes (Du et al., 1999; Yang & Du, 2003). We also found that the lacquer polysaccharide promoted blood coagulation at the concentration of 0.016 mg/ml, shortening the coagulation time of bovine plasma by more than one minute in comparison with that of a blank. The lacquer polysaccharide also had antitumor activity in vivo; that is, oral administration of the lacquer

polysaccharide to mice at the dose of 50 mg/kg halved the weights of sarcoma 180 tumors. In addition, after sulfation, sulfated lacquer polysaccharides had potent anti-HIV activity at concentrations as low as 0.1 μg/ml and moderate blood anticoagulant activity at 10 units/mg compared to those standard polysaccharides of curdlan and dextran sulfates (Lu et al., 2000).

In this paper, we report the separation of two lacquer polysaccharide fractions using Sephadex column chromatography to give pure polysaccharide fractions for the first time. The lacquer polysaccharides had two main molecular weights, $\overline{M}_n$ = 10 x 10^4, and 3.0 x 10^4 in the proportion of 25 mol% and 75 mol%. Then, structural analysis was carried out by high resolution NMR. Further, the fractions were treated with dilute alkaline solution to reduce the molecular weight of the polysaccharides to $\overline{M}_n$ = 3.0 x 10^4 and 1.4 x 10^4, respectively, with the same structure as each other. We then measured the interaction of these polysaccharides with poly-L-lysine using SPR to quantitatively elucidate their biological activities.

3.3 Experimental

3.3.1 Materials

Poly-L-lysine with a molecular weight of 1000-5000 was purchased from Sigma-Aldrich, Co. A CM5 sensor chip, an amine coupling kit, HBS-EP+ 10x 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)), and 50 mM NaOH solution were supplied by GE Healthcare Japan, Co. Ltd. The HBS-EP+ 10x buffer was diluted 10 times with Milli-Q water.

3.3.2Measurement

^{13}C NMR spectra were recorded on a JEOL JNM ECX-400 or JEOL ECX-600 spectrometer at 100 MHz or 150 MHz at 40°C in D_2O or DMSO-d_6 solvents, respectively. Chemical shifts are expressed as ppm downfield from 4, 4'-dimethyl-

4-silapentane-1-sulfonate (DSS) as an internal standard. Molecular weights of polysaccharides were determined at 40°C 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 mmol phosphate buffer, pH=6.68) with a Tosoh RI detector using pullulan standards. Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. 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 surface plasmon resonance (SPR) spectrum was taken on a Biacore X100 instrument at 25°C using a CM5 sensor chip.

3.3.3 Separation of lacquer polysaccharides by Sephadex G-100 column chromatography

Lacquer polysaccharides with a mixture of two molecular weight fractions ($\overline{M}_n$ = 10 x 10^4 and 3.0 x 10^4 in the proportion of 25 mol% and 75 mol%) were obtained from the acetone powder prepared from a Chinese lacquer sap

according to the method of Reinhammar (Reinhammar, 1970). The mixture of lacquer polysaccharides (10g) was dissolved in deionized water (20ml) and then applied to a Sephadex G-100 column (2.4 x 40 cm). Phosphate buffer (66.7 mmol, pH=6.68) was used as an elution solvent. The two light brown bands were separated and dialyzed by deionized water for 24h, respectively. Each dialysate was freeze-dried to give pure lacquer polysaccharide fractions with molecular weights of $\overline{M}_n$ = 10 x 10^4 and 3.0 x 10^4, respectively.

3.3.4 Treatment of lacquer polysaccharides with diluted alkaline solution.

The each lacquer polysaccharide fraction (200 mg) was dissolved in 5% NaOH solution (20 ml) and the mixture was stirred for 12 h at 60°C. After dialysis with deionized water for 24h, the lacquer polysaccharide was obtained by freeze-drying. The molecular weights of the lacquer polysaccharides obtained were $\overline{M}_n$ = 10 x 10^4 and 3.0 x 10^4 in the proportion of 85 mol% and 15 mol% from the pure $\overline{M}_n$ =

10 x 10^4 fraction, and $\overline{M}_n$ = 3.0 x 10^4 and 1.4 x 10^4 in the proportion of 70 mol% and 30 mol% from the pure $\overline{M}_n$ = 3.0 x 10^4 fraction. The lacquer polysaccharide with the lower molecular weight of $\overline{M}_n$ = 1.4 x 10^4 was purified by Sephadex G-50 column chromatography.

3.3.5 SPR measurement

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 (Fischer, 2010). The concentration was 5 mg/ml and the flow rate was 10 μl/min for 7 minutes. The value for the immobilized poly-L-lysine was 2000 response units (RU). A reference cell without poly-L-lysine was used. Lacquer polysaccharides (500 μg/ml) with different molecular weights were injected for 3 min (30 μl/min) over the poly-L-lysine immobilized sensor chip, and then the HBS-EP+ running buffer solution was injected for 12 min to determine the association and dissociation rate constants, as shown in Figure 3.4. A typical procedure for the weakly interacting sample was as follows.

The lacquer polysaccharide solution ($\overline{M}_n$ = 10 x 10^4, 500 μg/ml) was prepared in the HBS-EP+ buffer, and then the solution diluted to various concentrations with the HBS-EP+ buffer was injected over the sensor chip at a flow rate of 30 μl/min for 3 min for association, and then dissociation was carried out with injection of only the HBS-EP+ buffer for 12 min. For regeneration of the CM5 sensor chip, NaOH (50 mM) solution was used. The initial concentration of sulfated polysaccharides with high interaction was 5 μg/ml.

3.4. Results and Discussion

3.4.1 Isolation and structure of lacquer polysaccharides

The lacquer polysaccharide was isolated from the acetone powder that was obtained by adding acetone to the Chinese lacquer sap according to Reinhammar's method (Reinhammar, 1970). In previous reports (Oshima & Kumanotani, 1984; Lu & Yoshida, 2003), the lacquer polysaccharide had two molecular weight fractions with

number-average molecular weights ($\overline{M}_n$) of around $\overline{M}_n$ = 10 x 10^4 and 3.0 x 10^4, respectively, in the proportion of 25:75 mol%. However, we had observed that only the larger fraction was present in the sap isolated immediately after collection of Aizu lacquer from Fukushima Prefecture, Japan (Lu & Yoshida, 2003). The smaller fraction was contained only in Taiwan and Vietnam lacquer saps.

In the present work, two lacquer polysaccharide fractions were isolated from Chinese lacquer sap for the first time by using the Sephadex G-100 column chromatography to give two pure polysaccharides with different molecular weights and the same structure as each other according to the results of NMR measurements, as described below. Figure 3.1 shows the GPC profiles of (A) the original lacquer polysaccharides in the proportion of 25 mol% (larger fraction) and 75 mol% (smaller fraction) isolated from the Chinese lacquer sap, and pure fractions with the molecular weights of $\overline{M}_n$ = 10 x 10^4 (B), and 3.0 x 10^4 (C). The specific rotations of the three polysaccharides were almost the same, $[\alpha]_D^{25}$= -4.13°, -3.63°, and -6.23° (c1, H_2O) for the original, larger, and smaller polysaccharides, respectively, as shown in Figure 3.1.

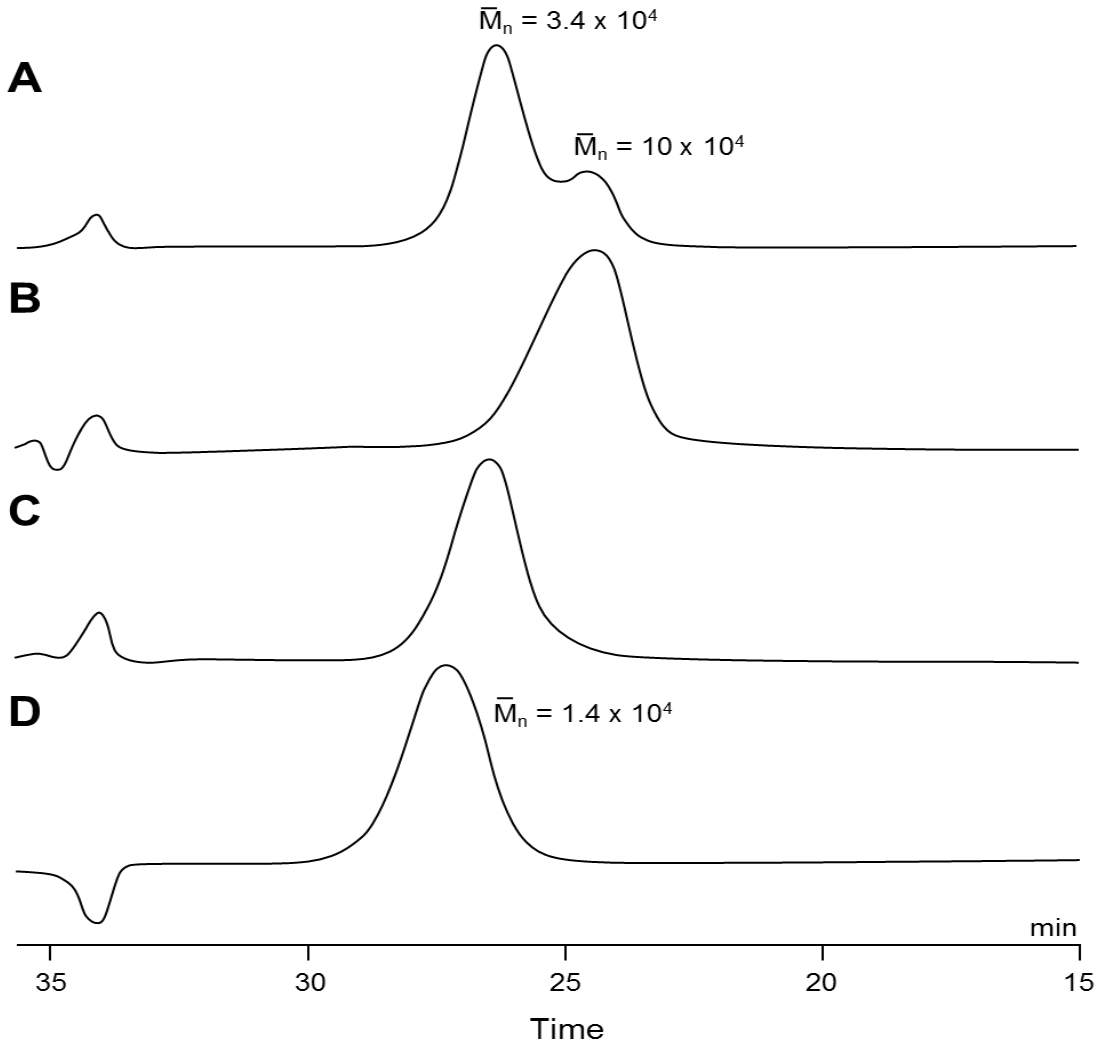

Figure 3.1. Aqueous GPC profiles of Chinese lacquer polysaccharides. (A) The original lacquer polysaccharide ($[\alpha]_D^{25}$= -4.13°(c1, H_2O)) with two molecular weight fractions of $\bar{M}_n$ = 10 x 10^4 and $\bar{M}_n$ = 3.4 x 10^4 in the proportion of 25 mol% and 75 mol%, (B) and (C) pure larger ($[\alpha]_D^{25}$= -3.63°(c1, H_2O)) and smaller ($[\alpha]_D^{25}$ = -6.23°(c1, H_2O)) fractions after Sephadex G-100 column chromatography of (A), and (D) pure fraction with $\bar{M}_n$ = 1.4 x 10^4 ($[\alpha]_D^{25}$= -8.91°(c1, H_2O)) after 5% NaOH treatment of (C).

In addition, the separated lacquer polysaccharides with $\overline{M}_n$ = 10 x 10^4 and 3.0 x 10^4 were treated with 5% NaOH solution for 12 h at 60°C to give two pure polysaccharides with $\overline{M}_n$ = 3.0 x 10^4 and 1.4 x 10^4 with homogeneous molecular weight distributions after separation by Sephadex G-50 column chromatography. Figure 3.4D shows the GPC profile of the lacquer polysaccharide with $\overline{M}_n$ = 1.4 x 10^4 and $[\alpha]_D^{25}$= -8.91°. Therefore, we assumed that the lacquer polysaccharide had an associated structure with the smaller molecular weight polysaccharide, but further treatment of the smaller lacquer polysaccharide with the diluted alkaline solution gave no lower molecular weight polysaccharides. These results do not contradict our previous results that the lacquer polysaccharide was one molecular weight fraction with $\overline{M}_n$ = 6.7 x 10^4 after immediate isolation from the fresh sap (Lu & Yoshida, 2003). After the sap was stored for a long time, a new fraction with the smaller molecular weight of $\overline{M}_n$ = 2.7 x 10^4 was observed.

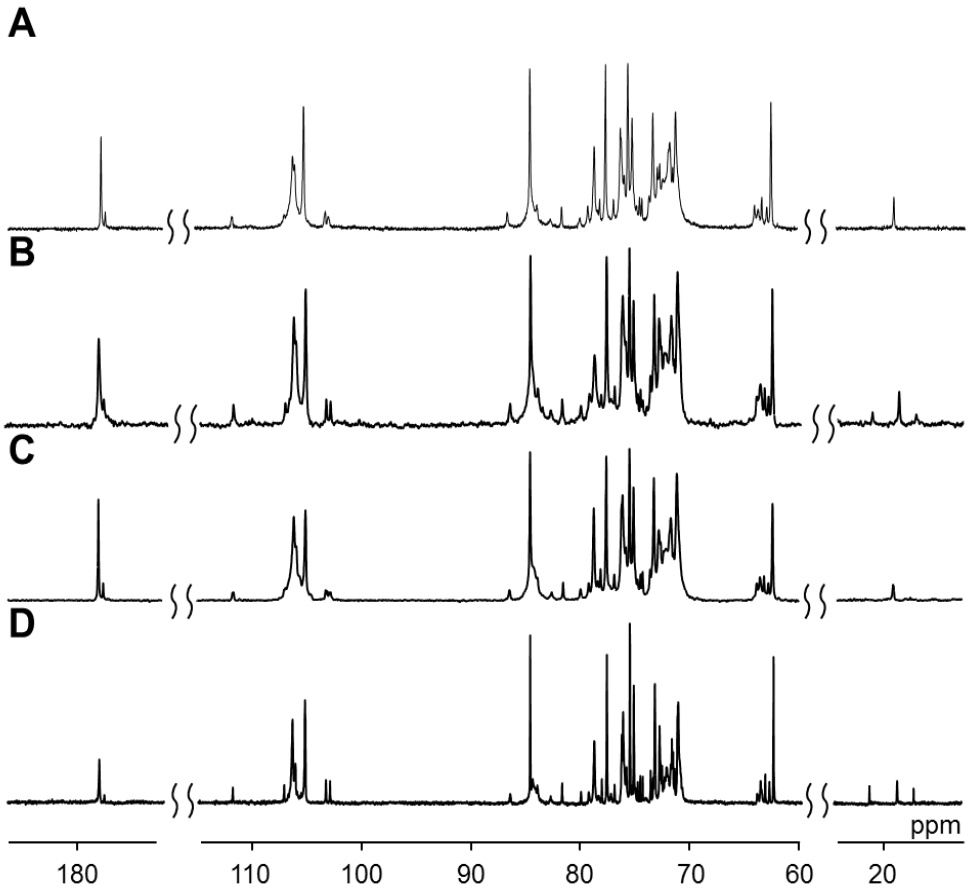

Figure 3.2. ^{13}C NMR spectra of lacquer polysaccharides at 100 MHz for (A), (B), and (C), and at 150 MHz for (D) in D_2O at 40℃.

Figure 3.2 shows the ^{13}C NMR spectra of lacquer polysaccharides that correspond to those in Figure 3.1. Previously, we assigned most of the complex signals (Figure 3.2A) by high resolution NMR measurements involving two dimensional DQF-COSY, TOCSY, HMQC, and HMBC experiments. The intensity and chemical shifts

of the carbon spectra in Figure 3.1 were similar to each other.

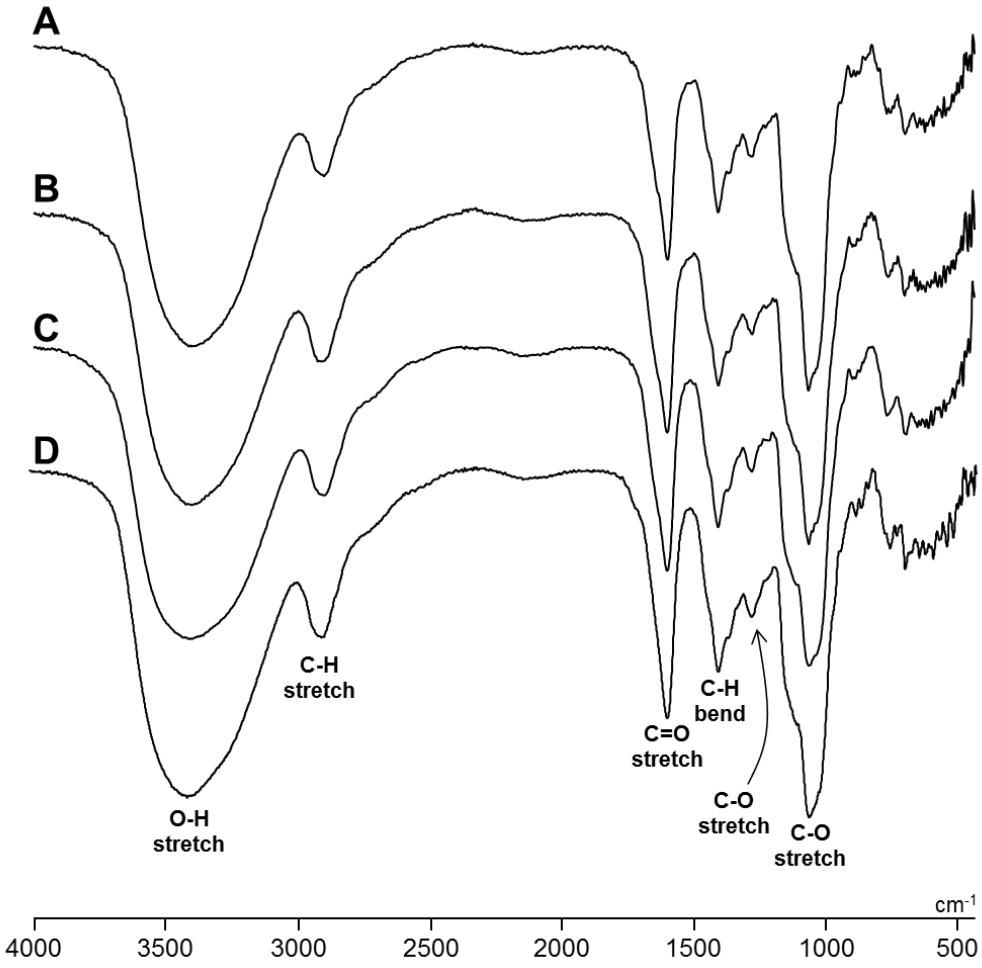

Figure 3.3. FI-IR spectra of lacquer polysaccharides (KBr-method). (A) Original, and (B), (C), and (D) pure fractions with $M_n = 10 \times 10^4$, 3.4×10^4, and 1.4×10^4, respectively, corresponding to those in Figure 1.

In the IR spectra of these fractions (Figure 3.3), absorption bands at 1610 cm^{-1}, 1250 cm^{-1}, and 1050 cm^{-1} due

to COO^- stretching vibration and the large absorption at 3400 cm^{-1} due to OH stretching vibration appeared. These characteristic absorptions as well as small bands in the finger print region gave the same shapes and intensities as the four fractions. The associated structure might be constructed by the alkaline metal and/or earth ions, for example, Ca^{2+} and/or Fe^{2+} (Aspinall, 1983; Klemm, 2010). With passing of time after collection, the association decreased and they divided into smaller polysaccharides. Further detailed investigations of the associated structure of the lacquer polysaccharides are in progress.

3.4.2 Binding of lacquer polysaccharides to poly-L-lysine

We found previously that lacquer polysaccharides have specific biological activities such as promoting blood coagulation and antitumor activities. After sulfation, sulfated polysaccharides have potent anti-HIV and moderate blood anticoagulant activities (Lu & Yoshida, 2000). Therefore, we predicted that the electrostatic

interaction of negatively charged carboxylic or sulfated groups of lacquer polysaccharides and positively carged amino groups of the cell surface glycoproteins between polysaccharides and cell surface glycoproteins play an important role in the biological activities (Uryu et al., 1992; Jeon et al., 1997; Jeon et al., 2000). The affinity of lacquer polysaccharides to poly-L-lysine as a model compound of proteins and peptides was examined to elucidate the interaction mechanism of the biological activities using surface plasmon resonance (SPR) measurement (Schasfoort & Tudos, 2008). We found that lacquer polysaccharides interacted weakly with poly-L-lysine. The RU values decreased with decreasing molecular weight of lacquer polysaccharides. The lacquer polysaccharide with the higher molecular weight of $\overline{M}_n$= 10 x 10^4 gave the higher association response of about 130 RU (Figure 3.4B) compared to the lower molecular weight polysaccharides with $\overline{M}_n$= 3.4 x 10^4 and $\overline{M}_n$= 1.4 x 10^4 (Figures 4C and 4D) at the concentration of 500 μg/ml, indicating that the interaction was dependent on the molecular weights of lacquer polysaccharides. Lacquer polysaccharides are acidic polysaccharides with glucuronic acids at the terminals of

Table 3.1 Kinetic results of lacquer polysaccharides[a), b)]

	$\overline{M}_n$ x 10^4	S %	k_a 1/M	k_d 1/s	K_D M
Original lacquer polysaccharide[c)]	5.1	-	4.99 x 10^2	3.54 x 10^{-4}	7.10 x 10^{-7}
Lacquer polysaccharide 1	10	-	8.23 x 10^2	2.94 x 10^{-4}	3.58 x 10^{-7}
Lacquer polysaccharide 2	3.4	-	3.02 x 10^2	3.08 x 10^{-4}	1.02 x 10^{-6}
Lacquer polysaccharide 3	1.4	-	1.69 x 10^2	4.75 x 10^{-4}	2.81 x 10^{-6}
Sulfated lacquer polysaccharide	0.7	11.0	4.31 x 10^4	1.74 x 10^{-4}	4.04 x 10^{-9}
Dextran sulfate	1.3	18.4	2.27 x 10^6	3.73 x 10^{-4}	1.64 x 10^{-10}

a) Lacquer polysaccharide was injected 90 μl for 180 sec at a flow rate of 30 μl/min of a HBS-EP running buffer at 25 °C and then the running buffer was further flowed for 600 sec. Concentration of lacquer polysaccharide was 500, 250, 125, 62.5, and 31.3 μg/ml, respectively. For sulfated polysaccharides, the concentration was 5.0, 2.5, 1.25, 0.62, and 0.31 μg/ml, respectively.

b) k_a; Association-rate, k_d; dissociation-rate constants, and dissociation constant $K_D = k_d/k_a$

c) Original lacquer polysaccharide had two molecular fractions with $\overline{M}_n = 10 \times 10^4$ and $\overline{M}_n = 3.4 \times 10^4$ in the proportion of 25 and 75 mol%.

the branches, but their affinity to poly-L-lysine was weaker than that of sulfated polysaccharides (Figures 4E and 4F). These results are comparable with the biological activities

of lacquer polysaccharides, the increased anti-HIV and blood coagulant activities we reported previously (Lu et al., 2000).

Table 3.1 shows the apparent kinetic constants of lacquer polysaccharides to poly-L-lysine calculated by the global fitting curves according to the 1 : 1 binding model. Dextran sulfate ($\overline{M}_n$ = 1.3 x 10^4, S = 18.4%), which has potent anti-HIV and blood anticoagulant activities, was used as a positive reference compound. Although the lacquer polysaccharide is an acidic polysaccharide with carboxylic acids, the association (k_a) and dissociation-rate (k_d) constants were low because of the low affinity to poly-L-lysine. These results are consisttent with the weak anti-HIV and blood coagulation promoting activities of lacquer polysaccharides (Lu et al., 2000). After sulfation, sulfated lacquer polysaccharides ($\overline{M}_n$ = 0.7 x 10^4, S = 11.0%) had higher association (k_a) and lower dissociation- (k_d) rate constants, indicating that sulfated lacquer polysaccharides had a high affinity to poly-L-lysine by the electrostatic interaction of the negative charges of sulfate groups and positive charges of amino groups. Therefore, the SPR measurement was found to support the elucidation of

biological activities.

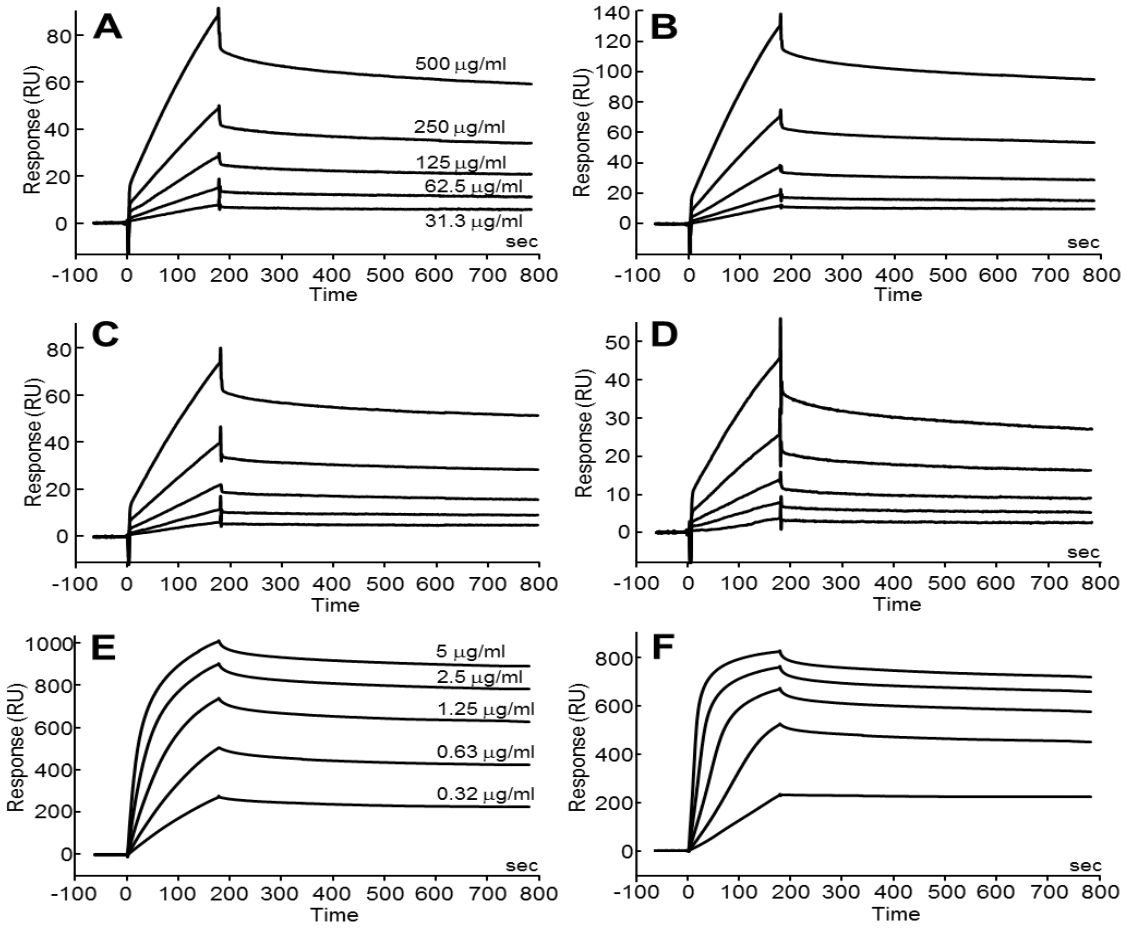

Figure 3.4 Binding curves of lacquer polysaccharides with M_n = 10 x 10^4 to immobilized poly-L-lysine. Binding of (A) original, and (B), (C), and (D) pure molecular weight fractions with M_n = 10 x 10^4, 3.4 x 10^4, and 1.4 x 10^4, respectively, corresponding to those in Figure 1. (E) Binding of sulfated lacquer polysaccharide with M_n = 0.7 x 10^4 and (F) dextran

sulfate with M_n = 1.3 x 10^4. Lacquer polysaccharide (90 ml) was injected for 180 sec at a flow rate of 30 ml/min of a HBS-EP running buffer at 25 C, and then the running buffer was further flowed for 600 sec. Concentrations oflacquer polysaccharide were 500, 250, 125, 62.5, and 31.3 mg/ml. For sulfated polysaccharides, the concentrations were 5.0, 2.5, 1.25, 0.63, and 0.32 mg/ml.

Figure 3.5 shows the relationship between the kinetic properties of lacquer polysaccharides and sulfated polysaccharides in which the affinity to poly-L-lysine differed with the molecular weights and acidic functional groups. The polysaccharides had almost the same dissociation rate constant (k_d), but the association rates (k_a) differed with their functional groups and molecular weights. The association rate of lacquer polysaccharides increased gradually with increasing molecular weights. On the other hand, the sulfated lacquer polysaccharide showed high affinity to poly-L-lysine, indicating that sulfated lacquer polysaccharide had potent biological activities due to the interaction between the sulfate groups and amino groups.

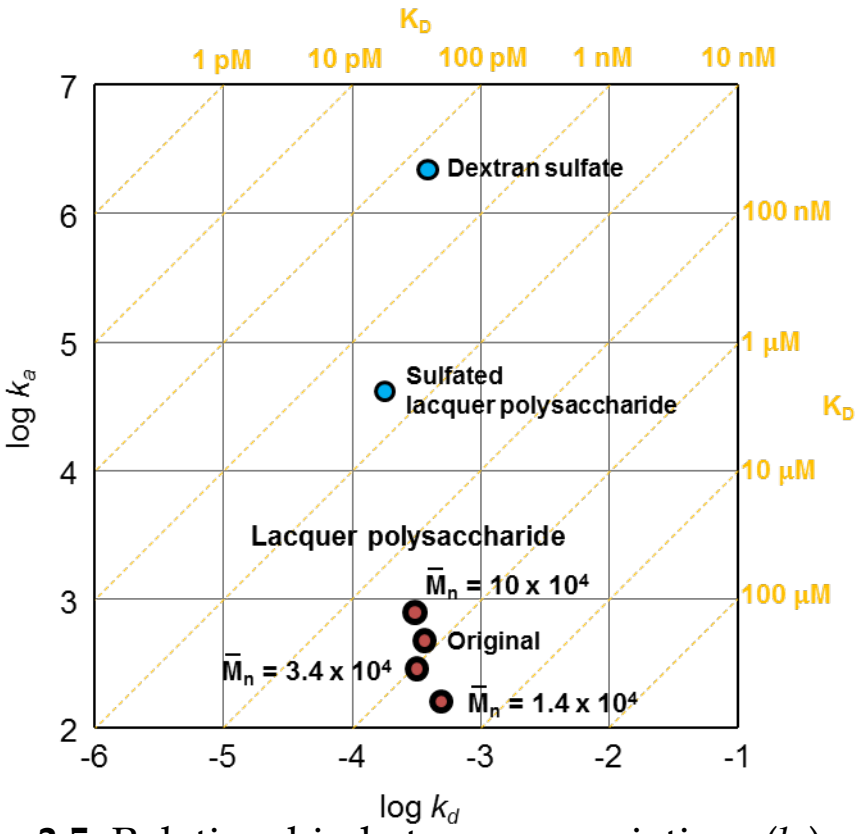

Figure 3.5. Relationship between association- (k_a) and dissociation- (k_b) rate constants of lacquer polysaccharides. The dotted line shows the dissociation constant, K_D.

3.5 Conclusion

We obtained three pure polysaccharide fractions of a Chinese lacquer polysaccharide with $\overline{M}_n$= 10 x 10^4, 3.0 x 10^4, and 1.4 x 10^4 by Sephadex column chromatography and dilute alkaline treatment of the fractionated lacquer polysaccharides. The structures of these polysaccharides

were almost the same according to NMR, IR, and specific rotations, assuming that the low molecular weight polysaccharides structures were associated through Ca^{2+} and/or Fe^{2+}.

In addition, the present work showed the interaction of these lacquer polysaccharides with poly-L-lysine using SPR measurement to calculate the apparent kinetic constants such as the k_a, k_d, and K_D constants, suggesting that lacquer polysaccharides had a weak affinity to poly-L-lysine compared to sulfated polysaccharides. These kinetic data support the hypothesis that the antitumor and blood coagulation-promoting activities are due to electrostatic interaction. However, sulfated lacquer polysaccharides have a high affinity to poly-L-lysine according to the SPR measurements, results that correspond to the potent anti-HIV and anticoagulant activities we reported previously. The mechanism of action is under further investigation to elucidate the specific biological activities of lacquer polysaccharides.

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Chapter 4

Interaction between sulfated lacquer polysaccharides with oligopeptides

4.1 Abstract

Three different passages of gp120 glycoprotein V3 loop sequence from 297 to 316, C-terminus i from 493 to 511 CD4 domain sequenced from 362 to 380, was synthesised as peptide A, B, C. Dextran sulfate, with molecular weight $6.0x10^4$, degree of sulfation 1.20 was used to evaluate interaction with peptide A, B, C as well as poly-*L*-lysine. The result shows that, poly-*L*-lysine as an all positive charged peptide gave strongest interaction with dextran

sulfate. And the peptide C, which is lack of positive charged amino acid, only one lysine in the sequence, gave almost no response on the SPR sensorgram, which is hardly interacted with dextran sulfate. Both peptide A and B interaction strongly with dextran sulfate, and peptide B has slightly stronger attraction that peptide was chosen to evaluate the interaction against lacquer polysaccharides sulfate Lacquer polysaccharides was sulfated by piperidine-textitsmall N-sulfate in the solvent of dimethylsulfoxide at nitrogen atmosphere, 1 to 3 times gave three sulfated lacquer polysaccharides A, B and C with different degree of sulfattion, together with the sulfated lacquer polysaccharides used in previous chapter lacquer polysaccharides sulfate D, the interaction with peptide B were determined. The result show that higher sulfated polysaccharides gave higher recognition (k_a), high stabilities (k_d), to the stronger interaction (K_D), against peptide B. The recognition changes apparently according to the change of sulfation degree, while stabilities of lacquer polysaccharides derivatives interaction against the peptide B differ slightly.

4.2 Introduction

Lacquer polysaccharides, which exist in the sap of Asian lacquer tree, are highly branched acidic polysaccharides with 1, 3-β-galactan backbone and glucuronic acid terminals. Lacquer polysaccharides were reported to be antitumor active and blood coagulation promoting effective, which specific activities might be caused from electrostatic attraction between negative-charged carboxyl groups of the uronic acid terminal of polysaccharides, and positive-charged amino groups of target proteins. The sulfated polysaccharides, like dextran sulfate was reported HIV active and anticoagulation active. The anticoagulation effect my cause severe danger to life, while a trauma occurred. That is the reason why focus on lacquer polysaccharide, which is coagulation promoting effective. And the sulfation derivatives of lacquer polysaccharides, which show high anti-HIV activity and low anticoagulation activity, need to be well studied. The anti-HIV activities was shown in (Lu, Yoshida, 1999)

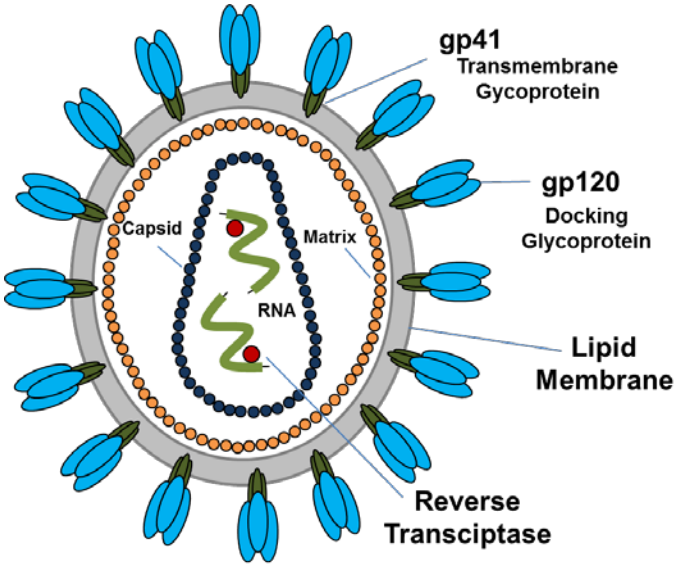


Figure 4.1 Structure of HIV virion

National Institutes of Allergy and Infectious Disease (NIAID) image

Since branched 1, 3-β glucans have potent anti-tumour activity, the lacquer polysaccharides with similar linkage were assayed for the anti-tumour activity by using Sarcoma 180 tumour in rat. When lentinan (1 mg/kg) provided to the rat by an intraperitoneal injection (i.p.), the tumour disappeared after 35 days of transplantation. For the natural lacquer polysaccharide, it was found that the weights of tumour decreased to 10, 11, and 13 g by 50 and 5 mg/kg by p.o., and 1 mg/kg by i.p., respectively.. These results indicate that the glucuronic acid in the terminal of the branches might play an important role in the anti-tumour activity of the lacquer polysaccharide.

The lacquer polysaccharide was revealed to promote the blood coagulation more than 1 min on the blank (4 min and 25 s). After sulfation, the lacquer polysaccharides showed weak anticoagulant activity, which increased slightly with increasing degree of sulfation.

However, the sulfated lacquer polysaccharides provided low anticoagulant activity, probably because of competition with anticoagulant activity originated from sulfonate groups and activation of coagulant factors by the branched structure.

4.3 Experimental

4.3.1 Materials

Poly-*L*-lysine with a molecular weight of 1000-5000 was purchased from Sigma-Aldrich, Co. A SA sensor chip, an amine coupling kit, HBS-EP+ 10x buffer (including 0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 30mM EDTA, and 0.5 v/v% polyoxyethylene (20)

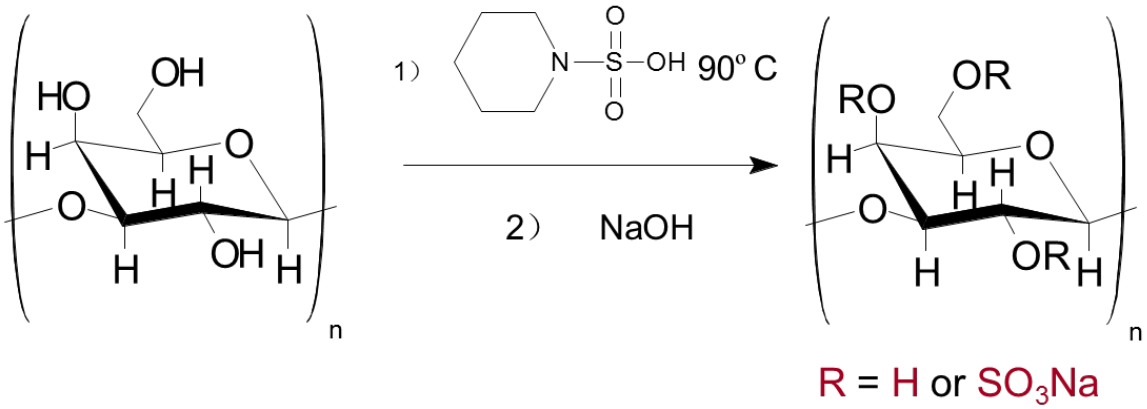
sorbitan monolaurate surfactant (surfactant P20)), and 50 mM NaOH solution were supplied by GE Healthcare Japan, Co. Ltd. The HBS-EP+ 10x buffer was diluted 10 times with Milli-Q water.

4.3.2 Measurement

Molecular weights of polysaccharides were determined at 40℃ 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 mmol phosphate buffer, pH=6.68) with a Tosoh RI detector using pullulan standards. Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. Specific rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at 25℃ in a water-jacketed 10 ml quartz cell. The surface plasmon resonance (SPR) spectrum was taken on a Biacore X100 instrument at 25℃ using a SA sensor chip.

4.3.3 Sulfation of lacquer polysaccharides

Lacquer polysaccharides 1000mg was sulfated by 3g of piperidine-N-sulfate at 90℃for 1 hour under a nitrogen atmosphere, then neutralize by 10% of sodium hydroxide aqueous solution, and dialysed for 48 hour against deionized water and froze dry to give 580 mg of lacquer polysaccharides sulfate A, yield 58%. Take 450 mg of lacquer polysaccharides sulfate A repeat sulfation as above get lacquer polysaccharides sulfate B 344 mg, yield 76%. Then take 213 mg of lacquer polysaccharides sulfate B repeat sulfation get 160mg of lacquer polysaccharides sulfate C.



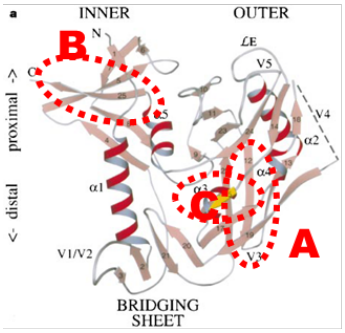
4.3.4 SPR measurement

peptides A, B, C were immobilized on the SA sensor chip using an amine coupling kit according to the Biacore protocols (Fischer, 2010). The concentration was 5 mg/ml and the flow rate was 10 µl/min for 7 minutes. The value for the immobilized oligopeptide was 1000 response units (RU). A reference cell without peptide was used. Lacquer polysaccharides sulfate(5µg/ml) with different sulfation rate were injected for 3 min (30 µl/min) over the oligopeptide immobilized sensor chip, and then the HBS-EP+ running buffer solution was injected for 12 min to determine the association and dissociation rate constants, The lacquer polysaccharide sulfate solution (5µg/ml) was prepared in the HBS-EP+ buffer, and then the solution diluted to various concentrations with the HBS-EP+ buffer was injected over the sensor chip at a flow rate of 30 µl/min for 3 min for association, and then dissociation was carried out with injection of only the HBS-EP+ buffer for 12 min. For regeneration of the SA sensor chip, NaOH (50 mM)

solution was used. The initial concentration of sulfated polysaccharides with high interaction was 5 µg/ml.

4.4 Results and Discussion

In this chapter, peptide A, B and C was synthesised according to the amino acid sequence of HIV virus surface docking glycoprotein, gp120. Three different passages of gp120 glycoprotein were selected. First is peptide A, called V3 loop sequence from 297 to 316, which is rich of positive charged amino acid such as lysine and arginine; second is peptide B, called C-terminus is also a positive charged amino acid rich passage sequenced from 493 to 511; and peptide C is called CD4 domain sequenced from 362 to 380, which is lack of positive charged amino acid.



Peptide A (V3 loop, 20AA, Lys/Arg rich)
[Biotin]-^{297}TRPNNNTRKRIRIQRGPGRA^{316}

Peptide B (C-terminus, 19AA, Lys/Arg rich)
[Biotin]-^{493}PLGVAPTKAKRRVVQREKR^{511}

Peptide C (CD4-binding domain, 19AA)
[Biotin]-^{362}KQSSGGDPEIVTHSFNCGG^{380}

(Chen 2005)

Figure 4.3 Structure of HIV surface docking glycoprotein gp120

Dextran sulfate, with molecular weight 6.0 x 104, degree of sulfation 1.20 was used to evaluate interaction with peptide A, B, C as well as poly-*L*-lysine. The result shows that, Poly-*L*-lysine as an all positive charged peptide gave strongest interaction with dextran sulfate. And the peptide C, which is poor in positive charged amino acid, only one lysine in the sequence, gave almost no response on the SPR sensorgram, which is hardly interacted with dextran sulfate.

Both peptide A and B interaction strongly with dextran sulfate, and peptide B has slightly stronger attraction that

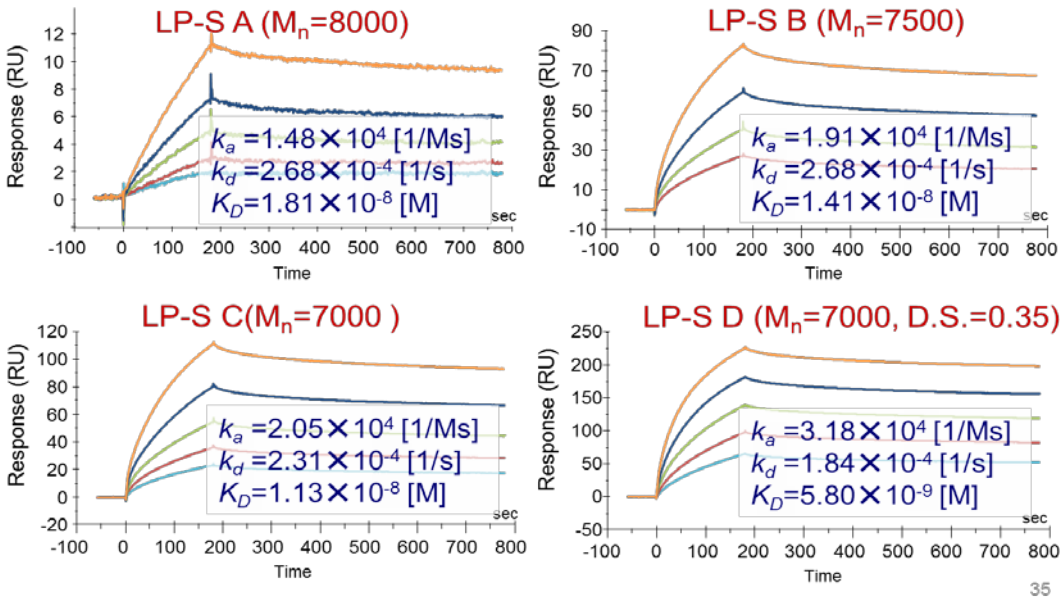
peptide was chosen to evaluate the interaction against lacquer polysaccharides sulfate series. The number of lysine and arginine in peptide A and B are same and the deference in interaction suggest that arrangement of amino acid also affect the interaction. Concentrated positive charge gave more electrostatic attraction power than scattered one.

Lacquer polysaccharides was sulfated by piperidine-*N*-sulfate in the solvent of dimethylsulfoxide at nitrogen atmosphere, 1 to 3 times gave three sulfated lacquer polysaccharides A, B and C with different degree of sulfattion.

The sulfated lacquer polysaccharides used in previous chapter, named lacquer polysaccharides sulfate D, was also discussed the interaction with oligopeptides, compared to these new three. Lacquer polysaccharides D was sulfated in the same condition but doubled the amount of piperidine-*N*-sulfate, reaction time.

Table 4.1 Result of comparison of three peptides interact with dextran sulfate.

ligand	Amino acid sequence	k_a ($\times 10^5$) [1/Ms]	k_d ($\times 10^{-4}$) [1/s]	K_D ($\times 10^{-10}$) [M]
Poly-L-lysine	**KKKKKKKKKKKKKKKKK**	9.08	1.44	1.59
Peptide A	T**R**PNNNT**RKRIRIQR**GPG**R**A	0.68	1.91	28.0
Peptide B	PLGVAPT**KA KRR**VVQ**R**E**KR**	8.84	2.44	2.76
Peptide C	**K**QSSGGDPEIVTHSFNCGG	NA	NA	NA



The result of the SPR determination of sulfated lacquer polysaccharides and peptide B shows that higher sulfated polysaccharides gave higher recognition (k_a), high stabilities (k_d), to the stronger interaction (K_D), against peptide B. The recognition changes apparently according to the change of sulfation degree, however stabilities of lacquer polysaccharides derivatives interaction against the peptide B differ slightly, between the different sulfated degree, and even the unsulfated derivatives despite the determine condition. But even small, the sulfation have affection on the dissociation rate constant k_d. The dissociation constant K_D is affected accordingly.

サンプル	$\bar{M}_n$ (×10^{-4})	D.S	k_a (×10^4) [1/Ms]	k_d (×10^{-4}) [1/s]	K_D (×10^{-8}) [M]
LP-S A	**0.80**	**-**	**1.48**	**2.68**	**1.81**
LP-S B	**0.75**	**-**	**1.91**	**2.68**	**1.41**
LP-S C	**0.70**	**-**	**2.05**	**2.31**	**1.13**
LP-S D	**0.70**	**0.35**	**3.18**	**1.84**	**0.58**

Table 4.2 SPR result of LP-S(lacquer polysaccharides sulfate) with peptide B.

4.5 Conclusion

In this chapter, peptides synthesised according to the amino acid sequence of HIV virus surface docking glycoprotein gp120. Three different passages of gp120 glycoprotein were selected. All positive charged Poly-*L*-lysine as an all positive charged peptide gave strongest

interaction with dextran sulfate. And the peptide C, CD4 domain sequenced from 362 to 380, which is poor in positive charged amino acid, gave no interaction with dextran sulfate as shown in Figure 4.

Both peptide A V3 loop sequence from 297 to 316, and peptide B, C-terminus from 493 to 511, interaction strongly with dextran sulfate, The number of lysine and arginine in peptide A and B are was the main reason of the stronger interaction.

peptides A and B have the same number of positive charged amino acid, give slightly different interaction, imply that arrangement of amino acid also affect the interaction strenth. Concentrated positive charge gave more electrostatic attraction power than scattered one.

By control the reaction time and piperine-N-sulfate amount, and multiple sulfonation, the lacquer polysaccharides with different sulfation rate was

synthesised. The result of the SPR determination of sulfated lacquer polysaccharides and peptide B shows that higher sulfated polysaccharides gave higher recognition (k_a), high stabilities (k_d), to the stronger interaction (K_D), against peptide B. The recognition changes apparently according to the change of sulfation degree, however stabilities of lacquer polysaccharides derivatives interaction against the peptide B differ slightly, between the different sulfated degree, and even the unsulfated derivatives despite the determine condition. But even small, the sulfation have affection on the dissociation rate constant k_d. The dissociation constant K_D is affected accordingly.

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Conclusions

In this study, the structure of lacquer polysaccharides that had not been well studied, was discussed. The association structure, which cause the special form of degradation of larger fraction of lacquer polysaccharide into one third molecular weight, including natural degradation in the lacquer sap by exposure to air and also acidic hydrolysis by diluted sulphuric acid, was well discussed.

In order to approach to the mechanism how structure affect the biological activities, Surface plasmon resonance (SPR), Nuclear magnetic resonance (NMR) as well as infrared spectroscopy (IR) etc. was utilized, and the relation between the structural details and interaction was well discussed. As model of compounds of glycoproteins that polysaccharides interact with, poly-*L*-lysine and

synthesised oligopeptides were immobilized on SPR sensor chip CM5 and SA respectively. And dynamic constant including association rate constant k_a, dissociation rate constant k_d, and dissociation constant K_D was calculated by using 1 to 1 binding fitting model.

Additionally, the sulfated lacquer polysaccharides with different degree of sulfation was synthesised, and affection of sulfation to saccharide-protein interaction, was discussed.

First, two pure fractions were first isolated in this study by using Sephadex size exclusive chromatography. It was found that both of the two fractions show the same structure by ^{1}H and ^{13}C NMR characterization. It was also found that addition of ferric or ferrous ion can lead to a gelation of lacquer polysaccharides with lower molecular weights in aqueous solution, suggesting that the degradation may cause by breakage of metal-carboxyl association linkages. Appearance at 1612 cm^{-1} due to COO^- ion of the IR spectra and alkaline degradation of $3.0x10^4$ fraction into $1.4x10^4$, which halved the molecular weight,

could also be a proof of existence of metal-carboxyl association.

Second, lacquer polysaccharides, which is highly branched 1, 3- β galactan backbone with glucuronic acid terminals, the biological activities such as antitumor and blood coagulation promoting effective, is considered to cause by electrostatic attraction between negative-charged carboxyl groups from the uronic acid terminal of polysaccharides, and positive-charged amino groups from target proteins. In the dynamic study, poly-L-lysine as an all-positive charged polypeptide model was used to be evaluated the activity of lacquer polysaccharides and their derivatives. It was found that the interaction increased with increasing molecular weight of lacquer polysaccharides.

Third, the sulfated derivatives showed significantly high interactions with dissociation-rate constant of k_d = 1.74x10-4 [1/s], association-rate constant of k_a = 4.31x10^4 [1/Ms] and dissociation constant of K_D=4.0x10^{-9}[M] respectively, while original lacquer polysaccharides gave

only k_d = 3.54 x10^{-4} [1/s], k_a = 4.99x10^2 [1/Ms] and K_D = 7.10\x10-7 [M]. These results suggest that the sulfate groups, compared to carboxyl groups give stronger attraction to amino groups, and the interaction should be the reason why sulfated lacquer polysaccharides had potent anti-HIV acctivity.

Fourth, in order to evaluate the affection of sulfation to anti-HIV activities, lacquer polysaccharides were sulfated 1, 2 and 3 times respectively, by piperidine-*a*-sulfate to gives sulfated lacquer polysaccharides with different degree of sulfations. According to the amino acid sequence of V3 loop, C-terminus, and CD4-binding domain of HIV surface glycoprotein gp120 were synthesised and immobilized to the sensor chip separately. The C-terminus gave the highest response that increased with increasing the degree of sulfation, also be the proof of interaction result from the electrostatic attraction.

Fifth, while the association-rate constant was different from the molecular weights, degree of sulfation, and

binding ligands, the dissociation-rate constant of lacquer polysaccharide derivatives was not affective so much and gave rather low values. This implies high stability of the interaction and sulfated polysaccharides were expected for long-term effectives and were possible candidates as an anti-HIV medicine in the future.

There are still numerous of issues not well understood as concerning to the lacquer polysaccharides, including structure and biological activities and its relation, and further more detailed study will be conveyed in the near future.

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My first and sincere appreciation goes to my supervisor Professor Dr. Takashi Yoshida for all I have learned from him and for his continuous help and support in all stages of this thesis. I will never have chance to study abroad and finish my works without the help of Professor Yoshida

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Yuting BAI

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