Direct Analysis of Cellulose in Polar Ionic Liquids

A Thesis Presented to
Tokyo University of Agriculture and Technology

 Kosuke KURODA

2014

Direct Analysis of Cellulose in Polar Ionic Liquids

Kosuke Kuroda Ohno Laboratory Tokyo Univesity of Agriculture and Technology

Acknowledgement

The research in this thesis was carried out between April 2009 and September 2014 at Tokyo University of Agriculture and Technology (TUAT), Department of Biotechnology under the direction of Professor Hiroyuki Ohno.

First, I would like to express my deep gratitude to my supervisor, Professor Hiroyuki Ohno. His continuous support and encouragement have greatly helped me to do the present research. This thesis would not be completed without his help.

I also would like to express my deeply appreciation to Professor Nobuhumi Nakamura for his continuous encouragement and valuable advices.

I am very grateful to Dr. Yukinobu Fukaya. He taught me how to do scientific research. I learned from him the basis of research, and technique of experiments.

I would like to show my gratitude to Dr. Kyoko Fujita and Dr. Takahiro Ichikawa for their helpful advices and valuable suggestions.

I would like to express my appreciation to Dr. Naomi Nishimura, Dr. Junko Kagimoto, Dr. Kenichi Murata, Dr. Yuki Kohno, Dr. Liu Yuan, Dr. Yuki Tsuji, Dr. Miyuki Masuda, Dr. Mitsuru Abe, Dr. Satomi Taguchi, Dr. Shohei Hayakawa, and Mr. Masato Suzuki. They encouraged me and gave me a lot of advices.

I want to express deeply thank to the fellows in the same grade, Mr. Shohei Saita, Mr. Kouta Takeda, Ms. Akiko Tsurumaki, Mr. Hiromitsu Hattori, Mr. Jun Kuwahara, Mr. Misaki Inukai, and Mr. Fumiaki Hanaoka for their encouragements and supports.

I also express my appreciation to Mr. Haruhito Kunimura, Mr. Masato Ueda, and Mr. Daiki Sato for their assistance and experiments. And I am also very grateful for all other members of the Ohno-nakamura group for their kind assistance.

I am very grateful to Ms. Patricia McGahan. She corrected my English as a native speaker. Especially, she helped me in international oral presentation.

I am grateful for financial support from the JSPS Research Fellowship for Young Scientists from April 2012 to September 2014.

Finally, I express my deep gratitude for my parents, Mr. Yuji Kuroda and Mrs. Kayoko Kuroda for their continuous support and powerful encouragement.

July 2014

Kosuke Kuroda

Department of Biotechnology
Tokyo University of Agriculture and Technology

Chapter 1

Polar Ionic Liquids as Cellulose Solvents

1-1. Ionic Liquids

Ionic liquids (ILs) are organic molten salts designed to melt below 100 °C or, preferably, near room temperature (Figure 1-1).[1-6]

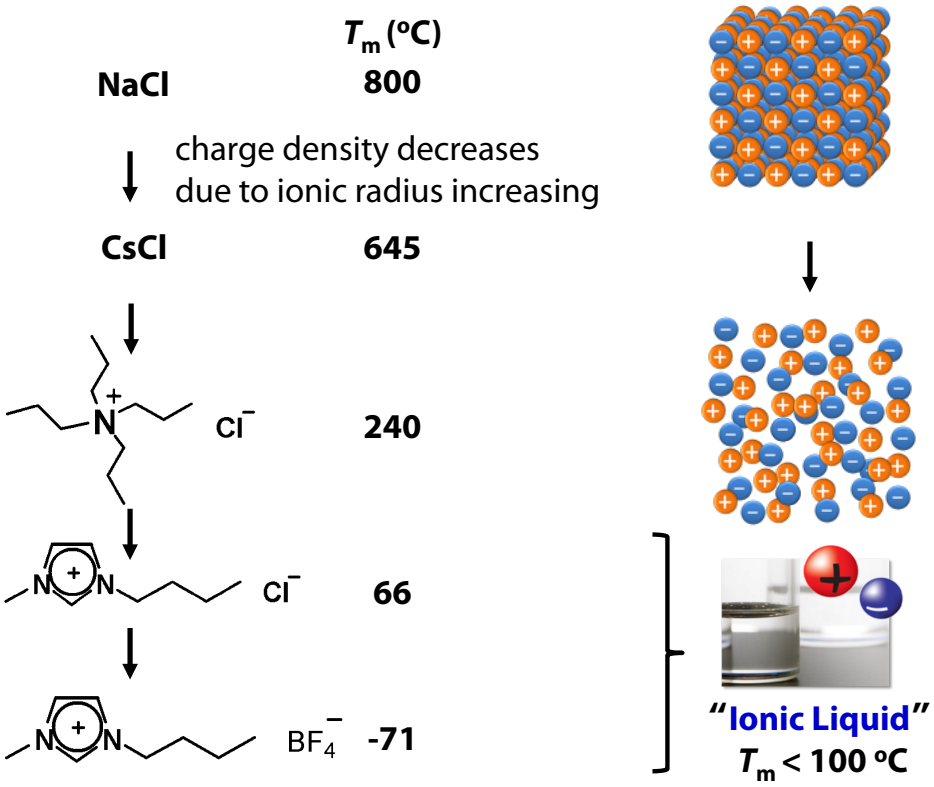


Figure 1-1 Melting points of salts.

Almost all the salts we usually see are solids at room temperature because they have high lattice energies. For example, the melting point (T_m) of sodium chloride is 801 °C. Lattice energy is represented by the following equation: $E = kQ_1Q_2/d$, where k is the Madelung constant, Q_1 *and* Q_2 are the charges on the ions, and d is the distance between the ions. Thus, increasing the distance between the ions by increasing the ionic radii makes the lattice energy lower, which results in a low T_m. For example, KCl and CsCl melt at 770 °C and 645 °C, respectively, due to their larger cations compared with sodium chloride. Salts containing organic cations have low T_m because of

their bulky structures. Tetrabutylammonium chloride melts at 240 °C, and 1-butyl-3-methylimidazolium chloride melts at 65 °C. On the other hand, Hurley *et al.* reported salts with low melting points using aluminum chloride ($AlCl_3$) and 1-ethylpyridium chloride.[7] In the 1970s and 1980s, Osteryoung *et al.*[8] and Hussey *et al.*[9, 10] investigated room temperature ILs based on chloride salt/$AlCl_3$ mixtures. However, chloroaluminate-type ILs are not stable in moisture and air.

Air- and moisture stable ILs were reported by Wilkes in 1992.[11] They reported 1-ethyl-3-methylimidazolium ($[C_2mim]^+$) salts combined with tetrafluoroborate ($[BF_4]^-$) or nitrate ($[NO_3]^-$) anions. These ILs are stable under ambient conditions and can be obtained as melted salts at room temperature. It is worthwhile to note that the melting point of $[C_2mim][BF_4]$ is −71 °C, and thus, this salt does not solidify even during winter in Moscow.

To date, various types of ILs have been reported. As representative cations, imidazolium, pyridinium, pyrroridinium, ammonium, sulfonium, and phosphonium are often used (Chart 1-1). On the other hand, a great number of organic and inorganic anions are utilized.

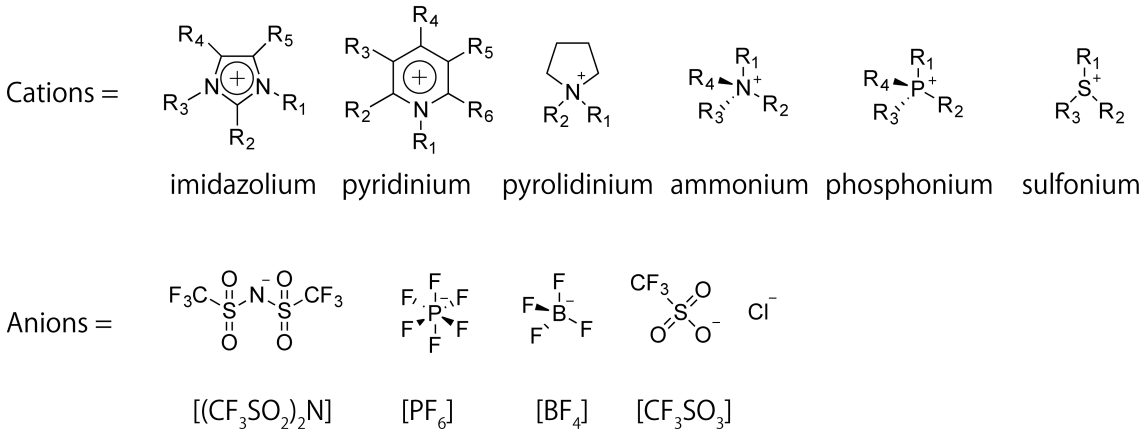


Chart 1-1 Ionic species widely used for ILs.

1-2. Features of Ionic Liquids

Because ILs are composed entirely of ions, they have various features. For example, they have negligible vapor pressure due to the strong interaction between the ions, indicating that they have high flame resistance. The features which "typical" ILs have are shown in Figure 1-2. Due to these features, ILs are called "green solvents".[2] Compared with traditional industrial solvents, most of which are volatile organic compounds, ILs are not emitted into the atmosphere. However, it should be noted that ILs are not intrinsically "green" as some are extremely toxic, but they have large potential benefits for sustainable chemistry.

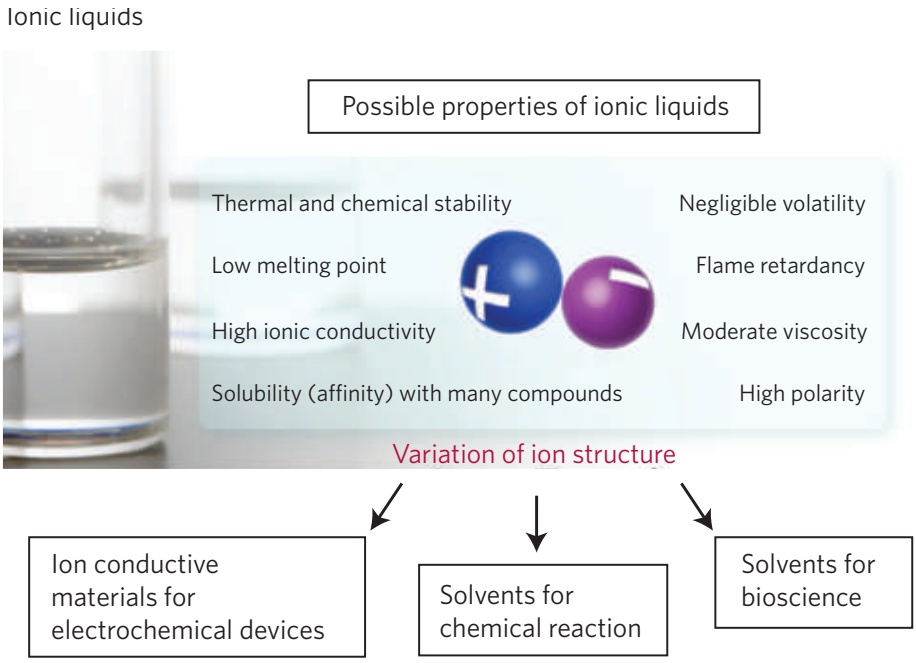


Figure 1-2 Features of ILs.[5]

As reason why I have mentioned the features that "typical" ILs have, their properties depend quite significantly on their structures. For example, the thermal stabilities of imidazolium salts significantly vary according to this order (based on the onset temperature for

decomposition): $[(CF_3SO_2)N]$ (440 °C) > $[BF_4]$ > $[PF_6]$ > Br > [SCN] > Cl (260 °C).[12] Furthermore, in some extreme cases, some ILs are distillable under harsh conditions.[13, 14] In other words, these facts strongly support that their physico-chemical properties, e.g., thermal properties, polarity, and viscosity, are controllable by exploiting diverse components, including organic cations and organic or inorganic anions. This potential possibility for variation has led to ILs being described as "designer solvents".[15]

1-3. Application of Ionic Liquids as Cellulose Solvents

Among the fascinating characteristics of ILs, their cellulose-dissolution ability is one of their most interesting and useful characteristics.[16-23] This characteristic has the potential to overcome the problems caused by the quite low solubility of cellulose in conventional molecular liquids.

1-3-1. Cellulose Dissolution with Polar Ionic Liquids

In 2002, Rogers *et al.* reported that some ILs can dissolve cellulose (Table 1-1).[24] Up to 10 wt% of cellulose (DP ≈ 1000) can be dissolved in [C_4mim]Cl by only heating the solution, and up to 25 wt% can be dissolved with microwaves. On the other hand, stirring the solution of cellulose/ILs under ambient conditions does not lead to dissolution, although wetting of the cellulose fibers with the ILs occur.

Table 1-1 Solubility of cellulose in [C_4mim] salts.

Anions	Methods	Solubility
Cl	Heating (at 70 °C)	3 wt%
Cl	Heating (at 100 °C)	10 wt%
Cl	Microwave	25 wt%
Br	Microwave	5-7 wt%
[SCN]	Microwave	5-7 wt%
[BF_4]	Microwave	insoluble
[PF_6]	Microwave	insoluble

In 2005, 1-allyl-3-methylimidazolium chloride ([Amim]Cl) was reported as a powerful solvent for cellulose.[25, 26] [Amim]Cl shows a lower melting point at ca. 17 °C and a considerably lower viscosity of

685 cP at 30 °C compared with [C_4mim]Cl, which has a melting point of 65 °C and a viscosity of 11000 cP at 30 °C.[27] The relatively lower melting point and viscosity of [Amim]Cl are due to suppression of crystallization by introducing an allyl group at the N-position.[26] [Amim]Cl has cellulose dissolution ability at 60 °C while cellulose is only swelled at room temperature.

In 2006, Fukaya *et al.* reported that ILs containing formate anion ([HCOO]).[28] It dissolves cellulose more easily than [Amim]Cl ([Amim][HCOO] and [Amim]Cl dissolve 10 wt% of cellulose at 60 ˚C and 100 ˚C, respectively, shown in Figure 1-3). The superior solubility is attributed to low viscosity ([Amim][HCOO]; η = 66 cP) and high polarity. However, carboxylate salts have a problem; they are not thermally stable.

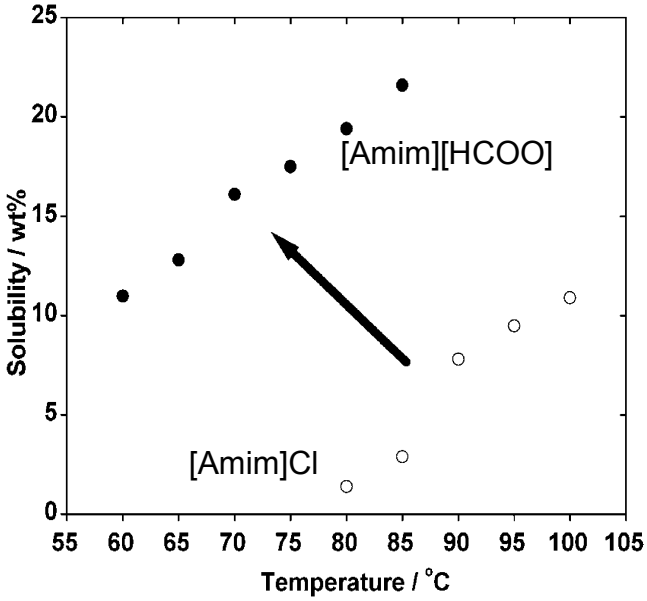


Figure 1-3 Temperature dependence of the solubility of cellulose in [Amim][HCOO] and [Amim]Cl.

In 2008, phosphonate type ILs were proposed as stable, less viscous, and polar ILs.[29] Furthermore, they dissolve cellulose at room temperature within several hours (2 wt% within 3 hours and 4 wt%

within 5 hours) as shown in Figure 1-4. These three types of ILs (chloride, carboxylate, and phosphonate type ILs) are known to be representative polar ILs that have been found to dissolve cellulose to date.

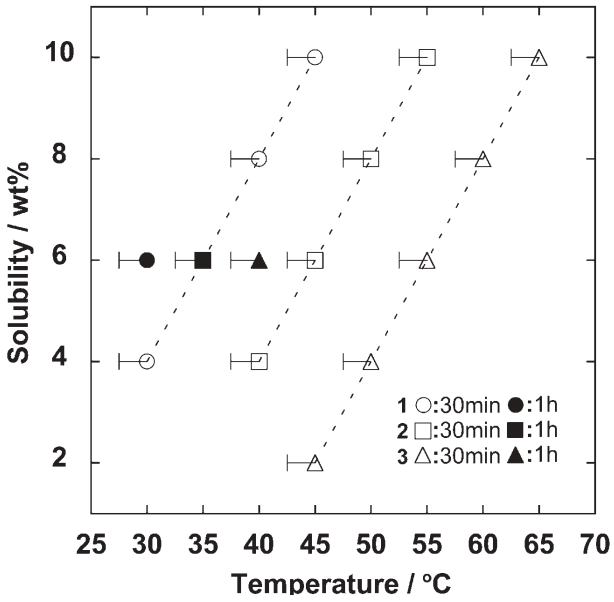


Figure 1-4 Solubility of cellulose in 1:[C_2mim][(MeO)(H)PO_2], 2: [C_2mim][(MeO)(Me)PO_2], and 3: [C_2mim][(MeO)(MeO)PO_2].

Interestingly, an even-odd effect on the cellulose solubility was reported.[30, 31] The number of carbon atoms in the alkyl chain of 1-alkyl-3-methylimidazolium chloride strongly affects the solubility of cellulose (Figure 1-5). A strong odd-even effect was especially observed for alkyl chains that were pentyl or shorter. Cellulose was more soluble in even-numbered ILs compared with odd-numbered ILs with chains with less than six carbon units. [C_4mim]Cl is the best even-numbered IL and dissolves 20 wt% of cellulose, whereas [C_7mim]Cl is the most efficient odd-numbered IL but only dissolves 5 wt% of cellulose. The reason for this effect is not clarified.

IL/organic solvent mixtures also dissolve cellulose.[32-36] As an efficient solvent for cellulose, a

[C_4mim]Cl/1,3-dimethyl-2-imidazolidinone system has been reported. When the IL fraction is 0.40 (by mol), 10 wt% of cellulose can be dissolved in only 3 min at 100 °C. In addition, [C_4mim]Cl/other organic solvent systems have also been found to dissolve cellulose despite the large fraction of the organic solvents. Reported [C_4mim]Cl/organic solvent systems are shown in Table 1-2.

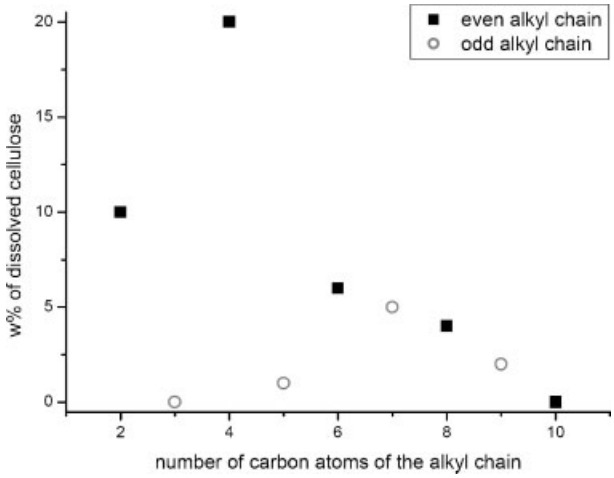


Figure 1-5 Dependency of the solubility of cellulose at 100 °C on the alkyl chain length of 1-alkyl-3-methylimidazolium chloride.

Table 1-2 Molar fraction of IL (x_{IL}) required for the dissolution of Avicel.

Entry	Solvent	χ_{IL}[a]	$E_T(30)$/ kcal mol^{-1}
1	*N*,*N*-Dimethylformamide (DMF)	0.10	43.5
2	*N*,*N*-Dimethylacetamide (DMA)	0.27	43.0
3	Pyrrolidinone	0.30	48.2
4	δ-Valerolactam	0.27	44.0
5	ε-Caprolactam	0.42	42.1
6	*N*-Methylpyrrolidinone (NMP)	0.16	42.5
7	1,3-Dimethyl-2-imidazolidinone (DMI)	0.18	42.4
8	*N*,*N*′-Dimethylpropylene urea (DMPU)	0.33	42.1
9	*N*,*N*,*N*′,*N*′-Tetramethylurea	0.59	41.2
10	Dimethylsulfoxide (DMSO)	0.08	45.1
11	Sulfolane	0.23	45.1
12	Acetylacetone	0.51	38.9
13	*tert*-Butanol	0.48	43.7
14	*tert*-Pentanol	0.49	41.2
15	EMIMAcO	—	49.8
16	BMIMCl	—	49.3

[a] $\chi_{IL} = n_{IL}/(n_{ms} + n_{IL})$, where χ_{IL} is the molar fraction of IL in the electrolyte solution, n_{ms} and n_{IL} are the amount in mol of molecular present and of IL in the final mixture, respectively.

Recently, tetrabutylphosphonium hydroxide (TBPH) has been reported as a superior solvent for the dissolution of cellulose.[37] It dissolves 20 wt% of cellulose within 5 min at 25 °C (Table 1-3). A more interesting advantage of TBPH is that it dissolves cellulose in the presence of a certain amount of water. The effective dissolution of cellulose can be performed under water content between 20 and 50 wt%. Additionally, it has been confirmed that TBPH dissolves cellulose by solvation and not by decomposition while TBPH is highly basic.

Table 1-3 Dissolution of cellulose in TBPH/water mixtures

Water content (wt%)	Cellulose (wt%)	Dissolution time (min)
50	15	5
40	1	1
	15	3
	20	5
30	15	5
20	5	7

As interesting ILs for cellulose dissolution, distillable and polar ILs have been reported.[38, 39] Some distillable or reversible ILs which are not polar enough but they have cellulose-dissolution ability with the aid of other cosolvent.[13, 39, 40] The distillable and polar ILs have potential for use in the recycling of ILs and the easy collection of extracts from biomass.

In addition, polar ILs dissolve other scarcely soluble polymers. According to polysaccharides, [C_2mim][HCOO] dissolves some polysaccharides such as inulin and pectin.[28] Cl type ILs are known to dissolve *bombyx mori* silk fibroin.[41] At 100 °C, [C_2mim]Cl, [C_4mim]Cl, and 1-butyl-2,3-dimethylimidazolium ([C_4mmim]) chloride dissolve 13.2 wt%, 23.3 wt%, and 8.3 wt% of silk fibroin, respectively. They mentioned that the ILs disrupt the hydrogen bonding present in the *β*-sheets, which results in dissolution. [C_4mim]Cl and cholinium

thioglycolate are found to be solvents for wool keratin or feather keratin fibers.[42-44] Cholinium alkanoates extract suberin from cork.[45, 46] Chitin and chitosan also dissolve polar ILs.[47-49] These results are summarized in Table 1-4.

Table 1-4 Scarcely soluble polymers and their solvents.

ILs	Solutes
$[C_2mim][HCOO]$	Inulin
$[C_2mim][HCOO]$	Xylan
$[C_2mim][HCOO]$	Pectin
$[C_4mim]Cl$	Silk fibroin
$[C_2mim]Cl$	Silk fibroin
$[C_4mmim]Cl$	Silk fibroin
[Amim][dca]	Wool keratin
$[C_4mim]Cl$	Feather and wool keratin
[Amim]Cl	Feather and wool keratin
cholinium thioglycolate	Feather and wool keratin
$[C_2mim][MeCO_2]$	Chitin
$[C_4mim]Cl$	Chitin chitosan
[Amim]Br	Chitin

1-3-2. Solubilization Mechanism

The mechanisms of the dissolution of cellulose in ILs are gradually revealed. To dissolve cellulose, it is known that the hydrogen bond between cellulose and the anion is very important. The hydrogen bonding basicity of ILs is measured by a solvatochromic measurement such as the Kamlet-Taft system. The Kamlet-Taft parameter quantifies the hydrogen bond basicity (β), hydrogen bond acidity (α), and dipolarity and polarizability (π^*). As shown in Figure 1-6, the β value of ILs and cellulose solubility are clearly correlated.[24, 29, 50-56]

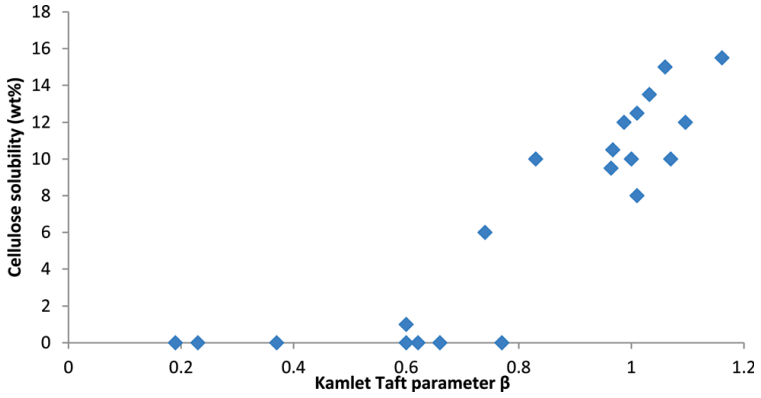


Figure 1-6 The solubility of cellulose in 1-ethyl-3-methylimidazolium and 1-butyl-3-methylimidazolium ILs as a function of the β value of the ILs.[16]

Recently, some other potential methods for the estimation of the hydrogen bonding basicity have been reported. Xu *et al.* focused on the hydrogen bond between the imidazolium cation and anion.[57] It is known that the hydrogen at the $C_{(2)}$-position is more acidic than other ring or aliphatic hydrogen atoms,[58, 59] and it strongly interacts with highly basic anions such as chloride.[60] Thus, Xu *et al.* investigated the relationship between the chemical shift of the hydrogen on ^{1}H NMR spectra and the solubility of cellulose because they think that more basic anions interact more strongly with the hydrogen. Cellulose solubility increases as the chemical shift of the hydrogen increases and this relationship is similar to the relationship between the β value and the cellulose solubility. Froschauer *et al.* used ethanol as a hydrogen bond donor (*pseudo*-OH of cellulose) and investigated the chemical shift of OH in ethanol/IL solutions.[61, 62] The relationship between the chemical shift of OH and the β value was observed as linear, indicating that it can be used as a conventional method for estimating hydrogen bond basicity of ILs. As another method, the computational calculation of the β value has also been

reported.[63] Furthermore, Pb^{2+} was also reported as a solvatochromic dye for measuring the hydrogen bond basicity.[63] These two methods are not a mainstream method, but they should be useful for some ILs, such as TBPH, which cannot be subjected to Kamlet-Taft experiments.

To clarify the specific interaction between ILs and cellulose precisely, various analyses have been examined. As mentioned above, the interaction between cellulose and ILs is mainly a hydrogen bond between the hydroxyl group and the anion. Remsing *et al.* confirmed this hydrogen bond using $^{35/37}$Cl NMR and cellulose.[64] This analysis also revealed that chloride ions interacted in a 1:1 ratio with the carbohydrate hydroxyl groups. Molecular dynamics (MD) simulation is also powerful tool for analyzing interactions. Gross *et al.* confirmed that Cl anions existed near OH groups at the 2, 3, and 6-positions.[65] Anions can form hydrogen bonds with the hydroxyl groups of glucan chains from either the equatorial or the axial directions while cations exist above and below ring of glucose. Furthermore, it was reported that 80 % of OH groups formed hydrogen bonds with anions in $[C_2mim][MeCO_2]$.[66] According to the coordination number, it was found that anions could form bridging hydrogen bonds with two inter- or intrachain hydroxyl groups depending on IL species.[67] Radibideau *et al.* calculated the number of hydrogen bonds between cellulose and ILs: $[C_2mim][MeCO_2]$ (2.75 per glucose unit) > $[C_2mim]Cl$ (2.48) > $[C_1mim][(MeO)_2PO_2]$ (2.39).[68, 69] Additionally, the average number of hydrogen bonds with a specific hydroxyl group was reported (Table 1-5).[68] Comparing $[MeCO_2]$ and $[(MeO)_2PO_2]$, it is observed that their contributions for individual hydrogen bond are similar. The major reduction in the overall hydrogen bonding in $[(MeO)_2PO_2]$ comes from a reduction in the bridging interactions. It is possible that $[(MeO)_2PO_2]$'s larger size and ensuing rotational freedom contribute to this reduction; the much smaller $[MeCO_2]$ ion is more agile and can maneuver toward these energetically favorable positions.

Interaction energy was also calculated.[67, 70] The electrostatic potential energy between cellulose and [$MeCO_2$] anion is -52.6 kcal/mol per glucose unit (around 14 kcal/mol per hydroxyl group) and the van der Waals potential energy is +0.56 kcal/mol. As reference, it is known that binding energy of dimer of water molecules and ethanol molecules is 4.4 kcal/mol and 5.3 kcal/mol, respectively.[71] This clearly shows quite strong electrostatic interaction between anions and hydroxyl groups is dominant factor for dissolution of cellulose.

Table 1-5 Average number of hydrogen bonds per glucose unit for the ILs.

IL		H_2	H_3	H_6	Total
[BMIM]Cl	H_2	0.32	0.35	0.19	0.87
	H_3	0.35	0.27	0.18	0.81
	H_6	0.19	0.18	0.42	0.79
[EMIM]Ac	H_2	0.24	0.59	0.19	1.02
	H_3	0.59	0.31	0.09	1.00
	H_6	0.19	0.09	0.63	0.92
[DMIM]DMP	H_2	0.27	0.42	0.11	0.80
	H_3	0.42	0.35	0.12	0.89
	H_6	0.11	0.12	0.55	0.79

Diagonal elements H_i–H_i represent individual hydrogen bonds with the respective atom; off-diagonal elements H_i–H_j refer to bridging hydrogen bonds between the respective atoms and includes bridges within the same glucose unit and between different glucose units.

Concerning the cations, their role in the dissolution of cellulose is under discussion. Some claim that cation forms hydrogen bonds with hydroxyl groups based on the results of a ^{13}C NMR experiment, NOESY (2D NMR), and MD simulation.[72-77] Additionally, Cho *et al.* reported that cations strongly interact with linker oxygen atoms.[78] However, some claim that the cation does not form hydrogen bonds with the hydroxyl groups. NOESY (2D NMR), relaxation time measurements by ^{13}C NMR, and MD simulations show that there are

no hydrogen bonds between cellulose and the ILs.[67, 79]

On the other hand, cation surely interacts with cellulose. Their interaction with cellulose is explained as being based on van der Waals forces.[67, 70] The van der Waals potential energy of cation is -17.3 kcal/mol and the electrostatic potential energy is only -6 kcal/mol (those concerning [$MeCO_2$] anions are +0.56 and -52.6 kcal/mol, respectively, as mentioned above). In addition, it was confirmed that there is no CH-π interaction between the cation and cellulose. As an interesting role of cation, preventing the re-association of strands, separated by interaction with the anions, has been reported (Figure 1-6).[68] Only cation enters the cavity between strands dissociated by the anions, and the cation keep the strands isolated.

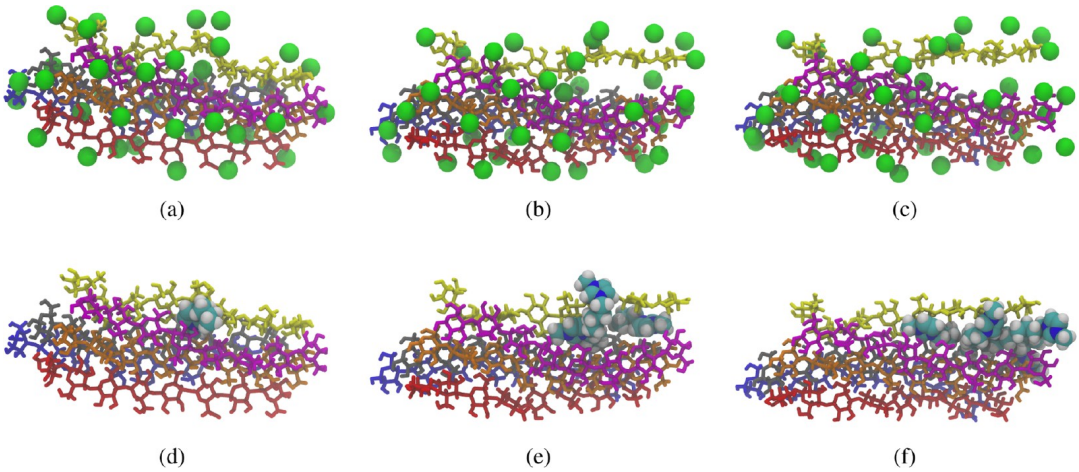


Figure 1-6 Cellulose I*α* bundle in [C_4mim]Cl showing contacting chlorides (top row, green) and penetrating cations (bottom row, cyan and white) after (a, d) 2 ns, (b, e) 50 ns, and (c, f) 55 ns.

Simultaneously, the dissolution mechanisms of IL/cosolvent solutions were reported.[80, 81] Useful solvents are aprotic and have high hydrogen bond basicity. They interact with the cation, resulting in isolation of the anion (likely to be bulk). The isolated anions apparently become more highly basic. This is one of the reasons for the superior solubility of cellulose into IL/cosolvent mixtures (of course,

decrease of viscosity is also a reason). Concerning some ILs which do not dissolve cellulose, they enable to dissolve cellulose only when a cosolvent is added.[39] These results should support the hypothesis, isolation of anions.

1-4. Application of Ionic Liquids in Cellulose Processing

1-4-1. Extraction of Cellulose from Biomass

The first reports of the extraction of cellulose from biomass with ILs were simultaneously published in 2007 by Kilpeläinen *et al.* and Fort *et al.* Kilpeläinen *et al.* reported that [Amim]Cl extracted up to 8 wt% of cellulose from biomass at 110 ˚C in 8 h.[78] Fort *et al.* reported that [C_4mim]Cl/DMSO extracted up to 50 wt% of cellulose from biomass.[34] After that, complete dissolution of wood biomass has also been achieved with [C_4mim][$MeCO_2$] in 2009.[82] Both softwood (southern yellow pine) and hardwood (oak) are completely dissolved at 110 ˚C in 46 h and 25 h, respectively. In addition, most of the wood (> 90 wt%) can be dissolved within 16 h. Furthermore, pretreatment with microwaves or ultrasound decreases the cooking time required for complete dissolution, especially microwaves which decrease the time from 46 h to 16 h. The effects of both treatments on the dissolution of cellulose in ILs have been confirmed.[24, 83]

The extraction of cellulose without heating has been performed by Abe *et al.*[84] Approximately 14 wt% of wheat bran is dissolved in [C_2mim] phosphinate at 25 ˚C. Due to its low viscosity, [C_2mim] phosphinate extracts cellulose more efficiently than other phosphonate type ILs. They also found the predominant factor for determining the extraction ratio to be the intrinsic viscosity of the ILs (at least in the series of phosphonate type ILs).

Aqueous TBPH, which is an efficient solvent for cellulose dissolution (mentioned above), extracts cellulose effectively (Figure 1-7).[85] Aqueous TBPH (water content: 40 wt%) extracts 37 wt% of polysaccharides after only 1 hour stirring at room temperature. After 24 h, more than 50 wt% of the polysaccharides is extracted. An advantage of aqueous TBPH is that it is suitable to use for process of wet biomass because it intrinsically includes water. It is known that

a certain amount of water (approximately 5-10 wt%) disables dissolution ability of traditional polar ILs (water-tolerant poloar ILs are repoted only recently[86]). Additionally, it is also indicated that the water content can be automatically recovered after processing wet biomass. TBPH containing 60 wt% of water gradually loses its water, and finally, its water content becomes 45 wt% in a room at 25 ˚C with 45 % humidity.

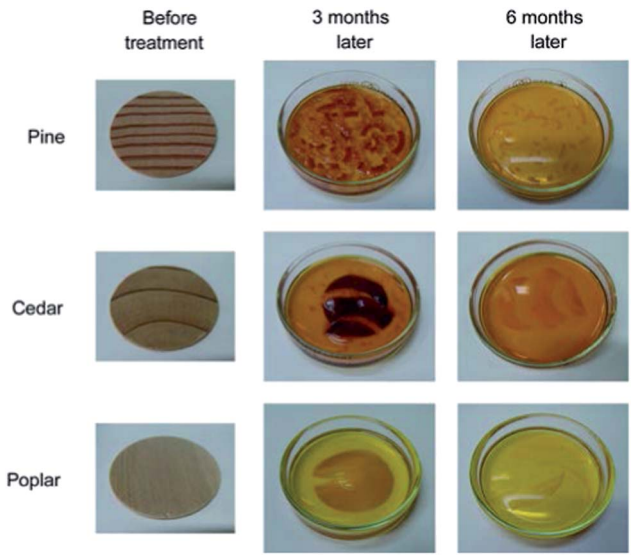


Figure 1-7 Changes in the shapes of wood disks in TBPH containing 40 wt% water. Wood disks (diameter: 34 mm, thickness: 1 mm, approximately 0.5 g) were soaked in TBPH containing 40 wt% water (15 g) without heating or stirring.

1-4-2. Hydrolysis of Cellulose

Methods to hydrolyze cellulose are categorized into three types based on the species of catalyst used:

(1) Mineral acid catalyst

(2) Solid acid catalyst

(3) Enzymatic catalyst

(1) Mineral acid catalyst

Mineral acid is the most widely used catalyst to hydrolyze cellulose.[87, 88] In chloride type ILs, cellulose confirmed to be hydrolyzed with hydrochloric acid (from 0.9 to 7.4 wt%).[89] Compared with water as a solvent, hydrolysis is facilitated by using ILs due to homogeneous reaction. Surprisingly, a 59 % yield of total reducing sugars (TRS) was achieved within 3 min (Table 1-6). This hydrolysis is also effective on lignocellulosic biomass.[90] It is known that the hydrolysis of lignocellulose is more difficult than cellulose, but a 66 % TRS yield was obtained in 30 min.

Table 1-6 Reaction conditions and yields from the hydrolysis of Sigmacell cellulose in $[C_4mim]Cl$ at 100 °C.

Entry	Acid/cellulose mass ratio	Time [min]	$Yield_{glucose}$ [%]	$Yield_{TRS}$ [%]
1	5	120	5	7
2	0.92	3	36	59
3	0.46	42	37	64
4	0.11	540	43	77
5[a]	0.92	1080	13	27

[a] Reaction was performed in water.

Rinaldi *et al.* reported a relationship between the hydrolysis activity of acids and their pK_a.[91] They found that cellulose was extensively cleaved with acids of pK_a < 1, and the degree of the depolymerization of cellulose is directly associated with the acid strength. According to the strength of the acids in ILs, it is already known that the acid strength rankings generally resemble the strengths observed in water.[14]

In addition, it is claimed that alkylimidazolium ILs contain weakly basic anions are not appropriate for acid-catalyzed hydrolysis.[91] Acetate or phosphonate anions are easily protonated by strong acids,

leading to forming acetic acid (pK_a = 4.78) or phosphonate acids (methylphosphonic acid, pK_a = 2.38). Consequently, cellulose cannot be sufficiently protonated for hydrolysis due to the same reason in these ILs.

(2) Solid acid catalyst

Solid acid is also useful catalyst due to easy recovery from the media after the reaction.[92, 93] Rinaldi *et al.* reported that solid acid catalysts such as Amberlyst 15DRY enable hydrolysis in ILs.[94] The hydrolysis activity of Amberlyst 15DRY is shown to be similar to that of *p*-toluenesulfonic acid. Further investigation of solid acid catalysts was reported in 2010.[91] It was mentioned that the hydrolysis is caused by free acid, generated through ion exchange between the ionic resin and the IL (Figure 1-8). Considering this mechanism, though recycle of catalyst was achieved, usefulness of solid catalyst is questionable.

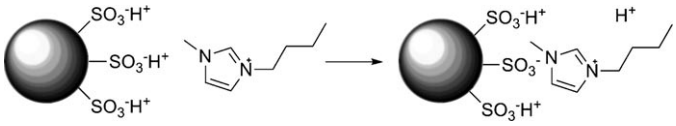


Figure 1-8 Ion-exchange process involving $[C_4mim]^+$ and H^+ species.

Addtionally, we have already found solid catalyst does not work in phosphonate type ILs (not published). This is due to weak acid (phosphonic acid) generated by ion exchange, supporting the mechanism of hydrolysis with solid catalysts.

(3) enzymatic catalyst

Enzymatic hydrolysis has attracted much interest as an energy-saving hydrolysis method because it can be performed under mild conditions.

The first report concerning the activity of cellulase in ILs was published by Turner *et al.,* but cellulase was denatured in $[C_4mim]Cl$.[95] Therefore, some approaches for retaining enzymatic activity in ILs have been reported. As an approach in ILs, $[HEMA][MeOSO_3]$ was proposed as a medium for cellulase, and it was confirmed that cellulase kept its structure in the IL.[96] However, $[HEMA][MeOSO_3]$ does not dissolve cellulose, while it does dissolve other carbohydrates. As an enzymatic approach, haloalkaliphilic and thermostable cellulase (*Hu*-CBH1) was found to be IL-tolerant.[97] While the activity of typical cellulase (from *T. reesei*) in 20 wt% polar IL solutions was from 60 to 20 % of that in 2 M aqueous NaCl, the activity of *Hu*-CBH1 was from 140 to 60 %. Wahlstrom *et al.* reported that IndiAge ONE is more stable cellulase.[98] The activity of cellulase in almost neat ILs reported was not enough to use, but these abovementioned literatures surely give an important indication of progress. Furthermore, as an approach of modification of enzyme, immobilization of cellulase was reported by Lozano *et al.*[99] Cellulase immobilized on Amberlite XAD4 and coated with hydrophilic ILs found to be somewhat IL-tolerant and showed activity in cellulose/IL solutions (Figure 1-9).

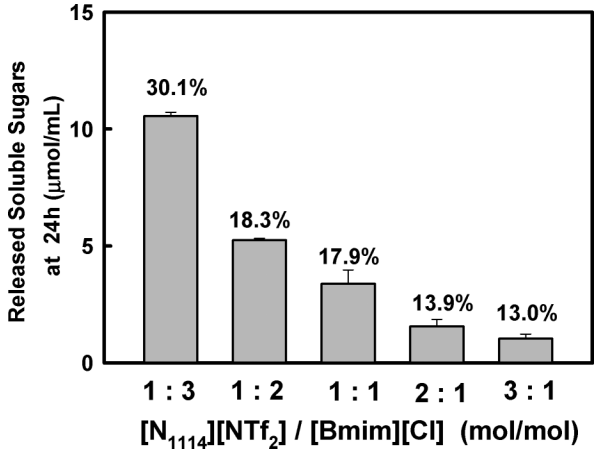


Figure 1-9 Enzymatic *in situ* saccharification of cellulose in $[C_4mim]Cl$ media for 24 h at 50 °C. Immobilized cellulase (30 mg) was first coated with $[N_{1114}][NTf_2]$, and then 1 wt% of cellulose in $[C_4mim]Cl$ was added.

However, it should be noted that cellulose was not dissolved in these reported solutions due to the high fraction of non-polar ILs.

1-4-3. Oxidation of Sugars

Only a few papers have reported on the oxidation of sugars in ILs. Fujita *et al.* reported that cellobiose was oxidized in hydrated choline dihydrogen phosphate ([ch][dhp]) with cellobiose dehydrogenase (CDH).[100] When the IL content was high (65 wt%), CDH oxidized cellobiose and transferred the extracted electron to the sodium salt of 2,6-dichlorophenolindophenol (DCIP).

In another trial, the direct conversion of cellulose to ethanol in a one-pot IL system was reported.[101] To this end, cellulase-displaying yeast was used (Figure 1-10). This yeast displays cellulase on its surface with an aid of α-agglutinin, and the cellulase hydrolyzes cellulose into glucose. The generated glucose is fermented by yeast. Cellulose was dissolved into $[C_2mim][(EtO)_2PO_2]$, and regenerated by acetate buffer (final IL concentration: 200 mM), and directly fermented with the yeast. The IL concentration did not significantly affect the cell viability, and ethanol production was considerably accelerated compared with a cellulose sample without IL-treatment.

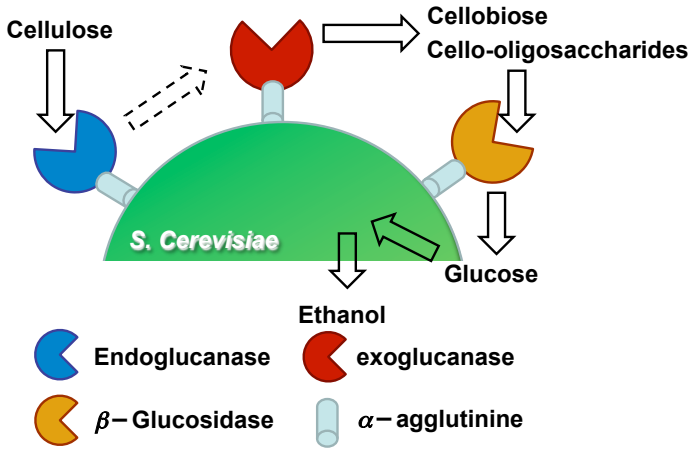


Figure 1-10 Cellulase-displaying yeast.

1-5. Analysis of Cellulose Dissolved in Ionic Liquids: Previous Methods

Direct analysis of the cellulose dissolved in ILs has been scarcely reported in spite of the great cellulose-dissolving abilities of ILs. Therefore, almost all researchers have performed the analysis through multi-step pretreatment procedures.[24, 25, 82, 89-91, 102-104] The analytical methods can be categorized as follows.

(1) precipitation → direct analysis (ONLY applicable to gravimetric determination, X-ray scattering, FT-IR)

This analysis is widely used for the quantification of the cellulose extracted from plant biomass (they hypothesize all of extracts is cellulose but it may be not, detail will be mentioned in chapter 3).[82, 84, 85, 105] After extraction, water or alcohol are added for precipitation, and then, the precipitated material is washed and dried under reduced pressure. After these pretreatment procedures, the precipitate is weighed. The extraction ratio can also be calculated from the residues treated with ILs. The dried samples can be used in X-ray scattering and FT-IR analyses.[24, 105-107]

(2) precipitation → degradation to water-soluble sugars

This method is used for analyzing the composition of extracted saccharides.[102, 103] Extracted polysaccharides are dispersed into water and hydrolyzed with sulfonic acid. After this pretreatment, the composition of the saccharides can be analyzed with GC, HPLC, or MS because degraded saccharides are water-soluble.

(3) precipitation → dissolution into other cellulose solvents

To investigate the intrinsic properties of cellulose, the dissolution of cellulose itself is necessary. For example, changes in the molecular weight of cellulose before and after dissolution into ILs were

investigated through dissolution into other solvents.[25, 108] Zhang *et al.* dissolved IL-treated cellulose into a cupriethylenediamine hydroxide solution, and then measured its intrinsic viscosity. Tetrahydrofuran (THF) is also an candidate[104], but it dissolves only the cellulose oligomer.

Currently, many solvents which dissolve cellulose are known, such as LiCl/dimethylacetamide (DMAc) and *N*-methylmorpholine-*N*-oxide. However, the only solvents that dissolve cellulose without derivatization and degradation are LiCl/DMAc and LiCl/dimethylimidazolidinone (DMI). Additionally, these solvents have some problems: several days are necessary for dissolution, and they are corrosive. Thus, even these solvents, they are used under only limited conditions.[109, 110]

(4) derivatization → precipitation → dissolution into common solvents

Polar ILs are known as reaction media for derivatization.[111-114] Derivatization of cellulose is analyzed by NMR after precipitation and dissolution into other common solvents. Based on the knowledge of derivatization, Zoia *et al.* performed a HPLC analysis of wood dissolved in ILs.[115, 116] The cellulose and lignin swelled with the ILs are benzoylated and precipitated. The benzoylated materials are then dissolved into THF and analyzed by HPLC. Salanti *et al.* developed this method by combining benzolylation and acetylation.[117]

The problem with these analyses is the complex pretreatment procedures. They may not be accurate because complete precipitation is difficult. This matter is shown in the mismatch of mass balances.[102] Additionally, derivatization may cause change of molecular weight distribution.[115, 118] To solve these problems, the direct analysis of cellulose in ILs is strongly requested.

1-6. Preliminary Study of Ionic Liquids as Solvents for Cellulose Analysis

Until now, "non-polar" ILs have been applied to some analytical systems[119-121] such as gas chromatography (GC)[122-126], scanning electron microscopy (SEM)[127-131], matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[132-135], and NMR spectroscopy[136-139], while "polar" ILs have not been applied into the analysis mentioned in *section 1-5*. In terms of the use of analytical systems with polar ILs, ^{13}C NMR analysis of cellulose in IL/DMSO mixture was attempted.[140] DMSO decreases the viscosity of cellulose/IL solutions, which are highly viscous. The addition of 15 wt% of DMSO into [C_4mim]Cl significantly improved the resolution of the spectra, and they claimed DMSO did not affect the chemical shifts compared with cello-oligosaccharides in neat [C_4mim]Cl.

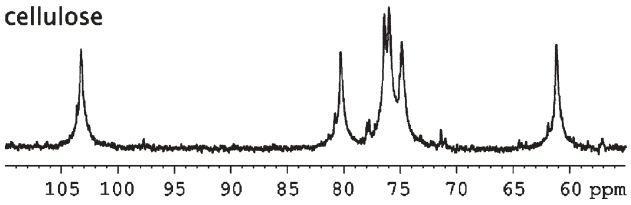


Figure 1-11 ^{13}C NMR spectra of cellulose (5 wt%) in [C_4mim]Cl/DMSO-d_6 solutions at 90 ˚C.

In addition, the sugars and starch extracted from green bananas using ILs were analyzed.[141] This analysis revealed the compositional variety of sugars in fruits. These data clearly show the potential use of neat ILs for cellulose analysis.

1-7. Aims of This Study

I mentioned the processing and analysis of cellulose in ILs above. In spite of the great progress of cellulose processing in ILs, the development of analytical methods for the processing have scarcely progressed. To realize efficient use of cellulose, basic insights such as physicochemical properties of cellulose and extraction ratio of cellulose from biomass are necessary. Therefore, we focused on the development of direct analytical methods for the evaluation of various properties of cellulose/IL solutions. For this purpose, two basic analytical systems of polymers, high performance liquid chromatography (HPLC) and ^{1}H NMR, were selected, and polar ILs that dissolve cellulose were applied to these systems. HPLC with ILs as eluents should be useful to reveal molecular weight distribution of cellulose. It leads the analysis of the hydrolysis of cellulose in ILs and the extracted cellulose from plant biomass with ILs. ^{1}H NMR should be valuable for the analysis of the interaction between cellulose and ILs and the quantification of the polysaccharides extracted from plant biomass with ILs.

1-8. References

1. T. Welton, *Chem. Rev.*, 1999, **99**, 2071-2083.

2. R. D. Rogers and K. R. Seddon, *Science*, 2003, **302**, 792-793.

3. P. Wasserscheid and T. Welton, *Ionic Liquids in Synthesis*, vol. 1, WILEY-VCH, Weinheim, 2008.

4. P. Wasserscheid and T. Welton, *Ionic Liquids in Synthesis*, vol. 2, WILEY-VCH, Weinheim, 2008.

5. M. Armand, F. Endres, D. R. MacFarlane, H. Ohno and B. Scrosati, *Nat. Mater.*, 2009, **8**, 621-629.

6. H. Ohno, *Development and view of ionic liquids (ionekitai no kaihatsu to ouyou in Japanese)*, CMC publisher, Tokyo, 2003.

7. F. H. Hurley and T. P. Weir, *J. Electrochem. Soc.*, 1951, **98**, 207-212.

8. H. L. Chum, V. R. Koch, L. L. Miller and R. A. Osteryoung, *J. Am. Chem. Soc.*, 1975, **97**, 3264-3265.

9. J. S. Wilkes, J. A. Levisky, R. A. Wilson and C. L. Hussey, *Inorg. Chem.*, 1982, **21**, 1263-1264.

10. D. Appleby, C. L. Hussey, K. R. Seddon and J. E. Turp, *Nature*, 1986, **323**, 614-616.

11. J. S. Wilkes and M. J. Zaworotko, *J. Chem. Soc. Chem. Commun.*, 1992, 965-967.

12. D. M. Fox, W. H. Awad, J. W. Gilman, P. H. Maupin, H. C. De Long and P. C. Trulove, *Green Chem.*, 2003, **5**, 724-727.

13. M. J. Earle, J. M. Esperanca, M. A. Gilea, J. N. Lopes, L. P. Rebelo, J. W. Magee, K. R. Seddon and J. A. Widegren, *Nature*, 2006, **439**, 831-834.

14. D. R. MacFarlane, J. M. Pringle, K. M. Johansson, S. A. Forsyth and M. Forsyth, *Chem. Commun.*, 2006, 1905-1917.

15. M. Freemantle, *Chem. Eng. News*, 1998, **76**, 32-37.

16. A. Brandt, J. Gräsvik, J. P. Hallett and T. Welton, *Green Chem.*, 2013, **15**, 550-583.

17. H. Wang, G. Gurau and R. D. Rogers, *Chem. Soc. Rev.*, 2012, **41**, 1519-1537.

18. S. Zhu, Y. Wu, Q. Chen, Z. Yu, C. Wang, S. Jin, Y. Ding and G. Wu, *Green Chem.*, 2006, **8**, 325-327.

19. A. Pinkert, K. N. Marsh, S. S. Pang and M. P. Staiger, *Chem. Rev.*, 2009, **109**, 6712-6728.

20. H. Ohno and Y. Fukaya, *Chem. Lett.*, 2009, **38**, 2-7.

21. Y. Cao, J. Wu, J. Zhang, H. Li, Y. Zhang and J. He, *Chem. Eng. J.*, 2009, **147**, 13-21.

22. N. Sun, H. Rodriguez, M. Rahman and R. D. Rogers, *Chem. Commun.*, 2011, **47**, 1405-1421.

23. S. S. Y. Tan and D. R. MacFarlane, *Ionic Liquids in Biomass Processing*, Springer, Berlin Heidelberg, 2009.

24. R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, *J. Am. Chem. Soc.*, 2002, **124**, 4974-4975.

25. H. Zhang, J. Wu, J. Zhang and J. S. He, *Macromolecules*, 2005, **38**, 8272-8277.

26. T. Mizumo, E. Marwanta, N. Matsumi and H. Ohno, *Chem. Lett.*, 2004, **33**, 1360-1361.

27. K. R. Seddon, A. Stark and M. Torres, *Clean Solvents: Alternative Media for Chemical Reactions and Processing*, American Chemical Society, Washington, DC, 2002.

28. Y. Fukaya, A. Sugimoto and H. Ohno, *Biomacromolecules*, 2006, **7**, 3295-3297.

29. Y. Fukaya, K. Hayashi, M. Wada and H. Ohno, *Green Chem.*, 2008, **10**, 44-46.

30. T. Erdmenger, C. Haensch, R. Hoogenboom and U. S. Schubert, *Macromol. Biosci.*, 2007, **7**, 440-445.

31. J. Vitz, T. Erdmenger, C. Haensch and U. S. Schubert, *Green Chem.*, 2009, **11**, 417-424.

32. R. Rinaldi, *Chem. Commun.*, 2011, **47**, 511-513.

33. K. Ohira, K. Yoshida, S. Hayase and T. Itoh, *Chem. Lett.*, 2012, **41**, 987-989.

34. D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, *Green Chem.*, 2007, **9**, 63-69.

35. S. Köhler, T. Liebert and T. Heinze, *J. Polym. Sci., Part A: Polym. Chem.*, 2008, **46**, 4070-4080.

36. M. Gericke, T. Liebert and T. Heinze, *Macromol. Biosci.*, 2009, **9**, 343-353.

37. M. Abe, Y. Fukaya and H. Ohno, *Chem. Commun.*, 2012, **48**, 1808-1810.

38. A. W. King, J. Asikkala, I. Mutikainen, P. Jarvi and I. Kilpeläinen, *Angew. Chem. Int. Ed.*, 2011, **50**, 6301-6305.

39. Y. Yang, H. Xie and E. Liu, *Green Chem.*, 2014, in press.

40. P. G. Jessop, D. J. Heldebrant, X. W. Li, C. A. Eckert and C. L. Liotta, *Nature*, 2005, **436**, 1102-1102.

41. D. M. Phillips, L. F. Drummy, D. G. Conrady, D. M. Fox, R. R. Naik, M. O. Stone, P. C. Trulove, H. C. De Long and R. A. Mantz, *J. Am. Chem. Soc.*, 2004, **126**, 14350-14351.

42. H. Xie, S. Li and S. Zhang, *Green Chem.*, 2005, **7**, 606-608.

43. A. Idris, R. Vijayaraghavan, U. A. Rana, D. Fredericks, A. F. Patti and D. R. MacFarlane, *Green Chem.*, 2013, **15**, 525-534.

44. A. Idris, R. Vijayaraghavan, U. A. Rana, A. F. Patti and D. R. MacFarlane, *Green Chem.*, 2014, in press.

45. R. Ferreira, H. Garcia, A. F. Sousa, M. Petkovic, P. Lamosa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. S. Pereira, *New J. Chem.*, 2012, **36**, 2014-2024.

46. R. Ferreira, H. Garcia, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. S. Pereira, *Ind. Crop. Prod.*, 2013, **44**, 520-527.

47. A. Takegawa, M. Murakami, Y. Kaneko and J. Kadokawa, *Carbohydr. Polym.*, 2010, **79**, 85-90.

48. Y. Qin, X. M. Lu, N. Sun and R. D. Rogers, *Green Chem.*, 2010, **12**, 968-971.

49. H. Xie, S. Zhang and S. Li, *Green Chem.*, 2006, **8**, 630-633.

50. T. V. Doherty, M. Mora-Pale, S. E. Foley, R. J. Linhardt and J. S. Dordick, *Green Chem.*, 2010, **12**, 1967-1975.

51. A. Brandt, M. J. Ray, T. Q. To, D. J. Leak, R. J. Murphy and T. Welton, *Green Chem.*, 2011, **13**, 2489-2499.

52. H. Zhao, C. I. L. Jones, G. A. Baker, S. Xia, O. Olubajo and V. N. Person, *J. Biotechnol.*, 2009, **139**, 47-54.

53. X. F. Tian, Z. Fang, D. Jiang and X. Y. Sun, *Biotechnol. Biofuels*, 2011, **4**:53.

54. J. L. Xu, C. Zhang, X. G. Wang, H. Ji, C. C. Zhao, Y. Wang and Z. H. Zhang, *Green Chem.*, 2011, **13**, 1914-1922.

55. S. Padmanabhan, M. Kim, H. W. Blanch and J. M. Prausnitz, *Fluid Phase Equilib.*, 2011, **309**, 89-96.

56. M. Zavrel, D. Bross, M. Funke, J. Buchs and A. C. Spiess, *Bioresour. Technol.*, 2009, **100**, 2580-2587.

57. A. R. Xu, J. J. Wang and H. Y. Wang, *Green Chem.*, 2010, **12**, 268-275.

58. C. Hardacre, S. E. J. McMath and J. D. Holbrey, *Chem. Commun.*, 2001, 367-368.

59. K. M. Dieter, C. J. Dymek, N. E. Heimer, J. W. Rovang and J. S. Wilkes, *J. Am. Chem. Soc.*, 1988, **110**, 2722-2726.

60. R. C. Remsing, J. L. Wildin, A. L. Rapp and G. Moyna, *J. Phys. Chem. B*, 2007, **111**, 11619-11621.

61. C. Froschauer, M. Hummel, G. Laus, H. Schottenberger, H. Sixta, H. K. Weber and G. Zuckerstatter, *Biomacromolecules*, 2012, **13**, 1973-1980.

62. A. Stark, M. Sellin, B. Ondruschka and K. Massonne, *Sci. China Chem.*, 2012, **55**, 1663-1670.

63. Q. Yang, H. Xing, Z. Bao, B. Su, Z. Zhang, Y. Yang, S. Dai and Q. Ren, *J. Phys. Chem. B*, 2014 in press.

64. R. C. Remsing, R. P. Swatloski, R. D. Rogers and G. Moyna, *Chem. Commun.*, 2006, 1271-1273.

65. A. S. Gross, A. T. Bell and J. W. Chu, *J. Phys. Chem. B*, 2011, **115**, 13433-13440.

66. H. Liu, G. Cheng, M. Kent, V. Stavila, B. A. Simmons, K. L. Sale and S. Singh, *J. Phys. Chem. B*, 2012, **116**, 8131-8138.

67. T. G. A. Youngs, J. D. Holbrey, C. L. Mullan, S. E. Norman, M. C. Lagunas, C. D'Agostino, M. D. Mantle, L. F. Gladden, D. T. Bowron and C. Hardacre, *Chem. Sci.*, 2011, **2**, 1594-1605.

68. B. D. Rabideau, A. Agarwal and A. E. Ismail, *J. Phys. Chem. B*, 2013, **117**, 3469-3479.

69. B. D. Rabideau, A. Agarwal and A. E. Ismail, *J. Phys. Chem. B*, 2014, **118**, 1621-1629.

70. H. B. Liu, K. L. Sale, B. M. Holmes, B. A. Simmons and S. Singh, *J. Phys. Chem. B*, 2010, **114**, 4293-4301.

71. E. E. Fileti, P. Chaudhuri and S. Canuto, *Chem. Phys. Lett.*, 2004, **400**, 494-499.

72. J. Zhang, H. Zhang, J. Wu, J. Zhang, J. He and J. Xiang, *Phys. Chem. Chem. Phys.*, 2010, **12**, 1941-1947.

73. J. Zhang, H. Zhang, J. Wu, J. Zhang, J. He and J. Xiang, *Phys. Chem. Chem. Phys.*, 2010, **12**, 14829-14830.

74. R. C. Remsing, I. D. Petrik, Z. Liu and G. Moyna, *Phys. Chem. Chem. Phys.*, 2010, **12**, 14827-14828.

75. T. G. A. Youngs, C. Hardacre and J. D. Holbrey, *J. Phys. Chem. B*, 2007, **111**, 13765-13774.

76. K. Okushita, E. Chikayama and J. Kikuchi, *Biomacromolecules*, 2012, **13**, 1323-1330.

77. R. S. Payal and S. Balasubramanian, *Phys. Chem. Chem. Phys.*, 2014, in press.

78. H. M. Cho, A. S. Gross and J. W. Chu, *J. Am. Chem. Soc.*, 2011, **133**, 14033-14041.

79. R. C. Remsing, G. Hernandez, R. P. Swatloski, W. W. Massefski, R. D. Rogers and G. Moyna, *J. Phys. Chem. B*, 2008, **112**, 11071-11078.

80. F. Huo, Z. Liu and W. Wang, *J. Phys. Chem. B*, 2013, **117**, 11780-11792.

81. Y. L. Zhao, X. M. Liu, J. J. Wang and S. J. Zhang, *J. Phys. Chem. B*, 2013, **117**, 9042-9049.

82. N. Sun, M. Rahman, Y. Qin, M. L. Maxim, H. Rodríguez and R. D. Rogers, *Green Chem.*, 2009, **11**, 646-655.

83. J.-P. Mikkola, A. Kirilin, J.-C. Tuuf, A. Pranovich, B. Holmbom, L. M. Kustov, D. Y. Murzin and T. Salmi, *Green Chem.*, 2007, **9**, 1229-1237.

84. M. Abe, Y. Fukaya and H. Ohno, *Green Chem.*, 2010, **12**, 1274-1280.

85. M. Abe, T. Yamada and O. Hiroyuki, *RSC adv.*, 2014, in press.

86. F. Boissou, A. Muhlbauer, K. D. Vigier, L. Leclercq, W. Kunz, S. Marinkovic, B. Estrine, V. Nardello-Rataj and F. Jerome, *Green Chem.*, 2014, **16**, 2463-2471.

87. N. S. Mosier, C. M. Ladisch and M. R. Ladisch, *Biotechnol. Bioeng.*, 2002, **79**, 610-618.

88. R. W. Torget, J. S. Kim and Y. Y. Lee, *Ind. Eng. Chem. Res.*, 2000, **39**, 2817-2825.

89. C. Li and Z. K. Zhao, *Adv. Synth. Catal.*, 2007, **349**, 1847-1850.

90. C. Li, Q. Wang and Z. K. Zhao, *Green Chem.*, 2008, **10**, 177-182.

91. R. Rinaldi, N. Meine, J. vom Stein, R. Palkovits and F. Schuth, *ChemSusChem*, 2010, **3**, 266-276.

92. Y. B. Huang and Y. Fu, *Green Chem.*, 2013, **15**, 1095-1111.

93. D. Yamaguchi, M. Kitano, S. Suganuma, K. Nakajima, H. Kato and M. Hara, *J. Phys. Chem. C*, 2009, **113**, 3181-3188.

94. R. Rinaldi, R. Palkovits and F. Schuth, *Angew. Chem. Int. Ed.*, 2008, **47**, 8047-8050.

95. M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem.*, 2003, **5**, 443-447.

96. S. Bose, D. W. Armstrong and J. W. Petrich, *J. Phys. Chem. B*, 2010, **114**, 8221-8227.

97. T. Zhang, S. Datta, J. Eichler, N. Ivanova, S. D. Axen, C. A. Kerfeld, F. Chen, N. Kyrpides, P. Hugenholtz, J. F. Cheng, K. L. Sale, B. Simmons and E. Rubin, *Green Chem.*, 2011, **13**, 2083-2090.

98. R. Wahlstrom, A. King, A. Parviainen, K. Kruus and A. Suurnakki, *Rsc Adv.*, 2013, **3**, 20001-20009.

99. P. Lozano, B. Bernal, J. M. Bernal, M. Pucheault and M. Vaultier, *Green Chem.*, 2011, **13**, 1406-1410.

100. K. Fujita, N. Nakamura, K. Igarashi, M. Samejima and H. Ohno, *Green Chem.*, 2009, **11**, 351-354.

101. K. Nakashima, K. Yamaguchi, N. Taniguchi, S. Arai, R. Yamada, S. Katahira, N. Ishida, H. Takahashi, C. Ogino and A. Kondo, *Green Chem.*, 2011, **13**, 2948-2953.

102. C. Froschauer, M. Hummel, M. Iakovlev, A. Roselli, H. Schottenberger and H. Sixta, *Biomacromolecules*, 2013, **14**, 1741-1750.

103. Z. Qiu, G. M. Aita and M. S. Walker, *Bioresour. Technol.*, 2012, **117**, 251-256.

104. R. Rinaldi, R. Palkovits and F. Schuth, *Angew. Chem. Int. Ed.*, 2008, **47**, 8047-8050.

105. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, *J. Agric. Food Chem.*, 2007, **55**, 9142-9148.

106. I. P. Samayam, B. L. Hanson, P. Langan and C. A. Schall, *Biomacromolecules*, 2011, **12**, 3091-3098.

107. T. Leskinen, A. W. T. King, I. Kilpeläinen and D. S. Argyropoulos, *Ind. Eng. Chem. Res.*, 2013, **52**, 3958-3966.

108. W. Lan, C.-F. Liu, F.-X. Yue, R.-C. Sun and J. F. Kennedy, *Carbohydr. Polym.*, 2011, **86**, 672-677.

109. T. Bikova and A. Treimanis, *Carbohydr. Polym.*, 2002, **48**, 23-28.

110. M. Yanagisawa, I. Shibata and A. Isogai, *Cellulose*, 2004, **11**, 169-176.

111. J. Wu, J. Zhang, H. Zhang, J. S. He, Q. Ren and M. Guo, *Biomacromolecules*, 2004, **5**, 266-268.

112. J. Wu, H. Zhang, J. Zhang and J. S. He, *Chem. J. Chinese. U.*, 2006, **27**, 592-594.

113. H. Xie, A. King, I. Kilpeläinen, M. Granstrom and D. S. Argyropoulos, *Biomacromolecules*, 2007, **8**, 3740-3748.

114. Y. Cao, J. Wu, T. Meng, J. Zhang, J. He, H. Li and Y. Zhang, *Carbohydr. Polym.*, 2007, **69**, 665-672.

115. L. Zoia, A. W. T. King and D. S. Argyropoulos, *J. Agric. Food Chem.*, 2011, **59**, 829-838.

116. T. Leskinen, A. W. T. King, I. Kilpeläinen and D. S. Argyropoulos, *Ind. Eng. Chem. Res.*, 2011, **50**, 12349-12357.

117. A. Salanti, L. Zoia, E. L. Tolppa and M. Orlandi, *Biomacromolecules*, 2012, **13**, 445-454.

118. R. Evans, R. H. Wearne and A. F. A. Wallis, *J. Appl. Polym. Sci.*, 1989, **37**, 3291-3303.

119. S. Pandey, *Anal. Chim. Acta*, 2006, **556**, 38-45.

120. W. E. Acree and L. M. Grubbs, *Encycl. Anal. Chem.*, 2012, 1-40.

121. J. L. Anderson, D. W. Armstrong and G. T. Wei, *Anal. Chem.*, 2006, **78**, 2892-2902.

122. D. W. Armstrong, L. F. He and Y. S. Liu, *Anal. Chem.*, 1999, **71**, 3873-3876.

123. J. L. Anderson and D. W. Armstrong, *Anal. Chem.*, 2005, **77**, 6453-6462.

124. J. Ding, T. Welton and D. W. Armstrong, *Anal. Chem.*, 2004, **76**, 6819-6822.

125. C. D. Tran and S. Challa, *Analyst*, 2008, **133**, 455-464.

126. R. J. Soukup-Hein, M. M. Warnke and D. W. Armstrong, *Annu. Rev. Anal. Chem.*, 2009, **2**, 145-168.

127. S. Kuwabata, A. Kongkanand, D. Oyamatsu and T. Torimoto, *Chem. Lett.*, 2006, **35**, 600-601.

128. Y. Ishigaki, Y. Nakamura, T. Takehara, N. Nemoto, T. Kurihara, H. Koga, H. Nakagawa, T. Takegami, N. Tomosugi, S. Miyazawa and S. Kuwabata, *Microsc. Res. Tech.*, 2011, **74**, 415-420.

129. T. Tsuda, N. Nemoto, K. Kawakami, E. Mochizuki, S. Kishida, T. Tajiri, T. Kushibiki and S. Kuwabata, *ChemBioChem*, 2011, **12**, 2547-2550.

130. S. Arimoto, H. Kageyama, T. Torimoto and S. Kuwabata, *Electrochem. Commun.*, 2008, **10**, 1901-1904.

131. S. Arimoto, D. Oyamatsu, T. Torimoto and S. Kuwabata, *Chemphyschem: Euro. J. Chem. Phys. Phys. Chem.*, 2008, **9**, 763-767.

132. D. W. Armstrong, L. K. Zhang, L. F. He and M. L. Gross, *Anal. Chem.*, 2001, **73**, 3679-3686.

133. M. Mank, B. Stahl and G. Boehm, *Anal. Chem.*, 2004, **76**, 2938-2950.

134. T. N. Laremore, F. M. Zhang and R. J. Linhardt, *Anal. Chem.*, 2007, **79**, 1604-1610.

135. Y. Fukuyama, S. Nakaya, Y. Yamazaki and K. Tanakat, *Anal. Chem.*, 2008, **80**, 2171-2179.

136. V. P. Ananikov, *Chem. Rev.*, 2011, **111**, 418-454.

137. R. Giernoth, D. Bankmann and N. Schlörer, *Green Chem.*, 2005, **7**, 279-282.

138. X. Creary, E. D. Willis and M. Gagnon, *J. Am. Chem. Soc.*, 2005, **127**, 18114-18120.

139. A. Durazo and M. M. Abu-Omar, *Chem. Commun.*, 2002, 66-67.

140. J. S. Moulthrop, R. P. Swatloski, G. Moyna and R. D. Rogers, *Chem. Commun.*, 2005, 1557-1559.

141. D. A. Fort, R. P. Swatloski, P. Moyna, R. D. Rogers and G. Moyna, *Chem. Commun.*, 2006, 714-716.

Chapter 2

^{1}H NMR Analysis of Cellulose Dissolved in Non-deuterated Ionic Liquids for the analysis of dissolved state

2-1. Introduction

To realize efficient extraction of cellulose from plant biomass, interaction between cellulose and ILs should be revealed. It is already known that ILs form hydrogen bond with hydroxyl groups of cellulose due to their high hydrogen bond basicity. For further investigation on interaction, NMR analysis is strong method for it. Until now, ^{13}C NMR measurement has been tried to detect signals of cellulose in ILs[1], but it is not appropriate for analyzing hydrogen bond because it cannot directly observe hydrogen bond; indirect factor should be included. From these reasons, we tried to develop ^{1}H NMR analysis of cellulose dissolved in ILs in this Chapter.

However, ^{1}H NMR analysis of dissolved cellulose in ILs has a problem to overcome. This is attributed to large proton signals derived from large excess amount of ILs compared to cellulose. They give rise to dropping sensitivity and overlapping to signals of cellulose. General solution for these problems is thought to be deuteration of ILs. However, deuteration of ILs is not desirable because ILs have structural diversity; deuteration of various ILs causes troublesome processes and consuming enormous time. Furthermore, deuteration sometimes leads to vanishing OH signals due to hydrogen-deuterium exchange. For example, fully deuterated pyridinium chloride makes OH signals undetectable[2], and it is known that the proton at 2-position of imidazolium ring is also exchangeable.[3] Whereas use of deuterated additives for shimming, lock, and decreasing viscosity is one of the potential methods to obtain ^{1}H NMR spectrum, it disrupts the interaction between cellulose and ILs. Considering these facts, development of direct ^{1}H NMR analysis of cellulose dissolved in non-deuterated pure ILs should be quite beneficial.

To this end, ^{1}H NMR measurement with no-deuterium (No-D) NMR technique[4-6] and a solvent suppression technique (water suppression enhanced through T_1 effect: WET) should be quite

attractive. In this study, ^{1}H NMR using No-D NMR and WET was performed in a non-deuterated IL, 1,3-dimethylimidazolium methyl methylphosphonate ([C_1mim][(MeO)(Me)PO_2]).

2-2. Materials and Methods

Materials and Instruments

1-Methylimidazole was purchased from Kanto Chemical Co., Inc., and dried over KOH and distilled before use. Dimethyl methylphosphonate was purchased from Tokyo Chemical Ind. Co., Ltd. and distilled before use. Toluene was purchased from Kanto Chemical Co., Ink and used as received. Microcrystalline cellulose powder (cellulose powder C) was purchased from Advantec Toyo Co. and used after drying under reduced pressure. Chitosan (Chitosan 100) was purchased from Wako Pure Chemical Industries, Ltd. and used as received. Xylan was purchased from Tokyo Chemical Ind. Co., Ltd. It was dissolved in DMSO and precipitated by water. The precipitation was washed with excess amount of water and dried before use. Deuterium oxide and DMSO-d_6 were purchased from Acros Organics and used as received. 3-(Trimethylsilyl)-propanesulfonic acid sadium salt was purchased from Merck KGaA and used as received. The amounts of water of IL samples were confirmed by Karl Fischer coulometric titration (Kyoto Electronics; MKC-510N).

^{1}H- and ^{13}C NMR measurements of saccharides were carried out on a JEOL ECX-400 (^{1}H base frequency 400 MHz) with a NM-40TH5AT/FG2 autotunable gradient probe (JEOL Ltd.). The analysis of NMR spectra was carried out with JEOL Delta (version 5.0.1).

Synthesis of [C_1mim][(MeO)(Me)PO_2]

[C_1mim][(MeO)(Me)PO_2] was synthesized as follows (Figure 2-1). Dimethyl methyl phosphonate (50 g; 0.40 mol) and 1-methylimidazole (36.4 g; 0.44 mol) were added into 100 ml of toluene under an argon gas atmosphere at room temperature. The reaction mixture was stirred at 120 °C for 48h. The resulting liquid was dried *in vacuo* at 80 °C and washed repeatedly with excess dehydrated diethyl ether. The

residual liquid was dissolved in dichloromethane, and the resulting solution was passed through a column filled with neutral activated alumina. After removal of dichloromethane, the residual liquid was dried *in vacuo* at 100 °C for 8h to give [C_1mim][(MeO)(Me)PO_2] with water content below 400 ppm. Structure of [C_1mim][(MeO)(Me)PO_2] was confirmed by ^{1}H- and ^{13}C NMR spectra (JEOL ECX-400; JEOL Ltd.) and elemental analysis (Elementar vario EL III; Elementar Analyzensysteme GmbH).

^{1}H-NMR (400 MHz; $CDCl_3$; Me_4Si) d = 1.26 (3H, d, J = 15.6 Hz, PCH_3), 3.57 (3H, d, J = 10.1 Hz, POCH_3), 4.05 (6H, s, NCH_3), 7.57 (2H, d, J = 1.8 Hz, NC*H*C*H*N), 10.73 (1H, s, NC*H*N). ^{13}C-NMR (100 MHz; $CDCl_3$; Me_4Si) d = 11.47, 12.79 (P*C*H_3), 36.06 (N*C*H_3), 51.18 (PO*C*H_3), 123.26 (NCH*C*HN), 139.79 (N*C*HN).

Elemental analysis: Found: C, 39.22, H, 7.77, N, 13.18. Calc. for $C_7H_{15}N_2O_3P\_0.5H_2O$: C, 39.07, H, 7.49, N, 13.02 %. [C_1mim][(MeO)(Me)PO_2] absorbed small amount of water during elemental analysis because it is very hygroscopic.

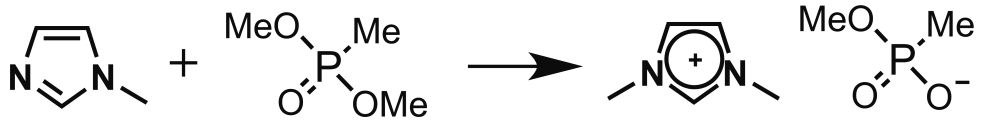


Figure 2-1 Synthesis of [C_2mim][(MeO)(Me)PO_2]

Analysis of cellulose with No-D NMR and WET

Cellulose (30 mg) was added into 1.0 g of dried [C_1mim][(MeO)(Me)PO_2]. The mixture was stirred gently 80 °C until the solution became homogeneous and clear. The resulting solution was transferred to 5 mm NMR tubes. The sample tube was capped with plastic lids, then the top was wrapped in a parafilm. All treatments were processed under an atmosphere of dry nitrogen gas. The sample was analyzed at 100 °C with 240 scans.

WET method: the measurements were performed based on the standard pulse sequence purchased by JEOL Ltd. The WET pulse of 11 ms and WET attenuator of 50 dB were used. The attenuation level required for a hard 90 degree proton pulse was 4.5 dB and the duration of the pulse was 12.8 μs. For suppression of ^{13}C satellite peaks, MPF8 was applied as a decoupling sequence.

Analysis of deuterated cellulose, and xylan and chitosan

We measured deuterated cellulose because three types of the protons of cellulose should be distinguished. Protons of cellulose are generally identified as backbone protons (C***H***s) or protons of hydroxyl groups (C-O***H***s). The signals of C-O***H***s of deuterated cellulose are thought to be invisible.

Cellulose (30 mg) was dissolved into [C_1mim][(MeO)(Me)PO_2] (1.0 g) and deuterium oxide (40 ml) was added to the sample solution. The resulting solution was heated 150 ^{o}C for 5min and transferred to 5 mm NMR tubes. This sample was measured by No-D NMR and WET as described above.

We measured xylan and chitosan dissolved in [C_1mim][(MeO)(Me)PO_2] to assign C-O***H***s. Because xylan has no C-O***H*** at 6-position and chitosan has no C-O***H*** at 2-position, we assigned these C-O***H***s by comparing these spectra and that of cellulose.

Xylan (30 mg) and chitosan (30 mg) were respectively added into 1.0 g of [C_1mim][(MeO)(Me)PO_2]s and the samples were stirred until homogeneous and clear. The resulting solutions were transferred to 5 mm NMR tubes, and coaxial inserts containing 3-(trimethylsilyl)-propane sulfonic acid sodium salt/DMSO-d_6 mixtures (1.2 wt%) were subsequently fitted as external standards (0 ppm). The sample tubes were capped with glass lids, then the tops were wrapped in parafilms. The resulting solutions were measured by No-D NMR and WET as described above.

Analysis of chemical shifts for cellulose in [C$_1$mim][(MeO)(Me)PO$_2$] and [C$_1$mim][(MeO)(Me)PO$_2$]/DMSO-d_6 mixtures

The mixtures of [C$_1$mim][(MeO)(Me)PO$_2$] and DMSO-d_6 in mixing ratio from 1:0 to 1:25 (mol:mol) were prepared. Cellulose (30 mg) was added into 1.0 g of [C$_1$mim][(MeO)(Me)PO$_2$]/DMSO-d_6 mixtures. The mixtures were stirred gently 80 ^{o}C until the solutions became homogeneous and clear. The resulting solutions were transferred to 5 mm NMR tubes, and coaxial inserts containing 3-(trimethylsilyl)-propane sulfonic acid sodium salt/DMSO-d_6 mixtures (1.2 wt%) were subsequently fitted as external standards (0 ppm). The sample tubes were capped with glass lids, then the tops were wrapped in parafilms. All treatments were processed under an atmosphere of dry nitrogen gas. The resulting solutions were measured by No-D NMR and WET as described above. While DMSO-d_6 was used as an additive, shimming and lock with deuterium were not used.

Measurement of β values of Kamlet-Taft parameters

β values of Kamlet-Taft parameters were measured as follows. The solvatochromic dyes, 4-nitroaniline (from Tokyo Chemical Industries Co., Ltd) and *N*,*N*-diethyl-4-nitroaniline (from Kanto Chem.), were used as received. These dyes were added to 0.2 ml of sample solutions. These solutions were placed into quartz cells with 1 mm light-path length. From the wavelength at the maximum absorption (λ_{max}) determined by UV-*vis* spectrophotometer (UV-2450; Shimadzu), the β values were calculated by use of the following equations:

$$\nu_{(dye)} = 1/(\lambda_{max(dye)} \times 10^{-4})$$

$$\beta = (1.035\nu_{(N,N\text{-diethyl-4-nitroaniline})} + 2.64 - \nu_{(4\text{-nitroaniline})})/2.80$$

H,H-COSY of glucose

Glucose (0.10 g) was added into 1.0 g of dried [C_1mim][(MeO)(Me)PO_2]. The mixture was stirred gently 80 ^{o}C until the solution became homogeneous and clear. The resulting solution was transferred to a 5 mm NMR tube. The sample tube was capped with a plastic lid, then the top was wrapped in a parafilm. This sample was analyzed at 60 ^{o}C with 60 scans.

WET method: the measurement was performed based on the standard pulse sequence purchased by JEOL Ltd. The WET pulse of 14 ms and WET attenuator of 50 dB were used. For suppression of ^{13}C satellite peaks, MPF8 was applied as a decoupling sequence.

COSY method: the correlation experiment was performed using a standard gradient-selected COSY pulse sequence purchased from JEOL Ltd.

2-3. Detection of ^{1}H Signals of Cellulose in Non-deuterated Ionic Liquids

Figure 2-2 shows ^{1}H NMR spectra of the $[C_1mim][(MeO)(Me)PO_2]$/cellulose solution measured by the method using No-D NMR and WET. In the spectrum obtained by the standard method (Figure 2-2a), no peaks of cellulose but those for $[C_1mim][(MeO)(Me)PO_2]$ were observed. To improve resolution, the solution was measured with No-D NMR (Figure 2-2b). While all peaks became relatively sharp and their intensities increased, any peaks of cellulose were not found. WET was applied to the peak of $[C_1mim][(MeO)(Me)PO_2]$ at 2.7 ppm for confirmation of suppression of IL peaks in $[C_1mim][(MeO)(Me)PO_2]$/cellulose solution (Figure 2-2c). It was found that the peak and its satellite peaks were suppressed locally even in the IL/cellulose solution, which is highly viscous. Then, all peaks for $[C_1mim][(MeO)(Me)PO_2]$ at 0.3, 2.7, 3.5, 7.7, and 9.6 ppm were suppressed (Figure 2-2d). The peaks of cellulose were observed at 2.4, 2.9, 3.1, 4.0, 5.2, 6.1, and 6.4 ppm. In the spectrum, since sufficient signal to noise ratio was obtained, a diluted solution was measured using No-D NMR and WET (Figure 2-3). ^{1}H signals of cellulose in 0.5 wt% solution were detected by the accumulation of only 12 scans. In the case of ^{13}C NMR, detection of signals of cellulose requires over 5000 scans (several hours) even in 5 wt% cellulose/IL solutions. Integrals of peaks near the suppressed peaks were not in stoichiometric ratio. Sharp peaks without arrows in Figure 2-2d were attributed to impurities of $[C_1mim][(MeO)(Me)PO_2]$ in spite that the $[C_1mim][(MeO)(Me)PO_2]$ was confirmed to be sufficiently pure by elemental analysis; C/N ratio was found to be 2.98 (3.00 in calculation).

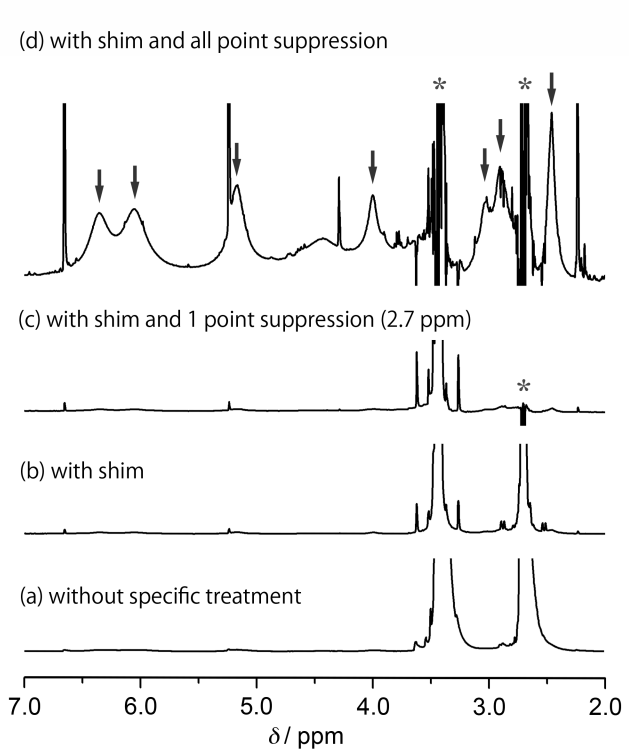


Figure 2-2 ^{1}H NMR spectra of cellulose in $[C_1mim][(MeO)(Me)PO_2]$ at 100 °C. Arrows indicate the signals of cellulose. *:suppressed signal

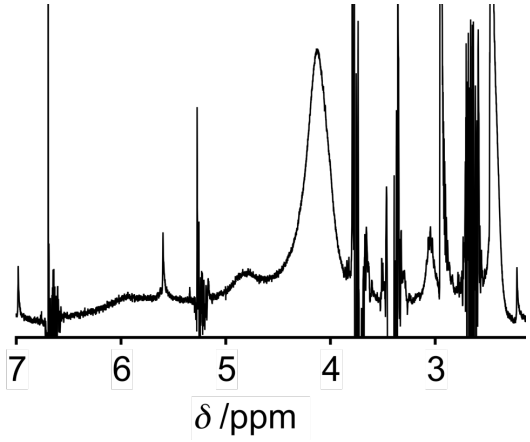


Figure 2-3 ^{1}H NMR spectra of 0.5 wt% of cellulose in $[C_1mim][(MeO)(Me)PO_2]$ at 100 °C.

2-4. Assignment of Signals

In Figure 2-2d, seven peaks for cellulose were observed. Generally, protons of cellulose are categorized into two types based on C***H***s and C-O***H***s. To assign the signals, we preliminarily measured the spectrum of cellulose in [C_1mim][(MeO)(Me)PO_2] before and after adding deuterium oxide (Figure 2-4). After addition of deuterium oxide, the intensities of the peaks at 5.2, 6.1, and 6.4 ppm decreased, showing that the three peaks should be assigned to C-O***H***s. Four remained peaks at 2.4, 2.9, 3.1, and 4.0 ppm should therefore be assigned to C***H***s while the peak at 4.0 ppm was not observed due to overlapping with the signal of water. For further assignment of C-O***H*** signals, we have measured ^{1}H NMR spectra of xylan and chitosan which lack the C-O$\boldsymbol{H}_{(6)}$ and C-O$\boldsymbol{H}_{(2)}$, respectively. The signal at 5.2 ppm can be assigned to C-O$\boldsymbol{H}_{(6)}$ which was not observed in the spectrum of xylan. The signal at 6.4 ppm was assigned to C-O$\boldsymbol{H}_{(2)}$ considering the spectrum of chitosan. Therefore, we believe that the C-O***H*** signal at 6.1 ppm is assigned to C-O$\boldsymbol{H}_{(3)}$.

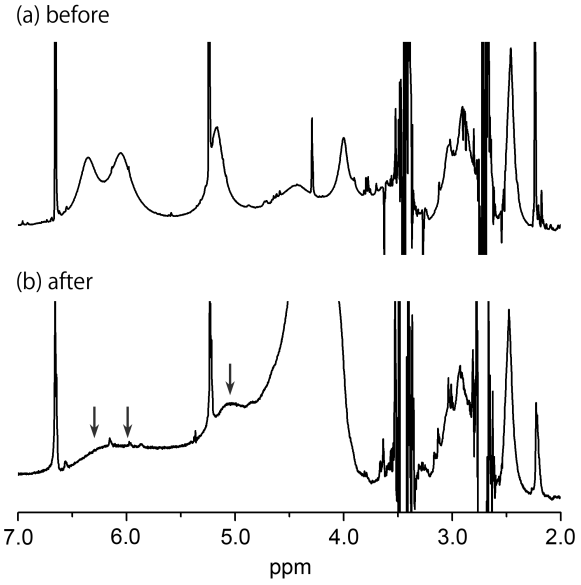


Figure 2-4 ^{1}H NMR spectra of cellulose in [C_1mim][(MeO)(Me)PO_2] (a)before and (b)after adding deuterium oxide. Arrows indicate the signals of C-O***H***s

2-5. Analysis of Interaction between Ionic Liquids and Cellulose

^{1}H NMR is one of the most suitable techniques for investigating hydrogen bond unlike other indirect techniques including ^{13}C NMR. The results in Figure 2-2d show that both C***H***s and C-O***H***s were observed by ^{1}H NMR, and hence the interaction between ILs and two types of protons can be analyzed. To confirm that ILs interact strongly with C-O***H***s, rather than C***H***s, we compared the chemical shifts of cellulose in [C_1mim][(MeO)(Me)PO_2] and those of cellobiose in DMSO-d_6 (Figure 2-5). The signals for C-O***H***s of cellulose in [C_1mim][(MeO)(Me)PO_2] (see Figure 2-2d, 5.0 to 6.6 ppm) were observed on much lower magnetic field side than those of cellobiose in DMSO-d_6 (4 to 5 ppm). On the other hand, the signals of C***H***s in both solutions were observed in the similar chemical shifts. From these results, it was confirmed that [C_1mim][(MeO)(Me)PO_2] interacted with C-O***H***s stronger than with C***H***s. [C_1mim][(MeO)(Me)PO_2] interacted with C-O***H***s due to a high hydrogen bond basicity of [C_1mim][(MeO)(Me)PO_2] (β value of Kamlet-Taft parameter of [C_1mim][(MeO)(Me)PO_2] was 1.10). This high β value of [C_1mim]-

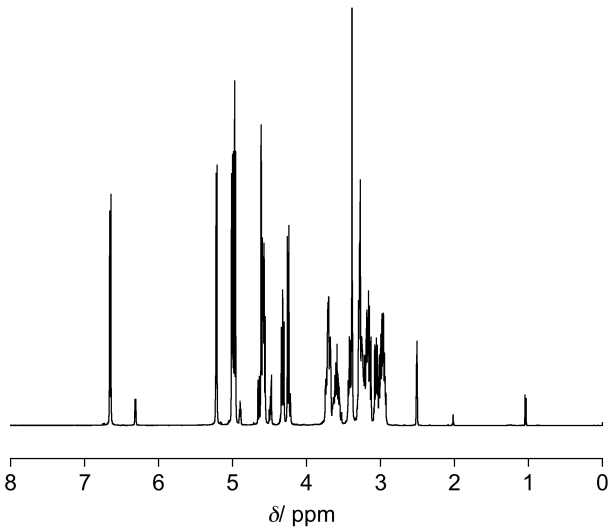


Figure 2-5 The spectrum of cellobiose in DMSO-d_6

[(MeO)(Me)PO_2] is the proof of high solubility of cellulose in [C_1mim][(MeO)(Me)PO_2]. This was confirmed by comparing its β value with that of DMSO-d_6 studied (0.71).

It is known that C-O***H***$_{(6)}$ plays an important role to make cellulose insoluble in ordinary molecular solvents.[7] To solubilize cellulose, interchain hydrogen bonds among C-O***H***$_{(6)}$s therefore should be destroyed by highly polar solvents. The ^{1}H NMR spectra of cellulose in [C_1mim][(MeO)(Me)PO_2] before and after adding different amounts of DMSO-d_6 were measured. Cellulose was found to be insoluble in [C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 mixture with the mixing ratio of 1:25 (by mol) or more. As shown in Figure 2-6, by increasing DMSO-d_6 fraction, the peaks for C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$ shifted to higher magnetic field side. On the other hand, no shift of the peak for C-O***H***$_{(6)}$ was found. These data pointed out that the interacted [C_1mim][(MeO)(Me)PO_2]s to C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$ were gradually replaced with DMSO-d_6, or C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$ formed intrachain hydrogen bond by increasing DMSO-d_6 fraction. Against these, the signals for C-O***H***$_{(6)}$ did not show any shift, suggesting solubilization of cellulose by the strong interaction of [C_1mim][(MeO)(Me)PO_2] and C-O***H***$_{(6)}$ even in the presence of excess DMSO-d_6. Through these experiments, it was confirmed that the strong interaction of [C_1mim][(MeO)(Me)PO_2] and C-O***H***$_{(6)}$ was the key step to dissolve cellulose. This is the first report about experimental proof related to the importance of interaction between C-O***H***$_{(6)}$s and ILs because thus far there are not any other methodologies for direct detection of hydrogen bonds between respective C-O***H***s and ILs. This is supported by the report that both C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$ are known to contribute to form *intrachain* hydrogen bonds whereas C-O***H***$_{(6)}$ leads to the formation of *interchain* hydrogen bonds.[8] It is worthily noted that reactivity of the respective hydroxyl groups should be different depending on the mixing ratio of [C_1mim][(MeO)(Me)PO_2] and

DMSO-d_6 due to the difference of their hydrogen bonds. As shown here, it can be estimated by ^{1}H NMR.

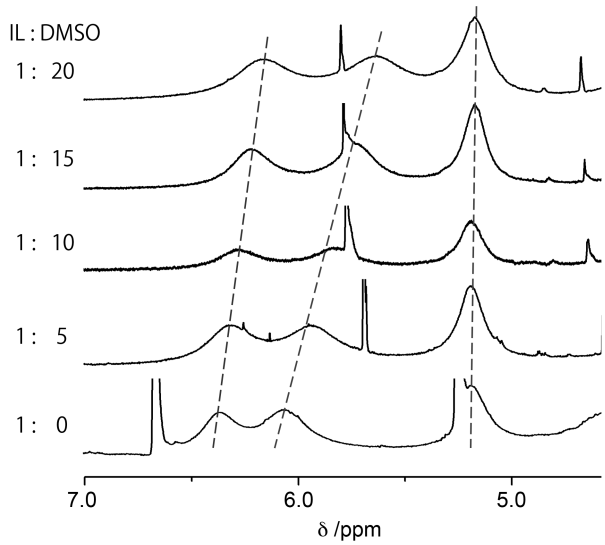


Figure 2-6 ^{1}H NMR spectra of cellulose in IL **1**/DMSO-d_6 mixtures (number of scans: 240, temperature: 100 °C).

Kamlet-Taft parameter is commonly used for estimating polarity of ILs, and it is well known that ILs which have high β values can form hydrogen bonds with cellulose and dissolve it. Using the ^{1}H NMR studied, the β values were compared with behavior of hydrogen bonds. Figure 2-7 shows the relations between β values of [C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 solutions and the chemical shifts of C-O***H***s. As increasing the fraction of DMSO-d_6, β value gradually decreased from 1.1 to 0.96 and the relations of C-O$\boldsymbol{H}_{(2)}$ and C-O$\boldsymbol{H}_{(3)}$ were linear. It was reconfirmed that β value is available for estimating intensity of hydrogen bond between ILs and C-O$\boldsymbol{H}_{(2)}$ and C-O$\boldsymbol{H}_{(3)}$. According to C-O$\boldsymbol{H}_{(6)}$, the shift of the β value may suggest that [C_1mim][(MeO)(Me)PO_2] interacted strongly to C-O$\boldsymbol{H}_{(6)}$ when the β value was higher than 0.96. Therefore, it was confirmed that the results about hydrogen bonds from ^{1}H NMR and solvatochromism were

similar, especially concerning C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$. Thsese results indicate that potential factors which affect estimation did not appeared in the case of cellulose and [C_1mim][(MeO)(Me)PO_2]. In some cases, there are potential factors affecting estimation such as steric hindrance. It is important to mention that β value of Kamlet-Taft parameter was useful even in mixtures. From λ_{max} of dyes related to the β value in [C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 solutions, it was confirmed that β value depended on the interaction with 4-nitroaniline (4N) more strongly rather than *N*,*N*-diethyl-4-nitroaniline. Because the relationships between λ_{max} of 4N and the chemical shifts of C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$ were also linear, it was confirmed that λ_{max} of 4N indicated the behavior of hydrogen bond with C-O***H***$_{(2)}$ and C-O***H***$_{(3)}$ (Figure 2-8). This is the first report about relations between hydrogen bonds of specific C-O***H***s and β value quantitatively.

It is important to mention that when β value was higher than 0.96 (at least), ILs strongly interacted to C-O***H***$_{(6)}$ and destroyed interchain hydrogen bonds. When β value is over 0.8, the effect of β value on degree of solubility is now under discussion. This result should be clue to clarify it.

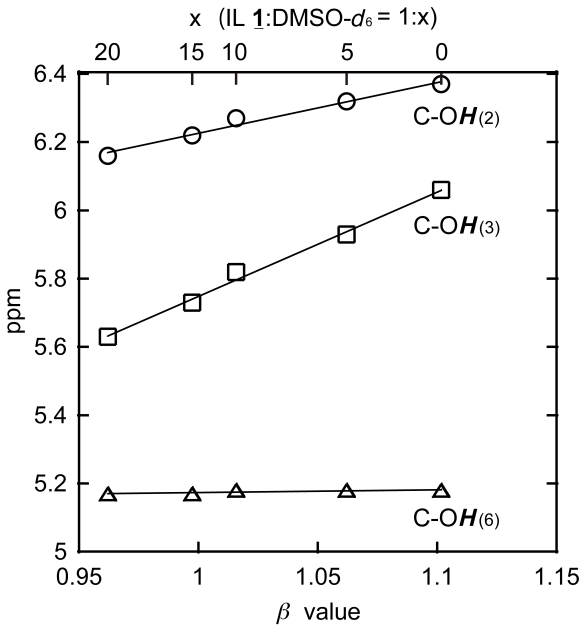


Figure 2-7 The relationship between β values of [C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 mixtures and chemical shifts of C-O***H***s in cellulose/[C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 mixtures

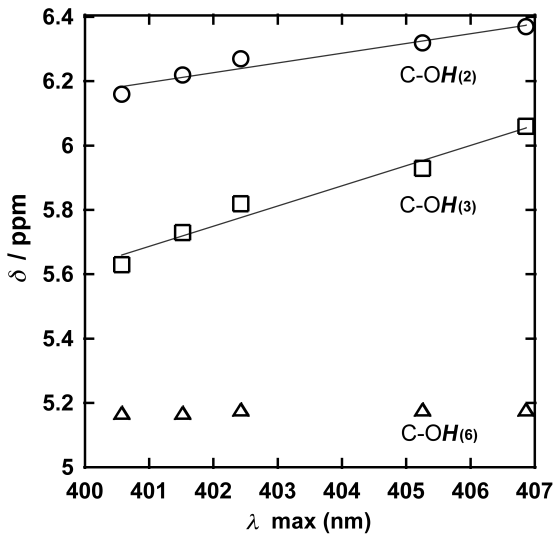


Figure 2-8 The relationship between λ_{max} of 4N in [C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 mixtures and chemical shifts of C-O***H***s in cellulose/[C_1mim][(MeO)(Me)PO_2]/DMSO-d_6 mixtures

2-6. Application of No-D NMR into Two-dimensional NMR

As a method for further investigation on the interaction of C-O***H***s and ILs, 2D NMR is an excellent candidate. 2D NMR is capable of analyzing compounds in view of interaction, conformation, spatial configuration and so forth. As a problem of 2D NMR, however, high sensitivity is essential for detection of correlation peaks. Therefore, even 2D NMR of glucose was only analyzed in deuterated ILs until now in the case of pure ILs.[9] As a popular 2D NMR, H,H-correlation spectroscopy (H,H-COSY) of 10 wt% glucose in $[C_1mim][(MeO)(Me)PO_2]$ was performed using No-D NMR and WET (Figure 2-9). Some correlation peaks for the coupling between C***H***s were detected due to the attenuation of the signals for $[C_1mim][(MeO)(Me)PO_2]$ by WET, though the signals of $[C_1mim][(MeO)(Me)PO_2]$ still remained. The structural identification was not completed because some correlation peaks were not observed between the anomeric proton and other C***H***s. However, no correlation peaks were observed in the case of usual H,H-COSY without No-D NMR and WET. Observation of the correlation peaks on 2D NMR spectra should contribute to investigating the profile of cellulose in ILs with 2D NMR measurements including NOESY.

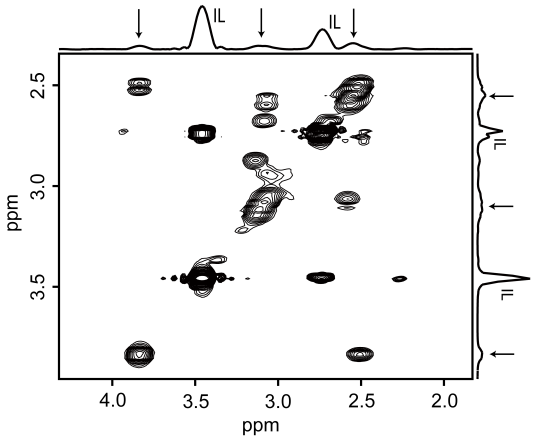


Figure 2-9 H,H-COSY spectrum of glucose in $[C_1mim][(MeO)(Me)PO_2]$ (number of scans: 60, temperature 60 °C). Arrows indicate the signals of C***H***s of glucose

2-7. Conclusion

^{1}H NMR spectroscopy of cellulose in a non-deuterated IL was performed with No-D NMR and WET. We successfully detected signals of cellulose dissolved in ILs which dissolve cellulose. Both signals of C***H***s and C-O***H***s were observed in non-deuterated ILs. From chemical shifts of the signals, hydrogen bonds between ILs and C-O***H***s were confirmed. Interaction between ILs and C-O$\boldsymbol{H}_{(6)}$ was confirmed to be the key step for dissolution of cellulose.

2-8. References

1. J. S. Moulthrop, R. P. Swatloski, G. Moyna and R. D. Rogers, *Chem. Commun.*, 2005, 1557-1559.
2. N. Jiang, Y. Q. Pu, R. Samuel and A. J. Ragauskas, *Green Chem.*, 2009, **11**, 1762-1766.
3. S. T. Handy and M. Okello, *J. Org. Chem.*, 2005, **70**, 1915-1918.
4. T. R. Hoye, B. M. Eklov, T. D. Ryba, M. Voloshin and L. J. Yao, *Org. Lett.*, 2004, **6**, 953-956.
5. T. R. Hoye, B. M. Eklov and M. Voloshin, *Org. Lett.*, 2004, **6**, 2567-2570.
6. X. Creary, E. D. Willis and M. Gagnon, *J. Am. Chem. Soc.*, 2005, **127**, 18114-18120.
7. T. Kondo, *J Polym Sci Pol Phys*, 1997, **35**, 717-723.
8. Y. Nishiyama, P. Langan and H. Chanzy, *J. Am. Chem. Soc.,* 2002, **124**, 9074-9082.
9. T. G. A. Youngs, J. D. Holbrey, C. L. Mullan, S. E. Norman, M. C. Lagunas, C. D'Agostino, M. D. Mantle, L. F. Gladden, D. T. Bowron and C. Hardacre, *Chem. Sci.*, 2011, **2**, 1594-1605.

Chapter 3

No-D NMR Analysis of Composition of Extracts from Wheat Bran with Ionic Liquids

3-1. Introduction

When polysaccharides are extracted with ILs, cellulose and hemicellulose should be extract selectively due to differences between these polysaccharides such as their physicochemical properties. However, many papers do not identify the components of polysaccharides; they report only entire weights of the extracts. There is a strong request on the convenient method to quantify these polysaccharides.

The composition of extracts from biomass using ILs has been analyzed via indirect methods.[1, 2] Unfortunately, this method involves complex pretreatments before analysis, including precipitation of polysaccharides with alcohol or water, washing, drying, and hydrolyzing the polysaccharides with sulfonic acid. The data obtained often contain considerable errors due to incomplete precipitation of all the extracts. The sum of the amount of precipitates and that of undissolved residues seldom reached 100 % of the untreated biomass.

In chapter 2, we have developed a ^{1}H NMR analysis of cellulose in ILs with No-D NMR and WET. These techniques enable detection of cellulose in variety of non-deuterated ILs. Comparing to previously reported studies on the NMR measurements with fully deuterated ILs (only few deuterated ILs were reported[3, 4]), these make direct NMR measurements of target materials in many ILs much easier. Thus, this method should be effective to analyze extracted cellulose and hemicellulose in ILs.

In this chapter, we selected three representative ILs for treatment of wheat bran and evaluated their abilities on extraction of cellulose and xylan (main hemicellulose of wheat bran).

3-2. Materials and Methods

Materials and Instruments

1-Methylimidazole was purchased from Kanto Chemical Co., Inc., and dried with KOH and distilled before use. Dimethyl methylphosphonate and dimethyl sulfate were purchased from Tokyo Chemical Ind. Co., Ltd. and distilled before use. Acetic acid and toluene were purchased from Kanto Chemical Co., Inc., and used as received. Microcrystalline cellulose powder (cellulose powder C) was purchased from Advantec Toyo Co. and used after drying under reduced pressure. Xylan was purchased from Tokyo Chemical Ind. Co., Ltd. It was dissolved in DMSO and precipitated by water. The precipitation was washed with excess amount of water and dried before use. 3-(Trimethylsilyl)-propanesulfonic acid sodium salt (TMS salt) was purchased from Merck KGaA and used as received. The amount of water of IL samples was confirmed by Karl Fischer coulometric titration (Kyoto Electronics; MKC-510N). Thermogravimetric analysis was performed on a SII TG/DTA 7200 (Seiko Instruments Inc.). ^{1}H- and ^{13}C NMR spectra for analysis of polysaccharides and confirmation of structures of ILs were performed with JEOL ECX 400 (JEOL Ltd.).

Preparation of ILs

$[C_1mim][(MeO)(Me)PO_2]$ was synthesised as described in chapter 2.

1,3-dimethylimidazolium acetate ($[C_1mim][MeCO_2]$) was synthesised as follows. Methyl iodide (30 g; 0.21 mol) and 1-methylimidazole (18 g; 0.22 mol) were added into 240 ml of tetrahydrofuran under an argon gas atmosphere at 0 ^{o}C. The reaction mixture was stirred at room temperature for 24h. The resulting solid was washed with excess of dehydrated diethyl ether and dried *in vacuo*. Iodide anion was converted into hydroxide by passing an aqueous

solution of the iodide salt through a column filled with anion exchange resin (Amberlite IRN 78A). This aqueous hydroxide solution was then neutralized with small excess of acetic acid, and weak base anion exchange resin (Amberlite IRA 67) was added and stirred for 48h for removing excess amount of acid. After filtration, resulting liquid was dried *in vacuo* at 80 °C. ^{1}H NMR (400 MHz; DMSO-d_6; Me_4Si) d = 1.61 (3H, s, CH_3CO), 3.89 (6H, s, NCH_3), 7.84 (2H, d, J = 1.6 Hz, NCHCHN), 10.02 (1H, s, NCHN). ^{13}C NMR (100 MHz; DMSO-d_6; Me_4Si) d = 28.80 (CH_3CO), 38.12 (NCH_3), 51.18 (POCH_3), 126.14 (NCHCHN), 141.00 (NCHN), 176.25 (CH_3CO).

1,3-dimethylimidazolium methylsulfonate ([C_1mim][$MeOSO_3$]) was synthesised as follows. Dimethyl sulfate was washed with 3.6 % Na_2CO_3 aq and dried with excess of $CaCl_2$. After distillation, dimethyl sulfate (10 g; 0.12 mol) and 1-methylimidazole (16.1 g; 0.13 mol) were added into 60 ml of tetrahydrofuran under an argon gas atmosphere at room temperature. The reaction mixture was stirred at 70 °C for 24h. The resulting liquid was dried *in vacuo* and washed repeatedly with excess dehydrated diethyl ether. The residual liquid was dissolved in dichloromethane, and the resulting solution was passed through a column filled with neutral activated alumina. After removal of dichloromethane, the residual liquid was dried *in vacuo* at 80 °C. ^{1}H NMR (400 MHz; DMSO-d_6; Me_4Si) d =3.40 (3H, s, SOCH_3), 3.86 (6H, s, NCH_3), 7.69 (2H, d, J = 1.6 Hz, NCHCHN), 9.04 (1H, s, NCHN). ^{13}C NMR (100 MHz; DMSO-d_6; Me_4Si) d =36.15 (NCH_3), 53.44 (SOCH_3), 123.95 (NCHCHN), 137.63 (NCHN).

Measurement of Kamlet-Taft parameters of ILs

Measurement of the Kamlet-Taft parameters of a series of ILs was carried out as follows. The solvatochromic dyes, (2,6-dichloro-4-(2,4,6-triphenyl-1-pyridinio)phenolate (Reichardt's dye #33, from Fluka), 4-nitroaniline (from Tokyo Chemical Industries Co.,

Ltd), and *N*,*N*-diethyl-4-nitroaniline (from Kanto Chemical Co., Inc.), were used as received. The dyes were added to 0.25 g of ILs as concentrated methanol solutions. The methanol was then carefully removed by vacuum drying. These IL solutions were placed into quartz cells with 1 mm light-path length. In the case of $[C_1mim][MeCO_2]$, temperature of the quartz sample cell was maintained at 60 °C by water circulation because it was solid under 60 °C. From the wavelength at the maximum absorption (λ_{max}) determined, the α, β and π^* values were calculated by use of the following equations:

$$\nu_{(dye)} = 1/(\lambda_{max(dye)}10^{-4})$$

$$E_T(30) = 0.9986\,(28\,592/\lambda_{max\ (Reichardt's\ dye\ \#33)}) - 8.6878$$

$$\pi^* = 0.314(27.52 - \nu_{(N,N\text{-diethyl-4-nitroaniline})})$$

$$\alpha = 0.0649E_T(30) - 2.03 - 0.72\pi^*$$

$$\beta = (1.035\nu_{(N,N\text{-diethyl-4-nitroaniline})} + 2.64 - \nu_{(4\text{-nitroaniline})})/2.80$$

Analysis of mixtures composed of cellulose and xylan by ^{1}H NMR

TMS salt was added into 20 g of DMSO to be final concentration of 0.5 wt%. A 0.70 g aliquot of the resulting DMSO/TMS salt solution and 0.30 g of ILs were mixed and stirred until the solutions became homogenous. Cellulose and xylan were added into the solutions and the resulting solutions were stirred until the solutions became homogeneous and clear. The samples were transferred to 5 mm NMR tubes. The sample tubes were capped with plastic lids, then the tops were wrapped in parafilm. The samples were analyzed at 100 °C with 12 scans. WET method is described in chapter 2.

Analysis of extracts from wheat bran with ILs by ^{1}H NMR

Wheat bran (70 mg, 42 - 50 mesh size) was dried under reduced pressure before use (the water content was 4.5 wt%, so that the final water concentration of IL/bran solutions was about 0.51 wt%). The dried bran (70 mg) was added into 1.0 g of dried ILs and stirred at 200 rpm in an oil bath. The resulting solutions were centrifuged at 14,800 rpm (16200 G) from 10 to 60 min for removing residue. The supernatants were mixed with 70 wt% of DMSO/TMS salt solutions mentioned above and the resulting solutions were stirred at 80 °C for 3min. After filtration with glass filter under reduced pressure, the samples were transferred to 5 mm NMR tubes after filtration. The sample tubes were capped with plastic lids, then the tops were wrapped in parafilm. The samples were analyzed with ^{1}H NMR at 100 °C with 240 scans. Yield of polysaccharides was calculated from the following eqn (1)

$$\text{yield (\%)} = \frac{weight\ of\ polysaccharides\ from\ NMR\ (mg)}{70\ (mg)} \times 100 \qquad (1)$$

When we extracted polysaccharides from IL-treated bran, the IL/bran solution after first extraction (extraction temperature: 80 °C, extraction time: 2h, feed bran: 70 mg, [C1mim][(MeO)(Me)PO2]: 1.0 g, stirring: 200 rpm) was centrifuged and the precipitation was collected. The precipitation was dispersed into 10 ml of DMSO and mixed with vortex mixer for 1min, to strip any dissolved substances adsorbed or trapped within the solid texture. The solution was centrifuged (16200 G) and the supernatant was removed. For further washing, 30 ml of methanol was added and the solution was mixed with vortex mixer for 1min. The solution was centrifuged (16200 G) and the supernatant was removed. Washing process with methanol was repeated 2 times. After drying under reduced pressure, the IL-treated bran was added into 1.0 g of fresh [C_1mim][(MeO)(Me)PO_2] and stirred (80 °C, 2h, 200

rpm). Bran treated with $[C_1mim][(MeO)(Me)PO_2]$ twice was obtained by repeating this process once more.

According to selective extraction, xylan fraction of extracts was calculated from the following equation:

$$\text{xylan fraction (\%)} = \frac{\text{xylan extracted (mol)}}{\text{cellulose extracted (mol)+xylan extracted (mol)}} \times 100 \quad (2)$$

^{13}C NMR analysis was performed at 100 ^oC with 5000 scans with the same sample we used for ^1H NMR analysis.

Measurement of weight of extracted polysaccharides through re-precipitation

We filtrated extract/$[C_1mim][(MeO)(Me)PO_2]$ sample (80 ^oC, 2h) with a glass filter. The solution was stirred with excess methanol to re-precipitate dissolving materials. The re-precipitated solid was washed with methanol repeatedly to remove residual ILs. The solid was collected by filtration and dried under reduced pressure. After these pretreatments, the resulting solid was weighed. Considering loss of sample during treatments, we calibrated the weight of polysaccharides as in 1.0 g of ILs.

Hydrolysis of cellulose included in the IL-treated bran and detection of generated glucose

Hydrolysis

The bran treated with $[C_1mim][(MeO)(Me)PO_2]$ twice (3.0 mg) and 10 mg of cellulase (celluclast, from Novozyme) were added into 0.40 ml of 100 mM acetate buffer (pH 5.0). The sample was stirred at 60 ^oC for 3h and the supernatant was obtained after centrifugation. Cellulase was used after purification with ultrafiltration.

Detection of glucose

Glucose oxidase (Toyobo Co., Ltd.) and DCIP (Wako Pure Chemical Industries, Ltd.) were dissolved into 100 mM acetate buffer (pH 6.0) to be 10mg/ml and 1 mM, respectively. We mixed 120 µl of the hydrolyzed sample, 20 µl of the glucose oxidase solution, and 100 µl of the DCIP solution and stirred the sample at room temperature. Absorbance of the resulting solution at 600 nm was measured with UV-*vis* spectrometry using quartz cells with 1 cm light-path length. As a control, a sample with 120 µl of acetate buffer as an alternative of the hydrolyzed sample solution was measured and compared.

3-3. Evaluation of the usual method: effect of ash on apparent weight of the extracted materials

A proportion of inorganic material is also expected to be dissolved during treatment with polar ILs. To determine the amount, we undertook a thermogravimetric analysis. Wheat bran (70 mg) was added to 1.0 g of dried $[C_1mim][(MeO)(Me)PO_2]$ and stirred at 80 °C for 2h. After centrifuging and separation, excess methanol was added to the supernatant so as to re-precipitate polysaccharides. The precipitate was washed repeatedly with methanol and dried *in vacuo* in order to collect re-precipitated solids. The weight of the re-precipitated solid was 25 mg, with a degree of extraction of 36 wt%. Thermogravimetric analysis was performed at a heating rate of 10 °C/min (Figure 3-1). Upon heating to 500 °C, about 45 wt% of solid materials remained intact. Cellulose and xylan were both fully decomposed up to 500 °C, suggesting that the undecomposed material was inorganic. This finding indicates that weighing re-precipitated solid is not recommended when studying extracted polysaccharides.

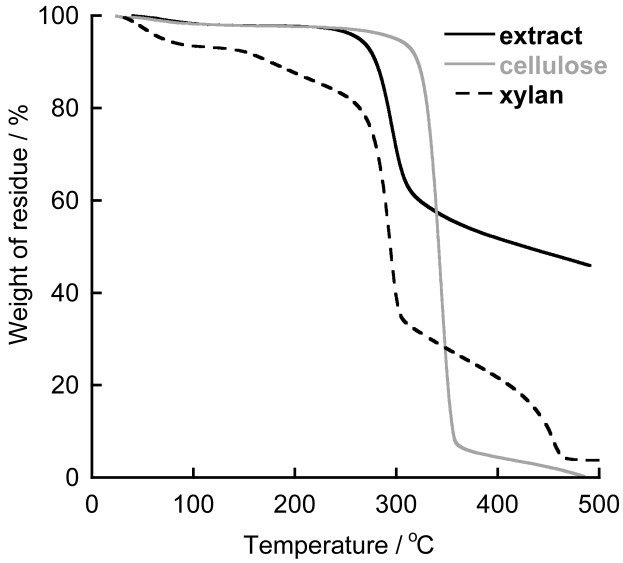


Figure 3-1 TGA curves of the extract from wheat bran with $[C_1mim][(MeO)(Me)PO_2]$, cellulose, and xylan.

For further analysis of the main components of the inorganic material, the sample was washed with water. An excess of milli-Q water was added to the dried extract, and the sample was stirred for 48 h. The resulting solution was filtered and the residue was dried and weighed. During this process 38 wt% of the re-precipitated solid was washed out, suggesting that these components were water-soluble inorganic salts (84 wt% of inorganic materials). The rest is believed to be mainly SiO_2.

3-4. Measurement of Mixtures of Cellulose and Xylan

As a preliminary study, cellulose and xylan were individually analyzed by ^{1}H NMR. As their solvents, we prepared three different ILs, composed of the 1,3-dimethylimidazolium cation and the [(MeO)(Me)PO_2] or [$MeCO_2$] or [$MeOSO_3$] anion. The structure of these is shown in Figure 3-2. [C_1mim][(MeO)(Me)PO_2] and [C_1mim][$MeCO_2$] both dissolve cellulose and xylan, because of their high hydrogen bond basicity (large β value in Table 3-1). [C_1mim][$MeOSO_3$] dissolves only xylan, because of its relatively low hydrogen bond basicity (β = 0.61 in Table 3-1). Since these ILs are highly viscous, DMSO was added to the IL solutions to facilitate the NMR measurements. In [C_1mim][(MeO)(Me)PO_2], proton signals from cellulose were observed at 3.0 - 3.1, 3.6 - 3.7, and 4.45 - 4.55 ppm. Proton signals from xylan were observed at 3.0 - 3.1, 3.5 - 3.6, and 4.3 - 4.4 ppm. These data suggest that signals from cellulose and xylan detected around 4.4 ppm should be observed even in arbitrary mixtures (Figure 3-3a, top and bottom). These signals are assigned to protons at the 1-position of cellulose and xylan (as shown in Figure 3-4), because the ^{1}H signal at the 1-position of cello-oligosaccharides is detected at almost the same chemical shift in conventional polar solvents.[5, 6] In the case of [C_1mim][$MeCO_2$], proton signals were observed at almost identical chemical shifts (Figure 3-4b, top and bottom). In [C_1mim][$MeOSO_3$], the proton signal of xylan was also detected at around 4.3 ppm (Figure 3-4c bottom). It was confirmed that the peaks were observed at almost the same chemical shifts in these three ILs in spite of their different polarities. It is known that ILs interact strongly with hydroxyl groups[5] rather than backbone protons and proton signals of hydroxyl groups were found to shift to their lower magnetic field side in the ILs.[7]

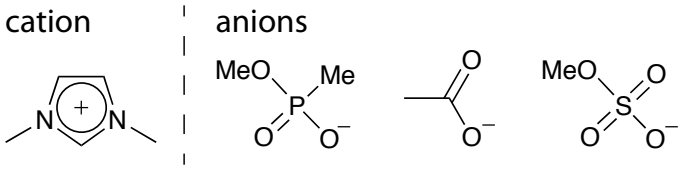


Figure 3-2 Structures of ILs used in this study.

Table 3-1 Kamlet-Taft parameters of ILs.

ILs	α	β	π^*
$[C_1mim][(MeO)(Me)PO_2]$	0.53	1.10	1.05
$[C_1mim][MeCO_2]$	0.54[a]	1.08[a]	1.07[a]
$[C_1mim][MeOSO_3]$	0.56	0.61	1.12

[a] measured at 60 °C.

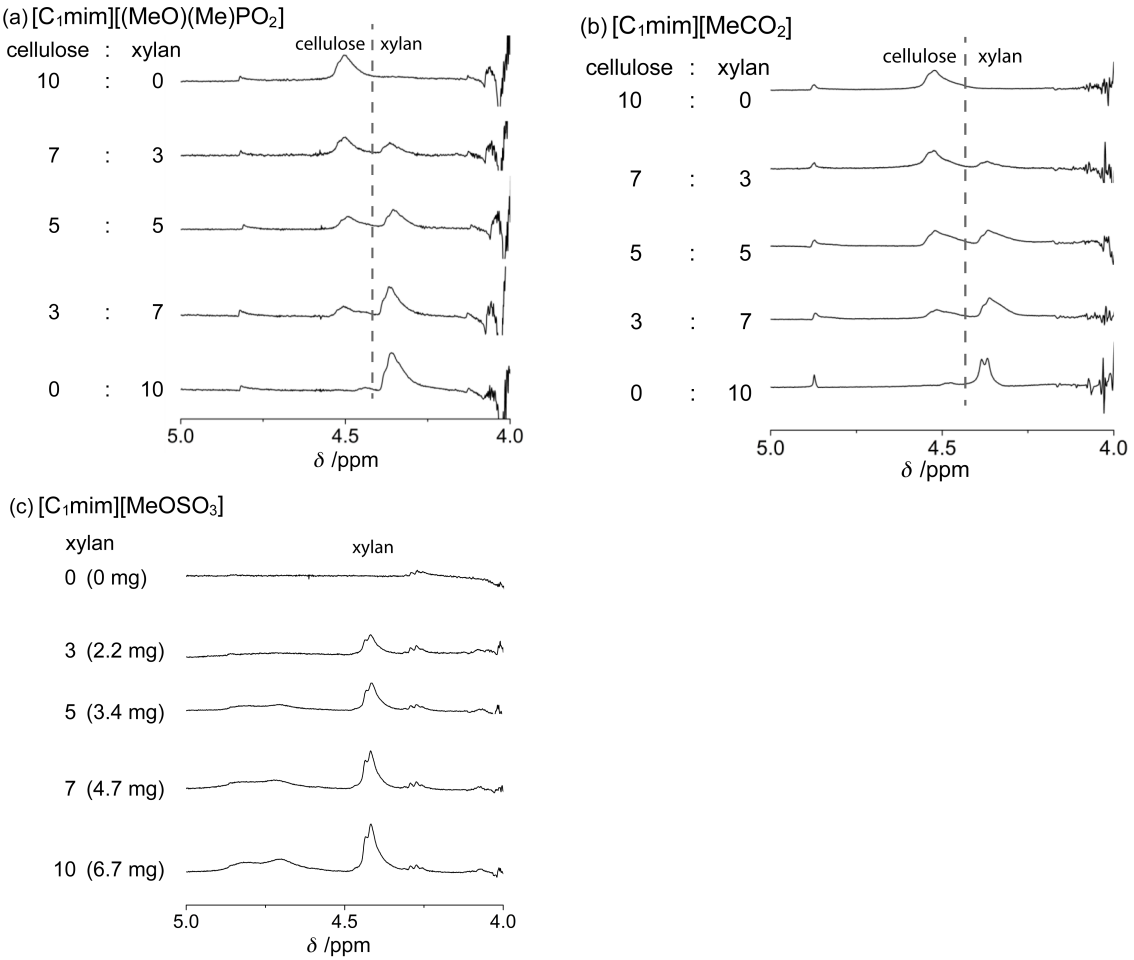


Figure 3-3 ^{1}H NMR spectra of cellulose/xylan mixtures (0:10 ~ 10:0 by molar ratio) in ILs.

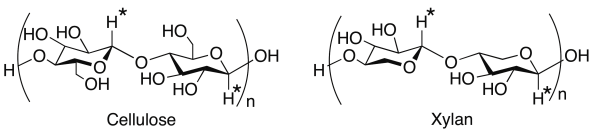


Figure 3-4 Structure of cellulose and xylan. *1-position

Acting as models of extracted major polysaccharides, mixtures of cellulose and xylan were dissolved in [C_1mim][(MeO)(Me)PO_2], and their NMR signals were detected (Figure 3-3a). The signals for cellulose and xylan were observed independently at around 4.3 and around 4.5 ppm respectively, as expected. The peak area increased

with increasing fraction of each polysaccharide. In $[C_1mim][MeCO_2]$ the signals (at around 4.4 ppm) partly overlapped, but the components could be distinguished (Figure 3-3b). In $[C_1mim][MeOSO_3]$ the signal for xylan was detected clearly at around 4.3 ppm (Figure 3-3c). The peak area changed with the sample concentration in both $[C_1mim][MeCO_2]$ and $[C_1mim][MeOSO_3]$. Signals from both cellulose and xylan, which have very similar chemical structures, were resolved separately regardless of IL species.

Figure 3-5 shows the relation observed between cellulose fraction and relative peak area. In the case of NMR, the peak area in different spectra cannot be compared directly without a common standard. We therefore used TMS salt as a standard. The peak area of polysaccharides was calculated relative to the TMS salt signal. Figure 3-5 reveals a linear relation between the relative peak area of polysaccharides and the cellulose fraction of the mixed samples in $[C_1mim][(MeO)(Me)PO_2]$. Therefore, it was possible to determine the proportions of cellulose and xylan in the mixed samples. As the signals from cellulose and xylan in $[C_1mim][MeCO_2]$ partly overlap, we analyzed deconvolution of the spectra as Lorentzian line shapes using grams/386 ver. 3.04 (Galactic Industrial Corporation). The relation between the cellulose fraction in a cellulose/xylan mixture and the peak area of cellulose and xylan in $[C_1mim][MeCO_2]$ and $[C_1mim][MeOSO_3]$ also proved to be linear, with a value of R^2 of more than 0.99. It is therefore possible to quantify the amounts of cellulose and xylan dissolved in various ILs using ^1H NMR.

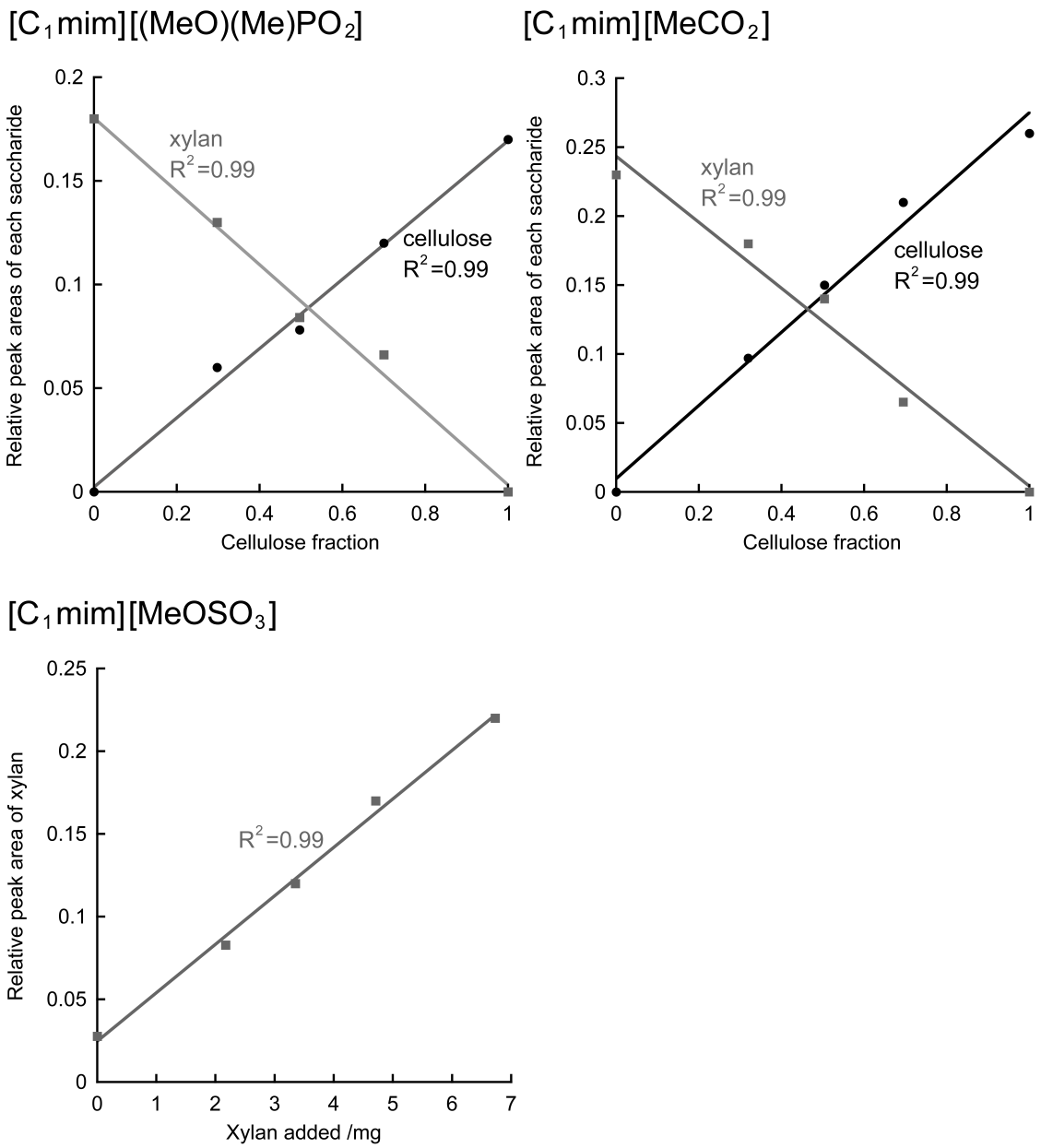


Figure 3-5 Relation between cellulose fraction of the mixed sample and relative peak area of xylan and cellulose dissolved in $[C_1mim][(MeO)(Me)PO_2]$, $[C_1mim][MeCO_2]$, and $[C_1mim][MeOSO_3]$.

Based on these results, we analyzed the components extracted from biomass. Figure 3-6 shows ^1H NMR spectra of the component of bran extracted with ILs. Polysaccharides were extracted from bran

using these ILs at 80 °C for 2h. As expected, signals of cellulose and xylan were both detected in each IL, $[C_1mim][(MeO)(Me)PO_2]$ and $[C_1mim][MeCO_2]$, between 4.2 and 4.7 ppm. Only the xylan signal was detected in the treated solution of $[C_1mim][MeOSO_3]$, at 4.4 ppm. Furthermore, lignin was not detected in these spectra, whereas lignin is observed at 6 - 8 ppm in polar IL solutions.[22]

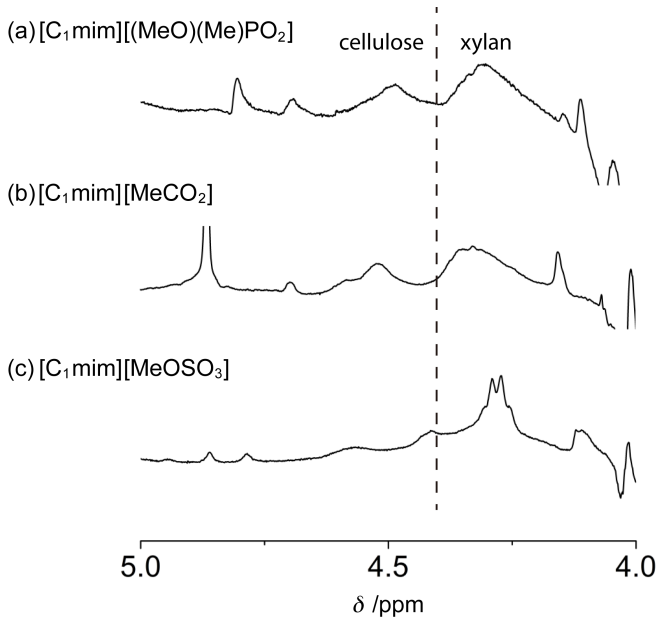


Figure 3-6 ^{1}H NMR spectra of extracts from bran treated with ILs.

^{13}C NMR analysis of cellulose in ILs has been reported by Rogers *et al.*[8] It is also a potential candidate for analysis of polysaccharides extracted by ILs. Then the extracted sample at 80 °C for 2h with $[C_1mim][(MeO)(Me)PO_2]$ (the same sample shown in Figure 3-6a) was analyzed by ^{13}C NMR at 100 °C with the accumulation of 5000 scans (about 4h), and it is shown in Figure 3-7. No signals of polysaccharides were detected in the spectrum while it is known that cellulose shows 6 signals from 60 to 110 ppm in polar ILs. This result was caused by lower sensitivity of ^{13}C NMR compared to that of ^{1}H NMR (about 1/100). In addition, concentration of polysaccharides of

this sample (1.1 wt%) was lower than that Rogers *et al.* used (5 wt%). We think that signals can be observed with a great number of accumulations. However, ^{1}H NMR surely has advantage in this measurement because quantification needs high signal to noise ratio.

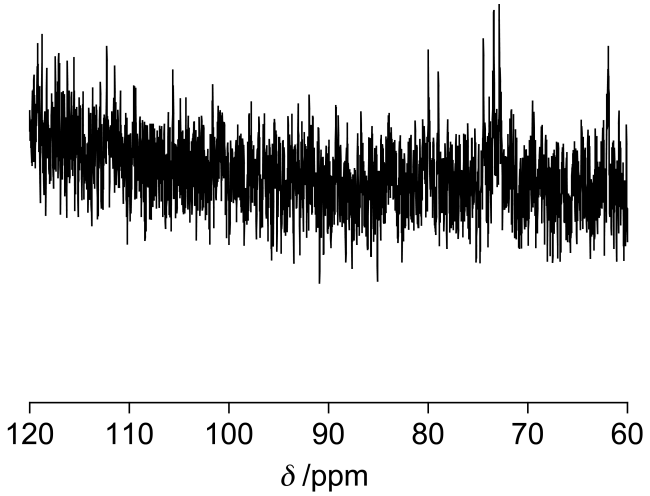


Figure 3-7 ^{13}C NMR spectrum of the extract from bran with $[C_1mim][(MeO)(Me)PO_2]$.

3-5. Quantification of Polysaccharides in Extracts

Figure 3-8 shows the amount and yield of cellulose and xylan extracted from wheat bran using three different ILs. We have quantified the amount of extracted polysaccharides using the standard relations shown in Figures 3-5. Polysaccharide was extracted from wheat bran, with 16.1% yield when bran was immersed in $[C_1mim][(MeO)(Me)PO_2]$ at 80 °C for 2h. The weights of cellulose and xylan extracted with $[C_1mim][(MeO)(Me)PO_2]$ were 4.2 and 7.1 mg, respectively. To consider this value further (11.3 mg; 4.2 + 7.1 mg), we compared it to the weight of re-precipitated solid from the IL solution used for extraction. As mentioned above, the re-precipitated solid was 25 mg, and its organic compound fraction was about 50 % (12.5 mg) from thermogravimetric analysis (see Figure 3-1). Thus, the values obtained (11.3 mg and 12.5 mg) were reasonably similar. The difference between the values can be explained by the presence of other organic compounds.

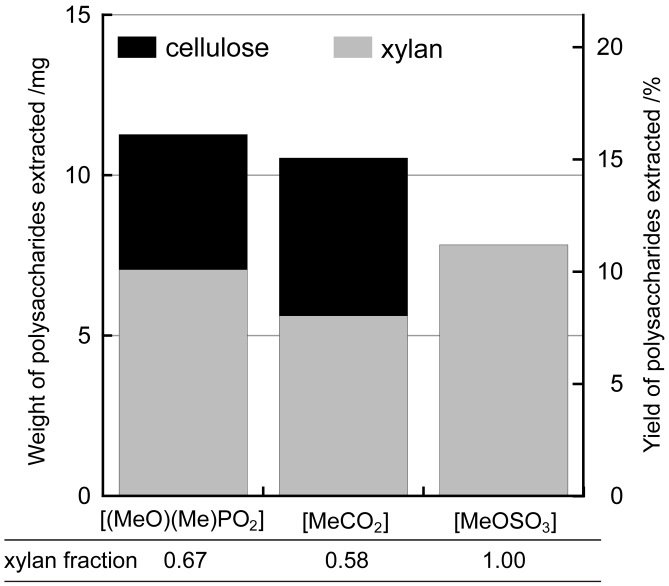


Figure 3-8 Weight and yield of the extracted cellulose and xylan with ILs at 80 °C for 2h.

The weights of cellulose and xylan extracted with $[C_1mim][MeCO_2]$ were 4.0 and 9.8 mg, respectively. The xylan fraction was 0.67 and 0.75 for $[C_1mim][(MeO)(Me)PO_2]$ and $[C_1mim][MeCO_2]$, respectively. $[C_1mim][MeOSO_3]$ extracted xylan selectively; its extraction ability was somewhat greater than that of $[C_1mim][(MeO)(Me)PO_2]$. These three ILs have almost the same α value (see Table 3-1), but β is lower for $[C_1mim][MeOSO_3]$ than for the other two ILs (Table 3-1). Dissolution of cellulose depends strongly on the β value, but the optimal properties of the ILs for dissolving xylan are not known.

We sought to extract further cellulose from the IL-treated bran, by adding fresh $[C_1mim][(MeO)(Me)PO_2]$ to it. Figure 3-9 shows the amount of extracted polysaccharides from bran (1st extraction) and from IL-treated bran (2nd extraction). Both extractions were undertaken at 80 °C for 2h with fresh $[C_1mim][(MeO)(Me)PO_2]$. To our surprise, almost no polysaccharides were extracted from IL-treated bran.

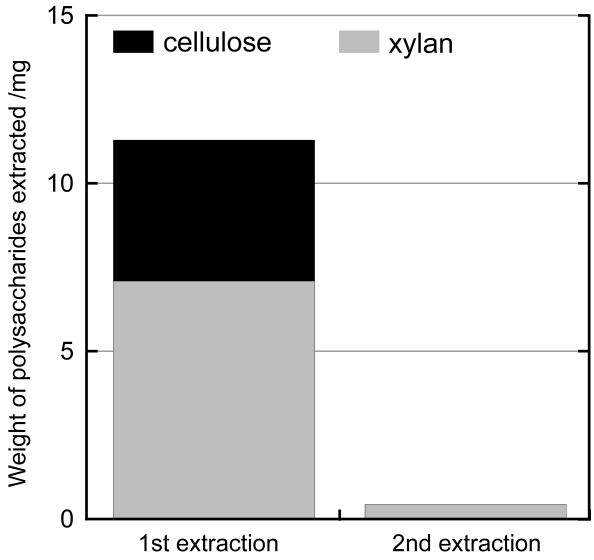


Figure 3-9 Weight of extracted polysaccharides from 70 mg of bran at 80 °C for 2h (1st extraction) and that from the IL-treated bran at 80 °C for 2h (2nd extraction) with $[C_1mim][(MeO)(Me)PO_2]$, calculated from NMR signals.

To confirm that there is cellulose in the bran after 2nd extraction, it was analyzed by thermogravimetric analysis (Figure 3-10). After heating up to 500 °C, about 80 % was lost. This result indicates that the IL-treated bran presumably contains polysaccharides whereas only little amount of polysaccharides was extracted in 2nd extraction. To confirm it more precisely, we performed hydrolysis of cellulose included in the bran treated with $[C_1mim][(MeO)(Me)PO_2]$ twice, and then generated glucose was detected by glucose oxidase (Table 3-2). The IL-treated bran was hydrolyzed with cellulase and the resulting solution was added into solution of 2,6-dichlorophenol-indophenol sodium salt (DCIP) and glucose oxidase. Absorbance of DCIP decreased by addition of the hydrolyzed sample because electron

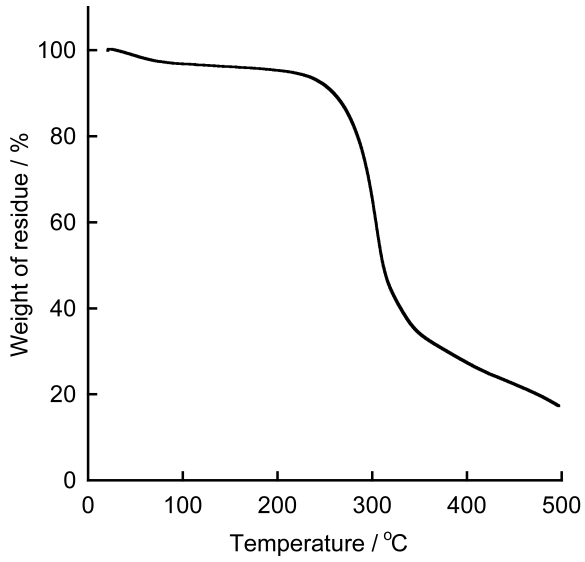


Figure 3-10 A TGA curve of the bran treated with $[C_1mim][(MeO)(Me)PO_2]$ twice.

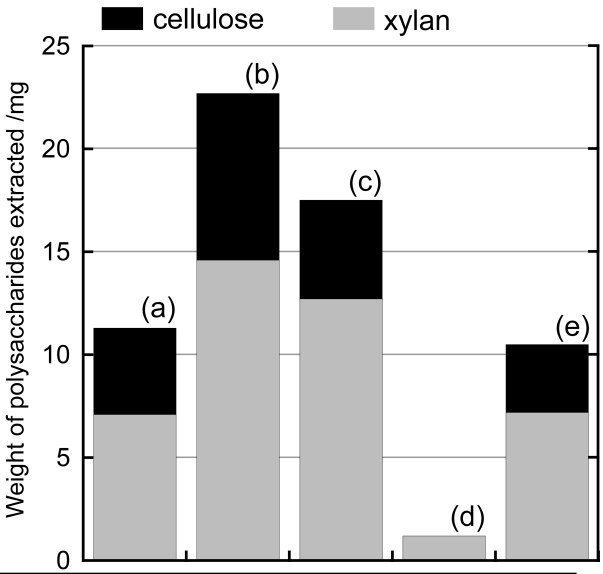
Table 3-2 Absorbance of 2,6-dichlorophenolindophenol sodium salt at 600 nm.

Sample	Absorbance at 600 nm
Control (buffer)	0.72
Hydrolyzed sample (hydrolyzed bran after treatment with $[C_1mim][(MeO)(Me)PO_2]$ twice)	0.32

was transferred from glucose to DCIP. It clearly shows that cellulose was included in the IL-treated bran. These results show that extraction of polysaccharides is limited despite existence of polysaccharides. This clearly claims that about yield of 6 and 10 wt% concerning cellulose and xylan is upper limit of extraction at this condition. In addition, it is shown that this limit is not attributed to solubility of polysaccharides or the increase of viscosity caused by dissolution of polysaccharides.

To investigate the extraction-limit further, we varied the amount of feed bran and the temperature (Figure 3-11). When we used double the quantity of bran (12.3 wt%), the amount of cellulose and xylan extracted also doubled (Figure 3-11b) despite a considerable increase in viscosity. This result also supports that the limit is not caused by solubility of polysaccharides nor increase of viscosity. The treatment temperature is known to affect the amount of polysaccharides extracted from bran or other biomass. As shown in Figure 3-11c, more was extracted at higher temperature (120 ^{o}C). In detail, more xylan was obtained (7.1 to 12.7 mg), but the amount of cellulose extracted did not increase. This increase in the amount of xylan extracted can be explained by the effect of lignin-carbohydrate complexes (LCCs). LCCs are composed of covalently bound hemicellulose and lignin, and they are known to suppress the solubilization of plant biomass.[9] Lignin partially decomposes[10, 11] and liquefies[12] at high temperature in polar ILs. The increase in xylan extracted is therefore attributable to partial decomposition and liquefaction of LCC. Furthermore, xylan was extracted at room temperature (1.2 mg, see Figure 3-11d). The xylan extracted was considered not to form LCCs at room temperature. We infer that the extra xylan extracted at 80 ^{o}C and 120 ^{o}C (difference between c and a) forms LCCs, but the xylan extracted at 25 ^{o}C does not. It is not easy to conclude the different extraction degree at 80 ^{o}C and 25 ^{o}C to be

partial decomposition of LCC at 80 °C. As for the effect of the duration of extraction, the amount of polysaccharides extracted in 30min and 2h was almost the same (see Figure 3-11, a and e). This shows that almost all polysaccharides extractable at 80 °C were extracted within 30min. In other words, no LCC was strongly suggested to be decomposed at 80 °C.



	(a)	(b)	(c)	(d)	(e)
temp (°C)	80	80	120	25	80
time (min)	120	120	120	120	30
bran (mg)	70	140	70	70	70
xylan fraction	0.67	0.69	0.77	1.00	0.73

Figure 3-11 Weight of extracted polysaccharides at various conditions using $[C_1mim][(MeO)(Me)PO_2]$, calculated from NMR signals.

The composition of extracts in ILs was easily obtained using ^1H NMR with No-D NMR and the WET technique. The results obtained will lead to real-time monitoring of extraction with polar ILs.

3-7. Conclusion

We analyzed performance of ILs on extraction from wheat bran using ^{1}H NMR. Both No-D NMR and WET technique were used for direct detection of extracted polysaccharides with non-deuterated ILs. This enables rapid analysis without troublesome pretreatments and accordingly reduces experimental errors. In addition, this analysis was not affected by the presence of ash in crude samples. The effect of LCCs on extraction with ILs and its temperature-dependence were confirmed.

3-8. References

1. C. Froschauer, M. Hummel, M. Iakovlev, A. Roselli, H. Schottenberger and H. Sixta, *Biomacromolecules*, 2013, **14**, 1741-1750.
2. Z. Qiu, G. M. Aita and M. S. Walker, *Bioresour. Technol.*, 2012, **117**, 251-256.
3. N. Jiang, Y. Q. Pu, R. Samuel and A. J. Ragauskas, *Green Chem.*, 2009, **11**, 1762-1766.
4. C. Hardacre, S. E. J. McMath and J. D. Holbrey, *Chem. Commun.*, 2001, 367-368.
5. J. Zhang, H. Zhang, J. Wu, J. Zhang, J. He and J. Xiang, *Phys. Chem. Chem. Phys.*, 2010, **12**, 1941-1947.
6. A. Isogai, *Cellulose*, 1997, **4**, 99-107.
7. K. Kuroda, H. Kunimura, Y. Fukaya and H. Ohno, *Cellulose*, 2014, **21**, 2199-2206.
8. J. S. Moulthrop, R. P. Swatloski, G. Moyna and R. D. Rogers, *Chem. Commun.*, 2005, 1557-1559.
9. T. Leskinen, A. W. T. King, I. Kilpeläinen and D. S. Argyropoulos, *Ind. Eng. Chem. Res.*, 2013, **52**, 3958-3966.
10. J. Y. Kim, E. J. Shin, I. Y. Eom, K. Won, Y. H. Kim, D. Choi, I. G. Choi and J. W. Choi, *Bioresour. Technol.*, 2011, **102**, 9020-9025.
11. J.-L. Wen, T.-Q. Yuan, S.-L. Sun, F. Xu and R.-C. Sun, *Green Chem.*, 2014, **16**, 181-190.
12. W. Y. Li, N. Sun, B. Stoner, X. Y. Jiang, X. M. Lu and R. D. Rogers, *Green Chem.*, 2011, **13**, 2038-2047.

Chapter 4

HPILC Analysis of Cellulose to Evaluate Extracts from Biomass with Ionic Liquids

4-1. Introduction

High performance liquid chromatography (HPLC) has some modes such as reverse phase chromatography and ion exclusion chromatography. We focused on size exclusion chromatography (same as gel permeation chromatography) to analyze molecular weight distribution (MWD) of cellulose. From a viewpoint of cellulose-dissolving ability of polar ILs, they are suitable for the eluents but they have not been applied until now due to their extremely high viscosity. For example, [C_4mim]Cl are 11000 cP (at 30 ^{o}C, under supercooling condition) and [Amim]Cl are 2090 cP at 25 ^{o}C. Then in this chapter, we searched less viscous polar ILs and developed HPLC with polar ILs as eluents (HPILC).

Furthermore, I think that HPILC has a potential to analyze MWD of extracted polysaccharides from biomass with ILs. Plant biomass is mainly composed of cellulose and hemicellulose and lignin and it is known that polar ILs can extract them.[1-3] However, relation between MWD (MWD) of extracts and extraction conditions has not reported whereas MWD affects physico-chemical properties of polymers. There is a strong request on the reliable method to analyze MWD of polysaccharides.

4-2. Materials and Methods

Materials and Instruments

1-Ethylimidazole was purchased from Kanto Chemical Co. and used after drying over KOH and distillation. Dimethyl phosphite was purchased from Tokyo Chemical Ind. Co. and was used after distillation. The amounts of water of IL samples were confirmed by Karl Fischer coulometric titration (Kyoto Electronics; MKC-510N). ^{1}H- and ^{13}C NMR spectra for analysis of polysaccharides and confirmation of structures of ILs were performed with JEOL ECX 400 (JEOL Ltd.).

Synthesis of [C_2mim][(MeO)(H)PO_2]

1-Ethylimidazole (100g, 1.04 mol) and dimethyl phosphite (126g, 1.14 mol) were slowly mixed under an argon gas atmosphere at room temperature without solvent. The reaction mixture was stirred at 80 ^{o}C for 24h. The resulting liquid was washed repeatedly with excess dehydrated diethyl ether. The residual liquid was dissolved in dichloromethane, and the resulting solution was passed through a column filled with neutral activated alumina. After removal of dichloromethane, the residual liquid was dried *in vacuo* at 80 ^{o}C for 24h to give [C_2mim][(MeO)(H)PO_2] as a colourless liquid.

Water content of [C_2mim][(MeO)(H)PO_2] was measured with Karl Fischer Coulometric Titrator (Kyoto Electronics; MKC-510N). The IL with water content of less than 2000 ppm was used as both eluent and solvent. Structure of [C_2mim][(MeO)(H)PO_2] was confirmed by ^{1}H- and ^{13}C- NMR spectra (JEOL ECX-400). ^{1}H-NMR d_H (400 MHz; $CDCl_3$; Me_4Si); 1.58 (3H, t, J = 7.3 Hz, NCH_2CH_3), 3.55 (3H, d, J = 11.9 Hz, $POCH_3$), 4.06 (3H, s, NCH_3), 4.36 (2H, q, J = 7.3 Hz, NCH_2CH_3), 6.92 (1H, d, J = 588.5 Hz, PH), 7.58 (2H, d, J = 11.3 Hz, NCHCHN), 10.66 (1H, s, NCHN). ^{13}C-NMR d_c (100 MHz; $CDCl_3$; Me_4Si); 15.22 (NCH_2CH_3), 35.87 (NCH_3), 44.98 (NCH_2CH_3), 50.05

(PO*C*H$_3$), 121.35 (NCH*C*HN), 123.17 (N*C*HCHN), 138.40 (N*C*HN).

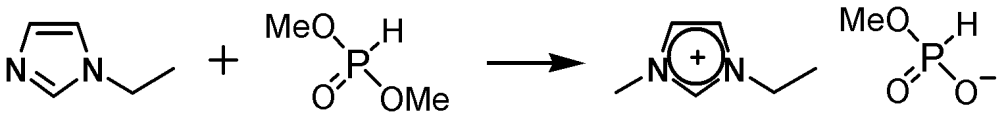


Figure 4-1 Synthesis of [C_2mim][(MeO)(H)PO_2]

Methods

HPLC setup

Components in the HPLC system used were high pressure durable pump (KHP-011; SIC), an injector (7725; Rheodyne) with a 5 mL loop, a UV-vis detector (only used for analysis of extracts from biomass, SPD-20AV; Shimadzu), and a refractive index detector (Shodex RI-71; Showa Denko). Columns filled with silica gel (Shodex KW-402.5-4F, 4.6 mm (inner diameter) × 300 mm, 3 mm, and KW-405-4B, 4.6 mm (inner diameter) × 50 mm, 5 mm; Showa Denko) were used in tandem. The pump and the columns were heated at 55 °C using a ribbon heater and a column oven (CTO-10Avp; Shimadzu). The RI detector cells were maintained at 40 °C. The flow rate was set at 0.01 mL·min^{-1}. Pullulan standards were used for calibration of the SEC system. For data acquisition and processing we used the software package SIC-480 II XP (SIC). [C_2mim][(MeO)(H)PO_2] with water less than 2000 ppm was used as an eluent under an argon atmosphere.

UV-vis spectroscopy of lignin solution

Lignin/[C_2mim][(MeO)(H)PO_2] solutions (0.005 wt%) were placed into quartz cells with 1 mm light-path length. Absorbance of the sample solution was measured with wavelength from 800 to 200 nm using UV-*vis* spectrophotometer (UV-2450; Shimadzu) at room temperature.

HPILC measurement of pullulan, cellulose and lignin

Suspensions of pullulan, cellulose or lignin (1.0 mg each) in 200 mg of

dried $[C_2mim][(MeO)(H)PO_2]$ were prepared under dry nitrogen gas atmosphere. The mixtures were gently stirred at room temperature until the solutions became homogeneous and clear. The solutions were directly injected into HPILC and measured.

Analysis of extracts from biomass with ILs by HPILC

The milled biomass from different sources were used; wheat bran (herbaceous plant, 42-50 mesh), pine (softwood, *Picea jezoensis*, 36-200 mesh), and beech (hardwood, *Quercus crispula*, 36-200 mesh) without defatting. Detailed procedure according to *Prunus* × *yedoensis* was described below. Biomass was dried under reduced pressure before use. The dried biomass (70 mg) was added into 1.0 g of dried $[C_2mim][(MeO)(H)PO_2]$ and stirred at 200 rpm in an oil bath. The resulting solutions were centrifuged at 14,800 rpm (16200 G) from 10 to 60 min for removing residue. The supernatants were mixed with 70 wt% of DMSO and the resulting solutions were stirred at 80 °C for 3min. After filtration with glass filter under reduced pressure, the samples were injected to HPILC.

When we extracted polysaccharides from IL-treated bran, the $[C_2mim][(MeO)(H)PO_2]$/bran solution after 1st extraction (50 °C, 2h, 200 rpm) was centrifuged and the precipitation was collected. The precipitation was dispersed into 40 ml of DMSO and mixed with vortex mixer for 2min, to strip any dissolved substances adsorbed or trapped within the solid texture. The solution was centrifuged (10000 G, 10min) and the supernatant was removed. For further washing, 40 ml of methanol was added and the solution was mixed with vortex mixer for 1min. The solution was centrifuged (10000 G, 10min) and the supernatant was removed. Methanol-washing process with methanol was repeated 2 times. After drying under reduced pressure at room temperature, the IL-treated bran (70 mg) was added into 1.0 g of fresh $[C_1mim][(MeO)(H)PO_2]$ and stirred (80 °C, 2h, 200 rpm).

Pretreatment of *Prunus* × *yedoensis*

Petal, leaf, and branch of *P.* × *yedoensis* were obtained in the ground of Tokyo University of Agriculture and Technology in April, 2014. They were freeze-dried, and bark of the branch was peeled. They were fragmented by hands, to be almost 1 mm^2.

4-3. Selection of Suitable Ionic Liquids as Eluents

Among a variety of ILs proposed as solvents for cellulose, we first selected a candidate IL. Such ILs should be able to dissolve cellulose, have sufficiently low viscosity, and be stable enough. One series of ILs proposed for dissolution of cellulose comprises the alkylimidazolium carboxylate salts, such as acetate and formate salts, which are known to be low viscosity ILs.[4] Unfortunately, these ILs are thermally unstable, and gradually decompose even at ambient temperature. We have, however, found a series of phosphate derivative ILs that is thermally stable and not excessively viscous, yet which retains the capability to dissolve cellulose (Figure 4-2).[5] From this series, we considered $[C_2mim][(MeO)(H)PO_2]$ is suitable as an eluent in HPLC, because it is easy to prepare and has low viscosity. We then attempted to pump $[C_2mim][(MeO)(H)PO_2]$ into an HPLC system as an eluent. To reduce its viscosity, and therefore the pressure, we heated it to 55 °C. At a very slow flow-rate of 0.01 ml min^{-1}, HPLC with $[C_2mim][(MeO)(H)PO_2]$ (η = 25 cP at 55 °C) was performed below the upper limit of the operating pressure (<10 MPa). The system was summarized in scheme 4-1.

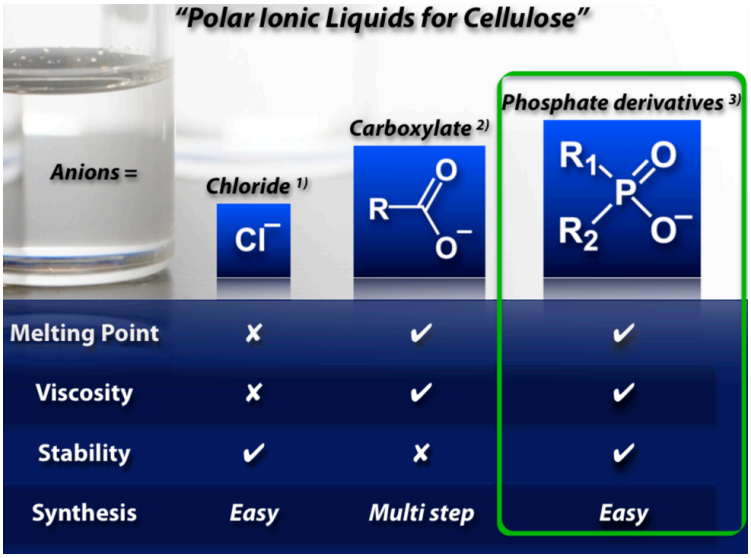
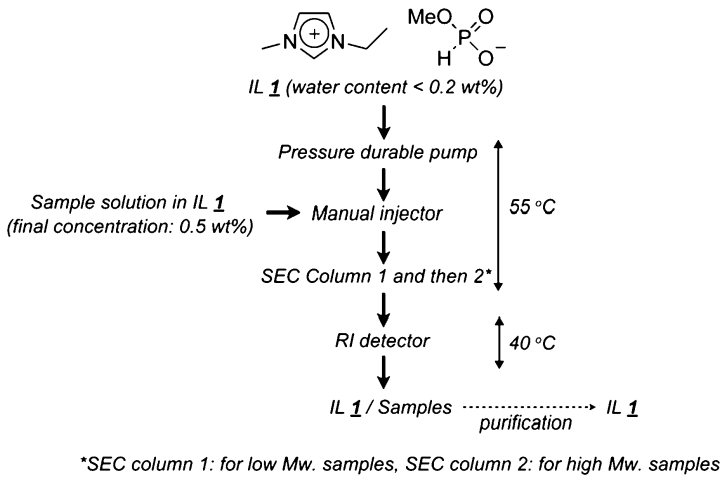


Figure 4-2 Selection of ILs.



Scheme 4-1 The system of HPILC and the measurement conditions

4-4. Verification of HPILC

4-4-1. Confirmation of Dissolution of Polysaccharides without Degradation

We confirmed that dissolution of polysaccharides in ILs does not affect to molecular weight and chemical structure of polysaccharides. We used a pullulan series as a standard polymer common in HPLC analysis; pullulan is a polysaccharide consisting of maltotriose units and includes *α*-glycosidic bond and *β*-glycosidic bond (Figure 4-3). Cellulose has *β*-glycosidic bond, and pullulan can be used as an alternative. We compared MWD of pullulan before and after dissolution using traditional HPLC (Figure 4-4). First, eight pullulan standards with different degrees of polymerization (MW: 0.59 x 10^4 to 78.8 x 10^4) were dissolved into milli-Q water respectively, and the samples were measured using HPLC with water as eluent (short columns were used: 402.5-4B and 405-4B). Next, we once dissolved same pullulan series into $[C_2mim][(MeO)(H)PO_2]$ respectively and precipitated it with methanol. The each precipitated pullulan was dissolved into milli-Q water and measured with the same system and compared. Two calibration curves of pullulan before and after dissolution into $[C_2mim][(MeO)(H)PO_2]$ was not change. This clearly shows that $[C_2mim][(MeO)(H)PO_2]$ does not affect molecular weight and chemical structure of pullulan, and it is confirmed that $[C_2mim][(MeO)(H)PO_2]$ is applicable as an eluent of HPLC.

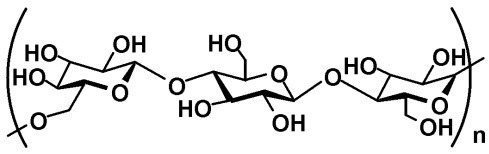


Figure 4-3 Structure of pullulan.

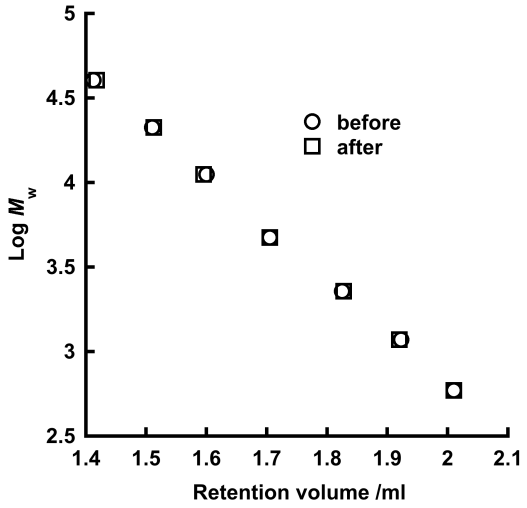


Figure 4-4 Calibration curves of pullulan between retention volume and molecular weight before and after dissolution into $[C_2mim][(MeO)(H)PO_2]$.

4-4-2. Confirmation of Size Exclusion Effect

Size exclusion effect of HPILC was confirmed. The pullulan standards were dissolved individually in $[C_2mim][(MeO)(H)PO_2]$ to be final concentration of 0.5 wt%, and the resulting solutions were directly injected into HPILC. Figure 4-5 shows the resulting chromatograms of this pullulan series. The elution peaks assigned to these standards were separated, and their elution volumes were in the order of their molecular weight. In order to analyze samples with a wide range of molecular weight, herein two columns suited to separate both low and high molecular weight samples were used. Open circles in Figure 4-6 show relation between retention volume and their M_w measured by HPILC, and it was confirmed a good relations. According to resolution, it is confirmed to be enough. It is noted that the plots of pullulan (M_w: 5,000) and glucose were well separated while the plot of glucose in the calibration curve is at a slightly lower retention volume than expected. To verify this calibration curve, we compared it to a calibration curve obtained with HPLC with water as an eluent (Figure 4-6, open square). While the slope of the standard relation of HPILC was slightly larger, two

standard relations were similar. This indicated that relatively high resolution was obtained even in the case of ILs, which are highly viscous. These results clearly show that samples were analyzed by the size exclusion effect by HPILC.

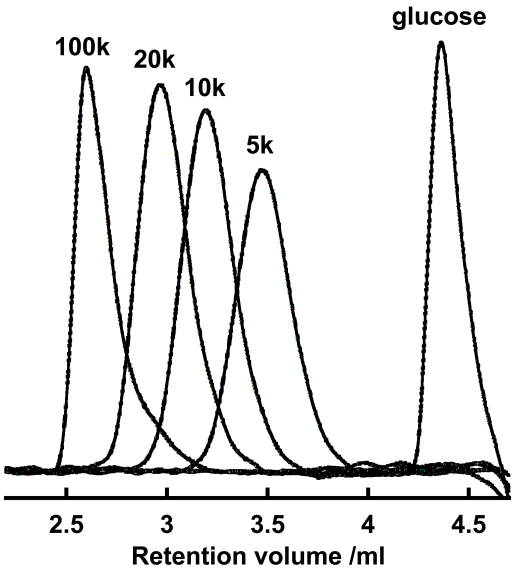


Figure 4-5 Chromatograms of pullulan standards and glucose measured with HPILC

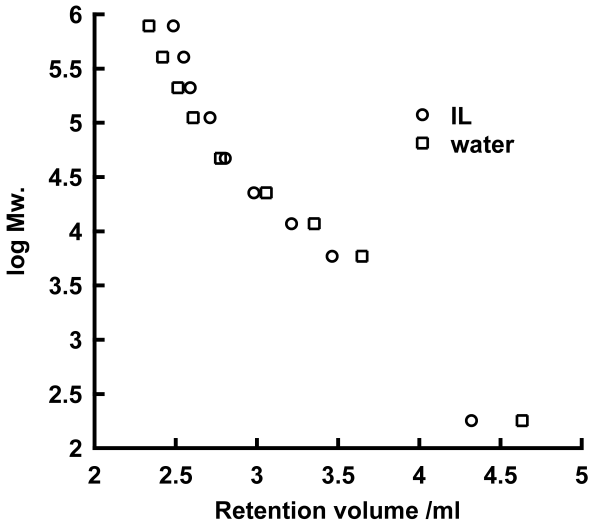


Figure 4-6 Standard relation between retention volume and logarithm of molecular weight using pullulan (Mw: 800,000 to 5,000) and glucose (Mw: 180) as standard substance. Measured with ○HPILC, □HPLC with water as eluent

4-5. Analysis of Cellulose

Three cellulose samples were analyzed: microcrystalline cellulose, ashless pulp, and bacterial cellulose. These cellulose samples were respectively mixed with $[C_2mim][(MeO)(H)PO_2]$ and stirred gently without heating, and they gave homogeneous solutions. The resulting clear solutions were directly analyzed by HPILC (Figure 4-7). Because their elution order consists with a literature[6], it is confirmed they are analyzed based on their molecular weight.

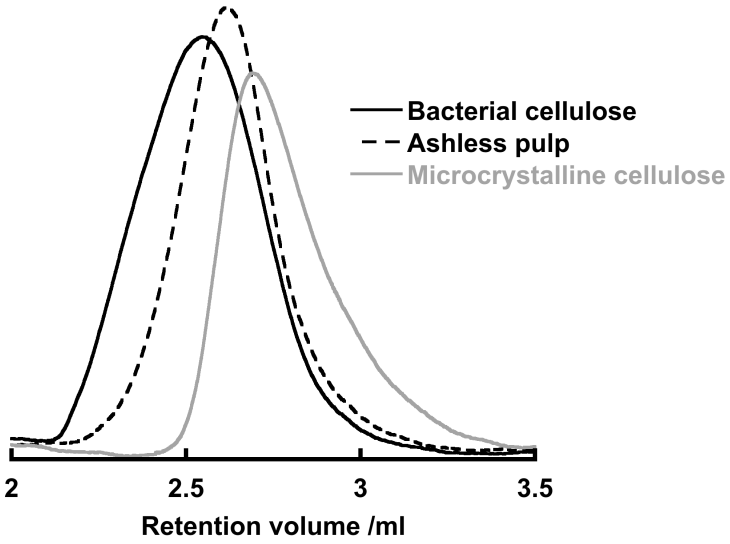


Figure 4-7 Chromatograms of cellulose dissolved in $[C_2mim][(MeO)(H)PO_2]$.

4-6. Setup of HPILC for Detection of Lignin with a UV Detector

Plant biomass is mainly composed of cellulose and hemicellulose and lignin and it is known that polar ILs can extract them.[1-3] However, relation between MWD of extracts and extraction conditions has not reported whereas MWD affects physico-chemical properties of polymers. There is a strong request on the reliable method to analyze MWD of polysaccharides.

To date, a method to analyze MWD of extracts from biomass through derivatisation has been reported.[7, 8] However, the method is not suitable for analysis of extracts as follow reasons; (1) MWD of the derivertized materials is changed (2) derivartized cellulose and specific hemicellulose (xylan etc.) have different UV absorbance due to different number of reactive hydroxyl groups (three and two, respectively). To overcome these problems, direct analysis of extracts in ILs without derivatization should be utilized. Thus, we thought that HPILC should be effective to analyze extraction of polysaccharides in ILs.

To analyze polysaccharides and lignin with identifying, combination of refractive index (RI) detector and UV detector should be effective. It is known that lignin has UV absorption, pointing that lignin is detectable with both RI detector and UV detector. Since cellulose and hemicellulose have no UV absorption, polysaccharides and lignin are expected to be distinguished by using both detectors. However, representative polar ILs also have UV absorption based on imidazolium ring. We preliminarily performed UV-*vis* spectrometry of lignin dissolved in 1-ethyl-3-methylimidazolium methylphosphonate ($[C_2mim][(MeO)(H)PO_2]$), as shown in Figure 4-8. $[C_2mim][(MeO)(H)PO_2]$ has UV absorption under 350 nm, and it was saturated under 260 nm. Lignin dissolved in $[C_2mim][(MeO)(H)PO_2]$ shows different spectrum; absorbance considerably increased in spite

of low concentration of lignin (0.005 wt%). We chose 300 nm for detection of lignin with UV detector because their absorbance was most different.

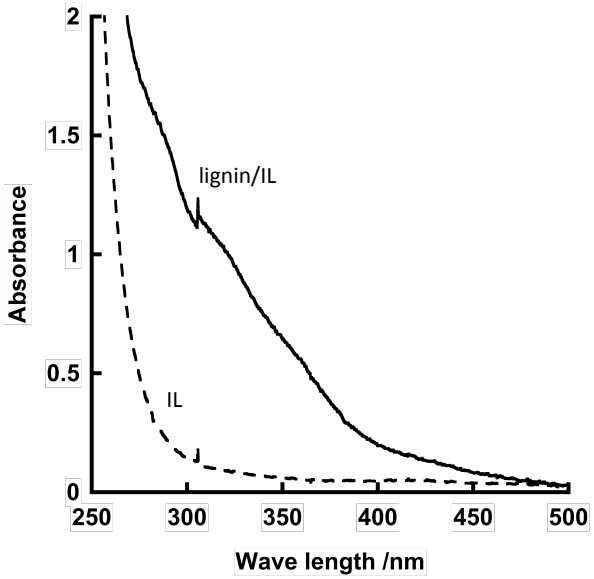


Figure 4-8 UV-*vis* stpectra of $[C_2mim][(MeO)(H)PO_2]$ and lignin/$[C_2mim][(MeO)(H)PO_2]$ solution.

To confirm that cellulose and lignin were distinguished with two detectors, solutions of cellulose or lignin in $[C_2mim][(MeO)(H)PO_2]$ (0.5 wt%) were measured using HPILC (Figure 4-9). As expected, the signals of cellulose and lignin were detected with RI detector (Figure 4-9, left). With UV detector, only lignin was detected (Figure 4-9, right). There was difference of intensity of the peaks between detectors depending on their sensitivity (7 mV with RI detector and 265 mV with UV detector). To compare these chromatograms easily, these signals were normalized based on the intensity of the peaks for lignin: maximum intensity of the peaks of lignin was calculated to be 100. Maximum intensity of the peak for cellulose was also almost 100, but it was coincidence.

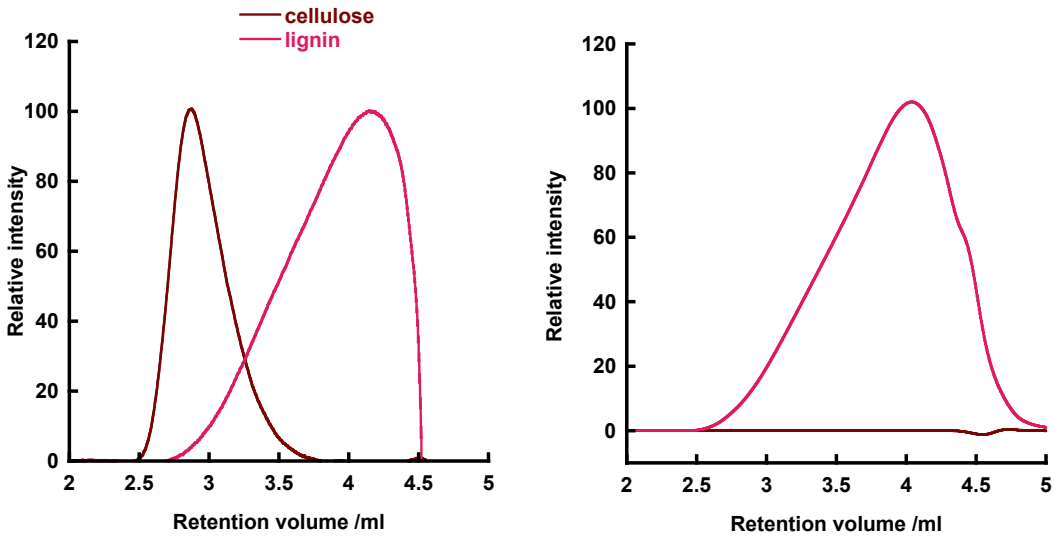


Figure 4-9 Chromatograms of cellulose and lignin dissolved in $[C_2mim][(MeO)(H)PO_2]$ after normalization (left: detected by RI detector, right: detected by UV detector).

4-7. Analysis of Extracts from Bran

Figure 4-10 shows chromatograms of extracts from wheat bran with $[C_2mim][(MeO)(H)PO_2]$ at various temperature. Wheat bran (70 mg) was added into 1.0 g of $[C_2mim][(MeO)(H)PO_2]$ and stirred for 2h. To decrease viscosity, dimethyl sulfoxide was added. The resulting solution was measured after filtration. Comparing the RI- and UV-chromatograms, the RI-chromatograms showed much higher intensity than UV-chromatograms (e.g. 170 vs 4 at 80 ^oC). This strongly indicates that RI-chromatograms do nearly not include signal of lignin. In RI-chromatograms, three peaks were observed at 2.4 ml, 2.8 ml, and 4.4 ml. Among them, the peak at 4.4 ml was assigned monomeric sugar and other low MW compounds. Between 2.0 and 3.5 ml, bimodal distribution was observed (molecular weight is $> 10^4$). In UV-chromatograms, while lignin was scarcely obtained, mainly two peaks were observed at high and low retention volume (> 3.5 ml and < 3.5 ml, respectively). The peaks at high retention volume should be assigned to lignin and low MW aromatic species. The peak at low retention volume was presumably assigned to lignin-carbohydrate complexes (LCCs).[8]

At lower temperature, only low-MW polysaccharides were obtained. As increasing extraction temperature, high MW polysaccharides were obtained with increase of extracted amount. At 120 ^oC, extracted amount of high MW components did not increase but low MW polysaccharides (3.0 to 4.2 ml) were more extracted. Additionally, decomposition of low MW polysaccharides was detected since intensity of the signal at 2.8 ml decreased. According to lignin, there is no change in MWD between 25 to 80 ^oC, but extracted amount increased at higher temperature. At 120 ^oC, decrease of the peak at 2.6 ml and appearance of new peak at 3.1 ml were observed. They are attributed to decomposition of lignin. It is known that partial decomposition of lignin occurs over 100 ^oC.[9, 10]

On the other hand, we have already investigated relation between extraction temperature and extracted amount of cellulose and xylan (main hemicellulose of wheat bran) with ^{1}H NMR (using a similar IL: 1,3-dimethylimidazolium methyl methylphosphonate) in chapter 3. In the chapter, it is reported that extracted amount of only xylan increases between 80 °C and 120 °C. Therefore, increased signal between 3.0 and 4.2 ml in RI-chromatogram was attributed to be xylan. Furtheremore, since decomposition of LCCs was observed at 120 °C in a UV-chromatogram, the increase of xylan signal was caused by degradation of LCCs.

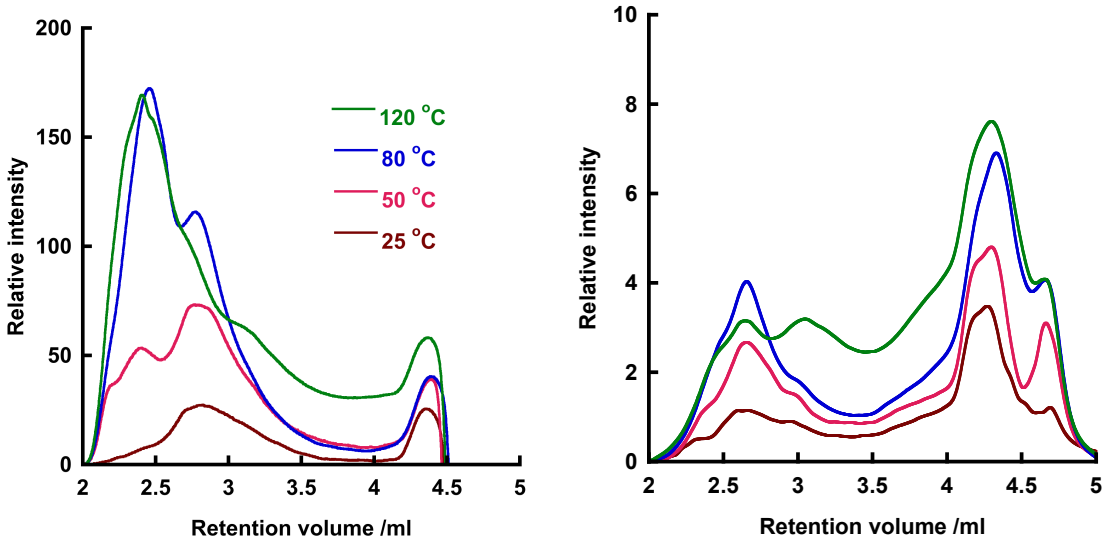


Figure 4-10 Chromatograms of extracts from wheat bran with [C_2mim][(MeO)(H)PO_2] at various temperature (load amount: 70 mg, IL amount: 1.0 g, extraction time: 2h, left: detected with RI detector, right: detected with UV detector).

Figure 4-11 shows MWD of extracted polysaccharides and lignin at various extraction time at 80 °C. At longer time, extracted amount and ratio of high MW cellulose increased. However, compared to effect of temperature (see Figure 4-10), extraction time less affected ratio of high MW polysaccharides. Extracted amount of

lignin also increased as increasing extraction time.

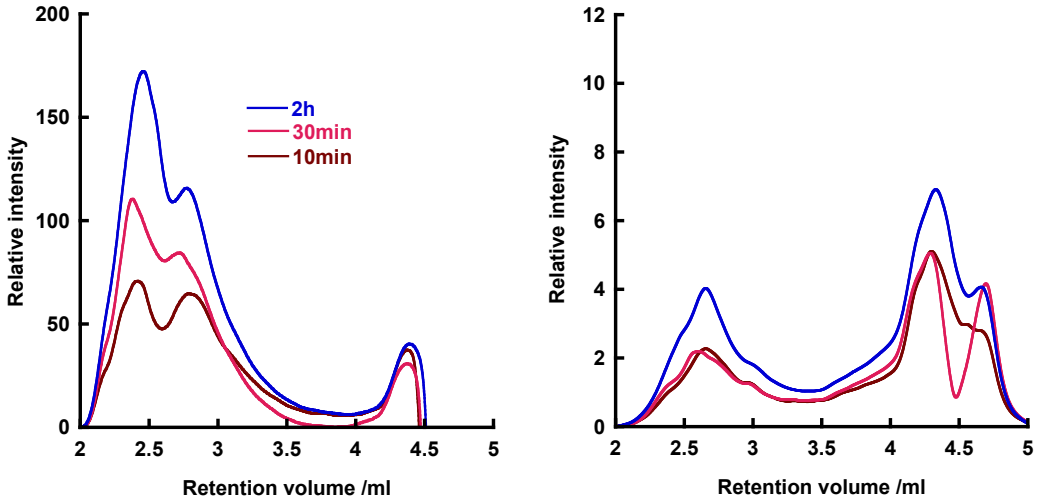


Figure 4-11 Chromatograms of extracts in various extraction time from wheat bran with $[C_2mim][(MeO)(H)PO_2]$ (load amount: 70 mg, IL amount: 1.0 g, extraction temperature: 80 °C, left: detected with RI detector, right: detected with UV detector).

Extraction at 25 °C was also performed (Figure 4-12). As mentioned above, only low MW polysaccharides were extracted when extraction time was 2h. However, longer extraction time (e.g. 96h) led to extraction of high MW polysaccharides. This result strongly suggests that $[C_2mim][(MeO)(H)PO_2]$ is capable of extraction of high MW polysaccharides even at 25 °C, but other factors such as viscosity of ILs and recalcitrance of high MW polymer affect the MWD of extracts. It should be noted, nevertheless, that low MW polysaccharides were main component of extracts at 25 °C even at 96h. Concerning extracted amount of polysaccharides, the extract at 25 °C for 96h was similar to those at 50 °C for 2h and at 80 °C for 10min. From this result, it was confirmed that elevating temperature significantly accelerated extraction of polysaccharides.

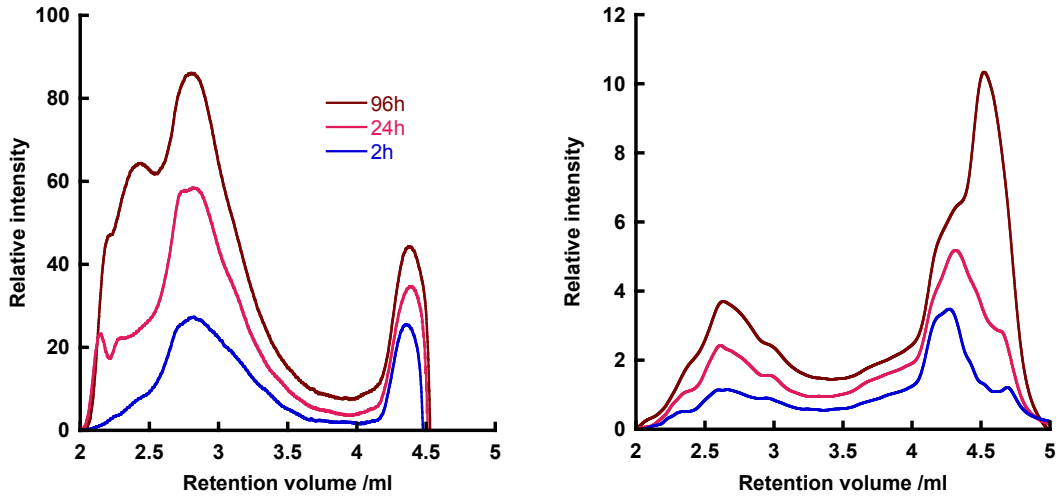
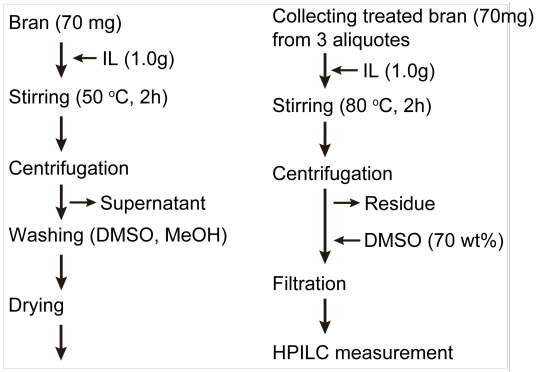


Figure 4-12 Chromatograms of extracts in various extraction time from wheat bran with $[C_2mim][(MeO)(H)PO_2]$ (load amount: 70 mg, IL amount: 1.0 g, extraction temperature: 25 °C, left: detected with RI detector, right: detected with UV detector).

From these results, we have found that ratio of high MW polysaccharides in extracts was more affected by extraction temperature than extraction time. Therefore, we expected that only high MW polysaccharides could be obtained from the bran by treatment at higher temperature after pretreatment at lower temperature. Bran was first treated at 50 °C for 2h, and successively the treated bran was immersed at 80 °C (summarised in Scheme 4-1). As shown in Figure 4-13, only high MW polysaccharides were confirmed to be extracted. Furthermore, lignin content in the sample was found to be low. These show that extracts predominantly composed of high MW polysaccharides were successfully obtained. Until now, there are no reports that single and pure IL enables to control MWD of extracted polysaccharides just by varying temperature. This fact should be an aid to solve problems concerning industrial process such as removing co-solvent.



Scheme 4-1 Extraction at 80 °C from bran treated with $[C_2mim][(MeO)(H)PO_2]$ at 50 °C.

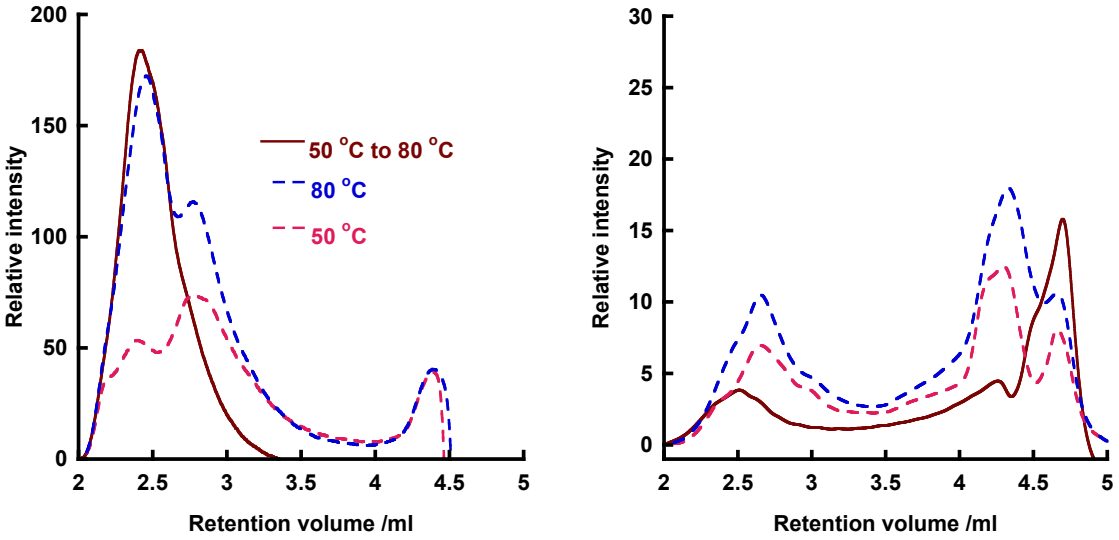


Figure 4-13 Chromatograms of the extract from wheat bran (80 °C) after treatment with $[C_2mim][(MeO)(H)PO_2]$ at 50 °C (left: detected with RI detector, right: detected with UV detector).

4-8. Analysis of Extracts from Woody Biomass: Species dependence

We extracted polysaccharides from pine (*Picea jezoensis*) as softwood and beech (*Quercus crispula*) as hardwood and analyzed them (Figure 4-14). Extraction from wood is known to be more difficult than herbaceous species, and this trend was also observed in this experiment; the intensity in RI-chromatograms was considerably lower (less than 1/10 at 80 °C). Conversely, lignin was confirmed to be more extracted, according to UV-chromatograms. Additionally, MW of both extracted lignin was larger than that from bran.

When we extracted polysaccharides from both woods at 80 °C for 2h, RI-chromatograms showed bimodal distribution. The peaks at lower- and higher retention volumes should be attributed to polysaccharides and lignin, in consideration of the UV-chromatograms. Between pine and beech, extracts from pine contained low MW polysaccharides and low MW lignin. The broad MWD of polysaccharides and narrow MWD of lignin were also seen in the case of cedar (Softwood, *Cyptomeria japonica*), shown in Figure 4-15. Thus it is possibly attributed to the characteristics of softwood. Additionally, it is known that molecular weight of sulfonated lignin isolated from softwood is smaller than that from hardwood. Investigation on various wood samples enables further discussion.

According to beech at 120 °C, increase of extracted amount of polysaccharides was confirmed. Lignin also increased so as to be similar polysaccharides/lignin proportion. In the extracts from pine, increase of polysaccharides was also confirmed. However, extracted amount of lignin was not changed. This indicates that polysaccharides can be preferentially obtained at higher temperature in the case of pine. From a viewpoint of MWD, minimum and maximum retention volume of polysaccharides was not changed between 80 °C and 120 °C while ratio of high MW polysaccharides

increased.

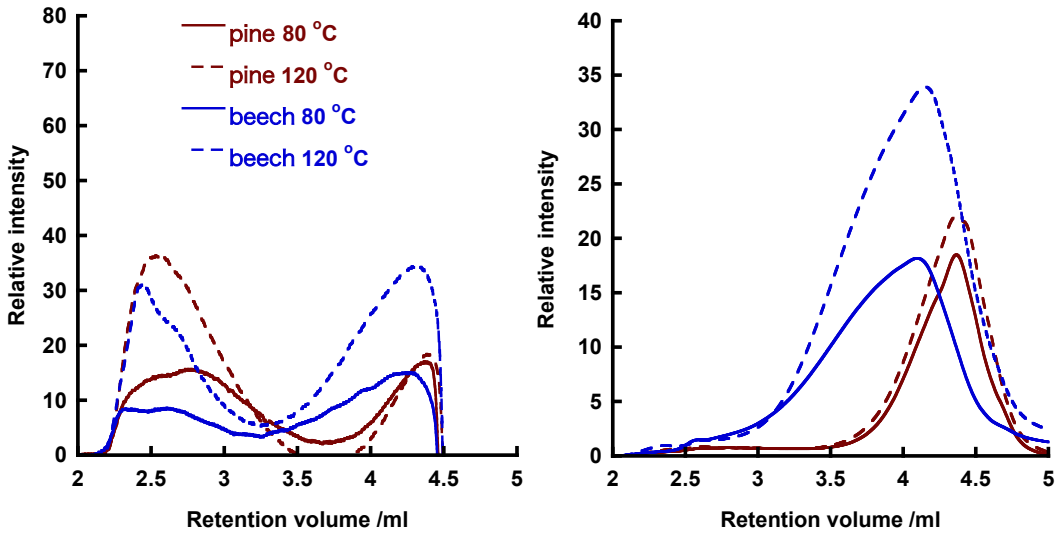


Figure 4-14 Chromatograms of extracts at 80 °C and 120 °C from wood biomass with $[C_2mim][(MeO)(H)PO_2]$ (load amount: 70 mg, IL amount: 1.0 g, extraction time: 2h, left: detected with RI detector, right: detected with UV detector).

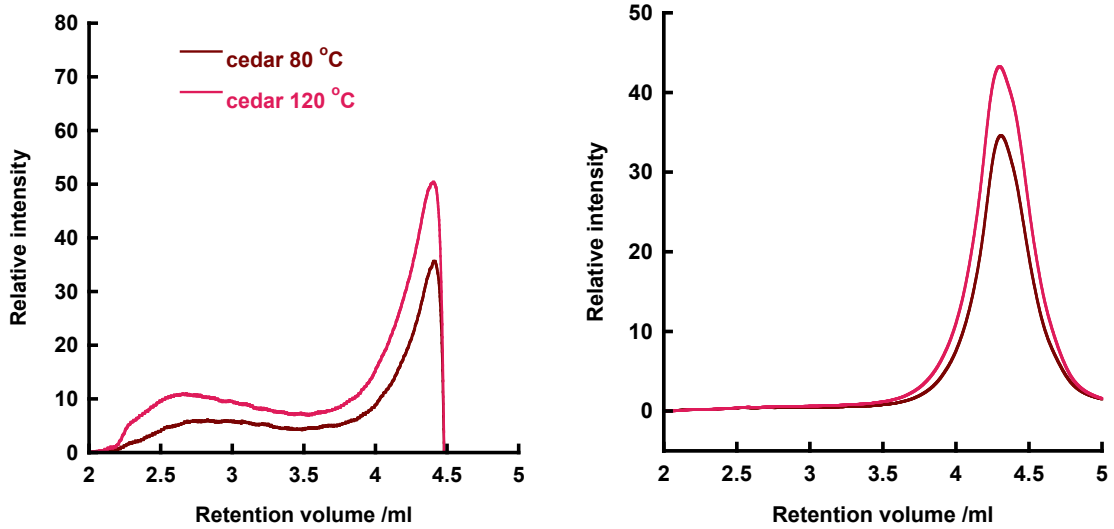


Figure 4-15 Chromatograms of extracts at 80 °C and 120 °C from cedar with $[C_2mim][(MeO)(H)PO_2]$ (load amount: 70 mg, IL amount: 1.0 g, extraction time: 2h, left: detected with RI detector, right: detected with UV detector).

We compared MWD of extracts at 2 and 6h at 80 °C (Figure 4-16). In the case of pine, no change was observed in the RI- and UV-chromatograms. It showed that extractable components at 80 °C were extracted within 2h. In the case of beech, intensity of the RI- and UV-chromatograms increased. Additionally, longer extraction time led to low polysaccharides/lignin ratio.

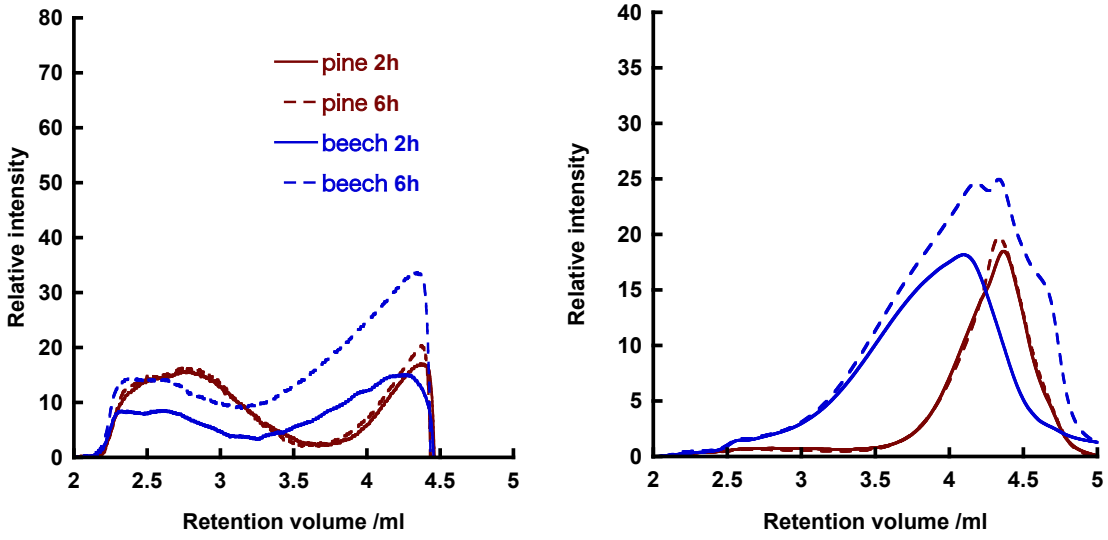


Figure 4-16 Chromatograms of extracts for 2h and 6h from wood biomass with $[C_2mim][(MeO)(H)PO_2]$ (load amount: 70 mg, IL amount: 1.0 g, extraction temperature: 80 °C, left: detected with RI detector, right: detected with UV detector).

4-9. Analysis of Extracts from Various Parts of Cherry Wood

For efficient use of plant biomass, various parts of plant biomass such as branch and leaf should be utilized. They are intrinsically different cells, and thus extracted polysaccharides should have different MWD and recalcitrance to extraction. We utilized leaf, petal, and branch of *Prunus* × *yedoensis 'Somei-yoshino'* as biomass. These were added into [C_2mim][(MeO)(H)PO_2] and stirred at 120 °C for 2h. From leaf, large amount of polysaccharides were extracted compared to that from branch (Figure 4-17). According to MWD of polysaccharides extracted from leaf, low MW polysaccharides were mainly extracted and extract amount was large. From branch, high MW polysaccharides were extracted as well as low MW polysaccharides. MWD of extracted polysaccharides from petal was similar to that from leaf but its amount was somewhat lower. Lignin extracted from branch was largest MW

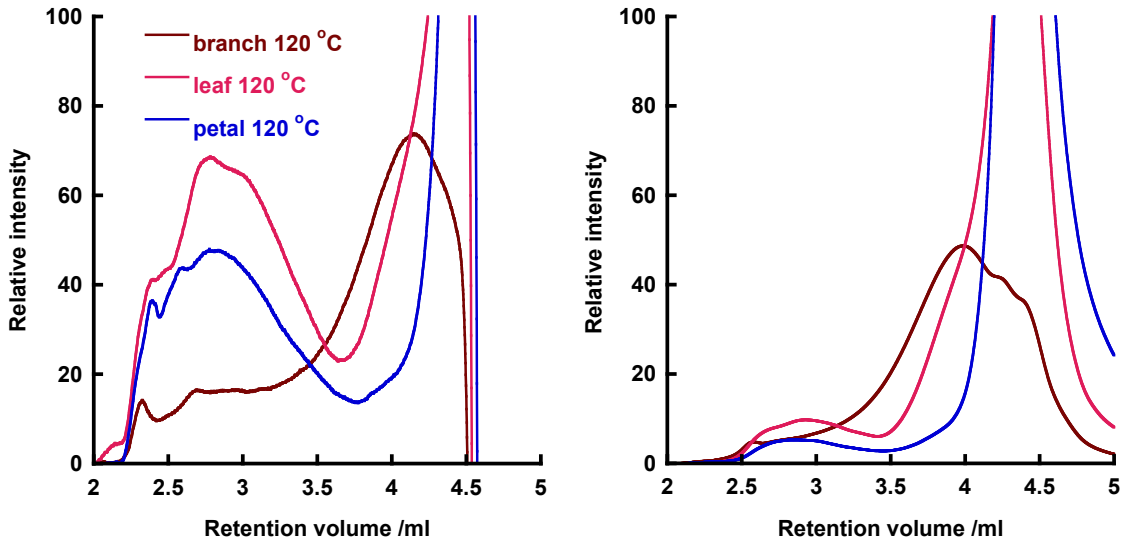


Figure 4-17 Chromatograms of extracts from various parts of cherry wood with [C_2mim][(MeO)(H)PO_2] (load amount: 70 mg, IL amount: 1.0 g, extraction temperature: 120 °C, extraction time: 2h, left: detected with RI detector, right: detected with UV detector).

among them. It is noted that UV-chromatograms of leaf and petal may include other aromatic compounds such as dye.

We also performed extraction at 80 °C and analyzed them (Figure 4-18). Except for increase of extracted amount from branch, significant change was not observed. This indicates that 80 °C was enough to extract polysaccharides from leaf and petal. On the other hand, much higher temperature (> 120 °C) should be required for efficient extraction from branch.

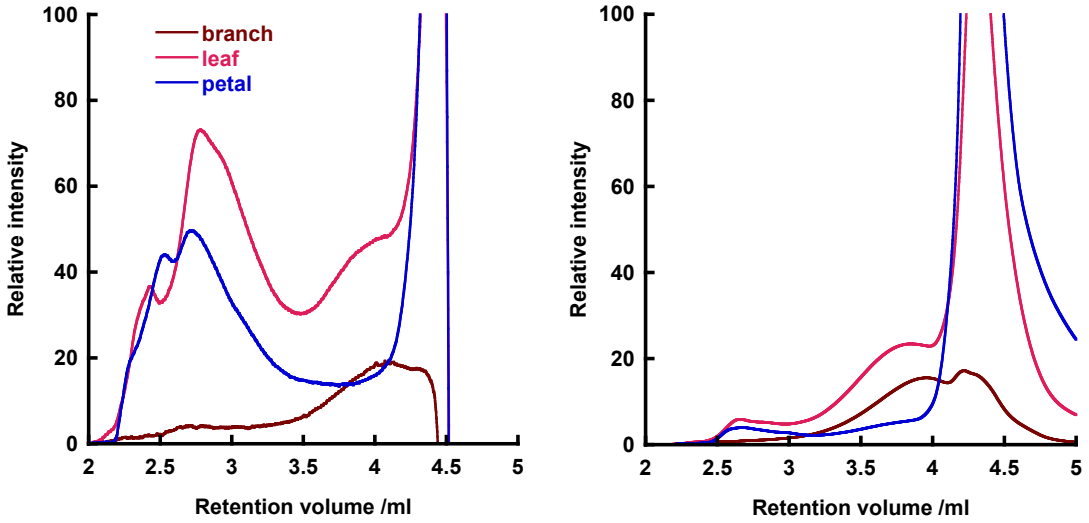


Figure 4-18 Chromatograms of extracts from various parts of cherry wood with $[C_2mim][(MeO)(H)PO_2]$ (load amount: 70 mg, IL amount: 1.0 g, extraction temperature: 80 °C, extraction time: 2h, left: detected with RI detector, right: detected with UV detector).

4-10. Conclusion

We have demonstrated the first case of HPLC with an IL as an eluent by using $[C_2mim][(MeO)(H)PO_2]$, which is relatively low viscous and polar ILs. Even in the case of ILs as eluents, size exclusion effect was confirmed. HPILC successfully analyzed three kinds of cellulose depending on their molecular weight.

By using HPILC, we analyzed MWD of polysaccharides and lignin in extracts from biomass. Higher extraction temperature led to increase of extracted amount and ratio of high MW polysaccharides. While longer extraction also did, it was less effective than temperature. Furthermore, we controlled MW of extracted polysaccharides. High MW polysaccharides were extracted at 80 °C from the bran pretreated at 50 °C with the same IL. Extracts from wood biomass were also investigated, and both extraction temperature and extraction time were confirmed to affect to extraction amount and MWD, similarly to bran. Polysaccharides extracted from different parts of *Prunus* × *yedoensis* were analyzed. It was observed that polysaccharides from leaf had low MW and those from branch had relatively high MW. Relatively mild condition (80 °C) was found to be enough to extract polysaccharides.

4-11. References

1. S. S. Y. Tan, D. R. MacFarlane, J. Upfal, L. A. Edye, W. O. S. Doherty, A. F. Patti, J. M. Pringle and J. L. Scott, *Green Chem.*, 2009, **11**, 339-345.
2. C. Froschauer, M. Hummel, G. Laus, H. Schottenberger, H. Sixta, H. K. Weber and G. Zuckerstatter, *Biomacromolecules*, 2012, **13**, 1973-1980.
3. A. Brandt, J. Gräsvik, J. P. Hallett and T. Welton, *Green Chem.*, 2013, **15**, 550-583.
4. Y. Fukaya, A. Sugimoto and H. Ohno, *Biomacromolecules*, 2006, **7**, 3295-3297.
5. Y. Fukaya, K. Hayashi, M. Wada and H. Ohno, *Green Chem.*, 2008, **10**, 44-46.
6. M. Yanagisawa, I. Shibata and A. Isogai, *Cellulose*, 2004, **11**, 169-176.
7. L. Zoia, A. W. T. King and D. S. Argyropoulos, *J. Agric. Food Chem.*, 2011, **59**, 829-838.
8. A. Salanti, L. Zoia, E. L. Tolppa and M. Orlandi, *Biomacromolecules*, 2012, **13**, 445-454.
9. J.-L. Wen, T.-Q. Yuan, S.-L. Sun, F. Xu and R.-C. Sun, *Green Chem.*, 2014, **16**, 181-190.
10. J. Y. Kim, E. J. Shin, I. Y. Eom, K. Won, Y. H. Kim, D. Choi, I. G. Choi and J. W. Choi, *Bioresour. Technol.*, 2011, **102**, 9020-9025.

Chapter 5

Direct HPILC Analysis of Hydrolysis of Cellulose dissolved in ionic liquids

5-1. Introduction

In this chapter, we tried to apply HPILC to analysis of hydrolysis of cellulose. There has been no method for direct analysis of change of MWD of cellulose in ILs during hydrolysis. Hydrolyzed cellulose in ILs has generally been evaluated stepwise with 3,5-dinitrosalicylic acid (DNS) assay[1] or by high performance liquid chromatography (HPLC)[2] with such complex treatments as changing solvents from ILs to water through ion exchange resin and other troublesome steps. These treatments are time-consuming and may change the composition of the samples. Furthermore, only a few sugars are detectable such as water-soluble sugars or reducing sugars. HPILC should have potential to overcome these problems, and then the possibility of HPILC was investigated in this chapter.

5-2. Materials and Methods

General

Pullulan standards were purchased from Showa denko, and were used as received. Microcrystalline cellulose powder (cellulose powder C) was purchased from Advantec Toyo Co., and was used as received.

Synthesis of $[C_2mim][(MeO)(H)PO_2]$

$[C_2mim][(MeO)(H)PO_2]$ was synthesized by the same method in chapter 4.

HPLC setup

We used the HPILC system mentioned in chapter 4, except for UV detector; it was not utilized in this chapter. Pullulan was used as polymer standard.

Solubilization of cellulose

Suspensions of cellulose (0.5 wt%) in dried $[C_2mim][(MeO)(H)PO_2]$ were prepared under an atmosphere of dry nitrogen gas. The mixtures were stirred gently at 25 ^oC under nitrogen gas until the solution became homogeneous and clear.

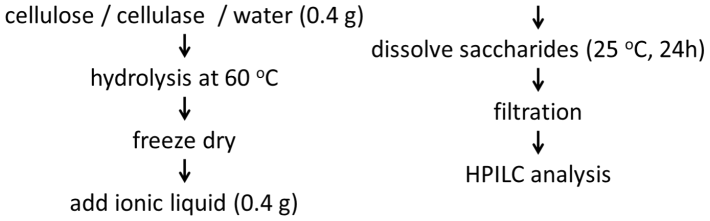
Immobilization of enzyme

Cellulase from *Trichoderma reesei* (Kyowa Hakko Kirin Co.) and methacrylic beads (EC-HA; Mitsubishi Chemical Corporation) and 25% glutaraldehyde aqueous solution (Tokyo Chemical Ind. Co.) were used as received. Methacrylic beads have hexamethylamine as the functional group. The beads (2.5 g) were added to 4.5 ml of 40 mM phosphate buffer (pH 7.5) with 2.5 % glutaraldehyde, and were stirred

for 2h at 25 °C. After washing of the beads with phosphate buffer, 3.75 ml of buffer solution was added and 2 g of cellulase was added to the solution and stirred for 10h. The beads were then washed with milliQ water and freeze dried.

HPILC analysis of enzymatic hydrolysis

The immobilized cellulase (8 wt%) and cellulose (0.5 wt%) were dispersed into 0.4 g of pure water. The resulting solution was stirred at 60 °C. After hydrolysis, the hydrolysates were frozen in liquid nitrogen to stop the reaction without disruption of the solutions. Each sample was freeze dried, and 0.4 g of $[C_2mim][(MeO)(H)PO_2]$ was added. To dissolve saccharides, the solutions were stirred for 24h at 25 °C, and passed through a PTFE filter prior to HPILC measurement. The procedure is summarised in scheme 5-1.



Scheme 5-1 Preparation of the hydrolyzed samples for HPILC analysis.

Determination of glucose concentration with a glucose test kit

The test kit consists of glucose dehydrogenase (GOx), peroxidase and mutarotase. Glucose was converted to gluconic acid and H_2O_2 with GOx. The hydrolyzed samples (20 ml) were added to 3ml of the solution of the test kit and held for 5min at 37 °C. The UV absorbance of the samples at 505 nm was measured with a UV-*vis* spectrometer.

Determination of the glucose concentration with HPILC

The glucose concentration was calculated from the standard relation between the glucose fraction in mixed samples (composed of cellulose and glucose) and each peak height (Fig. 5-3, right). In this calculation, the density of $[C_2mim][(MeO)(H)PO_2]$ was taken as 1.17 $g \cdot cm^{-3}$.

Depolymerization of cellulose with ultrasonication

A cellulose solution (0.5 wt%) in $[C_2mim][(MeO)(H)PO_2]$ was depolymerized with an ultrasonic stirrer (USS-1; Nihonseiki Ltd.) at 40 ^oC under a nitrogen gas atomosphere. The sonication power was 35 W and the frequency was 40 kHz.

5-3. Measurement of Models of Hydrolyzed Cellulose for Investigating Ability of HPILC

We already mentioned that HPILC enabled to analyze molecular weight distribution (MWD) of cellulose. As further potential of HPILC, we highlighted direct analysis of hydrolysis of cellulose in ILs. In this section, models of hydrolyzed cellulose were measured to evaluate ability of HPILC.

5-3-1. Resolution: a Mixture of Cellulose and Cello-oligosaccharides

As a typical model of depolymerized cellulose, a mixture of cellulose and several cello-oligosaccharides (glucose, cellobiose, cellotetraose, and cellohexaose) was analyzed with HPILC. These cello-oligosaccharides and cellulose (2.0 mg each) were mixed with 0.20 g of dried $[C_2mim][(MeO)(H)PO_2]$ and stirred gently at 25 ^oC resulting in a clear homogeneous solution. The solution was injected directly into the HPILC system. Figure 5-1 shows a HPILC chart of the injected solution. A broad peak for cellulose (from 2.5 to 3 ml) and some sharp peaks for cello-oligosaccharides (from 4 to 4.5 ml) were observed. The peaks for cello-oligosaccharides were observed in different retention volumes, respectively. Taking these results into account, we see that HPILC is an effective tool for the analysis of crude samples like hydrolyzed cellulose.

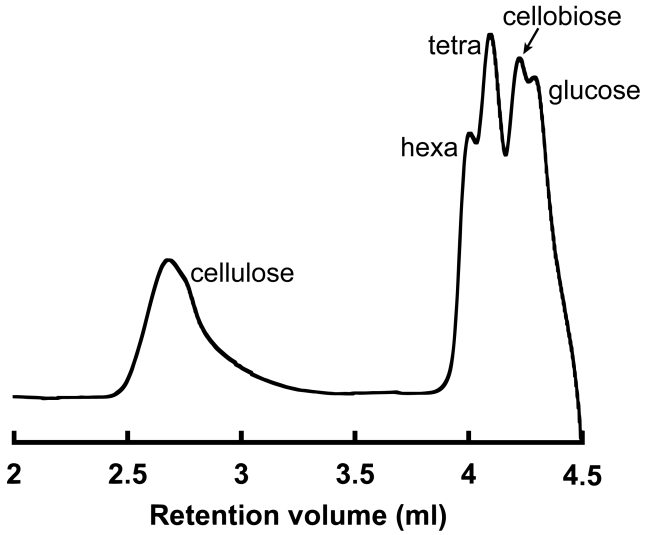


Figure 2-1 HPILC profile of a mixed sample composed of cellulose and cello-oligosaccharides.

5-3-2. Quantification: Mixtures of Cellulose and Glucose

In traditional HPLC analysis, quantification is possible from peak area or peak height. To confirm that HPILC can perform quantitative analysis, we prepared mixtures of cellulose and glucose and measured it (Figure 5-2). Mixtures of cellulose and glucose were added to 0.2 g of $[C_2mim][(MeO)(H)PO_2]$ to a final concentration of 0.5 wt% and the samples were gently stirred at 25 ^oC to obtain a clear homogeneous solution. The composition of glucose and cellulose was changed from 0 : 10 to 10 : 0 by weight. In the obtained chromatograms, the peaks of glucose and cellulose were completely separated and their peak area and peak height were calculated. As shown in Figure 5-3, there were linear correlations, and their concentration can be detected from the peak area or peak height. These results indicate that HPILC can analyze the cellulose and its hydrolysate quantitatively.

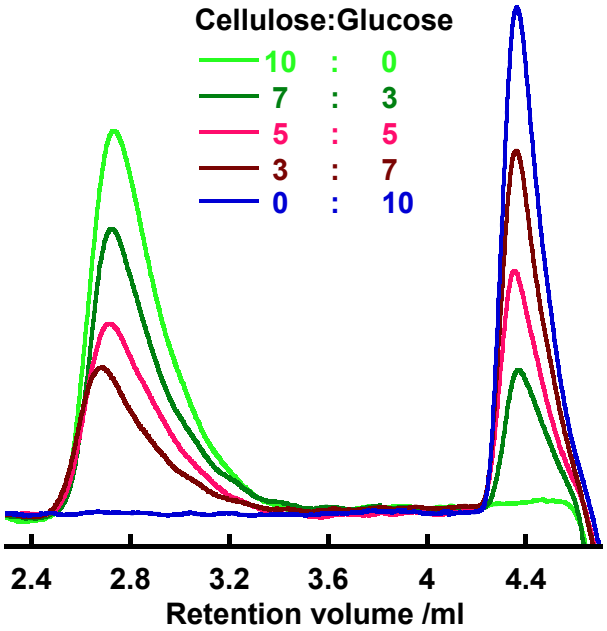


Figure 5-2 Chromatograms of mixtures of cellulose and glucose.

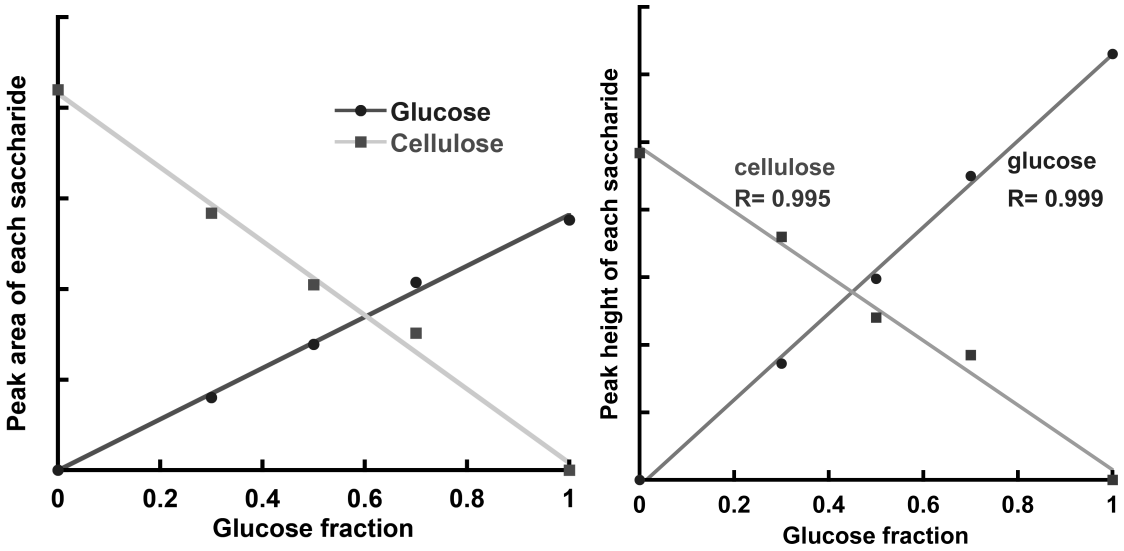


Figure 5-3 Relation between glucose fraction in mixed samples and (left) each peak area (right) each peak height.

5-4. Analysis of Hydrolyzed Cellulose in Water using Enzyme

Based on the results mentioned above, we expected that cellulose hydrolysis would be analyzed by HPILC through detection of time depending changes in the MWD of cellulose. To confirm this, enzymatic cellulose hydrolysis was carried out in an aqueous medium (conventional hydrolysis). In general, it is difficult to hydrolyze cellulose by cellulase due to high crystallinity of the cellulose. Thus we used cellulose with low crystallinity, which has been dissolved in $[C_2mim][(MeO)(H)PO_2]$, subsequently precipitated by addition of ethanol and dried under reduced pressure. This regenerated cellulose is known to be easily hydrolyzed by cellulase, because of its low crystallinity.[6, 7] For a biocatalyst, we used a cellulase mixture including endoglucanase (EG), cellobiohydrolase (CBH), and *β*-glucosidase (BGL). EG hydrolyzes cellulose at random, and CBH hydrolyzes cellulose to cellobiose. After hydrolysis of cellulose to cellobiose, BGL converts cellobiose into glucose. To avoid contamination of cellulase in the reaction media before HPILC measurement, we herein used cellulase immobilized onto acrylic beads and washed the samples with water.

Figure 5-4 shows chromatograms of hydrolyzed cellulose. The peak for cellulose and two peaks corresponding to glucose and cellobiose were observed together with uncertain peaks. The HPILC profiles clearly show mechanism of enzymatic hydrolysis of cellulose. At first, the cellulose peak decreased with increasing the peak for cellobiose. Then the cellobiose peak decreased and the glucose peak increased. In the retention volume from 3.5 to 4.0 ml, no peak was observed, indicating that oligosaccharides except for cellobiose were scarcely generated. These observations clearly reflect catalytic feature of enzymatic hydrolysis; mainly glucose and cellobiose are produced. These results also show that different activity of EG, CBH,

and BGL. First, EG hydrolyzed cellulose in an early stage, especially within 15min. After the first hydrolysis, a large amount of cellobiose was produced from cellulose by CBH in the interval from 15min to 1.5h, and glucose was produced by BGL. We believe that this hydrolytic order is influenced by the concentration of substrates in the solution; EG produced new reducing and non-reducing terminals of cellulose by hydrolysis, which can be bound by CBH, subsequently CBH produced a substrate of BGL. As lose enzymes from the acrylic beads, the peaks from 3 to 3.5 ml increased with increase of reaction time. These results show that cellulose hydrolysis by the three kinds of cellulase could successfully be analyzed by HPILC.

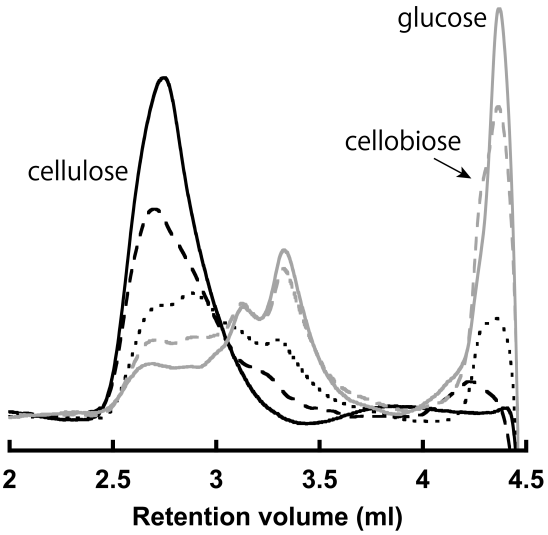


Figure 5-4 Chromatograms of hydrolyzed cellulose in water with immobilized cellulase at different reaction times: 0h (black continuous line), 5min(black dashed line), 15min (black dotted line), 1.5h (grey dashed line), 6h (grey continuous line).

For investigating the capability of HPILC in quantitative analysis, the glucose concentration was calculated from chromatograms of HPILC in Figure 5-4. The calculation was carried out based on the peak height attributed to glucose. The obtained

glucose concentration was compared to that calculated from conventional enzymatic methods (Figure 5-5). A similar trend on glucose concentration was observed, indicating that HPILC is able to trace the concentration of glucose *via* hydrolysis. From 30min to 3h, a slightly larger amount of glucose was detected by HPILC than that by the glucose test kit. This is presumably due to the contribution of the cellobiose peak, which partially overlaps with the peak for glucose. Since the cellobiose was consumed within 6h, almost the same concentration was detected with both methods. In addition, no increase in the amount of cellobiose was seen in spite of remaining some cellulose after 6h. It should be caused by denaturation of CBH, since the chromatogram at 24h (not shown here) nearly corresponded to that of 6h. These results show that HPILC is an effective tool to analyze the hydrolysis of cellulose with the advantages of HPLC, MWD analysis and quantitative analysis.

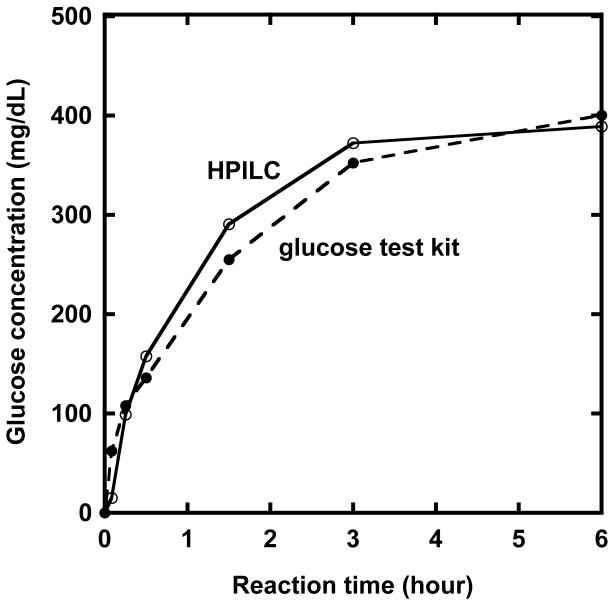


Figure 5-5 Glucose concentration of hydrolyzed cellulose samples at different reaction times determined by HPILC and a glucose test kit (Wako Pure Chemical Industries).

5-5. Analysis of Depolymerization of Cellulose in Ionic Liquids using Ultrasonication

According to the results in Figures 5-4 and 5-5, HPILC is expected to be effective to analyze cellulose depolymerization in ILs. Then we next analyzed the depolymerization of cellulose in $[C_2mim][(MeO)(H)PO_2]$ without pretreatment. There is however no report of efficient cellulose hydrolysis in ILs without disturbing samples. As an alternative method of hydrolysis, we focused on ultrasonication. Ultrasonication can depolymerize without change in the composition of the sample solutions, in contrast to catalytic reaction systems.

The samples containing cellulose (0.50 wt%) and 0.20 g of $[C_2mim][(MeO)(H)PO_2]$ were stirred at 25 °C until they became completely clear. The solutions were ultrasonicated at 40 °C to depolymerize cellulose, and the resulting samples were injected directly into our HPILC system. Figure 5-6 shows change in the profile of depolymerized cellulose as the function of ultrasonication

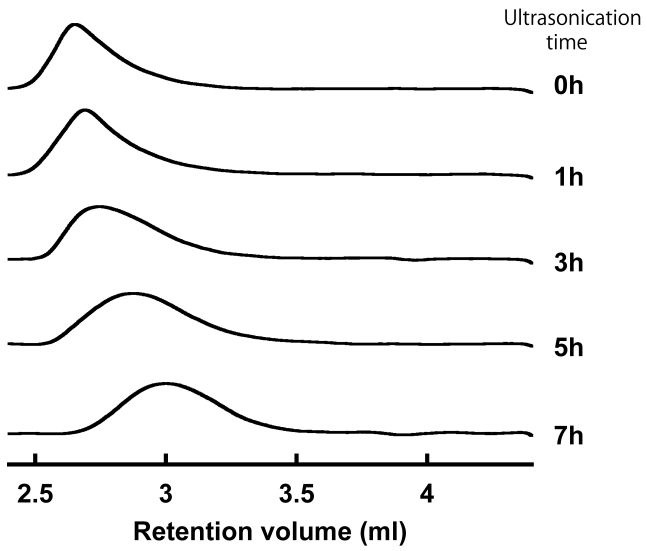


Figure 5-6 Chromatograms of cellulose depolymerized by ultrasonication at the different ultrasonication time.

time. The peak for cellulose shifted to higher retention volume side as the ultrasonication time increased. The average molecular weight of cellulose decreased with ultrasonication. No peaks for low molecular weight saccharides were found in Figure 5-6; retention volume from 4.0 to 4.4 ml, in contrast to enzymatic hydrolysis (see Figure 5-4). From this change of chromatograms, it was confirmed that the depolymerization of cellulose by ultrasonication occurred in random.

To study cellulose depolymerization in detail, the average molecular weight of cellulose was depicted as the function of ultrasonication time (Figure 5-7). Average molecular weight of cellulose was calculated with the standard relation obtained by the preliminary experiments with pullulan. The average molecular weight decreased with the increase of ultrasonication time. Our results show that depolymerization of cellulose in ILs can be analyzed directly by HPILC.

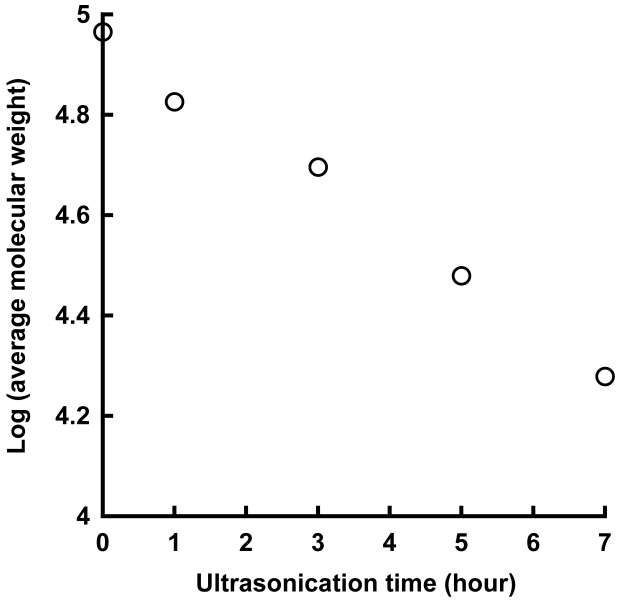


Figure 5-7 Relation between the ultrasonication time and the logarithm of the average molecular weight of depolymerized cellulose.

5-6. Conclusion

We found that HPILC is a powerful tool for the analysis of cellulose depolymerization in ILs. Cellulose and cello-oligosaccharides were analyzed by a single scan using HPILC, showing that this HPILC can detect dynamic change from cellulose to the final product (glucose) by hydrolysis. Hydrolysis in an aqueous medium with cellulase mixture was analyzed. It was confirmed that cellulose was hydrolyzed to glucose *via* cellobiose without generating other oligosaccharides. Depolymerization of cellulose in an IL with ultrasonication was also analyzed by HPILC. It was observed that the peak for cellulose simply shifted to high retention volume side, indicating random depolymerization, unlike hydrolysis by cellulase. These results show HPILC should be a potential tool for analyzing cellulose in ILs.

5-7. References

1. C. Li and Z. K. Zhao, *Adv. Synth. Catal.*, 2007, **349**, 1847-1850.
2. R. Rinaldi, R. Palkovits and F. Schuth, *Angew. Chem. Int. Ed.*, 2008, **47**, 8047-8050.
3. Y. Fukaya, A. Sugimoto and H. Ohno, *Biomacromolecules*, 2006, **7**, 3295-3297.
4. Y. Fukaya, K. Hayashi, M. Wada and H. Ohno, *Green Chem.*, 2008, **10**, 44-46.
5. M. Yanagisawa, I. Shibata and A. Isogai, *Cellulose*, 2004, **11**, 169-176.
6. A. P. Dadi, S. Varanasi and C. A. Schall, *Biotechnol. Bioeng.*, 2006, **95**, 904-910.
7. N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto and H. Takahashi, *Biotechnol. Lett.*, 2008, **30**, 1037-1040.

Chapter 6

Conclusions and Future Prospects

6–1. Conclusions

This thesis discusses novel methods for analyzing cellulose dissolved in ILs. These methods enabled the direct analysis of cellulose processing in ILs, in particular, analyses of polysaccharide extraction from plant biomass and hydrolysis of cellulose. Basic insights into these processes that are useful for the improvement of the chemical structure of ILs and optimization of reaction conditions were obtained.

In *Chapter 2*, we applied polar ILs to ^{1}H NMR as cellulose solvents to investigate the interaction between ILs and cellulose. For investigations performed in non-deuterated ILs, the No-D NMR technique and WET technique were applied. Cellulose signals were detected in non-deuterated [C_1mim][(MeO)(Me)PO_2]. Both types of signals of cellulose (C***H***s and C-O***H***s) were observed. Taking advantage of the direct observation of the proton state by ^{1}H NMR, we analyzed the hydrogen bonds between ILs and C-O***H***s. We confirmed that the hydrogen bond between C-O***H*** and [C_1mim][(MeO)(Me)PO_2] is stronger than that between DMSO and C-O***H***. Furthermore, we confirmed that solvation of C-O$\boldsymbol{H}_{(6)}$ is the most important factor concerning the dissolution of cellulose.

In *Chapter 3*, we analyzed cellulose and xylan extracted from wheat bran using ILs. By using the ^{1}H NMR technique established in Chapter 2, direct quantification was realized. The signals of these polysaccharides were individually detected in three non-deuterated ILs ([C_1mim][(MeO)(Me)PO_2], [C_1mim][$MeCO_2$], and [C_1mim][$MeOSO_3$]). The peak area of the signals was proportional to the added amount of the polysaccharides. Analysis of the extracts also revealed the detection of individual signals of polysaccharides. The extraction performance of ILs was found to be limited to a certain level at 80 °C, caused by lignin-carbohydrate complexes. Additionally, it was observed that almost all extractable polysaccharides at 80 °C were obtained within 30 min.

In *Chapter 4*, high-performance liquid chromatography with ILs as eluents (HPILC) was developed. $[C_2mim][(MeO)(H)PO_2]$ was determined to be a suitable eluent in terms of viscosity, thermal stability, and melting point. The size exclusion effect of HPILC was confirmed by measuring a pullulan standard. Three types of cellulose (bacterial cellulose, cotton linter, and microcrystalline cellulose) were analyzed based on their molecular weight by HPILC. Furthermore, using HPILC, we analyzed the molecular weight distribution (MWD) of polysaccharides and lignin in extracts obtained from biomass with ILs. Higher extraction temperature led to an increase in the extraction amount and the proportion of high-MW polysaccharides. Although longer extraction times yielded similar results, such long durations were less effective in extracting a large proportion of high-MW polysaccharides than was temperature. Furthermore, we controlled the MW of extracted polysaccharides by pretreatment. Only high-MW polysaccharides were extracted at 80 °C from bran pretreated at 50 °C with the same IL. Extracts from wood biomass were also investigated, and both extraction temperature and extraction time were confirmed to have affected the extraction amount and MWD, similarly to the results obtained for extracts from bran. Polysaccharides extracted from different parts of cherry wood were analyzed, and it was observed that polysaccharides from leaf had low MW and those from branch had relatively high MW.

In *Chapter 5*, HPILC was employed to analyze cellulose hydrolysis. As models of hydrolyzed cellulose, mixtures of cellulose and glucose were analyzed, demonstrating that HPILC could be applied for the quantitative analysis of cellulose hydrolysis. Hydrolysis in an aqueous medium with a cellulase mixture was analyzed. Enzymatic conversion, namely regioselective hydrolysis, was observed. Depolymerization of cellulose in an IL with ultrasonication was also analyzed by HPILC, and the signal peak for

cellulose simply shifted toward high retention volumes. This result indicates random depolymerization in contrast to enzymatic hydrolysis. These results demonstrate that HPILC is capable of not only estimating the efficiency of hydrolysis but also elucidating the mechanisms of hydrolysis.

6-2. Future Prospects: Potential and Limitations of the Methods Developed in this Study

^{1}H NMR and HPLC with polar ILs revealed properties of cellulose/IL solutions that have not been previously investigated. The potential and limitations of these analytical methods are herein discussed.

Concerning the potential of ^{1}H NMR with ILs, this NMR technique is applicable to many fields. Of course, various interactions between ILs and scarcely soluble polymers can be analyzed, but we must pay more attention to 2D NMR analysis. 2D NMR is capable of analyzing compounds in terms of interaction, conformation, spatial configuration and so forth. For example, the technique can reveal the structure of protein in ILs that dissolve proteins stably. Because the secondary structure of proteins in imidazolium-type ILs is not analyzable by circular dichroism (CD) spectroscopy, 2D NMR should be a useful method in this respect. Moreover, because ILs can dissolve proteins that are hardly soluble, the analytical method is applicable to the study of membrane proteins and aggregated proteins such as amyloids.

As a limitation of ^{1}H NMR with ILs, the difficulty of applying ILs that have many protons in their structure is highly problematic. In this thesis, we utilized the WET technique to suppress the signals of ILs, but this suppression was observed to have induced the distortion of the baseline. However, we also observed that IL signals were suppressed without distortion by distinguishing the IL signals from the signals of cellulose by T_1 (data are not shown here). Therefore, this method could be used to solve the aforementioned problem in some cases.

To realize the potential of the HPILC technique, various ILs should be applied. Because some ILs dissolve other scarcely soluble polymers (summarized in the introduction), these polymers are therefore analyzable. Additionally, ILs can be applied as stationary

phases for reversed-phase chromatography. Indeed, ILs have been utilized as stationary phases in gas chromatography (commercialized by Aldrich), exhibiting unique interactions with the chemical compounds and thus resulting in unique elution patterns. Based on reports of resin-supported ILs, such as silica-supported ILs, the application of ILs in chromatography can soon realize its full potential.

As limitations of the HPILC technique, the applicable measurement conditions and applicable types of ILs are the most problematic because the maximum pressure of the stationary phase for SEC is very low (<15 MPa/30 cm), compared to that employed in reversed-phase chromatography. Although there are great number of ILs, low-viscosity ILs (that also, of course, exhibiting a melting point below room temperature) are not as numerous. Indeed, functional ILs are likely to be highly viscous. To date, we have already tried to use 1-allyl-3-methylimidazolium chloride ([Amim]Cl) as an eluent of HPILC. The viscosity of [Amim]Cl is known to be greater than 2000 cP at 25 °C (viscosity of $[C_2mim][(MeO)(H)PO_2]$ is 107 cP). Thus for application as an eluent, [Amim]Cl must be heated to over 90 °C and eluted at a flow rate of 0.01 ml/min. As a result of applying high temperature, cellulose was not detected (perhaps it decomposed). However, it should be noted that the latest HPLC systems could solve this problem by applying a slower flow rate (minimum 0.001 ml/min). From the perspective of measurement conditions, a very slow flow rate represents one type of limitation. One measurement requires over 9 hours to complete, representing a barrier to making HPILC a commonly used method. We have already observed that a flow rate of 0.02 ml/min (a flow rate of 0.01 ml/min was used in Chapters 4 and 5) can be applied, but it is not sufficient for general use.

In summary, the analytical methods described in this thesis are potentially powerful tools in view of their adaptability and range of application and will be vital in polymer chemistry, biochemistry,

analytical chemistry, and other areas. We therefore believe that the analyses will have a great impact on chemistry.

List of Publications

Original Papers

1) Kosuke Kuroda, Haruhito Kunimura, Yukinobu Fukaya, and Hiroyuki Ohno "^{1}H NMR Analysis of cellulose in ionic liquids", *Cellulose*, **21**, 2199-2206 (2014) [Chapter 2]

2) Kosuke Kuroda, Haruhito Kunimura, Yukinobu Fukaya and Hiroyuki Ohno, "^{1}H NMR evaluation of polar and non-deuterated ionic liquids for selective extraction of cellulose and xylan from wheat bran", *ACS Sustain. Chem. Eng.,* in press (2014). **DOI:** 10.1021/sc500407a [Chapter 3]

3) Kosuke Kuroda, Yukinobu Fukaya, and Hiroyuki Ohno, "HPILC evaluation of polar ionic liquids for extraction of cellulose from biomass", *in preparation* [Chapter 4]

4) Yukinobu Fukaya, Atsushi Tsukamoto, Kosuke Kuroda, and Hiroyuki Ohno, "High performance "ionic liquid" chromatography", *Chem. Commun.*, **47**, 1994-1996 (2011) [Chapter 4]
Hot Article

5) Kosuke Kuroda, Yukinobu Fukaya, and Hiroyuki Ohno, "Direct HPILC Analysis on Cellulose Depolymerisation in Ionic Liquids", *Anal. Methods*, **5**, 3172-3176 (2013) [Chapter 5]
Cover Article & Hot Article & Most Downloaded Article

Other Publications

Books and Reviews

1. 松本拓朗、黒田浩介、税田祥平、鶴巻晃子、阿部充、田口怜美、藤田恭子、西村直美、中村暢文、大野弘幸、「イオン液体の略称」イオン液体サーキュラー　第 1 号、2013