Direct HPILC Analysis on Cellulose Depolymerisation in Ionic Liquids

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Change of average molecular weight and molecular weight distribution of cellulose in a polar ionic liquid (IL) were analysed by high performance liquid chromatography with the polar IL as an eluent (HPILC). 1-Ethyl-3-methylimidazolium methylphosphonate was used as the polar IL. As a model of partly depolymerised cellulose, a mixed sample composed of cellulose and cello-oligosaccharides (glucose, cellobiose, cellotetraose, and cellohexaose) was evaluated to test the resolution of the HPILC. In the resulting chromatograms, corresponding peaks for each saccharide were found. Hydrolysed cellulose catalysed by cellulase mixture in water was then prepared, dried, dissolved in the polar IL to analyse its molecular weight distribution. The molecular weight distribution changed depending on the enzymatic reaction time. The peak for cellulose was found to decrease with increase of that for cellobiose, and subsequently the peak for cellobiose decreased with increase of the peak for glucose. In addition, cellulose oligomers except for cellobiose were scarcely observed, showing the catalytic feature of cellulase. Depolymerisation of cellulose in the polar IL was also carried out using ultrasonication. The peak for cellulose in the HPILC profiles shifted to high retention volume side and broaden with the sonication time, strongly suggesting random depolymerisation of cellulose. Thus, HPILC was confirmed to be effective for the dynamic analysis of cellulose depolymerisation.

Introduction

Ionic liquids (ILs), organic salts with melting points below 100 °C, have attracted much interest as functional media, especially for the cellulose treatment so as to produce sustainable bioenergy.[1-4] To convert cellulose into energy in ILs, there are three essential processes: extraction of cellulose from biomass,[5, 6] hydrolysis of cellulose into glucose or cellobiose,[7-11] and energy conversion *via* alcohol fermentation[12] or biofuel cells.[13, 14] To this end, we have developed a new class of polar ILs comprising several derivatives of both carboxylate and phosphonate as anions;[11, 15-17] these ILs enable to extract cellulose at ambient temperature. In spite of these developments in biomass processing using ILs, there are few reports on analytical methods to detect profiles of cellulose dissolved in ILs. For instance, while NMR spectroscopy has been used to directly estimate components in the extracted polysaccharides,[18-20] there has been no method for dynamic analysis of cellulose in ILs during hydrolysis. Hydrolysed cellulose in ILs has generally been evaluated stepwise with 3,5-dinitrosalicylic acid (DNS) assay[7] or by high performance liquid chromatography (HPLC)[9] 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.

To overcome these problems, HPLC with ILs as eluents (HPILC) is a method for tracing cellulose hydrolysis in polar ILs in view of the molecular weight distribution (MWD) without pretreatment. We preliminary reported HPILC with 1-ethyl-3-methylimidazolium methylphosphonate ($[C_2mim][(MeO)(H)PO_2]$) as an eluent and MWD analysis of cellulose using the HPILC.[21] These observations suggest that HPILC should be well suited for the MWD analysis of time dependent change of cellulose including hydrolysis. It also makes possible to shorten the pretreatment process and to prevent disturbing sample composition due to direct injection of the samples. Here we analysed time depending change of the MWD of cellulose in $[C_2mim][(MeO)(H)PO_2]$. As a method for breaking cellulose down into low-molecular weight fragments in $[C_2mim][(MeO)(H)PO_2]$, ultrasonication was applied because this did not contaminate the sample solution unlike catalysts or other additives did.

Experimental

Materials

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. Cello-oligosaccharides purchased from Seikagaku Corporation and microcrystalline cellulose powder (cellulose powder C) purchased from Advantec Toyo Co. were used after drying under reduced pressure.

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

$[C_2mim][(MeO)(H)PO_2]$ was synthesised according to the

method as described previously.[21] 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. Viscosity of $[C_2mim][(MeO)(H)PO_2]$ at 25 and 55 °C was 107 and 25.4 cP, respectively.

Methods

Setup of HPILC

Components in the HPLC system used were high pressure durable pump (KHP-011; SIC), an injector (7725; Rheodyne) with a 5 μL loop, 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 μm, and KW-405-4B, 4.6 mm (inner diameter) × 50 mm, 5 μm; 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 $mL \cdot min^{-1}$. Pullulan standards from Showa Denko with molecular weight ranging from 5,000 to 800,000 Da 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. Typical pressure for driving HPILC was 11 MPa at 55 °C. For data acquisition and processing we used the software package SIC-480 II XP (SIC).

Solubilisation of saccharides

Suspensions of saccharides (cellulose, cello-oligosaccharides, pullulan) in dried $[C_2mim][(MeO)(H)PO_2]$ were prepared under dry nitrogen gas atmosphere. The mixtures were gently stirred at room temperature until the solution became homogeneous and clear.

Immobilisation of cellulase mixture onto polymer beads

Cellulase mixture from *Trichoderma reesei* (Kyowa Hakko Kirin Co.), methacrylic beads having hexamethylamine as the functional group (EC-HA; Mitsubishi Chemical Corporation), and 25% glutaraldehyde aqueous solution (Tokyo Chemical Ind. Co.) were used as received. The beads (2.5 g) were added to 4.5 ml of 40 mM phosphate buffer (pH 7.5) with 2.5% glutaraldehyde and the resulting mixture was stirred for 2h at 25 °C. After washing the beads with phosphate buffer, 3.75 ml of buffer solution was added and 2.0 g of cellulase mixture was added to the solution and stirred for 10h. The beads were then washed with Milli-Q water to remove impurities of cellulase mixture. The resulting clear solution was then freeze-dried.

HPILC analysis of enzymatic hydrolysis

The immobilised cellulase (8.0 wt%) and cellulose (0.50 wt%) were dispersed into 0.40 g of Milli-Q water. The resulting solution was heated at 60 °C in an oil bath and stirred. After hydrolysis, the hydrolysates were quickly frozen in liquid nitrogen to stop the reaction without contamination of the solutions. Each sample was freeze-dried, and subsequently 0.40 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 for removing cellulase prior to HPILC measurement.

Determination of glucose concentration with a glucose test kit (enzymatic method)

A method for determination of glucose concentration by a test kit (glucose CII-test; Wako Pure Chemical Industries) was follows. The hydrolysed samples (20 μl) were added to 3.0 ml of the solution of the test kit and held for 5min at 37 °C. At this time, glucose was converted to gluconic acid and H_2O_2 by glucose oxidase. As successive reaction arising from H_2O_2, phenol and 4-aminoantipyrine were coupled by peroxidase. The UV absorbance of the samples at 505 nm was measured with a UV-vis spectrophotometer (UV-2450; Shimadzu) and converted into the glucose concentration (r^2= 0.999).

Determination of the glucose concentration with HPILC

The glucose concentration was calculated from the standard relation between the peak height of glucose and the glucose concentration (see ESI† , Fig. S1). In this calculation, the density of $[C_2mim][(MeO)(H)PO_2]$ was measured by density/specific gravity meter (DA-100; Kyoto Electronics Manufacturing Co., Ltd.) as 1.19 $g \cdot cm^{-3}$.

Depolymerisation of cellulose with ultrasonication

Depolymerisation of cellulose was carried out using ultrasonication bath (USS-1; Nihonseiki Ltd.) with an average power output of 35 W and 40 kHz frequency. Cellulose (0.50 wt%) in 2.0 mg of $[C_2mim][(MeO)(H)PO_2]$ were depolymerised at 40 °C under stirring in a nitrogen gas atmosphere, and thus the sample solution was directly injected into HPILC. The average molecular weight of cellulose was calculated with software SIC-480 II XP (SIC) by comparing to the retention volumes of pullulan (as a model of polysaccharide) standards.

Results and Discussion

Investigation of resolution in HPILC using a model of depolymerised cellulose

As a typical model of depolymerised cellulose, a mixture of cellulose and several cello-oligosaccharides (glucose, cellobiose, cellotetraose, and cellohexaose) was analysed 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 °C resulting in a clear homogeneous solution. The solution was injected directly into the HPILC system. Fig. 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. For more investigation, we analysed the relation between molecular weight and retention volume, by measuring retention profile of pullulan (Mw: 800,000 to 5,000) and glucose (Mw: 180) as standard substances (see ESI† , Fig. S2). These plots are in different retention volumes, similar to usual HPLC measurements. Taking these results into account, resolution of HPILC was comparable to usual HPLC even when using a polar IL as an eluent, which is a highly viscous liquid. We see that HPILC is an effective tool for the analysis of crude samples like

hydrolysed cellulose.

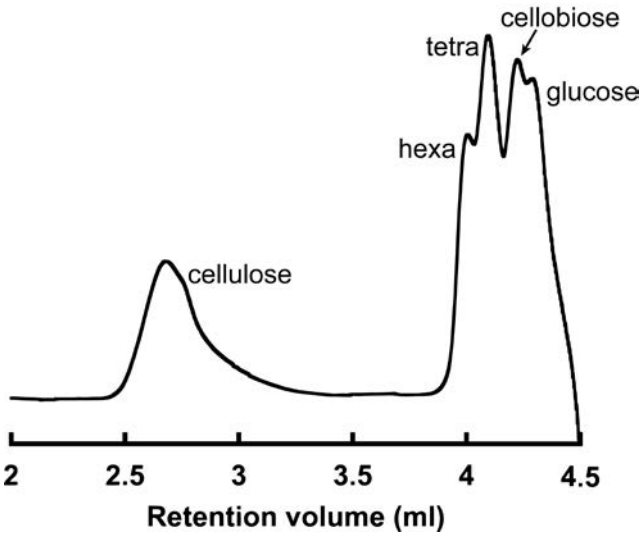

Fig.1 HPILC profile of a mixed sample composed of cellulose and cello-oligosaccharides.

Analysis of cellulose hydrolysis with cellulase mixture in water

Based on the results mentioned above, we expected that cellulose hydrolysis would be analysed 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 hydrolyse cellulose by cellulase due to high crystallinity of the cellulose. We used cellulose with low crystallinity, which was cellulose that has been dissolved in $[C_2mim][(MeO)(H)PO_2]$, subsequently precipitated by addition of ethanol and dried under reduced pressure. This regenerated cellulose can easily be hydrolysed by cellulase, because of its low crystallinity.[22, 23] For a biocatalyst, we used a cellulase mixture that included endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL). EG hydrolyses cellulose at random, and CBH hydrolyses cellulose to cellobiose. After hydrolysis of cellulose to cellobiose, BGL converts cellobiose into glucose. To avoid contamination of cellulase in the reaction medium before HPILC measurement, we herein used cellulase immobilised onto acrylic beads and filtrated the samples before measurements.

Fig. 2 shows chromatograms of hydrolysed cellulose, measured by HPILC. The peak for cellulose decreased, and two peaks corresponding to glucose and cellobiose appeared together with uncertain peaks. The HPILC profiles shown in Fig. 2 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 that produces mainly glucose and cellobiose. These results also show that different activity of EG, CBH, and BGL. First, EG hydrolysed 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

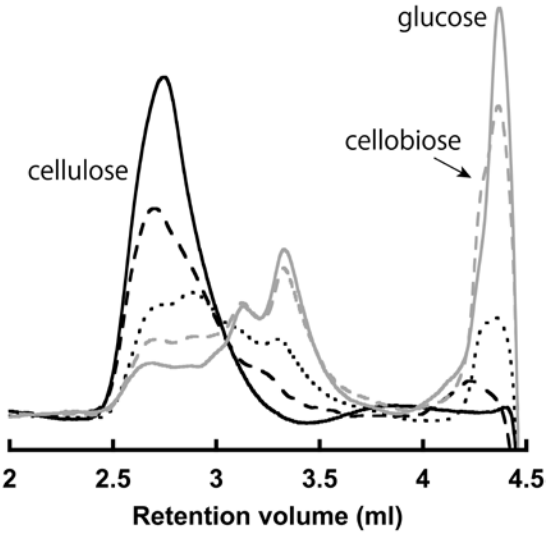

Fig. 2 Chromatograms of hydrolysed cellulose in water with immobilised cellulase at different reaction times: 0h (black continuous line), 5min(black dashed line), 15min (black dotted line), 1.5h (grey dashed line), 6h (grey continuous line).

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 analysed by HPILC. For investigating the capability of HPILC in quantitative analysis, the glucose concentrations were calculated from chromatograms of HPILC in Fig. 2 and two more chromatograms and they were compared to the glucose concentration calculated from conventional enzymatic methods (Fig. 3). We have already found that the glucose concentration can be calculated from the peak height of chromatograms of HPILC (see ESI† , Fig. S1). A similar trend on glucose concentrations 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 analyse the hydrolysis of cellulose, having the advantages of HPLC, MWD analysis and quantitative analysis.

Direct analysis of cellulose depolymerisation in ILs *via* ultrasonication

According to the results in Figs. 2 and 3, HPILC is expected to be effective to analyse cellulose depolymerisation in ILs. Then we next analysed the depolymerisation of cellulose in an IL without

pretreatment. There is however no report of efficient cellulose

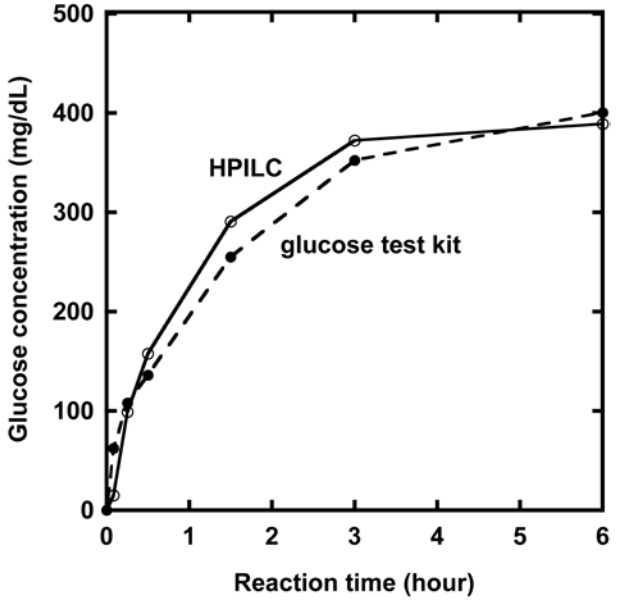

Fig. 3 Glucose concentration of hydrolysed cellulose samples at different reaction times determined by HPILC and a glucose test kit (Wako Pure Chemical Industries).

hydrolysis in ILs which can dissolve cellulose at room temperature. As an alternative method of hydrolysis, we focused on ultrasonication in tracing the depolymerisation of cellulose in ILs. Ultrasonication can depolymerise 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 depolymerise cellulose, and the resulting samples were injected directly into our HPILC system. Fig. 4 shows change in the profile of depolymerised cellulose as the function of ultrasonication time. The peak for cellulose shifted to higher retention volume side as the ultrasonication time increased. The average molecular weight of cellulose decreased with

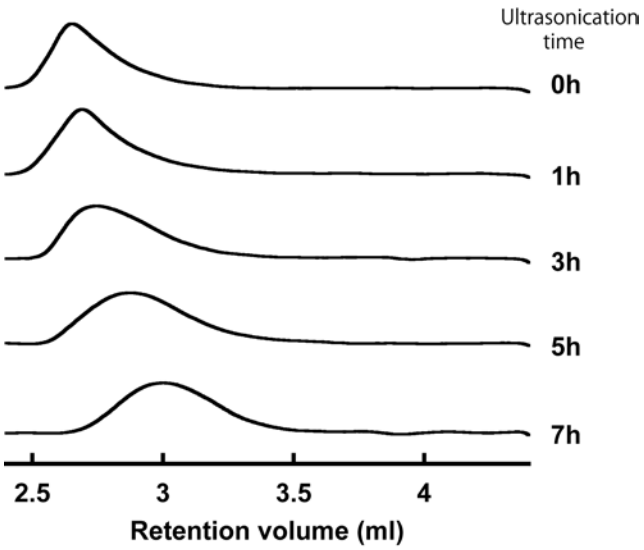

Fig. 4 Chromatograms of cellulose depolymerised by ultrasonication at the different ultrasonication time.

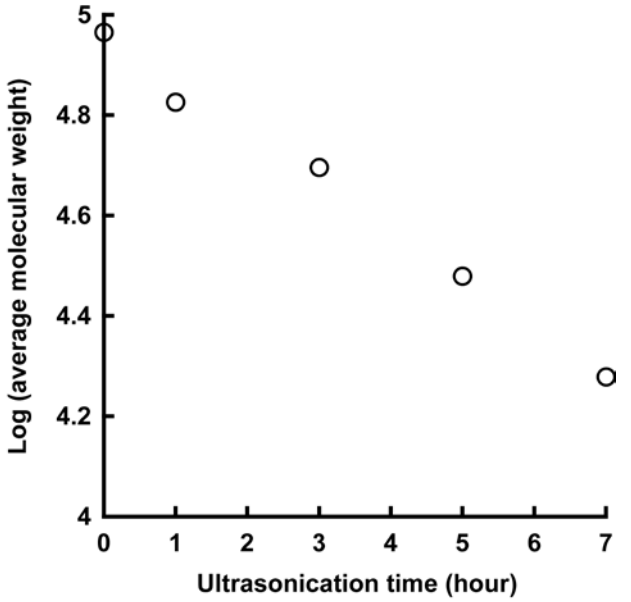

Fig. 5 Relation between the ultrasonication time and the logarithm of the average molecular weight of depolymerised cellulose.

ultrasonication. No peaks for low molecular weight saccharides were found as seen in Fig. 4; retention volume from 4.0 to 4.4 ml, in contrast to enzymatic hydrolysis (see Fig. 2). From this change of chromatograms, it was confirmed that the depolymerisation of cellulose by ultrasonication occurred in random.

To study cellulose depolymerisation in detail, the average molecular weight of cellulose was depicted as the function of ultrasonication time (Fig. 5). Average molecular weight of cellulose was calculated with the standard relation obtained by the preliminary experiments with pullulan (see Fig. S2). The average molecular weight decreased with the increase of ultrasonication time. Our results indicate that depolymerisation of cellulose in ILs can be analysed directly by HPILC.

It was found that HPILC was a powerful tool to study the dynamics of cellulose hydrolysis in ILs. Furthermore, since ILs can dissolve many other scarcely soluble polymers, HPILC should also be effective to analyse them.

Conclusions

We found that high performance liquid chromatography using ILs as eluents, called HPILC, is a powerful tool for the analysis of cellulose depolymerisation in ILs. Cellulose and cello-oligosaccharides were analysed 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 analysed. It was confirmed that cellulose was hydrolysed to glucose *via* cellobiose without generating other oligosaccharides. Depolymerisation of cellulose in an IL with ultrasonication was also analysed by HPILC. It was observed that the peak for cellulose simply shifted to high retention volume side and broaden, indicating random depolymerisation, unlike hydrolysis

by cellulase. These results show HPILC should be a potential tool for analysing cellulose in ILs.

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† Electronic supplementary information (ESI) available: Relation between glucose concentration and its peak height in mixed samples; standard relation between molecular weight of saccharides and their retention volume by measuring the retention volume of pullulan and glucose. See DOI:

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