Notes

A Transition from Cellulose Swelling to Cellulose Dissolution by *o*-Phosphoric Acid: Evidence from Enzymatic Hydrolysis and Supramolecular Structure

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Introduction

Cellulose is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable polymer produced in the biosphere (e.g., ~100 billion dry tons/year).[1−3] Cellulose, a common material in plant cell walls, is a linear condensation polymer consisting of D-anhydroglucopyranose joined together by β-1,4-glucosidic bonds with a degree of polymerization (DP) from 100 to 20 000.[1−3] Adjacent anhydroglucose molecules are rotated 180° with respect to their neighbors, and coupling of adjacent cellulose molecules by extensive hydrogen bonds[4] and van der Waals forces[5] results in a parallel alignment and a crystalline structure, which produces a straight, stable heterogeneous supramolecular structure and low accessibility to chemicals and cellulases.[1,6−8] In nature, cellulose is very stable with a half-life of 5−8 million years for β-1,4-glucosidic bonds at 25 °C.[9] Much faster cellulose degradation mediated by cellulase is a very important biological process to return carbon sediments to the atmosphere and would offer promises for the production of biobased products and bioenergy from cellulosic materials.[1,10,11]

Cellulose swelling and cellulose dissolution are two completely different processes. The cellulose swelling process maintains the gross structure of cellulose as a moiety of particles, fibers, or a film (i.e., solid cellulosic phase), despite significant changes of physical properties and an increase in sample volume due to uptake of the swelling agent. Cellulose dissolution is a transition from a two-phase system to a one-phase system (clear solution), in which the original supramolecular structure of cellulose is destroyed.[12] However, there is no clear-cut borderline between a swelling process and a dissolution process: the same system can act either as a swelling agent or as a dissolution agent, depending on the properties of cellulose and operation conditions.[12]

Walseth[13] first developed a procedure for producing a high-reactivity cellulose suitable for cellulase activity studies by swelling air-dried cellulose in 85% phosphoric acid. Since then, phosphoric acid swollen cellulose (PASC) has become one of the most common cellulose substrates for cellulase activity assays,[14−16] with small preparation modifications, such as the removal of phosphoric acid by filtration or centrifugation,[17,18] and cellulose origins—cotton linter or microcrystalline cellulose (Avicel).[14] Since PASC preparation is a cellulose swelling (heterogeneous) process, the quality of PASC varies greatly and strongly depends on operation conditions such as swelling time and temperature, blending time and severity, and efficiency in removing any cellulose lumps.[14,16] The fluctuating quality of PASC results in difficulty in comparing results of cellulase activity assays based on PASC from different laboratories around the world, and even from different batches in the same laboratory.[16]

In this study, we investigated the effects of phosphoric acid concentration on supramolecular structure of and enzymatic hydrolysis of the treated cellulose. A phase transition from cellulose swelling to cellulose dissolution was observed, when phosphoric acid concentration was greater than a critical value. On the basis of the above finding, we developed a simple procedure for producing a regenerated amorphous cellulose (RAC), precipitated from the dissolved homogeneous cellulose rather than from the swollen heterogeneous cellulose.

Experimental Section

Materials and Chemicals. All chemicals were reagent grade, purchased from Sigma (St. Louis, MO), unless otherwise noted. Microcrystalline cellulose, Avicel PH105 (20 μm), was obtained from FMC Co. (Philadelphia, PA). Whatman CC41 and Whatman no.1 filter paper were purchased from Fisher Scientific Co. (Pittsburgh, PA). Whatman no.1 filter paper was cut to 3.5-mm-diameter disks using a paper punch. A cellulase preparation from *Trichoderma reesei* (Spezyme) was a free gift from Dr. Moniruzzaman at Genencor International Co. (Palo Alto, CA), and β-glucosidase from *Aspergillus niger* (Novozyme 188) was purchased from Sigma. The cellulase and β-glucosidase activities were 58 filter paper unit (FPU)/ml and 385 IU/ml, respectively.[19]

Regenerated Amorphous Cellulose Preparation. Approximately 0.2 g of microcrystalline cellulose (FMC PH-105) was added to a 50-mL centrifuge tube, and 0.6 mL distilled water was added to wet the cellulose powder to form a cellulose-suspended slurry. Ten milliliters of ice-cold 86.2% H_3PO_4 was slowly added to the slurry with vigorous stirring so that the final phosphoric acid concentration was ca. 83.2%. Before the last 2 mL of phosphoric acid was added, the cellulose suspension solution must be mixed evenly. The cellulose mixture turned transparent within several minutes, and stood for about an hour on ice with occasional stirring. Approximately 40 mL of ice-cold water was added at a rate of approximately 10 mL per addition with vigorous stirring between additions, resulting in a white cloudy precipitate. The precipitated cellulose was centrifuged at ~5000 g and 4 °C for 20 min. The pellet was suspended by ice-cold water, followed by centrifugation to remove the supernatant containing phosphoric acid four times. Approximately 0.5 mL of 2 M Na_2CO_3 was added to neutralize the residual phosphoric acid, and then, 45 mL of ice-cold distilled water was used to suspend the cellulose pellet. After centrifugation, the pellet was suspended by distilled water and centrifuged twice or until pH 5−7. The regenerated amorphous (homogeneous) cellulose slurry can

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be kept for a long time at ~4 °C by adding a small amount of sodium azide. The carbohydrate concentration of RAC was calibrated by the phenol−H_2SO_4 method.[19] Sigmacell 20 can be used to replace FMC PH105, since both have the same quality (P. J. Weimer, personal communication).

Enzymatic Cellulose Hydrolysis. Enzymatic cellulose hydrolysis was carried out in a 50-mL reaction solution containing 50 mM citric acid buffer (pH 4.8) and 10 g/L cellulose, with an enzyme loading of 15 FPU Spezyme cellulase/g cellulose plus 60 IU cellobiase/g cellulose, until otherwise noted, in a rotary shaker at 200 rpm at 50 °C. One milliliter of the enzyme hydrolysate sample was withdrawn using a 1-mL pipet and transferred to a 2-mL centrifuge tube containing 10 μL of 10 N NaOH at time intervals as indicated. After centrifuging, the supernatant was measured for the soluble sugars released. The pellet was suspended in 1 mL of 1.1% SDS, boiled for 5 min, centrifuged, and washed in 75% (v/v) ethanol three times, followed by 1 mL of distilled water washing. The pellet was then suspended in distilled water and used for reducing end assay by the modified BCA method.[19]

Scanning Electron Microscopy and X-ray Diffraction. The supramolecular structure of cellulose materials was analyzed by high-resolution scanning electron microscopy (SEM, FEI XL-30) equipped with a field emission electron gun. The cellulose sample was deposited onto a polished silicon substrate by casting a droplet of cellulose suspension solution and then blowing to dry by nitrogen gas. Prior to the SEM experiment, a 2−3-nm Au thin film was evaporated onto the sample surface in order to avoid a charging effect during SEM testing. Images were taken at 15 kV with magnification as indicated in the figures. The crystallinity index of cellulose was determined by the X-ray diffraction method elsewhere.[1,20]

Cellulose Dissolution by DMAc/LiCl. Fifty milligrams of cellulose was added to a tube with a screw mouth. Cellulose was activated by being soaked in water, freeze-dried, soaked in acetone, and freeze-dried. Five milliliters of DMAc plus 8% LiCl was added to the tube. The tube was vacuumed and flushed by nitrogen two times and heated in a heat-block at 100 °C by vortexing frequently. The dissolved cellulose in DMAc/LiCl was precipitated to solid amorphous cellulose by adding acetone as described elsewhere.[21]

Sugar and DP Measurement. Total soluble sugar in the hydrolysate supernatant was measured by the phenol−sulfuric acid method after centrifugation.[19,22] The reducing end concentration in residual cellulose was determined by the modified 2,2′-bicinchoninate (BCA) method, which was conducted at 75 °C.[19] The number-average DP was calculated as the ratio of glucosyl monomer concentration, determined using the phenol−sulfuric acid method, divided by the reducing end concentration, determined using the modified BCA method.[19]

Results and Discussion

The exact concentration of commercial concentrated 85% phosphoric acid was 86.2% (wt/v), according to information from Fisher Scientific Co., and the specific gravity, determined by the Gay-Lussac specific gravity bottle, was 1.71 g/mL. For various concentrations of phosphoric acid (from 70% to 85%), cellulose slurrying in water, prior to addition of phosphoric acid, was developed for expediting phosphoric acid diffusion in the liquid phase and heterogeneous cellulose. When final phosphoric acid concentrations were greater than 81%, except when the dry cellulose was treated by 86.2% phosphoric acid directly, Avicel-suspended solutions turned transparent within several minutes (i.e., the cellulose was dissolved completely). When phosphoric acid concentrations were less than 80%, this transition was not observed. Phosphoric acid concentrations required for dissolving cellulose varied, depending on cellulose origins—microcrystalline cellulose, fibrous cellulose, filter paper, and Sigma α-cellulose, ratio of solvent/cellulose, and reagent temperature (data not shown). Ice-cold phosphoric acid (≥83%) can dissolve all tested cellulosic materials.

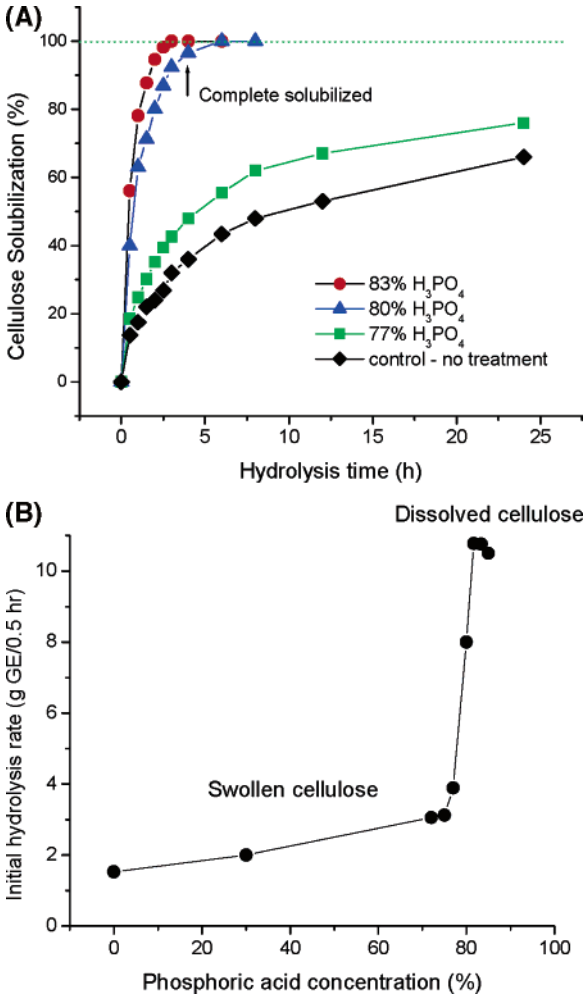

Figure 1. The effects of phosphoric acid concentration on enzymatic cellulose hydrolysis (A) and the initial hydrolysis rates (B). For clarity, not all experimental data are shown in part A.

Figure 1A shows the profiles of enzymatic hydrolysis of 10 g/L intact Avicel, and the samples treated by various concentrations of phosphoric acid at a cellulase loading of 15 FPU/g cellulose and 60 IU β-glucosidase/g cellulose at 50 °C. One hundred percent cellulose solubilization for regenerated amorphous cellulose was obtained after 3 h. At the same cellulase loading, 5 g/L RAC was completely hydrolyzed within a half-hour (data not shown), because of lesser sugar inhibition effects. Figure 1B clearly demonstrates the phosphoric acid concentration effects on the initial enzymatic hydrolysis rates measured within the first half-hour. When phosphoric acid concentrations were less than 80%, enzymatic hydrolysis rates were increased slightly. At a small acid concentration range from 80% to 81.7%, the hydrolysis rate increased to 10.7 g glucose-equivalent (GE)/L/h by 2.7-fold. More than 81.7% phosphoric acid treatment did not enhance hydrolysis rate any further.

To our knowledge, the above specific hydrolysis rate is the fastest hydrolysis rate reported, as compared to results on PASC,[13,23,24] and on other amorphous celluloses prepared by cellulose solvents (NaOH, Cadoxen, LiCl/DAMc)[21,25,26] or by mechanic treatments.[26,27] Phosphoric acid concentration effects on cellulose hydrolysis rates have been studied at high concentration ranges from 72.0% to 78.8%,[23] and 72% to 80%,[28] and the low concentration ranges from 0% to 2%.[29,30] Not surprisingly, an enhancement in hydrolysis rates was always observed with increasing H_3PO_4 levels. This may be the first report to

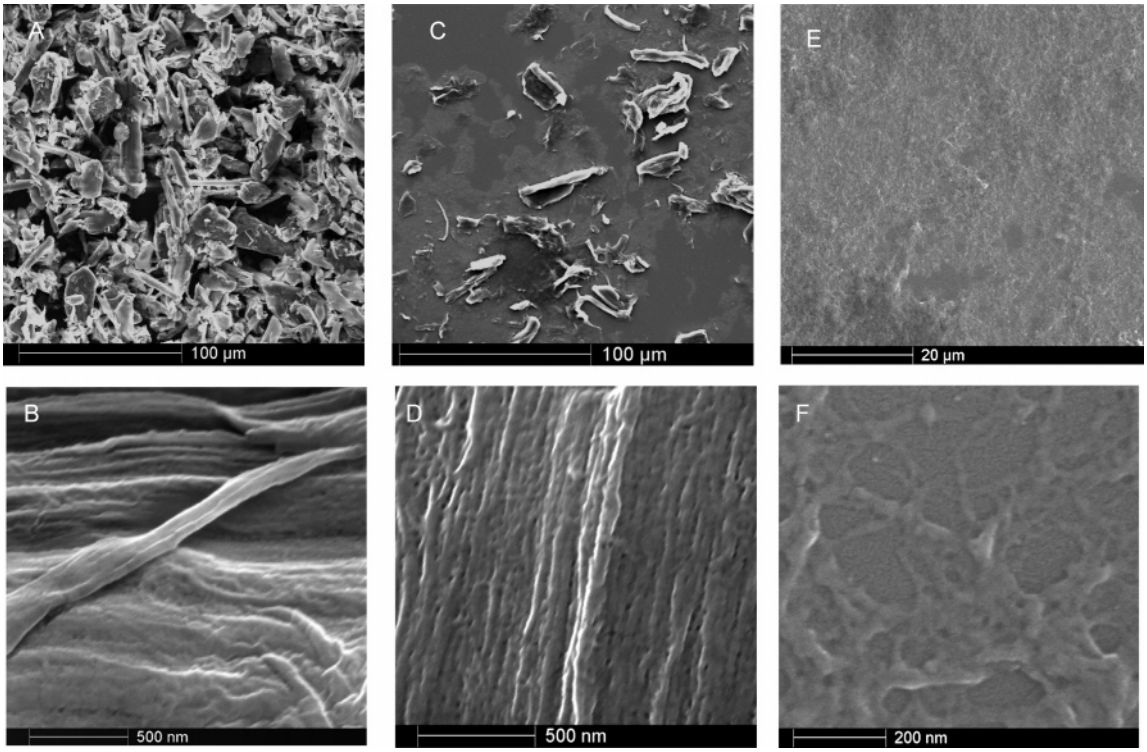

Figure 2. The SEM images of intact Avicel (A,B), the cellulose sample treated by 77% phosphoric acid (C,D), and the sample treated by 83% phosphoric acid (E,F), with two different magnifications as shown in the pictures.

demonstrate the phase transition from cellulose swelling to cellulose dissolution at higher H_3PO_4 levels, although cellulose dissolution by superphosphoric acid has been reported previously.[31]

Cellulose slurrying before addition of concentrated phosphoric acid was the most important modification for rapid cellulose dissolution, because it efficiently avoided the formation of highly viscous cellulose-dissolved gels outside the dry cellulose particles, which occurred often in the PASC preparation procedure—adding concentrated phosphoric acid to dry cellulose. The transition from cellulose swelling to dissolution occurred at a small phosphoric acid concentration range, depending on cellulose origin. When the phosphoric acid concentration was less than that narrow range, cellulose particles were swollen only and the rheology did not change significantly. Therefore, even mixing was easily achieved by manual mixing or by vortexing. Further addition of a small volume of concentrated acid caused the acid concentration to increase to a level high enough to dissolve cellulose. Cellulose slurrying can eliminate mechanic blending in the PASC preparation. Therefore, RAC, prepared from dissolved cellulose rather than swollen cellulose, had a constant quality.

The changes in the supramolecular structures of the treated cellulosic materials in terms of phosphoric acid concentration were also confirmed by the results from scanning electron microscopy (Figure 2). Figure 2A,B shows the supramolecular structure of nontreated Avicel at different magnifications. Nontreated Avicel has a particle size of around 20 μm and has a smooth surface at a higher magnification. Images of the swollen cellulose treated by 77% phosphoric acid (Figure 2C,D) show that a significant fraction of Avicel is digested by the acid, resulting in smaller particle sizes, and many small holes are found on the surface of the residual cellulose particles, resulting in a higher hydrolysis rate (Figure 1). When the phosphoric acid concentration was greater than a critical value

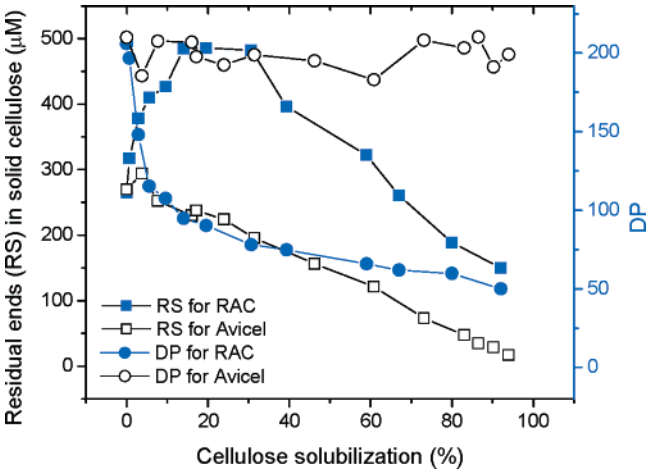

Figure 3. The relationship between the reducing ends and DPs in residual cellulose for RAC and Avicel in terms of cellulose solubilization. For RAC, the enzyme loading was reduced to 2 FPU/g cellulose.

(~80.5% for Avicel), it dissolved cellulose completely. Regenerated cellulose from the dissolved cellulose did not have any original supramolecular structure of the cellulosic particles or fibers after precipitation (Figure 2E). Under higher magnification, no orderly structured fibrillar form was observed, as shown in Figure 2F. The regenerated cellulose was entirely amorphous, as confirmed by X-ray diffraction (data not shown).

Figure 3 shows profiles of the reducing ends of solid cellulose and DP in terms of cellulose solubilization for regenerated amorphous cellulose and Avicel. The reducing end concentration of residual solid cellulose of RAC increased nearly twofold when cellulose solubilization was limited (less than 4%), resulting in a rapid reduction in DP from 206 to 115. Similar patterns have been widely observed in soluble cellulose deriva-

tives, carboxymethyl cellulose hydrolysis by endoglucanase and cellulase mixtures,[32] suggesting that regenerated amorphous cellulose is a homogeneous substrate that enables endoglucanase to cleave all β-glucosidic bonds randomly, resulting in a rapid reduction of DP. The greater number of ends of RAC generated by endoglucanase enabled other hydrolysis components including cellobiohydrolyase I and II to efficiently solubilize shorter insoluble cellulose fragments to soluble cellobiose, resulting in the fastest enzymatic hydrolysis rate reported. In contrast, heterogeneous cellulose (Avicel) hydrolysis had a different pattern, nearly constant DPs at various conversions, suggesting that endoglucanase cleaved β-glucosidic bonds only on external cellulose chains, followed by processive erosion by exoglucanases. Hydrolysis of internal β-glucosidic bonds did not occur until they were made accessible by the action of exoglucanases. The peeling or progressive surface erosion mechanism has been found for other heterogeneous celluloses, including pretreated softwood,[33] cotton fibers,[34] and bacterial microcrystalline cellulose.[35]

Cellulose dissolution in phosphoric acid involves two main processes: (1) an esterification reaction between alcoholic hydroxyl grounds of cellulose and phosphoric acid to form cellulose phosphate(H_3PO_4 + cellulose $\Leftrightarrow$ cellulose$-o$-PO_3H_2), and (2) a competition of hydrogen-bond formation between hydroxyl groups of cellulose chains and hydrogen-bond formation between one hydroxyl group of a cellulose chain and a water molecule or with a hydrogen ion.[12] Meanwhile, another by-reaction is acid hydrolysis of β-glucosidic bonds of cellulose, but such acid hydrolysis can be minimized by decreasing the dissolution temperature.[3,19,36] During the regeneration process by water, cellulose phosphate reversibly converts back to free phosphoric acid and amorphous cellulose without any significant substitution and recrystallization.

As compared to most of the other cellulose solvents,[20,37] phosphoric acid dissolves cellulose simply, fast, and at low temperature, because the hydrogen ion from phosphoric acid is very small and can easily diffuse into heterogeneous cellulose. In addition, the regenerated cellulose remains an amorphous structure and has high reactivity. We also tested another regenerated cellulose from the dissolved cellulose by lithium chloride/*N*,*N*-dimethylacetamide (LiCl/DMAc) followed by addition of acetone or water.[21] The regenerated cellulose formed lots of flocs, which were difficult to disperse and handle (data not shown).

The dramatic changes in the hydrolysis rates of the cellulosic samples before and after phosphoric acid treatment were due to the changes in supramolecular structures (accessibility) but not due to acid hydrolysis, because (1) there was no change in the DP before and after treatment (Figure 3), and (2) it was well-known that stronger ice-cold inorganic acids such as nitric acid can dissolve cellulose without any significant degradation. For example, Boerstoel et al. reported a limited hydrolysis of cellulose during cellulose dissolution in superphosphoric acid at elevated temperature.[31]

Conclusion

The RAC was prepared more easily by a series of steps, cellulose slurrying in water, dissolution in ice-cold phosphoric acid, and regeneration in water, because no mechanical blending was required for the most widely used PASC. As compared to PASC, the RAC had a constant quality because of regeneration from homogeneous dissolved cellulose, had easy handling and transferring properties, was a homogeneous substrate without any chemical substitution impacting both endoglucanase and exoglucanase activities, and had an extremely high reactivity even for very low cellulase activity assays. The dramatic increases in enzymatic hydrolysis rate and enzymatic digestibility for the regenerated amorphous cellulose suggests that increasing substrate accessibility by cellulose solvents including concentrated phosphoric acid could be a new potential approach for lignocellulose pretreatment.

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