

Design and Test Improved Assays for Endoglucanases and Exoglucanases
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Task 1. Further adapt the BCA reductometric technique to measuring activity of cellulases on insoluble high-molecular weight cellulose and lignocellulose, including cotton cellulose, bacterial cellulose from *Acetobacter xylinum*, and at least one lignocellulosic feedstock of interest to the NREL Ethanol Project.

The modified BCA method, which was developed and reported to NREL during the first two months of the subcontract, employed bacterial cellulose from *A. xylinum*. The work is now in progress to extend this procedure to cotton cellulose, which was obtained from Prof. Roy Cantrell, Department of , New Mexico State University.

Task 2. Apply the modified BCA method to characterizing endoglucanases selected by the NREL Research Monitor on insoluble high-molecular weight cellulose and lignocellulose. The substrates may include cotton cellulose or bacterial cellulose, and lignocellulosic feedstock(s) of interest to the NREL Ethanol Project. Differentiate between endo- and exo-mode of enzyme action based on the ratio between insoluble and soluble reducing end-groups formed during the enzymatic hydrolysis.

In our previous report we indicated that most of purified enzymes that we have were obtained 5-8 years ago, and recent measurements have shown that their activity significantly decreased over time. As a result, the quantity of purified enzymes is not sufficient to perform extensive kinetic studies employing both the BCA (Task 2) and the HPSEC-MALLS (Task 5) methods. The specific objective for this project, however, is to use BCA in combination with HPSEC-MALLS to contrast endo- and exo- mode of enzyme action on the basis of the $RS_{\text{insoluble}}/RS_{\text{soluble}}$ ratio measured by the BCA and molecular-weight distributions obtained by the HPSEC-MALLS.

To perform this work, we need milligram quantities of at least two purified cellulases, one endoglucanase and one cellobiohydrolase. We are asking the NREL Research Monitor to consider our request for such enzymes. The combinations of the enzymes that can be used for this study may include *T. reesei* EGI/*T. reesei* CBHI or *A. cellulolyticus* E1/*T. reesei* CBHI. The latter combination is of special interest because *A. cellulolyticus* E1 endoglucanase is one of the most active cellulases known (Himmel *et al.*, 1994). When used on cellulose in combination with the CBHI from *T. reesei*, E1 gave the highest saccharification and degree of synergism of all cellulase binary systems tested (Baker *et al.*, 1995; Himmel *et al.*, 1997).

Task 4. Develop the HPSEC-MALLS method for analysis of insoluble high-molecular weight cellulose using LiCl/DMAc solvent system. Substrates used for this task will include cotton cellulose or bacterial cellulose.

We have completed an extensive search of the literature for methods used to dissolve cellulose in DMAc/LiCl. The result of this work is summarized in Table 1. Based on this information, we decided that the method described by Timpa (1991) is the most promising for dissolution of high-molecular weight cellulose. This procedure requires preliminary activation of cellulose using hot vapors of DMAc. Another procedure used in our laboratory for dissolution of Avicel (DP 100-250), phosphoric-acid swollen cellulose (DP 166) and $ZnCl_2$ -treated cellulose (DP 129) requires pre-swelling the cellulose in water followed by solvent exchange to DMAc (Johnston, 1997). We have found that this procedure was not able to dissolve cotton or bacterial cellulose. Important advantage of the procedure reported by Timpa (1991) is that it is essentially a “one pot” procedure and therefore attractive for handling large number of samples.

In Table 2 we summarized conditions used by different research groups for GPC analysis of cellulose in DMAc/LiCl solvent system. Based on these data, we decided that Waters Styragel HT Columns would be the best choice for our application. These columns are designed for use in the mid-to-high molecular weight range, especially for applications requiring high-temperature solvents. Five different types of packing are available, with pore size ranges from low (100 D) to high (10,000,000 D) molecular weight. In addition, a mixed bed Styragel HT 6E column, ranging from molecular weight of 5000 to molecular weight of 10,000,000 is available as a general purpose workhorse column. A possible column choice for our application is a combination of one Styragel HT 2 column (MW range 100 - 10,000) with two Styragel HT 6E columns. While such a combination does not provide the highest resolution analysis, it is the best scouting tool for unknown samples or for samples with broad molecular weight distribution (Neue, 1999).

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Table 1. Dissolution of cellulose in DMAc/LiCl

Reference	Source of cellulose	Dissolution Procedure	Comments
McCormick, 1981	<p>Baker reagent grade, 75-150 μm particle size.</p> <p>Powdered cellulose (Polysciences, Cat. # 0230).</p>	<p>Preparation of cellulose solution.</p> <p>15 g of cellulose (Baker) was suspended in 1500 ml of DMAc, which contained 75 g of LiCl. The system was heated from room temperature to 150°C over 1-1.5 h with stirring, and kept at 140-150°C for 10-20 min. Then the system was cooled to room temperature slowly with stirring. The cellulose was dissolved after cooling to room temperature.</p> <p>Precipitation of cellulose from solution.</p> <p>2-3 grams of powdered cellulose was added to 100 ml of a DMAc/5% LiCl solvent system. The mixture was heated to 150°C with stirring until a dark brown color emerged and the solution was clear. The polymer was precipitated in absolute methanol and filtered through Whatman # 1 filter paper using a Buchner funnel and 500-ml vacuum Erlenmeyer flask. The cellulose precipitate was returned to another 100 ml of a DMAc/5% LiCl for redissolving and the filtrate was concentrated under vacuum with stirring (no heat). The concentrate was dried in a vacuum desiccator for 48 h. The resulting residue had a very high relative viscosity.</p>	<p>Specific dissolution technique was developed.</p> <p>The homogeneous solutions of cellulose in DMAc/LiCl are prepared by adding the cellulose (up to ~3% w/w) with stirring to DMAc containing up to 8% LiCl w/w and heating and maintaining the resulting mixture at ~150°C. If the cellulose does not dissolve, the mixture can be alternately heated to ~100°C and cooled to ~50°C until solution is obtained.</p> <p>It has also been discovered that not only purified cellulose but apparently cellulose from any source, including cotton linters, wood and paper, can be used. It appears that only the rate of dissolution changes with the different type of cellulose source due to the MW, crystallinity and lignin content.</p> <p>In additions, it has been discovered that by dissolving cellulose and lignin containing materials in DMAc/LiCl the lignin can be separated from the cellulose by differential precipitation using methanol.</p> <p>The LiCl and DMAc are required ingredients of the solvent mixture. The substitution of other lithium salts and organic solvents does not give comparable results.</p>
Turbak <i>et al.</i> , 1981	<p>Low molecular weight cellulose:</p> <p>Prehydrolyzed kraft pulp</p> <p>Cotton linter</p>	<p>Example 1.</p> <p>This example illustrates the activation of cellulose by the use of hot vapors of DMAc. Prehydrolyzed kraft pulp, cut to pass through a 0.125 inch screen, was placed in a flask to which DMAc was added. The mixture was heated for 30 min with continuous stirring at reflux temperature (165°C). The slurry was then allowed to slowly cool to 100°C, at which point anhydrous LiCl was added with constant stirring. The mixture was then allowed to cool to room temperature and left stirring overnight. The composition of mixture by weight percent of cellulose/LiCl/DMAc was 5.9/8.6/85.5. The following morning, the cellulose had completely dissolved. The solution was then diluted with 60 ml of DMAc, centrifuged for one hour and suction filtered. The filtrate was regenerated in water and the intrinsic viscosity determined. (Cotton linter was dissolved to give 3.1/8.8/88.1 solution).</p> <p>Example 2.</p> <p>This example illustrates the activation of cellulose with water, followed by solvent exchange and then dissolution. 280 g of prehydrolyzed kraft pulp were cut into 5x5 inch sheets and soaked in 1.2 L of water at room temperature for 10 min and then passed between two 6x6 inch stainless steel mesh screens with cellulose pulp sheets above and below acting as blotting media. 200 psig pressure was applied for 10 min. The pulp sheets were then soaked in 800 ml of DMAc at room temperature for 30 min and the activated pulp was analyzed for water content (8.5%). The pulp sheets were shredded and then mixed with a solution of 400 g of LiCl in 4000 g total DMAc at room temperature for 3 h. Dissolution was achieved after standing overnight. The solution contained 0.51% water. Cellulose concentrations of up to ~6.5 % were achieved using this technique.</p> <p>Example 4.</p> <p>Steam activation, followed by solvent exchange and dissolution was employed for the preparation of a 2.5 kg solution of cellulose/LiCl/DMAc of composition 6.0/8.5/85.5. Thus, 140 g of prehydrolyzed kraft pulp sheets were cut into 5x5 inch sheets and each was exposed to steam for 2 min, then soaked in 700 ml of DMAc for 1 h with constant agitation. The water content of the exchanged pulp was 5.24% after pressing the sheets at 15,000 psig. The sheets were shredded as described in Example 2 and then mixed with a solution of 200 g LiCl in 2000 g DMAc. Dissolution of the pulp occurred in less than 4 hours at room temperature. The solution contained 0.29% water.</p>	<p>Cellulose is activated by penetration of the cellulose with a polar medium (such as water, steam and liquid ammonia), which swells the cellulose to permit access by the solvent at ambient temperatures, or at least at temperatures low enough to avoid significant polymer degradation (<150°C). It was found that any polar medium used for activation, other than DMAc, would prevent access by the DMAc/LiCl solvent system and, therefore, should be removed to a final concentration less than that of the cellulose and generally less than 5% prior to the dissolution, or dissolution will not be complete.</p> <p>If the polar medium used for activation is not DMAc, the polar medium must then be removed by exchange with either a medium less polar than water or with DMAc. If it is removed by exchange with a less polar medium, it should then be dried prior to dissolution. Upon drying, the pores will not collapse, as they would do when drying cellulose impregnated with a polar medium. Examples of media less polar than water, which do not swell the cellulose, are acetone, methanol, ethanol, isopropanol, acetonitrile and tetrahydrofuran. If the polar medium is removed by exchange with DMAc, the cellulose will dissolve upon addition of LiCl.</p> <p>The cellulose may be soaked in water, squeezed free of excess water and then exchanged with DMAc to produce an activated cellulose ready for dissolution. Steaming the cellulose for several minutes, followed by DMAc exchange also produces activated cellulose, which will readily dissolve under ambient conditions.</p> <p>It has been found that cellulose may be activated by using hot vapors of DMAc. Cellulose mixed with DMAc and the slurry brought to a boil for a short period (~150°C). The heating is stopped, the slurry cooled to about 100°C, the LiCl added and the mixture stirred for several hours until complete dissolution.</p>

Reference	Source of cellulose	Dissolution Procedure	Comments
			<p>LiCl should be present in amounts ranging from 3 to 15% by weight of the solution. The process has been successful in obtaining solutions of as much as 16% by weight of cellulose. A wide variety of cellulose sources may be used in preparing the solutions such as chemical pulps, either sulfite, prehydrolyzed kraft, bleached or unbleached. Cotton linters, reprocessed cellulose are other typical sources of cellulose. Unpurified cellulose (sawdust) can also be utilized but with far less efficiency.</p> <p>The invention is specific to LiCl and either DMAc or 1-methyl-2-pyrrolidinone. A large number of halide salts were tried over various concentrations, temperatures and pressures with various solvents, and produced no dissolution.</p> <p>This procedure was not successful for dissolution of either the native cotton fiber or the extracted cotton fabric (Timpa, 1991).</p>
Turbak, 1983			<p>“Activation” of the cellulose, defined as opening up the cellulose structure to the solvent, is necessary for dissolution.</p> <p>The five best procedures are:</p> <ol style="list-style-type: none"> 1. Water activation – DMAc exchange. 2. Steam activation – DMAc exchange. 3. Water activation – Distillation to less than 2% H₂O. 4. Hot DMAc activation – Cooling prior to LiCl addition. 5. Liquid ammonia activation – DMAc exchange. <p>Any of these methods can be employed to make a solution containing 12% of cellulose (DP 550) and 10% LiCl in about 4-6 h. Only 4% solutions were obtained for higher molecular weight cellulose (DP 1700).</p> <p>Cellulose solutions in DMAc/LiCl were reported to be stable at room temperature for several years. Heating up to 100 °C was not detrimental.</p>
McCormick <i>et al.</i> , 1985	<p>Cat. # 0230 & 4853 from Polysciences, Inc.</p> <p>Cat. # 1525, 1528 & 1529 from Baker Chemical Co.</p> <p>Cellulose powder CF1 from Whatman Ltd.</p> <p>Regenerated cellulose viscose yarn, buckeye cottons 505 & ER-6500 from the Jam River Corp.</p>	<p>Method 1. Cellulose solutions were prepared by suspending cellulose (~1-3%) in DMAc/3-9% LiCl, followed by heating to 150 °C and cooling to room temperature.</p> <p>Method 2. Swelling the cellulose in water overnight followed by solvent exchange through methanol to DMAc and then adding the cellulose to DMAc/9% LiCl:</p> <p>20-50 g of cellulose powder or cotton linters were suspended overnight in 500 ml of DI water. The mixture was placed in a crucible filter, and excess water was removed. 400 ml of dried methanol were added for 30 min and then removed. Four such exchanges were done with dried methanol and, finally, five exchanges with DMAc. After the DMAc treatment, the sample was dried by a steam of nitrogen overnight.</p> <p>The swollen cellulose sample (0.1-30.0 g, approximately 0.05-15.0 g actual cellulose weight) was added to 100 ml DMAc/9% LiCl. The swollen cellulose went into solution at room temperature, and concentrations of up to 15% have been achieved. Complete solutions of 1-5% have been achieved in less than 1 h, while with 6-15% cellulose it took 24-48 h. The actual concentration of cellulose in the swollen material was determined by drying several 1-g samples in a vacuum oven at 60 °C for 48 h.</p>	<p>It has been shown that the DMAc/LiCl is a highly specific, nondegrading solvent capable of dissolving 15% cellulose. The mechanism of dissolution appears to involve hydrogen bonding of the hydroxyl protons of cellulose with the chloride ion, which is in turn associated with the Li⁺(DMAc) macrocation complex.</p> <p>DMAc/LiCl did not react with the cellulose and a true solution was formed.</p> <p>Only 2-3% loss in viscosity was observed after 30 days for a solution maintained at 30 °C.</p>

Reference	Source of cellulose	Dissolution Procedure	Comments
Ekmanis, 1986	Wood sulfate pulp: M_n/M_w 64,700/131,900 or 66,300/175,200 depending on what part of a chromatogram was included in calculations. Softwood sulfite pulp: M_n/M_w 131,900/790,000 or 158,800/795,700 or 265,400/834,900.	H₂O activation : 120 mg/10 ml H ₂ O in a 15 ml graduated centrifuge tube, shake for 30 min, centrifuge for 10 min, remove supernatant with a disposable pipette, compress the fibers with a glass stirring rod, centrifuge for 10 min, remove supernatant. DMAc exchange : add 10 ml dry DMAc, fluff up the sample with a spatula, shake for 15 min and treat as before to remove DMAc. Perform three more exchanges with DMAc. Cellulose dissolution : add DMAc/6% LiCl to a total volume of 10 ml and shake overnight to obtain 1.2% (w/v) cellulose solution. Dilute by a factor of 12 with DMAc to obtain 0.1% cellulose solution in DMAc/0.5% LiCl. Filter through a 0.5 μ Teflon membrane (Millex SR disposable filter unit).	
Ekmanis, 1987a	Wood pulp cellulose	The procedure was based upon Turbak's method (1981). Cellulose was dissolved in DMAc/LiCl by first swelling the cellulose with water and then solvent exchanging with DMAc four times.	The final concentration (0.1% cellulose in DMAc/0.5% LiCl) was compatible with commercial GPC columns.
Ekmanis, 1987b		The procedure was based upon Turbak's method (1981). Cellulose was activated by using hot vapors of DMAc at 150°C, cooled to about 100°C when the LiCl was added, and stirred for several hours until the cellulose was completely dissolved.	A simple "one pot" dissolution procedure was reported. Direct application of the procedure was not successful for preparation of cotton fiber samples for GPC analysis on a consistent basis; however, the results were the most promising of the literature procedures (Timpa, 1991).
Kvernheim & Lystad, 1988	Bleached sulfite pulps from Scandinavian spruce (for rayon production) containing ~3% hemicellulose. Whatman CF1 cellulose powder (M_n 27,500, M_w 68,800). Purified cotton (M_n 212,500, M_w 933,800).	Dissolution of pulp samples was carried out using the method of McCormick <i>et al.</i> , 1985, except for heating to 100°C for 1 h during preswelling with water. Dissolution of Whatman CF1 and cotton cellulose was performed using modification of the procedure by Turbak <i>et al.</i> , 1981. Cellulose (2g) was refluxed (165°C) in DMAc (50 ml) for 20-60 min under an N ₂ atmosphere. The reaction mixture was cooled to 100°C and LiCl (9% w/v) was added. The suspension was stirred for 30 min at 100°C, 2 h at 80°C and 24 h at room temperature. The cotton sample was adjusted to 1% cellulose and 9% LiCl by adding DMAc and LiCl, heated to 100°C for 2 h and stirred for another 48 h period at room temperature before being centrifuged. The concentration of cellulose was found to be 0.7% after the sample has undergone a seven-times repeated shaking, centrifugation and removal of water, followed by freeze drying of the precipitated material.	DMAc/LiCl is the only solvent known to be reasonably inert toward SEC column material. Two methods for cellulose dissolution have been used: (1) preswelling cellulose in water, and (2) activating cellulose using refluxing DMAc. Method (1) was the least degradative (less than 5% reduction in viscosity), but was not able to dissolve Whatman CF1 cellulose and cotton sample. Method (2) dissolved these samples but some degradation was observed.
Kennedy <i>et al.</i> , 1990	Cotton linters, MW 307,000 (by viscosity of carbanilate derivative). Spruce (softwood) sulfite pulp, MW 288,000. Birch (hardwood) sulfite pulp, MW	The cellulose sample (0.015 g) was manually shredded into a centrifuge tube and water (2 ml) was added. The mixture was vortexed for 10 min or stirred with a glass rod to totally wet the sample and was left to stand overnight. The sample was then centrifuged at 3500 rpm for 15 min. Excess water was removed with the aid of the pipette and the sample was squeezed to remove as much water as possible. DMAc (Baker, 2 ml) was added and with the aid of a glass rod, the fiber mass was loosened and then vortexed for 15 min. The mixture was left to stand for further 15 min, then centrifuged. DMAc was removed with the aid of a pipette and the pellet was thoroughly squeezed to be able to remove all the DMAc. The DMAc exchange was repeated a total of eight times. At the end of the last exchange, after the DMAc had been completely removed, 0.75 ml of 10% LiCl (anhydrous) in DMAc (or 1.5 ml for cotton linters) was added into the sample. The mixture was stirred briskly for 30 min and allowed to stand until the solution became clear. The length of time of dissolving varied with the	The amount of LiCl and the length of dissolution time required vary with the type of cellulose sample. A uni- or bimodal distribution of molecular weight may be obtained, depending on the origin of the sample.

Reference	Source of cellulose	Dissolution Procedure	Comments
	344,000.	sample type (from 3 h to overnight). The sample was diluted to 10 ml (or 20 ml for cotton samples) with DMAc to produce a final concentration of 0.15% cellulose or 0.075% for cotton in DMAc/0.75%LiCl. The solution was then filtered through a 0.45 µm Nylaflo (Gelman Sciences Ltd.) membrane filter.	
Timpa, 1991	Cotton fiber from Americal Upland cotton (<i>Gossypium hirsutum</i> L.) variety Texas Marker 1 (TM-1), 98% cellulose Desized, scoured, and bleached 80x80 cotton printcloth Acid-washed cellulose powder (Baker Cat. # 1525)	<ol style="list-style-type: none"> 1. Cotton fiber and fabric were ground in a Wiley mill to pass a 20-mesh screen. 2. A sample of cotton (0.8-1.2% w/v) was added to 5 ml of DMAc in a 10-ml ReactaVial (Pierce) in a heating block (Pierce). The temperature was raised to 150°C and maintained at that temperature with stirring (Teflon magnetic stirbars, 2.5 cm?) for activation of the cellulose (1-2 h). 3. The mixture was allowed to cool to 100°C. Dry LiCl (8% w/v) was added. The temperature was lowered to 50°C and maintained until the cellulose was dissolved (48 h). 4. The vials were shaken for 1-2 h on a laboratory shaker at room temperature and returned to the heating block at 50°C. The solution was quantitatively transferred to a 50-ml volumetric flask and diluted with DMAc. 5. The solution was then filtered through a Teflon solvent-resistant, disposable filter (Miller SR, 0.5 µm, Millipore) prior to injection onto the GPC system. An extraction apparatus (Baker 10) was employed with 10-cm³ glass syringes (BD) fitted onto filters with 4-ml glass vials (WISP, Waters) with Teflon septa held in the small volumetric holder. Vacuum pulled samples through. 6. The dissolution of filtered samples was assessed by GPC analysis (area under peaks), and total solids were obtained by drying 1 ml of sample in a vacuum oven at 60° for 48 h. 	<p>Grinding in the Wiley mill was necessary in order to obtain complete and consistent dissolution.</p> <p>The solvent DMAc/LiCl, which had previously been employed to dissolve cellulose from other sources and the short fibers of low MW cotton linters, has been used successfully to dissolve high MW cotton fibers for examination by GPC.</p> <p>8% LiCl in DMAc was necessary for dissolution of cotton cellulose.</p> <p>This is the first time that the universal calibration concept with the viscometer detector has been applied to cotton cellulose samples.</p>
Timpa & Triplett, 1992	Cotton fiber from Americal Upland cotton (<i>Gossypium hirsutum</i> L.) cultivar Texas Marker 1 (TM-1)	<p>Samples were dissolved according to the procedure reported by Timpa (1991) with the following modifications:</p> <ol style="list-style-type: none"> 1. 2. Ground fiber (1.5% w/v) was added to 3 ml of DMAc. Activation time was 1 h. 3. Dissolution time was 24-48 h. 4. No shaking at room temperature. Final sample concentration was 0.9-1.5 mg/ml in DMAc/0.5% LiCl 5. 6. 	Cellulose from fibers at primary cell wall stages had lower MW than the cellulose from fibers at the secondary wall stages.
Timpa & Ramey, 1994	Sets of well characterized cotton fiber samples	As previously described (Timpa, 1991; Timpa & Triplett, 1992)	Correlation of higher average MW with greater strength of cotton fibers has been established.
Benedict <i>et al.</i> , 1994	Crystalline microfibrillar fragments isolated by treating different cotton fibers with acetic acid/nitric acid reagent	<p>As reported by McCormick <i>et al.</i>, 1985.</p> <p>24 mg of the ground sample was hydrated in 5 ml H₂O for 24 h with five changes. The crystalline cellulose was treated with 5 ml methanol for 24 h with five changes followed by treatment with 5 ml DMAc for 24 h with five changes. The cellulose was solubilized in 4 ml 60 g/L LiCl in DMAc overnight. This preparation was diluted 1:12 with DMAc and filtered through a 0.45 µm teflon filter prior to GPC analysis.</p>	The M _w and MWD of the crystalline cellulose remaining after acid hydrolysis of cotton fibers is correlated to the bundle fiber strength.
Triplett & Timpa, 1995	<i>Gossypium hirsutum</i> TM-1 fiber cells from ovule culture	As previously described (Timpa, 1991; Timpa & Triplett, 1992)	

Reference	Source of cellulose	Dissolution Procedure	Comments
Striegel & Timpa, 1995	Cellulose 4 (J. T. Baker Chemical Co., Cat. # 1525-1) and cellulose 5 (Baker, Cat. #1528-1). Also, amyloses, amylopectins, arabinogalactan, curdlan, decalcified chitin, dextrans and pullalans. M _w range 20,000 - 5,000,000.	<p>Samples were dissolved generally following the procedure reported for cotton by Timpa, 1991 with the following modifications: No grinding. Initial polysaccharide concentration 6 mg/ml. Initial LiCl concentration 5% w/v. Final polysaccharide concentration 0.6 mg/ml in DMAc/0.5% LiCl. Cellulose activation was carried out for 1 h.</p> <p>Polysaccharide (30 mg) was added to 5 ml DMAc in 10-ml ReactaVials TM with a colical magnetic stirrer in a heating block. The temperature was raised to 150°C and maintained with stirring for 1 h.</p> <p>The mixture was allowed to cool to 100°C, and 0.250 g of dry LiCl was added. The vials were shaken by hand and returned to the heating block, where the mixture was maintained with stirring at 100 °C for 1 h. The temperature of the block was lowered to 50°C, and the samples were stirred at this temperature overnight.</p> <p>The solutions were quantitatively transferred to 50-ml volumetric flasks and diluted to volume with DMAc.</p> <p>The solutions were then filtered through a solvent-resistant Teflon disposable filter. An extraction apparatus was employed with 10-ml glass syringes fitted onto filters with 4-ml glass vials held in the small volumetric holder.</p>	<p>DMAc/LiCl has the capability of dissolving a wide variety of representative polysaccharides differing in molecular weight, branching, linkage, and anomeric configuration, without the need for prior extraction, derivatization, or fractionation.</p>
Striegel & Timpa, 1996	Cellulose 5 (J. T. Baker Chemical Co., Cat. # 1528-1). Pullulan (Pfanstiehl, Cat. # 12474).	As previously described (Striegel & Timpa, 1995).	
Johnston, 1997	Phosphoric-acid swollen cellulose (PSC), DP 166, MW 27,000. ZnCl ₂ -treated cellulose (ZTC), DP 129, MW 21,000.	<ol style="list-style-type: none"> Cellulose (4 mg) was wetted with 1 ml of water and allowed to stand overnight. Hydration could be accelerated by boiling the cellulose for 2 h with addition of more water as needed. The hydrated cellulose was centrifuged and the water removed by aspiration using a Pasteur pipette. One ml of DMAc (HPLC grade) was added and the suspension mixed by vortexing and “flicking the tube”. The sample was left to stand for 15 minutes and again centrifuged. The DMAc was removed by aspiration using a Pasteur pipette and 1 ml fresh DMAc was added. This process of addition and removal of DMAc was repeated a total of 8 times. When the last DMAc portion was removed, 100 µl of 10% LiCl in DMAc was added at 80°C. The solution was mixed and allowed to stand until clear (heating at 80 °C speeds dissolution). Clearing may take up to 48 h depending on the degree of crystallinity of the cellulose, but it typically dissolved rapidly. The sample was diluted with DMAc to 1 ml. The final concentration of LiCl was 1%. The final concentration of cellulose was determined using the Dubois method. 	<p>The amount of cellulose that can be dissolved appears to depend on cellulose chain length and crystallinity. Shorter chain lengths can give higher concentrations, and higher crystallinity decreases the final amount that can be dissolved.</p>
Silva & Laver, 1997	Softwood kraft, softwood sulfite, and hardwood kraft pulps bleached using the specified sequences. Unbleached hardwood kraft pulps. Rayon, straw fiber, cotton linters, commercial acid-washed cellulose.	<p>Cellulose solutions were prepared by a modified method described by Timpa (1991).</p> <p>Ground pulp samples were suspended in DMAc (1.2%) and heated to 150°C for 0.5 - 2.0 h. After the solutions were cooled to 100°C, dry LiCl (8%) was added. The mixture was then constantly stirred and cooled to 50°C for 12-48 h. The mixtures were stirred for another 6-24 h at 23°C until all solids were completely dissolved. The clear solutions were transferred to a 50-ml volumetric flask and diluted with DMAc. The solutions were filtered through a Millipore 0.45 µm Millex-HV filter unit before SEC injection.</p>	<p>Longer activation, dissolution, and stirring times were necessary in cellulose that was more crystalline, higher in MW, and higher in lignin content.</p>

Reference	Source of cellulose	Dissolution Procedure	Comments
Einfeldt & Klemm, 1997	Bacterial cellulose (BC) from <i>Acetobacter xylinum</i>	<p>After cultivation, the formed BC pellicles were collected from the medium and purified to remove the nutrient medium and the immobilized bacterial cells from the cellulosic network. The purified wet cellulose pellicles were freeze-dried. The water content in the obtained BC samples was 3%.</p> <p>Freeze-dried BC was swollen in DMAc for 1 h at room temperature. An adequate amount of dried LiCl was added to obtain 5-9% salt solutions, and the mixture was stirred for 24 h at room temperature again. Well-dried BC was dissolved by this procedure to give highly viscous 0.5% solutions. Sometimes no dissolution could be achieved at room temperature. In this case only highly swollen mixtures of BC in DMAc/LiCl were obtained. Heating of such mixtures at 120 °C for several hours was not successful for dissolving the BC.</p> <p>Alternatively, the swollen BC could be treated by solvent exchange from water to methanol to DMAc obtaining cellulose soluble in DMAc/LiCl at room temperature.</p>	This is the first time that cellulose samples (freeze-dried BC) were directly dissolved in DMAc/LiCl at room temperature. The authors assumed that unsuccessful dissolution attempts of BC were due to small differences in the moisture content of BC.
Strlic <i>et al.</i> , 1998	<p>Untreated and oxidised cellulose samples from two sources:</p> <p>Cellulose linters powder, M_n 93,500, M_w 449,000 (Fluka).</p> <p>Cellulose fibrous, long, M_n 49,500, M_w 96,500 (Sigma).</p>	<p>5 mg of sample was weighed into a 10-ml centrifuge tube into which was added 5 ml Milli-Q water, and left overnight to allow the fibers to swell thoroughly. The samples were centrifuged at 4000 rpm for 15 min, after which the supernatant was decanted and 5 ml of DMAc (Fluka) was added. After 15 min of heavy stirring with a PTFE bar, the centrifugation and decantation was repeated. The whole solvent exchange procedure was repeated three times. Finally 1.25 ml of 8% (w/v) LiCl in DMAc was added, stirred for 60 s and left for approximately 24 h to dissolve completely, with occasional mild stirring (100 rpm). The solutions were then transferred into 10-ml volumetric flasks and diluted with DMAc to give a concentration of 0.05% (w/v) cellulose and 1% (w/v) LiCl.</p>	
Emsley <i>et al.</i> , 2000	Cotton linters	<ul style="list-style-type: none"> • The samples were mulched in distilled DI water using a laboratory blender and soaked in water overnight. • The pulp was passed through a grade 2 sintered filter. • Rinsed thoroughly with methanol and mixed with methanol 2-3 h (sealed container to avoid evaporation). • Filtered as before and washed with DMAc. • Mixed for 2-3 h with dry DMAc. • Filtered as before. • The pulp removed from the sintered filter and added to DMAc/8% LiCl (20 ml). • Stirred until a clear solution had formed (2-48 h, depending on the sample). • Diluted with DMAc to final concentration of LiCl of 1% 	

Table 2. GPC analysis of cellulose dissolved in DMAc/LiCl

Reference	Analyzed materials	Columns	Temp, °C	Eluent	Flow rate, ml/min	Injection volume, μ l	Conc.	Run time, min	Standards / Calibration Procedure	Detection	Determined parameters
Ekmanis, 1986	Wood sulfate pulp with DP ~ 500. Softwood sulfite pulp.	Three 30-cm cross-linked SDVB* Ultrastyrigel columns, 10^3 , 10^4 , and 10^5 A (Waters). Efficiency 7700 p/ft.	80°C	DMAc/ 0.5% LiCl**	1.0	300 μ l (100 μ l per column)	0.1%	40	Polystyrene standards 370 - 2,880,000 D. MW vs. V_R .	Waters 410 RI detector	Wood sulfate pulp: M_n/M_w 64,700/131,900 or 66,300/175,200 depending on what part of a chromatogram was included in calculations. Softwood sulfite pulp: M_n/M_w 131,900/790,000 or 158,800/795,700 or 265,400/834,900 – close to exclusion volume.
Kvernheim & Lystad, 1988	Bleached sulfite pulps from Scandinavian spruce (for rayon production) containing ~3% hemicellulose. Whatman CF1 cellulose powder. Purified cotton.	Styrigel columns 10^3 to 10^6 A	30-45°C	DMAc/ 0.5% LiCl	1.0 ~1500 psi	40 μ l			Pullulan Shodex P-82 standard kit (Showa Denko KK). MW vs. V_R .	Waters 410 RI detector	Bleached sulfite pulps: M_n 37,100-68,100, M_w 383,200-420,400. Whatman cellulose: M_n 27,500, M_w 68,800. Purified cotton: M_n 212,500, M_w 933,800.
Kennedy <i>et al.</i> , 1990	Cotton linters, MW 307,000 (by viscosity of carbanilate derivative). Spruce (softwood) sulfite pulp, MW 288,000. Birch (hardwood) sulfite pulp, MW 344,000.	PL gel mixed A° column (Polymer Laboratories Ltd., UK), 10 μ , 0.75 x 30 cm. Guard column, 0.7x5 cm, packed with 10 μ 100 A° packing material (Polymer Laboratories Ltd.).	80°C	DMAc/ 0.5% LiCl	1.0				Pullulan standards (Polymer Laboratories Ltd.): MW 5,800 – 853,000 lgMW vs. V_R/V_{GlC} .	A Knauer GPC system equipped with a Model 98 differential refractometer	Cotton linters: MW_{Peak} 319,400. Spruce: MW_{Peak} 365,300/17,600 (bimodal MWD). Birch: MW_{Peak} 547,000/23,600 (bimodal MWD).
Timpa, 1991	Cotton fiber from Americal Upland cotton (<i>Gossypium hirsutum</i> L.) variety Texas Marker 1 (TM-1), 98% cellulose. Desized, scoured, and bleached 80x80 cotton printcloth. Acid-washed cellulose powder (Baker Cat. # 1525).	Ultrastyrigel 10^3 , 10^4 , 10^5 and 10^6 Waters columns. Guard column (Phenogel, linear; Phenomenex).	80°C	DMAc/ 0.5% LiCl	1.0	400 μ l (100 μ l per column)	0.08-0.12%	60	Polystyrene standards from Toyo Soda Manufacturing (10,300 – 2,890,000). Universal calibration (lg($MW \times [\eta]$) vs. V_R).	Viscometer detector (Viscotek Model 100). Waters 410 RI detector.	Cotton fiber: M_w 1,830,000, DP_w 11,300. Cotton cloth: M_w 1,310,000, DP_w 8,100. Cellulose powder: M_w 211,000, DP_w 1,300.
Timpa & Triplett, 1992	Cotton fiber from Americal Upland cotton (<i>Gossypium hirsutum</i> L.) cultivar Texas Marker 1 (TM-1)	Ultrastyrigel 10^3 , 10^4 , 10^5 and 10^6 Waters columns. Guard column (Phenogel, linear; Phenomenex).	80°C	DMAc/ 0.5% LiCl	1.0	400 μ l (100 μ l per column)	0.08-0.12%	65	Polystyrene standards from Toyo Soda Manufacturing (10,300 – 2,890,000). Universal calibration (lg($MW \times [\eta]$) vs. V_R).	Viscometer detector (Viscotek Model 100). Waters 410 RI detector.	M_w, DP_w

Reference	Analyzed materials	Columns	Temp, °C	Eluent	Flow rate, ml/min	Injection volume, μ l	Conc.	Run time, min	Standards / Calibration Procedure	Detection	Determined parameters
Timpa & Ramey, 1994	Sets of well characterized cotton fiber samples	Ultrastryragel 10^3 , 10^4 , 10^5 and 10^6 Waters columns. Guard column (Phenogel, linear; Phenomenex).	80°C	DMAc/ 0.5% LiCl	1.0	400 μ l (100 μ l per column)	0.08- 0.12%	65	Polystyrene standards from Toyo Soda Manufacturing (10,300 – 2,890,000). Universal calibration ($\lg(MW_x[\eta])$ vs. V_R).	Viscometer detector (Viscotek Model 100). Waters 410 RI detector.	M_w 1,500,000 – 3,000,000
Benedict <i>et al.</i> , 1994	Crystalline microfibrillar fragments isolated by treating different cotton fibers with acetic acid/nitric acid reagent	Three linear Ultrastryragel columns, 1×10^6 A to 500A (Waters)	80°C	DMAc/ 0.5% LiCl	1.0	200 μ l	100 mg / 200 μ l = 50% (?)		Polyisoprene standards (3,250 - 8,300,000). $\lg M_w$ vs. Retention time.	Waters 150C GPC	Crystalline cellulose from <i>Gossypium hirsutum</i> (TM-1): M_w 183,000, MWD range 800-2,000,000.
Triplett & Timpa, 1995	<i>Gossypium hirsutum</i> TM-1 fiber cells from ovule culture	Ultrastryragel 10^3 , 10^4 , 10^5 and 10^6 Waters columns. Guard column (Phenogel, linear; Phenomenex).	80°C	DMAc/ 0.5% LiCl	1.0	400 μ l (100 μ l per column)	0.9-1.5 mg/ml	65	Polystyrene standards from Toyo Soda Manufacturing (10,300 – 2,890,000). Universal calibration ($\lg(MW_x[\eta])$ vs. V_R).	Viscometer detector (Viscotek Model 100). Waters 410 RI detector.	M_w 3,400,000, M_n 109,000
Striegel & Timpa, 1995	Cellulose 4 (J. T. Baker Chemical Co., Cat. # 1525-1) and cellulose 5 (Baker, Cat. # 1528-1). Also, amyloses, amylopectins, arabinogalactan, curdlan, decalcified chitin, dextrans and pullalans.	Three 10 μ m Mixed-B columns (Burdick & Jackson/Polymer Laboratories). Guard column (Burdick & Jackson/Polymer Laboratories).	80°C	DMAc/ 0.5% LiCl	1.0	100 μ l and 150 μ l	0.6 mg/ml	34	Polystyrene standards from Toyo Soda Manufacturing (10,300 – 2,890,000). Universal calibration ($\lg(MW_x[\eta])$ vs. V_R).	Viscometer detector (Viscotek Model 100). Waters 410 RI detector.	Cellulose 4: M_w 180,000, M_n 27,000. Cellulose 5: M_w 330,000, M_n 42,000. Max M_w of 21,000,000 was determined for amylopectin from corn. Measured M_w were in the range from ~ 20,000 to 5,000,000.
Striegel & Timpa, 1996	Cellulose 5 (J. T. Baker Chemical Co., Cat. # 1528-1). Pullulan (Pfanstiehl, Cat. # 12474).	Three 10 μ m Mixed-B columns (Burdick & Jackson/Polymer Laboratories). Guard column (Burdick & Jackson/Polymer Laboratories).	80°C	DMAc/ 0.5% LiCl	1.0	100 μ l	0.6 mg/ml	34	Polystyrene standards from Toyo Soda Manufacturing (10,300 – 2,890,000). Universal calibration ($\lg(MW_x[\eta])$ vs. V_R). Absolute MW determination by MALLS.	MALLS detector (miniDAWN, Wyatt Technology Corp.). Viscometer detector (Viscotek Model 100). Waters 410 RI detector.	<u>Cellulose 5</u> Universal calibration: M_w 330,000, M_n 42,000, R_{g_w} 16.2. LS: M_w 320,000, M_n 28,000, R_{g_w} 14.5.

Reference	Analyzed materials	Columns	Temp, °C	Eluent	Flow rate, ml/min	Injection volume, μ l	Conc.	Run time, min	Standards / Calibration Procedure	Detection	Determined parameters
Johnston, 1997	Phosphoric-acid swollen cellulose (PSC), DP 166, MW 27,000. ZnCl ₂ -treated cellulose (ZTC), DP 129, MW 21,000.	TosoHaas G3000SW _{XL} and G2000SW _{XL} columns Guard column	35°C	DMAc/ 1% LiCl, filtered through a 0.22 μ m nylon filter, degassed	0.4	50–100 μ l, filtered through a 0.22 μ m Teflon syringe filter		80 - 90		MALLS detector (DAWN DSP-F, Wyatt Technology Corp.). ERMA 712 RI detector.	
Silva & Laver, 1997	Softwood kraft, softwood sulfite, and hardwood kraft pulps bleached using the specified sequences. Unbleached hardwood kraft pulps. Rayon, straw fiber, cotton linters, commercial acid-washed cellulose.	Ultrastyrigel 10 ³ , 10 ⁴ , 10 ⁵ and 10 ⁶ Millipore columns. 10 μ m Phenomenex guard column.	80°C	DMAc/ 0.5% LiCl	1.0	400 μ l (100 μ l per column)		62	Narrow distribution polystyrene standards MW vs. V _R	Waters 410 RI detector.	Range of measured MW: M _n 58,200 - 196,900, M _w 212,000 - 645,700, M _z 382,600 - 1,580,850.
Strlic <i>et al.</i> , 1998	Untreated and oxidised cellulose samples from two sources: Cellulose linters powder (Fluka). Cellulose fibrous, long (Sigma).	Crosslinked SDVB columns: PLgel 5 μ m MIXED C 7.5x300 mm (Polymer Laboratories). PLgel 5 μ m GUARD column 7.5x50 mm (Polymer Laboratories)	Room temp.	DMAc/ 1.0% LiCl	0.7 ~1500 psi	100 μ l	0.05%		Pullulan standards from Polymer Laboratories (180 - 1,600,000)	Waters R401 differential refractometer	Untreated cellulose linters powder: M _n 93,500, M _w 449,000. Untreated fibrous cellulose, long: M _n 49,500, M _w 96,500.
Emsley <i>et al.</i> , 2000	Cotton linters	Waters μ Styrigel HT 10 ³ , 10 ⁴ , 10 ⁵ A and Waters Ultrastyrigel 10 ⁶ A.	60°C	DMAc/ 0.5% LiCl, 0.9453 g/ml	0.8	400 μ l		90	Eight narrow MW polysaccharide standards from Polymer Laboratories (8,500-853,000). NBS 706 polystyrene broad standard. Universal calibration technique.	Waters 150-CV (GPCV) incorporating RI and viscometer detectors.	Range of measured M _n : 50,000 - 250,000. MW could be measured up to 630,000