Bioresource Technology 101 (2010) 7410–7415

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of acid-chlorite delignification on cellulose degree of polymerization

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ARTICLE INFO

Article history:
Received 12 November 2009
Received in revised form 3 April 2010
Accepted 13 April 2010
Available online 14 May 2010

Keywords:
Cellulose
Degree of polymerization (DP)
Delignification
GPC
Molecular weight

ABSTRACT

Two types of pure cellulose, Avicel PH-101 and Whatman filter paper, were treated with an acid-chlorite delignification procedure in the presence of varying amounts of incorporated lignin, and the molecular weight distributions and degrees of polymerization (DP) of derivatized cellulose were determined by gel permeation chromatography (GPC). Avicel samples with 0% added lignin showed a DP reduction of nearly 5% during acid-chlorite delignification, compared to a 1% drop in DP with 30% added lignin. Lignin-free filter paper samples showed a DP reduction of nearly 35% after hollocellulose delignification. This drop in DP was reduced to less than 12% for samples which contained 30% lignin. Thus, the presence of lignin in biomass samples minimized the DP reduction of cellulose due to acid catalyzed cleavage during acid-chlorite delignification.

1. Introduction

Delignification is a critical process towards the successful characterization of cellulose and hemicelluloses from lignocellulosic biomass. In addition to lignin removal, a better understanding of the structure and morphology of cellulose and hemicelluloses is key in the effort to reduce biomass recalcitrance, thus improving access to fermentable sugars for biofuel production (Ishizawa et al., 2009; Ragauskas et al., 2006). The presence of lignin in biomass reduces accessibility to cellulose microfibrils. Lignin can also hinder or mask molecular details evident to many spectroscopic techniques. For these reasons, it is essential to remove all or a majority of the incorporated lignin prior to analysis.

Biomass delignification can achieved by a variety of methods. Kraft pulping is the most common industrial process used for the removal of wood lignin; however, it is not preferred on a laboratory scale for isolating hollocellulose for many reasons, including degradation of polysaccharides, extraction of hemicelluloses and incomplete lignin removal (approximately 90–95%). Alkaline peroxide processes are effective for both delignification and removal of hemicelluloses but their use is limited due to extensive degradation of cellulose caused by the peroxide radical (Fang et al., 1999; Sun et al., 2004). Perhaps the most popular and established laboratory method for the removal of lignin from biomass is acid-chlorite delignification utilizing an aqueous solution of acetic acid and sodium chlorite. This method effectively bleaches and then solubilizes lignin at moderate temperatures. Acid-chlorite delignification is selective in the removal of lignin with only trace solubilization of glucan and xylan (Ahlgren and Goring, 1971). However, Kumar et al. (2009) have recently reported that the acid-chlorite delignification process has a significant detrimental effect on cellulose chain length. In the case of pure cellulose from filter paper, a reduction of nearly 75% in average degree of polymerization (DP) was found. The reduction was less, 15%, for lower molecular weight micro-crystalline cellulose. It should be noted that in both cases the acid-chlorite delignification process was conducted in the complete absence of lignin.

Acid-chlorite primarily acts on lignin in biomass, but it can also affect the polysaccharides. According to Grierer (1986), the two most likely scenarios for cellulose degradation during acid-chlorite delignification are acidic cleavage of the glycosidic bonds and/or oxidative degradation of the polysaccharides. Acid hydrolysis is a well established mechanism for the molecular weight reduction of cellulose under even mildly acidic conditions (Millett et al., 1954). The oxidation of cellulose with hypochlorite is non-specific and degradation proceeds most rapidly near a neutral pH (Lewin and Epstein, 1962). Oxidative degradation is limited under acidic conditions (Singh, 1982) and acetic acid is generally added to the delignification procedure to reduce pH. However, the addition of acetic acid increases the likelihood of chain degradation due to acid hydrolysis.

Cellulose chain length, measured as degree of polymerization by a variety of methods, is an important material characteristic that factors into the conversion of the biopolymer to fermentable sugars via enzymatic digestion. The accurate characterization of native cellulose chain length is critical to the study of cellulase performance, particularly in the case of exo-cellulases (Gupta and Lee, 2009). If the current method of acid-chlorite delignification is drastically altering the measured cellulose DP then the process may not

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doi:10.1016/j.biortech.2010.04.029

be suitable for proper DP determination. In the current study, acid-chlorite delignification was performed on a series of pure cellulose samples with varying amounts of incorporated lignin. It was hypothesized that the effects of acid hydrolysis and/or oxidative cleavage on cellulose would be reduced in the presence of lignin.

2. Methods

2.1. Materials

Avicel PH-101 micro-crystalline cellulose and sodium chlorite (80%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (glacial, ACS grade) and methanol (general laboratory grade) were purchased from BDH through VWR International (Westchester, PA, USA). Tetrahydrofuran (OmniSolv, HPLC grade), 1,4-dioxane (OmniSolv) and pyridine (anhydrous, DriSolv) were purchased from EMD Chemicals (Gibbstown, NJ, USA). Phenyl isocyanate (98+%) was purchased from Alfa Aesar (Ward Hill, MA, USA). All reagents were used as received. Polyester Kapak brand sealer bags and Whatman #4 filter paper were purchased from VWR International (Westchester, PA, USA) and a kraft softwood linerboard was acquired from a commercial facility in the southeastern USA. Deionized water was passed through a NANOpure water purification system (18.1 MΩ-cm). Unless otherwise noted the lignin used in this study was softwood lignin isolated from kraft cooking liquors obtained during the kraft pulping of a mature Loblolly pine tree (Froass et al., 1998). Milled wood lignin (MWL) isolated from extractive-free poplar wood was used as a comparison to the kraft lignin utilized in the rest of the study. The milled wood lignin was isolated and purified according to published literature methods (Guerra et al., 2004; Holtman et al., 2006).

2.2. Lignocellulosic sample preparation

Lignocellulosic samples were prepared by combining isolated softwood lignin with two types of pure cellulose, Avicel PH-101 micro-crystalline cellulose and Whatman filter paper. Avicel PH-101 samples (0.50 g total mass) were prepared in duplicate with lignin concentrations of 0%, 1%, 5%, 10%, 15%, 20% and 30% by weight. The appropriate amount of lignin was first dissolved in a mixture of 1,4-dioxane and water (10:1 *v/v*, 11 mL) with magnetic stirring in a 20 mL glass vial with screw cap. Avicel PH-101 was added and the mixture was allowed to stir at room temperature for 2 h. The slurry was then transferred to an aluminum weigh dish and allowed to dry overnight in a fume hood. Filter paper samples (0.50 g total mass) were prepared in duplicate with lignin concentrations of 0%, 1%, 5%, 10%, 15%, 20% and 30% by weight. The appropriate amount of lignin was first dissolved in a mixture of 1,4-dioxane and water (10:1 *v/v*, 11 mL) with magnetic stirring in a 20 mL glass vial with screw cap. Shredded filter paper was added and the mixture was allowed to stir at room temperature for 2 h. The slurry was then transferred to an aluminum weigh dish and allowed to dry overnight in a fume hood. A separate set of samples consisting of filter paper cellulose with 5% added lignin were prepared using milled wood lignin in the place of kraft lignin to compare the effects of each on cellulose DP reduction during acid-chlorite delignification.

2.3. Scanning electron microscopy

The starting Avicel PH-101 and filter paper samples and samples with 30% lignin content, were mounted onto a stage and then coated with gold for two minutes by an Electron Microscopy Services 350 sputter coater. SEM images were acquired via a Hitachi-3400SN scanning electron microscope from Hitachi High Technologies American, Inc. (Pleasanton, CA, USA) at 12 or 15 kV at various resolving powers.

2.4. Holocellulose treatment

Acid-chlorite treatment of cellulose and lignocellulosic samples prepared from Avicel cellulose and filter paper was performed in a reciprocating water bath using sodium chlorite and acetic acid at 70 °C according to a modified literature method (Hallac et al., 2009). Each sample was placed in a Kapak sealing pouch with deionized water (83.3 mL/g biomass), sodium chlorite (0.666 mg/g biomass) and glacial acetic acid (0.666 mL/g biomass). Each bag was then sealed and placed in a reciprocating water bath at 70 °C. After 2 h, a second dose of sodium chlorite and glacial acetic acid was added and the bags were resealed and placed back in the reciprocating water bath at 70 °C. After 2 h, a third dose of sodium chlorite and glacial acetic acid was added and the bags were resealed and placed back in the reciprocating water bath at 70 °C. After a total of 6 h and three doses of sodium chlorite and acetic acid, the samples were removed from the bath, the solids filtered through a sintered-glass filter and washed with copious amounts of deionized water until the filtrate pH was neutral. The samples were dried overnight in a fume hood. Acid-chlorite delignification was also conducted on kraft linerboard pulp. The pulp samples were subjected to two, three, four and five cycles of the above 2 h holocellulose treatment. The linerboard pulp samples were rinsed and dried overnight in a fume hood.

2.5. Klason lignin analysis

The Klason lignin content of the samples after delignification was determined using the Laboratory Analytical Procedures (LAPs) provided by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). This procedure was also used to assess the lignin content of the linerboard pulp starting material and the lignin content of the linerboard pulp after each of the delignification cycles.

2.6. Gel permeation chromatography (GPC) analysis of cellulose

The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were determined by GPC after tricarbanilation of cellulose (Wood et al., 1986; Cohen et al., 2002). Lignin-free cellulose (15 mg) from each sample was placed in separate test tubes equipped with micro stirbars and dried overnight under vacuum at 40 °C. The test tubes were then capped with rubber septa. Anhydrous pyridine (4.00 mL) and phenyl isocyanate (0.50 mL) were added sequentially via syringe. The test tubes were placed in an oil bath at 70 °C and allowed to stir for 48 h. Methanol (1.00 mL) was added to quench any remaining phenyl isocyanate. The contents of each test tube were then added dropwise to a 7:3 methanol/water mixture (100 mL) to promote precipitation of the derivatized cellulose. The solids were collected by filtration and then washed with methanol/water (1 × 50 mL) followed by water (2 × 50 mL). The derivatized cellulose was then dried overnight under vacuum at 40 °C. Prior to GPC analysis the derivatized cellulose was dissolved in THF (1 mg/mL), filtered through a 0.45 μm filter and placed in a 2 mL auto-sampler vial.

The molecular weight distributions of the cellulose tricarbanilate samples were then analyzed on a PSS-Polymer Standards Service (Warwick, RI, USA) GPC SECurity 1200 system featuring Agilent HPLC 1200 components equipped with four Waters Styragel columns (HR1, HR2, HR4, HR6), Agilent refractive index (RI) detector and Agilent UV detector (270 nm) using THF as the mobile phase (1.0 mL/min) with injection volumes of 20 μL. A calibration curve was constructed based on eight narrow polystyrene

standards ranging in molecular weight from 1.5×10^3 to 3.6×10^6 g/mol. Data collection and processing were performed using Polymer Standards Service WinGPC Unity software (Build 6807). Molecular weights (M_n and M_w) were calculated by the software relative to the universal polystyrene calibration curve. Number-average degree of polymerization (DP_n) and weight-average degree of polymerization (DP_w) were obtained by dividing M_n and M_w by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeat unit. Polydispersity index (PDI) was calculated by dividing M_w by M_n. All reported values for molecular weight and degree of polymerization were the average of duplicate samples, except in the case of the untreated material which was the average of six samples for each type of cellulose.

3. Results and discussion

3.1. Preparation of lignocellulosic samples

When preparing the lignocellulosic samples an effort was made to incorporate the lignin into the structure of the cellulose. Rather than creating a dry physical mixture of the two components—lignin and cellulose—the lignin was solubilized before the cellulose sample was added to form a slurry. In this way, the lignin molecules were allowed to closely interact with the cellulose fibrils, coating the outside of the fibrils and penetrating some of the larger pores. The purpose of this procedure was to model the structure of lignocellulosic biomass, in which the lignin is thought to be bundled in and around the cellulose and hemicellulose chains (Fengel and Wegener, 1983; Mozier et al., 2005).

In the case of the Avicel samples, it was difficult to determine the extent to which the lignin was incorporated into the structure of the cellulose particles as the Avicel cellulose is itself a powder. Nonetheless, the lignin treated celluloses particles visually appeared to be homogenous in lignin distribution. Upon examination of the untreated material and the 30% lignin content sample by SEM, it was apparent that the Avicel cellulose particles were indeed coated with lignin. The lignin appeared in the images as small spheres ranging in diameter from 500 nm to some a little over a micron. The lignin coverage was, for the most part, limited to the surface but some lignin particles appeared to have entered some of the larger pores. There were also a small number of larger, free lignin particles that were not bound to the surface of Avicel.

In the case of the filter paper samples it was evident to the eye that the lignin had coated the cellulose fibers. The resulting samples consisted of light brown fibers that became darker with increasing lignin content. Visually, there appeared to be no free lignin powder in the filter paper samples. Upon examination of the filter paper samples by SEM was evident that the lignin had indeed coated the surface of the fibers. The SEM images of the filter paper samples are shown in Fig. 1. Once again, the lignin particles appeared mostly as spheres ranging in diameter from submicron to several microns. Although the surfaces of the larger fibrils were coated with lignin, it appeared that the majority of the lignin particles tended to cluster in and around the smaller microfibrils and the channels between them.

3.2. Delignification

Delignification of the lignocellulosic samples was performed using the acid-chlorite process described in Section 2.4. All samples, regardless of the original lignin content, were subjected to the exact same reaction time (6 total hours), temperature (70 °C) and chemical loading (3 doses of acetic acid and sodium chlorite) during processing. The presence of lignin added a brown hue to the cellulose particles and caused the initial solution to be dark and cloudy. As the delignification process proceeded and the lignin is oxidized, the acid-chlorite solution turned a bright yellow color. In the case of the low lignin concentration samples, those which contained up to 10% lignin, the cellulose appeared white after the first two h treatment and there was no dark residue inside the treatment pouch. This was strictly visual evidence that the majority of the lignin had been removed during the first two hour treatment time. The higher lignin content samples still contained a dark residue and the cellulose had not returned to its original white color after the first of three treatments. After the second treatment, all of the samples except for 30% lignin content samples contained cellulose which was pure white without any trace of dark lignin residue. For the 30% lignin content samples, the cellulose had returned to the original white color and there was no indication as to the presence of lignin after the third acid-chlorite dose and two hour treatment time. The final lignin values after delignification for the originally 1%, 5%, 10%, 15%, 20% and 30% lignin Avicel samples were determined, by Klason lignin content analysis, to be 0.00%, 0.54%, 0.54%, 0.55%, 1.1% and 1.1%, respectively. The final lignin values after delignification for the originally 1%, 5%, 10%, 15%, 20% and 30% lignin filter paper samples were determined to be 0.09%, 0.53%, 0.53%, 0.53%, 1.0% and 1.6%, respectively.

3.3. GPC analysis of Avicel samples

It has been previously reported that the acid-chlorite delignification procedure has a tendency to reduce cellulose molecular weight and degree of polymerization (Kumar et al., 2009), most likely the result of acid hydrolysis and oxidative cleavage of the polymer chain. The degree of polymerization data are shown in Fig. 2. It is apparent that there was little reduction in molecular weight as a consequence of the acid-chlorite process on Avicel cellulose. The untreated material, which was derivatized for GPC but

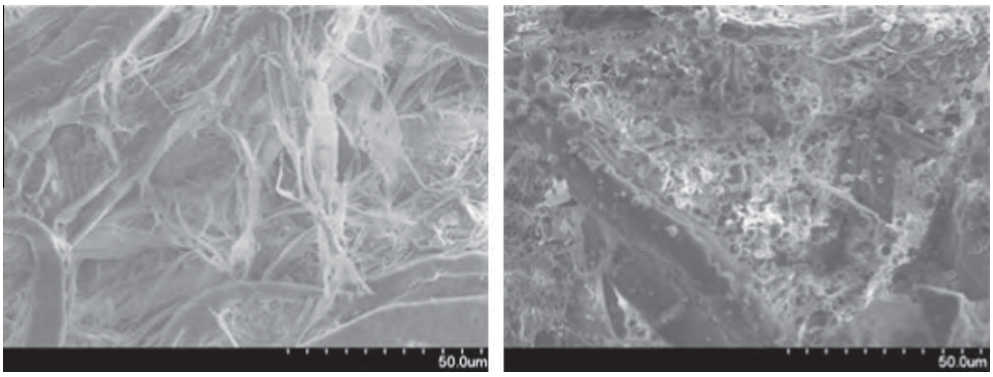

Fig. 1. SEM images of Whatman #4 filter paper samples containing 0% lignin (left) and 30% lignin (right).

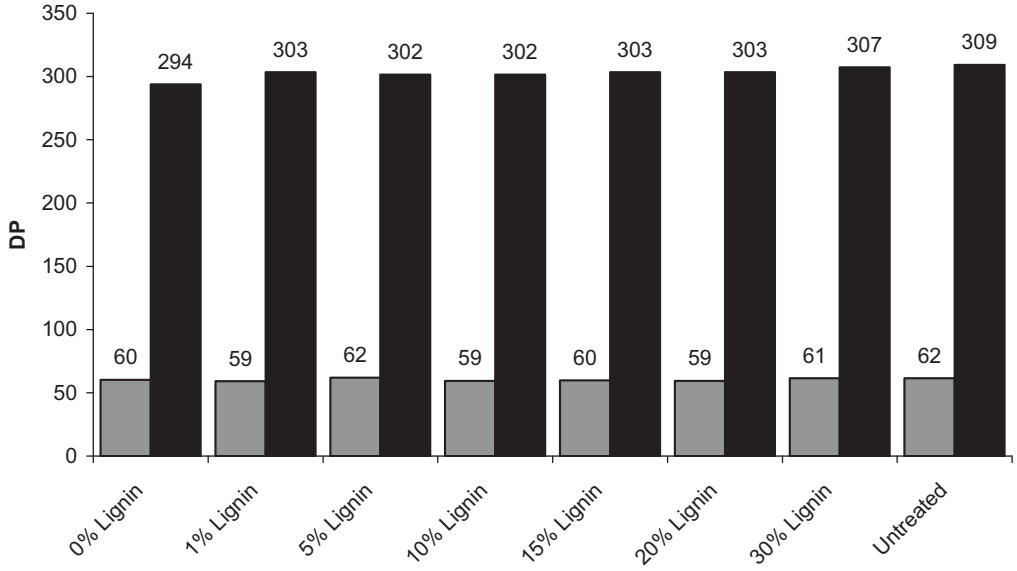

Fig. 2. Degree of polymerization data as a function of lignin content for Avicel PH-101 samples which were subjected to an acid-chlorite delignification procedure. DP_w is displayed in black; DP_n is displayed in grey.

did not receive an acid-chlorite treatment, had an average DP_w of 309. The control sample, pure Avicel PH-101 with no added lignin, which was subjected to the delignification procedure had an average DP_w of 294. The net result of holocellulose delignification caused a ~5% drop in cellulose degree of polymerization. The samples to which lignin had been added prior to delignification resulted in degrees of polymerization that were in between the untreated samples and the 0% lignin samples. The 30% lignin samples had an average DP_w of 307—a decrease of only 1%. The 1% lignin samples had an average DP_w of 303—a decrease of approximately 2%. It appears that the presence of even a very small amount of lignin to Avicel cellulose acts to inhibit chain degradation during acid-chlorite delignification. The polydispersity of the molecular weight distributions also remained nearly constant at a value near 5. This indicates there was little shift in the peak shape of the distribution during delignification.

In general, regardless of lignin content, Avicel cellulose appears to be resistant to drastic changes in degree of polymerization during acid-chlorite delignification. This was attributed to be a consequence of the already low DP of Avicel cellulose and, in part, to its crystallinity. Level-off degree of polymerization (LODP) is a well established theory for acid hydrolysis of cellulose. LODP theory states that acid hydrolysis occurs in two phases—a very rapid DP degradation phase and a slower plateau rate phase (Battista et al., 1956). Thus, there is a portion of cellulose that is more resistant to acid hydrolysis and further reduction in molecular weight (Sharples, 1958). For most native celluloses, this generally occurs in the DP range of several hundred (Immergut and Ranby, 1956; Millett et al., 1954).

3.4. GPC analysis of filter paper samples

In the study by Kumar et al. (2009), it was reported that the acid-chlorite delignification procedure caused a drastic reduction in the average degree of polymerization for cellulose from filter paper. The reported drop was from DP_v of 1500 for untreated samples to approximately 410 for the samples which went through the delignification procedure. That was a reduction of nearly 75% during acid-chlorite delignification as determined by dilute solution viscometry.

The degree of polymerization data for the filter paper cellulose samples are shown in Fig. 3. It is at once obvious that cellulose from filter paper has a much higher molecular weight than Avicel cellulose reported in the previous section. It is also important to notice that filter paper cellulose appears to be more sensitive to the acid-chlorite delignification procedure than was Avicel cellulose because larger changes in molecular weight are apparent between the untreated samples and the 0% lignin control samples. The untreated material, which was derivatized for GPC but did not receive an acid-chlorite treatment, had an average DP_w of 2046. The control sample, pure filter paper cellulose with no added lignin which went through the delignification procedure, had an average DP_w of 1347. The net result of holocellulose delignification caused a nearly 35% drop in cellulose degree of polymerization. Once again, however, the presence of lignin greatly retarded this effect. In the case of the 30% lignin samples, the cellulose DP_w was determined to be 1798—a reduction of approximately 12%. As the lignin content of the samples decreased, the delignification procedure had a more noticeable effect causing the DP to be reduced. But even in the case of the 1% lignin samples the average measured DP was 1706—a reduction of nearly 17%. Once again, hollocellulose delignification had little or no effect on polydispersity indicating that the procedure does not change the breadth or shape of the molecular weight distribution curve. As was the case with the Avicel cellulose, it appears that the incorporation of even a very small amount of lignin to the filter paper cellulose hinders the DP reduction effect of the acid-chlorite delignification procedure.

In an effort to verify the validity of using isolated kraft lignin as the source of lignin for the proposed lignocellulosic model, a set of samples were prepared consisting of filter paper cellulose with 5% added milled wood lignin. The average cellulose DP_w after delignification for the 5% lignin samples using kraft lignin was 1715. The same DP value for that of MWL was 1709. These values are nearly identical and it was concluded that kraft lignin is a suitable source of lignin for use in the model system of this study.

The presence of lignin during hollocellulose treatments reduced cellulose chain cleavage in both Avicel and filter paper. The SEM images of the untreated starting materials and the samples containing lignin show that the lignin is concentrated on the surfaces of the microcrystalline particles of Avicel, and on the surfaces of

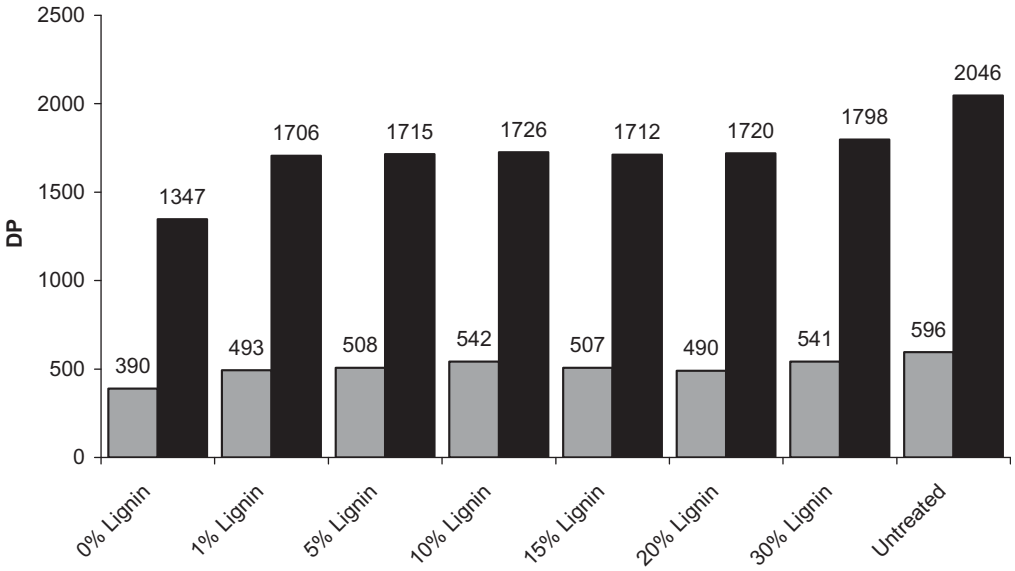

Fig. 3. Degree of polymerization data as a function of lignin content for Whatman #4 filter paper samples which were subjected to an acid-chlorite delignification procedure. DP_w is displayed in black; DP_n is displayed in grey.

the cellulose fibrils in filter paper. Any lignin on the surface of the particles or fibrils could be preferentially degraded before the cellulose chains that lie beneath. Therefore, samples containing more lignin would exhibit less of a cellulose DP reduction and this is exactly what was seen in the experiments.

The filter paper samples subjected to acid-chlorite delignification showed a greater reduction in cellulose DP than what was observed for the Avicel samples. One might attribute this disparity to the difference in crystallinity between micro-crystalline cellulose and cellulose from filter paper. The amorphous regions of the cellulose domain are more accessible to the reagents, thus contributing to an increase in chain degradation. However, a comparison of crystalline indexes for the two substrates show that they are similar. Park et al. (2009) presented crystalline indexes for various forms of cellulose using a variety of techniques and it was concluded that Avicel PH-101 and Whatman filter paper have very similar values. In every case, the Avicel PH-101 was only a few percentage points higher in crystallinity than the cellulose from filter paper. Thus, the difference in crystallinity may contribute to the large gap in DP reduction during acid-chlorite delignification but may not be the only factor.

True lignocellulosic biomass ultrastructure consists of the three primary components of the cell wall—lignin, cellulose and hemicelluloses—in a well-defined relationship in which the lignin serves to protect and add support to the cellulose and hemicelluloses. Lignin is covalently linked to hemicellulose which creates a crosslinked network of lignin with some plant polysaccharides, thus adding mechanical strength to the cell wall and the plant as a whole (Chabannes et al., 2001). The protective effect of lignin is thought to be due, in part, to its hydrophobicity. The incorporation of lignin into the cell wall of plants not only aids water transport during the life cycle but also serves to inhibit degradation of the polysaccharides by chemicals and pests (Sarkanen and Ludwig, 1971). The depressed effect of DP reduction observed in this study as a result of added lignin would most likely be enhanced in a natural lignocellulosic system. Thus, acid-chlorite delignification of natural lignocellulosic biomass should still be an effective method to remove lignin for subsequent DP testing of isolated celluloses. But the results in this study and those by Kumar et al. (2009) highlight a need to cautiously use this procedure and monitor the amount of lignin present. Over hollocellulose treatment of lignocellulosics samples to ensure the complete removal of lignin will lead to significant degradation of cellulose DP.

3.5. GPC analysis of kraft linerboard pulp samples

A series of kraft linerboard pulp samples were subjected to the acid-chlorite delignification procedure to gain greater insight into possible degradation of the polysaccharides during treatment. The Klason lignin content of the unbleached linerboard pulp starting material was 19.1%. The pulp samples were exposed to multiple cycles of the delignification procedure with the lignin content measured after each two hour treatment time. The molecular weight distribution curves for the linerboard pulp holocellulose samples are shown in Fig. 4. The major peak in each chromatogram corresponds to the cellulose component while the minor peak is assigned to hemicellulose. After two treatment cycles the Klason lignin was measured to be 3.2% and there was still a significant amount of hemicellulose present as indicated by the intensity of the secondary peak. After three treatment cycles the Klason lignin was 1.1% and there was a noticeable depreciation in the amount of hemicellulose present; however, the location of the primary cellulose peak had not shifted indicating little or no degradation of the cellulose chains. The weight-averaged molecular weight for the three cycle sample was 2.32×10^6 g/mol. After four cycles of acid-chlorite delignification the lignin content was measured to be 0.47%. At this point the hemicellulose peak had diminished in intensity and the location of the higher molecular weight cellulose peak had begun to shift towards lower weights indicating significant degradation of the cellulose chains. The weight-averaged molecular weight of the four cycle sample was measured to be 2.01×10^6 g/mol. After five treatment cycles the lignin content was reduced to 0.08% and the cellulose peak had shifted even farther to the left. The weight-averaged molecular weight of the five cycle sample was measured to be 1.53×10^6 g/mol, roughly a 25% reduction in cellulose DP in a single treatment cycle.

The above data suggest that lignin in a traditional lignocellulosic sample indeed protects the polysaccharides from degradation caused by acid hydrolysis and oxidative cleavage during acid-chlorite delignification. However, once the lignin content is reduced to below approximately one percent the polysaccharides are susceptible to degradation. At this point the acid-chlorite treatment

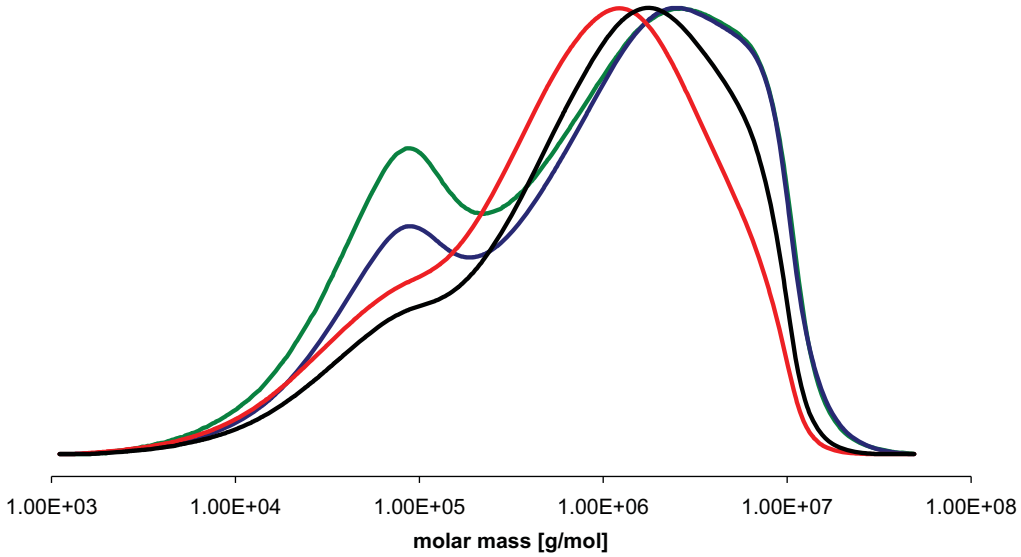

Fig. 4. Molecular weight distributions of derivatized kraft linerboard pulp holocellulose samples subjected to two (green curve), three (blue curve), four (black curve) and five (red curve) cycles of the acid-chlorite delignification procedure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

begins to affect the hemicelluloses and then finally begins to attack the cellulose component. This is further evidence that holocellulose treatment to levels below 1% lignin can cause significant damage to the polysaccharide components.

4. Conclusions

Acid-chlorite delignification of purely cellulosic biomass had a detrimental effect on cellulose degree of polymerization due to acid hydrolysis and/or oxidative cleavage of the cellulose chain. The introduction of even a small portion of lignin to the system greatly reduced this negative DP effect. Upon increased lignin content in the prepared lignocellulosic sample, the drop in DP from the untreated celluloses was minimized. Effective cellulose chain degradation was more prominent in the filter paper cellulose than Avicel cellulose, more than likely due to the higher starting DP of the former.

Acknowledgements

The research was made possible by support from the BioEnergy Science Center (BESC). The BioEnergy Science Center is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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