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Probing the nature of AFEX-pretreated corn stover derived decomposition products that inhibit cellulase activity

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Abstract

Sequential fractionation of AFEX-treated corn stover extracts was carried out using ultra-centrifugation, ultra-filtration, and solid phase extraction to isolate various classes of pretreatment products that are inhibitory to cellulases. (A) dark brown precipitates were removed during ultra-centrifugation that caused no appreciable enzyme inhibition; (B) ultra-filtration using a 10 kDa MWCO membrane removed additional high molecular weight components that contributed to 20-25% of the total observed enzyme inhibition caused by ultra-centrifuged AFEX-treated corn stover extractives, while a 3kDa MWCO removed up to 75%, suggesting appreciable inhibition by oligomeric materials; (C) solid phase extraction (SPE) adsorption of AFEX-treated corn stover extractives before or after ultra-centrifugation, or ultra-centrifugation followed by ultra-filtration, removed about 40% of the inhibitors added to the SPE column. SPE adsorption removed appreciable amounts of hydrophobic inhibitors, and several phenolic compounds identified by mass spectrometry.

‡ AFEX (Ammonia-fiber expansion) is a trademark of MBI Incorporated, Lansing, MI USA.

Keywords: AFEX, extraction, ultra-filtration, fractionation, inhibition
1. Introduction

Cellulosic ethanol is a promising next generation liquid biofuel that can avoid the food versus fuel and land use issues by using waste biomass, including corn stover or switchgrass grown on marginal lands unsuitable for farming (Dale et al., 2010; Service, 2007). To produce cellulosic ethanol, plant cell walls must first be hydrolyzed to monomeric sugars (glucose, xylose, arabinose and mannose) via enzymatic action. However, plant cell walls have evolved to resist enzymatic and microbial catalyzed deconstruction (Himmel, 2008). Recalcitrance to deconstruction can be overcome by disrupting the biomass structure by thermochemical pretreatments; these include treatments with steam, dilute acid, ionic liquids, lime, and ammonia (Chundawat et al., 2011a). Currently, processing improvements are necessary to make cellulosic ethanol cost-effective. Some of these improvements include optimizing pretreatment methods, reducing enzyme use, and improving fermentation methods/strains (Alvira et al., 2010;
Pretreatments enhance enzymatic digestibility of biomass by increasing enzyme access to cellulose. AFEX pretreatment breaks lignin–carbohydrate ester linkages, and partially solubilizes and relocates lignin, phenolics, and hemicellulose fragments to cell wall surfaces (Chundawat et al., 2011b). The resulting porous networks provide improved access to cellulose and enhance rates of cellulose digestion by enzymes (Chundawat et al., 2011b; Teymouri et al., 2005). AFEX also creates a highly fermentable hydrolysate by enriching the biomass with soluble nitrogenous compounds and preserving nutrients in the biomass, unlike most acidic pretreatments (Lau et al., 2008; Chundawat et al., 2010).

Pretreatments can also negatively impact digestion enzymes and fermentation microbes via the inhibitory action of degradation products formed during the process. Therefore, pretreated biomass may require additional processing to mitigate these effects; these methods include solvent-washing, nutrient supplementation, and detoxification, all of which may increase processing costs (Wyman et al., 2011; Sedlak and Ho, 2004; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000; Kazi et al., 2010; von Sivers et al., 1994). Although AFEX produces fewer inhibitors than other pretreatment methods, enzymatic hydrolysis could still be inhibited by certain compounds produced during AFEX. These compounds include phenolics, lignin fragments and a number of other compounds that have not yet been identified.
(Chundawat et al., 2011a; Chundawat et al., 2010; Humpula et al., 2011; Sharma et al., 2009).

In the present study ultra-centrifugation (UC), ultra-filtration (UF) with 10, 5 or 3 kDa molecular weight cutoff (MWCO) filters, and solid phase extraction (SPE) were used to separate the inhibitors in aqueous extracts of AFEX-treated corn stover (AFEX-CS) by density, molecular weight, and hydrophobicity. Cellulase activity assays and liquid chromatography/mass spectrometry (LC/MS) were used to shed light upon the physical and chemical properties of the materials that inhibited enzymes. The results indicate that materials with nominal molecular weights ≤ 10 kDa account for a majority of the inhibition, and both hydrophobic molecules (likely derived from lignin and Maillard reactions) and non-hydrophobic (non-SPE binding) molecules could be significant contributors. Solid phase extraction could offer a reusable means of removing and recovering inhibitors.

2. Materials and methods

2.1. Biomass and AFEX pretreatment

Dry, milled (4 mm particle size) MSU corn stover, harvested from the Michigan State University (MSU) campus was obtained from the MSU Department of Crop and Soil Science. AFEX pretreatment of MSU corn stover was performed as described previously (Chundawat et al., 2010). AFEX pretreatment conditions for corn stover were as follows: 60% biomass moisture content (dry weight basis; dwb), 130 ºC, 15 min
residence time, 1:1 ammonia to biomass loading (w/w). Avicel PH-101, (Fluka, St. Louis, MO) was also subjected to a variety of AFEX pretreatment conditions, ranging from greater severity (130 °C, 45 min reaction time, 1:1 or 3:1 ammonia to biomass loading, zero or 60% added moisture) to lesser severity (90-100 °C, 45 min reaction time, 1:1 ammonia to biomass loading, zero or 60% added moisture).

2.2. Aqueous extraction

All extractions were performed using a Dionex Accelerated Solvent Extractor (ASE 200, Dionex Corporation, Sunnyvale, CA). The moisture content of AFEX-CS or AFEX-treated Avicel (AFEX-A) prior to extraction was determined using a MA 35 gravimetric moisture analyzer (Sartorius, Edgewood, NY). Three grams of AFEX-CS or AFEX-A (to within 0.1 mg of dwb) was transferred into a 22 mL automated solvent extraction cell with a pre-weighed filter frit at the bottom of the cell. The biomass was then extracted at 70 °C and 1500 psi for 7 minutes using distilled water as a solvent, yielding about 30 mL extract and a final 10% solids loading equivalent extract (w/v). The extract was stored at 4 °C. The extracted biomass and filter frit were transferred to a pre-weighed weigh boat and allowed to dry completely in an incubator oven at 50 °C. After the sample was dried, the final weight was calculated to estimate percent mass loss.
2.3. Fractionation of water-soluble extractives

AFEX-CS extract (AFEX-CS-E) was ultra-centrifuged at 205,000 × g for 16 hours at 4 ºC using an Optima L-100 XP Ultracentrifuge equipped with a Type 45 Ti rotor (Beckman-Coulter, USA). The supernatant was collected using a Pasteur pipette. Aliquots of the collected supernatant were ultra-filtered using a 3 kDa, 5 kDa, or 10 kDa MWCO Vivaspin 20 centrifugal filter (GE Healthcare, Waukesha, WI). Ultra-filtered samples were produced by centrifugation at 5,000 × g for 6 hours at 4ºC. The retentate was re-suspended in a volume of distilled water equal to that of the original supernatant. The filtrates and retentates were stored at 4 ºC.

Analytical scale removal of inhibitors from AFEX-CS-E or its fractions was done by elution through SPE cartridges (Waters Corp. (Milford, MA), C-18 Sep-pak (3 ml bed volume, 820 mg sorbent) and Oasis HLB, MAX and MCX (6 ml bed volume, 500 mg sorbent) cartridges). Three cc C-18 Sep-pak cartridges were wetted with 4 mL HPLC grade methanol and rinsed with 4 mL of water. Sixteen mL of AFEX-CS-E or AFEX-A extract (AFEX-A-E) was applied to each cartridge, 4 mL at a time, followed by washing with 4 mL water, and in succession, 5 mL volumes of methanol, isopropanol and DMSO; the first 2 mL of eluate was discarded and the next 4 x 4 mL volumes were collected for triplicate enzyme assay (one column per assay). The organic solvent eluates were also collected. Bio-Beads SM-2 (XAD-2 polystyrene-divinylbenzene resin), employed without initial washing (Bio-Rad Laboratories, Hercules, CA) were also used to test SPE removal of inhibitors by placing 1 g of the resin in a fine mesh nylon bag in 10 mL of AFEX-CS-E in a 50 mL plastic tube. The
tube was placed in a shaker at 50 °C and 200 rpm for 1 h, at which point the bag with resin was removed and the extract was tested immediately for enzyme inhibition.

Preparative scale SPE adsorption was done with AFEX-CS-E directly and with aliquots of 5 kDa MWCO retentate and filtrate, using Sep-Pak Vac 35cc C-18 cartridges (Waters Corp., Milford MA). The cartridge was first flushed with 34 ml of HPLC Grade methanol. It was then rinsed with 34 ml of water. Up to 40 mL of AFEX-CS-E was applied and the first 34 mL was discarded before collecting the rest for enzyme assays and mass spectrometric analysis.

2.4. Enzyme assays

The enzyme assays were carried out using Avicel PH-101, (Fluka, St. Louis MO) at 1% glucan loading in either 15 mL vials (5 mL reaction volume) or in microplates (0.5 mL reaction volume) (Gao et al., 2010; Chundawat et al., 2008). The enzymatic assay was carried out at 6 mg/g glucan protein loading using commercial enzyme mixtures: Accellerase 1000 (Genencor, Palo Alto, CA) with and without an additional 3 mg/g glucan protein loading each of Multifect Xylanase and Multifect Pectinase (Genencor, Palo Alto, CA). Additional assays were conducted with 1:1 mixtures by protein weight of Spezyme CP (Genencor, Palo Alto, CA) and Novozyme 188 (Sigma-Aldrich, St. Louis MO) at a combined 6 to 12 mg/g glucan. The pH was maintained at 4.8 with 50 mM citrate buffer. When present, AFEX extract or fraction comprised 40% of the assay volume. Assay vials or micro-plates were incubated at 50 °C for 24 hours at 250 rpm. After hydrolysis the micro-plates or aliquots from the assay
vials were centrifuged and the supernatant was collected. Glucose released after hydrolysis were assayed using glucose oxidase enzymatic bio-assay kits purchased from R-Biopharm (Marshall, MI) (Gao et al., 2010).

2.5. Acid hydrolysis and sugar analysis by HPLC

Aliquots of each of the fractions collected during the extraction, UC, UF and SPE adsorption steps were analysed for sugar concentrations by HPLC. Monomeric sugar concentrations were measured before acid hydrolysis, while polysaccharide concentrations were calculated by taking sugar concentrations measured after acid hydrolysis and subtracting the monomeric sugar concentrations. For acid hydrolysis, 500 \( \mu \)L of sample and 17.6 \( \mu \)L of 72% sulfuric acid were placed in tightly capped 10 mL culture tubes which were vortexed and placed in a preheated dryblock heater at 121 \( ^\circ \)C for 1 hour, after which they were cooled on ice to room temperature. For sugar analysis, the fractions were filtered using 0.22 \( \mu \)m PES syringe filters (Whatman Inc., Piscataway, NJ), and were analysed using a Prominence HPLC system (Shimadzu, Columbia, MD) with an Aminex HPX-87H HPLC carbohydrate analysis column, a Cation H guard column (Bio-Rad, Hercules, CA) and a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). 5 mM \( \text{H}_2\text{SO}_4 \) was the mobile phase, run at a flow rate of 0.6 mL/min and 50 \( ^\circ \)C. The injection volume was 10 \( \mu \)L.
2.6. **LC/MS analysis**

Degradation products from AFEX-CS-E and its fractions were separated on an Ascentis Express C-18 column (2.1 × 50 mm, 2.7 µm, Sigma-Aldrich, St. Louis MO) using a 20-minute solvent gradient based on 0.15% aqueous formic acid (88%, J.T. Baker, USA) (solvent A) and methanol (solvent B). Eluted analytes were analysed using a LCT Premier TOF mass spectrometer for accurate mass measurement (Waters Corporation, Milford, MA) and electro-spray ionization (ESI) in negative ion mode. Gradient conditions were as follows: initial 99% solvent A/ 1% solvent B, held for 1 min; linear gradient to 1% solvent A/ 99% solvent B at 15 min with a hold until 17 min, going back to the initial condition immediately after 17 min with a hold until 20 min. Injection volume, column temperature and flow rate were 10 µL, 50 °C and 0.25 mL/min, respectively. Spectra were acquired over m/z 50 to 1000.

3. **Results**

3.1. **Inhibition of Avicel Digestion by Aqueous Extracts of AFEX-CS**

Figure 1 shows the effect of AFEX-CS-E on enzymatic digestion of 1% Avicel loading (w/v). In the absence of AFEX-CS-E, Accellerase at 6 mg/g glucan released an average of 44% of the glucose for Avicel after 24 h of digestion. 1:1 Spezyme/Novozyme at a total 8 to 10 mg/g glucan achieved the same degree of digestion (data not shown). Adding 50% additional protein concentrations of Multifect Xylanase and Multifect Pectinase (3 mg protein/g glucan of each enzyme set) to Accellerase increased
the glucose release to an average 56%, a 27% increase in final yield. Recent proteomic analysis has shown that Multifect Xylanase and Multifect Pectinase have significant endo-glucanase and exo-glucanase activity that may further complement cellulase activity present in Accellerase (Chundawat et al., 2011c).

Adding AFEX–CS-E reduced the 24 h glucan conversion by Accellerase to 30%, an inhibition of 32%; and had a similar effect on digestion by Spezyme + Novozyme (8 mg/g glucan; data not shown). When Multifect Xylanase and Multifect Pectinase were present with Accellerase, the higher degree of digestion was inhibited 24% by AFEX-CS-E.

3.2. Effect of UC on Inhibition by AFEX-CS-E

UC created three layers; a pellet, a dark brown lower layer with considerable cloudiness above it, and a yellow supernatant at the top. The supernatant made up about 90% of the volume. Adding the supernatant from UC AFEX-CS-E (Figure 1) caused the same degree of enzyme inhibition seen with non-ultra-centrifuged extract, i.e. there was no diminution in inhibitor concentration despite removal of the UC pellet and material in the dark brown cloudy layer. In a single complementary experiment, the dark brown cloudy layer above the UC pellet was tested and it also yielded the same amount of inhibition as the supernatant, so there was no increased concentration of inhibitors in this layer.
3.3. Fractionation of AFEX-CS-E Inhibitors by UF

The filtrates of UF-UC AFEX-CS-E obtained with progressively lower MWCO filters saw progressively greater removal of inhibitors. The degree of inhibition after ultrafiltration was reduced by 25-75% as the MWCO went from 10 kDa to 3 kDa (Figure 2). AFEX-CS-E exerted a greater extent of inhibition on Accellerase activity than on Accellerase in combination with Multifect Xylanase and Multifect Pectinase.

However, UF removed a greater fraction of the inhibition of the former than the latter enzyme system, resulting in similar degrees of inhibition by the ultrafiltrates. The % inhibition remaining after ultrafiltration may be calculated by multiplying the initial degree of inhibition by UC-AFEX-CS-E (32% inhibition of Avicel digestion by Accellerase, 24% inhibition of digestion by Accellerase plus Multifect Xylanase and Multifect Pectinase) by the % of inhibitor remaining shown in Fig. 2. Thus, inhibition of Avicel digestion by Accellerase was inhibited 23%, 11% and 8% by 10, 5 and 3 kDa MWCO ultrafiltrates of UC-AFEX-CS-E and digestion by Accellerase plus Multifect Xylanase and Multifect Pectinase was inhibited 19%, 14% and 11% by 10, 5 and 3 kDa MWCO ultrafiltrates of UC-AFEX-CS-E.

Selected assays using reconstituted UF retentates were carried out (one or two replicates with each type of filter). When the percentage of inhibition by the filtrate and retentate were added, the sums ranged from 71 to 145% relative to the UC AFEX-CS-E (data not shown). The sums for the relative inhibition by filtrate + retentate averaged 99% with a standard deviation of 29%.
3.4. Removal of AFEX-CS-E Inhibitors by SPE

Inhibitors were removed from the ultra-centrifuged AFEX-CS-E (UC-AFEX-CS-E) by passage through C-18 SPE columns. SPE adsorption removed 43% of the inhibition of Accellerase and 34% of the inhibition of Accellerase combined with Multifect Xylanase and Multifect Pectinase, caused by ultra-centrifuged aqueous extract of AFEX-treated corn stover (UC-AFEX-CS-E) (Figure 3). UC-AFEX-CS-E caused 32% inhibition of Accellerase digestion, which fell to 18% after passage through an SPE column; 24% inhibition of Accellerase + Multifect Xylanase and Multifect Pectinase, also fell to 18% after UC-AFEX-CS-E was passed through a C-18 SPE column.

The 5 kDa MWCO UF filtrates after UC were also subjected to C-18 SPE adsorption (Figure 3). The inhibition was reduced by over 40% when hydrolysis was catalyzed using Accellerase with or without added Multifect Pectinase and Xylanase. Experiments were also performed with AFEX-CS-E without UC or UF; eluting AFEX-CS-E through C-18 columns also removed about 40% of the inhibition of Avicel saccharification by Spezyme/ Novozyme (data not shown). Un-centrifuged AFEX-CS-E was also eluted through other types of SPE media. Waters Oasis HLB Cartridges with mixed hydrophobic and polar adsorption, Oasis MAX cartridges with quaternary amine groups added to HLB, and Oasis MCX cartridges with sulfonate groups added to HLB all reduced the inhibition by a comparable percentage to the C-18 silica-bonded cartridges. Bio-Rad Biobeads SM-2 (purified XAD-2 hydrophobic styrene-divinylbenzene resin) placed in nylon bags immersed in un-centrifuged AFEX-CS-E also
reduced inhibition, but less than if AFEX-CS-E was passed through an SPE cartridge directly (30% reduction instead of 40%).

3.5. *Sugar Concentrations in AFEX-CS-E and its Fractions*

Table 1 shows the concentrations of polysaccharides present in AFEX-CS-E before and after UC, in the filtrates and retentates after UF and in the SPE eluates. These polysaccharides include gluco-oligomers, xylo-oligomers, arabino-oligomers, xylo-glucans and phenolic glucosides (Chen et al., 2007). Free sugar concentrations (0.12, 0.09 and 0.07 g/L for glucose, xylose and arabinose, respectively) were 4.5 to 23 times lower than the corresponding polysaccharide concentrations. The concentrations of free sugars were mostly unchanged within experimental error in the initial AFEX-CS-E, the UC supernatant, the different MWCO UF filtrates, and the SPE eluates (data not shown). Therefore, free sugars are not part of the 40% or more of inhibition removed by UF or SPE.

The initial AFEX-CS-E contained 1.52 g dry weight biomass extractives per 100 mL of extract, or about 15% of the original weight of the biomass (approximately 3 g biomass/30 mL of extract). Free sugars totalled 0.028 g/100 mL of extract and polysaccharides 0.307 g/100 mL (Table 1), making monomeric + polymeric sugars 22% of the extracted dry weight in AFEX-CS-E.

About 25% of polymeric glucose and polymeric xylose and 35% of polymeric arabinose were removed by UC (Table 1). Since UC did not reduce enzyme inhibition, these polysaccharides removed by UC were not inhibitory to enzymatic digestion.
The 10 kDa MWCO ultra-filter removed 23%, 85% and 77% of the polymeric glucose, xylose and arabinose remaining after ultracentrifugation, respectively, while 70-80% of the inhibition remained. Since the 10 kDa MWCO ultra-filter reduced the inhibition by only 20-25% (Figure 2) while removing most of the polymeric xylose and arabinose, most of the polymeric xylose and arabinose-species can contribute only a minor part of overall enzyme inhibition.

Decreasing MWCO ultra-filters removed progressively greater amounts of the 75% of polysaccharides remaining in AFEX-CS-E supernatant after UC. The 3 kDa ultra-filter removed 49%, 94% and 90% of the glucose, xylose and arabinose, respectively, and corresponds to the removal of 75% of the inhibition of Accellerase digestion and 60% of the inhibition of digestion by Accellerase plus Multifect Xylanase and Multifect Pectinase. Polymeric glucose was removed more slowly than inhibition as the MWCO went from 10 to 5 to 3 kDa, while removal of the remaining small amounts of polymeric xylose and arabinose tracked removal of inhibitors more closely.

The sugar mass balance after UF was calculated by adding together the concentrations of the respective filtrates and retentates (Table 1). For polymeric glucose and arabinose, the sum of the sugars recovered in the filtrate and retentate were in the range of the original concentrations present in UC-AFEX-CS-E prior to UF. However, only 50-70% of the original polymeric xylose concentration was recoverable in the filtrate plus retentate, with the missing polysaccharides presumably remaining bound to the UF membrane. With all three polymeric sugars, the sums of sugars recovered in the filtrate plus retentate rose as the MWCO decreased. Since all three ultra-filters were
made of the same material, this suggests the missing polymeric sugars were presumably trapped within the ultra-filtration pores rather than being lost to sorption, with decreasing trapping as the pore-size decreased.

SPE adsorption performed after UC or UF did not remove any polysaccharides from the aqueous extract. On the other hand, SPE adsorption applied to the ultra-filtrates (or to AFEX-CS-E directly) decreased the enzyme inhibition by 40% or more (Figure 3), indicating that a significant amount of enzyme inhibition comes from substances that bind to the SPE adsorbent and are not associated with sugars.

3.6. LC/MS Analysis of AFEX-CS-E and Fractions

LC/MS profiles for AFEX-CS-E and fractions appear in Figure 4. UF with 5 kDa MWCO filters removed nearly 50% of the inhibitors and hence the 5 kDa MWCO filtrate was chosen for mass spectrometric analysis. AFEX-CS-E is a complex mixture, but the profiles for an AFEX-CS-E extract after UC and eluate after UC followed by UF through a 5 kDa MWCO filter, were largely identical to the profile for the original extract. The greatest change occurred after the UF eluate was subjected to C-18 SPE adsorption, which markedly simplified the profile by removing a large number of phenolic materials that eluted from the column between 3 and 12 minutes.

Coumaric acid (m/z 163, [M-H]- ion) was detected in all four LC/MS chromatograms from the initial extract and after UC, UF and SPE adsorption. AFEX-CS-E itself is mildly basic (pH 7.5-7.8) due to residual ammonia, and most carboxylic acids in this mixture (with pKa’s of 4-5) would be negatively charged during UC, UF
and SPE adsorption and therefore less likely to bind to a C-18 resin matrix unless they are substantially hydrophobic. Coumaric acid is insufficiently hydrophobic to be retained.

Coumaroyl amide ([m/z 162, [M-H]-]) was removed by SPE adsorption. Feruloyl amide ([m/z 192, [M-H]-]), diferulates (with m/z values of 385, 384 and 383 for negatively-charged di-acids, acid-amides and di-amides) and many other phenolics determined by integration of selected extracted ion chromatograms (e.g., m/z 237, 415, 517, 593, 637, 329) were also present after UF but were removed by SPE adsorption. In contrast to coumaric acid, any of these species containing negatively charged carboxylates were hydrophobic enough to be retained on the SPE column.

Ions with m/z 415, 517, 593, 329 and many other ions eluted in the retention time window of 3-12 minutes are considered phenolics for a couple of reasons. Accurate mass measurements of these ions show relative mass defects (RMD) values of 200-400 ppm which is the expected value for phenolic compounds owing to their relatively low hydrogen content (Chundawat et al., 2010; Stagliano et al., 2010). RMD is calculated as follows: 

\[ \frac{(\text{exact mass} - \text{nominal mass})}{\text{exact mass}} \times 10^6 \]

Another confirmation is the presence of ferulate or coumarate as fragment ions in the MS/MS spectra of some of these phenolics, which means the ions have a ferulic or coumaric acid core with other moieties attached. Some of these ions were also identified as flavonoids, such as m/z 637 which is determined as methylated rutin. More detailed analysis of AFEX-CS-E and AFEX-A-E which includes nitrogen containing and Maillard reaction products is published in a recent report (Chundawat et al., 2010).
3.7. Reduced Digestibility of AFEX-A and Inhibition of Enzymatic Digestion by AFEX-A-E

Avicel that underwent high severity (130 ºC, 45 min) AFEX treatment with 1:1 ammonia to Avicel (w/w) turned golden brown in color while Avicel treated with 3:1 ammonia to Avicel (w/w) under high severity turned dark brown. Avicel that underwent low severity AFEX treatment (90-100 ºC with 1:1 ammonia to Avicel (w/w)) became light yellow in color. Under high severity conditions, the degree of digestibility with 1:1 Spezyme/Novozyme varied greatly from one sample of AFEX-A to another, somewhat independent of whether the ammonia loading was 1:1 or 3:1. Hot water was used to extract chromophoric compounds from two highly browned Avicel batches that underwent high severity 3:1 ammonia to Avicel (w/w) pretreatment and were found to be minimally digestible (down to less than 20% of control Avicel). The extracted AFEX-A samples still remained brown-colored and saw an increase in digestibility to only about 33% of untreated Avicel control. The extracts, AFEX-A-E, which were darker brown than AFEX-CS-E, inhibited the digestion of untreated Avicel by 1:1 Spezyme/Novozyme by an average of 46% (44% and 48% for two samples), more than the 33% inhibition caused by AFEX-CS-E. AFEX-A produced under low severity conditions digested at essentially the same rate as untreated Avicel (data not shown).

When AFEX-CS-E was eluted through a Waters HLB SPE column, a substantial amount of the brown-colored extractives came through starting with the solvent front (Figure 5, top) and the eluate still contributed about 60% of the total inhibition (not shown). In sharp contrast, another aqueous extract of AFEX-A-E that caused a 50%
total inhibition of Avicel digestion by Accellerase was applied to a Waters HLB SPE column in parallel and no colored extractives came through except for a trace that appeared in the water wash applied after the sample (Figure 5, bottom). The inhibition of Avicel digestion by the SPE eluate dropped to only 5% from the initial 50%, hence SPE filtration removed 90% of the inhibition. In both cases (AFEX-CS-E and AFEX-A-E) the enzyme inhibitory contribution tracked with the color of the extract. Most of the color in the AFEX-A-E and most of the remaining color from the AFEX-CS-E eluted in the methanol and DMSO fractions, although some colored compounds still remained bound to the column.

4. Discussion


AFEX pretreatment cleaves linkages between lignin and hemicellulose and mobilizes lignin, while at the same time creating products of Maillard and Amadori reactions. The complex mixture of water extractable AFEX-CS-E inhibitors of Avicel digestion could contain large and small molecules derived from cellulose, hemicellulose and lignin. Given this complex composition (see Figure 4), AFEX-CS-E was fractionated with a simple batch processes using different separation principles to shed light on the nature of the inhibitors. Volumes were kept constant through each process to avoid dilution artefacts.
AFEX-CS-E caused a 32% decrease in the extent of enzymatic digestibility of Avicel by Accellerase or Spezyme plus Novozyme. Doubling the enzyme loading by adding Multifect Xylanase and Multifect Pectinase to Accellerase caused a 27% increase in the extent of Avicel digestion over 24 h. Avicel has been reported to contain some residual xylan and the addition of xylanase causes glucose release to increase well beyond what would be predicted from removal of just a few % of xylan and mannan (Várnai et al., 2011; Sloneker, 1971). The degree of increase we observed with added Multifect Xylanase was virtually identical to increase observed by Varnai et al. (2011) when they added xylanase. At the same time, the digestion became less sensitive to inhibition by AFEX-CS-E (percent inhibition dropped to 24%) when Multifect Xylanase and Multifect Pectinase were added. This decrease in inhibition would be consistent with either the additional digestion achieved with the extra enzymes being insensitive to the inhibitors, or the enzymes destroyed some of the inhibitors.

With Avicel lacking lignin and being largely free of hemicelluloses, subjecting Avicel to AFEX treatment was conceived as a model for creating the simpler set of inhibitors arising during AFEX treatment from the cellulosic component of biomass. Avicel subjected to AFEX treatment under conditions of high severity turned it brown in color and aqueous extracts of these samples also were brown colored. AFEX-A produced at 130 ºC digested poorly and its aqueous extracts were highly inhibitory to commercial cellulase mixtures, which corroborates previous findings (Chundawat et al., 2010).
Presumably, the dark coloration of cellulose subjected to AFEX treatment at high severity was due to the Maillard and Amadori type reactions between ammonia and reducing sugar ends. The reaction temperature was lower than what is needed for caramelization to occur (Hodge, 1967). The brown colored extractives and inhibitors in AFEX-A-E are likely to include solubilized products of Maillard reactions with cellulose and the degradation products of cellulose; some could also have arisen from the residual xylan present.

Maillard and Amadori product inhibitors similar to those produced in AFEX-A would presumably be formed by ammonia-driven reactions in AFEX treated corn stover under conditions of high severity. Although corn stover contains proteins and amino acids, these (<10 mg ammonia equivalent/g biomass) are present in minor quantities compared to the amount of ammonia used during AFEX (1 g or more of ammonia/g biomass) (Chundawat et al., 2010). Lowering the temperature of the AFEX pretreatment of Avicel to 100 ºC or less largely prevented inhibitor formation.

Maillard reaction based inhibitors created from Avicel could include intermediate products where a cellulose chain is altered at the reducing end with the rest of the chain remaining intact. If these chains were sizable in length, they would be indistinguishable from unchanged glucose polymers in the sugar analysis. Interference with exoglucanase activity operating at reducing ends of these intermediate products would be one likely inhibitory mechanism (Chundawat et al., 2011a). With CS having a full complement of xylan and arabinan, AFEX-CS-E could contain inhibitors in the form of xylan and arabinan oligomers whose ends have been modified by Maillard
reactions. It that case, to be consistent with the observations, cleavage of the intact part of the oligomer chain would have to reduce the overall inhibition.

AFEX pretreatment is also known to mobilize lignin. Brown colored lignin species are another possible set of inhibitors found in AFEX-CS-E.

4.2. Insights from UC

Prior to embarking on these experiments AFEX-CS-E was routinely filtered through 0.22 µm filters immediately after it was produced, but would still show slow aggregation and precipitation of particulate matter upon storage at 4 ºC. It was hypothesized that these particles might be lignocellulose fragments of cell walls that could bind and inhibit enzymes and they might be removed by UC (Petridis et al., 2011; Várnai et al., 2010). As it turned out, the materials removed by UC (16 h at 4 ºC) were minimally inhibitory.

The precise identity of these sedimenting precipitates is unknown, but the following can be inferred based on certain experimental observations. In AFEX-CS-E, high molecular weight hemicellulose and hemicellulose that is linked, either covalently or non-covalently, to lignin constituted up to a quarter of the dry mass of the extract, with the remainder consisting largely of lignin and ash (Chundawat et al., 2010). The sedimenting pellet and cloudy layer contained 25-35% of the total polymeric glucose, xylose and arabinose in the original AFEX-CS-E, so an appreciable fraction of the sugars sedimented, with or without prior aggregation. Lignin based components, Maillard intermediates with intact sugar chain moieties, and Maillard end products are
likely to be responsible for the brown color in AFEX-CS-E. Much of the color in the extract also appeared in the cloudy layer and pellet, with the UC supernatant becoming much less intensely colored.

Sedimentation velocity during UC follows the Svedberg equation, \( s = \frac{m(1-\bar{V}\rho)}{f} \) where \( m \) is the mass of the particle; \( \bar{V} \) is its partial specific volume, the inverse of density; \( \rho \) is the density of the solvent; and \( f \) is the frictional coefficient of the particle. The polysaccharides cellulose and hemicellulose have densities of 1.5 – 1.6 g/mL while lignin is much lower at 1.1 to 1.3 g/mL (Sun, 2005; Ramiah and Goring, 1965; Terashima et al., 2009). The brown colored pellet probably contained the highest contributions from polysaccharides, including Maillard intermediates and inorganic materials; lignin fragments of very high molecular weight and compact shape (low friction) could also be present. The dark brown cloudy layer would also contain materials of similar composition but with slightly lower molecular weight or density. Dynamic aggregation of smaller lignin fragments or crosslinking of Maillard intermediates would also result in sedimentation of these materials. The nascent aggregates were probably present in the cloudy layer in the ultra-centrifugation tube. With the likely presence of the highest molecular weight lignin fragments, polysaccharides and Maillard intermediates in the brown pellet and cloudy layer, it was unexpected that their removal would have little impact on enzyme inhibition. Simple insolubility would not be expected to eliminate non-productive interaction with cellulases, which are designed to operate on insoluble substrates, but the soluble
materials remaining in the UC supernatant account for nearly all the inhibitors present in AFEX-CS-E.

4.3. Insights from UF

The 85% removal of xylan from the extract by a 10 kDa MWCO ultra-filter (85% decrease in polymeric xylose concentration and 77% decrease in polymeric arabinose, but only 23% decrease in polymeric glucose) was associated with only 20-25% reduction in overall inhibition by UC-AFEX-CS-E. Large xylan species can be responsible for only a small part of the overall inhibition. Removal of smaller xylans by lower MWCO ultra-filters correlated with removal of larger fractions of the inhibition. A 5 kDa MWCO ultra-filter removed 64% of the inhibition of Accellerase by UC-AFEX-CS-E while a 3 kDa ultra-filter removed 75% of the inhibition, which correlated with removal of most of the residual polymeric xylose and arabinose but less than half of the polymeric glucose. Combined Accellerase plus Multifect Xylanase and Multifect Pectinase experienced less inhibition by AFEX-CS-E and ultra-filtration removed less of this inhibition compared to inhibition of Accellerase alone (Figure 2). These observations would be consistent with intermediate sized (3 kDa < MW < 10 kDa) xylans comprising an appreciable fraction of the inhibitors. It is not known if the inhibitory species that are removed by the progressively decreasing MWCO ultrafilters have ends that are altered by Maillard reactions or are composed/ coupled to lignin species.
Furans produced during thermochemical pretreatment of biomass are also known to be inhibitory to various cellulase activities (Jing et al., 2009). Potentially inhibitory small molecule species were observed in the mass spectra. However, compared to conventional acidic pretreatments, these are not the major degradation products formed during AFEX (Chundawat et al., 2010). With only 25% of inhibitors of Accellerase digestion left after passage through a 3 kDa MWCO ultra-filter, it would appear that small molecule Maillard and Amadori end products, small sugar oligomers and small lignin-derived species comprised a minority of the inhibitors. If added Multifect Xylanase and Multifect Pectinase destroyed inhibitory xylan, low MW species could then have been a larger fraction of the remaining inhibitor. Just 56% of inhibitors of Accellerase plus Multifect Xylanase and Multifect Pectinase digestion were removed by the 3 kDa ultra-filter while 75% of inhibitors of digestion by Accellerase alone were removed.

It should be noted that the nominal MWCOs are not inherently discriminatory to molecular partitioning based on molecular weights alone. In addition, linear molecules larger than the MWCO can pass through if they enter a pore end-on, while those smaller than the MWCO will not if there is a charge interaction or an interaction with higher MW materials that are trapped (Revchuk and Suffet, 2009). Lignin fragments are likely to remain highly cross-linked in addition to being prone to aggregation; nominal MWCOs could apply to lignin fragments since they are not linear molecules, except where small fragments that should pass through bind to each other or to the membrane. Hemicellulose fragments released from the cell walls during AFEX pretreatment would
no longer be covalently connected to each other or to lignin by ester linkages and the limited branching of hemicellulose chains may not prevent them from behaving like linear molecules, so the operational MWCO for oligosaccharide chains could be much higher than the nominal value (Saha, 2003). Retention of the vast majority of polymeric xylose and arabinose by the highest 10 kDa MWCO filter suggests that the hemicellulose fragments, including Maillard intermediates, present in AFEX-CS-E are rather large (≥ 10kDa/DP 55). This agrees with published data suggesting that AFEX-CS has glucan chains of average length DP 6600 (Kumar et al., 2009). Glucose chains would also be linear and not follow nominal MWCOs unless they are bound to lignin or became cross-linked as Maillard intermediates.

During UF, the membrane turned the same color as AFEX-CS-E, indicating lignin-derived or Maillard intermediate material were likely adsorbed to it. Smaller MW inhibitors could have been removed by membrane sorption. The sum of the inhibition in the filtrates plus retentates nearly equalled 100% of the pre-filtration value. This suggests that most of the inhibitors were recovered in the filtrate or retentate and did not bind irreversibly to the membrane, but weak sorption could have been enough to prevent inhibitors from passing into the filtrate. That would lead to an overestimation of the MW of the material removed. On the other hand, more than 35% of the inhibitor activity relative to the UC AFEX-CS-E passed through the 5 kDa MWCO ultra-filter and the mass spectrum of the 5 kDa filtrate remained largely the same as the mass spectrum prior to UF. The likely inhibitors appearing in the 5 kDa eluate include all the small molecules, including the hydrophobic and charged ones identified in the mass spectrum.
spectra (further discussion below). All three different MWCO membranes were made of the same material, so differential sorption would not be a factor for inhibitor removal in addition to differential pore size.

4.4. Insights from SPE Adsorption

When SPE treatment was done after UC, the inhibition by AFEX-CS-E was reduced by 34-43% (Figure 3), while none of the polymeric glucose, xylose or arabinose was removed (Table 1). Therefore, a minimum of about 35% of the inhibition is not associated with any of these sugars.

The mass spectra before and after SPE adsorption exhibited substantial differences due to the removal of lignin-derived materials from the eluate; these included multimeric lignin carboxylates (diferuloylate diamide and acid-amide) and monomeric phenolic amides (feruloylamide and coumaroylamide) but not monomeric carboxylates (coumarylate). Thus, uncharged lignin monomers and dimers were retained, singly charged monomers were not, but singly charged dimers were. The degree of SPE retention of lignin monomers and dimers, reflecting the countervailing effects of charged, polar and hydrophobic moieties is consistent with the observed partial binding to SPE materials of natural organic matter such as the more highly oxidized, polar and charged (compared to lignin) humic and fulvic acids (McElmurry et al. 2010). The same factors would influence the binding of lignin or Maillard materials.

The brown color in the SPE eluate of AFEX-CS-E (Figure 5, top) was probably a mixture of lignin-derived material and Maillard products. Methanol and DMSO eluted
deeply brown colored fractions that would contain small phenolic compounds, such as those identified by mass spectrometry, somewhat larger lignin-derived materials and most of the Maillard end products, all of which are hydrophobic. After DMSO elution, the SPE cartridges still retained some brown color, particularly around the top frit, but far less than what eluted with methanol and DMSO. The material retained in the SPE cartridges after DMSO elution was likely to be large lignin derived or Maillard materials, trapped in the frit or strongly attached to the sorbent by multi-site attachment.

Adding ion-exchange groups to the SPE media did not decrease inhibition beyond experimental error. Oasis HLB cartridges have polar in addition to hydrophobic interactions, but removed about the same amount of inhibition as C-18 sorption did. Therefore the bulk of inhibitors removed by SPE appear to bind through hydrophobic interactions.

Maillard products would be the predominant inhibitors in AFEX-A. Maillard inhibitors produced by ammonia bound very strongly to the SPE column (Figure 5, bottom), unlike part of the inhibitory fraction from AFEX-CS-E, which appeared in the eluate, and thus are likely to be polar and perhaps unique to AFEX or other pretreatments that employ ammonia.

The same removal of 30 to 40% or more of inhibition by SPE adsorption is seen after UF as before UF. This indicates that hydrophobic sorption to the UF membrane is not preventing hydrophobic materials from passing into the UF filtrate.

Maillard product formation is temperature dependent; although some reaction occurs even at ambient temperature, the rates increase rapidly as the temperature rises
towards 150 °C (Nursten, 2005). Careful temperature control during AFEX pretreatment is critical, when the AFEX reactor is brought to temperature, hot spots at or above 130 °C should be avoided.

4.5. Process Removal of Enzyme Inhibitors by SPE

Thermochemical pretreatments such as AFEX increase access to cellulose, decreasing recalcitrance to glucan conversion by enzymes, but also produce a complex mixture of lignin-based and Maillard products that could inhibit enzyme activity. These inhibitors need to be removed but many of these methods are expensive (e.g. over liming or adsorption on activated carbon). C-18 SPE cartridges are not meant to be reused or used with very large volumes, but XAD-type resins lend themselves to both types of use. Resins placed in mesh bags can be placed in large volumes to adsorb hydrophobic inhibitors if mixing and stirring is possible and the resins may be regenerated by washing with recoverable organic solvents. Hydrophobic resins are especially effective at removing colored Maillard intermediates (Figure 5, lower) and phenolic amides (Figure 4). Small, charged species like phenolic acids were not removed by SPE, but if the extract pH were lowered to 4.0-4.5 for optimum cellulase activity, better removal of these species would be expected.

5. Conclusions

Batch separation was used to determine the nature of the enzyme inhibitors present. The UC-AFEX-CS-E sediment included 25% of the sugar polymers and
significant amounts of lignin and/or Maillard products, but no observed reduction in inhibition.

The 10 kDa MWCO ultra-filter removed 25% of inhibitors, including most of the hemicellulose fragments, high molecular weight Maillard intermediates and unreacted chains. The lowest 3 kDa MWCO ultra-filter removed 75% of inhibitors, including sugar oligomers, lignin fragments and cross-linked ligno-oligomers. A mass spectrometric analysis of the final 25% of inhibitors indicated they may be low molecular weight phenolics and Maillard reaction products.

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Figures

**Fig. 1.** Percent glucan conversion of Avicel after 24 hours while in the presence or in the absence of AFEX-CS-E and supernatant of UC-AFEX-CS-E. Error bars depict standard deviation from average for triplicate assays. A) Avicel; B) AFEX-CS-E added to Avicel; C) UC-AFEX-CS-E added to Avicel.

**Fig. 2.** Percent inhibition remaining after UF-UC-AFEX-CS-E using different molecular weight cutoff ultra-filters. Error bars depict standard deviation from average for triplicate assays. The data are normalized to A = 100%. A) UC-AFEX-CS-E added to Avicel; B) 10 kDa UF-UC-AFEX-CS-E added to Avicel; C) 5 kDa UF-UC-AFEX-CS-E added to Avicel; D) 3 kDa UF-UC-AFEX-CS-E added to Avicel.

**Fig. 3.** Removal of inhibition of enzymatic digestion through solid phase extraction (SPE) of the supernatant of UC-AFEX-CS-E and UF-UC-AFEX-CS-E. The y-axis represents the percent inhibition remaining in the eluate after SPE (B or D) relative to the amount of inhibition present before SPE filtration (A or C). The data are normalized to A and C = 100%. Error bars depict standard deviation from average for duplicate assays. A) UC-AFEX-CS-E added to Avicel; B) UC-AFEX-CS-E passed through SPE, added to Avicel; C) UC-AFEX-CS-E, ultrafiltrate from 5 kDa MWCO ultrafiltration,
added to Avicel; D) UC-AFEX-CS-E, ultrafiltrate from 5 kDa MWCO ultrafiltration, then passed through SPE, added to Avicel.

**Fig. 4.** LC/MS total ion chromatograms of AFEX-CS-E and its fractions. A) AFEX-CS-E; B) UC-AFEX-CS-E; C) 5 kDa filtrate of UC-AFEX-CS-E; D) C-18 eluate of 5 kDa filtrate of UC-AFEX-CS-E. Asterisks (*) indicate unknown phenolics.

**Fig. 5.** Fractions collected from elution through 6 cc Waters Oasis HLB columns, with 4 x 4 mL of sample applied. A through I – AFEX-CS-E. J through R – AFEX-A-E. A and J) 4 mL of solvent front from the first 4 mL of sample application; B, C, D, K, L and M) 3 x 4 mL volumes as the rest of the extract was applied; E and N) 4 ml corresponding to a 4 mL water wash; F, G, O and P) 5 mL eluted by methanol; H and Q) 4 mL of isopropanol wash; I and R) 4 mL of DMSO wash.
Tables

Table 1

Polysaccharide concentrations in AFEX-CS-E and its ultra-centrifuged (UC) and ultra-filtered fractions. Polysaccharide concentrations are measured after acid hydrolysis and represent the differences in sugar concentrations before and after acid hydrolysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Polysaccharide Sugar Concentrations</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Glucose (g/L)</td>
</tr>
<tr>
<td>AFEX-CS-E</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>AFEX-CS-E: UC supernatant (UCS)</td>
<td>0.39 ± 0.03</td>
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<tr>
<td>AFEX-CS-E; UCS; 10 kDa filtrate</td>
<td>0.30 ± 0.02</td>
</tr>
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<td>AFEX-CS-E; UCS; 5 kDa filtrate</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>AFEX-CS-E; UCS; 3 kDa filtrate</td>
<td>0.20 ± 0.02</td>
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<tr>
<td>AFEX-CS-E; UCS; 10 kDa retentate</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>AFEX-CS-E; UCS; 5 kDa retentate</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>AFEX-CS-E; UCS; 3 kDa retentate</td>
<td>0.28 ± 0.03</td>
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<tr>
<td>AFEX-CS-E; UCS; SPE eluate</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>AFEX-CS-E; UCS; 10 kDa filtrate; SPE eluate</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>AFEX-CS-E; UCS; 5 kDa filtrate; SPE eluate</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>AFEX-CS-E; UCS; 3 kDa filtrate; SPE eluate</td>
<td>0.21 ± 0.02</td>
</tr>
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