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Inhibition of enzymatic cellulolysis by phenolic compounds

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ABSTRACT

Phenolics derived from lignin and other plant components can pose significant inhibition on enzymatic conversion of cellulosic biomass materials to useful chemicals. Understanding the mechanism of such inhibition is of importance for the development of viable biomass conversion technologies. In native plant cell wall, most of the phenolics and derivatives are found in polymeric lignin. When biomass feedstocks are pretreated (prior to enzymatic hydrolysis), simple or oligomeric phenolics and derivatives are often generated from lignin modification/degradation, which can inhibit biomass-converting enzymes. To further understand how such phenolic substances may affect cellulase reaction, we carried out a comparative study on a series of simple and oligomeric phenolics representing or mimicking the composition of lignin or its degradation products. Consistent to previous studies, we observed that oligomeric phenolics could exert more inhibition on enzymatic cellulolysis than simple phenolics. Oligomeric phenolics could inactivate cellulases by reversibly complexing them. Simple and oligomeric phenolics could also inhibit enzymatic cellulolysis by adsorbing onto cellulose. Individual cellulases showed different susceptibility toward these inhibitions. Polyethylene glycol and tannase could respectively bind and degrade the studied oligomeric phenolics, and by doing so mitigate the oligomeric phenolic's inhibition on cellulolysis.

1. Introduction

Detrimental effect from lignin-derived phenolics on enzymatic cellulolysis had long been suspected and targeted in the research and development efforts for biomass conversion (for recent reviews and reports, see Refs. [1–13]). In native plant cell wall, as well as minimally pretreated biomass feedstocks, most of the phenolics are found in lignin, a highly complex and heterogeneous biopolymer that is physically entangled with or chemically linked to cellulose and hemicelluloses [14,15]. During biomass-disrupting physicochemical pretreatment, native lignin is often modified, resulting in demethylation (and generation of exposed phenolic moieties), solubilization (or formation of simple/oligomeric phenolics), or other degradation changes [16–21]. Insoluble lignin may remain, and be co-present with cellulose or hemicellulose, in pretreated biomass materials, and it often hinders cellulase or hemicellulase's hydrolytic activity by unspecific adsorption [10,22–24]. Many simple phenolic compounds have been identified in the solution phase of pretreated biomass [16–20], and they often inhibit fermentative fungi or bacteria that convert glucose (Glc), xylose, or other carbohydrates into ethanol. These phenolic compounds can inhibit or deactivate lignocelluloses-hydrolyzing enzymes [8,9,11,25,26], especially at high phenolic concentrations pertinent to high biomass-loading (high consistency suspensions) enzymatic hydrolysis systems. In addition to simple/monomeric phenolics, oligomeric phenolics, such as tannin, may also inhibit or deactivate lignocelluloses-hydrolyzing enzymes [8,27,28]. To break up tannin–protein complexes or reactivate tannin-inhibited enzymes (including rumen cellulase), polymers of polyalkoxylates, nonionic surfactant, or other natures (such as polyethylene glycol (PEG), Tween 20, PVP) have been applied [27,29–31].

To further investigate the action of lignin-derivable phenolic compounds on enzymatic cellulolysis, we carried out a comparative study on how a series of simple phenolics (representing major lignin subunits), and tannins (both hydrolysable and condensed, mimicking solubilized lignin oligomers) interacts with various cellulases and impact the enzymes' reaction. We also probed potential ways to mitigate the detrimental effect from such phenolic inhibitors on enzymatic cellulolysis.

2. Materials and methods

2.1. Materials and instruments

Chemicals used as reagents or buffers were commercial products of reagent or purer grade unless specified otherwise. A dilute acid-pretreated corn stover (PCS) preparation (~59% glycan and 28% lignin), obtained after subjecting dried/chopped corn stover (at 28 wt%) to sulfuric acid (0.05 g acid per g of dry corn stover) for 1 min at 190 °C [32], was kindly provided by US National Renewable Energy Laboratory, ground and sieved, then extensively washed, then pH-adjusted to pH 5. PCS lignin residue was prepared previously [23]. Phosphoric acid-swollen cellulose (PASC) was

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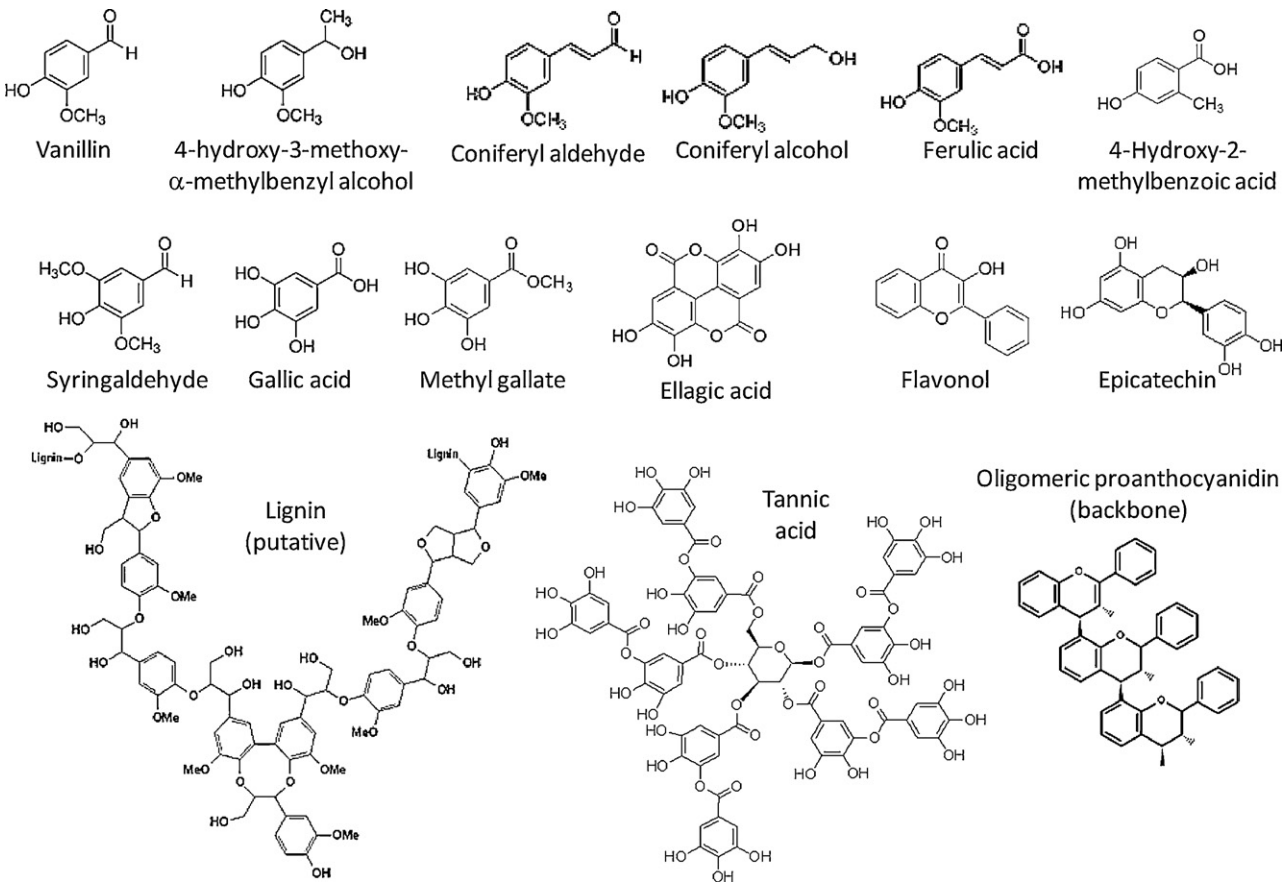

Fig. 1. Lignin and subunits models used in this study. For clarity, the hydroxyl substitutions on the benzene and chromane rings are not shown in the oligomeric proanthocyanidin sketch (adapted from [34]). Lignin sketch is from Wikipedia (http://en.wikipedia.org/wiki/Lignin).

prepared from Avicel (FMC, PH101) by a published method [33]. Carboxymethyl cellulose (CMC) was from Hercules (7L2 type, 70% substitution). One oligomeric proanthocyanidin (OPC) preparation was made from a vitamin supplement product (Nature's Way Masqueliar's Tru-OPCs, containing 75 mg/tablet of dried grape seed extract, of which ~65% was OPCs and 30% was other polyphenols; inactive ingredients included cellulose, maltodextrin, modified cellulose gum, stearic acid, cellulose, silica, glycerin, etc.), which was grinded by mortar/pestle and then solubilized in water (1 tablet or 0.45 g in 10 mL water, yielding ~17 mM OPC assuming a subunit molecular mass of 290 Da as that of catechin). Another OPC preparation was made from another vitamin supplement, TruNature grapeseed extract (soft gel), by dissolving the content of one soft gel (~150 mg) into 5 mL H_2O. Polyethylene glycol (PEG) 4000 and Glc pentaacetate were from Alfa Aesar. Stock solutions of studied simple phenolics and tannic acid (all from Sigma–Aldrich) were made in water. Fig. 1 shows the structure of tested phenolics.

An experimental cellulase preparation from Novozymes ("cellulase mix") was used for enzymatic cellulolysis. The mix contained extracellular *Hypocrea jecorina* (*Trichoderma reesei*) cellulases and *Aspergillus oryzae* Cel3A β-glucosidase (AspOr3A BG) (among the total proteins, cellobiohydrolase-I (CBH-I), cellobiohydrolase-II (CBH-II), β-1,4-endoglucanase-I (EG-I), β-1,4-endoglucanase-II (EG-II), and BG accounted to ~60, 15, 5, 6, and 2 wt%, respectively). Wild-type *H. jecorina* Cel7A CBH-I (HypJe7A), recombinant *H. jecorina* Cel6A CBH-II (HypJe6A), Cel7B EG-I (HypJe7B), Cel5A EG-II (HypJe5A), and Aspor3A BG were prepared as previously reported [33,35]. *A. oryze* tannase (or tannic acyl hydrolase, EC 3.1.1.20) was a commercial product from Novozymes (9650 U/g).

Spectrophotometric measurement was carried out on a Molecular Devices SpectraMax 340PC reader with Costar 96-well microplates. Sugar analysis was carried out on an Agilent 1100 HPLC instrument, equipped with a Bio-Rad Aminex HPX-87H column and a refractive index detector, under 5 mM H_2SO_4 elution. Temperature-controlled incubations were made in New Brunswick Scientific Innova 4080 incubation shakers. Polyacrylamide gel electrophoresis (PAGE) was carried out in a Bio-Rad Criterion cell with criterion precast gels, and stained with Bio-Rad's Bio-Safe coomassie kit.

2.2. Enzymatic cellulose hydrolysis

PCS hydrolysis was carried out in 2-mL 96-well VWR deep well plates, typically with 43.4 g/L PCS (dry weight), 0.25 g/L (~4 μM based on an average molecular mass of 60 kDa) or 9.8 mg/(g cellulose) cellulase mix cellulases, 50 mM Na-acetate, pH 5, in 1 mL suspensions and at 50 °C for up to 4 days (or otherwise stated), under 150 rpm shaking. Heat-sealing of the plates was made by an ABgene ALPS-300 device (160 °C for 2 s). Aliquots (80 μL) of the suspension were sampled and then filtered (on Millipore Multiscreen-HV 96-well plate filters), and the supernatants were analyzed for soluble sugars on HPLC. Hydrolysis extent was estimated from the observed Glc and cellobiose (longer cellodextrins were negligible), and based on the glucan content of the PCS. Hydrolysis of PASC, Avicel, and cellobiose was carried out under conditions similar to that of PCS. Typical cellulose dosing was 2, 23, and 2 g/L, respectively, and typical cellulase dosing was 0.25 g/L cellulase mix or 0.04 g/L (or ~0.7 μM) individual cellulase. At least duplicates were run in each experiment. CMC hydrolysis was made with 10 or 20 g/L CMC, 1–20 mg/L (or 0.05–1 mg/(g CMC)) cellulases, and 50 mM Na-acetate, at pH 5 and 50 °C. Aliquots of the suspension were sampled, and their supernatants were analyzed for soluble reducing sugars by *p*-hydroxybenzoic acid hydrazide (PHBAH) [23].

2.3. Inhibition of enzymatic cellulolysis

Inhibition study of enzymatic cellulolysis by selected phenolic compounds was carried out in hydrolysis of selected cellulosic substrates by either cellulase mix or individual cellulases. PCS was tested as a representative industrially feedstock (relevant to cellulosic fuel ethanol or other bio-based chemical productions), and as a heterogeneous substrate with which cellulases' action on cellulose could be studied in the presence of non-cellulosic substances such as lignin. Avicel was tested as an isolated cellulose preparation that could mimic (with the coexistence of both crystalline and amorphous cellulose parts) the cellulose in real biomass feedstocks, and have negligible background phenolics to interfere with the inhibition study of exogenous phenolics. PASC was tested as an isolated, amorphous cellulose preparation upon which individual cellulases could act with fast kinetics. CMC and cellobiose were tested as substrates specific to EG and BG, respectively. Targeted candidates were added to the hydrolysis suspension (with pH readjustment if needed) and their inhibitions were evaluated by impacted hydrolysis. The inhibitions were measured by the loss in hydrolysis extent, supplemented by the loss in initial hydrolysis rate (which might depend on not only the concentration of active cellulase, but also the number of enzyme-acting sites on cellulose).