Rapid Chromatography for Evaluating Adsorption Characteristics of Cellulose Binding Domain Mimetics

Nathan S. Mosier,1,2 Jonathan J. Wilker,3 Michael R. Ladisch1,2,4
1Department of Agricultural and Biological Engineering, 500 Central Drive, Purdue University, West Lafayette, Indiana 47907; telephone: 765-494-7022; fax: 765-494-7023; e-mail: mosiern@purdue.edu
2Laboratory of Renewable Resources Engineering, 500 Central Drive, Purdue University, West Lafayette, Indiana 47907
3Department of Chemistry, 500 Central Drive, Purdue University, West Lafayette, Indiana 47907
4Department of Biomedical Engineering, 500 Central Drive, Purdue University, West Lafayette, Indiana 47907

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Abstract: The cost of cellulosic enzymes is one barrier to the economic production of fermentable sugars from lignocellulosic biomass for the production of fuels and chemicals. One functional characteristic of cellulosic enzyme systems that improves reaction kinetics over mineral acids is a cellulose binding domain that concentrates the catalytic domain to the substrate surface. We have identified maleic acid as an attractive catalytic domain with $pK_a$ and dicarboxylic acid structure properties that hydrolyze cellulose while producing minimal degradation of the glucose formed. In this study we report results of a rapid chromatographic method to assess the binding characteristics of potential cellulose binding domains for the construction of a synthetic cellulase over a wide range of temperatures (20° to 120°C). Aromatic, planar chemical structures appear to be key indicators of cellulose adsorption. Indole, the sidechain of the amino acid tryptophan, has been shown to reversibly adsorb to cellulose at temperatures between 30° and 120°C. Trypan blue, a polyaromatic, planar molecule, was shown to be irreversibly adsorbed to cotton cellulose at temperatures of <120°C on the time scale of the experiments. These results confirm the importance of hydrophobic cellulose and the cellulose-binding component of cellulosidic enzymes and cellulosic enzyme mimetics.

Keywords: cellulose adsorption; cellulase; mimic; cellulose binding domain; indole; trypan blue

INTRODUCTION

A number of processes for hydrolyzing cellulose into fermentable glucose for the production of renewable fuels and chemicals have been developed over the years. The vast majority of processing schemes utilize either cellulosic enzymes or sulfuric acid of varying concentrations. Historically, enzymes have been too expensive for economical production of fuel ethanol from biomass (Lynd et al., 1996). Sulfuric acid is less expensive than cellulosic enzymes, although disposal costs associated with the use of sulfuric acid and the need for expensive, corrosion-resistant materials of construction significantly increase the cost of sulfuric acid hydrolysis at dilute acid concentrations. However, the single largest drawback to using sulfuric acid is that it also readily degrades glucose at the high temperatures required for cellulose hydrolysis (Bienkowski et al., 1987; McKibbins et al., 1962; Saeman, 1945). Glucose degradation lowers the yield of fermentable sugars from biomass, and the degradation products, 5-hydroxymethylfurfural (HMF), levulinic acid, and formic acid, are inhibitory to yeast fermentation (Delgenes et al., 1996; Jeffries and Sreenath 2000; Larsson et al., 1999; Taherzadeh et al., 1999).

Solutions for the three-dimensional structure of cellulosidic enzymes and the mechanisms of cellulose binding and hydrolysis (Divne et al., 1994; Kleywegt et al., 1997; Mosier et al., 1999; Rouvinen et al., 1990) have greatly increased the understanding of the interaction between enzyme structure and catalytic activity for cellulosidic enzymes. Preliminary successes with the synthesis of complexes that mimic galactose oxidase (Wang et al., 1998) suggest that an organic molecule mimicking cellulase activity could be generated. The goal of this research is to combine the cost advantage of mineral acid catalysts, such as sulfuric acid, with the selectivity of enzymes to develop catalysts that mimic the specific action of cellulosidic enzymes for converting plant biomass into fermentable sugars.

Most cellulosidic enzymes are composed of two functionally distinct domains. The cellulose-binding domain is responsible for the close association of the enzyme with solid cellulose through strong binding to crystalline cellulose (Gilkes et al., 1992), amorphous cellulose (Boraston et al., 2003), or both (Boraston et al., 2001; Coutinho et al.,

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Adsortion of the cellulose-binding domain is known to consist largely of entropically driven interactions between aromatic amino acids, especially tryptophan and tyrosine, and cellulose (Creagh et al., 1996; Tomme et al., 1996). The catalytic domain is responsible for catalyzing cellulose hydrolysis through a general acid mechanism. In inverting, but not in retaining, glucoside hydrolases, two acidic functional groups (usually glutamic acid), one as a proton donor and one as a proton acceptor, catalyze the hydrolysis of the O-glycosidic bonds in the cellulose chain (Davies and Henrissat, 1995; McCarter et al., 1994). The proposed enzyme mimetic, like cellulolytic enzymes, will have both structural and functional components that will allow cellulose binding and hydrolysis through general acid catalysts. The advantage of adsorbing the catalyst to the surface is that it increases the local acid concentration near the substrate, cellulose, to enhance the rate of reaction. For this concentration effect to occur, we propose that the solution pH be within a range in which the acid moieties of the mimetic are partially protonated so that interactions between protons, the acid, and the cellulose are more likely to occur. For this hypothesis to be tested, we must identify compounds that adsorb to cellulose as a chemical platform for synthesizing an enzyme mimetic.

Previously reported research into the development of a cellulolytic enzyme mimetic has focused on the identification of catalytic domains and the fundamental mechanisms of acid-catalyzed cellulose hydrolysis and glucose degradation (Mosier et al., 2000, 2002). This study describes experimental data and analyses for the identification of potential cellulose binding compounds that could be used to construct a cellulase mimic and the fundamental mechanisms influencing their adsorption to cellulose.

THEORY

The proposed cellulolytic enzyme mimetic is an organic compound, not a protein, which contains the same essential functional components as the naturally occurring enzymes. Cellulose hydrolysis occurs in a heterogeneous, two-phase system. The reaction kinetics are improved by adsorption of the enzyme to the cellulose surface through a cellulose binding domain that acts to concentrate the catalytic domain close to the substrate. The proposed enzyme mimetic, like most cellulases, will contain a cellulose-binding domain that selectively adsorbs to cellulose. This enzyme mimetic capitalizes on the advantage of enhanced surface concentration of the catalyst through adsorption.

Because catalyst concentration influences the rate of reaction, the partitioning of the mimetic to the cellulose surface will generate a much higher reaction rate than expected based on the bulk solution concentration. From the collision theory of reaction rates, the reaction rate will be increased due to increased collision rate of catalyst and reactant simply because there is a greater chance of colliding due to the increased concentration of the catalyst at the substrate surface. This approach also represents a more efficient use of catalyst because it is concentrated at the cellulose surface in close proximity to the reactant instead of being homogeneously distributed throughout the liquid phase.

Forms of the Langmuir adsorption model have commonly been used to model the adsorption of cellulases to cellulose (Bothwell and Walker, 1995; Gilkes et al., 1992; Lee and Fan 1982; Steiner et al., 1988, Stuart and Ristroph, 1985). However, the Langmuir adsorption model is true only under isothermal conditions. For a more complete understanding of adsorption, the effect of temperature on adsorption must be examined. A kinetic treatment presented by Davies and Rideal (1961) and Kipling (1965) predicts adsorption equilibria using an Arrhenius definition of the kinetic constant. This analysis has been applied by Ladisch et al. (1991) in the form:

$$n = \left[ n_0 K \right]_c = \left[ n_0 K \cdot e^{\Delta W/kT} \right]_c$$

(1)

where \( n \) is solute taken up by the solid phase, \( n_0 \) is the maximum surface coverage, \( K \) is equilibrium constant, \( c \) is solute concentration in solution, \( k \) is ratio of solute adsorption and desorption rates, \( \Delta W \) is the difference between energies of adsorption and desorption, \( k \) is a constant proportional to the universal gas constant, and \( T \) is absolute temperature for the prediction of adsorption equilibrium constants for aspartame on a methacrylic, macroporous, polymeric sorbent. An Arrhenius plot of the natural log of \( K \) from isothermal data versus inverse temperature yields the values of \( \Delta W/k \) from the slope and a value of \( k \) from the intercept. This analysis predicts that the isotherm at low concentrations falls with rising temperature, which corresponds to a decrease in the equilibrium constant with little or no change in maximum coverage (possible adsorption sites). This correlation also corresponds to a weakening of the attractive forces between the solute and the solid surface and an increase in solubility of the solute in the solvent (Kipling, 1965). Consequently, the adsorption of the cellulose binding domain of a cellulase mimetic must be strong at room temperature for sufficient adsorption at hydrolysis temperatures (>100°C) to receive the benefit of the concentration effect.

Adsorption equilibrium constants are often determined by contacting liquid of varying concentrations of solute with a known amount of adsorbent until equilibrium is reached. However, at elevated temperatures and pressures, this experimental scheme is difficult. An effective method for determining dynamic adsorption and the equilibrium constant(s) is through the use of liquid chromatography where a pulse of liquid containing the solute in question is introduced to the inlet stream entering the stationary/solid-phase adsorbent and the elution of the solute from the column is measured. As long as the solute concentration is within the linear region of the adsorption curve, the elution data can be used to calculate the adsorption equilibrium (Ladisch, 2001a).

By definition, the adsorption equilibrium constant (partition coefficient) is defined as the ratio of solute concentration in the solid phase to concentration in the liquid phase. The retention behavior of solutes in liquid chromatographic
systems is usually expressed in terms of capacity factor, $k'$, in terms of total moles of solute. The capacity factor is defined as (Ladisch, 2001a).

$$k' = \frac{\text{moles of } A \text{ in the stationary phase}}{\text{moles of } A \text{ in mobile phase}}$$

(2)

The capacity factor is therefore directly proportional to the adsorption partition coefficient, $k'$ (Small, 1989).

$$k' = k' \frac{V_s}{V_m}$$

(3)

where $k'$ is LC capacity factor, $k'_{0}$ is adsorption equilibrium constant (partition coefficient), $V_s$ is volume of stationary phase, and $V_m$ is volume of mobile phase. The capacity factor can easily be determined from retention times of single eluting peaks in chromatograms. It is defined as:

$$k' = \frac{t_r - t_0}{t_0}$$

(4)

where $k'$ is LC capacity factor, $t_r$ is peak retention time, and $t_0$ is retention time of excluded component (Ladisch, 2001a). This relationship can be used when the solute concentration is within the linear portion of the adsorption equilibrium curve and the mobile phase flow rate is low enough to allow equilibrium to be attained.

**MATERIALS AND METHODS**

All chemicals used in these experiments were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). General laboratory and high-performance liquid chromatography (HPLC) supplies were obtained from Fisher Scientific (Pittsburgh, PA). Stainless-steel tubing and Swagelok fittings were purchased from Indianapolis Valve and Fitting Co. (Indianapolis, IN). All other specialty supplies, tools, and equipment were obtained from the manufacturers.

**Rolled Cotton Stationary-Phase Chromatographic Screening**

The screening was conducted utilizing a quick chromatographic method. Cotton and cotton/polyester blend textiles in tightly rolled cylinders have been shown to make good continuous stationary-phase materials for rapid chromatography (Hamaker et al., 1996, 1998; Li et al., 2002). The material chosen for the adsorption chromatography screening was an untreated cotton print cloth, which is nearly 100% cellulose. The advantage of using cotton to assess binding capabilities is the homogeneity of composition. Tested compounds are known to adsorb to cellulose as opposed to other polymers found in plant lignocellulose, such as hemicellulose and lignin.

This rolled, continuous stationary phase consists of cellulose fibers (cotton) that are aligned in the form of yarns, with the yarns woven into textiles. The textile was tightly rolled into a cylinder, which was inserted into a liquid chromatography column. The resulting experimental column was 17.4 cm long with a 1.0-cm inner diameter (Table I). Buffer of 50 mM NaCl and 5 mM H₂SO₄ (pH 2.2) in water was used as the mobile phase at a flow rate of 2.00 mL/min. NaCl was required to shield the naturally occurring negative charges on the cellulose surface to prevent ion-exchange binding of the tested compounds. Sulfuric acid was used to lower the pH to levels common to acid-catalyzed hydrolysis. The column apparatus was connected to a solvent delivery system pump (Rainin HPXL, Rainin Instrument Co., Woburn, MA), a manual injector with a 10-μL sample loop, and a differential refractometer (R401, Waters) for sample elution detection. The system was controlled and data collected using an HPLC controller (Rainin Dynamax DA) and data acquisition software on a Macintosh Performa 6300 CD computer.

A series of compounds were injected into this chromatographic system to determine binding. For each candidate, volumes of 10 μL were injected. Each candidate was dissolved in buffer to a concentration of 50 mg/mL. Dextran, 66.3-kDa average molecular weight, and sodium chloride were used as probe compounds to assess the external and internal void fractions of the stationary phase. Dextran eluted in the shortest time, 2.6 min. The longest time for complete elution, for indole, occurred in <10 min.

**Avicel Microcrystalline Cellulose Chromatographic Screening**

Measurement of adsorption at temperatures of >100°C required the use of a more inert form of cellulose than cotton textile. Furthermore, the binding domains of cellubiohydrolases interact with crystalline forms of cellulose. Microcrystalline cellulose, a highly recalcitrant material with a typical crystallinity index of 85% to 90% (De Souza et al., 2002; Fan et al., 1980), was chosen as the stationary phase. A packed-bed chromatography column was constructed from 0.375-in. × 0.065-in. 316 stainless-steel tubing cut to 6-in. length. The tube ends were fitted with 0.375-in. Swagelok end fittings that had 0.0625-in. outlet fittings and a frit with 20-μm-diameter pores (Swagelok, Solon, OH). The column was packed with microcrystalline cellulose, Avicel, sieved to a particle size of between 25 and 40 μm. Avicel was used in this higher temperature system because microcrystalline cellulose is highly recalcitrant and will not degrade at the

| Table I. Rolled cotton stationary-phase and Avicel column operating parameters. |
|---|---|---|
| **Rolled stationary-phase column** | **Avicel column** |
| Column volumes (mL) | 13.7 | 5.5 |
| Length (cm) | 7.4 | 15.2 |
| Flow rate (mL/min) | 2 | 1.45 |
| Temp. | 55°C | N/A |
| $\varepsilon_p + \varepsilon_P$ | 0.54 | 0.65 |
| $\Phi$ | 0.87 | 0.53 |
| System dead volume (mL) | N/A | 1.8 |
proposed temperatures unless it is first pretreated. Because Avicel is >98% pure cellulose, adsorption was most likely to cellulose, not hemicellulose or lignin. The column was placed in a Varian 3700 GC oven for temperature control.

The remaining system components were similar to a conventional HPLC system (Fig. 1). A minipump device (16 to 160 mL/h) was used for pumping (Milton-Roy Co., Ivyland, PA). Solution elution was achieved using an absorbance detector (Model V4, Isco, Inc., Lincoln, NE) set to 260 nm (UV). Data from the UV absorbance detector were logged onto a PC using a data acquisition board (Strawberry Tree DAQ, Strawberry Tree, Inc., Sunnyvale, CA). A pressure check valve (Swagelok Co.) set to 100 psi at outlet of the cooling bath prevented the superheated liquid buffer from flashing to gas at the system outlet. A 1-mL injection loop connected to a Varian manual HPLC injector (Rainin Instrument Co., Inc., Woburn, MA) was used to inject the samples into the system. HPLC stainless-steel tubing (0.625 in.) was used for solvent delivery to and from the column. Twenty-four inches of tubing preceded the column in the gas chromatography (GC) oven to allow solvent heat-up. The length of tubing between the outlet of the GC oven and the UV detector was submerged in ice water to cool the solvent before the check valve. One drawback to this system is the significant dead volume in the tubing that must be subtracted to calculate the adsorption equilibrium constants over Avicel.

This dead volume was measured by replacing the column with a “zero-volume” union (Swagelok Co.) and measuring the elution of the probe compound, tris(hydroxymethyl)-aminomethane (Tris). Tris adsorsbs UV light and was found to elute at nearly the same retention volume as sodium chloride in the rolled cotton stationary-phase column. The key operational parameters of the Avicel chromatography system are given in Table I.

Binding was characterized at 50°, 80°, and 120°C to determine the Arrhenius constants of adsorption. Three injections of each candidate in aqueous solution were tested at each temperature. The equilibrium constants were calculated using the equations and methods described earlier.

RESULTS AND DISCUSSION

Molecular understanding of the relationship between structure and function in cellulolytic enzymes, especially in cellulose binding, offered a starting point for selecting candidates for screening. Point mutagenesis of the T. reesei carbohydrolase I gene has shown that aromatic residues, two tyrosines and one tryptophan on β-sheets on the cellulose-binding face of the enzyme, are likely important for binding (Linder et al., 1995). Replacing the tyrosines on this flat face with alanine showed a marked decrease in adsorption to crystalline cellulose. The family II and family III binding domains from bacterial cellases consist of a similar structure that is much larger than family I cellulose binding domains (100 to 170 amino acid residues) with three aromatic amino acid residues, mainly tryptophan, associated with the cellulose binding face of the enzyme (Tormo et al., 1996). Aromatic amino acids, especially tryptophan and tyrosine, provided initial candidates for screening potential cellulose binding candidates for generating an enzyme mimetic.

The microstructure of individual cellulose fibrils that compose a cotton cellulose thread impart some size-exclusion capability to a rolled stationary-phase column, as can be seen in the near-baseline separation between Dextran (66.3 kDa) and sodium chloride (Li et al., 2002). However, cellulose binding, not size-exclusion effects, is the property of interest for mimetic binding domain candidates. Two probe compounds were used to determine the internal and external void fractions of the column: Dextran (66.3 kDa) and sodium chloride. High-molecular-weight dextran is completely excluded from the internal pores, whereas sodium and chloride ions are able to penetrate nearly all of the internal pore volume. However, elution of solutes from the Avicel column was detected using UV adsorption. By definition, the total void fraction of the column, \( e_t \), is defined by the sum of the external void fraction and the pore void fraction or, more simply, the retention volume of a small molecule (NaCl or Tris), \( V_0 \), divided by the total column volume, \( V_{col} \):

\[
\epsilon_t = \epsilon_b + \epsilon_p = \frac{V_0}{V_{col}}
\]  

A more useful constant is the phase ratio, \( \phi \), defined as the ratio of stationary phase to mobile phase in the column [Eq. (6)]. Void fraction and phase ratio for the rolled stationary phase column are listed in Table I:

\[
\phi = \frac{1 - \epsilon_t}{\epsilon_t}
\]  

Because adsorption to cellulose, not size exclusion, was the key property being screened, the capacity factor, \( k' \), of a binding domain candidate was defined using the aforementioned total void fraction definition of phase ratio. From the aforementioned experimentally derived constants, the equilibrium constant, \( K \), can be calculated:

\[
k' = \frac{V_t - V_0}{V_0} = K\phi
\]

The results from selected binding domain candidates are listed in Table II. All equilibrium constants are calculated by defining the retention volume of sodium chloride as \( V_0 \).
The final column in Table II gives the theoretical plate heights for the solutes by the half-height method (Ladisch, 2001b). The amino acid tryptophan adsorbed well, but it was found that the side chain, indole, was by far the most retained candidate tested (Figs. 2 and 3). Tryptophan has been shown to be a critical amino acid in the binding of cellulytic enzymes to cellulose through hydrophobic adsorption. Tyrosine has been shown to be critical for enzymatic adsorption; however, in the chromatographic system, tyrosine—tryptophan dimer was less retained than tryptophan alone or tryptophan—tryptophan dimer (Fig. 2).

If hydrophobic adsorption is the mechanism of the retention of indole in the rolled cotton stationary phase, temperature should influence the binding characteristics/retention time. The effect of temperature on binding was examined by injecting an indole solution into the same column operating at 30°, 55°, and 80°C (Fig. 3). Unsurprisingly, increased temperature reduced the retention volume slightly while sharpening the elution profile. These results are indicative of hydrophobic adsorption driven by enthalpy.

If the adsorption can be modeled using the Langmuir assumptions that there is no interaction between the molecules of adsorbed solute on the surface, and that the equilibrium is governed by the way the solute is adsorbed and desorbed, an Arrhenius definition of the kinetic constants of adsorption can be used to predict adsorption equilibria at various temperatures. An Arrhenius plot of the natural log of $K_D$ from isothermal data versus inverse temperature yields the values of $\Delta W/k$ from the slope and a value of $x$ from the intercept. An Arrhenius plot of the data obtained from indole

<table>
<thead>
<tr>
<th>Compound</th>
<th>RV (column volume)</th>
<th>RT (min)</th>
<th>$k'$</th>
<th>$K_D$</th>
<th>$H$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole</td>
<td>0.85</td>
<td>5.82</td>
<td>0.59</td>
<td>0.68</td>
<td>2.41</td>
</tr>
<tr>
<td>l-DOPA</td>
<td>0.61</td>
<td>4.19</td>
<td>0.15</td>
<td>0.17</td>
<td>3.09</td>
</tr>
<tr>
<td>Phthalic acid</td>
<td>0.61</td>
<td>4.15</td>
<td>0.14</td>
<td>0.16</td>
<td>2.29</td>
</tr>
<tr>
<td>2,6-pyridine dicarboxylic acid</td>
<td>0.61</td>
<td>4.14</td>
<td>0.13</td>
<td>0.15</td>
<td>2.57</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.59</td>
<td>4.00</td>
<td>0.09</td>
<td>0.11</td>
<td>N/A</td>
</tr>
<tr>
<td>Imidazole</td>
<td>0.57</td>
<td>3.91</td>
<td>0.07</td>
<td>0.08</td>
<td>3.41</td>
</tr>
<tr>
<td>Indoline</td>
<td>0.56</td>
<td>3.86</td>
<td>0.05</td>
<td>0.06</td>
<td>3.46</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>0.55</td>
<td>3.78</td>
<td>0.03</td>
<td>0.04</td>
<td>3.18</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.55</td>
<td>3.75</td>
<td>0.02</td>
<td>0.03</td>
<td>3.28</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>0.54</td>
<td>3.69</td>
<td>0.01</td>
<td>0.01</td>
<td>2.50</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.54</td>
<td>3.66</td>
<td>0.00</td>
<td>0.00</td>
<td>2.07</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.54</td>
<td>3.66</td>
<td>0.00</td>
<td>0.00</td>
<td>2.05</td>
</tr>
<tr>
<td>Tris</td>
<td>0.53</td>
<td>3.64</td>
<td>0.00</td>
<td>0.00</td>
<td>3.68</td>
</tr>
<tr>
<td>1,4,5,8-naphthalene tetracarboxylate</td>
<td>0.53</td>
<td>3.61</td>
<td>-0.01</td>
<td>-0.02</td>
<td>10.88</td>
</tr>
<tr>
<td>Bicine</td>
<td>0.52</td>
<td>3.58</td>
<td>-0.02</td>
<td>-0.02</td>
<td>1.94</td>
</tr>
<tr>
<td>Bis-Tris</td>
<td>0.52</td>
<td>3.57</td>
<td>-0.02</td>
<td>-0.03</td>
<td>2.72</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>0.52</td>
<td>3.53</td>
<td>-0.04</td>
<td>-0.04</td>
<td>2.87</td>
</tr>
<tr>
<td>Bis (2-hydroxyethyl)-piperidine</td>
<td>0.50</td>
<td>3.42</td>
<td>-0.06</td>
<td>-0.07</td>
<td>3.95</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.50</td>
<td>3.42</td>
<td>-0.07</td>
<td>-0.08</td>
<td>3.22</td>
</tr>
<tr>
<td>1,3,6-naphthalene trisulfonic acid</td>
<td>0.48</td>
<td>3.25</td>
<td>-0.11</td>
<td>-0.13</td>
<td>3.55</td>
</tr>
<tr>
<td>Dextran, 39, 4 kDa</td>
<td>0.38</td>
<td>2.56</td>
<td>-0.30</td>
<td>-0.35</td>
<td>2.60</td>
</tr>
</tbody>
</table>

**Figure 2.** Chromatograms of tryptophan and various derivatives at 20°C over a rolled cotton stationary phase in 50 mM NaCl mobile phase.

**Figure 3.** Chromatograms of indole in rolled cotton stationary-phase column system at various column temperatures.
at various temperatures is shown in Figure 4a. If the slope, \( \Delta W/k \), and intercept, \( \kappa \), data are used to extrapolate the adsorption equilibrium, \( K_D \) will only be 0.33 at 175°C. Because the partition coefficient is directly proportional to the concentration effect of adsorption, a \( K_D \) of 0.33 will poorly concentrate the acid to the cellulose surface. This result suggests that indole will make a relatively poor binding domain for the enzyme mimetic at the elevated temperatures required for cellulose hydrolysis. One possible error in this extrapolation pertains to the fact that molecular interactions in aqueous solutions are almost always accompanied by a significant change in heat capacity that leads to a nonlinear dependence of \( \ln(K_D) \) on \( 1/T \) over a large temperature range. Although \( K_D \) for indole cannot be confidently predicted at 175°C based on the data presented, the affinity of indole to cellulose at elevated temperatures will be less than the relatively weak affinity measured at 20°C, making this a poor choice for a binding domain to selectively adsorb carboxylic acids near the cellulose surface.

Changing pH by varying sulfuric acid concentration in the buffer had no affect on retention of indole (data not shown). Another notable result (Table II) is that many compounds are excluded from the pore volume (\( K_D < 0 \)). One commonality between these various compounds is a negative charge at pH 2.2. Cellulose has a native cation-exchange capability (Hamerker et al., 1996), thus the solute exclusion is most likely due to the negative charge on the cellulose surface repelling these compounds from the pore volume.

Indole, the best candidate from the rolled cotton stationary-phase chromatographic screening, was tested at 30°, 80°, and 120°C in the Avicel chromatography system (Fig. 5). In addition, lignin derivatives, ferulic acid (4-hydroxy-3-methoxycinnamic acid), 4-hydroxycinnamic acid, and cinnamic acid, were also tested at 30°C (Fig. 6). The lignin derivatives were screened because, like indole, they all possess an aromatic structure and adsorption of these monomers has been suggested as a possible mechanism facilitating the close interaction of lignin to cellulose in the secondary cell walls of higher plants (Houtman and Atalla, 1995). Of the lignin monomers tested, ferulic acid adsorbed to Avicel nearly as well as indole and better than all of the other binding domain candidates reported in Table II.

The effect of temperature on the adsorption of indole to Avicel is similar to its adsorption to cotton. An Arrhenius plot of the indole adsorption equilibria for the three temperatures over Avicel is shown in Figure 4b. Although the value of \( \Delta W/k \) (the difference between energies of adsorption and desorption over a constant proportional to the universal gas constant) for indole adsorption over cotton is statistically different from Avicel at a 95% confidence interval, the difference is insignificant at confidence intervals of >95%.

The data suggest that indole, when linked with an acid catalyst, will not effectively increase the local catalyst concentration at the cellulose surface at hydrolysis temperatures (>150°C). Because indole is a planar, aromatic compound, a larger planar aromatic compound was selected as a candidate for the cellulose-binding domain (Fig. 7a). The hypothesis is that increased aromatic moieties will increase the adsorption equilibrium because hydrophobic interactions drive adsorption to cellulose as demonstrated by the Arrhenius plot. Trypan blue (Fig. 7b), commonly used to dye
cellular organelles for microscopy, is planar and has multiple aromatic moieties.

The negatively charged $-\text{SO}_3^-$ moieties improve the solubility of trypan blue in water, but the impact of these charges on adsorption may be negative. Hydrophilic and charged moieties on a complete cellulase mimetic will be required to make the solubility of the catalyst in water sufficient for effective delivery to the cellulose surface for hydrolysis. This must be balanced by the impact these charges have on the strength of binding. A significant change in solution pH that changes the net charge on the cellulase mimetic may significantly alter the adsorption properties of the catalyst. This suggests a possible mechanism for controlling the adsorption of the catalyst and recovery of the mimic from the remaining insoluble fiber after hydrolysis.

The rolled cotton stationary-phase chromatography system was used to assess the binding characteristics of trypan blue because the glass column allowed visual inspection of the migration of dye through the column. However, trypan blue adsorbed very strongly at 20°C. The dye visibly stained the rolled cotton stationary phase at the inlet of the column after injection. After 15 column volumes of buffer were used to flush the column, the visibly stained cotton was confined to the upper 10% of the column. Further flushing at 20°C failed to cause the migration of the dye through the stationary phase. Trypan blue was also injected into the Avicel column system at 120°C to determine if adsorption was low enough to see elution from the packed bed. Three separate 1-mL injections were made, each followed by 40 bed volumes of eluent. A small rise in UV absorbance was measured at the retention time corresponding to the system total void volume (corresponding to the elution of Tris). However, the total peak area was <3% of elution peaks of trypan blue in the system when the column was replaced with a zero-volume union (Fig. 8). These eluting compounds are most likely contaminants in the trypan blue (3% by weight per analysis from supplier). Physical examination of the Avicel packing the column after these experiments revealed blue-stained microcrystalline cellulose only in the top 10% of the packed bed.

The strong adsorption of trypan blue makes it a promising candidate for the cellulose binding domain for a constructed cellulase enzyme mimetic. Further experiments at elevated temperatures that should lower the adsorption equilibrium are required to assess the properties of trypan blue at hydrolysis conditions (temperature >150°C). Multiple planar, aromatic structures increase the total energy of adsorption for trypan blue, which may account for the strength of adsorption. The presence of amines and hydroxyl moieties on trypan blue offers opportunities for linking chemistries to connect acid catalysts to this cellulose-adsorbing compound.

Figure 6. Equilibrium constants for indole and lignin monomers from an Avicel stationary-phase chromatography column obtained at 30°C. Additional equilibrium constants for indole at higher temperatures included for comparison.

Figure 7. Structure of (a) indole and (b) trypan blue.

Figure 8. Chromatograms trypan blue and Tris from a high-temperature chromatography system with and without Avicel stationary-phase column.
The strength of adsorption is also the major drawback of trypan blue for constructing an enzyme mimetic catalyst. Nearly irreversible binding of the catalyst to the cellulose surface may limit hydrolysis effectiveness if the catalyst cannot desorb and readсорb at different locations as the cellulose is hydrolyzed. An ideal cellulose binding compound probably lies in between indole and trypan blue in terms of molecular weight and number of planar, aromatic moieties. Additional factors that must receive attention for further development of a cellulase mimetic are the recovery, reuse, and disposal of the enzyme mimetic. Carboxylic or other acid moieties on the mimetic may allow control of adsorption by changes in solution pH, which influences the net charge, and thus hydrophobicity, of the mimetic. Raising the solution pH, thus deprotonating the mimetic, may be a method of recovering the mimetic for recycling. Even if the mimetic can be recycled, a hydrolysis process utilizing an enzyme mimetic must include disposal of used or fouled catalyst. Toxicity to fermentative microorganisms and biodegradation are also important factors for consideration in the continued development of a cellulose-mimicking catalyst for industrial use.

CONCLUSIONS

The rolled stationary-phase chromatographic screening method outlined herein allows for a substantial number of compounds to be screened for cellulose adsorption. From these results, aromatic, planar chemical structures appear to be key indicators of cellulose adsorption. Similar results were also found for a form of microcrystalline cellulose, Avicel. Indole, the side-chain of the amino acid tryptophan, has been shown to reversibly adsorb to cellulose at temperatures between 30° and 120°C. The affinity of trypan blue to both cotton and Avicel was shown to be sufficiently high at temperatures of <120°C to appear to be irreversibly bound on the time scale of the experiments. These results show the importance of multiple hydrophobic interactions between cellulose and the cellulose-binding component of cellulolytic enzymes and enzyme mimetics for strong adsorption characteristics. However, simple hydrophobic adsorption is not the only governing parameter for cellulose adsorption. Other hydrophobic, planar molecules such as phenylalanine, L-dopamine, phthalic acid, and dipicolinic acid (2,5-pyrinedicarboxylic acid) adsorbed to cellulose with lower equilibrium constants than indole. A second important factor for enzyme adsorption to cellulose may be due to hydrogen bonding between the protein and the hydroxyl groups of the anhydroglucose units of the cellulose. These results suggest that a structure with multiple planar aromatic rings, of which at least one can be functionalized to ultimately covalently attach the catalytic domain, is needed to construct a cellulolytic enzyme mimetic.

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MOSIER ET AL.: EVALUATING ADSORPTION BY CHROMATOGRAPHY 763