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Adsorption of *Thermomonospora fusca* E_5 and *Trichoderma reesei* Cellobiohydrolase I Cellulases on Synthetic Surfaces

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Abstract

The interfacial behavior of *Thermomonospora fusca* E_5 and *Trichoderma reesei* cellobiohydrolase I (CBHI) cellulases were studied at synthetic surfaces. For this purpose, colloidal silica and polystyrene particles were used to prepare cellulase-particle suspensions that could be analyzed by solution-phase techniques. Circular dichroism spectroscopy of each cellulase, alone as well as in suspension with silica, was used to determine whether structural changes occurred on adsorption. Changes in spectra were observed for CBHI, but not for E_5. Gel-permeation chromatography of the cellulase-particle suspensions showed that neither cellulase binds to silica, suggesting that changes in spectra for CBHI were a result of solution-phase phenomena. Microfiltration of cellulase-polystyrene suspensions showed that both cellulases bind to polystyrene. However, circular dichroism experiments with polysterene proved unworkable, owing to excessive light absorption by the polystyrene. Adsorption kinetics of each cellulase were recorded, *in situ*, at hydrophilic and silanized, hydrophobic silica surfaces using ellipsometry. Ellipsometric data recorded for each cellulase at hydrophilic silica showed insignificant adsorption. Binding did occur between each cellulase and silanized silica, most likely mediated through hydrophobic associations. Adsorption in this case was irreversible to dilution.

Index Entries: *Thermomonospora fusca* E_5; *Trichoderma reesei* CBHI; cellulase adsorption; cellulase binding; hydrophobic effects.

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Introduction

Protein interactions with solid surfaces have been studied for decades, and several comprehensive reviews are available *(1–4)*. Protein adsorption occurs at virtually any natural or synthetic surface in contact with a protein-containing fluid and is thus important in many areas relevant to bioprocess and biomedical technology. In cellulase-cellulose systems, protein adsorption is an important event accompanying hydrolysis. The presence of multiple enzymes and the heterogeneous and transient nature of cellulose structures complicate this system.

Many important observations relevant to protein interfacial behavior in complex systems have been explained in terms of a protein's tendency to unfold and contact surface hydrophobicity. Past theoretical and experimental work with proteins at interfaces supports the notion that a given protein can adsorb in different structural states. Proteins are observed, in general, to change conformation to a greater extent on hydrophobic surfaces relative to hydrophilic surfaces, owing to the presence of hydrophobic interactions between the solid surface and hydrophobic regions in the protein. On a hydrophilic surface, forces acting between the surface and the protein may be smaller in magnitude. The resulting conformational change would likely be smaller, preserving a greater repulsive force among adsorbed proteins *(5,6)*.

Carbohydrate-protein interactions often involve aromatic residues on the binding face of the protein *(7–10)*. Studies with carbohydrate-binding proteins in general have shown that the interaction between these aromatic rings and sugars can be largely attributed to entropically driven, hydrophobic associations *(11,12)*. Site-directed mutagenesis studies have shown that aromatic residues present on the hydrophobic binding face of CBHI and endoglucanase I of *Trichoderma reesei* are important in binding *(11,13)*. Furthermore, study of the effects of ionic strength on CBHI binding pointed to the importance of hydrophobic interactions *(14)*. Hydrophobic intercations have been shown to play an important role in the binding of cellulases of bacterial origin as well. For example, isothermal titration microcalorimetry studies indicated that dehydration (hydrophobic) effects are the primary driving force for *Cellulomonas fimi* Cex binding to cellulose *(15)*. Hydrophobic interactions have been shown to play a role in binding of *C. fimi* CenC as well, a cellulase that binds only soluble cellulose *(16)*.

In the present study, we investigated interactions between *Thermomonospora fusca* E_5 and *T. reesei* CBHI, and well-characterized, synthetic solid surfaces. We used hydrophilic and hydrophobic (silanized) silica, as well as hydrophobic polystyrene, in order to study hydrophobic effects on binding. The model surfaces used here were not meant to mimic a cellulosic surface. In particular, we anticipate that once the influence of a selected factor on adsorption is understood at a model surface, experiments can then be extended to include more relevant surfaces. For example, model surfaces have been used extensively to study plasma protein adsorption phenomena, and this has proved instrumental in establishing our current

understanding of the biocompatibility of blood-contacting implants *(1–3)*. Thus, we believe that in the absence of mass transfer limitations, pore-size and other morphologic effects *(17)*, and hydrolysis, all of which accompany cellulase binding in natural circumstances, questions relating to hydrophobic influences on cellulase adsorption can be meaningfully and less ambiguously addressed.

Materials and Methods

Production and Purification of T. fusca E_5 *and* T. reesei *CBHI*

T. fusca E_5 cellulase was produced by transformed *Streptomyces lividans* TK24 carrying a plasmid (pGG74) bearing the E_5 gene *(18)*. The production and purification procedures were based on those described earlier *(17)*. In particular, the culture was initiated from frozen stock into 15 mL of tryptic soy broth medium containing 5 µg/mL of thiostrepton and incubated at 30°C for 48 h. This was subcultured into 150 mL of the same medium and incubated for 24 h. This culture was used to inoculate a 7-L fermentor containing 4.8 L of the tryptic soy broth medium. The fermentation was carried out at 30°C, agitated at 150 rpm, for 48 h. Cells were removed with centrifugation (Beckman J2-MI; Seattle, WA) and filtration (Millipore Pellicon Filter system with a 0.22-µ cassette; Millipore, Bedford, MA). The filtered supernatant was adjusted to 1.2 *M* $(NH_4)_2SO_4$ and 0.1 m*M* phenylmethylsulfonyl fluoride and loaded onto a 150-mL Phenyl Sepharose CL-4B column (Sigma, St. Louis, MO). After washing the column, bound cellulase was eluted with a series of buffers as described by Walker et al. *(19)*. All fractions containing E_5 were combined and concentrated by ultrafiltration with a PM 30 membrane. The cellulase was then diluted 1:1 with 0.01 *M* BisTris, pH 5.4, and passed through a 150-mL Q-Sepharose column (Sigma). The column was washed with 1 column vol of 0.02 *M* BisTris, pH 5.4. The protein was eluted with a linear gradient of 0–0.3 *M* NaCl in the same buffer. E_5 fractions were combined, ultrafiltered, and stored at –80°C until use.

T. reesei CBHI was purified from crude cellulase (Spezyme™-CP; Environmental BioTechnologies, Menlo Park, CA). The purification process was as described by Piyachomkwan et al. *(20)*. In brief, the crude cellulase was passed through a DEAE-Sepharose CL-6B column. Fractions containing CBHI were combined and passed through a *p*-amino-phenyl-1-thio-β-D-cellobioside affinity column with 0.1 *M* NaOAc, pH 5.0, and 1 m*M* D-glucono-δ-lactone as the mobile buffer. The cellulase was eluted by adding 0.01 *M* cellobiose to the buffer. The partially purified CBHI fractions were combined and concentrated prior to loading on a Phenyl Sepharose CL-4B column. The loading buffer was 25 m*M* NaOAc, pH 5.0, containing 0.85 *M* $(NH_4)_2SO_4$. The column was washed with 5 column vol of the buffer and then eluted with a linear gradient of 0.85–0.35 *M* $(NH_4)_2SO_4$ in buffer. The CBHI fractions were combined, and the buffer was exchanged to 50 m*M* NaOAc, pH 5.0, by ultrafiltration with a PM 10 membrane. The concentrated cellulase was stored at –80°C until use. Both cellulases

were judged to be 98 to 99% pure as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and carboxymethyl cellulase activity.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter with a xenon lamp in a manner similar to that described by Tian et al. *(21)*. In particular, CD spectra were recorded from 400- to 184-nm wavelengths using a quartz cuvet of 0.2-mm path length. Sample concentrations were 0.3 mg/mL of E_5 and 0.2 mg/mL of CBHI. Spectra presented are an average of three scans at 10 nm/min. The percentage of α-helix content was estimated using the ellipticity recorded at 222 nm (θ_{222nm}) according to Kondo et al. *(22)*:

$$\% \ \alpha\text{-helix} = \frac{-[\Theta_{222nm}]}{40{,}000} \times 100$$

Secondary structure of the proteins was predicted using the variable selection method *(23)*. This program combines singular value decomposition with the statistical method of variable selection to estimate the secondary structure of a protein based on its CD spectrum.

CD spectra were also obtained for each cellulase in the presence of 20-nm silica particles (EKA, Marietta, GA). Cellulase-particle suspensions were prepared by mixing cellulase with particles at a particle:protein ratio of 2:1 and allowing adsorption to occur for 90 min at 25°C and 125 rpm. All remaining procedures were the same as those described for the particle free samples.

Size Exclusion Chromatography

Size exclusion studies were carried out in sodium phosphate buffer, pH 7.0, with either hydrophilic silica or hydrophobic polystyrene particles and cellulase, as well as with cellulase alone. Cellulase concentrations used were 1.0 mg/mL of E_5 and 0.5 mg/mL of CBHI. Colloidal silica (20 nm) and 19-nm polystyrene particles (Duke Scientific, Palo Alto, CA) were used without modification. Cellulase-particle suspensions were prepared by mixing cellulase with particles at a particle:protein ratio of 2:1 and allowing adsorption to occur for 90 min at 25°C and 125 rpm. This suspension (1 mL) was passed through a Sephadex G-100 column (Pharmacia Biotech, NJ) at a flow rate of about 400 µL/min. The absorbance of each 0.8- to 1.0-mL fraction was recorded at 280 nm using a Spectronic 601 (Milton Roy) spectrophotometer. The same procedure was performed with cellulase in the absence of particles. All experiments were performed at least twice.

Microporous Membrane Separations

Cellulases were mixed with 0.21-µm-diameter polystyrene microspheres (Bangs, Fishers, IN) and allowed to adsorb for 90 min at 25°C and 125 rpm. Cellulase concentrations used were 0.23 mg/mL of E_5 and 0.31 mg/mL of CBHI. After incubation, samples were placed in Ultrafree

CL 0.1-µm Durapore membrane filter tubes (Millipore) and centrifuged at 4000*g* for 80 min at 4°C. The absorbance of the filtrate at 280 nm was recorded. Particle-only filtrate was used as a control. These samples were centrifuged at 4000*g* for 20 min at 4°C. All experiments were performed three times.

Ellipsometry

All surfaces were prepared from silicon (Si) wafers (hyperpure, type N, phosphorous doped, orientation 1-0-0) purchased from Wacker Siltronic (Portland, OR). Surfaces were oxidized in O_2 (1 atm) for 17 min at 1000°C. Next, they were cut into rectangles of 1×3 cm^2 using a tungsten pen. Each surface was then washed with a solution of NH_4OH and H_2O_2, rinsed with distilled and deionized water, washed with a solution of HCl and H_2O_2, and rinsed again with distilled and deionized water, according to procedures described elsewhere *(24)*. This treatment rendered the surfaces hydrophilic, as verified by their wettability. A set of these surfaces were made hydrophobic according to the procedure of Jönsson et al. *(25)*, as slightly modified by Krisdhasima et al. *(24)*. In particular, the surfaces were silanized with 0.1% dichlorodimethylsilane (Aldrich, Milwaukee, WI) in xylene. Silanized surfaces were then washed in sequence with xylene, acetone, and ethanol. Surface hydrophobicity was verified with contact angle methods *(5)*.

Protein adsorption was monitored continuously with an automated Garertner *in situ* ellipsometer (Gaertner, Chicago, IL) equipped with a thermostated cuvet and modified to allow for stirring and flow. The instrument was described in detail by Podhipleux *(26)*. Adsorbed mass was calculated from ellipsometrically determined values of film thickness and refractive index according to Cuypers et al. *(27)*, using a calculation procedure based on a one-film model *(28)*. The partial specific volume and the ratio of molecular weight to molar refractivity, both required to determine adsorbed mass, are 0.85 cm^3/g and 4.05 g/cm^3, respectively, for E_5, and 0.84 cm^3/g and 4.05 g/cm^3, respectively, for CBHI *(27,29)*.

Experiments were performed with 50 m*M* sodium acetate buffer, pH 5.5, at 325 rpm and 25°C. The pseudorefractive index of the bare surface was determined prior to the addition of enzyme. An experiment began with the addition of 1.0 mL of enzyme solution to the cuvet containing 5.0 mL of buffer, yielding a final protein concentration of 0.10 mg/mL of E_5 and 0.13 mg/mL of CBHI. Adsorption was monitored for 30 min. The cuvet was then rinsed with buffer for 5 min at a flow rate of 25 mL/min, and film properties were monitored for an additional 20 min. Each experiment was performed at least three times.

Results and Discussion

CD Spectroscopy

The CD spectra of E_5 and CBHI, in the absence and presence of colloidal silica, are shown in Fig. 1. The CD spectrum of E_5 was virtually unchanged in the presence of silica. This indicated that either no conforma-

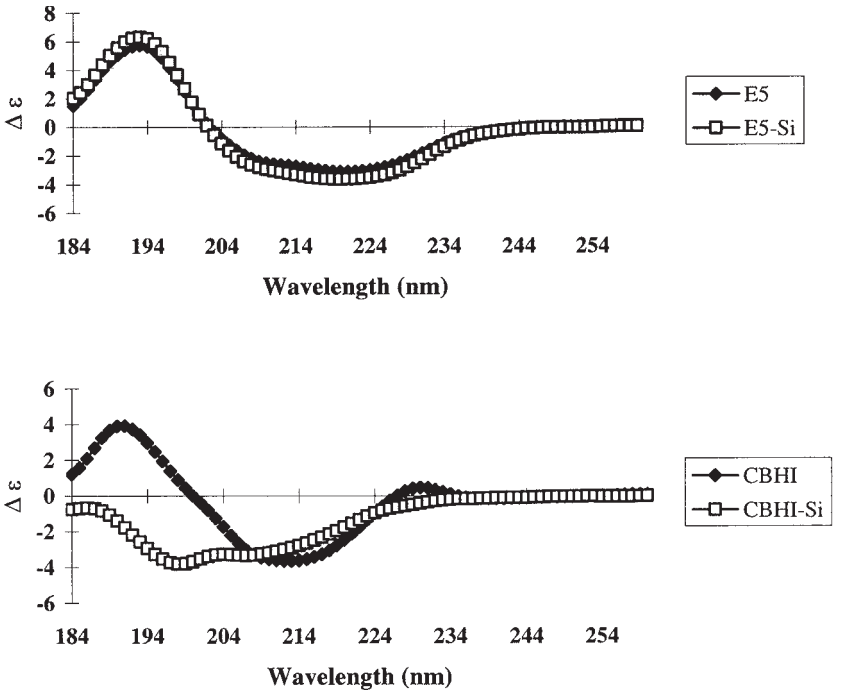

Fig. 1. CD spectra of cellulase in the absence (◆) and presence (□) of colloidal silica.

Table 1
Secondary Structure of Cellulases in Absence and Presence of Colloidal Silica[a]

	Secondary structure content (%)				
	α-Helix	Parallel β-sheet	Antiparallel β-sheet	β-Turn	Other
T. fusca E_5	26(1)	9(1)	19(1)	15(1)	30(1)
T. fusca E_5 + silica	31(1)	7(1)	17(1)	15(1)	30(1)
T. reesei CBHI	11(2)	14(1)	34(3)	10(1)	32(2)
T. reesei CBHI + silica	6(1)	8(1)	27(2)	20(1)	37(1)

[a]Standard deviations are given in parentheses.

tional changes took place upon adsorption or adsorption did not occur. Table 1 shows the secondary structure estimated for each cellulase in the absence and presence of silica. In the case of E_5, it is clear that the distribution of α-helix, β-sheet, and other structures changed little on the addition of silica. While α-helix content was determined to increase by 5% on addition of silica, there is no compelling reason to attribute this increase to an adsorption event. In fact, changes in secondary structure for a number of proteins upon adsorption to nanoparticles have been associated with only a decrease in α-helix content *(21,22,30,31)*. By contrast, the CD spectra of CBHI recorded in the absence and presence of silica were dissimilar, suggesting that CBHI did experience structural alteration.

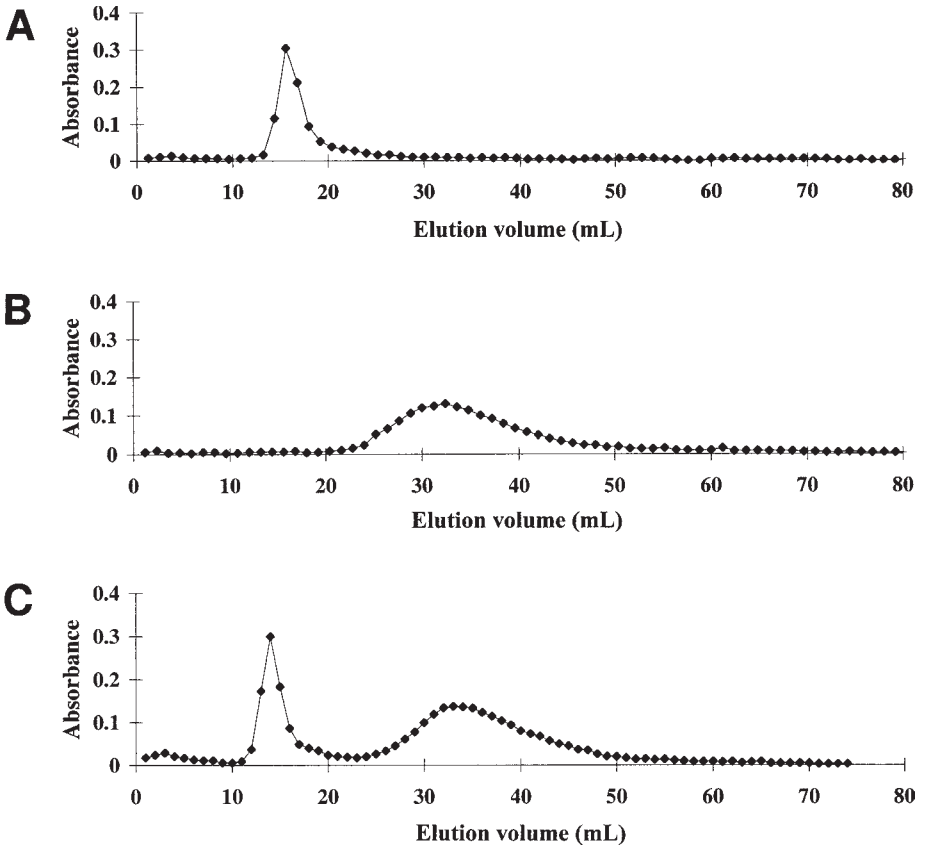

Fig. 2. Size exclusion chromatography elution profiles of **(A)** 20-nm-diameter silica particles; **(B)** E_5; and **(C)** E_5 in suspension with silica. Absorbance was measured at a wavelength of 280 nm.

CD measurements of cellulases in the presence of polystyrene particles were attempted as well. While other studies have reported protein structural changes upon adsorption to polystyrene *(22)*, we were unable to measure changes in ellipticity, apparently owing to the high light absorbance of the polystyrene particles. To interpret the data of Fig. 1 unambiguously, it was necessary to determine whether binding to silica actually occurred in each case.

Verification of Adsorption and the Importance of Hydrophobic Associations

Size exclusion chromatography provides a simple way to determine whether cellulase is bound to the silica particles *(30)*. If cellulase does not adsorb, two peaks should elute from the column, whereas one peak would be recorded if cellulase-particle complexes are formed. Elution profiles for silica particles, cellulase, and cellulase in the presence of silica particles are shown in Figs. 2 and 3 for E_5 and CBHI, respectively. Figures 2 and 3 show that individual silica and cellulase peaks are preserved on mixing, for each

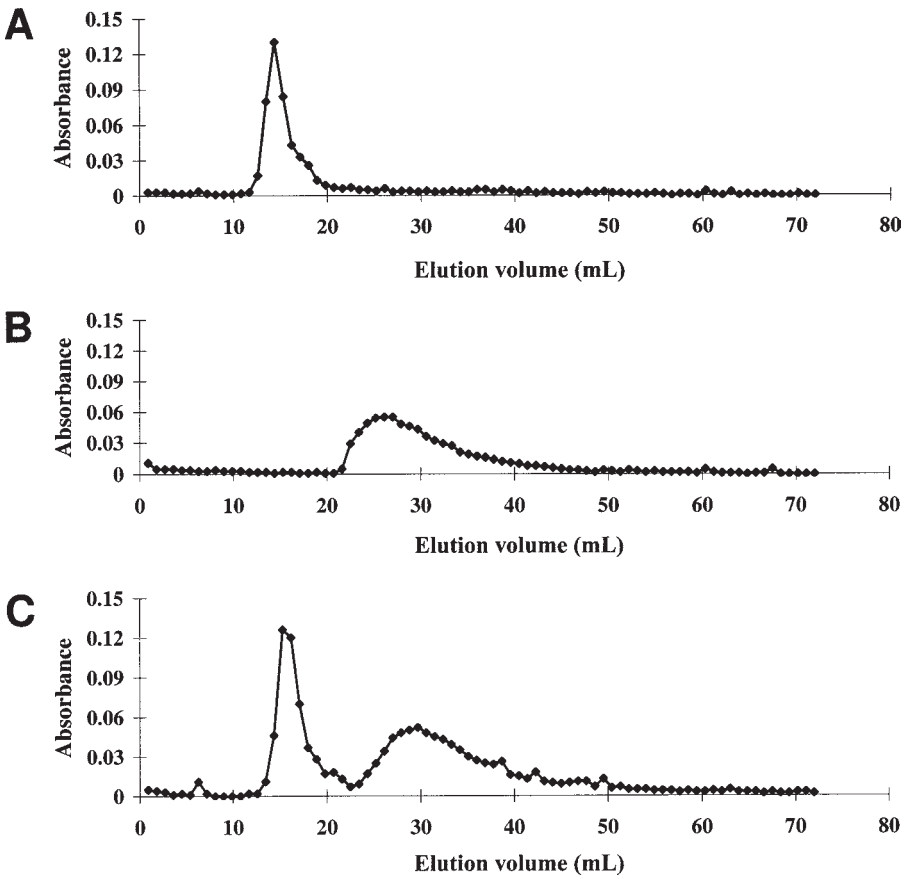

Fig. 3. Size exclusion chromatography elution profiles of **(A)** 20-nm-diameter silica particles; **(B)** CBHI; and **(C)** CBHI in suspension with silica. Absorbance was measured at a wavelength of 280 nm.

cellulase. This indicates that binding did not occur in either case. The CD spectrum of E_5 in the presence of silica is consistent with E_5 remaining unbound upon mixing with the particles. The changes in the spectrum recorded for CBHI in the presence of silica, however, cannot be attributed to surface-induced conformational changes on adsorption. The CD data in this case may be a result of solution-phase phenomena, e.g., protein aggregation occurring upon mixing with colloidal silica.

Gel filtration experiments carried out with polystyrene particles yielded different results. Figure 4 shows the elution profile of polystyrene microspheres, alone and in the presence of each cellulase. The small peak eluted between 50 and 60 mL (Fig. 4A) represents trace surfactants used by the manufacturer to maintain polystyrene microspheres in suspension. Only one large peak was recorded for each cellulase-particle suspension, suggesting that both E_5 and CBHI adsorb to the hydrophobic particles. The elution profiles display a long tail region, presumably owing to light absor-

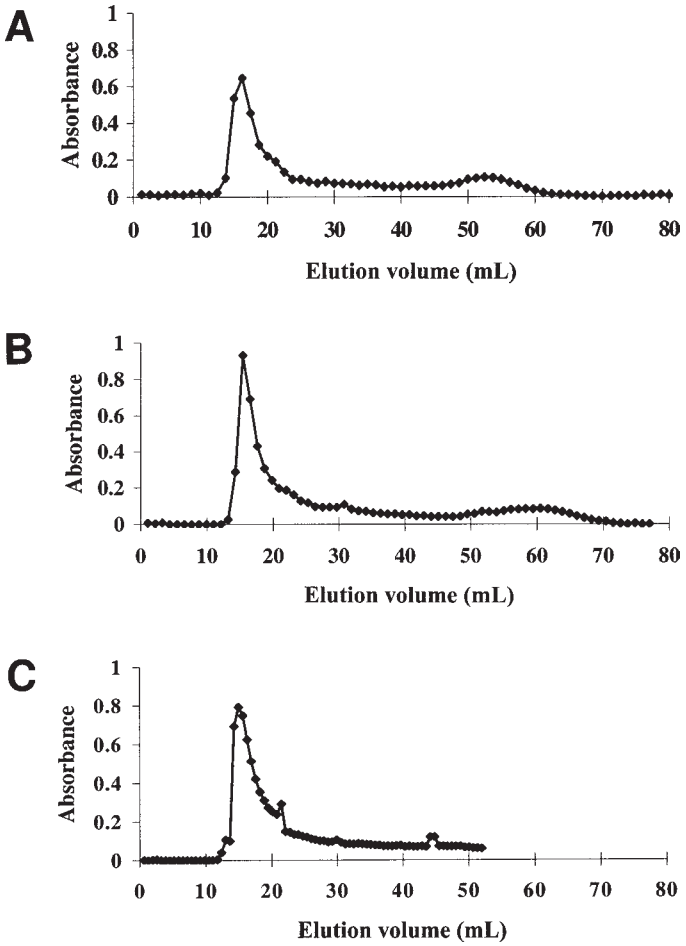

Fig. 4. Size exclusion chromatography elution profiles of **(A)** polystyrene microspheres; **(B)** E_5 in suspension with polystyrene; and **(C)** CBHI in suspension with polystyrene. Absorbance was measured at a wavelength of 280 nm.

bance by the polystyrene. This made it difficult to clearly verify the absence of a peak corresponding to free cellulase. Thus, while the data of Fig. 4 would suggest that E_5 and CBHI adsorb to polystyrene, adsorption was verified with microporous membrane separations. In particular, membranes were used to separate any free cellulase that may be present in a cellulase-polystyrene suspension. When cellulase-particle mixtures were filtered, nearly all the cellulase originally present remained with the particles such that 99.3 and 97.4% of E_5 and CBHI, respectively, were bound. For purposes of a controlled comparison, cellulase solutions were filtered in the absence of particles. This verified that each cellulase readily enters the filtrate, with negligible retention by the filter.

Ellipsometry can provide a direct measure of adsorbed mass, *in situ*, during protein film formation. Figure 5 shows the adsorption kinetics

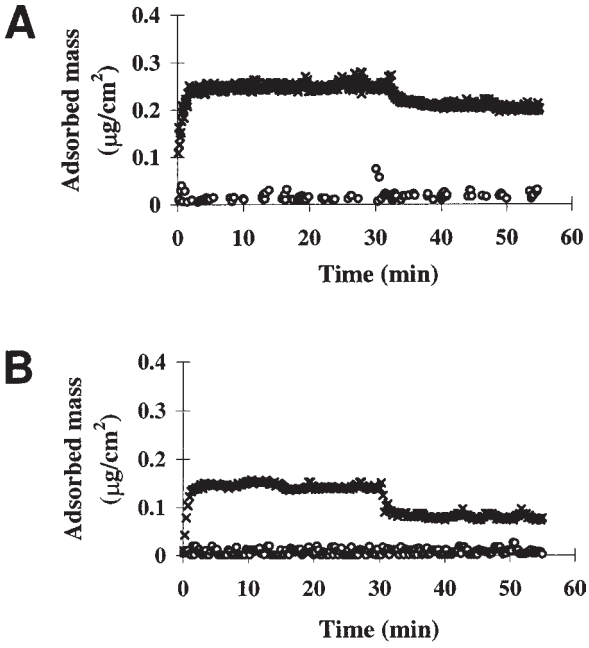

Fig. 5. Adsorption kinetics of **(A)** E_5 (0.10 mg/mL) and **(B)** CBHI (0.13 mg/mL) on hydrophilic (○) and hydrophobic (×) silica surfaces.

recorded for E_5 and CBHI on planar hydrophilic silica and silanized silica. The silanized silica exposes a uniform field of methyl groups to the solution and is hydrophobic. In each case, adsorption was allowed to occur for 30 min, at which time the surface was rinsed. The amount of adsorbed mass was monitored for an additional 30 min following the rinse. The kinetic plots show that each cellulase rapidly achieves a plateau in adsorbed mass on the hydrophobic surface. Adsorption to this surface was largely irreversible to dilution in both cases, and protein removed upon rinsing may have been part of a weakly bound outer layer. Adsorption to the hydrophilic surface is insignificant.

The observation that E_5 and CBHI do not adsorb to silica was unexpected. In particular, unlike surfactants and other small nonpeptide molecules, proteins undergo structural change upon adsorption *(21,22,30–32)*. These structural changes contribute to the adsorption free energy by increasing the entropy of the polypeptide chain, and thus contribute to the adsorption driving force *(33,34)*. For example, the fact that a protein and surface may both be negatively charged would generally have little to do with whether adsorption would occur. Nevertheless, the cellulases did not bind to hydrophilic silica but did bind to the hydrophobic surfaces. The ellipsometry results show that adsorption to silanized silica was irreversible to dilution as well. This is generally the case in protein adsorption, in which exchange between free protein (or other surface active molecules) and bound protein may readily occur, but spontaneous desorption would

not. These findings indicate that hydrophobic association plays an important role in cellulase adsorption. They also indicate that if hydrophobic interactions are dominant, the irreversibility of the process would need to be accounted for in the construction of any model proposed to describe cellulase adsorption *(35,36)*.

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