AN ABSTRACT OF THE THESIS OF

Worakrit Suvajittanont for the degree of Doctor of Philosophy in Bioresource Engineering presented on April 6, 1999. Title: Adsorption of *Thermomonospora fusca* E_3 and E_5, and *Trichoderma reesei* CBHI Cellulases on Cellulose and Silica.

Abstract approved: Redacted for Privacy
Michelle K. Bothwell

Adsorption of cellulolytic enzymes at solid-water interfaces were investigated. Selected cellulases were used for this purpose including E_3 and E_5 from *Thermomonospora fusca* and CBHI from *Trichoderma reesei*. Adsorption of both E_5 and CBHI were studied on synthetic surfaces to better separate binding from catalytic events. In particular, the adsorption kinetics and dodeceyltrimethyl-ammonium bromide-mediated elution of each were recorded *in situ*, at hydrophobic, silanized silica with ellipsometry. Experiments were performed at solution concentrations ranging from 0.001 to nearly 1.0 mg/ml. For E_5, adsorbed layers were only partially elutable in protein-free buffer. Treatment with surfactant removed more of the adsorbed enzyme, with the remaining adsorbed mass varying little among experiments. Adsorption kinetic data were interpreted with reference to a mechanism allowing for irreversible adsorption into two dissimilar states. These states were distinguished by differences in occupied interfacial area, and binding strength.

For CBHI, adsorbed layers were partially elutable upon rinsing and incubation in protein-free buffer, with the resistance to elution being generally lower at higher concentrations. The values of adsorbed mass remaining after elution with protein-free buffer were highest at intermediate solution concentrations. Resistances to surfactant-mediated elution were fairly high, but generally lower at intermediate concentrations. The concentration-dependent adsorption behavior of CBHI was described with reference to a mechanism allowing for adsorption of CBHI aggregates, or associated monomers, as well as free monomers.

Competitive adsorption of cellulases E_3, E_5, and CBHI was studied on avicel and bacterial microcrystalline cellulose (BMCC). Cellulase adsorption was quantified with reverse phase, high pressure liquid chromatography. Both binary and ternary mixtures of cellulases were studied at selected solution concentrations. Adsorption of E_3 and E_5 on avicel appeared independent of whether they adsorbed from single-enzyme or binary solutions. When either E_3 or E_5 was mixed with CBHI, however, CBHI adsorption was inhibited especially at high concentrations. Adsorption from solutions containing all three enzymes showed that E_3 and E_5 preferentially adsorbed, especially at high concentration. Adsorption data recorded with BMCC as the substrate was inconclusive as a substantial percentage of that substrate was degraded during the experiment.

Adsorption of *Thermomonospora fusca* E_3 and E_5, and *Trichoderma reesei* CBHI Cellulases on Cellulose and Silica

by

Worakrit Suvajittanont

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented April 6, 1999
Commencement June, 1999

Doctor of Philosophy thesis of Worakrit Suvajittanont presented on April 6, 1999

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Worakrit Suvajittanont, Author

ACKNOWLEDGEMENTS

There, my academic life finally comes to an end. After my long, and incredible journey, I would like to express my appreciation to all the people who have, in any way, touched and influenced my life. First of all, my profound appreciation goes to Dr. Michelle K. Bothwell, my major advisor and my friend, for her careness, encouragement, and wonderful guidance. She has always been kind, cheerful, and patient. I consider myself a fortunate person to have a chance to work with her. My equal gratitude also extends to Dr. Joe McGuire for being kind, helpful, and supportive. Their friendship and kindness will be remembered always. I as well sincerely thank Dr. Michael H. Penner, Dr. Sonia Anderson, and Dr Ashok Chandrashekar for serving on my committee.

I am also grateful to Diana Irwin, Dr. L.P. Walker, and Dr. D.B. Wilson (Cornell University) for their inputs and suggestions, especially the starting cellulases and microorganisms. My special thanks go to all my friends and colleagues; Carolyn, Lee, Nong, Sod, Steve, Billy, Ali, and others for their friendship. I also would like to acknowledge the Royal Thai Government Scholarship for financial support.

I am deeply, profoundly, absolutely grateful to my parents. No word could describe how wonderful and special they have been. Their emotional support and love are so precious and priceless and will be tattooed in my heart forever. I also give credits to my brothers and sisters for their encouragement throughout my

academic life. They used any means to push me, both forward and reverse psychology.

My special appreciation certainly belongs to the one and the only Kunruedee Sangseethong (Nonglek). She is my world, my life, my everything She always gives me joy and happiness. She really completes me. I would not have been who I am today if it is not for her. For that, I am completely grateful.

CONTRIBUTION OF AUTHORS

Carolyn S. Baker was involved in data collection, analysis, and writing of manuscript in Appendix. Dr. Michelle K. Bothwell and Dr. Joseph McGuire assisted in result interpretation and preparation of each manuscript.

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This thesis is dedicated

to my mother and father, my brothers and sisters,

and

to my love and my life, Nonglek.

ADSORPTION OF *THERMOMONOSPORA FUSCA* E_3 AND E_5, AND *TRICHODERMA REESEI* CBHI CELLULASES ON CELLULOSE AND SILICA

CHAPTER 1

INTRODUCTION

Cellulose is the most abundant carbohydrate on Earth, making up a large percentage of total lignocellulosic biomass, including hardwoods, grasses, and crop residues. This renewable resource can be converted into fuels and other value-added products by chemical and biological processes. The bioconversion of lignocellulosic materials can be divided into three steps: (i) biomass pretreatment; (ii) cellulose saccharification; and (iii) fermentation of the resulting sugar streams. Pretreatment of the biomass improves the accessibility of the substrate to cellulose-degrading enzymes, thus enhancing the results of the cellulose saccharification step. This increase in bioavailability is a result of removing the lignin encasement, solubilizing the hemicellulose, reducing the cellulose's crystallinity and increasing its pore volume (Weil, et al., 1994). The second step in the bioconversion process involves the degradation of the cellulose chains into individual glucose residues. This is accomplished by the action of cellulases, extracellular enzymes produced by several bacterial and fungal species (Coughlan, 1991, Robson and Chambliss, 1989, Tomme et al., 1995). The final step of the bioconversion process involves the fermentation of the xylose stream, recovered from the solubilization of the

hemicellulose, and the glucose stream, recovered from the hydrolysis of the cellulose, into value-added products. Work is underway to optimize these processes (Ooshima et al., 1990, Philippidis et al., 1993, Zhang et al., 1995) and includes substantial effort to elucidate the molecular mechanisms involved in cellulose saccharification.

Because the enzymatic hydrolysis of cellulose is heterogeneous in nature, diffusion of the cellulases into, and products out of, the substrate matrix, and cellulase adsorption on the cellulose surface are extremely important. Further, the efficient degradation of crystalline cellulose requires the action of a β-glucosidase, and a representative cellulase from each of the three cellulase classes: endoglucanases, and two types of exoglucanases (Irwin et al., 1993, Walker et al., 1993, Wood et al., 1989). The endo- and exoglucanases work synergistically to degrade cellulose into cellobiose (Bothwell et al., 1993, Medeve et al., 1994, Nidetzky et al., 1993, Walker et al., 1992). β-glucosidase then hydrolyzes the cellobiose into glucose residues. Given that the adsorption of cellulases on the cellulose surface is important to hydrolysis, and that at least three cellulases are needed to efficiently degrade cellulose, the importance of understanding how complex, multi-cellulase mixtures adsorb and function at the cellulose surface is evident.

In this study we investigated the adsorption of *Thermomonospora fusca* E_3 and E_5, and *Trichoderma reesei* CBHI cellulases on cellulose. In addition, we investigated the adsorption of E_5 and CBHI from single-enzyme solutions to a

hydrophobic synthetic surface. Non-porous, well-characterized silica was used for this purpose. The use of silica removes uncharacterized heterogeneous surface effects that may influence binding behavior, as well as catalytic events that accompany binding in natural circumstances.

Chapters 2 and 3 describe the adsorption kinetics and elution of E_5 and CBHI, respectively, at silainized silica surfaces. Chapter 2 is currently in review at *Biotechnology and Bioengineering*. In Chapter 4 the simultaneous adsorption of *T. fusca* E_3 and E_5, and *T. reesei* CBHI on two model cellulosic surfaces is described. Another manuscript, in review at *Applied Biochemistry and Biotechnology*, is appended. That work describes the importance of hydrophobic associations in cellulase binding.

CHAPTER 2

ADSORPTION OF *THERMOMONOSPORA FUSCA* E_5 CELLULASE ON SILANIZED SILICA

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Submitted to *Biotechnology and Bioengineering*
February, 1999, in review

2.1 ABSTRACT

The adsorption kinetics and dodeceyltrimethylammonium bromide-mediated elution of *Thermomonospora fusca* E_5 cellulase were recorded *in situ*, at hydrophobic, silanized silica. Experiments were performed at different solution concentrations, ranging from 0.001 to 0.70 mg/ml. Plateau values of adsorbed mass generally increased with increasing solution concentration, with the adsorbed layer being only partially elutable in protein-free buffer. Treatment with surfactant removed more of the adsorbed enzyme in each case, with the remaining adsorbed mass varying little among experiments. Adsorption of E_5 into this nonremovable state was suggested to occur early in the adsorption process and continue until some critical surface concentration was reached. Beyond this critical value of adsorbed mass, adsorption progressed with protein adopting more loosely bound states. Adsorption kinetic data were interpreted with reference to an adsorption mechanism allowing for irreversible adsorption into two dissimilar states. These states were distinguished by differences in occupied interfacial area, and binding strength, presumably a result of differences in structure. Comparison of the data to the kinetic model based on this mechanism showed that the fraction of adsorbed molecules present in the more tightly bound state decreased as adsorption occurred from solutions of increasing concentration. However, the absolute values of more tightly bound molecules were less dependent on adsorption conditions.

2.2 INTRODUCTION

Protein adsorption occurs at virtually any natural or synthetic surface in contact with a protein-containing fluid, and is thus important in many areas of industrial and medical relevance. Past theoretical and experimental work has shown that a given protein can adsorb at an interface in different structural states (Andrade et al., 1996; Brash and Horbett, 1995; Horbett and Brash, 1987; McGuire and Bothwell,1999). The concept that adsorbed proteins can exist in multiple states on a surface plays a role in interpretation of most if not all experiments in protein adsorption (Andrade et al., 1984; Brash and Horbett, 1995; Elwing et al., 1988; Fröberg et al., 1998; Horbett and Brash, 1987; Jönsson et al. 1987). For example, study of the interfacial structure and function of synthetic mutants of bacteriophage T4 lysozyme has shown that adsorption can be modeled as occurring such that molecules adopt one of only two states. These states can be characterized as differing in binding strength, occupied area, and function, with differences in behavior among the molecules attributable to the relative amounts adsorbed in each state (Billsten et al., 1995; Bower et al., 1998; Bower et al., 1999; McGuire et al, 1995a, 1995b; Tian et al., 1998; Wang and McGuire, 1997).

To-date, there is no comprehensive model of any real utility describing competitive cellulase binding. Adsorption data are often fit to "single-state," reversible Langmuir or Langmuir-type equations despite their substantial shortcomings in accurately describing protein adsorption in general (Beldman et al., 1987; Bothwell et al., 1997a, 1997b; Din et al., 1994; Gilkes et al., 1992; Kim

et al., 1994, 1997; Nedetzky et al., 1994; Srisodsuk et al., 1993; Stahlberg et al., 1991). While no reports of cellulase interfacial structure are available, it is probably reasonable to suspect that adsorbed cellulase can exist in multiple states. Kondo et al. (1995) measured the secondary structure of α-amylase, of a class of enzymes frequently compared to cellulases, before and after adsorption to silica nanoparticles. Structural changes upon adsorption were clearly observed in these experiments.

Several mechanisms and models for protein adsorption at solid surfaces have been described in the literature and most include some kind of structural alteration in the adsorbed protein (Lee et al., 1999; Lundström and Elwing, 1990; Wahlgren et al., 1995). If a multi-state model proved applicable to cellulase adsorption, knowledge of the tendency of a cellulase to attain a given state upon adsorption, and to undergo exchange reactions with cellulase from solution, would be sufficient to predict the time-dependent make-up of an adsorbed layer during competitive adsorption. In this work, we describe adsorption kinetic behavior of *Thermomonospora fusca* E_5 cellulase at a hydrophobic, silanized silica surface. Kinetic data are interpreted with reference to a multi-state adsorption mechanism.

2.3 MATERIALS AND METHODS

2.3.1 *Production and purification of T. fusca E_5*

Thermomonospora fusca E_5 cellulase was produced by transformed *Streptomyces lividans* TK24 carrying a plasmid (pGG74) bearing the E_5 gene (Ghangas and Wilson, 1987). The production and purification procedures were based on those described earlier (Bothwell et al., 1997a). In particular, the culture was initiated from frozen stock into 15 ml of tryptic soy broth (TSB) medium containing 5 μg/ml of thiostrepton (tsr) 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-liter fermenter containing 4.8 liters of TSB and tsr medium. A few drops of MAZU DF 204 (PPG Industries, Inc., Gurnee, IL) were added to the culture as an anti-foaming agent. The fermentation was carried out at 30 °C, agitated at 150 rpm, for 48 h. Cells were harvested with centrifugation (Beckman J2-MI, Seattle, WA) and filtration (Millipore Pellicon Filter system with a 0.22 micron cassette). The filtered supernatant was adjusted to 1.2 M $(NH_4)_2SO_4$ and 0.1mM phenylmethylsulfonyl fluoride, and loaded onto a 150 ml Phenyl Sepharose CL-4B column (Sigma Chemical Co., St. Louis, MO) pre-equilibrated with 1 M $(NH_4)_2SO_4$, 10 mM NaCl, 5 mM Kpi, pH 6. The column was washed with 300 ml of the loading buffer and then 600 ml of 0.3 M $(NH_4)_2SO_4$, 5 mM NaCl, 5 mM Kpi, pH 6. The cellulase was eluted with 600 ml of 5 mM Kpi, pH 6, followed by 600 ml of deionized-distilled water (DDW). The flow rate throughout

this procedure was 1 ml/min. All fractions containing E_5 were combined and concentrated by ultrafiltration with a PM 30 membrane, and adjusted to 10% glycerol. The cellulase preparation was then diluted 1:1 with 0.01 M BisTris, pH 5.4 and loaded on a 150 ml Q-Sepharose column (Sigma Chemical Co., St. Louis, MO) pre-equilibrated with 0.02 M BisTris, pH 5.4, containing 10% glycerol. The column was washed with one column volume of the loading buffer, followed by protein elution with a 0-0.3 M NaCl gradient in the same buffer. E_5 fractions were combined, and ultrafiltered to remove NaCl and glycerol and to exchange the buffer to 50 mM sodium acetate, pH 5.5. The final preparation was judged to be 98-99% pure as indicated by SDS-PAGE and CMCase activity, and its concentration was spectrophotometrically quantified at 280 nm using its molecular weight and extinction coefficient reported by Irwin et al.(1993). The enzyme was stored at -80°C until use.

2.3.2 *Surface preparation*

All surfaces were prepared from silicon (Si) wafers (hyperpure, type N, phosphorous doped, orientation 1-0-0) purchased from Wacker Siltronic Corporation (Portland, OR). Surfaces were oxidized in O_2 (1 atm) for 17 min at 1000 °C. Following, 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 DDW, washed with a solution of HCl and H_2O_2, and rinsed again with DDW, according to procedures described elsewhere (Krisdhasima et al., 1993). This

treatment rendered the surfaces hydrophilic, as verified by their wettability. These surfaces were made hydrophobic according to the procedure of Jönsson et al. (1982), as slightly modified by Krisdhasima et al. (1993). In particular, the surfaces were silanized with 0.1% dichlorodimethylsilane (Aldrich Chemical Co., Inc., Milwaukee, WI) in xylene. Silanized surfaces were then washed in sequence with xylene, acetone, and ethanol. Surface hydrophobicity was verified with contact angle methods (Krisdhasima et al., 1992b).

2.3.3 *Adsorption kinetics, isotherm construction and elution*

Protein adsorption was monitored continuously with an automated Garertner *in situ* ellipsometer (Gaertner Scientific Corp., Chicago, IL) equipped with a thermostatted cuvette and modified to allow for stirring and flow. The instrument was described in detail by Podhipleux (1998). Adsorbed mass was calculated from ellipsometrically-determined values of film thickness and refractive index according to Cuypers et al. (1983), using a calculation procedure based on a one film model (Krisdhasima et al., 1992a). The partial specific volume and the ratio of molecular weight to molar refractivity for E_5, both required to determine adsorbed mass, are 0.85 cm^3/g and 4.049 g/cm^3, respectively (Pethig, 1979; Cuypers et al., 1983).

Experiments were performed with 50 mM sodium acetate buffer, pH 5.5, at 25 °C. The pseudo-refractive index of the bare surface was determined prior to addition of enzyme. An experiment began with addition of 1.0 ml of enzyme

solution to the cuvette containing 5.0 ml of buffer, prepared to yield the desired enzyme concentration (0.001-0.7 mg/ml). The solution was mixed using a magnetic stirrer (325 rpm) during the first minute following introduction of the enzyme. Adsorbed mass was recorded every 15 s for 1 h, after which the cuvette was rinsed with enzyme-free buffer for 5 min at a flow rate of 25 ml/min. The solution was well mixed during rinsing. After rinsing, the stirring was stopped, and the film properties were monitored for an additional 20 min.

Isotherms were constructed using two methods, based on steady-state kinetic behavior. One method involved introduction of E_5 to the cuvette in a single step to reach a desired solution concentration. In the other method, E_5 was introduced to the cuvette successively, in order to reach a desired solution concentration. Adsorption was allowed to reach steady state after each successive addition of enzyme. It is important to note that with the planar surfaces used in ellipsometry, surface area available for adsorption is on the order of 1 cm^2. The mass of protein that would leave the solution phase and adsorb would then be on the order of 10^{-4} mg.

In elution experiments, adsorption and rinsing were carried out as described above. Dodecyltrimethyl-ammonium bromide (DTAB) was then introduced to the cuvette such that surfactant concentration was 0.18 M, twice the critical micelle concentration. Following 15 min of surfactant contact, rinsing and incubation in buffer proceeded as above. All experiments were performed at least three times.

2.4 RESULTS AND DISCUSSION

2.4.1 *Adsorption and elution*

Representative plots of E_5 adsorption and elution kinetics are shown in Figure 2.1. In each case, E_5 adsorption was allowed to occur for 60 min, at which time the surface was rinsed; surfactant was added at 85 min, and the final rinse was started at 100 min. The initial adsorption rates, as well as the plateau values of adsorbed mass generally increased with enzyme concentration. Adsorbed mass recorded at a concentration of 0.001 mg/ml did not achieve a plateau, and no enzyme was removed upon rinsing. At all other concentrations adsorbed mass decreased upon rinsing. Adsorption was largely irreversible to dilution in each case, and protein removed upon rinsing may have been part of a weakly bound outer layer. Introduction of DTAB resulted in a decrease in adsorbed mass, followed in each case by another decrease with the final buffer rinse.

The average values of adsorbed mass of E_5 remaining on the surface immediately before introduction of DTAB (85 min), and at the end of each experiment (125 min) are listed in Table 2.1. It is instructive to determine whether the adsorbed mass remaining after the first rinse is consistent with sub-monolayer, monolayer or multi-layer coverage, in each case. Knowledge of E_5 molecular dimensions in solution would allow estimation of a range of values of adsorbed mass consistent with monolayer coverage. While such information for E_5 is not known with certainty, its structure is similar to CenA from *Cellulomonas fimi*

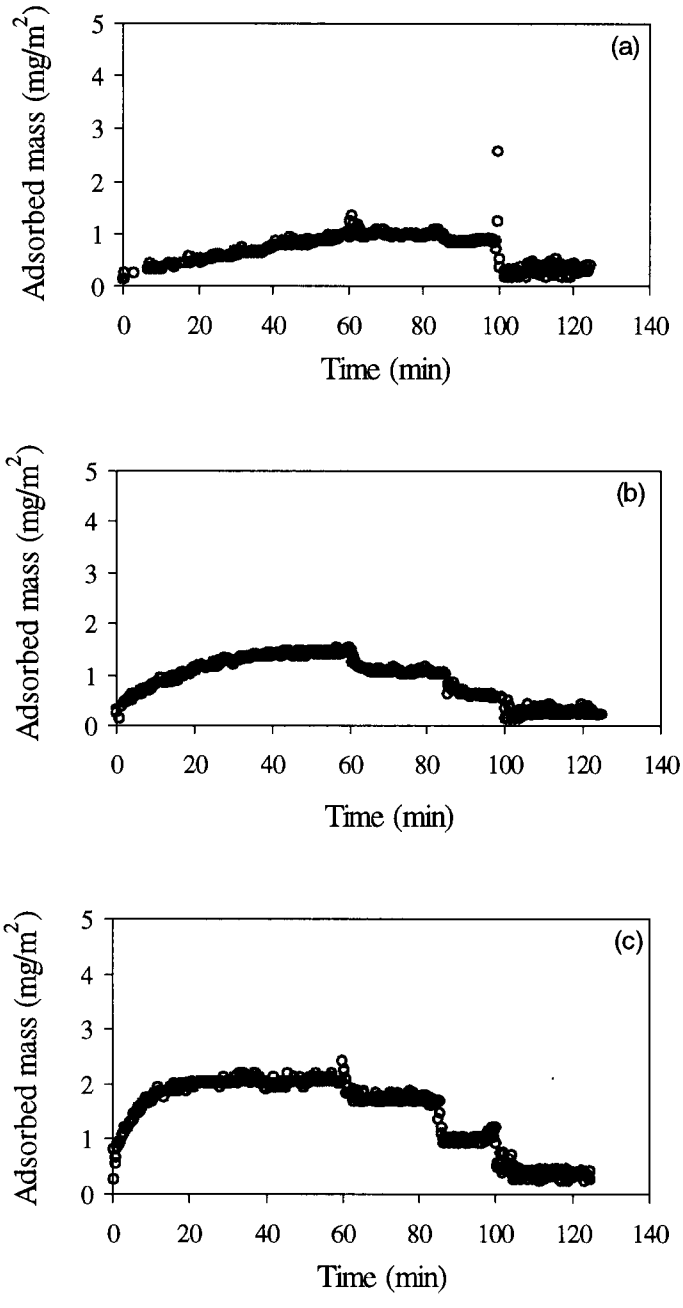
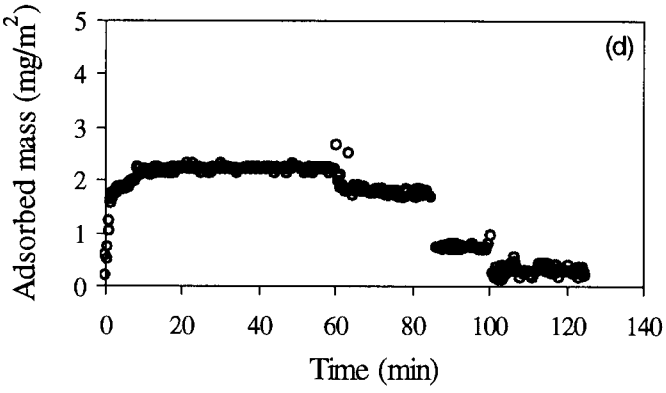
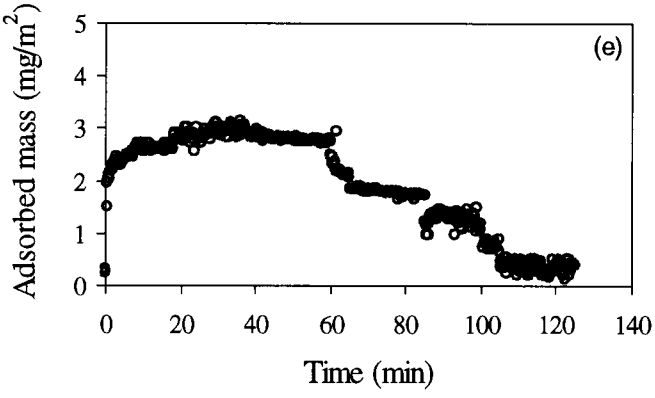


Figure 2.1 Representative adsorption and elution kinetic plots recorded for E_5 at hydrophobic silica. The cellulase concentrations are: (a) 0.001 mg/ml; (b) 0.002 mg/ml; (c) 0.005 mg/ml; (d) 0.01 mg/ml; (e) 0.02 mg/ml; (f) 0.05 mg/ml; (g) 0.2 mg/ml; (h) 0.5 mg/ml; (i) 0.7 mg/ml.





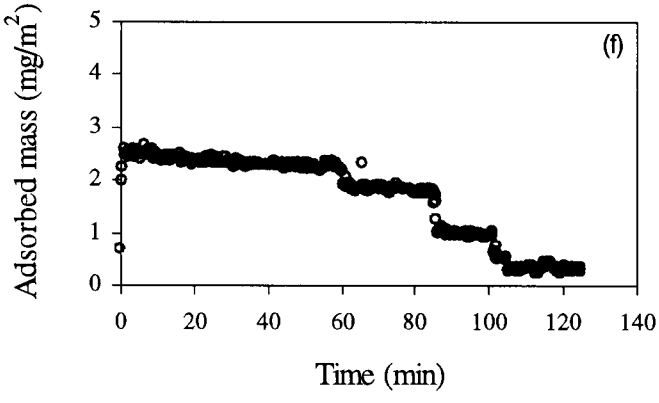


Figure 2.1 Continued

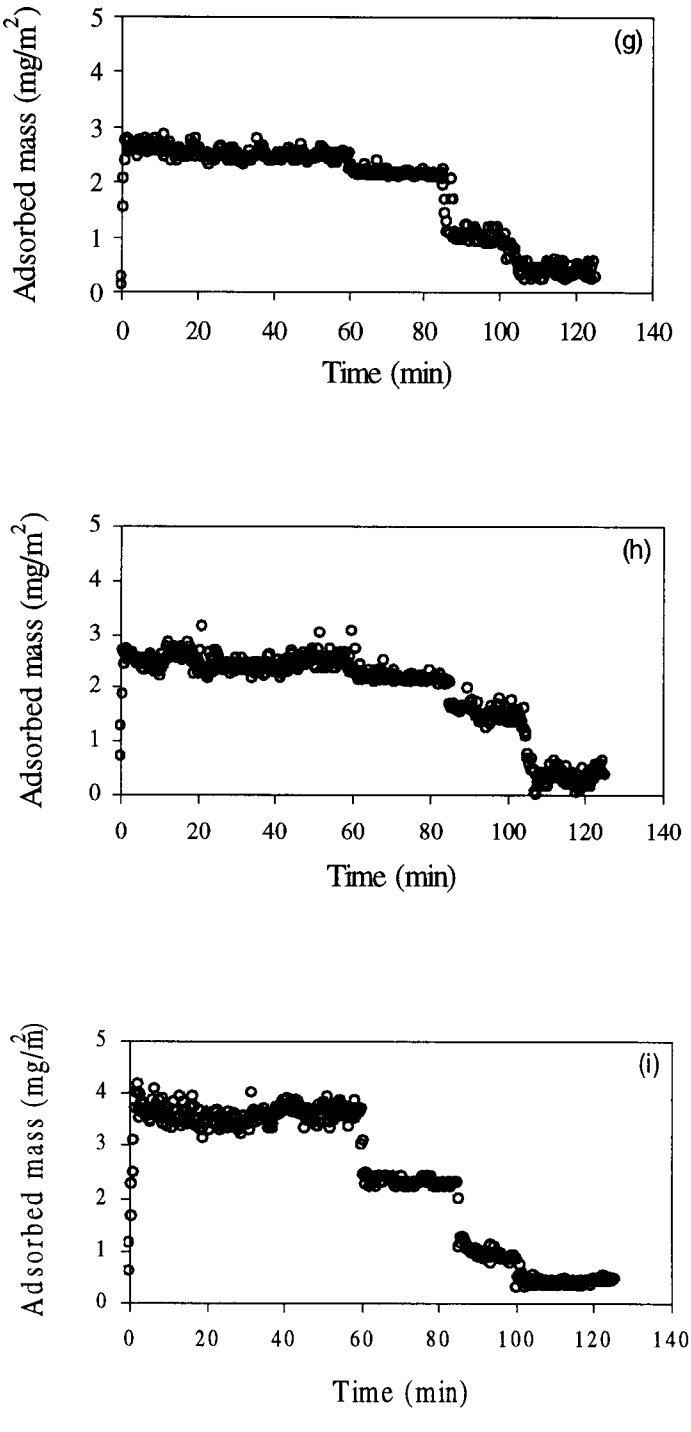


Figure 2.1 Continued

which is about 21.0 nm long with a maximum diameter of 4.5 nm (Pilz et al., 1990). Using these data as a fair estimate of the molecular dimensions of E_5, "end-on" adsorption of an E_5 molecule would require about 20.3 nm^2 (4.5 nm x 4.5 nm) and "side-on" adsorption would require about 94.5 nm^2 (4.5 nm x 21.0 nm). Limits in surface coverage expected for a monolayer of E_5 molecules adsorbed end-on and side-on would be 3.79 mg/m^2 and 0.81 mg/m^2, respectively. The amounts of protein remaining on the surface after the first rinse and incubation in buffer (85 min) would therefore correspond to monolayer coverage in each case.

The average resistances to elution associated with each concentration are also listed in Table 2.1. The resistance to elution was calculated as the mass of protein remaining after 125 min divided by that remaining after 85 min. While the adsorbed mass remaining on the surface after the first rinse increased with concentration, the amounts remaining after treatment with DTAB were similar, showing no clear relationship with concentration. Thus, resistance to elution, which we will consider here as an index of binding strength, decreased with increasing concentration in these experiments. This finding is in agreement with the generally well-accepted observation that at a less crowded interface, a protein will more readily alter its conformation upon adsorption. This in turn would allow establishment of more noncovalent bonds with the surface (Horbett and Brash, 1995; Andrade et al., 1996; McGuire and Bothwell, 1999). The interfacial region is likely to be less crowded when adsorption occurs from a solution of low concentration, resulting in a greater interfacial area occupied per molecule and

Table 2.1 Adsorbed mass of *T. fusca* E_5 following the first rinse after adsorption for 60 min, second rinse after contact with DTAB, and also the binding strength of the protein at various concentration.

Cellulase concentration (mg/ml)	Adsorbed mass (mg/m^2)		Resistance to elution with DTAB
	85 min	125 min	
0.001	1.015	0.331	0.326
0.002	1.398	0.294	0.210
0.005	1.606	0.353	0.220
0.01	1.769	0.323	0.183
0.02	1.833	0.388	0.212
0.05	1.876	0.405	0.216
0.2	2.030	0.390	0.192
0.5	2.239	0.343	0.153
0.7	2.230	0.390	0.175

greater resistance to elution. On the other hand, as the amounts remaining on the surface after treatment with DTAB were largely independent of solution concentration, it may be that adsorption into this nonremovable state occurs early in the adsorption process and continues until some critical surface concentration is reached. Beyond this critical value of adsorbed mass, adsorption progresses with protein adopting more loosely bound states.

It is instructive to interpret the E_5 adsorption isotherm after generating it through a single-step introduction of cellulase, and sequential introduction of the enzyme, to yield the desired steady-state solution concentration. In particular, if protein spreading at the interface occurs more readily at low solution

concentrations, we would expect attainment of a lower plateau value with successive additions than with single-step introduction of enzyme (Lundström, 1985). Figure 2.2 shows the adsorption isotherm for E_5 recorded by single-step enzyme introduction, in addition to a representative plot recorded during the successive addition of enzyme to determine the plateau value at 0.7 mg/ml. At all concentrations tested, the steady-state value of adsorbed mass was independent of the method used to measure it. This finding is consistent with the thought that adoption of different conformational states at the interface is governed by adsorbed protein concentration, and not protein concentration in solution. The presence of more spreading at low solution concentrations cannot entirely be dismissed, but if it occurs, its extent is insufficient to affect neighboring molecules. In any case, adsorption at this hydrophobic surface is possibly mediated through the binding domain, which is much smaller than the catalytic domain. In this case, the relatively mobile catalytic domain would determine the interfacial area required per molecule.

2.4.2 *Comparison to a model*

Protein adsorption involves both transport of protein to the interface, and binding. In order to begin to model the process, the issue of whether the adsorption rate is mass transfer-controlled or kinetically controlled must be addressed. If adsorption were transport-limited, the rate of adsorption would equal the rate of diffusion (Krisdhasima et al., 1992b):

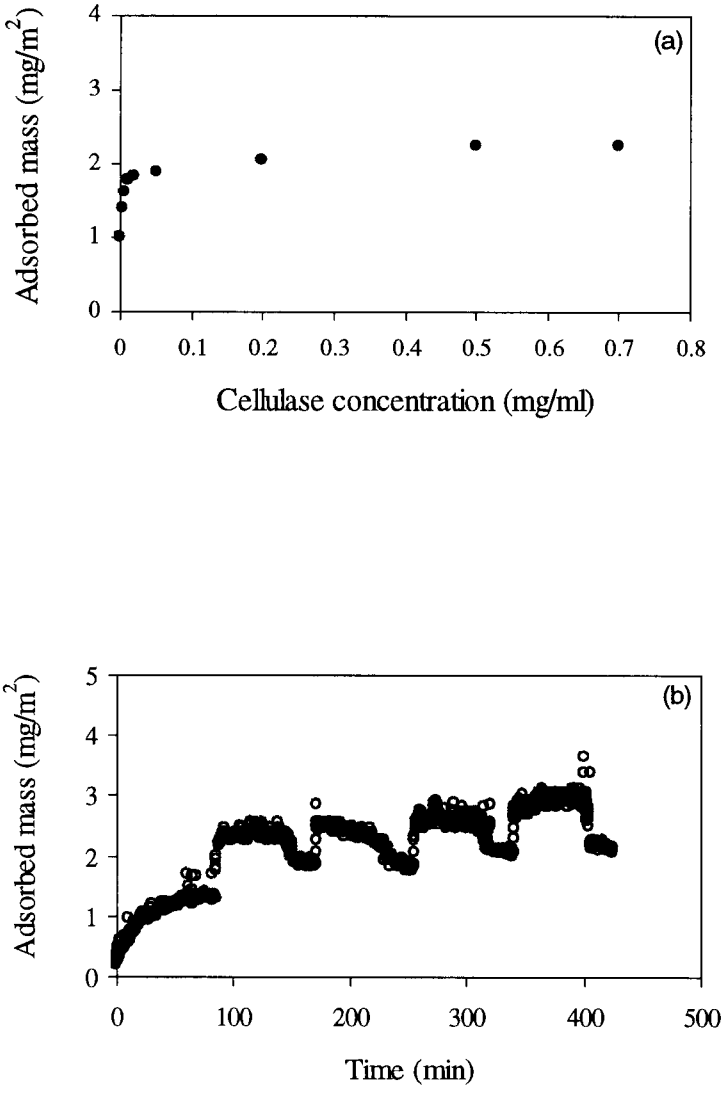


Figure 2.2 (a) Adsorption isotherm recorded by single-step introduction of E_5, at hydrophobic silica. (b) Representative data recorded during successive addition of enzyme to determine the plateau value at 0.7 mg/ml. The enzyme solution was sequentially injected in the order, 0.002, 0.01, 0.05, 0.2, and 0.7 mg/ml.

$$d\Gamma/dt = MC(D/\pi t)^{1/2} \quad [1]$$

or

$$\Gamma = 2MC(Dt/\pi)^{1/2} \quad [2]$$

where Γ is adsorbed mass, M is protein molecular weight, C is protein concentration in solution, D is the protein diffusion coefficient, and t is time.

Equation [2] indicates that the slope of a plot between Γ and $t^{1/2}$ would be proportional to $D^{1/2}$ if adsorption was diffusion-controlled. Such a plot was constructed for each concentration used in this study, using data recorded during the first 60 s of adsorption. Determination of the initial slopes yielded estimates of the diffusion coefficient ranging from 10^{-12} to 10^{-15} m^2/s corresponding to the lowest and highest concentrations used here. While the diffusion coefficient of E_5 is not known, diffusion coefficients of protein in solution generally fall between 10^{-10} and 10^{-11} m^2/s (Andrade et al., 1985). As the experimentally estimated values of D were several orders of magnitude lower than those generally recorded for protein, it is probably fair to consider E_5 adsorption as having been kinetically-controlled in these tests.

As discussed with reference to Figures 2.1 and 2.2, and Table 2.1, E_5 adsorption is characterized by the co-existence of multiple binding states. For modeling purposes, three states can be identified: one that is removable by elution with protein-free buffer (state 1); one that is removable by DTAB-mediated elution (state 2); and one that is not removable (state 3). In this case, expressions

describing the rate of change in fractional surface coverage of each state can be written as follows (Lee et al., 1999):

$$d\theta_1/dt = k_1C(1-\theta_1-\theta_2-\theta_3) \quad [3]$$

$$d\theta_2/dt = k_2C(1-\theta_1-\theta_2-\theta_3) \quad [4]$$

and

$$d\theta_3/dt = k_3C(\theta_{crit}-\theta_1-\theta_2-\theta_3) \quad [5]$$

where the k_i are kinetic rate constants for adsorption into state i, the θ_i are fractional surface coverages of protein in state i, and θ_{crit} refers to the critical surface coverage at which adsorption into state 3 no longer occurs. With appropriate boundary conditions, equations [3]-[5] could be solved in order to simulate each fractional surface coverage as a function of time. In addition, comparison of these equations to kinetic data would allow estimation of rate constants k_1, k_2 and k_3. The utility of such an analysis would be limited, however, as data relevant to generation of individual adsorbed states would be needed to estimate these constants with reasonable confidence. The ellipsometric data of Figure 2.1 provide an indication only of the sum of the fractional surface coverages (i.e., $\theta_1+\theta_2+\theta_3$) as a function of time.

Nevertheless, determination of rate constants governing adsorption into different states constitutes a worthy approach to modeling adsorption, as competitive adsorption behavior of a cellulase would surely be related to the distribution of its binding states. The simplest adsorption mechanism consistent with the fact that adsorbed proteins can exist in multiple states would include two

adsorbed states (McGuire et al., 1995a; Tian et al., 1998). In this case, protein may adopt one of two states, where state 1 molecules are less tightly bound (less resistant to removal) than those in state 2, but where molecules can be considered partially removable in either state. Additionally, a state 2 molecule is defined as occupying a greater interfacial area (A_2) than it would occupy in state 1 (A_1). While this mechanism allows for adoption of states 1 and 2 directly from solution, a more accurate and detailed mechanism might include a multi-step path to state 2. However, for engineering purposes, the actual path to state 2 is not consequential; we need only account for the different rates of generation of two functionally dissimilar forms of adsorbed enzyme.

Defining the maximum mass of molecules that could be adsorbed in a monolayer as Γ_{max}, we will define θ_1 as the mass of state 1 molecules adsorbed at any time divided by Γ_{max}, and θ_2 as the mass of state 2 molecules adsorbed at any time divided by Γ_{max}. Expressions for the rate of change in fractional surfaces coverages θ_1 and θ_2 can be solved such that (Tian et al., 1998):

$$\theta_1 = 1/(1 + ak_2/k_1)[1 - \exp(-k_1C - ak_2C)t] \qquad [6]$$

and

$$\theta_2 = [(k_2/k_1)/(1 + ak_2/k_1)][1 - \exp(-k_1C - ak_2C)t], \qquad [7]$$

where a is A_2/A_1, the ratio of interfacial areas occupied by state 2 and state 1 molecules, and k_1 and k_2 are rate constants governing adsorption into state 1 and state 2, respectively. The adsorbed mass at any time, Γ, is equal to $\Gamma_{max}(\theta_1 + \theta_2)$, or

$$\Gamma = \Gamma_{max}[(1 + k_2/k_1)/(1 + ak_2/k_1)][1 - \exp(-k_1C - ak_2C)t] \qquad [8]$$

With estimation of Γ_{max} and a, the kinetic data recorded by ellipsometry could be fit to Eq. [8] in order to estimate the rate constants. For the case of E_5, Γ_{max} can be estimated as 3.79 mg/m^2, corresponding to a monolayer of molecules adsorbed end-on. The value of parameter a cannot be determined with any certainty. But since plateau values of adsorbed mass in these experiments fell within the range expected for monolayer coverage of end-on and side-on molecules, A_1 and A_2 were taken as the specific interfacial areas occupied by end-on and side-on molecules, respectively, or parameter a was set equal to 4.68 (= 3.79/0.81).

The kinetic data of Figure 2.1 were fit to Eq. [8]. In each case, all protein removable during the first rinse was considered as having been present in an outer layer, such that only values of adsorbed mass less than or equal to the steady-state value recorded at 85 min were used for comparison to Eq. [8]. The values of k_1C and k_2C were determined for each experiment and are listed in Table 2.2. The ratio k_2/k_1 is equal to θ_2/θ_1, the ratio of tightly bound to less tightly bound molecules. Using this ratio, the fraction of adsorbed enzyme present in state 2 immediately prior to DTAB addition can be estimated directly. In particular, $\theta_2/(\theta_1 + \theta_2)$ is readily calculated as the reciprocal of the quantity $1 + k_1/k_2$. Data in Table 2.2 show that the fraction of adsorbed molecules in state 2 decreased as adsorption occurred from solutions of increasing concentration, and this is in keeping with the

Table 2.2 Kinetic rate constants estimated by analysis of adsorption kinetic data with Eq. [8], and the fraction of protein at the interface adsorbed in state 2 prior to contact with DTAB.

Cellulase concentration (mg/ml)	k_1C (s^{-1})	k_2C (s^{-1})	k_2/k_1	$\theta_2/(\theta_1 + \theta_2)$	Γ_2 @ 85 min
0.001	0.005	0.008	1.723	0.633	0.643
0.002	0.015	0.016	1.047	0.511	0.714
0.005	0.149	0.089	0.596	0.374	0.601
0.01	0.398	0.179	0.449	0.310	0.548
0.02	0.687	0.265	0.386	0.278	0.510
0.05	0.780	0.293	0.376	0.273	0.512
0.2	1.259	0.398	0.316	0.240	0.487
0.5	1.432	0.330	0.231	0.187	0.447
0.7	1.499	0.352	0.235	0.190	0.424

earlier discussion relevant to differences in resistance to elution. The absolute value of state 2 molecules present prior to DTAB addition (Γ_2 @ 85 min) can be estimated as well, by multiplying $\theta_2/(\theta_1 + \theta_2)$ by the total adsorbed mass present at that time (85 min). These estimates are also listed in Table 2.2. The finding that the adsorbed mass remaining at the end of each experiment was fairly constant (Table 2.1) would lead to an expectation that the amounts estimated as present in state 2 would vary little from one experiment to the next. Table 2.2 shows that these values decreased as solution concentration increased, although this change is considerably less pronounced than that of $\theta_2/(\theta_1 + \theta_2)$. This is probably a consequence of using a model allowing for only 2 states, and assuming that the

surface is covered at 85 min, at each concentration. At low solution concentrations, a monolayer may not have been achieved or preserved after rinsing, in which case, fitting to Eq. [8] would "force" k_2 to be greater than it otherwise would be.

Still, the fact that this model describes the adsorption kinetics in a physically realistic albeit simplified way lends some credibility to the two-state mechanistic approach described here. In particular, E_5 adsorption can be modeled as an irreversible process using a multi-state mechanism where molecules in each state may occupy different interfacial areas. Such a model ought to be extendible to quantifying competition in multi-cellulase mixtures, if adsorption in such cases is a function of a given protein's tendency to adopt each state (given by the adsorption rate constants), and state-dependent exchange constants. Exchange constants are experimentally accessible. In any event, it is anticipated that this approach could contribute to a foundation upon which other investigators could re-evaluate existing data, and conduct experiments yielding more meaningful data relevant to cellulase sequential and competitive adsorption behavior.

2.5 ACKNOWLEDGMENT

We are indebted to Professor David Wilson and co-workers at Cornell University for providing the bacterial strain in addition to the technical support needed for successful E_5 production.

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CHAPTER 3

ADSORPTION OF *TRICHODERMA REESEI* CBHI CELLULASE ON SILANIZED SILICA

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3.1 ABSTRACT

The adsorption kinetics and dodeceyltrimethylammonium bromide-mediated elution of *Trichoderma reesei* CBHI cellulase were recorded *in situ*, at hydrophobic, silanized silica. Experiments were performed at different solution concentrations, ranging from 0.001 to 0.98 mg/ml. Initial adsorption rates were extremely rapid, and plateaus in adsorbed mass were achieved at all concentrations tested. Adsorbed enzyme was partially elutable upon rinsing and incubation in protein-free buffer, with the resistance to elution generally lower at higher concentrations. The values of adsorbed mass remaining after elution with protein-free buffer were highest at intermediate solution concentrations. Introduction of surfactant followed by a final rinse resulted in a further decrease in adsorbed mass, with resistances to surfactant-mediated elution being fairly high in each case, but generally lower at intermediate concentrations. These observations could be explained by allowing for the possibility that some aggregation phenomena are involved in CBHI adsorption. In particular, the concentration-dependent adsorption behavior of CBHI was described with reference to a mechanism allowing for adsorption of CBHI aggregates, or associated monomers, as well as free monomers.

3.2 INTRODUCTION

Interactions between carbohydrates and proteins often involve aromatic residues on the binding face of the protein (Engle, 1994, Spurlino et al., 1992, Vyas, 1991). The origin of these interactions has been largely attributed to entropically driven, hydrophobic association (Linder et al., 1995a). Site-directed mutagenesis studies have shown that aromatic residues present on the hydrophobic binding face of the cellulase *Trichoderma reesei* CBHI are important in its binding to cellulose (Linder et al., 1995b, Reinikainen et al., 1992). Hydrophobic associations have been shown to play an important role in the binding of cellulases of bacterial origin as well (Creagh et al., 1996, Johnson, 1996).

In an earlier paper, we described the adsorption kinetic behavior of *Thermomonospora fusca* E_5 cellulase at a hydrophobic, silanized silica surface (Suvajittanont et al., 1999). Adsorption kinetic data and the results of elution with protein-free buffer and the surfactant dodecyltrimethylammonium bromide (DTAB) were interpreted with reference to a multi-state adsorption mechanism. In this work we carried out the same experiments performed earlier with E_5, but with CBHI isolated from *T. reesei*. In this case, however, adsorption data were not amenable to analysis with the kinds of models used earlier, based on mechanisms depicting adsorption of E_5 monomers into different structural states. Rather, CBHI adsorption was most credibly explained with reference to a mechanism allowing for adsorption of CBHI aggregates as well as free monomers.

3.3 MATEREALS AND METHODS

3.3.1 *Production and purification of T. reesei CBHI*

Trichoderma reesei cellobiohydrolase I (CBHI) was purified from crude cellulase (Spezyme™-CP, Environmental BioTechnologies Inc., Menlo Park, CA). Purification followed the procedure described by Piyachomkwan et al. (1997). In short, the crude mixture was loaded on a DEAE-Sepharose CL-6B, equilibrated with 50 mM NaOAc, pH 5. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in 50 mM NaOAc, pH 5. The CBHI peak was identified by 10% SDS-gel electrophoresis. Fractions containing CBHI were combined and concentrated by ultrafiltration (model 8200 with membrane PM10, Amicon, Inc., Beverly, MA) and then passed through a *p*-amino-phenyl 1-thio-β-D-cellobioside affinity column equilibrated with 0.1 M NaOAc, pH 5, 1 mM D-glucono-δ-lactone. 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 mM NaOAc, pH 5, containing 0.85 M $(NH_4)_2SO_4$. The column was washed with 5 vol of buffer and then eluted with a linear gradient of 0.85-0.35 M $(NH_4)_2SO_4$, 25 mM NaOAc, pH 5. The pure CBHI fractions were combined and the buffer was exchanged to50 mM NaOAc, pH 5, by ultrafiltration. The final preparation was judged to be 98-99% pure as indicated by SDS-PAGE. The enzyme was stored at -80°C until use.

3.3.2 *Surface preparation*

All surfaces were prepared from silicon (Si) wafers (hyperpure, type N, phosphorous doped, orientation 1-0-0), purchased from Wacker Siltronic Corporation (Portland, OR), as described previously (Suvajittanont, 1999).

3.3.3 *Adsorption and elution kinetics, and isotherm construction*

Protein adsorption was monitored continuously with an automated Garertner *in situ* ellipsometer (Gaertner Scientific Corp., Chicago, IL) equipped with a thermostatted cuvette and modified to allow for stirring and flow. Podhipleux (1998) described the instrument in detail. Adsorbed mass was calculated from ellipsometrically-determined values of film thickness and refractive index according to Cuypers et al. (1983), using a calculation procedure based on a one film model (Krisdhasima et al., 1992a). The partial specific volume and the ratio of molecular weight to molar refractivity for CBHI, both required to determine adsorbed mass, are 0.843 cm^3/g and 4.05 g/cm^3, respectively (Pethig, 1979; Cuypers et al., 1983).

Adsorption and elution kinetic data were recorded using the exact procedures described by Suvajittanont (1999). Bulk enzyme concentrations ranged between 0.001 and 0.98 mg/ml. As reported in earlier work (Suvajittanont, 1999), isotherms were constructed using two methods, based on steady-state kinetic behavior. In brief, one method involved introduction of CBHI to the cuvette in a single step to reach a desired solution concentration. In the other method, CBHI

was introduced to the cuvette successively, in order to reach a desired solution concentration. Adsorption was allowed to reach steady state after each successive addition of enzyme. All experiments were performed at least three times.

3.4 RESULTS AND DISCUSSION

3.4.1 *Adsorption kinetics and elution*

Representative plots of CBHI adsorption and elution kinetics are shown in Figure 3.1. In each case, CBHI adsorption was allowed to occur for 60 min, at which time the surface was rinsed; surfactant was added at 85 min, and the final rinse was started at 100 min. Initial adsorption rates were extremely rapid, and plateaus in adsorbed mass were achieved at all concentrations tested. Plateau values of adsorbed mass varied from 0.5 mg/m^2 at the lowest concentration tested to nearly 2 mg/m^2 at higher concentrations. This is in contrast to similar measurements recorded for *Thermomonospora fusca* E_5, which varied from about 1 to nearly 4 mg/m^2 over a similar concentration range (Suvajittanont, 1999). Adsorbed enzyme was partially elutable upon rinsing and incubation in protein-free buffer, with the resistance to elution generally lower at higher concentrations. Introduction of DTAB resulted in an increase of adsorbed mass in each case, most likely a result of DTAB binding to both CBHI and bare surface faster than enzyme removal through solubilization or displacement. Following the final rinse, adsorbed mass decreased

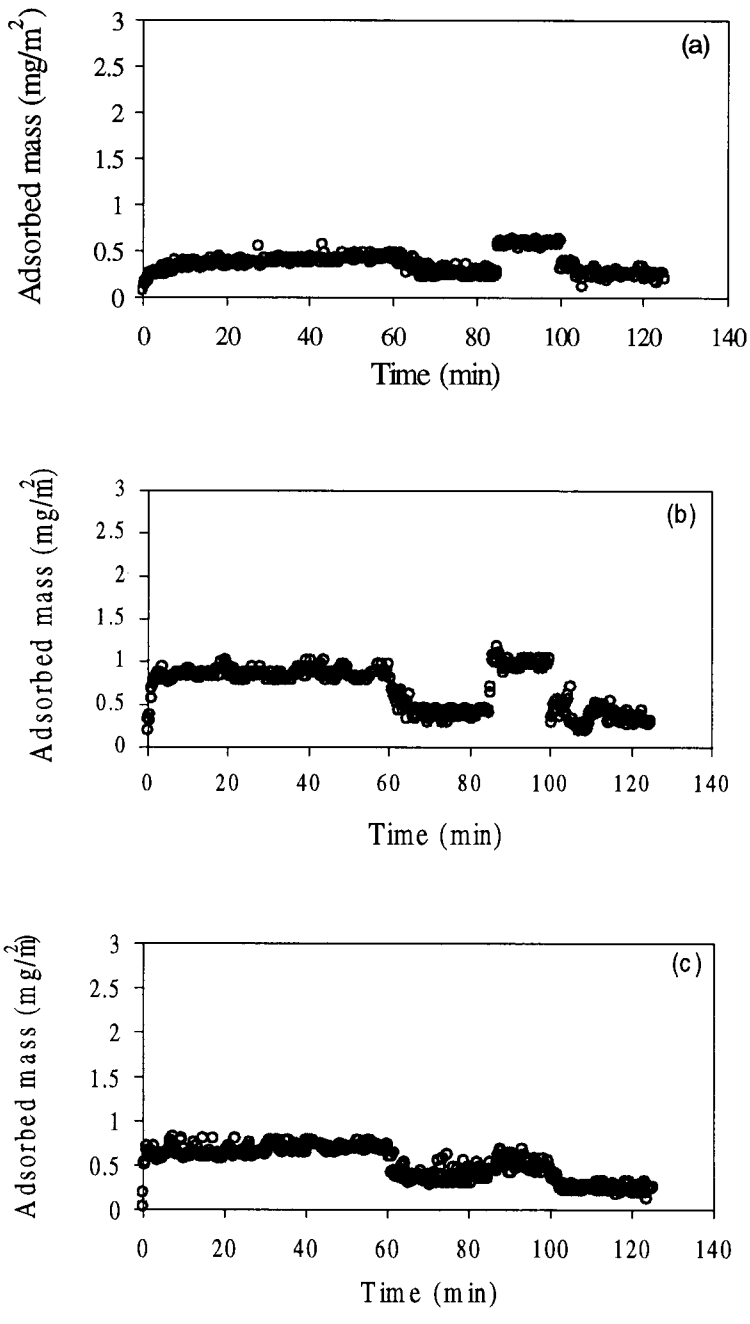
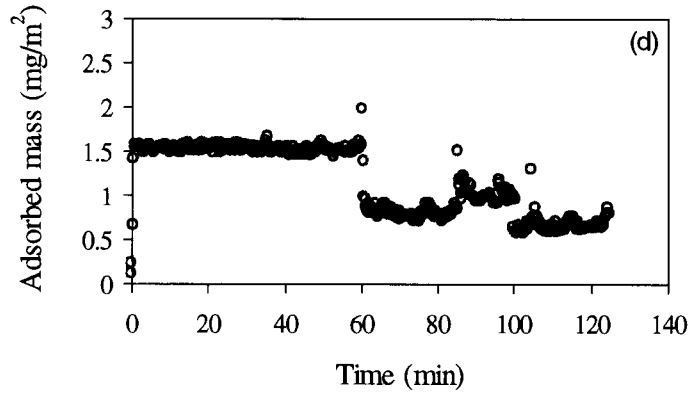
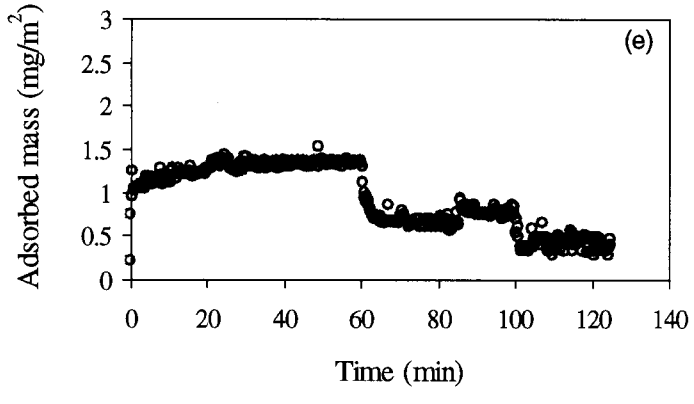


Figure 3.1 Representative adsorption kinetics and elution plots recorded for CBHI at hydrophobic silica. The cellulase concentrations are: (a) 0.001 mg/ml; (b) 0.01 mg/ml; (c) 0.07 mg/ml; (d) 0.13 mg/ml; (e) 0.28 mg/ml; (f) 0.35 mg/ml; (g) 0.40 mg/ml; (h) 0.46 mg/ml; (i) 0.70 mg/ml; (j) 0.98 mg/ml.





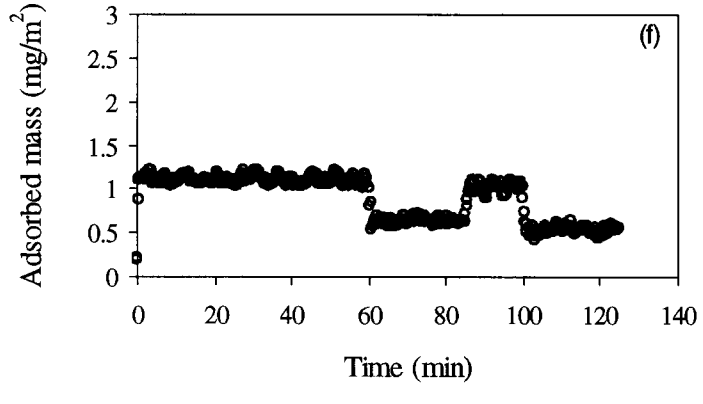


Figure 3.1 Continued

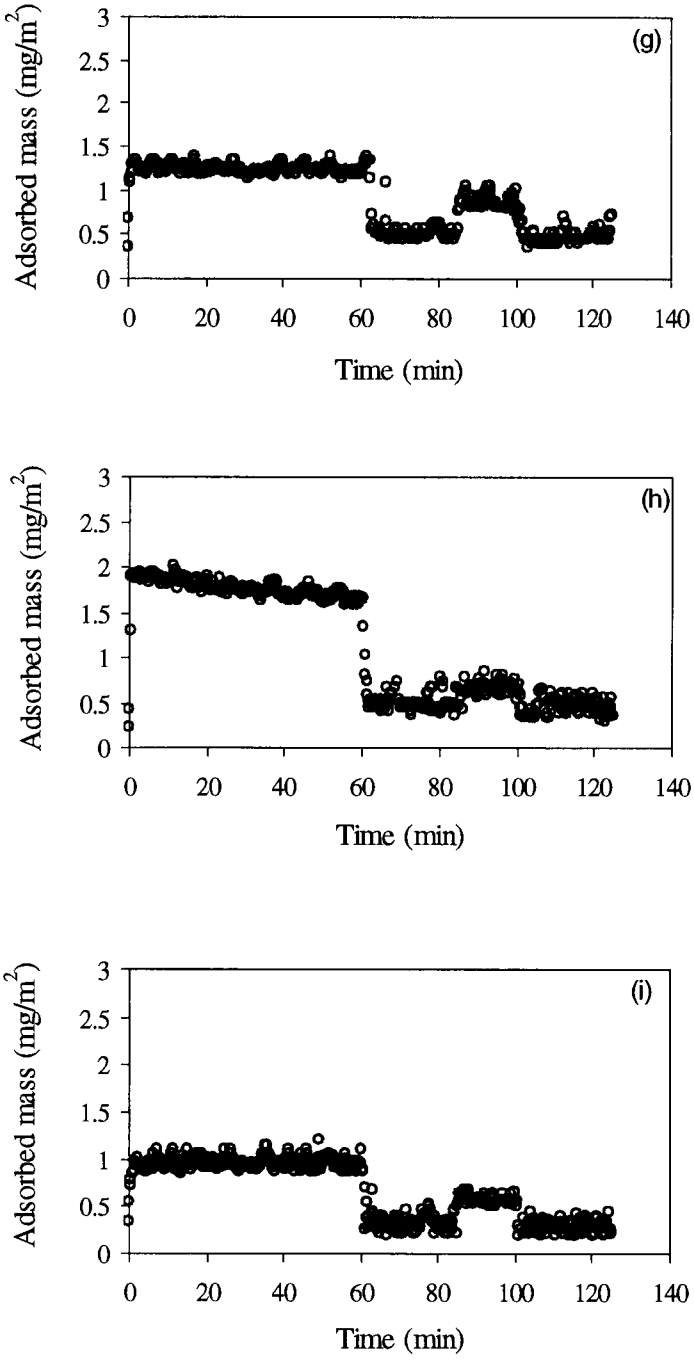


Figure 3.1 Continued

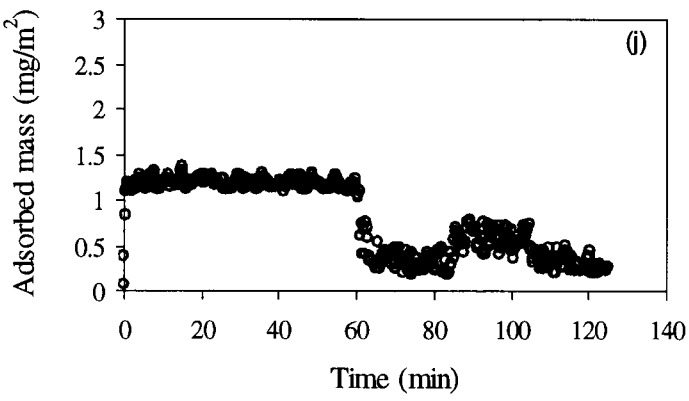


Figure 3.1 Continued

to a value lower than that present immediately prior to surfactant addition, with resistances to elution by DTAB being fairly high in each case.

The average values of adsorbed mass of CBHI remaining on the surface immediately before introduction of DTAB (85 min), and at the end of each experiment (125 min) are listed in Table 3.1. Based on the molecular dimensions of CBHI in solution (Abuja, 1988), the expected surface coverages for a monolayer of molecules adsorbed "end-on" and "side-on" would be 5.58 and 1.36 mg/m^2, respectively. The amounts of protein remaining on the surface after both the first rinse and incubation in buffer (85 min) thus correspond to submonolayer coverages in each case. The adsorbed mass remaining after the first and second rinses reached a maximum in tests conducted with intermediate enzyme concentrations; i.e. the adsorbed mass remaining increased upon changing cellulase concentration from 0.07 to 0.13 mg/ml, eventually decreasing as higher enzyme concentrations were

approached. The mass of protein remaining on the surface after elution with DTAB (125 min) divided by that remaining after elution with protein-free buffer (85 min) are also listed in Table 3.1 for each concentration tested. These values were greatest at the lowest and highest solution concentrations tested.

Table 3.1. Adsorbed mass of *T. reesei* CBHI following the first rinse after 60 min adsorption, second rinse after 15 min contacting with DTAB, and also the binding strength of the enzyme at various concentrations.

Cellulase concentration (mg/ml)	Adsorbed mass (mg/m^2)		Resistance to elution with DTAB
	First rinse	Second rinse	
0.001	0.32	0.284	0.888
0.01	0.385	0.344	0.894
0.07	0.401	0.299	0.745
0.13	0.743	0.589	0.793
0.28	0.752	0.498	0.662
0.35	0.635	0.546	0.860
0.40	0.536	0.464	0.865
0.46	0.535	0.437	0.816
0.70	0.365	0.360	0.984
0.98	0.337	0.319	0.947

The data of Figure 3.1 and Table 3.1 show trends that differ substantially from those observed for adsorption of *T. fusca* E_5 to hydrophobic silica (Suvajittanont, 1999). In that work, plateau values of adsorbed mass generally increased with increasing solution concentration, as did the amounts remaining on the surface following the first rinse in protein-free buffer (85 min). The adsorbed mass remaining after treatment with DTAB varied little among experiments. Those results could be explained with reference to an adsorption mechanism allowing for irreversible adsorption into two dissimilar states, distinguished by differences in occupied interfacial area, and binding strength. Such a simple model is insufficient for describing the present data. In particular, an acceptable explanation of the interfacial behavior of CBHI must account for: (i) the maxima in adsorbed mass observed in tests conducted with intermediate enzyme concentrations; (ii) the resistance to elution with buffer being generally lower at higher concentrations; and (iii) the resistance to elution with DTAB being generally lower at intermediate concentrations.

It is difficult to explain these observations without allowing for the possibility that some aggregation phenomena are involved in CBHI adsorption; i.e. the data suggest that CBHI may not simply adsorb as non-interacting monomers. Figure 3.2 depicts an adsorption scheme that would be consistent with the interfacial behavior observed for CBHI. In this scheme, CBHI is considered capable of self-association such that the adsorbed layer consists of CBHI monomers and aggregates. The relative amounts of monomers and aggregates in solution and

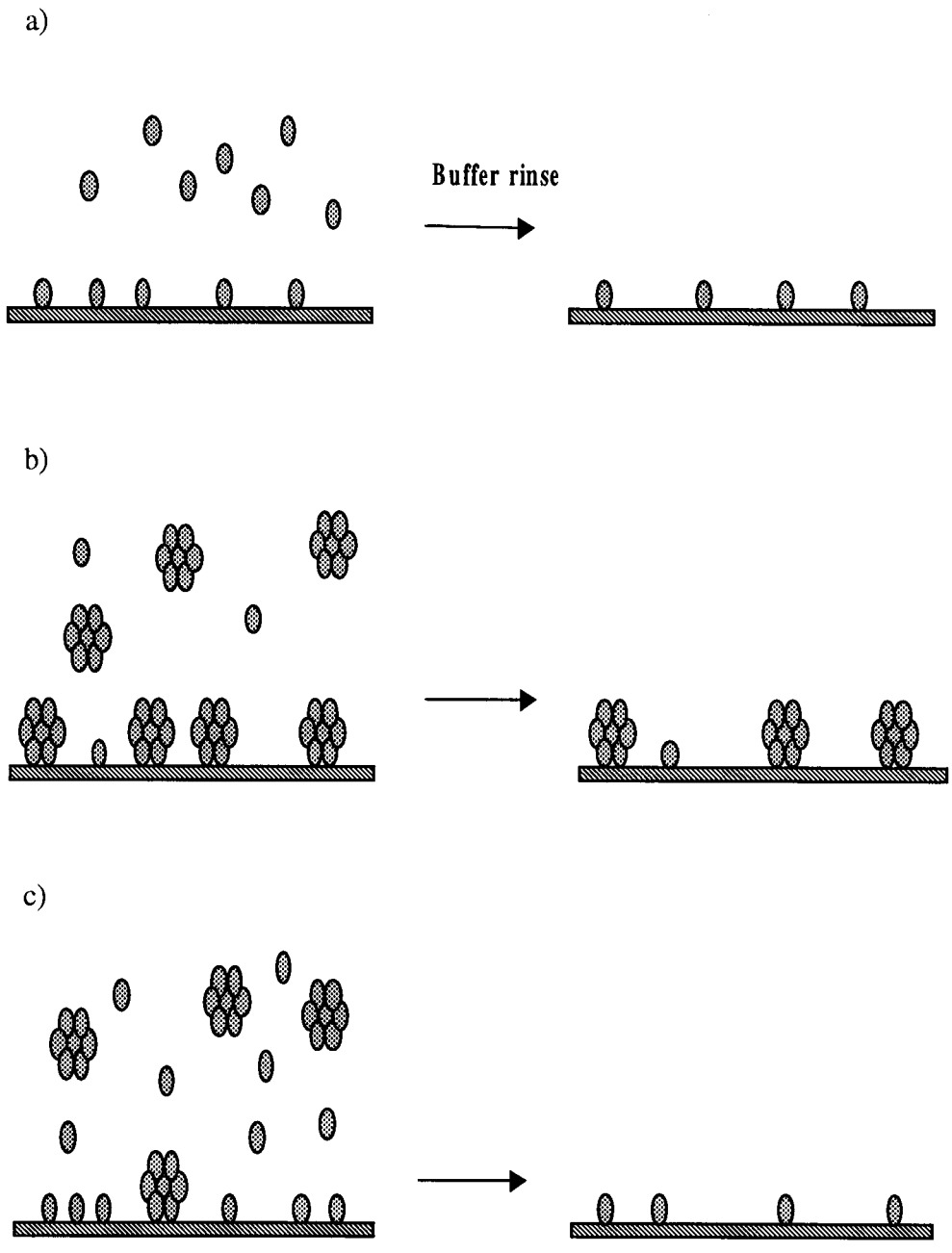


Figure 3.2 Illustration of the proposed concentration-dependent adsorption of CBHI, and subsequent elution of the adsorbed layer by protein-free buffer. CBHI concentrations in solution are: (a) low; (b) intermediate; and (c) high.

on the surface, and the size of the aggregates relative to monomers, are depicted in Figure 3.2 purely for illustrative purposes with no intent to be accurate in a quantitative sense. Once adsorbed, the aggregates may undergo a slow, surface-induced dissociation to monomers, although this is not essential to describing the data and so not depicted in Figure 3.2. While we have as yet no direct evidence of aggregate formation in CBHI solutions, it seems reasonable to allow for such behavior. For example, at all concentrations tested in this study, CBHI adsorption was extremely rapid. This suggests a high affinity for hydrophobic association. Studies with carbohydrate-binding proteins in general have shown that the carbohydrate-protein interaction can be largely attributed to hydrophobic associations (Linder et al., 1995a; Mattinen et al., 1997). Further, aromatic residues present on the binding domain of CBHI have been shown to be important in binding (Linder et al., 1995b; Reinikainen et al., 1992). Thus, it may be plausible that hydrophobic associations can be made among monomers in solution. Concerning this issue, Baker et al. (1999) recorded changes in circular dichroism spectra for CBHI upon addition of colloidal silica, and demonstrated that the spectral changes could not be attributed to any adsorption phenomena, suggesting that solution phase phenomena may have been involved. In any event, protein aggregation, whether in solution, or involving associations between adsorbed protein and protein entering the interface from solution, has been suggested to play a role in otherwise anomalous adsorption behavior of ferritin (Nygren and Stenberg, 1990), insulin

(Nilsson et al., 1991), and some T4 lysozyme variants (McGuire et al., 1995a, 1995b), among others.

At low concentrations, we will consider adsorption to occur from a solution of CBHI monomers, resulting in an adsorbed layer of CBHI monomers (Figure 3.2a). As solution concentration is increased from low to intermediate values, the population of associated monomers in solution is suggested to increase. The adsorbed layer is thus depicted in Figure 3.2b as containing a substantial population of CBHI aggregates. The amount of unoccupied interface would be higher than that expected if all protein in the adsorbed layer were in monomeric form, serving to reduce the degree of repulsive electrostatic forces between neighboring entities, whether monomeric or multimeric. Noted however that adsorbed mass could be high relative to that of an adsorbed layer of monomers at a similarly low surface coverage. As solution concentration is further increased, while aggregates may still be present in solution, the number of monomers available for adsorption would be expected to increase. The adsorbed layer is thus depicted in Figure 3.2c as containing a greater population of CBHI monomers than shown in Figure 3.2b, owing to their high diffusivity relative to that of a protein aggregate. The total fractional surface coverage of protein in this case would be higher than that proposed for adsorption from solutions of intermediate concentration. Therefore, we could fairly expect less resistance to elution with protein-free buffer for adsorbed layers like that illustrated in Figure 3.2c, as any repulsive, nearest neighbor interactions in this more crowded interface would serve to weaken binding strength.

After rinsing and incubation in buffer, the adsorbed layer formed at the lowest and highest solution concentrations (Figure 3.2a and 3.2c) would be more resistant to DTAB elution than that formed at intermediate concentrations. This is presumably due to protein bound directly to the surface being more tightly held than protein present in an aggregate and not directly bound to the surface. This kind of thinking is consistent with the data recorded in Table 3.1, which shows resistance to elution with buffer being generally lower at higher concentrations, and resistance to elution with DTAB being generally lower at intermediate concentrations.

3.4.2 *Adsorption isotherms*

Using methods described by Suvajittanont et al. (1999), the CBHI adsorption isotherm was constructed using both single-step and sequential introduction of enzyme to yield the desired steady-state solution concentration (Figure 3.3). Constructing the isotherm by single-step introduction of cellulase, we would expect adsorbed mass to be low at low concentrations, increasing to a maximum at intermediate concentration, and then decreasing as solution concentration is further increased. This is consistent with the discussion above, and in fact each point on the isotherm constructed by single-step addition and plotted on Figure 3.3, is listed in Table 3.1 as the adsorbed mass after the first rinse (85 min).

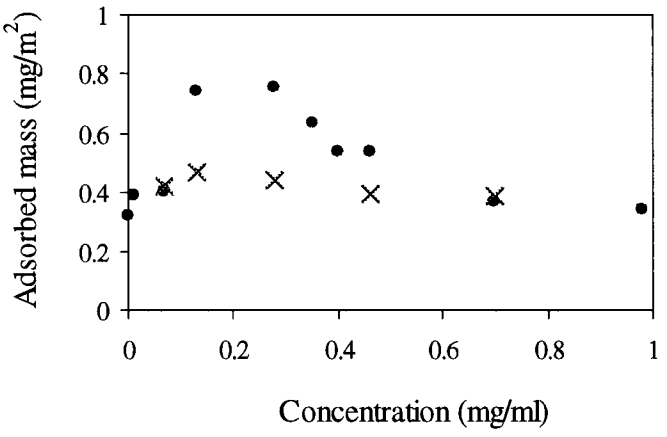


Figure 3.3 Adsorption isotherms recorded by (•) single-step introduction of CBHI, and (×) successive addition of enzyme.

On the other hand, we would expect the isotherm to exhibit a different shape if constructed by successive addition of CBHI. In particular, the isotherm would be expected to show a steep increase at low concentration, followed by attainment of a plateau. This corresponds well to the isotherm constructed by successive addition of enzyme determined experimentally, and plotted in Figure 3.3. While successive addition of CBHI would eventually lead to formation of aggregates in solution, the interface would already be occupied by monomers that had adsorbed from solutions of lower concentration. At all solution concentrations tested, CBHI surface coverages were well below that expected for a monolayer of molecules in any orientation. This suggests that some minimal amount of unoccupied interfacial area must be available for adsorption into a state that would resist buffer elution. This

amount is possibly a function of repulsive force interactions among neighboring molecules (or aggregates). Thus, upon successively increasing solution concentration, further adsorption would not be expected to occur.

3.5 ACKNOWLEDGMENT

We are grateful to Kunruedee Sangseethong for providing the APTC-affinity column essential for successful CBHI purification.

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CHAPTER 4

QUANTIFICATION OF *THERMOMONOSPORA FUSCA* E_3 AND E_5, AND *TRICHODERMA REESEI* CBHI IN RECONSTITUTED MIXTURES AND ITS APPLICATION TO STUDY OF COMPETITIVE BINDING

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4.1 ABSTRACT

The adsorption behavior of *Thermomonospora fusca* E_3 and E_5, and *Trichoderma reesei* CBHI on avicel and bacterial microcrystalline cellulose was investigated. Both binary and ternary mixtures of these cellulases were studied at three different total cellulase concentrations. Cellulase concentrations were chosen such that low, intermediate, and high cellulose surface coverages would result. Reverse phase, high pressure liquid chromatography was found to be a reliable and accurate means to separate and quantify the individual cellulases in the reconstituted mixtures. Mixtures of E_3 and E_5 did not demonstrate competitive adsorption behavior at any of the cellulase concentrations investigated, suggesting that these cellulases bind to specific sites. By contrast, the cellulases in the mixtures of E_3 and CBHI, and E_5 and CBHI competed for surface sites at the highest cellulase loading. In these circumstances, E_3 or E_5 showed preferential binding over CBHI. Significant binding competition was noted for the ternary cellulase mixture at all cellulase concentration levels investigated.

4.2 INTRODUCTION

The attention to enzymatic hydrolysis of cellulose has been increasing partly due to the fact that cellulose is a renewable resource which can be used to produce valuable products. Cellulose can be hydrolyzed by enzymes to yield monomeric sugars. In turn, these sugars can be converted into fuels and chemicals by way of fermentation.

Cellulases from several bacterial and fungal systems have been characterized and studied for their capability to work cooperatively in hydrolyzing cellulose (Beldman et al., 1988, Fagerstam et al., 1980, Henrissat, et al., 1985). To obtain efficient hydrolysis of microcrystalline cellulose, at least three functionally different types of cellulase are essential, including an endocellulase and two types of exocellulases (Walker et al., 1992, Wood et al., 1989). The cellulases work synergistically during hydrolysis of cellulosic materials; the endoglucanase hydrolyzes internal glucosidic bonds in the cellulose chains after which cellobiohydrolase splits off cellobiose units from the chain ends (Goyal et al., 1991). Several investigators have suggested that the synergistic actions of the cellulases must be regarded as a phenomenon that is related to the competitive binding between cellulase components. They have hypothesized that for hydrolysis of cellulose, the optimum initial endoglucanase:exoglucanase ratio is greatly dependent on the binding behavior of these enzymes (Kim et al., 1992, Kyriacou et al., 1989, Ryu et al., 1984).

The hydrolysis process is a complex, heterogeneous reaction. Since the interfacial adsorption of the enzymes is a prerequisite step to hydrolysis, the elucidation of the molecular mechanisms of cellulase adsorption is of importance to understand the entire hydrolysis process. Until recently, it was not possible to investigate the adsorption of individual cellulases, nor controlled cellulase mixtures, because of the difficulty in purification process and quantification techniques. To date, relatively few multi-component adsorption studies have been conducted using mixtures of purified cellulases (Irwin et al., 1993, Kim et al., 1994, Walker et al., 1993). Medve et al. (1994) reported that CBHI and CBHII from *Trichoderma reesei* competed for some adsorption sites and bound to specific sites as well. Furthermore, CBHI had a higher affinity for the adsorption sites. Evidence for both common and distinct adsorption sites for cellulases have also been reported by Kyriacou et al.(1989). Ryu et al. (1984) also indicated stronger adsorption affinity of cellobiohydrolases over endoglucanases of *T. reesei.* Several studies have shown preferential binding of cellobiohydrolases which have higher affinity (Kim et al., 1994, Kyriacou et al., 1989, Medve et al., 1994, Ryu et al., 1984). In addition, Walker and co-workers (1993) investigated the cellulose (Avicel) binding behavior of *Thermomonospora fusca* E_3 and E_5, and *Trichoderma reesei* CBHI and reported that competition for binding sites did not occur between E_5 and exocellulases. However, the cellulase concentrations used in this study were below the saturation binding level.

Many investigators have been trying to quantify cellulases when in mixtures. Without a reliable technique, it is difficult to distinguish one cellulase from another in solution. Data interpretation would thus be tentative at best. Most techniques to date rely on indirect measurements for this purpose, e.g. activity assays (Kim et al., 1992, Ryu et al., 1984, Nidetzky and Claeyssens, 1994, Nidetzky et al., 1994a, Nidetzky et al., 1994b). In addition, total protein measurement by the Lowry method has been widely used (Kim et al., 1988, Nidetzky and Steiner, 1993, Ooshima et al., 1983), but it fails to provide specific information about individual enzymes in the mixture. Finally, a few researchers have used chromatographic techniques to either quantify or purify from *T. reesei* (Ellouz et al., 1987, Hayn and Esterbauer, 1985, Medve et al., 1994, Medve et al., 1998). These techniques probably constitute the methods of choice, being the only methods allowing for direct measurement of dissimilar proteins in solution.

In this work we used two types of cellulase from *Thermomonospora fusca* (E_3 and E_5) and CBHI cellulase from *Trichoderma reesei*. We chose to investigate these three cellulases because they represent each cellulase class; E_5 is an endoglucanase, and E_3 and CBHI represent the two classes of exoglucanases. The adsorption behavior exhibited by these cellulases in binary and ternary mixtures was recorded, using a solution depletion method. High pressure liquid chromatography (HPLC) was used to directly determine cellulase concentration in the supernatant.

4.3 MATERIALS AND METHODS

4.3.1 *Cellulase production and purification*

Thermomonospora fusca E_3 and E_5, and *T. reesei* CBHI cellulases were a gift from Drs. L.P. Walker and D.B. Wilson (Cornell University, NY). The growth conditions, harvesting techniques, and purification methods used to isolate *these enzymes* are exactly as described by Bothwell et al. (1997).

4.3.2 *Adsorption studies on avicel*

The cellulase mixtures investigated include E_3+E_5, E_3+CBHI, E_5+CBHI, and E_3+E_5+CBHI. The initial concentrations of each cellulase component were tabulated in Table 4.1a. Three levels of initial cellulase concentration were used: low (a level that results in 25% of the maximum binding level for each cellulase, as estimated from isotherms constructed and described by Bothwell et al.(1997)); high (a level that results in the maximum binding level for each cellulase, as estimated from isotherms constructed and described by Bothwell et al.(1997)); and, an intermediate, equimolar loading. All experiments were performed in micro-centrifuge tubes with screw caps. Avicel PH 102 (4.0% w/v) was suspended in 50 mM sodium acetate buffer, pH 5.5, and incubated with end-over-end rotation in a 50°C incubator (VWR Scientific Inc., Philadelphia, PA) for 30 minutes. Reconstituted mixtures of cellulases were added to the pre-incubated solutions to achieve one of the three

cellulase concentrations investigated. The tubes were rotated to ensure proper mixing in a 50°C incubator for 2 hours. Following, the avicel with bound cellulases was removed from the supernatant by repeated centrifugation (Eppendorf 5415C, Brinkmann Instruments Inc., Westbury, NY) at 14,000 × g for a total of 15 minutes. The filtrate was analyzed for the free cellulase concentration using HPLC equipped with a reverse phase column (Delta PAK, 5 μ, C18, 300 A°, Waters Corporation, Milford, MA). The amount of bound cellulase was calculated by subtracting the free cellulase concentration from the initial concentration. Each experiment was done in duplicate.

4.3.3 *Adsorption studies on BMCC*

The experimental procedures were similar to the one described above (for avicel). Three levels of initial concentrations were tabulated in Table 4.1b according to the isotherms for BMCC constructed by Bothwell et al. (1997). BMCC (0.1% w/v) was suspended in 50 mM sodium acetate buffer, pH 5.5 and incubated in 50°C incubator for 30 min. After adding mixtures of cellulases, the incubation continued for 30 min. Following, the BMCC was removed and the filtrate was injected to HPLC. The analysis was the same as for avicel studies.

Table 4.1. Initial concentrations of each cellulase corresponding to the three solution loading levels used in this study.

a) Avicel studies

Cellulase	Total concentration (μM)		
	Low	Equimolar	High
E_3	4.7	15	50
E_5	7.2	15	70
CBHI	6.5	15	55

b) BMCC studies

Cellulase	Total concentration (μM)		
	Low	Equimolar	High
E_3	4.5	10	40
E_5	4.5	10	50
CBHI	1.8	10	20

4.3.4 *Cellulase quantification*

The free cellulase concentration of each component in the reconstituted mixtures were determined using reverse phase, high pressure liquid chromatography (Waters 600, Waters Corporation, Milford, MA. The sample (10 μl) was loaded on to the column equilibrated with 64% 0.1 M sodium acetate, pH 7.0 and 36% methanol. The mobile solution was changed in a step-wise manner, from an initial methanol concentration of 36% to 57%. The flow rate was kept constant at 1 ml/min. The absorbance at 280 nm was used to detect the presence of cellulase.

Free cellulase concentration was determined using calibration curves based on peak areas.

4.3.5 *Analysis of substrate degradation*

The supernatant from each adsorption experiment was assayed for reducing sugar using the DNS method (Miller et al., 1960) with glucose as the standard. The extent of substrate degradation which occurred during the adsorption experiments was determined by mass balance. Each assay was carried out in triplicate.

4.4 RESULTS AND DISCUSSION

A representative chromatogram of the ternary mixture (E_3, E_5, and CBHI) is shown in Figure 4.1. All three cellulases were completely separated, with CBHI and E_3 eluting first when the mobile phase contained 36% methanol. E_5 eluted last by increasing the percentage of methanol to 57%. Reverse phase HPLC thus proved effective in quantifying the concentration of different cellulases present in reconstituted mixtures.

The relative abundance of each cellulase present at the avicel surface was determined for all three levels of initial cellulase concentration tested. The adsorbed mass of a single component cellulase system (E_3, E_5, and CBHI) can be estimated for any initial total cellulase concentration using the Langmuir isotherms

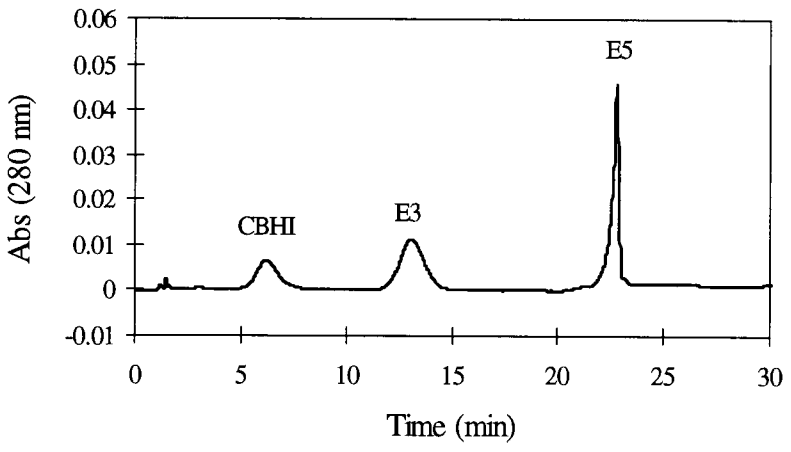

Figure 4.1 A representative chromatogram of ternary mixture of E_3+E_5+CBHI

described by Bothwell et al. (1995). These values were used to evaluate the binding behavior of cellulases acting in reconstituted mixtures. Specifically, the adsorbed mass of a given cellulase acting in a mixture was determined. A ratio of this value to the value of adsorbed mass that would be expected if the cellulase was adsorbing from a single cellulase solution was obtained and converted into a percentage. If the cellulase did not compete with other cellulases in the mixture for surface sites, the percentage would be close to 100. However, if the cellulases were competing for surface sites, the percentage would drop depending on their adsorption affinity toward the cellulose surface sites.

The competitive adsorption results of the cellulases in various reconstituted mixtures are tabulated in Table 4.2a-d. The mixtures of E_3 and E_5 did not indicate

any binding competition at any levels of initial cellulase concentration investigated (Table 4.2a). This may imply that E_3 and E_5 react to specific sites on the cellulose surface. Nevertheless, this was not the case for mixtures including CBHI. At high initial cellulase concentration, CBHI competed for binding sites with both E_3 and E_5 (Table 4.2b and c). However, E_3 and E_5 clearly showed preferential binding over CBHI. By contrast, there was no binding competition at low initial concentration. The result indicated that CBHI might share common binding sites with E_3 and E_5. At low initial concentration, there were still enough binding sites for both cellulases; thus, no competition occurred. Once the sites were taken up by increasing the initial cellulase concentration in the mixtures, the higher adsorption affinity cellulase dominated the surface sites. In addition, the adsorption of the three cellulase mixtures was also recorded (Table 4.2d). Competitive adsorption took place at all cellulase concentration levels investigated. The competition among cellulases was not as pronounced at low initial cellulase concentration as at the higher concentrations. It is interesting to note that at the equimolar loading, E_3 was highly competitive, but E_5 became dominant at the high loading.

In experiments of this kind, in which cellulase binding to its natural substrate is quantified, the fact that hydrolysis occurs concurrently cannot be dismissed. A desorption of enzyme could take place as a result of substrate degradation. Therefore, it is of importance to determine the amount of substrate degradation following the adsorption studies in order to verify the validity of the results. The reducing sugars in samples were measured and converted into the

Table 4.2 The competitive adsorption results of binary and ternary mixtures of reconstituted mixtures of cellulases on avicel at three concentration levels.

a) E_3+E_5

Concentration level	Percentage of bound cellulase compared to its individual component system (%)	
	E_3	E_5
Low	107±2	100±0.9
Equimolar	100±2	93±2
High	99±8	97±4

b) E_3+CBHI

Concentration level	Percentage of bound cellulase compared to its individual component system (%)	
	E_3	CBHI
Low	112±3	98±0.7
Equimolar	100±3	81±6
High	63±6	48±9

c) E_5+CBHI

Concentration level	Percentage of bound cellulase compared to its individual component system (%)	
	E_5	CBHI
Low	103±2	105±3
Equimolar	100±0.2	95±3
High	98±6	74±11

Table 4.2 Continued

d) E_3+E_5+CBHI

Concentration level	Percentage of bound cellulase compared to its individual component system (%)		
	E_3	E_5	CBHI
Low	103±0.8	87±2	95±4
Equimolar	88±3	74±0.2	65±6
High	73±15	91±17	31±6

amount of avicel degraded (Table 4.3a). The data show less than 15% of total avicel degraded during adsorption studies. These results assured that the adsorption behaviors established in the study were mainly a result of binding competition as opposed to substrate degradation. The adsorption data recorded for BMCC were inconclusive, as substantial cellulose degradation (up to 86%) occurred during the course of the experiments (Table 4.3b).

Table 4.3 The degradation of cellulosic substrates during the competitive adsorption experiments.

a) Avicel

Cellulase mixtures	Percentage of substrate degradation (%)		
	Low	Equimolar	High
E_3+E_5	3.66	6.02	5.69
E_3+CBHI	6.77	12.99	14.18
E_5+CBHI	3.97	5.94	5.09
E_3+E_5+CBHI	5.45	10.26	9.57

Table 4.3 Continued

b) BMCC

Cellulase mixtures	Percentage of substrate degradation (%)		
	Low	Equimolar	High
E_3+E_5	27.73	31.78	40.99
E_3+CBHI	58.45	69.17	86.05
E_5+CBHI	22.94	32.82	26.28
E_3+E_5+CBHI	59.00	63.08	64.61

4.5 ACKNOWLEDGMENT

We are indebted to Drs. Larry P. Walker and David B. Wilson at Cornell University for providing cellulases used throughout the study.

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CHAPTER 5

CONCLUSIONS

The individual adsorptions of E_5 and CBHI were monitored on silica surfaces with *in situ* ellipsometry. Both enzymes were found to adsorb on hydrophobic silica while only insignificant binding on hydrophilic silica took place, emphasizing the importance of hydrophobic interaction between the enzymes and the surfaces. The adsorption behaviors of the enzymes on hydrophobic, silanized silica were also described by using a simple, parallel adsorption kinetic mechanism allowing the protein to adopt one of two dissimilar conformational states upon adsorption at the interfaces. E_5 data could well be interpreted by this model and showed that the fractions of more tightly bound state molecules changed inversibly with the increasing solution concentrations. The elution studies with surfactant indicated the early accumulation of molecules with high binding strength. After a critical value was reached, the incoming adsorbed molecules were more likely to be more loosely bound due to the limited of available surface areas. CBHI, on the other hand, did not behave like proteins in general and could not be described by this adsorption mechanism. The high adsorbed mass and elutability by surfactant at intermediate solution concentrations implied that molecular self-association phenomena might highly influence the adsorption behaviors at these concentration levels. Nevertheless, more studies are needed to confirm the existence of this solution-phase phenomenon.

Competitive adsorption of *Thermomonospora fusca* E_3 and E_5, and *Trichoderma reesei* CBHI cellulases were as well investigated using avicel and BMCC as cellulosic substrates. For this purpose, an analytical technique was developed to quantitate the amount of individual cellulase concentration both in binary and tertiary mixtures. A method using reverse phase HPLC was found to be reliable quantitatively. The competitive adsorption results on avicel indicated the existing of specific sites for E_3 and E_5. However, they did compete for the surface sites with CBHI and demonstrated preferential binding on the surface. The avicel degradation during the adsorption was also observed but in a very low percentage. For the studies on BMCC, however, the competitive binding data were inconclusive due mainly to a substantially high substrate degradation during the adsorption studies

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APPENDIX

ADSORPTION OF *THERMOMONOSPORA FUSCA* E_5 AND *TRICHODERMA REESEI* CBHI CELLULASES ON SYNTHETIC SURFACES

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January 1999, in review

ABSTRACT

The interfacial behavior of *Thermomonospora fusca* E_5 and *Trichoderma reesei* 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 upon 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 polystyrene proved unworkable, due 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.

INTRODUCTION

Protein interactions with solid surfaces have been studied for decades, and several comprehensive reviews are available (Andrade et al., 1996, Brash and Horbett, 1995, Horbett and Brash, 1987, McGuire and Bothwell, 1999). 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, due 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 (Krisdhasima et al., 1992b, Lu and Park, 1991).

Carbohydrate-protein interactions often involve aromatic residues on the binding face of the protein (Din et al, 1994, Engle et al., 1994, Spurlino et al., 1992, Vyas, 1991). 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 (Linder et al., 1995, Mattinen et al., 1997). Site-directed mutagenesis studies have shown that aromatic residues present on the hydrophobic binding face of CBHI and endoglucanase I of T*richoderma reesei* are important in binding (Linder et al., 1995, Reinikainen et al., 1992). Further, study of ionic strength effects on CBHI binding pointed to the importance of hydrophobic interactions (Reinikainen et al., 1995). Hydrophobic interactions have been shown to play an important role in 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 (Creagh et al., 1996). Hydrophobic interactions have been shown to play a role in binding of *C. fimi* CenC as well, a cellulase that binds only soluble cellulose (Johnson et al., 1996).

In this work 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 (Andrade et al., 1996, Brash and Horbett, 1995, Horbett and Brash, 1987). Thus, we believe that in the absence of mass transfer limitations, pore-size and other morphological effects (Bothwell et al., 1997) 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

Thermomonospora fusca E_5 cellulase was produced by transformed *Streptomyces lividans* TK24 carrying a plasmid (pGG74) bearing the E_5 gene (Ghangas and Wilson, 1987). The production and purification procedures were based on those described earlier (Bothwell et al., 1997). 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-liter fermenter containing 4.8 liters 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 micron cassette). The filtered supernatant was adjusted to 1.2 M $(NH_4)_2SO_4$ and 0.1mM phenylmethylsulfonyl fluoride, and loaded onto a 150 ml Phenyl Sepharose CL-4B column (Sigma Chemical Co., St. Louis, MO). After washing the column, bound cellulase was eluted with a series of buffers as described in Walker et al. (1992). 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 Chemical Co., St. Louis, MO). The column was washed with one column volume 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.

Trichoderma reesei CBHI was purified from crude cellulase (Spezyme™ - CP, Environmental BioTechnologies Inc., Menlo Park, CA). The purification process was as described in Piyachomkwan et al. (1997). 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, 1 mM 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 mM NaOAc, pH 5, containing 0.85 M $(NH_4)_2SO_4$. The column was

washed with 5 column volumes 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 exchanged to 50 mM NaOAc, pH 5, by ultrafiltration with a PM 10 membrane. The concentrated cellulase was stored at –80°C until use. Both cellulases were judged to be 98-99% pure as indicated by SDS-PAGE and CMCase 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 in Tian et al. (1998). In particular, CD spectra were recorded from 400 nm to 184 nm wavelengths using a quartz cuvette of pathlength 0.2 mm. Sample concentrations were 0.3 mg/ml E_5 and 0.2 mg/ml CBHI. Spectra presented are an average of three scans at 10 nm/min. The percent α-helix content was estimated using the ellipticity recorded at 222 nm (θ_{222nm}) according to Kondo et al. (1992):

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

Secondary structure of the proteins was predicted using the variable selection method (Manavalan and Johnson, 1987). 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 Chemicals Inc, Marietta, GA). Cellulase-particle suspensions were prepared by mixing cellulase with particles at a 2:1 particle:protein ratio and allowing adsorption to occur for 90 minutes at 25° C and 125 rpm. All remaining procedures were the same as described above.

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 E_5 and 0.5 mg/ml CBHI. Colloidal silica (20 nm), and 19 nm polystyrene particles (Duke Scientific Corporation, Palo Alto, CA), were used without modification. Cellulase-particle suspensions were prepared by mixing cellulase with particles at a 2:1 particle:protein ratio and allowing adsorption to occur for 90 minutes at 25 °C and 125 rpm. This suspension (1 ml) was passed through a Sephadex G-100 column (Pharmacia Biotech. Inc, New Jersey) at a flow rate of about 400 µl/min. The absorbance of each 0.8-1.0 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 Laboratories, Fishers, IN), and allowed to adsorb for 90 minutes at 25 °C and 125 rpm. Cellulase concentrations used were 0.23 mg/ml E_5 and 0.31 mg/ml CBHI. After incubation, samples were placed in Ultrafree CL 0.1 μm Durapore membrane filter tubes (Millipore Corporation, Bedford, MA), and centrifuged at 7,000 rpm 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 7,000 rpm 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 Corporation (Portland, OR). Surfaces were oxidized in O_2 (1 atm) for 17 min at 1000 °C. Following, 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 (DDW), washed with a solution of HCl and H_2O_2, and rinsed again with DDW, according to procedures described elsewhere (Krisdhasima et al., 1993). 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. (1982), as slightly modified by Krisdhasima et al. (1993). In particular, the surfaces were silanized with 0.1% dichlorodimethylsilane (Aldrich Chemical Co., Inc., Milwaukee, WI) in xylene. Silanized surfaces were then washed in sequence with xylene, acetone, and ethanol. Surface hydrophobicity was verified with contact angle methods (Krisdhasima et al., 1992).

Protein adsorption was monitored continuously with an automated Garertner *in situ* ellipsometer (Garertner Scientific Corp., Chicago, IL) equipped with a thermostatted cuvette and modified to allow for stirring and flow. The instrument was described in detail by Podhipleux (1998). Adsorbed mass was calculated from ellipsometrically-determined values of film thickness and refractive index according to Cuypers et al. (1983), using a calculation procedure based on a one film model (Krisdhasima et al., 1992a). 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 (Cuypers et al., 1983, Pethig, 1979).

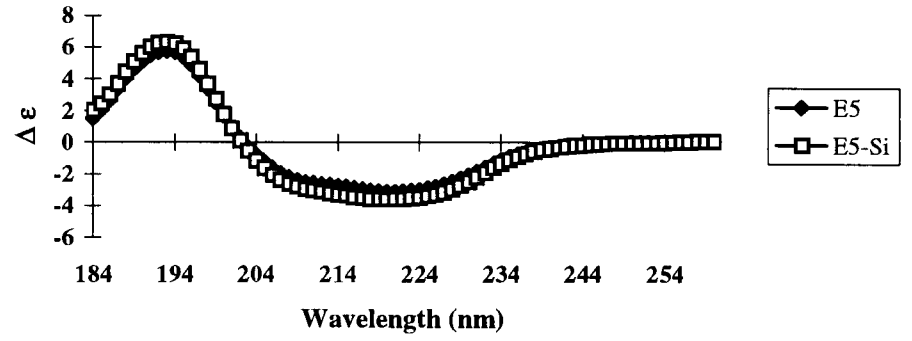
Experiments were performed with 50 mM sodium acetate buffer, pH 5.5, at 325 rpm and 25 °C. The pseudo-refractive index of the bare surface was determined prior to addition of enzyme. An experiment began with addition of 1.0 ml of enzyme solution to the cuvette containing 5.0 ml of buffer, yielding a final protein concentration of 0.10 mg/ml E_5 and 0.13 mg/ml CBHI. Adsorption was monitored for 30 min. The cuvette 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

Circular dichroism spectroscopy

The CD spectra of E_5 and CBHI, in the absence and presence of colloidal silica, are shown in Figure A. The CD spectrum of E_5 was virtually unchanged in the presence of silica. This indicated that either no conformational changes took place upon adsorption, or adsorption did not occur. Table 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 upon the addition of silica. While α-helix content was determined to increase by 5% upon 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 (Billsten et al., 1995, Kondo et al., 1992, Norde and Favier, 1992, Tian et al., 1998). In contrast, the CD spectra of CBHI recorded in the absence and presence of silica were dissimilar, suggesting that CBHI did experience structural alteration.



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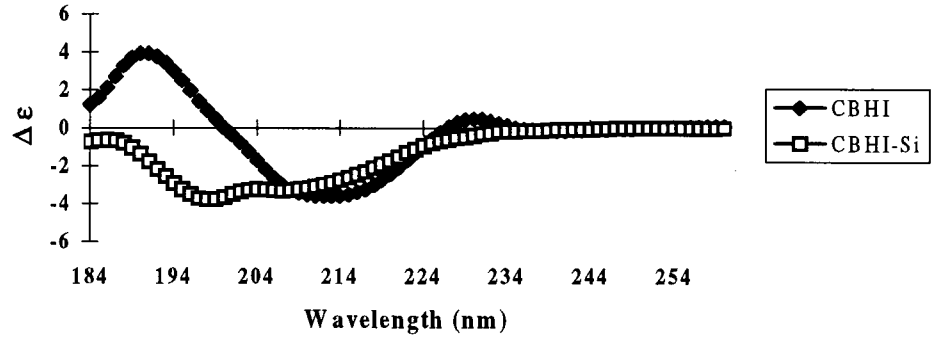


(b)

Figure A. Circular dichroism spectra of cellulases in the absence and presence of colloidal silica: (a) *T. fusca* E_5; (b) *T. reesei* CBHI

Table Secondary structure of cellulases in the absence and presence of colloidal silica. Standard deviations are shown in parentheses.

Cellulase/Particles	Secondary structure content (%)				
	α-helix	Parallel β-sheet	Anti-parallel 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)

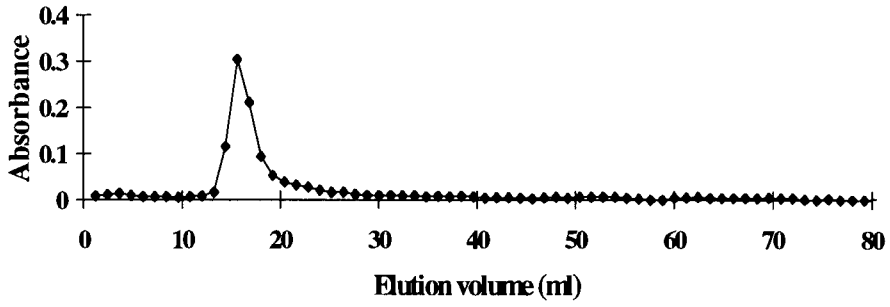
CD measurements of cellulases in the presence of polystyrene particles were attempted as well. While other studies have reported protein structural changes upon adsorption to polystyrene (Kondo et al., 1992), we were unable to measure changes in ellipticity, apparently due to the high light absorbance of the polystyrene particles. In order to unambiguously interpret the data of Figure A, 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 (Billsten et al., 1995). If cellulase does not adsorb, two peaks should elute from the column, while 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 Figure B and C for E_5 and CBHI, respectively. These figures show that individual silica and cellulase peaks are preserved upon mixing, for each 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 upon 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 D shows the elution profile of polystyrene microspheres, alone and in the presence of each cellulase. The small peak eluted between 50 and 60 ml (Figure Da) 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 due to light absorbance by the polystyrene. This made it difficult to clearly verify the absence of a peak corresponding to free cellulase. So while the data of Figure D 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



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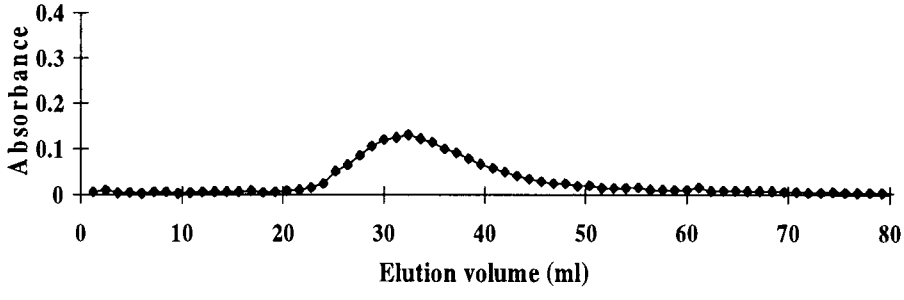

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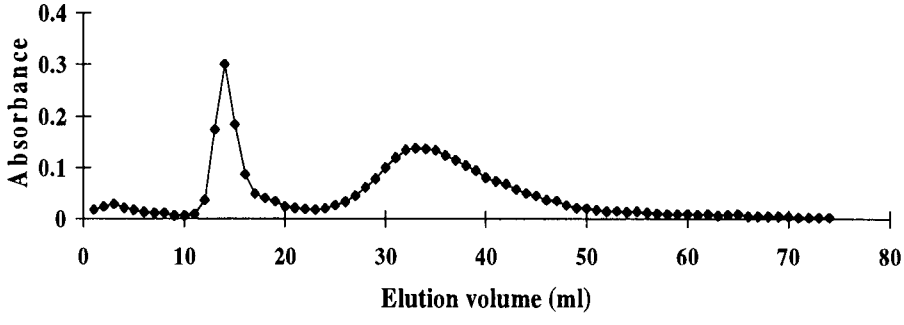


(c)

Figure B. 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.

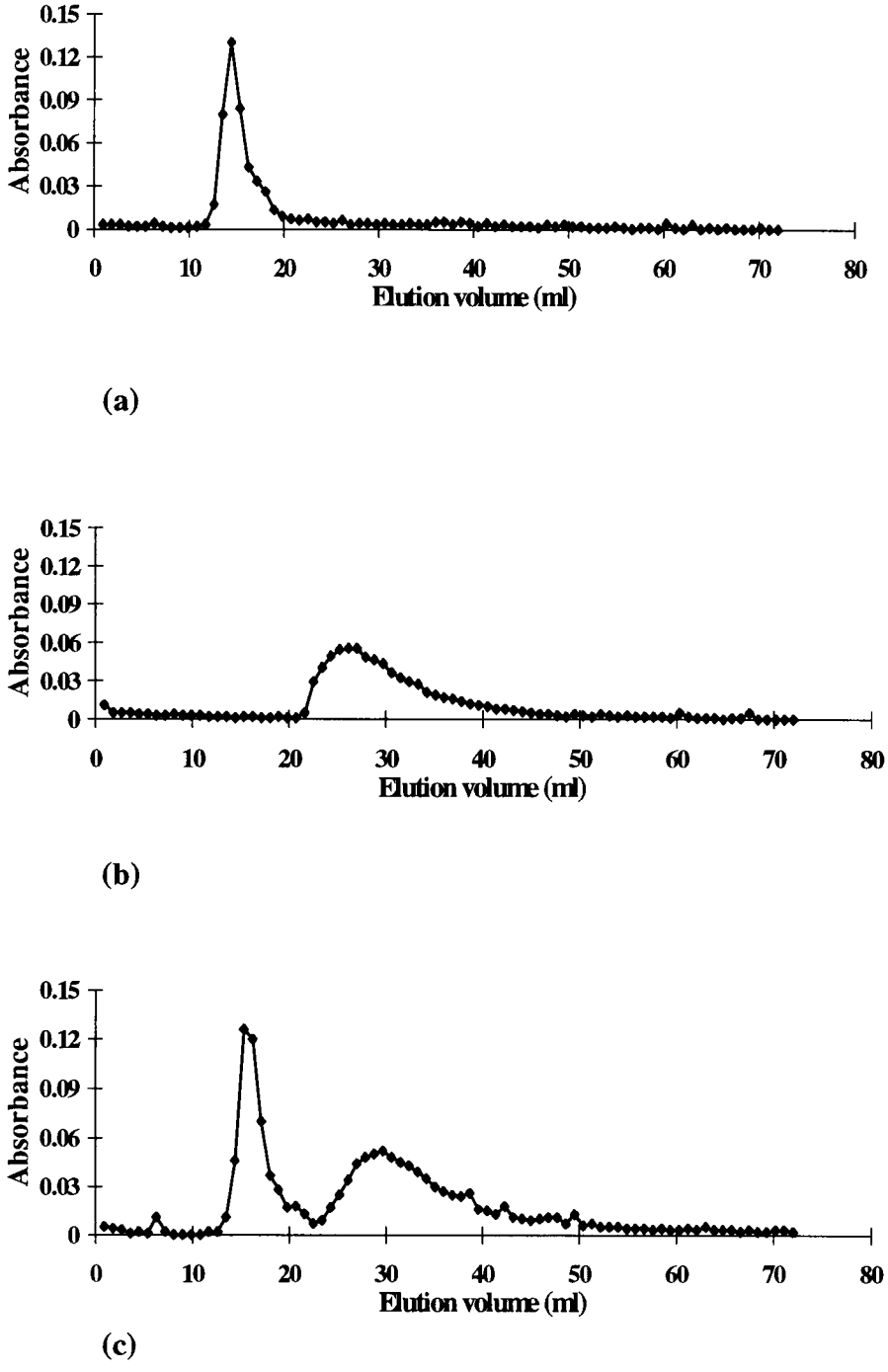
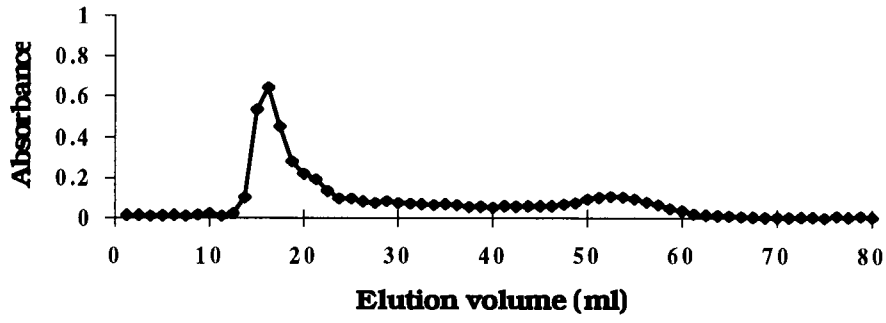


Figure C. Size exclusion chromatography elution profiles of: (a) 20 nm diameter silica particles; (b) CBHI; and (c) CBHI in suspension with silica. Absorbance was measured at a wavelength of 280 nm.



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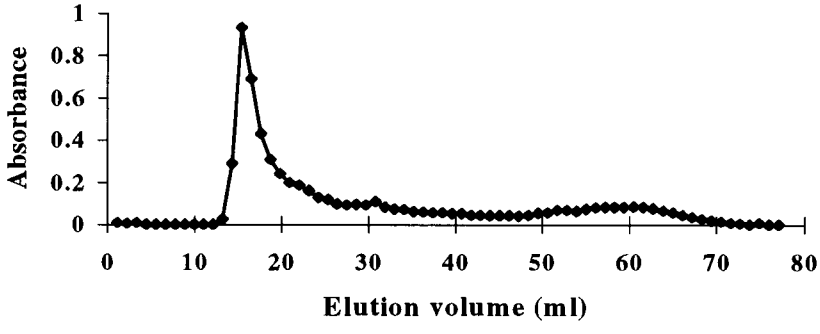

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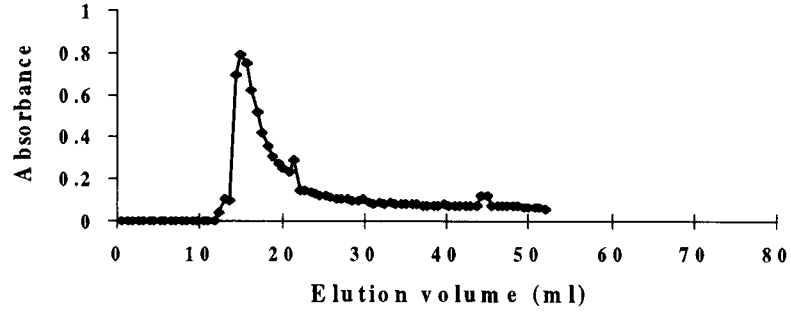


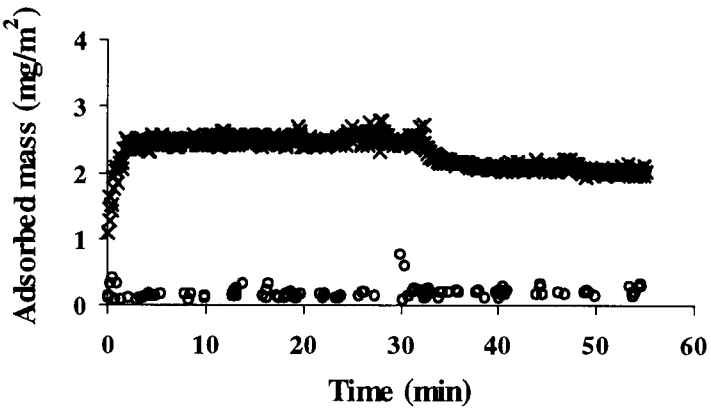
(c)

Figure D. 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.

suspension. When cellulase-particle mixtures where 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 particular, membranes were used to separate any free cellulase that may be present in a cellulase-polystyrene suspension. When cellulase-particle mixtures where 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 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 E shows the adsorption kinetics 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. The kinetic plots show that each cellulase rapidly achieves a plateau in adsorbed mass on the hydrophobic surface, with this layer being only partially removable by rinsing. 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 non-peptide molecules, proteins undergo structural change upon adsorption (Billsten et al., 1995, Fröberg et al., 1998, Kondo et al., 1992, Norde and Favier, 1992, Tian et al., 1998). These structural changes contribute to the adsorption free energy by increasing the



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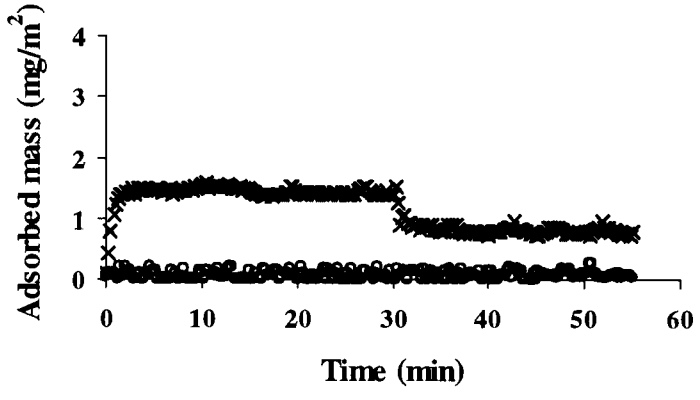

(b)

Figure E. Adsorption kinetics of: (a) E_5 (0.10 mg/ml) and (b) CBHI (0.13 mg/ml) on hydrophilic (o) and hydrophobic (×) silica surfaces.

entropy of the polypeptide chain, and thus contribute to the adsorption driving force (Haynes and Norde, 1995, Norde, 1986).The fact that a protein and surface may both be negatively charged, for example, 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, where 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. These results indicate that if hydrophobic interactions are dominant, the irreversibility of the process would need to be accounted for in construction of any model proposed to describe cellulase adsorption (Suvajittanont et al., 1999).

ACKNOWLEDGMENT

We are indebted to Professor David Wilson and co-workers at Cornell University for providing the bacterial strain in addition to the technical support needed for successful E_5 production. Financial assistance from the STAR Fellowship Program, Environmental Protection Agency, is gratefully acknowledged.

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