

AN ABSTRACT OF THE THESIS OF

Carolyn S. Baker for the degree of Master of Science in Bioresource Engineering presented on October 6, 1998. Title: Adsorption of *Trichoderma reesei* CBHI and *Thermomonospora fusca* E₅ Cellulases on Model Solid Surfaces.

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Michelle K. Bothwell

In this research, the interfacial behavior of *Trichoderma reesei* CBHI and *Thermomonospora fusca* E₅ cellulases were studied at synthetic surfaces. For this purpose, colloidal silica and polystyrene particles were used to prepare cellulase-particle suspensions that were analyzed by several solution-phase techniques. These included circular dichroism spectroscopy, size exclusion chromatography and filtration, and a spectrophotometric assay for cellulase activity. All techniques were performed in the presence and absence of particles. Circular dichroism spectroscopy (CD) and size exclusion chromatography showed, however, that binding did not occur between either cellulase and silica, presumably because silica is hydrophilic and negatively charged. Binding did occur between each cellulase and polystyrene, most likely mediated through hydrophobic associations. Cellulase-polystyrene complexes were not analyzed using CD because of high light absorption by the polystyrene nanoparticles. Upon adsorption to polystyrene, the activity of the E₅ dropped about 95% relative to that of the free enzyme. While this substantial loss in activity may have been the result of binding being mediated through the catalytic domain, strong evidence supporting the thought that adsorption occurs through hydrophobic associations, mediated through the binding domain, suggests that structural or steric factors were partly responsible for the loss.

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Adsorption of *Trichoderma reesei* CBHI and
Thermomonospora fusca E₅ Cellulases on Model Solid Surfaces

by

Carolyn S. Baker

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APPROVED:

Redacted for Privacy

Major Professor, representing Bioresource Engineering

Redacted for Privacy

Chair of Department of Bioresource Engineering

Redacted for Privacy

Dean of Graduate School

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Carolyn S. Baker, Author

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Dedication

This thesis is dedicated to the loving memory of my NaNa.

Adsorption of *Trichoderma reesei* CBHI and *Thermomonospora fusca* E₅ Cellulases on Model Solid Surfaces

Chapter 1: Introduction

Fundamental studies on cellulases are driven by the potential use of abundant lignocellulosic biomass as a renewable resource. Cellulosic biomass can be converted into fuels and other value-added products by biological processes. The bioconversion of lignocellulosic materials can be divided into three general phases: 1) biomass pretreatment; 2) cellulose saccharification; and 3) fermentation of the resulting sugar streams. This study focuses on phenomena relevant to the cellulose saccharification phase that involves the degradation of cellulose chains into glucose residues by a system of cellulases and β -glucosidase. Due to the heterogeneous nature of the reaction, cellulase adsorption on the cellulose surface is extremely important. In fact, the adsorption of cellulases on the cellulose surface is often viewed as the controlling step (Beldman et al. 1987, Stahlberg et al. 1991, Nidetzky and Steiner 1993, Kurakake et al. 1995). Understanding the complex interactions among various cellulases and the cellulose adsorption surface will aid in optimizing the overall conversion process.

In this work we investigated cellulase interactions with model solid surfaces, using *Trichoderma reesei* CBHI and *Thermomonospora fusca* E₅ cellulases. Model surfaces have been used extensively for decades to study protein adsorption (Billsten et al. 1995, Andarade et al. 1996, Andrade 1985, Elwing et al. 1988). We used hydrophilic silica, as well as hydrophobic polystyrene, in order to study binding events in the absence

of catalytic events. The use of such model surfaces would allow less ambiguous analysis of hydrophobic effects in cellulase adsorption.

Considerable effort was devoted to determining cellulase secondary structure in the presence and absence of hydrophobic and hydrophilic nanoparticles. In addition, CMCase assays were used to compare the activity of cellulase bound to polystyrene microspheres to the activity of free cellulase. Gel filtration and microporous membrane separations were used to determine the extent to which these cellulases adsorbed to the model surfaces used in this study.

Chapter 2: Literature Review

2.1 Cellulose

Cellulose is an abundant carbohydrate synthesized by plants to provide mechanical strength and by some microorganisms to provide a support matrix on which to grow. Unlike many carbohydrates, cellulose is not a storage polymer, but a polymer of β -1,4 linked glucose units that has a complex physical structure, making it difficult to degrade (Tomme et al. 1995). The chains are linked by intra- and inter-molecular hydrogen bonds to form rigid microfibrils. Multiple types of bonds and varying chain lengths create heterogeneity in cellulose structure (Tomme et al. 1995). This heterogeneity complicates investigation of the enzymatic degradation process.

2.2 Cellulases

The breakdown of cellulose requires several enzymes: endoglucanases, exoglucanases (cellobiohydrolases), and β -glucosidases (Gilkes et al. 1991). Endoglucanases are cellulases that cleave interior bonds of cellulose chains. Exoglucanases are cellulases that cleave cellobiose units from the ends of cellulose chains. Both bacteria and fungi produce cellulase systems capable of degrading cellulose.

Like other enzymes involved in insoluble polymeric carbohydrate hydrolysis, cellulases from aerobic microorganisms have a discrete functional domain structure (Gilkes et al. 1991). Most have two functional domains: a catalytic domain or core

protein that contains the active site, and a cellulose binding domain (CBD) that serves to anchor the protein to the substrate matrix. Some studies have attributed a destabilizing function to some CBDs, suggesting that the CBD may "peel" cellulose chains from the top layer of crystalline microfibrils (Reinikainen et al. 1993, Knowles et al. 1988, and Din et al. 1991). A glycosylated linker region serves as a flexible connection between the two domains.

Cellulases are catalogued into families by the amino acid sequence and hydrophobic cluster analysis (HCA) of the catalytic and cellulose binding domains (Gilkes et al. 1991). A family was originally defined as a group of proteins with the same polypeptide-folding pattern. Some proteins may not fit in their assigned families because HCA may not detect all pattern similarities if the amino acid sequences of two cellulases are very different (Béguin and Aubert 1994). It is interesting to note that cellulases in the same family, and thus of similar amino acid sequence, may have different functionalities and specificities (Gilkes et al. 1991). For example, the family membership of a catalytic domain does not disclose whether a cellulase is an endoglucanase or exoglucanase. Further, cellulases with catalytic domains in different families can have CBDs from the same family (Gilkes et al. 1991). One organism can produce enzymes from several catalytic families, but only one kind of CBD per organism has been found so far (Gilkes et al. 1991).

The specificity of the catalytic domain of a cellulase can vary in three ways, according to: 1) mode of action (endo- or exo-glucanase), 2) substrate morphology (amorphous or crystalline cellulose), and 3) substrate chain length (Béguin and Aubert 1994). Because cellulose structure is at once heterogeneous and dynamic, a diverse

system of cellulases with different specificities is needed. In particular, the substrate structure changes during hydrolysis. Cellulase catalytic domains are grouped into 12 families (Tomme et al. 1995). Each family contains enzymes from a wide range of organisms from different kingdoms. The relatedness of catalytic domains of cellulases does not imply the relatedness of the organism that produces them.

2.3 Cellulose Binding Domains and the Importance of Binding in Cellulose Saccharification

CBDs are categorized into families, according to their structural homology. Cellulases with family I and II CBDs were used in this study. Family I includes the CBDs of fungal cellulases only. NMR reveals three aligned aromatic amino acid residues are present on the flat face of family I CBDs (Kraulis et al. 1989, Xu et al. 1995). Family I CBDs only bind to crystalline cellulose (Mattinen et al. 1997), and are about 30 amino acid residues in size (Tomme et al. 1995). Specificity of the CBD from this family has been attributed to the idea that the flexible oligosaccharide chains of amorphous cellulose do not provide a rigid enough surface for binding (Mattinen et al. 1997).

Family II includes the CBDs of bacterial enzymes: cellulases, xylanases, arabinofuranosidases, and chitanases (Béguin and Aubert 1994). The CBDs of this Family are 100 residues in size, and four tryptophans are highly conserved (Béguin and Aubert 1994). Family II CBDs bind both crystalline and amorphous cellulose (Xu et al. 1995, Din et al. 1995). Three aligned aromatic amino acid residues are present on family II CBDs (Kraulis et al. 1989, Xu et al. 1995). These aromatic residues have been shown

to play a key role in cellulase binding to cellulose (Reinikainen 1992, Linder et al. 1995, Din et al. 1994).

CBHI, the fungal exoglucanase used in this study, has a family I CBD. NMR spectroscopy of the C-terminal CBD of CBHI, a 36 residue polypeptide, was used to determine the 3-D structure of the binding domain in solution (Kraulis et al. 1989). The 3-D structure showed that the CBD has a wedge like shape with a hydrophilic face and a hydrophobic face (Kraulis et al. 1989). Both faces present a flat surface, the hydrophobic face containing a slight indentation in the middle (Kraulis et al. 1989). The overall dimensions of the CBD are $30 \times 18 \times 10 \text{ \AA}$ (Kraulis et al. 1989).

The CBD of the bacterial endoglucanase E_5 is a family II CBD (Lao et al. 1991). E_5 hydrolyzes amorphous and crystalline cellulose. A study of the DNA sequence of E_5 shows that the N-terminal region shares homologous sequences with family II CBDs: 40% homology with the endoglucanase A (CenA) and 32% with the exoglucanase (Cex) of *C. fimi* (Lao et al. 1991). Although the structure and binding of E_5 CBD have not been studied, the structure and binding of *C. fimi* exoglucanase CBD have been studied (Xu et al. 1995, Creagh et al. 1996). The CBD of Cex is about $45 \times 25 \times 25 \text{ \AA}$ in size, and contains a cone shaped cavity (Xu et al. 1995). Although it does not have two distinct hydrophilic and hydrophobic faces like the CBD of *T. reesei* CBHI, it does contain a hydrophobic core.

Although different CBD structures bind different types of cellulose, hydrophobic interactions between aromatic residues and cellulose are important, sometimes the primary driving force in the binding of many CBDs (Creagh et al. 1996, Linder et al. 1995, Reinikainen et al. 1992). Previous studies suggest hydrophobic interactions are

significant in the binding of the CBDs of proteins used in this study (Creagh et al. 1996, Din et al. 1994, Reinikainen et al. 1995).

2.4 Protein Adsorption

Protein adsorption occurs at virtually any natural or synthetic surface in contact with a protein containing fluid. Protein adsorption is important in many areas relevant to food, pharmaceutical and biomedical technology. In cellulase-cellulose systems protein adsorption is important because the cellulose surface not only provides an interface for binding, but is the substrate as well. The presence of multiple enzymes and the heterogeneous and transient nature of cellulose structure complicate this system.

Proteins often change conformation and/or orientation during or after adsorption (Lundström 1985). Sometimes proteins undergo surface induced conformational changes as a protein relaxes into a more energetically favorable conformation. As the number of contact points change, protein-binding strength may change over time (Lundström and Elwing 1990). In addition to undergoing conformational change, a protein may reorient to allow more hydrophobic regions of the protein to interact with the interface. These structural and orientation changes may occur spontaneously or over a period of time.

Protein molecular conformations, the nature of the surface, the nature of the solution, and the presence of other proteins in solution are important factors affecting protein behavior at an interface. A hydrophobic interface is generally associated with a stronger influence on protein binding than a hydrophilic interface (Lundström 1985). In general for negatively charged proteins, less protein adsorbs to hydrophilic than to

hydrophobic silica surfaces (Lundström 1985) and the higher the degree of surface hydrophobicity, the greater the protein adsorption (Krisdhasima et al 1992).

When adsorption isotherms and kinetics are examined, it is evident that simple Langmuir type models cannot be used to describe protein adsorption. Adsorption isotherms often show a plateau at low protein concentrations, then a slight increase over a large range of protein concentrations (Lundström 1985). In measurement of protein adsorption isotherms, adsorbed mass is dependent on how the protein is added to solution. When protein is added in increments, less adsorbs than if all the protein is added at once (Lundström 1985). Proteins often undergo conformation or orientation changes that can alter binding area. When all the protein is added at once there is little time for conformational changes before the surface is covered, thereby allowing more protein to adsorb. For this reason a dynamic multiple state model is used to account for multiple states (Tian et al. 1998).

Past theoretical and experimental work with enzymes at interfaces, including cellulases at interfaces, supports the hypothesis that a given protein can adsorb in different structural states. In general, these states are perhaps best characterized by exhibiting different binding strengths (Brash and Horbett 1995, Andrade et al. 1996, Horbett and Brash 1987) and therefore, different functionality (Bower et al. 1998) or conformational state (Tian et al. 1998) at the interface. In summary, hydrophobicity and structural changes are very important in protein adsorption.

2.5 Evidence of Importance of Hydrophobicity in Cellulase Binding

Carbohydrate-protein interactions often involve aromatic residues on the binding face of the protein (Engle 1994, Vyas 1991, Spurlino et al. 1992). 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). Aromatic residues have also been shown to play an important role in cellulase binding (Linder et al. 1995, Reinikainen et al. 1992).

Site-directed mutagenesis studies have shown that aromatic residues present on the hydrophobic binding face of CBHI and endoglucanase I cellulases of *T. reesei* are important in binding (Linder et al. 1995, Reinikainen et al. 1992). These studies suggest that an intact hydrophobic surface is required for efficient binding. Further, CBHI adsorption is affected by high ionic strength, again suggesting the importance of hydrophobic interactions (Reinikainen et al. 1995).

It has been shown that similar variables determine the chromatographic behavior of proteins and the intermolecular interactions between proteins (Regnier 1987). A study of the chromatographic behavior of several endo-cellulases suggested that hydrophobic interactions are the principal force between endocellulases and polysaccharide ligands (Golovchenko 1992).

Many hydrophobic residues are present on the binding face of *C. fimi* Cex. Isothermal titration microcalorimetry studies indicated that dehydration (hydrophobic) effects are the primary driving force for *C. 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 1996). In summary, there is an abundance of evidence suggesting that hydrophobic interactions play a significant role in binding of family I and II CBDs.

2.6 Model Surfaces

Most cellulase adsorption studies used cellulose as the binding surface. The use of cellulose complicates matters for many reasons. First, the nature of the surface changes with time because the binding surface is also the substrate. Second, cellulose is porous and can act as a sieve, limiting cellulase access to surfaces (Bothwell 1997, Tanaka et al. 1988, Grethlein 1985). Third, kinetic and equilibrium constants determined using cellulose are affected by diffusion and convection phenomena. The use of nonporous model surfaces allows the circumvention of some of these limitations.

Model surfaces are not meant to mimic the lignocellulosic surface, but to simplify the system. Once the influence of a selected factor on adsorption is understood at model surfaces, the approach can be extended to include more relevant surfaces. Understanding fundamentals of adsorption contributes to the starting point for modeling more practically relevant systems. For example, model surfaces have been used extensively to study the role of adsorption in the performance of blood-contacting implants. Both model interfaces and simplified simulants of blood have been instrumental in establishing our current understanding of blood-material compatibility (Brash and Horbett 1995, Andrade 1996, Horbett and Brash 1987, Andrade 1985). Studying non-specific adsorption is also important because cellulases adsorb to lignin during degradation of lignocellulosic

material (Ooshima et al. 1990, Converse et al. 1990, Chernaglazov et al. 1988). This non-specific adsorption has the net effect of "inactivating" some cellulases and reducing activity.

Chapter 3: Materials and Methods

3.1 Production and Purification of *Thermomonospora fusca* E₅ and *Trichoderma reesei* CBHI

Thermomonospora fusca E₅ cellulase was produced by transformed *Streptomyces lividans* TK24 carrying a plasmid (pGG74) bearing the E₅ gene (Ghangas and Wilson 1987). The production and purification procedures were, in part, as described in Irwin et al. (1993) and Bothwell et al. (1997). In short, the culture was initiated from frozen stock into 15 ml of tryptic soy broth medium containing 5 mg/ml of thiostrepton and incubated at 30 ° C for 48 h. The culture was then subcultured into 150 ml of the same medium and incubated for 24 h. This culture was used to inoculate an autoclaved 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 harvested with centrifugation (Beckman J2-MI, Seattle, WA) and filtration (Millipore Pellicon Filter system with a 0.22 micron cassette). The filtered supernatant was prepared with 1.2 M (NH₄)₂SO₄ and 0.1mM phenylmethylsulfonyl fluoride. This solution was loaded directly 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 E₅ 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. E₅ fractions were combined, then ultrafiltered and stored at -80°C.

Trichoderma reesei cellobiohydrolase I (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, DEAE-Sepharose CL-6B was used to separate CBHI from other cellulases (Beldman et al. 1985). The CBHI fractions were then passed through a *p*-amino-phenyl 1-thio-β-D-cellobioside affinity column with 0.1 M NaOAc, pH 5, containing 1 mM D-glucono-δ-lactone as a 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 Phenyl Sepharose CL-4B column. The loading buffer was 25 mM NaOAc, pH 5, containing 0.85 M (NH₄)₂SO₄. The column was washed with 5 column volumes of the buffer, and then eluted with a linear gradient of 0.85-0.35 M (NH₄)₂SO₄ in buffer. The CBHI fractions were combined and the buffer was exchanged to 50 mM NaOAc, pH 5 buffer by ultrafiltration with a PM 10 membrane. The concentrated cellulase was then stored at -80°C.

3.2 Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter with a xenon lamp. Data were recorded online with a Gateway PC. CD spectra were recorded from 400 nm to 184 nm wavelengths using a quartz cuvette of pathlength 0.2 mm. In each case, the absorbance spectrum was scanned from 400 nm to

184 nm on a Cary 15 scanning spectrophotometer to check for noise before recording CD spectra. The cuvette was rinsed with water and ethanol, and dried between measurements. Spectra presented are an average of three scans at 10 nm/min. A 2 s response time was used for optimal signal to noise ratio. Ellipticity readings were made every 1 nm. Sample concentrations were 0.2 mg/ml CBHI and 0.3 mg/ml E₅. The α -helix content was estimated with the ellipticity at 222 nm using the following formula (Kondo et al. 1992).

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

Secondary structural composition of the proteins was predicted using the VARSLC program (Johnson 1988). This program combines singular value decomposition, a variation of least squares, with the statistical method of "variable selection" to estimate the secondary structure of a protein based on its CD spectrum.

3.3 Size Exclusion Chromatography

Size exclusion studies were carried out in 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 0.5 mg/ml CBHI and 1.0 mg/ml E₅. Colloidal silica particles, 20 nm diameter (EKA Chemicals Inc, Marietta, GA), 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 20 ° C. A volume of 1 ml of this suspension was then passed through a

Sephadex G-100 column (Pharmacia Biotech. Inc, New Jersey) at a flow rate of about 400 $\mu\text{l}/\text{min}$. The absorbance of each 0.8-1.0 ml fraction was recorded manually using a Spectronic 601 (Milton Roy) spectrophotometer at 280 nm. The same procedure was performed with cellulase in the absence of particles as well. All experiments were performed at least twice.

3.4 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 20 $^{\circ}\text{C}$ on a shaker at 100 rpm. Cellulase concentrations used were 0.312 mg/ml CBHI and 0.229 mg/ml E₅. The surface area available for adsorption was $8.0 \times 10^{-4} \mu\text{m}^2/\text{cellulase}$. After incubation, samples were placed in Ultrafree CL 0.1 μm Durapore membrane filter tubes (Millipore Corporation, Bedford, MA). Particle-free samples were centrifuged at 7,000 rpm at 4 $^{\circ}\text{C}$ for 20 minutes and cellulase-particle suspensions were centrifuged for 80 minutes. The absorbance of the filtrate at 280 nm was recorded. Particle-only filtrate was used as the control.

3.5 Cellulase activity assay

CMCase assays were started by adding NaOAc buffer (pH 5.5), cellulase and CMC sequentially to microcentrifuge tubes. The final concentrations in the reaction

volumes were between 1.875 and 300.75 mM cellulase and 1% CMC. Reaction mixtures were incubated at 50 °C for 15 min in a circulating water bath (Blue M, Asheville, NC).

The amount of reducing sugar produced was measured by using a modified dinitrosalicylic acid (DNS) method (Irwin 1993). Cellulase activities were calculated using a DNS standard curve for glucose and a conversion for cellobiose production to glucose production (Irwin 1993). The CMC target level of digestion was 6.6% (Irwin 1993). Assays were carried out for a fixed time, at enzyme concentrations above and below the target level for digestion. The amount of cellulase required to reach target digestion was found by plotting cellobiose produced versus protein used.

CMCase assays were also carried out to determine the activities of adsorbed cellulase. Hydrophobic, surfactant-free, 53 nm diameter polystyrene particles (Duke Scientific Corporation, Palo Alto, CA) were mixed with E₅ cellulase. A 2:1 particle:protein ratio was used, in a total volume of 200 µl. Cellulase and substrate concentrations were the same as above. In these suspensions the surface area available for adsorption was 0.0157 µm²/cellulase. Cellulases were allowed to adsorb to the hydrophobic particles for 90 minutes at 25 °C and 125 rpm. The cellulase-particle mixtures were used in place of the cellulase in the CMC assays. All remaining procedures were the same as described above. These experiments were replicated at least three times.

Chapter 4: Results and Discussion

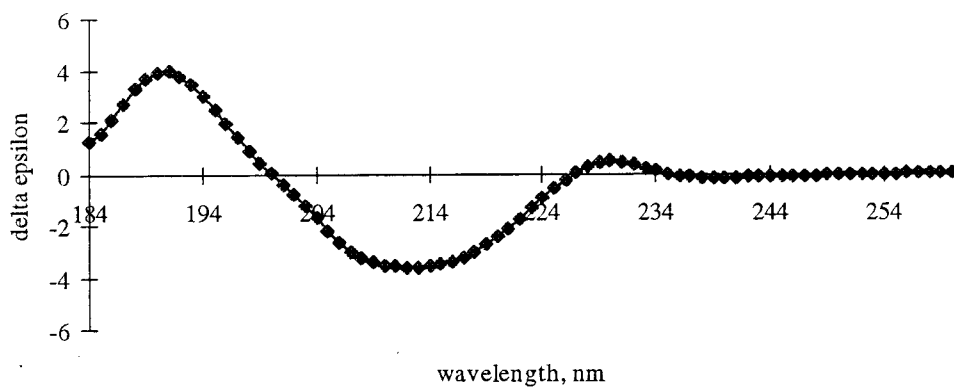
4.1 Circular Dichroism Spectroscopy

Circular dichroism spectra can be analyzed to get information about the secondary structure of protein and peptides in solution. CD spectra of CBHI and E₅ are shown in Figure 1. These spectra were used to determine the secondary structure of both cellulases using the VARSLC program (Table 1). Both enzymes have a high β -sheet content.

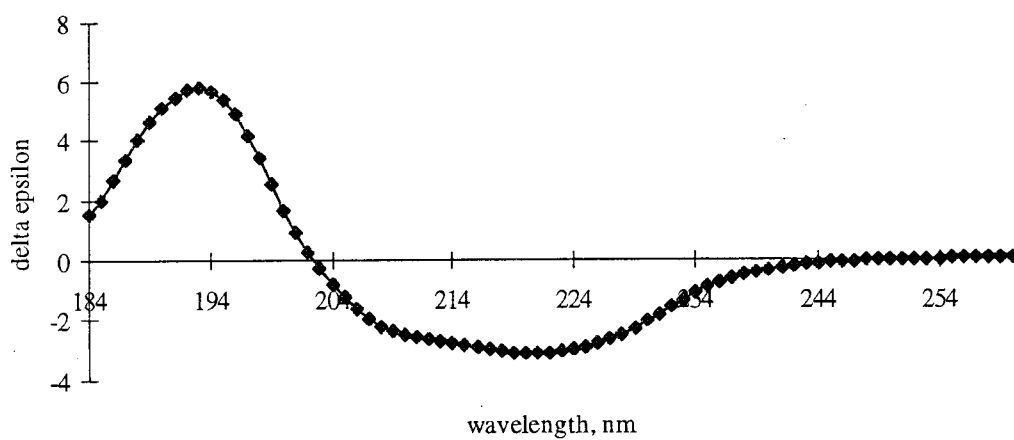
Table 1. Secondary structural composition (%) of cellulases from CD.

Cellulase	α -helix	Parallel β -sheet	Anti-parallel β -sheet	β -turn	Other
<i>T. reesei</i> CBHI	11	34	14	10	32
st.dev.	2	3	1	1	2
<i>T. fusca</i> E ₅	26	19	9	15	30
st.dev.	1	1	1	1	1

Cellulase structural changes upon adsorption were studied using colloidal (hydrophilic) silica. The CD spectrum of E₅ was virtually unchanged in the presence of silica (Figure 2). This indicated that either no conformational changes took place upon adsorption or that adsorption did not occur. In contrast, the CD spectrum of CBHI was changed in the presence of silica (Figure 2). This indicated that either CBHI was undergoing conformational changes upon adsorption to the silica or undergoing self association, or grouping, due to high concentration of hydrophilic silica particles in solution.

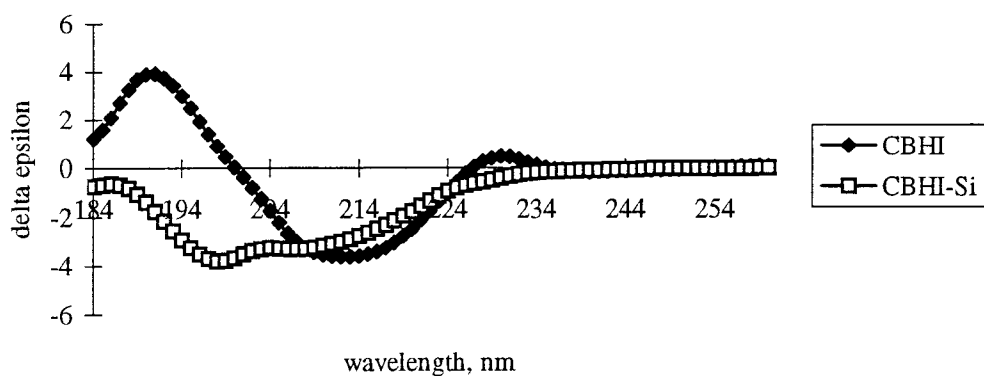


(a)

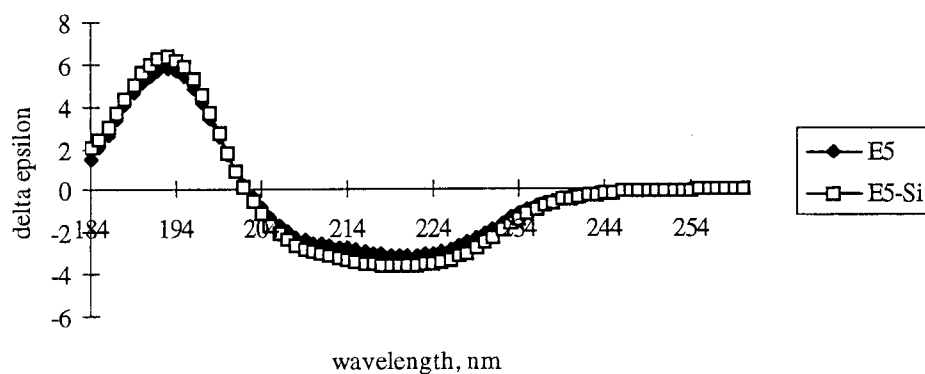


(b)

Figure 1. Circular dichroism spectra of CBHI(a) and E₅ (b).



(a)



(b)

Figure 2. Circular dichroism spectrum of cellulase and cellulase in presence of silica particles.

CD measurements of cellulases in the presence of hydrophobic polystyrene particles were attempted as well. While other studies have successfully measured protein structural changes upon adsorption to polystyrene particles using CD (Kondo et al.1992,

Tian et al. 1998), we were unable to measure CD due to the high light absorbance of the polystyrene particles. Effort was therefore directed to verifying whether binding even occurred to silica and polystyrene.

4.2 Size Exclusion Chromatography

Size exclusion, or gel filtration, chromatography has been used to determine protein-particle binding by Billsten et al. (1995). Size exclusion studies separate particles or molecules according to size: the larger particles leaving the column first and the smaller later. This method can be a simple way to determine whether or not two molecules bind. If the cellulases do not adsorb to the particles two peaks should elute from the column. The elution profile should show only one peak if the cellulase binds to the particle. Elution profiles of silica particles, CBHI and CBHI in the presence of silica particles are shown in Figure 3. Figure 3a shows that the silica particles began to exit the column at a volume of 13 ml. Figure 3b shows that CBHI began to elute at a volume of 23 ml. The elution profile of the particle-CBHI suspension (Figure 3c) clearly shows two peaks corresponding to those observed for particles and cellulase alone. Similar results were obtained with E₅ as shown in Figure 4. These size exclusion chromatographs clearly demonstrated that neither CBHI nor E₅ adsorbed to colloidal silica.

We initially assumed that CBHI and E₅ would adsorb to silica because many proteins adsorb to hydrophilic silica even though they carry a net negative charge. The observation that cellulases do not bind to hydrophilic silica suggests that hydrophobic association is a very important mechanism in adsorption. There is increasing evidence

that hydrophobic interaction between CBDs and the cellulose surface do indeed play a major role in CBD binding (Golovchenko et al. 1992, Engle et al. 1994, Din et al. 1994).

Gel filtration experiments carried out with hydrophobic polystyrene particles yielded different results. Figure 5 shows the elution profile of hydrophobic polystyrene microspheres. The polystyrene began to elute at about 13 ml volume. The small peak at an initial rise of 48 ml represents trace surfactants used by the manufacturer to maintain polystyrene microspheres in suspension.

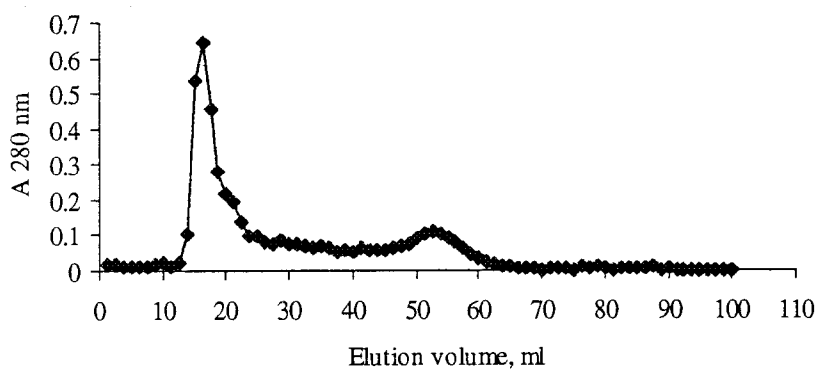
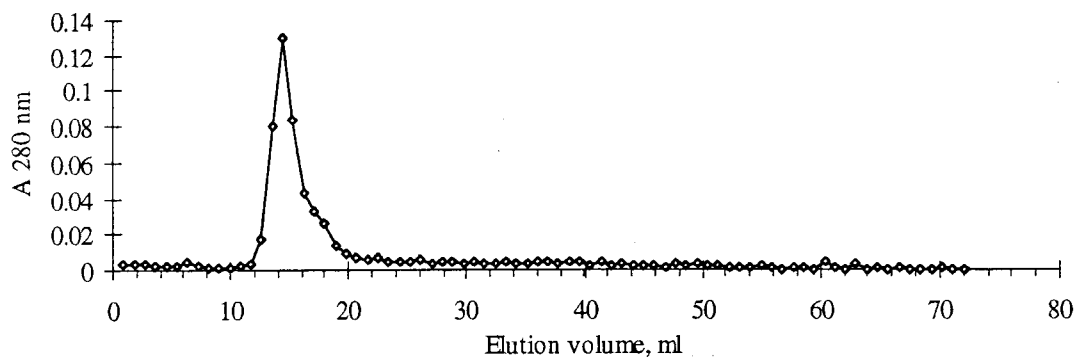
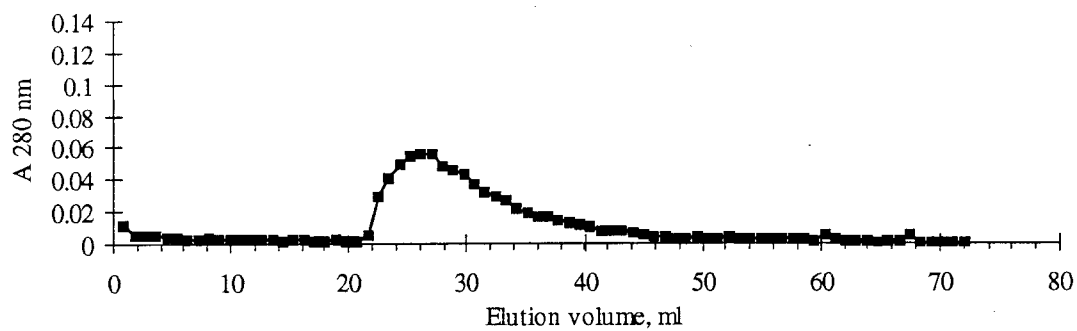


Figure 5. Size exclusion chromatography elution profile of polystyrene microspheres.

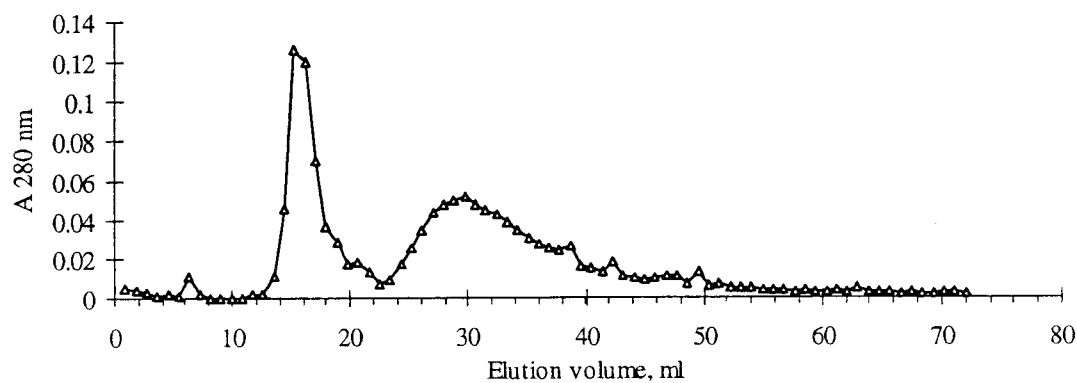
Figures 6 and 7 show the elution profiles of the CBHI-polystyrene and E₅-polystyrene suspensions. Only one large peak was eluted and no peak corresponding to the protein was detected. We would expect the protein to have started to elute at a volume of about 23 ml if it did not adsorb. These results suggest that the CBHI and E₅ do adsorb to the hydrophobic particles.



(a)

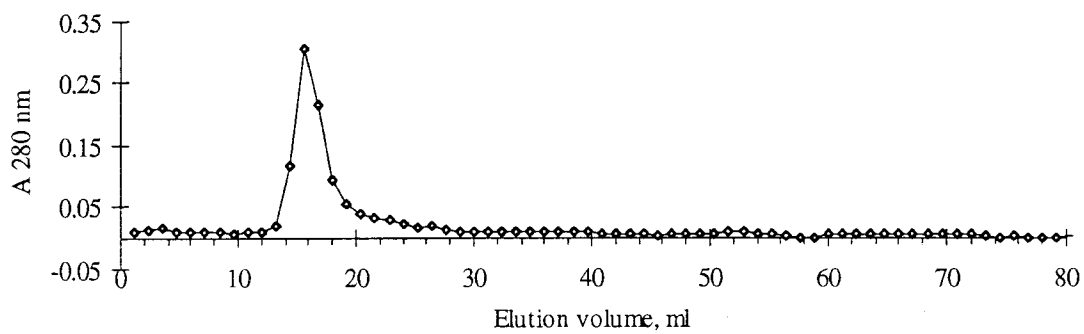


(b)

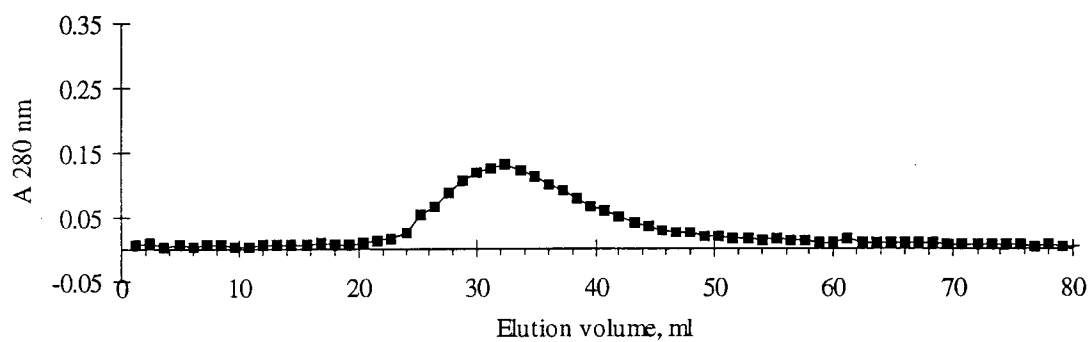


(c)

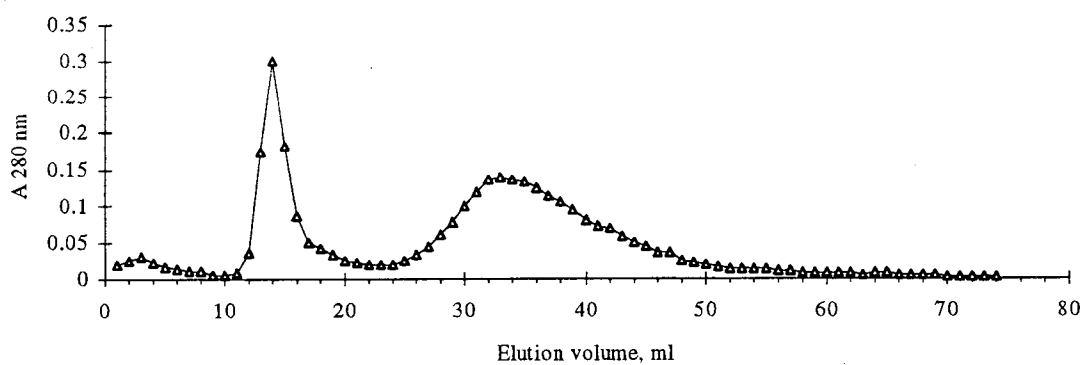
Figure 3. Size exclusion chromatography elution profiles of (a) 20 nm diameter silica particles, (b) 0.5 mg CBHI, and (c) CBHI in suspension with silica.



(a)



(b)



(c)

Figure 4. Size exclusion chromatography elution profiles of (a) 20 nm diameter silica particles, (b) 1.0 mg E₅, and (c) E₅ in suspension with silica.

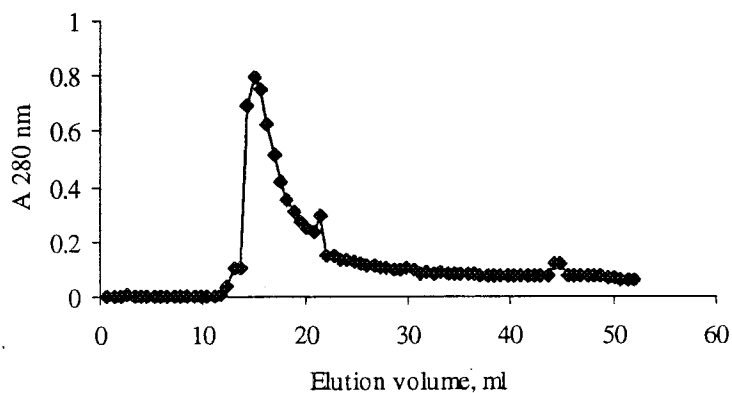


Figure 6. Size exclusion chromatography elution profile of CBHI in suspension with polystyrene microspheres.

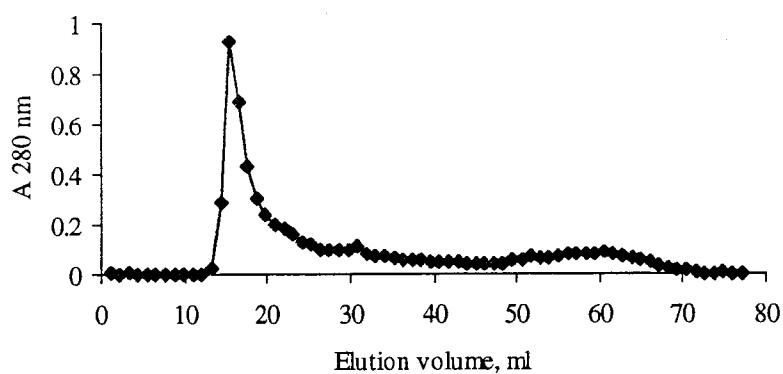


Figure 7. Size exclusion chromatography elution profile of E_5 in suspension with polystyrene microspheres.

The elution profiles display a long tail region, presumably due to light absorbance by the polystyrene. The much greater light absorption of the polystyrene microspheres relative to the cellulases made it difficult to clearly verify the absence of a peak corresponding to free cellulase. Although the elution profiles suggest that CBHI and E_5 adsorb to polystyrene, adsorption could not be unambiguously verified and another method was employed.

4.3 Microporous Membrane Separations

Microporous membranes were used to separate cellulase bound to 0.21 μm polystyrene particles from any unbound cellulase that may have been present in the suspension. The membrane pores were large enough to allow free cellulase molecules to pass through, but small enough to retain the particles and any cellulase-particle complexes. Figure 8 is a schematic of the membrane system. Two possible outcomes of the membrane separation are shown: i) the cellulase adsorbs to the particles, and thus retained by the membrane; or ii) the cellulase does not adsorb to the particles and pass through the membrane with the filtrate. Cellulase adsorption to the particles was detected by a reduction in filtrate concentration relative to the initial cellulase concentration. Results showed a 97.4 % and 99.3% reduction in filtrate CBHI and E₅ concentrations, respectively, in the presence of particles (Table 2).

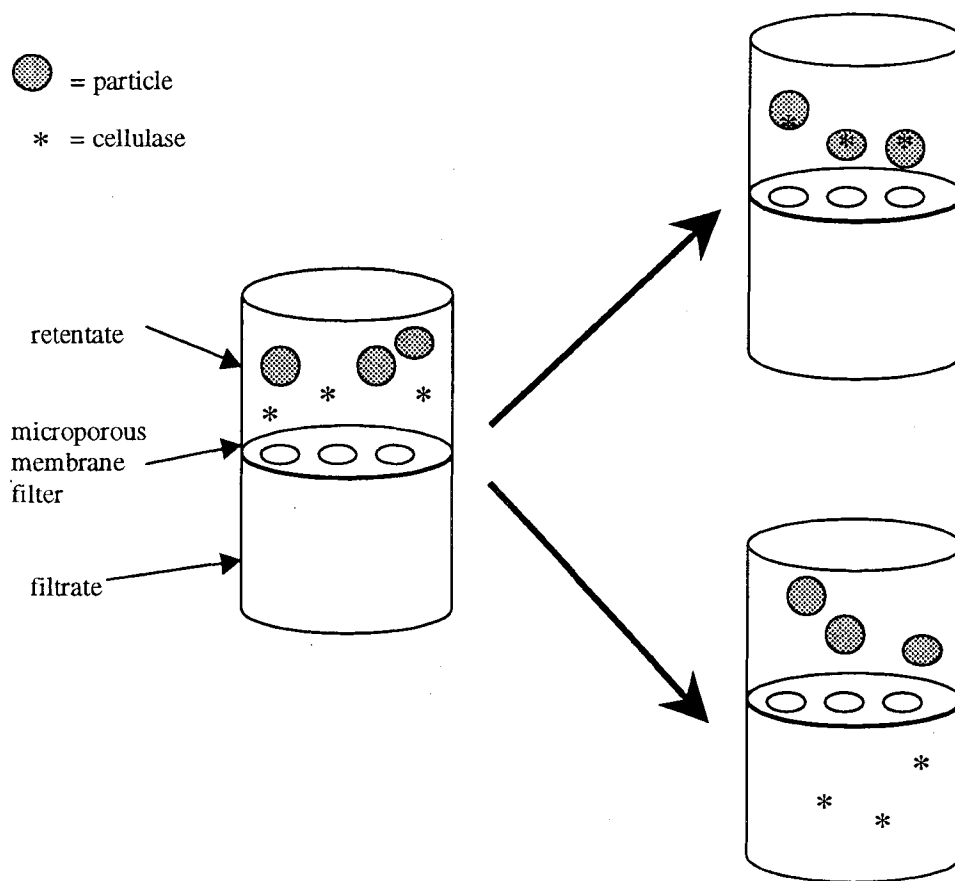


Figure 8. Schematic of microporous membrane filtration system.

Table 2. Cellulase concentration before and after filtration through a 0.1 μm microporous membrane filter.

Solution	before separation [cellulase] mg/ml	after separation [cellulase] mg/ml	after separation SD	% cellulase adsorbed
CBHI	0.317	0.312	0.0049	-
CBHI-Polystyrene	0.317	0.008	0.0037	97.43
E5	0.238	0.229	0.0034	-
E5-Polystyrene	0.238	0.002	0.0008	99.31

Microporous membrane separations demonstrated that both CBHI and E₅ adsorb to hydrophobic polystyrene. Other studies have shown that hydrophobic interactions are a major factor in cellulase adsorption to cellulose and oligosaccharides (Mattinen et al. 1997, Linder et al. 1995). Our results show that surface hydrophobicity is a critical factor for CBHI and E₅ adsorption. Since hydrophobicity is an important factor in cellulase adsorption, model hydrophobic surfaces may be useful for study of fundamental aspects of adsorption. An understanding of cellulase adsorption gained from such simplified studies might eventually be extended to more relevant surfaces. This method was not used to verify gel filtration results obtained with silica because silica microspheres of the appropriate concentration and size were not available.

4.4 Cellulase Activity Assays

The importance of hydrophobic interactions in cellulase binding seen here, along with the lack of any evidence supporting an important role for other types of non covalent binding, would imply that adsorption is mediated through the CBD. However this ought to be more directly verified in order to apply confidently results obtained using model solid surfaces to describing cellulase behavior at cellulosic surfaces. If the binding domain is primarily responsible for cellulase adsorption to the model surface, then the catalytic core should be free to act on substrate that enters the interface. Activity of bound E_5 was investigated because previous studies have shown that the catalytic domain of E_5 , alone, has similar activity on CMC as the native cellulase (Irwin 1993). As the catalytic domain of E_5 maintains full activity on CMC in the absence of its binding domain, E_5 should maintain activity if the CBD mediates binding to polystyrene.

Table 3 shows the activities of E_5 and the E_5 -polystyrene complex. The activity of E_5 adsorbed to polystyrene was almost 20 times less than that of unbound E_5 . While there was considerable variation in the activities of the E_5 -polystyrene complexes (SD=0.0-0.005 μmol cellobiose/min μmol), all specific activity values of the complex were at least 10 times lower than the specific activity of E_5 . These data suggest that E_5 lost most of its activity upon adsorption.

Table 3. Specific activities of E₅ and E₅-polystyrene complex.

Sample	Specific activity (μmol cellobiose/min μmol)
E ₅	3546
E ₅ +Polystyrene*	188.5

*Average specific activity values are reported because target digestion was not achieved.

While the data in Table 3 do not prove that adsorption is mediated by the CBD, the data do not disprove it either. There are many possible explanations for the loss of activity upon adsorption. The presence and size of the polystyrene microspheres may interfere with the ability of the substrate to reach the catalytic site. Another possibility is that CBD structure at the interface may affect linker mobility, inhibiting catalytic activity. Or the orientation of the cellulase bound to the particle may block the active site. It has been shown that conformational changes may greatly reduce activity (Bower et al. 1988).

Chapter 5: Conclusions

These studies clearly showed that CBHI and E₅ do not adsorb to hydrophilic silica but do adsorb to hydrophobic polystyrene. Hydrophobic interactions seem to mediate binding at the interface, whether natural or synthetic. In the future we hope to use hydrophobic model surfaces to study cellulase adsorption events. Adsorption studies at model interfaces have provided information about the molecular origins of binding of many other proteins, such as blood components. We hope that similar studies with cellulases and model surfaces will provide information about the molecular origins of adsorption. Once adsorption behavior is better understood at model surfaces the knowledge can be used to study real systems.

BIBLIOGRAPHY

- Abuja, P. M., Schmuck, M., Pilz, I., Tomme, P., Claeysens, M., and H. Esterbauer (1988) Structural and functional domains of cellobiohydrolase I from *Trichoderma reesei*. *Eur. Biophys. J.* **15**: 339.
- Andrade, J.D., *et al.*, *Proteins at Interfaces: Principles, Problems, and Potential*, in *Interfacial Phenomena and Bioproducts*, J.L. Brash and P.W. Wojciechowski, Editors. 1996, Marcel Dekker, Inc.: New York.
- Andrade, J.D., ed. *Principles of Protein Adsorption*. Surface and Interfacial Aspects of Biomedical Polymers, ed. J.D. Andrade. Vol. 2. 1985, Plenum Press: New York.
- Béguin, P., and Aubert, J. P. (1994) The biological degradation of cellulose. *FEMS Microbiol. Rev.* **13**: 25.
- Beldman, G., Searle-Van Leeuwen, M.F., Romgouts, F.M., and Voragen, F.G.J. (1985) The cellulase of *Trichoderma viride*. Purification, characterization, and comparison of all detectable endoglucanases, exoglucanases, and β -glucosidases. *Eur. J. Biochem.* **146**: 301.
- Beldman, G., Voragen, A.G.J, Rombouts, F.M., Searle-van Leeuwen, M.F., and Pilnik, W. (1987) Adsorption and kinetic behavior of purified endoglucanases and exoglucanases from *Trichoderma viride*. *Biotechnol. and Bioeng.* **30**: 251.
- Billsten, P., Wahgren, M., Arnebrant, T., McGuire, J., and Elwing, H. (1995) Structural changes of T4 lysozyme upon adsorption to silica nanoparticles measured by circular dichroism. *J. Colloid Interface Sci.* **175**: 77.
- Bothwell, M. K., Daughhetee, S. D., Chau, G. Y., Wilson, D. B., and Walker, L. P. (1997) Binding capacities for *Thermomonospora fusca* E3, E4, and E5 binding domain, and *Trichoderma reesei* CBHI on avicel and bacterial microcrystalline cellulose. *Bioresource Technology* **60**: 169.
- Bower, C. K., Xu, Q., and McGuire, J. (1998) Activity losses among T4 lysozyme variants after adsorption to colloidal silica. *Biotechnol. and Bioeng.* **58**: 657.
- Brash, J.L. and T.A. Horbett, *Proteins at Interfaces*, in *Proteins at Interfaces II*, T.A. Horbett, J.L., and Brash, Editors. 1995, ACS Symposium Series 602: Washington D.C.
- Chernoglazov, V.M., Ermolova, O.V., and A.A. Klyosov (1988) Adsorption of high-purity eno-1,4- β -glucanases from *Trichoderma reesei* on components of lignocellulosic materials: cellulose, lignin, and xylan. *Enzyme Microb. Technol.* **10**: 899.

- Converse, A.O., Ooshima, H., and D.S. Burns (1990) Kinetics of enzymatic hydrolysis of lignocellulosic materials based on the surface area of cellulose accessible to enzyme and enzyme adsorption on lignin and cellulose. *Appl. Biochem. Biotechnol.* **24/25**: 67.
- Creagh, A. L., Ong, E., Jervis, E., Kilburn, D. G., and Haynes, C. A. (1996) Binding of the cellulose-binding domain of exoglucanase Cex from *Cellulomonas fimi* to insoluble microcrystalline cellulose is entropically driven. *Proc. Natl. Acad. Sci.* **93**: 12,229.
- Din, N., Coutinho, J. B., Gilkes, N. R., and Jervis, E. (1995) Interactions of cellulases from *Cellulomonas fimi* with cellulose. *Progress in Biotechnology* **10**:261.
- Din, N., Forsyth, I.J., Burtnick, L.D., Gilkes, N.R., Miller, Jr., R.C., Warren, R.A.J. and D.G. Kilburn (1994) The cellulose-binding domain of endoglucanase A (CenA) from *Cellulomonas fimi*: evidence for the involvement of tryptophan residues in binding. *Mol. Microbiol.* **11**: 747.
- Din, N., Gilkes, N.R., Tekant, B., Miller, R.C. Jr., Warren, R.A.J. and D.G. Kilburn (1991) Non-hydrolytic disruption of cellulose fibers by the binding domain of a bacterial cellulase. *Bio/Techn.* **9**: 1096.
- Elwing, H., Welin, S., Askendal, A. and I. Lundström (1988) Adsorption of fibrinogen as a measure for the distribution of methyl groups on silicon surfaces. *J. Colloid Interface Sci.* **123**: 306.
- Engle, A.R., Purdie, N. and Hyatt, J.A. (1994) Induced circular dichroism study of the aqueous solution complexation of cello-oligosaccharides and related polysaccharides with aromatic dyes. *Carbohydr. Res.* **265**: 181.
- Ghangas, G.S. and D.B. Wilson (1987) Expression of a *Thermomonospora fusca* cellulase gene in *Streptomyces lividans* and *Bacillus subtilis*. *Appl. Environ. Microb.* **53**: 1470.
- Gilkes, N. R., Henrissat, B., Kilburn, D.G., Miller, R.C., and R.A.J. Warren (1991) Domains in microbial b-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**: 303.
- Gilkes, N. R., Jervis, E., Henrissat, B., Tekant, B., Miller, R.C. Jr., Warren, R.A.J., and D.G. Kilburn (1992) The adsorption of a bacterial cellulase and its two isolated domains to crystalline cellulose. *J. Biol. Chem.* **267**: 6743.
- Golovchenko, N.P., Kataeva, I.A., and V.K. Akimenko (1992) Elucidation of the role of hydrophobic interactions in the adsorption of endo-1,4- β -glucanases on polysaccharides. *Enzyme Microb. Technol.* **14**: 327.

- Grethlein, H.E. (1985) The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. *Bio/Tecnol.* **3**:155.
- Henrissat, B. and Bairoch, A. (1993) New Families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical J.* **293**: 781.
- Henrissat, B., M. Glaeysens, P. Tomme, L. Lemesie, and J. Mornon. (1989) Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81**: 83.
- Horbett, T.A. and J.L. Brash. *Proteins at Interfaces: Physicochemical and Biochemical Studies.* in *ACS Symposium Series 343.* 1987. Anaheim, CA.
- Irwin, D.C., Spezio, M., Walker, L.P., and D.B. Wilson (1993) Activity studies of eight purified cellulases: specificity, synergism, and binding domain effects. *Biotechnol. Bioeng.* **42**: 1002.
- Johnson, P.E., Tomme, P., Joshi, M.D., and McIntosh, L.P. (1996) Interaction of soluble cellooligosaccharides with the N-Terminal cellulose-binding domain of *Cellulomonas fimi* CenC. 2. NMR and ultraviolet absorption spectroscopy. *Biochemistry* **35**: 13906.
- Johnson, P.E., Joshi, M.D., Tomme, P., Kilburn, D.G., and McIntosh, L.P. (1996) Structure of the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC determined by nuclear magnetic resonance spectroscopy. *Biochemistry* **35**: 14381.
- Klyosov, A. A. (1990) Trends in biochemistry and enzymology of cellulose degradation. *Biochemistry* **29**: 10577.
- Knowles, J., Teeri, T.T., Lehtovaara, P., Penttila, M. and M. Saloheimo. *The use of gene technology to investigate fungal cellulolytic enzymes,* in *Biochemistry and Genetics of Cellulose Degradation.* Aubert, J.-P., Beguin, P. and J. Millet, Editors. 1988 Proceedings of FEMS Symposium No. 43. Academic Press, London and New York.
- Kondo, A., Murakami, F., and Higashitani, K. (1992) Circular dichroism studies on conformational changes in protein molecules upon adsorption on ultrafine polystyrene particles. *Biotechnol. Bioeng.* **40**: 889.
- Kraulis, P.J., Clore, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J., and Gronenborn, A.M. (1989) Determination of the three-dimensional solution structure of the C-terminal domain of Cellobiohydrolase I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry-dynamical simulated annealing. *Biochemistry* **28**: 7241.
- Krisdhasima, V., McGuire, J., and Sproull, R. (1992) Surface hydrophobic influences on β -lactoglobulin adsorption kinetics. *Journal of Colloid and Interface Science* **154**: 337.

- Kurakake, M., Shirasawa, T., Ooshima, H., Converse, A. O., and Kato, J. (1995) An extension of the Harano-Ooshima rate expression for enzymatic hydrolysis of cellulose to account for changes in the amount of adsorbed cellulose. *Applied Biochem. and Biotech.* **50**: 231.
- Lao, G., Gurdev, S.G., Jung, E.D., and D.B. Wilson (1991) DNA Sequences of Three β -1,4-Endoglucanase Genes from *Thermomonospora fusca*. *J. of Bacteriology* **173**: 3397.
- Lee, S.B., and Kim, I.H. (1983) Structural properties of cellulose and cellulase reaction mechanism. *Biotechnol. Bioeng.* **25**: 33.
- Linder, M., Lindeberg, G., Reinikainen, T., Teeri, T.T., and Petterson, G. (1995) The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. *FEBS Letters* **372**: 96.
- Lundström, I. (1985) Models of protein adsorption on solid surfaces. *Progr. Colloid. & Polymer Sci.* **70**:76.
- Lundström, I., and Elwing, H. (1990) Simple kinetic models for protein exchange reactions on solid surfaces. *Journal of Colloid and Interface Science* **136**:68.
- Mattinen, M-L., Linder, M., Teleman, A., and Annala, A. (1997) Interaction between cellobiose and cellulose binding domains from *Trichoderma reesei* cellulases. *FEBS Letters* **407**:291.
- Nidetzky, B., Steiner, W., and Claeysens, M. (1993) Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: adsorptions of two cellobiohydrolases, two endocellulases and their core proteins on filter paper and their relation to hydrolysis. *Biochem. J.* **303**: 817.
- Ooshima, J., Burns, D.S., and A.O. Converse (1990) Adsorption of cellulase from *Trichoderma reesei* on cellulose and lignocellulosic residue in wood pretreated by dilute sulfuric acid with explosive decompression. *Biotechnol Bioeng.* **36**: 446.
- Piyachomkwan, K., Gable, K.P., and M.H. Penner (1997) *p*-Aminophenyl 1-thio- β -D-cellobioside: Synthesis and application in affinity chromatography of exo-type cellulases. *Carbohydr. Res.* **303**: 255.
- Reinikainen, T., Ruahonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T. A., Knowles, J. K. C., and T. Teeri (1992) Investigation of the function of mutated cellulose-binding domains of *Trichoderma reesei* cellobiohydrolase I. *Proteins: Structure, Function, and Genetics* **14**: 475.

- Reinikainen, T., Srisodsuk, M., Jones, A, Teeri, T.T. (1993) Enzymatic hydrolysis of Crystalline cellulose by *Trichoderma reesei* cellobiohydrolase I. *Protein Engineering* **6**: 49.
- Spurlino, J.C., Rodseth, L.E., and F.A. Quijoch (1992) Atomic interactions in protein-carbohydrate complexes, tryptophan residues in the periplasmic maltodextrin receptor for active transport and chemotaxis. *J. Mol. Biol.* **226**: 15.
- Stahlberg, J., Johansson, G., and Petterson, G. (1991) A new model for enzymatic hydrolysis of cellulose based on the 2-domain structure of cellobiohydrolase I. *Bio/Technology* **9**: 286.
- Tanaka, M., Ikesaka, M., Matsuno, R., and Converse, A. O. (1988) Effect of pore size in substrate and diffusion of enzyme on hydrolysis of cellulosic materials with cellulases. *Biotechnol. Bioeng.* **32**: 698.
- Tian, M., Lee, W.-K., Bothwell, M. K., and McGuire, J. (1998) Structural stability effects on adsorption of bacteriophage T4 lysozyme to colloidal silica. *Journal of Colloid and Interface Science* **200**: 146.
- Tomme, P., Driver, D.P., Amandoron, E.A., Miller, Jr., R.C., Antony, R., Warren, J., and D.G. Kilburn (1995) Comparison of a fungal (Family I) and bacterial (Family II) cellulose-binding domain. *Journal of Bacteriology* **177**: 4356.
- Vyas, N. Atomic features of protein-carbohydrate interactions. (1991) *Curr. Opinion Struct. Biol.* **1**: 732.
- Walker, L.P., Wilson, D.B., Irwin, D.C., McGuire, C., and M. Price (1992) Fragmentation of cellulose by the major *Thermomonospora fusca* cellulases, *Trichoderma reesei* CBHI, and their mixtures. *Biotechnol. Bioeng.* **40**: 1019.
- Xu, G-Y, Ong, E., Gilkes, N.R., Kilburn, D.F., Muhandiram, D.R., Harris-Brandts, M, Carver, J.P., Kay, L.E., and Harvey, T.S. (1995) Solution structure of a cellulose-binding domain from *Cellulomonas fimi* by nuclear magnetic resonance spectroscopy. *Biochemistry* **34**: 6993.

APPENDIX

Appendix: An Interactive, Multimedia Bioengineering Ethics Module

Motivation

As biotechnology continues to push the limits of science, engineers and scientists will be required to make more and more ethical decisions. Ethics are not only important in high-tech laboratories, but are an integral part of how professionals in many fields make decisions. It is important that we empower engineers with the tools and framework needed to assess dilemmas and make ethical decisions.

A requirement that all programs address ethics was issued by ABET in 1980. While the requirement has been in place for over a decade, many engineering programs fail to address ethics in a substantive manner. Those programs that do teach ethics usually deliver the material in freestanding courses or integrate the material into the engineering curriculum itself. Freestanding ethics courses are usually electives and as such can make ethics look optional rather than integral. Curriculum integration is perhaps the best means of delivery, as integration allows students to see ethics as a core component of engineering.

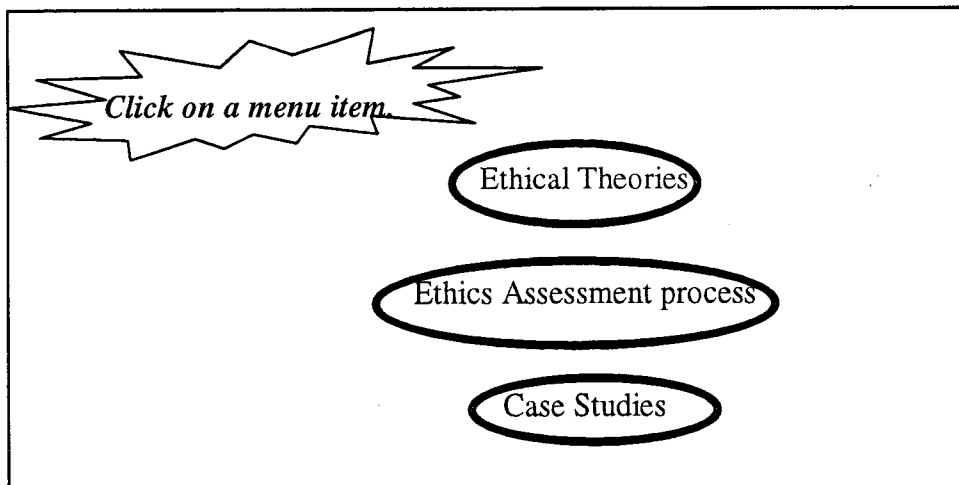
Teaching strategies for ethics in an engineering curriculum include role-playing, analysis of engineering cases, and computer assisted instruction. Engineering faculty are weary of using the first two methods due to a perceived lack of expertise in ethical theory and practice. For this reason we are developing an interactive, multimedia bioethics module that will be accessible via the WWW. This stand-alone module can be integrated into appropriate biological and agricultural science and engineering courses across the nation. The goal of this activity is to provide accessible instructional materials that can be readily incorporated into existing engineering and science curricula.

Bioethics Module

The interactive bioethics module was written using Authorware (Macromedia, Inc., San Francisco, CA). Through the use of menus, buttons, arrows and hot spots, the user is able to navigate the module at his or her own pace. At any time the user can go back and review information already covered. A computer-assisted instruction method, such as this, provides opportunities to make choices and receive feedback, and promotes problem solving skills.

Topics addressed in the interactive bioethics module include ethical theories, an ethics assessment process, and applied ethics. The treatment is not meant to provide in depth analysis of ethical theories, but an introduction to ethical theories and ethics assessment. The goal of the interactive module is to provide an ethical framework and assessment process with which the student can approach an ethical dilemma and make a decision.

Each of the following boxes represents the content of a full screen. The arrows are used to navigate forward and backward. After a brief introduction the program allows users to choose from the following menu ("main menu"):



If they choose "Ethical Theories" from the menu the following series of screen are displayed.

Quit
Main Menu
◀▶

ETHICS:

- * derived from *ethos*, Greek:
 meaning custom, usage, or character
- * a branch of Philosophy which studies human normative behavior
- * considers "rights" and "wrongs" of human conduct

Quit
Main Menu
◀▶

A Theory of Ethics is a set of principles that provide a framework for human action.

Quit
Main Menu
◀▶

Human action can be viewed in 3 parts:

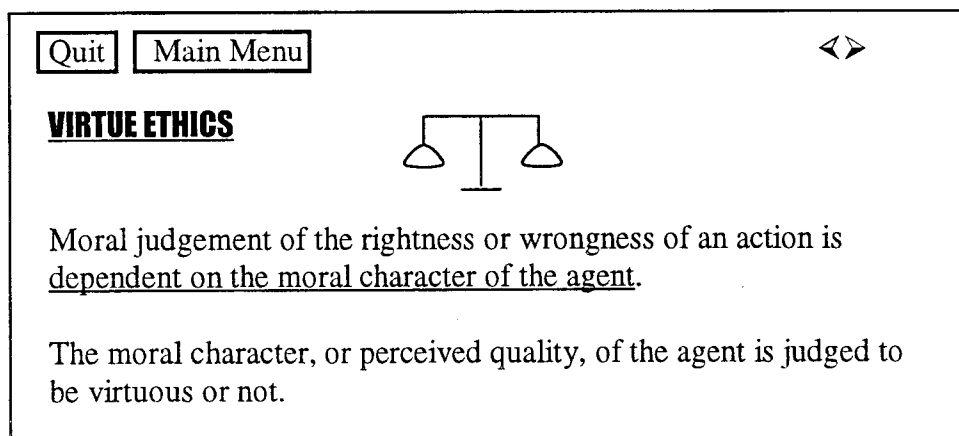
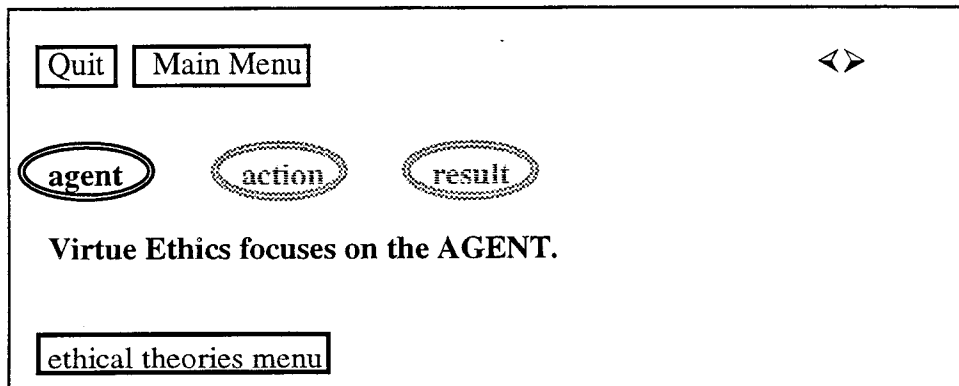
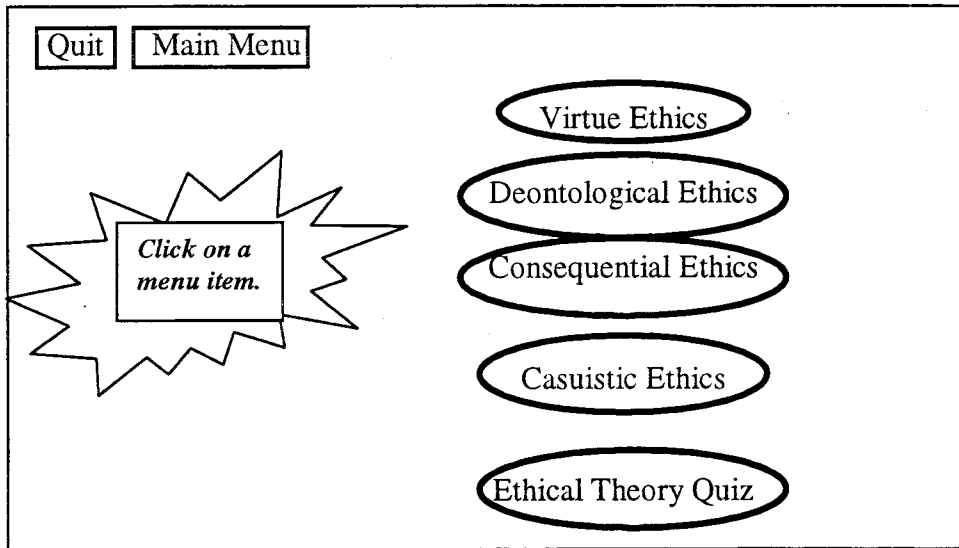
agent
action
result

#1 the agent
the moral agent can be a person , a company, a government, any entity capable of making and acting on a decision

#2 the action
the action taken as a resolution to an ethical dilemma

#3 the result
the consequences or outcomes of an action or decision

The following menu can be used to access a series of pages dedicated to each ethical theory. The following is an example of the pages the user will encounter if they click on Value Ethics. All ethical theories are treated similarly.



Quit

Main Menu



Characteristics of moral virtue

- * intentions to act virtuously
- * motivations
- * character traits
- * overall pattern of conduct

Quit

Main Menu



What or who is a moral agent?

- ❶ A moral agent can be any entity that is capable of making moral decisions.
- ❷ A moral agent can be a person, a corporation, or a group of people.

Quit

Main Menu



The questions to ask:

- ◇ Who is the moral agent?
- ◇ What is the agent's moral character?

Quit

Main Menu



Priority List for Virtue Ethicist:

- ❶ moral character of the agent
- "good" results
- do the right thing

Once the user feels he or she has an understanding of the ethical theories they can take the "Ethical Theory Quiz". The following is an example of one of the quiz questions.

Quiz Part 1

Read the following ethical dilemma then click on "quiz question".

You are a beginning engineer in a large company. You are responsible for interviewing college students for an intern position. The applicant with the highest credentials, most experience, and most enthusiasm, has a speech impediment (a noticeable stutter). You know that your supervisor, who has the last word in hiring, would look unfavorably upon the applicant's speech habit. You are aware that such discrimination is against company policy.



Click on the ethical theory used to decide on the following alternative.

You highly value the principles of equality and respect for persons. You decide to recommend the applicant for the position, intentionally neglecting to mention the speech habit.

Virtue Ethics

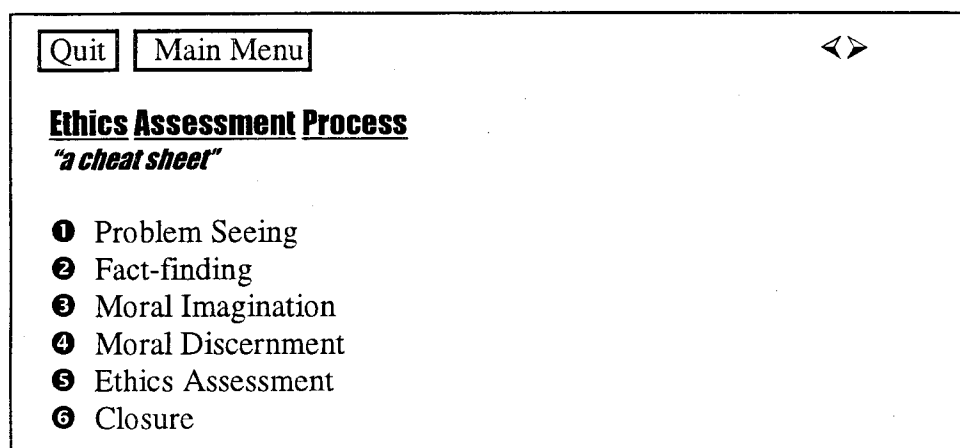
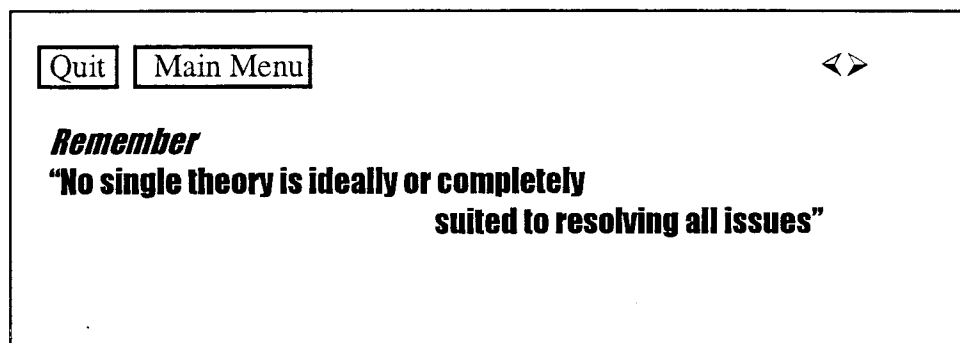
Deontological Ethics

Consequential Ethics

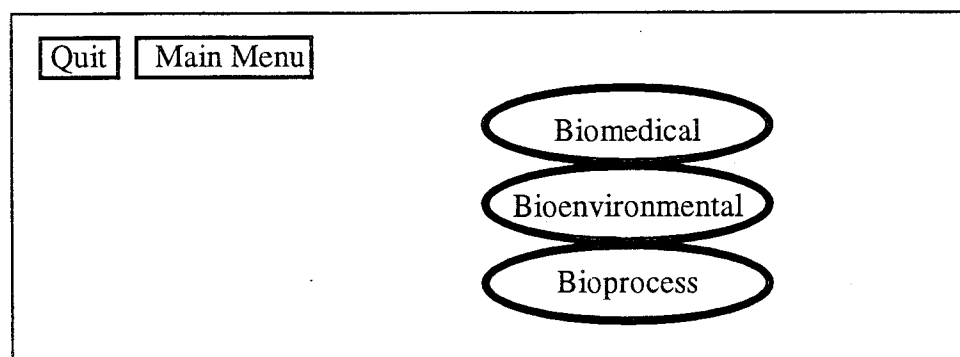
Casuistical Ethics

The program will respond depending on what answer the user gives. The correct answer will allow the user to progress to the next quiz question. After completing the quiz, the is

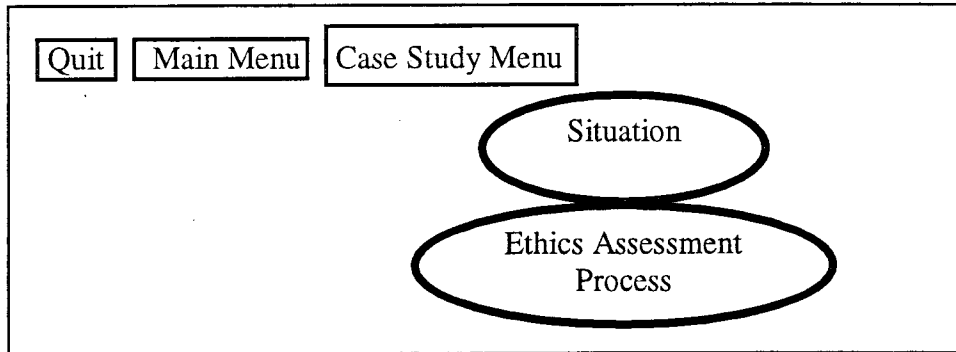
encouraged may return to the main menu and click on the “Ethical Assessment Process” button. The following screens introduce an ethical assessment process.



Each step of the assessment process is explained in greater detail when the student clicks on the number. After exploring the steps, the user may return to the main menu and click on “Case Studies”. Ethical assessments of case studies are accessed through the following menu. The following menu appears, allowing access to ethical assessments of case studies.



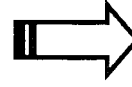
The following is a fictional Bioprocess case study based on a real medical condition, Diabetes Insipidus, and the pharmaceutical hormonal (DDAVP) replacement treatment. The method of production of DDAVP and the engineer, Jane and the situation are fictional.



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Ethical Situation:

Jane is a biological engineer responsible for quality control for DDAVP production. DDAVP is a synthetic anti-diuretic peptide hormone. This hormone is used for replacement therapy for those suffering from Diabetes Insipidus, a medical condition in which ADH (anti diuretic hormone) is no longer produced by the pituitary gland. While this condition is very rare, it is a common side effect of patients with brain tumors. The primary symptom of the condition is dehydration. Patients must drink about 1 liter of water every 45 minutes to replenish water loss. Without the drug the patient cannot live a normal life.

[Quit](#)[Main Menu](#)[Biomedical Case Study Menu](#)**Ethical Situation continued...**

Jane's company produces the drug using a transformed bacteria. FDA guidelines require 99.5% purity for use. Jane has found a large batch of product has only 99.3% purity. It is contaminated with a by-product of the fermentation, a peptide that causes a severe allergic reaction in a very small percentage of the population. Jane's company is the sole producer of the drug, and without this batch there may be a shortage of the drug for ADHD deficient patients, as well as financial loss for the company.

Jane voices concern about the contamination to the line manager. He says it is close enough, and the batch should be used. *What should Jane do?*

One method Jane could use to determine the best course of action is an ethics assessment of the situation. The following is an example of how Jane might approach the ethical dilemma. To step through the process, a student may return to the Case Study Menu and click on "Ethics Assessment Process".

[Quit](#)[Main Menu](#)[Bioprocess Case Study Menu](#)**Ethical Assessment Process:****① Problem-seeing****② Fact-Finding****③ Moral Imagination****④ Moral Discernment****⑤ Ethic Assessment****⑥ Closure**

By clicking on each number the following screens will be shown.

[Quit](#)[Main Menu](#)[Biomedical Case Study](#)[Ethics Assessment](#)**① PROBLEM-SEEING****What is the ethical conflict in this case?**

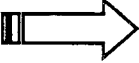
*The ethical conflict Jane is faces is between company loyalty and public safety.

*Should the drug be distributed and sold?

What is the end result Jane wants to achieve?

*Ideally Jane would like to maintain her professional integrity, prevent great financial loss to her company, and provide adequate amounts of safe quality DDAVP.

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Fact-Finding

What information do you need to know so that you can make a sound decision?

A great deal of information is missing from the problem definition. The best decision cannot be made without sufficient factual information. Some information that would be helpful include:

- ✦ How severe is the allergic reaction to the peptide contaminant?
- ✦ How large is the financial effect this loss would have on the company?
- ✦ What are the penalties the company and Jane will face if the FDA discovers that the drug does not meet purity requirements?

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Fact-Finding Continued...

- ✦ What legal action would the company be subjected to if a patient has an intense or fatal reaction to the contaminant?
- ✦ Can the batch be further purified with FDA approval ?
- ✦ How long would it take for the FDA to approve changes in the process?
- ✦ Is there a reduced therapeutic value to the DDAVP that does not meet FDA purity guidelines?
- ✦ As the engineer responsible for production, what legal responsibility does she have for complications DDAVP users may have due to use of the less pure batch?

Quit Main Menu Biomedical Case Study Ethics Assessment

➊ **Moral Imagination**

What are three viable alternative solutions to the problem?

Keep in mind the following alternatives are only a few of many possible alternatives.

Alternative A

Alternative B

Alternative C

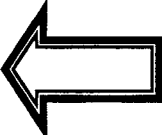
Advantages and Disadvantages

The user clicks on alternative A, then B, then C.

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Alternative A

Jane would recommend that the batch be re-purified to the required purity level. This action would require FDA approval.



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Alternative B



Jane would recommend that a warning label be placed on the bottles of DDAVP from this batch to warn potential users of the difference in purity and increased risk of allergic reaction. This action would require FDA approval.

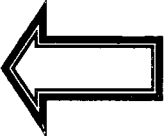
Quit Main Menu Biomedical Case Study

Alternative C



Jane would not approve the contaminated batch for medical use because it does not meet FDA requirements.

After reviewing the three alternatives the advantages and disadvantages of each are summarized in a table.

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What are the benefits and disadvantages of these alternatives in terms of reaching the desired end result?

	Benefits	Disadvantages
A. Re-purification	Increase purity of DDAVP Don't waste batch.	Takes time to get FDA approval. May be shortage of DDAVP.
B. Warning label	Honesty (consumers and FDA). Reduce number of allergic reactions due to awareness.	Take time to get FDA approval.
C. Reject batch	Follow FDA guidelines.	Financial loss for company. Disrupt lives of DDAVP dependent due to shortage.

Then back to the Ethics Assessment Menu.

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④ Moral Discernment

Determine which professional values are relevant to this case.

Public Safety

Company Loyalty

Compliance with FDA requirements

Efficiency

Quit

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Biomedical Case Study

Ethics Assessment

Ⓞ Ethics Assessment

Evaluate your three alternatives according to the values you have deemed relevant to the case. Mark positive responses with a (+), negative responses with a (-), and leave the box blank (or a (0)) if the outcome is unknown.

Alternative	Value: Public safety	Value: Company loyalty	Value: Compliance with FDA	Value: Efficiency	Total
A. Re- purification	+	0	+	0	++
B. Warning label	0	+	-	+	+
C. Reject batch	+	-	+	-	0

Quit

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Ethics Assessment

Ⓞ Closure

Based on this assessment, **Jane's decision is** to attempt to get FDA approval for re-purification of the batch of DDAVP to ensure proper purity.

Assuming that FDA approval for further purification is quick (less time than it takes to make a new batch), **this is the best solution because** the pharmaceutical will be considered safe by the FDA and the company will not completely waste the batch.

The plan to implement this solution is for Jane to petition the FDA for one time approval to re-purify the batch of DDAVP. This may not be feasible in which case she will have to re-evaluate the situation.

Included in the program is a similar sample case study for the student to complete and submit answers to the instructor. This interactive module can currently be used on any computer by downloading the appropriate files. An HTML version is currently being built for use on the WWW. In the web version the student will be able to submit responses and case studies to the instructor via e-mail.