## AN ABSTRACT OF THE THESIS OF

Kuakoon Piyachomkwan for the degree of Doctor of Philosophy in Food Science and Technology presented on June 25, 1997. Title: Interaction of *Trichoderma reesei* Exo-Acting Cellulases With Immobilized *p*-Aminophenyl 1-Thio-β-D-Cellobioside.

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Michael H. Penner

Cellulolytic enzymes capable of efficiently degrading crystalline cellulose are a complex mixture of endo- (endoglucanases) and exo-acting (cellobiohydrolases) enzymes. One approach to separating these enzymes is affinity chromatography. A new ligand, *p*-aminophenyl 1-thio-β-D-cellobioside (APTC), is introduced for this purpose. The property of APTC in affinity chromatography is demonstrated using *Trichoderma reesei* cellulases. The behavior of these enzymes on APTC-affinity column was essentially equivalent to that reported for the same enzymes on *p*-aminobenzyl 1-thio-β-D-cellobioside (ABTC)-columns; ABTC being the traditional ligand for affinity chromatography of exocellulases. The primary advantage of the APTC ligand is its ease of preparation.

The affinity between CBHs and APTC may be considerably affected by nonspecific interactions. In this study, the significance of nonspecific protein/matrix interactions in affinity chromatography of cellulolytic enzymes is evaluated. The role of

pH, NaCl, coupling conditions and stationary phase functional groups (N-hydroxysuccinimide ester and cyanogen bromide) on the affinity purification of *Trichoderma reesei* CBHs has been systematically determined. The results suggest that the apparent discrepancies in existing methods for the affinity purification of CBHs are due to nonspecific interactions, i.e. ionic interactions, between the enzymes and the stationary phase matrix.

Exocellulases can be classified into two classes, based on their hydrolytic specificities. Class I enzymes preferentially hydrolyze cellulose from the reducing end, while Class II enzymes preferentially hydrolyze cellulose from the nonreducing end. *Trichoderma reesei* CBH I is a class I enzyme and CBH II is a class II enzyme. CBH I and CBH II are both retained on the APTC-affinity column; showing that both CBH classes bind to immobilized APTC. To further understand the differences in the two CBH classes, the behavior of CBH I and CBH II on the APTC-affinity column was compared. The affinity of CBH I for immobilized APTC was found to decrease when glucose was present in the system. In contrast, glucose was found to increase the affinity of CBH II for immobilized APTC. An outcome of this difference is that in the presence of glucose CBH I can be selectively eluted from the column. Equilibrium binding studies with each enzyme clearly reflect that CBH II has a higher affinity for immobilized APTC than CBH I.

# Interaction of *Trichoderma reesei* Exo-Acting Cellulases With *p*-Aminophenyl 1-Thio-$\beta$-D-Cellobioside

by

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Head of Department of Food Science and Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kuakoon Piyachomkwan, Author

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## CONTRIBUTION OF AUTHORS

Dr. Michael H. Penner was involved in the design, analysis and writing of each chapter. Dr. Kevin P. Gable was also involved in preparation of chapter 3.

## TABLE OF CONTENTS

## TABLE OF CONTENTS (CONTINUED)

## TABLE OF CONTENTS (CONTINUED)

## TABLE OF CONTENTS (CONTINUED)

## LIST OF FIGURES

## LIST OF FIGURES (CONTINUED)

Figure Page

## LIST OF FIGURES (CONTINUED)

## LIST OF TABLES

# INTERACTION OF *Trichoderma reesei* EXO-ACTING CELLULASES WITH IMMOBILIZED *p*-AMINOPHENYL 1-THIO-β-D-CELLOBIOSIDE

## CHAPTER 1

## INTRODUCTION

Cellulose is an insoluble biopolymer mostly found as a principal structural component of plant cell walls. Due to the availability of cellulose in large quantity, it has received considerable attention as a potential renewable carbon source for the production of liquid fuels. Cellulose is a long linear chain of glucopyranose residues linked by β-1,4 glycosidic linkage. The chains are oriented in parallel to form highly ordered structures, called crystalline regions, and less ordered structures, called amorphous regions. The efficient and complete hydrolysis of native cellulose is normally catalyzed by microbial enzyme systems which are a mixture of several hydrolases, each of which has limited hydrolytic activity when used alone. Hydrolases common to these enzyme systems include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Enari *et al.*, 1983). The mechanisms of cellulolytic enzyme action have been extensively studied. However, the precise chemistry that dictates rates of hydrolysis of different celluloses is not clearly understood. This is largely due to the complexities of both the substrate and the enzymes. To simplify these experimental systems it is common to study the mode of action of individual enzyme components acting on model substrates.

Cellulases obtained from the filamentous fungus *Trichoderma reesei* are the most widely studied. This fungus produces at least two or three different endoglucanases (EG I, EG II, and EG III) and two cellobiohydrolases (CBH I and CBH II) (Teeri *et al.*, 1992). Although most of these *Trichoderma reesei* cellulases have been purified and characterized for their hydrolytic specificities, there are still discrepancies between the reported properties of individual enzymes. This discrepancy may be due to different degrees of contamination of individual enzymes used in different studies. It is very difficult to obtain a homogenous cellulase preparation from a natural mixture of cellulases due to many of the enzymes having similar physicochemical properties and similar activities.

The similarity of these enzymes often makes the purification of individual components difficult. Affinity chromatography using the appropriate complementary ligand has proved to be particularly useful in this regard. In this technique, only the target enzymes that have the specific interactions with the ligands can be adsorbed on the column whereas other non-interacting enzymes cannot. The ligand, *p*-aminobenzyl-1-thio-$\beta$-D-cellobioside (ABTC) was first introduced by van Tilbeurgh *et al.* (1984) for the separation of exo- versus endo-acting cellulases from *Trichoderma reesei*. These affinity ligands would undoubtedly be widely used if they were more easily synthesized. In this study we present a relatively simple synthetic protocol for the preparation of an affinity ligand, *p*-aminophenyl 1-thio-glycoside of cellobiose (APTC), and we demonstrate the behavior of *Trichoderma reesei* cellulases on APTC-derivatized supports. The separation of desired compounds in affinity chromatography is achieved based on their exceptional

abilities to bind specifically to their complementary ligands. Nevertheless, the effectiveness and selectivity of ligand-target enzyme interactions can be largely affected by other nonspecific interactions, especially ionic interactions between the supporting matrix and the proteins in the enzyme preparation. These nonspecific interactions can influence either adsorption or desorption of the target compounds. All interactions, specific and nonspecific, are sensitive to the conditions used in the chromatography. A variety of different conditions have been used during the affinity purification of exo-acting cellulases from *Trichoderma reesei* (Tomme *et al.*, 1988; Orgeret *et al.*, 1992; Irwin *et al.*, 1993). In fact, on first observation the variety of conditions used is confusing. For example, salt is reportedly required for enzyme desorption (Orgeret *et al.*, 1992), for enzyme adsorption (Irwin *et al.*, 1993), or not at all (Tomme *et al.*, 1988) when working with *Trichoderma reesei* cellulases. Similarly, the working pHs for these affinity systems differ between laboratories (Tomme *et al.*, 1988; Irwin *et al.*, 1993; Orgeret *et al.*, 1992).

The intent of this study was to establish the significance of nonspecific protein/matrix interactions in the affinity chromatography of cellulolytic enzymes and to define the chemical basis for the apparent discrepancies in published experimental protocols. A rational approach to the optimization of these chromatographic systems was also provided. Two types of enzymes, *Trichoderma reesei* CBH I and CBH II, and two types of matrix, N-hydroxysuccinimide ester and cyanogen bromide activated agarose, were used to demonstrate the role of nonspecific interactions between enzyme and ligand-coupled matrix. We have systematically evaluated the role of pH, ionic strength,

coupling conditions and stationary phase functional groups on the adsorption and desorption of these exo-acting cellulases in APTC-derivatized affinity chromatography.

Exo-acting cellulases have been traditionally considered to release cellobiose from the nonreducing ends of cellulose chains. Recently, several studies on the hydrolytic specificities of exocellulases from various microorganisms reveal an exocellulase type that preferentially degrades cellooligosaccharides and cellulose from their reducing ends (Arai *et al.*, 1989; Vrsanska and Biely, 1992; Shen *et al.*, 1994; Barr *et al.*, 1996). These studies indicate the existence of two functional classes of exocellulases. One class, referred to as "class I", favorably hydrolyzes cellulose substrates from the reducing ends, whereas the other class, referred to as "class II", preferentially hydrolyzes cellulose from the nonreducing ends. The two major exocellulases from *Trichoderma reesei* are apparently in different classes; CBH I being a class I exoenzyme and CBH II being a class II exoenzyme. Both CBHs can interact with immobilized APTC in affinity chromatography. This is of interest because immobilized APTC is prepared by coupling it via its reducing end to agarose beads. Since agarose beads are considerably larger than the dimensions of exocellulases, it effectively blocks the reducing end of APTC, not allowing it to enter the enzyme active site. Only the nonreducing end of APTC is available for entering the active site tunnel of exoenzymes. Thus, it is of interest to determine whether or not the two exoenzyme classes may be differentiated by their behavior with immobilized APTC. The interactions of CBHs and immobilized APTC were qualitatively characterized by affinity chromatographic methods. The relative affinities of the two enzymes for immobilized APTC were also quantitatively determined

by traditional partition equilibrium experiments. The results clearly indicate a difference in the interaction of CBH I and CBH II with immobilized APTC.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. CELLULOSE

Cellulose is the most abundant polysaccharide on earth. It is normally found as the major structural component of plant cell walls. Cellulose is one of three major components of wood and agricultural wastes along with hemicellulose and lignin. Due to the considerable amount of cellulose produced annually, it has received considerable attention as a renewable carbon resource for the production of liquid fuels. The product of enzymatic cellulose saccharification is glucose, which can be converted to ethanol by yeast fermentation. The glucose resulting from cellulose degradation can also be utilized for the production of other substances, including single cell protein and industrial chemicals.

Cellulose is naturally synthesized by plants as the principal component of their cell walls. The molecular weight and degree of polymerization of cellulose vary depending on its source, as shown in Table 2.1. In native cellulose, 3,500-10,000 glucose residues are linked to form a long chain molecule (Fan *et al.*, 1980). Cellulose is also produced by non-plant organisms, including bacteria, algae (*Valonia ventricosa* and *Boergesenia forbesii*), and animals (tunicin). *Acetobacter xylinum* is an example of a prokaryote that synthesizes cellulose, known as bacterial microcrystalline cellulose (BMCC), which is used in the food industry (Reinikainen, 1994).

Table 2.1. Molecular weight and degree of polymerization of various cellulosic materials.

| Source | Molecular Weight | Degree of Polymerization |
|---|---|---|
| Native cellulose | 600,000-1,500,000 | 3,500-10,000 |
| Chemical cottons | 80,000-500,000 | 500-3000 |
| Wood pulps | 80,000-340,000 | 500-2100 |

From Fan *et al.*, 1980.

Cellulose is a high molecular weight linear polymer consisting of $\beta$-D-(+)-glucopyranose residues linked by 1,4-glucosidic linkages. Each consecutive glucose residue is rotated by 180° compared to its adjacent glucose molecule, therefore cellobiose is in fact the repeating unit of a cellulose polymer, as shown in Figure 2.1.

Most of glucose residues in a cellulose chain have the chair conformation with the hydroxyl groups in equatorial and the hydrogen atoms in axial positions. The hydroxyl groups in a glucose unit can form a hydrogen bond between the sugar rings, causing the intramolecular hydrogen-bonding interactions of cellulose molecules. These intramolecular interactions cause the linear cellulose to adopt a ribbon conformation (Rees, 1977). The linear extension of the ribbon conformation allows many cellulose molecules to align and pack with favorable hydrogen bonding and other non-covalent interactions, i.e. Van der Waal's interaction between chains. A cellulose fiber, which is composed of multiple cellulose chains, is held together by both inter and intramolecular hydrogen bonding. These interactions cause the fully extended chains to fit closely together over much of their lengths, and arrange in highly ordered structures called crystalline structures. Such compact and tightly bonded aggregation of cellulose chains in crystalline regions accounts for their insoluble and rigid fibrous characters. The crystalline regions of cellulose chains can be interrupted by less ordered structures called paracrystalline or amorphous regions, as shown in Figure 2.2. The proportion of crystalline versus amorphous structures in cellulose fibers varies in different cellulosic materials; in general, native cellulose is approximately 70% crystalline (Fan *et al.*, 1980).

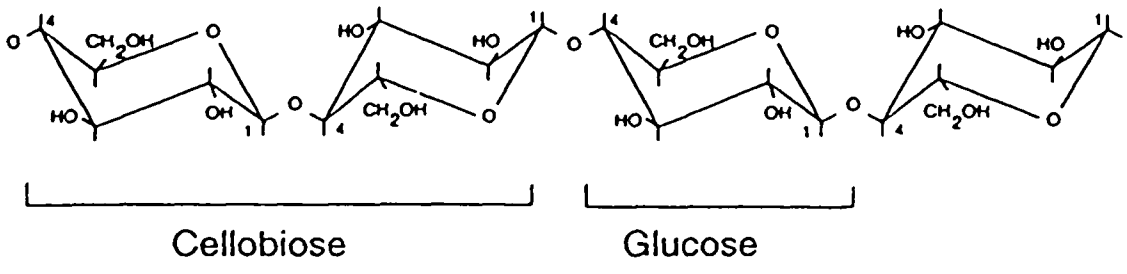



Figure 2.1. Chemical structure of cellulose with cellobiose as a smallest repeating unit.

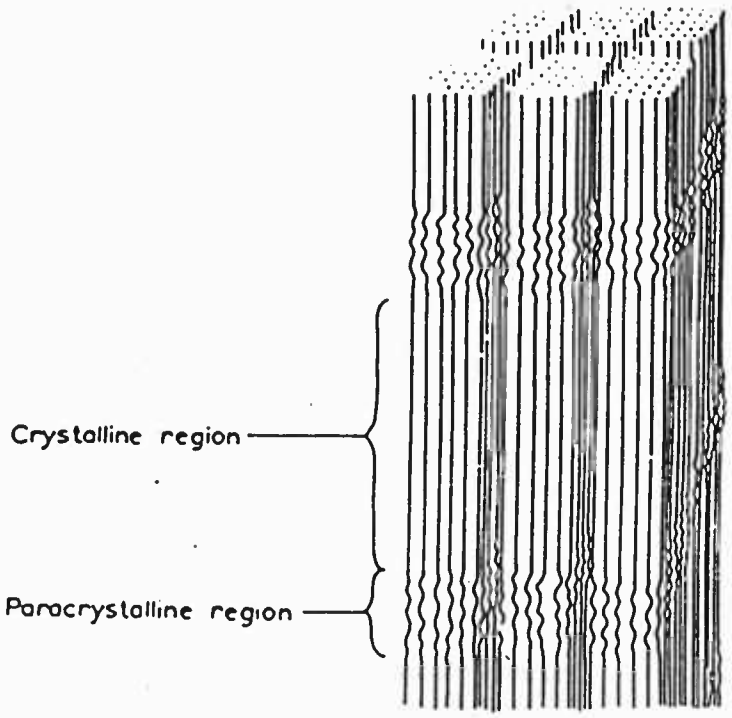
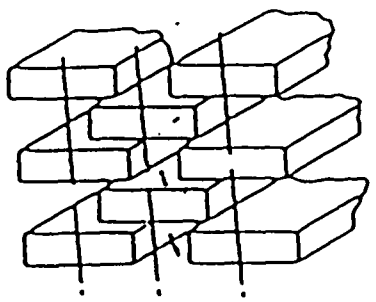


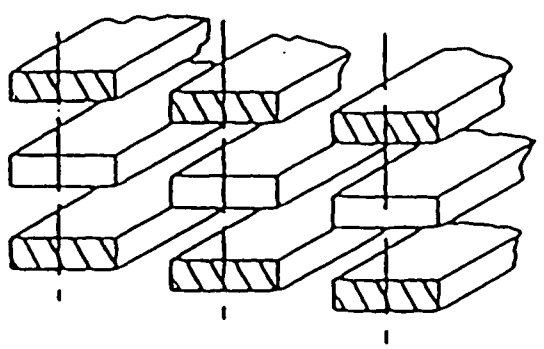

Figure 2.2. The structure of cellulose molecules containing highly ordered structure (called crystalline region) and less ordered structure (called paracrystalline or amorphous region) (Cowling, 1975).

Cellulose polymers can be classified into two major allomorphs, i.e. cellulose I and cellulose II, based on the difference in cellulose chain packing. In cellulose I, the ribbons are layered in sheets and staggered between sheets. The linear $\beta$-1,4 glucan chains are oriented parallel to one another with the same polarity, i.e. the chain ends at one end of a bundle are all of one type, to form microfibrillar structure. Cellulose I is the predominant form in nature. In cellulose II, the ribbons are piled in more obvious stacks, and consist of antiparallel chains, i.e. chain ends at the end of a bundle are of both types as illustrated in Figure 2.3. Cellulose II, known as regenerated cellulose, results from the conversion of the parallel chain structure present in cellulose I. The conversion of cellulose I to cellulose II can be achieved by chemical treatments such as strong alkali, i.e. mercerization. Only a few organisms can synthesize cellulose II allomorph in nature (Lee *et al.*, 1994).

In nature, cellulose chains are oriented in a parallel alignment and firmly bound together to form elementary fibrils called protofibrils. Elementary fibrils are the smallest structural unit of microfibril and fiber, and many of elementary fibrils are aggregated into long slender bundles to form microfibrils. In cell walls of higher plants, the microfibrils are seen as the bundles in lamella, and usually embedded in a matrix of hemicellulose and lignin as shown in Figure 2.4.



(a)



(b)

Figure 2.3. Packing of cellulose chains in natural cellulose I and regenerated cellulose II; (a) in cellulose I, chains are parallel rather than antiparallel, i.e. chain ends at one end of a bundle are all of one type (not hatched), (b) in cellulose II, chains are antiparallel, i.e. chain ends at the end of a bundle are of both types hatched and not hatched) (Rees, 1977).

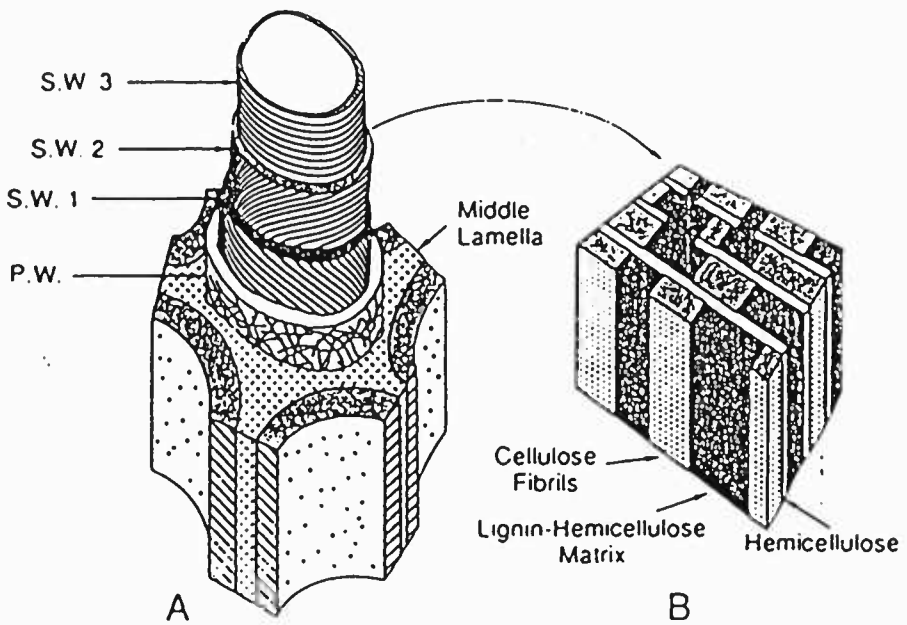



Figure 2.4. Schematic illustration of (A) cell wall layers in woody fiber and (B) the structure of microfibrils embedded in lignin and hemicellulose in the secondary cell wall. P.W., primary cell wall; S.W.1-S.W.3, secondary cell walls (Beguin and Aubert, 1992).

The biological degradation of cellulose frequently occurs by enzymatic hydrolysis. The susceptibility of cellulose to enzymatic saccharification is attributed to various factors especially the physical and chemical properties of cellulose substrates (Cowling, 1975; Fan *et al.*, 1980). These include the accessible surface area, degree of crystallinity, and degree of polymerization (DP). Any structural characters that limit the accessibility of cellulose to enzymes will diminish the susceptibility of cellulose to hydrolysis. Cellulosic materials from different sources can have a great variation in their molecular structures, and hence, have differ in enzyme accessibility. So far many celluloses have been used for studying cellulase hydrolysis, including native and modified celluloses. Inexpensive sources of relatively pure cellulose are commercially available, such as cotton, filter paper, microcrystalline cellulose (known as Avicel). Another type of microcrystalline cellulose that is widely used for studies of cellulolytic enzymes is produced by the bacteria *Acetobacter xylinum*; this cellulose is known as bacterial microcrystalline cellulose (BMCC). Some chemical modifications are introduced to produce cellulose derivatives that increase the solubility of cellulosic substrates. The most widely used of these cellulose derivatives are carboxymethyl cellulose (CMC), and hydroxyethyl cellulose (HEC).

Due to the complication and diversity of cellulosic materials, soluble celloligosaccharides and their chromogenic derivatives are also widely used to study cellulolytic hydrolysis. The chromogenic derivatives of these cellooligosaccharides increase the ease and sensitivity of determining reaction products due to the presence of

functional groups such as 2′-cholro-4′-nitrophenyl, methylumbelliferyl, or radiolabeled compounds (Claeyssens *et al.*, 1989).

## 2.2. THE HYDROLYSIS OF CELLULOSE

The biological degradation of cellulosic materials is most frequently caused by cellulolytic microorganisms. The ultimate product of enzymatic hydrolysis of cellulose is glucose which is an important chemical intermediate for many products including ethanol from yeast fermentation, single cell protein and other industrial chemicals.

### 2.2.1. Cellulolytic Enzymes

Cellulolytic enzymes, produced by microorganisms, are usually a mixture of several glycosidases, consisting of at least three discrete classes of enzymes, including endoglucanases (1,4-$\beta$-D-Glucan-4-glucanohydrolase, EC 3.2.1.4), cellobiohydrolases (1,4-$\beta$-D-Glucan cellobiohydrolase or exoglucanase, EC 3.2.1.91), and $\beta$-Glucosidases ($\beta$-D-Glucoside glucohydrolase or cellobiase, EC 3.2.1.21).

### 2.2.2. The Enzymatic Hydrolysis of Cellulose

The process of enzymatic hydrolysis of cellulose consists of two steps, i.e. the adsorption of cellulases onto the surface of insoluble cellulose, and the breakdown of cellulose by enzymatic catalysis.

2.2.2.1. Adsorption of cellulolytic enzymes

The enzymatic hydrolysis of cellulose is a soluble enzyme/insoluble substrate heterogeneous system. Therefore, the adsorption of enzyme on the cellulose surface is the initial step in the reaction. The initial rate of enzymatic hydrolysis is evidently proportional to the amount of cellulase adsorbed on cellulose (Steiner *et al.*, 1988; Nidetzky and Steiner, 1993). Studies of cellulase adsorption on insoluble cellulose show that enzyme adsorption can be described by the Langmuir adsorption isotherm (Ooshima, 1990; Beldman *et al.*, 1987). Contradictory, the deviation from Langmuir isotherm of cellulase adsorption was observed by the concaveness rather than the linearity in the Scatchard plots derived from the Langmuir binding isotherm (Gilkes *et al.*, 1992; Stahlberg *et al.*, 1991; Woodward *et al.*, 1988a). The deviation from Langmuir isotherm of cellulase adsorption can be explained by the heterogeneity of cellulase-cellulose binding sites and/or cellulase-cellulase interactions (Gilkes *et al.*, 1992; Woodward *et al.*, 1988a).

The adsorption of cellulases onto cellulose can be affected by many factors, including the type and concentration of enzymes, the physicochemical properties of the cellulose substrate, pH, ionic strength and temperature (Kyriacou *et al.*, 1988; 1989; Steiner *et al.*, 1988).

2.2.2.2. The hydrolysis of cellulose

Following enzyme adsorption, a mixture of enzymes catalyzes the hydrolytic breakdown of cellulose to yield glucose. All components of the cellulolytic enzyme mixture are essential for complete hydrolysis, as shown in Figure 2.5.

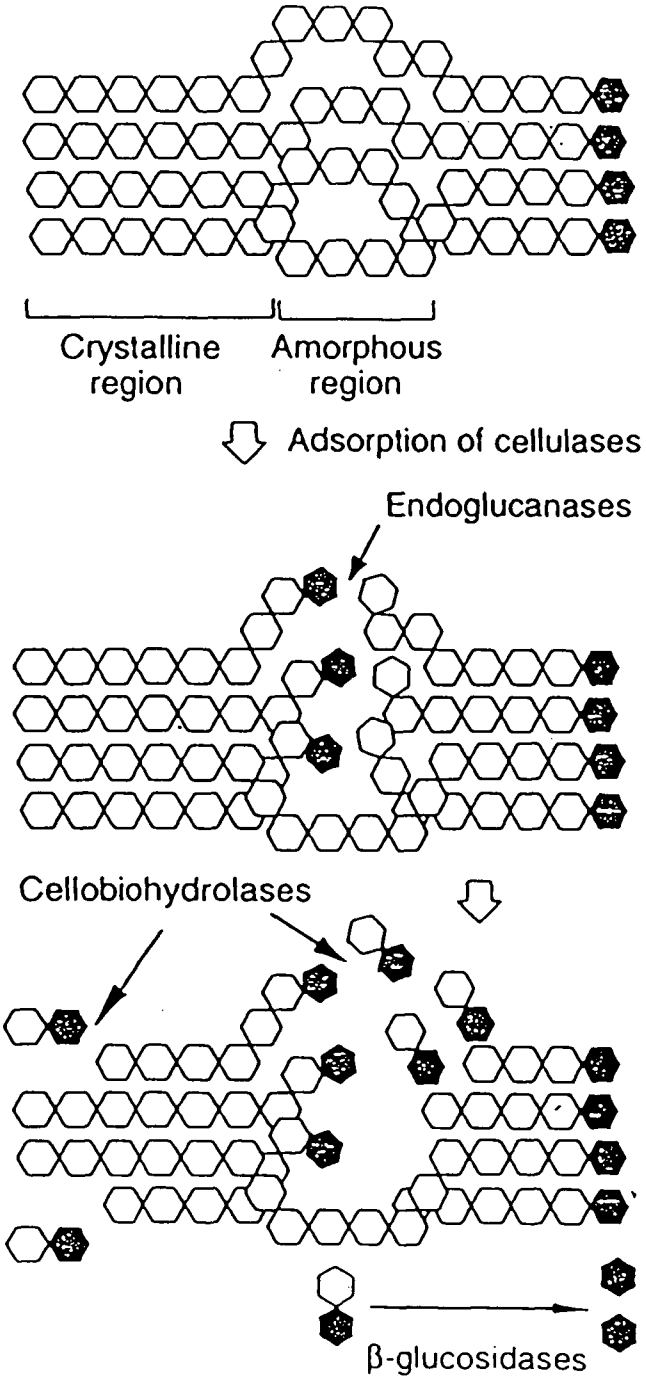



Figure 2.5. A scheme presents enzymatic hydrolysis of cellulose. Glucose residues are indicated by hexagons with reducing ends shown in black (Beguin and Aubert, 1992).

Endoglucanases catalyze hydrolysis at internal $\beta$-1,4-glycosidic linkages in amorphous regions of cellulose chains. Cellobiohydrolases, the exo-acting enzymes, are capable of degrading amorphous and crystalline cellulose by removing cellobiose molecules from chain ends. $\beta$-glucosidases hydrolyze cellobiose and other low molecular weight soluble cellodextrins to yield glucose molecules.

2.2.3. Synergism

Synergism is a phenomenon that when enzyme components act simultaneously, the extent of hydrolysis is higher than the sum of that produced by each enzyme acting alone. This phenomenon is frequently found in enzymatic hydrolysis of cellulose by cellulolytic enzymes (Nidetzky *et al.*, 1994; Woodward *et al.*, 1988b). Two types of synergism have been found so far for cellulose hydrolysis by fungal cellulases, i.e. endo-exo synergism, and exo-exo synergism. For example, in *Trichoderma reesei* cellulase, synergism between CBHs and EGs, and between CBH I and CBH II have been demonstrated (Nidetzky *et al.*, 1994). In addition, cross synergism between the major cellulases of *Thermomonospora fusca* and *Trichoderma reesei* CBH I and CBH II have been reported (Walker *et al.*, 1993). However, *Trichoderma koningii* cellobiohydrolases do not hydrolyze synergistically with all endoglucanases of the same species (Beguin and Aubert, 1992). These observations infer a specificity of synergism to the types and sources of cellulolytic enzymes. The degree of synergism of cellulolytic enzymes depends on many factors including types and concentrations of enzymes and substrates used in hydrolysis. Synergism is trivial in hydrolysis of soluble substrates, but appears

considerable when the endo- and exo-acting enzyme mixtures work simultaneously on insoluble solid substrates such as Avicel or filter paper (Fujii and Shimizu, 1986).

The synergistic action between endo- and exo- type enzymes can be simply explained by a sequential mechanism of enzyme actions as described in Figure 2.5. Initially, endoglucanases attack on the amorphous regions of cellulose, and cause the breakdown of substrates resulting in more new chain ends for the actions of cellobiohydrolases. Once the endo-acting enzymes generate a saturation of chain ends for the exo-acting enzymes, the reaction kinetics obey only the rate of the reaction of exoenzymes. The degree of synergism also falls off at low enzyme concentrations due to the saturation of chain ends initially present for the reaction by exo-acting enzymes (Converse and Optekar, 1993). The presence of $\beta$-glucosidases also increases the effect of synergism by preventing the accumulation of cellobiose which is an inhibitor for endo- and exo-acting enzymes.

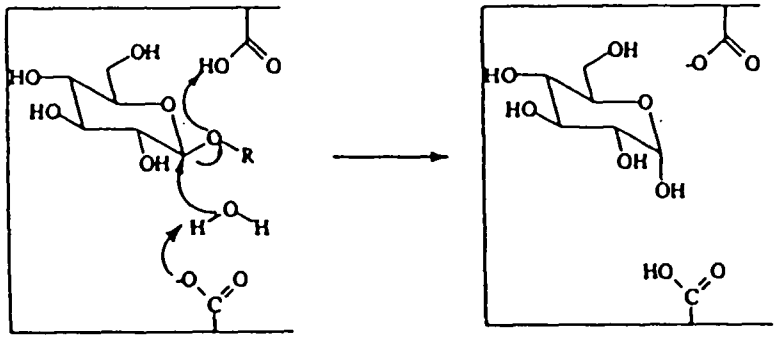
Nevertheless, a simple explanation for endo- and exo-acting synergistic action cannot be used as a rationale for exo-exo type synergism. Many models have been proposed in order to explain this type of synergism. One of them is the formation of a loose complex between CBH I and II of *Trichoderma reesei* occurring prior to adsorption, and hence increases the adsorption of enzymes to substrates (Tomme *et al.*, 1990). Another explanation for exo-exo type synergism was proposed by Henrissat *et al.* (1985) that CBH I had the endo-type adsorption pattern on cellulose and also exhibited the endo-type hydrolysis pattern on barley $\beta$-D-glucan. A synergistic effect of CBH I and II from *Penicillium pinophilum* has been accounted for by different orientations of the

nonreducing end groups in crystalline cellulose which require two cellobiohydrolases with different stereochemical specificities (Medve *et al.*, 1994). Recently, exo-exo type synergism has been explained by the existence of two functional classes of exocellulases; one class preferentially hydrolyzes cellulose from the reducing end whereas the other class prefers to hydrolyze cellulose from the non-reducing end (Barr *et al.*, 1996). Nevertheless, it is still not clear how exo-exo synergism occurs.

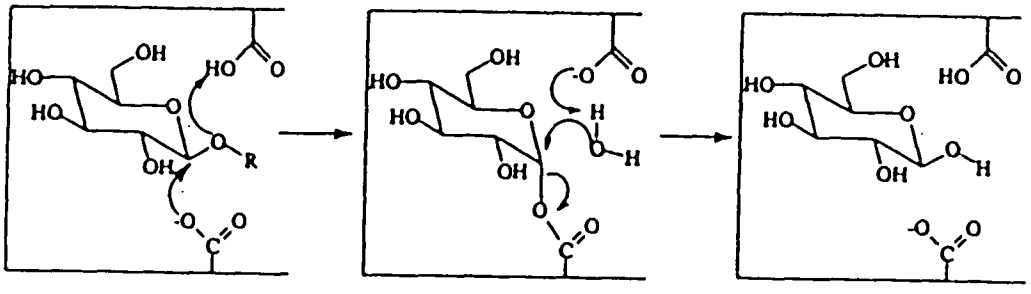
## 2.3. CELLULOLYTIC ENZYMES

Cellulolytic enzymes are glycosidases that catalyze the hydrolysis of glycosidic linkages by general acid/base catalysis via two different mechanisms; one with inversion of anomeric configuration, and the other with retention of anomeric configuration.

Inverting glycosidases function by a single displacement mechanism ($S_N2$-reaction) in which a water molecule directly displaces at the anomeric center of the substrate molecule. One of amino acids in active site of these enzymes acts as general base to help the nucleophilic water molecule deprotonate, while the other amino acid acts as a general acid to protonate the departing oxygen atom, as shown in Figure 2.6a. For retaining glycosidases, the enzymes generally operate through a double displacement mechanism ($S_N1$-reaction), in which a glycosyl enzyme intermediate is formed and hydrolyzed via oxocarbonium ion-like transition state (Figure 2.6b). In both mechanisms, the acid and base catalytic groups are normally the carboxylic side chain of aspartic and/or glutamic acid of the enzyme active sites (Reinikainen, 1994; Withers, 1995).



(a)



(b)

Figure 2.6. The hydrolytic mechanism of glycodiases by general acid/base catalysis with (a) inversion and (b) retention of anomeric configuration (Withers, 1995).

2.3.1. Classification and Properties of Cellulolytic Enzymes

Microorganisms, especially fungi, are the predominant producers of cellulolyltic enzymes in nature, including *Trichoderma*, *Fusarium*, *Penicillium*, *Phanerochaete* and *Schizophyllum*. Bacteria that are capable of producing cellulolytic enzymes are *Clostridium thermocellum*, *Pseudomonas fluorescens*, *Cellulomonas sp.*, *Thermomonospora sp.*, *Thermoctinomyces sp.*, and *Sporatrichum thermophilum*. Some protozoa living in the hindgut of lower termites can also produce cellulolytic enzymes to digest cellulose. In addition, plants synthesize cellulases which play a role in morphogenesis and development processes, such as the fruit ripening (Beguin and Aubert, 1982). Fungi and bacteria can produce many types of cellulolytic enzymes which are commonly classified into three categories: 1,4-$\beta$-D-Glucan-4-glucanohydrolase (EC 3.2.1.4, endoglucanases), 1,4-$\beta$-D-glucan-cellobiohydrolases (EC 3.2.1.91, cellobiohydrolases), and $\beta$-D-Glucoside glucohydrolases (EC 3.2.1.21, $\beta$-Glucosidases). However, the classification of cellulases into these groups does not explain the multiplicity in the mechanistic actions of these enzymes when degrading cellulosic materials. A "hydrophobic cluster analysis" of cellulase amino acid sequences has indicated that these enzymes fall into seven families, as shown in Table 2.2 (Henrissat *et al.*, 1989; Henrissat and Mornon, 1990).

The *Trichoderma reesei* cellulase mixture is comprised of at least four endoglucanases (EGs, approximately 15% of total cellulolytic enzyme mass), two cellobiohydrolases (CBH I and CBH II, approximately 60% and 25% of total cellulolytic enzyme mass) and $\beta$-glucosidases (Chirico and Brown, 1987). CBH I catalyzes the

Table 2.2. Classification of $\beta$-glycanases in families of homologous proteins.

| Family | Code | Enzyme | Source | Stereoselectivity | Type |
|---|---|---|---|---|---|
| A | $A_1$ | EG | *Bacillus subtilis* | | b |
| | $A_2$ | EG C | *Clostridium thermocellum* | | b |
| | $A_3$ | EG III | *Trichoderma reesei* | Retention | f |
| | $A_4$ | EG E | *Clostridium thermocellum* | | b |
| | $A_5$ | EG B | *Clostridium thermocellum* | | b |
| | $A_6$ | EG | *Bacillus* sp. strain 1139 | | b |
| | $A_7$ | EG | *Bacillus* sp. strain N-4 gene pNK1 | | b |
| | $A_8$ | EG | *Bacillus* sp. strain N-4 gene pNK2 | | b |
| | $A_9$ | EG Z | *Erwinia chrysanthemi* | | b |
| | $A_{10}$ | EG I | *Schizophyllum commune* | | f |
| | $A_{11}$ | EG | *Clostridium acetobutylicum* | | b |
| | $A_{12}$ | EG/CBH | *Caldocellum saccharolyticum* | | b |
| | $A_{13}$ | EG A | *Clostridium cellulolyticum* | | b |
| | $A_{14}$ | EG | *Xanthomonas campestris* | | b |
| | $A_{15}$ | EG | *Bacillus* sp. strain N-4 (gene pNK3) | | b |
| B | $B_1$ | CBH II | *Trichoderma reesei* | Inversion | f |
| | $B_2$ | EG A | *Cellulomonas fimi* | | b |
| | $B_3$ | EG | *Streptomyces* sp. (KSM-9) | | b |
| | $B_4$ | EG A | *Microbispora bispora* | | b |
| C | $C_1$ | CBH I | *Phanerochaete chrysosporium* | | f |
| | $C_2$ | CBH I | *Trichoderma reesei* | Retention | f |
| | $C_3$ | EG I | *Trichoderma reesei* | Retention | f |
| D | $D_1$ | EG | *Cellulomonas uda* | | b |
| | $D_2$ | EG A | *Clostridium thermocellum* | | b |
| | $D_3$ | EG Y | *Erwinia chrysanthemi* | | b |
| E | $E_1$ | EG D | *Clostridium thermocellum* | | b |
| | $E_2$ | EG | *Pseudomonas flurescens* | | b |
| | $E_3$ | EG | *Persea americana* | | p |
| F | $F_1$ | CBH | *Cellulomonas fimi* | | b |
| | $F_2$ | Xyn Z | *Clostridium thermocellum* | Retention | b |
| | $F_3$ | Xyn | *Cryptococcus albidus* | | f |
| | $F_4$ | Xyn A | *Bacillus* sp. | | b |
| | $F_5$ | EG/CBH | *Caldocellum saccharolyticum* | | b |
| G | $G_1$ | Xyn | *Bacillus subtilis* | Retention | b |
| | $G_2$ | Xyn A | *Bacillus pumilus* | | b |
| | $G_3$ | Xyn | *Bacillus circulans* | | b |

Type: b = bacteria; f = fungi; p = plant

From: Henrissat and Mornon (1990).

hydrolysis of cellodextrins and cellulose with retention of anomeric configuration. CBH II catalyzes the hydrolysis of cellodextrins and cellulose with inversion of configuration (Claeyssens *et al.*, 1990; Knowles *et al.*, 1988; Konstantinidis *et al.*, 1993). All of the *Trichoderma reesei* enzymes belong to one of three families. The two CBHs are in different families eventhough they are both exo-acting enzymes; CBH I is in family C, CBH II is in family B. Endoglucanase I (EG I) also belongs to family C, while endoglucanase III (EG III) is in family A.

Some properties of cellobiohydrolases, endoglucanases and $\beta$-glucosidases from different microorganisms are summarized in Tale 2.3, 2.4 and 2.5, respectively.

Table 2.3. Some properties of cellobiohydrolases from different microorganisms.

| Microorganism | Enzyme | MW (KDa) | pI | %Carbohydrate | Reference |
|---|---|---|---|---|---|
| *Trichoderma reesei* | CBH I | 64 | 3.9 | 5.6 | 1 |
| | CBH II | 53 | 5.9 | 18 | 1 |
| *Trichoderma viride* | Exo I | 53 | 5.3 | + | 2 |
| | Exo II | 60.5 | 3.5 | + | 2 |
| | Exo III | 62 | 3.8 | + | 2 |
| *Thermomospora fusca* | $E_3$ | 65 | 4.2 | nd | 3,4 |
| | $E_4$ | 90.2 | nd | nd | 3 |
| | $E_6$ | 106 | nd | nd | 3 |
| *Trichoderma koningii* | CBH 1 | 62 | 3.8 | 33 | 5 |
| | CBH 2 | 62 | 3.95 | 9 | 5 |
| *Penicillium funiculosum* | CBH | 46.3 | 4.36 | nd | 6 |

nd = not reported
+ = carbohydrate presented, but not reported
References: 1) Bhikhabhai *et al.*, 1984, 2) Beldman *et al.*, 1985, 3) Irwin *et al.*, 1993, 4) Walker *et al.*, 1992, 5) Fogarty, 1983, 6) Wood *et al.*, 1980.

Table 2.4. Some properties of endoglucanases from different microorganisms.

| Microorganism | Enzyme | MW (KDa) | pI | %Carbohydrate | Reference |
|---|---|---|---|---|---|
| *Trichoderma reesei* | EG II | 55 | 4.5 | 6 | 1 |
| | EG III | 48 | 5.5 | 10 | 1 |
| *Trichoderma viride* | Endo I | 50 | 5.3 | + | 2 |
| | Endo II | 45 | 6.9 | + | 2 |
| | Endo III | 58.5 | 6.5 | + | 2 |
| | Endo IV | 23.5 | 7.8 | - | 2 |
| | Endo V | 57 | 4.4 | + | 2 |
| | Endo VI | 52 | 3.5 | + | 2 |
| *Thermomospora fusca* | $E_1$ | 101.2 | -- | nd | 3 |
| | $E_2$ | 43 | 5.2 | nd | 3,4 |
| | $E_5$ | 46.3 | 5.02 | nd | 3,4 |
| *Trichoderma koningii* | $E_1$ | 13 | 4.72 | nd | 5 |
| | $E_3$ | 48 | 4.32 | nd | 5 |
| | $E_4$ | 31 | 5.09 | nd | 5 |

nd = not reported
+ = carbohydrate presented, but not reported
- = no carbohydrate presented
References: 1) Bhikhabhai *et al.*, 1984, 2) Beldman *et al.*, 1985, 3) Irwin *et al.*, 1993, 4) Walker *et al.*, 1992, 5) Fogarty, 1983.

Table 2.5. Some properties of $\beta$-glucosidases from different microorganisms.

| Microorganism | MW (KDa) | pI | %Carbohydrate | Reference |
|---|---|---|---|---|
| *Trichoderma reesei* | 47.7 | 5.74 | nd | 1 |
| | 81.6 | 8.5 | <1 | 2 |
| *Trichoderma viride* | 76 | 3.9 | + | 3 |
| | 47 | 5.74 | 0 | 4 |
| *Trichoderma koningii* | 39.8 | 5.53 | 0 | 1 |
| | 39.8 | 5.83 | 1-2 | 1 |
| *Aspergillus oryzae* | 218 | 4.3 | 10 | 1 |
| *Aspergillus niger* | 150 | nd | 0.03 | 1 |

nd = not reported
References: 1) Fogarty, 1983, 2) Chirico and Brown, 1987, 3) Beldman *et al.*, 1985, 4) Berghem and Pettersson, 1974.

2.3.2. Purification of Cellulolytic Enzymes

Cellulolytic enzymes are usually a mixture of several discrete enzymes which differ in their modes of action, molecular weights, amino acid sequences, and isoelectric points. Some components also occur as multiple isozymes which appear to differ with respect to their glycosylation. Due to this complexity of these cellulolytic systems, it is difficult to separate individual components. A combination of different purification methods is generally used to fractionate the individual components, including size exclusion chromatography, adsorption chromatography, ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, isoelectrofocusing, and immunochemical method. The individual components of cellulolytic enzymes, i.e. endoglucanases, cellobiohydrolases, and $\beta$-glucosidase obtained from different microorganisms including *Trichoderma viride*, *Trichoderma koningii*, and *Penicillium funiculosum* have been isolated by using size exclusion chromatography, ion exchange chromatography and isoelectrofocusing techniques (Berghem and Pettersson, 1973; Berghem *et al.*, 1975; 1976; Wood and McCrae, 1978; Wood *et al.*, 1980; Olama *et al.*, 1993).

The use of affinity chromatography has been successfully and extensively used to separate the individual components of cellulolytic enzymes, based on their specific affinities toward their complementary ligands. Only the enzymes of interest can bind to their complementary ligands, whereas the others cannot. The adsorbed target enzymes can be subsequently desorbed by changing the conditions of the system to decrease the interactions between the enzyme-ligand complex. Protein-ligand complexes can be

disrupted by modifications in temperature, pH, ionic strength, or by displacing with competitive compounds. Affinity chromatography using crystalline cellulose has been employed to separate the two cellobiohydrolases from *Trichoderma viride* (Beldman *et al.*, 1985). Immunoaffintiy chromatography has been used for isolating cellobiohydrolases from *Trichoderma reesei* (Nummi *et al.*, 1983). Concanavalin A (con A) is a specific ligand that has affinity for the carbohydrate moieties, $\alpha$-D-manopyranosyl and $\alpha$-D-glucopyranosyl of glycoproteins. Based on this specific property, con A immobilized on agarose, has been successfully used to separate non-glycosylated cellobiases from endoglucanases of *Trichoderma viride*; the latter being glycoproteins containing mannose and glucose as sugar moieties (Gong *et al.*, 1979; Kminkova and Kucera, 1982). Affinity chromatography of cellulase enzymes is practical, and powerful when thioglycosides are used as complementary ligands since they are known to be competitive inhibitors for glycosidases (Matta *et al.*, 1975). A thio compound of $\beta$-D-glucopynose was first introduced to separate $\beta$-glucosidases from *Stachybotrys atra*, and subsequently from *Alcaligenes faecalis* (De Gussem *et al.*, 1978; Holme *et al.*, 1988). A simple method for separating exocellulases by using affinity chromatography has been introduced by van Tilbeurgh *et al.* (1984). In this method, an aryl thioglycosides of cellobiose, *p*-aminobenzyl 1-thio-$\beta$-D-cellobioside is used as an immobilized ligand on agarose beads. Exocellulases from cellulase enzyme mixtures produce by *Trichoderma reesei*, *Penicillium pinophilum*, and *Thermomonospora fusca* have been successfully separated by the use of affinity chromatography with immobilized *p*-aminobenzyl 1-thio-$\beta$-D-cellobioside (Tomme *et al.*, 1988; Wood *et al.*, 1989; Walker *et al.*, 1993). A

modification of this technique employed a thioglycosidic linkage into the cellobiose portion of the ligand, *p*-aminophenyl 1,4-dithio-β-D-cellobioside (Orgeret *et al.*, 1992). The thio linkage between glucose units of this ligand makes it more resistant to hydrolysis of β-glucosidases, and thus prolongs the lifetime of the ligand (Orgeret *et al.*, 1992).

### 2.3.3. Assays for Determining Activities of Cellulolytic Enzymes

Due to the structural variability of cellulosic substrates and the complexity of cellulase enzyme systems, considerable problems have been established in measuring the activities of cellulolytic enzymes. Many assays have been developed in an attempt to meet the needs of enzymologists for quantifying the enzyme activities. The measurement of cellulolytic enzymes can be determined for a complete enzyme system or individual components by using either natural or modified substrates as summarized in Table 2.6 (Wood and Bhat, 1988).

The complete activity of cellulolytic enzymes can be measured by using crystalline cellulose such as filter paper, cotton and Avicel. The assay with filter paper, recommended by the Commission on Biotechnology (IUPAC) is widely used for presenting the total activity of cellulase mixtures, and the activity can be reported as the filter paper unit, FPU, based on the amount of enzyme that releases exactly 2.0 mg of glucose equivalents from a 50 mg sample of filter paper under defined conditions (Wood and Bhat, 1988).

The activity of individual components can be determined based on the differences in their modes of action. Exo-acting enzymes hydrolyze cellulose by removing cellobiose

Table 2.6. The methods for measuring cellulase activities.

| Enzyme | Substrate | Assay |
|---|---|---|
| Cellulase mixture | Cotton | Reducing sugar released<br>Weight loss<br>Loss in tensile strength |
| | Filter paper<br>Hydrocellulose<br>Avicel<br>Solka Floc | Release of reducing sugar |
| | Dyed Avicel | Release of dyed soluble fragments |
| Exoglucanase (Cellobiohydrolases) | Avicel<br>Hydrocellulose | Release of reducing sugar |
| | Dyed Avicel | Release of dyed cellobiose |
| | Amorphous cellulose | Release of reducing sugar or decrease in turbidity |
| | Cellooligosaccharides | Increase in reducing power or analysis by HPLC |
| Endoglucanases | Carboxymethylcellulose<br>Hydroxyethylcellulose | Release of reducing sugar<br>Decrease in viscosity |
| | Cellooligosaccharides | Increase in reducing power or analysis by HPLC |
| | Cotton | Swelling in alkali |
| | Amorphous cellulose | Release of reducing sugar or decrease in turbidity |
| $\beta$-Glucosidase | *o*- or *p*-Nitrophenyl-β-D-glucosides | Release of *o*- or *p*-nitrophenol |
| | Salicin or esculin glycosides | Release of glucose |
| | Cellobiose<br>Cellooligosaccharides | Increase of reducing power |

From: Wood and Bhat (1988).

from cellulose chain ends whereas endoglucanases can hydrolyze amorphous cellulose randomly, but hardly degrade the crystalline cellulose. Therefore, Avicel is the commonly used substrate for determining the activity of exoglucanases. Substituted cellulose substrates, formed by replacing the hydrogen of hydroxyl groups of cellulose with reactive groups such as methyl, carboxymethyl, and hydroxyethyl groups, are less susceptible to attack by exoglucanases since exoglucanases stop hydrolysis when encountering substituted residues after progressively removing unsubstituted residues from the end, due to their end-wise mechanism (Gilkes *et al.*, 1984). Hence, these substituted cellulose, such as carboxymethylcellulose and hydroxyethylcellulose, can be used as substrates for determining activities of endo-acting enzymes.

Synthetic substrates, such as *p*-nitrophenyl and methylumbelliferyl derivatives of glycosides, can also be used for determining cellulase activities. The activity can be measured based on the release of the chromophoric groups. The most commonly used synthetic substrate is *p*-nitrophenyl-$\beta$-D-glucopyranoside for determining the activity of $\beta$-glucosidases.

Enzyme activity can be monitored by measuring either a change in some substrate property, such as viscosity, turbidity and solubility, or the amount of products produced as a result of hydrolysis, i.e. the sugar or chromphoric groups from chromogenic substrates. Endo-acting enzymes hydrolyze modified cellulose randomly at the internal glycosidic linkages, thus causing dramatic changes in the degree of polymerization, and hence the viscosity of solutions containing these substrates. The relationship between polymer length (as viscosity) and reducing sugar released during the hydrolysis of a

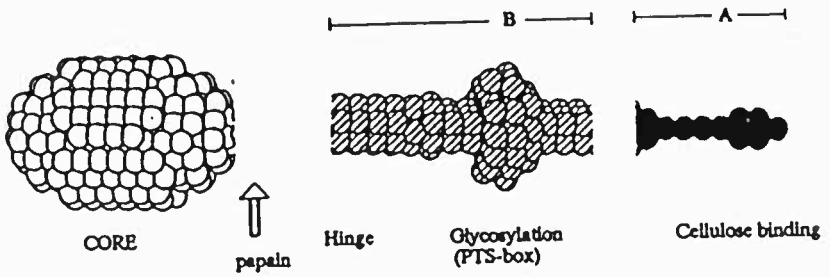
modified cellulose can help reveal the mode of enzyme action. Endoglucanases generate a steep curve between fluidity of the solution and the amount of reducing sugar released, whereas a much lower slope is expected for exoglucanases (Gilkes *et al.*, 1984). Therefore, viscometric methods are extremely sensitive for measuring the activity of endoglucanases, but not for exoglucanases. Since exoglucanases hydrolyze cellulose by removing cellobiose from the chain ends, the viscosity of the solution does not change at a significant rate compared with the number of hydrolytic events.

Enzyme activity can also be monitored by measuring the amount of products released during hydrolysis, i.e. glucose, cellobiose, and some cellooligosaccharides. Different techniques can be used to measure the amount of sugar released during a reaction, including high performance liquid chromatography, thin layer chromatography and colorimetric methods. Colorimetric methods are most commonly used for the estimation of sugars. Total sugars can be measured by the phenol-sulfuric method, while many assays can be used to quantify the amount of reducing sugar released due to enzymatic hydrolysis, including Somogyi-Nelson (Nelson, 1944), dinitrosalicylic acid (Ghose, 1987), disodium 2,2′-bicinochoninate (Garcia *et al.*, 1993), and *p*-hydroxybenzoic acid hydrazide (Lever, 1972).

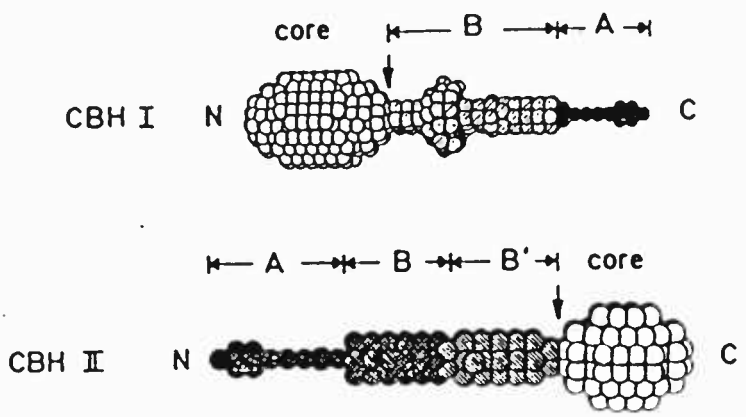
### 2.3.4. Three-Dimensional Structures of Cellulolytic Enzymes

Cellulases obtained from *Trichoderma reesei* are the most extensively studied among all cellulolytic enzymes. The tertiary structures of cellobiohydrolases from this fungi have been investigated by small angle X-ray scattering, and clearly elucidate the

typical tadpole structure of the intact enzyme with a more isotropic and ellipsoid shape for the head portion as shown in Figure 2.7 (Abuja *et al.*, 1988; Kubicek *et al.*, 1990). All *Trichoderma reesei* cellulases except EG III have similar structures; they consist of two different domains. The intact enzymes can be cleaved into two fragments by partial proteolysis, resulting in a core fragment with the catalytic domain and a tail fragment with the binding domain. These two fragments are linked together by a proline, serine and threonine rich O-glycosylated hinge region (Teeri *et al.*, 1992). The binding domains can be located at either the C-terminus of the primary sequence (i.e. CBH I) or at the N-terminus (i.e. CBH II), as shown in Figure 2.7. Similarities in the structure of CBH I and CBH II exists, however, there are significant differences in size and shape. CBH II has more elongated shape with a longer tail part, resulting from a duplication of block B in this fragment (Abuja *et al.*, 1988).




(a)




(b)

Figure 2.7. (a) The three-dimensional structure of *Trichoderma reesei* cellobiohydrolases as elucidated by small angle X-ray scattering. (b) Comparison between the tertiary structure of *Trichoderma reesei* CBH I and CBH II (Abuja *et al.*, 1988).

2.3.4.1. The catalytic domain of cellulolytic enzymes

To date, intact cellulases have not been crystallized, presumably due to the presence of the flexible tail fragment. However, the catalytic core of the enzymes have been successfully crystallized, which has allowed more studies on their three dimensional structures (Bergfors *et al.*, 1989; Divne *et al.*, 1993). The three dimensional structure of *Trichoderma reesei* CBH II core reveals $\alpha$-$\beta$ proteins consisting of seven strands with folding into a barrel topology essentially similar to the structure of triose phosphate isomerase. The active site tunnel is formed by two extended loops at the C-terminal end of the barrel. CBH II core contains cysteine residues resulting in a disulfide linkage that is involved in the formation of the tunnel shape of the enzyme active site. This tunnel shape presumably restricts the enzyme to an endwise hydrolysis mechanism. Two aspartic acid residues, Asp 175 and Asp 221, located in the center of the tunnel are the essential amino acids involved in hydrolysis of glycosidic linkages by acid/base catalysis (Rouvinen *et al.*, 1990).

Recently, the three-dimensional structure of the CBH I core from *Trichoderma reesei* was also determined. It consists of two large antiparallel $\beta$ sheets that stack face-to-face to form a $\beta$ sandwich, and has loops connecting $\beta$ strands. Similar to the structure of the CBH II core, these loops are partly stabilized by disulfide bridges. The CBH I core has a structure comparable to that of proteins in a family of bacterial $\beta$-glucanases with the folding topology of the legume lectins. Two glutamic acid residues, Glu 217 and Glu 212 are the important amino acids for glycosidic hydrolysis (Divne *et al.*, 1994). The three dimensional structures of these cellobiohydrolases provide a good explanation for

the difference in the modes of actions between endo- and exo-acting enzymes. By comparing the sequences of cellobiohydrolases and the homologous endoglucanases, several sequences corresponding to the tunnel loops have been lost during the evolution of those related endoglucanases, resulting in more open structures in active sites of these endo-acting enzymes. Thus, endoglucanases can hydrolyze the internal glycosidic bonds of the substrate molecules anywhere along the chain. In contrast, a restrict tunnel structure of cellobiohydrolase active site requires that the cellulose chain has to be threaded into the tunnel of active site for effective hydrolysis as shown in Figure 2.8 (Divne *et al.*, 1994).

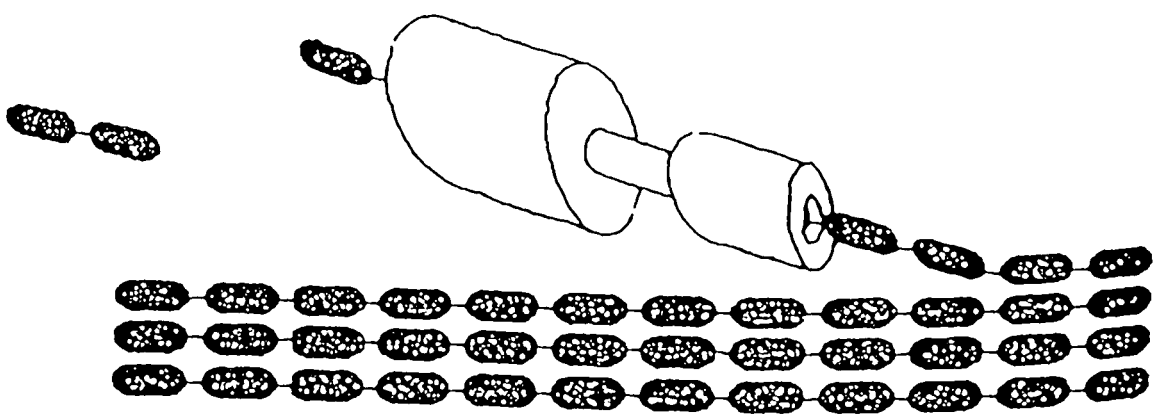


Figure 2.8. Schematic illustration of cellobiohydrolases acting processively on cellulose with an endwise mechanism (Coughlan, 1992).

The active sites of the CBHs have also been investigated. Early studies on the interaction of CBH II with small substrates and ligands suggested an active site containing four subsites, referr-ed to as A, B, C, and D. The glycosidic linkage to be cleaved is between the second and third subsites (van Tilbeurgh *et al.*, 1985). Binding experiments of crystallized core proteins of CBH II with small inhibitors and ligands, including *o*-iodobenzyl-1-thio-β-D-cellobioside, 4-methylumbelliferyl-β-cellobioside, and glucose-cellobiose complex support this model, i.e. CBH II active site comprises of four binding sites for glucosyl units (A-D), and the site of cellulose cleavage is likely to be between B and C (Rovuinen *et al.*, 1990). Initial binding studies of CBH I with small substrates and ligands suggest only three glucosyl binding subsites in the enzyme active site (Claeyssens *et al.*, 1989). Later studies indicate that the CBH I active site contains at least six glucosyl binding subsites (Shieh *et al.*, 1989). A study of crystallized core CBH I structure also suggests an extended active site with seven glucosyl binding subsites (A-G); the glycosidic bonds between subsites B and C is the one that is cleaved (Divne *et al.*, 1994).

2.3.4.2. The binding domains of cellulolytic enzymes

The binding domains of cellulolytic enzymes, located at either C- or N- terminus of enzymes, are linked to catalytic domains with a proline, serine and threonine rich O-glycosylated hinge region. The three-dimensional structure of the binding domains from *Trichoderma reesei* CBH I and CBH II, determined by NMR, exhibits a wedge-like shape with an amphiphilic character. The hydrophilic face contains tyrosine at conserved

positions, and this aromatic amino acid residue, demonstrated by specific modification, is somehow involved in binding of enzymes on insoluble cellulose. The binding domains of CBH I and CBH II contain some disulfide bridges, resulting in a compact structure (Claeyssens and Tomme, 1989). The native disulfide-bond in this three-dimensional structure also appears to be responsible for enzyme binding (Johansson *et al.*, 1989). The role of the enzyme binding domain has been examined by proteolytic removal of this fragment from the intact enzymes of two *Trichoderma reesei* cellobiohydrolases and two *Thermomonospora fusca* endoglucanases, $E_2$ and $E_5$. The resulting core fragments were still active on small soluble substrates, but a decrease in their adsorption capacities and activities on insoluble substrates are evidently observed, compared to the intact enzymes (Tomme *et al.*, 1988; Stahlberg *et al.*, 1993). These suggest a significant role of cellulase binding domains for effectively degrading crystalline cellulose.

2.3.5. Modes of Action of Cellulolytic Enzymes and Their Specificities.

A complete and effective hydrolysis of cellulosic material undoubtedly requires a cellulase mixture that consists of different enzymes that work in concert. Many studies have been done to investigate the actions of each component on the hydrolysis of cellulosic materials. The use of cellulose substrates for mechanistic studies still has some disadvantages since they are insoluble and do not have sufficiently well-defined structures. Due to the complexity and diversity of cellulose molecules in nature, soluble cellooligosaccharides and their derivatives have been used to investigate the modes of action and specificities of cellulolytic enzymes. The reaction patterns for hydrolysis of

some chromophoric glycosides by CBHs, and EGs from *Trichoderma reesei* and *Pennicillium phinophilum* have been studied and compared. CBH II has a specific hydrolysis pattern for cellobiose removal from the nonreducing end of glycosides, whereas CBH I attacks these substrates with less specificity than CBH II. EGs also exhibit a different hydrolysis pattern from those of CBHs (van Tilbeurgh *et al.*, 1982; 1985; 1988; Claeyssens *et al.*, 1989). Interestingly, a clear difference in specificities of these enzymes can be used to distinguish the type of enzymes. Only *Trichoderma reesei* CBH I and EG I can hydrolyze 4-methylumbelliferyl-$\beta$-D-cellobioside and lactoside. Therefore, CBH I and EG I can be differentiated from CBH II by the hydrolytic ability of this compound. Moreover, CBH I can be distinguished from EG I, based on a specific and selective inhibition by cellobiose for CBH I, but not for EG I (van Tilbeurgh *et al.*, 1988; Claeyssens and Aerts, 1992).

The hydrolysis specificity of exocellulases has been further studied by using modified cellooligosaccharides and/or cellulose. Reduced cellooligosaccharide and reduced alkali-swollen cellulose were used to investigated the action property of two exocellulases from *Aspergillus aculeatus*. The results suggested the different actions of two exocellulases toward these substrates; one exocellulase acting on the reducing end of the cellulose chain, whereas the other one acting on the nonreducing end of the same substrate (Arai *et al.*, 1989). Further study, using cellooligosaccharides labeled with tritium in the reducing moiety, was done to investigate the action pattern of CBH I derived from *Trichoderma reesei*; CBH I exhibited a marked preference to attack the first three linkages at the reducing ends of cellooligosaccharides (Vrasnska and Biely, 1992).

A hydrolysis study of Cbh B (previously called Cen E) from *Cellulomonas fimi* also exhibited the enzyme's ability to degrade cellodextrins from their reducing ends (Shen *et al.*, 1994). Recently, the hydrolytic specificities of different exo-acting celluases, two from *Trichoderma reesei* (CBH I and CBH II), and three from *Thermomonospora fusca* ($E_3$, $E_4$, and $E_6$) were investigated. The results suggested two different types of exocellulases based on their specificities; one class cleaves cellooligosaccharides from their reducing ends, including CBH I, $E_4$ and $E_6$. The other preferentially cleaves cellooligosaccharides from their nonreducing ends, including CBH II and $E_3$ (Barr *et al.*, 1996). All of these studies indicate the existence of two functional classes of exocellulases; one class preferentially hydrolyzing cellulose from the reducing end, the other preferentially hydrolyzing cellulose from the nonreducing end.

## 2.4. AFFINITY CHROMATOGRAPHY

Affinity chromatography is a very powerful technique in the separation of biological compounds due to their exceptional abilities to bind specifically and reversibly to their complementary compounds, called ligands. It is a type of adsorption chromatography in which the desired compounds to be separated are adsorbed on the ligands with the specific interactions, based on their biological functions, or individual chemical structures. These specific interactions result in ligand-substrate complex formation, a complex can also occur between enzymes and their inhibitors, substrates, cofactors or effectors; antibodies with antigens or haptens; lectins with carbohydrates; nucleic acids with nucleotides; hormones and toxins with receptors; transport proteins

with vitamins or sugars etc. (Turkova, 1978). Only the target compounds having specific interactions with the complementary ligands can bind, whereas other compounds cannot. These affinities between the desired compounds and ligands can subsequently be weakened by changing elution conditions that can no longer facilitate the formation of target compound-ligand complex.

In affinity chromatography, the complementary ligands, with respect to the isolated substances, are immobilized on the solid support. Different types of solid supports can be used including agarose, cellulose, dextran, polyacrylamide, silica and glass beads. Among these, crosslinked agarose is most commonly used due to the availability of many simple and convenient ligand coupling methods on this matrix. In general, the matrix support is initially activated to be reactive towards the functional groups of the ligands by chemical reactions. The activation of supporting matrix can be accomplished by the reactions with cyanogen bromide, bisepoxirane, sulphonyl chloride, carbonyldiimidazol, N-hydroxysuccinimide ester etc. These activated matrices are now ready to couple selectively with ligands, based on the functional groups of ligands, e.g. $-NH_2$, -OH, -SH, -COOH groups. Currently, many types of activated matrices are commercially available for coupling with different functional groups of ligands.

An important consideration in affinity chromatography is the strength of the interaction between the isolated compounds and their complementary ligands. Too weak an interaction will result in no absorption. In contrast, too strong an interaction will present difficulties in eluting the adsorbed compounds. In addition, the effectiveness and selectivity of affinity ligands are affected by nonspecific interactions, especially

hydrophobic and ionic interactions. These nonspecific interactions can influence either absorption or desorption of the isolated compounds. All of the interactions between the desired substances and their complementary ligands depend on many factors including steric accessibility, concentration of ligands and the isolated compounds, equilibration time, temperature, pH, and ionic strength.

# CHAPTER 3

# *p*-AMINOPHENYL 1-THIO-β-D-CELLOBIOSIDE; SYNTHESIS AND APPLICATION IN AFFINITY CHROMATOGRAPHY OF EXO-TYPE CELLULASES

## 3.1. ABSTRACT

One approach to the separation of cellulolytic enzymes is affinity chromatography. Affinity chromatography using the ligand *p*-aminobenzyl 1-thio-β-D-cellobioside (ABTC) is currently the most efficient method for separating exo- and endo-acting cellulases. We are introducing a new ligand, *p*-aminophenyl 1-thio-β-D-cellobioside (APTC), for this purpose. APTC is prepared by direct attachment of *p*-aminobenzenethiol to 2,3,6-tri-O-acetyl- 4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl bromide, and subsequent deacetylation. APTC has been coupled to N-hydroxysuccinimide activated agarose column for affinity chromatography. We have applied this support to the separation of the cellulolytic enzymes produced by *Trichoderma reesei*, a fungus which produces a complex mixture of endo-acting (endoglucanases) and exo-acting (cellobiohydrolases) acting enzymes. *Trichoderma reesei* cellulases appear to behave the same on APTC- and ABTC-derivatized columns, the major cellobiohydrolases bind and the major endoglucanases do not. Cellobiohydrolases can be eluted from the APTC-derivatized column by including cellobiose in the eluent. The primary advantage of the APTC-ligand versus the ABTC-

ligand is ease of preparation, the former requiring approximately one-half the number of synthetic steps as the latter.

## 3.2. INTRODUCTION

Microbial enzyme systems capable of efficiently degrading crystalline cellulose are composed of a mixture of hydrolases, each having their own specificity. Hydrolases common to these enzyme systems include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and $\beta$-glucosidases (EC 3.2.1.21) (Enari, 1983). The similarity of these enzymes often makes the purification of individual components difficult. Several methods have been used toward this end, including ion exchange chromatography, isoelectric focusing, immunochemical and affinity chromatography (Schulein, 1988; Fagerstam and Pettersson, 1979; Tomme *et al.*, 1988). Affinity chromatography using *p*-aminobenzyl-1-thio-$\beta$-D-cellobioside (ABTC) as the complementary ligand has proved to be particularly useful in this regard. This ligand was first introduced by van Tilbeurgh *et al.* (1984) for the separation of endo- versus exo-acting cellulases from *Trichoderma reesei*. They found that the major exo-acting enzymes were retained on the ABTC-derivatized support while the major endo-acting enzymes were not. This same trend has been observed for exo- and endo-acting cellulases produced by other microorganisms, e.g. *Penicillium pinophilum* (Wood *et al.*, 1989) and *Thermomonospora fusca* (Walker *et al.*, 1993).

Affinity chromatography of cellulases is most commonly performed as originally described; the crude enzyme mixture containing exo-cellulases is applied to the ABTC-

derivatized column in a neutral to slightly acidic buffer containing inhibitors of β-glucosidase. The exo-acting cellulases are retained on the column while other non-interacting proteins pass through. The exo-cellulases are then desorbed from the column by the addition of cellobiose to the eluting buffer. Inhibitors of β-glucosidases are routinely included in the mobile phase in order to extend the lifetime of the column. Orgeret *et al.* (1992) have introduced an alternative affinity ligand, *p*-aminophenyl 1,4-dithio-β-D-cellobioside (APDC), which alleviates the need for inclusion of β-glucosidase inhibitors in the mobile phase, because the 1,4 thio glycosidic linkage of APDC is resistant to β-glucosidase catalyzed hydrolysis.

These affinity ligands would undoubtedly be used by more laboratories if they were either commercially available or more easily synthesized. Unfortunately, the procedure used to prepare ABTC ligand for this use is difficult and laborious as shown in Figure 3.1 (van Tilbeurgh *et al.*, 1984). We have sought to identify a less labor intensive means of obtaining an affinity ligand for the purification of exo-cellulases. Our approach was to prepare an affinity column using the *p*-aminophenyl 1-thio-glycoside of cellobiose (APTC) as the complementary ligand. We anticipated APTC to be an effective ligand based on the use of corresponding derivatives for the purification of other glycosidases (Iino and Yoshida, 1976) and on the observed affinity of exocellulases for the *p*-aminophenyl 1-thio derivative of 4-thiocellobiose (Orgeret *et al.*, 1992). In this study we present a relatively simple synthetic protocol for the preparation of APTC as shown in Figure 3.2, and we show that the chromatographic behavior of representative cellulolytic

enzymes on APTC-derivatized supports is essentially the same as that reported on ABTC-derivatized supports.

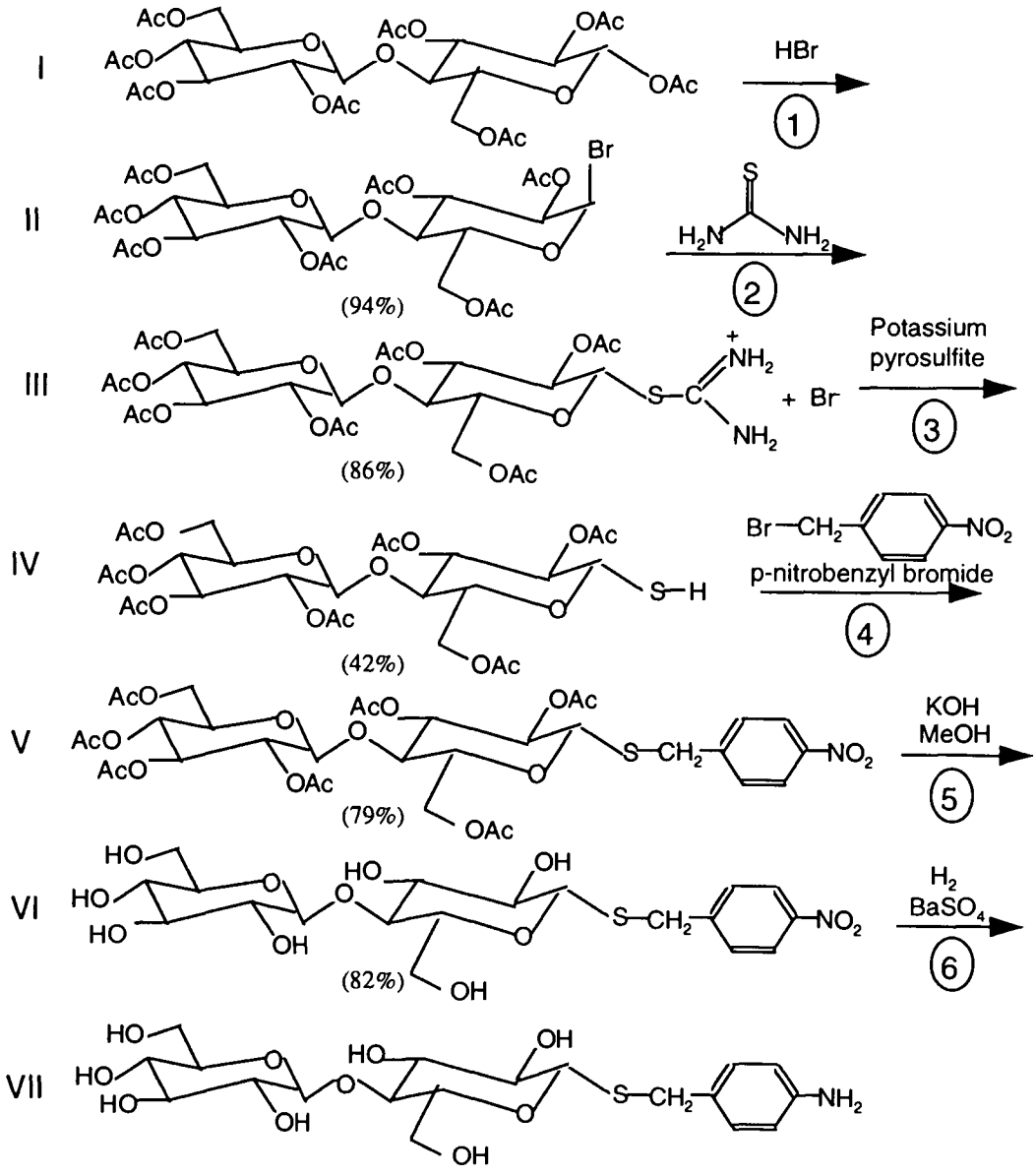



Figure 3.1. Preparation of *p*-aminobenzyl 1-thio-β-D-cellobioside (ABTC) by van Tilbeurgh *et al.* (1984). The numbers in parenthesis represent the yield of each compound.

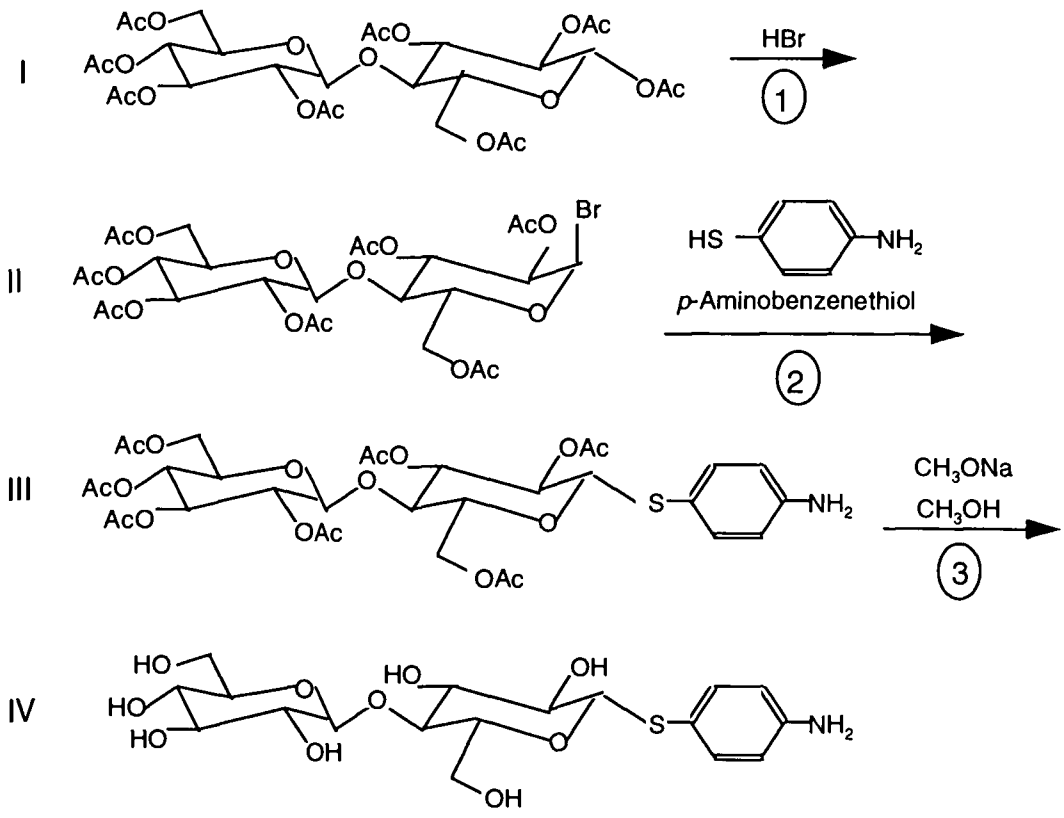



Figure 3.2. New preparation of *p*-aminophenyl 1-thio-β-D-cellobioside (APTC).

## 3.3. MATERIALS AND METHODS

### 3.3.1. Materials

$\alpha$-D-Cellobiose octaacetate, HBr (30% wt solution in acetic acid), and *p*-aminobenzenethiol were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Affigel 10 was obtained from BioRad Laboratories (Hercules, CA). DEAE-Sepharose CL 6B and phenyl Sepharose FF (low sub) were purchased from Pharmacia Inc. (Piscataway, NJ). *Trichoderma reesei* crude cellulase, Spezyme™-CP, was obtained from Environmental BioTechnologies, Inc. (Menlo Park, CA).

### 3.3.2. General Methods

IR spectra of intermediates were obtained using a Nicolet-510P spectrophotometer. The $^1$H nmr spectra were recorded at 400 MHz with a Bruker AM 400 spectometer, using tetramethylsilane as an internal standard. Assignments were confirmed by double resonance irradiation and 2D-COSY experiments. Enzyme-containing fractions resulting from chromatography were identified by their UV absorbance (Shimadzu UV 160U spectrophotometer).

### 3.3.3. Synthesis of *p*-Aminophenyl 1-Thio-$\beta$-D-Cellobioside (APTC)

#### 3.3.3.1. 2,3,6-Tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-$\beta$-D-glucopyranosyl)-$\beta$-D-glucopyranosyl bromide (II)

30 mL of HBr (30% wt solution in acetic acid) was added to a cold solution of 2.00 g (2.95 mmol) $\alpha$-D-cellobiose octaacetate (I) dissolved in 25 mL dichloromethane, then stirred for 5 hr at 0°C. The mixture was then diluted with 50 mL dichloromethane

and successively washed with equal volumes of ice-cold aqueous 10% $KHSO_4$ (2x), aqueous saturated $NaHCO_3$ (2x) and water (2x). The washed dichloromethane solution was dried over anhydrous $Na_2SO_4$ and evaporated at room temperature under reduced pressure to give compound II (2.05 g, 99%): m.p. 182-184°C; lit. m.p. 185°C (Scheurer and Smith, 1954).

3.3.3.2. *p*-Aminophenyl 2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-*β*-D-glucopyranosyl) -*β*-D-glucopyranoside (III)

Glycosyl bromide (II) (approximately 2 g, 2.9 mmol) was dissolved in 80 mL acetone; the resulting solution was mixed with 56 mL of an aqueous solution containing 1.76 g (12.7 mmol) $K_2CO_3$ and 1.6 g (12.7 mmol) *p*-aminobenzenethiol. This reaction mixture was stirred overnight at room temperature. The solvent was subsequently removed by evaporation at room temperature under reduced pressure. The resulting residue was dissolved in 100 mL dichloromethane; the dichloromethane solution was then washed, dried and evaporated as described above. The target compound was purified by silica gel chromatography (70-230 mesh, SIGMA Chemical Co., St. Louis, MO), 2.5 x 40 cm column, using an ethyl acetate:hexane (1:1,v/v) eluent. Column fractions were monitored by absorbance at 260 nm. Significant peaks were subsequently tested by thin layer chromatography using silica plates (LK6DF, Whatman Inc., Clifton, NJ) and the same mobile phase as used for column chromatography. The compound of interest was identified by positive tests with both *p*-anisaldehyde- (sugar) and ninhydrin-based (amine) visualizing reagents (Bolliger *et al.*, 1965). Fractions containing the target compound were pooled, evaporated to dryness under reduced pressure and then

crystallized from methanol to yield compound III (1.1 g, 53%): m.p. 190-193°C; IR (NaCl): ν 1757 and 1226 (ester) and 3468 and 3395 $cm^{-1}$ (amine); $^1H$ nmr ($CDCl_3$): δ 1.97-2.1 (s, 21 H, OAc), 3.55 (m, 1 H, H-5), 3.64 (m, 1 H, H-5′), 3.69 (m, 1 H, H-4), 3.78 (s, 2 H, NH), 4.0 (dd, 1 H, $J_{5'6a'}$ 1.9 Hz, $J_{6a'6b'}$ 12.5 Hz, H-6a′), 4.05 (dd, 1 H, $J_{56a}$ 5.1 Hz, $J_{6a6b}$ 11.9 Hz, H-6a), 4.35 (dd, 1 H, $J_{5'6b'}$ 4.1 Hz, $J_{6a'6b'}$ 12.5 Hz, H-6b′), 4.48 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.5 (d, 1 H, $J_{1'2'}$ 10.2 Hz, H-1′), 4.58 (dd, 1 H, $J_{56b}$ 1.4 Hz, $J_{6a6b}$ 11.9 Hz, H-6b), 4.8 (t, 1 H, $J_{2,3}$ 9.7 Hz, H-2), 4.9 (t, 1 H, $J_{2'3'}$ 8.5 Hz, H-2′), 5.05 (t, 1 H, $J_{4'5'}$ 9.6 Hz, H-4′), 5.1 (t, 1 H, $J_{3'4'}$ 9.2 Hz, H-3′), 5.15 (t, 1 H, $J_{3,4}$ 9.2 Hz, H-3), 6.5 (d, 2 H, $J$ 8.2 Hz, ArH), and 7.27 (d, 2 H, $J$ 8.2 Hz, ArH).

3.3.3.3. *p*-Aminophenyl 1-thio-β-D-cellobioside (IV)

The protected thioglycoside (III) was deacetylated by treatment with sodium methoxide in methanol as described by Thompson and Wolfrom (1972). A half mL of 1M sodium methoxide in methanol was added to 0.5 g (0.67 mmol) of compound III dissolved in 150 mL methanol and the mixture was stirred overnight at room temperature. It was then stirred with Amberlite IR-120 (H+) (SIGMA Chemical Co., St. Louis, MO), until the solution was neutral. The resin was removed by filtering and the solvent removed by evaporation under reduced pressure. The resulting residue was dissolved in 5 mL distilled water, mixed with 0.01 g decolorizing carbon and filtered. The filtrate was dried at room temperature by evaporating under reduced pressure; the target compound (IV) was then crystallized from methanol (0.26 g, 85%): m.p. 247-250°C. Complete deacetylation was confirmed by the absence of acetyl-proton resonances in NMR

experiments. $^1$H nmr ($Me_2SO$-d6): δ 3.0 (m, 6 H,), 3.24 (d, 1 H, *J* 5.8), 3.3 (m, OH), 3.65 (m, 4 H), 4.25 ( d, 1 H, *J* 9.6), 4.25 ( d, 1 H, *J* 6), 4.5 (m, 2 H), 4.7 (d, 1 H), 5 (dd, 2 H, *J* 11.6), 5.2 (dd, 4 H), 6.50 (d, 2 H, *J* 8.4 Hz, ArH), and 7.20 (d, 2 H, *J* 8.4 Hz, ArH),

### 3.3.4. Separation of *Trichoderma reesei* Cellobiohydrolases

#### 3.3.4.1. Anion-exchange chromatography

Crude cellulases were initially separated by DEAE-Sepharose chromatography as described by Beldman *et al.* (1985) Crude cellulase (200 mg) in 5 mL starting buffer (50 mM sodium acetate, pH 5) was applied to the column (2.5x16 cm) at 4°C. The column was washed with starting buffer prior to running a gradient from 0-0.5 M NaCl in starting buffer. Fractions containing cellulase activity were dialyzed against 50 mM sodium acetate, pH 5, and concentrated using an Amicon dialysis cell equipped with a PM-10 membrane (Molecular weight cut off = 10,000, Amicon Co., Danvers, MA). The partially purified DEAE-fractionated cellulases were used for affinity chromatography experiments.

#### 3.3.4.2. Affinity chromatography

APTC was coupled to Affigel 10 (BioRad Laboratories) as suggested by the manufacturer. Ten mL of Affigel 10 was added to 20 mL of cold 0.05 M $NaHCO_3$, pH 8.3, containing 0.09 g (0.2 mmol) of APTC. The suspension was gently agitated at 4°C for 4 hr. The ligand-coupled gel was then washed with fresh $NaHCO_3$ coupling buffer. The washed gel was mixed with 75 mL of 1 M ethanolamine, pH 8, and agitated at room

temperature for 2 hr. The gel was then washed with fresh coupling buffer, loaded into silica glass columns (1 x 10 cm) and subsequently equilibrated with the initial mobile phase buffer.

Crude exocellulase preparations, CBH I and CBH II, obtained from DEAE-Sepharose chromatography were applied to APTC-derivatized affinity columns. The initial mobile phase buffer for CBH I chromatography was 0.1 M sodium acetate, pH 5, containing 1 mM gluconolactone; the initial buffer for CBH II chromatography was the same as that for CBH I with the exception that the buffer was also 0.2 M glucose. All solutions were 1 mM in gluconolactone in order to retard ligand hydrolysis in the presence of $\beta$-glucosidases (van Tilbeurgh *et al.*, 1984). Exo-cellulases were applied to columns and the columns were subsequently washed with the initial mobile phase buffer until a background baseline, based on absorbance at 280 nm, was established. Mobile phase flow rates were approximately 0.5 mL/min and fractions were collected for 10 min. Adsorbed CBHs were eluted from the affinity columns by making the starting buffer 0.01 M in cellobiose.

3.3.4.3. Hydrophobic interaction chromatography (HIC)

CBH I- and CBH II-containing fractions from APTC-derivatized affinity chromatography, in 50 mM sodium acetate buffer (pH 5), were chromatographed on a phenyl Sepharose FF column (1 x 10 cm) to separate intact enzymes from catalytic core fragments. CBH I and CBH II were applied to the columns in 25 mM sodium acetate buffer at pH 5, made either 0.85 or 0.35 M ammonium sulfate, respectively. Columns

were washed with starting buffer prior to eluting enzymes with a gradient from 0.85 - 0.35 M ammonium sulfate, for CBH I, or from 0.35 - 0.01 M ammonium sulfate, for CBH II, in 25 mM sodium acetate buffer, pH 5.

3.3.5. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 10% acrylamide gels (Hoefer Scientific Instruments, 1986).

3.3.6. Activity Measurements

Endo-type cellulase activity was measured with either carboxymethyl cellulose (CMC; CMC7HOF, Aqualon Co., Wilmington, DL) or hydroxyethyl cellulose (HEC; medium viscosity, Fluka Chemical Corp., Ronkonkoma, NY) substrates. Enzyme solution, 0.2 mL, was added to 0.8 mL of 0.5% CMC, or 1.8 mL of 0.5% HEC, in 50 mM sodium acetate buffer, pH 5. Enzyme-substrate mixtures were incubated at 50°C for 30 min. Reactions were terminated by boiling 5 min; new reducing ends were estimated by comparing total reducing sugars in reaction mixtures versus those in substrate blanks using the alkaline-copper assay of Nelson (1944). Results are reported in units of mg reducing sugar $min^{-1}$ mg $protein^{-1}$.

Exo-type cellulase activity was measured with a microcrystalline cellulose (MCC; Avicel PH 101, FMC Cor. Philadelphia, PA) substrate. Enzyme solution, 0.3 mL, was added to 1.5 mL 50 mM sodium acetate buffer, pH 5, containing 1.8 mg MCC. Enzyme-substrate mixtures were incubated at 50°C for 4 hr with orbital shaking at 160 rpm.

Reactions were terminated by centrifuging to pellet the insoluble MCC, and then removing the soluble phase. Total reducing sugar liberated into the soluble phase was then determined by the alkaline-copper assay of Nelson (1944). Results are reported in units of mg reducing sugar $hr^{-1}$ mg $protein^{-1}$.

$\beta$-glucosidase activities were determined at 50°C by measuring rates of *p*-nitrophenyl-$\beta$-D-glucoside hydrolysis as described by Wood and Bhat (1988).

## 3.4. RESULTS AND DISCUSSION

The synthetic approach summarized in Figure 3.2 for APTC represents a relatively simple means of obtaining an affinity ligand for exo-acting cellulases. The glycosyl bromide (II) of commercially available cellobiose octaacetate (I) is prepared in step one. This intermediate is then reacted with commercially available *p*-aminobenzenethiol, yielding peracetylated APTC (III). The structure of peracetylated APTC (III) is determined by $^1H$ and COSY nmr (Figure 3.3 and 3.4, respectively). APTC (IV) is then prepared by deacetylation in basic methanol. The structure of the ultimate compound, APTC (IV), is also investigated by $^1H$ and COSY nmr (Figure 3.5 and 3.6, respectively). The total synthesis requires only three steps, which is a significant reduction compared to six steps required for the synthesis of ABTC, the traditional affinity ligand for exo-cellulases (van Tilbeurgh *et al.*, 1984). The overall synthetic yield of APTC was 45%, which compares favorably to the 22% yield reported for the synthesis of ABTC (Irwin, 1994). Amino-containing ligands can be readily coupled to a variety of commercially

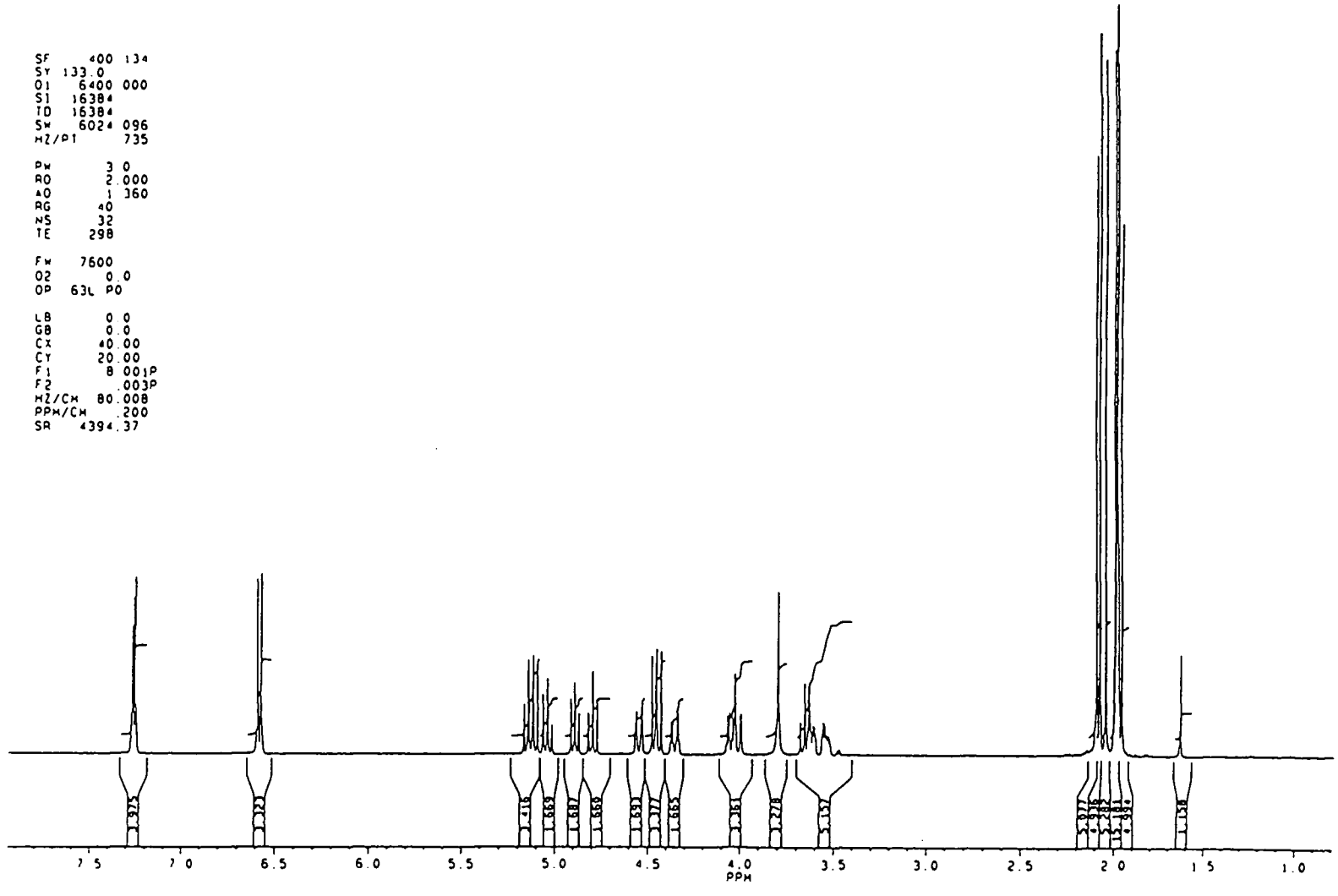



Figure 3.3. 400-MHz $^{1}H$ NMMR spectrum of peracetylated compound (III) in $CDCl_3$.

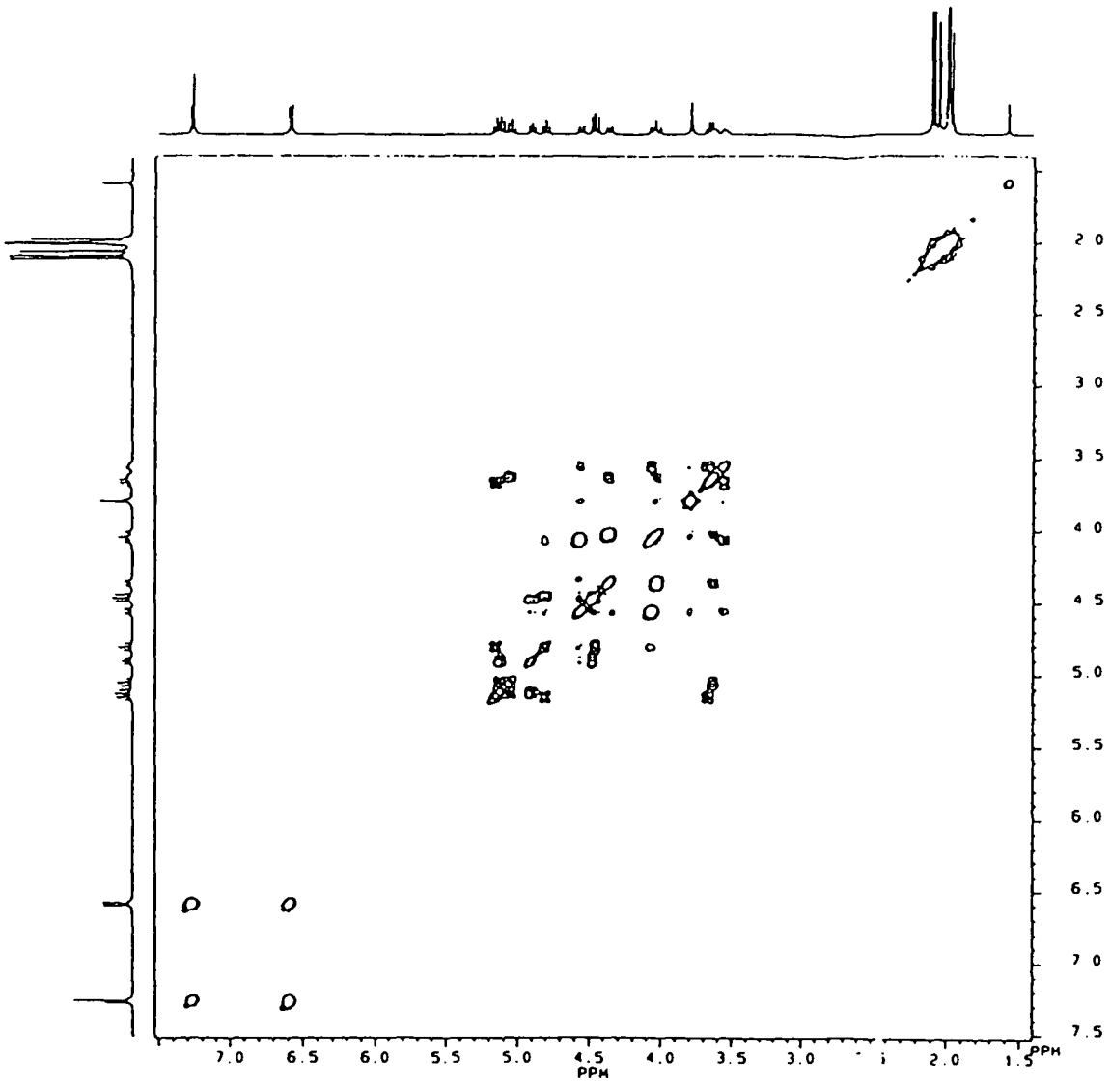



Figure 3.4. 400-MHz COSY spectrum of peracetylated compound (III) in $CDCl_3$.

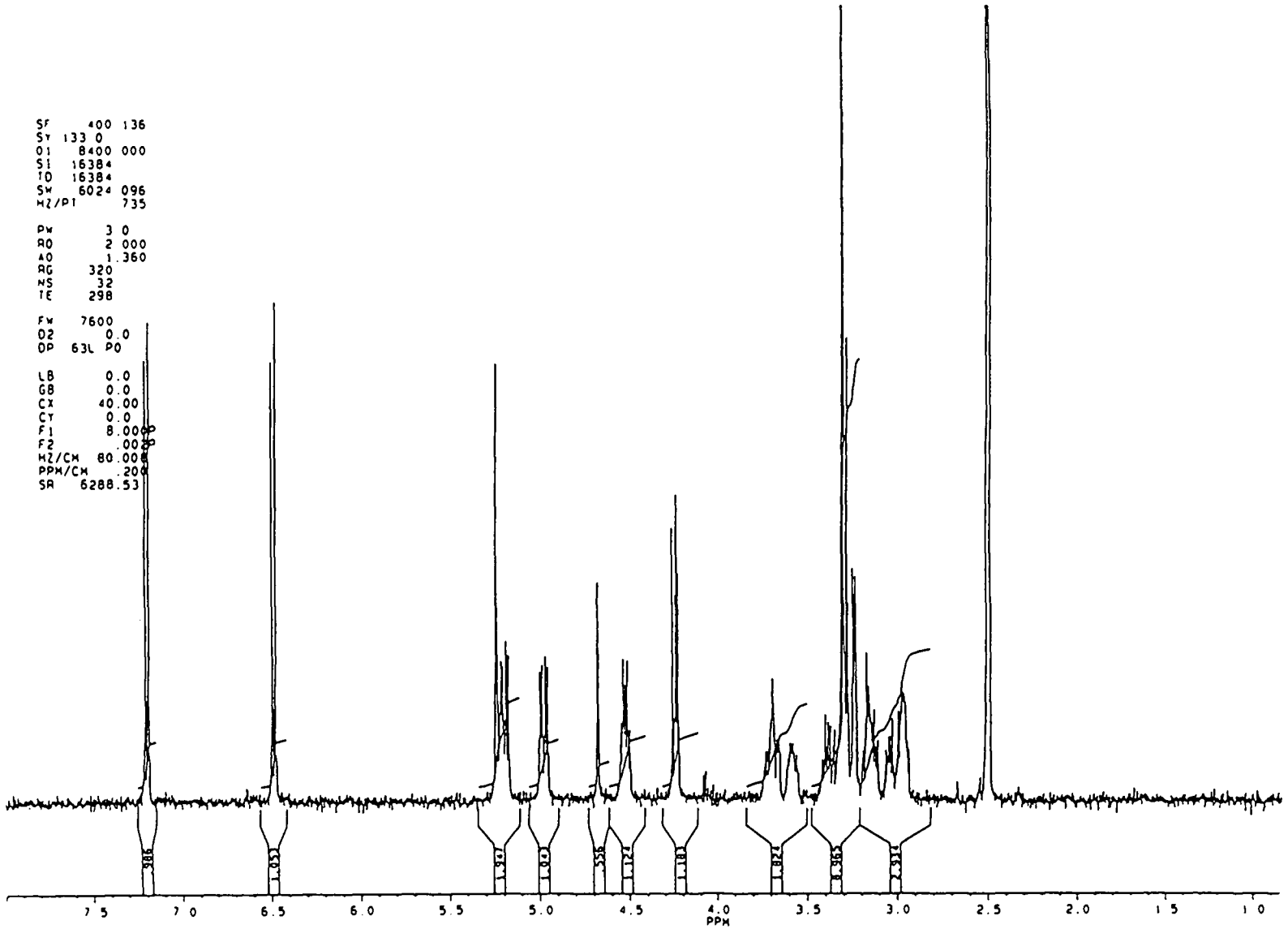



Figure 3.5. 400-MHz $^1$H NMMR spectrum of APTC (IV) in $Me_2SO$.

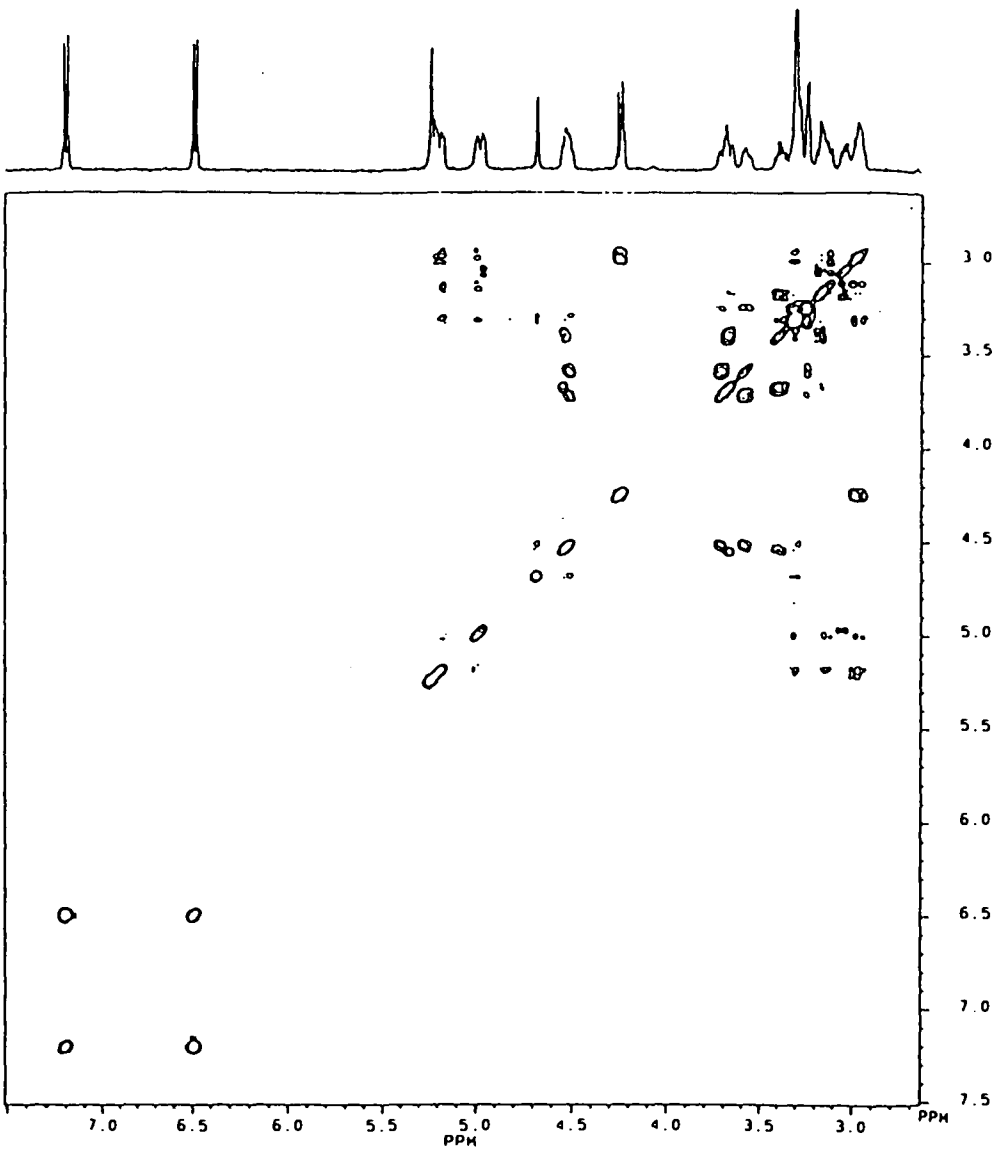



Figure 3.6. 400-MHz COSY spectrum of APTC (IV) in $Me_2SO$.

available supports (Carlsson *et al.*, 1989). In this work the APTC ligand was coupled to NHS-activated agarose. These APTC-derivatized supports had maximum binding capacities, based on tests with CBH I, of approximately 4-5 mg protein per mL gel.

Affinity chromatography is widely recognized as an important tool for the purification of exo-acting cellulase. In a typical purification protocol, crude cellulase mixtures are initially fractionated by DEAE-anion exchange chromatography (*Tomme et al.*, 1988). Fractions containing exo-cellulase activity are then applied to an affinity column in order to separate the exo-cellulases from contaminating non-cellulolytic proteins, endo-cellulases and $\beta$-glucosidases. This protocol was first applied to the purification of exo-acting cellulases from *Trichoderma reesei* (van Tilbeurgh *et al.*, 1984). The *Trichoderma reesei* enzyme system contains two principle exo-acting cellulases, cellobiohydrolase I (CBH I) and cellobiohydrolase II (CBH II) (Teeri *et al.*, 1992). It also contains endo-acting cellulases and $\beta$-glucosidases. CBH I and CBH II are readily separated in the initial anion-exchange purification step; the pIs of CBH I and CBH II being 3.9 and 5.9, respectively (Bhikhabhai *et al.*, 1984). However, the cellobiohydrolase preparations resulting from this step are generally not functionally pure, due to coelution of endo-acting enzymes. Coelution is the result of corresponding endo- and exo-acting cellulases being quite homologous (Divne *et al.*, 1994). These homologous pairs are difficult to separate by ion exchange, size exclusion and immunochemical methods; affinity chromatography being the most widely recognized approach for their separation (Tomme *et al.*, 1988). The application of APTC as an appropriate affinity ligand for this type of separation is here demonstrated by using this

ligand in the same manner as reported for the ABTC-based purification of *Trichoderma reesei* cellobiohydrolases (Tomme *et al.*, 1988).

A chromatogram showing fractionation of the crude enzyme mixture by anion-exchange chromatography is presented in Figure 3.7, activities corresponding to the major peaks are given in Table 3.1. The breakthrough peak contains cellobiohydrolase (CBH II), endoglucanase and $\beta$-glucosidase activities. The trailing peak contains the other cellobiohydrolase (CBH I) along with some endoglucanase activity.

Figures 3.8 and 3.9 demonstrate the use of the APTC affinity ligand for purification of the two cellobiohydrolases obtained from anion-exchange chromatography. Pooled fractions containing either CBH I or CBH II were applied to the affinity column. Non-retained proteins were then washed through with buffer, and cellobiohydrolases were then eluted by addition of cellobiose in the mobile phase. Experiments with partially purified endoglucanases and $\beta$-glucosidases showed that these enzymes were not retained on the affinity column (Figure 3.10 for endoglucanase I). The activities of the affinity-purified cellobiohydrolases, CBH I and CBH II (Table 3.1), demonstrate the functional purity of these preparations. The electrophoretic purity of the two cellobiohydrolases resulting from APTC-derivatized affinity chromatography is shown in lanes D and F of Figure 3.11. The relatively weak staining bands of lower molecular weight are due to the presence of catalytically active exocellulase core fragments that result from proteolysis of the intact enzymes (Teeri *et al.*, 1992). The core fragments can be separated from the intact enzymes by hydrophobic interaction chromatography (HIC; Figure 3.12 and 3.13 for CBH I and CBH II, respectively); the

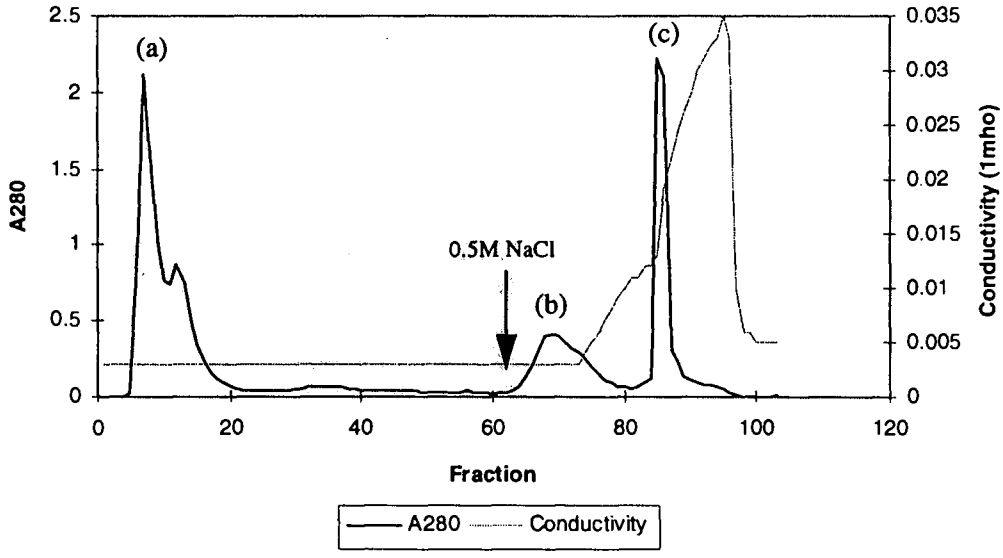



Figure 3.7. DEAE-Sepharose chromatography of *Trichoderma reesei* cellulases. Crude cellulase in 50 mM sodium acetate buffer pH 5 was applied to the DEAE-Sepharose column, and eluted with 0-0.5 M NaCl. Peak a: $\beta$-glucosidase + endoglucanases + CBH II; Peak b: endoglucanases; Peak C: endoglucanases + CBH I.

Table 3.1. Specific activities of affinity-purified cellobiohydrolases.

| Enzyme | β-Glucosidase (mg *p*-nitrophenol $min^{-1}$ mg $protein^{-1}$) | HECase* (mg reducing sugar $min^{-1}$ mg $protein^{-1}$) | Avicelase** (mg reducing sugar $hr^{-1}$ mg $protein^{-1}$) |
|---|---|---|---|
| Crude cellulase | 0.029 | 0.270 | 1.674 |
| Anion exchange column | | | |
| 1st peak (a) | 0.065 | 0.151 | 0.368 |
| 2nd peak (b) | 0.000 | 0.137 | 0.015 |
| 3rd peak (c) | 0.001 | 0.059 | 0.294 |
| APTC- affinity column | | | |
| Unretarded CBH I (d) | 0.003 | 0.227 | 0.004 |
| CBH I (e) | 0.000 | 0.016 | 0.126 |
| Unretarded CBH II (f) | 0.109 | 0.380 | 0.065 |
| CBH II (g) | 0.000 | 0.024 | 0.189 |

*Hydroxyethylcellulose was used to determine the endocellulase activity.
**Avicel was used to determine the exocellulase activity.

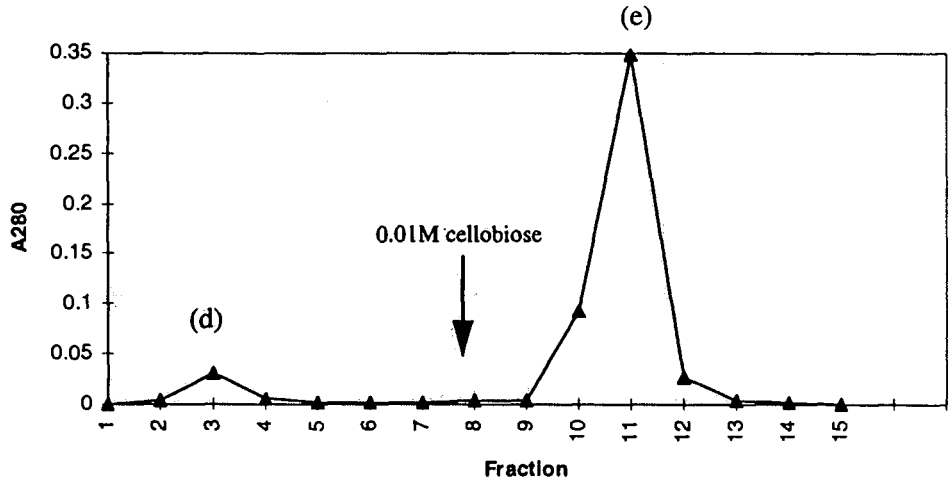



Figure 3.8. APTC-derivatized affinity chromatography of CBH I. CBH I fraction obtained from DEAE-Sepharose column was applied to APTC-derivatized affinity column, and eluted with 0.01 M cellobiose in 0.1 M sodium acetate buffer pH 5, containing 1 mM gluconolactone. Peak D: unretarded CBH I (endoglucanases); Peak E: CBH I.

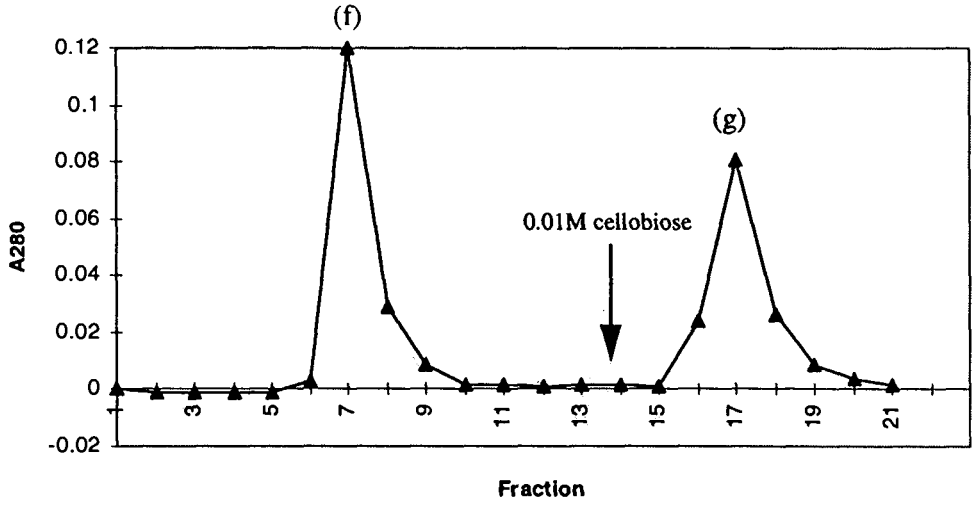



Figure 3.9. APTC-derivatized affinity chromatography of CBH II. CBH II fraction obtained from DEAE-Sepharose column was applied with 0.2 M glucose to APTC-derivatized affinity column, and eluted with 0.01 M cellobiose in 0.1 M sodium acetate buffer pH 5, containing 1 mM gluconolactone. Peak F: unretarded CBH II ($\beta$-glucosidase + endoglucanases); Peak G: CBH II.

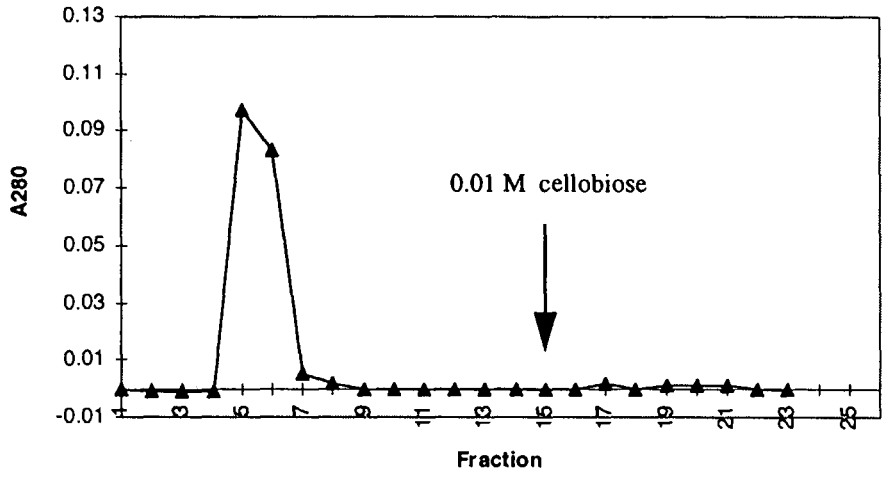



Figure 3.10. APTC-derivatized affinity chromatography of endoglucanase I. Endoglucanase I obtained from DEAE-Sepharose column was applied to APTC-derivatized affinity column, and eluted with 0.01 M cellobiose in 0.1 M sodium acetate buffer pH 5, containing 1 mM gluconolactone.

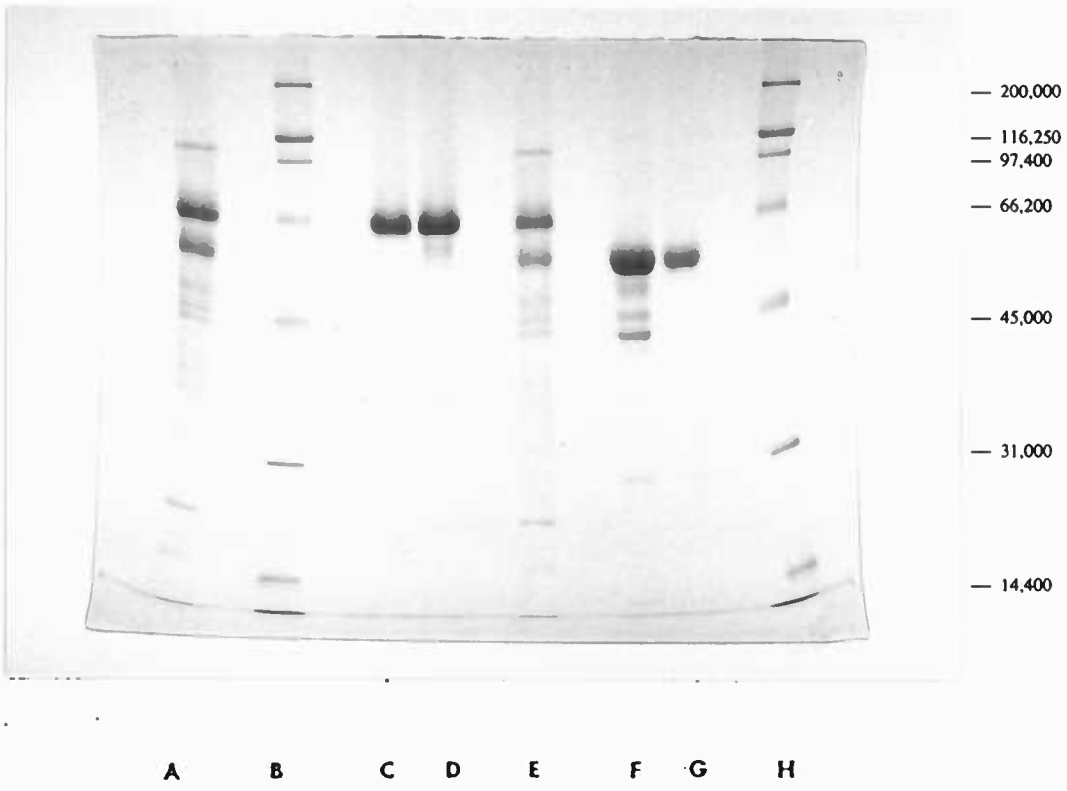



Figure 3.11. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cellulase before and after purification. Lane A and E: crude cellulase; Lane B and H: Protein standard; Lane C: CBH I received from APTC-derivatized affinity and HIC column; Lane D: CBH I received from APTC-derivatized affinity column; Lane F: CBH II received from APTC-derivatized affinity column; and Lane G: CBH II received from APTC-derivatized affinity and HIC column.

intact enzymes are retained on the column while the core fragments pass through with the void volume (Siika-Aho, 1996). The electrophoretic purity of the two intact cellobiohydrolases, following HIC, is shown in lanes C and G of Figure 3.11. The activities of purified CBHs are summarized in Table 3.2.

In conclusion, APTC-derivatized affinity columns appear to have the same chromatographic properties as those reported for standard ABTC-derivatized affinity columns and thus, could be used interchangeably. The primary advantage of using APTC-derivatized columns is in the ease of preparation of the ligand. As described in the Experimental section, preparation of the APTC ligand requires half the number of synthetic steps needed for the traditional ABTC ligand.

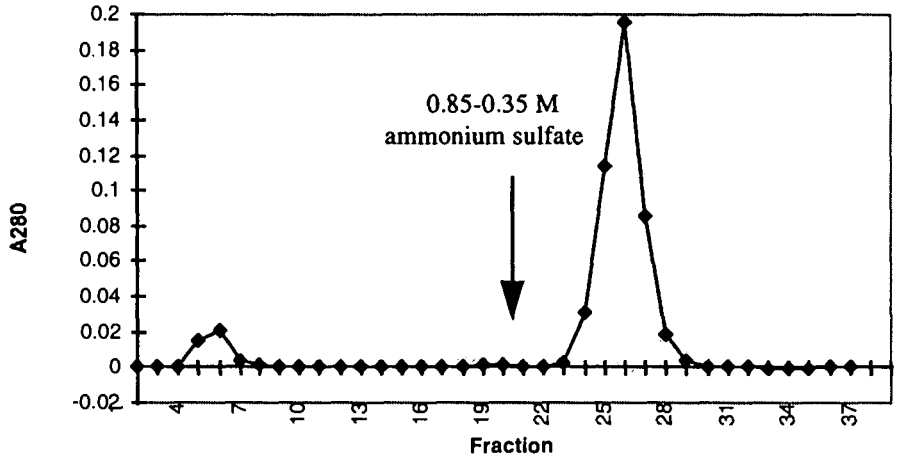



Figure 3.12. Hydrophobic interaction chromatography of CBH I. CBH I fraction obtained from DEAE-Sepharose and APTC-derivatized affinity column was applied to phenyl Sepharose FF column with 0.85 M ammonium sulfate, and then eluted with a gradient from 0.85-0.35 M ammonium sulfate in 25 mM sodium acetate buffer at pH 5.

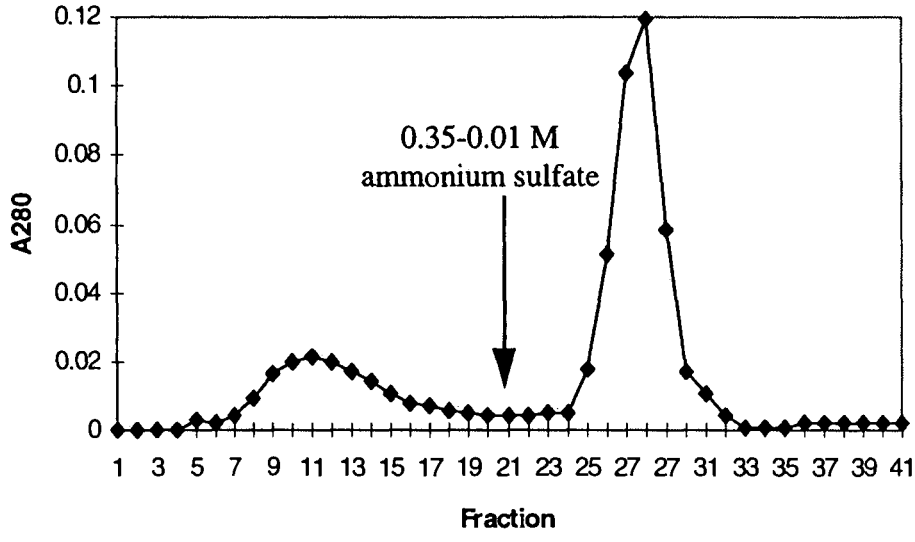



Figure 3.13. Hydrophobic interaction chromatography of CBH II. CBH II fraction obtained from DEAE-Sepharose and APTC-derivatized affinity column was applied to phenyl Sepharose FF column with 0.35 M ammonium sulfate, and then eluted with a gradient from 0.35-0.01 M ammonium sulfate in 25 mM sodium acetate buffer at pH 5.

Table 3.2. Specific activity of purified cellobiohydrolases after anion exchange, affinity and hydrophobic interaction chromatography.

| Enzyme | β-Glucosidase (mg *p*-nitrophenol $min^{-1}$ mg $protein^{-1}$) | Endocellulase* (mg reducing sugar $min^{-1}$ mg $protein^{-1}$) | Exocellulase** (mg reducing sugar $hr^{-1}$ mg $protein^{-1}$) |
|---|---|---|---|
| Crude cellulase | 0.027 | 0.300 | 1.580 |
| CBH I | 0.000 | 0.001 | 0.205 |
| CBH II | 0.000 | 0.017 | 0.209 |

* Hydroxyethylcellulose was used to determined the endocellulase activity.
** Avicel was used to determined the exocellulase activity.

## 3.5. ACKNOWLEDGMENTS

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# CHAPTER 4

# AFFINITY CHROMATOGRAPHY OF CELLULOLYTIC ENZYMES; THE ROLE OF NONSPECIFIC INTERACTIONS

## 4.1. ABSTRACT

The purpose of this study was to establish the significance of nonspecific protein/matrix interactions in the affinity chromatography of cellulolytic enzymes and to provide a basis for the experimental optimization of these chromatographic systems. *p*-Aminophenyl 1-thio-$\beta$-D-cellobioside was used as a representative affinity ligand to which exo-acting cellulases (cellobiohydrolases, CBHs) preferentially bind. A "crude" cellulase preparation from the fungus *Trichoderma reesei* served as an enzyme source. The adsorption properties of the two principle exo-acting CBHs in this preparation, CBH I and CBH II, were distinctly different under most scenarios. Their relative affinities were shown to be highly dependent on the functional groups employed for ligand coupling and on the extent of functional group hydrolysis. This dependency on the chemistry of the supporting matrix was illustrated using agarose supports with either cyanate ester or N-hydroxysuccinimide functional groups. The behavior of each of the CBHs could be rationalized, and predictably modified, by considering that the columns were functioning as both affinity and ion-exchange columns.

## 4.2. INTRODUCTION

Affinity chromatography is a powerful separation technique that has been successfully applied to the purification of selected cellulolytic enzymes from microbial enzyme mixtures (van Tilbeurgh *et al.*, 1984; Orgeret *et al.*, 1992; Piyachomkwan *et al.*, 1997). These naturally occurring enzyme mixtures often include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and $\beta$-glucosidase (EC 3.2.1.21). Affinity chromatography is primarily used with these systems for the separation of nearly homologous endo- and exo-acting $\beta$-glucanases (Tomme *et al.*, 1988). The affinity ligands used for this purpose have been thio-derivatives of cellobiose or cellotriose. These ligands are coupled to a stationary phase support via their reducing ends, such that the non-reducing terminus is free for interaction with target enzymes. Exo-acting $\beta$-glucanases, cellobiohydrolases (CBH), are retained on these affinity columns while endo-acting $\beta$-glucanases, endoglucanases (EG), and other noncellulolytic enzymes pass through.

Thioglycoside affinity systems are clearly useful for obtaining purified CBH preparations, but the literature is somewhat confusing with respect to the appropriated working conditions necessary for the successful application of this technique. For example, one laboratory reports that 1M NaCl is required for enzyme desorption (Orgeret *et al.*, 1992), another laboratory indicates that 0.25-1.0 M NaCl (or $(NH)_2SO_4$) is required for enzyme adsorption (Irwin, 1994), and yet another laboratory uses no salt during either the adsorption or desorption phase of these experiments (van Tilbeurgh *et al.*, 1984). Similarly, the working pHs for these affinity systems differ between laboratories (van

Tilbeurgh *et al.*, 1984; Orgeret *et al.*, 1992; Irwin, 1994). In each of these cases the same, or very similar, affinity ligands were used. These discrepancies in working conditions suggest that costly and time consuming trial-and-error experimentation is often required prior to the successful application of this purification approach. This need for trial-and-error experimentation also adds to the frustrations that undoubtedly arise when newly synthesized ligands/stationary phases, used according to published procedures, perform below expectations.

The intent of the study presented in this paper was to define the chemical basis for the apparent discrepancies in published experimental protocols and to suggest a rational approach to the optimization of these affinity based columns. The experimental system used in this study included *Trichoderma reesei* cellulases (Kubicek *et al.*, 1990) and *p*-aminophenyl thiocellobioside, an exo-specific complementary ligand used for the purification of CBHs (Piyachomkwan *et al.*, 1997). We have systematically evaluated the role of pH, NaCl, coupling conditions and stationary phase functional groups on the affinity purification of CBH I and CBH II; with CBH I and CBH II being the two principle CBHs in the mixture of cellulolytic enzymes produced by *T. reesei*. The results show that the apparent contradictions in existing methods for the affinity purification of CBHs are due to nonspecific interactions between the enzymes and the stationary phase matrix, and that the behavior of the system can be rationalized by considering the net charge on the supporting matrix, the pI of the enzymes and the composition of the mobile phase.

## 4.3. MATERIALS AND METHODS

### 4.3.1. Materials

N-hydroxysuccinimide activated cross-linked agarose (Affigel 10) was purchased from BioRad Laboratories (Hercules, CA). DEAE-derivatized cross-linked agarose (DEAE-Sepharose CL6B) and CNBr-activated cross-linked agarose (CNBr-activated Sepharose 4B) were obtained from Pharmacia (Piscataway, NJ). *Trichoderma reesei* crude cellulase Spezyme$^{TM}$-CP was obtained from Environmental BioTechnologies, Inc. (Menlo Park, CA).

### 4.3.2. Enzyme Preparations

Crude cellulases were initially fractionated by anion-exchange chromatography as described by Beldman *et al.* (1985). Crude cellulase (200 mg) in 5 mL starting buffer (50 mM sodium acetate, pH 5) was applied to a DEAE-column (2.5x16 cm), previously equilibrated with starting buffer, at 4°C. The column was washed with 300 mL starting buffer (flow rate 0.65 mL/min) prior to running a 160 mL gradient ranging from 0-0.5 M NaCl in starting buffer. Eluted protein was detected by absorbance at 280 nm. Fractions containing cellulase activities were pooled based on resolved peaks and then dialyzed against 50 mM sodium acetate buffer, pH 5, and/or concentrated using an Amicon dialysis cell with PM-10 membrane (molecular weight cut off = 10,000, Amicon Co., Danvers, MA).

The DEAE-fractionated, partially purified cellobiohydrolases were used as starting material for APTC-affinity chromatography experiments. The DEAE-derived

CBH I preparation included those proteins eluted in the major peak following application of the salt gradient (Beldman *et al.*, 1985). The DEAE-derived CBH II preparation included those proteins which were not retained on the DEAE column.

4.3.3. Preparation of APTC-Affinity Columns

The affinity ligand, *p*-aminophenyl 1-thio-$\beta$-D cellobioside (APTC), was synthesized as described previously (Piyachomkwan *et al.*, 1997). APTC was then coupled to either NHS ester-activated agarose or CNBr-activated agarose as suggested by the manufacturers using ligand concentrations of 20 μmoles per mL gel.

4.3.3.1. APTC/NHS-activated agarose

Ligand coupling was accomplished by adding 10 mL of NHS-activated agarose to 20 mL of cold 0.05 M $NaHCO_3$, pH 8.3, containing 0.09 g APTC and gently agitating at 4°C for 4 hr. The APTC-coupled gel was then washed with fresh $NaHCO_3$ coupling buffer (3 x 20 mL). The remaining NHS esters were then chemically blocked by mixing the coupled, washed, gels with 75 mL 1.0 M ethanolamine, pH 8, and agitating for 2 hr at room temperature. The coupled, blocked, gel was then washed with fresh coupling buffer (3 x 20 mL), loaded into a 1x10 cm column and equilibrated with the initial mobile phase for cellobiohydrolase chromatography.

In one set of experiments the effect of excessive functional group hydrolysis was evaluated. In this case ligand coupling and subsequent washing was done as above; the resulting gel was then left in coupling buffer at 4°C for 15 hr.

4.3.3.2. APTC/CNBr-activated agarose

Three g of CNBr-activated agarose was suspended in 75 mL of cold 1 mM HCl for 5 min. The gel was then washed with another 200 mL of cold 1 mM HCl and subsequently suspended in 30 mL cold 0.1 M $NaHCO_3$, pH 8.3, containing 0.09 g of APTC. The suspension was gently agitated for 2 hr at room temperature. The APTC-coupled gel was then washed with fresh coupling buffer (3x20 mL). Unreacted cyanate functional groups were then chemically blocked by mixing the coupled, washed, gels with 75 mL of 1.0 M ethanolamine, pH 8, and agitating for 2 hr at room temperature. The coupled, blocked gel was then washed with fresh coupling buffer (3 x 20 mL), loaded into a 1x10 cm column and equilibrated with the initial mobile phase for cellobiohydrolase chromatography.

4.3.4. Affinity Chromatography of CBH I and CBH II

Experiments were done with low pressure columns and peristaltic pumps in order to simulate the approach most commonly used in biochemical laboratories. DEAE-derived CBH I and DEAE-derived CBH II were applied to affinity columns at 3-5 mg per mL of the initial mobile phase. The initial mobile phase for CBH I chromatography was 0.1 M sodium acetate, 1 mM gluconolactone. The initial mobile phase for CBH II chromatography was 0.1 M sodium acetate, 1 mM gluconolactone and 0.2 M glucose. Gluconolactone is an inhibitor of $\beta$-glucosidases and glucose is necessary for CBH II adsorption (Piyachomkwan *et al.*, 1997). Following enzyme application, the column was washed with the initial mobile phase until a background baseline, based on absorbance at

280 nm, was established. Retained CBHs were eluted from the column by the addition of cellobiose (final concentration = 0.01 M) to the mobile phase. The pH and salt (NaCl) content of the mobile phases were adjusted as described in the text. Flow rates were kept at 0.5 mL per min and fractions were collected every 10 minutes.

## 4.4. RESULTS AND DISCUSSION

The results presented here demonstrate that the interaction between CBHs and their complementary ligand, *p*-aminophenyl thiocellobioside (APTC), are markedly influenced by nonspecific, enzyme-support interactions. Our interpretation of these results is based on the chemistry of the supports, as illustrated in Figure 4.1 and 4.2, and the net charge properties of the two target enzymes; CBH I, pI ~ 3.9; CBH II, pI ~ 5.9 (Bhikhabhai *et al.*, 1984; Tomme *et al.*, 1988).

### 4.4.1. pH Effects

The effect of pH on the interaction of CBH I with APTC coupled to NHS-activated agarose is shown in Figures 4.3a-d. In this case the overall net charge on the support is expected to be negative, due to the hydrolysis of the NHS esters (Figure 4.1; Cuatrecasas and Parikh, 1972). The number of ionizable carboxyl groups on the support will be a function of the efficiency of ligand coupling and functional group blocking; greater efficiency in these steps will result in reduced numbers of ionizable carboxyl groups. The CBH I enzyme will also have a net negative charge at all of the pH values illustrated in Figure 4.3 (pH 4.5-6). The net negative charge on the enzyme, and on the

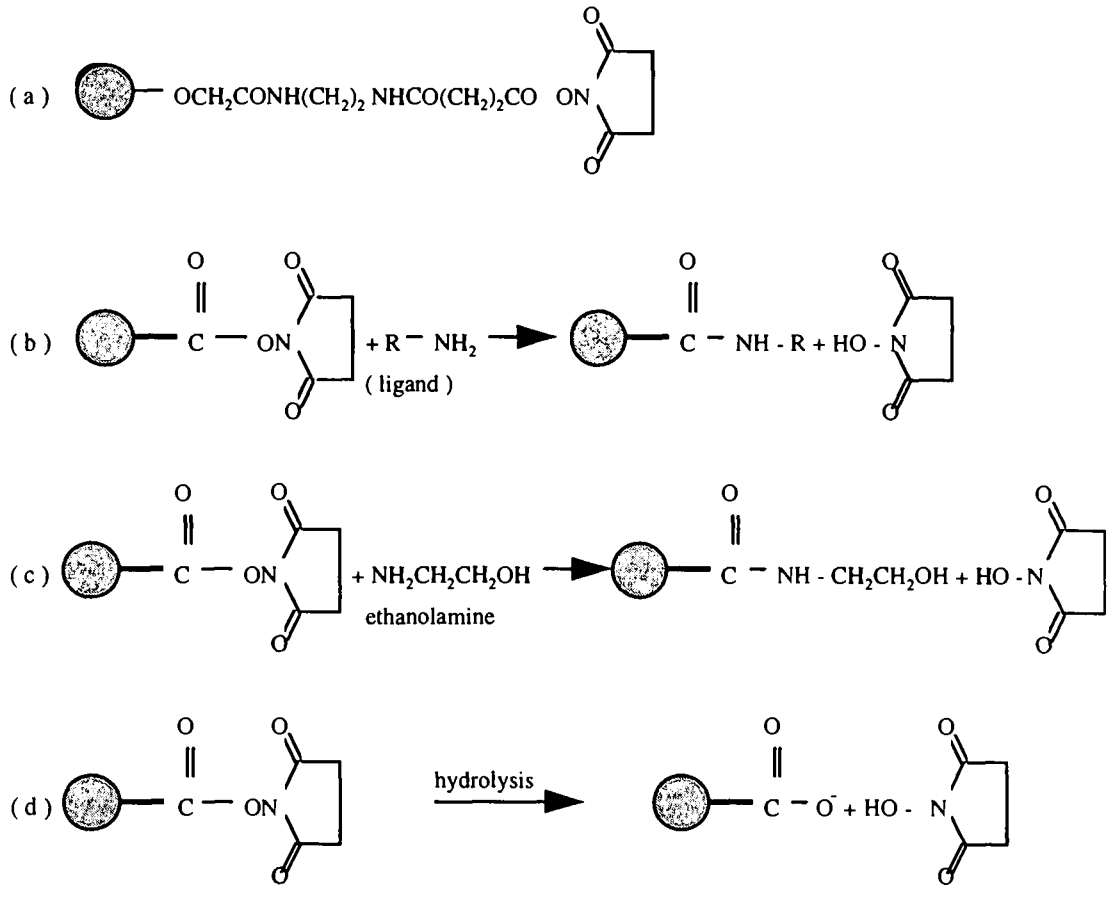



Figure 4.1. The chemical reactions of (a) N-hydroxysuccinimide ester (NHS) activated agarose bead when (b) coupled with the amine-containing ligand, (c) blocked any excess active groups with ethanolamine, and (d) hydrolyzed. ◯ = Agarose bead, ▬ = Spacer arm.

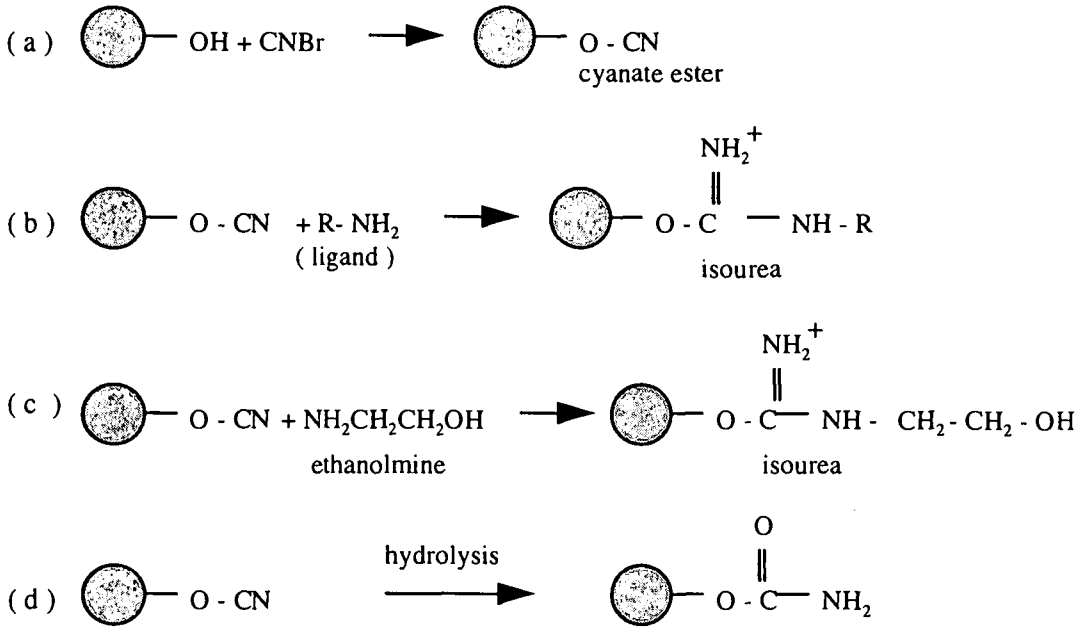
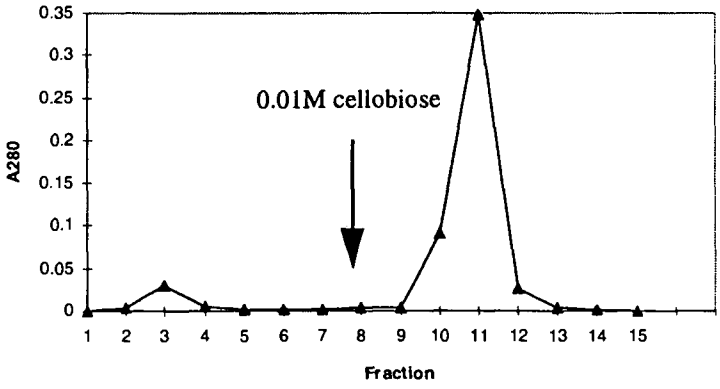
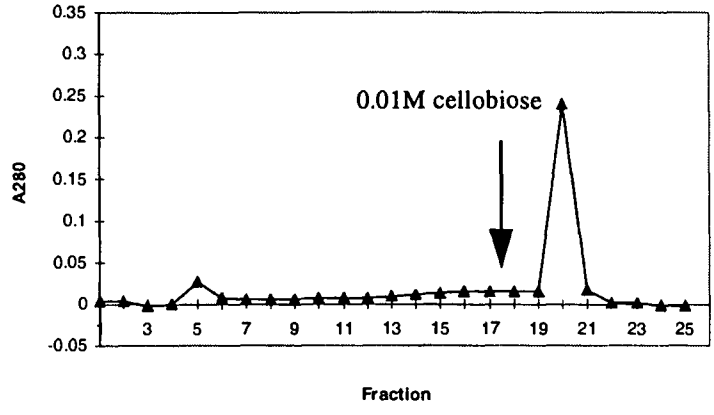



Figure 4.2. The chemical reactions of (a) cyanogen bromide (CNBr) activated agarose bead when (b) coupled with the amine-containing ligand, (c) blocked with ethanolamine, and (d) hydrolyzed. ◎ = Agarose bead.




(a)




(b)

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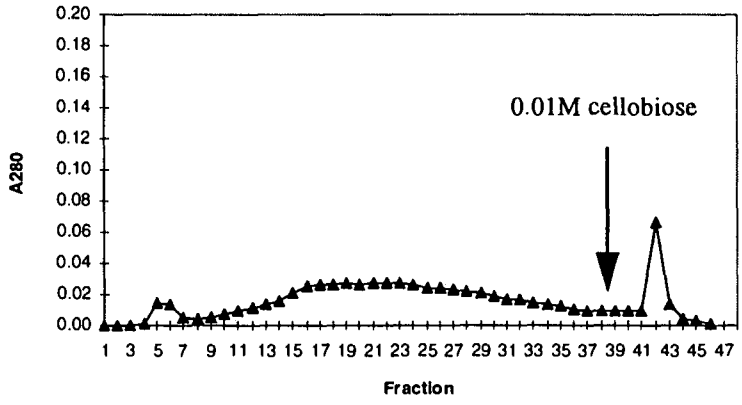
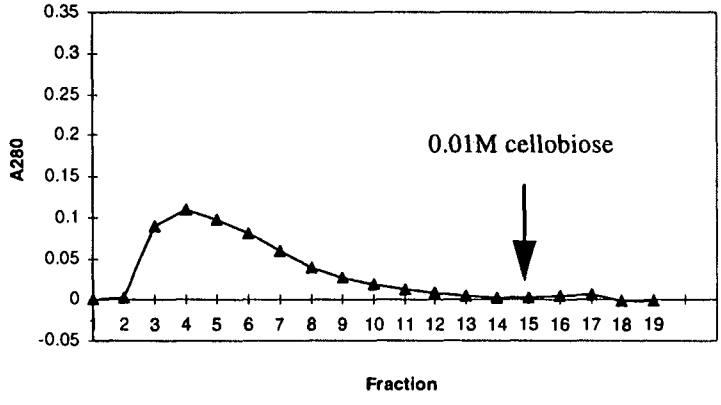



(c)



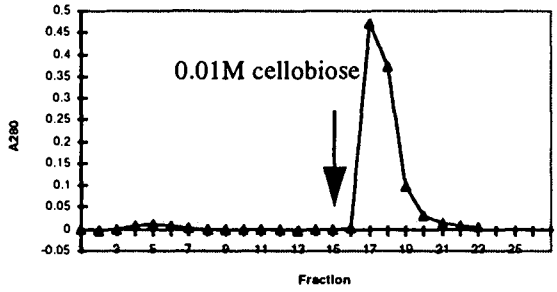

(d)

Figure 4.3. Affinity chromatography of CBH I by using APTC/NHS-activated agarose column at different pH (a) 4.5, (b) 5, (c) 5.6, and (d) 6. CBH I preparations from anion exchange column were applied to APTC/NHS-activated agarose column with 0.1M sodium acetate buffer containing 1mM gluconolactone, and eluted with 0.01M cellobiose in the same buffer.

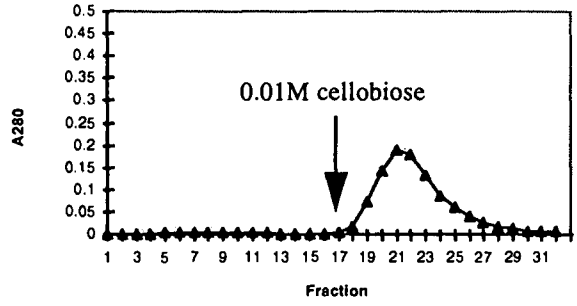
support, will increase as the pH of the system increases, resulting in an increase in ionic repulsion between the supporting matrix and CBH I. Above some critical pH, which is somewhat column specific, ionic repulsion will begin to measurably effect the ability of the column to retain CBH I. The critical pH for the CBH I-APTC system depicted in Figure 4.3 is between 4.5 and 5.0, since leaching of CBH I is first observed when the system is at pH 5.

The small peak corresponding with the initial fractions of the chromatograms (Figure 4.3) is an endoglucanase (EG) contaminant present in CBH I preparations obtained from DEAE chromatography (see "Methods"). This EG has a net negative charge (pI ~ 4.5, Bhikhabhai *et al.*, 1984) at the pHs depicted and it does not have appreciable affinity for APTC; thus, it is not retained on these columns by either specific enzyme-ligand or nonspecific enzyme-matrix interactions. If the pH of the system is increased to 6, then CBH I coelutes with the EG in the breakthrough fractions due to the increased magnitude of the ionic repulsion between CBH I and the supporting matrix.

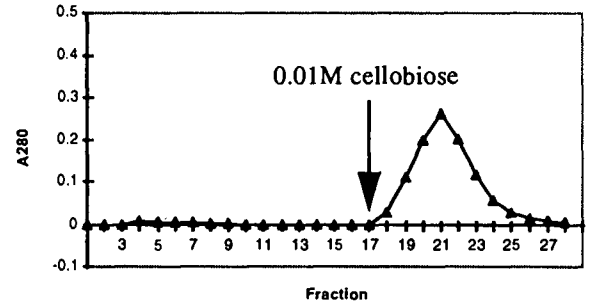
The effect of pH on the interaction of CBH I with APTC coupled to CNBr-activated agarose is shown in Figure 4.4a-c. In this case, the support is expected to have a net positive charge due to the formation of ionizable isourea groups (Figure 4.2; Kohn and Wilchek, 1981; Carlsson *et al.*, 1989). As with NHS-activated agarose, the density of ionizable functional groups on the support will be a function of the efficiency of ligand coupling and functional group blocking. However, in contrast to NHS-activated agarose, the density of ionizable functional groups will increase as coupling and blocking efficiency increases. The net charge on the CNBr-activated support will be positive under




(a)




(b)

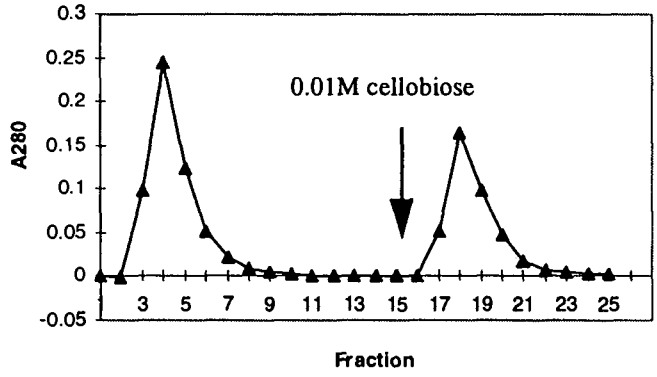



(c)

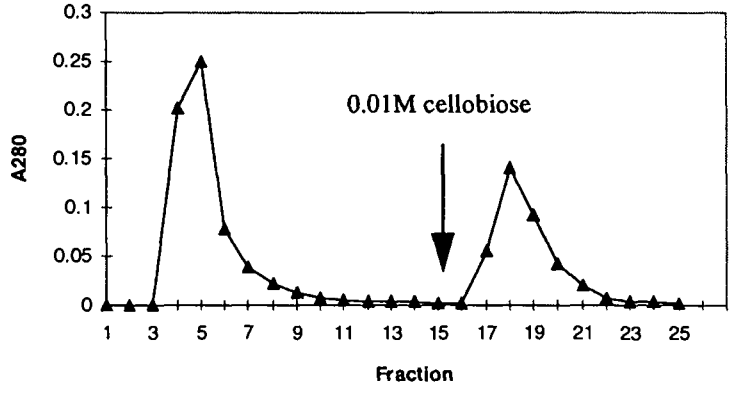
Figure 4.4. Affinity chromatography of CBH I by using APTC/CNBr-activated agarose column at different pH (a) 4, (b) 5, and (c) 6. CBH I preparations were applied to APTC/CNBr-activated agarose column with 0.1M sodium acetate buffer, and eluted with 0.01M cellobiose in the same buffer.

all conditions typically used for CBH purification (Figure 4.4). The net negative charge on CBH I increases as the pH of the system is raised from pH 4 to 6 (pI ~ 3.9). The chromatograms of Figure 4.4 are consistent with the development of progressively stronger ionic attractions, between the stationary support and CBH I, as the pH is increased from 4 to 6. The pH 4 chromatogram has a relatively narrow peak resulting from elution of CBH I by the addition of cellobiose to the mobile phase. In contrast, cellobiose elution of CBH I at higher pHs results in a much broader peak. CBH I peak broadening at higher pHs is most likely attributable to ionic attractions between CBH I and the supporting matrix, since the true "affinity" function of the column is negated by the addition of cellobiose to the mobile phase (Piyachomkwan *et al.*, 1997). The clear indication is that under the conditions depicted in Figure 4.4c, the column is functioning as both an affinity and an ion-exchange column. It is interesting to note the behavior of the small EG contaminant under these chromatographic conditions. The EG peak, which is easily seen in the chromatograms of Figure 4.3, is not as obvious in the chromatograms of Figure 4.4, primarily due to peak broadening as a result of retention of the EG enzyme on the positively charged column. An obvious concern under these conditions is that there may be contaminating proteins, including EGs, that are retained on the column by nonspecific ionic interactions and later coelute with CBH I.

The effect of pH on the chromatography of CBH II on APTC coupled to NHS-activated agarose is shown in Figure 4.5a-d. The nonretained peak is relatively large in these chromatograms, compared to the CBH I chromatograms of Figure 4.3, due to the higher percentage of non-CBH proteins in the DEAE-derived CBH II preparation




(a)

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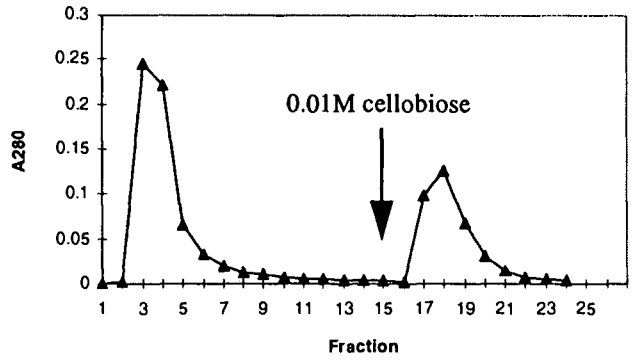
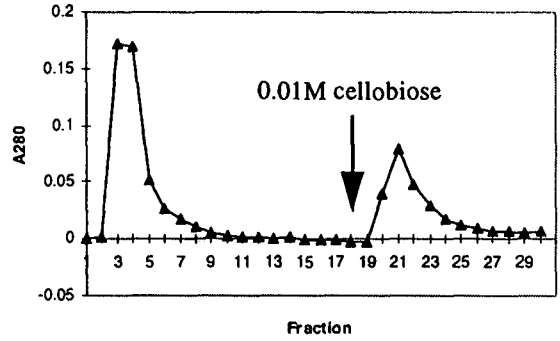



(c)




(d)

Figure 4.5. Affinity chromatography of CBH II by using APTC/NHS-activated agarose column at different pH (a) 5, (b) 6, (c) 7, and (d) 8. CBH II preparations from anion exchange column were applied to APTC/NHS-activated agarose column with 0.2M glucose in 0.1M sodium acetate buffer containing 1mM gluconolactone, and eluted with 0.01M cellobiose in the same buffer.

compared to the DEAE-derived CBH I preparation used as starting materials (see "Methods"). CBH II has a net positive charge at pH 5, is approximately neutral at pH 6, and negative at pHs 7 and 8. In contrast to CBH I, CBH II is retained on the APTC/ NHS-activated agarose column at pHs well above its pI (Figure 4.5d). This behavior is consistent with the formation of the CBH II-ligand complex being energetically more favorable than the corresponding CBH I-ligand complex (compare chromatograms 4.3d and 4.5d).

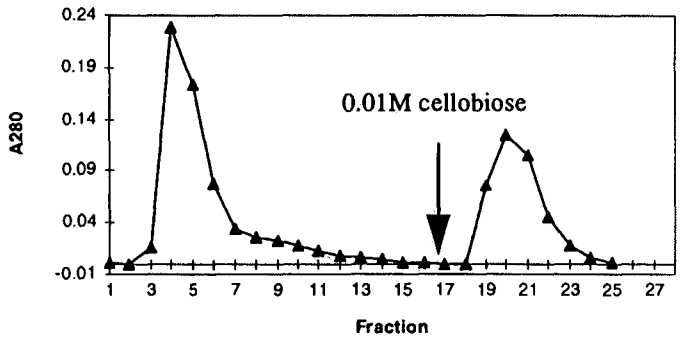
The DEAE-derived CBH II preparation traditionally used for the affinity purification step of this enzyme is composed of the nonretained proteins that pass through a DEAE column at pH 5 (Bhikhabhai *et al.*, 1984; Voragen *et al.*, 1988). Thus all of the proteins in this preparation, including CBH II and non-CBH II proteins, are expected to behave on ion-exchange columns as though their net charge is neutral to positive at pH 5; a pH commonly used for CBH II affinity purification (van Tilbeurgh *et al.*, 1984; Tomme *et al.*, 1988; Pere *et al.*, 1995). The net negative charge associated with the supporting matrix of APTC/NHS-activated agarose columns creates the potential problem of contaminating proteins in the CBH II preparation being retained on these columns due to ionic effects; the likelihood of this occurring increases as the anion density of the supporting matrix increases. The chromatograms of Figure 4.5 are consistent with the nonspecific retention of a portion of the non-CBH II proteins in the original DEAE-derived CBH II preparation, particularly at the lower pHs. The rationale for this statement comes from the observation that the percentage of total protein applied to the column recovered in the nonretained fractions increases in going from pH 5, ~59% of

total protein in nonretained fractions, to the pH 8, ~66% of total protein in nonretained fractions. The most elementary explanation is that at pH 8 a higher percentage of the non-CBH II proteins are anionic and, thus, not retained on the column.

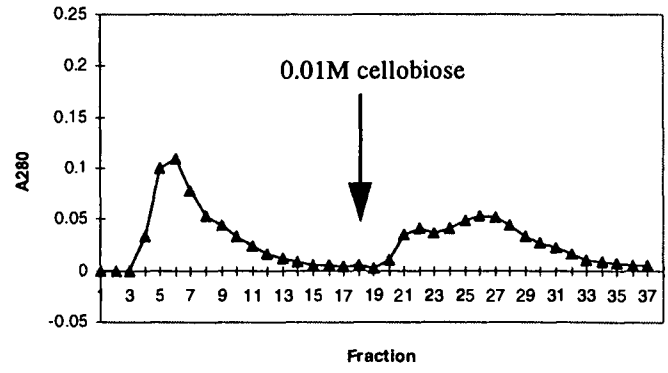
The effect of pH on the chromatography of CBH II on APTC coupled-CNBr-activated agarose support is shown in Figure 4.6a-d. In this case the stationary phase is cationic. Thus, as the pH of the system increases, and the net negative charge on the protein increases, one may predict that the enzyme will be retained on the column simply due to nonspecific ionic interactions. This appears to be the case based on the relatively broad peak observed when cellobiose is used to elute CBH II from the column at pH 7 (Figure 4.6d) versus pH 4 (Figure 4.6a). At low pH values, ~pH 4, there is undoubtedly some ionic repulsion due to CBH II and the supporting matrix being of like charge. However, this repulsion does not appear to be of sufficient magnitude to disrupt the CBH II-APTC ligand complex, as evidenced by the ability of the column to retain the enzyme under these conditions.

### 4.4.2. Salt Effects

In light of the pH affects presented above, the affect of salt on the behavior of these affinity systems is most simply explained in terms of nonspecific ionic interactions between the CBHs and the supporting matrixes used for APTC coupling. The influence of salt on the behavior of CBH I-APTC affinity systems is illustrated in Figure 4.7 and 4.8. Figure 4.7 shows that relatively high salt concentrations (2 M NaCl) are required for enzyme adsorption to APTC/NHS activated agarose column when the chromatography is




(a)




(b)

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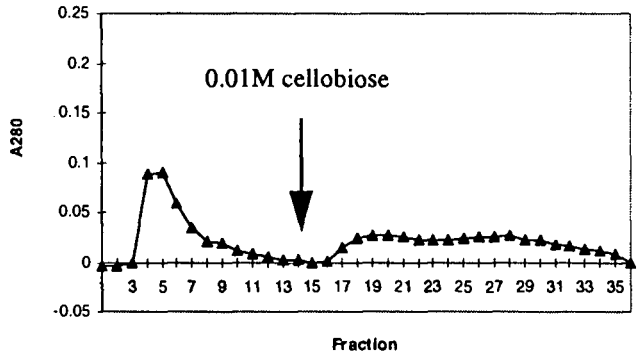


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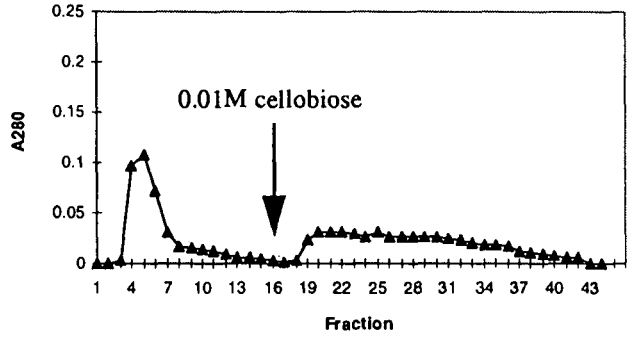



(d)

Figure 4.6. Affinity chromatography of CBH II by using APTC/CNBr-activated agarose column at different pH (a) 4, (b) 5, (c) 6, and (d) 7. CBH II preparations from anion exchange column were applied to APTC/CNBr-activated agarose column with 0.2M glucose in 0.1M sodium acetate buffer containing 1mM gluconolactone, and eluted with 0.01M cellobiose in the same buffer.

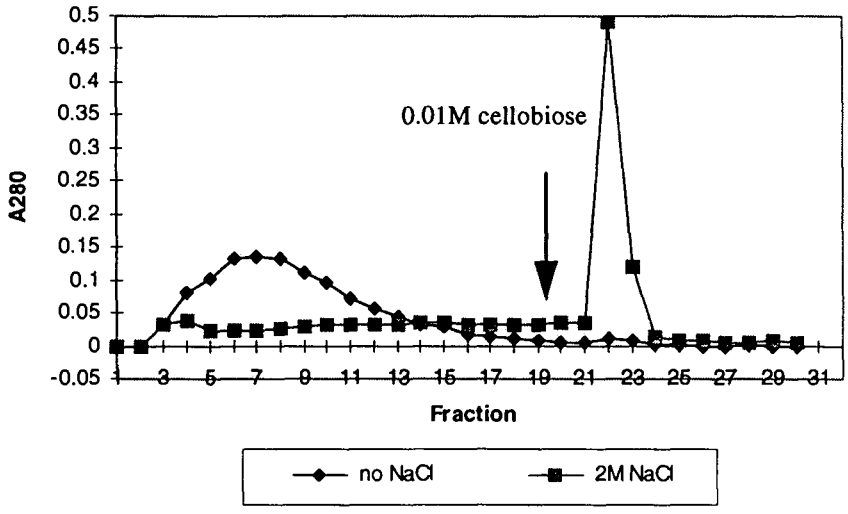
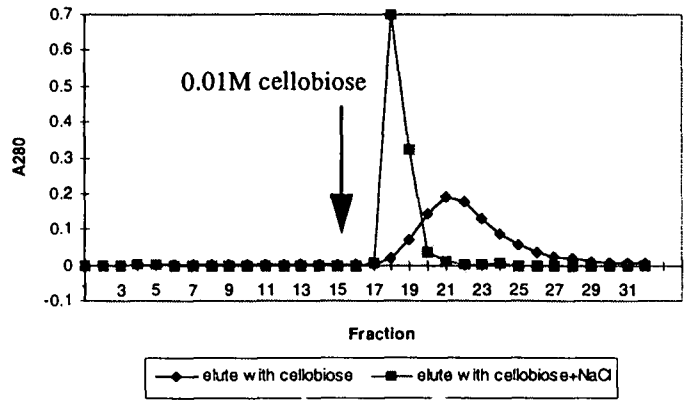
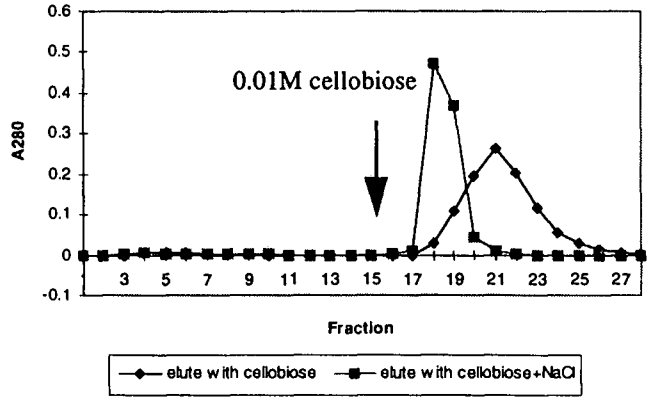



Figure 4.7. The effect of salt on the affinity chromatography of CBH I by using APTC/ NHS-activated agarose column at pH 6. CBH I preparations from anion exchange column were applied to APTC/NHS-activated agarose column without and with (2M) NaCl in 0.1M sodium acetate buffer, pH 6, containing 1mM gluconolactone, and eluted with 0.01M cellobiose in the same buffer.




(a)




(b)

Figure 4.8. The effect of salt on the affinity chromatography of CBH I by using APTC/ CNBr-activated agarose column at pH (a) 5 and (b) 6. CBH I preparations from anion exchange column were applied to APTC/CNBr- activated agarose column in 0.1M sodium acetate buffer containing 1mM gluconolactone, and eluted with 0.01M cellobiose, without and with (1M) NaCl, in the same buffer.

done at pHs significantly above the pI of the enzyme. Figure 4.8 illustrates that NaCl significantly improves the enzyme's desorption characteristics when working with APTC/CNBr-activated agarose columns. Hence, apparent discrepancies (Orgeret *et al.*, 1992; Irwin *et al.*, 1993) with respect to the effect of salt on CBH I adsorption/desorption can be rationalized by focusing on the supporting matrix, rather than on the properties of the affinity ligand itself. For CBH I, salt may be used to improve enzyme adsorption when working with NHS-activated matrixes and enzyme desorption when working with CNBr-activated matrix.

Figure 4.9 is an illustration of how salt may be used to improve the desorption properties of CBH II when working with CNBr-activated matrix. The addition of salt to the eluting buffer narrowed the elution peak of both CBH II (Figure 4.9) and CBH I (Figure 4.8); the similarity in the behavior of CBH I and CBH II in this case is expected since both experiments were done under conditions in which the pH of the system was above the pI of the target enzyme.

### 4.4.3. Role of Functional Group Blocking

The chemistry of the supporting matrix (Figure 4.1 and 4.2) is such that the density of ionizable groups will be dependent on the efficiency of ligand coupling and subsequent functional group blocking with ethanolamine. This, in turn, is expected to have a strong influence on the relevance of nonspecific ionic interactions during affinity chromatography. The influence of blocking efficiency is illustrated in Figure 4.10a, b and c. In this experiment ligand was coupled to the NHS-activated support as in other

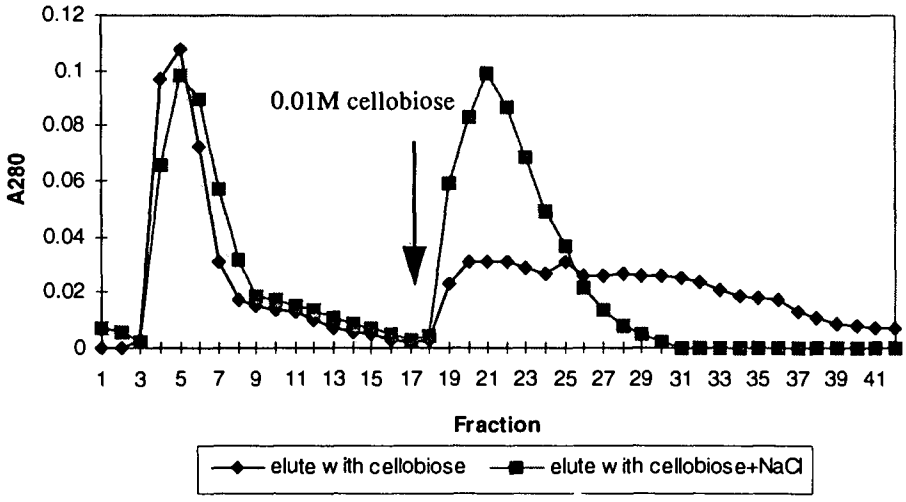
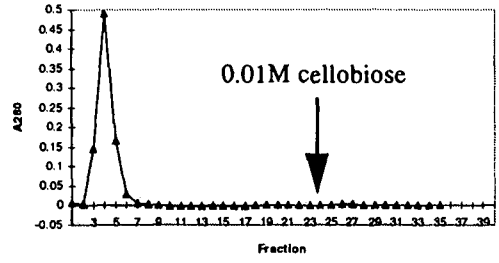
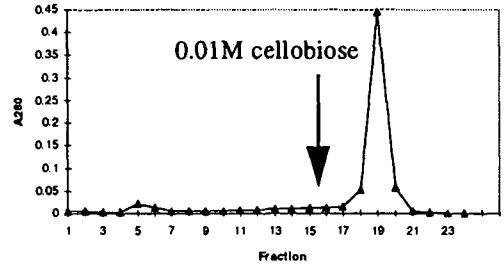


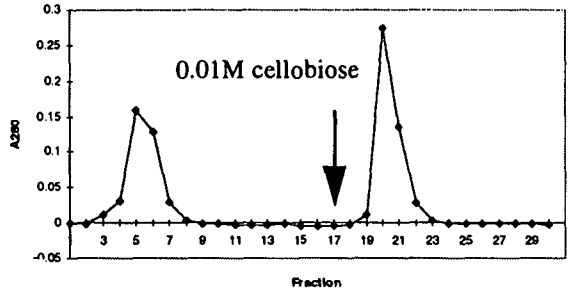

Figure 4.9. The effect of salt on the affinity chromatography of CBH II by using APTC/ CNBr-activated agarose column at pH 7. CBH II preparations from anion exchange column were applied to APTC/CNBr-activated agarose column with 0.2M glucose in 0.1M sodium acetate buffer containing 1mM gluconolactone, and eluted with 0.01M cellobiose, without and with (1M) NaCl in the same buffer.




(a)




(b)




(c)

Figure 4.10. Affinity chromatography of (a) CBH I at pH 5, (b) CBH I at pH 4, and (c) CBH II at pH 5 by using APTC/NHS-activated agarose column with hydrolysis after ligand coupling. CBH I and CBH II preparations from anion exchange column were applied to APTC/NHS-activated agarose column without and with 0.2M glucose in 0.1M sodium acetate buffer containing 1 mM gluconolactone, respectively, and eluted with 0.01M cellobiose in the same buffer.

experiments, but following ligand coupling the functional groups were hydrolyzed by leaving the coupled matrix in coupling buffer for an extended period of time (see "Methods"). The result of allowing excessive hydrolysis to occur is an increase in the density of ionizable carboxyl groups on the supporting matrix. Comparison of the chromatograms of Figure 4.10a and Figure 4.3b shows that reduced blocking efficiency, which corresponds to increased functional group hydrolysis, results in increased ionic repulsion between CBH I and the supporting matrix. In the case depicted in Figure 4.10a, the ionic repulsion is of such magnitude that the column can no longer be used for affinity purification under conditions analogous to those depicted in Figure 4.3b. Figure 4.10b illustrates that a column having a relatively high anion density, as a result of excessive hydrolysis, can still be used for the affinity purification of CBH I if the pH of the system is adjusted accordingly.

The chromatogram of Figure 4.10c, which corresponds to a column having a relatively high anion density, appears to retain a higher percentage (65%) of the DEAE-derived CBH II preparation proteins than the corresponding column with a relatively low anion density (40%, Figure 4.5a), even though the chromatography conditions for Figures 4.10c and 4.5a were identical. This behavior is in agreement with the trends discussed above with respect to the amount of protein retained in the CBH II/APTC NHS-activated systems run at different pHs (Figure 4.5).

4.4.4. Implications

The data presented in this paper is intended to serve as a guide for those developing affinity systems for the purification of exo-acting cellulases. However, the rationale used to interpret the data presented here is applicable to most other enzyme-ligand affinity systems. The results of this study are to be interpreted in a qualitative manner, since the affinity matrixes prepared in different laboratories will differ somewhat with respect to ligand and charge density.

The obvious implication from this work is that affinity chromatography systems widely used for the purification of CBHs are likely to function as both affinity and ion-exchange columns under a variety of scenarios. It appears essential that precautions be taken to ensure that the ion-exchange properties of the column do not significantly reduce the usefulness of the affinity properties of the column. In some cases ionic repulsion may be of such magnitude that the target enzyme no longer associates with the affinity matrix. In other cases ionic interactions may result in the nonspecific retention of both the target enzyme and contaminant proteins. This latter case will be associated with difficulties in eluting the target enzyme. Problems in enzyme elution have previously been circumvented by the addition of salts to the eluting buffer (Orgeret *et al.*, 1992). However, this approach must be used with caution since conditions that reduce the nonspecific interactions between the target enzyme and the matrix, thus giving a narrower elution band upon addition of the competitive ligand to the eluting buffer, will likely result in coelution of the retained non-CBH proteins.

The role of nonspecific interactions in the affinity chromatography of CBHs has been shown to be dependent on the pI of the target enzyme, the chemistry of the

supporting matrix and the conditions under which the experiment is done. With reference to experimental conditions, it is generally advisable to adjust the pH and ionic strength of the mobile phase such that nonspecific ionic interactions are minimized throughout the entire experiment. An example of the application of this reasoning is presented in Table 4.1 for the target enzymes and affinity supports used in this study.

Table 4.1. Guidelines for affinity chromatography of cellobiohydrolases.

| Enzyme | Supporting matrix | Potential problems | Remedies |
|---|---|---|---|
| CBH I[a] | CNBr (+)[c] | 1. Retention of non-CBH I protein (at pH >5) | 1.1 use lower pH<br>1.2 include salt in all mobile phase |
| CBH I | NHS ester (-) | 1. Desorption of CBH I from affinity column (at pH >5) | 1.1 use lower pH<br>1.2 include salt in all mobile phase |
| CBH II[b] | CNBr (+) | 1. Retention of non-CBH II protein (at pH > 5)<br>2. Desorption of CBH II from affinity column (at pH <5) | 1.1 use lower pH<br>1.2 include salt in all mobile phase<br>2.1 use higher pH<br>2.2 include salt in all mobile phase |
| CBH II | NHS ester (-) | 1. Retention of non-CBH II protein (at pH <5)<br>2. Desorption of CBH II from affinity column (at pH > 5) | 1.1 use higher pH<br>1.2 include salt in all mobile phase<br>2.1 use lower pH<br>2.2 include salt in all mobile phase |

[a] CBH I (pI ~ 3.9)
[b] CBH II (pI ~ 5.9)
[c] represents the net charge of the support matrix.

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# CHAPTER 5

# CAN IMMOBILIZED *p*-AMINOPHENYL 1-THIO-β-D-CELLOBIOSIDE BE USED TO DISTINGUISH CLASS I AND CLASS II EXOCELLULASES?

## 5.1. INTRODUCTION

Exocellulases have been traditionally considered to release cellobiose by general acid-base catalysis from the nonreducing ends of cellulose chains. However, this mode of action does not explain the behavior observed when exocellulases are acting on soluble cellooligosaccharides. Several studies have been performed to elucidate the polarity of these enzymes. A study based on the hydrolysis of reduced cellooligosaccharides and reduced alkali-swollen cellulose by exocellulases from *Aspergillus aculeatus* showed that two exocellulases exhibited opposite polarity; one enzyme appeared to initiate hydrolysis of cellulose polymers at the reducing end, and the other enzyme appeared to initiate hydrolysis of cellulose polymers at the nonreducing end (Arai *et al.*, 1989). The actions of *Trichoderma reesei* CBH I on [1-$^3$H] cellooligosaccharides were also investigated, and the results suggested the hydrolytic preference of this enzyme to the reducing end of labeled substrates (Vrsanska and Biely, 1992). Another study with *Cellulomonas fimi* Cbh B also suggested that this exocellulase is capable of hydrolyzing cellodextrins at their reducing end (Shen *et al.*, 1994). Recently, some exocellulases from *Thermomonospora fusca*, $E_4$ and $E_6$, when tested with labeled cellooligosaccharides, indicated a preference to cleave substrates from the reducing end, whereas other

exocellulase, $E_3$, indicated a preference to degrade substrates from the nonreducing end (Barr *et al.*, 1996). All of these studies indicate the existence of two functional classes of exocellulases. One class, referred to here as "Class I", preferentially hydrolyzes cellulose substrates form the reducing ends, whereas the other class, referred to here as "Class II", preferentially hydrolyzes cellulose from the nonreducing ends.

Exocellulases bind specifically and reversibly to thio derivatives of cellobiose and cellotriose (van Tilbeurgh *et al.*, 1982; Claeyssens *et al.*, 1989; Orgeret *et al.*, 1992). Hence, these derivatives may be used for separating these enzymes from other proteins via affinity chromatography (van Tilbeurgh *et al.*, 1984, Tomme *et al.*, 1988; Orgeret *et al.*, 1992; Piyachomkwan *et al.*, 1997). Cellobiohydrolases bind to these columns, while endoglucanases, $\beta$-glucosidase, and other noncellulolytic enzymes do not bind. Following the binding of exocellulases to these immobilized ligands, the enzyme-ligand complex can be disrupted by adding compounds which compete for the enzyme's active site.

In affinity chromatography, the thio derivatives of cellobiose and cellotriose are coupled to the solid support via their reducing ends. Immobilized *p*-aminophenyl 1-thio-$\beta$-D-cellobioside (APTC) is prepared by coupling it via its amino moiety to NHS-activated agarose beads. In this case, only the nonreducing terminus of APTC is available for exoenzymes to interact with. An important question is whether or not the two exoenzyme classes will interact with immobilized APTC by different modes. Since we have available to us purified exocellulases which are separately from different classes (CBH I, class I; CBH II, class II), we attempted to identify differences in how these two

exocellulases interact with immobilized APTC. Exocellulase and immobilized APTC interactions were characterized qualitatively by evaluation of the enzymes under chromatographic conditions, and quantitatively by traditional partition equilibrium experiments.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Materials

N-hydroxysuccinimide activated cross-linked agarose (Affigel 10) was purchased from BioRad Laboratories (Hercules, CA). DEAE-Sepharose CL6B and phenyl Sepharose FF (low sub) were purchased from Pharmacia (Piscataway, NJ). *Trichoderma reesei* crude cellulase Spezyme$^{TM}$-CP was obtained from Environmental BioTechnologies, Inc. (Menlo Park, CA). CBH I from *Trichoderma reesei* was used as an example of class I exoenzymes (having the preference for the reducing end), whereas CBH II from the same source was used as an example of class II exoenzymes (having the preference for the nonreducing end).

### 5.2.2. Preparation of Immobilized APTC

The ligand, *p*-aminophenyl 1-thio-$\beta$-D cellobioside (APTC), was synthesized as described previously (Piyachomkwan *et al.*, 1997). APTC was then coupled to NHS ester-activated agarose as suggested by the manufacturers using ligand concentrations of 20 μmoles per mL gel.

Ligand coupling was accomplished by adding 10 mL of NHS-activated agarose to 20 mL of cold 0.05 M $NaHCO_3$, pH 8.3, containing 0.09 g APTC and gently agitating at 4°C for 4 hr. The APTC-coupled gel was then washed with fresh $NaHCO_3$ coupling buffer (3 x 20 mL). The remaining NHS esters were then chemically blocked by mixing the coupled, washed, gels with 75 mL 1.0 M ethanolamine, pH 8, and agitating for 2 hr at room temperature. The coupled, blocked, gel was then washed with fresh coupling buffer (3 x 20 mL), and 0.1 M sodium acetate pH 5, 1 mM gluconolactone, 0.02% $NaN_3$. The immobilized APTC agarose was used for affinity chromatography and binding experiments.

5.2.3. Enzyme Preparations

Crude cellulases were initially fractionated by anion-exchange chromatography as described by Beldman *et al.* (1985). Crude cellulase (200 mg) in 5 mL starting buffer (50 mM sodium acetate, pH 5) was applied to a DEAE-column (2.5x16 cm), previously equilibrated with starting buffer, at 4°C. The column was washed with 300 mL starting buffer (flow rate 0.65 mL/min) prior to running a 160 mL gradient ranging from 0-0.5 M NaCl in starting buffer. Eluted protein was detected by absorbance at 280 nm. Fractions containing cellulase activities were pooled based on resolved peaks and then dialyzed against 50 mM sodium acetate buffer, pH 5, and/or concentrated using an Amicon dialysis cell with PM-10 membrane (molecular weight cut off = 10,000, Amicon Co., Danvers, MA).

The DEAE-fractionated, partially purified cellobiohydrolases were used as starting material for APTC-affinity chromatography experiments. The DEAE-derived CBH I preparation included those proteins eluted in the major peak following application of the salt gradient (Beldman *et al.*, 1985). The DEAE-derived CBH II preparation included those proteins which were not retained on the DEAE column.

### 5.2.4. Adsorption Experiments of CBH I and CBH II on APTC-Derivatized Affinity Chromatography

Experiments were done with low pressure columns and peristaltic pumps. Immobilized APTC agarose was loaded into a 1x10 cm column and equilibrated with the initial mobile phase for cellobiohydrolase chromatography. The initial mobile phase was 0.1 M sodium acetate, 1 mM gluconolactone. Sugars, including glucose, xylose, mannose, galactose, and gluconolactone (final concentration = 0.2 M) were added to the mobile phase in order to determine their effects on enzyme adsorption. DEAE-derived CBH I and DEAE-derived CBH II in initial mobile phase were applied to affinity columns. Following enzyme application, the column was washed with the initial mobile phase until a background baseline, based on absorbance at 280 nm, was established. Retained CBHs were eluted from the column by the addition of cellobiose (final concentration = 0.01 M) to the mobile phase. Flow rates were kept at 0.5 mL per min and fractions were collected every 10 minutes.

5.2.5. Desorption Experiments of CBH I and CBH II on APTC-Derivatized Affinity Chromatography

Experiments were done similarly as described for adsorption experiments except the initial mobile phase for CBH II included 0.2 M glucose. Retained CBHs were eluted from the column by the addition of different eluting sugars including cellobiose, lactose, methyl β-D-glucopyranoside (final concentration = 0.01 M, 0.1 M and 0.2 M, respectively) to the mobile phase. The effects of glucose on the desorption of bound CBH II with different eluting sugars were also investigated by the inclusion of glucose (final concentration = 0.2 M) to the eluting buffers.

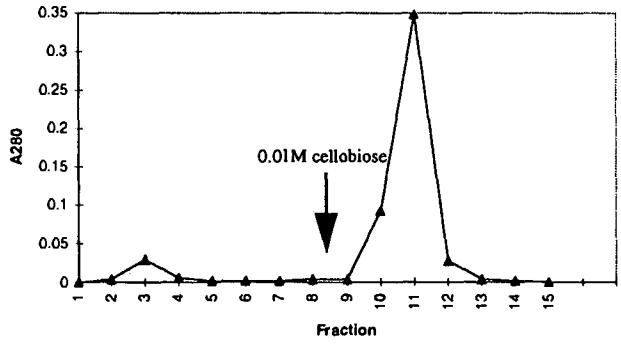
5.2.6. Partition Equilibrium Experiments

Relative binding affinities were estimated based on partition coefficients, K, from the adsorption isotherm. CBH I and CBH II used in this study were purified as described previously (Piyachomkwan *et al.*, 1997). The binding studies of cellobiohydrolases were done in 50 mM sodium acetate pH 5 for CBH I, and 0.2 M glucose in 50 mM sodium acetate pH 5 for CBH II, with and without salt (1 M NaCl). In this assay, 1.5 mL of enzyme solution with a known protein concentration (2-30 μM) was mixed with 0.04 g of immobilized APTC agarose in a 1.7 mL microcentrifuge tube by rotation at 4°C for 2 hr to reach equilibrium. After incubation, the mixture was centrifuged (10,000 rpm, 5 min), and the amount of unbound protein in the supernatant was then quantified by measuring the absorbance at 280 nm (molar absorptivities at 280 nm are 73000 $M^{-1}cm^{-1}$ for CBH I and 75000 $M^{-1}cm^{-1}$ for CBH II; Claeyssens *et al.*, 1989). Underivatized agarose was prepared by coupling and blocking the NHS-activated agarose with ethanolamine, and

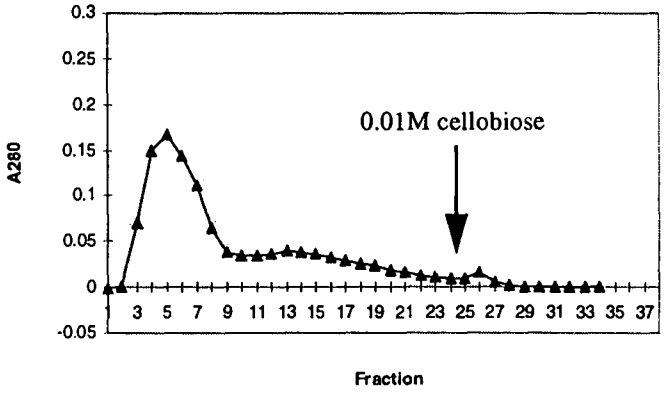
used as a control to account for nonspecific interactions between the enzyme and the agarose matrix. The amounts of bound enzymes on immobilized APTC agarose were determined from the difference between the initial amount of protein added and the unbound protein, and corrected for nonspecific interactions with underivatized agarose.

## 5.3. RESULTS

The interactions of two exocellulases from *Trichoderma reesei*, CBH I and CBH II, on immobilized *p*-aminophenyl 1-thio-β-D-cellobioside (APTC) were evaluated qualitatively by affinity chromatographic method under different conditions. The chromatographic technique was used in this study because it was an easy, fast and convenient method to determine enzyme-ligand interactions, in term of enzyme adsorption and desorption. When CBH I and CBH II preparations were applied to the APTC-derivatized column, as shown in Figure 5.1, CBH I was retained on the column to a greater extent than CBH II. Glucose has been demonstrated to increase the affinity between CBH II and soluble cellodextrins (van Tilbeurgh *et al.*, 1989) and, thus, the effect of glucose on the retention of CBHs by immobilized APTC was evaluated. When CBH I was applied to the APTC-derivatized column in the presence of glucose, there was an obvious reduction in affinity, CBH I slowly leaching from the column, as shown in Figure 5.2. In contrast, addition of glucose to the mobile phase dramatically increased the retention of CBH II, as shown in Figure 5.3.

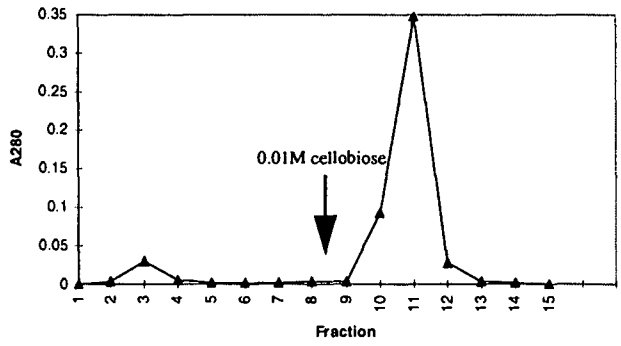



(a)

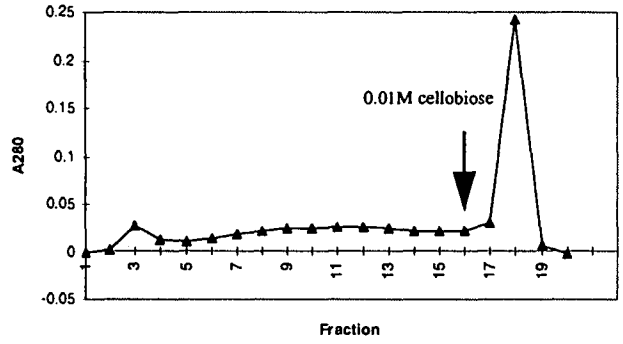



(b)

Figure 5.1. APTC-derivatized affinity chromatography of (a) CBH I and (b) CBH II. CBH I and CBH II fractions obtained from DEAE-Sepharose column was applied to APTC-derivatized column in 0.1 M sodium acetate pH 5, 1 mM gluconolactone, and eluted with 0.01 M cellobiose in the same buffer.

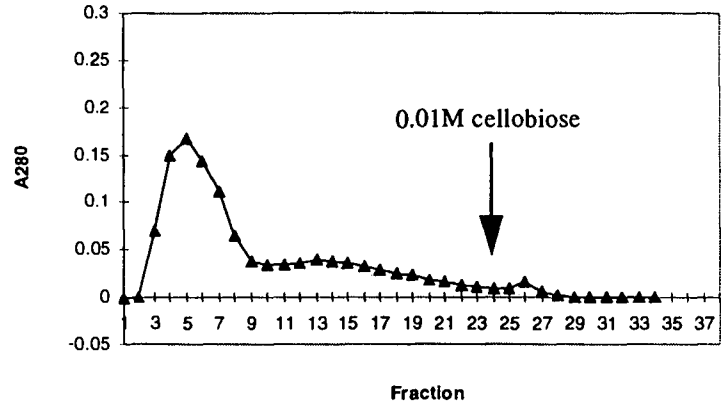



(a)

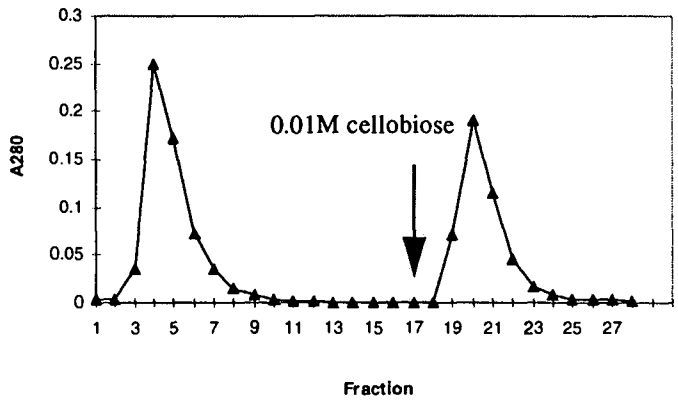



(b)

Figure 5.2. The effect of glucose on the adsorption of CBH I on APTC-derivatized affinity chromatography. CBH I fraction obtained from DEAE-Sepharose column was applied to APTC-derivatized column with (a) no glucose and (b) 0.2 M glucose in 0.1 M sodium acetate pH 4.5, 1 mM gluconolactone, and eluted with 0.01 M cellobiose in the same buffer.




(a)



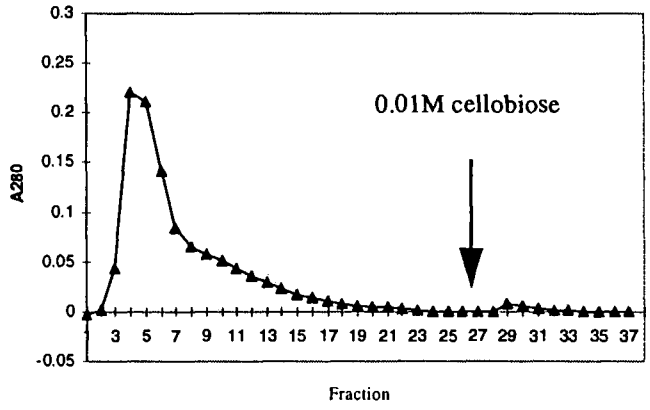

(b)

Figure 5.3. The effect of glucose on the adsorption of CBH II on APTC-derivatized affinity chromatography. CBH II fraction obtained from DEAE-Sepharose column was applied to APTC-derivatized column with (a) no glucose and (b) 0.2 M glucose in 0.1 M sodium acetate pH 5, 1 mM gluconolactone, and eluted with 0.01 M cellobiose in the same buffer.

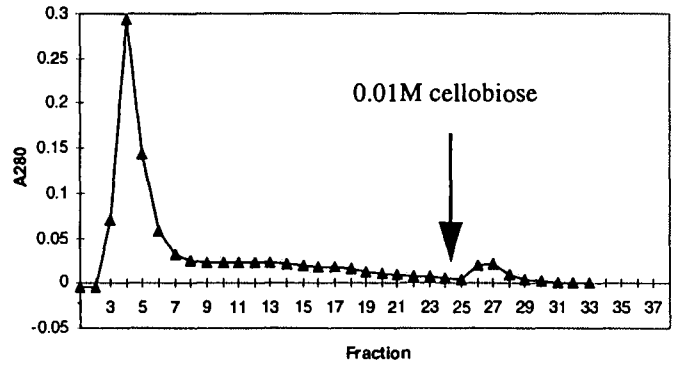
The effect of other sugars on CBH II affinity chromatography was also studied. Galactose, mannose, xylose, and gluconolactone were tested, but none of these sugars appeared to increase the affinity of CBH II for immobilized APTC (see Figure 5.4 and 5.5). Thus, an increase in the affinity of CBH II for immobilized APTC was only observed in the presence of glucose.

Different glycosides were tested for their abilities to elute CBHs from APTC-affinity columns including, cellobiose, lactose, and methyl β-D-glucopyranoside. These potential eluting compounds were tested in the presence and absence of glucose when working with CBH II, due to the demonstrated effect of glucose on CBH II affinity. These same compounds were tested only in the absence of glucose when working with CBH I. Making the mobile phase 0.01M cellobiose resulted in the elution of both CBHs under all conditions tested (Figure 5.6). Both CBH I and CBH II were eluted from the APTC-derivatized columns with lactose and methyl β-D-glucopyranoside when glucose was absent from the mobile phase (Figure 5.7 and 5.8). However, in the presence of glucose, neither lactose nor methyl β-D-glucopyranoside caused elution of CBH II from this affinity column.

The interaction of the two CBHs with immobilized APTC was also determined quantitatively by partition equilibrium experiments, in order to obtain a partition coefficient for each enzyme. A partition coefficient (K) is defined as the ratio of bound enzyme to free enzyme, and can be estimated from the slope of the straight line obtained at low total enzyme concentration in the adsorption isotherm (Mohr and Pommerening, 1985). Figure 5.9 is an illustration of adsorption isotherms for CBH I and CBH II with

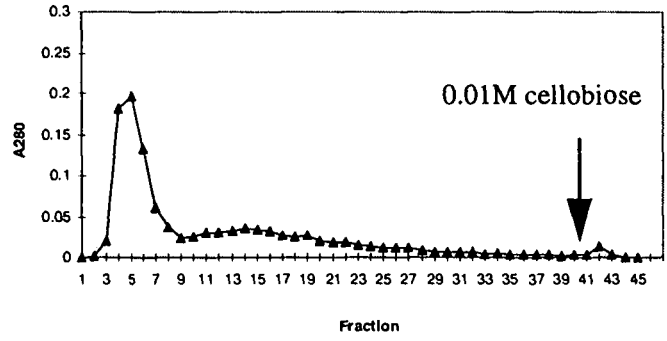



(a)

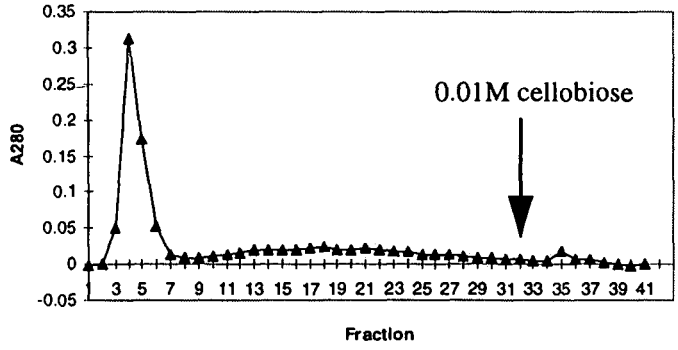



(b)

Figure 5.4. The effect of galactose and mannose on the adsorption of CBH II on APTC-derivatized affinity chromatography. CBH II fraction obtained from DEAE-Sepharose column was applied to APTC-derivatized column with (a) 0.2 M galactose and (b) 0.2 M mannose in 0.1 M sodium acetate pH 5, 1 mM gluconolactone, and eluted with 0.01 M cellobiose in the same buffer.

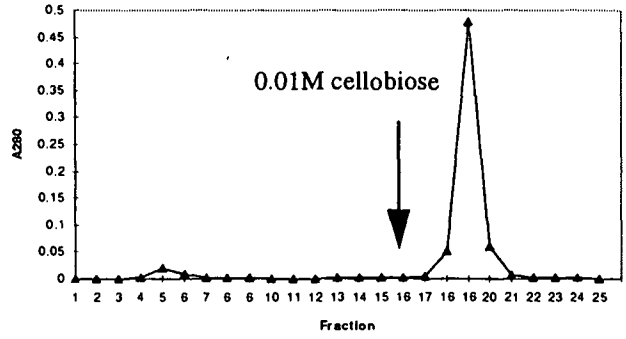



(a)

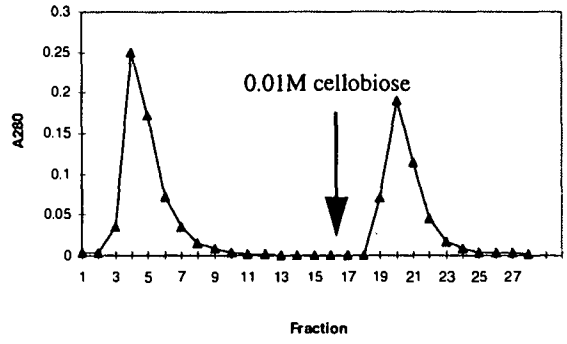



(b)

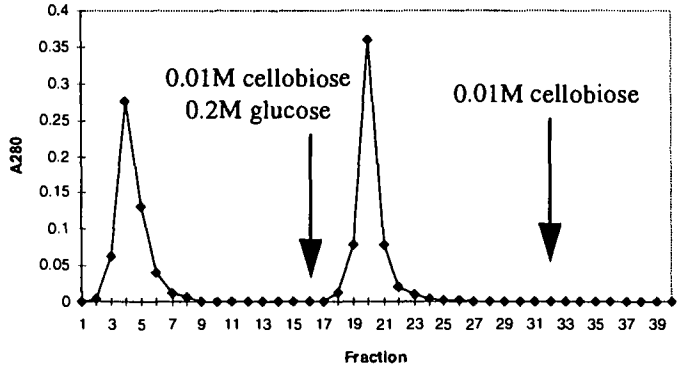
Figure 5.5. The effect of xylose and gluconolactone on the adsorption of CBH II on APTC-derivatized affinity chromatography. CBH II fraction obtained from DEAE-Sepharose column was applied to APTC-derivatized column with (a) 0.2 M xylose and (b) 0.2 M gluconolactone in 0.1 M sodium acetate pH 5, 1 mM gluconolactone, and eluted with 0.01 M cellobiose in the same buffer.




(a)

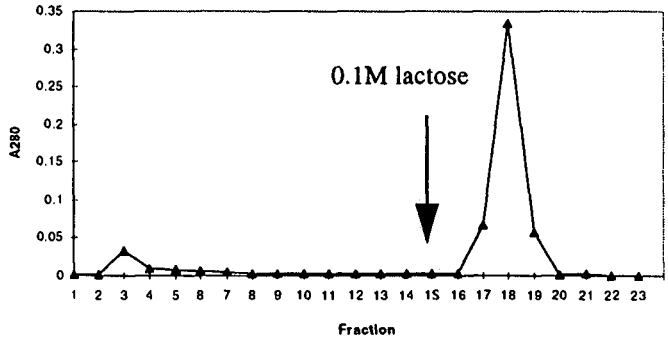



(b)

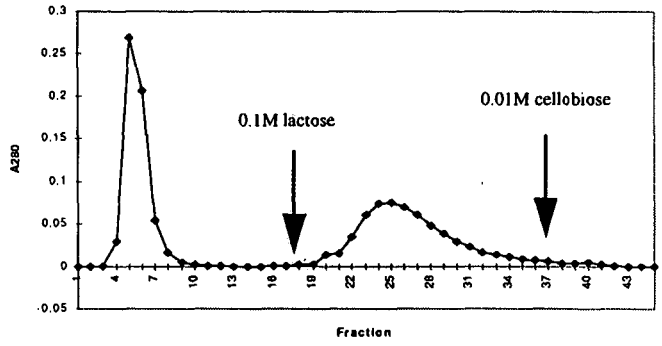



(c)

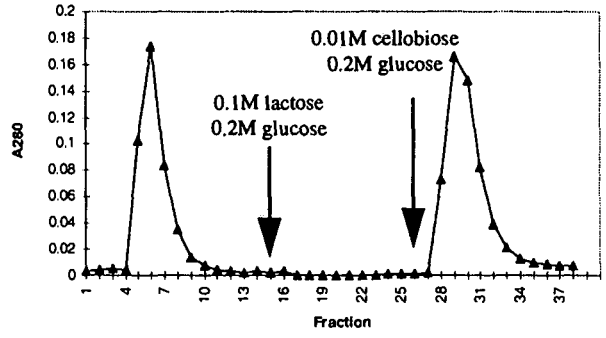
Figure 5.6. The effect of cellobiose on the desorption of CBH I and CBH II from APTC-derivatized affinity chromatography. (a) Retained CBH I and (b) CBH II were eluted from the column by 0.01 M cellobiose. (c) Retained CBH II was first eluted with 0.2 M glucose and 0.01 M cellobiose, and later with 0.01 M cellobiose.




(a)

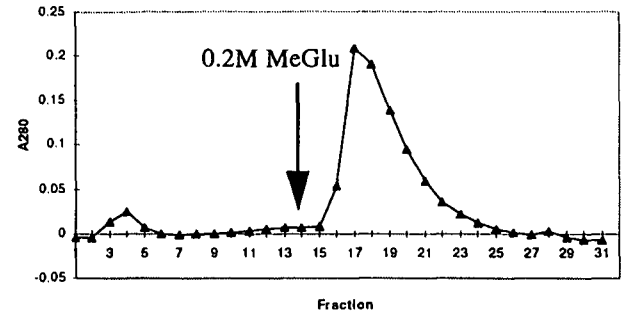



(b)

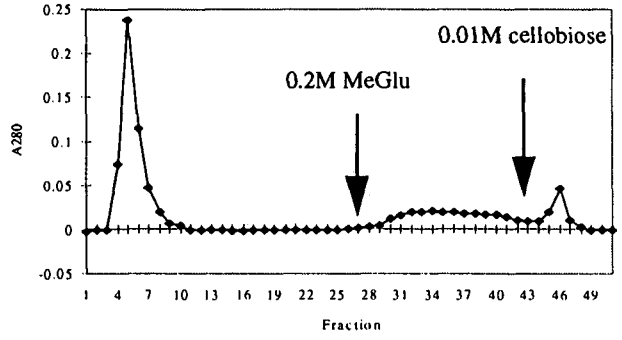



(c)

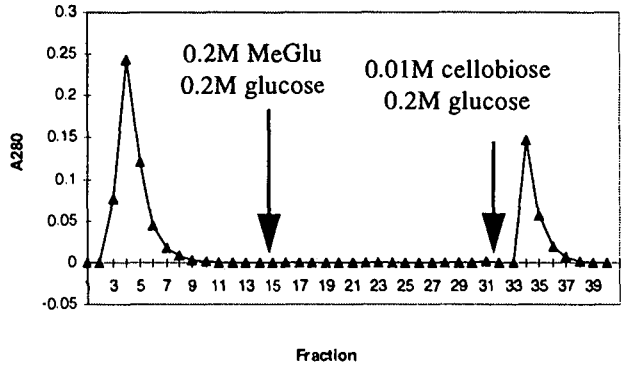
Figure 5.7. The effect of lactose on the desorption of CBH I and CBH II from APTC-derivatized affinity chromatography. (a) Retained CBH I was eluted from the column by 0.1 M lactose. Retained CBH II was first eluted by 0.1 M lactose (b) without glucose, and (c) with 0.2 M glucose, and later with 0.01 M cellobiose in the same buffer.




(a)




(b)




(c)

Figure 5.8. The effect of methyl $\beta$-D-glucopyranoside (MeGlu) on the desorption of CBH I and CBH II from APTC-derivatized affinity chromatography. (a) Retained CBH I was eluted from the column by 0.2 M MeGlu. Retained CBH II was first eluted by 0.2 M MeGlu (b) without, and (c) with 0.2 M glucose, and later with 0.01 M cellobiose.

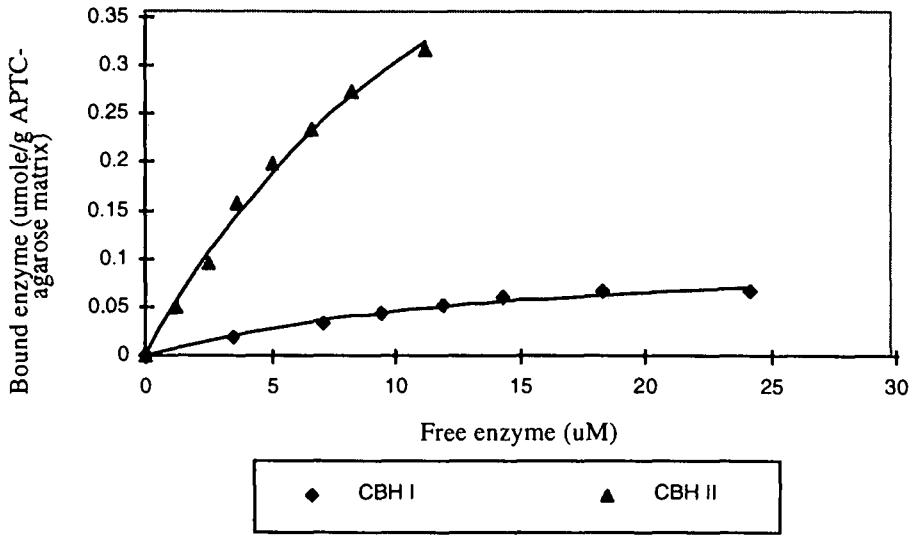



Figure 5.9. Adsorption isotherm of cellobiohydrolases on immobilized APTC agarose at 4°C. 1.5 mL of enzyme solutions with known protein concentrations (in 50 mM sodium acetate pH 5, for CBH I, and 0.2 M glucose, 50 mM sodium acetate pH 5, for CBH II) were mixed with 0.04 g of immobilized APTC agarose at 4°C for 2 hr.

immobilized APTC at 4°C. These adsorption isotherms were obtained under apparent optimum conditions for CBH I and CBH II binding, i.e. CBH I in 50 mM sodium acetate pH 5, CBH II in 0.2 M glucose, 50 mM sodium acetate pH 5. Under these conditions, the partition coefficient for CBH II ($K_{II}$) is approximately 10 times higher than that of CBH I ($K_I$): $K_I = 0.0038 \pm 0.0001$ $lg^{-1}$, and $K_{II} = 0.034 \pm 0.006$ $lg^{-1}$ (Table 5.1). CBH I has a lower partition coefficient when glucose is added to the system: $K_I = 0.0038 \pm 0.0001$ $lg^{-1}$ (in the absence of glucose) versus $K_I = 0.0020 \pm 0.0004$ $lg^{-1}$ (in the presence of glucose).

The role of nonspecific interactions between the enzymes and ligand-coupled matrix were discussed in chapter 4. In these binding experiments, the effect of nonspecific interactions were minimized by efficient coupling and blocking of N-hydroxysuccinimide ester groups. The nonspecific interactions are mainly due to ionic interaction, thus, salt was added to the system to account for this effect. In the presence of salt, the partition coefficients of both enzymes increased with the value of CBH II still being about 10 times higher than that of CBH I: $K_I = 0.0044 \pm 0.0001$ $lg^{-1}$, and $K_{II} = 0.043 \pm 0.004$ $lg^{-1}$.

## 5.4. DISCUSSION

Immobilized APTC is prepared by coupling it via its reducing end to agarose beads, thus only its nonreducing end can be accessed by exocellulases, as shown in Figure 5.10. It is interesting to determine whether the two exoenzyme classes will interact with immobilized APTC differently. When tested with exocellulases from *Trichoderma reesei*, the interactions of two CBH classes with immobilized APTC seemed to be

Table 5.1. Partition coefficients (K) of *Trichoderma reesei* cellobiohydrolases for immobilized APTC agarose.

| Conditions | Partition coefficient, K ($lg^{-1}$)[a] | |
|---|---|---|
| | CBH I | CBH II |
| Buffer[b] | 0.0038±.0001 | n.d.[c] |
| Buffer + 0.2M Glucose | 0.0020±.0004 | 0.034±0.006 |
| Buffer + 1M NaCl | 0.0044±0.0001 | 0.043±0.004[d] |

[a] Partition coefficients were determined from the slope of the straight line of the adsorption isotherm.
[b] The buffer used in the binding experiment was 50 mM sodium acetate pH 5.
[c] not determined.
[d] The buffer for CBH II always contained 0.2 M glucose in 50 mM sodium acetate pH 5.

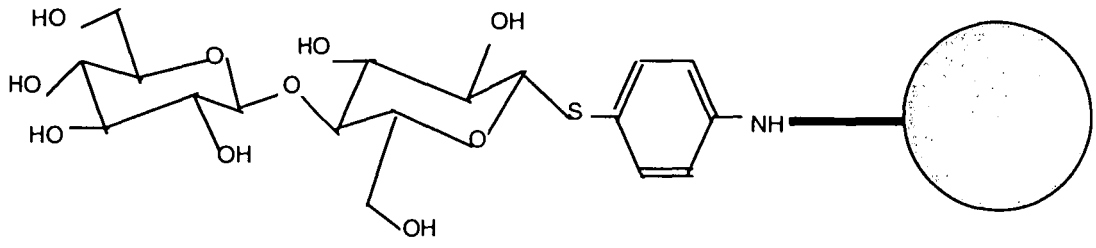
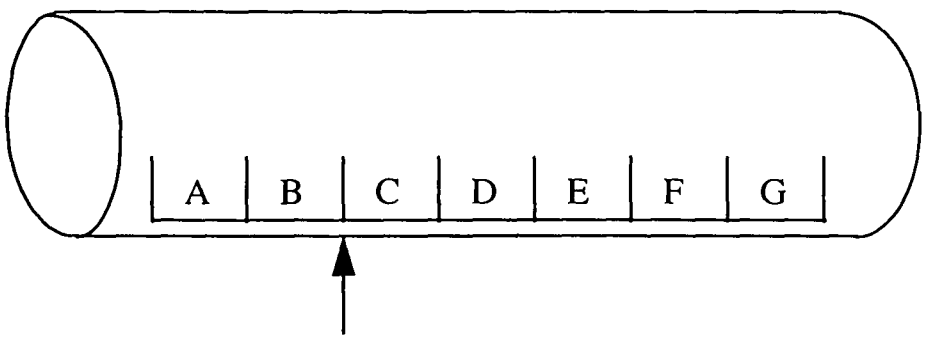



Figure 5.10: *p*-Aminophenyl 1-thio-β-D-cellobioside (APTC), when immobilized on agarose bead. Note that the figure is not drawn to scale, in that the bead is extensively larger than APTC. ◎ = Agarose bead.

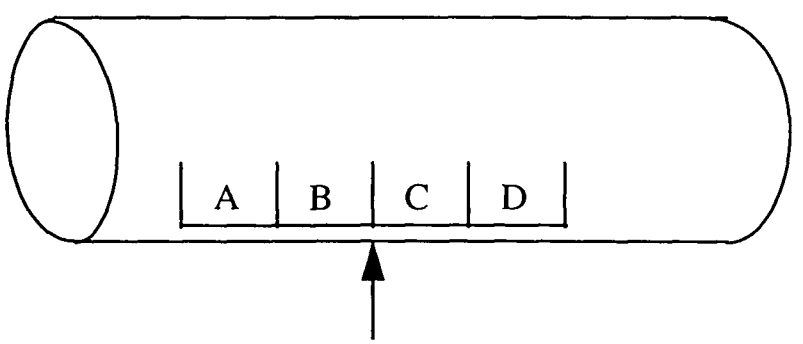
different. The affinity of CBH I for immobilized APTC decreased when glucose was present in the system. In contrast, glucose enhanced the affinity of CBH II for immobilized APTC.

It is of interest to consider this behavior in terms of the "subsite" theory of polymer degrading enzymes. The active site of exo-acting enzymes is considered to be composed of several subsites. Based on three-dimensional crystal structure, the active site of *Trichoderma reesei* CBH I consists of 7 glucosyl subsites referred to as A-G (Figure 5.11A). Site G is at the entrance of the active site tunnel while site A is at the exit of the active site tunnel. The catalytic residues are likely located between subsites B and C (Divne *et al.*, 1994). The active site of *Trichoderma reesei* CBH II is composed of 4 subsites (A-D), with the catalytic residues presumably locate between subsites B and C (van Tilbeurgh *et al.*, 1985; Rouvinen *et al.*, 1990) (Figure 5.11B). An increase in CBH II affinity for immobilized APTC may be explained by the same rationale as used by other investigators to explain the increase in the affinity of CBH II for soluble cellodextrins, i.e. binding of glucose in the A subsite of CBH II induces a conformational change in CBH II resulting in concomitant and comparable increases in the affinity of the other subsites in enzyme active site (van Tilbeurgh *et al.*, 1989).

Figure 5.12A represents the probable mode of interaction of CBH II with free APTC in the presence of glucose, based on the published three-dimensional structure of CBH II-cellodextrin complex, and the binding studies of CBH II with soluble cellodextrins (van Tilbeurgh *et al.*, 1989; Rouvinen *et al.*, 1990; Teleman *et al.*, 1995). Figure 5.12B illustrates the mode of interaction between CBH II and immobilized APTC.



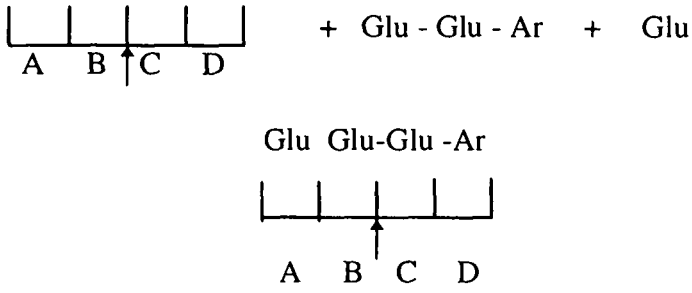

(A)




(B)

Figure 5.11. A model of enzyme active site for (A) CBH I and (B) CBH II, based on their three-dimensional structures (Divne *et al.*, 1994; Rouvinen *et al.*, 1990). A-G represents each subsite in enzyme active site. The arrow represents the catalytic site in enzyme active site.

(A) Free APTC




(B) Immobilized APTC

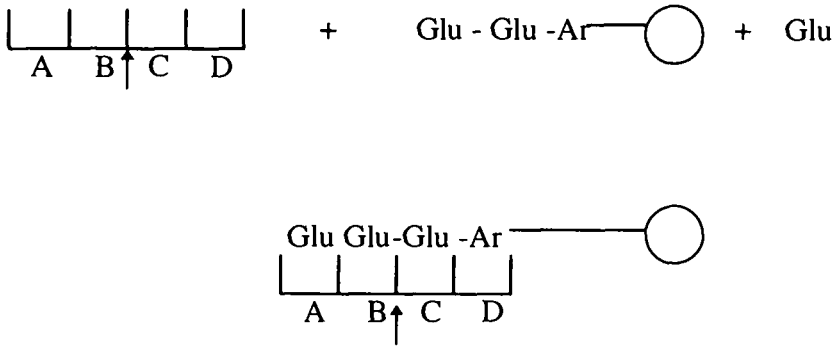



Figure 5.12. The interaction of CBH II with (A) free APTC and (B) immobilized APTC, in the presence of glucose. A-D represents each subsite of CBH II active site, and the arrow represents the catalytic site.

The binding of immobilized APTC was analogous to the binding of free APTC, based on the fact that CBH II preferentially initiates the hydrolysis of cellodextrins from the nonreducing end.

Once exoenzymes bind to immobilized APTC, other compounds can be added to the mobile phase which will compete with the ligand for the enzyme active sites, thus eluting the enzymes. Cellobiose, lactose, and methyl β-D-glucopyranoside exhibited the ability to elute both of the CBHs in the absence of glucose. However, lactose and methyl β-D-glucopyranoside failed to elute CBH II when glucose was present in the mobile phase. Cellobiose may be use to elute CBH II in the presence or absence of glucose. A binding study of CBH II with soluble cellodextrins suggested that methyl β-D-glucopyranoside binds to subsite A of the enzyme, which then displaces cellodextrins from the CBH II active site. The methyl group of methyl β-D-glucopyranoside, which replaces the hydroxyl group at the anomeric center of glucose, prevents the binding of cellodextrins at subsite B (van Tilbeurgh *et al.*, 1985; van Tilbeurgh *et al.*, 1989). Thus, glucose and methyl β-D-glucopyranoside compete for the same subsite within enzyme active site, i.e. subsite A. Therefore, the inability of methyl β-D-glucopyranoside to elute CBH II from the APTC-derivatized column in the presence of glucose is explained by competitive nature of glucose and methyl β-D-glucopyranoside binding at subsite A; glucose having the higher affinity for this subsite.

A binding study of CBH II with soluble cellodextrins suggests that cellobiose competes with other cellodextrins for subsites of enzyme active site, by binding at subsites A and B, B and C, or C and D (van Tilbeurgh *et al.*, 1985). Hence, cellobiose

directly competes with cellodextrins, and by analogy APTC, for subsites B, C, and D even in the presence of glucose. Therefore, cellobiose is capable of eluting CBH II from the APTC-derivatized column in the presence of glucose.

It appears that methyl β-D-glucopyranoside and cellobiose desorb CBH II from immobilized APTC by different mechanism, i.e. methyl β-D-glucopyranoside competing with glucose for the same subsite, whereas cellobiose directly competing with ligand subsites. It is not clear which mechanism applies to the elution of CBH II by lactose. Clearly, lactose does not elute CBH II in the presence of glucose. It may be that lactose competes with glucose for the subsite A, analogous to methyl β-D-glucopyranoside, which would explain its inability to elute CBH II in the presence of glucose. However, it is also possible that lactose also competes for subsites B, C, and D, analogous to cellobiose. In this case, the inability to elute CBH II in the presence of glucose would be explained by the much higher affinity of CBH II for APTC, compared to lactose, in the presence of glucose. It is well known that glucose increases the affinity of CBH II for APTC, and cellobiose (van Tilbeurgh *et al.*, 1985; van Tilbeurgh *et al.*, 1989). Data on the effect of glucose on CBH II-lactose interaction is not available. The presumption made in this second mechanism is that there is little to no increase in the binding constant for CBH II and lactose due to the presence of glucose. Therefore, lactose does not desorb CBH II from the APTC-derivatized column, when glucose is in the system.

The relative affinities of CBH I and CBH II for immobilized APTC were estimated in terms of partition coefficients. CBH II exhibited a relative higher affinity for immobilized APTC than CBH I under apparent optimum conditions for each enzyme.

This result is not expected based on the affinities of CBH I and CBH II for free cellobiose in solution as summarized in Table 5.2. CBH I has a much higher affinity for soluble cellobiose than CBH II, even when CBH II is evaluated in the presence of glucose (van Tilbeurgh *et al.*, 1985; van Tilbeurgh *et al.*, 1989). Clearly, APTC and cellobiose are different compounds and so the direct comparison of relative affinities may be in error. However, cellobiose is used in this study for comparison with APTC, based on the assumption that the aromatic group of APTC does not have a large effect on the affinity of this compound for the CBHs (see $K_a$ for cellobiose and 2′,4′-dinitrophenyl 1-thio-β-D-cellobioside in Table 5.2).

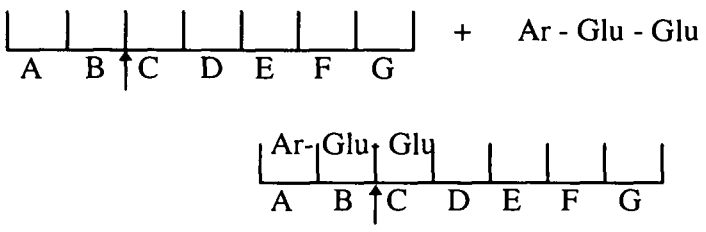
Since immobilized APTC no longer has the reducing end available for enzyme interaction, one may predict that the mode of interaction between CBH I and immobilized APTC may be altered significantly, since CBH I preferentially works at the reducing end. In contrast, the mode of interaction between CBH II and immobilized APTC may not change significantly, since CBH II preferentially works at the nonreducing end. The change in relative affinities of CBH I and CBH II, when considering soluble versus immobilized ligands, suggests a difference in the mode of binding of at least one of the enzymes, most probably CBH I due to its chain end specificity. An X-ray diffraction study of a CBH I and O-iodobenzyl-1-thio-β-D-cellobioside complex suggests that the aromatic group of free APTC will bind in subsite A, and the two glucosyl units in subsites B and C (Figure 5.13A). This assumes that free APTC and O-iodobenzyl-1-thio-β-D-cellobioside bind in the same way, both being soluble aromatic cellobiosides. The mode of binding of immobilized APTC to CBH I may not be the same as that of free

Table 5.2. Association constants for binding of *Trichoderma reesei* CBH I and CBH II with non-chromophoric ligands from literature[a].

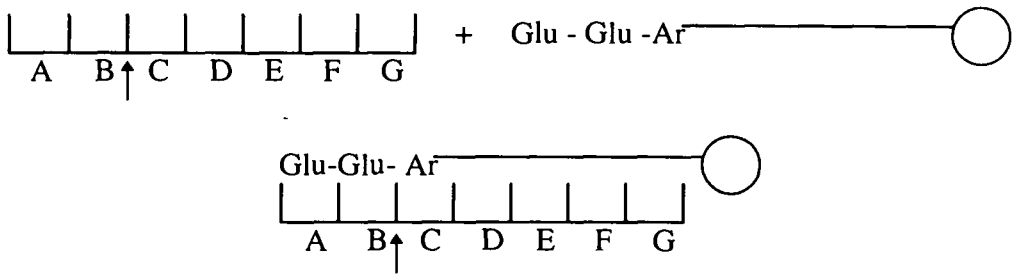
| Ligand | Association constants, $K_a$ ($M^{-1}$)[b] | |
|---|---|---|
| | CBH I | CBH II |
| Cellobiose | $(5.4\pm0.7)\times10^4$ | $(5.4\pm0.05)\times10^2$ (no glucose)<br>$(4.8\pm0.01)\times10^3$ (with glucose) |
| $N_2PhS(Glu)_2$[c] | $(6.1\pm0.4)\times10^4$ | n.r.[d] |

[a] from Claeyssens *et al.* (1989) and van Tilbeurgh *et al.* (1989).
[b] association constants were determined by ligand difference spectrophotometry and by displacement titration in 50 mM sodium acetate buffer (pH 5.0) at 25°C.
[c] $N_2PhS(Glu)_2$ = 2′,4′-dinitrophenyl 1-thio-β-D-cellobioside.
[d] not reported.

(A) Free APTC

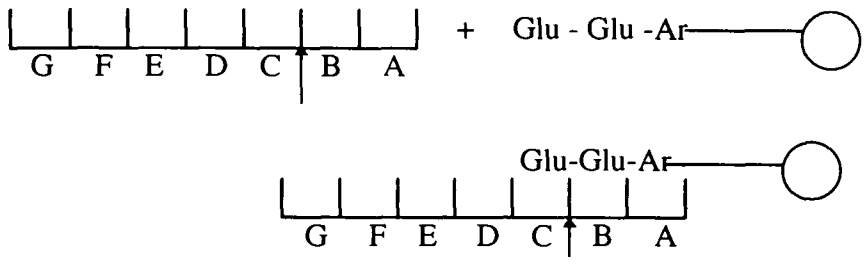



(B) "Forward" mechanism for immobilized APTC




(continued)

(C) "Backward" mechanism for immobilized APTC




(D) "Backward" mechanism with a frame shift

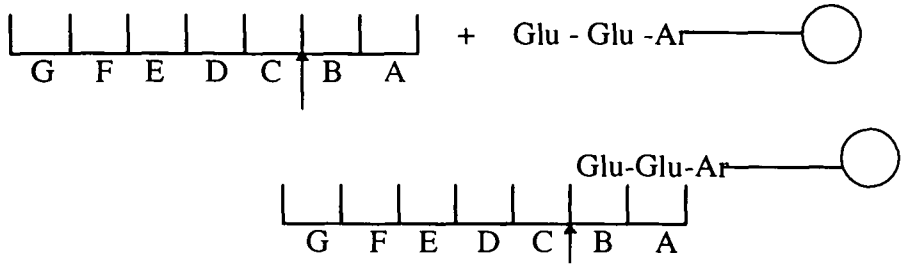



Figure 5.13. Two proposed models for the binding of CBH I with immobilized APTC. The binding of CBH I with (A) free APTC, (B) immobilized APTC by "forward" model, (C) immobilized APTC by "backward" model, and (D) immobilized APTC by "backward" model with a frame shift, ◯ = agarose bead. A-G represents each subsite of CBH I active site, and the arrow represents the catalytic site.

APTC. Two models are here proposed for the mode of binding of immobilized APTC with CBH I. The two models are referred to as the "forward" and "backward" models, in reference to whether immobilized APTC enters the active site tunnel via the "normal entrance" (the forward model) to the enzyme tunnel or whether immobilized APTC enters the active site tunnel of the enzyme via the "normal exit" (the backward model).

In the "forward" model, APTC enters the enzyme active site tunnel at subsite G, aligning with subsites as shown in Figure 5.13B. The attractive thing about this model is that the ligand still enters the active site via the "normal entrance", defined as the entrance through which soluble cellodextrins enter the active site of this enzyme. This model is also capable of explaining the reduced affinity of CBH I, relative to CBH II, for immobilized APTC, since the polarity (position of C4 relative to C1) of each of the glucosyl residues of the ligand will be the reverse of that which best fits the respective subsite. This reverse polarity, in terms of mode of binding, may make this mechanism unlikely since relatively small differences in the structure of the glucosyl subunit are expected to significantly effect ligand binding even when the ligand is bound with the correct polarity. This reverse polarity is not likely to occur with CBH II since it typically binds APTC via its nonreducing end, and nonblocked terminus.

In the "backward" model, APTC enters the enzyme active site tunnel at its traditional exit, that is at the end at which products resulting from the hydrolysis of soluble cellodextrins leave the tunnel, i.e. subsite A. In this model, APTC aligns with the subsites as shown in Figure 5.13C. This model is attractive in that the polarity of the bound ligand is the same as that observed upon the binding of free (not immobilized)

ligands. This is possible because the immobilized ligand "backs" its way into the active site tunnel. It is not clear whether this is feasible based on general principles of substrate/product migration into and out of enzyme active site tunnels. It is clear, however, that this mechanism allows for stable interactions between CBH I and immobilized APTC, even though the reducing end of the ligand is blocked. It is difficult to explain the reduction in the relative affinity of CBH I, compared to CBH II, for immobilized APTC if the binding is as described in Figure 5.13C, since this binding mode corresponds to that observed for soluble cellodextrins. It may be that the reduction in CBH I binding is due to a frame shift of the ligand due to its being immobilized. In this case, the relative affinity may decrease due to non-optimum alignment of the ligand in subsites A through G (Figure 5.13D).

CBH I and CBH II, representing the two classes of exocellulases, may be separated on an APTC-derivatized affinity column by making use of their different behaviors in the presence of glucose (Figure 5.14). In this case, a mixture of CBH I and CBH II was applied to the column in 0.2 M glucose, in 0.1 M sodium acetate buffer, pH 5. The mobile phase was then made 0.1 M in lactose, while maintaining a glucose concentration of 0.2 M. Under these conditions, CBH I was eluted from the column, while CBH II remains associated with the column. CBH II was then eluted by making the mobile phase 0.01 M in cellobiose (with or without glucose). The ability to separate the two classes of CBHs obviously broadens the range of applications for this chromatographic method. This behavior is not unique to APTC, in that CBH I and CBH II of *Trichoderma reesei* can also be separated using a *p*-aminobenzyl 1-thio-$\beta$-D-

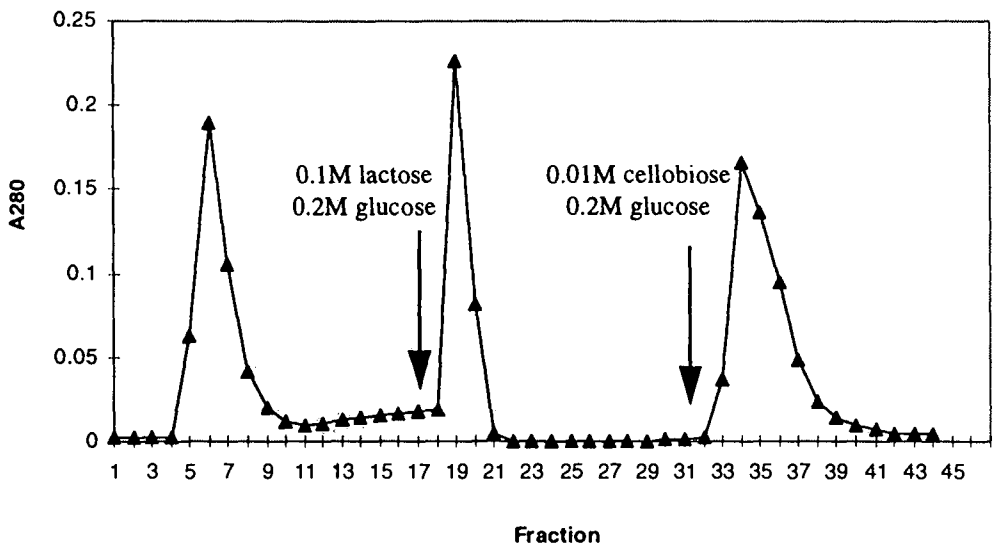



Figure 5.14. APTC-derivatized affinity chromatography of a mixture of CBH I and CBH II. A mixture of CBH I and CBH II obtained from DEAE-Sepharose column was applied with 0.2 M glucose, 0.1M sodium acetate pH 5, 1 mM gluconolactone, to APTC-derivatized affinity column, and first eluted with 0.1 M lactose, and later with 0.01 M cellobiose containing 0.2 M glucose in the same buffer.

cellobioside (ABTC) affinity column (van Tilbeurgh *et al.*, 1984). The drawback to this approach, however, is that glucose affects the binding of these two enzymes in opposite ways, such that chromatographing CBH I in the presence of glucose results in lower affinity, concomitant leaching of enzyme and, thus, lower recoveries (see Figure 5.4).

This study is a preliminary investigation which demonstrates that immobilized APTC has the potential to be used for differentiating between the two classes of exocellulases; class I being those enzymes that preferentially work at the reducing terminus, and class II being those enzymes that preferentially work at the nonreducing terminus. The data presented here shows that CBH I and CBH II interact with immobilized APTC differently. It is not clear whether this difference is characteristic of the differences between class I and class II enzymes, per se, or whether the observed differences simply reflect the variation in naturally occurring exocellulases. Further studies with class I and class II enzymes from other microorganism, such as *Thermomonospora fusca*, *Cellulomonas fimi* and *Aspergillus aculeatus* will provide insight into the answer to this question.

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# CHAPTER 6

## CONCLUSIONS

The ligand, *p*-aminophenyl 1-thio-β-D-cellobioside (APTC) can be used to separate exo-acting cellulases from an enzyme mixture in affinity chromatography. Only cellobiohydrolases are retained on the APTC-affinity column, while endo-acting cellulases are not. The affinity between CBHs and APTC is also affected by nonspecific interactions, i.e. ionic interactions between the enzyme and ligand-supporting matrix. The behavior of CBHs on APTC-affinity column can be rationalized by considering that the columns are functioning as both affinity and ion-exchange columns. Both classes of exocellulases, class I and class II, bind to immobilized APTC. However, the behavior of these two exoenzymes on the APTC-affinity column is different; which suggests that immobilized APTC has the potential to be used for differentiating between the two classes of exocellulases.

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