AN ABSTRACT OF THE THESIS OF

Kunruedee Sangseethong for the degree of Doctor of Philosophy in Food Science and Technology presented on May 7, 1999. Title: Immobilized Cellooligosaccharides in the Study of *Trichoderma reesei* Cellobiohydrolases.

Abstract approved: _____
Michael H. Penner

A novel type of model substrates, i.e. immobilized *p*-aminophenyl-β-D-cellooligosaccharides, was developed and used in the study of exocellulases. The two major cellobiohydrolases from *Trichoderma reesei*, CBH I and CBH II were used as representative enzymes. *p*-Aminophenyl derivatives of cellobiose ($PAPG_2$), cellotriose ($PAPG_3$), and cellotetraose ($PAPG_4$) were synthesized from the reaction of *p*-nitrophenol and peracetylated glycosyl bromide of the corresponding cellooligosaccharides under the phase-transfer catalyzed conditions, followed by deacetylation and catalytic hydrogenation. *p*-Aminophenyl cellooligosaccharides were then tethered via their amino functional groups to N-hydroxy succinimide-activated agarose. The ability of CBH I and CBH II to associate with and catalyze the hydrolysis of reducing end tethered cellooligosaccharides was tested. CBH I catalyzed the hydrolysis of free $PAPG_2$ but CBH II did not. Both CBH I and CBH II reversibly bound, but did not hydrolyze, immobilized $PAPG_2$. Hence, the immobilized $PAPG_2$ was tested for the affinity chromatographic application.

$PAPG_2$ is shown to be an effective ligand for the chromatographic fractionation of cellobiohydrolases (CBHs). The $PAPG_2$-derivatized agarose specifically retained the CBH component of relatively complex cellulase mixtures. The purity of the resulting CBH preparation was comparable to that of corresponding enzyme preparations obtained using more traditional thioglycoside-based affinity ligands. The application of $PAPG_2$ as an affinity ligand suggests that the immobilized reducing end-blocked ligand associate with the *T. reesei* CBHs in a catalytically nonproductive mode.

The catalytic activity for the hydrolysis of free and immobilized aryl-cellodextrins by the CBH I and CBH II were determined. CBH II attacked free and immobilized $PAPG_3$ and $PAPG_4$ in a typical exo manner in which cellobiose is a major hydrolytic product released from the nonreducing end. The rate of hydrolysis increases with increasing chain length suggesting the extended binding sites (at least 4 binding sites). Like CBH II, CBH I preferentially cleaved immobilized $PAPG_3$ and $PAPG_4$ at a second glycosidic linkage from the nonreducing end; the rate of hydrolysis increases as a function of chain length. However, it attacked free aryl-cellodextrins in a random manner. The rate of hydrolysis increases only from $PAPG_2$ to $PAPG_3$ and significantly drops in PAPG4. This suggests that CBH I interacts with free and immobilized substrates in different modes.

Immobilized Cellooligosaccharides in the Study of *Trichoderma reesei* Cellobiohydrolases

by

Kunruedee Sangseethong

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented May 7, 1999
Commencement June, 1999

Doctor of Philosophy thesis of Kunruedee Sangseethong presented on May 7, 1999

APPROVED:

Major Professor, representing Food Science and Technology

Chair 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.

Kunruedee Sangseethong, Author

ACKNOWLEDGEMENTS

As my graduate student life has come to an end, I would like to express my appreciation to people who have helped make my dream come true. First and foremost, I would like to thank Dr. Michael H. Penner, my major advisor for his kindness, encouragement and invaluable advice throughout my study. I came to his lab thinking that I didn't know and couldn't do so many things, but little by little he has changed my attitude. After being trained in his lab in the past four years, I know if I try really hard, I can accomplish almost everything. Mike helps me find the confidence I once thought I never had.

I sincerely thank Dr. Kevin P. Gable, Dr. Ronald E. Wrolstad, Dr. Ajoy Velayudhan, and Dr. James A. Moore for serving on my committee.

I would like to thank all my labmates for their friendship and help in several ways. Their enjoyable conversation and scientific discussion are appreciated.

I am deeply grateful to my parents, as well as my brothers and sisters, for their unconditional love and emotional support throughout my life. Just a thought of them can refresh my mind and get me through the endless time of stress and frustration.

My deepest appreciation goes to Worakrit Suvajittanont for his emotional support, endless patience and understanding. He has been my optimistic half since we met. Whenever I feel depressed about my research, he has always reminded me that there is the light at the end of the tunnel. He always finds the way to cheer me

up and make me stronger. Without his support, it would have been a lot harder to accomplish this study.

CONTRIBUTION OF AUTHORS

Dr. Michael H. Penner was involved in the design, data interpretation, and writing of each manuscript. Lisbeth Meunier-Goddik, Usicha Tuntasucharit, Ean-Tun Liaw were involved in data collection, analysis, and writing of manuscript in Appendix A.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF APPENDICES

LIST OF APPENDIX FIGURES

LIST OF APPENDIX TABLES

This thesis is dedicated

to my mother and father, my brothers and sisters,

and

to my love, Worakrit.

IMMOBILIZED CELLOOLIGOSACCHARIDES IN THE STUDY OF *TRICHODERMA REESEI* CELLOBIOHYDROLASES

CHAPTER 1

INTRODUCTION

As a major constituent in plant, cellulose is the most abundant organic polymer on earth. It is constantly replenished to our biosphere by photosynthesis from solar energy, carbon dioxide and water. Since cellulose is made up of glucose monomers, it is considered a major supply of fermentable sugar for the production of food, fuel and industrial chemicals. Cellulose is a structural component of plant cell wall which gives the plant strength and rigidity. It has a high degree of crystallinity and it is embedded in a matrix of hemicellulose and lignin. Hence, the hydrolysis of cellulose is rather difficult.

Industrially, cellulose can be hydrolyzed into soluble sugars using either acid or enzyme catalysts. Because of increasing demands for environmentally friendly processes, the enzymatic hydrolysis of cellulose has become the more desirable process. The efficient hydrolysis of cellulose requires cooperative action between several enzymes; each of which has a distinct function. Current applications tend to use small amounts of crude cellulase due to the high cost of enzyme production and purification. Thus, high specificity and unique mode of action of individual cellulolytic enzymes is not often exploited by industry. In order to develop new products and processes based on the utilization of cellulolytic

enzymes, a detailed understanding of the modes of action of individual enzymes is a prerequisite.

Detailed characterization of a particular enzyme in a cellulolytic system requires that it is free from other enzyme components. This often proves to be difficult because cellulolytic systems produced by most microorganisms contain a multiplicity of enzyme components; some of which are closely related and difficult to separate.

A novel type of model substrates has been developed and used in this work to overcome some of the basic challenges encountered in studies concerning the mechanistic function of cellulolytic enzymes. The first part of this study deals with the preparation and use of *p*-aminophenyl β-D-cellobioside in the chromatographic purification of exo-type cellulases. The ease of preparation and effectiveness of this ligand was compared with that of the more traditional affinity ligands, i.e. *p*-aminobenzyl1-thio-β-D-cellobioside and *p*-aminophenyl1-thio-β-D-cellobioside, in terms of relative purity and specific activity of purified enzymes. The second part of this study deals with the synthesis and application of immobilized, reducing end-blocked cellooligosaccharides to elucidate the modes of action of two exocellulases, cellobiohydrolase I and cellobiohydrolase II produced by the filamentous fungus *Trichoderma reesei*. In addition, another aspect of cellulolytic study concerning with the heterogeneity of microcrystalline cellulose, another type of a model substrate that is widely used by many laboratories, is included in

Appendix A. Specifically, it describes the rationale for particle size effect on rates of enzymatic saccharification of microcrystalline cellulose.

CHAPTER 2

LITERATURE REVIEW

2.1 Cellulose

Cellulose is the major carbohydrate synthesized by plants. It is the most abundant biopolymer on earth. Depending on the source, the plant cell wall consists of 40-60% cellulose. Most native cellulose is found in cell walls in the form of structural microfibrils. Cellulose microfibrils do not exist alone in nature. It is usually closely associated with hemicellulose and lignin, forming very complex structure as shown in Figure 2.1. Hemicellulose and lignin impose a physical barrier impeding the biodegradation of cellulose.

Cellulose is a linear polymer of β-1,4-D-glucopyranose. The glucopyranosyl units in the cellulose molecule exist in a chair configuration ($^4C_1$), which is the lowest energy conformation. Degree of polymerization (DP), i.e. the number of glucose units per molecule, of cellulose varies from 500 to 14,000 depending on the source (Richmond, 1991). Because of the nature of the β-linkage, each glucopyranosyl unit is oriented 180° relative to its neighbors. Thus, the disaccharide cellobiose is the repeating unit of the polymer (Figure 2.2). In nature, cellulose forms bundles, or microfibrils, in which the molecules are packed and tightly held together by intra- and inter-molecular hydrogen bonds and multiple van der Waals interactions. The intra-molecular hydrogen bonding between adjacent

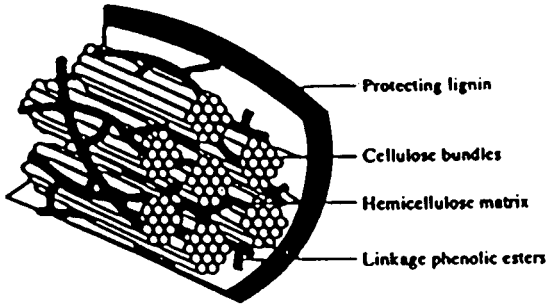


Figure 2.1 Schematic illustration of the probable relationship of lignin and hemicellulose to cellulose microfibrils in plant tissue (van Zyl et al., 1998)

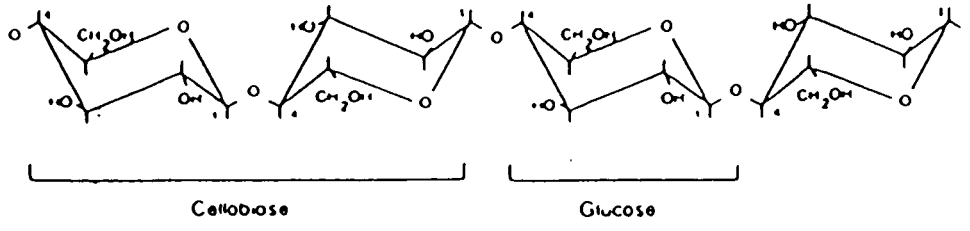


Figure 2.2 Chemical structure of cellulose

residues confers rigidity and causes the polymer to adopt a flat, extended, ribbon-like shape. The intermolecular hydrogen bonding, together with van der Waal's forces, cause cellulose chains to pack very tightly together, forming highly ordered crystalline region. The crystalline regions are interspersed with loosely packed, less ordered amorphous regions (Figure 2.3). The closely packed crystalline structure is the main reason why cellulose is highly resistant to hydrolysis. The crystallinity of cellulose microfibrils depends on the origin and handling/processing of the plant material. It can vary from 0% for amorphous, acid-swollen cellulose to nearly 100% for the cellulose isolated from algae (*Valonia macrophysa*). Cellulose from cotton is approximately 70% crystalline. The size of microfibrils also varies depending on source. The lateral dimension of microfibrils ranges from 3-4 nm in higher plants to about 20 nm for unicellular algae *Valonia macrophysa* (Clarke, 1997).

Cellulose can exist in at least four allomorphs or crystal lattice types, celluloses I-IV (Richmond, 1991). Native cellulose occurs in the form of cellulose I, in which the glucan chains within a microfibril have a parallel orientation, i.e. an arrangement in which the reducing ends of all glucan chains are located at the same end of a microfibril. Cellulose II, also called regenerated cellulose, is another lattice type obtained upon recrystallization of swollen or solubilized cellulose I. Cellulose II may be produced by alkali treatment (mercerization) of cellulose I. The transition of cellulose I to cellulose II is irreversible, suggesting that cellulose I is metastable whereas cellulose II is the more thermodynamically stable form (Sarko, 1978;

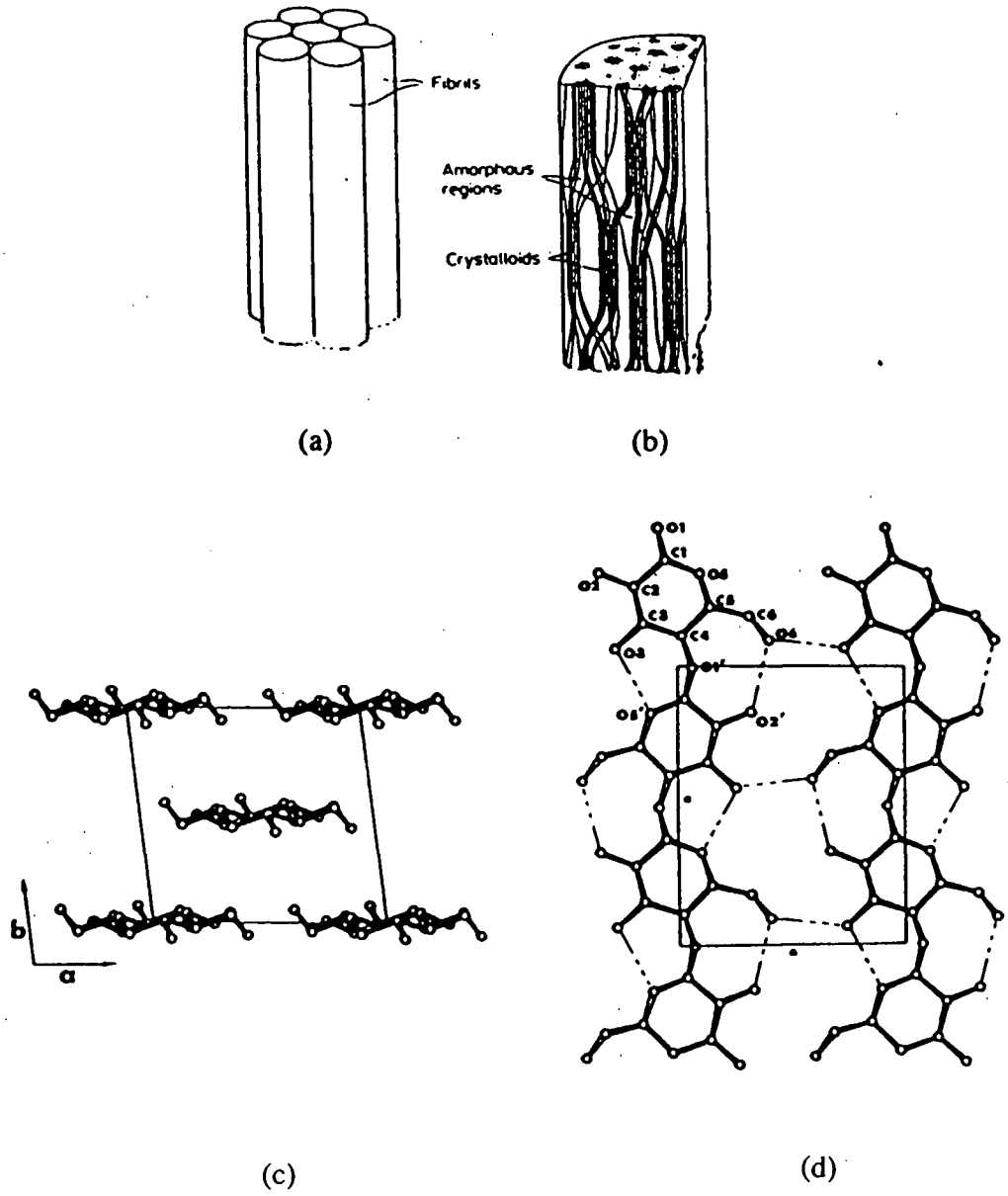


Figure 2.3 Organization of cellulose microfibrils.

(a) Bundle of parallel fibrils held together crosswise by hydrogen bonds.
(b) Lateral sectional view of one fibril showing crystalline regions interspersed by amorphous regions (Enari, 1983).
(c) Alignment of cellulose chain viewed perpendicular to the ab plane.
(d) Alignment of cellulose chain viewed perpendicular to the ac plane; hydrogen bonds are shown by dashed lines (Gardner and Blackwell, 1974)

Blackwell, 1982). Although rare, there is some evidence that cellulose II is produced in nature (Lee et al., 1994). The other two allomorphs of cellulose, III and IV, are reversibly produced by chemical treatments of either cellulose I or II (Sarko, 1978)

2.2 Enzymatic degradation of cellulose

Due to its crystalline structure and its interaction with other cell wall components, the hydrolysis of cellulose is not easy. This is why cellulose, the most abundant renewable resource, is industrially underutilized. In general, cellulose can be hydrolyzed into soluble sugars either enzymatically or chemically. In many applications, enzymatic hydrolysis becomes increasingly desirable due to many advantages. The process can be operated at mild conditions; enzymatic action is very specific and does not produce undesirable by-products. Since the enzymatic process does not use the corrosive chemicals and does not produce the hazardous waste, it is more environmentally friendly. The main product of cellulose hydrolysis is glucose which is an important starting material for many potential products such as ethanol, single cell protein and other chemicals.

2.3 Cellulolytic enzymes

The efficient hydrolysis of native cellulose involves a combination of several cellulases. These enzymes act synergistically to generate soluble

cellooligosaccharides, yielding glucose as the final product. The main producers of cellulolytic enzymes are fungi and bacteria. Protozoa found in the hindgut of lower termites can also produce cellulases. In addition, plants also synthesize cellulases to play an important role during ripening and morphogenesis of many fruits (Beguin and Aubert, 1992).

At least three major types of enzymes are required for the complete degradation of native cellulose to glucose. These are endoglucanases (endo-1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4), exoglucanases or cellobiohydrolases (exo-1,4-β-D-glucan-4-cellobiohydrolase, EC 3.2.1.91) and β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21). Each of these enzymes has different specificity and mode of action, as shown in Figure 2.4. Endoglucanases randomly cleave internal glucosidic linkages on the amorphous regions of cellulose. The action of endoglucanases causes a rapid drop in the degree of polymerization of the substrate chain and a relatively slow increase in soluble reducing groups. Cellobiohydrolases cleave cellobiose from the chain ends. They are capable of hydrolyzing crystalline cellulose. β-Glucosidases hydrolyze cellobiose and other low molecular weight cellooligosaccharides to glucose (Singh and Hayashi, 1995). The general view regarding the activity of these enzymes on different substrates is summarized in Table 2.1.

The modes of action of different cellulolytic enzymes on cellulose are dictated by the shape of their active sites. From the three-dimensional structures of glycosyl hydrolases, the active site topologies can be divided into three classes

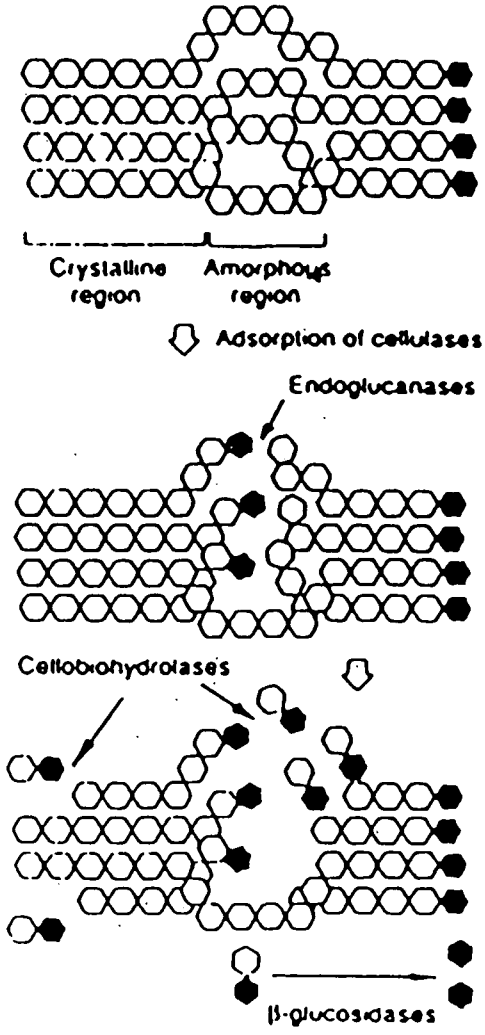


Figure 2.4 Modes of action of different cellulolytic enzymes. Glucose residues are indicated by hexagons and reducing end are shown in black (Béguin and Aubert, 1992).

Table 2.1 Action of cellulase components on different substrates (Wood and Campayo, 1990).

Enzyme	Crystalline cellulose	Amorphous swollen cellulose	CM-Cellulose	Cellooligo-saccharides	Cellobiose
Cellobiohydrolase	Slow	Very active	Nil	Active	Nil
Endoglucanase	Nil	Very active	Very active	Active	Nil
β-Glucosidase	Nil	Nil	Nil	Active	Active

(Davies and Henrissat, 1995). Pocket-active sites are found in enzymes hydrolyzing monosaccharide units from chain ends, such as β-glucosidases and glucoamylases (Figure 2.5a). In the second class, active sites have tunnel-liked shape (Figure 2.5b). This type of active site is found in exoglucanases, such as CBHI and CBHII from *T. reesei*. The activities of exoglucanases with tunnel-shaped active sites are confined to the chain ends. The third class of active sites is found in endo-acting enzymes. In this case, the active site is situated in an open cleft or groove (Figure 2.5c). With open active sites, endoglucanases can bind to and cleave the internal linkages in the middle of cellulose chain.

2.4 Cellobiohydrolase

Cellobiohydrolases are often the most abundant proteins found in the potent cellulolytic system. They are regarded as the key enzymes for the complete saccharification of crystalline cellulose (Beguin and Aubert, 1992; Divne et al.,

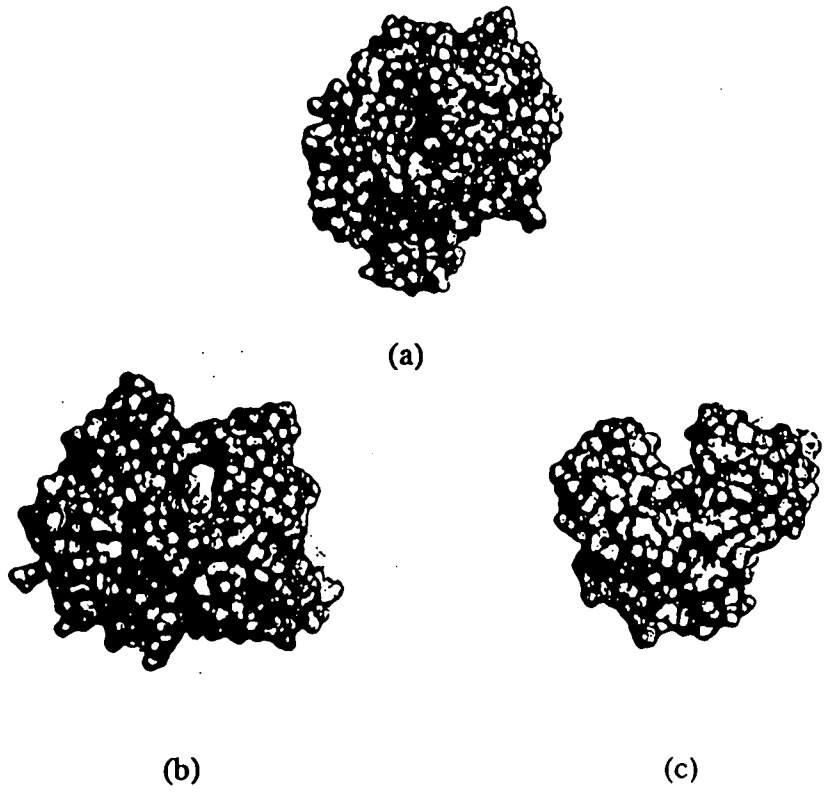


Figure 2.5 Three different active sites topologies of glycosyl hydrolases.
(a) The pocket active site of *A. awamori* glucoamylase.
(b) The tunnel-shaped active site of *T. reesei* CBH II.
(c) The cleft of *T. fusca* E_2 (Davies and Henrissat, 1995).

1994; Teeri, 1997). The filamentous fungus *Trichoderma reesei* produces one of the most efficient cellulolytic enzyme mixtures. It is comprised of 60% CBH I and 20% CBH II accounting for 80% of total enzyme produced (Teeri, 1997). Even though both CBH I and CBH II share some common features, such as tunnel-shaped active site, high activity toward crystalline cellulose and binding to the same affinity column, their modes of action on modified soluble cellodextrins differ considerably(Biely et al., 1993; Claeyssens et al., 1989; Barr et al., 1996). CBH I and CBH II are immunologically unrelated (Fagerstam and Pettersson, 1979). Both are glycoproteins differing in the amount of carbohydrate and the amino acid composition (Enari and Niku-Paavola, 1987; Wood et al., 1988). They hydrolyze glycosidic linkages via different mechanisms. Some properties of *T. reesei* cellobiohydrolase are listed in Table 2.2.

Table 2.2 Properties of *Trichoderma reesei* cellobiohydrolases (Bhikhabhai et al., 1984; Teeri, 1997).

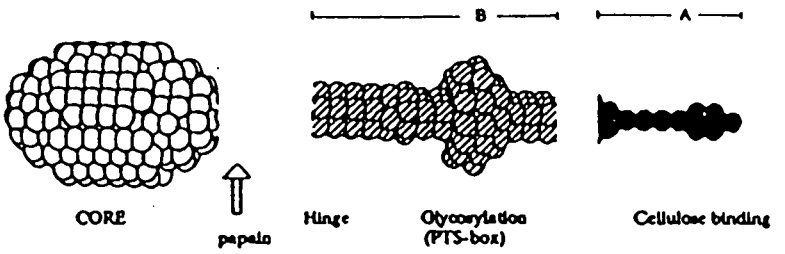
Enzyme	Family	Mw (kDa)	pI	% Carbohydrate	Mechanism	End specificity
CBH I	7	64	3.9	5.6	Retaining	Reducing
CBH II	6	53	5.9	18	Inverting	Non-reducing

2.4.1 Domain structure

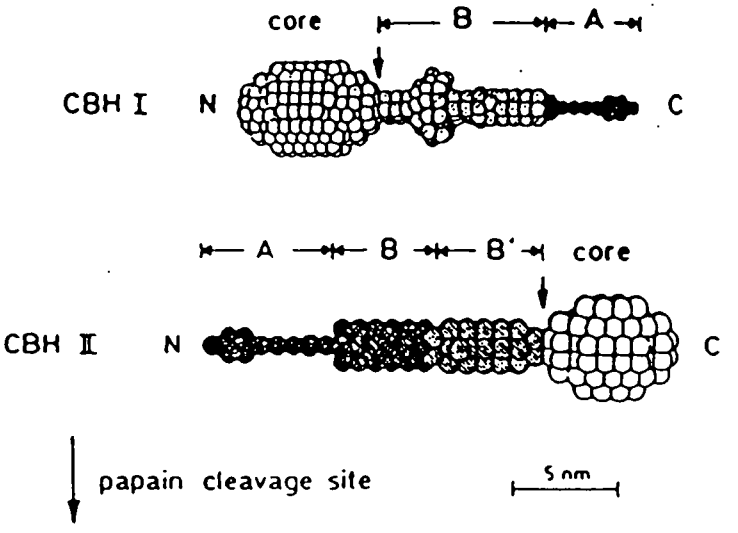
The common feature of most cellulolytic enzymes is the domain structure (Wood and Garcia-Campayo, 1990; Tomme et al, 1995a). Both CBH I and CBH II consist of a relatively large catalytic domain joined to a relatively small cellulose binding domain (CBD). These two domains are attached by a flexible *O*-glycosylated linker peptide as shown in Figure 2.6 (Abuja et al., 1988). So far no structure has been obtained for intact cellulases, probably due to flexible linker peptide. However, based on the partial proteolysis of the intact enzymes, the individual domains can be crystallized and studied; thus, providing an insight into the possible role of each domain.

2.4.1.1 *Cellulose binding domain (CBD)*

It has been shown that removal of CBD from CBH I and CBH II dramatically reduces the activity of both enzymes on crystalline cellulose but not on soluble substrates (van Tilbeurgh et al., 1986; Tomme et al., 1988; Gilkes et al., 1991). NMR spectroscopy reveals that the CBD of cellobiohydrolase I from *Trichoderma reesei* has a wedge-shape (Figure 2.7), with an amphiphilic character. Two flat surfaces can be distinguished: one predominantly hydrophobic, the other predominantly hydrophilic (Kraulis et al., 1989). Three aromatic amino acids, i.e. tyrosine, on the hydrophobic surface are thought to be responsible for the relatively tight binding of enzyme to cellulose (Linder et al., 1995). The precise role of CBD



(a)



(b)

Figure 2.6 Domain structure of *T. reesei* cellobiohydrolases as elucidated by small-angle X-ray scattering.

(a) Structure of *T. reesei* CBH I showing different domains and the papane cleavage site.

(b) Comparison between the domain arrangement of *T. reesei* CBH I and II (Abuja et al., 1988)

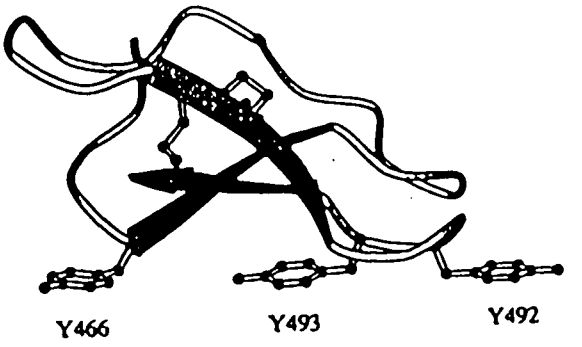


Figure 2.7 Ribbon diagram of the structure of cellulose binding domain from *T. reesei* CBH I. β-Sheets are represented as arrows. Aromatic residues implicated in binding to cellulose and disulfide bridges are shown in "ball and stick" form (Tomme et al., 1995b).

is not yet well understood. However, it is commonly assumed that CBD enhance activity by increasing the local enzyme concentration on the substrate surface, or by disrupting the intermolecular interactions of crystalline cellulose, thus increasing the substrate accessibility (Knowles et al., 1987; Teeri et al., 1992).

2.4.1.2 *Catalytic domain*

Recent three-dimensional crystal structure determination of the catalytic domains of *T. reesei* CBH I and CBH II show that both enzymes have extended active site tunnels for substrate binding and catalysis (Rouvinen et al., 1990; Divne

et al., 1994; Divne et al., 1998). The tunnel structure is partially stabilized by disulfide bridges. The active site tunnel of CBH I is approximately 50 Å long containing ten well-defined subsites (-7 to +3) for binding with glucosyl units (Divne et al., 1998). Binding of glucosyl units to subsites in the tunnel occurs via protein-mediated hydrogen bonding and stacking onto tryptophan residues W40, W38, W367, and W376 in subsite –7, -4, -2 and +1 respectively. Two glutamic acid residues (E212 and E217) and one aspartic acid residue (D214) between sites –1 and +1 play a crucial role in the catalytic process (Stahlberg et al., 1996).

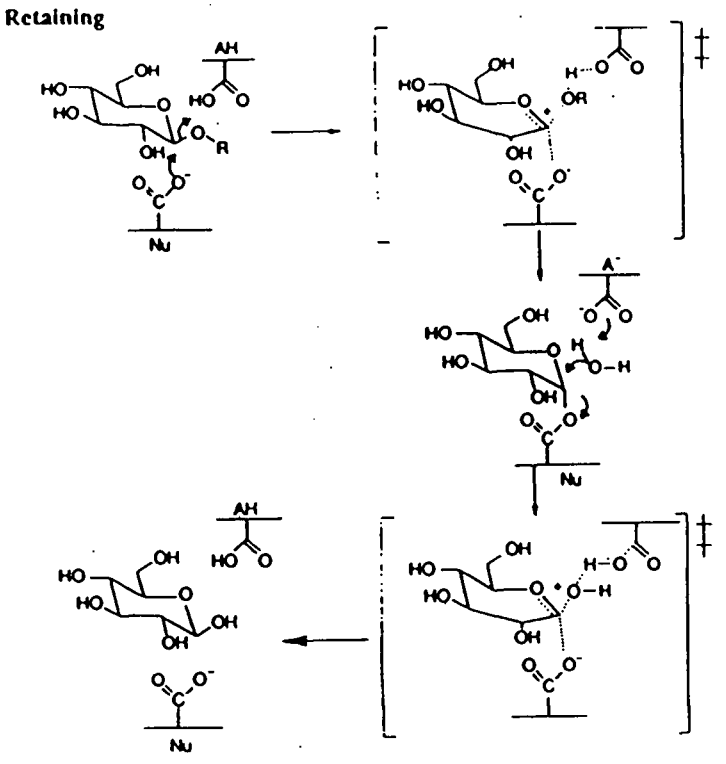
The active site tunnel of CBH II is much shorter than that of CBH I; it is about 20 Å long. Four glucosyl-binding subsites (-2 to +2) have been identified. Besides hydrogen bonding between amino acid side chains and glucosyl units, three tryptophan residues W135, W367 and W269 in subsites –2, +1 and +2 respectively, make significant contribution to the binding of enzyme and substrate (Rouvinen et al., 1990). The catalytic reaction involves two aspatate residues (D175 and D221) and one tyrosine (Y169) (Koivula et al., 1996).

2.4.2 Catalytic reaction mechanism

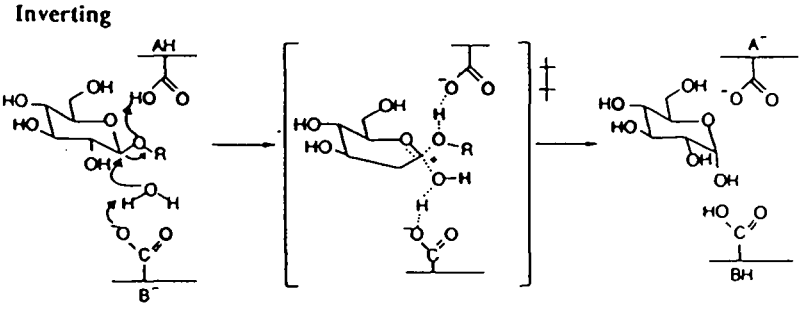
Cellulases, like all glycosidases, catalyze the hydrolysis of glycosidic bonds by general acid/base catalysis via two different mechanisms: one with net inversion of anomeric configuration, the other with net retention. It has been shown that the catalytic residues involved in both mechanisms are usually the carboxylic side chains of aspartic or glutamic acids. *T. reesei* CBH I is a retaining enzyme

(Knowles et al., 1988; Claeyssens et al., 1990). It is believed to function by a double displacement mechanism (S_N1-type nucleophilic substitution) in which a glucosyl-enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition state (Figure 2.8a). In this mechanism, one amino acid residue acts as general acid and general base while the other acts as a nucleophile and a leaving group. In the first step, a deprotonated amino acid residue acting as a nucleophile (Nu) attacks at the anomeric center. A general acid catalytic residue (AH) assists in this process by donating a proton to the glycosidic oxygen. In a second step, a water molecule attacks at the anomeric center with an assistance of a general base catalytic residue (A-), thus displacing a nucleophile (Nu) and releasing the product (Sinnott, 1990; Whithers, 1995).

T. reesei CBH II is an inverting enzyme (Knowles et al., 1988; Claeyssens et al., 1990; Konstantinidis et al., 1993). It is believed to function by a single-displacement mechanism (S_N2-type nucleophilic substitution) in which a water molecule directly displaces the glycosidic leaving group from the anomeric center (Figure 2.8b). Inverting mechanism involves two active site amino acid residues; one acts as a general base, the other as a general acid. A general base (B^-) helps to deprotonate the nucleophilic water molecule and a general acid (AH) protonates the departing glycosidic oxygen in a concert fashion as the bond cleaves.



(a)



(b)

Figure 2.8 Proposed catalytic mechanism for glycosidases: (a) for retaining enzyme; (b) for inverting enzyme (modified from Withers and Aebersold, 1995).

2.4.3 Mode of action and chain-end specificity

Traditionally, it has been thought that all cellobiohydrolases are exoglucanases that release cellobiose from the nonreducing end of cellulose chains (Wood and Garcia-Campayo, 1990; Schomburg and Salzmann, 1991). This presumed mode of action does not provide an explanation for the synergism, i.e. mixtures giving higher activity than the sum of activities of the enzymes acting alone, observed between different exocellulases (Irwin et al., 1993; Nidetzky et al., 1994a). The exoglucanases with the same specificity would compete for the limited hydrolysis sites rather than cooperating to give synergistic action. However, recent experimental evidence suggests that there are two classes of cellobiohydrolases with opposite chain-end specificities. A study based on activities of two exoglucanases from *Aspergillus aculeatus* on reduced alkali-swollen cellulose showed that one enzyme attacks cellulose reducing end while the other attacks nonreducing end (Arai et al., 1989). Using cello-oligosaccharides labeled with ^{3}H or ^{18}O at their reducing ends as model substrates, it has been shown that *T reesei* CBH I, as well as *Thermomonospora fusca* E_4 and E_6 preferentially attack the reducing end while CBH II and E3 act at the non-reducing end (Vrsanska and Biely, 1992; Biely et al., 1993; Barr et al., 1996).

The orientation and directionality of different oligosaccharides in the active sites of CBH I and CBH II, as revealed by high-resolution crystal structures, support the proposed chain-end specificities (Rouvinen, 1990; Divne et al., 1994; Divne et al., 1998). The active sites of these enzymes have a tunnel-shape and are

confined to cellulose reducing end for CBH I and nonreducing end for CBH II. This tunnel structure is restricted and designed to accommodate a single glucan chain in a defined orientation. It has been proposed that a single glucan chain enters the active site tunnel from one end, followed by their threading through the entire length of the tunnel to where it is bound by protein-carbohydrate interactions. The catalytic residues are located at the far end of the tunnel where bond cleavage occurs and cellobiose product was released. After the catalytic reaction, the cellulose chain remains bound and threads further into the tunnel, thus leading to another cycle of catalytic action. Based on this, CBH I and CBH II are believed to have a processive or "multiple-attack" mode of action in which several consecutive hydrolytic events occur without dissociation of the enzyme and the substrate (Rouvinen, 1990; Divne et al., 1994; Divne et al., 1998).

2.5 Model substrates: Cellodextrin based substrates and ligands

The detailed characterization of the mode of action of cellulolytic component has proven to be difficult and inconsistent from different laboratories due to the complexity and diversity of both the enzymes and the natural substrates. Cellulolytic enzyme systems normally contain several distinct enzyme components, including multiple exocellulases and multiple endocellulases. Some of these enzymes are closely related and so they are difficult to separate. This problem has been partially overcome by advances in enzyme purification. Affinity chromatography has been shown to be particularly effective for this application

(van Tilbeurgh et al., 1984; Piyachomkwan et al., 1997). The analysis of cellulolytic enzymes requires highly purified preparation due to the high degree of synergistic effect between different cellulolytic enzymes, which means that a small amount of contaminant could lead to a wrong conclusion.

Cellulose, the natural substrate for cellulases, is poorly characterized and too complicated to be useful for detail enzymatic studies. It is insoluble and heterogeneous containing both crystalline and amorphous regions. Many physical properties of cellulose, such as extent of crystallinity, porosity, degree of polymerization, particle size, and surface area, can influence the experimental result. In order to overcome these problems, many well-defined soluble cellooligosaccharides and their derivatives have been developed over the years.

Perhaps, unmodified cellooligosaccharides are the best soluble model substrates to be used to investigate the modes of action and kinetic parameters of cellulolytic enzymes (Cole and King, 1964; Hsu et al., 1980; Nidetzky et al., 1994b). They have the same chemical structure as native cellulose; they are simply shorter. Cellooligosaccharides can be obtained by acid hydrolysis of cellulose, followed by chromatographic purification to separate each fraction. Separation and analysis of enzymatic reaction products are usually done either by HPLC or TLC. The problem with this substrate is that the glycosidic bonds of cellooligosaccharides that are cleaved can not be precisely identified because an identical pair of products is formed from hydrolysis at different glycosidic bonds.

The confusion of the cleavage site does not exist when reduced cellooligosaccharides are used (Cole and King, 1964; Bhat et al, 1990; Schou et al., 1993a). This group of substrates is prepared by reduction of cellooligasaccharides with sodium borohydride, $NaBH_4$ (Schou et al., 1993a). Since the reducing moiety of these substrates has been modified, an open alditol unit at the reducing end might effect the catalytic activity of investigated enzymes. In addition, the low sensitivity of both normal and reduced cellooligosaccharides makes them less practical in some applications.

Alternatively, chromophoric cellooligosaccharides have been used as substrates. Chromophoric aglycons used for this purpose include *p*-nitrophenyl, 2,3-di-nitrophenyl, or 4-methylumbelliferyl. Since the aglycon is coupled to the reducing end of the cellodextrin, the actual linkage cleaved can be identified unequivocally. The major advantage of these substrates is that the kinetics can be determined directly from the measurable increase in fluorescence or UV absorbance following the hydrolysis of the aglycon linkage. Chromophoric cellooligosaccharides are sensitive tools in many aspects of enzymatic studies including determination of the number of binding site, study of binding kinetics, cleavage site pattern, differentiation and detection of cellulolytic enzymes (Capon et al., 1979; van Tilbeurgh, et al, 1982; Deshpande et al., 1984; van Tilbeurgh, et al., 1988; Claeyssens, et al., 1989; Bhat et al, 1990). However, the use and interpretation of the results obtained from this group of substrates should be done

cautiously because it has been shown that introducing aglycon on to the normal cellooligosaccharides can alter the apparent mode of hydrolysis (Bhat et al., 1990).

The use of cellooligosaccharides labeled with ^{3}H, ^{14}C or ^{18}O at their reducing end overcomes the limitations encountered in other substrates mentioned earlier. The isotopic label does not change the chemical structure of the cellooligosacchrides and it also allows sensitive detection. This group of substrates have been used mostly to monitor end-product distribution, thus providing information about cleavage site specificity of the enzyme (Chirico and Brown, 1987; Vrsanska and Biely, 1992; Biely et al. 1993; Barr et al., 1996). Nevertheless, there is some technical difficulties associated with using radioactive (^{3}H or ^{14}C labeled) compounds.

There is another category of modified cellooligosacchrides that is not intended to be substrates, i.e. they are non-hydrolysable substrate analogues. The most used compounds in this category are 4-thiocellooligosacchrides. The substitution of the *S*-linkage for the *O*-glycosidic linkage of cellooligosaccharides makes them resistant to cellulolytic attack. Sometimes the substitution is selectively done on a particular linkage that is enzymatically susceptible. These thio-analogues find their valuable use as affinity ligands for the chromatographic purification of exo-type cellulases (van Tilbeurgh et al., 1984; Orgeret et al., 1992; Piyachomkwan et al., 1997) and in the study of inhibition characteristics (Schou et al., 1993b). They also have been used in studies of cellulase structure and function. Because of their resistance to enzymatic attack, they are used in X-ray crystallography to

obtain complexes of oligosaccharides bound to enzyme active-site providing the information about how enzyme works (Sulzenbacher et al., 1996; Davies, 1998).

2.6 Chemical synthesis of cellooligosaccharide-based substrates

2.6.1 Preparation of cellooligosaccharides

Even though it has been shown that chemically cellobiose, cellotriose, and cellotetraose can be synthesized by the Koenigs-Knorr reaction (Takeo et al., 1983), the process is quite tedious, difficult and required some degree of expertise. The method is also not practical for longer cellooligosaccharides. As a result, cellooligosaccharides are more commonly obtained from fragmentation of cellulose molecules.

Cellooligosaccharides are obtained from cellulose either by acetolysis followed by deacetylation (Dickey and Wolfrom, 1949; Miller et al., 1960) or by acid hydrolysis (Miller et al., 1960, Pereire et al., 1988). In acetolysis, cellulose is treated with one part of glacial acetic acid, one part of acetic anhydride, and a tenth part of concentrated sulfuric acid. The crude oligomer peracetates obtained is then deacetylated to yield a mixture of cellooligosaccharides.

Acid hydrolysis of cellulose is done either by HCl or H_2SO_4 (Pereira et al., 1988). The mixture is then neutralized by addition of $NaHCO_3$. Sodium chloride salt formed is then removed by ion-exchange resin, Dowex-3 (OH^-) and Dowex W-X8 (H^+) (Streamer et al., 1975). Alternatively, the crude cellooligosaccharides is

neutralized by consecutive washing with 1-propanol and ethanol (Hamacher et al., 1985). The mixture of cellooligosaccharides can then be fractionated by several techniques of liquid chromatography (for review see Pereira et al., 1988).

2.6.2 Glycoside formation: Synthesis of chromophoric cellooligosaccharide

The laboratory synthesis of glycoside is often difficult. The methods for glycoside bond formation usually start with an activation of anomeric center that leads to a strong glycosyl donor property. The method, called Koenigs-Knorr reaction, is particularly useful for preparing β-glycosides. It involves the formation of acetylated glycosyl bromide, followed by nucleophilic substitution by the appropriate nucleophile in the presence of metal ion as promoter. Both α-and β-anomers of acetylated glycosyl bromide give the same β-glycoside product. The mechanism is best explained by the neighboring-group effect. After the acetylated glycosyl bromide spontaneously releases bromide ion, the oxygen atom of the ester group at C_2 attacks the cationic center at C_1 to form a stabilized oxonium ion intermediate. Since the oxonium ion formed is on the bottom of the glycosyl ring, the nucleophile, or glycosyl acceptor in this case, has to attack C_1 on the other side via S_N2 displacement, yielding a β-glycoside and regenerating the acetate ester group at C_2 (McMurry, 1992). The schematic representation of the mechanism is shown in Figure 2.9. In spite of the generality of the method, the requirement of at least an equimolar amount (often up to 4 eq) of metal salt and the problems concerning the disposal of the waste material (e.g., mercury) could be limiting

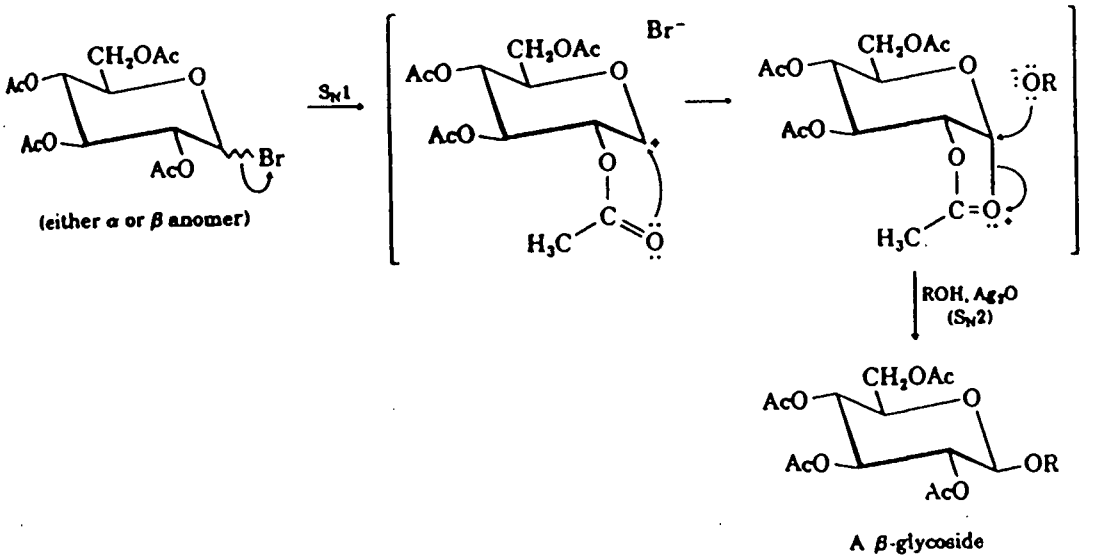


Figure 2.9 Mechanism of the Koenigs-Knorr reaction showing the neighboring-group effect of the acetate ester group at C_2 (McMurry, 1992).

factors for some application (Schmidt and Jung, 1997).

Many chromophoric cellooligosaccharides are obtained from the reaction of acetylated glycosyl bromide with nucleophilic aglycon in the mixture of acetone and alkaline solution such as sodium hydroxide and potassium carbonate (Capon and Thomson 1979; Day and Withers, 1985; van Tilbeurgh et al., 1988). The yield from this method is quite low and in some cases the reaction takes up to 5 days. The method called phase-transfer catalysis has been successfully used in the preparation of some chomophoric glycosides (Roy and Tropper, 1991; Roy et al.,

1992; Tropper et al., 1992). The reaction takes place in an immisible mixture of organic and alkaline aqueous phase, which is vigorously stirred, in the presence of catalyst such as tetrabutylammonium hydrogen sulfate. The method is proven to be high stereospecific, i.e. no α-glycoside detected, provide reasonable yield, and complete within a reasonable time period.

2.7 References

Abuja, M.P., Pilz, I., Claeyssens, M., and Tomme, P. 1988. Domain structure of cellobiohydrolase II as studied by small angle X-ray scattering: close resemblance to cellobiohydrolase I. *Biochem. Biophys. Res. Comm.* 156:180-185.

Arai, M., Sakamoto, R., and Murao, S. 1989. Different action by two avicelases from *Aspergillus aculeatus*. *Agric. Biol. Chem.* 53:1411-1412.

Barr, B.K., Hsieh, Y., Ganem, B., and Wilson, D.B. 1996. Identification of two functionally different classes of exocellulases. *Biochemistry* 35: 586-592.

Beguin, P. and Aubert, J.P. 1992. Cellulases. *Encyclopedia of Microbiology* 1: 467-477.

Bhat, K.M., Hay, A.J., Wood, T., and Claeyssens, M. 1990 Study of the mode of action and site-specificity of the endo-1,4-β-D-glucanases of the fungus *Penicillium pinophilum* with normal, 1-^{3}H-labelled, reduced and chromogenic cello-oligosaccharides. *Biochem. J.* 266:371-378.

Bhikhabhai, R., Johansson, G., and Pettersson, G. 1984. Isolation of cellulolytic enzymes from *Trichoderma reesei* QM 9414. *J. Applied Biochem.* 6:336-345.

Biely, P., Vrsanska, M., and Claeyssens, M. 1993. Mode of action of *Trichoderma reesei* β-1,4-glucanases on cellooligosaccharides. In *TRICEL Symposium on Trichoderma reesei Cellulases and other Hydrolases*, (P. Suominen and T. Reinikainen, eds.), pp. 99-108. Foundation for Biotechnical and Industrial Fermentation Research , Espoo, Finland.

Blackwell, J. 1982. The macromolecular organization of cellulose and chitin. In *Cellulose and Other Natural Polymer Systems.* (R.M. Brown Jr., ed.), pp. 403-428, Plenum Press, New York.

Capon, B. and Thomson, J. W. 1979. Studies on cellulolytic enzymes I. The use of 3,4-dinitrophenyl glycosides as substrates. *Bioor. Chem.* 8: 147-173.

Chirico, W. and Brown, R.D., Jr. 1987. β-Glucosidase from *Trichoderma reesei*. Substrate-binding region and mode of action on [1-^{3}H] cello-oligosaccharides. *Eur. J. Biochem.* 165:343-351.

Claeyssens, M., Tomme, P., Brewer, C.F., and Hehre, E.J. 1990. Stereochemical course of hydrolysis and hydration reactions catalyzed by cellobiohydrolases I and II from *Trichoderma reesei*. *FEBS Lett.* 263:89-92.

Claeyssens, M., van Tilbeurgh, H., Tomme, P., Wood, T.M. and McCrae, S.I. 1989. Fungal cellulase systems. Comparison of the specificities of the cellobiohydrolases isolated from *Penicillium pinophilum* and *Trichoderma reesei*. *Biochem. J.* 261: 819-825.

Clarke, A.J. 1997. *Biodegradation of Cellulose*, pp.1-7, Enzymology and Biotechnology. Technomic Publishing Co., Inc.

Cole, F.E. and King, K.W. 1964. Site of hydrolysis of cellulodextrins and reduced cellulodextrins by purified cellulase components. *Biochem. Biophys. Acta* 81: 122-129.

Davies, G. J. 1998. Substrate distortion in glycoside hydrolysis: the use of modified cellooligosaccharides to study cellulase structure and function. In Carbohydrases from *Trichoderma reesei* and Other Microorganisms. Structure, Biochemistry, Genetics and Applications.(Claeyssens, M., Nerinckx, W., and Piens, K., eds.). pp. 166-173. The Royal Society of Chemistry, Cambridge, UK

Davies, G. and Henrissat, B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* 3:853-859

Day, A.G. and Withers, S.G. 1985. A simple kinetic test to distinguish exo-glucosidase and β-glucosidase activities. *Biochem. and Biophys. Res. Comm.* 133:628-632.

Deshpande, M. V., Eriksson, K. E., and Pettersson, L. G. 1984. An assay for selective determination of exo-1,4-β-glucanases in a mixture of cellulolytic enzymes. *Anal. Biochem.*, 138: 481-487.

Dickey, E.E. and Wolfrom, M.L. 1949. A polymer-homologous series of sugar acetates from the acetolysis of cellulose. *J. Am. Chem. Soc.* 71:825-828.

Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, K.C., Teeri, T.T., and Jones, T.A. 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. *Science* 265: 524-528.

Divne, C., Stahlberg, J., Teeri, T.T., and Jones, T.A. 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. *J. Mol. Biol.* 275: 309-325.

Enari, T.M. 1983. Microbial cellulases. In *Microbial Enzymes and Biotechnology* (W.M. Fogarty, ed), pp.183-223, Applied Science Publishers, New York.

Enari, T.M. and Niku-Paavola, M.L. 1987. Enzymatic hydrolysis of cellulose: Is the current theory of the mechanisms of hydrolysis valid? *CRC Crit. Revs. Biotechnol.* 5:67-87.

Fagerstam, L.G. and Pettersson, L.G. 1979. The cellulolytic complex of *Trichoderma reesei* QM 9414: an immunochemical approach. *FEBS Lett.* 98(2):363-367.

Gardner, K.H. and Blackwell, J. 1974. The structure of native cellulose. *Biopolymers*, 13:1975-2001.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55: 303-315.

Hamacher, K., Schmid, G., Sahm, H., and Wandrey, Ch. 1985. Structural heterogeneity of cellooligomers homogeneous according to high-resolution size-exclusion chromatography, *J. Chromatogr.* 319:311-318.

Hsu, T. A., Gong, C. S., and Tsao, G. T. 1980. Kinetic studies of cellodextrins hydrolyses by exocellulase from *Trichoderma reesei*. *Biotechnol. Bioeng.* 22: 2305-2320.

Irwin, D.C., Spezio, M., Walker, L.P., and Wilson, D.B. 1993. Activity studies of eight purified cellulases: specificity, synergism, and binding domain effects. *Biotechnol. Bioeng.*, 42:1002-1013.

Knowles, J.K.C., Lehtovaara, P., Murray, M., and Sinnot, M.L. 1988. Stereochemical course of the action of cellobioside hydrolases I and II of *Trichoderma reesei*. *J. Chem. Soc., Chem. Commun.* pp. 1401-1402.

Knowles, J., Lehtovaara, P., and Teeri, T. 1987. Cellulase families and their genes. *TIBTECH* 5: 255-261.

Koivula, A., Reinikainen, T., Rouhonen, L., Valkeajarvi, A., Claeyssens, M., Teleman, O., Kleywegt, G.J., Szardenings, M., Rouvinen, J., Jones, T.A.,

and Teeri, T.T. 1996. The active site of *Trichoderma reesei* cellobiohydrolase II: the role of tyrosine-169. *Protein Eng.* 9: 691-699.

Konstantinidis, A., Marsden, I., and Sinnott, M.L. 1993. Hydrolyses of α- and β-cellobiosyl fluorides by cellobiohydrolases of *Trichoderma reesei Biochem. J.* 291:883-888.

Kraulis, P.J., Clore, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J.K.C., and Gronenborn, A.M. 1989. Determination of the three dimensional structure of the C-terminal domain of cellobiohydrolases I from *Trichoderma reesei.* A study using nuclear magnetic resonance and hybrid distance geometry-dynamical simulated annealing. *Biochemistry* 28: 7241-7257.

Lee, J.H., Brown, R.M., Kuga, S., Shoda, S., and Kobayashi, S. 1994. Assembly of synthetic cellulase I. *Proc. Natl. Acad. Sci.* 91:7425-7429.

Linder, M., Mattinen, M.L., Kontteli, M., Lindeberg, G., Stahlberg, J., Drakenberg, T., Reinikainen, T., Pettersson, G., and Annila, A. 1995. Identification of functionally important amino acids in the cellulose-binding domain of *Trichoderma reesei* cellobiohydrolase I. *Protein Sci.* 4:1056-1064.

McMurry, J. 1992. *Organic chemistry*, Third edition, pp. 931-934, Brooks/Cole Publishing Co., California.

Miller, G.L., Dean, J., and Blum, R. 1960. A study of methods for preparing oligosaccharides from cellulose, *Arch Biochem. Biohpys.* 91:21-26.

Nidetzky, B., Steiner, W., Hayn, M., and Claeyssens, M. 1994a. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. *Biochem. J.* 298:705-710.

Nidetzky, B., Zachariae, W., Gercken, G., Hayn, M., and Steiner, W. 1994b. Hydrolysis of cellooligosaccharides by *Trichoderma reesei* cellobiohydrolases: experimental data and kinetic modeling. *Enzyme Microb. Technol.* 16: 43-52.

Orgeret, C., Seillier, E., Gautier, C., Defaye, J., and Driguez, H. 1992. 4-Thiocellooligosaccharides. Their synthesis and use as ligands for the separation of cellobiohydrolases of *Trichoderma reesei* by affinity chromatography. *Carbohydr. Res.* 224: 29-40.

Pereira, A.N., Mobedshahi, M., and Ladisch, M.R. 1988. Preparation of cellodextrins, *Methods Enzymol.* 160:26-38.

Piyachomkwan, K., Gable, K.P., and Penner, M.H. 1997. *p*-Aminophenyl 1-thio-β-D-cellobioside: synthesis and application in affinity chromatography of exo-type cellulases. *Carbohydr. Res.* 303: 255-259.

Richmond, P.A. 1991. Occurrence and functions of native cellulose. In Biosynthesis and Biodegradation of Cellulose, (C.H. Haigler and P.J. Weimer, eds.) pp. 5-23, Marcel Dekker, Inc., NewYork.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C., and Jones, T.A. 1990. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei. Science* 249:380-386.

Roy, R. and Tropper, F.D. 1991. Carbohydrate protein interactions. Syntheses of agglutination inhibitors of wheat germ agglutinin by phase transfer catalysis, *Can. J. Chem.* 69:817-821.

Roy, R., Tropper, F.D., and Romanowska, A. 1992. New strategy in glycopolymer syntheses. Preparation of antigenic water-soluble poly(acrylamide-co-*p*-acrylamidophenyl β-lactoside), *Bioconjugate Chem.* 3:256-261.

Sarko, A. 1978. What is the crystalline structure of cellulose? *Tappi* 61(2):59-61.

Schmidt, R.R. and Jung, K. 1997. Oligosaccharide synthesis with trichloroacetimidates. In *Preparative carbohydrate chemistry*, (S. Hanessian, ed), pp. 283-312, Marcel Dekker, Inc., New York.

Schomburg, D. and Salzmann, M., eds. 1991. *Enzyme Handbook*, Springer-Verlag, New York.

Schou, C., Rasmussen, G., Kaltoft, M., Henrissat, B., and Schulein, M. 1993a. Sterochemistry, specificity and kinetics of the hydrolysis of reduced cellodextrins by nine cellulases. *Eur. J. Biochem.* 217:947-953

Schou, C., Rasmussen, G., Schulein, M., Henrissat, B., and Driguez, H. 1993b. 4-Thiocellooligosaccharides: their synthesis and use as inhibitors of cellulases. *J. Carbohydr. Chem.* 12: 743-752.

Singh, A. and Hayashi, K. 1995. Microbial cellulases: protein architecture, molecular properties, and biosynthesis. *Advs Appl. Microb.* 40:1-44.

Sinnot, M.L. 1990. Catalytic mechanisms of enzymic glycosyl transfer. *Chem. Rev.* 90:1171-1202.

Stahlberg, J., Divne, C., Koivula, A., Piens, K., Claeyssens, M., Teeri, T.T., and Jones, T.A. 1996. Activity studies and crystal structures of catalytically deficient mutants of cellobiohydrolase I from *Trichoderma reesei. J. Mol. Biol.*, 264:337-349.

Streamer, M., Eriksson, K., and Pettersson, B. 1975. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. Functional characterization of five endo-1,4-β-glucanases and one exo-1,4-β-glucanase, *Eur. J. Biochem.* 59:607-613.

Sulzenbacher, G., Driguez, H., Henrissat, B., Schulein, M., and Davies, G. J. 1996. Structure of the *Fusarium oxysporum* endoglucanase I with nonhydrolyzable substrate analogue: substrate distortion gives rise to the preferred axial orientation for the leaving group. *Biochemistry* 35: 15280-15287.

Takeo, K., Okushio, K., Fukuyama, K, and Kuge, T. 1983. Synthesis of cellobiose, cellotriose, cellotetraose, and lactose, *Carbohyd. Res.* 121:163-173.

Teeri, T.T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *TIBTECH* 15: 160-167.

Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Ruohonen, L., Lehtiö, J., Reinikainen, T., Srisodsuk, M., Kleman-Leyer, K., Kirk, T.K., and Jones, T.A. 1998. Cellulose-cellulase interactions of native and engineered cellobiohydrolases from *Trichoderma reesei*. In *Carbohydrases from Trichoderma reesei and Other Microorganisms. Structures, Biochemistry, Genetics and Applications*, (M. Claeyssens, W. Nerinckx, and K. Piens, eds), pp. 3-12, The Royal Society of Chemistry Information Services, Cambridge, UK.

Teeri, T.T., Reinikainen, T., Rouhonen, L., Jones, T.A., and Knowles, J.K.C. 1992. Domain function in *Trichoderma reesei* cellobiohydrolases. *J. Biotechnol.* 24:169-176.

Tomme, P., van Tilbeurgh, H., Pettersson G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. 1988. Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* 170:575-581.

Tomme, P., Warren, R.A.J., and Gilkes, N.R. 1995a. Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol.* 37:1-81.

Tomme, P., Warren, R.A.J., Miller, Jr., R.C., Kilburn, D.G., and Gilkes N.R. 1995b. Cellulose-binding domains: Classification and properties. In *Enzymatic Degradation of Insoluble Carbohydrates*, (J.N. Saddler and M.H. Penner, eds.) pp. 142-163, ACS Symposium Series 618, American Chemical Society, Washington, DC.

Tropper, F.D., Andersson, F.O., Grand-Maître, C., and Roy, R. 1992. Phase transfer catalyzed synthesis of 4-nitrophenyl 1-thio-β-D-glycobiosides, *Carbohyd. Res.* 229:149-154.

van Tilbeurgh, H., Bhikhabhai, R., Pettersson, L.G., and Claeyssens, M. 1984. Separation of endo-and exo-type cellulases using a new affinity chromatography method. *FEBS Lett.* 169: 215-218.

van Tilbeurgh, H., Claeyssens, M., and de Bruyne, C. K. 1982. The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes. FEBS Lett. 149:152-156.

van Tilbeurgh, H., Loontiens, F. G., de Bruyne, C. K., and Claeyssens, M. 1988. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. *Methods Enzymol.* 160: 45-59.

van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R., and Pettersson, G. 1986. Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*. *FEBS Lett.* 204: 223-227.

van Zyl, W.H., la Grange, D.C., Crous, J.M., Luttig, M., van Rensburg, P., and Pretorius, I.S. 1998. Cloning of hydrolases from *Trichoderma reesei* and other microorganisms in yeast. In *Carbohydrases from Trichoderma reesei and Other Microorganisms. Structures, Biochemistry, Genetics and Applications*, (M. Claeyssens, W. Nerinckx, and K. Piens, eds.), pp. 280-287, The Royal Society of Chemistry Information Services, Cambridge, UK.

Vrsanska, M. and Biely, P. 1992. The cellobiohydrolase I from *Trichoderma reesei* QM 9414: action on cello-oligosaccharides. *Carbohydr. Res.* 227: 19-27.

Withers, S.G. 1995. Enzymatic cleavage of glycosides: how does it happen? *Pure & Appl. Chem.* 67:1673-1682.

Withers, S.G. and Aebersold, R. 1995. Approaches to labeling an identification of active site residues in glycoidases, *Protein Sci.* 4:361-372.

Wood, T.M. and Garcia-Campayo, V. 1990. Enzymology of cellulose degradation. *Biodegradation.* 1: 147-161.

Wood, T.M., McCrae, S.I., Wilson, C.A., Bhat, K.M., and Gow, L.A. 1988. In *Biochemistry and Genetics of Cellulose Degradation*, (J.P. Aubert, P. Béguin, and J. Millet, eds.), pp. 31, Academic Press, Orlando, FL.

CHAPTER 3

p-AMINOPHENYL β-D-CELLOBIOSIDE AS AN AFFINITY LIGAND FOR EXO-TYPE CELLULASES

Kunruedee Sangseethong and Michael H. Penner

Department of Food Science and Technology,
Oregon State University, Corvallis, OR

Published in *Carbohydrate Research*
April, 1999, In Press.

3.1 Abstract

p-Aminophenyl β-D-cellobioside (PAPC) is shown to be an effective affinity ligand for the chromatographic fractionation of cellobiohydrolases (CBHs). A "crude" cellulase preparation from the filamentous fungus *Trichoderma reesei* served as a representative source of enzymes for this study. Prior to chromatography, PAPC was tethered via its amino functional group to N-hydroxy succinimide-activated agarose. The resulting affinity matrix specifically retained the CBH component of relatively complex cellulase mixtures. The purity of the resulting CBH preparations, based on measured specific activities, was comparable to that of corresponding enzyme preparations obtained using more traditional thioglycoside-based affinity ligands. The application of PAPC as an affinity ligand illustrates that the tethered ligand associates with the *T. reesei* CBHs in a catalytically nonproductive mode. In contrast, the free ligand is readily hydrolyzed by *T. reesei* CBH I.

3.2 Introduction

Microbial cellulolytic enzyme systems capable of degrading crystalline cellulose typically contain at least three classes of enzymes: endoglucanases (E.C. 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91) and β-glucosidases (E.C. 3.2.1.21). Studies aimed at elucidating the properties of the individual enzymes comprising these mixtures are often hampered by the difficulties encountered when trying to obtain functionally pure enzymes. Affinity chromatography has proven to be a most valuable approach to the separation of nearly homologous endo- and exo-acting cellulases [1, 2]. *p*-Aminobenzyl 1-thio-β-D-cellobioside (ABTC) was the first cellooligosaccharide-based affinity ligand used for the fractionation of endo- and exo-cellulases [3]. Other cellooligosaccharide derivatives have subsequently been used for the same application. Orgeret *et al.* [4] introduced *p*-aminophenyl 1,4-dithio-β-D-cellobioside as an alternative to ABTC; the advantage of this ligand being that the 1,4 thioglycosidic linkage is resistant to β-glucosidase catalyzed hydrolysis. Piyachomkwan *et al.* [5] then demonstrated the use of *p*-aminophenyl 1-thio-β-D-cellobioside (APTC) for the fractionation of endo- and exo-acting cellulases; the advantage of this ligand being the relative ease with which it can be prepared. The common feature of all of these ligands is the substitution of an *S*-linkage for an *O*-linkage at the aglyconic bond of the cellobioside derivative, thus making all of these ligands resistant to exo-cellulase (cellobiohydrolase, CBH)-catalyzed hydrolysis.

Chromogenic derivatives of cellobioside, e.g., nitrophenyl glycosides, 4-methylumbelliferyl glycoside, etc., have long been used as model substrates for the study of CBHs [6, 7, 8]. These substrates are susceptible to CBH-catalyzed hydrolysis at the agluconic linkage, although CBHs differ markedly in their ability to catalyze this reaction [9]. Considering the *Trichoderma reesei* cellulase system, CBHI catalyzes this reaction and CBHII does not. Thus far, these compounds have only been considered as potential substrates for the CBHs. However, the following two observations suggest that compounds of this type have potential as affinity ligands for cellulase fractionation. First, CBHII does not catalyze the hydrolysis of these compounds and yet the enzyme clearly associates with their immobilized thio analogs, e.g., ABTC [3] and APTC [5]. Second, although these compounds are substrates for CBHI, this enzyme preferentially acts at the reducing terminus of cellooligosaccharide chains [10, 11, 12]; so the reducing-end-tethered form of these substrates (as used in affinity chromatography) would not necessarily form a catalytically productive complex.

The intent of this communication is to demonstrate that amino terminus-tethered *p*-aminophenyl β-D-cellobioside (PAPC) associates with the major *T. reesei* CBHs in a catalytically nonproductive manner, thus making it a convenient affinity ligand. This finding has implications with respect to the mode of binding of similar thio analogs which are currently used as affinity ligands for exo-acting cellulases.

3.3 Results and Discussion

As expected [8, 9], CBH I catalyzed the hydrolysis of the aglyconic linkage in *p*-nitrophenyl β-D-cellobioside and CBH II did not. Neither enzyme catalyzed the hydrolysis of the compound's interglycosidic linkage. The two enzymes showed the same specificities with PAPC, the reduced form of *p*-nitrophenyl β-D-cellobioside. However, coupling of PAPC, via its amino functional group, to an agarose matrix rendered it resistant to hydrolysis by either of the CBHs. CBH I and CBH II exhibited no measurable activity toward either of the *O*-glycosidic linkages in tethered PAPC over the course of a 37 h reaction period at 50°C, pH 5 (optimum conditions for CBH activity).

To test the application of PAPC as an affinity ligand for the fractionation of CBHs, partially purified CBH I and CBH II were chromatographed using PAPC-coupled agarose as the stationary phase (see chromatograms in Figures 3.1 and 3.2). In both cases, the CBHs were retained on the stationary phase while non-CBH proteins passed through the column. The CBHs were eluted from the column only after making the mobile phase 0.01 M cellobiose. The electrophoretic purity of the adsorbed CBHs was essentially the same as that of the purified enzymes prepared using APTC-derivatized agarose [5]. The relative functional purity of the resulting

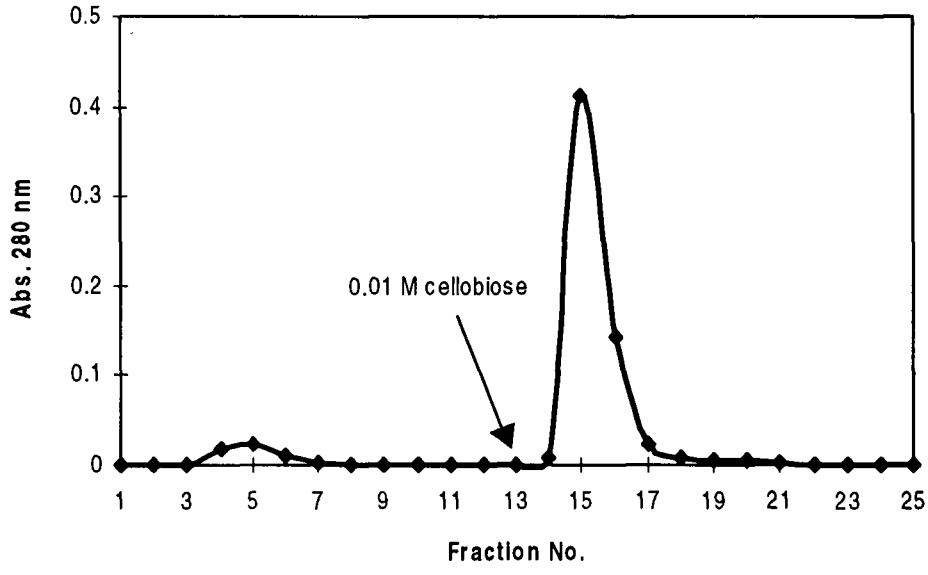


Figure 3.1 Affinity chromatography of CBH I using PAPC-derivatized agarose. A partially purified CBH I preparation was applied to the PAPC-affinity column in 0.1 M NaOAc, pH 5 containing 1 mM gluconolactone. CBH I was eluted by making the mobile phase 0.01 M cellobiose.

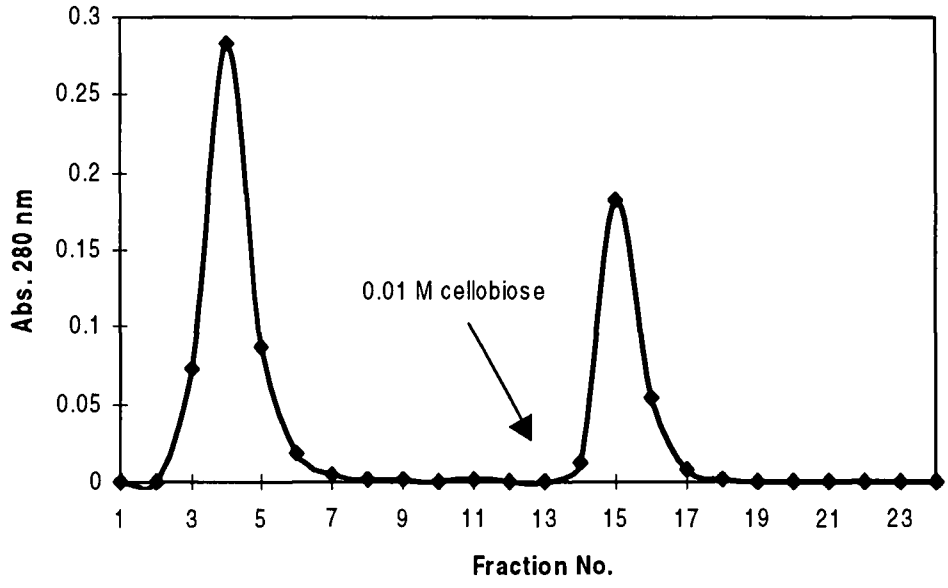


Figure 3.2 Affinity chromatography of CBH II using PAPC-derivatized agarose. A partially purified CBH II preparation was applied to the PAPC-affinity column in 0.1 M NaOAc, pH 5 containing 1 mM gluconolactone and 0.2 M glucose. CBH II was eluted by making the mobile phase 0.01 M cellobiose.

CBH preparations was evaluated by comparing their specific activities with those of analogous preparations resulting from the more traditional aryl thioglycoside-based affinity chromatography [5]. The specific activities of the CBH I and CBH II preparations resulting from PAPC-based affinity chromatography were found to be similar to those obtained from aryl thioglycoside-based affinity chromatography (Table 3). The preparations had no detectable β-glucosidase activity and their corresponding endo-type activities decreased dramatically. The decrease in the specific activity of the two CBH preparations toward microcrystalline cellulose is caused by the relative absence of synergism that results from enzyme fractionation (13).

The mechanistic explanation for the lack of activity of CBH I on amino terminus-tethered PAPC is not obvious. CBH I is a retaining exo-cellulase which preferentially acts at the reducing end of cellooligosaccharide chains [10, 11, 12]. The obvious difference between tethered- and free-PAPC is that the terminus analogous to the reducing end, the amino terminus in this case, is sterically blocked only in the case of the tethered ligand. The implication of this is that the tethered-ligand must "back" into the active site tunnel via the opening which is thought to primarily serve as an "exit" for generated product. Free PAPC may enter the active site tunnel via either end. CBH I is known to have ten well-defined glucosyl binding subsites within its active site tunnel [14] and cellobiose is known to preferentially bind at the product binding subsites (subsites +1 and +2, as described by Stahlberg *et al.* [15]). It is likely that the two glucosyl units of the tethered-

Table 3. Specific activities of purified cellobiohydrolase fractions.

Enzyme	Specific activities		
	β-Glucosidase[a] (μmol *p*-nitrophenol min^{-1} mg $protein^{-1}$)	Endocellulase[b] (μmol reducing sugar. min^{-1} mg $protein^{-1}$)	Exocellulase[c] (μmol reducing sugar min^{-1} mg $protein^{-1}$)
Crude cellulase	0.125	0.279	0.036
Anion exchange column			
• CBH I fraction[d]	0.001	0.099	0.013
• CBH II fraction	0.373	0.470	0.019
CBH I / affinity column			
• APTC[d]	0	0.014	0.011
• PAPC[e]	0	0.011	0.010
CBH II / affinity column			
• APTC[d]	0	0.034	0.011
• PAPC[e]	0	0.030	0.011

[a] *p*-nitrophenyl β-D-glucopyranoside was used to determine β-glucosidase activity.
[b] Hydroxyethylcellulose was used to determine endocellulase activity.
[c] Avicel was used to determine exocellulase activity.
[d] Activity of purified CBH I (or CBH II) after anion-exchange and APTC-affinity column; APTC was synthesized as described by Piyachomkwan et al. [5].
[e] Activity of purified CBH I (or CBH II) after anion-exchange and PAPC-affinity column.

ligand also associate with the product-binding subsites of CBH I. In contrast, the glucosyl units of free PAPC, when positioned for hydrolysis, are presumably bound in subsites -1 and -2, with the aminophenyl group being alligned at subsite +1. Tethered-PAPC may not be able to form the analogous catalytically productive complex, covering subsites -2 to +1, due to steric limitations arising from the bulky matrix to which it is affixed. A second possibility is that a kinetic barrier restricts the activity of CBH I on tethered-PAPC. This scenario may result as a consequence of the ligand "backing" into the active site tunnel, as required for the tethered form. In this case, the CBH I-ligand complex would have been formed without the ligand ever passing through the catalytically productive complex (-2 to +1).

In this study PAPC was prepared in four synthetic steps. However, PAPC can be obtained relatively easily by the reduction of commercially available *p*-nitrophenyl β-D-cellobioside (PNPC). PNPC is relatively inexpensive, currently selling for approximately 60 U.S. dollars per 100 mg (Sigma Chemical Co., St. Louis), and PAPC yields following PNPC reduction are typically >95%. The affinity supports demonstrated in this work were prepared in coupling reaction mixtures containing approximately 10 mg of ligand per ml agarose gel. This resulted in affinity gels having maximum CBH I (ε =73000 $M^{-1}cm^{-1}$, Mw = 65000 [16]) binding capacities of approxiately 4 mg protein per mL gel. PAPC-based columns may be reused as necessary; our laboratory having now used a single column for eight chromatographic runs of *T. reesei* CBHs without observing appreciable changes in column performance. The reality that PAPC can be obtained

from an inexpensive commercially-available precursor in but a single, high-yielding, synthetic step makes it the most readily accessible of any of the currently available affinity ligands for this purpose.

In conclusion, this work demonstrates that the affinity ligands commonly used for the fractionation of endo- and exo-acting cellulases associate with prototypical CBHs in a catalytically nonproductive mode. Hence, in many cases there may be relatively little advantage in incorporating a thio linkage at the aglyconic bond.

3.4 Experimental

3.4.1 General methods

All organic solutions were dried with anhydrous Na_2SO_4. Solvents were evaporated at reduced pressure (below 45°C). The ^1H NMR spectra were recorded at 400 MHz with a Bruker AM 400 spectrometer, using tetramethylsilane as an internal standard. Reactions were monitored by TLC on precoated plates of Silica Gel 60 F_{254} (Whatman, Clifton, NJ). The following solvent systems (v/v) were used for TLC: *A*, 1:1 EtOAC-hexane with 0.5% 2-propanol; *B*, 5:4:1 chloroform-MeOH-H_2O. TLC separated compounds were visualized by exposure to UV light, reacting with *p*-anisaldehyde-sulfuric acid visualizing reagent, and reacting with a ninhydrin-based visualizing reagents [17].

3.4.2 *p*-Nitrophenyl 2,3,6-tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranoside (**2**)

Compound **2** was prepared under phase-transfer catalyzed conditions [18]. A solution of hepta-O-acetylcellobiosyl bromide (**1**) [5] (1.01g, 1.44 mmol), tetrabutylammonium hydrogen sulfate (0.49 g, 1.44 mmol) and *p*-nitrophenol (0.4 g, 2.88 mmol) in CH_2Cl_2 (10 mL) was mixed with M aq NaOH (10 mL). The mixture was stirred and gently warmed until all solids dissolved. The two-phase reaction mixture was vigorously stirred at room temperature for 3 h. TLC (solvent *A*) indicated disappearance of **1** and the presence of **2**. The organic phase was successively washed with cold M NaOH (2 x 20 mL) and water (2 x 20 mL). The washed organic phase was then dried (Na_2SO_4), filtered and concentrated. A solution of residue in 1:1:1.5 EtOAc-2-propanol-CH_2Cl_2 (14 mL) was filtered through a layer of silica gel (230-400 mesh, SIGMA Chemical Co., St. Louis, MO), which was washed with 1:1 EtOAc-hexane containing 0.5% 2-propanol (200 mL). The combined filtrate and washings were evaporated to dryness and crystallized from 95% EtOH to give **2** (0.48 g, 44%): mp 245-247 C; ^{1}H-NMR ($CDCl_3$): δ 8.2 (d, 1 H, J 9.4 Hz, H-meta), 7.05 (d, 1 H, J 9.4 Hz, H-ortho), 5.29 (t, 1 H, $J_{3,4}$ 8.5 Hz, H-3), 5.24-5.18 (2 H, H-1,2), 5.16 (t, 1 H, $J_{3',4'}$ 9.5 Hz, H-3'), 5.08 (t, 1 H, $J_{4',5'}$ 9.6 Hz, H-4'), 4.94 (t, 1 H, $J_{2',3'}$ 9.2 Hz, H-2'), 4.55 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1'), 4.53 (dd, 1 H, $J_{5,6a}$ 1.8 Hz, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.36 (dd, 1 H, $J_{5',6a'}$ 4.6 Hz, $J_{6a',6b'}$ 12.5 Hz, H-6a'), 4.13 (dd, 1 H, $J_{5,6b}$ 5.5 Hz, $J_{6a,6b}$ 12.3 Hz, H-6b), 4.07 (dd, 1 H,

$J_{5',6b'}$ 2.16 Hz, $J_{6a',6b'}$ 12.5 Hz, H-6b'), 3.91-3.83 (m, 2 H, H-4,5), 3.71-3.67 (m, 1 H, H-5'), 2.1-1.99 (cluster of S, 21 H, 7 OAc).

3.4.3 *p*-Nitrophenyl β-D-cellobioside (**3**)

The acetylated glycoside **2** (0.48 g, 0.63 mmol) was deacetylated with sodium methoxide (1 M in MeOH, 0.36mL) in MeOH (80 mL). The mixture was stirred overnight at room temperature, neutralized with Amberlite IR-120 (H^+) resin, filtered and concentrated. Compound **3** was crystallized from MeOH (0.28 g, 95%): mp 253-255°C, lit. 255-256°C [19]. NMR spectra of synthesized compound **3** matched that of commercially available compound **3** (Sigma Chemical Co., St. Louis)

3.4.4 *p*-Aminophenyl β-D-cellobioside (PAPC) (**4**)

Compound **4** was obtained by catalytic hydrogenation of **3** using 10% Pd on activated charcoal as a catalyst. TLC (solvent *B*) indicated the complete conversion of the nitro compound **3** into its amino derivative **4**.

3.4.5 Preparation of PAPC-derivatized agarose

PAPC was coupled to Affigel 10 (BioRad Laboratories, Hercules, CA), under anhydrous condition to minimize non-specific protein-matrix interactions [2],

as recommended by the manufacturer. The gel (10 mL), after being washed with cold 2-propanol (50mL), was added to a solution of PAPC (200 μmol) in anhydrous MeOH (15 mL). The slurry was rotated end-over-end for 3 h at room temperature. Ten mL of 1 M ethanolamine, pH 8, was then added to the slurry and allowed to react for 1 h in order to block active ester groups remaining on the gel. The resulting gel was washed with 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone (100 mL) and loaded into 1 x 8 cm glass columns.

Protein binding capacities of the PAPC-gels were determined in partition equilibrium experiments under chromatographic conditions. Control experiments, using non-PAPC-coupled / ethanolamine-blocked gels, demonstrated negligible binding of *T. reesei* proteins to the underivatized matrix.

3.4.6 Affinity chromatography of cellobiohydrolases

Crude cellulase produced by *T. reesei* (Spezyme™-CP, Environmental BioTechnologies Inc., Menlo Park, CA) was initially fractionated by anion-exchange chromatography using DEAE-Sepharose [20]. The resulting partially purified CBH I and CBH II preparations (10-25 mg) were further fractionated using PAPC-derivatized agarose as the affinity matrix. CBH I was applied to the column in 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone. CBH II was applied to the column in 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone and 0.2 M glucose. Gluconolactone is included in the mobile phase for suppression of β-glucosidase activity [3]. Glucose is included in the specified mobile phases to enhance the

association of CBH II with the ligand [2, 21]. Chromatography was performed at 4 C with the mobile phase flow rate at 0.5 mL/min. Adsorbed CBHs were eluted by the addition of cellobiose, 0.01 M, to the mobile phase. Protein-containing fractions were detected by monitoring absorbance at 280 nm.

Endo- and exo- type cellulase activities were measured as described previously [5] using hydroxyethylcellulose (HEC; medium viscosity, Fluka Chemical Corp., Ronkonkoma, NY) and microcrystalline cellulose (MCC; Avicel PH 101, FMC Cor. Philadelphia, PA) as substrates. The amount of reducing sugar liberated was determined by a colorimetric method (*p*-hydroxybenzoic acid hydrazide, PAHBAH) [22]. β-Glucosidase activities were measured using *p*-nitrophenyl β-D-glucopyranoside as substrate [23]. Enzyme activities were measured at 50 C in 50 mM NaOAc buffer, pH 5.

3.5 Acknowledgements

This study was supported in part by funds (award number 98-35103-6558) from the NRI Competitive Grants Program/United States Department of Agriculture. This paper is Oregon State University Agricultural Experiment Station Technical Publication Number 11,427.

3.6 References

[1] P. Tomme, S. McCrae, T.M. Wood, and M. Claeyssens, *Methods Enzymol.*, 160 (1988) 187-193.

[2] K. Piyachomkwan and M.H. Penner, *Anal. Biochem.,* 255 (1998) 223-235.

[3] H. van Tilbeurgh, R. Bhikhabhai, L.G. Pettersson, and M. Claeyssens, *FEBS Lett.*, 169 (1984) 215-218.

[4] C. Orgeret, E. Seillier, C. Gautier, J. Defaye, and H. Driguez, *Carbohydr. Res.*, 224 (1992) 29-40.

[5] K. Piyachomkwan, K.P. Gable, and M.H. Penner, *Carbohydr. Res.*, 303 (1997) 255-259.

[6] B. Capon and J.W. Thomson, *Bioorg. Chem.*, 8 (1979) 147-173.

[7] M.V. Deshpande, K.E. Eriksson, and L.G. Pettersson, *Anal. Biochem.*, 138 (1984) 481-487.

[8] M. Claeyssens, H. van Tilbeurgh, P. Tomme, T.M. Wood, and S.I. McRae, *Biochem. J.*, 261 (1989) 819-825.

[9] D.C. Irwin, M. Spezio, L.P. Walker, and D.B. Wilson, *Biotechnol. Bioengr.*, 42 (1993) 1002-1013.

[10] M. Vrsanska, and P. Biely, *Carbohydr. Res.*, 227 (1992) 19-27.

[11] C Divne, J. Stahlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J.K.C., Knowles, T.T. Teeri, and T.A. Jones, *Science,* 265 (1994) 524-528.

[12] B. K. Barr, Y. Hsieh, B. Ganem, and D.B. Wilson, *Biochemistry*, 35 (1996) 586-592.

[13] B. Nidetzky, W. Steiner, and M. Claeyssens, *Synergistic Interaction of Cellulases from* Trichoderma reesei *During Cellulose Degradation*, in J.N. Saddler and M.H. Penner (Eds.), *Enzymatic Degradation of Insoluble Carbohydrates*, American Chemical Society, Washington, DC, 1995, pp 90-112.

[14] C. Divne, J. Stahlberg, T.T. Teeri, and T.A. Jones, *J. Mol. Biol.*, 275 (1998) 309-325.

[15] J. Stahlberg, C. Divne, A. Koivula, K. Piens, M. Claeyssens, T.T. Teeri, and T.A. Jones, *J. Mol. Biol.*, 264 (1996) 337-349.

[16] P. Tomme, H. van Tilbeurgh, G. Pettersson, J. van Damme, J. Vandekerckhove, J. Knowles, T. Teeri, and M. Claeyssens, *Eur. J. Biochem.*, 170 (1988) 575-581.

[17] D. Waldi, *Spray Reagents for Thin-Layer Chromatography* in E. Stahl (Ed.), *Thin Layer Chromatography. A Laboratory Handbook*, Academic Press Inc., New York, 1965, pp 485-502.

[18] R. Roy, F.D. Tropper, and A. Romanowska, *Bioconjugate Chem.*, 3 (1992) 256-261.

[19] A.G. Day and S.G. Withers, *Biochem. and Biophys. Res. Comm.*, 133 (1985) 628-632.

[20] G. Beldman, M.F. Searle-Van Leeuwen, F.M. Rombouts, and F.G.J. Voragen, *Eur. J. Biochem.*, 146 (1985) 301-308.

[21] H. van Tilbeurgh, G. Pettersson, R. Bhikabhai, H. De Boeck, and M. Claeyssens, *Eur. J. Biochem.*, 148 (1985) 329-334.

[22] M. Lever, *Anal. Biochem.*, 47 (1972) 273-279.

[23] T.M. Wood and K.M. Bhat, *Methods Enzymol.*, 160 (1988) 87-113.

CHAPTER 4

THE ACTION OF *TRICHODERMA REESEI* CELLOBIOHYDROLASES ON IMMOBILIZED CELLOOLIGOSACCHARIDES

Kunruedee Sangseethong and Michael H. Penner

Department of Food Science and Technology,
Oregon State University, Corvallis, OR

4.1 Abstract

The catalytic activity of cellobiohydrolase I (CBH I) and cellobiohydrolase II (CBH II) from *Trichoderma reesei* on reducing end-tethered cellodextrins (immobilized cellodextrins) was investigated. *p*-Amiphenyl derivatives of cellobiose ($PAPG_2$), cellotriose ($PAPG_3$) and cellotetraose ($PAPG_4$) were covalently tethered to activated agarose supports via their amino functional groups. The hydrolytic activity of the investigated enzymes on free and immobilized aryl-cellodextrins was compared. CBH II attacked free and immobilized $PAPG_3$ and $PAPG_4$ in a typical exo manner in which cellobiose is a major hydrolytic product released from the nonreducing end. The rate of hydrolysis increases with increasing chain length suggesting the extended binding sites (at least 4 binding sites). Like CBH II, CBH I preferentially cleaved immobilized $PAPG_3$ and $PAPG_4$ at a second glycosidic linkage from the nonreducing end; the rate of hydrolysis increases as a function of chain length. However, it attacked free aryl-cellodextrins in a random manner. The rate of hydrolysis increases only from $PAPG_2$ to $PAPG_3$ and significantly drops in $PAPG_4$ suggesting that CBH I interacts with free and immobilized substrates in different modes.

4.2 Introduction

Enzyme systems that efficiently catalyze the hydrolytic degradation of native cellulose are composed of several functionally distinct enzymes. A prototypical enzyme system of this type is that produced by the filamentous fungus *Trichoderma reesei*. It produces at least four endoglucanases (EGs), two exoglucanases (cellobiohydrolases, CBHs) and a β-glucosidase (Tomme et al., 1995). The two major cellobiohydrolases, CBHI and CBHII, are believed to play a predominant role in the hydrolysis of crystalline cellulose (Chanzy et al., 1983; Divne et al., 1994). These two enzymes typically make up ~80% (molar basis) of the total cellulolytic enzymes present (Teeri, 1997). Originally, both of these CBHs were assumed to catalyze the release of cellobiose units from the non-reducing end of cellulose chains (Schomburg and Salzmann, 1991). This view still holds for CBH II. However, relatively recent studies suggest that CBH I preferentially acts at the reducing terminus (Vrsanska and Biely, 1992; Biely et al., 1993; Divne et al., 1994; Barr et al, 1996). Studies making use of isotope-labeled cellodextrins have shown that the distribution of label in reaction products is indicative of preferential hydrolysis at the reducing terminus (Vrsanska and Biely, 1992; Biely et al.; 1993; Barr et al, 1996). Divne et al. (1994) presented the crystal structure of the catalytic core of CBHI in the presence of the substrate analog *o*-iodobenzyl 1-thio-β-D-cellobioside. The crystal structure revealed the ligand residing at the catalytic end of the active site tunnel, and the ligand was positioned such that its polarity was consistent with

cellobiose being liberated from the reducing end. Thus, available data strongly supports the notion that CBHI preferentially hydrolyzes cellobiose units from the reducing terminus of cellulose chains.

An important question related to this finding is how specific is the apparent reducing end → nonreducing end polarity of CBH I action. Is CBH I limited to acting at the reducing terminus, or can it also hydrolyze cellobiose units from the nonreducing end? This is particularly relevant to the biodegradation of native cellulose due to the crystalline, recalcitrant, nature of this substrate. It is clear that CBH I can at least associate with the nonreducing end of cellulose chains, as this behavior has been the basis for the affinity-based purification of CBHs for many years (see Piyachomkwan and Penner (1998) and references therein). CBH-affinity ligands are typically aryl-thioglycosides of cellobiose. For enzyme purification purposes, the ligand is tethered to an insoluble support via the aglycone end, thus leaving the nonreducing end of the cellobiose moiety exposed for interaction with the CBHs (van Tilbeurgh et al., 1984; Orgeret et al, 1992; Piyachomkwan et al., 1997). The heteroglycosidic linkage of the thioglycoside affinity ligands is resistant to hydrolysis, thus it is not known if these ligands associate with the enzyme in a catalytically productive manner. It was recently shown that a reducing end-tethered aryl–o-glycoside of cellobiose (p-aminophenylcellobioside, PAPC) was stable in the presence of CBH I, and CBH II, under typical chromatographic conditions (Sangseethong and Penner, 1999) – suggesting that the tethered ligand does not form a catalytically productive complex under these conditions.

In this study we have used reducing end-tethered cellodextrins (G_2 thru G_4) to test the reducing end- specificity of CBH I. Steric constraints make it such that the tethered substrates can only enter the enzyme's catalytic tunnel via their nonreducing end, thus making them particularly well suited to answer this question. CBH II has been included in the study for comparative purposes. Action patterns observed with the tethered substrates were compared with those observed when the enzyme was acting on the analogous soluble substrates. The data presented in this paper demonstrates that CBH I is capable of acting at the nonreducing end of cellulose.

4.3 Materials and Methods

4.3.1 Substrate preparation

4.3.1.1 *General methods*

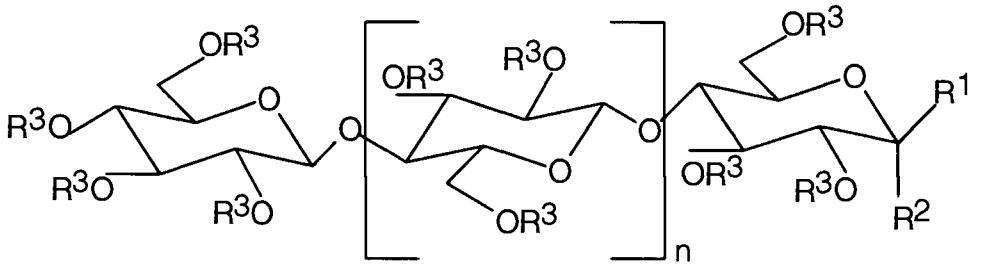
All organic solutions were dried with anhydrous Na_2SO_4. Solvents were evaporated at reduced pressure (below 45°C). The ^{1}H-NMR spectra were recorded at 400 MHz with a Bruker AM 400 spectrometer, using tetramethylsilane as an internal standard. Assignments were confirmed by COSY experiments. TLC was performed on precoated plates of SilicaGel $60F_{254}$ (Whatman, Clifton, NJ). Spots on TLC were detected by exposure to UV light and by spraying with *p*-anisaldehyde-sulfuric acid visualizing reagent. The detection of amino group after catalytic

hydrogenation of *p*-nitrophenyl glycosides were done by spraying with ninhydrin-based visualizing reagent (Waldi, 1965). Flash column chromatography was performed on Silica Gel 60 (230-400 mesh, SIGMA Chemical Co., St. Louis, MO). The following solvent systems (v/v) were used: *A*, 1:1 EtOAc-toluene; *B*, 5:2 EtOAc-hexane; *C*, 2:1 EtOAc-hexane; *D*, 5:4:1 chloroform-MeOH-H_2O. The chemical structures of compounds synthesized are shown in Figures 4.1 and 4.2.

Cellobiose octaacetate (**1**) was obtained commercially (Aldrich Chemical Co. Inc., Milwaukee, WI). The derivatives of cellobiose (**2**,**3**,**4**,**5**) and immobilized cellobiose (**16**) were prepared as previously described (Sangseethong and Penner, 1999).

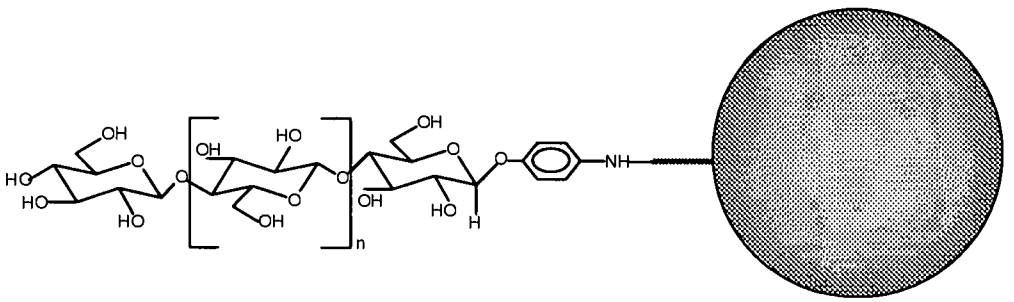
4.3.1.2 *Peracetylated α-cellooligosaccharides* (**6** and **11**)

α-cellotriose undecaacetate (**6**) and α-cellotetraose tetradecaacetate (**11**) were prepared by controlled acid-catalyzed acetolysis of cellulose (Dickey and Wolfrom, 1949; Miller et al., 1960). In brief, 80 g of cellulose powder (medium fibrous, Aldrich Chemical Co. Inc., Milwaukee, WI) was added to a mixture of AcOH (310 mL), Ac_2O (310 mL), and H_2SO_4 (33 mL). The temperature of reaction mixture, during the addition of cellulose powder, was kept less than 40° C by external cooling with ice-H_2O. After standing at room temperature for 60 h, the reaction mixture was poured into ice-cold H_2O to precipitate the peracetylated cellooligosaccharides. The solid was resuspended in H_2O and the suspension was



1	n=0	R^1=H,	R^2=OAc,	R^3=Ac
2	n=0	R^1=H,	R^2=Br,	R^3=Ac
3	n=0	R^1=$OC_6H_4NO_2$,	R^2=H,	R^3=Ac
4	n=0	R^1=$OC_6H_4NO_2$,	R^2=H,	R^3=OH
5	n=0	R^1=$OC_6H_4NH_2$,	R^2=H,	R^3=OH
6	n=1	R^1=H,	R^2=OAc,	R^3=Ac
7	n=1	R^1=H,	R^2=Br,	R^3=Ac
8	n=1	R^1=$OC_6H_4NO_2$,	R^2=H,	R^3=Ac
9	n=1	R^1=$OC_6H_4NO_2$,	R^2=H,	R^3=OH
10	n=1	R^1=$OC_6H_4NH_2$,	R^2=H,	R^3=OH
11	n=2	R^1=H,	R^2=OAc,	R^3=Ac
12	n=2	R^1=H,	R^2=Br,	R^3=Ac
13	n=2	R^1=$OC_6H_4NO_2$,	R^2=H,	R^3=Ac
14	n=2	R^1=$OC_6H_4NO_2$,	R^2=H,	R^3=OH
15	n=2	R^1=$OC_6H_4NH_2$,	R^2=H,	R^3=OH

Figure 4.1 Chemical structures of compounds **1-15**



16 n=0
17 n=1
18 n=2

Figure 4.2 Schematic illustration of immobilized cellodextrins

neutralized with $NaHCO_3$. The suspension was allowed to stand overnight. The precipitate was then filtered, washed with water, and dried in a vacuum oven at 40° C. Crude acetates (168 g) were mixed with anhydrous MeOH (700 mL) in a Waring blender. The suspension was filtered and the solid was resuspended in anhydrous MeOH (700 mL) and refiltered. The filtrate was taken to dryness using a rotary evaporator. The gummy residue was then dissolved in a minimum amount of hot chloroform and poured with rapid stirring into 3 L of ice-cold hexane (Feather and Harris, 1967). After filtration, the white solid was dried in a vacuum oven (below 40°C). Peracetylated cellooligosaccharides (23.8 g) were then separated by silica gel column chromatography (70-230 mesh, SIGMA Chemical Co. St. Louis, MO) with solvent *A* as eluent. The column fractions were monitored by TLC. Fractions containing cellotriose and cellotetraose peracetates were pooled, evaporated to dryness and crystallized from 95% EtOH to yield α-cellotriose undecaacetate (**6**, 2.5 g): mp 223-225° C, lit. 223-224° C (Dickey and Wolfrom, 1949); and α-cellotetraose tetradecaacetate (**11**, 1.92 g): mp 228-231° C, lit. 230-234° C (Dickey and Wolfrom, 1949).

4.3.1.3 *Glycosidation of peracetylated glycosyl bromides*

p-Nitrophenyl glycosides (**8** and **13**) were prepared using phase transfer catalysis (Roy et al., 1992). A solution of peracetylated glycosyl bromide, **7** (1.42 g, 1.44 mmol) or **12** (1.52 g, 1.19 mmol), prepared as described by Piyachomkwan et

al. (1997), tetrabutylammonium hydrogen sulfate (1 equiv), and *p*-nitrophenol (3 equiv) in CH_2Cl_2 (10 mL) was mixed with M aq NaOH (10 mL). The mixture was stirred and gently warmed until all solids dissolved. The reaction mixture was vigorously stirred at room temperature for 4 h. Reaction was monitored by TLC (solvent *B*). The organic phase was successively washed with cold M NaOH (2 × 20 mL) and then water (2 × 20 mL). The washed organic phase was then dried (Na_2SO_4), filtered and concentrated. The concentrated mixture was dissolved in a minimum amount of EtOAc-2-propanol-CH_2Cl_2 (1:1:1.5) (total of 15 mL) and filtered though a layer of silica gel (30 mL), which was then washed with 200 mL of solvent *B*. The combined filtrate and washing were evaporated to dryness. Flash chromatography (solvent *C*, compound **8**; solvent *B*, compound **13**) followed by crystallization from 95% EtOH afforded compound **8** (0.451 g, 30%); ^{1}H-NMR ($CDCl_3$): δ 8.2 (d, 2 H, J 9.1 Hz, H-meta), 7.04 (d, 2 H, J 9.1 Hz, H-ortho), 5.28 (t, 1 H, $J_{3,4}$ 8.3 Hz, H-3), 5.2 (2 H, H-1,2), 5.14 (t, 1 H, $J_{3',4'}$ 9.3 Hz, H-3′), 5.13 (t, 1 H, $J_{3'',4''}$ 9 Hz, H-3′′), 5.06 (t, 1 H, $J_{4'',5''}$ 9.6 Hz, H-4′′), 4.9 (t, 1 H, $J_{1'',2''}$ 8.5 Hz, H-2′′), 4.88 (t, 1 H, $J_{1',2'}$ 8.5 Hz, H-2′), 4.53 (dd, 1 H, $J_{6a,6b}$ 11.1 Hz, H-6a), 4.51 (d, 1 H, $J_{1'',2''}$ 8.5 Hz, H-1′′), 4.49 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1′), 4.47 (dd, 1 H, $J_{6a',6b'}$ 11.1 Hz, H-6a′), 4.36 (dd, 1 H, $J_{5'',6a''}$ 4.2 Hz, $J_{6a'',6b''}$ 12.5 Hz, H-6a′′), 4.15-4.09 (m, 2 H, H-6b,6b′), 4.05 (dd, 1 H, $J_{5'',6b''}$ 2.1 Hz, $J_{6a'',6b''}$ 12.5 Hz, H-6b′′), 3.9-3.82 (m, 2 H, H-4,5), 3.78 (t, 1 H, $J_{3',4'}$ 9.3 Hz, H-4′), 3.66-3.58 (m, 2 H, H-5′,5′′), 2.16-1.98 (cluster of S, 30 H, 10 OAc), and compound **13** (0.40 g, 27%); ^{1}H-NMR ($CDCl_3$): δ 8.2 (d, 1 H, J 9.1 Hz, H-meta), 7.05 (d,1 H, J 9.1 Hz, H-ortho), 5.28 (t,

1 H, $J_{3,4}$ 8.3 Hz, H-3), 5.22-5.08 (m, 5 H, H-1,2,3′,3″,3‴), 5.05 (t, 1 H, $J_{4''',5'''}$ 9.6 Hz, H-4‴), 4.9 (t, 1 H, $J_{2',3'}$ 8.5 Hz, H-2′), 4.85 (t, 1 H, $J_{2'',3''}$ 8.5 Hz, H-2″), 4.83 (t, 1 H, $J_{2''',3'''}$ 8.5 Hz, H-2‴), 4.52 (dd, 1 H, $J_{6a,6b}$ 12.4 Hz, H-6a), 4.49 (d, 1 H, $J_{1',2'}$ 9.7 Hz, H-1′), 4.48 (d, 1 H, $J_{1'',2''}$ 9.2 Hz, H-1″), 4.44 (d, 1 H, $J_{1''',2'''}$ 10.6 Hz, H-1‴), 4.41 (dd, 2 H, J 11 Hz, H-6a′,6a″), 4.35 (dd, 1 H, $J_{5''', 6a'''}$ 4.4 Hz, $J_{6a''',6b'''}$ 12.6 Hz, H-6a‴), 4.14-4.07 (m, 3 H, H-6b,6b′,6b″), 4.03 (dd, 1 H, $J_{5''',6b'''}$ 2.1 Hz, $J_{6a''',6b'''}$ 12.6 Hz, H-6b‴), 3.88-3.83 (m, 2 H, H-4,5), 3.76 (t, 2 H, J 9.1 Hz, H-4′,4″), 3.66-3.54 (m, 3 H, H-5′,5″,5‴), 2.16-1.96 (cluster of S, 39 H, 13 OAc).

4.3.1.4 *Deacetylation of nitrophenyl glycosides*

The acetylated glycosides (**8**, 0.35 g, 0.34 mmol; **13**, 0.415 g, 0.31 mmol) were dissolved in MeOH (75 mL) containing 1 M NaOMe in MeOH (0.21 mL). The solution was stirred overnight at room temperature; deacetylated glycosides (**9**, **14**) were partially crystallized. Ether (40 mL) was added to cooled solutions to ensure complete crystallization. After filtration (0.45 μm, HV membrane, Millipore Corp., PA) and drying, pure **9** (0.19 g, 90%); ^{1}H-NMR (D_2O): δ 8.2 (d, 2 H, J 9.1 Hz, H-meta), 7.22 (d, 2 H, J 9.1 Hz, H-ortho), 5.14 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.36 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.24 (d, 1 H, $J_{1'',2''}$ 7.8 Hz, H-1″), 3.8-2.96 (m, 18 H), and **14** (0.2 g, 82%) were obtained; ^{1}H-NMR (D_2O): δ 8.2 (d, 2 H, J 9.3 Hz, H-meta), 7.22 (d, 2 H, J 9.3 Hz, H-ortho), 5.14 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.36 (d, 1 H, $J_{1',2'}$ 7.8

Hz, H-1′), 4.31 (d, 1 H, $J_{1'',2''}$ 8 Hz, H-1′′), 4.23 (d, 1 H, $J_{1''',2'''}$ 7.9 Hz, H-1′′′), 3.8-2.95 (m, 24 H).

4.3.1.5 *Preparation of immobilized cellooligosaccharides* (**17** and **18**)

The amino derivatives (**10** and **15**) were obtained by catalytic hydrogenation of the corresponding nitro compounds (**9** and **14**) in 80% MeOH using 10% Pd on activated charcoal as a catalyst. TLC (solvent *D*) indicated complete conversion.

After solvent removal, the amino derivatives (**10** and **15**) were coupled to Affigel 10 (BioRad Laboratories, Hercules, CA), under anhydrous conditions as previously described (Sangseethong and Penner, 1999), with the following exceptions. The amino derivative of cellotriose (**10**) was dissolved in DMSO-MeOH (1:3) and the amino derivative of cellotetraose (**15**) was dissolved in DMSO-MeOH (3:1). Affigel 10 (7 mL) was washed with cold 2-propanol, then added to 21 mL of the solvent containing the amino derivative (140 μmol). The suspension was rotated end-over-end for 3 h at room temperature. Ethanolamine (1 M, 7 mL) was added to the slurry and allowed to react for 1 h in order to block unreacted functional groups remaining on the gel. The resulting gel was washed with 50 mM NaOAc, pH 5 and stored in this buffer at 4°C.

4.3.2 Enzyme purification

Cellobiohydrolases were purified as described by Piyachomkwan et al. (1997). Crude cellulases produced by *T. reesei* (Spezyme™-CP, Evironmental BioTechnologies Inc., Menlo Park, CA) was initially fractionated on anion-exchange chromatography using DEAE-Sepharose (Beldman et al., 1985). The partially purified CBHI and CBHII fractions were further fractionated on affinity chromatography using APTC-derivatized agarose as the affinity matrix. CBHI fraction was applied to the affinity column in 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone. CBHII fraction was applied to the column in 0.1 M NaOAc, pH5, containing 1 mM gluconolactone and 0.2 M glucose. Adsorbed CBHs were eluted by addition of cellobiose, 0.01M, to the mobile phase.

CBHI and CBHII fractions from affinity chromatography were further purified on a hydrophobic interaction chromatography using a phenyl Sepharose FF column (Pharmacia Inc., Piscataway, NJ) to separate the intact enzymes from catalytic-core fragments. CBHI and CBHII preparations were applied to the column in 25 mM NaOAc, pH 5, containing 0.85 M ammonium sulfate for CBHI or 0.35 M ammonium sulfate for CBHII. The adsorbed enzymes were eluted from the column with a gradient of 0.85-0.35 M ammonium sulfate for CBHI or 0.35-0.01 M ammonium sulfate for CBHII. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and the enzyme assay for specific activities of the enzymes were performed to monitor purity of the resulting enzymes (Piyachomkwan et al., 1997).

4.3.3 Cellobiohydrolase activity experiments

The activity of CBHI and CBHII on free and immobilized cellooligosaccharides was determined. In this study, the free substrates mean *p*-nitrophenyl cellobioside ($PNPG_2$), *p*-nitrophenyl cellotrioside ($PNPG_3$) and *p*-nitrophenyl cellotetraoside ($PNPG_4$). The immobilized substrates refer to the *p*-aminophenyl glycosides of cellobiose, cellotriose and cellotetraose that are attached to the agarose beads via the amino group on the aglycone (reducing ends).

All enzymatic experiments were done at 50° C in 50 mM NaOAc buffer, pH 5. The reaction was initiated by addition of the enzyme (CBHI or CBHII) to the solution of free or immobilized cellooligosaccharide (substrate concentration was 1 mM and enzyme concentration was 0.5 μM). The reaction mixture was rotated end-over-end at 50°C. At appropriate time intervals, the reaction mixture was diluted (2:1) with stop reagent (Teleman et al., 1995) which consisted of EtOH and 1 M glycine buffer, pH 11 (9:1).

The concentration of immobilized substrate was defined as the amount of enzyme accessible cellodextrin per mL of agarose bead. This was determined by exhaustive hydrolysis of the cellodextrin-coupled gel with the appropriate enzyme, (either CBHI or CBHII). Enzyme concentrations were determined from absorbance at 280 nm using a molar absorption coefficient of 73000 $M^{-1}cm^{-1}$ for CBHI and 75000 $M^{-1}cm^{-1}$ for CBHII (Tomme et al., 1988).

Products generated in experiments with free substrates were analyzed by HPLC using an Aminex HPX-87H column (7.8 mm × 300 mm, BioRad Laboratories, Hercules, CA) with isocratic elution of 15% acetonitrile in 5 mM H_2SO_4 (65° C, 0.4 mL/min) and detected at 330 nm (corresponding to the *p*-nitrophenyl moiety). Assay containing $PNPG_4$ were also chromatographed in 5 mM H_2SO_4, without acetonitrile, in order to resolve $PNPG_3$ and $PNPG_4$. Cellodextrins resulting from the hydrolysis of the immobilized substrates were chromatographed on an Aminex HPX-87P column s(7.8 mm × 300 mm), using water as eluent (85°C, 0.2 mL/min). The cellodextrin products were detected with a differential refractometer (model 410, Waters Assoc., Milford, MA).

4.4 Results and Discussion

The recent crystallographic studies reveal that CBHI and CBHII have similar active site structure. Both enzymes contain a long cellulose-binding tunnel consisting of several glucosyl-binding subsites: six binding sites in the CBHII active site tunnel (Teeri, 1997) and ten subsites in CBHI tunnel (Divne et al., 1998). This tunnel-shaped active site supports the notion that CBHs confine their activity at cellulose chain ends. Even though both CBHs were originally thought to cleave cellulose from nonreducing end, recent studies show that CBHI prefers to act at reducing end. Immobilized cellodextrins were used as model substrates in this study to determine the capability of CBHI on catalyzing the hydrolysis of cellulose at the

nonreducing end. Immobilized cellodextrins were prepared by attaching the reducing end of the cellodextrins to the large agarose support. Since the agarose bead is much larger than the active site tunnel of the CBHs, the reducing ends of cellodextrins are unavailable for the enzymes. CBHII, reported to consistently show traditional exocellulase activity, was used in the study as a control. Under the optimum condition both CBHI and CBHII had no apparent activities on the immobilized phenyl cellobioside ([S] = 1 mM and [E] = 0.5 μM). However, when the enzyme concentration were increased (12 μM), small activity of CBHI on the substrate was observed (data not shown). The activities of CBHI and CBHII on RE-blocked cellodextrins, as seen in Figures 4.3 and 4.4, are apparent on the longer substrates (cellotriose and cellotetraose). This indicates that the activity of CBHI is not confined to the reducing end of cellulose but it is capable of initiating the hydrolysis at the nonreducing end of the substrates. The hydrolytic profile of immobilized cellotriose and cellotetraose by CBHI and CBHII are similar, with cellobiose as the major product. Cellotriose was produced in small amount. There was no glucose produced in the initial stage of hydrolysis. It could be detected only in the later stage as a result of enzymatic attack on the cellotriose formed earlier. Because of the zigzag nature of glycosidic bonds in cellulose chain, i.e. 180° rotation of every alternating glycosyl unit, it has been suggested that half of the initial product would be cellobiose and the other half would be cellotriose. Cellobiose would only predominate as product in the later stage of cellulose hydrolysis as a result of processive action on the cellulose chain by exo-cellulases

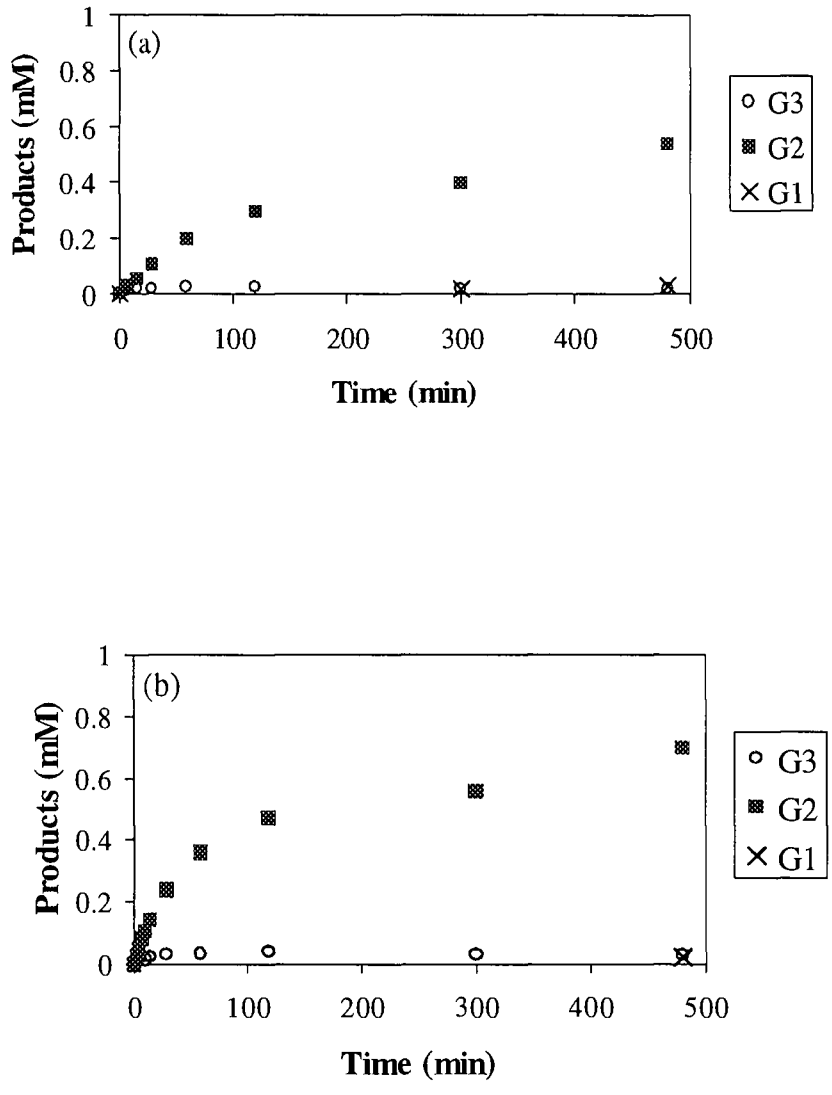


Figure 4.3 CBH I activities on immobilized cellodextrins: (a) immobilized G3; (b) immobilized G4

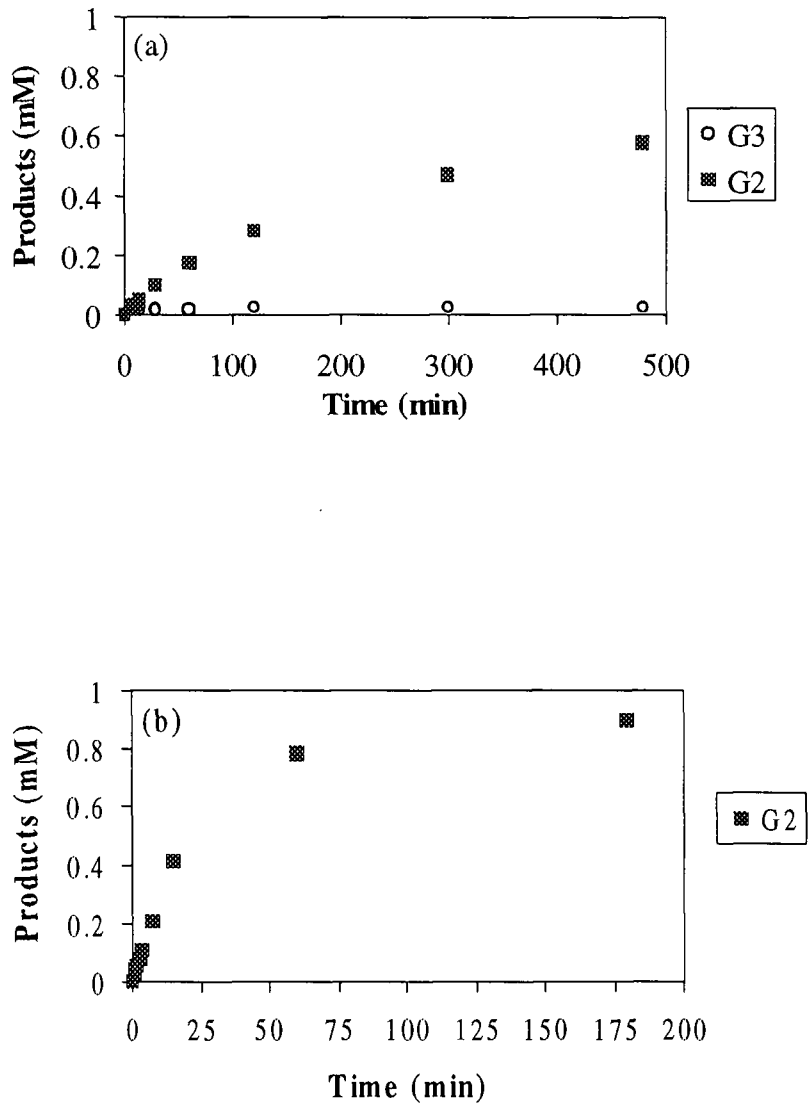


Figure 4.4 CBH II activities on immobilized cellodextrins: (a) immobilized G3; (b) immobilized G4

(Divne et al., 1998). Surprisingly, our results do not support this notion. Cellobiose was the major product in all cases even though the multiple-attack or processivity is not expected on these immobilized cellotriose and cellotetraose.

The cleavage sites and the enzymatic rates of CBHI and CBHII on the immobilized cellodextrins were determined and compared with those on the *p*-nitrophenyl glycosides (free substrates). The data obtained for CBHII are summarized in Figure 4.5. The results support the suggestion that at least three contiguous glucosyl units on substrate molecule are required for hydrolysis by CBHII (van Tilbeurgh and Claeyssens, 1985; Claeyssens et al., 1989). The enzyme does not hydrolyze either free or immobilized aryl β-D-cellobioside. The patterns of the enzymatic attack on both free and immobilized cellodextrins (cellotriose and cellotetraose) are similar with the second linkage from the nonreducing end as the major cleavage site. Small but significant activity on the third bond from nonreducing end was also observed on the immobilized cellodextrins. Since CBHII has shown preference to nonreducing end of cellulose (Biely et al., 1993; Barr et al., 1996), the similar rate of hydrolysis from free and reducing end-blocked cellodextrins would be expected. However, due to the difference in the nature of the substrates, i.e. steric or diffusion limitation that might associate with the agarose support in the case of immobilized substrates, the direct comparison of the hydrolysis rates between free and immobilized system might not be appropriate. This might be the case for the hydrolysis of modified cellotriose where the hydrolysis rate of free aryl cellotriose is almost twice that of immobilized one (15.2

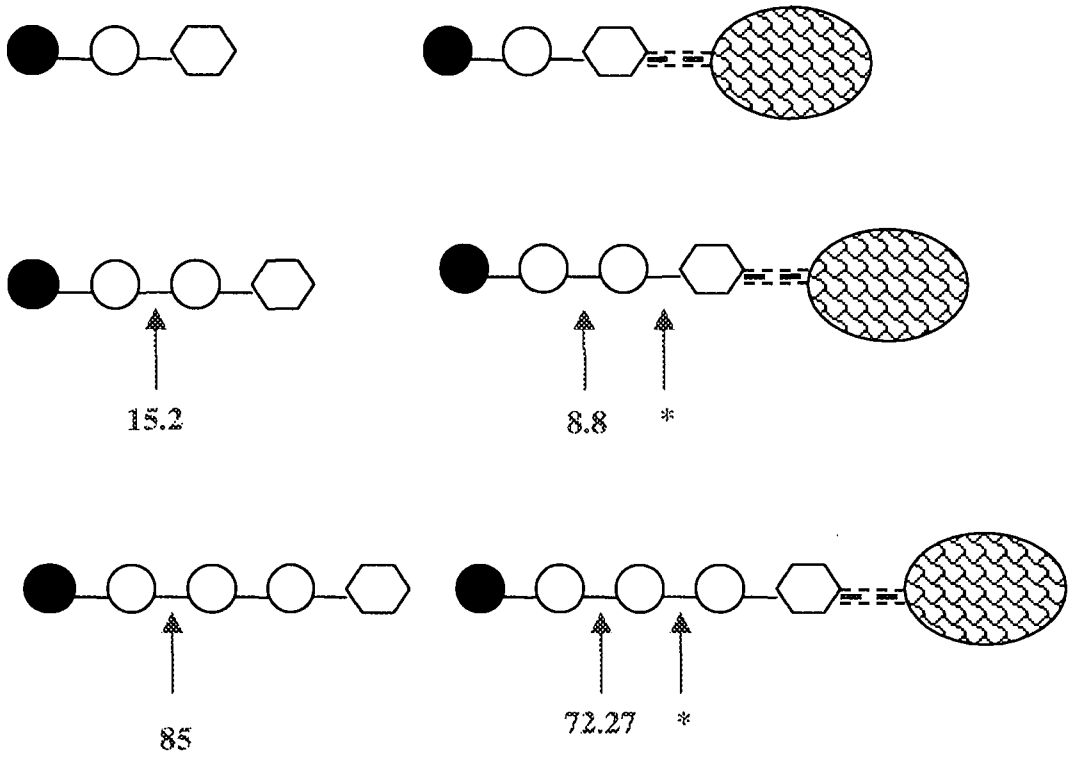


Figure 4.5 Activities of CBH II on free and immobilized cellodextrins (μmol/min/μmol protein)

Glucosyl unit
Non-reducing end
p-Nitrophenyl group
Agarose bead
Spacer arm
* Very low activity

and 8.8 μmol/min/μmol protein). The steric limitation of agarose bead becomes insignificant, when cellodextrin chain gets longer as that the rates for aryl cellotetraoside in both free and immobilized substrates are very close (85 and 72.27 μmol/min/μmol protein). This result confirms the specificity of CBHII at nonreducing end of cellulose.

The cleavage sites and the enzymatic rates of CBHI on free and immobilized aryl β-D-glycosides of cellobiose, cellotriose and cellotetraose are summarized in Figure 4.6. Under the condition used in this experiment, CBHI hydrolyzes free but not immobilized aryl cellobioside. This could be due to the more favorable formation of the catalytically non-productive complex between
CBHI's active site tunnel and immobilized aryl cellobioside, as previously discussed (Sangseethong and Penner, 1999). Even though the major cleavage sites of CBHI on both free and immobilized cellotrioside and cellotetraoside are very similar, the enzymatic rates between the two systems are significantly different. The activity on the immobilized cellodextrin increases with increasing chain length. The same trend is not observed on the free aryl cellooligosaccharides. The rate increases with the substrate polymerization degree only in going from cellobioside to cellotrioside. Cellotetraoside, however, is found to be hydrolyzed at a much slower rate than cellotrioside. The total hydrolysis rate on cellotetraoside is six times lower than that for cellotrioside. This is not an artifact since similar trend were observed in the earlier hydrolysis studied of *T. reesei* CBHI on small soluble 4-methylumbelliferyl glycosides of cellooligosaccharides (Claeyssens et al., 1989).

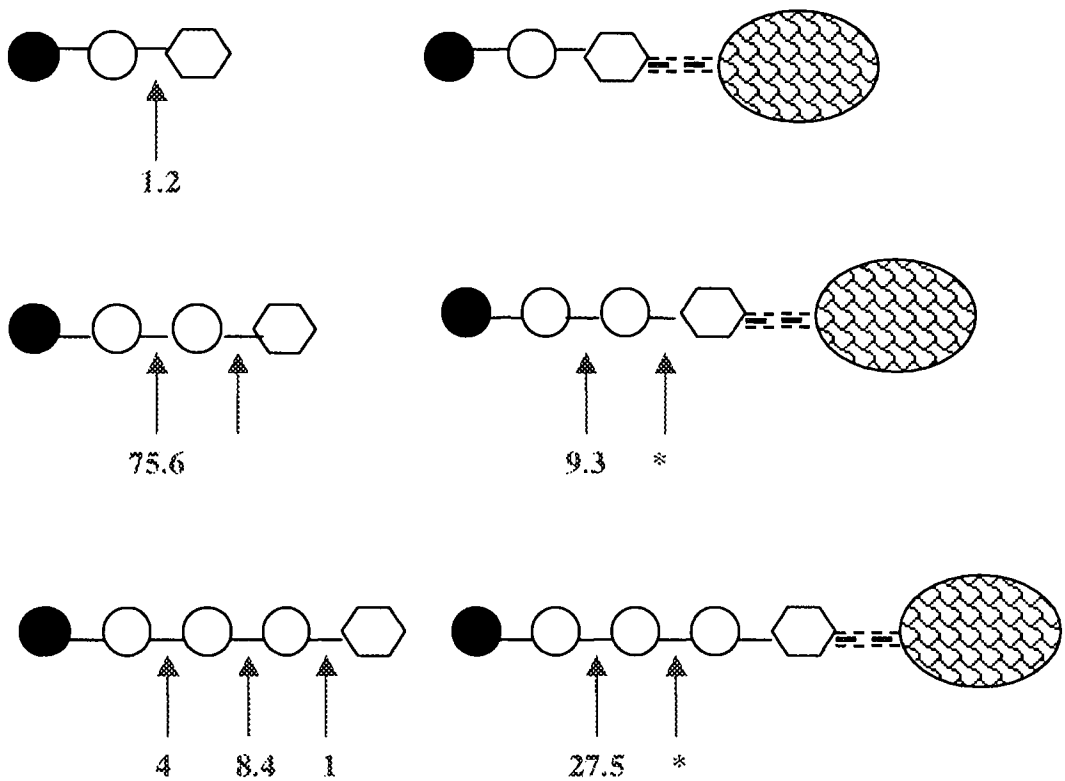


Figure 4.6 Activities of CBH I on free and immobilized cellodextrins (μmol/min/μmol protein)

○ Glucosyl unit
● Non-reducing end
⬡ *p*-Nitrophenyl group
Agarose bead
Spacer arm
* Very low activity

Based on the crystallographic studies, CBHI's active site is a long tunnel consisting of ten glucosyl binding subsites, ie. subsites -7 to +3, with the catalytic residues located between subsites -1 and +1 (Divne et al., 1998). The same study also shows that cellooligosaccharides bound in the CBHI's active site have the same directionality and orientation with reducing end pointing to subsite +3 and nonreducing end pointing to subsite -7. Since CBHI never cleaves glucose from nonreducing end, we believe that, with the substrate molecule crossing over catalytic site, subsites -1 and -2 have to be occupied by glucosyl units for the hydrolysis to occur. The phenomenon observed in the activity of CBHI on small soluble cellooligosaccharides could be explained by the non-productive binding of aryl cellotetraoside to CBHI active site covering subsites -6 to -2; the same subsites occupied by cellopentaose (Divne et al., 1998), as illustrated in Figure 4.7. The formation of such complex could hamper the efficiency of CBHI catalytic activity. The non-productive binding effect is not seen in the case of aryl cellotriose. This might be because the twisting orientation of aryl cellotrioside between subsites -4 to -2 could not provide the interaction strong enough to stabilize the substrate-enzyme complex covering such subsites. Aryl cellotrioside would prefer a, more energetically favorable, relaxed conformation and bind to the enzyme's active site in the same way that unsubstituted cellotetraose does, i.e. occupying subsites -7 to -4 and -2 to +2 (Divne et al., 1998). This would leave the catalytic end of the active site tunnel free and available for productive hydrolysis of other molecules of substrate.

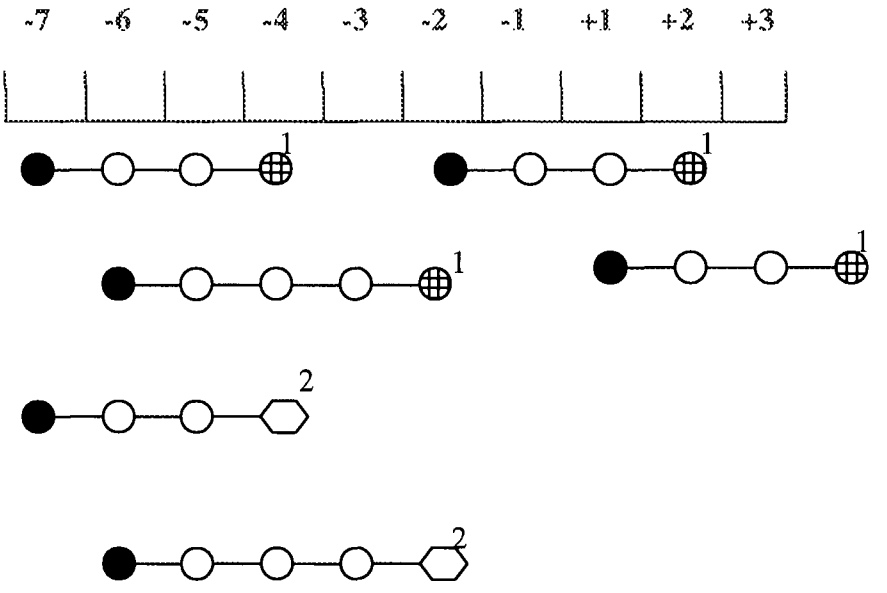


Figure 4.7 The binding of cellodextrins in CBH I's subsites.

○ Glucosyl unit
● Non-reducing end
⊕ Reducing end
⬭ *p*-Nitrophenyl group
1 Divne et al., 1998
2 Proposed

The information obtained from the hydrolysis of immobilized cellodextrins could be pertinent for the elucidation of CBHs interaction with the loose, highly hydrated nonreducing ends of polymer chain in the native cellulose. Based on several biochemical studies and crystallographic structure, it has been proposed that CBHI cleaves the cellulose from reducing end. However, in this study we used immobilized reducing end-blocked cellodextrins to unequivocally show that CBHI's activity is not confined to reducing end; it is capable of catalyzing the hydrolysis at the nonreducing end as well. We speculate that the reducing end-blocked cellooligosaccharides were forced to back into the enzyme tunnel via the opening, close to subsite +3, which is thought to primarily serve as an exit for the generated product. This proposed mechanistic mode seem to be realistic since the effect of non-productive binding observed in the activity of CBHI on free aryl cellooligosaccharides does not seem to exist in the case of immobilized ones.

4.5 References

Barr, B.K., Hsieh, Y., Ganem, B., and Wilson, D.B. 1996. Identification of two functionally different classes of exocellulases. *Biochemistry* 35: 586-592.

Beldman, G., Searle-Van Leeuwen, M.F., Rombouts, F.M., and Voragen, F.G.J. 1985. The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. *Eur. J. Biochem.* 146: 301-308.

Biely, P., Vrsanska, M., and Claeyssens, M. 1993. Mode of action of *Trichoderma reesei* β-1,4-glucanases on cellooligosaccharides. In *TRICEl Symposium on Trichoderma reesei Cellulases and other Hydrolases*, (P. Suominen and T. Reinikainen, eds.), pp. 99-108. Foundation for Biotechnical and Industrial Fermentation Research , Espoo, Finland.

Chanzy, H., Henrissat, B., Vuong, R., and Schulein, M. 1983. The action of 1,4-β-D-glucan cellobiohydrolases on *Valonia cellulose* microcrystals. *FEBS. Lett.* 153: 113-118.

Claeyssens, M., van Tilbeurgh, H., Tomme, P., Wood, T.M. and McCrae, S.I. 1989. Fungal cellulase systems. Comparison of the specificities of the cellobiohydrolases isolated from *Penicillium pinophilum* and *Trichoderma reesei. Biochem. J.* 261: 819-825.

Dickey, E.E. and Wolfrom, M.L. 1949. A polymer-homologous series of sugar acetates from the acetolysis of cellulose. *J. Am. Chem. Soc.* 71: 825-828.

Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, K.C., Teeri, T.T., and Jones, T.A. 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei. Science* 265: 524-528.

Divne, C., Stahlberg, J., Teeri, T.T., and Jones, T.A. 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei. J. mol. Biol.* 275: 309-325.

Feather, M.S. and Harris, J.F. 1967. Chromatographic isolation of some cellulose oligosaccharides. U.S. Forest Servise Research Note-FPL-0161.

Miller, G.L., Dean, J., and Blum, R. 1960. A study of methods for preparing oligosaccharides from cellulose. *Archives of Biochemistry and Biophysics.* 91: 21-26.

Orgeret, C., Seillier, E., Gautier, C., Defaye, J., and Driguez, H. 1992. 4-Thiocellooligosaccharides. Their synthesis and use as ligands for the separation of cellobiohydrolases of *Trichoderma reesei* by affinity chromatography. *Carbohydr. Res.* 224: 29-40.

Piyachomkwan, K., Gable, K.P., and Penner, M.H. 1997. *p*-Aminophenyl 1-thio-β-D-cellobioside: synthesis and application in affinity chromatography of exo-type cellulases. *Carbohydr. Res.* 303: 255-259.

Piyachomkwan, K. and Penner, M.H. 1998. Aryl thioglycoside-based affinity purification of exo-acting cellulases. *Anal. Biochem.*, 255:223-235.

Roy, R., Tropper, F.D., and Romanowska, A. 1992. New strategy in glycopolymer syntheses. Preparation of antigenic water-soluble poly(acrylamide-co-*p*-acrylamidophenyl β-lactoside). Bioconjugate Chem. 3: 256-261.

Sangseethong, K. and Penner, M.H. 1999. *p*-Aminophenyl β-D-cellobioside as an affinity ligand for exo-type cellulases. *Carbohydr. Res.* In press.

Teeri, T.T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *TIBTECH* 15: 160-167.

Teleman, A., Koivula, A., Reinikainen, T., Valkeajarvi, A., Teeri, T.T., Drakenberg, T., and Teleman, O. 1995. Progress-curve analysis shows that glucose inhibits the cellotriose hydrolysis catalysed by cellobiohydrolase II from *Trichoderma reesei*. *Eur. J. Biochem.* 231: 250-258.

Tomme, P., van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. 1988. Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* 170: 575-581.

Tomme, P., Warren, R.A.J., and Gilkes, N.R. 1995. Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol.* 37: 1-81.

van Tilbeurgh, H., Bhikhabhai, R., Pettersson, L.G., and Claeyssens, M. 1984. Separation of endo-and exo-type cellulases using a new affinity chromatography method. *FEBS Lett.* 169: 215-218.

Vrsanska, M. and Biely, P. 1992. The cellobiohydrolase I from *Trichoderma reesei* QM 9414: action on cello-oligosaccharides. *Carbohydr. Res.* 227: 19-27.

Waldi, D. 1965. Spray reagents for thin-layer chromatography. In *Thin Layer Chromatography. A Laboratory Handbook*, (E. Stahl, ed.) pp. 485-502, Academic Press Inc., New York.

CHAPTER 5

CONCLUSION

The synthesis of *p*-aminophenyl derivatives of cellodextrins ($PAPG_2$, $PAPG_3$, $PAPG_4$) and their application in the study of *Trichoderma reesei* cellobiohydrolases were demonstrated in this thesis.

p-Aminophenyl β-D-cellobioside ($PAPG_2$) has been shown to be an effective ligand for the chromatographic fractionation of cellobiohydrolases. The $PAPG_2$-derivatized agarose specifically retains the CBH component of relatively complex cellulase mixtures. The analysis based on gel-electrophoresis and specific activities of resulting CBH preparations indicates that $PAPG_2$ can be used interchangeably with more traditional thioglycoside-based affinity ligands. The application of $PAPG_2$ as an affinity ligand illustrates that the tethered ligand associates with the *T. reesei* CBHs in catalytically nonproductive mode. Hence, in many cases there may be relatively little advantage in incorporating a thio linkage at the aglyconic bond.

The catalytic action patterns of CBH I and CBH II on free and immobilized *p*-aminophenyl cellodextrins were used to elucidate the chain-end specificity and/or preference of these enzymes. CBH II attacks free and immobilized $PAPG_3$ and $PAPG_4$ in a typical exo-glucanase manner by cleaving off cellobiose from the nonreducing end of substrates. Results from immobilized cellodextrins indicate that CBH I is capable of initiating the hydrolysis at the nonreducing end of substrates

with a major activity at the second linkage from the nonreducing end. However, it hydrolyzes free $PAPG_2$, $PAPG_3$, and $PAPG_4$ in a random manner at the first three linkages from the chromophoric group located at the reducing terminus of cellodextrins. This random pattern could be due to a combination of two different modes of action of this enzyme acting on these free substrates; one acting at the reducing end, and the other acting at the nonreducing end.

BIBLIOGRAPHY

Abuja, M.P., Pilz, I., Claeyssens, M., and Tomme, P. 1988. Domain structure of cellobiohydrolase II as studied by small angle X-ray scattering: close resemblance to cellobiohydrolase I. *Biochem. Biophys. Res. Comm.* 156:180-185.

American society for testing and materials. 1986. Standard test method for intrinsic viscosity of cellulose. *Annual book of ASTM standards.* 15.04, 360-366. American Society for Testing and Materials, Pennsylvania.

Arai, M., Sakamoto, R., and Murao, S. 1989. Different action by two avicelases from *Aspergillus aculeatus. Agric. Biol. Chem.* 53:1411-1412.

Atalla, R. H. 1983. The structural of cellulose: recent developments. In *Wood and Agricultural Residues*, (J. Soltes, ed.) pp.59-77, Academic Press, New York.

Barr, B.K., Hsieh, Y., Ganem, B., and Wilson, D.B. 1996. Identification of two functionally different classes of exocellulases. *Biochemistry* 35: 586-592.

Beguin, P. and Aubert, J.P. 1992. Cellulases. *Encyclopedia of Microbiology* 1: 467-477.

Beldman, G., Searle-Van Leeuwen, M.F., Rombouts, F.M., and Voragen, F.G.J. 1985. The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. *Eur. J. Biochem.* 146: 301-308.

Bertran, M. S. and Dale, B.E. 1985. Enzymatic hydrolysis and recrystallization behavior of initially amorphous cellulose. *Biotechnol. Bioeng.* 27:177-181.

Bhat, K.M., Hay, A.J., Wood, T., and Claeyssens, M. 1990 Study of the mode of action and site-specificity of the endo-1,4-β-D-glucanases of the fungus *Penicillium pinophilum* with normal, 1-^{3}H-labelled, reduced and chromogenic cello-oligosaccharides. *Biochem. J.* 266:371-378.

Bhikhabhai, R., Johansson, G., and Pettersson, G. 1984. Isolation of cellulolytic enzymes from *Trichoderma reesei* QM 9414. *J. Applied Biochem.* 6:336-345.

Biely, P., Vrsanska, M., and Claeyssens, M. 1993. Mode of action of *Trichoderma reesei* β-1,4-glucanases on cellooligosaccharides. In *TRICEL Symposium on*

Trichoderma reesei Cellulases and other Hydrolases, (P. Suominen and T. Reinikainen, eds.), pp. 99-108. Foundation for Biotechnical and Industrial Fermentation Research , Espoo, Finland.

Blackwell, J. 1982. The macromolecular organization of cellulose and chitin. In *Cellulose and Other Natural Polymer Systems,* (R.M. Brown Jr., ed.), pp. 403-428, Plenum Press, New York.

Bothwell, M.K., Daughhetee, S.D., Chaua, G.Y., Wilson, D.B., and Walker, L.P. 1997. Binding capacities for *Thermomonospora fusca* E3, E4 and E5, the E3 binding domain, and *Trichoderma reesei* CBHI on Avicel and bacterial microcrystalline cellulose. *Bioresour. Technol.* 60:169-178.

Capon, B. and Thomson, J.W. 1979. Studies on cellulolytic enzymes I. The use of 3,4-dinitrophenyl glycosides as substrates *Bioorg. Chem.*, 8:147-173.

Chanzy, H., Henrissat, B., Vuong, R., and Schulein, M. 1983. The action of 1,4-β-D-glucan cellobiohydrolases on *Valonia cellulose* microcrystals. *FEBS. Lett.* 153: 113-118.

Chirico, W. and Brown, R.D., Jr. 1987. β-Glucosidase from *Trichoderma reesei.* Substrate-binding region and mode of action on [1-^{3}H] cello-oligosaccharides. *Eur. J. Biochem.* 165:343-351.

Claeyssens, M., Tomme, P., Brewer, C.F., and Hehre, E.J. 1990. Stereochemical course of hydrolysis and hydration reactions catalyzed by cellobiohydrolases I and II from *Trichoderma reesei. FEBS Lett.* 263:89-92.

Claeyssens, M., van Tilbeurgh, H., Tomme, P., Wood, T.M. and McCrae, S.I. 1989. Fungal cellulase systems. Comparison of the specificities of the cellobiohydrolases isolated from *Penicillium pinophilum* and *Trichoderma reesei. Biochem. J.* 261: 819-825.

Clarke, A.J. 1997. *Biodegradation of Cellulose*, pp.1-7, Enzymology and Biotechnology. Technomic Publishing Co., Inc.

Cole, F.E. and King, K.W. 1964. Site of hydrolysis of cellulodextrins and reduced cellulodextrins by purified cellulase components. *Biochim. Biophys. Acta* 81: 122-129.

Coughlan, M.P. 1992. Enzymic hydrolysis of cellulose: an overview. *Bioresour. Technol.* 39:107-115.

Cowling, E.G. and Kirk, T.K. 1976. Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes. *Biotechnol. Bioeng.* Symp. No. 6:95-124.

Davies, G. J. 1998. Substrate distortion in glycoside hydrolysis: the use of modified cellooligosaccharides to study cellulase structure and function. In Carbohydrases from Trichoderma reesei and Other Microorganisms. Structure, Biochemistry, Genetics and Applications.(Claeyssens, M., Nerinckx, W., and Piens, K., eds.). pp. 166-173. The Royal Society of Chemistry, Cambridge, UK

Davies, G. and Henrissat, B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* 3:853-859

Day, A.G. and Withers, S.G. 1985. A simple kinetic test to distinguish exo-glucosidase and β-glucosidase activities. *Biochem. and Biophys. Res. Comm.*, 133:628-632.

Deshpande, M.V., Eriksson, K.E., and Pettersson, L.G. 1984 An assay for selective determination of exo-1,4,-β-glucanases in a mixture of cellulolytic enzymes *Anal. Biochem.*, 138:481-487.

Dickey, E.E. and Wolfrom, M.L. 1949. A polymer-homologous series of sugar acetates from the acetolysis of cellulose. *J. Am. Chem. Soc.* 71: 825-828.

Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, K.C., Teeri, T.T., and Jones, T.A. 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei. Science* 265: 524-528.

Divne, C., Stahlberg, J., Teeri, T.T., and Jones, T.A. 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei. J. Mol. Biol.* 275: 309-325.

Ehrman, T. 1992. Two stage sulfuric acid hydrolysis for determination of carbohydrates. Chemical analysis and testing standard procedure No. 002; National Renewable Energy Laboratory. Golden, CO.

Ek, R., Alderborn, G., and Nystrom, C. 1994. Particle analysis of microcrystalline cellulose: Differentiation between individual particles and their agglomerates. *Int. J. of Pharm.* 111:43-50.

Enari, T.M. 1983. Microbial cellulases. In *Microbial Enzymes and Biotechnology* (W.M. Fogarty, ed), pp.183-223, Applied Science Publishers, New York.

Enari, T.M. and Niku-Paavola, M.L. 1987. Enzymatic hydrolysis of cellulose: Is the current theory of the mechanisms of hydrolysis valid? *CRC Crit. Revs. Biotechnol.* 5:67-87.

Fan, L.T., Lee, Y.H. and Beardmore, D.H. 1980. Mechanism of the enzymatic hydrolysis of cellulose: effects of major structural features of cellulose on enzymatic hydrolysis. *Biotechnol. Bioeng.* 22:177-199.

Fagerstam, L.G. and Pettersson, L.G. 1979. The cellulolytic complex of *Trichoderma reesei* QM 9414: an immunochemical approach. *FEBS Lett.* 98(2):363-367.

Feather, M.S. and Harris, J.F. 1967. Chromatographic isolation of some cellulose oligosaccharides. U.S. Forest Service Research Note-FPL-0161.

FMC Corporation. 1986. The avicel advantage (L-0786 AAP 5), Philadelphia, Pennsylvania.

Gardner, K.H. and Blackwell, J. 1974. The structure of native cellulose. *Biopolymers*, 13:1975-2001.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55: 303-315.

Grethlein, H.E. 1985. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. *Bio/Technology* 3:155-160.

Hamacher, K., Schmid, G., Sahm, H., and Wandrey, Ch. 1985. Structural heterogeneity of cellooligomers homogeneous according to high-resolution size-exclusion chromatography, *J. Chromatogr.* 319:311-318.

Hsu, T. A., Gong, C. S., and Tsao, G. T. 1980. Kinetic studies of cellodextrins hydrolyses by exocellulase from *Trichoderma reesei. Biotechnol. Bioeng.* 22: 2305-2320.

Hsu, J.C. and Penner, M.H. 1991. Preparation and utilization of cellulose substrates regenerated after treatment with hydrochloric acid. *J. Agri. Food Chem.* 39:1444-1447.

Irwin, D.C., Spezio, M., Walker, L.P., and Wilson, D.B. 1993. Activity studies of eight purified cellulases: specificity, synergism, and binding domain effects. *Biotechnol. Bioengr.*, 42:1002-1013.

Knowles, J.K.C., Lehtovaara, P., Murray, M., and Sinnot, M.L. 1988. Stereochemical course of the action of cellobioside hydrolases I and II of *Trichoderma reesei*. *J. Chem. Soc., Chem. Commun.* pp. 1401-1402.

Knowles, J., Lehtovaara, P., and Teeri, T. 1987. Cellulase families and their genes. *TIBTECH* 5: 255-261.

Koivula, A., Reinikainen, T., Ruohonen, L., Valkeajärvi, A., Claeyssens, M., Teleman, O., Kleywegt, G.J., Szardenings, M., Rouvinen, J., Jones, T.A., and Teeri, T.T. 1996. The active site of *Trichoderma reesei* cellobiohydrolase II: The role of tyrosine-169. *Protein Eng.* 9:691-699.

Konstantinidis, A., Marsden, I., and Sinnott, M.L. 1993. Hydrolyses of α- and β-cellobiosyl fluorides by cellobiohydrolases of *Trichoderma reesei*. *Biochem. J.* 291:883-888.

Kraulis, P.J., Clore, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J.K.C., and Gronenborn, A.M. 1989. Determination of the three dimensional structure of the C-terminal domain of cellobiohydrolases I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry-dynamical simulated annealing. *Biochemistry* 28: 7241-7257.

Lee, J.H., Brown, R.M., Kuga, S., Shoda, S., and Kobayashi, S. 1994. Assembly of synthetic cellulase I. *Proc. Natl. Acad. Sci.* 91:7425-7429.

Levenspiel, O. 1972. *Chemical Reaction Engineering,* Second Edition, pp. 460-477, John Wiley & Sons, Inc., New York.

Lever, M. 1972. A new reaction for colorimetric determination of carbohydrates. *Anal. Biochem.*, 47:273-279.

Linder, M., Mattinen, M.L., Kontteli, M., Lindeberg, G., Stahlberg, J., Drakenberg, T., Reinikainen, T., Pettersson, G., and Annila, A. 1995. Identification of functionally important amino acids in the cellulose-binding domain of Trichoderma reesei cellobiohydrolase I. *Protein Sci.* 4:1056-1064.

McMurry, J. 1992. *Organic chemistry*, Third edition, pp. 931-934, Brooks/Cole Publishing Co., California.

Medve, J., Stahlberg, J., and Tjerneld, F. 1994. Adsorption and synergism of cellobiohydrolase I and II of *Trichoderma reesei* during hydrolysis of microcrystalline cellulose. *Biotechnol. Bioeng.* 44:1064-1073.

Miller, G.L., Dean, J., and Blum, R. 1960. A study of methods for preparing oligosaccharides from cellulose. *Archives of Biochemistry and Biophysics.* 91: 21-26.

Nidetzky, B., Steiner, W., and Claeyssens, M. 1995. Synergistic interaction of cellulases from *Trichoderma reesei* during cellulose degradation, In *Enzymatic Degradation of Insoluble Carbohydrates*, (J.N. Saddler and M.H. Penner, eds.), pp 90-112, American Chemical Society, Washington, DC.

Nidetzky, B., Steiner, W., Hayn, M., and Claeyssens, M. 1994a. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. *Biochem. J.* 298:705-710.

Nidetzky, B., Zachariae, W., Gercken, G., Hayn, M., and Steiner, W. 1994b. Hydrolysis of cellooligosaccharides by *Trichoderma reesei* cellobiohydrolases: experimental data and kinetic modeling. *Enzyme Microb. Technol.* 16: 43-52.

Orgeret, C., Seillier, E., Gautier, C., Defaye, J., and Driguez, H. 1992. 4-Thiocellooligosaccharides. Their synthesis and use as ligands for the separation of cellobiohydrolases of *Trichoderma reesei* by affinity chromatography. *Carbohydr. Res.* 224: 29-40.

Penner, M.H. and Liaw, E.T. 1994. Kinetic consequences of high ratios of substrate to enzyme saccharification systems based on *Trichoderma* cellulase. In *Enzymatic Conversion of Biomass for Fuels Production*, (M. E. Himmel, J. O. Baker and R. P. Overend, eds.) pp. 363-371, ACS Symposium Series 566, American Chemical Society, Washington, DC.

Pereira, A.N., Mobedshahi, M., and Ladisch, M.R. 1988. Preparation of cellodextrins, *Methods Enzymol.* 160:26-38.

Piyachomkwan, K., Gable, K.P., and Penner, M.H. 1997. *p*-Aminophenyl 1-thio-β-D-cellobioside: synthesis and application in affinity chromatography of exo-type cellulases. *Carbohydr. Res.* 303: 255-259.

Piyachomkwan, K. and Penner, M.H. 1988. Aryl thioglycoside-based affinity purification of exo-acting cellulases. *Anal. Biochem.*, 255:223-235.

Poulsen, O.M. and Petersen, L.W. 1992. Degradation of microcrystalline cellulose: synergism between different endoglucanases of *Cellulomomas sp.* ATCC 21399. *Biotechnol. Bioeng.* 39:121-123.

Puri, V.P. 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. *Biotechnol. Bioeng.* 26:1219-1222.

Richmond, P.A. 1991. Occurrence and functions of native cellulose. In Biosynthesis and Biodegradation of Cellulose (C.H. Haigler and P.J. Weimer, eds.), pp. 5-23, Marcel Dekker, Inc., NewYork.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, K.C., and Jones, T.A. 1990. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei. Science* 249:380-386.

Roy, R. and Tropper, F.D. 1991. Carbohydrate protein interactions. Syntheses of agglutination inhibitors of wheat germ agglutinin by phase transfer catalysis, *Can. J. Chem.* 69:817-821.

Roy, R., Tropper, F.D., and Romanowska, A. 1992. New strategy in glycopolymer syntheses. Preparation of antigenic water-soluble poly(acrylamide-co-*p*-acrylamidophenyl β-lactoside), *Bioconjugate Chem.* 3:256-261.

Sangseethong, K. and Penner, M.H. 1999. *p*-Aminophenyl β-D-cellobioside as an affinity ligand for exo-type cellulases. *Carbohydr. Res.* In press.

Sarko, A. 1978. What is the crystalline structure of cellulose? *Tappi* 61(2):59-61.

Schmidt, R.R. and Jung, K. 1997. Oligosaccharide synthesis with trichloroacetimidates. In *Preparative carbohydrate chemistry*, (S. Hanessian, ed), pp. 283-312, Marcel Dekker, Inc., New York.

Schomburg, D. and Salzmann, M., eds. 1991. *Enzyme Handbook*, Springer-Verlag, New York.

Schou, C., Rasmussen, G., Kaltoft, M., Henrissat, B., and Schulein, M. 1993a. Sterochemistry, specificity and kinetics of the hydrolysis of reduced cellodextrins by nine cellulases. *Eur. J. Biochem.* 217:947-953

Schou, C., Rasmussen, G., Schulein, M., Henrissat, B., and Driguez, H. 1993b. 4-Thiocellooligosaccharides: their synthesis and use as inhibitors of cellulases. *J. Carbohydr. Chem.* 12:743-752.

Segal, L., Creely, J.J., Martin, Jr., A.E., and Conrad, C.M. 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Text. Res. J.* 29:786-794.

Singh, A. and Hayashi, K. 1995. Microbial cellulases: protein architecture, molecular properties, and biosynthesis. *Advs Appl. Microb.* 40: 1-44

Sinnot, M.L. 1990. Catalytic mechanisms of enzymic glycosyl transfer. *Chem. Rev.* 90:1171-1202.

Stahlberg, J., Divne, C., Koivula, A., Piens, K., Claeyssens, M., Teeri, T.T., and Jones, T.A. 1996. Activity studies and crystal structures of catalytically deficient mutants of cellobiohydrolase I from *Trichoderma reesei. J. Mol. Biol.*, 264:337-349.

Streamer, M., Eriksson, K., and Pettersson, B. 1975. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. Functional characterization of five endo-1,4-β-glucanases and one exo-1,4-β-glucanase, *Eur. J. Biochem.* 59:607-613.

Steiner, W., Sattler, W., and Esterbauer, H. 1988. Adsorption of *Trichoderma reesei* cellulase on cellulose: experimental data and their analysis by different equations. *Biotechnol. Bioeng.* 32, 853-865.

Stone, J.E. and Scallan, A.M. 1968. A structure model for the cell wall of water-swollen wood pulp fibers based on their accessibility to macromolecules. *Cellulose Chem. Technol.* 2:343-358.

Sulzenbacher, G., Driguez, H., Henrissat, B., Schulein, M., and Davies, G. J. 1996. Structure of the *Fusarium oxysporum* endoglucanase I with nonhydrolyzable substrate analogue: substrate distortion gives rise to the preferred axial orientation for the leaving group. *Biochemistry* 35: 15280-15287.

Takeo, K., Okushio, K., Fukuyama, K, and Kuge, T. 1983. Synthesis of cellobiose, cellotriose, cellotetraose, and lactose, *Carbohyd. Res.* 121:163-173.

Tantasucharit, U. 1995. Porosity, surface area and enzymatic saccharification of microcrystalline cellulose. M.S. thesis. Oregon State University.

Teeri, T.T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *TIBTECH* 15: 160-167.

Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Ruohonen, L., Lehtiö, J., Reinikainen, T., Srisodsuk, M., Kleman-Leyer, K., Kirk, T.K., and Jones, T.A. 1998. Cellulose-cellulase interactions of native and engineered cellobiohydrolases from *Trichoderma reesei.* In *Carbohydrases from Trichoderma reesei and Other Microorganisms. Structures, Biochemistry, Genetics and Applications*, (M. Claeyssens, W. Nerinckx, and K. Piens,

eds), pp. 3-12, The Royal Society of Chemistry Information Services, Cambridge, UK.

Teeri, T.T., Reinikainen, T., Rouhonen, L., Jones, T.A., and Knowles, J.K.C. 1992. Domain function in *Trichoderma reesei* cellobiohydrolases. *J. Biotechnol.* 24:169-176.

Teleman, A., Koivula, A., Reinikainen, T., Valkeajarvi, A., Teeri, T.T., Drakenberg, T., and Teleman, O. 1995. Progress-curve analysis shows that glucose inhibits the cellotriose hydrolysis catalysed by cellobiohydrolase II from *Trichoderma reesei. Eur. J. Biochem.* 231: 250-258.

Thompson, D.N., Chen, H.C., and Grethein, H.E. 1992. Comparison of pretreatment methods on the basis of available surface area. *Bioresour. Technol.* 39:155-163.

Tomme, P., Heriban, V., and Claeyssens, M. 1990. Adsorption of two cellobiohydrolases from *Trichoderma reesei* to Avicel: Evidence for "exo-exo" synergism and possible "loose complex" formation. *Biotechnol. Lett.* 12(7):525-530.

Tomme, P., McCrae, S., Wood, T.M., and Claeyssens, M. 1988. Chromatographic separation of cellulolytic enzymes. *Methods Enzymol.*, 160:187-193.

Tomme, P., van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. 1988. Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* 170: 575-581.

Tomme, P., Warren, R.A.J., and Gilkes, N.R. 1995a. Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol.* 37:1-81.

Tomme, P., Warren, R.A.J., Miller, Jr., R.C., Kilburn, D.G., and Gilkes N.R. 1995b. Cellulose-binding domains: Classification and properties. In *Enzymatic Degradation of Insoluble Carbohydrates*, (J.N. Saddler and M.H. Penner, eds.) pp. 142-163, ACS Symposium Series 618, American Chemical Society, Washington, DC.

Tropper, F.D., Andersson, F.O., Grand-Maître, C., and Roy, R. 1992. Phase transfer catalyzed synthesis of 4-nitrophenyl 1-thio-β-D-glycobiosides, *Carbohyd. Res.* 229:149-154.

van Tilbeurgh, H., Bhikhabhai, R., Pettersson, L.G., and Claeyssens, M. 1984. Separation of endo-and exo-type cellulases using a new affinity chromatography method. *FEBS Lett.* 169: 215-218.

van Tilbeurgh, H., Claeyssens, M., and de Bruyne, C. K. 1982. The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes. FEBS Lett. 149:152-156.

van Tilbeurgh, H., Loontiens, F. G., de Bruyne, C. K., and Claeyssens, M. 1988. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. *Methods Enzymol.* 160: 45-59.

van Tilbeurgh, H., Pettersson, G., Bhikabhai, R., De Boeck, H., and Claeyssens, M. 1985. Studies of the cellulolytic system of *Trichoderma* reesei QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-β-glucan cellobiohydrolase II. *Eur. J. Biochem.*, 148:329-334.

van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R., and Pettersson, G. 1986. Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei. FEBS Lett.* 204: 223-227.

van Zyl, W.H., la Grange, D.C., Crous, J.M., Luttig, M., van Rensburg, P., and Pretorius, I.S. 1998. Cloning of hydrolases from *Trichoderma reesei* and other microorganisms in yeast. In *Carbohydrases from Trichoderma reesei and Other Microorganisms. Structures, Biochemistry, Genetics and Applications*, (M. Claeyssens, W. Nerinckx, and K. Piens, eds.), pp. 280-287, The Royal Society of Chemistry Information Services, Cambridge, UK.

Vrsanska, M. and Biely, P. 1992. The cellobiohydrolase I from *Trichoderma reesei* QM 9414: action on cello-oligosaccharides. *Carbohydr. Res.* 227: 19-27.

Waldi, D. 1965. Spray reagents for thin-layer chromatography. In *Thin Layer Chromatography. A Laboratory Handbook*, (E. Stahl, ed.) pp. 485-502, Academic Press Inc., New York.

Walker, L.P. and Wilson, D.B. 1991. Enzymatic hydrolysis of cellulose : an overview. *Bioresour. Technol.* 36:3-14.

Weimer, P.J. and Weston, W.M. 1985. Relationship between the fine structure of native cellulose and cellulose degradability by the cellulase complexes of *Trichoderma reesei* and *Clostridium thermocellum. Biotechnol. Bioeng.* 27:1540-1547.

Withers, S. 1995. Enzymatic cleavage of glycosides: How does it happen? *Pure & Appl. Chem.* 67(10):1673-1682.

Withers, S.G. and Aebersold, R. 1995. Approaches to labeling an identification of active site residues in glycoidases, *Protein Sci.* 4:361-372.

Wood, T.H. 1988. Preparation of crystalline, amorphous, and dyed cellulase substrates. *Methods Enzymol*, 160:19-25.

Wood, T.M. and Bhat, K.M. 1988. Methods for measuring cellulase activities. *Methods Enzymol.*, 160:87-113.

Wood, T.M. and Garcia-Campayo, V. 1990. Enzymology of cellulose degradation. *Biodegradation.* 1: 147-161.

Wood, T.M., McCrae, S.I., Wilson, C.A., Bhat, K.M., and Gow, L.A. 1988. In *Biochemistry and Genetics of Cellulose Degradation*, (J.P. Aubert, P. Béguin, and J. Millet, eds.), pp. 31, Academic Press, Orlando, FL.

APPENDICES

APPENDIX A

RATIONALE FOR PARTICLE SIZE EFFECT ON RATES OF ENZYMATIC SACCHARIFICATION OF MICROCRYSTALLINE CELLULOSE

Kunruedee Sangseethong, Lisbeth Meunier-Goddik, Usicha Tantasucharit, Ean-Tun Liaw and Michael H. Penner

Department of Food Science and Technology
Oregon State University, Corvallis, OR

Published in *Journal of Food Biochemistry,*
22: 321-330 (1998)

Abstract

Commercially available microcrystalline cellulose (MCC) preparations are widely used as substrates for the characterization of cellulolytic enzymes. The intent of this paper is to demonstrate the importance of considering particle size when using these substrates and to provide a rationale for particle-size related phenomena. Rates of saccharification of MCC substrates having average particle sizes of 20, 50 and 100 μm were compared under typical *Trichoderma reesei* cellulase-based saccharification conditions. The results demonstrate that MCC substrates of different particle size are not equivalent with respect to their kinetics of enzymatic saccharification. The three MCC substrates were shown to be essentially equivalent with respect to physical and chemical parameters commonly correlated with rates of saccharification. Hence, the influence of particle size on rates of MCC saccharification is likely due to the relative importance of mass transfer limitations within the agglomerate particles that make up these substrates.

Introduction

Microcrystalline celluloses (MCC) are widely used as model substrates in cellulose saccharification studies (Wood, 1988). Commercially available MCC preparations are generally prepared from wood pulp. The pulp is cut into small pieces, hydrolyzed in mineral acid at high temperature and pressure, solidified into a cake by filtration, and spray dried (FMC Corp., 1986). The common use of MCC in cellulase research is undoubtedly due to its crystallinity, its perceived purity and its commercial availability. A knowledge of the relative advantages and disadvantages of using MCC as a "standard" cellulose substrate for the characterization of cellulolytic enzymes is important (IUPAC Nomenclature Meeting-Cellulases, Lake Tahoe, CA, June 1994). One aspect of concern with regard to any "standard" substrate is the extent and consequences of variations among different preparations. This is a particularly interesting question with reference to the average particle size of MCC substrates, since preparations differing with respect to particle size are commercially available and widely used (Tomme *et al.*, 1990; Poulsen and Petersen, 1992; Medve *et al.*, 1994; Penner and Liaw, 1994; Bothwell *et al.*, 1997) without reference to this parameter.

In this study we have evaluated the enzymatic susceptibility of three MCC preparations that differ with respect to their average particle size. Enzymatic susceptibility was compared under standard saccharification conditions using a *Trichoderma reesei* cellulase preparation. The results demonstrate that MCC

preparations, apparently differing only with respect to particle size, are not equivalent on the basis of their rates of saccharification. A rationale for this behavior is presented based on the chemical and physical properties of these substrates.

Materials and Methods

Reagents Three microcrystalline cellulose (MCC) preparations differing with respect to their average particle size, 20 μm (Avicel PH 105), 50 μm (Avicel PH 101), and 100 μm (Avicel PH 102), were obtained from FMC Corp. (Philadelphia, PA). Dextrans were obtained from Fluka Chemical Corp. (Ronkonkoma, NY), Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and Polysciences Inc. (Warrington, PA). Glucose and raffinose were obtained from Sigma Chemical Co. (St. Louis, MO), the maltose from Fisher Scientific (Pittsburgh, PA). The glucose oxidase/peroxidase assay system was obtained from Sigma Chemical Co. (St. Louis, MO). Cupriethylenediamine was from Olin (Pisgah Forest, NC).

Neutral sugar composition The neutral monosaccharide composition of the constituent polysaccharides in each MCC preparation was quantified by an HPLC-based assay of the hydrolysate resulting from a two-stage sulfuric acid treatment (Ehrman, 1992). Sugars were separated using a Bio-Rad Aminex HPX-

87P column and quantified based on the signal from a Waters 401 refractive index detector.

Degree of polymerization The viscosity average degree of polymerization (DP) was measured according to ASTM standard D 1795 (ASTM, 1986; Hsu and Penner, 1991) using a size 75 Cannon-Fenske viscometer (Thomas Scientific, Swedesbora, NJ) at 25±0.1°C. Measured efflux times of cellulose in 0.5 M cupriethylenediamine solutions at concentrations of 0.05, 0.1, 0.15, and 0.2% (w/v) were used to calculate intrinsic viscosities. DP values were obtained by multiplying the intrinsic viscosities by 190.

Crystallinity index (CrI) X-ray diffractograms were obtained with an automated Philips X-ray diffractometer using nickel-filtered CuKα radiation. The diffraction intensity was measured between Bragg angles (2θ) of 10° and 30°. The crystallinity indexes were determined using the following empirical relationship (Segal *et al.*, 1959):

$$\text{Crystallinity index (\%)} = (1-I_{am}/I_{002}) \times 100$$

where I_{002} denotes the maximum intensity of the 002 lattice diffractions at 2θ = 22.5°and I_{am} is the intensity of diffraction at 2θ = 18.5°.

Internal enzyme-accessible surface area - solute exclusion approach The pore size distribution and, subsequently, the associated internal surface area of each substrate was determined by the solute exclusion approach. The technique is based on determining the volume of substrate-associated solvent, within the capillary structure of the particulate substrate, accessible to probes of different sizes (Stone and Scallan, 1968). Sugars and dextrans with molecular diameters ranging from 8 to 560 Å were used as probes (Thompson *et al.*, 1992). Assay mixtures containing 0.7 % (w/w) probe and 10% (w/w) MCC in a total volume of 10 ml were allowed to equilibrate for 5 h. Particulate-free solvent was obtained by centrifugation and subsequent filtration (0.22 μm membrane; Millipore Corp., Bedford, MA). The probe concentration of particulate-free solvents was obtained by comparing their optical rotations (Polarimeter, Perkin-Elmer model 141) with that of standard solutions (Tantasucharit, 1995). Internal surface areas were calculated assuming parallel plate pore geometry as originally proposed by Stone and Scallan (1968).

External surface area External surface areas were estimated by assuming the particles can be represented as solid spheres of radius one-half their average particle size. The density of individual particles was taken as 1.35 g/cm^3 based on the positioning of the particles in a sucrose gradient ranging from 1.0 to 1.4 g/cm^3.

Total enzyme-accessible surface area - enzyme adsorption approach Maximum enzyme adsorption capacities were determined under saturating

conditions; saturating conditions were identified from adsorption isotherms. Enzyme was incubated with 1.5 % (w/v) MCC in 50 mM sodium acetate, pH 5.0, at 50°C. Bound enzyme was determined following a 20 min incubation period; 20 min was identified as the time required to achieve maximum adsorption. Non-adsorbed enzyme was determined from A280 measurments of the MCC-free solvent obtained following centrifugation and filtration of reaction mixtures. Adsorbed enzyme was calculated as the difference between total enzyme and non-adsorbed enzyme. Enzyme accessible surface areas for each MCC were calculated from their respective maximum binding capacities with the following assumptions (Steiner *et al.* 1988): maximum enzyme adsorption corresponds to monolayer coverage of the enzyme accessible surface area, an average molecular weight for *T. reesei* cellulases of 48,000 g/mol and an average cellulase diameter of 65.8 Å.

Enzymatic saccharification The enzymatic susceptibility of each MCC substrate was determined using a *Trichoderma reesei* cellulase preparation (Environmental Biotechnology Inc., Santa Rosa, CA). Assays were done in 50 mM sodium acetate, pH 5.0, at 50°C. Substrate concentrations ranged from 0.1 to 2 % (w/v), enzyme loads were 0.008 FPU/mL reaction mixture. Reactions were initiated by the addition of enzyme to a temperature equilibrated suspension of cellulose in buffer. Reactions were allowed to proceed for 1 hr with gentle end-over-end agitation in a conventional incubator. Reactions were terminated by immersion in boiling water for 5 min, followed by filtration through a 0.22 μm filter. The extent of

saccharification was determined by measuring the glucose content of the filtrate with a glucose oxidase/peroxidase assay system.

Results and Discussion

Rates of saccharification of three microcrystalline cellulose (MCC) substrates, which differed with respect to particle size, were determined using a *T. reesei* enzyme preparation under apparent optimum conditions for enzyme catalysis. The resulting substrate-velocity profiles are shown in Figure A. Rates of saccharification per unit weight substrate were clearly different for the three substrates. The MCC preparation having the smallest average particle size (20 μm) was digested at the fastest rate; the MCC preparation having the largest average particle size (100 μm) was digested at the slowest rate. This trend, the smaller the particle size the faster the rate of saccharification, was also observed when enzyme concentrations and/or reaction times were systematically varied. The obvious implication is that particle size has a significant impact on the rate of saccharification of MCC substrates.

The preceding statement is based on the knowledge that the three substrates were not appreciably different with respect to other chemical and structural parameters thought to influence rates of saccharification. The particles which define the "particle size" of MCC substrates are agglomerates of smaller, individual, microcrystalline cellulose "subparticles" (Ek *et al.*, 1994). Thus, MCC

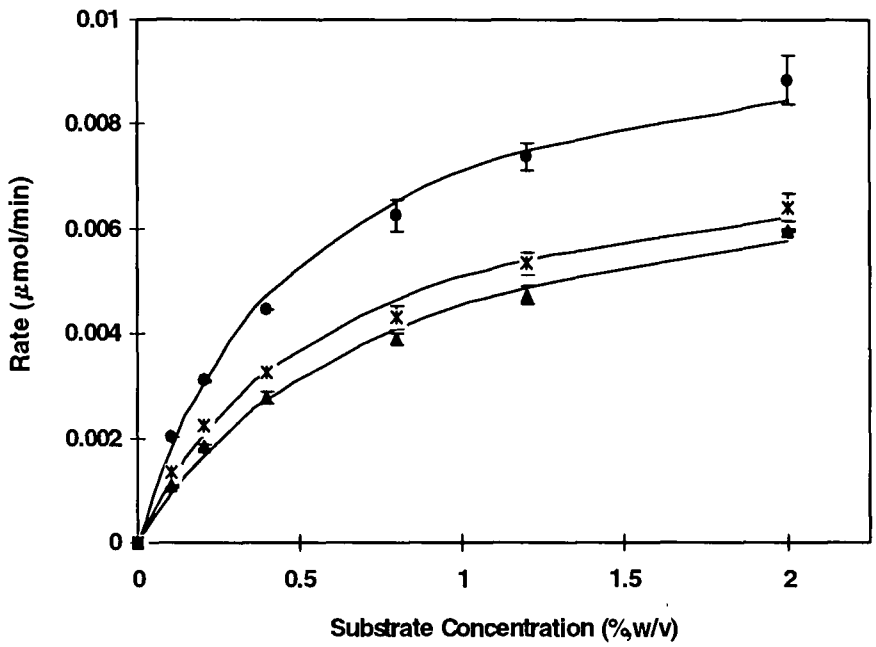


Figure A. Substrate-velocity profiles for enzymatic saccharification of microcrystalline celluloses: Avicel PH101 (50 μm), ×; Avicel PH 102 (100 μm), ▲; Avicel PH 105 (20 μm), •. Experiments were conducted in 50 mM sodium acetate (pH 5.0) at 50 °C with an enzyme concentration of 0.008 FPU/mL for 1 h.

preparations of different particle size differ with respect to the size of the agglomerate particles; they are not necessarily different with respect to the characteristics of the individual microcrystalline cellulose subparticles which make up the agglomerates. The properties of MCC substrates which are expected to be directly dependent on the individual microcrystalline cellulose subparticles include the relative crystallinity of the substrate, the degree of polymerization of the constituent cellulose, and the chemical composition of the substrate. Each of these parameters is considered important relative to the enzymatic susceptibility of cellulosic substrates (Fan *et al.*, 1980; Puri, 1984; Bertran and Dale, 1985). The data of Table A1 shows that the three substrates used in the Figure A experiments are essentially the same in terms of these parameters. Not indicated in Table A1 is that the X-ray diffractograms for each of the substrates was characteristic of cellulose I (Atalla, 1983). Taken together, the data indicates that the differences observed in the rates of saccharification of the three MCC substrates are not due to differences in the fundamental chemical and structural properties so widely recognized to affect rates of saccharification.

The enzyme-accessible surface area of cellulosic substrates has been shown to correlate well with the susceptibility of these substrates to enzymatic saccharification (Grethlein, 1985; Weimer and Weston, 1985; Thompson *et al.*, 1992). The total enzyme-accessible surface area is the sum of the substrate's external surface area plus the "internal" surface area associated with pores of sufficient dimensions to allow enzyme access. External surface area is expected to

Table A1. Physical characteristics of commercial microcrystalline cellulose preparations.

Microcrystalline cellulose	Particle size (μm)	% Glucose equivalents	% Xylose equivalents	Degree of polymerization	Crystallinity index
Avicel PH105	20	93.5	1.9	200	86
Avicel PH101	50	94.1	2.2	219	89
Avicel PH102	100	94.6	2.0	218	88

be a function of particle size, so external surface area per unit mass is expected to be different for each of the MCC preparations. The external surface area values given in Table A2 are based on an elementary model which assumes the particles approximate solid spheres, the different MCC preparations differing only with respect to their average radius. This simple model will underestimate the actual external surface area of analogous irregularly shaped particles and may overestimate the actual external surface area of highly porous particles. Estimates of the internal enzyme-accessible surface area were based on solute exclusion experiments (Table A2); with enzyme accessible pores being defined as those with a nominal diameter equal to or larger than 51 Å (Cowling and Kirk, 1976). Comparison of the external and internal enzyme-accessible surface areas of Table A2 suggests that the vast majority of the enzyme accessible surface is within the pore structure of these substrates. Hence, the predicted differences in the external surface area of the three MCC preparations is likely to be negligible with respect to the total surface area accessible to cellulolytic enzymes.

Further support for the similarity in the enzyme accessible surface area of the three substrates comes from cellulase adsorption experiments. The enzyme adsorption capacity of the three MCC preparations, in mg protein adsorbed per unit weight substrate, differed by less than 5% (Table A2). An enzyme-accessible surface area can be estimated from the enzyme adsorption capacity by assuming an enzyme monolayer and a value for the area covered per mole enzyme (see Methods).

Table A2. Estimated specific surface area of microcrystalline cellulose preparations.

Microcrystalline cellulose	Particle size (μm)	External surface area[a] (m^2/g)	Accessible internal surface area[b] (m^2/g)	Enzyme adsorption[c] (mg/g)	Accessible surface area[d] (m^2/g)
Avicel PH105	20	0.222 (1.3 %)	17 (98.7 %)	55	23.5
Avicel PH101	50	0.089 (0.6 %)	15 (99.4 %)	56	23.9
Avicel PH102	100	0.044 (0.3 %)	17 (99.7 %)	54	23.0

[a] Based on the assumption of solid spherical particle.
[b] Based on the solute exclusion technique; the values represent the internal surface area accessible to probes of size equal to or larger than 51 Å.
[c] The maximum amount of enzyme adsorbed on the substrate.
[d] Enzyme accessible surface area calculated from the maximum enzyme adsorption.

The apparent enzyme-accessible surface area of the three MCC substrates were similar, averaging 23.5 m^2 per gram substrate. Enzyme accessible surface areas calculated in this way are somewhat higher than those estimated from the solute exclusion experiments (Table A2). However, the most relevant point, with respect to the data of Figure A, is that both approaches suggest that any differences in the enzyme-accessible surface area of the MCC substrates used in this study are relatively small. Hence, differences in the rates of saccharification of these substrates are not likely to be a simple function of enzyme-accessible substrate.

A structural feature which is expected to differ for the three MCC substrates used in this study, and one which can impact saccharification kinetics, is the relative depth of the enzyme-accessible pores within the agglomerate particles. Pore-depth is expected to increase as agglomerate particle size increases, and increases in pore-depth will impact rates of saccharification if mass transfer in and/or out of these pores becomes rate limiting. This indeed appears to be the case with the MCC substrates used in this study. A classical approach for demonstrating mass transfer limitations in heterogeneous reaction systems with porous particles is to show the influence of pore depth on reaction rates (Levenspiel, 1972), assuming all other reaction parameters are held constant. To the extent possible, this approach has been used in the current study. The analytical data clearly indicates that the three MCC substrates used in this work are essentially the same with respect to chemical and structural parameters previously shown to correlate with rates of cellulose saccharification (Walker and Wilson, 1991; Coughlan, 1992; and references therein).

This implies that the overriding difference in the three MCC substrates, with respect to enzymatic saccharification, is in the size of their component particles. In light of this, the behavior demonstrated in Figure A is consistent with the rationale that observed differences in the rates of saccharification of the three MCC substrates are a result of mass transfer phenomena within the agglomerate particles. The altered kinetics may be a result of either product and/or enzyme mass transfer.

A knowledge of the kinetic consequences of mass transfer limitations within the agglomerate particles of MCC substrates is important for several reasons. The most obvious is that MCC preparations of different particle size are not equivalent substrates with respect to the fundamental rate constants that dictate the observed rates of saccharification. The rate constants associated with mass transfer phenomena appear to be more "rate limiting" in saccharification systems employing the larger particle size substrates. This obviously limits the comparison of kinetic data obtained with MCC substrates of different particle size. It also suggests that saccharification systems based on MCC substrates of different particle size may respond differently to equivalent changes in the properties of a cellulolytic enzyme. For example, MCC substrates which are minimally influenced by mass transfer limitations are potentially more sensitive substrates for assays aimed at detecting differences in saccharification rates associated with enzymes mutagenized for purposes of modifying a particular rate constant. In an applied sense, the data of this study suggests that particle size is an important parameter that should be considered when correlating the structural properties of a cellulosic substrate with

that substrate's susceptibility to enzymatic saccharification. This latter point is important in that particle size is often not addressed when discussing biomass-to-glucose saccharification systems.

References

AMERICAN SOCIETY FOR TESTING AND MATERIALS. 1986. Standard test method for intrinsic viscosity of cellulose. *Annual book of ASTM standards*. 15.04, 360-366. American Society for Testing and Materials, Pennsylvania.

ATALLA, R. H. 1983. The structural of cellulose: recent developments. In *Wood and Agricultural Residues*, (J. Soltes, ed.) pp.59-77, Academic Press, New York.

BERTRAN, M. S. AND DALE, B. E. 1985. Enzymatic hydrolysis and recrystallization behavior of initially amorphous cellulose. Biotechnol. Bioeng. 27, 177-181.

BOTHWELL, M. K., DAUGHHETEE, S. D., CHAUA, G. Y., WILSON, D. B. AND WALKER, L. P. 1997. Binding capacities for *Thermomonospora fusca* E3, E4 and E5, the E3 binding domain, and *Trichoderma reesei* CBHI on Avicel and bacterial microcrystalline cellulose. Bioresour. Technol. 60, 169-178.

COUGHLAN, M. P. 1992. Enzymic hydrolysis of cellulose: an overview. Bioresour. Technol. 39, 107-115.

COWLING, E. G. AND KIRK, T. K. 1976. Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes. Biotechnol. Bioeng. Symp. No. 6, 95-124.

EK, R., ALDERBORN, G. AND NYSTROM, C. 1994. Particle analysis of microcrystalline cellulose: Differentiation between individual particles and their agglomerates. Int. J. of Pharm. 111, 43-50.

EHRMAN, T. 1992. Two stage sulfuric acid hydrolysis for determination of carbohydrates. Chemical analysis and testing standard procedure No. 002; National Renewable Energy Laboratory. Golden, CO.

FAN, L. T., LEE, Y. H. AND BEARDMORE, D. H. 1980. Mechanism of the enzymatic hydrolysis of cellulose: effects of major structural features of cellulose on enzymatic hydrolysis. Biotechnol. Bioeng. 22, 177-199.

FMC CORPORATION. 1986. The avicel advantage (L-0786 AAP 5), Philadelphia, Pennsylvania.

GRETHLEIN, H. E. 1985. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. Bio/Technology 3, 155-160.

HSU, J. C. AND PENNER, M. H. 1991. Preparation and utilization of cellulose substrates regenerated after treatment with hydrochloric acid. J. Agri. Food Chem. 39, 1444-1447.

LEVENSPIEL, O. 1972. *Chemical Reaction Engineering,* Second Edition, pp. 460-477, John Wiley & Sons, Inc., New York.

MEDVE, J., STAHLBERG, J. AND TJERNELD, F. 1994. Adsorption and synergism of cellobiohydrolase I and II of *Trichoderma reesei* during hydrolysis of microcrystalline cellulose. Biotechnol. Bioeng. 44, 1064-1073.

PENNER M. H. AND LIAW, E. T. 1994. Kinetic consequences of high ratios of substrate to enzyme saccharification systems based on *Trichoderma* cellulase. In *Enzymatic Conversion of Biomass for Fuels Production,* (M. E. Himmel, J. O. Baker and R. P. Overend, eds.) pp. 363-371, ACS Symposium Series 566, American Chemical Society, Washington, DC.

POULSEN, O. M. AND PETERSEN, L. W. 1992. Degradationof microcrystalline cellulose: synergism between different endoglucanases of *Cellulomomas sp.* ATCC 21399. Biotechnol. Bioeng. 39, 121-123.

PURI, V. P. 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. Biotechnol. Bioeng. 26, 1219-1222.

SEGAL, L., CREELY, J. J., MARTIN, JR., A. E. AND CONRAD, C. M. 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Text. Res. J. 29, 786-794.

STEINER, W., SATTLER, W. AND ESTERBAUER, H. 1988. Adsorption of *Trichoderma reesei* cellulase on cellulose: experimental data and their analysis by different equations. Biotechnol. Bioeng. 32, 853-865.

STONE, J. E. AND SCALLAN, A. M. 1968. A structure model for the cell wall of water-swollen wood pulp fibers based on their accessibility to macromolecules. Cellulose Chem. Technol. 2, 343-358.

TANTASUCHARIT, U. 1995. Porosity, surface area and enzymatic saccharification of microcrystalline cellulose. M.S. thesis. Oregon State University.

THOMPSON, D. N., CHEN, H. C. AND GRETHEIN, H. E. 1992. Comparison of pretreatment methods on the basis of available surface area. Bioresour. Technol. 39, 155-163.

TOMME, P., HERIBAN, V. AND CLAEYSSENS, M. 1990. Adsorption of two cellobiohydrolases from *Trichoderma reesei* to Avecel: evidence for "exo-exo" synergism and possible "loose complex" formation. Biotechnol. Lett. 12(7), 525-530.

WALKER, L. P. AND WILSON, D. B. 1991. Enzymatic hydrolysis of cellulose : an overview. Bioresour. Technol. 36, 3-14.

WEIMER, P. J. AND WESTON, W. M. 1985. Relationship between the fine structure of native cellulose and cellulose degradability by the cellulase complexes of *Trichoderma reesi* and *Clostridium thermocellum.* Biotechnol. Bioeng. 27, 1540-1547.

WOOD, T. H. 1988. Preparation of crystalline, amorphous, and dyed cellulase substrates. *Methods Enzymol.* 160:19-25.

APPENDIX B

NMR Spectra for Cellooligosaccharide Derivatives

The structures of the synthesized cellooligosaccharide derivatives, their purity, and the anomeric configuration of glycosidic bonds were confirmed by NMR data (Figure B1-B10). NMR-spectra were recorded at 400 MHz with a Bruker AM 400 spectrometer.

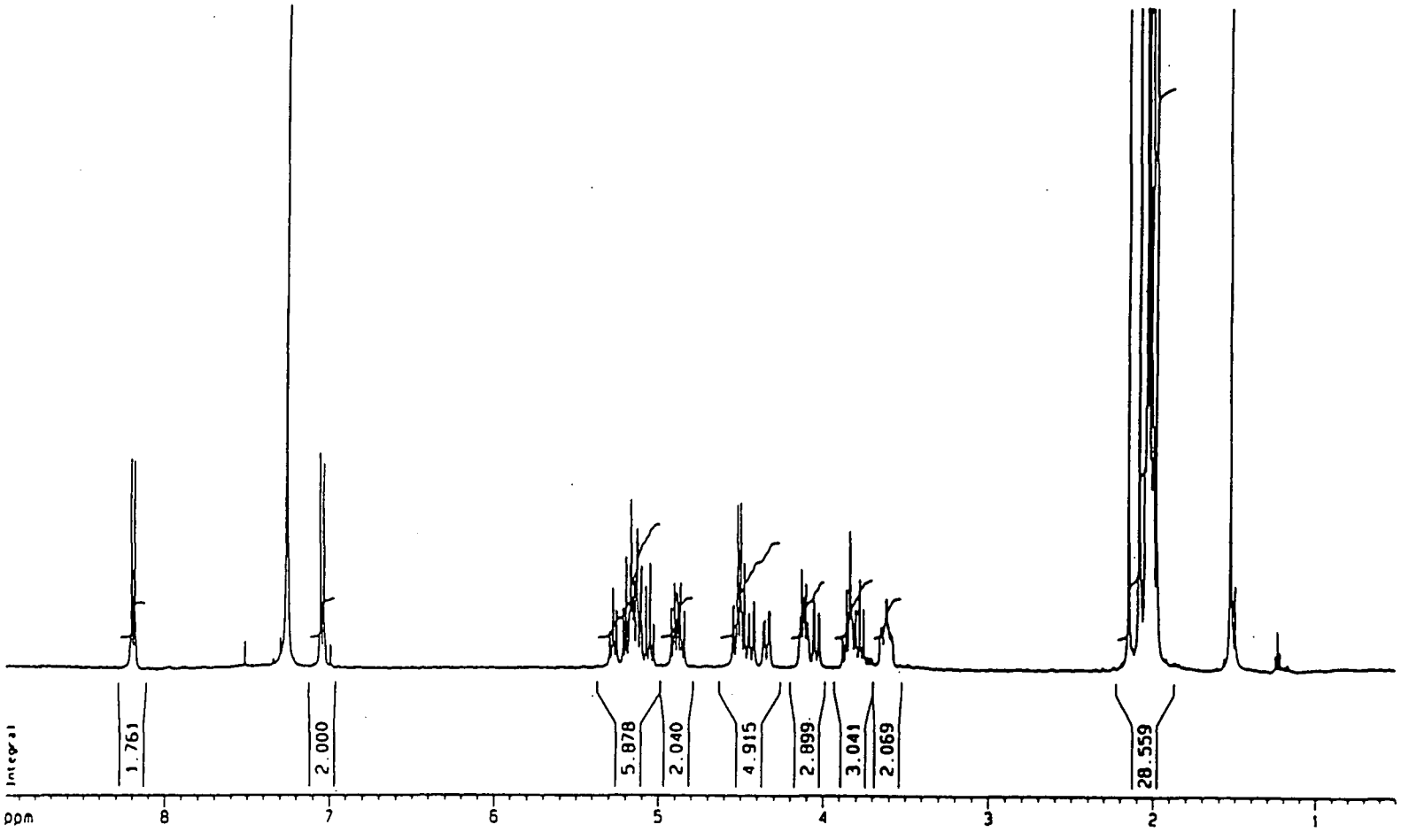


Figure B1. ^{1}H-NMR spectrum (400 MHz) of *p*-nitrophenyl deca-*O*-acetyl-β-cellotrioside (compound **8**) in $CDCl_3$

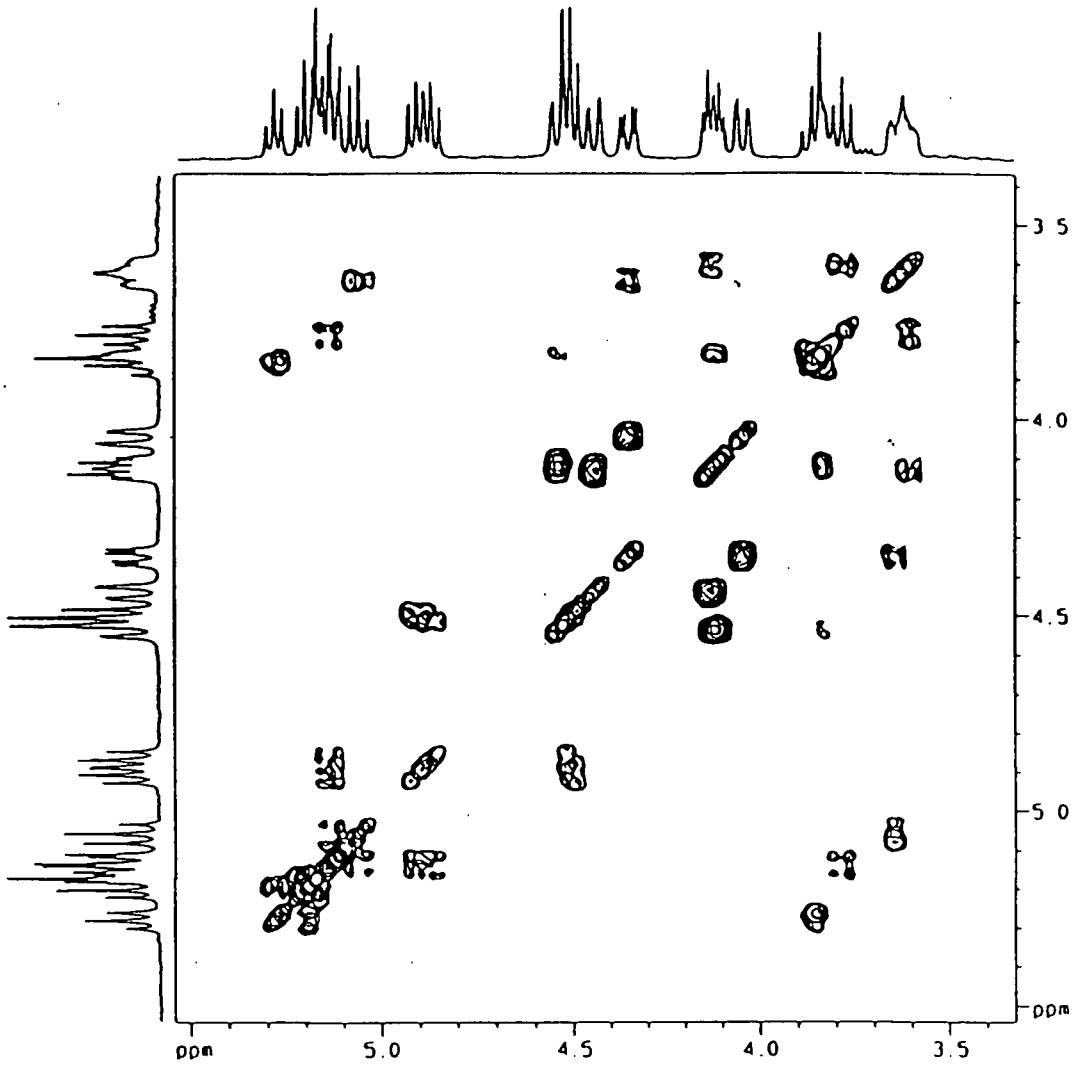


Figure B2. COSY spectrum (400 MHz) of *p*-nitrophenyl deca-*O*-acetyl-β-cellotrioside (compound **8**) in $CDCl_3$

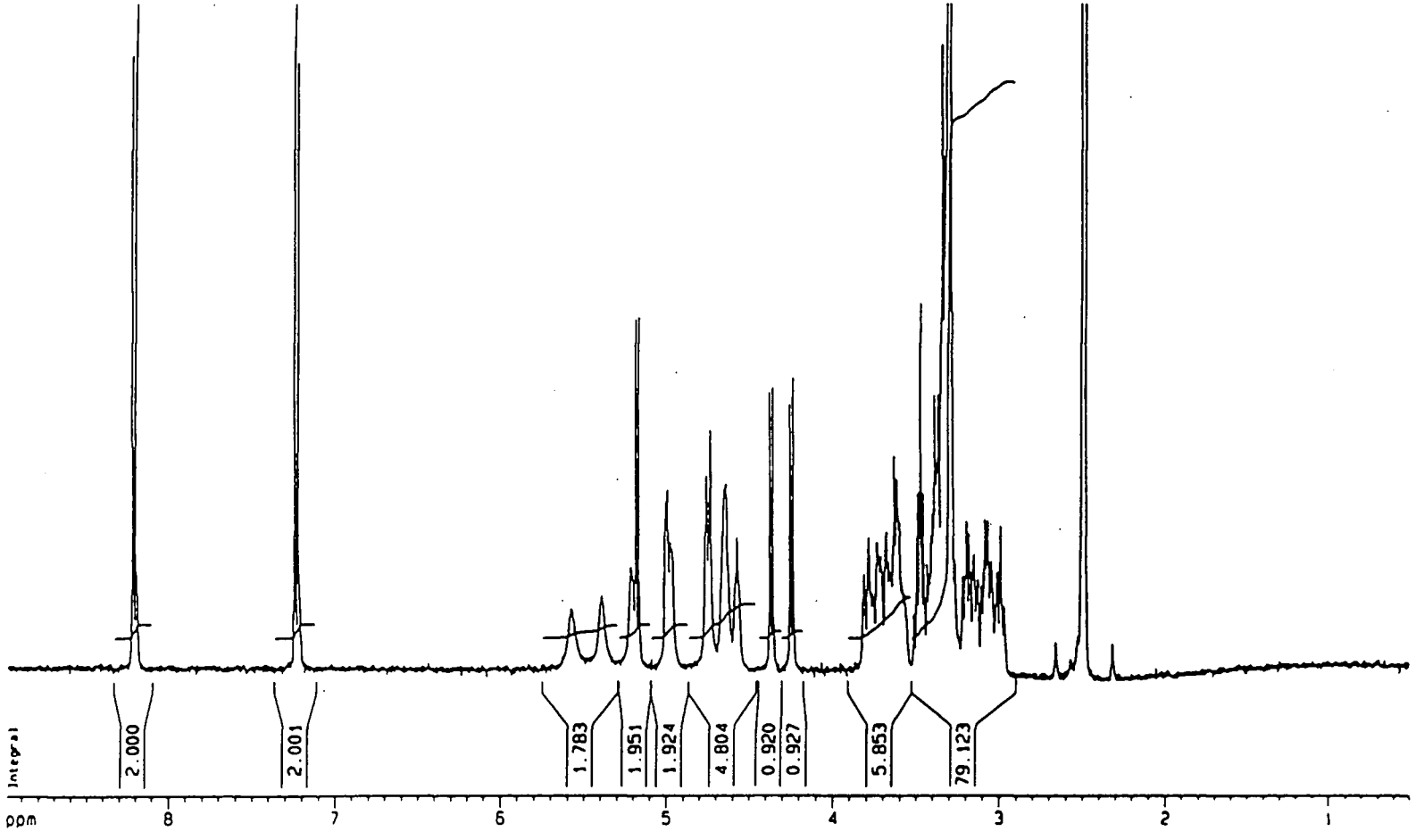


Figure B3. ^{1}H-NMR spectrum (400 MHz) of *p*-nitrophenyl-β-D-cellotrioside (compound **9**) in Me_2SO-d_6

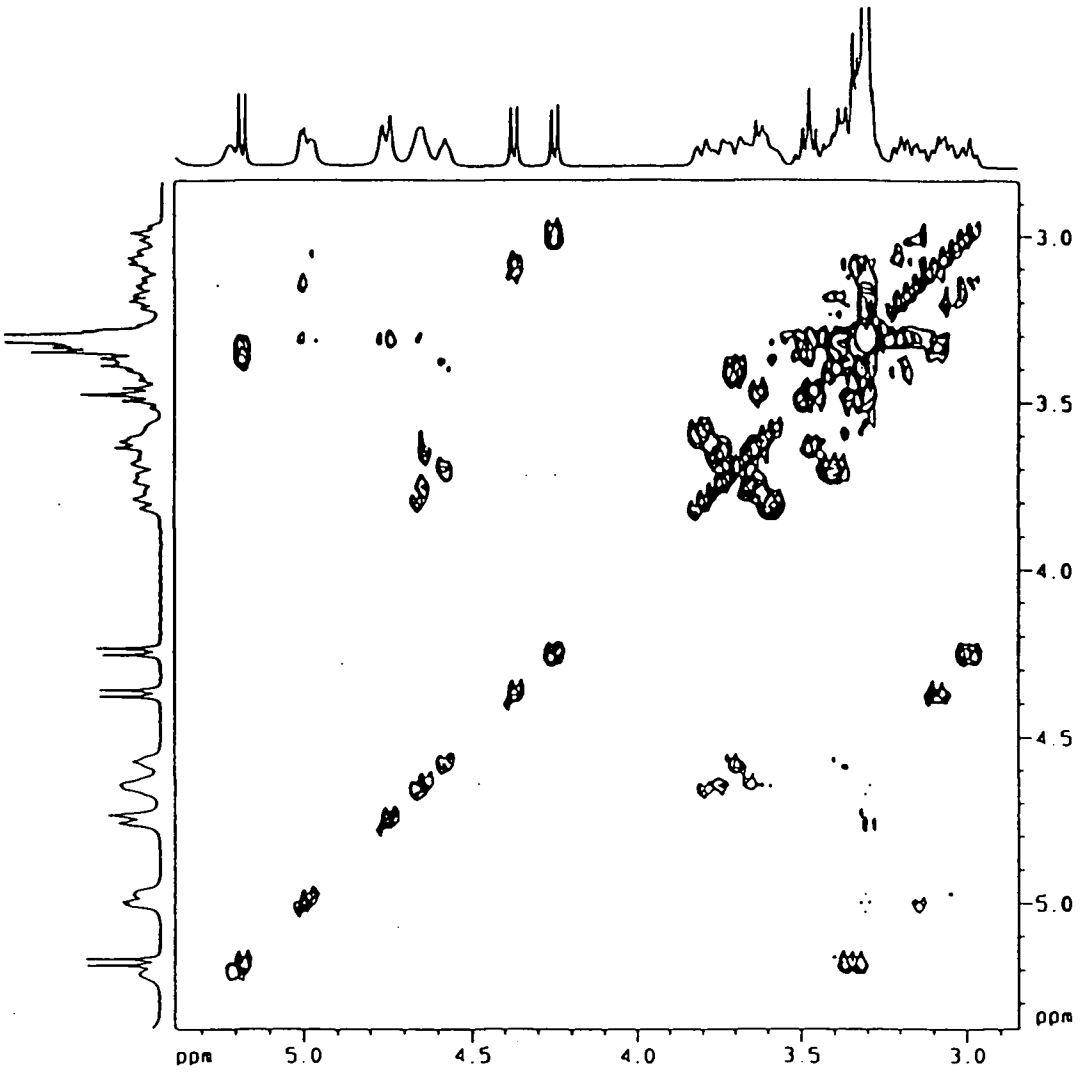


Figure B4. COSY spectrum (400 MHz) of *p*-nitrophenyl-β-D-cellotrioside (compound **9**) in Me_2SO-d_6

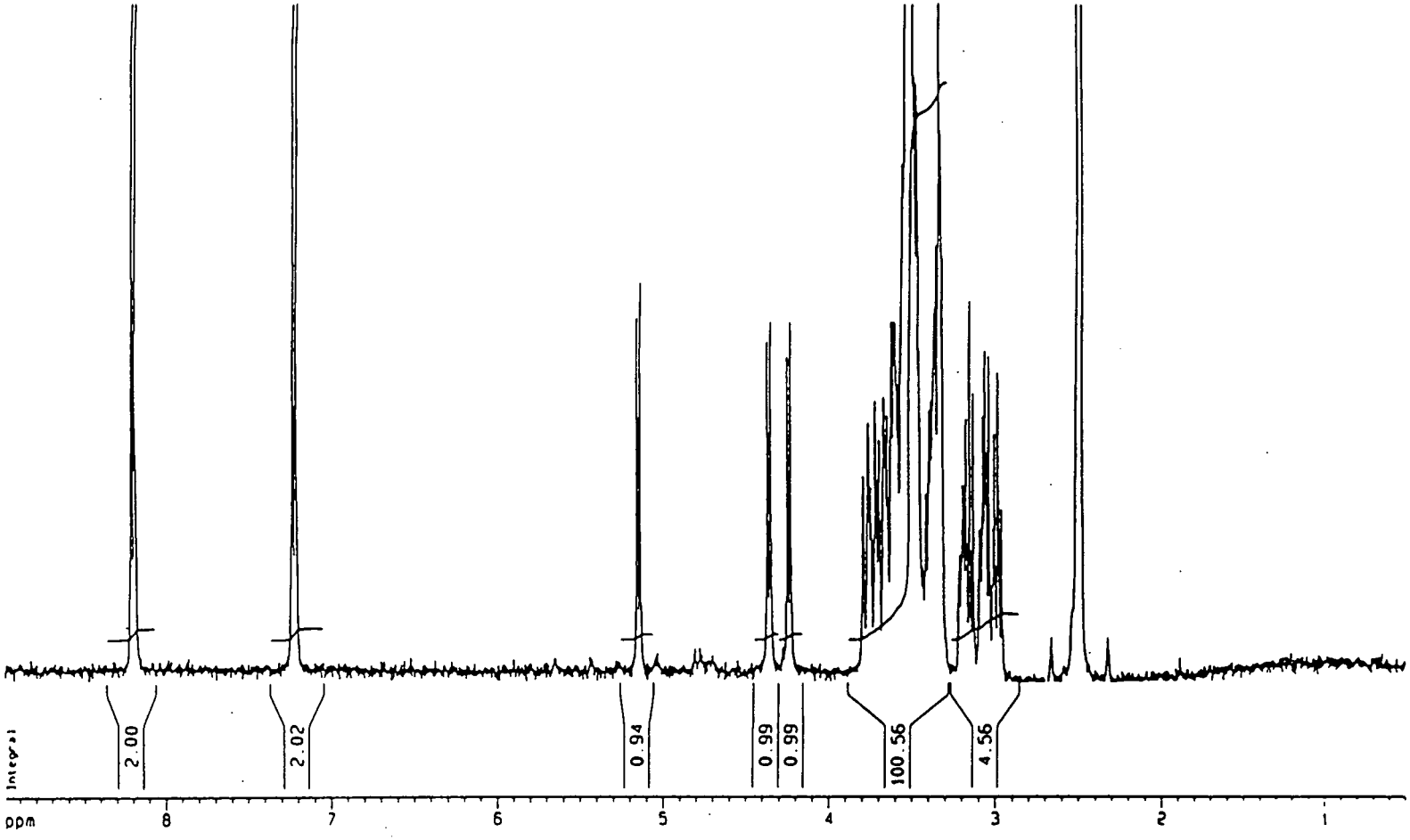


Figure B5. ^{1}H-NMR spectrum (400 MHz) of *p*-nitrophenyl-β-D-cellotrioside (compound **9**) in D_2O

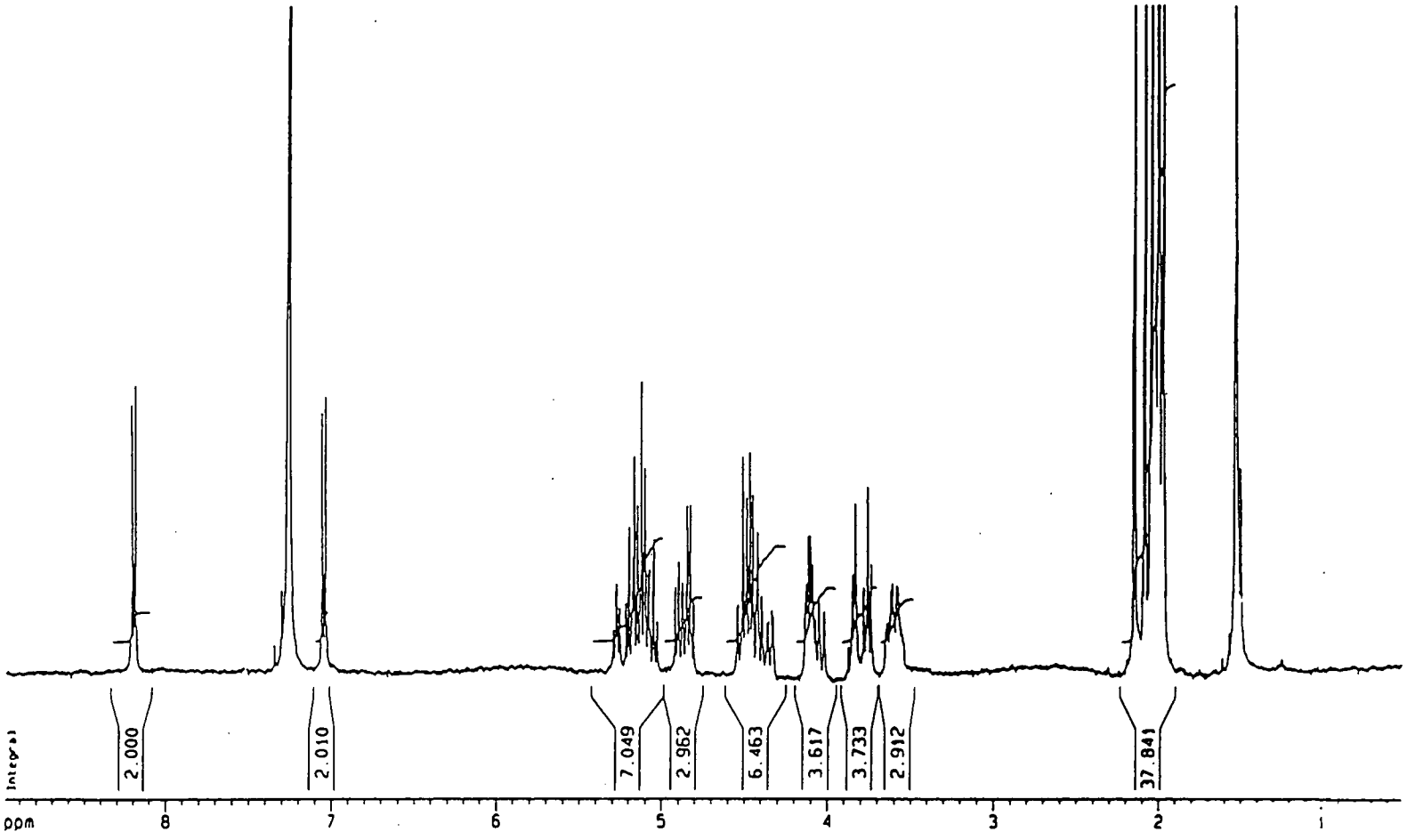


Figure B6. ^{1}H-NMR spectrum (400 MHz) of *p*-nitrophenyl trideca-*O*-acetyl-β-cellotetraoside (compound **13**) in $CDCl_3$

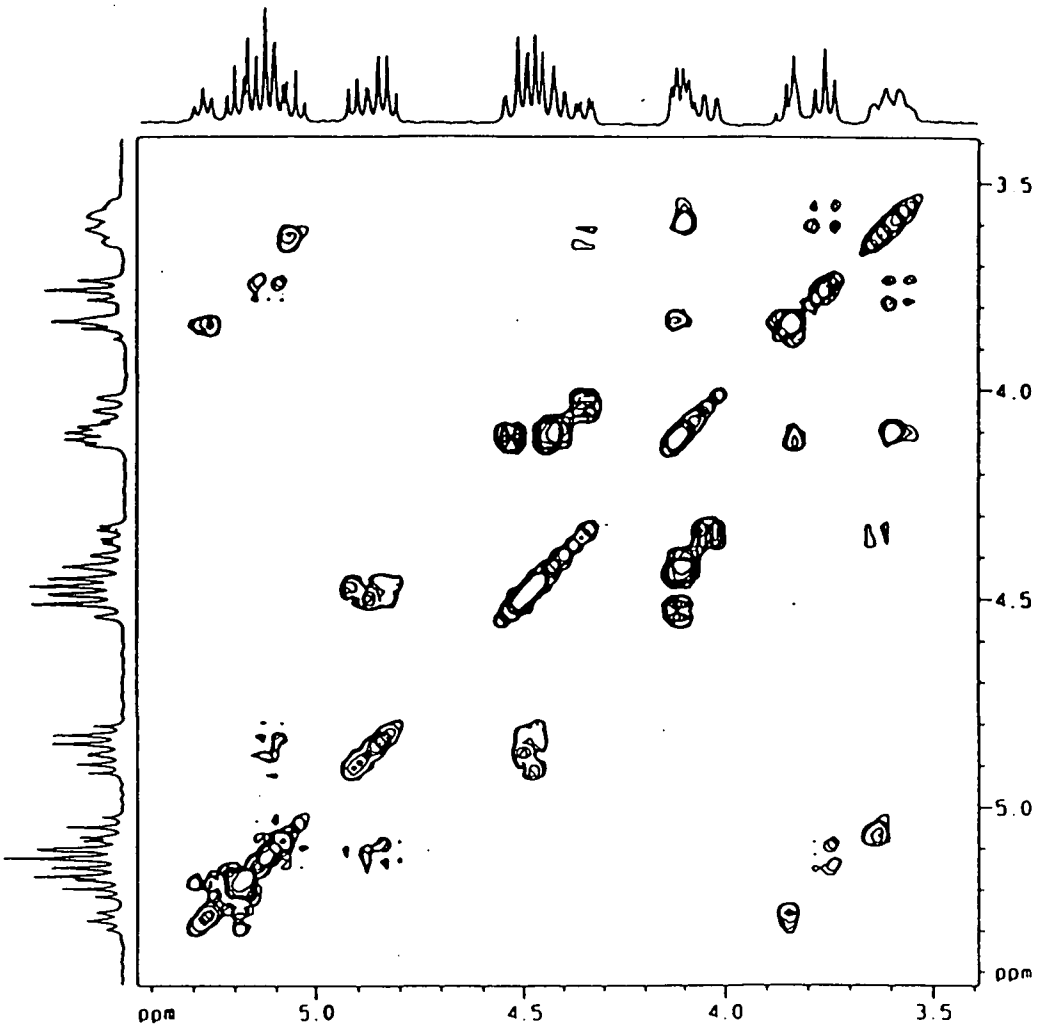


Figure B7. COSY spectrum (400 MHz) of *p*-nitrophenyl trideca-*O*-acetyl-β-cellotetraoside (compound **13**) in $CDCl_3$

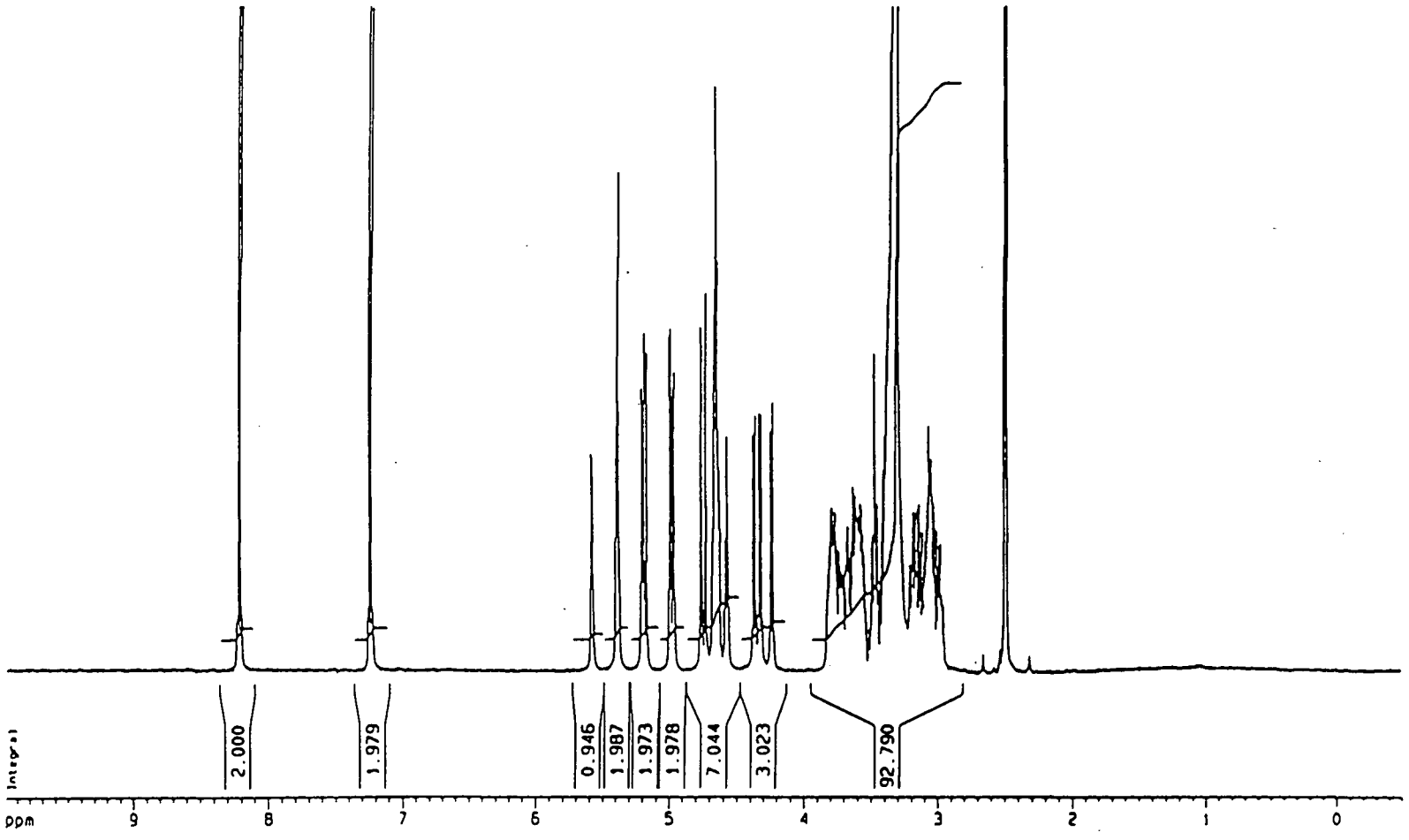


Figure B8. ^{1}H-NMR spectrum (400 MHz) of *p*-nitrophenyl-β-D-cellotetraoside (compound **14**) in Me_2SO-d_6

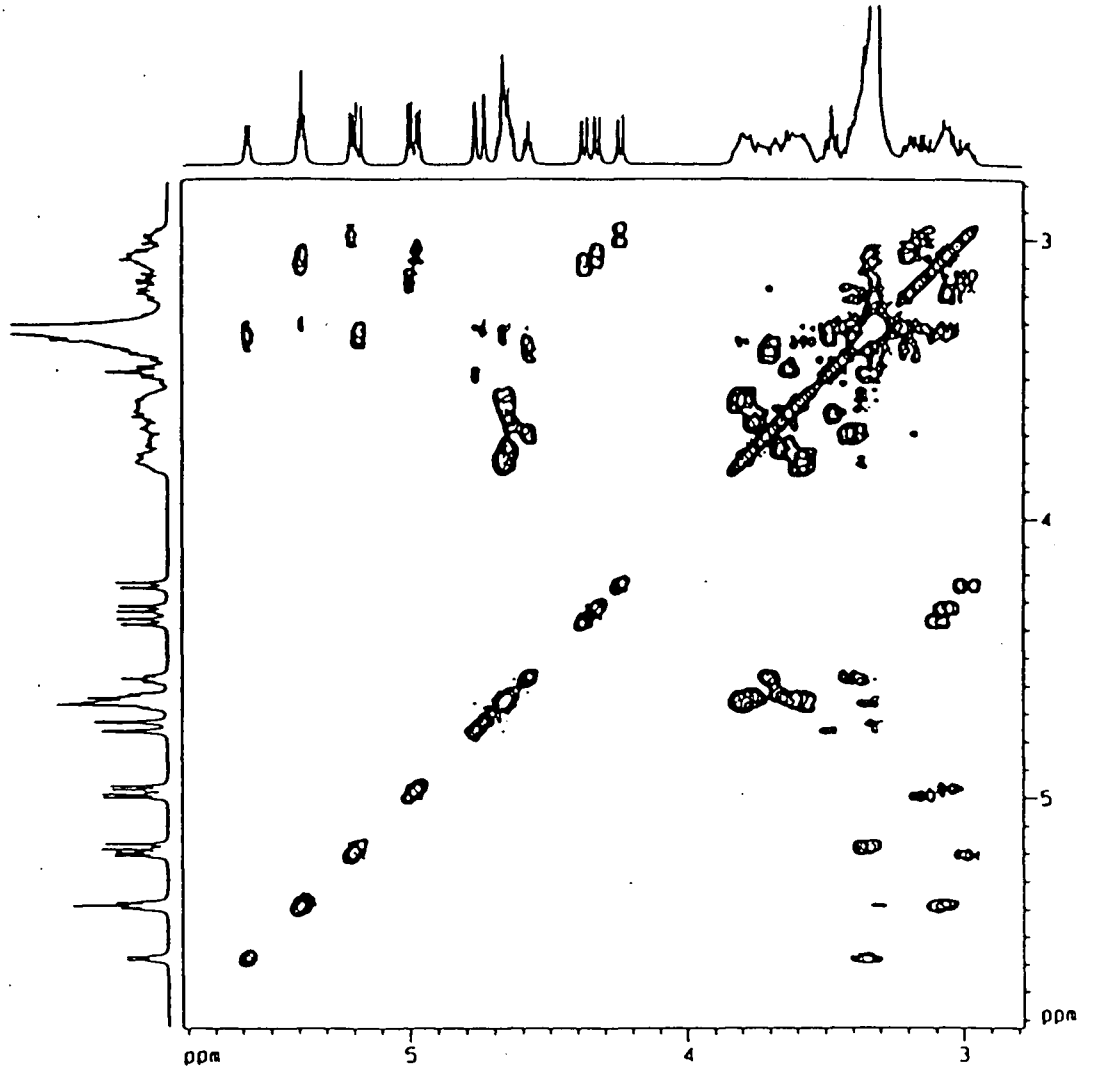


Figure B9. COSY spectrum (400 MHz) of *p*-nitrophenyl-β-D-cellotetraoside (compound **14**) in Me_2SO-d_6

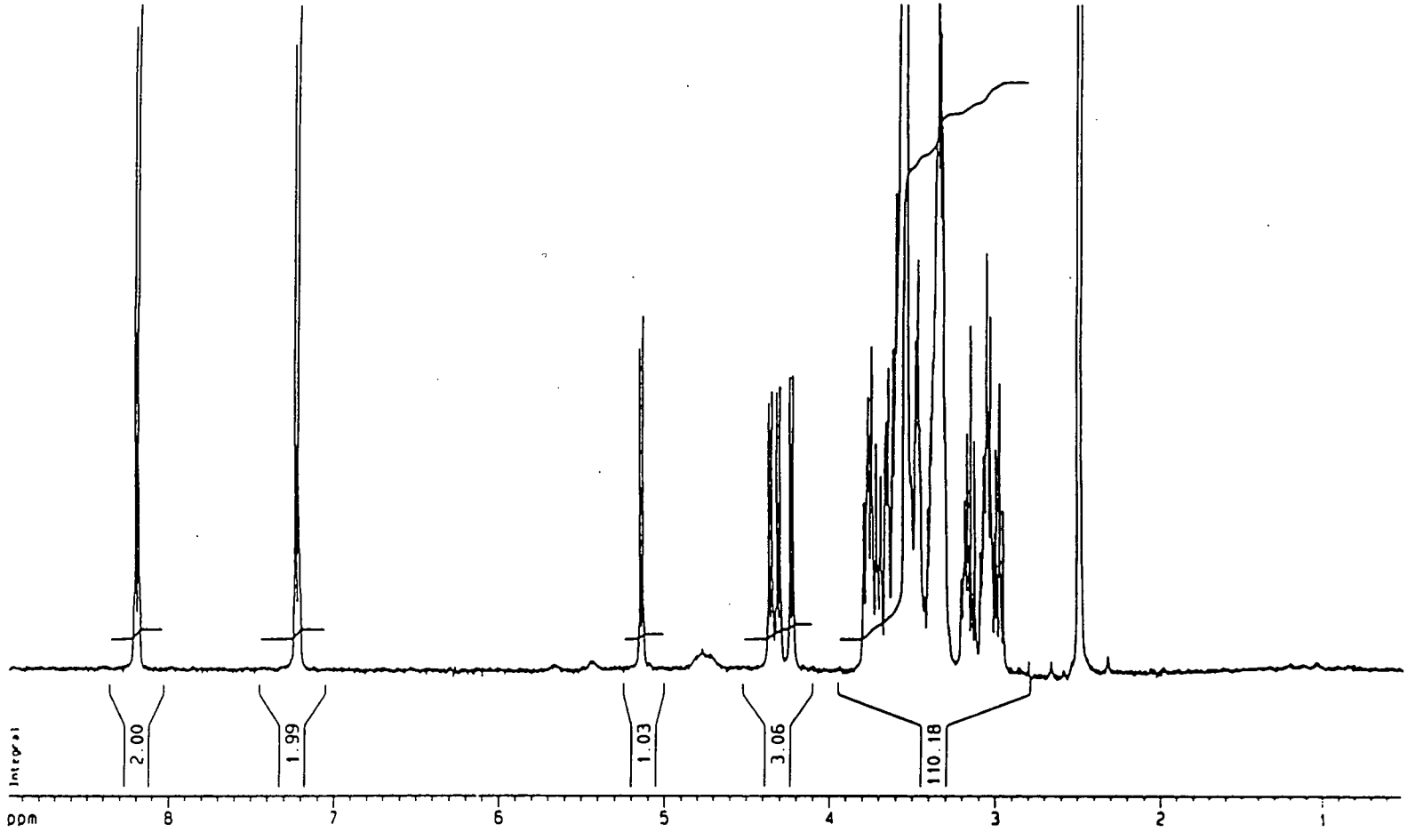


Figure B10. ^{1}H-NMR spectrum (400 MHz) of *p*-nitrophenyl-β-D-cellotetraoside (compound **14**) in D_2O

APPENDIX C

Purification of *Trichoderma reesei* CBH I and CBH II Cellulases

CBH I and CBH II were purified from commercial crude cellulase produced by filamentous fungus *Trichoderma reesei*. For this purpose, three types of column chromatography were used as described in section 4.3.2. Experimental condition for the chromatographic methods are tabulated in Table C1. The summarized scheme for the purification of CBHs is depicted in Figure C. Besides, the specific activity of purified enzymes is listed in Table C2.

Table C1. Chromatograhpic conditions for cellobiohydrolase purification

Parameter	Chromatographic technique		
	Anion-exchange	Affinity column	HIC
Packing material	DEAE-Sepharose	APTC derivatized agarose	Phenyl Sepharose FF
Column dimensions	2.5 × 15 cm	1.5 × 18.5 cm	2.5 × 12 cm
Starting buffer	50 mM NaAc, pH 5	CBH I: 0.1 M NaAc, pH 5 + 1 mM gluconolactone CBH II: 0.1 M NaAc, pH 5 + 0.2 M glucose + 1 mM gluconolactone	CBH I: 25 mM NaAc, pH 5 + 0.85 M $(NH_4)_2SO_4$ CBH II: 25 mM NaAc, pH 5 + 0.35 M $(NH_4)_2SO_4$
Eluting condition	Gradient of NaCl (0-0.5 M NaCl in 50 mM NaAc, pH 5)	0.01 M cellobiose in the starting buffer of CBH I	Gradient of $(NH_4)_2SO_4$ CBH I: 0.85-0.35 M CBH II: 0.35-0.01 M
Flow rate	0.7 mL/min	0.5 mL/min	0.75 mL/min
Temperature	4°C	4°C	4°C
Fraction volume	7 mL	5 mL	7.5 mL
Detection	280 nm	280 nm	280 nm

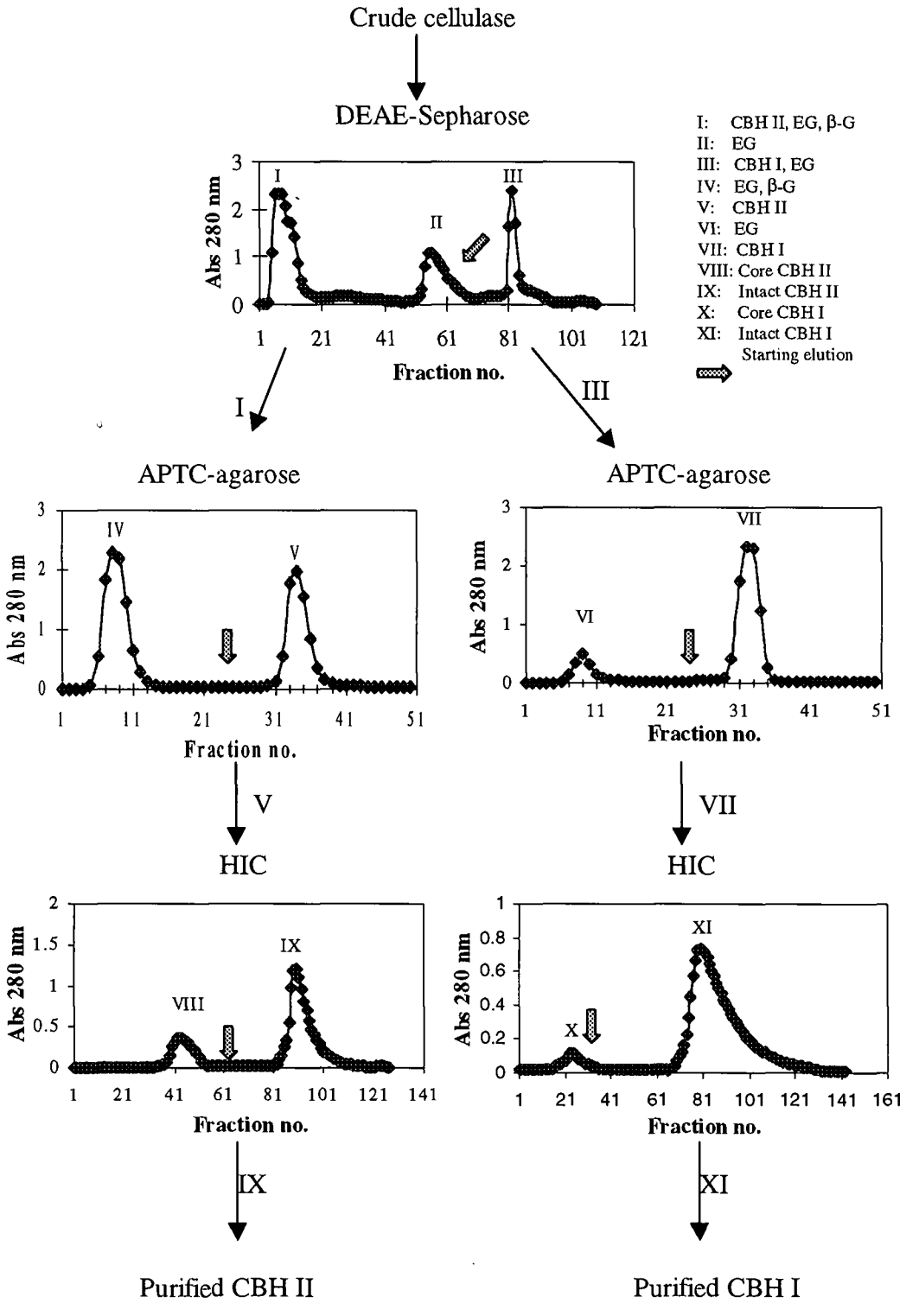


Figure C. A scheme for purification of CBHs from *T. reesei* crude cellulase

Table C2. Specific activity of CBHs fraction after each purification step

Enzyme	Specific activity		
	β-Glucosidase (μmol/min/mg)	HECase (μmol/min/mg)	Avicelase (μmol/min/mg)
Crude	0.122	0.319	0.038
CBH I			
-DEAE	0.001	0.108	0.014
-APTC	0	0.01	0.012
-HIC	0	0	0.01
CBH II			
-DEAE	0.353	0.405	0.02
-APTC	0.001	0.034	0.012
-HIC	0	0.013	0.007