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Strategy For Cellulase Immobilization And Its Partial Purification And Characterization

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by

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Abstract

Strategy for Cellulase Immobilization and its Partial Purification and Characterization

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Conversion of cellulose to glucose units by cellulases, called hydrolysis, is a very complex step in ethanol production. It requires the mixing of aqueous suspensions of cellulose/cellulases so that cellulases (majority composed of the active site domain and the binding site domain) can attach to cellulose chains, cut or hydrolyze β(1-4)glycosidic bonds between glucose units, de-attach and move to another location. Mixing extent (insufficient or excessive agitation) might influence the attachment of cellulases and possibly lead to lower glucose yields. A long-term goal of this research is to determine the strength of mixing required to be applied during the cellulose-cellulase mixing cycle. For that purpose, one of the objectives was to purify CBH I exocellulase from the commercial cellulase mixture. A partial purification of the CBH I that was performed on a much smaller scale with uncontrolled flow rate was successful. Another objective was to propose a scheme that would covalently immobilize CBH I exocellulase via its active site domain (ASD) on an atomic force microscopy-compatible support, a silicon support. A theoretically-developed hypothetical scheme was constructed (with the provided detailed procedure). The approach of immobilizing the inhibitor specific to the ASD of CBH I enzyme led to the possibility that no purification of CBH I could be required. Skipping CBH I purification step would save time and hassle associated with purification step. Once the ASD of CBH I is immobilized on a silicon support, the AFM force profile between the free-floating CBD and substrate cellulose could be established.

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First and foremost, I would like to thank my supervisor, Dr. Ginette Turcotte, for providing priceless training opportunities in research, guidance, expertise and encouragement; I am forever grateful.

I would also like to thank co-supervisor, Dr. Darrick Heyd, for guidance, Dr. John Marshall for providing necessary chemicals and his lab for this research, Dr. Ron Pushchak for the support and motivation, Dr. Gideon Wolfaardt for guidance, and Dr. Russell Viire for the suggestions. I am also truly grateful to all the faculty members in the program of Environmental Applied Science and Management.

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Chapter 1 – Introduction

Much of the world, including Canada and United States, is facing complicated environmental and economic problems associated with energy use. Gasoline is one type energy provider that has powered cars for the last century. Burning of gasoline by our car engines leads to the release of greenhouse gases, volatile organic compounds, and air toxins. All these pollutants cause air pollution, and emission of greenhouse gases results in global warming, which is a problem today. Spills and underground storage tank leaks of gasoline can cause water pollution. Therefore, it is believed that ethanol would be a step away from gasoline dependence and a step towards cleaner air and water.

Different legislation in Canada and United States such as Canada's Alternative Fuels Act (1995), Canada's Ethanol Fuel Act (2002), the Canada-U.S. Air Quality Agreement (1996), the U.S. Clean Air Act Amendments (1990), the U.S. Alternative Motor Fuels Act (1988), the U.S. Energy Policy Act (2003), and other legislation have put pressure on Canada and the U.S. to use alternative fuels. In 2002, Canada formally approved the Kyoto Protocol treaty that came into force in 2005, requiring Canada to reduce greenhouse gas emissions between 2008 and 2012 to 6% below the level of 1990.

Canada and the U.S. rely on energy derived from petroleum, most of which is imported. The goal of independence from outside sources of energy has resulted in the development of new sources of energy that are cost-effective and environmentally sound. One renewable liquid fuel that has the potential to substitute petroleum is bioethanol. Bioethanol-blended gasoline is used today as a fuel source for the transportation sector in Canada and the U.S. Both countries hope that the use of gasoline blended with ethanol as a fuel will solve emission-related environmental problems; for example, it will reduce greenhouse gas emissions and, therefore, diminish climate change. Due to rising prices of crude oil, ethanol production from corn initially attracted a lot of attention since the mid-1970s as a "non-complicated and potential" process. Recently, the ethanol-from-corn process has been criticized as environmentally damaging and costly; therefore, ethanol-from-cellulosics such as wheat, corn stalks, and grasses have started to attract more attention.

Some of the car-fuel stations in Canada and the U.S. sell alternative fuels, and ethanol-blended gasoline is one of them that usually contains 5-10% of ethanol by volume. Bioethanol, rich in oxygen, is added to gasoline to promote more complete combustion of gasoline. The Canadian Parliament recently passed Bill C-33, requiring that all gasoline sold in Canada must contain 5% ethanol by 2010. Therefore, ethanol production in Canada will be a continuously growing industry.

Bioethanol can be produced from various sources of biomass, for example, woody and herbaceous crops such as wheat, corn, grass as well as agricultural forestry residues and wastepaper (Wyman, 1996). However, a significant amount of ethanol in Canada and the U. S. is derived from corn. According to critics, there are various environmental impacts associated with the production of corn. Some of the major ones are soil erosion, dependence on herbicides, insecticides, and fertilizers. All those chemical additives can cause water pollution, air pollution and aquifer depletion (Pimentel, 2003; Patzek *et al.*; 2005; Ferguson, 2003; Hodge, 2002). Critics of ethanol as a source of fuel argue that it takes a lot of energy from oil, methane, and coal to grow corn, and even more fossil energy to convert the corn feedstock into ethanol (Patzek *et al.* 2005; Ferguson, 2003). Patzek *et al.* (2005) report that about 65% of the fossil fuel energy input is lost during the conversion process of corn to ethanol. Pimentel (2003) and Ferguson (2003) state that ethanol production energy input is about 20-30% greater than corn ethanol energy output.

Since the production of ethanol from corn requires a lot of energy input, recent studies have concentrated on using cellulosics for ethanol production. Cellulosics require no special conditions and little space for their growth. On the other hand, the production of ethanol itself from cellulosics is considered to be a costly (requiring enzymes to break down lignin and cellulose) and a complicated process. Therefore, a lot of research has to be done in order for cellulosics-to-ethanol production system to be widely accepted as the 'renewable' fuel production machine.

Cellulosics such as grasses have a complex lignocellulosic structure. Therefore, their degradation is not simple. Major components of the lignocellulose include cellulose (45% w/w), hemicellulose (30% w/w), and lignin (25% w/w) (Wood *et al.*, 1997; Wyman, 1996). Cellulose consists of long chains of glucose molecules linked together with β(1-4)glycosidic bonds. Hemicellulose is a complex polymer of pentoses (5-carbon

sugars) and hexoses (6-carbon sugars) (Van Wyk, 2001; Badger, 2002), the exact composition of which varies among plants. Hemicellulose contains more pentoses than hexoses (Badger, 2002). Lignin is a very complex polymer of phenylpropane units, the exact structure of which is not known (Lignin Institute, 2005). Cellulose is only comprised of glucose sugars that are readily fermented by some yeasts and bacteria to ethanol; also, it constitutes a major part in cellulosics and is the substrate of digestion for all cellulases. Consequently, cellulose is used in this research as a substrate for cellulases.

Industrial processes of the conversion of cellulose to ethanol require various steps. At first, lignin that contains no sugars and provides structural plant support has to be removed from cellulose and hemicellulose by chemical and/or mechanical means. Obtained hemicellulose and cellulose are converted to pentoses and hexoses with glucose as a major product, using enzymes called cellulases. Released glucose is finally converted by some yeasts and bacteria into ethanol. Conversion of cellulose to glucose units by cellulases is a very complex step called hydrolysis, which requires mixing of aqueous suspensions of cellulose/cellulases so that cellulases (majority composed of the active site domain (ASD) and the carbohydrate binding domain (CBD)) can attach to cellulose chains, cut or hydrolyze β(1-4)glycosidic bonds between glucose units, de-attach and move to another location. Mixing extent (insufficient or excessive agitation) might influence the attachment of cellulases and possibly lead to lower glucose yields. Therefore, it will be beneficial to determine the strength of mixing required to be applied during cellulose-cellulase mixing cycle. This strength could be directly related to the binding force between the CBD and cellulose. The bioethanol industry could benefit from developing a mixing index that would be related to the cellulose-cellulase binding force.

Although there have been some studies reported on cellulose surface topography and thickness determination, and on the conformation of some cellulases and their amino acid sequences, the author could find no information about the related value nor the measurement of this binding force. Consequently, using atomic force microscopy (AFM) to measure and evaluate such a binding force profile seemed to be appropriate. It was thought that immobilizing the hydrolyzing part of a cellulase (active site domain, ASD) onto the platform of the AFM would leave the attaching part of that cellulase (cellulose binding domain, CBD) free to bind with a cellulose-covered tip (functionalized tip) of the

AFM. The first step was thus to determine how to immobilize the ASD onto the AFM platform, which is totally contrary to all reported immobilization techniques (the ASD remains to be free when cellulases are immobilized on a support). This research then had two objectives:

1. Propose a scheme that would covalently immobilize a cellulase via its active site domain on a silicon support; and
2. Purify a commercial cellulase mixture to obtain one cellulase with an ASD and a CBD.

Chapter 2 - Literature Review

A number of discoveries about cellulases have recently been made concerning their type, structure, purification, mechanism of their action, synergism, and immobilization. Each aspect of the previous discoveries creates a new question that needs to be answered. Although most of the discoveries were concentrated on cellulase structure, mechanism, synergism and purification, there is not much known about cellulase immobilization at the active site that this research is aimed at, and that consequently can be applied in the future to avaluate cellulase-cellulose binding force profile. This section reviews the current understanding of the cellulose-cellulase relationships, such as composition of cellulosics, types of cellulases and mechanism of the enzymatic hydrolysis of cellulose. To potentially measure the cellulose-cellulase binding force by atomic force microscopy, a pure cellulase enzyme must first be immobilized on a silicon support. These two steps, cellulase purification and immobilization, are also described below.

2.1 Composition of Cellulosics and Ethanol Production

Cellulosics represent a plant-based biomass such as grasses, softwoods, and hardwoods that have a complex lignocellulosic structure; therefore, their degradation is not simple. The lignocellulose composition varies between different biomass types; however, in general, major components of the lignocellulose include cellulose (25-55% w/w), hemicellulose (25-50% w/w), and lignin (10-35% w/w) (Wood *et al.*, 1997; Wyman, 1996). Cellulose consists of long chains of glucose molecules linked together with β(1-4)glycosidic bonds. Hemicellulose is a complex polymer composed of different pentoses (5-carbon sugars), hexoses (6-carbon sugars), and glucoronic acid (Van Wyk, 2001; Badger, 2002), the exact composition of which varies among plants. Lignin is a very complex polymer of phenylpropanoid units, the exact structure of which is not completely known (Lignin Institute, 2005). It contains no sugars and provides structural plant support. Cellulose chains usually associate with each other by hydrogen bonding and van der Waals interactions to form microfibrils. These cellulose microfibrils are surrounded by hemicellulose and lignin. In ethanol-from-cellulosics production, an initial pretreatment step is performed to separate one or more components of cellulosic biomass.

Because lignin protects hemicellulose and cellulose from degradation, it must be separated from hemicellulose and cellulose by chemical and/or mechanical means (Kadam, 1996). After the pretreatment, the hydrolysis step is performed that utilizes acid or enzymes to produce fermentable sugars. The final step is fermentation that involves yeasts and bacteria to convert sugars to ethanol.

Both hemicellulose and cellulose are used in industry to produce sugars which can be converted to ethanol. The problem with hemicellulose is that pentoses comprise a major part of it, and final degradation products are mainly pentoses. Their recovery and fermentation are not efficient because microorganisms that are capable of fermenting both pentoses and hexoses have low production rates and low tolerance for the end-product ethanol. A lot of research has been and has to be done in order to develop a genetically-engineered microorganism that would be able to efficiently ferment both pentoses and hexoses (Badger, 2002). In contrast to hemicellulose, cellulose is only comprised of glucose sugars that are released after cellulose is subjected to cellulase digestion and readily fermented by some yeasts and bacteria. This research involves work with cellulases; therefore, cellulose is used throughout the research as their substrate.

2.2 Mechanisms of Enzymatic Hydrolysis of Cellulose by Cellulases

One of the most important steps in cellulose-to-ethanol processing is the conversion of cellulose to glucose that can be achieved by using acids (acid hydrolysis) or by using enzymes generally called cellulases (enzymatic hydrolysis). There are a number of disadvantages associated with acid hydrolysis; the most important of which are the formation of by-products and the requirement of relatively high temperatures (Kadam, 1996; Zaldivar *et al.*, 2001). Consequently, a considerable attention has been paid to the enzymatic hydrolysis of cellulose.

Complete hydrolysis of cellulose requires the presence of three types of cellulases that belong to the class of glycoside hydrolases. Cellulases are classified as **endocellulases** (1,4-β-D-glucan glucanohydrolases or endoglucanases), **exocellulases** (1,4-β-D-glucan cellobiohydrolases or cellobiohydrolases) and **β-glucosidases** (β-D-glucoside glucohydrolases), based on their mode of action and on the fact that all are involved in the hydrolysis of the β(1-4)glycosidic bond. What makes them functionally

different includes their substrate specificities, the location where they attack the substrate, and their mode of action. For example, endocellulases are able to make internal cuts in the amorphous regions of the microfibril chains of cellulose, thereby reducing the chain length and producing free chain ends (reducing and non-reducing) for further attack by exocellulases that produce cellobiose residues. β-Glucosidase, on the other hand, is required for the degradation of cellobiose into glucose units. Cellobiose is considered to be a strong competitive inhibitor of endo- and exocellulases, therefore the presence of β-glucosidase is a requirement for the efficient degradation of cellulose.

Endocellulases can be identified using carboxymethylcellulose (CMC) or amorphous cellulose, toward which they show high activity (Macarron *et al.*, 1993; Beldman *et al.*, 1985; Pere *et al.*, 1995; Garcia *et al.*, 1993; Vlasenko *et al.*, 1998; Klesov *et al.*, 1981; Ghose, 1987). Exocellulases, on the other hand, can be identified using p-nitrophenyl-β-D-cellobioside (Woodward *et al.*, 1990; Deshpande *et al.*, 1984; Evans *et al.*, 1993; Lassig *et al.*, 1995). β-Glucanase activity can be determined using cellobiose as a substrate (Gong *et al.*, 1989; Ghose, 1987).

Most cellulose-degrading enzymes have a similar structure. The majority are comprised of two domains that are structurally and functionally distinct (Gerber *et al.*, 1997; Nidetzky *et al.*, 1994; Shoemaker *et al.*, 1983; Pere *et al.*, 1995; Bhikhabhai *et al.*, 1984; Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986): a) an active site domain (ASD), which is involved in the hydrolysis of cellulose and constitutes the major part of the enzyme, and b) a cellulose binding domain (CBD), which is responsible for the binding of the enzyme to the substrate and constitutes a smaller portion of the enzyme. Both parts of the enzyme (ASD and CBD) are linked by a glycosylated flexible peptide chain that enables both domains to approach the substrate. Some of the cellulose-degrading enzymes are comprised of only one domain, an ASD. Endo- and exocellulases usually have a two-domain structure and β-glucosidases usually have a one-domain structure. A two-domain cellulase was chosen for this research (described later in this section) because the long-term goal of this research is to establish the force of binding between a CBD and cellulose, therefore the presence of a CBD in a chosen cellulase is required.

Once the CBD of cellulase binds to cellulose, the hydrolysis of the β(1-4)-glycosidic bond in cellulose by the ASD begins. Figure 2.2.1 represents two major

mechanisms of enzymatic glycosidic bond hydrolysis by cellulases, the overall retention and inversion. Those two mechanisms result either in overall retention or inversion of hydrolysis product's stereochemistry with respect to the substrate (Davies and Henrissat, 1995; CAZy, 2008). Generally, the hydrolysis of the β(1-4)-glycosidic bond by cellulases requires two catalytic parts on the ASD of the enzyme: a proton donor (HA) and a nucleophile/base (B-) (Davies and Henrissat, 1995). The retaining mechanism (Figure 2.2.1 (a)), in which oxygen in the glycosidic bond is protonated by the catalytic proton donor (HA), and aglycon (ROH) departure is provided by the catalytic nucleophile/base (B-). The resulting glycosyl-enzyme complex is then hydrolyzed by a water molecule. The generated product has the same stereochemistry (same configuration) as the substrate. In the inverting mechanism (Figure 2.2.1 (b)) a water molecule is activated by the catalytic nucleophile/base (B-). An activated water molecule attacks carbon that is involved in the glycosidic bond formation, followed by the protonation of oxygen in the glycosidic bond by the catalytic proton donor (HA) (Davies and Hanrissat, 1995). The generated product has a different stereochemistry (different configuration) than the substrate. The distances between the HA and B- within enzyme's active site required for both mechanisms to take place are shown in Figure 2.2.1 as 5.5Å and 10.0Å. The distances between a proton donor and glycosidic oxygen in both mechanisms are identical – within hydrogen-bonding distance. However, the distance between the catalytic nucleophile/base and glycosidic oxygen in the inverting mechanism is larger due to the necessity of accommodation of a water molecule between the nucleophile/base and the substrate.

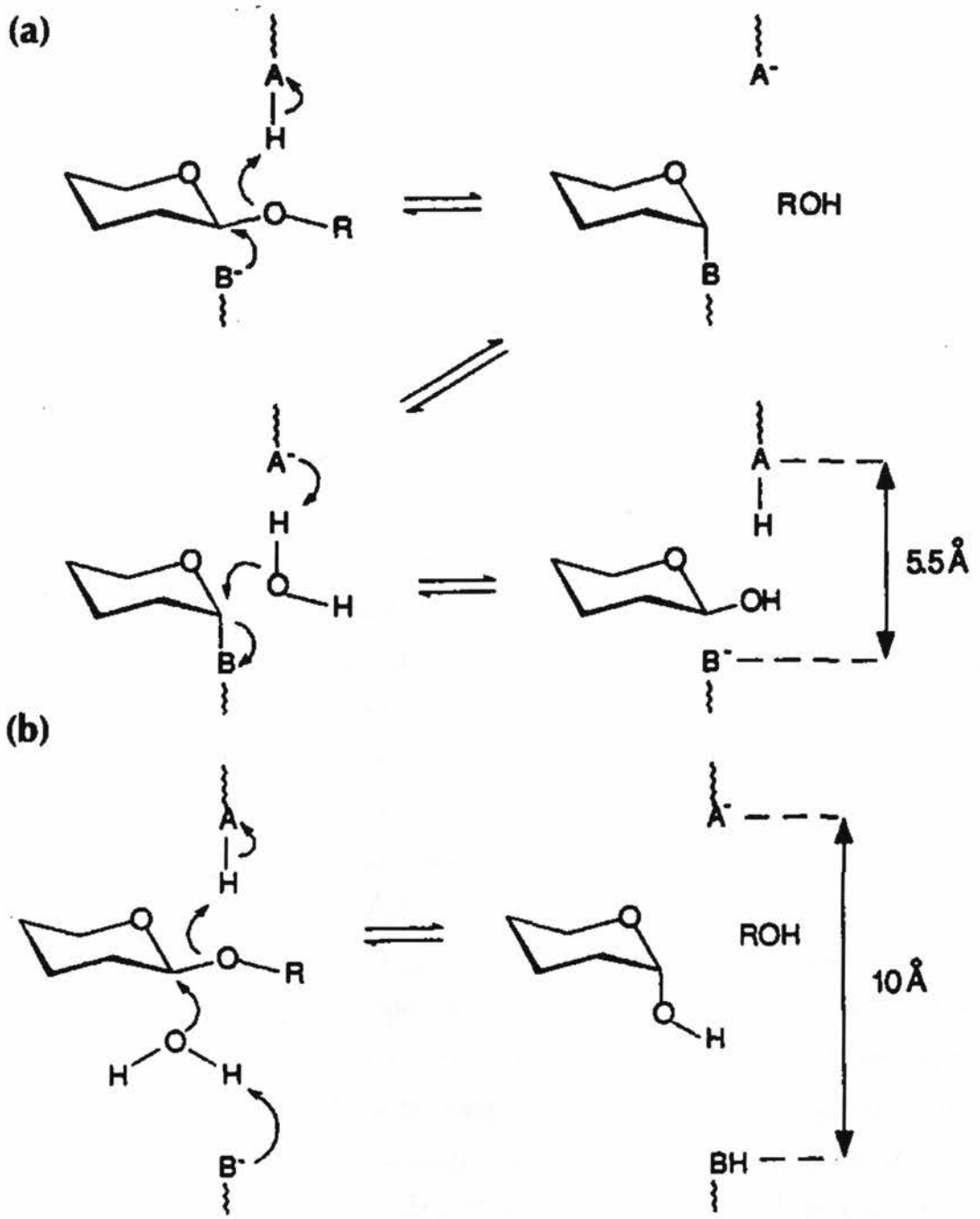

Figure 2.2.1. Two major mechanisms, (a) retaining and (b) inverting, that are involved in the hydrolysis of β(1-4)glycosidic bond by cellulases (Davies and Henrissat, 1995).

According to the Carbohydrate-Active Enzymes database (CAZy), most endocellulases and exocellulases belong to glycoside hydrolases families 6 and 7, out of a total of 112 families (CAZy, 2008), based on their amino acid sequence similarities and

3D structures. The glycoside hydrolases family 6 includes endocellulases and non-reducing end-acting exocellulases that all possess the inverting mechanism with the aspartate (Asp) amino acids acting as both the catalytic nucleophile/base (B-) and the catalytic proton donor (AH). The glycoside hydrolases family 7 includes endocellulases and reducing end-acting exocellulases that all possess the retaining mechanism with the glutamate (Glu) amino acids acting as both the catalytic nucleophile/base and the catalytic proton donor.

For this research, exocellulase I (CBH I) from fungus *Trichoderma reesei* was chosen due to its effective contribution of the enzymatic activity to degradation of cellulose, its commercial availability, its abundance in the commercial mixture (Wenger, 2006), and because it represents a classical cellulase that is composed of two domains: an ASD and a CBD (Pere *et al.*, 1995; Schulein, 1988; Divne *et al.*, 1993; Tomme *et al.*, 1988; Divne *et al.*, 1994; Klarskov *et al.*, 1997; Van Tilbeurgh *et al.*, 1986). The molecular weight of the CBH I is ~65kDa and isoelectric point (pI) is between 3.8-4.1 (Pere *et al.*, 1995; Tomme *et al.*, 1988; Gerber *et al.*, 1997). CBH I is classified as 'exo' type glycoside hydrolase (more specifically exocellulase) that belongs to the family 7 of glycoside hydrolases, and so possesses the retaining mechanism (CAZy, 2008; Divne *et al.*, 1994). It is comprised of 497 amino acids, where 434 amino acids represent an ASD with the approximate dimensions of 60Å by 50Å by 40Å, 27 amino acids represent linker, and 36 amino acids represent a CBD (Evans *et al.*, 1993; Divne *et al.*, 1994). The 434-residue ASD is arranged in a way that ~40Å long tunnel is created, which is the probable active site and the binding site of inhibitors (Divne *et al.*, 1994). The glutamate amino acid at position 217 (Glu-217) in the ASD tunnel of CBH I acts as the catalytic proton donor (HA), while the glutamate amino acid at position 212 (Glu-212) in the ASD tunnel of CBH I acts as the catalytic nucleophile/base (B-). Both Glu-217 and Glu-212 are required for the glycosidic bond cleavage. These amino acids are located in the tunnel of the CBH I' ASD on opposite sides, where the reducing end of cellulose fits in. Cleavage of cellobiose units occurs from that end.

The course of the cellulose hydrolysis reaction can be measured reductometrically (due to the formation of reducing end-groups after cellulose is cleaved by cellulases) or viscometrically (due to the reduction in cellulose viscosity after cellulose is cleaved by

cellulases). The reductometric method is considered to be more sensitive than the viscometric (Vlasenko *et al.*, 1998), because it detects all reducing groups formed in the solution as a result of cellulose attack by cellulases (Klesov *et al.*, 1981; Vlasenko *et al.*, 1998; Garcia *et al.*, 1993; Ghose, 1987). The most widely used reagents for the determination of reducing groups are 3,5-dinitrosalicylic acid (DNS), Nelson-Somogyi, and disodium 2,2'-bicinchoninate (BCA) (Vlasenko *et al.*, 1998; Garcia *et al.*, 1993; Ghose, 1987; Klesov *et al.*, 1981). In this research, the DNS method was used as a reagent for the determination of reducing groups after the cellulose hydrolysis reaction.

2.3 Cellulase Immobilization

There are various methods used to immobilize (attach) enzymes to a support, such as covalent binding, adsorption, encapsulation, cross-linking, and entrapment (Bickerstaff, 1996). Covalent immobilization of an enzyme to a support is the strongest attachment that permanently (irreversibly) restricts enzyme deattachment; therefore covalent immobilization for the CBHI' ASD was chosen for the research. The most common support materials for covalent immobilization are: polysaccharide polymers, porous glass, silica, and silicon. The advantages of using porous glass, silica, and silicon are the durability and the strength of the materials; silicon was chosen for the research. The most common amino acid functional groups on the enzyme that can be involved in covalent bond formation are: the carboxyl group (COOH) of glutamic acid or aspartic acid, the amino group (NH_2) of arginine or lysine, the sulfydryl group (SH) of cysteine, and the hydroxyl group (OH) of threonine or serine (Bickerstaff, 1996). There are two main steps involved in covalent immobilization of enzymes on a support: a) the support should be activated by a reagent in order to make the functional groups on the support electron-deficient (strongly electrophilic); b) the enzyme should be coupled to the activated support by reacting electron-deficient groups on a support with electron-donating groups (strong nucleophiles) such as functional groups of certain amino acids on the surface of the enzyme. For the chosen exocellulase CBH I, glutamate amino acids that are specifically located on the ASD were assumed to perform as functional groups or targets for covalent immobilization.

Covalent immobilization of cellulases has normally been done in the past with the purpose of retaining their enzymatic activity (Table 2.4.1). Researchers assumed that those cellulase immobilizations were via the CBD. Therefore, with the research purpose of immobilizing the ASD, the author searched for an immobilization technique that would reduce cellulase activity after the immobilization to a very low value. In the Table 2.4.1 the post-immobilization activity of a purified cellulase ranges from 3% to 192% compared to the activity of native cellulase. In the case of post-immobilization cellulase activity of 0% it could be assumed that cellulase was covalently attached through its ASD to the support (one of the goals of this research). Post-immobilization low cellulase activity of 3% (Table 2.4.1) could be a good start only if researchers of previously performed covalent cellulase immobilization had not given a reason for such a low post-immobilization activity, which is the de-attachment of cellulases from the support. Therefore, another approach was chosen using a non-competitive (covalently binding) inhibitor that is specific to CBH I' ASD.

For the purpose of covalent immobilization of CBH I' ASD stated above, the CBH I enzyme has to be primarily purified from the commercial cellulase mixture. The presence of two domains (~55kDa ASD and ~10kDa CBD) has to be subsequently proved by digesting CBH I with trypsin or papain somewhere in the linker (Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986; Divne *et al.*, 1993; Divne *et al.*, 1994; Abuja *et al.*, 1988). It was expected that two bands would be shown on SDS-polyacrylamide gel after digestion: CBD and ASD. For further covalent immobilization purposes, a CBH I inhibitor, (R,S)-3,4-epoxybutyl β-cellobioside, was chosen to covalently bind glutamate amino acid in position 212 (Glu-212), which acts as the active site nucleophile (B-) (Klarskov *et al.*, 1997). Klarskov *et al.* (1997) suggested that the CBH I inactivation most likely occurs at the active site. The proof was: once the inhibitor was covalently bound to the Glu-212 amino acid, the complete amino acid sequence of CBHI was broken into peptides with electrospray ionization mass spectrometry equipment. The analysis of those peptides, before and after inactivation with (R,S)-3,4-epoxybutyl β-cellobioside inhibitor, proved that Glu-212 on ASD of CBH I enzyme covalently bound this inhibitor. With the aim of finding a covalently binding inhibitor for the ASD of CBH I, the author chose an (R,S)-3,4-epoxybutyl β-cellobioside inhibitor as a potential tool for the CBH I

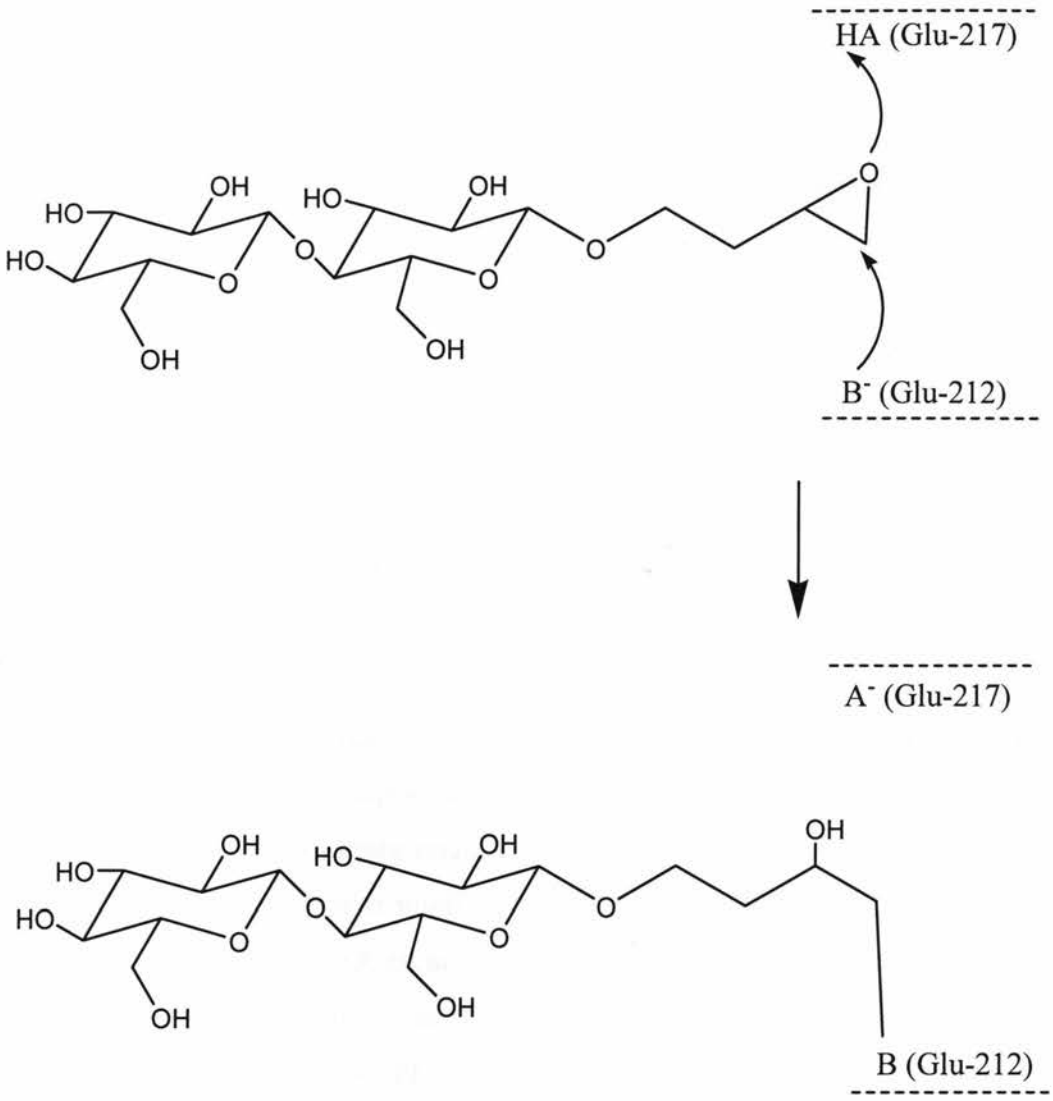

Figure 2.4.2. Covalent attachment of the (R,S)-3,4-epoxybutyl β-cellobioside inhibitor to the ASD of CBHI via Glu-212 with a help of Glu-217.

Glutamate at the position 217 (Glu-217) should act as a proton donor (HA) and glutamate at the position 212 (Glu-212) should act as a catalytic nucleophile/base (B-) (Klarskov *et al.*, 1997; Divne *et al.*, 1994). First, the inhibitor's cellobioside part is recognized by a CBH I enzyme, then Glu-217 in the ASD of CBH I protonates oxygen in the epoxide part of the inhibitor. Epoxyde, as a very reactive group, becomes the target of the nucleophilic attack by Glu-212, and a covalent ester bond is formed between Glu-212

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Table 2.3.1. Previously performed immobilization of cellulases via their CBDs.

Immobilized enzymes	Support used for immobilization	Chemical used to activate platform	Immobilization site (ASD or CBD)	Immob. cellulase activity (% of native cellulase)	References
Cellulases from *Trichoderma viride* and *Aspergillus niger*	>porous silica glass >ceramics (alumina and titania) and their silanized derivatives	>titanium tetrachloride >γ-aminopropyltriethoxy-silane (coupled through glutaraldehyde)	CBD	3-53	Shimizu and Ishihara, 1987
Cellulases from *Aspergillus terreus*	>glass beads	>3-aminopropyltriethoxy-silane (coupled through glutaraldehyde) >3-glycidoxypropyltri-methoxysilane (coupled through $NaBH_4$)	CBD	71.6-98.1	Rogalski *et al.*, 1985
Cellulases from *Trichoderma viride*	>sepharose 4B >glass beads	>CNBr >concanavalin-A	CBD	68-192	Fadda *et al.*, 1984
Cellulases from *Trichoderma viride*	>sepharose 4B (no spacer) >CH-sepharose 4B (with 7-atom spacer) >affi-gel 15 (with 16-atom spacer)	>CNBr >$NaHCO_3$ >$NaHCO_3$ + NaCl	CBD	17.2-32.7	Chim-anage *et al.*, 1986
Cellulases from *Trichoderma reesei*	>nylon >nylon with glass	>hydrochloric acid (coupled through glutaraldehyde) >N,N-dimethylamino-propylamine (coupled through glutaraldehyde)	CBD	~60	Jain and Wilkins, 1987

immobilization via ASD.

2.4 Irreversible Inhibition of CBH I Active Site Domain

The epoxide-based glycosyl inhibitor, (R,S)-3,4-epoxybutyl β-cellobioside, binds to the ASD of the CBH I enzyme irreversibly by resembling the enzyme's corresponding sugar substrate (Withers and Aebersold, 1995). The structure of (R,S)-3,4-epoxybutyl β-cellobioside inhibitor is shown in Figure 2.5.1. The R and S are diastereoisomers of the inhibitor molecule and are a pair of isomers that have opposite configurations at one or more of the chiral centers (R and S, clockwise and counterclockwise substitution with respect to the chirality center). The cellobiose unit of the inhibitor is a saccharide moiety that is specific for recognition by the ASD of CBHI, and the epoxide group is the target for nucleophile Glu-212 (B-) attack by the ASD of CBHI. This mechanism is shown in Figure 2.4.2. It should be noted that both diastereoisomers of 3,4-epoxybutyl β-cellobioside are effective in inhibition of CBH I (Klarskov *et al.*, 1997).

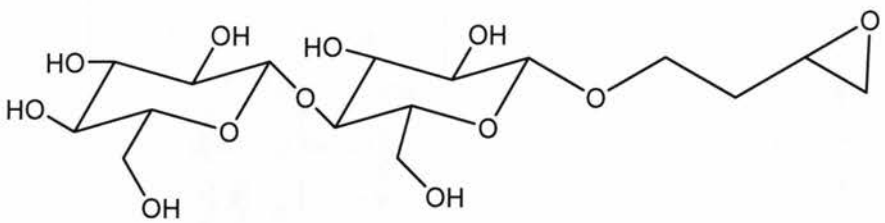


Figure 2.4.1. (R,S)-3,4-epoxybutyl β-cellobioside inhibitor that is composed of a cellobiose unit attached to an epoxybutyl unit.

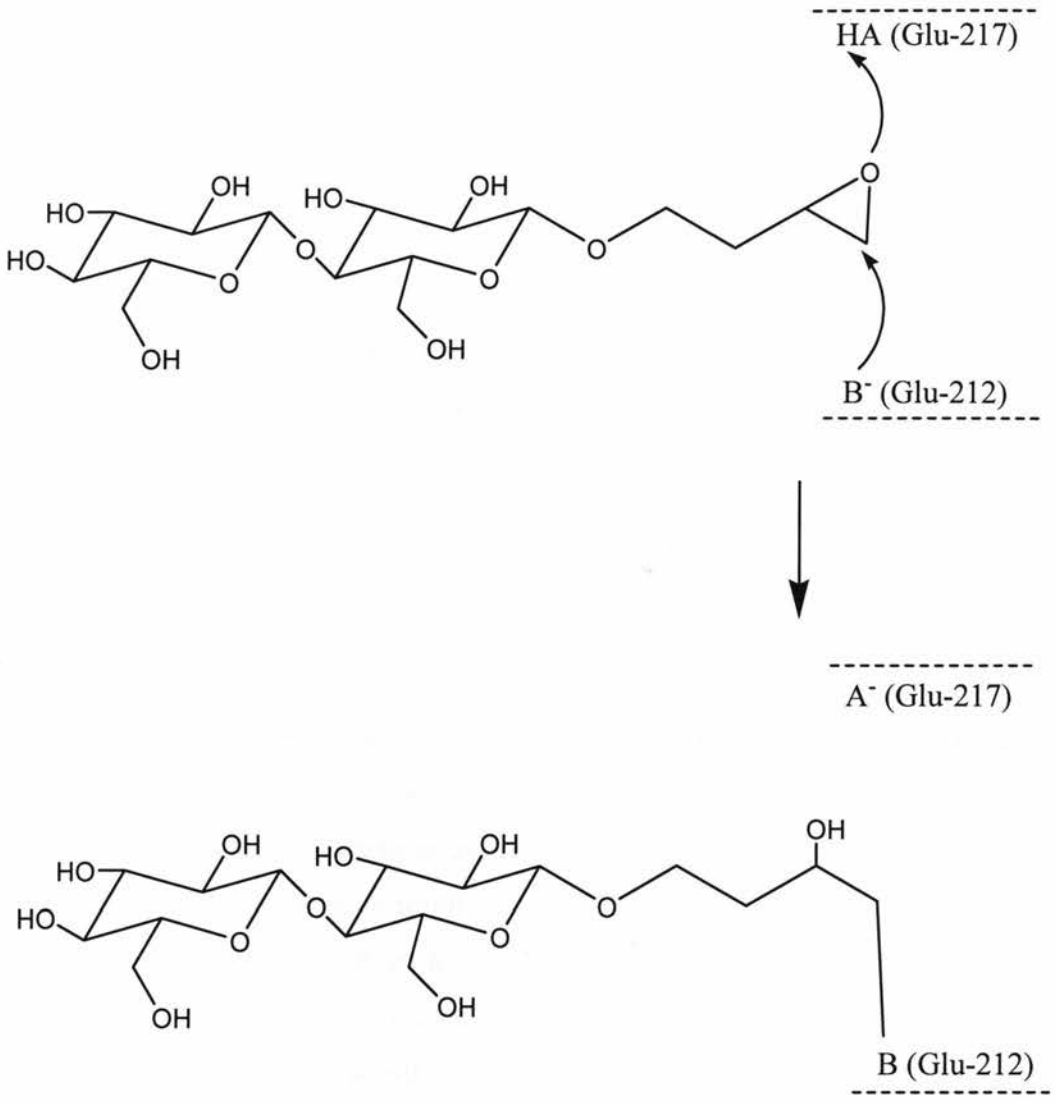


Figure 2.4.2. Covalent attachment of the (R,S)-3,4-epoxybutyl β-cellobioside inhibitor to the ASD of CBHI via Glu-212 with a help of Glu-217.

Glutamate at the position 217 (Glu-217) should act as a proton donor (HA) and glutamate at the position 212 (Glu-212) should act as a catalytic nucleophile/base (B-) (Klarskov *et al.*, 1997; Divne *et al.*, 1994). First, the inhibitor's cellobioside part is recognized by a CBH I enzyme, then Glu-217 in the ASD of CBH I protonates oxygen in the epoxide part of the inhibitor. Epoxyde, as a very reactive group, becomes the target of the nucleophilic attack by Glu-212, and a covalent ester bond is formed between Glu-212

and epoxyde (Klarskov *et al.*, 1997). It was proposed that Glu-212 and Glu-217 are located on the opposite sites of the ASD tunnel and close to its entrance.

2.5 The Approach of CBH I Immobilization via ASD

The main goal of the research was to covalently immobilize CBH I on the AFM compatible material, a silicon platform, through the non-compatitive (irreversibly-binding) inhibitor (3,4-epoxybutyl β-cellobioside) specific to the ASD of the CBHI enzyme. Because the inhibitor does not offer sufficient molecular spacing between the silicon platform and the ASD of CBH I to enable covalent target binding, the covalent attachment of the inhibitor through a spacer arm became necessary. The spacer arm chosen was 11-bromo-undecyltrichlorosilane (long enough to allow CBHI to approach the immobilized inhibitor) that has to be covalently attached at one end through a silanization method to a silicon platform leaving a bromine end exposed for further attachment of the inhibitor. Once the inhibitor is immobilized on the spacer arm, the following covalent link is established: silicon-bromosilane-inhibitor (functionalized platform). At this point, the functionalized silicon platform can be incubated with the CBH I enzyme. It is expected that since the inhibitor is specific to the ASD of CBH I, a covalent immobilization of the CBH I enzyme via its ASD on the functionalized silicon platform can be achieved. A detailed synthesis of the inhibitor and its covalent attachment to the silicon platform followed by the immobilization of the ASD are described in the Results and Discussion section with the provided detailed procedure in Appendix A.

2.6 Purification of Cellulases

Usually cellulase-producing species secrete more than one type of cellulase; therefore, purification is required. Previously performed purifications of cellulases included an initial desalting step on Bio-Gel P10 or Sephadex G-25 chromatography resins. Table 2.3.1 summarizes cellulase purifications with the emphasis on which type(s) of chromatography resin(s) was/were used to purify a particular type of cellulase. It can be noted that anion exchange, cation exchange, affinity and size exclusion are the main approaches used to purify cellulases. The purification schemes of Pere *et al.* (1995) and

Bhikhabhai *et al.* (1984) were chosen as references due to their logic and relatively simple approaches, and due to their clarity of explanation.

The purity and the approximate molecular weight of each cellulase solution can be subsequently verified with sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) using standards with known molecular weights (Laemmli, 1970; Suvajittanont *et al.*, 2000; Beldman *et al.*, 1985; Nidetzky *et al.*, 1994; Tomme *et al.*, 1988).

The purification of CBHI is necessary in this research so that a pure enzyme is obtained for its subsequent covalent attachment (immobilization) to an AFM compatible support via ASD.

Table 2.6.1. Examples of chromatographic material used for the purification of cellulases from *Trichoderma* yeasts.

Cellulases separated	Cellulase-producing species	Types of purification used	References
Endocellulases I, II; exo-cellulases I, II	*T. reesei*	DEAE Sepharose FF (anion exchange) p-aminobenzyl 1-thio-β-D-cellobioside Sepharose 4B (affinity) CM Sepharose FF (cation exchange) phenyl Sepharose FF (hydrophobic interaction)	Pere et al., 1995
Endocellulases I,II,III,IV,V,VI; exocellulases I,II,III; β-glucosidase I (cellobiase)	*T. viride*	DEAE-Bio-Gel A (anion exchange) Bio-Gel P100 (size exclusion) SE-Sephadex (cation exchange) Avicel cellulose (affinity)	Beldman et al., 1985
Endocellulase III	*T. reesei*	DEAE-Sepharose CL-6B (anion exchange) Ultrogel AcA-44 (size exclusion)	Macarron et al., 1993
Exocellulases I, II; endo-cellulases I, II	*T. reesei*	DEAE-Sepharose CL-6B (anion xchange)	Divne et al., 1993
Exocellulases I, II; endo-cellulases II, III	*T. reesei*	SP-Sephadex (cation exchange) DEAE-Sepharose CL-6B (anion exchange)	Bhikhabhai et al., 1984
endocellulase I	*T. reesei*	Affigel-10 sensitized with purified anti-EG I monoclonal antibodies	Nieves et al., 1990
Low molecular weight endocellulase (18 kDa); high molecular weight endocellulase (52 kDa); exocellulase (71 kDa); cellobiase (76 kDa)	*T. reesei*	DEAE-Sepharose (anion exchange) DEAE-cellulose (anion exchange) Sephadex G-75 (size exclusion) Sephadex G-100 (size exclusion)	Gong et al., 1979
Endocellulase (25.8 kDa); exocellulase (36 kDa)	*T. reesei*	DEAE-Sepharose CL-6B Sephacryl S200 (size exclusion)	Massiot., 1992
Endocellulase I; exocellulase I; cellobiase I	*T. reesei*	DEAE-Sepharose CL-6B (anion exchange) SP-Sephadex C-50 (cation exchange)	Shoemaker et al., 1983
Endocellulases I, II; exo-cellulases I, II	*T. reesei*	Rotofor (isoelectric focusing) Sephacryl 100 HR (size exclusion) Mono Q (anion exchange) Mono S (cation exchange) Mono P (chromatofocusing)	Gama et al., 1998
Exocellulase I; endocellulases I, II	*T. reesei*	Mono Q (anion exchange) DEAE-Sephadex (anion exchange) Sephacryl S-200 (size exclusion)	Schulein., 1988

Chapter 3 – Material and Methods

There are two parts in this research: the hypothetical immobilization of CBH I inhibitor on a silicon platform via ASD and the experimental purification of CBH I.

Cellulases

Two commercial cellulase mixtures (NS50013 CCN03067 and NS50013 CCN03105) from *Trichoderma reesei* were kindly donated by Novozymes (Denmark). They were brown, viscous liquids with a protein content of 1-40% (w/v) in water, both containing approximately the following types of enzymes: CBH I (60%), CBH II (15%), EG I and II (20%), and BG (2%) (Wenger, 2006). The mixtures were stored at 4°C until used. Protein and reducing sugars concentration as well as total activity (CMC) of the mixtures were determined according the procedures described in Analyses. The number of enzymes in the mixtures and their molecular weights were determined using SDS/PAGE electrophoresis described in Analysis.

Immobilization

A procedure for the hypothetical immobilization of CBH I via ASD is described in Appendix A.

CBH I Purification

Two purification procedures ((Pere at al. (1995) and Bhikhabhai *et al.* (1984)) were attempted to purify CBH I from the commercial cellulase mixture. The details and procedures are provided below.

Column chromatography was performed on the following resins: Bio-Gel P-10 (fine, 45-90μm mesh; Bio-Rad Laboratories), Sephadex G-25 (superfine; Pharmacia LKB), and DEAE-Bio-Gel A (hydrated, Bio-Rad). Concentration of the eluted fractions was performed with a DNA Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY, Model No: DNA 110-120, Serial No: DNA 110-4J4405301E).

In gel filtration chromatography (desalting) Bio-Gel P-10 and Sephadex G-25 resins were used. The protein sample was 1.5 mL and the column bed volume was 4.0

mL (according to the Bio-Rad Manual, a protein sample can be as large as 30-35% of the bed volume). In ion-exchange chromatography, enzymatic separation was performed on DEAE-Bio-Gel A; the protein sample was 0.10 mL and the column bed volume was 4.0 mL (according to the Bio-Rad Manual, a protein sample should not be larger than 1-5% of the bed volume). Exclusion limits for Sephadex G-25 and Bio-Gel P-10 were 5,000 Da and 1,500-20,000 Da, respectively. Protein binding capacity of DEAE-Bio-Gel A was 45±10 mg of hemoglobin per mL of resin, determined in 5 mM Tris-HCL buffer, pH 8.6.

Gel Filtration Chromatography

Pere *et al.* (1995): Dry powder (1g) of Sephadex G-25 was added to 15mL of 10mM sodium phosphate buffer (pH 7.2) in a beaker and stirred. This mixture was hydrated for 24 hrs at room temperature and was ready for column packing. The Bio-Gel P-10 packed column (0.4 x 8.0 cm (4.0 mL)) was washed with 4 bed volumes of 10mM sodium phosphate buffer, and 1.5mL of the commercial cellulase mixture were applied on the Sephadex G-25 packed column. All collected eluant fractions were pooled into one fraction (total volume: 16 mL) and concentrated to 1.5mL. Protein and glucose concentration as well as a total activity on CMC were determined of the desalted commercial cellulase mixture. Gel filtration was performed in duplicate. Column flow rate was 2.0 mL/hr.

Bhikhabhai *et al.* (1984): Dry powder (1g) of Bio-Gel P-10 was added to the 15mL of 0.01M ammonium acetate buffer (pH 5) in a beaker and stirred. This mixture was hydrated for 24 hrs at room temperature and was ready for column packing. The Bio-Gel P-10 packed column (0.4 x 8.0 cm (4.0 mL)) was washed with 4 bed volumes of 0.01M ammonium acetate buffer and 1.5mL of the commercial cellulase mixture were applied on the Bio-Gel P-10 packed column. All collected eluant fractions were pooled into one fraction (total volume: 16 mL), concentrated to 1.5mL and protein and glucose concentration as well as a total activity on CMC were determined of the desalted commercial cellulase mixture. Gel filtration was performed in duplicate. Column flow rate was 2.0 mL/hr.

Ion Exchange Chromatography

Pere *et al.* (1995): The hydrated DEAE-Bio-Gel A resin was mixed with 10mM sodium phosphate buffer, pH 7.2 (50% v/v of each) and the resin was ready for column packing. The DEAE Bio-Gel A packed column (0.4 x 8.0 cm (4.0 mL)) was washed with 4 bed volumes of 10mM sodium phosphate buffer and 0.1mL of the desalted commercial cellulase mixture was applied on the DEAE Bio-Gel A packed column. The column was washed with four buffers of various salt content: 4 bed volumes of 10mM sodium phosphate starting buffer (pH 7.2), 4 bed volumes of 25mM NaCl in the starting buffer pH (7.2), 4 bed volumes of 120mM NaCl in the starting buffer (pH 7.2), and 4 bed volumes of 150mM NaCl in the starting buffer (pH 7.2). Consequently, four fractions were collected (16mL each). The purification was performed in triplicate. The SDS/PAGE of each fraction was performed. Column flow rate was 6.5 mL/hr.

Bhikhabhai *et al.* (1984): The hydrated DEAE-Bio-Gel A resin was mixed with 0.01M ammonium acetate buffer (50% v/v of each) and the resin was ready for column packing. The DEAE Bio-Gel A packed column (0.4 x 8.0 cm (4.0 mL)) was washed with 4 bed volumes of 0.01M ammonium acetate buffer and 0.1mL of the desalted commercial cellulase mixture was applied on the DEAE Bio-Gel A packed column. One hundred and twenty five eluant fractions (0.2mL each) were collected, giving a total volume of 25mL. The first 40 fractions were eluted with 0.01M ammonium acetate buffer (pH 5), another 40 fractions were eluted with 0.25M ammonium acetate buffer (pH 5), and the last 45 fractions were eluted with 0.5M ammonium acetate buffer (pH 5). The purification was performed in triplicate. The protein concentration was checked in all 125 fractions, and the total activity (CMC) was checked only in fractions that contained protein. The SDS/PAGE of each pooled peak was performed. Column flow rate was 6.5 mL/hr.

Analyses

Total Protein Concentration

The protein concentration was measured following instructions of the Quick Start Bradford Assay from Bio-Rad Laboratories Inc. (Hercules, CA). Bovine Serum Albumin (BSA) was used as a standard. BSA and protein measurements were done in duplicate. Protein samples were diluted such that the resulting absorbance readings at 595nm on a Perkin Elmer UV/VIS Spectrometer (Lambda 20, Norway, USA; Model No: BUV20200, Serial No: 101N9072623) were between 0.10 and 0.85. The calibration curve and raw data for the protein concentration are found in Appendix B.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS/PAGE)

A polyacrylamide gel (11% v/v) was prepared and the buffer system of Laemmli (1970) was used to determine the molecular weight of proteins/cellulase enzymes. Precision Plus Protein Standards (Bio-Rad Laboratories) were used with the following molecular weights: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa respectively. Electrophoresis apparatus was obtained from Bio-Rad Laboratories Inc. (Model No: Mini Trans-Blot Cell; Serial No: 153BR25635). All protein samples were diluted 2 times with a sample buffer, consisting of 20% (v/v) 0.5M Tris-HCl/0.4% (w/v) SDS, pH 6.8, 8% (w/v) SDS, 24% (v/v) glycerol, 3.1% (w/v) dithiothreitol (DTT), 0.02% (w/v) Coomassie Blue G-250 and subsequently boiled for 5 min to denature proteins. After cooling to room temperature, protein samples were ready for gel-loading. The sample loading volume was 10μL per well. Samples were electrophoresed for 30 min with 50V, followed by 130V for 2 hrs. After that time, gels were stained (~30min) for protein with Coomassie Blue Staining Solution (50mL of Staining Solution per gel in a covered plastic container), consisting of 0.5g per liter Coomassie Blue R-250, 500mL per liter methanol, 100mL per liter acetic acid, and 400mL per liter of ultra filtrated (UF) water. After staining, gels were destained (overnight) with Destaining Solution (100mL of Destaining Solution per gel in covered plastic container), consisting of 5% methanol and 7% acetic acid in UF water. After destaining, protein bands could be seen with the naked eye. Gels were stored in the destaining solution at room temperature.

Total Cellulase Activity

This type of activity is based on the spectrophotometric measurement of released sugars after enzymatic hydrolysis of the cellulose by the commertial cellulase mixture, using glucose as a standard. A 1% (w/v) carboxymethylcellulose (CMC) substrate was used to measure total cellulase activity.

The CMC was prepared according to the procedure described in Vlasenko *et al.* (1998) with the following modifications. One gram of 60-mesh low viscosity CMC with an average degree of substitution of 0.7-0.8 (BDH Chemicals Ltd.) was dispersed into 50mL of hot water (80°C) with vigorous stirring for 1h at 80°C. The solution was then cooled to room temperature and diluted to 100mL with a 0.1M sodium citrate buffer (pH 4.8). This 1% CMC was stored at 4°C for not more than 2 days.

Enzymatic activity on CMC was measured according to the procedure of Ghose (1987) with the following modifications. Fifty microliters of 1% CMC solution were added to 50μL of five glucose standards, a buffer blank (0.05M sodium citrate, pH 4.8), and unknown cellulase-containing solutions (diluted 100 and 125 times), mixed well, and incubated in a water bath at 50°C for 30min. After 30min, 0.3mL of Dinitrosalicylic Acid reagent (DNS) were added to each tube, mixed, boiled for 5min to stop the reaction, and 2mL of distilled water were added to each tube and mixed. The formed color was measured against the blank at 540nm on a Perkin Elmer UV/VIS Spectrometer. A standard curve was constructed from glucose concentrations between 0.50 and 2.0 mg/mL, and is found in Appendix B. Reducing sugars, liberated as a result of the action of cellulases on CMC, were read from the standard curve. All protein samples were diluted such that the resulting absorbance readings at 540nm were between 0.07 and 0.4. Total cellulase activity was expressed as International Units (IU) per mg of protein. One International Unit (IU) of enzyme activity was defined as the amount of enzyme liberating reducing sugars corresponding to 1μmol of glucose per min under assay conditions (Ghose, 1987). Total cellulase activity measurements were done in duplicate.

Chapter 4 – Results and Discussion

This section covers the following results: theoretically derived hypothetical pathway of a covalent immobilization of exocellulase I (CBH I) via its Active Site Domain (ASD) (section 4.1), and experimental results on CBH I purification (section 4.2).

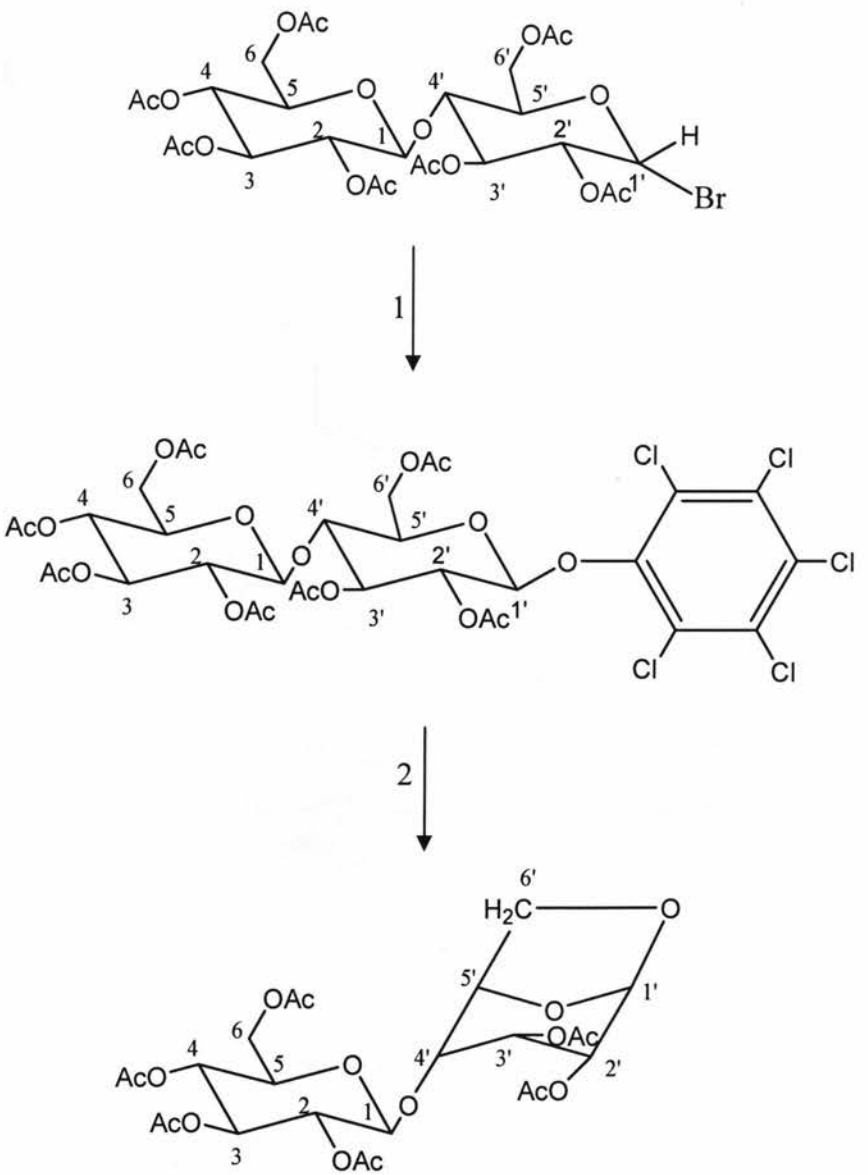
4.1 Covalent Immobilization of CBH I via its Active Site Domain

This subsection covers a hypothetical procedure for a covalent immobilization of 3,4-epoxybutyl-β-cellobioside (an irreversible inhibitor of CBH I) on a silicon platform via 6' carbon (Figure 4.1.1) and a covalent immobilization of ASD of CBH I on that inhibitor (Figure 4.1.2).

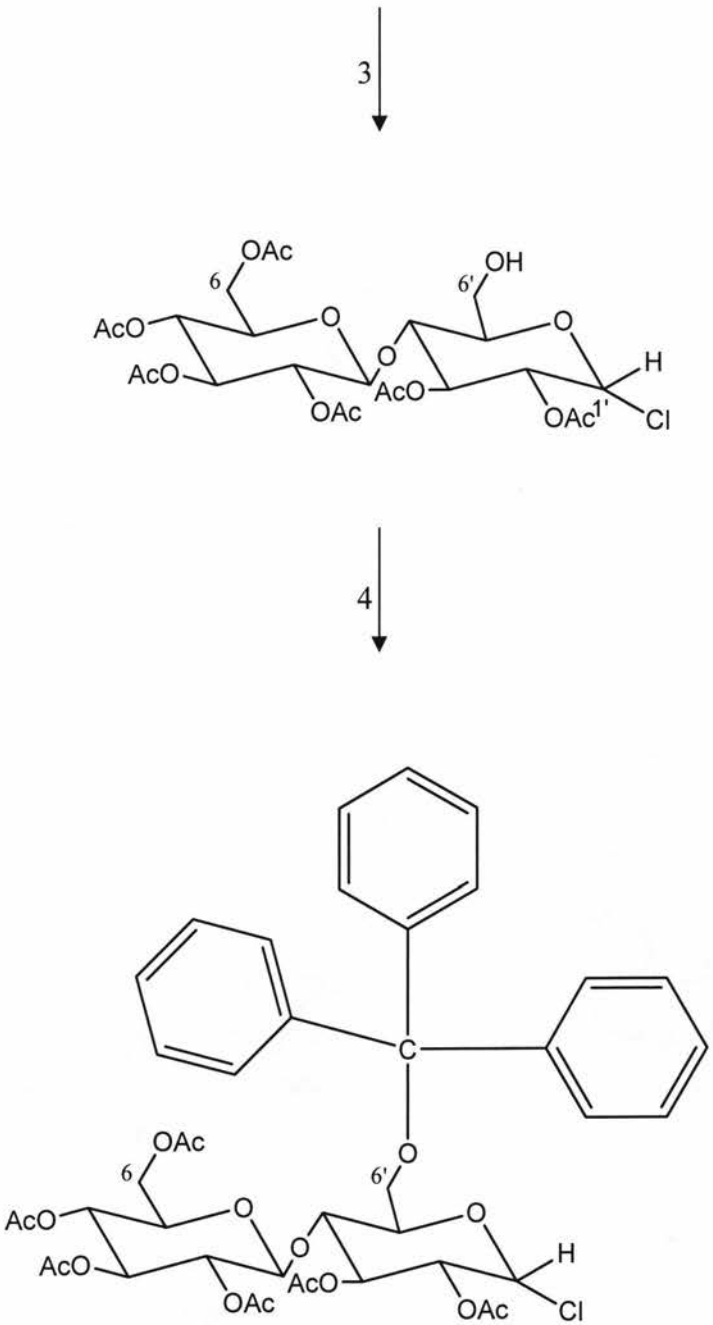
Figure 4.1.1 presents the proposed hypothetical immobilization pathway that the author developed for the attachment of the Active Side Domain (ASD) of a CBH I on a silicon platform. Nine steps are thought to be required starting with a commercially available α-acetobromocellobiose, and finishing with a covalently-immobilized inhibitor (3,4-epoxybutyl-β-cellobioside) that the ASD will recognize and covalently attach to. Figure 4.1.2 presents the final expected outcome of the ASD being covalently immobilized on the immobilized inhibitor. The logical approach and the steps are described below. The detailed hypothetical procedure can be found in Appendix A.

For the purpose of covalently attaching the ASD of an exocellulase to a platform of an AFM, the author searched for a molecule that could covalently and irreversibly attach the ASD. A covalent inhibitor, 3,4-epoxybutyl-β-cellobioside (R and S diastereoisomers), specific to the ASD of a CBH I exocellulase seemed to be a reasonable molecule. The 3,4-epoxybutyl-β-cellobioside is composed of a cellobiose unit which is a saccharide moeity that is specific for recognition by the ASD of CBH I, and the epoxy group is the target for the nucleophilic attack by the Glutamate-212 amino acid located on the ASD of CBH I (Withers and Aebersold, 1995). The next steps to think about were at which point and how to immobilize that inhibitor so that a CBH I enzyme has enough space to approach and grab the inhibitor. Knowing the approximate dimensions of the ASD of CBH I enzyme (60 Å by 50 Å with a 40 Å tunnel) and the idea that amino acids

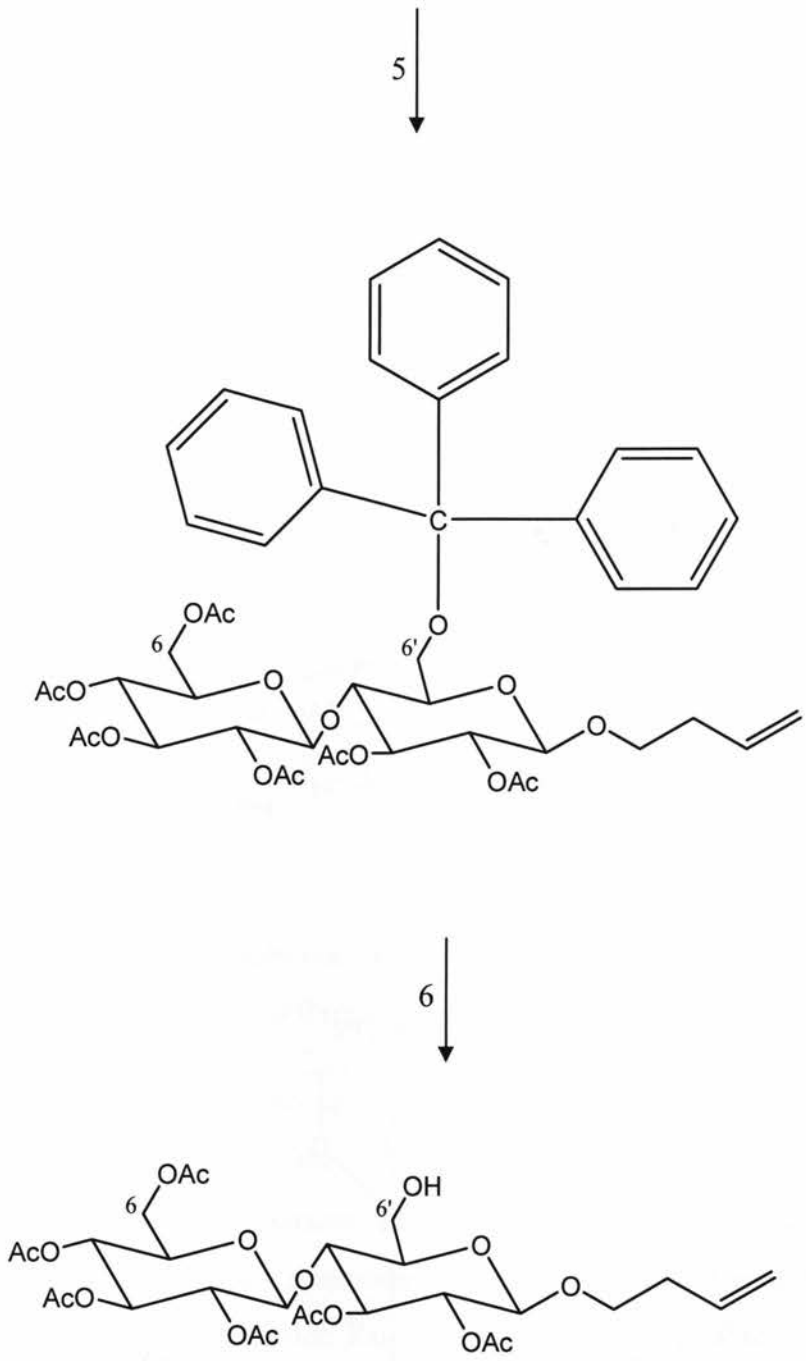
that are involved in inhibitor binding are located close to the surface of the ASD (Divne *et al.*, 1994)), the author thought of immobilizing the inhibitor on a bromosilane spacer arm (~17.4 Å), which must be initially immobilized on the AFM compatible platform (silicon platform). It was thought that once the immobilized inhibitor is treated with CBH I, the inhibitor would covalently attach the ASD of the enzyme. The following steps reflect the logic of the reactions necessary to perform in order to covalently immobilize ASD of CBH I that correspond to the steps shown in Figure 4.1.1 and to the procedure steps described in Appendix A.



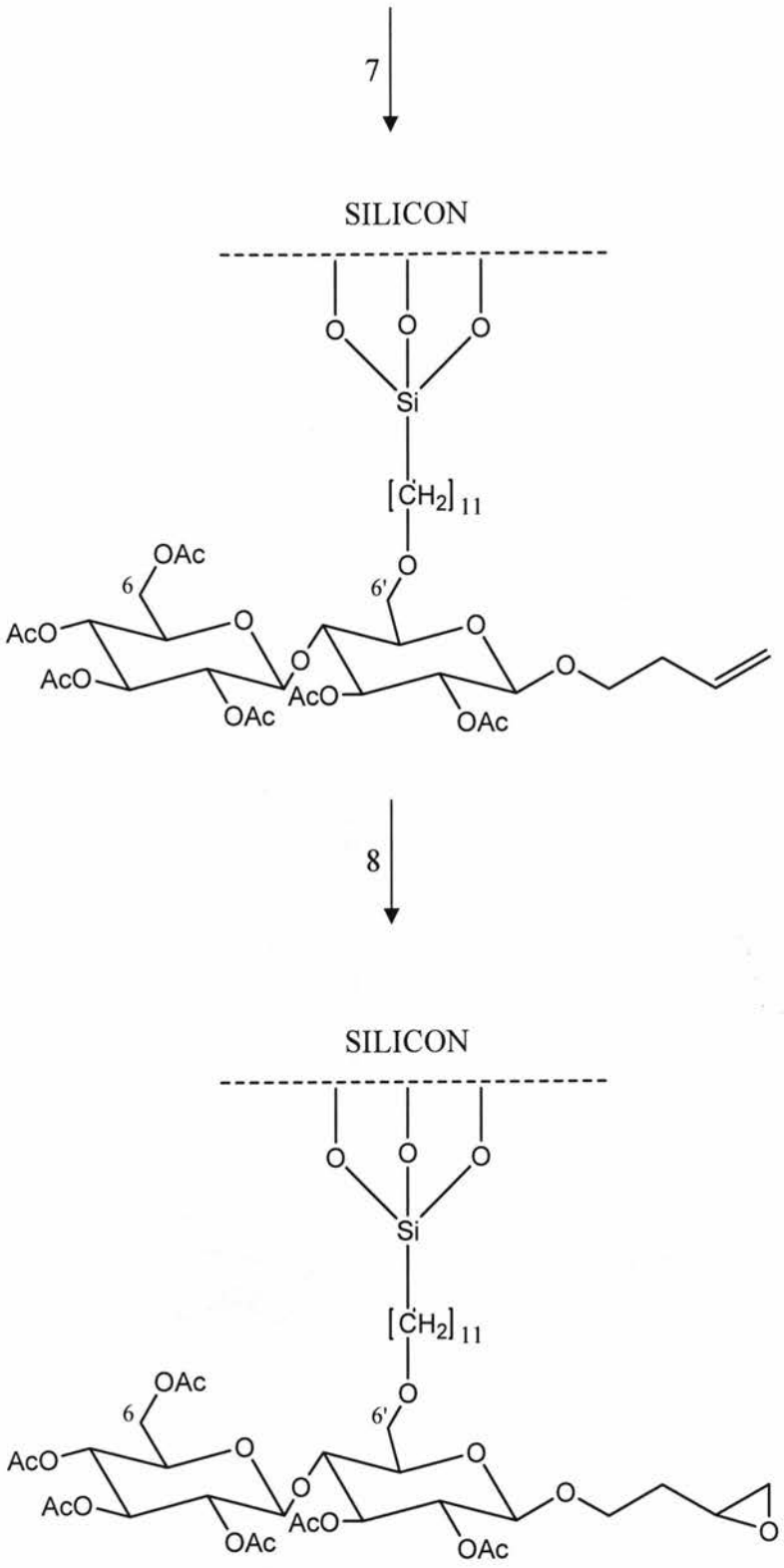
Steps 1 and 2 of Figure 4.1.1.



Steps 3 and 4 of Figure 4.1.1.



Steps 5 and 6 of Figure 4.1.1.



Steps 7 and 8 of Figure 4.1.1.

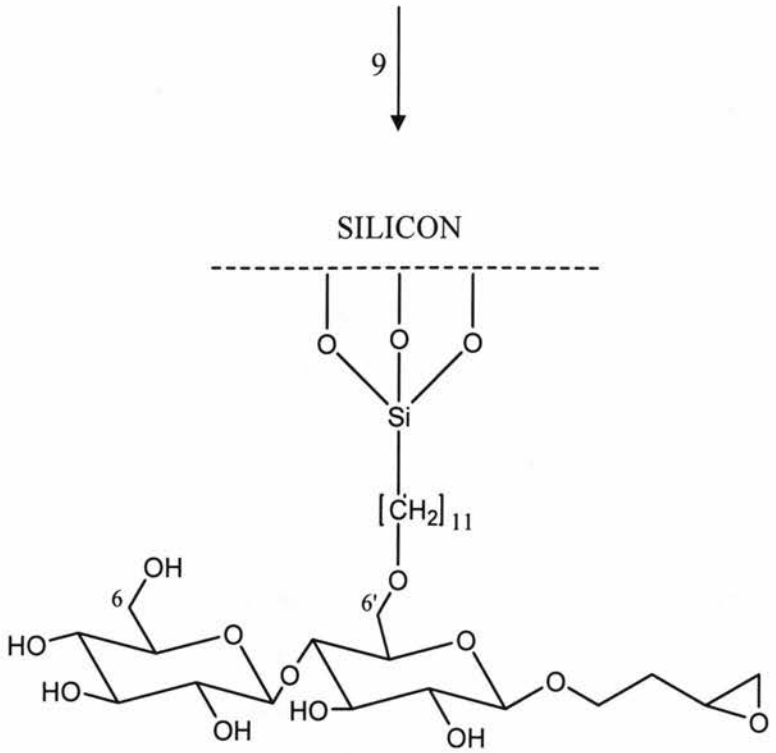


Figure 4.1.1. Hypothetical immobilization of 3,4-epoxybutyl-b-cellobioside (irreversible inhibitor of CBH I) on a silicon platform via 6' carbon.

(1) This Koegins-Knorr reaction would involve an S_N2 displacement of a bromide ion in α-acetobromocellobiose from the α-configuration at carbon 1' by a pentachlorophenoxide ion (an alkoxide ion) to the β-configuration at carbon 1'. The expected product would be pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-β-cellobioside. A pentachlorophenoxide ion was chosen due to its ability to be cleaved faster in the following step (compared to other alkoxide ions tried by other researchers) so that the 1',6'-anhydro ring can be formed faster (Tejima and Okamori, 1972), saving time and offering a simplified procedure for the anhydro ring formation.

(2) This step would involve simultaneous deacetylation of the pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-β-cellobioside and the formation of the 1',6'-anhydro

ring, followed by acetylation of 1',6'-anhydro-β-cellobiose to form 2',3',2,3,4,6-hexa-O-acetyl-1',6'-anhydro-β-cellobiose or cellobiosan. Only the β-configuration of pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-β-cellobioside would give the 1',6'-anhydro ring (Montgomery *et al.*, 1943; Fujimaki *et al.*, 1982; Tejima and Okamori, 1972). When the O-pentachlorophenyl group at carbon 1' and initially deacetylated CH_2OAc group (CH_2OH) at carbon 5' are in the *cis* position (β-configuration), the 1',6'-anhydro ring would be formed by the elimination of pentachlorophenol through the action of the hot alkali, aqueous potassium hydroxide. Oxygen at carbon 1' would close the anhydro cycle at carbon 6'. The yield of the expected product, 2',3',2,3,4,6-hexa-O-acetyl-1',6'-anhydro-β-cellobiose or cellobiosan, should to be ~61% (Fujimaki *et al.* 1982) and the reason of such a low yield is that some α-configuration of pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-α-cellobioside would be present in the reaction mixture and, consequently, the 1',6'-anhydro ring would not be formed.

(3) Titanium tetrachloride would act in the reaction as an electrophile that accepts an electron pair from a nucleophile (2',3',2,3,4,6-hexa-O-acetyl-1',6'-anhydro-β-cellobioside (cellobiosan)), breaking the 1',6'-anhydro ring in cellobiosan and adding chloride to carbon 1' in the α-configuration (Johansson *et al.*, 1963; Koizumi and Utamura, 1978; Delaney and Johnstone, 1986). The resulting product would be 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride. Formation of the α-configuration is a part of the reaction sequence and will be changed in the step 5 to a necessary β-configuration.

(4) An electrophile, trityl chloride, would react with the only available nucleophile, the primary alcohol, at carbon 6' of 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride . The resulting product would be 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride. Tritylation does not cause enantiomerization (Koizumi and Utamura, 1978), therefore the final product of this reaction would remain in α-configuration. The purpose of adding a trityl group to the only available primary alcohol is to protect it from reacting with other chemicals while carrying out the desired reactions. This protecting group has to be

removed when required to regenerate the primary alcohol needed in the upcoming step (7b) through which the inhibitor would be immobilized.

(5) The next product 1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-β-cellobioside would be prepared by a modified Koenigs-Knorr condensation of 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride with unsaturated alcohol (3-buten-1-ol). This is a substitution reaction that would be performed in the presence of the base, silver carbonate, as a promoter (McMurry, 2000). A protecting trityl group would remain to be attached to the structure in the basic environment. An iodide base would be used to deprotonate primary alcohol, 3-buten-1-ol, to form an alkoxide ion. The oxygen atom at this alkoxide ion is nucleophilic and is prone to be attacked by an electrophile. The 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride is a weak electrophile that undergoes a loss of chloride, followed by internal reaction with the ester group (OAc) at carbon 2', promoted by silver carbonate, to form an oxonium ion. The displacement of the oxonium ion by the alkoxide ion attack at carbon 1' occurs with the usual inversion of the configuration known as the S_N2 displacement, and the stereochemical outcome of this reaction would result in a 1',2'-trans stereochemical arrangement (β-configuration) (McMurry, 2000) of covalently attached 1'-O-but-3-enyl arm at carbon 1' with respect to the regenerated acetate at carbon 2'. The resulting product would be an ether, 1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-β-cellobioside.

(6) A weakly acidic environment provided by aqueous acetic acid would make the trityl group leave, but would not break the β(1-4)glycosidic bond. A proton would attach to the oxygen at carbon 6', making it the only -OH available on the acetylated pre-inhibitor through which it would be covalently attached to a functionalized silicon platform.

(7a, 7b) A functionalized silicon platform (11-bromo-undecyltrichlorosilane silicon) would be prepared according to Barbot *et al.* (2007) (see Appendix A, 7a) leaving bromine at the end of the bromosilane arm available to react with the only primary alcohol on the previously synthesized acetylated pre-inhibitor. This substitution reaction would be initiated by the strong base, sodium hydride (NaH), that removes hydrogen

from the primary alcohol at carbon 6' forming an alkoxide ion in the form of a sodium salt of that alcohol (McMurry, 2000). A nucleophilic alkoxide ion (an alkoxide form of 1^{o} alcohol on an acetylated pre-inhibitor) would attack CH_2 next to the bromine at the end of the 11-bromo-undecyltrichlorosilane arm causing bromide to leave and attach itself in place of the bromine to CH_2 of that arm via ether link. The product of this reaction would be the covalently immobilized acetylated pre-inhibitor.

(8) Oxidation of the alkene at the end of the 1'-O-but-3-enyl arm of immobilized acetylated pre-inhibitor with m-chloroperbenzoic acid (peroxyacid) would result in an epoxide formation at the end of that arm. Peroxyacid transfers oxygen to the alkene through a one-step mechanism without intermediates (McMurry, 2000). The resulting product would be a covalently immobilized acetylated epoxide pre-inhibitor.

(9) Methanol (MeOH), and an alkali metal alkoxide catalyst, sodium methoxide (NaOMe), would be used for the deacetylation of the covalently immobilized acetylated epoxide pre-inhibitor. First, acetoxy groups on the covalently immobilized acetylated pre-inhibitor would react with sodium methoxide to form methyl acetate and a sodium derivative of cellulose having the functionality -ONa. Next, a molecule of methanol would react to replace the sodium atom with hydrogen (McMurry, 2000). The final product would be a deacetylated and covalently immobilized inhibitor on silicon platform.

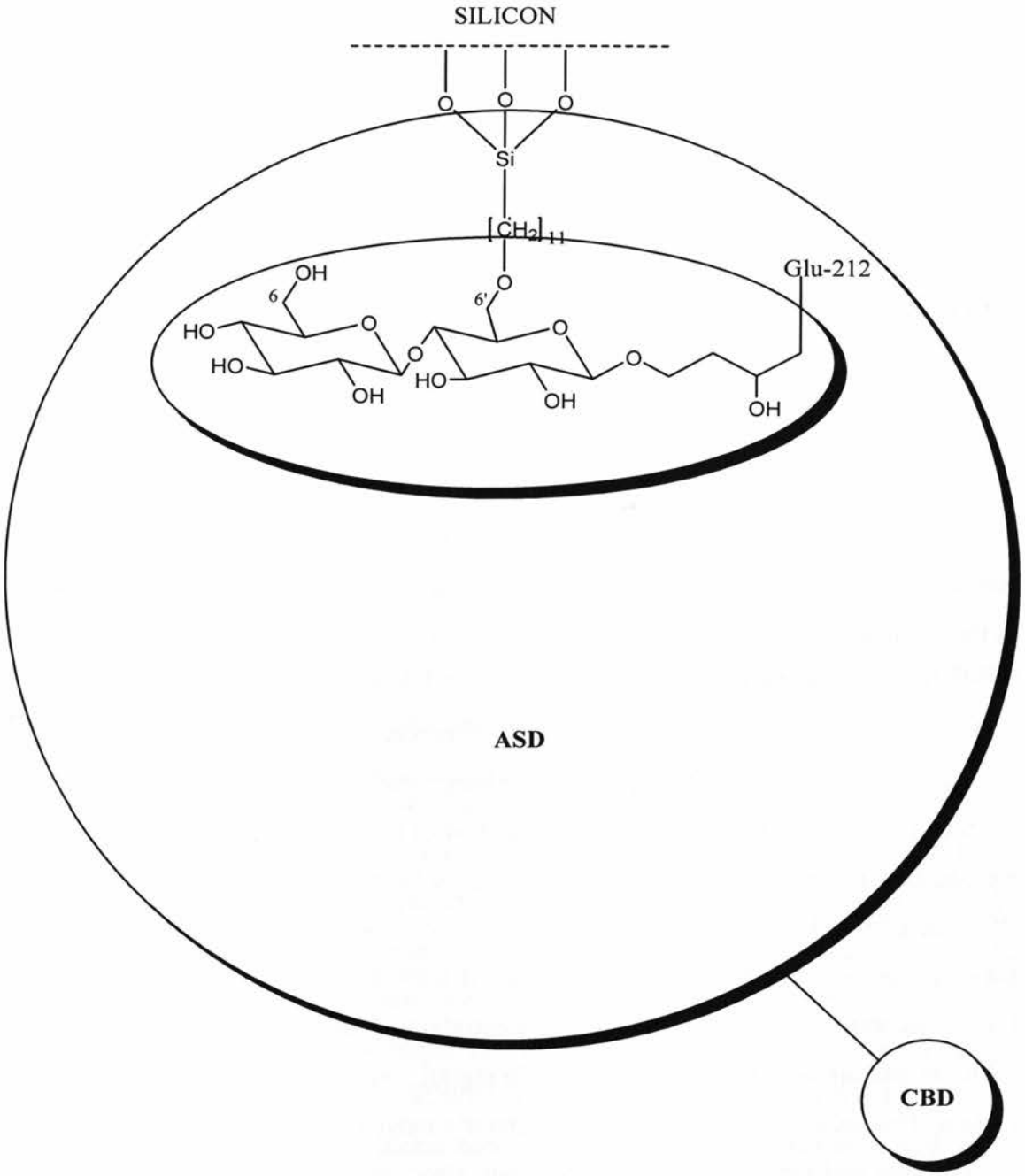


Figure 4.1.2. Hypothetical immobilization of CBH I' ASD on the irreversible inhibitor - not to scale.

At this point, incubation of the covalently immobilized inhibitor with CBH I enzyme would result in covalent immobilization of the ASD of the enzyme on the silicon

platform (Figure 4.1.2) leaving the CBD free-floating and available for the evaluation of a force profile between the CBD and the substrate cellulose. All reaction steps were created so that the inhibitor would end up being attached to the spacer arm via the 6' carbon and not 6 carbon, so that CBH I enzyme can recognize and approach a saccharide moiety of the inhibitor. At the same time, an epoxy group of the inhibitor was planned to be available to covalently link the amino acid on ASD. The next step to follow was the purification of CBH I which is discussed below.

4.2 Purification of CBH I from the Commercial Cellulase Mixture

Some specifications of the commercial cellulase mixture such as protein concentration, molecular weight, and activity on carboxymethyl cellulose (CMC), as well as the purification of CBH I are covered in this section.

The calibration curve shown in Figure 4.2.1 was used to follow the recovery of CBH I during its purification from the commercial cellulase mixture. Each point represents a mean value of the duplicate absorbance readings at 595nm. The original data are found in Table B.1 of Appendix B. A method of least squares was applied to obtain the equation for the best fit line that was A_{595nm}=0.8618[BSA](mg/mL)+0.0196 with an R^2 value of 0.9948. The R^2 value range from 0 to 1, with 1 representing a perfect fit between the data and the line. The R^2 value of 0.9948 indicated excellent correlation. Using this standard curve, the average concentration of total protein in the commercial cellulase mixture was determined to be 46.76 mg/mL, which corresponds to 4.676 % w/v protein. This value agrees with the manufacturer's range of 1-40 % w/v (Wenger, 2006). All raw data and sample calculations on mean, standard deviation about regression, and standard deviation of protein concentration can be found in Appendix B.

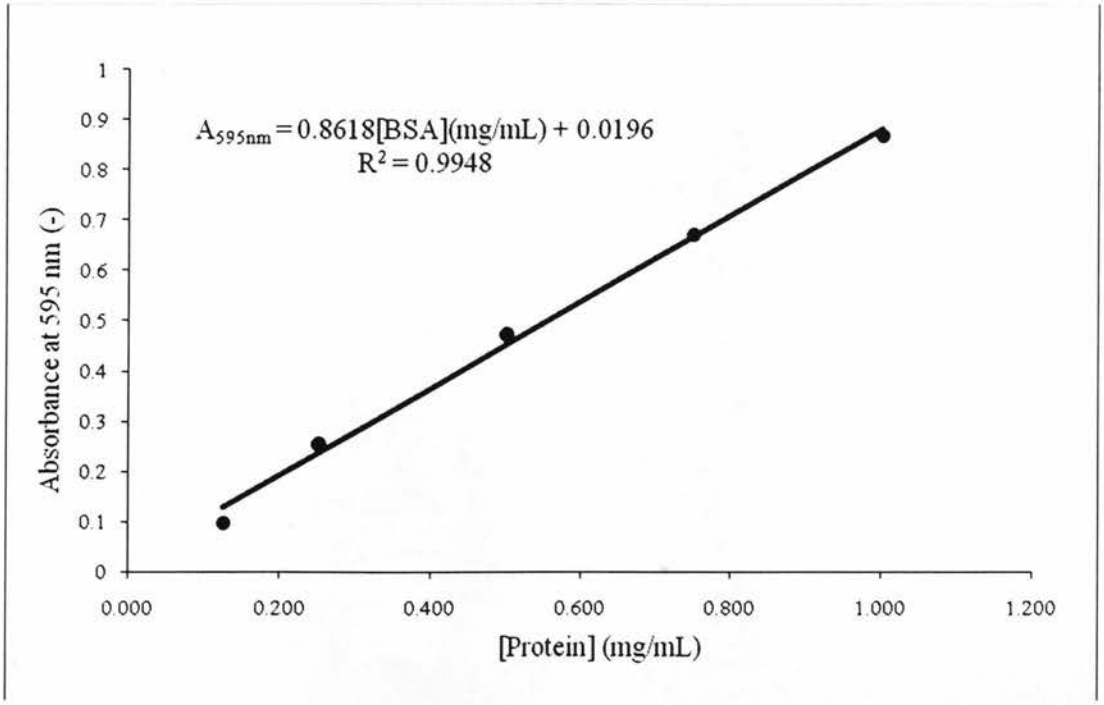


Figure 4.2.1. Calibration curve for protein concentration with Bovine Serum Albumin (BSA) at 595nm. Mean values of duplicate readings are shown.

Figure 4.2.2 shows the molecular weight (MW) distribution of proteins from an SDS/PAGE separation of the commercial cellulase mixture #NS50013 CCN03067 (Novozymes). The first column represents protein standards ranging in weight from 10 kDa to 250 kDa. The other columns contain the cellulase mixture diluted 20 and 40 times, respectively. The largest band is expected to be the CBHI enzyme. The cellulase manufacturer mentioned that the major enzyme in the commercial cellulase mixture is ~60% w/v CBHI (Wenger, 2006). SDS/PAGE results confirmed that CBH I is a major protein in the mixture, giving a thicker band on the gel, with an approximate MW of 65 kDa that also corresponds to the literature values reported (Shoemaker *et al.*, 1983; Pere *et al.*, 1995; Bhikhabhai *et al.*, 1984; Tomme *et al.*, 1988; Gerber *et al.*, 1997; Van Tilbeurgh *et al.*, 1986). Other bands are not clearly seen on the Figure 4.2.2, but are clearly seen on the gel. They represent three other minor proteins present in the commercial cellulase mixture: ~20% w/v endocellulase I (EGI) and endocellulase II (EGII), 55 kDa and 48 kDa respectively, and ~15% w/v exocellulase II (CBHII), 53 kDa.

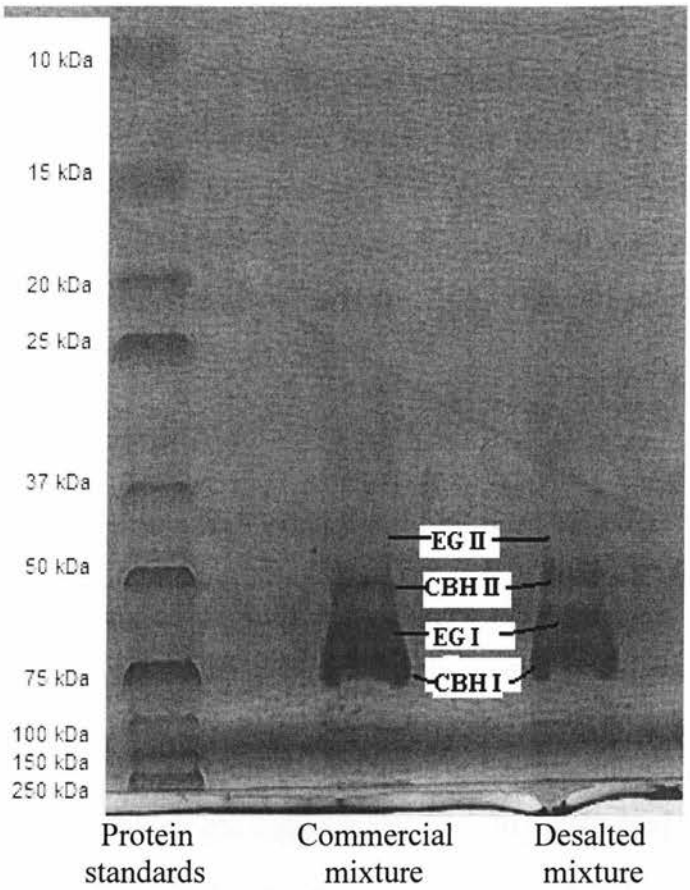


Figure 4.2.2. SDS/PAGE of the commercial cellulase mixture #NS 50013 CCN03067.

Total enzyme activity of the mixture on 1% CMC was 0.9887 International Units per mg of a total protein (IU/mg) (see calibration curve of reducing sugars and the calculation of activity in Appendix B). In comparison, Saddler *et al.* (1985) reported the value of the 1% CMC activity of the cellulase mixture from *Trichoderma reesei* (Rutgers University, United States) to be 1.0 IU/mg (the same as this author obtained).

Using the commercial cellulase mixture, the Pere *et al.* (1995) purification scheme was performed to obtained pure CBH I. Initially the commercial cellulase mixture was desalted to remove salts and pigments using Sephadex G-25. After the desalting, the concentration of the desalted commercial cellulase mixture was 4.408% w/v compared to 4.676% before desalting. It can be concluded that almost all proteins were recovered. After the desalting step, protein separation followed.

Figure 4.2.3 represents the SDS/PAGE of the eluted fractions after the commercial cellulase mixture was subjected to a DEAE-Bio Gel A anion-echange column according to the Pere *et al.* (1995) purification scheme. It should be noted that purification was performed on a much smaller scale, 97.5% smaller scale than in Pere *et al.*, (1995). Four fractions were collected. Each fraction represents the eluted protein from DEAE-Bio Gel A with a buffer containing a required salt concentration. The first column to the right includes protein standards ranging in weight from 10 kDa to 250 kDa. The other four columns represent the presence of a major protein (CBH I) in eluted fractions #1-#4. All four fractions contained CBH I protein, but fraction #4 contained relatively purified (partially) CBH I.

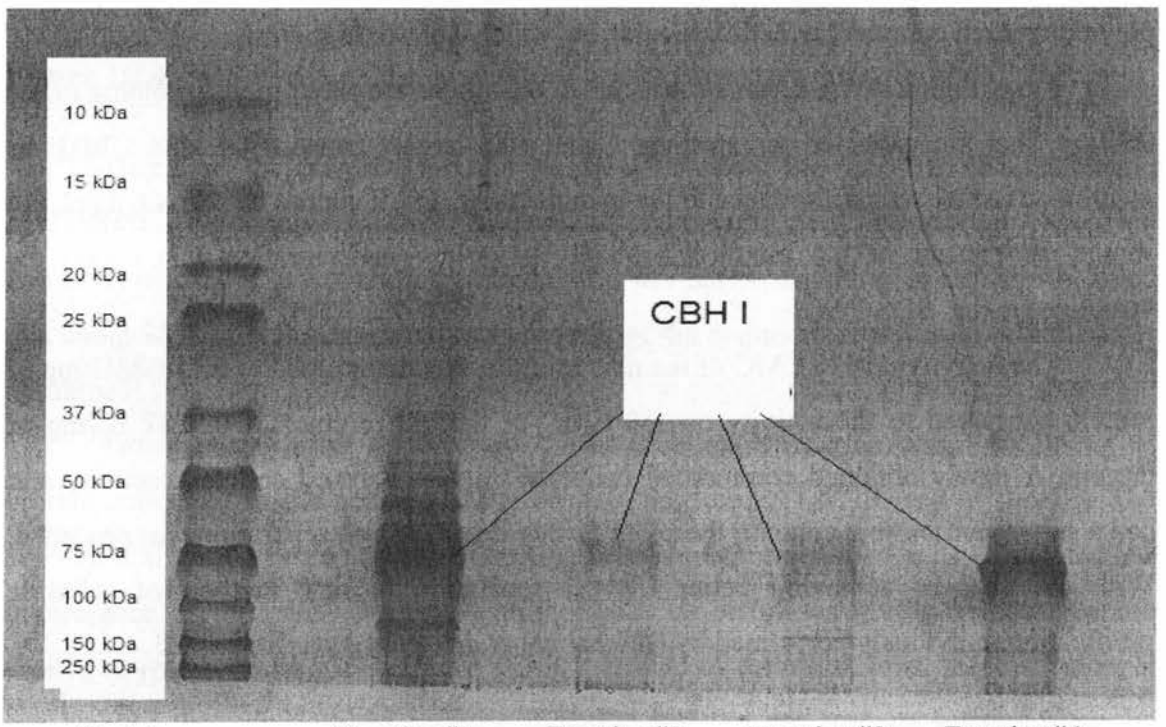


Figure 4.2.3. SDS/PAGE of fractions after separation on DEAE-Bio Gel A of a desalted cellulase mixture #NS50013 CCN03067.

According to the Pere *et al.* (1995) purification scheme, it was expected that CBH I would be eluted in fraction #4, while fraction #1 would contain endocellulase II (EG II), fraction #2 exocellulase II (CBH II), and fraction #3 endocellulase I (EG I). Contrary to expectations, all four fractions contained CBH I enzyme, but fraction #4 contained relatively purified (partially) CBH I.

At this point, more of the commercial cellulase mixture was required, therefore a new mixture was ordered. Analysis such as protein concentration, SDS/PAGE, and total activity were performed on the new cellulase mixture and is described below.

The average concentration of protein in the cellulase mixture was determined to be 42.40 mg/mL, which corresponds to 4.240 % w/v protein. This value agrees with the manufacturer's range of 1-40 % w/v (Wenger, 2006). Protein concentration of a new commercial cellulase mixture reflects similarities with the protein concentration of a previously used commercial cellulase mixture, which was 46.76 mg/mL.

From Figure 4.2.4 it can be concluded that there are three major proteins in the mixture, that are expected to represent CBHI (the largest band), EGI, and CBHII as mentioned earlier with accordance to the manufacturer. EG II cannot be seen on a gel, but should be present in the mixture, probably at a low concentration that cannot be visualized using SDS/PAGE.

The activity on 1% CMC of the new mixture was determined to be 1.07 IU/mg of protein compared to the activity on 1% CMC of the old mixture of 0.9887 IU/mg of protein. A newly obtained commercial cellulase mixture showed cellulase activity and had a significant protein content, therefore further desalting and separation was proceded. With the goal of achieving better CBH I separation, a new method of cellulase purification, previously performed by Bhikhabhai *et al.* (1984), was attempted.

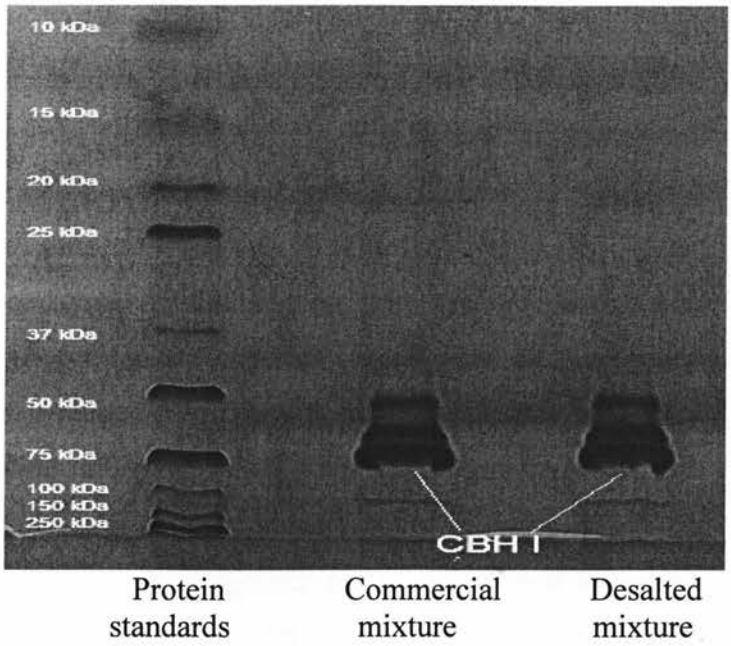
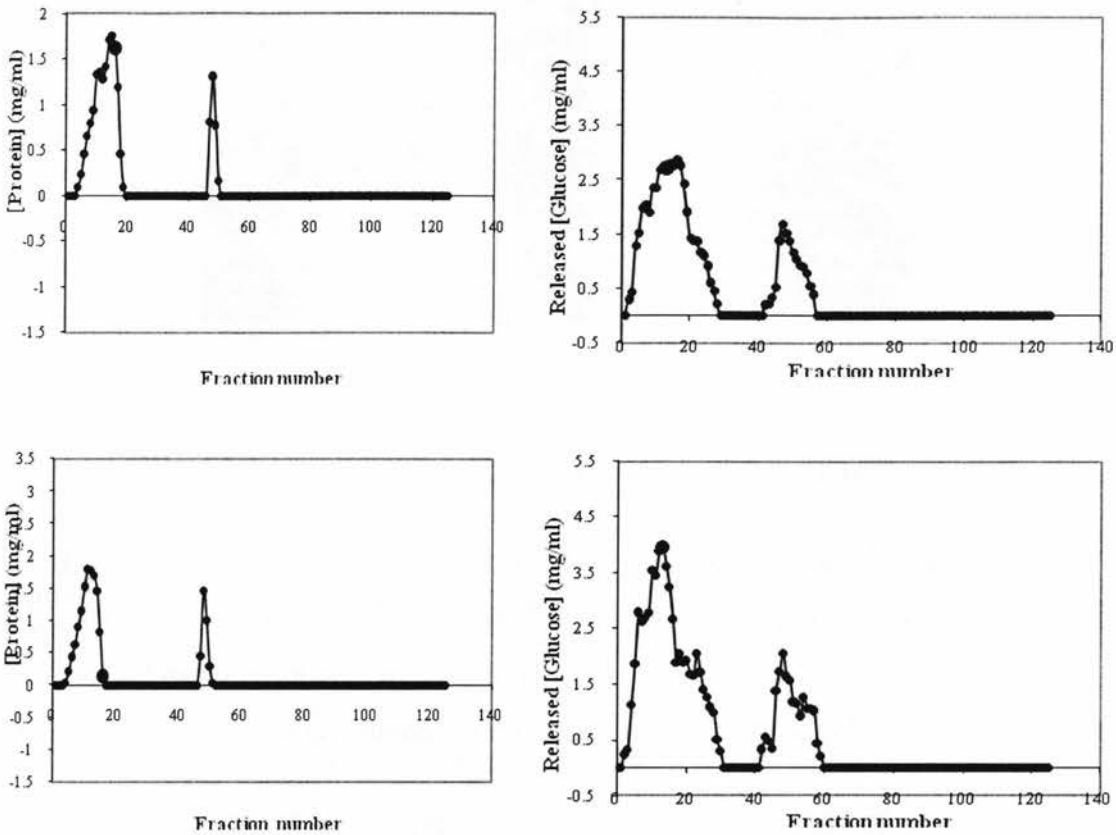


Figure 4.2.4. SDS/PAGE of the commercial cellulase mixture #NS 50013 CCN03105.

This time, the cellulase mixture was desalted using Bio-Gel P-10 (Bhikhabhai *et al.* (1984)). The concentration of the desalted commercial cellulase mixture (based on two runs) was 4.466% w/v compared to 4.540% w/v before desalting. It can be concluded that about 98% of cellulases were recovered from the commercial cellulase mixture after desalting.

Total activity after desalting of the new cellulase mixture was 1.30 IU/mg of protein compared to the activity of the original mixture of 1.07 IU/mg of protein. The activity is slightly higher after the desalting because the removal of salts eliminated the interference of the salts' ions with amino acids of cellulases giving cellulases more chance to interact with the substrate (CMC) instead of the salts' ions. In conclusion, it can be said that the desalted mixture is active and did not lose its activity after the application on Bio-Gel P-10. After an initial desalting step, primarily to remove impurities, anion-exchange chromatography according to Bhikhabhai *et al.* (1984) followed.

One hundred and twenty five fractions were collected. The protein concentration and released glucose of all fractions were measured after DEAE Bio Gel A separation. Figure 4.2.5 shows the concentration profile vs. eluted fractions for both protein and released glucose.



(a) (b)

Figure 4.2.5. Anion-exchange chromatography on replicate DEAE Bio Gel A of desalted commercial cellulase mixture: (a) the protein concentration vs. fraction number of two replicates, and (b) the glucose concentration vs. fraction number of two replicates. One hundred and twenty five fractions (0.2mL each) were collected in each of the two compared runs.

Two major peaks are observed in both runs of protein concentration profile (Figure 4.2.5 (a)). During the first run (top of the Figure 4.2.5 (a)), protein elution started at fraction 4, reaching maximum concentration peak at fraction 15, and ended at fraction 20. Therefore, fractions ranged from 4 to 20 represent first peak of eluted proteins from the first run. Second peak protein elution from the first run started at fraction 51, reaching

maximum concentration peak at fraction 48, and ended at fraction 55. Therefore, fractions ranged from 51 to 55 represent second peak of eluted proteins from the first run. During the second run (bottom of the Figure 4.2.5 (a)), protein elution started at fraction 4, reaching maximum concentration peak at fraction 11, and ended at fraction 16. Therefore, fractions ranged from 4 to 16 represent first peak of eluted proteins from the second run. Second peak protein elution from the second run started at fraction 51, reaching maximum concentration peak at fraction 48, and ended at fraction 55. Therefore, fractions ranged from 51 to 55 represent second peak of eluted proteins from the second run. One run is the reliable replicate of another run, both giving two major peaks of eluted proteins in corresponding fractions.

Two major peaks are also observed in both runs of total activity profile (Figure 4.2.5 (b)). During the first run (top of the Figure 4.2.5 (b)), released glucose as a result of the digestion of CMC by eluted proteins was initially determined in fraction 2, reaching maximum activity peak in fraction 16, and ending in fraction 28. Fractions 2 to 28 represent first peak of total activity from the first run. Second peak of total activity from the first run started at fraction 42, reaching maximum activity at fraction 47, and ending at fraction 56. Fractions from 42 to 56 represent second peak of total activity from the first run. During the second run (bottom of the Figure 4.2.5 (b)), released glucose was initially determined in fraction 2, reaching maximum activity peak in fraction 13, and ending in fraction 30. Fractions 2 to 30 represent first peak of total activity from the second run. Second peak of total activity from the second run started at fraction 42, reaching maximum activity at fraction 48, and ending at fraction 59. Fractions from 42 to 59 represent second peak of total activity from the second run. Two major peaks of activity reflect two major peaks of eluted proteins, providing the information about the total activity of eluted proteins.

There was enzymatic activity found in some fractions while no protein concentration was detected. Probably, protein content was very low to be detected but was significantly active on CMC substrate, resulting in broader activity peaks but narrower protein concentration peaks.

Concentration profile vs. eluted fractions for both protein and released glucose only gave information about the presence of total protein concentration and its total

activity; both concentration profiles did not give information about what type of protein was eluted in each peak. Therefore, at this point, each peak was pooled and SDS/PAGE was performed to determine what type of protein was represent each peak. From the information in Figure 4.2.5 it can be concluded that all cellulases were eluted in the first peak (all bands are shown) and second peak contained no major cellulase, at least not enough protein to be shown on the gel. The second peak still showed the activity because it doas contain some cellulases that are not detectable by SDS/PAGE but are very active on CMC substrate.

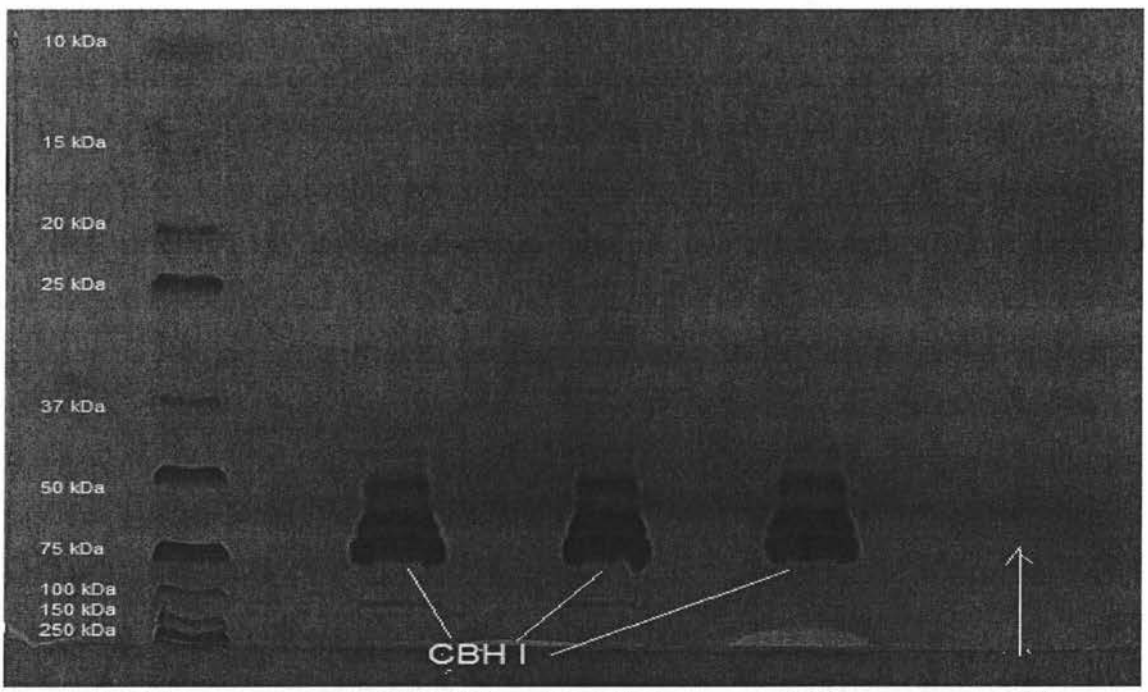


Figure 4.2.6. SDS/PAGE of fractions after separation on DEAE-Bio Gel A of a desalted cellulase mixture #NS50013 CCN03105.

If to compare Bhikhabhai *et al.* (1984) cellulase purification results with the results obtained in this research, Bhikhabhai *et al.* (1984) obtained three peaks for both protein and released glucose concentration, with fractions 1-40 representing the first peak, fractions 50-90 representing the second peak, and fractions 95-125 representing the third peak. CBH I was expected to be eluted in the third peak. In this research, cellulases

were eluted in two peaks, with fractions 2-28 representing the first peak and fractions 42-56 representing the second peak. As almost all cellulases were eluted in the first peak, and the second peak only contained traces of cellulases, no major separation was obtained with Bhikhabhai *et al.* (1984) purification scheme.

Based on the attempts of CBH I purification from the commercial cellulase mixture according to the two purification schemes, it follows that partial CBH I purification according to Pere *et al.* (1995) purification scheme was achieved, while no CBH I purification was achieved when Bhikhabhai *et al.* (1984) purification scheme was used. The reason could be that the researchers of the original purification procedures used properly equipped and elution rate controlled (1 mL/h) anion-exchange columns. In this research, gravity controlled the elution rate resulting in much faster elution rate (6.5 mL/h). Probably, slower rate of cellulase elution gave enough time for cellulases to attach to the resin and to be eluted in a required order.

In the case of partially purified CBH I, the following proof was thought in order to prove the presence of CBH I. A recovered fraction that contain relatively pure CBH I should be subjected to a trypsin or papain digestion that would cut in the linker of CBH I. Application of the digests on SDS/PAGE should prove the presence of two domains (~55kDa ASD and ~10kDa CBD) (Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986; Divne *et al.*, 1993; Divne *et al.*, 1994; Abuja *et al.*, 1988). Specific activity of CBH I that is significantly active on p-nitrophenyl-β-cellobioside would be another proof of CBH I presence in a recovered fraction.

In the case of CBH I immobilization, the following proof was thought in order to prove an immobilization of CBH I. The topology and density of immobilized proteins on a silicon wafer can be examined and characterized. The topology of a silicon wafer surface-containing immobilized protein can be performed with an AFM. A combination of an AFM with the immobilization kinetics can provide the surface density of the protein monolayer (Liao *et al.*, 2004). Measurements of the density and thickness of immobilized protein layers can be also performed using ellipsometry, surface plasma resonance (SPR), and scanning electron microscopy (SEM) (Barbot *et al.*, 2007; Hodneland *et al.*, 2002; Murphy *et al.*, 2004; Liao *et al.*, 2004).

With the two lengthy CBH I purifications attempted the author developed a thought of having no need to purify CBH I from the commercial cellulase mixture. As the chosen inhibitor is selective and specifically as well as covalently binds ASD of CBH I exocellulase, there might be no need to purify CBH I. Once the inhibitor will be immobilized on a silicon support, further experiments have to be performed in order to prove that thought.

Chapter 5 – Conclusion and Recommendations

One of the objectives was to purify CBH I exocellulase from the commercial cellulase mixture. A partial purification of the CBH I that was performed on a much smaller scale with uncontrolled flow rate was successful. Another objective was to propose a scheme that would covalently immobilize CBH I exocellulase via its ASD on a silicon support. A theoretically developed hypothetical scheme was constructed with the provided detailed procedure. The approach of immobilizing the inhibitor specific to the ASD of CBH I enzyme led to the possibility that no purification of CBH I could be required. Since the chosen inhibitor is selective and irreversible, it is expected that upon immersing silicon wafer with the immobilized inhibitor into the commercial cellulase mixture containing CBH I, the inhibitor would selectively bind the ASD of CBH I enzyme. Once the ASD of CBH I is immobilized on the inhibitor, the AFM force profile between the free-floating CBD and substrate cellulose could be established. Next step of the future research would be to perform, experimentally, a covalent immobilization of the inhibitor specific to the ASD of CBH I enzyme according to the provided hypothetical procedure, followed by a covalent immobilization of the ASD of CBH I enzyme on the inhibitor, followed by the establishment of a binding force between the free-floating CBD of immobilized CBH I enzyme and a substrate cellulose by the AFM.

Appendix A – Covalent Immobilization of CBH I via its Active Site Domain (Procedure)

Each of the numbered steps presented below correspond to the reactions shown in Figure 4.1.1 and 4.1.2.

(1) Synthesis of pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-β-cellobioside (Fujimaki *et al.*, 1982).

Add 157g of commercial 95% sodium pentachlorophenoxide to a solution of 230g α-acetobromocellobiose in 1.0L acetone. Boil the mixture for 6h under reflux (with stirring), cool and filter. Evaporate filtrate to dryness, and extract the residue with dichloromethane. Wash the dichloromethane extract with ice-cold 1M aqueous sodium hydroxide, then water, and evaporate. Recrystallize the crude product from ethyl acetate-hexane to give pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-β-cellobioside. About 200g expected, 61%, melting point 212-212.5°C, specific optical rotation: $[\alpha]_D^{28}$-12° (*c* 1.0, chloroform); the specific rotation, $[\alpha]_D$, of a compound is a physical constant characteristic of a given optically active compound and is defined as the observed rotation when the sample pathlength is 1 dm, the sample concentration is 1g/mL, and light of 589nm wavelength (D) is used. The number 28 refers to the temperature in Celsius at which the measurement is performed and the solvent in which the material is dissolved is chloroform, (*c* 1.0, chloroform) means the concentration of chloroform solvent is 1.0g/mL (McMurry, 2000). The formal unit of the specific rotation is in degrees (circle based-360°). Negative value means a β-configuration and positive value means an α-configuration.

(2) Synthesis of 2',3',2,3,4,6-hexa-O-acetyl-1',6'-anhydro-β-cellobiose (cellobiosan) (Fujimaki *et al.*, 1982).

Heat a 80g of pentachlorophenyl 2',3',6',2,3,4,6-hepta-O-acetyl-β-cellobioside in 500mL of 4M aqueous potassium hydroxide for 20h at 120° C with stirring, cool, treat with 3M sulfuric acid to pH 3-4, and filter. Adjust the pH of the filtrate to 7 by adding sodium hydrogen carbonate, and evaporate the solution to dryness. Add 60g of anhydrous sodium acetate and 500mL acetic anhydride to the residue. Boil the mixture for 3h under reflux, cool, pour into ice water, stir at room temperature, keep overnight, and extract

with chloroform. Wash the extract with aqueous sodium hydrogen carbonate, then water, dry, and evaporate to a syrup. Dissolve in ethanol, decolorize with charcoal, and then recrystallize from ethanol to produce 2',3',2,3,4,6-hexa-O-acetyl-1,6-anhydro-β-cellobiose (cellobiosan), (~35g, 61%), melting point 142-143°C, $[\alpha]_D^{20}$-54° (*c* 1.3, chloroform).

(3) Conversion of 2',3',2,3,4,6-hexa-O-acetyl-1',6'-anhydro-β-cellobiose (cellobiosan) to 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride (Johansson *et al.*, 1963).

Add 10g of cellobiosan, 4mL titanium tetrachloride and 0.3mL acetic acid (0.3mL) to 200mL of anhydrous, ethanol-free chloroform. A yellow precipitate is expected to be formed immediately. Reflux the mixture for 10h (bath temperature 65°C), cool and wash successively with ice-water, saturated aqueous sodium hydrogen carbonate, then water, dry over calcium chloride, decolorize with a small amount of activated carbon and concentrate. Crystallize the residue from chloroform-ethyl ether to yield 7.2g of crude material, melting point 220-224°C. Crystallize from acetone-isopropyl ether to yield pure 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride, melting point 231-232°C, $[\alpha]_D^{20}$ + 79° (*c* 2.0 in chloroform).

(4) Protection of the only primary alcohol in 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride with trityl group to produce 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride (Koizumi and Utamura, 198).

Stir 2g of well-dried and powdered compound 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride in 200mL pyridine for 15-30min at 100°C and dissolve as far as possible. Then add 1.6g of trityl chloride and stir the mixture continuously for 1h at 100°C. Evaporate off the solvent under reduced pressure, dissolve the residue in a minimum volume of methanol, and pour the solution into ice-water. Collect the precipitate by filtration, wash with cold water, and dry to yield ~3g of a mixture of trityl ethers in a powder form. Two compounds are expected: unreacted 2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride and its tritylated version 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride. Use TLC (silica gel 60, 0.25mm) with chloroform-acetone-methanol-water 58:20:20:2 (v/v) solvent to indicate the presence of both. Unreacted

2',3',2,3,4,6-hexa-O-acetyl-α-cellobiosyl chloride can be recovered from its tritylated version by silica gel chromatography using chloroform-methanol 9:1 as a solvent (the more polar unreacted compound is expected to be more strongly adsorbed to the surface of silica gel, retaining it longer on the silica gel than its tritylated version). The purity of separated compounds can be checked with TLC using chloroform-acetone-methanol-water 58:20:20:2 as a solvent. Compound 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride is useful for the research, a powder form of which should be made.

(5) The substitution of the chloride in 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride with the inhibitor arm, 3-buten-1-ol, to yield 1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-β-cellobioside (Rodriguez and Stick, 1990; Legler and Bause, 1973).

Dissolve 0.2g of 2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-α-cellobiosyl chloride in 5mL of dry dichloromethane and add to a mixture of 0.6mL 3-buten-1-ol (3.1mmol), 255mg silver carbonate (0.92mmol), a crystal of iodine, 750mg anhydrous calcium sulfate and 750mg powdered molecular sieve 4A in 5mL of dry dichloromethane previously stirred at room temperature with the exclusion of light and moisture for 30min. Stir the mixture for 24h and after a normal workup[1] followed by flash chromatography[2] (EtOAc/petrol, 2:1) about 1.5g of 1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-β-cellobioside will be obtained. The permanganate test for double bonds presence can be performed: place a drop of the reaction mixture onto filter paper and spray with 0.5% $KMnO_4$ in 5% Na_2CO_3 (Test for Double Bonds, Sigma-Aldrich).

[1]Normal workup means extraction into an organic solvent (here dry dichloromethane), sequential washing of the organic extract with saturated 0.5M aqueous sodium bicarbonate solution and water, drying ($MgSO_4$), and concentration on a rotary evaporator at water aspirator pressure and bath temperature of 45°C. All solvents are purified by distillation.

[2]Flash chromatography is performed on columns of silica gel (<230 mesh) under positive pressure (30-40psi).

(6) Removal of trityl group from 1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-β-cellobioside with weak acid to make primary alcohol at 6' carbon of partially synthesized acetylated pre-inhibitor (1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-β-cellobioside) available for covalent attachment to the functionalized silicon platform (Koizumi and Utamura, 1981).

Dissolve 0.1mmol of 1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-6'-O-trityl-β-cellobioside in 10mL of 80% aqueous acetic acid and heat for 30min at 100°C. Remove the solvent under reduced pressure, and remove traces of acetic acid from the residue by repeated codistillation with methanol. TLC with solvents benzene-ethyl acetate 2:1 or 1:1 can be used to check the purity of the formed acetylated pre-inhibitor (1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-β-cellobioside).

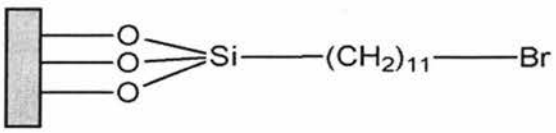
(7a) Preparation of functionalized silicon platform: 11-bromo-undecyltrichlorosilane silicon (Barbot *et al.*, 2007).

Pretreatment of silicon platforms (wafers). Use silicon wafers with an oxide layer. Sonicate them in acetone/methanol mixture, blow-dry in nitrogen and immerse in a freshly prepared "piranha" solution (70/30 v/v, concentrated sulfuric acid, 30% hydrogen peroxide) for 1h at 80-100°C. This procedure removes hydrocarbon contaminants from the surface and yields a 2nm thick native oxide layer that can be determined by ellipsometry. Ellipsometry is used for measuring layer thicknesses (Barbot *et al.*, 2007). Repeat until the surface is perfectly hydrophilic. Subsequently, rinse surface with large amounts of distilled water, dry under nitrogen stream and use immediately.

Synthesis of 11-bromo-undecyltrichlorosilane. Place 10mmol of undecyl-10-enyl bromide in a heavy walled vessel containing about 2mg of H_2PtCl_6 (hydrogen hexachloroplatinate) hexahydrate dissolved in 200µl of isopropanol. Add a 3M excess of trichlorosilane with stirring. Seal the flask and heat for 6h at 60°C. Remove excess trichlorosilane and isolate the residue by Kugelrohr distillation, identify by ^1H NMR.

Silanization of silicon wafers. In a vial, immerse a freshly pre-treated wafer under nitrogen atmosphere in a cold 1% solution of freshly distilled 11-bromo-undecyltrichlorosilane in hexane at 4°C. Stir the solution with a rod, cap the vial and place at 4°C for 3h. Remove the wafer from the adsorbate solution and sonicate it. Then,

immerse the wafer in chloroform and rinse with toluene and ethanol. Finally, sonicate the wafer in ethanol, rinse with ethanol and blow-dry in nitrogen. The final product is a covalently attached 11-bromo-undecyltrichlorosilane to a silicon platform:



Barbot *et al.* (2007) calculated thickness of bromosilane arm monolayer to be 17.4 angstroms. Ellipsometry can be used to determine a thickness of monolayer experimentally.

(7b) Substitution reaction between partially synthesized acetylated pre-inhibitor (1'-O-but-3-enyl-2',3',2,3,4,6-hexa-O-acetyl-β-cellobioside) at primary alcohol (carbon 6') and 11-bromo-undecyltrichlorosilane arm at bromine to produce immobilized acetylated pre-inhibitor.

The reaction involves sodium hydride (NaH) where $:H^-$ serves as a strong base (McMurry, 2000):

ROH + **NaH** → **RO:$^-$** + **Na^+** + **H_2**

1^o alcohol (acetylated pre-inhibitor) — sodium hydride — alkoxide

RO:$^-$ + **$R'CH_2Br$** $\xrightarrow{THF^1}$ **$ROCH_2R'$** + **Br^-**

alkoxide — immobilized 11-bromo-undecyl-trichlorosilane arm — immobilized acetylated pre-inhibitor

[1]Tetrahydrofuran solvent.

The conditions of this reaction have to be found in the literature. The expected product is a covalently immobilized acetylated pre-inhibitor.

(8) Oxidation of double bond in the end of the arm of the immobilized acetylated pre-inhibitor to produce immobilized acetylated epoxide pre-inhibitor (Rodriguez and Stick, 1990).

Heat at reflux for 8h the immobilized acetylated pre-inhibitor (145mg immobilized, weigh by mass difference) with 80% m-chloroperbenzoic acid and a few crystals of 2,6-di-t-butyl-4-methylphenol in dichloromethane. Flush the platform with 0.5M saturated aqueous sodium bicarbonate solution then water to wash away unbound compounds to give immobilized acetylated epoxide pre-inhibitor. Determine the epoxide content by Kerckow's method (Kerckow, 1937).

(9) Deacetylation of immobilized acetylated epoxide pre-inhibitor to produce the covalently immobilized inhibitor (3,4-epoxybutyl-β-cellobioside) via 6' carbon (Hoj *et al.*, 1989).

The immobilized acetylated epoxide pre-inhibitors have to be stored as acetates in the dark at 4°C. Prior to binding to the ASD of CBH I, suspend the inhibitor in anhydrous MeOH (methanol) to reach a concentration of 100mg/mL and deacetylate with an equal volume of 1M NaOMe (sodium methoxide) at room temperature for 45min with continuous vortex mixing. Desalt and neutralize the sample by addition of Amberlite IRC-50(H+) ion-exchange resin (40mg/mg inhibitor) previously washed in MeOH. After 30min of vigorous shaking, wash the IRC-50 ion exchanger away with MeOH. Dry wafers at 45°C under reduced pressure. Use the deacetylated immobilized inhibitor immediately or store in sealed container at -80°C up to 5 days prior to use. ATR-FTIR can be used to characterize functionalized groups immobilized on surfaces (Barbot *et al.*, 2007).

Immobilization of CBH I on the attached inhibitor via ASD (Klarskov *et al.*, 1997)

Flash wafers with immobilized inhibitors on them with 50mM sodium acetate buffer, pH 5, then incubate wafers for 10min at 37°C with CBH I in the same buffer (CBH I concentration can be chosen from the fact that one mol of inhibitor is incorporated per mol of CBH I core protein). After the attachment of the ASD of CBH I to the immobilized inhibitor, treat the surface with SDS (0.5mg/mL). SDS is a detergent

that removes non-covalently immobilized protein from the surface (Hodneland *et al.*, 2002).

Appendix B – Purification of CBH I from the Commercial Cellulase Mixture

Determination of protein concentration

Table B.1. Calibration data for BSA concentration.

	Absorbance for BSA standard (595nm)				
	0.1250 mg/mL	0.2500 mg/mL	0.5000 mg/mL	0.7500 mg/mL	1.0000 mg/mL
1st replicate	0.0970	0.2556	0.5020	0.6839	0.8640
2nd replicate	0.0974	0.2532	0.4413	0.6569	0.8683
mean	0.0972	0.2544	0.4717	0.6704	0.8662

Calculation of the mean for absorbance data at 0.1250mg/mL:

Mean = $\frac{\sum x_i}{n}$, where x_i = absorbance value of replicate *i*, and *n* = number of replicates.

Mean = $\frac{0.0970 + 0.0974}{2}$ = 0.0972, which is the value shown in Table B.1 for this BSA concentration.

Table B.2. Total protein concentration of cellulases.

Dilution factor for protein (-)	A_{595nm} of diluted protein (-)	[Diluted protein] (mg/mL)	[Protein] (mg/mL)	$[Protein]_{ave}$ (mg/mL)	$[Protein]_{ave}$ %(w/v)	S_c (mg/mL)
Commercial cellulase mixture #NS50013 CCN03067 (old)						
100	0.4249	0.4703				
100	0.4078	0.4505				
		mean=0.4604	46.04			0.025
125	0.335	0.3689		46.76	4.676	
125	0.3524	0.3909				
		mean=0.3799	47.49			0.025
Commercial cellulase mixture (old) after desalting on Sephadex G-25						
100	0.3833	0.4299				
100	0.3964	0.4464				
		mean=0.4381	43.81			0.025
125	0.3135	0.3411		44.08	4.408	
125	0.3371	0.3685				
		mean=0.3548	44.35			0.026
Commercial cellulase mixture #NS50013 CCN03105 (new)						
100	0.4164	0.4604				
100	0.4199	0.4645				
		mean=0.4625	46.25			0.025
125	0.3038	0.3298		45.4	4.54	
125	0.3497	0.383				
		mean=0.3564	44.55			0.026
Commercial cellulase mixture (new) after desalting on Bio-Gel P-10						
X100	0.3876	0.427	42.7			
X100	0.4019	0.4436	44.36	44.66	4.466	0.025
X125	0.3246	0.3539	44.24			
X125	0.3459	0.3786	47.33			0.026

The equation of the standard curve in Figure 4.1 Absorbance = 0.8618[Diluted Protein] + 0.0196 was used to calculate the diluted cellulase mixture concentration. For the Commercial cellulase mixture #NS50013 CCN03067 with a dilution factor of 100, the [Diluted Protein] is:

$0.4249 = 0.8618[\text{Diluted protein}] + 0.0196$

$$[\text{Diluted protein}] = \frac{(0.4249 - 0.0196)}{0.8618} = 0.4703\ (\text{mg/mL})$$

The protein concentration of the cellulase mixture ([Protein]) was obtained by multiplying $[\text{Diluted protein}]_{ave}$, 0.4604 mg/mL, by the dilution factor of 100.

The protein concentration in %(w/v) was calculated as:

$$\%(w/v)\ [\text{Protein}]_{ave} = 46.76\ \frac{mg}{mL} \bullet \frac{1g}{1000mg} \bullet \frac{100}{100} = \frac{4.676g}{100mL} = 4.676\%$$

The column S_c represents the standard deviation of the diluted protein concentration obtained from the calibration curve:

$$S_c = \frac{S_r}{m}\sqrt{\frac{1}{M}+\frac{1}{N}+\frac{(\bar{y}_c-\bar{y})^2}{m^2 S_{xx}}}$$

S_r is the standard deviation about the regression on the calibration data shown below; m is the slope of the linear regression of the calibration data; $\bar{y}_c$ is the mean absorbance value of a set of M replicate of unknown solutions (here 2); $\bar{y}$ is the mean absorbance value for the N calibration standards; and S_{xx} is the sum of the squares of the deviations from the mean for individual values of x.

[BSA] (mg/mL) x_i	A_{595nm} of [BSA] (-) y_i	x_i^2	y_i^2
0.125	0.0972	0.015625	0.00944784
0.250	0.2544	0.0625	0.06471936
0.500	0.4717	0.25	0.22250089
0.750	0.6704	0.5625	0.44943616
1.000	0.8662	1	0.75030244
2.625	2.3599	1.890625	1.49640669

The regression line was: A = 0.8618[BSA] + 0.0196; so the slope, m was 0.8618.

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} = 1.49640669 - \frac{5.56912801}{5} = 0.382581088$$

$$S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N} = 1.890625 - \frac{6.890625}{5} = 0.5125(mg/mL)^2$$

$$S_r = \sqrt{\frac{S_{yy}-m^2 S_{xx}}{N-2}} = \sqrt{\frac{0.382581088-(0.8618)^2\times 0.5125}{5-2}} = 0.025480237 \approx 0.025$$

$$\bar{y}_c = \frac{0.4249+0.4078}{2} = 0.41635$$

$$\bar{y} = \frac{0.0972+0.2544+0.4717+0.6704+0.8662}{5} = 0.47198$$

So,

$$S_c = \frac{0.025480237}{0.8618}\sqrt{\frac{1}{2}+\frac{1}{5}+\frac{(0.41635-0.47198)^2}{0.74269924\times 0.5125}} = 00024880183 \approx 0.025 mg/mL$$

which is the value shown in Table B.2 for the Commercial cellulase mixture #NS50013 CCN03067 with a dilution factor of 100.

Determination of reducing sugars concentration

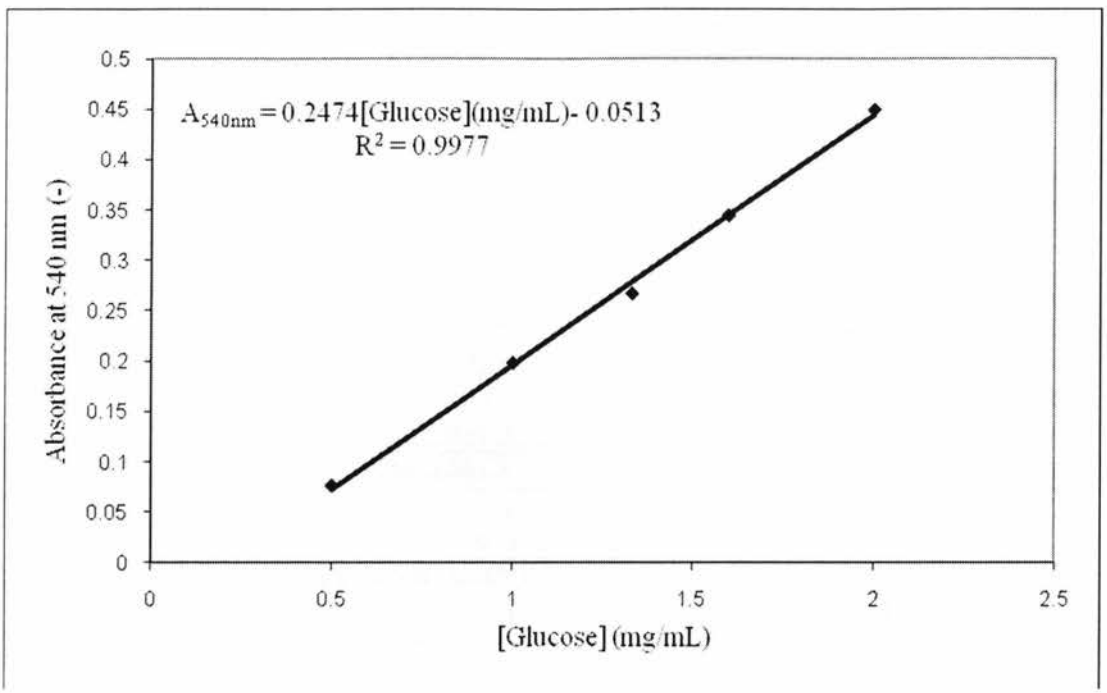


Figure B.1. DNS standard curve for the determination of reducing sugar concentration with glucose as a standard at 540nm. (♦): mean values of duplicate readings.

Table B.3. Data for DNS standard curve.

	Absorbance measurements for glucose standard (540nm)				
	0.500 mg/mL	1.000 mg/mL	1.333 mg/mL	1.600 mg/mL	2.000 mg/mL
1st set	0.0751	0.1828	0.2702	0.3335	0.4552
2nd set	0.0772	0.2138	0.2637	0.3552	0.4435
mean value	0.0761	0.1983	0.2670	0.3444	0.4493

Table B.4. Reducing sugar concentration produced by commercial cellulase mixtures #NS50013 CCN03067 and #NS50013 CCN03105 after digestion of CMC.

Dilution factor for protein (-)	A_{540nm} of glucose (-)	[Glucose] (mg/mL)	Activity (IU/mg)	$Activity_{ave}$ (IU/mg)	S_c (mg/mL)
Commercial cellulase mixture #NS50013 CCN03067 (old)					
100	0.5099	2.2683			
100	0.5027	2.2391	0.9057		0.063
		mean=2.2537		0.9887	
125	0.4774	2.1369			
125	0.5088	2.2638	1.0716		0.06
		mean=2.2003			
Commercial cellulase mixture #NS50013 CCN03105 (new)					
100	0.525	2.3292			
100	0.5516	2.4371	0.9533		0.07
		mean=2.3831		1.0733	
125	0.532	2.3575			
125	0.5029	2.2399	1.1933		0.065
		mean=2.2987			
Commercial cellulase mixture #NS50013 CCN03105 after desalting on Bio Gel P-10					
1st run					
100	0.6372	2.7829	1.2058		
125	0.6185	2.7073	1.4154	1.3106	0.092
2nd run					
100	0.6464	2.8201	1.1762		
125	0.649	2.8305	1.3833	1.27975	0.091

Specific activity (activity per mg of protein) of the cellulase mixture was calculated by converting mg/mL of glucose into μmol/mL glucose, dividing by the time of hydrolysis and by mg protein used:

$$\text{Specific activity (IU/mg)} = \frac{(\text{glucose mg/mL})}{(\text{MW of glucose})\,(\text{time of reaction})\,(\text{mg of diluted protein})} \bullet \frac{1000\ \mu\text{mol}}{1\ \text{mmol}}$$

For example, as can be found in Table B.4 the specific activity (IU/mg) of the cellulase mixture diluted 100 times (see Table B.2) that generated [Glucose] mean of 2.2537 mg/mL after digestion of CMC was calculated as following:

$$\text{Specific activity (IU/mg)} = \frac{(2.2537\ \text{mg/mL})}{(180.16\ \text{mg/mmol})(30\ \text{min})(0.4604\ \text{mg/mL})} \bullet \frac{1000\ \mu\text{mol}}{1\ \text{mmol}}$$

$$= 0.9057\ \mu\text{mol/min*mg} = 0.9057\ \text{IU/mg}$$

Separation data

Table B.5. Separation data (protein and released glucose concentration) after desalted commercial cellulase mixture (new) was applied on DEAE-Bio Gel A according to Bhikhabhai *et al.* (1984) purification scheme.

	Fraction # (-)	[Protein] (mg/mL)	Released [Glucose] (mg/mL)		Fraction # (-)	[Protein] (mg/mL)	Released [Glucose] (mg/mL)
Run #1	1	0	0	Run #2	1	0	0
	2	0	0.294		2	0	0.2395
	3	0	0.4318		3	0	0.3219
	4	0.1004	1.2739		4	0.0421	1.1272
	5	0.235	1.5214		5	0.2129	1.8638
	6	0.464	1.9828		6	0.4467	2.8004
	7	0.6618	2.0415		7	0.6324	2.6329
	8	0.7994	1.8947		8	0.9138	2.6935
	9	0.9427	2.3564		9	1.1599	2.7968
	10	1.3374	2.3567		10	1.5396	3.548
	11	1.3651	2.6888		11	1.8065	3.4569
	12	1.2878	2.7337		12	1.7935	3.8903
	13	1.4185	2.7161		13	1.702	3.9641
	14	1.7194	2.8102		14	1.4672	3.6263
	15	1.7576	2.7501		15	0.8276	3.2432
	16	1.6228	2.8718		16	0.1384	2.6703
	17	1.1933	2.7657		17	0	1.8957
	18	0.458	2.4184		18	0	2.0492
	19	0.1	1.9132		19	0	1.902
	20	0.0003	1.4243		20	0	1.925
	21	0	1.3713		21	0	1.6838
	22	0	1.3669		22	0	1.6672
	23	0	1.1539		23	0	2.0494
	24	0	1.1005		24	0	1.7178
	25	0	0.9114		25	0	1.4096
	26	0	0.6013		26	0	1.272
	27	0	0.4414		27	0	1.0913
	28	0	0.204		28	0	0.9814
	29	0	0		29	0	0.5113
	30	0	0		30	0	0.3001
	31	0	0		31	0	0
	32	0	0		32	0	0
	33	0	0		33	0	0
	34	0	0		34	0	0
	35	0	0		35	0	0
	36	0	0		36	0	0
	37	0	0		37	0	0
	38	0	0		38	0	0
	39	0	0		39	0	0
	40	0	0		40	0	0
	41	0	0		41	0	0
	42	0	0.2001		42	0	0.3319
	43	0	0.2143		43	0	0.5583
	44	0	0.3333		44	0	0.4783
	45	0	0.5145		45	0	0.339
	46	0	1.3864		46	0	1.377
	47	0.8161	1.6847		47	0.4546	1.7308
	48	1.3147	1.527		48	1.4759	2.0575
	49	0.779	1.3736		49	1.0201	1.6469
	50	0.1655	1.1622		50	0.2978	1.5715
	51	0.0009	1.0507		51	0.032	1.1858
	52	0	0.9287		52	0	1.1565
	53	0	0.8967		53	0	0.9342
	54	0	0.7899		54	0	1.2721
	55	0	0.5418		55	0	1.0513
	56	0	0.3887		56	0	1.0524
	57	0	0		57	0	1.0254
	58	0	0		58	0	0.4401
	59	0	0		59	0	0.2117
	60	0	0		60	0	0

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