AN ABSTRACT OF THE THESIS OF

Ozlem Akpinar for the degree of Doctor of Philosophy in Food Science and Technology presented on September 5, 2002. Title: Preparation and Modification of Cellooligosaccharides

Abstract approved: _____

Michael H. Penner

Cellooligosaccharides are the reaction intermediates produced during the hydrolysis of cellulose to glucose. Hence, they have the same chemical structure as cellulose, just shorter chain lengths. Cellooligosaccharides up to DP eight are soluble in water. The soluble cellooligosaccharides can be used "as is" in the food industry as non-digestible oligosaccharides and in the laboratory as representative substrates for cellulolytic enzymes. The soluble cellooligosaccharides may also be chemically modified for use in the laboratory, in this case serving as affinity ligands, reporter groups, or model substrates.

A number of methods are available for the separation of cellooligosaccharides differing only with respect to DP. This type of separation is relevant to both laboratory and industrial applications. A new approach to the

chromatographic separation of cellooligosaccharides is presented in this thesis. It is shown that cellulose stationary phases, in conjunction with ethanol-water mobile phases, may be used for cellooligosaccharide fractionation. The system appears to behave as an affinity/partition system, with retention times increasing as the DP of the cellooligosaccharides increase. The feasibility of using such a chromatographic system for the "clean-up" of cellooligosaccharide mixtures is demonstrated.

The relative merit of different chromatographic approaches putatively used for the fractionation of cellooligosaccharides was determined. Affinity-, adsorption-, ion-mediated- and molecular exclusion-approaches were tested. Adsorption chromatography, using a charcoal-celite stationary phase, was the most generally applicable method for the preparation of near gram quantities of pure cellooligosaccharides. Cellulose-based affinity/partition chromatography was found to be the least time consuming and most economical method for the preparation of cellotetraose and cellopentaose.

Studies using chemically modified cellooligosaccharides are typically limited to derivatives whose aglycone group is conjugated to the reducing end of the sugar. This is because the chemistry involved in modifying the reducing end is typically much easier than that involved in selectively modifying other sites on the oligosaccharides. A portion of the studies presented herein was aimed at exploring approaches for the modification of the non-reducing end of cellooligosaccharides. Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside was synthesized by reacting methyl 4,6-

O-p-nitrobenzylidine-β-Dglucoside with N-bromosuccinimide. This method has potential as a general method for the modification of the reducing terminus of oligosaccharides, including, cellooligosaccharides.

Preparation and Modification of Cellooligosaccharides

by
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APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

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

Ozlem Akpinar, Author

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CONTRIBUTION OF AUTHORS

Dr. Michael H. Penner and Dr. Robert J. McGorrin were involved in the design, analysis and writing of each chapter.

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CHAPTER 1

INTRODUCTION

Cellulose is the most abundant organic polymer in the biosphere. It is the major constituent in plant cell walls. It is a linear chain of β-D-glucose units linked together by 1,4-glycosidic linkages. The chains are oriented in parallel to form highly ordered structures called microfibrils. These structures form crystalline paracrystalline regions. Due to its widespread availability, cellulose has been used in many applications, such as polymer construction materials, natural textile fibers, paper and lumber. Also, hydrolysis of cellulose into soluble sugars is a starting material for the production of food, fuel and industrial chemicals. Cellulose can be hydrolyzed into soluble sugars either enzymatically or chemically. Enzymatic hydrolysis is more desirable because it is more specific, it goes at mild conditions, it does not produce undesirable by-products and it does not require corrosive chemicals. The enzymatic hydrolysis of cellulose is catalyzed by microbial cellulases. In nature, cellulolytic enzyme systems consist of endocellulases (EC 3.2.1.4), exocellulases or CBHs (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). All these enzymes are necessary for the complete hydrolysis of crystalline cellulose to

glucose. To study any one of these enzymes in detail requires that the enzyme of interest be free from other enzyme activities.

Affinity chromatography has been successfully used to purify the exocellulases from contaminating non-cellulolytic proteins, endocellulases and β-glucosidases. Exocellulases are often the most abundant proteins found in potent cellulolytic enzyme systems. They appear to be the key enzymes required for the complete hydrolysis of crystalline cellulose.

The structural complexity of cellulose limits its usefulness for detailed mechanistic studies aimed at understanding the mode of action of cellulolytic enzymes. Therefore many model substrates have been developed. Most of these substrates are modified cellooligosaccharides.

Cellooligosaccharides are produced by the controlled hydrolysis of cellulose. Thus, they have the same chemical structure as cellulose, only shorter chain lengths. They serve as model substrates for kinetic studies and they are the starting material for the synthesis of modified soluble substrates and affinity ligands. In addition, they have potential applications as non-digestible oligosaccharides in food systems, since they are natural compounds that are not hydrolyzed by human enzymes or absorbed in the human gastrointestinal tract.

Cellooligosaccharides are currently being investigated as nondigestible oligosaccharides (NDO) for food applications. In this application, cellooligosaccharides are used without further modification. The widespread use of

cellooligosaccharides in food systems will require cost effective methods of producing them. One aspect of this production scheme will be the fractionation of cellooligosaccharides of different chain lengths (degree of polymerization, DP). Chapter 3 of this thesis presents a novel approach to the fractionation of cellooligosaccharides.

Laboratories dealing with cellulolytic enzymes typically have heavy demands for cellooligosaccharides. They are used extensively as model substrates in mechanistic studies and as affinity probes for both analysis and purification. The preparation of these oligosaccharides is not trivial; so laboratories spend considerable amounts of time and/or money to obtain them. Chapter 4 of this thesis is a comparative study looking at chromatographic approaches used in the preparation of cellooligosaccharides.

As stated above, many of the applications of cellooligosaccharides require that they be chemically modified. This includes their use as immobilized affinity ligands, as reporter groups in structural studies, and as substrates in kinetic and specificity studies. In the vast majority of cases, the approach used has been to modify the cellooligosaccharides via their reducing end. This is to be expected due to the relative ease of working at the reducing end, compared to the non-reducing end. However, the lack of information on the interaction of exo-acting cellulases with non-reducing end modified cellooligosaccharides limits our understanding of the chain-end specificity of this important class of enzymes. Chapter 5 of this thesis

explores synthetic approaches that may be used to modify the non-reducing end of cellooligosaccharides.

CHAPTER 2

LITERATURE REVIEW

2.1. CELLULOSE

Cellulose is the most abundant organic polymer, produced by nature at an annual rate of 10^{11}-10^{12} tons. It is produced both in pure form, such as cellulose in the seed hair of the cotton plant, and in combined form with lignin and other polysaccharides, so-called hemicellulose in the cell wall of woody plants. It is also produced by non-plant organisms including bacteria (*Acetobacter xylinum*), algae (*Valonia ventricosa and Boergesenia forbesii*), and animals (tunicate) (Reinikainen, 1994; Brown, 1978).

Due to its solubility, rigidity, and lack of toxicity, cellulose is used in many applications. It serves as a polymer construction material, mainly in the form of intact wood, as natural textile fibers like cotton and flax, as paper and as board. It is a starting material for subsequent chemical conversion for the production of artificial cellulose based threads and films as well as for the production of a variety of soluble cellulose derivatives to be used in many areas of industry and domestic life (Klemm et al., 1998). Also, hydrolysis of cellulose into soluble sugars is a starting material for the production of food, fuel and industrial chemicals. Bacterial microcrystalline cellulose, produced extracellularly by strains of *Acetobacter xylinum*, has been used in the food industry.

Cellulose is a linear polymer composed of D-glucose units linked together by β-1,4-glycosidic linkages (Figure 2.1). Each glucopyronoside unit exists in the chair conformation ($^4C_1$). In this conformation, the free hydroxyl groups are equatorial and hydrogen groups are axial. Each glucopyronosyl unit is oriented 180^0 relative to its neighbors and the glycosidic oxygens point alternatively up and down. Thus, the repeating unit of the polymer is cellobioside (Klemm et al., 1998).

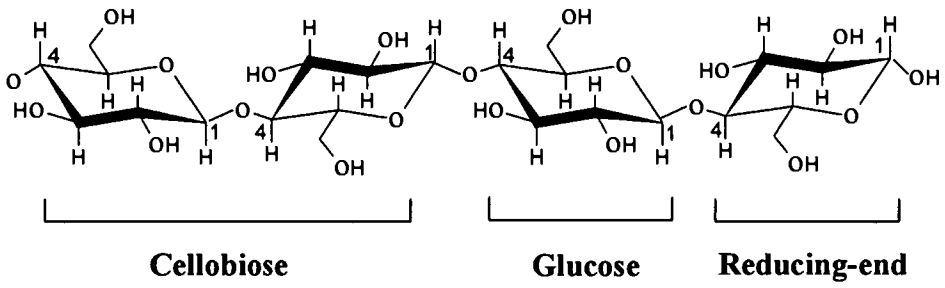


Figure 2.1. Structure of cellulose consisting of repeating units of cellobiose.

The molecular size of cellulose is expressed by its average degree on polymerization (DP). The average molecular mass results from the product of DP and the molecular weight of repeating glucose units minus water. Depending on origins and pretreatment, the DP value of cellulose varies (Klemm et al., 1998). It can be as high as 12, 000. The DP values of several types of cellulose are shown in Table 2.1.

Table 2.1. Degree of polymerization of various cellulose materials (Klemm et al., 1998).

Material	**Range of DP**
Native Cotton	up to 12000
Scoured and bleached cotton linters	800-1800
Wood pulp (dissolving pulp)	600-1200
Man-made cellulose filaments and fibers	250-500
Cellulose powders (prepared by partial hydrolysis and mechanical disintegration)	100-200

The structure of cellulose is stabilized by intra and inter molecular hydrogen bonds and van der Waals interactions as shown in Figure 2.2. There are two intramolecular hydrogen bonds between adjacent glucose residues, and the hydrogen bonding network consists of all polar functional groups except glycosidic oxygens. The intramolecular hydrogen bonding affects the relative position of each repeating glycoside residue in the chain which causes the polymer to an adopt an extended, ribbon like shape with a repeating distance of 1.03 nm along the chain axis, which is equal to one-cellobiose unit (Gardner and Blackwell, 1974). Therefore, the smallest repeating unit of the polymer is cellobiose rather than glucose.

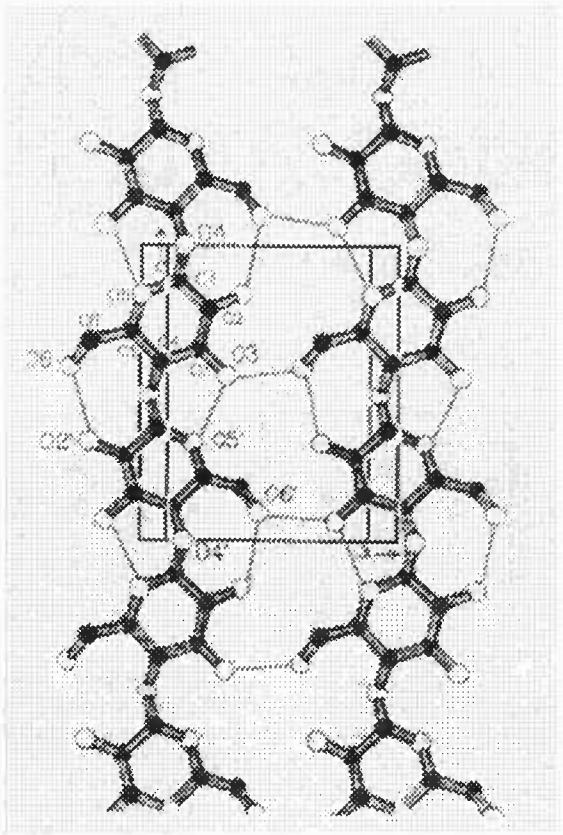


Figure 2.2. Parallel β-1,4-D-glucose chains form intra and intermolecular hydrogen bonds (http://www.ap.univie.ac.at/users/vogl/inst/actualcellulose.htm)

The intermolecular hydrogen bonding and van der Waals forces cause cellulose chains to aggregate into microfibrils which in turn can pack very tightly together to form larger fibrils and finally cellulose fibrils. These interactions form a highly ordered structure, called crystalline regions. X-ray diffraction and

spectroscopic studies on the crystal structure of microfibrils in different native and chemically threaded celluloses showed that there are four distinct crystalline cellulose structures named cellulose I, II, III and IV (Sarko, 1987). The main difference between these structures is in the cellulose chain packing. Cellulose I is also called native cellulose in which the chains have a parallel orientation and they are arranged side by side in hydrogen bonded sheets. The sheets are held together by van der Waals interactions. Cellulose I has two different forms, Iα and Iβ, which differ in their intermolecular hydrogen bonding patterns (Atalla and VanderHart, 1984). Most natural cellulose has both of them and the proportions depend on the source. Cellulose II, also called regenerated cellulose, is obtained from cellulose I by sodium hydroxide treatment. In cellulose II, the chains are orientated in an anti-parallel way with consecutive formation of extensive hydrogen bonding which makes cellulose II more stable than cellulose I. The process of transformation of cellulose I to cellulose II is generally thought irreversible. Cellulose II is formed naturally by a mutant strain of *Gluconacetobacter xylinum* (Kuga et al., 1993) and also occurs in the algae *Halicystis* (Sisson, 1938). Cellulose III is obtained from cellulose II or cellulose I by liquid ammonia treatment or with some amines such as ethylene diamine. Cellulose IV is the predominant form in plant primary cell walls. It is known as a disordered form of cellulose I. Also, it can be obtained from cellulose III by boiling in glycerol.

Although cellulose has a highly ordered structure, it also contains less ordered regions called paracrystalline or amorphous regions. The crystalline regions are interrupted by amorphous regions. This has been described as a degree of crystallinity which is expressed as a percentage and is determined by wide range X-ray scattering (WAXS) pattern, and ^{13}C high resolution cross-polarization magic angle spinning (CP-MAS) solid state NMR. The proportion of crystalline and amorphous regions in cellulose fibres varies in different celluloses (Figure 2.3). The degree of crystallinity of different cellulose depends on the origin and the treatment of the sample. In general, native cellulose is 70% crystalline. Valonia cellulose is the most crystalline, while acid swollen cellulose is the least crystalline (Beguin and Aubert, 2000).

In nature, cellulose chains align in parallel and tightly bound together to form elementary fibrils. Elementary fibrils have been considered the smallest morphological units. Elementary fibrils aggregate together to form microfibrils, which in turn form macrofibrils. Micro and macrofibrils are the construction units of the cellulose fiber cell-wall architecture. The fiber consists of different layers as shown in Figure 2.4. Most of the cellulose is located in the secondary cell (S1 and S2) wall, usually embedded in a matrix of hemicellulose and lignin (Figure 2.4) (Klemm et al., 1998).

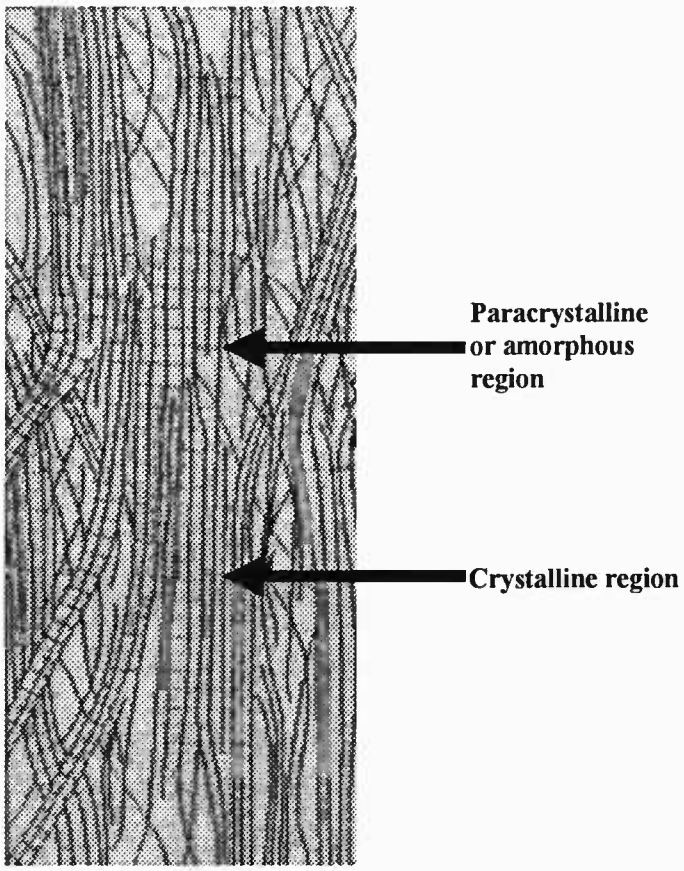


Figure 2.3. The structure of cellulose microfibril molecules containing crystalline and paracrystalline regions (http://www.ap.univie.ac.at/users/vogl/inst/actualcellulose.htm)

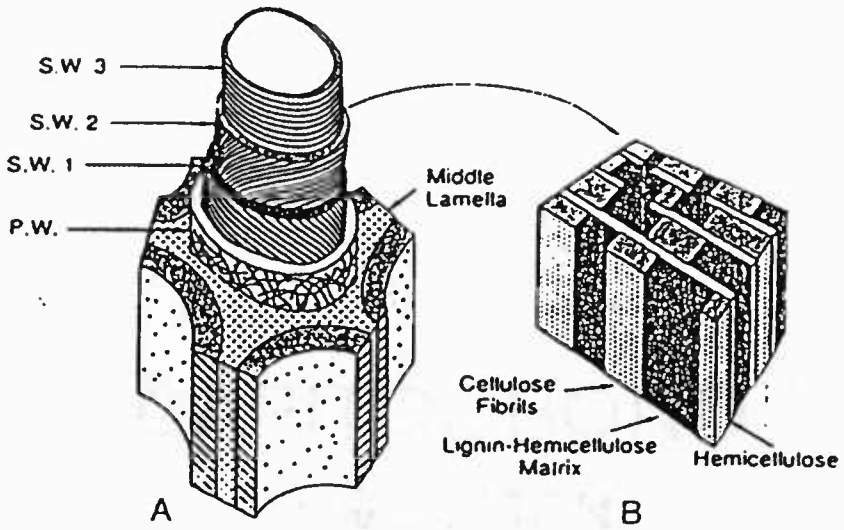


Figure 2.4. Schematic illustration of cell wall and structure of microfibrils A) cell wall layers; B) the structure of microfibrils in the secondary cell wall. PW: primary cell wall; SW 1, SW 2, SW 3: secondary cell walls (Beguin and Aubert, 2000).

2.2. THE HYDROLYSIS OF CELLULOSE

Cellulose can be hydrolyzed into soluble sugars either enzymatically or chemically. Enzymatic hydrolysis of cellulose is more desirable because it is more specific and the reaction occurs under mild conditions. Unlike chemical hydrolysis, enzymatic hydrolysis does not require corrosive chemicals and does not produce undesirable by-products. At least three major cellulolytic enzymes are required to complete the enzymatic hydrolysis of cellulose. Cellulolytic enzymes, cellulases, are enzymes which hydrolyze β-1,4 glycosidic linkages in cellulose. In nature, cellulolytic enzymes systems consist of endocellulases (endo-1,4-ß-D-glucan-4-glucanohydrolases, EC 3.2.1.4), exocellulases (exo-1,4-ß-D-glucan-4-cellobiohydrolases,EC 3.2.1.91) and β-glucosidases (ß-D-glucoside glucohydrolases, EC 3.2.1.21). Each enzyme has different specifity and action on cellulose.

2.2.1. Cellulases

Cellulases are enzymes that hydrolyze cellulose into glucose oligomers by cleaving of β-(1→4) glycosidic bonds. Hydrolysis of cellulose is of interest because hydrolysis products, such as soluble sugar, are the starting material for the production of food, fuel and industrial chemicals. Cellulases have been classified into 13 structural families based on protein sequence comparison (Countinho and

Henrissat, 1999). These enzymes have different activities on cellulolytic substrates (Table 2.2).

Table 2.2. The substrates for cellulases (Wood and Garcia-Campayo, 1990)

	Cellulose	**Amorphous cellulose**	**Modified cellulose (HEC and CMC)**	**Soluble cellooligosaccharides (unmodified and modified)**
Exocellulases	**active**	**active**	------	**active**
Endocellulases	------	**active**	**active**	**active**
β-glucosidase	------	------	------	**active**

Some microorganisms produce an efficient set of enzymes for the complete degradation of cellulose. White-rot and soft-rot fungi, such as *Trichoderma, Fusarium, Humicola, Penicillium* and *Schizophyllum*, produce efficient cellulase systems (Wood and Garcia-Campayo, 1990; Nevalainen and Penttila, 1995; Clarke et al., 1997; Mackenzie et al., 1997; Schulein, 1997). The number and the amount of enzyme components are dependent on the fungus and cultivation conditions. These systems contain various endo and exocellulases and one β-glucosidase.

Trichoderma reesei, a soft rot fungus, is one of the most efficient producers of cellulolytic enzymes. It produces two cellobiohydrolases (CBHI and CBHII), four endocellulases (EGI, EGII, EGIII and EGV) and one β-glucosidase. *Trichoderma reesei* celluloytic enzymes have been studied extensively due to their efficiency in degrading cellulose substrates. The properties of these enzymes (for reviews see Goyal et al., 1991) are given in Table 2.3.

Table 2.3. Properties of cellulolytic enzymes from *Trichoderma reesei*

	%[1]	Number of residues	Isoelectric point	Family	Ref
CBHI	60	497	3.5-4.2	7	Shoemaker et al., 1983
CBHII	20	447	5.1-603	6	Chen et al., 1987
EGI	10	437	4.0-6.0	7	Penttila et al., 1986
EGII	5-10	397	5.5	5	Saloheimo et al., 1988
EGIII		218	7.5	12	Ward et al., 1993
EGV		225		45	Saloheimo et al., 1994
β-glucosidase		713	7.8	3	Barnett et al., 1991

[1] The percent ratio of individual enzymes in total cellulolytic enzymes produced by *Trichoderma reesei*.

Endocellulases cleave bonds within chains located in the amorphous and disordered regions of cellulose (Figure 2.5.A). They rapidly decrease the degree of polymerization of the substrate, and create new chain ends on the cellulose surface. Exocellulases, CBHI and CBHII, (now called Cel7A and Cel6A) are believed to hydrolyze cellulose chains from their ends (Figure 2.5.B) liberating cellobiose as the primary product. Therefore, they are also called cellobiohydrolases. They are thought to be more responsible for the hydrolysis of the more inaccessible, crystalline regions of cellulose (Divne et al., 1998). β-glucosidases are exoenzymes which act to release D-glucose units from cellobiose and low molecular weight cellooligosaccharide (Figure 2.5.C). The exoactivity of β-glucosidase is important because accumulation of cellobiose strongly inhibits the cellobiohydrolases.

It should be noted that the rate of enzymatic hydrolysis of cellulose depends on the physical nature of the substrate, the nature of cellulase complex, and the inhibitory effects of both substrates and products (Table 2.2). The total hydrolysis of cellulose by cellulase endo and exocellulases is synergistic (Henrissat et al., 1985; Nidetsky et al., 1993; Irwin et al., 1993; Divne et al., 1994; Barr et al., 1996; Beguin and Aubert, 2000).

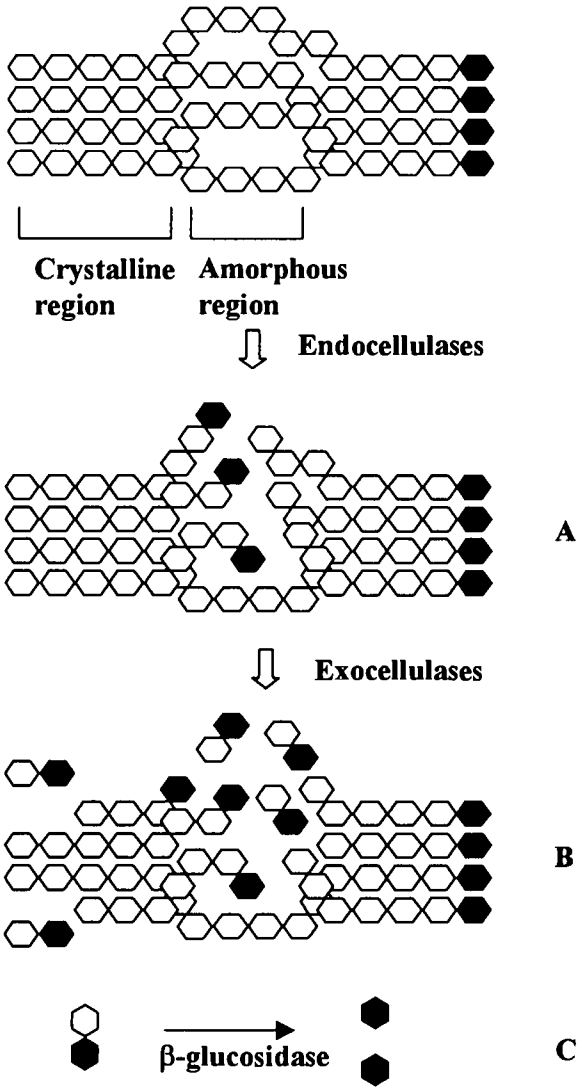


Figure 2.5. Enzymatic hydrolysis of cellulose by cellulolytic enzymes. Glucose residues are indicated by hexagons and reducing ends are shown in black (adapted from Beguin and Aubert, 2000).

2.2.2. Endocellulases

T. reesei produces four endocellulases (EGI, EGII, EGIII and EGV). The properties of these enzymes were given in Table 2.3. EGI is the major endo acting cellulase. It is 10% of the total cellulases secreted by this fungus. It hydrolyzes the β-1,4-glycosidic linkages with retention of configuration (Table 2.5). It also has transfer activity as well as xylanase activity (Claeyssens et al., 1990; Biely et al., 1993). *Trichoderma reesei* EGI is the most extensively studied endocellulase with a defined 3D structure as determined by X-ray diffraction (Kleywegt et al., 1997).

2.2.3. Exocellulases

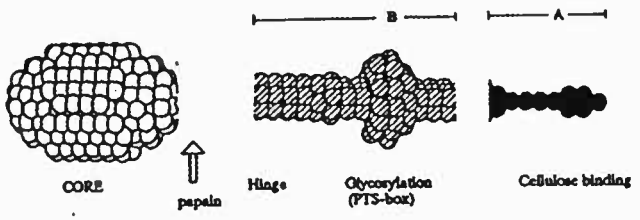
Exocellulases or cellobiohydrolases (CBHs) are the key enzymes for the hydrolysis of crystalline cellulose. *Trichoderma reesei*, a filamentous fungus, produces one of the most efficient cellulolytic enzyme mixtures. It produces two cellobiohydrolases, 60% CBHI and 20% CBHII of total cellulolytic enzymes (Teeri, 1997). Because of their technological importance, they have been well studied. Both of them has been cloned and sequenced. They have been expressed in different system for continuous production of active enzyme complex (Teeri et al., 1983; Shomaker, 1984; Penttila et al., 1988; Aho et al., 1996; van Zyl et al., 1998; Godbole et al., 1999). CBHI and CBHII act on the crystalline regions of cellulose and yield cellobiose on hydrolysis. Both of them have tunnel shaped active sites and bind to the same affinity column (Piyachomkwan et al., 1997). Although both of them are glycoproteins, the amount of carbohydrate and their amino acid

composition are different (Wood et al., 1988). Also, their modes of action on modified soluble substrates (Biely et al., 1993) and their mechanism of hydrolysis of glycosidic linkages are different (Davies and Henrissat, 1995). CBHII inverts the configuration at the glycosidic bond so the hydrolysis product is the α anomer while CBHI proceeds with retention of configuration, so the hydrolysis product is the β anomer (Rouvinen et al., 1990).

2.2.4. Domain Structure of Cellobiohydrolases from *T. reesei*

T. reesei CBHI and CBHII have two-domain organizations with a large catalytic domain connected to a small cellulose binding domain (CBD) via a glycosylated linker peptide (Figure 2.6) (Tomme et al., 1988). The structure of an intact cellulase has not been determined by crystallography since they have not been crystallized due to flexible linker peptide. On the other hand, since individual domains can be separated by proteolysis, they can be crystallized and the role of each domain can be studied (Figure 2.6) (Abuja et al., 1988).

Cellulose binding domains (CBD) from all fungal organisms so far studied are small, compact, domains with flat binding surfaces. The role of CBD has been well studied (Stahlberg et al., 1993; Reinikainen et al., 1995; Carrard and Linder, 1999). However, it has not been understood very well. It was found that the removal of the CBD does not generally affect the activity of soluble substrates but decreases the affinity and activity of enzymes on crystalline cellulose



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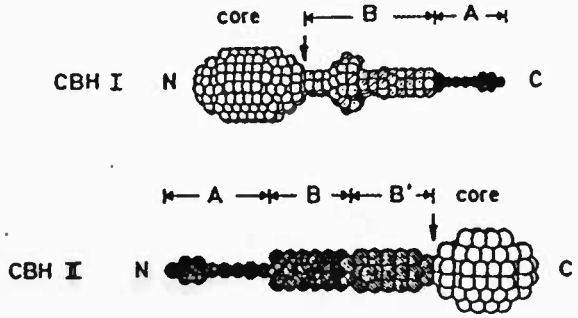

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Figure 2.6. The three dimensional structure of *Trichoderma reesei* cellobiohydrolases A) Tertiary structure of CBH and papain cleavage site B) Comparison between the tertiary structure of CBHI and CBHII (Abuja et al., 1988).

(van Tilbeurgh et al., 1986; Tomme et al., 1988). It is assumed that the CBD increases the substrate accessibility by disturbing the intermolecular interactions of cellulose, or it maintains a high local enzyme concentration by binding cellulose surface, thus it enhances the enzyme activity (Teeri et al., 1998)

CBHI core is a single domain with overall dimensions of approximately 60 Å by 50 Å by 40 Å. It contains ten well defined subsites (-7 to +3) for binding glucosyl units (Divne et al., 1994; 1998). The CBHII catalytic core is almost half as long as that observed in CBHI. It is about 20 Å long and has four glucosyl-binding subsites (-2 to +2) (Rouvinen et al., 1990)

Divne et al. (1998) estimated that the tunnel contained ten glucosyl-binding sites (-7, +3). Binding of the glucose units occurs via hydrogen bonding and in four subsites –7, -4, -2 and +1, stacking onto tryptophan residues W40, W38, W367 and W376, respectively. CBHII has four clearly defined glucosyl binding sites (-2, -1, +1, +2) within the active site tunnel. In subsites –2, +1, +2, W135, W367, W269 respectively, make a significant contribution to the formation of the sugar binding sites (Rouvinen et al., 1990).

Besides tryptophan residues, the active site tunnel of CBHI and CBHII contains amino acid residues which play an important role in the catalytic process. Two glutamic acid residues (E212 and E 217) and one aspartic acid residue (D214) of CBHI (Stahlberg et al.,1996) and two asparatic acid residues (D175 and D221)

and one tyrosine (Y169) of CBHII (Koivula et al., 1996) are important amino acid residues during catalytic reactions.

2.2.5. Mechanism of Hydrolysis

Glycosyl hydrolases hydrolyze glycosidic bonds with general acid base catalysis. Catalysis is performed with either inversion or retention of the anomeric configuration of the substrate. The hydrolysis mechanism involves two active site amino acid residues: one acts as a general base, the other as a general acid.

Inverting enzymes use a single displacement mechanism (SN2) to catalyze the hydrolysis with inversion of configuration at the anomeric center. A general base (B) assists in the nucleophilic attack of water by deprotonating a water molecule and a general acid (AH) protonates the leaving glycosidic oxygen in a concerted fashion as the bond cleaves and the water molecule directly displaces the glycosidic leaving group from the anomeric center (Figure 2.7).

Retaining enzymes are believed to use a double displacement mechanism (SN1) to catalyze the hydrolysis with retention of configuration at the anomeric center. Glucosyl-enzyme intermediate is formed and hydrolyzed via an oxocarbenium ion-like transition state. One amino acid acts as a general acid and a general base, the other acts as a nucleophile and a leaving group. In the first step, a deprotonated amino acid residue, acting as a nucleophile (Nu), attacks the anomeric center. A catalytic residue, acting as a general acid (AH), assists that process by

donating a proton to glycosidic oxygen. In the second step, a water molecule attacks the anomeric center with the base catalytic assistance (A-), thus displacing a nucleophile (Nu) and releasing the product (Figure 2.7) (Withers and Aebersold, 1995). Furthermore, in the second step instead of water molecules, another oligosaccharide attack the anomeric center with the assistance of general base catalytic residue (A-), thus resulting in elongation of the oligosaccharide chain. This is known as a tranglycosylation reaction and it can be used for enzymatic synthesis of different oligosaccharides (Harjunpaa, 1998).

All CBHs were thought to catalyze the hydrolysis of cellobiose units from the non-reducing end of cellulose chains (Schomburg and Salzmann, 1991). However, the orientation and directionality of different oligosaccharide in the active sites of CBHI and CBHII, studied by high-resolution crystallography shows CBHI preferentially acts on cellulose via the reducing end while CBHII preferentially acts at the non reducing end (Figure 2. 9) (Divne et al., 1998).

However, CBHI can bind to the non-reducing end and can catalyze the hydrolysis at the non-reducing end as well. Even though the activity is less than CBHII, it is capable of catalyzing the hydrolysis of reducing end blocked cellooligosaccharides from the non-reducing end (Sangseethong, 1999). This explains why CBHI can be purified by the same affinity ligand, reducing end blocked affinity ligand, used for CBHII (van Tilhbeurgh et al., 1984; Orgeret et al., 1992; Piyachomkwan et al., 1997; Sangseethong and Penner, 1998).

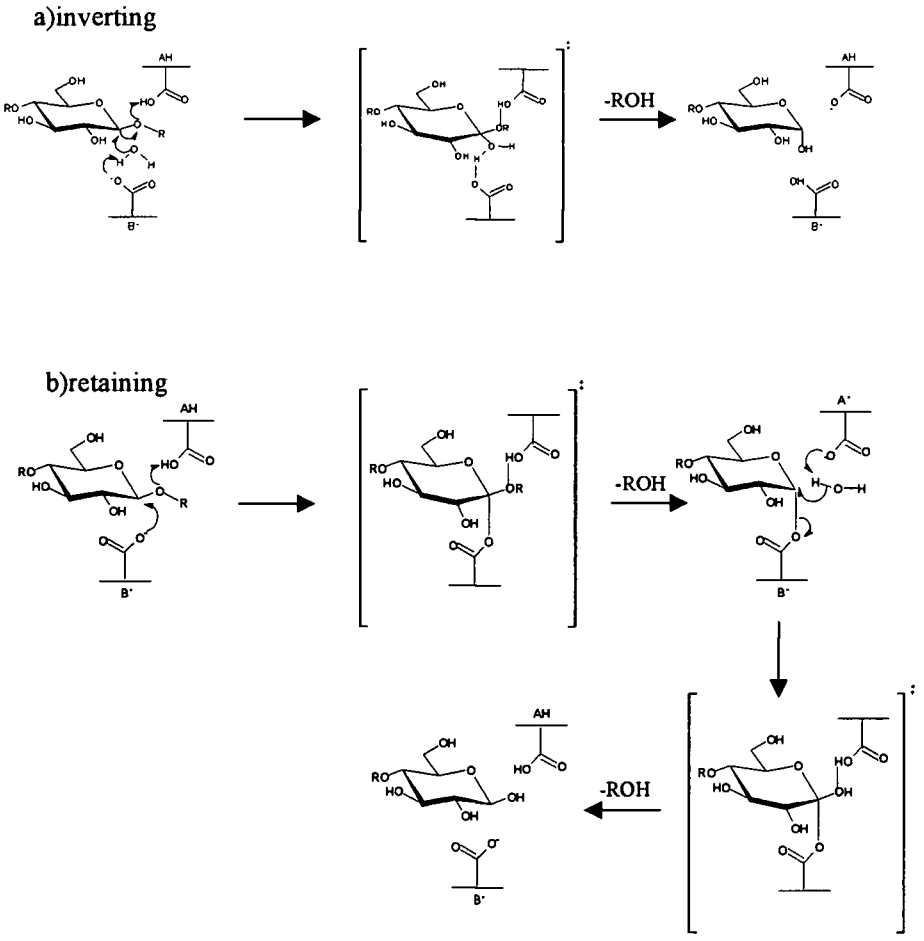

Figure 2.7. The mechanism of hydrolysis of inverting and retaining glucosyl hydrolases: a) for inverting enzymes; b) for retaining enzymes (adapted from Withers and Aebersold, 1995).

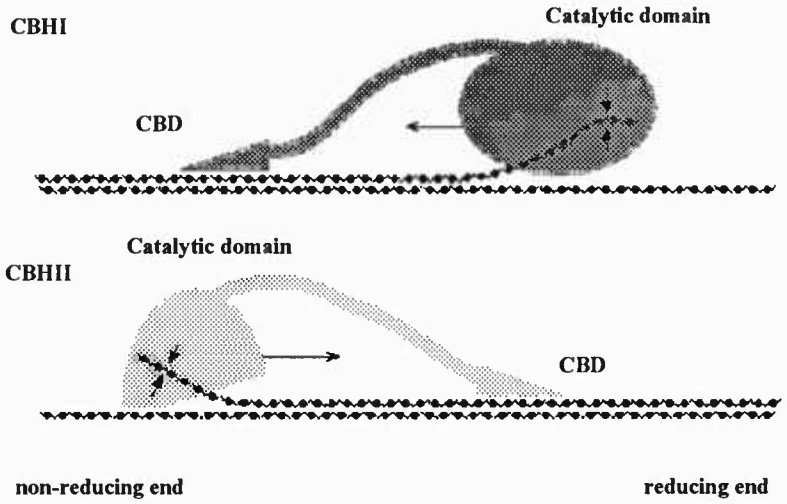

Figure 2.8. Schematic picture of intact CBHI and CBHII and how they work at the surface of cellulose chain. CBHI hydrolyzes cellulose chain from the reducing end while CBHII hydrolyzes cellulose chain from the non-reducing end (adapted from http://xray.bmc. uu.se/markh/ cbh/ EU_ abstract.html)

2.3. SUBSTRATES FOR CELLOBIOHYDROLASES

Cellulose is the natural substrate for cellulolytic enzymes. Since it is insoluble and heterogeneous, containing both crystalline and amorphous regions, it is too complicated to be useful for detailed enzymatic studies. The reactions between cellulases and cellulose have been studied by following changes in the properties of substrates such as viscosity, turbidity, solubility and molecular size or production of

reducing sugars. Numerous analytical methods have been used to follow the production of reducing oligosaccharides. These include the DNS-method (3,5-dinitrosalicylic acid) (Summer and Somers, 1949), the Somogyi-Nelson method (Somogyi, 1952), and enzymatic redox systems (Canevascini, 1985). Another method is to monitor the hydrolysis products of cellulose by liquid chromatography (Nidetzky et al., 1994). This technique provides more information than the others. However, since the exact structure of cellulose is unknown, characterization of enzymatic activity of cellulases is complicated and experimental data must be analyzed with great care. Therefore, many well defined substrates have been developed, such as unmodified cellooligosaccharide (Hsu et al., 1980), radioactively labeled cellooligosaccharides (Chirico and Brown, 1987), chromophoric cellooligosaccharide (van Tilbeurgh et al., 1982; Deshpande et al., 1984; Claeyssens and Aerts 1992), fluoregenic cellooligosaccharides (Armand et al., 1997; Varrot et al., 1999; Barr and Holewinski, 2002) and 4-thio-cellooligosaccharides (Schou et al., 1993b; Sulzenbacher et al., 1996; Davies, 1998).

2.4. CELLOOLIGOSACCHARIDES

Soluble cellooligosaccharides, also called cellodextrins, have the same chemical structure as cellulose with degrees of polymerization between 2 and 7. Their structure and behavior in aqueous solvents has been well studied because they serve as models for cellulose (Inhat and Goring, 1967; Gast et al., 1980; Amu, 1981;

Bosso et al., 1984; Hato and Minamikawa, 1996; Flugge et al., 1999). Since they have a rodlike shape in aqueous solution (Inhat and Goring, 1967), they are classified as "extended ribbon" type oligosaccharides (Hato and Minamikawa, 1996). They have limited solubility in aqueous solvents and their solubility decrease with increasing molecular weight. The properties of cellooligosaccharides (Huebner et al., 1978) are summarized in Table 2.4.

Cellooligosaccharides are the reaction intermediates produced during the hydrolysis of cellulose into soluble sugar. They serve as model substrates for cellulolytic enzymes and they are also starting materials for the synthesis of modified soluble substrates and affinity ligands for cellulolytic enzymes. In addition, since they are not hydrolyzed or absorbed by the human gastrointestinal tract, these non-digestible oligosaccharides may have potential uses as a non-caloric bulking agents in food products (Satouchi et al., 1996; Watanabe, 1998; van Loo et al.,1999; Stahl, 2001). The major methods for preparation of cellooligosaccharides are chemical synthesis (Takeo et al.,. 1983; Nakatsubo, 2001), enzymatic synthesis (Kobayashi et al., 1993; Tanaka, 1993; Yan and Liau, 1998; Fort et al., 2000; Shoda and Fujita, 2000) and fragmentation of cellulose by enzymes (Sasaki et al., 1989; Watanabe et al., 1993; 1994; 1995; Hishimoto and Matsumoto, 1994; Sato and Yashiro, 1996; Xia and Cen, 1999; Arai et al., 2001) or acid (Dickey and Wolfrom, 1949; Miller et al., 1960).

Table 2.4. Properties of cellooligosaccharides (Huebner et al., 1978; Wolfrom and Dacons, 1952)

Compound	Mol Wt.	Optical rotation $[\alpha]_D$	Melting point (^{0}C)	Solubility (g/liter)			Diffusion coefficient, D_0x10^6 (cm^2sec^{-1})	Instrinsic viscosity $[\eta]$ ($dl\ g^{-1}$)	Molecular dimensions		
				H_2O	C_2H_5OH	CH_3OH			L	X	d
Cellobiose	342.3	36.0	225	125-147	insol	-	5.7-5.9	0.027	14.6		6.42
Cellotriose	504.45	23.5	206-209	very sol	insol	slowly sol	4.8-4.9	0.030	20.2		6.56
Cellotetraose	666.59	17.3	252-253	78 at 25^{0}C	insol	insol	4.2-4.28	0.036	26.2		6.60
Cellopentaose	828.73	13.8	266-268	40 (slowly sol. in warm water, 4.8 at 25 ^{0}C	insol	insol	3.82	0.038	31.8		6.66
Cellohexaose	990.86	10.3	275-278	10 (slowly sol. warm water)	-	-	3.38	0.047	37.6		6.68
Celloheptaose	1152.90		283-286	1 (slowly sol. in warm water)	-	-	-	-	-		-

2.4.1. Chemical Synthesis

Chemical synthesis of cellooligosaccharides is performed under Koenigs-Knorr conditions (Takeo et al., 1983) which involves the treatment of glucose pentaacetate with HBr, followed by the addition of the appropriate sugar which has a free –OH in the C-4' position and selectively protecting the rest of the hydroxly groups in the presence of heavy metals such as silver or mercury.

Cellulose oligomers are peracetylated with acetic anhydride in the presence of pyridine. Prior to addition of the sugar, the acetylated sugar is converted into a more reactive bromosugar (donor) by using 30% HBr in glacial acetic acid. The second reaction involves with the addition of the appropriate sugar (acceptor) which has a free –OH in the C-4' position in the presence of heavy metals. Acetylated glucosyl bromide spontaneously releases bromide ion followed by internal reaction of the cationic center at C-1 with an oxygen atom of the ester group at C-2 to form a stabilized oxonium ion intermediate (see reaction mechanism depicted in Figure 2.9). Nucleophilic displacement of the oxonium ion by back site attack at C-1 then occurs yielding β-glycosides and regenerating the acetate ester group at C-2 (Figure 2.9) (McMurry, 1992). After formation of acetylated cellooligosaccharides, they are deacetylated in basic methanol.

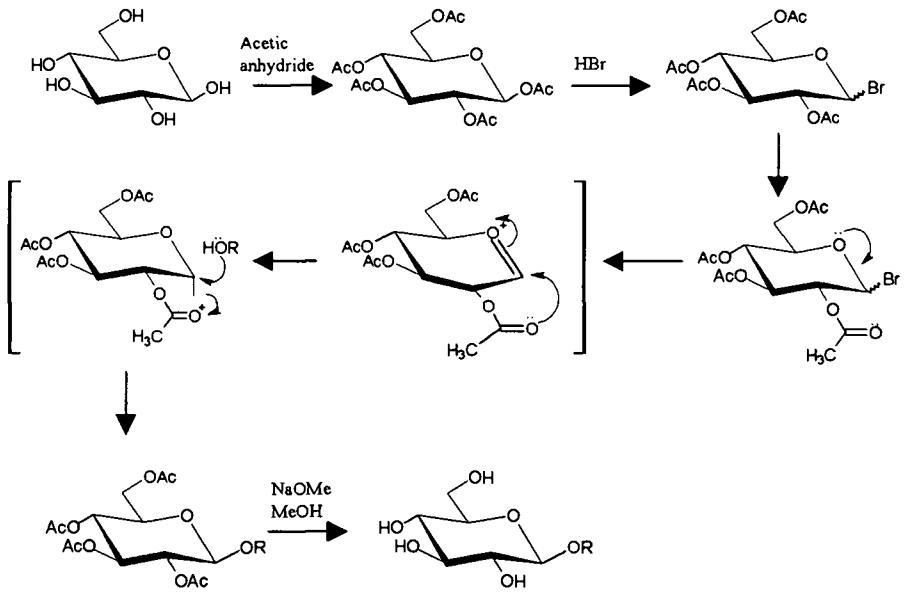

Figure 2.9. The chemical synthesis of cellooligosaccharides

2.4.2. Enzymatic Synthesis

Another method, which is quite specific and highly effective for the production of cellooligosaccharides, is enzymatic synthesis (Fort et al., 2000). The use of enzymes for the formation of glycosidic bonds in oligosaccharide synthesis is theoretically a straightforward approach. The enzymes, glycoside hydrolases, and the requisite donor (aryl glycosides, alkyl glycosides, glycosyl fluorides or disaccharides) are readily available and inexpensive. Among them, glycosyl fluoride,

a sugar derivative whose anomeric center is replaced by a fluoride atom is the most commonly used donor. Retaining glycosidases are known to be effective for oligosaccharide synthesis. At the first step, the fluoride atom is protonated by a carboxylic acid in the catalytic site to give an oxocarbenium ion intermediate. Oligosaccharides are synthesized when a sugar competes with water as an acceptor during the deglycosidation step (Figure 2.10). The enzymatic synthesis of oligosaccharides is very specific and under appropriate conditions, oligosaccharides can be isolated with good yields (Fort et al., 2000).

2.4.3. Fragmentation of Cellulose by Enzymes

Cellulase treatment of cellulose can also produce cellooligosaccharides. Generally cellulase, which has an endo activity, in the presence of gluconolactone to inhibit β-glucosidase activity is incubated with cellulose, resulting in a supernatant containing a mixture of cellooligosaccharides with different DPs (Aoyanagi et al., 1992). Enzymatic fragmentation of cellulose is very specific and it produces cellooligosaccharides with high yield. For example, the yield of cellooligosaccharide mixtures in the supernatant is 0.5 mg/mL glucose, 3.7 mg/mL cellobiose, 2.3 mg/mL cellotriose, and 1.2 mg/mL cellotetraose (Sato and Yashiro, 1996).

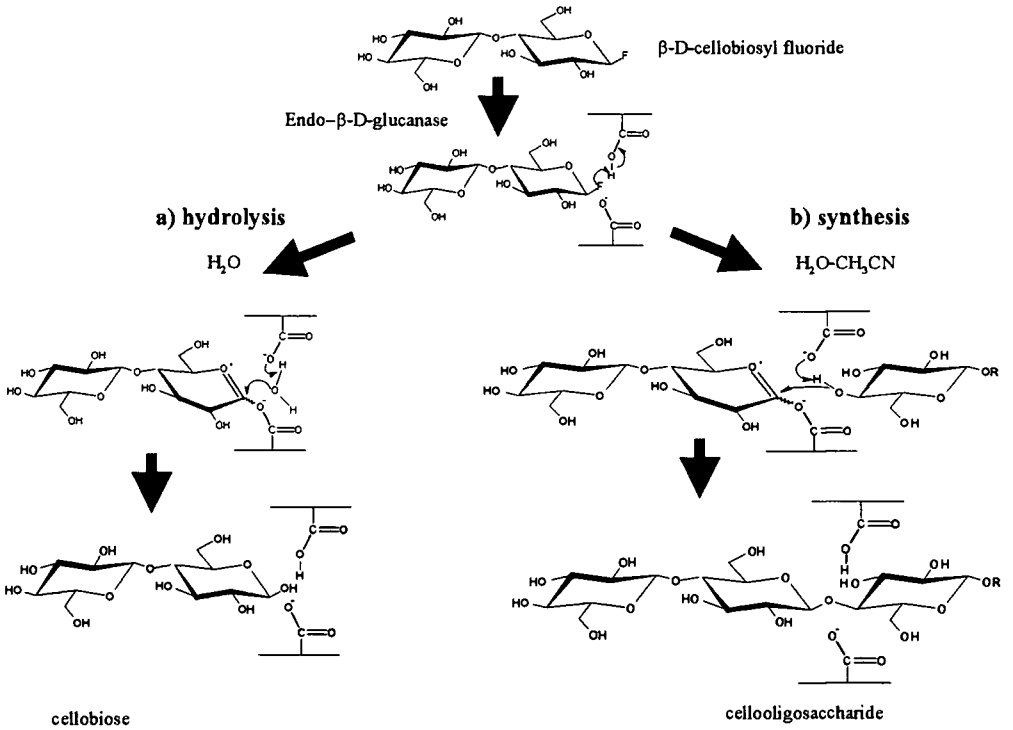

Figure 2.10. The enzymatic synthesis of cellooligosaccharides: a) hydrolysis mechanism; b) synthesis mechanism by endocellulases (adapted from http://www.glycoforum.gr.jp/science/word/glycotechnology/GT-B01E.html)

2.4.4. Fragmentation of Cellulose by Acid

The chemical synthesis approach has been used for the production of cellobiose, cellotriose and cellotetraose. This method is not practical for the preparation of longer cellooligosaccharides and it requires the disposal of heavy metals. Although enzymatic synthesis has been reported to be very specific and highly effective for the production of oligo and polysaccharide, like chemical synthesis, it is quite difficult, tedious and requires special expertise. Enzymatic fragmentation of cellulose is a fairly new method and there are some technical difficulties related with dissolution of cellulose (Arai et al., 2001) and the fractionation of large quantities of enzyme which have appropriate endo activity in the absence of β-glucosidase activity (Hishimoto and Matsumoto, 1994).

Currently the easiest way to produce cellooligosaccharides is by fragmentation of cellulose by acetolysis followed by deacetylation (Dickey and Wolfrom, 1949; Miller et al., 1960) or by direct acid hydrolysis (Miller et al., 1960; Pereira et al., 1988). Cellulose, such as avicel, cotton, and filter paper, which is inexpensive and commercially available in relatively pure form, has been used as the starting material for preparation of cellooligosaccharides. Sulfuric acid (Voloch et al., 1984), trifluoroacetic acid (Wing and Freer, 1984), or hydrochloric acid (Miller et al., 1960) have been used as a catalyst for hydrolysis of the cellulose. Acid hydrolyzes the glycosidic bonds between adjacent glucose units (Figure 2.11).

After fragmentation of the cellulose to cellooligosaccharides having different degrees of polymerization, the cellooligosaccharides can be separated using suitable chromatographic methods. The separation methods include adsorption chromatography with charcoal-celite columns (Miller et al., 1960), size exclusion chromatography on polyacrylamide or crosslinked dextrans (John et al., 1969; Sabbagh and Fagerson, 1976), ion-exchange chromatography (Ladisch et al., 1978), and partition chromatography using silica gel (Streamer et al., 1975).

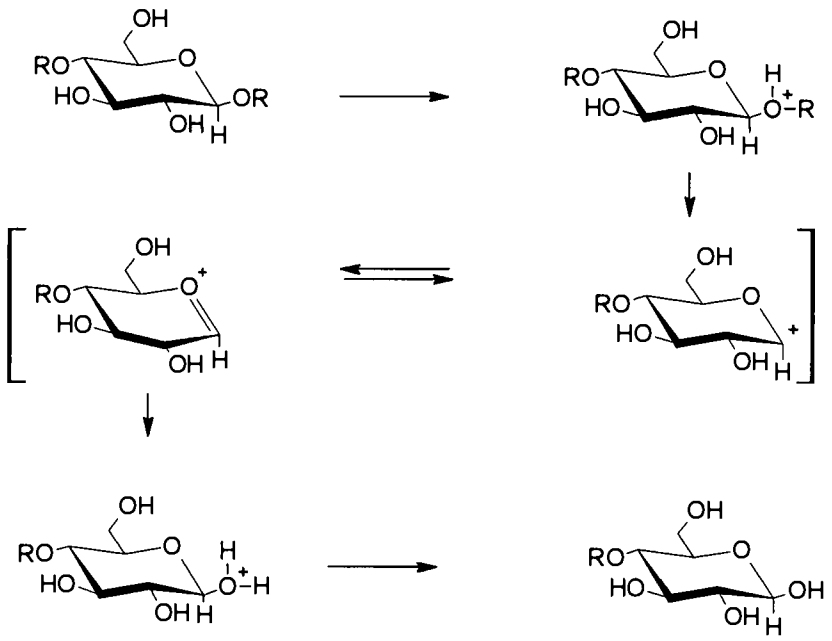

Figure 2.11. Acid catalyzed hydrolysis of cellulose (analogous to the hydrolysis of glycosidic linkages in cellulose)

2.5. UTILIZATION OF CELLOOLIGOSACCHARIDES

Modified and unmodified cellooligosaccharides have been used as model substrates for the characterization of cellulases. Also, they are the starting material for the synthesis of affinity ligands for cellulolytic enzymes. They have also started to receive attention in the food industry as non-digestible oligosaccharides.

2.5.1. Model Substrates

Pure soluble cellooligosaccharides (Figure 2.12.A) with DP 3 to 7 have been used for the characterization of cellulases (Hsu et al., 1980). They are the best mimics of cellulose since there are no structural changes. The only difference being that cellooligosaccharides have shorter chain lengths than cellulose and, thus, they are more soluble. However, the number of studies where these substrates have been used is small since these studies require advanced high performance liquid chromatography (HPLC) for the analysis of hydrolysis products. Another problem of using these substrates is that since the same pair of products is formed from hydrolysis at different glycosidic bonds, the glycosidic bonds of cellooligosaccharides hydrolyzed can not be precisely identified. Therefore, instead of using pure soluble cellooligosaccharides, derivatized or modified analogs of them are preferred.

Reduced oligosaccharides (Figure 2.12.B) are the first example of the derivatized anologs of cellooligosaccharides (Bhat et al., 1990; Schou et al., 1993a)

These substrates have been used to determine the cleavage patterns of enzymes. They are prepared by reduction of cellooligosaccharides with sodium borohydride, $NaBH_4$. The open alditol end at the reducing end of the cellooligosaccharides might affect the catalytic activity of the enzymes.

Another group of soluble substrates are chromophoric or fluorogenic cellooligosaccharides (Figure 2.12.C). Chromophoric or fluorescent groups such as p-nitrophenyl, 2,4-di-nitrophenyl, or 4-methylumbelliferyl are coupled to the reducing end of the cellooligosaccharides. The actual linkage hydrolyzed can be identified unequivocally. The main advantage of using these substrates is that kinetics can be determined by monitoring the change in fluorescence or UV absorbance resulting from the hydrolysis of the aglycon linkage at very low concentrations (van Tilbeurgh et al., 1982; Deshpande et al., 1984; Vozynki, et al., 1987; Nanjo and Sakai, 1990; Claeyssens and Aerts 1992; Armand et al., 1997). Also, when the glycosidic linkage between cellobiose and the chromophoric or the fluorescent groups are replaced with a sulfur atom, these substrates serve as a soluble inhibitor. These inhibitory thio analogs are very useful for the study of enzyme inhibition and enzyme-ligand interaction (Schou et al., 1993b; Barr and Holewinski, 2002). However, it should be noted that, these are modified substrates and the results must be analyzed with great care since the introduction of aglycon group onto the cellooligosaccharides can change enzyme-substrate interactions.

Isotope labeled cellooligosaccharides (Figure 2.12.D), such as ^{3}H, ^{14}C, and ^{18}O, overcome the limitations encountered in other substrates due to their being modified. Isotope labeling does not change the chemical structure of the cellooligosaccharides and it provides for sensitive detection (Chirico and Brown, 1987; Barr et al., 1996). Labeled compounds are easily detected by counting radioactivity of the sample or by mass spectrometry (MS). They are very useful for analysis of cleavage pattern (Chirico and Brown, 1985; Biely et al., 1993; Barr et al., 1996). Also, thin layer chromatography (TLC), has been used for the separation and analysis of different reaction products (Chirico and Brown, 1985; Biely et al., 1993).

Another group of model susbtrates for cellobiohydrolases are the reducing end tethered cellooligosaccharides (Figure 2.12.E). In this category, the substrates are covalently attached to a solid phase via functional group at their reducing end. Since the reducing end is blocked, the enzymes are forced to interact from the non-reducing end of substrates. These types of substrates have been used to understand how strict is the chain end specifity of enzymes (Sangseethong, 1999).

The last category of modified cellooligosaccharides are the thio cellooligosaccharides (Figure 2.12.F). Thio oligosaccharides have been used as tools in glycobiology to study the actions of cellobiohydrolases. These substrate inhibitors have at least one of the interglycosidic oxygen atoms substituted by a sulfur atom

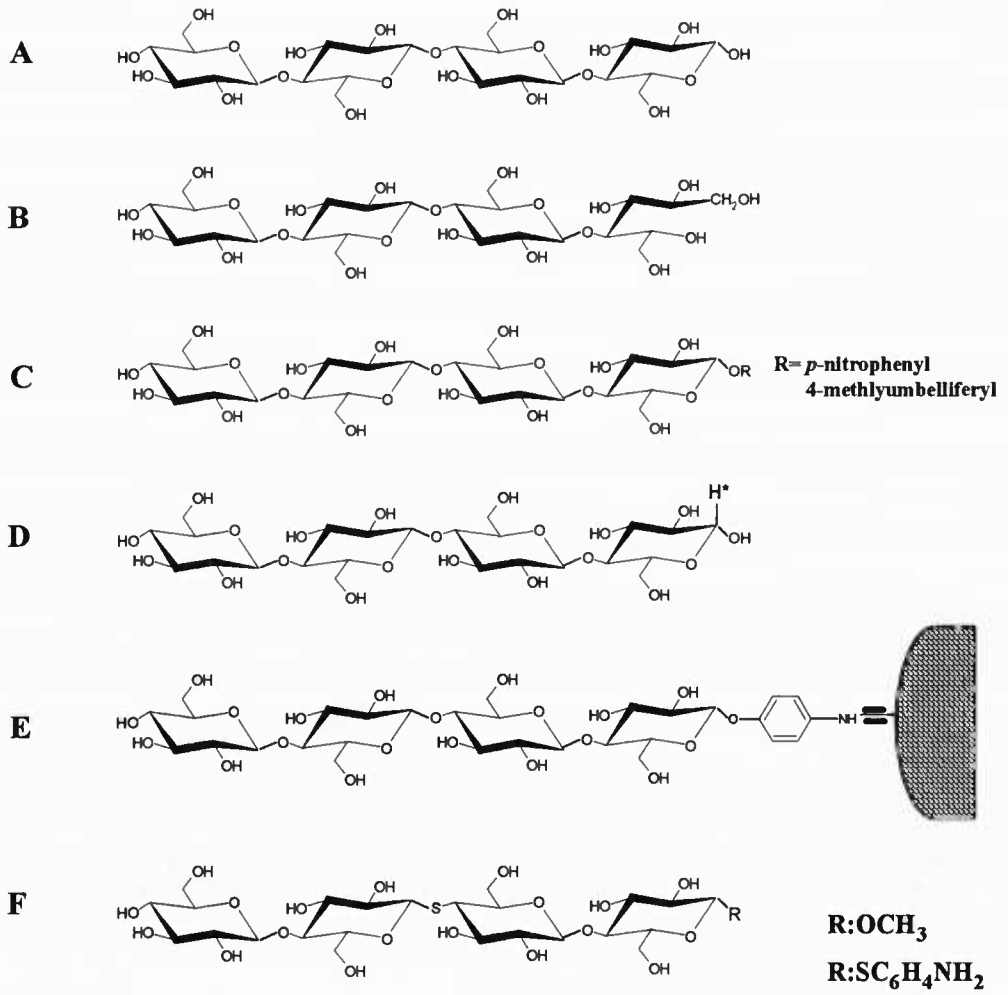

Figure 2.12. Substrates for cellobiohydrolases. A: unmodified cellooligosaccharides; B: reduced cellooligosaccharides; C: chromophoric and fluoregenic cellooligosaccharides; D: isotope labeled cellooligosaccharides; E: reducing-end tethered cellooligosaccharides; F: thio-cellooligosaccharides.

(Driguez, 1997). The substitution of an O linkage with an S linkage in cellooligosaccharides makes them resistant to cellulolytic attack. Because of their resistance to enzymatic attack, they have been used (1) in X-ray crystallography to obtain complexes of oligosacharides bound to enzyme active-site providing the information on how enzymes work (Sulzenbacher et al., 1996; Davies, 1998), (2) as an affinity ligand for purification of exo-type cellulases (Orgeret et al., 1992; Piyachomkwan et al., 1997) and (3) in the studies of enzyme inhibition (Schou et al., 1993b).

2.5.2. Affinity Ligand

Cellulolytic enzymes are usually a mixture of several discrete enzymes and the ratio of the components of this mixture depends on the organism producing the enzyme mixture and its method of cultivation. In addition, it may vary because the purification of the enzyme is difficult and the enzymes differ in modes of action, molecular weight, amino acid sequence, and isoelectric point. A combination of different purification methods is generally used to fractionate the individual components. In a typical purification protocol, crude cellulase mixtures are initially fractionated by DEAE-anion exchange chromatography. Fractions containing exocellulase activity are then applied to affinity columns in order to separate the exocellulases from contaminating non-cellulolytic proteins, endocellulases and β-glucosidases (Piyachomkwan et al., 1997).

Individual components of cellulolytic enzymes have been separated by using affinity chromatography. The target enzyme has specific interactions with the complementary affinity ligands and thus can bind whereas other compounds do not. These affinities between the desired compounds and ligands can subsequently be weakened by changing elution conditions such that they no longer facilitate the formation of enzyme-ligand complex (Robyt and White, 1990).

All affinity ligands used for the purification of cellobiohyrolases are oligosaccharides in which the reducing end has been modified. The first affinity ligand for purification of exocellulases was introduced by van Tilbeurgh et al. (1984). They were able to separate CBHI and CBHII from endocellulase by the use of affinity chromatography with immobilized *p*-aminobenzyl 1-thio-β-D-cellobioside (Figure 2.13.A). The second affinity ligand (Figure 2.13.B) was 4-aminophenyl 1,4-dithio-β-cellobioside (Orgeret et al., 1992). It has a thio linkage between glucose units which makes it more resistant to hydrolysis of β-glucosidase. Piyachomkwan et al. (1997) showed that *p*-aminophenyl 1-thio-β-D-cellobioside (Figure 2.13.C) was a functional affinity ligand for the separation of cellobiohydrolases. The main advantage of this ligand is that the chemistry involved with the synthesis is simpler than the previous two. Later, it was found that *p*-aminophenyl β-cellobioside (Figure 2.13.D) substrate for cellobiohydrolases could serve as an affinity ligand when it was immobilized onto agarose beads (Sangseethong and Penner, 1998).

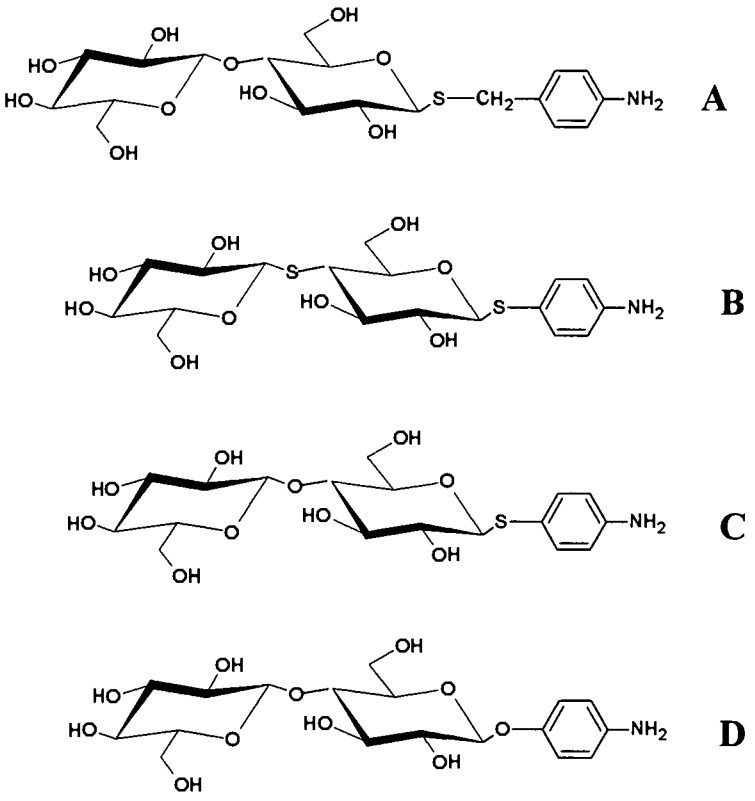

Figure 2.13. Affinity ligands for cellobiohydrolases: A: *p*-aminobenzyl-1-thio-β-D-cellobioside by van Tilbeurgh et al., 1984; B: *p*-aminophenyl 4-S-β-D-glucopyronosyl-1,4-dithio-β-D-glucopyronoside by Orgeret et al., 1992; C: *p*-aminophenyl 1-thio-β-D-cellobioside by Piyachomkwan et al., 1997; D: *p*-aminophenyl β-D-cellobioside by Sangseethong and Penner 1998.

A potential drawback of using *p*-aminobenzyl 1-thio-β-D-cellobioside, *p*-aminophenyl 1-thio-β-D-cellobioside, and *p*-aminophenyl β-cellobioside is that the glycosidic linkage of the cellobiose part of the ligand is not resistant to β-glucosidase. This problem can be minimized by using a β-glucosidase inhibitor in the mobile phase.

2.5.3. Non-digestible Oligosaccharides

General interest in the functionallity of food (functional food) has been important over the last decade. Functional food is defined by the Food and Nutrition Board of National Academy of Sciences as "any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains" (Thomas and Earl, 1994). Nowadays, functional foods receive enormous attention and they have become one of the fastest growing divisions of the world food industry. Researches have begun to focus on non-digestible oligosaccharides (NDO's) and resistant starch as functional foods. The use of NDO's as food ingredients has increased in some European countries and Japan (van Loo et al., 1999).

Non-digestible oligosacharides (NDO's) are oligosacharides that are resistant to hydrolysis by human enzymes and containing between 3 and 10 sugar moieties. Hence, they are not digested in the upper gastrointestinal tract and they are often referred to as prebiotics. Prebiotics are non-digestible food ingredients that

beneficially affects the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon and thus improve health (Roberfroid, 1998). However, not all NDO's are considered to be prebiotics. For a food ingredient to be a prebiotic (Table 2.5) it should have the following properties

1. It should be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal track.
2. It should be a selective substrate for one or limited number of bacteria in the colon thus selectively simulating the growth of bacteria.
3. It should alter the colonic microflora toward a healthier composition (Collins and Gibson, 1999).

At present, most studies for prebiotics have focused on the growth of lactic-acid producing microorganisms due to their health-promoting properties. However, in the future, the study of prebiotics may include aspects of their effect on pathogenic flora (Collins and Gibson, 1999). Cellobiose is an example of the latter. It was found that cellobiose repressed virulance of *Listeria monocytogenes* (Park and Kroll, 1993).

NDO's can serve as prebiotics as well as non-caloric bulking agents and soluble dietary fibers. The physiological importance and health benefits of NDO's are given in Table 2.6. NDO's naturally occur in various plants, processed foods and human breast milk. They have been commercially produced by extraction of

Table 2.5. Chemical composition and characteristics of prebiotic carbohydrates (Macfarlane and Cummings, 1999).

Oligosaccharides	Chemical Composition
Fructo-oligosaccharides (Raftilose P95)	95% oligosaccharides β(2-1) fructan, 60% glucose fructose(n), 40% fructose(n) dp 2-8, average 4-5
Inulin	>99% oligosaccharides β(2-1) fructan, average dp 10-12
Pyrodextrins	Complex mixtures of glucose-containing oligosaccharides
Transgalactosylated oligosaccharides (Oligomate 55)	Mainly 6' galactosyllactose, dp of oligosaccharide fraction 2-5 (primarily dp 3); 55% pure
Galacto-oligosaccharides	Oligogalactose (85%), small amounts of glucose, galactose and lactose
Soya oligosaccharides	Stachyose (fructose, galactose, glucose) and rafinose (fructose, galactose, glucose), dp 3-4
Xylo-oligosaccharides	β(1-4) linked xylose; 70% pure, dp of oligosaccharide fraction 2-4
Isomalto-oligosaccharides	Mixture of α(1-6) linked glucose oligomers (isomaltose, panose, isomaltotriose)
Lactulose	Galactose and fructose-containing disaccharides

Table 2.6. Physiological importance and health benefits of non-digestible oligosaccharides (Macfarlane and Cummings, 1999)

Physiological effects	**Health factors**
Stimulate carbohydrate metabolism in colonic bacteria: increased bacterial mass, short chain fatty acids and fermantation gases	Through short chain fatty acids, they provide energy sources for the colonic epithelium and control of differentiation. Flatulence may be a problem. Laxative effects
Selection of bifidobacterial and lactic acid bacterial growth in large bowel	Enhanced resistance to invading pathogens
Not hydrolysed by oral micro-organism	Protection against caries
Not glycaemic	Potentially useful for diabetics
Non-specific stimulation of immune function	Resistance to infection
Modulation of carcinogen metabolism	Anticancer properties
Reduced hepatic synthesis of very low density lipoprotein cholesterol and serum triglycerides	Coronary heart disease
Increased absorption of Mg and Ca	Osteoporosis

naturally occurring NDO's, chemical condensation reactions, transglycosylation reactions, are controlled hydrolysis of polysaccharides (van Laere et al., 2000).

Cellooligosaccharides are starting to receive attention as non-digestible oligosaccharides. Cellooligosaccharides are sugar oligomers, which are made up of D-glucose units linked together by β-1,4-glycosidic linkages. They have the same chemical structure as cellulose with degrees of polymerization between 2 and 7. They are typically produced by depolymerization .of cellulose.

Since cellooligosaccharides are not hydrolyzed in the upper gastrointestinal tract or absorbed from gastrointestinal tract, they are a type of NDOs. Studies on sugar tolerance tests of cellooligosaccharides showed that cellobiose served as an indigestible sugar in healthy humans, so it did not increase blood glucose levels (Satouchi et al., 1996; Watanabe, 1998). The digestibility, absorptive, and physiological effects of cellooligosaccharides in human beings showed that cellooligosaccharides affect carbohydrate metabolism and have potential application in the prevention of diabetes and obesity (Watanabe, 1998).

The simple way to prepare cellooligosaccharides is to fragment cellulose by either acid or enzyme. In general, food-grade oligosaccharides are not pure products, rather they are mixtures containing oligosaccharides with different degrees of polymerization. For food applications, cellobiose (DP=2) is considered to be a cellooligosaccharide, even if for other purposes the concept oligo is defined as that having a higher DP. The separation of cellooligosaccharides within a given DP range

can be carried out by using membrane techniques in order to remove cellooligosaccharides within an undesired DP range (Sasaki et al., 1989; Watanabe et al., 1993). Removal of simple sugars, such as glucose, provides oligosaccharide products with several advantages. These mixtures have increased viscosity and reduced swettness and cause less Mailard reactions during heat processing (Crittenden and Playne, 2002). In addition, for cellooligosaccharides, the absence of glucose lowers caloric value and allows the oligosaccharides to be included in diabetic foods.

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CHAPTER 3

CELLULOSE-BASED CHROMATOGRAPHY FOR CELLOOLIGOSACCHARIDE PRODUCTION

3.1. ABSTRACT

The chromatographic separation of cellooligosaccharides using cellulose as a stationary phase was studied. The driving force of the work is the current interest in using cellooligosaccharides as functional non-digestible oligosaccharides in foods. The studies presented here illustrate the potential of using ethanol-water mobile phases in conjunction with cellulose stationary phases for cellooligosaccharide fractionation. Cellooligosaccharide solubility in ethanol-water mixtures and their elution order from cellulose-based columns using ethanol-water mobile phases were shown to be in line with their degree of polymerization (DP); the higher DP cellooligosaccharides being less soluble and having longer retention times. Microcrystalline cellulose and fibrous cellulose were shown to work as chromatographic stationary phases. The results demonstrated that cellotetraose and cellopentaose could be obtained in relatively pure form using a cellulose stationary phase with a 20% ethanol mobile phase at room temperature. The application experiments demonstrate the potential of using cellulose stationary phases for clean-up and fractionation of cellooligosaccharide mixtures generated via acid-catalyzed hydrolysis of cellulose.

3.2. INTRODUCTION

Functional foods defined as those "...consumed as a part of usual diet and demonstrated to have physiological benefits and/or reduce the risk of chromic disease beyond basic nutritional functions" (Dentali, 2002) are important internationally from both health and economic perspectives. Nowadays, within the general class of functional foods, prebiotics receive enormous attention and they have become a popular food ingredient for the world food industry (Milner, 2000; Sloan, 2000; Shah, 2001). Prebiotics are defined as non-digestible food ingredients that beneficially effect the host by selectively stimulating the growth or activity of one or a limited bacteria in the colon and thus improve the health (Roberfroid, 1998; Macfarlane and Cummings, 1999). Non-digestible oligosacharides (NDOs) are oligosacharides containing between 3 and 10 sugar moieties that are resistant to digestion by human gastric and pancreatic enzymes. They are considered candidate prebiotics (Cummings et al., 2001). NDO's naturally occur in various plants, processed foods and human breast milk. They have been commercially produced by extraction, chemical condensation, transglycosylation reactions, or controlled hydrolysis of parent polysaccharides (van Laere et al., 2000). A number of such preparations have been investigated (van Loo et al., 1999).

Cellooligosaccharides are sugar oligomers, which are made up of β-D-glucose units linked together by 1,4-glycosidic linkages. They are potentially the most widely available of the NDO's, in that the parent polysaccharide is the largest carbon sink in the biosphere. Studies with humans and rodents have suggested beneficial affects related to carbohydrate metabolism, diabetes and obesity associated with cellooligosaccharides intake (Satouchi et al., 1996; Watanabe, 1998). The widespread technological application of cellooligosaccharides in foods is dependent on practical routes for the preparation of products with defined chemical/physical properties.

Cellooligosaccharides are produced from cellulosic materials by fragmentation of cellulose using acid or enzyme. The resulting mixed cellooligosaccharide are then separated by based on their DP by using suitable chromatographic methods (Miller et al., 1960; John et al., 1969; Streamer et al., 1975; Sabbagh and Fagerson, 1976; Ladisch et al., 1978, Hamacher et al., 1985). However, food-grade oligosaccharides are not pure products, rather they are mixtures containing oligosaccharides with different degrees of polymerization. In this study, we explore the potential of using cellulose as the chromatographic stationary phase for cellooligosaccharides fractionation. The rationale is to exploit the affinity of cellooligosaccharides for its parent polysaccharide, cellulose. Cellulose itself is a relatively inexpensive stationary phase and it would be readily available at cellooligosaccharides processing facilities. The results demonstrate that

cellulose stationary phase, can be used to obtain cellooligosaccharides preparations with unique degree of polymerization profiles and, hence, functional properties.

3.3. MATERIALS AND METHODS

3.3.1. Materials

The Aminex HPX-42A column (dimension: 300x7.8 mm; average particle size: 25 μm) was purchased from Bio-Rad Laboratories, Hercules, California; fibrous cellulose powder (CF 11) and LK5D (150 Å) TLC plates from Whatman Chemical Division, Clifto, New Jersey; microcrystalline cellulose (Avicel PH 105) from FMC Corp., Rockland, ME; 4,4' dicarboxy-2,2'-biquinoline (Disodium salt of Bicinchonic acid) from Sigma Chemical Co., St Louis, MO; *p*-anisaldehyde from Aldrich Chemical Co, Milwaukee, WI.

3.3.2. Preparation of Water Soluble Cellooligosaccharides

Soluble cellooligosaccharides were prepared according to Miller et al. (1960) with slight modification. Ten grams of fibrous cellulose powder was dissolved in 100 mL concentrated HCl, pre-cooled to –30 ^{0}C, and stirred for 15-20 min. The temperature was then raised to 25 ^{0}C and the stirring continued for 2-3 hr. The solution was then slowly added to 725 mL 4 ^{0}C 1-propanol and stirred for 15 min. The resulting precipitate was collected after centrifugation at 5,000xg for 5 min. The pellet was washed with technical grade ethanol until the pH reached 5-6. Finally, the pellet was extracted with 400 mL cold double-distilled water. The insoluble material was removed by centrifugation and decantation. The

cellooligosaccharides in the aqueous phase were concentrated by rotary evaporation and freeze dried. The DP profile of the resulting cellooligosaccharide mixture was determined by HPLC analysis using an Aminex HPX-42A column at 85°C, water mobile phase, and refractive index detector.

3.3.3. Ethanol and Cellulose Affect on Cellooligosaccharide Solubility

One mg of cellooligosaccharide preparation was added to 1 ml of 0, 20, 40, 60, 80 or 90 % ethanol-in-water solutions. Solutions were mixed thoroughly and allowed to stand at ambient (~22 °C) temperature for 1 hour. The resulting solutions/suspensions were then centrifuged at 5000xg for 5 min and the DP profile of the soluble phase determined as described above. The affect of microcrystalline cellulose on cellooligosaccharides solubility was investigated by adding 9 mg of the appropriate cellulose with 1 mg of cellooligosaccharides preparation to 1 mL of 0, 20, 40 and 60% ethanol-in-water solutions. The resulting suspensions were mixed and allowed to stand at ambient temperature for 2 hrs. The resulting suspensions were processed and analyzed as described for those test solutions not containing cellulose.

3.3.4. Cellooligosaccharide Chromatography with Cellulose Stationary Phases

Fibrous and microcrystalline celluloses were tested as chromatographic media in conjunction with water/ethanol mobile phases. Cellulose preparations were washed repeatedly to remove impurities and fines prior to their being packed, using

gravity feed, into 2 x 25 cm columns. The columns were then equilibrated with the starting mobile phase. A representative cellooligosaccharide preparation, ~100 mg, was dissolved in 10 ml of the same mobile phase and then introduced onto the column. Column eluate was monitored for the presence of cellooligosaccharides, as reducing sugars, using a 2,2'-bicinchoninate-based assay (Garcia et al., 1993). Fractions showing the presence of reducing sugars were further analyzed by thin layer chromatograpy (silica plates, ethyl acetate:methanol:water 40:20:15 mobile phase, *p*-anisaldehyde-sulfuric acid visualizing reagent) to determine the DP of the cellooligosaccharides therein. On the basis of the TLC results, fractions containing homologous cellooligosaccharides were pooled and cellooligosaccharides quantified via HPLC analyses, as described above. Following each chromatographic run, columns could be regenerated by washing with water at room temperature until no further reducing sugars could be detected.

3.3.5. Application Experiments

3.3.5.1. Experiment 1

Soluble cellooligosaccharides were prepared as described above with the exception that following the 2-3 hr reaction period at 25 °C the solution was poured into 400 mL ice-cold water and subsequently neutralized, to ~ pH 6, with solid $NaHCO_3$. The resulting solids were removed via centrifugation and the supernatant made 60% in ethanol. Any additional precipitate formed at this time was removed

prior to chromatography. Two hundred ml of the neutralized cellooligosaccharides in 60%-ethanol solution was then loaded on a 60 % ethanol-equilibrated 5 X 25 cm column packed with fibrous cellulose. The column was run as a one step gradient, using 60% ethanol as the initial eluent (for elution of lower DP cellooligosaccharides and salts) and water as the final eluent. Eluate was monitored via reducing sugar assays, TLC and HPLC as described above.

3.3.5.2. Experiment 2

Soluble cellooligosaccharides were prepared as described above with the exception that the cellooligosaccharides and cellulose containing pellet obtained following centrifugation of the isopropanol-diluted hydrolysis mixture was suspended in 100% ethanol. This suspension was packed into an empty 2.5x 45 cm column, thus providing a uniform mixture of cellulose stationary phase and associated cellooligosaccharides. The column was then washed with ethanol until the eluent pH was 5-6. Soluble cellooligosaccharides were then eluted using an ethanol (100%) → water gradient. Eluate was monitored via reducing sugar assays, TLC and HPLC as described above.

3.4. RESULT AND DISCUSSION

Initial experiments were aimed at determining the extent to which ethanol, a relatively safe and inexpensive solvent affects cellooligosaccharide solubility. The experiments were performed using a representative cellooligosaccharide mixture, the composition of which is given in Table 3.1. Figure 3.1 illustrates the large decline in cellotetraose, cellopentaose and cellohexaose solubility as ethanol concentrations were increased above 60%; cellopentaose and cellohexaose being essentially insoluble at ethanol concentrations ≥80%. The results demonstrate that moderate-to-high ethanol concentrations profoundly influence cellooligosaccharide solubility, the extent of the affect being dependent on the degree of polymerization of the cellooligosaccharides. This lends credence to ethanol-based clean-up protocols applied to heterogeneous cellooligosaccharide preparations and indicates that ethanol-water mixtures have potential as mobile phases for the chromatographic fractionation of cellooligosaccharides differing only with respect to degree of polymerization.

The addition of small amounts of cellulose to cellooligosaccharide containing ethanol/water solutions had a significant impact on the relative solubility of the different cellooligosaccharides (Figure 3.2). Cellohexaose, which showed appreciable solubility in the both 40 and 60% ethanol solutions in the absence of cellulose, was effectively adsorbed from these solutions due to the addition of relatively small amounts of microcrystalline cellulose. The addition of

microcrystalline cellulose also resulted in decreases in the cellopentaose content of the 40 and 60% ethanol solutions, although the extent of adsorption of cellopentaose was significantly less than that observed for cellohexaose. The effect of cellulose addition on cellotriose and cellotetraose solubility, in these same solutions, was negligible. The cellulose effects are consistent with the relative solubility of the different cellooligosaccharides; cellooligosaccharide solubility decreases as the degree of polymerization increases (Taylor, 1957; Huebner et al., 1978). The cellulose component of the reaction mixtures provides a template upon which cellooligosaccharides may adsorb. The cellooligosaccharide-cellulose complexes presumably have interaction geometries equivalent to, or very similar to, those of cellooligosaccharide aggregates formed in the absence of cellulose. This being a consequence of cellulose fiber/microcrystal surfaces being comprised of arrays of cellulose molecules – the surfaces thus approximating a two-dimensional array of cellooligosaccharide domains (Kuutti et al., 1995). When considering cellooligosaccharide adsorption, we presume that the change in entropy associated with cellooligosaccharide-cellulose complex formation is more favorable than the analogous change in entropy for the association of two soluble cellooligosaccharides – since the cellulose molecule itself has a relatively limited degree of freedom.

Table 3.1. The concentration of individual components of cellooligosaccharides mixtures in water

Cellooligosaccharide mixtures	Cellotriose (mM)	Cellotetraose (mM)	Cellopentaose (mM)	Cellohexaose (mM)
1 mg/mL	0.23	0.40	0.33	0.23

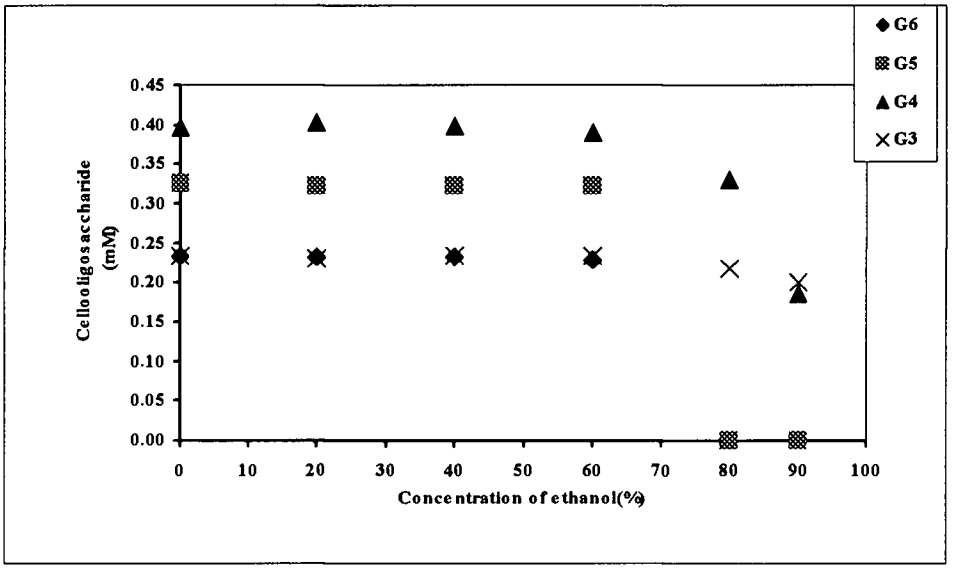

Figure 3.1. The effect of ethanol on cellooligosaccharides solubility (G3: cellotriose; G4: cellotetraose; G5: cellopentaose; G6: cellohexaose).

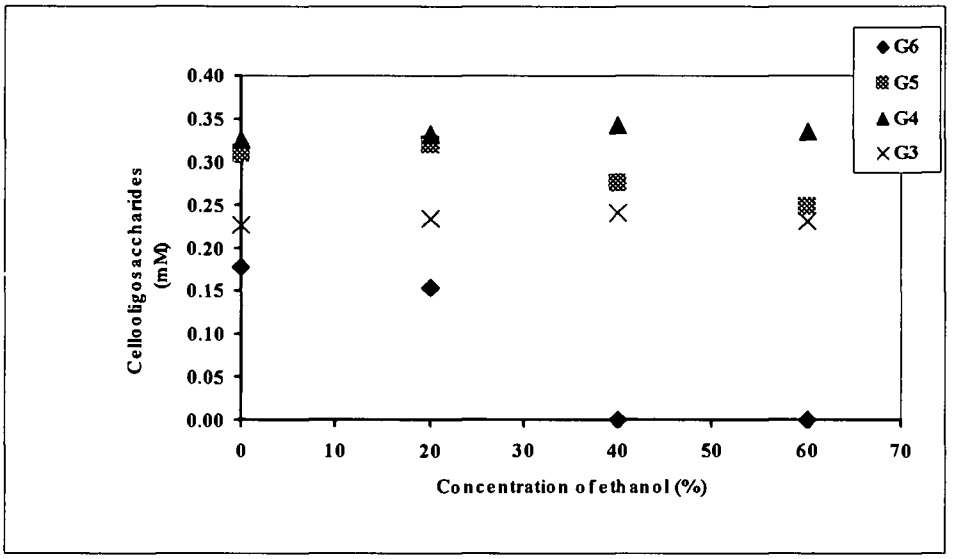

Figure 3.2. The effect of microcrystalline cellulose on cellooligosaccharide solubility in different ethanol concentration (G3: cellotriose; G4: cellotetraose; G5: cellopentaose; G6: cellohexaose).

The chromatogram presented in Figure 3.3 illustrates the potential of using water/ethanol mobile phases in conjunction with cellulose stationary phases for the fractionation of cellooligosaccharides. The stationary phase in this case was a commercially available microcrystalline cellulose. The results demonstrate that a 20% ethanol mobile phase can be used to effectively separate the lower DP cellooligosaccharides (DP $\leq$3) from the higher DP cellooligosaccharides. Under these conditions, cellopentaose was obtained with baseline resolution. The elution

volume of the lower DP cellooligosaccharides (predominantly cellotriose, see Table 3.1) was found to be greater than the void volume for the column; the implication being that cellooligosaccharides as short as cellotriose have demonstrable affinities for cellulose stationary phases. This is in agreement with the results of Chitumbo and Brown (1971), who reportedly observed chemisorption between lower DP cellooligosaccharides and cross-linked cellulose gels.

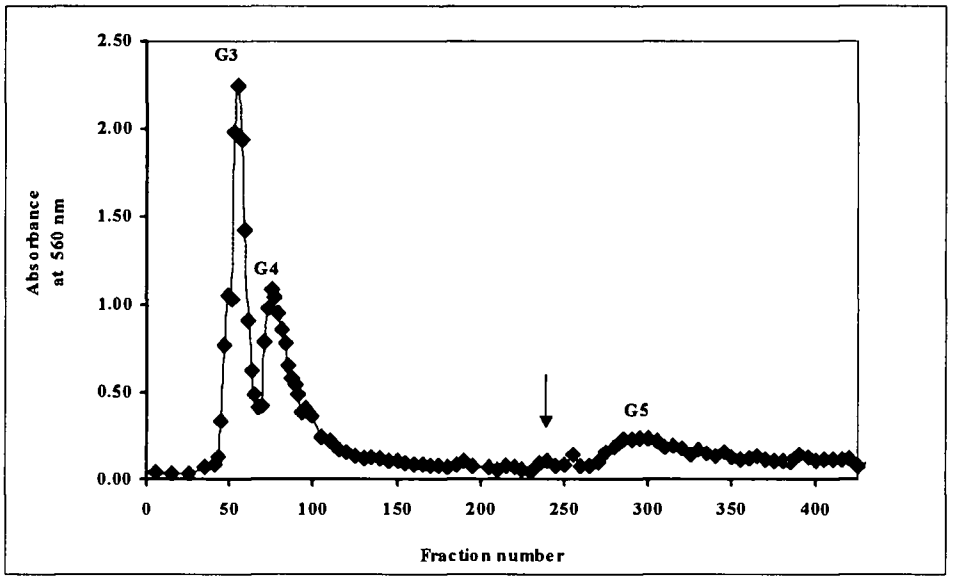

Figure 3.3. Elution profile of cellooligosaccharides from microcrystalline cellulose (Avicel PH105) with 20% ethanol-water. Arrow indicates that mobile phase was replaced with water (G3: cellotriose; G4: cellotetraose; G5: cellopentaose).

The results reported thus far were obtained using a microcrystalline cellulose as the solid phase. The question arises as to whether or not a less crystalline cellulose preparation would give similar results. This question was addressed by running the same experiment as depicted in Figure 3.3 with the exception of using traditional fibrous cellulose as the stationary phase. The results are presented in Figure 3.4. The chromatograms of Figures 3.3 and 3.4 are similar, suggesting that either fibrous or microcrystalline cellulose preparations may be used for cellooligosaccharide fractionation. However, the retention times for the higher cellooligosaccharides were consistently greater when working with the microcrystalline stationary phases. Thus, the more crystalline stationary phases seem to favor cellooligosaccharide chemisorption.

The effect of temperature on the partitioning of the cellooligosaccharides was studied using the microcrystalline stationary phase with water as the mobile phase (Figure 3.5a). Increased temperatures corresponded with increased retention volumes for all cellooligosaccharides. The temperature effect was slightly more pronounced for the longer cellooligosaccharides (cellotetraose and cellopentaose), thus improving the resolving power of the column (Figure 3.5b). The retention volume of the lower DP cellooligosaccharides, which appear to have relatively low affinities for microcrystalline cellulose, also increased with temperature. Again suggesting that at least minimal chemisorption occurs between the lower DP cellooligosaccharides and microcrystalline cellulose.

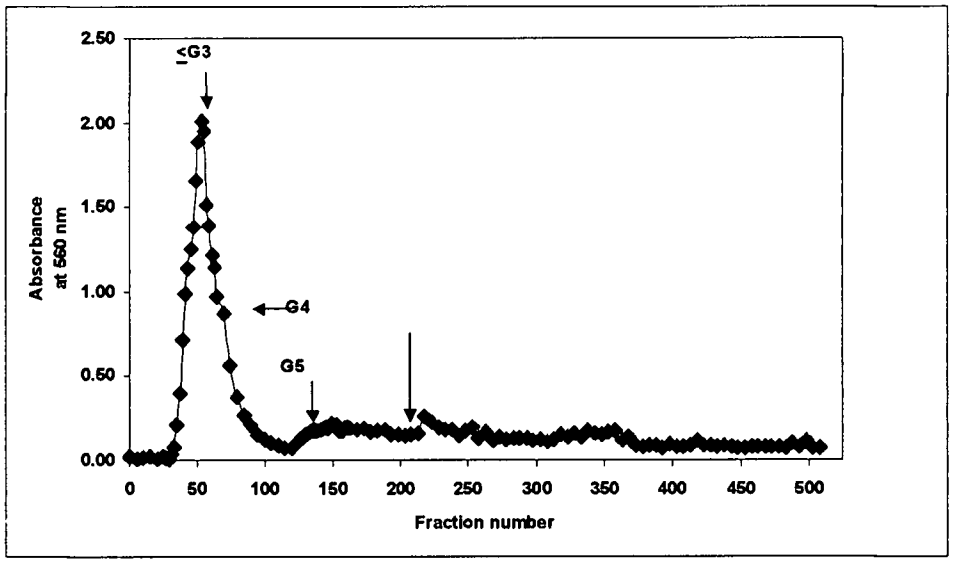

Figure 3.4. Elution profile of cellooligosaccharides with 20% ethanol-water from fibrous cellulose (CF11) Arrow indicates that mobile phase was replaced with water (G3: cellotriose; G4: cellotetraose; G5: cellopentaose).

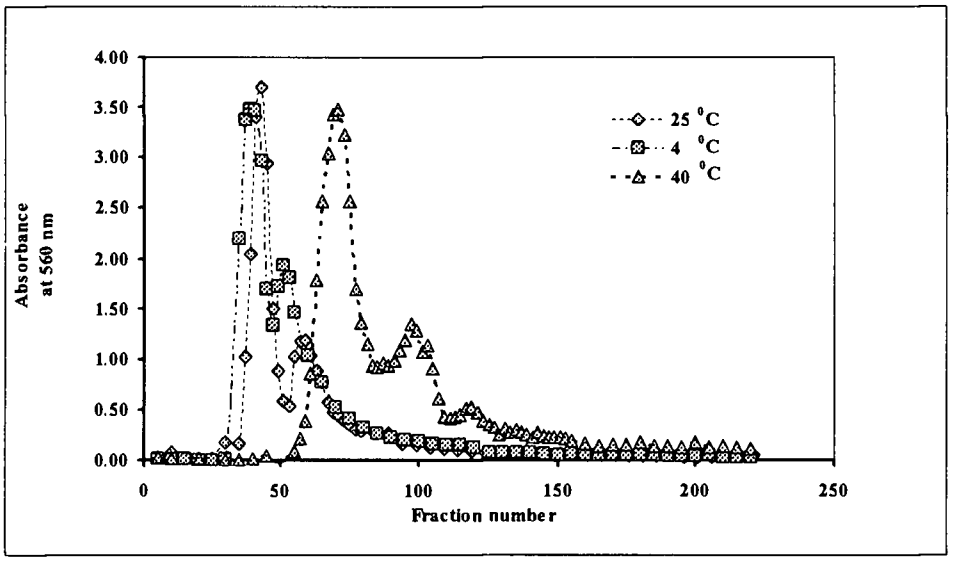

Figure 3.5a. The potential of using water mobile phase along with cellulose stationary phases for the fractionation of cellooligosaccharides.

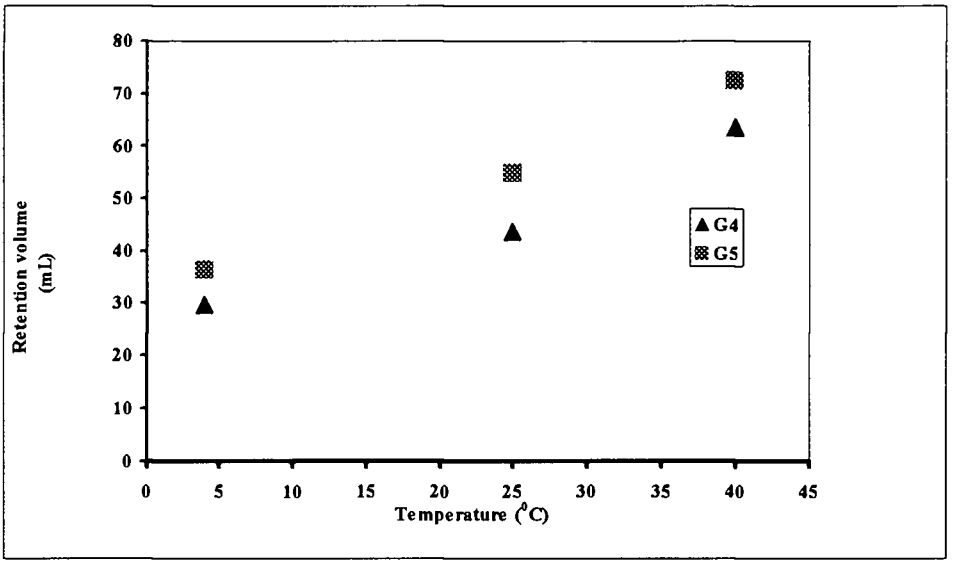

Figure 3.5b. The effect of temperature on the elution of cellotetraose and cellopentaose from the column (G4: cellotetraose; G5: cellopentaose)

Figures 3.6 and 3.7 provide illustrations of how cellulose-based columns may be used for making cellooligosaccharide preparations with differing DP profiles. The scheme of Figure 3.6a summarizes the case where a cellulose hydrolysate mixture, containing the complete range of cellooligosaccharides plus unhydrolyzed cellulose, is diluted with alcohol to precipitate the cellooligosaccharides and subsequently the liquid phase removed along with the majority of the glucose and cellobiose. The remaining insoluble phase is suspended in ethanol, loaded into an empty column, washed with ethanol, and then the cellooligosaccharides eluted from the accompanying cellulose using an ethanol/water gradient. The unique aspect of this approach is that the cellulose remaining after acid-catalyzed hydrolysis is used as the stationary phase in the column. The stationary phase in this case is expected to be less crystalline than either the microcrystalline cellulose or the fibrous cellulose, since the dissolution process preceding cellulose hydrolysis is similar to that use for the preparation of amorphous cellulose (Hsu and Penner, 1991). The chromatogram of Figure 3.6b illustrates that two crude fractions, one that is predominantly cellotriose and the other dominated by higher DP cellooligosaccharides, can be easily obtained. Interestingly, the higher DP cellooligosaccharides were eluted from this column at higher ethanol concentrations than with the corresponding columns discussed above. This is consistent with the higher DP cellooligosaccharides having a greater affinity for crystalline cellulose.

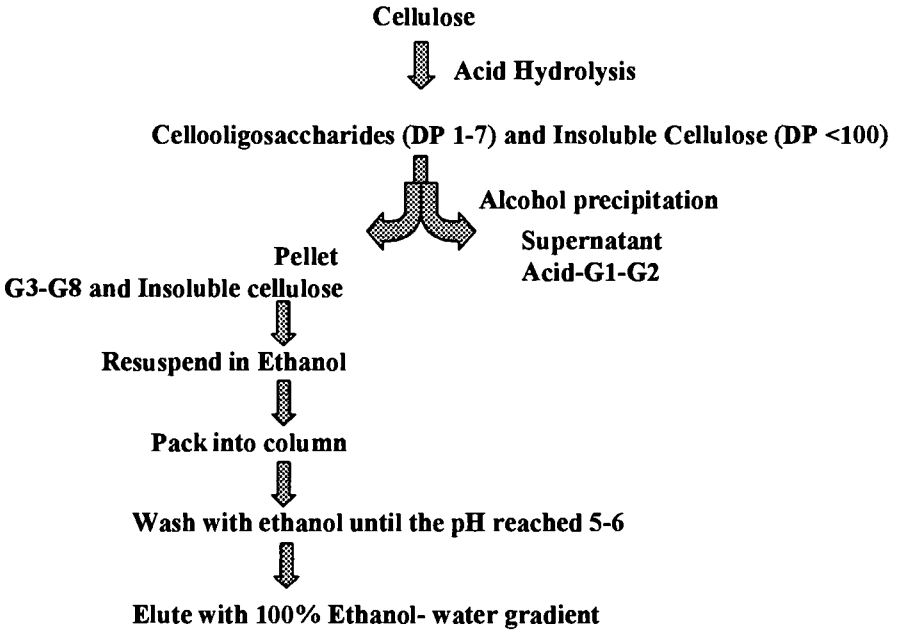

Figure 3.6a. Application scheme of cellulose based materials for preparation of cellooligosaccharides

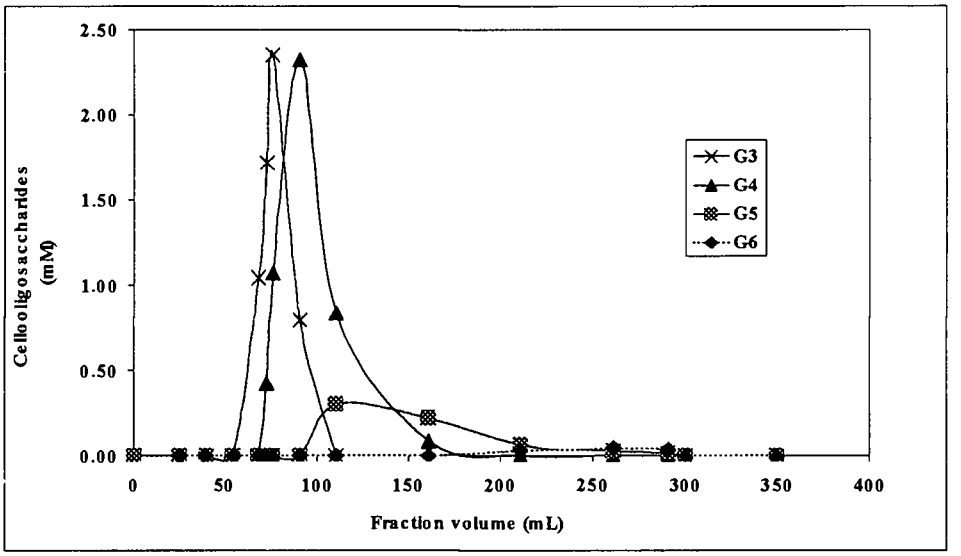

Figure 3.6b. Quantification of eluted cellooligosaccharides with ethanol and water from cellulose remaining after acid-catalyzed hydrolysis

The scheme of Figure 3.7a summarizes the case where the liquid phase resulting from the cellulose hydrolysis process (see "Methods") is neutralized, made 60% in ethanol, and then directly applied to a cellulose column. In this case the solution to be chromatographed contained substantial amounts of glucose and cellobiose, these being unavoidable side-products generated during the making of cellooligosaccharides via acid-catalyzed hydrolysis. These sugars are not present to any significant extent in the previously discussed cellooligosaccharide preparations because they were removed, prior to chromatography, by repeated alcohol washes, as is traditionally done (Pereira et al., 1988). In the present case, the complete mixture resulting from the hydrolysis of cellulose was chromatographed. It can be seen that the glucose and cellobiose were eluted from the column in the initial carbohydrate-containing fractions; the higher molecular weight cellooligosaccharides coming in later fractions (Figure 3.7b). Hence, the data illustrates the potential of using cellulose columns, as a possible alternative to alcohol washes, for the separation of lower molecular weight sugars from cellooligosaccharides. This type of separation is expected to be a prerequisite step for many applications of cellooligosaccharides.

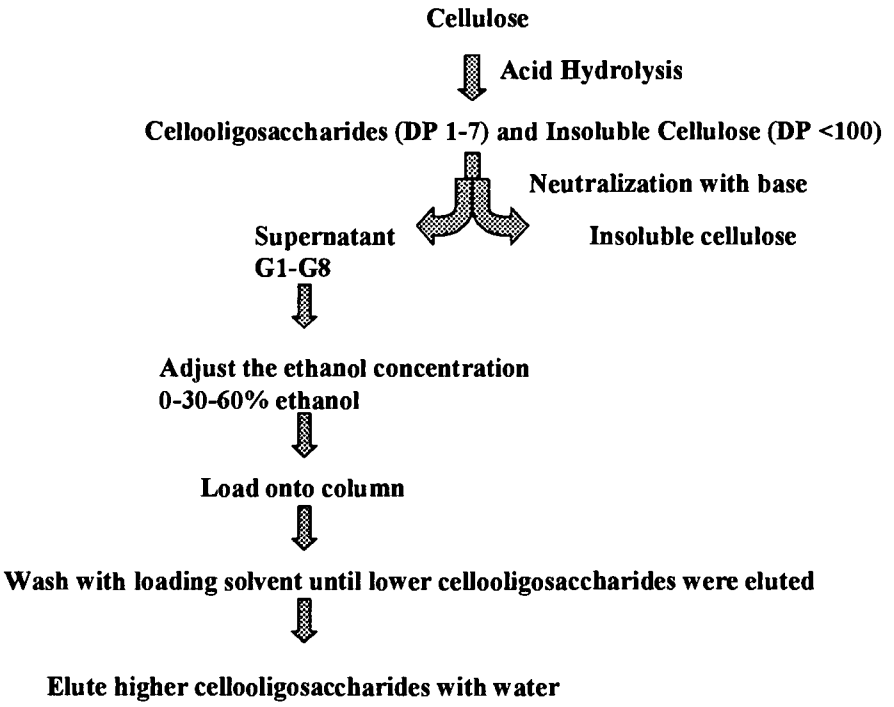

Figure 3.7a. Application scheme of cellulose based materials for preparation cellooligosaccharides.

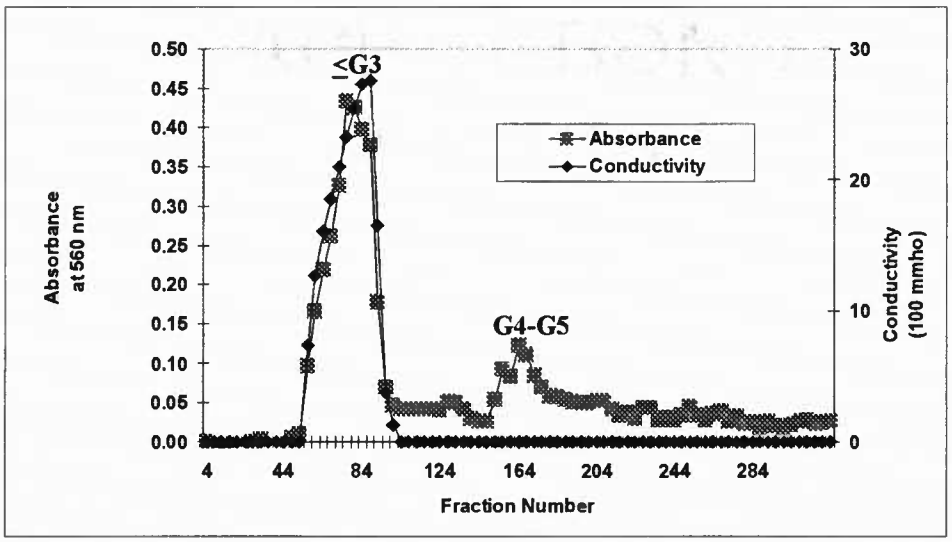

Figure 3.7b. Elution profile of cellooligosaccharides from fibrous cellulose (CF 11) with 60% ethanol–water (G3: cellotriose; G4: cellotetraose; G5: cellopentaose).

In nature cellulose forms microfibrils where the molecules are packed tightly together by intra and inter molecular hydrogen bonds and van der Waals interactions. The intermolecular hydrogen bonding and van der Waals forces cause cellulose chains to pack very tightly together and form highly ordered structures. Similarly, the structural identity and the formation of hydrogen bonds between cellulose and cellooligosaccharides causes strong chemisorption.

Adsorption between a solute and a adsorbent is related to the solubility of the solute. Typically, among a homologous series, adsorption increases strongly and regularly with increasing molecular weight of the solute (Freundlich, 1926). Figure 3.2 illustrates that the adsorption of cellooligosaccharides to cellulose becomes more important with increasing chain length of the cellooligosaccharides since the solubility of the cellooligosaccharides decreases with increasing chain length. This is thought to be the result of an increased number of intermolecular interactions (Chitumbo and Brown, 1971; Huebner et al., 1978).

The choice of solvent is very important for interaction of cellooligosaccharides and cellulose. Solvents that favor the solubility of cellooligosaccharides such as DMSO, interfere with the adsorption of cellooligosaccharide to the cellulose support. In such a case, interactions between the solute and the solvent are stronger than the interactions between the solute and the adsorbent. The separation observed in these latter cases are based on liquid-liquid partition chromatography (Chimota and Brown, 1971; 1973). The opposite

effect occurs when water or ethanol-water is used as the mobile phase. Incorporating ethanol into eluting solvent makes cellooligosaccharides less soluble, thus causing cellooligosaccharides to bind strongly on cellulose chains (Figure 3.2). This effect becomes more noticeable with increasing molecular weight.

The partition of solute with adsorbent is determined by the net interaction between adsorbent-solute and solute-solvent. Therefore, when the temperature is altered, it may favor or inhibit adsorption to the stationary phase (Brown, 1972). The interaction between cellooligosaccharides and cellulose decreased as the temperature decreased. It is difficult to interpret this relationship because there may be unobserved changes in the character of the cellulose stationary phase as a function of temperature (Chitumbo and Brown, 1973).

This study shows that cellooligosaccharides at chain length greater than three units showed adsorption on cellulose support depending on the conditions. The elution profiles of these four different conditions showed that the elution profile of adsorbed cellooligosaccharides became flattened and broad contrasting with the sharp profiles of non-adsorbed cellooligosaccharides. The results are consistent with the retained cellooligosaccharides participating in chemisorption with the cellulose support. The results show that cellulose can be used as a stationary phase for the preparation of cellotetraose and cellopentaose.

Since cellulose has been used in food industry to stabilize foam, to replace fat, to control ice crystal growth and to form a gels (BeMiller and Whistler, 1996),

the use of it as a support for the separation of cellooligosaccharides with different chain length provide as an advantage for preparation of food grade cellooligosaccharide. The results showed that the insoluble part after acid hydrolysis of cellulose could serve as stationary phases as post hydrolysis processing aids for the production of cellooligosaccharides and removing of glucose. Also, by using any kind of cellulose support, different combination of cellooligosaccharides with desired DP range could be obtained with low cost and these mixture can be useful to be used in the food industry as non-digestible oligosaccharides.

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CHAPTER 4

PREPARATION OF CELLOOLIGOSACCHARIDES: COMPARATIVE STUDY

4.1. ABSTRACT

Cellooligosaccharides having a specific degree of polymerization are commonly used in mechanistic studies of cellulolytic enzymes. They are typically produced by the controlled hydrolysis of cellulose, which produces a mixture of cellooligosaccharides differing with respect to their degree of polymerization, followed by chromatographic fractionation to obtain preparations homogeneous with respect to degree of polymerization. The following chapter summarizes a comparative study of suggested fractionation methods. Charcoal-celite adsorption, molecular exclusion, ion-mediated, silica and cellulose affinity/partition chromatography were evaluated. Adsorption chromatography with charcoal-celite stationary phases was the most generally applicable method for the preparation of near gram quantities of pure cellooligosaccharides in the DP range of three to seven. Cellulose affinity/partition chromatography was found to be the least time consuming and most economical method for the preparation of cellotetraose and cellopentaose. Ion-mediated chromatography was the most readily applicable for analytical applications.

4.2. INTRODUCTION

Cellulolytic enzymes, which consist of endocellulases (EC 3.2.1.4), exocellulases (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21), catalyze the hydrolysis of cellulose, one of the most abundant organic polymer on Earth. All of these enzymes are necessary for the hydrolysis of cellulose to glucose which can then be used for the production of food, fuel and industrial chemicals. Exocellulases, or cellobiohydrolases (CBHI and CBHII), are often the most abundant proteins found in cellulolytic systems. They appear to be the key enzymes for the saccharification of crystalline cellulose.

Cellulose is the natural substrate for cellulolytic enzymes. It is insoluble and heterogeneous, containing both crystalline and amorphous regions, so it is too complicated for detail enzymatic studies. Therefore, many well-defined substrates have been developed, such as unmodified cellooligosaccharide (Hsu et al., 1980), radioactively labeled cellooligosaccharides (Chirico and Brown, 1987; Bhat et al., 1990), chromophoric cellooligosaccharide (van Tilbeurgh et al., 1982; Deshpande et al., 1984; Vozynki, et al., 1987; Nanjo and Sakai, 1990; Claeyssens and Aerts, 1992) and 4-thio-cellooligosaccharides (Schou et al., 1993b; Sulzenbacher et al., 1996; Driguez, 1997; Davies, 1998), for the study of cellulolytic enzymes.

Cellooligosaccharides, also called cellodextrins, are soluble substrates and that may be used as the starting material for the synthesis of modified soluble

substrates and affinity ligands (Orgeret et al., 1992; Piyachomkwan et al., 1997; Sangseethong and Penner, 1998) for cellulolytic enzymes. In addition, since they are not hydrolyzed or absorbed by the human gastrointestinal tract, (non-digestible oligosaccharides) they may have potential as non-caloric bulking agents in food products (Satouchi et al., 1996; Watanabe, 1998; van Loo et al.,1999; Stahl et al., 2001).

The major methods for the preparation of cellooligosaccharides are chemical synthesis (Takeo et al.,. 1983; Nakatsubo, 2001), enzymatic synthesis (Tanaka, 1993; Kobayashi et al., 1993; Yan and Liau, 1998; Fort et al., 2000; Shoda and Fujita, 2000) and fragmentation of cellulose (Sato et al., 1996; Arai et al 2001). The chemical synthesis has been used for production of cellobiose, cellotriose and cellotetraose. However, this method is not practical for the preparation of longer cellooligosaccharides. Enzymatic synthesis has been reported to be very specific and highly effective for the production of oligo and polysaccharides (Fort et al., 2000). However, both enzymatic and chemical synthesis is quite difficult, tedious and requires special expertise. As a result, cellooligosaccharides are mainly obtained from the hydrolytic fragmentation of cellulose with enzyme or acid catalysts. In contrast to the acid fragmentation of cellulose, enzymatic fragmentation of cellulose is a fairly new method (Sasaki et al., 1989; Watanabe et al., 1993; 1994; 1995; Hishimoto and Matsumoto, 1994; Sato et al., 1996; Xia et al., 1999; Arai et al., 2001). In the enzymatic fragmentation of cellulose, the cellulose is incubated with an

endo-glucanase to produce cellooligosaccharide mixtures. Although enzymatic fragmentation of cellulose is very specific and produces cellooligosaccharides with high yield, there are some technical difficulties related with the dissolution of cellulose and the fractionation of large quantities of enzyme with the correct endo activity.

The general method to produce cellooligosaccharides is to initially fragment cellulose either by acetolysis/deacetylation (Dickey and Wolfrom, 1949; Miller et al., 1960) or direct acid hydrolysis (Miller et al., 1960; Pereira et al., 1990) and then separate cellooligosaccharide products by chromatography. Cellulose, such as avicel, cotton, filter paper, all of which are inexpensive and commercially available in relatively pure form, have been used as starting materials for the preparation of cellooligosaccharides. The separation methods employed after fragmentation of cellulose by acid, includes adsorption chromatography using a charcoal-celite column (Miller et al., 1960), gel permeation chromatography on polyacrylamide or crosslinked dextran (John et al., 1969; Sabbagh and Fagerson, 1976), chromatography on ion-exchange resin (Ladisch et al., 1978) and partition chromatography using silica gel (Streamer et al., 1975). These methods are quite satisfactory for preparation of cellooligosaccharides in small quantities. However, considering the heavy demand for cellooligosaccharides in many cellulase/cellulose laboratories there is a need for the development of more efficient, less expensive and less time consuming methods for the separation of cellooligosaccharides. In our

laboratory we developed a method for the separation of cellooligosaccharides using cellulose stationary phases. The objective of this study was to compare the major methods available for the separation of cellooligosaccharide mixtures.

4.3. MATERIALS AND METHODS

4.3.1. Materials

Aminex HPX-42A column (dimension: 300x7.8 mm; average particle size: 25 μm) and Bio-gel P2 were purchased from Bio-Rad, Life Science Research 2000 Alfred Nobel Drive Hercules, California. Cellulose powder (CF 11), LK5D (150 Å) and LK6DF (60 Å) TLC plates were obtained from Whatman Chemical Division, Clifto, New Jersey. Cellulose powder (microcrystalline), Avicel PH 105 was obtained from FMC Corp., Rockland, ME. Silica gel (70-230 mesh), 4,4' dicarboxy-2,2'-biquinoline (Disodium salt of Bicinchonic acid) and stearic acid were purchased from Sigma Chemical Company, St Louis, Missouri. Glacial acetic acid, hydrochloric acid, sulfuric acid, pyridine (certified ACS) were obtained from Fisher Scientific, Fair Lawn, New Jersey. Darco G-60, celite 545 and *p*-anisaldehyde were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI.

4.3.2. Preparation of Cellooligosaccharides

4.3.2.1. Preparation of cellooligosaccharides with aceteolysis

Acetylated cellooligosaccharides were prepared by controlled acid catalyzed aceteolysis of cellulose (Dickey and Wolfrom, 1949). Cellulose powder, 25 g, was added in portions to a mixture of 100 mL of glacial acetic acid, 100 mL of acetic

anhydride and 10 mL of concentrated sulfuric acid. The temperature of the reaction was kept below 40 ^{0}C by external cooling with ice water during the addition of cellulose powder. After standing at room temperature for 60 hr, the reaction mixture was poured into ice-cold water, which precipitated the mixed acetylated oligosaccharides. The precipitate was removed by filtration. The solid was resuspended into H_2O and excess acid was neutralized by $NaHCO_3$. The suspension was allowed to stand overnight. The precipitate was then removed by filtration, washed with water and dried in a vacuum oven at 40 ^{0}C. Crude acetates were mixed with anhydrous methanol (275 mL) and stirred with the aid of a Waring blender. The suspension was filtered and the solid was washed by resuspending in anhydrous methanol (275 mL) and refiltered. The filtrate was evaporated until dryness using a rotary evaporator. The gummy residue was dissolved in the minimum amount of hot chloroform and poured into 1000 mL of ice-cold hexane to precipitate acetylated cellooligosaccharides. After filtration, the solid was dried in a vacuum oven at 40 ^{0}C.

4.3.2.2. Preparation of cellooligosaccharides with acid hydrolysis

Soluble cellooligosaccharides were prepared according to Miller et al. (1960) with slight modification. Ten grams of cellulose powder was dissolved in 100 mL of concentrated HCl, pre-cooled to –30 ^{0}C. After the reaction mixture was stirred for 15-20 min at this temperature the temperature of the solution was

brought to 22 ^{0}C and stirred for another 2 hr. Cellulose/cellooligosaccharide mixtures were diluted and neutralized with the method described below.

4.3.2.2.1. Neutralization with $NaHCO_3$

The solution was poured into 600 mL ice-cold water and neutralized with $NaHCO_3$. The insoluble part was removed by centrifugation 5,000xg for 5 min and the supernatant containing the celloligosaccharide mixture was used for chromatographic fractionation.

4.3.2.2.2. Neutralization by consecutative washing by 1-propanol and ethanol

The solution was slowly added to 725 mL of 4 ^{0}C 1-propanol and stirred for 15 min, the precipitate was collected via centrifugation at 5,000xg for 5 min. The pellet was washed with technical grade ethanol until the pH reached 5-6. Finally, the pellet was extracted with 400 mL cold double distilled water. The insoluble material was removed with centrifugation and soluble cellooligosaccharides were concentrated to 50 mL by rotary evaporation and freeze dried.

4.3.3. Separation of Cellooligosaccharides with Column Chromatography

4.3.3.1. Separation of cellooligosaccharides with cation exchange resin

A representative cellooligosaccharide mixture, 1 mg, was dissolved in 1 mL water, and chromatographed using an HPLC system equipped with a refractometric detector (Water, Milford, USA). Samples were eluted from analytical Aminex HPX-42A cation exchange column in the silver form with a complementary deashing cartridge packed with Aminex resin (Bio-Rad) by using a mobile phase of MiliQ water at 85 ^{0}C and a flow rate of 0.4 mL/min .

4.3.3.2. Separation of cellooligosaccharides with charcoal celite column

A representative cellooligosaccharides mixture, neutralized with $NaHCO_3$, was loaded directly onto a column of radius 5 cm, length 95 cm. The column resin consisted of 1:1 Darco G-60/celite 545 equilibrated successively with solutions of 2.5% stearic acid in absolute ethanol, and 50% (v/v) aqueous ethanol saturated with stearic acid. The resin was packed into the column and equilibrated with water prior to introduction of the oligosaccharides. The oligosaccharides were eluted with a water→ethanol gradient (0-45%) (Miller et al., 1960). Fractions were monitored by analyzing with the BCA reducing sugar assay (Garcia et al., 1993). Fractions were also analyzed by silica TLC plate using a *p*-anisaldehyde-sulfuric acid visualizing reagent with EtOAc:MeOH:H_2O (40:20:15) as a mobile phase. On the basis of

results obtained from reducing sugar assays and TLC plates, the fractions corresponding to each oligosaccharide were combined and freeze dried.

4.3.3.3. Separation of cellooligosaccharides with Biogel-P2

A homogenous salt-free cellooligosaccharide mixture, 300 mg, was dissolved in 10 mL of water and loaded onto a column of radius 5 cm, length 95 cm. The oligosaccharides were eluted with water at 40 ^{0}C and monitored by analyzing each fraction as described above. Fractions corresponding to each oligosaccharide were combined and freeze dried.

4.3.3.4. Separation of peracetylated cellooligosaccharides with silica gel

Peracetylated cellooligosaccharides were separated on silica gel column with EtOAC:toluene (1:1) as eluant (Sanseethong, 1999). The column fractions were monitored by thin layer chromatography using silica plates with the same mobile phase used for column chromatography. The fractions corresponding to each oligosaccharide were combined and then evaporated until dryness using a rotary evaporator.

4.3.3.5. Separation of cellooligosaccharides with cellulose

Cellooligosaccharides were separated on cellulose column (microcrystalline cellulose). Avicel PH105 was used as chromatography media. Prior to use, the fines were removed from cellulose by stirring 50 g of Avicel with 1 L of water and allowing settling. The supernatant liquid along with the fines was then removed and

this procedure was repeated five times. After that the resin was packed into 2x25 cm column. The column was washed with water and equilibrated with 20% (v/v) ethanol. A representative cellooligosaccharide mixture, 100 mg, was dissolved in 10 mL 20% (v/v) ethanol and loaded onto the column. Samples were eluted with 20% (v/v) ethanol and water. Eluate fractions were analyzed by BCA reducing sugar assay (Garcia et al., 1993). Fractions were also analyzed by reacting with *p*-anisaldehyde-sulfuric acid visualizing reagent after separation on silica TLC plate with EtOAc:MeOH:H_2O (40:20:15) as mobile phase. On the basis of results obtained from reducing sugar assay and TLC plate, fractions corresponding to each oligosaccharide were combined and freeze dried.

4.4. RESULT AND DISCUSSION

4.4.1. Preparation of Cellooligosaccharides

The general method to produce cellooligosaccharides is fragmentation of cellulose by either aceteolysis followed by deacetylation (Dickey and Wolfrom, 1949; Miller et al., 1960) or direct acid hydrolysis (Miller et al., 1960; Pereira et al., 1990). First, cellooligosaccharides were produced from cellulose by aceteolysis (Dickey and Wolfrom, 1949). Since hydrolysis of cellulose took place in the presence of acetic anhydride, the resulting product was peracetylated cellooligosaccharides with different degrees of polymerization (Figure 4.1). From 25 g of cellulose powder, a total of 8.02 g of acetylated cellooligosaccharides was obtained. After this step the acetylated cellooligosaccharides were either separated on silica gel or deacetylated and separated by using various chromatographic methods described above.

Acid hydrolysis of cellulose was introduced by Miller et al. (1960). Acid hydrolysis of cellulose could be done using different acids, including hydrochloric, sulfuric, trifluoroacetic or a combination of different acids. The most common acids used for fragmentation of cellulose are hydrochloric acid and sulfuric acid (Pereira et al., 1988). Voloch et al. (1984) showed that the yield of cellooligosaccharides with sulfuric acid was lower than that with hydrochloric acid.

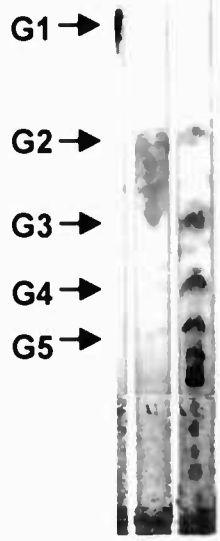

Figure 4.1. Acetylated cellooligosaccharides, prepared by aceteolysis. Solvent: EtOAc:Toluene (1:1). (G1: Glucose pentaacetate; G2: Cellobiose octacetate; G3: Cellotriose undecaacetate; G4: Cellotetraose tetradecaacetate; G5: Cellopentaose heptadecaacetate)

After cellulose hydrolysis, the next step is the neutralization of excess acid. Neutralization could be achieved by using Amberlite IRA-93 macroreticular weak-base anion-exchange resin (Huebner et al., 1978), addition of $NaHCO_3$ (Miller et al., 1960) or consecutative washing with 1-propanol and ethanol (Hamacher et al., 1985). Amberlite IRA-93 macroreticular weak-base anion-exchange resin is quite effective for neutralizing cellooligosaccharides in a short time by exchanging OH- with Cl- ions. However, considering the high concentration of acid in the starting material, there is localized heating in the resin bed during this ion exchange. Combination of heat and the basic environment caused partial hydrolysis of the sugars (Ladisch et al., 1978). The most common neutralization method is addition of $NaHCO_3$. However, the addition of such a base produces neutralization products, such as sodium chloride. To avoid formation of neutralization products crude cellooligosaccharides can be neutralized by consecutative washing with an appropriate solvent, such as 1-propanol and/or ethanol (Hamacher et al., 1985). The main hydrolysis products, glucose and cellobiose, are soluble in the acid-alcohol-water phase while the higher DP cellooligosaccharides (DP3-8) tend to precipitate with the insoluble cellulose. A mixture of salt-free cellooligosaccharides mixture can be obtained by water extraction of this solvent-washed precipitate (Figure 4.2).

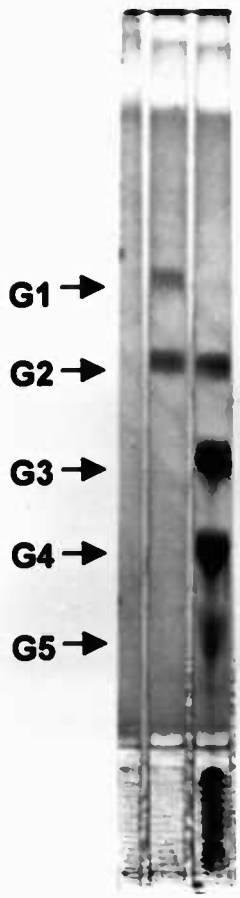

Figure 4.2. Celloligosaccharide mixtures, prepared by acid hydrolysis. Solvent: EtOAc:Methanol:Water (40:20:15). (G1: glucose; G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose).

The different neutralization methods have disadvantages and advantages. In some chromatographic applications, the presence of sodium chloride in cellooligosaccharide mixture interferes with the interaction of the cellooligosaccharides with the stationary phase and/or it co-elutes with lower molecular weight sugars. Neutralization by ethanol washing successfully removes glucose and cellobiose but it also decreases the amount of cellotriose with respect to the other cellooligosaccharides (Table 4.1) and reduces total yield of celloligosaccharide.

Table 4.1. The percent ratio of representative cellooligosaccharide preparations after acid hydrolysis of cellulose and A)Neutralization of excess acid with base; $NaHCO_3$ B)Neutralization of excess acid with ethanol washing (determined as described in Section 4.3.3.1).

Component	A	B
Glucose	13.7±1.3	----
Cellobiose	14.4±1.8	----
Cellotriose	19.9±1.9	13.7±1.2
Cellotetraose	22.7±1.9	27.9±0.5
Cellopentaose	14.1±2.3	31.4±1.4
Cellohexaose	15.2±0.9	27.0±0.6

4.4.2. Separation of Cellooligosaccharides

After fragmentation of the cellulose to cellooligosaccharides having different degrees of polymerization, the cellooligosaccharides was separated using suitable chromatographic methods. Separation of cellooligosaccharides was carried out either analytically or preperatively using adsorption chromatography on charcoal-celite, Aminex HPX-42A cation exchange resin, Biogel P2 size exclusion resin, silica gel and cellulose column.

4.4.2.1. Cation exchange resin

HPLC is a powerful technique for analysis and preparative isolation of oligosaccharides. Cellooligosaccharides can be separated in a short time easily on an analytical and preparative scale by using a cation exchange column.

Aminex columns, consisting of sulfonated polystyrene-divinylbenzene copolymer matrixes, are strong cation exchange resins. The separation on these columns use the mechanism of ion exclusion, ion exchange, ligand exchange, size exclusion, reversed phase and normal phase partitioning. These multiple mechanisms, called "ion-moderated partitioning" (Jupille et al., 1981), offer a unique ability for separating compounds. Complete ion exclusion would require that all the components elute within the total column volume since mobile ions with like charge can not penetrate the resin bead. The sample molecules are distributed between a polar aqueous phase and the nonpolar resin. Non-polar compounds are retained

longer in the column than these similar polar compounds, which suggests that reverse phase mechanisms can be important with Aminex columns. Size exclusion mechanism also plays an important role in the separation on Aminex columns. The Aminex columns have a crosslinked sulfonated polystyrene-divinylbenzene copolymer lattice, thus the amount of resin crosslinking determines the bead pore size. A resin with lower crosslinking has more open structure and it is more permeable to higher molecular weight substances. Another mechanism involved with the separation mechanism of Aminex columns is ligand exchange which involves the binding of the hydroxyl groups of the sugar with the fixed counterion of the resin. Ligand exchange mechanisms are affected by the nature of the counter ion and the conformation (axial vs equatorial) of hydroxyl groups (Talmadge et al., 1997). This means that coordinating ability of adjacent hydroxyl groups of the sugars with the fixed counterion of the resin is different (Figure 4.3).

The first study for the separation of cellooligosaccharides on the analytical scale with HPLC was introduced by Ladisch and Tsao (1978). They used a 4% cross-linked Aminex resin in the calcium form. Later Bonn et al. (1984), introduced a cation exchange resin in the Ag^{+} form for separation of cellooligosaccharides. These systems were found to have excellent potential for scale up to preparative levels (Hicks et al., 1994). The calcium form of the Aminex HPX-42C column separate oligosaccharides up to DP 4-5 while the silver form can separate oligosaccharides to DP 10 (Talmadge et al., 1997). It was found that Aminex HPX-

42A column separated malto-, xylo- and cello-oligosaccharides up to DP 9 (Schmidt et al., 1981). In our laboratory we have used Aminex HPX-42A cation exchange resin in the Ag^+ form in quantitative and qualitative analysis of cellooligosaccharides. It can resolve cellooligosaccharides up to DP 6 in less than an hour with water as an eluant (Figure 4.4).

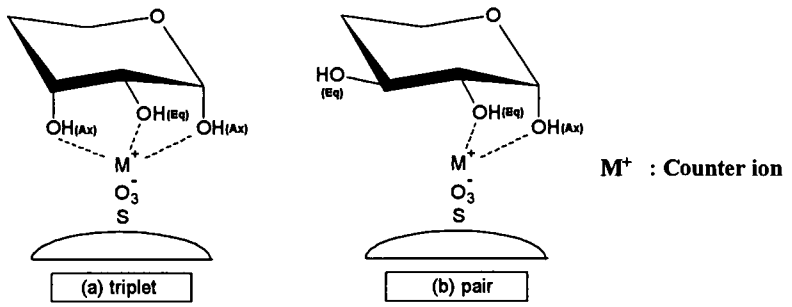

Figure 4.3. Ligand exchange mechanism of cation exchange resin (http://www.sdk.co.jp/shodex/english/dc030104.htm)

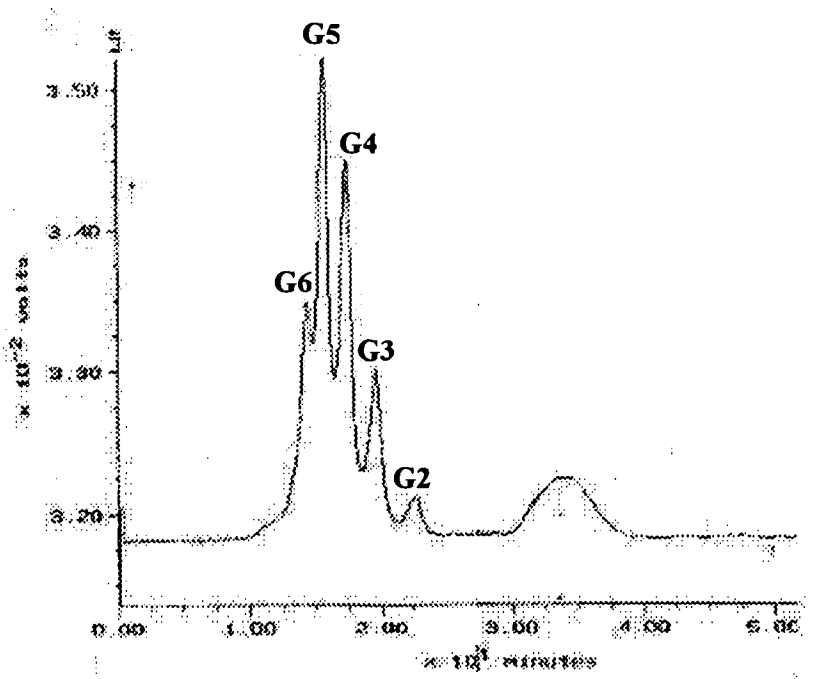

Figure 4.4. Separation of cellooligosaccharides with cation exchange resin, Aminex HPX-42A, Biorad (G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose; G6: cellohexaose).

4.4.2.2. Charcoal-celite column

Charcoal-celite columns are capable of separating gram quantities of cellooligosaccharides with good resolution. This stationary phase has another benefit. It is inexpensive and the elution solvents are available in high purity. This method has been applied to the separation of maltooligosaccharides and cellooligosaccharides (Miller et al., 1960). In this work, the separation of cellooligosaccharides was achieved on a charcoal surface. Celite was used to improve flow characteristics of the charcoal because of the granulation of charcoal (Motl and Novotny, 1979). The extent of adsorption between solutes and the adsorbent increases strongly and regularly with increasing molecular weight among homologous series (Freundlich, 1926). Thus, the order of the affinities of cellooligosaccharides increases with increasing degree of polymerization (celloheptaose>cellohexaose>cellopentaose>cellotetraose> cellotriose>cellobiose>glucose). Adsorbed cellooligosaccharides on charcoal are desorbed by using a water→ethanol gradient. The oligosaccharides and the eluting agent compete for the same active sites on the charcoal surface. The adsorbed substances are desorbed from the column in order of their ease of elution by varying different concentration of ethanol. To improve the desorption and eliminate the irreversible adsorption of higher cellooligosaccharides, the charcoal is treated with stearic acid, called co-adsorbent (Alm et al., 1952; Hassler, 1974). The function of co-adsorbates is a bit uncertain. Depending on the characteristics of the system

involved, it may act as a modifier, deactivator, mordant and complexing agent. In cellooligosaccharides separations, it deactivates the surface sites having strongest adsorptive power (Alm, 1952; Jermyn, 1957; Hassler, 1974). It was found that charcoal treated with stearic acid allowed the isolation of cellohexaose and celloheptaose (Miller et al., 1960; Jermyn, 1957).

The separation of cellooligosaccharides was accomplished by chromatography on charcoal-celite column using gradient elution with ethanol-water as eluant. Isolation of cellooligomers from controlled acid hydrolysis of cellulose was achieved using a charcoal-celite column chromatography method based on that of Miller et al. (1960). Sodium chloride formed during neutralization of the acid is removed from the column by washing with water. Glucose and cellobiose are separated from the rest of the cellooligosaccharides by elution with water and 5-7.5% ethanol. The elution of the rest of the cellooligosaccharides, adsorbed onto the charcoal surface, is achieved by increasing the concentration of ethanol. Over a five day period, cellooligosaccharides from glucose to celloheptaose were separated (Figure 4.5). This method yielded oligosaccharides ranging from 964 mg of cellotriose to 158 mg cellohexaose (Figure 4.5). The main disadvantage of the using charcoal-celite column for preparation of cellooligosaccharides is that, once used, the column-packing material has diminished separation capability. Hence, before each run, new adsorbent had to be prepared (Huebner et al., 1978).

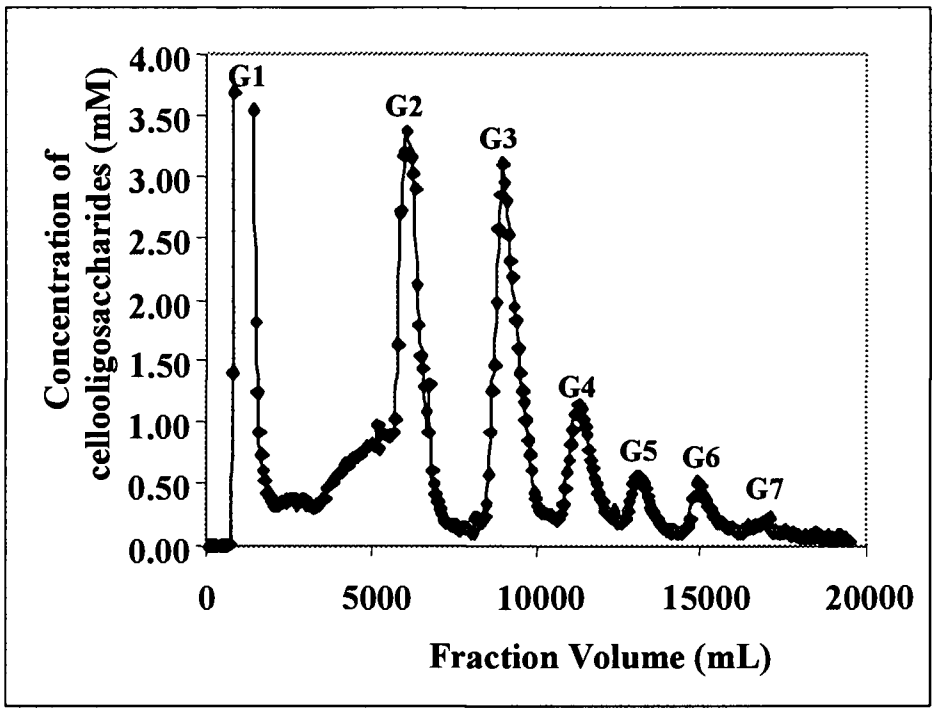

Figure 4.5. Elution profile of cellooligosaccharides from charcoal-celite column (G1: glucose; G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose; G6: cellohexaose; G7: celloheptaose).

4.4.2.3. Bio-gel P2

Size exclusion chromatography is based on the ability of molecules to move through a column of a gel that has pores of clearly defined sizes. Cellooligomers have been separated by using size exclusion chromatography on polyacrylamide (John et al., 1969; Sabbagh and Fagerson, 1976), and crosslinked dextran (Ladisch et al., 1978). The larger molecules can not enter the pores and are eluted first while the smaller molecules can enter the pores and are eluted last. The main advantage of this method is that it is simple, isocratic, and water can be used as the mobile phase. However, the matrix used as size exclusion media is quite expensive.

Gel permeation chromatography with crosslinked polycrylamide was employed for fractionating cellooligosaccharides. This method was able to separate only cellotriose and cellotetraose. To achieve the same resolution with charcoal celite column, three times longer column was required. Only one fifth of the sample size loaded on the charcoal celite column could be loaded on the size exclusion column. The yield of cellotriose and cellotetraose was 22 mg and 30 mg, respectively (Figure 4.6).

Since size exclusion chromatography on Bio-gel P2 is a relatively low resolution method, the oligomers isolated did not have high purity (Hamacher et al., 1985). The main advantage of using size exclusion columns with polyacrylamide stationary phase for the isolation of cellotriose and cellotetraose is that these are reusable matrices. Hence it does not require the preparation of a new stationary phase before each run.

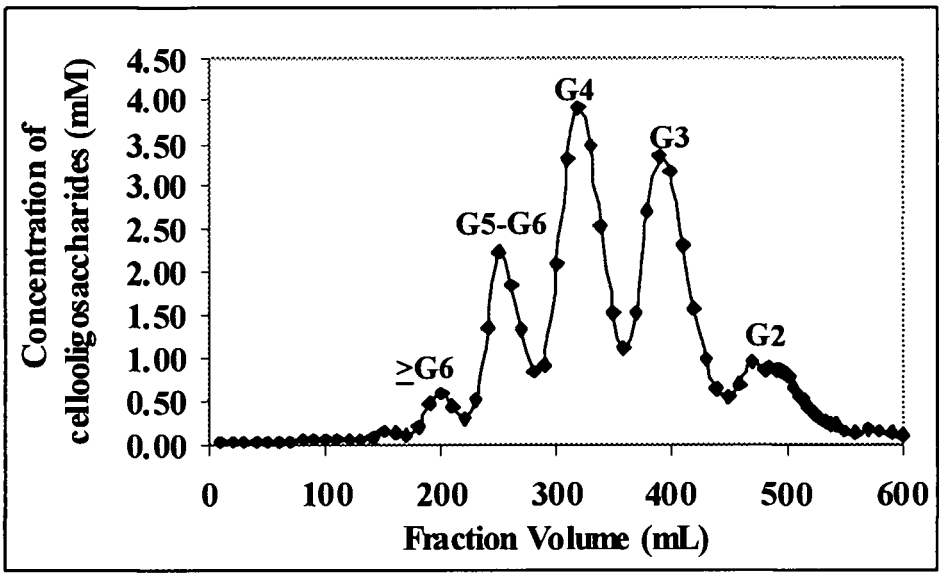

Figure 4.6. Elution profile of cellooligosaccharides from Bio-gel P2 column (G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose; G6: cellohexaose).

4.4.2.4. Silica gel

Silica gel (SiO_2) is one of the most common stationary phases for column chromatography. The separation mechanism of cellooligosaccharides on silica gel is mainly via polar interactions. The surface of silica gel is covered by OH groups bonded to a SiO_2 skeleton. The presence of hydroxyl groups makes the surface of silica gel highly polar. Polar functional groups in oligosaccharides (OH groups) interact strongly with the surface of the gel, while non-polar groups interact weakly. Oligosaccharides bind to the surface through hydrogen bonds. It should be noted that the conformation of OH groups, such as axial vs equatorial, of oligosaccharides play an important role in the strength of interaction with silica gel surface. The mobile phase for this type of chromatography is an organic solvent or a mixture of organic solvents. As the mobile phase moves past the surface of silica gel, it transports the oligosaccharides with the solvent. The oligosaccharides which weakly bind to the silica gel surface elute before those strongly bind to the silica surface. The elution order for cellooligosaccharides is cellobiose> cellotriose> cellotetraose> cellopentaose> cellohexaose (Figure 4.7).

Cellooligosaccharides either acetylated (Dickey and Wolfrom, 1949; Feather and Harris, 1967) or deacetylated (Saif ur Rahman et al., 1968; Chricico and Brown, 1985) could be separated with thin layer and column chromatography on silica gel (Dickey and Wolfrom, 1949; Saif ur Rahman et al., 1968; Chricico and Brown, 1985) In our laboratory, we have been using thin layer chromatography and

column chromatography with silica gel for the qualitative analysis of acetylated and deacetylated cellooligosaccharides (Figure 4.7 and 4.8) and for the purification of acetylated cellooligosaccharides. Both thin layer chromatography and column chromatography are closely related with each other and they have the same separation mechanism. Thin layer chromatography is carried out on a layer of silica gel while column chromatography is carried out on a column of silica gel.

The separation method of acetylated cellooligosaccharides with silica gel allowed the convenient isolation of each member of the series as the crystalline acetates. Cellotriose undecaacetate and cellotetraose tetradecaacetate were isolated with this method using EtOAc:toluene (1:1) as the eluant (Figure 4.7). The isolation of acetylated cellooligomer could be advantageous. Direct isolation of acetylated cellooligosaccharides eliminates some steps during the chemical modification of cellooligosaccharides.

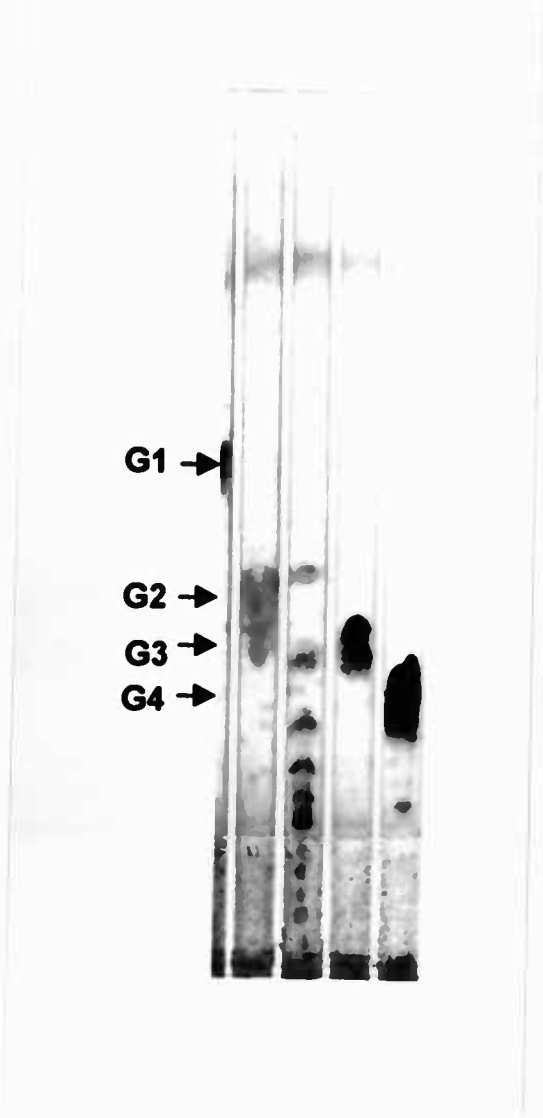

Figure 4.7. Separation of acetylated cellooligosaccharides on silica gel column. Fractions monitored by thin layer chromatography by using the same mobile phase as used for the column. (G1: Glucose pentaacetate; G2: Cellobiose octacetate; G3: Cellotriose undecaacetate; G4: Cellotetraose tetradecaacetate).

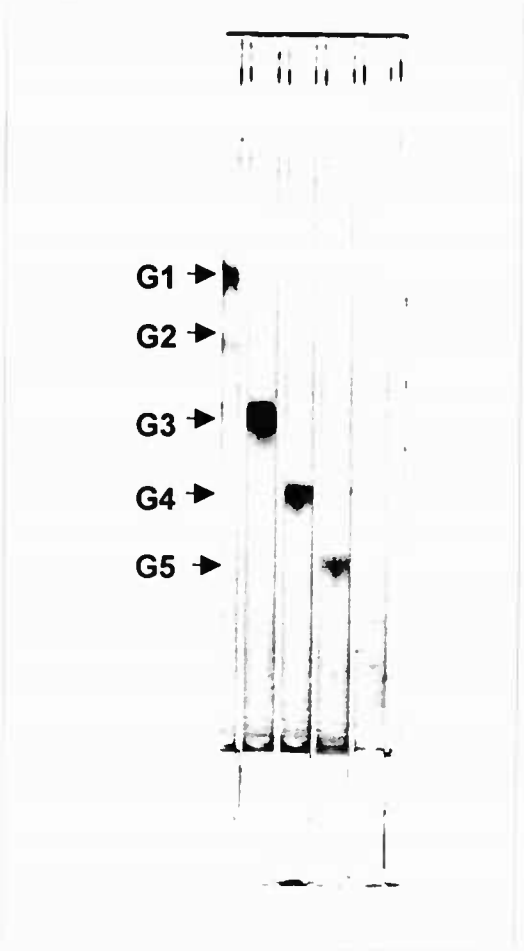

Figure 4.8. Qualitative analysis of cellooligosaccharides by thin layer chromatography. Solvent: EtOAc:Methanol:Water (40:20:15). (G1: glucose; G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose; G6: cellohexaose).

4.4.2.5. Cellulose column

Cellulose is a linear polymer of β-1,4-D glucopyronoside. It has been used as a supporting material for normal phase partition chromatography for the separation of oligosaccharides from wort, beer, brewing syrups (Otter et al., 1970), and starch hydrolysates (Thoma et al., 1959). The separation of cellooligosaccharides on cellulose supports is presumably based on adsorption rather than partition as a result of the structural correspondence between the adsorbent and the adsorbate. In nature cellulose forms microfibrils where the molecules are packed tightly together by intra and inter molecular hydrogen bonds and van der Waals interactions. The intermolecular hydrogen bonding and van der Waals forces cause cellulose chains to pack very tightly together, forming a highly ordered structure. The same type of interactions between cellulose and cellooligosaccharides appear to be responsible for the strong chemisorption for cellooligosaccharides to cellulose.

In our laboratory we have developed a method for the separation of cellooligosaccharides by using cellulose as the stationary phase. The cellulose column is run at room temperature with a 20% ethanol mobile phase. The elution profile shows that cellooligosaccharides with the chain lengths of four or more glucose units are retained on the cellulose support. The cellulose column is able to isolate cellotetraose and cellopentaose. Cellooligosaccharides with chain lengths smaller than four showed little adsorption to the cellulose support. Chain lengths bigger than five were strongly adsorbed to the column and desorption of these higher DP cellooligosaccharides from the cellulose surface required lower ethanol

concentration than those used for the desorption of cellopentaose and cellotetraose. The main advantage of using the cellulose column was that the isolation of cellooligomers was accomplished on a reusable matrix, and 6 mg of cellotetraose and 10 mg of cellopentaose could be obtained with relatively low expenses from 100 mg of cellooligosaccharide mixture (Figure 4.9). The column was quite stable and did not require repacking after each run. The use of cellulose as a support for separation cellooligosaccharides with different chain length provides an advantage for the preparation of food grade cellooligosaccharides.

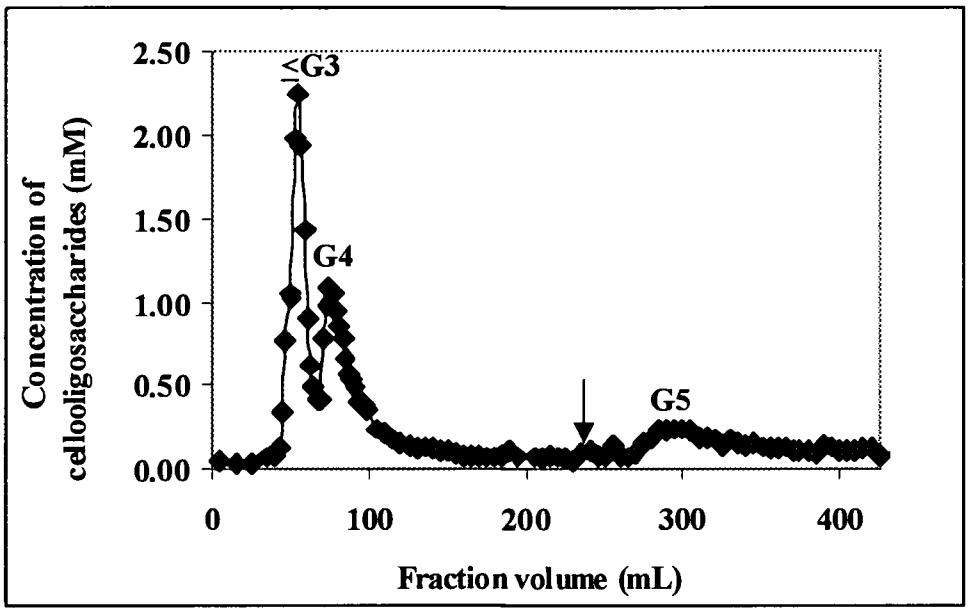

Figure 4.9. Elution profile of cellooligosaccharides from cellulose column. Stationary phase: microcrystalline cellulose (Avicel PH105, FMC); Mobile phase: 20% ethanol-water; Arrow indicates that mobile phase was switched to water.

4.5. CONCLUSION

Table 4.2 summarizes the advantages and disadvantages of the methods tested for the fractionation of cellooligosaccharides. Biogel-P2 is capable of easily isolating cellotriose and cellotetraose. The cellulose column, which does not require organic solvents, allows one to isolate cellotetraose and cellopentaose with low costs on a reusable matrix. The charcoal-celite column appears to be the most efficient method for obtaining near gram quantities of purified cellooligomers from DP3 to DP7. Depending on the purpose for obtaining cellooligosaccharides (affinity ligand, kinetic studies or food ingredient), one of the methods from Table 4.2 should be applicable.

Table 4.2. Comparison of methods for the separation of cellooligosaccharides

	Aminex-HPX42A.	Silica gel	Charcoal-celite	Biogel-P2	Cellulose
Starting material	Cellulose	Cellulose	Cellulose	Cellulose	Cellulose
Hydrolysis	Acid	Aceteolysis	Acid	Acid	Acid
Neutralization	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	Ethanol	Ethanol
Separation	G1-G7	G1-G4	G1-G7	G1-G4	G4-G5
Cost of stationary phase	High	High	Low	High	Low
Suggested use	Analytical	Analytical-preparative	Preparative	Preparative	Preparative
Elution	Water	EtOAc:Toluene (1:1)	0-45% Ethanol	Water	20-0% Ethanol
Temperature	Room	Room	Room	40 ^{0}C	Room
Column reusability	Reusable	Not usable	Not usable	Reusable	Reusable

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CHAPTER 5

AN APPROACH FOR PREPARATION OF NON-REDUCING END MODIFIED CELLOOLIGOSACCHARIDES

5.1. ABSTRACT

Model substrates are widely used to elucidate mechanisms of enzyme action. Chemically-modified cellooligosaccharides are used in this way for the study of cellulolytic enzymes. The vast majority of modified cellooligosaccharides used in cellulase research have been derivatized via their reducing end – due to the relative ease of working at this end of the molecule. The present study investigates the derivatization of such substrates via the non-reducing end. The modification of the non-reducing end of glucosides is described. Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside was synthesized by reacting methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside with N-bromosuccinimide (NBS), First, methyl β-D-glucoside was converted into methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside with p-nitrobenzaldehyde. Later, methyl 4,6-O-*p*-nitrobenzylidine-β-D-glucoside was opened oxidatively with NBS to give, non-reducing end modified, methyl 6-*O*-p-nitrobenzoyl-β-D-glucoside. The method described here could be used as a general method for the modification of cellooligosaccharides for use as model substrates and/or affinity ligands.

5.2. INTRODUCTION

Cellulolytic enzyme systems are composed of several discrete enzymes; endoglucanases (EC 3.2.1.4), exocellulases (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). A combination of different purification methods are generally used to fractionate the individual enzyme components. In a typical purification protocol, crude cellulase mixtures are first fractionated by DEAE-anion exchange chromatography. Fractions containing exo-cellulase (CBHs) activity are then applied to an affinity column in order to purify the exocellulases (CBHI and CBHII) from contaminating non-celluloytic proteins, endoglucanases and β-glucosidases (Figure 5.1). Affinity chromatography is the most valuable approach for purification of endo and exo-acting cellulases. At present, all of the immobilized affinity ligands used for the purification of CBHs, are prepared by modifying the reducing end of cellooligosaccharides, such as *p*-aminobenzyl 1-thio-β-D-cellobioside (van Tilbeurgh et al., 1984); *p*-aminophenyl 1,4-dithio-β-cellobioside (Orgeret et al., 1982); *p*-aminophenyl 1-thio-β-D-cellobioside (Piyachomkwan et al., 1997); *p*-aminophenyl β-cellobioside (Sangseethong and Penner, 1998). This approach is used because these reducing end modified ligands are easier to prepare and they work adequately.

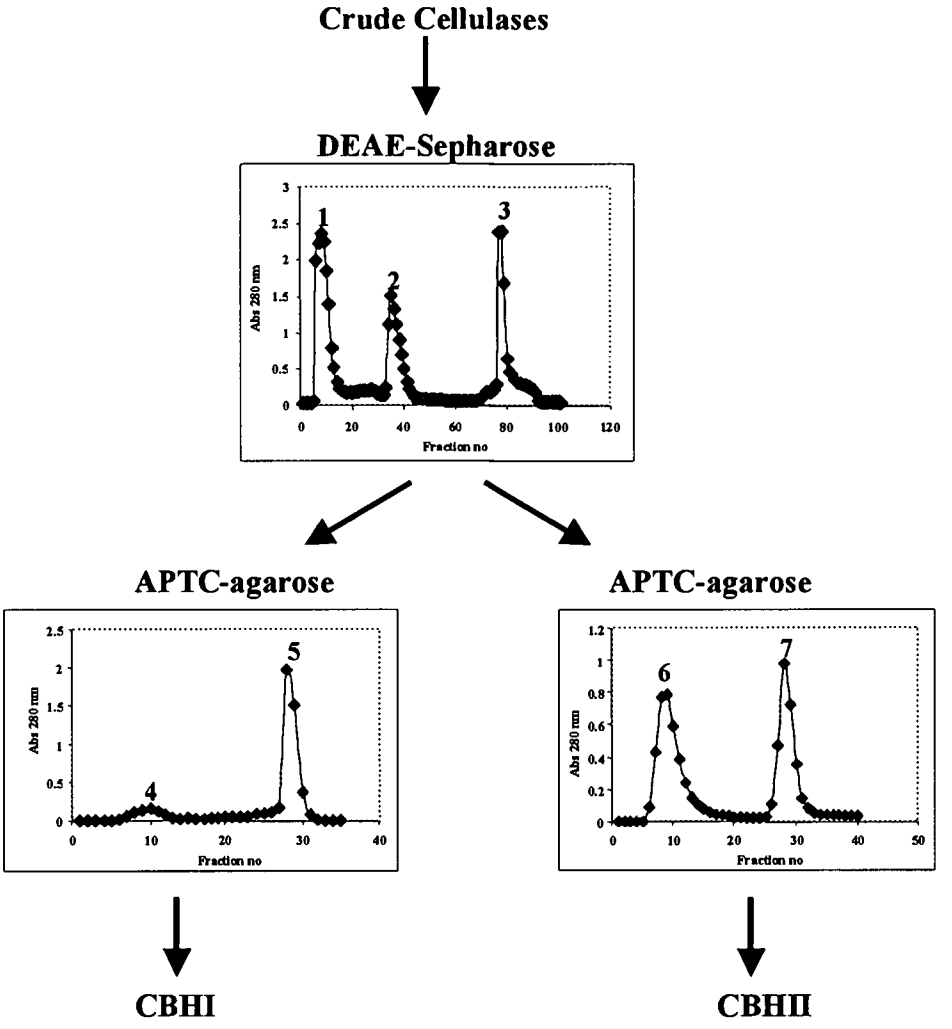

Figure 5.1. The purification scheme for CBHI and CBHII. APTC-agarose: *p*-aminophenyl 1-thio-β-D-cellobioside derivatized agarose; Peak 1: CBHII, endoglucanases and β-glucosidase; Peak 2: endoglucanases; Peak 3: CBHI and endoglucanases; Peak 4: endoglucanases; Peak 5: CBHI; Peak 6: endoglucanases; Peak 7: CBHII.

The orientation and directionality of different oligosaccharides in the active sites of CBHI and CBHII by high-resolution crystalline structures show that CBHI preferentially acts on cellulose via the reducing end while CBHII preferentially acts at the non reducing end (Divne et al., 1998). However, CBHI can bind to non-reducing ends and catalyze hydrolysis as well. Even though the activity is less than CBHII, it is capable of catalyzing reducing end blocked cellooligosaccharides from the non-reducing end (Sangseethong, 1999). This explains why CBHI can be purified by the same affinity ligand, reducing end blocked affinity ligand, used for CBHII (van Tilbeurgh et al., 1984; Orgeret et al., 1992; Piyachomkwan et al., 1997; Sangseethong and Penner, 1998). Therefore the distinction between reducing end versus non-reducing end-acting CBHs is not clear.

The purpose of this study was to explore possible approaches for the preparation of non-reducing end modified cellooligosaccharides. As far as is known, no simple procedures have been reported for the direct immobilization of sugar chains solid supports. Therefore, the non-reducing end of the substrate will be derivatized to give a *p*-nitro group. The *p*-nitro group can then be reduced with catalytic hydrogenation under hydrogen atmosphere in the presence of palladium on activated carbon, to give the non-reducing end of the substrate a *p*-aminophenyl group. This compound can be immobilized to the insoluble support by using CnBr or NHS activated agarose.

The chemistry of modifying the non-reducing end of cellooligosaccharides is considerably more difficult than that associated with the reducing end. The majority of the effort given in this research is focused on developing a synthetic approach for the preparation of modified cellooligosaccharides having a *p*-nitro phenyl moiety at the non-reducing end.

5.3. MATERIALS AND METHODS

5.3.1. Materials

Silica gel (70-230 mesh) and 4,4' dicarboxy-2,2'-biquinoline (Disodium salt of Bicinchonic acid), NBS (N-bromosuccinimide), 4,4' dicarboxy-2,2'-biquinoline (Disodium salt of Bicinchonic acid), *p*-dioxane and *p*-nitrobenzaldehyde were purchased from Sigma Chemical Company, St Louis, Missouri. LK6DF (60 Å) TLC plates was obtained from Whatman Chemical Division, Clifto, New Jersey. *p*-Anisaldehyde, 30% HBr in glacial acetic acid, calcium sulfate hemihydrate ($CaSO_4.1/2H_2O$), mercury oxide (HgO), mercury bromide ($HgBr_2$), celite 545 and sodium methoxide ($NaOCH_3$) were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Dichloromethane was purchased from Mallinckrocdt Baker Inc., Paris, Kentucky. Sulfuric acid (certified ACS) was obtained from Fisher Scientific, Fair Lawn, New Jersey. Carbontetrachloride, CCl_4, was obtained from Stock Room, Food Science and Technology Department, Oregon State University, Corvallis, OR.

5.3.2. General Methods

All organic solutions were dried with anhydrous Na_2SO_4. The ^1H NMR spectra recorded at 400 MHz with a Bruker AM 400 spectrometer using tetramethylsilane as an internal standard. Assignments were confirmed 2D-COSY,

HMBC and HSQC experiments. Spots on TLC were detected by exposure to UV light and by spraying with *p*-anisaldehyde-sulfuric acid visualizing reagent.

5.3.3. Preparation of Methyl Glycosides

Methyl glycosides were prepared using Koenigs-Knorr conditions. A solution of peracetylated glycosyl bromide was prepared by adding 10 equivalent of 30% HBr in glacial acetic acid to a cold solution containing 1 equivalent peracetylated glycosides in dichloromethane. The mixture was stirred under N_2 for 1 hr. The solution was diluted with dichloromethane and the organic layer was washed with ice water, ice/saturated $NaHCO_3$ and ice water. The organic layer was then dried and the solvent was removed under reduced pressure using a rotary evaporator. The product was dissolved in $CHCl_3$ containing $CaSO_4.1/2H_2O$ (5.25 equiv), HgO (4.47 equiv), $HgBr_2$ (0.03 equiv), CH_2Cl_2 and CH_3OH (370 equiv) and stirred under N_2 for 36 hr. The reaction mixture was filtered through a pad of Celite 545. The solvent was removed under reduced pressure and resulting solid was dissolved in $CHCl_3$. The solution was washed with ice water, ice/saturated $NaHCO_3$, again ice water, dried with Na_2SO_4 and then the solvent was evaporated to dryness under reduced pressure (Flugge et al., 1999).

Acetylated methyl glycosides (1.5 equiv) were dissolved in 1 equivalent of 1 M NaOMe in MeOH. The solution was stirred overnight at room temperature,

neutralized with amberlite IR-120 (H+) resin, filtered, concentrated and crystallized from MeOH (Sanseethong, 1999).

5.3.4. Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside

Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside was prepared according to the method described by Collins and Oparaeche (1974). Methyl-β-glucoside (2.04 g), *p*-nitrobenzaldehyde (1.46 g), and concentrated sulfuric acid (0.8 ml) in *p*-dioxane (20 ml) were stirred together at room temperature for 2 hr. The solution was then diluted with dichloromethane (50 ml), neutralized with solid sodium carbonate, filtered and concentrated under reduced pressure. The concentrate was dissolved in dichloromethane, washed with water and then dried. The solution was evaporated and crystallized from ethanol as a white crystal (0.9635 g).

5.3.5. Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside

Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside (30 mg) and NBS (214 mg), in carbon tetrachloride containing 2 equiv of water (2 equiv) was refluxed for 2 hr. The solution was filtered and concentrated under diminished pressure. The concentrate was dissolved in ethyl acetate and washed with saturated $NaHCO_3$, cold water, dried with Na_2SO_4 and evaporated. The target compound was purified by silica gel chromatography (70-230 mesh, SIGMA Chemical Co., St. Louis, MO), using ethyl acetate:ethanol:water (30:9.5:0.5). Column fractions were monitored by

thin layer chromatography using silica plates ($LK6DF_{254}$, Whatman Inc., Clifton, NJ) and the same mobile phase used for column chromatography. The compound of interest was identified on TLC plate by exposuring to UV light (phenyl group) and by spraying with *p*-anisaldehyde-sulfuric acid visualizing reagent (carbohydrate). Fractions containing the target compound were pooled, evaporated to dryness under reduced pressure and then crystallized from ethanol to yield methyl 6-O-*p*-nitrobenzoyl-β-D-glucoside (6.75 mg).

5.4. RESULT AND DISCUSSION

Methyl glycosides were prepared under Koenigs-Knorr conditions (Flugge et al., 1999). The first step involved preparation of the peracetylated glycosides. Prior to the methylation reaction, the acetylated sugar is converted into the more reactive bromosugar by using 30% HBr in glacial acetic acid. The second reaction involves addition of alcohol in the presence of heavy metals. Methylation of the reducing end of the glycosides was accomplished under Koenigs-Knorr conditions using methanol and methylene chloride in the presence of $CaSO_4.1/2H_2O$, HgO, and $HgBr_2$. Acetylated methyl glycosides were deacetylated with sodium methoxide. The resulting compound exhibited a strong ^{1}H NMR signal at δ 3.37 (s, 3H- OCH_3) characteristics of methyl group.

The next reaction involved selectively protecting the hydroxyl groups at C-4 and C-6 position with *p*-nitrobenzaldehyde. Methyl β-D-glucoside was directly mixed with *p*-nitrobenzaldehyde in the presence of an acid catalyst to give methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside. The ^{1}H NMR spectra of this compound showed signals at δ 8.24 (d $J_{meta,ortho}$ = 8.82 Hz Hmeta) and δ 7.71 (d $J_{meta,ortho}$ = 8.82 Hz Hortho) characteristic of the aromatic ring and at δ 5.73 (s PhCH) resulting from the proton belonging to the *p*-nitrobenzylidene group. These assignments were confirmed with 2D-COSY, HMBC and HSQC experiments.

The last reaction was the regioselective opening of methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside with NBS (N-bromosuccinimide). When methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside was oxidized with NBS (N-bromosuccinimide) in the presence of water, the resulting reaction mixture gave three different products on TLC plate (Figure 5.2). The first one was unreacted starting material, the second one was an unknown side reaction product and the third one was methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside, confirmed by NMR. The ^{1}H NMR spectra of this compound showed that the signal at δ 5.73 (s PhCH) arising from the proton belong to *p*-nitrobenzylidene group disappeared. The lowest field sugar proton resonance at δ 4.57 (dd, 1H, $J_{6a,6b}$= 11.7 Hz, $J_{6b,5}$= 2.10 Hz H-6b) indicated *p*-nitrobenzoate group at position 6 of the glucose ring. The other 2D-COSY, HMBC and HSQC experiments supported this conclusion.

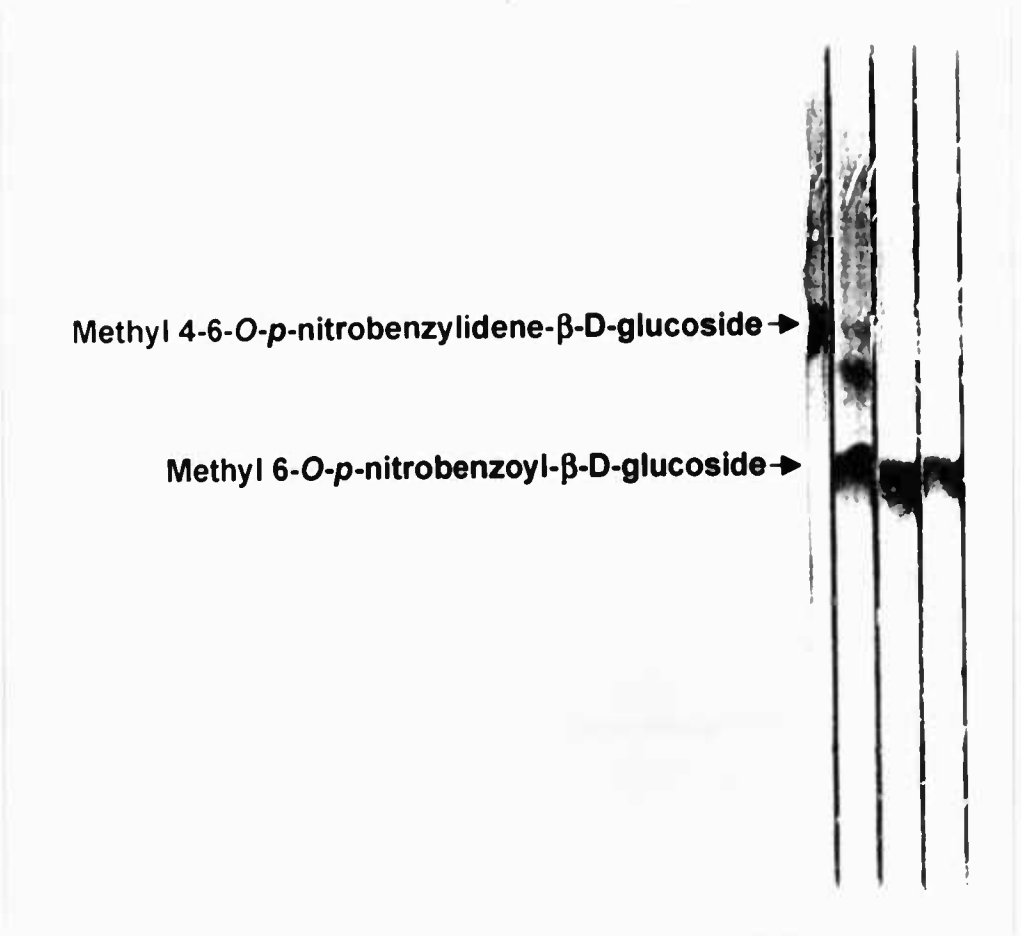

Figure 5.2. Oxidation of methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside with NBS. LN1: Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside; LN2: reaction mixture. LN3: methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside; LN4: same as LN3.

Cyclic acetals have been used in carbohydrate chemistry for protecting groups. The main advantage of cyclic acetals is that they can block a pair of diols in one step (Zhang, 1996). Generally an aldehyde and a sugar are mixed, either directly (benzaldehyde) or in solution by using a solvent (dioxane) in the presence of catalyst. The catalyst can be either soluble acid (sulfuric acid, *p*-toluonesulfonic acid) or an insoluble one (amberlyst resins) (Calinaud and Gelas, 1996). Treatment of unprotected hexopyronoside with aldehyde in the presence of catalytic acid gives selectively protected 4,6-O-benzylide acetals since the least hindered hydroxyl group (the primary at C-6) is more reactive than other hydroxyl groups which leads the formation of 4,6-O-benzylide acetals (Figure 5.3) (Binkley, 1988). Another alternative are *p*-nitrobenzylidene acetals (Figure 5.4).

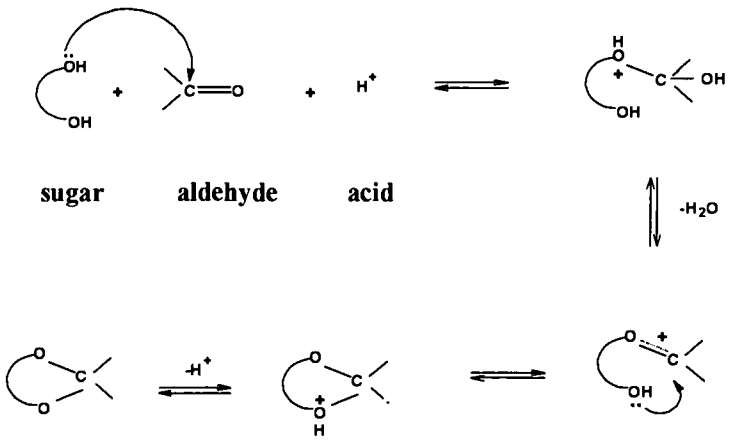

Figure 5.3. Formation of cyclic acetals (Calinaud and Gelas, 1996)

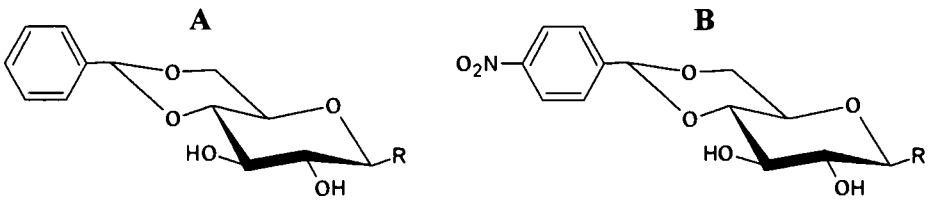

Figure 5.4. 4,6-*O*-benzylide acetals (A); 4,6-*O*-*p*-nitrobenzylidine acetals (B)

Benzylidene acetals can be opened selectively by acid catalyzed reduction to yield either 4-O or 6-O benzyl ether monohydroxyl, derivatives. The selectivity of the reduction is determined by the reducing agents (metal hydride), acid catalysts, solvents, and steric effects (Zhang, 1996). Reduction of 4,6-O-benzylidene acetals with lithium aluminium hydride-aluminium chloride gives 4-O-benzyl ethers. Lewis acids like aluminium chloride coordinate with the less hindered 6-O to give a reactive complex that is reduced by lithium aluminium hydride to produce 4-O-benzyl derivatives (Figure 5.5). Proton catalyzed reduction (sodium cyano borohydride-ethereal hydrogen chloride) gives 6-O-benzyl derivatives (Figure 5.5). This is because the 4-O oxygen is more basic than the 6-O oxygen and the proton is small enough to reach the 4-O oxygen; thus protonation of 4-O oxygen produces an

intermediate which reacts with sodium cyanoborohydride to give the 6-O benzyl derivative (Garegg, 1996).

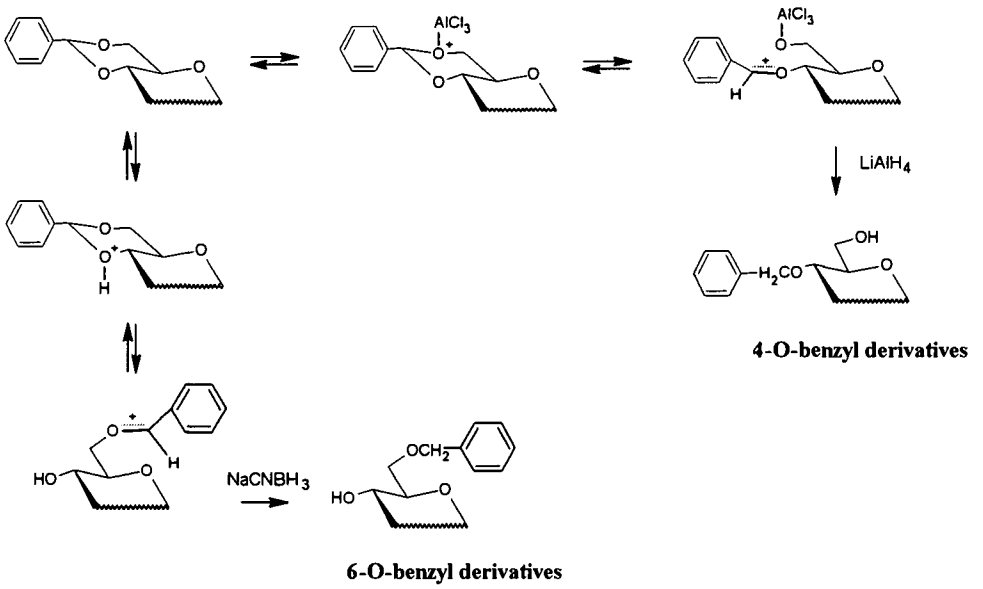

Figure 5.5. Opening of benzylidene acetals selectively by acid catalyze reduction (Binkley, 1988)

However, *p*-nitrobenzylidene acetals are more resistant to acid catalyzed reduction than are simple benzylidene acetals. The nitro substituents on the aromatic rings makes them less reactive toward electrophilic atack. Electrons can flow from the benzene ring to the substituent, thus leaving a positive charge in the ring,

destabilizing carbocation intermediates and thus making the ring less susceptible to the electrophilic attack (McMurry, 1992). Early in this study we attempted to reduce *p*-nitrobenzylidene acetals with lithium aluminium hydride-aluminium chloride to give 4-O-benzyl ethers. This reaction seemed attractive since lithium aluminium hydride has the potential to reduce nitro group simultaneously to an amino group (Walker, 1976). Unlike the nitro group, amino substituents on aromatic rings makes them more reactive toward electrophilic attack (McMurry, 1992). However, attempts to reduce *p*-nitro-4,6-O-benzylidene acetals with lithium aluminium hydride-aluminium chloride were not successful (data not shown). The reaction mixture was applied to TLC plate and when it was exposured to UV light, it gave positive result. It was concluded that the aromatic group was on the sugar ring. It gave a purple color (positive for amino groups) with a ninhydrin based visualizing reagent on TLC plate. It was concluded that *p*-nitro group was reduced to the *p*-amino group. However, it gave a mixture of green color (positive for carbohydrates) and white discolorization spot with *p*-anisaldehyde-sulfuric acid visualizing reagent (carbohydrate) on TLC plate. The suspect compound was purified by silica gel chromatography (70-230 mesh, SIGMA Chemical Co., St. Louis, MO). The fractions containing the suspect compound gave white precipitate and unmeaningful results with ^{1}H NMR experiments. Therefore it was concluded that acid catalyzed reduction of *p*-nitro-4,6-O-benzylidene acetals did not result in appreciable yields of the desired methyl 4-O-*p*-nitrobenzyl-β-D-glucoside.

Another way to open benzylidene acetals is by oxidative ring opening. Benzylidene acetals can be oxidized with NBS (N-bromosuccinimide) in the presence of water. The mechanism of this reaction has been studied extensively for the synthesis of 4-O-benzoyl-6-bromo-6-deoxy-α-D-glucopyranoside (Hanessian, 1966; Hanessian and Plessas, 1969a;b; Hanessian, 1987). This reaction has not been tried for regioselective ring opening for 4,6-*O*-*p*-nitrobenzylidene acetals in carbohydrate chemistry. As shown in the Figure 5.6. free radical bromination of the benzylidene acetal carbon atoms gives a bromo derivative **A,** which generates stabilized carbocation **B**. The carbocation intermediate is attacked by water to form very reactive orthoacetal **C** which may undergo ring opening by either of two paths depending on the conditions used. Path "a" leads to the formation of 6-O-NO_2Bz ester, whereas path "b" gives the 4-O-NO_2Bz ester.

Although the origin of the regioselectivity of the ring opening has not been fully explained, steric factors may affect this reaction. The nitro group plays an important role in this reaction, since the selectivity is somewhat different from that of other *O*-benzylidene acetals. Since the formation 4-O oxygen is more basic than 6-O, enhancing the acidity of the reaction solution is expected to increase the percentage of 4-O-NO_2Bz ester. However, the dominant product is 6-O-NO_2Bz ester. In addition, excess acid may lead to a general glycoside hydrolysis reaction.

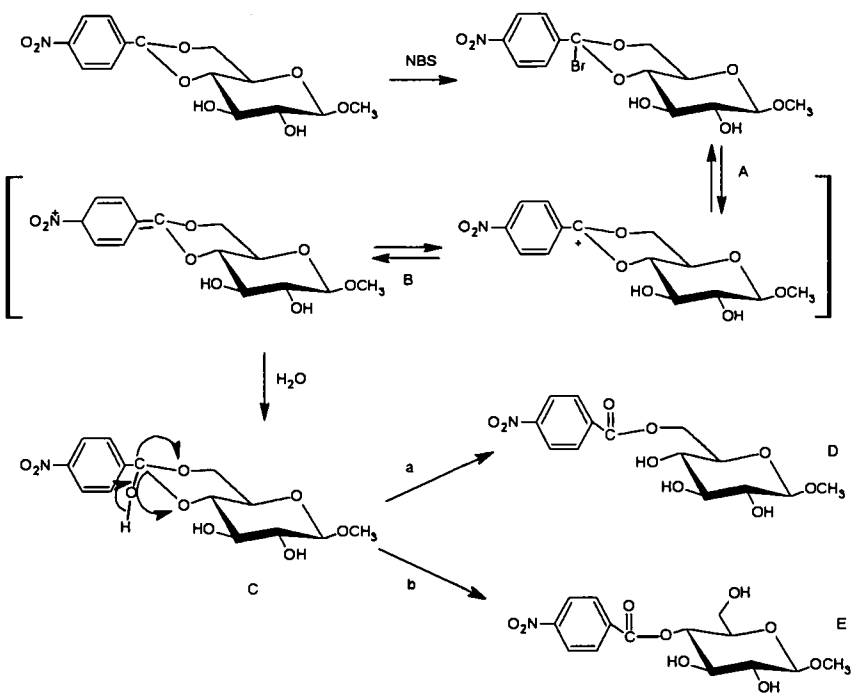

Figure 5.6. Opening of benzylidene acetals selectively by NBS oxidation

The synthetic method described here can be used as a general method for the modification of the non-reducing terminus of oligosaccharides (Figure 5.7). In this method the anomeric carbon needs to be protected due to its high reactivity. This was accomplished by blocking with a methyl group due to its stability under various reaction conditions. It is not expected to block the enzyme-ligand interactions because of its relatively small size. Protection of 4'-6'-positions of methyl cellooligosaccharides with *p*-nitrobenzylidene acetals followed by regioselective ring

opening gives non-reducing end modified cellooligosaccharides. This method is simple because it does not require many protection and deprotection steps. Such modified oligosaccharides might be used for testing of the chain end specifity of exoenzymes. Thus, modified oligosaccharides can also be used as affinity ligands for the separation of exo-cellulases from endocellulases.

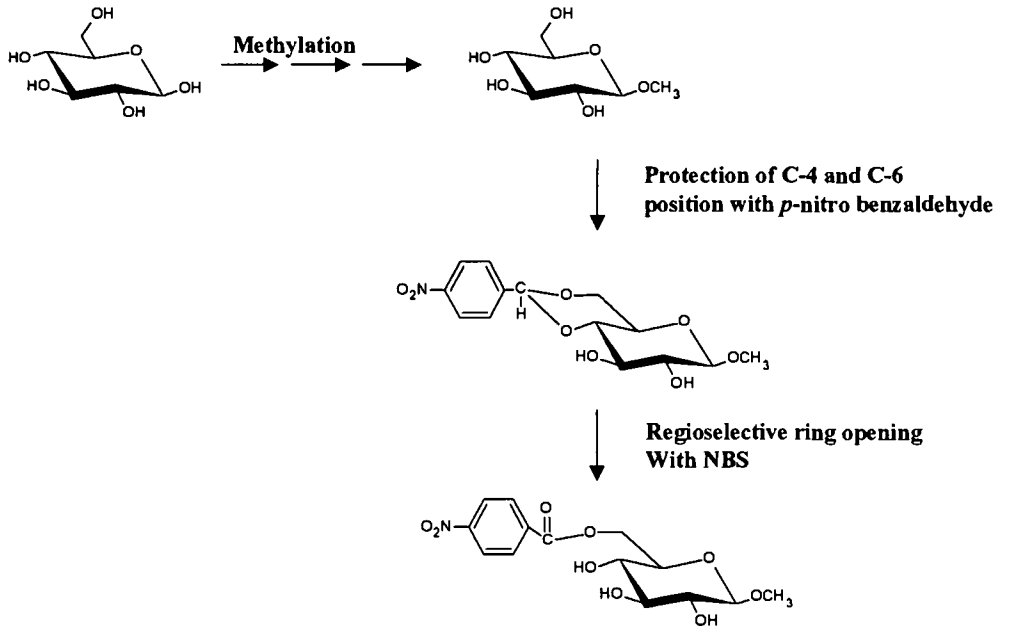

Figure 5.7.The scheme of preparation of non-reducing end modified glycosides.

5.5. SUPPORTING INFORMATION

5.5.1. NMR data for Methyl β-D-glucoside

^{1}H (Me_2SO-*d6*) data: δ 4.02 (d, 1H, $J_{1,2}$= 7.62 Hz H-1), δ 3.66 (dd 1H J= 10.1 Hz, J= 4.27 Hz), δ 3.43 (1H J= 10.3 Hz), δ 3.12-2.90 (m 4H), δ 4.99 (OH), δ 4.88 (OH), δ 4.86 (OH), δ 4.47 (OH),δ 3.37 (s, 3H- OCH_3).

5.5.2. NMR data for Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside

^{1}H (Me_2SO-*d6*) data: δ 4.22 (s, 1H, $J_{1,2}$= 7.7 Hz H-1), δ 4.23 (dd,1H $J_{6a,6b}$= 10.3 Hz, $J_{6a,5}$= 3.88 Hz H-6a), δ 3.73 (dd,1H $J_{6a,6b}$= 10.3 Hz H-6b), δ 3.43-3.41 (m 3H, H-3,4,5), δ 3.05 (dd,1H $J_{1,2}$= 7.7 Hz, $J_{2,3}$= 13.1 Hz H-2), δ 5.37 (OH), δ 5.35 (OH), δ 3.39 (s, 3H- OCH_3), δ 5.73 (s PhCH), δ 8.24 (d $J_{meta,ortho}$ = 8.82 Hmeta), δ 7.71 (d $J_{meta,ortho}$ = 8.82 Hortho). ^{13}C: δ 106 (C-1), δ 74.7 (C-2), δ 76.2 (C-3), δ 82.6 (C-4), δ 67.5 (C-5), δ 70.1 (C-6), δ 58.5 (OCH3), δ 101 (PhC), δ 130 (Ortho), δ 125(Meta).

5.5.3. NMR data for Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside

^{1}H (Me_2SO-*d6*) data: δ 4.11 (d, 1H, $J_{1,2}$= 7.7 Hz H-1), δ 3.24-3.15 (m, 2H, H-2,3), δ 2.985 (1H H-4), δ 3.50 (1H, H-5), δ 4.37 (dd, 1H, $J_{6a,6b}$= 11.7, $J_{6a,5}$= 6.1 Hz H-6a), δ 4.57 (dd, 1H, $J_{6a,6b}$= 11.7, $J_{6b,5}$= 2.10 Hz H-6b), δ 3.29 (s, 3H- OCH_3), δ 8.36

(d Hmeta), δ 8.17 (d Hortho). ^{13}C: δ 104 (C-1), δ 70.0 (C-2), δ 76 (C-3), δ 72.2 (C-4, C-5), δ 60.1 (C-6), δ 55.3 (OCH3), δ 126 (Ortho), δ 132 (Meta).

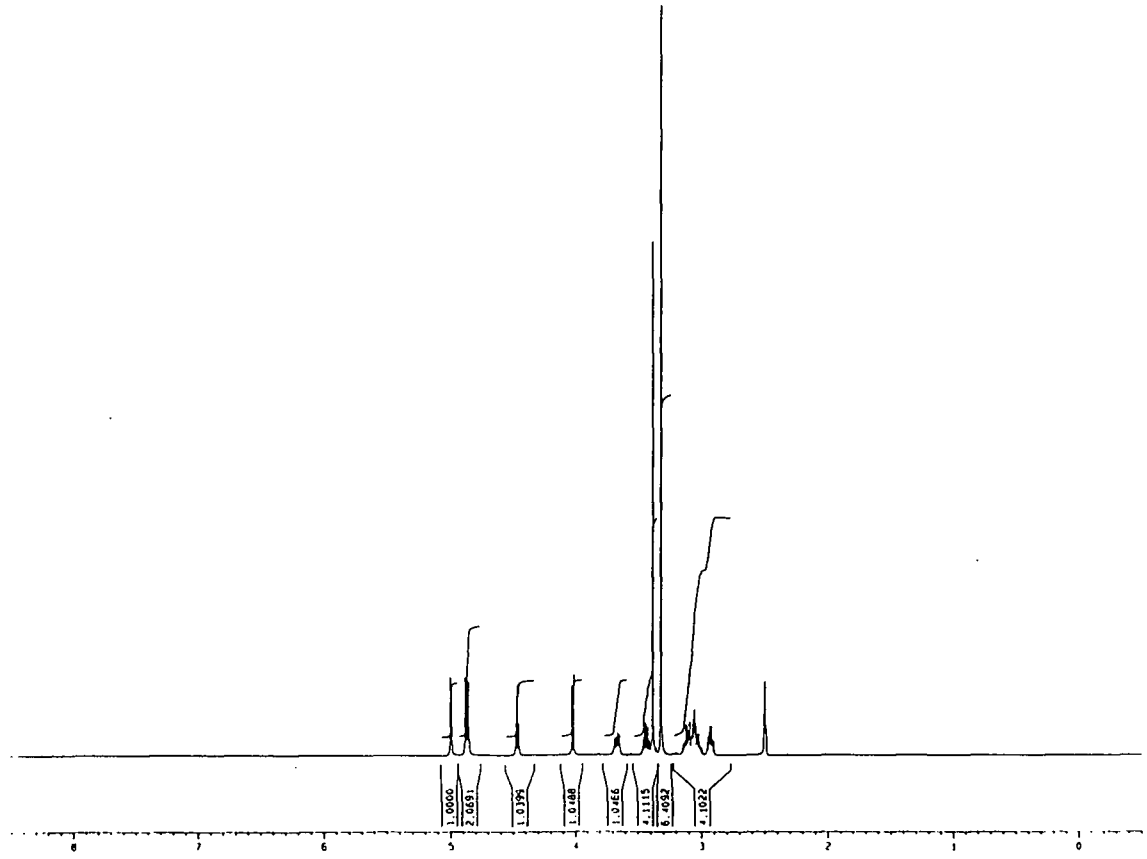

Figure 5.8. ^{1}H NMR spectrum for Methyl-β-D-glucoside (Me_2SO-*d6*).

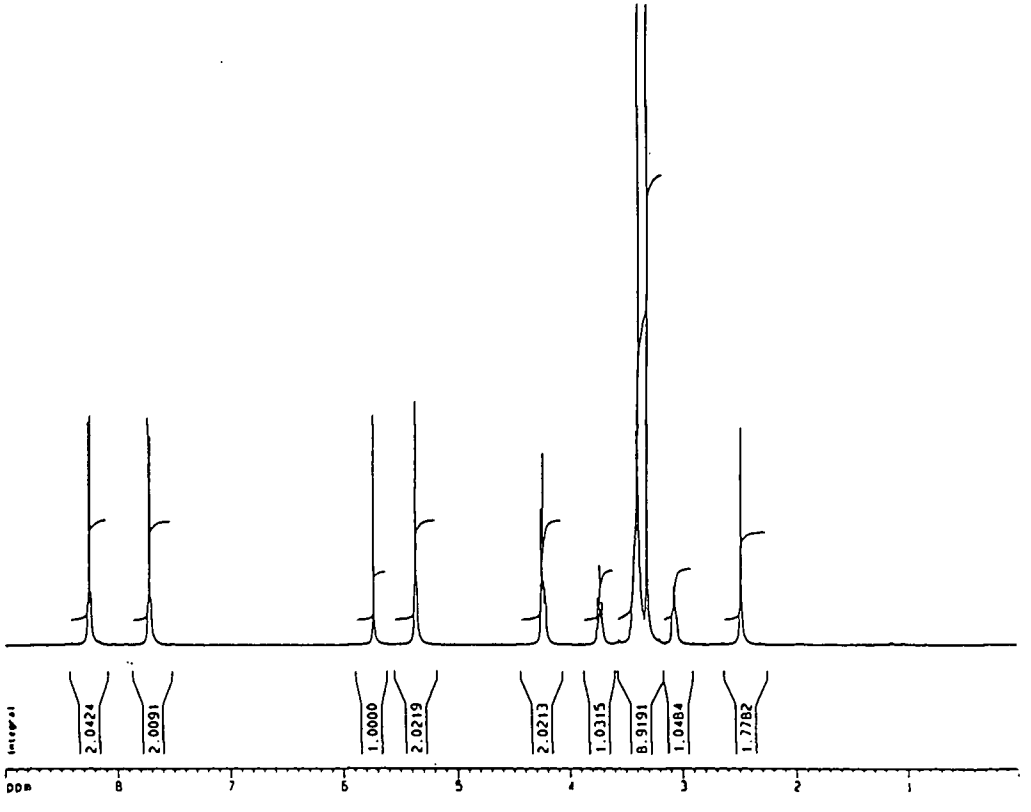

Figure 5.9. ^{1}H NMR spectrum data for Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside (Me_2SO-*d6*).

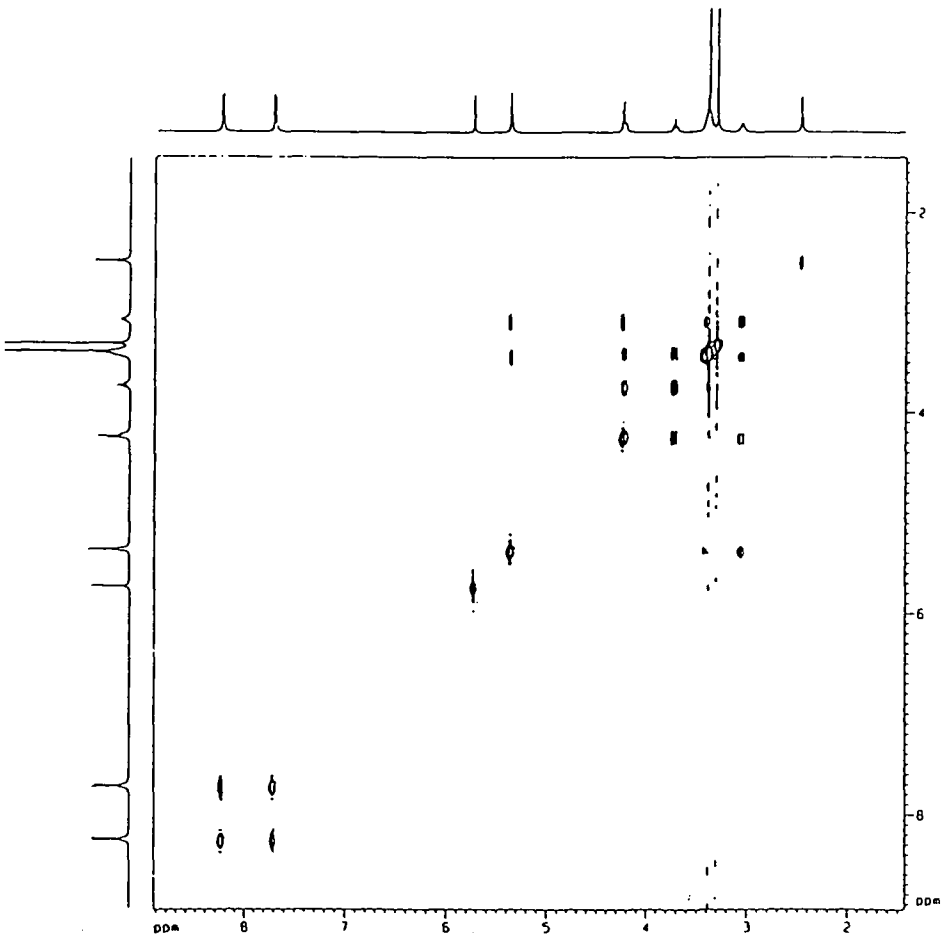

Figure 5.10. 2D COSY spectrum for Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside (Me_2SO-*d6*).

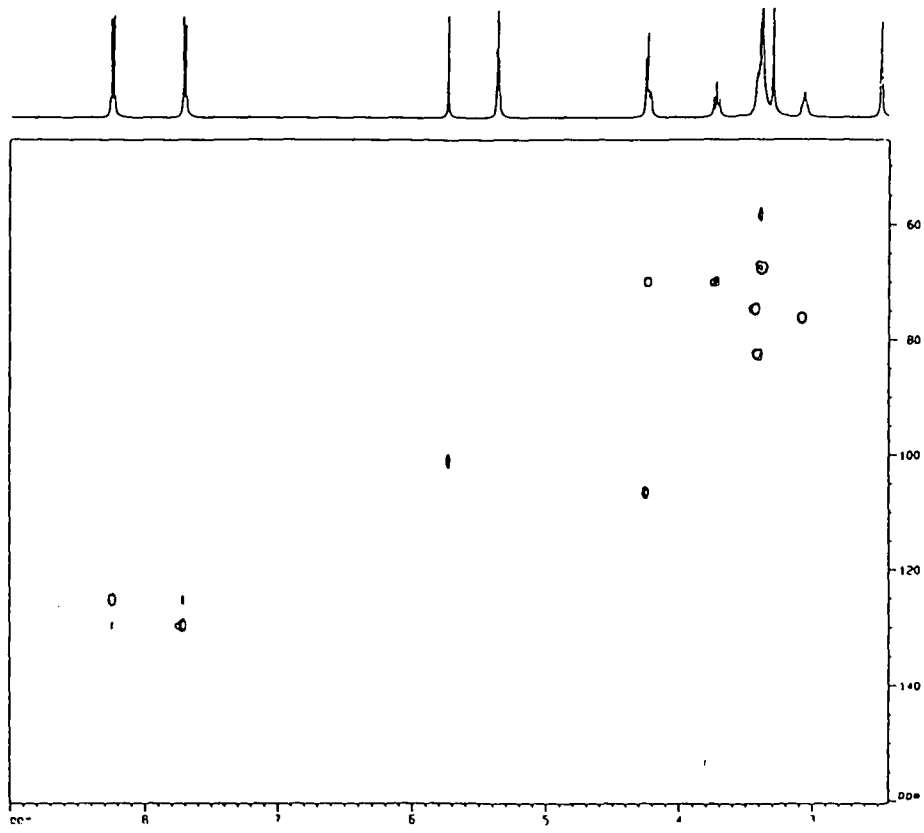

Figure 5.11. HSQC spectrum for Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside (Me_2SO-*d6*).

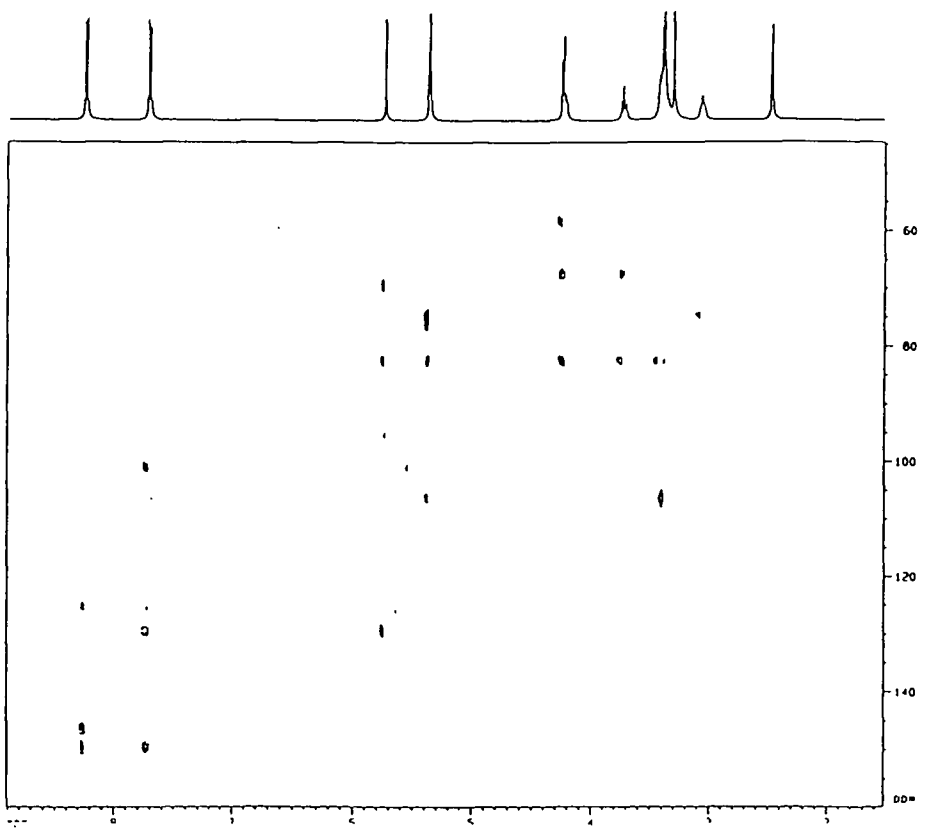

Figure 5.12. HMBC spectrum for Methyl 4,6-*O*-*p*-nitrobenzylidine-β-D-glucoside (Me_2SO-*d6*).

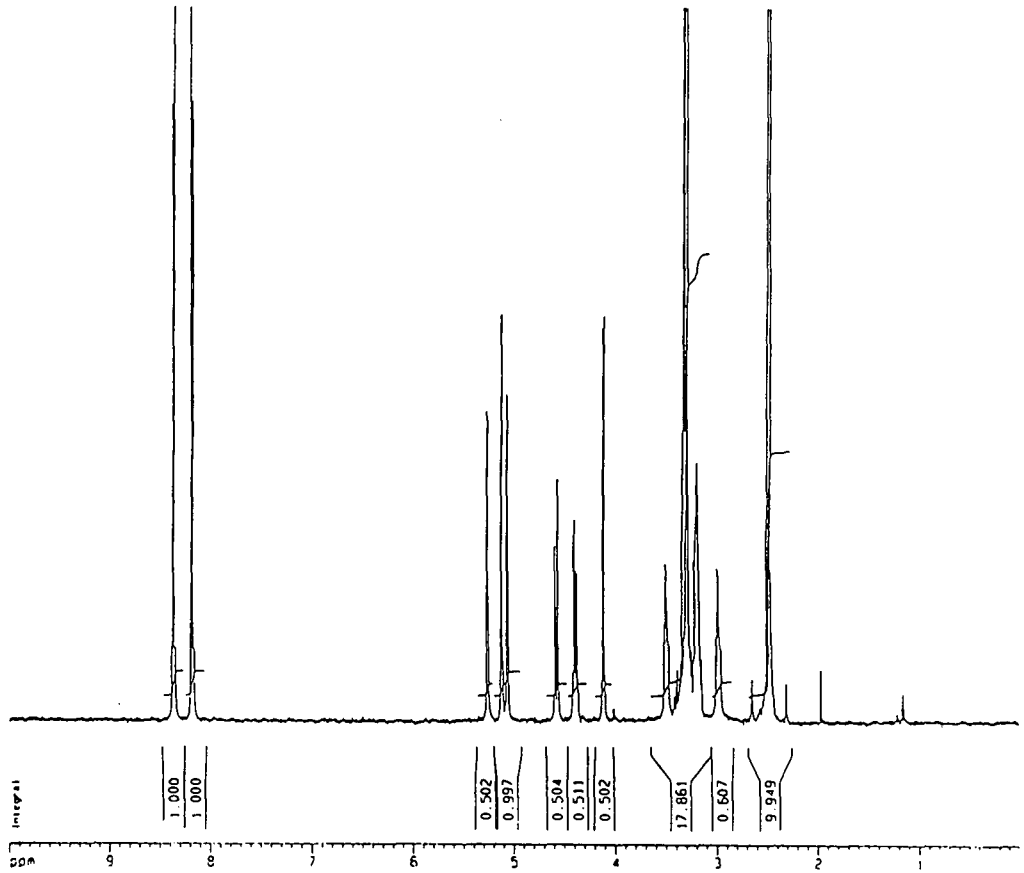

Figure 5.13. ^{1}H NMR spectrum for Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside (Me_2SO-*d6*).

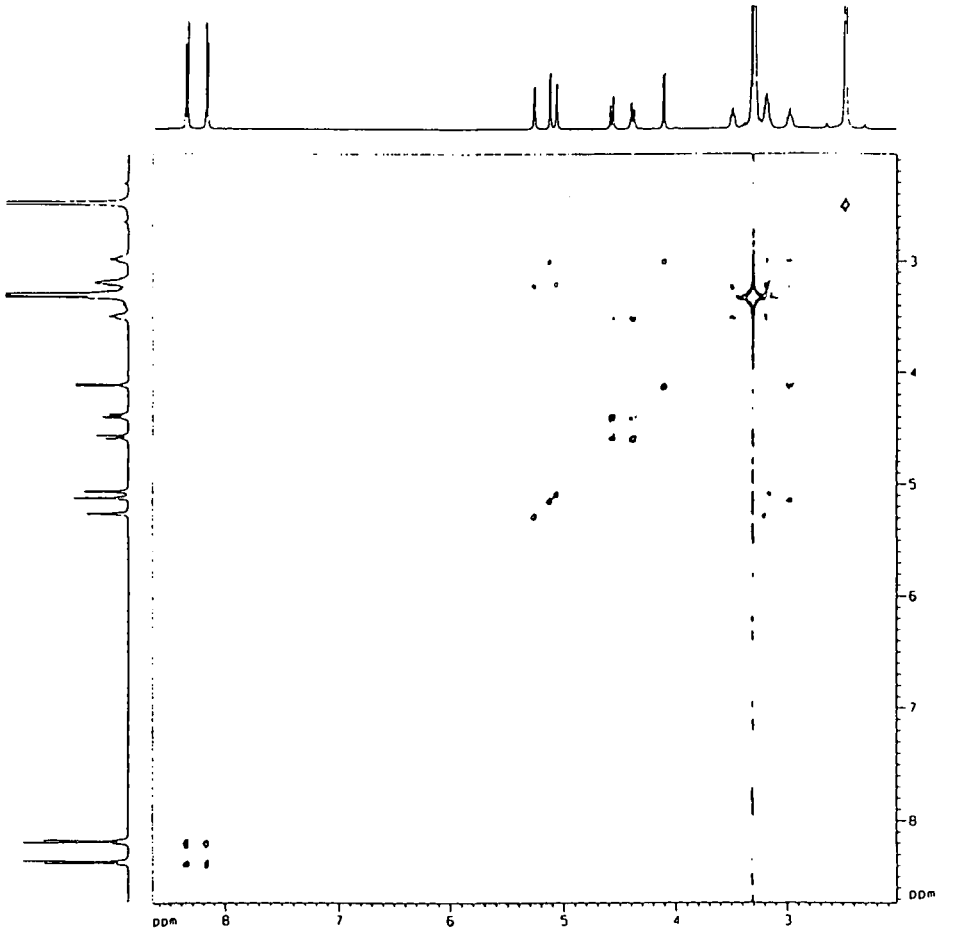

Figure 5.14. 2D COSY spectrum for Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside (Me_2SO-*d6*).

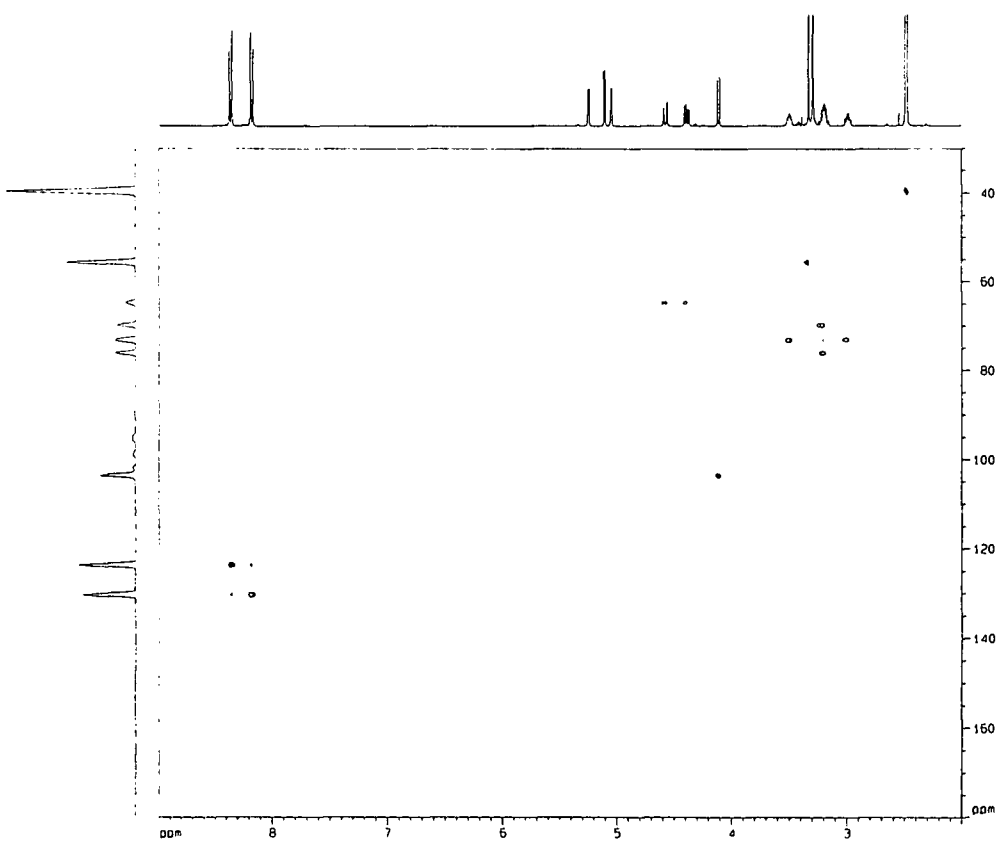

Figure 5.15. HSQC spectrum for Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside (Me_2SO-*d6*).

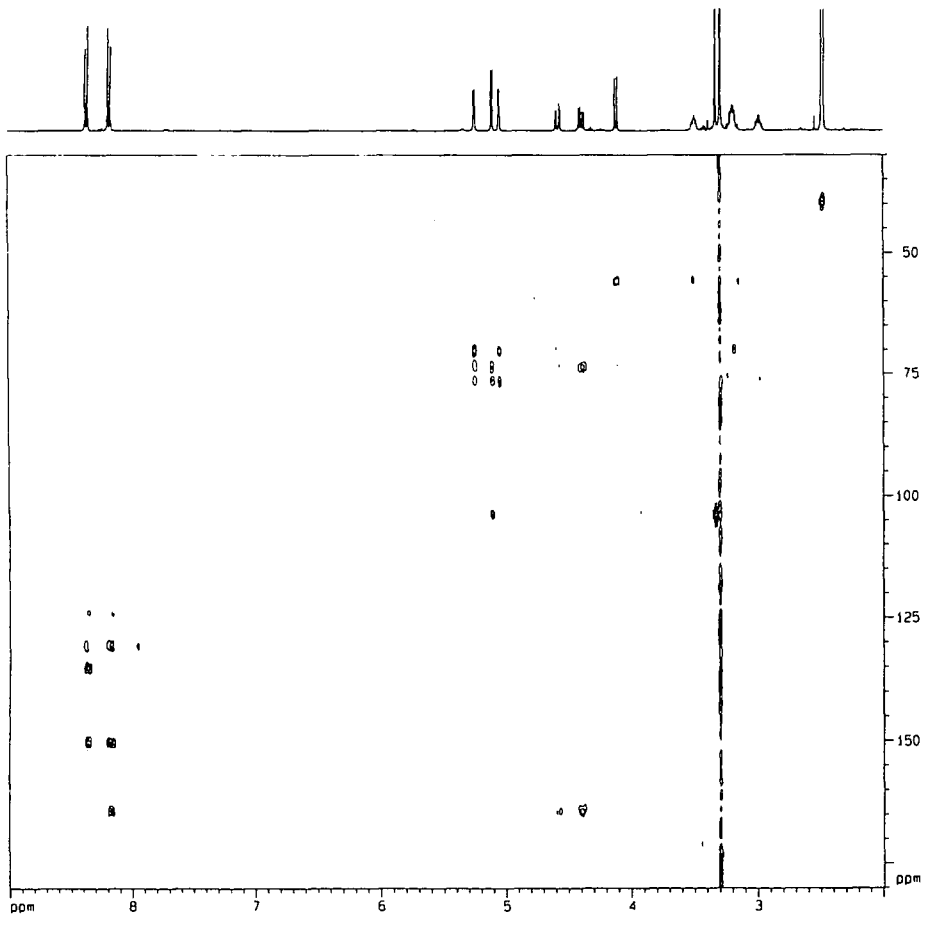

Figure 5.16. HMBC spectrum for Methyl 6-*O*-*p*-nitrobenzoyl-β-D-glucoside (Me_2SO-*d6*).

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CHAPTER 6

CONCLUSION

The general objective of the work described in this thesis was to make it easier for laboratories to use cellooligosaccharides in the study of cellulolytic enzymes. Two current difficulties were addressed, (1) methods of obtaining pure cellooligosaccharide preparations and (2) methods for the selective modification of cellooligosaccharides. The comparative experiments, looking at various chromatographic approaches to the fractionation of cellooligosaccharides, resulted in tabulated data appropriate for determining the chromatographic approach most suited for specific cellooligosaccharide applications. These fractionation studies also led to the development of a new approach for the fractionation of cellooligosaccharides - the use of cellulose stationary phases in conjunction with water-ethanol mobile phases for cellooligosaccharide fractionation. This approach seems most applicable to those cases where low cost, low resolution, separations are appropriate, such as may be likely for the food industry. Lastly, a general method is described for potential application in the selective modification of the non-reducing terminus of cellooligosaccharides. This type of modification will be useful for the preparation of affinity ligands and model substrates for cellulolytic enzymes.

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