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Sasithorn Kongruang for the degree of Doctor of Philosophy in Bioresource Engineering presented on October 28, 2003.
Title: Quantification and Reactivity of Cellulose Reducing Ends: Implication for Cellulose/Cellulase Saccharification.

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The primary purpose of this study was to (1) develop methods for the analysis of and (2) provide information on the chemical nature of reducing ends in typical cellulose substrates used for the study of cellulolytic enzymes. The studies were designed such that values obtained for cellulose substrates were compared with those obtained for a series of soluble cellooligosaccharides. The initial phase of the study tested the validity of using established colorimetric reducing sugar assays, developed for the measurement of reducing sugars in solution, for the quantification of reducing ends on insoluble substrates. The results demonstrate that published methods give widely differing values for the number of reducing ends per unit weight cellulose. The Cu^{++}-based assay, using bicinchoninic acid (BCA) as a color yielding chelator of Cu^{+}, is shown to provide values that appear most consistent the properties of the substrates. A method was developed using the Cu^{++}-BCA reagent, following a mild sodium borohydride treatment, to provide an estimate of the number of solvent accessible reducing ends on insoluble

substrates. The kinetics of sodium borohydride reduction of reducing ends on crystalline cellulose, amorphous cellulose and soluble cellooligosaccharides were compared in order to ascertain the relative reactivity of these reducing ends. The apparent second order rate constants for the reduction of reducing ends associated with the crystalline celluloses were significantly lower than those for the reduction of reducing ends associated with either the insoluble amorphous celluloses or the soluble cellooligosaccharides. These results indicate the reducing ends associated with crystalline celluloses are not extended out from the surface as though mimicking solution phase reducing ends. The relevance of this, as well as the other results, to the behavior of cellulolytic enzymes is discussed. The final phase of the study was the demonstration of both a reducing sugar-based and a viscosity-based assay for the detection of a prototypical polysaccharide depolymerizing glycosyl hydrolase, polygalacturonase.

Quantification and Reactivity of Cellulose Reducing Ends: Implication for Cellulose/Cellulase Saccharification.

by

Sasithorn Kongruang

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CONTRIBUTION OF AUTHORS

Dr. Michael H. Penner was involved in the design, data interpretation, and writing of each manuscript. Myung Joo Han and Claudia Isela Gil Breton assisted in the collection of quantification analysis of cellulose reducing ends in Chapter 3.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF APPENDIX FIGURES

LIST OF APPENDIX TABLES

This thesis is dedicated to my mother, Jamneng Kongruang

QUANTIFICATION AND REACTIVITY OF CELLULOSE REDUCING ENDS: IMPLICATION FOR CELLULOSE/CELLULASE SACCHARIFICATION SYSTEMS

CHAPTER 1

GENERAL INTRODUCTION

Lignocellulosic biomass is a remarkably diverse and abundant renewable resource, coming from hardwoods, grasses, agricultural materials and municipal waste. The major component of lignocellulosic materials is cellulose. It coexists with hemicellulose, lignin and various inorganics. Lignocellulosic materials have tremendous potential for conversion to fuels and other valuable-added products. Conversion schemes for the production of these products can be based on mechanical, biological and/or chemical processes. Bio-based processes typically make use of biologically derived cells and/or catalysts in at least one of three steps: (i) biomass pretreatment, (ii) cellulose saccharification; and (iii) fermentation.

The cellulose saccharification step, defined here as the enzyme-catalyzed conversion of cellulose to glucose, is an example of an soluble enzyme/insoluble substrate system. The kinetics of this process are complex and not well understood. In fact, current mechanism-based models of this process are extremely elementary due to the lack of fundamental information on the factors that govern enzyme-substrate interactions. The process is known to be catalyzed by a complex mixture of polysaccharide (cellulose) depolymerizing glycosyl

hydrolases, collectively referred to as "cellulases". A "complete" cellulase system is comprised of endoglucanases (enzymes that randomly hydrolyze the cellulose molecule), exoglucanases (typically the predominant enzymes in the mixture, act via either the reducing or non-reducing end), and β-glucosidases. These enzymes are known to work synergistically to efficiently degrade cellulose (Medve et al., 1994; Nidetzky et al., 1994; Sild et al., 1998). The primary objective of the experiments described in this thesis was to further our understanding of the properties of cellulose that dictate the behavior of the exoglucanases.

A lot known about the structure and chemical mechanism (inverting versus retaining mechanism) of the exocellulases (Divne et al., 1994; Divne and Henrissat, 1995; Medve et al., 1998; Vajamae et al., 1998; Becker et al., 2001; Boisset et al., 2001). However, little is known with regard to how they interact with actual cellulose substrates. They clearly have a preference for either the reducing or the non-reducing end of the cellulose chains. The reducing end of the cellulose molecule is distinct in that it contains a free C-1 semialdehyde group. In contrast, the C-1 position of the glucosyl residue at the non-reducing end is involved in a glycosidic linkage. The glucosyl residue at the non-reducing end is unique in that it has a free 4-OH group, while the corresponding position at the reducing end is involved in a glycosidic linkage. The major exocellulase (Cellobiohydrolase I) from the prototypical *Trichoderma* cellulase system preferentially works via the reducing end. This fact, along with the relative ease

with which this end can be studied, is the reason the study focused on the properties of the reducing ends of typical cellulose substrates.

Chapter 2 of this dissertation provides an extensive review of the literature related to the structure and function of lignocellulosics, cellulose and cellulases. The chapter has been divided into 7 sections.

Chapter 3 presents a study whose objective was two-fold; first, to compare the relative merit of methods used to determine the number of reducing ends on cellulose substrates and, second, to obtain a quantitative measure of the reducing ends on commonly employed cellulose substrates. The study also addresses the issue of total versus enzyme- or reagent-accessible reducing ends.

The study described in Chapter 4 was aimed at determining the relative reactivity of cellulose chain ends in insoluble crystalline versus insoluble amorphous versus soluble celluloses. "Reactivity" was defined as the relative rate at which these reducing reacted with sodium borohydride. The approach was to obtain time courses for the reduction of each of the test celluloses and subsequently fit the data with a second order rate model. The resulting best-fit second order rate constants were then compared to determine the relative reactivity of the different celluloses.

The study described in Chapter 5 deviated somewhat from those described in Chapters 3 and 4 in that it dealt with the enzyme polygalacturonase. This enzyme is similar to the cellulases in that it is a glycosyl hydrolase. However, its preferred substrate is polygalacturonic acid. The objective of the study was to

establish the relative merits of methods used for the detection of this enzyme. This research was initiated at the request of a local food processor interested in developing a quality control system for their fruit processing facility. Two assay approaches were investigated, one based on the generation of new reducing sugars as a result of polygalacturonase activity and the other based on the reduction in viscosity of polygalacturonic acid containing solutions that occurs as a result of polygalacturonase activity.

An overall conclusion to dissertation is provided in Chapter 6.

CHAPTER 2

LITERATURE REVIEW

2.1 Cellulose

Cellulose is the most plentiful carbohydrate polymer in the world. It has been exploited as an essential material in industry. Although cellulose has been studied extensively for decades, the research in this field is still a progressive quest for understanding. Cellulose is found in the protective cell walls of plants as the main microfibril structure. It is also present in bacteria, fungi, algae and in animals (e.g., tanicates) (Tomme et al., 1995).

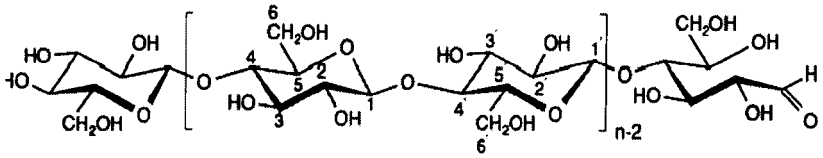
The cellulose content of the plants naturally ranges between 35 and 50 % of plant dry weight (Lynd et al., 1999). However, in cotton balls, cellulose is present in a nearly pure state. In nature, cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses (20-35%) and lignin (5-30%) of plant dry weight respectively (Lynd et al., 2002). The average degree of polymerization (DP) of native cellulose varies roughly between 1,000 and 14,000 glucose units, depending on the source (Fan et al., 1980). For example, in wood cellulose, chains have a degree of polymerization of approximately 10,000-glucopyranose units and reach 15,000 in native cotton cellulose. In certain algae, cellulose crystals with a DP as high as 23,000 are produced (Sugiyama et al., 1985; Brown, 1999).

Structure of cellulose

Cellulose is composed of β-D-glucopyranose units linked by (1→4) glycosidic bonds. In the conformational form, the pyranose rings have been found to be in the chair conformation $^4C_1$, the lowest free energy conformation of the molecule with the hydroxyl group in the equatorial position (Figure 2.1 a), while the hydrogen atoms are in the vertical position (axial). The polymer contains free hydroxyl groups at the C-2, C-3, and C-6 atoms. Based on the OH groups and the oxygen atoms of both the pyranose ring and the glycosidic bond, ordered hydrogen bond systems form various types of supramolecular semi-crystalline structures (Krassig, 1985, Klemm et al., 1998). The cellulose chains have a strict polarity with one end containing a free C-1 semialdehyde group (reducing end) and the other containing a free 4-OH group (nonreducing end) as portrayed in Figure 2.1 (a).

Celluloses and cellooligosaccharides share a common structure in that they are linear β-1, 4-glucosidically linked polymers with glucose as the monomer. Soluble celloligosaccharide have three to six glucosyl residues per molecule. Cellulose forms a long unbranched polymer with a highly intra- and inter-chain hydrogen bonds forming crystallinity (Figure 2.1 b,c). Studies of the structure of cellooligosaccharides and cellulose revealed that these structure are analogous (Bernet et al., 2000). X-ray diffraction data shows the arrangement of the individual chains within elementary cellulose fibrils, and statistical models have

been used to calculate the structure based on minimum conformational energy. Researchers from different institutes (Koyama et al., 1997; Kroon-Batenburg and Kroon, 1997; O'Sullivan, 1997) do not agree about the structural models, although the fundamental orientation of adjacent chains are parallel up versus parallel down. Regardless of their origination, cellulose chains in the microfibrils are stiff due to both intrachain and interchain hydrogen bonds (Figure 2.1 c). Adjacent sheets of cellulose chains overlie one another and are held together by weak intersheet van der Waals forces. The cellulose structures are reviewed extensively by these investigators (Koyama et al., 1997 and Hayashi et al., 1998).



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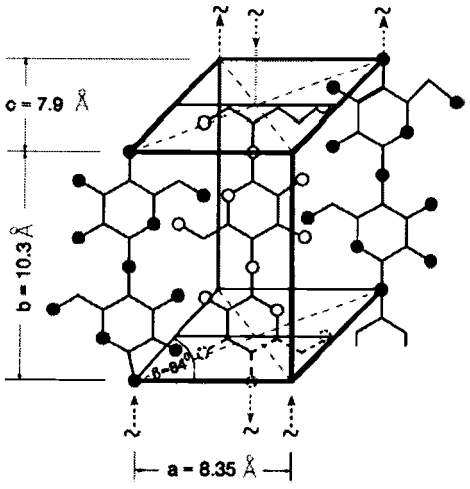

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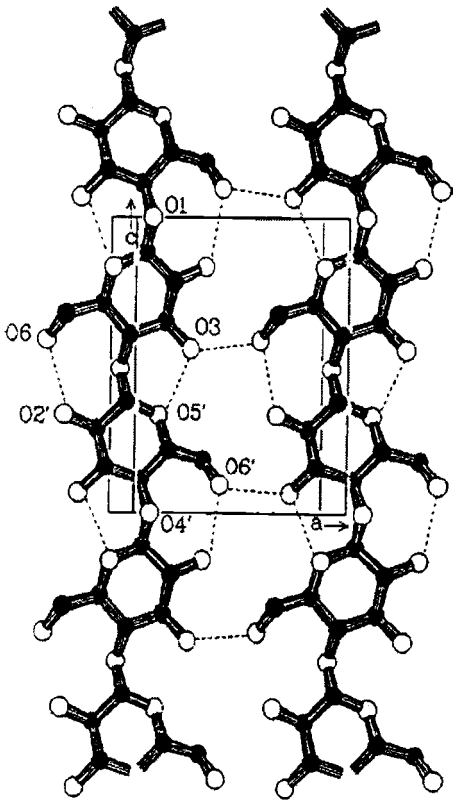


(c)

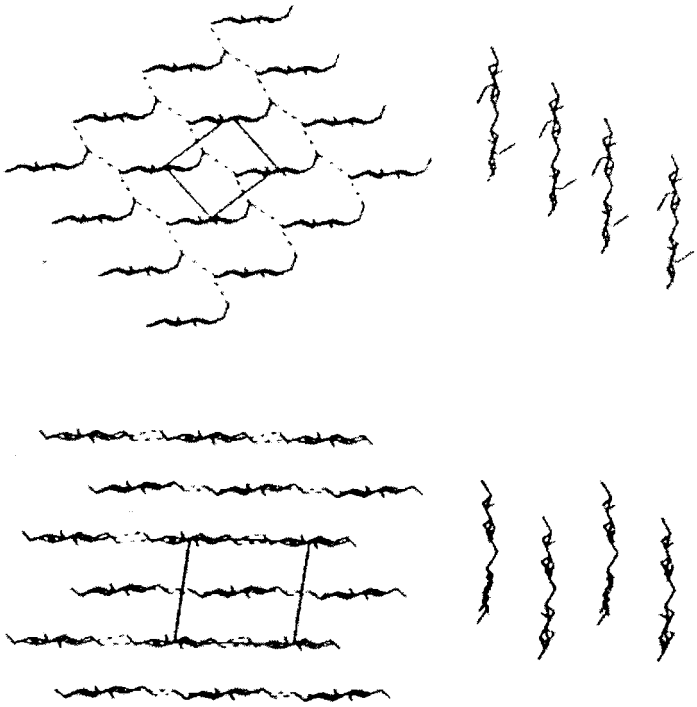
Figure 2.1. (a) Fragment (repeating unit) of a cellulose chain (Klemm et al., 1998). (b) Unit cell of cellulose I according to the Meyer-Misch model (Kolpak and Blackwell, 1976, 1978) and (c) Most probable bond pattern of cellulose I, a retangular form shows hydrogen bonding of cellulose I (Klemm et al, 1998).

Organization and polymorphy of cellulose

Cellulose structures can be defined in terms of three organizational levels (Attalla, 1993). The primary level is simply covalent bonds of a homopolymer of β-1,4-linked anhydroglucose. The secondary level describes molecular conformation, e.g. the spatial relationship of the repeat units to one another, as illustrated by the updated crystal structures in Figure 2.2 a,b. The tertiary level defines the association of molecules into ordered aggregates such as crystalline lattices. The secondary and tertiary levels of organization of most polymeric systems are not usually considered independently because only one secondary structure is compatible with a given tertiary structure, but there are at least two stable secondary structures for cellulose: k_I and k_{II} (Figure 2.3 a). These differ in the arrangement of their intramolecular hydrogen bonds but are able to coexist within a particular lattice because they occupy similar regions of space (Attalla, 1993; Tomme et al., 1995). Therefore, cellulose I and cellulose II are different lattice types that can accommodate two types of secondary structure; k_I is the predominant structure in cellulose I while k_{II} is predominant in cellulose II (Attalla and VanderHart, 1984).

The polymorphy of cellulose and its derivatives has been well documented (Tomme et al., 1995; O'Sullivan, 1997). Six polymorphs of cellulose (I, II, III_I, III_{II}, IV_I and IV_{II}) can be interconverted as shown in Figure 2.3b. Particularly, cellulose I, or native cellulose, is the form found in nature. Cellulose II, the second most extensively studied form, may be obtained from cellulose I by either

of two processes: (a) regeneration, which is the solubilization of cellulose I in a solvent followed by precipitation by dilution in water to give cellulose II; or (b) mercerization, which is the process of swelling native fibres in concentrated sodium hydroxide, to yield cellulose II on the removal of the swelling agent. Cellulose III_I and III_{II} (Marrinan and Mann, 1956; Hayashi et al., 1975) are formed in a reversible process, from cellulose I and II by treatment with liquid ammonia or some amines and the subsequent evaporation of excess ammonia (Davis et al., 1943; Sarko et al., 1976; Sarko, 1987). Polymorphs IV_I and IV_{II} (Gardiner and Sarko, 1985) may be prepared by heating cellulose III_I and III_{II} to 206 ^{0}C in glycerol.



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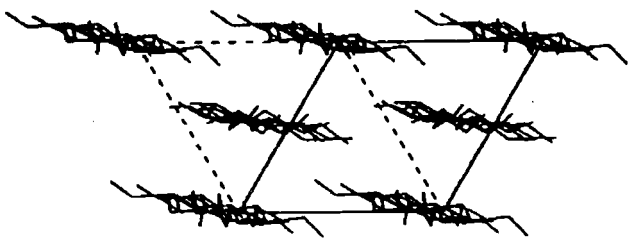
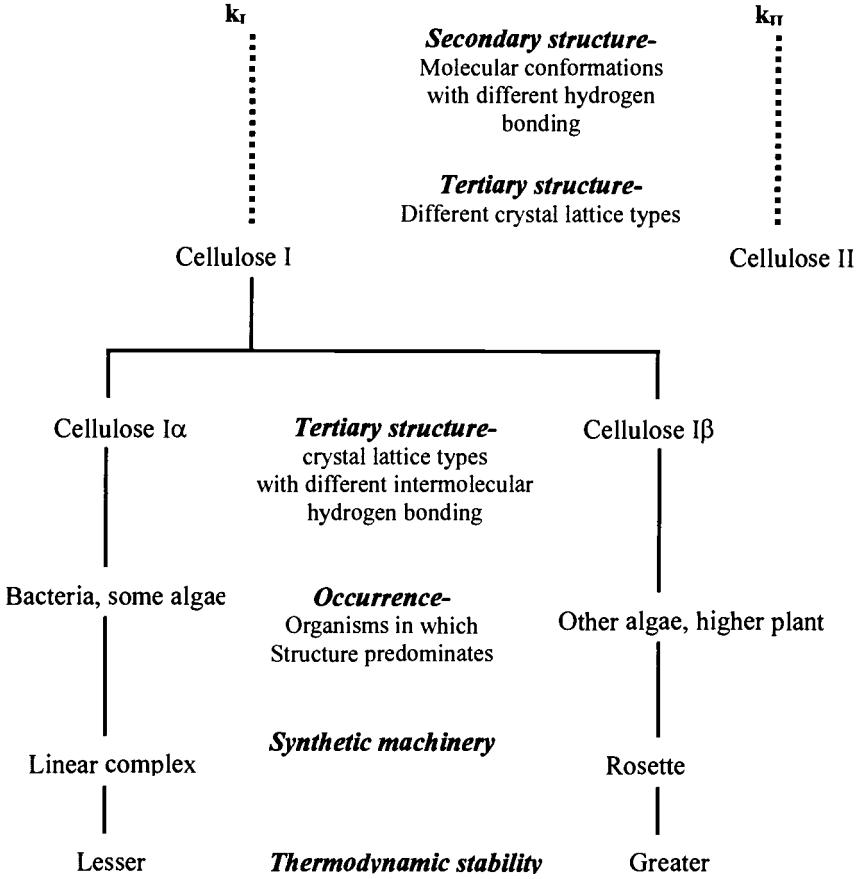


(b)

Figure 2.2. Projection of the crystal structures: (a) cellulose I_α (top) and I_β (bottom) on a plane perpendicular to the fiber axis. The respective chain along the fiber axis are illustrated on the right; (b) β-D-cellotetraose on the ab plane. The unit outline of cellulose II is shown in a dashed line. (Sarko, 1987; Hardy and Sarko, 1996)



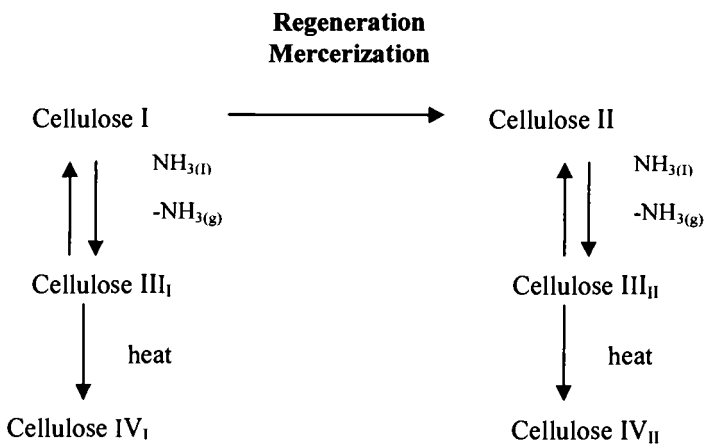


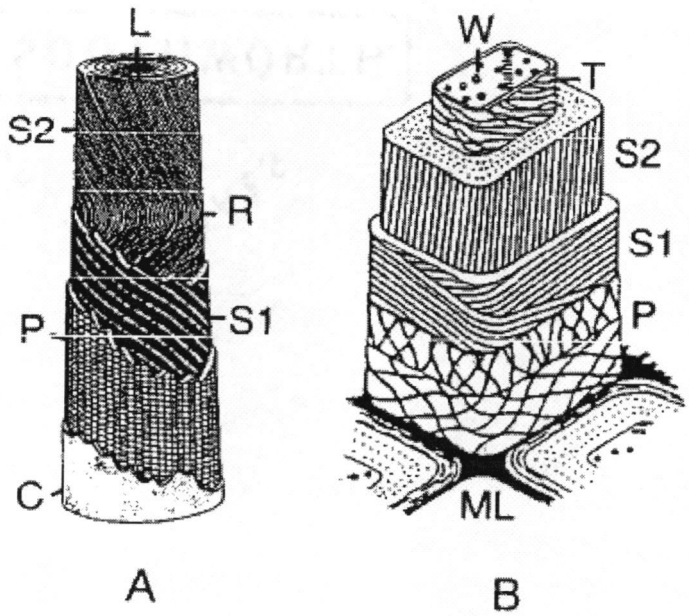
Figure 2.3 (a) Summary of structures and properties of cellulose I and II (Adapted from Coughland, 1991; Tomme et al., 1995), (b) Interconversion of the polymorphs of cellulose (O'Sullivan , 1997).

2.2 Model substrates

Model substrates used for studies of cellulose hydrolysis and microbial utilization vary extensively in the cellulose-cellulase research. Barley β-glucan (soluble, unsubstituted, mixed linkage glucans), carboxymethylcellulose (soluble, substituted celluloses), cellooligosaccharides and commercially available insoluble celluloses are often used to investigate specific aspects of cellulase action. The substrates differ in their fine structural features, which affects the results obtained. Celluloses, like filter paper or cotton (Figure 2.4), are also most often used to study the hydrolytic properties of cellulases or their adsorption on the substrate because they are considered representative of highly crystalline cellulose I and are readily available (Nidetzky et al., 1994; Zhang et al., 1999; Kim et al., 1998). Furthermore, microcrystalline celluloses (e.g. Avicel and Sigmacell)-- which are nearly pure cellulose resulting from a dilute-acid treatment that removes both hemicelluloses and the more extensive amorphous regions of the cellulose fibers-- are the most common model substrate.

Insoluble celluloses are sometimes modified and then used as substrates; for example, Avicel and cotton which have been swollen in concentrated phosphoric acid (Stahberg et al., 1993; Stalbrand et al., 1998) are often regarded as a convenient source of amorphous (noncrystalline) celluloses, but their structure are unclear and recent investigations describe they as a low crystallinity form of cellulose II. Amorphous celluloses also can be prepared by regeneration of these substrates using a solution in dimethyl sulphoxide-sulfphur dioxide-ethylene

diamine as a preferable alternative (Atalla, 1993, Isogai and Attala, 1991). Other types of cellulose, bacterial cellulose and bacterial microcrystalline cellulose, synthesized by the aerobic bacterium *Acetobacter xylinum* (Figrue 2.5) have been tremendously useful as a model system for studying cellulose biosynthesis and understanding cellulose biodegradation (Delmer, 1999; Boisset et al., 1999; Polonen et al., 1999; Valjamae et al., 2001). These substrates have advantages over those that come from plants because they have a more homogeneous structure, higher crystallinity and are available in a never dried form.



(A) a cotton fiber
(B) a delignified spruce wood fiber
C = cuticle (rich in pectins and waxes); L= lumen;
ML = middle lamella (mainly lignin); P = primary wall;
R = reversal of the fibril spiral; S1 = secondary wall ("winding layer");
S2 = secondary wall (main body); T= tertiary wall; W= wart layer.

Figure 2.4. Scheme of the "morphological architecture" according to Krässig (1993).

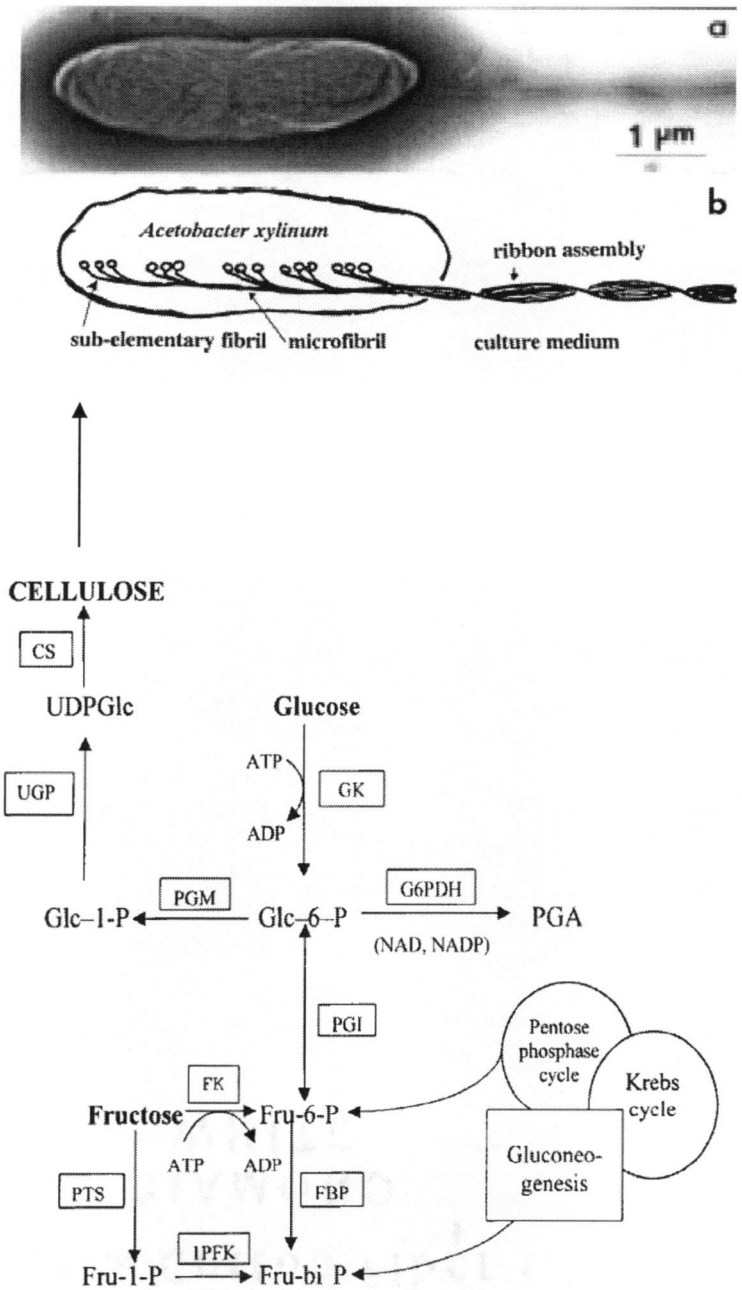


Figure 2.5. Pathways of carbon metabolism in *A.xylinum*. CS, cellulose synthase (EC 2.4.2.12); FBP, fructose-1,6-biphosphate phosphatase (EC 3.1.3.11); FK, glucokinase (EC 2.7.1.2); G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 1PKF, fructose-1-phosphate kinase (EC 2.7.1.56); PGI, phosphoglucoisomerase; PMG, phosphoglucomutase (EC 5.3.1.9); PTS, system of phosphotransferases; UGP, pyrophosphorylase UDPGlc (EC 2.7.7.9); Fru-bi-P, fructose-1,6-bi-phosphate; Fru-6-P, fructose-6-phosphate; Glc-6(1)-P, glucose-6(1)-phosphate; PGA, phosphogluconic acid; UDPGlc, uridine diphosphoglucose (Delmer and Amor, 1995).

2.3 Cellulolytic enzymes

Enzymatic degradation of cellulose

A large number of saprophytic soil bacteria, rumen bacteria and plant pathogens produce cellulolytic enzymes, but relatively few can utilize crystalline cellulose as a carbon source (for reviews on microbial cellulose utilization see Beguin and Aubert, 1994; Lynd et al., 2002). Recent progress in three-dimension structure of cellulases and cellulose binding domains from different families has been described (Bayer et al., 1998; Henrissat and Davies, 1997). Cellulolytic microorganisms that have been studied are *Trichoderma reesei*, *Thermomonospora fusca, Cellulomonas fimi, Clostridium thermocellulum,* and *Erwinia chrysanthemi* (Divne et al., 1998; Sakon et al., 1997; Notenboom et al., 1998; Gal et al., 1997; Brun et al., 1997; Henrissat et al., 1998).

Studies of the synergism of the cellulase enzymes have been reported from several groups (Woodward et al., 1988; Nidetzky et al., 1994; Beldman et al., 1988; Tomme et al., 1995; Medve et al., 1998). It turns out that the efficient hydrolysis of crystalline cellulose requires synergism between three kinds of enzymes as illustrated in Figure 2.7. As shown, they are cellobiohydrolases (E.C.3.2.1.91, 1,4-β-D-glucan cellobiohydrolase, exoglucanse); endoglucanse (E.C. 3.2.1.4, endo-1,4-β-D-glucan 4-glucanohydrolase); and β-glucosidase (E.C. 3.2.1.21, β-D-glucoside glucohydrolase). In terms of cooperative actions, cellobiohydrolases act as exoglucanases, releasing cellobiose as the main product, and endoglucanases act randomly along the cellulose chains, thus producing new

sites for the cellobiohydrolases and β-glucosidase hydrolyses cellobiose;thus, they remove a strong inhibitor of the cellobiohydrolases from the reaction mixture (Medve et al., 1998; Becker et al., 2001).

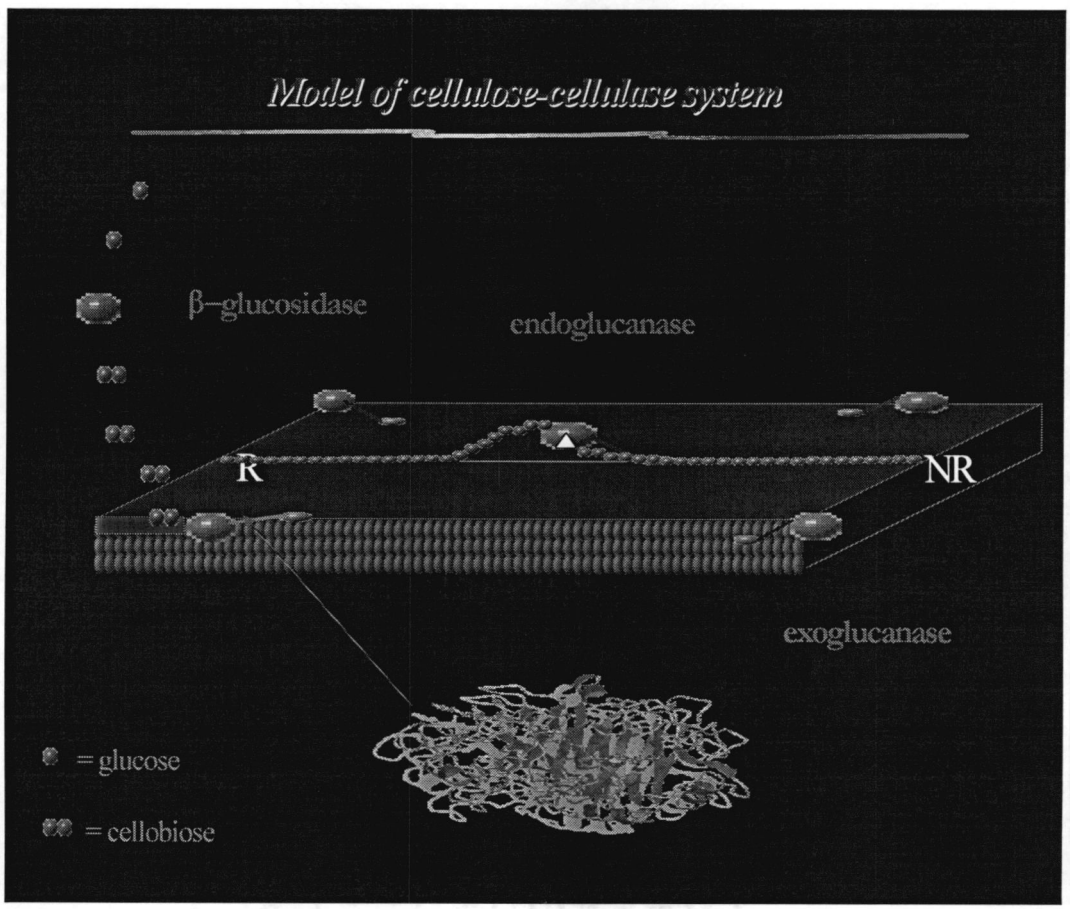
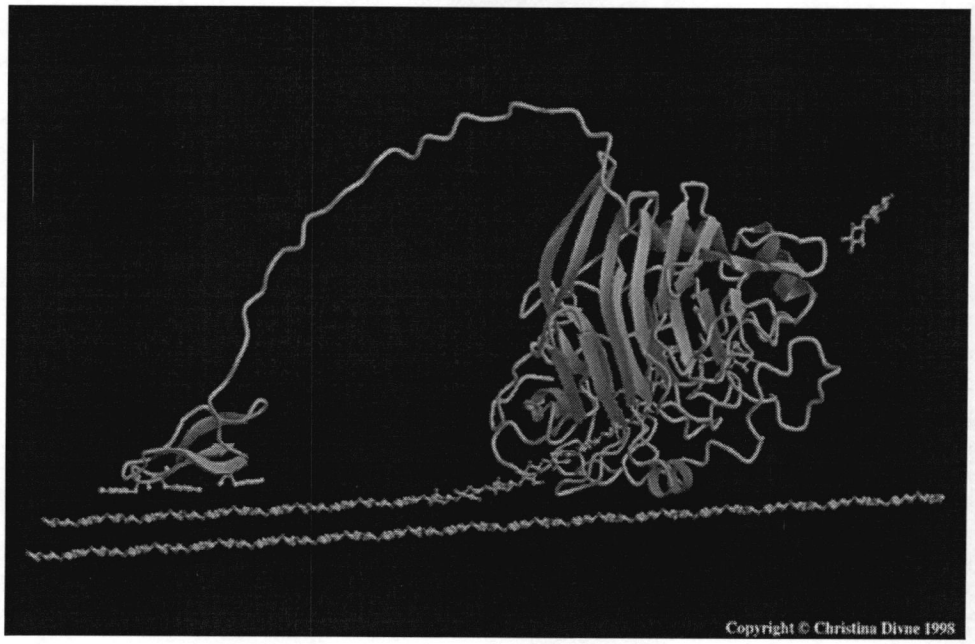


Figure 2.6. The synergism of cellulase enzymes for crystalline cellulose hydrolysis.

Trichoderma reesei

The soft rot fungus *Trichoderma reesi* produces an effective mixture of cellulases. The most abundant enzymes are two cellobiohydrases, Cel 7A and Cel 6A, formerly called CBHI and CBHII, and these are also the enzymes that are most efficient on highly crystalline cellulose. *Trichoderma reesei* also produces four endoglucanses (EGI (Cel 7B), EGII (Cel 5A), EGIII (Cel 12A), and V). The two cellobiohydrases have active sites enclosed in tunnels formed by long loops on the surface of the catalytic domains. These cellobiohydrolases therefore act primarily at the cellulose chain ends as shown in Figure 2.7a, whereas those of endoglucanses (Figure 2.8b) are open clefts (Rouvinen et al., 1990; Spezio et al., 1993; Divne et al., 1994; Kleywegt et al., 1997). A characteristic feature of most cellulolytic enzymes is a structural organization based on a catalytic domain linked by a flexible region to a cellulose-binding domain, as illustrated in Figure 2.7 a,b (Van Tilbeurgh, 1986; Johansson et al., 1989). In particular, Cel 7A catalytic domain has a β-sandwich fold, and the active site tunnel can put up to 10 sugar units (subsite –7 → +3) (Divne et al., 1994; Divne et al., 1998). The glycosidic bond is cleaved by retention of the configuration (between the subsite –1 and +1).



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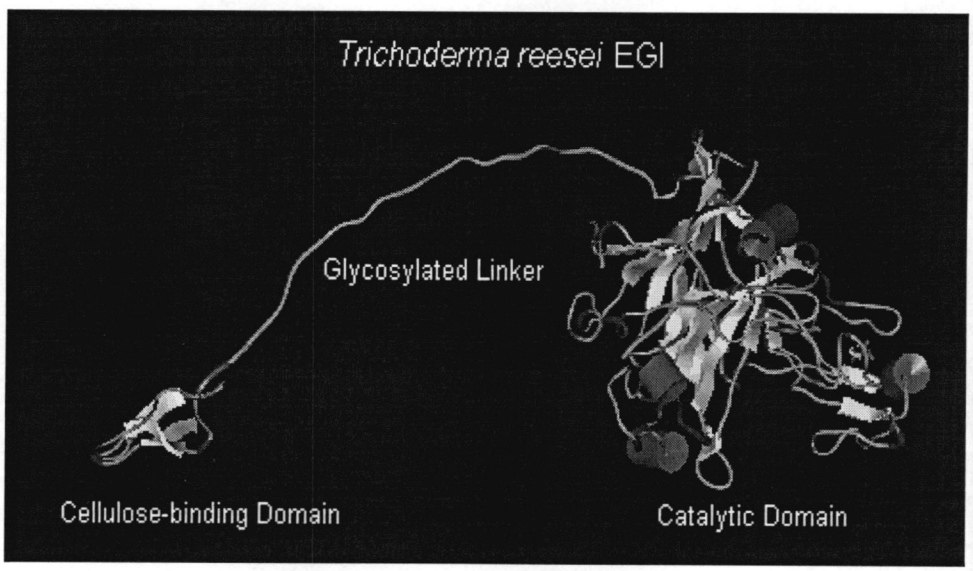

(b)

Figure 2.7. A hypothetical model of cellulases: (a) CBHI acting on the crystalline cellulose (Divne et al., 1998); (b) EGI structure (Kleywegt et al., 1997).

2.4 Measurement of substrate concentration

2.4.1. Reducing sugar assay

Typical colorimetric assays used for measuring enzyme-catalyzed cellulose saccharification are based on the generation of soluble reducing sugar equivalents. Quantitative tests for "reducing sugars" include the dinitrosalycilic acid (DNS) assay, the p-hydroxybenzoic acid hydrazide (PAHBAH) assay, the Cu^{2+}/arsenomolybdate (Nelson) assay, Cu^{2+}/bicinchoninic acid (BCA) assay and 3-Methyl-2-benzothiazolinonehydrazone (MBTH). These assays were originally developed to measure soluble phase reducing sugars; however, the assays have recently been adopted to measure reducing sugars in the solid phase (Kruus et al., 1995; Irwin et al., 1993; Kim et al., 1998; Zhang et al., 2000).

The reduction of alkaline 3,5-dinitrosalicylate (DNS) was adopted to measured the reducing groups produced as a result of cellulase and amylase activity although this assay was originally developed to determine the amount of glucose in blood and urine (Summer, 1925; Ghose, 1987). The redox reaction of DNS is depicted in Figure 2.8a. This method is still used for detecting the reducing sugar, although the method is not sensitive and gives an inaccurate result because the amount of oxidation is dependent of the chain length. Many investigations have shown that the DNS assay gives molar absorbance reponses that increase with increasing degree of polymerization of a series of homologous oligosaccharides (Robyt and Whelan, 1972, Miller et al., 1960; Breuil and Saddler, 1985). Another method used to measure the reducing sugars is p-hydroxybenzoic

acid hydrazide, as illustrated in Figure 2.8 b. This method has been used in carbohydrate determination in which the reaction is based on the reduction of acid hydrazides by reducing sugars (Lever, 1972).

The Nelson and BCA methods of reducing sugar quantification employ the same reaction pathway; that is, they are based on the reduction of Cu^{++} to Cu^{+} (Figure 2.9 a). In particular, the oxidation of the aldehyde is conducted under an alkaline condition of pH 10-11. The reduced metal cation forms a complex with a chromogenic cheator and this complex can then be monitored spectrophotometrically. A Nelson-modified Somogyi procedure (Nelson, 1944; Robyt and Whelan, 1968) measures the oxidation of the reducing carbohydrate by measuring the amount of Cu_2O formed. The cuprous oxide reacts with an arsenomolybdate reagent that gives a blue-green color. The reaction pathway of the copper bicinchoninate is illustrated in Figure 2.9. These two methods have been used extensively for measuring the product formed during the action of cellulolytic enzymes (Medve et al., 1998; Vajame et al., 1999; Ståhlberg et al., 1993; Zhang et al., 2000).

3-Methyl-2-benzothiazolinonehydrazone (MBTH) was orignally developed to determine aliphatic aldehydes in the atmosphere (Hauser and Cummin, 1964). In particular, in this reaction an aldehyde combines with two molecules of MBTH in a two-step process. In the first step, which occur at neutral pH, the aldehyde condenses with a single MBTH to form an adduct. In the second step, which occurs under acid and oxidizing conditions, this adduct reacts with a second MBTH to form a highly colored final product. This method can be used to

determine a number of aldehydes and is specific for aldehydes versus ketones (Gordon and Barrett, 2002).

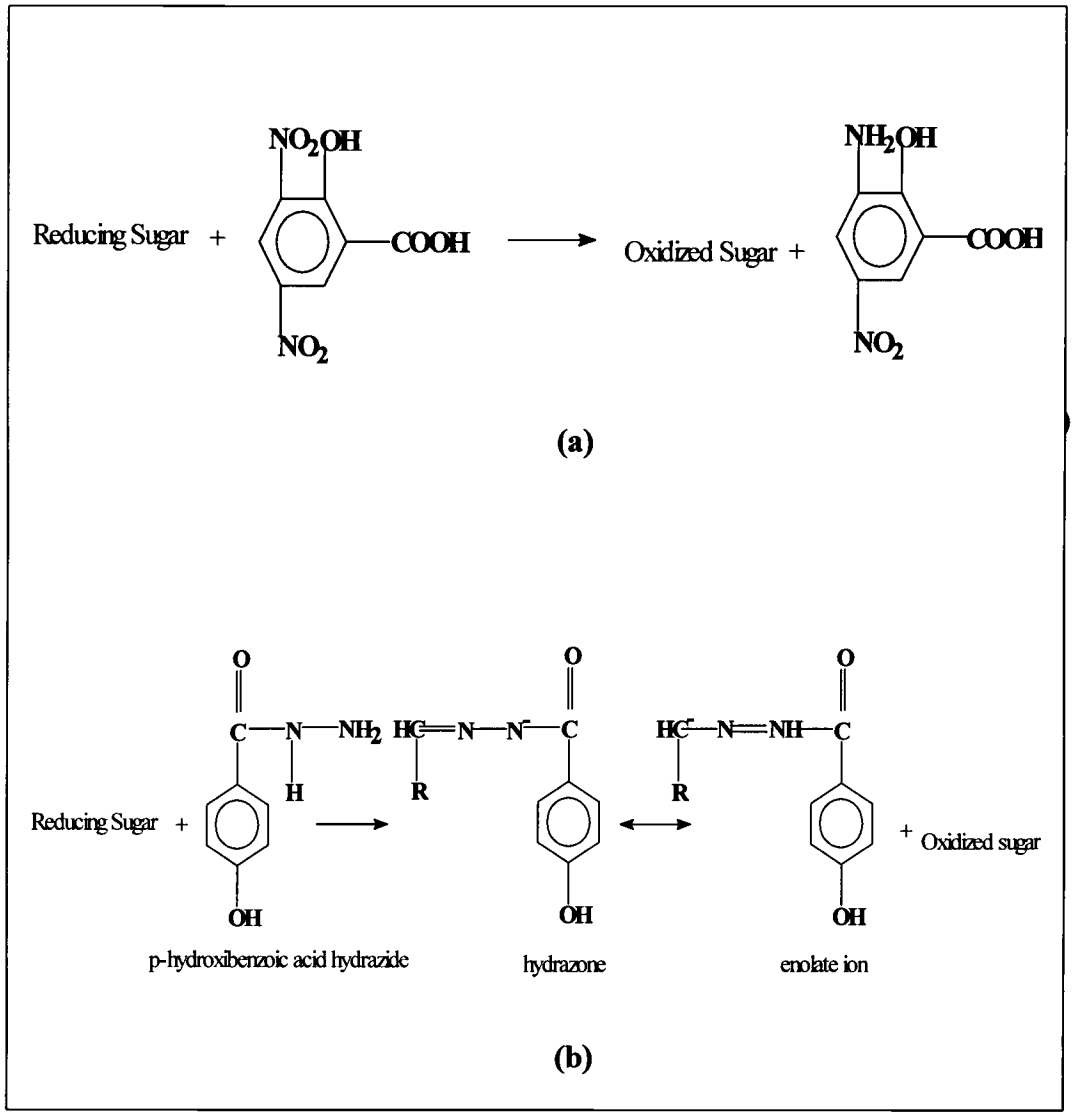

Figure 2.8. The reaction schemes of the title compounds of reducing sugar assay: (a) the dinitrosalycilic acid assay, (b) the p-hydroxybenzoic acid hydrazide.

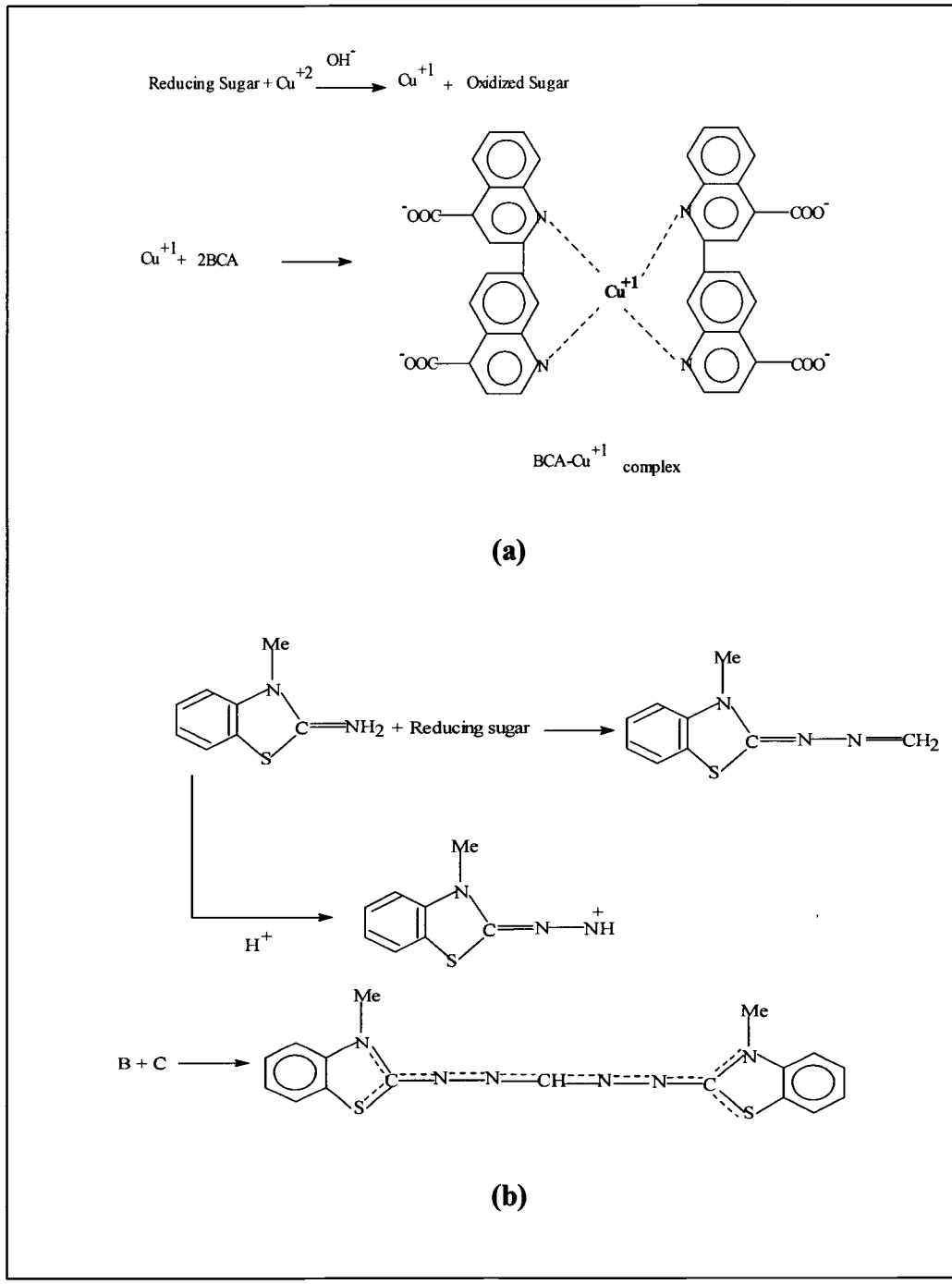

Figure 2.9. The reaction schemes of reducing sugar assays: (a) Bicinchoninic acid complex formation with Cu^{+1}resulting a highly purple colored chromophore (Smith, 1985), (b)Reaction of the reducing sugar with 3-methyl-2-benzothiazolone hydrazone, A, to form the azine, B; oxidation of A to a reactive cation, C; and formation of the blue cation, D (Sawicki, 1961).

2.4.2. Gel permeation chromatography

Gel permeation chromatography (GPC) or a size exclusion chromatography (SEC) is a technique where by separation takes place based on the size or hydrodynamic volume of a macromolecule relative to the pore size of stationary phase. This method has been utilized to measure the degree of polymerization and characterize a number of representative polysaccharides (i.e. dextran, pullulans, celluloses, arabinogalactans, amylose, and amylopectin (Striegel and Timpa, 1995; Striegel and Timpa, 1996). Many types of celluloses have been measured by this method: spruce and birch (Kennedy et al., 1990), cotton fiber and acid-washed cellulose powder (Timpa, 1991; Timpa and Triplett, 1992; Timpa and Ramey, 1994; Benedict et al., 1994; Emsley et al., 2000), Rayon and straw fiber (Silva and Laver, 1997), bacterial cellulose (Einfeldt and Klemm, 1997) and commercial celluloses (Strlic et al., 1998). GPC requires dissolution of the material under study without degradation. For this reason, it is preferable to avoid derivatization before dissolution to prevent possible changes in the material. This approach has been used for the study of degree of polymerization after the cellulolytic enzyme treatment of celluloses (Valjamae et al., 1999; Kleman-Leyer et al., 1992; Kleman-Leyer et al., 1994).

2.5 Sodium borohydride

Reduction mechanism of $NaBH_4$

Sodium borohydride ($NaBH_4$) is used as a probe for study of the reactivity of aldehydes, ketones, as well as for Schiff bases (Jentoft and Dearborn, 1979; Bunn and Higgins, 1981). The reaction is base-initiated with a hydride ion as the nucleophile. Theoretically, one mole of sodium borohydride reduces four moles of aldehyde or ketone and this reaction follows the same pattern as with lithium aluminum hydride where the reaction mixture is then acidified to produce the neutral alcohol (Brown and Krishnamurthy, 1979; Chaikin and Brown, 1949).

The mechanism of borohydride reduction of aldehydes was initially thought to proceed by a stepwise hydride ion transfer to the carbonyl carbon atom, resulting in formation of a tetraalkoxyborate containing the substrate being reduced and the subsequent hydrolysis of the complex eventually generated the alcohol as the product (Rickborn and Wuesthoff, 1970). Even though the carbonyl is found in only small percentages in solutions, and hemiacetals cannot be reduced directly because they lack a pi bond, the ring opening reaction is fast and reduction can take place completely through the carbonyl (Brown et.al, 1957; Bruce and Wuesthoff, 1970; Pagliaro, 1998). Particularly, the reduction of an aldose to a glycitol will be preceded by ring opening of the cyclic modification of the aldose to the aldehydo-form in the acyclic staggered zig-zag conformation (Bragg and Hough, 1957).

Employing of sodium borohydride for the reduction of reducing sugars to the corresponding polyols as the labeled substrates have been adopted to study the mode of action of enzymes for many years (Chirico and Brown, 1987; Suganuma et al., 1978; Takeda et al., 1990; Bhat et al., 1990; Takeda et. al, 1992; Hayashi et al., 1994; Barr et al., 1996; Han and Robyt, 1998; Boraston et al., 2002). Specifically, both normal and reduced cellooligosaccharides were modified by the reaction with borohydride to make the substrates available for the investigation of cellulolytic enzymes mode of action (Chirico and Brown, 1987; Sakamoto et al., 1989; Bhat et al., 1990; Nidetzky et al., 1994).

Properties of $NaBH_4$

The stability of sodium borohydride in the solution (water or slightly alkaline solution) is dependent upon the temperature and pH. As the reaction proceeds, causing a rise in the pH value, the rate of decomposition therefore decreases. The kinetics of the hydrolysis of hydroborate under a wide range of pH, temperature and buffer conditions has been studied (Mesmer and Jolly, 1961; Gardiner and Collat, 1964; Davis et al., 1962; Stockmayer et al., 1961; Davis and Swain, 1960). The decomposition seems to be dependent on many environmental factors.

Prokoptchik and Salkauskiene, (1970, 1971) studied the catalytic decomposition of $NaBH_4$ using metallic Cu. They proposed that the rate of catalytic reaction is directly proportional to the catalyst quantity and the overall

reaction mechanism can be explained as the following equation:

$$BH_4^- + 4\,H_2O \xrightarrow{Cu} B(OH)_4^- + 4\,H_2$$

Consequently, the decomposition of sodium borohydride can be described as a first order process where the rate of disappearance of borohydride, $-d[BH_4^-]/dt$, is given by the expression:

$$-d[BH_4^-]/dt = k_d\,[BH_4^-] \quad \text{.....[1]}$$

Where $[BH_4^-]$ is the concentration of borohydride at time t and k_d is the rate constant for decomposition at the experimental condition. This proposed rate of degradation is applicable under the assumption that the amount of boric acid formed (any intermediate in the process) has no effect on the reaction. This is because the total reducing power determination cannot account for the individual steps in the hydrolysis when the total concentration of intermediates is less than a few percent of the hydroborate concentration (Gardiner and Collat, 1965). Hence, the concern about a true estimation of the rate of degradation resulting from the forming of intermediate species BH_3OH^-, $BH_2(OH)_2^-$ and $BH(OH)_3^-$ in the hydroborate solution undergoing hydrolysis, which has been reported by these investigators (Goubeau and Kallfass, 1959 and Mikheeva, et al., 1954), can be disregarded. Evidently, Mesmer and Jolly, (1961) estimated the rate of degradation the same way as the investigation in this study because their

explanation that all of their " first-order" plots (log reducing power vs time), some of which were carried as far as 90 % reduction, gave straight lines, with no suggestion of the formation of intermediates in amounts greater than 5% of the hydroborate initially present (Mesmer and Jolly, 1961).

2.6 Sodium boro [^{3}H] hydride

Sodium boro [^{3}H] hydride is a reducing agent that reacts with cellulose reducing ends where the reaction proceeds stoichiometrically to introduce radioactivity to C-1 position at the reducing ends of cellulose molecule. The assay is based on the measurement of reducing group, which is reduced with sodium boro [^{3}H] hydride to the equivalent alcohol with the incorporation of a tritium atom from the borotritide into the final product. This simple quantitative estimation of reducing groups assay has been reported as a specific and sensitive assay (Conrad et al., 1966; Chirico and Brown, 1987; Bhat et al., 1990; Takeda et al., 1992; Richard and Whelan, 1973). Comparatively few studies applied the tritium labeling techniques to assess the enzymatic catalysis of insoluble celluloses (Chawla et al., 1985; Sild et al., 1998; Sinnott, 1998; Sinnott and Sweilem, 1998) while several investigators adopted this method to use with the oligosaccharides and polysaccharides (Conrad et al., 1966; Richard and Whelan, 1973; Kratky and Biely, 1980; Biely et al., 1981a,b; Chirico and Brown, 1987; Bhat et al., 1990; Takeda et al., 1992; Vrsanská and Biely, 1992; Hayashi et al., 1994).

2.7 Mathematical approach of the simulation program (DYNAFIT)

The DYNAFIT computer program was developed for fitting either the initial velocities or the time course of enzyme reactions to an arbitrary molecular mechanism represented symbolically by a set of chemical equations (Kuzmic, 1996). The explanation of the mathematical theory in the program is according Kuzmic, (1996).

Computation of reaction progress curves:

A modification of the Livermore solver of ordinary differential equation (LSODE (Hindmarsh, 1983)) was employed to compute progress curves. The backward differentiation formula with full analytical Jacobian was used, along with stringent error tolerances (absolute error 10^{-20} M, relative error 10^{-8}). Changes in LSODE, inspired by the design of the differential-algebraic solver DASSL (Petzold, 1983), were introduced to prevent negative concentrations from arising during the computation.

Least-square regression:

A validation of the Levenberg-Marquardt algorithm due to Reich (Reich, 1992) was further modified, to allow optional restarts whenever the weighted sum of square derivations increased. Restart attempts occur when the algorithm reaches a minimum on the least-squares hypersurface (Seber, 1989). In some cases, these restarts guide the Levenberg-Marquardt-Reich minimization out of

a shallow false minimum. The following physical quantities can be treated as adjustable parameters: (i) rate constants, (ii) analytic concentration of reactants, (iii) molar response coefficients (e.g., the molar absorbance coefficients in spectrophotometry), and (iv) the instrumental offset (e.g. absorbance at Time 0). Some authors have used a rational power function (Mannervik, 1982) to describe the experimental variances; we use instead a cubic polynomial. *Numerical tests of goodness of fit*:

Seven numerical tests are used to diagnose the goodness of fit to a given model.

(1) The incomplete gamma distribution (Press *et al.*, 1992) measures the probability that the reduced χ^2 (Bevington, 1969) could arise by pure chance.

(2) The average deviation measures the systematic bias of the fit.

(3) The randomness in the runs of the same signs of residuals (Rawlings, 1988) is expressed as a probability that any given run of signs is entirely random.

(4) The continuous criterion for the goodness of fit (Rayner and Best, 1989) is a pass-fail test, at the 5% confidence level, that the residuals of fit come from the normal distribution.

(5) The Kolmogorov-Smirnov statistic (Press et al., 1992) also measures the probability that the residuals follow the normal or Gaussian distribution.

(6) The Durbin-Watson statistic (Seber and Wild, 1989 and Draper and Smith, 1981) measures the probability that the residuals of fit might be serially correlated, which indicates a lack of fit.

(7) The Tukey statistics $T_{1,1}$ and $T_{1,2}$ (Draper and Smith, 1981) diagnose various types of systematic misfit.

Graphical tests of goodness of fit:

Five graphical techniques were used to diagnose lack of fit, manifested by nonrandom patterns in the residuals.

(1) For a successful fit, the residuals plotted against the independent variable should be symmetrically distributed about the horizontal axis (Mannervik, 1981).

(2) Even more expressive are plots of residuals against the dependent variable (e.g. absorbance or velocity) (Rawling, 1988; Mannervik, 1981).

(3) The histogram of standardized residuals should have the characteristic bell shape.

(4) The normal plot of standardized ordered residuals (D'Agostino, 1986).

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CHAPTER 3

QUANTITATIVE ANALYSIS OF CELLULOSE REDUCING ENDS

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3.1 ABSTRACT

Methods for the quantification of total and accessible reducing ends on traditional cellulose substrates have been evaluated due to their relevance to enzyme-catalyzed cellulose saccharificaion. For example, quantification of accessible reducing ends is likely to be the most direct measure of substrate concentration for the exo-acting, reducing end preferring, cellobiohydrolases. Two colorimetric assays (dinitrosalicyclic acid (DNS) and bicinchoninic acid assay (BCA)) and a radioisotope approach (NaB^3H_4 labeling) were evaluated for this application. Cellulose substrates included microcrystalline celluloses, bacterial celluloses and filter paper. Estimates of the number of reducing ends per unit mass cellulose were found to be dependent on the assay system (i.e. the DNS and BCA assays gave strikingly different results). DNS-based values were several-fold higher than those obtained using the BCA assay, with fold-differences being substrate specific. Sodium borohydride reduction of celluloses, using cold or radiolabelled reagent under relatively mild conditions, was used to assess the number of surface (solvent accessible) reducing ends. Results indicate that 30 to 40% of the reducing ends on traditional cellulose substrates are not solvent accessible, i.e. they are buried in the interior of cellulose structures and thus not available to exo-acting enzymes.

Index Entries: Cellulose, cellobiohydrolase, reducing sugar assays, insoluble reducing ends, solvent accessible reducing ends.

3.2 INTRODUCTION

Typical kinetic analyses of enzyme-substrate systems are based on observed rates of enzyme-catalyzed reactions over a range of enzyme and substrate concentrations. Analyses of this type are commonplace for soluble enzyme-soluble substrate systems in which measures of substrate and enzyme concentrations are rather straightforward. However, the biological world is replete with soluble enzyme-insoluble substrate systems for which measurements of substrate concentration can be rather ambiguous. Systems of this type include lipases, proteases and many of the glycosyl hydrolases that act on polysaccharides. In this paper we address the concern of how to best measure the substrate concentration in cellulase-cellulose systems. More specifically, we focus on the substrate parameters that are of most relevance to the activity of exo-acting cellulases, hereafter referred to as cellobiohydrolases (CBH).

Fungal cellulase enzyme systems capable of efficiently catalyzing the hydrolytic degradation of crystalline cellulose are typically composed of endo-acting cellulases (EGs), exo-acting cellulases (CBHs) and at least one cellobiase (1-6). The CBHs are typically the predominant enzymes, on a mole fraction basis, in such systems (7). Consequently, the CBHs have been the focus of many studies (8). The 3-D structure of prototypical CBHs are known (9,10,11,12) and their specificities are, in general, well characterized (13,14). However, mechanism-based kinetic analyses of CBH-catalyzed cellulose saccharification are rather limited (15,16). Studies of this latter type are particularly difficult due to the

inherent complexity of native cellulose substrates.

A fundamental dilemma that must be addressed when analyzing CBH-cellulose reaction mixtures is how to express substrate concentrations. Substrate concentrations for cellulase-cellulose reaction mixtures in general are most commonly expressed in units of mass (such as mg cellulose per reaction mixture) or enzyme accessible surface area (17). The latter values are typically obtained from solute exclusion experiments (17-19). Expressing substrate concentrations in terms of surface area is theoretically appealing in that it seems to better represent the amount of substrate actually available to the enzymes (20,21). The same reasoning suggests that it would be beneficial, at least for some analyses, if measures of substrate concentration for reaction mixtures containing only exo-acting cellulases were based on cellulose chain ends.

This paper addresses the issue of determining substrate concentrations based on cellulose chain-ends. Typical insoluble cellulose substrates were evaluated. The study focuses on the analysis of reducing ends, rather than non-reducing ends, due to the relative ease with which reducing ends can be detected and due to their importance with respect to reducing end-specific CBHs. The results are expected to be of relevance to those considering factors that dictate rates of CBH activity on typical cellulose substrates.

3.3 MATERIALS AND METHODS

3.3.1 *Celluloses Substrate Preparation*

Microcrystalline cellulose (MCC) was obtained commercially (Avicel, PH 101, FMC, Philadelphia, PA). Amorphous microcrystalline cellulose (AMCC) was produced from MCC by the method of Isogai and Atalla (22) using an SO_2-diethylamine-DMSO solvent for cellulose dissolution. Phosphoric acid swollen cellulose (PSC) was prepared according to Ståhlberg et al. (23). Bacterial microcrystalline cellulose (BMCC) was prepared from cultures of *Acetobacter xylinum* (ATCC #23769). Cultures were incubated in nutrient medium-containing trays for 10 days at 30 ^{0}C without shaking (24). Surface layers of cellulose were harvested and purified by the method of Gilkes et al., (25). Amorphous bacterial microcrystalline cellulose (ABMCC) was prepared by first soaking BMCC in DMSO for 2 hours, followed by centrifugation and then 2 additional washes with DMSO. The BMCC was then treated as in the preparation of amorphous MCC; first being dissolved in the SO_2-diethylamine-DMSO solvent (22) and then precipitated, and subsequently washed, with cold water. Filter Paper was obtained commercially (Whatman No.# 1,Whatman International Ltd, Maidstone, England). Filter paper was added to reaction mixtures as 7mm diameter discs, the average weight of which was 3.5 mg. The AMCC, BMCC, and ABMCC preparations were kept in water containing 0.02% sodium azide until used. Amorphous celluloses, including PSC, were prepared on a minimum of two occasions to insure reproducibility.

3.3.2 *Colorimetric Reducing End Assays*

All samples were analyzed in triplicate on at least two separate occasions.

3.4.2.1 Soluble phase reducing groups. The dinitrosalicylic acid assays (DNS and DNS_{sg}) were performed as described by Ghose (26). A finite amount of reducing sugar is consumed during the course of the traditional DNS assay. Hence, for some applications it is helpful to supplement traditional reaction mixtures (and blanks) with a known amount of glucose. Assays including "supplemental glucose", 100 μg, are herein referred to as DNS_{sg}. The Nelson-Somogyi copper-based assays were performed as described by Nelson (27) and Robyt and Whelan (28). The traditional assay results in a final reaction mixture volume, prior to measuring absorbance, of 10 mL. This study also included a series of assays for which final reaction mixture volumes were 5 mL (results corresponding to this latter approach are herein referred to as $Nelson_{5mL}$). The *p*-hydroxybenzoic acid assay was done according to Lever (29). The assay based on bicinchoninic acid chelation of cuprous ions (BCA) was conducted as described by Garcia *et al.* (30).

3.4.2.2 Insoluble phase reducing groups. The "BCA" assay, as applied to the quantification of insoluble reducing ends, was taken from Johnston *et al.* (31). One mL of BCA "working reagent" (prepared as in Garcia *et al.*, (30) was added to glass tubes containing one mL of test cellulose suspension. Tubes were mixed, capped with glass marbles, and incubated at 80^0C for 30 min. Tubes were then cooled to room temperature by standing in water, mixed, transferred to two mL microcentrifuge tubes, and the cellulose pelleted by centrifugation at 3,000 rpm for

5 min. The absorbance, at 560 nm, of the cellulose-free supernatants was then determined. nMoles of reducing groups per mg cellulose were calculated from measured color yields for standard glucose samples (calibration curve from 0-55 nmoles glucose per mL) treated per the aforementioned protocol.

The "DNS" assay, as applied to the quantification of insoluble reducing ends, was taken from Irwin *et al.* (32). DNS reagent, 1 mL, was added to 0.4 mL cellulose suspension containing 200 nmoles cellobiose (cellobiose addition being analogous to using supplemental glucose in the DNSsg assay, as discussed above). Reaction mixtures were then mixed, capped with glass marbles, and incubated in boiling water for 15 min. Following heating, tubes were cooled to room temperature, again mixed, transferred to two mL microcentrifuge tubes, and centrifuged at 3,000 rpm for 5 min. The absorbance of the resulting cellulose-free supernatants was measured at 600 nm. nMoles of reducing groups per mg cellulose were calculated from measured color yields for standard cellobiose solutions (calibration curves ranged from 0-800 nmoles).

3.3.3 *Solvent Accessible Reducing Ends*

All samples were analyzed in triplicate on at least two separate occasions. Sodium borohydride solutions were prepared just prior to initiating experiments.

3.4.3.1 Sodium borohydride reduction. The "mild" $NaBH_4$ reduction was done at 22°C (room temperature) and pH 8. The slightly alkaline conditions were necessary to maintain reasonable stability for the $NaBH_4$ reagent. Sodium borohydride, 0.1 mL of 0.25 M $NaBH_4$ in 0.1 M NaOH, was added to test

solutions containing 1 mg cellulose suspended in 0.785 mL 0.1 M sodium phosphate, pH 8. Reaction mixtures were mixed and allowed to react for up to 90 min at room temperature. Reactions were terminated at selected times by the addition of 20 μL 37% (w/v) HCl. Terminated reaction mixtures were typically allowed to stand for 30 min prior to neutralization by the addition of 95 μL 2 N NaOH. Separate experiments showed that residual sodium borohydride could not be detected following the low-pH thirty-minute incubation period.

3.4.3.2 Sequential sodium borohydride treatments. MCC was used as a representative cellulose. The cellulose was initially reduced as described above. The terminated, neutralized, reaction mixture was then centrifuged at 3,000 rpm for 5 min. and the supernatant removed. To the resulting cellulose pellet was added 0.785 mL reaction buffer and 0.1 mL fresh sodium borohydride (all concentrations as given above). The cellulose was suspended by mixing and the reaction allowed to proceed for another 90 min, at which time the reaction was terminated, neutralized, and the cellulose again separated via centrifugation. The same cycle was repeated a third time. In total, test celluloses received three sequential $NaBH_4$ treatments; each treatment using fresh reagents.

The "mild" reduction protocol (above) was modified to simulate reaction conditions used in the colorimetric assays for reducing ends. This test included three treatments: (a) the "mild" protocol in 0.1 M sodium phosphate, pH 8, and 22°C; (b) the "high pH" treatment in 0.25 M sodium carbonate/bicarbonate, pH 10, and 22°C; and (c) the "high pH/high temperature" treatment in 0.25 M sodium carbonate/bicarbonate, pH 10, and 80°C. As above, reactions were initiated by

adding 0.1 mL of 0.25 M $NaBH_4$ in 0.1 M NaOH to 0.785 mL cellulose suspension (containing 1 mg cellulose). Reactions were allowed to go for 90 min. The cellulose was then separated from the soluble phase, as described above, and the treatment repeated. Samples received a maximum of five sequential $NaBH_4$-treatments (up to 450 min). Following the last treatment, reactions were terminated by the addition of HCl and subsequently neutralized with NaOH.

3.3.3.3 Sodium borotritiide reduction. All reductions and subsequent treatments up to scintillation counting were done in a fume hood. Reaction mixtures contained 0.1 to 1.0 mg cellulose per reaction mixture, with the exception that filter paper ranged from 3.5 to 17.5 mg. Reactions were initiated in 20 mL scintillation vials by the addition of 0.1 mL reducing agent, containing 50 nmoles NaB^3H_4 (specific activity 370 mCi/mmole; ICN Biomedicals, Irvine, CA) mixed with 25 μmoles $NaBH_4$ (final specific activity 0.738 Ci/mole) in 0.1 N NaOH, to 0.88 mL cellulose suspension in 0.1 M sodium phosphate, pH 8. Initiated reactions were mixed and the reaction allowed to proceed for the desired time at 22 ^{0}C. Reactions were terminated by the addition of 20 μL 37% HCl. The acidified reaction mixture was allowed to stand open for a minimum of 30 min. Reaction mixtures were then evaporated to dryness on a hot plate at 50 ^{0}C (approximately 1 h). The evaporation step was necessary to drive off residual labeled $^3H_2$ gas (33). The solid residue remaining was then resuspended in 1 ml phosphate buffer and to that suspension was added 10 mL scintillation cocktail (Scintisafe Gel, Fisher Scientific, Pittsburgh, PA). Samples were counted in a Beckman LS 6500 Scintillation System (Beckman Instrument, Inc., Fullerton, CA)

at 20 min per vial. Blanks containing no reducing sugar and "zero-time" samples to which the acid was added prior to the addition of reducing agent were included in all experiments. Corrected counts per minute were converted to μmoles reducing ends per gram cellulose using calibration curves, 0-40 nmoles, with glucose as the calibration standard (tests showed that results based on cellobiose as the calibration standard gave equivalent results). As for previous experiments, all samples were done in triplicate on a minimum of two occasions.

3.3.3.4 Quantification of reducing ends on $NaBH_4$-treated celluloses.

Reduced and neutralized cellulose preparations were assayed using the BCA assay as described above for insoluble reducing ends using glucose as the calibration standard.

3.4 RESULTS AND DISCUSSION

3.4.1 Soluble Phase Reducing Ends

The present study was initiated in response to the desire to know the number of chain ends available to exo-acting enzymes in typical cellulose/cellulase reaction mixtures. Several laboratories have published data of relevance to this question. In each case the number of reducing ends associated with a particular cellulose preparation was estimated by adapting a colorimetric reducing sugar assay traditionally used with aqueous solutions. Colorimetric assays adapted in this way include those based on dinitrosalicylic acid (DNS; Irwin et al, (32), *p*-hydroxybenzoic acid (PAHBAH; Boraston et al., (34)), Cu-arsenomolybdate (Nelson-Somogyi; Ståhlberg et al., (23)) and Cu-bicinchoninic acid (BCA; Johnston et al. (31)). Published studies give little indication of how results obtained with the different assays compare, making assay selection and data comparisons difficult. Thus, an objective of the present study was to provide such information, along with providing estimates of the number of reducing ends associated with traditional cellulose substrates.

Initial experiments were designed to obtain analytical parameters for common colorimetric reducing sugar assays when used to quantify soluble reducing-sugars generated as a consequence of cellulose saccharification. The assays were run as published, with the exception of the more concentrated Nelson (5 mL) assay. The results are presented in Tables 3.1, 3.2 and 3.3.

Table 3.1. Detection limit, linear range and working range for the dinitrosalicyclic acid (DNS), modified dinitrosalicylic acid (DNS_{sg}), Nelson-Somogyi (Nelson 10 mL), modified Nelson-Somogyi (Nelson 5 mL), ρ-hydroxybenzoic acid (PAHBAH) and bicinchoninic acid assay (BCA) assays.

Assay	Detection limit (μg glu)	Associated standard error	Linear range upper limit (μg glu)	Working range[b] (μg glu)
DNS	55	0.058	>4000	80-257
DNS_{sg}	1.9	0.157	>4000	25-187
Nelson 10 mL	1.1	0.050	~400	22-170
Nelosn 5 mL	0.66	0.024	~200	12-86
PAHBAH	0.22	0.024	>20	2-16
BCA	0.12	0.007	~10	1-8

[a] The PAHBAH calibration curve is not linear over a wide range, but for small ranges appears linear (with slope changes). The range here reported was used for determination of sensitivity and detection limit.
[b] Based on linear regression parameters, slope and intercept.

Table 3.2. Calibration sensitivity, analytical sensitivity and standard errors for the dinitrosalicylic acid (DNS), modified dinitrosalicylic acid (DNS_{sg}), Nelson-Somogyi, modified Nelson-Somogyi, ρ-hydroxybenzoic acid (PAHBAH) and bicinchoninic acid assay (BCA) assays.

Assay	Calibration sensitivity (Abs/μg glu)		Analytical sensitivity[a] (Δ Abs/μg glu)			
	Calibration sensitivity x 10^{-3}	Associated std. error x 10^{-3}	Analytical sensitivity	Associated std. Error x 10^{-2}	Std. Dev[b] x 10^{-3}	Reference conc [c]
DNS	3.95	0.042	1.780	1.91	2.22	90
DNS_{sg}	4.31	0.067	0.896	1.60	4.81	90
Nelson10ml	4.73	0.023	0.783	1.12	6.04	90
Nelson 5 ml	9.37	0.030	1.770	3.03	5.33	50
PAHBAH	50.40	0.880	3.880	7.38	13.4	12
BCA	106.00	1.070	15.400	40.33	6.87	5

[a] Signal change is defined as absorbance change for the assays.
[b] Pooled standard deviation from different experiments.
[c] The concentration at which replicate measurement were analyzed to obtain pooled standard deviations.

Table 3.3. Cellodextrin reponses for the dinitrosalicyclic acid (DNS), Nelson-Somogyi (Nelson) and bicinchoninic acid (BCA) assays.

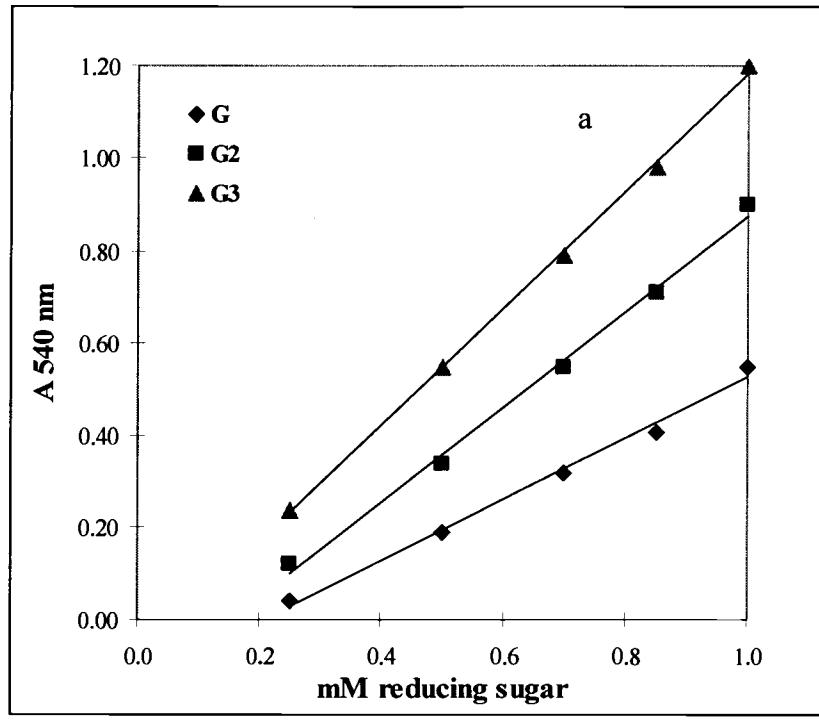
Assay*	Similar response of equimolar amounts of G, G2, G3	G		G2		G3	
		m_{ratio}[a]	b_{ratio}[b]	m_{ratio}	b_{ratio}	m_{ratio}	b_{ratio}
DNS	No	1.0	1.0	1.5	1.0	1.8	0.9
Nelson	Yes	1.0	1.0	1.0	1.0	1.0	1.0
BCA	Yes	1.0	1.0	1.0	1.0	1.0	1.0

*Not determined for ρ-hydroxybenzoic acid assay (PAHBAH)

[a] Calculated as the ratio of the calibration sensitivities (or slope m) of the different cellodextrin standard curves to the glucose standard curve. Thus, a ratio of 1 is expected, theoretically, for a true reducing sugar assay, which has the same molar color yield for a series of saccharides.

[b] Calculated as the ratio of the intercepts of the different cellodextrin standard curves to the glucose standard curve. Thus, a ratio of 1 indicates agreement in the intercepts between the cellodextrin and the glucose calibration curves.

Detection limits for all of the assays are near 1 μg (although values in this range differ by nearly 16-fold), with the exception of the traditional DNS assay, which is known to consume a fixed amount of analyte (26). The combined data provides a ready means by which to compare and select appropriate assays for application in cellulase-catalyzed cellulose saccharification experiments. Products in such experiments are expected to include glucose, cellobiose and, potentially, some cellooligosaccharides. Optimum reducing sugar assays would have equivalent molar color yields for these soluble products. As shown in Table 3.3, this optimum situation only applies to the two copper based assays (Nelson, BCA). Due to their importance with respect to the analysis of insoluble cellulose (see below), calibration curves reflecting the molar color yields for the DNS and BCA assays are presented in Figure 3.1.



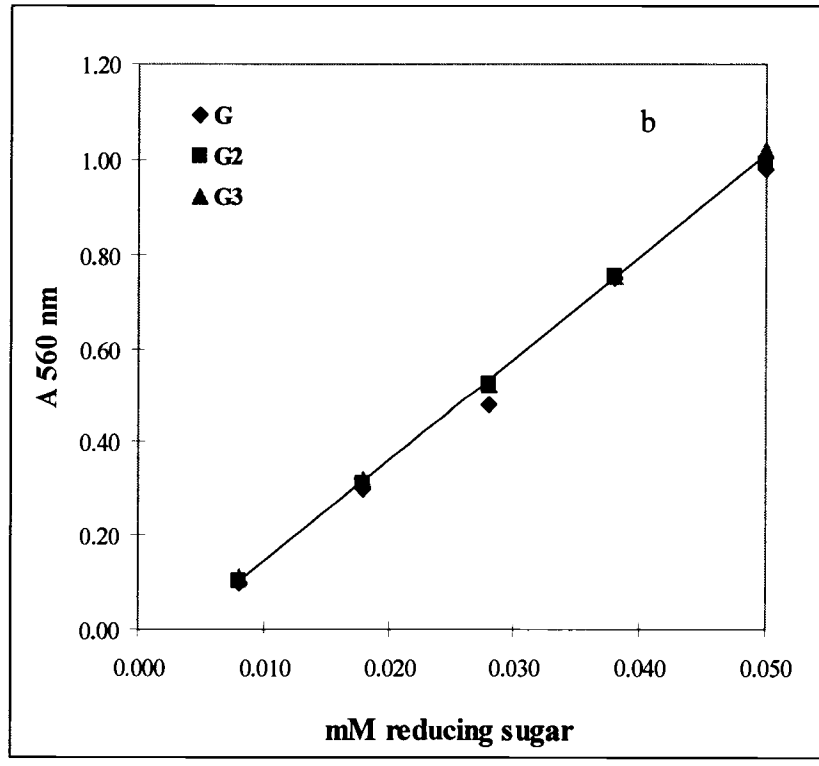


Figure 3.1. Absorbance response of equimolar amounts of soluble cellulose saccharification products, glucose (G), cellobiose (G2) and cellotriose (G3) by (a) the DNS assay, (b) the BCA assay.

3.4.2 Insoluble Phase Reducing Ends

The DNS- and BCA-based assays are frequently used for the quantification of insoluble reducing ends in cellulose/cellulase systems (31, 32, 35-40). The relative merit of using the assays for this determination was evaluated by applying the assays, as published, to traditional cellulose substrates (Table 3.4). The results from both assays had relative errors of approximately 3%. Figures 3.2 through 3.5, from which the values of Table 3.4 were calculated, illustrate the linear relationship between mass of cellulose and number of reducing ends for both assays. The linear relationships suggest that either method may be used to determine relative numbers of reducing ends for a given cellulose preparation (for example, relative values for MCC-derived substrates). The similar values obtained for MCC, AMCC, and PSC, and for BMCC and ABMCC, further support this conclusion. The similarity in the results for the microcrystalline celluloses and the corresponding amorphous preparations demonstrates that the solid-state structure of the cellulose, typically expressed in terms of relative crystallinity (41), had little effect on color yield. This conclusion is based on the premise that the method used for the preparation of the amorphous celluloses did not result in significant depolymerization and concomitant production of new reducing ends (as shown by Isogai and Attala, (22)). The ordinates of the BCA-based and DNS-based plots of Figures 3.2-3.5 are in units of glucose and cellobiose equivalents, respectively, due to the use of the different sugars for the preparation of standard curves (see "Methods").

Table 3.4. Reducing ends per amount cellulose as determined by BCA and DNS assays.

Cellulose preparation[a]	Amount (mg)	Reducing ends (μmoles/g) (Standard Error)	
		BCA[b]	DNS[c]
MCC	0.1 –1.0	32.13 (0.38)	188.36 (3.27)
AMCC	0.1 –1.0	30.40 (0.68)	197.39 (8.80)
PSC	0.1 –1.0	30.02 (0.77)	179.27 (5.80)
BMCC	0.1 –1.0	18.26 (0.25)	60.98 (1.00)
ABMCC	0.1 –1.0	20.51 (1.93)	67.73 (1.12)
FP	3.5-17.50	1.25 (0.03)	10.50 (0.43)

[a] Microcrystalline cellulose (MCC), amorphous microcrystalline cellulose (AMCC), phosphoric acid swollen cellulose (PSC), bacterial microcrystalline cellulose (BMCC), amorphous bacterial microcrystalline cellulose (ABMCC), filter paper (FP).
[b]Glucose equivalents.
[c]Cellobiose equivalents.

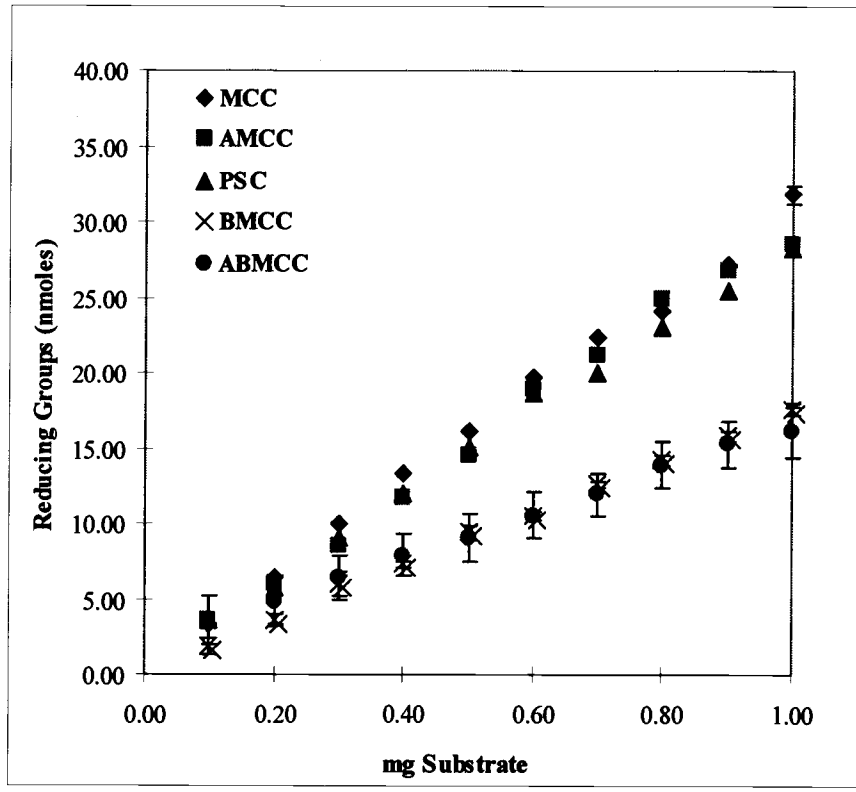


Figure 3.2. Reducing groups per amount cellulose determined by BCA assay for microcrystalline cellulose (MCC, ◆), amorphous microcrystalline cellulose (AMCC, ■), phosphoric acid swollen cellulose (PSC, ▲), bacterial microcrystalline cellulose (BMCC,×), amorphous bacterial microcrystalline cellulose (ABMCC, ●).Values represent the mean of triplicate determinations; error bars represent ± the standard errors. When not shown, the error bars fall within the symbols.

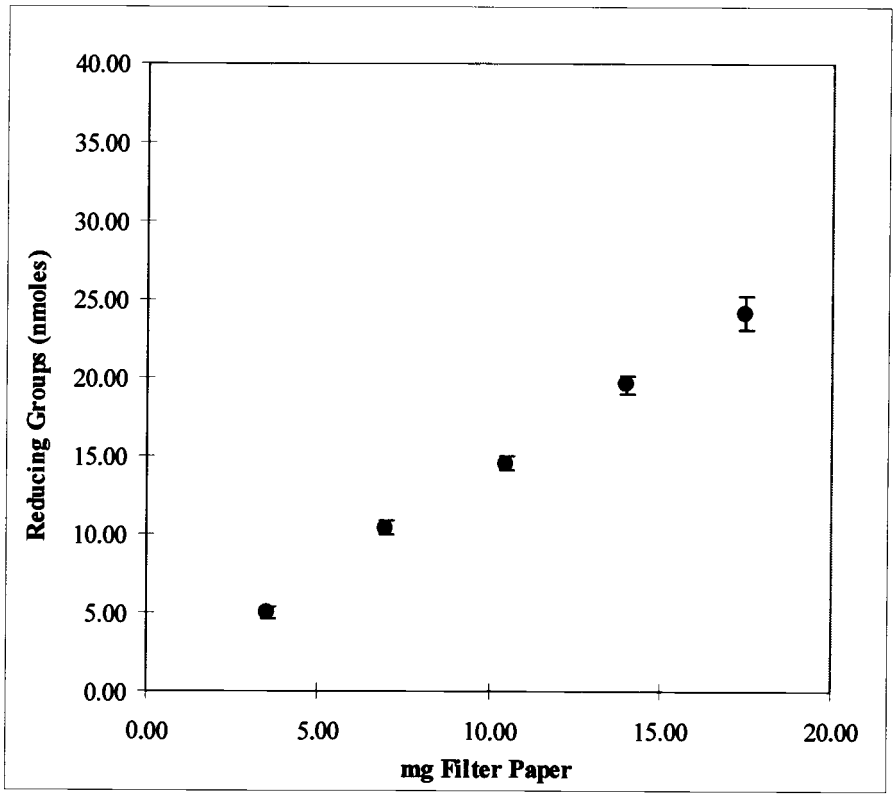


Figure 3.3. Reducing groups per amount filter paper determined by BCA assay. statistical parameters as in Figure 3.2.

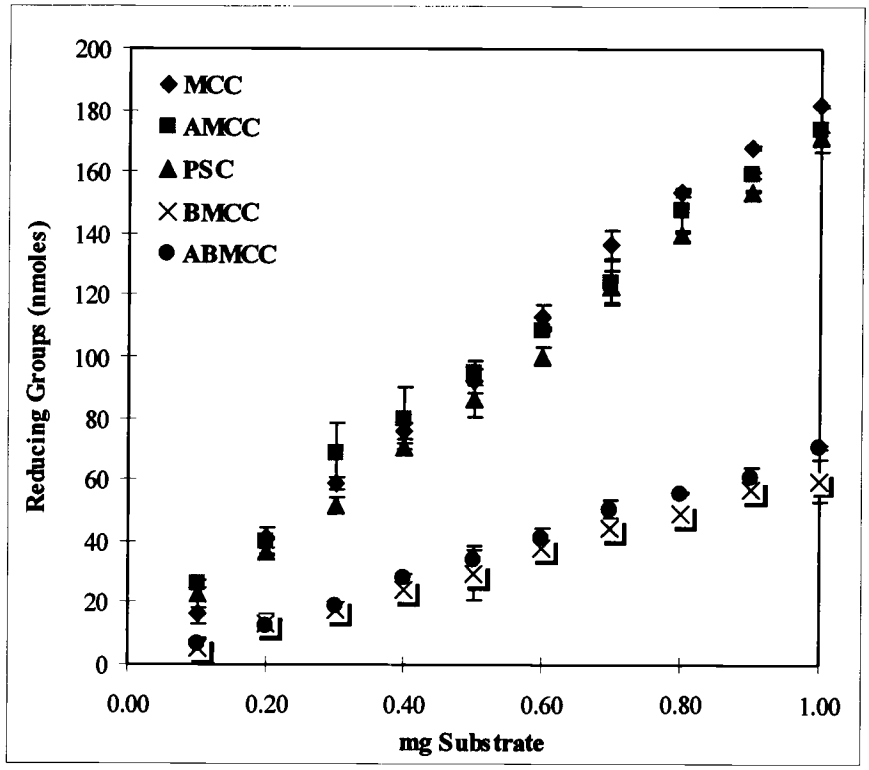


Figure 3.4. Reducing groups per amount cellulose determined by DNS assay for microcrystalline cellulose (MCC, ◆), amorphous microcrystalline cellulose (AMCC, ■), phosphoric acid swollen cellulose (PSC, ▲), bacterial microcrystalline cellulose (BMCC, ×), amorphous bacterial microcrystalline cellulose (ABMCC, ●). Statistical parameters as in Figure 3.2.

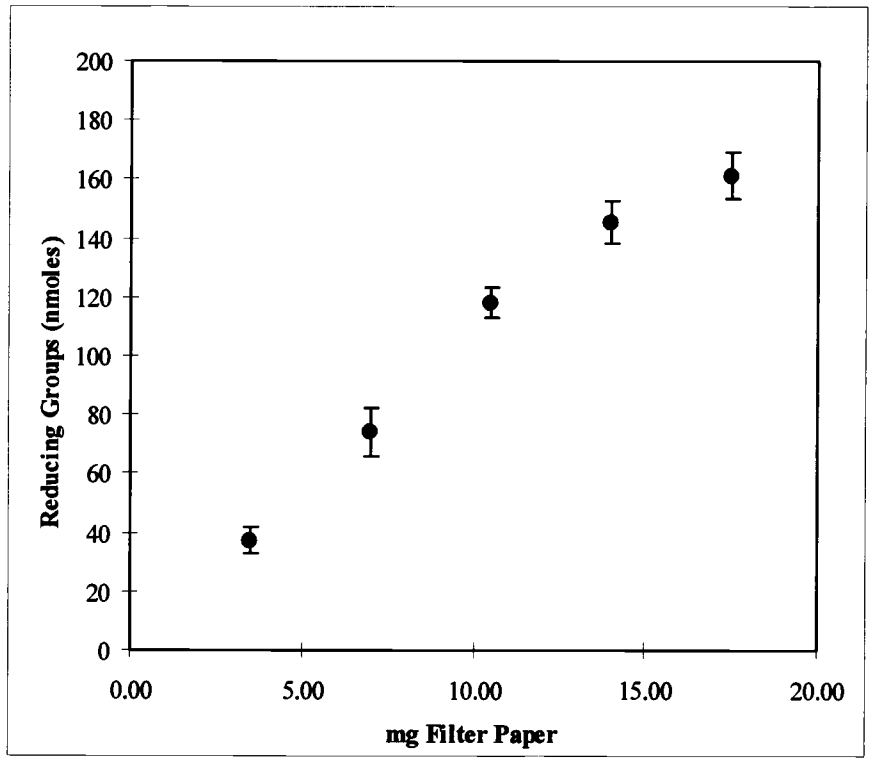


Figure 3.5. Reducing groups per amount filter paper determined. Statistical parameters as in Figure 3.2.

An obvious conclusion from the data of Table 3.4 is that the DNS assay, compared to the BCA assay, consistently gives higher values for equivalent cellulose preparations. The fold difference between the two assays was dependent on the source of the cellulose. Values for the microcrystalline cellulose-based substrates (MCC, PSC, and AMCC) differed approximately 6-fold. Those for filter paper differed approximately 8-fold. The DNS values for the bacterial cellulose-based preparations (BMCC, ABMCC) were just over 3-fold higher than the corresponding BCA values. The high DNS-obtained values, relative to the BCA-obtained values, are likely attributable to over-consumption of the DNS reagent as a result of the generation of reactive side products during the assay (42). The reactive side products being a result of cellulose degradation under the relatively harsh conditions used for the DNS assay (100°C, pH 13, 15 min). The conditions for the BCA assay, by comparison, are relatively mild (80°C, pH 10, 30 min).

It is not clear why the fold-differences between the DNS- and BCA-obtained values vary to the extent they do for the different celluloses. As discussed above, the fold-differences are not likely attributable to differences in the crystallinity of the substrates – or to properties thought to be associated with crystallinity, such as porosity and surface area. The differences appear to be attributable to characteristics originating with the starting material, since MCC-based substrates have similar values, BMCC-based substrates have similar values, etc. This suggests that the substrate-specific fold-differences between the DNS- and BCA-assays are related to the degree of polymerization of the substrates. The average

DP of the filter paper (~1640) is clearly greater than that of the microcrystalline substrates (~220) (43). The average DP for the different MCC- and BMCC-derived substrates is expected to be similar; and yet the corresponding fold-differences for these substrates differ nearly two-fold. This apparent discrepancy may be explained by the DP profiles of the different substrates. Stålbrand *et al.* (44) have shown that although the average DP values for an MCC- and a BMCC-dervied substrate were similar, their DP profiles were significantly different. The BMCC substrate was shown to have a higher mole-fraction of its constituent molecules in the low DP range. As discussed above with relation to Figure 1, the DNS assay is DP sensitive when working with cellooligosaccharides and, presumably, also with cellulose – while the BCA assay is not. Hence, it seems that if the observed substrate-specific fold-differences are a function of the cellulose preparations' DP, as is suspected, then that function is best considered in terms of the overall DP profiles of the substrates rather than simply average DP values.

The BCA-assay results presented here for the MCC substrates are comparable to those obtained by Ståhlberg et al. (23) using the Nelson-Somogyi method. They reported ~37 μmoles reducing ends/g cellulose for their microcrystalline (MCC) and phosphoric acid regenerated cellulose (PSC) preparations. Similarly, Johnston et al. (31), using the BCA assay, reported 30 to 35 μmoles reducing ends/g phosphoric acid regenerated cellulose. (The similar results for the Nelson-Somogyi and the BCA assays are expected since both are based on quantification of the cupric-to-cuprous reduction.) The DNS-results for the FP substrate are in general agreement with that reported by Irwin et al. (32). The similarity of the

values obtained in our laboratory with those scattered in the literature indicates that the inter-laboratory reproducibility of the assays, at least when applied to substrates for which we can make a comparison, is good.

3.4.3.Insoluble Phase/Solvent Accessible Reducing Ends

Values obtained using the DNS and BCA assays are presumably a function of the total number of reducing ends per unit weight cellulose. This is an important number for many purposes, but it may not be the most relevant value when considering enzyme-accessible reducing ends. The latter value is important when asking questions related to effective substrate concentrations for reducing end-specific exo-acting cellulases, a prototypical enzyme in this category being *Trichoderma reesei* cellobiohydrolase I (10). An approach to getting these enzyme-applicable values is to determine the fraction of reducing ends exposed to solvent under conditions more conducive to enzyme activity. In the DNS and BCA assays the cellulose is in a highly alkaline solution (pH $\geq$ 10) at elevated temperatures (T $\geq$ 80°C). These conditions are conducive to cellulose swelling and thus increase solvent accessibility (45), and they are clearly well outside those compatible with typical enzyme systems. The above suggested "enzyme-applicable" approach was implemented in this work by first reducing the cellulose with $NaBH_4$ under relatively mild conditions ($NaBH_{4,mild}$), and then assaying the $NaBH_{4,mild}$ –treated cellulose for remaining reducing ends (using the BCA assay as described for insoluble cellulose). The number of solvent accessible reducing ends could then be calculated by taking the difference in the number of reducing ends

associated with the $NaBH_{4,mild}$–treated and the untreated substrates. Results from such assays applied to the test celluloses are presented in Table 3.5. While these values are not optimum for assessing the number of CBH-accessible reducing ends, the optimum would be an "enzyme accessible" value rather than a "solvent accessible" value, they are a significant improvement over the "total" reducing end values obtained by traditional colorimetric assays. A second assay was developed for this study in order to check the validity of the values obtained using the combined $NaBH_4$-BCA assay. In this second approach the initial reduction was done using sodium borotritiide and then the number of solvent accessible reducing ends determined by the extent of tritium incorporation into cellulose (see "Methods" for details). It can be seen that results obtained using this isotope uptake approach are in general agreement with those obtained using the $NaBH_4$-BCA colorimetric approach (Table 3.5). The similarity in the values lends credibility to the numbers obtained from either assay. A comparison of the relative accessibility of the different substrate's reducing ends, as determined by the combined $NaBH_4$–BCA assay, is also included in Table 3.5. The tabulated values were taken from the plateau region of the progress curves presented in Figure 3.6. The time-courses for reduction of the soluble substrates (glucose and cellobiose) show that all reducing ends are readily susceptible to $NaBH_4$ reduction under the chosen "mild" conditions. In contrast, the insoluble substrates all show a minimum of two classes of reducing ends; for simplicity the two classes are heretofore referred to as those that are generally susceptible to

Table 3.5. Solvent accessible reducing ends per amount cellulose as determined by combined $NaBH_4$-BCA and NaB^3H_4-tritium uptake assays.

Cellulose preparation[a]	Solvent accessible reducing ends (μmoles/g)		Solvent accessibility (percent of total)[b,c]
	$NaBH_4$- BCA[b]	NaB^3H_4-tritium uptake[b]	
Glucose	-	-	100
Cellobiose	-	-	100
MCC	17.12 ± 0.55	13.85 ± 0.50	60.00 ± 3.59
AMCC	23.63 ± 0.28	22.50 ± 0.64	72.37 ± 0.66
PSC	24.08 ± 0.99	20.93 ± 0.73	72.36 ± 2.03
BMCC	8.64 ± 0.46	14.55 ± 0.26	45.32 ± 2.34
ABMCC	10.29 ± 0.21	15.71± 0.73	49.04 ± 1.52
FP	0.773 ± 0.06	1.33 ± 0.08	59.22 ± 4.49

[a] as in Figure 3.4
[b] ± standard error.
[c] Calculated from BCA obtained values in Table 3.4 and $NaBH_4$-BCA values of Table 3.5.

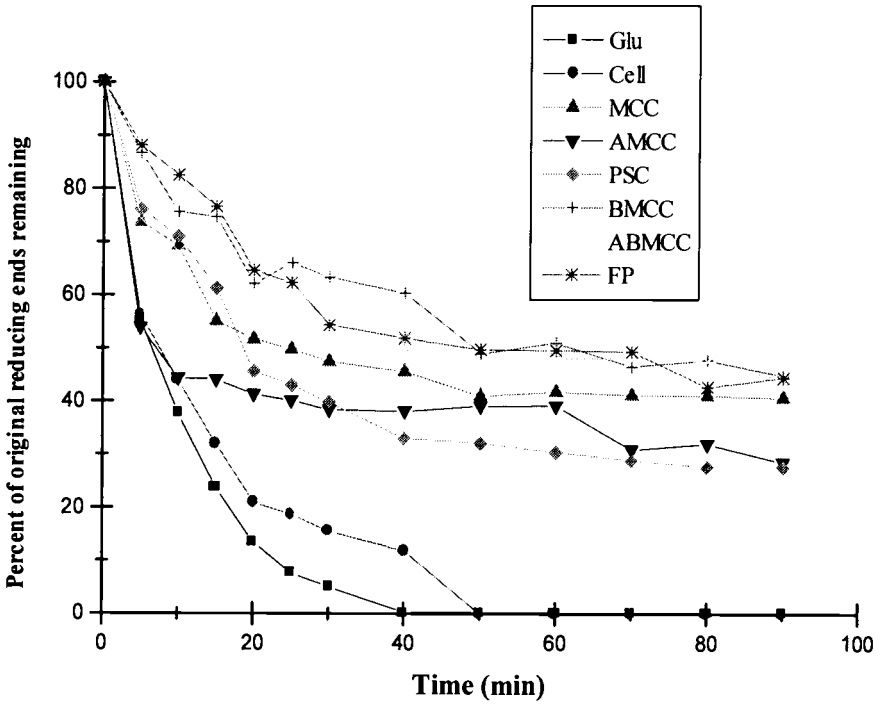


Figure 3.6. Time-courses of celluloses reduction by $NaBH_4$ at pH 8, 22 ^{0}C. Unreacted reducing ends determined by BCA assay. Celluloses as in Table 3.4.

$NaBH_4$ reduction and those that are not. The percents of reducing ends susceptible to $NaBH_4$ reduction were found to be similar for all of the celluloses, ranging from 45% (BMCC) to 72% (AMCC, PSC).

A small increase (~ 20 %) in solvent-accessible reducing ends was observed for the amorphous MCC-based preparations (AMCC and PSC) compared with the MCC starting material. This result can be rationalized based on the documented enhanced reactivity, presumably corresponding to a more open structure, of amorphous versus crystalline cellulose (21, 46). The fact that 30% of the reducing ends of the AMCC preparation are not susceptible to $NaBH_4$ reduction shows that, even with amorphous substrates, a significant fraction of the "total" reducing ends (as measured by traditional colorimetric assays) are not available as sites for initiation of reducing end-specific cellobiohydrolase saccharification. The two BMCC based substrates had essentially the same percentage of total reducing ends unavailable for reaction with $NaBH_4$. This result was not anticipated based on the presumed higher surface area associated with amorphous, versus crystalline, substrates. The somewhat unexpected lower accessibility of the reducing ends in the amorphous preparation (versus BMCC) suggests that amorphous aggregates formed during ABMCC preparation are not particularly solvent ($NaBH_4$) permeable; interestingly, they are less so than those in the analogous AMCC substrate preparation. The difference in the aggregation states of the two amorphous cellulose preparations (ABMCC and AMCC), detected, as a difference in the percent of $NaBH_4$-susceptible reducing ends, is likely a result of complex aggregation events that occur during cellulose regeneration. The percent of

reducing ends accessible in the filter paper substrate was in line with that observed for the MCC and BMCC substrates.

Several experiments were conducted to evaluate the effect of reaction conditions on $NaBH_4$-accessible reducing ends. An initial concern in the pH 8/22°C reductions was that the observed plateaus in reduction time courses (Figure 3.6) were the result of reagent decomposition. If this were the case, then the observed plateaus would reflect a lack of reagent rather than inaccessible reducing groups. The experiment depicted in Figure 3.7 illustrates that this is not the case. In this experiment the MCC preparation went through the mild $NaBH_4$ treatment, as did the preparations depicted in Figure 3.6, but at the completion of the typical time course the cellulose was washed and then again treated with fresh $NaBH_4$ reagent. Subsequent to this second $NaBH_4$ treatment the cellulose was again washed and $NaBH_4$ treated. The consequence of the three sequential $NaBH_4$ treatments was only a minimal decrease in residual BCA-detectable reducing ends when compared to the celluloses that had received only the single treatment (as in Figure 3.6). This result provides strong support for the notion that the plateaus observed in Figure 6 are due to the $NaBH_4$-inaccessible nature of a significant fraction of the reducing ends associated with these cellulose preparations.

Time courses of $NaBH_4$-reduction of the MCC substrate under different pH/temperature conditions were compared to test the hypothesis that reaction conditions corresponding to those of the colorimetric assays are consistent with measuring "total" reducing ends (Figure 3.8). Three reaction conditions were compared: (a) the "mild" conditions as employed above, (b) those analogous to the

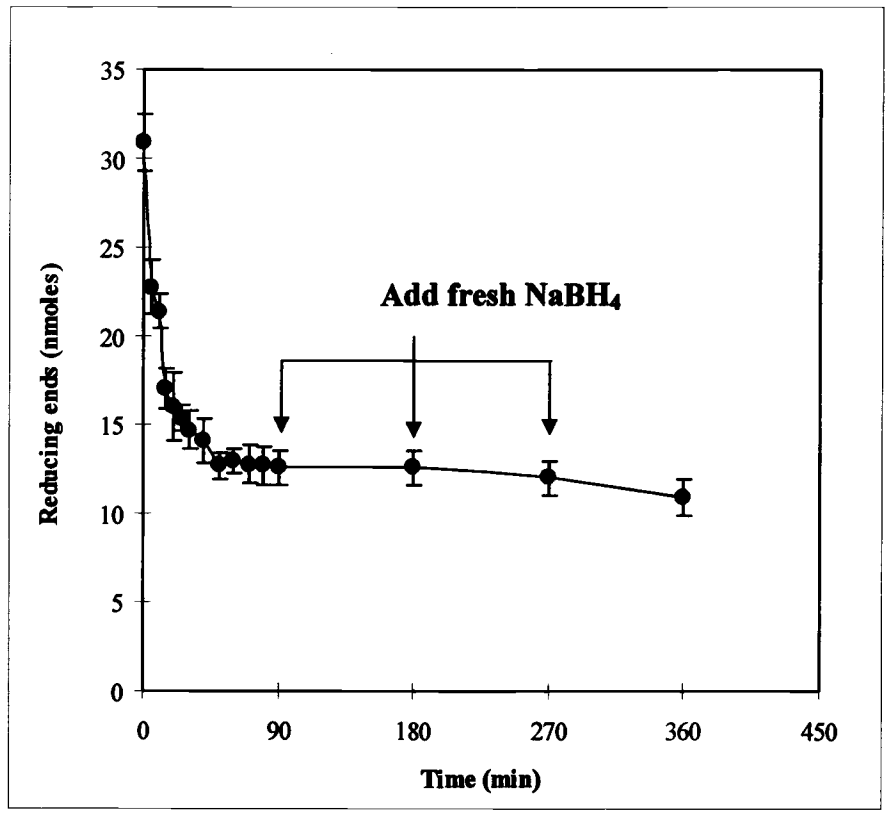


Figure 3.7. Time course of microcrystalline cellulose (MCC) sequential $NaBH_4$ treatments at pH 8, 22 ^{0}C. Unreacted reducing ends determined by BCA assay.

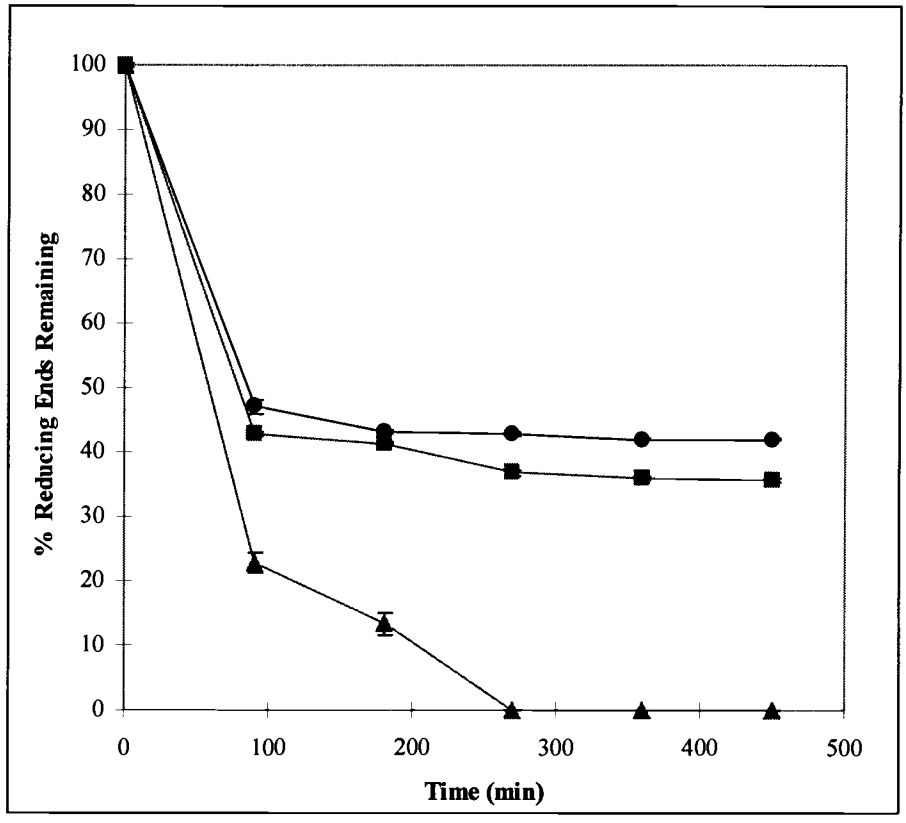


Figure 3.8. Time courses of microcrystalline cellulose (MCC) reduction by $NaBH_4$ at different reaction conditions: pH = 8, T = 22 ^{0}C (●); pH =10, T= 22 ^{0}C (■); pH =10, T= 80 ^{0}C (▲).

BCA assay, *i.e.* pH 10 and 80°C, and (c) conditions of intermediate severity. A plateau in the extent of reduction is evident in the time courses corresponding to the two milder conditions. In contrast, reductions under the more severe conditions resulted in complete depletion of BCA-detectable reducing ends. This result supports the contention that conditions commonly used for traditional colorimetric assays are consistent with measuring "total" numbers of reducing ends. The results from this experiment also demonstrate that the color generated in the BCA assay is dependent on the presence of reducing ends, since no reducing ends could be detected after exhaustive $NaBH_4$ reduction. This was also shown to be the case for color generation in the DNS assay (data not shown). This is significant in that it indicates that potentially color yielding side products generated during the course of reducing sugar assays (as suggested above for the DNS assay) are a result of the presence of reducing ends per se – and not due to side reactions occurring at sites away from the reducing end termini.

3.5 CONCLUSION

The colorimetric assays discussed in this paper are most commonly used in cellulose/cellulase studies to quantify the number of reducing sugars associated with the soluble phase (glucose, cellobiose, low DP cellooligosaccharides). However, they are increasingly being used to determine the number of reducing ends associated with the insoluble phase, cellulose. The combined results from this study show the merits of the different reducing sugar assays when used with

either the soluble or the insoluble substrates (detection limits, sensitivities, etc.) and suggest that the limitations of these assays, with regard to the analysis of celluloses per se, should be considered. The DNS assay tends to overestimate absolute numbers of reducing ends per unit mass cellulose and it appears likely that the degree of overestimation is a function of the cellulose's DP profile. Hence, the BCA assay looks to be the more appropriate assay for obtaining absolute values for cellulose reducing ends. Both the BCA and the DNS assays are shown to relate to the "total" number of cellulose-associated reducing ends. One can obtain reasonable estimates of the absolute number of solvent accessible reducing ends by using either the combined $NaBH_4$/BCA assay or the sodium borotritiide/tritium uptake assay as presented herein. Values obtained with the latter two assays will be particularly relevant in studies concerned with the number of available catalytic sites for exo-acting cellulases and for those studies examining general changes in the surface properties and/or accessibility of cellulose substrates.

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CHAPTER 4

BOROHYDRIDE REACTIVITY OF CELLULOSES REDUCING ENDS

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Submitted in *Carbohydrate Polymer*

4.1 ABSTRACT

A comparative study of the kinetics of sodium borohydride reduction of reducing ends on model celluloses and soluble cellooligosaccharides (COS) was conducted to better understand the nature of reducing ends encountered by reducing end-specific exo-acting cellobiohydrolases. Apparent second order rate constants for the reduction of glucose, COS (degree of polymerization from two to five) and typical microcrystalline and amorphous cellulose substrates were determined. In general, rate constants for the reduction of the cellulose-associated borohydride-accessible reducing ends were similar to those describing the reduction of the COS. Thus, no significant differences were observed between the amorphous celluloses and COS. The reactivity of the microcrystalline cellulose preparation was found to be significantly ($p = 0.0018$) lower (~ 20%) than that of the reference COS. All of the celluloses and COS had significantly lower rates of reaction than glucose ($p<0.0001$). The results indicate that the reagent-accessible reducing ends on typically employed amorphous cellulose substrates behave similar to those of cellooligosaccharides free in solution.

Keywords: cellooligosaccharide; cellulose; reduction; reducing ends; kinetics; sodium borohydride.

4.2 INTRODUCTION

Cell-free fungal enzyme systems capable of efficiently catalyzing the saccharification of cellulose are typically comprised of a mixture of endo- and exo-acting cellulases. The predominant enzymes in these mixtures are usually the exo-cellulases. Exo-cellulase catalysis is dependent on an initial seating of an appropriate cellulose chain end in the enzyme's catalytic site. The appropriate end, be it either the reducing end or the non-reducing end of the cellulose molecule, is dependent on the nature of the enzyme (Davies & Henrissat, 1995, Barr et al., 1996; Nutt et al., 1998; Valjamae et al., 1998; Becker, 2001; Koivula et al., 2002). Those exo-acting cellulases that preferentially act via the reducing end are presumably dependent on acquired mechanisms for interaction with such ends. Interactions of this type will of course be dependent on the complimentary chemistry of the enzyme and the substrate's reducing end. The present study was designed to provide information on the nature of such reducing ends in typically employed cellulose substrates. An important related question is whether or not the reducing ends of soluble cellooligosaccharides adequately represent the reducing ends in typically employed cellulose substrates, since both classes of substrates are used to study exo-cellulase activity (Vrsanska & Biely, 1992; Mattinen et al., 1997; Medve et al., 1998; Sild et al, 1998). The experimental approach was to compare the borohydride-reactivity of a series of cellooligosaccharides (COS) with that of celluloses typically employed in the study of exo-acting cellulases.

"Reactivity" is here based on each of the compounds, all of which have reducing end terminal glucosyl residues, susceptibility to reduction by sodium borohydride.

4.3 MATERIALS AND METHODS

4.3.1 Substrates

Glucose was purchased from Sigma Chemical Co. (St. Louis, MO). Cellooligosaccharides were prepared by acid hydrolysis of the microcrystalline cellulose and subsequent fractionation using stearic acid-treated charcoal-based columns (Miller et al., 1960). Microcrystalline cellulose (Avicel, PH 101) was from FMC (Philidelphia, PA). Bacterial microcrystalline cellulose (BMCC) was prepared from *Acetobacter xylinum* (ATCC #23769). Cultures were incubated in nutrient medium-containing trays for 10 days at 30 ^{0}C without shaking (Hestrin, 1963). Surface layers of cellulose were harvested and purified by the method of Gilkes et al., (1992). Amorphous celluloses were produced from MCC and BMCC by the method of Isogai and Atalla (1991) using a SO_2-diethylamine-dimethylsulfoxide solvent system for cellulose dissolution. Phosphoric acid swollen cellulose (PSC) was prepared from MCC PH-101 according to Ståhlberg et al., (1993). Amorphous and phosphoric acid swollen celluloses were kept in water containing 0.02% sodium azide until used.

4.3.2. Hydrolysis of sodium borohydride

Rates of hydrolytic decomposition of sodium borohydride ($NaBH_4$) were determined as the change in total reducing power of the borohydride containing

solution with time. Reaction conditions were 25 mM sodium borohydride, 100 mM sodium phosphate, pH 8.0, and 22°C. Total reducing power was quantified as the capacity for the cupric to cuprous ion conversion, as measured using the bicinchoninic acid reagent (BCA, Garcia et al., 1993). The time course of the reaction was followed for 600 minutes and experiments were conducted in duplicate on separate occasions. Total reducing power was converted to sodium borohydride concentrations by the use of calibration curves prepared using standard sodium borohydride solutions.

4.3.3 Reduction of glucose, cellooligosaccharides, and celluloses

Reaction mixtures were designed to be 25 mM $NaBH_4$ and 0.033 mM reducing ends (total reaction volume of 1 mL). The molar concentration of reducing ends in the glucose and the cellooligosaccharide solutions was taken as the molar concentration of each compound itself. For the celluloses, the molar concentration of reagent-accessible ($NaBH_4$-accessible) reducing ends was taken as the difference in the total reducing ends per unit weight cellulose before and after exhaustive reduction by sodium borohydride under the given reaction conditions (Kongruang et al., 2003). Reductions were done in 100 mM sodium phosphate, pH 8.0, at 22°C. Time courses for the reaction were determined by monitoring the decrease in reducing ends over a 90-minute reaction period. At predetermined times (0, 5, 10, 15, 20, 25, 30, 40, 50, 60, and 90 min) the reaction was terminated by lowering the pH (w/ 20 μL HCl) to rapidly decompose the borohydride reducing agent. The number of reducing groups remaining in reaction mixtures was then determined by the copper-based bicinchoninic acid method

(Garcia et al., 1993).

4.3.4 Rate of incorporation of sodium boro [^{3}H] hydride

Solid sodium boro [^{3}H] hydride, specific activity 370 mCi/mmol (13.7 GBq/mmol), was obtained from ICN Biomedicals (Irvine, CA). A 0.2505 M NaB^3H_4–working solution (specific activity 0.738 mCi/mmole), in 0.1 M NaOH, was prepared using the commercial NaB^3H_4 preparation combined with cold $NaBH_4$. Reductions were initiated by the addition of 100 μL NaB^3H_4-working solution to a 20 mL scintillation vial containing the reducing carbohydrate suspended in 0.88 mL 0.1 M sodium phosphate, pH 8. Initiated reactions were allowed to proceed at 22°C for specified times and then terminated by the addition of 20 μL 37% HCl. Acidified reaction mixtures were allowed to stand open for a minimum of 30 min. Reaction mixtures were then evaporated to dryness on a hot plate at 50 ^{0}C (approximately 1 h). The evaporation step was necessary to drive off residual labeled $^3H_2$ gas (Conrad et al., 1966). The solid residue remaining was then resuspended in 1 mL phosphate buffer and to that suspension was added 10 mL scintillation cocktail (Scintisafe Gel, Fisher Scientific, Pittsburgh, PA). Samples were counted in a Beckman LS 6500 Scintillation System (Beckman Instrument, Inc., Fullerton, CA) at 20 min per vial. Time courses of isotope uptake were developed using reaction times of 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 120 min. Controls included the treatment of previously $NaBH_4$ reduced celluloses as described above to assess the extent of non-specific isotope incorporation. Experiments were done in triplicate and all reductions and subsequent treatments involving tritium, up to the scintillation counting, were done in a hood.

4.3.5 Curve fitting and statistical analyses

Progress curves of all reactions were analyzed using DYNAFIT, a kinetic simulation program (Kuzmič, 1996). Concentration changes, with time, were fitted assuming the borohydride/carbohydrate reaction obeys second order kinetics. Changes in borohydride composition due to hydrolytic degradation were modeled using first order kinetics. Thus, time courses were obtained by solving an initial value problem described by the following system of differential equations:

$$d[BH_4^-]/dt = -k_d\,[BH_4^-] - k_{app}\,[BH_4^-][G]$$

$$d[B(OH)_4]/dt = k_d[BH_4^-] + k_{app}[BH_4^-][G]$$

$$d[G]/dt = -k_{app}[BH_4^-][G]$$

$$d[GH]/dt = k_{app}[BH_4^-][G]$$

where BH_4^- is borohydride; $B(OH)_4$, borate; G, aldose form of carbohydrate; and GH, alditol form of carbohydrate. Parameter k_d (sec^{-1}) is the first order rate constant describing borohydride decomposition and k_{app} ($M^{-1}sec^{-1}$) the second order rate constant describing the reaction between borohydride and the aldose form of the carbohydrate. A modification of the Livermore solver of ordinary differential equations was used to numerically solve the above equations and compute progress curves. Progress curves, and thus the corresponding rate constants, were optimized by least-squares regression of the calculated and real data (Reich, 1992 & Marquardt, 1993). Statistical differences between best-fit second order rate constants obtained for the reduction of the different test

compounds were determined using multiple regression analyses (extra-sum-of-squares F-test) by SAS software (SAS Institute, Inc., 1990), yielding two-sided "p-values". Significant differences were defined as $p < 0.002$. Cellotriose was used as the prototypical cellooligosaccharide for statistical comparison of rates of reduction among cellooligosaccharides and celluloses.

4.4 RESULTS AND DISCUSSION

Time courses for the reduction of the different test compounds are presented in Figures 4.1a-j. Included in each figure is the corresponding simulated time course generated using the second order rate constants that "best fit" (least-squares optimization, Table 4.1) the real data. The summary of the raw data used for plotting the time course of reduction of each compound is summarized in appendix F. The general agreement between the calculated and real data indicates the second order rate expressions are adequate for such analyses. Rate constants for the reduction of the different cellooligosaccharides were found to be similar, all of them being significantly below the corresponding value for glucose. The relative values of the rate constants for glucose and cellobiose are in general agreement with a previous report showing glucose to be the more reactive (Bragg & Hough, 1957); values for the other cellooligosaccharides are not in the available literature. It is important that no significant differences were detected among the rate constants for cellooligosaccharides of differing degree of polymerization (DP).

Table 4.1. Best-fit second order rate constants, with 95% confidence intervals, obtained from minimum least-square regression of time course data for sodium borohydride reduction of test compounds (Figures 4.1 a through j).

Test compound	Rate constant (M^{-1} sec^{-1})	Statistical analysis p-value[a]
Glyceraldehyde	>>19.070 ± 0.100	-
Glucose	0.0781 ± 0.0250	<0.0001
Cellobiose	0.0466 ± 0.0052	0.5765
Cellotriose	0.0463 ± 0.0032	-
Cellotetraose	0.0480 ± 0.0035	0.8371
Cellopentaose	0.0465 ± 0.0038	0.2776
MCC[b]	0.0355 ± 0.0029	0.0018
AMCC[c]	0.0527 ± 0.0043	0.3129
PSC[d]	0.0502 ± 0.0033	0.7292
BMCC[e]	0.0379 ± 0.0031	0.0014
ABMCC[f]	0.0609 ± 0.0075	0.1862

[a] Multiple regression (extra-sum-square F-test); $P < 0.002$ indicates the associated rate constant is significant different from the reference compound (cellotriose).
[b] microcrystalline cellulose; [c] amorphous microcrystalline cellulose; [d] phosphoric acid swollen-cellulose; [e] bacterial microcrystalline cellulose; [f]amorphous bacterial microcrystalline cellulose.

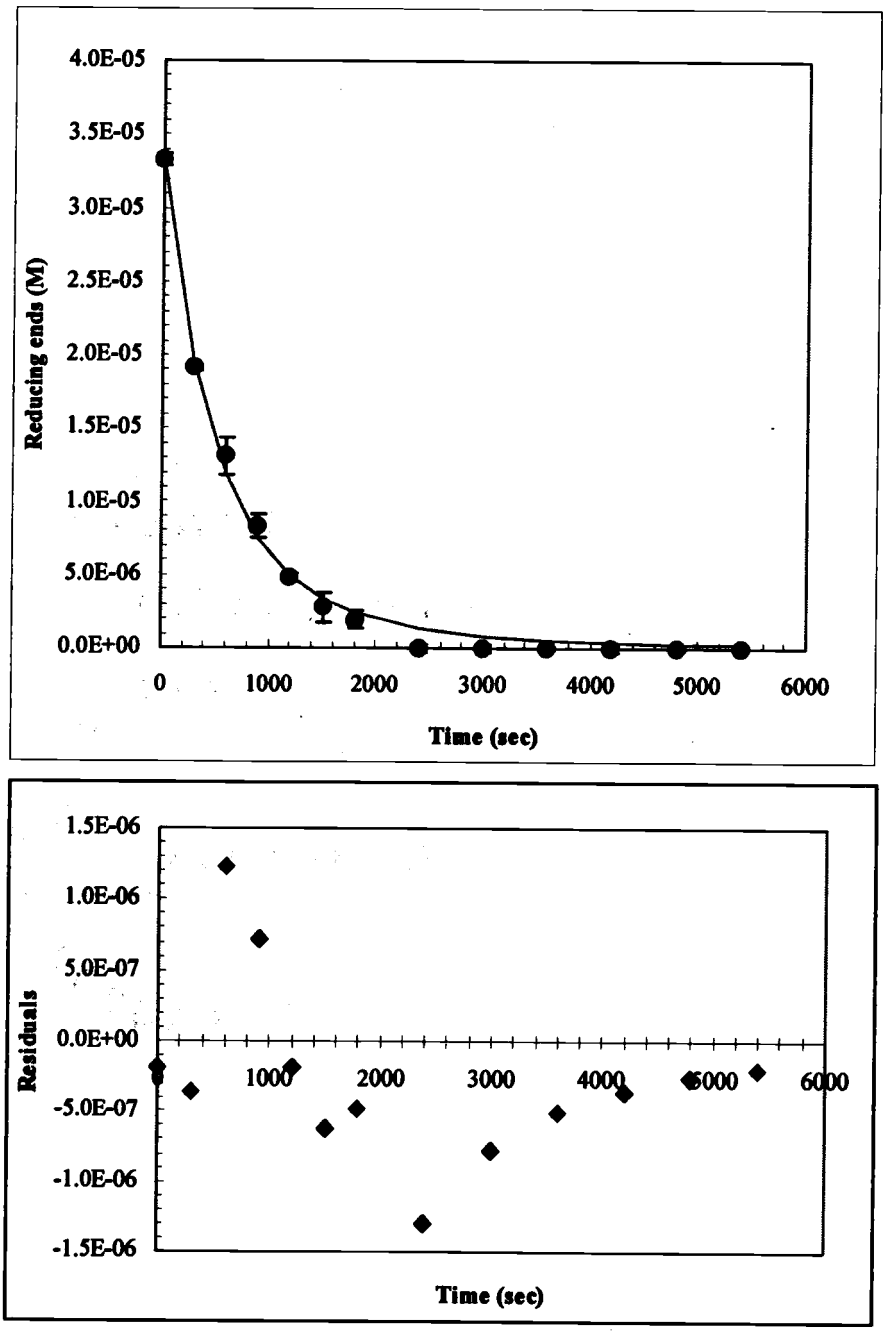
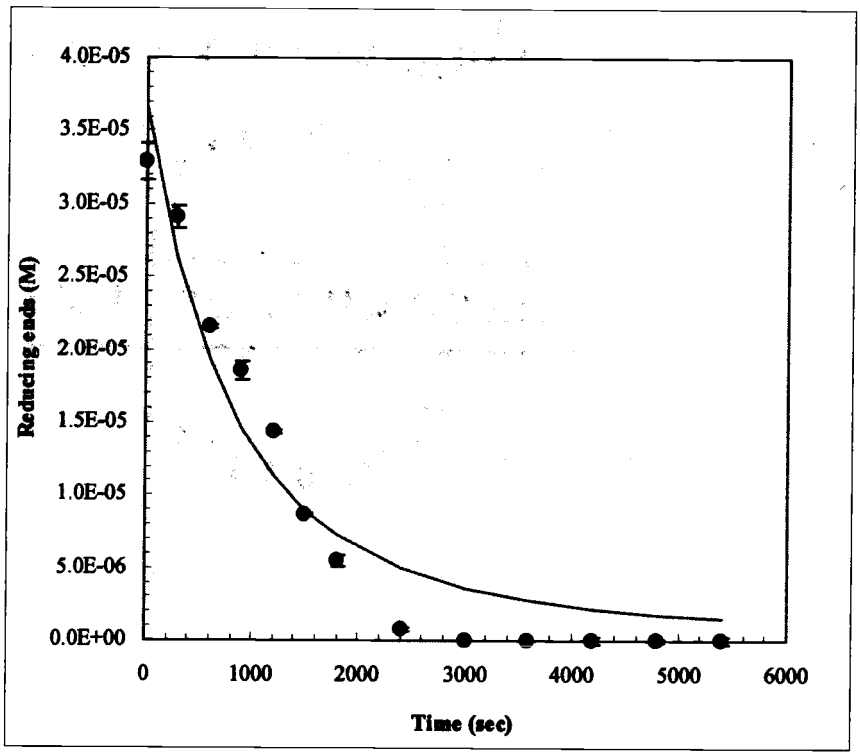


Figure 4.1 a Time course, including experimental data (●) and fitted curves (--), for the reduction of glucose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.033 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.



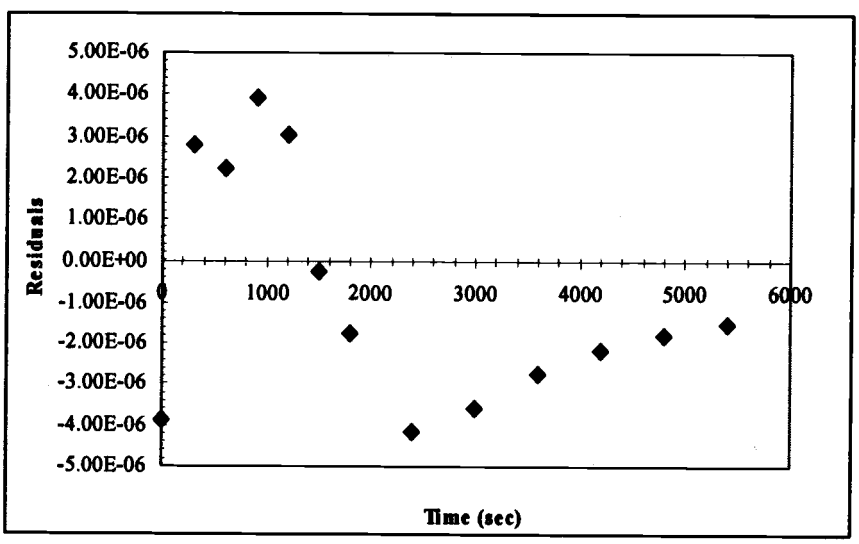


Figure 4.1 b Time course, including experimental data (●) and fitted curves (--), for the reduction of cellobiose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.033 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

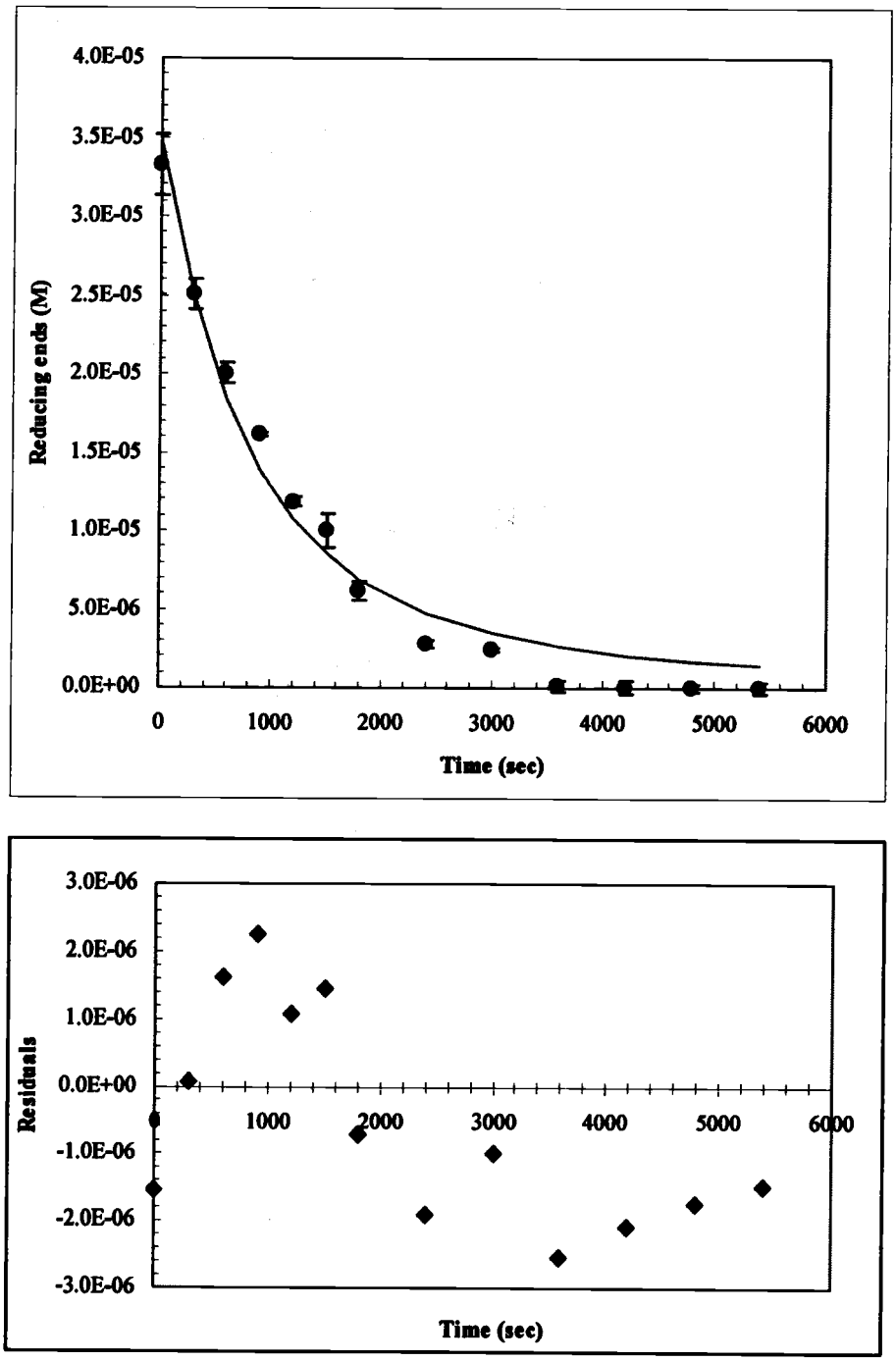


Figure 4.1 c Time course, including experimental data (●) and fitted curves (--), for the reduction of cellotriose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.033 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

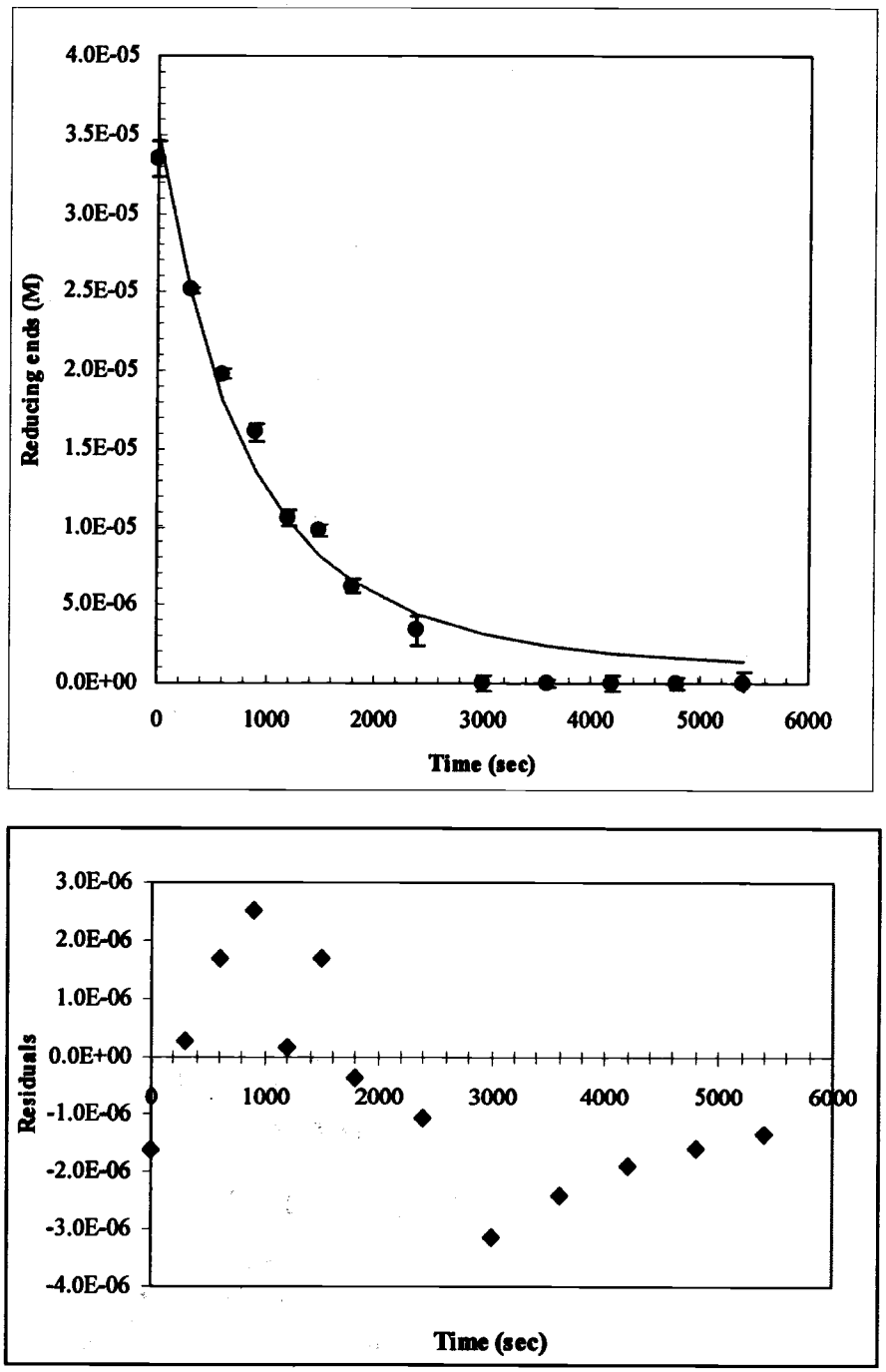


Figure 4.1 d Time course, including experimental data (●) and fitted curves (--), for the reduction of cellotetraose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.033 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

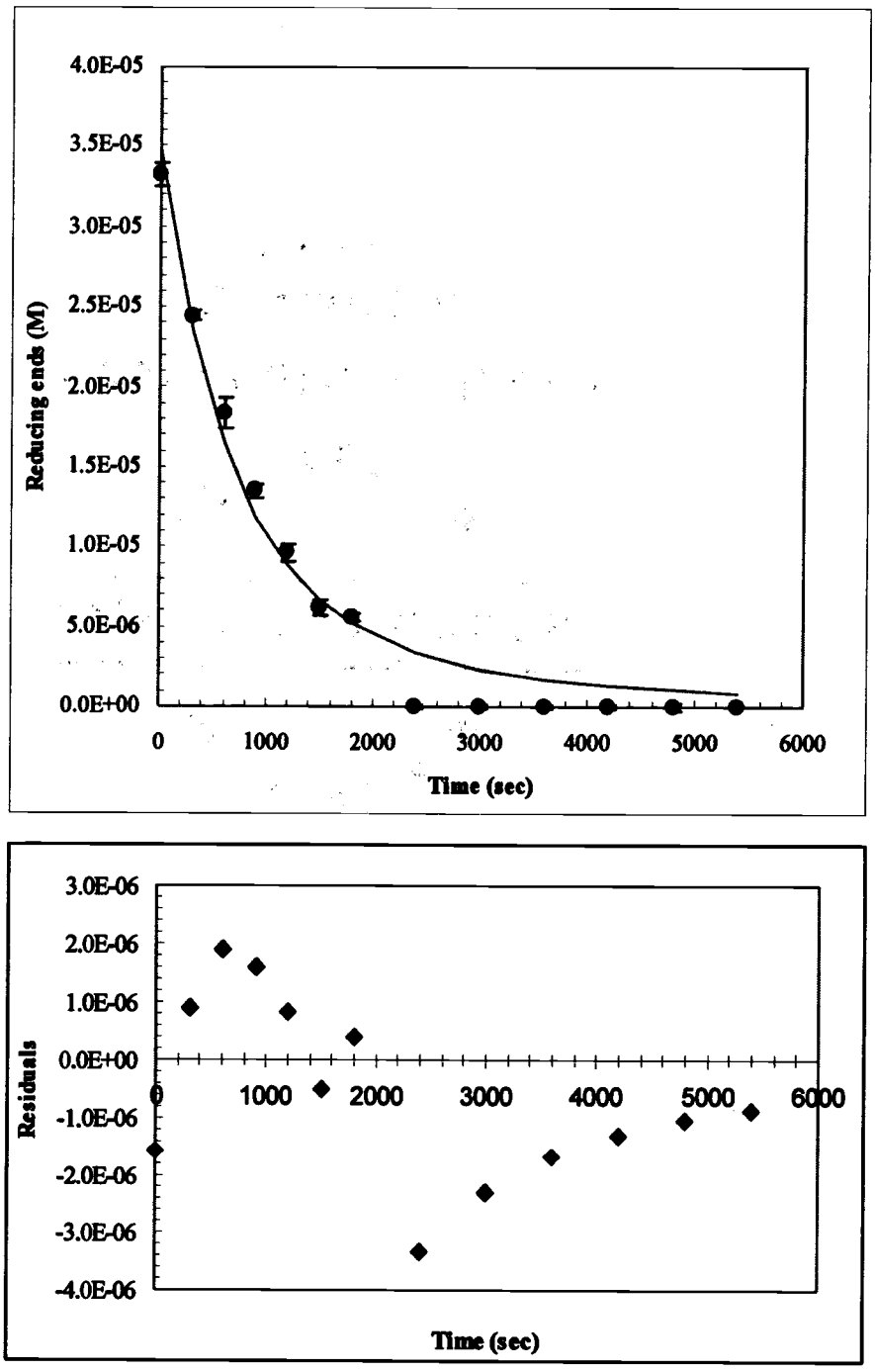


Figure 4.1 e Time course, including experimental data (●) and fitted curves (--), for the reduction of cellopentaose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.033 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

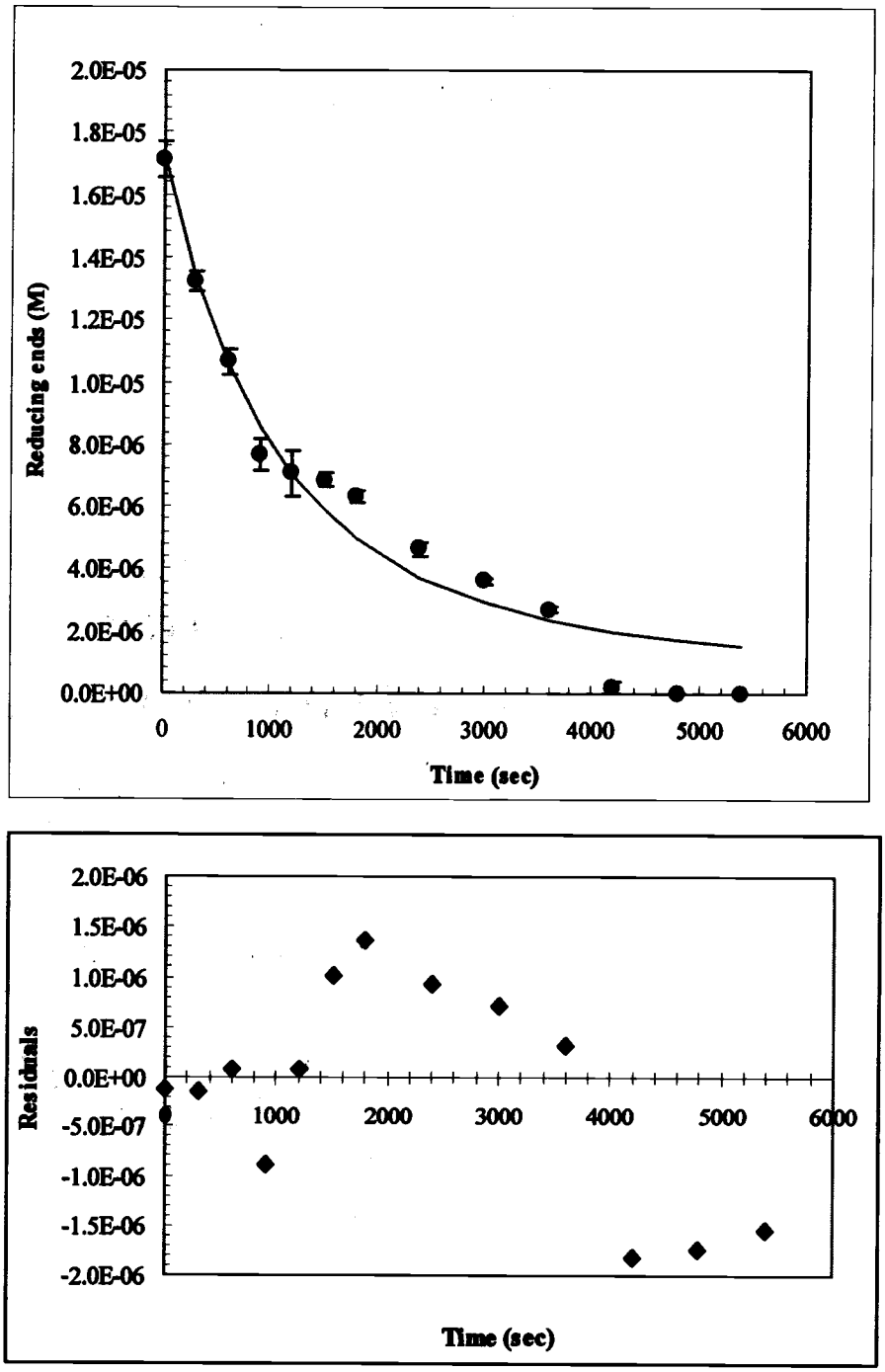


Figure 4.1 f Time course, including experimental data (•) and fitted curves (--), for the reduction of microcrystalline cellulose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.017 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

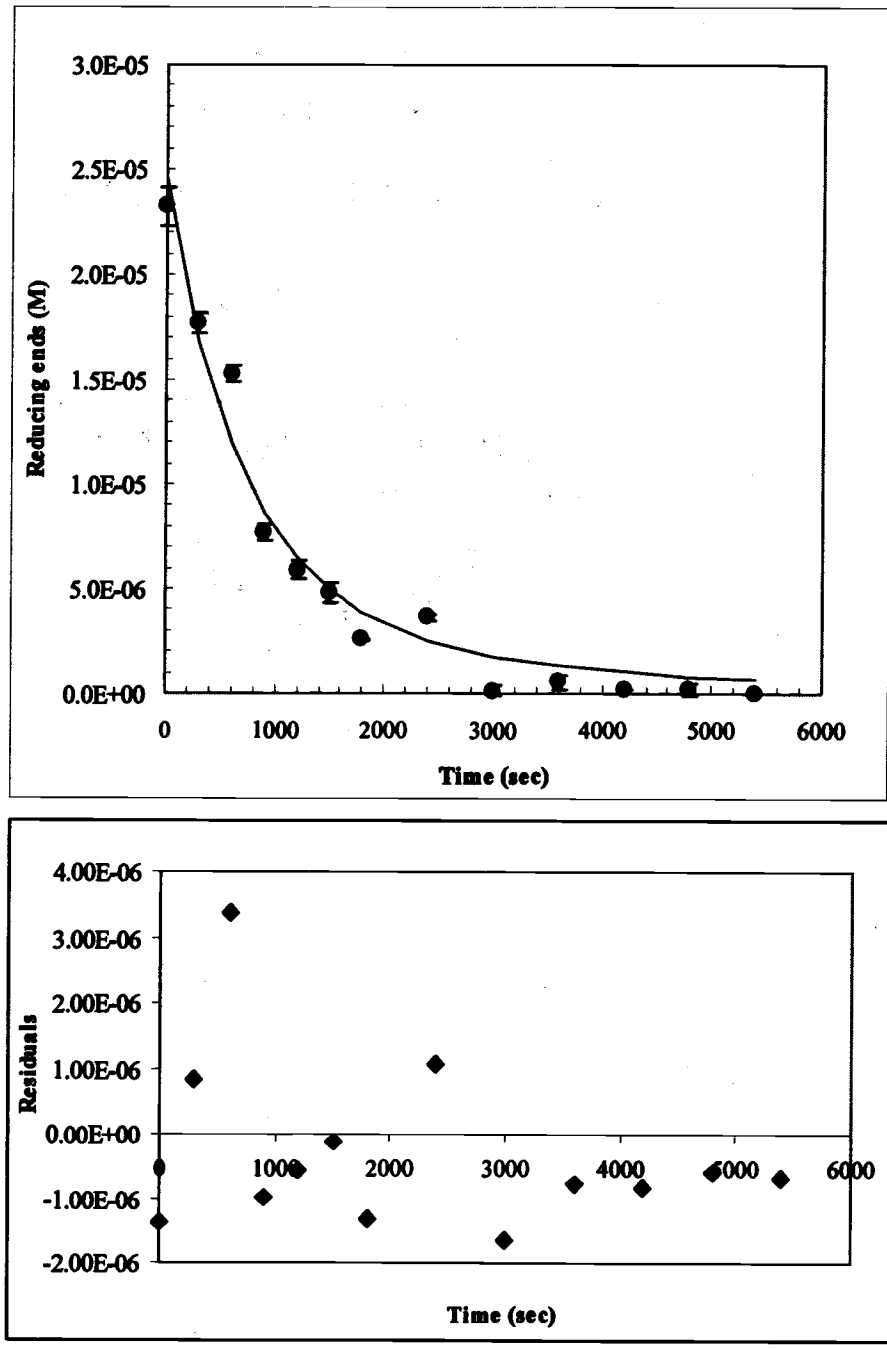


Figure 4.1 g Time courses, including experimental data (•) and fitted curves (--), for the reduction of amorphous microcrystalline cellulose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.023 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

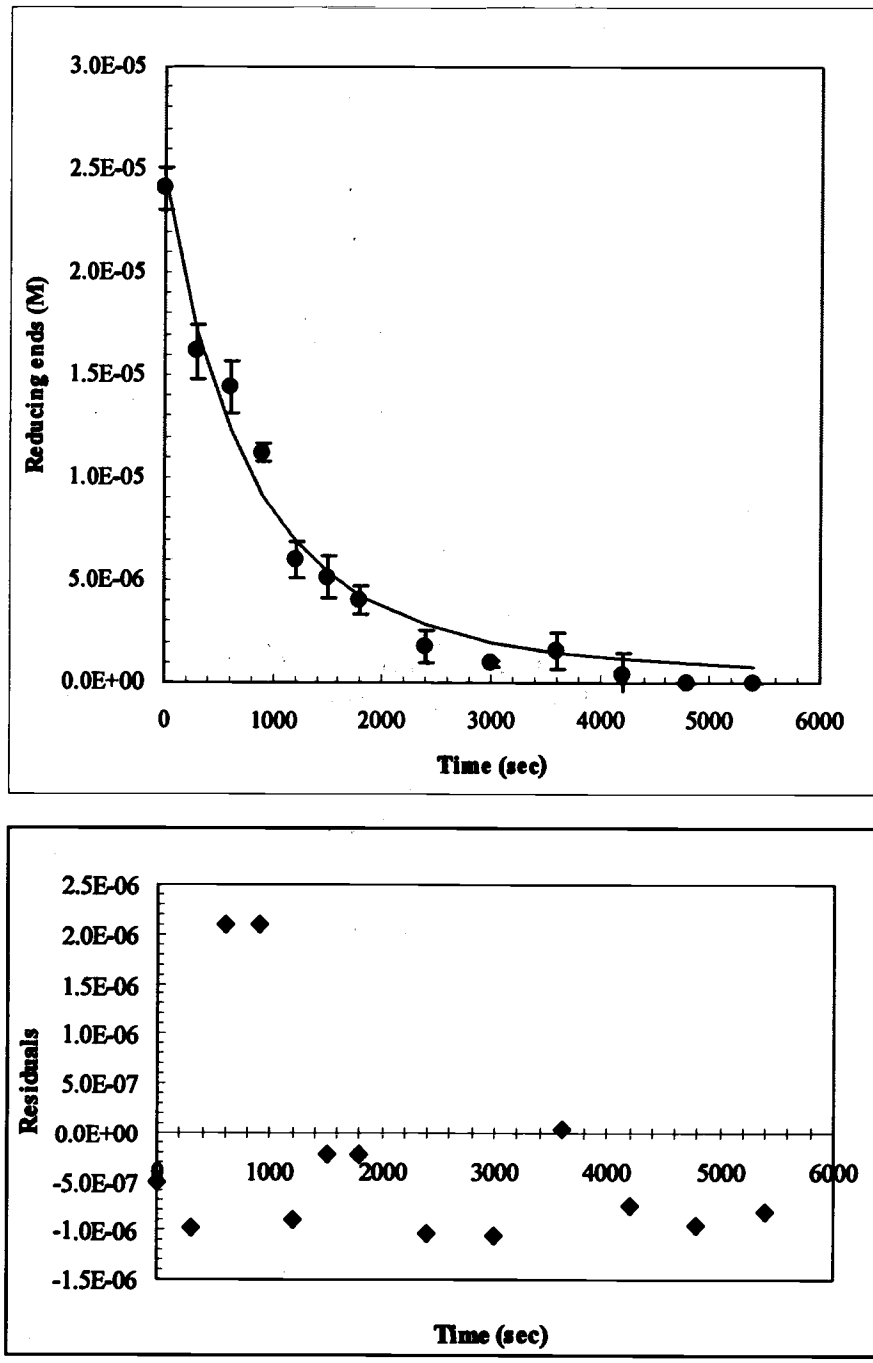


Figure 4.1 h Time course, including experimental data (●) and fitted curves (--), for the reduction of phosphoric acid swollen cellulose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.024 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

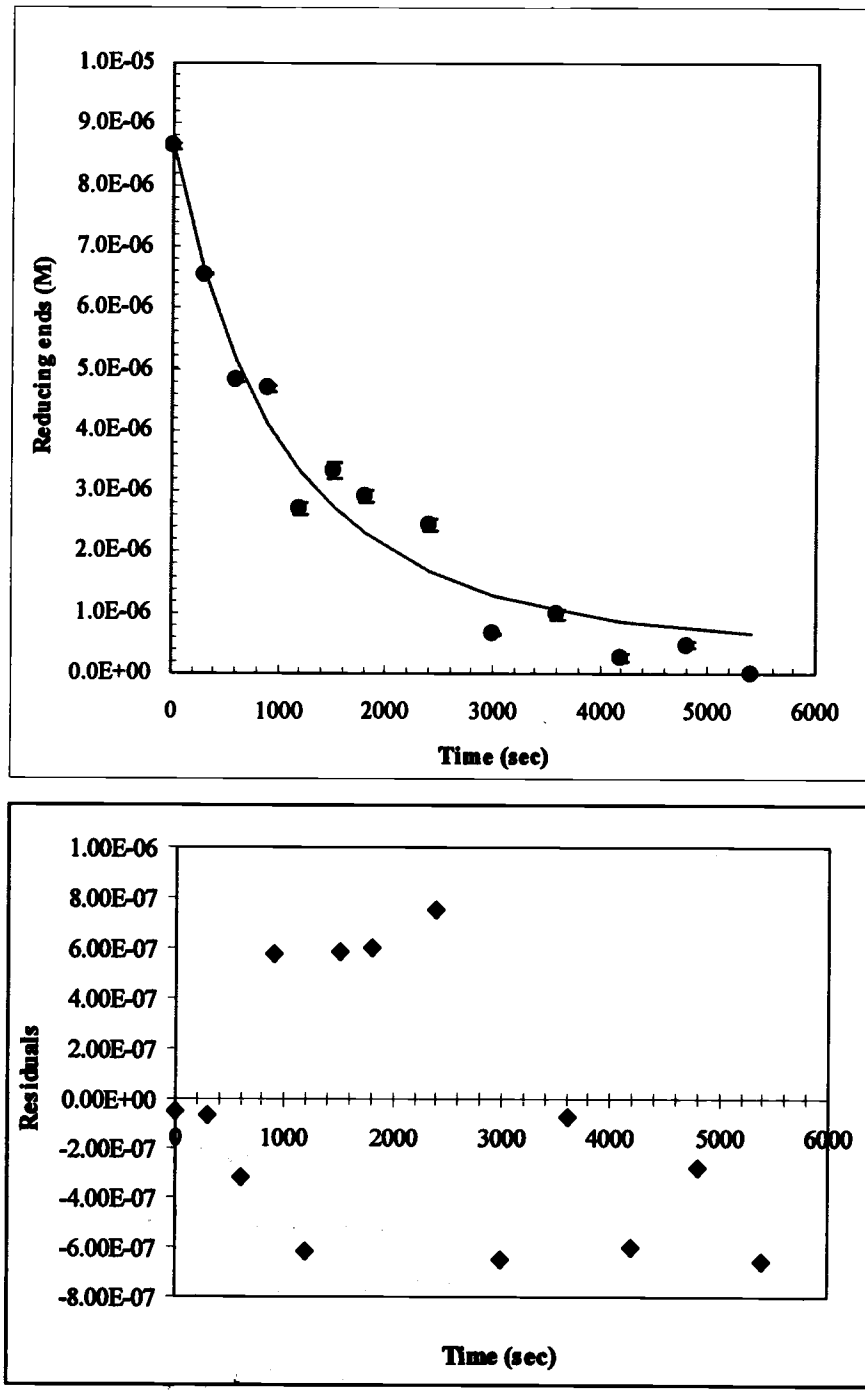


Figure 4.1 i Time course, including experimental data (•) and fitted curves (--), for the reduction of bacterial microcrystalline cellulose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.009 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

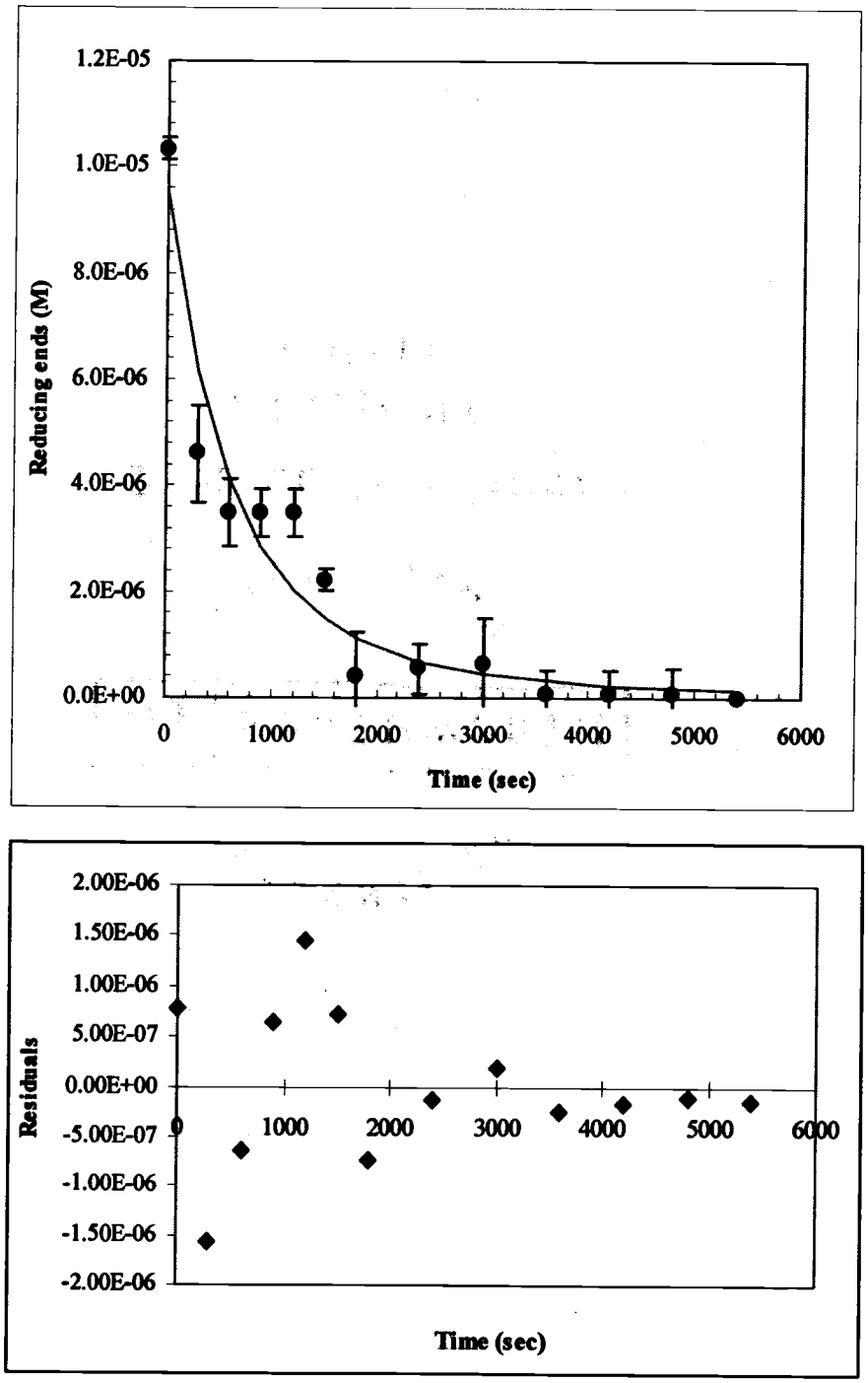


Figure 4.1 j Time course, including experimental data (•) and fitted curves (--), for the reduction of amorphous bacterial microcrystalline cellulose. Extents of reduction are expressed in terms of the amount of reducing sugar (aldose) remaining in reaction mixtures. Data for each of the associated residual plots were obtained as described in "Materials and methods" section of text. Reaction conditions were 25 mM sodium borohydride, 0.010 mM test compound, 100 mM sodium phosphate, pH 8.0 and 22°C.

This observation leads to the conclusion that rate constants describing the reduction of celluloses, i.e. those polymers with much higher DP, will also be in this general range provided the reaction is not hindered by factors associated with the insoluble nature of the celluloses.

Comparison of the rate constants obtained for the reduction of the different celluloses indicates a demonstrable difference between the reactivity of the reducing ends associated with amorphous versus microcrystalline celluloses. Rates of reduction of the reducing ends in the amorphous cellulose preparations were not significantly different from those obtained for the soluble cellooligosaccharides ($p = 0.3129$). In contrast, the corresponding rate constant for the microcrystalline cellulose was significantly lower ($p = 0.0018$). The cellulose-associated rate constants obviously reflect only the reactivity of the "reagent-accessible" reducing ends (Kongruang et al., 2003); these ends presumably being on the surface of cellulose aggregates/fibers.

Included in table 4.1 is a minimal value for the rate constant describing the time course of glyceraldehyde reduction. This minimal value reflects the fact that glyceraldehyde was essentially consumed in the reaction prior to the initial time point used to monitor the reduction of the other test compounds. The relatively rapid rate of reaction with glyceraldehyde, relative to the other compounds, indicates the importance of the chemistry of the carbohydrate moiety in determining the overall rate of borohydride reduction. A major difference between glyceraldehyde and the other compounds is its inability to form the hemiacetal ring structure. One may surmise that the slower reaction rates for glucose, the

cellooligosaccharides, and the celluloses is largely attributable to their existing, predominantly, in the ring form – which is not reduced by sodium borohydride (Figure 4.2). In the acyclic form, the saccharides would be expected to react at rates approaching that of glyceraldehyde. This rationale for the difference in reaction rates for glyceraldehyde and glucose minimizes the importance of potential steric effects limiting borohydride-glucose interactions.

The pH chosen for this study, pH 8.0, was a compromise between that necessary for sodium borohydride ($NaBH_4$) stability and relevance to the enzymatic saccharification of cellulose. Sodium borohydride decomposition is acid catalyzed (Davis & Swain, 1960), thus the reagent is far more stable at highly alkaline pHs. Fungal cellulases typically have pH optima below 7, pHs at which borohydride rapidly hydrolyzes. Thus, pH 8.0 was chosen since it is within the technologically relevant range and yet the rate of borohydride decomposition at this pH is such that it can be accounted for (see "*Materials and methods*" section). The actual time course of $NaBH_4$ hydrolysis under these experimental conditions is presented in Figure 4.3. The simulated data was generated using a first order rate expression with $k = 0.000291\ sec^{-1}$ ($0.017451\ min^{-1}$).

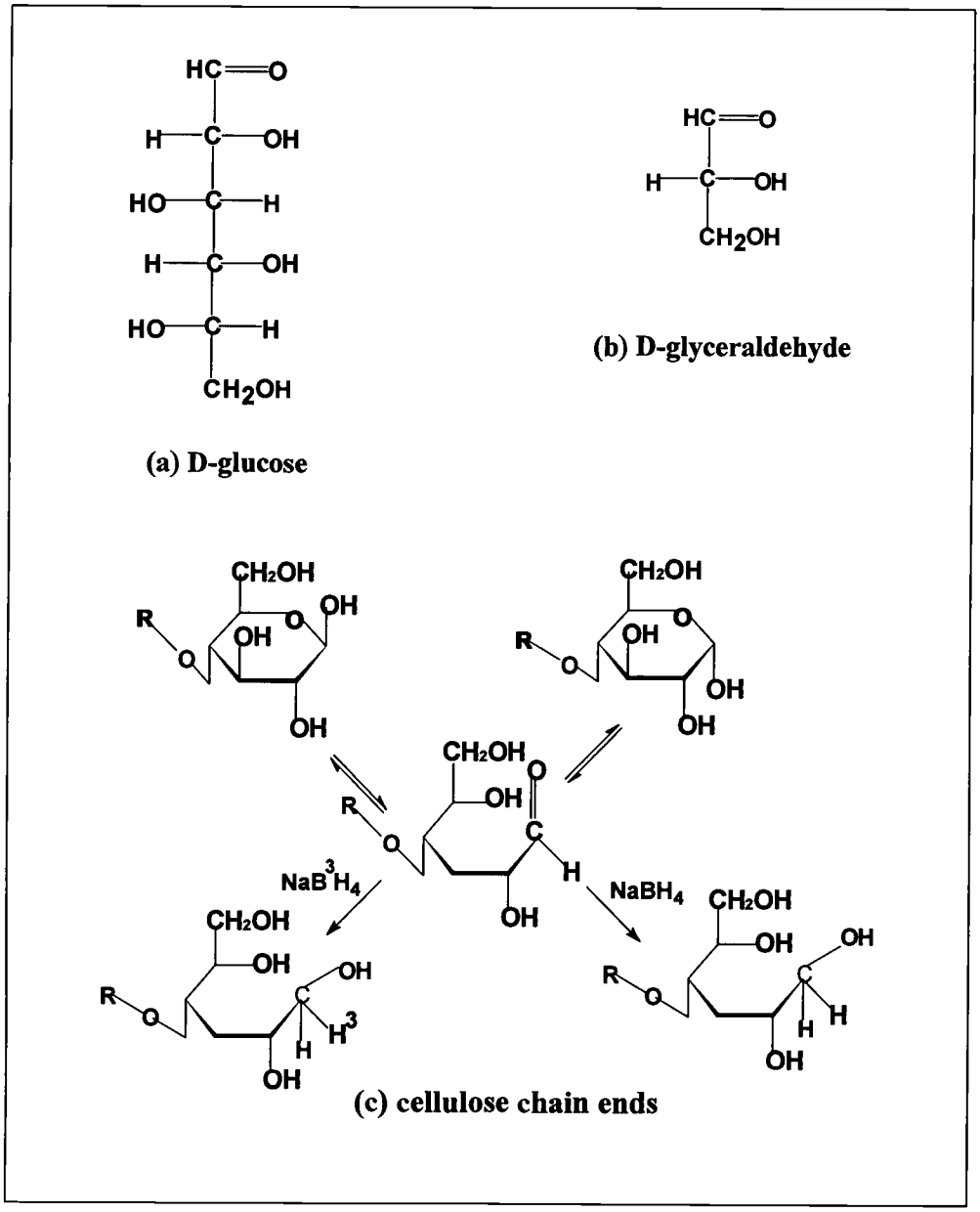
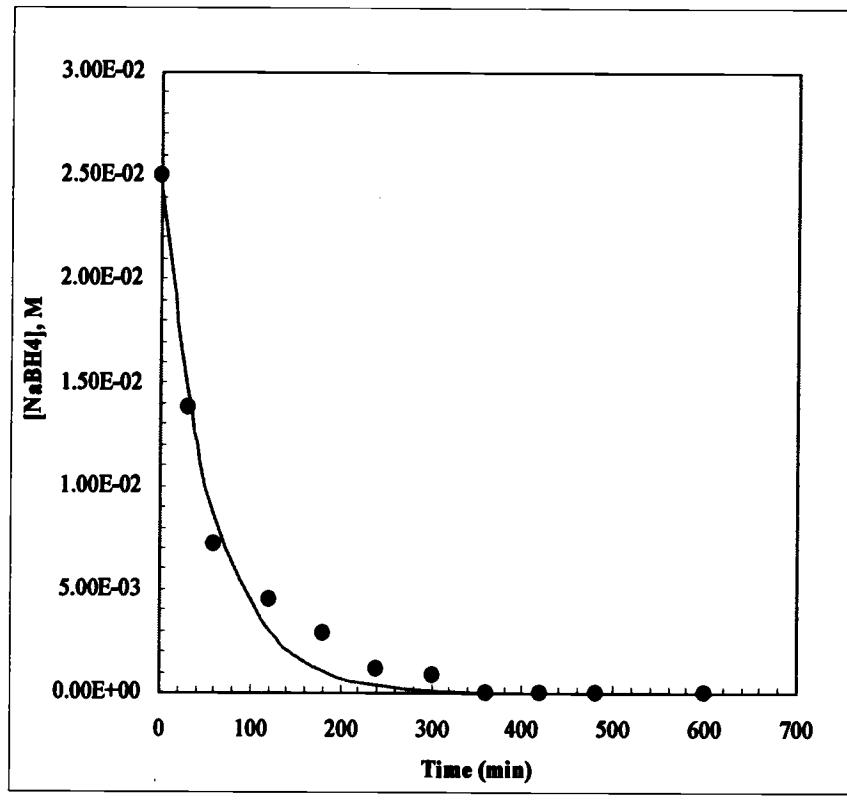


Figure 4.2 Structural compounds involved in the sodium borohydride reaction: (a) D-glucose, (b) D-glyceraldehyde, (c) cellulose chain ends (the reduction of an aldose to a glycitol will be preceded by ring-opening of the cyclic modification of the aldose to the aldehydo-form in the acyclic conformation).



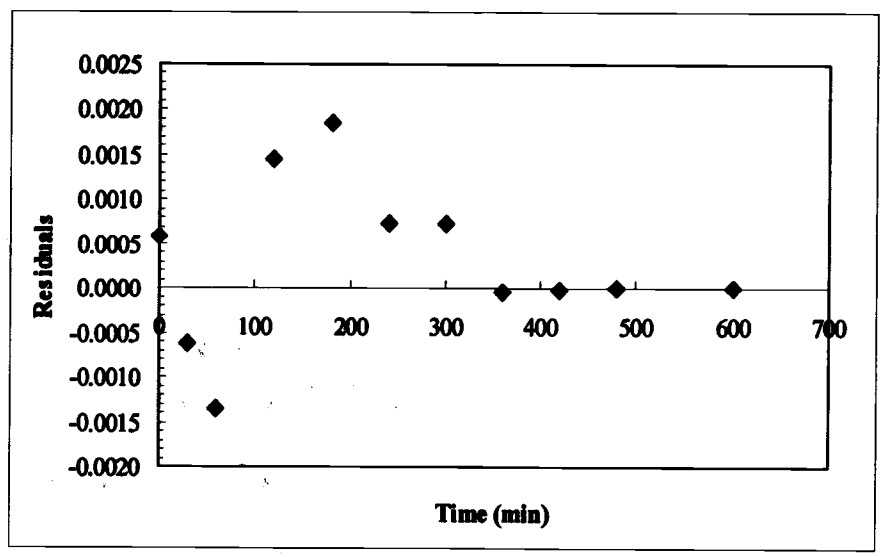


Figure 4.3 Time course, including experimental data (●) and fitted curves (--), for hydrolytic degradation of sodium borohydride in 100 mM sodium phosphate buffer, pH 8.0, 22°C. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

To check the validity of the experimental approach used to generate the previously discussed data an analogous set of experiments, focusing on glucose and the experimental celluloses, was done using tritiated sodium borohydride. In this case the extent of reduction was determined by isotope uptake at the C-1 position (Mclean et al., 1973; Richards & Whelan, 1973; Evans et al., 1974; Chirico & Brown, 1985; Bhat et al., 1990), rather than by measuring the amount of reducing carbohydrates remaining, over the course of the reaction. Time courses illustrating the results from this set of experiments are presented in Figures 4.4 a-d. The corresponding "best fit" rate constants are tabulated in Table 4.2. The general agreement between the values presented in Tables 4.1 and 4.2 provide further credibility to the data presented herein.

Several "fitting" approaches, all based on the DYNAFIT regression analysis system, were compared in order to ascertain the significance of experimental assumptions made during the determination of "best-fit" second order rate constants for the borohydride reduction of test compounds. The relevant assumptions relate to the concentration of the test compounds at the initiation of the reaction ("time zero") and the relevance of negative concentration values measured as the reactions neared completion.

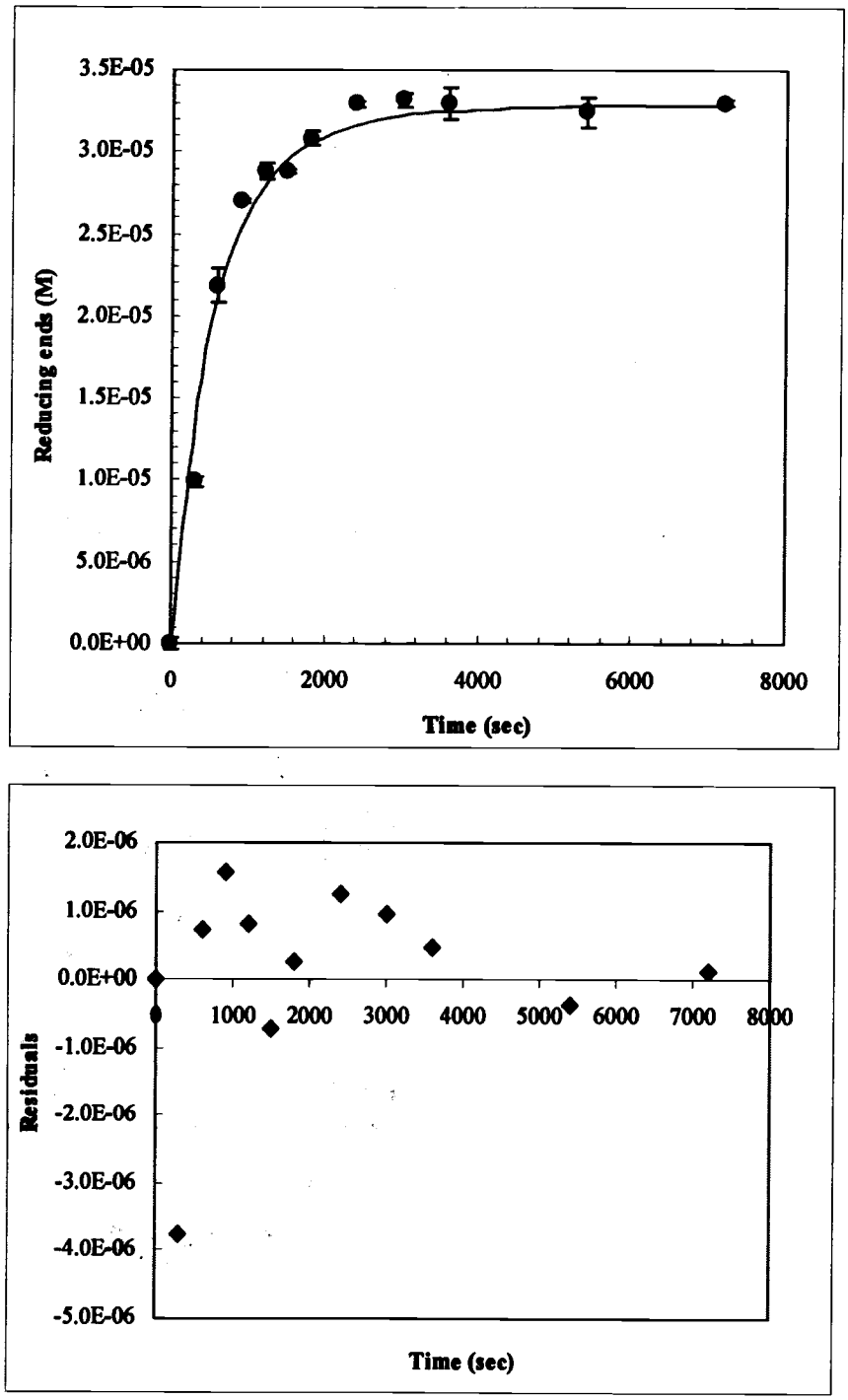
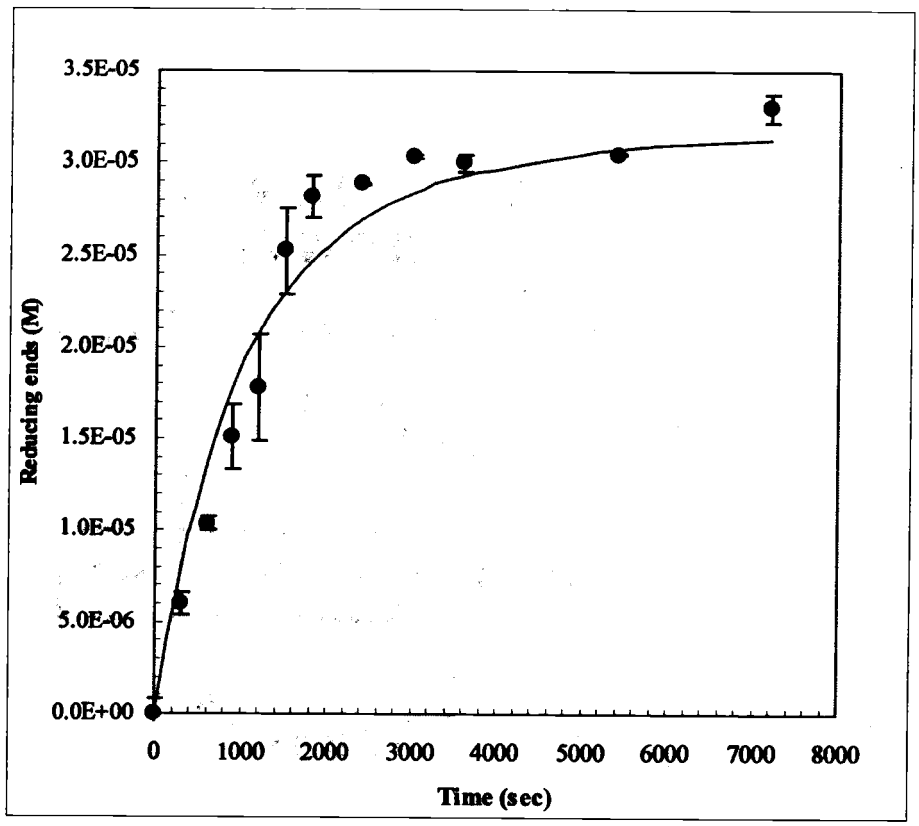


Figure 4.4 a Time course, including experimental data (●) and fitted curves (--), for the incorporation of tritium into glucose resulting from treatment with ^{3}H-labeled sodium borohydride. Isotope incorporation is expressed in terms of moles reducing sugar (aldose) reduced. Reaction conditions were as in Figure 4.1. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.



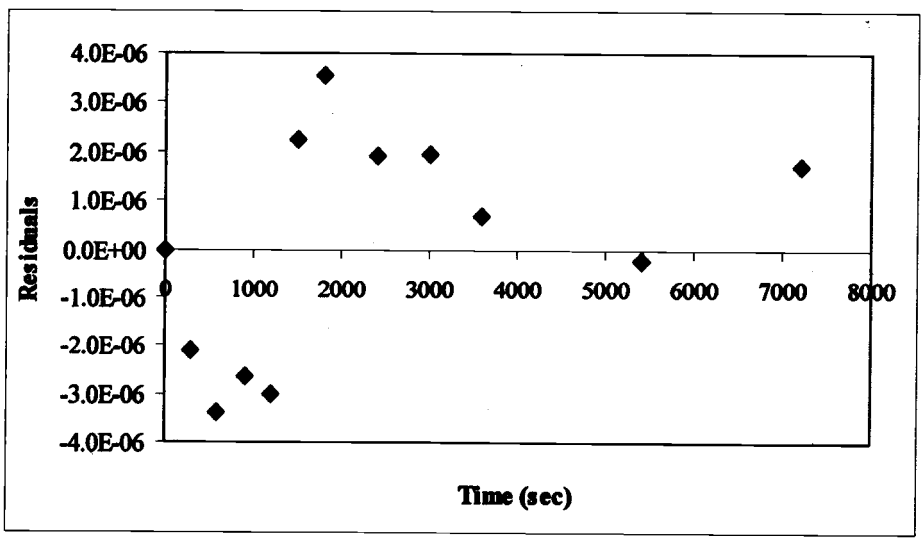
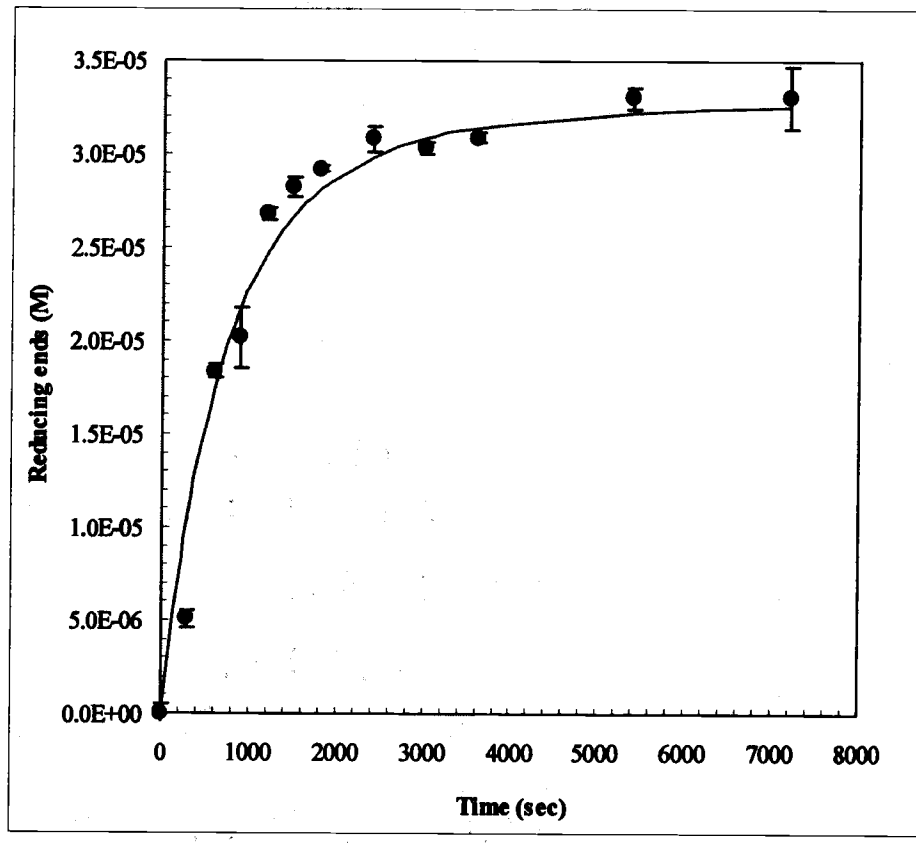


Figure 4.4 b Time course, including experimental data (●) and fitted curves (--), for the incorporation of tritium into microcrystalline cellulose resulting from treatment with ^{3}H-labeled sodium borohydride. Isotope incorporation is expressed in terms of moles reducing sugar (aldose) reduced. Reaction conditions were as in Figure 4.1. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.



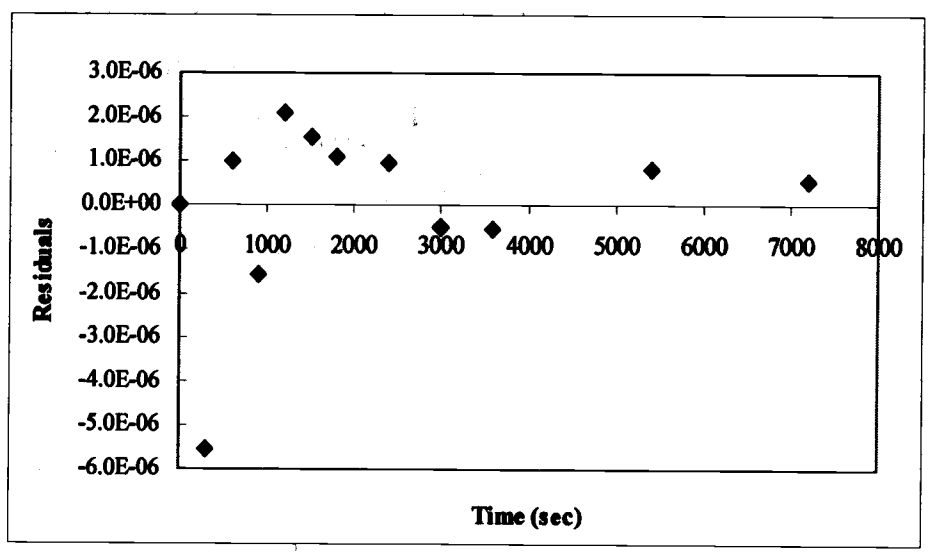
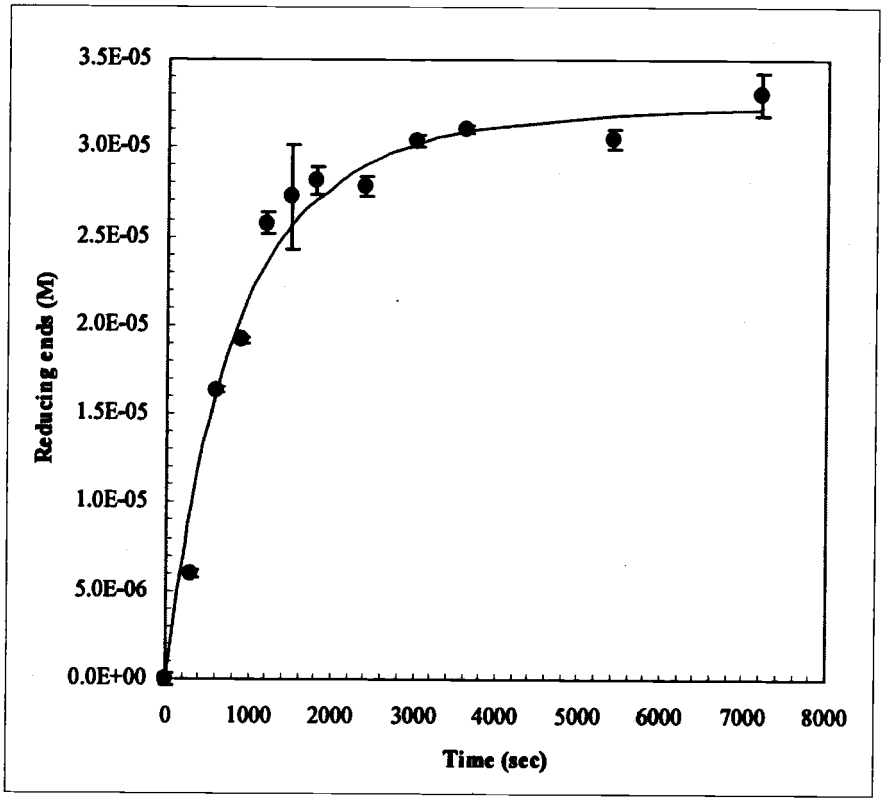


Figure 4.4 c Time course, including experimental data (●) and fitted curves (--), for the incorporation of tritium into amorphous microcrystalline cellulose resulting from treatment with ^{3}H-labeled sodium borohydride. Isotope incorporation is expressed in terms of moles reducing sugar (aldose) reduced. Reaction conditions were as in Figure 4.1. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.



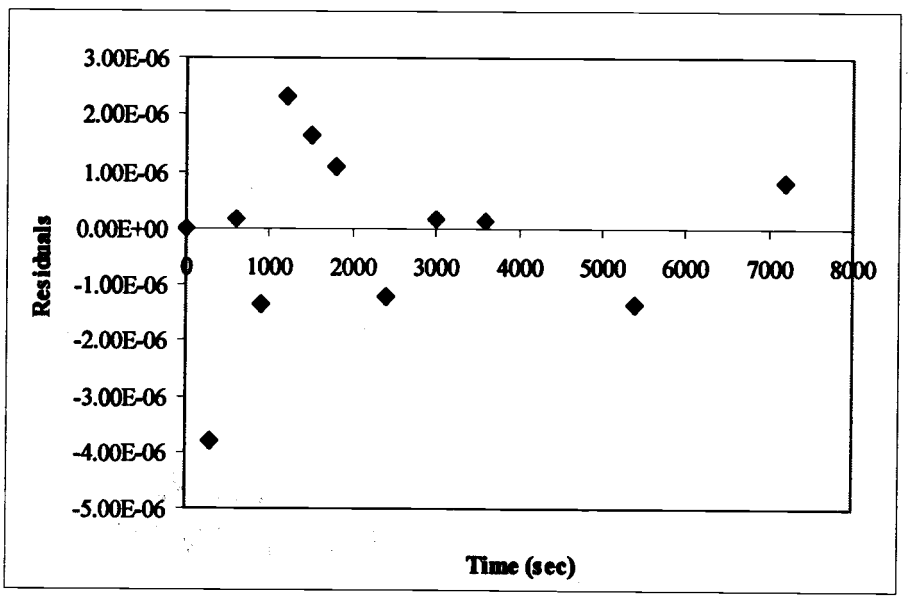


Figure 4.4 d Time course, including experimental data (•) and fitted curves (--), for the incorporation of tritium into phosphoric acid swollen cellulose resulting from treatment with ^{3}H-labeled sodium borohydride. Isotope incorporation is expressed in terms of moles reducing sugar (aldose) reduced. Reaction conditions were as in Figure 4.1. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

Table 4.2. Best-fit second order rate constants, with 95% confidence intervals, obtained from minimum least-square regression of time course data for tritium incorporation resulting from ^{3}H-labelled sodium borohydride treatment of test compounds (Figures 4.4 a through d).

Test compound	Rate constant (M^{-1} sec^{-1})
Glucose	0.0744 ± 0.0042
MCC[a]	0.0392 ± 0.0029
AMCC[b]	0.0544 ± 0.0039
PSC[c]	0.0491 ± 0.0027

[a] microcrystalline cellulose
[b] amorphous microcrystalline cellulose
[c] phosphoric acid swollen-cellulose

The question of concentration at time zero arises because the "theoretical concentration" (zero time^{T}) of the reaction mixture, as prepared, does not match the concentration measured in the "zero time" experiment (zero time^{E}; *i.e.* the concentration measured if the acid is added to the reaction mixture prior to the addition of sodium borohydride; the assumption here being that the borohydride will decompose rapidly at the low pH and thus not be present for a long enough time to significantly reduce the glucose concentration - the zero time^{E} value is expected to be somewhat lower than the theoretical value, zero time^{T}, if significant glucose is reduced prior to the degradation of borohydride). The issue of negative concentration values occurring at the completion of the time course is whether or not to adjust the data to account for these values, or to simply record all negative values as zero. Three fitting approaches were compared, in order to ascertain the importance of these data handling issues. "Method 1" used theoretical concentrations as the starting point (zero time^{T}) for regression analyses and assigned all negative concentration values to zero. "Method 2" adjusted all experimentally determined concentrations upward by summing the measured values with the opposite of the mean of the negative values obtained for the last three measurements in the time course. The starting point for regression analyses was taken as the value obtained experimentally (zero time^{E}). "Method 3" was done as for "Method 2" with the exception that all experimentally derived data points were adjusted by multiplying the experimental value by a common factor such that zero time^{E} was equivalent to zero time^{T}. The best fit second order rate constants obtained by each of these approaches to the analysis are presented in

Table 4.3. In general, the trends obtained using the different approaches were similar. In particular, "Methods 2" and "3" gave essentially the same results; as may be expected since each data point was proportionally modified. In each case, the rate constant describing glucose reduction was significantly higher than the corresponding rate constants describing the reduction of the cellooligosaccharides. The observed decreases in apparent rate constants attributable to switching from the assumptions of Method 1 to those of either Method 2 or Method 3 were 35 % for glucose; 10%, cellobiose; 16%, cellotriose; 7%, cellotetraose; and 11%, cellopentose. Representative time courses showing the application of Methods "2" and "3" are presented in Figure 4.5 a-f.

The aim of this work was to generate information pertaining to the relative reactivity of the terminal glucosyl residues of cellooligosaccharides and those celluloses typically employed in saccharification studies. The premise being that the terminal anomeric carbon of each of these glucose-based oligosaccharides/polysaccharides is expected to have similar reactivity. Support for this notion comes from the similar reactivities of the entire series of soluble cellooligosaccharides tested. The slower rate of reduction of the cellooligosaccharides, relative to glucose, is attributable to the substitution of the terminal glucosyl units at the 4 position (Rickborn & Wuesthoff, 1970). The number of glucosyl units appended at this position appears to have a relatively minor affect on borohydride reactivity – provided the molecule stays in solution.

Table 4.3. Rate constants using different experimental assumptions

Compound	Reduction rate of solvent accessible reducing ends ($M^{-1}sec^{-1}$)		
	Method 1	Method 2	Method 3
Glucose	0.0781 ± 0.0250 (33.24×10^{-6})[a]	0.0519 ± 0.033 (26.09×10^{-6})	0.0519 ± 0.033 (33.24×10^{-6})
Cellobiose	0.0466 ± 0.0052 (32.89×10^{-6})	0.0433 ± 0.0058 (29.60×10^{-6})	0.0433 ± 0.0058 (32.86×10^{-6})
Cellotriose	0.0463 ± 0.0032 (33.24×10^{-6})	0.0419 ± 0.0420 (27.67×10^{-6})	0.0419 ± 0.0420 (32.77×10^{-6})
Cellotetraose	0.0480 ± 0.0035 (33.47×10^{-6})	0.0438 ± 0.0038 (30.98×10^{-6})	0.0438 ± 0.0038 (39.98×10^{-6})
Cellopentaose	0.0465 ± 0.0038 (33.28×10^{-6})	0.0478 ± 0.0043 (29.74×10^{-6})	0.0478 ± 0.0043 (38.37×10^{-6})

[a]The initial substrate concentrations for finding the reduction rate (M) based on the DYNAFIT regression analysis.

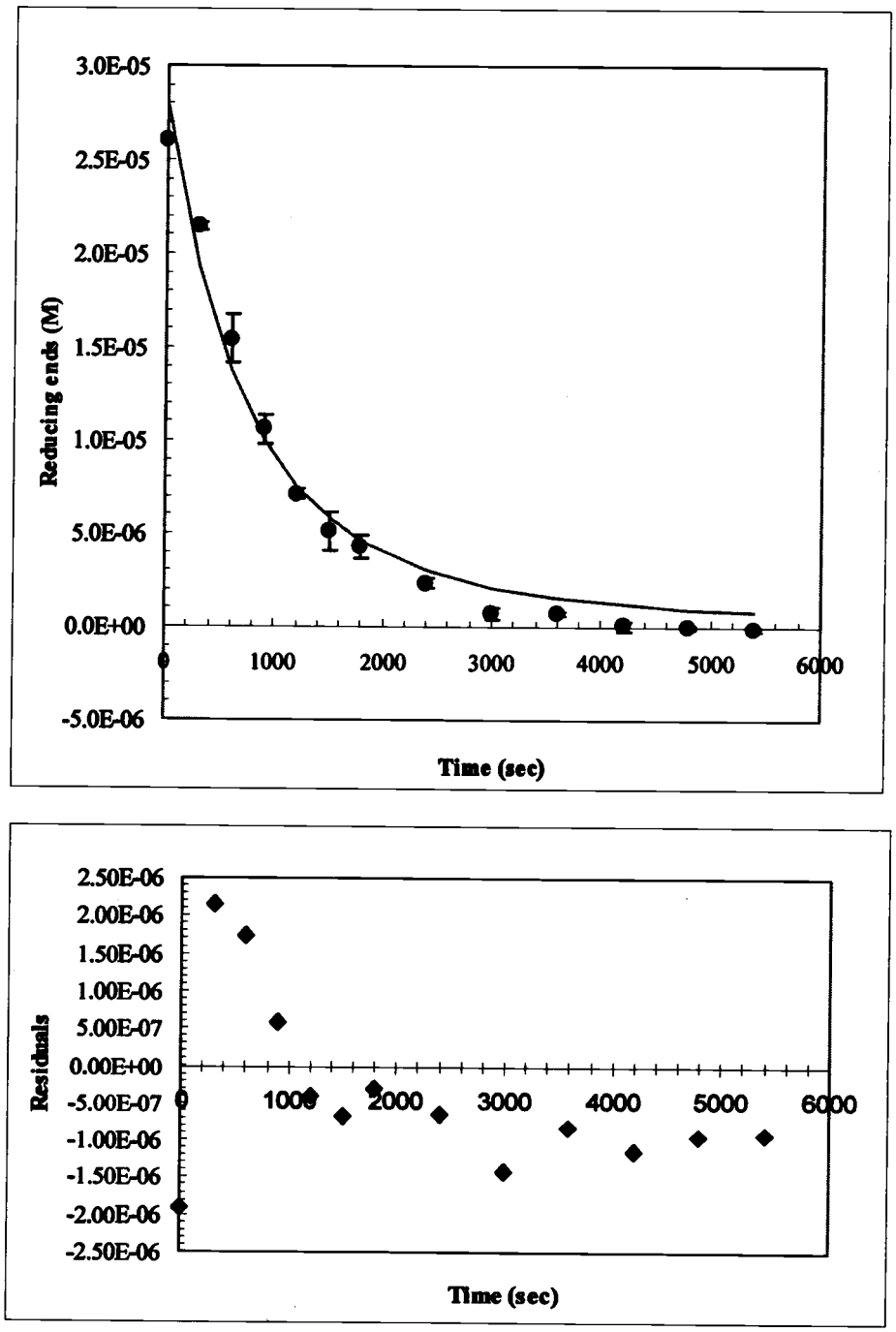


Figure 4.5 a Representative time course of glucose showing the application of Method 2. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

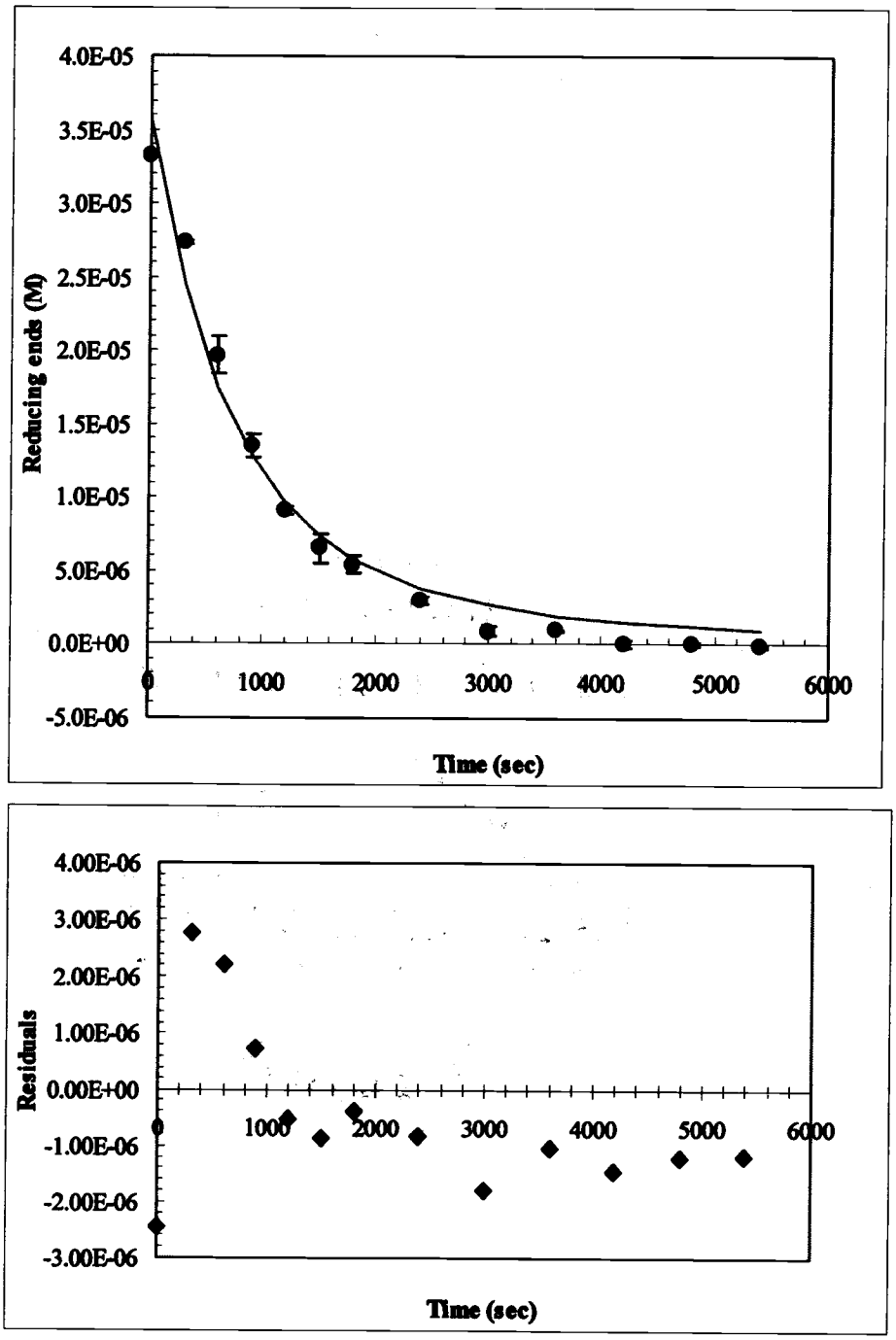


Figure 4.5 b Representative time course of glucose showing the application of Method 3. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

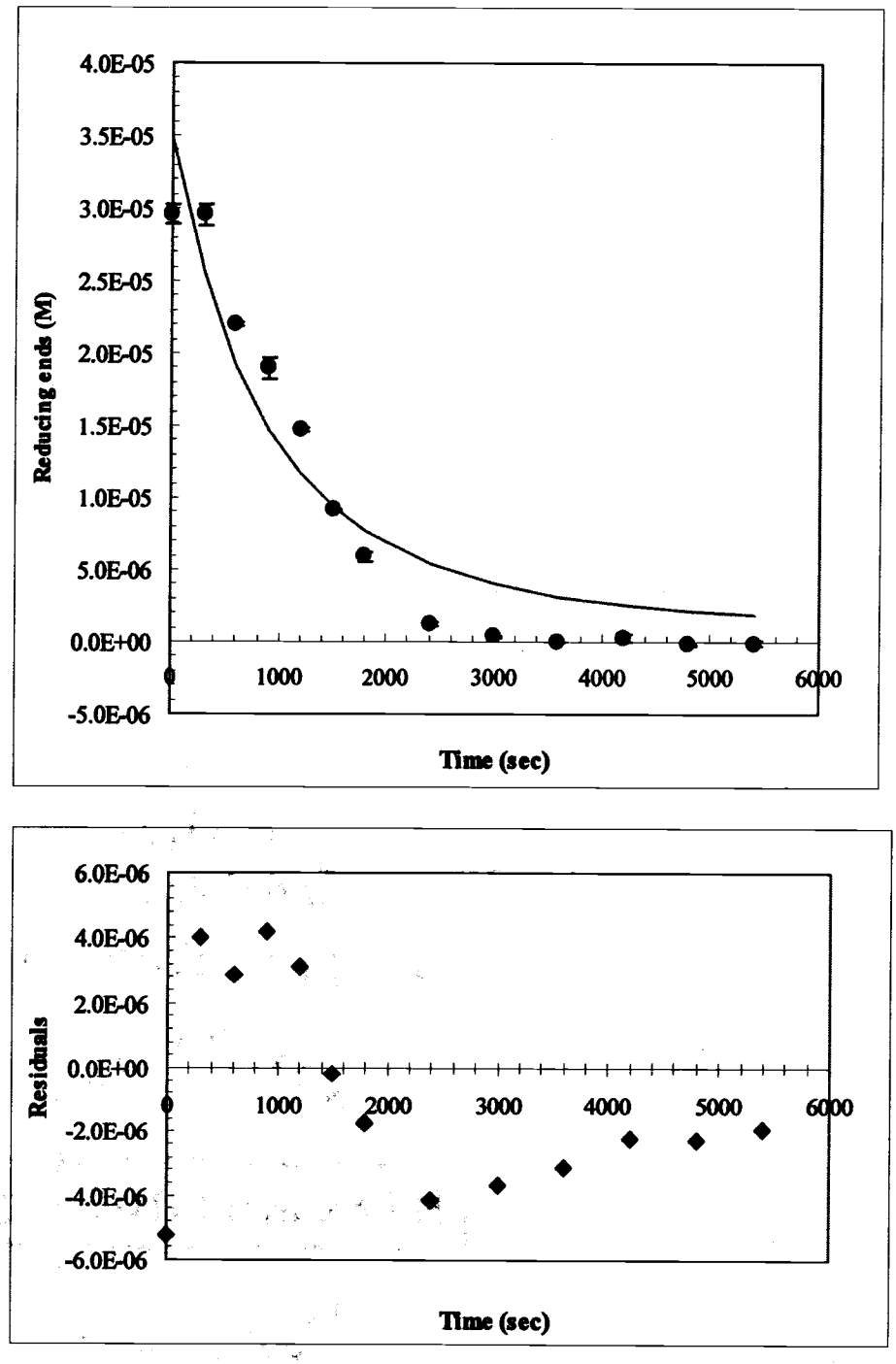


Figure 4.5 c Representative time course of cellobiose showing the application of Method 2. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

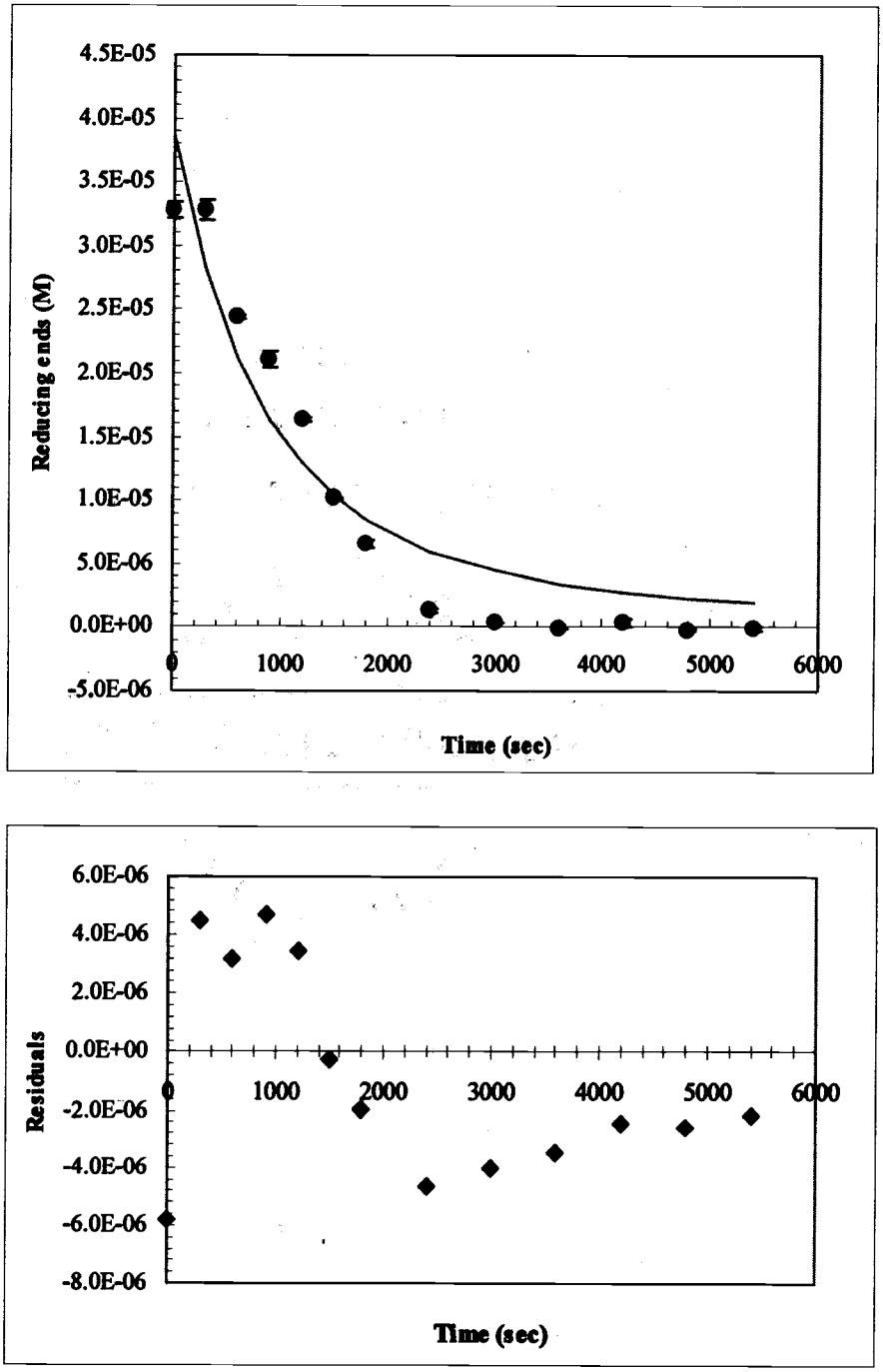


Figure 4.5 d Representative time course of cellobiose showing the application of Method 3. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

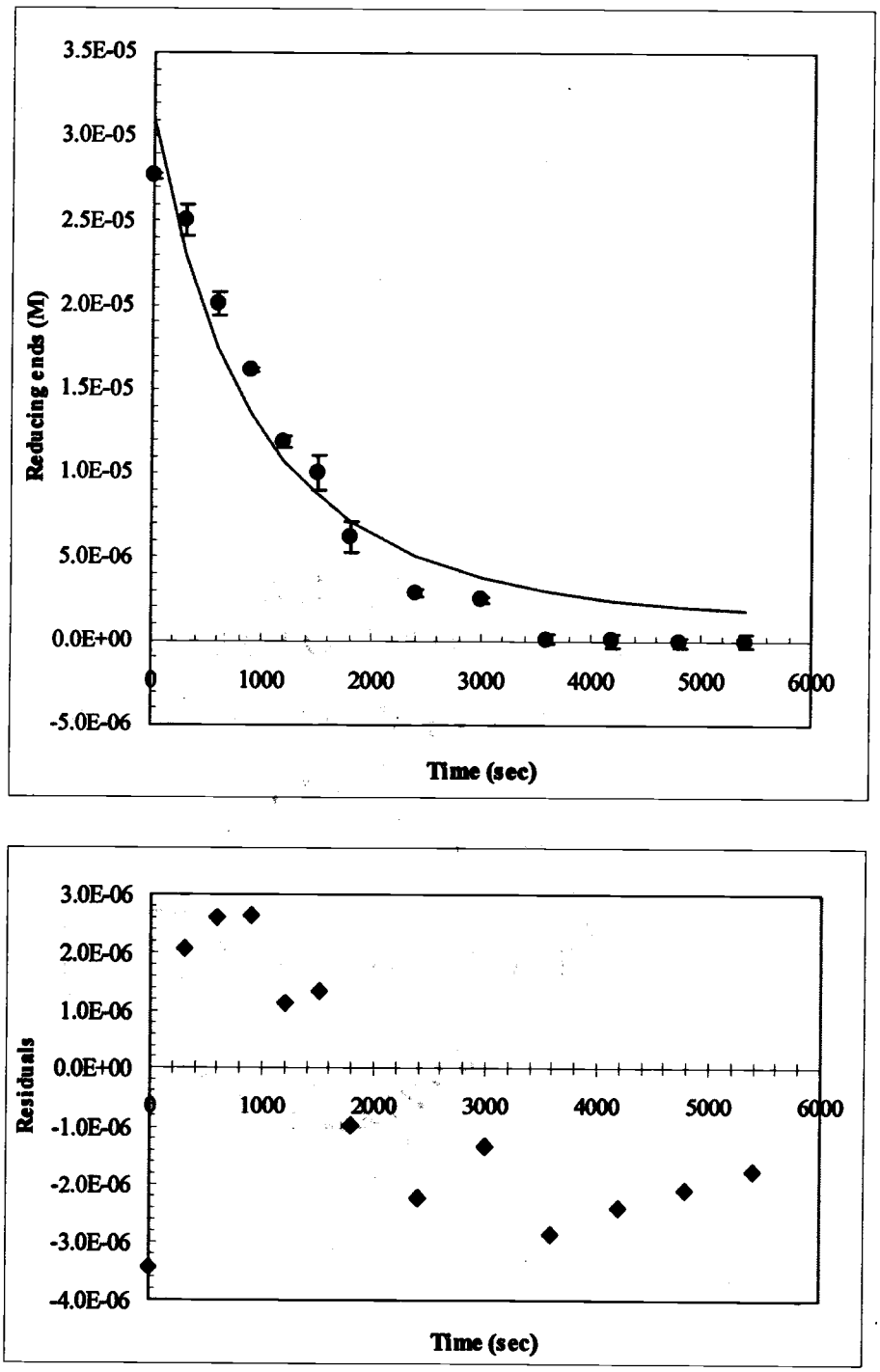


Figure 4.5 e Representative time course of cellotriose showing the application of Method 2. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

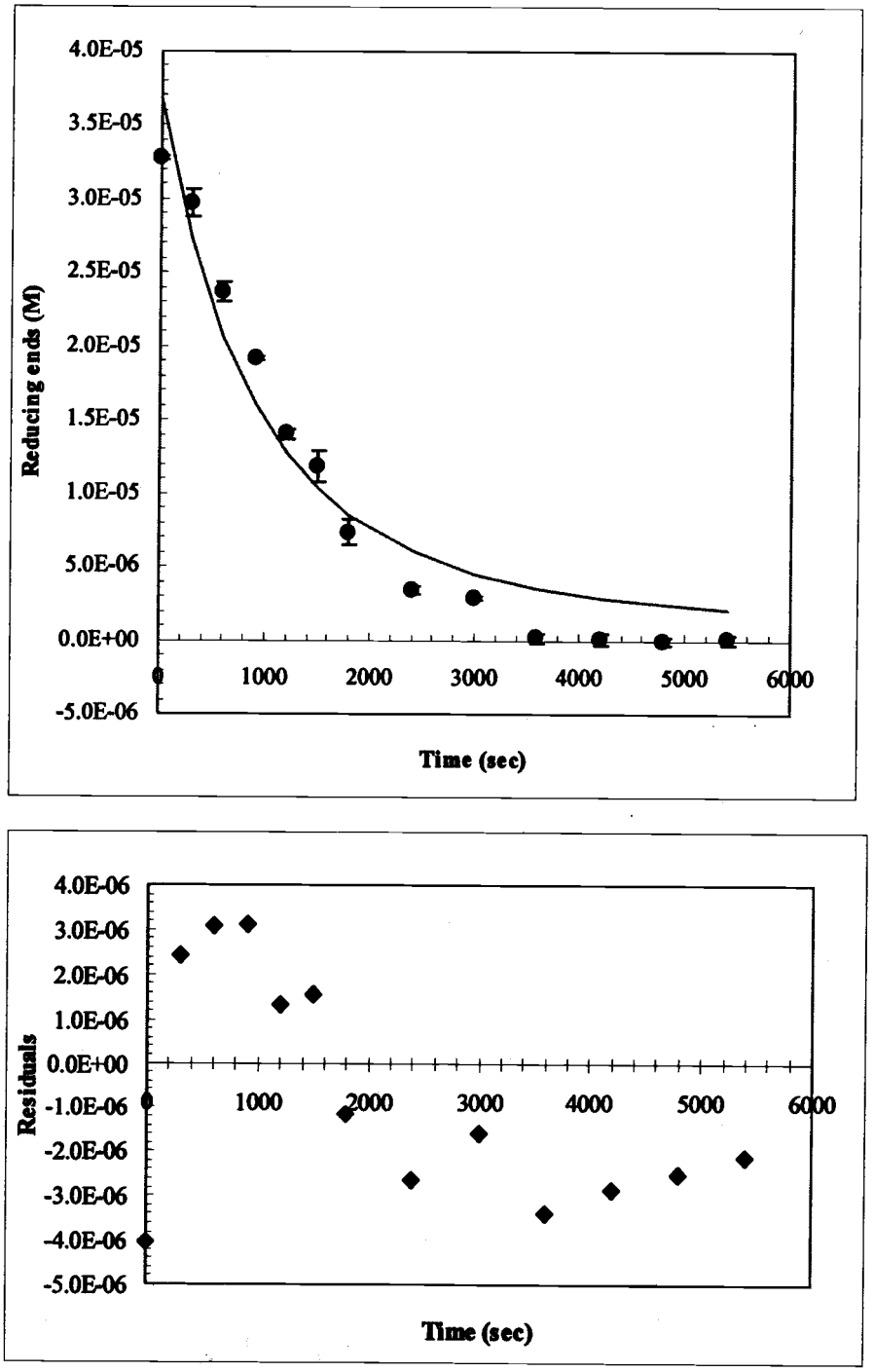
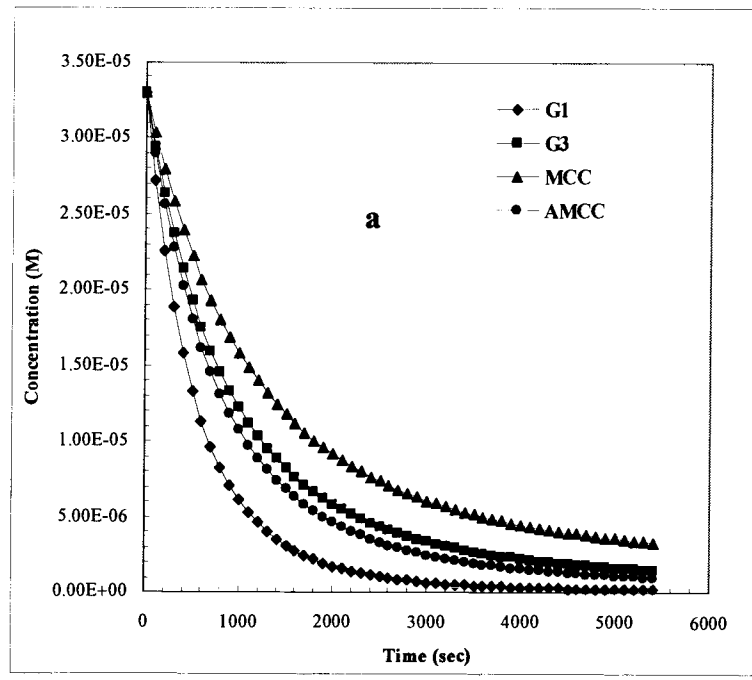


Figure 4.5 f Representative time course of cellotriose showing the application of Method 3. Data for each of the associated residual plots were obtained as described in "Materials and Methods" section of text.

The implication being that the celluloses, having much greater DPs, will be reduced at rates similar to those of the soluble cellooligosaccharides provided the terminal glucosyl units behave in a manner analogous to that of terminal residues that remain in solution. If the terminal glucosyl units of the celluloses are found to have significantly lower reaction rates, then one may surmise that the reaction has been perturbed as a result of the nature of the insoluble substrate. Whether this perturbation is the result of an electronic inductive effect or some steric effect, such as may restrict $NaBH_4$ access to the terminal *aldehydo* group, is not distinguished in this study.

The relative reactivity of the different classes of test compounds (glucose, cellooligosaccharides, amorphous cellulose, and microcrystalline cellulose) are illustrated in Figures 4.6 a and b. The solvent-accessible reducing ends of the amorphous celluloses behave, with respect to $NaBH_4$ reduction, as though they are free in solution. This result was not a given in that amorphous celluloses are insoluble and are expected to have regions of partial ordering (Newman & Hemmingson, 1994). The terminal glucosyl units of the microcrystalline cellulose, however, are herein shown to be chemically distinct from those of their soluble cellooligosaccharide analogs. This finding seems reasonable in that a higher degree of order may be expected at the surface of microcrystalline cellulose preparations (Newman & Hemmingson, 1994).



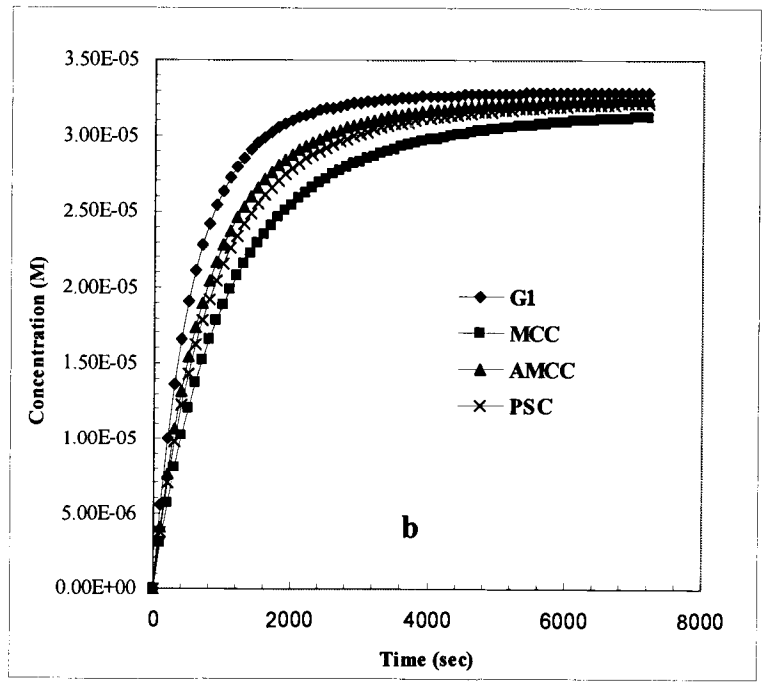


Figure 4.5 Comparison of simulated time courses for the reaction of test compounds with (a) sodium borohydride and (b) ^{3}H-labelled sodium borohydride. Time courses were generated using the best-fit second order rate constants of Tables 4.1 and 4.2. Extents of reaction for glucose (G1), cellotriose (G3), microcrystalline cellulose (MCC), amorphous microcrystalline cellulose (AMCC) and phosphoric acid swollen cellulose (PSC) are expressed as described in Figures 4.1 and 4.4.

4.5 CONCLUSION

These results give chemical evidence in support of the concept that the energetics of the initial interactions between exo-acting cellulases and the reducing ends of typical cellulose substrates will be at least partially dependent on the crystalline nature of those substrates. The implication is that the reactivity of the terminal residues of surface molecules in amorphous substrates, but not crystalline substrates, approaches that of the terminal residues of the soluble cellooligosaccharides.

4.6 REFERENCES

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CHAPTER 5

DETECTING ENZYME ACTIVITY: A CASE STUDY OF POLYGALACTURONASE

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5.1 ABSTRACT

Chemical and physical approaches to monitoring enzyme activity are illustrated using polygalacturonase as the focus enzyme. Polygalacturonase is a depolymerase that catalyzes the hydrolysis of 1,4-glycosidic linkages in linear homogalacturonan regions of pectic polymers. Activity measurements of this enzyme may be based on the generation of new product as a consequence of catalysis, such as the generation of reducing sugars. Two basic assay protocols, reducing sugars- and viscosity-based assays, are presented here. Discussion of approaches to enzyme extraction and critical parameters for maintaining assay specificity are included.

Keywords: polygalacturonase, polygalacturonic acid, viscosity, reducing sugar, depolymerase, enzyme, extraction

5.2. INTRODUCTION

Polygalacturonase (PGase) is the trivial name commonly used in reference to poly-α-1,4-galacturonide glycanohydrolases (EC 3.2.1.15). These enzymes are glycosyl hydrolases, splitting the 1,4 glycosidic linkage between galacturonic acid residues in their linear polygalacturonic acid substrate. PGases can be either endo- or exo-acting. The endo-acting enzymes, those that are capable of catalyzing the hydrolysis of linkages in the interior of the polysaccharide chain, appear to be the most common. The PGase enzymes are most commonly assayed using either reducing sugar- or viscosity-based methods. The former is based on the generation of a new reducing sugar with each catalytic event. The latter is based on the decrease in viscosity that occurs as a result of the enzyme-catalyzed hydrolytic degradation of polygalacturonic acid (Basic Protocol 2). The Support Protocol describes a method for isolating PGase from tomatoes.

STRATEGIC PLANNING

The two methods described herein are inherently different in that one is a traditional initial velocity assay that attempts to quantitatively measure rates of product formation (see Basic Protocol 1) whereas the other correlates the activity of an enzyme preparation with its ability to change the rheological properties (i.e., viscosity) of a substrate solution (see Basic Protocol 2). For both assays, it is presumed that the analyst is using soluble substrate and enzyme preparations, appropriate buffer systems, and a method to control the reaction mixture

temperature. The ultimate goal of both assays is the same: to obtain a quantitative estimate of the PGase activity of test solution.

Basic protocol 1 makes use of the fact that one of the products resulting from the PGase-catalyzed reaction is a reducing sugar (Figure 5.1). Thus, in the simplest case, a determination of the number of reducing ends generated during a given reaction period will directly correspond to the number of catalytic events during that period. The assay requires a measurement of the number of reducing ends present at the initiation of the reaction and at a sufficient number of time points to establish an initial velocity. The assay is based on measurements at predetermined time points, rather than continuously monitoring the extent of reaction, because of the relatively harsh conditions necessary for the detection of reducing-sugars. The assay is equally sensitive to endo- and exo-acting PGases, because both enzymes generate a single reducing end per catalytic event.

Basic protocol 2 is based on the viscosity change that occurs in a polygalacturonic acid containing solution as a result of enzyme activity. The assay does not directly measure the number of catalytic events, as is attempted in Basic Protocol 1, but rather a physical parameter that is a function of the number of catalytic events. Viscometric assays are typically done using capillary viscometers of the Ostwald type at a fixed predetermined temperature. Units for these assays are reported as either a function of the time required to reduce the viscosity of a given solution by a fixed percentage (Bateman, 1963) or as that amount of enzyme that will reduce the viscosity of the given solution by a fixed percentage in a fixed amount of time (Tagawa and Kaji, 1988).

The substrate prescribed for these assays is commercially available polygalacturonic acid. It was chosen based on its availability and on the observation that PGase enzymes are typically specific for glycosidic linkages between de-esterified galacturonic acid units (Schols and Voragen, 2002). Other substrates, such as citrus pectin, may be used in some cases, but overall PGase activities will likely be lower on the esterified pectins and results from assays using the esterified pectins are more likely to be influenced by nonPGase enzymes, particularly pectin lyase and pectin methyl esterase (see Commentary).

The reaction conditions chosen for the assays are based on published optimum conditions for PGase enzymes. These enzymes typically have maximum activities at slightly acidic pH (Tucker and Seymour, 2002) and, in general, appear to be relatively stable at temperatures from 30 to 40°C. Optimum reaction conditions are likely to be enzyme specific, so one may have to alter the conditions to match the properties of the enzyme of interest. In all cases, the analyst should take into account the properties of the substrate, particularly solubility, as well as the properties of the enzyme. For example, because solutions of polygalacturonic acid tend to gel as the pH is lowered below 3, viscometric assays (Basic Protocol 2) at these relatively low pHs are often not feasible.

The PGase extraction scheme outlined below (see Support Protocol) is based on the properties of the enzyme from tomato (Pressey, 1986). Enzymes from other sources will likely require different extraction conditions. A more detailed discussion of common approaches to enzyme extraction is included (see Background Information, discussion of samples for pectic enzyme assays).

5.3 MATERIALS AND METHODS

5.3.1 BASIC PROTOCOL 1

DETERMINATION OF POLYGALACTUROASE ACTIVITY USING 2,2'BICINCHONINIC ACID

Polygalacturonase (PGase) is most commonly assayed by measuring the liberation of reducing groups in reaction mixtures containing the enzyme and a suitable polygalacturonic acid substrate. The reaction conditions used in such assays correspond to those optimum for the enzyme, those pertinent to a particular physiological/food system or, (for comparative purposes) those previously established. In this assay we prescribe conditions that are in the functional range of typical PGases.

There are several colorimetric assay reagents available for measuring the number of reducing groups generated over the course of the PGase reaction. Those include the following: Nelson copper-arsenomolybdate (Nelson, 1944; Takawa and Kaji, 1988), 2-cyanoacetamide (Honda *et al.*, 1980; Gross, 1982; Bach and Schollmeyer, 1992), 3-methyl-2-benzothiazolinone hydrozone (Anthon and Barrett, 2002), 2,2'-bicinchoninate (Doner and Irwin, 1992; Gracia *et al.*, 1993), modified dinitrosalicylic acid (Wang *et al.*, 1997) and 4-hydroxybenzoic acid hydrazide (Lever, 1972; Atkinson *et al.*, 2002). The assay presented here, "Basic Protocol 1", uses the 2,2'-bicinchoninate acid method to quantify reducing ends.

The assay is presented as though a single unknown enzyme preparation is being tested for PGase activity. The assay includes the determination of reaction rates at two enzyme loads (plus corresponding enzyme and a substrate blanks) to verify that the measured activity is proportional to the amount of enzyme in the reaction mixture. It suggests measuring four time points per enzyme load, in order to ascertain appropriate initial velocities. The assay does not indicate the number of replicates for each measurement, because this is partially dictated by familiarity with the assay. The authors suggest doing all assays in either duplicate or triplicate.

The assay contains both enzyme and substrate-blanks. For the purpose of this unit, the enzyme-blank contains all reaction mixture components except the enzyme preparation, and .the substrate blank contains all reaction mixture components except the substrate.

Materials

0.25 % (w/v) polygalacturonic acid solution (see recipe)
20 mM sodium acetate, pH 5.0
BCA working reagent (see recipe)
Enzyme preparation (see Strategic Planning and see Support Protocol)
1mM galacturonic acid (see recipe)
12 x 75-mm glass test tubes
37 ^{0}C and 80 ^{0}C water baths

Set up reaction tubes

1. Label five 12 x 75-mm glass test tubes as follows:

   Enzyme Load 1 and 2,
   Substrate Blank 1 and 2,
   Enzyme Blank.

   If checking the PGase activity associated with a single enzyme preparation, there will be five test solutions. The enzyme load and substrate blanks are done at two different enzyme concentrations. With duplicate and triplicate analyses, there will thus be ten or fifteen tubes in total.

   The substrate blanks and enzyme blank are used to account for any apparent rates of reaction that are attributable to factors other than the specific interaction of the PGase enzyme with the polygalacturonic acid substrate.

2. To each of the substrate-containing tubes (enzyme load and enzyme blank) add 0.2 ml of 0.25% polygalacturonic acid solution and the appropriate amount of 20 mM sodium acetate such that the final volume of the reaction mixture will be 1.0 ml following the addition of the enzyme preparation (step 6).

3. To the two substrate blank tubes, add the appropriate amount of 20 mM sodium acetate such that the final volume of the reaction mixture will be 1.0 ml following the addition of enzyme preparation (step 6).

4. Place all tubes in a 37°C water bath and allow time for them to equilibrate to that temperature.

Initiate reaction and collect time points

5. Set up a series of four 12 x 75-mm glass test tubes for each of the five test solutions described above. Label tubes within each set to correspond to an

appropriate time point (e.g., 0, 30, 60, and 120 min). Add 1 ml cold BCA working reagent to each tube and place tubes on ice.

These tubes will be used for termination of the reaction and for color development of the reducing sugar.

6. Initiate the reaction in the enzyme-containing tubes (all tubes except the enzyme blank) by adding the appropriate amount of enzyme preparation with gentle mixing. Maintain reaction mixture at 37°C throughout the reaction period.

   The amount of enzyme preparation added will depend on the relative activity of the enzyme preparation. A convenient starting point would be to add 40 µl of enzyme preparation to enzyme load 1 and substrate blank 1 and 80 µl of enzyme load 2.and substrate blank 2.

7. At the specified times (e.g., 0, 30, 60 and 120 min), remove 0.1 ml reaction mixture (or blank solution) and add it to its corresponding tube containing BCA reagent (step 5). Terminate the 0 time point immediately after the reaction is initiated and place on ice. Proceed until the final time points are collected.

   The high pH of the resulting solution, combined with the relatively low temperature, will effectively terminate the reaction.

8. Include with the five sets of termination tubes a series of five tubes to be used to establish the calibration curve. Place in the calibration tubes a mixture containing 0, 5, 10, 15, or 20 µl of 1 mM galacturonic acid, then 20 mM sodium acetate to bring volume to 0.1 ml, next add 1 ml BCA working reagent (total volume 1.1 ml per tube). The calibration points can

be done in either duplicate or triplicate, depending on the experience of the analyst.

The standards correspond to 0, 5, 10, 15, and 20 nmol galacturonic acid.

Perform color development

9. Transfer all of the BCA-containing reaction mixtures to an 80°C water bath to initiate color development. Cover each tube with a glass marble, Parafilm, or other suitable covering to minimize evaporation, and leave tubes in bath for 30 min.
10. Transfer to cold water for 10 min and centrifuge 5 min at 14,000 x g, room temperature, to clarify solutions.
11. Read absorbance with a spectrophotometer set at 560 nm using water as the reference.

Calculate results

12. For each test solution, plot time versus absorbance and obtain the slope (i.e., the reaction rate) by linear regression.
13. Correct the rate of reduction for the enzyme/substrate solution by subtracting the values obtained for the corresponding substrate and enzyme blanks.
14. To generate a calibration curve, use data from the galacturonic acid-containing tubes (step 8) and plot moles galacturonic acid versus absorbance.
15. Convert absorbance values to moles galacturonic acid equivalent using the calibration curve.

The PGase activity of the unknown enzyme preparation, in terms of reducing groups generated per unit time, is based on the corrected rate of increase in galacturonic acid equivalents for the experimental enzyme/substrate test solutions. One unit of enzyme activity (katals) is defined as that amount of enzyme that liberates 1 mole of reducing sugar per second under the defined conditions.

5.3.2 BASIC PROTOCOL 2

VISCOSITY-BASED ASSAY FOR POLYGALACTURONASE ACTIVITY

Polygalacturonase (PGase) is an example of a polymer depolymerase enzyme, and the activity of this type of enzyme has been measured by viscosity-based methods for many years. These assays are based on the presumption that the viscosity of a polymer-containing solution is a function of the molecular weight of its component polymers. Thus, in this assay the degradation of polygalacturonic acid, as catalyzed by PGase, results in a lowering of the viscosity of the polygalacturonic acid containing solution. In this assay the viscosity of the substrate solution is determined as a function of the efflux time required for the solution to pass between the indicated marks on an Ostwald viscometer. The assay has evolved for over forty years (Mill and Tuttobello, 1961; Bateman, 1963; Tagawa and Kaji, 1988; and Gusakov *et al.*, 2002); the one presented here is adapted from Tagawa and Kaji (1988) and Gusakov *et al.* (2002).

Materials

0.7% to 1.0% (w/v) sodium pectate in 20 mM sodium acetate, pH 5.0.
Enzyme preparations (see Support Protocol), both active and heat inactivated, in 20 mM sodium acetate, pH 5.0.
Ostwald viscometer (capillary diameter of 0.5 mm; Fisher Scientific)
Constant- temperature mechanism (e.g. appropriate water bath)

1. Pipet 5.0 ml of 0.7% to 1.0% sodium pectate solution into an Ostwald viscometer and bring to 30°C.

   The efflux time of the substrate solution in the viscometer should be in the range of 115-120 seconds.

2. Add 100 µl active enzyme preparation to the viscometer with mixing. Start a timer to monitor reaction time.

   Adding the enzyme initiates the degradation of polygalacturonic acid.

3. Determine an efflux time immediately after initiating the reaction (using a second timer) and then at suitable interval to allow graphical determination of the time to reach a 50 % reduction in viscosity.

   If the reaction proceeds too quickly, the enzyme preparation should be diluted with buffer and the assay repeated. One should attempt to obtain a minimum of two time points prior to there being a 50% reduction in the relative viscosity of the reaction mixture.

4. Set up a second reaction as described above but in this case use a heat-inactivated enzyme preparation. The efflux time of this reaction mixture is to be used as the zero-time data point, "V_0".

   In general, enzymes can be thermally inactivated by bring the enzyme-containing solution to 95-100^{0}C for 5 minutes. However, one should always verify that this treatment does indeed result in complete inactivation (via a check for residual activity), since some enzymes are particularly heat stable.

5. To obtain the solvent efflux time, "V_s", prepare yet another reaction mixture as above with the exception that the mixture, of equivalent final volume, contains only buffer and inactivated enzyme.

6. Determine the percentage fall in viscosity (A), which is defined as follows (Roboz et al., 1952).

$$A = [(V_0 - V_t) / (V_0 - V_s)] \times 100$$

Where V_0 is the efflux time of sodium pectate solution plus inactivated-enzyme, V_t is the efflux time of sodium pectate solution plus enzyme, and V_s is the efflux time of inactivated enzyme plus buffer solution. All efflux times are measured in seconds.

A unit of activity is defined arbitrarily as that amount of enzyme that will reduce the viscosity of the sodium pectate solution by 50% in a 5-min period.
The time required to reach a value for A = 50% is typically inversely proportional to the concentration of enzyme (Mill and Tuttobello, 1961; Gusakov et al., 2002).

5.3.3 SUPPORT PROTOCOL

POLYGALACTURONASE ENZYME PREPARATION

In most food applications the analyst is working with rather complex matrixes. This raises the question of how to quantitatively extract a representative fraction of the enzyme to be assayed. In the best-case scenario, all or a representative fraction of the active target enzyme will be obtained in a solution devoid of other components that may hinder the assay (compounds that may affect either the enzyme itself or some other aspect of the assay). The objective of many assays is to measure the total amount of enzyme activity associated with a particular sample; this determination is dependent on the quantitative extraction of the target enzyme. In all cases, it is essential that the enzyme preparation step be clearly defined when reporting the amount of enzyme associated with a given product, since this step is likely to have a major impact on measured activities. Furthermore, it should not be assumed that an enzyme preparation protocol

optimized for a particular sample/product is necessarily optimum for a different sample.

Optimum conditions for the extraction of polygalacturonase (PGase) depend on the sample matrix (type of food product) and the source of the enzyme (plant versus microbial origin). The extraction scheme presented below is that developed for the PGase of tomato (Pressey, 1986).

Materials

Tomato, slice
Water, 4 ^{0}C
1.2 M NaCl, pH 6.0
Homogenizer
Additional reagents and equipment for dialysis or size-exclusion chromatography.

1. Add 100 g sliced fruit to 100 ml 4°C water and homogenized.
2. Adjust the homogenate pH to 3.0 with 0.1 N HCl and leave to settle for 15 min at 4°C.
3. Centrifuge20 min at 8,000 x g and the discard supernatant.
4. Wash pellet twice, each time suspending the pellet in cold water pH ~ 3.0 (adjust pH as necessary with 0.1 N HCl), followed by centrifugation at 8,000 x g for 20 min and subsequent decantation of the supernatant.
5. Extract PGase from the washed pellet by suspensing pellet in 100 ml of 1.2 M NaCl at pH 6.0 and centrifuging 20 min at 8,000 x g. Collect supernatant.
6. Remove salt while exchanging the reaction mixture with an appropriate buffer system by dialysis or size-exclusion chromatography.

The molecular weights of PGases from different sources are expected to vary. For example, tomato PGase exists in two forms, PG1 and PG2. PG1 and PG2 have molecular weights of 84 and 44 kD, respectively (Wong, 1995). Hence, one should not assume a molecular weight for novel polygalacturnases-this being important when choosing separation techniques based on size-exclusion principles.

5.3.4 REAGENTS AND SOLUTIONS

Use deionized and/or distilled water in all recipes and protocol steps.

2,2'Bicinchoninic acid solution (BCA)

For assay solution A: Dissolve (in order) 12.1 g sodium bicarbonate ($NaHCO_3$), 27.4 g sodium carbonate (Na_2CO_3) and 971 mg bicinchoninic acid sodium salt in 450 ml distilled water, in order, with stirring. Bring to 500 ml with distilled water in the volumetric flask. Store in the dark bottle up to 1 month at room temperature

For assay solution B: Dissolve 624 mg copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and 631 mg L-serine with stirring. Bring to 500 ml with distilled water in the volumetric flask. Store in the dark bottle up to one month at 4 ^{0}C.

Working BCA reagent: On the day of the experiment by mixing equal volumes of "assay solution A" and "assay solution B".

Galacturonic acid, 1 mM

Prepare stock 10 mM galacturonic acid solution by dissolving 0.2122 g D-galacturonic acid monohydrate in 100 ml 20 mM sodium acetate buffer, pH 5.0. Dilute this stock solution 10-fold with buffer with buffer. Store up to 1 week at 4 ^{0}C.

Polygalacturonic acid, 0.25 % (w/v)

Disperse 0.5 g of polygalacturonic acid (Sigma) in 100 ml distilled water by stirring at room temperature in a 1-liter beaker. With continued stirring, add 400 ml 95 % ethanol to precipitate the polygalacturonic acid (free galacturonic acid will remain in solution). Collect the precipitate by vacuum filtration of the wash-suspension through Whatman #1 paper. Dry the washed polygalacturonic acid at 22 ^{0}C overnight.

Dissolve 0.25 g ethanol-washed polygalacturonic acid in 70 ml 20 mM sodium acetate buffer. With continued stirring, adjust the pH to 5.0 with 0.1 N NaOH; then bring to a final volume of 100 ml with buffer. Divide into aliquots and store at 0^{o}C to 2 months.

5.4 RESULTS AND DISCUSSION

5.4.1COMMENTARY (Background Information)

Pectin polysaccharides

Pectins are a group of polysaccharides found in the primary cell walls and intercellular regions of higher plants; they are typically the most abundant polymers in these regions of fruit (Willats *et al.*, 2001). They can be extracted with relatively mild aqueous solvents (such as mildly acidic or basic and/or chelator containing water). Their function appears to be to maintain the integrity and rigidity of cell walls, to enhance water retention, and to act as an adhesive between cells (Schols and Voragen, 2002). Polygalacturonase (PGase) is a hydrolytic enzyme that acts on pectic polysaccharides.

Pectic polysaccharides are heterogeneous with respect to chemical structure and size. The major components are polymers comprised primarily of α–D-galactopyranosyluronic acid building blocks. Linear polymers containing only these moieties, associated via covalent (1→4)-linkages, are termed homogalacturonans. Varying percentages of the individual building blocks in homogalacturonans are typically esterified at the 6-position carboxyl group with methanol. More complex pectic polysaccharides, having neutral sugar-containing branched segments interspersed along the linear homogalacturonan polymer, have also been obtained from several fruit tissues (Schols *et al*, 1990). The different pectin polymers are typically divided into several different groups: homogalacutronans, xylogalacturonans, rhamnogalacturonans, arabinans, arabinogalactans, and apiogalacturonans. The key feature for the major pectic polysaccharides is the presence of linear chain regions comprised of (1→4)-linked α–D-galactopyranosyluronic acid units (BeMiller, 1986).

Pectin enzymes

Pectic enzymes are produced by plants and microorganisms. The major depolymerase enzymes-that is, those that split the α- (1→4)-glycosidic linkages in the backbone of the homogalacturonans, are polygalacturonase (PGase) and pectic lyase (PL). The other major pectic enzyme is pectinesterase (PE). PE catalyzes the removal, via hydrolysis, of methoxy groups from methylated pectic substances. PGase (EC 3.2.1.15) is hydrolytic pectin depolymerase produced by both

microbial and plant tissues. It catalyzes the hydrolysis of the α- (1→4)-linkages between two adjacent galacturonic acid residues within the homogalacturonan backbone (Figure 1). Generally, PGases act at glycosidic linkages adjoining two de-esterified galacturonic acid resides. The enzymes normally have pH optima in the acid range (pH 4-6) and can be either exo- or endo- acting; endo-acting PGAase being the most common (Tucker and Seymour, 2002).

PL (EC 4.2.2.2; sometimes called pectate transeliminase) is a pectin depolymerase like PGase, but it is believed to be produced only by fungi and bacteria, not higher plants. The PL-catalyzed reaction goes via a β-eliminative cleavage. The reaction generates a new reducing end and a new non-reducing end (Figure 5.2). The new non-reducing end differs from that produced in the PGase-catalyzed reaction in that the terminal sugar unit is a 4-deoxy-α-D-galacto-4-enuronosyl group. The pectate lyases are typically subdivided based on their specificity for pectinic versus pectic acid and whether they are exo- or endo-acting. The pH optima of the pectate lyases is generally in the range of 8.5 to 9.0 and all appear to require Ca^{2+} for activity (Whitaker, 1994).

Pectic enzyme assays

Reducing end-based assays are widely used to measure pectin depolymerases. These assays take advantage of the fact that the primary depolymerase enzymes, PGase and PL, generate a new reducing end as a consequence of each catalytic event. Therefore, enzyme activity can be followed

by monitoring the increase in reaction mixture reducing ends as the depolymerases act on the appropriate substrate, either pectic or pectinic acid. Quantification of reducing ends is typically done using a colorimetric assay under highly alkaline conditions. Hence, they are discontinuous assays – in which the reaction is terminated and the number of reducing ends determined. There are several different colorimetric assays for the quantification of reducing ends (Anthon and Barrett, 2002; Gross, 1982; Nelson, 1944; Doner and Irwin, 1992).

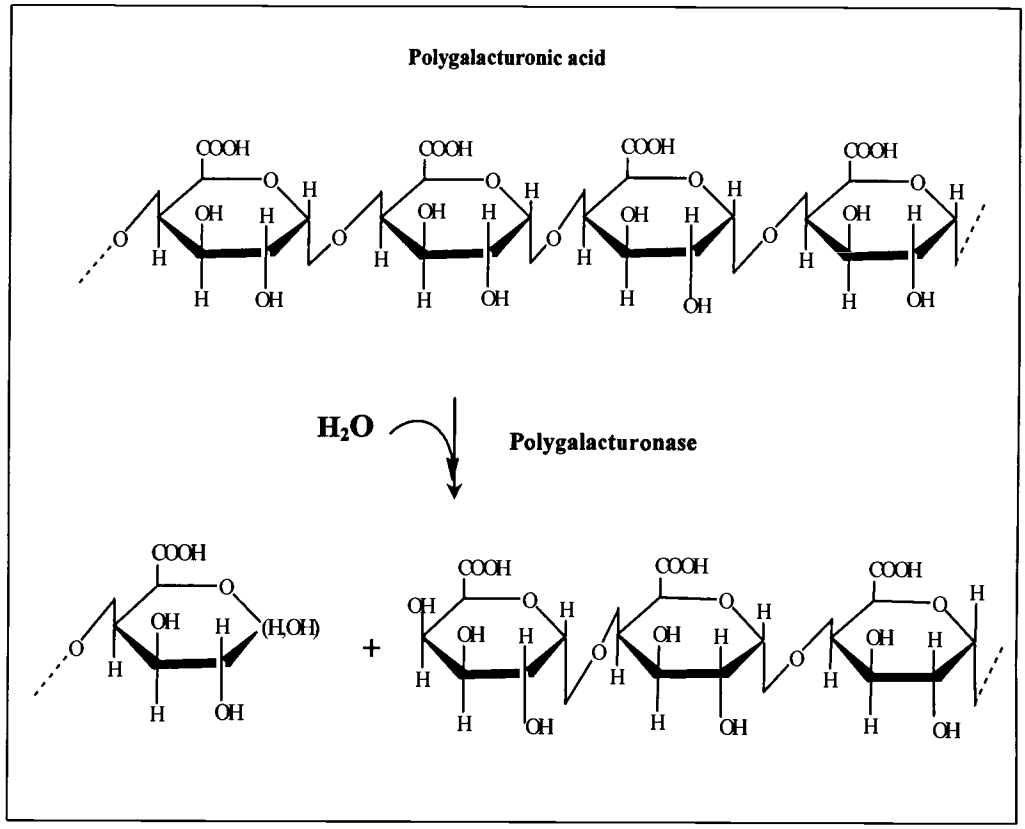

Figure 5.1. Reaction catalyzed by polygalacturonase

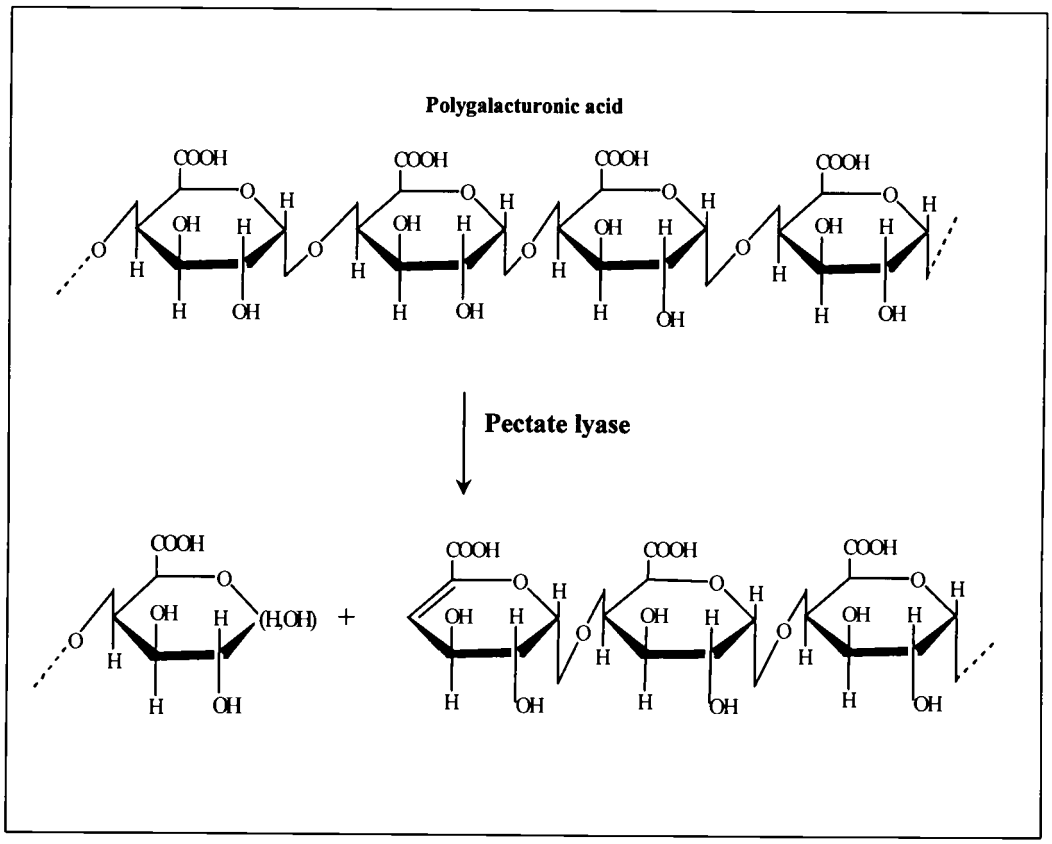

Figure 5.2. Reaction catalyzed by pectate lyase

When working with pure PGase, the results of the reducing sugar assay presented here will clearly reflect the inherent activity of that enzyme on the chosen substrate. However, in many cases analysts are working with rather crude enzyme preparations that may contain a combination of PGases and PLs. In such cases, reducing sugar-based assays would be expected to reflect a combined PGase and PL activity. Hence, if one wants to focus only on PGase, then it is important to account for potential lyase activity. This is done by choosing reaction conditions that favor PGase activity; such as using a low methoxy pectin substrate (i.e. polygalacturonic acid), a relatively low reaction mixture pH, and avoiding the presence of calcium chelator (to sequester Ca^{2+}). Even with these optimized conditions, however, it is possible that lyase activity is occurring. To check for this, a rather simple spectrophotometric assay, based on the fact that the lyase reaction yields, along with the new reducing end, a 4,5-unsaturated non-reducing end that absorbs radiation of 232 nm, can be used (Collmer *et al.*, 1988).

PGase and PL, as stated above, are both depolymerases. Viscosity assays have been used to measure combined depolymerase activity for over fifty years (Owens *et al.*, 1946). The viscosity of pectin-containing solutions, as well as that of other polymer containing solutions, is a function of the shape and molecular weight of the component polymers. Depolymerase activity is expected to lower the viscosity of a polymer containing solution, provided that other parameters affecting viscosity remain constant, due to their activity resulting in a lowering of the polymer's molecular weight. Hence, the rate of reduction in the viscosity of a

polymer containing solution can be used to measure depolymerase activity. Viscosity-based assays are more sensitive to endo-acting depolymerases, compared to exo-acting depolymerases, since on average, there is a greater change in molecular weight per endo-depolymerase catalyzed reaction.

Traditional viscosity assays use either a capillary or a rotating spindle viscometer (Sherwood and Kelman, 1964; Gusakov *et al.*, 2002). The viscometric assay presented in Basic Protocol 2, is intended to measure PGase activity, with minimal interference from PL. PGase activity is favored by using polygalacturonic acid as the substrate rather than pectins with higher methoxy content, by using a relatively low reaction mixture pH, and by avoiding the addition of Ca^{2+} to the reaction mixture. We recommend that PL activity be monitored, as discussed above (Collmer *et al.*, 1988), at least during the initial phases of the analysis to confirm that one is not measuring combined PGase/PL depolymerization is not measured.

Samples for pectic enzyme assays

In the Support Protocol, the tomato puree is first washed with water to remove water-soluble components, particularly reducing sugars, and then extracted with an equal weight of 1.2 M NaCl. The procedure is appropriate for this application because the tomato enzyme is sparingly soluble in water at pH 3, but is soluble in relatively high salt solutions. The advantage of this procedure is that the water-soluble components can be easily removed prior to extracting the enzyme. This is particularly nice for the reducing sugar-based assay because the soluble

reducing sugars inherent in the enzyme-containing sample add unwanted background signal in the final assay (*i.e.* they give a high "blank" absorbance reading).

The authors have applied this same procedure to peach purees, although at the pH of the peach product (~3.9). In that case however, the PGase activity associated with the peach puree was relatively soluble in water. Hence, the initial water extracts had significant PGase activity. Therefore, one may choose to assay the water extract itself. As noted above for removal of salt, the soluble reducing sugars in the water extract can be removed by techniques such as dialysis or size-exclusion chromatography.

Difficulties in extracting enzyme from different sample matrixes may be the result of the enzyme (1) is covalently or non-covalently associated with insoluble components, (2) is physically entrapped in the tissue matrix such that it is solvent inaccessible, or (3) is itself being insoluble in the chosen solvent. Several papers suggest that a percentage of the PGase inherent in the tomato is immobilized, and thus insoluble, in the cell wall of that fruit (Pressey, 1986; Hobson, 1964; Jackman *et al.*, 1995). Researchers have developed two experimental approaches to getting this enzyme into solution. First, some of the "immobilized", or in-extractable, enzyme can be freed from the fruit by disrupting the tissue's ionic complexes (via addition of high salt or increasing the pH of the extraction buffer to >6). The ionic complexes probably include negatively charged pectate polymers with cationic regions on the PGase enzyme. Second, some of the

immobilized enzyme can be solubilized by disrupting the physical structure of the cell wall thru chelation of Ca^{2+}.

It is not clear if the types of interactions that dictate the behavior of tomato PGase are common to other PGase-food systems. Certainly one may expect that microbial PGase, for instance those associated with rotting fruit, will have somewhat different associations with the food matrix. Enzyme-tissue ionic complexes are likely to be important in the case of exogenous microbial PGase, but probably not physical entrapment.

5.4.2 Critical Parameters

The assays for PGase activity presented here are based on measurements of reaction rates. Hence, all experimental parameters that may affect the rate of an enzyme-catalyzed reaction (including pH, ionic strength, buffer composition and temperature) need to be defined. Theses parameters are typically chosen to coincide with optimum or biologically relevant conditions. The protocols presented here specify reaction conditions that are appropriate for typical PGases. An analyst may choose to change these conditions to better reflect the properties of a novel enzyme or to better simulate the conditions present in a particular food product.

The reducing sugar-based assay is intended to provide initial velocity kinetics. This means that the analyst must establish, for all experimental permutations, that initial rates are indeed being measured. These topics, as well as

other information related to the design of enzyme assays, are discussed in *UNIT C1.1*.

The substrate chosen for the assays presented in this chapter is polygalacturonic acid. As explained previously, this substrate is advisable in that PGases typically have higher activity, and lyases lower activity, toward this low methoxy substrate. However, an analyst may want to use a more highly esterified substrate in order to simulate pectins of a particular product. If this is the case, then the analyst must consider two potential complications. First, the PGase activity toward the more esterified substrate is likely to be sensitive to the presence of pectin methylesterase activity. This type of interaction is not a problem when working with pure enzymes, but can be a problem when using crude enzyme preparations. Significant methylesterase activity will create regions of low methoxy content in the esterified substrates, and these low methoxy regions are likely to be more susceptible to PGases. The net result of simultaneous methyl esterase activity would be an increase in measured PGase activity. The second potential complication is that common reducing sugar assays make use of relatively high temperatures (80–100°C) and alkaline reaction mixtures (pH > 10). These conditions are conducive to the production of non-enzyme-derived reducing sugars via a base-catalyzed β-elimination reaction (Keijbets and Pilnik, 1974). This may lead to the production of non-polygalacturonidase-derived reducing sugars. This complication can be avoided by using Basic Protocol 2.

In some cases, particularly when working with samples containing low PGase activity, it may be necessary to run assays for extended reaction periods.

One of the concerns with extended assays is the potential for microbial growth to interfere with assay results. This may be avoided by including an antimicrobial agent in the reaction mixture. The stipulation that goes with this approach is that the antimicrobial must not significantly interfere with the assay. We have used thimerosal and sodium azide as antimicrobial agents in PGase assays. Thimerosal was initially chosen as the antimicrobial because it has been shown to be effective in other enzyme assays without having an adverse effect on enzyme activity. However, a problem with thimerosal is that it reacts with the BCA reagent; so when using this antimicrobial an alternative reducing sugar assay, such as the MBTH procedure (Anthon and Barrett, 2002), must be employed. Sodium azide is another widely used antimicrobial and it does not appear to interfere with the BCA assay.

5.4.3 Anticipated Results

The results from Basic Protocol 1 are expected to be consistent with traditional initial velocity assumptions for enzyme kinetics (see *Unit C1.1*). The assay, as presented, includes four time points (along with a zero-time value) in order to establish the relationship between reaction time and product formed. Representative data, demonstrating the hyperbolic nature of this relationship, is presented in Figure 5.3. In this case, only the initial time points at the lowest enzyme concentrations are consistent with the linear initial velocity assumption. If that relationship is required for estimating enzyme activity, then one may try to improve in the progress of the reaction or by further diluting the enzyme

preparation and re-assaying over the same time frame. As will be seen in the following paragraph, a linear relationship between enzyme load and product generated per specified time is obtained in the present case without measurements of true initial velocities.

The assays presented herein are based on there being a linear relationship between the amount of active enzyme present in a reaction mixture and the observed activity for that reaction mixture. To verify this, the analyst must assay a minimum of two enzyme concentrations (as suggested in Basic Protocol 1). The expected relationship is demonstrated in Figure 5.4; in this case four enzyme loadings were assayed and, as expected, the measured activity is shown to be proportional to the amount of enzyme present.

The reducing sugar-based assays are predicated on the correct measurement of enzyme-generated product. A typical calibration curve for the BCA assay, using galacturonic acid as the calibration standard, is presented in Figure 5.5. Note that the assay is linear for the analysis of up to approximately 20 nMoles reducing ends. If an enzyme assay generates product extending beyond this range, then the reaction mixture should be appropriately diluted prior to quantification of reducing sugars.

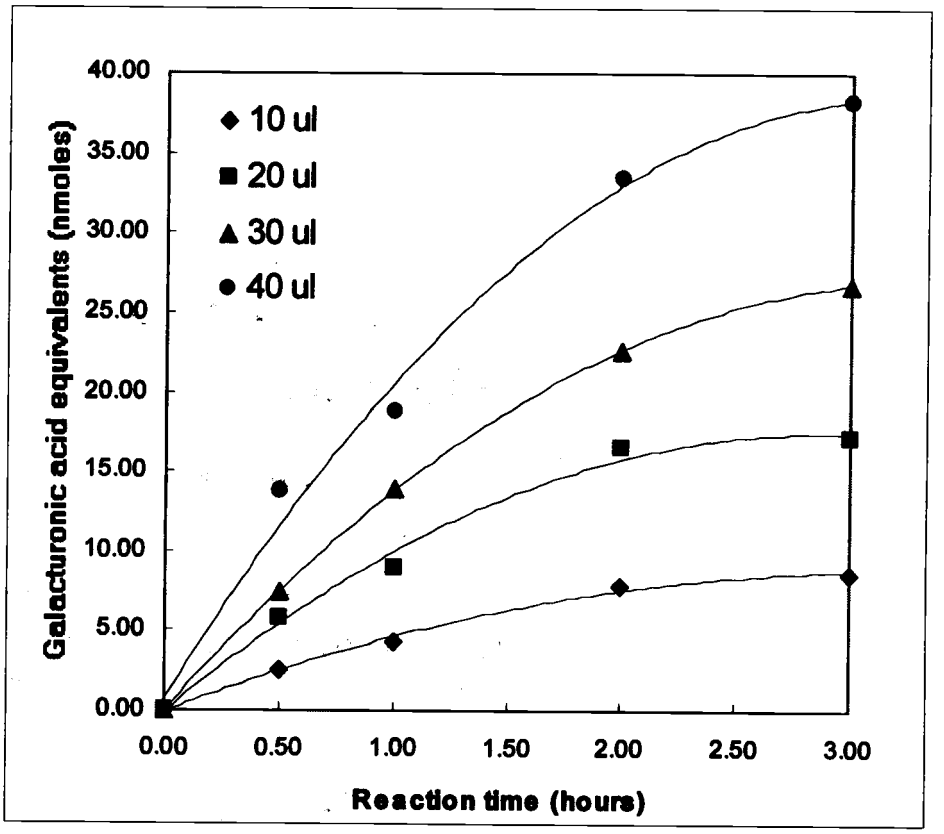

Figure 5.3. Reaction progress curves for the production of new reducing ends (measured as galacturonic acid equivalents) at different enzyme loads.

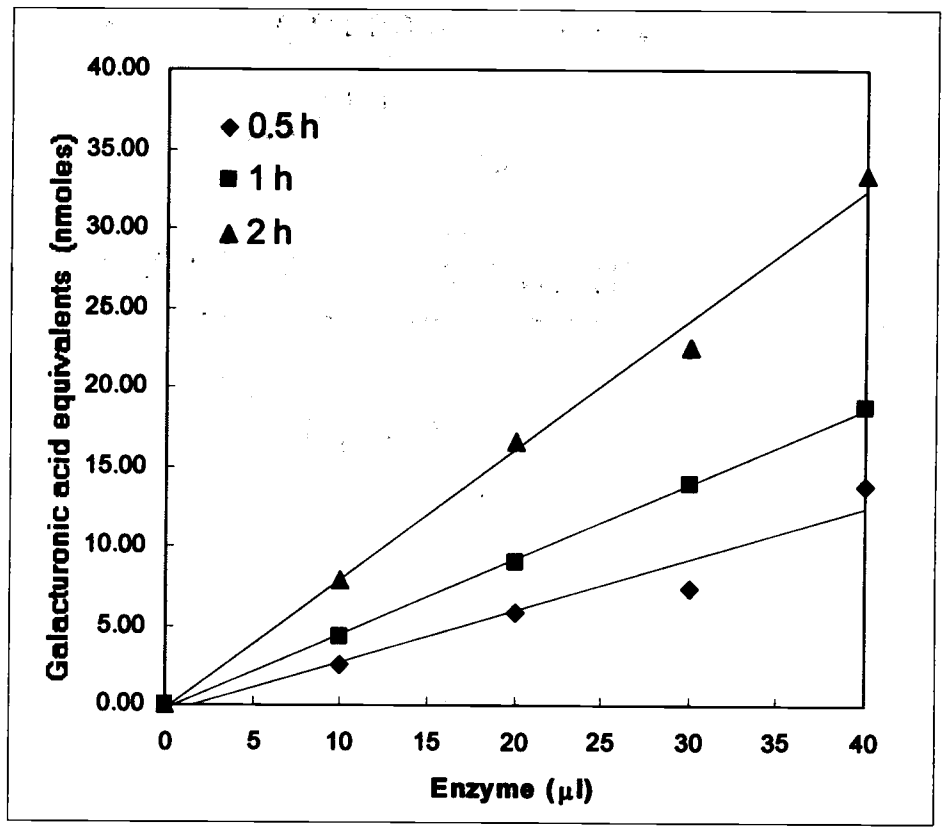

Figure 5.4. Relationship between enzyme load and amount of product generated (new reducing ends) at different extents of reaction.

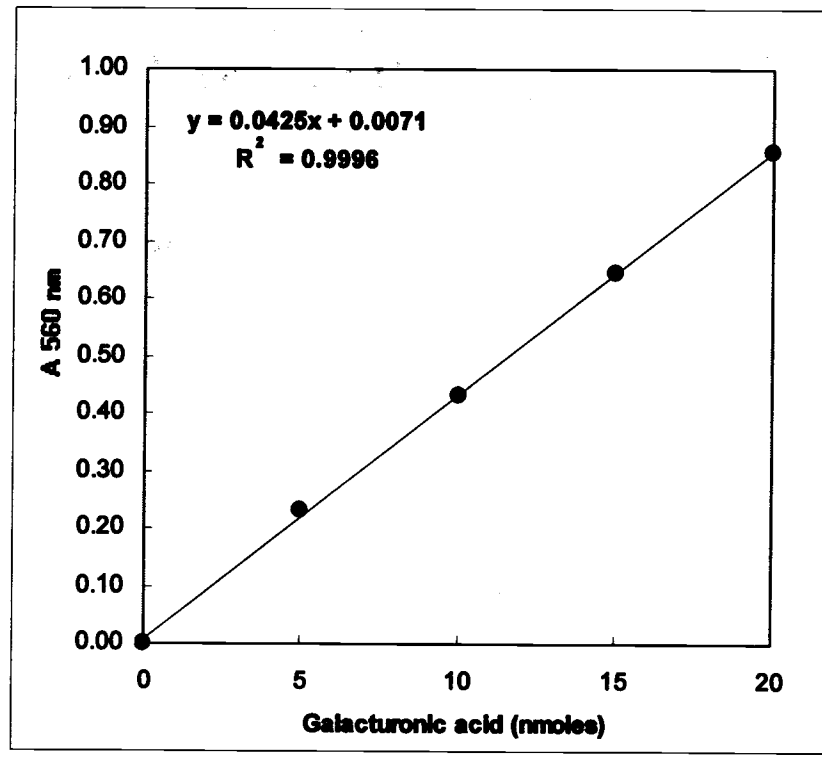

Figure 5.5. Calibration curve for the BCA reducing sugar assay with galacturonic acid as the calibration standard and A_{560} as the analytical signal.

5.4.4 Time Considerations

A typical reducing sugar-based assay (Basic Protocol 1) takes approximately ~4 hr following reagent and enzyme preparation. The assay is designed for use with enzyme preparations having relatively low activities. Enzyme preparations with higher activities could shorten the enzyme/substrate incubation period; the limitation being the need to generate sufficient reducing sugars for detection. The time required for the viscosity-based assay (Basic Protocol 2) is again somewhat dependent on the activity of the enzyme preparation being tested. As presented, the assay takes ~1 hr following enzyme and substrate preparation. To prepare an enzyme sample from tomatoes (Support Protocol)~ 5 hr is needed.

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This book contains a wealth of information on pectins and pectinases. Chapters cover pectin structures, enzyme properties, and enzyme applications.

Gusakov, A.V., Markov, A.V., Grishutin, S.G., Semenova, M.V., Kondratyeva, E.G., and Sinitsyn, A.P. 2002.Viscometric method for assaying of total endodepolymerase activity of pectinases. *Biochemistry* (Moscow) 67: 676-682.

This is a current paper with representative viscometric results and extended discussions of data handing.

Pressey, R. 1986. Extraction and assay of tomato polygalacturonases. *Hort Sci.* 21: 490-492.

The paper covers many aspects of polygalacturonase preparation, including an appropriate experimental design for working with these enzymes.

CHAPTER 6

GENERAL CONCLUSION

Renewable resources, a major part of which is cellulosic biomass, are expected to play a significant role in meeting the world's energy requirements. The efficient bioconversion of cellulosic biomass to liquid fuel, *i.e.* ethanol, requires that the complex cellulose molecule be converted to its constituent sugar, glucose, prior to fermentation. The conversion of cellulose to glucose, termed saccharification, is a technologically complex process that is not well understood. At the heart of this process is the treatment of the biomass with cellulolytic enzymes. Thus there is international interest in developing mechanism-based kinetic models capable of predicting rates of sugar production from various celluloses. The work presented in this thesis is directed at providing information necessary for developing such models. Specifically, the work focuses on the characteristics of the cellulosic substrate that are likely to effect the rates at which exo-acting cellulases form the initial enzyme-substrate complex. These exo-acting enzymes, called cellobiohydrolases, are the dominant enzymes in typical commercial cellulase preparations.

The first phase of the study was directed at developing a method to estimate the number of reducing ends associated with the traditional cellulose substrates used in cellulose bioconversions studies. This parameter is extremely important for model development because it is the most direct measure of the

number of potential catalytic sites for reducing end-preferring exo-acting cellulases. The approach was to measure substrate concentration, in terms of reducing ends, by quantification of total and accessible reducing ends. The estimation of total reducing ends per unit mass was evaluated using two colorimetric assays (a dinitrosalicyclic acid-based and a bicinchoninic acid-based assay) and one radioisotope-based assay (NaB^3H_4 labeling). The results demonstrate that the bicinchoninic acid (BCA)-based assay is likely to provide the most reliable estimate of total reducing ends per unit mass cellulose for the least amount of experimental difficulty. The absolute number of solvent accessible reducing ends was shown to be most reliably estimated by doing a combined $NaBH_4$/BCA assay or the NaB^3H_4–based radioisotope assay. The value for solvent accessible reducing ends is here argued to be the most relevant in terms of available catalytic sites for reducing end-specific exo-acting cellobiohydrolases, and thus the value that should be used in modeling the behavior of these systems.

The reactivity on the reducing ends of soluble cellooligosaccharides and celluloses was further investigated by comparison of the kinetics of sodium borohydride reduction. Kinetic rate constants for each reaction, obtained through regression analyses of time course data, were based on the plausibility of this being a second order reaction. The reactivity of the reducing ends associated with cellooligosaccharides and amorphous celluloses were shown to be similar. Thus, the solvent accessible reducing ends of the amorphous celluloses tend to behave as though they are free in solution. In contrast, the terminal glucosyl units of the microcrystalline celluloses are shown to be less reactive than those on the other

substrates. This result is important in terms of applying kinetic parameters obtained using soluble cellooligosaccharides to models of insoluble cellulose saccharification. The data suggests that this practice has some merit when working with the amorphous celluloses, but not when working with microcrystalline celluloses.

The last phase of this study dealt with the development of a quality control polygalacturonase assay for use in commercial fruit processing facilities. Assays of this type are extremely important for monitoring processing lines in order to produce products that meet consumer's expectations. For example, in fruit puree products, decreases in viscosity caused by the polygalacturonase enzyme are unacceptable for many markets. The source of enzyme activity in such products may be microbial contamination during processing and/or residual activity from enzyme naturally present in the fruit. A critical point of control in dealing with such products is to have a reliable quantitative assay for polygalacturonase so that sources of detrimental activity can be identified in the processing line. The work described here demonstrates the application of a reducing sugar-based assay and a viscosity-based assay for application in such quality control situations. The reducing sugar-based assay measures the combined activity of both endo- or exo-acting polygalacturonases; the viscosity-based assay is more specific for the endo-acting enzymes. Appropriate approaches to enzyme extraction, as well as other intricacies of the assays, are also presented.

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APPENDICES

APPENDIX A
METHOD FOR CELLOOLIGOSACCHARIDE AND CELLULOSE PREPARATION

Isolation of Cellooligosaccharides

The cellulose hydrolysis was described in Miller et al., (1960). In brief, microcrystalline cellulose (Avicel, PH105, 10g) was first suspended in a 100 mL saturated solution of HCl (specific.gravity =.1.19) in 250-mL Erlenmeyer flask at room temperature (22 ^{0}C) to disperse the cellulose particles. A 100 mL of precooled HCl acid with solid CO_2 (-30 ^{0}C) was added to a solution mixture before stirring to dissolve the cellulose for 20 min in order to avoid the formation of degradation products (Halliwell and Vincent, 1981). The mixture was stirred until become a slightly viscous-yellow homogenous solution. The solution was then warmed and allowed to stand at 25 ^{0}C for 2 h. The solution mixture then was poured into 600 mL of ice-cold distilled water and neutralized with 220g Na_2CO_3 to pH 5.0. The clear supernatant solution was collected by centrifugation at 5,000 rpm for 10 min. The supernatant was then loaded into a charcoal-celite column of radius 2.9 cm, length 100 cm. The column resin consisted of 1:1 charcoal Darco G-60/Celite 545 (Aldrich Chemical Company, Milwaukee, WI), equilibrated with 1% stearic acid in absolute ethanol. After equilibration for 2 h, the mixture was filtered, resuspended in 50 % ethanol, refiltered and resuspended in 10 % ethanol. The resin was packed in the column and equilibrated with water before introduction of the cellooligosaccharides. The soluble cellooligosaccharides

purification process was followed as the method of Flugge et al. (1999) with slight modifications. The soluble cellooligosaccharides were introduced into the column and washed with 4 liters of water to remove salt and glucose. Specifically, the cellooligosaccharides were eluted with a linear water/ethanol gradient of 0-60 % ethanol (250 mL each) and monitored by analyzing each fraction with a reducing sugar (disodium 2,2'-bicinchoninate (BCA) assay. Each fraction was checked for the contamination of glucose by the glucose oxidase/peroxidase assay obtained from Sigma (St. Louis, MO). The corresponding fractions to each oligosaccharide were identified by Rf values on thin-layer chromatography (Whatman Chemical Division, Clifto, NJ) by using an ρ-anisaldehyde-sulfuric acid visualizing reagent with EtOHAc: MeOH: H_2O (40:20:15) as a mobile phase as described in Chirico and Brown, (1985). The pure fractions were pooled and concentrated by a rotary evaporator at 45 ^{0}C. The molar absorptivity of the fraction cellotriose, cellotetrose and cellopentose was verified again with BCA assay resulting in the equimolar responses. The residues were then freeze-dried.

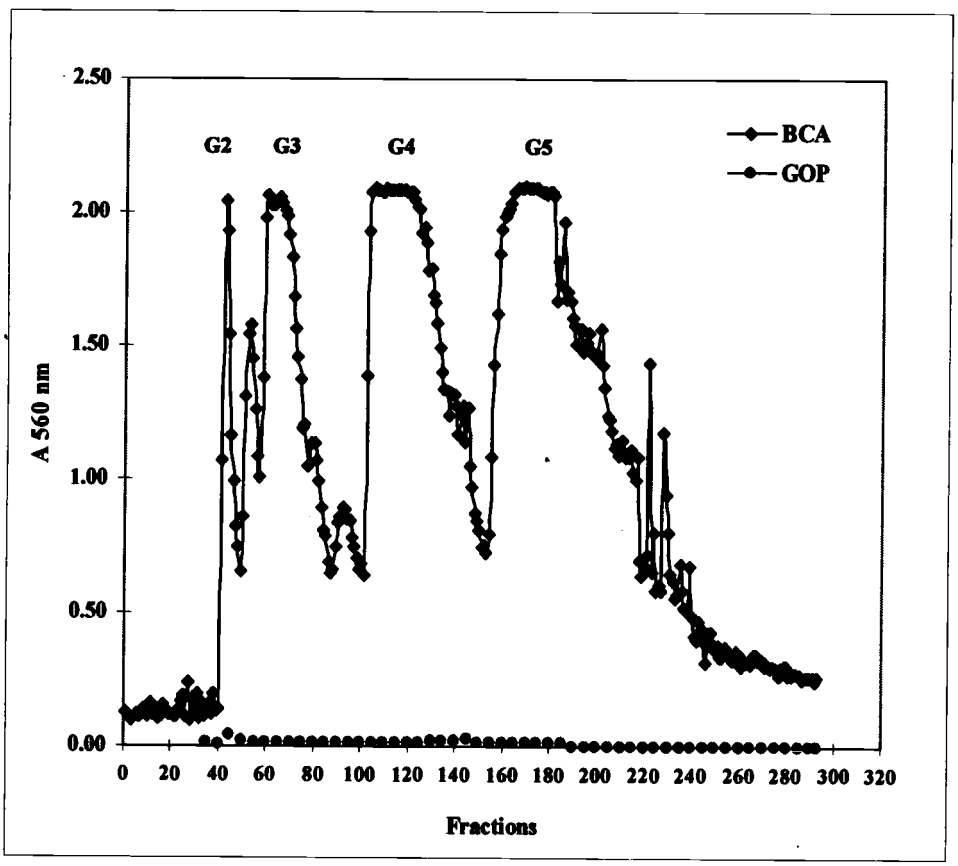


Figure A1. Absorption of cellulose oligomers versus fraction number of the gradient elution during chromatographic run: 1st peak—cellobiose, 2nd peak—cellotriose, 3rd peak cellotetraose, 4th peak cellopentose.

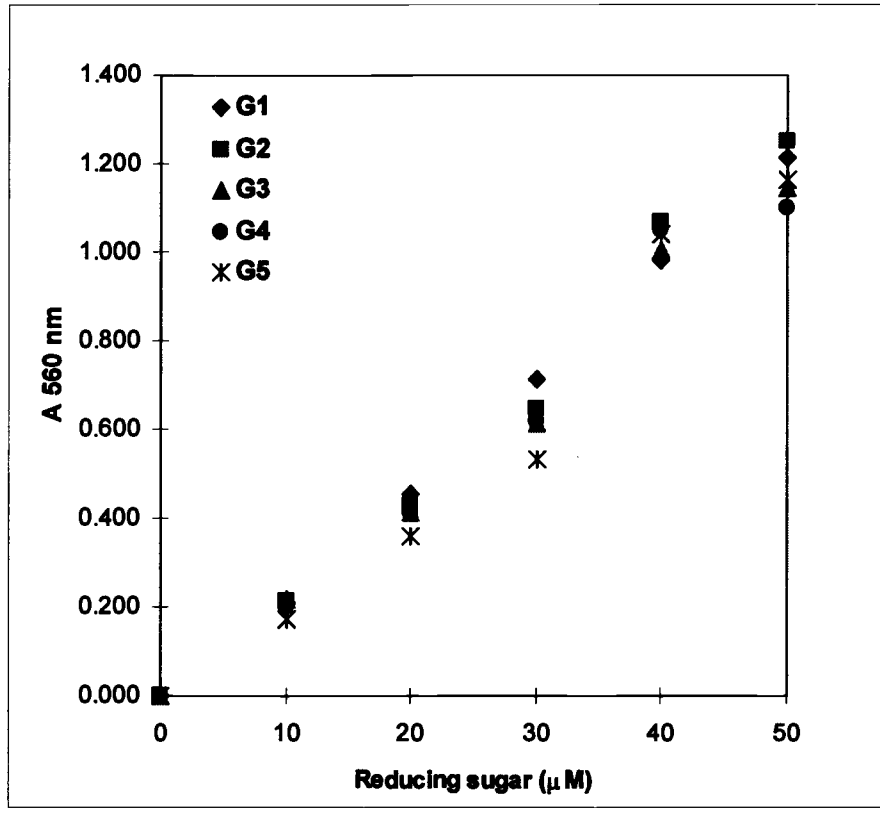


Figure A2. The equimolar responses of the purified fraction (◆) Glucose, (■) cellobiose, (▲) cellotrisoe, (●) cellotetraose, (*) cellopentose using BCA assay.

Production of bacterial microcrystalline cellulose

Bacterial microcrystalline cellulose (BMCC) was prepared with cultures of *Acetobacter xylinum* (ATCC 23769) using the production process described by Gilkes et al., (1992). In particular, cultures were grown on peptone/yeast extract/glucose medium and incubated in trays (Hestrin, 1963) for 10 days at 30 ^{0}C in the incubator without shaking. Surface layers of bacterial cellulose were harvested, cut into approximately 1-cm squares, and washed with distilled water. The cellulose pellets were then extracted with 2 L of 4 % NaOH at 4 ^{0}C overnight and the extraction was repeated five times with NaOH. The cellulose pieces were then extensively washed with distilled water and neutralized with HCl. The pellets were then hydrolyzed by refluxing in 2.5 M HCl for 1 hour. The cellulose mixture was collected after cooling for 3 hours for homogenization in the warning blender for 20 seconds. The homogenized cellulose was then recovered by filtration on a glass fiber filter and resuspended in the fresh 2.5 M HCl. The reflux process was repeated twice for 30 minutes each. The cellulose was then collected and washed with distilled water and recovered by centrifugation at 10,000 rpm for 10 min. The bacterial microcrystalline cellulose was suspended in distilled water. The sample was then taken for dry weight determination. The final concentration of BMCC was resuspened in phosphate buffer containing 0.02 % NaN_3 and stored at 4 ^{0}C.

Production of amorphous microcrystalline cellulose and amorphous bacterial microcrystalline cellulose

Amorphous microcrystalline cellulose (AMCC) and amorphous bacterial microcrystalline cellulose (ABMCC) were produced from MCC and BMCC respectively by the method of Isogai and Atalla (1991) using SO_2-diethylamine-dimethylsulfoxide (SO_2-DEA-DMSO) solvent system for cellulose dissolution. The procedure of preparation of SO_2 solution in DMSO was according to Isogai and Atalla (1991). In particular, the bubble of 99.9 % of SO_2 gas from a cylinder (Aldrich Chemical Company, WI) was passed through a $CaCl_2$ tube into 40 mL DMSO for 20 min. Then, 1 mL of the SO_2 solution was poured into 100 mL water after cooling to room temperature. To determine the concentration of SO_2/ DMSO in solution, the solution was tritrated with 0.5 N NaOH using phenolphthalein. The solution was then kept at room temperature in the dark bottle. To prepare these cellulose solutions, MCC or BMCC (1 g) and DMSO (60 mL) were placed in an Erlenmeyer flask with a universal stopper. The mixture was kept for 5 h to insure sufficient penetration of DMSO into celluloses. The 1.19 g of SO_2 preparation was added to the mixture and followed by 1.35 g diethylamine (DEA). The mixture was then shaken vigorously. A clear cellulose solution was obtained within 3 hours and the celluloses were completely dissolved in the solvent system. The celluloses were then subjected to the regeneration process. In brief, the reaction mixture was transferred to 200 mL of low speed stirred cold distilled water for 10 min. The regenerated celluloses were exchanged four times with fresh distilled water. The celluloses were then dispersed in a blender for 1 min.

The final solution was kept in phosphate buffer pH 8 containing 0.02 % NaN_3 and stored at 4 ^{0}C after the dry weight determination.

Production of phosphoric acid swollen

Phosphoric acid swollen cellulose (PSC) was prepared according to Ståhlberg et al., (1993). In brief, the 4 g of MCC was slowly added into 400 mL of 85 % phosphoric acid under stirring on ice for 2 h. The remaining particles were then filtered through glass wool into 4 L of ice-cold distilled water. The supernatant of cellulose was decanted after being allowed to sediment at 4 ^{0}C overnight. The cellulose was then washed with distilled water once each day for 7 days. The sample was kept in phosphate buffer with 0.02 % NaN_3 after the dry weight determination.

APPENDIX B

A REACTIVITY OF GLUCOSE ACCOUNTING FOR RATE OF MUTAROTATION

Reaction Mechanism

The chemical system analyzed in here is described by the following reaction mechanism:

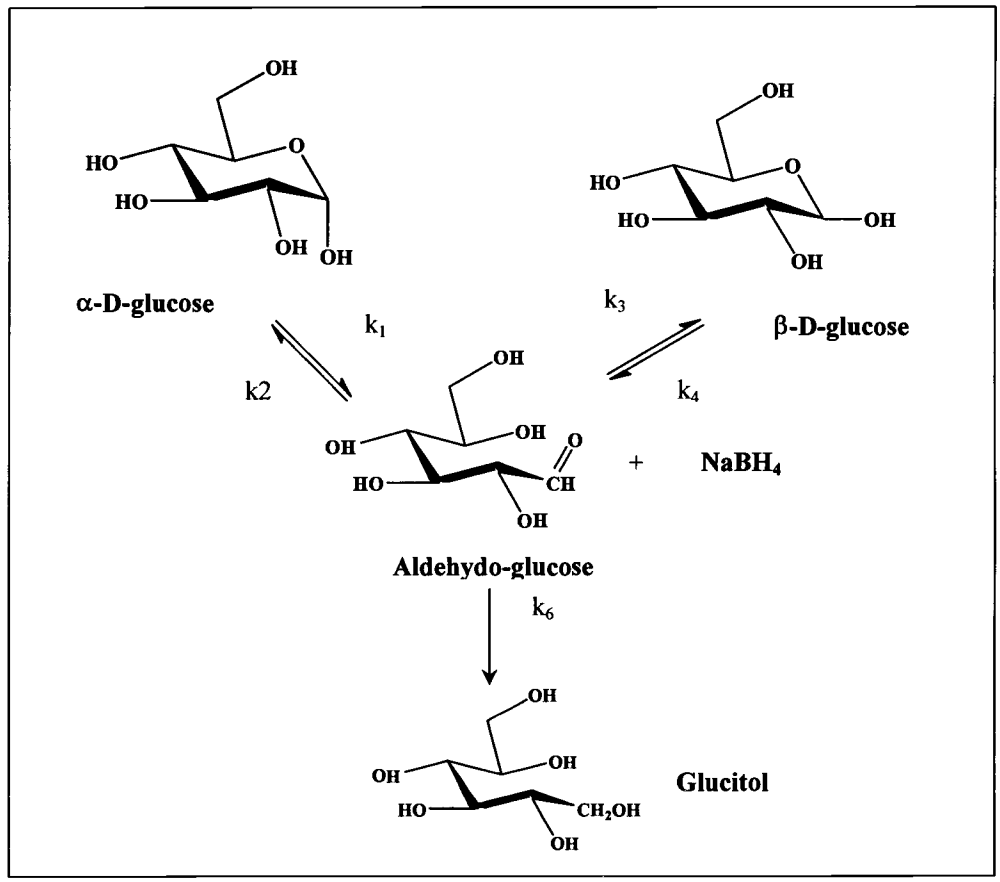


Figure B1. Reaction mechanism of glucose reactivity

Differential Equation:

The changes in concentrations of chemical species over time are computed by solving and initial value problem described by the following system of differential equations:

$$d[\beta]/dt = -k_1[\beta]+k_2[\gamma]$$

$$d[\gamma]/dt = +k_1[\beta]-k_2[\gamma] -k_3[\gamma]+k_4[\alpha]-k_6[\gamma][BH_4^-]$$

$$d[\alpha]/dt = +k_3[\gamma]-k_4[\gamma][BH_4^-]$$

$$d[BH_4^-]/dt = -k_5[BH_4^-]-k_6[\gamma][BH_4^-]$$

$$d[B(OH)_4]/dt = +k_5[BH_4^-]$$

$$d[R]/dt = +k_6[\gamma][BH_4^-]$$

Parameters:

β = the initial concentration of the β-D-glucose anomer (M)

γ = the initial concentration of the aldehydro-glucose anomer (M)

α = the initial concentration of the α-D-glucose anomer (M)

BH_4^- = the initial concentration of borohydride in the solution (M)

$B(OH)_4$ = the initial concentration of a product of borohydride after hydrolysis in the solution (M)

R = the concentration of glucitol (M)

k_1 = the forward rate of mutarotation from α-D-glucose to aldehydro-glucose (7.6 x 10^3 sec.).

k_{-1} = the reverse rate of mutarotation from α-D-glucose to aldehydro-glucose (108 sec.).

k_2 = the forward rate of mutarotation from β-D-glucose to aldehydro-glucose (2.7 x 10^3 sec.).

k_{-2} = the reverse rate of mutarotation from β-D-glucose to aldehydro-glucose (66 sec.).

[a]**Condition:** The rate of mutarotation was determined by polarographic measurement (Pigman and Isbell, 1968). The experiment were conducted at 25 ^{0}C in 0.112 M phosphate buffer of pH 6.9 and gave the parameters of reaction constants for mutarotation of α-D-Glucose as tabulated below.

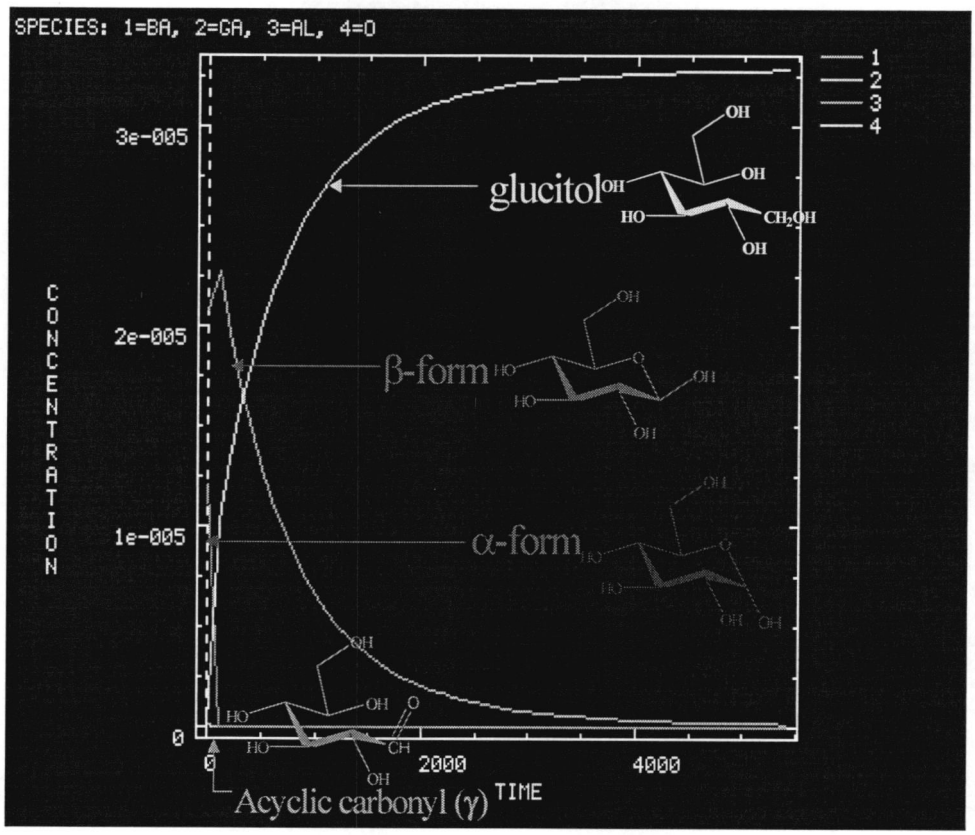


Figure B2. Calculated values of time course of reaction of glucose typed reaction.

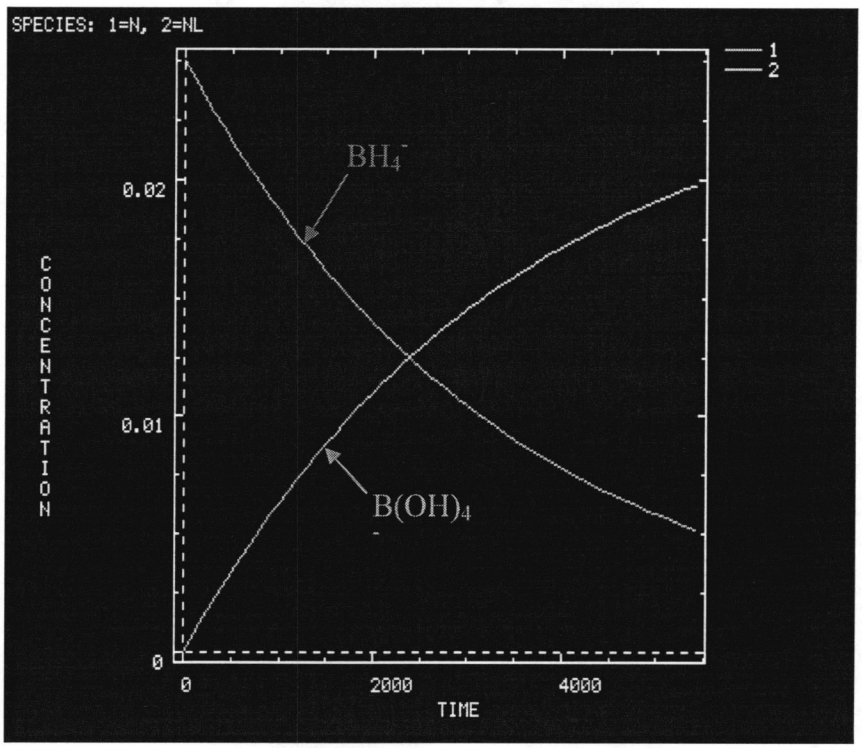
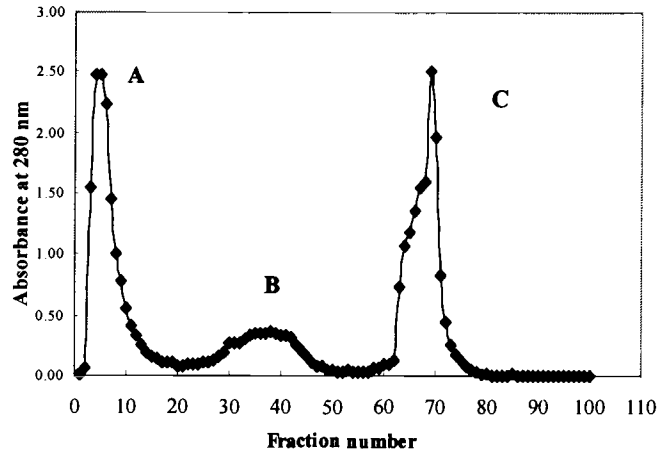


Figure B3. Calculated values of time course of reaction of sodium borohydride.

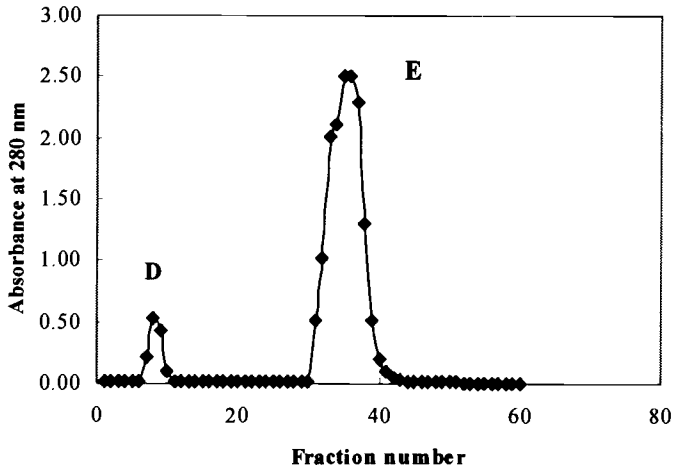
APPENDIX C
PURIFICATION OF CELLOBIOHYDROLASE I FROM *TRICHODERMA REESEI*

Trichoderma reesei CBHI was purified from crude cellulase (Spezyme^{TM}–CP, Environmental BioTechnologies Inc., and Menlo Park), using the purification process described by Piyachomkwan et al. (1997). In brief, the crude cellulase was passed through a DEAE Sepharose CL-6B column preequilibrated with 50 mM NaOAc, pH 5. The cellulase was eluted with a linear gradient of 0 - 0.5 M NaCl in the same buffer. Fractions containing CBHI were combined, and loaded on a *p*-aminophenyl 1- thio-β-D-cellobioside affinity column with 1 mM D-glucono-δ-lactone, 0.1 M NaOAc, pH 5, as the mobile buffer. The cellulase was eluted by adding 0.01 M cellobiose to the buffer. The partially purified CBHI fractions were combined and concentrated prior to loading on a Phenyl Sepharose CL-4B column. The loading buffer was 0.85 M $(NH_4)_2SO_4$ and 25 mM NaOAc, pH 5. The column was washed with 5 column volumes of the buffer, and then the protein was eluted with a linear gradient of 0.85 - 0.35 M $(NH_4)_2SO_4$ in buffer. The CBHI fractions were combined, and the buffer exchanged to 50 mM NaOAc, pH 5 by ultrafiltration with a PM 10 membrane. The concentrated cellulase was stored at -80 ^{0}C until use. Both cellulases were judged to be 98-99 % pure as indicated by SDS-PAGE and CMCase activity.



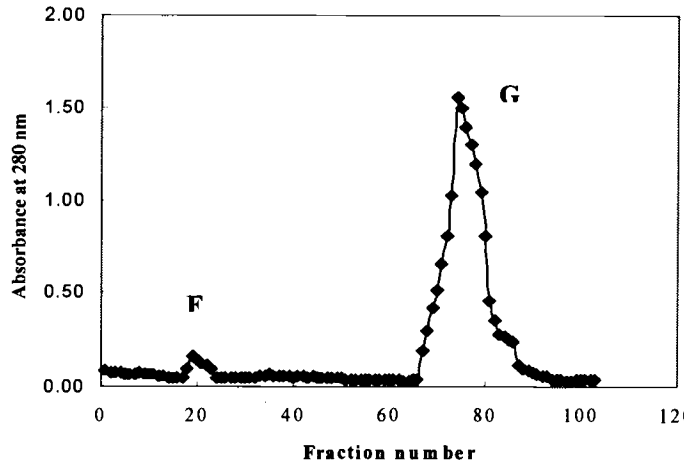
EAE-Sepharose

A: CBHII, EG,β-G
B: EG
C: CBHI, EG



APTC-agarose

D: EG
E: CBHI



Phenyl Sepharose

F: Core CBHI
G: Intact CBHI

Figure C. Purification steps for cellobiohydrolase I from *Trichoderma reesei*: (a) DEAE Sepharose CL-6B column, (b) ρ-aminophenyl 1-thio-β-D-cellobioside affinity column, (c) Phenyl Sepharose CL-4B column.

APPENDIX D

CALIBRATION AND ANALYTICAL SENSITIVITY OF [^{3}H] BOROHYDRIDE METHOD

Table D1.The calibration sensitivity of assay response in the [^{3}H]borohydride method (NaB^3H_4) for determination of the number of soluble and insoluble reducing ends. Cellulose substrates are microcrystalline cellulose (MCC), amorphous microcrystalline cellulose (AMCC), phosphoric acid swollen cellulose (PSC), bacterial microcrystalline cellulose (BMCC) and amorphous bacterial microcrystalline cellulose (ABMCC) and filter paper (FP).

Substrate	Calibration sensitivity (ΔCpm/Δmg[a]) x 10^3	Associated standard error x 10^3	R^2
Glucose	0. 236	0.0002	0.9998
Cellobiose	0.240	0.0007	0.9987
MCC	2.729	0.204	0.9890
AMCC	4.760	0.186	0.9970
PSC	4.877	0.175	0.9974
BMCC	2.975	0.169	0.9961
ABMCC	3.176	0.099	0.9981
FP	0.373	0.022	0.9930

[a]In this study, the signal change in terms of the radioactive responses versus and increasing amount of celluloses was reported from the value obtained from a linear regression analysis using Microcial Origin. The data showed based on slope and the standard error associated with the regression.

Table D2.The analytical sensitivity of assay response in the [^{3}H]borohydride method (NaB^3H_4) for determination of the number of soluble and insoluble reducing ends. Cellulose substrates are microcrystalline cellulose (MCC), amorphous microcrystalline cellulose (AMCC), phosphoric acid swollen cellulose (PSC), bacterial microcrystalline cellulose (BMCC) and amorphous bacterial microcrystalline cellulose (ABMCC) and filter paper (FP).

Substrate	Analytical sensitivity (ΔCpm/Δmg[a]) x 10^3	Std. Dev x 10^3	Coefficient of variation
Glucose	0.003	0.072	30.31
Cellobiose	0.001	0.217	90.56
MCC	0.016	0.171	6.25
AMCC	0.031	0.156	3.27
PSC	0.033	0.146	3.00
BMCC	0.027	0.110	3.70
ABMCC	0.038	0.083	2.61
FP	0.001	0.029	8.19

APPENDIX E

POLYGALACTURONASE AND PECTATE LYASE ASSAYS

Sigma Enzyme:

The Polygalacturonase (PGase) stock (Sigma, P-3304, Sigma Chemical Co., St. Louis, MO) contains PGase, purified from *Aspergillus japonicus pectolyase*, suspended in 3.2 M $(NH)_4SO_4$, pH 4.5, containing 1 mM PMSF. The suspension contains 0.445 units per µl suspension (suspension contains 1.0 mg protein per mL, Lowry method). A unit of enzyme is defined by Sigma as that amount of enzyme that will release 1.0 µmole of reducing sugar, measured as D-galacturonic acid, from polygalacturonic acid per minute at pH = 5.0, 30 ^{0}C using the final concentration of 0.5% (w/v) polygalacturonic acid in 50 mM sodium acetate in presence of 0.002 % (w/v) bovine serum albumin by Nelson colorimetric method.

Enzyme Extraction for Enzyme Testing:

Peach purees are obtained commercially. They were used directly without modification. Apples or Pears (2 out of 5 fruits from the sampling plan in the plant processing line) were diced into ~ 1 cm cubes, and stored on ice. The hundred grams of fruit was homogenized in a Warning blender using the puree mode for 2 minutes. The homogenate were then kept on ice for enzyme activities without any

further treatment. A 5 g of each sample was transferred to a 50 ml centrifuge tube. The 25 ml of water was added and mixed with the sample. The sample was then centrifuged at 10,000 rpm (11950 x g) in the centrifuge at 0 ^{0}C (GS-15 R, Beckman Instruments, Inc, CA). The supernatant was discarded and the pellet was resuspended in 25 mL water. The centrifugation process was repeated. After the supernatant was removed the pellet was suspended in 4 ml of 1 M NaCl. The sample was then recentrifuged using the same speed and the supernatant was saved on ice as the high salt extract. All extraction samples were kept on ice. The gel filtration (Sephadex G-25) was prepared by equilibrated with 10 mM sodium acetate buffer (pH 5) and centrifuged for 1 min at 2,000 rpm. After removed the buffer, a 1 ml of the high extract was then desalted by centrifugation through Sephadex G-25 equilibrated with 10 mM sodium acetate buffer (pH 5) for 1 minute at 2,000 rpm in a tabletop centrifuge. The desalting material was saved on ice for the reducing sugar assay. The washing, equilibration, and predesalting centrifugations of Sephadex G-25 were repeated before applying the next sample for desalting. In detail, the upper top section of column were rinsed with water twice and followed by 95 % ethanol. After that, the column was washed two times with 10 mM sodium acetate buffer (pH5) and then set aside with buffer for a day.

Enzyme Extraction in Washed Cell-Wall Residues of Peaches:

Peach purees were frozen and stored in –4 ^{0}C. All of steps of this extraction procedure were conducted at low temperature (samples were on ice).

Peach puree from each lot was thawed. 5 g of each puree was added to 25 ml of cold double distilled water. The suspension was gently mixing the centrifuged at 10,000 rpm for 10 min. The supernatant was kept as a 1st extract. The sample was then resuspended in 25 ml of double distilled water and centrifuge again with the same speed and time. The supernatant was kept as a 2nd extract. The pellet was then further resuspended in 4 ml of 1M NaCl. The supernatant was kept as a 3rd extract after centrifuge. All the extraction samples in each step were then passed through a size exclusion chromatography column (Sephadex G-25). The columns were centrifuged at 2000 rpm for 1 min. The retentates of each sample were kept for analysis of enzyme activity.

Determination of Polygalacturonase Activity by using MBTH Method:

The standards blanks contained 0.1 ml of substrate stock and spiked with the stock of 10 mM galacturonic acid. The standard curve was prepared in triplicate by varies the amount of galacturonic acid (0-38.46 nmoles). For the samples, the 0.4 ml enzyme extracts were mixed with 0.1 ml substrate stock and incubated in the water bath at 37 ^{0}C for 3 hours. All samples were mixed before further analysis. Samples (0.1 ml) were taken form the incubations and mixed with 0.1 ml of 0.5 N NaOH to stop the reaction, both after the mixing (zero time: enzyme blank) and after 3 hours of incubation. The amount of reducing sugar then was determined colorimetrically by reaction with MBTH. The reagent was prepared by mixing equivalent volumes of 3 mg/ml MBTH and 1 mg/ml DTT

immediately before use. In a 12 x 75-mm test tube, the standard curve samples containing 0 to 38.46 nmol of galacturonic acid (100 µl) were mixed with 100 µl of 0.5 N NaOH and followed by 100 µl of the mixture of MBTH and DTT reagent. The samples and standards were then heated for 15 min at 80 ^{0}C in a water bath. As the samples were removed form the heat 200 µl of a solution containing 0.5 % ($FeNH_4(SO_4)_2$) 12 H_2O, 0.5 % sulfamic acid, and 0.25 N HCl was added and the samples allowed to cool to room temperature. The color development from orange to green was completed in 15 minutes. After the color development and extensively mixing, a 1 ml of water was added to the samples. The sample was then measured the absorbance at 620 nm, and the nmoles of reducing sugar per was calculated by comparing with the standard curve. All samples were analyzed in duplicate. The MBTH and DTT stock solution solutions could be stored in the refrigerator for at least 1 week at 4 ^{0}C. The acidic Fe solution was stable at room temperature. Anthon and Barrett, (2002).

Determination of Polygalacturonase Activity by using BCA Method:

To reaction mixtures, 10 µl (20,30,40 µl in other concentrations) of "1500X" sigma enzyme and 0.1 ml of substrate stock in 10 mM sodium acetate buffer pH 5.0 were mixed in vortex briefly. Samples were subsequently incubated in a shaking water bath at 37 ^{0}C with a marble over each tube. Enzyme reaction was stopped by taking 0.1 ml of the reaction mixture at time interval, either immediately after mixing (zero time) or after 1, 2, 3, and 4 hours for incubation

and immediately mixed with 1 ml of BCA reagent to stop the reaction (a high alkaline condition, pH 11) to both reaction mixtures and standard curve, after that the samples were incubated in water bath for 30 min at 80 ^{0}C with a marble on top of each tube to reduce the evaporation. The reaction mixture was transferred to ice-cold water for 10 min before the amount of reducing sugar were determined spectrophotometrically by reading the absorbance at 560 nm. The increase in absorbance over time in reaction samples were then subtracted from the sample blanks at each time interval. The difference in terms of absorbance was then calculated from the reference of standard curve of D-galacturonic acid. All enzyme activities were expressed in International Units. One unit of enzyme activity (katals) is defined as the amount of enzyme that liberates 1 mol product s^{-1}.

Detecting the Pectate Lyase Activity:

Sigma enzyme and peach puree extract were subjected to test for pectate lyase activity according to Collmer et al., 1988. For Sigma enzyme, the enzyme suspension were prepared to "1500X" from original supplied stock in 10 mM sodium acetate, pH 5.0. For peach puree enzyme extraction, a whirlbag sample (Sample # 1, from customer compliant: thining) was chosen to detect the amount of pectate lyase enzyme. Five grams of puree was suspension in 25 ml of water and centrifuged for 10 min at 10,000 rpm. The supernatant was kept for analysis. A 2.5 ml of substrate stock containing 60 mM Tris-HCl pH 8.5, 0.6 mM calcium

chloride and 0.24 % (w/v) of polygalacturonic acid was mixed with 0.5 ml of enzyme samples (either from sigma preparation or a puree enzyme extract with water) in 10 mM sodium acetate buffer pH 5.0 was separately incubated in water bath for 1 hour at 30 ^{0}C. To start the reaction, the samples of substrate and enzyme were rapidly mixed in a 3-ml cuvette with a 1-cm light path. The subsequent increase in absorbance at 232 nm was monitored as a function of time with a recording spectrophotometer. The enzyme without a substrate and the substrate without enzyme sample in the final volume 3 ml were run simutaneously. One unit of enzyme forms 1 μmol of 4,5-unsaturated product in 1 min under the conditions of the assay. The molar extinction coefficient for the unsaturated product at 232 nm is 4600 M^{-1} cm^{-1}

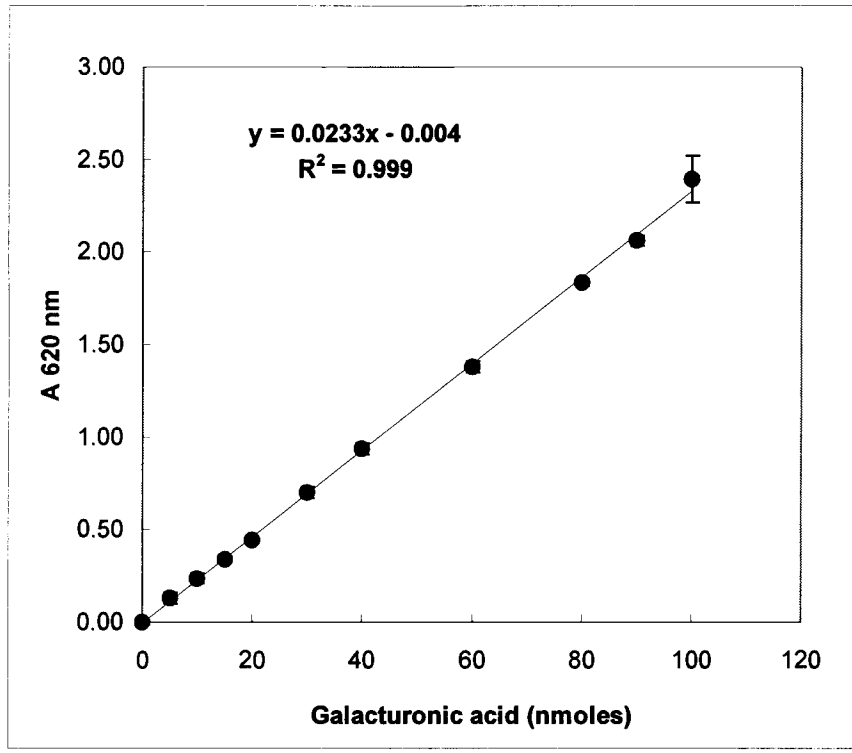


Figure E1. Calibration curve for the MBTH reducing sugar assay with galacturonic acid as the calibration standard and A_{620} as the analytical signal.

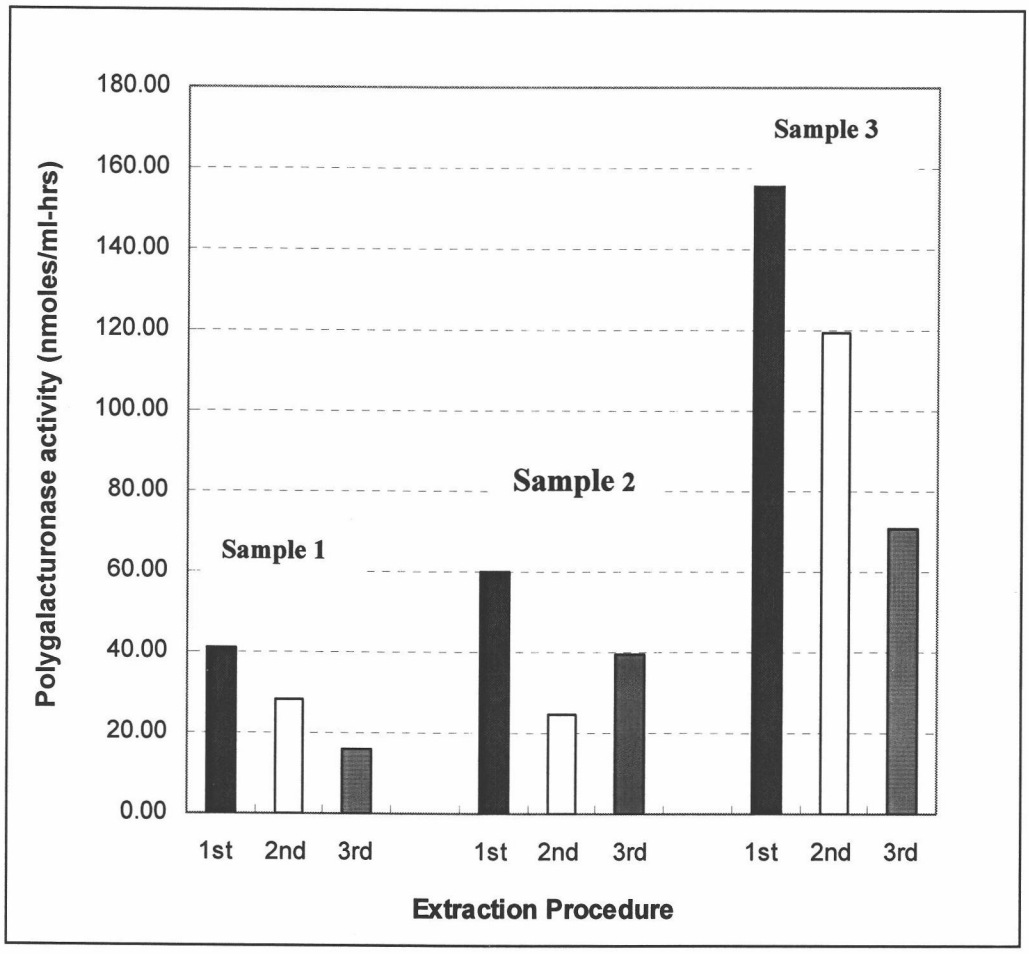


Figure E2. Comparison the step of peach extraction procedure (■) 1st extract with H_2O, (□) 2nd extract with H_2O, (▩) 3rd extract with 1 M NaCl from the sample 1: peach puree A, sample 2: peach puree B, and sample 3: peach puree C.

Figure E2 illustrates the verification of analysis method steps as followed the UC-Davis (as the method to analysis tomatoes- Pressey, 1986). Results showed that the highest polygalacturonase in all three samples were detected in the 1st wash by distilled water while the 3rd washed with 1 M NaCl had a lowest PG_{ase} activity.

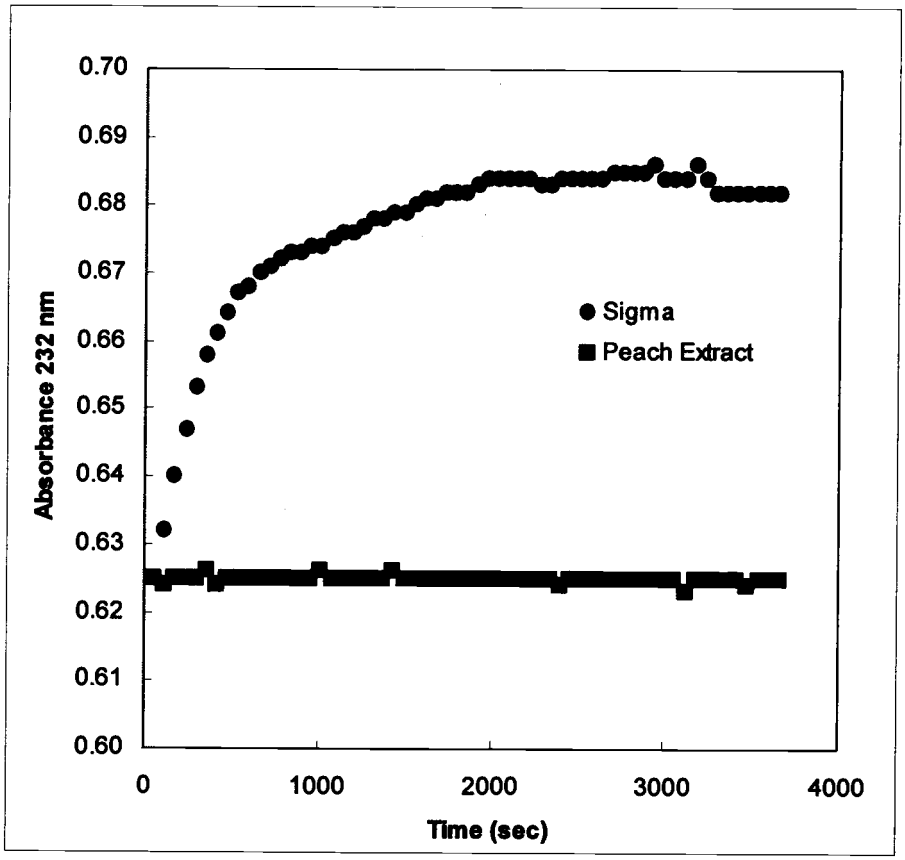


Figure E3. Reaction progress curve from the pectate lyase activity (the production of oligosaccharides with terminal 4-deoxy-6-methoxy-α-D-galact-4-enuronosyl groups as the changing of A_{232} over time).

Table E. Specific activity for polygalacturonase at 1 h.

Enzyme (μl/reaction mixture)	Activity [a] (pkat)	Protein [b] (mg/reaction mixture)	Specific activity (nkat/mg protein)
0	0	0	0
10	1.195	0.00016	7.466
20	2.488	0.00032	7.774
30	3.863	0.00048	8.048
40	5.212	0.00064	8.143

[a] Measured by BCA reagent, pkat = 10^{-12}

[b] Calculated value, protein concentrations were determined by Lowry method (Sigma).

APPENDIX F

RAW DATA FOR DYNAFIT ANALYSIS

1) The combined $NaBH_4$/BCA assay

Table E (a) glucose

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
Glucose	0-T	33.28	32.51	33.95	33.24	0.722
	0-E	23.69	23.31	24.27	23.76	0.484
	5	19.43	18.80	19.14	19.13	0.313
	10	15.68	11.88	11.79	13.12	2.221
	15	9.81	7.22	7.79	8.28	1.363
	20	5.10	4.24	5.05	4.80	0.486
	25	4.77	1.35	2.27	2.79	1.767
	30	0.87	2.99	1.93	1.93	1.058
	40	-0.43 (0)	0.44	0.01	0.01	0.433
	50	-2.21 (0)	-1.10 (0)	-1.63 (0)	-1.64 (0)	0.553
	60	-1.63 (0)	-1.44 (0)	-1.72 (0)	-1.60 (0)	0.147
	70	-2.54 (0)	-1.72 (0)	-2.54 (0)	-2.27 (0)	0.472
	80	-2.40 (0)	-2.06 (0)	-2.40 (0)	-2.29 (0)	0.194
	90	-2.35 (0)	-2.40(0)	-2.54(0)	-2.43(0)	0.100

Table E (b) cellobiose

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
Cellobiose	0-T	32.99	30.74	34.94	32.89	2.10
	0-E	30.29	27.64	29.56	29.16	1.12
	5	30.63	29.60	27.07	29.10	1.34
	10	21.70	21.81	21.26	21.59	0.28
	15	18.50	19.75	17.40	18.55	1.18
	20	14.60	14.42	13.98	14.33	0.23
	25	8.72	8.57	8.76	8.69	0.09
	30	5.01	5.05	6.26	5.44	0.62
	40	0.71	1.00	0.60	0.77	0.20
	50	0.08	-0.10 (0)	-0.25 (0)	-0.09 (0)	0.09
	60	-0.43(0)	-0.47 (0)	-0.51 (0)	-0.47 (0)	0.02
	70	-0.51 (0)	-0.43 (0)	0.56 (0)	-0.13 (0)	0.51
	80	-0.29 (0)	-0.84 (0)	-0.76 (0)	-0.63 (0)	0.11
	90	-0.69 (0)	-0.84 (0)	-0.10 (0)	-0.54 (0)	0.37

Table E (c) cellotriose

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
Cellotriose	0-T	35.15	35.19	29.40	33.24	3.33
	0-E	27.60	27.40	27.89	27.63	0.24
	5	26.97	24.01	24.09	25.03	1.68
	10	21.34	19.28	19.44	20.02	1.15
	15	16.00	16.40	16.08	16.16	0.21
	20	12.03	12.31	11.22	11.85	0.57
	25	11.18	7.86	10.94	9.99	1.85
	30	6.73	6.77	4.97	2.84	6.77
	40	2.31	3.08	3.04	2.81	0.43
	50	2.72	2.31	2.27	2.43	0.25
	60	0.29	0.57	-0.56 (0)	0.10	0.59
	70	0.25	-0.80 (0)	0.57	0.01	0.72
	80	-0.16(0)	-0.52 (0)	0.41	-0.09 (0)	0.47
	90	-0.76(0)	0.41 (0)	0.29	-0.02(0)	0.65

Table E (d) cellotretraose

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
Cellotetraose	0-T	33.11	35.61	31.70	33.47	1.98
	0-E	30.50	29.20	30.84	30.18	0.87
	5	24.74	25.20	25.46	25.13	0.37
	10	20.44	19.68	19.26	19.80	0.60
	15	16.98	16.22	14.97	16.06	1.02
	20	9.68	10.94	11.13	10.58	0.79
	25	10.25	9.11	10.22	9.86	0.65
	30	6.45	6.76	5.31	6.17	0.76
	40	4.82	3.68	1.55	3.35	1.66
	50	0.29	0.75	-0.96(0)	0.03	0.89
	60	-1.08 (0)	-0.28 (0)	-0.62 (0)	-0.66 (0)	0.40
	70	0.29(0)	-1.23(0)	-1.08 (0)	-0.67 (0)	0.84
	80	-1.12 (0)	-1.61(0)	-0.47 (0)	-1.06 (0)	0.57
	90	0.56	-2.14(0)	-0.47(0)	-0.68 (0)	1.36

Table E (e) cellopentaose

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
Cellopentose	0-T	32.98	32.20	34.65	33.28	1.25
	0-E	29.29	28.68	28.23	28.73	0.53
	5	24.00	24.85	24.57	24.47	0.44
	10	19.55	19.10	16.53	18.39	1.63
	15	13.96	13.79	12.69	13.48	0.69
	20	10.16	10.16	8.53	9.62	0.94
	25	6.32	5.26	6.94	6.17	0.85
	30	6.08	5.26	5.39	5.58	0.44
	40	-0.57	-0.41 (0)	0.00	-0.33(0)	0.29
	50	-0.94	-0.49(0)	-0.45 (0)	-0.63(0)	0.27
	60	-0.21	-0.21(0)	0.00 (0)	-0.14 (0)	0.12
	70	-0.90	-0.49(0)	-1.02(0)	-0.81(0)	0.28
	80	-1.68	-1.19(0)	-1.02 (0)	-1.30(0)	0.34
	90	-0.98	-0.90(0)	-0.86 (0)	-0.91(0)	0.06

Table E (f) Data for insoluble cellulose: microcrystalline cellulose

1) Original data

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
MCC	0	30.29	32.37	34.28	32.31	1.99
	5	26.09	29.00	30.17	28.42	2.20
	10	23.38	26.17	28.00	25.85	2.33
	15	20.67	22.99	24.87	22.84	2.10
	20	20.00	22.00	24.87	22.29	2.45
	25	19.59	22.77	23.81	22.06	2.20
	30	19.05	22.28	23.28	21.54	2.21
	40	17.00	20.82	21.69	19.84	2.50
	50	16.00	19.85	20.63	18.83	2.48
	60	15.26	18.88	19.57	17.90	2.32
	70	12.99	16.34	16.81	15.38	2.08
	80	12.40	16.34	16.81	15.18	2.42
	90	12.44	16.34	16.81	15.20	2.40

2) Solvent accessible reducing ends

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
MCC	0	17.85	16.03	17.47	17.12	0.96
	5	13.65	12.66	13.36	13.22	0.51
	10	10.94	9.83	11.19	10.65	0.72
	15	8.23	6.65	8.06	7.65	0.87
	20	7.56	5.66	8.06	7.09	1.27
	25	7.15	6.42	7.00	6.86	0.38
	30	6.61	5.94	6.47	6.34	0.35
	40	4.56	4.48	4.88	4.64	0.21
	50	3.56	3.51	3.82	3.63	0.17
	60	2.82	2.53	2.76	2.70	0.15
	70	0.55	0.00	0.00	0.18	0.32
	80	-0.04	0.00	0.00	-0.01	0.02
	90	0.00	0.00	0.00	0.00	0.00

Table E (f) Data for insoluble cellulose: amorphous microcrystalline cellulose

1) Original data

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
AMCC	0	33.09	32.13	34.00	33.07	0.94
	5	27.57	27.33	27.64	27.52	0.16
	10	25.40	24.98	25.02	25.13	0.23
	15	17.22	17.74	17.61	17.53	0.27
	20	15.92	15.57	15.75	15.75	0.18
	25	14.23	14.93	14.84	14.67	0.38
	30	12.64	12.97	11.59	12.40	0.72
	40	13.56	13.83	12.97	13.45	0.44
	50	10.54	10.56	8.80	9.97	1.01
	60	10.96	11.19	8.97	10.37	1.22
	70	10.32	10.55	9.22	10.03	0.71
	80	10.39	10.99	8.81	10.06	1.13
	90	10.09	10.34	9.09	9.84	0.66

2) Solvent accessible reducing ends

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
AMCC	0	23.00	21.79	24.91	23.23	1.57
	5	17.48	16.99	18.55	17.68	0.80
	10	15.31	14.64	15.93	15.29	0.65
	15	7.13	7.40	8.52	7.69	0.73
	20	5.83	5.23	6.66	5.91	0.72
	25	4.14	4.59	5.75	4.83	0.83
	30	2.55	2.63	2.50	2.56	0.07
	40	3.47	3.49	3.88	3.61	0.23
	50	0.45	0.22	-0.29	0.13	0.38
	60	0.87	0.85	-0.12	0.53	0.57
	70	0.23	0.21	0.13	0.19	0.05
	80	0.30	0.65	-0.28	0.22	0.47
	90	0.00	0.00	0.00	0.00	0.00

Table E (f) Data for insoluble cellulose: phosphoric acid swollen

1) Original data

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
PSC	0	32.48	34.10	33.23	33.27	0.81
	5	24.14	27.00	24.93	25.36	1.48
	10	22.73	25.34	22.77	23.61	1.50
	15	20.78	20.53	19.91	20.41	0.45
	20	14.55	15.43	15.63	15.20	0.57
	25	13.56	15.30	14.10	14.32	0.89
	30	13.02	13.39	13.23	13.21	0.19
	40	10.49	11.28	11.15	10.97	0.42
	50	11.24	9.24	9.95	10.14	1.01
	60	10.07	10.61	11.48	10.72	0.71
	70	8.75	10.49	9.62	9.62	0.87
	80	10.28	8.54	8.75	9.19	0.95
	90	10.28	8.54	8.75	9.19	0.95

2) Solvent accessible reducing ends

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
PSC	0	22.20	25.56	24.48	24.08	1.72
	5	13.86	18.46	16.18	16.17	2.30
	10	12.45	16.80	14.02	14.42	2.20
	15	10.50	11.99	11.16	11.22	0.75
	20	4.27	6.89	6.88	6.01	1.51
	25	3.28	6.76	5.35	5.13	1.75
	30	2.74	4.85	4.48	4.02	1.13
	40	0.21	2.74	2.40	1.78	1.37
	50	0.96	0.70	1.20	0.95	0.25
	60	-0.21	2.07	2.73	1.53	1.54
	70	-1.53	1.95	0.87	0.43	1.78
	80	0.00	0.00	0.00	0.00	0.00
	90	0.00	0.00	0.00	0.00	0.00

Table E (f) Data for insoluble cellulose: bacterial microcrystalline cellulose

1) Original data

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
BMCC	0	16.12	15.87	14.96	15.65	0.61
	5	14.10	12.49	14.10	13.56	0.93
	10	11.83	12.28	11.43	11.85	0.43
	15	11.68	12.13	11.27	11.70	0.43
	20	12.18	7.84	9.15	9.73	2.23
	25	8.70	9.30	13.04	10.35	2.35
	30	9.66	11.12	8.95	9.91	1.11
	40	8.24	10.67	9.46	9.46	1.21
	50	8.04	7.28	7.69	7.67	0.38
	60	7.23	7.28	9.46	7.99	1.27
	70	7.08	7.74	7.03	7.28	0.39
	80	7.23	7.74	7.49	7.49	0.25
	90	7.64	6.38	7.03	7.02	0.63

2) Solvent accessible reducing ends

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
BMCC	0	8.48	9.49	7.93	8.64	0.79
	5	6.46	6.11	7.07	6.55	0.49
	10	4.19	5.91	4.39	4.83	0.94
	15	4.04	5.76	4.24	4.68	0.94
	20	4.55	1.46	2.12	2.71	1.62
	25	1.06	2.93	6.01	3.33	2.50
	30	2.02	4.75	1.92	2.90	1.60
	40	0.61	4.29	2.42	2.44	1.84
	50	0.40	0.91	0.66	0.66	0.25
	60	-0.40	0.91	2.42	0.98	1.42
	70	-0.56	1.36	0.00	0.27	0.99
	80	-0.40	1.36	0.45	0.47	0.88
	90	0.00	0.00	0.00	0.00	0.00

Table E (f) Data for insoluble cellulose: amorphous bacterial microcrystalline cellulose

1) Original data

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
ABMCC	0	21.69	20.42	20.90	21.00	0.64
	5	15.98	17.17	15.66	16.27	0.79
	10	14.87	15.42	15.10	15.13	0.28
	15	14.87	14.79	14.79	14.81	0.05
	20	14.87	14.79	14.79	14.81	0.05
	25	13.60	12.72	13.60	13.30	0.50
	30	11.77	12.96	12.01	12.25	0.63
	40	11.93	11.85	11.77	11.85	0.08
	50	12.01	11.77	10.02	11.27	1.08
	60	11.45	11.37	11.53	11.45	0.08
	70	11.45	11.37	11.53	11.45	0.08
	80	11.45	11.37	11.21	11.35	0.12
	90	11.37	9.79	10.98	10.71	0.83

2) Solvent accessible reducing ends

Compound	Time (min)	Amount (nmoles)			Ave	SD
		Replication 1	Replication 2	Replication 3		
ABMCC	0	10.32	10.63	9.92	10.29	0.36
	5	4.60	7.38	4.68	5.56	1.58
	10	3.49	5.63	4.13	4.42	1.10
	15	3.49	5.00	3.81	4.10	0.79
	20	3.49	5.00	3.81	4.10	0.79
	25	2.22	2.94	2.62	2.59	0.36
	30	0.40	3.17	1.03	1.53	1.46
	40	0.56	2.06	0.79	1.14	0.81
	50	0.63	1.98	-0.95	0.56	1.47
	60	0.08	1.59	0.56	0.74	0.77
	70	0.08	1.59	0.56	0.74	0.77
	80	0.08	1.59	0.24	0.63	0.83
	90	0.00	0.00	0.00	0.00	0.00

2) Time course reaction of NaB^3H4 uptake

Compound	Time (min)	Radioactive reading (cpm)			Ave	SD
		Replication 1	Replication 2	Replication 3		
Glucose	0	9809	9400	8999	9402	405
	5	11579	11247	11790	11538	274
	10	21229	20056	19177	20154	1029
	15	23900	24009	23685	23864	164
	20	25000	25680	24767	25149	474
	25	25178	25087	25271	25178	92
	30	26120	26970	26772	26621	444
	40	28333	27990	28038	28120	185
	50	28188	28790	27934	28304	439
	60	28800	28654	26985	28146	1008
	90	27770	28666	26908	27781	878
	120	28000	28142	28437	28193	223
Control 1	Without HCl	1617666	1666587	167809	165411	32085
Control 2	With HCl	9339.00	8266.6	8802	8802	536.2

Compound	Time (min)	Radioactive reading (cpm)			Ave	SD
		Replication 1	Replication 2	Replication 3		
MCC	0	13378	13259	14591	13743	737.13
	5	13753	13578	14591	13974	541.64
	10	16275	16867	16734	16625	310.52
	15	20999	19567	17962	19509	1519.24
	20	25879	21699	15698	21092	5117.71
	25	27587	25678	23499	25588	2045.58
	30	27987	27900	26240	27376	984.25
	40	27758	27785	27787	27777	16.22
	50	28749	28555	28699	28668	100.79
	60	28750	28000	28654	28468	408.11
	90	28789	28730	28763	28761	29.57
	120	30990	29600	30276	30289	695.08
Control 1	Without HCl	1679680	1669620	1689690	1679663	10035.
Control 2	With HCl	10496	10747	11567	10937	560.02

Compound	Time (min)	Radioactive reading (cpm)			Ave	SD
		Replication 1	Replication 2	Replication 3		
AMCC	0	9921	10821.00	10151	10298	467.6
	5	10998	11098	10253	10783	461.6
	10	13789	13486	13052	13442	370.3
	15	15876	14386	17568	15943	1591.8
	20	19190	19888	19543	19540	349.0
	25	21098	22037	21932	21689	514.4
	30	23800	23478	23579	23619	164.7
	40	25876	25600	24616	25364	662.4
	50	26134	26533	26678	26448	281.9
	60	27568	27990	28076	27878	271.9
	90	29138	29654	28501	29098	577.4
	120	30098	29999	27196	29098	1647.3
Control 1	Without HCl	1661880	177771	1689990	1176547	865079.6
Nonspecific	With HCl	10429	10661	9999	10363	335.9

Compound	Time (min)	Radioactive reading (cpm)			Ave	SD
		Replication 1	Replication 2	Replication 3		
PSC	0	10221	9684.45	10251	10052	318.7
	5	13456	13678	13261	13465	208.7
	10	19234	19538	19369	19380	152.3
	15	20953	20761	21166	20960	202.5
	20	24697	24111	25380	24729	634.9
	25	24996	26999	21240	24412	2923.6
	30	26000	26905	25386	26097	764.3
	40	26548	27000	25876	26475	565.5
	50	27300	27654	26990	27315	332.2
	60	27600	27960	27527	27696	231.8
	90	27896	27444	26867	27402	516.0
	120	28900	30001	27627	28843	1188.2
Control 1	Without HCl	1699780	1869710	1689110	1752867	101329.8
Nonspecific	With HCl	9704.6	10029	8795	9509	639.9