AN ABSTRACT OF THE THESIS OF

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Title: The Relative Activity of the Cellulase Enzyme System of *Trichoderma reesei* with Native and Modified Cellulosic Substrates

Abstract approved by :_____

Michael H. Penner

The relative merit of assays commonly applied to cellulase/cellobiase assays have been investigated. A *Trichoderma reesei* cellulase preparation and a cellobiase derived from *Aspergillus niger* were used as saccharification catalysts. Microcrystalline cellulose, filter paper and cellobiose were used as representative substrates. Quantification of products by enzyme-based, reducing sugar-based and HPLC-based assays were compared. Antimicrobials and methods of terminating cellulolytic reactions were tested for their compatibility with different assay schemes. Enzymatic methods of product analysis had analytical sensitivities greater than 12-fold higher than that of reducing sugar assays and approximately double that of the HPLC-refractive index-based assays. A product analysis scheme which calls for two glucose determinations (glucose oxidase/peroxidase assay), one prior to and one following a cellobiase treatment of the terminated reaction mixture, is presented.

Specific combinations of commonly used reagents and assay protocols which significantly reduce the precision and/or accuracy of cellulase assays are identified.

Trichoderma derived cellulase systems are known to exhibit an apparent substrate inhibition. This phenomena has been evaluated using a microcrystalline cellulose substrate and a complete *T. reesei* enzyme preparation. Rates of saccharification were defined as total glucose equivalents solubilized per unit time. The extent of substrate inhibition, at 50°C, pH 5.0, was shown to be dependent on the ratio of total enzyme to total substrate. The properties associated with this type of inhibition are consistent with the general concept that there is a kinetic advantage when independent enzymes occupy/act at the same substrate loci.

The time course of *Trichoderma reesei* cellulase catalyzed saccharification of structurally distinct celluloses has been compared. The degree of polymerization (DP), crystallinity index (CrI) and apparent surface area of the substrates clearly effects the kinetics of saccharification. There was an apparent substrate inhibition associated with approximately 40% of the substrates tested. With one exception, all substrates exhibiting "substrate inhibition" had a relatively high crystallinity index ($CrI > 80$) and low degree of polymerization ($DP < 250$).

The Relative Activity of the Cellulase Enzyme System of *Trichoderma reesei* with Native and Modified Cellulosic Substrates

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CHAPTER 1. INTRODUCTION

Cellulose is the major carbohydrate synthesized by plants. It has been estimated that there are 4 x 10^{10} metric tons generated annually (Coughlan, 1985). This vast resource is of considerable economic interest due to its potential use for the production of liquid fuels and chemical feedstocks. The enzymatic hydrolysis of cellulose by microbial cellulases has been studied extensively. The cellulolytic enzyme system from *Trichoderma reesei* has been the focus of many studies due to its relatively high activity on crystalline cellulose. Cellulase enzyme systems are composed of different groups of enzymes which act together to solubilize cellulose. In general, three classes of enzymes are involved in the hydrolysis of cellulose to glucose. They are classified as (I) 1,4-ß-D-glucan glucanohydrolase (endoglucanase or EG; EC 3.2.1.4), (II) 1,4-ß-D-glucan cellobiohydrolase (exoglucanase or CBH; EC 3.2.1.91), and (III) ß-glucosidase (cellobiase; EC 3.2.1.21). These enzymes act in a cooperative manner to degrade crystalline cellulose.

Cellulose is relatively simple polymer in terms of its composition, it being a linear homopolymer of glucose linked ß-1,4. However, the physical properties of cellulose are quite variable due to differences in the packing of individual polymers within a microfibril. Cellulose is made up of closely associated glucose chains which pack in either a highly ordered crystalline arrangement or a more dissociated amorphous arrangement. The more ordered the packing of cellulose chains, the higher the crystallinity of cellulose and the less susceptible that cellulose will be to enzymatic degradation.

The relative rate of saccharification of a given cellulose is dependent on many factors, including the activity and specificity of the enzymes and the structure of the substrate. Parameters affecting the activity of the enzymes relate to the relative quantity of *endo* and *exo* acting enzymes, the degree of product inhibition, and substrate to enzyme ratios. Properties of the substrate which are thought to affect the rate of hydrolysis include the relative crystallinity, available surface area, and degree of polymerization.

This thesis is divided into two major sections. The first section provides a review of the current literature on cellulolytic enzymes. The second part includes experimental work on the saccharification of cellulose by the cellulase enzyme system of *Trichoderma reesei*. The experimental section is divided into three chapters; (1) a comparative analysis of methods used to monitor cellulose saccharification, (2) a study of the kinetic consequences of diverse enzyme to substrate ratios, and (3) a comparative look at the substrate-activity profiles of a range of native and modified cellulosic substrates. The thesis abstract provides a brief, general summary of the major observations discussed in the experimental section.

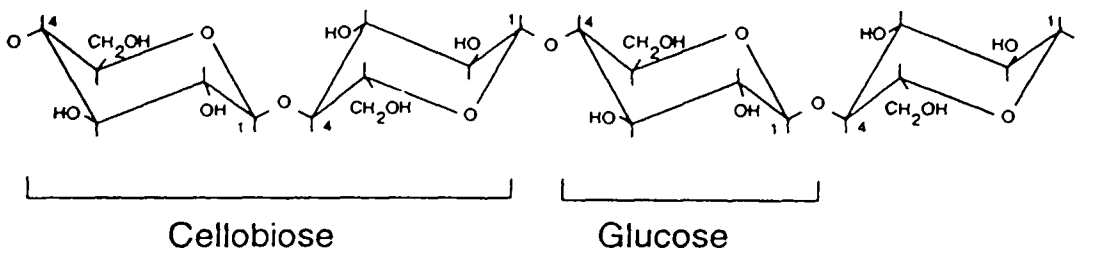
CHAPTER 2. LITERATURE REVIEW

2.1. CELLULOSE SUBSTRATE

2.1.1. Structure

Cellulose is synthesized by all higher plants as well as by a wide variety of other organisms. The worldwide aggregate amount of synthesis is enormous, making cellulose the most abundant biopolymer on earth (Coughlan, 1985). Most cellulose is found in cell walls in the form of structural microfibrils. Cellulose is a linear polymer made of glucose subunits linked by ß-1,4 bonds (Fig. 1). Each glucose residue is rotated by 180° relative to its neighbors, so that the basic repeating unit is in fact cellobiose. Chain lengths vary between 100 and 14,000 residues (Marx-Figini, 1982). Cellulose chains form numerous intra- and intermolecular hydrogen bonds, which account for the formation of rigid, insoluble microfibrils. Microfibrils range in lateral dimension from 3-4 nm in higher plants (Preston, 1974) and up to 20 nm for microfibrils of the alga *Valonia macrophysa* (Frey-Wyssling, 1976). The chains are oriented in parallel and form highly ordered, crystalline domains interspersed by more disordered, amorphous regions. The biological significance of these two regions is that the amorphous component is digested more readily by enzymatic attack than the crystalline component. Thus, the formation of this structure is directly related to the availability of the cellulosic material to enzymatic hydrolysis.

Two models have been proposed to described the structure of cellulose within microfibrils: (1) the fringed fibrillar model (Fig. 2A; Scallan,1971) and (2) the folding chainmodel (Fig. 2B; Chang, 1971). In the fringed fibrillar model, the cellulose



Cellulose chain: D-glucose units are in ß(1,4) linkage

Figure 1. Structure of cellulose (cellobiose unit).

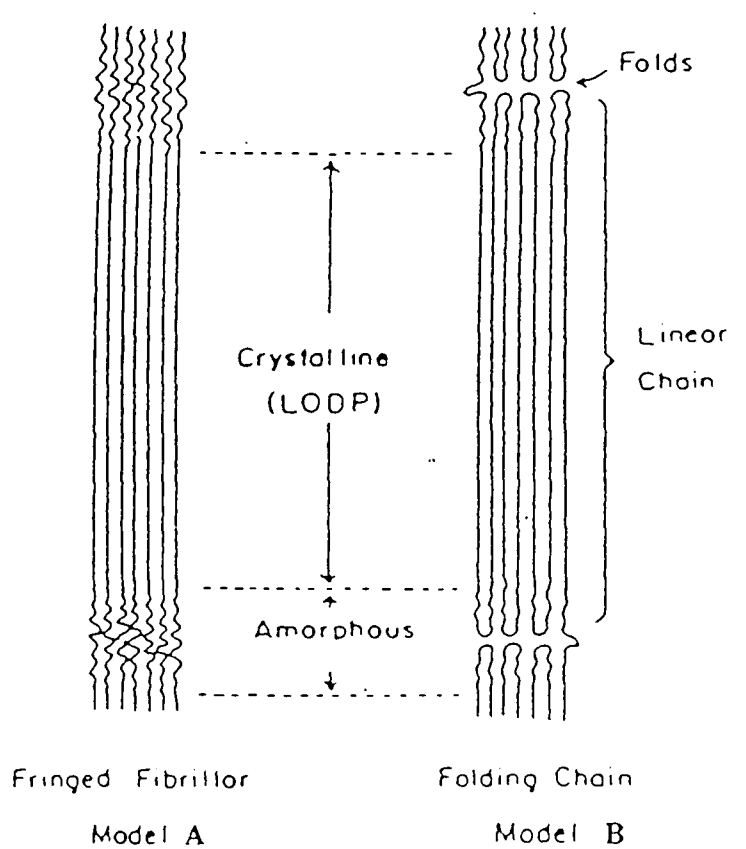


Figure 2. Cellulose structure models. (from Scallan, 1971; Chang, 1971)

molecules in the basic fibril are fully extended with molecular direction in the line with the fibril axis. The crystalline regions and amorphous regions are formed naturally along the cellulose fiber. The crystalline regions are separated by amorphous regions. In the folding chain model, the cellulose molecules fold back and forth along the fibrillar axis within the 101 plane of the crystalline lattices to form a sheet-like "platellite" unit (Chang, 1980; Atalla, 1983).

Microscopic and crystallographic examination of cellulose microfibrils from a variety of organisms has demonstrated that the width and crystallite size of microfibrils varis greatly between organisms and within organisms at different stages of development. Raman spectroscopy and solid-state ^{13}C-NMR have shown that crystalline cellulose occurs in various form. The native, crystalline form of cellulose has a structure designated as cellulose I, which can be converted into cellulose II by alkaline treatment (Figure 3). Cellulose I and cellulose II differ in their intrachain hydrogen bonding pattern. Most native celluloses are composed of two slightly different forms of type I cellulose, termed I_α and I_β, which differ with respect to their intermolecular hydrogen bonding pattern. The ratio of I_α to I_β is dependent on the source of the cellulose (Coughlan, 1992; Atalla and VanderHart, 1984; Kugasnf Brown, 1991; Richmond, 1991). The degree of crystallinity of cellulose is dependent on its source and the methods used to prepare it. Cellulose isolated from *Valonia macrophysa* (Chanzy et al 1983) has a degree of crystallinity of nearly 100%. Cellulose from cotton is about 70% crystalline (Wood, 1988). The degree of crystallinity of most commercially available cellulose preparations form wood or cotton varies between 30 and 90%. Acid swollen celluloses prepared in the laboratory may have a degree of crystallinity approaching zero.

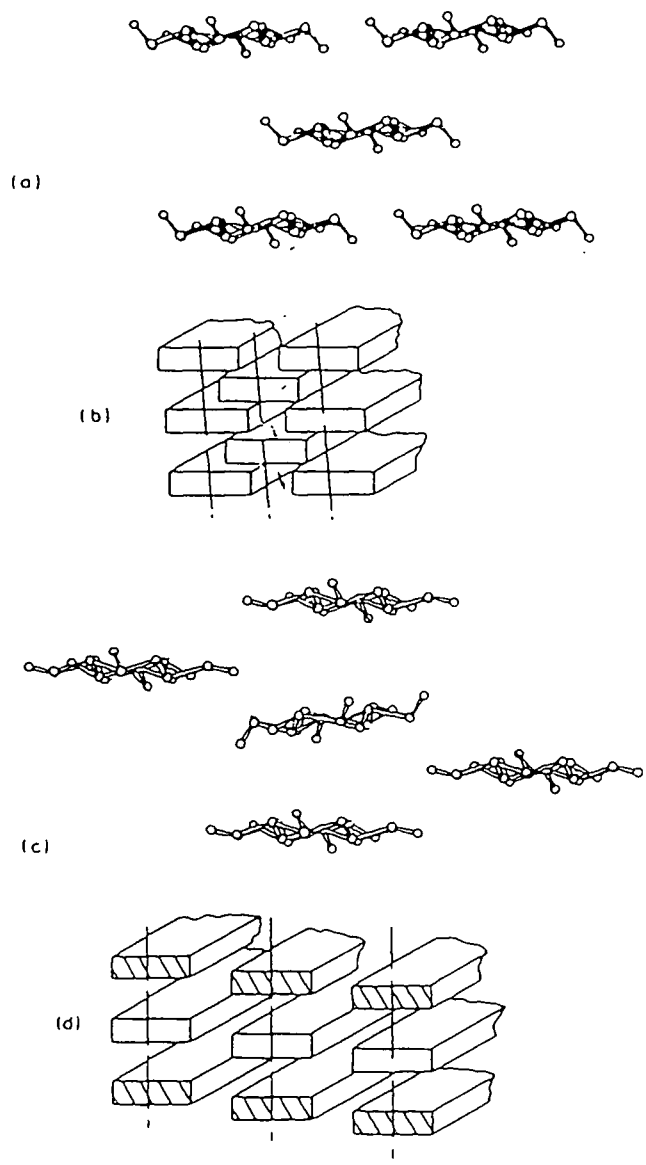


Figure 3. Packing of cellulose chains in the natural and regenerated states: all views are end-on , down a bundle of chains. (a) computer plot of chain packing in natural cellulose (Cellulose I) (b) chains are parallel rather than anti-parallel i.e. the chain ends at on end of bundle are all of one type (not hatched) (c) computer plot of chains packing in regenerated cellulose (Cellulose II) (d) chains are antiparallel i.e. chain ends at the end of a bundle are of both types (hatched and not hatched). (from Rees, 1977)

The wide distribution of cellulose in nature is correlated with significant diversity in the size and crystallinity of microfibrils. The large microfibrils in the walls of many algae, which are woven together into very strong cell walls, are probably an adaptation to the physical stresses as unicells or filaments in aquatic environments. The mechanism of coupled polymerization and crystallization, which provides the basis for microfibril formation by cell-directed self-assembly, has allowed different organisms to synthesize microfibrils with different sizes and crystallinities and, thereby, to be better adapted to different environments.

In contrast to starch, which serves as a storage polymer for glucose, the role of cellulose is exclusively structural. The high tensile strength of cellulose enables plant cells to withstand osmotic pressure and is responsible for the resistance of plants to mechanical stress. The mechanical strength of cellulose is particularly obvious in the case of wood and textile fibers (cotton, ramie), which consist of the walls of elongated, empty cells. In the secondary wall of plant cells, cellulose forms several sheets in which microfibrils are organized in parallel, each sheet having a different orientation. The microfibrils are usually embedded in a matrix of hemicellulose which is made up of branched short, heteropolymers of pentose and hexose, such as xylose, galactose, mannose , arabinose as well as uronic acid and lignin which is a complex aromatic polymer based on phenylpropane (Figures 4 and 5).

2.1.2. Substrates used in cellulase studies

The diversity and heterogeneity of cellulosic substrates contributes to the difficulty of interpreting the results of cellulase studies. There are many substrates available for the study of cellulolytic enzymes (Table 1) and they differ widely in their resistance to

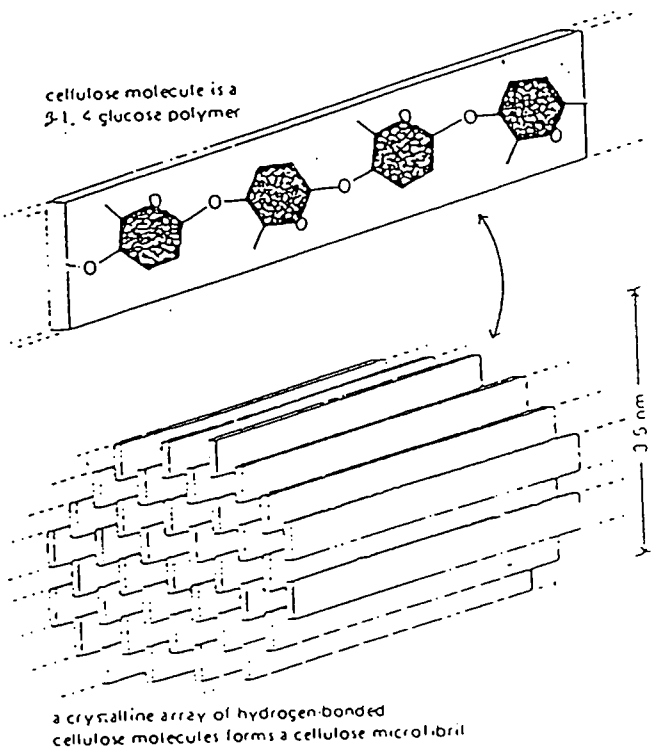


Figure 4. The structure of cellulose microfibril. (from Chang et al. 1980)

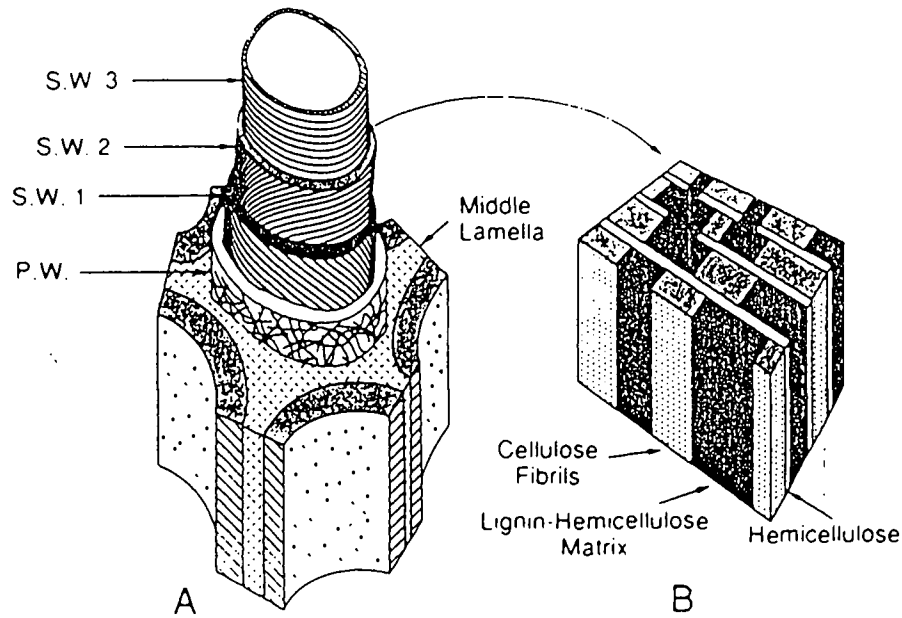


Figure 5. Cutaway view showing the organization of the cell wall layers composing woody fibers. (from Wood, 1992)

enzymatic hydrolysis. This resistance to hydrolysis is directly correlated with their likeness to natural cellulose (Nagieb et al. 1985, Weimer and Weston, 1985).

2.1.2.1. Native cellulose

Pure cellulose is commercially available in several forms (cotton, filter paper, microcrystalline). These cellulose substrates are generally used to assess the efficiency of a complete cellulase system. However, their physical heterogeneity (degree of crystallinity, available surface area, pore size) complicates detailed enzymological studies. Furthermore, they are not adequate for the study of incomplete cellulase systems or individual cellulolytic enzymes, which are only weakly active on these relatively crystalline substrates.

2.1.2.2. Amorphous cellulose and soluble cellulosic derivatives

Amorphous cellulose is prepared by treatment of native cellulose with phosphoric acid (Walseth cellulose), hydrochloric acid (Hsu and Penner, 1991), DMSO/SO_2 (Isogai and Atalla, 1991) or by ball-milling (Van Dyke, 1972). Soluble celluloses are prepared by introducing either carboxymethyl or hydroxyethyl substituents into the chain. Amorphous forms such as acid-swollen cellulose and soluble carboxymethlylcellulose (CMC) are frequently used for assays, because of their high susceptibility to enzymatic hydrolysis. In the case of CMC, the degree of substitution negatively influences the rate of hydrolysis, resulting in a rapid deviation of hydrolysis from linear kinetics. For the same reason, CMC is a poor substrate for exo-acting enzymes. The extent to which the crystallinity of a substrate is disrupted by a physical or chemical treatment has an effect on the rate and extent of its enzymatic hydrolysis. These substrates are difficult to prepare in a reproducible fashion which creates problems for workers trying to compare data.

Table 1. Commonly used cellulosic substrates

Substrate	Ref.
Biomass	
Untreated wood	Fan and Lee 1980
Pretreated wood	Ryu et al. 1982
Agricultural residues	Tanaka et al. 1988
Pure cellulose	
Valonia cellulose	Chanzy et al. 1983
Cotton linters	Okazaki and Moo-Young 1978
Filter paper	Ghose 1987
Bacterial cellulose	Gusakov et al. 1985
Microcrystalline cellulose	Ohmine et al. 1983
Amorphous cellulose	Isogai and Atalla 1991
Reprecipitated cellulose	Stone et al. 1969
Cellodextrins	Weimer and Weston 1985
Modified cellulose	
Covalently dyed cellulose	Henrissat et al. 1987
Nonionic-substituted cellulose (e.g., hydroxyethylcellulose)	Wood 1988
Ionic-substituted cellulose (e.g., carboxymethylcellulose)	Wood 1988
Covalently dyed cellulose derivatives	Eriksson and Pettersson 1968

2.1.2.3. Soluble oligosaccharides

Several low molecular mass substrates can also be used for cellulase studies. Soluble oligodextrins comprising three to six glucosyl residues can be obtained by partial hydrolysis of cellulose (Miller et al. 1960). Chromogenic and fluorogenic substrates can be made from these dextrins, in which colored or fluorescent compounds are linked to the anomeric carbon of the cellodextrin by a ß-glucosidic bond (van Tilbeurgh et al. 1982). The most frequently used compounds, p-nitrophenyl-ß-cellobioside and methylumbelliferyl-ß-D-cellobioside (MUC) are commercially available. However, homologues with a higher degree of polymerization have proved useful in characterizing the specificity of enzymes and in defining the topology of substrate binding sites, in particular the number of subsites and the position of the cleavage site (Macarron et al. 1993; Claeyssens and Aerts, 1992).

2.1.3. Characteristics of substrates related to enzymatic degradation

The action of enzymes on native cellulose can be assessed by a number of different parameters. Catalytic activity is usually assayed by measuring the release of soluble reducing sugars, which are expressed as glucose equivalents. Alternatively, residual insoluble cellulose can be determined gravimetrically (Tailliez et al. 1989), colorimetrically (Updegraff, 1969), or by turbidimetry (Johnson et al.,1982). Relatively few studies deal with alterations of the physico-chemical properties of the insoluble substrate during the course of substrate saccharification. Physico-chemical properties of interest include the substrates crystallinity index, degree of polymerization, available surface area, and particle size. These properties are known to have considerable influence on hydrolysis kinetics (Table 2). Electron microscopy has been used in several studies, showing sites of cellulase adsorption (Chanzy et al., 1983), patterns of erosion (Chanzy

and Henrissat,1985), and alterations of fibre or microfibril structure (Din et al. 1991; Sprey and Bochem 1991; 1992; 1993).

Table 2. Substrate-related factors which affect rate and extent of cellulose biodegradation

Factor	Ref.
Degree of polymerization	Fan and Lee 1980
Degree of substitution	Vanderhart et al. 1985
Crystallinity	Taylor et al. 1983
Available surface area	Lee and McCaskey 1983
Pore volume and its distribution	Focher et al. 1981
Association with hemicellulose and lignin	Lee and McCaskey 1983

2.2. CELLULASE ENZYME

2.2.1. Introduction

In catalyzing the decay of lignocellulosic residues, cellulolytic microorganism are of major importance in our ecosystem. The cellulase enzymes synthesized by the microorganism have a crucial role to play in recycling nutrients, in maintaining soil fertility, and in preserving the carbon cycle in nature. Not surprisingly, cellulolytic enzymes have been the subject of many investigators. The growing need for energy, food and chemicals and the problem of disposing of industrial wastes has focused attention on the importance of cellulases, and fungal cellulase in particular. These enzymes are of particular interest for the generation of glucose feedstocks to be used in further processes. ß-glucosidases are also of interest for the conversion of cellobiose to glucose. Strictly speaking, these enzymes are not cellulases. However, since the ß-glucosidases have an extremely important role to play in the rate and extent of degradation of cellulose by fungal cellulases, their properties are also reviewed.

2.2.2. Cellulase complex

The cell-free cellulase that can degrade crystalline cellulose is often described as a "cellulase complex", "a true cellulase" or a "complete cellulase system". Relatively few such cell-free cellulases are known, but notable in this group are those from *Trichoderma reesei* (formerly *T. viride QM6a*), *T. koningii*, *Penicillum pinophilum*, and *Neocallimastix frontalis* (Wood and McCrae, 1986). As currently understood, the cellulase complex consists of a number of different enzymes, which by acting sequentially and in concert effectively render cellulose soluble (Wood et al. 1979). There are many reports which characterize the component enzymes of different cellulase systems, but it is still not known with any degree of certainty how the different enzymes

interact to degrade crystalline cellulose (Wood, 1985). Notwithstanding these uncertainties, there is some consensus that the enzymes of the complex comprise three major types: endo-1,4-ß-glucanases (endo-1,4-ß-D-gluconohydrolase, EC 3.2.1.4), exo-1,4-ß-glucanases (normally 1,4-ß-D-glucan cellobiohydrolase, EC 3.2.1.91), and ß-D-glucoside glucohydrolase (EC 3.2.1.21). The systematic nomenclature has now completely displaced that used previously, which described the enzyme system in terms of a nonhydrolytic chain-disaggregating enzyme (so-called C1) and hydrolytic enzymes (so-called Cx) (Reese et al. 1950) (Figure 6). The use of the systematic names implies that the various enzymes fall neatly into one or another of the categories mentioned above. However, some literature (Chanzy et al. 1983; Henrissat et al. 1985) indicates that the mode of action of the enzymes described as cellobiohydrolases and endoglucanases are ambiguous. A complete reclassification of the enzymes may be necessary.

2.2.3. Isolation and purification of individual components

Separation and purification of the individual components of several cellulase systems have been reported. Each of these studies has commented on the difficulty of obtaining a homogeneous enzyme preparation due to the presence of several proteins having very similar physicochemical properties. Earlier methods of separation were dependent on differential adsorption and ion exchange, but all the traditional methods of protein purification including gel filtration, electrophoresis, isoelectric focusing, chromatofocusing, and hydrophobic interaction chromatography are now used. Repeated application of one or another of these methods is required to obtain a single cellulase protein species. Invariably, these methods involve repeated concentration, precipitation, or desalting steps as preliminary to chromatography or electrophoresis, and clearly there is a risk that this may result in partial denaturation of the enzyme.

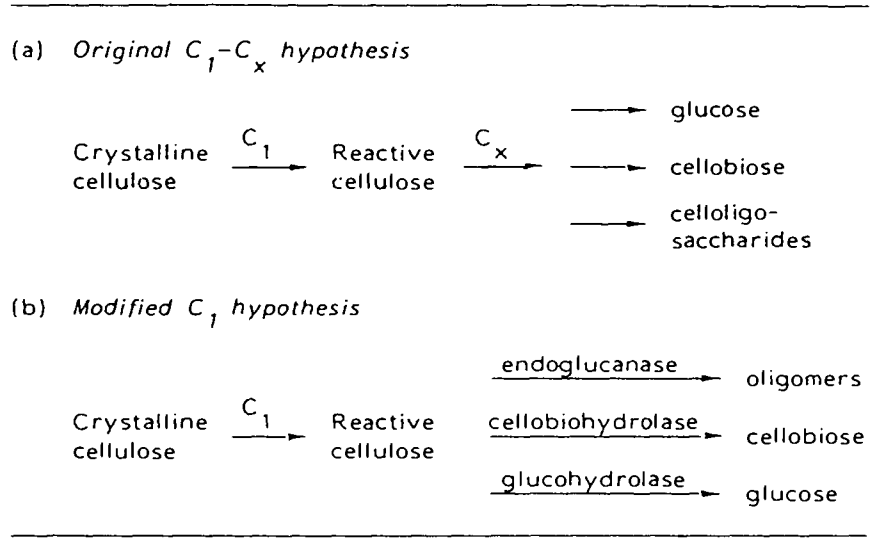


Figure 6. Original and amended hypotheses for the mechanism of cellulases action involving a C1 component. (from Reese et al. 1950)

More gentle methods of separation involving affinity chromatography have been used successfully. The complete separation of ß-glucosidase of *T. viride* (Gong et al 1977) or *T. koningii* (Wood et al. 1982) from the rest of the cellulase system was obtained using the lectin concanavalin A immobilized on agarose or Sepharose. More elegant procedures involving affinity chromatography have been used to provide highly purified cellobiohydrolase from *T. reesei* (Nummi et al. 1983; Van Tilbeurgh et al. 1984). Using these various methods of purification, a large number of components have been judged to be pure by criteria which include isoelectric focusing, electrophoresis in denaturing conditions, ultracentrifugation, and immunoelectrophoresis. However, Sprey and Lambert (1983) have demonstrated that cellulase components of *T. reesei,* which are apparently homogeneous by isoelectric focusing, could be separated into at least six proteins after treatment of the preparation with urea-octylglucoside, indicating that the criteria normally used for protein purity may not be sufficient when working with cellulases. The implication of this study in terms of the published substrate specificities is clear; substrate specificities previously attributed to a single enzyme may actually relate to a group of enzymes. The existence of enzyme-enzyme complexes may explain many of the anomalous results and unusual substrate specificities recorded in the literature. It was suggested that the more gentle affinity methods of purification may result in the isolation of enzymes with substrate specificities more close to those found in culture filtrates (Enari and Niku-Paavola, 1987). However, the more drastic the purification procedures used, the more likely that the complexes will be disrupted.

2.2.3.1. Endoglucanases

Endoglucanases hydrolyze cellulose chains at random to effect a rapid change in their degree of polymerization with a slow increase in reducing power. Substrates

include carboxymethylcellulose and cellulose swollen in phosphoric acid or alkali. Neither cotton fiber (Wood et al 1979) or microcrystalline cellulose is thought to be hydrolyzed extensively by endoglucanases. However, a purified endoglucanase from *T. reesei* was reported to have no capacity for degrading an amorphous cellulose prepared by ball-milling (Niku-Paavola et al. 1985), while *T. viride* endoglucanase could solubilize microcrystalline cellulose extensively (Beldman et al. 1985). Some properties of purified endoglucanases are listed in Table 3.

Glucose, cellobiose, and longer chain oligosaccharides are the normal products of the reaction of endoglucanases. However, as transglycosylation has been reported to be a property of *T. viride* endoglucanase (Shoemaker and Brown, 1978), under some conditions other oligosaccharides will be found in the solution. A feature of most fungal cellulases is the multiplicity of forms in which the endoglucanase activity exists. These various forms differ in their mode of action (Wood et al. 1978; Shoemaker and Brown, 1978), in the extent to which they are adsorbed on cellulose (Rabinovich et al. 1984; Klyosov et al. 1986), and in their capacity for acting synergistically with cellobiohydrolase in solubilizing cellulose (Wood et al. 1978). The endoglucanases are typically glycoproteins of acidic pI and range in size from 12 to approximately 60 KD.

2.2.3.2. Cellobiohydrolase

It has been held for some time that fungal cellobiohydrolases act by removing cellobiose from the nonreducing end of the cellulose chain (Wood et al. 1979). This was demonstrated by the fact that the degree of polymerization of acid-treated cellulose decreased very slowly during hydrolysis by *T. viride* cellobiohydrolase (Berghem et al. 1976). Similar results were obtained with the cellobiohydrolase of *F. solani* , *T.*

Table 3. Some properties of selected endoglucanases.

source	Mr (KDa)	pI	Avicel[a]	CMC[a]	Ref.
T. reesei					
Endo I	52	4.0~5.0	--	--	1
Endo II	48	7.0	--	--	1
Endo II	55	4.5	--	--	2
Endo III	48	5.5	--	--	2
EG I	54	4.7	--	--	3
Endo	43	4.0	--	--	4
T. viride					
EGI	50	5.5	.013	13.1	5
EGII	45	6.9	.007	20.1	5
EGIII	58.5	6.5	.016	3.2	5
EGIV	23.5	7.7	.003	9.6	5
EGV	57	4.4	.007	14.7	5
EGVI	52	3.5	.004	15.8	5

[a] specific activity. CMC, carboxymethylcellulose, U/mg.

References: 1) Schulein, 1988; 2) Bhikhabhai et al. 1984; 3) Shoemaker et al. 1983; 4) Niku-Paavola et al. 1985; 5) Beldman et al. 1985.

koningii, and *P. pinophilum/funiculosum* (Wood et al. 1986), using cotton which had been swollen in phosphoric acid. Recently, however, it was suggested that some cellobiohydrolases may attack linkages some distance from the nonreducing end of the chain (Enari et al. 1987): some workers indeed interpret their data to indicate that an enzyme, often described as a cellobiohydrolase, is in fact an endoglucanase (Enari and Niku-Paavola. 1987; Henrissat et al. 1985; Kyriacou et al. 1987). Thus, interpretation of the mode of action of the cellobiohydrolases is currently in some state of confusion.

Most "pure" cellobiohydrolases are reported to release small quantities of glucose along with cellobiose. In *S. pulverulentum* (Streamer et al. 1975), the amount of glucose released is a significant proportion of the total soluble sugars released, but the enzyme is still described as a cellobiohydrolase or exoglucanase. When present in culture filtrates, cellobiohydrolase constitutes the major protein. In *T. reesei* cellulase it amounts to approximately 50~80% of the extracellular protein (Montenecourt, 1985). The substrate specificity of the cellobiohydrolases from different sources appear to differ markedly. Thus, degradation of cotton fibers reported to be extensive (Chanzy et al. 1983; Nummi and Niku-Paavola, 1987) by the cellobiohydrolase of *T. reesei* acting alone but negligible in *T. koningii* cellobiohydrolase (Wood et al. 1979; Wood et al. 1982). Whatman # 1 filter paper, which is prepared from cotton fiber was also broken down into short fragments by *T. reesei* cellobiohydrolase (Enari and Niku-Paavola, 1987), but no breakdown was observed with either *T. koningii* (Wood et al. 1982) or *P. pinophinum* (Wood et al. 1980). These differences in enzyme specificity may relate to the existence of a variety of enzyme-enzyme complexes, as indicated by Sprey and Lambert (1983).

Microcrystalline cellulose (Avicel) is reported to be a substrate for most cellobiohydrolases isolated, although the rate and extent of hydrolysis varies widely. Avicelase is now regarded to be synonymous with cellobiohydrolase, and even the smallest release of reducing sugar is considered to indicate the presence of cellobiohydrolase. This is unfortunate, however, for some endoglucanases can also hydrolyze Avicel. Indeed, the specific activity for Avicel for some endoglucanases of *T. reesei* and *T. viride* was reported to be higher than the cellobiohydrolase. In this circumstance, synergistic activity between cellobiohydrolase and endoglucanase would give completely erroneous results.

Carboxymethylcellulose is reported not to be a substrate for cellobiohydrolase (Wood et al. 1979), and this characteristic is widely held as being the property that distinguishes these enzymes from endoglucanases. However, a scan of the literature reveals that this distinction has become more confused. Thus, cellobiohydrolase isolated from *T. reesei* (Nummi et al. 1983; Kyriacou et al. 1987) and *T. viride* (Beldman et al. 1985) culture filtrates are reported to be able to hydrolyze CMC to an extensive degree. The molecular weights of purified cellobiohydrolases range from 41-65 KD, and isoelectric points are generally acidic. All except the exoglucanase of *S. pulverulentum* are glycoproteins, with proportions of carbohydrate ranging from near 0 to 33%: mannose being the principal sugar present (Wood et al. 1980). Some properties of selected purified cellobiohydrolases are listed in Table 4.

2.2.3.3. ß-glucosidase

Fungal ß-glucosidase can catalyze the hydrolysis of both alkyl- and aryl-ß-D-glucosides as well as glucosides containing only carbohydrate (Shewale and Sadana

Table 4. Some properties of selected CBH I and CBH II.

source	Mr (KDa)	pI	Avicel[a]	CMC[a]	Ref.
CBH I					
T. reesei	68	4.4	.09	nd	1
T. reesei	65	3.8-4.0	--	--	2
T. reesei	--	3.9	.0175	.0099	3
T. reesei	64-68	4.05-4.25	--	.037	4
T. reesei	64	3.9	--	--	5
T. reesei	66	4.2	.04	<.01	6
T. reesei	65	3.6-4.12	--	.01	7
CBH II					
T. reesei	--	5.9	.0391	.0165	3
T. reesei	53	5.9	--	--	5
T. reesei	--	5.9	--	--	6
T. reesei	58	6.3	--	--	7

[a] specific activity, U/mg protein.

References: 1) Huang and Penner, 1991; 2) Schulein, 1988; 3) Tomme et al. 1988; 4) Riske et al. 1986; 5) Bhikhabhai et al. 1984; 6) Shoemaker et al. 1983; 7) Nummi and Niku-Paavola et al. 1985.

1978; Woodward et al. 1981 & 1986). Some properties of selected purified ß-glucosidase are listed in Table 5. Many ß-glucosidases are not specific for the ß-1,4 linkage, and ß-1,2, ß-1,3, and ß-1,6 linkages are attacked with ease (Shewale and Sadana, 1978). The ß configuration is retained on hydrolysis and this property distinguishes them from exoglucanases, which act by inversion (Withers et al. 1986; Gebler et al. 1992). ß-glucosidases can also be transferases acting on glucose units and forming alcohols, or other sugar molecules such as dimers, trimers, and higher oligosaccharides (Wood et al. 1982).

Most ß-glucosidase enzymes are strongly inhibited by glucose and gluconolactone, and by excess substrate (Shewale and Sadana, 1978; Woodward et al. 1981). End-product inhibition was reported to be either competitive (Dekker, 1986) or noncompetitive (Gong et al. 1977) depending on the source. Nojirimycin (5-amino-5-deoxy-D-glucopyranose) is a powerful inhibitor of ß-glucosidase of *T. reesei* (Reese and Parrish, 1971). Because ß-glucosidase enzymes are the only enzymes of the cellulase system that can hydrolyze cellobiose, they have a vital role to play in cellulose degradation. In cellulase systems deficient in ß-glucosidase activity, the concentration of cellobiose increases during hydrolysis, and this results in inhibition of the cellobiohydrolases and endoglucanases enzymes. *Trichoderma reesei*, although a good producer of cellulase, produces ß-glucosidase in only small quantities (Mandels, 1982). The black *aspergilli* are the best sources of ß-glucosidase (Woodward et al. 1981), and have been used to supplement the *T. reesei* cellulase system in attempts to produce an enzyme system that could be used commercially for the production of glucose from lignocellulosic materials (Sternberg, 1976). Several of the purified enzymes have been found to be glycoproteins with molecular weights ranging from 40 to 400 KD.

Table 5. Some properties of selected ß-glucosidase.

Source		Km (mM) state	pNPG	G2	KDa[1]	pI	Ref.
A. niger	purified	1.22		1.59	116/SDS	4.2	1
A. niger	purified	0.8		1.8	84/SDS	--	2
A. niger	crude	1.03		5.63	--	5.0	3
A. niger	purified	0.22		--	170/SDS	--	4
A. niger	purified (I-III)	0.24-0.67		1.1-1.64	>200/GF	--	5
A. niger	crude	--		3.6	--	--	6
T. reesei	purified	0.3		0.5	70/SDS	8.4	7
T. reesei	purified	0.1		1.25	81.6/SDS	8.5	8
T. reesei	purified	--		--	115/SDS	6.0	9
T. viride	purified (I-III)	2.50-2.74		--	76/SDS	--	10
T. viride	purified	0.33		2.68	47/SDS	--	11
T. viride	purified	0.28		1.5	47/SDS	5.74	12

[1] SDS, SDS gel electrophoresis; GF, gel filtration chromatography.

References: 1) Huang and Penner, 1991; 2) Enari and Niku-Paavola, 1981; 3) Dekker, 1986; 4) Adikane and Patil, 1985; 5) King and Smibert, 1963; 6) Woodward, 1982; 7) Schmid and Wandrey, 1987; 8) Chirico and Brown, 1987; 9) Shoemaker et al. 1983; 10) Gong et al. 1977; 11) Maguire, 1977; 12) Berghem and Pettersson, 1974.

2.2.4. Multiplicity in cellulase activity

Detailed fractionation studies have shown that multiple forms of each type of the cellulase enzyme exist in fungal enzyme systems. The nature and origin of, and the need for, the various forms has been the subject of much discussion. Heterogeneity in the cellulases of *Trichoderma* species has been particularly well studied. These cellulases have been shown to be heterogeneous with respect to ß-glucosidase, endoglucanase, and cellobiohydrolase. Thus the cellulase of *T. koningii* was found to contain two ß-glucosidases, four major endoglucanases, and two cellobiohydrolases (Wood et al. 1979; 1982; 1985). The cellulase of *T. viride* (Bieley et al 1985) was shown to have three ß-glucosidases, six endoglucanases and three exoglucanases.

2.2.4.1. Cellobiohydrolase multiplicity

Heterogeneity with respect to cellobiohydrolase is of particular current interest. The cellobiohydrolase with the more acidic pI has been designated cellobiohydrolase I; the more basic was called cellobiohydrolase II. Studies on the heterogeneity of cellobiohydrolase I have been the most extensive. The principal differences between the two enzymes were in the isoelectric pH and in the composition and content of covalently bound carbohydrate. Cellobiohydrolase I and II have been shown to be markedly different in many fungal enzyme systems, based on amino acid sequence analysis (Enari and Niku-Paavola, 1987; Wood et al. 1986; Fagerstam and Pettersson, 1980), which showed clearly that there was no apparent relationship between the two enzymes.

An extension of these sequence analyses has given further insight into the active sites of the cellulase components. Evidence was obtained (Yaguchi et al. 1983) demonstrating a similarity between the respective catalytic sites of lysozyme, a

cellobiohydrolase of *T. reesei* and an endoglucanase (EG-1) of *S. commune*. Through sequence analysis, a certain homologous section in different enzymes was observed. In establishing these homologies, the hypothesis was put forward that cellulases and lysozyme use a common catalytic mechanism. The involvement of carboxyl groups in the active site of *S. commune* endoglucanase was confirmed by chemical modification and kinetic experiments (Clarke and Yaguchi, 1985).

Further interesting homologies have now been established between cellobiohydrolase I and II and the main endoglucanase I (EG-I) of *T. reesei* cellulase (Enari and Niku-Paavola, 1987; Pentilla et al. 1986). This was derived from sequence analysis of the cloned genes. A common amino acid sequence of 34 residues was found in all the three enzymes at the end of the molecule. This sequence appeared to be joined to the other part of the enzyme by a short region in which the amino acids were highly O-glycosylated. Surprisingly, however, whereas in cellobiohydrolase II the 34-long amino acid sequence was found at the N-terminal end of the enzyme, in both cellobiohydrolase I and endoglucanase I, it was localized at the C-terminal end (Figure 7).

These structural features suggest that the 34 -amino-acid region and possibly the carbohydrate region may be important determinants of enzyme action on crystalline cellulose (Enari and Niku-Paavola, 1987). Support for this conclusion was provided by the discovery that cellobiohydrolase I, when treated with papain, released a 10-KD, highly glycosylated fragment from the C-terminal end (van Tilbeurgh et al. 1986) which was necessary for activity on crystalline cellulose. The loss of the peptide, with its associated carbohydrate, resulted in a concomitant loss in the capacity of the high molecular weight peptide (56-KD) to adsorb to crystalline cellulose. However, there was

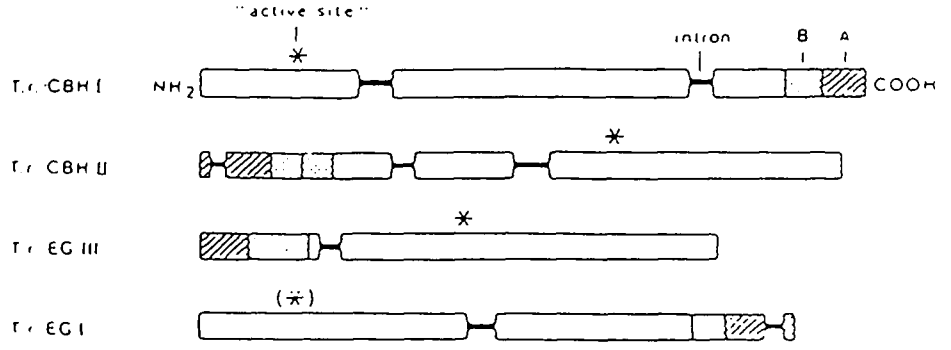


Figure 7. Structural organization of the cellulase genes from *T. reesei* . B is the O-glycosylated region that joins the A region to the main body of the enzyme (from Knowles et al. 1988)

no loss in its activity on soluble chromophoric glycosides. Based on these results, it was suggested that cellobiohydrolase I consisted of two domains. The domain corresponding to the C terminal was considered to involved in the binding of enzyme to insoluble cellulose, while the other contained the active site. Similar results have been obtained with cellobiohydrolase II (Tomme et al. 1988).

2.2.4.2. Endoglucanase multiplicity

As already stated, multiplicity in terms of endoglucanase is also the rule in most cellulase preparations investigated so far (Wood et al. 1979). However, it seems that opinion as to the nature and origin of the heterogeneity is particularly diverse and controversial. The various endoglucanases found in any one cellulase are reported to differ in their isoelectric points, their molecular weights, and their association with carbohydrate (Wood, 1985). Evidence that heterogeneity may result either from proteolytic modification or from the formation of artifacts during purification is provided by Nakayama et al. (1976), who has observed that partial proteolysis of endoglucanases yielded enzymes with altered substrate specificities. In contrast, some other data (Labudova and Farkas, 1983) indicate that the ability to produce multiple endoglucanase is a characteristic property of *T. reesei.* Thus, the nature and origin of the heterogeneity continues in both endoglucanase and cellobiohydrolase activity to be uncertain. Wood (1986) argued from theoretical consideration of the stereochemistry of cellulose chains in the cellulose crystallite that two stereospecific endoglucanase and two stereospecific cellobiohydrolases may be required for maximum efficiency in terms of solubilizing crystalline cellulose (Figure 8 and 9).

2.2.4.3. ß-glucosidase multiplicity

Multiple forms of ß-glucosidase exist in many fungi. Some of them produce up to five extracellular forms (Wood et al. 1982).

2.2.5. Biochemical properties of cellulase system

Since cellulose cannot get into the cells, cellulolytic enzymes are by necessity secreted into the medium or bound to the outside surface of cellulolytic microorganisms. Furthermore, cellulase systems generally display a set of typical properties. The systems contain a multiplicity of enzyme components showing a marked synergism against crystalline cellulose. These enzymes often possess a substrate-binding site independent from the catalytic site, and are often associated with each other and with the surface of cellulolytic microorganisms. Sequence analysis of cellulase genes and the biochemical characterization of wild-type and truncated enzymes have shown that many cellulolytic enzymes are multifunctional proteins composed of distinct domains which can be arranged in various combinations. Whenever investigated, domains of the same family appear to share similar biochemical properties (Claeyssens and Aerts, 1992).

All cellulolytic microorganisms have evolved a battery of enzymes having different specificities with respect to endo/exo mode of action, activity towards amorphous or crystalline regions, or preference for substrates of different chain length. Such a diversity may be needed, in part, to cope with the physical heterogeneity of the substrate. Moreover, the structure of cellulose changes during the process of degradation, requiring different enzymes at different times during the course of saccharification.

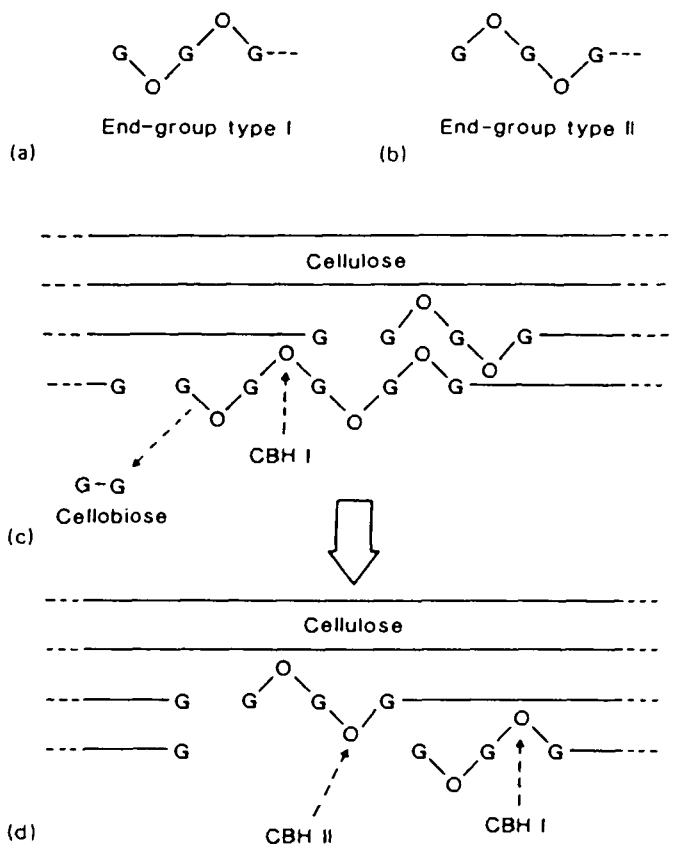


Figure 8. Proposed mechanism of synergistic action of endoglucanase and cellobiohydrolase on cellulose. (from Wood, 1980)

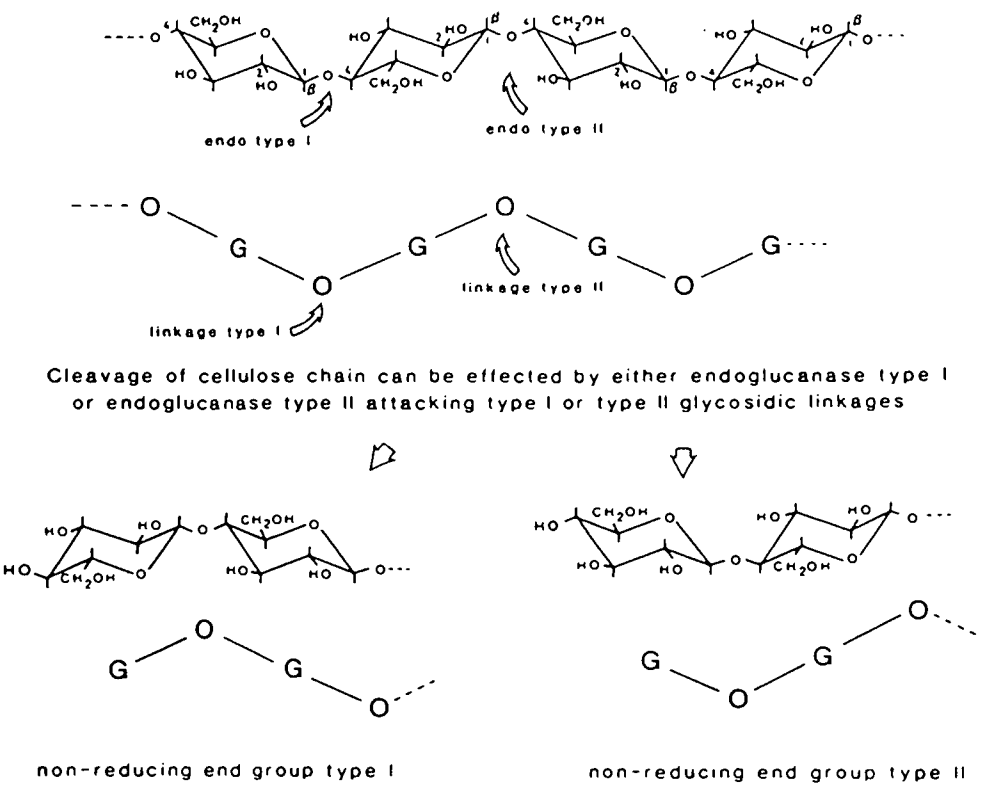


Figure 9. Proposed explanation for synergism between cellobiohydrolase I and II. (from Wood, 1980)

2.2.5.1. Three-dimensional structure of cellulases

The tertiary structure of CBH I from *T. reesei* was elucidated by small-angle X-ray scattering by Abuja et al. (1988) (Figure 10). The three-dimensional structure of some cellulases have also been determined by X-ray diffraction, including *T. reesei* cellobiohydrolase (CBH) II (Rouvinen et al. 1990), and *Clostridium. thermocellum* endoglucanase CelD (Juy et al. 1992). The folding pattern of *T. reesei* CBH II consists of a 7-stranded ß-barrel. The active site is formed by two extended loops at the C-terminal end of the barrel. *C. thermocellum* CelD comprises an N-terminal domain composed of two ß-sheets, and a large, C-terminal catalytic domain composed of 12 helices forming an α-barrel. Three of the loops, connecting six of the helices on the same side of the barrel, form the active site. The geometry of the active site of the various enzymes provides an elegant explanation of their *endo* or *exo* specificity. In the case of endoglucanases and of the xylanase, the substrate comes to lie in an open cleft, which can straddle cellulose or xylan molecules anywhere along the chain, in agreement with the endo mode of action of these enzymes. The *exo* mode of action of CBH II is explained by the fact that the active site forms an almost perfectly enclosed tunnel through which the cellulose chain has to be threaded from the non-reducing end (Figure 11). Modelling after the structure of CBH II suggests that in the case of bacterial endoglucanases related to CBH II the loops forming the reaction center are too short to form a closed structure (Gilkes et al. 1993).

2.2.5.2. Cellulose-binding domains

Many cellulolytic enzymes contain non-catalytic cellulose-binding domains (CBDs). These are usually located at the NH_2 or COOH terminus of the enzymes, and are often separated from the catalytic domains by glycosylated, Pro/Thr/Ser rich linker

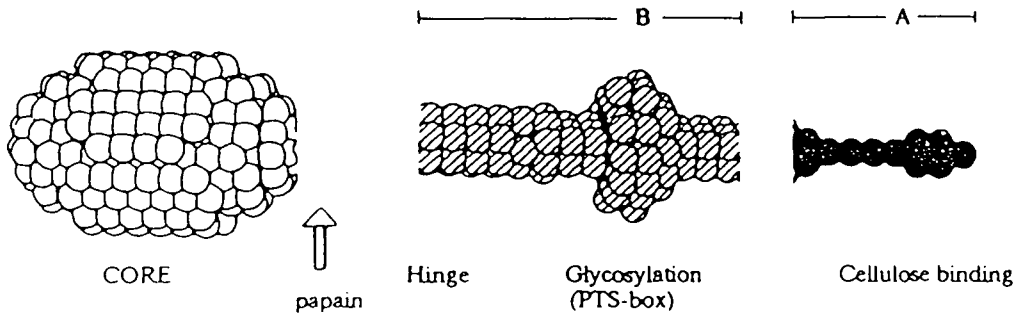


Figure 10. Tertiary structure of CBH I from *Trichoderma reesei* as elucidated by small-angle X-ray scattering. (from Abuja et al. 1988)

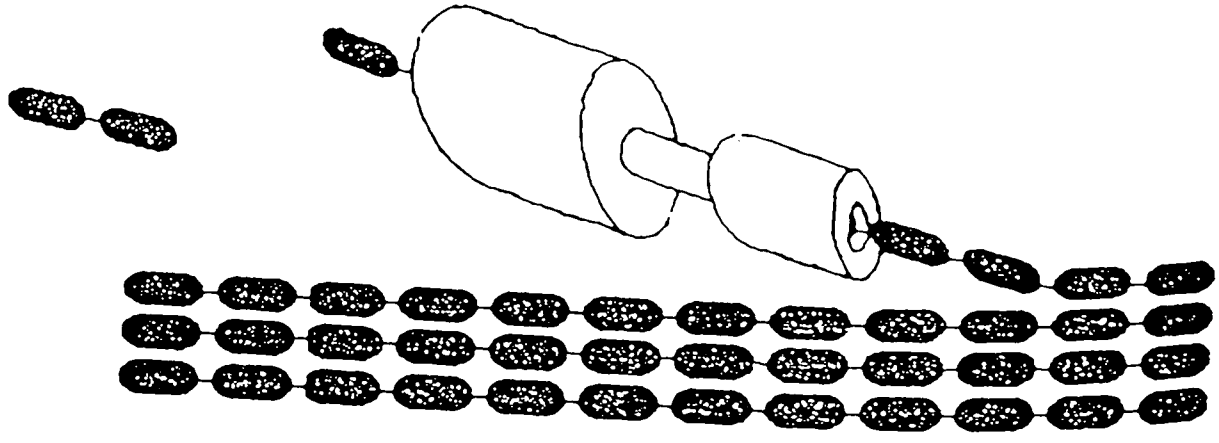


Figure 11. Speculative diagrammatic illustration of a cellobiohydrolase acting processively on cellulose. (from Coughlan, 1992)

segments. Cellulose-binding properties have actually been demonstrated for only a fraction of the putative domains that can be identified by sequence similarity. So far, there is no reason to doubt that the latter are functional, although quantitative differences in binding affinity are to be expected. Most CBDs identified to date share a highly conserved sequence of about 30 residues. The CBD of *T. reesei* CBH I was synthesized chemically and its 3-D structure was determined by NMR spectroscopy (Figure 12). The domain is shaped like wage, whose dimensions are approximately 30 x 18 x 10 Å. The secondary structure is composed of an irregular ß-sheet with three antiparallel strands and two disulfide bridges (Kraulis et. al. 1989).

2.2.5.3. Role of cellulose-binding domains in cellulose degradation

There is a strong correlation between the capacity of cellulolytic enzymes to degrade crystalline cellulose and their affinity for cellulose (Klyosov, 1990). In addition, all of the cellulases that are active against crystalline cellulose, and whose sequence has been determined, possess a CBD or are associated with a cellulose-binding protein. This suggested that the presence of a CBD enhances the activity of cellulolytic enzymes towards crystalline cellulose. Indeed, the presence of an independent CBD should allow cellulases to perform many catalytic cycles while remaining anchored to the substrate (Figure 13-A). For this, the link between the CBD and the catalytic site must be sufficiently flexible to permit cleavage of more than one bond without desorption of the CBD, and binding of the CBD to cellulose must be reversible in order to allow movement of the enzyme along the substrate. Limited diffusion of the enzymes on the surface of the substrate should help to focus hydrolysis at defined locations, and take advantage of the local disruption of the structure already induced by previous hydrolytic events.

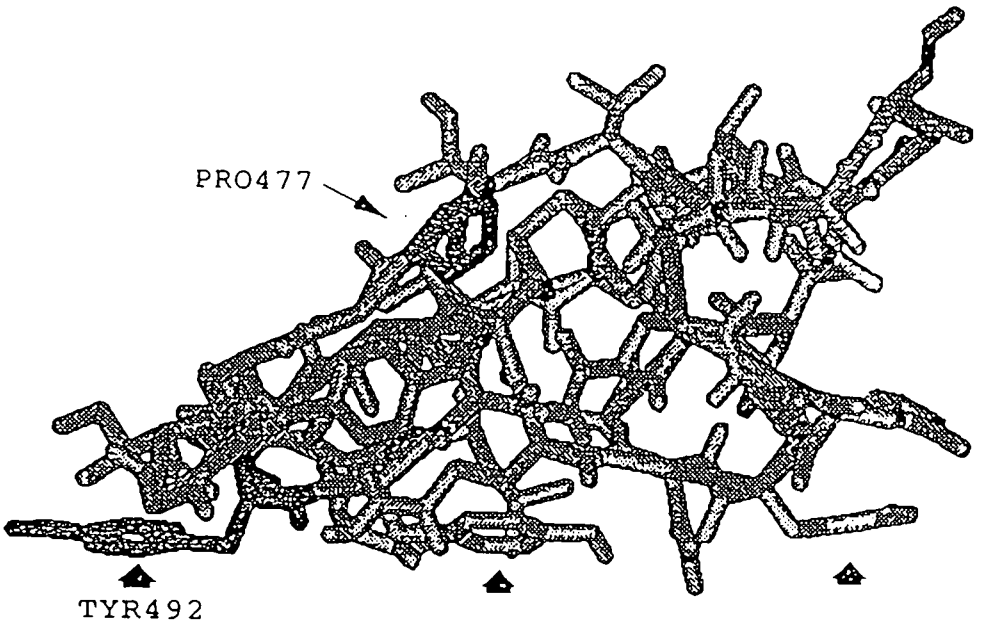


Figure 12. The overall structure of CBH I cellulose-binding domain. The hydrophilic surface is partly created by three observed tyrosines (indicated by arrows). The mutated residues are labelled. (from Reinikainen et al. 1992)

None of the available evidence contradicts these views, but a considerable variation was observed when the effect of the CBD on enzyme activity was determined for different enzymes. This was achieved either by comparing intact and truncated forms of cellulases naturally containing a CBD, or by inserting foreign CBDs by means of recombinant DNA technology. For *T. reesei* CBH I and CBH II, removal of the CBD by proteolytic cleavage reduced the activity towards microcrystalline cellulose by 85% and 40-60%, respectively, whereas activity toward chromogenic oligosaccharides remained unchanged. (Tomme et al. 1988; van Tilbeurgh et al. 1986).

The effect of the CBD varies considerably according to reaction conditions (incubation time and enzyme concentration) (Stahlberg et al. 1991). Proper joining of the CBD to the core enzyme can affect the adsorption/desorption behavior of the enzyme and its catalytic activity (Shen et al. 1991). Finally, little is known about the destabilizing activity that different CBDs might have on crystalline cellulose structure. It has been proposed that the wedge-like shape of fungal CBDs not only promotes binding to the substrate, , but also helps to peel off cellulose chains form the top layer of cellulose microfibrils (Knowles et al. 1988) (Figure 13-B). A destabilizing effect on the structure of cellulose fibers was observed when cotton or ramie fibers were incubated with the CBD derived from *C. fimi* CenA. The surface of the fibers became rough, showing signs of exfoliation of the surface layers. The fibers also became more permeable to penetration by and labelling with fluorescein isothiocyanate-labelled CBD (Din et al. 1991).

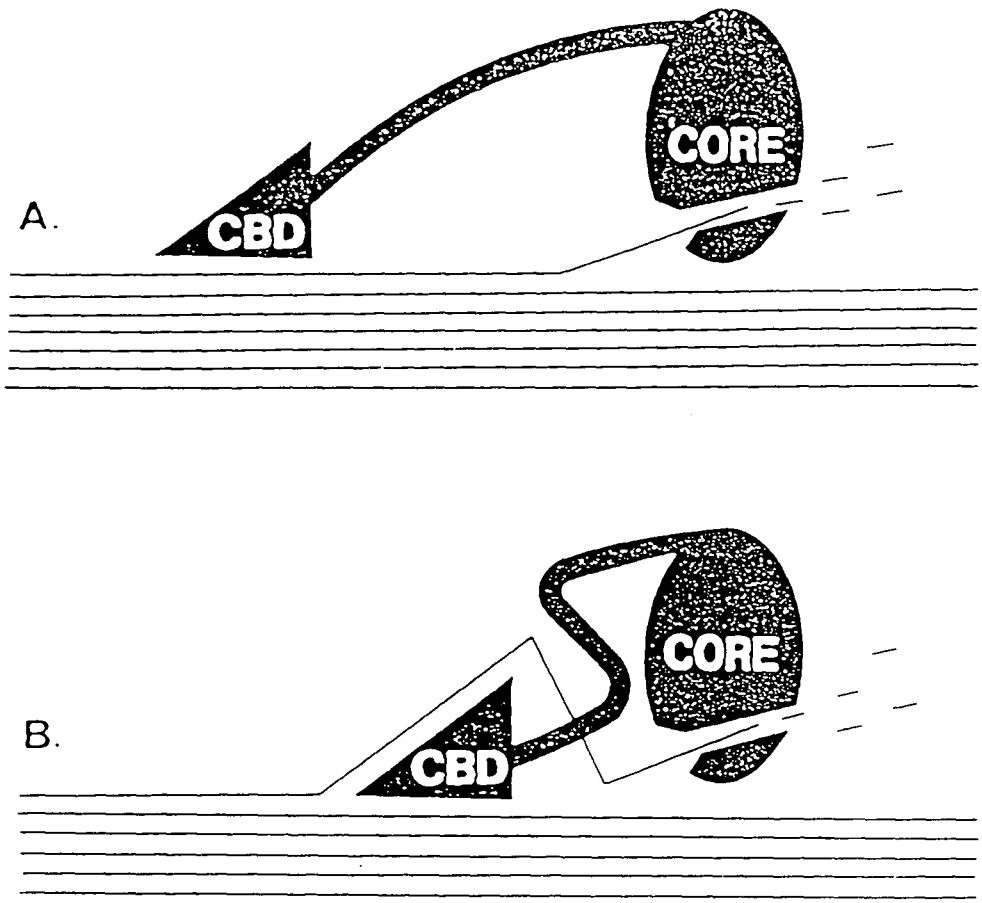


Figure 13. Hypotheses of the function of the cellulose-binding domain (A) crystal binding site model and (B) crystal disrupting model. (form Reinikainen et al. 1992)

2.3. CELLULOSE DEGRADATION

2.3.1. Quantitative measurements of cellulose biodegradation

The complex structure of cellulose and cellulosic materials introduces considerable problems to the researcher attempting to measure its biodegradation. The rate and extent of cellulose degradation by microorganisms and their enzymes is dependent in part on physical and chemical parameters such as temperature, pH, and composition of the reaction mixture. Moreover, because cellulose is a solid substrate of complex structure, a number of substrate-related factors are major determinants of degradability (Table 2). The field of cellulose biodegradation has attracted the interest of researchers from many different scientific disciplines and has involved the study of a wide variety of different organisms. It is thus no wonder that a large number of methods for measuring cellulose biodegradation have been developed, each seemingly tailored to the unique needs of the individual experimenter. It is necessary to present an overview of these different methods, along with a discussion of their particular advantages and drawbacks. Two general types of substrates are used to measure cellulose biodegradation (Table 1). The first group includes relatively unaltered natural substrates such as pure crystalline cellulose or biomass; the second includes modified cellulosic substrates whose degradation occurs more rapidly (eg, substituted cellulose) and is often more easily measured (eg, dyed cellulose). Within each class there is a continuity of degradability which reflects the structural similarity or dissimilarity of each substrate to native cellulose.

2.3.2. Assay of cellulolytic enzymes

Assays of the activity of cellulolytic enzymes include the determination of specific activities, kinetic parameters, and the location of individual cleavage sites within a

polymer chain. The optimum measurement of specific activity is determination of the number of 1,4-ß-glucan bonds hydrolyzed per unit enzyme per unit time. Such measurements are complicated by the fact that fragments of different lengths display differences in chemical reactivity and in hydrodynamic behavior, and thus respond differently to measurement techniques. No analytical method overcomes this problem entirely, although some do a better job than others. As might be expected, the results and interpretation of different assays vary tremendously and they are largely dependent on the substrate used.

In many cases it is not necessary to perform a detailed analysis of the individual cellulolytic enzymes, but only to assay the net activity of the mixture of individual enzymes which together make up what might loosely be called the "cellulase complex". However, attempts to develop a widely accepted standard assay for total cellulase activity have met with considerable resistance. Among these, the filter paper assay of Mandels *et al.* (1976) has enjoyed the widest use and has provided a way to gather a large amount of data on cellulose biodegradation. The method is useful only for assaying exoglucanase-containing cellulase complexes of sufficient activity to overcome the kinetic constraints imposed by the very low surface area of the substrate. In recent years there has been a pronounced trend toward the use of microcrystalline cellulose powder as a substrate for assaying the activity of the cellulase complex. Regardless of the substrate used, the preferred method of measuring activity involves measurement of the release of soluble reducing sugar.

In the last decades, CMC has come to be used as a substrate for endoglucanases, which randomly hydrolyze cellulose chain; microcrystalline celluloses (such as Avicel)

have come to be used as substrate for exoglucanases, which sequentially liberate cellobiose from the nonreducing end of the polymeric chain (Table 6). However, as mentioned in chapter II, at least some purified endoglucanases may display considerable activity toward Avicel and some exoglucanases have been reported to hydrolyze CMC. Furthermore, the possibility of mechanistic differences within each class of cellulase components has thrown the whole matter of cellulase assays into a state of confusion.

2.3.2.1.Reducing sugar

With minor exceptions, the component monosaccharides of cellulosic materials are classified as reducing sugars in that they are capable of reducing Fehling's solution. The application of total reducing sugar methods for quantitative measurement of cellulose degradation has evolved in the direction of assay simplicity, versatility, and reproducibility. Reducing sugar methods involve removal of subsamples from reaction mixtures, followed by separation of the soluble saccharides from residual insoluble substrates by filtration or centrifugation, followed by destructive conversion of the soluble saccharides to colored reaction products whose concentration may be measured colorimetrically. These methods have found wide acceptance due to their simplicity and the fact that minimal skills and equipment are required. The most commonly used detection agent for reducing sugars formed from cellulose is 3,5-dinitrosalicylic acid (DNS) . Although its use for this purpose was first described by Sumner and Sisler (1944), the most widely used modification of the method is that of Miller (1959). Advantages of the assay include relative simplicity, a reasonably linear relationship between absorbance and concentration, and good stability of the colored product (Miller, 1959). The major disadvantages of the assay are (I) variable color formation with

Table 6. Action of cellulase components on different substrates.

Substrate	Enzyme		
	Exoglucanase	Endoglucanase	ß-glucosidase
crystalline cellulose	slow	nil	nil
Amorphous "swollen" cellulose	very active	very active	nil
CM-cellulose	nil	very active	nil
Cellooligosaccharides	active	active	active
Cellobiose	nil	nil	active

(from Wood, 1989)

variation in boiling time, (II) relatively poor sensitivity, and (III) differential response of glucose, cellodextrins, xylose, and xylodextrins (Miller et al. 1960).

The Nelson modification (Nelson, 1944) of the Somogyi method (Schaffer and Somogyi, 1933) is also widely employed for measurement of reducing sugars, particularly glucose. The sensitivity of the method is only slightly greater than that of DNS assay (Hodge and Hofreiter, 1962). The major advantages of this procedure are its linearity and the stability of the molybdenum blue complex. Disadvantages include the requirement for deproteinizing the sample and the instability of the alkaline cupric working solution. In addition, the widely varying response of different sugars makes the assay impractical for analysis of hydrolytic products of complex natural biomass materials (Weimer, 1991). Glucose can also be measured in the presence of cellobiose based on differences in the extinction coefficients of these sugars following their reaction with the Nelson-Somogyi reagent (Peiji, 1987). Glucose can also be assayed enzymatically using hexokinase coupled to glucose-6-phosphate dehydrogenase (Bergmeyer and Bernt, 1979) or glucose oxidase coupled to peroxidase (Lloyd and Whelan, 1969).

2.3.2.2. Dye release methods

The use of chromogenic substrates to measure enzymatic activity toward soluble substrates has been extensively studied. Only recently, however, has this technique been adapted to measurement of cellulase activity. If the dyed substrates are derived from microcrystalline or amorphous celluloses, the activity measured is analogous to the "total cellulase" activity described above; dyed substrates derived from substituted celluloses or cellodextrins can be used to estimate the activity of specific enzymatic components (Cleary, 1980). Considerable differences have been observed among different celluloses

and cellulose derivatives with respect to their reactivity with various dyes (Leisola & Linko, 1976). The most sensitive assays use fluorescent dyes attached to soluble cellulose derivatives (Cleary et al. 1980). Chromogenic substrates, 4-methylumbelliferylglycosides of cellooligosaccharides, have been used to study chain cleavage by the cellobiohydrolase I (CBH I) and endoglucanase of *T. reesei* (van Tilbeurgh et al. 1982). This study demonstrated that CBH I does not act as a true cellobiohydrolase, and that the endoglucanase displayed a certain specificity with respect to cleavage site (i.e., a nonrandom attack of substrate).

2.3.2.3. Viscometric methods

Viscometric methods for measuring cellulose degradation involve measurement of inherent, intrinsic, or specific viscosities of reaction mixtures under precisely controlled physical and chemical conditions. Viscometric methods are extremely sensitive for endoglucanases since relatively few bond scissions yield large decreases in viscosity; by contrast, viscosity changes are minimal during attack by exoglucanases, particularly when high-DP celluloses are used as substrates.

Due to concerns over the lack of assay uniformity between laboratories, a committee of the International Union of Pure and Applied Chemistry (IUPAC) recommended a set of standard procedures for the measurement of cellulase activities (Ghose, 1987). Included within the recommendations are specific, step-by-step protocols for measurement of endoglucanase activity (using both CMC and HEC as substrates), filter paper activity, and cellobiase, as well as procedures for protein (by Folin method) and reducing sugar (by DNS method). Recently, measurement of individual products present in cellulose hydrolysates by HPLC method have been

reported (Schwald et al. 1988). These studies suggest that quantification of individual cellodextrins is the best way to measure the products of the reaction mixture if the results are to be used for predicting the hydrolytic potential of a cellulase preparation.

2.3.3. Mechanism of cellulase action

2.3.3.1. Initial attack

One of the most controversial aspects of the mechanism of cellulose degradation has been which enzyme actually initiates the attack on crystalline cellulose. The hypothesis put forward by Reese et al. in 1950 (so-called C1/Cx hypothesis) stated that attack was initiated by a nonhydrolytic chain-separating enzyme (C1) which produced a reactive cellulose which could then be attacked of different assays by the hydrolytic enzymes (Cx) (Figure 6). There is no good evidence as yet for the existence of a C1-type enzyme. There has been a report on the isolation, from a commercial cellulase preparation (*T. reesei*), of a nonenzymic factor capable of generating microfibrils and short fibers from filter paper (Griffin et al. 1984). Koenigs (1975) suggested that brown rot fungi, which do not appear to use a cellobiohydrolase-endoglucanase system for hydrolysis, initiate attack crystalline cellulose via an H_2O_2/Fe^{2+} system. An interesting feature of the involvement of cellobiose oxidase (Ayers et al. 1978) in cellulose degradation by *S. pulverulentum* cellulase is the generation of superoxide anion as a product of the reaction.

Purified cellobiohydrolase I from the *T. reesei* produced no visible change in crystalline cellulose when acting alone, but when acting in conjunction with endoglucanase it brought about the rapid dissolution of the cellulose (White, 1982). These results, however, are in complete contrast to those of Chanzy et al. (1983), who

claimed in their studies using electron microscopy that cellobiohydrolase I from *T. reesei* could effect some disaggregation of the cellulose in microfibrils. They also interpreted their electron micrographs to show that cellobiohydrolase I, when acting alone, could cause the complete dissolution of crystals of cellulose from the alga *Valonia macrophyta* (Chanzy et al. 1983) and that endoglucanase action was confined to the amorphous areas of cellulose (Chanzy & Henrissat, 1985). As a result of these and other studies (Chanzy et al. 1983 and 1984), it was suggested that cellobiohydrolase I has an endoglucanase mode of action, and only cellobiohydrolase II (Chanzy & Henrissat, 1985) acted as a typical exolgucanase. However, Enari and Niku-Paavola (1987) are of the opinion that cellobiohydrolase II from the same fungus also has some of the properties of an endoglucanase. Thus, the questions regarding which enzyme initiates the attack on the cellulose crystallite and the nature of that attack are far from resolved.

2.3.3.2. Synergism between enzyme components

One of the most interesting phenomena in cellulose hydrolysis is the synergistic action between the individual components of these enzyme mixtures (Figure 14). The definition of synergism states that the extent of hydrolysis by combined fractions is somehow greater than the calculated sum of the extents of hydrolysis by the individual fractions. Synergism between the enzymes found in the cellulases of fungi was first demonstrated by Gilligan and Reese in 1954. Despite the passage of time, the understanding of the various interactions involved in the synergistic process is still unclear, although there has been no shortage of published data on the subject.

The degree of synergistic activity shown by various "purified" components is obviously largely dependent on the extent to which the individual components are capable

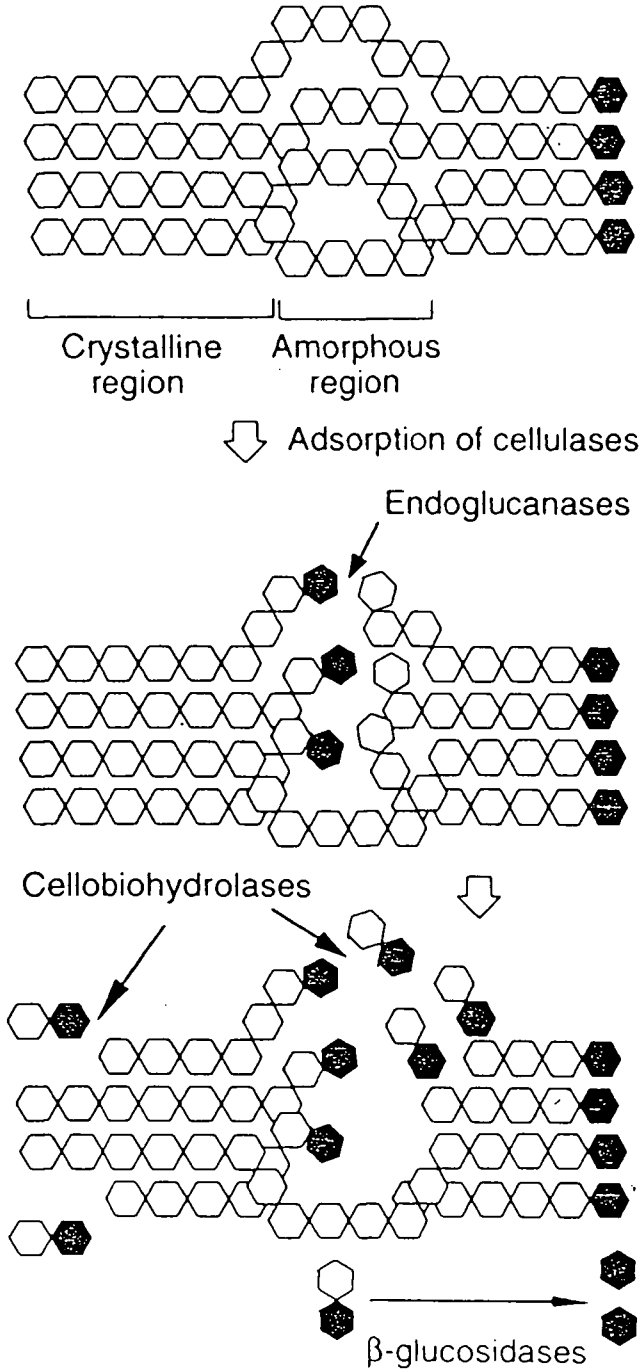


Figure 14. Synergism between endoglucanases, CBHs, and ß-glucosidases in fungal cellulase system. Glucose residues are indicated by hexagons; reducing ends are shown in black. (from Beguin and Aubert, 1992)

of solubilizing crystalline cellulose when acting in isolation. As state above, opinions on this particular aspect differ widely. Some maintain that only one enzyme is required for extensive hydrolysis (Berghem et al. 1976). Wood and McCrae (1979), however, showed that when the enzymes are highly purified and the substrate is dewaxed cotton fiber, which has been subjected to the minimum of pretreatment, degradation of crystalline cellulose in this form is virtually completely dependent on the cooperative action of the various enzymes. Synergistic effects between endoglucanase, cellobiohydrolase, and ß-glucosidase were also demonstrated with enzymes isolated from *P. pinophilum* (Wood et al .1980), *T. viride* (Selby, 1969), and *S. pulverulentum* (Streamer et al. 1975), but the degree of synergistic activity observed between the various enzymes varied widely (Table 7).

The results of Table 7 show that synergism between fungal cellulases is most marked when crystalline cellulose (particularly cotton fiber) is the substrate, but it is low or nonexistent with amorphous, extensively hydrated cellulose, and it is absent with soluble cellulose derivatives (Wood et al. 1979). Further, it would appear that the synergistic effect is dependent on the ratio of cellobiohydrolase to endoglucanase (Wood et al 1989; Henrissat et al. 1985) and on the type of endoglucanase acting in concert with the cellobiohydrolase (Wood et al. 1978). Thus, it was observed that two of the four major endoglucanases of *T. koningii* cellulase had no apparent capacity for acting synergistically with the cellobiohydrolase. In contrast, all four endoglucanases of *T. emersonii* (Moloney et al. 1985) acted equally efficiently with the cellobiohydrolase in solubilizing crystalline cellulose. Other interesting observations on the enzymes have been made upon mixing cellobiohydrolase from one cellulase with endoglucanase from another (Wood et al. 1979; 1980; 1982). It was found by Wood and his colleagues that

Table 7. Synergistic effect on different cellulosic substrates among cellulase components.

source	component[1]	substrate used	DSE[2]	Ref.
T. viride	EGs	microcrystalline cellulose	1.3-2.5	Rabinovich et al. 1986
T. reesei	CBHs	Avicel (1%)	2.0	Fagerstam and Pettersson 1980
T. reesei	CBHs EGs	Avicel PH105 bacterial MCC	1.8-2.6	Henrissat et al. 1985
T. reesei	EGs Exos	Avicel	1.0-2.1	Beldman et al. 1988
T. reesei	CBHs EGs	Avicel PH105	1.1-1.7	Woodward et al. 1988
T. reesei	CBH I,II EG I	Amorphous cellulose filter paper	>1.0	Heitz et al. 1991
T. reesei	CBH I, II EGII	Avicel PH105	1.1-2.0	Woodward et al. 1988
T. koningii	CBHs EGs	dewaxed cotton	2.7-3.8	Wood and McCrae 1978
T. koningii	CBHs	Cotton fiber Avicel	1.9-3.5 1.0-1.7	Wood et al. 1988
T. koningii	EGs Exos	Avicel	2.1-4.8	Coughlan 1987
P. pinophilum	EGs CBH I, II	Avicel PH101	1.3-3.2	Wood and McCrae 1986
Celluomonas	EGA,B &C	Avicel	1.1-2.1	Poulsen and Pettersson 1992

[1] EG, endoglucanases; CBH, cellobiohydrolases; Exo, exoglucanases.

[2] DSE, degree of synergistic effect.

some--but not all -- endoglucanases and cellobiohydolases acted synergistically to solubilize crystalline cellulose and that synergism was only significant when the enzymes were obtained from a fungal source that was capable of extensive hydrolysis of crystalline cellulose.

The question of why all endoglucanases are not able to act synergistically with the cellobiohydrolase to the extent was addressed by Wood and his colleagues (1982). They suggested that synergism is likely to be explained by a sequence of events in which endoglucanases cleave cellulose chains thereby creating new chain ends for the cellobiohydrolase to attack (Wood et al. 1975; 1979). The original model has been modified to account for the "anomalous" results, and two postulates have been made by Wood and his coworkers. Thus, they have suggested that (I) rapid sequential action between endoglucanase and cellobiohydrolase is necessary to prevent the reformation of glycosidic linkages between glucose residues rigidly held in position by hydrogen bonds, and that this can best be effected by those endoglucanases and cellobiohydrolases that form a "loose" endolgucanase-cellobiohydrolase complex on the face of the cellulose crystallite (Wood et al. 1978), and (II) the end group generated by the endoglucanase in the first phase of the hydrolysis must have the correct configuration for attack by the stereospecific cellobiohydrolase involved in the second stage of hydrolysis (Figure 8, 9 and Table 8). As yet there is no evidence to support any of these possibilities, but theoretical considerations of the stereochemistry of the cellulose chains in the cellulose crystallite should favor the latter (Wood et al. 1981).

Another factor relating to the stereospecificity of the enzymes, and hence synergistic action, is adsorption of the enzymes to the cellulose crystallite. Adsorption of

endoglucanases is very variable. Ryu et al. (1984) examined the differences in adsorption of the separated components of the cellulase complex of *T. reesei MCG 77* on Avicel and interpreted their observations in terms of synergistic action. They suggested that the endoglucanases and cellobiohydrolase adsorb at different sites and that these sites corresponded to the sites of hydrolysis. Endoglucanase consisted of adsorbable and nonadsorbable components, but cellobiohydrolase had the strongest adsorption affinity. Significant was the suggestion that adsorption of the cellulase components was competitive and that this could explain the synergistic interaction of the components in solubilizing crystalline cellulose. The rate and extent of desorption and hence the synergistic activity varied according to the substrate used. Henrissat *et al.* (1985) also showed synergism to vary according to substrate. Interestingly, the degree of competitive adsorption observed by Ryu *et al.* (1984) was highest when cellobiohydrolase and endoglucanase components were present in the ratio in which they were present in the crude culture filtrate. These data could be interpreted to support the hypothesis that endoglucanase-cellobiohydrolase complexes are required for the hydrolysis of crystalline cellulose.

In all of these hypotheses, it is the synergism between endoglucanase and cellobiohydrolase that is discussed. However, the recent isolation of two immunologically unrelated cellobiohydrolases from *T. reesei* and *P. pinophilum*, and the demonstration of synergistic activity between the enzymes (Wood & McCrae, 1986; Fagerstam and Pettersson, 1980), opened up a new and important aspect of the mechanism of cellulase action. Some workers interpret their data to indicate that most of the synergistic activity in *T. reesei* cellulase may indeed be attributed to the cooperation of the two immunologically unrelated cellobiohydrolase (Heitz et al. 1991). Synergism

between two endwise-acting enzymes is difficult to explain, but Wood (1985; 1986) suggested that a reasonable speculation might be that cellobiohydrolase I and II could exist different substrate stereospecificities, each attacking one of the two types of naturally-occurring nonreducing group that might be found in the cellulose crystallite (Figures 8,9 and Table 8). The synergistic interaction could then be understood if one realize the successive removal of cellobiose from one type of nonreducing chain end could expose on a neighboring chain an end group with the correct configuration for attack by the other stereospecific cellobiohydrolase. Thus the actual mechanism of cellulase action continues to be uncertain although progress has clearly been made.

2.3.4. Structural features of cellulose and their effects on enzymatic hydrolysis

Correlations between structural features of the substrate and its enzymatic degradation are required for a better understanding of the mechanisms of saccharification. These correlations are of practical importance because they indicate the type of pretreatment necessary to make native cellulose susceptible to enzymatic hydrolysis. Pretreatments of cellulosic materials are designed to facilitate their enzymatic hydrolysis. Pretreatments can be of a physical type, such as dry and wet milling (Vanderhart and Atalla, 1984), high-pressure treatment (Tayor et al. 1983) and high-energy radiation (Focher e al. 1981) or of a chemical type, such as alkaline or acidic treatments using NaOH (Warwicker & Wright, 1967) or phosphoric and sulfuric acid (Grohmann et al. 1985).

2.3.4.1. The mode of enzymatic degradation of cellulose

The enzymatic hydrolysis of cellulose by cellulase is rather complex owing to (I) the heterogeneity of the system, (II) the dual nature of the insoluble substrate (crystalline

Table 8. Stereochemical courses of hydrolysis catalyzed by sixteen ß-1,4-glucanases and -xylanases representative of families A-C and E-G.

Family	Subtype	Organism	Enzyme	Stereoselectivity
A	3	*C. thermocellum*	CelC	Retention
A	4	*C. thermocellum*	CelH	Retention
A	5	*T. reesei*	EGIII	Retention
A	2	*T. fusa*	E5	Retention
B	1	*C. fimi*	CenA	Inversion
B	2	*T. reesei*	CBHII	Inversion
B	1	*T. fusa*	E2	Inversion
C		*T. reesei*	CBHI	Retention
C		*T. reesei*	EGI	Retention
E	2	*C. fimi*	CenB	Inversion
E	1	*C. thermocellum*	CelD	Inversion
E	1	*T. fusa*	E1	Inversion
F		*C. fimi*	Cex	Retention
F		*C. thermocellum*	XynZ	Retention
G		*B. subtilis*	Xyn	Retention
G		*S. commune*	Xyn	Retention

(from Gelber et al. 1992)

and amorphous), and (III) the component multiplicity of the enzymatic system. This complexity is increased by the fact that the structure of the substrate changes during saccharification and the different enzyme components act in a synergistic fashion. Three factors mainly affect the enzymatic hydrolysis of insoluble cellulose: the properties and mode of action of cellulase, the structural properties of cellulose, and the mode of interaction between the enzymes and the cellulose molecules.

2.3.4.2. Relationships between structural features and enzymatic hydrolysis

Referring to the influence of structural features of cellulosic materials on their bioconversion, the more recent results emphasize the importance of the surface area available to the enzymes and of the pore size and distribution inside these materials with respect to such parameters as crystallinity index and degree of polymerization of cellulose (Tanaka et al. 1988; Dermoun & Belaich, 1988; Wong et al. 1988). As a result of all the above observations, the search for a preferential structural parameter that essentially governs the rate of enzymatic hydrolysis has been unsuccessful. Difficulties in this research arise from the fact that it is virtually impossible to vary a single structural feature without affecting the others.

A fundamental topic that deserves more attention is the conformational aspects of cellulose-cellulase interactions which dictate the formation of catalytically active complexes. Maybe it is necessary assumes that cellulose macromolecules have a peculiar conformation that does not create steric hindrance to the attack of the glycosidic linkage and at the same time allows a successful interaction with enzyme molecules (Atalla, 1983).

2.3.5. Substrate inhibition

Substrate inhibition exists in many enzyme systems. Substrate inhibition is generally defined as any apparent decrease in the reaction rate that accompanies an increase in substrate concentration. Many studies have been conducted on substrate inhibition due to its importance in enzyme kinetics and reactor design. However, the mechanism of substrate inhibition in many enzyme systems is still unknown. Several possible mechanisms related to substrate inhibition have been proposed, such as the non-productive, binding of two substrates per active site or the binding of substrate to a peripheral nonactive site that modifies enzyme activity (Webb, 1963; Dixon et al. 1979).

Substrate inhibition mechanisms encountered in classical soluble enzyme/soluble substrate systems are generally attributed to the formation of dead-end or abortive complexes (Fromm, 1975). In a single substrate reaction , the substrate inhibition can be interpreted in terms of the existence of two types of substrate-binding site in the enzyme. Occupation of the first, high-affinity type at low substrate concentration leads to "normal" kinetic behavior; at high substrate concentration, the second, low affinity type of site becomes occupied and this is presumed to inhibit the catalytic reaction taking first type of site.

Substrate inhibition caused by a reduction in available water in the reaction system with increases in substrate concentration has been demonstrated for invertase (Nelson & Schubert, 1928). Another case in which substrate inhibition is observed is when the concentration of an inhibitor in the reaction mixture is constantly proportional to the variable substrate concentration (Cleland et al. 1973). These inhibitors will cause an apparent substrate inhibition due to an uncompetitive or noncompetitive pathway. In

general, it seems that the mechanism of substrate inhibition may vary with enzyme system and the substrate properties. For detail information on this subject, refer to references by Webb (1963), Cleland (1979) and Dixon et al. (1979). In these cases, the reactions occur in the mixture of soluble enzyme versus soluble substrate, that is , in a homogeneous system.

Substrate inhibition has been observed in the Trichoderma *cellulase* system (Van Dyke, 1972; Howell and Struck, 1975; Okazaki and Moo-Young, 1978). In a previous study, a commercial *T. viride* cellulase preparation was shown to exhibit substrate inhibition when acting on a microcrystalline cellulose (Avicel) but not on a powdered cellulose substrate (Solka-Floc BW200 NF) (Liaw and Penner, 1990). Huang and Penner (1991) have also observed substrate inhibition for the cellulase system from *T. reesei* when acting on microcrystalline cellulose.

CHAPTER 3. COMPARATIVE ANALYSIS OF ASSAY METHODS APPLIED TO THE STUDY OF CELLULOSE SACCHARIFICATION

3.1. INTRODUCTION

The enzymatic hydrolysis of cellulose plays a key role in many natural systems and is critical to several industrial processes. For this reason, there is a great deal of interest in the properties of natural cellulolytic enzyme systems. Properties such as hydrolytic potential, pH activity profiles and temperature stability are of general interest for industrial applications. The hydrolytic potential of cellulase enzyme preparations is generally based on a series of assays with different cellulosic substrates. These substrates often vary in physicochemical properties which are thought to significantly effect their susceptibility to enzymatic degradation (Ramos et al. 1993; Sinitsyn et al. 1989; Ryu and Lee, 1986; Grethlein, 1985; Cowling, 1975). The International Union of Pure and Applied Chemists (IUPAC) has recommended an assay which is based on the degradation of filter paper cellulose (Ghose, 1987). In the IUPAC assay, activity is measured in filter paper units (FPU), 0.37 FPU equals that amount of enzyme which will generate (solubilize) 2mg of reducing sugar equivalents from a 50 mg strip of filter paper under the defined assay conditions. Other cellulosic substrates commonly included in studies attempting to characterize a given enzyme preparation's activity include a form of microcrystalline cellulose and one of several amorphous cellulose preparations. In general, the activity of an enzyme preparation with any of these substrates is determined by a measurement which reflects the amount of cellulose solubilized per unit time.

In cellulase assays, the value obtained for the extent of saccharification of a substrate is dependent on the method used to measure the cellodextrins liberated into the

aqueous phase of the reaction mixture. Cellodextrins having a degree of polymerization greater than seven are essentially insoluble (Okazaki and Moo-Young, 1978). Saccharification assays based on the quantification of solubilized glucose, solubilized reducing sugar equivalents and total sugar solubilized are in the current literature. The assays based on the measurement of glucose per se will not measure the higher oligosaccharides which have been liberated from the substrate. The assays based on the measurement of reducing sugar equivalents will reflect values related to the total number of saccharides solubilized. Assays based on the measurement of total sugar solubilized are estimating the mass of saccharides liberated from the insoluble cellulose. The total sugar assays, generally based on the degradation of sugars in strong mineral acids, are not as widely used to quantify the products of cellulase saccharification due to their inherently low analytical sensitivity.

The relatively large number of assays appearing in the cellulase literature may sometimes contribute to the confusion encountered when attempting to choose the most relevant assay for a particular experiment. The task of selecting an appropriate quantification method for estimating solubilized saccharides would be simplified if a systematic comparison of pertinent parameters for each of the common assays were available. In this regard, the experiments included in this paper were designed to provide comparative data for the most common methods used to measure saccharification products. Methods were compared with respect to their sensitivity, detection limit and use with reagents common to cellulase reaction mixtures. Reagents commonly included in cellulase reaction mixtures include antimicrobials and secondary buffers used to adjust the pH when terminating the reaction. Consideration is given to the amount of information which can be obtained form the different quantification methods. The

cellulase enzyme system used in this study was from *Trichoderma reesei* and the cellobiase preparation from *Aspergillus niger*. These two enzyme preparations were chosen due to their widespread use in applied and basic work on the conversion of lignocellulosics to glucose. We conclude the paper with a general recommendation for a specific glucose-based assay to be used in conjunction with a cellobiase treatment following termination of the primary cellulolytic reaction.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and enzymes

D(+)-cellobiose (C-7252), adenosine 5'-triphosphate (A-3377), ß-nicotinamide adenine dinucleotide (N-0632), hexokinase (HK, H-5000), glucose-6-phosphate dehydrogenase (G6PDH, G-5760), thimerosal (ethyl[2-mercaptobenzoato-S]mercury sodium salt, T-5125), sodium azide (S-2002), δ-gluconolactone (D-gluconic acid lactone, G-4750), and glucose oxidase/peroxidase (GO/P, 510A) were obtained from Sigma Chemical Co (St. Louis, MO). Glucose (D-1017) was purchased from Spectrum Chemical MFG CORP. (Gardena, CA). *Aspergillus niger* cellobiase (Novozym 188)was generously donated by NOVO Nordisk Bioidustrials, Inc. (Danbury, CT). The commercial cellobiase product was partially purified in our laboratory using gel filtration as described by Huang (PhD dissertation, 1992). A complete cellulase preparation was prepared in our laboratory from shake-flask cultures of *Trichoderma reesei* as described by Mandels *et al* (1981).

3.2.2. Instrumentation

Spectrophotometer, model DU-40 (Beckman Instruments, Inc., Fullerton, CA), was used for all enzyme-based and reducing sugar-based assays. A Waters HPLC system (Millipore, model 501, Milford, MA) with a refractive index (RI) detector (Waters model 410) was used in all HPLC assays.

3.2.3. Enzyme working reagents

A concentrated hexokinase/glucose-6-phosphate dehydrogenase (HK/G6PDH) working reagent having the following composition was prepared for all HK/G6PDH assays: 0.4 units hexokinase, 0.4 units G-6-P dehydrogenase, 1.0 μmol ATP, 1.5 μmol

ß-NAD and 2.1 µmol maganism per ml. The working reagent for the glucose oxidase/peroxidase (GO/P) assay was prepared fresh daily with the following composition: 13.41 units glucose oxidase, 2.69 units peroxidase and 0.1 mg o-dianisidine per ml.

3.2.4. Enzyme assays

To initiate cellobiase reactions, 0.1 ml of temperature-equilibrated cellobiase (3 IU/ml) in buffer was added to 0.9 ml of 8 mM cellobiose in 50 mM sodium acetate buffer, pH 5.0, 50°C. The reaction was terminated at 10 minutes by either adjusting the pH to 9.0 (Chen et al. 1992; Gilkes et al. 1984), addition of the inhibitor δ–glucono lactone (Bolobova and Rabinovich 1984; Sternberg et al. 1977), or by immersion of the reaction mixture in boiling water for 5 min (Hoh et al. 1992; Bock and Sigurskjold 1989). To initiate cellulose saccharification assays, 0.016 FPU cellulase in 0.1 ml is added to 3.9 ml of a 1% (w/v) suspension of microcrystalline cellulose in 50 mM sodium acetate buffer, pH 5.0, 50°C. At designated time points, aliquots of the reaction mixture were removed, filtered and terminated by adjustion the pH of the filtrate to 9.0. The pH adjusted sample was then immersed in boiling water for 5 min. Soluble products generated in cellulase and cellobiase reaction mixtures were determined by either enzymatic methods, HK/G6PDH (Lee et al. 1988; Zabriskie et al. 1980; Hsuanyu & Laidler 1984) and GO/P (Sinitsyn et al. 1989; Kwon et al. 1992; Hoh et al. 1992), colorimetric reducing sugar methods, Nelson (Nelson 1944; Somogyi 1952) and DNS (Ghose, 1987; Miller et al. 1960) or by an HPLC-based assay (Sinitsyn et al. 1989; Converse and Optekar, 1993; Schwald et al. 1988). All experiments included substrate and enzyme blanks. Glucose solutions prepared in 50 mM sodium acetate buffer, pH 5.0 were used as calibration standards.

3.2.5. Termination of cellulolytic enzyme assay

Termination methods included (1) immersion of the reaction mixture in boiling water for 5 minutes. (2) addition of 0.1 ml 1M Tris or carbonate buffer, pH 11.0 or (3) addition of δ-gluconolactone to 40 mM.

3.2.6. Antimicrobials

The effect of two antimicrobials, thimerosal (Streamer et al. 1975; Vallander & Eriksson 1991) and sodium azide (Chirico and Brown, 1987; Mandels et al. 1981; Tanaka et al. 1988), on *T. reesei* and *A. niger* enzyme activities was determined. The time course of saccharification of microcrystalline and amorphous cellulose and the rate of cellobiose hydrolysis were determined in the presence and absence of either 0.001% thimerosal or 5 mM sodium azide.

3.2.7. Sensitivity, detection limit and precision of assays

Sensitivity, detection limit and relative error for all assays were determined using standard glucose solutions, ranging in concentration from 1 to 100 μM in 50 mM sodium acetate, pH 5.0. Optimization of reducing sugar assays was accomplished by varying the concentration of working reagent solutions and modifying subsequent dilution schemes. The HPLC system used in all analyses was a Waters HPLC system (Model 501), equipped with a Waters 410 refractive index detector and a 200 μl injection loop. The principle column used was a 30 cm Bio-Rad HPX-87P column at 85°C which was downstream from a standard deashing guard column (Bio-Rad Lab. Hercules, CA). Deionized water was used as the eluent (Pettersen et al. 1984; Vallander & Eriksson 1991; Grous et al. 1985; Wayman & Chen 1992).

3.2.8. Kinetic parameters study

Apparent kinetic constants, *Km* and *Vmax*, for *A. niger* and *T. reesei* cellobiases were determined at 50°C, pH 5.0. The extent of reaction was determined by quantification of glucose using both the HK/G6PDH and GO/P assays. Substrate concentrations for these assays ranged from 0.3 to 15 mM cellobiose. Enzyme concentrations were 0.003 IU and 0.064 FPU per ml reaction mixture for *A. niger* and *T. reesei*, respectively.

3.3. RESULTS AND DISCUSSION

3.3.1. Product quantification

The most common assays for the enzymatic saccharification of cellulose include the quantification of solubilized saccharides as either reducing sugar equivalents, glucose or as individual cellodextrins following separation by HPLC. In many cases the primary product in the reaction mixture is glucose. This is particularly true in studies on the applied aspects of lignocellulosic conversion where complete cellulase systems, such as that from *T. reesei*, are supplemented with cellobiase to insure the rapid conversion of soluble products to glucose. In these cases, glucose is essentially the only product. Table 9 and Figure 15 include assay parameters related to the quantification of glucose in 50 mM sodium acetate buffer, pH 5.0 (traditional reaction conditions for cellulase saccharification). Working definitions of calibration sensitivity, analytical sensitivity, relative error and detection limit are given in Table 10. The sensitivity of an assay provides an indication of the ability of the assay to discriminate between small differences in analyte concentration. With respect to the methods included in Table 9, the higher the sensitivity the more likely the assay will allow the detection of relatively small changes in reaction mixture glucose concentrations. The calibration sensitivity is essentially the slope of the calibration curve, thus it is most easily visualized as depicted in Figure 15. The calibration sensitivities of the enzyme based assays are approximately 6-fold greater than that of the reducing sugar assays. Comparison of the calibration sensitivity of the HPLC-based assay with corresponding values for the other assays of Table 9 is not valid due to the use of a different analytical signal in the HPLC-based assay, i.e. changes in refractive index versus absorption of electromagnetic radiation.

The analytical sensitivity of an assay is a more refined measure of the ability of an assay to discriminate between small analyte concentrations due to the fact that it accounts for the precision of a method. This is done by defining the analytical sensitivity as a function of the calibration sensitivity and the standard deviation of the method (Table 10). The analytical sensitivity of the enzyme-based assays are approximately 12-fold higher than that of Nelson's reducing sugar assay and approximately 2-fold higher than that of the HPLC-based assay (Table 9). The analytical sensitivity of an assay may be concentration dependent if the standard deviation shows a concentration dependence. However, no strong correlations are observed between analyte concentration and analytical sensitivity for the assays of Table 9. The relative error (relative standard deviation) may also be concentration dependent, as appears to be the case for the reducing sugar and HPLC-based assays. As expected, the ranking of the methods with respect to relative error is the opposite to their ranking with respect to analytical sensitivity. The enzyme-based assays have the lowest relative error, followed by the HPLC-based assay, the reducing sugar-based assays having the highest relative error.

The detection limits listed in Table 9 indicate the lowest glucose concentrations which can realistically be distinguished from the background signal. This is generally defined as the analyte concentration which corresponds to an analytical signal three standard deviations above the background signal (Skoog and Leary, 1992). The enzyme-based assays are capable of detecting glucose concentrations somewhat less than 1.0 μM, which was the lowest detection limit observed. The other extreme was the DNS reducing sugar assay which, as is clear from Figure 15, has a detection limit of greater than 100 μM. In this regard, the DNS method of reducing sugar quantification is specified in the IUPAC filter paper assay. However, in that particular assay the targeted end-point for

reaction termination corresponds to glucose concentrations of approximately 7.4 mM. The detection limits reported for the reducing sugar assays were obtained in optimization studies which attempted to identify working reagent concentrations and dilution volumes which correspond to maximum analytical sensitivities. Attempts to lower the detection limit beyond that reported in Table 9 were unsuccessful in that improvements in the calibration sensitivity were negated by corresponding increases in the standard deviation of the measurements.

The parameters given in Table 9 are based on the measurement of glucose. Glucose is the primary product found in saccharification systems using cellobiase-supplemented *Trichoderma* cellulase preparations. In these systems, cellobiase supplementation is adjusted so that glucose is essentially the only product observed in the aqueous phase. In saccharification systems which are not supplemented with cellobiase, cellodextrin products may be observed. Cellobiose and glucose are the soluble products which accumulate in saccharification systems using *Trichoderma* cellulase preparations not supplemented with cellobiase (Schwald et al. 1985; Huang, PhD dissertation, 1992). The molar ratio of cellobiose to glucose in these reaction mixtures is generally greater than one. The quantity of soluble saccharide measured in reaction mixtures containing more than one product will be dependent on the quantitative method used for the analysis. For example, the five assays listed in Table 9 will not give the same quantitative measure of soluble saccharides. The two enzymatic assays, as presented above, would measure only glucose. The reducing sugar assays will measure total reducing sugar equivalents, which are most often converted to "glucose equivalents" due to the common practice of using glucose as a calibration standard. HPLC-based assays may be used to quantify individual cellodextrins, thus having the potential to provide information on the complete product

profile. This is the major advantage of using HPLC-based assays with *Trichoderma* enzyme preparations not supplemented with cellobiase. A disadvantage of the HPLC-based assays is that they require the use of relatively expensive instrumentation, which limits the number of laboratories that can use these assays on a routine basis.

An alternative, relatively simple, analytical scheme which allows the measurement of reaction mixture cellobiose and glucose concentrations is presented in Figure 16. The assay consists of two glucose measurements on the terminated reaction mixture. Assay #1 is made directly on the reaction mixture immediately following termination of the cellulolytic reaction. The results of assay #1 are a measure of the glucose concentration of the reaction mixture at the endpoint of the reaction. Glucose assay #2 is done after the terminated reaction mixture is treated with a cellobiase preparation designed to convert essentially all of the cellobiose to glucose. The results of assay #2, after being corrected for the glucose present in the original reaction mixture prior to cellobiase treatment (from assay #1), are used to calculate reaction mixture cellobiose concentrations at the endpoint of the reaction. This analytical approach provides the same product profile as that of the HPLC-based assay for saccharification systems employing native (non-cellobiase supplemented) *T. reesei* enzyme preparations. This is because non-cellobiase supplemented *Trichoderma* saccharification systems do not appear to accumulate products of DP greater than two (Schwald et al. 1985; Huang, PhD dissertation, 1992). If a saccharification system is employed which generates a spectrum of cellodextrin products, then the assay outlined in Figure 16 would provide information on the glucose concentration of the reaction mixture (assay #1) and a concentration term which is a function of the total amount of cellodextrins present (assay #2). In which case, an HPLC based assay would be necessary to obtain the complete product profile. The sensitivity,

relative error, and detection limit of the quantification scheme depicted in Figure 16 will be similar to that of the enzyme-based assays of Table 9. Representative saccharification time courses, obtained using the proposed assay for non-supplemented *Trichoderma* enzyme preparations acting on microcrystalline cellulose and filter paper are presented in Figure 17. Representative time courses of cellobiose and glucose yields from filter paper in reaction mixtures containing either a cellobiase-supplemented or a non-supplemented *T. reesei* enzyme preparation are presented in Figure 18.

3.3.2. Antimicrobials

Saccharification reaction mixtures are often supplemented with antimicrobials due to the relatively long incubation periods at warm temperatures. Two of the more common antimicrobial components used in cellulase research are sodium azide (Chirico and Brown, 1987; Mandels et al. 1981; Tanaka et al.) and thimerosal (Streamer et al. 1975; Vallander and Eriksson, 1991). Figure 19A compares the time course for microcrystalline cellulose saccharification without antimicrobials, in the presence of sodium azide and in the presence of thimerosal. Corresponding curves for the saccharification of amorphous cellulose are presented in Figure 19B. The data suggests that these two antimicrobials should have no significant effect on the time course of cellulose saccharification under the conditions employed in these assays. However, experiments using thimerosal indicated that this antimicrobial is not compatible with the HK/G6PDH-based glucose assay if the primary cellulase (cellobiase) reaction is terminated by boiling the reaction mixture. The incompatibility was associated with the observation that boiling the thimerosal containing reaction mixture resulted in highly variable absorbance readings at 340 nm, the wavelength of choice for monitoring the

HK/G6PDH-based assay. Thus, it is recommended that the GO/P assay be used for glucose quantification when using thimerosal as an antimicrobial.

3.3.3. Termination of cellulolytic reactions

An appropriate method for terminating a timed assay is one which instantaneously terminates the reaction of interest and does not effect the subsequent quantitative measurement of product and/or substrate. In the case of cellulase assays there are a number of termination methods in the literature. The termination methods considered in this study are presented in Table 11. The most common method of terminating classical saccharification assays is to immerse the reaction mixture in boiling water for 5 min. Our tests indicate that *T. reesei* cellulases and *A. niger* ß-glucosidase preparations are completely inactivated under following these treatments. The 5 min. termination period represents a relatively small error when compared with reaction times of up to 72 hrs, such as are encountered in cellulase saccharification studies. In other cases, particularly with measurements of cellobiase activity, reaction times are of a shorter duration, often ranging from 10 to 30 min. In these cases a 5 min termination period may become significant relative to the overall kinetics. For example, 10 min cellobiase assays terminated by immersion of the reaction mixture in boiling water overestimated the actual cellobiase activity by approximately 11% relative to methods which appear to terminate the cellobiase reaction instantaneously (Table 11). In such cases, the actual extent of overestimation will depend on the rate of loss of enzyme activity, which will be some function related to the heat transfer characteristics of the system. The low heat transfer coefficient of polycarbonate was used to demonstrate the importance of this parameter; the cellobiase activity of reaction mixtures contained in polycarbonate tubes was overestimated by an average of 33%. This value is to be compared with the 11%

overestimation, as discussed above, for the same reaction mixtures in borosilicate glass tubes terminated by the same protocol. Obviously, the applicability of a termination method based on heat inactivation must be considered with reference to the relative amount of time for the primary cellulolytic reaction and the termination period.

A second method used in terminating timed assays is to jump the pH to a value which results in essentially a complete loss of enzyme activity. The data of Table 12 indicates that cellulase/cellobiase activities from *A. niger* and *T. reesei* have relatively low activities at pH 8.0 and are effectively quenched at pH 9.0. In these tests the pH was adjusted using a 1M solution of either Tris-chloride of sodium-carbonate. Each of the buffers behaved the same in this phase of the study. The apparently instantaneous termination of the reaction makes this a very effective method for short term assays (Table 11). A potential drawback to this approach is that it does not irreversibly inactivate the enzymes. Thus, if the pH of the terminated reaction mixture is again changed to a value compatible with enzyme activity, the reaction of interest will likely continue. This may be the case in circumstances where, following termination, the reaction products are assayed by one of the enzyme-based assay. For instance, the enzyme-based glucose assays of Table 9 are run at near neutrality. To circumvent this problem we recommend that reactions be terminated by pH adjustment followed by immersion of the sample in a boiling water bath for 5 min. The pH jump will instantaneously terminate the reaction and the heat treatment will serve to irreversibly inactivate the enzyme. In using this approach we found that carbonate buffers, when used for pH adjustment prior to heat treating, were associated with highly variable overestimates of enzyme activity. Further tests indicate that glucose is generated from cellobiose in carbonate solutions, pH 9.0, at temperatures greater than 70°C. Quantitative measurements indicate one mol glucose

generated per mol cellobiose degraded, suggesting a mechanism similar to the "peeling" of polysaccharides in alkaline solution (Brooks and Thompson, 1966). In contrast to the results observed for carbonate containing solutions, no glucose was detected in Tris buffered solutions incubated at the same pH, temperature and cellobiose concentrations. Therefore, Tris-based solutions are recommended for termination of saccharification systems in cases where reaction mixtures will undergo a form of heat treatment. Specifically, we recommend the addition of 0.1 ml 1M Tris, pH 11, for the termination of 1 ml reaction volumes, assuming the traditional saccharification buffer of 50 mM sodium acetate, pH 5.0 is used.

Termination of cellulase/cellobiase assays by the addition of inhibitors is also reported in the literature (Bolobova and Rabinovich, 1984; Sternberg et al. 1977). δ–Gluconolactone appears to be the most common inhibitor used for reaction termination. It was our experience that the complete inhibition of cellobiase activity did not occur until reaction mixtures were made 40 mM in δ–gluconolactone (Table 13). This high level of δ–gluconolactone resulted in high absorbance backgrounds at 450 nm, which is the wavelength of choice for the GO/P assay recommended for product quantification. The outcome of using this inhibitor in conjunction with the GO/P assay is that the precision of the final data will be compromised.

3.3.4. Summary.

The assay scheme presented in Figure 16 is recommended for *T. reesei*/ *A. niger* enzyme based saccharification systems. This assay protocol makes use of the highly sensitive enzyme-based analytical methods for production quantification, it allows

determination of reaction mixture cellobiose to glucose ratios and it provides a value for the total glucose equivalents solubilized during the course of saccharification. The method is appropriate for monitoring cellulase saccharification assays, as described above (Figures 18 and 19). The assay is also of general use when assessing kinetic constants associated with cellobiase activity, as illustrated for both *A. niger* and *T. reesei* enzyme preparations (Table 14). In the course of testing alternative product quantification methods, termination methods, and antimicrobials we identified several combinations of reagents/protocols which were not compatible. These are summarized in Table 15 along with suggestions for alternative methods which should provide superior results.

Table 9. Calibration sensitivity, analytical sensitivity, relative error and detection limit for glucose assay under *Trichoderma reesei* saccharification conditions.[a]

Assay	Calibration sensitivity[b]	Analytical sensitivity(μM)$^{-1}$ [G] μM 15	40	100	Relative error(%) [G] μM 15	40	100	Detection limit (μM [G])
GO/P	4.5	4500	2250	2250	0.6	0.6	0.3	~1.0
HK/G6PDH	4.7	2350	2350	2350	0.8	0.5	0.2	~1.0
Nelson	0.71	237	178	178	7.2	5.9	2.8	~10.0
DNS	0.004	-	-	-	-	-	-	>100
HPLC	NA(1.28)	1280	780	1320	4.9	2.1	0.4	~6.0

[a]: glucose solution in 50 mM Na acetate, pH 5.0.
[b]: signal changed in this study was defined as absorbance changed in each method except millivolt per sec changed for HPLC method.
NA: not applicable.

Table 10. Definition of calibration sensitivity, analytical sensitivity, detection limit and relative error.

Calibration Sensitivity (m):

the slope of the calibration curve, assuming a linear relationship between analytical signal and sample concentration

$$S = m\ C + S_{bl}$$

S: measured signal
C: concentration of the analyte
S_{bl}: instrumental signal for assay blank
m: slope of the calibration curve

Analytical Sensitivity (γ):

$$\gamma = m\ /s_s$$

m: slope of the calibration curve
s_s: standard deviation of the signal at the concentration of interest

Detection Limit (C_M):

$$C_M = (S_M - S_{bl})\ /m$$
$$S_M = S_{bl} + 3\ s_{bl}$$

C_M: minimum concentration that can be detected at a confidence level of > 90%
m : slope of the calibration curve
S_{bl}: mean blank signal
s_{bl}: standard deviation of blank
S_M: minimum distinguishable analytical signal

Relative Error (RE):

$$RE = (S_m/\text{mean}) \times 100$$

S_m: standard deviation/square root of number of replications

Table 11. Effect of reaction termination method on measured cellulolytic enzyme activity.[1]

termination method	*Aspergillus niger* [a] measured activity (n mol G/min)	relative error(%)[6]	*Trichoderma reesei* [b] measured activity (*n* mol G/min)	relative error(%)[6]
boiling[2]	5.96±.04	11.4	nd	nd
pH[3]	5.07±.01	0	2.60 ± .01	0
pH/boiling[4]	5.11±.01	0.8	2.63 ± .02	1.1
inhibitor[5]	5.13±.02	1.6	nd	nd

1 product determined by glucose oxidase/peroxidase assay.
2 immersion of reaction mixture in boiling water bath for 5 min.
3 pH adjusted to 9.0 by addition of 1 M Tris.
4 pH adjusted as in 3 followed by heat treatment as in 2.
5 reaction mixture made 40 mM in delta-glucono lactone concentration.
6 relative error based on comparison with assay terminated by pH adjustment.
a enzyme concentration : 5×10^{-5} mg/ml.
b enzyme concentration : 0.01 mg /ml.
nd: not determined.

Table 12. Termination of cellulolytic enzyme activity by pH adjustment.[1]

buffer used for pH adjustment	pH	% activity relative to pH 5.0 *A. niger*[2]	*T. reesei*[3]
Carbonate	8.0	8.2	nd[4]
Carbonate	9.0	0	nd[4]
Tris	9.0	0	0

[1] reaction conditions: 0.05 μg cellobiase per ml in 8 mM cellobiose, 50 mM Na acetate. Reaction time = 10 min.
[2] product determined by HK/G6PDH assay.
[3] product determined by GO/P assay.
[4] nd:not determined.

Table 13. Termination of cellobiase activity by inhibitor.[1,2]

inhibitor	concentration	% inhibition
δ–gluconolactone	4 mM	17
δ–gluconolactone	40 mM	100
nojirimycin[3]	0.6 μM	50
nojirimycin[4]	0.56 mM	99

[1] reaction conditions: 0.05 μg cellobiase per ml in 8 mM cellobiose, 50 mM Na acetate. Reaction time = 10 min.
[2] product determined by HK/G6PDH assay.
[3] data from Reese et al. 1971, Carbohydrate Research, 18:381-388.
[4] data from Atsumi et al. 1990, The J. of Antibiotics, 43:1579-1585.

Table 14. Apparent kinetic constants for *Trichoderma reesei* and *Aspergillus niger* cellobiases.

	Km(mM)		*Vmax* (IU/mg prep)	
assay	*T. reesei*	*A. niger*	*T. reesei*	*A. niger*
HK/G6P DH	1.041±.018	1.040±.009	.0156±.0004	71.25±.35
GO/P	1.083±.007	1.078±.060	.0157±.0001	73.40±3.39

Table 15. Reagents combination interfering with soluble product quantification in cellulose saccharification assays.

Reagents	use	Effect	Suggestions
carbonate (w/heating)[a]	• buffer • pH adjustment to terminate saccharification	• Δ product profile • decreased cellobiose • increased glucose	• use Tris buffer for pH adjustment to terminate saccharification
thimerosal (w/heating)[a]	• antimicrobial	• decreased precision for traditional HK/G6PDH assay used for glucose quantification	• use GO/P assay for glucose quantification • use sodium azide as an antimicrobial
citrate	• buffer	• decreased sensitivity for Nelson's alkaline copper reducing sugar assay	• use enzymatic assays as described in text • use DNS assay for reducing sugar equivalents

[a]: w/heating-- the reaction mixture is placed in boiling water for 5 minutes.

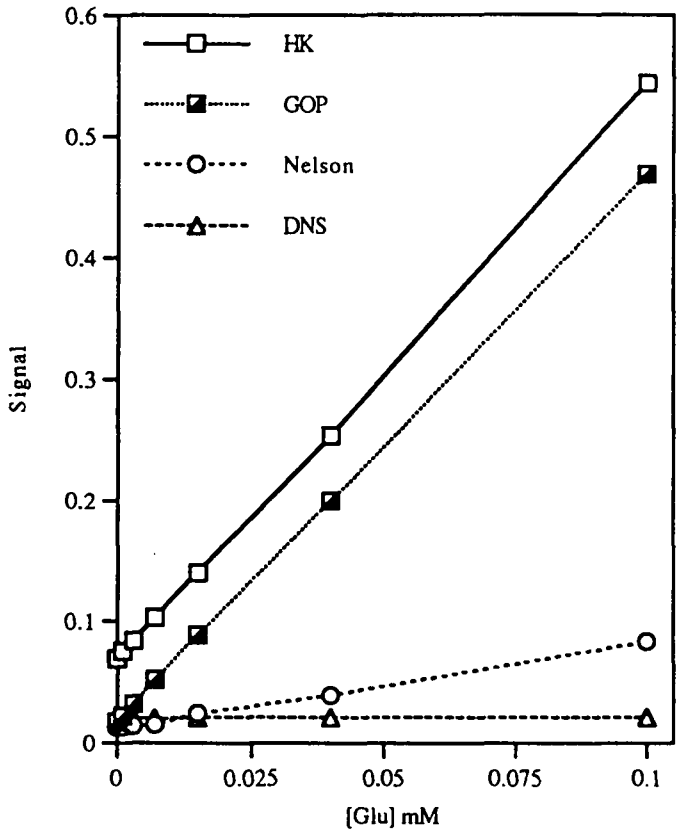
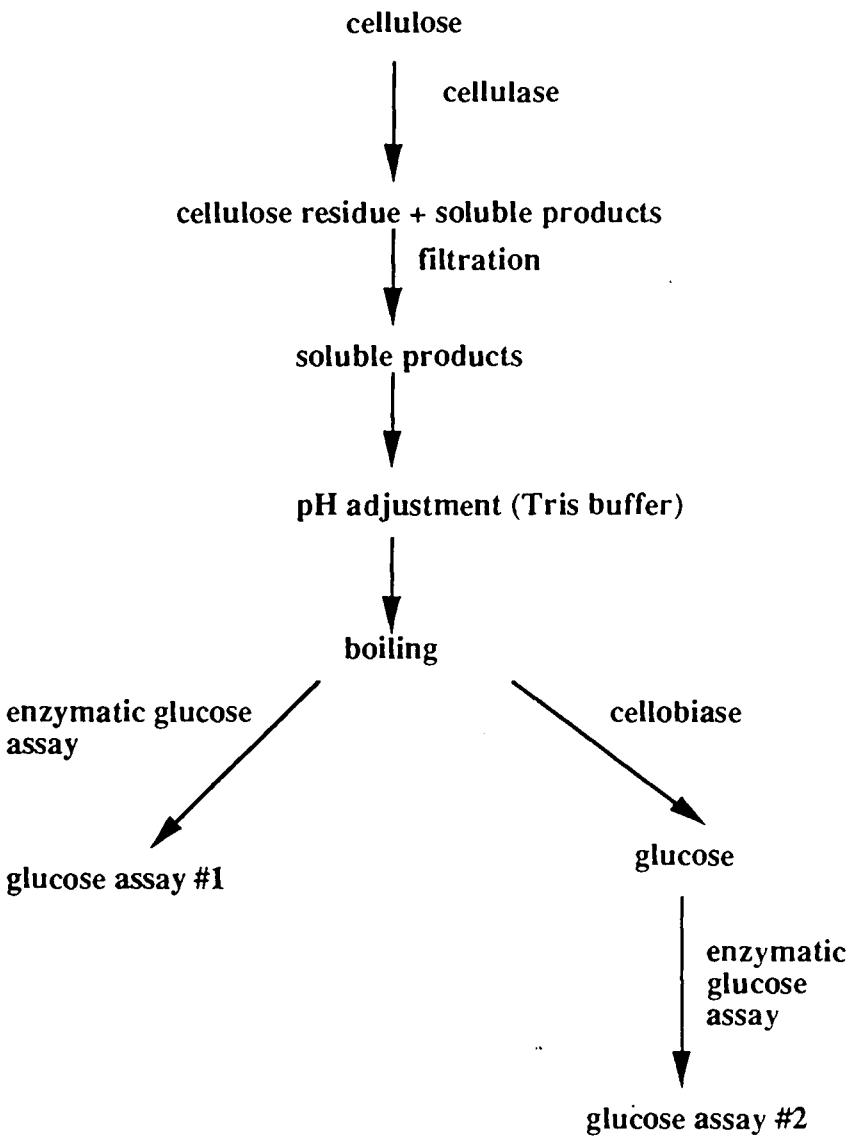


Fig.15 Calibration curves for reducing sugar and enzymatic Assays



Cellobiose (G2) = (#2-#1) / 2

Figure 16. Cellulose saccharification assay protocol

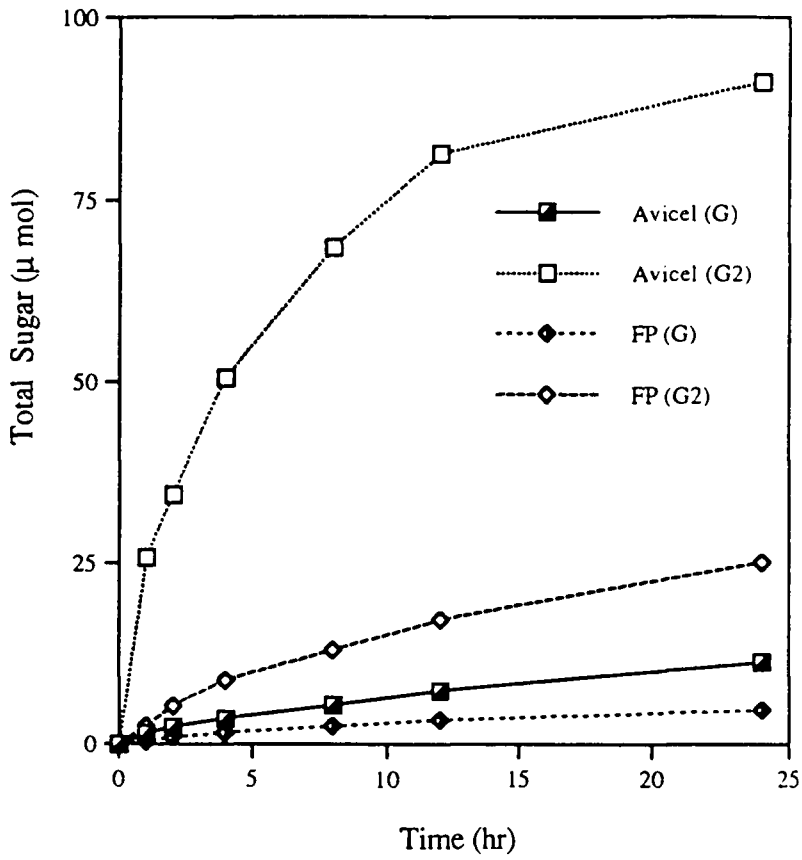


Figure 17. Product profile of Avicel and filter paper degradation by *T. reesei*. Reaction conditions were performed either in 50 mM Na acetate, pH 5.0 for Avicel or 50 mM Na citrate, pH4.8 for filter paper at 50°C. Enzyme concentration, 0.127 FPU per reaction mixture.G, glucose; G2, cellobiose.

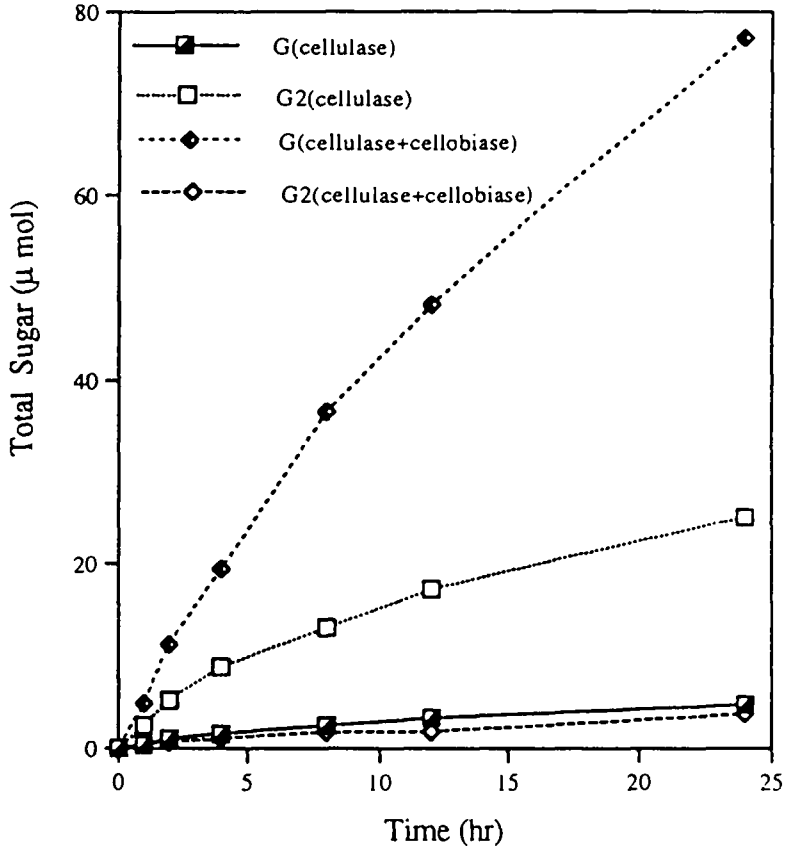


Figure 18. Product profile of Filter paper degradation by native *T. reesei*. cellulase or supplemented with cellobiase from *A. niger*. Reaction conditions were performed in 50 mM Na citrate, pH 4.8. at 50°C. Enzyme loads: cellulase, 0.1 FPU per ml reaction mixture; cellobiase, 0.77 IU per ml reaction mixture. G, glucose; G2, cellobiose.

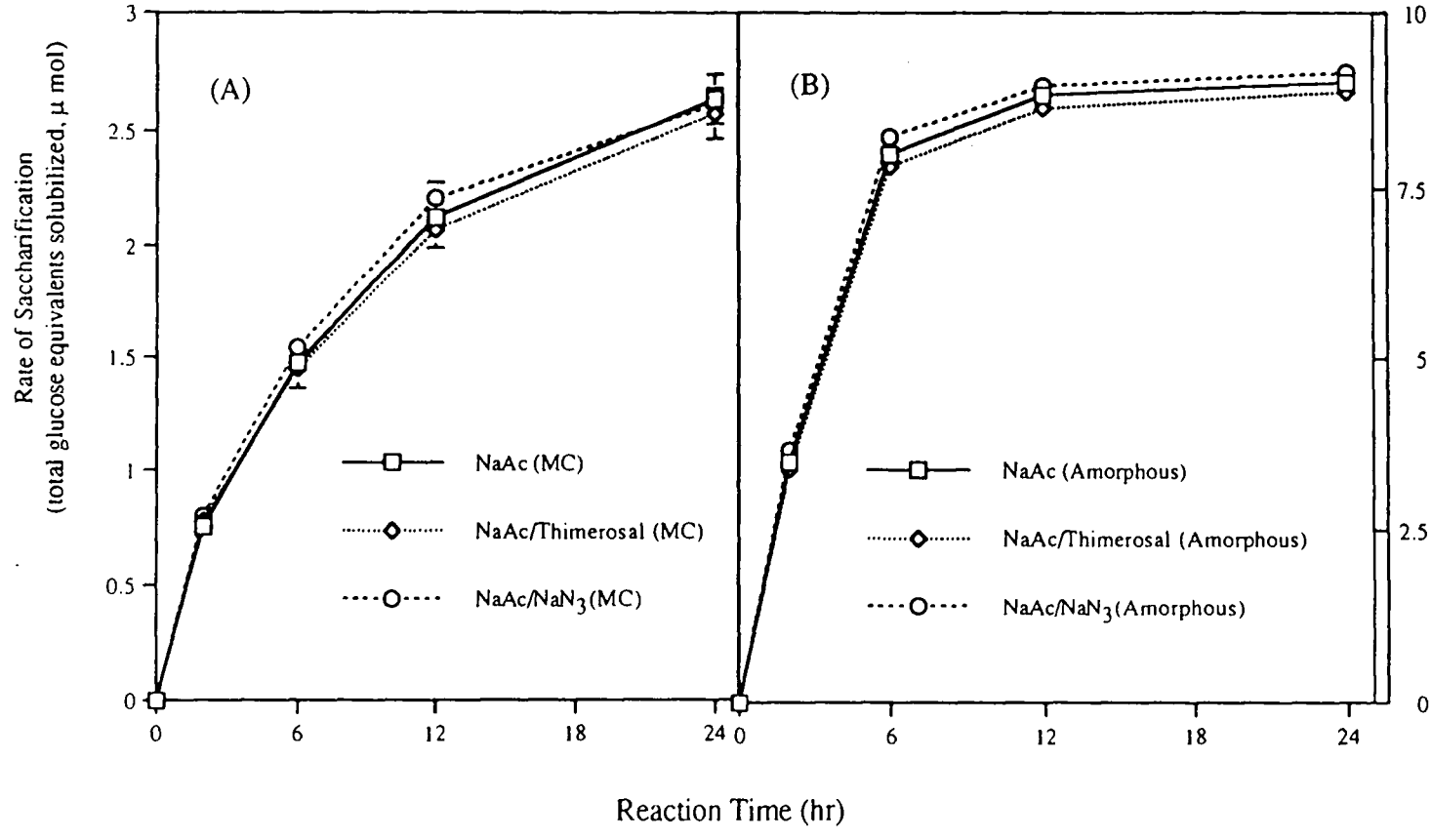


Figure 19. Effect of antimicrobials on (A) microcrystalline and (B) amorphous cellulose saccharification.

CHAPTER 4. KINETIC CONSEQUENCES OF HIGH SUBSTRATE TO ENZYME RATIOS IN TRICHODERMA CELLULASE BASED SACCHARIFICATION SYSTEMS

4.1. INTRODUCTION

Complete cellulase preparations from *T. reesei* and *T. viride* have been shown to exhibit an "apparent substrate inhibition". "Apparent substrate inhibition" in this case refers to any decrease in the rate of cellulose saccharification which accompanies an increase in substrate concentration. This behavior is noteworthy with respect to both basic and applied aspects of cellulose saccharification. In an applied sense, such behavior may prove to be an important factor in terms of saccharification reactor design. From a more basic perspective, it is of interest to determine the mechanistic basis for this behavior. Substrate inhibition may be viewed as a fundamental kinetic property and, hence, reflect a mechanism that is germane to the enzyme system as a whole. The inhibition of *Trichoderma* cellulase catalyzed cellulose saccharification at relatively high substrate concentrations has been observed in several laboratories. Substrate inhibition of *T. viride* derived cellulase systems have been observed with ball-milled (Van Dyke, 1972), microcrystalline (Liaw and Penner, 1990) and unspecified cellulose substrates (Okazaki and Moo-Young, 1978). The enzyme system from *T. reesei* has similarly been observed to exhibit substrate inhibition in apparent initial velocity studies (Lee and Fan, 1982; Huang and Penner, 1991) and in time dependent assays (Ryu and Lee, 1986).

Apparent substrate inhibition is not uncommon when enzymes are acting at relatively high substrate concentrations. In classical soluble enzyme/soluble substrate systems this behavior is generally attributed to the formation of dead-end or abortive complexes in which two substrates bind per active site (Fromm, 1975). However, these

types of complexes may be less important for heterogeneous systems involving insoluble substrates. It has been postulated that the observed substrate inhibition of cellulose saccharification is the result of decreasing the bulk aqueous phase of reaction mixtures as substrate concentrations increase (Lee and Fan, 1982). This mechanism is most probable for reaction mixtures in which there are very large decreases in reaction mixture "free water" due to the association of reaction mixture water with the additional substrate. Although this explanation appears plausible under some conditions, it does not appear to be a likely mechanism for all the systems in which substrate inhibition has been observed. For example, an apparent substrate inhibition has been observed in reaction mixtures containing a microcrystalline cellulose substrate at concentrations in which greater than 80% of the reaction mixture aqueous phase is apparently still "free water" (Liaw and Penner, 1990; Huang and Penner, 1991). Similarly, the observation that the optimum substrate concentration for some reaction mixtures is enzyme dependent (Liaw and Penner, 1990; Huang and Penner, 1991) and that a purified component of the *T. reesei* enzyme system does not show substrate inhibition under similar conditions (Huang and Penner, 1991) also indicate that the phenomena is not solely a function of the reaction mixture bulk aqueous phase. The complexity of the observed substrate inhibition is further illustrated by the observation that, under apparently equivalent experimental conditions, not all cellulose substrates generate substrate-activity profiles indicative of substrate inhibition (Liaw and Penner, 1990).

4.2. MATERIALS AND METHODS

4.2.1. Enzyme and substrate

Complete cellulase was produced by *Trichoderma reesei* QM9414 in our laboratory using shake-flask cultures as described by Mandels *et al.* (Mandels et al.

1981). The stock *Trichoderma* culture used for enzyme production was graciously provided by M. Mandels (U.S. Army Natick Research and Development Command, Natick, MA). Powdered cellulose, Solka-Floc SW40 (James River Inc., Berlin, NH) was used as the primary energy source for cellulase induction. Enzyme was separated from mycelia by filtration after 7 days of incubation. The pH of the enzyme solution was adjusted to 4.8 and the solution concentrated approximately 20-fold using a Millipore PM7178 membrane. The enzyme was then precipitated by the addition of 2 volumes of acetone at 4°C, separated by centrifugation, washed twice with cold acetone, and dried under vacuum. The resulting powder constituted the complete cellulase preparation which has a specific activitl;y of 1.27 FPU per gram solids. The microcrystalline cellulose starting material, Avicel PH105, was commercially obtained from FMC Corp. (Philadelphia, PA).

4.2.2. Enzymatic saccharification assays

The standard enzymatic saccharification assay was performed in 50 mM sodium acetate buffer, pH 5.0, at 50°C. Cellulose substrate was added to the reaction flask (25 ml, Erlenmeyer) containing buffer and equilibrated to 50°C. The reaction was then initiated by the addition of buffered enzyme solution. The final volume for each reaction mixture was 10 ml. Reaction mixtures were agitated at 160 rpm in a constant-temperature, orbital shaking water bath. Saccharification reactions were terminated at 2 hours by filtering the reaction mixture through a 0.22 μm pore size membrane filter followed by immediate assay for total glucose equivalents using a coupled glucose oxidase/peroxidase assay following treatment of the filtrate with *A. niger* cellobiase.

4.3. RESULTS AND DISCUSSION

4.3.1. Substrate-activity profiles for *T. reesei* cellulases

Representative substrate-activity profiles which reflect the apparent substrate inhibition of the complete *T. reesei* cellulase system acting on a microcrystalline cellulose substrate are provided in Figure 20. Each curve of Figure 20 represents a series of reaction mixtures containing equivalent amounts of enzyme, but differing with respect to substrate concentration. The data clearly illustrate that this enzyme/substrate system does not obey classical saturation kinetics. Instead, the rate of saccharification increases to a maximum with increasing substrate concentrations, after which further increases in substrate concentration result in corresponding decreases in rates of saccharification. In the context of this paper, the rate of saccharification refers to the total amount of saccharide solubilized per unit time. The substrate concentration corresponding to the fastest rate of saccharification may, for many practical purposes, be considered the "optimum" substrate concentration. The optimum substrate concentration for a given reaction mixture is likely to be dependent of the enzyme source, substrate, and reaction conditions. Optimum substrate concentrations for the conditions employed in this study ranged from 0.25 to 1.0%. The curves of Figure 20 indicate that the reaction rate decreases asymptotically with increasing substrate concentrations above the optimum. The maximum amount of substrate inhibition being approximately 75%. The "extent of substrate inhibition" referred to in this work was calculated as follows:

$$ESI_i = [(MORS - RS_i) / MORS] \, 100$$

where: ESI_i = extent of substrate inhibition as substrate concentration "i"

MORS = maximum observed rate of saccharification

RS_i = rate of saccharification at substrate concentration "i"

Clearly, this working definition is only applicable for substrate concentrations greater than that which corresponds to the maximum observed rate of saccharification. Figure 20 also suggests that increasing the enzyme concentration of a given reaction mixture results in an apparent shift in the optimum substrate concentration, the optimum substrate concentration being higher for those reaction mixtures containing more enzyme, as reported previously (Liaw and Penner, 1990; Huang and Penner, 1991).

The relationship between enzyme concentration and optimum substrate concentration is more easily visualized when the rate of saccharification is presented with respect to the ratio of total enzyme (FPU) to total substrate (g) in the reaction mixture. A plot of this type is presented in Figure 21. The Figure illustrates that the maximum rate of saccharification for a reaction mixture containing a constant amount of enzyme is dependent on the ratio of total enzyme to total substrate. This behavior is in contrast to well behaved Michaelis-Menton systems in which substrate concentrations above that required for enzyme saturation have little or no effect on the reaction rate. The optimum enzyme to substrate ratio is likely to be dependent on the particular substrate and enzyme used in any given study. In the system presented here, the optimum enzyme to substrate ratio was approximately 0.8 FPU per gram substrate.

4.3.2. Enzyme concentration and the rate of saccharification

The rate of saccharification of microcrystalline cellulose is expected to be a function of the amount of enzyme adsorbed on the substrate surface. A relatively simple scenario would be that the rate of saccharification is directly proportional to the amount of bound enzyme and that the relationship between bound enzyme and the enzyme concentration of the reaction mixture is described by a Langmuirian type sorption

isotherm. In this case, a plot of initial saccharification rate versus enzyme concentration for a given substrate load would yield a linear relationship between enzyme concentration and reaction rate at relatively low enzyme concentrations. However, as enzyme concentrations increase this relationship will break down, such that further increases in enzyme concentration will result in progressively smaller increases in saccharification rates. Eventually, it is expected that the substrate surface will be saturated and, hence, further increases in enzyme concentration will have relatively little effect on the rate of saccharification. Graphically, this behavior would take the form of a right rectangular hyperbola. Experimentally, this simple relationship is not observed. The experimental data reflects a sigmoidal relationship between enzyme concentration and rates of cellulose saccharification (Figure 22). The sigmoidal curve is a consequence of the same behavior which was termed "substrate inhibition" when referring to Figure 20. A data set consisting of the four lowest enzyme concentrations included in Figure 22 is plotted in Figure 23a. This plot demonstrates the linear relationship between enzyme concentration and rate of reaction at these low enzyme concentrations. The change in saccharification rate per unit change in enzyme concentration is approximately 9.5 under these conditions. Each data point in Figure 23a corresponds to a total enzyme (FPU) to total substrate (g) ratio of less than 0.05 (for reference, see Figure 21). A second data set, consisting of the next four higher enzyme concentrations from Figure 22, corresponding to total enzyme (FPU) to total substrate (g) ratios ranging from 0.1 to 1.4, are plotted in Figure 23b. The change in saccharification rate per unit change in enzyme concentration at these enzyme to substrate ratios was 34.4, approximately 3.6-fold greater than that observed for the data of Figure 23a. The higher slope for the data set of Figure 23b relative to that of Figure 23a can be rationalized with reference to Figure 21, which indicates that the extent of

"substrate inhibition" progressively declines as reaction mixture enzyme to substrate ratios are increased approximately 0.08 to approximately 0.6.

4.3.3. Mechanistic basis of apparent substrate inhibition

Sigmoidal curves, such as that of Figure 22, are commonly observed for biological systems demonstrating positive cooperativeity. A form of positive cooperativeity which exists for cellulolytic enzyme systems is synergism. Synergism between cellulolytic enzymes is widely recognized (Wood and McCrae, 1978; Wood et al, 1988; Irwin et al. 1993) and has been the focus of several studies with the *Trichoderma* cellulases (Henrissat et al. 1985, Woodward et al. 1988; Heitz et al. 1991). A mechanism which is consistent with the data presented above is one which embraces the concept of synergism between component enzymes. The mechanism is based on the assumption that each enzyme component has an equal affinity for all loci on the substrate surface, and that the maximum kinetic efficiency for a pair of potentially synergistic enzymes occurs only when the two enzymes occupy the same locus. The mechanism does not necessitate that the pair of synergistic enzymes be at the same locus simultaneously. If there is a kinetic advantage for two enzymes to act sequentially at the same locus, then a form of substrate inhibition may also be exhibited.

A mechanism which provides a kinetic advantage for enzymes acting at a "common loci" suggests that the observed substrate inhibition is the result of a competitive binding of the pertinent enzyme components between common loci, where they would express relatively higher activity, and physically distinct loci where the activity associated with the enzymes acting independently would be relatively lower. In this mechanism, additional substrate above that required for enzyme saturation would

promote the formation of physically distinct single enzyme-substrate complexes, which for this system are kinetically inferior. The mechanism suggests that substrate inhibition would not be observed for the purified enzyme components acting independently, which has been demonstrated for the major component of the *T. reesei* system (Huang and Penner, 1991). The mechanism also suggests an asymptotic decrease in activity with increasing substrate concentration, as depicted in the data of Figure 20. The activity observed at relatively high substrate concentrations and, thus, the maximum attainable substrate inhibition, would be dictated by the activity of each of the enzymes acting independently.

The mechanism suggested in this paper is presented in its most general form. A better understanding of the basic enzymology which governs this system, including information on possible enzyme-enzyme interactions at common loci, the relative importance of sequential versus simultaneous action of multiple enzymes at common loci and the values of applicable kinetic and thermodynamic constants for enzymes acting at various loci, will obviously be helpful in terms of developing a comprehensive mechanistic model which can account for the kinetic behavior reported here.

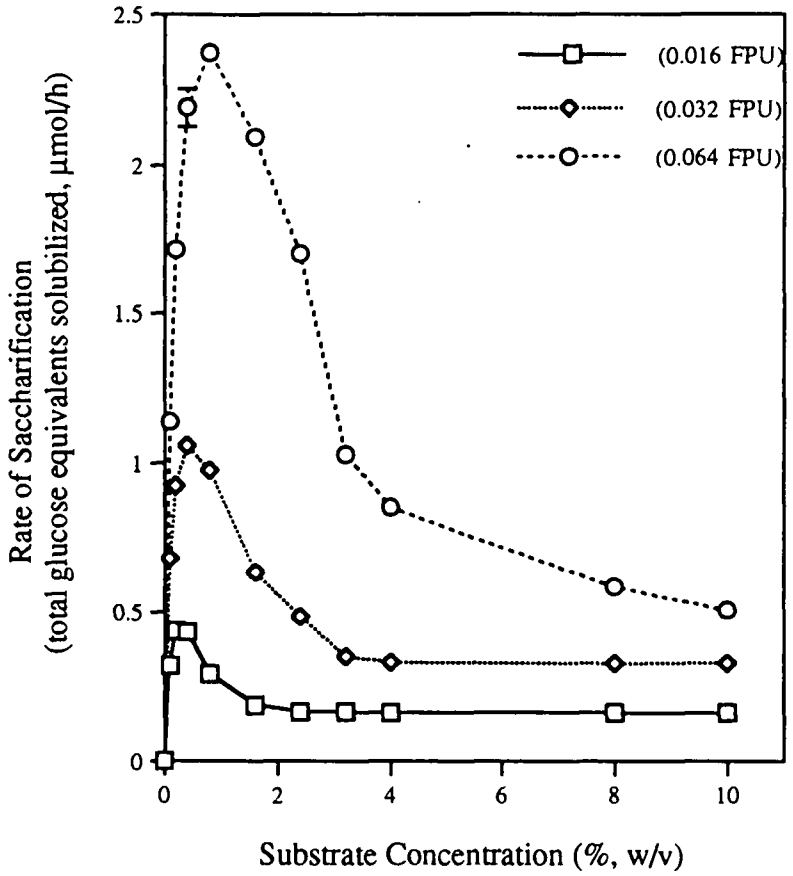


Figure 20. Substrate-activity profile for the *Trichoderma reesei* cellulase system acting on a microcrystalline cellulose substrate. Reaction conditions were 50 mM sodium acetate, pH 5.0 at 50°C, with a total reaction volume of 10 ml. Total enzyme loads were 0.016, 0.032 or 0.064 FPU per reaction mixture. Substrate concentrations were as indicated along the abscissa. "Rate of saccharification", as indicated along the ordinate, is measured as umol glucose equivalents solubilized per hr. per 10 ml reaction mixture.

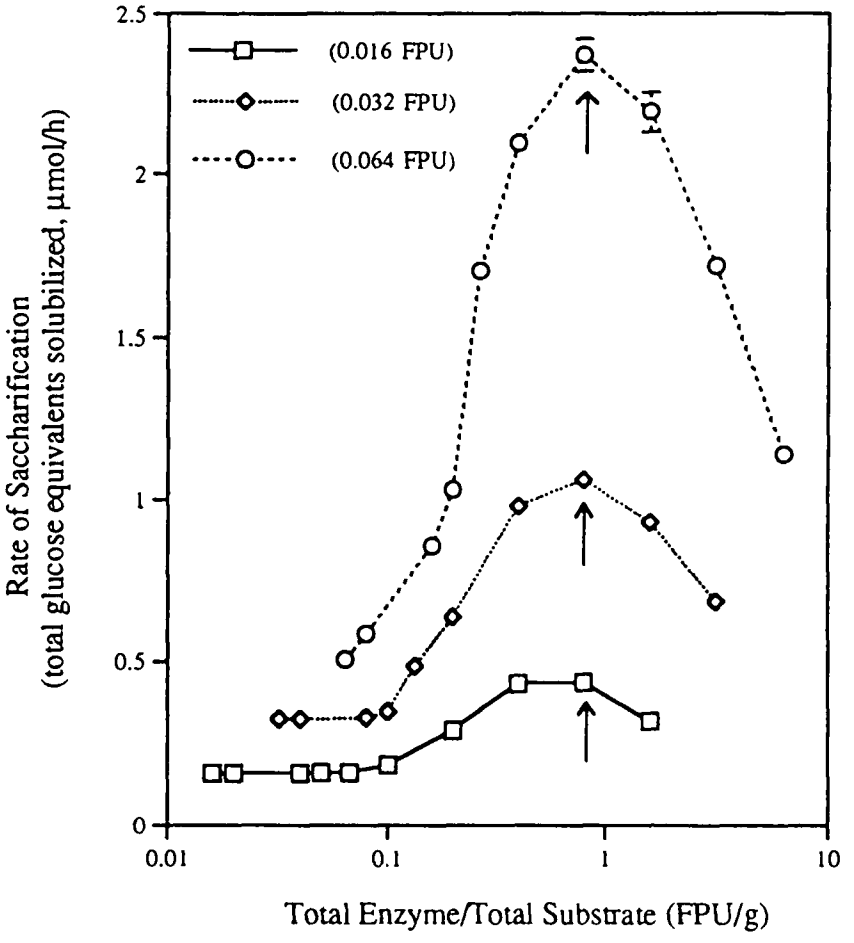
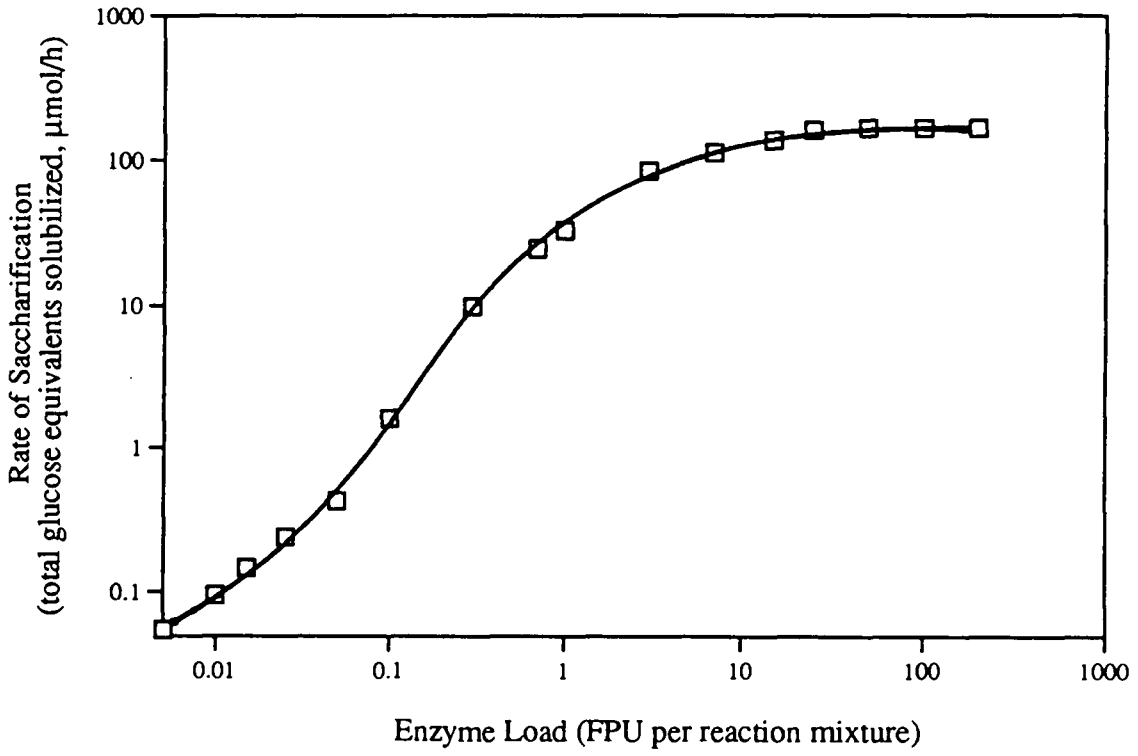


Figure 21. Effect of enzyme to substrate ratio on rate of saccharification of microcrystalline cellulose. Reaction conditions, enzyme loads and substrate concentrations were as defined in Figure 20.



Figurre 22. Effect of total enzyme load on rate of saccharification of microcrystalline cellulose. Reaction conditions as defined in Figure 20 with a substrate concentration of 5 % (w/v). Enzyme loads (total enzyme, FPU, per reaction mixture) varied as indicated along the abscissa.

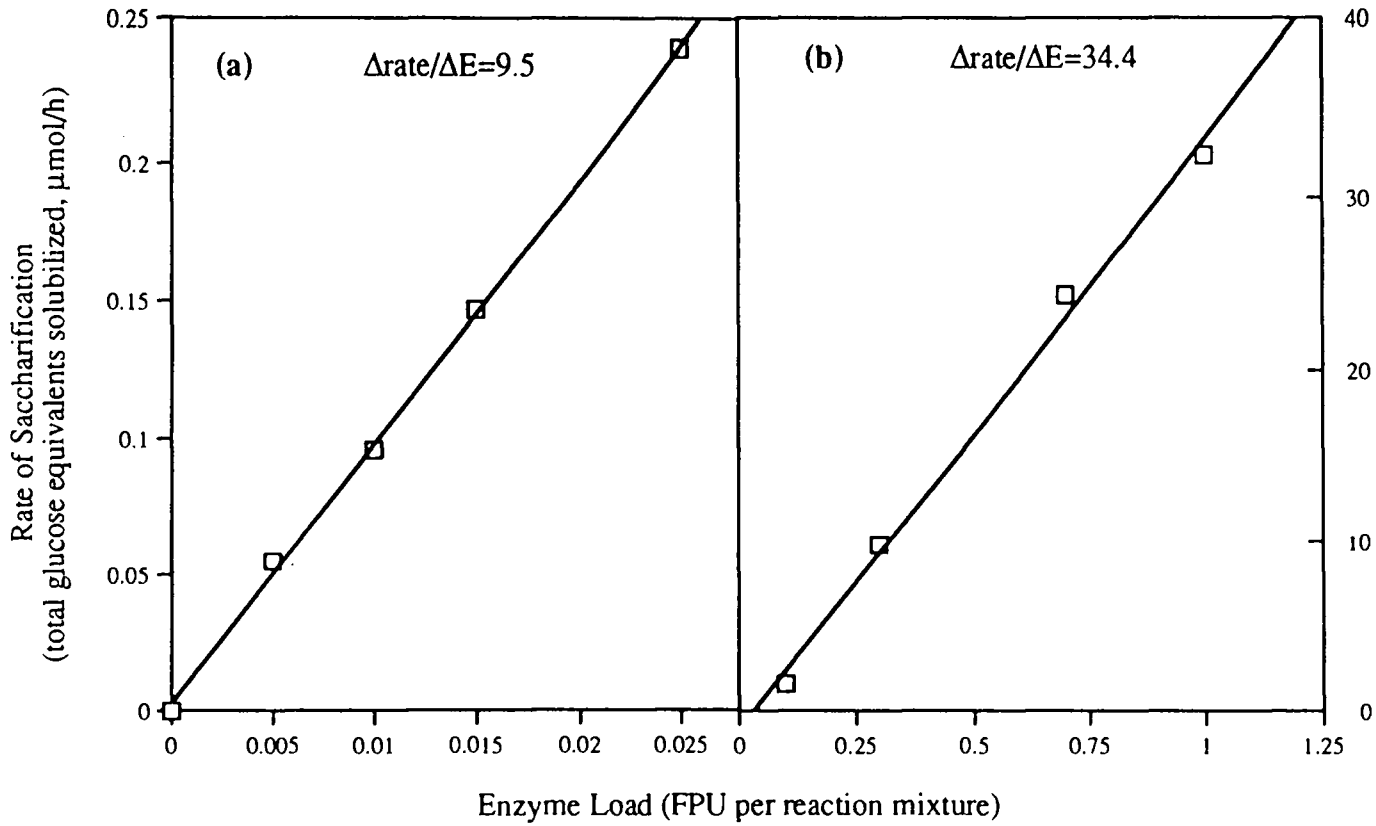


Figure 23. Correlations between enzyme load and rate of saccharification. Reaction conditions as defined in Figure 20 with a substrate concentration of 5 % (w/v). Enzyme loads (total enzyme, FPU, per reaction mixture) ranged from (a) 0.005 to 0.025 and (b) 0.1 to1.0.

CHAPTER 5. THE SUBSTRATE-ACTIVITY RELATIONSHIPS OF *TRICHODERMA REESEI* CELLULASE WITH NATIVE AND MODIFIED CELLULOSIC SUBSTRATES

5.1. INTRODUCTION

The rate of degradation of a cellulosic substrate is affected by several parameters, including the source of the cellulase enzymes (Coughlan et al. 1988), the physicochemical properties of the substrate (Weimer and Weston, 1985), and the extent of product (Mandels et al. 1978) and substrate (Lee and Fan, 1982) inhibition. Of these parameters, substrate inhibition has received the least amount of experimental attention. Substrate inhibition is a fundamental kinetic property which reflects a deviation in the expected saturation kinetics of enzyme-catalyzed reactions. Apparent substrate inhibition is not uncommon when enzymes are acting at relatively high substrate concentrations, and the property may be the effect of several causes (Dixon and Webb, 1979). Along with its inherent mechanistic information, substrate inhibition is of importance relative to several applied aspects of cellulase and cellulose research. When the relative maximum cellulolytic capacity of a microbial enzyme system is evaluated, it is essential to consider the potentially unique substrate inhibition properties of that particular enzyme. Similarly, substrate inhibition is of relevance to the design of experiments that analyze native and modified cellulosic substrates to identify potential pretreatments that are capable of increasing the reactivities of cellulosic materials.

Despite the large number of comparative and mechanistic studies done with *Trichoderma* enzymes, there are relatively few studies which have considered their apparent substrate inhibition properties. Okazaki and Moo-Young (1978) have presented a generalized mechanistic model for the enzymatic hydrolysis of cellulose which, based

on concurrent random and endwise attack of the substrate, predicts substrate inhibition. They also stated that they observed apparent substrate inhibition in their studies. Lee and Fan (1982) have presented initial velocity data which reflect apparent substrate inhibition of the *T. reesei* enzyme. They attributed the inhibition to hydrodynamic factors and, therefore, focused their initial velocity study on reaction conditions which did not exhibit substrate inhibition. Apparent substrate inhibition of *Trichoderma* and *Aspergillus* cellulase complexes by a relatively complex cellulosic substrate, leached beet cosette, has also been reported (Contreras et al. 1982). Although further characterization related to substrate inhibition have been conducted in our previous studies (Liaw and Penner, 1990; Huang and Penner, 1992), properties of substrate inhibition are still unclear.

In the present work, the substrate-activity profile of the *T. reesei* cellulase enzyme system are characterized by screening native and modified cellulosic substrates either differing in crystallinity or degree of polymerization. The results presented here demonstrate that the apparent substrate inhibition properties of this enzyme are indeed complex and are dependent upon the cellulosic substrate that is used. The effect of bulk aqueous phase on rate of saccharification on microcrystalline cellulose was also investigated.

5.2. MATERIALS AND METHODS

5.2.1. Substrates

The microcrystalline cellulose starting materials, Avicel PH101, PH102, and PH105 were commercially obtained from FMC Corp. (Philadelphia, PA), and Solka-Floc BW 200 was from James River Co. (Berlin, NH). Carded cotton sliver was a gift from Dr. Bertoniere (Southern Regional Research Center, USDA, New Orleans, LA). Whatman #1 filter paper and Whatman CF1 cellulose powder were obtained from Whatman Labsales Inc. (Hillsboro, OR). Celufil was obtained from United States Biochemical (Cleveland, OH) and α-cellulose was purchased from Sigma Chemical Co. (St. Louis, MO).

5.2.2. Enzymes

Trichoderma reesei cellulase was cultured by our laboratory as described in Huang and Penner (1991). The same ß-glucosidase preparation as described in chapter 3 was used in the present study.

5.2.3. Chemicals and reagents

Hydrochloric acid, reagent grade, and standardized sodium hydroxide solution were obtained from Fisher Scientific, (Pittsburgh, PA). Glucose oxidase/Peroxidase, dimethylsulfoxide (DMSO) and sodium hydrosulfite were purchased from Sigma Chemical Co. (St. Louis, MO). Cupriethylenediamine hydroxide solution was obtained from Synmet Inc. (Baton Rouge, LA). Diethylamine (DEA) and sodium anthraquinone-ß-sulfonate were obtained from Eastman Fine Chemicals (Rochester, NY) and Pfaltz &

Bauer, Inc. (Waterbury, CT) respectively. Sulfur dioxide gas was purchased from Aldrich Chemical Inc. (Milwaukee, WA).

5.2.4. Substrate Preparation

5.2.4.1. Hydrochloric acid regenerated Avicel PH101 (HCR-Avicel PH101)

Hydrochloric acid regenerated Avicel PH101 was prepared by dissolution of cellulose powder in HCl as described by Hsu and Penner (1991). The regenerated cellulose was solvent-dried by washing with methanol and acetone twice.

5.2.4.2. Ball-milled Avicel PH101 (BM-Avicel PH101)

A 40-g batch of ball-milled cellulose was prepared by milling microcrystalline cellulose for 24 hours at 23°C in a 1.3 L capacity jar containing 1 kg of burundum cylinders rotating at 45 rpm. Upon completion of milling , the resulting product had an average particle size of less than 50 μm.

5.2.4.3. Enzyme modified Avicel PH101 (EM-Avicel PH101)

Enzyme-treated Avicel PH101 was prepared by initiating the reaction with a high loading cellulase enzyme (Environmental Biotechnologies Inc. Santa Rosa, CA), 65 FPU per gram cellulose. The reaction mixture was incubated at 50°C, 160 rpm, shaker bath for 5 hours. The reaction was terminated by filtering with glass microfiber membrane and the residue was resuspended in 0.1 M Tris buffer at pH 9.0. The suspension was placed in boiling water bath for 5 min to inactivate the cellulase enzyme. The reaction mixture was filtered and dried by washing with 100% ethanol and acetone. The resulting material was approximately 27 % less weight compared with the original sample weight.

5.2.4.4. Amorphous cellulose

Amorphous cellulose was exactly prepared by dissolving the microcrystalline cellulose, Avicel PH101, in DMSO/SO_2 solution and was regenerated by excess water and followed by freeze-drying as described by Isogai and Atalla (1991).

5.2.4.5. Acid-treated Solka-Floc BW200 (AT-SF)

Acid-treated Solka-Floc BW200 NF was made by cooking the powder cellulose in 2.5 N HCl at 105°C for 30 min as described by Battista (1950). The sample was vacuum oven-dried at 105°C for 5 hours and was grounded with a constant-speed grinder (Arthur H. Thomas Co., Philadelphia, PA). The fine particle was sieved through 40 mesh screen and was homogeneously collected as a starting material for the present study. The final product was approximately 20% less weight compared with the initial sample weight.

5.2.4.6. Enzyme-modified Solka-Floc BW200 (EM-SF)

Enzyme-modified Solka-Floc BW200 NF was prepared as described in enzyme-treated Avicel PH101 section (5.2.4.3.). The resulting material was approximately 40% less weight compared with the initial sample weight.

5.2.4.7. Dewaxed cotton cellulose

Dewaxed cotton cellulose was made from carded cotton sliver by removing wax in a Soxhlet apparatus with chloroform and followed by 95% ethanol as described by Wood (1988). The dewaxed cotton was solvent-dried as described in 5.2.4.3. The resulting material was regarded as a substrate for experiment.

5.2.4.8. Filter paper

A uniform size of Whatman #1 filter paper (0.7 diameter) was prepared by a single hole hand-puncher. The round shape of filter paper was punched and collected as one of the tested substrates.

5.2.5. Substrate Characterization

5.2.5.1. Degree of Polymerization (DP)

The degree of polymerization was determined using a size of 75 Cannon-Fenske viscometer (Thomas Scientific, Swedesbora, NJ) at 25.0 $\pm$ 0.1°C. Cellulose was dissolved in 0.5 M cupriethylenediamine to give a final cellulose concentration of 0.05, 0.1, 0.15 and 0.2% (w/v) and the efflux time was measured according to ASTM standard D 1795 (ASTM, 1986). The degree of polymerization was then obtained by multiplying the intrinsic viscosity by 190 (Lupton and Villalba, 1988).

5.2.5.2. Crystallinity index (CrI)

The crystallinity index of substrates were calculated from diffractograms obtained with an automated Philips X-ray diffractometer using nickel-filtered Cu Kα radiation. The diffraction intensity was measured between Bragg angles (2θ) of 10 and 30. Crystallinity index (CrI) values were determined as described by Hsu and Penner (1989).

5.2.5.3. Particle size measurement (Image analysis)

Cellulose particle images were captured via a Cohu camera (Cohu Inc., San Diego, CA) mounted on as Epistar incident light microscope (Cambridge Instruments). The video signal was digitized to eight bits of accurance per pixel by a "frame grabber" (a Visionplus-AT board, Imaging Technology Inc.) before storage in frame memory. The

frame grabber's display logic then converted the pixels back into an analog format for video monitor display. Each stored image had a resolution of 640 x 480 pixels with a range of 256 gray values. Particles were enumerated, and their lengths measured and averaged by Image-Pro plus (Silver Spring, MD) processing software which also contained a descriptive statistics program.

5.2.6. Enzymatic saccharification assays

The standard enzymatic saccharification assay was performed in 50 mM sodium acetate buffer (pH 5.0) at 50°C. Cellulose substrate was added to the reaction flask (10 ml, Erlenmeyer) containing buffer and equilibrated to 50°C. The reaction was then initiated by the addition of 0.016 FPU enzyme solution. The final volume for each reaction mixture was either 4 ml or 10 ml. Reaction mixtures were agitated at 160 rpm in a constant-temperature, orbital shaking water bath (model 3540; Lab-Line, Melrose Park, Ill.). Saccharification reactions were terminated at 2 hours by filtering the mixture through a 0.22 μm pore size membrane filter (Millipore Corp., Cambridge, Mass.), followed by pH adjustment to 9.0 with 1 M Tris-buffer. The total glucose equivalents were measured by glucose oxidase/peroxidase assay as described in methods section in chapter 3.

5.2.7. Volume Effect on Cellulose Saccharification

Cellulose substrate, Avicel PH101 (lot no.1716) was employed in current study. The fixed amount of cellulose (400 mg) was added to the reaction flask (10, 25 or 125 ml Erlenmeyer flask) containing temperature-equilibrated buffer, ranging from 4 ml to 80 ml. Reaction conditions, total enzyme loads and saccharification assay was as defined in section 5.2.6.

5.3. RESULTS AND DISCUSSION

5.3.1. Volume effect on cellulose saccharification

Since the mass transfer retardation has been reported by Lee and Fan (1982) if the relatively high substrate concentration was employed. It is necessary and worthy to examine this problem by varying the bulk aqueous volume. The volume effect on microcrystalline cellulose saccharification was investigated and the result is illustrated in Figure 24. The data demonstrated that saccharification rate for *T. reesei* cellulase on microcrystalline cellulose was independent on the total volume, at least ranging from 4 ml to 80 ml under specified conditions. In other words, the reaction rate is dependent upon the total enzyme to total substrate ratio. The results indicated that the reaction rate is close if a homogeneous suspension can be held during the reaction period (Liaw and Penner, 1990). However, mass transfer retardation may not be a case in the present study and can be ignored here. Berg and Purcell (1977) suggested that ß-amylase molecules weakly attached to the surface of starch-gel have a fairly good chance of diffusing back to the surface and being readsorbed. Similarly, the principle can be applied to this insoluble substrate-soluble enzyme system, cellulose and cellulase. The cellulase enzymes remain close to the surface of the cellulose substrate after breaking glycosidic bonds. They do not migrate far from surface of the substrate, not into the bulk aqueous phase. Our data agree with that of Berg and Purcell's since the volume of bulk aqueous phase has little effect on microcrystalline cellulose saccharification.

5.3.2. Effect of particle size on cellulose saccharification

In an insoluble substrate system, particle size, surface area and pore size/volume concerns become more important with respect to saccharification rate of substrates

(Tanaka et al. 1988; Grethlein, 1985). Three different particle sizes of microcrystalline cellulose 20, 50, and 90 microns, were selected and designated as MC20, MC50, and MC90, respectively. The effect of microcrystalline cellulose particle size on saccharification rate is shown in Figure 25. The relative surface per gram substrate for these microcrystalline celluloses is 1:1.8:4.5 for MC90, MC50 and MC20, respectively. On a per unit of mass basis such particles will show relative surface areas inversely proportional to their respective particle sizes. This relationships holds for particles of any similar shape so long as each particle size has the same shape (e.g. between spherical particles, or between cubic particles, etc.). Crystallinity index and degree of polymerization (DP) of these substrates are characterized and tabulated in Table 16. No significant differences were observed among these physical parameters.

The data show a substrate inhibition for each of the microcrystalline substrates. The graph suggested that the optimum substrate concentration which corresponds to a maximum saccharification rate was shifted from 0.4, 0.8 to 1.6% when the particle size was increased from 20, 50 to 90 microns respectively. The magnitude of substrate inhibition was 49, 52 and 69% for MC90, MC50 and MC20. Theoretically, the surface area of the substrate decreases with increasing particle size, then the smallest particle (MC20) with most surface area will reach the maximum reaction rate at lower substrate concentration than those have a bigger particle size with less surface area.

Effect of total enzyme to total substrate ratio on rate of saccharification acting on different microcrystalline celluloses is shown in Figure. 26. The ratio were shifted from 0.2, 0.4 to 0.8 when average particle size of substrate was changed from 90, 50 to 20 microns. However, the ratio is kept constant and independent of enzyme concentration if

the same substrate was used (see chapter 4, Figure 21). The total enzyme/total substrate ratio for maximum reaction rate of MC90 is 0.2 FPU enzyme per gram cellulose which is 4-fold less than that of MC20, 0.8 FPU per gram cellulose. Incidentally, the total enzyme/total substrate ratio change for maximum reaction rate seems proportional to the relative surface area ratio change of MC90 to MC20 (1:4.5). A positive relationship was also found between MC90 and MC50. However, the particle size (surface area) factor can not explain the different maximum saccharification rates for these microcrystalline celluloses.

5.3.3. Microcrystalline cellulose and enzyme-modified cellulose

A substrate-activity profile for *T. reesei* cellulase acting on microcrystalline cellulose and enzyme-pretreated microcrystalline cellulose is presented in Figure 27. The results exhibit a similar substrate inhibition phenomenon as our previous data when the microcrystalline cellulose was used as a substrate. The magnitude of substrate inhibition was 72% with 0.016 IU enzyme per reaction mixture. Crystallinity and degree of polymerization of both substrates are characterized and listed in Table 16. The data indicated no significant change in these two parameters following enzyme treatment. The particle size of both substrates was visualized by the Image-Pro system with incident light microscopy. No significant change on particle size between native and modified substrates was observed. The result appeared to indicate that enzyme-pretreated Avicel PH101 does not show substrate inhibition behavior under conditions equivalent to that which showed substrate inhibition for the nonenzyme-treated sample. The enzyme behavior on this pretreated substrate obeyed Michaelis-Menten kinetics, exhibiting a hyperbolic curve when the enzyme was saturated by the substrate.

As demonstrated in our previous study (Liaw and Penner, 1990), the determinants of substrate inhibition are not only governed by substrate itself but also enzyme-substrate interactions. Enzyme adsorption and desorption on insoluble substrate has been extensively studied (Lee and Fan 1982; Rabinovich et al. 1984; Kyriacou and Neufeld, 1989; Hoshino et al. 1992). From the adsorption affinity point of view, reversible and irreversible adsorption coexist at the same time. With respect to hydrolysis reaction, the productive and nonproductive adsorptions are also involved in the adsorption process (Ryu et al. 1984; Tomme et al. 1990; Klyosov, 1986; Converse, et al. 1988; Henrissat et al. 1988; Otter, et al. 1989; Gilkes, et al. 1992; Nidetzky and Steiner, 1993). Thus, the adsorption of cellulase components depends on the structural properties of the adsorbents. Ryu et al. reported that cellulase components appear to have distinctly different adsorption sites on cellulose chains. As a consequence, strong-affinity cellulase adsorbed on a specific locus of a cellulose chain may blocked the productive-binding of a second enzyme. This phenomenon may explain the lower saccharification rate observed at lower substrate concentrations when the enzyme-pretreated Avicel PH101 was used as a substrate compared with that of untreated substrate. Thereafter, the rate of saccharification of the enzyme treated substrate increases with increasing substrate concentration, since the probability of the cellulase enzyme interacting with the blocking loci of cellulose chain is decreasing.

Based on the mechanism we proposed here (Figure 34), even though some productive-binding sites have been sterically hindered by the cellulase, a number of productive-binding sites have been prehydrolyzed by the enzyme so that they are still available for enzyme attack. Consequently, the reaction rate of enzyme-pretreated substrate are approximately 4.5-fold higher than that of untreated microcrystalline

cellulose at 0.4 gram total substrate. Several authors (Wood, 1975; Sinistyn et al. 1989) demonstrated that enzymatic cellulolysis occurs first at amorphous regions of native cellulose by preferential random attack of endo-type cellulases, resulting in exposure of crystalline regions, which become more rigid to resist enzymes hydrolysis. Some laboratories (Coughlan, 1992; Henrissat et al. 1985) have reported that cellulase enzymes have functions on cellulose chain-loosening and surface roughening. The result is conversion of the cellulose substrate into a more susceptible state for enzyme attack. However, due to little effect on crystallinity and DP of native and modified substrates, the enzyme-nicked (enzyme-prehydrolyzed) mechanism becomes a reasonable rationale for interpreting the results we show here. Nidetzky et al. (1994) also reported that individual cellulase components have a distinct effect on enzyme-pretreated filter paper. Their results suggested that only a limited number of substrate sites in filter paper are available for hydrolysis by endoglucanases. This concept is in agreement with the curves shown in Figure 27, with respect to irreversible enzyme adsorption and productive site blocking by cellulase enzyme (as mentioned earlier in this section). A significant conversion rate (27%) with no difference in DP values of enzyme-modified cellulose (Table 16) also indicates that *T. reesei* cellulase prefer a "single chain-multiple attack" action on microcrystalline cellulose or that they act their peeling of single layers of substrate prior to attacking interior or "buried" cellulose molecules..

5.3.4. Hydrochloric Acid-Regenerated cellulose and ball-milled cellulose

Substrate-activity profile of *Trichoderma reesei* acting on microcrystalline cellulose, ball-milled cellulose and hydrochloric acid-regenerated cellulose is depicted in Figure 28. All of these substrates have approximately the same DP. However, they differ considerably in crystallinity, decreasing from 90% to 40%, were observed in acid-

regenerated cellulose (Table 16). No substrate inhibition was observed when solvent-dried regenerated cellulose was used as a substrate. The particle size of regenerated cellulose is bigger than that of microcrystalline cellulose (Figures 38 and 39). Theoretically, the surface area of regenerated cellulose decreases with increasing particle size, the resulting reaction rate of regenerated cellulose should be expected lower than that of microcrystalline cellulose. But, on the contrary, the reaction rate of the regenerated cellulose, even at lower substrate concentrations is still higher than that of microcrystalline cellulose. The explanation of this phenomenon is probably either due to the structure change from cellulose I to cellulose II or a less compact structure forms during cellulose gel regeneration (Sinitsyn et al 1989) resulting in more susceptible features for enzymatic hydrolysis or even though the external surface area decreases with particle size increase, the internal surface area increase with structure change due to the regenerated cellulose having a bigger porosity. In this case, pore size distribution, and diffusion of enzymes into the cellulose substrate should be considered (Grethlein, 1985; Tanaka et al. 1988; Weimer and Weston, 1985). In small pores, only the smaller components of cellulase complex can enter. This tends to segregate the cellulase components to reduce the overall hydrolysis rate because the synergistic action between the cellulase components is reduced. In large pores, however, all the components can enter, and since the available surface area is increased, the hydrolysis rate is increased. In short, small pores hinder and large pores enhance, the hydrolysis. As expected, the surface area of ball-milled Avicel PH101 increased with decreasing particle size (Figure 40) and the optimum substrate concentration for maximum reaction rate was shifted to 0.5% instead of 1% for untreated cellulose. To ball-milled MC50, there was slight effect on DP with a considerable decrease in crystallinity, approximately 65% (Figures 35, 36 and Table 16). Some authors (Caulfield and Moore, 1974; Bhama Iyer et al. (1984)

suggest that the effect of particle size tends to mask the effect of varying crystallinity, especially since amorphous character and decreased particle size are often linked. The overall increase in the maximum saccharification rate of those pretreated substrates is clearly a result of decreased particle size and increased available surface area rather than a result of reduced crystallinity. There is no apparent correlation between crystallinity of the substrate and the pattern of substrate inhibition.

5.3.5. Acid-treated and enzyme-modified Solka-Floc BW200

Substrate-activity profile of *Trichoderma reesei* cellulase acting on powdered cellulose, Solka-Floc BW200 and its pretreated substrates, was presented in Figure 29. No substrate inhibition were observed when Solka-Floc BW200 or enzyme-pretreated Solka-Floc was used as a substrate. Substrate inhibition behavior was found when acid-treated Solka-Floc was employed as a starting material. The crystallinity of each of the Solka-Floc substrates was similar but the DPs were significantly different (Table 16). In enzyme-pretreated Solka-Floc , the DP is approximately 10% less than untreated Solka-Floc, but the average length of acid-treated Solka-Floc was 90% less than that of untreated Solka-Floc. It seems the data demonstrated here suggested that the DP of substrate itself play a critical role on substrate inhibition. It is reasonable to infer that some pretreated substrates do not show substrate inhibition because of changes in particle size, resulting in insufficient surface area to exhibit substrate inhibition. The particle size of acid-treated Solka-Floc was relatively larger than that of the untreated substrate (Figure 41), then the particle size effect may be critical by evidence of this study. A fringed fibrillar model presented in Figure 2A may explain the approximately 90% decrease in DP with only a 20% conversion in acid-treated Solka-Floc BW200. Because of the amorphous region are evenly distributed in middle of microfibrillar cellulose chain and are

hydrolysis by acid. Again, 40% conversion with 10% DP decrease of enzyme-treated Solka-Floc BW200 indicated that a "single chain-multiple attack" is a possible mechanism when cellulase enzyme acting on particular cellulose as mentioned in section 5.3.3.

5.3.6. Cotton cellulose

It is necessary to evaluate the substrate-activity profile of *T. reesei* by investigating another source of cellulose, and the result was shown in Figure 30. As we mentioned in chapter 4, the substrate inhibition were found in some other cellulosic materials (Van Dyke, 1972; Okazaki et al. 1978; Contreras et al. 1982). This data provided another evidence and indicated that the substrate inhibition can be observed in different cellulose substrate with various magnitude of substrate inhibition, 72% for softwood cellulose (Avicel PH101) and 64% for cotton cellulose (Whatman CF1). The two-fold higher reaction rate in softwood cellulose than that of cotton cellulose may be related to the larger particle size, resulting in less surface area for the cotton cellulose. The crystallinity index of both substrates shown in Figure 30 are similar (Table 16). Klyosov (1986) reported that adsorption capacity of endoglucanase appears to play a critical role on the difference in the reactivity of the crystalline and amorphous cellulose. Hoshino and his coworkers (1992) suggested that maximum amount of adsorbed enzyme obtained from the Langmuir plots showed an inverse correlation to the crystallinity of the substrate with endo- and exo-cellulases from *Iprex lacteus*.

5.3.7. Solka-Floc BW200, Celufil and α–cellulose

Longer DP celluloses (~700) were selected to test their substrate inhibition (Figure 31). Crystallinity and DP of these cellulose substrates are tabulated in Table 16. Clearly, all these longer DP substrates exhibit no substrate inhibition, although they show

different maximum reaction rates. At this point, I believe that substrate inhibition properties may attributed to the short DP of cellulose substrate.

5.3.8. Dewaxed cotton fiber and filter paper

Substrate-activity profile of dewaxed cotton fiber and filter paper were examined with respect to their basic and applied aspects and the results are illustrated in Figure 32. The crystallinity of filter paper and dewaxed cotton are approximately 70% (Wood, 1988) and the DP values, approximately 7.5 to 9,5-fold longer than microcrystalline cellulose Avicel PH101 (Table 16). Since the DP of the substrate is a critical determinant for substrate inhibition properties, no substrate inhibition was observed on both longer substrates.

5.3.9. Highly amorphous cellulose

A substrate-activity profile of microcrystalline and amorphous cellulose was demonstrated in Figure 33. No significant DP change in the amorphous cellulose preparation (Table 16), provides an advantage and allows us to compare the difference between the crystalline and amorphous cellulose (Figures 35 and 37). In contrast to microcrystalline cellulose, no apparent substrate inhibition was observed when amorphous cellulose was used as a substrate. The saccharification rate of this regenerated cellulose obeyed the normal kinetics. The reaction rate levels off when a relatively high substrate concentration is employed. The different reaction rates on these two substrates can be attributed to structural changes (from crystalline to amorphous form), surface area increase, pore size/volume increase and enzyme adsorption increase (Klyosov et al. 1986). As a consequence, the reaction rate of amorphous cellulose is much faster than that of microcrystalline cellulose.

The more interesting question was brought up in this paper is why the substrate inhibition occurs in short DP substrate instead of long DP substrate. The data presented here demonstrates that there is only one parameter, that is the DP value, which different significantly between microcrystalline cellulose, Avicel PH101, and powdered cellulose, Solka-Floc BW200 ((Figures 27, 29 and Table 16). A putative mechanism was proposed for interpretation of substrate inhibition (Figure 34). The rationale of substrate inhibition behavior in cellulase/cellulose system was detailed described in chapter 4. DP change in the course of enzymatic hydrolysis is dependent on different fraction of enzyme, various source of enzyme and the initial DP of starting material (Sinitsyn et al. 1989). Theoretically, based on the random-attack mechanism, the possibility of dramatic DP changes should be found in longer DP cellulose substrate. Surprisingly, Sinitsyn's data demonstrated that 4% of overall hydrolysis of the long DP substrate (DP=1,600; Ball-milled cotton cellulose), only decrease DP 12.5%, while approximate 33% DP decrease in short DP substrate (DP=150; hydrocellulose). Their results also implied that a "single chain-multiple attack" action occurs in long DP cellulose substrate but a "multiple chain-single attack" behavior functions in short DP cellulose substrate.

Importantly, not only low DP but also highly crystalline structure are two synchronously required determinants for the substrate itself to govern the substrate inhibition. Absence of one of these required determinants exhibit no substrate inhibition (Table 17). Therefore, even though the low DP were observed in amorphous cellulose and hydrochloric acid regenerated cellulose, the structure of these two substrates has been changed and resulted in no substrate inhibition. It is reasonable to infer that physical properties changed after various pretreatments, the single enzyme-substrate complex (Figure 34) may have different reaction rates to generate the products. Consequently, no

substrate inhibition behavior will be expressed when amorphous or hydrochloric acid regenerated cellulose is used as a substrate.

Table 16. Summary of subtrate inhibition properties of various cellulosic substrates.

Substrate	Crystallinity Index Cellulose I	DP	Sub Inh	Opt [S]	% Inh.
Avicel PH101	89	219±3	+	1%	72
BM-Avicel PH101	22	194±5	+	0.5%	65
HCR-Avicel PH101 (solvent-dried)	40	214±3	-	-	-
Amorphous Avicel PH101	4	201±5	-	-	-
Enz-treated Avicel PH101	90	217±6	-	-	-
Avicel PH102	88	218±11	+	2%	62
Avicel PH105	86	200±5	+	0.5%	83
Solka-Floc BW200	81	703±20	-	-	-
Enz-modified Solka-Floc	87	634±8	-	-	-
Acid-Treated Solka-Floc	84	76±1	+	1%	68
Whatman CF1	94	246±2	+	1%	64
Dewaxed cotton	nd	2084±42	-	-	-
Filter Paper	nd	1641±19	-	-	-
Celufil	63	790±18	-	-	-
α–cellulose	77	650±11	-	-	-

nd: not determined
+: show substrate inhibition
-: no substrate inhibition

Table 17. Correlations between physical properties of cellulosic substrates and substrate inhibition.

Substrate	Crystallinity Index Cellulose I (>80)	DP(<250)	Substrate inhibition
Avicel PH101	Yes	Yes	Yes
Amorphous Avicel PH101	No	Yes	No
Solka-Floc BW200	Yes	No	No
Celufil	No	No	No

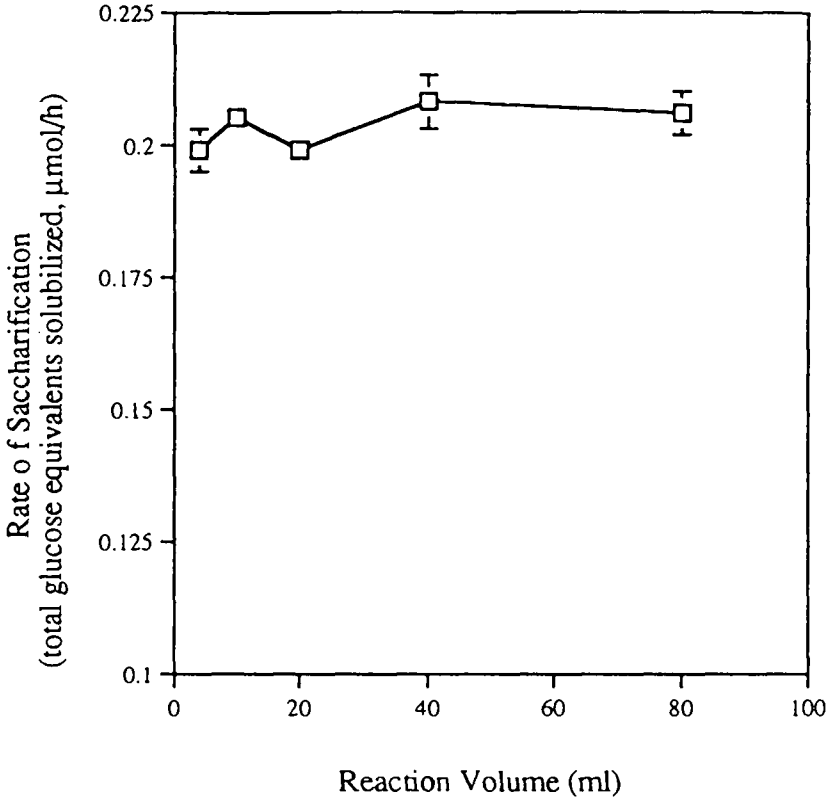


Figure 24. Volume effect on rate of microcrystalline celllulose saccharification (Avicel PH101). Experiment was performed in 50 mM sodium acetate, pH 5.0 at 50°C, 160 rpm agitation for 2hr, with a 0.016 IU enzyme load and 400 mg of substrate.

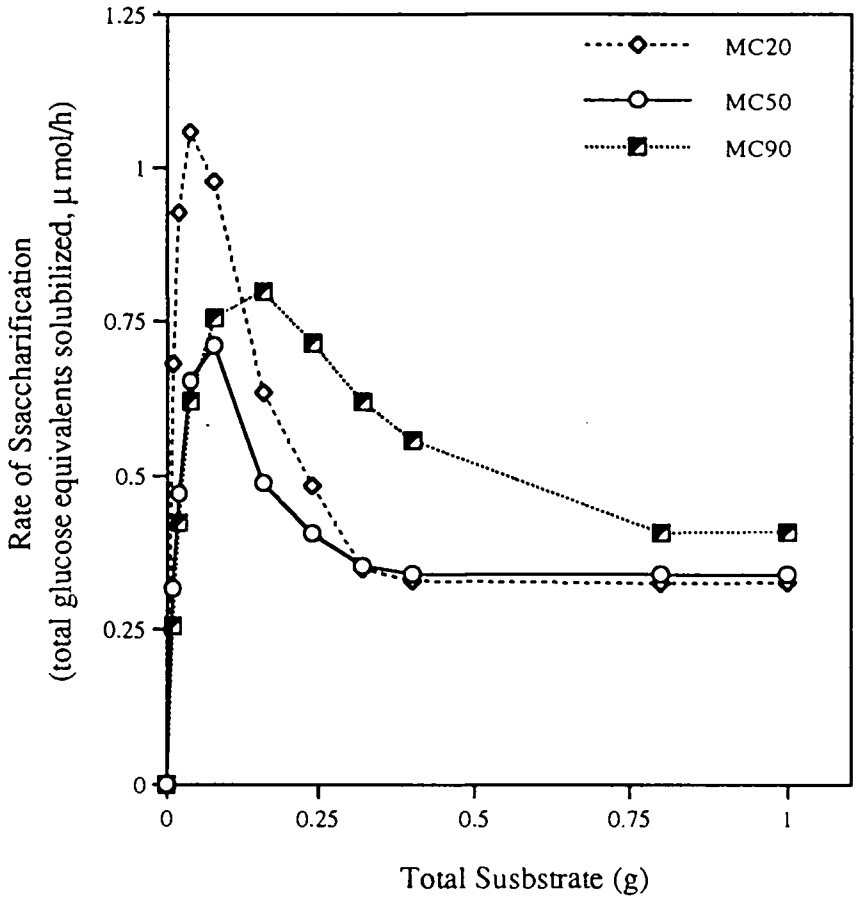


Figure 25. Effect of particle size on the rate of microcrystalline cellulose saccharification. The reaction was performed in 50 mM Na acetate buffer, pH5.0, 50°C, 160 rpm for 2hours. Enzyme loading was 0.032 IU and the substrate quantities were as indicated along the abscissa. Particle size of 20, 50, and 90 micron are designated as MC20, MC50 and MC90 respectively.

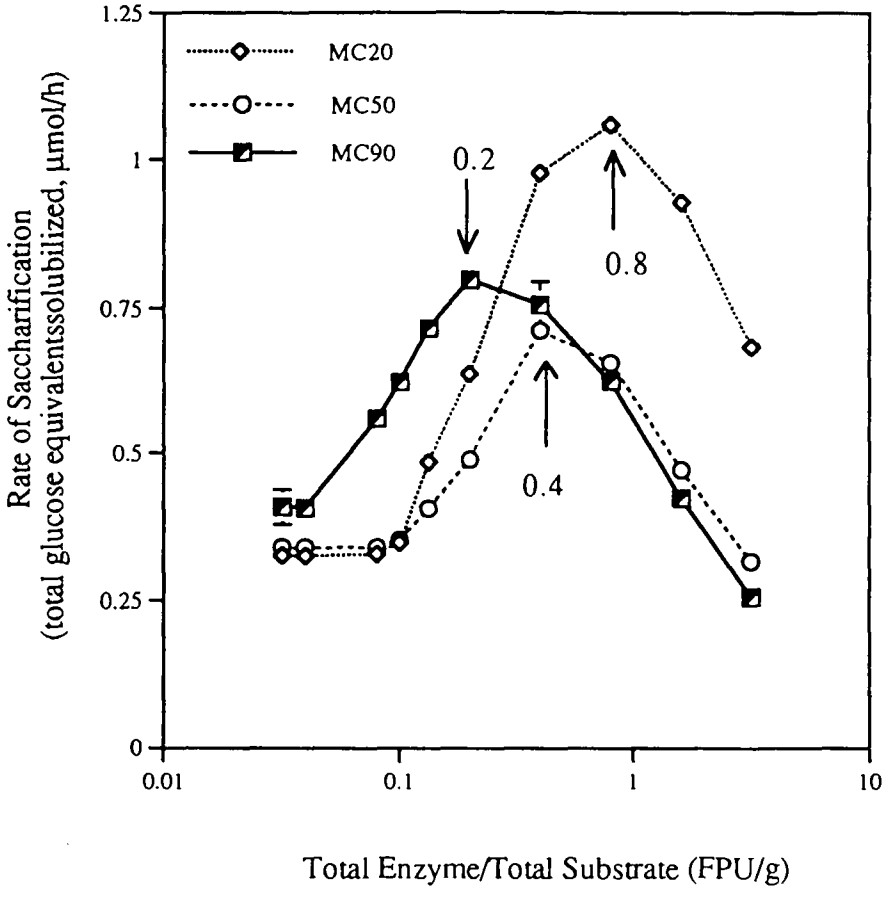


Figure 26. Effect of total enzyme to total substrate ratio on the rate of microcrystalline cellulose saccahrification. Reaction conditions, enzyme load, and substrate concentrations were as defined in Figure 25.

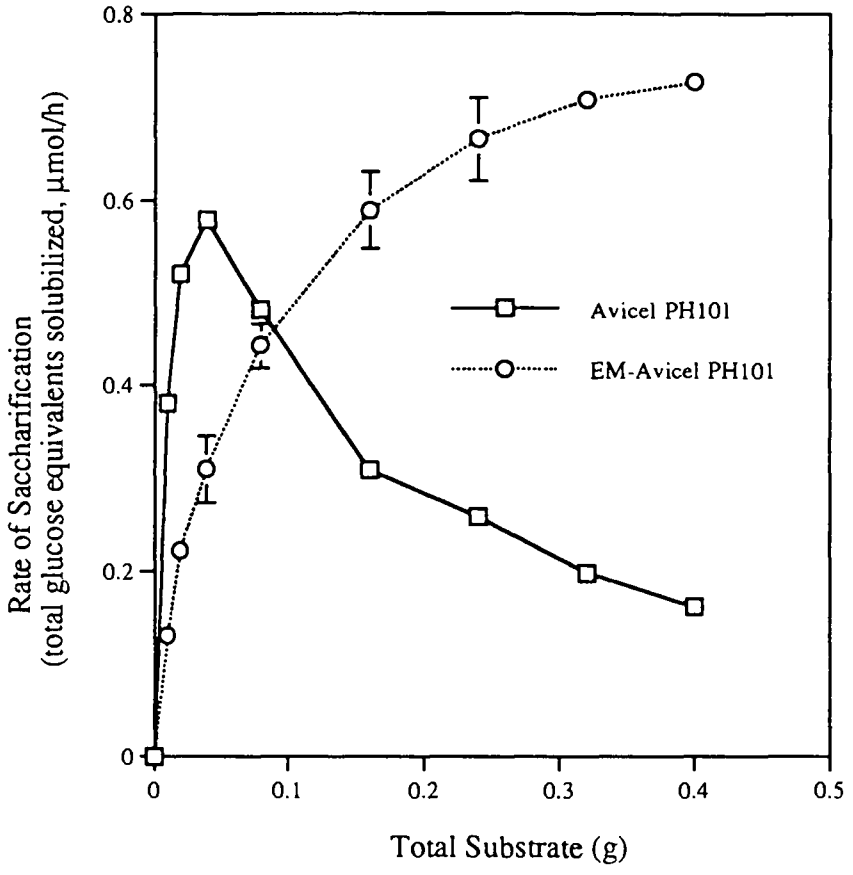


Figure 27. Rate of saccharification of *Trichoderma reesei* cellulase on microcrystalline cellulose and enzyme-modified microcrystalline cellulose. Reaction conditions were performed in 50 mM Na acetate, pH5.0, at 50°C, 160 rpm for 2h with various substrate quantities, ranging from 0.01 to 0.4 g. Enzyme load was 0.016 IU.

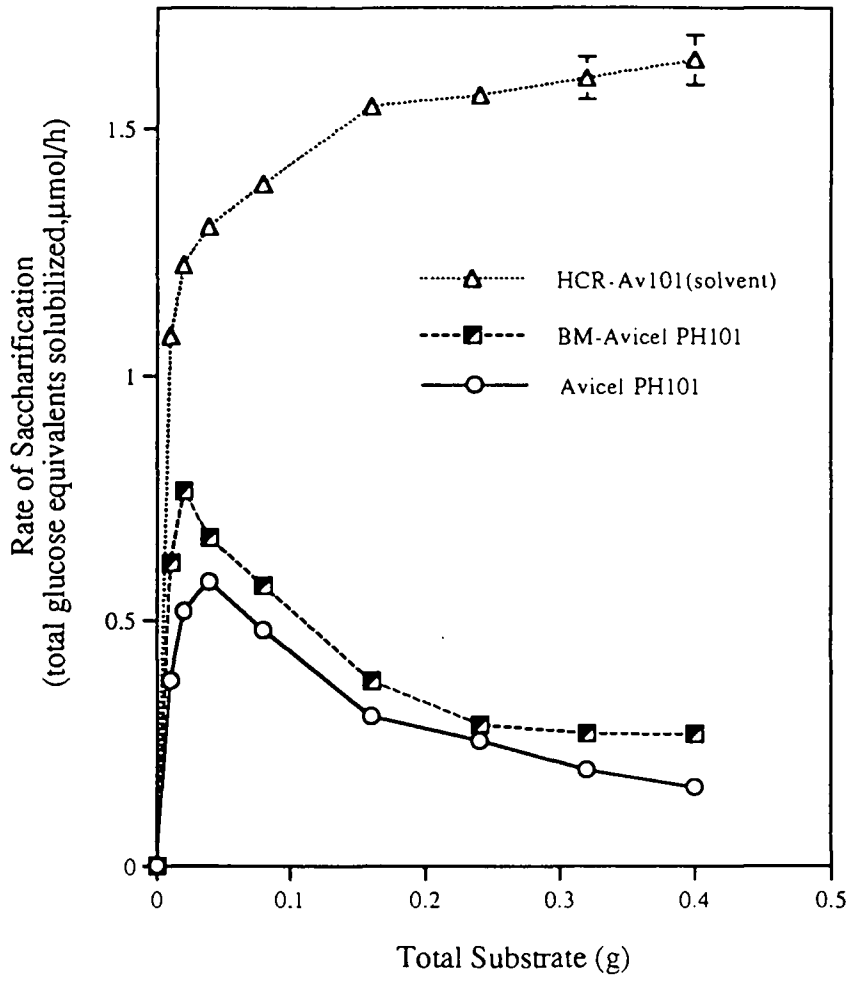


Figure 28. Rate of saccharification of *Trichoderma reesei* cellulase on microcrystalline cellulose, ball-milled cellulose and hydrochloric acid regenerated cellulose. Reaction conditions, enzyme loads and substrate quantities were as defined in Figure 27.

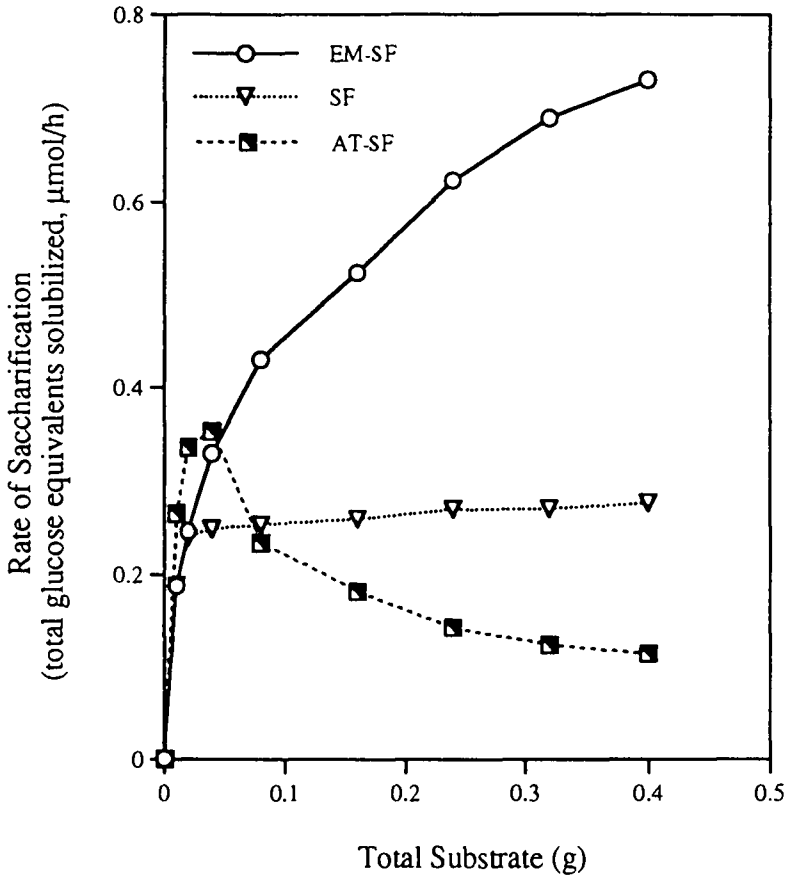


Figure 29. Rate of saccharification of *Trichoderma reesei* cellulase acting on powdered cellulose and pretreated powdered cellulose. Reaction conditions, enzyme loads, and substrate quantities were as defined in Figure 27.

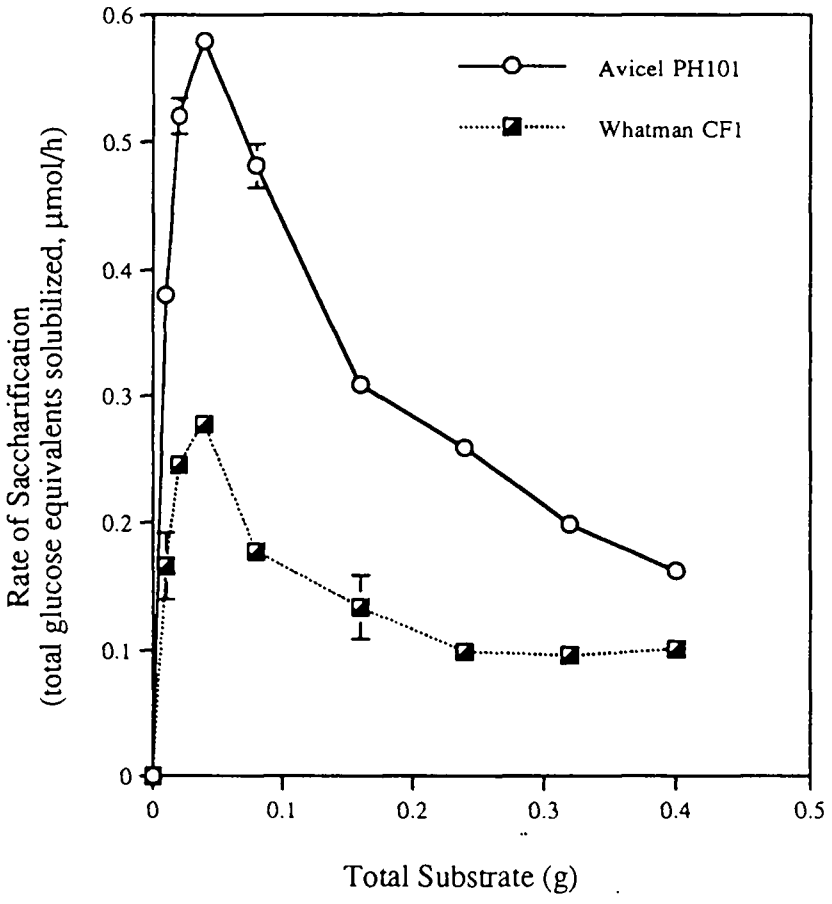


Figure 30. Substrate-activity profile of *Trichoderma reesei* cellulsase acting on mocrocrystalline cellulose from soft wood and cotton. Reaction conditions, enzyme loads, and substrate quantities were as defined in Figure 27.

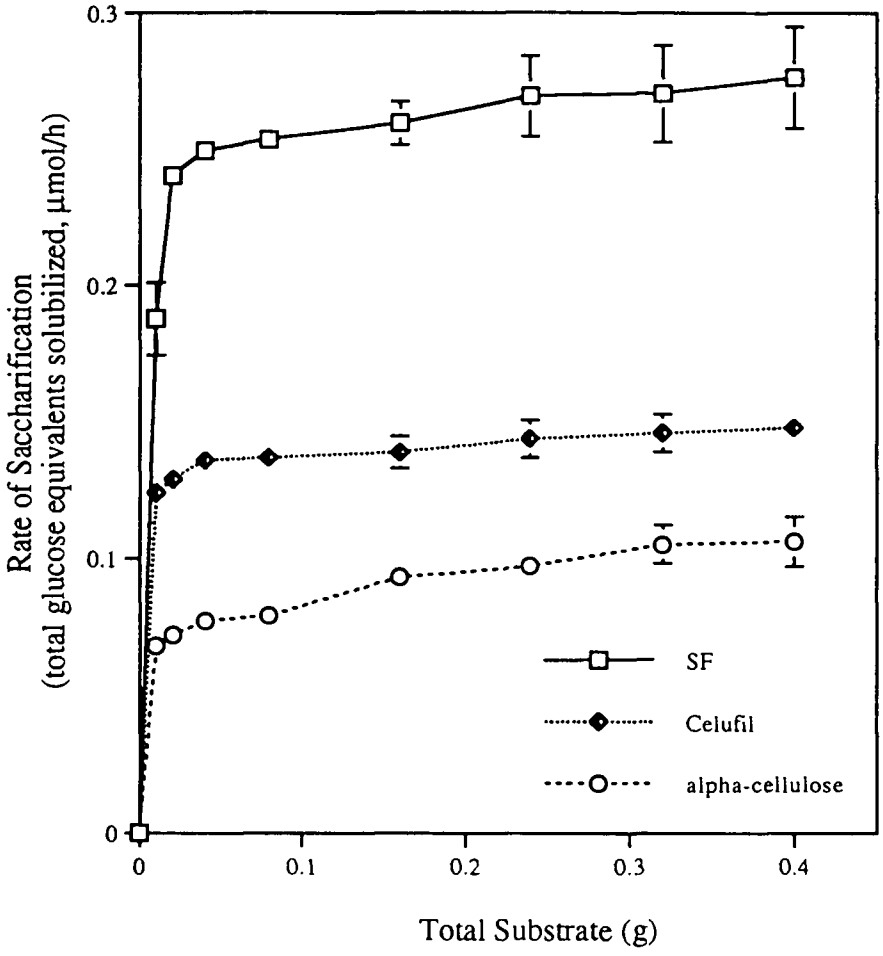


Figure 31. Substrate-activity profile of *Trichoderma reesei* cellulase acting on longer DP (~700) powdered cellulose . Reaction conditions, enzyme loads and substrate quantities were as defined in Figure 27.

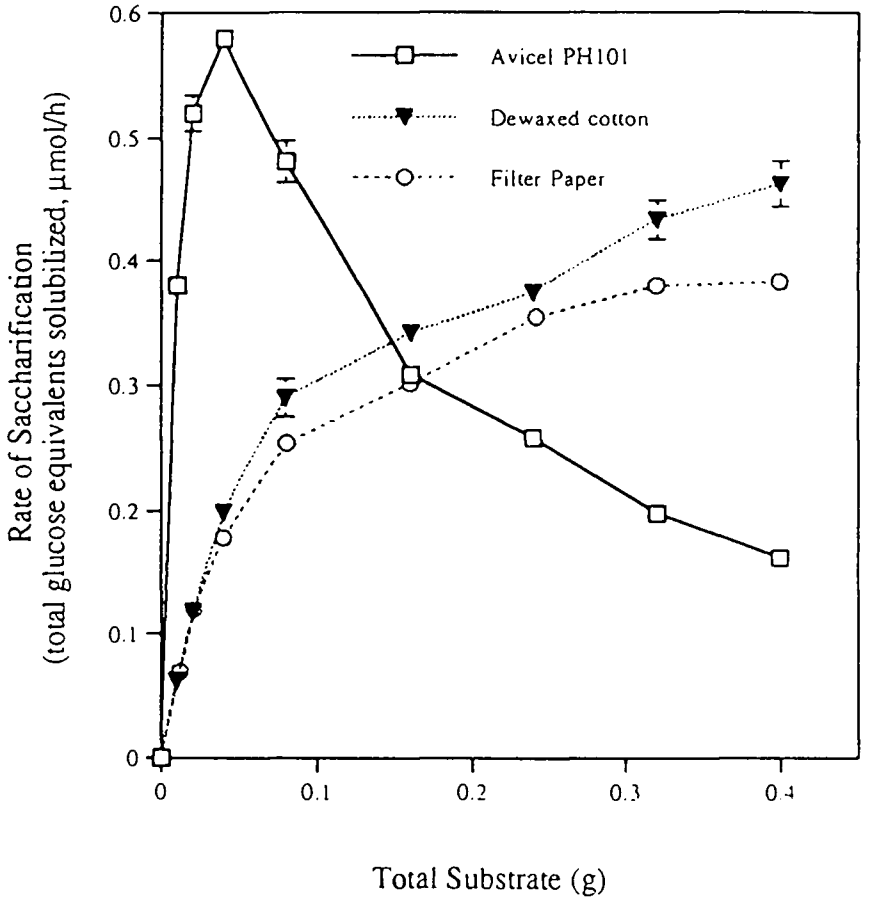


Figure 32. Substrate-activity profile of Trichoderma reesei cellulase acting on microcrystalline cellulose, dewaxed cotton and filter paper. Reaction conditions, enzyme loads and substrate quantities were as defined in Figure 27.

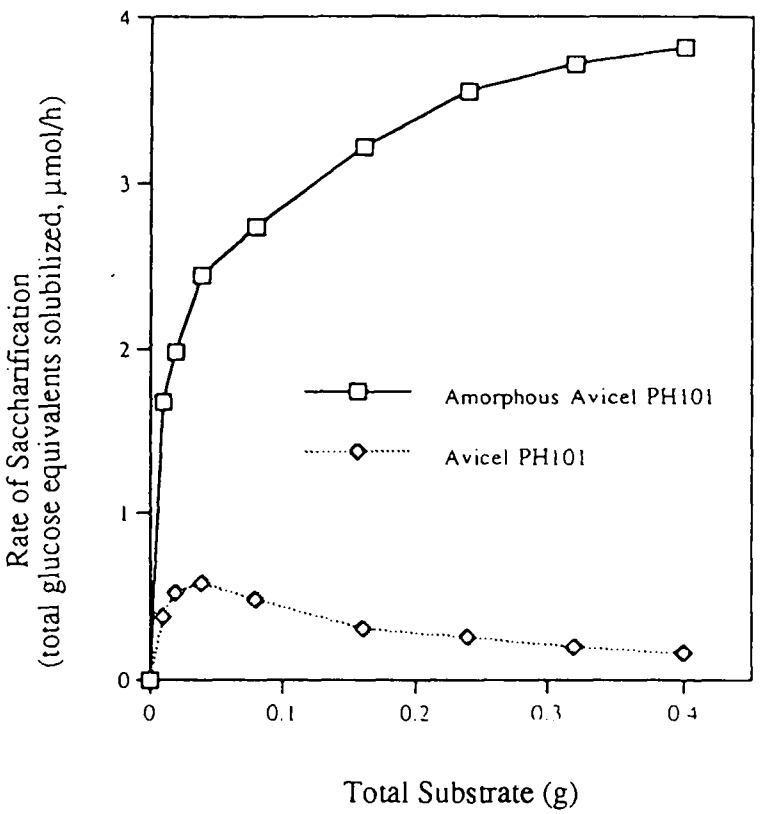
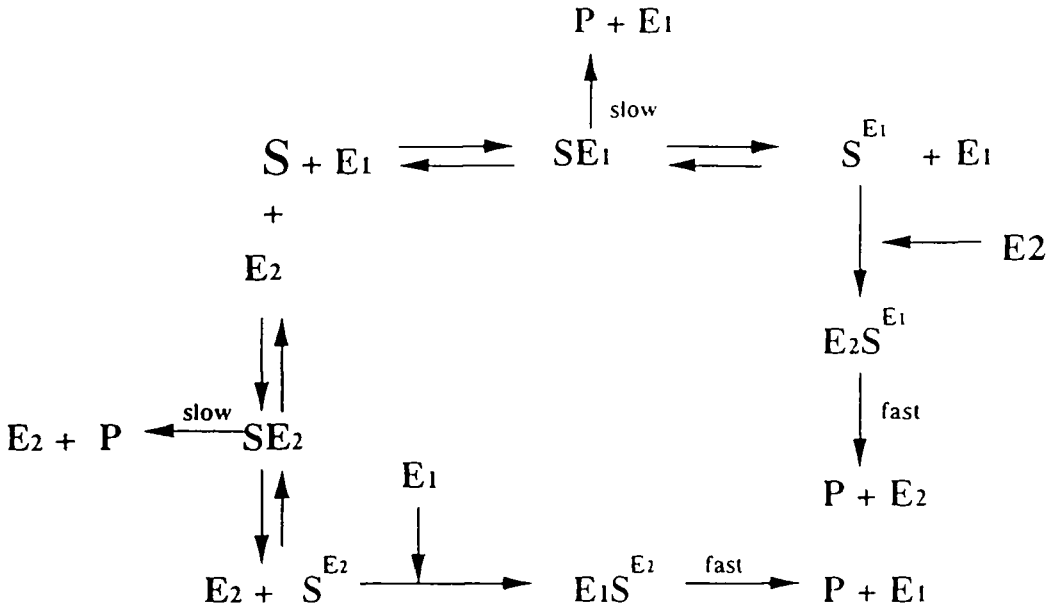


Figure 33. Substrate-activity profile of Trichoderma reesei cellulase acting on microcrystalline and amorphous celluloses. Reaction conditions, enzyme loads and substrate quantities were as defined in Figure 27.



S^{E1} : nonproductive intermediate which has been nicked by E1

S^{E2} : nonproductive intermediate which has been nicked by E2

E_2S^{E1} : productive intermediate which E2 reacting with nonproductive intermediate S^{E1}

E_1S^{E2} : productive intermediate which E1 reacting with nonproductive intermediate S^{E2}

Figure 34. Potential mechanism of substrate inhibition.

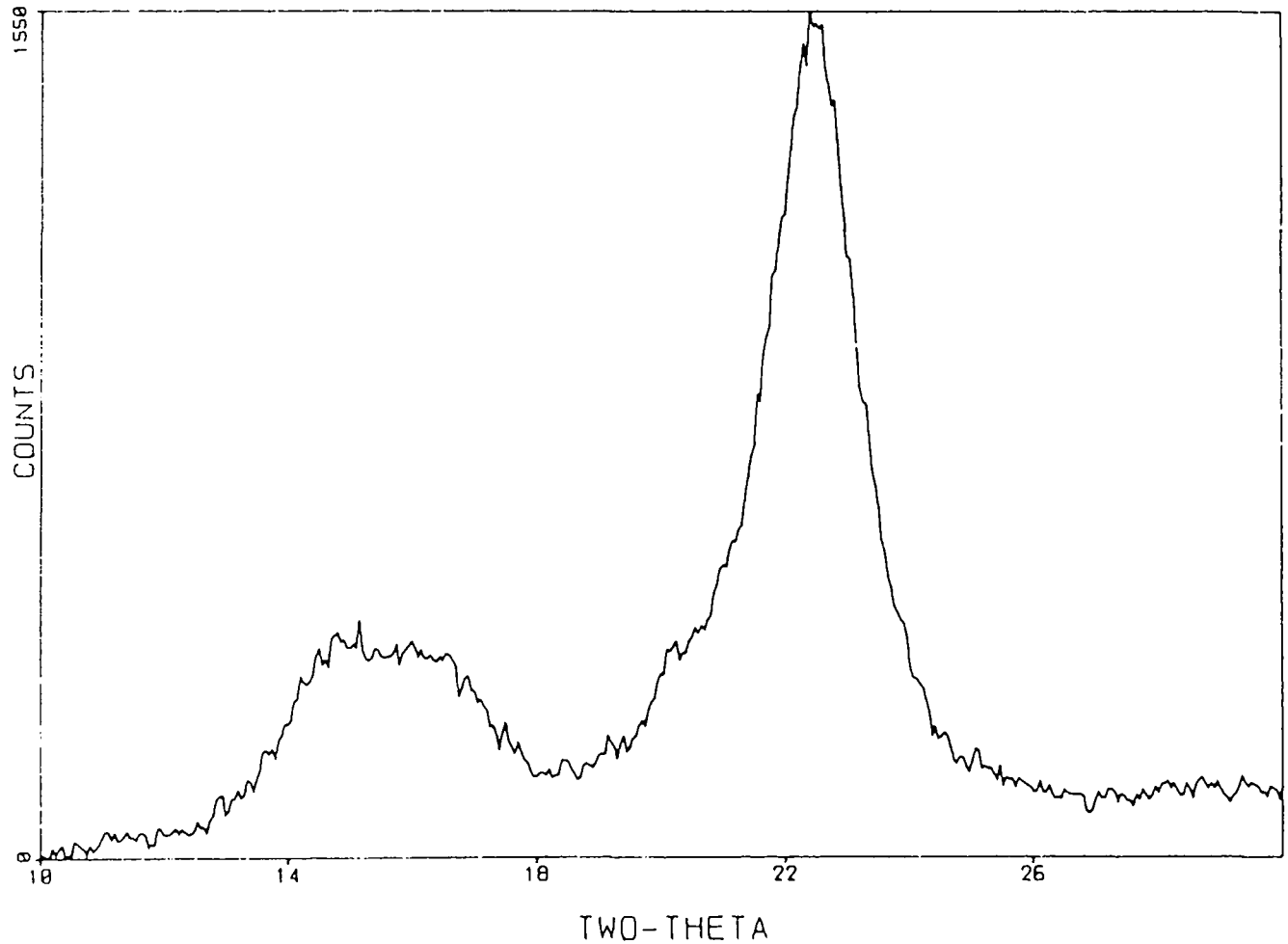


Figure 35. X-ray diffractogram of microcrystalline cellulose, Avicel PH101.

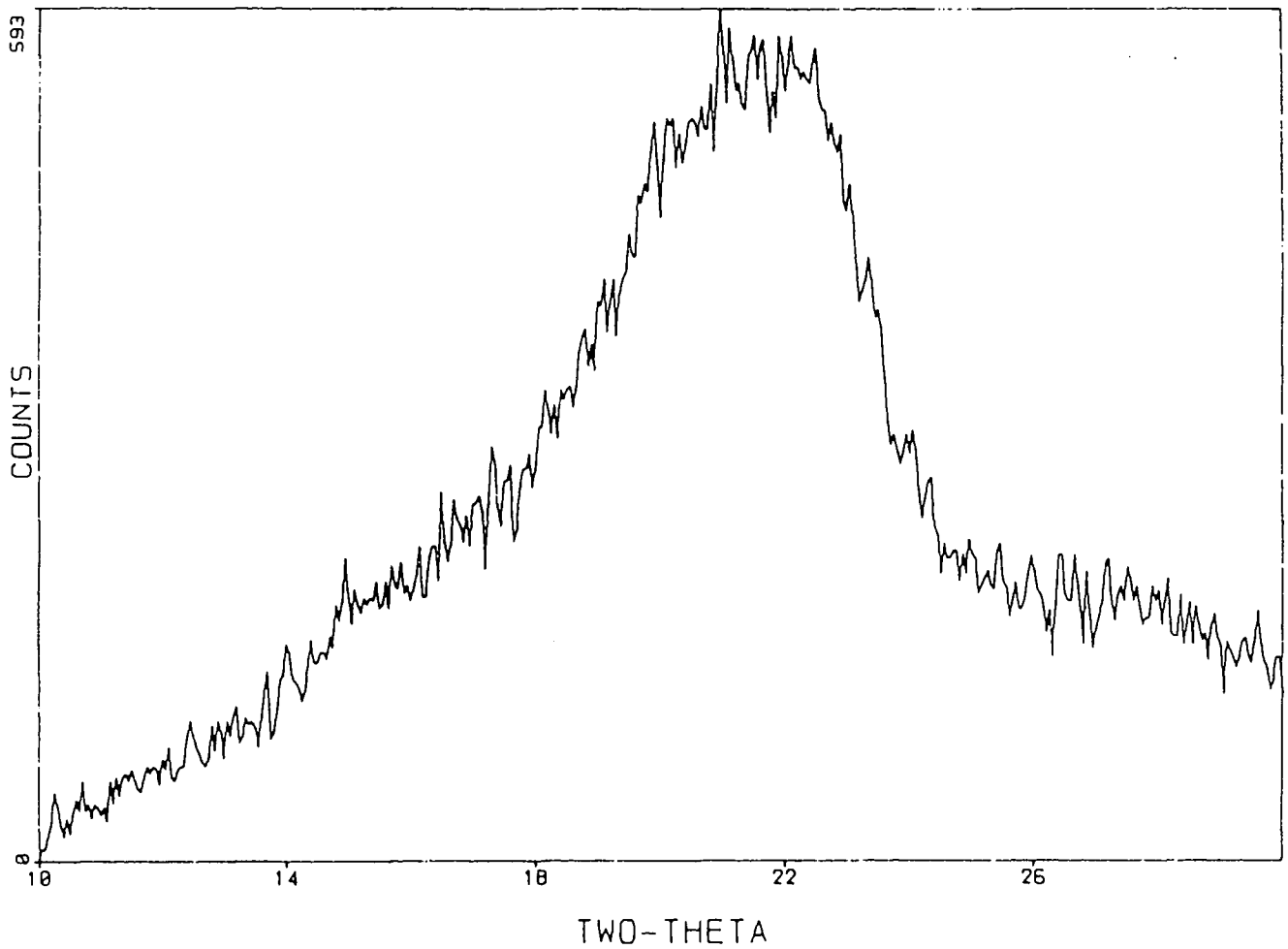


Figure 36. X-ray diffractogram of hydrochloric acid regenerated cellulose.

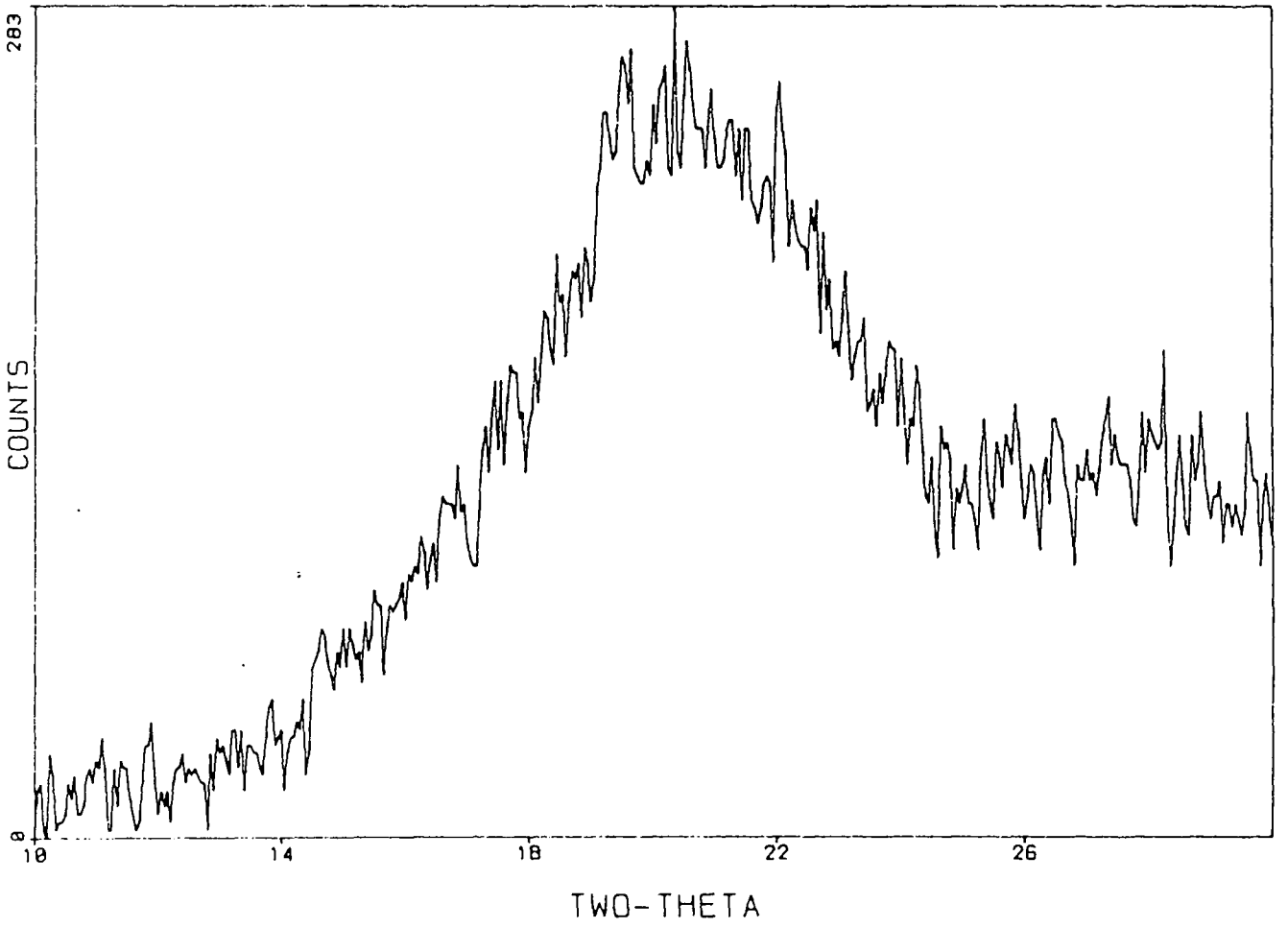


Figure 37. X-ray diffractogram of amorphous cellulose.

Figure 38. Electronically acquired image of microcrystalline cellulose.

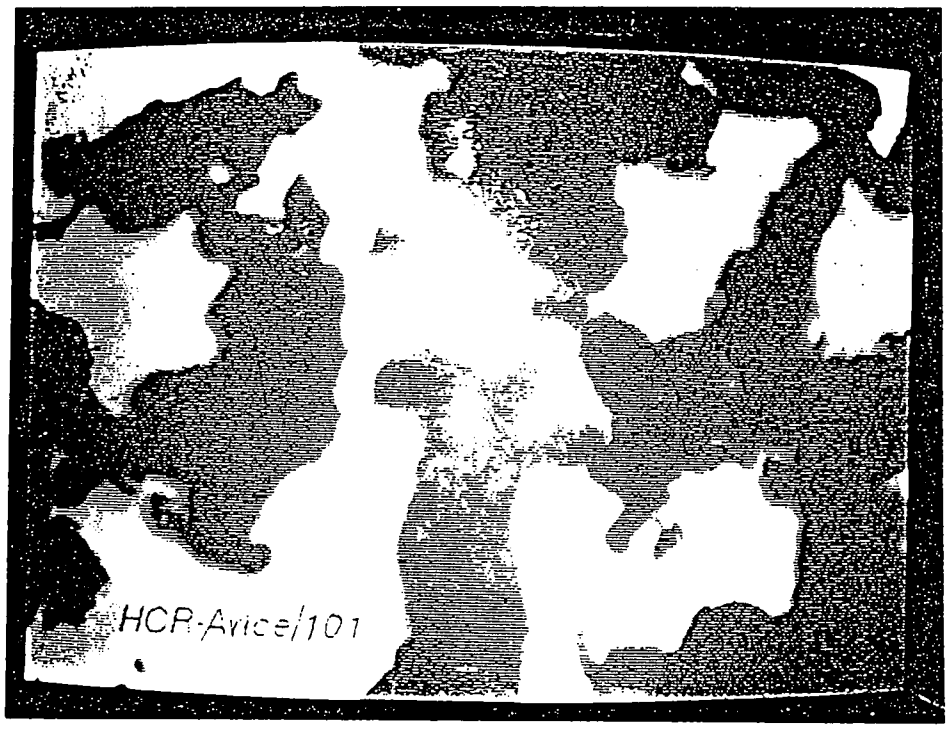
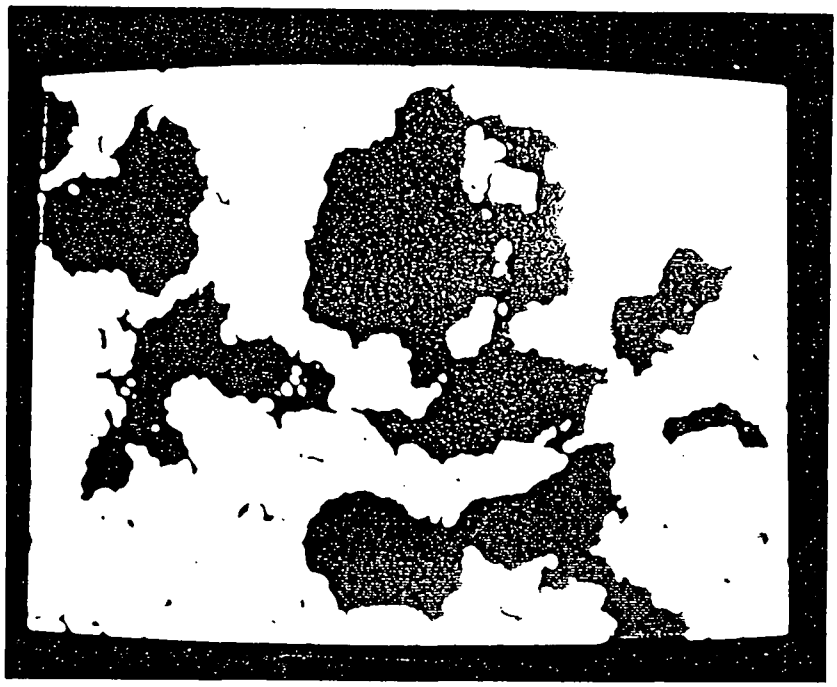


Figure 39. Electronically acquired image of hydrochloric acid regenerated cellulose.

Figure 40. Electronically acquired image of ball-milled cellulose.



Fibure 41. Electronically acquired image of acid-treated Solka-Floc BW200 cellulose.

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