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Title: The Cellulase System of *Trichoderma reesei* QM9414: A Study of its Apparent Substrate Inhibition

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The apparent substrate inhibition properties of the cellulase enzyme system from *Trichoderma reesei* QM9414 have been studied. Rates of saccharification were quantified by measuring solubilized sugars released from an insoluble, microcrystalline, cellulose substrate. The enzyme system does not obey classical saturation kinetics. Increasing substrate concentrations corresponded to increasing rates of solubilization of reducing sugar equivalents up to an optimum, above which

the rate appeared to decrease asymptotically. The optimum substrate concentration is directly proportional to the enzyme concentration used. In contrast to the complete cellulase system, a cellobiohydrolase (CBHI) purified from the complete cellulase mixture was found to obey saturation kinetics under equivalent assay conditions. The purified CBHI was found to exist in multiple forms but the predominant species has a molecular mass of 68 kDa, pI 4.2, and constitutes about 25% protein mass of the complete cellulase preparation. Addition of the CBHI isoenzyme to the complete enzyme system resulted in corresponding increases in the rate of saccharification without noticeably affecting the optimum substrate concentration for the reaction mixture.

The interrelationships of reaction product composition, cellobiase activity and apparent substrate inhibition have been determined. The reaction products were quantified by HPLC and reducing sugar methods. Supplementation of the native cellulase preparation with a purified cellobiase from *Aspergillus niger* results in similar substrate-activity profiles to that of the native cellulase preparation, both exhibiting an apparent substrate inhibition without affecting the optimum substrate concentration. Substrate-activity profiles based on the different reaction products, glucose and /or cellobiose or solubilized reducing sugar equivalents, all followed the same general curvature.

The Cellulase System of *Trichoderma reesei* QM9414: A Study of its Apparent Substrate Inhibition

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TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

THE CELLULASE SYSTEM OF *Trichoderma reesei* QM9414: A STUDY OF ITS APPARENT SUBSTRATE INHIBITION

INTRODUCTION

Cellulose is an abundant carbohydrate source. It has been estimated that there are $4x10^{10}$ metric tons fixed annually (Coughlan, 1985). The economic utilization of this vast renewable resource is of great interest due to increasing demands for expanded energy and food supplies. In this context the enzymatic hydrolysis of cellulose by microbial cellulases has been studied extensively. *Trichoderma reesei* cellulases have been the focus of a large percentage of these studies due to its relatively high activity on crystalline cellulose and, consequently, its great economic potential.

The cellulase system from *Trichoderma reesei* is a complex enzyme mixture consisting of up to 10 cellulase species which have been purified and characterized (Beldman et al. 1985). Each of these enzymes belongs to one of three general classes of cellulolytic enzymes; (1) 1,4-β-D-glucan glucanohydrolase (endoglucanase or EG; EC 3.2.1.4), (2) 1,4-β-D-glucan cellobiohydrolase

(exoglucanase or CBH; EC 3.2.1.91) or (3) β-glucosidase (cellobiase; EC 3.2.1.21). Enzymes from each of these general groups act cooperatively to hydrolyze crystalline cellulose.

The molecular events which dictate the observed rates of cellulose saccharification in the presence of cellulolytic enzyme systems are not known in spite of the large number of studies that have been conducted on the kinetic and structural properties of fungal cellulases. One kinetic property which has received little attention and yet is known to be applicable to cellulose saccharification is substrate inhibition. Substrate inhibition of *Trichoderma* cellulase catalyzed cellulose saccharification has been reported by several laboratories (Howell and Struck, 1975; Van Dyke, 1972, Okazaki and Moo-Young, 1978). However, the mechanistic basis of this behavior is not understood. An understanding of the molecular basis of the observed substrate inhibition would undoubtedly aid our understanding of the hydrolytic process in general. The substrate inhibition porperties of this enzyme system are not only of academic interest, but are also likely to be relevant to the design of efficient enzyme based reactors for cellulose saccharification.

The principal aim of the studies presented in this thesis is to advance our understanding of the apparent substrate inhibition of *T. reesei* cellulases. The thesis begins with a literature review covering the cellulose substrate, the nature of the cellulolytic enzymes and properties associated with the enzymatic hydrolysis of cellulose. The experimental section of the thesis follows the

literature review and it is divided into three chapters. The first chapter summarizes a study of the primary component of the *T. reesei* enzyme system, CBHI. The enzyme was shown to exist in multiple forms which differed in selected physical or kinetic porperties. The second chapter summarizes a study of the substrate-activity properties of the complete enzyme system and evaluates the role of CBHI in the observed substrate inhibition. The final chapter of the experimental section summarizes a study in which correlations between the observed substrate inhibition, reaction mixture product profiles and total reaction mixture cellobiase activities were investigated.

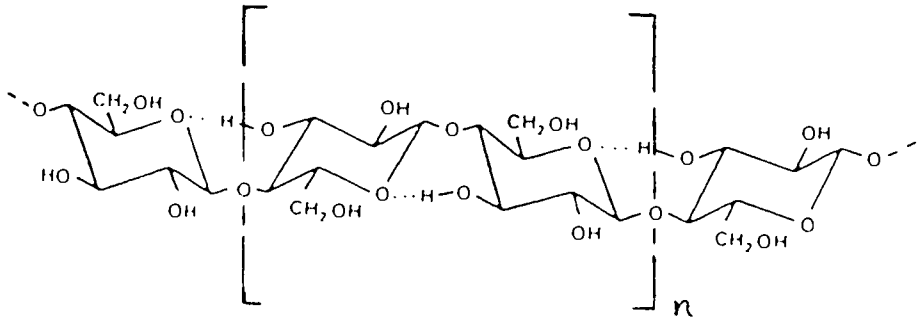
PART I. LITERATURE REVIEW

CHAPTER I. CELLULOSE SUBSTRATE

Cellulose

Cellulose is a long-chain water-insoluble linear polymer of pure anhydroglucose linked by 1,4-β-glucosidic bonds with the basic repeating unit of cellobiose (Fig 1). The average molecular length ranges of cellulose from plant source from 1,000 to 10,000 glucose units (Chang et al. 1980). Naturally, the individual cellulose chains form microfibrils through inter- and intramolecular hydrogen bonding in which they arrange to form two distinct types of structural components: a highly ordered, crystalline region and a less-ordered, amorphous region. 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 utilization of the cellulosic material by enzymatic hydrolysis.

Two models have been proposed to describe the cellulose structure: 1) the fringed fibrillar model (Fig 2a; Scallan, 1971); and 2) the folding chain model (Fig. 2b; Chang, 1971). In the fringed fibrillar model, the cellulose molecules in the basic fibril are fully extended with the molecular direction in the line with the fibril axis. The crystalline regions and amorphous regions are formed naturally



CELLULOSE CHAIN:

D-glucose units are in $\beta(1-4)$ linkage

Fig. 1. Cellobiose unit of cellulose.

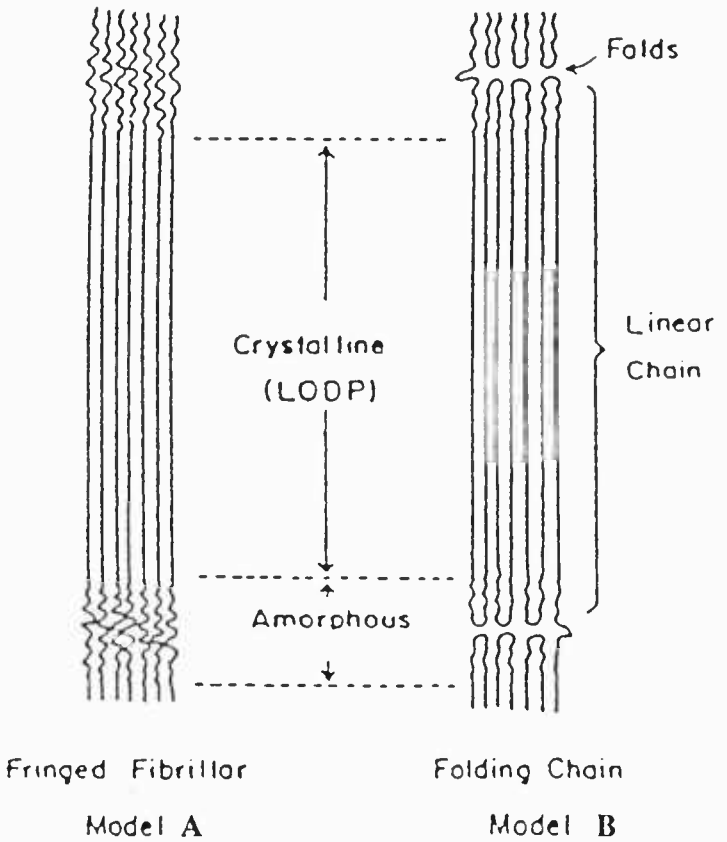


Fig. 2. Cellulose structure models.

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. The corresponding portions of molecule connecting two platellites are a single stranded chains and hang loose from the crystalline structure. For the detail information about the structure of cellulose, refer to the excellent reviews of Chang et al. (1980), Atalla (1982) and Marsden and Gray (1986).

Recently, it has been reported in Chemical & Engineering News (1985) that Colvin and his associates studied the structure of bacterial cellulose and cotton cellulose by scanning electron microscopy and transmission electron microscopy and the same morphology was observed from those two celluloses. They proposed that microfibrils are extensively cross-linked to form three-dimensional interconnected structures with coherent wholes rather than simply intertwined or superimposed.

In the cell wall of a higher plant, cellulose is associated with lignin -- a complex aromatic polymer based on phenylpropane units, and hemicellulose which is made up of branched short, heteropolymers of pentose and hexose, such as xylose, galactose, mannose, arabinose as well as uronic acids. The favored model is depicted in Fig. 3, in which all the cellulose chains are oriented in the same direction, lying parallel, bonded together to form a cellulose microfibril and then cross-linked with other saccharides and protein to form the cell wall of

higher plants. Cellulose is the major constituent, varied from 30-60%, in the lignocellulosic material. The ratio of cellulose, lignin and hemicellulose in lignocellulosic material varies with the materials from different sources. These components form a complex structure in which lignin and hemicellulose serve to protect the cellulose from breakdown by either chemical or biological agents. Fan et al. (1980) concluded that the resistance of lignocellulosic material to enzymatic attack can be attributed to three major factors: 1) cellulose in lignocellulosic material possesses highly resistant crystalline structure; 2) lignin surrounding cellulose forms a physical barrier; 3) sites available for enzymatic attack are limited due to the insolublity of cellulose. Thus, for efficient hydrolysis of cellulose by cellulases, it is necessary to remove lignin and hemicellulose from the lignocellulose material as well as to reduce the crystallinity of the cellulose.

Pretreatment of cellulosic material has been commonly employed to increase the cellulosic material suscepbility. The methods of pretreatment can be divided into physical methods and chemical methods, depending on their modes of action on the substrate. Generally speaking, the physical pretreatments are more applicable to cellulosic substrates while the chemical pretreatments have a great effect on lignocellulosic materials (Focher et al. 1991). The physical methods include dry milling, wet milling, high-pressure steam treatment and radiation. The chemical pretreatment can be classified into several methods, mainly depending on the solvents used. The common used solvents are HCl, H_2SO_4, H_3PO_4, NaOH, NH_4OH etc.

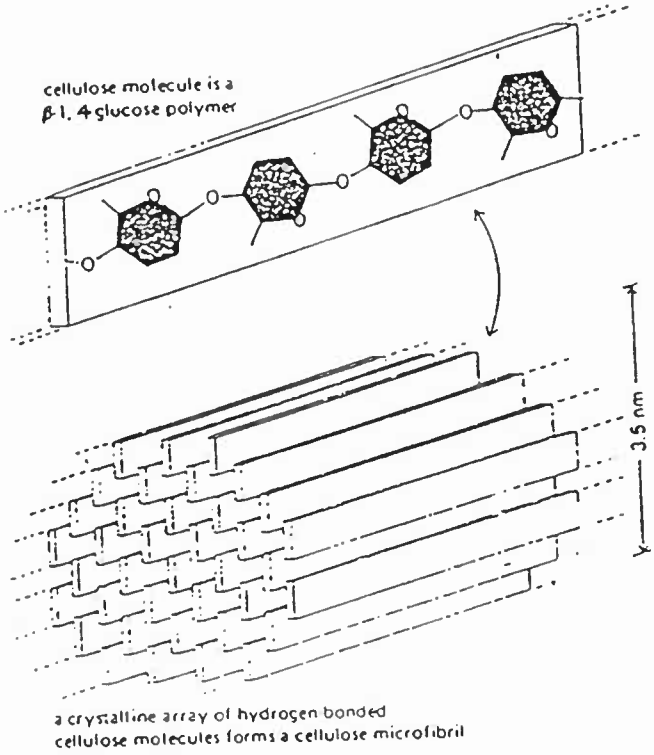


Fig. 3. The structure of cellulose microfibril (From Chang et al. 1980).

The structural properties of cellulose are among the most important factors governing their enzymatic hydrolysis. Although different pretreatment is used, the general purpose is to enhance the susceptibility of cellulose to enzymatic action by the means of: 1) reduction in crystallinity, 2) reduction in lignin content, 3) alteration of cellulose conformation, 4) increase in surface area, and 5) combinations of the above listed. It has been found that any means that increases the amorphous content will enhance the hydrolysis rate. Several reviews in this subject are available (Chang et al. 1980; Fan et al. 1981a and 1981b; Marsden and Gray 1986; Wood and Saddler 1988; and Focher et al. 1991).

Properties of substrate related to enzymatic hydrolysis

Factors related to enzymatic hydrolysis of cellulose are the following:

1. Degree of crystallinity. The crystallinity of cellulose is an important factor related to the susceptibility of cellulose to enzymatic hydrolysis (Fan et al. 1980). There is an inverse relationship between the rate of hydrolysis of cellulose and its crystallinity index (Ryu and Lee, 1986). The degree of crystallinity of cellulose is usually indicated by crystallinity index (CrI), based on X-ray diffractogram (Segal et al. 1959), that is:

$$CrI=(I_{002}-I_{am})x100/I_{002}$$

where I_{002} is the intensity of the 002 peak (at about $2\theta=22^{\circ}$) which corresponds to the crystalline fraction and I_{am} is the intensity at $2\theta=18^{\circ}$ which corresponds to the amorphous fraction.

The crystalline polymorphs of cellulose have generally been distinguished on the basis of their X-ray patterns. Four types of celluloses have been designated. 1) cellulose I. The cellulose is isolated in states approximating native cellulose form; 2) cellulose II. The cellulose has been regenerated from caustic solutions (mercerization) at ambient temperature; 3) cellulose III. The cellulose is produced by treatment in anhydrous ammonia or one of a variety of amines; 4) cellulose IV. The cellulose is regenerated from solution at an elevated temperature.

2. Specific surface area. There is a linear relationship between the initial reaction rate and the surface area (Stone et al., 1969; Fan et al., 1980). The surface area is important because direct physical contact between cellulase and the surface of cellulose is a prerequisite step for subsequent catalytic reaction. Specific surface area is measured using a sorptometer and applying the BET equation (Carberry 1976). Nitrogen is used as the adsorbate gas and helium as the carrier gas in the determination. The desorptions at different partial pressures are determined and a three-point BET graph is plotted. The specific surface area is calculated from the plot. It is important to bear in mind that this surface area measurement, based on nitrogen adsorption, gives an inflated value for the available surface area due to the relatively small size of the nitrogen molecule. Actually, enzyme molecules are much larger and hence inaccessible to the surfaces in small pores. Therefore, considering enzyme molecular shape and size, enzyme accessible surface area is more meaningful to indicate the properties of cellulose on the behavior of enzymatic cellulose hydrolysis. Alternatively, a

method for determining small-molecule accessibility of the substrate has been used to evaluate the enzyme accessibility to the substrate (Nickerson, 1951).

3. Degree of polymerization (DP). There is very limited information available on the relationship between the DP of the cellulose substrate and the hydrolysis rate catalyzed by the cellulase system. Puri (1984) observed that a reduction in DP influenced the rate and extent of enzymatic hydrolysis. It is reported that cellooligosaccharides are hydrolysed by cellobiohydrolases with increasing rate as the degree of polymerization increases (Eriksson and Wood, 1985). However, it was reported that DP does not influence either the rate or the yield of the hydrolysis reaction (Focher et al. 1991). Since only soluble substrates were used in these studies, the conclusions may not necessarily fit the case of insoluble substrates. Thus, developing a methodology to study the insoluble fraction of cellulose is needed. The common method for the measurement of the DP is provided by the American Society for Testing and Materials (1986). The DP of cellulose is estimated by multiplying its intrinsic viscosity in 0.5 M cupriethylenediamine by 190.

4. Cellulosic material composition. As mentioned in the previous section, lignin, hemincellulose, and cellulose form a complex structure which is very resistant to hydrolysis. In particular, the percentage of each component in cellulosic material may affect the rate of cellulose hydrolysis by cellulase. Therefore, a compositional analysis of cellulosic material will provide important information on the properties of the substrate. The methods for determination of

lignin, hemincellulose, cellulose content from plant source are availiable in the literature (Goering and Soest, 1971; Soest, 1973; Burtscher and Bobleter, 1987).

Other factors related to cellulose hydrolysis are water holding capacity (WHC) and degree of substitution (DS) for synthestic substrates. These factors are less studied and their roles in the cellulose hydrolysis are not fully understood.

All factors listed above would affect, more or less extendly, the hydrolysis rate of cellulose. However, it appears that CrI, surface area, and composition of the cellulose are considered as the most important factors governing the rate of cellulose hydrolysis (Fan et al. 1980; Lee et al. 1983). It should be pointed out, here, that a thorough knowledge of the structure of the substrate is vital for an understanding of the roles of enzymes involved in cellulose hydrolysis. However, accurate relationships between structural features and hydrolysis rate have not yet been found despite large numbers of studies focused on the subject. The difficulties in this area arise from the fact that it is virtually impossible to vary a single structural feature without affecting the others.

Commonly used substrate in cellulase study.

The substrates used in cellulase studies can be classed into two major groups: microcrystalline cellulose and amorphous cellulose. Microcrystalline cellulose is used to measure true cellulase activity and exoglucanase activity on native cellulose. The most commonly used crystalline celluloses are the following:

1. Dewaxed cotton cellulose. A natural microcrystalline cellulose with virtually pure cellulose, DP=10,000-15,000.

2. Avicel. A commercially available microcrystalline cellulose. For example, Avicel PH101 has a DP=219 , CrI=80, Cellulose=99.6% (Liaw and Penner, 1990). Avicel is made from wood pulp. After chopping, the small particle size of wood pulp is hydrolyzed with diluted acid under high temperature and pressure. Then, the cellulose is bleached, washed, and spray-dried as powder form. For more detail, refer to the review of Penner and Liaw (1990).

3. Solka Floc. A commercially available microcrystalline cellulose made from wood. Solka Floc BW200, for instance, has a DP=703, CrI=73, Cellulose=91.8% (Liaw and Penner, 1990).

4. Filter Paper (Whatman #1). Filter paper is prepared from cotton and is widely used to measure overall hydrolysing activity of cellulase on native cellulose.

Amorphous cellulose can be divided into water insoluble and water soluble celluloses. These substrates are offen used to measure the activities of endoglucanases and β-glucosidases. They are listed as the following:

1. Water insoluble amorphous cellulose. These substrates are prepared by treatment of native crystalline cellulose with acid (swollen) or ball-milling. These treatments disrupt the crystalline structure of cellulose and therefore make the cellulose more accessible to enzyme attack. These substrates are poorly defined and the properties are dependent on the conditions used.

2. Water soluble cellulose substrates. Soluble cellulose substrates are prepared by introducing either carboxymethyl or hydroxyethyl substituents into the cellulose chains. The examples are CMC (carboxymethyl cellulose) and HEC (hydroxyethyl cellulose). The solubility of the substrate is directly related to the degree of substitution (DS). The higher the DS, the more water soluble the cellulose will be. These substrates are widely used to estimate endoglucanase activity.

3. Cellodextrins. These substrates are prepared by extensive hydrolysis of cellulose with acids. The DP of the substrates are usually 2-6 glucose units. These substrates are very defined and often used to study the mechanism of the enzyme reaction. Cellobiose (DP=2) is widely used to determine cellobiase activity.

Other substrates used in cellulase study are: (1) dyed-cellulose, in which dye is bound to the cellulose. The amount of released dye in the solution is proportional to the cellulase activity (McCleary, 1980); and (2) p-nitrophenol glucoside (pNPG) is widely used to measure β-glucosidase activity.

It should be noted that most substrates used in cellulase studies are very poorly defined. Due to the poorly defined substrate, the substrate specificity of the individual cellulase appears to be very difficult to compare between laboratories. Therefore, the cellulase activity can only be compared by using exactly the same substrate under the same conditions.

Some studies have focused on the practical utilization of cellulosic materials catalyzed directly by cellulases. Those substrates are straw (Fan et al. 1981a),

sugar cane waste (Dekker and Wallis, 1983) and newspaper (Castanon and Wilke, 1980). It is obvious that these substrates are far more complex than that of pure cellulose and thus, these offer more challenges for cellulase study.

CHAPTER II CELLULASE

Cellulase system

Cellulase is a complex enzyme system. This cellulase system consists of a number of enzyme forms. Fagerstam and Pettersson (1979) and Farkas et al. (1982) found that at least 30 components are readily detectable from *Trichoderma reseei* QM 9414 cellulase by isoelectric focusing. Fractionation studies on culture filtrates of the fungi have demonstrated that there are three major classes of enzymes involved in hydrolysis of crystalline cellulose to glucose: (1) 1,4-β-glucan cellobiohydrolases (CBH) or exoglucanases (EC 3.2.1.91), which act on the cellulose from the non-reducing end of the cellulose chains and release cellobiose as the predominant product; (2) endo-1,4-β-D-glucanases or endoglucanases (EG) (EC 3.2.1.4), which randomly attack cellulose chains and produce new non-reducing ends from cellulose; and (3) 1,4-β-D-glucosidases or cellobiases (EC 3.2.1.21) which act on soluble oligosaccharides, particularly on cellobiose to produce glucose. It should be noted here that cellulase systems, derived from different microorganisms differ markedly in their ratio of these constituent enzymes (Coughlan and Ljungdahl, 1988). Furthermore, cellulases isolated from various sources differ in their molecular characteristics (molecular weight, amino acid composition and sequence, pI and carbohydrate content), adsorption properties on cellulose, catalytic activity, substrate specificity (Klyosov, 1990). A general cellulase action scheme is shown in Fig. 4. These enzymes act

synergistically in cellulose hydrolysis. This synergism is essential for the hydrolysis of microcrystalline cellulose.

Complexity of cellulase

As mentioned above, the cellulase system consists of multiple forms of enzymatic components and can be classified into three distinct classes. Furthermore, each of the distinct classes of cellulases usually exists in multiple forms. For example, five endoglucanases and three exoglucanases have been isolated from the *T. viride* cellulase system (Beldman et al., 1985) and three β-glucosidases have been isolated and purified from the *T. viride* cellulase system (Gong et al., 1977). Two immunological distinct CBHs, namely CBHI and CBHII have been found in the *T.reesei* cellulase system (Fagerstam and Pettersson, 1979). Despite numerous studies focusing on the isolation and purification of the cellulases, at the present time, it is still unknown how many cellulase components exist in a cellulase system.

The reason for the multiplicity of the cellulases has long been conjectured and debated. Several questions have arise: Is each of the individual forms genetically determined to have a separate function in cellulose hydrolysis? Do they arise from differential modification by proteolysis or glycosylation inside or outside the cell? Do they arise from artifacts in the extensive purification process? Wood(1981) suggests that from a stereochemistry point of view, at least two types of endoglucanases and exoglucanases should be required for cellulose hydrolysis

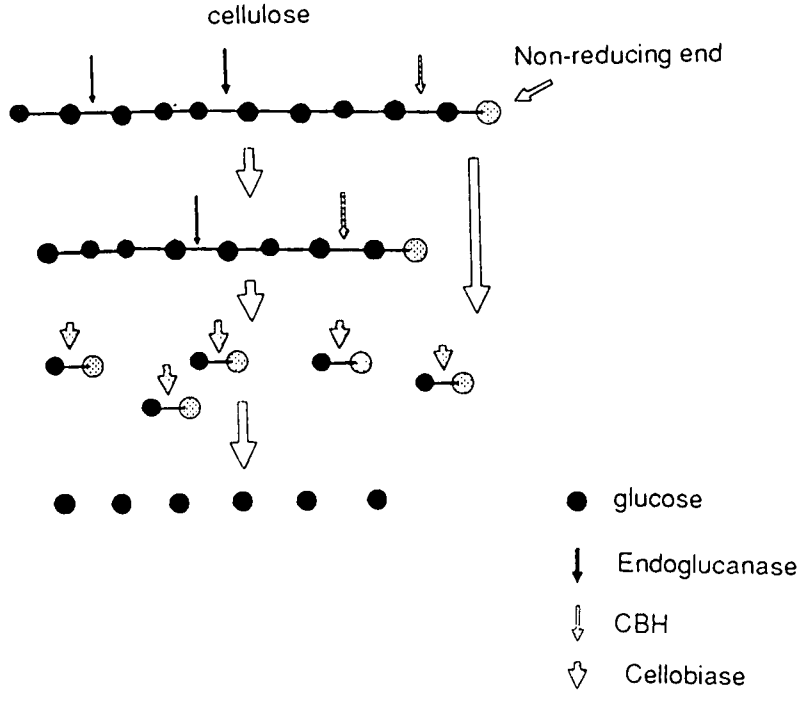


Fig. 4. Enzymatic hydrolysis of cellulose.

due to the stero-type structure of cellulose. This theory is consistent with the fact that two immunologically unrelated exoglucanases, CBHI and CBHII, have been isolated from a *T. reesei* cellulase preparation (Fagerstam and Pettersson, 1980), and no homology exists between the first 20 amino acid residues of these enzymes (Pettersson et al. 1981). This result suggestes that the information necessary for the synthesis of these forms would certainly seem to be encoded in different genes. This conjecture has been confirmed by the study of Knowles et al. (1987) who found that there are at least two genetically different CBHs (CBIII and CBHII) and two EGs (EGI and EGIII) based on the cDNA sequences.

The protease modification of cellulase has been considered as a causative factor in the multiple forms of cellulase. Proteases have been found in the cellulase preparations from *Trichoderma reesei* and *S. pulverulentum* (Sheir-Neiss and Montenecourt, 1984; Eriksson and Pettersson, 1982). Nakayama et al. (1976) found that treatment of an endoglucanase isolated from *T. viride* cellulase with a protease from the same source yielded a number of forms with minor alterations in substrate specifity. The protease activity seems to increase with the culture age. Recently, Hagspiel et al. (1989) reported that at late cultivation stages, CBHI and CBHII become partially modified to lower molecular weight components, whereas β-glucosidase and endoglucanase I appeared to remain largely intact. However, Dunne (1982) and Labudova and Farkas (1983) argue against proteolysis as the sole arbiter of multiplicity. They suggested that production of multiple forms of cellulase components of *Trichoderma reesei* may be an intrinsic property of the

fungus.

Enari and Niku-Paavola (1987) pointed out that the difference in cellulase properties might be due to the modification and inactivation caused by rigorous purification treatments and too many purification steps. Another possible cause may result from the different glycosylation of the cellulase (Gum and Brown, 1977; Moloney et al., 1985) since almost all cellulases are glycoproteins. It has been found that most cellulases contain carbohydrate content in the range of 1-10% (Enari, 1983). Some higher carbohydrate-containing cellulases have been reported (McHale and Coughlan, 1981). The amount of the carbohydrate moiety in the cellulase may be responsible for the differences in activity, pI value and molecular mass.

It is very interesting that different carbon sources in the cultural medium also affect the composition of the cellulase produced. Gritzali and Brown (1979) used sophorose to induce cellulase synthesis by *Trichoderma reesei* grown on dextrose. Only one endoglucanase and two exoglucanases were produced under these conditions. Only one cellobiohydrolase was produced, when *Trichoderma reesei* grew under "controlled broth condition", (Gong and Tsao, 1979). Saddler et al. (1986) listed several factors which influence cellulase production and hydrolytic efficiency. These include type of inoculum, nature and concentration of the substrate, composition of the media, pH and duration of incubation. It is also reported (Cochent, 1991) that culture conditions can influence the enzymatic profile. Cellulase synthesis appears to be regulated at the level of mRNA

transcription. It was found that the levels of *Trichoderma reesei* CBHI mRNA increased in the presence of Avicel or sophorose, and were repressed upon addition of glucose (El-Gogary et al., 1989).

In summary, the reason for the complexity of the cellulases is not fully understood. It is possible that the factors listed above are more or less responsible for the complexity.

Purification and characterization of cellulases

In order to understand the cellulase system, it is necessary to isolate and purify each enzyme component and study its kinetic and physicochemical properties in a simple system. Detailed study of the purified cellulase component will provide useful information in understanding the behavior of the enzyme in the complex system. Furthermore, this study will help us to understand the mechanism of the complex cellulase in cellulose hydrolysis.

So far, there are no standard protocols for the isolation and purification of an individual cellulase component from the cellulase complex though many attempts are being made. The similarity in physicochemical properties of several cellulase components causes the difficulty in the isolation and purification. Commonly employed methods in the isolation and purification of cellulase are the following: (1) conventional chromatography. This includes column chromatography (molecular exclusion, ion exchange) and preparative isoelectro focusing (Berghem and Pettersson, 1973; Beldman et al., 1985; Sheomaker et al., 1984). Normally,

repeated application of one or another of these methods is required to obtain a single cellulase protein species; (2) affinity chromatography. Examples of affinity matrices are Avicel for CBH (Gum and Brown, 1976; Halliwell and Griffin, 1978; Beldman et al., 1985), Concanavalin A for β-glucosidase (Woodward et al., 1986), p-aminobenzyl-1-thio-β-D-cellobioside coupled to Affigel 10 for CBH's (Fagerstam and Pettersson, 1979) and affinity chromatography (Van Tilbeurgh et al., 1984). (3) immunoaffinity chromatography. Monoclonal antibodies and immobilized antibodies have been used in the purification of CBH's (Riske et al., 1986; Schulein, 1988).

Various results on the properties of purified cellulase components have been reported in the literature. Some properties of selected purified endo's, CBH's, and β-glucosidases are listed in Tables 1, 2, 3, respectively. Clearly, these variations may reflect differences in purity of the enzymes, differences in substrate specifity or difference in accessibility of substrate. A general substrate specificity of cellulase enzyme components (Table 4) was summarized by Wood (1989).

Another reason for the various results is the diversity of cellulase assay methods used in different laboratories. This includes different reaction conditions (temperature, pH, and time, for example) and substrate used. This diversity of assay methods has been a considerable impediment to the meaningful quantitative comparison of results between laboratories. In an attempt to rectify the situation the Commission on Biotechnology (International Union of Pure and Applied

Chemistry or IUPAC) published a number of standard procedures for the measurement of cellulase activities (Ghose, 1987). These procedures are also collected in Methods in Enzymology (1988).

The complexity of both the cellulase enzyme system and cellulose substrate bring numerous problems to the charaterization of cellulase and even purified cellulase components. Generally, several methods have been used to distinguish the cellulase and/or cellulase components. (1) Physicochemical properties, such as molecular mass, pI value, optimum pH and temperature, amino acid composition, and amino acid sequence or gene sequence analysis; (2) Enzyme activity, including substrate specificity and product profiles; (3) Kinetic constants K_m, V_{max} and K_i with various substances; and (4) Immunogical properties. However, in one way or another, problems still remain in cellulase studies due to a lack of sophisticated methods at present time. One example is that cellulase components of *Trichoderma reesei,* apparently homogeneous by isoelectric focusing, could be separated into at least six proteins after treatment with urea-octyl glucoside (Sprey and Lambert, 1983).

Table 1. Some properties of selected CBHI and CBHII

source	Mr. (kDa)	pI	Sp. Activity[a] Avicel	CMC	pNPG	Ref
CBHI						
T. reesei	68	4.4	.09	n.d	0	1
T. reesei	65	3.8-4.0	--	--	--	2
T. reesei	--	3.9	.0175	.0099	--	3
T. reesei	64-68	4.05-4.25	--	.37	--	4
T. reesei	64	3.9	--	--	--	5
T. reesei	66	4.2	.04	<.01	<.01	6
T. reesei	65	3.6-4.12	--	.01	--	7
CBHII						
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) Shulein, 1988, 3) Tomme et al., 1988, 4) Riske et al., 1986, 5) Bhikabhai et al., 1984, 6) Shoemaker et al., 1983, 7) Nummi et al., 1983.

Table 2. Some properties of selected endoglucanases

Source	Mr(kDa)	pI	Avicel[a]	CMC[a]	ref
T. reesei					
Endo1	52	4.0-5.0	--	--	
Endo2	48	7.0	--	--	1
EndoII	55	4.5	--	--	
EndoIII	48	5.5	--	--	2
EGI	54	4.7	--	--	3
Endo	43	4.0	--	--	4
T. viride					
EGI	50	5.5	.013	13.1	
EGII	45	6.9	.007	20.1	
EGIII	58.5	6.5	.016	3.2	
EGIV	23.5	7.7	.003	9.6	
EGV	57	4.4	.007	14.7	
EGVI	52	3.5	.004	15.8	5

[a] specific activity. CMC, Carboxymethyl-cellulose, 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.

Table 3. Some properties of selected β-glucosidase

Source	state	K_m (mM) pNPG	G2	Mr. kDa[a]	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/GF	5.74	12

[a] SDS, SDS-PAGE.
GF, gel filtration chromatography

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

Table 4. Action of cellulase components on different substrates[a].

Substrate	Enzyme		
	Exoglucanase	Endoglucanase	β-glucosidase
Crystalline cellulose	slow	nil	nil
Amorphous "swollen" cellulose	very active	very active	nil
CM-cellulose	nil	very active	nil
Cello-oligo sacchrides	active	active	active
Cellobiose	nil	nil	active

[a] Wood, T. M. (1989).

Structure-function of cellulases

The amino acid sequences of CBHI, CBHII, EGI and EGII of *Trichoderma reesei* cellulase have been extensively studied (Terri et al, 1983; Shomaker et al, 1983; Terri et al, 1987; Chen et al, 1987; Saloheimo et al, 1988) with a view to understanding structure-function relationships in cellulose hydrolysis. Amino acid sequence analysis shows that the sequences of these cellulases are different from each other (Bhikhabhai et al., 1984; Fagerstam et al., 1984; Shoemaker et al., 1983) and these results were confirmed later by sequencing the corresponding genes (Knowles et al. 1987). It has been found that a region of 30 amino acids is highly conserved in all four enzymes. However, the highly conserved homology region is present at the C-terminal of EGI and CBHI, while it is at the N-terminal of EGII and CBHII in the mature protein. The major function of the conserved domain appears to be substrate binding (Beguin, 1990). Knowles et al. (1987) suggested that the function of the conserved domain might be, in addition to substrate binding, to "plough" or "unzip" the crystalline structure of cellulose in order to facilitate the hydrolytic step. In particular, glycine and cysteine residues have been found to be highly conserved in this region. The structural organizations of genes coding for EG's and CBH's are presented in Fig. 5.

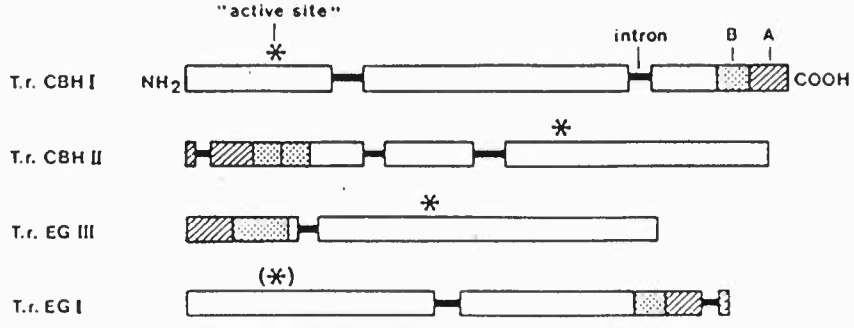


Fig. 5. Structural organization of the cellulase genes from *T. reesei*. B is the O-glycosylated region that joins the A region to the main body of the enzyme (From Knowles et al. 1988).

The CBH'S have been studied extensively due to their unique properties in hydrolysis of cellulose and their role as predominant components (30-60%) in the cellulase system. CBHI consists of 497 amino acid residues. The degree of glycosylation is about 5-10%. The molecular mass found by different methods is 59-68 kDa. CBHII consists of 447 amino acid residues with a glycosylation estimated at 8-18% and a size of 58 kDa by SDS-PAGE. No homology has been found to exist in the amino acid sequence in *Trichoderma reesei* CBHI and CBHII (Pettersson et al., 1981). It has been proven that both enzymes are encoded by different genes (Knowles et al., 1987).

It has been proposed that cellulases apparently share a common structural organization which is characterized by a central core (for example, CBHI contains a size of 54 kDa and CBHII 45 kDa) containing the catalytic active domain and a tail containing a highly glycosylated region and cellulose binding domain (Esterbauer et al., 1991; Tomme et al., 1988 and Salovuari et al., 1987). The three-dimensional structure studies of CBHI and CBHII by small angel X-ray diffraction show that the intact molecules of the CBH's have a similar tadpole-shaped structure (Schumuck et al., 1986; Abuja et al., 1988). The structure of CBHI and CBHII are shown in Fig. 6.

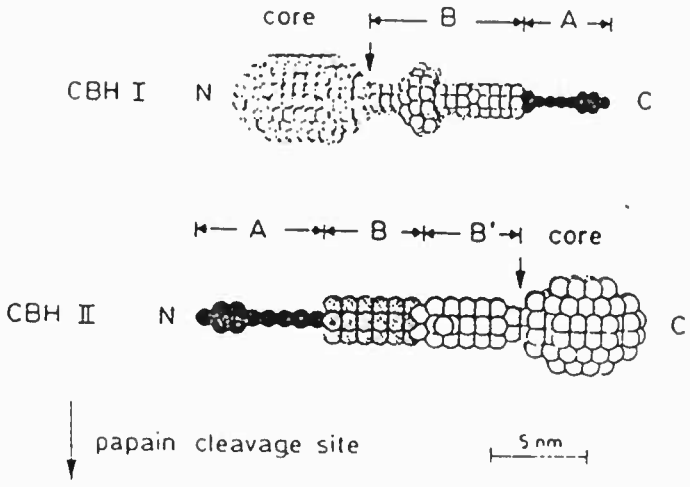


Fig. 6. Models of CBHI and CBHII deduced from small angel X-ray scaning measurements (From Abuja et al. 1988).

The two domain theory has been further supported by limited proteolytic studies (Tomme et al., 1988). Treatment with papain cleaves the polypeptide chain of CBHI at about residue 430, producing a small (10 kDa) C-terminal glycoprotein which strongly binds to cellulose, and a large 54 kDa N-terminal core protein which contains the active center. The dimension of isolated core protein is in excellent agreement with that of the intact CBHI head. (Esterbauer et al., 1991). Further catalytic studies show that these core proteins of CBHI and CBHII retain the activity (100%) on soluble substrates such as MeUmb$(Glc)_3$, but their activity against a crystalline substrate such as Avicel is almost completely (CBHI core protein 90%), or partially (CBHII core protein 60%) lost (Van Tibeurgh et al., 1986; Tomme, et al., 1988).

Both CBHI and CBHII yield cellobiose on hydrolysis of crystalline cellulose, however, they proceed via different stereochemical pathways: CBHI proceeds by retention (β-anomer), while CBHII by inversion (α-anomer) (Knowles et al., 1987).

Based on the definition, Endo's randomly act on amorphous cellulose chains and release short chain oligosaccharides. It is not clear, however, why endo I and endo II, which do not hydrolysze crystalline cellulose, require the conserved domain except for substrate binding.

The comparison of homology in the active sites between cellulase and lysozyme demonstrates that the two different enzymes have a similar acid catalyzed mechanism (Yaguchi et al. 1983; Wood, 1991). Aspartic acid and

glutamic acid residues are possibly involved in the active center (Esterbauer et al., 1991). However, Claeyssens and Tomme (1989) proposed that CBHII may utilize a beta-amylase-like, single displacement mechanism. Recently, CBHII core protein from *Trichoderma reesei* has been crystallized and the three dimensional structure of CBHII core protein was fully determined by X-ray diffraction (Rouvinen et al., 1990). It was found that the active site of CBHII is located at the carboxyl-terminal end of a parallel β-barrel in an enclosed tunnel through which the cellulose threads. Two aspartic acid residues are probably involved in the active center.

CHAPTER III CELLULOSE HYDROLYSIS

Adsorption and desorption of cellulase

The adsorption of cellulase enzymes is a prerequisite step for the hydrolysis of insoluble cellulose substrate. A dynamic adsorption-desorption mechanism plays a key role during the hydrolysis reaction. Moreover, an understanding of the adsorption characteristics of each component in the cellulase system may help to elucidate the complex hydrolytic mechanism. The adsorption of enzyme on the substrate is also interesting from the viewpoint of recovery of enzyme after the reaction and recycling, since a large fraction of the operating cost of enzymatic hydrolysis of cellulose is due to the production of the enzymes (Mandels, 1985).

The Langmiur-type adsorption isotherm is widely used to study the adsorption kinetics of cellulase on cellulose. The cellulases used in adsorption studies include a complete cellulase preparations (Steiner et al., 1988; Peitersen et al., 1977), partially purified cellulases (Ryu et al., 1984), purified endoglucanases (Klyosov et al., 1986; Beldman et al., 1987), and purified CBH's (Beldman et al., 1987). It was found that the rate of enzymatic hydrolysis of crystalline cellulose is often determined by the rule: the better the adsorption, the better the catalysis. Furthermore, the behavior of adsorption of cellulase is directly related to the synergistic behavior with other components (Klyosov, 1990). It is found that adsorption, in the initial rapid phase, is greatest under the conditions of pH and temperature that are optimal for hydrolysis (Mandels et al., 1971; Moloney and

Coughlan, 1983).

Ryu et al. (1984) found that the endoglucanases and CBH's appare to have distinctly different adsorption sites on cellulose chain. However, competitive adsorption on the cellulose (Avicel) was observed when both components are present in the system. Kyriacou et al. (1989) studied the adsorption reversibility and competitive adsorption between fractionated cellulase components. They found that, in a sequential adsorption study, interactions between enzyme components largely determine the degree of adsorption and concluded that endo and CBH occupy both common and distinct adsorption sites, depending on which components are involved. The study by Chanze et al. (1984) demonstrated that CBHI preferably binds on the crystal edges instead of the crystal surface, and the binding of the CBHI is not specific for the crystal tips where the cellulose chain ends are supposedly located.

Factors affecting the adsorption of cellulase to cellulose may include the nature of the cellulose (crystallinity, surface area), enzyme/substrate ratio and the nature of enzyme system as well as reaction conditions (temperature, pH, etc.).

Although many studies have been conducted on the adsorption behavior of cellulase on cellulose, the mechanism of adsorption is still not completely understood. It is not clear how individual cellulase components adsorb on the heterogenous surface of cellulose and how the cellulase components interact with each other following adsorption.

Synergism of cellulase

One of the interesting phenomena in cellulose hydrolysis by cellulase is the synergistic action between the individual components of these enzyme mixtures. The definition of synergism is that the extent of hydrolysis by combined fractions is somewhat greater than the calculated sum of the extents of hydrolysis by the individual fractions. A typical synergistic effect among cellulase components is listed in Table 5. It has been found that the synergistic effect exists between endoglucanase and CBH (Wood and McCrae, 1979; Henrissat et al., 1985; Beldman et al., 1985), endoglucanases (Klyosov., 1990), CBHI and CBHII (Fagerstam and Pettersson, 1980; Wood, 1985; Woodward et al., 1988a), and CBH and β-glucosidase (Halliwell and Griffin, 1973).

The degree of synergism may be affected by the following factors. First, the physical properties of the cellulose are important. It was found that the synergistic action of cellulase is most significant on native crystalline cellulose, and low on amorphous cellulose substrates, and absent on soluble derivatives (Wood and McCrae, 1979; Ryu et al. 1984). Henrissat et al.(1985) studied the synergism of cellulase on Avicel, CM-cellulose, filter paper, homogenized Avicel, bacterial MCC, and Valonia microcrystals by using purified CBH I and EG I cellulase components and found a different synergism pattern between those substrates. There was no synergism for the Valonia microcrystals and CM-cellulose. The highest synergism occurred with homogenized Avicel and bacterial MCC. This study may indicate that different synergism patterns may

Table 5. Synergistic action of cellulases[a]

Enzyme	% Cotton Solubilization
Cellobiohydrolase (CBH)	7
Endoglucanase (EG)	12
β-Glucosidase (βG)	3
CBH+EG	50
CBH+βG	22
CBH+EG+βG	59
Original culture filtrate	63

[a] *F. solani* cellulases (From Wood 1969).

occur with different cellulose substrates.

The ratio of cellulase components also affects the synergism. The enzymes known to play a main role in synergism are the endo and exo glucanases. Henrissat et al. (1985) found that the maximum synergism of enzymatic hydrolysis on Avicel was at a 1:1 proportion of endo:exo. This endo-exo cooperation and 1:1 ratio in synergism may indicate that a tertiary complex may be formed in the reaction. Beldman et al. (1988) also suggested that a maximal synergism very likely occurred at a endo- and exo glucanase complex in a 1:1 ratio. However, Woodward et al. (1988b) found that synergism is not dependent upon the exo/endo ratio but rather the total enzyme concentration. Wood (1975) reported that synergism is at a maximum when the components are used in the ratios in which they occur in the original fungal filtrates.

The concentration of product (glucose and cellobiose) is known to affect the observed synergism. Wood and McCrae (1978) found that both glucose and cellobiose had a strong inhibitory effect on synergistic activity.

The mechanism of synergism is still in debate. Wood (1985) proposed that the two cellobiohydrolases are stereospecifically different and the synergism between two cellobiohydrolases could be explained by their stereospecificities on cellulose. Because cellobiose is the repeating unit in the cellulose chain which is rigidly held in the position by intra- and intermolecular hydrogen bonds, it is possible to predict that two types of nonreducing end groups will exist in the cellulose crystallite. These end groups will require two different stereospecific

cellobiohydrolases for hydrolysis.

Klyosov (1990) suggested that the synergism results from the different binding abilities between two cellulase components in the enzyme system. Normally, the synergism occurs in enzyme systems which contains two types of cellulase components: a tightly bound component, and a weakly bound component. In the reaction, the tightly bound components penetrate into intercrystalline regions and induce a dispersion of the crystallites, and open new sites for the action of weakly bound cellulase components which act rapidly on amorphous regions of cellulose. This action results in the observed synergistic action between these components. This apparently is the mechanism of the synergism not only between endoglucanases but also between cellobiohydrolases as well as between their combinations.

The synergism may not be observed in some cases. Enari and Niku-Paavola (1987) reported that the synergism can not be observed between CBHI or CBHII and EG; it is necessary to have combinations of CBH I, CBH II, and EG for the synergism. Moreover, the cross-synergism between CBH and endo from different microbial sources, or that between CBH and different endo's from the same source, is not often observed (Wood and McCrae,1979; Beldman et al., 1988; Wood, 1989).

Substrate inhibition

Substrate inhibition exits in many enzyme systems. Substrate inhibition in this

study is defined as any apparent decrease in reaction rate that accompanies an increase in substrate concentration. Limited studies have been conducted on substrate inhibition in the cellulase system. However, substrate inhibition is an important property, relevent to cellulose saccharification.

The mechanism of substrate inhibition in many enzyme systems is still unknown and debated. Several mechanisms for substrate inhibition have been proposed, such as the simultaneous, non-productive, the binding of two substrates per active site or 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, substrate inhibition can be interpreted in terms of the existence of two types of substrate-binding sites in the enzyme. Occupation of the first high-affinity type of site 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 occurring in the first type of site. Substrate inhibition of invertase, a hydrolytic enzyme, caused by reduction of available water in the reaction mixture was reported (Nelson and Schubert, 1928). Yet, another mechanism which may account for substrate inhibition is the condition in which the concentration of an uncompetitive or noncompetitive inhibitor is kept in constant proportion to the variable substrate concentration (Cleland et al. 1973). Several reviews on

substrate inhibition are available (Webb, 1963; Cleland, 1979; and Dixon et al. 1979).

Substrate inhibition has been observed for the *Trichoderma* cellulase system (Van Dyke, 1972; Howell and Struck, 1975; Okazaki and Moo-Young, 1978). Recently, 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 (Solke Floc) (Liaw and Penner 1990). Moreover, Huang and Penner (1991) studied the cellulase system from *T. reesei* and found that substrate inhibition also exists in the cellulase system when acting upon Avicel. A fractionation study of the cellulase on the behavior of substrate inhibition demonstrated that purified CBHI, a major component (25%) in the cellulase system, does not exhibit substrate inhibition under the same reaction conditions. This result indicates that CBHI alone can not account for the apparent substrate inhibition, and if CBHI is involved in substrate inhibition, then it must be acting in conjunction with another component. Based on these findings, an enzyme ternary complex model has been proposed in our laboratory. It is possible that an optimum CBH-EG complex is forming on the substrate surface while the reaction occurs. The mechanism underlying the apparent substrate inhibition has not been determined. The possible mechanisms described above for a homogeneous system may not be applicable to the heterogeneous cellulase-cellulose system.

Mechanism of cellulase action

The mechanisms governing the enzymatic hydrolysis of cellulose are not fully understood in spite of a large numbers of studies in this area. Current concepts of cellulose hydrolysis are base on sequential cellulase component attack studies and kinetic studies.

1. Sequential attack theory.

It has long been recognized that the disruption of the crystalline structure of cellulose is a crucial step in the hydrolysis. In the early 1950's, Reese et al.(1950) proposed a Cx/C1 theory to describe a sequential enzyme action on crystalline cellulose. In the model, the C1 cellulase component (now named CBH) initially attacks cellulose and creates a reactive cellulose which could then be attacked by the Cx cellulase component (now named EG). Although the theory has been somewhat disapproved by many workers, based on recent cellulase fractionation studies, the model is still considered relevant to the breakdown of cellulose by some workers.

Since the finding that the combination of purified CBH and purified EG can completely hydrolyze crystalline cellulose, many studies have focused on how they act synergistically and which component initiates the hydrolytic action. Streamer et al. (1975) and Gong et al. (1979) reported that the EG initiated the attack on cellulose due to the fact that EG pretreated-cellulose followed by CBH action resulted in a large increase in reducing sugar release but not *vice versa*. This conclusion was further supported by the study of White and Brown (1981) who

used electron microscopy to study the action of the *Trichoderma reesei* cellulase on cellulosic ribbons. In contrast, Chanzy et al. (1983) demonstrated that CBHI from *Trichoderma reesei* acting alone affected the complete dissolution of highly crystalline cellulose from *Valonia macrophyta.*

More recently, Klyosov (1990) summarized their studies in the relationship between the adsorption of EG's on the cellulose and the activity of cellulase and suggested that both EG and CBH can bring about the dispersion of cellulose and the dispersion can result from hydrolytic and mechanical action on cellulose. Furthermore, several research groups (Leatherwood, 1969; Emert et al., 1974; Wood, 1991) proposed the formation of an enzyme "loose complex" between EG and CBH, and the complex then acts on cellulose.

Disruption of crystalline cellulose by other factors, rather than CBH or EG, have also been reported by several groups. Koenigs (1975) reported that an H_2O_2/Fe^{2+} system generated by the enzyme in brown rot fungi might initiate the disruption of the cellulose crystalline structure. Griffin et al. (1984) found that a nonenzymatic factor in *T. reesei* cellulase was capable of generating microfibrils and short fibers from filter paper. Furthermore, a short-fiber-forming C2 factor was found in the filtrates of *T. koningii* (Halliwell 1975). More recently, Krull et al. (1988) isolated a microfibril-generating factor from *T. reesei* cellulase. The factor is a peptide and requires ferric iron for increasing activity. The activity of the factor varied on different substrates. Reese (1977) modified his early C1/Cx theory and suggested that the existence of a C1 component, other than CBHI, was

responsible for initiating the disruption of crystalline cellulose and creating a reactive cellulose which was more accessible to CBHs and EGs.

2. Kinetic study

Kinetic studies have been used to evaluate the potential mechanisms of the cellulose-cellulase system, mostly based on the Michaelis-Menton type kinetics. The important parameters considered in these studies are substrate properties, enzyme properties, product inhibition, and substrate inhibition. Van Dyke (1972) studied the hydrolysis kinetics on Solka Floc cellulose and found that the substrate appeared to be made up of four fractions: an initially soluble fraction (0.9 wt%), insoluble amorphous cellulose (2.3 wt%), accessible crystalline cellulose (11.4 wt%), and inaccessible cellulose (85.4 wt%). The rates of hydrolysis were first order with the substrate and were proportional to the enzyme concentration. However, substrate inhibition was observed in this enzyme system (*T. viride*).

Howell and Stuck (1975) found that a kinetic model including substrate and product inhibition would probably best describe cellulose hydrolysis. Okazaki and Moo-Young (1978) incorporated product inhibition into a model of cellulose hydrolysis by a CBH-EG cellulase system and found that the synergism between the components resulted from several factors such as DP of the cellulose, the concentration of the two components, product inhibition and substrate concentration.

A kinetic study by Lee and Fan (1982) demonstrated that the initial rate of

hydrolysis on Solka Floc was affected by the structural features of cellulose, the surface reaction between the enzyme and substrate, and product inhibition. With extended hydrolysis times, the decrease in the hydrolysis rate was possibly due to the change of substrate into a less digestible form, the decrease in surface area, and increase in product inhibition.

Based on the finding of the relationships between enzyme concentration and hydrolysis rate reported by Woodward et al. (1988b), Bailey (1989) questioned the adaption of the kinetic model based on Michaelis-Menton assumptions for studying cellulose hydrolysis and proposed a model in which the rate of cellulose hydrolysis is expressed as a function of enzyme concentration, rather than substrate concentration as in the Michaelis-Menton equation. He conwuded that the reaction rate as a function of substrate concentration is artificial when the reaction takes place on a hydrated solid within which it is impossible to change the concentration of substrate sites.

Transferase activity of cellobiase has been observed at high concentrations of cellobiose (114 mM)(Wood and McCrae ,1982). Schmid and Wandrey (1989) have also found transferase activity with cellobiase, even at a substrate concentration as low as 10 mM. Ladisch et al. (1980) reported minor formation of a reversion product, cellotriose, at higher cellobiose concentration (90mM) in the presence of an endoglucanase. The effect of transferase activity in a complete cellulase system on the cellulose hydrolysis has not been systematically studied. If transferase activity exists in the cellulase system, the apparent hydrolysis rate should be

interpreted carefully. These observations indicate that transferase activities of cellulase and cellobiase may produce reversion products which would result in an apparent decrease in hydrolysis rate, particularly measured by reducing sugar method.

CHAPTER IV. MULTIPLE FORMS OF CBHI IN *Trichoderma reesei* CELLULASE SYSTEM: ISOLATION, PURIFICATION AND CHARACTERIZATION.

Introduction

The cellulolytic enzyme system of *Trichoderma reesei* has received considerable attention due to its high activity on relatively crystalline cellulose. The enzyme system is composed of three primary classes of enzymes; endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and beta-glucosidases (EC 3.2.1.21). Fractionation studies of this system have demonstrated that the cellulase mixture contains more than 30 protein components, analyzed by Isoelectric focusing (Fagerstam and Pettersson, 1979; Farkas et al. 1982). Ten of these 30 components have been identified as distinct cellulase components present in the active enzyme mixture (Beldman et al. 1985). It is clear from these fractionation studies that the predominant enzyme of this cellulase system is an exoglucanase, cellobiohydrolase I (CBHI). This enzyme constitutes from 25 to 60% of the total enzyme mass of crude enzyme preparations derived from *Trichoderma* cultures (Huang and Penner, 1991; Shoemaker et al. 1984).

CBHI is currently under active study due to the progress being made in elucidating the structural features of this enzyme. The protein is composed of a single polypeptide chain which appears to fold into a tadpole shape; the head being the catalytic core unit of the protein and the "tail" region functions as the

cellulose binding domain (Tomme et al. 1988, Esterbauer 1991). CBHI has been purified by a variety of methods over the past two decades. The most popular methods being those involving ion-exchange chromatography and preparative electrophoresis (Berghem and Petterson, 1973; Sheomaker et al. 1984; Bhikhabhai et al. 1984; Beldman et al. 1985). A complicating factor in the purification of CBHI is that the enzyme is known to exist in multiple forms (Montenecourt et al., 1980). Different CBHI species were isolated by a combination of ion-exchange and affinity chromatography methods (previously referred to as CBH A, B and C) and the enzymes were shown to differ with respect to their associated carbohydrates (Gum and Brown, 1977). Immunochemical techniques and electrophoretic methods have also been used to identify and separate distinct species of CBHI (Riske et al, 1986; Fagerstam and Pettersson, 1979). More recent studies have demonstrated the use of ion-exchange methods for the separation of different species of CBHI (Bhikhabhai et al., 1984; and Tomme et al., 1988; Witte et al. 1991). Each of these studies have demonstrated the existence of the multiple forms of CBHI, but these multiple forms of CBHI have not been well characterized and it is not clear whether the different forms are kinetically differentiable. If the different CBHI enzymes are kinetically distinct, then it will undoubtedly complicate the interpretation and comparison of studies utilizing CBHI purified by different methods. Conversely, if the enzyme forms behave in a similar fashion, then it may not be necessary to quantitatively separate the isozymes prior to model studies on the role of CBHI in cellulose saccharification.

In this report we illustrate a simple low pressure liquid chromatographic method for the separation of different molecular species of CBHI and we provide specific activity data which suggests that the kinetic properties of the different species are nearly indistinguishable on microcrystalline cellulose, but different on soluble substrates.

Materials and methods

Cellulase preparation. Complete cellulase was produced from *T. reesei* QM9414 in our laboratory using shake-flask cultures as described by Mandels et al. (1981). The properties of the complete cellulase preparation were reported by Huang and Penner (1991).

DEAE sepharose chromatography. DEAE-Sepharose A50 (Pharmacia Fine Chemicals Co.) column (50x200 cm) was prepared in 50 mM acetate buffer at pH 5.0 at 4°C. 600 mg of the complete cellulase powder was dissolved in 30 ml of the 50 mM acetate buffer, pH 5.0 and centrifuged for 10 min. The supernatant was applied into the column and washed with the same buffer until there was no absorbance at 280 nm and then eluted with a 0 - 0.5 M NaCl gradient in 50 mM acetate buffer (pH 5.0). The four sequential peaks, namely, I, II, III, and IV, respectively, based on A280 were pooled and dialyzed against distilled water and concentrated by a Amicon ultrafiltration cell (membrane cut off = 10,000 dalton). The concentrate of DEAE IV was freeze dried and served as crude CBHI.

SP-Sephadex chromatography. The crude CBH I was further purified on a sp-

sephadex C50-120 (Sigma Chemical Co.) column (2.4x7.0 cm), equilibrated with 50 mM ammonium acetate, pH 3.5. Approximately 30 mg of crude CBH I powder was dissolved in 5 ml of the ammonium acetate, pH 3.5. After centrifugation, the supernatant was applied onto the column and eluted with 100 ml of the ammonium acetate buffer, followed by a 600 ml gradient from pH 3.5 to 4.5. The peaks, namely a, b, c, respectively, were pooled and freeze-dried. This dried powder served as CBHIa, CBHIb, CBHIc.

Substrates. The microcrystalline substrate was Avicel pH101 (FMC Corp.), the soluble substrate, CMC, was carboxymethyl cellulose 7HOF (Aqualon Co., Wilmington, Delaware), and p-nitrophenyl-β-D-glucopyranoside (pNPBG) was used for β-glucosidase assays (Sigma Chemical Co.). Cellotriose was purchased from Pfanstichl Laboratories Co.(Wankegan, IL).

Preparation of amorphous cellulose. Five games of Avicel was dissolved in 60 ml of H_2SO_4 (60%) and incubated at 25°C for 5 min. The cellulose solution was dispersed into ice-water, using a blender. The cellulose solution was then filtrated with a membrane (0.45 μm) and washed twice with 400 ml of ice-water. The pellet was dispersed into the ice-water and the pH was adjusted to 6.0 with 1 M NaOH. The pretreated cellulose was filtered and washed three times with ice-water, and the final pellet was dissolved in cold water and dried by lyophilization. The acid-pretreated amorphous cellulose has a degree of polymerization (DP) 200, determined by the intrinsic method (ASTM 1986), and has a Crystallinity index (CrI) of 30, determined by X-ray diffraction method (Segal et al. 1959).

Protein determination. Protein content was determined by the BCA method (Smith et al. 1985), using BSA (Sigma Chemical Co.) as standard.

Electrophoresis. Gradient gel electrophoresis under denaturing conditions and isoelectric focusing (pH 3.0-5.0) (ampholyte from Pharmacia) followed the methods described by Huang and Penner (1991).

Product analysis by HPLC. The products in the reaction mixtures were analyzed by HPLC (Shimadzu, Japan), using an Aminex HPX-87H column (300x7.8 mm, BioRad, CA). The column temperature was 65°C and the mobile solvent was 0.005 M H_2SO_4 at a flow rate of 0.6 ml/min. Glucose, cellobiose and cellotriose were used as standards.

Specific activities and kinetic constants. Carboxymethyl (CM)-cellulase activity, β-glucosidase activity, and Avicelase actvity were measured as described by Huang and Penner (1991).

The kinetic constants, K_m and V_{max}, were determined with the microcrystalline cellulose at substrate concentrations ranging from 0.1 to 0.5% (w/v) and with the amorphous cellulose at substrate concentrations ranging from 0.1-2.0%. The reaction conditions were 50 mM sodium acetate, pH 5.0, 50°C, agitating at 160 rpm. The reactions were terminated at 1 h for amorphous cellulose and 5 h for crystalline cellulose by heating in boiling water. Then, the product was determined by reducing sugar method (Nelson 1944, Somogyi 1952). The K_m and V_{max} were calculated by an Eadie-Hofstee plot.

The time course of CBHI with cellotriose as substrate. Assay conditions were

50 mM sodium acetate, pH 5.0, at 50°C with the designated enzyme (20 μg/ml) and substrate concentration (1.0 mM cellotriose) in a total volume of 0.45 ml. Reactions were initiated by the addition of 0.05 ml of enzyme solution to 0.4 ml temperature equilibrated substrate solution. Aloquits of 0.06 ml were taken from the reaction mixture at different time intervals. The reaction was stopped by heating for 5 min in a boiling water-bath. The reaction product was analyzed by HPLC as described above, using glucose, cellobiose, and cellotriose as standard.

N-terminal sequencing and amino acid analysis. The CBHIa, CBHIb, and CBHIc were deblocked by removing the pyroglutamic acid residue with calf-liver pyroglutamyl aminopeptidase following the procedure described by Podell and Abraham (1978). After the reaction, the solutions were dialyzed against distilled water and lyophilized. The lyophilized powder was dissolved in distilled water and the enzyme was sequenced up to 4 amino acid residues by Edman degradation, using a Model HPPL Biosystems (470A seqencer, 120A analyzer, and 900A data control) at the Central Service Laboratory, OSU.

Amino acid analysis of CBHIa, CBHIb, and CBHIc was obtained by hydrolyzing the proteins with 6 M HCl/1% phenol at 110°C for 20 h. The amino acids in the hydrolysates were quantified with a Bechman 126 AA System Gold HPLC Amino Acid Analyzer at the Central Service Laboratory, OSU.

Statistical methods. The results of pairwise t tests, all based on the pooled estimate of experimental error, were used to test the significance of differences found among the group means of CBHIa, CBHIb, and CBHIc. Differences among

means were considered significant at the 95% confidence level (Bates and Watts, 1988).

Results

Separation of CBHI species. Chromatographic separation of the three CBHI forms is depicted in Fig. 7. The DEAE-sephadex chromatogram of the fractionation of the complete cellulase preparation is similar to that reported by others (Bergherm and Pettersson, 1973; Bhikhabhai et al. 1984; Beldman et al. 1985; Kyriacou et al. 1989). Crude CBHI was identified as fraction IV (Fig. 7a), which accounted for approximately 50% of the total protein starting material. The crude CBHI preparation displayed a single band following electrophoresis under denaturing conditions and at least two bands following isoelectric focussing (Fig.8). The DEAE-derived crude CBHI preparation was then chromatographed on SP-sephadex using a pH gradient as mobile phase (Fig. 7b). The three major fractions resulting from SP-sephadex chromatography were denoted CBHIa, CBHIb and CBHIc; these fractions constituted approximately 9%, 10% and 25%, respectively, of the total protein mass in the original complete cellulase preparation. The purified CBHIc was rechromatographed on SP-sephadex, giving a single elution band indicating that the CBHIb and CBHIc, which have a same pI value, are not in simple equilibrium.

Physicochemical properties of CBHI species. The three CBHI species all had a molecular mass of approximately 68 kDa, identical N-terminal sequences through

5 residues (Table 6) and similar amino acid profiles (Table 7). The N-terminal sequences and the amino acid compositions, based on mole percentages, are in good agreement with those previously reported for CBHI (Gum and Brown, 1977; Shoemaker et al., 1983; Bhikhabhai et al., 1984) with the exception that our proline values, 12%, were noticeably higher than that indicated previously, 6%. The isoelectric point of CBHIa, 4.0, was found to be somewhat lower than that of CBHIb and CBHIc, 4.2. The chromatogram of Fig. 7b illustrates that, under the defined conditions, the three CBHI species differed in their interaction with the SP-sephadex ion-exchanger.

Kinetic parameters of CBHI species. The specific activities of the three CBHI species acting on 5 different substrates are compared in Table 8. In general, the three enzymes were similar in that they had the highest specific activity on cellotriose, no detectable activity toward β-p-nitrophenylglucoside and their specific activities toward the microcrystalline cellulose were approximately 0.1 IU per mg protein. Statistically, there were no significant differences in the specific activities of the three enzymes acting on the microcrystalline substrate ($P<.05$). However, each of the three enzymes was distinct with respect to their observed activity toward the cellotriose and carboxymethyl cellulose substrates. The enzymes were similar with respect to their activity on amorphous cellulose, extreme specific activity values differing by less than 1.5-fold.

Initial velocity parameters K_m and V_{max} were determined for each of the enzymes with respect to the two insoluble substrates (Table 9). The estimated V_{max}

values for each of the enzymes was approximately 10-fold higher for the amorphous substrate compared to the microcrystalline substrate. The K_m values for the enzymes ranged from 1.3 to 1.8-fold higher for the amorphous compared to the microcrystalline substrate. Statistical analyses between enzyme species detected no significant differences in either the K_m or V_{max} of the different enzymes acting on the microcrystalline substrate ($P<.05$). Similarly, there were no significant differences in the K_m values of the different enzymes when acting on the amorphous substrate. A small but significant difference was observed between the V_{max} value for CBHIa and CBHIc acting on the amorphous substrate. The V_{max} of CBHIb was not significantly different from either CBHIa or CBHIc.

The solubilized products resulting from the CBHIa, CBHIb or CBHIc catalyzed hydrolysis of the microcrystalline substrate were essentially identical. Each of the reaction mixtures contained cellobiose as the predominant product, with glucose representing from 15-20% of product on a molar basis. Cello-oligosaccharides higher than cellobiose (DP>2) could not be detected in any of the reaction mixtures. Following a 10 h reaction period, the product ratio of cellobiose to glucose (molar basis), for reaction mixtures containing CBHIa, CBHIb, and CBHIc, was 7.0±0.4, 5.7±0.5, and 6.4±0.8, respectively. The product ratios remained approximately the same when reaction mixtures were analyzed following a 15 h reaction period; 6.3±0.5, 5.2±0.2, and 5.7±0.8 for reaction mixtures containing CBHIa, CBHIb and CBHIc, respectively. None of these values were significantly different at the 95% confidence level.

The time course for cellotriose hydrolysis by each of the enzyme preparations is presented in Fig.9. The time course is expected to be influenced by a range of kinetic constants as substrate is depleted and product accumulates. The expected products of cellotriose hydrolysis, cellobiose and glucose, were found in equimolar concentrations at each of the time points tested. The data is consistent with the specific activity data of Table 8 and clearly indicates differences in the kinetic properties of these enzyme preparations when acting on cellotriose.

Discussion

In this study three apparently distinct forms of CBHI have been separated by low pressure ion-exchange chromatography. Each of the proteins was identified as CBHI based on their amino acid composition, N-terminal sequences and relative activities on selected substrates. Two of the proteins had essentially the same pI and molecular mass, but differed in their interaction with the SP-sephadex exchanger. Witte et al. (1990) recently reported the separation of two CBHI species with equivalent pIs, also using ion-exchange chromatography. The multiplicity of CBHI has also been proven by other purification methods. Schulein (1988) reported that a CBH I purified by HPLC shows multiple bands on IEF-PAGE with pI values of 4.05 to 4.25 although the purified CBH I shows a single band on SDS-PAGE. Riske et al. (1986) found that a CBHI purified by monoclonal antibodies can be further separated into three proteins with pI values of 4.05, 4.15, 4.25 on IEF though the purified CBHI yielded a single band on

SDS-PAGE. It appears clear that multiple forms of CBHI exist in the cellulase system. However, it is not clear how many forms of the enzyme can be expected or whether the CBHI species identified in different laboratories represent the same enzyme forms. This is because, in most cases, the different species of CBHI have been identified by isoelectric focusing and, as demonstrated in this study, at least some of the CBHI forms have apparently equivalent pIs. It is reasonable to assume that the CBHI forms separated in any given study will depend on the purification protocol used.

The amino acid composition and sequence data may indicate CBHIa, b and c are composed of the same polypeptide chain; indicating that the enzymes differ due to post-translational modifications. The nature of the differences in the species analyzed in this study was not determined. Gum and Brown (1977) have shown that at least some CBHI species differ with respect to the carbohydrate associated with the protein. It has also been suggested that different forms of the cellulolytic enzymes may arise from proteolytic processing (Nakayama et al. 1976; Hagspiel et al. 1989). The amino acid composition and sequence data reported in this study appear most consistent with differences in the carbohydrate moieties.

The kinetic properties of the three CBHI enzymes appear virtually identical with respect to the microcrystalline substrates. The specific activity, K_m, V_{max} and product profiles suggest that the different CBHI forms have essentially the same role in microcrystalline cellulose saccharification. Their similarity also indicates that studies evaluating the kinetic parameters of a single CBHI species are likely,

at least, applicable to other CBHI species obtained through different purification methods. The kinetic properties of CBHI with respect to the microcrystalline substrate are of particular importance due to the unique ability of the cellobiohydrolases to efficiently catalyze its hydrolysis to cellobiose/glucose.

The results obtained for the other substrates are more difficult to interpret than those described for the microcrystalline substrate. This is because potentially contaminating cellulolytic enzymes, such as the endoglucanases, are relatively active on these modified substrates (Wood, 1989). With these substrates, the only "clean" result would have been if all three of the enzyme species were found to have essentially the same kinetic properties. This was not the case. It is, therefore, not possible to distinguish whether the observed activity differences were the result of actual differences in the enzymes per se or whether the observed differences resulted from minor contaminants possessing relatively high activities on these particular substrates. Note that these contaminants would not be expected to affect the experiments using microcrystalline cellulose since the likely contaminants have extremely low activities toward that substrate. Endoglucanase-type contaminants would, however, be expected to have relatively high activities on CMC and cellotriose. In this study, the specific activities of the CBHI preparations showed the largest differences with these two substrates. Relative to the soluble substrates, potential contaminants would be expected to show relatively lower activities toward the insoluble amorphous cellulose preparation. Consistent with this, the three CBHI species showed similar kinetic properties

when acting on the amorphous substrate; the K_m's of each enzyme being essentially equivalent and the extreme V_{max} values differing by less than 40%. Gum and Brown (1977) have reported similar specific activities, ranging from 0.53-1.01 IU/mg protein, for the four CBH species on phosphoric acid-swollen cellulose. Although there was a significant difference at the 95% confident level, differences in the interaction of CBHI species with amorphous cellulose substrates have more recently been reported based on substrate dispersion studies (Witte et al., 1990). However, just as in this study, it is difficult to rule out the presence of a minor, but significant, contaminant when non-equivalence is observed. In experiments of this type, further purification steps will do little to validate apparent kinetic differences since the possibility of a trace contaminant can not be excluded.

In summary, the data presented demonstrates the presence of three distinct forms of CBHI which may differ as a result of post-translational modifications. The CBHI species were shown to be readily separated by ion-exchange chromatography. The enzymes appear to behave similarly when acting on insoluble substrates. The kinetic properties were essentially identical when acting on the microcrystalline substrate and showed only minimal differences, if at all, with respect to their activity on the amorphous substrate. The results of experiments utilizing modified soluble substrates were inconclusive in that real differences in specific activities were observed but it could not be ruled out that undetectable contaminants were the source of these differences.

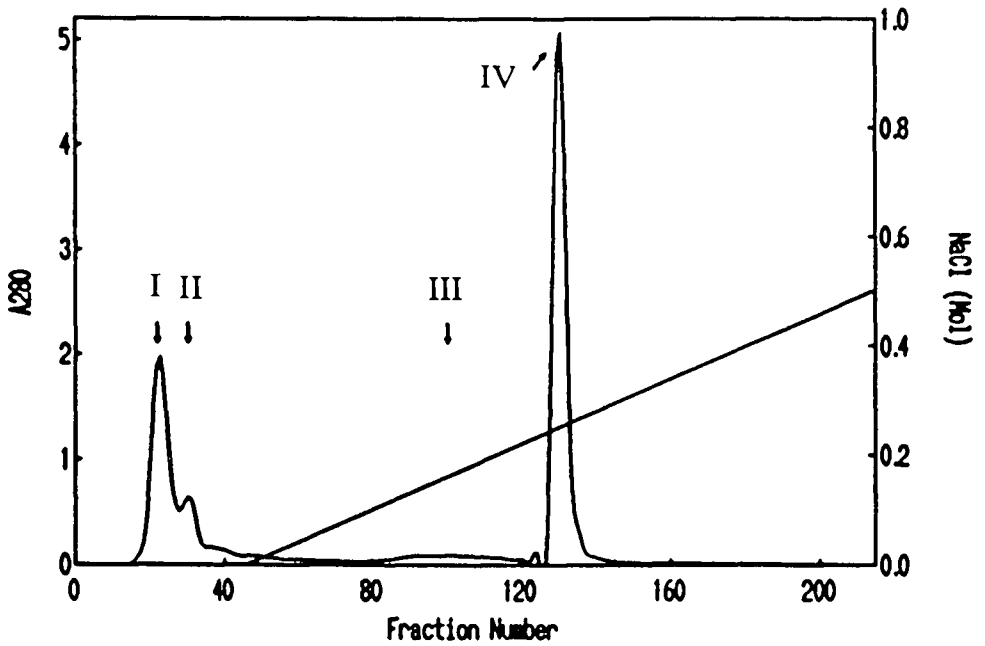


Fig. 7. a) Chromatography of complete cellulase preparation on DEAE-sepharose. The relative protein content of fraction was measured as A280. The flow rate was 30 ml/h and fraction volume was 7.5 ml.

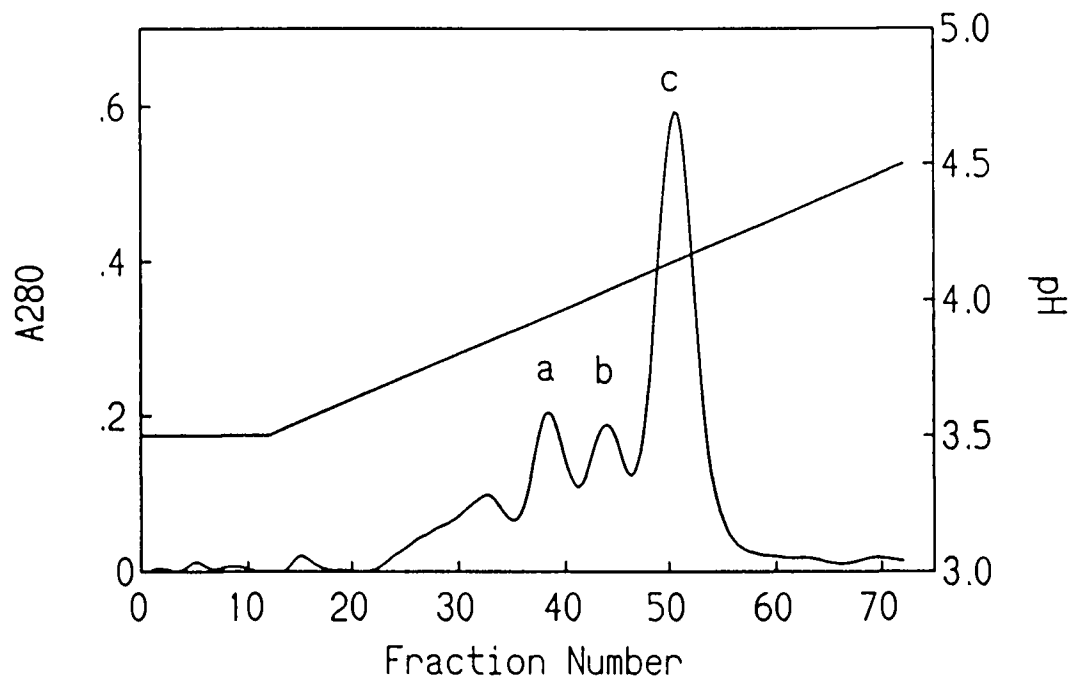


Fig. 7 b) Chromatography of DEAE-sepharose fraction IV on SP-sephadex. Protein was measured as A280. The flow rate was 30 ml/h and fraction volume was 7.5 ml.

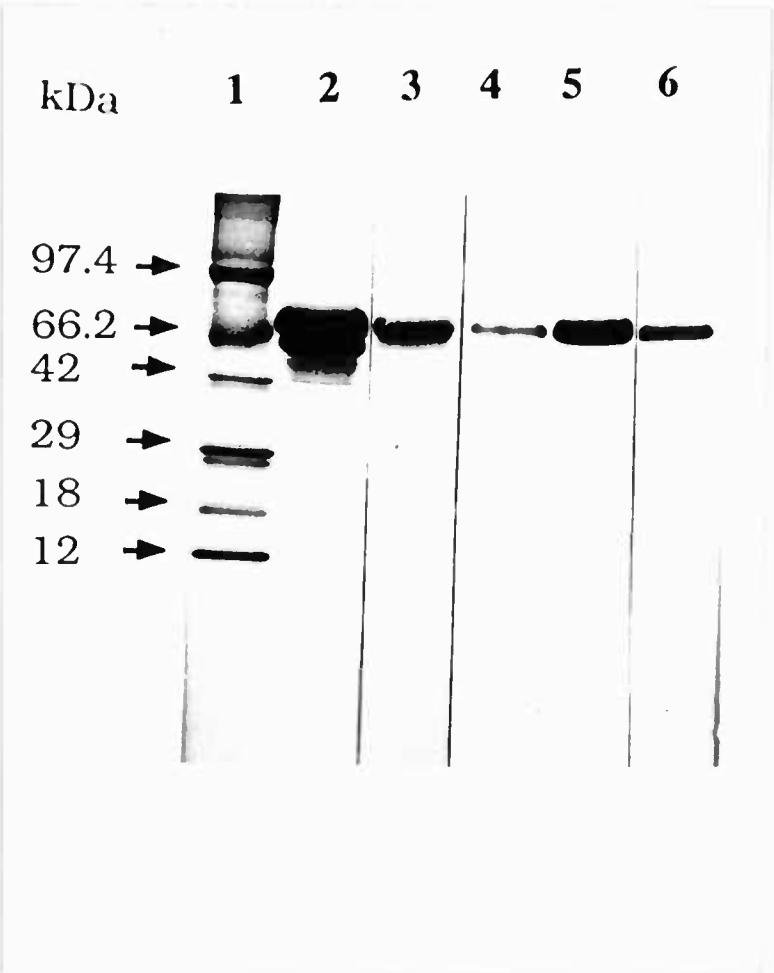


Fig. 8 a)SDS-gel electrophoresis of cellulase. (1). protein markers, (2). complete cellulase preparation (60 μg), (3). DEAE IV fraction (25 μg), (4). CBHIa (20 μg), (5). CBHIb (25 μg), (6). CBHIc (20 μg).

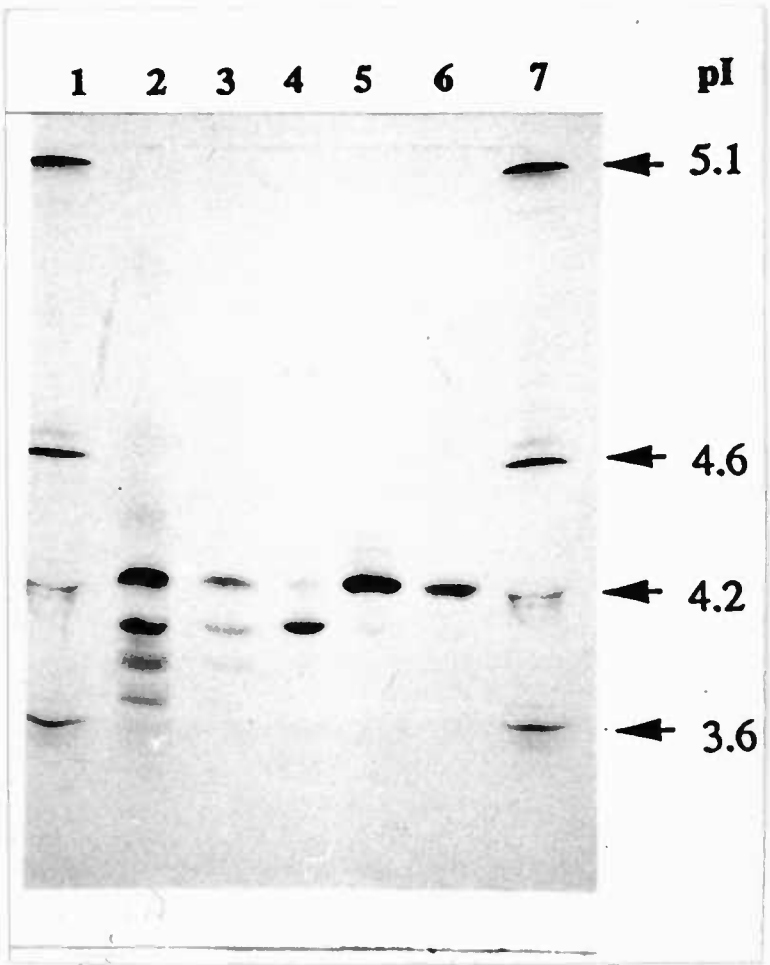


Fig. 8 b) Analytic isoelectric focusing of cellulases in the pH range 3.0-5.0. (1)&(7). pI markers, (2). complete cellulase preparation (120 μg), (3). DEAE IV (40 μg), (4). CBHIa (25 μg), (5). CBHIb (25 μg), (6). CBHIc (25 μg).

Table 6. Physicochemical properties of CBHI enzymes

Enzyme	Mr[a]	pI[b]	N-terminal sequence
CBHIa	68	4.0	PyrGlu-Ser-Ala-Cys(?)-Thr
CBHIb	68	4.2	PyrGlu-Ser-Ala-Cys(?)-Thr
CBHIc	68	4.2	PyrGlu-Ser-Ala-Cys(?)-Thr

[a] Mr, molecular mass (kilodaltons) estimated by gradient SDS-PAGE.
[b] pI was estimated by analytic isoelectric focusing at pH 3.0-5.0.

Table 7. Amino acid composition of CBHI enzymes[a]

	mol percent		
Amino acid	CBHIa	CBHIb	CBHIc
Aspartic acid[b]	12.1	11.1	11.3
Threonine	11.4	10.6	10.8
Serine	10.7	10.0	10.1
Glutamic acid[c]	9.0	8.5	8.5
Proline	11.1	12.2	13.2
Glycine	13.3	12.8	12.6
Alanine	6.0	6.0	5.9
Valine	4.2	4.5	4.5
Methionine	1.1	1.1	1.2
Isoleucine	2.1	2.0	2.0
Leucine	5.6	5.3	5.3
Tyrosine	5.0	4.8	4.8
Phenylalanine	3.1	3.0	3.0
Lysine	2.7	2.6	2.6
Histidine	1.1	1.0	1.1
Arginine	2.0	1.8	1.9
Cystine	--	2.6	1.3

[a] hydrolysis in 6 N HCl/1% phenol at 110°C for 20 h *in vacuo*. Tryptophan was not determined.
[b] Sum of Aspartic acid and Aspargine.
[c] Sum of Glutamic acid and Glutamine.

Table 8. Specific activities on different substrates[1]

Enzyme	Specific Activity[2] AC	MC	CMC	pNPG	G3
CBHIa	0.97^{b} ±0.08	0.12^{a}±.01	0.17^{b}±.07	0	252^{b}±37
CBHIb	$0.84^{a,b}$±0.06	0.09^{a}±.01	0.35^{c}±.03	0	540^{c}±48
CBHIc	0.68^{a} ±0.06	0.09^{a}±.01	0.02^{a}±.01	0	136^{a}±7

[1] Specific activities measured at conditions of 50 mM sodium acetate, pH 5.0, 50°C. The reaction time were different with the given substrates. All values reported in units of micromolar of product produced per minute per milligram of protein with the given substrate. AC, amorphous cellulose at the concentration of 1% (w/v), 1 hour; MC, microcrystalline cellulose, at 1% (w/v), 5 hour; CMC, carboxymethylcellulose, 0.25%, 30 minute; pNPG, p-nitrophenoyl-β-D-glucopyranoside, 3.3 mM, 10 minute; G3, cellotriose, 1 mM, 15 minute.

[2] Values are means±SEM, n=3. Column means with a common supercript are not significantly different (P>0.05). Significance based on pooled variance pairwise t test.

Table 9. Kinetic constants on different substrates[1,2]

Enzyme	A-cellulose K_m	A-cellulose V_{max}	M-cellulose K_m	M-cellulose V_{max}
CBHIa	0.51^{a}±0.09	1.37^{b} ±0.09	0.38^{a}±0.07	0.13^{a}±0.02
CBHIb	0.63^{a}±0.07	$1.18^{a,b}$±0.08	0.35^{a}±0.06	0.12^{a}±0.02
CBHIc	0.47^{a}±0.10	0.96^{a} ±0.12	0.32^{a}±0.07	0.10^{a}±0.01

[1] Reaction conditions were 50 mM sodium acetate buffer, pH 5.0, 50°C. A-cellulose, amorphous cellulose, 0.1-2.0% (w/v), 1 h; M-cellulose, microcrystalline cellulose, 0.1-0.5% (w/v), 5 h. K_m (percent), V_{max} (micromoles per minute per milligram of protein) were calculated by Eadie-Hofstee plot.

[2] Values are means±SEM, n=4. Column means with a common supercript are not significantly different (P>0.05). Significance based on pooled variance pairwise t test.

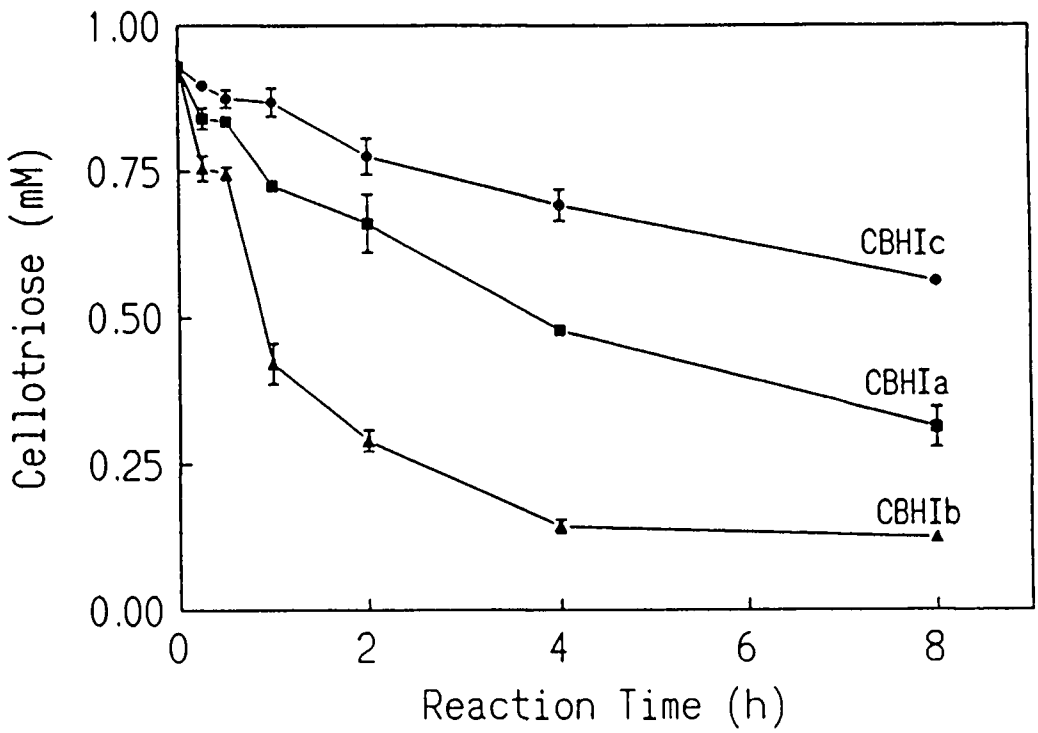


Fig. 9. Time course of CBHI's on cellotriose. Enzyme concentrations were 20 μg/ml for CBHIa, CBHIb, CBHIc. Reaction conditions were 50 mM sodium acetate, pH 5.0, at 50°C, using cellotriose substrate. The reaction was terminated at 15 min. The decrease in the substrate concentration were measured by HPLC.

CHAPTER V. APPARENT SUBSTRATE INHIBITION IN *T. reesei* CELLULASE

Introduction

The enzymatic conversion of cellulose to glucose is of continuing interest due to the potential production of energy and/or chemical feedstocks from cellulosic biomass. This saccharification process is catalyzed by a complex enzyme system which typically includes at least three distinct classes of enzymes: endoglucanases (EC 3.2.1.4), cellobiohydrolases (3.2.1.91) and β-glucosidases (EC 3.2.1.21). Cellulase enzyme systems derived from different microorganisms differ markedly in their ratio of these constituent enzymes and, consequently, in their ability to degrade native cellulose, (Coughlin and Ljungdahl, 1988). The *Trichoderma reesei* cellulase system is one which has received considerable attention due to its economic potential (Mandels, 1985). This potential rests in the fact that it is a complete, extracellular enzyme system capable of catalyzing the hydrolysis of crystalline cellulose.

The kinetic mechanisms governing the full time course of cellulose hydrolysis have not been determined. However, several kinetic models capable of predicting a large portion of the reaction time course, under specified conditions, have been presented, (Lee and Fan, 1983, Okazaki and Moo-Young, 1978, and Huang, 1975). These kinetic models have been based on classical Michaelis-Menton assumptions. The Michaelis parameters derived for this system are difficult to interpret

mechanistically due to its heterogeneous, multienzyme nature (Lee and Fan, 1982). In this regard, Beldman et al. (1985) have characterized 10 enzymes from the cellulase system of *Trichoderma viride*.

A kinetic property applicable to cellulose saccharification which has not yet been adequately characterized is substrate inhibition. Substrate inhibition, in general, is not uncommon for enzymes acting at relatively high substrate concentrations and it is ordinarily attributed to dead end complex formation between the substrate and one or more enzyme forms (Fromm, 1975). The inhibition of cellulose saccharification by excess substrate has been observed for *Trichoderma* (Howell and Struck, 1975, and Van Dyke, 1972), and mixed *Aspergillus/Trichoderma* (Contreras et al., 1982) derived cellulase systems. Apparent substrate inhibition of *T. viride* derived cellulase complexes has been observed with ball-milled (Howell and Struck, 1975, and Van Dyke, 1972), and unspecified cellulose substrates (Okazaki and Moo Young, 1978). More recently, a commercial *T. viride* cellulase preparation was shown to exhibit substrate inhibition when acting upon a microcrystalline substrate, but not a powdered cellulose substrate under apparently equivalent conditions (Liaw and Penner, 1990). The enzyme complex from *T. reesei* has similarly been observed to exhibit substrate inhibition. Lee and Fan (1982) have presented data indicating substrate inhibition at high substrate concentrations relative to those used in initial velocity studies. Ryu and Lee (1986) demonstrated a time dependent decrease in the rate of cellulose hydrolysis at high substrate concentrations which was not observed at

lower substrate levels. These studies collectively indicate that the apparent substrate inhibition of cellulose hydrolysis is neither restricted to a single cellulase system nor to a single substrate.

In the present paper we characterize the substrate inhibition properties of the *T. reesei* derived cellulase system. The substrate-activity profiles for this enzyme system over a range of enzyme concentrations are presented. The major component of the complete enzyme system, a cellobiohydrolase (CBHI) constituting 25% of total enzyme mass, was isolated and its substrate-activity relationships determined. The influence of supplemental CBHI activity on the observed substrate inhibition properties of the complete cellulase system are also reported.

Materials and methods

Cellulase preparation. Complete cellulase was produced by *T. reesei* QM9414 in our laboratory using shake-flask cultures as described by 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 two 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. The cellulase concentration in all reaction mixtures is given in IU per ml based on the preparation's filter paper activity.

CBHI preparation. CBHI was isolated from the complete cellulase preparation described above. The cellulase powder was first chromatographed on DEAE-Sepharose (Pharmacia Inc.) according to Beldman et al. (1985) eluting with a 0 to 0.5 M NaCl gradient. The predominant cellobiohydrolase fraction (Fraction IV) was further chromatographed on SP-Sephadex (Sigma Chemical Co.). Approximately 30 mg of crude CBHI was applied to a 2.4 X 7 cm column, washed initially with 100 ml of 50 mM ammonium acetate, pH 3.5, followed by a 600 ml gradient from pH 3.5 to 4.5. The major component, purified CBHI, was lyophilized and stored dessicated at 4°C. The purified CBHI concentration in all reaction mixtures is given in μg protein per ml.

Gradient gel electrophoresis under denaturing conditions was done according to Laemmli (1970) as modified by Malencik and Anderson (1987). Molecular weight standards ranging from 12,000 to 97,400 daltons were used for molecular weight estimations. Isoelectric focusing was done with a Bio-Rad Horizontal Electrophoresis System equipped with a Model 1405 electrophoresis cell according to the application note provided by the manufacturer. The pI was estimated by comparison with protein standards ranging in pI from 2.9 to 5.0 (Sigma Chemical

Co.)

Substrates. The microcrystalline substrate was Avicel pH101 (FMC Corp.), the soluble substrate, CMC, was carboxymethyl cellulose 7HOF (Aqualon Co., Wilmington, Delaware), and p-nitrophenyl-β-D-glucopyranoside (pNPG) was used for β-glucosidase assays (Sigma Chemical Co.).

Enzymatic hydrolysis of microcrystalline cellulose. Assay conditions were 50 mM sodium acetate buffer, pH 5.0, at 50°C with the designated enzyme and substrate concentrations in a total volume of 4 ml. Enzyme concentrations ranged from 2.2 to 16.6 x 10^{-3} IU per ml for the complete preparation and 1.65 to 13.2 μg per ml for purified CBHI. Substrate concentrations ranged from .25 to 10%. Reaction mixtures, in 10 ml flasks, were agitated at 160 RPM. Reactions were initiated by the addition of 0.1 ml enzyme solution to 3.9 ml temperature equilibrated substrate solution. Substrate concentrations are expressed in percent, (w/v). Protein concentrations are expressed as μg enzyme per ml; determined by the method of Smith et al. (1985) using bovine serum albumin as the calibration standard. Reactions were terminated at 5 h by centrifugation and immediate assay of supernatant for solubilized reducing sugar equivalents, (Somogyi, 1952; Nelson, 1944) or total sugar equivalents (Roe, 1955) using glucose as the calibration standard. Assays contained control reaction mixtures consisting of substrate alone, enzyme alone and substrate plus enzyme terminated at zero time.

Specific activities and kinetic constants. Carboxymethyl (CM)-cellulase and β-

glucosidase activities were measured as described by Beldman et al. (1985) using a 30 min reaction period for the CM-cellulase assay. Filter paper activities were determined as described by Mandels et al. (1976). Reaction conditions for the determination of specific activities with microcrystalline cellulose were 50 mM sodium acetate, pH 5.0, 50°C, 1% substrate (w/v), and enzyme concentrations of 6.6 and 8.5 μg per ml for the cellobiohydrolase and complete cellulase preparation, respectively. The reaction mixture volume was 4 ml and the agitation rate 160 RPM for the 5 h reaction period. The kinetic constants, K_m and V_{max}, were determined with the microcrystalline substrate at substrate concentrations ranging from 0.1 to 0.5% (w/v) using the reaction conditions given above.

Results

Effect of substrate concentration on the rate of saccharification catalyzed by the *T. reesei* enzyme system. The kinetic parameters applicable to the complete *T. reesei* cellulase preparation are presented in Table 10. The filter paper-based specific activity of 1.05 IU per mg protein was comparable to that observed for other *Trichoderma* enzyme preparations (Liaw and Penner, 1990). The measured K_m, 0.33%, and V_{max}, 0.54 μmole per min per mg protein, were specific to the microcrystalline substrate. Analysis of the enzyme preparation by chromatographic fractionation, isoelectric focusing and electrophoresis under denaturing conditions indicated it was composed of a minimum of 15

enzymes/proteins ranging in molecular weight from 20,000 to 100,000 daltons. Fractionation studies indicated essentially all the observed proteins possess cellulase activity. Beldman et al. (1985) have similarly reported the presence of at least 10 cellulolytic enzymes present in *Trichoderma viride* cellulase preparations. The extent to which post translational modification during enzyme production and isolation may account for the different enzymes/isozymes has not been established.

The *T. reesei* cellulase system does not obey classical saturation kinetics (Fig. 10). Instead, the rate of saccharification increases with increasing substrate concentrations to a maximum, after which further increases in substrate concentration result in a decrease in the rate of saccharification. Saccharification, in the context of this paper, refers to the solubilization of reducing sugar equivalents from the insoluble substrate. In this regard, analysis of reducing sugar equivalents or total sugar equivalents solubilized results in similar substrate-activity profiles (Fig. 10b). The substrate concentration corresponding to the maximum rate of saccharification, referred to as the optimum substrate concentration, was affected by the enzyme concentration of the reaction mixture. The optimum substrate concentrations for reaction mixtures containing 2.2, 4.4, and 8.8 x 10^{-3} IU of enzyme per ml were approximately 1%, 1-2% and 2-4%, respectively (Fig. 10). The maximum rate of saccharification at 17.6 x 10^{-3} IU enzyme per ml was first attained at approximately 6% substrate, following the trend observed at the lower enzyme

concentrations. Substantial substrate inhibition was not observed at the highest enzyme concentration, presumably because the highest substrate concentrations tested were not sufficiently greater than the apparent 6% optimum. Substrate concentrations greater than 10% were not tested due to inherent mixing problems (Liaw and Penner, 1990). The extent of inhibition observed at 10% substrate was similarly dependent on the enzyme concentration of the reaction mixture. At the lowest enzyme concentration tested, 2.2 x 10^{-3} IU per ml, the reaction rate decreased asymptotically to a rate approximately 70% less than the maximum observed. The reaction rate of the reaction mixture containing 4.4 x 10^{-3} IU enzyme per ml appeared to similarly decrease with increasing substrate concentrations.

The maximum rate of saccharification at the different enzyme levels was roughly proportional to the amount of enzyme present, even though the substrate concentration corresponding to that maximum differed. The maximum rate of saccharification, obtained from the data in Fig. 10, was 0.34, 0.39, 0.52 and 0.43 μmoles reducing sugar equivalents solubilized per IU enzyme per min for curves (a) thru (d), respectively. The amount of substrate solubilized during any assay period was always less than 14% of the total substrate available; the maximum conversion of substrate to product occurred for reaction conditions of 0.25% substrate and 17.6 x 10^{-3} IU enzyme per ml.

Effect of substrate concentration on the rate of cellobiohydrolase catalyzed saccharification. The predominant enzyme of the *T. reesei* cellulase system,

CBHI, was isolated and its substrate-activity interrelationships characterized for comparison with those of the complete enzyme system. The physical and kinetic properties of the purified enzyme, homogeneous based on native and denaturing gel electrophoresis and isoelectric focusing (Fig. 11), are presented in Table 10. The relatively high activity of the enzyme toward microcrystalline cellulose, the negligible activity toward CMC, the predominance of the enzyme in the complete enzyme preparation, its molecular weight and its isoelectric point are indicative of CBHI. The enzyme's properties compare well with the CBHI fraction from *T. reesei* reported by Bhikhabhai et al. (1984) and Shoemaker et al. (1983). The isolated CBHI comprised approximately 25% by weight of the total enzyme preparation.

CBHI obeyed classical saturation kinetics under the conditions tested (Fig. 12). At all enzyme levels the rate of saccharification approached a maximum asymptote as the substrate concentration increased. The rate of saccharification at each substrate concentration was directly proportional to the amount of enzyme present, in agreement with classical enzyme behavior. Consequently, there is no apparent shift in the substrate concentration corresponding to V_{max} and no "optimum" substrate concentration, as observed for the complete enzyme preparation.

Effect of additional CBHI on the rate of saccharification by the complete cellulase preparation. Graded amounts of CBHI were added in a stepwise manner to a constant amount of complete enzyme preparation to determine the

significance of CBHI relative to the substrate inhibition properties observed for the complete enzyme preparation. The quantity of complete enzyme preparation added to each reaction mixture, 4.2 μg per ml (4.4×10^{-3} IU per ml), contained approximately 1.05 μg of endogenous CBHI per ml. The quantity of purified CBHI added to the complete enzyme preparation ranged from zero to 6.6 μg per ml. Consequently, the CBHI concentration in the reaction mixtures tested ranged from the endogenous level up to a six-fold excess above the endogenous level. The addition of purified CBHI to the complete enzyme preparation increased the rate of saccharification at all substrate concentrations (Fig. 13). The added CBHI had little or no effect on the optimum substrate concentration. Therefore, the addition of CBHI increased the optimum rate of saccharification but did not measurably affect the substrate concentration corresponding to that optimum. The similarity of the substrate-activity profiles in Fig. 13 also indicates that additional CBHI does not influence the change in the rate of saccharification with respect to substrate concentration at substrate concentrations above the optimum. The actual difference in reducing sugar equivalents solubilized at the optimum substrate concentration (approximately 2.0%) and that solubilized at 10% substrate was nearly equivalent for each of the enzyme concentrations tested, corresponding to a net reduction of approximately 0.22 μmoles of reducing sugar equivalents per ml reaction mixture.

A synergistic effect was observed for those reaction mixtures containing both complete enzyme preparation and additional CBHI. The observed synergism was

evident in that the rate of saccharification for the combined enzymes was greater than that for the sum of the two enzyme preparations acting independently. The average degree of synergism at 2% substrate, calculated from the data presented in Figures 10b, 12 and 13, was 1.5.

Discussion

The cellulase enzyme system produced by *T. reesei* QM9414 is of primary importance due to its ability to degrade crystalline cellulose (Mandels, 1985). It is therefore of particular relevance that the apparent substrate inhibition properties reported here were observed on a microcrystalline substrate. To our knowledge, the presented data is the first documentation of this behavior for this enzyme system. In the context of this paper, the term "substrate inhibition" refers to any apparent decrease in the rate of the reaction which accompanies an increase in substrate concentration. Lee and Fan (1982) have noted substrate inhibition of *T. reesei* QM9414 cellulase activity on a hammer-milled cellulose substrate. Their observation differs from the present results in that they observed substrate inhibition at substrate to enzyme ratios (g cellulose/IU enzyme) of approximately 0.2. In the present study substrate inhibition was only observed at ratios greater than 10, representing a 50-fold difference in reaction conditions. Ryu and Lee (1986), using a powdered cellulose substrate and reaction conditions similar to those of Lee and Fan (1982), observed a similar substrate inhibition of the cellulase system produced by *T. reesei* MCG-77. The substrate inhibition

observed with powdered cellulose substrates, as in the above studies, has been attributed to the hydrodynamic properties of the substrate, (Lee and Fan, 1982). The substrate inhibition observed with the powdered substrates showed no apparent sensitivity to changes in enzyme concentration, (Lee and Fan, 1982 and Ryu and Lee, 1986). The substrate inhibition properties of a commercial cellulase enzyme preparation from *T. viride* have recently been characterized (Liaw and Penner, 1990). The properties of that enzyme system and the *T. reesei* QM9414 system of this study are very similar, both showing a direct relationship between the total enzyme activity of a reaction mixture and its corresponding optimum substrate concentration.

The role of CBHI in substrate inhibition is of particular relevance due to its independent activity on crystalline cellulose and because it is the predominant enzyme in the complete system. CBHI reportedly constitutes from 24 to 60 percent, by weight, of *T. reesei* enzyme preparations (Riske, et al., 1986, Shoemaker, et al., 1983). The results of the present study demonstrate that CBHI alone can not account for the observed substrate inhibition. The kinetics of the purified enzyme demonstrate that if CBHI is involved in substrate inhibition, then it must be acting in conjunction with another component. This is further supported by the combined results depicted in Figs. 10 and 13 showing that addition of complete enzyme to the reaction mixture shifts the optimum substrate concentration while addition of CBHI has little or no effect on optimum substrate concentrations.

The substrate inhibition properties of the *T. reesei* enzyme system further illustrate the complex nature of cellulose hydrolysis. 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). Commonly discussed mechanisms, such as the simultaneous, non-productive, binding of two substrates per active site or the binding of substrate to a peripheral non-active site which modifies enzyme activity (Webb, 1963), may not be applicable to this heterogeneous system. The mechanism underlying the observed kinetic behavior in this complex system, consisting of an insoluble substrate which upon hydrolysis results in soluble and insoluble "products" that are themselves substrates, is not known. Due to the complexity of this heterogeneous system, it is imperative that the results not be considered only in terms of classical soluble substrate systems. Substrate inhibition of cellulose hydrolysis has previously been rationalized by mechanisms involving decreases in the movable aqueous phase of the reaction mixture which results in diffusional limitations (Lee and Fan, 1982), or decreases in the extent of concurrent action on the same chain by component enzymes (Van Dyke, 1972). The contrasting substrate-activity profiles for the complete cellulase system and purified CBHI indicate that the observed substrate inhibition is not based simply on the general hydrodynamic properties of the substrate.

Table 10. Kinetic and physical constants of emzyme preparations

Enzyme	Specific Activities[1] (IU/mg protein)				Physical Constants[2]		Kinetic Constants[3]	
	F.P	M.C.	CMC	pNPG	Mr (KDa)	pI	K_m (%)	V_{max}
Complete Cellulase	1.05	0.51	8.12	0.11	--	--	0.33	0.54
CBHI	--	0.09	ND[4]	0	68	4.3	0.30	0.10

[1] Specific activities measured at conditions of 50 mM NaAcetate, pH 5.0, 50°C. All values reported in units of μmoles reducing sugar equivalent produced per min per mg protein with the given substrate. F.P., filter paper; M.C., microcrystalline cellulose; CMC, carboxymethyl cellulose; pNPG, p-nitrophenol-β-D-glucopyranoside.

[2] Molecular mass estimated by electrophoresis at denaturing conditions and pI by isoelectric focusing.

[3] K_m (%) and V_{max} (μmole/min/mg protein) values determined for the microcrystalline cellulose substrate at 50 °C, pH 5.0, 50 mM NaAcetate buffer.

[4] Not detectable. The value is less than instrument detection limit (0.02 μmole reducing sugar equivalent/ml).

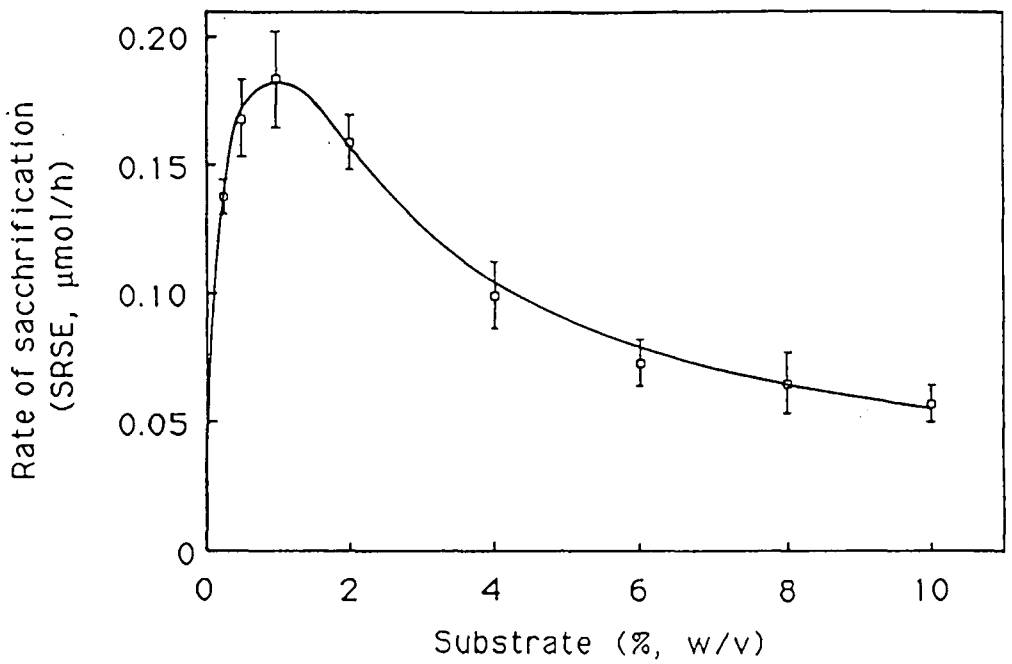


Fig. 10 a) Substrate-activity profiles for the complete cellulase system at enzyme concentration of 2.2 X 10^{-3} IU per ml. Reaction conditions were 50 mM sodium acetate, pH 5.0, at 50°C using the microcrystalline cellulose substrate. The reaction was terminated at 5 h. SRSE, solubilized reducing sugar equivalents.

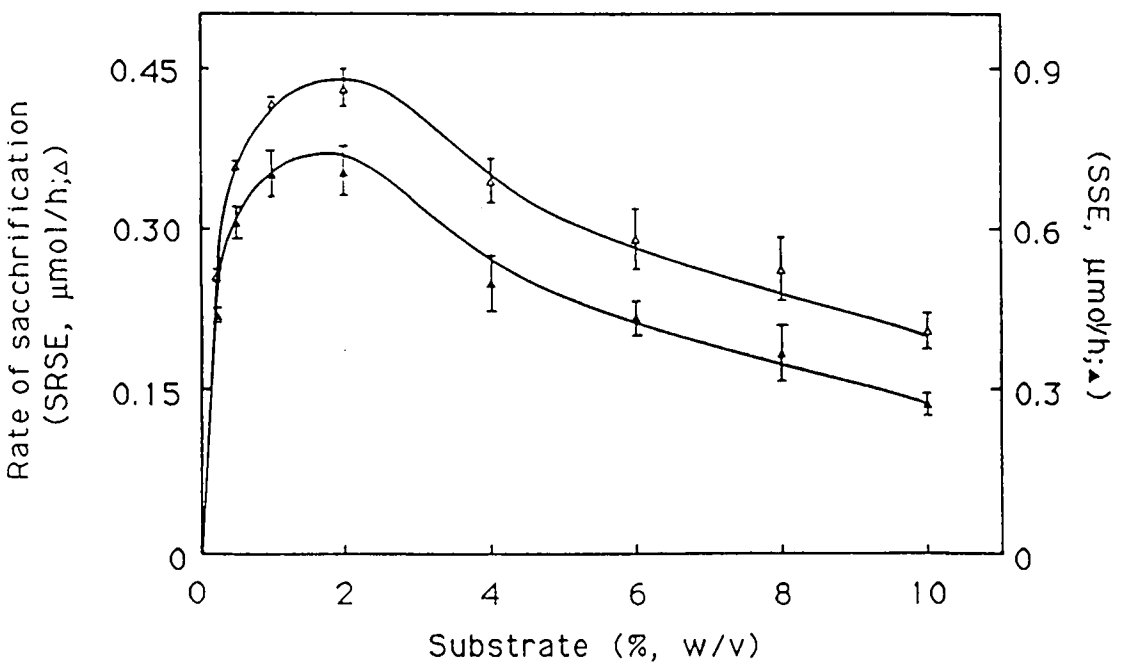


Fig. 10 b) Substrate-activity profiles for the complete cellulase system at enzyme concentration of 4.4 X 10^{-3} IU per ml. The reaction conditions were as in Figure 10a. SRSE, solubilized reducing sugar equivalents; SSE, total solubilized sugar equivalents.

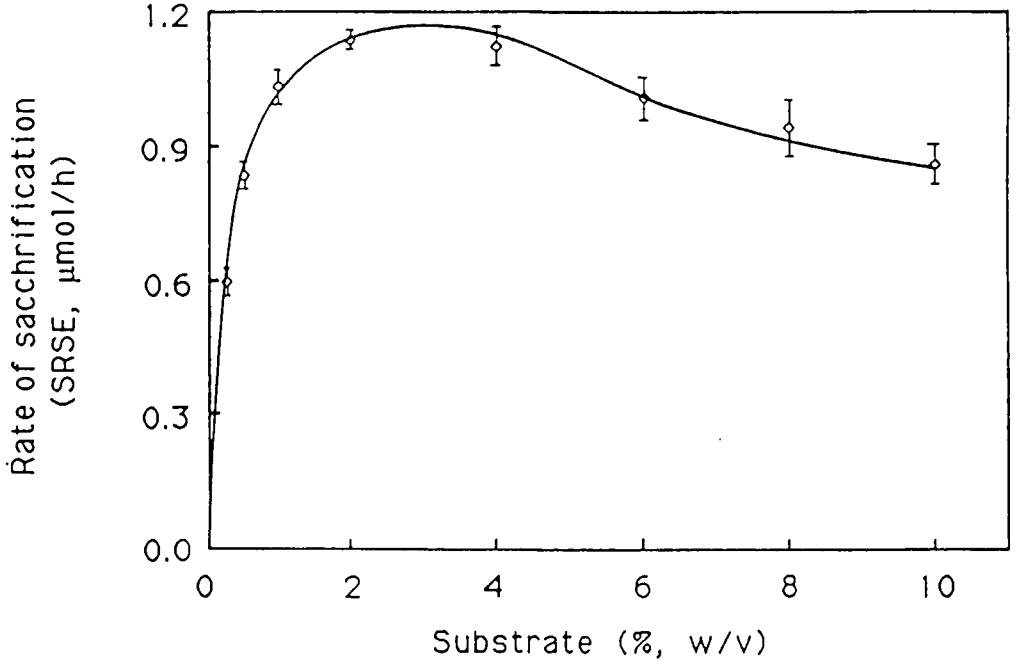


Fig. 10 c) Substrate-activity profiles for the complete cellulase system at enzyme concentration of 8.8 X 10^{-3} IU per ml. Reaction conditions were as in Figure 10a. SRSE, solubilized reducing sugar equivalents.

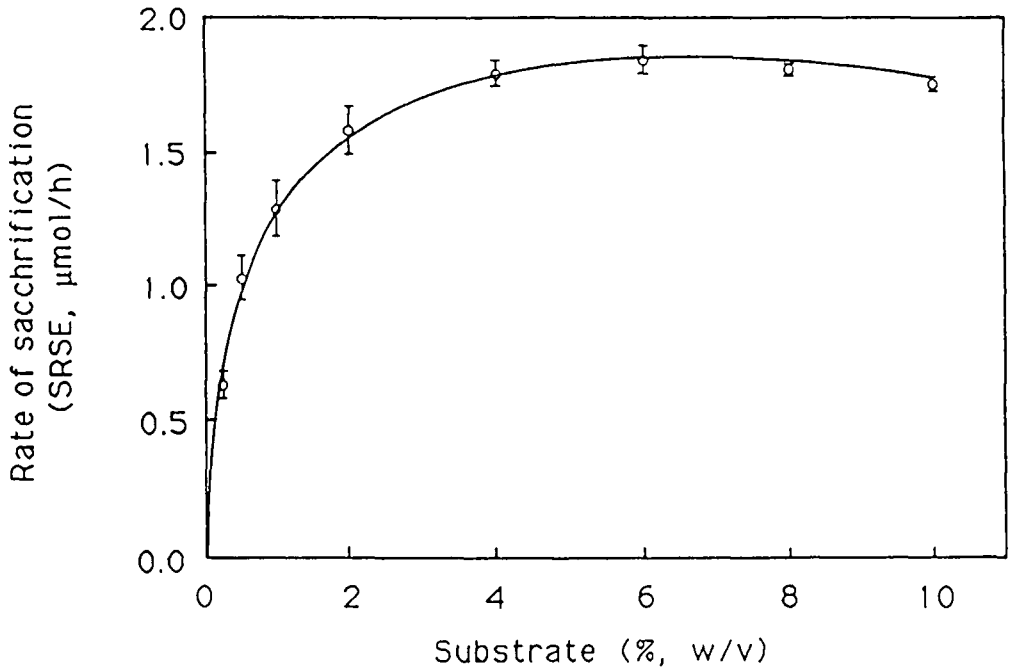


Fig. 10 d) Substrate-activity profiles for the complete cellulase system at enzyme concentration of 17.6 X 10^{-3} IU per ml. Reaction conditions were as in Figure 10a. SRSE, solubilized reducing sugar equivalents.

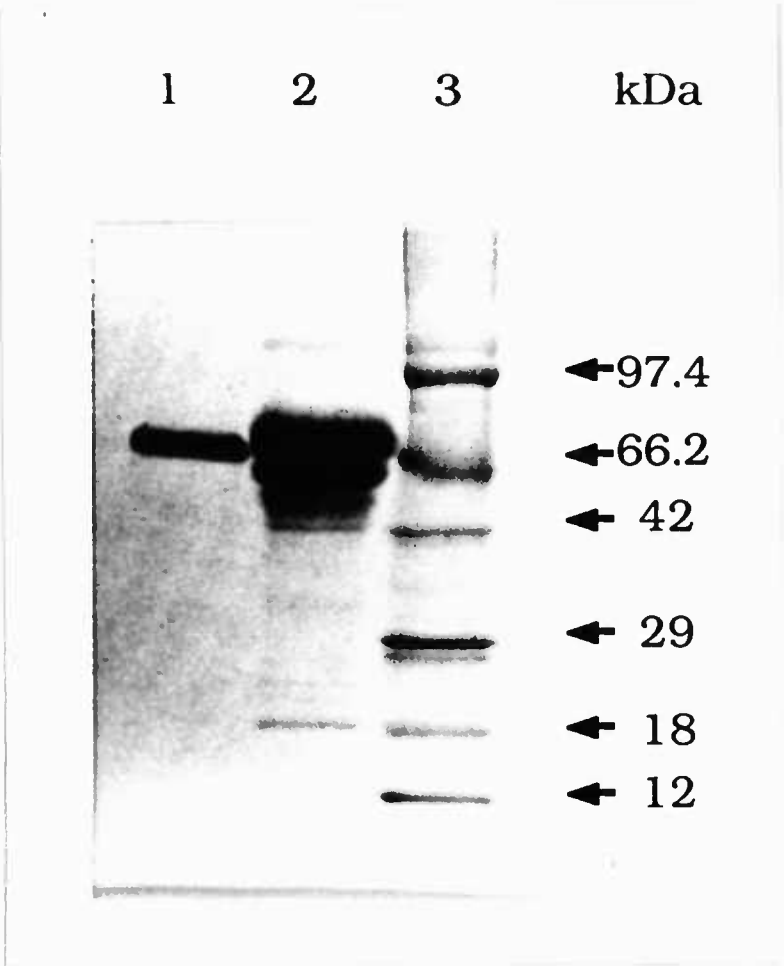


Fig. 11 a) SDS-polyacrylamide gradient gel electrophoresis of cellulases. (1) CBHI (20 μg); (2) Complete cellulase (60 μg); (3) Standard proteins (phosphorylase, BSA, actin, carbonic anhydrase, TnC, cytochrome C, 97.4 to 12 KDa).

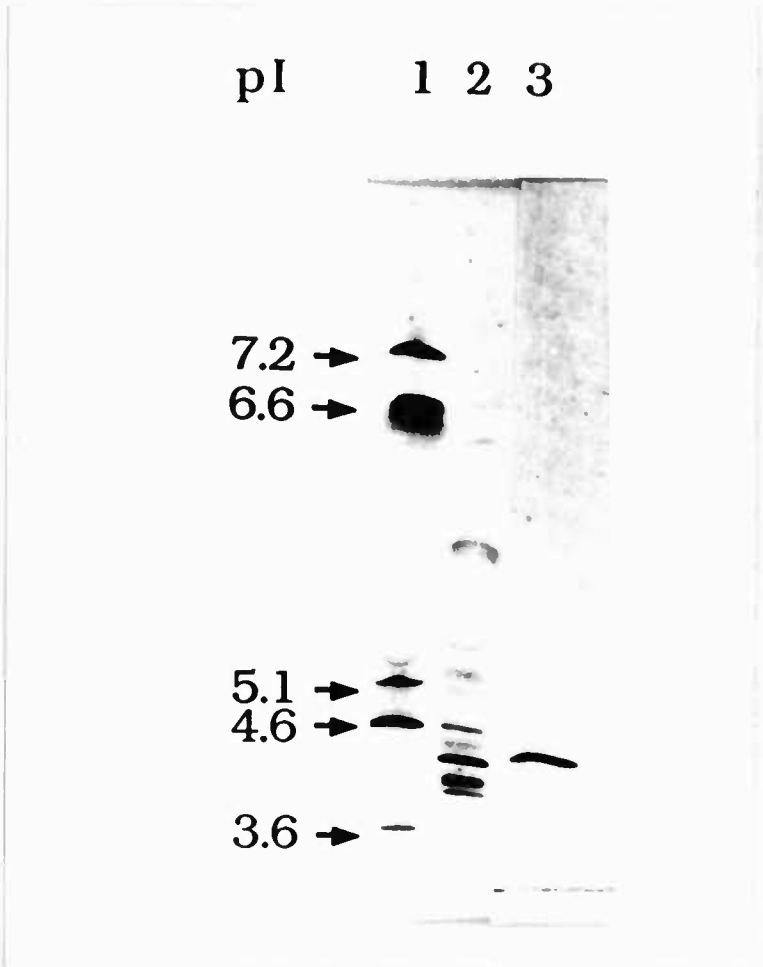


Fig. 11 b) Analytic isoelectric focusing of cellulases in the pH range 3.0-10.0. (1) pI markers (myoglobin, carbonic anhydrase I, β-lactoglobulin A, trypsin inhibitor, amyloglucosidase, pI 7.2 to 3.6);1 (2) Complete cellulase (120 μg); (3) CBHI (15 μg).

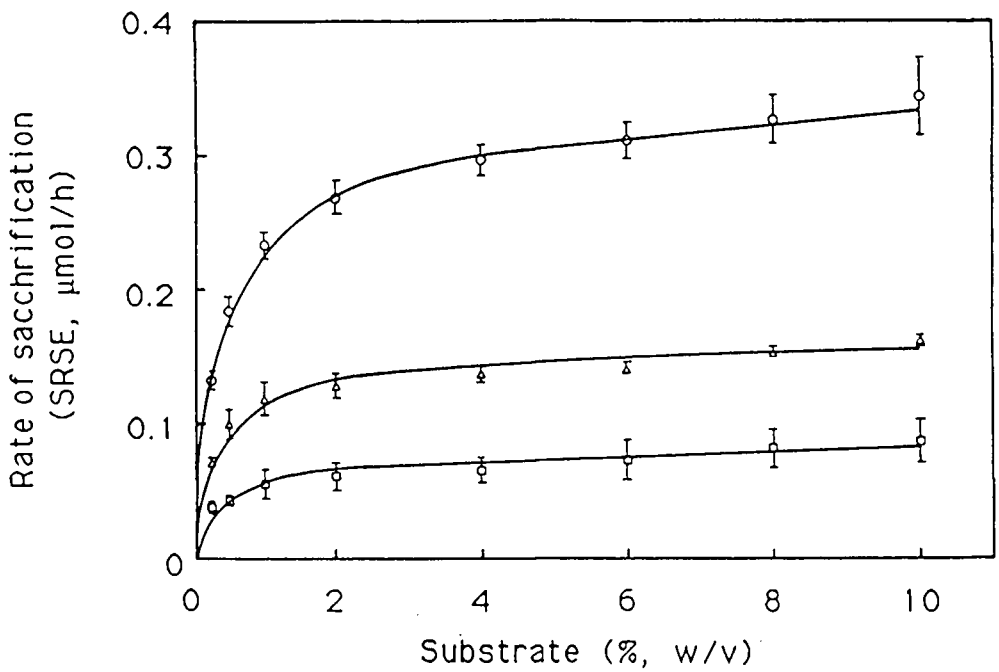


Fig. 12 a) Substrate-activity profiles for cellobiohydrolase at different enzyme concentrations. Enzyme concentrations were 3.3(□), 6.6 (△) and 13.2 (○) µg per ml. Reaction conditions were as in Figure 10a. SRSE, solubilized reducing sugar equivalents.

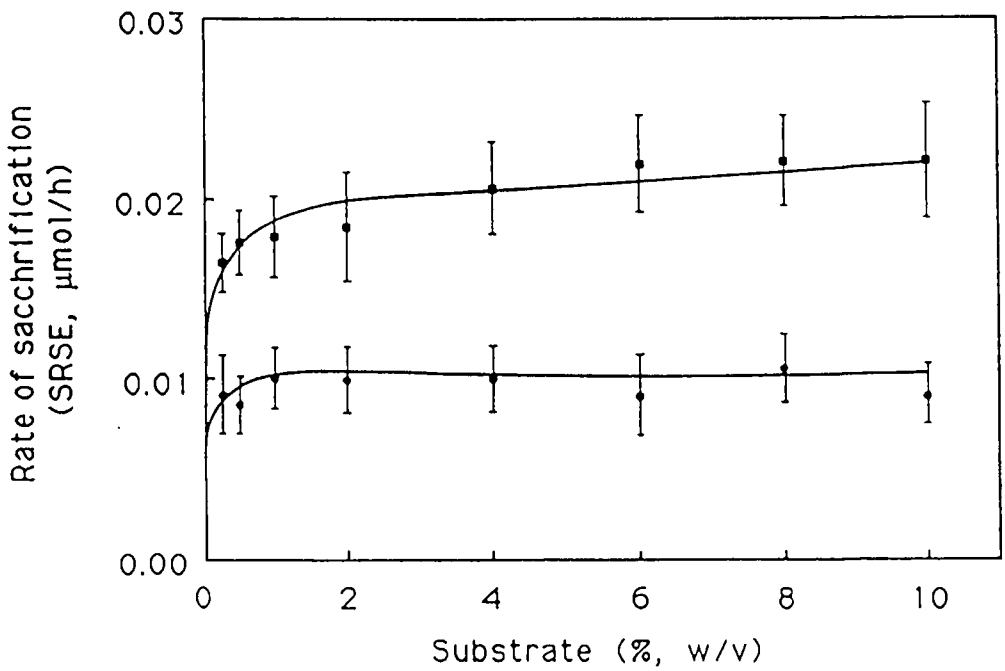


Fig. 12 b) Substrate-activity profiles for cellobiohydrolase at different enzyme concentrations. Enzyme concentrations were 0.8 (•), 1.6 (■) µg per ml. Reaction conditions were as in Figure 10a. SRSE. solubilized reducing sugar equivalents.

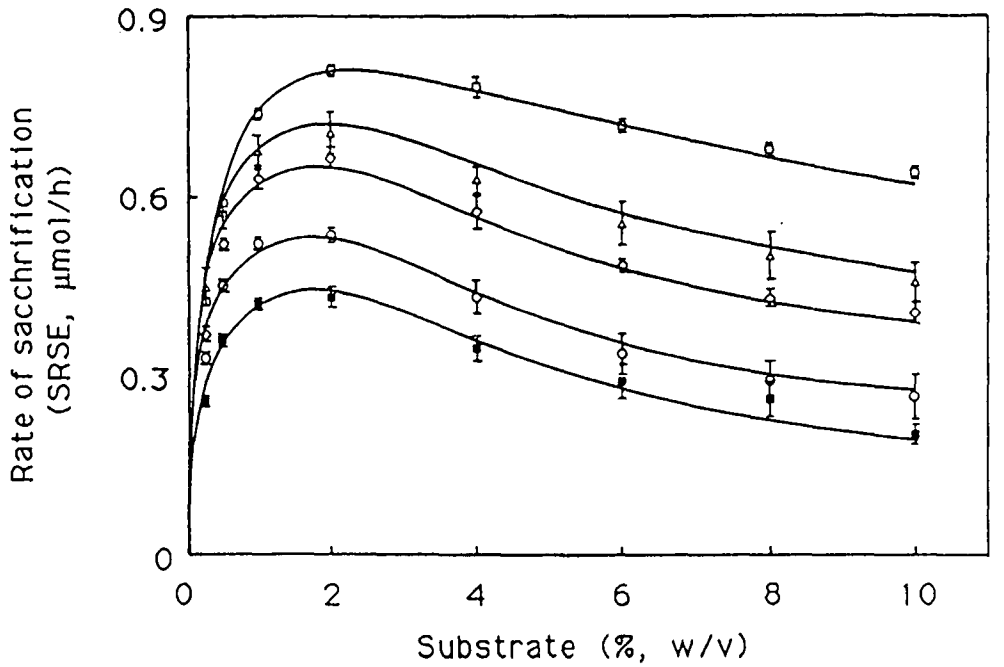


Fig. 13 Effect of added cellobiohydrolase on substrate-activity profiles for the complete cellulase system. The complete cellulase concentration was 4.2 µg protein per ml (4.4 X 10^{-3} IU per ml) in all cases. The added cellobiohydrolase concentration was 0 (■), 1.65 (○), 3.31 (◇), 4.96 (△) or 6.62 (□) µg per ml. Reaction conditions were as in Figure 10a. SRSE, solubilized reducing sugar equivalents.

CHAPTER VI. INTERRELATIONSHIPS OF PRODUCT RATIOS, CELLOBIASE ACTIVITY, AND SUBSTRATE-ACTIVITY PROFILES OF *T. reesei* CELLULASE

Introduction

Trichoderma cellulases have been the focus of extensive research due to their potential economic importance with respect to the saccharification of biomass cellulose. Their potential is largely based on their high rates of production and their ability to hydrolyze crystalline cellulose (Mandels, 1985). The exact mechanism(s) through which these enzyme systems catalyze the hydrolysis of relatively inert crystalline cellulose is not clear. *Trichoderma* enzyme systems are generally considered to be composed of three principal enzyme activities; endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and cellobiase (EC 3.2.1.21) (Coughlan and Ljungdahl, 1988). It is generally accepted that the endoglucanase and cellobiohydrolase components act synergistically on the crystalline substrate, producing cellobiose and glucose. The cellobiose is then hydrolyzed to glucose in the presence of sufficient cellobiase activity (Chan et al. 1989; Ferchak and Pye, 1983; Sternberg, 1976).

The kinetics of cellulose saccharification are complicated, as may be expected for a heterogeneous reaction system consisting of several enzymes. A kinetic property of particular relevance to cellulose saccharification is substrate

inhibition, which has been observed by several groups (Howell and Struck, 1975; Lee and Fan, 1982; Okazaki and Moo-Young, 1970; Ryu and Lee, 1986; Wood and McCrae, 1975) and further characterized in our laboratory (Huang and Penner, 1991; Liaw and Penner 1990). Substrate inhibition is important with respect to the interpretation of comparative studies contrasting cellulase preparations, lignocellulosic pretreatments and reactor designs (Liaw and Penner, 1990). The molecular mechanism(s) underlying the observed substrate inhibition has not been experimentally defined. Previous studies showing substrate inhibition have been based on empirical methods of product analysis, such as reducing sugar and total sugar assays, which do not provide information on the product composition of reaction mixtures. Therefore, very little is known about how product profiles change when reaction mixture conditions change from those showing traditional saturation kinetics to those showing substrate inhibition. This is of interest due to the differences observed in the inhibitory properties of the expected products, glucose and cellobiose (Ferchak and Pye, 1983; Ohmine et al. 1983; Van Dyke, 1972; Wood and McCcrae, 1978). Compared to glucose, cellobiose is a strong inhibitor of cellulose saccharification (Halliwell and Griffin, 1973; Mangat and Howell, 1978; Holtzapple et al. 1989; Ryu and Lee, 1986). Cellobiose is generally present in reaction mixtures due to the limiting cellobiase activity of most *Trichoderma* cellulase preparations (Mandels et al. 1981). Reaction mixtures are often supplemented with a source of cellobiase in order to decrease the cellobiose to glucose ratio and, hence, increase the overall rate of

saccharification.

In this paper we address three questions relevant to the apparent substrate inhibition reflected in the substrate-activity profiles of the *T. reesei* cellulase system. First, studies demonstrating substrate inhibition have not identified reaction mixture products. This makes it difficult to establish if, and how, changes in product profiles are associated with substrate-activity profiles. In this study we have demonstrated that although product ratios differ for reaction mixtures at different substrate concentrations, these differences do not appear to account for the apparent substrate inhibition. Second, in practical cellulose saccharification systems utilizing *Trichoderma* cellulases, the reaction mixture is traditionally supplemented with additional cellobiase activity. Previous studies have not addressed the question of how cellobiase supplementation modifies the observed substrate-activity profiles. In this study we have demonstrated that although reaction rates are increased due to cellobiase supplementation, this supplementation does not elevate the apparent substrate inhibition. Third, Michaelis-Menton kinetics predict that when certain classes of inhibitors are present as contaminants of a substrate preparation they will cause an apparent substrate inhibition. The role of this type of inhibition had not been determined with regard to *Trichoderma* cellulases acting on microcrystalline substrates. In this study we show that soluble inhibitors of this type do not appear to influence the observed substrate-activity profiles.

Materials and methods

Cellulase preparation. The complete cellulase preparation was produced by *T. reesei* QM9414 in our laboratory using shake flask cultures as described by Mandels et al. (1981). The properties of the enzyme preparation were reported previously (Huang and Penner,1991).

Cellobiase purification. A commercial cellobiase preparation (Cellobiase, NOVO) derived from *Aspergillus niger* was used as the starting material for the following purification. Fifty ml of cellobiase solution was diluted to 550 ml with 50 mM sodium acetate buffer, pH 5.0, then concentrated to 70 ml with a Millipore PM7178 membrane. The dilution/concentration step was repeated twice. Protein was then precipitated by the addition of two volumes cold acetone, 4°C, pelleted by centrifugation, washed twice with cold acetone, and dried under vacuum. The resulting powder represented crude cellobiase. Six hundred mg of crude cellobiase in 4 ml of 50 mM ammonium acetate, pH 5.0, was chromatographed isocratically on a Sephadex G100 column (2.6 x 46 cm), at 4°C, using a flow rate of 20 ml/h (Fig. 14a). Fractions comprising peak 1, which had the highest specific activity, were pooled and freeze-dried. Ninety mg of freeze-dried powder was dissolved in 6 ml 50 mM ammonium acetate, pH 3.5, and loaded on an SP-Sephadex cation exchange column (2.6 x 20 cm), equilibrated with the same buffer, at 4°C. The column was washed with starting buffer until eluent was protein free based on A_{280}, approximately 130 ml. A 400

ml gradient of 50 mM ammonium acetate buffer, pH 3.5 to 6.0, was run, followed by washing with 0.5 M NaCl, 50 mM sodium acetate, pH 6.0. Fractions comprising the major protein peak, peak II (Fig. 14b), were pooled and freeze-dried. The resulting enzyme powder was utilized as purified cellobiase.

Cellobiase characterization. The purified cellobiase was characterized with respect to specific activity, mol. wt. and isoelectric point. Substrates used for activity measurements were microcrystalline cellulose (Avicel PH 101, FMC Corp.), carboxymethylcellulose (CMC7HOF, Aqualon Co. Wilmington, Delaware), p-nitrophenyl-β-D-glucopyranoside (pNPG) and cellobiose (Sigma Chemical Co.). Protein was measured with bicinchoninic acid as described by Smith et al. (1985), using bovine serum albumin (Sigma Chemical Co.) as the reference standard. All activity measurements were performed at 50°C in 50 mM sodium acetate, pH 5.0. Cellobiase activity was determined by measuring glucose production from cellobiose as follows. The reaction was initiated by adding 0.05 ml enzyme solution to 0.45 ml temperature-equilibrated 8 mM cellobiose in buffer. Reactions were terminated after 10 min by immersion in a boiling water-bath for 5 min. Glucose produced was then determined by glucose oxidase (Glucose Diagnostic Kit, No. 510, Sigma Chem. Co), and HPLC. Cellobiase activity is reported as μmoles substrate hydrolyzed per min. Specific activities with respect to the pNPG, CMC and microcrystalline substrates were determined as described previously (Huang and Penner, 1991) and expressed in terms of μmoles of product produced per min. Michaelis parameters, K_m and V_{max}, were determined

for the pNPG and cellobiose substrates. Substrate concentrations for these assays ranged from 0.1 to 1.6 mM for pNPG and 1.0 to 8.0 mM for cellobiose. The kinetic constants were estimated from double-reciprocal plots.

Electrophoretic analysis of the enzyme preparation was based on SDS gradient (9-18%) gel electrophoresis and analytical isoelectric focusing (IEF). The methodology and instrumentation have been described (Huang and Penner, 1991).

The stability of the purified cellulase preparation was verified by testing the linear nature of the reaction, under the defined conditions, witl the pNPG substrate. Reaction mixtures contained 3.3 mM substrate and either 7.34 X 10^{-6}, 1.47 X 10^{-5}, or 2.9 X 10^{-5} IU enzyme per ml in 50 mM sodium acetate, pH 5.0, at 50°C. Reaction mixtures were intermittently sampled and assayed for product over a 40 min reaction period. Reactions were terminated by addition of 2.5 ml 0.1 M sodium carbonate to 0.25 ml of reaction mixture. The extent of reaction was determined based on A_{400} using p-nitrophenol (Sigma Chem. Co.) as a calibration standard. Enzyme stability in the absence of substrate was similarly determined with the exception that substrate was added after a given incubation period (up to 18 h) of enzyme in buffer at 50°C.

Enzymatic hydrolysis of microcrystalline cellulose. The standard assay was performed at 50°C in 50 mM sodium acetate buffer, pH 5.0 and 4.8 X 10^{-3} IFPU of cellulase per ml. Substrate concentrations ranged from 0.25 to 10%. Selected reaction mixtures were supplemented with an additional 0.16 or 0.64 IU of

cellobiase activity per ml. Reactions were initiated by addition of enzyme and agitated at 160 RPM as described by Huang and Penner (1991). Reactions were terminated at 5 h by immersion of the reaction mixture in a boiling water-bath for 5 min, followed by filtration through a Whatman GF 0.45 μm membrane. The supernatant was then analyzed for solubilized reducing sugar by the method of Nelson (1944) and Somogyi (1952), using glucose as the calibration standard, and for glucose, cellobiose and higher cellodextrins by HPLC, using glucose, cellobiose and cellotriose as reference standards.

High Performance Liquid Chromatography (HPLC). Reaction mixture products were analyzed by HPLC (Shimadzu Model LC-6A), equipped with an RID-6A differential refractometer and a CR 601 data processor. Following filtration, reaction mixture products were routinely chromatographed on an Aminex HPX-87H column, 300 x 7.8 mm (BioRad, Richmond, CA) using a 0.005M H_2SO_4 mobile phase. The column temperature was 65°C and the flow rate 0.6 ml/min. Glucose, cellobiose and cellotriose reference standards (Pfanstiehl Laboratories, Inc., Waukegan, IL) were used for detector calibration. Reaction mixtures were also chromatographed on an HPX-45A column (BioRad, Richmond, CA) in studies questioning the presence of higher cellodextrins. Terminated, filtered reaction mixtures (1 ml) were treated with 0.4 g AG 501-X8 resin (BioRad) for acetate removal prior to chromatography on the HPX-45A column.

Assay for soluble inhibitory impurities in the microcrystalline substrate. A

10% substrate solution of microcrystalline cellulose in standard buffer was incubated/agitated at 50°C for 1 h and then filtered (Whatman #1). The resulting filtrate containing the original buffer components plus any extracted species was then used to prepare 2% substrate solutions containing fresh microcrystalline cellulose, i.e. microcrystalline cellulose that had not been previously exposed to buffer. The time course of saccharification for these reaction mixtures was compared to that of controls. Control reaction mixtures contained 2% fresh microcrystalline cellulose in the standard buffer. The standard buffer composition and reaction conditions were equivalent to those described previously. The enzyme concentration for each of these reaction mixtures was 9.6 X 10^{-3} IFPU per ml. Reactions were terminated at selected times by immersing 0.5 ml of reaction mixture in a boiling water bath. Terminated samples were centrifuged at 10,000 g for 10 minutes and the supernatants assayed by HPLC as described above. Replicate time courses were assayed in triplicate for the test and control reaction mixtures.

Results

Effect of substrate concentration on rates of saccharification and product profiles. The data of Fig. 15 demonstrates the apparent substrate inhibition of *T. reesei* catalyzed saccharification of microcrystalline cellulose. "Apparent substrate inhibition", as used in this report, refers to any apparent decrease in

the rate of saccharification which accompanies an increase in substrate concentration. The substrate-activity profile is similar to that reported previously for *T. reesei* (Huang and Penner, 1991) and *T. viride* (Liaw and Penner, 1990) cellulase preparations. It is readily apparent that the rate of solubilization of reducing sugar equivalents increases as the substrate concentration is increased to an optimum of approximately 2% (w/v) under these reaction conditions. However, increasing substrate concentrations above this optimum resulted in apparent decreases in the rate of saccharification.

To better understand the basis of this kinetic behavior we have further analyzed the product profiles produced under the reaction conditions given in Fig. 15. HPLC analysis of terminated reaction mixtures indicated the predominant product was cellobiose in each of the reaction mixtures. All reaction mixtures contained glucose. Cellodextrins larger than cellobiose were not detected under these reaction conditions. The predominance of cellobiose agrees with observations suggesting that cellobiase is limiting in *Trichoderma* cellulase preparations (Mandels et al. 1981). The substrate-activity profiles of Fig. 16, each representing different products, are consistent with the reducing sugar assays of Fig. 15. It is of interest to compare the relative concentration of cellobiose to glucose in the different reaction mixtures since cellobiose is reportedly a more potent inhibitor of cellulose saccharification than glucose (Halliwell and Griffin, 1973; Mangat and Howell, 1978; Holtzapple et al. 1989; Ryu and Lee, 1986). Our results suggest that the cellobiose to glucose ratio is a function of the rate of the

reaction (Fig. 17). The ratio was highest at the optimum substrate concentration (Fig. 17a). The product ratio decreases as the reaction rate is decreased, regardless of whether the reaction rate is decreased by increasing or decreasing the substrate concentration relative to that at the optimum. However, it is apparent from the data presented in Fig. 17b that the relationship between the ratio of the products and the rate of the reaction, as modified in these experiments, is not strictly equivalent for reaction mixtures with substrate concentrations above versus below the optimum.

Effect of cellobiase supplementation on rates of saccharification. The consequences of cellobiase supplementation and the importance of product inhibition with respect to the observed substrate-activity profiles were tested by supplementing the enzyme preparation with graded levels of cellobiase activity. The supplemental cellobiase used for these experiments was purified to near homogeneity based on SDS gel electrophoresis and IEF (Fig. 18). The diagnostic properties of the cellobiase preparation are presented in Table 11. Specific activity measurements on microcrystalline cellulose and CMC suggest negligible activities corresponding to traditional endoglucanase and cellobiohydrolase enzymes. The rate of cellobiase catalyzed hydrolysis of pNPG was found to be linear over a 40 min reaction period, indicating the apparent stability of the enzyme under reaction conditions. Further analyses suggested reaction mixture cellobiase activities were stable for greater than 18 h.

Cellobiase supplementation was done at two levels, corresponding to an

additional 0.16 and 0.64 IU of cellobiase activity per ml. Reaction mixtures containing the non-supplemented *T. reesei* enzyme contained only 5.1 X 10^{-4} IU cellobiase activity per ml. In each case, cellobiase supplementation effectively eliminated cellobiose as a detectable reaction mixture product (Fig. 19). The addition of purified cellobiase to the complete cellulase system increased the rate of saccharification at all substrate concentrations. Cellobiase supplementation did not appear to have a significant effect on the substrate concentration corresponding to the maximum rate of saccharification. Consequently, the substrate-activity profile for the non-supplemented and supplemented enzyme preparations are shifted relative to each other with respect to rates of saccharification at given substrate concentrations, but the general contour of the profiles remains the same. Therefore, cellobiase supplementation does not alleviate the apparent substrate inhibition of this enzyme system.

Assay for inhibitory contaminants in the microcrystalline substrate. Our experimental design tested for soluble inhibitors by comparing the time course of saccharification for reaction mixtures, (prepared with buffer previously used for extraction of potential contaminants in the microcrystalline substrate) with that of reaction mixtures prepared with fresh buffer. The time course of saccharification was essentially identical for reaction mixtures composed of the fresh buffer and the buffer containing potential contaminants (Fig. 20). The predominant product of each reaction mixture was cellobiose, in agreement with the data of Fig. 16. However, in this experiment, which had double the enzyme concentration of those

discussed previously, higher cellodextrins could be detected in each reaction mixture. In this experiment a decrease in the rate of saccharification for reaction mixtures containing the "test" buffer relative to those containing the "control" buffer would be expected if the cellulose substrate were contaminated with a soluble inhibitor(s). This result was not observed in the present study.

Discussion

The cellobiose to glucose ratio resulting from the saccharification of cellulose is expected to have a significant effect on the rate of saccharification due to the differences in the inhibitory properties of glucose and cellobiose, cellobiose being the more potent inhibitor. The results of this study show that this product ratio changes over the substrate range tested and that the change appears to be a function of the rate of the reaction. It is likely that, under the defined conditions, the rate of the reaction governs the product ratio and not *vice versa*. This conclusion appears justified due to the relatively strong inhibitory properties of cellobiose and the observation that the cellobiose to glucose ratio, and the cellobiose concentration *per se*, are highest at the optimum substrate concentration. The apparent relationship between the rate of saccharification and the resulting product ratio is depicted in Fig. 17b. It may be expected that an increase in the rate of saccharification reflects an increase in the rate of cellobiose production and, since cellobiase activity is limiting for this enzyme system, will

result in an increase in the ratio of cellobiose to glucose. This reasoning is in general agreement with the observed trends in the data. However, it does not explain the difference in product ratios observed for reaction mixtures produced at nearly equivalent saccharification rates but different substrate concentrations (i.e., the difference in the curve defined by points 1 through 4 versus that defined by points 5 through 8 of Fig. 17b).

Cellobiase supplementation had little effect on the general contour of the substrate-activity profiles. Consistent with other reports (Chan et al. 1989; Ferchak and Pye, 1983; Ghose and Bisaria, 1979; Sternberg, 1976), the data shows that the rate of saccharification is enhanced by cellobiase supplementation. However, the enhanced activity is consistently observed at all substrate concentrations, having little effect on the apparent optimum substrate concentration. Consequently, the substrate inhibition properties of this enzyme system do not appear to be dependent on the cellobiase activity of the enzyme preparation. Increases in saccharification activity with cellobiase supplementation are well documented and presumably the result of lowering the cellobiose concentration. The observed percent increase in rate of saccharification due to cellobiase supplementation is dependent on the method used to assay the reaction products. In our experiments, the addition of 0.64 IU cellobiase activity per ml reaction mixture resulted in an apparent 71% increase in saccharification rate based on solubilized glucose equivalents (calculated from HPLC measurements as 2 times cellobiose plus glucose) and an apparent 178% increase in

saccharification rate based on solubilized reducing sugar equivalents. The discrepancy can be accounted for by the increase in reducing sugar equivalents resulting from the hydrolysis of existing cellobiose to glucose. Therefore, the actual % increase in saccharification is best based on the assay of individual products rather than the more general reducing sugar method. This reasoning is, of course, based on the premise that "saccharification" refers to the solubilization of cellulose as saccharide, be mono, di, tri, etc. The reason for the observed differences in saccharification rates for reaction mixtures supplemented with different levels of cellobiase is not readily apparent. No cellobiose was detected in any of the supplemented reaction mixtures. Therefore, if increased saccharification rates were only the result of decreased cellobiose inhibition, then additional cellobiase supplementation above that observed at the lowest level required to remove cellobiose should have no effect. That was not the case. It is possible that the cellobiase supplemented saccharification rates above that predicted based on alleviation of cellobiose inhibition are associated with contaminating non-cellobiase enzyme activities or unexpected properties of the cellobiase enzyme itself.

A mechanism which may account for substrate inhibition in certain cases is that of an inhibitor being present in all reaction mixtures in constant proportion to the variable substrate. It has been shown for homogeneous enzyme systems that linear uncompetitive inhibition, hyperbolic uncompetitive inhibition and liner noncompetitive inhibition will all produce an apparent substrate inhibition under

these conditions; such as occurs when the inhibitor is present as an impurity in the substrate (Cleland et al. 1973). The potential for this mechanism was of particular interest for this system since the enzyme preparation does not exhibit substrate inhibition with all cellulose substrates (Liaw and Penner). However, the data of Fig. 20 suggests that this type of soluble inhibitor is not responsible for the observed behavior.

Table 11. Properties of the purified cellobiase

Item	
Mol. Mass (kDa)	116
pI	4.2
Opt. pH[a]	4.2-4.5
pH stability[a,b]	4.2-5.2
Opt. Temperature (°C)[a,c]	60
Stability at 50°C (h)[a]	>18
Activation energy (kJ/mol/°K)[a,d]	50.5
K_m (mM)	
Cellobiose[e]	1.59±0.10
pNPG[f]	1.22±0.15
V_{max}[g] (IU/mg protein)	
Cellobiose[e]	292±25
pNPG[f]	255±27
Sp. activity (IU/mg protein)	
Cellobiose[h]	202.9
pNPG[i]	141.0
Avicel	0.01
CMC	0.02

[a]Using pNGP as substrate.
[b]Cellobiase solutions were in 50 mM Citrite-phosphate buffer pH range from 2.6-8.0 and incubated at 4°C for 24 h. Cellobiase activity was then assayed in 50 mM NaAc, pH5.0, at 50°C as described in the text.
[c]Temperature range: 30-80°C.
[d]Temperature range: 30-60°C.
[e]Substrate concentration 1.0-8.0 mM.
[f]Substrate concentration ranged 4.1-1.6 mM.
[g]V_{max} values calculated from doube-reciprocal plots.
[h]Substrate concentration at 8.0 mM.
[i]Substrate concentration at 3.3 mM.

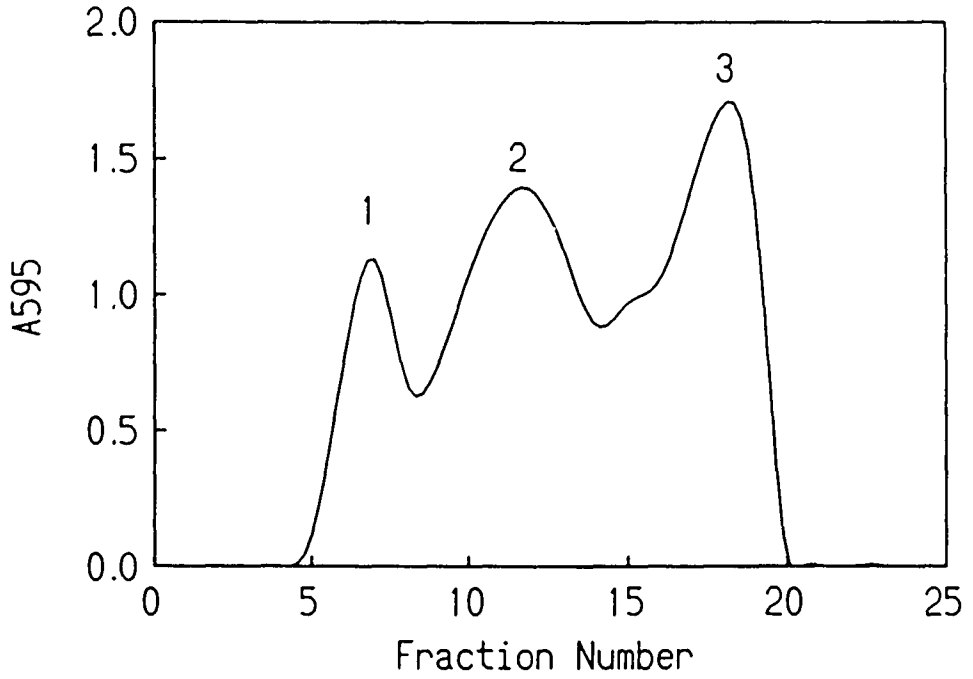


Fig. 14 a) Chromatography of original cellobiase preparation on Sephadex G100. The relative protein content of fractions was measured as A595 based on the Bradford dye-binding procedure (Bio-Rad Protein Assay). The flow rate was 20 ml/h and fraction volume was 7.5 ml.

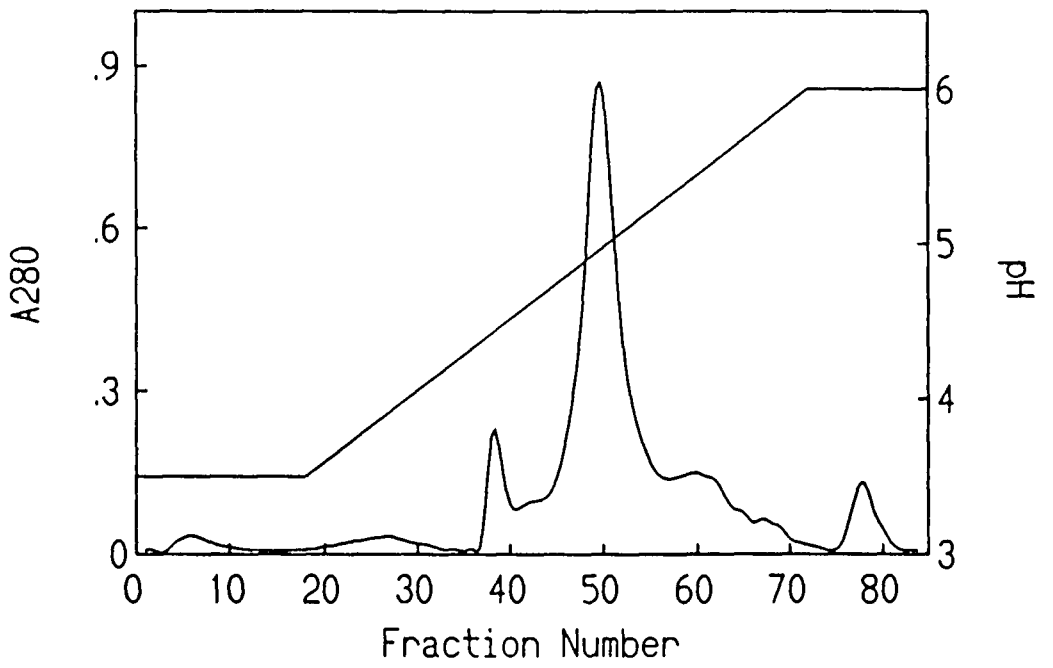


Fig. 14 b) Chromatography of Sephadex G-100 fractionated peak1 cellobiase on SP-sephadex. Relative protein content of fractions was determined by A280. The flow rate was 30 ml/h and fraction volume was 7.5 ml. Fractions 18 - 72 correspond to a 400 ml pH gradient, pH 3.5 to 6.0, in 50 mM sodium acetate. Fractions 73-85 were eluted with 0.5 M NaCl in 50 mM sodium acetate pH 6.0.

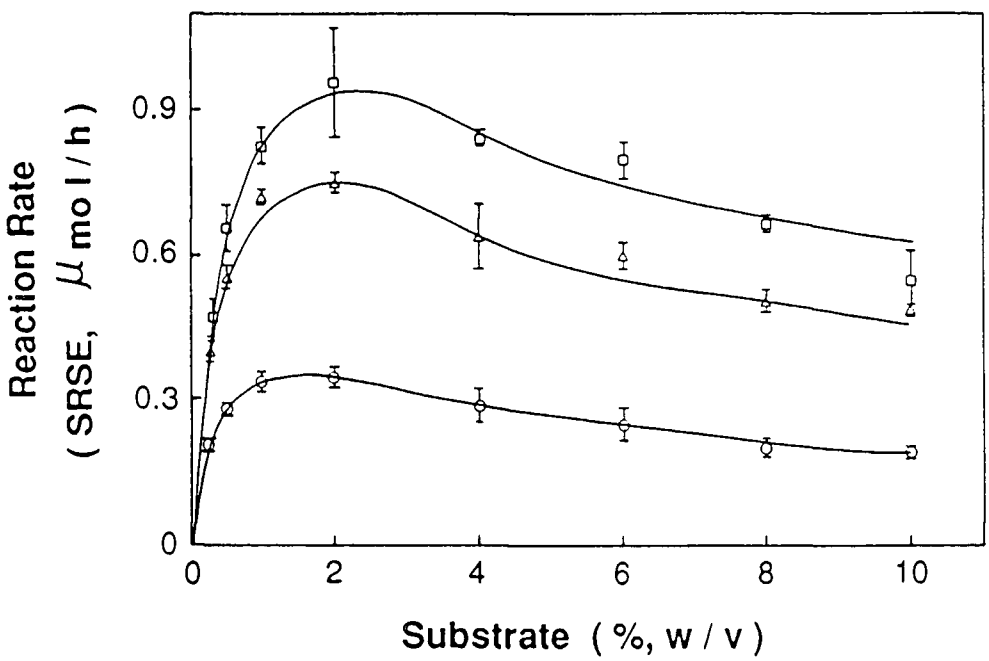


Fig. 15 Substrate-activity profiles for cellulase saccharification of microcrystalline cellulose at different levels of cellobiase supplementation. *Trichoderma* cellulase (4.8×10^{-3} IFPU per ml) was supplemented with either 0.0 (○), 0.16 (△), or 0.62 (□) IU cellobiase per ml. Reaction conditions were as in Figure 10a. Products were measured as solubilized reducing sugar equivalents (SRSE). Each data point represents the mean±SEM of three experiments.

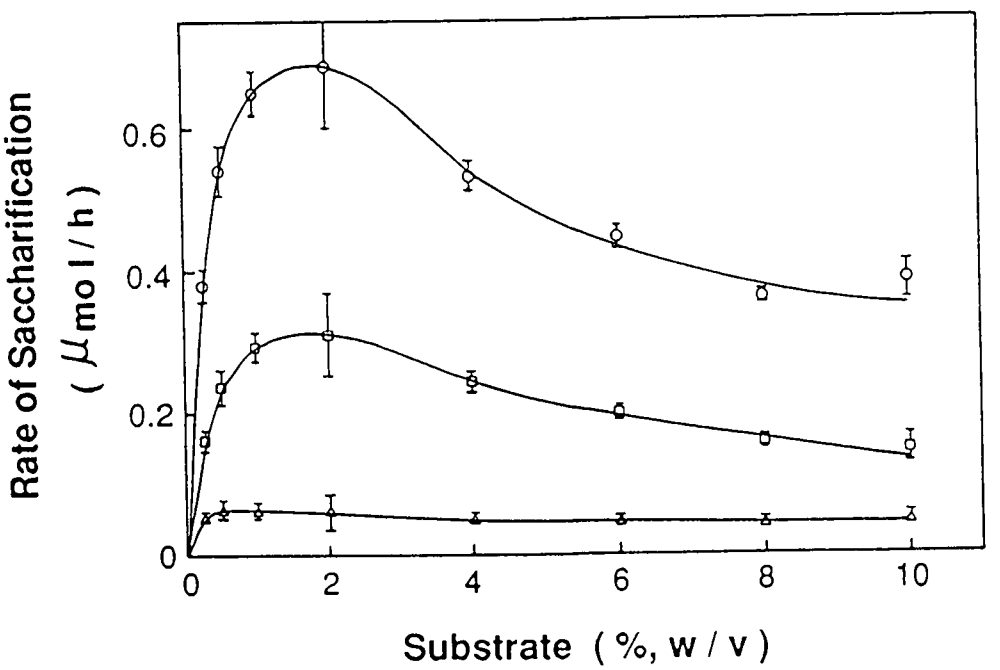


Fig. 16 Substrate-activity profiles based on solubilization of glucose (▵), cellobiose (□) and theoretical glucose equivalents (○) from microcrystalline cellulose. Reaction conditions were as in Figure 10a with 4.8×10^{-3} IFPU cellulase per ml. Theoretical glucose equivalents were calculated as (2 x cellobiose) + glucose. Reaction conditions were as in Figure 10a. Each data point represents the mean±SEM of three experiments.

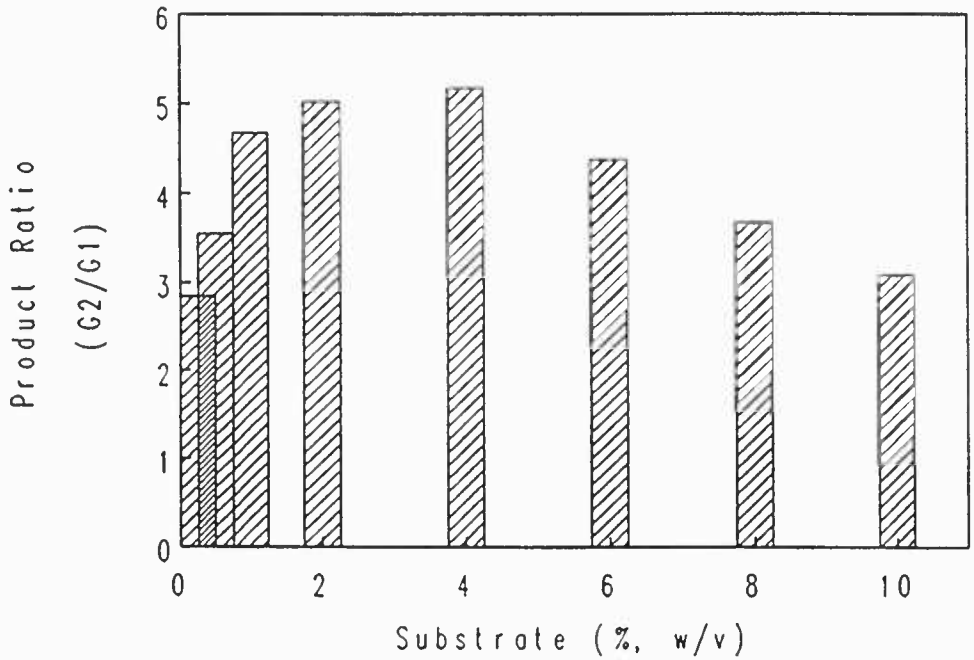


Fig. 17 a) Relationship between initial substrate concentration and reaction mixture product ratio, cellobiose (G2) to glucose (G1). Reaction conditions were as in Figure 10a.

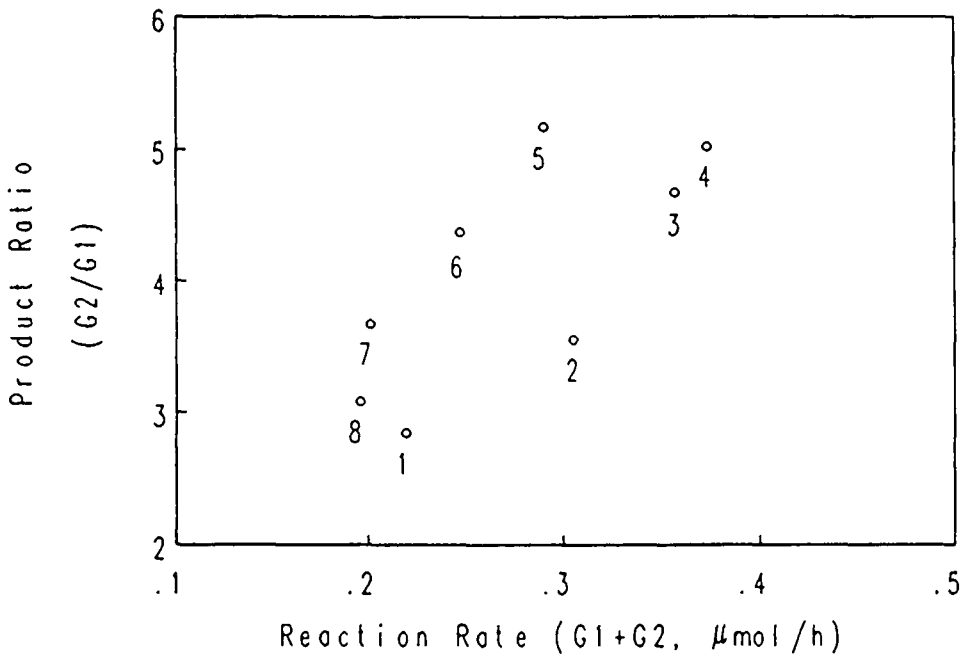


Fig. 17 b) Relationship between reaction rate and reaction mixture product ratio, cellobiose (G2) to Glucose (G1). Reaction rate values calculated as (μmol G2 + μmol G1) per h. Points 1, 2, 3, and 4 (0.25, 0.50, 1.0 and 2.0%, respectively) correspond to increasing substrate concentrations below the optimum. Points 5, 6, 7 and 8 (4.0, 6.0, 8.0, and 10.0%, respectively) correspond to increasing substrate concentrations above the optimum.

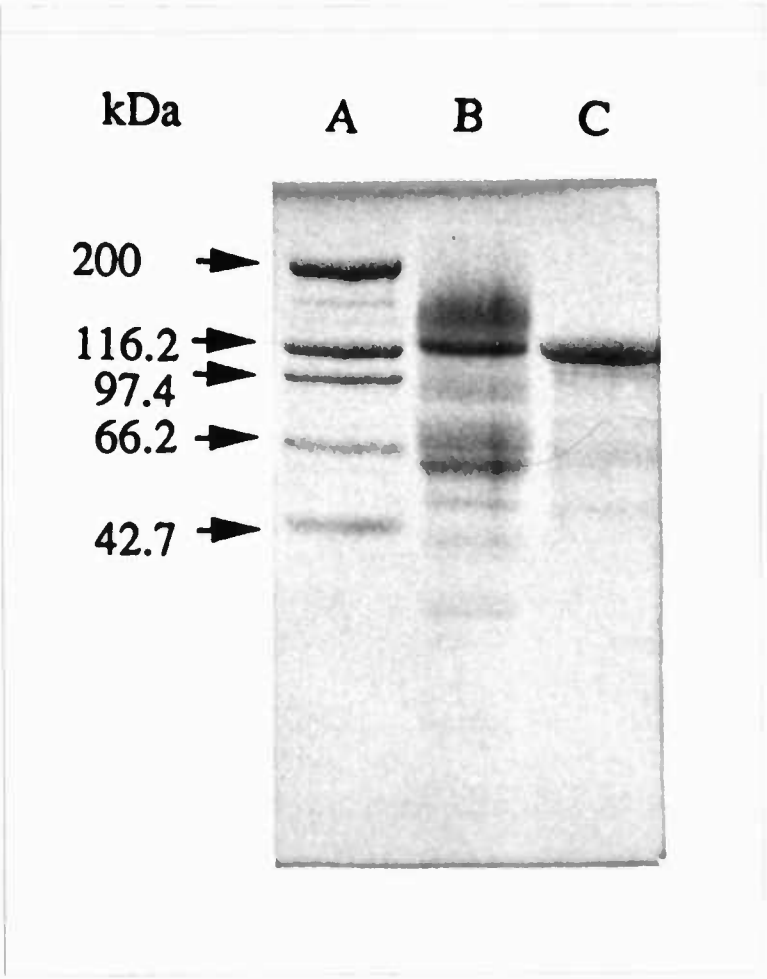


Fig. 18 a) SDS-polyacrylamide gradient gel electrophoresis of cellobiase. Lanes 1 and 4 are standards, 12 to 97.4 kDa (phosphorylase, BSA, actin, carbonic anhydrase, TnC, cytochrome c); 2, purified cellobiase (25 μg); 3, commercial cellobiase (60 μg).

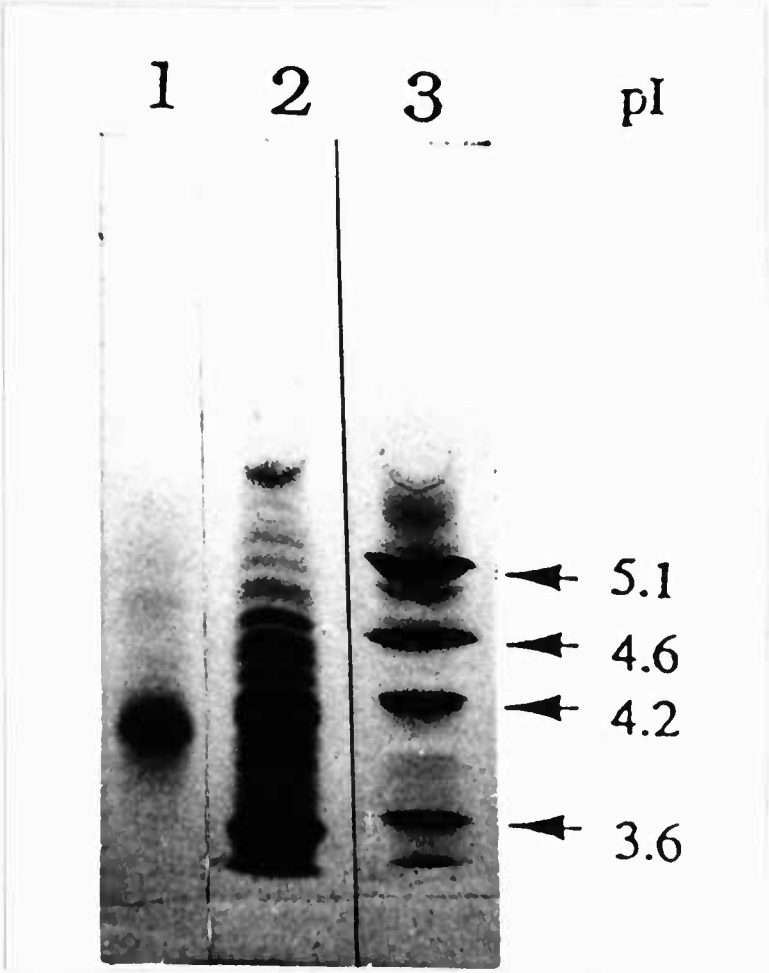


Fig. 18 b) Analytical isoelectric focusing of cellobiase within a pH gradient from 3.0 to 10. (1) purified cellobiase (25 μg); (2) crude cellobiase (100 μg); (3) standards of pI 3.6 to 5.1.

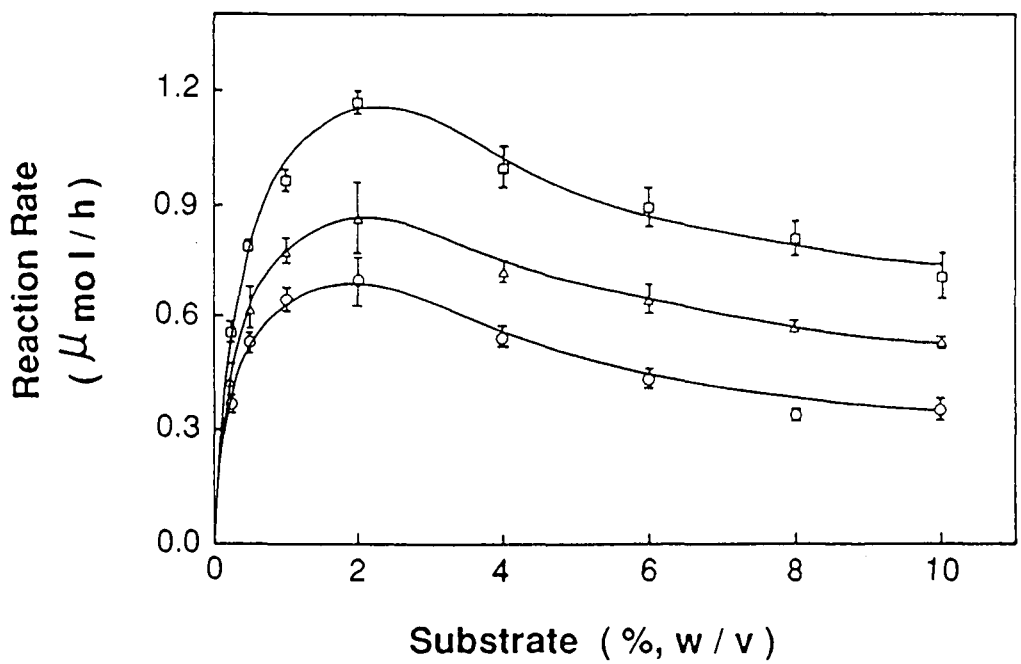


Fig. 19 Substrate-activity profiles for cellulase saccharification of microcrystalline cellulose at different levels of cellobiase supplementation; 0.0 (○), 0.16 (△), and 0.62(□) IU supplemental cellobiase per ml. The non-supplemented curve represents theoretical glucose equivalents, calculated as described in Fig. 17.

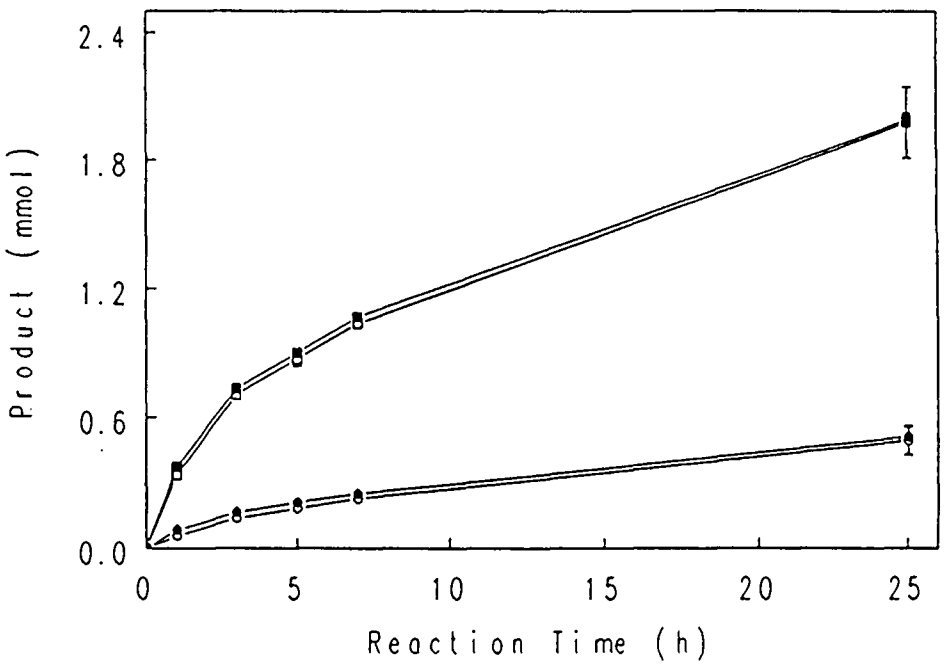


Fig. 20 Time course of saccharification for reaction mixtures composed of fresh buffer (o glucose, □ cellobiose) and test buffer (• glucose, ■ cellobiose). Reaction products were determined by HPLC methods.

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