Apparent Substrate Inhibition of the *Trichoderma reesei* Cellulase System[†]

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The apparent substrate inhibition properties of the cellulase enzyme system from *Trichoderma reesei* have been characterized. Rates of saccharification were measured by quantification of solubilized reducing 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. Graded increases in the reaction mixture enzyme concentration caused approximately proportional increases in the apparent optimum substrate concentration. The maximum inhibition observed was approximately 70% relative to that rate observed at the optimum substrate concentration. A cellobiohydrolase, CBHI, representing approximately 25% of the total enzyme mass was purified and found to obey saturation kinetics under equivalent assay conditions. Addition of CBHI 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.

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 (EC 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 that 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; 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; 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; 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 restricted neither 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 were 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

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Command, Natick, MA). Powdered cellulose, Solka Floc SW40 (James River Inc., Berlin, NH), was used as the primary energy source for cellulase induction. Enzyme was separated from mycelia by filtration after 7 days of incubation. The pH of the enzyme solution was adjusted to 4.8 and the solution concentrated approximately 20-fold using a Millipore PM7178 membrane. The enzyme was then precipitated by the addition of 2 volumes of acetone at 4 °C, separated by centrifugation, washed twice with cold acetone, and dried under vacuum. The resulting powder constituted the complete cellulase preparation. The cellulase concentration in all reaction mixtures is given in IU per milliliter 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 the method of Beldman et al. (1985) eluting with a 0–0.5 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 CBH, was lyophilized and stored desiccated at 4 °C. The purified CBHI concentration in all reaction mixtures is given in micrograms of protein per milliliter.

Gradient gel electrophoresis under denaturing conditions was done according to the method of Laemmli (1970) as modified by Malencik and Anderson (1987). Molecular mass standards ranging from 12 000 to 97 400 daltons were used for molecular mass 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 p*I* was estimated by comparison with protein standards ranging in p*I* from 2.9 to 5.0 (Sigma).

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

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/mL for the complete preparation and from 1.65 to 13.2 μg/mL for purified CBHI. Substrate concentrations ranged from 0.25 to 10%. Reaction mixtures, in 10-mL flasks, were agitated to 160 rpm. Reactions were initiated by the addition of 0.1 mL of enzyme solution to 3.9 mL of temperature-equilibrated substrate solution. Substrate concentrations are expressed in percent (w/v). Protein concentrations are expressed as micrograms of enzyme per milliliter, determined according to 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. Carboxymethylcellulase 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 (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/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.

Table I. Kinetic and Physical Constants of Enzyme Preparations

enzyme	sp act.,[a] IU/mg of protein				physical constants[b]		kinetic constants[c]	
	FP	MC	CMC	pNPG	*M*_r	p*I*	*K*_m	*V*_max
complete cellulase	1.05	0.51	8.12	0.11			0.33	0.54
CBHI		0.09	ND[d]	0	68	4.3	0.30	0.10

[a] Specific activities measured at conditions of 50 mM sodium acetate, pH 5.0, 50 °C. All values reported in units of micromolar of reducing sugar equivalent produced per minute per milligram of protein with the given substrate. FP, filter paper; MC, microcrystalline cellulose; CMC, carboxymethylcellulose; pNPG, *p*-nitrophenyl β-D-glucopyranoside. [b] Molecular mass (kilodaltons) estimated by electrophoresis at denaturing conditions and p*I* by isoelectric focusing. [c] *K*_m (percent) and *V*_max (micromoles per minute per milligram of protein) values determined for the microcrystalline cellulose substrate at 50 °C, pH 5.0, 50 mM sodium acetate buffer. [d] Not detectable. The specific activity is less than 0.02 IU/mg of protein.

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 I. The filter paper based specific activity of 1.05 IU/mg of 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 μmol min⁻¹ (mg of protein)⁻¹, 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 mass from 20 000 to 100 000 daltons. Fractionation studies indicated essentially all of the observed proteins possess cellulase activity. Beldman et al. (1985) have similarly reported the presence of at least 10 cellulolytic enzymes present in *T. viride* cellulase preparations. The extent to which posttranslational 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 (Figure 1). 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 (Figure 1B). 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/mL were approximately 1%, 1–2%, and 2–4%, respectively (Figure 1). The maximum rate of saccharification at 17.6 X 10⁻³ IU of enzyme/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
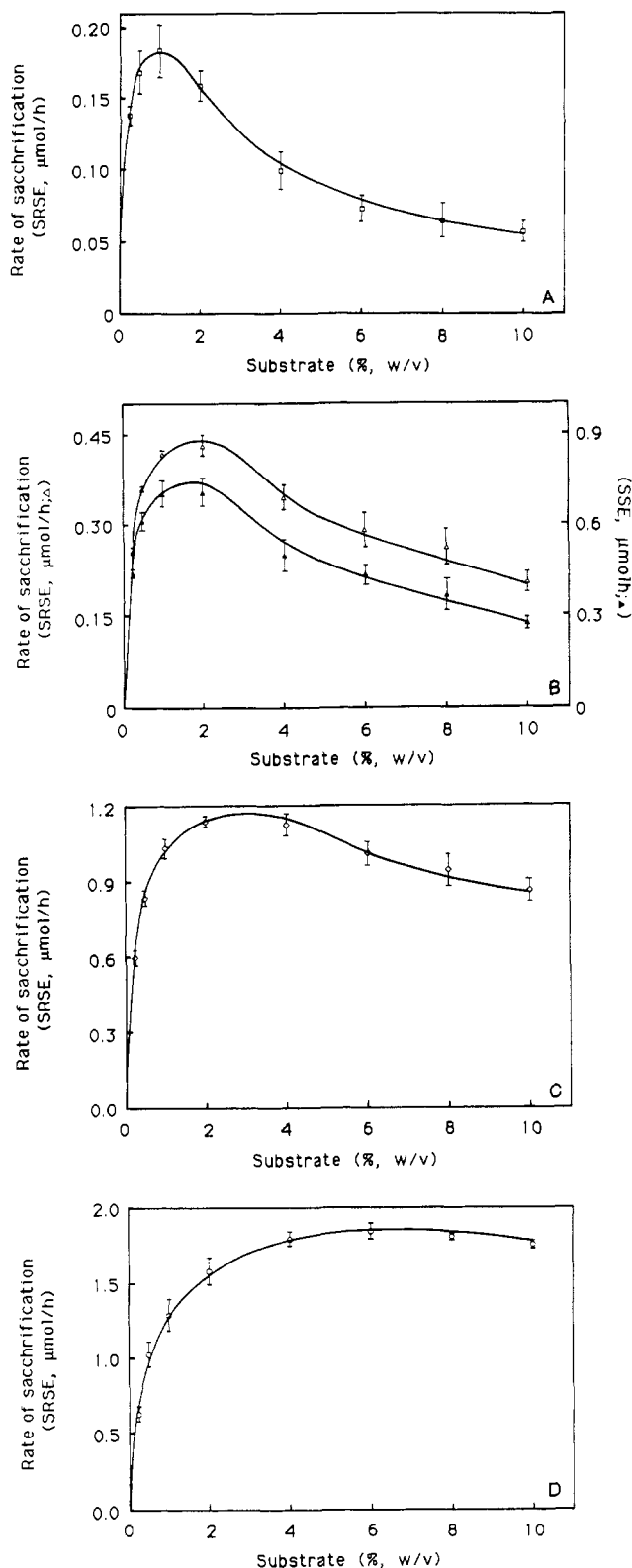
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Figure 1. Substrate–activity profiles for the complete cellulase system at different enzyme concentrations. Reaction conditions were 50 mM sodium acetate, pH 5.0, at 50 °C utilizing the microcrystalline cellulose substrate. The reaction was terminated at 5 h. Enzyme concentrations were 2.2 (A), 4.4 (B), 8.8 (C), and 17.6 (D) × 10^{-3} IU/mL. SRSE, solubilized reducing sugar equivalents; SSE, total solubilized sugar equivalents.

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 × 10^{-3} IU/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 × 10^{-3} IU of enzyme/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 rates of saccharification, obtained from the data in Figure 1, were 0.34, 0.39, 0.52, and 0.43 μmol of reducing sugar equivalents solubilized (IU of enzyme)$^{-1}$ min^{-1} for curves a–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 × 10^{-3} IU of enzyme/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 were characterized for comparison with those of the complete enzyme system. The physical and kinetic properties of the purified enzyme, homogeneous on the basis of native and denaturing gel electrophoreses and isoelectric focusing (Figure 2), are presented in Table I. 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 (Figure 3). 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/mL (4.4 × 10^{-3} IU/mL), contained approximately 1.05 μg of endogenous CBHI/mL. The quantity of purified CBHI added to the complete enzyme preparation ranged from 0 to 6.5 μg/mL. Consequently, the CBHI concentration in the reaction mixtures tested ranged from the endogenous level up to a 6-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 (Figure 4). 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 Figure 4 also indicaes that additional CBHI
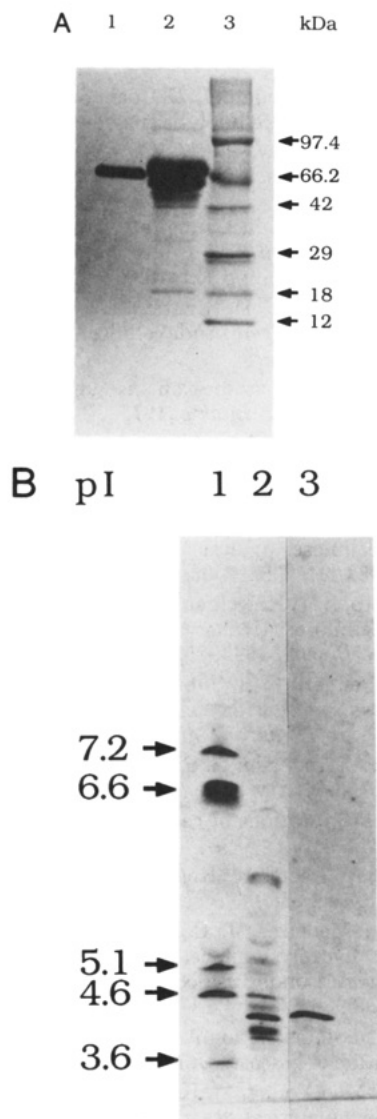
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Figure 2. (A) SDS–polyacrylamide gradient gel electrophoresis of cellulases. (a) CBHI (20 µg); (2) complete cellulase (60 µg); (3) standard proteins (phosphorylase, BSA, actin, carbonic anhydrase, TnC, cytochrome *c*, 97.4 to 12 kDa). (B) Analytic isoelectric focusing of cellulases in the pH range 3.0–10.0. (1) p*I* markers (myoglobin, carbonic anhydrase I, β-lactoglobulin A, trypsin inhibitor, amyloglucosidase, p*I* 7.2–3.6); (2) complete cellulase (120 µg); (3) CBHI (15 µg).

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 µmol of reducing sugar equivalents/mL of 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 1B, 2, and 3, was 1.5.

DISCUSSION

The cellulase enzyme system produced by *T. reesei* QM9414 is of primary importance due to its ability to
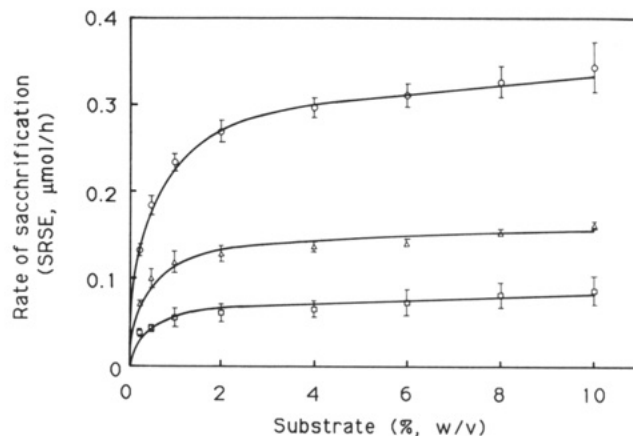

Figure 3. Substrate–activity profiles for cellobiohydrolase at different enzyme concentrations. Reaction conditions were as in Figure 1. Enzyme concentrations were 3.3 (□), 6.6 (△), and 13.2 (○) µg/mL. SRSE, solubilized reducing sugar equivalents.
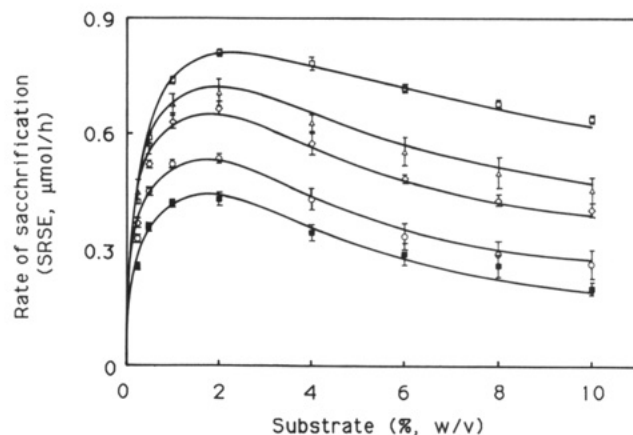

Figure 4. Effect of added cellobiohydrolase on substrate–activity profiles for the complete cellulase system. Reaction conditions were as in Figure 1. The complete cellulase concentration was 4.2 µg of protein/mL (4.4×10^{-3} IU/mL) in all cases. The added cellobiohydrolase concentration was 0 (■), 1.65 (○), 3.31 (◇), 4.96 (△), or 6.62 (□) µg/mL. SRSE, solubilized reducing sugar equivalents.

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 are 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 that 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 (grams of cellulose/international units of enzyme) of approximately 0.2. In the present study substrate inhibition was only observed at ratios greater than 5, representing a 25-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

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concentration (Lee and Fan, 1982; 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%, 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 cannot 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 Figures 1 and 3, showing that addition of complete enzyme to the reaction mixture shifts the optimum substrate concentraton 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, nonproductive, binding of two substrates per active site or the binding of substrate to a peripheral nonactive site that 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.

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