Substrate-Velocity Relationships for the \textit{Trichoderma viride} Cellulase-Catalyzed Hydrolysis of Cellulose†

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The influence of substrate and enzyme concentrations on the rate of saccharification of two defined insoluble cellulose substrates, Avicel (FMC Corp., Philadelphia, Pa.) and Solka-Floc (James River Co., Berlin, N.H.), by the cellulase enzyme system of \textit{Trichoderma viride} was evaluated. In the assays, enzyme concentrations ranging from 0.004 to 0.016 IU/ml and substrate concentrations up to 10% (wt/vol) were used. Analysis by initial velocity methods found the maximum velocity of saccharification to be nearly equivalent for the two substrates and the $K_m$ for the two substrates to be of a similar magnitude, i.e., 0.20% (wt/vol) for Solka-Floc and 0.63% (wt/vol) for Avicel. Studies in which relatively high substrate concentrations (greater than 15 times the $K_m$) were used demonstrated that the enzyme exhibited very different apparent substrate inhibition properties for the two substrates. The rate of saccharification of Avicel at relatively high substrate concentrations was up to 35% lower than the maximum rate which was observed at lower substrate concentrations. The Avicel concentration corresponding to the maximum rate of saccharification was dependent on the enzyme concentration. In contrast to the results with Avicel, the enzyme did not exhibit substrate inhibition with the Solka-Floc substrate. Potential differences in the degree of substrate inhibition with different substrates, as reported here, are particularly relevant to the experimental design of comparative studies.

The enzymatic saccharification of cellulose is catalyzed by a complex cellulase enzyme system which typically includes at least three distinct classes of enzyme: endoglucanases (EC 3.2.1.4), celllobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). The relative activities of the component enzymes in a given cellulase preparation are dependent on the source of the enzyme (6). The principal methods used to characterize the saccharification properties of these enzymes are based on kinetic analyses. Kinetic studies are used to compare the relative cellulosolytic capacity of enzyme systems from different sources (14, 26) as well as the relative enzymatic susceptibility of different cellulosic substrates (9, 24). Generally, these comparative studies include a measure of the time course of saccharification over some initial reaction period as well as a measure of the total extent of hydrolysis after a relaxed, relatively long reaction period. Kinetic studies are also used to evaluate potential mechanisms of the component cellulase enzymes (2, 3) and to develop kinetic models which can be used to predict the rate of saccharification of a particular substrate (11, 12, 19). The latter studies have resulted in mechanistically distinct models, each of which is capable of simulating a portion of the time course of saccharification for a given substrate under defined conditions. The models differ with regard to the assumptions made relative to the composition of cellulase enzymes, the structural complexity of the substrate, product inhibition, and enzyme stability. Although many kinetic studies have been reported, the complete mechanism of the enzyme is not known.

The rate of degradation of a cellulosic substrate is affected by several parameters, including the source of the cellulase enzymes (6), the physicochemical properties of the substrate (31), and the extent of product (18) and substrate (15) inhibition. Of these parameters, substrate inhibition has received the least amount of experimental scrutiny. Substrate inhibition is a fundamental kinetic property which reflects a deviation in the expected saturation kinetics of enzyme-catalyzed reactions. Apparent substrate inhibition is not uncommon when enzymes are acting at relatively high substrate concentrations, and the property may be the effect of several causes (7). Along with its inherent mechanistic information, substrate inhibition is of importance relative to several applied aspects of cellulase and cellulose research. When the relative maximum cellulosolytic capacity of a microbial enzyme system is evaluated, it is essential to consider the potentially unique substrate inhibition properties of that particular enzyme. Similarly, substrate inhibition is of relevance to the design of experiments that analyze native and modified cellulosic substrates to identify potential pretreatments that are capable of increasing the reactivities of cellulosic materials.

The cellulase enzyme complexes from \textit{Trichoderma viride} and \textit{Trichoderma reesei} have been the focus of considerable research because of their commercial potential. The heightened interest in these enzymes is largely because they are complete, extracellular enzyme systems that are capable of hydrolyzing crystalline cellulose and because they can be obtained in relatively high yields. The complete nature of these enzyme systems, coupled with their historical use in cellulase research, has made them among the primary enzymes against which newly discovered enzyme complexes are compared (4, 16). Their complete nature has also made these enzymes a primary focus of studies attempting to model the hydrolysis of crystalline cellulose (19, 22). Despite the large number of comparative and mechanistic studies done with \textit{Trichoderma} enzymes, there are relatively few...
studies which have considered their apparent substrate inhibition properties. Okazaki and Moo-Young (23) have presented a generalized mechanistic model for the enzymatic hydrolysis of cellulose which, based on concurrent random and endwise attack of the substrate, predicts substrate inhibition. They also stated that they observed apparent substrate inhibition in their unpublished studies. Lee and Fan (15) have presented initial velocity data which reflect apparent substrate inhibition of the T. reesei enzyme. They attributed the inhibition to hydrodynamic factors and, therefore, focused their initial velocity study on reaction conditions which did not exhibit substrate inhibition. Apparent substrate inhibition of Trichoderma and Aspergillus cellulase complexes by a relatively complex celluloseic substrate, leached beet cossette, has also been reported (5). In the present study, we extended these previous observations by characterizing the apparent substrate inhibition properties of the T. viride enzyme system with respect to two substrates that are commonly used in cellulase and cellulose research.

In the present study, the substrate inhibition properties of the cellulase enzyme system from T. viride were characterized through analysis of substrate-velocity profiles for two substrates, Avicel and Solka-Floc, over a range of enzyme and substrate concentrations. The results presented here demonstrate that the apparent substrate inhibition properties of this enzyme are indeed complex and are dependent on the cellulose substrate that is used. The importance of these results relative to appropriate experimental designs in cellulase and cellulose research is discussed.

MATERIALS AND METHODS

Materials. Substrates were obtained commercially; Avicel PH101 was from FMC Corp. (Philadelphia, Pa.), and Solka-Floc BW 200 NF was from James River Co. (Berlin, N.H.). The following potentially variable chemicals and reagents were obtained from the designated suppliers: BCA protein assay reagent, Pierce Chemical Co. (Rockford, Ill.); bovine serum albumin, Sigma Chemical Co. (St. Louis, Mo.); formic acid (95 to 97%) and soluble starch, Aldrich Chemical Co., Inc. (Milwaukee, Wis.); and cupriethylenediamine hydroxide solution, Synmet Corp. (Baton Rouge, La.).

Enzyme preparation. A commercially available cellulase enzyme system from T. viride (Cellulysin; Calbiochem Corp., San Diego, Calif.) was used without modification. The activity of the enzyme preparation, which was determined by the filter paper method of Mandels et al. (17), as modified by Ghose (10), was 0.50 IU/mg of enzyme preparation. The protein content, which was determined by the modified biuret method of Smith et al. (27), was 0.53 mg of bovine serum albumin equivalents per mg of enzyme preparation.

Substrate characterization. The percent cellulose content of the two substrates was determined by extraction of the noncellulosic polysaccharides with acetic and nitric acid as described by Updegraff (30). This was followed by quantitation of residual cellulose as described by Southgate (29) by using the anthrone reagent with glucose as the calibration standard.

The crystallinity index of the substrates was determined by X-ray diffraction methods as described by Hsu and Penner (13). The degree of polymerization of substrates was calculated by multiplying their experimentally determined intrinsic viscosity in 0.5 M cupriethylenediamine by 190 (1). The small-molecule accessibility of the substrates was determined by the Eberstadt formylation method (8) as described by Nickerson (21). Briefly, cellulose substrates were formylated in 90% formic acid for various lengths of time at 25°C to establish a time course of formylation. The time course was then extrapolated back to zero time to estimate the accessibility of the native substrate prior to any modification resulting from formylation. The percent accessibility of the substrates was then determined by quantitative comparison of the extent of formylation of the analyzed substrate with the extent of formylation of an equivalent weight of soluble starch. The water retention volume, which is defined as the weight of water retained per unit weight of substrate following centrifugation at 1,300 × g for 5 min, was determined as described by Lee and Fan (15) by using reaction mixture buffer as the aqueous phase.

Enzymatic saccharification assays. The standard enzymatic saccharification assay was performed in 50 mM sodium acetate buffer (pH 5.0) at 40°C. Cellulose substrate was added to the reaction flask (125 ml, Erlenmeyer) containing buffer and equilibrated to 40°C. The reaction was then initiated by the addition of buffered enzyme solution. The final volume for each reaction mixture was 62 ml. Reaction mixtures were agitated at 140 rpm in a constant-temperature, orbital shaking water bath (model 3540; Lab-Line, Melrose Park, Ill.). Substrate concentrations, expressed as percent (wt/vol) cellulose, and enzyme concentrations, expressed as IU per ml, were varied as described in the Results. In experiments in which we evaluated bulk mass transfer, the standard assay conditions were used, except that the amount of agitation was varied as described in the Results.

Saccharification reactions were terminated at appropriate times (2 or 6 h) by filtering the reaction mixture through a 0.22-μm-pore-size membrane filter (Millipore Corp., Cam-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Cellulose</th>
<th>$\text{Ctrl}^b$</th>
<th>$\text{Small-molecule accessibility}^b$</th>
<th>$\text{DP}^d$</th>
<th>$\text{WRV}^e$</th>
</tr>
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<tbody>
<tr>
<td>Avicel</td>
<td>99.6 ± 0.5</td>
<td>80</td>
<td>16.5</td>
<td>219 ± 3</td>
<td>2.17 ± 0.05</td>
</tr>
<tr>
<td>Solka-Floc</td>
<td>91.8 ± 2.0</td>
<td>73</td>
<td>26.0</td>
<td>703 ± 20</td>
<td>3.06 ± 0.06</td>
</tr>
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</table>

$^a$ Values are means ± standard errors of the mean.

$^b$ Ctrl, Crystallinity index, which was obtained by X-ray diffraction methods.

$^c$ Values reflect the percentage of substrate hydroxyl groups esterified in 90% formic acid.

$^d$ DP, Degree of polymerization, which was obtained from intrinsic viscosity measurements.

$^e$ WRV, Water retention volume, which is grams of water retained per gram of cellulose after centrifugation.

<table>
<thead>
<tr>
<th>Enzyme concn (IU/ml)</th>
<th>$K_m$ (wt/vol)</th>
<th>$V_{max}$ (μmol of RSE/h)$^b$</th>
<th>$K_m$ (wt/vol)</th>
<th>$V_{max}$ (μmol of RSE/h)$^b$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Avicel</td>
<td></td>
<td>Solka-Floc</td>
</tr>
<tr>
<td>0.004</td>
<td>0.63 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.08 ± 0.02</td>
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<tr>
<td>0.008</td>
<td>0.63 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>0.016</td>
<td>0.62 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.33 ± 0.01</td>
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</table>

$^a$ Experimental conditions were 50 mM sodium acetate buffer (pH 5.0) at 40°C and a 2-h reaction time, and the variable substrate concentration ranged from 0.2 to 2% (wt/vol). Values are means ± standard errors of the mean.

$^b$ Values are micromoles of reducing sugar equivalents (RSE) solubilized per hour.
FIG. 1. Effect of substrate concentration on rate of saccharification. Experiments were performed in 50 mM sodium acetate (pH 5.0) at 40°C, 140 rpm agitation, and an enzyme concentration of 0.004 IU/ml. The substrate was either Avicel (A) or Solka-Floc (B). Total sugar equivalents solubilized and reducing sugar equivalents solubilized were measured at 2-h (○) and 6-h (■) reaction times.

Compositional and structural parameters of cellulose substrates. The compositional data for the two substrates indicate their similarity in that both substrates contained greater than 90% cellulose (Table 1). However, with these substrates it is informative to compare the relative amount of noncellulosic material. In this regard, the Solka-Floc substrate contains approximately 20-fold more noncellulosic

bridge, Mass.), followed by immediate assay for reducing sugar and total sugar equivalents. Reducing sugars were measured by the cupric ion reduction methods of Nelson (20) and Somogi (28), and total sugars were measured by the anthrone method of Roe (25), as described by Southgate (29). Glucose was used as the calibrating standard for both reducing and total sugar assays. Kinetic constants were determined from double-reciprocal plots of experimental data (7).
material than that in the Avicel substrate. Technical information obtained from the supplier indicated that the noncellulose material is primarily hemicellulose. Selected structural parameters of the two substrates are also given in Table 1. The crystallinity index values reported in Table 1 are empirical estimates of the ratio of crystalline to amorphous structure in the cellulose samples and, therefore, should not be interpreted as a finite measure of the percent crystalline cellulose. Avicel had a slightly higher crystallinity index than that of Solka-Floc, indicating that a greater percentage of the cellulose molecules in the Avicel substrate are involved in highly ordered, crystalline regions of low reactivity. Both cellulose substrates, however, showed much more crystalline character than did experimentally prepared amorphous substrates, such as ball-milled cellulose (13). The small-molecule accessibility of the cellulosomes, which was estimated by measuring the extent of formylation of potentially reactive hydroxyl groups, suggested that the majority of hydroxyl groups in both substrates is unavailable for reaction with solvent molecules. These results, which were consistent with the crystallinity index values, indicate that the accessible surface area of Avicel is somewhat less than that of Solka-Floc. The degree of polymerization of the substrates reflected an apparent 3.2-fold greater molecular weight for the cellulose molecules in Solka-Floc compared with those in Avicel. The water retention volume of a fibrous substrate is an estimate of the amount of water which may be entrapped within the substrate. The results indicate that Solka-Floc absorbs roughly 40% more water per unit weight than Avicel does under the defined reaction conditions.

**Analysis of kinetic constants.** Initial velocity methods were used to determine the $K_m$ and the $V_{max}$ of the enzyme with respect to both of the substrates (Table 2). The $K_m$'s of the two substrates were of a similar magnitude, 0.20% for Solka-Floc and 0.63% for Avicel. Consistent with the assumptions of Michaelis-Menten kinetics, the $K_m$ was not affected by changes in the enzyme concentration. The $V_{max}$ values obtained for the two substrates were nearly identical when equivalent enzyme concentrations were compared. As predicted by Michaelis-Menten kinetics, the $V_{max}$ was proportional to the enzyme concentration in the reaction mixture. The amount of sugar solubilized in each of the reaction mixtures corresponded to less than 2.5% of the initial substrate that was present.

**Effect of substrate concentration on the rate of saccharification.** The effects of the substrate concentrations on the quantity of solubilized reaction products released from the Avicel and Solka-Floc substrates, after 2- and 6-h reaction times, are depicted in Fig. 1. With Solka-Floc as the substrate, the enzyme appeared to obey classical saturation kinetics over the substrate concentration ranges studied. In contrast, when Avicel was the substrate, the enzyme deviated substantially from this classical behavior. The marked decrease in the apparent reaction rate at relatively high Avicel concentrations resulted in an optimum substrate concentration, above which substrate inhibition was observed. The maximum amount of substrate inhibition observed corresponded to an approximate 35% decrease in the rate of saccharification relative to that observed at the optimum substrate concentration. The profiles of the substrate-velocity curves for the respective substrates were similar whether the extent of the reaction was measured by total sugar solubilized or by solubilized reducing sugars (Fig. 1). The average ratio of total solubilized glucose equivalents to solubilized reducing sugar equivalents was 1.4. Since the ratio was greater than 1 and yet less than 2, it suggests that the measured reaction products contain cellobiose, other cellooligosaccharides, or both, along with glucose. The lack of conversion of all solubilized products to glucose was consistent with previous studies in which the relatively low cellobiase activity in *Trichoderma* cellulase enzyme preparations has been demonstrated, although its specific activity in this preparation was not determined. Comparison of the shape of the substrate-velocity curves determined at 2- and 6-h reaction times indicated that the observed substrate
inhibition properties were expressed consistently throughout this time period.

The relevance of bulk mass transfer to the observed reaction rates was estimated by measuring the rate of saccharification of Avicel at various degrees of reaction mixture agitation (Fig. 2). It was visually obvious that substrate was settling out of suspension in the reaction mixtures that received the lowest amount of agitation (100 rpm), and the corresponding rates of saccharification in those reaction mixtures were consistently lower than those in reaction mixtures which were more aggressively agitated. Substrate in reaction mixtures agitated at 140 or 180 rpm remained in suspension, and the corresponding rates of saccharification of these reaction mixtures were similar, suggesting that under the standard assay conditions used in this study there was a relatively small bulk mass transfer influence on the rate of cellulose saccharification.

**Effect of enzyme concentration on the substrate-velocity profiles.** The substrate-velocity profiles for the Solka-Floc and Avicel substrates at different enzyme concentrations are presented in Fig. 3. The cellulase system with Solka-Floc as the substrate behaved as expected, with a given substrate concentration providing nearly equivalent V/Vmax ratios at the different enzyme concentrations. However, the relation-
ship between enzyme concentration, substrate concentration, and the rate of saccharification was more complex with the Avicel substrate. The rate-versus-substrate-concentration curves at the two lower enzyme concentrations (0.004 and 0.008 IU/ml) both demonstrated significant substrate inhibition, while the corresponding curve at 0.016 IU/ml did not. The curves presented in Fig. 3 show that the Avicel concentration required for the maximum rate of saccharification is dependent on the enzyme concentration of the reaction mixture. When the enzyme concentration was doubled from 0.004 to 0.008 IU/ml, the Avicel concentration required for maximum activity also approximately doubled, from 2 to 4%. Similarly, the reaction mixture containing 0.016 IU/ml appeared to reach its maximum rate of saccharification at an Avicel concentration of approximately 8%.

**DISCUSSION**

The cellulase enzyme system of *T. viride* appears to obey Michaelis-Menten kinetics at relatively low substrate concentrations when either the Solka-Floc or the Avicel substrate is used. However, at relatively high substrate concentrations, the rate of saccharification of Avicel decreased, which is indicative of substrate inhibition. Substrate inhibition of the enzyme was not observed with the Solka-Floc substrate under equivalent reaction conditions. The apparent substrate inhibition of the enzyme complex by the Avicel substrate demonstrates that any mechanistic interpretation of the kinetic constants $K_m$ and $V_{max}$ is indeed complex and that these parameters are best used only for comparative purposes. The term “substrate inhibition,” as used in this report, refers to any apparent decrease in the rate of the reaction which accompanies an increase in substrate concentration. The cause of the substrate inhibition characterized in this study is not clear. Previous observations of substrate inhibition have been rationalized by mechanisms that involve a decrease in the extent of concurrent action on the same chain by component enzymes (23; B. H. Van Dyke, Jr., Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1972) or a decrease in the movable aqueous phase of the reaction mixture (15). The kinetic model developed by Okazaki and Moo-Young (23), which is based on concurrent random and endwise attack of the substrate by the endo- and exo-component enzymes similarly predicts substrate inhibition. Further studies are required to determine conclusively the actual mechanism(s) which governs this behavior.

Knowledge of the substrate inhibition properties of a cellulase enzyme complex can most obviously be applied to the design of reactors for the saccharification of cellulosic substrates. When considering reactor conditions, it is clear that classical saturation kinetics should not be assumed and that a potential optimum substrate-to-enzyme ratio must be considered. The differences in the substrate inhibition properties of the enzyme with the two cellulose substrates used in this study strongly suggest that each cellulosic material must be analyzed independently to ascertain its optimum concentration. In the present study, we compared relatively refined cellulose substrates derived from wood pulp. It is not known how the inhibitory properties of these substrates compare with those of cellulosic substrates derived from other sources by other methods. Our results, considered in conjunction with previously observed substrate inhibition by leached beet cosset (5) and ball-milled Solka-Floc (Van Dyke, Ph.D. thesis), suggest that the extent of substrate inhibition is dependent on the structural properties of the substrate and that it is not a unique property of Avicel.

Substrate inhibition, as described here, is of importance relative to the appropriate experimental design for comparative studies which analyze the rate of digestion of different cellulose-type substrates. Cellulose substrates with a range of structural properties are often used to analyze the degree to which specific physical properties of cellulose are of relevance to their susceptibilities to cellulolytic enzymes (31). In related studies, cellulose substrates are compared to
identify appropriate pretreatments to increase the enzymatic susceptibilities of lignocellulosic materials (9). In comparative studies of this nature, a single set of reaction conditions, including a single substrate and enzyme concentration, is often used to determine which of the cellulose substrates is most susceptible to saccharification. The present data suggest that the conditions chosen for the assay may significantly affect the results of such a study. This point is illustrated in Fig. 4, which depicts the rate of saccharification of the two celluloses, Solka-Floc and Avicel, used in this study, as determined by total sugars produced after a 6-h reaction period. Figure 4 illustrates that the substrate with which the enzyme shows the most activity is a function of the substrate concentration used in the comparative assay. Assays with 2% substrate indicate that Avicel is degraded at a rate approximately 38% greater than that of Solka-Floc. However, assays with 8% substrate indicate that the rate of degradation of Solka-Floc is approximately twofold greater than that of Avicel.

Substrate inhibition properties may similarly affect the conclusions drawn from studies designed to compare the relative activities of different cellulase enzyme preparations. This type of study is commonly done to evaluate the industrial potential of novel cellulase enzyme systems (4, 16). The Avicel substrate is often used in these studies because of its microcrystalline character. Figure 5 illustrates the influence that the substrate concentration may have on the results from this type of study. In the example provided in Fig. 5, the enzyme concentration was increased fourfold, from 0.004 to 0.016 IU/ml, and the rate of saccharification was determined at two substrate concentrations. At the lower substrate concentration (2%), there was nearly a linear relationship between the enzyme concentration and the rate of saccharification. However, at the higher substrate concentration (8%), the relationship was nonlinear, so that the actual fourfold increase in enzyme concentration was measured to be nearly ninefold. The date presented here demonstrate how the conclusions drawn from comparative studies used to measure the relative Avicelase activity of two enzyme preparations may unintentionally be biased if substrate-velocity interrelationships are not considered.

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LITERATURE CITED


FIG. 5. Effect of substrate concentration on the linearity of the enzyme-velocity profile for saccharification of Avicel. The values are taken from the data presented in Fig. 3A and represent substrate concentrations of 2% (•) and 8% (□).


