0273-2289/92/3435—0061$02.80

On the Use of Enzyme Adsorption and Specific Hydrolysis Rate to Characterize Thermal-Chemical Pretreatment

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ABSTRACT

The pseudo enzyme adsorption isotherm of the pretreated substrate is used to estimate the pretreatment's effectiveness in generating accessible adsorption sites. The specific hydrolysis rate (i.e., the hydrolysis rate divided by the adsorbed enzyme) is used as a measure of substrate reactivity. Poplar, subjected to partial acid hydrolysis at 180 and 200°C in 1% H_2SO_4 for 9 s, was contacted with GC 123 (a *Trichoderma reesei* cellulase produced by Genencor, Inc., South San Francisco, CA). Both adsorption capacity and specific reaction rate increase significantly at the higher pretreatment temperature. The absolute rate and the specific hydrolysis rate fall off rapidly with conversion, and the absolute rate, but not the specific rate, exhibits a maximum as enzyme concentration is increased.

Index Entries: Pretreatment; enzyme hydrolysis; enzyme adsorption.

INTRODUCTION

This article takes a new approach to studying the effect of thermal-chemical pretreatment on the subsequent enzymatic hydrolysis. Rather than measuring the pore volume distribution (*1*) in order to estimate the surface area accessible to the enzyme (*2*), the amount of adsorbed enzyme

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has been measured. This, after all, is what is important about the surface area. Furthermore, it automatically senses only the surface that is available to the enzyme, and it includes the external surface area, which is neglected by the solute exclusion measurement of the pore volume.

The second quantity studied is the specific hydrolysis rate (3), i.e., the hydrolysis rate divided by the concentration of adsorbed enzyme. When all the adsorption sites are occupied (i.e., at the high enzyme loading), the specific rate becomes a measure of substrate reactivity.

Thus, through these experiments, the effect of pretreatment conditions on substrate adsorption capacity and reactivity can be studied. We recommend that adsorption and reactivity be separated in this manner in all studies of the kinetics of enzyme-catalyzed reactions of insoluble substrates.

MATERIALS AND METHODS

Materials

Poplar wood chips were donated by the Solar Energy Research Institute (Golden, CO). The cellulase, GC123 from Genencor Inc., was found to contain 1.45 mg/mL glucose, 5.02 mg/mL carbohydrate, 43.4 mg protein/mL (by methods described later in this section), and 116 U/mL filter paper activity as determined by the method of (4) with citrate buffer replaced by acetate buffer of the same concentration. Other chemicals were of reagent grade.

Pretreatment of Poplar Wood

The poplar chips were ground to 60 mesh in a Wiley mill. The milled wood was pretreated by partial acid hydrolysis with explosive decompression in a continuous plug flow reactor developed by McParland et al. (5), using 5 wt% wood slurry containing 1 wt% H_2SO_4. The temperature was controlled to 200 and 180°C by direct steam injection at a pressure significantly above the saturation pressure. The residence time, 9 s, was based on the volume of the reactor, as measured by liquid displacement, divided by the volume of slurry collected in a known time; the steam was assumed to condense instantaneously. These two pretreated poplar substrates are referred to as PTP200 and PTP180, respectively, in the remainder of the article. The pretreated wood was neutralized and washed on filter paper with distilled water until the pH of the supernatant was 5–7. The wet substrate was stored at 5°C in 1% NaN_3 solution. Care was taken to keep the wood substrate wet at all times. Before use, the substrate was washed with distilled water and filtered to a water content between 70–80%. The pretreated substrates were found, by quantitative saccharification (4), to con-

tain 63.6 and 61.6% potential glucose by weight for PTP200 and PTP180, respectively.

Hydrolysis of Pretreated Poplar (PTP) Wood Substrate

Hydrolysis was carried out in 158-mL serum bottles at 40°C with vigorous shaking for the first minute to agitate the reaction mixture thoroughly, followed by gentle shaking in a water-batch incubator using pH 5.0, 0.05*M* Na-acetate buffer. Initial substrate concentration (S_o) of 1% w/v (dry basis) was reacted with different initial enzyme concentration (EZ_o) such that all reaction vessels contained the same amount of substrate in a total reaction volume of 100 mL. Approximately 1-mL samples were withdrawn from the reaction mixture at various time intervals, and analyzed for glucose and enzyme protein concentration.

Measurement of Enzyme Protein in Solution

The protein in solution was determined by the Bradford colorimetric assay from Bio-Rad Co. Bovine serum albumin was used as the protein standard. By taking the difference between the initial concentration and the concentration at a particular time during the hydrolysis, the amount of adsorbed enzyme was calculated.

Cellulase Adsorption

The concentration of crude cellulase protein was measured as described above. The amount of adsorbed protein was computed from the difference between the measured concentration in solid-free solution and the known amount of enzyme added originally. Normally, reaction and adsorption occurred simultaneously. In selected studies, in order to study adsorption without simultaneous reaction, the enzyme mixture of 0.1 to 20 mg/mL and 1 g of pretreated wood PTP200/180 was incubated for 1 h at 3°C in pH 5.0, 0.05*M* Na-acetate buffer. At this temperature, there was little enzymatic hydrolysis.

Measurement of Sugars in Solution

The glucose in solution was determined by the hexokinase/glucose-6-phosphate dehydrogenase reagent from Sigma Chemicals as employed by Lynd and Grethlein (*6*). The total potential glucose (TPG) of both solids and liquids was measured by first acid hydrolysing samples, using 0.0357 mL 72% H_2SO_4/mL sample solution at 121°C and 24 psi, for 1 h. The acid-hydrolyzed samples were then analyzed for glucose as described above. The cellobiose in solution was calculated by the difference between TPG and glucose in solution.

Reaction Rate

In order to study the reaction rate, an empirical equation developed by Ooshima (7) was applied to the glucose and TPG data. The function is of the form:

$$G = k * \ln(1 + m * t) \quad (1)$$

where G represents production (mg/mL), and t represents time of hydrolysis elapsed (min), and k and m are arbitrary constants.

The constants k and m were determined by a nonlinear least square search. Differentiation of G in eq. (1) leads to the following expression for the reaction rate:

$$\text{Rate} = (k * m / 1 + m * t) \quad (2)$$

The initial rate at time zero is:

$$\text{Initial rate} = k * m \quad (3)$$

This method of processing the concentration data was employed in order to eliminate the scatter that occurs when numerical differentiation is employed. It also provides a measure of the initial rate that does not depend on the arbitrary choice of the initial time increment.

RESULTS AND DISCUSSION

Enzyme Adsorption Capacity

The concentration of enzyme protein, adsorbed on PTP200, is presented as a function of time in Fig. 1 for initial enzyme concentrations that range from 0.5 mg/mL to 20 mg/mL. The concentration of the pretreated wood substrate was only 10 mg/mL; hence, the ratio of enzyme to substrate was quite large at the higher enzyme concentrations. At the lowest enzyme level, about 90% of the enzyme was adsorbed at the point of maximum enzyme adsorption. At the highest enzyme concentration, only about 5% was adsorbed at the peak. The corresponding values for PTP180 (Fig. 2) were 50% and 4%, respectively.

In Fig. 3, the concentration of enzyme protein adsorbed on PTP200 and PTP180 (PTP200-40 and PTP180-40), averaged the first 3 h, is presented as a function of the enzyme concentration in solution. Four interesting observations can be made. First, at low enzyme concentrations, a very large fraction of the enzyme is adsorbed. Second, there is no absolute saturation, i.e., the concentration of adsorbed enzyme continues to increase as the total enzyme concentration is increased. Third, assuming that the curve must go through the origin, it exhibits a sharp change in the slope at the lowest enzyme concentration studied. Finally, the adsorption on PTP200 is greater than on PTP180.

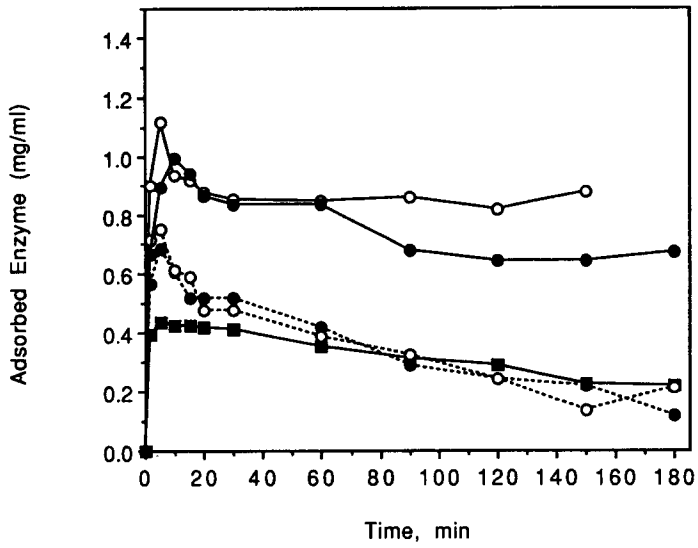

Fig. 1. Adsorbed enzyme protein, mg/mL, as a function of time during the hydrolysis of PTP200 at T=40°C. Substrate concentration=10 mg/mL. —■— EZ_o=0.5 mg/mL, --●-- EZ_o=2.5 mg/mL, ---○--- EZ_o=5.0 mg/mL, —●— EZ_o=10 mg/mL, —○— EZ_o= 20 mg/mL.

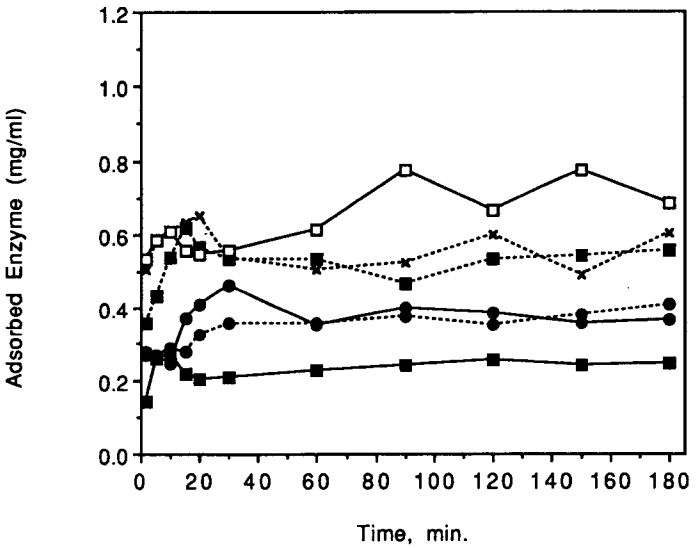

Fig. 2. Adsorbed enzyme protein, mg/mL, as a function of time during the hydrolysis of PTP180 at T=40°C. Substrate concentration=10 mg/mL. —■— EZ_o=0.5 mg/mL, ---●--- EZ_o=2.5 mg/mL, —●— EZ_o=5.0 mg/mL, ---■--- EZ_o=10 mg/mL, --X-- EZ_o=15 mg/mL, —□— EZ_o=20 mg/mL.

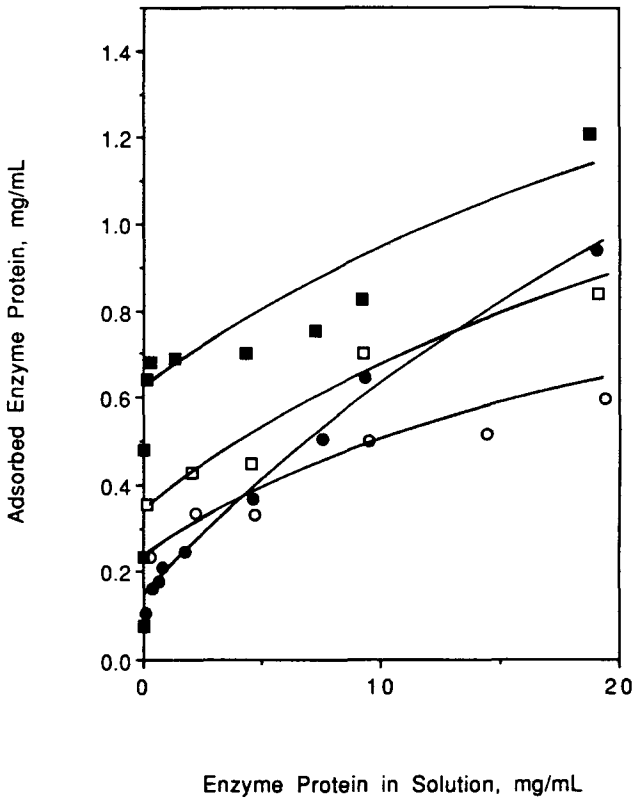

Fig. 3. Adsorbed enzyme protein, mg/mL, average over the first 3 h of hydrolysis, for PTP200 and PTP180 at T = 40°C and after 1 h of contact at 3°C, as a function of enzyme protein concentration in solution. Substrate concentration = 10 mg/mL. □ PTP200-40, ○ PTP180-40, ■ PTP200-3, ● PTP180-3.

In the above study, the temperature was 40°C; hence, considerable hydrolysis of the cellulose occurred, particularly of the PTP200. In order to avoid the effect of the hydrolysis reaction on the adsorption, experiments were also run at 3°C (PTP200-3 and PTP180-3 in Fig. 3). For the PTP200, the knee in the adsorption curve is very apparent. Even though there is an abrupt change in the slope, the concentration of adsorbed enzyme continues to increase as the concentration of total enzyme is increased, as was observed at the higher temperature. For PTP180, the change in slope is more gradual. Adsorption of the enzyme on the PTP200 is much stronger than on PTP180. For example, at a total enzyme concentration of 0.5 mg/mL, 96% of the enzyme is adsorbed on the PTP200 whereas only 30% is adsorbed on the PTP180.

The curves presented in Fig. 3 are not true equilibrium isotherms because (1) reaction occurred during the hydrolysis at 40°C, and (2) the same curve is not followed upon dilution (work in progress). Hence, in the Abstract, they are referred to as pseudo adsorption isotherms.

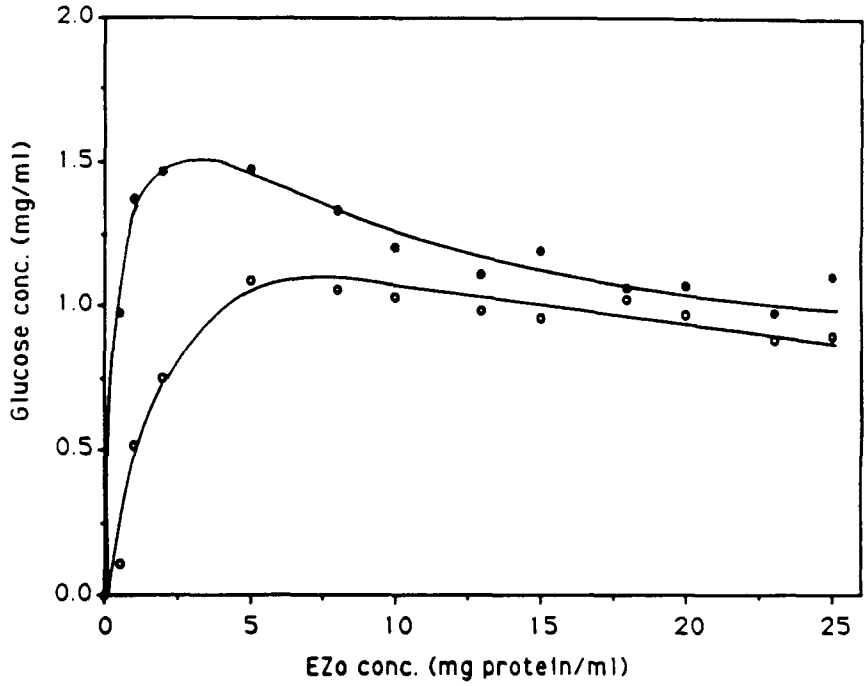

Fig. 4. Glucose and TPG concentrations after 5 min of hydrolysis as a function of the initial enzyme concentration. Substrate concentration = 10 mg/mL. ○ Glucose, ● TPG.

Our objective in these experiments was not to characterize the adsorption fully, but only to estimate the concentration of available adsorption sites as a means of characterizing the pretreatment. Since saturation did not occur, it was not possible to infer the concentration of adsorption sites from the saturation concentration of adsorbed enzyme. Although the reasons for the observed behavior are not clear, it may be that strong site-specific adsorption of the enzyme on the cellulose dominates at low enzyme concentration and then saturates, whereas nonspecific hydrophobic adsorption occurs on the lignin without saturation. If this is the case, the cellulase capacity could be estimated approximately from the intercept of the extrapolation of the data as shown in Fig. 3. However, it has been pointed out that it may be owing to different proteins in the crude enzyme having different adsorption characteristics. More study with purified enzyme and substrate components is needed to clarify the matter.

Substrate Reactivity

In an attempt to develop a simple measurement of substrate reactivity, the glucose and TPG concentrations were measured after 5 min of reaction time (Fig. 4). The TPG data display a maximum when plotted against the total enzyme concentration.

The cellulase enzyme, GC123 as supplied by Genencor, contains glucose and potential glucose. It is therefore possible that the reduced conversion at the higher enzyme concentration is owing to inhibition by the glucose in the enzyme preparation. In order examine this possibility, the hydrolysis of PTP200 was carried out with initial enzyme concentrations (EZ_o) of 5 and 20 mg protein/mL, and an initial enzyme concentration of 5 mg protein/mL plus 0.62 mg/mL glucose, the difference in the glucose concentration when the enzyme concentration is increased from 5 to 20 mg/mL. Glucose inhibition of glucose production was observed (*8*) (data not shown). However, the conversion, with EZ_o of 5 mg protein/mL plus 0.62 mg/mL glucose, was significantly higher than that of reaction with EZ_o of 200 mg protein/mL. The effect of added glucose on TPG production was very slight compared to the effect of the higher enzyme concentration. Hence, we conclude that the reduced conversion at the higher enzyme level is not an artifact caused by the glucose in the enzyme.

As explained in a previous section, in order to study the rate of hydrolysis, glucose and TPG concentration vs time data were fit with Ooshima's empirical relationship. This was then differentiated analytically to determine the rate, including the initial rate. This procedure eliminates the arbitrary choice of step size and the scatter caused by numerical differentiation. All the corresponding parameter values and standard deviations are presented in Table 1 and by Wang (*8*).

The reaction rate, based on TPG formation, for PTP200 is presented as a function of conversion in Fig. 5. In agreement with the 5-min conversion data (Fig. 4), the rate goes through a maximum at an enzyme concentration of 5 mg/mL. The initial rate is particularly sensitive to enzyme concentration.

Presumably, the rate increases with increased total enzyme concentration because more of the enzyme is adsorbed. It is tempting to attribute the decline in reaction rate at higher enzyme concentrations to competitive adsorption between the enzyme components, with a corresponding fall-off in the rate because of a loss of synergy between the enzyme components, as discussed by Woodward et al. (*9*). However, cellobiohydrolase is more strongly adsorbed than endogluconase (*10,11*). The cellobiohydrolase acts on the cellulose polymer chain ends, and immediately following pretreatment, one might expect the cellulose chain ends to be present in excess. Hence, it is difficult to explain the behavior of the initial rate by competitive adsorption. Another possible explanation is that, when surface coverage is extensive, the large enzyme molecule sterically hinders access to the chain ends.

In order to separate substrate reactivity from enzyme adsorption capacity, the rate data have been converted to specific rate data, i.e., rate divided by adsorbed enzyme concentration. The effect of enzyme concentration on the specific rate of PTP200 is shown in Fig. 6. In contrast with the rate data (Fig. 5), the specific rate is largely independent of total enzyme concentra-

Table 1
Least Squares Fit to Hydrolysis Data

	k, mg/mL	m, −1 min	Initial rate	Standard deviation
PTP200				
Glucose production				
EZ_0=0.5 mg/mL	0.912	0.175	0.160	0.119
EZ_0=2.5 mg/mL	1.31	0.392	0.511	0.0576
EZ_0=5.0 mg/mL	1.30	0.488	0.635	0.0706
EZ_0=10 mg/mL	1.38	0.405	0.558	0.162
EZ_0=20 mg/mL	1.45	0.223	0.322	0.150
TPG production				
EZ_0=0.5 mg/mL	1.45	0.247	0.357	0.0775
EZ_0=2.5 mg/mL	1.15	0.602	0.690	0.0723
EZ_0=5.0 mg/mL	1.14	0.712	0.813	0.0920
EZ_0=10 mg/mL	1.20	0.493	0.594	0.140
EZ_0=20 mg/mL	1.19	0.396	0.472	0.156
PTP180				
Glucose production				
EZ_0=0.5 mg/mL	1.19	0.0544	0.0646	0.0832
EZ_0=2.5 mg/mL	1.48	0.0831	0.123	0.0567
EZ_0=5.0 mg/mL	1.35	0.0986	0.133	0.0920
EZ_0=10 mg/mL	1.36	0.0956	0.130	0.0711
EZ_0=15 mg/mL	1.24	0.102	0.126	0.0932
EZ_0=20 mg/mL	1.20	0.0936	0.112	0.0892
TPG production				
EZ_0=0.5 mg/mL	1.71	0.0502	0.0859	0.0726
EZ_0=2.5 mg/mL	1.45	0.0884	0.128	0.0656
EZ_0=5.0 mg/mL	1.39	0.0945	0.131	0.106
EZ_0=10 mg/mL	1.37	0.0897	0.123	0.0738
EZ_0=15 mg/mL	1.11	0.133	0.148	0.0990
EZ_0=20 mg/mL	1.08	0.122	0.132	0.104

tion for the three lowest concentrations and then decreases as the enzyme concentration is increased. This behavior has been observed by Converse et al. (*12*) and Bernardez (*13*).

The corresponding rate and specific rate data for PTP180 are presented in Figs. 7 and 8, respectively. The trends are the same as in the case of the PTP200, but much less clear-cut. PTP180 exhibits a much lower hydrolysis rate; however, the specific rate is reduced to a much smaller extent, indicating that the reduced enzyme adsorption on PTP180 is of greater importance than the difference in substrate reactivity.

One possible explanation of the decline in the specific rate at high total enzyme concentration is that the increased adsorption at high enzyme concentrations is on lignin or in multiple layers. In either case, the enzyme

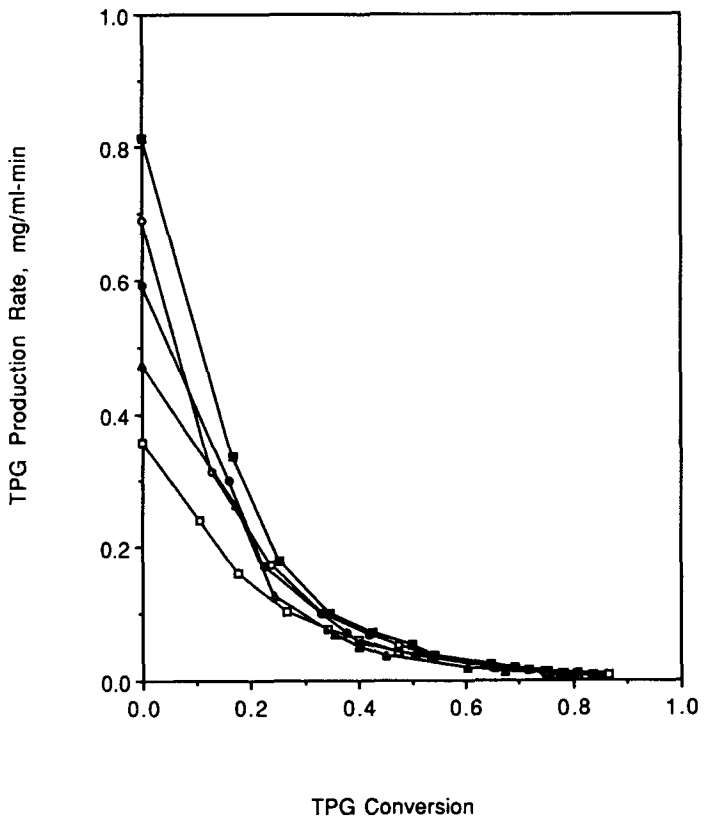

Fig. 5. Hydrolysis rate of PTP200, based on TPG formation, as a function of conversion. —□— EZ_o=0.5 mg/mL, —○— EZ_o=2.5 mg/mL, —■— EZ_o=5 mg/mL, —●— EZ_o=10 mg/mL, —▲— EZ_o=20 mg/mL.

adsorbed in this fashion would not be able to reach the reactive cellulose portion of the substrate. This is consistent with the first interpretation of the adsorption data.

Specific rate vs conversion data (Figs. 5 and 7) show a large decrease in specific rate as conversion proceeds. This behavior is consistent with a synergistic mechanism if the pretreatment generates a large concentration of cellulose chain ends. The rate of sugar formation would be expected to be proportional to the concentration of adsorbed enzyme times the concentration of chain ends. Thus, the initial specific rate would be increased by pretreatment conditions that generate more chain ends. Furthermore, these chain ends might then be consumed at a greater rate than they could be subsequently produced by the action of the endogluconase component, thus explaining the decline of the specific rate with conversion. This points to the need to follow the degree of polymerization during pretreatment and to develop the necessary kinetics model. Likewise, studies of cellulose chain end concentrations during hydrolysis are needed.

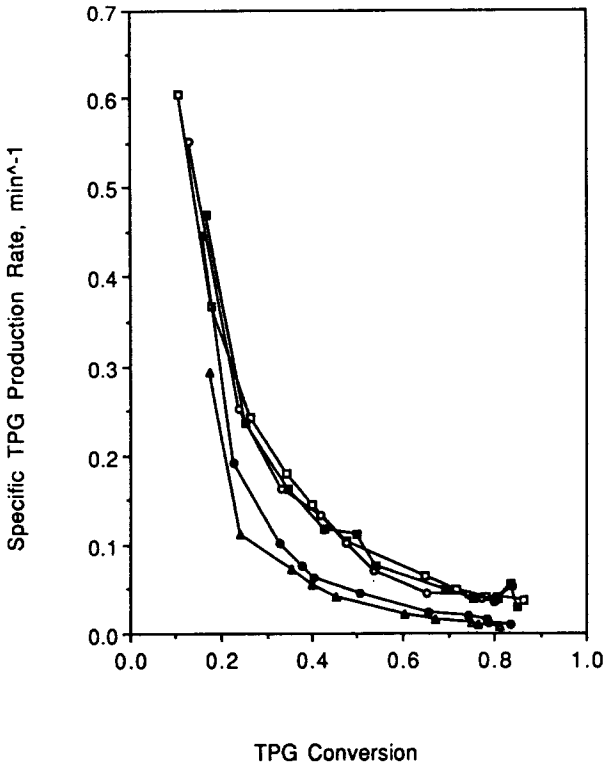

Fig. 6. Specific hydrolysis rate of PTP200, based on TPG formation, as a function of conversion. —□— EZ_o=0.5 mg/mL, —○— EZ_o=2.5 mg/mL, —■— EZ_o=5 mg/mL, —●— EZ_o=10 mg/mL, —▲— EZ_o=20 mg/mL.

CONCLUSIONS

Over the wide range of enzyme concentration studied, the concentration of adsorbed enzyme does not reach a limit. It does however exhibit a "knee" in the adsorption curve. We recommend that the intercept of the extrapolation of the 3°C isotherm above the knee, as shown in Fig. 3, be used as a measure of the concentration of the available adsorption sites on the cellulose surface.

The hydrolysis rate goes through a maximum as the total enzyme concentration is increased. The specific hydrolysis rate is largely independent of enzyme concentration for the lower three enzyme concentrations studied and then declines with increasing enzyme concentration at the higher enzyme levels. Thus, the increase in the hydrolysis rate at low enzyme level is the result of increased enzyme adsorption. The decline at the higher levels is not well understood. It may be owing to competitive adsorption of the cellulase components or possibly steric hindrance of the cellulose chain ends by the adsorbed enzyme.

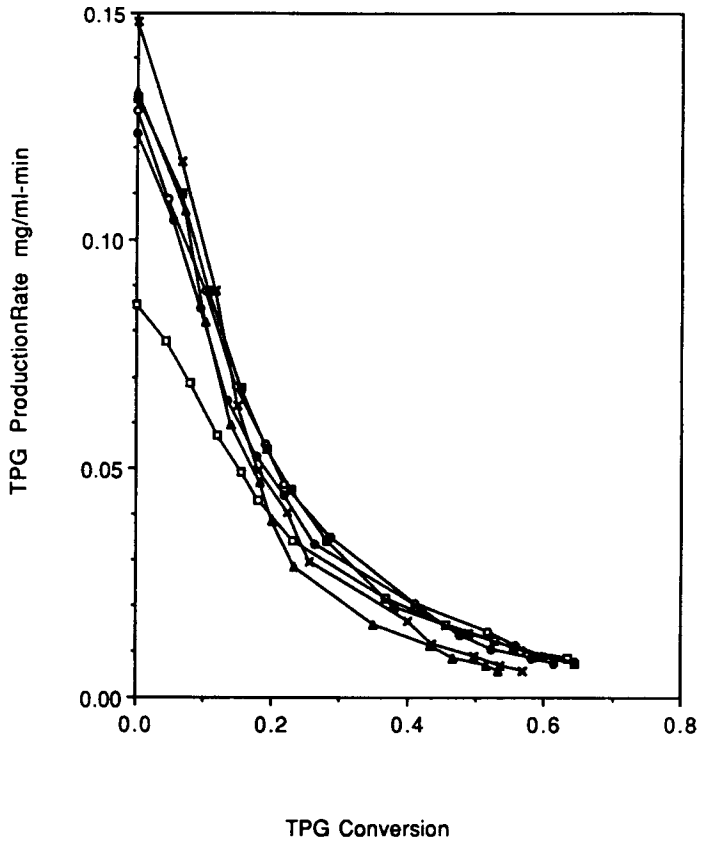

Fig. 7. Hydrolysis rate of PTP180, based on TPG formation, as a function of conversion. —□— $EZ_o = 0.5$ mg/mL, —○— $EZ_o = 2.5$ mg/mL, —■— $EZ_o = 5.0$ mg/mL, —●— $EZ_o = 10.0$ mg/mL, -X- $EZ_o = 15.0$ mg/mL, —▲— $EZ_o = 20.0$ mg/mL.

The specific reaction rate declines greatly as conversion increases. This is consistent with a hypothesis that pretreatment generates a large number of cellulose chain ends, which are depleted through reaction with cellobiohydrolase at a rate greater than they are formed through the action of endogluconase.

ACKNOWLEDGMENTS

The authors wish to express their appreciation for the funding of this work by the National Science Foundation, Contract Number BCS-8919335. We also wish to thank the members of the Biotechnology and Biochemical Engineering Group at the Thayer School of Engineering. In particular, we would like to acknowledge the suggestion from Professor Lee R. Lynd that the *specific* hydrolysis rate should be useful in separating adsorption and kinetic effects.

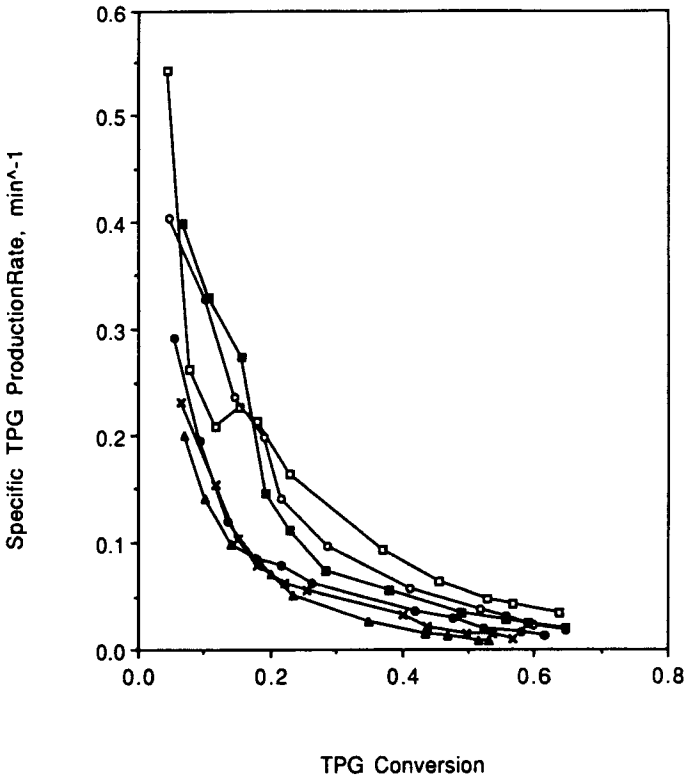

Fig. 8. Specific hydrolysis rate of PTP180, based on TPG formation, as a function of conversion. —□— $EZ_o = 0.5$ mg/mL, —○— $EZ_o = 2.5$ mg/mL, —■— $EZ_o = 5.0$ mg/mL, —●— $EZ_o = 10.0$ mg/mL, —▲— $EZ_o = 20.0$ mg/mL.

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