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The Effect of Enzyme and Substrate Levels on the Specific Hydrolysis Rate of Pretreated Poplar Wood

JAMES R. K. NUTOR AND ALVIN O. CONVERSE*

Thayer School of Engineering, Dartmouth College, Hanover, NH 03755

ABSTRACT

The hydrolysis of pretreated poplar wood was carried out with initial concentrations of 1.26, 2.52, 5.04 mg protein/mL of GC123 *Trichoderma reesei* cellulase and substrate concentrations of 2.5% w/v, 5% w/v, and 10% w/v at pH 4.8 and 40°C. The concentration of enzyme protein remaining in solution, the glucose concentration, and the total potential glucose concentrations were measured as a function of time during the hydrolysis. The enzyme rapidly adsorbed initially, reaching a maximum in about 30 min. About 55–75% of the cellulase returned to solution as the remaining cellulose was hydrolyzed. Dilution of the unhydrolyzed residue, largely lignin, did not cause additional desorption of the cellulase. The specific hydrolysis rate (i.e., the rate/amount of adsorbed enzyme) declined significantly with increased conversion, even when corrected for glucose inhibition. At a given initial substrate concentration, the specific rate was found to be largely independent of the total enzyme concentration. However, at a given fractional conversion, the specific rate was found to be reduced by increased substrate concentration.

Index Entries: Pretreated lignocellulosics; enzyme hydrolysis; adsorption; *Trichoderma reesei;* glucose inhibition.

INTRODUCTION

Since the enzymatic hydrolysis of cellulose is a heterogeneous reaction, it is necessary to understand the adsorption of the enzyme in order

*Author to whom all correspondence and reprint requests should be addressed.

to be able to formulate an adequate model of the overall reaction kinetics. Although several adsorption-based models have been developed (*1–4*), only one (*4*) has been tested by its ability to predict the amount of adsorbed enzyme as well as conversion. In this case, data from the action of Meicelase on Avicel (*5*) was used. It was necessary to assume, not only that the concentration of adsorption sites fell off in proportion to the square of the substrate concentration, but also that the adsorbed enzyme deactivated during the hydrolysis. Unfortunately, this assumption weakens the model discrimination aspect of the work, since a direct measurement of the active adsorbed enzyme is not available.

In the case of lignocellulosic substrates, one must contend with the added complication that some of the enzyme may adsorb on the lignin, as well as the cellulose. Tatsumoto et al. (*6*) followed the loss from solution of five enzymatic activities during the first 3 h of digestion of alpha cellulose and four pretreated lignocellulosic substrates. During this time, the digestion was probably less than 10% complete, although the digestion studies were conducted in separate runs. They found that the adsorption of filter paper activity decreased, and that the adsorption of β-glucosidase increased, with increased lignin content of the substrate, steam-exploded aspen being the exception to the general correlation. They concluded that "native lignin appears to have little effect on the substrate-specific adsorption of cellulose depolymerizing enzymes." Thus, they imply that native lignin does not have the ability to adsorb CMC and filter paper activities; however, this was not tested directly since even the substrate, which had been previously subjected to extensive enzyme hydrolysis, still contained 34% anhydroglucose. The work reported here differs considerably from the above study in that the adsorption of the enzyme was followed by measuring protein concentration, rather than activities, and it was continued throughout the course of the hydrolysis. Thus, it covers the period during which enzyme returns to solution toward the end of hydrolysis, as well as the initial period of adsorption. Furthermore, the substrate, prepared by partial acid hydrolysis, was not dried prior to use. In addition, enzyme and substrate loadings were both varied in order to obtain data needed for the development of a kinetics model.

The relationship between enzyme activity and protein concentration was investigated by Ooshima et al. (*7*) in a study of pretreated hardwood. He found that Avicelase and CMCase activities approximately tracked the protein concentration during the hydrolysis; β-glucosidase activity however, did not, being adsorbed to a increasing degree as the reaction proceeded. Ooshima et al. (*8*) previously found that β-glucosidase does not adsorb on cellulose; hence, the above result indicates adsorption on lignin. This is in agreement with the β-glucosidase aspect of the study by Tatsumoto et al. (*6*). The work reported in this study extends the results of Ooshima et al. (*7*) to a new substrate and pretreatment temperature; furthermore, it includes the effect of substrate, as well as enzyme level.

In addition to its importance in understanding the reaction kinetics, enzyme adsorption is also of interest because the high cost of enzymes dictates the development of lower enzyme loadings, and/or higher enzyme recovery, to make the process more economical. Several studies have been conducted on the desorption of cellulases from lignin or hydrolysis residue (*9–12*). Consistent with the results reported in the present study, in most studies (*9,10*). enzyme recovery was difficult. Ooshima (*7*) has found that the amount of enzyme adsorbed on the lignin residue is reduced when the temperature of the pretreatment is increased, possibly because of the agglomeration of the lignin. Two studies (*7,12*) report equilibrium adsorption relationships; however, work reported in the present study indicates that studies, in which desorption as well as adsorption is carried out, do not confirm the same apparent equilibrium behavior.

In the present study, we report an investigation of the enzymatic hydrolysis of pretreated poplar wood at three different substrate concentrations (2.5%, 5.0%, and 10.0% w/v) and three different initial enzyme concentrations (1.26, 2.52, and 5.04 mg protein/mL) for each substrate concentration. The desorption of the cellulase from lignin at the end of hydrolysis was also studied at three different initial enzyme concentrations.

MATERIALS AND METHODS

Materials

Poplar wood chips were obtained as a gift from SERI. The cellulase was from Genencor Inc. (*Trichoderma reesei GC123*). Other chemicals were of reagent grade. The enzyme was found to have a filter paper activity of 116 U/mL of enzyme solution and an Avicelase activity of 4.2 mg glucose/min/mL, by a modification of standard methods (*13*).

Pretreatment of Poplar Wood

The poplar wood chips were milled to 60 mesh size by knife milling in a Wiley mill. The milled wood was pretreated by acid hydrolysis, using 5wt% wood slurry containing 1wt% H_2SO_4 for a residence time of $9.5s \pm 0.5s$. This was done at $220 \pm 0.5°C$ in a continuous plug-flow reactor developed by McParland et al. (*14*). The pretreated wood was neutralized and washed on filter paper with distilled water and 0.2% NaN_3 solution until the pH of the supernatant was between pH 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 substrate was found by quantitative saccharification (*13,17*) to contain 51% potential glucose by weight.

Hydrolysis of Pretreated Poplar Wood Substrate (PTP)

Hydrolysis of the PTP was carried out in 158 mL pressure bottles at 40°C with gentle shaking in a water bath or controlled temperature room and at a pH of 4.8, using 0.05*M* Na acetate buffer. Initial substrate concentrations of 2.5, 5.0, and 10.0 wt% PTP(dry basis) were reacted with initial enzyme concentrations of 1.26, 2.52, and 5.04 mg/mL, such that all reaction vessels contained the same amount of substrate in a total reaction vol of 100 mL. Approx 1 mL samples were withdrawn from the reaction mixture at various time intervals and analyzed for total potential glucose, and enzyme protein concentrations.

Desorption of Enzyme from Lignin

The PTP was reacted for 8 d to ensure maximum hydrolysis of the cellulose. The reaction mixture at the end of hydrolysis was filtered off and the solid residue diluted with various amounts of 0.5*M* Na acetate buffer. This mixture was observed for any changes in enzyme concentration by measuring the amount of protein in solution at differing times. By measuring the initial and final enzyme concentrations, the amount of hydrolysate filtered, and the vol of diluent added, it is possible to evaluate the amount of enzyme desorbed by the dilution.

Measurement of Enzyme in Solution

This was determined as the amount of protein in solution by the Bradford colorimetric assay from Bio-Rad Co. (*15*). Bovine serum albumin was used as the protein standard, and the units were mg protein/mL. 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.

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 (*16*). The total potential glucose (TPG) was determined by first acid hydrolyzing samples with 72% H_2SO_4 at 121°C for 1h. The acid hydrolyzed samples were then analyzed for glucose as described above. The cellobiose in solution was then calculated by the difference between TPG and glucose in solution.

Glucose Inhibition

Glucose, in concentrations of 0, 2.0, 5.0, 10.0, and 20.0 mg/mL, was added to solutions of 5.0 wt% PTP220; 2.52 mg protein/mL of GC123

cellulase was then added to each, and the hydrolysis carried out as indicated above.

RESULTS AND DISCUSSION

Soluble Products

Figures 1–4 show amounts of glucose and TPG in solution, as fractions of the TPG in the substrate, and the adsorbed enzyme, as fraction of the total enzyme, during the course of hydrolysis. The data are given in this normalized form so that the fractional conversion and degree of enzyme adsorption are apparent. Corresponding data, in terms of absolute glucose concentrations, are presented in Fig. 5. In Figs. 1 and 2, the initial concentration of enzyme is 1.26 mg protein/mL; in the parts a, b, and c, the initial substrate concentrations are 2.5%, 5.0%, and 10.0% respectively. Figure 2 presents an expanded version of the initial portion of the data presented in Fig. 1. The data obtained with an initial enzyme concentration of 2.52 and 5.04 mg protein/mL are presented in Figs. 3 and 4, respectively. For an increase in enzyme concentration, with a fixed substrate concentration (e.g., Figs. 1b, 3b, and 4b), the time for complete hydrolysis is shorter, indicating a higher conversion rate. In all cases, it is seen that the TPG and glucose fractions are not very different. This implies that the enzyme system is able to break down cellobiose effectively, preventing inhibition of by the accumulation of cellobiose.

It is evident from these figures that 2 d is usually sufficient for a conversion of 80% or more. However, this is not always the case, and the conversion is dependent on initial substrate loadings, as can be seen in Figs. 1–4. In some cases, appreciable conversions of 20–40% occur rapidly, within the first 2 h of reaction.

Enzyme Adsorption

From Figs. 1–4, a general trend of rapid enzyme adsorption, followed by desorption back into solution as conversion increases, is evident. In the case of S_o (initial substrate concentration) = 10.0%, the amount of adsorbed enzyme remains almost constant until about 70% conversion. This suggests that all the adsorbable enzyme, about 80%, is adsorbed, and that the substrate is in excess. For the case of $S_o = 2.5\%$, all the values of E_o (the initial enzyme concentration) are in excess, making the resolution of adsorbed fractions difficult. With increased substrate concentration, greater fractions of enzyme remain adsorbed on the unhydrolyzable substrate (predominantly lignin). For economical applications, the substrate concentration must be relatively high and the enzyme concentration, low. This implies that high fractions of about 50% enzyme would remain adsorbed

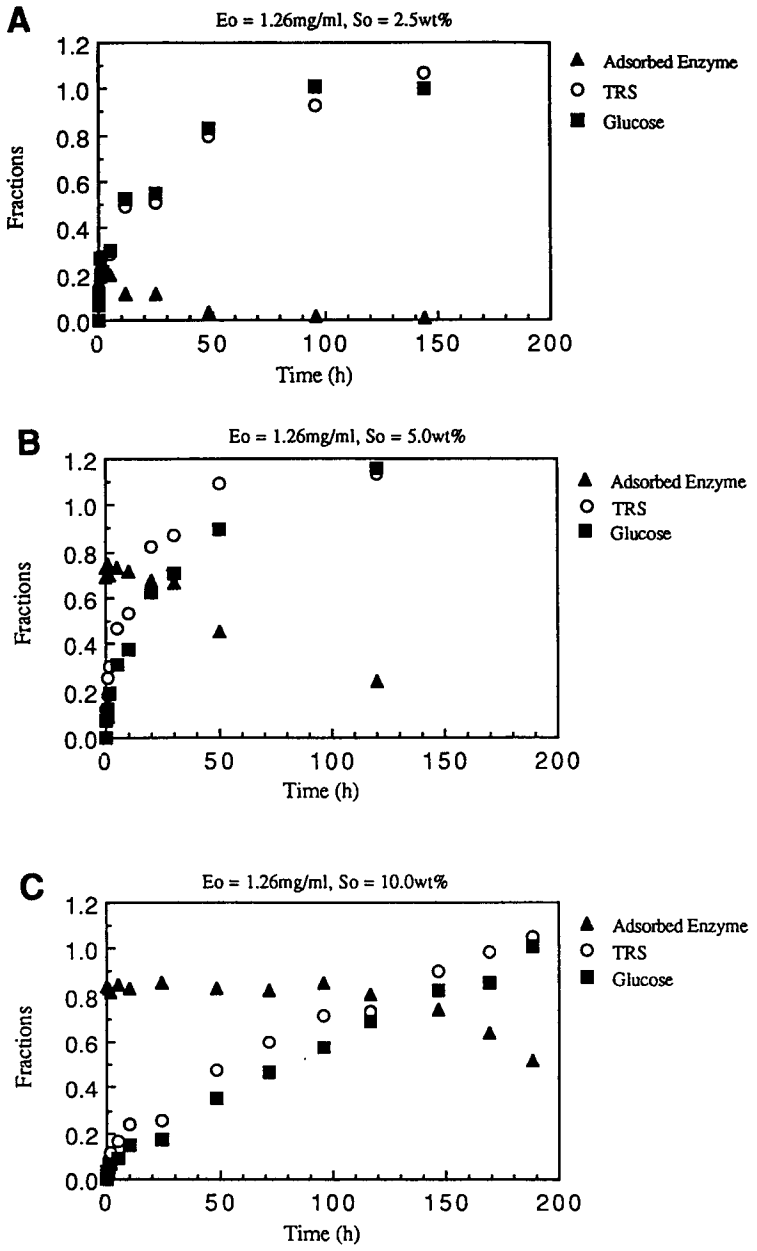


Fig. 1. Total potential glucose of soluble sugars (TPG) and glucose as fractions of the total potential glucose of the initial substrate; and adsorbed enzyme, as fraction of the total enzyme, during the course of hydrolysis. The initial concentration is 1.26 mg/mL, and substrate concentrations are as shown.

(Fig. 1c). There is, therefore, a need to reclaim the adsorbed enzyme to reduce total enzyme costs.

The data in Table 1 indicate that the adsorbed enzyme does not desorb when the residue is washed with fresh buffer. To explain Table 1, we introduce the following nomenclature:

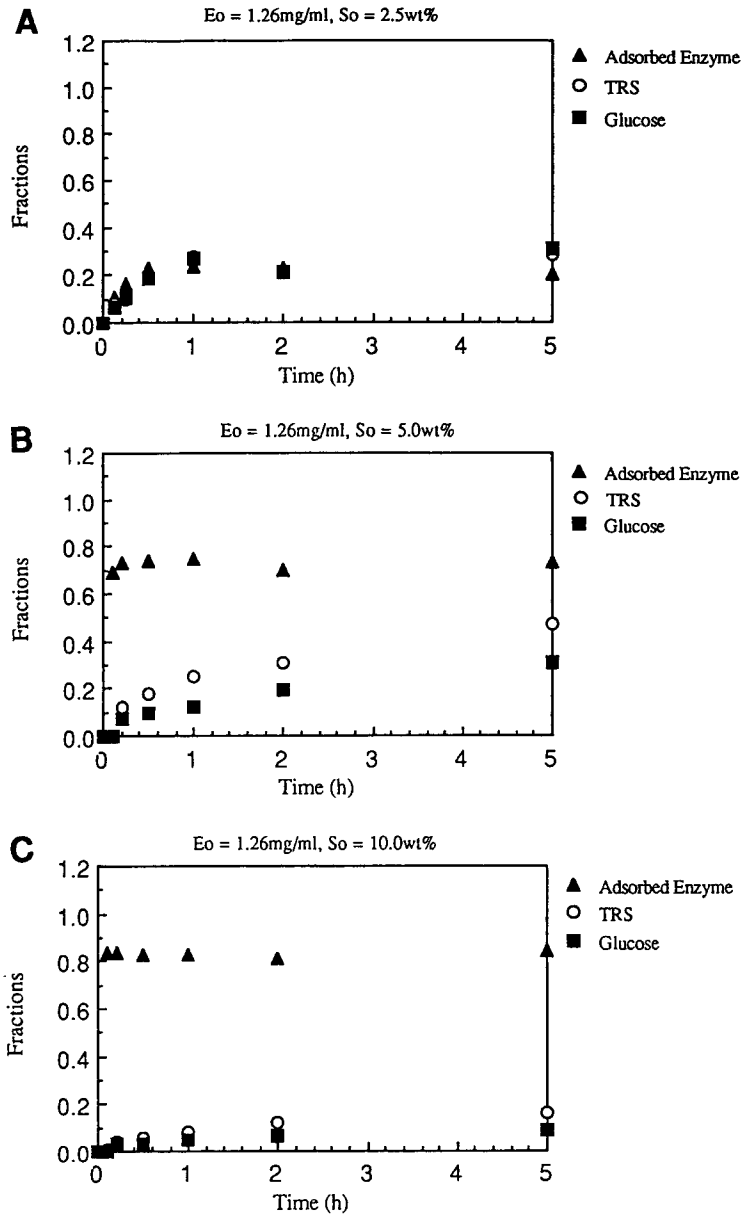


Fig. 2. Same data as in Fig. 1, but expanded so that the initial behavior can be observed.

E_d = enzyme concentration if no desorption occurs during dilution, mg/mL,
E_{dd} = enzyme concentration if complete desorption occurs during dilution, mg/mL,
E_o = initial enzyme concentration, mg/mL,
E_t = enzyme concentration at end of hydrolysis, mg/mL,
E_z = enzyme concentration at the end of dilution, mg/mL,

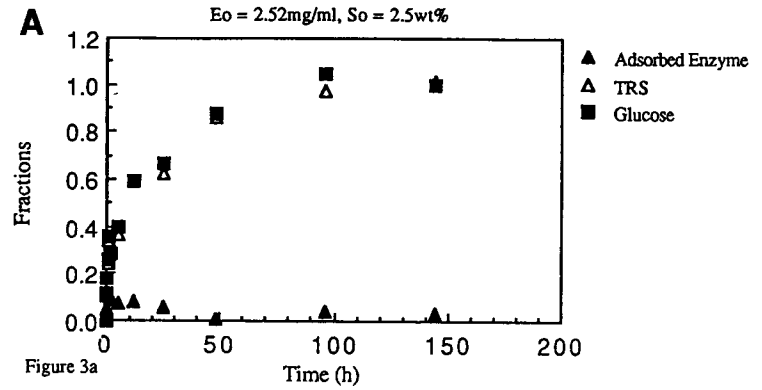


Figure 3a

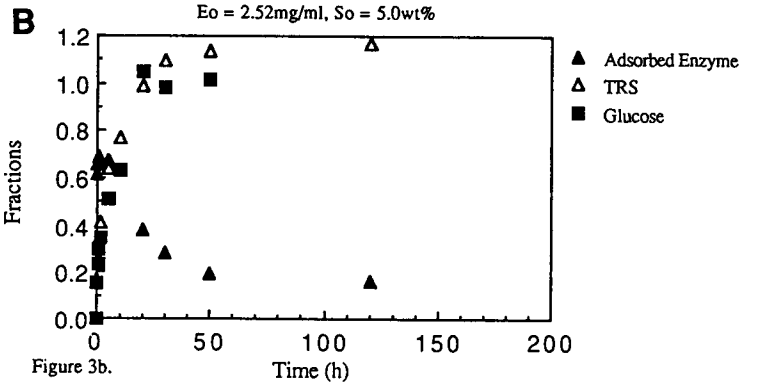


Figure 3b.

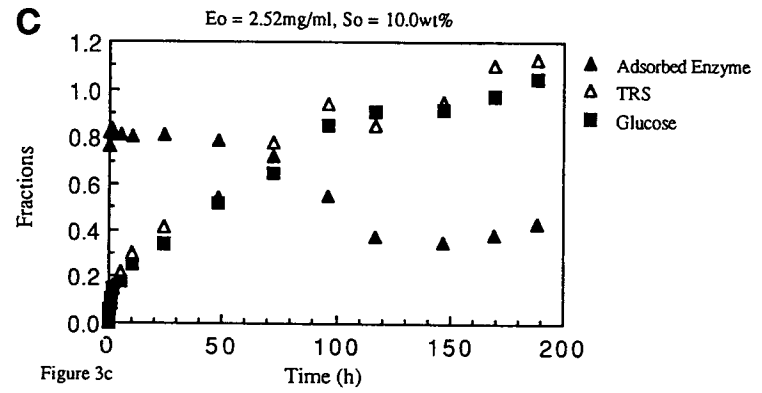
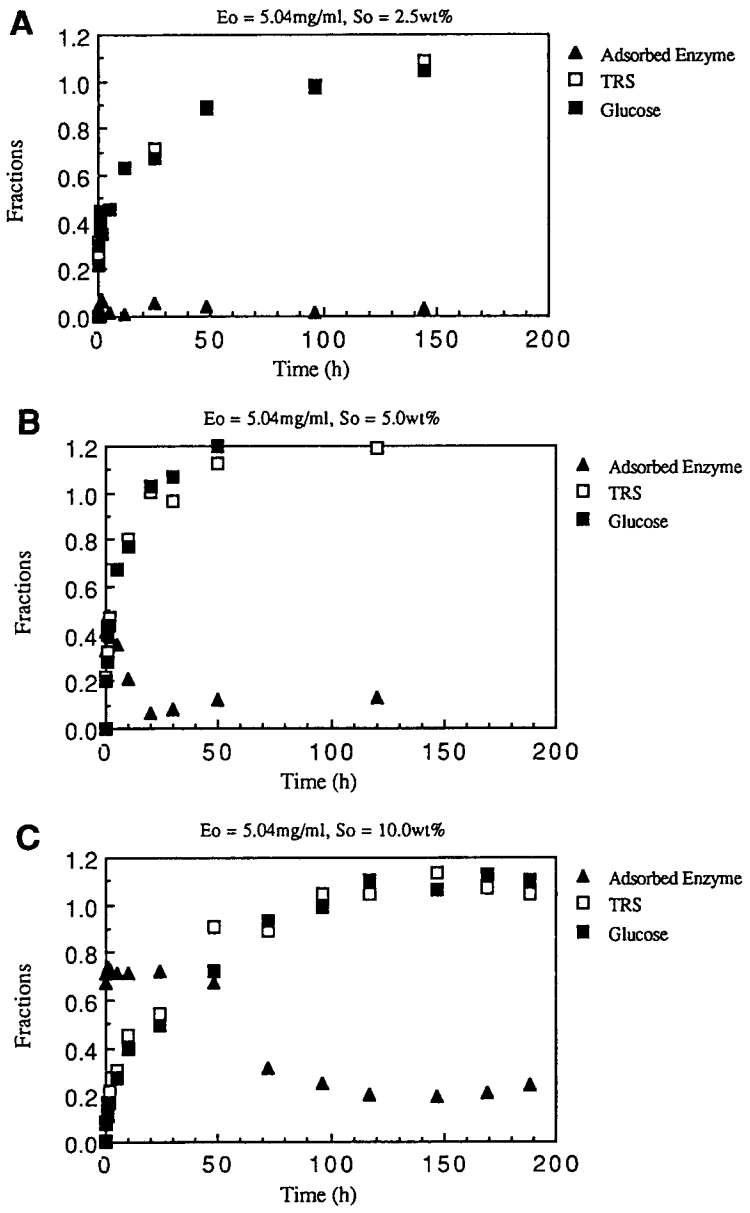


Figure 3c

Figs. 3a, b, and c. Same quantities as in Fig. 1, but at an initial enzyme concentration of 2.52 mg/mL.

V_d = volume of buffer added, mL,
V_f = volume of liquid removed during filtration step, mL,
V_l = volume of residue, calculated from the lignin content of the original substrate and an assumed density of 1g/mL, mL,
V_o = initial volume of liquids at beginning of hydrolysis, mL,
V_r = measured total volume at the end of hydrolysis, mL, and
V_t = volume of liquids at end of hydrolysis, mL.



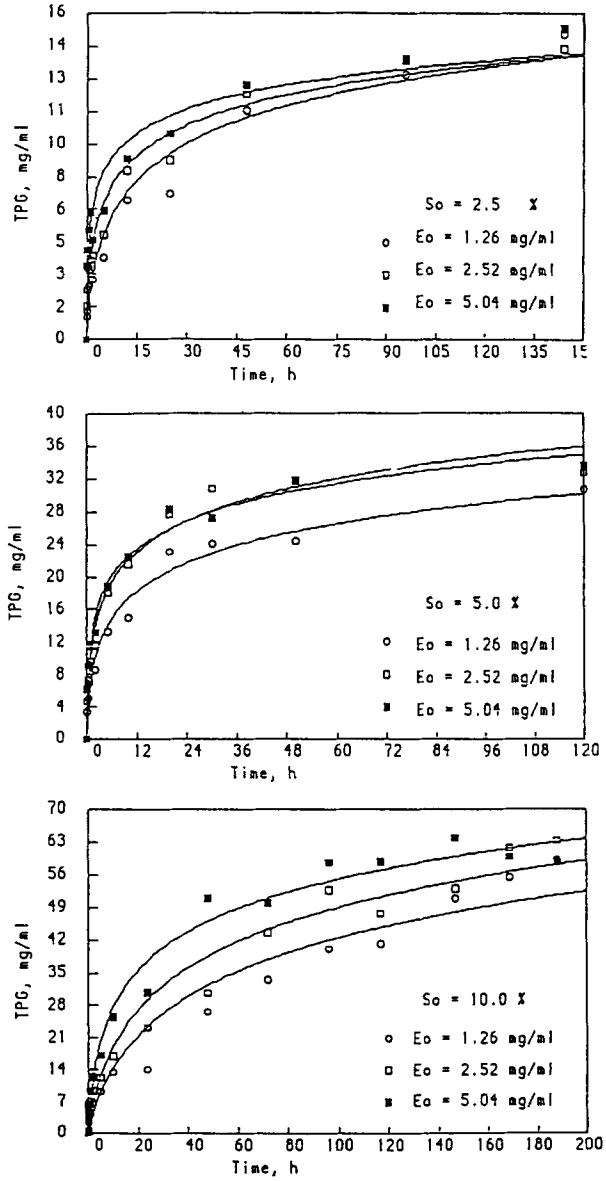
Figs. 4a, b, and c. Same quantities as in Fig. 1, but at an initial enzyme concentration of 5.04 mg/mL.

The values of E_o are presented in column 1 of Table 1. E_{dd}, presented in column 2, is calculated from the following equation:

$$E_{dd} = \{E_t[V_t - V_f] + [V_oE_o - V_tE_t]\} / [V_t - V_f + V_d] \quad (1)$$

E_d, presented in column 3, is calculated from the following equation:

$$E_d = E_t[V_t - V_f] / [V_t - V_f + V_d] \quad (2)$$



Figs. 5a, b, and c. Total potential glucose of soluble sugars (TPG) in mg/mL during the course of hydrolysis, with initial enzyme and substrate concentrations as shown. The curves are computed from Eq. (3) with the parameter values, as given in Table 2, obtained from a nonlinear least squares fit. Standard deviations are given in Table 2.

The measured values of the enzyme concentrations after dilution, presented in column 4, are very close to the computed values of E_d. Hence, it is concluded that the enzyme does not desorb under these conditions.

In an effort to further study enzyme adsorption on the lignin in the hydrolysis residue, Nutor (*17*) washed residue, obtained from runs for which complete conversion was indicated by the sugar analysis, with dis-

Table 1
Cellulase Desorption Study

E_o, mg/ml	E_{dd}, mg/ml	E_d, mg/ml	E_z, mg/ml	V_d, ml
0.63	0.41	0.06	0.04	80
0.63	0.33	0.05	0.04	110
1.26	0.86	0.09	0.08	82
1.26	0.69	0.07	0.06	110
2.52	1.55	0.20	0.17	90
2.52	1.33	0.18	0.17	110

E_d = enzyme concentration if no desorption occurs during dilution, mg/ml,

E_{dd} = enzyme concentration if complete desorption occurs during dilution, mg/ml,

E_o = initial enzyme concentration, mg/ml,

E_z = enzyme concentration at the end of dilution, mg/ml, and

V_d = volume of buffer added, ml.

tilled water, 1 *M* NaOH, and distilled water, in that order. This was contacted with fresh enzyme solution. He found that the enzyme adsorption appeared to reach equilibrium well within 3 h, and that the concentration of adsorbed enzyme, mg enzyme/mg residue, was proportional to the enzyme concentration in the remaining solution (data not shown). However, as described above, this adsorbed enzyme does not appear to desorb when diluted with fresh buffer. Furthermore, when this residue was added to a solution of fresh substrate, but no fresh enzyme, cellulase activity was detected, as indicated by glucose formation. It is as if the cellulase can be transferred in an active state from the residue to the fresh substrate directly without first desorbing into the solution. These observations indicate: (1) that desorption, as well as adsorption experiments, are needed to establish a true equilibrium relationship for cellulase adsorption, and (2) that a solid-to-solid transfer mechanism may be important in the recovery of cellulase from the hydrolysis residue. These subjects obviously need further study.

Reaction Rates

To be able to calculate the rate of reaction for the various hydrolysis runs, an empirical function, developed by Ooshima (*8*), was applied to the absolute TPG values. The function is of the form:

$$y = k*1n\,(1 + m*t) \qquad (3)$$

where: y= total potential glucose, TPG, t= time of hydrolysis elapsed, and k and m are arbitrary constants.

The absolute values of TPG are presented as a function of time in Fig. 5, the curves are computed from Eq. (3), using the least squares parameter values given in Table 2. The various standard deviations are also given in Table 2.

Table 2
Least Squares Fit of Hydrolysis Data

S_0	E_0	k	m	Std. Deviation
wt. %	mg/ml	mg/ml	h^{-1}	mg/ml
2.5	1.26	2.734	1.0941	1.106
2.5	2.52	2.172	4.188	0.925
2.5	5.04	1.719	24.016	1.148
5.0	1.26	5.25	2.594	1.234
5.0	2.52	5.625	4.906	1.520
5.0	5.04	4.953	9.422	1.100
10	1.26	15.109	0.156	4.262
10	2.52	15.047	0.250	3.901
10	5.04	12.609	0.781	3.257

S_0 = initial substrate concentration,

E_0 = initial enzyme concentration

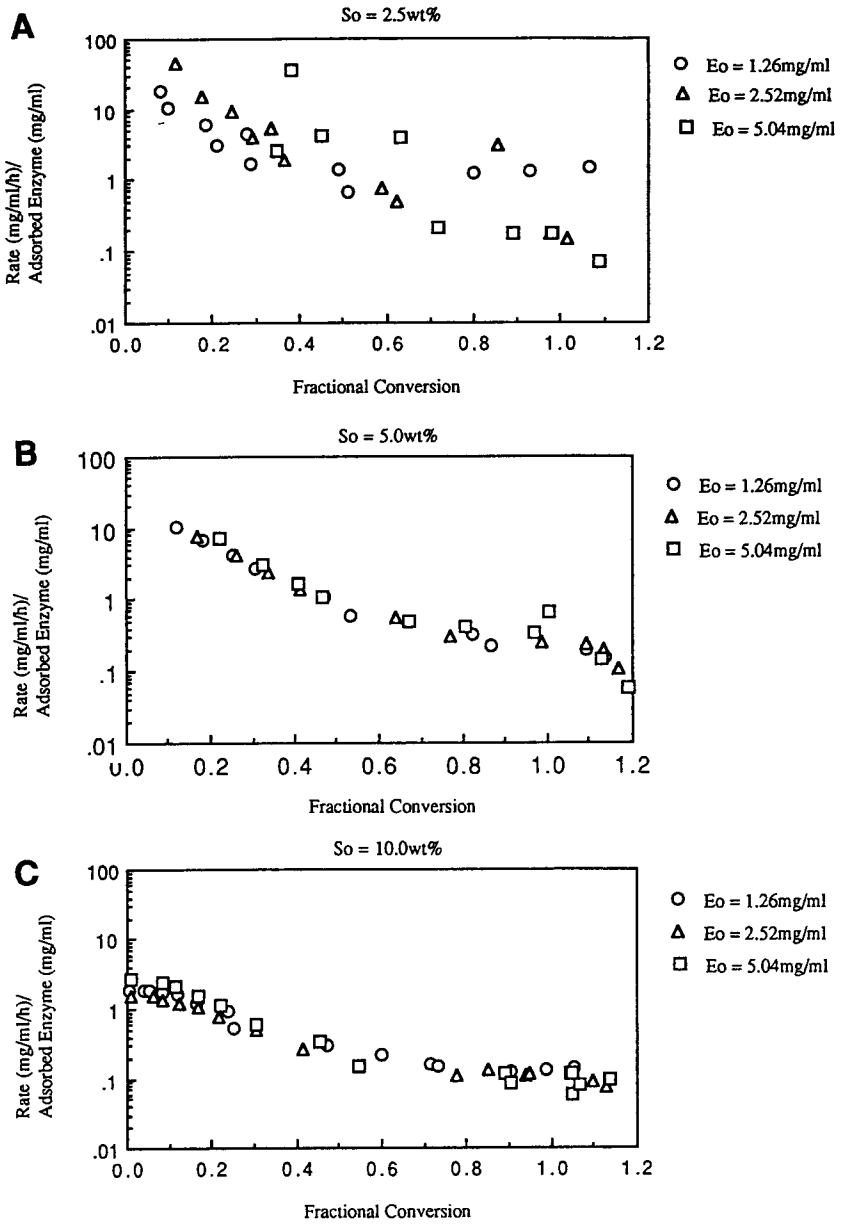
k,m = parameters defined by Eq.(3)

Differentiation of Eq. (3) yields the following expression for the hydrolysis rate:

$$r = k*m / (1+ m*t) \quad (4)$$

This expression was used to calculate the hydrolysis rates that were divided by the concentration of adsorbed enzyme to give the specific rate, in mg glucose/mL-h/mg protein/mL, presented in Fig. 6. No mechanistic justification for Eq. (4) is offered; it is simply an empirical expression that has been able to fit a wide variety of hydrolysis data over the full range of the hydrolysis (*8*). As such, it provides a uniform method for evaluating the rates that is not sensitive to local scatter.

All plots of the specific rate vs the fractional conversion, in Fig. 6, show a significant decline in the specific rate as conversion increases. The data for the case in which $S_0 = 2.5\%$ show considerable scatter, partially owing to the small amount of the enzyme adsorbed when the substrate concentration is low. The data for $S_0 = 5.0$ and 10.0% are much tighter, and indicate that the use of the specific rate eliminates the effect of the enzyme level. In other words, for a given substrate concentration, at a given conversion, the rate is proportional to the amount of adsorbed enzyme. In this very limited way, the adsorbed enzyme behaves as the activated complex in Michaelis-Menton homogeneous enzyme kinetics. Comparison of the results in Fig. 6b with those in Fig. 6c indicates that, at a given conversion, the specific rate is reduced when the substrate concentration is increased. Since the adsorbed enzyme is measured as a concentration in terms of mg protein per mL of solution, the ratio of adsorbed enzyme to substrate is reduced when the substrate concentration is increased, and the adsorbed enzyme concentration is held constant. Thus, comparison of Figs. 6b and 6c indicates that the adsorbed enzyme is less effective



Figs. 6a, b, and c. Specific hydrolysis rate in mg glucose/mL-h/mg adsorbed enzyme/mL as a function of the fractional conversion, with initial enzyme and substrate concentrations as shown.

when it is more widely separated on the substrate surface, an effect that is consistent with synergistic behavior of enzyme components.

Glucose Inhibition

The effects of added glucose concentration on the fractional conversion vs time, and on the fraction of enzyme adsorbed vs fractional conversion, are shown in Figs. 7a and b. The effect on the specific rate vs fractional

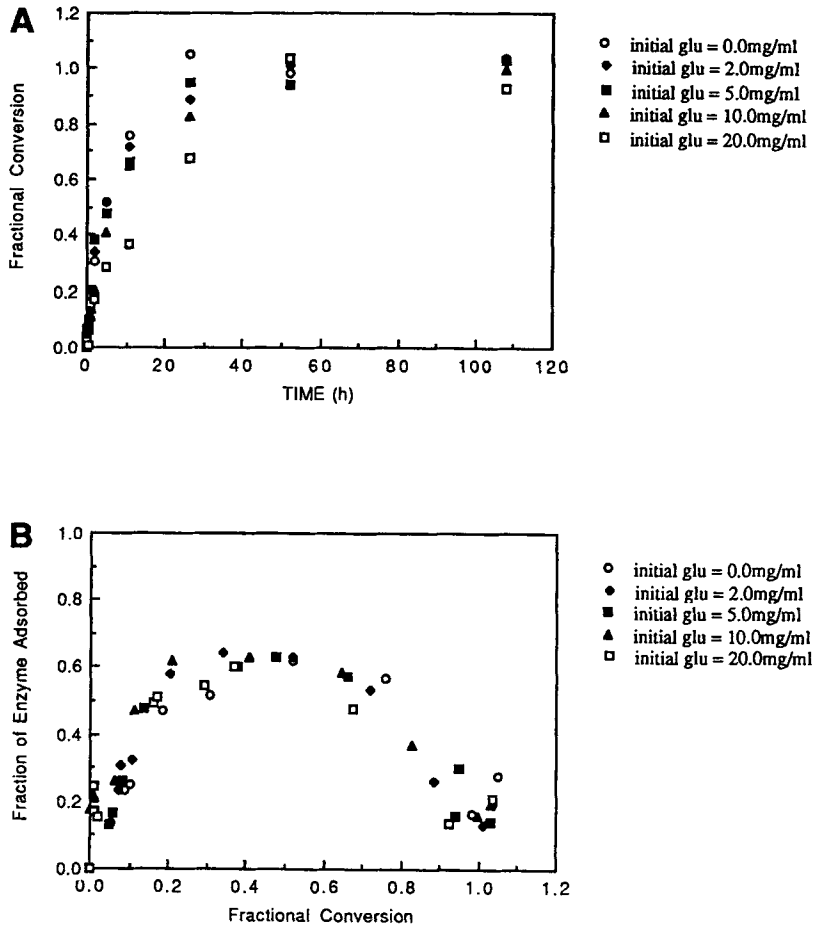


Fig. 7. Data from glucose inhibition study. Fig. 7a. Fractional conversion vs time, with added glucose concentrations as shown. Fig. 7b. Adsorbed enzyme vs fractional conversion, with the added glucose concentrations as shown.

conversion is shown in Fig. 7c. If it is assumed that the glucose in solution can combine reversibly with the adsorbed enzyme to form an inactive adsorbed enzyme complex, the concentration of active adsorbed enzyme would be given by the following equation:

$$E_{ad} = [E_o - E] / [1 + K_i{*}P] \quad (5)$$

where E_{ad} = the concentration of active adsorbed enzyme, mg/mL, E_o = the initial concentration of enzyme, mg/mL, E = the concentration of enzyme remaining in solution, mg/mL, K_i = the experimentally-determined inhibition constant, mL/mg, and P = the concentration of glucose owing to the amount added as well as the amount produced, mg/mL.

The concentration of adsorbed enzyme was recalculated, using Eq. (5) with K_i being set by trial and error in order to eliminate the glucose

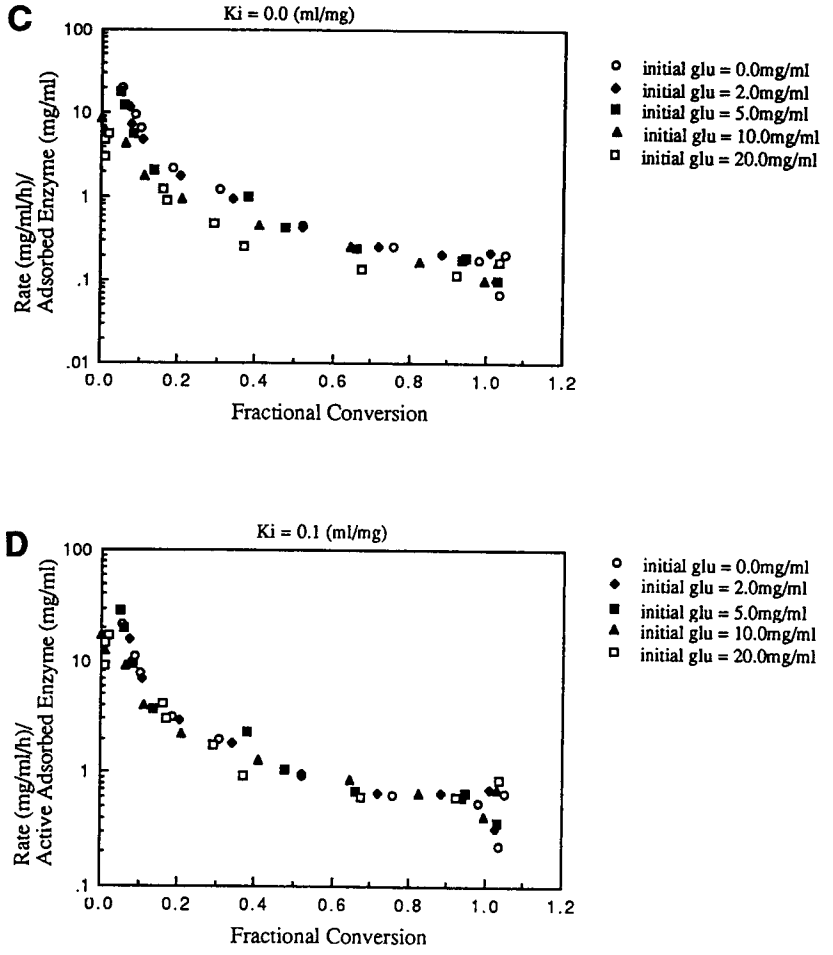


Fig. 7 (*cont'd*). Fig. 7c. Specific rate vs fractional conversion, without correcting for glucose inhibition. Fig. 7d. Specific rate vs fractional conversion, with correction for glucose inhibition according to Eq. (5). $K_i = 0.1$ mL/mg.

effect from the Fig. 7c. The result with $K_i = 0.1$ is presented in Fig. 7d. From the behavior shown in Fig. 7d, we conclude that the decline in the specific rate with conversion is not caused by glucose inhibition.

One other mechanism that might account for the decline in the specific rate is the gradual adsorption of enzyme on lignin, where it is inactive, rather than on cellulose, as the reaction proceeds. Nutor (*17*) attempted to account for this mechanism by subtracting the amount of adsorbed enzyme that remains on the final residue from the total adsorbed enzyme, this difference being the minimum amount that would be adsorbed on the cellulose. The specific rate was recalculated, using this difference as the adsorbed enzyme. However, the specific rate still declined (data not shown). This behavior indicates a decline in the reactivity of the substrate or denaturation of the enzyme as the hydrolysis proceeds.

CONCLUSIONS

This study found that: (1) GC 123 cellulase adsorbs rapidly, reaching a maximum in about 30 min, and then about 55–75%, depending on the enzyme to substrate ratio, returns to solution near the end of hydrolysis, (2) dilution of the unhydrolyzed ligancious residue with buffer does not cause additional desorption of the cellulase, (3) the specific hydrolysis rate (i.e., the rate/amount of adsorbed enzyme) declines significantly with increased conversion, even when corrected for glucose inhibition, (4) at a given initial substrate concentration, the specific rate is largely independent of the total enzyme concentration, and (5) at a given fractional conversion, the specific hydrolysis rate is reduced at increased substrate concentration.

REFERENCES

1. Fan, L. T. and Lee, Y-H. (1983), *Biotechnol. Bioengr.* **25,** 2707.
2. Ryu, D. D. Y., Lee, S. B., Tassinari, T., and Macy, C. (1982), *Biotechnol. Bioeng.* **24,** 1047.
3. Holtzapple, M. T., Caram, H. S., and Humphrey, A. E. (1984), *Biotechnol. Bioeng.* **26,** 775.
4. Converse, A. O., Matsuno, R., Tanaka, M., and Taniguchi, M. (1988), *Biotechnol. Bioeng* **32,** 38.
5. Tanaka, M., Nakamura, H., Taniguchi, M., Morita, T., Matsuno, R., and Kamikubo, T. (1986), *Appl. Microbiol. Biotechnol.* **23,** 263.
6. Tatsumoto, K., Baker, J. O., Tucker, M. P., Oh, K. K., Mohagheghi, A., Grohmann, K., and Himmel, M. E. (1988), *Appl'd Biochem. Biotechnol.* **18,** 159.
7. Ooshima, H., Burns, D. S., and Converse, A. O. (1990), *Biotechnol. Bioeng.* **36,** 446.
8. Ooshima, H., Sakata, M., and Harano, Y. (1983), *Biotechnol. Bioeng.* **25,** 3103.
9. Deshpande, M. V. and Eriksson, K. E. (1984), *Enzyme Microb. Technol.* **6,** 338.
10. Clesceri, L. S., Sinitsyn, A. P., Saunders, A. M., and Bungay, H. R. (1985), *Appl'd. Biochem. Biotechnol.* **11,** 433.
11. Sutcliff, R. and Saddler, J. N. (1986), *Biotechnol. Bioeng. Symp.* #17, 749.
12. Chernoglazov, V. M., Ermolova, O. V., and Klyosov, A. A. (1988), *Enzyme Microbiol. Technol.* **10,** 503.
13. Saeman, J. F. (1945), *Ind. Engr. Chem.* **37,** 47.
14. McParland, J. J., Grethlein, H. E., and Converse, A. O. (1982), *Solar Energy* **28,** 55.
15. Bio-Rad Chemical Division, Richmond, CA.
16. Lynd, L. R. and Grethlein, H. E. (1987), *Biotechnol. Bioeng.* **29,** 92.
17. Nutor, J. R. K. (1990), "Enzyme hydrolysis of pretreated poplar wood," M. E. thesis, Thayer School of Engineering, Dartmouth College, Hanover, NH.