0273-2289/94/4546—0407$03.00

Evaluation of the Enzymatic Susceptibility of Cellulosic Substrates Using Specific Hydrolysis Rates and Enzyme Adsorption

DORA LEE, ALEX H. C. YU,
KEN K. Y. WONG, AND JOHN N. SADDLER*

Forest Products Biotechnology, Faculty of Forestry, University of British Columbia, #270-2357 Main Mall, Vancouver, Canada V6T 1Z4

ABSTRACT

Adsorption of cellulases to cellulose is a critical step in the hydrolysis of cellulosic substrates. However, the importance of adsorption in determining the hydrolysis rate is unclear. The accessibility to cellulases and specific hydrolysis rates were measured for various substrates. No correlation was found between the amount of enzyme adsorbed and the initial hydrolysis rate for different substrates. Specific hydrolysis rates were found to differ among substrates. Furthermore, both accessibility to cellulases and the specific hydrolysis rate of substrates were found to be changed by chemical and physical pretreatment of the substrate.

Index Entries: Cellulase; specific hydrolysis rate; adsorption; cellulosic substrates; cellulose hydrolysis.

INTRODUCTION

The susceptibility of cellulosic substrates to enzymatic hydrolysis depends on various structural features. The crystallinity of cellulose (*1–4*), the degree of polymerization of cellulose (*2*), the lignin content and distribution (*4*), and the surface area available to the enzymes (*4–10*) have all

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

been implicated. Other factors such as enzyme adsorption, enzyme inactivation, and product inhibition are also known to affect the rate of cellulose hydrolysis.

Of the many structural features of the substrate, the surface area available for enzyme binding is expected to be an important factor determining the hydrolysis rate because the cellulases must be able to adsorb onto the insoluble substrate in order to carry out hydrolysis. Several reports have suggested that the rate of hydrolysis is proportional to the amount of adsorbed protein (*11–13*), whereas others have suggested that it depends only on the surface area of the substrate (*5–9*). However, most of these studies were carried out over an extensive period of hydrolysis during which the substrate might have undergone substantial changes. To study the relationship between the hydrolysis rate and the adsorbed enzyme, the hydrolysis rate should be measured early in hydrolysis, when product inhibition, enzyme inactivation, and substrate recalcitrance can be considered to be not so important.

It is expected that a better understanding of the relationship between enzyme adsorption and the corresponding hydrolysis rate will lead to a more efficient use of enzymes in cellulose hydrolysis. This relationship is the focus of our study on various cellulosic and pretreated lignocellulosic substrates. The maximal extent of cellulase adsorption was the parameter used to measure the accessibility of a substrate, whereas the specific hydrolsis rate (i.e., the initial rate per amount of adsorbed cellulases) was used to measure the reactivity of the substrate.

MATERIALS AND METHODS

Substrates

Avicel PH101 (AV), a microcrystalline cellulose, and Solka-floc BW300 (SF), a hammer-milled sulfite pulp, were purchased from Fluka (Buchs, Switzerland) and James River Corp. (Bern, New Hampshire), respectively. Acid-swollen Avicel (ASA) was prepared by treating AV with 85% phosphoric acid (*14*). Portions of ASA were either air-dried for 4 d to give the "AD" substrate or oven-dried overnight at 80°C to give the "OD" substrate. A sulfite pulp (SP) was provided by Q. Nguyen (Tembec, Témiscaming, Québec) and water-washed steam-exploded birch (WB) by J. Tolan (Iogen, Ottawa, Ontario). Alkaline extraction of the steam-exploded birch was carried out using 0.4% NaOH (*15*) to give the "AB" substrate, and a portion of the AB substrate was treated with 1% hydrogen peroxide (*16*) to produce the "PB" substrate. The cellulose content of these substrates was determined by measuring glucose content in their sulfuric acid hydrolysates (*10*).

Cellulases

Celluclast (80 IU/mL, 80 mg protein/mL; batch #CCN 3027, Novo-Nordisk A/S, Bagsvaerd, Denmark) was the cellulase used in all experiments. For cellulose hydrolysis, it was supplemented with Novozym (792 CBU/mL, 73 mg protein/mL; batch #DCN 0012, Novo-Nordisk A/S) at 3.5 CBU/IU Celluclast to alleviate end-product inhibition by cellobiose (*17*).

Determination of Cellulase Adsorption Kinetics and Isotherms

Preliminary experiments were conducted to determine the time required for Celluclast to reach adsorption equilibrium using the various substrates. Celluclast at a final protein concentration of 3 mg/mL (i.e., 150 U/g substrate) was added to tubes containing a fixed amount of substrate. The final substrate concentration was 2% (w/v) in 50 m*M* sodium acetate (pH 4.8) buffer. The tubes were incubated at 4°C with agitation. Aliquots of the mixture were sampled at different times and centrifuged to remove the substrate. The amount of protein in the supernatants was measured by the Bradford assay (BioRad, Richmond, CA). The time required to attain adsorption equilibrium was determined to be: 10 min for ASA, 90 min for AV and SF, and 60 min for SP, AD, OD, and the birchwood-derived substrates.

For determining the adsorption isotherms, varying amounts of Celluclast were added to each substrate as described for the kinetics experiments. Free Celluclast was determined by measuring the amount of protein in the supernatant after equilibrium was attained. Bound Celluclast was calculated as the difference between free protein and the total protein initially added. The experimental data were fitted to the Langmuir adsorption isotherm using the following linearized form of the equation:

$$P / P_{ads} = 1 / P_{max}K_p + (1 / P_{max})\, P$$

where P = concentration of unadsorbed Celluclast (mg Celluclast/mL); P_{ads} = concentration of adsorbed Celluclast (mg Celluclast/mg cellulose); P_{max} = the maximal adsorbed Celluclast (mg Celluclast/mg cellulose); K_p = equilibrium constant (mL/mg Celluclast). Regression analysis was performed to determine the slope and y-intercept, thus allowing calculations of P_{max} as slope^{-1}, and K_p as (P_{max} × y-intercept)$^{-1}$.

Measurement of Initial Hydrolysis Rates

Hydrolysis by the Celluclast/Novozym mixture was carried out using 2% (w/v) of each substrate in 50 m*M* sodium acetate (pH 4.8) at a cellulase loading of 0.2 mg/mL (10 IU/g substrate). For each substrate, the hydrolysis reaction was allowed to proceed for 5 min at 50°C, stopped by boiling for 5 min, and then centrifuged to remove the insoluble material. The

release of glucose and cellobiose was determined by HPLC using the Bio-Rad HPX-87H column. Hydrolysis was calculated as the amount of reducing sugar released per h, with reducing sugar determined as: mg glucose + 0.5 (mg cellobiose × 1.053).

Measurement of the Specific Hydrolysis Rate of Cellulases

Adsorption of cellulase and subsequent hydrolysis were carried out as described in the previous subsections. Before hydrolysis was carried out, an aliquot of the reaction mixture was removed and the protein in the supernatant measured. The specific hydrolysis rate was calculated as the hydrolysis rate after 5 min of reaction divided by the amount of protein adsorbed prior to the initiation of hydrolysis. The protein adsorbed on to cellulosic substrate was assumed to be from Celluclast as protein adsorption was not observed with Novozym alone.

RESULTS AND DISCUSSION

Cellulase Adsorption and Hydrolysis of Different Substrates

The adsorption isotherms of Celluclast on four substrates are shown in Fig. 1. The good fit of the experimental data to the model indicated that adsorption of the proteins present in Celluclast can be represented by the Langmuir isotherm. The equilibrium constant (K_p) and maximal adsorbed protein (P_{max}) for all nine substrates studied, along with the corresponding initial hydrolysis rates, were determined (Table 1). These initial hydrolysis rates were measured before substrate conversion reached 10%. When the different substrates were arranged in the order of decreasing P_{max}, the corresponding initial rates did not decrease accordingly. This indicated a poor correlation between these two parameters (Fig. 2A). It appeared that the initial hydrolysis rate does not depend solely on the total surface area available for enzyme binding. A plot of the initial hydrolysis rate with the actual amount of adsorbed Celluclast (Fig. 2B) also indicated no correlation between these two parameters. It was apparent that substrates with similar amount of adsorbed enzyme were not hydrolyzed at similar rates. This result suggested that the specific hydrolysis rates differ among cellulosic substrates.

Specific Hydrolysis Rate

As the specific hydrolysis rate is a measure of the ease with which a substrate can be hydrolyzed by bound cellulases, the possibility that the structural features of different substrates might affect this rate is suggested

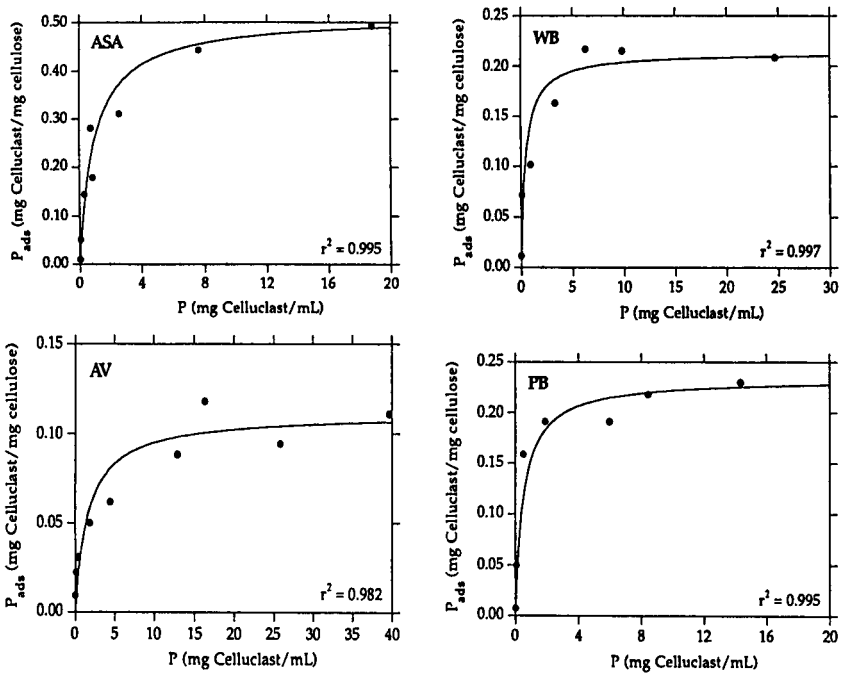

Fig. 1. Adsorption isotherms for Celluclast on selected cellulosic substrates (ASA, AV, WB, PB). The theoretical curve was generated using the adsorption parameters K_p *and* P_{max} determined from the experimental data. The coefficient of determination (r^2) shows a good fit of the data to the Langmuir adsorption model.

by the data shown in Table 1. The specific hydrolysis rates were found to be different for most substrates, with all the birch-derived substrates showing the lowest rates. Since the surface coverage of a substrate by a given quantity of Celluclast can be expressed as a percent of the substrate's maximal binding capacity for Celluclast (P_{max}), the dependency of specific hydrolysis rate on percent surface coverage was assessed (Fig. 3). It was apparent that the specific hydrolysis rate decreased as more of the substrate was covered by adsorbed enzymes, suggesting that the specific hydrolysis rate is a function of P_{ads}. A power law relationship for these two parameters has been suggested by other workers (*18*). When the data obtained in our study were fitted to the power law equation ($v = a \times P_{ads}^{b}$; where v = specific rate, a and b = constants), the exponent, b, for the WB, PB, and AV substrates were found to be between 0.56–0.6. This is in agreement with the previous finding that the reaction order is less than unity (0.66–0.68) (*18*). Thus, it would appear that the initial specific hydrolysis rate drops when more enzymes are adsorbed on the substrate. In addition, the specific hydrolysis rate of a particular substrate has been shown to decrease as hydrolysis progresses (*19,20*). This is most likely due to

Table 1
Adsorption Parameters for Celluclast on Various Cellulosic Substrates and the Corresponding Initial Hydrolysis Rates and Specific Hydrolysis Rates[a]

Substrate	Abbreviation	P_{max} mg Celluclast/ mg cellulose	K_p, ml/mg Celluclast	Initial hydrolysis rate,[b] mg/h/ mg cellulose	Specific hydrolysis rate,[b] mg glucose/h/ mg Celluclast
Acid swollen avicel	(ASA)	0.513 ± 0.015	1.0 ± 0.2	1.39 ± 0.15	101 ± 18
Air-dried ASA	(AD)	0.192 ± 0.002	14.1 ± 41.4	1.25 ± 0.04	91 ± 11
Oven-dried ASA	(OD)	0.113 ± 0.005	3.5 ± 6.8	1.02 ± 0.05	70 ± 8
Avicel	(AV)	0.111 ± 0.006	0.6 ± 0.3	0.56 ± 0.11	45 ± 14
Water-washed steam-exploded birch	(WB)	0.214 ± 0.005	2.1 ± 1.1	0.76 ± 0.02	38 ± 4
Alkaline-washed WB	(AB)	0.237 ± 0.008	2.8 ± 1.8	0.54 ± 0.03	36 ± 4
Peroxide-treated AB	(PB)	0.233 ± 0.007	1.9 ± 0.8	0.61 ± 0.01	47 ± 4
Solka-Floc	(SF)	0.048 ± 0.003	1.3 ± 0.4	0.68 ± 0.03	58 ± 8
Sulfite pulp	(SP)	0.119 ± 0.007	2.1 ± 2.1	0.87 ± 0.02	72 ± 10

[a]Hydrolysis rates were determined at a low coverage of the substrate surface by Celluclast.
[b]Means and standard deviations (n = 2–4) are provided.

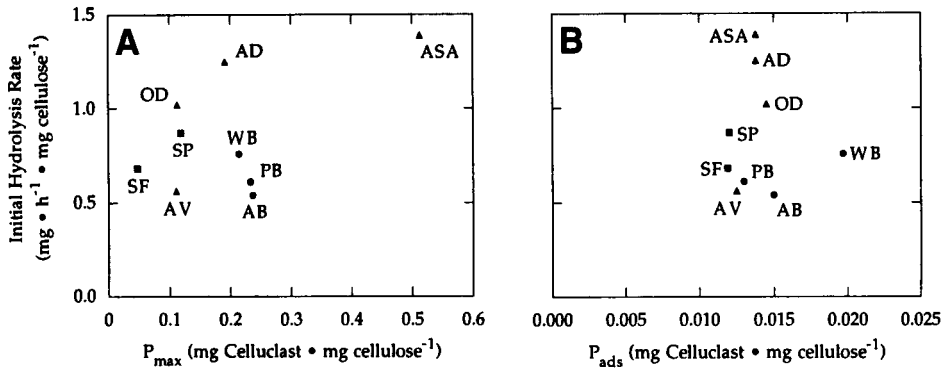

Fig. 2. Plots of the initial hydrolysis rates of different cellulosic substrates against (**A**) the maximal adsorption capacity for Celluclast (P_{max}) and (**B**) the Celluclast adsorbed (P_{ads}).

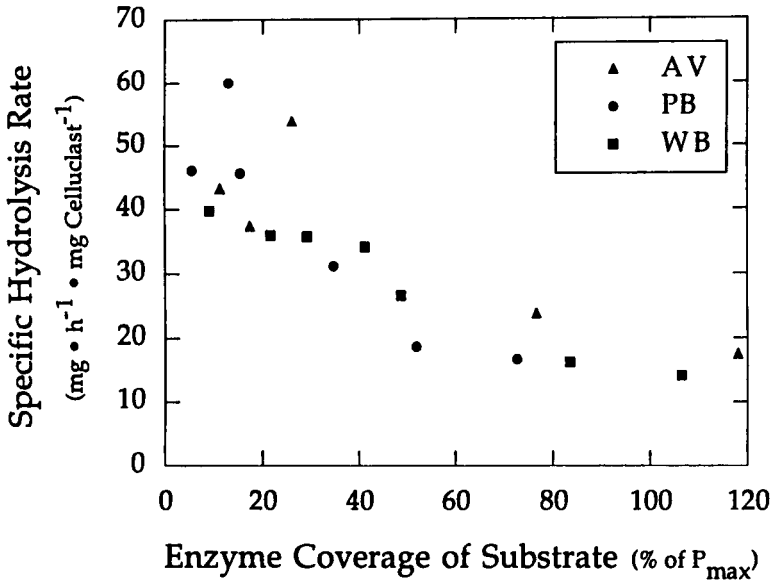

Fig. 3. Effect of surface coverage by Celluclast on the specific hydrolysis rates of selected cellulosic substrates (AV, PB, WB).

multiple factors, including substrate recalcitrance, enzyme inactivation, and product inhibition. In our study, it was found that there was a 10% decrease in the specific rate after about 20% substrate conversion (data not shown).

Effect of Pretreatments

Chemical and physical pretreatments of substrates can result in increased hydrolysis rates and yields. One of the major benefits of pretreatment has been attributed to increases in the surface area available to

cellulases (*9*). Stone et al. (*8*) found that the glucose yield was linearly correlated with the available surface area when a single substrate, cotton linters, was swollen to increasing extents using phosphoric acid. However, they did not consider the effect that this treatment might have on the specific hydrolysis rate. Our results show that both the amount of bound cellulases and specific hydrolysis rate were increased when Avicel was swollen with phosphoric acid to produce ASA (Table 1). It would seem that acid swelling increases the surface area of the substrate available for enzyme adsorption as well as disrupting the highly ordered state of the cellulose in Avicel. When the ASA substrate was subsequently dried, the swelling action of water is removed, and the accessibility of the substrate probably decreased as the pores collapsed. Thus, the OD and AD substrates bound less cellulases than the ASA substrate. The collapse of many of the pores might also serve to bring neighboring surfaces together resulting in their tighter alignment. This may be responsible for the lower specific hydrolysis rate of the OD substrate (Table 1).

Similarly, pretreatment of the birch substrates resulted in changes in the specific hydrolysis rate and P_{max}. When alkali was used to extract the WB substrate (containing 33% lignin) to produce the AB substrate (10% lignin), a slight increase in the cellulase binding capacity without any change in the specific hydrolysis rate was achieved. Peroxide treatment of the AB substrate further reduced the lignin content of the PB substrate to 5%. This treatment resulted in an increase in the specific hydrolysis rate and did not affect the cellulase binding capacity when compared to the AB substrate.

In conclusion, both substrate accessibility to cellulases and the specific hydrolysis rate have to be considered in any kinetic studies of cellulose hydrolysis. Both of these two parameters should be used in evaluating cellulose pretreatment methods in terms of their action, as has been suggested previously (*19*). From our study of nine different substrates, it is apparent that certain pretreatment methods might increase both the substrate accessibility and the specific hydrolysis rate (e.g., phosphoric acid treatment), whereas others might only change one of the two parameters (e.g., alkaline extraction, peroxide treatment). By considering only the hydrolysis yield, as in previous studies, the effect of the pretreatment step cannot be fully determined.

ACKNOWLEDGMENTS

We thank J. Tolan (Iogen, Ontario) and Q. Nguyen (Tembec, Québec) for providing pretreated birch and sulfite pulp, respectively. This work was partly financed by the Dept. of Energy, Mines and Resources and the Natural Sciences and Engineering Research Council.

REFERENCES

1. Fan, L. T., Lee, Y.-H., and Beardmore, D. H. (1980), *Biotechnol. Bioeng.* **22,** 177–199.
2. Puri, V. P. (1984), *Biotechnol. Bioeng.* **26,** 1219–1222.
3. Sasaki, T., Tanaka, T., Nanbu, N., Sato, Y., and Kainuma, K. (1979), *Biotechnol. Bioeng.* **21,** 1031–1042.
4. Gharpuray, M. M., Lee, Y.-H., and Fan, L. T. (1983), *Biotechnol. Bioeng.* **25,** 157–172.
5. Grethlein, H. E. (1985), *Bio/Technology* **3,** 155–160.
6. Grous, W. R., Converse, A. O., and Grethlein, H. E. (1986), *Enzyme Microb. Technol.* **8,** 274–280.
7. Sinitsyn, A. P., Gusakov, A. V., and Vlasenko, E. Y. (1991), *Appl. Biochem. Biotechnol.* **30,** 43–59.
8. Stone, J. E., Scallan, A. M., Donefer, E., and Ahlgren, E. (1969), *Adv. Chem. Ser.* **95,** 219–241.
9. Thompson, D. N., Chen, H. C., and Grethlein, H. E. (1992), *Bioresource Technol.* **39,** 155–163.
10. Wong, K. K. Y., Deverell, K. F., Mackie, K. L., Clark, T. A., and Donaldson, L. A. (1988), *Biotechnol. Bioeng.* **31,** 447–456.
11. Lee, Y.-H. and Fan, L. T. (1983), *Biotechnol. Bioeng.* **25,** 939–966.
12. Converse, A. O., Ooshima, H., and Burns, D. S. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 67–73.
13. Wald, S., Wilke, C. R., and Blanch, H. W. (1984), *Biotechnol. Bioeng.* **26,** 221–230.
14. Walseth, C. S. (1952), *TAPPI* **35(5),** 228,229.
15. Saddler, J. N., Mes-Hartree, M., Yu, E. K. C., and Brownell, H. H. (1983), *Biotechnol. Bioeng. Symp.* **13,** 225–238.
16. Gould, J. M. (1984), *Biotechnol. Bioeng.* **26,** 46–52.
17. Breuil, C., Chan, M., Gilbert, M., and Saddler, J. N. (1992), *Bioresource Technol.* **39,** 139–142.
18. Iogen Corporation. Kinetic Study of Enzymatic Hydrolysis of Exploded Wood. DSS Contract No. 51SZ.23283-7-6031. Efficiency and Alternative Energy Technology Branch, Energy, Mines, and Resources Canada, Ottawa, Ontario. 1990.
19. Wang, S. S. and Converse, A. O. (1991), *Appl. Biochem. Biotechnol.* **34/35,** 61–74.
20. Ooshima, H., Kurakake, M., Kato, J., and Harano, Y. (1991), *Appl. Biochem. Biotechnol.* **31,** 253–266.