Cotton Cellulose: Enzyme Adsorption and Enzymatic Hydrolysis

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Synopsis

Adsorption of a crude cellulase complex from *Trichoderma viride* on variously pretreated cotton celluloses has been studied in the framework of the Langmuir approach, in the temperature range 2–8°C. The saturation amount of adsorbed enzyme has been related to their susceptibility to hydrolysis. In every case the adsorption process was found to be faster by 2–3 orders of magnitude than the hydrolysis step to give end products. For one substrate, the Langmuir parameters were found to be fairly well correlated with the value of the Michaelis constant K_m, measured for its enzymatic hydrolysis, and the adsorptive complex $(ES)_{ad}$ was indistinguishable from the complex (ES) of the Michaelis–Menten model for the hydrolysis.

INTRODUCTION

The study of enzyme adsorption on cellulosic surfaces allows one to gain insight into the degradation mechanism of insoluble cellulose since this process is the first step of the enzymatic hydrolysis.[1,2] Unfortunately, the literature on this very important subject contains few data.[1–8]

Here the characteristics of the adsorption of a cellulase complex $C_1 + C_x$ on chemically pretreated cotton celluloses at low temperature are presented, and their role on the hydrolysis process is discussed. The characterization of the substrates as well as their enzymatic degradation kinetics in the range 30–60°C have been previously reported.[9]

A Langmuir-type approach is employed with an adsorption equilibrium represented by the equation

$$E + S \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} (ES)_{ad} \tag{1}$$

$(ES)_{ad}$ is the adsorptive complex between the enzyme E and the substrate S, and k'_1 and k'_{-1} are the rate constants of adsorption and desorption, respectively.

Cellulolytic enzyme adsorption of this type has been considered as a prerequisite step for the enzymatic hydrolysis of insoluble cellulose.[2,4,5,8] However, mechanistic models that do not involve explicitly the enzyme adsorption on substrate are also reported.[10–12] In these models the reaction between enzyme and substrate is assumed to be of the common Michaelis–Menten type:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES) \tag{2}$$

$$(ES) \xrightarrow{k_2} P + E \tag{3}$$

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Both assumptions of a fast preequilibrium [reaction (1) or (2)], followed by the slow reaction (3) to give end products are reported.[4,11] A relation between the observed Michaelis constant K_m and the adsorption equilibrium parameters might exist, since $1/K_m$ represents a near approximation of the equilibrium constant of the reaction (2). Here such a relation is suggested.

EXPERIMENTAL

Cotton fiber celluloses were washed with 1*N* HCl solution and with water. After sieving the 200–400 mesh fraction was collected.

Portions of the homogenized material underwent one of the following pretreatments:

Sodium hydroxide treatment: the cotton was treated with a 18% (w/w) NaOH solution, with a bath ratio (solid substrate/solution) of 1:50 (w/v), at 20 ± 1°C for 2 h.

Zinc chloride treatment: the cotton was treated with a 70% (w/w) $ZnCl_2$ solution [bath ratio 1:50 (w/v)] at 20 ± 1°C, for 2 h under slow stirring (50 rpm).

Other details of the above pretreatments were previously reported.[9]

Enzymes

Crude cellulase from *Trichoderma viride*, with a declared activity of 210 IU/g towards Whatman cellulose CC31, was used. It was kindly supplied by Novo Enzyme Co.

Enzyme activity: C_1, β-1,4-exocellobiohydrolase (EC 3.2.1.91) and C_x, β-1,4-endoglucanase (EC 3.2.1.4.) activities were measured towards Whatman powder CC31 and carboxymethylcellulose (D.S.O.7.; MW 80,000), respectively. The measured activity values were 114 IU/g (as reducing sugars) for C_1 and 57 IU/g for C_x. β-Glucosidase (EC 3.2.1.21.) activity, determined in citrate buffer (pH 4.8) towards cellobiose, was negligible.

Enzyme Analysis

The amount of free enzyme was determined with the Folin–Ciocalteau reagent[13] by using a Jasco–Uvidec 505 spectrophotometer and determining the absorbance at 600 nm. Cellulase was used for calibration.

Reducing Sugars Analysis

The reducing sugars produced in the enzymatic hydrolysis of the cellulose samples were determined with the ferricyanide method,[14] using glucose for calibration.

Adsorption Measurements

The runs were performed in a reactor, placed in a Lauda UKT 60 cryostat, in the temperature range 2–8°C, under mechanical stirring (400 rpm), by using 0.05*M* citrate buffer (pH 4.8). Most measurements were carried out at 5.0 ± 0.1°C.

The substrate concentration was in every case $C_S^\circ = 7.5$ mg/mL, while the enzyme concentration was varied in the range 0.10–3.00 mg/mL by adding successive aliquots (2 mL) of concentrated enzyme solution to the initial mixture (150 mL). The mixture volume was kept constant by a previous withdrawing of an equivalent amount (2 mL) of the suspension for analysis. These samples were quickly filtered, the solid weighed and the filtrate analyzed for free enzyme and reducing sugars content. In most cases 1 h was allowed to elapse before any further addition.

In some cases the time course of the adsorption was measured. This procedure was commonly followed when studying the enzyme adsorption on the $ZnCl_2$-treated cotton cellulose. A thorough mixing of the cotton cellulose suspension with the enzyme solution and the filtration required not less than 50–60 s. Thus, the first measurement of the free enzyme concentration was carried out after 1 min.

Desorption Measurements

A run was performed at 5.0 ± 1°C with the $ZnCl_2$-treated cotton cellulose as substrate ($C_S^\circ = 7.5$ mg/mL; $C_E^\circ = 3$ mg/mL). After the equilibrium was reached, 120 mL of buffer solution was added to the mixture (40 mL) and the desorption measured as free enzyme concentration increase during 6 h.

Stirring

The influence of stirring on the initial rate of hydrolysis was determined at 50.0 ± 0.1°C with $C_S^\circ = 7.5$ mg/mL and $C_E^\circ = 3$ mg/mL. Measurements were carried out under mechanical stirring in the range 50–600 rpm. No influence of external diffusion hydrolysis rate was observed for values higher than 350 rpm.

Hydrolysis

The occurrence of hydrolysis during the adsorption measurements was tested and its extent evaluated by analyzing total reducing sugars, as previously reported,[9] and by determining the mass decrease of the filtered insoluble cotton cellulose.

RESULTS AND DISCUSSION

The adsorption behavior of the enzymatic system ($C_1 + C_x$) on variously treated insoluble cotton celluloses has been determined in the framework of the Langmuir approach[15] by using

$$\frac{C_E}{x/m} = \frac{1}{b(x/m)_{max}} + \frac{C_E}{(x/m)_{max}} \tag{4}$$

Here C_E is the free enzyme concentration and (x/m) the amount of enzyme adsorbed per unit weight of cellulose at any equilibrium condition. From a set of C_E and (x/m) values, by applying eq. (4), the values of the two parameters $b = k_1'/k_{-1}'$, the adsorption equilibrium constant, and $(x/m)_{max}$, the saturation amount of adsorbed enzyme, were obtained.

The Langmuir approach seems to be particularly adequate since it can fit chemical as well as physical adsorption involving the formation of a monolayer. Evidence for the physical nature of adsorption of exo- and endoglucanase on bagasse has been recently reported.[1]

Most measurements have been carried out at 5.0 ± 0.1°C with enzyme concentration C_E° = 0.75–3.00 mg/mL and a substrate concentration C_S° = 7.5 mg/mL.

Three substrates have been examined:

—*untreated cotton cellulose*, which may be considered as a suitable model of crystalline native cellulose (cellulose I);

—*18% NaOH-treated cotton cellulose*, which presents a change in the crystalline unit cell (from cellulose I to cellulose II) and in morphological characteristics;

—*70% $ZnCl_2$-treated cotton cellulose*, which shows a remarkable structural lateral order variation without changes of the type of crystalline unity and a great modification of the initial morphology.

In every case stirring was properly adjusted (400 rpm) in order to prevent any influence of external diffusion.

A comparison of the adsorption trends for the three considered substrates is shown in Figure 1 in terms of C_E dependence on time. When the interaction enzyme-untreated cotton cellulose was examined, the adsorption equilibrium was reached within a time not measurable in our experimental conditions. When NaOH-treated and even more when $ZnCl_2$-treated cotton cellulose were considered, the adsorption process was slower. However, the required time to reach more than 70% of the final value resulted in every case shorter than 10 min.

When some hours were allowed to elapse in order to obtain a correct measure of the equilibrium value of the free enzyme concentration, the intervention of hydrolysis was taken into account in the evaluation of the uncertainties that affected experimental results. In the extreme case, i.e., when the enzyme ad-

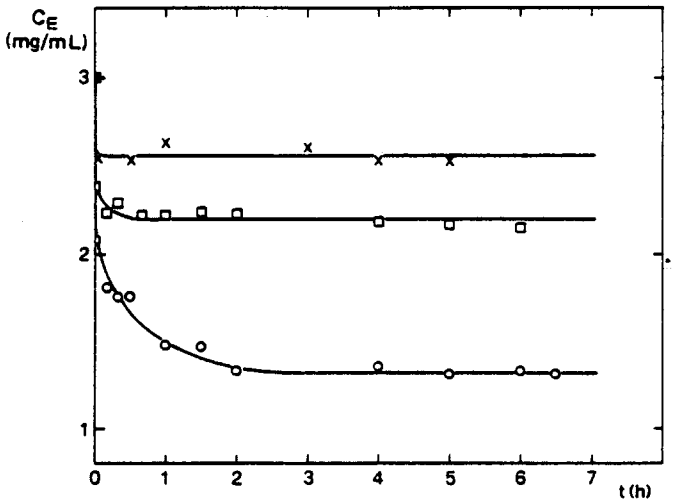

Fig. 1. Kinetics of interactions of the crude cellulase complex from *Trichoderma viride* (C_E° = 3.00 mg/mL) with variously treated cotton celluloses (C_S° = 7.5 mg/mL). (○) $ZnCl_2$-treated cotton cellulose; (□) NaOH-treated cotton cellulose; (X) untreated cotton cellulose. Conditions: pH 4.8, 5.0 ± 0.1°C.

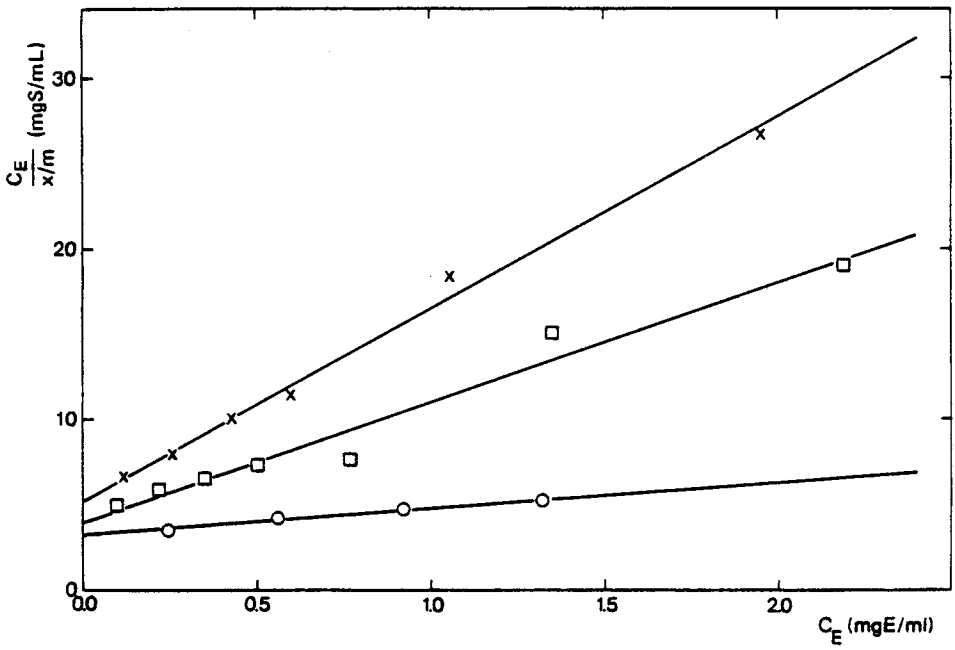

Fig. 2. Adsorption Langmuir isotherms for the crude cellulase complex from *Trichoderma viride* on variously treated cotton celluloses (C_S° = 7.5 mg/mL). (O) $ZnCl_2$-treated cotton cellulose; (□) NaOH-treated cotton cellulose; (X) untreated cotton cellulose. Conditions: pH 4.8, 5.0 ± 0.1°C.

sorption on the $ZnCl_2$-treated cotton cellulose was examined with C_E° = 3 mg/mL and t = 6.5 h, a maximum value of 12% hydrolysis was observed. This substrate was found to be[9] the most susceptible one to hydrolysis.

The values of $C_E/(x/m)$ plotted vs. C_E are shown in Figure 2 and the resulting Langmuir parameters are collected in Table I.

The adsorption measurements provide a significant parameter, the $(x/m)_{max}$ value, of the variously pretreated cotton celluloses. This parameter is a measure of the number of disposable sites on the cellulose surface: hence it is related to the susceptibility of the various substrates to enzymatic hydrolysis. The $(x/m)_{max}$ ratios 10:2.2:1.4 obtained from Table I for the sequence $ZnCl_2$-, NaOH-treated, and untreated cotton cellulose are in a rough agreement with the observed order of ease of hydrolysis reaction. So, for C_S° = 7.5 mg/mL, the ratios

TABLE I
Langmuir Parameters for Enzyme Adsorption on Cotton Celluloses[a]

Cotton celluloses	T (°C)	$(x/m)_{max}$ (mg E/mg S)	b [(mg E/mL)$^{-1}$]	$1/b(x/m)_{max}$ (mg S/mL)
$ZnCl_2$-treated	2.2	0.50 ± 0.10	0.67 ± 0.11	3.0 ± 0.4
	5.0	0.66 ± 0.07	0.47 ± 0.05	3.3 ± 0.1
NaOH-treated	5.0	0.14 ± 0.01	1.8 ± 0.2	4.0 ± 0.6
Untreated	2.3	0.078 ± 0.010	2.7 ± 0.4	4.8 ± 1.0
	5.0	0.089 ± 0.004	2.2 ± 0.2	5.2 ± 0.5
	8.2	0.079 ± 0.006	2.6 ± 0.3	4.9 ± 0.6

[a] $(x/m)_{max}$ = saturation amount of adsorbed enzyme; b = adsorption equilibrium constant; E = enzyme: crude cellulase complex from *Trichoderma viride*; S = substrate.

10:2:1 may be evaluated for the hydrolysis initial rates in the temperature range 30–60°C,[9] while, in terms of the more significant specificity constant k_2/K_m, values somewhat lower (10:1.2:0.5) are obtained. The relative $(x/m)_{max}$ ratios can be reasonably assumed little dependent on temperature.

With regard to the dependence of adsorption on temperature, some measurements have been carried out in the narrow range 2–8°C to prevent a large extent of hydrolysis. For the untreated cotton cellulose the dependence of Langmuir parameters on temperature was not observed (Table I). The values of $\ln [1/b(x/m)_{max}]$ at 2.2 and 5.0°C, obtained in the case of $ZnCl_2$-treated cotton cellulose, are reported in Figure 3. However, these measurements have been obtained by exploring a very narrow range of temperature and experimental errors greatly affect the significance of the difference between the parameter values.

The measurable time required to obtain a complete adsorption might make questionable the occurrence of a fast adsorption preequilibrium. From a kinetic point of view, the protein adsorption on polymer surfaces is far from a simple process.[16] In this case, as a consequence of the various pretreatments of the cellulosic material, internal diffusion may play a decisive role: so, the observed slow approach to equilibrium in the final stage is probably due to the decreasing accessibility of internal sites, during the adsorption, after an initial fast adsorption on the more external sites. In fact, in going from $ZnCl_2$- to NaOH-treated and to untreated cotton cellulose, that is, from an almost amorphous cellulose to more crystalline ones, the time required for a complete adsorption of enzyme markedly decreases. The opposite sequence observed in the hydrolysis rate[9] suggests that the role of internal diffusion becomes negligible at the temperature at which hydrolysis does work extensively.

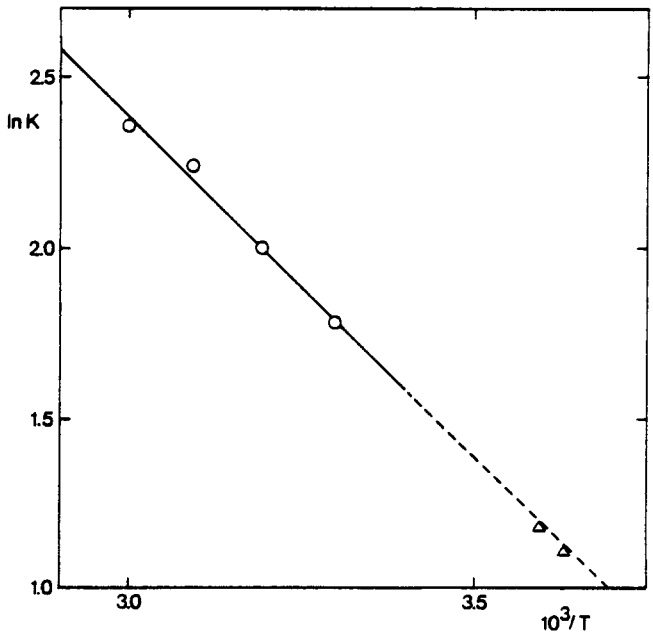

Fig. 3. Temperature dependence of K_m value [(○) $K = K_m$] for the enzymatic hydrolysis of $ZnCl_2$-treated cotton cellulose and values of $1/b(x/m)_{max}$ [(△) $K = 1/b(x/m)_{max}$] from measurements of enzyme adsorption on the same substrate. Enzyme: crude cellulase complex from *Trichoderma viride*.

The kinetic considerations that are invoked to justify the slow free-enzyme disappearance in the final stage do not affect the reliability of the equilibrium results.

A desorption batch measurement has revealed the presence of almost irreversible steps in the process, just confirming its complexity. The irreversibility, which arises after a first reversible step, may be due to a subsequent slow conformation change of the protein[16] or/and to an evolution of the substrate surface.[8]

The occurrence of a fast adsorption preequilibrium in the cellulose hydrolysis can be supported by the comparison of k_2 with k'_{-1} values. If $k_2 \ll k'_{-1}$, the decomposition of the enzyme–substrate complex to give end products is much slower than the attainment of adsorption equilibrium. In principle, this does not imply necessarily that the decomposition is the rate determining step in hydrolysis since the adsorption process might lead to an adsorptive complex $(ES)_{ad}$ different from the complex (ES) involved in the final step to give end products. The formation of (ES) may be either slow or fast after the formatioin of $(ES)_{ad}$. Only if (ES) is rapidly formed does the result $k_2 \ll k'_{-1}$ mean that the decomposition of the enzyme–substrate complex is the rate determining step.

The k'_{-1} value can be obtained from the equation $k'_{-1} = k'_1/b$, where b is given from Table I, and k'_1 can be evaluated from the adsorption initial rate according to

$$-[dC_E/dt]_{t=0} = k'_1 C^\circ_E C^\circ_S \tag{5}$$

By plotting the initial rate from Figure 4 vs. C°_E and dividing the resulting slope by $C^\circ_S = 7.5$ mg/mL and by b, one obtains, for the $ZnCl_2$-treated cotton cellulose at 5.0°C, $k'_{-1} = 7.7$ (mg E/mg S)·h^{-1}. This desorption constant can be expressed

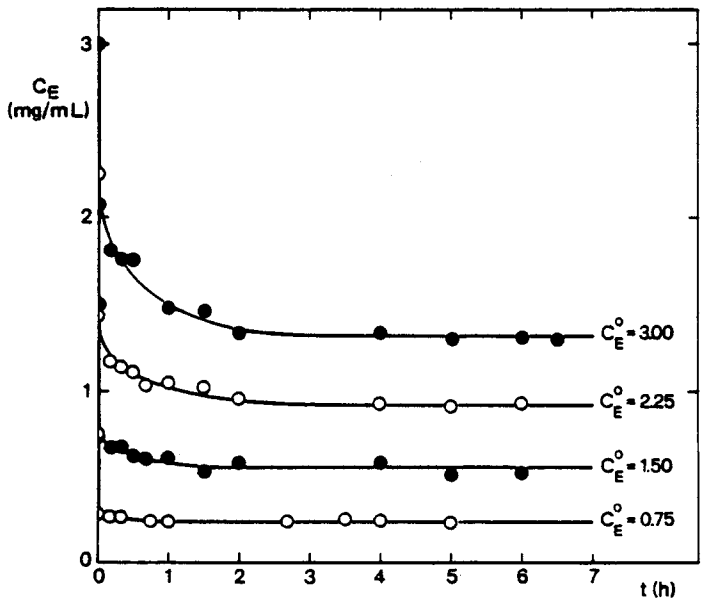

Fig. 4. Kinetics of interaction of the crude cellulase complex from *Trichoderma viride* with the $ZnCl_2$-treated cotton cellulose at various initial enzyme concentrations. Conditions: pH 4.8, 5.0 ± 0.1°C.

in the proper first order dimension (h^{-1}) in the following way. Let M_E be the average equivalent weight of the enzyme and M^* the average amount of the substrate corresponding to one adsorption site. Then

$$x_{max}/M_E = m/M^* \tag{6a}$$

that is

$$(M_E/M^*) = (x/m)_{max} \tag{6b}$$

So, dividing k'_{-1} expressed as (mg E/mg S)·h^{-1} by (M_E/M^*), that is, by $(x/m)_{max}$, one obtains $k'_{-1} = 12\ h^{-1}$. An analogous procedure has been followed for the other two substrates. All the resulting k'_{-1} values are shown in Table II.

The k_2 value can be obtained by dividing the extrapolated V_{max} value from hydrolysis measurements[9] by enzyme concentration according to

$$V_{max} = k_2 C_E^\circ \tag{7}$$

The extrapolated V_{max} values at 5.0°C (C_E° = 3 mg/mL) are 0.751, 1.05, 0.461 (mg S/mL)·h^{-1} for $ZnCl_2$-, NaOH-treated, and untreated cotton cellulose, respectively. The V_{max}/C_E° values obtained as (mg S/mg E)·h^{-1} have now to be multiplied by (M_E/M^*), that is, $(x/m)_{max}$, according to eq. (6b), to give the rate constants k_2 expressed in h^{-1}. In this case some observations need to be made. Equation (6b) holds for the adsorptive complex $(ES)_{ad}$, and does not for the complex (ES) involved in the final step to give end products. Since adsorption may occur in a specific as well as in a nonspecific manner, while the interaction in the productive complex (ES) is only specific, it can be reasonably argued that the mass ratio enzyme/substrate is lower for (ES) than for $(ES)_{ad}$. So, the above procedure gives the maximum value that the rate constant k_2 may assume. The k_2 coefficients, evaluated in the aforementioned manner, as well as the ratios k'_{-1}/k_2 are shown in Table II.

Taking into account that the k'_{-1} values are underestimated, since it was assumed $V^\circ \simeq V_{1\ min}$, while the k_2 values are overestimated, it may be concluded that for all substrates the decomposition reaction to give end products is slower by 2–3 orders of magnitude than the adsorption process.

The V_{max} and K_m values previously obtained[9] for the hydrolysis carried out with the same enzymatic system here employed on $ZnCl_2$-treated cotton cellulose are reported in Table III in the range 30–50°C. The values at 5°C have been obtained by extrapolating ln V_{max} and ln K_m vs. $1/T$. From these data, when C_E° = 3 mg/mL, a hydrolysis initial rate at 5°C (V° = 0.52 mg/mL·h) can be evaluated in a good agreement with the observed value (V° = 0.53 mg/mL·h).

The K_m dependence on temperature is reported in Figure 3. On the same graph the values of ln $[1/b(x/m)_{max}]$ obtained here for $ZnCl_2$-treated cotton

TABLE II
Comparison between Values of Enzyme Desorption Constant (k'_{-1}) and of Rate Constant of Enzymatic Reaction (k_2) for Cotton Celluloses at 5.0°C

Cotton celluloses	k'_{-1} (h^{-1})	k_2 (h^{-1})	k'_{-1}/k_2
$ZnCl_2$-treated	12	0.16	75
NaOH-treated	7	0.050	140
Untreated	6	0.014	430

TABLE III
Kinetic Parameters for Enzymatic Hydrolysis of $ZnCl_2$-treated Cotton Cellulose[a]

T (°C)	60	50	40	30	5
K_m[b] (mg/mL)	10.53	9.38	7.37	5.93	3.32[c]
V_{max}[b] (mg/mL h)	18.95	12.62	7.79	3.60	0.75[c]

[a] Enzyme E: crude cellulase complex from *Trichoderma viride:* $C_E^* = 3$ mg/mL.
[b] For K_m: $\Delta H = 3.9$ kcal/mol ($r = 0.993$) (correlation factor); for V_{max}: $\Delta E = 11.0$ kcal/mol ($r = 0.992$).
[c] Extrapolated value.

cellulose at 5.0 and 2.2°C are also reported. As it may be seen, there is a very good agreement of the extrapolated K_m value (3.32 mg/mL) with the value of $1/b(x/m)_{max}$ (3.25 mg/mL) at 5.0°C.

It is noteworthy that, by multiplying the adsorption equilibrium constant b by (M_E/M^*), that is, by $(x/m)_{max}$, one obtains the equilibrium constant of the reaction (1) in terms of substrate concentration. As a consequence, the observed equivalence

$$b(x/m)_{max} = 1/K_m \tag{8}$$

suggests that the adsorptive complex $(ES)_{ad}$ is indistinguishable from the enzymatic complex (ES) of the Michaelis–Menten model and, at the same time, that $K_m \simeq k_{-1}/k_1$.

The equivalence (8) implies that the adsorption interaction is specific, i.e., the interaction occurs between the substrate and the active center of the enzyme.[8]

So the assumed occurrence of a fast preequilibrium,[11] as well as the proposed adsorption nature of this preequilibrium[4] seems to be confirmed in the case of the $ZnCl_2$-treated cotton cellulose.

However, also for the untreated one the more reliable ln K_m values at 30 and 40°C[9] and the ln $[1/b(x/m)_{max}]$ value at 5°C are fairly aligned when plotted vs. $1/T$. For the NaOH-treated cellulose no secure indication can be drawn owing to the uncertainty of K_m values, but the order of magnitude of these values and their scarce dependence on temperature seem to rule out in this case the equivalence (8). It must be noted that the NaOH treatment, unlike the $ZnCl_2$ treatment, induces a change in the crystalline unit cell of cellulose.

CONCLUSIONS

The Langmuir parameter $(x/m)_{max}$ that measures the number of disposable sites of a substrate has been correlated with the susceptibility to hydrolysis of the variously treated cotton celluloses.

In every case the adsorption process has resulted faster by 2–3 orders of magnitude than the final step to give end products as suggested by a comparison of the kinetic coefficients k_2 and k'_{-1}.

When the mechanism of the enzymatic hydrolysis of an insoluble substrate involves a fast specific adsorption preequilibrium followed by a slow reaction to give end products, the following equivalence holds between the parameters obtained from hydrolysis and from adsorption measurements:

$$K_m = 1/b(x/m)_{max}$$

This equivalence has resulted for the hydrolysis of a $ZnCl_2$-treated cotton cellulose carried out with a $C_1 + C_x$ enzyme system, just confirming for this substrate the above mechanism.

For the other two substrates, the NaOH-treated and the untreated cotton cellulose, this mechanism is questionable and needs further support.

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References

1. T. K. Ghose and V. S. Bisaria, *Biotechnol. Bioeng.*, **21**, 131 (1979).
2. N. Peitersen, J. Medeiros, and M. Mandels, *Biotechnol. Bioeng.*, **19**, 1091 (1977).
3. M. Mandels, J. Kostick, and R. Parizeck, *J. Polym. Sci. C*, **36**, 445 (1971).
4. A. A. Huang, *Biotechnol. Bioeng.*, **17**, 1421 (1975).
5. A. E. Humphrey, A. Moreira, M. Ladish, W. Armiger, and D. Zabriskie, Paper No. 7 US/USSR Joint Conference on Single Cell Protein, 1975.
6. A. Binder and T. K. Ghose, *Biotechnol. Bioeng.*, **20**, 1187 (1978).
7. G. Halliwell and M. Griffin, *Biochem. J.*, **169**, 713 (1978).
8. A. A. Klyosov and L. Rabinowitch, in *Enzyme Engineering, Future Directions*, L. B. Wingard Jr., I. V. Berezin, and A. A. Klyosov, Eds., Plenum, New York, 1980.
9. B. Focher, A. Marzetti, M. Cattaneo, P. L. Beltrame, and P. Carniti, *J. Appl. Polym. Sci.*, **26**, 1989 (1981).
10. J. A. Howell and J. D. Stuck, *Biotechnol. Bioeng.*, **17**, 873 (1975).
11. M. Okazaki and M. Moo-Young, *Biotechnol. Bioeng.*, **20**, 637 (1978).
12. N. Peitersen and E. W. Ross, *Biotechnol. Bioeng.*, **21**, 997 (1979).
13. O. H. Lowry, N. Y. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14. H. C. Hagedorn, *Biochem. Z.*, **135**, 46 (1932).
15. I. Langmuir, *J. Amer. Chem. Soc.*, **38**, 2267 (1916).
16. M. E. Soderquist and A. G. Walton, *J. Colloid Interface Sci.*, **75**, 386 (1980).

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