

ENZYMATIC HYDROLYSIS OF CELLULOSE (I): RELATIONSHIP BETWEEN KINETICS AND PHYSICO-CHEMICAL PARAMETERS

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Several purified celluloses (Avicel PH 101; Sigmacell type 100; Whatman CF-11; Cotton; Amorphous cotton) were physically characterised. The crystallinity, particle size and degree of polymerisation were analysed during successive enzymatic digestions with a commercial cellulase of *Trichoderma reesei* under conditions of high enzyme to substrate ratio.

The variation in the degree of polymerisation after 30 min of enzymatic digestion, as measured by the variation of the reducing power of the insoluble fibres, correlated with the cellulose solubilised after 12 h. The results obtained suggest that the overall kinetics of digestion are governed by the amorphous area of the fibres which is accessible to endoglucanases.

The effect of the substrate and enzyme concentrations on the initial kinetics of hydrolysis was also studied. The relative susceptibility of the different substrates to enzymes depended on the enzyme to substrate ratio, owing to the different adsorption properties of the tested celluloses. Analysis of the effect of the physical properties of the substrate cannot therefore be done neglecting the enzyme to substrate ratio.

Keywords: Cellulose; Cellulase; Crystallinity; Degree of polymerisation; Kinetics; Adsorption

INTRODUCTION

Research on the application of cellulases for bioconversions is well established (Hinman *et al.*, 1992). More recently, other applications have

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considered not only the hydrolytic capabilities of these enzymes but also their strong affinity for cellulose through the core binding domain (CBD), which alone is claimed to alter the properties of fibres. For instance, deinking and fibre modification in the paper industry, and biopolishing in textile industry, seem to depend on the adsorption process to some extent. The development of these applications still demands fundamental research, namely on the relationship between the properties of the substrates, the adsorption process and the reaction rate.

In nature, cellulose is mixed with other compounds, mainly lignin and hemicelluloses. The enzymatic hydrolysis of cellulose with cellulases becomes more difficult in the presence of these polymers, because the accessibility of the enzymes is reduced and because of irreversible enzyme adsorption on lignin (Ooshima *et al.*, 1990). However, even working with purified celluloses, it is not always understood why a particular cellulose is more easily hydrolysed than another (Tanaka *et al.*, 1988; Puri, 1984; Ramos *et al.*, 1993; Lee *et al.*, 1983; Gharpuray *et al.*, 1983).

Kinetic work and the study of physico-chemical parameters affecting the kinetics using crude cellulases has been extensively reported in the literature (Wald *et al.*, 1984; Gusakov *et al.*, 1985; Lee and Fan, 1982), and several tentative kinetic models have been produced. These models should address three factors:

- the nature of the enzymes, namely, the synergism between endoglucanolytic and exoglucanolytic enzymes (Henrissat *et al.*, 1985).
- the interaction between substrate and enzyme, i.e., the adsorption process (Hoshino *et al.*, 1992).
- the physical structure of the substrate, namely crystallinity, surface area, degree of polymerisation (Gama *et al.*, 1994).

The relative importance of the several physical parameters on the reaction rate remains arguable. Different authors, analysing different substrates, ranked differently the crystallinity, surface area, porosity, etc. The influence of these properties on hydrolysis cannot be dissociated from the degree of synergism, strength of adsorption and concentration at non-effective sites which are often not considered when analysing the effect of the physical properties and might explain some apparent contradictions in the literature over the past 30 years.

In this work, we aim to study the interdependence of these factors, and introduce a somewhat different perspective in the discussion of this subject.

METHODS

Materials

The cellulase from *Trichoderma reesei* Multifect L-250 was a kind gift from Finnish Sugar Co., Ltd, Finland.

Five kinds of cellulose were used: Sigmacell type 100; Avicel PH-101; fibrous Whatman CF-11; commercial Cotton, free of loose fibres and amorphous cotton (AmCotton), which was prepared from the latter by solubilisation in 85% H₃PO₄, water reprecipitation and solvent drying according to Lee *et al.* (1982). These substrates were selected because they are structurally different regarding each relevant property for the enzymatic hydrolysis, and because of the low level of contaminating compounds like hemicellulose and lignin.

Degree of Polymerisation (DP)

This was calculated from the initial cellulose weight, the total solubilised sugars and the Insoluble Fibres Reducing Power (Gama *et al.*, 1991; 1993), according to the following equation:

$$DP = (TC \cdot 1.1) / IFRP,$$

where:

- TC – total cellulose dry weight, determined by weighing after overnight drying at 100°C.
- 1.1 – correction factor for expressing cellulose as glucose equivalents.
- IFRP – insoluble fibres reducing power, in glucose equivalents.

The IFRP value was determined by applying the Nelson–Somogy method to cellulose suspensions, after washing the fibres three times with cold water (4°C) in a refrigerated centrifuge (5,000 rpm, 5 min). In the case of the enzymatically digested celluloses, the total residual cellulose was calculated as the difference between initial cellulose and solubilised glucose and cellobiose equivalents, as measured by ion chromatography.

Enzyme Hydrolysis

Studies on the Degree of Polymerisation and Successive Digestions

The reaction mixture, containing 20 mg of cellulose, 1.5 mL of 50 mM acetate buffer, pH 5.0, and 0.5 mL of diluted enzyme (1 : 100), was placed

for 0.5 h (for Insoluble Fibres Reducing Power – IFRP – variation measurements only) and periods ranging between 10 and 14 h in a 50 mL flask and incubated at 50°C in a reciprocating shaker (100 rpm). The samples were then transferred with an additional 1 mL of water to a centrifuge tube. The reaction medium was then centrifuged (5,000 rpm, 3 min), and the supernatant analysed for soluble sugars as described below. The insoluble fibres reducing power (IFRP) was then determined as described previously (Gama *et al.*, 1991; 1993).

In order to achieve high conversion yields, successive digestions were performed. In between digestions, the insoluble centrifuged residue was washed twice with a Tween 85 solution (0.1%) to remove the adsorbed enzyme, the supernatants were discarded, and the reaction was restarted under the same conditions only with the residual cellulose. Two independent experiments were carried out, with samples running in duplicate. For each digestion stage, samples were withdrawn from parallel experiments for crystallinity and particle size distribution analysis.

Effect of the Substrate and Enzyme Concentration

This was analysed running similar experiments with substrate and enzyme concentrations ranging, respectively, between 50 and 1500 mg, and between 0.18 and 100 mg, in a total volume of 20 mL. To determine the reducing sugars concentration, 1 mL samples were analysed by the dinitrosalicylic acid (DNS) method after 1, 3 and 24 h reaction. Before analysis, the samples were centrifuged and the supernatants were left for 1 h at room temperature to allow for the conversion of cellobiose to glucose.

X-Ray Diffraction

X-ray diffraction was recorded using a Philips PW1710 diffractometer, operated at 40 kV tube voltage and a 20 mA tube current. The samples were scanned for a range of 2θ from 10° to 30°. The crystallinity index (CrI) was calculated by applying the empirical equation of Segal *et al.* (1959).

Particle Size Distribution

The size distribution of Avicel and Sigmacell particles during digestion was obtained in previous work, utilising three different particle sizers: Galai Cis 100, Coulter Counter and Malvern (Gama *et al.*, 1997).

Soluble Sugars Analysis

Soluble sugars were determined with a Dionex DX 300 equipped with a CarboPac PA-1 column, using 100 mM NaOH as eluent, with a sodium

acetate gradient (0 to 66 mM in 20 min). The eluent flowrate was 1 mL/min. A pulsed amperometric detector with a gold electrode was used, operating according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The Effect of Structural Properties

Many laboratories apply microcrystalline celluloses in the study of cellulose properties versus enzymatic reactivity. However, the initial kinetics of digestion may not be representative of the digestibility of a cellulose, due to the heterogeneity of the cellulose particles.

In this work, successive enzymatic digestions of several cellulosic samples were carried out with high enzyme concentrations, and the structural modifications of the fibres and sequential conversion rates were analysed (Table I). Together with the structural properties of the substrates, product inhibition may also influence the enzymatic solubilisation of cellulose. In order to reduce the possible effects of product inhibition, the addition of cellobiase is recommended. The presence of contaminant compounds like hemicelluloses, which may occupy a large area on the surface of the fibres, can also affect the kinetic analysis. However, as no xylose was detected in the chromatographic analysis of the solubilised sugars, it was concluded that, if any hemicellulose was present, its concentration was small.

The following observations can be noted from the results in the Table I.

Crystallinity

All celluloses except Amcotton display the X-ray diffraction pattern characteristic of the cellulose I polymorph (Isogai *et al.*, 1989). The crystallinity, as measured by the Segal empirical equation (Segal *et al.*, 1959), remains nearly constant during digestion, rising only in the case of Sigmacell at high degrees of conversion. This result, which agrees with several others in the literature (Lee *et al.*, 1983; Fan *et al.*, 1980), suggests that the cellulose fibres of Sigmacell, Avicel and Whatman (the case of cotton is discussed below) are solubilised layer by layer, i.e., the amorphous fraction is not hydrolysed first. The crystallite thickness at the half maximum height for the 002 diffraction plane ($2\theta = 22^\circ$), expressed as a fraction of the value for the non-digested cellulose, had the following values in the successive digestions:

Sigmacell – 1.0/0.9/0.7;

Whatman – 1.0/0.9/0.9;

Avicel – 1.0/0.9/1.0/1.0.

TABLE I Polymerisation degree, crystallinity, solubilisation and increase of particle size (d_{50}) of particular and fibrous celluloses as a function of digestion time with a cellulase from *Trichoderma reesei*

Digestion time (h)	0	0.50	12	24	36	48	60	72	84	96
Polymerisation degree	Sigmacell	236	173	132	58	51				
	Avicel	150	133	118	112	135	154			
	Whatman	182	196	203	188	202	172	186	167	163
	Cotton	2138	1229	1151	891	630				
	Amcotton	2193	204							
Crystallinity (%)	Sigmacell	79.0		78.8	80.5	86.6				
	Avicel	91.0		89.8	90.7	91.6	90.3	90.6		
	Whatman	95.3		95.6	95.6	96.1	95.9	95.3		
	Cotton	93.0		92.9						
	Amcotton	47.3								
Solubilisation (%)	Sigmacell			44	80	89				
	Avicel			27	38	42	51			
	Whatman			9	12	15	22	24	27	29
	Cotton			7	15	26				
	Amcotton			83						
Particle size increase (% d_{50})	Sigmacell			10		44				
	Avicel			16		32				

These values reveal a decrease of the Sigmacell average crystallite thickness in the 002 diffraction plane, which was not detected for the other celluloses, possibly due to their lower conversion.

The digestibility of the various celluloses increased roughly with the inverse order of their crystallinity. However, not all the examples follow this general trend (Avicel vs. Cotton, for instance) which means that other structural parameters must also be taken into account to explain the different susceptibility to cellulases.

As the Amcotton was solubilised to a very high degree (83%), it may be assumed that in the case of the other celluloses the conversion was restricted mainly because of structural limitations, and not because of product inhibition.

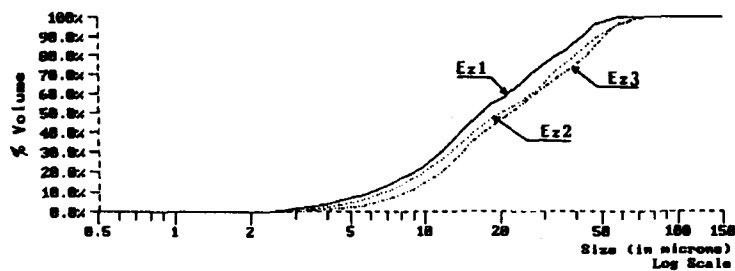
Particle Size

Typically, the cellulose hydrolysis rate slows down as the reaction proceeds, which can in part be explained as the result of product inhibition. However, the structure of the substrate may also give rise to this type of kinetics. In fact, the existence of two different fractions of cellulose (crystalline/amorphous regions; more accessible/less accessible microfibrils) is a basic assumption of several of the kinetic models presented in the literature (Lee *et al.*, 1983; Wald *et al.*, 1984; Nidetzky and Steiner, 1993), to explain the two stage kinetics generally found.

As the crystallinity of the celluloses remains constant during enzymatic digestion, mean particle size variation, and hence accessibility, may thus be associated with the biphasic kinetics (Fig. 3(A)). In the case of the particulate celluloses, Avicel and Sigmacell, an increase in d_{50} was observed during conversion to soluble sugars. Table I and Fig. 1 show the increase in particle size with digestion time, expressed as a percentage of the initial d_{50} in the case of the results presented in Table I. The smaller particles are therefore hydrolysed faster than the others (Schell *et al.*, 1989).

The difference in the susceptibility to hydrolysis in the first 12 h between Avicel and Cotton may be explained by their different accessibility. As shown elsewhere (Gama *et al.*, 1994), Avicel has a larger surface area than Cotton (0.080 and 0.040 m²/gr, respectively). However, for Avicel, after the initial digestion steps, there is a reduction in the reaction rate, due to the increased particle size and the reduced susceptibility to endoglucanolytic depolymerisation. In turn, defibrillation of Cotton (Din *et al.*, 1991), which is evident by the end of the second digestion step, allows for an increased accessibility, and consequently for a higher reaction yield.

A



B

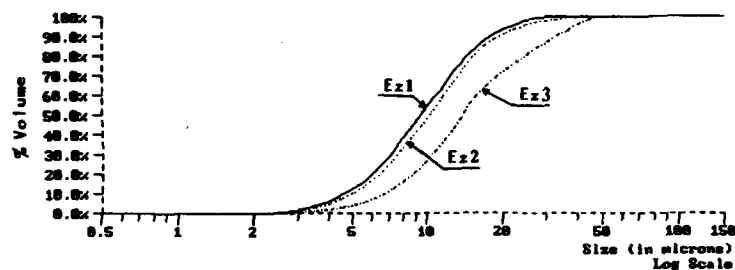


FIGURE 1 Cumulative size distribution of Avicel (A) and Sigmacell (B) particles at several stages of enzymatic treatment. Ez1, Ez2 and Ez3 correspond, respectively, to enzymatic treatment periods of 0.5, 12 and 36 h.

Degree of Polymerisation

Size exclusion chromatography seems to be the best available method to measure the mean DP and the polydispersity of cellulose molecules. Its sensitivity in detecting endoglucanolytic activity at the level of the fibre surface is, however, questionable. The cellobiosyl repeating unit in the cellulose crystallite measures 0.53×1.04 nm, and considering the case of Sigmacell (surface area = $3 \text{ m}^2/\text{gr}$, by N_2 adsorption measurement) (Gama *et al.*, 1994), one can estimate a concentration of 1.089×10^{19} glycosyl residues on this cellulose surface, per gram of anhydrous cellulose. Since there are 3.718×10^{21} glycosyl residues in one gram of anhydrous cellulose, only

0.3% of the glycosyl residues are exposed at the cellulose surface. As shown in previous work (Gama *et al.*, 1994), the enzymes are not able to penetrate the pores of these substrates. Fragmentation of the fibres by the enzymes has been shown, elsewhere, not to occur in the case of Sigmacell, while in the case of Avicel a considerable reduction in particle size is detected, but still this substrate remains less accessible than Sigmacell (Gama *et al.*, 1997). Hence, it is reasonable to assume that only a very small fraction of cellulose molecules are likely to undergo a levelling-off DP, at any time, if the cellulose is solubilised layer by layer. This seems to be the case of Sigmacell, Avicel and Whatman celluloses, in this work, and of the bleached eucalyptus Kraft pulp treated with the *Celluclast* preparation by Ramos *et al.* (1993). On the other hand, Kleman-Leyer *et al.* (1992; 1995) measured large DP variations in cotton cellulose degraded both by recombinant endoglucanases from *Cellulomonas fimi* and by the brown rot fungus *P. placenta*, by comparing the elution profile of the carbanilate derivatives of cellulose, using size exclusion chromatography. These variations were similar to the DP reduction of Cotton obtained in this work, and suggest that the enzymes are able to deeply cleave the cotton molecules in the less-ordered amorphous sites. Otherwise, the variation in DP would not be detected by size exclusion chromatography. Which of the two types of enzymatic degradation (e.g. layer by layer or deep attack on the amorphous zones of the fibres) is found, in any particular study, depends not only on the cellulose morphology, but also on the cellulase system utilised, as shown by Puls and Wood (1991).

The variation in DP at the beginning of digestion is a measure of the endoglucanolytic action, as discussed before (Gama *et al.*, 1993). In the case of Avicel, after two digestion steps with an overall degree of conversion of 38%, and a reduction of the average DP from 150 to 110, the extent of conversion drops in the subsequent digestions and the DP rises again to the former value of 150, which means that the endoglucanase activity has decreased. The increase in DP cannot be caused by transglycosylation, because this is expected to occur at higher concentrations of soluble sugars. In the case of Avicel, the first digestion step has the highest degree of conversion and is accompanied by a drop in the value of DP. Therefore, it is improbable that transglycosylation reactions would take place in the subsequent digestion steps, where the soluble sugars concentration was lower.

The endoglucanolytic action seems to be proportional to the overall cellulolytic action, as first shown by Reese *et al.* (1957). Figure 2 shows the correlation between the initial variation in the IFRP and the degree of

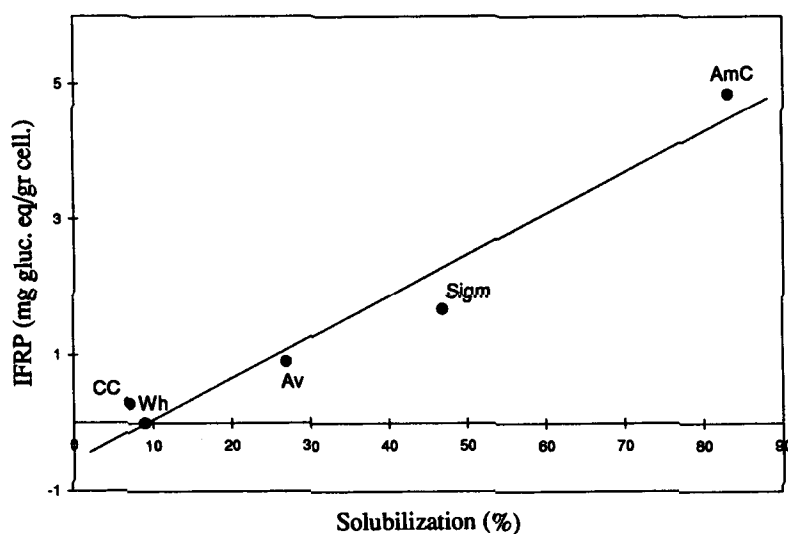


FIGURE 2 Correlation between the Insoluble Fibres Reducing Power (IFRP) and the sugars solubilised after, respectively, 0.5 and 12 h of enzymatic digestion of five different kinds of cellulose (Gluc. eq. = glucose equivalent; CC = commercial cotton; Av = avicel; Sigm = sigmacell; AmC = amorphous cotton).

conversion of the different celluloses after 12 h digestion. It seems that, when there is no variation in the initial IFRP (e.g. Whatman), the release of soluble sugars is also negligible. With increasing variation in IFRP (Avicel, Sigmacell, AmCotton), which corresponds to higher depolymerisation by endoglucanase, the overall activity increases probably due to more efficient synergism. The variation in IFRP is likely to be dependent on the accessible amorphous surface area, which is a function of the accessibility and crystallinity. It is however difficult to correlate directly the conversion with these parameters, due to desegregation phenomena (Walker *et al.*, 1992), which makes the true accessible area difficult to measure.

The Effect of the Substrate and Enzyme Concentrations

According to the literature (Lee and Fan, 1982), a linear correlation between the hydrolysis rate and enzyme or substrate concentration (first order reaction), followed by a plateau corresponding either to substrate or enzyme saturation (zero order reaction) are expected. The order of the reaction with respect to the enzyme and substrate concentrations was studied in this work to examine how these profiles compare for different celluloses. These experiments were carried out with Avicel, Sigmacell and

Whatman. The cottons were not studied because of constraints in medium agitation at high concentrations.

After measuring the soluble reducing sugars, the reaction rate was calculated for each enzyme and substrate concentration. Figure 3 shows some of the data obtained. Graph A shows the non-linear reaction rate with increasing digestion time. The reduction in the reaction rate was unexpectedly more evident in the case of the more amorphous Sigmacell. Figure 3(B) provides evidence for the saturation of the enzyme by the substrate at a low enzyme to substrate ratio. Moreover, this graph shows that the reaction rate order for the different substrates depends on the cellulose concentration. Saturation of the substrate by the enzyme was also demonstrated in some cases, particularly in the assays with low cellulose concentrations (Fig. 3(C)). However, a decline of the reaction rate at the higher enzyme concentrations, as detected by Wang and Converse (1992), was not observed in this work.

The kinetic parameters were calculated from double-reciprocal plots of the Michaelis–Menten equation, and the values of V_{\max} and K_m obtained are plotted in Fig. 4. The following observations can be drawn from the analysis of the V_{\max} values:

- the maximum reaction rate increases almost linearly with the enzymatic load for low enzyme concentrations (up to 0.1 mg/mL). A dramatic increase of several orders of magnitude is reached at higher concentrations in the case of Sigmacell.
- the order of magnitude of the V_{\max} increase with enzyme concentration is substantially different for each cellulose (132 for Sigmacell; 40 for Avicel; 8 for Whatman).
- the order of the cellulose susceptibility depends on the enzyme concentration: it follows the order Whatman > Avicel > Sigmacell at the lower-enzyme concentrations and Sigmacell > Avicel > Whatman for higher enzyme concentrations.

The order of the reactivity at high enzyme concentrations can be explained considering the structural properties of the substrates, as discussed above. More difficult to understand is the reversed order of reactivity when the enzyme is scarce. The Whatman cellulose has a lower effective concentration, i.e., fewer reactive sites than the other celluloses, per weight, owing to its lower surface area and a higher crystallinity. A plausible hypothesis for a higher reactivity at low enzyme concentrations, as compared with Sigmacell, is that it depends on the adsorption dynamics. Several authors suggested that the cellulases would remain immobilised on

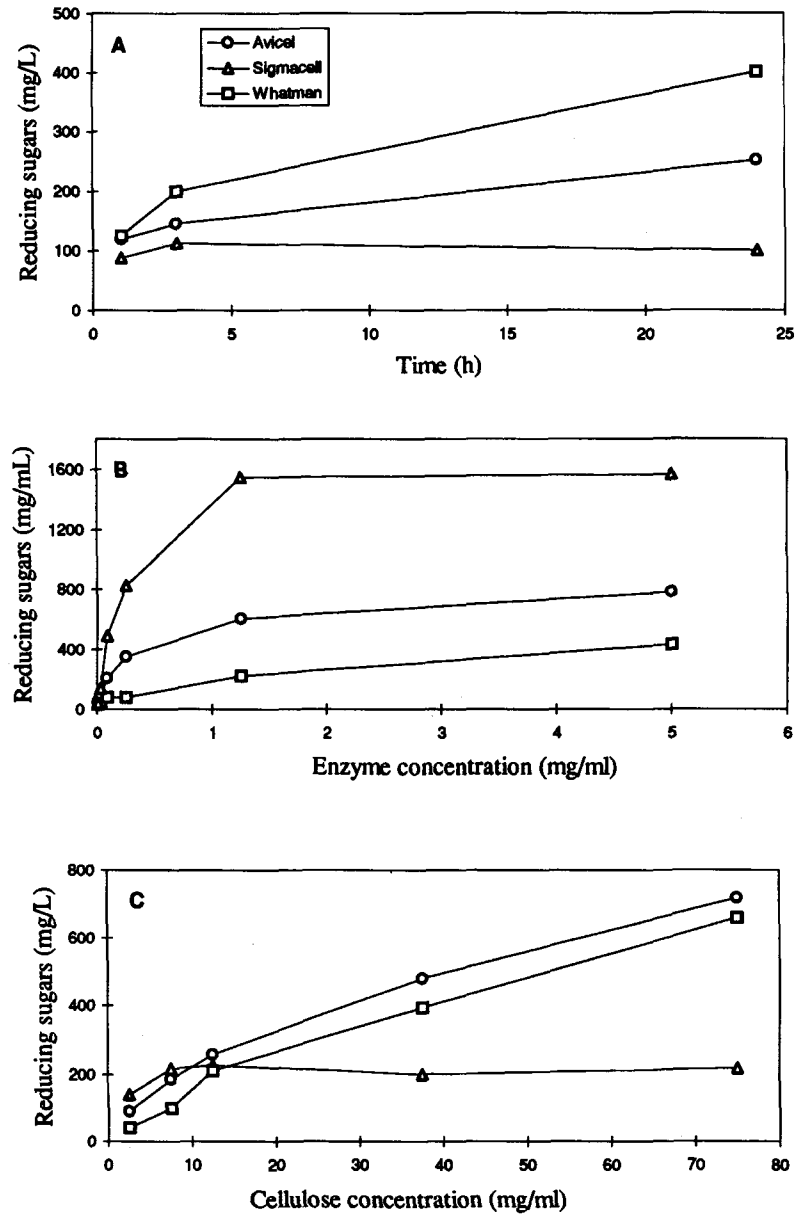


FIGURE 3 Reducing sugar concentration of the cellulose hydrolysis reaction media as a function of (A) digestion time, (B) enzyme concentration and (C) cellulose concentration. The data displayed correspond to (A) an enzyme concentration of 0.0088 mg/mL and cellulose concentration of 37.5 mg/mL, (B) enzyme concentration of 0.0293 mg/mL and soluble sugars measured after 3 h of reaction, and (C) cellulose concentration of 2.5 mg/mL and reducing sugars measured after 3 h of reaction.

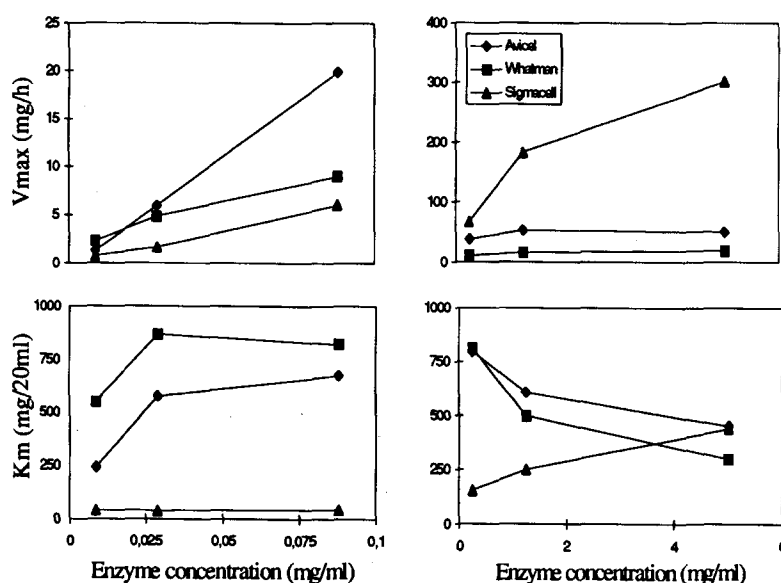


FIGURE 4 Kinetic parameters for the enzymatic hydrolysis of cellulose, obtained by plotting double-reciprocal representations of the Michaelis-Menten equation, for different enzyme concentrations. Plots on the left side correspond to the values for the lower enzymatic concentrations.

the surface of the fibres following adsorption, performing several catalytic cycles without leaving the surface (Ooshima *et al.*, 1990; Lee *et al.*, 1983). However, other results suggest the existence of an adsorption equilibrium (Wald *et al.*, 1984; Klyosov, 1990).

According to our results, the sequence of adsorption and desorption events, or of the lateral diffusion of the adsorbed enzymes, is probably different for the different celluloses. Indeed, the low activity of the enzyme upon Sigmacell at low enzyme concentration must be the result of either an irreversible adsorption onto this less crystalline, hence more highly hydrated cellulose, or of a lower mobility of the proteins on the cellulose surface. Furthermore, part of the adsorption is non-productive, in terms of hydrolysis (Lee *et al.*, 1982). An enzyme concentration of 0.0088 mg/mL is already saturated with substrate at 7.5 mg/mL, but after 3 h of reaction, the release of soluble sugars is very low (Fig. 3(A)). Apparently, the enzyme can hydrolyse only the molecules where it first adsorbs on.

In contrast, in the case of the rather crystalline Whatman cellulose, the enzymes have an increased chance of finding reactive sites on the cellulose surface, either by quickly adsorbing and desorbing or by lateral diffusion

(Gaspers *et al.*, 1994). The last hypothesis is a reasonable one, considering that Tilton *et al.* (1990) found that bovine serum albumin molecules adsorbed at a solid-liquid interface were able to move over distances of the order of several micrometers while remaining surface bound. The lateral diffusion of cellulases may be particularly favoured by their three domain structure (catalytic core, core binding domain, linker). In practice, the simultaneous desorption of the core binding domain and of the catalytic core is unlikely. As the enzyme concentration rises, the cellulose surface becomes fully coated and the reaction rate becomes proportional to the concentration of reactive sites on each cellulose surface (effective concentration), the order of reactivities being consequently reversed (Fig. 4). Furthermore, the competition of several molecules for the surface (Ryu *et al.*, 1984) probably enhances the adsorption and desorption rates, thus increasing the probability of successful connections between the enzyme and the substrate. If the enzyme adsorbs irreversibly and moves slowly along the surface, the synergism between endo and exoenzymes is possible only for high enzyme concentrations, since the probability of a consecutive action on the same molecule by an endoglucanase and an exoglucanase would otherwise be very low.

As mentioned previously, analysis of the kinetic parameters cannot be made with reference to the Michaelis-Menten model. Indeed, the strong dependence of the K_m values on the enzyme concentration contradicts the model.

The data presented here demonstrate that the study of the influence of the physical properties of the substrates on the reaction rate cannot be dissociated from their influence on the adsorption properties. Therefore, the effect of the enzyme to substrate concentration ratio should be analysed carefully, since the actual reactivity of the substrate might be masked by adsorption of enzyme molecules to non-effective sites. This topic is analysed with more detail in the part II of this work.

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