Role of the Activity and Adsorption of Cellulases in the Efficiency of the Enzymatic Hydrolysis of Amorphous and Crystalline Cellulose

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Received May 8, 1985; Revised Manuscript Received August 22, 1985

ABSTRACT: With several different cellulase preparations from various microbial sources (fungi *Trichoderma*, *Geotrichum*, *Myrothecium*, *Sporotrichum*, and *Aspergillus* and actinomycete *Thermomonospora*), it was shown that the mechanisms of enzymatic hydrolysis of a crystalline and an amorphous cellulose are different. The major factor related to cellulases that control the difference in the reactivity of the crystalline and amorphous cellulose appears to be the adsorption capacity of endoglucanase on cellulose. Taking this factor into account as a partition coefficient of cellulases between the substrate surface and the bulk solution allows a quantitative prediction to be made of the enzymatic hydrolysis rate for both amorphous and crystalline celluloses.

One of the most puzzling phenomena in the biochemistry of cellulose degradation is that cellulolytic enzymes from various microbial sources, equally active with respect to soluble cellulosic substrates like carboxymethylcellulose (CMC) and similar in activity to amorphous cellulose, differ solely in their capacity to hydrolyze native (highly ordered or crystalline) cellulose (Reese, 1976; Klyosov et al., 1982; Berezin & Klyosov, 1981). For instance, cellulases from the fungus *Trichoderma* almost completely hydrolyze a crystalline cellulose to glucose and cellobiose within a fairly short time whereas under the same conditions (and with equal activities of the enzymes with respect to CMC) cellulases from the fungus *Aspergillus* can only hydrolyze 7–9% of the substrate (Berezin & Klyosov, 1981; Rabinowitch et al., 1981; Klyosov, 1983) (this corresponds to the content of amorphous cellulose in the substrate).

This phenomenon was revealed in the initial studies of cellulose multienzymatic hydrolysis, and at the beginning of the 1950s, it was suggested (Reese et al., 1950) that the subdivision of cellulase complexes into "valuable" and "nonvaluable" ("full-value" and "low value" ones) may be due to the fact that the "full-value" enzymes contain a so-called C_1 factor, which can degrade a highly ordered cellulose, thus preparing it for the follow-up action of other enzymes in the complex. According to this hypothesis, the enzymes of low-value cellulase complexes, lacking the C_1 factor, can only degrade soluble and amorphous cellulose entities.

This paper shows the C_1 factor not as an individual substance or enzyme with a particular specificity but rather as a property of already known enzymes, namely, the capacity of cellulases (endo-1,4-β-glucanases, EC 3.2.1.4) to be adsorbed on the surface of insoluble cellulose.

Materials and Methods

The endoglucanase activities of enzyme preparations under study are given in Table I. Other properties of these preparations have been described in detail elsewhere (Klyosov et al., 1982; Klyosov & Rabinowitch, 1980). The concentration of the enzyme preparations used in cellulose hydrolysis studies was varied from 0.03 g/L (*Trichoderma viride*, *Trichoderma longibrachiatum*, and *Geotrichum candidum*, *Aspergillus terreus*) to 0.5 g/L (*Aspergillus niger*, *Myrothecium verrucaria*, and *Sporotrichum dimorphosporum*). The concentration of protein in the cellulolytic culture filtrates usually varies from 1 to 20 g/L (of which endoglucanase forms 10–20%), which is in general significantly higher than that used in our studies. The viscometric method of determining the endoglucanase activity was described before (Klyosov & Rabinowitch, 1980); cellobiase activity was determined by measuring glucose formation from cellobiose (Klyosov & Rabinowitch, 1980) by the glucose oxidase–peroxidase method (Klyosov et al., 1980). Reducing sugars were measured by the modified method of Nelson and Somogyi (Klyosov et al., 1980). The efficiency of endoglucanase adsorption on the surface of the cellulose was characterized by the partition coefficient (K_p) of the enzyme between the substrate surface and the water phase (Klyosov et al., 1982, 1983).

K_p is numerically equal to the ratio of the quantity of the enzyme adsorbed by a unit of mass, usually 1 g (or surface, usually 1 m^2), of the adsorbent (microcrystalline cellulose in

Table I: Partition Coefficient Values (K_p) of Endoglucanases from Various Microbial Sources under the Adsorption on Microcrystalline Cellulose

no.	source	endoglucanase activity (IU/g)	K_p (L/g)
1	*T. viride*[a]	1000	0.37
2	*T. viride*[b]	700	0.37
3	*T. longibrachiatum*[a]	610	0.35
4	*G. candidum*[c]	2000	0.30
5	*A. terreus*[c]	2400	0.13
6	*M. verrucaria*[d]	300	0.10
7	*S. dimorphosporum*[e]	430	0.040
8	*A. niger*[f]	100	0.035
9	*T. viride*[g]	550	0.030

[a] USSR commercial preparations. [b] Onozuka R10, Serva. [c] Kindly given by Dr. V. I. Maximov, Research Institute of Biotechnology, Moscow. [d] A gift from Dr. N. A. Rodionova, Bach Institute of Biochemistry, USSR Academy of Sciences. [e] Rapidase. [f] Koch-Light. [g] Boehringer Mannheim.

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0006-2960/86/0425-0540$01.50/0

our case) to the equilibrium concentration of the enzyme in the bulk solution under conditions of linearity of the adsorption isotherm (Rabinowitch et al., 1981). The K_p values given in Table I were determined as the slope of the initial (linear) portion of a plot of endoglucanase activity in the solution vs. endoglucanase activity on the surface (the latter was determined as the difference between the endoglucanase activity in the solution before and after addition of a given amount of cellulose, usually 5 g/L).

The concentration of the cellulase preparations (Table I) in these experiments varied between 0.05 (*A. terreus*) and 1.5 (*A. niger*) g/L, and in terms of endoglucanase activity usually from 0.03 to 0.2 IU/mL. The K_p values were measured at pH 4.5 (0.1 M sodium acetate buffer), 40 °C, in 10-mL glass cells with a magnetic stirrer. In a typical experiment, 50 mg of cellulose in 5 mL of the buffer was stirred in a cell for 1 h; then, 5 mL of the enzyme solution was added and stirred for 10 min (the adsorption equilibrium under these conditions was reached in 1–3 min). After this, cellulose (with adsorbed cellulases) was separated by centrifugation at 5000 rpm for 5 min at 40 °C, and the residual endoglucanase activity was determined in the supernatant. The difference between the initial (control) and residual activity corresponds to the amount of the endoglucanase adsorbed. The meaning of K_p values determined according to the procedure has been discussed in detail in Rabinowitch et al. (1983).

A microcrystalline cellulose (Chemapol, Czechoslovakia) was used as a substrate for adsorption of cellulases and for enzymatic hydrolysis. Since the cellulosic substrate contains about 10% amorphous cellulose (Klyosov et al., 1983), the hydrolysis of the *amorphous* part was studied at low degrees of the substrate conversion (below 2%, at a substrate concentration of 30 g/L), whereas the hydrolysis of the *crystalline* part of the cellulose was studied at large degrees of conversion: 30–50% (except the hydrolysis catalyzed by cellulases with a low adsorption capacity, see below) at a substrate concentration of 5 g/L to avoid a marked inhibition by hydrolysis products. We took the stationary hydrolysis rate (Δ[glucose]/t = constant) established after the initial burst on the kinetic curve (due to the more rapid hydrolysis of the amorphous cellulose fraction) as representing the cellulase hydrolytic activity to the crystalline cellulose. In all the cases, kinetic studies were conducted in the linear region of the dependence of the cellulose hydrolysis velocity on the concentration of cellulase preparations. Cellulases characterized by low adsorption on cellulose afforded a hydrolysis of microcrystalline cellulose no deeper than 10–15% for the time of the experiment, which was also followed by the low stationary hydrolysis rate established that was attributed to a crystalline cellulose fraction. The stationary rate of glucose and reducing sugar formation was usually established 50–60 h after hydrolysis started. Microcrystalline cellulose was enzymatically hydrolyzed at pH 4.5, 40 °C, in 20-mL cells with a magnetic stirrer. To evaluate the effect of cellulase inactivation on the kinetics of their hydrolytic action, the enzymatic hydrolysis of microcrystalline cellulose by cellulases from *T. viride* and *S. dimorphosporum* has been studied in the presence of 0.01 g/L poly(propylene glycol), which, as was shown earlier (Reese, 1980) and confirmed by our studies, significantly reduces the inactivation of cellulases. In the presence of poly(propylene glycol), the stationary hydrolysis rates were equal to 2.1 and 0.58 μM/min, respectively, while the control experiments [without poly(propylene glycol)] gave 1.7 and 0.48 μM/min; that is, the ratio between the velocities was the same, and the possible inactivation did not affect the principal difference between the reactivity of the cellulose toward the cellulase complexes under study.

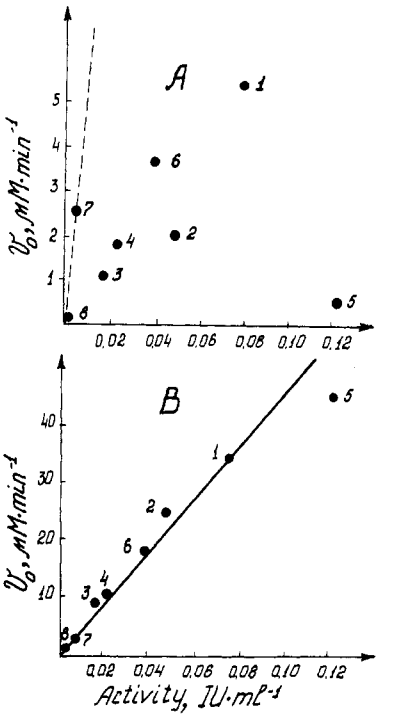

FIGURE 1: Dependence of the stationary rate of glucose formation during the initial period of enzymatic hydrolysis of the amorphous part of microcrystalline cellulose on the endoglucanase content in the reaction system. Dependences are expressed (A) for initial cellulase complexes and (B) for cellulase complexes with an excess of purified cellobiase from *A. foetidus*. The cellulase preparations are denoted by the following numbers: (1) *G. candidum*; (2) *T. reesei*; (3) *T. longibrachiatum*; (4) *T. lignorum*; (5) *S. dimorphosporum*; (6) *A. niger*; (7) *A. foetidus*; (8) *Thermomonopora* sp.

To change the rate-limiting step of glucose formation from cellobiase action to endoglucanase action on insoluble cellulose (see Results and Discussion), an excess of purified cellobiase from *Aspergillus foetidus* (0.2 g/L of cellobiase with a specific activity of 2050 IU/g) was added to the reaction system.

Results and Discussion

The hydrolysis of the *amorphous* part of microcrystalline cellulose catalyzed by cellulases showed no correlation between the stationary rate of glucose formation (in the initial phase of hydrolysis) and the activity of endoglucanase in the reaction system (Figure 1A). This is fairly clear because the endoglucanase activity does not limit the rate of glucose formation for most of the cellulase complexes used in enzymatic cellulose hydrolysis (Klyosov & Grigorash, 1981a). Figure 1B shows that the excess of cellobiase increased the rate of glucose formation to different extents under the action of most of the cellulase complexes studied (to reach the level shown by the broken line in Figure 1A) and finally this rate becomes proportional to the endoglucanase activity in the reaction system.

So, for all the cellulase complexes studied in this work and chosen from a number of various sources, the hydrolysis rate of the *amorphous* part of the cellulose is solely determined by the endoglucanase activity when the rate of glucose formation is limited by the endoglucanase activity [the latter condition is easy to achieve by adding an excess of cellobiase to the reaction system (Klyosov & Grigorash, 1981a,b)]. The fairly important conclusion follows from this that the hydrolysis rate of *amorphous* cellulose catalyzed by cellulases may, under certain conditions, be well predicted quantitatively, solely from the endoglucanase activity (Figure 1B). However, the situation

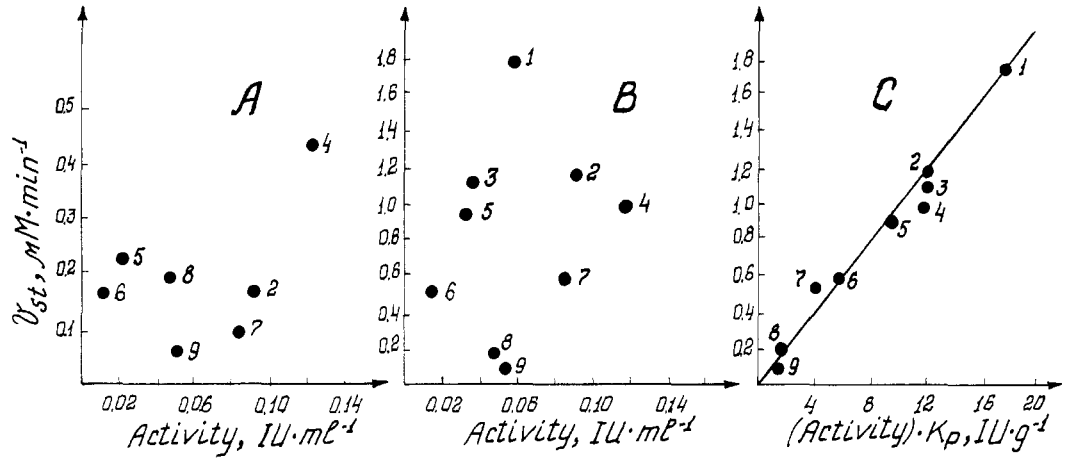

FIGURE 2: Dependence of the stationary rate of glucose formation under the enzymatic hydrolysis of the crystalline part of microcrystalline cellulose on the endoglucanase content (A) in the initial reaction system, (B) with an excess of purified cellobiase from *A. foetidus*, and (C) taking into account the adsorption capacity of endoglucanase on cellulose (in the form of K_p). Numeration of the cellulase preparations: (1) *T. viride*; (2) *A. terreus*; (3) *T. longibrachiatum*; (4) *M. verrucaria*; (5) *T. viride* (Onozuka R-10); (6) *G. candidum*; (7) *S. dimorphosporum*; (8) *A. niger*; (9) *T. viride* (Boehringer Mannheim).

was the reverse with the hydrolysis of *crystalline* cellulose. Figure 2A,B shows that the excess of cellobiase in the reaction system increased the glucose formation rate by 6–8-fold but led to no correlation between the hydrolysis rate and the endoglucanase activity (Figure 2B). Otherwise, during the hydrolysis of crystalline cellulose, unlike amorphous cellulose, the endoglucanases from various sources behave like *different* enzymes, and the endoglucanase activity (measured with respect to the hydrolysis of soluble cellulose) is still not a sufficiently pronounced factor to predict the reactivity of *crystalline* cellulose with respect to cellulases.

We have found that, in this respect, the efficiency of endoglucanase adsorption is a necessary factor that can be expressed as the partition coefficient (K_p) of the enzyme between the cellulose surface and the water phase (Table I). If the activity of endoglucanases (A) in the reaction system (in the presence of excess cellobiase) presented in Figure 2A were multiplied by the partition coefficient values given in Table I (i.e., AK_p), a fairly distinct correlation is observed between this parameter and the rate of glucose formation during hydrolysis of the crystalline part of cellulose (Figure 2C).

So, for all cellulase complexes studied in this work, the hydrolysis rate of the crystalline part of microcrystalline cellulose depends on two parameters: (a) the endoglucanase activity (when the endoglucanase action limits the rate of glucose formation, usually with the excess cellobiase in the reaction system) and (b) the adsorption capacity of endoglucanase on insoluble substrate. The measurement of these two parameters allows the hydrolysis rate to be predicted quantitatively both for the amorphous and for the crystalline cellulose parts for cellulases from various microbial sources.

It should be stressed that the role of cellulase adsorption on cellulose in the efficiency of enzymatic catalysis is by no means just the "effect of concentration", i.e., to an increase in the surface concentration of the enzyme on insoluble substrate for the cellulases having a higher adsorption capacity. We especially showed elsewhere (Rabinowitch et al., 1981; Klyosov et al., 1982) that it is the "tightness" of adsorption, suggesting the quality of binding the enzyme to the substrate (and not the surface concentration of the enzyme) plays a crucial role in degrading the crystalline cellulose. The mechanism of this phenomenon—"the tighter the binding, the better the catalysis"—is still unclear, but the very fact of discovering this effect means that it can be used to predict the reaction ability of insoluble substrates with respect to enzymatic hydrolysis.

Registry No. Cellulose, 9004-34-6; endoglucanase, 74191-29-0; cellulase, 9012-54-8.

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