Adsorption Characteristics of Cellulases from a Constitutive Mutant of *Trichoderma reesei*

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Received 9 September 1997/Accepted 21 May 1998

The adsorption parameter, A_{max} (maximum protein adsorbed/g substrate) and K_A (adsorption equilibrium constant) of *Trichoderma reesei* C-5 cellulases were 58.8 mg protein/g cellulose and 11.6×10^4 l/mol respectively. The activation energies for the adsorption rate constants of cellobiohydrolases and endoglucanases of C-5 were 30% and 11% lower respectively than that in the parent *T. reesei* QM9414 enzymes indicating the greater binding ability of the former. This was also reflected in its increased saccharification efficiency.

[Key words: cellulases, adsorption, cellulose hydrolysis]

The cellulases produced by the aerobic fungus *Trichoderma reesei* are among the most efficient enzymes for cellulose hydrolysis (1). The three types of enzymes that are associated with cellulose hydrolysis are the cellobiohydrolases (CBH, EC 3.2.1.91), endoglucanases (EG, EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). Synergistic action of all three is essential for maximal hydrolysis. The adsorption of cellulases on to cellulose depends on the enzyme characteristics and the structural features of cellulose, as well as on pH, temperature and mass-transfer effects (1–4).

Addition of L-sorbose to partially constitutive cellulase-producing cultures of *T. reesei* C-5 mutants growing on cellobiose (or glucose), has been found to enhance extracellular endoglucanase and β-glucosidase activities (5), with altered cellulase profiles. It was therefore, considered of interest to study the adsorption properties of the C-5 cellulases produced on sorbose-supplemented cellobiose medium as well as their efficiency in hydrolysing microcrystalline cellulose, and compare them with those of the parent QM9414 cellulases produced on 1% Avicel cellulose medium.

The strains were maintained as reported previously (5). The cellulases were produced by batch cultivation in 500 ml flasks containing an appropriate carbon source in 100 ml of Vogel's minimal medium containing other supplements (5). After 72 h of growth, the culture filtrate containing the enzymes was obtained by separating the mycelia from the culture broth by centrifugation. The cellulases used for the adsorption studies and cellulose hydrolysis were acetone-precipitated from the culture filtrate as described before (5). The filter paper units (FPU) and endoglucanase (EG) activities were assayed according to Ghose (6), and the cellobiohydrolase (CBH) activity according to Van Tilbeurgh *et al.* (7). The total protein concentration was estimated by Lowry's method.

The adsorption studies were carried out in 100 ml flask containing 20 ml of sodium citrate buffer (50 mM, pH 4.8), 2% w/v microcrystalline cellulose powder, (MCCP, Cellulose Products of India Ltd., Ahmedabad) and a fixed concentration of cellulase protein (1 mg/ml). The enzyme preparation was allowed to be in contact with cellulose for 30 min in an orbital shaker (100 rpm), during which samples were removed at regular intervals. The samples were centrifuged immediately in a microfuge (Eppendorf) at $10,000 \times g$ for 10 min and the clear supernatant analyzed for the presence of unadsorbed CBH and EG activities. From the difference in the original CBH and EG activities and that obtained at specific time intervals in the supernatant, the percent adsorption at each time point was calculated. Since the adsorption at and after 15 min contact was approximately the same, it was considered as representing the saturation value of the adsorbed component. The supernatant in each sample was analyzed for the presence of reducing sugars to calculate the extent of cellulose hydrolysis accompanying the adsorption process. Adsorption rate constants (k_a) were computed at different temperatures based on first-order rate kinetics (4).

$$\frac{dC_a}{dt} = k_a(C_s - C_a) \quad (1)$$

$$k_a = \frac{1}{t} \cdot \ln \frac{C_s}{C_s - C_a} \quad (2)$$

where C_a is the concentration of the adsorbed component at time t (IU/g substrate), C_s, the saturation concentration of the adsorbed component (IU/g substrate), considered as the amount of adsorbed component after 15 min-contact with the substrate and k_a, the adsorption rate constant.

The hydrolysis of cellulose was carried out in 100 ml flasks containing 20 ml citrate buffer (50 mM, pH 4.8), a cellulosic substrate (5% w/v) and different concentrations of the enzyme preparation, to give the requisite filter paper units per g cellulose substrate at 50°C and at 150 rpm. Samples were removed at regular time intervals, heated to 100°C to inactivate the enzyme, and centrifuged at $10,000 \times g$ for 5 min to remove the insoluble substrate. The supernatant was assayed for reducing sugars (8).

For measurement of the saccharification efficiency of the enzyme preparations on MCCP and Solka Floc (SF), various FPUs of the enzyme were incubated with the substrate at a fixed concentration (5% w/v) for 48 h. The total amount of reducing sugars produced was measured and the percent saccharification calculated as follows:

$$\% \text{ saccharification} = \left(\frac{\text{mg sugar produced} \times 0.9}{\text{mg initial substrate}}\right) \times 100$$

Both the adsorption and the hydrolysis experiments were performed in duplicate flasks, in three independent runs. The reported values are the average values with individual variations of less than ±3%.

The CBH and EG activities of *T. reesei* C-5 grown in 4% sorbose-supplemented 1% cellobiose cultures were increased 6-fold and 5-fold respectively, when compared with cultures grown in media not supplemented with sorbose. The presence of sorbose enhanced these activities in the parent QM9414 cultures also but the overall specific activities were higher in the C-5 cultures. An altered cellulase profile, especially with respect to increased CBHI and low-molecular-weight endoglucanases, was also observed in sorbose-supplemented C-5 cultures (unpublished data). The adsorption characteristics and effectiveness of this cellulase preparation in hydrolysing cellulosic substances were, therefore, studied. Figures 1a and b show the percent adsorption of CBH from C-5 and QM9414, respectively, at different temperatures. At all temperatures, adsorption equilibrium was attained within a 15 min contact period for both the enzyme preparations, and this was taken as representing maximum adsorption. The percent adsorption was maximum at 10°C while the initial rate of adsorption was maximum at 30°C. At all temperatures, the percent adsorption of C-5 CBH was more than that of QM9414. At 30°C, k_a for C-5 CBH was 9.78 min^{-1} and for QM9414 CBH, 10.6 min^{-1}. Energy of activation, E_a, was calculated from the Arrhenius plot (Fig. 2) and found to be 4351 J/mol for C-5 CBH and 6276 J/mol for QM9414 CBH. The results of adsorption of EG from C-5 and QM9414 were similar (data not shown). The decrease in the rate of adsorption after 30°C was most probably due to the hydrolysis of cellulose at those temperatures which affected the adsorption behaviour of cellulase. For this reason, low-temperature or short periods of incubation have been used (4, 9). The only exception reported was by Lee *et al.* (10), who found nearly the same binding capacity of *T. reesei* MCG-77 cellulases at 4°C and 50°C on Solka Floc cellulose. The adsorption of EGs of both the strains was higher than that of the CBHs. Similar observations have been made earlier (9). On the other hand, Kyriacou *et al.* (11) have reported preferential adsorption of CBH using ^3H and ^{14}C-labeled enzyme. In the present study, the computed activation energies of EGs from C-5 and QM9414 were 3324 J/mol and 3740 J/mol, respectively, which are lower than the values obtained for the CBHs of the two strains.

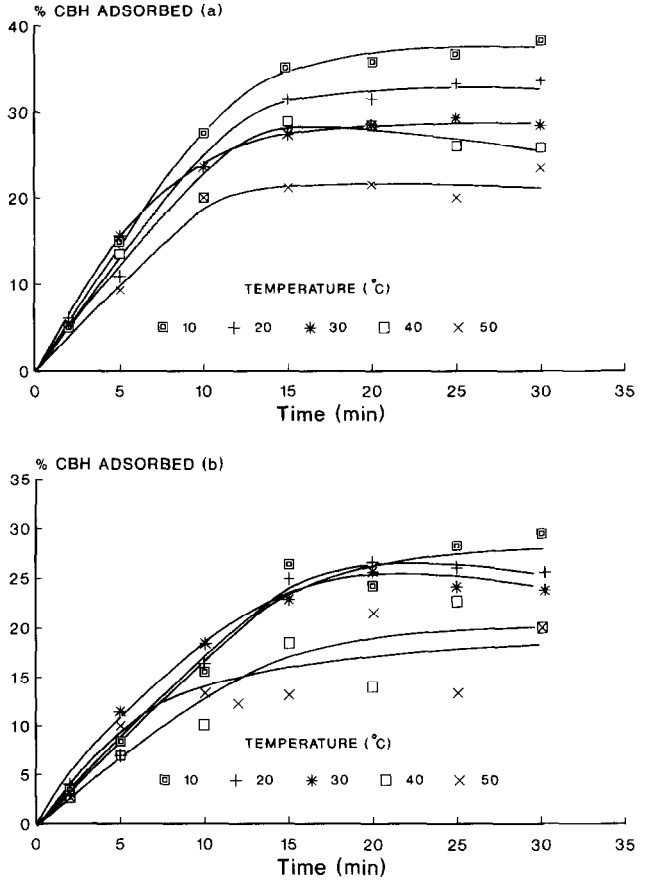

FIG. 1. (a) Time-course of adsorption of cellobiohydrolases of *T. reesei* C-5 cellulases on to 2% MCCP at different temperatures. (b) Time-course of adsorption of cellobiohydrolases of *T. reesei* QM9414 cellulases on to 2% MCCP at different temperatures.

The adsorption parameters, A_{max} and K_A, were computed from the modified form of the Langmuir equation:

$$\frac{E}{A} = \frac{1}{K_A A_{max}} + \frac{E}{A_{max}} \qquad (3)$$

where E is the equilibrium concentration of protein (mg/ml) and A is the specific adsorption of protein (mg/g substrate). The adsorption was carried out at 5°C to minimize the hydrolytic effect. The concentration of the protein was varied from 0.2 to 1.0 mg/ml. The specific adsorption of protein, A, was measured at different values of E. The specific adsorption increased linearly with respect to enzyme concentration at lower values, but decreased as the concentrations were increased. A Scatchard plot of the data is shown in Fig. 3. The values of A_{max} and K_A were 58.8 mg protein/g cellulose and 11.6×10^4 *l*/mol, respectively, for C-5 cellulases, and 50.0 mg protein/g cellulose, and 8.9×10^4 *l*/mol, respectively, for QM9414 cellulase, assuming the average molecular weight of cellulase as 6×10^4 (9). These values match closely with the values reported earlier. Wood-

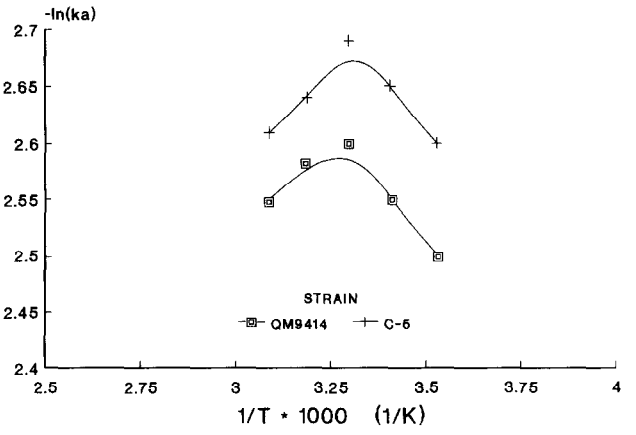

FIG. 2. Arrhenius plot for cellobiohydrolases of *T. reesei* C-5 and QM9414.

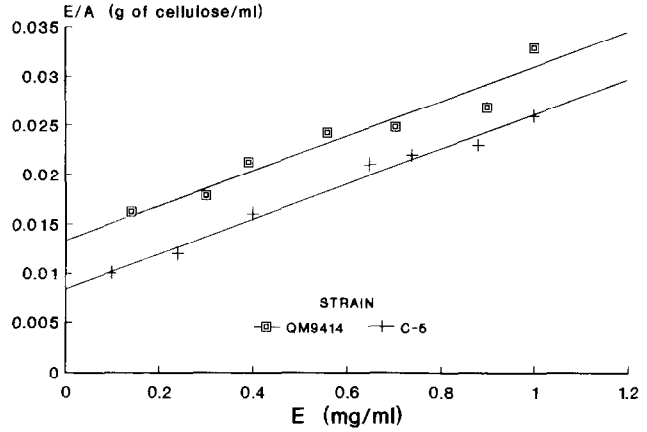

FIG. 3. Scatchard plot for the adsorption of cellulases of *T. reesei* C-5 and QM9414.

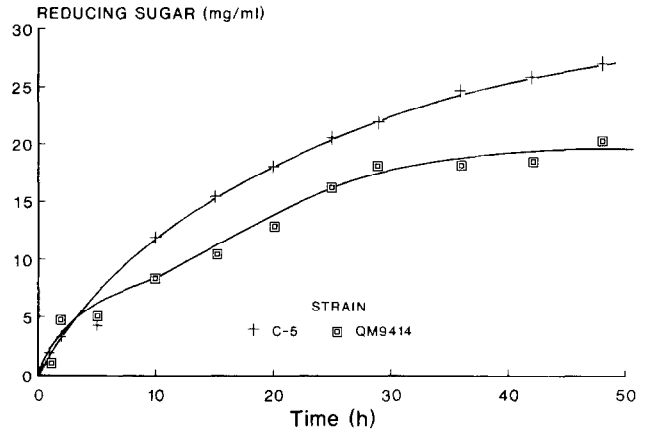

FIG. 4. Time-course of hydrolysis of 5% microcrystalline cellulose powder at an enzyme loading of 16 FPU/g cellulose, by cellulases of *T. reesei* C-5 and QM9414.

ward *et al.* (12) have reported a low value of K_A (2–10 mg/g cellulose), which perhaps resulted from the non-equilibrium binding of cellulases. Klyosov *et al.* (13) have characterized the affinity of enzyme adsorption by the partition coefficient *K*p and found a strict proportionality between the adsorption of endoglucanase and hydrolyzability of crystalline cellulose. A linear relationship between adsorption of CBH II and EG II and hydrolysis rates have also been reported by Nidetzky *et al.* (14). In the present study, the specific adsorption values of *T. reesei* C-5 CBH and EG were higher than those of QM9414 CBH and EG, and therefore the cellulases produced by the C-5 strain are expected to be more efficient in hydrolyzing cellulose. Observations consistent with this assumption are discussed below.

The efficacy of C-5 cellulases produced on sorbose-supplemented cellobiose was determined by hydrolyzing MCCP. Cellulases produced by QM9414 on 1% Avicel cellulose were used as the control. The time-course of hydrolysis at an enzyme loading of 16 FPU/gm substrate is shown in Fig. 4 for both the cellulases. After 48 h, the reducing sugars released by C-5 cellulases were 27 mg/ml, compared to 20.4 mg/ml obtained with QM9414 cellulases corresponding to 48.6% and 36.7% conversion efficiencies respectively. The enzyme loading, *i.e.* FPU/g substrate was also varied to evaluate the effectiveness of the two enzyme preparations for achieving maximum percent conversion of MCCP. At all enzyme loadings, higher percent conversion was achieved with the C-5 cellulases (data not shown). Enzyme loading per g of Solka Floc was also varied to see the effect of the two enzyme preparations on saccharification, and C-5 produced enzyme preparations were found to be superior. While the molecular characteristics of the cellulase enzymes produced by the two strains, albeit on different substrates, are not expected to be different, the increased saccharolytic activity of the C-5 cellulases might make greater number of sites available on the substrate which could contribute to the increased binding affinity observed.

In conclusion, the data presented herein indicate the superior adsorption capacity and hydrolytic efficiency of *T. reesei* C-5 cellulases. Since cellulase enzymes were produced by C-5 on a soluble carbon source (sorbose-cellobiose) rather than an insoluble cellulosic substrate (Avicel cellulose), the system provides a better alternative for the cultivation of the fungus in a bioreactor for large-scale enzyme production.

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