Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction

Bernd NIDETZKY,*§ Walter STEINER,* Marianne HAYN† and Marc CLAEYSSSENS‡

*Institute of Biotechnology, Technical University of Graz, Petersgasse 12/I, A-8010 Graz, Austria, †Institute of Biochemistry, University of Graz, Schubertstrasse 1, A-8010 Graz, Austria, and ‡Laboratory of Biochemistry, Department Biochemistry-Fysiology-Microbiology, University of Gent, K.L.-Ledeganckstraat 25, B-9000 Gent, Belgium

The hydrolysis of Whatman no. 1 filter paper by purified cellulolytic components from *Trichoderma reesei* and the synergistic action of binary combinations of these enzymes on the same substrate were investigated. At 20 g/l filter paper, enzyme concentrations needed to obtain half-maximal hydrolysis rates (K_m values) were in the 3–4 μ M range for the cellobiohydrolases (CBHs) and 0.05–0.10 μ M for the endoglucanases (EGs). Catalytic-core proteins of CBH I and EG III, lacking the cellulose-binding domain, exhibit K_m values 2.3 and 5.1 times higher than those of the intact enzymes. In synergistic combi-

nations of two cellulases, the K_m value of at least one enzyme was 3–10-fold reduced. CBH I/CBH II and CBH I/EG III combinations showed the most powerful synergism, and optimal ratios were a function of the total protein concentration. Results obtained in activity and adsorption assays using filter paper pretreated with one component, followed by inactivation and subsequent hydrolysis with the same or another cellulase component, point to a sequential enzymic attack of the cellulose and seems consistent with the mathematical model presented.

INTRODUCTION

Traditionally, hydrolysis of (semi)crystalline cellulose by fungal cellulase systems is thought to require the cooperative action of so-called endoglucanases (EGs; EC 3.2.1.4) and cellobiohydrolases or exocellulases (CBHs; EC 3.2.1.91). Since the rôle of each component in the hydrolysis of the insoluble substrate is not well understood and, also, no clear-cut distinction between the two cellulase types exists (Henrissat et al., 1985; Van Tilbeurgh and Claeysens, 1985; Vrsanska and Biely, 1992), hypothetical models for synergism have to be re-evaluated.

For fungal cellulases, two types of synergism have been put forward. So-called endo-exo-synergism is generally interpreted by a sequential mechanism of enzyme action: endoglucanases in an initial attack on the amorphous regions of the cellulose provide new chain ends for the action of CBHs (Wood and McCrae, 1979; Henrissat et al., 1985; Beldman et al., 1988). Another model assumes competition among individual cellulases for adsorption sites on cellulose (Ryu et al., 1984; Kyriacou et al., 1987), but its relevance for synergism remains unclear.

For exo-exo-synergism, described by several authors (Wood and McCrae, 1986; Fägerstam and Pettersson, 1980; Henrissat et al., 1985; Kyriacou et al., 1987; Tomme et al., 1988), two mechanistic models have been proposed. One is based on experimental evidence that between the enzymes from *Trichoderma reesei* (CBH I and CBH II) a loose complex is formed in solution and that adsorption of the individual components to cellulose is maximal in optimal synergistic admixtures (Tomme et al., 1990). Wood and McCrae (1986), on the other hand, tentatively ascribed synergism between CBH I and CBH II from *Penicillium pinophilum* to different orientations of the non-reducing end groups in crystalline cellulose, requiring two cello-

biohydrolases with different stereochemical specificities. However, elucidation of the three-dimensional structure of the core protein of CBH II from *T. reesei* and modelling of the substrate-binding site (Rouvinen et al., 1990) showed that the cellulose chain may enter the active-site tunnel in two different orientations.

Therefore plausible mechanistic concepts for cellulase synergistic action cannot be advanced as results published in the literature are inconclusive and sometimes contradictory. In part, this may be due to the unsatisfactory degree of purity of the individual cellulases (Van Tilbeurgh et al., 1984; Wood and Garcia-Campayo, 1990). Nevertheless some significant findings are: (i) synergism between cellulases depends on the ratio of the individual enzymes (Henrissat et al., 1985; Tomme et al., 1988), (ii) the importance of the degree of substrate saturation (Woodward et al., 1988) and (iii) the influence of the physico-chemical properties of the substrate itself (Henrissat et al., 1985).

The present paper deals with the synergistic action of the four major cellulases from *T. reesei*, namely CBH I, CBH II, EG I and EG III on Whatman no. 1 filter paper with a crystallinity index of about 45% (Henrissat et al., 1985). Furthermore, we present a mathematical and mechanistic model for the co-operative action of the cellulases.

EXPERIMENTAL

Materials

Filter paper no. 1 was from Whatman (Maidstone, Kent, U.K.). D-Glucose was determined either by the hexokinase assay (Chemie Linz, Linz, Austria) or the D-glucose oxidase (GOD)/peroxidase (POD) method (Boehringer, Mannheim, Germany). 2'-Chloro-4'-nitrophenyl (CNP) β -glycosides were prepared as

Abbreviations used: CBD, cellulose-binding domain; CBH, cellobiohydrolase, 1,4- β -D-glucan cellobiohydrolase; CNP, 2'-chloro-4'-nitrophenyl; CNPLac, 2'-chloro-4'-nitrophenyl lactoside; EG, endoglucanase, 1,4- β -D-glucan glucanohydrolase; i.e.f., isoelectric focusing; GOD, D-glucose oxidase; POD, peroxidase.

§ Present address and address for correspondence: Institut für Lebensmitteltechnologie, Universität für Bodenkultur, Peter-Jordan-Strasse 82, A-1190 Wien, Austria.

described by Van Tilbeurgh et al. (1988). β -Glucosidase from *Aspergillus niger* (Novozym 188; Novo, Bagsvaerd, Denmark) was purified by Bio-Gel P6 gel filtration (Bio-Rad, Richmond, VA, U.S.A.). Its activity was determined with 4-nitrophenyl β -D-glucoside (Ghose, 1987).

Enzyme purification

CBH I, CBH II and EG I were prepared from the culture filtrate of *T. reesei* MCG 77 using consecutive ion-exchange chromatography on DEAE- and CM-Sephadex. CBH I, CBH II and EG I were further purified by affinity chromatography (Van Tilbeurgh et al., 1984). The core protein and the cellulose-binding domain (CBD) of CBH I were prepared by limited proteolysis as described by van Tilbeurgh et al. (1986). EG III from *T. reesei* QM 9414 and its core, purified by reported methods (Macarron et al., 1993; Stahlberg et al., 1988), were gifts from respectively Dr. R. Macarron and Dr. G. Pettersson. The purity of the individual enzymes was checked and verified by SDS/PAGE, isoelectric focusing (i.e.f.)/PAGE and by the very sensitive measurement of the specific activity (50 °C) against small chromogenic substrates (Van Tilbeurgh et al., 1988). So, under these conditions, no appreciable activity of CBH II or EG III against CNP lactoside (CNPLac) could be detected (less than 0.01 % EG I and 0.1 % CBH I activity). No contamination (less than 0.1 %) of CBH I by EG I could be detected using activity measurements on 1 mM CNPLac in the presence of 20 mM cellobiose, which completely inhibits CBH I, but not EG I, activity. No release of the chromophore by CBH I, CBH II, and EG I was measured when using CNP cellotrioside as a substrate, thus ruling out contamination by EG III (less than 0.05 %).

The concentration of the individual cellulases was determined spectrophotometrically (280 nm) using the following molar absorption coefficients ($M^{-1} \cdot cm^{-1}$): CBH I (67200), CBH I core (62500), CBH II (79100), EG I (54800), EG III (77000) (Saloheimo et al., 1988), and EG III core (63600) (Stahlberg et al., 1988).

Enzymic hydrolysis

To prevent strong product inhibition by cellobiose during cellulose hydrolysis, individual enzymes or combinations were dissolved in 0.05 M sodium acetate buffer (pH 4.8) supplemented with β -glucosidase (0.2 unit/ml). No cellulolytic activity of the β -glucosidase against filter paper was detected. The reaction was started by adding a piece of filter paper (10 ± 0.5 mg) to 0.5 ml of preincubated (50 °C, 10 min) solution containing either individual enzyme or binary enzyme combinations in the following concentration ranges: CBH I (0.2–8.0 μM); CBH I core (0.4–15.0 μM); CBH II (0.1–7.0 μM); EG I (0.01–0.50 μM); EG III (0.02–0.90 μM); EG III core (0.02–0.90 μM). Further incubation was at 50 °C and agitation at 200 rev./min (Aquatron; Infors, Bottmingen, Switzerland). At stated times (0.5–5 h), samples were taken (15 μl), centrifuged [10000 rev./min (r_{av} , 7.5 cm), 5 min] and further incubated at 50 °C for 30 min to complete the conversion of soluble hydrolysis products into D-glucose. The latter was determined by the hexokinase assay measuring absorbances at 340 nm. The rates of hydrolysis were calculated from the linear range of D-glucose produced versus reaction time or, in case of significant deviation from linearity, by taking the amount of D-glucose released after 0.5 h.

Shaking intensities higher than 200 rev./min did not increase the reaction rates, thus indicating no mass-transfer limitation of

hydrolysis under the conditions used. Suitable test experiments proved that the addition of β -glucosidase did not alter the amount of D-glucose released by individual cellulases or combinations of the enzymes after 1 h (no synergism between the cellulases and the β -glucosidase).

Enzymic pretreatment of filter paper

The effect of substrate pretreatment was examined in a two-step experiment.

Step 1

Filter-paper discs (5.05 mm diameter; 2.0 ± 0.5 mg) in 100 μl 0.05 M sodium acetate buffer, pH 5.6, containing 0.2 unit of β -glucosidase/ml and different cellulases (either 2.6 μM CBH I, 2.6 μM CBH I core, 2.6 μM CBH II, 0.4 μM EG I or 0.7 μM EG III) were shaken (Thermomixer 5436; Eppendorf, Munich, Germany; 50 °C and 1300 rev./min). After 1 h, 70 μl of the reaction mixture were withdrawn and the same amount of buffer was added. The tubes were heated (90 min at 95 °C) to inactivate the enzyme adsorbed to the cellulose and, after cooling to room temperature, the enzymically pretreated filter paper was washed several times with 70 μl fresh buffer. Controls containing buffer and filter paper only were treated similarly.

Step 2

The enzyme used in the second step was added to the preincubated (50 °C, 10 min) solutions containing the pretreated filter paper (final volume 100 μl). Conditions and final concentrations were as in step 1. After 15, 30, 45 and 60 min, samples were taken (7 μl), further incubated for 30 min at 50 °C and analysed for D-glucose by the GOD/POD assay (100 μl reagent), measuring absorbances at 405 nm in microtitre plates (Easy Reader; SLT Lab Instruments, Graz, Austria). The complete inactivation of the enzyme used in step 1 was checked by addition of buffer and β -glucosidase to the pretreated filter paper and measuring formation of D-glucose as described above.

Adsorption

Adsorption of individual cellulases on to pretreated filter paper was determined by measuring the specific activity of non-adsorbed enzyme (1 h incubation) in the supernatants (Tomme et al., 1990). CBH I (core), EG I, and EG III activities were determined at 50 °C by on-line monitoring of the release of 2-chloro-4-nitrophenol ($\epsilon \approx 1.8 \times 10^4 M^{-1} \cdot cm^{-1}$ at 405 nm and pH 5.6) from the CNP β -glycosides of lactose (1 mM), cellobiose (1 mM), and cellotriose (0.25 mM) respectively (Van Tilbeurgh et al., 1988). Prior to these activity measurements any cellobiose present in the samples was hydrolysed by adding β -glucosidase as described above. In the subsequent activity measurement, the β -glucosidase (and contaminating β -galactosidase) activities in the samples had to be totally inhibited by addition of 10 mM glucono- δ -lactone and galactono- δ -lactone. Residual CBH II after adsorption was determined by measuring activities of supernatant samples against untreated filter paper (20 g/l).

Computational methods

Parameters in the mathematical models (see the Appendix) were estimated by non-linear least-squares regression using the BMDP statistical software package (BMDP statistical software, Los Angeles, CA, U.S.A.).

Table 1 Kinetic constants calculated by non-linear regression according to the models in the Appendix for individual cellulases and their binary, synergistic combinations acting on filter paper (results are means $\pm$ S.D.)

(a)					
Enzyme	Range (μM)	V_{max} (g/h per litre)	K_E (μM)	V_{max}/K_E	Ratio
CBH I	0.20–8.00	0.448 ± 0.072	4.243 ± 1.285	0.106 ± 0.036	2.17
CBH I core	0.40–15.00	0.471 ± 0.101	9.663 ± 2.548	0.049 ± 0.016	
CBH II	0.10–7.00	0.518 ± 0.052	3.301 ± 0.644	0.157 ± 0.034	
EG I	0.01–0.50	0.172 ± 0.021	0.095 ± 0.034	1.810 ± 0.503	—
EG III	0.02–0.90	0.299 ± 0.010	0.047 ± 0.007	6.361 ± 0.971	6.65
EG III core	0.02–0.90	0.271 ± 0.012	0.241 ± 0.031	1.125 ± 0.153	

(b)					(c)	
Enzyme 1/enzyme 2	$V_{\text{syn,max}}$ (g/h per litre)	$V(E_1) + V(E_2)$ (g/h per litre)	K_1 (μM)	K_2 (μM)	Calculated optimal ratios (total enzyme concentration)	
					(1 μM)	(10 μM)
CBH I/CBH II	2.183 ± 0.141	0.966 ± 0.089	4.243*	0.297 ± 0.063	62/38	70/30
CBH I/EG I	0.494 ± 0.053	0.620 ± 0.075	4.243*	0.022 ± 0.010	74/26	90/10
CBH I/EG III	1.277 ± 0.080	0.747 ± 0.073	1.488 ± 0.193	0.008 ± 0.002	82/18	92/8
CBH II/EG III	0.635 ± 0.069	0.817 ± 0.053	0.803 ± 0.216	0.042 ± 0.009	74/26	86/14

* Set constant during regression analysis because of highly linear correlation between $V_{\text{syn},\max}$ and K_1 .

RESULTS AND DISCUSSION

Hydrolysis of cellulose by individual cellulases and their core proteins

Under the conditions used and for each of the six enzymes studied (always in the presence of β -glucosidase), initial hydrolysis rates of Whatman No. 1 filter paper proved to be linear (first 30 min) when D-glucose was measured by the coupled assay (see the Experimental section). At longer reaction times a steady decrease in reaction rate was observed for EG I and EG III (core), probably indicating a rapid depletion of available substrate sites (results not shown).

Adsorption of cellulases on to the surface of their insoluble substrate is generally assumed to be a prerequisite step for hydrolysis. Therefore it seemed appropriate to analyse initial-rate data of cellulose hydrolysis at various enzyme concentrations and constant substrate concentration and to use equations deviating from classical Michaelis–Menten kinetics (see the Appendix). For the individual, intact cellulases and for the respective core proteins of CBH I and EG III, kinetic constants $V_{\max}$ and K_E were estimated by non-linear regression analysis (Table 1a). The resulting 'catalytic efficiencies' point to 10–50-fold higher values for the endocellulases compared with those for the exo-type enzymes and 2–6-fold reduced values for the core proteins compared with those for intact CBH I or EG III. The maximal hydrolysis rate values agree with those found for the same enzymes with other types of cellulosic substrates (Henrissat et al., 1985; Tomme et al., 1988).

Within statistical significance, maximal hydrolysis rates ($V_{\max}$ in Table 1a) are not influenced by proteolytic modification. Thus one function of the CBD can simply be to enhance the enzymes' efficiency by increasing the adsorption partition coefficient on crystalline cellulose (Tomme et al., 1988; Teeri et al., 1992). It seems thus improbable that the fungal CBD induces any additional 'active' property to the enzyme, as suggested by some

authors (Knowles et al., 1987), at least not in the case of filter-paper hydrolysis. Furthermore, no synergistic effect on filter-paper hydrolysis was observed when CBH I core and the isolated CBD of CBH I were admixed either in equimolar amounts or with a 5-fold molar excess of CBD.

Hydrolysis of cellulose by binary combinations of intact cellulases

Four combinations, CBH I, CBH II, EG I and EG III, showed positive co-operativity (Table 1b). This was investigated at various total enzyme concentrations and different ratios of the individual components. In all cases the synergistic factors, which were defined as the ratio of the activity of a given combination to the sum of the individual activities, were found to be constant during hydrolysis of filter paper up to 5 h reaction time. From a large amount of hydrolysis-rate data, parameters could be extracted by using the mathematical model outlined in the Appendix (eqns. A2a and A2b).

Values for $V_{\text{syn},\max}$, and the respective constants, K_1 and K_2 , were determined by non-linear regression for different combinations of the cellulases (Table 1b). Experimental hydrolysis rates were fitted to the model (eqns. A2a and A2b) using the parameter values from Table 1a. As shown in Figs 1(a)–1(d), no serious trend deviation can be detected, indicating that the model is valid to describe the experimental data.

The highest synergistic rates were observed with the combinations CBH I/CBH II and CBH I/EG III (Table 1b). The half-saturation constant of at least one enzyme in a synergistic combination (K_1 or K_2 in Table 1b) is reduced drastically when compared with the results in Table 1(a). This may indicate an increased affinity of the individual enzymes for the substrate when acting in a synergistic combination.

From eqns. (A2a) and (A2b) the maximal hydrolysis rates for different ratios of the components at a given total enzyme concentration can be calculated (Table 1c). It is evident that the

Figure 1 Action of the four synergistic, binary enzyme combinations (enzyme concentrations in μM) on 20 g/l filter paper at 50 °C (broken lines indicate model predictions according to eqns. A2a and A2b in the Appendix)

optimal ratio of enzymes is strongly influenced by the total protein concentration. At 10 μM , the estimated optimal ratios for EG/CBH combinations are in good agreement with those expected for endo-exo synergism (Henrissat et al., 1985; Wood et al., 1989). An optimal ratio of 4:1 for the combination CBH I/CBH II from *T. reesei* on Avicel was found by Henrissat et al. (1985) and Tomme et al. (1990). In case of CBHs I and II from *P. pinophilum*, this ratio was 1:1 on cotton fibres, requiring traces of EG activity for efficient hydrolysis (Wood and McCrae, 1986). We found a value of 7:3 (Table 1c; 10 μM total protein). The deviation from the results of Henrissat et al. (1985) and Tomme et al. (1988) may stem from different experimental conditions used (different substrate; addition of β -glucosidase, which prevents inhibition by cellobiose). Our results contradict the conclusions of Woodward et al. (1988) that synergism is not a function of the ratio of the individual enzymes.

The main conclusions from our results (Table 1a–1c) are as follows.

(1) For individual cellulases and their binary combinations hydrolysis rates and synergism are maximal at saturation of substrate with enzyme (hyperbolic relationships of reaction rate versus enzyme concentrations in eqns. (A1), (A2a), and (A2b) of the Appendix).

(2) Optimal degrees of synergism (= maximal synergistic factors) appear at non-saturating enzyme concentrations (compare K_E values in Tables 1a and 1b).

(3) Optimal ratios of the two cellulases (to obtain maximal hydrolysis rates) are determined, among other factors, by the total enzyme concentration reflecting the degree of substrate saturation (compare Table 1c).

(4) For a given total enzyme concentration, the maximal hydrolysis rate or the maximal synergistic factor may appear at different ratios of the two cellulases. This is deduced from the fact that the overall hydrolysis rate $[V(E_1, E_2)]$ can be described (see the Appendix) by the sum of the individual rates $[V(E_1)]$ and $[V(E_2)]$ and the synergistic rate $[V_{\text{syn}}(E_1, E_2)]$.

Table 2 Effect of enzymic substrate pretreatment by enzyme 1 on the activities of the individual, subsequently added cellulases (enzyme 2)

Activities are given in g of D-glucose/h per litre. The concentrations of the individual cellulases in each of both steps (enzyme 1 or 2) were: 2.6 μ M CBH I, 2.6 μ M CBH II, 0.4 μ M EG I and 0.7 μ M EG III. Further details are given in the Experimental section.

Enzyme 2	Activity					
	Enzyme 1 (pretreatment) ...	CBH I	CBH II	EG I	EG III	Buffer
CBH I		0.165	0.248	0.436	0.326	0.158
CBH II		0.302	0.110	0.159	0.334	0.127
EG I		0.080	0.038	0.016	0.008	0.044
EG III		0.144	0.143	0.113	0.036	0.146

Effect of enzymic pretreatment

The initial hydrolysis rates observed when individual cellulases act on enzymically pretreated filter paper were compared with those found in the control experiments (untreated filter paper). The increase in specific activities after substrate pretreatment (Table 2) seen in some cases seems to corroborate the synergistic effects observed above (Table 1a–1c). Effects of competition for or interaction between adsorption sites, which are present only when the components are acting simultaneously, may hamper a direct comparison. Within experimental error (3–5%), adsorption of the individual enzymes was unchanged by enzymic substrate pretreatment (results not shown). Therefore synergism cannot be interpreted in terms of an increase or *de novo* creation of adsorption sites for the individual enzymes.

When the substrate was pretreated with EG I or EG III and the same enzymes used for further hydrolysis, a decrease in hydrolysis rate was observed as compared with the controls (Table 2). This result confirms the observation that only a limited number of substrate sites in filter paper are available for hydrolysis by the EGs (see above). By contrast, for CBH I and CBH II, almost identical rates on untreated filter paper and on filter paper pretreated with these enzymes were found, proving that substrate sites were not depleted (Table 2). Subsequent additions of enzymes resulted in enhanced activity of the component added in the second step when compared to the controls (Table 2). The relevance of these results for synergism models are discussed below.

(a) Exo-exo-synergism

According to our results (Table 2), CBH I prepares a more readily hydrolysable substrate for CBH II and vice versa. Thus a simultaneous action of both enzymes is not required to observe 'synergism', since a sequential order of enzyme action is as effective. This result does not rule out the possibility that CBH I–CBH II complexes may exist (Tomme et al., 1990), but their formation does not seem to be a prerequisite for synergistic action on filter paper. From Table 2 one may see that the rate-enhancing effect of substrate pretreatment is much more pronounced when CBH II is acting on filter paper preincubated with CBH I than vice versa.

(b) Endo-exo synergism

In the case of EG III/CBH I and EG III/CBH II synergism, the theory of a sequential mechanism of enzyme action (Wood and

McCrae, 1979) may be valid: EG III prepares a more easily hydrolysable substrate for CBH I and CBH II. In contrast, EG III activity is not influenced after substrate pretreatment with the CBHs (Table 2).

EG I, on the other hand, acting synergistically with CBH I and, to a much lesser extent, with CBH II, only shows higher activity on filter paper pretreated with CBH I and not by CBH II (Table 2). This effect was reciprocal, and significantly enhanced activity was observed only for CBH I when acting on substrate pretreated by EG I (Table 2).

It is important to note that the increase of hydrolysis rate by substrate pretreatment reflects the degrees of synergism observed when the binary combinations were acting on untreated filter paper (Table 1b; Figures 1a–1e). Thus pretreatment of the substrate with EG I increased the activity of CBH II only slightly (about 15%), whereas the activity of the latter became 2.6-fold higher than the control after pretreating the filter paper with EG III. Pretreatment of filter paper with EG III increased the activity of CBH I 2.1-fold.

Although the physical effects of the enzymic pretreatment on the substrate are unknown, our results clearly indicate a transformation which may be the key to understand cellulase–cellulase synergism. The action of the cellulases does not significantly change the macroscopic physicochemical properties of the cellulose, such as crystallinity or specific surface area (Ohmine et al., 1983; Lenz et al., 1990), and also the action of the individual cellulases is strongly dependent on the type of cellulose used for hydrolysis (Nidetzky et al., 1993). This may be compatible with the finding of other authors (Henrissat et al., 1985) that the optimal ratio of the cellulases in a binary combination is influenced strongly by the substrate itself.

Our results show that the simultaneous concerted action of two cellulases is not intrinsically necessary to observe synergistic action, and therefore a sequential attack seems to be possible. The analogy of eqn. (A2b) to the steady-state rate equation for sequential two-substrate reactions (Cornish-Bowden, 1976) can be considered a further, albeit formal, confirmation for the hypothesis of sequential enzymic attack.

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REFERENCES

- Beldman, G., Voragen, A. G. J., Rombouts, F. M. and Pilnik, W. (1988) *Biotechnol. Bioeng.* **31**, 173–178
- Cornish-Bowden, A. (1976) *Principles of Enzyme Kinetics*, Butterworths, London
- Fägerstam, L. G. and Pettersson, L. G. (1980) *FEBS Lett.* **119**, 97–101
- Henrissat, B., Driguez, H., Viet, C. and Schülein, M. (1985) *Bio/Technology* **3**, 722–726
- Ghose, T. K. (1987) *Pure Appl. Chem.* **59**, 257–268
- Knowles, J., Lehtovaara, P. and Teeri, T. (1987) *Trends Biotechnol.* **5**, 255–261
- Kyriacou, A. K., MacKenzie, C. R. and Neufield, R. J. (1987) *Enzyme Microb. Technol.* **9**, 25–32
- Lenz, J., Esterbauer, H., Sattler, W., Schurz, J. and Wrentschur, E. (1990) *J. Appl. Polym. Sci.* **41**, 1315–1326
- Macarron, R., Acebal, C., Castillon, M. P., Dominguez, J. M., de la Mata, J., Pettersson, G., Tomme, P. and Claeysens, M. (1993) *Biochem. J.* **289**, 867–873
- Nidetzky, B., Macarron, R., Hayn, M. and Steiner, W. (1993) *Biotechnol. Lett.* **15**, 71–76
- Ohmine, K., Ooshima, H. and Harano, Y. (1983) *Biotechnol. Bioeng.* **25**, 2041–2053
- Rouvainen, J., Bergfors, T., Teeri, T., Knowles, J. K. C. and Jones, T. A. (1990) *Science*, **249**, 380–386
- Ryu, D. D. Y., Kim, Ch. and Mandels, M. (1984) *Biotechnol. Bioeng.* **26**, 488–496
- Saloheimo, M., Lehtovaara, P., Penttilä, M., Teeri, T. T., Ståhlberg, J., Johansson, G., Pettersson, G., Claeysens, M. and Knowles, J. K. C. (1988) *Gene* **63**, 11–21
- Ståhlberg, J., Johansson, G. and Pettersson, G. (1988) *Eur. J. Biochem.* **173**, 179–183
- Teeri, T., Reinikainen, T., Ruohonen, L., Jones, T. A. and Knowles, J. K. C. (1992) *J. Biotechnol.* **24**, 169–176

- Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. and Claeyssens, M. (1988) *Eur. J. Biochem.* **170**, 575–581
- Tomme, P., Heriban, V. and Claeyssens, M. (1990) *Biotechnol. Lett.* **12**, 525–530
- Van Tilbeurgh, H. and Claeyssens, M. (1985) *FEBS Lett.* **187**, 283–288
- Van Tilbeurgh, H., Bhikhabhai, R., Pettersson, G. and Claeyssens, M. (1984) *FEBS Lett.* **169**, 215–218
- Van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R. and Pettersson, G. (1986) *FEBS Lett.* **204**, 223–227

- Van Tilbeurgh, H., Loontjens, F. G., de Bruyne, C. K. and Claeyssens, M. (1988) *Methods Enzymol.* **160**, 46–59
- Vrsanska, M. and Biely, P. (1992) *Carbohydr. Res.* **227**, 19–27
- Wood, T. M. and Garcia-Campayo, M. (1990) *Biodegradation* **1**, 147–161
- Wood, T. M. and McCrae, S. I. (1979) *Adv. Chem. Ser.* **181**, 181–209
- Wood, T. M. and McCrae, S. I. (1986) *Biochem. J.* **243**, 93–99
- Wood, T. M., McCrae, S. I. and Bhat, K. M. (1989) *Biochem. J.* **260**, 37–43
- Woodward, J., Lima, M. and Lee, N. E. (1988) *Biochem. J.* **255**, 895–899

APPENDIX

A rate equation (eqn. A1) describing enzyme-dependent hydrolysis rates was proposed by Bailey (1989). Sattler et al. (1989) used a similar approach to describe hydrolysis of cellulose by a crude cellulase preparation:

$$V(E) = V_{\max} \frac{E}{(K_E + E)} \quad (\text{A1})$$

where $V(E)$ is the initial rate (enzyme-concentration-dependent) (g of glucose/h per litre), $V_{\max}$ is the maximal hydrolysis rate (at substrate saturation) (g of glucose/h per litre), E is the initial enzyme concentration (μM) and K_E is the half-saturation constant (μM).

The overall hydrolysis rate for a binary combination of the cellulases was described by the sum of the individual rates and an additional synergistic hydrolysis rate (eqn. A2a).

The expression for the synergistic hydrolysis rate (eqn. A2b) shows formal analogy to sequential two-substrate reactions where a ternary transitory complex of the enzyme and the two substrates is formed (Cornish-Bowden, 1976). In this case (eqn. A2b), the concentrations of two substrates in the steady-state rate equation (Cornish-Bowden, 1976) are substituted by these of the enzymes in the binary combination. However, the analogy to the

Michaelis–Menten theory must not be overstated, because the reaction system is heterogeneous in the case of cellulose hydrolysis.

$$V(E_1, E_2) = V(E_1) + V(E_2) + V_{\text{syn.}}(E_1, E_2) \quad (\text{A2a})$$

$$V_{\text{syn.}}(E_1, E_2) = V_{\text{syn., max.}} \frac{E_1 E_2}{(K_1 K_2 + K_1 E_2 + K_2 E_1 + E_1 E_2)} \quad (\text{A2b})$$

where E_1, E_2 are concentrations of enzymes 1 and 2 in a binary combination (μM), $V(E_1, E_2)$ is the hydrolysis rate of a combination of two cellulases (g of glucose/h per litre), $V(E_1)$, $V(E_2)$ are hydrolysis rates of the individual cellulases (see Table 1a), $V_{\text{syn.}}$ is the synergistic hydrolysis rate (g of glucose/h per litre), $V_{\text{syn., max.}}$ is the maximal synergistic hydrolysis rate (g of glucose/h per litre), and K_1, K_2 are half-saturation constants corresponding to enzymes 1 and 2 in a binary combination (μM).

REFERENCES

- Bailey, C. J. (1989) *Biochem. J.* **262**, 1001–1002
- Cornish-Bowden, A. (1976) *Principles of Enzyme Kinetics*, Butterworths, London
- Sattler, W., Esterbauer, H., Glatter, O. and Steiner, W. (1989) *Biotechnol. Bioeng.* **33**, 1221–1234