REVIEW

Toward an Aggregated Understanding of Enzymatic Hydrolysis of Cellulose: Noncomplexed Cellulase Systems

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Abstract: Information pertaining to enzymatic hydrolysis of cellulose by noncomplexed cellulase enzyme systems is reviewed with a particular emphasis on development of aggregated understanding incorporating substrate features in addition to concentration and multiple cellulase components. Topics considered include properties of cellulose, adsorption, cellulose hydrolysis, and quantitative models. A classification scheme is proposed for quantitative models for enzymatic hydrolysis of cellulose based on the number of solubilizing activities and substrate state variables included. We suggest that it is timely to revisit and reinvigorate functional modeling of cellulose hydrolysis, and that this would be highly beneficial if not necessary in order to bring to bear the large volume of information available on cellulase components on the primary applications that motivate interest in the subject. © 2004 Wiley Periodicals, Inc.
Keywords: adsorption; cellulose; cellulase; hydrolysis; kinetic model

INTRODUCTION

The potential importance of cellulose hydrolysis in the context of conversion of plant biomass to fuels and chemicals is widely recognized (Lynd et al., 1991, 1999; Himmel et al., 1999), and cellulose hydrolysis also represents one of the largest material flows in the global carbon cycle (Falkowski et al., 2000). The quantity of scientific information on components of cellulose-hydrolyzing enzyme system has expanded dramatically in recent years. Over the 12-year period from 1991 to 2003, for example, the number of known glycosyl hydrolases gene sequences has increased from ~300 to >10,000, and the number of cellulase crystal structures has increased from several to ~230 (H. Henrissat, pers. commun.). Also during this period, extensive structurally based classification schemes have been introduced for both catalytic and cellulose-binding modules, and have led to new insights and hypotheses with respect to the evolution of cellulase systems (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996), updated frequently at http//:afmb.cnrs.mrs.fr/CAZY.

In order for the large volume of available information on cellulase components to be brought to bear on the primary applications that motivate interest in cellulose hydrolysis, e.g., conversion of renewably produced biomass to fuels and commodity chemicals, it is necessary to incorporate this information into an understanding of cellulase systems comprised of multiple components with distinct modes of action. The situation is further complicated because the action of cellulase enzyme systems is impacted by substrate properties in addition to concentration—such as degree of polymerization, crystallinity, accessible area, the presence of lignin—which depend on the particular substrate being investigated and change as the reaction proceeds. In the course of seeking an "aggregated" understanding of enzymatic hydrolysis of cellulose that incorporates information about cellulase components and substrate features in addition to concentration, quantitative models are tremendously valuable. Of particular importance, measured parameters for cellulase components and substrates could in principle be incorporated into models used to predict the behavior of multicomponent cellulase enzyme systems. Comparison of such predictions to experimental measurements is the most systematic and rigorous means available by which to test whether understanding of cellulase components and their interactions is sufficient to explain a given observation. In addition, once a quantitative model is validated, it can be used to rapidly formulate new hypotheses of significance in both fundamental and applied contexts.

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This article reviews available information on enzymatic hydrolysis by noncomplexed cellulase systems; that is, systems based on components that act discretely rather than as stable complexes (Lamed et al., 1983; Tomme et al., 1995a). A considerable portion of this review is spent on the properties of cellulose in light of the central role such properties play in mechanistically based quantitative models of cellulose hydrolysis. In particular, the following section considers crystallinity, degree of polymerization, accessibility, preparation and properties of model substrates, and pretreated lignocellulosic materials. The section Cellulase Adsorption is devoted to adsorption leading to the formation of cellulose–cellulase complexes, including adsorption models, reversibility, and enzyme mobility, as well as inferred accessibility of cellulose from cellulase adsorption. Thereafter, mechanistic understanding of cellulose hydrolysis by noncomplexed systems is addressed in Cellulose Hydrolysis, with attention given to conceptual understanding of cellulose hydrolysis, features of the widely studied *Trichoderma reesei* cellulase system, documentation and understanding of synergism among cellulase components, and a summary of current mechanistic understanding. The section Quantitative Models presents a classification scheme and summarizes features of models reported in the literature. The final section offers concluding perspectives and outlines outstanding challenges associated with understanding and modeling noncomplexed cellulase systems. Since our primary focus is on the function of cellulases rather than their structure, we use the older, functionally defined nomenclature rather than the newer nomenclature based on amino-acid sequence and molecular structure.

CELLULOSE

All cellulose is produced biosynthetically. While cellulose production by photosynthetic higher plants and algae is thought to be by far the most important in terms of global carbon flows, cellulose production by nonphotosynthetic organisms (certain bacteria, marine invertebrates, fungi, slime molds and amoebae) has also been documented (Coughlan, 1985; Jarvis, 2003; Lynd et al., 2002; Tomme et al., 1995a). Cellulose is a linear condensation polymer consisting of D-anhydroglucopyranose joined together by β-1,4-glycosidic bonds. Anhydrocellobiose is the repeating unit of cellulose, since adjacent anhydroglucose molecules are rotated 180° with respect to their neighbors (Fig. 1a). This rotation causes cellulose to be highly symmetrical, since each side of the chain has an equal number of hydroxyl groups. Coupling of adjacent cellulose molecules by hydrogen bonds and van der Waal's forces results in a parallel alignment and a crystalline structure. The extensive hydrogen bonds of interchain (2 per anhydroglucopyranose) and intrachain (2 ~ 3 per anhydroglucopyranose) produces straight, stable supramolecular fibers of great tensile strength (Gardner and Blackwell, 1974a,b; Krassig, 1993; Nevell and Zeronian, 1985). In contrast, starch contains amylose and amylopectin connected by α-1,4 and to some extent α-1,6 glucosidic bonds, forming a tightly coiled helical structure maintained by interchain hydrogen bonds (Buleon et al., 1998; Calvert, 1997). Native cellulose, referred to as cellulose I, has two distinct crystallite forms, I_α, which is dominant in bacterial and algal cellulose, and I_β, which is dominant in higher plants (Atalla and Vanderhart, 1984). Native cellulose (cellulose I) can be converted to other crystalline forms (II–IV) by various treatments (Klein and Snodgrass, 1993; Krassig, 1993; O'Sullivan, 1997).

Cellulose exist as sheets of glucopyranose rings lying in a plane with successive sheets stacked on top of each other to form a three-dimensional particle. Because of this arrangement, the surface of a cellulose particle has distinct "faces" that interact with the aqueous environment and cellulase enzymes. The six carbons in the glucopyranose ring and internal β-glucosidic bonds lie in the ab plane or "110" face, whereas the ac plane or $1\bar{1}0$ face consists of the edges of rings (see Fig. 1b). Additional faces present reducing and nonreducing ends, respectively. The repeating unit of the 110 face is the cellobiose lattice, which measures 1.04 nm along the axis of the cellulose molecule and 0.54 nm in the perpendicular direction. About 100 cellulose glucans are aggregated into elementary fibrils with a crystalline width of 4–5 nm (O'Sullivan, 1997), and bunches of elementary fibrils are embedded in a matrix of hemicellulose with a thickness of 7–30 nm. The lignification process occurs late in the process of synthesizing natural fibers, so lignin is located primarily on the exterior of microfibrils where it covalently bonds to hemicellulose (Fig. 1c; Klein and Snodgrass, 1993).

The relationship between structural features of cellulose and rates of enzymatic hydrolysis has been the subject of extensive study and several reviews (Converse, 1993; Cowling and Kirk, 1976; Lynd et al., 2002; Mansfield et al., 1999; McMillian, 1994), but is still incompletely understood. Structural features of cellulose commonly considered as rate-impacting factors include crystallinity index, degree of polymerization, and accessible area.

Crystallinity Index (CrI)

Crystallinity has often been thought of as providing an indication of substrate reactivity, and is prominently featured in the model of Wood (1975) as well as other models. The crystallinity of dried cellulose samples can be quantitatively measured from the wide-range X-ray diffraction pattern (Krassig, 1993). In the case of cellulose-I, the crystallinity index (CrI) is calculated using the formula:

$$CrI = 1 - h_{am}/h_{cr} = 1 - h_{am}/(h_{tot} - h_{am}) \qquad (1)$$

based on the ratio of the height of crystalline cellulose in the 002 reflection at $2\theta = 22.5°$ (h_{cr}) to the height of amorphous cellulose (h_{am}), and $h_{tot} = h_{cr} + h_{am}$. Cotton

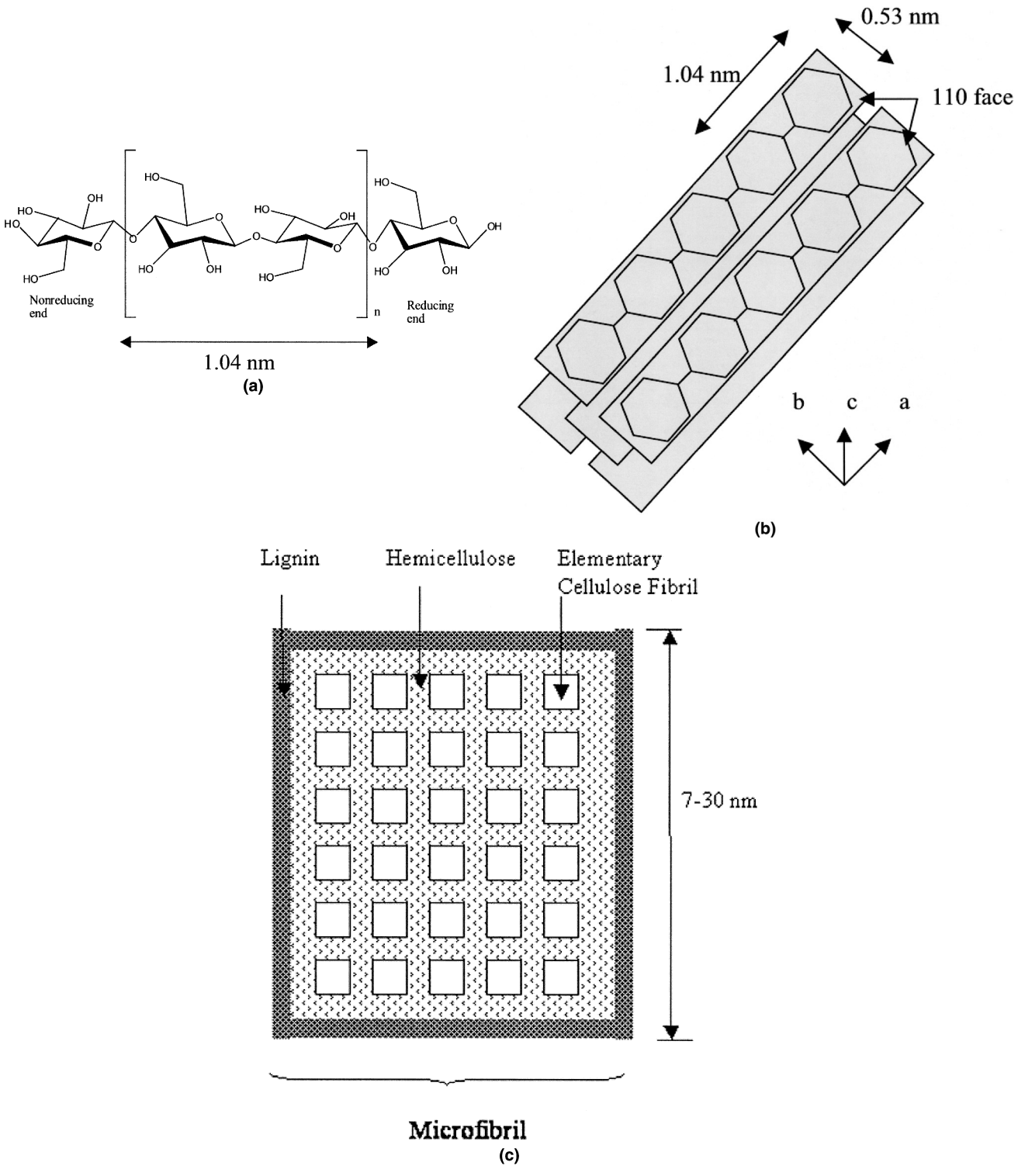

Figure 1. **a**: Structure of cellulose featuring repeating β 1,4-linked anhydrocellobiose units. **b**: Cellulose I crystal. The axes of the repeating unit (cellobiose) are: a = 0.817 nm, b = 1.04 nm, and c = 0.786 nm. The faces of the glucopyranose rings are parallel to the ab plane (110 face) of the crystal (Mosier et al., 1999). **c**: Organization of lignocellulose origanization into elementary fibrils and microfibrils (Klein and Snodgrass, 1993).

(Hoshino et al., 1997; Lee et al., 1982; Sinitsyn et al., 1991), bacterial cellulose from *Aacetobacter xylinum* (Boisset et al., 1999; Gilkes et al., 1992; Valjamae et al., 1999), and cellulose from the alga *Valonia ventricosa* (Boisset et al., 1999; Fierobe et al., 2002) provide examples of highly crystalline cellulose, while phosphoric acid swollen cellulose and ball-milled cellulose are regarded as amorphous cellulose (Hoshino et al., 1997; Lee et al., 1982; Ooshima

et al., 1983). Common model substrates derived from bleached commercial wood pulps, such as Avicel (Wood and Bhat, 1988; Wood, 1988), filter paper (Henrissat et al., 1985), and Solka Floc (Bertrain and Dale, 1985; Fan et al., 1980; Lee et al., 1982; Sinitsyn et al., 1991) are regarded as a blend of amorphous and crystalline forms (Gilkes et al., 1991). Typical values of CrI for various model cellulosic substrates are presented in Table I. The CrI value of cellulose increases after a period of water swelling due to recrystallization (Fan et al., 1980; Lee et al., 1983; Fengel and Wegener, 1984), and the variations in drying condition prior to measurement of CrI may cause differences between substrates arising from the method of substrate preparation rather than properties of the substrate per se (Lenze et al., 1990; Weimer et al., 1995). The presence of residual cells and proteins can also result in artifacts in the CrI assay (Converse, 1993).

Cellulose hydrolysis rates mediated by fungal cellulases are typically 3–30 times faster for amorphous cellulose as compared to high crystalline cellulose (Lynd et al., 2002; Table III). This observation led investigators in the 1980s to postulate a model for cellulose structure consisting of amorphous and crystalline fractions (Fan et al., 1980, 1981; Lee et al., 1983). If this hypothesis were correct, it would be expected that crystallinity should increase over the course of cellulose hydrolysis as a result of preferential reaction of amorphous cellulose (Betrabet and Paralikar, 1977; Ooshima et al., 1983). However, several studies have found that crystallinity does not increase during enzymatic hydrolysis (Lenze et al., 1990; Ohmine et al., 1983; Puls and Wood, 1991; Schurz et al., 1985; Sinitsyn et al., 1989). Considering both the uncertainty of methodologies for measuring CrI as well as conflicting results on the change of CrI during hydrolysis, it is difficult to conclude at this time that CrI is a key determinant of the rate of enzymatic hydrolysis (Lynd et al., 2002; Mansfield et al., 1999).

Future studies aimed at developing and applying improved methods would be useful to more definitively resolve the role of CrI in impacting hydrolysis. In interpreting crystallinity data, and indeed data for all cellulose physical properties, care must be taken to distinguish correlation from cause and effect. For example, several treatments that decrease crystallinity also increase surface area, and it has been suggested that the increased hydrolysis rates observed with substrates arising from such treatments may be due to increasing adsorptive capacity rather than substrate reactivity (Caulfield and Moore, 1974; Howell and Stuck, 1975; Lee and Fan, 1982). Comparing the hydrolysis rates on various sources of model cellulosic substrates, Fierobe et al. (2002) concluded that accessibility of cellulose is a more important factor than crystallinity index in determining the hydrolysis rate.

Table I. Summary of some physical properties of model cellulosic substrates.

Substrate[1]	CrI[2]	SSA[2] (m^2/g)	DP_N[2]	F_{RE} (%)
Avicel	0.5–0.6	20	300	0.33
BC	0.76–0.95	200	2000	0.05
PASC	0–0.04	240	100	1.0
Cotton	0.81–0.95	na.	1000–3000	0.1–0.033
Filter Paper	–0.45	na.	750	0.13
Wood pulp	0.5–0.7	61–55	500–1500	0.06–0.2

[1]BC, bacterial cellulose; PASC, phosphoric acid swollen cellulose; CrI denotes crystallinity index; SSA denotes specific surface area by BET; DP_N denotes the number-average degree of polymerization; F_{RE} denotes the fraction of reducing ends.

[2]References in text.

Degree of Polymerization

The degree of polymerization (DP) of cellulosic substrates determines the relative abundance of terminal and interior β-glucosidic bonds, and of substrates for exo-acting and endo-acting enzymes, respectively. DP may be defined in terms of the number average DP (DP_N), weight average DP (DP_W), or DP inferred from viscosity (DP_V):

$$DP_N = \frac{M_n}{MW_{glu}} = \frac{\sum N_i M_i}{\sum N_i} / MW_{glu} \quad (2)$$

$$DP_W = \frac{M_W}{MW_{glu}} = \frac{\sum N_i M_i^2}{\sum N_i} / MW_{glu} \quad (3)$$

$$DP_V = \frac{M_V}{MW_{glu}} = \frac{\sum N_i \eta}{\sum N_i} / MW_{glu} \quad (4)$$

where N_i is the number of moles of a given fraction i having molar mass M_i, M_N is the number-average molecular weight, M_w is the weight-average molecular weight, M_V is the viscosity-average molecular weight, MW_{glu} is the molecular weight of anhydroglucose (162 g/mol), and η is viscosity. Measurement of DP begins with dissolution of cellulose using a technique that does not alter chain length. Several such methods appear satisfactory, including: 1) metal complex solutions such as Cuam solution (Klemm et al., 1998) and cupriethylenediamine (Klemen-Leyer et al., 1992, 1994, 1996); 2) forming cellulose derivatives by reacting with organic solvents (Ng and Zeikus, 1980) or inorganic acids such as nitric acid (Whitaker, 1957); and 3) ionic solutions such as N,N-dimethylacetamide (DMAc)/LiCl (Striegel, 1997). After dissolution, DP_N can be measured by membrane or vapor pressure osmometry, cryoscopy, ebullioscopy, determination of reducing end concentration, or electron microscopy (Krassig, 1993). DP_W can be measured based on light scattering, sedimentation equilibrium, and X-ray small angle scattering, and DP_V is measured based on viscosity. The viscosity of dissolved cellulose or cellulose derivatives has been found to equal:

$$\eta = K_m M_i^{\alpha+1} \quad (5)$$

in which K_m = constant, with the value of α for cellulose and cellulose derivatives in most cases ranging from 0.75 to 1 (Krassig, 1993). Therefore, DP_V can be written as:

$$DP_V = \frac{\sum N_i M_i^{1.75-2}}{\sum N_i} / MW_{glu} \quad (6)$$

Since cellulose is polydisperse, $DP_W \geq DP_V > DP_N$. The DP_N values are adequate in dealing with cellulose hydrolysis, and DP_W and DP_V frequently show a good correlation to polymer properties (Klemm et al., 1998; Krassig, 1993). The distribution of DPs among a population of cellulose molecules can be measured by size exclusion chromatography (Yau et al., 1979). The reciprocal of DP corresponds to the fraction of reducing ends relative to all glucan units present (F_{NR}, unitless).

Cellulose solubility decreases drastically with increasing DP due to intermolecular hydrogen bonds. Cellodextrins with DP from 2–6 are soluble in water (Klemm et al., 1998; Miller, 1963; Pereira et al., 1988), while cellodextrins from 7–13 or longer are somewhat soluble in hot water (Zhang and Lynd, 2003; Schmid et al., 1988). A glucan of DP = 30 already represents the polymer "cellulose" in its structure and properties (Klemm et al., 1998).

The DP of cellulosic substrates varies greatly, from <100 to >15,000, depending on substrate origin and preparation, as shown in Figure 2. The DP of wood after pulping is reduced to 500–1,500 (Bertrain and Dale, 1985; Klein and Snodgrass, 1993; Lee et al., 1982; Swatloski et al., 2002). After partial acid hydrolysis, the DP of Avicel is further decreased to 130–800 (Hoshino et al., 1997; Ng and Zeikus, 1980; Ross-Murphy, 1985; Steiner et al., 1988; Wood, 1985), depending on hydrolysis conditions (Dong et al., 1998) and the DP of the original substrate (Wood, 1988). Similarly, the DP of natural cotton can be as high as 15,000, but is reduced to 1,000–3,000 or less in the preparation of cotton linters involving treatment to accomplish dewaxing and whitening (Kleman-Leyer et al., 1992, 1996; Okazaki and Moo-Young, 1978; Ryu and Lee, 1982), and filter paper made from cotton pulp has a DP of 500–1,000 or higher (Nisizawa, 1973; Kongruang et al., 2004). Bacterial cellulose (BC) has an average DP of 2,000–3,000 (Hestrin, 1963; Fierobe et al., 2002; Valjamae et al., 1999), while bacterial microcrystalline cellulose (BMCC) prepared by treatment of BC with acids ranges from 130–1,300, depending on hydrolysis conditions (Valjamae et al., 1999). The DP of phosphoric-acid swollen cellulose (PASC) ranges from 30 to more than 1,000 (Fan et al., 1980; Krassig, 1985; Petre et al., 1981; Wood and McCrae, 1972), depending on the DP of the starting substrate (Wood, 1988; Hoshino et al., 1997), as well as the phosphoric acid incubation time and temperature (Krassig, 1993).

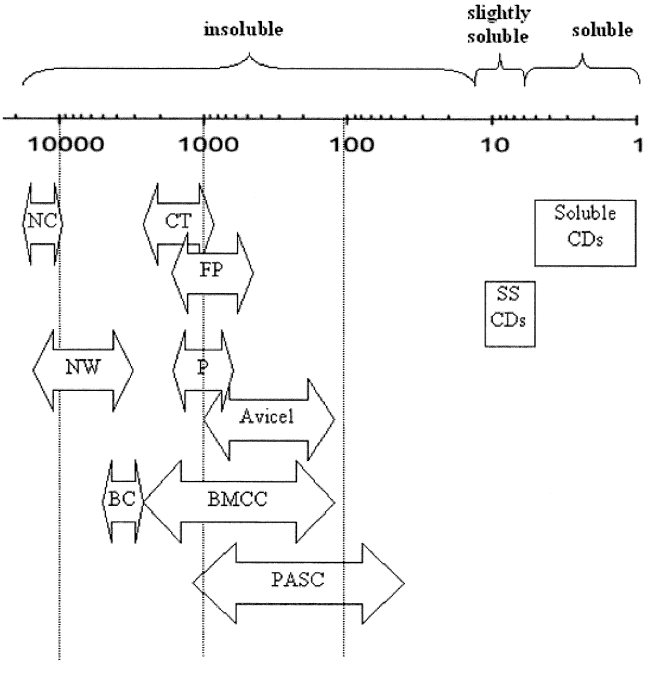

Figure 2. Typical DP values of cellulose and soluble cellodextrins. NC, natural cotton; NW, natural wood; P, pulp; CT, cotton linter; FP, filter paper.

The change in DP over the course of hydrolysis for cellulosic substrates is determined by the relative proportion of exo- and endo-acting activities and cellulose properties. Exoglucanases act on chain ends, and thus decrease DP only incrementally (Kleman-Leyer et al., 1992, 1996; Srisodsuk et al., 1998). Endoglucanases act on interior portions of the chain and thus rapidly decrease DP (Kleman-Leyer et al., 1992, 1994; Selby, 1961; Srisodsuk et al., 1998; Whitaker, 1957; Wood and McCrae, 1978). Exoglucanase has been found to have a marked preference for substrates with lower DP (Wood, 1975), as would be expected given the greater availability of chain ends with decreasing DP. It is well known that endoglucanase activity leads to an increase in chain ends without resulting in appreciable solubilization (Irwin et al., 1993; Kruus et al., 1995; Reverbel-Leroy et al., 1997). We know of no indication in the literature that the rate of chain end creation by endoglucanase is impacted by substrate DP.

Accessibility

Cellulase enzymes must bind to the surface of substrate particles before hydrolysis of insoluble cellulose can take place. The 3D structure of such particles (including microstructure) in combination with the size and shape of the cellulase enzyme(s) under consideration determine whether β-glucosidic bonds are or are not accessible to enzymatic attack. Cellulosic particles have both external and internal surfaces. In general, the internal surface area of cellulose is 1–2 orders higher than the external surface area (Chang et al., 1981), but this is not always the case, for example, in the case of bacterial cellulose. The internal surface area can be measured by small angle X-ray scattering (SAXS), mercury porosimetry, water vapor sorption, and size exclusion (Grethlein, 1985; Neuman and Walker, 1992; Stone et al.,

1969). The internal surface area of porous cellulose particles depends on the capillary structure and includes intraparticulate pores (1–10 nm) as well as interparticulate voids (>5 μm) (Marshall and Sixsmith, 1974). Grethlein (1985) found linear correlations between the initial hydrolysis rate of pretreated biomass and the pore size accessible to a molecule with a diameter of 51 Å, similar to the size of *T. reesei* cellulase components. But the surface exposed to dextran cannot distinguish the specific active cellulose surface area at which enzymatic hydrolysis occurs from the surface area which is not a site for enzymatic attack (Chanzy et al., 1984; Gilkes et al., 1992; Lehtio et al., 2003), resulting in potential overestimation of effective cellulase-accessible area. Techniques for measuring internal surface generally do not estimate external area (Converse, 1993).

External surface area is closely related to shape and particle size, and can be estimated by microscopic observation (Gilkes et al., 1992; Henrissat et al., 1988; Reinikainen et al., 1995b; Weimer et al., 1990; White and Brown, 1981). For example, the external surface area of BMCC is ~115 m^2/g (Gilkes et al., 1992) whereas that of Avicel is ~0.3 m^2/g (Weimer et al., 1990). Increasing cellulase adsorption and cellulose reactivity with decreasing particle size has been reported (Kim et al., 1992; Mandels et al., 1971). However, this may be due to causes other than increased external area, perhaps decreasing mass transfer resistance, since external surface is thought to be a small fraction of overall surface area for most substrates.

The gross cellulose accessibility is generally measured by the sorption of nitrogen, argon or water vapor, dimensional change or weight gain by swelling in water or organic liquids, and exchange of H to D atoms with D_2H. The most widely used procedure for specific surface area (SSA) is the Brunauer-Emmett-Teller (BET) method using nitrogen adsorption. Due to variations in the experimental conditions such as adsorption time, vacuum time and vacuum pressure (Marshall and Sixsmith, 1974), sample preparation (Grethlein, 1985; Lee et al., 1983), and sample origin and features (Marshall and Sixsmith, 1974; Weimer et al., 1990), a wide range of gross area values have been reported in the literature even for the same substrate. The specific area of Avicel PH 102 increases from 5.4 m^2/g surface area to 18 m^2/g after a long time of water swelling, because the capillary structure of air-dried cellulose from the water-swollen state collapses, resulting in drastic changes in physical parameters (Grethlein, 1985; Lee et al., 1983). To keep substrate capillary structure as it exists in the hydrated state, it is recommended that SSA be measured using solvent-dried samples (Grethlein, 1985; Lee et al., 1983). The typical SSA of BMCC, Avicel, and wet pulp are ~200 m^2/g BMCC (Bothwell et al., 1997), 1.8–22 m^2/g Avicel (Fan et al., 1980; Lee et al., 1983; Marshall and Sixsmith, 1974), and 55–61 m^2/g pulp (Fan et al., 1980; Kyriacou et al., 1988). The specific surface area of PASC from Solka Floc increases from 19.5 to 239 m^2/g when phosphoric acid concentration increases from 75% to 85% (Lee et al., 1982). Because a nitrogen molecule is much smaller than cellulase, it has access to pores and cavities on the fiber surface that cellulase cannot enter. Therefore, there is limited basis to infer that SSA measured using the BET method is a key determinant of enzymatic hydrolysis rate (Mansfield et al., 1999).

Preparation and Properties of Model Substrates

Wood pulp is made from wood using several steps, including shredding, delignification, bleaching, and washing (Klemm et al., 1998). For example, Solka Floc is made from SO_2-bleached spruce pulp by ball milling (Ghose, 1969). Avicel, also called hydrocellulose and microcrystalline cellulose, is prepared from cellulosic fibers (wood pulp) by partial acid hydrolysis and then spray drying of the washed pulp slurry, but microcystalline cellulose (Avicel) still contains a substantial amount (~30–50%) of amorphous cellulose (Krassig, 1993). Bacterial cellulose (BC) is prepared from the pellicle produced by *Acetobacter xylinum* (ATCC 23769) (Hestrin, 1963) or from Nata de Coco (Daiwa Fine Produces, Singapore; Boisset et al., 2000). Bacterial microcrystalline cellulose (BMCC) is prepared from BC by partial acid hydrolysis to remove amorphous cellulose (Valjamae et al., 1999). Cotton cellulose is made from natural cotton after removing impurities such as wax, pectin, and colored matter (Corbett, 1963). Whatman No. 1 filter paper is made from cotton pulp (Dong et al., 1998). Homogenous amorphous cellulose can be made from various pure cellulose powders, e.g., Avicel, cotton linters, by swelling treatments such as phosphoric acid, alkali, DMSO, DMAc/LiCl. Phosphoric acid swollen cellulose (PASC) is most commonly made by swelling cellulose powder using concentrated phosphoric acid, resulting in decreased crystallinity (Wood, 1988). Typical values for CrI, DP, gross surface area values (SSA by BET), and fraction of reducing ends (F_{NR}, reciprocal of DP) for model cellulosic substrates are presented in Table I.

Characteristics of Pretreated Lignocellulose

Natural cellulose molecules occur in elementary fibrils closely associated with hemicellulose and other structural polysaccharides as well as lignin (Fig. 1c). Such lignocellulose typically contains cellulose (35–50 wt. %), hemicellulose (20–35 wt. %), and lignin (5–30 wt. %) (Chang et al., 1981; Klein and Snodgrass, 1993; Lynd et al., 2002; Mansfield et al., 1999). A detailed consideration of enzymatic hydrolysis of native lignocellulose may be found elsewhere (Hatfield et al., 1999). Since enzymatic hydrolysis of native lignocellulose usually results in solubilization of ≤20% of the originally present glucan, some form of pretreatment to increase amenability to enzymatic hydrolysis is included in most process concepts for biological conversion of lignocellulose. Pretreatment, under appropriate conditions, retains nearly all of the cellulose present in the original material and allows close to theoretical yields upon enzymatic hydrolysis. Proposed pretreatment

processes include dilute acid, steam explosion at high solid concentration, "hydrothermal" process, "organosolv" processes involving organic acid solvents in an aqueous phase, ammonia fiber explosion (AFEX), strong alkali process (Lynd et al., 2002), as well as mechanical treatments such as hammer and ball milling (Millett et al., 1976; Sun and Cheng, 2002). Comparative features of these processes as well as consideration of substrate factors impacting the hydrolysis rate are reviewed elsewhere (Chang et al., 1981; Converse, 1993; Cowling and Kirk, 1976; Dale, 1985; Hsu, 1996; Ladisch et al., 1983; Mansfield et al., 1999; McMillian 1994; Lynd, 1996; Sun and Cheng, 2002; Weil et al., 1994; Wood and Saddler, 1988).

Hydrolysis of lignocellulosic biomass is more complicated than that of pure cellulose due to the presence of nonglucan components such as lignin and hemicellulose. Lignin removal and/or redistribution are thought to have a significant effect on observed rates of enzymatic hydrolysis (Chernoglazov et al., 1988; Converse, 1993; Lynd et al., 2002). Lignin has been implicated as a competitive cellulase adsorbent which reduces the amount of cellulase available to catalyze cellulose hydrolysis (Bernardez et al., 1993; Ooshima et al., 1990; Sutcliffe and Saddler, 1986). In addition, it has been suggested that residual lignin blocks the progress of cellulase down the cellulose chain (Eriksson et al., 2002; Mansfield et al., 1999).

The measured crystallinity index of lignocellulose is impacted by the presence of lignin and hemicellulose. Thus, care must be taken in comparing CrI values for lignocellulosic substrates to values for cellulosic substrates, and also in comparing the CrI of lignocellulosic substrates before and after pretreatment. Reported CrI values for pretreated materials are generally in the range of 0.4–0.7 (Chang and Holtzapple, 2000; Gharpuray et al., 1983; Koullas et al., 1992; Sinitsyn et al., 1989, 1991). Pretreatment by either dilute-acid or steam explosion under conditions that are quite effective in enhancing hydrolysis has been found to increase the composite CrI of lignocellulose (Deschamps et al., 1996; Kim et al., 2003; Knappert et al., 1980; Meunier-Goddik et al., 1999). Consistent with this, a negative correlation between hydrolysis rate and CrI has been shown in experiments that involved chemical pretreatments followed by ball milling (Chang and Holtzapple, 2000; Gharpuray et al., 1983; Knappert et al., 1980; Koullas et al., 1992; Sinitsyn et al., 1989, 1991), and also experiments that examined various pretreatment conditions (Chang and Holtzapple, 2000). In contrast to the trend observed for other pretreatment processes, AFEX pretreatment has been reported to result in a decrease in CrI (Gollapalli et al., 2002). Several investigators have implicated accessible surface area as an important factor in determining the effectiveness of pretreatment (Gharpuray et al., 1983; Grethlein, 1985; Grethlein and Converse, 1991; Sinitsyn et al., 1991). A significant difficulty in interpreting the effects of pretreatment at a mechanistic level is that exposure of substrates to conditions that cause one potential determinant of reactivity to change usually bring about changes in other such potential determinants. For example, Sinitsyn et al. (1991) found a strong negative correlation between CrI and accessible surface area accompanying several pretreatment processes. We suspect that the impact of increased surface area accompanying pretreatment may in many cases be more important than changes in CrI, although further work will be needed to establish this point and the relative significance of these and other factors may well be different for different processes.

DP values of lignocellulosic substrates such as bagasse, wheat straw, and *Eucalyptus regnans* pretreated using steam explosion, supercritical CO_2, alkali, and ozone mostly fall in the range of 600–1,100, although values as high as 3,000 have been recorded for *Pinus radiata* chips (Puri, 1984; Sinitsyn et al., 1991). During dilute acid-catalyzed cellulose hydrolysis, the DP of cellulosic materials decreases rapidly initially and achieves a nearly constant value thereafter called the level-off DP (LODP) (Klemm et al., 1998; Krassig, 1993; Wood, 1988). LODP values in the range of 100–300 have been measured, depending on the substrate and conditions such as temperature and acid concentration (Krassig, 1993; Wood, 1988). This LODP value may limit the rates of hydrolysis that can occur with dilute acid pretreated lignocellulose, although this has not been investigated experimentally. Different conclusions about the importance of DP in determining hydrolysis rates of pretreated cellulosic biomass have been drawn, with Sinitsyn et al. (1991) concluding that DP is relatively unimportant, but Puri (1984) concluding that it is quite important.

CELLULASE ADSORPTION

Adsorption

Cellulase adsorption is rapid compared to the time required for hydrolysis, with many studies finding that adsorption reaches steady-state within half an hour (Lynd et al., 2002). The most common description of cellulase adsorption is the Langmuir isotherm (Eq. [7]), derived assuming that adsorption can be described by a single adsorption equilibrium constant and a specified adsorption capacity. The Langmuir isotherm may be represented as:

$$E_a = \frac{W_{\max} K_P E_f}{1 + K_P E_f} \tag{7}$$

in which E_a is adsorbed cellulase (mg or μmol cellulase/L), W_{max} is the maximum cellulase adsorption = A_{max}*S (mg or μmol cellulase/L), A_{max} is the maximum cellulase adsorption per g cellulose (mg or μmol cellulase / g cellulose), S is cellulose concentration (g cellulose/L), E_f is free cellulase (mg or μmol cellulase/L), and K_P is the dissociation constant ($K_P = \frac{E_a}{E_f S}$) in terms of L/g cellulose. The distribution coefficient or partition coefficient, R, is defined as:

$$R = K_P W_{\max} \tag{8}$$

R has dimensions of L/g cellulose and corresponds to the ratio of E_a/E_f when substrate is excess, and hence $E_f = 0$ (Beldman et al., 1987; Klyosov, 1988, 1990; Kyriacou et al., 1988; Medve et al., 1997). In addition to equilibrium adsorption models, a dynamic adsorption model has been used by some investigators (Converse et al., 1988; Converse and Optekar, 1993; Nidetzky and Steiner, 1993; Nidetzky et al., 1994c).

The Langmuir equation is widely used because it provides a good (and often very good) fit to the data in most cases, and it represents a simple mechanistic model that can be used to compare kinetic properties of various cellulase–cellulose systems. But it is evident that cellulase binding does not comply with assumptions implicit in the Langmuir model due to one or more of the following: 1) partially irreversible cellulase adsorption (Palonen et al., 1999); 2) interaction among adsorbing cellulase components, especially at high concentrations (Jeoh et al., 2002); 3) multiple types of adsorption sites, even for one cellulase molecule (Linder and Teeri, 1997; Carrard and Linder, 1999); 4) cellulase entrapment by pores of cellulose (Lee et al., 1983); and 5) multicomponent cellulase adsorptions in which each component has different constants (Beldman et al., 1987). In light of these considerations, several equilibrium models representing alternatives to simple Langmuir adsorption have been proposed, including two-site adsorption models (Linder et al., 1996; Medve et al., 1997; Stalhberg et al., 1991; Woodward et al., 1988a), Freundlich isotherms (Medve et al., 1997), and combined Langmuir Freundlich isotherms (Medve et al., 1997).

Langmuir parameters for cellulase adsorption are presented in Table II, with an emphasis on noncomplexed cellulase systems. Although wide variations are observed in the values of parameters for different combinations of enzyme, substrate, and temperature, reproducibility among measurements from different labs taken for the same enzyme under a given set of conditions is rather good. Consider, for example, values from different sources for *T. reesei* cellulases listed in Table II, including CBH1 on BMCC at 4°C (Reinikainen et al., 1995b; Srisodsuk et al., 1993) and 50°C (Bothwell et al., 1997; Tomme et al., 1995b), CBH1 on Avicel at 20–25°C (Kim and Hong, 2000; Stahlberg et al., 1991; Tomme et al., 1990), and unfractionated cellulase adsorbing to Avicel at 4°C (Lee et al., 1982; Lu et al., 2002; Ooshima et al., 1983). This reproducibility suggests that experimental methods for measurement of adsorption parameters may be sufficiently standardized such that values from different labs can be meaningfully compared. We suggest that it may be useful to calibrate techniques with measurements made under well-characterized conditions when reporting adsorption data. In addition to experimental variables, different regression methods can lead to different values for parameters (Bothwell and Walker, 1995).

Ghose and Bisaria (1979) found that endoglucanases adsorb preferentially relative to cellobiohydrolases. But Ryu et al. (1984) found that cellulase contained tightly adsorbed cellobiohydrolases, some loosely bound EG1, and nonadsorbed endoglucanases other than EG1. Ooshima et al. (1983) found that the relative adsorption of *T. viride* endoglucanases and cellobiohydrolases was temperature-dependent, with endoglucanases preferentially adsorbed at 5°C, and cellobiohydrolases preferentially bound at 50°C. By contrast, Kyriacou et al. (1989) found that adsorption of *T. reesei* CBH1 was stronger than adsorption of EG1-3 on Solka Floc at 5°C, but that preferential adsorption of CBH1 was diminished at 50°C, and such preferential adsorption was also observed to be less pronounced with decreasing ionic strength. Joeh et al. (2002) reported that the combined adsorption of *T. fusca* cellulases Cel5A, Cel6B, and Cel9A was lower than the sum of individual adsorption at low temperature but higher at 50°C on BMCC.

Most early published studies have dealt with the reversibility of cellulase adsorption by measuring the amount of enzyme released into solution as cellulose hydrolysis progressed (Huang, 1975; Lee and Fan, 1982; Mandels et al., 1971; Moloney and Coughlan, 1983). But Beltrame et al. (1982) determined that the adsorption of protein consisted of irreversible steps, which were thought to arise from conformational changes of protein upon adsorption. Wald et al. (1984) contradicted Beltrame's finding by reporting that adsorbed cellulase can be removed by washing with buffer. Using fractionated cellulase, Kyriacou et al. (1989) found cellulase adsorption was irreversible, while Beldman et al. (1987) found cellulase adsorption to be partially reversible. Using radiolabeled cellulases from *T. reesei*, Palonen et al. (1999) found that desorption of CBH2 in response to sample dilution showed hysteresis (60–70% reversible), while desorption of CBH1 was more than 90% reversible. Nidetzky et al. (1994b) suggested that *T. reesei* CBH1 adsorption is partially reversible due to its bifunctional structure. The adsorption of *T. reesei* CBH1 CBM on microcrystalline cellulose was reported to be reversible (Linder and Teeri, 1996), while *T. reesei* CBH2 CBM could not be dissociated from cellulose (Carrard and Linder, 1999). Adsorption of CBMs from *T. fusca* Cel5A, Cel6B, Cel48A onto BMCC was reversible at low concentration but irreversibility was observed at high cellulase concentrations, apparently due to interstitial entrapment (Jung et al., 2002).

In an agitated batch reactor, the intensity of agitation has little effect on cellulose hydrolysis as long as cellulose particles are completely suspended (Huang, 1975). Jervis et al. (1997) studied surface diffusion of *Cellulomonas fimi* cellulases Cex and CenA on the surface of *Valonia ventricosa* microcrystalline cellulose using fluorescence recovery after photobleaching (FRAF). Based on comparison of the value of diffusion coefficient and specific cellulase activity, these investigators inferred that external diffusion of cellulase is not a rate-limiting factor for the whole reaction. In general, experiments examining stirring rate also suggest that external diffusion of cellulase on the surface is not rate-limiting (Fan et al., 1981; Fan and Lee, 1983). But when internal area is far larger than external surface, which

Table II. Summary of Langmuir cellulase adsoprtopm parameter for noncomplex cellulases and their sole cellulose-binding domains.

Strain	Cellulase	Substrate[a]	Temp. (°C)	A_{max} mg/g (μmol/g)	Kp L/g (L/μmol)	R L/g Cellulose	Reference
T. reesei	CBH1	BMCC	50	(4.6)	(0.28)	1.29	Bothwell et al., 1997
T. reesei	CBH1	BMCC	4	(6.0)	(8.33)	50	Reinikainen et al., 1995b
T. reesei	CBH1	BMCC	4	(4.2)	(7.14)	30	Srisodsuk et al., 1993
T. reesei	CBH1	BMCC	50	(2.63)	(4.03)	10.6	Tomme et al., 1995b
T. reesei	CBH1	Avicel	50	(0.48)	(0.09)	0.043	Bothwell et al., 1997
T. reesei	CBH1	Avicel	20	69 (1.1)	(0.278)	0.30	Stahlberg et al., 1991
T. reesei	CBH1	Avicel	25	70 (1.07)	(0.01)	0.011	Tomme et al., 1990
T. reesei	CBH1	Avicel	4	48 (0.74)	(0.93)	0.69	Medve et al., 1997
T. reesei	CBH1	Avicel	20	51.8	0.0192	0.99	Kim and Hong, 2000
T. reesei	CBH1	Avicel	40	40	0.0123	0.53	Kim and Hong, 2000
T. viride	CBH3 (CBH1)	Avicel	30	63	6.92	0.436	Beldman et al., 1987
T. reesei	CBH1	Filter Paper	50	(0.17)	(1.41)	0.24	Nidetzky et al., 1994c
T. reesei	CBH2	Avicel	25	64 (1.10)	(0.01)	0.011	Tomme et al., 1990
T. reesei	CBH2	Avicel	4	28 (0.52)	(1.92)	1.0	Medve et al., 1997
T. reesei	CBH2	Avicel	20	54.3	0.0071	0.039	Kim and Hong, 2000
T. reesei	CBH2	Avicel	20	48.9	0.0066	0.033	Kim and Hong, 2000
T. viride	CBH2	Avicel	30	6.6	4.96	0.037	Beldman et al., 1987
T. reesei	CBH2	Filter Paper	50	(0.258)	(0.95)	0.246	Nidetzky et al., 1994
T. reesei	EG1	Filter Paper	50	(0.166)	(0.56)	0.093	Nidetzky et al., 1994
T. viride	EG1	Avicel	30	126	0.88	0.111	Beldman et al., 1987
T. viride	EG2	Avicel	30	90	0.28	0.025	Beldman et al., 1987
T. viride	EG3	Avicel	30	26	11.67	0.303	Beldman et al., 1987
T. viride	EG4	Avicel	30	2.8	2.5	0.007	Beldman et al., 1987
T. viride	EG5	Avicel	30	105	0.89	0.094	Beldman et al., 1987
T. viride	EG6	Avicel	30	4.1	3.44	0.014	Beldman et al., 1987
T. reesei	EG3	Filter Paper	50	(0.308)	(0.91)	0.28	Nidetzky et al., 1994
T. reesei	total	Avicel	5	55.6	3.21	0.178	Ooshima et al., 1983
T. reesei	total	Avicel	4	64	1.23	0.079	Lee et al., 1982
T. reesei	total	Avicel	4	95.2	0.3	0.029	Lu et al., 2002
T. reesei	total	PSAC	4	1224	0.06	0.073	Lee et al., 1982
T. viride	total	Cotton	2–8	78–89	1.3–1.48	0.2	Beltrame et al., 1982
C. thermocellum	CBM_{CipA}	Avicel	25	10 (0.54)	(2.5)	1.35	Morag et al., 1995
C. thermocellum	CBM_{CipA}	PSAC	25	200 (1.08)	—	—	Morag et al., 1995
C. cellulovorans	CBM_{CbpA}	Avicel	37	(2.1)	(1)	2.1	Goldstein et al., 1993
C. cellulovorans	CBM_{CbpA}	Ab. Ct.	37	(6.4)	(1.25)	8	Goldstein et al., 1993
C. cellulovorans	CBM_{CipA}	Fb. Cellulose	37	(0.2)	(1.4)	0.28	Goldstein et al., 1993
C. thermocellum	CBM_{Ce1K}	PASC		(17.1)	—	(2.33)	Ketaeva et al., 2001
C. thermocellum	CBM_{Ce1K}	BMCC		(3.95)	—	(9.87)	Ketaeva et al., 2001
C. fimi	CBM_{Cex}	PASC	22	40	—		Ong et al., 1993
C. fimi	CBM_{Cex}	Avicel	22	3			Ong et al., 1993
C. fimi	CBM_{Cex}	BMCC	22	13.3			Ong et al., 1993
T. fusca	CBM_{E3}	BMCC	50	(1.65)	(0.124)	2.05	Bothwell et al., 1997
T. fusca	CBM_{E3}	Avicel	50	(1.77)	(0.182)	0.322	Bothwell et al., 1997
T. reesei	CBM_{CBH1}	BMCC	22			1.5	Palonen et al., 1999
T. reesei	CBM_{CBH2}	BMCC	22			1.0	Palonen et al., 1999

[a]Ab. Ct., absorbent cotton; Fb cellulose, fibrous cotton.

is the case for most cellulosic substrates, it is likely that some cellulase is entrapped in pores, resulting in lower hydrolysis rates.

Spatial Analysis of Adsorption and Inferred Accessibility of Cellulose

Analysis of adsorption in spatial terms is a prerequisite for understanding cellulose hydrolysis at a mechanistic level, and also provides a potentially powerful approach to evaluating the accessibility of cellulase enzymes. The area occupied by an adsorbed cellulase molecule is much larger that the area of the repeating cellobiose lattice (shown in Fig. 1b) for all cellulases for which information is available. As a result, the number of cellulase molecules that can bind to a cellulose surface is in general substantially smaller than the number of accessible cellobiose lattices on that surface. Adsorption of cellulase exhibits a preference for the 110 face (Fig. 1b) in both *T. reesei* CBH1 (Chanzy et al., 1984; Lehtio et al., 2003) and *C. fimi* cellulases (Gilkes et al., 1992). It seems reasonable to hypothesize that this is generally true since this is the face on which β-glucosidic bonds are accessible by cellulase.

Table III. Specific activities of *Trichoderma* cellulase components on insoluble cellulose substrates.

Strain	Enzyme	Temp. (°C)	Specific activity (substrate) (μmol GE/mg/min)	Reference
T. viride	CBH	40	0.42 (Av)	Berghem and Pettersson, 1973
T. viride	CBH	40	0.53–1.0 (AC)	Gum and Brown, 1977; Gritzali and Brown, 1978
T. reesei	CBH	50	0.08 (FP)	Ryu et al., 1984
T. reesei	CBH1	50	0.014 (Av), 0.039 (AC)	Tomme et al., 1988
T. reesei	CBH1	50	0.22 (FP)	Nidetzky et al., 1994c
T. reesei	CBH1	40	0.0175 (Av)	van Tilbeurgh et al., 1984
T. reesei	CBH1	50	0.065 (Av)*	Baker et al., 1998
T. reesei	CBH1	45	0.04 (Av), 0.6 (AC)	Shoemaker, 1983
T. reesei	CBH1	40	0.012 (Av)*, 0.0046 (FP)*	Henrissat et al., 1985
T. viride	CBH III (CBH1)	30	0.019 (Av), 0.03 (AC)	Beldman et al., 1985
T. reesei	CBH2	50	0.36 (FP)	Nidetzky et al., 1994c
T. reesei	CBH2	40	0.0391 (Av)	van Tilbeurgh et al., 1984
T. reesei	CBH2	50	0.027 (Av), 0.052 (AC)	Tomme et al., 1988
T. reesei	CBH2	50	0.065 (Av)*	Baker et al., 1998
T. reesei	EG	50	0.18 (FP)	Ryu et al., 1984
T. reesei	EG	50	3.6 (AC)	Niku-Paavola et al., 1985
T. reesei	EG1	45	0.17 (Av), 26 (AC)	Shoemaker, 1983
T. reesei	EG1	40	0.0046 (Av)*, 0.0023 (FP)*	Henrissat et al., 1985
T. viride	EG 3 (EG1)	40	0.13 (Av), 9.9 (AC)	Gritzali and Brown, 1978; Shoemaker, 1978
T. viride	EG 3 (like EG1)	30	0.196 (Av), 0.45 (AC)	Beldman et al., 1985
T. reesei	EG1	50	0.045 (Av)*	Baker et al., 1998
T. reesei	EG1	50	1.20 (FP)	Nidetzky et al., 1994c

*Long incubation time.

Gilkes et al. (1992) defined parameters consistent with a spatial interpretation of adsorption and incorporated these parameters into a modified Langmuir equation:

$$E_a = \frac{N_0 K_P' E_f}{1 + \alpha K_P' E_f} \quad (9)$$

where N_0 = μmol accessible cellobiose lattices/g cellulose, α = cellobiose lattices occupied/bound cellulase molecule, $K_P' = K_P/\alpha$.

It may be noted that the cellobiose lattices occupied/bound cellulase molecule, α, may be calculated from:

$$\alpha = N_0/A_{max} \quad (10)$$

For a cellulase with a given value of α, the surface area accessible to that cellulase (AS, m_2/g) may be calculated from the maximum adsorption capacity as follows:

$$AS = A_{max} N_A \alpha A_{G2} \quad (11)$$

where N_A = Avogadro's constant (6.023×10^{23} molecules/mol), A_{G2} = area of the cellobiose lattice (0.53×1.04 nm = 5.512×10^{-19}, m^2; Gardner and Blackwell, 1974a).

The value of *AS* is dependent on the value of α, which will vary depending on which enzyme is under consideration. For pure cellulose substrates, the fraction of β-glucosidic bonds accessible to cellulase relative to the total number of glucosidic bonds (F_a) is defined as:

$$F_a = 2\alpha A_{max} MW_{anhydroglucose} \quad (12)$$

where $MW_{anhydroglucose}$ = 162 g/mol anhydroglucose.

BMCC has been used in most studies aimed at determining parameter values for spatial analysis of adsorption. This is likely because the geometry of BMCC is well established, in contrast to most other cellulosic substrates. In particular, BMCC exists as a microfiber ribbon with a cross section of 15 × 40 nm, in which the narrower of the two faces is the reactive surface. Based on a cellulose density of 1.5–1.63 g/cm^3, Gilkes et al. (1992) and Reinikainen et al. (1995b) estimated N_0 for BMCC at 93–100 μmol cellobiose lattice/g.

At this time, the largest body of information relevant to estimating values for α is available for *C. fimi* and *T. reesei.* For *C. fimi*, Gilkes et al. (1992) estimate values of 32.9, 39.2, and 27.9 for CenA, the cellulose binding domain of CenA, and Cex, respectively. The catalytic domain of CBH1 is believed to occupy about 48 cellobiose lattices on a totally anisotropic surface (Sild et al., 1996), based on structural information inferred from X-ray crystallography (Divne et al., 1994). The CBM of CBH1 from *T. reesei* is thought to occupy about 10 cellobiose lattices based on nuclear magnetic resonance data (Kraulis et al., 1989; Reinikainen et al., 1995b). Reinikainen et al. (1995b) reported a range of values for A_{max} for CBH1 binding to BMCC, from which values of α from 15–40 can be calculated using Eq. [10]. These authors estimate a value of about 40 for α, which is very close to the value of 38.7 estimated by Tomme et al. (1995b) and is intermediate between the size of the catalytic domain and the CBM. Since binding of CBHI occurs primarily to the reactive face of BMCC (Chanzy et al., 1984; Gilkes et al., 1992; Lehtio et al., 2003), the value of α may also be estimated from the ratio of the reactive surface area to total surface

area, 15/(15 + 40) = 0.27. Based on this value, α for BMCC can be calculated as follows:

$$\alpha = N_0/A_{max} = 0.27 * S/(A_{max} * A_{G2} * N_A) \qquad (13)$$

where S is the total external surface area of BMCC from its geometric shape = 1 kg BMCC/(1.5–1.63 × 10^3 kg/m^3)/(15 × 10^{-9} m * 40 × 10^{-9} m) * 2* (15 + 40) × 10^{-9} m = 122–112 m^2 per g BMCC. If A_{max} = 6 μmol CBH1/g BMCC (Reinikainen et al., 1995b), α = 15.3–16.7 cellobiose lattice per adsorbed CBH1. It is important to note that the inferred value of α is influenced by experimental conditions such as temperature and ionic strength (Reinikainen et al., 1995b).

Based on a representative A_{max} value of 4.6 μmol/g for CBH1 adsorption to BMCC at 50°C (Table II) and α in the range of 15–40, AS values for BMCC of 23–61 m^2/g may be calculated using Eq. [11]. This value corresponds to 18–50% of the total external surface area of the MBCC ribbon (15 m^2/g). Regardless of the α value, it appears that cellulase does not adsorb to a significant fraction of the external surface of BMCC.

For Avicel (FMC PH105), 0.48 μmol/g is a representative A_{max} value for CBH1 adsorption at 50°C (Table II), from which the AS_{CBH1} of Avicel is found to be 6.4 m^2/g using Eq. [10] with a value of 40 assumed for α. The AS_{CBH1} value of Avicel PH 105 is much larger than the external surface area (0.3 m^2/g; Weimer et al., 1990), indicating that >~95% accessible area is internal. However, AS_{CBH1} is much smaller than the total surface area accessible to nitrogen, ~20 m^2 for Avicel (Marshall and Sixsmith, 1975), indicative of the presence of extensive internal surface area in pores too small to be accessed by cellulase molecules. Consideration of Avicel and BMCC clearly shows that the magnitude of external, internal, and gross surface area, as well as the relative importance of these, is quite different for different substrates. Using Eq. [12] with α = 40, F_a is found to be 6.0% for BMCC and 0.62% for Avicel.

Available data suggest that the area accessible to cellulase enzymes, as indicated, for example, by AS_{CBH1}, varies widely depending on the substrate. In addition to the 10-fold difference for AS_{CBH1} noted above for BMCC as compared to Avicel, studies using unfractionated *T. reesei* cellulase have reported a 3-fold higher cellulase adsorption capacity for Solka Floc SW40 compared to Avicel (Steiner et al., 1988), and a 20-folder higher capacity for PASC compared to Avicel (Lee et al., 1982; Morag et al., 1995). Accessible area in the order Avicel < BMCC < PASC is also supported by data from the CBMs isolated from *C. fimi* (Ong et al., 1993) and from CelK of *C. thermocellum* (Kataeva et al., 2001).

For pretreated lignocellulosic materials, adsorption to lignin typically occurs at the same time as adsorption to cellulose. Ooshima et al. (1990) estimated the maximum adsorption capacity for unfractionated *T. reesei* cellulase with respect to both cellulose and lignin present in dilute-acid-pretreated hardwood. They found the adsorption capacity for cellulose (as distinct from lignin) increased from 14.1 to 80.6 mg cellulase per gram cellulose as the pretreatment temperature increased from 180–220°C, while the capacity for lignin decreased from 100 to 12.3 mg cellulase/g lignin over the same temperature range. Lu et al. (2002), also working with unfractionated *T. reesei* cellulase, reported cellulase adsorption capacities of 180 mg/g cellulose relative to the cellulose fraction of Douglas fir prepared by SO_2-catalyzed steam explosion followed by peroxide treatment, and 95.2 mg cellulase/g Avicel. These results suggest that the accessibility of cellulose present in pretreated biomass can vary significantly as a function of conditions, but is often of a magnitude comparable to Avicel.

CELLULOSE HYDROLYSIS

On the Mechanism of Cellulose Hydrolysis (Noncomplexed Systems)

Beginning with Reese's original hypothesis for the action of C1 (Reese et al., 1950, 1968; Reese, 1976), there have been suggestions that the mechanism of cellulose hydrolysis involves physical disruption of insoluble cellulose in addition to endo- and exo-acting enzymes. The importance of such disruption, as well as the cellulase components responsible for it, is still not entirely clear. Coughlan (1985) used the term "amorphogenesis" to describe physical changes (i.e., swelling, segmentation, or destratification of cellulose) that enhance enzymatic hydrolysis and render crystalline cellulose more accessible to cellulase. Increased cellulose accessibility during enzymatic hydrolysis has been attributed to many factors. These include H_2O_2 production in the presence of Fe ion (Koenigs, 1975), or the short-fiber-forming factor in filtrates of *T. koningii* (Halliwell and Riaz, 1970), or *T. reesei* CBH1 (Chanzy et al., 1983; Lee et al., 2000) or its catalytic domain (Lee et al., 1996) or the CBH2 catalytic domain (Woodward et al., 1992), *T. reesei* endoglucanase–exoglucanase complex (Sprey and Bochem, 1993), *Humicola insolens* CBH2 (Boisset et al., 2000), *Thermomonospora fusca* cellulases E3 and E5 (Walker et al., 1990, 1992), some noncatalytic domains of cellulase such as the CBM of *C. fimi* endoglucanase A (Din et al., 1991, 1994), a short fiber-generating polypeptide from *T. pseudokoningii* (Wang et al., 2003), a *T. reesei* fibril-forming protein (MW = 11.4 kD) (Banka et al., 1998), and a novel *T. reesei* protein called swollenin (MW = 49 kD) (Saloheimo et al., 2002).

It is widely observed that the heterogeneous structure of cellulose gives rise to a rapid decrease in rate as hydrolysis proceeds, even when the effects of cellulase deactivation and product inhibition are taken into account (Zhang et al., 1999; Valjamae et al., 1999). Explaining this observation at a mechanistic level is an outstanding issue, with important fundamental and applied implications. Although very little work has been done involving detailed characterization, it would seem logical to expect that the declining reactivity

of residual cellulose during enzymatic hydrolysis is a result of factors such as less surface area and fewer accessible chain ends and/or adsorption of inactive cellulase on the surface of cellulose (or lignocellulose) particles which block further hydrolysis. At a macroscopic level, both the accessible area of cellulose (based on the BET assay; Fan et al., 1980) and cellulase adsorptive capacity (Ooshima et al., 1983) per gram cellulose have been reported to decrease with increasing hydrolysis. We speculate that the availability of glucan and chain ends per gram may also decrease with conversion. At a microscopic level, the *T. reesei* CBH1 disrupts fibers, resulting in more surface area (Lee et al., 1996), while EG II appears to smooth fiber surface, resulting in less surface area (Lee et al., 2000). Fresh addition of substrates can stimulate more soluble sugar release (Carrard et al., 2000), also indicating the loss of cellulose reactivity at the end of hydrolysis and/or increased reactivity for "new" cellulase/cellulose encounters as compared to "old" encounters.

When cellulase enzyme systems act in vitro on insoluble cellulosic substrates, three processes occur simultaneously: 1) chemical and physical changes in the residual (not yet solubilized) solid-phase cellulose; 2) primary hydrolysis, involving the release of soluble intermediates from the surface of reacting cellulose molecules; and 3) secondary hydrolysis, involving hydrolysis of soluble intermediates to lower molecular weight intermediates, and ultimately to glucose, as shown in Figure 3. Chemical changes in residual cellulose are manifested as changes in the DP and chain end concentration. Endoglucanase increases the concentration of chain ends and significantly decreases DP by attacking interior portions of cellulose molecules. Exoglucanases shorten DP incrementally and only occasionally decrease the concentration of chain ends. Thus, endoglucanase activity is thought to be primarily responsible for chemical changes in solid-phase cellulose that occur over the course of hydrolysis, but plays a minor role in solubilization relative to exoglucanase, while exoglucanase activity is thought to be primarily responsible for solubilization but plays a minor role in changing the chemical properties of residual cellulose. Physical changes in residual cellulose are manifested as changes in accessible surface area due to geometrical changes resulting from the consumption or enlargement of accessible surface of cellulose due to progressive solubilization. More speculatively at present, components of cellulase enzyme systems may make additional surface area available by mechanisms other than hydrolysis per se.

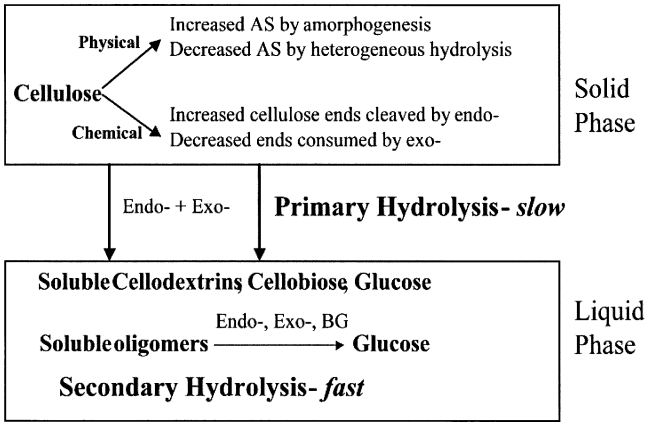

Figure 3. Mechanistic hypothesis of enzymatic hydrolysis for cellulose by *T. reesei* cellulase.

Since the rate of secondary hydrolysis is much faster than the rate of primary hydrolysis, it is possible—although at this point speculative—that soluble cellodextrins could account for a significant fraction of the immediate products of primary hydrolysis but not be detected. During enzymatic hydrolysis, cellodextrins with DP > 4 are present in the solid phase associated with crystalline cellulose (Kleman-Leyer et al., 1994, 1996; Srisodsuk et al., 1998; Stalbrand et al., 1998), and it has been suggested that this association impedes release of such cellodextrins to solution. However, cellodextrins with DP > 4 are not found associated with amorphous cellulose (Stalbrand et al., 1998). Thus, enzymatic hydrolysis of cellodextrins of length 4–6 associated with the solid phase may be an important part of the overall solubilization process for crystalline substrates, but not for amorphous substrates.

Most of the available data on cellulose hydrolysis concerns the rate of solubilization (process 2) above, often based on release of reducing sugars or soluble glucose equivalent. In our opinion, better characterization of chemical and physical changes associated with residual cellulose as well as secondary hydrolysis are promising areas of inquiry in order to improve fundamental understanding of cellulose hydrolysis.

Trichoderma reesei Cellulase System

Cellulases of the genus *Trichoderma* have received intensive attention due in significant part to the high levels of cellulase secreted. *Trichoderma viride* is a valid species aggregate, which is used for all unknown *Trichoderma* species; while all *T. reesei* are developed from a single isolate (QM6a), named in recognition of the pioneering contributions of Elwin Reese. Most commercial cellulases are produced from *Trichoderma* spp., with a few also produced by *Aspergillus niger* (Esterbauer et al., 1991; Nieves et al., 1998). The reader is referred to recent comprehensive reviews that address features of noncomplexed cellulase/hemicellulase systems produced by organisms other than *T. reesei* (Bhat and Bhat, 1997; Broda et al., 1996; Ito, 1997; Shallom and Shoham, 2003; Singh et al., 2003; Subramaniyan and Prema, 2000; Tomme et al., 1995a; Warren, 1996; Wilson, 2004).

The *T. reesei* cellulase mixture consists of many catalytically active proteins. At least two cellobiohydrolases (CBH1-2), five endoglucanases (EG1–5), β-glucosidases, and hemicellulases have been identified by 2D electrophoresis (Vinzant et al., 2001). CBH1, CBH2, and EG2 are the three main components of the *T. reesei* cellu-

lase system, representing 60 ± 5%, 20 ± 6%, and 12 ± 3% of total cellulase protein, respectively (Goyal et al., 1991; Gritzali and Brown, 1978; Knowles et al., 1987; Kyriacou et al., 1987; Nidetzky and Claeyssens, 1994). Reconstituted cellulase preparations based on purified components in these proportions exhibit specific activity equivalent to unfractionated preparations (Baker et al., 1998). The structure of CBH1, CBH2, and EG1 features a catalytic domain and a cellulose-binding domain connected by a glycolysated peptide linker (Gilkes et al., 1991; Lee and Brown, 1997; Linder and Teeri, 1997).

The catalytic domain structures of CBH1 and CBH2 are entirely different but both feature tunnel-shaped structures formed by disulfide bridges. In CBH2, two well-ordered loops form a 20 Å long tunnel adjacent to an α/β-barrel structure (Rouvinen et al., 1990). In CBH1, four surface loops form a tunnel of 50 A adjacent to a β-sandwich structure (Divne et al., 1993, 1994). The tunnel-shaped topology of CBH1 and CBH2 allows for a structural interpretation of the processive action of exoglucanase. The catalytic sites of both cellobiohydrolases are within the tunnel near the outlet, so that β-glucosidic bonds are cleaved by retaining (CBH1) or inverting (CBH2) mechanisms. Structural analyses, as opposed to measurement of hydrolysis products, provides direct evidence that cellobiose is the primary product of hydrolysis mediated by CBH1 and CBH2 (Divne et al., 1993, 1994; Davies et al., 1997). The *T. reesei* CBH1 and CBH2 can cleave several bonds following a single adsorption event before the dissociation of the enzyme substrate complex (Imai et al., 1998; Teeri et al., 1998a,b; Valjamae et al., 1998). Therefore, the action of CBH1 and CBH2 result in a gradual decrease in the degree of polymerization (DP) of cellulose (Kleman-Leyer et al., 1992, 1996; Srisodsuk et al., 1998). Cellobiohydrolase activity is often measured by reducing sugar release from Avicel, often called "Avicelase" activity. Avicel is a good substrate for measuring exoglucanase activity, although not exclusively, because it has the highest ratio of chain ends to accessible internal β-glucosidic bonds among model cellulosic substrates (see Table I and Adsorption, above).

EG1 and CBH1 have significant homology (45% identity, Penttila et al., 1986), belong to the same family (Cel7), and both use a retaining mechanism. The active site of EG1 is a groove rather than a tunnel (Henriksson et al., 1996), allowing glucan chains to be cleaved randomly to two shorter chains resulting in a rapid decrease in DP (Kleman-Leyer et al., 1992, 1994; Srisodsuk et al., 1998; Whitaker, 1957; Selby, 1961; Wood and McCrae, 1978). Endoglucanase activity is most often measured based on the rate of change of the viscosity of a soluble cellulose derivative such as carboxymethylcellulose (CMC) (Miller et al., 1960; Wood and McCrae, 1972). It may be noted that CMCase activity has been shown to correlate poorly with the ability to hydrolyze insoluble cellulose even for purified endoglucanases (Himmel et al., 1993; Klyosov, 1988; Klyosov, 1990). Of three purified *T. viride* endoglucanases obtained by Shoemaker and Brown (1978), the one exhibiting the highest rates of Avicel hydrolysis had the lowest CMCase activity. Klyosov (1990) clearly pointed out that the specific endoglucanase activities from many microorganisms measured on CMC do not correlate with activities against insoluble cellulose.

It is apparent that the division into endo- and exoglucanases is in many cases not absolute (Barr et al., 1996; Irwin et al., 1993; Henrissat and Davies, 1997; Teeri, 1997; Teeri et al., 1998a,b). Irwin et al. (1993) documented a processive endoglucanase in *T. fusca* E4. Some endoglucanase activity is exhibited by the *T. reesei* CBH2 (Enari and Niku-Paavolar, 1987; Kyriacou et al., 1987) and CBH1 (Schmid and Wandrey, 1990), as well as the *H. insolens* CBH2 (Boisset et al., 2000). Stahlberg et al. (1993) concluded that all *T. reesei* cellulases had some endo-acting activity. It has been suggested that exoglucanase could exhibit some endoglucanase activity due to temporary conformational changes of loops on the tunnel structure that expose their active sites (Warren, 1996; Zhang and Wilson, 1997). This hypothesis is supported by the observation that disruption of the loops comprising the tunnel of exoglucanase results in increased endoglucanase activity as well as higher k_{cat} (Kleywegt et al., 1997; Meinke et al., 1995). In addition, it may be observed that CBH2 contains fewer loops along the catalytic tunnel and exhibits greater endoglucanase activity relative to CBH1.

Removal of the CBM of *Trichoderma* cellulases results in a several-fold reduction in the rate of hydrolysis of insoluble cellulose but has little effect on hydrolysis of soluble substrates (Glikes et al., 1988; Irwin et al., 1994; Reinikainen et al., 1992; Srisodsuk et al., 1997; Stahlbeg et al., 1993; Tomme et al., 1988). The *T. reesei* CBMs belong to family 1 (CBM1), characterized by a small wedge-shaped fold featuring a cellulose binding surface with three exposed aromatic residues (Hoffren et al., 1995; Lehtio et al., 2003; Kraulis et al., 1989). These aromatic residues are thought to be critical for the binding of a CBM1 onto crystalline cellulose. The spacing of the three aromatic residues coincides with the spacing of every second glucose ring on a glucan chain, and it has been postulated that the aromatic amino acids of the CBMs form van der Waals interactions and aromatic ring polarization interactions with the pyranose rings on the surface of cellulose (Lehtio et al., 2003).

Specific activities of *T. reesei* EG1, CBH1, and CBH2 on various insoluble cellulosic substrates are presented in Table III. The data exhibit substantial variability even for apparently similar enzyme preparations and substrates. Notwithstanding this variation, the data support the following observations: 1) some early values for exoglucanase and endoglucanase activity were higher than values reported more recently, possibly due to use of lower purity enzyme preparations in earlier studies; 2) rates measured at longer reaction times are much slower than those at shorter times, which appears due at least in part to cellulose heterogeneity (Klyosov, 1990; Valjamae et al., 1998; Zhang et al., 1999); and 3) the rate of generation of soluble

reducing sugars by EG1 relative to CBH1 is $\gg 1$ for amorphous cellulose, ≤ 1 for Avicel, and $\ll 1$ for BMCC and cotton. The relatively low rate of reducing sugar release exhibited by EG1 on crystalline cellulose is consistent with most of the reducing ends generated by endoglucanase activity remaining in the solid phase, and does not necessarily imply a lower rate of β-glucosidic bond cleavage. The specific activity of CBH2 has been found to be nearly twice that of CBH1 in most (Henrissat et al., 1985; Medve et al., 1994; Nidetzky et al., 1994c; Tomme et al., 1988) but not all (Baker et al., 1998) studies.

Table IV presents the specific activities of the *T. reesei* EG1, CBH1, CBH2, and β-glucosidase on soluble glucans. While variability is again evident, the following trends may be noted: 1) the rate of reaction catalyzed by exoglucanase and endoglucanase increases with increasing soluble substrate chain length, whereas decreasing activity of β-glucosidase with increasing chain length is observed in the single study for which comparative data are available; 2) significantly higher rates are observed for EG1 as compared to CBH1 and CBH2. Comparing data in Tables III and IV, it may be seen that the specific activities of exoglucanases and endoglucanases acting on soluble substrates are higher by at least an order of magnitude than activities on insoluble substrates. Thus, the rate of primary hydrolysis (from cellulose to soluble glucans) is much slower than secondary hydrolysis (from soluble glucans to cellobiose and glucose).

Synergism

Synergism is said to occur when the activity exhibited by mixtures of components is greater than the sum of the activity of these components evaluated separately (Walker and Wilson, 1991; Wood and McCrae, 1979; Wood and Garcia-Campayo, 1990; Woodward, 1991). Quantitative representation of the extent of synergism is usually expressed in terms of a "degree of synergism" (DS)—equal to the ratio of the activity exhibited by mixtures of components divided by the sum of the activities of separate components. Types of synergism proposed in the cellulose hydrolysis literature include: 1) endoglucanase and exoglucanase; 2) exoglucanase and exoglucanase (Fagerstam and Pettersson, 1980; Tomme et al., 1988, 1990; Wood and McCrae, 1986; Wood and Garcia-Campayo, 1990); 3) endoglucanase and endoglucanase (Mansfield et al., 1998; Tuka et al., 1992; Walker et al., 1992); 4) exoglucanase or endoglucanase and β-glucosidase, which reduces inhibition by cellobiose (Lamed et al., 1991; Woodward, 1991); 5) intramolecular synergy between catalytic domain and CBM (Din et al., 1994) or two catalytic domains (Riedel and Bronnenmeier, 1998; Te'o et al., 1995; Warren et al., 1987; Zverlov et al., 1998); 6) cellulose-enzyme-microbe (CEM) synergism (Lynd et al., 2002); and 7) a proximity synergism due to formation of cellulase complexes (Fierobe et al., 2001, 2002; Mandels, 1985; Schwarz, 2001). Not all synergies are necessarily operative in any given situation. For example, synergism between the catalytic domain and CBM was reported for CenA of *C. fimi* on cotton fibers but not on BMCC (Din et al., 1994). Cell-enzyme-microbe synergism has been postulated for systems in which a metabolically active cell together with adhered cellulase binds to cellulose (Lynd et al., 2002), but has not been quantitatively evaluated.

Synergism between endoglucanases and exoglucanases is the most widely studied type of synergy and is among the most quantitatively important for hydrolysis of crystalline cellulose. As shown in Table V, the highest reported DS values are for BC (5–10) and cotton (3.9–7.6). Less pronounced but still significant synergism is exhibited for Avicel (DS 1.4–4.9), while the smallest synergistic effects (DS 0.7–1.8) have been reported for phosphoric acid-swollen and other acid-treated amorphous celluloses. DP appears to play an important and quite possibly dom-

Table IV. Specific activity of *Trichoderma* cellulase components on soluble substrates.

			Specific activity (μmol bond-breaking/mg/min) Substrate DP						
Strain	Enzyme	Temp. (°C)	G2	G3	G4	G5	G6	G7	Reference
T. viride	CBH	39		0.013					Li et al., 1965
T. reesei	CBH	50		0.1	2.7				Hsu et al., 1980
T. reesei	CBH1	25		0.23					van Tilbeurgh et al., 1982
T. reesei	CBH1	25		0.013					Claeyssens et al., 1989
T. reesei	CBH1	50			0.41	0.49	0.98	0.81	Nidetzky et al., 1994a
T. reesei	CBH2	27		0.056	3.78	1.01	12.9		Koivula et al., 1998 & 2002
T. reesei	CBH2	27		0.074	2.86	0.74	11.0		Harjunpaa et al., 1996
T. reesei	EG	50					17.5		Niku-Paavola et al., 1985
T. reesei	EG1	25		11					Claeyssens et al., 1989
T. viride	EG III (EG1)	40		24.4	66.7				Shoemaker and Brown, 1978
T. viride	BG	40	33	19					Berghem and Pettersson, 1974
T. viride	BG	50	58						Gong et al., 1977
T. reesie	BG1	45	31.4						Shoemaker et al., 1983
T. reesie	BG1	50	43.5						Chen et al., 1992
	BG2	50	9.8						

Table V. Maximum reported degree of exo/endo synergism for various model substrates.*

Strain	Enzyme combination	Maximum degree of synergism				Reference
		< 2	2 to 5		> 5	
C. stercorarium	Exo/Endo	0.7 (a)	1.4–2.1 (Av)			Riedel et al., 1997
C. thermocellum	Exo/Endo		2.5 (Av)	2.9 (FP)		Tuka et al., 1992
Humicola insolens	(CBH1+CBH2)/EG1				6.8 (BC)	Boisset et al., 2001
S. rolfsii	CBH/EG	1.8 (AC)	4.9 (Av)	3.9 (ct)		Sadana, 1985
S. pulverulentum	Exo/Endo	1 (AC)				Streamer et al., 1975
T. koningii	CBH/EG				7.6 (ct)	Wood and McCrae, 1978
T. reesei	CBH1/EG1		1.3–1.4 (Av)			Medve et al., 1998
T. reesei	(CBH1+CBH2)/EG1		$1.5–2^2$ (Av)			Woodward et al., 1988a
T. reesei	CBH1,CBH2/EG1		~2 (Av)			Baker et al., 1998
T. reesei	CBH1/EG1		~1.5–2 (b)			Srisodsuk et al., 1998
T. reesei	CBH1/EG1	1.7 (c)	4.1 (c)		7.8 (BC)	Valjamae et al., 1999
T. reesei	CBH1/EG2	1.5 (AC)	2.1 (Av)	3.2 (ct)		Hoshino et al., 1997
T. reesei	CBH1/EG2				~6 (BC)	Valjamae et al., 1999
T. reesei	CBH1/EG1		2.2 (Av), 2.5 (d)	3 (FP)	5 (BC)	Henrissat et al., 1985
T. viride	CBH1/EG2	1.2 (e)		5 (f)	10 (BC)	Samejima et al., 1998
T. viride	Exo/Endo	1.8 (AC)	1.7–3.5 (Av)			Beldman et al., 1988
T. viride	Exo/Endo		2.1 (Av)			Kim et al., 1992

*Av, Avicel; FP, filter paper; ct, cotton; BC, bacterial cellulose; AC, amorphous cellulose; a, acid-treated Avicel; b, acid-treated cotton; c, acid-treated BC; d, homogenized Avicel; e, acid-treated BC; f, SO_2-treated BC.

inant role in determining whether the DS is large or small. In support of this interpretation, we note that the above-listed ordering of cellulosic substrates with respect to DS is the same as the ordering with respect to degree of polymerization (see Cellulose, above) and is also consistent with modeling results (Okazaki and Moo-Young, 1978). Higher endo-exo synergy has been reported for substrates that have been treated to reduce CrI, for example, homogenized Avicel vs. Avicel (Henrissat et al., 1985) and ball-milled Solka Floc vs. Solka Floc (Fan et al., 1981). However, Hoshino et al. (1997) observed higher DS as CrI increased. As noted previously in our discussion of CrI, it is difficult to attribute observed changes to CrI based on work involving treatments that also change accessible surface area.

In addition to substrate properties, experimental conditions also affect the extent of synergy observed. It has been reported that endo-exo synergy increases with an increase in enzyme loading below saturation but decreases with oversaturated enzyme loading (Tuka et al., 1992; Watson et al., 2002; Woodward et al., 1988a,b; Woodward, 1991). In addition, such synergy is reported to be greater under conditions chosen to minimize inhibition by soluble hydrolysis products in some (Fierobe et al., 2001, 2002; Srisodsuk et al., 1998) but not all (Eriksson et al., 2002; Medve et al., 1998) studies.

Comparison of Cellulose and Starch Hydrolysis Rates

For the purpose of understanding factors limiting hydrolysis of cellulose by cellulases, it is informative to consider enzymatic hydrolysis of starch. As noted by several authors (Mandels, 1985; Klyosov, 1988), rates of starch hydrolysis can be about 100-fold faster than hydrolysis rates for cellulose under conditions anticipated for industrial processes and/or using crystalline model substrates.

In addition to any difference in the intrinsic reactivity of β-linked glucans as compared to α-linked glucans, three properties of cellulose and starch influence their hydrolysis rates: 1) the fraction of bonds accessible for insoluble substrates, 2) the availability of chain ends for insoluble substrates, and 3) the solubilities of hydrolysis products. The fraction of accessible glucose-glucose bonds, F_a, ranges from less than 0.002 to 0.12 for cellulose (based on Eq. [11] with $\alpha = 40$). This is 8–500-fold lower than for soluble starch ($F_a = 1$), soluble malto-oligosaccharides, or soluble cellulose derivatives like CMC ($F_a = 1$), and is 5–200-fold lower than insoluble starch ($F_a = \sim 0.2$; Fujii et al., 1981). The low fraction of accessible bonds is thought to limit rates of interior bond cleavage. Chain end availability (e.g., per unit mass) is lower for cellulose than for starch because of the high DP of cellulose as well as the incidence of branching in starch. For cellulose, the ratio of glucosyl units per chain end is equal to the DP and ranges from 300–2,000 (see Degree of Polymerization, above). For starch, which exhibits branches every 17 to 26 glucose units (Bertoldo and Antranikian, 2002; Bueleon et al., 1998), each branch gives rise to a new chain end and the ratio of glucosyl units to chain ends is thus ~22. Cellulose hydrolysis rates are thought to limited by the availability of chain ends for cellobiohydrolase (Schulein, 2000; Valjamae et al., 2001; Zhang and Wilson, 1997), and chain-end limitation has also been proposed for the action of glucoamylase on maltosaccharides (Mazur and Nakatani, 1993). Whereas cellodextrins are essentially insoluble at DP > 6–10 (Miller, 1963; Pereira et al., 1988; Zhang and Lynd, 2003), malto-oligosaccharides are soluble at DP up to 60 (John et al.,

1982). This difference can be attributed to the planar linear structure of cellodextrins as compared to the helical branched structure of starch. As a result of these differences in the solubility of hydrolysis product, many fewer bond cleavages need occur before soluble hydrolysis products are generated from starch as compared to cellulose, and a correspondingly larger fraction of bonds can be cleaved by enzymes acting in the liquid rather than solid phase for starch as compared to cellulose. In summary, most crystalline cellulosic substrates exhibit a $\geq$10-fold smaller fraction of accessible bonds, a $\geq$10-fold smaller frequency of chain ends, and a much smaller fraction of bonds cleaved in the soluble phase during enzymatic hydrolysis as compared to starch.

In contrast to the markedly different properties of cellulose and starch as substrates for enzymatic hydrolysis, available data suggests that the specific rate of solubilization exhibited by exo-acting saccharolytic enzymes appears rather similar on comparable substrates. Thus, the specific activity of CBH2 on cellohexaose at 27°C (k_{cat} = 14 s^{-1}; Harjunpaa et al., 1996; Koivula et al., 1998, 2002) is quite comparable to that for *Aspergillus awamori* glucoamylase on maltohexaose (G_6) at 45°C (49 s^{-1}; Fierobe et al., 1998), particularly when the different measurement temperatures are considered. The 3.5-fold higher value of k_{cat} observed for glucoamylase at 45°C relative to CBH at 27°C is very close to what would be expected based on the widely observed trend of doubled activity for every 10°C increase in temperature (Godfrey and West, 1996).

In light of these considerations, it appears to us that the large difference in the relative hydrolysis rates of cellulose and starch is due primarily to differences in substrate characteristics rather than to β-linked glucosidic bonds being intrinsically more difficult to hydrolyze than α-linked glucosidic bonds. Consistent with this hypothesis, we recently found that the initial hydrolysis rate of PASC is more than 100-fold higher than that of Avicel.

QUANTITATIVE MODELS

A classification scheme for quantitative models of enzymatic hydrolysis of cellulose is proposed in Table VI. We use the term "nonmechanistic models" for models based on data correlation without an explicit calculation of adsorbed cellulase concentration. While such models may be useful for correlating data, they are unlikely to be reliable under conditions different from those for which the correlation was developed, and they have limited utility for testing and developing understanding. Models featuring a defensible adsorption model but which are based on concentration as the only variable describing the state of the substrate and/or are based on a single cellulose hydrolyzing activity are termed "semimechanistic." In particular, models featuring concentration as the only substrate state variable are referred to as "semimechanistic with respect to substrate," whereas models with a single cellulose hydrolyzing activity are referred to as "semimechanistic with respect to enzyme." Most of the hydrolysis models proposed to date for design of industrial systems fall into the category of semimechanistic models. Semimechanistic models can be useful in the context of exercises motivated by including the minimal information necessary for descriptive purposes. However, semimechanistic models with respect to substrate cannot describe or lend insight into behaviors determined by substrate features other than concentration. Similarly, semimechanistic models with respect to enzyme cannot describe or lend insight into behaviors determined by multiple hydrolyzing activities. Models featuring an adsorption

Table VI. Classification scheme for models of enzymatic cellulose hydrolysis.

Model category	Defining feature & basis	Utility	Limitations
Nonmechanistic	Not based on a defensible adsorption model	• Data correlation	• Reliability under conditions different from those used to develop the correlation • Does not enhance understanding
Semimechanistic a. with respect to substrate b. with respect to enzyme	Based on a defensible adsorption model Concentration the only substrate state varible One solubilizing activity	• Data correlation • Reactor design • Identification of essential features	• Understanding at the level of substrate features and multiple enzyme activities
Functionally based	Includes an adsorption model, substrate state variables in addition to concentration, multiple solubilizing activities	• Testing and developing understanding at the level of substrate features and multiple enzyme activities • Identifying rate-limiting factors • Reactor design (potentially)	• Molecular design • State of model development and data availability currently limit application to design
Structurally based	Based on structural information pertaining to cellulase components	• Molecular design • Testing and developing understanding of structure/function relationships	• Challenging to develop meaningful kinetic models based on structural information

model, substrate state variables in addition to concentration, and multiple enzyme activities are denoted "functionally based models." Functionally based models are particularly useful for developing and testing understanding at the level of substrate features and multiple enzyme activities, including identification of rate-limiting factors and strategies to alleviate such factors. While functionally based models could conceivably be used for bioreactor design, application in this context to date is quite limited. A further limitation of functionally based models is that they provide little by way of guidance relative to design of cellulases at the molecular level. Finally, models based on structural features of cellulase components and their interaction with their substrates are termed "structurally based models." To a much greater extent than models in other categories, structurally based models are useful for molecular design as well as testing and developing understanding of the relationship between cellulase structure and function. Derivation of meaningful kinetic models based on structural models cannot be done at this time, and awaits major advances in the general field of inferring protein function from structure. We note that the vast majority of available kinetic models do not take into consideration changes in hydrolysis rate over the course of hydrolysis, and those that do represent such changes using empirically fitted parameters rather than mechanistically based parameters.

Nonmechanistic Models

Nonmechanistic models in the literature provide correlations for either fractional conversion or the rate of reaction as a function of various factors. Factors incorporated into models with conversion as the output include enzyme loading and substrate concentration (Sattler et al., 1989) as well as pretreated biomass properties (Chang and Holtzapple, 2000; Gharpuray et al., 1983; Koullas et al., 1992). Factors incorporated into models with rate as the output include hydrolysis time (Karrer et al., 1925; Miyamoto and Nisozawa, 1945), enzyme loading (Miyamoto and Nisozawa, 1945), and cellulose conversion (Ooshima et al., 1982). A few nonmechanistic models are considered here by way of example. Nonmechanistic models developed prior to the early 1980s are considered in detail in the reviews of Lee et al. (1980) and Ladisch et al. (1981).

An example of a model with conversion extent as an output is that proposed by Gharpuray et al. (1983). Those authors used regression to develop an exponential model to describe the influence of characteristics of pretreated wheat straw on the conversion of cellulose (X = 1 – [final cellulose concentration]/[initial cellulose concentration]) measured after 8 h:

$$X = 2.044(SSA)^{0.998}(100 - CrI)^{0.257}(L)^{-0.388} \qquad (14)$$

in which SSA is surface area measured by BET, and L is residual lignin content. Their results indicated that an increase in surface area and a decrease in the crystallinity and lignin content enhance hydrolysis, with specific surface area the most influential of the structural features, followed by lignin content. Chang and Holtzapple (2000) report a model to correlate maximum conversion in relation to residual lignin, crystallinity index, and acetyl content. Those authors found that lignin content and CrI have the greatest impact on final conversion, whereas acetyl content had a smaller effect. Koullas et al. (1992) also attempted to relate maximum conversion with CrI and degree of delignification, and obtained a similar conclusion about CrI and lignin effects.

Sattler et al. (1989) developed the following equation to describe final fractional conversion after enzymatic hydrolysis of pretreated poplar in relation to cellulase loading:

$$\frac{Y}{C_0} = \frac{Y_{\max}}{C_0}\frac{[E]}{K + [E]} \qquad (15)$$

where Y/C_0 gives the fraction of substrate hydrolyzed; [E] is given in FPU/g initially added substrate (FPU/g substrate); and Y_{max}/C_0 is the fraction of substrate which could maximally be hydrolyzed at an infinite enzyme loading, i.e., maximum digestibility. Later, Adney et al. (1994) applied this model to describe hydrolysis of cellulose such as Sigmacell 50 and various pretreated wood-powders.

An example of a model with reaction rate (V) as an output is that proposed by Holtzapple et al. (1984c):

$$V = \frac{(X_{\max} - X)^2}{t_{1/2}X_{\max}} \qquad (16)$$

where X_{max} is the maximum conversion, X is conversion, and $t_{1/2}$ is the time reaching $0.5*X_{max}$. Prompted by the observation that rate declines with increasing conversion, Ooshima et al. (1982) proposed the relationship:

$$\frac{dV}{dX} = -kV \qquad (17)$$

in which V is the hydrolysis rate and X is conversion.

Semimechanistic Models

Following the classification scheme presented in Table VI, semimechanistic models with respect to substrate and enzyme are based on an adsorption model but use a single variable to describe the state of the substrate and describe the action of cellulase in terms of a single solubilizing activity.

A representative model in this category is the HCH-1 model developed by Holtzapple et al. (1984a,b), which describes the initial rate of hydrolysis by:

$$V = \frac{k[S][E]i}{(\alpha + [S] + \varepsilon[E])} \qquad (18)$$

in which k is a rate constant; α, a lumped affinity constant; ε, the number of cellulose sites covered by an adsorbed

enzyme molecule, and i the fraction of total enzyme which is not inhibited by product. The quantity i represents inhibition by glucose (G) and cellobiose (G_2) according to:

$$i = 1/(1 + [G]/K_{I1} + [G_2]/K_{I2}) \quad (19)$$

in which K_{I1} and K_{I2} are inhibition constants. This model was used to simulate a total of 50 different hydrolysis conditions with a 10-fold range in enzyme concentration and a 30-fold range in cellulose concentration. Agreement with experimental data was rather good, and appeared better than some older models (Howell and Stuck, 1975; Huang, 1975).

Semimechanistic models with respect to enzyme only involve variables in addition to concentration to describe the state of the substrate. The widely observed trend of declining rate with increasing conversion appears to be a central motivation for many models in this category. Models describing an assumed change in shape and surface area over the course of hydrolysis have been proposed (Converse and Grethlein, 1987; Converse et al., 1988; Luo et al., 1997; Movagarnjad et al., 2000; Oh et al., 2001; Philippidis et al., 1992, 1993). However, none of these models have been tested against experimental data (e.g., for surface area) to our knowledge. Several "two-substrate" models have been proposed that partition cellulose into a less reactive highly crystalline fraction, and a more reactive amorphous fraction (Fan and Lee, 1983; Gonzalez et al., 1989; Gusakov et al., 1985a,b; Nidetzky and Steiner, 1993; Peitersen and Ross, 1979; Ryu and Lee, 1982; Scheiding et al., 1984). Although such models have met with some success in terms of correlating data, the trend of increasing CrI with increasing conversion—which would be expected if amorphous cellulose in fact reacts first—has not been conclusively confirmed by experimental data. An example of a two-substrate model is that of Wald et al. (1984), which includes shrinking cellulose spheres with an amorphous shell and a shrinking core as well as inhibition of cellulose hydrolysis by cellobiose and liquid-phase hydrolysis of cellobiose by β-glucosidase with inhibition by glucose. South et al. (1995) used a conversion-dependent rate constant to account for declining specific activity of cellulase–cellulose complexes over the course of hydrolysis:

$$k(X) = k\{(1 - X)^n + c\} \quad (20)$$

Those authors found an empirically determined value of n equal to 5.3, indicative of the very strong decline in rate with increasing conversion and in general agreement with direct specific activity measurements (Nutor and Converse, 1991; Ooshima et al., 1991).

Semimechanistic models with respect to substrate (only), involve concentration as the only substrate state variable and two or more solubilizing activities. Examples of models in this category in the literature to date are based on endoglucanase and exoglucanase. Nidetzky et al. (1994b) described saturation of the hydrolysis rate in terms of the concentration of a particular cellulase component, E_i, as follows:

$$V(E_i) = V_{\max} \frac{E_i}{K_{Ei} + E_i} \quad (21)$$

in which i is either 1 (for exoglucanase) or 2 (for endoglucanase), and K_{Ei} is a half-saturation constant. Based on this relationship, the following equation was proposed for the rate of hydrolysis in the presence of both exoglucanase and endoglucanase:

$$V(E_1, E_2) = V(E_1) + V(E_2) + V_{\text{syn,max}} \frac{E_1 E_2}{K_1 K_2 + K_1 E_2 + K_2 E_2 + E_1 E_2} \quad (22)$$

in which $V_{\text{syn,max}}$ is the maximum synergistic hydrolytic rate, and K_1,K_2 were the half-saturation constants corresponding to enzyme 1 and 2 in binary combination. The experimental results and model prediction clearly showed that the optimal ratio of exoglucanase to endoglucanase is a function of the total cellulase concentration, with higher enzyme concentrations needing less endoglucanase to achieve the maximum synergistic effect. The model of Beltrame et al. (1984) accounts for exoglucanase, endoglucanase, and β-glucosidase on textile cotton and cellulose pulp at various temperatures. The variable values can be adjusted depending on experimental conditions to fit experimental data well.

Half of the semimechanistic models listed in Table VII are based on the Michaelis-Menten model. The Michaelis-Menten model is only valid for the limiting case of substrate being in excess relative to enzyme (Lynd et al., 2002). In light of the small fraction of β-glucosidic bonds accessible to enzymatic attack, this condition is particularly limiting for cellulosic substrates. Excess substrate may be achieved in fundamentally oriented work, e.g., to characterize specific activity under laboratory conditions, but is seldom achieved in applications involving cellulose hydrolysis. Models based on a Langmuir adsorption model do not implicitly assume excess in either enzyme or substrate, and thus have a considerably broader range of potential application. The Langmuir model is subject to criticism on mechanistic grounds as noted in Cellulase Adsorption (above). Although adsorption models other than the Langmuir model have been proposed, few have been incorporated into kinetic models that lead to a prediction of hydrolysis rate.

Inhibition of the hydrolysis rate by soluble products has been incorporated into a substantial number of models. Competitive inhibition is the most common mechanism in the literature, but other uncompetitive and noncompetitive mechanisms have also been proposed (Table VII). Both the structural information (Davies et al., 1997; Teeri et al., 1998a,b) and a considerable body of experimental data indicate that individual cellulase enzymes are inhibited

Table VII. Mathematical models of enzymatic cellulose hydrolysis.

A. Nonmechanistic models	Independent variable	Dependent variables: Time	Enzyme	Substrate	>1 substrate variable
Miyamoto and Nisozawa, 1942	Conversion	Variable	Variable	Fixed	No
Holtzapple et al., 1984c- model 1	Conversion	Variable	Fixed	Fixed	No
Gharphuray et al., 1983	Conversion (8 hrs)	Fixed	Fixed	Fixed	Yes
Sattler et al., 1989	Conversion (max)	Fixed	Fixed	Variable	No
Adney et al., 1994	Conversion (max)	Fixed	Fixed	Variable	No
Koullas et al., 1992	Conversion (max)	Fixed	Fixed	Fixed	Yes
Chang and Holtzapple, 2000	Conversion (max)	Fixed	Fixed	Fixed	Yes
Karrer et al., 1925	Hydrolysis rate	Variable	Fixed	Fixed	No
Ooshima et al., 1982	Hydrolysis rate	Fixed	Fixed	Fixed	No
Holtzapple et al., 1984c- model 2	Hydrolysis rate	Fixed	Fixed	Fixed	No

B. Semimechanistic models	Substrate features*	Enzyme features	E-S interaction: Adsorption/MM	Inhibition
i. Semimechanistic models with respect to substrate and enzyme (1 substrate state variable, 1 solubilizing activity)				
Huang, 1975			M-M	competitive
Howell and Mangat, 1978			Langmuir	competitive
Beltrame et al., 1982			Langmuir	
Holtzapple et al., 1984a and b, 1990			Langmuir	non-competitive
Nakasaki et al., 1988			M-M	
Steiner et al., 1988			Langmuir	
Howell and Stuck, 1975		plus BG	M-M	
Dwivedi and Ghose, 1979		plus BG	M-M	competitive
Ghosh et al., 1982		plus BG	M-M	competitive
Asenjo, 1984		plus BG	M-M	competitive
Caminal et al., 1985		plus BG	M-M	competitive
Borchert and Buchholz, 1987		plus BG	Langmuir	competitive
Guaskov and Sinitsyn, 1992		plus BG	M-M	non-competitive
Moldes et al., 1999		plus BG	M-M	competitive
Belkacemi and Hamoudi, 2003		plus BG	M-M	
ii. Semimechanistic models with respect to enzyme only (>2 substrate state variables, 1 solubilizing activity)				
Converse and Grethlein, 1987	AS + [S]		M-M	
Converse et al., 1988	AS + [S]		Langmuir	competitive
Movagarnejad et al., 2000	AS + [S]		Langmuir	
South et al., 1995	[S] + X		Langmuir	
Ryu and Lee, 1982	A + C		Langmuir	
Philippidis et al., 1992 and 1993	AS + [S]	plus BG	Langmuir	non-competitive
Luo et al., 1997	AS + [S]	plus BG	Langmuir	non-competitive
Oh et al., 2001	AS + [S]	plus BG	Langmuir	non-competitive
Peitersen and Ross, 1979	A + C	plus BG	M-M	
Fan and Lee, 1983	A + C	plus BG	Langmuir	uncompetitive
Scheiding et al., 1984	A + C	plus BG	Langmuir	non-competitive
Guaskov et al., 1985a and b	A + C	plus BG	Langmuir	competitive
Gonzalez et al., 1989	A + C	plus BG	M-M	competitive
Nidetzky et al., 1993	A + C	plus BG	Langmuir	
Wald et al., 1984	AS + (A + C)	plus BG	Langmuir	competitive
Gan et al., 2003	AS + (A + C)	plus BG	Langmuir	competitive
iii. Semi mechanistic models with respect to substrate only (1 substrate state variable and 2 solubilizing activities)				
Beltrame et al., 1984	only [S]	Endo+Exo+BG	M-M	non-competitive
Nidetzky et al., 1994b	only [S]	Endo+Exo	Langmuir	

C. Functionally based models (≥2 substrate state variables, ≥2 solubilizing activities)				
Suga et al., 1975	[S], DP	Endo+Exo	M-M	
Okazaki and Moo-Young, 1978	[S], DP	Endo+Exo	M-M	non-competitive
Converse and Optekar, 1993	[S], AS	Endo+Exo	Dynamic adsorption	
Fenske et al., 1999	[S], AS, DP	Endo+Exo	Langmuir	

*AS = surface area; [S], substrate concentration; X = cellulose conversion; A, amorphous cellulose; C, crystalline cellulose.

competitively by cellobiose and glucose. However, it appears that mixtures of cellulase components can exhibit behavior consistent with mechanisms other than competitive inhibition under some conditions (Gusakov et al., 1985a,c; Gusakov and Sinitsyn, 1992; Holtzapple et al., 1984b, 1990). The mechanistic basis for this phenomenon is incompletely understood and has received little if any examination in the light of structural information gleaned during the 1990s.

Functionally Based Models

A few functionally based models, involving multiple substrate variables and solubilizing activities, have been proposed in the literature. Moo-Young and co-workers (Okazaki and Moo-Young, 1978; Suga et al., 1975) developed models based on the Michaelis-Menten model and assuming that all β-glucosidic bonds are accessible that incorporated two solubilizing activities (endoglucanase and exoglucanase) as well as β-glucosidase. In addition, these investigators used concentration and DP as substrate variables. The model predicts (Suga et al., 1975) that substrate DP changes as a function of time in the presence of endoglucanase, and that exoglucanase and endoglucanase synergism occurs for the degradation of longer chain cellulose molecules. Later, the model of Okazaki and Moo-Young (1978) predicted that the degree of endo-exo synergism is strongly impacted by DP. Converse and Optekar (1993) considered competitive adsorption of exoglucanase and endoglucanase for a limited number of sites, and predicted a lower DS under oversaturating conditions—that is, when cellulase is in substantial excess relative to the substrate. Fenske et al. (1999) modeled and observed a decline in hydrolysis rate with increasing cellulose concentration (Huang and Penner, 1991; Valjamae et al., 2001) in terms of decreased synergism when cellulase components with complementary activities bind at a distance from each other.

Table VIII. Important outstanding questions in the area of enzymatic hydrolysis of cellulosic biomass.

A. Fundamentally defined questions with applied implications
1) What is the role of substrate properties in determining the effectiveness of cellulase components?
2) What is the role of interactions among enzyme components in determining the effectiveness of cellulose hydrolysis?
3) What is the mechanistic basis for differences in the action of cellulase systems of different architectures (e.g., noncomplexed, complexed)? and how do such differences respond to substrate properties?
B. Application-defined questions with fundamental implications
1) What substrate features are of particular importance to change in order to effectively pretreat cellulosic biomass? How should these features be evaluated?
2) What principles and strategies guide the combination of cellulase components, potentially from different organisms, to achieve effective hydrolysis?
3) What principles and strategies guide the design of improved cellulase enzymes to more effectively achieve hydrolysis?

CONCLUDING PERSPECTIVES AND CHALLENGES

There is much about enzymatic hydrolysis that is not yet fully understood. Important outstanding questions include those presented in Table VIII. Consistent with the notion that advances in fundamental understanding and applied capability are potentially convergent rather than divergent activities (Stokes, 1998), questions in Table VIII are categorized in terms of fundamentally defined questions with applied implications and application-defined questions with fundamental implications.

All of these questions require aggregated understanding of the action of cellulase enzyme systems and their substrates—that is, understanding involving multiple cellulose-hydrolyzing activities and substrate features in addition to concentration. Quantitative modeling represents a powerful tool for advancing understanding and testing hypotheses involving aggregated understanding at the level implicit in Table VIII. Indeed, such advancement and testing can be carried out to only a very limited extent without quantitative models. As depicted in Figure 4, progress in this domain can be sought via two levels of inquiry: structural modeling and functional modeling.

Although there has been an explosion in documentation of structural features of cellulase enzyme components, as outlined in the Introduction, activity in the area of structural modeling of cellulase enzyme systems has been very limited to date. Progress in such structural modeling is constrained by the rate of advancement in the general field of predicting protein function from structure. There do not appear to be

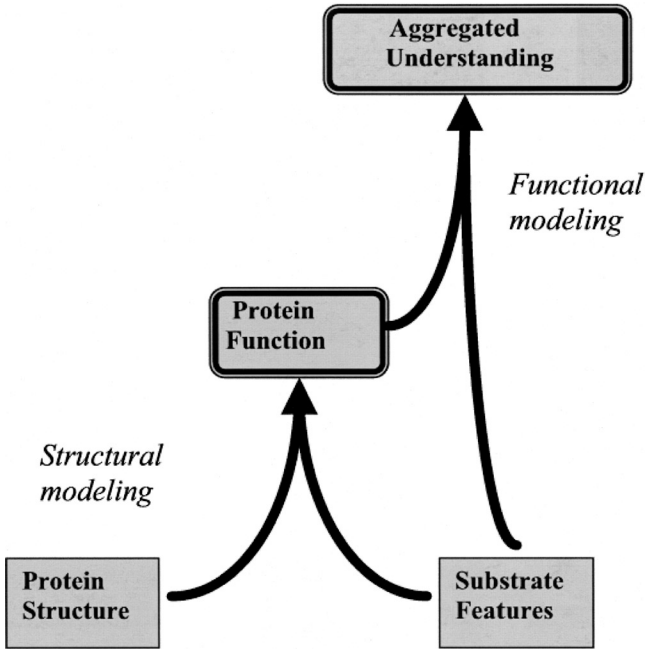

Figure 4. Levels of inquiry pertaining to enzymatic cellulose hydrolysis.

similar fundamental barriers to development of functional models. Moreover, functional models, informed as possible by information on structural features of cellulase enzymes, would appear at this time to be the most productive route by which to develop and test aggregated understanding of the enzymatic hydrolysis of cellulose. Yet, activity to date in the functional modeling area has also been very limited. We find it striking, for example, that of the four functional models listed in Table VII, only one was reported within the last 5 years and only two within the last 10 years.

We suggest that it is timely to revisit and reinvigorate functional modeling of enzymatic hydrolysis of cellulose. In addition to focusing the attention of investigators and research sponsors on this goal, advances in the area of functional modeling will benefit from development and application of new methodologies. Among such methodologies are improved procedures for determination of substrate properties including DP, crystallinity (without potential artifacts due to drying), and cellulase accessibility. Models based on application of these and other methodologies to relate changes in substrate properties to rates of primary and secondary hydrolysis (see On the Mechanism of Cellulose. . ., above) mediated by various cellulases and cellulase systems over the course of reaction appear to be a promising direction for future research.

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