Synergistic interactions in cellulose hydrolysis

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Cellulases acting on crystalline cellulose in synergistic mixtures have higher combined activities than the sum of their individual activities. The mechanisms by which different types of cellulases enhance each other's activities are complex and not completely understood, and the published data is often inconsistent. We discuss the role of the substrate and experimental factors in synergism experiments and consider the various modes of synergism that have been proposed. New oxidoreductase enzymes that participate in novel synergistic reactions are also discussed. Finally, we propose a general encompassing synergism model, based on the observations described in the review.

In 1950 Reese *et al.* proposed a mechanism for cellulose hydrolysis, which involved two general components, $C_1$and C_x, acting in sequence [1]. According to the model, the C_1 component first disrupted and swelled the crystalline cellulose, possibly releasing soluble oligosaccharides into solution. The C_x component, which was shown to have endoglucanase activity, was then able to effectively hydrolyze the previously inaccessible substrate along with the soluble oligosaccharides. Furthermore, the activity of the mixture was found to be higher than the activity of each component acting alone, indicating that the components were acting synergistically. In the following years, a number of groups began to identify and characterize the specific enzymes present in these components. It became clear that in addition to the endoglucanase activity demonstrated originally for the C_x component, exoglucanase activity was also present in the filtrates of cellulolytic organisms and that the combination of exo- and endo- activities resulted in the synergistic hydrolysis of cellulose [2–5]. In 1979, Wood and McCrae summarized the findings and proposed what became the classical endo–exo model of enzymatic cellulose hydrolysis [6]. According to this model, endocellulases attack the bulk cellulose, creating new chain ends that are susceptible to exocellulase digestion. Exocellulases in turn create more substrate for endocellulases by disrupting the crystalline substrate and/or by exposing previously inaccessible less ordered substrate that is susceptible to attack by endocellulases. With time it became evident that some exocellulases are also able to act synergistically with each other [7–10], giving rise to the suggestion that there are two classes of exocellulases that preferentially attack either the reducing or the nonreducing end of the cellulose chain. This was definitively demonstrated by Barr *et al.* in 1996 using ^{14}O- and ^{14}C-labeled cellooligosaccharides [11].

It seems likely that **synergism** occurs only when two cellulases attack different regions of the cellulose microfibril and that each enzyme creates new sites of attack for other enzymes in the mixture. However, this is an oversimplification of a complex process that is still not completely understood. There is no current evidence that synergism requires interactions between the synergizing cellulases, since cellulases from unrelated organisms, which would not have sites for binding to each other, often show cross synergism. However, it is not known if synergizing cellulases interact when they are bound to cellulose, and there could be interactions caused by two enzymes binding to a specific site on the cellulose. β-glucosidases, which cleave cellobiose and, to lesser extent, other oligosaccharides [12] to glucose,

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Key terms

Synergism: Cooperative enhanced activity of different types of cellulases acting together.

Crystallinity index: Quantitative parameter that reflects the relative amount of crystalline regions present in bulk cellulose.

Processivity: Number of successive cleavages by a cellulase before the cellulose chain is released.

Processive endocellulase: Endocellulase that attacks the cellulose chain at random locations and processively cleaves it similar to an exocellulase.

Carbohydrate binding module: Indpendent protein domain with affinity for polysaccharides, such as cellulose.

have been shown to enhance cellulase activity [10,13,14], presumably by relieving product inhibition. Although this is not certain, there is no evidence for other synergistic interactions between β-glucosidases and cellulases. Therefore, these enzymes are not discussed here.

Since the endo–exo model was proposed, it has been tested by numerous groups using enzymes from different cellulolytic bacteria and fungi. While most of the data provide support for this model, there is often evidence that the model is not sufficient to describe all aspects of the synergistic interactions between cellulases. It is evident that the methods used to carry out synergistic studies have an impact on the results and their interpretation. Therefore, published data on synergism are often inconsistent and at times contradictory, despite the fact that most experiments support the endo–exo model. This is likely due to the fact that synergism between cellulases involves multiple types of cooperative interactions, which we discuss in this review. In addition, the different factors that affect synergistic experimental data and their interpretation are discussed.

Substrate effect

The most common pure insoluble substrates used for studying cellulases, listed in the order of increasing crystallinity (which can vary slightly, depending on methods used to measure crystallinity), are phosphoric acid swollen cellulose (PASC), cotton, filter paper (FP), Avicel® and bacterial microcrystalline cellulose (BMCC). All these substrates are heterogeneous and have variable degrees of crystalline order. Due to their complexity it has not been possible to thoroughly characterize the substrates and the most widely used parameter for comparison has been the **crystallinity index** (CI), which reflects the average value of multiple regions in the substrate. While CI can be a useful parameter for comparing different substrates to each other, it is not a precise or an easily obtainable measurement. CI values depend on the method used to obtain them and variation can be over 30% for the same substrate [15–17]. In addition, there is evidence that cellulose may have more than two strictly amorphous and crystalline phases. Using nuclear magnetic resonance spectroscopy, Larsson *et al.* demonstrated that various model celluloses contain a significant amount of paracrystalline material, which has intermediate order between pure crystalline and amorphous regions [18]. According to their data, both wood and cotton have over 30% of paracrystalline material. The presence of paracrystalline material along with the specific method chosen to measure the CI may explain why the extent of substrate digestion often does not result in the expected change in CI [15]. Measured CI values can also partly depend on other substrate properties, such as particle size and surface to volume ratio, which themselves can affect enzyme behavior. Hence, using changes in CI values during enzymatic digestion to determine enzymatic properties and synergistic mechanisms should always be done with caution [15,17].

Synergistic properties of cellulases are commonly determined from their activities on different substrates when acting alone and together. It has been shown that in a synergistic mixture acting on highly ordered substrate the activities of all components are enhanced [13,19]. In a study by Irwin *et al.* this was demonstrated by the comparison of **processivity** of the individual enzymes and their mixtures on FP [13], while in research by Valjamae *et al.* this was done by the pretreatment of bacterial cellulose with one synergistic partner, either an endo- or an exo-cellulase, before the addition of the other enzyme [19]. However, in another instance, using FP, only pretreatment by endocellulases resulted in the enhancement of the subsequently added exocellulases, while the reverse was not true [10]. Activities and characteristics of the synergistic mixtures are affected by the substrate used in the studies, as demonstrated by Henrissat *et al.* [8]. The authors looked at synergistic combinations of *Trichoderma reesei* Cel7A (CBHI), Cel6A (CBHII), Cel7B (EGI), and Cel5A (EGII) on FP, Avicel, homogenized Avicel, BMCC and *Valonia* microcrystals. For the same total enzyme loads and substrate concentrations, the extent of synergism and optimum enzyme ratios varied substantially between the different substrates. *Valonia* cellulose, which is very highly ordered, showed no synergism at all. Lower synergism on higher ordered cellulose was also observed by Valjamae *et al.* [19] and Jeoh *et al.* [20]. In both cases, the authors compared the effect of removing amorphous fraction of bacterial cellulose with either acid treatment [19] or with digestion by an endocellulase [19,20]. They found that the degree of synergism was reduced for the more crystalline material. In some cases, pretreatment with acid or with an endocellulase may result in lower synergism between classical endo- and exo-cellulases due to the decrease of the average degree of polymerization (DP), as predicted by the functional model of Zhang and Lynd [21]. Lower DP, however, is unlikely to account for the lack of synergism on *Valonia* cellulose [8], or decreased synergism of mixtures that

include a **processive endocellulase** capable of degrading crystalline material [20], discussed below.

It is important to note that bulk substrate properties other than crystallinity have a direct impact on hydrolysis rates by individual cellulases and their mixtures. In particular, DP and the total surface area available to cellulases have been shown in various models to affect hydrolysis and, in some cases, the expected synergistic factors [21–23]. However, these properties specifically affect the kinetic aspects of cellulose digestion, as reviewed in [14], and are not likely to affect the mechanisms of synergistic interaction, which are the focus of this review. According to the most recent mechanistic model of enzymatic cellulose hydrolysis to date, the placement of the individual cellulose chain into the active site is the limiting step in the digestion of insoluble substrates [22]. This is also supported experimentally by high speed atomic force microscopy (AFM) data obtained by Igarashi *et al.* [24]. The ability of a given cellulase to access an individual cellulose chain should depend primarily on the mechanistic properties of that cellulase and on the local environment of the chain. Therefore, synergism between cellulases is likely to result, at least in part, from the enhanced access to the individual cellulose chains provided by the cooperative action of different types of cellulases. Given the demonstrated effects of the chosen substrate on synergism, it is likely that better characterization of the heterogeneities present within the commonly used substrates will lead to a more comprehensive understanding of synergistic mechanisms during the digestion of cellulose.

Experimental factors

In addition to the substrate, many other experimental parameters affect the extent of synergism. These include the specific enzymes in a mixture, the enzyme to substrate ratio, the molar ratios of the enzymes and the extent of substrate digestion.

The endo–exo model of synergism is based on the assumption that there are two distinct classes of cellulases and that some properties are common to all cellulases within either class. Thus, endocellulases are generally presumed to attack at random sites along a cellulose chain, to be nonprocessive and to attack only the amorphous regions of the substrates. Exocellulases, on the other hand, are presumed to attack only chain ends and are believed to be processive, generating cellobiose as the main product. A number of researchers, however, claim that some exocellulases may have the ability to initiate the attack in an endotype manner [6,8,25–27]. Regardless of whether some exocellulases are in fact able to initiate hydrolysis by an endotype attack, it is clear that different exocellulases have different abilities to digest crystalline substrate. For example, while *T. reesei* Cel7A is able to completely digest at least some forms of crystalline cellulose [28], *Thermobifida fusca* exocellulase Cel48A, which is the bacterial equivalent of Cel7A, cannot. In fact, Cel48A is unable to digest more than a few percent of any crystalline substrate [29, Kostylev M, Wilson D, Unpublished Data]. Yet, both Cel48A and Cel7A attack the reducing end of the cellulose chain [11], show strong synergism with nonreducing end-directed exocellulases, endocellulases, and processivie endocellulases, and no synergism with each other [13]. They also make up large fractions of the total secreted cellulases by their respective organisms [30,31]. It is not clear whether the mechanism by which Cel48A and Cel7A synergize with other enzymes is the same.

There are also a number of identified processive endocellulases [32–34]. The most studied of these is *T. fusca* Cel9A, and similar enzymes are present in many cellulolytic bacteria. The catalytic domain (CD) of Cel9A is tightly attached to a family 3c **carbohydrate binding module** (CBM) [34]. Based on structural analysis, it is believed that the role of CBM3c is to disrupt the crystalline cellulose surface and then feed the individual cellulose chain to the active site of the CD. This is likely the reason for the relatively high activity of Cel9A on crystalline substrate [13,34,35]. CBM3c is also responsible for the observed processivity of Cel9A, as it provides additional substrate binding sites in line with the substrate binding cleft of the CD [34–36]. Importantly, Cel9A is able to synergize with all other *T. fusca* cellulases, as well as with *T. reesei* Cel7A and Cel6A [13]. As a distinct type of a cellulase that combines the properties of classical exo- and endo-cellulases, Cel9A and other processive endocellulases may have a unique role in synergistic mixtures, which remains to be investigated thoroughly. Other processive endoglucanases, containing GH5 catalytic domains, have been identified in *Saccharophagus degradans*, which mainly produces GH5 cellulases and no known exocellulases [32]. However, early studies have not shown these enzymes to synergize with other cellulases.

Both, the enzyme to substrate ratio [37,38] and the molar ratios of the enzymes [8,20] in synergistic mixtures are variable parameters that can influence the rate and extent of substrate digestion and, thus, affect the interpretation of the obtained data. Substrate inhibition of synergistic mixtures has been reported by a number of groups [38–42]. In one study it was found that *T. reesei* crude cellulases show strong substrate inhibition but that Cel7A, which is the main cellulase in the mixture, was not inhibited [40]. This suggests that synergistic interactions between enzymes rather than individual enzyme activities were inhibited. It is proposed that at very low enzyme loads the rate-limiting

Key term

Cellulosome: A multienzyme complex containing catalytic domains of cellulases and other glycoside hydrolases.

step in synergism becomes the dissociation of cellulases bound unproductively to cellulose and their rebinding to a cleavage site from a synergistic partner. On the other hand, at higher enzyme to substrate ratios, diffusion of enzymes in solution to productive binding sites becomes rate limiting [38]. The change in apparent rate-limiting steps may impact the synergistic behavior of various mixtures of cellulases. Substrate inhibition may be a major cause of the high enzyme load requirement in the industrial production of cellulosic ethanol, as hydrolysis must be carried out at high substrate levels (i.e., 20–40%) to achieve an economical process. This is both in order to obtain a high volumetric productivity to reduce fixed costs and to obtain high sugar concentrations, which yield high initial ethanol concentrations and reduce subsequent concentration costs.

According to the endo–exo model proposed by Wood and McCrae the optimum ratio of endo- to exocellulases should be low, as their function in the model is to create new ends for the exocellulases that carry out most of the hydrolysis [6]. This is consistent with what is seen in nature, as exocellulases make up approximately 70% of the total cellulase produced by both *T. reesei* [31] and *T. fusca* [30]. In addition, the most abundant enzyme in the **cellulosome** of *Clostridium thermocellum* is an exocellulase [43]. Experimentally, high optimum exocellulase/endocellulase ratios have been demonstrated in many cases, but not always. Henrissat *et al.* determined optimum ratios of *T. reesei* exocellulase Cel6A and endocellulases Cel5A and Cel7B acting on various crystalline substrates to be approximately 95:5 [8]. On the other hand, the optimum ratio of *T. reesei* Cel7A and the same endocellulases acting on the same substrates was found to be approximately 1:1. According to the data reported by Jeoh *et al.* [20] the optimum fraction of *T. fusca* endocellulase Cel5A acting together with nonreducing end-directed exocellulase Cel6B on BMCC shifted from 0.1 at low extent of digestion to 0.3–0.5 after approximately 20% of the substrate was hydrolyzed, as predicted by the functional model of Zhang and Lynd [21]. However, when Cel5A was acting together with Cel9A, a processive endocellulase, its optimum ratio remained 0.1 up to 30% hydrolysis. The reasons for such observed differences and what they imply about the mechanisms of synergistic interactions remain unclear. Another surprising result is that for many enzyme pairs there is a broad plateau in the plot of activity versus percent of the added enzyme where the activity is independent of the composition. Given the very different activities of these enzymes this is difficult to explain.

Cellulose binding in synergistic mixtures has been studied by several different groups. Both synergism [20,44] and competition [20,45–47] in binding has been reported. Enhanced binding is usually explained as the creation of new binding sites by the enzymes for each other. Competition is presumed to occur because different enzymes can bind at the same sites on the substrate. This is certainly possible since cellulases usually bind via the CBM, whose binding properties do not correlate with the type of the CD to which it is attached. Another possible explanation, however, is that the apparent decrease in binding is the release of unproductively bound enzymes on the substrate surface by the synergistic interaction of the enzymes. We recently demonstrated that the majority of *T. fusca* Cel9A acting on BMCC is unproductively bound via its CBM2, with an unoccupied CD [48]. If some fraction of the unproductively bound enzyme is bound irreversibly due to obstacles on the surface of the substrate, a synergistic partner may help release the unproductive enzyme by hydrolyzing that obstacle. This would result in apparent binding competition as measured by the bound fraction of the total added enzyme.

Given the above observations, it is likely that the differences and inconsistencies in the reported data on synergism reflect different modes of synergism. In addition to correlating the derived mechanistic explanations of synergism with the specific substrates and enzymes used, it is important to consider the effect of the extent of substrate digestion on the synergistic interaction. A number of studies have, in fact, illustrated the importance of monitoring synergistic interactions in correlation with the extent of substrate digestion [19,20,46,49]. Using acid (HCl) hydrolysis or an endocellulase, Valjamae *et al.* removed amorphous fractions of bacterial cellulose to various extents before testing the behavior of *T. reesei* Cel7A and Cel7B, alone and together, on the pretreated substrate [19]. Short treatment of cellulose with HCl resulted in reduced activity of Cel7B, but strongly improved the activity of Cel7A. As the treatment time with acid increased, however, Cel7B activity was restored to above the untreated substrate levels, while that of Cel7A was reduced to just above the untreated substrate levels. Both, the net activity and the synergistic factors of the Cel7A/Cel7B mixture decreased with increasing acid treatment time. A similar pattern was observed when the substrate was pretreated with Cel7B instead of acid. All activities were monitored in time course experiments, revealing the different kinetic behavior of the cellulases on pretreated substrates. Based on this data and previously published Monte Carlo kinetic simulations [50], the authors make a convincing argument for a novel synergistic mechanism, which is complementary to the

classical endo–exo model. According to their model, as the exocellulase processively hydrolyzes individual cellulose chains from the bulk surface, the surface becomes eroded, with obstacles made up of solitary chains that limit the processivity and efficiency of the exocellulases. Endocellulases are able to efficiently remove the solitary chains left on the surface and thus increase the efficiency of the continued substrate hydrolysis by exocellulases. Our time course data using *T. fusca* Cel9A, a processive endocellulase, and Cel48A, a reducing end-directed exocellulase, suggest a similar interaction between these two enzymes [Kostylev M, Wilson D, Unpublished Data]. A recent study using high-speed atomic force microscopy to monitor activity of *T. reesei* Cel7A and Cel6A on highly crystalline cellulose from *Cladaphora* sp. provides additional support for this model [51]. Continuous monitoring of Cel7A activity alone revealed that the processive enzymes were regularly halted by obstacles on the substrate. In the presence of both Cel7A and Cel6A, however, a much larger fraction of the monitored enzymes was mobile. In another study, Josefsson *et al.* looked at real time interaction between several endo- and exo-cellulases and ultrathin cellulose films using a high resolution quartz crystal microbalance [49]. The physical action of cellulases was monitored from the initial binding to the point where the rate of hydrolysis was almost zero. An important finding in this study was that endocellulases not only create more ends for exocellulases to act on, but that the action of endocellulases appears to significantly swell the film, which would make the new ends much more accessible for the attack by exocellulases. Interestingly, the two endocellulases tested (Cel7B from *T. reesei* and Cel45A from *Humicola insolens*) show different abilities to swell the cellulose film. The possible reasons for these differences, however, were not discussed. Also, only Cel7B was tested in a synergistic mixture with two exocellulases. It would be interesting to determine the effect of individual endocellulase activity in this system on the characteristics of the synergistic mixtures.

Cellulosomes

Many anaerobic bacteria produce large multienzyme complexes called cellulosomes, which play a major role in cellulose degradation [43,52]. Most cellulosomal cellulases do not contain a CBM. Instead, the CBM is located on the scaffoldin protein to which the cellulases bind. Cellulosomal cellulases belong to the same families as the free cellulases produced by aerobic bacteria, but GH6 cellulases are not present in most anaerobic bacteria. It has been possible to produce small cellulosomes with a defined structure *in vitro* (designer cellulosomes) and they have been used to demonstrate synergistic activity for cellulosomal cellulases [53]. Mini designer cellulosomes were also used to show that most *T. fusca* free cellulases can function in cellulosomes, although it is interesting that the GH6 exocellulase was not able to function in the same setup [53]. The published literature on cellulosomal synergism remains limited, however, and it is unclear whether the physical proximity of the cellulosomal cellulases provides a distinct mode of synergistic interaction. It is possible that the relatively high specific activity of cellulosomes may result from a lack of substrate inhibition (discussed previously) as all of the different types of enzymes are close together on a cellulosome.

Synergism on biomass

When grown on cellulose, most cellulolytic bacteria produce hemicellulases, which act to remove hemicellulose bound to cellulose and thus allow the cellulases to access their substrate. In free enzyme systems, many of these hemicellulases contain cellulose binding CBMs presumably to direct the enzyme to cellulose-bound hemicellulose. A number of commonly used biomass pretreatment methods do not remove completely the hemicellulose present in the original biomass [54,55] and it has been shown that the residual hemicellulose can have a negative impact on cellulose hydrolysis by cellulases [56,57]. As a result, it appears that hemicellulases are able to act synergistically with cellulases on pretreated biomass, as has been demonstrated in recent studies [58,59]. Similarly, cellulosomes from cellulose grown anaerobic bacteria, contain hemicellulases that also act synergistically on biomass substrates [60,61]. Additional studies of synergistic interactions between cellulases and other enzymes that act on biomass are likely to have important implications for the industrial production of biofuels, as efficient utilization of such enzymes may significantly reduce costs associated with both biomass pretreatment and enzymatic cellulose hydrolysis.

The C_1 component

After the proposal of the C_1 component by Reese *et al.* a lot of effort was invested to identify the function and the enzymes involved in this component [1]. By the time Wood and McCrae proposed the endo–exo model of synergism, this was still an unresolved issue [6]. Reese and others believed that C_1 disrupts the crystalline surface in order to make the substrate more accessible to endo- and exo-cellulases [62]. Wood and McCrae, on the other hand, argued that the C_1 component is composed of exocellulases and that the initial attack on the substrate is carried out by the endocellulases in the C_x component. In their endo–exo model, the C_x component carries out the function proposed by Reese for the C_1

component. After it was discovered that cellulases usually have a CBM attached to a CD by an unstructured, flexible linker [63], some researchers proposed that the CBM may carry out the role of disrupting the substrate, making it more accessible for the CD [64–66]. While a number of studies have demonstrated some cellulose-disrupting ability by various CBMs from native cellulases, their ability to synergize with CDs appears to be weak at best [10,66].

In recent years, however, a number of disruptive proteins with strong synergistic properties have been discovered. These include a chitin-binding protein CBP21 produced by the chitinolytic bacterium *Serratia marcescens* [67,68], two *T. fusca* proteins E7 and E8 [69], CelS2 protein from *Streptomyces coelicolor* [70], and family 61 glycoside hydrolases (GH61) produced by plant degrading fungi [71,72]. CBP21, E7, E8 and CelS2 are classified in family CBM33 (E8 and CelS2 also contain a family 2 CBM attached to the CBM33). CBP21 has been shown to enhance the activity of chitinases on chitin [67], especially in the presence of a reducing agent [68]. In a similar manner, E7, E8, CelS2 and some GH61 proteins, enhance the activity of cellulases acting on crystalline cellulose in the presence of various small molecules [69–71]. In two studies of GH61 from *Thermoascus aurantiacus* [73] and *Neurospora crassa* [72] it was shown that the ability of GH61 to enhance activity of cellulases can also be stimulated by the presence of cellobiose dehydrogenase instead of a small molecule. All of the disruptive proteins produce oligosaccharides of various degrees of polymerization and seem to require oxygen for activity. Oxidized products have been detected for CBP21 [68], CelS2 [70] and GH61 proteins [73,74]. The x-ray structures for CBP21 [75] and GH61 proteins [71,74,76] reveal that there are none of the catalytic acid/base residues that are required for cellulase activity. The highly conserved residues in these proteins are found around a metal binding site, which may play a structural and/or a functional role. A number of divalent metal ions have been suggested, but the most thorough study in this regard to date, using GH61 proteins from *T. aurantiacus,* provides strong evidence that it is copper [74]. The authors suggest that GH61 family enzymes should be classified as copper oxidoreductases rather than glycoside hydrolases. While the detailed mechanism of these disruptive proteins remains to be determined, it is generally agreed that they disrupt crystalline cellulose, most likely via oxidoreductive cleavage of the cellulose chains in the bulk crystalline substrate. These proteins thus make cellulose more accessible to attack by cellulases and appear to carry out the function of the C_1 component predicted by Reese *et al.* in 1950 [1].

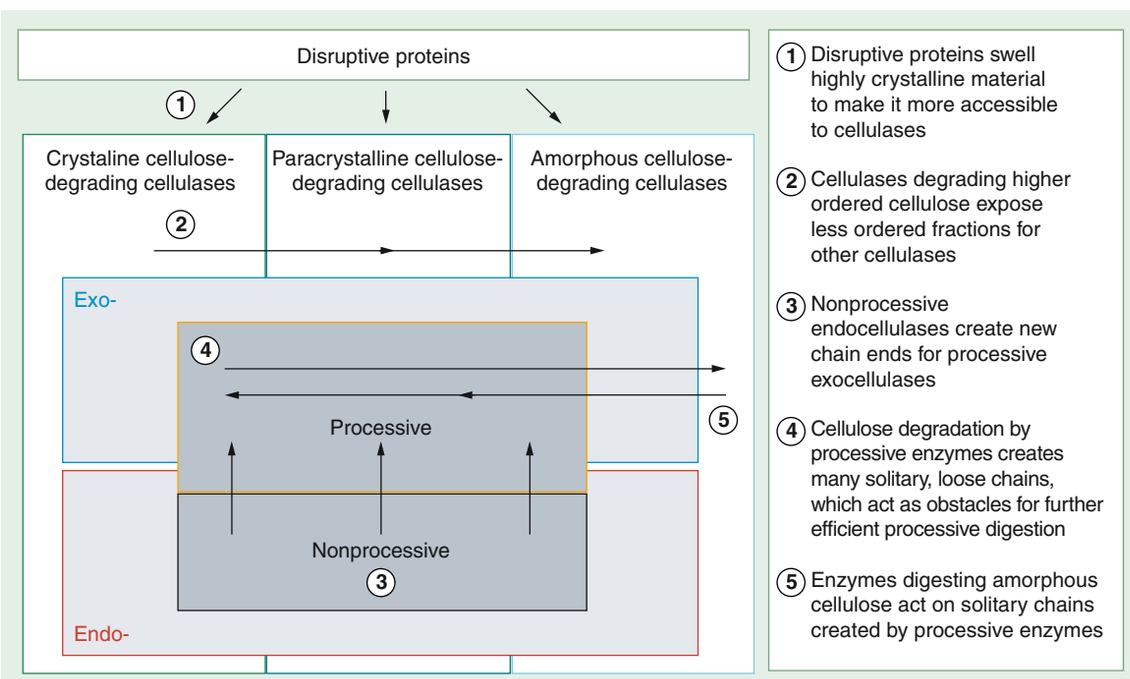

Figure 1. Tentative model of synergistic interactions in enzymatic cellulose hydrolysis. Each box represents a distinct cellulase property and overlapping boxes indicate that those properties can be shared by a single enzyme. Properties believed to be nondiscrete in nature are represented by touching boxes. Synergistic interactions are represented by arrows and brief descriptions are provided next to corresponding numbers.

Conclusion

Based on the observations discussed above, we propose a model of synergism that incorporates different modes of interactions between cellulose-degrading enzymes (Figure 1). This model is based on the idea that cellulose contains a spectrum of crystalline order ranging between strictly amorphous and crystalline material. In this context, it is likely that different enzymes preferentially bind to, and possibly act on, the different fractions of the substrate. This may explain why many cellulolytic organisms produce enzymes with apparently redundant activities.

It is generally accepted that the highly ordered fractions of cellulose are responsible for its recalcitrance to complete and efficient digestion, as most cellulases appear to preferentially attack the lesser ordered regions. Given the recent data obtained for proteins from CBM33 and GH61 families, it is likely that these enzymes disrupt the more recalcitrant portions of cellulose and make the substrate more accessible to digestion by cellulases. Efficient digestion by cellulases requires simultaneous action on all parts of the substrate including more- and less-ordered fractions. An optimum enzymatic mix attacks reducing and nonreducing ends of the chains, as well as regions within the chains. As endocellulases attack regions within the chains, they create new chain ends that are accessible to exocellulases. As processive cellulases (exocellulases and processive endocellulases) digest individual chains, they are likely to create an eroded surface covered by more exposed, and thus less ordered, chains. The exposed chains may act as obstacles to processive cellulases, but are susceptible to attack by cellulases that are unable to digest more ordered material. Optimum ratios of the different enzymes are likely to depend on the properties of the substrate, especially in the initial stages of digestion.

Future perspective

It has been proposed that physical interactions between cellulases play a role in the synergistic digestion of cellulose [9], but no definitive experimental evidence for this is available. Newly developed atomic force [24,51,77] and fluorescence [78,79] microscopy techniques are likely to reveal whether such interactions, or at least the physical proximity of the synergistic partners on the cellulose surface, are necessary for some forms of synergism. In addition, by monitoring the interactions between cellulases and the substrate, high-resolution microscopy studies may improve our understanding of substrate recalcitrance to digestion and the way synergistic mixtures are able to overcome recalcitrance. The emerging research on disruptive proteins from the CBM33 and GH61 families is particularly exciting, as these proteins appear to have a novel mechanism to attack cellulose and synergize with classical cellulases. Determination of the detailed mechanisms by which these enzymes function will contribute significantly to our understanding of cellulose digestion in nature and to the application of cellulose degrading enzymes in the processing of cellulosic feedstock for biomaterials and bioenergy.

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Executive summary

Substrate effect

- Interpretation of synergism data should correlate with the known substrate properties.
- More effort is needed to improve common cellulose substrate characterization, specifically with respect to the heterogeneities present in model substrates.
- It is important to distinguish kinetic and mechanistic effects in the interpretation of synergism data.

Experimental factors

- Different conclusions from synergism experiments likely reflect different modes of synergism. Individual enzyme properties, molar ratios of the different enzymes and enzyme to substrate ratios are likely to affect the extent of synergism.
- It is important to monitor the evolution of synergistic interactions with the extent of substrate digestion, as various modes of synergism may become more or less prominent at different times during digestion.

Synergism on biomass

- Demonstrated synergism between hemicellulases and cellulases on pretreated biomass suggests that there is a need for additional research into synergistic interactions between cellulases and other enzymes that act on biomass in nature.

The C_1 component

- Recently discovered disruptive proteins from the CBM33 and GH61 families strongly enhance cellulose degradation by cellulases and thus appear to have an important role in overcoming substrate recalcitrance.

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