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MINI-REVIEW

Processive and nonprocessive cellulases for biofuel production—lessons from bacterial genomes and structural analysis

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Abstract Cellulases are key enzymes used in many processes for producing liquid fuels from biomass. Currently there many efforts to reduce the cost of cellulases using both structural approaches to improve the properties of individual cellulases and genomic approaches to identify new cellulases as well as other proteins that increase the activity of cellulases in degrading pretreated biomass materials. Fungal GH-61 proteins are important new enzymes that increase the activity of current commercial cellulases leading to lower total protein loading and thus lower cost. Recent work has greatly increased our knowledge of these novel enzymes that appear to be oxidoreductases that target crystalline cellulose and increase its accessibility to cellulases. They appear to carry out the C1 activity originally proposed by Dr Reese. Cellobiose dehydrogenase appears to interact with GH-61 proteins in this function, providing a role for this puzzling enzyme. Cellulase research is making considerable progress and appears to be poised for even greater advances.

Keywords Synergism · Processivity · Molecular modeling · Swollenin · Oxidoreductase · Cellobiose dehydrogenase

Introduction

Cellulases are important enzymes in many proposed processes for producing fuels and chemicals from plant biomass (Adsul et al. 2011; Wilson 2009). Biochemical research on cellulases started in the 1940s and has been accelerating recently with the growing interest in industrial biotechnology. The determination of the structures of cellulases and carbohydrate-binding modules has been a major tool in understanding cellulase mechanisms and in engineering more active cellulases. Another major advance in our understanding of cellulose degradation came from the determination of genome sequences of cellulolytic microorganisms coupled with proteomic and transcriptomic studies of cellulose grown cells, which have identified new proteins that function in cellulose degradation (Zhou et al. 2010; Martinez et al. 2008). Most known cellulolytic organisms produce multiple cellulases that act synergistically on native cellulose (Wilson 2008a, b) as well as producing some other proteins that enhance cellulose hydrolysis (Wang et al. 2011a, b).

Structural studies

In 1990, the first structure of a cellulase, exocellulase Cel6A from *Trichoderma reesei*, was determined, and it showed that its active site was in a tunnel and that there were multiple subsites for binding glucose residues in the active site (Rouvinen et al. 1990). Many of the subsites contain aromatic residues, usually tryptophan, that stack against the glucose residue, as has been seen in many carbohydrate-binding proteins (Tam and Saier 1993). Later the structure of an endocellulase, *Thermobifida fusca* Cel6A, was determined and its active site was in an open cleft due to one of the tunnel closing loops present in *T. reesei* Cel6A being shorter and the other loop being turned away (Spezio et al. 1993) Subsequently, structures were determined for the other two families of exocellulases GH-7 and GH-48 and their active sites were also inside tunnels

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even though the GH-48 tunnel did not cover the +1 and +2 subsites (Divne et al. 1998; Parsiegla et al. 1998). These structures explain the clear differences between the mechanisms of exocellulases, which processively cleave cellobiose from one end of a cellulose chain and endocellulases, which randomly cleave cellulose molecules. All endocellulases have open active sites as is expected since they are able to bind at any point along a cellulose molecule (Sandgren et al. 2005). Structural studies played a key role in explaining the mechanism of a novel cellulase, *T. fusca* Cel9A, which displayed properties that were intermediate between exo- and endocellulases. The structure showed that its weak binding family 3c CBM was aligned with the active site cleft in the catalytic domain, suggesting that this enzyme could bind initially at any point along a cellulose chain but that after cleavage, the nonreducing end fragment that was bound to the CBM could move into the empty −4 to −1 subsites allowing processive cleavage of cellotetraose from the nonreducing end (Sakon et al. 1997). This mechanism was confirmed when it was shown that a modified enzyme lacking this CBM was no longer processive (Irwin et al. 1998). This type of cellulase is present in most cellulolytic bacteria and appears to replace the nonreducing end attacking GH-6 exocellulase in cellulosomes produced by many anaerobic bacteria (Gilad et al. 2003). This may be due to cellotetraose providing more ATP to the bacterium than cellobiose as it takes one ATP to transport an oligosaccharide into a bacterium, and if a phosphorylase is used to cleave the oligosaccharide, cellotetraose gives two more ATPs than cellobiose (Lynd et al. 2002). In fact, most anaerobic cellulolytic bacteria do not produce family 6 exocellulases. Recently, a new class of processive endocellulases, which is in family GH-5, was identified from the marine bacterium *Saccharophagus degradans* (Taylor et al. 2006). This organism does not contain any known processive cellulase genes and has 13 endocellulase genes of which 9 are in family GH-5. These processive cellulases do not require an additional domain for processivity, which suggests that their processivity may result from unusual subsite affinities but this has not been proven and no structures have been determined for these enzymes (Watson et al. 2009).

A novel cellulase fold was determined for a *Clostridum thermocellulum* cellulosomal enzyme. This enzyme had low activity by itself but acted synergistically with other cellulases (Brás et al. 2011). It is unusual in that there is no evidence for a catalytic base although it has a catalytic acid. There are only a few homologues of this enzyme so that it does not appear to be used by most cellulolytic microorganisms. There is an excellent recent review of structural mechanistic studies of cellulases, and it includes a detailed pathway for the bond cleavage step (Vocadlo and Davies 2008). However it is important to realize that the rate-limiting step for crystalline cellulose hydrolysis is not bond cleavage but the binding of a cellulose molecule into the active site of the catalytic domain of a cellulase, and we are just beginning to understand the residues that participate in this step (Koivula et al. 1998). A complete understanding of this step is needed to carry out protein engineering to try to produce cellulases with higher activity on specific pretreated biomass materials, which could reduce the cost of producing cellulosic biofuels. The detailed pathway for bond cleavage by exocellulase Cel7A has been predicted by molecular modeling (Li et al. 2010) which is a new approach to cellulase structure-based studies, whose use is increasing and this approach is discussed in a recent review (Beckham et al. 2011).

Genomic studies

Determination of the DNA sequences of two cellulolytic bacteria, *Cytophaga hutchinsonii* and *Fibrobacter succinogenes*, shows that they must use novel mechanisms for degrading cellulose as neither organism has any processive cellulase genes, and most of their cellulases do not contain CBMs unlike most well-studied cellulolytic microorganisms (Wilson 2008a, b). A number of proteins have been identified that increase the activity of synergistic cellulase mixtures on native cellulose. Swollenin is an expansin-like protein that was first found in *T. reesei*, but it has been found in a number of cellulolytic fungi (Saloheimo et al. 2002: Chen et al. 2010). It appears to disrupt hydrogen bonds in native cellulose, making it easier for the cellulases to bind to individual chains. Some bacteria that colonize plants also produce bacterial expansins that act like swollenin (Kerff et al. 2008). GH-61 proteins, which were first classified as weak endocellulases, have been shown to significantly stimulate fungal cellulase mixtures and are currently included in some commercial cellulases (Harris et al. 2010). Structures have been determined for two GH-61proteins, and they contain a metal-binding site that is present on a flat surface that lacks most of the residues seen in cellulase active sites (Karkehabadi et al. 2008: Quinlan et al. 2011). CBM33 proteins are produced by many aerobic bacteria, and they were originally thought to be chitin-binding proteins that stimulated chitinase hydrolysis of crystalline chitin (Vaaje-Kolstad et al. 2005a). It was recently shown that CP21 from *Serratia marcescens*, which binds chitin but not cellulose, stimulates chitinase activity to a much greater extent when ascorbate or certain other small molecule reductants are present (Vaaje-Kolstad et al. 2010). Furthermore, CP-21 was shown to carry out oxidative cleavage of crystalline chitin but not soluble chitosan, if and only if a reductant was present. The structure of CP-21 has been determined, and it has a

divalent metal-binding site on its flat surface (Vaaje-Kolstad et al. 2005b). The metal-binding site in CP21 is structurally very similar to the metal-binding site in a GH-61 protein from *T. reesei*, even though there is no overall similarity in their structures or sequences and it also lacks potential cellulase catalytic residues (Vaaje-Kolstad et al. 2005b). The activities of both GH-61 and CBM33 proteins are strongly inhibited by EDTA. *T. fusca*, an aerobic cellulolytic soil bacterium, produces large amounts two CBM33 proteins E7 and E8, when it is grown on cellulose (Yang et al. 2007). E7 contains only a family 33 CBM domain while E8 contains an additional family 2 CBM. These proteins were shown to bind to both cellulose and chitin, and they gave a small stimulation of cellulose hydrolysis by several cellulases as well as stimulating chitin hydrolysis by a chitinase (Moser et al. 2008). A recent paper showed that *Streptomyces coelicolor* produces only an E8-like protein that can cleave Avicel or filter paper in the presence of a reducing agent and a metal ion, producing cellulooligosaccharides from cellotriose to cellohexose along with a similar set of oxidized oligosaccharides (Forsberg et al. 2011). These products are unlike those produced by any other cellulase, since most cellulases rapidly cleave oligosaccharides larger than cellotriose so they do not appear in cellulose digests. This protein acts synergistically with cellulases in the presence of ascorbate on both amorphous and crystalline cellulose. From Blast searches it appears that there are at least two subfamilies of CBM33 proteins: one including those produced by cellulolytic bacteria that stimulate crystalline cellulose hydrolysis and the other produced by chitinolytic bacteria that stimulate chitin hydrolysis. It is not clear how these proteins cleave cellulose to produce unoxidized oligosaccharides since there is no evidence for a normal cellulase active site.

Recent studies of GH-61 proteins have shown that they also require a small molecule for their activity, and ascorbate, gallate, glutathione, and dehydroascorbate have all worked. In addition copper appears to be the metal ion that binds most tightly to the family 61 metal-binding site and it is required for activity. An interesting finding is that cellobiose dehydrogenase, a flavo-protein secreted along with cellulases by many cellulolytic fungi, acts synergistically with a GH-61 protein in cellulose hydrolysis producing oxidized oligosaccharides (Langston et al. 2011; Phillips et al. 2011). This reaction does not seem to require a small molecule, and this combination was able to significantly stimulate cellulose hydrolysis by any tested cellulase as well as by β-glucosidase. The stimulation of β-glucosidase is very surprising as by itself this enzyme has very low activity on cellulose, while a mixture of the three proteins was able to hydrolyze 30% of the cellulose. This combination of proteins did not stimulate the activity of a xylanase. The role of cellobiose dehydrogenase in cellulose hydrolysis has been puzzling, but the finding of its synergism with family 61 proteins now provides an important role for it in fungal cellulose degradation. When the expressed cellobiose dehydrogenase gene, which contains a CBM, was deleted from *Neurospora crassa*, the activity of the secreted cellulase was about 45% lower than wild type and activity was restored completely by addition of pure cellobiose dehydrogenase (Phillips et al. 2011). The activities of GH-61 and CBM33 proteins are similar to those proposed by Dr. Reese in 1950 for the C1 component of fungal cellulase that was suggested to be required for crystalline cellulose hydrolysis (Gilligin and Reese 1954).

An attempt to predict the pH optimum of cellulases from their sequence using computational methods gave about 75% accuracy, which is a promising start but still leaves room for improvement (Yan 2011). A crystallographic study of a family 5 cellulase along with several mutant cellulases complexed with cellotetraose provided evidence for the mechanism of bond cleavage by this enzyme (Kim et al. 2011a). An impressive cryo-EM structural study at 35 A resolution has provided some information on a structure of a minicellulosome containing three cohesins with an MW of about 200,000. In this structure the three bound enzymes alternated in their orientation relative to the scaffoldin. Since there are long flexible linkers that join each enzyme to a cohesin domain, there are many potential structures possible for these domains (García-Alvarez et al. 2011).

Improving cellulases

There are a number of approaches being used to identify or produce cellulases with higher activity on native cellulose to reduce the cost of producing biofuels: these include screening genomes and metagenomes for new cellulases, protein engineering, and directed evolution. A set of 55 endocellulases genes were screened for high-level expression in *Aspergillus niger*, and three of those that were expressed at the highest level were assayed for activity and compared to an active *T. reesei* endocellulase. Although some of these enzymes had higher activity on CMC and amorphous cellulose, none of them had higher activity in a synergistic mixture with an exocellulase on crystalline cellulose, which is the best model substrate for biomass activity although when possible it is better to screen for improved activity on the actual biomass substrate that will be used in the process (Tambor et al. 2011). A study of several β-glucosidases for their ability to give synergy with *T. reesei* crude cellulase showed that two of the tested enzymes were able to nearly double the hydrolysis of filter paper compared to *T. reesei* crude cellulase (Ng et al. 2011). A novel gene with weak cellulase activity was isolated from a bacterial metagenomic library prepared from abolone gut

contents, but the enzyme was not extensively characterized (Kim et al. 2011b). The thermostability of a family 7 exocellulase was improved by identifying stabilization domains by a computational method (Heinzelman et al. 2010a). The most stable constructed chimera was about 5°C more stable than the most stable WT enzyme. In a later study, it was found that changing a single cysteine to serine was responsible for most of the increase in thermostability (Heinzelman et al. 2010b).

A proteomic and transcriptional analysis of the anaerobic rumen fungus, *Neocallimastix patriciarum* W5, found that it produced a large number of different cellulases including family 48 and family 9 enzymes which are not found in any other fungi as well as several family 6 cellulases. In addition many of these enzymes contained family 10 CBM domains, which also have not been found in other fungi (Wang et al. 2011a, b). These results may result from horizontal gene transfer, as family 48 and family 9 cellulases are common in rumen bacteria; however, none of the most similar proteins in protein Blast search are from rumen bacteria. It is also unusual to find GH-6 cellulases in anaerobic microorganisms, as most anaerobic bacteria do not have cellulases from this family. This is thought to be due to the fact that exocellulases produce mainly cellobiose and there is a GH-9 processive endoglucanase that produces mainly cellotetraose, which appears to replace the GH-6 exocellulase in anaerobic bacteria, thus increasing the average size of the oligosaccharides produced during growth on cellulose, which would produce more ATP (Lynd et al. 2002). However, even though *N. patriciarum* contains two GH-9 cellulase genes, neither contains a family 3 CBM domain which is required for processivity, so that the exocellulase may be needed in this organism for efficient cellulose hydrolysis.

A surprising finding is that binding several CBM4 modules to a nanoparticle along with an endocellulase can increase endocellulase activity on CMC, amorphous cellulose, and Avicel (Kim and Ishikawa 2011). The increase in amorphous cellulose activity was quite high. Normally CBMs only increase activity on crystalline substrates and they rarely increase activity on CMC, so that this result seems odd. It would be interesting to see what would happen if an exocellulase and an endocellulase were present on such a CBM containing nanoparticle. It was reported that cellulase adsorbed to silica nanoparticles gave a higher yield of ethanol in simultaneous saccharification and fermentation due to increased cellulose hydrolysis (Lupoi and Smith 2011). However the reason for the increase in cellulose hydrolysis was not determined.

Single-molecule approaches and processivity

A very interesting study used scanning atomic force microscopy to visualize individual molecules of the exocellulase Cel7A from *T. reesei* on cellulose crystals (Igarashi et al. 2009). WT enzyme molecules moved at a steady rate along the crystal as expected for a processive cellulase. An inactive mutant enzyme showed no movement but remained bound to the cellulose, showing that activity was needed for movement as had been suggested. A mutant enzyme missing a residue required for introducing a cellulase chain into the active site did not move along the crystal, but the catalytic domain (CD) did move around showing that the CBM was able to stably bind to cellulose without the CD being bound. The catalytic domain by itself only rarely bound to the crystal, but when it did, it was able to move processively along the crystal at a slightly higher rate than the intact enzyme, showing that the CBM facilitated CD binding but was not required for processivity. Another approach to visualize individual cellulase molecules is to fluorescently label them and use confocal microscopy to visualize them on cellulose (Dagel et al. 2011; Moran-Mirabal et al. 2011). Unfortunately at this time, the resolution of this technique is not high enough to detect processive movement but continued improvements should make this a valuable approach.

Detailed studies of exocellulase processivity also have been reported using several different indirect methods based either on the assumption that the initial cleavage by an exocellulase produces equal amounts of cellobiose and cellotriose, while all other cleavages produce only cellobiose, or by modifying the reducing end and then measuring how many new ends were produced versus the amount of soluble sugars that were produced under single-burst conditions (Vuong and Wilson 2009; Kurasin and Väljamäe 2011). The processivity of the exocellulases studied that *T. fusca* Cel6B and *T. reesei* Cel7A were fairly low relative to that calculated from the off rate of the enzyme and the extensive interactions seen in the active site tunnel between the enzyme and bound substrate as well as that measured by scanning AFM. One possibility is that movement of an enzyme along a cellulose molecule is often blocked by another cellulose molecule or even by another enzyme molecule on an adjacent cellulose chain (Igarashi et al. 2011). However, it is also possible that errors in the assumptions of these methods underestimate processivity.

Future directions

Structural approaches have and will continue to play an important role in understanding the mechanism of enzymatic cellulose hydrolysis. This includes site-directed mutagenesis of key residues in cellulases. Molecular modeling should continue to gain in importance as computer speed increases and better algorithms are developed. In addition, the use of genomics and metagenomics

will continue to identify novel proteins that participate in this process. Finally the development of improved imaging techniques and higher resolution single-molecule methods has the potential to significantly improve our understanding of this complex process.

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