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CELLULOSE DEGRADATION BY OXIDATIVE ENZYMES

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Abstract: Enzymatic degradation of plant biomass has attracted intensive research interest for the production of economically viable biofuels. Here we present an overview of the recent findings on biocatalysts implicated in the oxidative cleavage of cellulose, including polysaccharide monooxygenases (PMOs or LPMOs which stands for lytic PMOs), cellobiose dehydrogenases (CDHs) and members of carbohydrate-binding module family 33 (CBM33). PMOs, a novel class of enzymes previously termed GH61s, boost the efficiency of common cellulases resulting in increased hydrolysis yields while lowering the protein loading needed. They act on the crystalline part of cellulose by generating oxidized and non-oxidized chain ends. An external electron donor is required for boosting the activity of PMOs. We discuss recent findings concerning their mechanism of action and identify issues and questions to be addressed in the future.

MINI REVIEW ARTICLE

1. Introduction

The increasing global demand for energy, coupled with diminishing reserves and global warming have made imperative the gradual replacement of fossil fuels by alternative resources such as renewable energies [1]. Among these, biomass is one of the most promising sources for the production of transportation fuels. Biomass-derived ethanol is currently the most widely used biofuel in the United States and is mainly produced from starch or sugar [2]. However, since the latter are also food sources, the production of second-generation bioethanol, mainly derived from lignocellulosic feedstocks, has been a goal for government and private industry for the last three decades [3]. The conversion of lignocellulosics to ethanol involves two processes: degradation of biomass to fermentable sugars, usually catalyzed by cellulolytic enzymes, and fermentation of the sugars to ethanol by yeasts or bacteria. Depending on the composition of the starting material, various pretreatment techniques have been developed in order to prepare it for the subsequent step of enzyme hydrolysis [4]. One of the main obstacles for the financially competitive production of ethanol has been the high cost of both pretreatment and hydrolysis steps, resulting from the increased biomass recalcitrance [5]. Dedicated efforts have been therefore focused on the development of cost-effective and robust biocatalysts used for breaking down lignocellulose to fermentable sugars.

Lignocellulosic biomass is mainly composed of plant cell walls that vary substantially in their contents depending on the species, variety and climate. Their main component is cellulose, the most abundant natural polymer on earth. The primary structure of cellulose is an unbranched glucan chain of repeating β-(1,4)-D glucose units. Many parallel glucans snap into crystalline microfibrils. Native cellulose occurs in two different crystal forms, a single-chain triclinic phase ($I\alpha$) and a two-chain monoclinic phase ($I\beta$) [6] and is highly resistant to enzymatic attack [7]. Cellulosic fibrils are embedded in a complex matrix involving hemicelluloses and lignin that hamper the way to cellulases and hemicellulases. Hemicelluloses are heterogeneous polymers of pentoses (e.g. xylose and arabinose), hexoses (e.g. mannose, glucose and galactose) and sugar acids (e.g. acetic, galacturonic and glucuronic). Contrary to cellulose, hemicelluloses are random and amorphous and more easily degraded to single sugars [8]. Hardwood hemicellulases contain mainly xylans, while softwood hemicellulases contain mainly glucomannans. Lignin is a complex aromatic polymer constructed of phenylpropane units derived from sinapyl, *p*-coumaryl and coniferyl alcohol. Lignin, hemicellulose and cellulose are linked by chemical bonds, forming a complex matrix that hampers the way to hemicellulases and cellulases [9,10].

Plant biomass degradation by fungi has been studied extensively since the middle of the previous century, however, our knowledge on the enzyme system used to degrade cellulose has changed dramatically just in the last three years. Traditionally, cellulose was thought to be degraded by three main types of enzyme activity: 1) endoglucanases (EC 3.2.1.4), 2) exoglucanases, including cellodextrinases (EC 3.2.1.74) and cellobiohydrolases (EC 3.2.1.91 for the non-reducing end acting cellobiohydrolases and EC 3.2.1.176 for the reducing end acting ones) and 3) β-glucosidases (EC 3.2.1.21) [11]. Endo-acting hydrolases introduce random breaks in the amorphous regions of the polysaccharide chain, exo-acting hydrolases cut processively cellooligosaccharides from chain ends and β-glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose. In spite of the cooperative activity exhibited by the aforementioned biocatalysts, the impressive biomass degrading efficiency demonstrated by various microorganisms in nature cannot be solely attributed to this endo-exo hydrolytic mechanism. Extracting and processing a single cellulose chain from its compact environment is energetically demanding considering the high crystallinity of cellulose and its tight association to other cell wall polysaccharides. Systems releasing small molecular weight oxidants such as the hydroxyl free radical that randomly attack the substrate via Fenton type chemistry reactions have been thought to act in conjunction with common cellulases in lignocellulose

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degradation. These include cellobiose dehydrogenase, quinone redox cycling and glycopeptide-based Fenton reaction [12,13].

Since the mid-20th century, researchers have suggested the presence of an additional non-hydrolytic factor that renders biomass less recalcitrant to enzymatic attack [14]. According to the proposed mechanism, cellulose hydrolysis was accomplished by the synergistic activity of two components, the first (C_1) swelling and disrupting cellulose and the second (C_x) having endoglucanase activity. In spite of many years of research, the nature of component C_1 has long remained an unresolved issue [15]. Previous studies have suggested components such as carbohydrate binding modules, expansins and expansin-like proteins (e.g. swollenin) as potential candidates for the C_1-mediated disruption of highly-ordered cellulose matrix [16]. A more recent study complemented this list with some fungal proteins with homology to glycosyl hydrolase (GH) family 61 of the continuously updated Carbohydrate Active enZyme database (CAZy; http://www.cazy.org), exhibiting cellulolytic enhancing ability when combined with common cellulases [17]. Interestingly, most of these proteins share a potential carbohydrate-binding surface; the exact mechanism, however, that renders recalcitrant lignocellulosic biomass accessible to degrading enzymes is yet to be fully elucidated.

2. GH61s: a cellulase-enhancing factor

To date, GH family 61 comprises approximately 250 members, widely distributed in the genome of most ascomycetous and basidiomycetous (white-rot and brown-rot) fungi [18,19]. Expression levels of most GH61 genes increase considerably during growth on lignocellulosic substrates, as compared to glucose media, suggesting their active involvement in cellulose decomposition. [20]. Even though the existence of these proteins has been long known, it was not until very recently that their physiological function was unraveled. Initial studies on GH61s reported a weak endoglucanase activity that could not be considered as their main role *in vivo* [21,22]. In 2007, it was reported that some GH61 members could boost cellulase activity resulting in increased lignocellulose conversion [17]. These findings launched intensive research efforts towards understanding the function of this enigmatic family. In 2010, *Harris et al.* identified three *Thielavia terrestris* GH61s as potential cellulase-enhancing factors [23]. The same group incorporated a *Thermoascus aurantiacus* GH61 encoding gene (*Ta*GH61A) in the genome of *Trichoderma reesei*, a common cellulase producer, resulting in a strain with improved cellulolytic efficiency. More precisely, the protein loading required to degrade lignocellulosic biomass was reduced two-fold [23]. It was also reported that this cellulase-boosting function was metal-ion dependent and eliminated when the mixture of cellulases/GH61 was applied on substrates composed solely of cellulose. One step further, the synergistic effect exhibited by *St*Cel61a, a GH61 from *Myceliopthora thermophila* (synonym *Sporotrichum thermophile*) was related to the lignin content and the antioxidant activity of an array of lignocellulosic materials [24]. Several hypotheses were put forward to explain GH61 mechanism such as the targeting of an unknown bond found in lignocellulose, but no definite answer was given regarding the interpretation of the enhancing effect.

The first crystal structure of a GH61 member, Cel61B from *Hypocrea jecorina* (anamorph *T. reesei*) was determined in 2008 at 1.6 Å resolution [25], followed by the 1.9 Å structure of *T. terrestris* GH61E [23]. Both GH61s fold into a beta-sandwich, where the two antiparallel twisted beta-sheets are connected through loops of varying length and conformation. The majority of conserved residues are clustered on the surface of the protein (Figure 1A). Cel61B structure comprises three nickel ions located in the two molecules of the asymmetric unit. Two of them are near the N-terminal of the two monomers and coordinated by highly conserved residues among GH61 family members (His1, His 89 and Tyr 176) (Figure 1B). In the case of GH61E, the corresponding ions are zinc or magnesium. In both structures, the authors did not manage to locate any polysaccharide binding cleft or typical glycoside hydrolase active site. A structural comparison search revealed that the most similar structure was that of CBP21 from *Serratia marcescens* [26], a protein that can be classified in carbohydrate-binding module family 33 (CBM33) of CAZy database and is known to be implicated in chitin degradation (Figure 1C). Interestingly, the two histidines coordinating the metal ion in GH61s superimposed nicely with the two highly conserved histidine residues in CBP21 structure (Figure 1D). It was suggested that GH61s and CBP21 could share a similar, even though at that time unknown, mechanism of action that led to increased hydrolysis rates of recalcitrant polysaccharides.

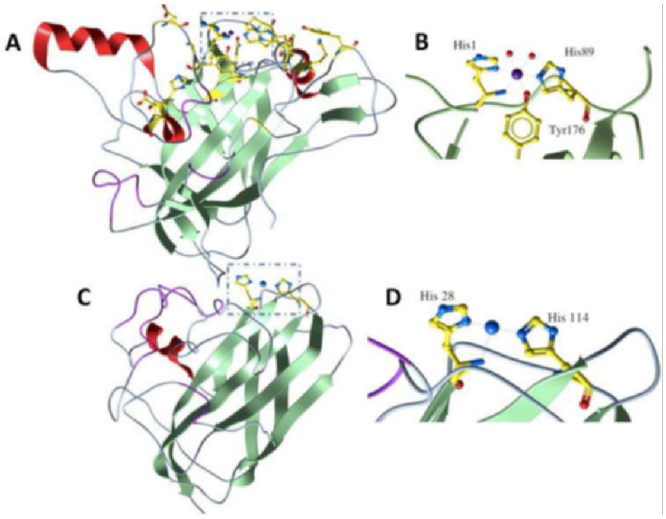

Figure 1. A. The figure shows the structure of Cel61B (molB, PDB code 2VTC) in cartoon representation. Conserved residues on the surface of the molecule are shown in ball and stick representation. **B.** The nickel ion (purple sphere) coordinated by His1, His 89, Tyr 176 and two water molecules (red spheres) in Cel61B structure. **C.** The structure of CBP21 (molC, PDB code 2BEM) in cartoon representation. Highlighted in ball and stick are the highly conserved residues His114 and His28, and a bound sodium ion (blue sphere). **D.** The sodium ion (blue sphere) coordinated by His28 and His 114 in molC of CBP21 structure. All figures were prepared with Molsoft [27].

3. CBP21 – oxidative cleavage of chitin

Chitin is a crystalline analogue of cellulose composed of β-(1,4) linked units of *N*-acetyl-D-glucosamine (GlcNAc). It is widely distributed in nature, particularly in the cuticle of arthropods and the cell walls of fungi and yeast. Similarly to cellulose-degrading enzymes, chitinases can be divided into two major categories: endochitinases that cleave chitin randomly at internal sites and exochitinases that involve chitobiosidases and β-(1,4) *N*-acetyl glucosaminidases [28]. CBM33 proteins were originally thought to be involved in substrate recognition due to the fact that they were secreted upon growth on lignin, bound on it and had no detectable hydrolytic activity [29]. More recently, it was shown that CBM33s such as CBP21 from *S. marcescens* could boost the hydrolytic activity of chitinases, indicating a more active involvement in chitin degradation [30]. However, the exact enzyme mechanism remained elusive until 2010, when in a