

Chapter 2

Cellobiohydrolases: Role, Mechanism, and Recent Developments

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Abstract Cellobiohydrolases or exoglucanases are produced by various bacteria and fungi with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases, which act at the chains end of cellulose resulting in release of glucose as well as cellobiose. The CBH I and II works processively from reducing and nonreducing ends of the cellulose chain, respectively. The catalytic module of CBHs is the tunnel structure formed by two surface loops that may covers entirety or part of active site evidenced that the mode of action proceeds in a processive manner as cellobiohydrolase progresses along the cellulose chain. CBHs are able to work actively in the crystalline region of cellulose, probably peeling them from the microcrystalline structure of Avicel. Although several assays have been proposed, no specific substrate as well as assay method to measure exoglucanases has been described till date. In recent days, several new approaches such as δ -sequence mediated integration, SCHEMA, and FoldX and a ‘consensus’ sequence have been developed to improve activity and stability of CBHs.

2.1 Introduction

Considering the plant biomass, the main component from plant cell wall is the cellulose, which is the more abundant carbohydrate constituted by glucopyranose monomers linked by β -1,4 glycosidic bonds with two distinct regions such as

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crystalline and amorphous regions (Dashtban et al. 2009). Cellulosic biomass is the largest amount of waste generated through human activities, which is the most attractive substrate for 'biorefinery strategies' to produce high-value products such as fuels, bioplastics and enzymes through fermentation processes (Mazzoli et al. 2012). The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Bhat 2000). In recent days, there is an increasing interest on use of biomass for biofuel production is pointed-out as an important alternative for reduction of energetic and environment problems.

Cellulases are classified into three major categories based on their substrate specificities and hydrolysis mechanism as (i) 1,4 β -D-glucanases or endoglucanases (EC 3.2.1.4), (ii) exoglucanases or cellobiohydrolases (EC 3.2.1.91), and (iii) cellobiase or β -glucosidases (EC 3.2.1.21). The most efficient hydrolysis of cellulose is the result of combined synergistic actions of cellulases, whereby the enzymatic activity of an enzyme mixture is significantly higher than individual enzyme activity (Wood 1992; Irwin et al. 1993; Nidetzky et al. 1994).

2.2 Exoglucanases or Cellobiohydrolases (CBH) (EC 3.2.1.91)

Exoglucanases, also known as cellobiohydrolases (CBHs) that act at the end of cellulose chains and releasing glucose as well as cellobiose as product in a processive fashion (Medve et al. 1998). CBHs are the most-studied exoglucanase, which is about 70 % of the secreted cellulases by cellulolytic fungi belongs to glycoside hydrolases (GH) 6 and 7, as well as 48 families (Teeri 1997). Different CBHs are produced by various bacteria and fungi, with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases. Aerobic fungal CBHs are belongs to families 6 and 7; aerobic bacterial CBHs belong to families 6 and 48; anaerobic fungal CBHs are in family 48 and anaerobic bacterial CBHs are in family 9 as well as 48. In general, family 7 CBHs originate only from fungi, whereas family 48 CBHs from bacteria (Zhang and Zhang 2013).

2.3 CBH I and II

CBHs mainly release cellobiose from cellulose derivatives, and the presence of cellobiose competitively inhibits the rate of hydrolysis. The fungal CBHs belong to glycosyl hydrolase families 6 and 7 (GH-6 and 7) and act most efficiently on highly ordered crystalline cellulose, hydrolyzing from either reducing or non-reducing end to liberate predominantly cellobiose (C2) with a minor amount of cellotriose (C3) (Boer et al. 2000; Takahashi et al. 2010). There are two types of cellobiohydrolase:

CBH I and II that works processively from the reducing and non-reducing end of the cellulose chain respectively. The cellobiohydrolases Cel6A (a CBH II) and Cel7a (a CBH I) are much studied enzymes of fungal origin and are usually expressed by the ascomycete *Trichoderma reesei*, accounts for 40 % of total protein and 70 % of cellulytic activity in the industrially relevant fungus *Hypocrea jecorina* (*T. reesei*) which is used for industrial-scale production due to its ability to produce more sugars from biomass through hydrolysis (Suominen et al. 1993).

2.4 Structure and Mode of Action of CBHs

The most significant topological feature of CBHs catalytic module is the tunnel structure, formed by two surface loops that may cover the entirety (family 7 CBHs) or part of the active site (family 48 CBHs) (Koivula et al. 2002; Vocadlo and Davies 2008; Zhang and Zhang 2013). The CBH I (TrCBH I) is the major component of the *T. reesei* cellulase system that belongs to the GH-7 family and hydrolyzes the β -1,4-linkages of a cellulose chain from its reducing end (Claeysens et al. 1990). The catalytic core domain (CCD) structures of TrCBH I suggested that a central structural motif constituted mainly by an antiparallel β -sandwich motif that bears four surface loops, which decorate the concave face of the sandwich forming the cellulose-binding tunnel (Divne et al. 1998; Stahlberg et al. 1996). During enzymatic catalysis, the cellulose chain slides through the substrate-binding tunnel, and every second glycosidic bond is correctly presented to the catalytic apparatus located at the far end of the tunnel to progressively liberate disaccharide units (cellobiose) from cellulose (Divne et al. 1994).

The three-dimensional (3D) structures of two GH-6 family members including CBH of *T. reesei* and that of *Humicola insolens* in complex with glucose, cellobiosaccharide, and a non-hydrolyzable substrate analog (Rouvinen et al. 1990; Varrot et al. 2002). It seems that the significant amino acids associated with catalytic core domain, where the catalytic site is buried inside a tunnel-shaped cavity and an enzyme–cello-oligosaccharide hydrogen bond network. This typical structure suggests that the mode of action proceeds in a processive manner as cellobiohydrolase progresses along the cellulose chain (Boisset et al. 2000; Henrissat 1998; Reverbel-Leroy et al. 1997).

Cellobiohydrolases or exoglucanases is able to work effectively on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri 1997). In general, CBHs are active on the crystalline regions of cellulose; whereas, endoglucanases (1,4- β -glucanases) are typically active on the more soluble amorphous region of the cellulose crystal (Rouvinen et al. 1990; Divne et al. 1998). It seems that there is a high degree of synergy observed between exo- and endoglucanases, which is required for efficient hydrolysis of cellulose crystals (Abdel-Shakour and Roushdy 2009).

2.5 Substrates and Assay Methods

In general, enzymes in cellulase mixture which show relatively high activity on Avicel and little activity on carboxymethyl cellulose (CMC) are identified as exoglucanases (CBHs) (Maki et al. 2009). The processive activity of CBHs on CMC is blocked or inhibited by the occurrence of derivatized glucose residues which leads to severe substrate limitation. Avicel (microcrystalline cellulose or hydrocellulose) is mainly used for measuring exoglucanase activity because of its low degree of polymerization. However, Avicel contains some amorphous cellulose and soluble cellodextrins which can act as substrates for both exo- and endoglucanases. Thus, there is no highly specific substrate to measure exoglucanase activity (Wood and Bhat 1988).

There are several different assays have been proposed to measure exoglucanase (CBH I and CBH II) activity; however, all of these assays have some sort of limitations. CBHs acting from nonreducing end of the substrate can be assayed with chromogenic arylcellobiosides, from which the chromogenic aryl residue, attached to the reducing end of the substrate liberated. However, the processive enzymes acting from the reducing end of the carbohydrate chain have been identified recently, but chromogenic substrates to identify these enzymes are not yet commercially available (Boisset et al. 1998). Measurement of the production of cellobiose directly via high-pressure liquid chromatography (Gum and Brown 1976) or via a cellobiose oxidoreductase assay (Kelleher et al. 1987) is complicated in that the endoglucanases that are present in crude cellulase mixtures also produce cellobiose. The substrate 4-methylumbelliferyl- β -D-lactoside was identified as an effective substrate for assaying CBH I of *T. reesei* as lactose, phenol and 4-methylumbelliferone (a fluorescent signal molecule) are the hydrolysis products (van Tilbeurgh et al. 1985). But, the activity of CBH II was not able to detect thus the resulting activity is not an effective representation of true exoglucanase activity. Alternatively, an assay for quantification of exoglucanase activity using *p*-nitrophenyl- β -D-cellobioside as substrate to yield cellobiose and *p*-nitrophenol has been developed; but (i) CBH II activity cannot be measured using *p*-nitrophenyl- β -D-cellobioside, (ii) the specific activity of the available purified endoglucanases may not be representative for all existing endoglucanases in the mixture, and (iii) the product ratio from endoglucanase actions may be influenced by the presence of exoglucanases (Deshpande et al. 1984).

A double-antibody sandwich enzyme-linked immunosorbent assay was developed for quantifying CBH I in cellulase complex of *T. reesei*. The antibody configuration resulted in the highest specificity for the assay of CBH I employed a monoclonal antibody to coat wells in polystyrene plates and peroxidase-labeled polyclonal antibody to detect CBH I bound to the immobilized monoclonal antibody. Since there was no direct assay of CBH I activity in the presence of the other enzymes in complex till date, it would be useful for quantifying this enzyme between the ranges of 0.1–0.8 μ g/ml (Riske et al. 1990).

2.6 Recent Developments

Recently, the cellulolytic yeast (*Saccharomyces cerevisiae*) with enhanced CBH activity was developed by integrating three types of CBH-encoding genes (cbh1 from *Aspergillus aculeatus*, cbh1, and cbh2 from *T. reesei*) with a strong constitutive promoter Ptpi were sequentially integrated into the *S. cerevisiae* W303-1A chromosome via δ -sequence mediated integration. The three recombinant (W1, W2, and W3) and control strains (W303-1A and AADY) produced ethanol (g/l) from acid-and alkali-pretreated corncob was about 5.92 ± 0.51 , 18.60 ± 0.81 , 28.20 ± 0.84 , 1.40 ± 0.12 , and 2.12 ± 0.35 , respectively (Hong et al. 2014).

The thermostable CBHs could offer potential benefits in the hydrolysis of pre-treated lignocellulosic substrates because the harsh conditions often required by several pretreatments can be harmful for conventional biocatalysts. Heinzelman et al. (2010) investigated an efficient SCHEMA recombination-based approach for screening homologous enzymes to identify stabilizing amino acid sequence blocks which has been used to generate active, thermostable CBH I enzymes from the 390,625 possible chimeras that can be made by swapping eight blocks from five fungal homologs. A total of 16 predicted thermostable chimeras, with an average of 37 mutations relative are more thermostable ($>65^\circ\text{C}$) than the most stable parent CBH I of thermophilic fungus *Talaromyces emersonii*.

Likewise, Komor et al. (2012) attempted to produce thermostable chimeric fungal CBH I by structure-guided recombination through FoldX and a 'consensus' sequence approach to identify individual mutations present in the five homologous parent CBH I enzymes which further stabilize the chimeras. With an alignment of 41 CBH I sequences, effects on ΔG (Folding) was calculated using amino acid frequencies at each candidate position and the mutations chosen using these methods increased the $T(50)$ of the most thermostable chimera by an additional 4.7°C , to yield a CBH I with $T(50)$ of 72.1°C , which is 9.2°C higher than that of native CBH I, from *Talaromyces emersonii*.

2.7 Perspectives

The interest in enzymatic hydrolysis of cellulosic biomass for biofuel production is increasing continuously due to availability and reutilization as cheaper source. CBHs are one among the important enzymes which could produce more sugars such as glucose and cellobiose from crystalline cellulose. (i) It is essential to develop easy and suitable method with specific substrate in order to measure the CBHs activity in the cellulose mixture. (ii) Engineering of CBHs is also needed in order to enhance the specific activity to reduce the use of CBHs and also to increase thermostability and reusability of exoglucanases. These approaches mentioned above to enhance the activity and stability of CBHs would be useful in biorefinery and biofuel industries.

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