Biotechnology Advances 31 (2013) 936–944

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Cellulosome-based, *Clostridium*-derived multi-functional enzyme complexes for advanced biotechnology tool development: Advances and applications

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ARTICLE INFO

Available online 4 April 2013

Keywords:
Clostridium cellulovorans
Cellulosome
Designer minicellulosome
Cohesin–dockerin interaction
Biosensor
Carbohydrate binding module
One-step CBM purification
Cell surface anchoring
Whole-cell biocatalyst
Consolidated bioprocessing

ABSTRACT

The cellulosome is one of nature's most elegant and elaborate nanomachines and a key biological and biotechnological macromolecule that can be used as a multi-functional protein complex tool. Each protein module in the cellulosome system is potentially useful in an advanced biotechnology application. The high-affinity interactions between the cohesin and dockerin domains can be used in protein-based biosensors to improve both sensitivity and selectivity. The scaffolding protein includes a carbohydrate-binding module (CBM) that attaches strongly to cellulose substrates and facilitates the purification of proteins fused with the dockerin module through a one-step CBM purification method. Although the surface layer homology (SLH) domain of CbpA is not present in other strains, replacement of the cell surface anchoring domain allows a foreign protein to be displayed on the surface of other strains. The development of a hydrolysis enzyme complex is a useful strategy for consolidated bioprocessing (CBP), enabling microorganisms with biomass hydrolysis activity. Thus, the development of various configurations of multi-functional protein complexes for use as tools in whole-cell biocatalyst systems has drawn considerable attention as an attractive strategy for bioprocess applications. This review provides a detailed summary of the current achievements in *Clostridium*-derived multi-functional complex development and the impact of these complexes in various areas of biotechnology.

Contents

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0734-9750/$ – see front matter
http://dx.doi.org/10.1016/j.biotechadv.2013.03.009

1. Introduction

Society today faces the challenging problem of finding renewable, alternative energy sources to the conventional and still widely used fossil fuels (Armaroli and Balzani, 2007). The development of a processing combination based on renewable substrates, such as plant biomass comprising plant cell walls, is required because of the current energy crisis. Annually, approximately 1011 tons of plant biomass are hydrolyzed by microbes, releasing energy corresponding to 640 billion barrels of crude oil (Fontes and Gilbert, 2010). Hence, the conversion of cellulosic biomass into fermentable sugars may represent a viable means of producing renewable fuels, such as ethanol (Cardona and Sanchez, 2007; Sanchez and Cardona, 2008). Because the rate-limiting step in this process is the hydrolysis of biomass, the development of more efficient enzyme systems is required (Fontes and Gilbert, 2010). The cellulosome may be useful in solving the hydrolysis problem related to the recalcitrant and complex structure of the plant cell wall. (Doi and Kosugi, 2004; Hyeon et al., 2010; Jeon et al., 2012). Research and an understanding of the microbial physiology related to the utilization of cellulose as a resource are important for the development of consolidated bioprocessing (CBP)-enabling industrial strains. (Lynd et al., 2005). CBP is a highly integrated configuration process containing the following three biologically mediated transformations in a single step: the production of saccharolytic enzymes, the hydrolysis of carbohydrate components and the fermentation of sugars (Cardona and Sanchez, 2007). The development of a hydrolysis enzyme complex is a useful strategy for the construction of CBP-enabling microorganisms that involves the engineering of non-cellulolytic organisms with the ability to produce a valuable and high-yield product, thereby expressing a heterologous enzyme complex system that utilizes cellulose as a carbon source (Olson et al., 2012). The use of the minicellulosome as a multi-functional enzyme complex leads to the colocalization of synergistic combinations of hydrolytic enzymes (Hyeon et al., 2010).

The utilization of a *Clostridium*-derived, multi-functional enzyme complex by microorganisms involves advanced biotechnology applications beyond the bioprocesses associated with the enzymatic hydrolysis of biomass (Fontes and Gilbert, 2010). This architectural protein complex has led to innovative molecular engineering approaches with diverse research and industrial applications (Doi and Kosugi, 2004; Fontes and Gilbert, 2010). This review explains these developments, focusing on the following: 1) the protein modules of the cellulosome, 2) the advanced applications of the cellulosome system in biotechnology, 3) the engineering of multi-functional enzyme complexes and 4) strain development as a whole-cell biocatalyst. Possible future directions include designer cellulosomes and microbial cell-based strategies, which are summarized herein.

2. Cellulosomes for the degradation of lignocellulosic biomass

Cellulosomes are high-activity multienzyme complexes that hydrolyze the crystalline cellulose and polysaccharides in plant cell walls (Beguin and Lemaire, 1996; Schwarz, 2001). In nature, cellulosomes have been identified in certain anaerobes, such as cellulolytic clostridia and ruminal bacteria. The cellulosomes act synergistically with various enzymes to hydrolyze intractable cellulosic and hemicellulosic polymers in the plant cell wall (Murashima et al., 2002). The synergistic interaction of multiple enzymes and their substrates helps overcome the rate-limiting step of converting crystalline forms of cellulose to cellobiose, leading to the efficient degradation of crystalline polysaccharides in plants. (Doi et al., 2003; Fontes and Gilbert, 2010). *Clostridium cellulovorans*, an anaerobic, mesophilic, spore-forming bacterium, is a cellulolytic clostridia that produces a cellulosome with a molecular weight of approximately 1 million Daltons, in which several cellulases interact tightly with the scaffolding protein CbpA (Cho et al., 2004; Sleat et al., 1984). The cellulosome structure of *C. cellulovorans* is shown as a schematic image in Fig. 1. This strain is capable of a multi-step conversion of insoluble cellulosic substrates into fermentable end-products by producing a cellulosome that is tightly controlled by the expression level of different extracellular enzymes (Han et al., 2004; Mechaly et al., 2000; Murashima et al., 2002). Like other *Clostridium* strains, *Clostridium thermocellum* has two types of cohesin–dockerin interactions with scaffoldin CipA and an anchoring protein. The Type I interaction is an interaction between an enzyme-borne type I dockerin and a scaffoldin-borne type I dockerin. The interaction between the type II dockerin on the cellulosomal scaffoldin and the type II cohesins on the anchoring scaffoldin is a Type II interaction (Fan et al., 2012).

2.1. Cohesin–dockerin interaction

The cohesin–dockerin interaction is a high-affinity protein–protein interaction between enzymes containing duplicated sequences (dockerin domains) and a non-catalytic scaffolding protein containing repeated sequences (cohesin domains) (Jeon et al., 2012; Mechaly et al., 2000). The *C. cellulovorans* protein CbpA is an example of such a scaffolding protein; it has nine cohesin domains. (Kosugi et al., 2004; Takagi et al., 1993). The cohesin domains of the scaffolding protein bind strongly to the dockerin domains of several catalytic subunits (Fig. 1A), resulting in the assembly of a cellulosome. Many studies emphasize the importance of the cohesin–dockerin interaction in cellulosome assembly and biotechnological applications (Fontes and Gilbert, 2010; Hyeon et al., 2011; You et al., 2012). The main reasons for the heterogeneous composition of cellulosomes are the differences in the cohesin–dockerin interactions, such as species-specific variations, and the differences in the number of cohesin repeats in each scaffolding protein (Doi and Kosugi, 2004; Fierobe et al., 2005). Depending on which enzymes are bound to the scaffolding protein, there is the potential to make a variety of cellulosomes, each with a different composition, within a single microorganism (Bomble et al., 2011; Koukiekolo et al., 2005).

2.2. Carbohydrate-binding module (CBM)

The non-catalytic scaffolding protein of the cellulosome includes a carbohydrate-binding module (CBM) that facilitates the degradation of cellulose. (Lavan et al., 2009; Murashima et al., 2002). For example, the CBM in the CbpA protein from *C. cellulovorans* belongs to the CBM3a family and is able to bind preferentially to crystalline cellulose (Fig. 1B) rather than to amorphous cellulose (Boraston et al., 2004). CBM3a includes a planar linear strip of aromatic and polar residues that is proposed to bind to crystalline cellulose (Yaniv et al., 2012). The CBM of a scaffolding protein plays a crucial role in effective hydrolysis by facilitating the strong binding of the scaffolding proteins to cellulose to increase the concentration of the hydrolysis enzyme near the substrate (Doi et al., 2003; Lavan et al., 2009). When CBM and a cellulose substrate bind, the assembled cellulosomal enzymes are brought into close proximity with the cellulose, making cellulose degradation more efficient than is possible with individual free enzymes (Tamaru et al., 2000). For example, when ExgS and EngH, the main cellulosomal enzymes in *C. cellulovorans*, are assembled in a complex with mini-CbpA, they are 1.5- to 3.0-fold more effective at hydrolyzing crystalline cellulose than when they are separate (Murashima et al., 2002).

2.3. Surface layer homology (SLH) domain

The surface layer homology (SLH) domains of the scaffolding proteins in cellulosome-producing bacteria are highly conserved and share homology with other proteins (Desvaux et al., 2006). These domains are found in many microorganisms and form a protein layer that lies outside of the cell wall (Kern et al., 2011). In *C. cellulovorans*, the scaffolding protein CbpA contains repeated domains with bacterial