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Research review paper

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

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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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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

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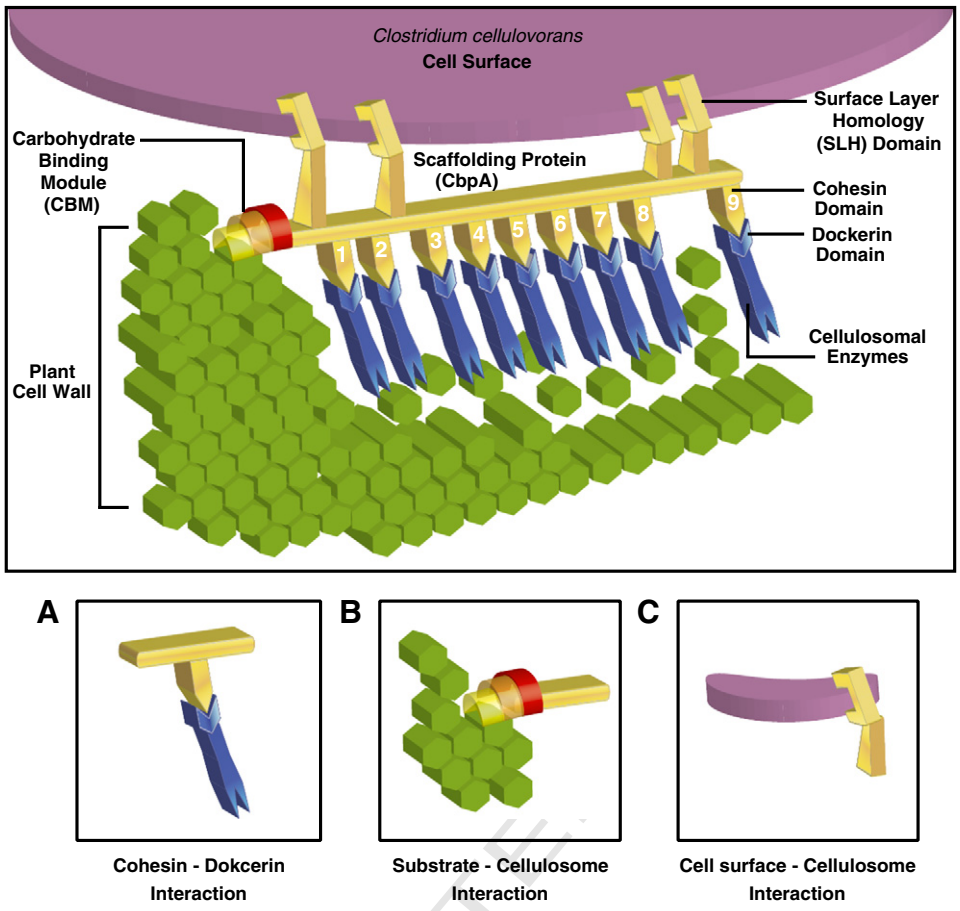

Fig. 1. Schematic illustrations of a *C. cellulovorans* cellulosome. The cellulosome structure, including the protein components and the modules related to the cellulosome system, is shown here. The scaffolding protein CbpA has a carbohydrate-binding module (CBM), nine cohesin domains and four surface layer homology (SLH) domains. The binding of the cohesin domain to the dockerin domain of cellulosomal enzymes leads to the assembly of the cellulosome. Each protein module's related cellulosome formation for advanced application in biotechnology is shown. (A) The high affinity, high-sensitivity and high-selectivity cohesin–dockerin interaction. (B) The substrate-cellulosome interaction using the CBM in a scaffolding protein. (C) The cell surface–cellulosome interaction using the SLH domains for the proximity effect.

SLH homology-related anchoring functions (Fig. 1C). These domains contribute to the cell surface anchoring of the cellulosome in *C. cellulovorans*. (Doi et al., 2003; Tamaru et al., 2000).

3. Advanced biotechnological applications of the cellulosome system

3.1. Biosensors based on the cohesin–dockerin interaction by cellulosomics

The main role of the cohesin–dockerin interaction is the assembly of the cellulosome, which contains scaffolding protein and cellulosomal enzymes (Doi, 2008). Because the dockerin domains in each strain can be selectively recognized by their cohesin domains, sensing techniques using cohesin–dockerin interactions would be beneficial for the development of single- or multi-target biosensors (Jeon et al., 2012). In addition, the high-affinity of the cohesin–dockerin interaction relative to other protein–protein interactions implies that a variety of affinity-based processes can be explored (Craig et al., 2006; Haimovitz et al., 2008). Dockerin domains are a pair of 22 amino acid residue segments that are highly conserved among species, whereas cohesin domains are weakly conserved among different species or different scaffolding proteins in the same species (Han et al., 2003; Park et al., 2001; Sakka et al., 2011). In particular, the *C. cellulovorans* CbpA contains more divergent cohesin domains with a higher percentage of amino acid substitutions than those found in scaffolding proteins in other clostridia, such as *C. thermocellum* and *Clostridium cellulolyticum* (Doi et al., 2003; Pages et al., 1996; Yaron et al., 1995). The general species-specific cohesin–dockerin interaction contributes to the heterogeneous cellulosome composition controlled by the relative level of the available cellulosomal enzymes (Fendri et al., 2009). In contrast, an anion-exchange chromatography experiment that examined the cellulosomal enzymes of *C. cellulovorans* in cellulose-containing medium culture suggested a different explanation (Doi et al., 2003; Han et al., 2005). These results showed several separated peaks that indicated that several factors, such as a region that is specifically surrounded by certain enzymes with the specificity for cohesin domains, control the cellulosomal populations and the ratio of the subpopulations (Cho et al., 2010; Han et al., 2005; Jeon et al., 2012). The dockerin domain is small in size (approximately 8–10 kDa), allowing proteins of interest to be easily marked with a dockerin domain using genetic modification; a dockerin-based affinity tag has been successfully developed with various potential applications (Demishtein et al., 2010). In addition, cohesin markers fused with fluorescent substance have high specificity and sensitivity (Fig. 2A) and can be used in powerful biosensor-based tools for identifying target proteins without an impact on the microbial system (Cho et al., 2010; Jeon et al., 2012). By combining the cohesin–dockerin domain interaction with immobilization techniques, label-free transducer technologies can be developed (Brouard et al., 2011; Pu et al., 2009).

3.2. One-step purification based on the carbohydrate-binding module

The CBM of a cellulosome system can be used as a tag for a one-step purification method using cellulose as a support because of

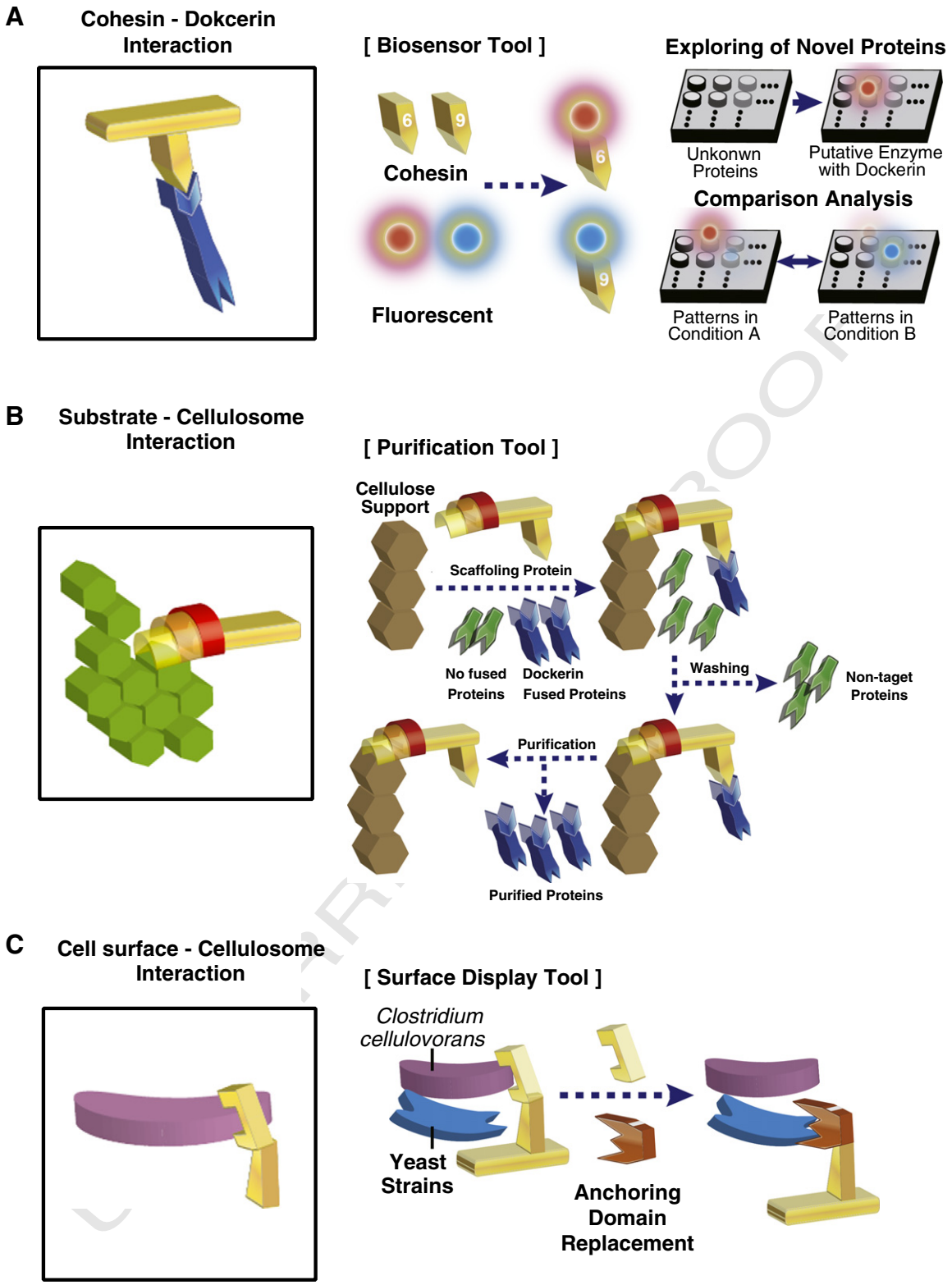

Fig. 2. Schematic illustrations of advanced applications in biotechnology of the cellulosome system. Each protein module of the cellulosome system can be used for advanced application in biotechnology. (A) A biosensor based on a cohesin–dockerin interaction is developed by combining with fluorescent substances. It can be utilized for exploring novel proteins and comparing related cellulosomics. (B) A one-step purification using the carbohydrate-binding module (CBM) is performed for easy purification of dockerin-fused enzymes. It enhances the cost-effectiveness of enzyme purification. (C) A surface display tool using a surface layer homology (SLH) domain is shown. The replacement of an anchoring domain in a scaffolding protein leads to the immobilization of enzymes or complexes on cell surfaces, such as on yeast. It can be used to construct a whole-cell biocatalyst system.

CBM's high affinity and specificity for cellulose (Mateo et al., 2001). Cellulose is an ideal matrix with large-scale affinity because of its low cost, excellent physical properties and low non-specific affinity with most proteins (Boraston et al., 2004; Hyeon et al., 2012a). Thus, a CBM-containing recombinant scaffolding protein miniCbpA, which was designed with the CBM of CbpA fused to its N-terminus, was easily purified by cellulose matrix chromatography (Hyeon et al., 2011). This method facilitated the easy purification of useful hydrolysis

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enzymes by fusing a dockerin domain to the desired enzyme and inducing complex formation with miniCbpA (Fig. 2B). This technique can replace multi-step purification techniques and help reduce the loss of product and the cost of enzyme purification (Hyeon et al., 2012a). Many attempts have been made to enable the utilization of CBM in a one-step purification process (Hong et al., 2008; Kwan et al., 2005; Shoseyov et al., 2006), and the utility of a CBM-based one-step purification technique using miniCbpA as the CBM-containing subunit has been confirmed (Hyeon et al., 2012a).

3.3. Cell surface display by replacing the surface layer homology domain

The recombinant scaffolding protein miniCbpA, which was derived from *C. cellulovorans* CbpA, possesses one SLH domain with a high functional affinity for cell wall polymers (Kosugi et al., 2002). Although these domains allow the cellulosome to bind to the *C. cellulovorans* cell surface, this cell surface anchoring system has not been applied to other strains, such as the yeast cell wall (Kosugi et al., 2004). The other corresponding anchoring domain is required for applying cellulosome anchoring system to the immobilization of proteins or complexes on foreign strains (Fig. 2C). The successful development of a cell surface display system on *Bacillus subtilis* or *Saccharomyces cerevisiae* has been reported (Hasunuma and Kondo, 2012; Lee et al., 2003).

4. Engineering multi-functional enzyme complexes

A minicellulosome, which consists of a recombinant scaffolding protein and cellulosomal enzymes, is an efficient multi-functional enzyme complex for use in industrial bioprocesses (Hyeon et al., 2011). The concept of a multi-functional complex containing a mix-and-match configuration using parts from different cellulosomes in a suitable industrial host cell system has drawn considerable attention as an attractive strategy for various biotechnology applications (Fig. 3). Thus, it is very important to construct these engineered complexes such that they have functions specific for the industrial process of interest. Enzyme complexes containing the cellulosomal cellulase gene and the recombinant scaffolding protein miniCbpA gene from *C. cellulovorans* were successfully co-expressed and formed in vivo in *B. subtilis, S. cerevisiae* and *Corynebacterium glutamicum* by harnessing the interaction between the cohesin and dockerin domains (Cho et al., 2004; Hyeon et al., 2011). In addition, cellulosomes derived from other *Clostridium* strains such as *C. thermocellum* and *C. cellulolyticum*, have been used to design mini-cellulosomes (Fan et al., 2012; Tsai et al., 2010; Wen et al., 2010). There has been interest in developing multi-functional complexes based on the cellulosome system of clostridia for several purposes, including the analysis of the synergy effect between various enzymes and the optimization of enzyme efficiency on biomasses of various compositions (Fierobe et al., 2005).

4.1. Design of a scaffolding protein containing cohesin domains

To construct a multi-functional complex, the *C. cellulovorans* scaffolding protein CbpA and the *C. thermocellum* scaffolding protein CipA were spliced into a small recombinant scaffolding protein (Fan et al., 2012; Hyeon et al., 2010). For example, the miniCbpA protein derived from the *C. cellulovorans* scaffolding protein CbpA carries two cohesin domains, one SLH domain and a CBM (CBM-SLH-Coh1-Coh2). The nucleotide sequence of the miniCbpA coding region is 1647 bp in length and encodes a polypeptide of 539 amino acids (Fig. 3A). As described above, the miniCbpA containing a CBM at its N-terminus was easily purified in a single step using a CBM-utilizing purification method (Hyeon et al., 2011). The cohesin domains of the *C. cellulovorans* miniCbpA protein, which serve as binding moieties for the assembly of other multi-subunit enzyme complexes, retain their complementary binding properties when expressed in many industrial strains, such as *S. cerevisiae*, *C. glutamicum* and *Escherichia coli* (Hyeon et al., 2010, 2011, 2012a).

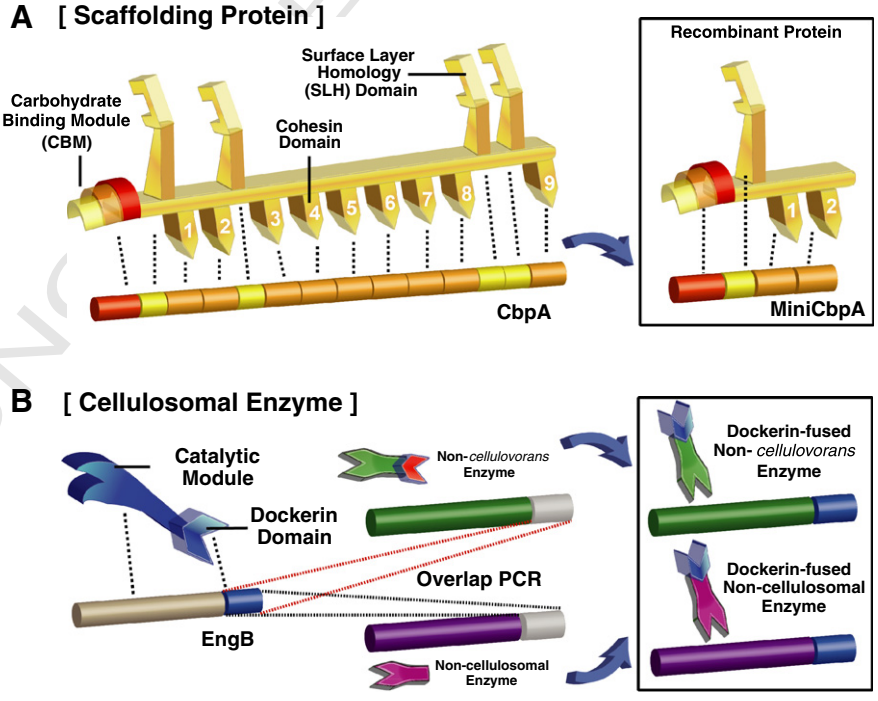

Fig. 3. Schematic illustrations of an artificial multi-functional enzyme complex. This complex is composed of a recombinant scaffolding protein and dockerin-fused chimeric enzymes. (A) The recombinant scaffolding protein for complex formation includes two cohesin domains and a CBM. The cohesin domain serves as a binding moiety for the assembly of a multi-subunit enzyme complex. (B) Two types of dockerin-fused enzymes derived from a non-*cellulovorans* and a non-cellulosomal enzyme, respectively, are constructed using the overlapping PCR method. Each chimeric enzyme is created by the fusion of a dockerin domain to endoglucanase EngB from *C. cellulovorans*.

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4.2. Design of chimeric hydrolysis enzymes containing the dockerin domain

To assemble a multi-functional complex via the cohesin–dockerin interaction, chimeric enzymes were constructed with the catalytic domains of target enzymes fused to the C-terminus of a dockerin domain (Hyeon et al., 2010). Because the cohesin–dockerin interaction has species-specificity among different cellulolytic bacterial species (Fierobe et al., 2005), many attempts have been made to successfully enable the assembly of enzymes from other cellulolytic clostridia species or the assembly of non-cellulosomal enzymes from other species (Hyeon et al., 2012a; Tsai et al., 2010). For example, a chimeric gene containing an exogenous type dockerin was constructed using pairs of overlapping primers with a widely used multistep PCR strategy (Horton et al., 1989; Hyeon et al., 2011). The catalytic domain fragment and the dockerin fragment of endoglucanase EngB from *C. cellulovorans* were amplified (Fig. 3B).

The endoglucanase CelE from the thermophilic anaerobic bacteria *C. thermocellum* is of considerable biotechnological interest because its high stability could greatly reduce enzyme replacement costs or permit processes to be performed at high temperatures (Kohli et al., 2001; Lamed et al., 1983; Song and Rhee, 2000). The chimeric CelE-doc gene contains the *C. thermocellum* endoglucanase CelE backbone fused to the dockerin region of *C. cellulovorans* EngB; this chimeric protein can be assembled with miniCbpA (Hyeon et al., 2011). The non-cellulosomal endoglucanase EngD from *C. cellulovorans* possesses multiple enzymatic activities, including endoglucanase, exoglucanase and xylanase activities (Jeon et al., 2009a). Although this enzyme exists as an independent non-cellulosomal enzyme because it does not interact with the scaffolding protein CbpA (Foong and Doi, 1992; Hamamoto et al., 1992), chimeric EngD containing a dockerin domain at its C terminus can be assembled with the recombinant scaffolding protein miniCbpA (Hyeon et al., 2010; Jeon et al., 2009a). Non-cellulase enzymes, such as agarase, that can be used to degrade marine biomass can also be fused with a dockerin module and assembled into an enzyme complex (Hyeon et al., 2012a).

5. Production of a multi-functional enzyme complex for biomass utilization

A whole-cell biocatalyst with the ability to degrade biomass and produce valuable products was developed by applying the *Clostridium*-derived multi-functional complex system (Ryu and Karim, 2011). Whole-cell biocatalysts have several advantages, such as reducing the carbon catabolite restriction effect, lowering sterilization costs and utilizing a single reactor because the cells immediately utilize the sugar (Balasubramaniam et al., 2012; Ryu and Karim, 2011). Many attempts to develop metabolically engineered industrial strains to produce a more valuable material have been reported (Olson et al., 2012; Yu et al., 2012a, 2012b). Most notably, strains that produce an efficient biofuel, such as bioethanol and microbial lipids, have been engineered by many researchers (Jarboe et al., 2010; Yu et al., 2010; Zhang et al., 2009). However, for a viable and cost-effective strategy to produce biomaterial using metabolically engineered strains, microorganisms with the ability to hydrolyze biomass, including lignocellulosic and marine biomass, are required (Guedon et al., 2002; Wood and Ingram, 1992; Zhou et al., 2001). Thus, the development of hydrolysis enzymes with high activity, such as multi-functional enzyme complexes, is important for the conversion of the resulting sugars to desirable products via a cellulolytic microorganism or consortium (Fig. 4).

5.1. Utilizing lignocellulosic biomass

Cellulose comprises a major part of the biomass on the earth, and its synthesis and utilization contributes to the environmental cycle (McKendry, 2002). Although the structure and the composition of the plant cell wall are different among various plant species, the cell wall structural composition among tissues of a single species and even among individual cells commonly consists of β-1-4-linked D-glucopyranosyl residues that constitute a fibrous, insoluble and crystalline polysaccharide (McNeil et al., 1984). Hemicellulose, pectin and lignin linked with cellulose fibers comprise the complex structure of the plant cell wall. Because the composition of the plant cell wall structure is very complex, the cooperative action of a multitude of cellulolytic enzymes, such as endoglucanase, exoglucanase and b-glucosidase, is required for its degradation (Fierobe et al., 2005; Murashima et al., 2002). In nature, it is therefore likely that this cooperative action also occurs among a number of different microorganisms to degrade plant cell wall materials (de Vries and Visser, 2001). Cellulases have been found either as part of the cellulosome or as free enzymes that work synergistically and contribute to the turnover of cellulosic materials (Tamaru et al., 2000). There have been many attempts to assemble a hydrolysis enzyme complex to enhance enzyme activity toward cellulosic substrates (Hyeon et al., 2011; Murashima et al., 2002). For example, the development of a multi-functional enzyme complex containing chimeric CelE bound to the recombinant scaffolding protein miniCbpA from *C. cellulovorans* industrial strains that can hydrolyze cellulosic materials has been reported (Hyeon et al., 2010). The resulting complexes efficiently degraded cellulose at a rate that was approximately 2.8-times faster than the corresponding enzyme alone (Hyeon et al., 2010).

In addition, a number of attempts have been made to utilize lignocellulosic biomass substrates to produce a valuable product by expressing cellulolytic enzymes in industrial strains, such as *S. cerevisiae* and *C. glutamicum* (Hyeon et al., 2010, 2011). One of the most effective ethanol-producing yeasts, *S. cerevisiae*, has several advantages, including a high efficiency of hexose to ethanol conversion and a high tolerance to ethanol or inhibitory compounds derived from acid hydrolysates after pretreatment of the lignocellulosic biomass (Hyeon et al., 2010). *C. glutamicum* was isolated as an aerobic, biotin-auxotrophic, nonpathogenic and Gram-positive soil bacterium during a screen for bacteria producing L-glutamate (Han et al., 2007, 2008; Hyeon et al., 2012b). *C. glutamicum* is able to grow on a variety of carbohydrates and organic acids using single or combined sources of carbon and energy, including glucose and acetate (Han et al., 2007). Because these industrial strains cannot use cellulosic materials directly, and the expansion of the substrate assortment is required, a polymeric biomass must first undergo hydrolysis to release monomeric hexose prior to fermentation (Hyeon et al., 2011). *Saccharomycopsis fibuligera* β-glucosidase (Bgl1) and cellulosomal endoglucanase were introduced into *S. cerevisiae*, the preferred industrial organism for ethanol fermentation, in an attempt to develop cellulose-degrading yeast cells that could convert a cellulose substrate into ethanol (Hahn-Hagerdal et al., 2001; Jeon et al., 2009a). The resulting strains demonstrated effective cellulosic substrate utilization and produced a maximum ethanol concentration of approximately 11.03 g/L using carboxymethyl cellulose (CMC) as a substrate (Jeon et al., 2009a). This result suggests that various cellulosic substrates, including CMC, β-glucan and phosphoric acid swollen cellulose (PASC), are sequentially degraded to glucose, and the glucose is immediately utilized by the yeast to produce ethanol (Jeon et al., 2009b). A yeast strain capable of consolidated conversion of cellulosic materials to ethanol has also been reported (Jeon et al., 2009b).

As described above, these results indicate that the hydrolysis of cellulosic materials by cellulolytic enzymes is a major factor in the production of ethanol by recombinant yeast. Efficient direct fermentation of cellulosic materials to ethanol was achieved by developing a yeast strain that expresses a multi-enzyme complex containing a recombinant scaffolding protein and an endoglucanase (Hyeon et al., 2010). The construction of the multi-enzyme complex affected cellulose hydrolysis by combining cellulases with the scaffolding subunit (Hyeon et al., 2010). These results demonstrated a significantly higher concentration of ethanol production

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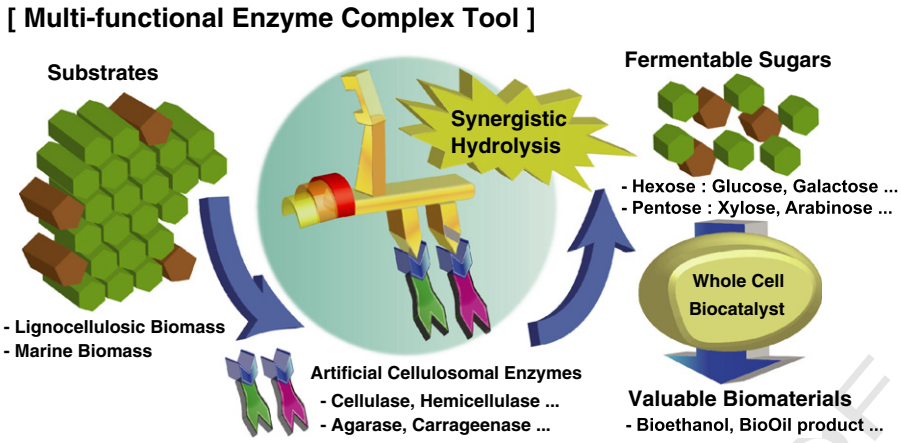

Fig. 4. Schematic illustrations of a multi-functional enzyme complex for biomass utilization. The multi-functional enzyme complex assembly with a recombinant scaffolding protein and a chimeric dockerin-fused enzyme has a high level of hydrolysis activity and can convert the various biomasses to valuable biomaterials.

than that reported in a free enzyme system and demonstrated the feasibility of a multi-enzyme complex with a synergic effect on fermentation using cellulosic materials.

5.2. Utilizing marine biomass

Marine algal polysaccharides such as agar, which is a phycocolloid extracted from the cell wall of a group of red algae, are promising alternative resources to land biomass (O'Sullivan et al., 2010). Agar consists of oligosaccharides, such as agarooligosaccharides and neoagarooligosaccharides, with high economic value (Schroeder et al., 2003). The degradation of marine biomass by enzymatic reactions has several notable advantages, including mild reaction conditions, simple facilities, excellent efficiency, low energy costs, controllable products and minimal environmental impact (Fu and Kim, 2010). Agarase degrades agarose polymer into various types of oligosaccharides that can be used as the first enzyme in the agar catabolic pathway to produce carbon sources for biotechnological development (Hyeon et al., 2012a).

To utilize marine biomass, an agarolytic complex was assembled via a cohesin–dockerin interaction using dockerin-fused chimeric *Zobellia galactanivorans* agarase cAgaB and the recombinant scaffolding protein miniCbpA from *C. cellulovorans* (Hyeon et al., 2012a). The resulting multi-enzyme complex increased the agarase activity level by approximately 1.4-fold compared with the activity level reported for the corresponding enzymes alone and caused a noticeable increase in the amount of sugar produced (Hyeon et al., 2012a). These results demonstrated that the correct folding of subunits with high-affinity interactions is sufficient to assemble the complex and that the cellulosome is a feasible system for efficiently utilizing marine biomass (Hyeon et al., 2012a). The development of a non-cellulase multi-enzyme complex such as the agarolytic complex is a key step in utilizing various biomasses and will assist in the designing of a useful whole-cell biocatalyst that can convert various biomasses to valuable products (Fu and Kim, 2010).

6. Concluding remarks

This review describes the development of a *Clostridium*-derived, multi-functional enzyme complex. The multipurpose potential of this cellulosome-like system suggests its utility as a tool in various challenging biotechnological applications. The CBP-related application of the developed heterologous multi-enzyme complex is a recognized biomass utilization strategy (Lynd et al., 2005). Additionally, the biosensor strategy based on the cohesin–dockerin interaction, the simple cellulose-supported protein purification method using CBM, and the cell surface display strategy employing an anchoring domain, such as the SLH domain, have been developed as tools to advance biotechnology (Hyeon et al., 2012a; Jeon et al., 2012; Kosugi et al., 2002). The mechanistic principles of the *Clostridium*-derived cellulosome, such as the regulation of each enzyme and the synergism of various enzymes, are currently under investigation (Han et al., 2005). A complete understanding of cellulosome formation is important and can be applied to a variety of biotechnology area. The utility of a multi-functional protein complex that is suitable for expression in various industrial host cell systems has drawn considerable attention as an attractive and powerful strategy for achieving viable and cost-effective biomaterial production and whole-cell biocatalysts with various functions (Hyeon et al., 2011; Yu et al., 2012b).

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