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Research Report

Towards an Artificial Cellulosome: Construction of a Hybrid Nano-cellulosome and Identification of Self-assembling Peptides

Takashi Matsuyama

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ABSTRACT Metabolically-engineered microorganisms useful for producing bioplastics or biofuels have been investigated for over 20 years to reduce the increased levels of atmospheric carbon dioxide caused by human activity. Saccharification of refractory crystalline cellulose is important for using biomass feedstock comprising mainly cellulose and hemicellulose. Cellulose is a water-insoluble polysaccharide consisting of a linear chain of several hundred to many thousand β-1,4 linked D-glucose units. Cellulose is converted into water-soluble short-chain sugars by pretreatment and enzymatic hydrolysis by cellulases, and the amount of cellulase used critically impacts the production cost of the target chemicals. Currently, cellulases are commercially supplied as the culture filtrate of *Trichoderma reesei* cells which contains many types of secreted cellulases. There are three approaches for reducing the cost of cellulases: (i) improve cellulase production, (ii) enhance the thermal stability of cellulases, and (iii) increase the activity of the cellulases. To address the last issue, we propose an "artificial cellulosome" with much higher cellulose hydrolysis activity than that of an individual cellulose. In this review, we first investigate the clustering of different cellulase domains on organic and inorganic nano-carriers to generate a hybrid nano-cellulosome, and then identify self-assembling peptides for integrating cellulase domains without subsequent need for nano-carriers.

KEYWORDS Biomass, Cellulose, Cellulase, Artificial Cellulosome, Hybrid Nano-cellulosome, Self-assembling Peptide

1. Introduction

Metabolically-engineered microorganisms that can be used to produce bioplastics or biofuels have been investigated for the last two and half decades as a means of reducing the increased levels of carbon dioxide in the atmosphere caused by human activity.[1] Saccharification of refractory crystalline cellulose is an important issue for using biomass feedstock, in which carbon is mainly in the form of cellulose and hemicellulose. Cellulose is a water-insoluble polysaccharide consisting of a linear chain of several hundred to many thousands of β-1,4 linked D-glucose units. Cellulose is converted into water-soluble short-chain sugars by pretreatment and enzymatic hydrolysis by cellulases, and the amount of cellulase used in this process is a critical factor impacting the production cost of the target chemicals. At present, cellulases are commercially supplied in the form of the culture filtrate of *Trichoderma reesei* cells which contains many types of secreted cellulases. There are three feasible approaches for reducing the cost of cellulases: (i) improve cellulase production,[2,3] (ii) enhance the thermal stability of cellulases,[4] and (iii) increase the activity of the cellulases. To address the last issue, we propose the concept of an "artificial cellulosome" with much higher cellulose hydrolysis activity than that of an individual cellulase (**Fig. 1**).

Many cellulolytic bacteria effectively hydrolyze recalcitrant cellulose substrates by assembling multiple cellulases to form supramolecular complexes, termed cellulosomes.[5,6] The cellulase activity of cellulosomes has often been observed to increase synergistically rather than additively and this may be due to a proximity effect. To reconstitute the extraordinary high cellulose activity of native cellulosomes, we first investigated the clustering of different cellulase domains from various organisms on organic or inorganic nano-carriers to generate hybrid nano-cellulosomes (box with a horizontal arrow in Fig. 1).[7-9] Next, to decrease the cost of the nano-carriers, we screened self-assembling peptides with hydrophobic ionic liquids (box with a vertical

arrow in Fig. 1).[10] We identified two tryptophan-rich "SW peptides", and observed that SW peptide-fused green fluorescent protein (GFP) localized at the water/hydrophobic ionic liquid interface at concentrations below the critical micelle concentration (CMC). We also found that the fusion protein multimerized and self-assembled in water at concentrations above the CMC. Combination of these two distinct technologies allow the formation of "artificial cellulosomes" (upper-right image in Fig. 1) composed of several different cellulase domains fused with a self-assembling peptide. These artificial cellulosomes will be useful for the low-cost generation of bioplastics or biofuels.

2. Construction of Hybrid Nano-cellulosome

Cellulases are classified into several types based on the type of enzyme reaction: endoglucanases, exoglucanases, possessive endoglucanases, and beta-glucosidases.[11] Endoglucanases (e.g., EC 3.2.1.4) randomly hydrolyze internal β-1,4 linkages of cellulose at amorphous sites. Exoglucanases or cellobiohydrolases (e.g., EC 3.2.1.91, EC 3.2.1.176) cleave two to four cellooligosaccharides, such as the cellobiose disaccharide, from the ends of the cellulose produced by endoglucanases. Exoglucanases are classified into type I and type II: type I hydrolyze processively from the reducing end of the cellulose, and type II hydrolyze processively from the nonreducing end. Possessive endoglucanases[12,13] cleave both internal bonds and the reducing end of cellulose to produce cellodextrins or cellobiose. Beta-glucosidase (e.g., EC 3.2.1.21) hydrolyze short-chain soluble cellodextrins released from cellulose by the other cellulases. In fungi, most cellulases have two different functional domains: the catalytic domain (CD) and the carbohydrate-binding module (CBM).[14] A previous classification termed the latter domain the cellulose-binding domain (CBD) and this domain is absent in some endoglucanases.

Multiple cellulases assemble on scaffold proteins, called scaffoldins, to form the cellulosome. Typical scaffoldins have three distinct domains: one or two CBMs to bind cellulose, one or more type I cohesin domains to non-selectively bind the cellulases through interaction with their corresponding type I dockerin, and one type II dockerin domain to anchor the cellulosome on the bacterial cell surface.[15] Cellulosomal cellulases containing type I dockerin are composed of various classes of cellulases and xylanases. The effective degradation of cellulose by cellulolytic bacteria is believed to be due to synergetic attack on the cellulose substrate by multiple cellulases in the cellulosome exhibiting different catalytic activities. The synergetic effect was demonstrated by in vitro reconstitution experiments with mini-cellulosomes that contained

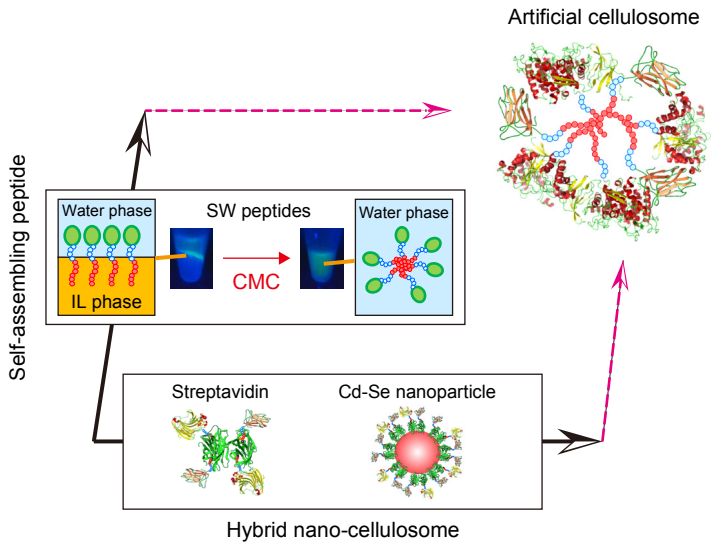

Fig. 1 Strategy towards a concept of "artificial cellulosome". Fusion of two distinct technologies (self-assembling peptide and hybrid nano-cellulosome) allows realizing the concept. In the box of vertical arrow, green ellipse means green fluorescent protein (GFP). A sequential red circles means an SW peptide while a sequential blue circles means a linker-peptide. CMC: critical micelle concentration.

various combinations of a scaffoldin containing one CBM and two cohesions, and two cellulases with one cognate dockerin module.[16] In *Saccharomyces cerevisiae*, *in vivo* reconstitution of functional cellulosome with one CBM and three or five cellulases has been reported.[17]

From the aspect of protein engineering, the catalytic domain and carbohydrate-binding module are considered to be distinct functional module proteins. Recent advances in bioconjugate technology have supplied various concepts for covalent and noncovalent linkages between proteins, which allow us to artificially assemble functional proteins. For example, the coupling between a specific pair of fluorescent proteins can transfer fluorescence energy so as to detect intermolecular interactions,[18] and the assembly of antibody fragments via protein-protein interactions increases the valence of antigen-binding sites to improve therapeutic effects in treating cancer tumors.[19] Furthermore, nanoscale structural designs obtained by molecular assembly and by downsizing of inorganic materials are enabling the fabrication of higher-order functional devices by utilizing the nanomaterials as scaffolds for assembling functional proteins. Protein cages, such as ferritin and viruses, have been widely used as nanoscale building blocks for higher-order nanostructures.[20-22]

Here we focused on a method to cluster these modules not on scaffoldin, but rather on streptavidin or nano-particles to construct hybrid nano-cellulosome complexes exhibiting higher activity than a simple cellulase (**Fig. 2**). The type of modules described in this review are listed in **Table 1**.

2. 1 Hybrid Nano-cellulosome: CD_{EglA} and CBM4[7]

First, we used a set of CD_{EglA} and CBM4 to establish the experimental systems: heterologous expression of CD and CBM in *E. coli*, preparation of these proteins, clustering on carriers, and assay of cellulase activity. CD_{EglA} is the catalytic domain of endoglucanase A (EglA) from *Aspergillus niger*,[23] and CBM4 is the N-terminal CBD in endoglucanase C from *Cellulomonas fimi*.[24,25] Streptavidin and streptavidin-immobilized inorganic CdSe nanoparticles were used as carriers.

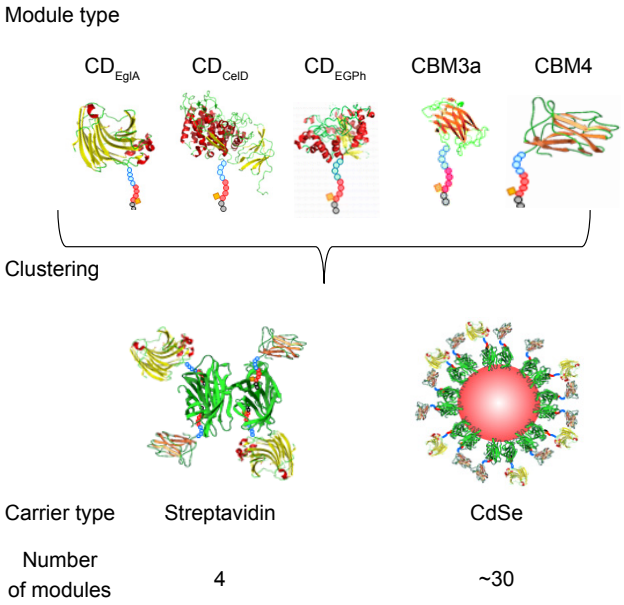

Fig. 2 Schematic illustration of the clustering of CD and CBM on organic or inorganic carriers.

Table 1 List of catalytic domains of cellulases (CDs) and cellulose-binding modules (CBMs) used in this review.

CDs and CBMs	Source organisms	References
CD_{EglA} (endoglucanase)	*Aspergillus niger*	*Small* (2011)
CBM4 (non-cellulosome)	*Cellulomonas fimi*	
CD_{CelD} (endoglucanase)	*Clostridium thermocellum*	*Catal. Sci. Technol.* (2012)
CBM3a (cellulosome)	*Clostridium thermocellum*	
CBM4 (non-cellulosome)	*Cellulomonas fimi*	
CD_{CelD} (endoglucanase)	*Clostridium thermocellum*	*ACS Catal.* (2013)
CD_{EGPh} (possessive endoglucanase)	*Pyrococcus horikoshii* OT3	
CBM3a	*Clostridium thermocellum*	
CBM4	*Cellulomonas fimi*	

The clustering of EglA together with CBD on streptavidin resulted in little improvement of EglA's degradation activity for water-soluble substrates, but the activity of EglA-CBD clusters for water-insoluble substrates increased as the valence of CBD increased in the clustered complexes on streptavidin. Consequently, clustering on streptavidin and on CdSe nanoparticles resulted in significant improvement of degradation activity for PSC, and clustering on streptavidin and on the nanoparticles enabled the degradation of Avicel (**Fig. 3** and Original **Fig. 4**). Cellulases in cellulosomes show effective activity for cellulose material degradation, but individual cellulases separated from cellulosomes have low degradation activity.[16,26] Carrard et al. generated several recombinant CBD molecules with a cohesin domain: the cohesin-fused CBD spontaneously binds to dockerin-containing cellulases at a ratio of 1:1 to activate the enzymes for water-insoluble substrates.[27] In this study, we drastically increased the activation of the endoglucanase EglA by clustering it with CBD on streptavidin and on nanoparticles. Our design not only improved the degradation activity of EglA owing to its conjugation with CBD, but also showed the avidity effect caused by the multivalence of CBD on the degradation activity.

2. 2 Hybrid Nano-cellulosome: CD_{CelD}, CBM3a, and CBM4[8]

In a previous study, we demonstrated that the substrate-targeting effect by CBM4 was important in the degradation of the crystalline cellulose Avicel. CBM4 has been reported to have affinity for amorphous celluloses, whereas CBM3a, which is a module of the scaffoldin from *Clostridium thermocellum*,[28] binds to the surface of microcrystalline cellulose.[27] Thus, we investigated whether or not these CBMs could result in differences in the degradation of cellulose by the corresponding hybrid nano-cellulosomes. CD_{CelD}, which is a catalytic domain of endoglucanase D from *Clostridium thermocellum*,[29] was used as a CD module because it exhibits higher cellulose hydrolysis activity than does CD_{EglA}.

Figure 4 shows the degradation of Avicel substrates by using the CD–CBM clusters. Consequently, although the enhancement of CD_{CelD} activity for Avicel is less drastic than that for PSC, the clustering of CD_{CelD} with CBM was also effective for the degradation of Avicel (Fig. 4), and the correlation between clustering format and activity enhancement denoted the same tendency of the degradation of PSC: the clustering with CBM3a on streptavidin more enhanced CD_{CelD} activity than on nanoparticles, while CBM4 enhanced CD_{CelD} activity

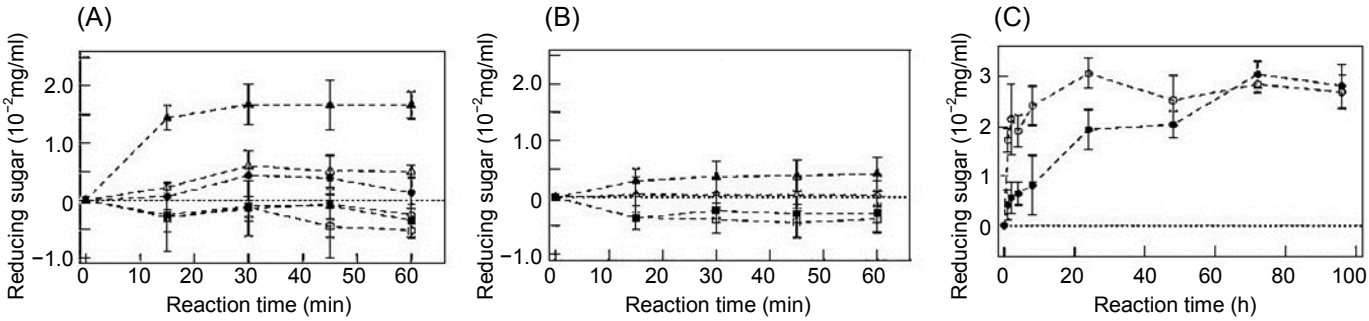

Fig. 3 Amounts of reducing sugars produced from 1 mg mL^{-1} avicel in a 50 mM sodium acetate solution (pH 5.0, 200 mM NaCl) at 40°C. (A) Degradation reaction for 60 min in the presence of 2 μM EglA (open circles), 2 μM EglA and 6 μM CBD (closed circles), and EglA–CBD clusters containing 2 μM EglA, 0 μM CBD, and 0.5 μM streptavidin (open squares), 2 μM EglA, 0.67 μM CBD, and 0.67 μM streptavidin (closed squares), 2 μM EglA, 2 μM CBD, and 1 μM streptavidin (open triangles), and 2 μM EglA, 6 μM CBD, and 2 μM streptavidin (closed triangles). (B) Degradation reaction for 60 min in the presence of 13.3 nM CdSe nanoparticles with 0.4 μM EglA (open squares), 17.8 nM CdSe with 0.4 μM EglA and 0.13 μM CBD (closed squares), 26.7 nM CdSe with 0.4 μM EglA and 0.4 μM CBD (open triangles), 53.3 nM CdSe with 0.4 μM EglA and 1.2 μM CBD (closed triangles). (C) Degradation reaction for 96 h in the presence of EglA–CBD clusters containing 0.4 μM EglA, 1.2 μM CBD, and 0.4 μM streptavidin (open circles), and 53.3 nM CdSe nanoparticles with 0.4 μM EglA and 1.2 μM CBD (closed circles). All experiments were conducted three times and average values were plotted with error bars of standard variation.

by clustering on nanoparticles. The use of one type of endoglucanase usually enables degradation of simple substrates, but our clustering design using streptavidin and nanoparticles was effective for the degradation of crystalline cellulose by using only one type of endoglucanase. Addition of a second type of enzyme to the complex might be helpful in further promoting the degradation of crystalline substrates.

2. 3 Hybrid Nano-cellulosome: CD_{CelD}, CD_{EGPh}, CBM3a, and CBM4[9]

In this study, we investigated the synergetic effects of the degradation of amorphous (**Fig. 5**) and crystalline (**Fig. 6**) cellulose by two different ypes of CDs. We used CDs from two endoglucanases: endoglucanase D, a glucoside hydrolase (GH) 9-family endoglucanase from *Clostridium thermocellum* (CD_{CelD}),[29] and endoglucanase EGPh, a GH5-family processive endoglucanase from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 (CD_{EGPh}),[30,31] and the two CDs were clustered on separate nanoparticles or on the same nanoparticle.

Nanocellulosomes constructed by separately clustering each type of CD with multiple CBMs on nanoparticles showed 5-fold enhancement in cellulase degradation activity relative to that of the corresponding free CDs, and mixtures of the two types of nanocellulosomes gradually and synergistically enhanced cellulase degradation activity as the CBM valency increased (finally, 2.5 times). Clustering the two types of CD together on the same nanoparticle resulted in a greater synergistic effect that was independent of CBM valency; consequently, nanocellulosomes composed of equal amounts of the endo and endoprocessive CDs clustered on a nanoparticle along with multiple CBMs (CD/CBM = 7:23) showed the best cellulose degradation activity, producing 6.5 and 2.4 times the amount of reducing sugars produced from amorphous and crystalline cellulose, respectively, by the native free CDs and CBMs in the same proportions. Our results demonstrate that hybrid nanocellulosomes constructed from the building blocks of cellulases and cellulosomes modules have the potential to serve as high-performance artificial cellulosomes.

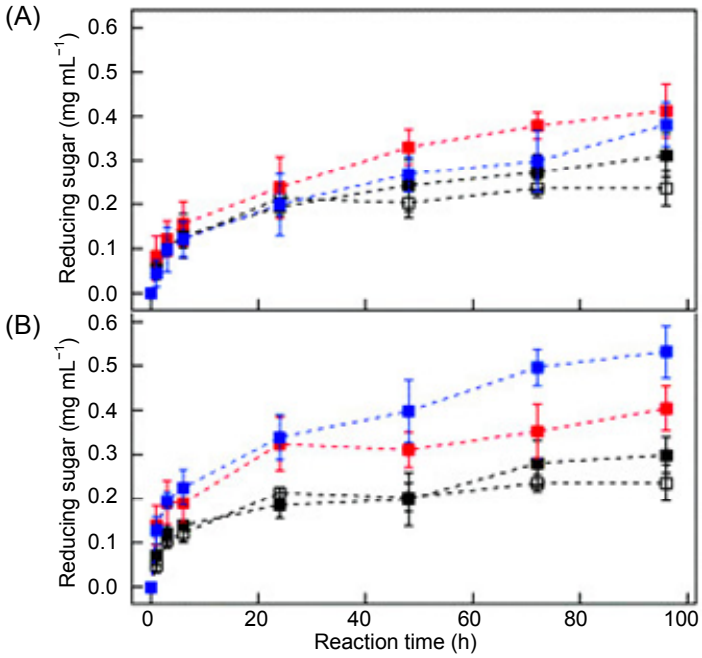

Fig. 4 Amounts of reducing sugars produced from 10 mg ml^{-1} avicel in a 50 mM MES buffer solution (pH 6.0, 10 mM $CaCl_2$) at 45°C for 96 h in the presence of CD_{CelD}–CBM3a clusters (A) and CD_{CelD}–CBM4 clusters (B). Each reaction was performed in the presence of CD_{CelD} (open black squares), a mixture of 1 CD_{CelD} and 3 CBM (closed black squares), CD_{CelD}–CBM clusters at a ratio of 1 CD_{CelD} to 3 CBM per streptavidin molecule (closed red squares), and CD_{CelD}–CBM clusters at a ratio of 7 CD_{CelD} to 23 CBM per nanoparticle (closed blue squares). All the experiments were carried out at the CD_{CelD} concentration of 2.5 μM. Each experiment was conducted three times, and the average values are plotted with error bars representing standard variation.

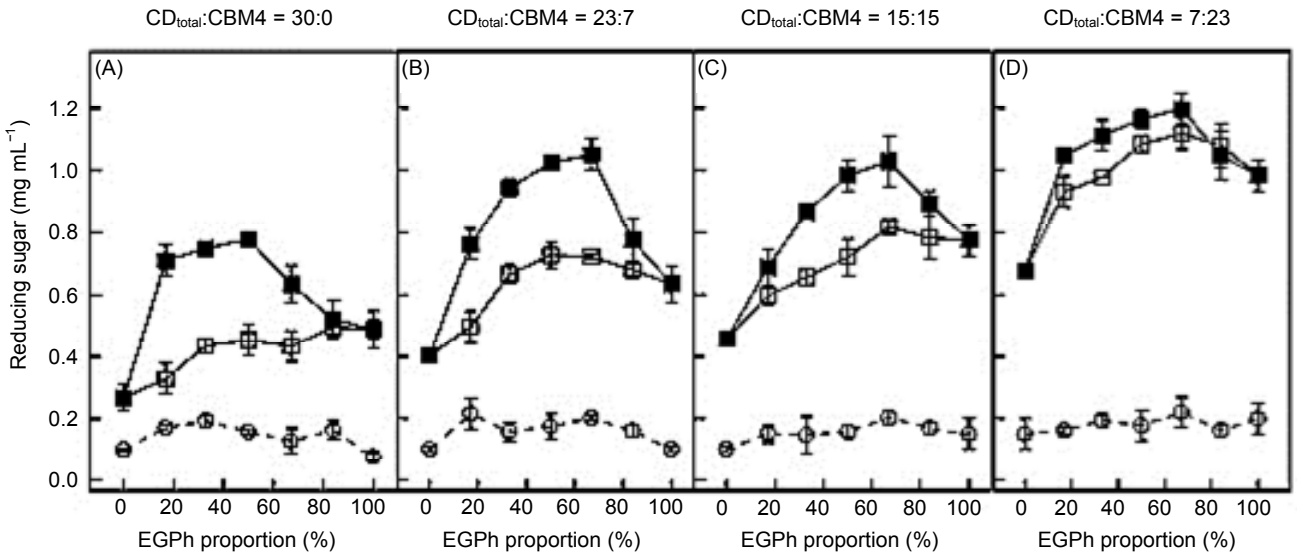

Fig. 5 CD_{EGPh} proportion dependence of reducing sugar production from PSC (3.5 mg mL^{-1}) in a 50 mM acetate buffer (pH 5.0, 200 mM NaCl) at 45°C at 96 h with a mixture of free CDs and CBM4 (open circles), with a mixture of CD_{CelD}–CBM4 and CD_{EGPh}–CBM4 clusters on separate streptavidin-conjugated CdSe nanoparticles (open squares), and with CD_{CelD} and CD_{EGPh} clustered on the same nanoparticle with CBM4 (closed squares). All the experiments were done at a total CD concentration (CD_{CelD} + CD_{EGPh}) of 40 nM and CD/CBM4 ratios were 30:0 (A), 23:7 (B), 15:15 (C), and 7:23 (D). Each experiment was conducted three times, and average values are plotted with error bars representing standard variations.

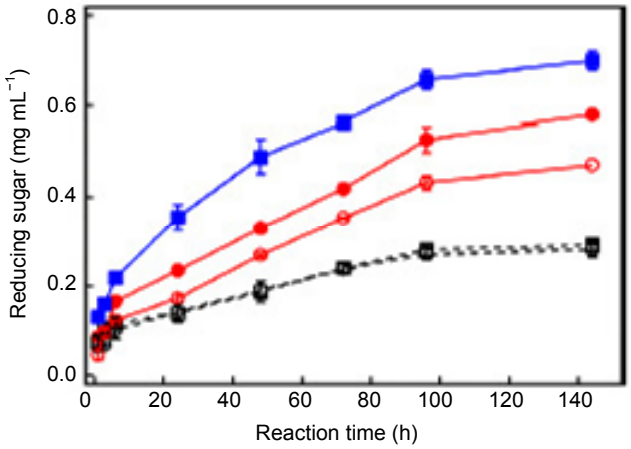

Fig. 6 Production of reducing sugars from Avicel (10 mg mL^{-1}) in a 50 mM acetate buffer (pH 5.0, 200 mM NaCl) at 45°C for 144 h with a mixture of free CD_{EGPh} and CBM4 (open black circles), a mixture of free CD_{CelD} and CD_{EGPh} (in equal proportions), and CBM4 (open black squares), CD_{EGPh} clustered with CBM3a on streptavidin-conjugated CdSe nanoparticles (open red circles), CD_{EGPh} clustered with CBM4 on streptavidin-conjugated CdSe nanoparticles (closed red circles), and CD_{CelD} and CD_{EGPh} (in equal proportions) clustered with CBM4 on the same nanoparticle (closed blue squares). All the experiments were done at a total CD concentration of 2.5 μM and a CD/CBM4 ratio of 7:23. Each experiment was conducted three times, and average values are plotted with error bars representing standard variations.

3. Identification of Self-assembling Peptides[10]

The above and other studies showed the synergetic effects of multiple types of CDs and CBMs on the degradation of crystalline cellulose.[16,26,32] Biotin, streptavidin, and the nanoparticles used in our studies are too expensive to be used in industrial processes. An alternative approach is to cluster heterologous cellulases on scaffoldin for industrial cellulase complexes, but the low number of clustered cellulases is problematic. For example, the native form of *Clostridium thermocellum* scaffoldin containing 9 cohesin domains has yet to be successfully overexpressed to reconstitute the cellulosome *in vitro* or *in vivo*. This difficulty is likely due to CipA being a large glycoprotein of 210000 Da with several hydrophobic regions.[28] These results suggest that utilization of the synergetic effect observed in cellulosomes for industrial processes requires alternative ways to assemble multiple types of cellulases. On the other hand, the self-organization of supramolecules through either hydrophobic or hydrogen bonds in the peptide secondary structure, such as α-helical coils and β-sheets, have been reported.[33,34]

Here we identified and characterized several peptides that self-assemble when fused with soluble proteins (**Fig. 7**). Two tryptophan-rich peptides, "SSSWWSWWWW" (SW1) and "SWWWWSWWWW" (SW2), show affinity for a hydrophobic ionic liquid (IL), and the fluorescence intensity (FI) of green fluorescent protein (GFP) fused to these peptides was observed at the IL/water interface (**Fig. 8**). To clarify the localization mechanism of SW1-GFP at the water/IL interface, we analyzed the localization behavior of SW1-GFP at various concentrations (Fig. 8). As SW1-GFP concentration increased from 0.9 μM to 7.1 μM, the FI at the interface intensified while the FI of the water phase remained unchanged (Figs. 8(A) and (B)). These results indicate that most of the SW1-GFP proteins were localized at the interface. Interestingly, at a concentration of 14.3 μM, the interfacial FI decreased markedly (Fig. 8(A)). We considered that this decrease could be attributed to a kind of phase transition, such as the formation of micelle-like structures in the water phase, at protein concentrations between 7.1 and 14.3 μM. Another possible explanation was that the quenching of GFP fluorescence occurred at the interfaces because SW1-GFPs were so accumulated at the interface. At SW1-GFP concentrations of more than 14.3 μM, FI at the interface was constant but the FI in the water gradually increased. At 57 μM, FI became

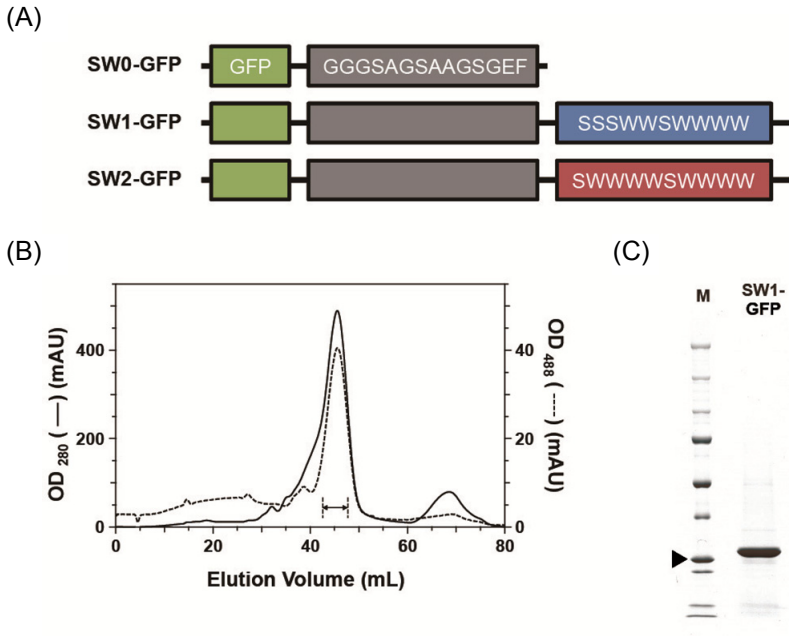

Fig. 7 Purification of SW1-GFP. (A) Schematic structure of the SW-peptide-fused GFP proteins (top, SW0-GFP; middle SW1-GFP; bottom, SW2-GFP). (B) Chromatogram of SW1-GFP from anion exchange column chromatography. The collected fractions are indicated by an arrow. (C) SDS-PAGE of the purified SW1-GFP. Left lane (M) molecular weight marker (250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa from the top to the bottom). Arrow indicates the 25 kDa marker.

comparable to that observed at the interface (Figs. 8(A) and (B)). These behaviors imply that the interface area was saturated by SW1-GFP at concentrations between 7.1 and 14.3 μM (Fig. 8(C)), and also supports our suggestion that SW1-GFP behaves like surfactant.

4. Conclusion

Our studies pave the way for the development of artificial cellulosomes, but true artificial cellulosomes have yet to be achieved. A database of Carbohydrate-Active enZYmes (CAZYmes) in 2013 included 131 GH families, including enzymes that degrade cellulose or xylan, and 64 CBM families.[35] Combinations of CDs and CBMs in various proportions should be optimized by rational methods and exploratory approaches such as combinatorial screening. There is an issue with self-assembling peptides in that SW peptides are so hydrophobic that SW-fused proteins can aggregate into inclusion bodies. It may be possible to control artificial-cellulase supramolecular behavior by pH regulation.[36] Most cellulases have peak cellulose hydrolysis activity at pH 5 while the pH of cytoplasm is about 7. Aspartic acid (pKa = 3.9) and glutamic acid (pKa = 4.1) are protonated at pH 5 and deprotonated at pH 7. Therefore, either the addition or substitution of these acidic amino acids into SW peptides should allow the design of self-assembling peptides. Specifically, these peptide-fused cellulases could form cellulosome-like supramolecular complexes in the reaction medium at pH 5, whereas their positive charge would cause them to separate from each other before secretion. Further investigation will provide artificial cellulosomes with high activity applicable for the bio-production of valuable chemicals.

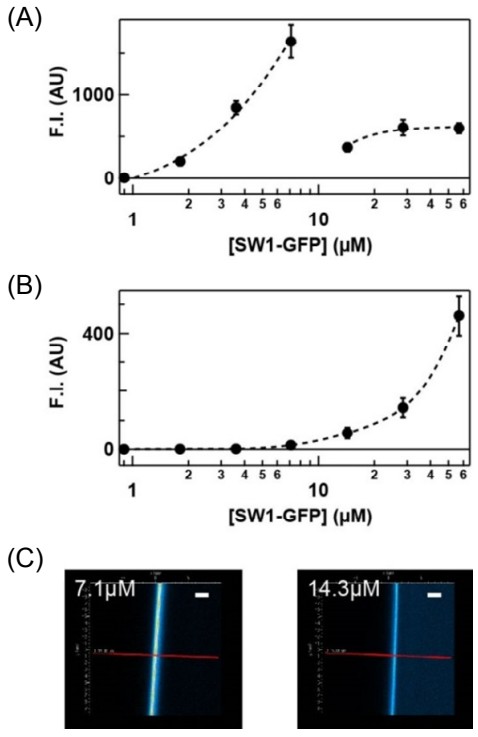

Fig. 8 Concentration dependence of SW1-GFP localization at the interface (A) and in the water phase (B). Each average value of FI and errors was calculated as well as described in Fig. 3. The FIs were measured at 0.9, 1.8, 3.6, 7.1, 14.3, 28.5 and 57 μM. Dotted lines were interpolated. (C) Representative GFP fluorescence images at discontinuous points. Scale bar (white line) represents 2 μm.

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Takashi Matsuyama
Research Fields:
- Molecular Biology in *Saccharomyces cerevisiae*
- Metabolic Engineering
- Synthetic Biology

Academic Degree: Ph.D.
Academic Societies:
- The Japan Society for Bioscience, Biotechnology, and Agrochemistry
- The Society for Biotechnology, Japan
- Yeast Genetics Society of Japan
- The Japanese Society of Plant Morphology

Award:
- Encouragement Award of the Japanese Society of Plant Morphology, 1999

