ACS Catalysis

Research Article

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Hybrid Nanocellulosome Design from Cellulase Modules on Nanoparticles: Synergistic Effect of Catalytically Divergent Cellulase Modules on Cellulose Degradation Activity

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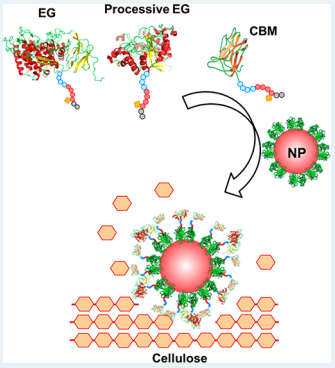
Supporting Information

ABSTRACT: Cellulosomes, which are assemblies of cellulases with various catalytic functions on a giant scaffoldin protein with a carbohydrate-binding module (CBM), efficiently degrade solid cellulosic biomass by means of synergistically coupled hydrolysis reactions. In this study, we constructed hybrid nanocellulosomes from the biotinylated catalytic domains (CDs) of two catalytically divergent cellulases (an endoglucanase and a processive endoglucanase) and biotinylated CBMs by clustering the domains and modules on streptavidin-conjugated nanoparticles. 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.



KEYWORDS: *cellulase, cellulosome, cluster effect, green chemistry, nanoparticles, protein assembly*

INTRODUCTION

Cellulose, a water-insoluble polysaccharide of glucosyl units connected by β-1,4 linkages, is an abundant carbon resource and a renewable biomass that can be used to produce alternative fuel. Cellulolytic bacteria and fungi hydrolyze cellulose to low-molecular-weight sugars by means of cellulolytic enzymes called cellulases,[1] which can be classified into three types: endoglucanases, exoglucanases, and processive endoglucanases.[2,3] Endoglucanases (e.g., E.C.3.2.1.4) randomly hydrolyze celluloses to cellooligosaccharides, exoglucanases (e.g., E.C.3.2.1.91 and E.C.3.2.1.176) degrade the chain ends of cellulose to cellobiose or glucose, and processive endoglucanases directly release short soluble cellodextrins, such as cellobiose and cellodextrin, after an endotype attack on cellulose.[4] Cooperative hydrolytic reactions involving these cellulases can degrade cellulose with low energetic and environmental loads, that is, cellulose can be degraded near room temperature in relatively neutral solution (pH 5−8).

At present, ∼1000 cellulases have been reported and 30% of them have carbohydrate-binding modules (CBMs) that promote the degradation of cellulose by substantially increasing the substrate concentration around the catalytic domain (CD).[5] Some bacteria produce complicated protein complexes called cellulosomes, in which 5−10 cellulases are tandemly arranged, by means of cohesin−dockerin interactions, on a giant scaffold protein containing a CBM,[6,7] and the clustering of catalytically divergent CDs in cellulosomes leads to synergistic degradation of biomass materials by means of coupled hydrolysis reactions.[8] Taking advantage of the high degradation efficiency of cellulosomes for the production of useful glucosyl units from biomass is an attractive possibility of cellulases for industrial use, but the currently available processes for extracting intact cellulosomes from native bacteria or preparing cellulosomes from recombinant proteins are not practical for industrial applications.

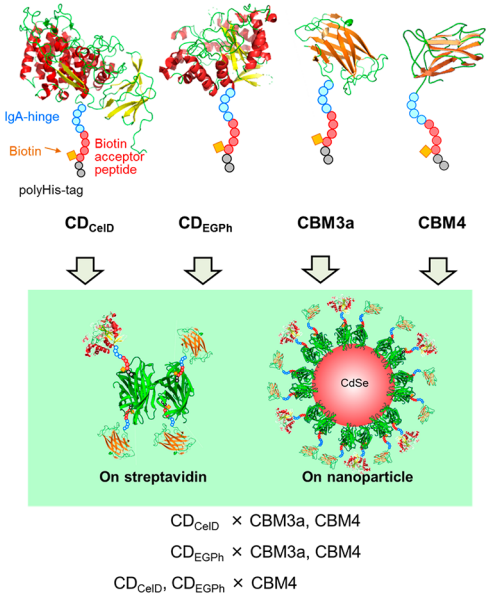
To facilitate the degradation of cellulose, chimeric cellulosomes have been designed and fabricated by attaching individual building blocks to several small scaffoldins modeled

Received: January 4, 2013
Revised: May 8, 2013
Published: May 10, 2013

after cellulosome-derived scaffold modules to form minicellulosomes with two or three CDs.[9−13] Recently, we designed novel artificial cellulosomes by using cellulosome building blocks that are separately prepared and then reassembled in vitro on the surface of streptavidin-conjugated CdSe nanoparticles.[14,15] Use of a nonbiological nanostructure in place of the scaffoldin eliminates the need to prepare the giant cellulosome-derived scaffold protein and,[16−18] furthermore, allows the design of structures that are more highly clustered than native cellulosomes. Clusters of endoglucanases with CBMs prepared by this method show substantially enhanced catalytic activity (7−10-fold) relative to that of free enzymes.[14,15]

In this study, we improved our hybrid nanocellulosome design, where CDs and CBMs are homogeneously oriented on inorganic nanoparticles, by mimicking the concept of native cellulosome that the clustering of several catalytically divergent CDs to allow for synergistic degradation reactions (Scheme 1).

Scheme 1. Clustering of CDs and CBMs on Streptavidin and on Streptavidin-Conjugated Cdse Nanoparticles[a]



[a]The protein data bank (PDB) data of number 1CLC, 2ZUM, 1NBC, and 1CX1 were referred for describing the protein structures of CD_{CelD}, CD_{EGPh}, CBM3a, and CBM4, respectively.

We used CDs from two endoglucanases: endoglucanase D, a GH9-family endoglucanase from *Clostridium thermocellum* (CD_{CelD}),[19] and endoglucanase EGPh, a GH5-family processive endoglucanase from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 (CD_{EGPh}),[20,21] and the two CDs were clustered on separate nanoparticles or on the same nanoparticle. Clustering with multiple CBMs on nanoparticles dramatically enhanced its degradation activity, and clustering of CD_{CelD} and CD_{EGPh} together with multiple CBMs synergistically enhanced the degradation activity further. Our results demonstrate that hybrid nanocellulosomes constructed from the building blocks of cellulase and cellulosome modules have the potential to serve as high-performance artificial cellulosomes.

EXPERIMENTAL SECTION

Substrates. Microcrystalline cellulose (Avicel PH101) was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan) and phosphoric-acid swollen cellulose (PSC) was prepared from Avicel according to a previously published method.[22] Briefly, Avicel (4 g) was dissolved in 100 mL of phosphoric acid and the solution was stirred for 1 h at 4 °C, diluted with 1900 mL of cold water, and then stirred for 1 h at 4 °C. The amorphous cellulose was collected by filtration with filter paper and was washed four times with ultrapure water, twice with 1% $NaHCO_3$ (neutralization), and then three more times with ultrapure water. The resulting cellulose paste was homogenized (2 min × 3) with a Multibrander mill (BLA-501, Nihonseiki Kaisha LTD, Tokyo, Japan), and the slurry was resuspended in 50 mM sodium acetate buffer (pH 5.0) for enzyme assay and stored at 4 °C.

Construction of Expression Vectors and Preparation of Biotinylated Cellulase Modules. Plasmids encoding (1) CD_{CelD}, (2) family 3a CBM from a cellulosome-integrating protein of *Clostridium thermocellum* (CBM3a),[23] and (3) family 4 CBM from the second N-terminal domain of endoglucanase C from *Cellulomonas fimi* (CBM4),[24] all with an IgA hinge linker (SPSTPPTPSPSTPP),[25] a biotin acceptor peptide (AviTag; GGLNDIFEAQKIEWH),[26] and a polyhistidine tag (HHHHHH), in that order, at the C-termini (Figure S1, Supporting Information), were used as previously produced (pRA2b-bioCelD, pRA2b-bioCipA, and pRA2b-bioCBD2endc, respectively),[14,15] to prepare CD_{CelD}, CBM3a, and CBM4 with AviTag.

To prepare CD_{EGPh} with AviTag, we synthesized the gene fragment encoding CD_{EGPh} from several oligonucleotides and external primers by means of overlap extension polymerase chain reaction with LA-taq DNA polymerase, and the fragment was inserted into the *Nco*I−*Sac*II site of pRA2b vectors containing an IgA hinge linker, an AviTag, and a polyhistidine tag (pRA2b-bioEGPh) to generate the plasmid.

Using the prepared plasmids, we expressed the biotinylated CDs and CBMs in *Escherichia coli* and purified the recombinant proteins as described in previous reports.[14,15] Briefly, we first transformed *E. coli* BL21 (DE3) by the plasmid of pBIRAcm encoding biotin ligase (Avidity Inc., Aurora, CO) and then transformed the same cells by the plasmids of pRA2b-bioCelD, pRA2b-bioCipA, pRA2b-bioCBD2endc, and pRA2b-bioEGPh, respectively. The transformed *E. coli* cells were incubated in the medium containing 50 μM of D-biotin (Sigma, St. Louis, MO), so that a biotin molecule was simultaneously labeled on the AviTag of CD and CBM expressed in *E. coli* cell. The expressed biotinylated CDs and CBMs were purified by means of a metal−chelate chromatography column and gel filtration chromatography (Hi-Load 16/60 Superdex 75 size exclusion column, GE Healthcare, Little Chalfont, U.K.), and the fractionated CD and CBM with biotinylated tag were collected after the presence of biotin was confirmed in the proteins by means of western-blotting analysis using streptavidin−horseradish peroxidase (GE Healthcare).

Clustering of Biotinylated CDs and CBMs on Streptavidin and on Streptavidin-Conjugated CdSe Nanoparticles. The biotinylated CDs and CBMs were mixed with (1) streptavidin (4 biotin binding sites) at various molar ratios in 50 mM sodium acetate (pH 5.0) with 200 mM NaCl at 4 °C for 24 h or (2) streptavidin-conjugated CdSe nanoparticles (size = 20 nm, biotin binding site = 30 sites,

dx.doi.org/10.1021/cs400012v | ACS Catal. 2013, 3, 1342−1348

Invitrogen, Carlsbad, CA) at various molar ratios in sodium acetate at 4 °C for 24 h, under the restriction that biotinylated cellulase modules (sum of CD and CBM) and scaffolds (streptavidin or nanoparticles) were mixed at the module/scaffold molar ratio of 4:1 (streptavidin) or 30:1 (nanoparticle) to theoretically saturate the biotin binding sites on streptavidin or nanoparticles with biotinylated CDs and CBMs.

Cellulase Degradation Activity Assays. CD−CBM clusters were added to 50 mM sodium acetate (pH 5.0, 200 mM NaCl) containing 3.5 mg mL^{-1} PSC and 10 mg mL^{-1} Avicel at 45 °C. The final concentrations of the CDs in the reaction solutions were adjusted to 40 nM and 2.5 μM for degradation activity assays against PSC and Avicel (CelD = 0.74 mg CD/g PSC and 16.3 mg/g Avicel, EGPh = 0.62 mg CD/g PSC and 13.3 mg/g Avicel), respectively. Each concentration of biotinylated CDs and CBMs was estimated using the following extinction coefficients (L mol^{-1} cm^{-1}): CD_{CelD} = 120200, CD_{EGPh} = 133300, CBM3a = 40700, CBM4 = 26100. After incubation intervals of 1−96 h or 1−120 h, 5 μL of the supernatant was mixed with 195 μL of tetrazolium blue chloride (TZ) assay buffer (1 mg mL^{-1} TZ, 0.5 M sodium tartrate, 200 mM NaOH, pH 5.0) at 100 °C for 3 min.[27] After the reaction, the solutions were rapidly cooled in an ice bath, and their absorbances at a wavelength of 655 nm were measured. The concentrations of the reducing sugars produced were estimated from the absorbance by normalization against the results of assays of glucose in TZ buffer.

RESULTS

Homoclustering of the Endoprocessive CD (CD_{EGPh}). In our previous study, a recombinant endo CD (CD_{CelD}) with a IgA 80 hinge linker and an biotinylated AviTag at the C-terminus was clustered on streptavidin and streptavidin-conjugated CdSe nanoparticles (20 nm).[15] Here, we clustered CD_{EGPh}, an endoprocessive CD, by using the same method to investigate the utility of the clustering format for this endoprocessive enzyme. The homoclustering of biotinylated CD_{EGPh} on streptavidin and on nanoparticles increased the degradation of PSC: the clustered CD_{EGPh} on streptavidin and on nanoparticles continuously produced reducing sugars from PSC for 96 h (red marks in Figure 1) and they had produced 3.3 and 3.8 times the amount of sugars produced by free CD_{EGPh}, respectively. In contrast, the clustering of CD_{CelD} initially increased the degradation of PSC; however, the rate plateaued 4 h after the reaction started, and consequently the amount of reducing sugars produced for 96 h was only about twice that produced by free CD_{CelD}.[15] The clustering format using streptavidin and nanoparticles via biotin−avidin interaction was also available for endoprocessive CD, but changing hydrolysis behavior of enzyme influenced the continuance of degradation by clustered CD.

In these assays, the amount of produced sugars was normalized by the mole concentration of CDs. If we regarded clustered CDs on scaffold (streptavidin or nanoparticles) as single enzyme, apparent activity enhancement because of multiple catalytic active site design in single enzyme would be gotten over the results. Therefore, our results indicate that the cluster effect by clustering on streptavidin and nanoparticles is different from that by simple multiple catalytic active site design.

Heteroclustering of CD_{EGPh} with CBM3a or CBM4. We previously reported that heteroclustering of CD_{CelD} with CBM enhances cellulose degradation activity more than homoclustering of CD_{CelD} does.[14,15] Here, CD_{EGPh} was clustered with CBM3a or CBM4 on streptavidin and on nanoparticles (Figure 2). Addition of CBM3a without streptavidin improved CD_{EGPh} activity only slightly, but clustering of CD_{EGPh} with CBM3a on streptavidin further enhanced the degradation activity of CD_{EGPh} and the enhancement increased with the increase of CBM3a valency (Figure 2a): for the reaction time of 96 h, CD_{EGPh}−CBM3a clusters with one CD_{EGPh} and three CBM3as per streptavidin produced 0.6 mg mL^{-1} of reducing sugars, 2.9 times the amount produced by a mixture of free CD_{EGPh} and CBM3a at the same ratio. Similar results were observed when the domains and modules were clustered on nanoparticles

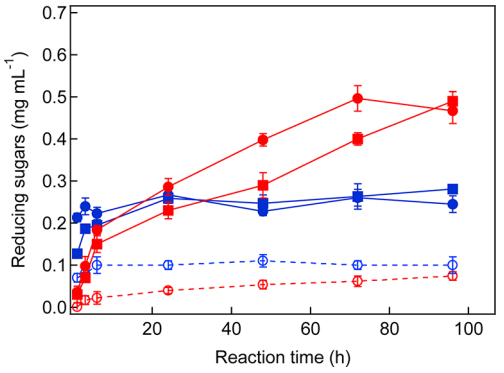


Figure 1. Production of reducing sugars from phosphoric-acid swollen cellulose (PSC, 3.5 mg mL^{-1}) in a 50 mM acetate buffer (pH 5.0, 200 mM NaCl) at 45 °C for 96 h in the presence of CD_{EGPh} (red) or CD_{CelD} (blue) clusters on streptavidin (closed circles) and on streptavidin-conjugated CdSe nanoparticles (closed squares). All the experiments were carried out at a CD concentration of 40 nM. Each experiment was conducted three times, and average values are plotted with error bars representing standard variations. Free CD_{EGPh} and CD_{CelD} (open circles) were used as references.

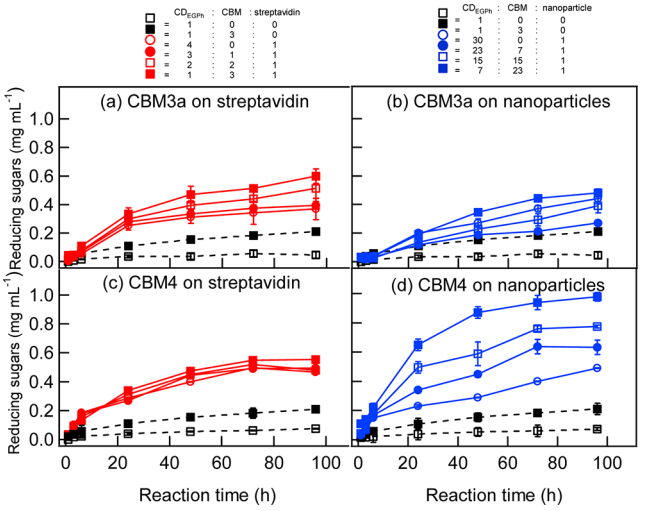


Figure 2. Production of reducing sugars from PSC (3.5 mg mL^{-1}) in a 50 mM acetate buffer (pH 5.0, 200 mM NaCl) at 45 °C for 96 h in the presence of clusters of CD_{EGPh} and CBM (a, b CBM3a; c, d CBM4) on streptavidin (a, c) and on streptavidin-conjugated CdSe nanoparticles (b, d). All the experiments were done at a CD_{EGPh} concentration of 40 nM. Each experiment was conducted three times, and average values are plotted with error bars representing standard variations. Free CD_{EGPh} and free CD_{EGPh}−CBM mixtures were used as references.

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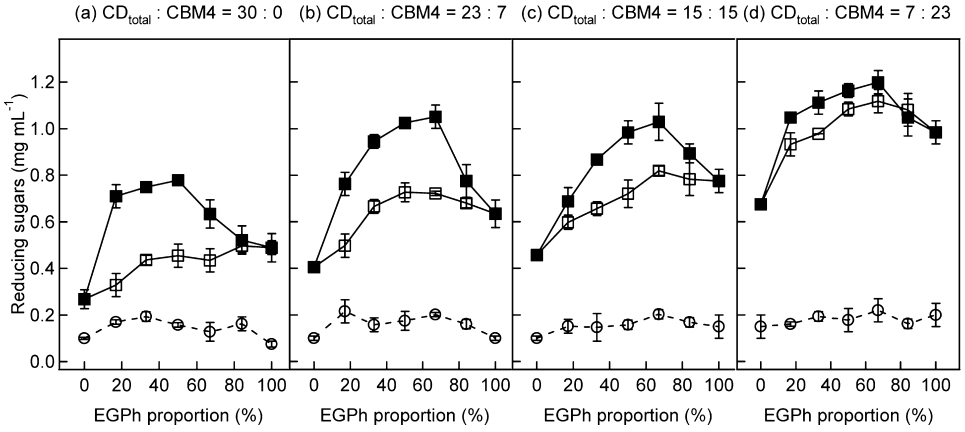

Figure 3. CD_{EGPh} proportion dependence of reducing sugar production from PSC (3.5 mg mL^{-1}) in a 50 mM acetate buffer (pH 5.0, 200 mM NaCl) at 45 °C at 96 h with a mixture of free CDs and CBM4 (open circles), with a mixture of CD_{CelD}−CBM4 and CD_{EGPh}−CBM4 clusters on separate streptavidin-conjugated 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.

(Figure 2b). The fluorescence from CdSe nanoparticles was observed on the surface of PSC (Figure S3, Supporting Information), indicating that the binding of CD_{EGPh}−CBM3a clusters on nanoparticles enhanced the degradation activity of CD_{EGPh}.

As with CBM3a, clustering of CD_{EGPh} with CBM4 on streptavidin enhanced the degradation activity relative to that observed with the free domains and modules; however, the enhancement was independent of the CBM valency (Figure 2c). In contrast, when CD_{EGPh} was clustered with CBM4 in equal proportions on nanoparticles, the increase in degradation activity was higher than that observed for homoclustered CD_{EGPh}, and increasing the CBM4 valency resulted in a gradual enhancement of degradation activity (Figure 2d). Consequently, for 96 h, the clusters with a CD_{EGPh}/CMB4 molar ratio of 7:23 had produced 4.7 times the amount of reducing sugars produced by a mixture of free CD_{EGPh} and CBM4. In our previous study of CD_{CelD}, clusters with a 7:23 CD_{CelD}/CBM4 molar ratio on nanoparticles also showed the highest degradation activity.[15] The result that the increase of CBM valency in the clustering format using nanoparticles significantly enhanced the degradation activity of clustered CD was observed for both endotype and endoprocessive cellulases. In comparison with CBM3a and CBM4, the clustering with CBM4 on nanoparticles more enhanced the CD activity than with CBM3a. This implies that this enhancement might depend on the binding specificity of the CBM chosen in combination with the substrate used.

Clustering of CD_{CelD} and CD_{EGPh} on Separate Nanoparticles and on the Same Nanoparticle. To investigate the effect of clustering endo and endoprocessive CDs together with CBMs on cellulose degradation activity, we first clustered CD_{CelD} and CD_{EGPh} separately with CBM4 on different nanoparticles at various CD/CBM molar ratios and then added nanoparticles with identical CD/CBM molar ratios to PSC suspensions at a range of CD_{EGPh} proportions (0−100%; Figure S4a, Supporting Information). We chose CBM4 over CBM3a because CBM4 enhanced the degradation activity of CD_{CelD} and CD_{EGPh} on nanoparticles more than CBM3a did (see Figure 2 and our previous report[15]). Under all the tested conditions, the PSC degradation behavior was similar to that observed for CD_{CelD}−CBM4 and CD_{EGPh}−CBM4 clusters separately (Figure 2d): PSC degradation increased with the increase of CBM valency. However, CD_{CelD}−CBM4 and CD_{EGPh}−CBM4 clusters together produced more reducing sugars than they did separately, indicating that coexistence of the two clusters synergistically increased PSC degradation.

We also clustered CD_{CelD} and CD_{EGPh} on the same nanoparticle at various CD/CBM4 molar ratios and CD_{EGPh} proportions (Figure S4b, Supporting Information). Under these conditions, coexistence of CD_{CelD} and CD_{EGPh} synergistically increased PSC degradation with the increase of CBM valency. However, comparison of the amounts of sugars produced over the course of 96 h showed that the dependence of the synergy on CBM valency differed between CDs clustered on separate nanoparticles and CDs clustered on the same nanoparticle (Figure 3): mixtures of CD−CBM clusters on separate nanoparticles showed a synergistic effect on PSC degradation as the CBM valency increased (opened squares), whereas CD−CBM clusters on the same nanoparticle showed a different synergistic effect, one that was independent of CBM valency (closed squares). When the amounts of CD_{CelD} and CD_{EGPh} were equal (CD_{EGPh} proportion = 50%) and no CBM4 was present, the amount of sugars produced was 1.7 times the amount produced by an equal mixture of individual CD clusters, whereas clusters of 7 CDs (CD_{EGPh} proportion = 50%) and 23 CBM4 units produced only 1.1 times the amount of sugars produced by the corresponding mixture of CD clusters on separate nanoparticles. Thus, although mixing the separately clustered catalytically divergent CDs had a synergistic effect on cellulase degradation activity,an effect that increased as the CBM valency on each nanoparticle increased, clustering of the two CDs on the same nanoparticle resulted in greater activity enhancement than that observed with a mixture of individual clustered CDs at the same proportions of CD_{CelD}, CD_{EGPh}, and CBM4.

Degradation of Microcrystalline Cellulose by CD_{EGPh} and CD_{CelD} Clustered on Nanoparticles. We investigated the activity of clustered CD_{EGPh} and clustered CD_{EGPh} and CD_{CelD} for the degradation of microcrystalline cellulose (Avicel, Figure 4). The degradation activity of a mixture of free CD_{EGPh} and CBM3a for Avicel was similar to that of CD_{EGPh} alone

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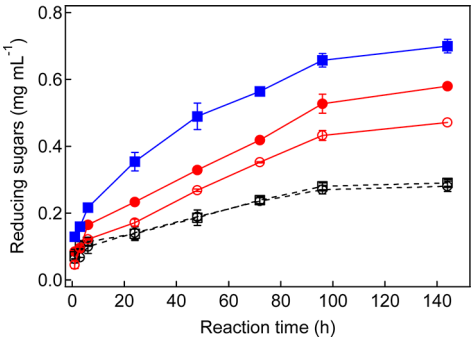

Figure 4. Production of reducing sugars from Avicel (10 mg mL^{-1}) in a 50 mM acetate buffer (pH 5.0, 200 mM NaCl) at 45 °C for 144 h with a mixture of free CD_{EGPh} and CBM4 (open black circles), a mixture of free CD_{CelD} and CD_{EGPh} (in equal proportions), and CBM4 (open black squares), CD_{EGPh} 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.

(data not shown). In contrast, nanoparticles with clusters of 7 CD_{EGPh}s and 23 CBM3as produced 1.7 times the sugars produced by a mixture of free CDs and CBMs. The use of CBM4 also showed the similar enhancement, but the CD_{EGPh}-CBM4 clusters enhanced cellulose degradation activity more than the clusters with CBM3a. In our previous study on CD_{CelD}, the clustering with CBM4 also enhanced the degradation activity of CD for PSC and Avicel more than that with CBM3a.[15] This implies that CBM4 can enhance the activity of CD_{EGPh} and CD_{CelD} more than CBM3a.

Addition of CBM4 to a mixture of free CD_{CelD} and CD_{EGPh} had little effect on the amount of sugars produced (data not shown), but clustering of CD_{CelD} and CD_{EGPh} together on nanoparticles increased the degradation of Avicel more than did the clustering of CD_{EGPh} with the same valency of CBM. Clusters prepared from 7 CDs (CD_{CelD} and CD_{EGPh} in equal proportions) and 23 CBM4s produced 1.2 times the amount of sugars produced by clustered CD_{EGPh} at the same 7:23 CD/CBM molar ratio and 2.4 times the amount produced by a mixture of the two free CDs and CBMs.

DISCUSSION

Use of a Processive Endoglucanase in Hybrid Nanocellulosomes. Endoglucanases randomly attack and hydrolyze amorphous cellulose, whereas processive endoglucanases have a processive function like that of exotype cellobiohydrolase;[4,28] that is, endoprocessive enzymes not only randomly attack cellulose but also attack them processively to release cellobiose. Previously, biotinylated CDs derived from endoglucanases were clustered with CBMs on streptavidin (4 domains or modules on a streptavidin) and streptavidin-conjugated CdSe nanoparticles (30 modules on a nanoparticle) via biotin−avidin interactions and the activity enhancement was estimated at the same mole concentration of CDs in reaction solution. The resulting CD−CBM clusters showed dramatically enhanced activity of clustered CD for the degradation of both amorphous and crystalline celluloses because the design with multivalent CBMs increases the contact frequency of enzyme.[14,15] In this study, we used the same clustering strategy for the CD of a processive endoglucanase (EGPh). The degradation activity of the clustered CD_{EGPh} for PSC increased as the CBM valency of the clusters was increased, and the multivalent CBM design was also effective for the degradation of Avicel. This result shows the versatility of our cluster design for enhancing the activity of various types of CDs.

We also evaluated two CBM modules, which enhance the degradation activity of CDs when they are combined on a native scaffoldin structure. Although the addition of CBM without scaffold (streptavidin and nanoparticles) slightly enhanced the degradation activity of CD for PSC maybe because CBM physically disrupts cellulosic fibers, the clustering on the scaffold critically enhanced the degradation activity of CD. We found that CBM3a slightly promoted CD_{EGPh} activity for PSC when the domain and module were clustered on the same streptavidin, whereas CBM4 dramatically increased the activity of CD_{EGPh} when they were clustered on nanoparticles. The superiority of CBM4 was also observed for the degradation of Avicel. In our previous study, we observed a similar trend for CD_{CelD}−CBM clusters against PSC and Avicel and attributed it to sustained degradation activity of cellulase over long reaction times.[15] Various CBMs have different specificities and affinities for cellulose surfaces, and these differences influence the catalytic properties of CD−CBM clusters.[29] CBM3a is reported to bind to the surface of microcrystalline substrates,[30] and CBM4 appears to bind to amorphous substrates,[24,31] However, our binding assay of CBM showed that CBM3a and CBM4 were adsorbed on PSC and Avicel, although CBM3a had higher affinity for Avicel than CBM4.[15] In the previous and current studies, the clusters formed from $CD_{CelD,\ EGPh}$ and CBM3a on streptavidin and nanoparticles showed less enhancement of cellulose degradation activity for PSC and Avicel than did clusters with CBM4, and our previous results showed no clear correlation between CBM binding affinity and cluster activity.[15] We are working on determining the critical properties of CBM necessary for sustained degradation activity of cellulase by CD−CBM clusters on nanoparticles.

In terms of crystal structure of CBM and modeling, CBM3a can bind three cellulose chains:[23] H57, Y67, and W118 have hydrophobic interaction and salt bridge with cellulose chain 1, and the group of N10, N16, and Q110 and the pair of S12 and S133 form hydrogen bonding to cellulose chain 2 and chain 3, respectively (Figure S5, Supporting Information). Especially, the result that CBM3b where the Y67 and W118 were not conserved fails to bind onto crystalline cellulose,[32,33] suggests that the binding to the cellulose chain 1 is critical. Whereas, the NMR analysis for CBM4 showed that CBM4 has a single site for cellulose chain.[31] The binding of a number of cellulose chains on a CBM might reduce the cluster effect when CBM3a is used.

Synergism between Endo and Endoprocessive CDs Clustered on Nanoparticles. Cellulosomes efficiently degrade solid substrates by taking advantage of the synergistic effects of multiple hydrolysis reactions catalyzed by several cellulases with different catalytic functions, and cellulosomal enzymes were assembled on minimized scaffold proteins by means of cohesin−dockerin interactions to construct mini-cellulosome.[9−13] Fierobe et al. prepared chimeric scaffoldins with a CBM and two or three cohesins, on which several pairs of CDs from endoglucanases and processive endoglucanases were assembled.[10,12] Although they did not distinguish between synergistic effects and clustering effects, they found that a

dx.doi.org/10.1021/cs400012v | ACS Catal. 2013, 3, 1342−1348

combination of endoglucanases and processive endoglucanases provided the most-efficient minicellulosomes for amorphous and crystalline celluloses. In this study, we used the 20-nm nanoparticles with 30 biotin binding sites (average distance between the sites ≈ 6 nm) and observed a synergistic effect on cellulose degradation activity when we combined 7−30 endo and endoprocessive CDs on nanoparticles. Our results indicated that, under the conditions tested, the clustering did not interfere with the cellulose degradation activity of the CDs. Furthermore, comparison of the degradation activities of mixtures of individual CD clusters on separate nanoparticles and clusters of two CDs on the same nanoparticle demonstrated the importance of proximally positioning catalytically divergent CDs to enhance the synergistic effect: the latter showed higher degradation activity than the former at all ratios of the components. Interestingly, even with the mixture of individual CD clusters, the synergistic effect was gradually shown as the number of CBMs increased (open squares in Figure 4). Increasing the CBM valency on the nanoparticles may have concentrated the nanoparticles on the substrate surface, decreasing the distance between the nanoparticles and thus generating the synergistic effect. Recently, other artificial cellulosomes using inorganic particles have been reported but showed no clear enhancement of degradation activity by clustering effect. Our cluster format may be a useful platform for artificial cellulosomes using inorganic nanoparticles.

In another study, cellulosomal endo- and exotype enzymes were assembled on a minimized scaffoldin that was derived from CbpA of *Clostridium cellulovorans* and has a CBM and two cohesins:[11] an assembly of an endoglucanase and a processive exoglucanase showed a synergistic effect, and minicellulosomes composed of an endoglucanase and hemicellulases also synergistically degraded corn fibers.[13] We are currently working on various systems of coupled enzymes for degradation reactions of solid substrates.

Degradation of Microcrystalline Celluloses. In this study, the clustering design with multivalent CBMs also enhanced the degradation activity of processive endoglucanase (CD_{EGPh}) for Avicel, as well as PSC. Considering that endoglucanases hydrolyze amorphous celluloses, the multivalent design can increase the contact frequency of CD_{EGPh} onto amorphous area in Avicel, so that the acceleration of cellulose degradation might unfold part of crystal structures to increase the active site for in CD_{EGPh} in Avicel. Synergistic effect of catalytically divergent CDs on cellulose degradation activity was also observed for Avicel. This acceleration of cellulose degradation also might be due to the degradation beyond amorphous area.

To summarize, we clustered two catalytically divergent biotinylated CDs with CBMs on streptavidin-conjugated CdSe nanoparticles to construct hybrid nanocellulosomes with high degradation activity for solid substrates. Clustering of the CDs with multiple CBMs significantly enhanced the degradation activity of both endo and endoprocessive CDs, and clustering of the two CDs on the same nanoparticle provided the most efficient hybrid nanocellulosomes as a result of a strong synergy between the CDs. Although we cannot make a simple comparison between our results and previously reported results, the enhance rate is comparable or superior to those of reported minicellulosomes and other artificial cellulosomes. In this hybrid nanocellulosome format, the various building blocks of cellulosomes can be easily combined in many ways to fabricate artificial cellulosomes, and the design may also be a useful platform for testing promising new building blocks for artificial cellulosomes.

■ ASSOCIATED CONTENT

Supporting Information

Amino acid sequences of biotinylated CDs and CBMs (Figure S1), absorption spectra of streptavidin-coated CdSe nanoparticles (Figure S2), fluorescence spectra and analysis of streptavidin-coated CdSe nanoparticles (Figure S3), reducing sugar production analysis from PSC (3.5 mg mL−1) in the presence of a mixture of heteroclustered hybrid nanocellulosome (Figure S4), and crystal structures of CBM3a and CBM4 (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partly supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan (M.U.), by a Scientific Research Grant from the Ministry of Education, Science, Sports, and Culture of Japan (M.U., I.K.), and by an Advanced Low Carbon Technology Research and Development Program Grant from the Japan Science and Technology (JST) Agency (M.U.).

■ ABBREVIATIONS

AviTag, biotin acceptor peptide; CBM, carbohydrate-binding module; CD, catalytic domain; CD_{EGPh}, catalytic domain of endoglucanase EGPh; CD_{CelD}, catalytic domain of endoglucanase D; PSC, phosphoric-acid swollen cellulose; TZ, tetrazolium

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