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An enhanced protein–protein interaction based on enzymatic complex through replacement of the recognition site

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ABSTRACT

Clostridium cellulovorans, produce multi-enzymatic complexes known as cellulosomes, which assemble via the interaction of a dockerin module in the cellulosomal subunit with one of the several cohesin modules in the scaffolding protein, to degrade the plant cell wall polymer. An enhanced cohesin–dockerin interaction was demonstrated by modified certain cellulosomal enzymes with altered amino acid residues at the crucial binding site, 11th and 12th positions in dockerin module. In fluorescence intensity analyses using the cellulosome-based biomarker system, the modified cellulosomal enzymes (EngE SL to AI and EngH SM to AI) showed an increased intensity (1.4- to 2.2-fold) compared with the wild-type proteins. Conversely, modified ExgS (AI to SM) exhibited a reduced intensity (0.6- to 0.7-fold) compared with the wild type. In enzyme-linked and competitive enzyme-linked interaction assays, the some modified protein (EngE SL to AI and EngH SM to AI) showed their increased binding affinity toward the cohesins (Coh2 and Coh9). Surface plasmon resonance analysis quantitatively demonstrated the binding affinity of these two modified proteins toward cohesins showed similar or higher affinity comparing with its with wild type proteins. These results suggest the replacement of amino acid residues in the certain recognition site significantly affects the binding affinity of the cohesin–dockerin interaction.

1. Introduction

The strictly anaerobic bacterium *Clostridium cellulovorans* is able to grow on the recalcitrant substrate crystalline cellulose. The higher efficiency of its extracellular hydrolytic machinery over that of other microorganisms is due to the formation of a massive enzyme complex known as the cellulosome [1]. Cellulosomes are multicomponent, multienzyme machines that include a scaffolding protein containing several cohesin modules and other cellulosomal enzymes, such as cellulase, hemicellulase and polysaccharide lyases, which are specifically assembled through their dockerin modules. The cohesin–dockerin interaction involved in the assembly of the cellulosome is a high-affinity protein–protein interaction ($>10^9\ M^{-1}$) [2]. Cohesin modules are highly conserved within a particular scaffolding protein and moderately conserved between different scaffolding proteins. Dockerin modules contain a pair of well-conserved 22-amino-acid residue segments separated by a linker of 8–18 residues. It has been reported that these amino acid sequences are well conserved between bacteria species [3].

To identify the mechanism of cellulosome formation and assembly, proteomic analyses of the cellulosome have been performed in cellulosome-producing bacteria species. In *C. thermocellum*, the entire draft genome sequence was analyzed to detect putative cellulosomal genes, and the resulting list was matched with the expressed proteins via two-dimensional SDS-PAGE (2D-PAGE) gel electrophoresis of the subproteome of purified cellulosomes [1]. In *C. cellulolyticum*, the putative enzymes were analyzed and subsequently compared with the sets of cellulosomal components determined experimentally following growth of the strain on different substrates, which included crystalline cellulose, xylan, and wheat straw [4]. In a previous study, we successfully developed a cohesin biomarker from anaerobic bacteria that can be utilized for proteomic analysis of the cellulosome of *C. cellulovorans.* Using this cohesin biomarker, we revealed the diversity of the cellulosomal enzymes and the production pattern of cellulosomal enzymes in the presence of different carbon sources [5]. Moreover, differences in the binding between cohesin and dockerin in cellulosomal enzymes was detected using a biomarker, and the results were verified via several analytical methods. For example, some cellulosomal enzymes, such as endoglucanase E (EngE) and endoglucanase H (EngH) which have different amino acid residues at 11th and 12th positions in the dockerin from *C. cellulovorans*, as targets of the

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Table 1
Primers used in overlap extension PCR to generate the mutants.

Primer	Sequence (5′–3′)[a]
EngE AI-F	GTAGTTAATGCTATAGATTTTGAGTTATTAAAGAA
EngE AI-R	CTCAAAATCTATAGCATTAACTACATCATTACCGT
EngE SM-F	GTAGTTAATTCAATGGATTTTGAGTTATTAAAGAA
EngE SM-R	CTCAAAATCCATTGAATTAACTACATCATTACCGT
EngH AI-F	ATCGTGAATGCTATAGATTTAGCGATGTTAAAGAA
EngH AI-R	CGCTAAATCTATAGCATTCACGATACCATTATTGT
EngH SL-F	ATCGTGAATTCACTTGATTTAGCGATGTTAAAGAA
EngH SL-R	CGCTAAATCAAGTGAATTCACGATACCATTATTGT
ExgS SM-F	AAAGTAAATTCAATGGATTTAGCTATATTAAAGAA
ExgS SM-R	AGCTAAATCCATTGAATTTACTTTAGCATCAGAGT

[a] Mutagenesis sites are underlined.

biomarker, presented a relatively low binding affinity for cohesin2 (Coh2) and cohesin9 (Coh9) compared with cohesin6 (Coh6). These results indicated that the diversities of amino-acid residues in common dockerin sequences alter their binding affinities with certain cohesin modules. Based on these findings, we hypothesized that this binding selectivity between the cohesin markers and cellulosomal enzymes in biomarker system might be affected by variations in the amino acids within the recognition site (11th and 12th residues) of dockerin [6].

In the present work, to demonstrate the recognition site in response to the selective cohesin–dockerin interaction from cellulosome, we tested the cellulosomal enzymes such as Exoglucanase S (ExgS), EngE and EngH containing modifications in the putative recognition site. The 11th and 12th amino acid residues of dockerin modules in the cellulosomal enzymes were replaced with other amino acid sequences (AI, SL and SM). AI which belongs to the dockerin modules of ExgS is common sequence among the cellulosomal enzymes in *C. cellulovorans*, whereas SL and SM belong to the dockerin modules of EngE and EngH, respectively [7]. The cohesin biomarkers were employed to detect the differences between the wild-type and modified dockerin modules. Additionally, analytical approaches, such as an enzyme-linked interaction assay (ELIA), a competitive enzyme-linked interaction assay (cELIA), and surface plasmon resonance (SPR) analysis, were applied as quantitative measurements for biological analysis. We believe that this approach provides a critical technique for modulating the sensitivity and selectivity of cellulosome-related proteomic analyses.

2. Materials and methods

2.1. Construction and bacterial expression of cohesin and cellulosomal enzymes

The wild-type forms of cellulosomal enzymes and Coh2 and Coh9 from cellulose binding protein A of *C. cellulovorans*, produced by *Escherichia coli*, were previously described [5,8]. Modified target proteins from EngE, EngH and ExgS with changes in the amino acid residues at the 11th and 12th positions of dockerin (EngE AI, EngE SM, EngH AI, EngH SL and ExgS SM) were prepared through site-directed mutagenesis with different combinations of PCR primers (Table 1) [9]. The wild-type recombinant and mutant proteins were produced in *E. coli* as previously described [5,6]. The purification process was performed as described elsewhere. The purified proteins were dialyzed against phosphate-buffered saline (PBS, pH 7.3), and the recombinant protein was concentrated via ultrafiltration (Millipore; 10-kDa cutoff). The cohesin proteins (100 μg) were fluorescently labeled on their primary amines with a succinimidyl ester moiety using the Alexa Fluor 647 protein-labeling kit (Molecular Probes) according to the manufacturer's instructions [6]. Alexa Fluor 647 dye-labeled cohesin displayed excitation and fluorescence emission maxima of approximately 650 nm.

2.2. Fluorescence detection using the biomarkers in 2DE analysis

The concentrations of the purified recombinant cellulosomal enzymes were measured via the Bradford assay using a kit from Bio-Rad that employs bovine serum albumin as the standard [10]. 2-D PAGE of the target proteins (2 μg per sample, wild-type and modified proteins) was performed as previously described [6]. The proteins were transferred to a PVDF membrane to detect the marker–target protein interaction using fluorescently labeled Coh2 and Coh9, as described elsewhere [5]. The membranes were blocked in a solution of TBS-T (10 mmol Tris–HCl, pH 7.4, 100 mmol NaCl and 0.1% Tween 20) with 3% non-fat milk, followed by an additional wash in TBS-T. The membranes were incubated with 2 μg of fluorescently labeled Coh2 and Coh9. The detected spots were analyzed using ImageMaster 2D Platinum Software (GE Healthcare BioScience) [11].

2.3. Affinity-based enzyme-linked interaction assay (ELIA)

Microtiter plates (SPL Lifescience) were coated with the cohesin proteins (100 μl/well, 1.3 μg of cohesin) for 2 h at 25 °C. The plates were then blocked for 2 h with blocking solution (300 μl/well of 3% bovine serum albumin in TrisNC buffer; 50 mmol Tris, 100 mmol NaCl, 2 mmol $CaCl_2$, and 0.02% sodium azide, pH 7.5) and washed three times with TrisNC buffer (300 μl/well). The protein–protein interaction was initiated upon the addition of the cellulosomal enzymes (100 μl/well, 10 μg of enzyme), and the plates were incubated for 2 h. After washing five times, the bound target proteins were detected by means of enzyme activity. Substrate solutions (100 μl/well, 0.5% carboxymethyl cellulose for EngE, EngH and 0.5% cellulose for ExgS in 100 mmol sodium acetate buffer, pH 5.0) were added, followed by incubation at 37 °C for 1 h. Finally, the dinitrosalicylic acid reagent (100 μl) was added, and the mixture was heated in a boiling water bath for 5 min. The absorbance was measured at 540 nm, as described previously [5].

2.4. Competitive enzyme-linked interaction assay (cELIA)

Microtiter plates were coated overnight with cohesin samples (200 μl/well, 270 nM of Coh9). The plates were then blocked for 2.5 h with the above-described blocking solution and washed three times with TrisNC buffer (50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, and 0.02% sodium azide, pH 7.5). The interaction was determined by the addition of 100 μl of the desired competitor samples (the ExgS wild-type dockerin protein, up to a maximum of 1.3 μM), immediately followed by the addition of 100 μl of a solution of wild-type and modified EngH, ExgS and EngE to a final concentration of 47 nM. The running buffer contained 10 mM $CaCl_2$ because calcium ions are required for the binding of cellulosomal enzymes to the cohesin modules [12]. Dilutions of the competitor were obtained using TrisNC buffer containing BSA to maintain a constant protein concentration. After incubation for 2.5 h, the wells were washed five times, and the amount of the target proteins bound to the coating cohesin (marker) was detected by means of enzyme activity, as described above. The results are presented as the percentage of binding, which was derived from the mean optical density values of three repetitions for each competitor concentration (percentage of binding = 100 × optical density of the test competitor concentration/optical density without the competitor). The data were analyzed using a 4-parameter fit with Grafit 5 software [13].

2.5. Surface plasmon resonance analysis of the cohesin and dockerin interaction

The interactions between the marker and target proteins were measured via SPR analysis using a Biocore 2000 (GE Healthcare

BioSci.). The preparation of sensor chips and the SPR analysis were performed as previously described, with minor modifications [7]. Marker proteins were immobilized on a dextran matrix with free carboxylic groups (CM5 chip) using conventional carbodiimide-coupling chemistry. The subsequent deactivation of excess active esters was accomplished using ethanolamine (EDC/NHS coupling kit; GE Healthcare BioSci.). The target proteins were diluted in running buffer (50 mM PBS buffer, 10 mM $CaCl_2$, and 0.005% surfactant P20, pH 6.5) and allowed to interact with the sensor surface during a 240-s injection. In all cases, at least three different concentrations (0.57–100 nM) of the ligand were injected at a flow rate of 30 μL/min. The association rate constant (k_{on}) and the dissociation rate constant (k_{off}) were calculated using BIAEVALUATION 3.2 software (GE Healthcare BioSci.) [6].

3. Results

3.1. Design of the modulated cohesin marker system

As illustrated in Fig. 1, the cohesin marker was generated by labeling a fluorescent substance [5]. The cellulosomal enzymes, which contain a dockerin capable of selectively interacting with cohesin, were modified via alteration of the amino acids within the recognition site in this study [3]. The binding affinity of the cellulosomal enzymes could be enhanced by changing the amino acids within the putatively critical recognition site. In a previous study, the crystal structures of cohesin and the dockerin protein from *C. thermocellum* were reported, and the results clearly showed that cohesin recognition occurs predominantly through an α-helix (α3) in the second segment of the dockerin [14]. The sequence duplication of dockerin modules is reflected in a near-perfect, two-fold structural symmetry, and each binding region recognizes its partner differently [15]. The 'AI' motif is a common amino acid sequence in the first segment, found at the 11th and 12th positions in the cohesin recognition sites of *C. cellulovorans*. Therefore, replacement of these positions (AI, SL and SM) would not disturb the tertiary structure of the domain but would alter the amino acid motifs that are important for specific recognition and induce minute variance in the protein–protein interaction [7]. The modified proteins were subjected to 2D-PAGE and analyzed based on the resultant fluorescence intensity by adding labeled cohesin modules. Cellulosomal enzymes such as the EngE, EngH and ExgS were subjected to the enzyme-linked interaction assay (ELIA) [6].

3.2. 2DE analysis through biomarker and fluorescence measurements

In a previous study, we developed an optical biomarker using fluorescently labeled cohesin and analyzed its intensity. The results revealed diverse profiles of binding to dockerin-containing cellulosomal proteins produced by *C. cellulovorans* grown on different carbon sources, such as Avicel, xylan and AXP (Avicel:xylan:pectin (3:1:1)) [6]. In the present study, we analyzed mutant EngE (AI and SM), EngH (AI and SL) and ExgS (SM) using the cohesin biomarkers

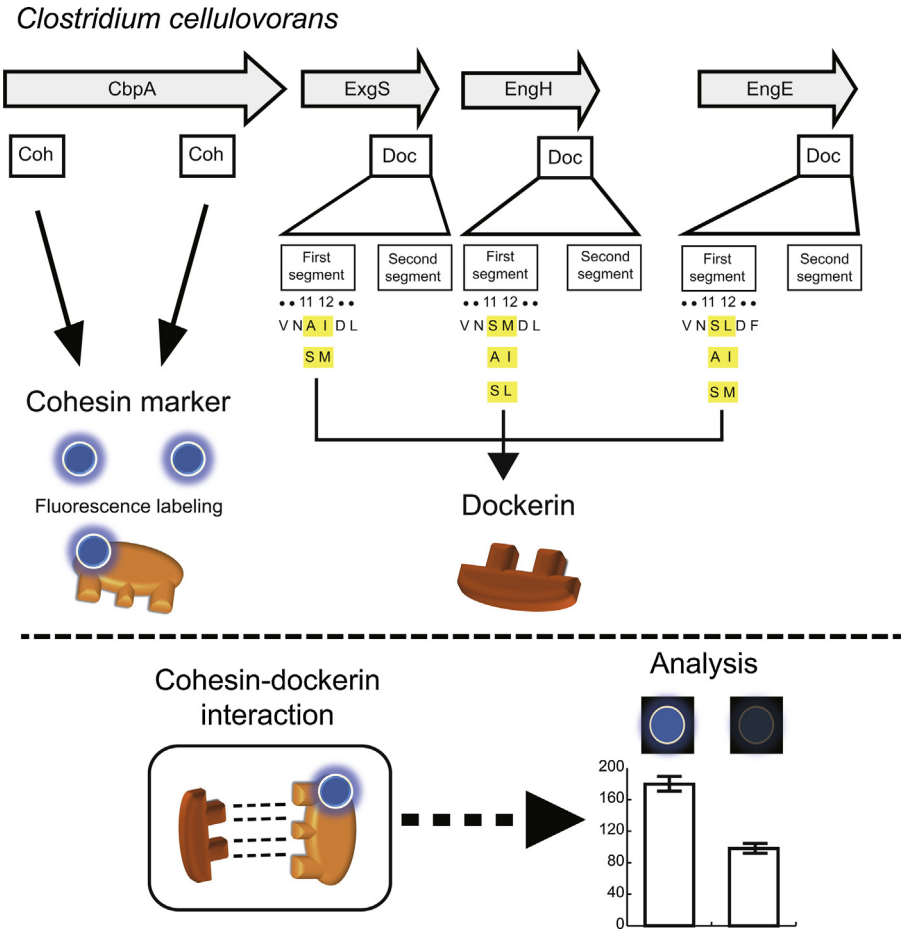

Fig. 1. The cohesin modules and cellulosomal enzymes of *C. cellulovorans* were selected to generate an improved biomarker system via increasing the affinity of the protein–protein interaction. The cohesin markers were generated with an attached fluorescent dye. The cellulosomal enzymes (EngE, EngH and ExgS) were modified through alteration of the amino acids at the 11th and 12th positions (AI, SL and SM) of first segment in the dockerin module to enhance binding affinity. Enhanced cohesin–dockerin binding affinity was measured via 2D-PAGE and fluorescence intensity analysis.

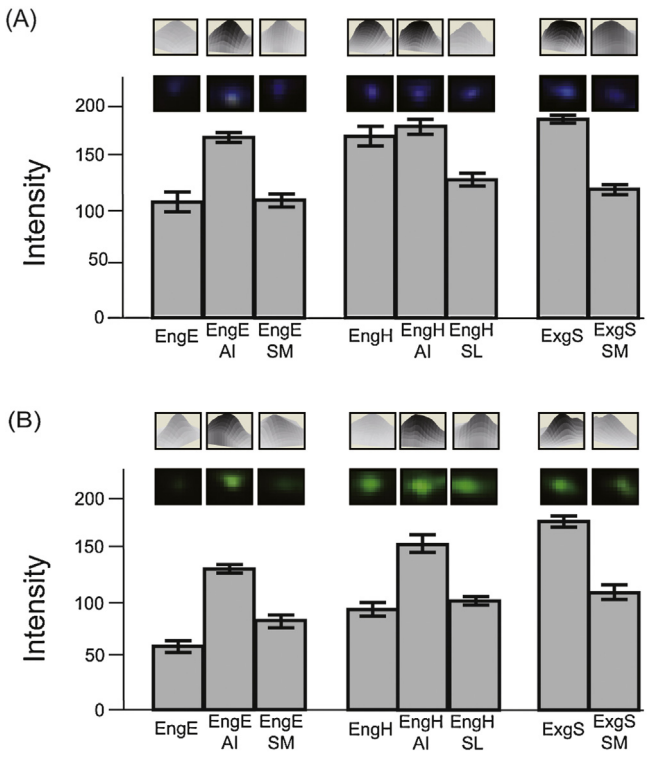

Fig. 2. 2D gel electrophoresis with fluorescence detection of wild-type (EngE, EngH and ExgS) and modified cellulosomal enzymes (EngE AI, EngE SM, EngH AI, EngH SL and ExgS SM) and quantitative intensity analysis using cohesin markers; (A) Coh2 and (B) Coh9. The spot images were analyzed via densitometry, and the results are indicated by the intensity bar. The spot images were rendered using ImageMaster 2D Platinum 6.0 (GE Healthcare).

(Fig. 2). The parameters employed for spot detection were set to a minimal area of 5 pixels, a smooth factor of 2, and a saliency of 1. The protein–protein interaction between EngH (SL and AI) and Coh2 biomarkers presented a similar intensity to the wild-type protein. However, with the Coh9 biomarker, EngH AI showed a 1.8-fold higher intensity than the wild-type interaction (Fig. 2B). On the other hand, the EngE AI displayed a 1.5- to 2.3-fold higher intensity for Coh9 as well as Coh2. In particular, EngE AI showed a similar intensity for Coh2 and Coh9 compared with the Coh6 biomarker [6]. We tested the modified ExgS (ExgS SM) as a target protein to analyze the effects of amino acid changes within the critical recognition site of a common target protein. Indeed, the interaction between ExgS SM and the Coh2 biomarker was significantly reduced (0.7 fold), and it exhibited a 0.6-fold lower intensity than Coh9 compared with the wild-type target. We demonstrated that alteration of the amino acids at the 11th and 12th positions within the cohesin recognition site had a significant impact on the cohesin–dockerin interaction in *C. cellulovorans*. According to these data, EngE (SL to AI) and EngH (SM to AI) modulated at these specific positions showed higher binding affinity during biomarker detection, which showed specific amino acids are essential for retaining high protein–protein affinity.

3.3. Determination of the binding affinity and preference of cohesin and modified cellulosomal enzymes

To estimate the binding affinity of the cohesin biomarkers for modulated cellulosomal enzymes, a simple and direct ELIA was performed (Fig. 3). The interaction of the EngH SL and AI with Coh2 biomarkers showed a similar binding affinity to the wild-type target. However, for the Coh9 biomarker, EngH AI showed a 1.9-fold higher affinity than the wild-type interaction (Fig. 3C and D). In contrast, the EngE AI mutant presented a 2.0-fold higher affinity for Coh9 as well as Coh2 (Fig. 3A and B). Notably, EngE AI exhibited a similar intensity for Coh2 and Coh9 compared with the intensity of Coh6 biomarker (Fig. 3B and D) [6]. The affinity of ExgS SM for the Coh9 biomarker was significantly reduced (2.2-fold), and Coh2 displayed a 0.4-fold lower affinity than the wild-type target (Fig. 3E and F). These results indicate that altering the binding affinity between the cohesin markers and dockerins triggers different fluorescence intensities.

Table 2
The association and dissociation constants for the binding of the modified target proteins to cohesin markers.

Target proteins	K_D (M)[a]	
	Coh2	Coh9
EngE wild	7.62×10^{-8}	5.71×10^{-7}
EngE AI	2.49×10^{-9}	5.30×10^{-8}
EngE SM	1.38×10^{-7}	6.78×10^{-8}
EngH wild	3.83×10^{-9}	6.12×10^{-8}
EngH AI	3.42×10^{-9}	3.71×10^{-9}
EngH SL	5.42×10^{-9}	5.23×10^{-9}
ExgS wild	5.33×10^{-9}	4.98×10^{-9}
ExgS SM	8.08×10^{-8}	4.18×10^{-7}

[a] K_D was determined using the formula k_{off}/k_{on}.

Additionally, to determine the preference of the cohesins for the modified cellulosomal enzymes, a competitive form of the assay (cELIA) was applied in which high binding constants can be determined in a comparative manner. For these assays, microtiter plates were coated with Coh9, which was used as biomarker, followed by mixing with the target protein [16]. The tendency of Coh2 to bind to the target protein was similar to that of Coh9 (data not shown). The wild-type proteins were used as a competitors, and when the concentration of the modified proteins was equivalent, we confirmed that the binding preference inhibited assembly (Fig. 4). EngE AI bound to Coh9 with more than an 80% efficiency, while wild-type EngE and EngE SM bound to Coh9 at a nearly 50% efficiency when the ratio of the wild type to the competitor was 1:1 (Fig. 4A). Similarly, EngH AI bound more efficiently to Coh9 (85%) than wild-type EngH and EngH SL (Fig. 4B). When we compared the wild-type protein ExgS to modified ExgS, the mutant was found to dissociate from Coh9 more easily than the wild type (12%) (Fig. 4C). These results indicated that the modified protein containing the AI motif bound easily to the cohesin markers because of the relatively higher binding affinity.

3.4. Quantitative affinity analysis via surface plasmon resonance

SPR offers a highly sensitive measure of bio-molecule interactions [17]. The SPR method was used to study the real-time interactions between the cohesin modules and modified dockerins in cellulosomal enzymes to compare the association and disassociation constants of binding. Two Coh2 and Coh9 proteins were covalently immobilized to the dextran carboxylmethyl groups on the surface of a CM5 sensor chip via the amine coupling method. Table 2 displays the affinity constants for the binding of the cohesins and modified dockerins. The constants between the wild-type cellulosomal enzymes and cohesins were referred to in a previous study [6]. Coh2 associated with the modified EngE AI, EngE SM, EngH AI, EngH SL, and ExgS SM with K_D values of 2.49×10^{-9}, 1.38×10^{-7}, 3.42×10^{-9}, 5.42×10^{-9}, and 8.08×10^{-8}, respectively. EngE AI displayed a higher affinity for Coh2 than the wild type (7.62×10^{-8}), indicating an approximately 31-fold difference in the affinity between EngE and EngE AI. The two modified EngH proteins showed a preference for Coh9 compared with the wild-type protein. EngH AI associated with Coh9 with a K_D value of 3.71×10^{-9}, and EngH SL showed a K_D value 5.23×10^{-9}, which represents an

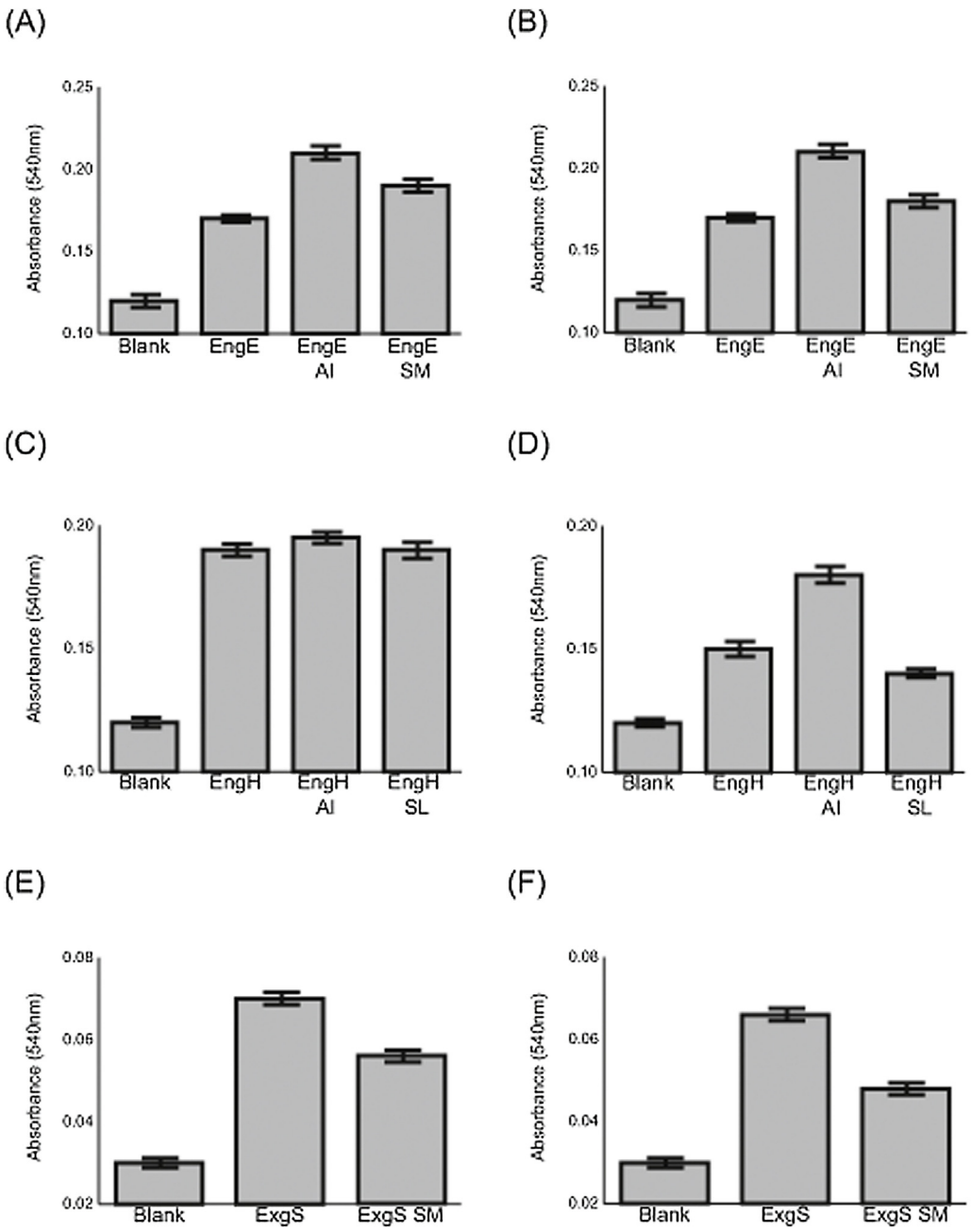

Fig. 3. Quantification of the affinity of the cellulosomal enzymes for the cohesin biomarkers. Enzyme-linked interaction assay (ELIA) plates were coated with a solution of cohesin (marker), and the immobilized sample was allowed to interact with a solution of the target protein. The interactions between the target protein and Coh2 (A, C and E) and Coh9 (B, D and F) were analyzed. The data are presented as the means ± SD ($n = 3$).

approximately 12- to 16-fold difference from their affinity with the wild type. ExgS SM exhibited a significantly reduced affinity (4.18×10^{-7}) for Coh9, while the K_D value of wild-type ExgS indicated its high affinity for Coh9. The finding indicated that different affinities exist between the modified dockerins in certain cellulosomal enzymes and cohesins in *C. cellulovorans*, some enhanced protein–protein interaction between cohesins and cellulosomal enzymes was proved with quantitative affinity analysis.

4. Discussion

The results regarding the cohesin–dockerin interaction in *C. thermocellum* and *C. cellulolyticum* showed that within an individual scaffolding, the cohesin modules are unable to discriminate between the dockerins present in the various cellulosomal enzymes. Both cohesins and dockerins are highly homologous within a given species, and the residues that are directly involved in protein:protein recognition are highly conserved within a species. For example, CelS (Cel48A) dockerin, the major catalytic component of the cellulosome, can bind any of the cohesins of CipA, the *C. thermocellum* scaffolding [2]. However, in a previous study, we showed that some cellulosomal enzymes, such as EngE and EngH, also exhibit a different binding affinity with associated cohesin modules. These results indicated that some cellulosomal enzymes present binding selectivity toward the cohesin modules within CbpA [6]. The amino acid differences in the dockerin modules may affect this interaction. For example, in typical *C. thermocellum* dockerin modules, residue 11 is a Ser, and residue 12

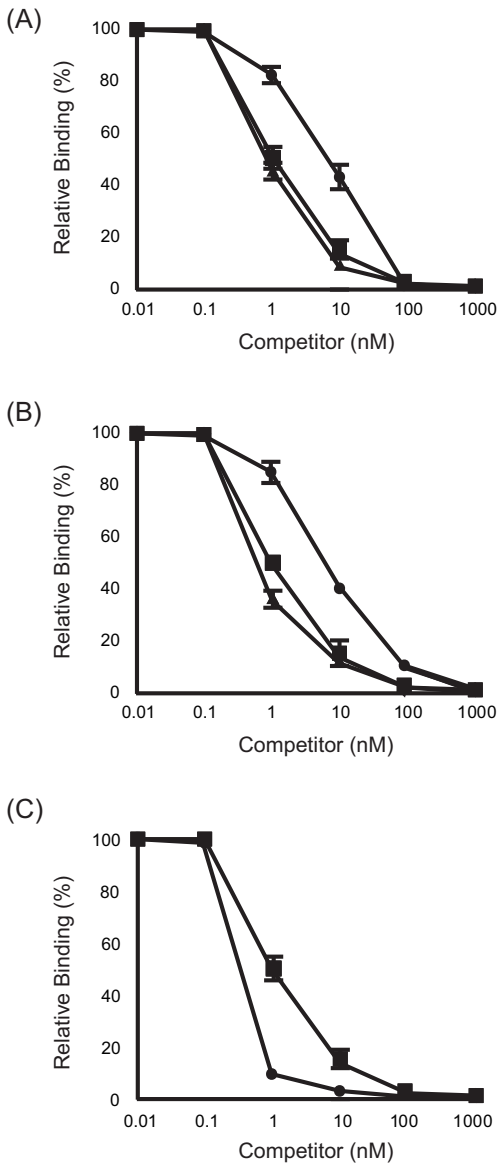

Fig. 4. Measurement of the relative binding affinity between the cohesins and cellulosomal enzymes. Microtiter plates were coated with Coh9. The immobilized cohesins (Coh9) and the target proteins (A) EngE (square), EngE AI (circle), and EngE SM (triangle); (B) EngH (square), EngH AI (circle) and EngH SL (triangle); and (C) ExgS (square) and ExgS SM (circle) were subjected to competitive enzyme-linked interaction assays (cELIA) in the presence of competitors. The error bars represent the standard deviation of triplicate measurements.

is either a Ser or Thr. The conserved residues in dockerin modules play an important role in controlling binding affinities [7]. In contrast, in the *C. josui*, *C. cellulolyticum* [18] and *C. cellulovorans* dockerin modules, residue 11 is an Ala, and residue 12 is a hydrophobic residue, usually a Leu or Ile [19]. In the case of EngE and EngH, the SL and SM amino acid sequences found at the 11th and 12th residues of dockerin, respectively, may be the cause of the discrimination by certain cohesin modules within CbpA from *C. cellulovorans*. In summary, it is thought that the scaffolding protein of *C. cellulovorans* binds some cellulosomal enzymes selectively because of its critical binding site in dockerin modules.

The cohesin–dockerin interactions observed in anaerobic microbes have been thought to represent promising biological tools for proteomic investigation of the cellulosome. We generated markers from cohesins and analyzed their enhanced fluorescence performance in relation to modified dockerin modules using 2D-PAGE. Furthermore, ELIA, cELIA, and SPR analyses were performed to quantitatively measure the protein–protein interactions between the cohesins and dockerins. Using these analytical methods, we demonstrated that different fluorescence intensities resulted from the binding affinity between the cohesins and dockerins and that the sensitivity of the cohesin markers could be improved via amino acid changes in protein recognition sites. Moreover, the enzyme-linked, protein complex-based interaction showed a potential for application to enzyme-linked analytical methods. In conclusion, altering critical positions in dockerin modules can change the affinity with certain cohesin modules in scaffolding proteins of *C. cellulovorans*, indicating that some cohesins in scaffolding proteins infrequently bind certain cellulosomal enzymes during cellulosome assembly.

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