NMR Analysis on Molecular Interaction of Lignin with Amino Acid Residues of Carbohydrate-Binding Module from *Trichoderma reesei* Cel7A

Yuki Tokunaga, Takashi Nagata, Takashi Suetomi, Satoshi Oshiro, Keiko Kondo, Masato Katahira & Takashi Watanabe

Lignocellulosic biomass is anticipated to serve as a platform for green chemicals and fuels. Nonproductive binding of lignin to cellulolytic enzymes should be avoided for conversion of lignocellulose through enzymatic saccharification. Although carbohydrate-binding modules (CBMs) of cellulolytic enzymes strongly bind to lignin, the adsorption mechanism at molecular level is still unclear. Here, we report NMR-based analyses of binding sites on CBM1 of cellobiohydrolase I (Cel7A) from a hyper-cellulase-producing fungus, *Trichoderma reesei*, with cellohexaose and lignins from Japanese cedar (C-MWL) and *Eucalyptus globulus* (E-MWL). A method was established to obtain properly folded TrCBM1. Only TrCBM1 that was expressed in freshly transformed *E. coli* had intact conformation. Chemical shift perturbation analyses revealed that TrCBM1 adsorbed cellohexaose in highly specific manner via two subsites, flat plane surface and cleft, which were located on the opposite side of the protein surface. Importantly, MWLs were adsorbed at multiple binding sites, including the subsites, having higher affinity than cellohexaose. G6 and Q7 were involved in lignin binding on the flat plane surface of TrCBM1, while cellohexaose preferentially interacted with N29 and Q34. TrCBM1 used much larger surface area to bind with C-MWL than E-MWL, indicating the mechanisms of adsorption toward hardwood and softwood lignins are different.

Lignocellulosic biomass is the most abundant renewable carbon resource. It consists of structural polysaccharides, cellulose, and hemicelluloses coated with a heterogeneous aromatic polymer, lignin. Recently, the production of bio-based fuels and chemicals from lignocellulosic biomass has attracted increasing attention due to the depletion of fossil resources and environmental issues. To produce biofuels and chemicals by enzymatic saccharification and the fermentation of lignocelluloses, it is necessary to realize pretreatments exposing plant cell wall polysaccharides and subsequent hydrolysis of polysaccharides with a cellulolytic enzyme cocktail simultaneously or prior to fermentation. Highly efficient enzymatic saccharification of lignocellulose with cellulolytic enzymes in a hydrolytic process is a primary key step in achieving lignocellulosic biorefinery process. Typical fungal cellulolytic enzymes, such as cellobiohydrolase and endoglucanase, are composed of catalytic domain (CD) and carbohydrate-binding modules (CBMs) connected with highly glycosylated linker. CBMs play a role in bringing catalytic domains in close proximity to the substrate to improve enzymatic activity. However, CBMs of polysaccharide hydrolases also bind to lignin. The efficiency of enzymatic saccharification, therefore, is strongly decreased. Because the pretreated biomass is usually hydrolyzed by cellulolytic enzymes in the presence of lignin fragments, methods have been extensively explored for protecting enzymes from the unfavorable binding with lignin. The approaches include the addition of masking agents, such as bovine serum albumin, polyethylene glycol, and surfactants, as well as the incorporation of ionic functional groups into lignin. However,
no fundamental theories have discussed how to alter the enzyme to avoid the unfavorable binding with lignin because the binding sites of lignin in enzymes are still not understood clearly.

Filamentous fungus *Trichoderma reesei* is known as a hyper producer of cellulolytic enzymes and widely used for commercial-scale production of cellulases and hemicellulases. Up to 60% of totally secreted cellulase is cello-biohydrolase I (*Tr* Cel7A) that bears family 1 CBM as the C-terminal domain (Fig. 1)9. Hence, there is a need for detailed understanding of the interaction between *Tr* CBM1 and lignin to solve the nonproductive binding issue and to establish a low-cost, highly efficient enzymatic saccharification process. However, both homologous and heterologous expressions of *Tr* CBM1 as well as its isolation are difficult due to its small molecular size (around 5-kDa). Because of these challenges, there has been no reports on the identification of amino acid residues of *Tr* CBM1 that are involved in binding with lignin without using site-directed mutagenesis that may cause conformational changes of such a small protein. It should be noted that the comparison of intact and *Tr* CBM1-deficient *Tr* Cel7A gives indirect information due to the interference of glycosylated linker10.

NMR titration analysis, such as chemical shift perturbation (CSP), is a powerful experimental strategy to identify substrate-binding sites of proteins at amino acid residue resolution11. CSP enables comprehensive analysis of interaction sites on the proposed structure of a protein without crucial conformational change. This approach has been used previously for binding site analysis of CBMs with poly- and oligosaccharides, including the interaction site and binding specificity between CBM56 and β-1,3-glucan12, CBM32 and chitosan oligosaccharides13, as well as CBM6 and xylohexaose14.

In this study, we applied CSP to analyze the interaction sites of *Tr* CBM1 against lignins from Japanese cedar and *Eucalyptus globulus*, using15N-labeled *Tr* CBM1 prepared as a single protein with correct folding. In addition, interaction of *Tr* CBM1 with cellohexaose was also analyzed by CSP to elucidate differences in the binding mechanisms of *Tr* CBM1 between polysaccharides and lignin. Enhanced understanding these differential interactions will lead to fundamental theory to develop hydrolases having high specificities toward carbohydrates having decreased binding affinity to lignin.

**Results**

**Expression and purification of 15N-labeled *Tr* CBM1.** 15N-labeled His-tag-*Tr* CBM1-GFP fusion protein was expressed using *Escherichia coli* BL21(DE3) (Fig. 2a). *Tr* CBM1 was cleaved off from His tag and GFP by proteolytic cleavage using enterokinase and thrombin, respectively. Finally, *Tr* CBM1 was purified to a single protein as demonstrated in SDS-PAGE (Fig. 3a) and MALDI-TOF-MS (Fig. 3b). The MALDI-TOF-MS spectrum gave the evidence that the obtained 15N-labeled *Tr* CBM1 possessed the correct molecular mass of 5255 expected for 15N incorporated protein (Fig. 2b).

The structures of 15N-labeled *Tr* CBM1 were assessed by observing the signal patterns of 2D 1H-15N SOFAST-HMQC spectra15. The 15N-labeled *Tr* CBM1 sample that was prepared using *E. coli* whose competent cell was stocked for more than five months showed a mixture of 2D 1H-15N SOFAST-HMQC spectra for both
folded and unfolded proteins (Fig. 4). The signals of the folded proteins appeared in the $^1$H-chemical shift range of 6.0–10.0 ppm. The signals of disordered proteins were observed only in the $^1$H-chemical shift range of 8.0–8.5 ppm$^{16,17}$. We conclude that this sample contained folded as well as either partially or fully disordered forms, although the theoretical molecular mass for $^{15}$N-labeled $\text{Tr}^{\text{CBM1}}$ was exhibited in MALDI-TOF-MS. $^{15}$N-labeled $\text{Tr}^{\text{CBM1}}$ prepared using fresh competent cell gave merely the correctly folded protein signals. The correctly folded $^{15}$N-labeled, $^{13}$C/$^{15}$N-labeled, and unlabeled $\text{Tr}^{\text{CBM1}}$ with single molecular weight were used in this study.

Spectral assignments of $^{13}$C/$^{15}$N-labeled $\text{Tr}^{\text{CBM1}}$. Spectral assignments of $^{13}$C/$^{15}$N-labeled $\text{Tr}^{\text{CBM1}}$ were achieved using a standard sequential assignment procedure. The $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-labeled $\text{Tr}^{\text{CBM1}}$ is shown in Fig. 5a with signal assignments. Backbone assignments of $\text{Tr}^{\text{CBM1}}$ were 89% accomplished with the exception of eight residues. Their signals were not observed because of line broadening that is mainly related to their locations in flexible loop regions. The chemical shifts of backbone atoms ($^1$HN, $^{15}$N, $^{13}$C$_\alpha$, $^{13}$C$_\beta$, and $^{13}$C') of $\text{Tr}^{\text{CBM1}}$ are listed in Supplementary Table S1. The amino acid residues that are in the secondary structures were predicted using TALOS$^+$ software based on Table S1$^{18}$, (Fig. 2-b). As a result, $^{15}$N-labeled $\text{Tr}^{\text{CBM1}}$ was predicted to have three $\beta$-strands: $\beta$1 (C8 to G10), $\beta$2 (T24 to V27), $\beta$3 (Y32 to L36). Previously, Kraulis et al.19.
determined the three-dimensional solution structure of the unlabeled TrCBM1 peptide (36 amino acid residues) that was chemically synthesized. According to their report, TrCBM1 has an anti-parallel β-sheet that comprised three β-strands: β1 (Q7 to G9), β2 (C25 to N29), and β3 (Y32 to C35). The structures of TrCBM1s prepared herein and by Kraulis et al., therefore, are consistent. Accordingly, we used the solution structure of TrCBM1 determined by Kraulis et al. to visualize the results of our NMR titration analyses.

**Analysis of interaction sites of TrCBM1 with MWLs and cellohexaose.** The interactions of TrCBM1 with lignin and cellohexaose were comparatively analyzed by NMR titration experiments using 1H-15N SOFAST-HMQC. We used highly purified milled wood lignins (MWLs) from a softwood, Japanese cedar (Cryptomeria japonica) (designated as C-MWL), and a hardwood, Eucalyptus globulus (E-MWL). Cellohexaose...
is an oligosaccharide having the minimum chain length recognizable by TrCBM120, which was used as a model compound of cellulose in the CSP analysis. The addition of excess amounts of MWLs resulted in the disappearance of NMR signals of TrCBM1, which hindered the assignments. Hence, the maximum concentrations of MWLs used for analyses were 2695 and 1200 μM for C-MWL and E-MWL, respectively. The signals of TrCBM1 were still found at these concentrations. Incremental titration to the TrCBM1 solution was carried out using different concentrations of C-MWL (1000, 1839, and 2695 μM), E-MWL (300, 900, and 1200 μM), and cellohexaose (700, 2800, and 5600 μM).

$^{1}$H-$^{15}$N SOFAST-HMQC spectra of 100 μM $^{15}$N-labeled TrCBM1 alone and in the presence of 2695 μM C-MWL were superimposed and are shown in Fig. 5. Upon incremental addition of the titrants to the solution of $^{15}$N-labeled TrCBM1, several signals clearly exhibited perturbation with the reduction in signal intensity. Further perturbation of the signals was caused by increasing amounts of the titrant. Chemical shift change ($\Delta \delta$) calculated by the formula (1) is summarized in Fig. 6. The incremental addition of cellohexaose continuously increased $\Delta \delta$.

$^{1}$H-$^{15}$N SOFAST-HMQC signals of G6, Q7, S14, T17, V18, A20, and L28 perturbed greatly upon the addition of C-MWL, while those of H4, G6, I11, T17, V18, T24, L28, C35, and L36 perturbed when E-MWL was added. $^{1}$H-$^{15}$N SOFAST-HMQC signals of G6 and S33 were line broadened in the presence of 900 and 1200 μM E-MWL, respectively, whereas the $^{1}$H-$^{15}$N SOFAST-HMQC signals were not line broadened in the presence of C-MWL. Therefore, distinct binding specificity toward hardwood and softwood lignins was found in the amino acid residues of TrCBM1. When C-MWL and E-MWL were added with concentrations higher than 2695 and 700 μM, respectively, $^{1}$H-$^{15}$N SOFAST-HMQC signals of Q7 resulted in line broadening. Q7, thus, was involved in direct or indirect interactions with MWLs.

The interaction sites of TrCBM1 revealed by NMR titration experiments were mapped on the solution structure of TrCBM1 determined by homonuclear NMR experiments (Fig. 7)19. As shown in Fig. 1, TrCBM1 has two major subsites, i.e., the flat plane surface and cleft. Triplet tyrosine (Y5, Y31, and Y32) of TrCBM1 is located on its flat plane surface, which plays a major role in the binding with cellulose. The triplet tyrosine is expected...
to be a main binding site with lignin due to its hydrophobic nature. However, the perturbations of the $^1$H-$^{15}$N SOFAST-HMQC signals of $Y_{31}$ and $Y_{32}$ became small, while the assignment of $Y_{5}$ was not accomplished. The small $\Delta \delta$ of the triplet tyrosine in the $^1$H-$^{15}$N SOFAST-HMQC spectra is due to the distant location between the aromatic ring in the side chain of tyrosine and $^1$H-$^{15}$N of the main chain, because specific detection of spin coupling of $^1$H-$^{15}$N in peptide bonds was monitored. Although the direct evidence of lignin binding via aromatic ring was not obtained, amino acid residues of the flat plane surface ($H_{4}$, $G_{6}$, $Q_{7}$, $I_{11}$, $L_{28}$, $N_{29}$, $Q_{34}$, and $L_{36}$) exhibited large $\Delta \delta$s upon the addition of MWLs and cellohexaose (Fig. 7). Thus, $TrCBM1$ interacted with both MWLs and cellohexaose on the flat plane surface. We also found that $G_{6}$ and $Q_{7}$ were line broadened upon the addition of MWLs, supporting the theory that MWLs strongly bound to $TrCBM1$ through the flat plane surface. The cleft composed of $T_{17}$, $V_{18}$, and $T_{24}$ also interacted with MWLs and cellohexaose. By extensive titration experiments, larger $\Delta \delta$s were consistently observed for $T_{17}$ and $V_{18}$ than $T_{24}$.

**Figure 7.** Mapping of cellohexaose and lignin binding sites identified by CSP on $TrCBM1$. Binding sites of (a) cellohexaose, (b) C-MWL, and (c) E-MWL are shown on the $TrCBM1$ surface. The residues that exhibited large $\Delta \delta$s are color coded as described in Fig. 6. Two representative views, lateral and bottom faces of $TrCBM1$, are shown with three concentrations of each titrant. Triplet tyrosine ($Y_{5}$, $Y_{31}$, and $Y_{32}$) and cleft ($T_{17}$, $V_{18}$, and $T_{24}$) are shown using purple stick. The residue $Y_{5}$ was not assigned.

**Binding affinity of $TrCBM1$ toward cellulose and lignin.** Adsorption experiment using Langmuir adsorption model was carried out using MWLs and Avicel. The latter is a commercially available cellulose rich in crystalline regions. A mixture of $TrCBM1$ with 1.0% (w/v) of either MWLs or Avicel was incubated at 50°C for 1 h. The amount of adsorbed $TrCBM1$ was calculated by subtracting nonadsorbed $TrCBM1$ from initial loading. The adsorption parameters that were calculated by the formula (2) are summarized in Table 1. The values of $TrCBM1$ adsorption by Avicel are similar to the previously obtained values using synthesized $TrCBM1$ analogs. Among these titrants, Langmuir affinity constant against $TrCBM1$ was in the order of E-MWL > C-MWL > Avicel. Therefore, $TrCBM1$ was found to possess higher affinity toward MWLs than Avicel. The highest $\Gamma_{\text{max}}$ was given by Avicel, indicating that it has a wide surface area and MWLs aggregated in water solution.
Lignin, which is a good indicator of binding\textsuperscript{29}. Additionally, irregular increasing and decreasing of CSP (Fig. 6b,c) by both (1) on- and off-rates of the complex formation and (2) diverse binding states due to the heterogeneity of Tr observed for cellohexaose. When MWLs were added to CBM1 solution, line broadening was mainly caused and in the flat pated in the specific electrostatic interactions and hydrogen bonding with cellulose chains.

MWLs were much less remarkable. The hydrophilic side chains of these amino acid residues, therefore, partici- pated in the binding with cellohexaose and MWLs, i.e., the flat plane surface and cleft, (Fig. 7). Previous studies based on site-directed mutagenesis tyrosine, i.e., Y5, Y31, and Y32, that are exposed in the flat plane surface by hydrophobic interaction, CH–π, and π–π stacking, respectively, although substitution of the tyrosine residues affected alignment of neighboring amino acid residues, especially when the protein of interest is small, such as in the case of TrCBM1\textsuperscript{17}. The use of stable isotope labeled proteins in combination with the adapted NMR titration experiments in this study is extraneous from such a disadvantage, giving direct information on the ligand and protein interaction at a molecular level in amino acid resolution.

Our NMR experiments indicated that two subsites of TrCBM1 were the major interaction sites with cellohexa- ose and MWLs, i.e., the flat plane surface and cleft, (Fig. 7). Previous studies based on site-directed mutagenesis suggested that pyranose rings of cellulose and aromatic rings of lignin bound to TrCBM1 through their triplet tyrosine, i.e., Y5, Y31, and Y32, that are exposed in the flat plane surface by hydrophobic interaction, CH–π, that are located on the flat plane surface as well as L17, and neighboring amino acid residues with adsorbed cellohexaose or MWLs. Aliphatic OH groups in cellohexa- ose as well as both aliphatic and phenolic OH groups in MWLs are also the potential binding sites with

|                      | Langmuir affinity constant \( K_0 \) (ml/mg) | Amount of adsorption at saturation \( I_{\text{max}} \) (µg/mg) |
|----------------------|---------------------------------------------|-------------------------------------------------|
| C-MWL                | 3.19                                        | 54.7                                            |
| E-MWL                | 5.93                                        | 48.8                                            |
| Avicel               | 2.65                                        | 63.7                                            |

Table 1. Adsorption parameters of TrCBM1 for C-MWL, E-MWL, and Avicel determined by Langmuir adsorption isotherm.

Discussion

\textit{T. reesei} is one of the most important industrial microorganisms for producing cellulolytic enzymes due to its high productivity and high activity for the produced enzymes. Using the cellulolytic enzyme system of \textit{T. reesei}, the production of CBH1 (Cel7A) reaches up to 60% of the total enzymes\textsuperscript{6}. CBH1 plays a major role in the catalysis. Its molecular functions including TrCBM1, therefore, have been studied extensively\textsuperscript{7}. In cellulose hydrolysis, TrCBM1 plays a crucial role in bringing enzyme close to the substrate, cellulose. However, due to the difficulties of expressing small proteins in \textit{E. coli}, the molecular functions of TrCBM1 have been studied using a chemically synthesized analog or as fusion proteins between TrCBM1 and catalytic domain of \textit{T. reesei} or other microbes, such as \textit{Talaromyces emersonii} and \textit{Melanocarpus albomyces}\textsuperscript{23,24}. The exceptions are the studies of Guo and Arslan. They studied the affinities of TrCBM1 to various cellulose substrates\textsuperscript{25} as well as the binding behavior of TrCBM1 to lignocellulosic substrates using an atomic force microscope\textsuperscript{26}. These reports described the expression and purification of TrCBM1. However, the molecular mass of the obtained TrCBM1 and whether the obtained TrCBM1 was correctly folded were not presented. These are crucial points, because we found that expression conditions greatly affected the correct folding of TrCBM1. In this study, we focused on the experimental scheme that the binding behavior of TrCBM1 at the molecular level was analyzed using a correctly folded single protein, TrCBM1, as revealed by MALDI-TOF-MS and 2D \(^{1}H-\text{\textsuperscript{15}N}\) SOFAST-HMQC (Figs 3 and 4).

In general, point mutation has been extensively applied for protein-ligand interaction analysis. Indeed, this approach enabled us to identify the key amino acid residues involved in either ligand binding or catalytic activity. In some cases, the substitution of amino acid residues caused undesired changes in the conformation of proteins either partially or entirely. These unwanted structural changes may distort understanding of the actual roles of amino acid residues, especially when the protein of interest is small, such as in the case of TrCBM1\textsuperscript{17}. The use of stable isotope labeled proteins in combination with the adapted NMR titration experiments in this study is extraneous from such a disadvantage, giving direct information on the ligand and protein interaction at a molecular level in amino acid resolution.

Our NMR experiments indicated that two subsites of TrCBM1 were the major interaction sites with cellohexa- ose and MWLs, i.e., the flat plane surface and cleft, (Fig. 7). Previous studies based on site-directed mutagenesis suggested that pyranose rings of cellulose and aromatic rings of lignin bound to TrCBM1 through their triplet tyrosine, i.e., Y5, Y31, and Y32, that are exposed in the flat plane surface by hydrophobic interaction, CH–π, π–π stacking, respectively, although substitution of the tyrosine residues affected alignment of neighboring amino acid residues\textsuperscript{23,24,27}. Our NMR study without the mutagenesis clearly indicated that the amino acid residues around triplet tyrosine (H4, G6, Q7, I11, L28, N29, Y31, Q34, and L36) constituting the flat plane surface exhibited large \( \Delta \delta \). This CSP is explained by changes in shielding effects caused by the interactions of the tyrosine and neighboring amino acid residues with adsorbed cellohexaose or MWLs. Aliphatic OH groups in cellohexa- ose as well as both aliphatic and phenolic OH groups in MWLs are also the potential binding sites with

Interestingly, a differential binding pattern was observed between cellohexaose and MWLs. N29 and Q34 showed large \( \Delta \delta \) upon the addition of cellohexaose and thereby were identified as the interaction sites for cel- lohexaose. This result is consistent with a previous report by Mattinen et al.\textsuperscript{28}. It was reported that the substitution of N29 and Q34 to alanine reduced the affinity toward cellulose over lignin, indicating that N29 and Q34 interacted with cellulose more effectively than lignin\textsuperscript{29}. In our NMR study, the interactions of N29 and Q34 with MWLs were much less remarkable. The hydrophilic side chains of these amino acid residues, therefore, participated in the specific electrostatic interactions and hydrogen bonding with cellulose chains. G6 and Q7 in the flat plane surface of TrCBM1 were line broadened upon the addition of MWLs. This phenomenon, however, was not observed for cellohexaose. When MWLs were added to TrCBM1 solution, line broadening was mainly caused by both (1) on- and off-rates of the complex formation and (2) diverse binding states due to the heterogeneity of lignin, which is a good indicator of binding\textsuperscript{29}. Additionally, irregular increasing and decreasing of CSP (Fig. 6b,c) support the diverse binding states between lignin and TrCBM1. Therefore, the line broadening of G6 and Q7 suggests that the flat plane surface of TrCBM1 played a central role in the binding with lignin.

Cellohexaose bound to the flat plane surface and cleft with high specificity (Fig. 8a). In comparison, MWLs bound to various high surface sites, including the flat plane surface and cleft, from much lower concentrations of tiritants (Fig. 8b). The cumulative binding of MWLs on multiple exposed sites increased the overall binding affinity to the lignin although the observed CSPs at each site are small (Table 1).

Recently, we found that lignin-binding peptides that can recognize lignin specifically changed their conformation upon the addition of softwood and hardwood lignins to adopt their molecular shapes along with the surface of lignins\textsuperscript{29}. Differences in the absorptivity toward softwood and hardwood lignins were also observed for

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|----------------------|---------------------------------------------|-------------------------------------------------|
| C-MWL                | 3.19                                        | 54.7                                            |
| E-MWL                | 5.93                                        | 48.8                                            |
| Avicel               | 2.65                                        | 63.7                                            |
TrCBM1. In addition to the flat plane surface and cleft, C-MWL interacted with the surface of TrCBM1 comprising amino acid residues of T1, S14, A20, and S22. E-MWL interacted with A20 and S33, which are also outside of the flat plane surface and cleft (Fig. 8). Therefore, we conclude that TrCBM1 recognized structural differences of softwood and hardwood lignins having similar weight-average molecular weights (C-MWL: 6254, E-MWL: 5776).

The binding of cellulase to lignin is affected by the structures of exposed surfaces of residual lignin, which results from structural differences of original biomass and pretreatment methods31,32. Guo et al. compared lignins from six different plants species and concluded that low S/G ratio induced high adsorption capacity, which is consistent with our results (Table 1)33. Moreover, high hydrophobicity, phenolic OH groups, and condensed structure of lignin increased adsorption capacity of cellulase, whereas aliphatic OH groups decreased adsorptivity28,34,35. Our NMR titration experiments indicated that TrCBM1 bound to lignins through various outer surfaces of the protein, including the flat plane surface and cleft. We found the differences of binding sites between softwood and hardwood lignins. Structural differences caused by pretreatments should also affect the binding behaviors. Thus far, the involvement of triplet tyrosine in lignin binding has been suggested by a combination of point-mutation and adsorption experiments23,24,27. These studies suggest the participation of the triplet in lignin binding; however, the role of other protein surfaces in the lignin binding cannot be analyzed and the point mutation may cause conformational changes of the protein. Our CSP study enables comprehensive analysis of interaction sites between the proposed structure of a protein and lignin without crucial conformational change.

Understanding of the flexible molecular recognition mechanism of TrCBM1 bound to polysaccharides and lignins from pretreated biomass softwood and hardwood could contribute to the molecular design of cellulolytic enzymes having controlled affinity to lignin and polysaccharides. The molecular design is indispensable for enzymatic saccharification with the minimum enzyme dosage.

Conclusion
Nonproductive binding of cellulolytic enzymes to lignin has been a serious issue for enzymatic saccharification of lignocellulosics. Understanding of the adsorption mechanism at the molecular level, however, is still limited. In the present study, we analyzed the interaction sites of correctly folded 15N-labeled TrCBM1 with MWLs and cellohexaose through NMR titration experiments. TrCBM1 bound to cellohexaose through the flat plane surface comprising triplet tyrosine as well as cleft with high site specificity. In high contrast, the interaction sites of TrCBM1 with MWLs were spread on the protein surface including the flat plane surface and cleft. Line broadening of G6 and Q7 suggests that the flat plane surface of TrCBM1 strongly interacted with MWLs, while hydrophilic amino acid residues, N29 and Q34, interacted with cellohexaose preferentially. The NMR approach using stable isotope labeling could lead to the development of a fundamental theory to design hyper enzymes that preferentially bind to polysaccharides without inactivation by coexisting lignin.
Materials and Methods

Materials. E. coli BL21 (DE3) was purchased from Merck (Darmstadt, Germany). The pRSET-EmGFP vector was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Enterokinase and thrombin were purchased from New England Bio Labs (Ipswich, MA, USA) and GE Healthcare (Chicago, IL, USA), respectively. Cellohexaose was obtained from Toronto Research Chemicals (Toronto, Canada). Other laboratory reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Wako Pure Chemical Ltd. (Osaka, Japan), nacalai tesque (Kyoto, Japan), and Cambridge Isotope Laboratories (Tewksbury, MA, USA).

Preparation of MWLs. Japanese cedar (Cryptomeria japonica) and Eucalyptus globulus woods were used for the preparation of C-MWL and E-MWL, respectively. The wood meal was extracted using a toluene and ethanol (2:1, v/v) mixture via a Soxhlet extractor at reflux temperature for 10 h. The extracted wood meal was dried at 105 °C for 12 h and finely divided in a vibratory ball mill having constant cooling water under a nitrogen atmosphere for 48 h. The milled wood was extracted using 96% aq. dioxane at room temperature for 24 h. The extract was allowed to evaporate and then freeze dried. The crude MWL was dissolved in 90% aq. acetic acid and then precipitated from distilled water and dissolved in a 1,2-dichloroethane and ethanol (2:1, v/v) mixture before they were added to diethyl ether. The precipitates were washed using petroleum ether and allowed for solvent evaporation to give MWL fractions. Molecular weight of MWLs was determined by gel permeation chromatography on three TSK gel supermixture HZ-M columns (Tosho, Tokyo, Japan) using a Shimadzu instrument equipped with an LC-20AD pump, an SPD M20A diode array detector (Kyoto, Japan). Tetrahydrofuran was used as the eluent at a flow rate of 0.35 ml/min at 40 °C.

Plasmid construction. The gene of TrCBM1 in Cel7A from T. reesei and thrombin recognition site was inserted into pRSET-EmGFP vector. The vector map of His-tag-TrCBM1-GFP expression plasmid is shown in supplementary (Fig. S1).

Expression and purification of 15N-labeled TrCBM1. E. coli BL21 (DE3) was transformed by heat shock with His-tag-TrCBM1-GFP expression plasmid. The transformant was inoculated to a 15N-labeling M9 medium (10 ml), containing 15N-NH4Cl as the sole nitrogen source and 100 μg/ml ampicillin, before it was precultured at 37 °C for 18 h with shaking at 200 rpm. The culture was used to inoculate 15N-labeled M9 medium (750 ml) and further incubated at 37 °C with shaking at 200 rpm, until OD 600 reached 1.2. Protein expression was induced using 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C for 5 h with shaking at 200 rpm as well.

After centrifugation at 7000 rpm for 15 min (HITACHI, himac CR21GII, R13A rotor), the cells were resuspended by a buffer containing 50 mM sodium phosphate (pH 7.5) and 500 mM NaCl to make a 10% weight per volume solution. This suspension was sonicated, centrifuged at 12000 g for 60 min, and filtered through a 0.45 μm filter. Then, cOmplete His Tag Purification Resin (Roche, Basel, Switzerland) equilibrated with the same buffer was mixed into the solution. The mixture was gently shaken for 15 min on ice and loaded into an open column (Bio Rad, CA, USA). The target protein containing His-tag-TrCBM1-GFP was eluted with the same buffer but containing 250 mM imidazole. The fractions containing His tag-TrCBM1-GFP were collected and diluted by tenfold using a 20 mM Tris-HCl buffer (pH 8.0), before they were applied to a 5 ml Hi Trap Q FF column (GE Healthcare, IL, USA) equilibrated with the same buffer. The protein was then eluted from the column using a 0–500 mM NaCl gradient in 20 mM Tris-HCl buffer (pH 8.0) on AKTA prime (GE Healthcare, IL, USA).

Using 10-kDa molecular weight cut off (MWCO) Vivaspin turbo ultrafiltration devices (Sartorius, Göttingen, Germany), the target fraction including His-tag-TrCBM1-GFP expression plasmid was concentrated to 1.0 mg/ml in a buffer containing 20 mM Tris-HCl and 50 mM NaCl (pH 8.0). The obtained His-tag-TrCBM1-GFP solution was treated by enterokinase (33 U/mg of protein) at 23 °C for 20 h without shaking to cleave the His tag. To remove the cleaved His tag, the reaction mixture was diluted by fivefold using 50 mM sodium phosphate buffer (pH 7.5) containing 500 mM NaCl and incubated with cOmplete His Tag Purification Resin for 15 min on ice. The obtained TrCBM1-GFP solution was then centrifuged at 500 g for 1 min, before the supernatant was filtered through a 0.45 μm filter. Subsequently, the solution was concentrated using 10-kDa MWCO ultrafiltration devices as well to obtain 2.6 mg/ml TrCBM1-GFP dissolved in PBS (-) buffer. The TrCBM1 was separated from GFP by treating with thrombin (50 U/mg of protein) at 22 °C for 20 h without shaking.

The reaction mixture was applied on a 1 ml Hi Trap Benzamidine FF column (GE Healthcare, IL, USA) to remove GFP and thrombin, which were bound to the column. The run-through fraction containing TrCBM1 was collected and buffer exchanged into 20 mM citric acid buffer (pH 3.0) using 3-kDa MWCO ultrafiltration device. The obtained solution was then applied to a 1 ml Hi Trap SP HP column (GE Healthcare, IL, USA) on AKTA prime. Before TrCBM1 was eluted using a 0–1 M NaCl gradient in a 20 mM citric acid buffer (pH 3.0). Finally, the obtained TrCBM1 was buffer exchanged into a 100 mM citric acid buffer (pH 5.0) using 3-kDa MWCO ultrafiltration devices.

We used LB medium, a M9 medium containing only 15N-H4Cl (99%, Cambridge Isotope Laboratories), and a M9 medium containing [U-13C] glucose (99%, Cambridge Isotope Laboratories)/15N-H4Cl, respectively, to obtain each of the nonlabeled, 15N-labeled, and 13C/15N-labeled TrCBM1s.

The protein concentration was determined by reading the absorbance at 280 nm and using extinction coefficient (11960 M⁻¹ cm⁻¹), proposed by Pace et al. Molecular mass and purity of the purified 15N-labeled TrCBM1 were analyzed by SDS-PAGE and MALDI-TOF-MS using Autollex III (Bruker Daltonics, MA, USA), respectively. The structures of the purified TrCBM1s were evaluated using 2D 1H-15N SOFAST-HMQC.

NMR spectroscopy and spectral assignment of TrCBM1. For NMR experiments, we used the 13C/15N-labeled TrCBM1 of 150 μM dissolved in 45 mM sodium acetate buffer (pH 5.0), containing 10% D2O and 20 μM 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS). All NMR spectra were recorded at 298 K on a Bruker

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NMR chemical shift perturbation analysis. $^{15}$N-labeled TrCBM1 of 100 μM was dissolved in a 100 mM citric acid buffer (pH 5.0), 90% H$_2$O/10% D$_2$O, and 20 μM DSS. Three different titrants, C-MWL, E-MWL, and cellohexaose, were individually titrated into the $^{15}$N-labeled TrCBM1 solution with incremental concentrations, i.e., C-MWL (600, 1000, 1839, 2695 μM), E-MWL (200, 600, 900, 1200 μM), and cellohexaose (700, 1400, 2800, 5600 μM). To identify the amino acid residues of $^{15}$N-labeled TrCBM1, which were involved in binding, chemical shift change $\Delta$δ (ppm) for each amino acid was calculated using the following equation:

$$\Delta\delta_{\text{ppm}} = \sqrt{(0.17\Delta\delta_{15N})^2 + (\Delta\delta_{1H})^2}$$

(1)

where $\Delta\delta_{15N}$ and $\Delta\delta_{1H}$ are chemical shift changes in $^{15}$N-axis and $^1$H-axis, respectively. Because MWL titrants were dissolved in $d_6$-DMSO, a control titrant containing $d_6$-DMSO without MWL was also prepared. The chemical shift changes obtained for MWL titrants were subtracted by those obtained for control titrant to obtain the actual $\Delta$s of TrCBM1 residues for MWLs. The amino acid residues that showed $\Delta$δ values larger than the average value ($\Delta\delta$) were mapped on the proposed TrCBM1 solution structure, which were color coded in pink. The amino acid residues whose signals disappeared upon the addition of titrants were mapped in red. TrCBM1 solution structure of the TrCBM1 was shown using molecular graphics software, PyMOL (Schrodinger, NY, USA).

Adsorption experiment. Adsorption experiment was employed to evaluate the affinities of TrCBM1 with each of the MWLs and Avicel using Langmuir adsorption isotherm. Sample solutions contained TrCBM1 and one of 1% (w/v) C-MWL, E-MWL, and Avicel in 50 mM citric acid buffer (pH 5.0). The concentration of TrCBM1 was varied as 40, 80, 160, 320, 640, 1280, and 2000 μg/ml with the total volume of 50 μl in a 1.5 ml micro tube. The sample solutions were incubated at 50 °C and were shaken at 1000 rpm for 60 min using thermomixer comfort (Eppendorf, Hamburg, Germany). Subsequently, the sample solutions were centrifuged at 12000 g for 10 min. The free TrCBM1 content in the supernatant was quantified based on the Bradford method using Bio-Rad Protein Assay (Bio-rad, CA, USA). The amount of adsorbed TrCBM1 was calculated by subtracting the amount of free TrCBM1 from that of the initially loaded TrCBM1. C-MWL, E-MWL, or Avicel (1%, w/v) without TrCBM1 were used as a blank. Experiments were carried out at least two times and the results were expressed as average values. Langmuir affinity constant was calculated by the following formula:

$$\Gamma_C = \frac{\Gamma_{C_{\text{max}}} K_C \Gamma}{1 + K_C \Gamma}$$

(2)

where $\Gamma_C$ is the amount of adsorbed TrCBM1 and $\Gamma_{C_{\text{max}}}$ is the amount of adsorbed TrCBM1 at saturation to MWLs and Avicel. $K_C$ is the Langmuir affinity constant to MWLs and Avicel. C is the concentration of free TrCBM1 in the supernatant.

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Author Contributions
Designed the experiments: Y.T., T.N., M.K., T.W. Performed the experiments: Y.T., T.S. Analyzed and interpreted the NMR data: Y.T., K.K., T.N., M.K. Contributed protein expression and purification: S.O. Wrote the paper: Y.T., T.W. T.N., M.K.

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