The First Identification of Carbohydrate Binding Modules Specific to Chitosan*

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Shoko Shinya1,2, Takayuki Ohnuma1, Reina Yamashiro‡, Hisashi Kimoto5, Hideo Kusakoë, Padmanabhan Anbazhagan1, André H. Juffer8, and Tamo Fukamizo13

From the 1Department of Advanced Bioscience, Kinki University, Nara 631-8505 Japan, 2Department of Bioscience, Fukui Prefectural University, Fukui 910-1195, 3Department of Environmental and Biotechnological Frontier Engineering, Fukui University of Technology, Fukui 910-8505, Japan, and 4Biocenter Oulu and Department of Biochemistry, University of Oulu, Oulu, FI-90014 Finland

Background: Carbohydratebinding modules (CBMs) specific to chitosan have yet to be identified.

Results: Two CBMs located at the C terminus of a chitosanase from Paenibacillus sp. IK-5 specifically bound chitosan oligosaccharides.

Conclusion: Individual CBMs can accommodate at least two glucosamine units at loops extruded from the core β-sandwich.

Significance: The synergistic action of the two CBMs appears to facilitate chitosan hydrolysis.

Two carbohydrate binding modules (DD1 and DD2) belonging to CBM32 are located at the C terminus of a chitosanase from Paenibacillus sp. IK-5. We produced three proteins, DD1, DD2, and tandem DD1/DD2 (DD1+DD2), and characterized their binding ability. Transition temperature of thermal unfolding (Tm) of each protein was elevated by the addition of cello-, laminar-, chitin-, or chitosan-hexamer (GlcN)n. The Tm elevation (ΔTm) in DD1 was the highest (10.3 °C) upon the addition of (GlcN)6 and was markedly higher than that in DD2 (1.0 °C). A synergistic effect was observed (ΔTm = 13.6 °C), when (GlcN)n was added to DD1 + DD2. From isothermal titration calorimetry experiments, affinities to DD1 were not clearly dependent upon chain length of (GlcN)n; ΔG° values were −7.8 (n = 6), −7.6 (n = 5), −7.6 (n = 4), −7.6 (n = 3), and −7.1 (n = 2) kcal/mol, and the value was not obtained for GlcN due to the lowest affinities. DD2 bound (GlcN)n with the lower affinities (ΔG° = −5.0 (n = 3) −5.2 (n = 6) kcal/mol). Isothermal titration calorimetry profiles obtained for DD1 + DD2 exhibited a better fit when the two-site model was used for analysis and provided greater affinities to (GlcN)n for individual DD1 and DD2 sites (ΔG° = −8.6 and −6.4 kcal/mol, respectively). From NMR titration experiments, (GlcN)n (n = 2–6) were found to bind to loops extruded from the core β-sandwich of individual DD1 and DD2, and the interaction sites were similar to each other. Taken together, DD1 + DD2 is specific to chitosan, and individual modules synergistically interact with at least two GlcN units, facilitating chitosan hydrolysis.

Chitosanases (EC 3.2.1.132) are glycosidases acting toward the β-1,4-glycosidic linkages of glucosamine (GlcN) polysaccharide chitosan (1), which is related to chitin and cellulose with respect to their chemical structures. Artificial chitosan has been prepared from chitin on an industrial scale by de-N-acetylation with sodium hydroxide (2). Natural chitosan is often found in fungal cell walls, which are remodeled or modified by the action of fungal chitosanases (3). The artificial and natural chitosans do not exist as homogeneous polysaccharides but as heterogeneous ones containing both GlcN and N-acetylglucosamine (GlcNAc) residues (4). In addition, chitin and cellulose mostly occur as a crystalline state, whereas chitosans are amorphous. Therefore, in enzymatic degradation of the chitosan polysaccharide, there may be some aspects that are different from those found in chitin and cellulose degradation (5,6). However, the enzymatic degradation of chitosan has not been fully understood with respect to its structure and mechanism.

Carbohydrate binding modules (CBMs)4 are involved in the enzymatic degradation of crystalline chitin and cellulose by recognizing the crystalline state of the substrates (7). According to the CAZy database (8), CBMs have been classified into 67 families based on their amino acid sequences. However, Boraston et al. (7) categorized CBMs into three types, Type A, Type B, and Type C, based on the state of the carbohydrate binding site. Type A CBMs bind to the flat surface of insoluble polysaccharides, but polysaccharides bind to the long-extended carbohydrate binding groove of Type B CBMs. Type C CBMs bind a small sugar, such as a mono-, di-, or trisaccharide. Insoluble and highly crystalline chitin and cellulose provide the flat surface, which is complementary to the planar architecture composed of some aromatic side chains of CBMs (9). This type of CBM

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1 Both authors equally contributed to this work.
2 Supported by a Research Fellowship for Young Scientists from Japan Society for the Promotion of Science (25-3639).
3 To whom correspondence should be addressed: Dept. of Advanced Bioscience, Kinki University, 3327-204 Nakamachi, Nara 631-8505 Japan. Tel.: 81-742-43-8237; Fax: 81-742-43-8976; E-mail: fukamizo@nara.kindai.ac.jp.
4 The abbreviations used are: CBM, carbohydrate binding module; DD, discolidin domain; DD1, the second CBM32 module from the C terminus of a chitosanase from Paenibacillus sp. IK-5; DD2, the first CBM32 module from the C terminus of the chitosanase; DD1 + DD2, a protein comprising DD1 and DD2 in tandem; (GlcNAc)n, β-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of n; (GlcN)n, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of n; ITC, isothermal titration calorimetry; HSQC, heteronuclear single quantum correlation.
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belongs to Type A. If a CBM specific to chitosan is present, it is unlikely to belong to Type A but instead to B or C because of the amorphous nature of chitosan. However, the chitosan binding modules have yet to be identified.

Discoidin domains (DDs) are structural modules comprising ~150 amino acids and are involved in interactions with a wide variety of ligand molecules, such as phospholipids, carbohydrates, and collagen (10–13). A subgroup of DD possessing binding ability toward carbohydrates has been classified as CBM family 32 (CBM32). Structure and binding mode of CBM32 proteins, such as discoidins I and II from Dictyostelium discoideum (14, 15) and a CBM from Clostridium perfringens N-acetyl-β-hexosaminidase (16), were analyzed by means of crystallography, and their mechanistic details are now available. Kimoto et al. (17) showed that a chitosanase from Paenibacillus sp. IK-5 has two DDs (DD1 and DD2) belonging to CBM32 at its C terminus as shown in Fig. 1A. This chitosanase was also found to lose its chitosan binding ability upon truncations of DDs (18). It appears that DD1 and DD2 are a novel type of CBM32 protein specific to chitosan. In this study we produced three proteins, DD1, DD2, and a protein comprising DD1 and DD2 in tandem (DD1 + DD2), using an Escherichia coli expression system. The three proteins were employed for thermal unfolding experiments, isothermal titration calorimetry (ITC), and NMR titration experiments to elucidate their function.

EXPERIMENTAL PROCEDURES

Materials—Chitosan oligosaccharides (GlcN)$_n$ $(n = 2–6)$, chitin hexasaccharide (GlcNAc)$_6$, cello-hexasaccharide 1–4(GlcNAc)$_6$, and laminari-hexasaccharide 1,3(Glc)$_6$ were purchased from Seikagaku Biobusiness Co. (Tokyo, Japan). E. coli BL21(DE3) pLacI and Rosetta (DE3) pLacI cells and the expression vector pETBlue-1 were from Novagen (Madison, WI). Nickel affinity resin, COSMOGEL His-Accept, was purchased from Nacalai Tesque Co. (Tokyo, Japan). Sepacryl S-100 HR was from GE Healthcare. All other reagents were of analytic grade.

Gene Cloning and Plasmid Construction—The gene encoding DD1, DD2, or DD1 + DD2 fused with a His$_6$ tag was amplified by PCR, which was conducted using the full-length gene of a GH8 chitosanase from Paenibacillus sp. IK-5 (formerly Paenibacillus fukuiensis D2) (17) as a template with the following primer sets: 5’-ATGATCGACCATCACCATGACACATG-GCCCTGAAACAAACCGGCCACC-3’ (forward) and 5’-TTA- CCGTACACCTGAAATCCAGAG-3’ (reverse) for DD1, 5’-ATGATCGACCATCACCATGACACATG-GCCCTGAAACAAACCGGCCACC-3’ (forward) and 5’-TTATCGTATA-CCTGAAATCCAGAG-3’ (reverse) for DD2, and 5’-ATGCG- ATCACCATACCATACATCAATCTGCGCTGGTAAACACAGCGGCCACC-3’ (forward) and 5’-TTATCGTATA-CCTGAAATCCAGAG-3’ (reverse) for DD1 + DD2. PCR products were purified and ligated into the pETBlue-1 vector by TA-cloning (pETB-DD1, pETB-DD2, and pETB-DD1 + DD2). After confirmation of the cDNA sequences, pETB-DD1, pETB-DD2, and pETB-DD1 + DD2 were introduced into E. coli BL21(DE3) pLacI, Rosetta(DE3) pLacI, and Rosetta(DE3) pLacI, respectively.

Protein Expression and Purification—E. coli cells harboring the plasmid, pETB-DD1, pETB-DD2, or pETB-DD1 + DD2 were grown to attain 0.6 optical density at 600 nm before induction with 1 mM isopropyl 1-thio-galactopyranoside. After induction, growth was continued for 18 h at 18 °C. The cells were harvested by centrifugation, suspended in a 10 mM Tris-HCl buffer (pH 8.0), and disrupted with a sonicator. After cell debris was removed by centrifugation (20,000 g, 15 min), the supernatant was dialyzed against the same buffer and applied onto a nickel-affinity column (1 × 2 cm) equilibrated with dialysis buffer. The column was washed with the same buffer, and adsorbed proteins were eluted with a 10 mM Tris-HCl buffer (pH 8.0) containing 250 mM imidazole. The fractions eluted were pooled and further separated using a column of Sepacryl S-100 HR equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. Fractions exhibiting a single protein band on SDS-PAGE were collected as a purified recombinant protein.

Protein Determination—Protein concentrations were determined by reading absorbance at 280 nm using the extinction coefficients 39,420 (DD1), 40,190 (DD2), and 80,330 (DD1 + DD2) M$^-1$ cm$^-1$, calculated from the equation proposed by Pace et al. (19).

Thermal Unfolding Experiments—To obtain the thermal unfolding curve of the protein, the CD value at 222 nm was monitored using a Jasco J-720 spectropolarimeter (cell length 0.1 cm), whereas the solution temperature was raised at a rate of 1 °C/min using a temperature controller (PTC-423L, Jasco). To facilitate comparisons between unfolding curves, experimental data were normalized as follows. The fraction of unfolded protein at each temperature was calculated from the CD value by linearly extrapolating pre- and post-transition base lines into the transition zone, and those were plotted against the temperature. Final concentrations of the enzyme and (GlcN)$_n$ were 5 μM and 5 mM, respectively.
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ITC Experiments—The DD1 or DD2 solution (80–90 μm) in 50 mM sodium acetate buffer (pH 5.0) was degassed, and its concentration was determined. Individual (GlcN)$_n$ (n = 1, 2, 3, 4, and 6) (1.5 mM) were dissolved in the same buffer, and the solution pH was adjusted to 5.0. Then the (GlcN)$_n$ solution was degassed and loaded into a syringe, whereas the protein solution (0.2028 ml) was loaded into the sample cell after confirming the solution pH 5.0. Protein and ligand concentrations for DD1+DD2 were set at 31 μM and 0.5 mM, respectively. In measurements at pH 7.0, 10 mM sodium phosphate buffer was used instead of the acetate buffer. Calorimetric titration was performed with an iTC200 system (Microcal, Northampton, MA) at 25 °C. Aliquots (1.0–2.0 μl) of the ligand solution were added to the sample cell with a stirring speed of 1000 rpm. Titrations were completed after 19–39 injections. When titration experiments were performed with c values from 10 to 100 (c = n$K_{assoc}$[M]/t), where n is the stoichiometry, $K_{assoc}$ is the association constant, and [M] is the initial protein concentration, $K_{assoc}$ values obtained can be regarded as reliable (20). Titration of GlcN to DD1 yielded a c value of 0.2 and those for DD2 yielded 0.3–3.4, whereas the c values for the other titration experiments were in the region 10 < c < 100. Binding thermodynamics can be obtained using ITC even when c is in the range 0.01 < c < 10 if the requirements proposed by Turnbull and Daranas (21) are satisfied. We confirmed that all requirements were fulfilled in our experiments except the titration of GlcN monomer to DD1. Thus, the thermodynamic data for the GlcN titration to DD1 are not presented (Table 2), but only the ITC profile is presented here (see Fig. 3F).

For analysis of the ITC data, the Origin software installed in an ITC instrument was used. The One Set of Sites model was employed to fit the data for DD1 and DD2, and the Two Sets of Sites model was employed for DD1+DD2. The n values for (GlcN)$_3$ and (GlcN)$_6$ to DD2 were fixed at 1.0 in the optimization process because the n values for (GlcN)$_3$ and (GlcN)$_6$ to DD1 were determined to be about 1.0 (Table 2). Sequential Assignment of the Backbone NMR Signals of DD1 and DD2—NMR samples contained 0.2–0.5 mM protein in 50 mM sodium acetate buffer pH 5.0 (90% H$_2$O/10% D$_2$O). All NMR spectra were acquired at 300 K using a Bruker AV500 spectrometer controlled with TopSpin 3.0 software and equipped with a triple-resonance pulsed-field-gradient cryoprobe head. 1H chemical shifts were referenced to HDO (4.64 ppm at 30°C) relative to trimethylsilyl propanoic acid. 15N and 13C chemical shifts were indirectly calibrated from each gyromagnetic ratio (22). Sequential assignments were performed using 15N/13C-labeled DD1 and DD2 from two-dimensional 1H,15N HSQC and three-dimensional HNCACB, CBCA-(CO)NH, HNCA, HNCA CO, HNCO, and HNCOCA experiments; All spectra were processed using NMRPipe software (23) and were analyzed using Sparky software (24).

Titrations of (GlcN)$_n$—Two-dimensional 1H,15N HSQC spectra were recorded for 0.25 mM 15N-labeled DD1 or DD2 in 50 mM sodium acetate buffer (pH 5.0; 90% H$_2$O/10% D$_2$O) in the presence of various concentrations of (GlcN)$_n$. Chemical shift changes induced by oligosaccharide binding ($\Delta \delta$) were calculated by the equation

$$\Delta \delta = \frac{(\Delta \delta^2 + \Delta \delta^2/25)^{1/2}}{\Delta \delta_{max}}$$  

where $\Delta \delta$ and $\Delta \delta_{max}$ represent the observed shifts in the 1H axis and 15N axis, respectively. Amino acid residues, whose resonances disappeared or shifted more than 0.05 ppm ($\Delta \delta$) at the saturated concentration of the ligand, were visualized on the protein surface of the modeled DD1 and DD2 structures to estimate the interaction sites.

Because the interaction between the protein and (GlcN)$_n$ was a fast or intermediate exchange, $\Delta \delta$ values relative to the maximum shift ($\Delta \delta_{max}$) were plotted against the free oligosaccharide concentrations, and the association constant, $K_{assoc}$, was estimated by nonlinear curve-fitting based on the equation

$$\Delta \delta/\Delta \delta_{max} = [S]_{free}/([S]_{free} + 1/K_{assoc})$$  

Free oligosaccharide concentrations [S]$_{free}$ were obtained by subtracting the bound oligosaccharide concentration [ES] from the total oligosaccharide concentration [S]$_{total}$.

Molecular Modeling—To select an appropriate template for molecular modeling of the three-dimensional structures of DD1 and DD2, the DD1 sequence was submitted to BLAST searches with respect to the PDB database. The x-ray crystal structure of a CBM32 domain of GH84 N-acetyl-β-hexosaminidase from C. perfringens (PDB code 2V5D) (16) was found to be the most suitable as the structural template. The target and template sequences were aligned using ClustalW (25), and alignment was then manually edited using JALVIEW (26) taking into account the position of secondary structural elements for gap positioning. Three-dimensional models of DD1 and DD2 were built by homology modeling with the MODELLER 9v1 version (27). Energy minimization was performed using the GROMACS 4.0 software (28) with a GROMOS 96 53a6 force field with a flexible SPC water model in a cubic box of the size 1 Å (29). The models created were evaluated for their secondary structures relative to those deduced based on the torsion angles predicted from the NMR chemical shifts by the TALOS software (30).

Docking Simulation—DD1 has two acidic residues in the binding region estimated from the NMR titration experiments. Thus, four different protonation states were assumed for DD1 as follows: (i) both residues are deprotonated, (ii) Glu-14 is protonated and Glu-36 is deprotonated, (iii) Glu-14 is deprotonated and Glu-36 is protonated, or (iv) both residues are protonated. In the case of DD2, because there is only one acidic residue (Glu-14) in the binding region, two protonation states were assumed: (i) Glu-14 is protonated, or (ii) Glu-14 is deprotonated. The α-glucosamine dimer, (GlcN)$_2$, was employed as a ligand. Its structure was obtained from the crystal structure of Amycolatopsis orientalis exo-chitosanase (CsxA) complexed with (GlcN)$_2$ (PDB code 2VZV) (31) and protonated using the PRODRG2 server (32). Docking simulations were conducted using AutoDock Version 4.2 and ADT Suite to prepare the systems for calculations (33). A gridbox was created to overlap with the (GlcN)$_2$ binding region estimated from the NMR titration experiments. Fifty docking runs using Lamarckian genetic algorithm (34) with the default parameters were performed, assuming the protein was rigid and the ligand was flexible. The lowest energy structures and structures with a
higher frequency were then selected for each protonation state. The selected structures were superimposed using PyMOL v.0.9.9.

RESULTS

Thermal Unfolding Experiments in the Presence of Ligands—Thermal unfolding curves of DD1, DD2, and DD1+DD2 were obtained by monitoring CD at 222 nm, as shown in Fig. 2. The transition temperature of thermal unfolding ($T_m$) of DD1 was elevated upon the addition of (GlcN)$_6$ ($\Delta T_m = 10.3 \, ^\circ C$), cello-hexamer ($\Delta T_m = 2.5 \, ^\circ C$), laminari-hexamer ($\Delta T_m = 6.0 \, ^\circ C$), or chitin hexamer ($\Delta T_m = 1.3 \, ^\circ C$). However, the values of $\Delta T_m$ for DD2 were much lower than those for DD1 as listed in Table 1. The interactions of the oligosaccharides with DD1 were markedly stronger than those with DD2. Among the oligosaccharides tested, (GlcN)$_3$ enhanced the stability of DD1 most intensively, indicating that DD1 is most specific to chitosan. The transition temperature of thermal unfolding ($T_m$) of DD1 was also $63.1 \, ^\circ C$ again indicating the specificity to chitosan.

Table 1

| Ligand      | $T_m$ | $\Delta T_m$ | $T_m$ | $\Delta T_m$ | $T_m$ | $\Delta T_m$ |
|-------------|-------|-------------|-------|-------------|-------|-------------|
| None        | 52.8  | 10.3        | 66.5  | 13.6        | 52.9  | 13.6        |
| (GlcN)$_6$  | 63.1  | 10.3        | 63.0  | 1.0         | 66.5  | 13.6        |
| (GlcN)$_5$  | 55.3  | 2.5         | 63.2  | 1.2         | 57.5  | 4.6         |
| (GlcN)$_4$  | 58.8  | 6.0         | 64.2  | 2.3         | 59.2  | 6.3         |
| (GlcNAc)$_6$| 54.1  | 1.3         | 62.9  | 0.9         | 56.2  | 3.3         |

**TABLE 1**

Thermal unfolding temperatures of DD1, DD2, and DD1+DD2 in the presence or absence of various hexameric ligands.

The protonation state of the amino groups of (GlcN)$_n$ appears to affect the thermodynamic parameters obtained by ITC. Thus, ITC experiments were conducted at pH 7.0 (Fig. 3I and J). We found that both the favorable contribution from an enthalpy change ($\Delta H^*$ = $-13.8$ kcal/mol) and unfavorable energy changes were lower than those of DD1 by 2.6 kcal/mol for both (GlcN)$_6$ and (GlcN)$_5$. The lower binding ability was mainly derived from the higher entropic penalty ($-\Delta T = 4.8$ ~ $5.1$ kcal/mol).
contribution from an entropy change (−TΔS° = 5.8 kcal/mol) in DD1 increased from those obtained at pH 5.0; finally the binding free energy change (−8.0 kcal/mol) did not change due to the elevation in pH (the data designated by footnote a in Table 2). Similarly, also in DD2, both contributions increased due to the elevation in pH. However, the favorable contribution from an enthalpy change in this case was more enhanced than the unfavorable contribution from an entropy change, resulting in higher affinity than that obtained at pH 5.0 (ΔΔG° = −1.1 kcal/mol).

The thermograms shown in Fig. 3 were successfully fitted using a non-linear least-squares algorithm and the One Set of Sites binding model employed by the Origin software accompanying an iTC200 instrument. However, the One Set of Sites binding model did not give satisfactory fits to the ITC data for DD1+DD2 (Fig. 4). Using the Two Sets of Sites binding model, the quality of the fit evaluated from the χ² value improved by a factor of 1.3 over that obtained by the One Set of Sites model. Thus, we presented here the thermodynamic data for DD1 obtained by the Two Sets of Sites model. Theoretical fits are shown in Fig. 4, and the values are listed in Table 3. Binding to the first binding site was clearly enthalpy-driven with a slight contribution from an entropy change. The favorable free energy changes of binding of (GlcN)₆ and (GlcN)₄ (ΔG° = −8.6 and −8.8 kcal/mol, respectively) were higher than those of the second binding site (ΔG° = −6.4 kcal/mol for

### TABLE 2

Thermodynamic parameters for chitosan oligosaccharide binding to DD1 and DD2 obtained from ITC profiles shown in Fig. 3.

| Protein | Ligand | n | K_{assoc}a | ΔH° | ΔS° | −TΔS° | ΔG° |
|---------|--------|---|-----------|-----|-----|-------|-----|
| DD1     | (GlcN)₆ | 1.2 ± 0.1 | (5.3 ± 0.2) × 10⁻⁵ | −10.0 ± 0.1 | −7.2 | 2.2 | −7.8 |
| DD1     | (GlcN)₅ | 1.0 ± 0.1⁵ | (8.0 ± 0.2) × 10⁻⁵ | −13.8 ± 0.1⁶ | −19.4⁵ | 5.8⁶ | −8.0⁶ |
| DD1     | (GlcN)₄ | 1.2 ± 0.1 | (3.7 ± 0.1) × 10⁻⁵ | −8.2 ± 0.1 | −2.0 | 0.6 | −7.6 |
| DD1     | (GlcN)₃ | 1.2 ± 0.1 | (3.6 ± 0.3) × 10⁻⁵ | −8.2 ± 0.1 | −1.8 | 0.5 | −7.6 |
| DD1     | (GlcN)₂ | 1.3 ± 0.1 | (3.9 ± 0.2) × 10⁻⁵ | −7.5 ± 0.1 | 0.6 | −0.1 | −7.6 |
| DD1     | (GlcN)₁ | 1.1 ± 0.1 | (1.4 ± 0.1) × 10⁻⁵ | −8.9 ± 0.1 | −6.1 | 1.8 | −7.1 |
| DD2     | (GlcN)₆ | 1.0⁷ | (6.5 ± 0.1) × 10⁻⁵ | −10.0 ± 0.1 | −16 | 4.8 | −5.2 |
| DD2     | (GlcN)₅ | 0.9 ± 0.1 | (3.9 ± 0.2) × 10⁻⁵ | −12.2 ± 0.2⁸ | −20.0⁹ | 5.9⁹ | −6.3⁹ |
| DD2     | (GlcN)₄ | 1.0⁹ | (5.2 ± 0.2) × 10⁻⁵ | −10.1 ± 0.2 | −6.9 | 5.1 | −5.0 |

a S.E. were obtained from the nonlinear least square fitting shown in the bottom figures of the panels of Fig. 3.

⁵ The data were obtained in 10 mM sodium phosphate buffer, pH 7.0. Protein concentrations were 80–90 μM for DD1 and DD2.

⁶ The n values were fixed in the optimization process because the values for (GlcN)₃ and (GlcN)₆ to DD1 were determined to be ~1.0.
each) due to the higher entropic penalty of the second site. The binding abilities of the individual sites were independent of the chain length.

**1H,15N HSQC Spectra and Resonance Assignments**—Fig. 5, A and B, show 1H,15N-HSQC spectra of DD1 and DD2. Resonances were fully separated, indicating that the proteins are rich in β-strands. Analysis of the three-dimensional spectra resulted in the nearly complete assignment of the backbone 1H,13C and 15N resonances. A total of 92% of the backbone 1HN and 15N, 91% of the 13Cα, 90% of the 13Cβ, and 91% of the 13Cγ resonances were assigned for DD1; three of the missing 15N assignments correspond to proline residues. A total of 92% of the backbone 1HN and 15N, 92% of the 13Cα, 93% of the 13Cβ, and 94% of the 13Cγ resonances were assigned for DD2; two of the missing 15N assignments correspond to proline residues. The assignments are labeled in the spectra shown in Fig. 5, A and B. All assignment data for DD1 and DD2 were deposited in the BMRB database as accession codes; 11513 and 11514, respectively.

**GlcN₆-binding Site**—When the (GlcN)₆ solution was titrated into the enzyme solution, several NH resonances in the spectrum (Fig. 5, C and D) shifted gradually from the free state (red resonances for both figures; molar ratio of (GlcN)₆/protein = 0) to the bound state (blue resonances in Fig. 5C, molar ratio of (GlcN)₆/DD1 = 5; blue resonances in Fig. 5D, (GlcN)₆/DD = 20) with line-broadening to some extent. No further change was observed when the molar ratio of (GlcN)₆/protein was more than 5 (DD1) or 20 (DD2), indicating that the proteins were saturated at these molar ratios. The continuous chemical shift changes suggested that the rate of exchange between the free and bound states was fast or intermediate. Thus, we calculated ΔG° according to Equation 1 for individual resonances affected by the (GlcNAc)₆ titration and attempted to estimate the interaction site of the individual proteins based on the chemical shift perturbation.

Before estimating the interaction site, the three-dimensional structures of DD1 and DD2 were created by homology modeling with the MODELLER 9v1 version (27). The final structures are shown in Fig. 1B. Both DD1 and DD2 exhibited a typical β-sandwich fold consisting of eight or nine distinct β-strands and loop structures extruded from the β-core structures. The secondary structures of these modeled structures were found to be consistent with those deduced by the TALOS software based on the 13C chemical shift data of the individual α-carbons (Fig. 6, A and B). This indicates that the modeled structures obtained were reliable.
Amino acid residues whose resonances were affected by the addition of \((\text{GlcN})_6\) were mapped on the surface of the modeled structure of DD1 and DD2. As shown in Fig. 7, \((\text{GlcN})_6\) was found to bind to the loop regions forming the roof of the core \(\beta\)-sandwich structure in both DD1 and DD2. In the loop region (top view of Fig. 7), the oligosaccharide appears to bind to the lower portion including residues 14, 33–38, and 117–123 of DD1 and DD2, respectively. Additional chemical shift perturbations were found at residues 22, 60–64, and 93–95 for DD1 and at residues 15 and 18 for DD2. When \((\text{GlcN})_n\) with various chain lengths \((n = 2 \text{ to } 5)\) were added to the individual proteins, no significant difference was found in amino acid residues whose resonances were affected (data not shown).

**Docking Simulations**—To more clearly identify the interaction site, we conducted docking simulations of \((\text{GlcN})_2\) binding to DD1 and DD2. Based on the clustering histogram provided by the AutoDock (Version 4.2) software, we selected the lowest energy structures (four structures for DD1 and two structures for DD2) and structures with a higher frequency (seven structures for DD1 and four structures for DD2) from the results obtained for individual protonation states. All of the selected structures were then superimposed on the surface of the individual modeled structures. The results are shown in Fig. 8. Although the superimposed structures included the results obtained by docking simulations under all protonation states, \((\text{GlcN})_2\) binding sites were found to be restricted to the shallow binding cleft including the residues 14, 33–38, and 121–123 for both DD1 and DD2.

**NMR-based Determination of Association Constants**—We also determined the association constants and binding free energy changes of individual \((\text{GlcN})_n\) \((n = 1 \text{ to } 6)\) based on the chemical shift changes of the Asp96 NH resonance for DD1 and the Ala-33 resonance for DD2. These resonances were fully separated from the others, allowing us to correctly determine the association constants based on the chemical shift changes \((\Delta \delta)\). Titration curves were obtained as shown in Fig. 9 and were used for nonlinear curve fitting to determine \(K_{\text{assoc}}\) values based on Equation 2. The results are listed in Table 4. The free energy changes of binding to DD1 were weakly dependent upon the chain length \((\Delta G = -6.2 \text{ to } -7.3 \text{ kcal/mol})\). \((\text{GlcN})_2\) was found to bind with the lowest affinity, and \((\text{GlcN})_5\) was found to bind...
with the highest affinity. Dependence upon the chain length was also weak in the binding free energy changes of DD2. From \((\text{GlcN})_2\) to \((\text{GlcN})_4\), the binding affinity gradually increased but was almost unchanged from \((\text{GlcN})_4\) to \((\text{GlcN})_6\).

**DISCUSSION**

**Specificity**—The specificities of DDs belonging to the CBM32 family have been recognized to be diverse (35). Specificity to galactose and lactose was demonstrated for the CBM of sialidase from *Micromonospora viridifaciens* (36). A CBM32 protein specific to polygalacturonic acid was also found in *Yersinia enterocolitica* (37). Specificity to LacNAc \((\alpha\text{-D-galactosyl-1,4-\beta\text{-D-N-acetylglucosamine}) was also shown for a CBM32 of N-acetylhexosaminidase from C. perfringens* (16). More recently, a mannan binding module belonging to the CBM32 family was found in a \(\beta\text{-1,4-mannanase from Clostridium thermocellum})* (38). In this study thermal unfolding experiments suggested that DD1 in a chitosanase from *Paenibacillus* sp. IK-5 strain was found to be specific to chitosan oligosaccharides and also to have less specificity toward laminari-hexamer (Fig. 2, Table 1). Thus, DD1 is a novel type of carbohydrate binding module belonging to the CBM32 family. Although the specificity of DD2 was unclear from the thermal unfolding experiments, the elevations in \(T_m\) \((\Delta T_m)\) observed for DD1+DD2 clearly suggested that the tandem DD1/DD2 is highly specific to \((\text{GlcN})_6\) \((\Delta T_m = 13.6 \, ^\circ\text{C}) and is likely to assist the action of chitosanase by leading the chitosan polysaccharide chain into the catalytic cleft of the chitosanase. This is the first identification of carbohydrate binding modules specific to chitosan.

**Binding Affinities of \((\text{GlcN})_n\)**—A recent study on a mannan binding module belonging to CBM32 revealed a greater contribution of the entropic penalty to interactions with \(\beta\text{-1,4-mannno-oligosaccharides} (- T \Delta S^c \approx 5.0 \text{ kcal/mol})\) (38). This situation resulted in less favorable free energy changes in

**FIGURE 6.** Comparison of the secondary structures of DD1 (A) and DD2 (B) predicted from NMR chemical shifts (green arrows) to those of the modeled structure (black arrows). The \(\Phi\) (red circles) and \(\Psi\) (black circles) angles were predicted by the TALOS software. The \(\beta\)-strands labeled as \(\beta 1\text{-}\beta 9\) correspond to those of DD1 and DD2 shown in Fig. 18.
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the interactions ($\Delta G^\circ = -5.5 \text{ kcal/mol}$) than those obtained for (GlcN)$_n$ ($n \geq 2$) interactions with DD1 ($\Delta G^\circ = -7.0 \sim -8.0 \text{ kcal/mol}$). This difference may be due at least partly to the amino groups in the C-2 positions of GlcN, which possibly make hydrogen bonds or electrostatic interactions with the proteins. On the other hand, the thermodynamic parameters obtained for the mannann binding module were similar to those for DD2 ($-T\Delta S^\circ = 5.0 \text{ kcal/mol}, \Delta G^\circ = -5.0 \sim -5.2 \text{ kcal/mol}$). Some interaction of DD1 with the amino group of GlcN residue may be disrupted in DD2. In fact, Glu-36 of DD1, which may be involved in electrostatic interaction with the amino group of GlcN residue, is substituted with tyrosine in DD2.

The binding free energy changes of (GlcN)$_2$ (GlcN)$_n$ for both DD1 and DD2 were not clearly dependent on the chain length (Table 2). A similar independency was also observed in the mannann binding module from C. thermocellum. It is well known that in interactions of oligosaccharides with a long-extended binding cleft of proteins, the binding free energy changes are dependent upon the chain length of oligosaccharides (39, 40). Thus it appears that DD1 and DD2 do not have such a long-extended binding cleft. However, the binding affinities of GlcN and (GlcN)$_n$ were dependent upon the chain length (Fig. 3, E and F). It is very likely that DD1 and DD2 can accommodate at least two GlcN units. Extra GlcN residues of (GlcN)$_n$ (GlcN)$_n$ may be extended beyond the ligand binding region and not involved in the interaction. This is consistent with the fact that the binding free energy changes determined by NMR titrations were not clearly dependent on the chain length (Table 4). Thus, DD1 and DD2 are Type C CBMs.

All data sets obtained by thermal unfolding experiments, ITC, and NMR titrations (Tables 1, 2, and 4) revealed that the affinity to (GlcN)$_n$ of DD1 is significantly higher than that of DD2. It is likely that DD1 dominates the specificity and binding affinity of DD1 + DD2.

Synergy—Similar binding studies were also conducted with DD1 + DD2 comprising the two modules in tandem (Fig. 1A). As listed in Table 1, the interaction ability with (GlcN)$_n$ deduced from the elevation in $T_m$ ($\Delta T_m$) for DD1 + DD2 (13.6 °C) was higher than the sum of those for DD1 and DD2 (10.3 and 1.0 °C, respectively). The free energy changes of binding of (GlcN)$_1$ to DD1 + DD2 were determined as −8.6 and −6.4 kcal/mol for individual binding sites, which corresponded to the DD1 and DD2 sites, respectively (Fig. 4, Table 3). Because the free energy changes of (GlcN)$_n$ binding to DD1 and DD2 were −7.8 and −5.2 kcal/mol, respectively, binding affinities were found to be synergistically enhanced in both binding sites of DD1 + DD2. A similar effect of the tandem CBMs was reported by Boraston et al. (41). They found that a protein comprising CBM17 and CBM28 in tandem derived from an alkalophilic Bacillus sp. 1139 cellulase (Cel5A) bound to amorphous cellulose with a significantly higher affinity than the sum of the affinities of individual CBM17 and CBM28. It has been recognized that the synergies often found in the cellulose-degrading enzyme system are derived from proximity effect (42, 43). Glycosyl hydrolases comprising CBMs in tandem were often found in various living organisms (44–47). Proximity effects resulting from the tandem CBMs may be advantageous for the enzyme action toward polysaccharide chains. A similar explanation is possible for the synergy of DD1 + DD2.

Interaction Sites—NMR titration experiments and docking simulation were conducted to identify the interaction site for DD1 and DD2. In both DD1 and DD2, amino acid residues whose resonances were affected by (GlcN)$_n$ binding were localized at the loops extruded from the β-sandwich core structure (Fig. 7). The interaction sites for the other CBM32 investigated thus far are similar to those of DD1 and DD2. However, closer examination of the complexed structures obtained by the docking simulation (top view of Fig. 8) revealed that (GlcN)$_2$ binds to the shallow binding cleft formed by the loops containing the residues 14, 33–38, and 121–123 for both DD1 and DD2. The residues 33–38 and 121–123 correspond to the loop before β1
and the loop between β7 and β8 for DD1 and to the loop between β1 and β2 and the loop between β8 and β9 for DD2, respectively (Fig. 6, A and B). These two loops are closely located, forming the (GlcN)$_n$-binding site, in both DD1 and DD2. However, the amino acid residues interacting with (GlcN)$_n$ could not be defined in this study. Crystal structure analysis of DD1, DD2, and DD1/DD2 is now under progress.

A CBM32 of N-acetylhexosaminidase from C. perfringens (CpCBM32) recognizes LacNAc (16). A sialidase from M. viridifaciens also has a CBM32 (MvCBM32), which recognizes galactose (36). Alignment of the amino acid sequences of DD1, DD2, CpCBM32, and MvCBM32 revealed that consensus sequences are mostly found in the regions corresponding to the β-strands, which form the core structure of CBM32s, and that the interaction sites with ligands were located in the loop region, which do not exhibit any sequence homology (data not shown). This situation may bring about the ligand diversity in CBM32s.

**Effect of pH**—Because the pK$_a$ value of the amino group of GlcN residue was reported to be 6.4 (48), the residue is protonated (positively charged) at pH 5.0 and deprotonated (uncharged) at pH 7.0. Thus, the electrostatic interactions mediated by amino groups of GlcN residues may be disrupted at pH 7.0, but the effect of pH elevation from 5.0 to 7.0 on the thermodynamic data (Table 2) was quite complicated and could not simply be interpreted by the disruption of the electrostatic interactions mediated by GlcN residues. On the other hand, it should be noted that the resonances of Glu-14 and Glu-36 responded to the (GlcN)$_n$ binding in DD1 and that the resonance of Glu-14 responded in DD2 (Fig. 7). Because ligands (GlcN)$_n$ are positively charged at the acidic pH region, the protonation states of these glutamic acid residues may strongly affect the binding mode of the ligands. In fact, closer inspection of the structures selected by docking simulation (Fig. 8) revealed that the binding mode of (GlcN)$_2$ slightly fluctuated depending on the protonation state. This indicates that the protonation states of glutamic acids do not greatly alter the binding site but slightly rearrange the binding mode. Electrostatic interactions may be significantly involved in (GlcN)$_n$ binding to DD1/DD2. However, at present it is difficult to correctly interpret the effect of pH on the binding ability of (GlcN)$_n$ to DD1/DD2.

**TABLE 4**

| Ligand | $K_{assoc}$ | $\Delta G$ | $K_{assoc}$ | $\Delta G$ |
|--------|-------------|------------|-------------|------------|
| (GlcN)$_6$ | $6.6 \times 10^4$ | $-6.6$ | $5.3 \times 10^3$ | $-5.1$ |
| (GlcN)$_5$ | $2.1 \times 10^5$ | $-7.3$ | $4.5 \times 10^3$ | $-5.0$ |
| (GlcN)$_4$ | $9.0 \times 10^4$ | $-6.8$ | $5.0 \times 10^3$ | $-5.1$ |
| (GlcN)$_3$ | $5.0 \times 10^4$ | $-6.4$ | $3.1 \times 10^3$ | $-4.8$ |
| (GlcN)$_2$ | $3.0 \times 10^4$ | $-6.2$ | $1.1 \times 10^3$ | $-4.1$ |

**FIGURE 9.** Titration curves of DD1 and DD2 with chitosan oligosaccharides based on the chemical shift perturbations obtained from NMR titration experiments. The chemical shift changes ($\Delta \delta$) of the resonances of Asp96 for DD1 and Ala-33 for DD2 were used for obtaining the titration curves.
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Biotechnological Significance—The first identification of the CBMs specific to chitosan suggested that the enzymatic degradation of chitosan may be facilitated by CBMs in a similar manner to those reported for enzymatic degradation of the other polysaccharides, such as chitin and cellulose (5, 6). However, because of the amorphous nature of chitosan, the binding mode of chitosan to DD1 and DD2 may be different from those reported for CBMs specific to chitin and cellulose. In fact, aromatic residues often found in the binding surface of the CBMs specific to chitin and cellulose (49, 50) are unlikely involved in the sugar recognition in DD1 and DD2. Further details of the chitosan recognition mechanism may be obtained by the structural analysis of the full-length protein of Paenibacillus sp. IK-5 chitosanase.

As described above, the artificial and natural chitosans do not exist as homogeneous polysaccharides but contain chitin to some extent. When the CBMs specific to chitosan are immobilized onto an appropriate matrix, the adsorbent may be useful in biological and biochemical studies related to chitosan.

Conclusions—DD1 + DD2 located at the C terminus of a chitosanase from Paenibacillus sp. IK-5 specifically bind chitosan oligosaccharides. Individual CBMs can accommodate at least two GlcN units at loops extruded from the core β-sandwich. Although DD1 dominates the chitosan binding ability of DD1 + DD2, the synergistic action of the two CBMs appears to facilitate the enzymatic hydrolysis of chitosan.

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