Interaction of di-N-acetylchitobiosyl moranoline with a family GH19 chitinase from moss, *Bryum coronatum*

Shoko Shinya2, Atsushi Urasaki2, Takayuki Ohnuma2, Toki Taira3, Akari Suzuki4, Makoto Ogata5, Taichi Usui4, Outi Lampela6, André H Juffer6, and Tamo Fukamizo1,2

1Department of Advanced Bioscience, Kinki University, 3327-204, Nakamachi, Nara 631-8505, Japan; 2Department of Bioscience and Biotechnology, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan; 3Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, 836 Ohyau, Suruga-ku, Shizuoka 422-8529, Japan; 4Department of Chemistry and Biochemistry, Fukushima National College of Technology, 30 Nagao, Iwaki, Fukushima 970-8034, Japan; 5Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, 836 Ohyau, Suruga-ku, Shizuoka 422-8529, Japan; 6Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu 90014, Finland

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Tri-N-acetylttriosyl moranoline, (GlcNAc)3-M, was previously shown to strongly inhibit lysozyme (Ogata M, Umemoto N, Ohnuma T, Nomata T, Suzuki A, Usui T, Fukamizo T. 2013). A novel transition-state analogue for the catalytic reaction of chitin oligosaccharides catalyzed by *BcChi19A* revealed that the complex is quite stable and the binding mode does not significantly change during the simulation. The moranoline moiety of (GlcNAc)2-M did not fit into the catalytic cleft (subsite −1) but was rather in contact with subsite +1. This situation may result in the moderate inhibition toward the *BcChi19A*-catalyzed hydrolysis.

Keywords: chitin oligosaccharide / family GH19 chitinase / isothermal titration calorimetry / moranoline / NMR

Introduction

Chitinases (EC 3.2.1.14) hydrolyze the β-1,4-glycosidic linkages of chitin, a β-1,4-linked polysaccharide of N-acetylglucosamine (GlcNAc). The enzymes are widely distributed in living organisms and are responsible for self-defense, growth, morphogenesis, cuticle destabilization and stress tolerance (Kasprzewska 2003; Donnelly and Barnes 2004; DuocChuan 2006; Bhattacharya et al. 2007; Ankan and Muthukrishnan 2010). A number of chitinase genes have been isolated and sequenced, and the gene products have been characterized. The enzymes are divided into families GH18 and GH19, according to the CAZy database (http://www.cazy.org/; Henrissat and Bairoch 1993; Fukamizo 2000). GH18 chitinases have been intensively studied with respect to their structure and function and have attracted public attention as possible targets for biological control. In fact, allosamidin, an inhibitor for family GH18 chitinases, has been used to control the chitinase-mediated biological events, such as infection with protozoa (Shahabuddin et al. 1993), insect ecdisis (Blattner et al. 1997) and self-defense in plants (Takenaka et al. 2009). Allosamidin has also been used frequently for structural and functional studies of GH18 enzymes (van Aalten et al. 2001; Bortone et al. 2002; Rao et al. 2003; Karasuda et al. 2004; Cederkvist et al. 2007).

On the other hand, GH19 enzymes are found only in plants and some bacteria, and their structure and function remain poorly understood. Although crystallographic analyses of GH19 chitinases have been conducted by several groups (Hart et al. 1993; Hoell et al. 2006; Ubhayasekera et al. 2007, 2009; Huet et al. 2008; Ohnuma et al. 2012, 2013), the mechanistic details underlying their catalytic reaction remain unclear. This may be due to the absence of an efficient inhibitor that mimics the transition state of the catalytic reaction for GH19 chitinases. Ogata et al. (2013) recently reported the novel lysozyme
inhibitor, tri-N-acetylchitotriosyl moranoline (GlcNAc)3-M, which strongly binds to subsites −4, −3, −2 and −1 of the binding cleft. The moranoline moiety of (GlcNAc)3-M was found to fit well into the catalytic cleft (subsite −1), and may mimic the transition state of the catalytic reaction. GH22 lysozymes and GH19 chitinases have been shown to share a similar fold containing two α-helices and a three-stranded β-sheet in their catalytic cleft (Monzingo et al. 1996). Thus, the moranoline derivatives of chitin oligosaccharides, (GlcNAc)n-M, may inhibit GH19 enzyme-catalyzed reactions.

Taira et al. (2011) isolated and characterized a GH19 chitinase from the moss Bryum coronatum, designated as BcChi19A. They showed that BcChi19A is smaller (22 kDa) than the GH19 chitinases from barley, rye and papaya (26 kDa), due to the deletion of several loop structures. Similar loop deletions have also been identified in family GH19 chitinase from Streptomyces coelicolor (Hoell et al. 2006) and class IV chitinase from Norway spruce (Ubhayasekera et al. 2009). The crystal structure of BcChi19A in complex with (GlcNAc)4 was recently solved by Ohnuma et al. (2014), and demonstrated that substrate-binding cleft of BcChi19A is composed of four subsites, −2, −1, +1 and +2. The backbone resonances of the 1H–15N two-dimensional heteronuclear single quantum correlation (HSQC) spectrum of BcChi19A were also completely assigned using three-dimensional nuclear magnetic resonance (NMR) approach (Shinya et al. 2012). Thus, BcChi19A is a highly suited enzyme for structurally examining interactions with various ligands.

In the present study, we analyzed the interaction of (GlcNAc)2-M with BcChi19A by thermal unfolding experiments, isothermal titration calorimetry (ITC), NMR spectroscopy, inhibition experiments and molecular dynamics simulation. (GlcNAc)2-M was found to interact more strongly than (GlcNAc)2 and moderately inhibit GH19 chitinase-catalyzed reaction.

Results and discussion

Thermal unfolding experiments

The thermal unfolding curves of BcChi19A and BcChi19A-E61A were obtained by monitoring circular dichroism (CD) at 222 nm in the absence or presence of the ligands, (GlcNAc)2, (GlcNAc)3, or (GlcNAc)2-M, as shown in Figure 1. Most data points for the individual unfolding experiments fitted well to the corresponding theoretical curves, assuming two-state transition. We could not determine the thermodynamic parameters because the unfolding transitions were found to be irreversible; therefore, we evaluated structural stability only from the transition temperatures (Tm) of the major transition zones. The results are listed in Table I. According to the elevations in Tm (ΔTm), the thermal stability increased only by 1.0°C, when (GlcNAc)2 was added to the BcChi19A solution. The addition of (GlcNAc)3 to BcChi19A did not elevate the Tm value. (GlcNAc)3 was shown to be slowly hydrolyzed by BcChi19A (Ohnuma et al. 2011), and the probability of (GlcNAc)3 occupying subsite −1, which has an unfavorable positive binding free energy change (Sasaki et al. 2003), is higher than that of (GlcNAc)2. These situations may decrease the affinity of (GlcNAc)3 to BcChi19A. However, the Tm value was considerably elevated in the presence of (GlcNAc)2-M (ΔTm = 4.3°C). When the inactive mutant BcChi19A-E61A was used instead of the wild type, ΔTm values for (GlcNAc)2 and (GlcNAc)3 were 1.4 and 3.4°C. However, (GlcNAc)2-M elevated Tm of BcChi19A-E61A by 5.8°C. The stabilization effects of (GlcNAc)2 and (GlcNAc)2-M in BcChi19A were similar to those in BcChi19A-E61A. The effect of (GlcNAc)3 in BcChi19A-E61A was much higher than that in BcChi19A, probably due to the inactivity and the altered catalytic center of BcChi19A-E61A. The binding mode of (GlcNAc)2-M may be markedly different from that of (GlcNAc)2, and the moranoline moiety of (GlcNAc)2-M may enhance the affinity to BcChi19A. (GlcNAc)3-M was not tested, because the (GlcNAc)3 moiety of the compound was hydrolyzed by BcChi19A.

Fig. 1. Thermal unfolding curves of BcChi19A (left panel) and BcChi19A-E61A (right panel) in the absence or presence of ligands. Unfolding curves were obtained in 50 mM sodium acetate buffer, pH 5.0, monitoring CD at 222 nm. Final concentrations of proteins and ligands were 8 μM and 8 mM, respectively.
Isothermal titration calorimetry analysis of (GlcNAc)₂-M binding to BeChi19A-E61A

We initially attempted to obtain ITC-binding data using the wild-type BeChi19A. Although the ITC measurements were conducted under various pHs, various temperatures and various concentrations of protein and ligands, the interaction of the ligands with the wild-type enzyme did not induce heat release or absorption. Thus, we used only the inactive mutant of BeChi19A (BeChi19A-E61A) in the ITC experiments. Binding of (GlcNAc)₂, (GlcNAc)₃ and (GlcNAc)₂-M to BeChi19A-E61A was examined at 25°C and pH 5.0. Figure 2 shows ITC thermograms and theoretical fits to the experimental data for the binding of individual ligands. Theoretical fits to the experimental data were obtained using a non-linear least-squares algorithm by varying the binding affinity constant ($K_a$), number of binding sites, i.e. the stoichiometry of the reaction ($n$), and enthalpy change of ligand binding ($\Delta H^o_r$). The ITC profile obtained for (GlcNAc)₂ (Figure 2A) did not provide reliable thermodynamic data because the titration did not lead to complete saturation. However, individual heat releases by the addition of (GlcNAc)₂ were less intensive than those by (GlcNAc)₃ and (GlcNAc)₂-M (Figure 2B and C), suggesting clearly that the affinity of (GlcNAc)₂ was lower than those of the others. Regarding (GlcNAc)₂ and (GlcNAc)₂-M, both fits yielded $n$ between 1.2 and 1.3 indicating a one to one stoichiometry (Table II). A two-site-binding model was also tried to confirm their stoichiometry, but did not provide a satisfactory fit between experimental and theoretical data.

(GlcNAc)₃ was found to bind to the enzyme with $K_{assoc}$ of $6.7 \times 10^3$ M⁻¹ and the binding free energy change, $\Delta G^o_r$, of $-5.2$ kcal/mol at 25°C and pH 5.0 (Table II). The (GlcNAc)₂ binding was clearly enthalpy driven ($\Delta H^o_r$=-7.0 kcal/mol) with a small unfavorable entropy ($-T\Delta S^o_r=1.8$ kcal/mol and $\Delta S^o_r=-6.1$ cal/K mol). The value was consistent with that reported previously (Ohnuma et al. 2011). Although the molecular size of (GlcNAc)₂-M is almost identical to that of (GlcNAc)₃, the binding ability of (GlcNAc)₂-M was higher than that of (GlcNAc)₃; the $K_{assoc}$ was larger than that of (GlcNAc)₃ (7.2 × 10⁴ M⁻¹), and $\Delta G^o_r$ was $-6.6$ kcal/mol. The (GlcNAc)₂-M binding was driven by a favorable enthalpy contribution ($-6.1$ kcal/mol) with a small but favorable entropy ($-T\Delta S^o_r=-0.5$ kcal/mol and $\Delta S^o_r=1.8$ cal/K mol). These results suggest again that the moranoline moiety of (GlcNAc)₂-M may enhance affinity to BeChi19A-E61A.

**Table I.** Stabilization effects on BeChi19A and BeChi19A-E61A by the addition of ligands

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ligand</th>
<th>$T_m$</th>
<th>$\Delta T_m$</th>
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<tbody>
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<td>76.7</td>
<td>-</td>
</tr>
<tr>
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<td>77.7</td>
<td>1.0</td>
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<td>(GlcNAc)₂-M</td>
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<td>4.3</td>
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<tr>
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<td>None</td>
<td>75.8</td>
<td>-</td>
</tr>
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<td></td>
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<td></td>
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<td></td>
<td>(GlcNAc)₂-M</td>
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<td>5.8</td>
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</table>

$T_m$ values were determined from the unfolding curves obtained by CD.

**NMR titration experiments**

Chemical shift perturbations upon the binding of (GlcNAc)₂ or (GlcNAc)₂-M were observed in a wide region of the $^1$H–$^{15}$N–HSQC spectra of $^{15}$N-labeled wild-type BeChi19A. Typical examples are shown in Figure 3A and B, overlaid spectra in the absence or presence of various concentrations of the ligands.

Fig. 2. ITC thermograms and theoretical fits for the interactions between BeChi19A-E61A and (GlcNAc)₂ (A), (GlcNAc)₃ (B) or (GlcNAc)₂-M (C). Titration experiments were performed in 50 mM acetate buffer, pH 5.0, using a MicroCal ITC200 system at 25°C.
The resonances of Thr24, Ser32, Glu37, Asn164, Gly174 and Thr189 gradually shifted with increase in the (GlcNAc)_2 concentration, whereas the resonances of Gly42, Gly56 and Gly90 remained unaffected (Figure 3A). The mode of the shifts in resonances revealed that the exchange rate between the free and bound states is fast or intermediate with respect to the NMR time scale. When (GlcNAc)_2-M was added instead of (GlcNAc)_2, however, the exchange rate evaluated from the shifting mode in the Asn164 resonance became slower, and the Thr24 resonance lost its intensity due to strong line broadening (Figure 3B). The molar ratios of the enzyme–ligand are listed in the right side of the panels.

The resonances of Thr24, Ser32, Glu37, Asn164, Gly174 and Thr189 gradually shifted with increase in the (GlcNAc)_2 concentration, whereas the resonances of Gly42, Gly56 and Gly90 remained unaffected (Figure 3A). The mode of the shifts in resonances revealed that the exchange rate between the free and bound states is fast or intermediate with respect to the NMR time scale. When (GlcNAc)_2-M was added instead of (GlcNAc)_2, however, the exchange rate evaluated from the shifting mode in the Asn164 resonance became slower, and the Thr24 resonance lost its intensity due to strong line broadening (Figure 3B). The molar ratios of the enzyme–ligand for the individual spectra listed in right side of the panels suggested that the resonances were found to be more sensitive to (GlcNAc)_2-M than to (GlcNAc)_2. The affinity of (GlcNAc)_2-M appears to be higher than that of (GlcNAc)_2. Similar effects were observed in other regions of the spectra. (GlcNAc)_3 was not tested for the binding cleft (left panels) and the hinge region located behind the binding cleft (right panels). The bound (GlcNAc)_4 is illustrated according to the crystal structure (PDB code, 3WH1).

Amino acid residues, whose resonances lost intensities or shifted more than 0.1 ppm (Δδ), were mapped on the surface of the crystal structure of BcChi19A-E61A in complex with (GlcNAc)_4 (Ohnuma et al. 2014; PDB code, 3WH1). The results are shown in Figure 5, which represents the substrate-binding cleft (left panels) and the hinge region located behind the binding cleft (right panels). The bound (GlcNAc)_4 is illustrated according to the crystal structure (PDB code, 3WH1). When (GlcNAc)_2 was titrated into the BcChi19A solution, the main chain resonances of Glu70, Gln100, Ser102 and Leu167 lost their intensities due to strong line broadening, while those of Trp103 and Asn164 shifted. All of these amino acids were shown to be involved in sugar residue interactions at subsite −2 and −1 (Ohnuma et al. 2014). (GlcNAc)_1 was reported to bind to subsites −2, −1 and +1 (Ohnuma et al. 2011), and the sugar residue affinity of subsite −2 was markedly higher than that of subsite +1 (Sasaki et al. 2003). Therefore, (GlcNAc)_2 is most likely to bind to subsites −2 and −1. The (GlcNAc)_2 binding may have affected the main chain dynamics of Glu70, Gln100, Ser102 and Leu167 as well as their conformation. When (GlcNAc)_2-M was added to the BcChi19A solution; however, additional effects were observed in the resonances of Gly64 and Gln71, which lost their intensities. (GlcNAc)_2-M appears to be in contact with subsite +1 in addition to subsites −2 and −1.

In the NMR study on the chitin oligosaccharide binding to BcChi19A (Ohnuma et al. 2014), we demonstrated that the amino acids located in the hinge region behind the substrate-binding cleft were also affected by oligosaccharide binding. These effects were explained by domain motion induced by oligosaccharide binding (Ohnuma et al. 2013). As shown in Figure 5 (right panels), amino acids, whose resonances were affected, are restricted to the hinge region in both cases. However, the number of amino acids, whose resonances lost intensities upon addition of (GlcNAc)_2-M, was markedly larger than that observed upon the addition of (GlcNAc)_2. (GlcNAc)_2-M binding considerably affected the main chain dynamics of the amino acids colored in blue, and this was attributed to enhanced domain motion of the BcChi19A enzyme.

Similar titration experiments were conducted for ^15N-labeled BcChi19A-E61A using (GlcNAc)_2 and (GlcNAc)_2-M. The results were basically similar to those obtained for wild-type
BcChi19A, except that the exchange rate between the free and ligand-bound states was slow in the inactive mutant enzyme. To quantitatively evaluate the binding abilities of (GlcNAc)₂ and (GlcNAc)₂-M, relative chemical shift changes (Δδ/Δδ_max) or intensity changes (I_bound/(I_free + I_bound)) of Glu37 at various ligand concentrations were calculated and plotted against free ligand concentrations (Figure 6). However, the mathematical analysis to obtain dissociation constant was not successful because of the abnormal line shape of the titration curves, especially for (GlcNAc)₂. Nevertheless, it was clear that (GlcNAc)₂-M has markedly higher affinity than (GlcNAc)₂ toward both BcChi19A and BcChi19A-E61A. The NMR data were consistent with the results obtained by thermal unfolding and ITC experiments.

Inhibition experiments

The higher affinity of (GlcNAc)₂-M to BcChi19A prompted us to examine the inhibitory activity of (GlcNAc)₂-M toward BcChi19A-catalyzed hydrolysis, using (GlcNAc)₄ and (GlcNAc)₆ as the substrates. As shown in Figure 7, inhibitory activity was moderate under the conditions employed (pH 5.0, 25°C). The half maximal (50%) inhibitory concentration, IC₅₀, of (GlcNAc)₂-M, which was determined based on the rate of (GlcNAc)₆ degradation, was 620 μM, while the value based on the rate of (GlcNAc)₄ degradation was 130 μM. Control experiments were conducted using (GlcNAc)₃ and (GlcNAc)₃ instead of (GlcNAc)₂-M. Neither oligosaccharide inhibited the enzymatic hydrolysis. The higher affinity of (GlcNAc)₂-M resulted in the moderate inhibition of GH19 chitinase-catalyzed hydrolysis.

Molecular dynamics simulation

To rationalize the moderate inhibition of (GlcNAc)₂-M, we attempted to solve the crystal structure of BcChi19A in complex with (GlcNAc)₂-M. However, we failed to obtain a crystal of the complex. Thus, we conducted a molecular dynamics simulation of (GlcNAc)₂-M binding to wild-type BcChi19A. We initially selected the lowest energy structure of BcChi19A in complex with (GlcNAc)₂-M based on the clustering histogram provided by the AutoDock (version 4.2) software, then the selected structure was submitted for the molecular dynamics simulation. Figure 8A shows a snapshot of the simulated structure of BcChi19A in complex with (GlcNAc)₂-M at 2 ns. From comparison with (GlcNAc)₄ bound to BcChi19A-E61A in the crystal structure, three individual units of (GlcNAc)₂-M were found to bind to subsites −2, −1 and +1. However, the conformation of the bound (GlcNAc)₂-M (magenta) was considerably different from that of the bound (GlcNAc)₄ (yellow), especially in the −1 sugar and the moranoline moiety of (GlcNAc)₂-M. The close-up view of the bound (GlcNAc)₂-M is shown in Figure 8B. The average distance from the Glu70 side chain to the moranoline O4 was 7.17 Å, and the root mean square fluctuation was 0.56 Å. The distance to the moranoline N5 was 5.77 Å, and the fluctuation was 1.22 Å. Thus, the complex can be regarded as stable during the simulation, because the ligand stays at the binding cleft. The binding mode of (GlcNAc)₂-M to subsites −2, −1 and +1 did not significantly change during the simulation and appeared to be consistent with the NMR titration data shown in Figure 5 (left-lower panel).

Fig. 4. Responses of the main chain resonances of BcChi19A to the titrations of (GlcNAc)₂ (molar ratio of the enzyme–ligand = 1:75) (A) and (GlcNAc)₂-M (molar ratio of the enzyme–ligand = 1:5) (B). The vertical axis represents the magnitude of chemical shift changes (Δδ) for individual resonances, which shifted upon titrations. Light green and green bars are amino acids whose resonances shifted >0.1 and 0.15 ppm, respectively. Blue bars are amino acids whose resonances lost intensities due to line broadening. Titration conditions were the same as those in Figure 3.
Fig. 5. Amino acid residues in BcChi19A, whose resonances shifted (green) or lost intensities due to line broadening (blue) in response to the titrations of (GlcNAc)$_2$ (molar ratio of the enzyme–ligand = 1:75) (A) or (GlcNAc)$_2$-M (molar ratio of the enzyme–ligand = 1:5) (B). Left panels: views of the binding cleft of BcChi19A. The bound (GlcNAc)$_4$ was illustrated according to the crystal structure (PDB code, 3WH1) by a stick model in yellow. The catalytic residue E(A)61 is colored in red. Right panels: views of the hinge region located behind the binding cleft. Titration conditions were the same as those in Figure 3.

Fig. 6. Titration curves of (GlcNAc)$_2$ and (GlcNAc)$_2$-M based on chemical shift changes in the Glu37 resonance of BcChi19A (left panel) and BcChi19A-E61A (right panel). Titration conditions were the same as those in Figure 3.
In the lysozyme-(GlcNAc)_3-M-complex (Ogata et al. 2013), the moranoline moiety appeared to fit into the catalytic cleft (subsite −1) to strongly interact with the carboxyl side chains of the catalytic residues, Glu35 and Asp52, and was likely to be the transition state analog for lysozyme. However, the moranoline moiety of (GlcNAc)_2-M bound to BcChi19A was found to stay outside the catalytic cleft (subsite −1), and interact with the main chain of Glu60 and the side chain of Glu61 at subsite +1 (Figure 8B). For −1 and −2 sugars of (GlcNAc)_2-M, a number of amino acids, such as Glu70, Leu101, Trp103, Asn106, Ile163, Asn164 and Glu168, are involved in the hydrogen bonds with the sugar residues, in a similar manner to that observed in the BcChi19A-E61A-(GlcNAc)_4 complex (Ohnuma et al. 2014). The BcChi19A enzyme is unlikely to recognize the moranoline moiety as a transition state analog for the GH19 chitinase. The non-distorted conformation of the moranoline moiety may not have been complementary to the catalytic cleft, resulting in moderate inhibition toward the GH19 chitinase. This indicates that the transition state experienced in the hydrolytic reaction catalyzed by GH22 enzymes may be essentially different from that experienced during the catalysis by GH19 enzymes. This situation may have resulted in the different inhibitory actions of (GlcNAc)_n-M toward GH22 and GH19 enzymes.

Conclusion

Di-N-acetylcobitosyl moranoline, (GlcNAc)_2-M, bound more strongly to BcChi19A than (GlcNAc)_2. This is analogous to the action of (GlcNAc)_2-M on GH22 lysozyme. However, the affinity of (GlcNAc)_2-M to BcChi19A was lower than that of (GlcNAc)_3-M to lysozyme. The lower affinity of (GlcNAc)_2-M to BcChi19A may be derived from the low complementarity between the moranoline moiety and the catalytic cleft, resulting in moderate inhibition to BcChi19A.

Materials and methods

Materials

Chitin oligosaccharides, (GlcNAc)_n (n = 2–6), were obtained by the acid hydrolysis of chitin (Rupley, 1964), and purified by gel filtration on Cellulose Gel-25m (JNC Co., Tokyo). (GlcNAc)_2-M was synthesized by transglycosylation catalyzed by hen egg white lysozyme and purified by the method previously described (Ogata et al. 2013). Escherichia coli BL21 (DE3) cells were purchased from Novagen (Madison, WI). Q Sepharose and Sephacryl S-100 HR were from GE Healthcare (Tokyo, Japan). Other reagents were of analytical grade commercially available.

Protein expression and purification

The wild-type BcChi19A and inactive mutant (BcChi19A-E61A) were obtained by the methods previously described (Ohnuma et al. 2011; Taira et al. 2011). Briefly, the wild-type and mutated plasmids, pET-BcChi19A and pET-BcChi19A-E61A, respectively, were introduced into Escherichia coli BL21 (DE3). E. coli cells harboring the plasmid were grown to OD600 nm = 0.6 before induction with 1 mM isopropyl thiogalactoside. After cultivation for 24 h at 18°C, cells were disrupted in 20 mM Tris–HCl buffer (pH 7.5) with a sonicator. The supernatant fraction obtained after the acid treatment (pH 4.0) was dialyzed against 10 mM sodium acetate buffer (pH 5.0) and applied to Q Sepharose Fast Flow column chromatography, followed by gel-filtration on Sephacryl S-100 HR. The purity of the enzyme preparation was confirmed by SDS–PAGE. Protein concentrations were determined by reading the absorbance at 280 nm, using the extinction coefficient of BcChi19A (49,390 M⁻¹ cm⁻¹) obtained from the equation proposed by Pace et al. (1995).

Thermal unfolding experiments

To obtain the thermal unfolding curves of BcChi19A and BcChi19A-E61A, the CD value at 222 nm was monitored using a Jasco J-720 spectropolarimeter (cell length 0.1 cm), while the solution temperature was raised at a rate of 1°C/min using a temperature controller (PTC-423L, Jasco). The protein solution dialyzed against 50 mM sodium acetate buffer (pH 5.0) was mixed with the ligand solution in the same buffer to give the final concentrations of the enzyme and ligands, 8 μM and 8 mM, respectively. To facilitate comparison 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 the

![Fig. 7. Dose–response curves of (GlcNAc)_2-M for the BcChi19A-catalyzed hydrolysis. The enzymatic reaction was performed in 50 mM sodium acetate buffer, pH 5.0, at 25°C, using (GlcNAc)_4 and (GlcNAc)_6 as the substrate. Enzyme and substrate concentrations were 0.13 μM and 5 mM, respectively. The enzymatic hydrolysis was monitored by HPLC.](image-url)

![Graph showing the relationship between concentration of GlcN₆M (mM) and rate of substrate degradation.](graph-url)
Isothermal titration calorimetry experiments

The protein solutions (150, 87 and 87 μM for the titrations with (GlcNAc)2, (GlcNAc)3, and (GlcNAc)2-M, respectively) in 50 mM sodium acetate buffer (pH 5.0) were degassed and their concentrations were determined. Individual ligands (10 mM) were dissolved in the same buffer, and the solution pH was adjusted to 5.0. The ligand solution was then degassed and loaded into a syringe, while the protein solution (0.2028 mL) was loaded into the sample cell after confirming the solution pH as 5.0. Calorimetric titration was performed with an iTC200 system (Microcal Northampton, MA) at 25°C. Aliquots (0.25–0.5 μL) of the ligand solution were added to the sample cell with a stirring speed of 1000 rpm. Titrations were completed after 50–79 injections. When titration experiments are performed with \( c \) values from 10 to 100 (\( c = nK_{assoc} [M] \); where \( n \) is the stoichiometry, \( K_{assoc} \) the association constant and \([M]\) the initial protein concentration), the \( K_{assoc} \) values obtained can be regarded as reliable (Wiseman et al. 1989). In this study, however, the titration of (GlcNAc)3 to BcChi19A-E61A yielded a \( c \) value of 0.6, and that of (GlcNAc)2-M yielded 6.2. 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 (2003) are satisfied. We confirmed that all requirements were fulfilled in our experiments, except for the titration of (GlcNAc)2 to BcChi19A-E61A. Although reliable thermodynamic data for the (GlcNAc)2 titration to BcChi19A-E61A were not obtained,

pre- and post-transition baselines into the transition zone, and plotted against the temperature.

**Fig. 8.** Stereoviews of a snapshot (at 2 ns) of the simulated structure of the BcChi19A-(GlcNAc)2-M complex obtained by molecular dynamics simulation. The modeled structure of wild-type BcChi19A was initially constructed by the MODELLER 9v1 software using the crystal structure of BcChi19A-E61A (PDB code, 3WH1) as a template. Docking simulations of wild-type BcChi19A with (GlcNAc)2-M were then conducted using AutoDock version 4.2 and ADT Suite. The structure of BcChi19A in complex with (GlcNAc)2-M was employed for molecular dynamics simulation. Gromacs molecular dynamics package version 4.6 (Berendsen et al. 1995) was used to simulate the complex dynamics in explicit water with TIP3 water model. GAff force field was used for the ligand and amber99sb forcefield for the protein. (A) The bound (GlcNAc)2-M is shown in magenta, and the bound (GlcNAc)4 in the crystal structure (PDB code, 3WH1) is also shown in yellow for comparison. (B) Hydrogen bonds between BcChi19A and (GlcNAc)2-M were indicated by dotted lines. Amino acid residues involved in the interaction with (GlcNAc)2-M are colored in cyan. The bound (GlcNAc)2-M is shown in magenta.
only the ITC thermogram is presented in this study (Figure 2A).

ITC data were collected automatically using the Microcal Origin v.7.0 software accompanying the iTC200 system (Wiseman et al. 1989). Prior to analysis, data were corrected for the heat of dilution by subtracting the heat remaining after the saturation of binding sites on the enzyme. These heat values had same magnitudes as those of the titrating ligand into the buffer alone. Data were fitted using a non-linear least-squares fitting algorithm and single-site-binding model. Data from the calorimetric titrations of (GlcNAc)3 and (GlcNAc)2-M fitted well with the single-site-binding model yielding the stoichiometry (n), equilibrium binding association constant (K_{assoc}) and enthalpy change (ΔH_f) of the reaction. The values of n were found to be 1.2 for (GlcNAc)3 and 1.3 for (GlcNAc)2-M (Table II). The reaction free energy change (ΔG_f) and the entropy change (ΔS_f) were calculated from the relationship described in the following equation.

\[
\Delta G_f = -RT \ln K_{assoc} = \Delta H_f - T \Delta S_f
\]

NMR titration experiments

Cells harboring the plasmid pET-BCChi19A or pET-BCChi19A-E61A were grown in M9 medium containing 15N NH4Cl to produce 15N-labeled BCChi19A and BCChi19A-E61A. The production and purification of the labeled enzyme were conducted in the same procedure as described in the Protein expression and purification section. NMR samples contained 0.4 mM protein in 50 mM sodium acetate buffer, pH 5.0 (90% H2O/10% D2O). 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. H-chemical shifts were referenced to HDO (4.64 ppm at 30°C) relative to TSP. The 15N-chemical shift was more accurately calibrated from the gyromagnetic ratio (Wishart et al. 1995). All spectra were processed using NMRpipe software (Delaglio et al. 1995) and analyzed using Sparky software (Goddard and Kneller). Two-dimensional 1H–15N HSQC spectra were recorded in the presence of various concentrations of ligands. Chemical shift changes induced by oligosaccharide binding (Δδ) were calculated using the equation,

\[
\Delta \delta = \left\{ \frac{(\Delta NH^2 + \Delta N^2/25)}{2} \right\}^{1/2}
\]

where ΔNH and ΔN represent the observed shifts in the 1H- and 15N-axis, respectively. Amino acids, whose resonances were affected by the ligand titration, were identified by referring to previous assignments (Shinya et al. 2012, BMRB Entry 11441; Ohnuma et al. 2014, BMRB Entry 11466), and mapped on the surface of the crystal structure reported previously (PDB code, 3WH1; Ohnuma et al. 2014). The structures were illustrated using PyMOL v.0.9.9 software.

Inhibition experiments

The inhibitory activity of (GlcNAc)2-M toward chitin oligosaccharide hydrolysis catalyzed by BCChi19A was examined by HPLC. The enzymatic reaction was performed in 50 mM sodium acetate buffer, pH 5.0, at 25°C, according to the method previously reported (Krokeide et al. 2007) using (GlcNAc)4 and (GlcNAc)6 as the substrates. Enzyme and substrate concentrations were 0.13 μM and 5 mM, respectively. To completely terminate the enzymatic reaction at a given point in time, a portion of the reaction mixture was mixed with acetonitrile (to give 75% acetonitrile), and immediately placed in liquid nitrogen. The resultant solution was applied to a gel-filtration column of TSK-GEL Amide 80 (Tosoh, Tokyo) and eluted with 75% acetonitrile at a flow rate of 0.7 ml/min. Chitin oligosaccharides were detected by measuring ultraviolet absorption at 220 nm. To determine the inhibitory activity of (GlcNAc)2-M, various concentrations of the compound was mixed with the enzyme solution before the enzymatic reaction, so that the final concentrations of (GlcNAc)2-M in the reaction mixture were in the range of 0–1 mM. (GlcNAc)2 and (GlcNAc)3 (0.75 mM) were also tested for inhibitory activity for comparison. The rates of substrate degradation determined from the reaction time-course were plotted against inhibitor concentrations to obtain the half maximal (50%) inhibitory concentration, IC50.

Molecular dynamics simulation

Before molecular dynamics simulation, we constructed the structure of wild-type BcChi19A by homology-modeling software MODELLER 9v1 version (Sali and Blundell 1993) using the crystal structure of BcChi19A-E61A liganded with (GlcNAc)4 (PDB code, 3WH1) as a template. The structure of (GlcNAc)2-M was obtained from the crystal structure of hen egg white lysozyme in complex with (GlcNAc)2-M (PDB code: 4HPI) (Ogata et al. 2013). Docking simulations were conducted using AutoDock version 4.2 and ADT Suite to prepare the systems for calculations (Goodsell et al. 1996). A gridbox was created to overlap with the (GlcNAc)2-M-binding region estimated from the NMR titration experiments (Figure 5, left-lower panel). Fifty docking runs were performed using the Lamarckian genetic algorithm (Morris et al. 1998) with default parameters, assuming the protein was rigid and the ligand was flexible. The lowest energy structure was then selected and submitted to molecular dynamics simulation.

Topologies for the ligands were created with antechamber (Wang et al. 2006) by means of Chimera and the cpptraj code (Sousa Da Silva et al. 2012). Gromacs molecular dynamics package version 4.6 (Berendsen et al. 1995) was used to simulate the complex dynamics in explicit water with TIP3 water model. GAFF force field (Wang et al. 2004) was used for the ligand and amber99sb forcefield for the protein. Protein ligand complex was solvated into water, in an octahedron box leaving 1.0 nm between the molecules and box boarders. Counter ions were added to neutralize the system. The system was relax by energy minimization using steepest descent algorithm until forces converged and allowing the volume and pressure to stabilize in 100 ps NVT and NPT molecular dynamics runs constraining the heavy atoms. Unconstrained molecular dynamics (NPT) was performed with leap-frog integrator for 11 ns. Time step was set to 0.002 ps and particle mesh ewald PME algorithm was used to treat long-range electrostatics. All cutoffs (coulomb, short-range VdW and neighborlist) were set to 1.0 nm. V-rescale modified Berendsen thermostat was used for temperature coupling and Parinello–Rahman for pressure coupling. Bonds were constrained using the LINCS algorithm (Hess et al. 1997). The trajectories were analyzed using programs available in the gromacs package and VMD visualization.
program version 1.9.1 (Humphrey et al. 1996). The structures were illustrated using PyMOL v.0.9.9.

Conflict of interest statement
None declared.

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Abbreviations
BeChi19A, a family GH19 chitinase from moss, *Bryum coronatum*; BeChi19A-E61A, a mutated BeChi19A, in which Glu61 is substituted with alanine; GlcNAc, β-D-glucosamine; (GlcNAc)n, β-1,4-linked oligosaccharide of GlcNAc; (GlcNAc)2-M, di-N-acetylchitobiosyl moranoline; NMR, nuclear magnetic resonance; HSOC, two-dimensional heteronuclear single quantum correlation; ITC, isothermal titration calorimetry; CD, circular dichroism.

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