

Introduction of a tryptophan side chain into subsite +1 enhances transglycosylation activity of a GH-18 chitinase from *Arabidopsis thaliana*, AtChiC

Naoyuki Umemoto^{2,†}, Takayuki Ohnuma^{2,†},
Mamiko Mizuhara², Hirokazu Sato², Karen Skriver³, and
Tamo Fukamizo^{1,2}

²Department of Advanced Bioscience, Kinki University, 3327–204 Nakamachi, Nara 631-8505, Japan and ³Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, 2200 Copenhagen, Denmark

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A tryptophan side chain was introduced into subsite +1 of family GH-18 (class V) chitinases from *Nicotiana tabacum* and *Arabidopsis thaliana* (NtChiV and AtChiC, respectively) by the mutation of a glycine residue to tryptophan (G74W-NtChiV and G75W-AtChiC). The specific activity toward glycol chitin of the two mutant enzymes was 70–71% of that of the wild type. Using chitin oligosaccharides, (GlcNAc)_n (*n* = 4, 5 and 6), as the substrates, we found the transglycosylation reaction to be significantly enhanced in G74W-NtChiV and G75W-AtChiC when compared with the corresponding wild-type enzymes. The introduced tryptophan side chain might protect the oxazolinium ion intermediate from attack by a nucleophilic water molecule. The enhancement of transglycosylation activity was much more distinct in G75W-AtChiC than in G74W-NtChiV. Nuclear magnetic resonance titration experiments using the inactive double mutants, E115Q/G74W-NtChiV and E116Q/G75W-AtChiC revealed that the association constant of (GlcNAc)₅ was considerably larger for the latter. Amino acid substitutions at the acceptor binding site might have resulted in the larger association constant for G75W-AtChiC, giving rise to the higher transglycosylation activity of G75W-AtChiC.

Keywords: GH-18 chitinase / chitin oligosaccharide / tryptophan residue / transglycosylation / NMR spectroscopy

Introduction

Chitinases (EC 3.2.1.14) hydrolyze β-1,4-glycosidic linkages of chitin, an insoluble β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc) residues. The enzymes are divided into families GH-18 and GH-19 according to the classification of the CAZy database (Henrissat and Davies 1997). The GH-19 chitinases consist of two α-rich domains (Hart et al. 1995; Huet et al. 2008; Ubhayasekera et al. 2009), whereas the GH-18 chitinases consist of a main (α/β)₈ barrel domain and an additional functional domain, such as an insertion domain, a chitin-binding domain or both (Perrakis et al. 1994; Terwisscha van Scheltinga et al. 1994; Fusetti et al. 2002). The GH-19 enzymes catalyze a hydrolytic reaction with anomer inversion (Brameld and Goddard 1998; Sasaki et al. 2003), whereas in GH-18 chitinases, the catalytic reaction takes place with anomer retention (Terwisscha van Scheltinga et al. 1995; Tews et al. 1997; Brameld et al. 1998; van Aalten et al. 2001; Sasaki et al. 2002). Several GH-18 chitinases are known to catalyze not only the hydrolysis but also a transglycosylation reaction resulting in products with a chain length longer than that of the initial substrate (Fukamizo et al. 2001; Aguilera et al. 2003; Taira et al. 2010). The transglycosylation reaction can be employed for production of useful chitin oligosaccharides with, for example, antitumor properties (Wang et al. 2007), the ability to control of cell growth (Semino and Allende 2000; Snaar-Jagalska et al. 2003), antioxidant effects (Ngoa et al. 2008) and the capacity to trigger defense systems in plants (Kaku et al. 2006; Miya et al. 2007).

Enhancement of the transglycosylation activity of chitinolytic enzymes has been attempted by several investigators and accomplished with partial success. Chemical modification of Trp62 and Asp101 of a hen egg white lysozyme impaired the binding of sugars at subsites –4, –3 and –2 and enhanced transglycosylation activity (Fukamizo et al. 1989). Aronson et al. (2006) reported the enhancement of transglycosylation activity by mutating a tryptophan residue located at subsite –3 (Trp167) of family GH-18 *Serratia marcescens* chitinase A (SmChiA). These strategies are based on the relative enhancement of acceptor binding to the positively numbered subsites by lowering the ability to bind to the negatively numbered subsites. An opposite strategy, introduction of the tryptophan side chain into the positively numbered subsites, might enhance the transglycosylation activity. However, there are no earlier reports on introducing the tryptophan side chain for enhancing

¹To whom correspondence should be addressed: Tel: +81-742-43-8237; Fax: +81-742-43-8976; e-mail: fukamizo@nara.kindai.ac.jp

[†]These two authors contributed equally to this work.

the transglycosylation activity. Recently, Zakariassen et al. (2011) reported another strategy for enhancing the transglycosylation activity of SmChiA and SmChiB; that is, the mutated SmChiA and SmChiB, in which the aspartic acid in the middle of the catalytic triad (the DXDXE motif) of both enzymes was mutated to asparagine, exhibited strong transglycosylation activity. Similar effects were also observed in *Bacillus circulans* chitinase A and *Trichoderma harzianum* Chit42 (Martinez et al. 2012). The mutation of the middle aspartic acid in the DXDXE motif might alter the electrostatic conditions in the catalytic cleft leading to an increased probability of nucleophilic attack by a sugar acceptor molecule.

Plant chitinases have been classified into at least six classes, classes I, II, III, IV, V and VI, according to the amino acid sequences (Neuhaus et al. 1996). Classes I, II and IV correspond to family GH-19 and classes III and V to family GH-18. We recently reported the crystal structure of family GH-18 (class V) chitinases from *Nicotiana tabacum* and *Arabidopsis thaliana* (NtChiV and AtChiC, respectively; Ohnuma et al. 2011a; 2011b), whose amino acid sequences are similar to each other (57%). As shown in Figure 1A, C and E, the enzymes exhibit an $(\alpha/\beta)_8$ fold (cyan) with an

insertion domain (magenta) as in the case of SmChiB, except that the plant enzymes do not have a chitin-binding domain (orange). Glu115 and Glu116 are the catalytic acids of the individual plant enzymes, respectively (Figure 1D and F). Two or three subsites have been recognized at both sides of the catalytic center (-2, -1, +1, +2 and +3; Figure 1B, D and F). NtChiV and AtChiC hydrolyze the chitin polysaccharide through a non-processive manner (Ohnuma et al. 2011a). The amounts of transglycosylation products obtained from the oligosaccharide substrates, $(\text{GlcNAc})_n$, were negligible in the reactions catalyzed by the plant enzymes. Horn et al. (2006) reported that Trp97 of SmChiB plays an important role in the processive action of the enzyme (Figure 1B). In NtChiV and AtChiC, the amino acid residue corresponding to Trp97 of SmChiB is substituted with glycine (Gly74 in NtChiV and Gly75 in AtChiC; Figure 1D and F). Thus, we tried to induce the processive action in NtChiV and AtChiC by mutating the glycine residue to tryptophan (G74W-NtChiV and G74W-AtChiC). However, no experimental data indicating the processivity of the mutant enzymes have not been obtained yet. Instead, the mutant enzymes were found to exhibit enhanced transglycosylation activity. We report here a new strategy for

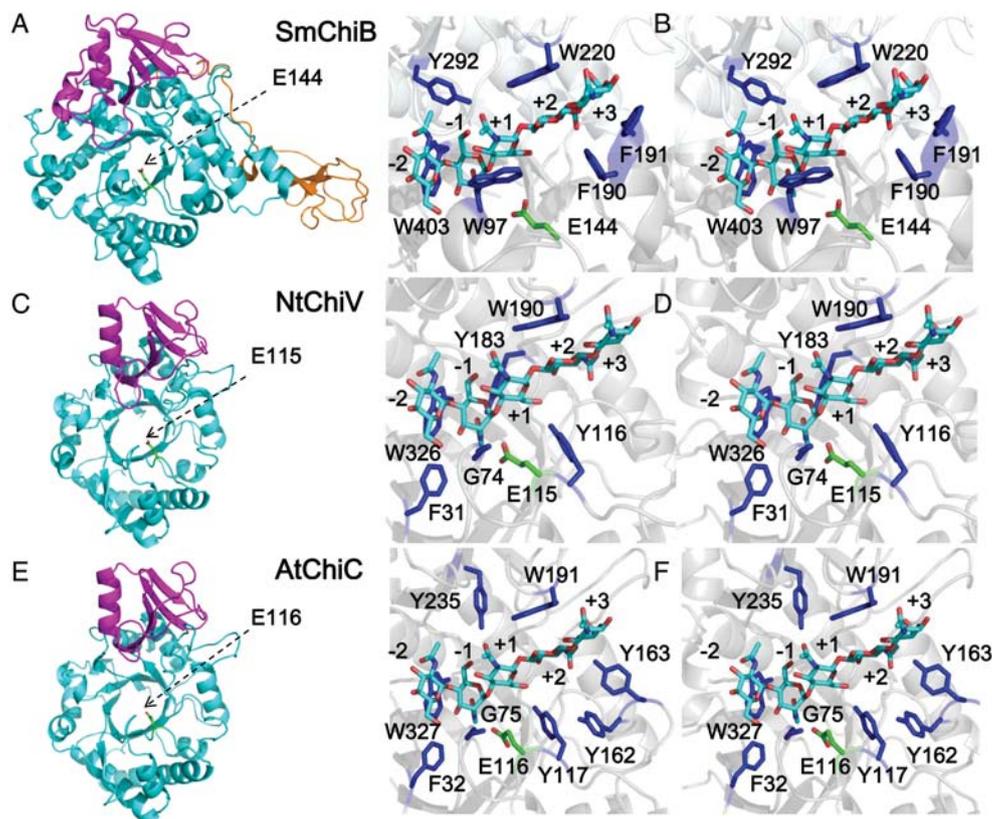


Fig. 1. Crystal structures of SmChiB (A), NtChiV (C) and AtChiC (E). A $(\alpha/\beta)_8$ barrel domain (cyan), an insertion domain (magenta) and chitin-binding domain (orange). Stereo view of the substrate-binding cleft of SmChiB (B), NtChiV (D) and AtChiC (F). For SmChiB, the structure is in a complex with $(\text{GlcNAc})_5$ (PDB code, 1e6n) (van Aalten et al. 2001). Since the complex structure was obtained only for SmChiB, the complex structures for NtChiV and AtChiC were obtained by docking simulation using the conformation of the bound $(\text{GlcNAc})_5$ in the SmChiB complex and the structure of free NtChiV (PDB code, 3alf) (Ohnuma et al. 2011a) and free AtChiC (PDB code, 3aqu) (Ohnuma et al. 2011b), respectively, by the method reported in the previous paper (Ohnuma et al. 2011b). The stick representations colored green are the catalytic acids (Glu144, Glu115 and Glu116) for individual enzymes. The $(\text{GlcNAc})_5$ molecules located in the binding cleft are indicated in cyan. The aromatic side chains involved in the oligosaccharide binding are colored deep blue.

enhancing transglycosylation; that is, the introduction of a tryptophan side chain into subsite +1.

Results

Enzyme production

The mutated enzymes (G74W-NtChiV and G75W-AtChiC) were successfully expressed in the *E. coli* expression system as in the case of the wild-type enzyme (NtChiV and AtChiC; Ohnuma et al. 2011a; 2011b). The glycine mutations to tryptophan did not significantly affect the expression level of the proteins. After purification of the enzymes, the individual proteins exhibited a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Supplementary Fig. S1). CD spectra of the mutant enzymes were almost identical to that of the wild type (Supplementary Fig. S2), indicating that the individual mutations do not significantly affect the global conformation of the enzyme.

Enzymatic activity toward glycol chitin

The glycine mutations to tryptophan did not strictly impair the enzymatic activity in either case. The specific activity of G74W-NtChiV (5.30 $\mu\text{M}/\text{min}/\text{mg}$) was reduced to 70% of that of NtChiV (7.53 $\mu\text{M}/\text{min}/\text{mg}$), and the specific activity of G75W-AtChiC (13.2 $\mu\text{M}/\text{min}/\text{mg}$) to 71% of that of AtChiC (18.4 $\mu\text{M}/\text{min}/\text{mg}$).

Enzymatic activity toward chitin oligosaccharides (GlcNAc)_n

First, we tested NtChiV and G74W-NtChiV using (GlcNAc)₄ as the substrate. The results are shown in Figure 2A and B. NtChiV produced only (GlcNAc)₂, indicating a simple hydrolysis of (GlcNAc)₄ into (GlcNAc)₂ + (GlcNAc)₂ (Figure 2A). A similar profile was obtained when G74W-NtChiV was used instead of NtChiV (Figure 2B). However, a small but a significant

amount of (GlcNAc)₃ was detected in the later stage of the reaction catalyzed by G74W-NtChiV. GlcNAc was not detected at all in the entire course of the reaction. (GlcNAc)₃ production from the substrate (GlcNAc)₄ without the production of GlcNAc was interpreted previously as a transglycosylation reaction (Taira et al. 2010); that is, (GlcNAc)₄ was cleaved into a (GlcNAc)₂ oxazolinium ion intermediate [(GlcNAc)₂*] and (GlcNAc)₂, then the glycosyl donor (GlcNAc)₂* was transferred to the acceptor (GlcNAc)₄ producing (GlcNAc)₆, which was further decomposed into (GlcNAc)₃ + (GlcNAc)₃. Next, we tested AtChiC and G75W-AtChiC with respect to the mode of action toward (GlcNAc)₄. As shown in Figure 2C and D, (GlcNAc)₃ production was enhanced considerably by the G75W mutation. The enhancement is much more intensive than that in NtChiV. A small amount of (GlcNAc)₆ was also detected in the early stage of the reaction catalyzed by G75W-AtChiC. When the enzymatic reaction was conducted with a much higher substrate concentration (80 mM) for 2 h, we observed clearly the precipitate in the reaction mixture of G75W-AtChiC, but not in the mixture of AtChiC (Figure 3A). The precipitate was isolated from the reaction mixture, dissolved with a larger volume of distilled water (300 μL) and employed for matrix-assisted laser-desorption ionization-time-of-flight MS (MALDI-TOF MS) analysis. The result is shown in Figure 3B. A considerable amount of the transglycosylation product (GlcNAc)₆ was produced and precipitated because of its lower solubility. A smaller amount of (GlcNAc)₈ was also produced by the transglycosylation reaction.

The substrates (GlcNAc)₅ and (GlcNAc)₆ were tested only for AtChiC and G75W-AtChiC. The results are shown in Figure 4A–D. The profiles of the time-courses for AtChiC (Figure 4A and B) were completely different from those for G75W-AtChiC (Figure 4C and D). AtChiC appears to act toward the substrates with simple hydrolysis; that is, (GlcNAc)₅

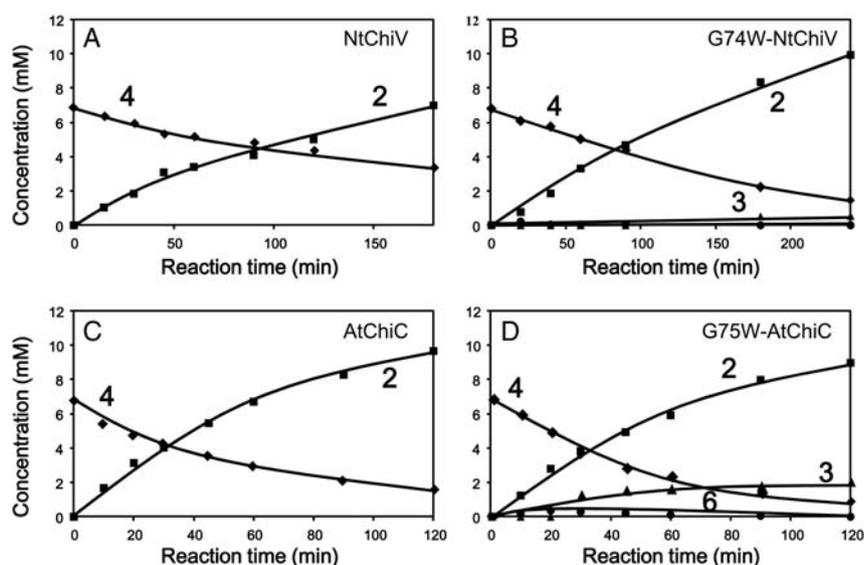


Fig. 2. Time-courses of (GlcNAc)₄ hydrolysis catalyzed by NtChiV (A), G74W-NtChiV (B), AtChiC (C) and G75W-AtChiC (D). The enzymatic reactions were conducted in 20 mM sodium acetate buffer pH 5.0 at 40°C. Concentrations used for the reactions were 0.32 μM for NtChiV, 0.16 μM for AtChiC and 6.8 mM for (GlcNAc)₄. The substrate and product concentrations at a given reaction time were determined by gel-filtration HPLC using a column of TSK-GEL G2000PW (Tosoh). Squares, (GlcNAc)₂; triangles, (GlcNAc)₃; diamonds, (GlcNAc)₄; filled circles, (GlcNAc)₆. Lines were obtained by roughly following the experimental data points. Figure 2C is cited from Ohnuma et al. (2011b).

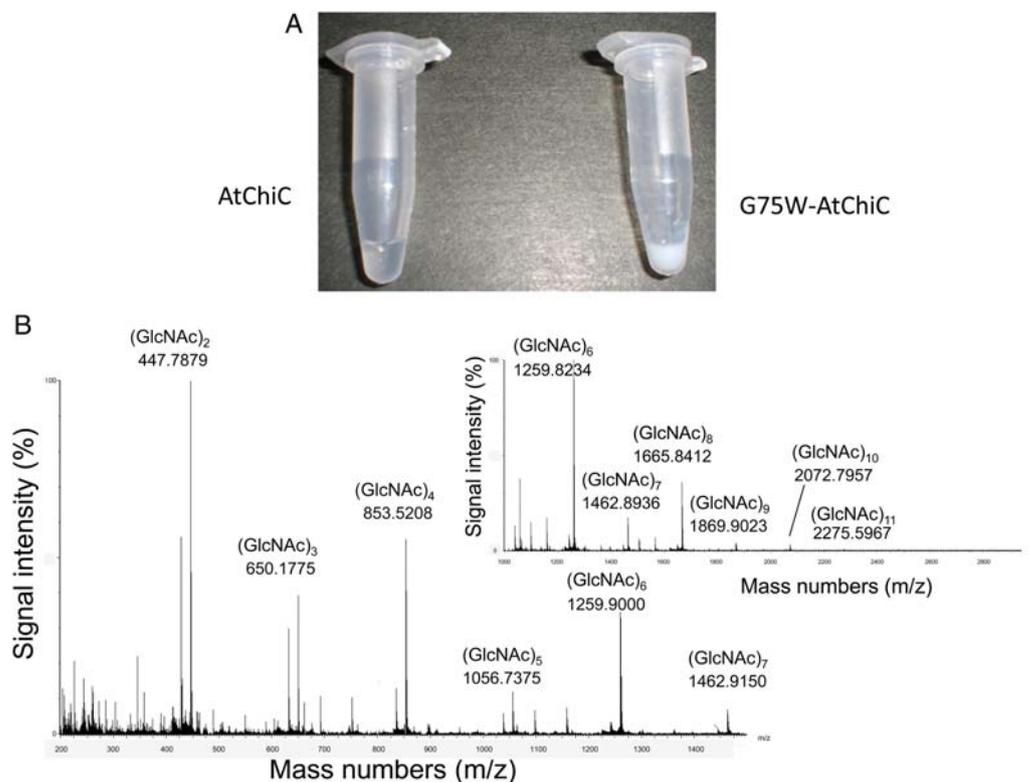


Fig. 3. (A) Reaction mixture obtained by 2 h incubation of 80 mM (GlcNAc)₄ with the AtChiC enzymes. Left, obtained with the wild-type AtChiC. Right, obtained with G75W-AtChiC. Other reaction conditions were the same as in Figure 2. (B) MALDI-TOF MS of the precipitate produced by G75W-AtChiC.

was converted into (GlcNAc)₂ + (GlcNAc)₃, and (GlcNAc)₆ into (GlcNAc)₂ + (GlcNAc)₄ and less frequently (GlcNAc)₃ + (GlcNAc)₃. For G75W-AtChiC, however, the amount of (GlcNAc)₂ produced was suppressed in both cases (Figure 4C and D), probably due to consumption as a glycosyl donor for transglycosylation. Instead, (GlcNAc)₇ was produced from (GlcNAc)₅ and (GlcNAc)₈ from (GlcNAc)₆. The production of (GlcNAc)₈ from (GlcNAc)₆ was confirmed by MALDI-TOF MS, as shown in Figure 4E. The time-course of Figure 4C could be explained as follows; (GlcNAc)₆ bound to subsites -2, -1, +1, +2, +3 and +4, is at first split into (GlcNAc)₂ + (GlcNAc)₄ and the product (GlcNAc)₂ acting as a donor substrate is transferred to the acceptor (GlcNAc)₆ producing the product (GlcNAc)₈. Thus, the preferred binding mode of (GlcNAc)₆ to G75W-AtChiC appears to be identical to that of the wild type (Ohnuma et al. 2011b). All of these results clearly indicated that the mutation of Gly75 to tryptophan enhances the transglycosylation reaction without changing the substrate-binding mode. However, the transglycosylation products appear to be immediately hydrolyzed again into the oligosaccharides with a lower degree of polymerization in the reaction catalyzed by G75W-AtChiC.

Binding experiments using nuclear magnetic resonance

To rationalize the difference in transglycosylation activity between G74W-NtChiV and G75W-AtChiC, we conducted (GlcNAc)₅-binding experiments using inactive double mutants, E115Q/G74W-NtChiV and E116Q/G75W-AtChiC, by means

of nuclear magnetic resonance (NMR) spectroscopy. As shown in Figure 5A, the ¹H-¹⁵N heteronuclear single-quantum coherence (HSQC) spectra of E115Q-NtChiV and E115Q/G74W-NtChiV were almost identical, indicating that the G74W mutation did not affect significantly the conformation of the enzyme. Closer examination of the spectra revealed an additional HSQC resonance (designated by a broken arrow) in the tryptophan indole region of the E115Q/G74W spectrum (bottom). This resonance could be assigned to the Trp74 side-chain NH. When the (GlcNAc)₅ solution was titrated into the E115Q/G74W-NtChiV solution, the Trp74 side-chain resonance shifted upon the addition of (GlcNAc)₅ (Figure 5B). Based on the chemical shift change of the Trp74 resonance, the association constant was calculated to be $1.13 \times 10^3 \text{ M}^{-1}$, and the free energy change of binding, -4.2 kcal/mol (Figure 7). In the control experiment using E115Q-NtChiV, the addition of (GlcNAc)₅ did not cause any changes in the spectrum.

Similar experiments were conducted using E116Q-AtChiC and E116Q/G75W-AtChiC. The spectra exhibited almost identical profiles, as shown in Figure 6A, indicating again no significant change in the conformation of the enzyme. In the spectrum of E116Q/G75W-AtChiC (bottom), an additional tryptophan side-chain resonance (designated by a broken arrow) was assigned to the side-chain NH resonance of Trp75. The intensity of the Trp75 resonance decreased gradually upon the addition of (GlcNAc)₅ with slightly changing its chemical shift, and the resonance completely disappeared when the molar ratio of the enzyme:(GlcNAc)₅ was 1:25 (Figure 6B). The association constant obtained from the

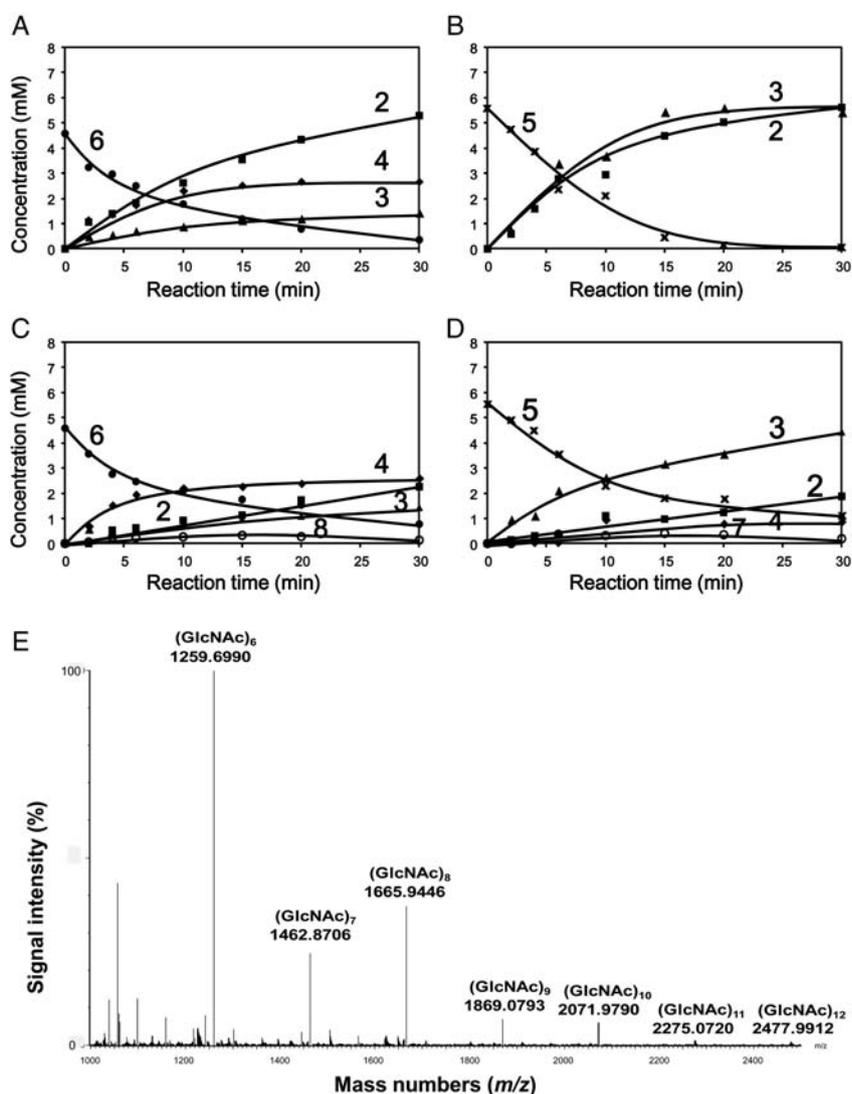


Fig. 4. Time-courses of the reactions catalyzed by AtChiC (A and B) and G75W-AtChiC (C and D). Substrates were 4.6 mM (GlcNAc)₆ (A and C) and 5.8 mM (GlcNAc)₅ (B and D). Other reaction conditions and analytical conditions were the same as in Figure 2. Squares, (GlcNAc)₂; triangles, (GlcNAc)₃; diamonds, (GlcNAc)₄; crosses, (GlcNAc)₅; filled circles, (GlcNAc)₆; open circles in (C), (GlcNAc)₈; open circles in (D), (GlcNAc)₇. Lines were obtained by roughly following the experimental data points. Figure 4A and B is cited from Ohnuma et al. (2011b). (E) MALDI-TOF MS of the reaction products from the substrate (GlcNAc)₆ by G75W-AtChiC. Reaction conditions were the same as in Figure 4C.

change in the Trp75 side-chain NH resonance was $6.12 \times 10^3 \text{ M}^{-1}$, and the free energy change of binding was calculated to be -5.2 kcal/mol (Figure 7). For E116Q-AtChiC, no significant change was observed in the spectrum upon the addition of (GlcNAc)₅.

Discussion

Chitin oligosaccharides, (GlcNAc)_n, are now drawing attention because of their potential biological functions (Semino and Allende 2000; Snaar-Jagalska et al. 2003; Kaku et al. 2006; Miya et al. 2007; Wang et al. 2007; Ngoa et al. 2008). Although (GlcNAc)_n have been produced by acid or enzymatic hydrolysis of chitin (Rupley 1964; Berkeley et al. 1972;

Woo and Park 2003), enzymatic synthesis (transglycosylation) using GH-18 chitinases seems to be much more advantageous than hydrolysis for controlling the polymerization degree of (GlcNAc)_n. The transglycosylation activity of the chitinases has been successfully enhanced by site-directed mutagenesis (Aronson et al. 2006; Zakariassen et al. 2011; Martinez et al. 2012). We reported here an alternative strategy for producing a mutant chitinase possessing higher transglycosylation activity from plant family GH-18 (class V) chitinases.

As described in *Introduction*, plant family GH-18 chitinases are subdivided into classes III and V (Melchers et al. 1994). The enzymes employed in this study, NtChiV and AtChiC, belong to class V. The mutation target, glycine residue (Gly74 in NtChiV and Gly75 in AtChiC), is conserved in most class V chitinases, which are likely non-processive enzyme with

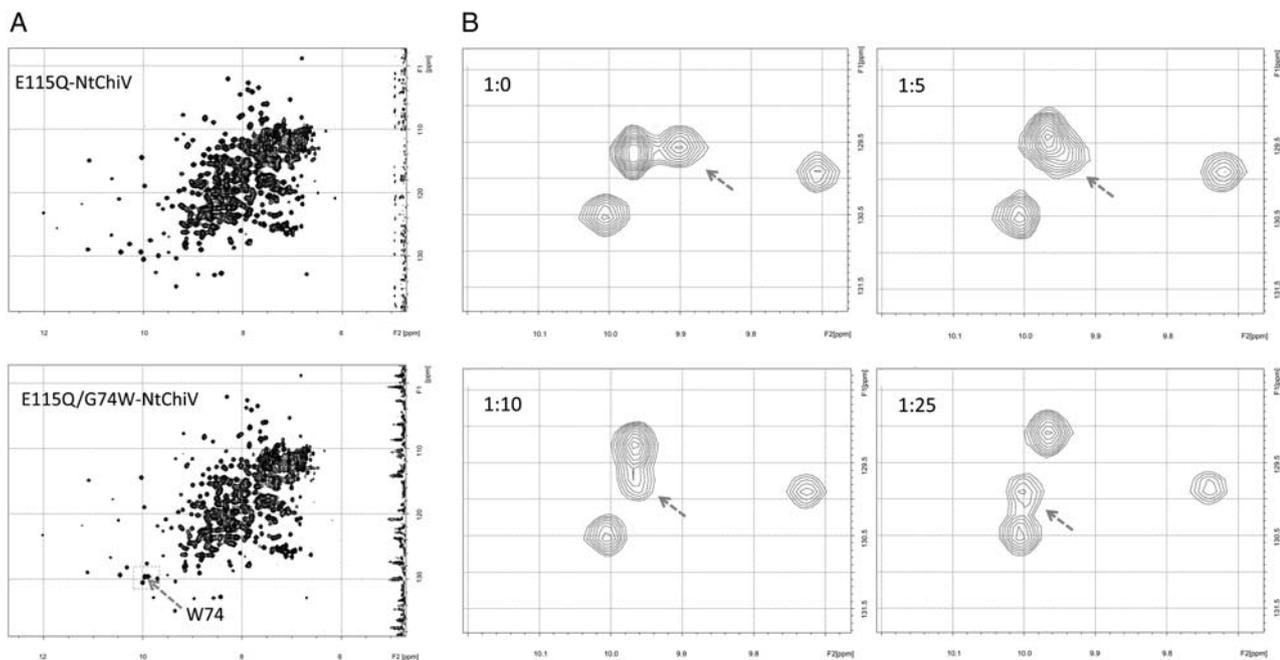


Fig. 5 (A) ^1H - ^{15}N -HSQC spectra of E115Q-NtChiV (top) and E115Q/G74W-NtChiV (bottom). NMR samples contained 0.4 mM ^{15}N -labeled protein in 50 mM sodium acetate buffer, pH 5.0 (90% $\text{H}_2\text{O}/10\%$ D_2O). The spectra were acquired at 300 K using a Bruker AV500 spectrometer. An additional resonance designated by a broken arrow was found in the tryptophan indole NH region and assigned to the Trp74 side chain. (B) Change in the Trp74 indole NH signal upon the addition of $(\text{GlcNAc})_5$. The boxed region of the HSQC spectrum (bottom of Figure 5A) was enlarged for individual profiles. The numerals in the upper-left corner of the individual spectra indicate the molar ratio of the enzyme to $(\text{GlcNAc})_5$.

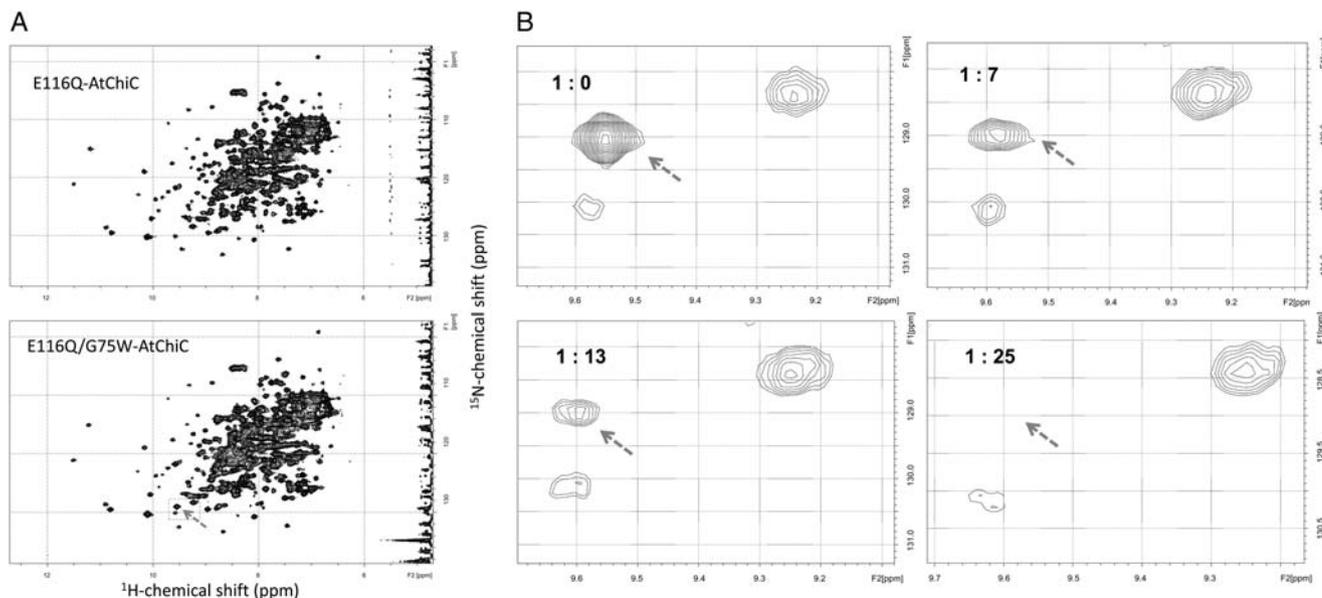


Fig. 6. (A) ^1H - ^{15}N -HSQC spectra of E116Q-AtChiC (top) and E116Q/G75W-AtChiC (bottom). Experimental conditions were the same as in Figure 5. An additional resonance designated by a broken arrow was found in the tryptophan indole NH region and assigned to the Trp75 side chain. (B) Change in the Trp75 indole NH signal upon the addition of $(\text{GlcNAc})_5$. The boxed region of the HSQC spectrum (bottom of Figure 6A) was enlarged for individual profiles. The numerals listed in the upper-left corner of the individual spectra indicate the molar ratio of the enzyme to $(\text{GlcNAc})_5$.

low intrinsic transglycosylation activity. On the other hand, human chitotriosidase having a similar fold to those of class V chitinases has a tryptophan residue at the glycine position (Fusetti et al. 2002) and is known to hydrolyze chitin

processively (Eide et al. 2012) and to catalyze transglycosylation reaction (Aguilera et al. 2003). Thus, it is reasonable to expect that the Gly \rightarrow Trp mutation of plant class V chitinases confers processivity on the chitin hydrolysis and enhances the

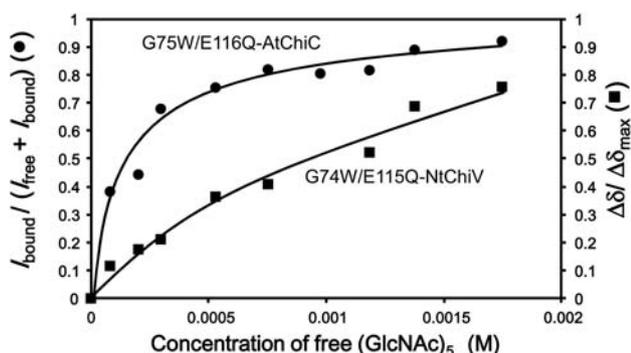


Fig. 7. Titration curves of $(\text{GlcNAc})_5$ into E115Q/G74W-NtChiV (filled squares) and E116Q/G75W-AtChiC (filled circles). Experimental data points were obtained from Figures 5B and 6B.

transglycosylation activity. However, we failed to observe processivity in the reactions catalyzed by the mutant enzymes. Instead, positive data were obtained in the AtChiC enzyme with respect to the transglycosylation reaction.

As shown in Figures 2D and 4C and D, transglycosylation activity was considerably enhanced by the G75W mutation of AtChiC, whereas the activity decrease in G75W-AtChiC was not so much; the activity toward glycol chitin was 71% and that toward $(\text{GlcNAc})_6$ was 72% of that of the wild-type enzyme. In the X-ray crystal structure of AtChiC (Ohnuma et al. 2011b) enough space appears to exist at the Gly75 site for accommodating a tryptophan side chain without affecting the local conformation. In addition, as deduced from the crystal structure of SmChiB, the introduced tryptophan side chain would be involved in the +1 sugar interaction, but located outside the binding cleft (Figure 1B). This situation would have resulted in only moderate loss of the enzymatic activity (71–72%) after the G75W mutation. In fact, the enzymatic products obtained by the glycosyl transfer catalyzed by G75W-AtChiC appear to be immediately decomposed to the final products with lower degree of polymerization, as described in Results. Further accumulation of the transglycosylation products might be possible by lowering the enzymatic activity through an additional mutation.

Hurtado-Guerrero et al. (2009) reported the structure of a family GH-72 β -1,3-glucano-transglycosylase and proposed a molecular mechanism that controls the balance between hydrolysis and transglycosylation. They found that the hydrolytic products remain associated with the enzyme, with an occlusion of the catalytic base (“base occlusion”). This situation protects the newly formed enzyme-oligosaccharide intermediate from nucleophilic attack by a water molecule, resulting in a high efficiency of transglycosylation. In the family GH-18 enzymes employed in this study, hydrolytic reaction takes place through a substrate-assisted mechanism, in which the catalytic residue Glu (DXDXE) can serve as both acid and base (van Aalten et al. 2001). The enzymatic hydrolysis is completed with a nucleophilic attack by a water molecule on the oxazolium ion intermediate stabilized by the middle Asp residue (DXDXE). In the mutated enzymes employed in this study, the tryptophan side chain introduced into subsite +1 is likely to repel the water molecule,

preventing the nucleophilic attack on the oxazolium ion intermediate. Thus, the first requirement for efficient glycosyl transfer to the acceptor molecule is to control the nucleophilic attack by a water molecule on the intermediate state.

In this study, NtChiV was also tested with respect to enhancement of transglycosylation by introducing a tryptophan side chain into subsite +1. However, the transglycosylation activity of G74W-NtChiV was not as distinct as that of G75W-AtChiC (Figure 2B and D). To further examine the difference between G74W-NtChiV and G75W-AtChiC, NMR titration experiments were conducted using $(\text{GlcNAc})_5$ and ^{15}N -labeled double mutants, E115Q/G74W-NtChiV and E116Q/G75W-AtChiC. The side-chain resonance of the introduced tryptophan was successfully assigned by comparison between the ^1H - ^{15}N -HSQC spectra of the double-mutant enzyme and the corresponding single mutant (Figures 5A and 6A). However, the binding free energy changes calculated based on the changes in the HSQC resonance of the introduced tryptophan side chain ($-4.2 \sim -5.2$ kcal/mol) were considerably lower than those obtained by isothermal titration calorimetry (ITC) for GH-19 and GH-18 chitinases (-7.9 kcal/mol, Norberg et al. 2011; -8.3 kcal/mol, Ohnuma et al. 2011c), probably due to a synergistic effect of the double mutations. Unfortunately, we failed to obtain the binding data supporting the NMR results presented here by other methods, such as ITC and fluorescence. Nevertheless, the values of the association constants differed considerably between E116Q/G75W-AtChiC ($6.12 \times 10^3 \text{ M}^{-1}$) and E115Q/G74W-NtChiV ($1.13 \times 10^3 \text{ M}^{-1}$), as described in Results. This might be due to the amino acid substitutions in the acceptor binding site. In fact, as shown in Figure 1F, Tyr162 and Tyr163 of AtChiC were found to be involved in sugar residue binding at subsites +2 and +3 (Ohnuma et al. 2011b). However, these two tyrosines are substituted with valine and asparagine, respectively, in NtChiV (Figure 1D). This situation would have resulted in the higher affinity of AtChiC for the transglycosylation acceptor, bringing about the increased transglycosylation activity of G75W-AtChiC relative to G74W-NtChiV.

In the titration experiments using NMR spectroscopy, the resonance of the introduced tryptophan side chain was most responsive in both cases (E115Q/G74W-NtChiV and E116Q/G75W-AtChiC). However, the mode of signal response in E115Q/G74W-NtChiV is different from that in E116Q/G75W-AtChiC (Figures 5B and 6B). The resonance of tryptophan side chain shifted without a change in intensity in E115Q/G74W-NtChiV, but in E116Q/G75W-AtChiC, the signal intensity was reduced gradually, eventually fading away with only a slight change in the chemical shift. The binding of $(\text{GlcNAc})_5$ to E116Q/G75W-AtChiC with higher affinity might have affected more strongly the state of the tryptophan side chain, resulting in the intensive broadening of the resonance. It is evident that the state of the introduced tryptophan residue of G74W-NtChiV differs from that of G75W-AtChiC in the $(\text{GlcNAc})_5$ -bound state of the enzymes. This situation might also contribute partly to the difference in transglycosylation activity between G74W-NtChiV and G75W-AtChiC.

In conclusion, the introduction of a tryptophan side chain into subsite +1 of AtChiC enhances the transglycosylation activity. The enhancement might be due to protection of the

oxazolium ion intermediate from nucleophilic attack by a water molecule and also due to higher affinity for the acceptor-binding site of the enzyme.

Materials and methods

Materials

Chitin oligosaccharides [(GlcNAc)_n, *n* = 1–6] were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Glycol chitin was synthesized by the method of Yamada and Imoto (1981). *Escherichia coli* Tuner(DE3) pLacI cells and the expression vector pETBlue-1 were obtained from Novagen (Madison, WI), SP-Sepharose and Sephacryl S-100 HR from GE Healthcare (Tokyo, Japan) and a TSK-GEL G2000PW column from Tosoh (Tokyo, Japan). All other reagents were of analytic grade.

Expression and purification of recombinant enzymes

The expression plasmids for pET-Blue1-NtChiV and pET11a-AtChiC were constructed as described previously (Ohnuma et al. 2011a; 2011b). Site-directed mutagenesis was conducted by the method of Wang and Malcolm (1999) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The nomenclature of the mutants and oligonucleotide primers used for mutagenesis are shown in Supplementary Table S1. The double-mutant genes were produced using the single-mutant gene (G → W) as a template and a primer containing an additional mutation (E → Q). The wild-type enzymes (NtChiV and AtChiC) and the mutated enzymes (G74W-NtChiV and G75W-AtChiC) were produced in *E. coli* cells and purified by a SP-Sepharose column followed by a gel-filtration on Sephacryl S-100, according to the methods described previously (Ohnuma et al. 2011a; 2011b). The proteins (E115Q-NtChiV, E115Q/G74W-NtChiV, E116Q-AtChiC and E116Q/G75W-AtChiC) employed for NMR spectroscopy were obtained with the same expression system using an M9 minimal medium containing ¹⁵N-NH₄Cl (0.5 g/L).

Chitinase activity

Chitinase activity was assayed colorimetrically using glycol-chitin as the substrate. Ten microliters of the enzyme solution was added to 500 μL of 0.4% (w/v) glycol chitin in a 50 mM sodium acetate buffer, pH 5.0. After incubation of the reaction mixture at 37°C for 15 min, the reducing sugar concentration of the reaction mixture was determined based on the decrease in absorbance at 420 nm ($\Delta A_{420\text{nm}}$) using the ferri-ferrocyanide reagent by the method of Imoto and Yagishita (1971). The values of $\Delta A_{420\text{nm}}$ were converted into molar concentrations using the standard curve obtained by GlcNAc. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmol of GlcNAc per min at 37°C.

Protein measurement

Protein concentrations were determined by reading absorbance at 280 nm, using an extinction coefficients, 84,340 (NtChiV), 89,840 (G74W-NtChiV), 87,780 (AtChiC) and 93,280 (G75W-AtChiC) M⁻¹ cm⁻¹, calculated from the equation proposed by Pace et al. (1995).

Electrophoresis

SDS-PAGE was carried out by the method of Laemmli (1970) using a 15% acrylamide gel. Proteins on the gel were stained with Coomassie Brilliant Blue R250. The molecular mass was measured in the presence of 2-mercaptoethanol.

CD spectroscopy

The protein solution was dialyzed against 20 mM sodium acetate buffer pH 5.0, and far UV CD spectra were recorded using a Jasco J-720 spectropolarimeter (cell length 0.1 cm) at 20°C. The protein concentration was 4 μM.

High-performance liquid chromatography-based determination of the reaction time-course

The reaction products from the chitinase-catalyzed hydrolysis of (GlcNAc)_n (*n* = 4, 5 or 6) were quantitatively determined by gel filtration high-performance liquid chromatography (HPLC) according to the method of Fukamizo et al. (2001). The reaction mixture (100 μL) containing enzyme (0.32 μM for NtChiV and 0.16 μM for AtChiC) and substrate [6.8 mM for (GlcNAc)₄, 5.8 mM for (GlcNAc)₅ and 4.6 mM for (GlcNAc)₆] in 20 mM sodium acetate buffer, pH 5.0, was incubated at 40°C. To completely terminate the enzymatic reaction at a given incubation time, a portion of the reaction mixture was mixed with an equal volume of 0.1 M NaOH solution and immediately frozen in liquid nitrogen. The resultant solution was applied to a gel filtration column of TSK-GEL G2000PW (Tosoh) and eluted with distilled water at a flow rate of 0.3 mL/min. Oligosaccharides were detected by measuring ultraviolet absorption at 220 nm. Peak areas obtained for individual oligosaccharides were converted to molar concentrations, which were then plotted against reaction time to obtain the reaction time-course.

MALDI-TOF MS of the enzymatic products

Products obtained from the enzymatic reaction were identified by MALDI-TOF MS. Reaction conditions were the same as in HPLC determination described in subsection *HPLC-based determination of the reaction time-course* in the *Materials and methods* section. A portion (1 μL) of the reaction mixture was mixed with an equal volume of 2,5-dihydroxy benzoic acid (20 mg/mL in CH₃CN:water, 80:20, v:v). After addition of 0.1 μL of 0.1% trifluoroacetic acid, the mixture was placed onto a plate in a MALDI micro MX (Waters Corporation, Milford, MA) and then dried. Mass spectra were obtained in positive-ion reflection mode. (GlcNAc)_n (*n* = 1–6) were used as standard *m/z*.

Binding experiments using NMR spectroscopy

The binding experiments were conducted using catalytic acid-deficient mutants, E115Q/G74W-NtChiV and E116Q/G75W-AtChiC, to avoid hydrolytic effects. The uniformly ¹⁵N-labeled proteins were produced and purified as described in subsection *Expression and purification of recombinant enzymes* in the *Materials and methods*. NMR samples contained 0.4 mM protein in 50 mM sodium acetate buffer, pH 5.0 (90% H₂O/10% D₂O). ¹H-¹⁵N-HSQC 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. The Trp74 indole NH resonance of E115Q/G74W-NtChiV was assigned by comparison between the ^1H - ^{15}N -HSQC spectra of E115Q-NtChiV and E115Q/G74W-NtChiV. Similarly, the Trp75 indole NH resonance of E116Q/G75W-AtChiC was assigned by comparison between the spectra of E116Q-AtChiC and E116Q/G75W-AtChiC. When $(\text{GlcNAc})_5$ was titrated into the ^{15}N -labeled E115Q/G74W-NtChiV solution, the Trp74 indole NH signal gradually shifted without a change in signal intensity. The chemical shift changes induced by the oligosaccharide binding ($\Delta\delta$) were calculated by the equation,

$$\Delta\delta = \left\{ \frac{\Delta\text{NH}^2 + \Delta\text{N}^2/25}{2} \right\}^{1/2} \quad (1)$$

where ΔNH and ΔN represent the observed shifts along the ^1H -axis and ^{15}N -axis, respectively. The relative $\Delta\delta$ values ($\delta/\delta_{\text{max}}$) were plotted against the free oligosaccharide concentration to obtain the experimental titration curve, which was analyzed by the following equation,

$$\frac{\Delta\delta}{\delta_{\text{max}}} = \frac{[\text{S}]_{\text{free}}}{[\text{S}]_{\text{free}} + 1/K_{\text{assoc}}} \quad (2)$$

The free oligosaccharide concentrations $[\text{S}]_{\text{free}}$ was obtained by subtracting the bound oligosaccharide concentration $[\text{ES}]$ from the total oligosaccharide concentration $[\text{S}]_{\text{total}}$. The association constant K_{assoc} was calculated from the experimental data by a non-linear curve fitting procedure. In the case of E116Q/G75W-AtChiC, the intensity of the Trp75 indole proton resonance gradually decreased as the $(\text{GlcNAc})_5$ concentration increased, and then faded away. Thus, the relative decrease in signal intensity $[(I_{\text{free}} - I_{\text{obs}})/I_{\text{free}}]$ was plotted against the free oligosaccharide concentration, and the association constant, K_{assoc} , was calculated using the equation,

$$\frac{I_{\text{free}} - I_{\text{obs}}}{I_{\text{free}}} = \frac{[\text{S}]_{\text{free}}}{[\text{S}]_{\text{free}} + 1/K_{\text{assoc}}} \quad (3)$$

Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

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Conflict of interest

None declared.

Abbreviations

AtChiC, a GH-18 (class V) chitinase from *Arabidopsis thaliana*; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(\text{GlcNAc})_n$, β -1,4-linked oligosaccharide of GlcNAc with a polymerization degree of n ; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum coherence; MALDI-TOF MS, matrix-assisted laser-desorption ionization-time-of-flight MS; NMR, nuclear magnetic resonance; NtChiV, a GH-18 (class V) chitinase from *Nicotiana tabacum*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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