Multiple Roles of Asp313 in the Refined Catalytic Cycle of Chitin Degradation by Vibrio harveyi Chitinase A

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Three acidic residues in the DXDXE sequence motif are suggested to play a concerted role in the catalysis of Vibrio harveyi ChiA. An increase in the optimum pH of 0.8 units in mutant D313A/N indicates that Asp313 influences the $p_K_a$ of the ionizing groups around the cleavage site. D313A showed greatly reduced $k_{cat}/K_m$ and increased $K_m$, suggesting that Asp313 participates in catalysis and ligand binding. Investigation of the enzyme-substrate interactions of V. harveyi ChiA and Serratia marcescens ChiB revealed two conformations of Asp313 and (−1)GlcNAc. The first conformation, likely to be the initial conformation, showed that the $\beta$-COOH of Asp313 only interacted with the $-\text{C}=\text{O}$ of the N-acetyl group in the distorted sugar. The second conformation, formed from the first by concerted bond rotations, demonstrated hydrogen bonds between the Asp313 side chain and the $-\text{NH}$ of the N-acetyl group and the $-\text{COOH}$ of Glu315. Here we propose a further refinement of the catalytic cycle of chitin hydrolysis by family-18 chitinases that involves four steps: Step 1: Priming. An acidic pair is formed between Asp311 and Asp313. Step 2: Substrate binding. The Asp313 side chain detaches from Asp311 and rotates to form a H-bond with the $\text{C}=\text{O}$ of the 2-acetamido group of (−1)GlcNAc. Step 3: Bond cleavage. The side chain of Asp313 and the 2-acetamido group simultaneously rotate, permitting Asp313 to interact with the side chain of Glu315 and facilitating bond cleavage. Step 4: Formation of reaction intermediate. The transient (−1) C$_1$-GlcNAc cation readily reacts with the 2-acetamido group, forming an oxazolinium ion intermediate. Further attack by a neighboring water results in retention of $\beta$-configuration of the degradation products.

Key words: chitin degradation; family-18 chitinases; Vibrio harveyi; substrate-assisted mechanism

Chitinases are a diverse class of glycosyl hydrolases that cleave the $\beta$-(1,4) linkages in chitin polymer. In nature, chitin degradation is achieved by two classes of chitin hydrolysing enzymes: endochitinases (EC 3.2.1.14), which cleave chitin chains at accessible points along their lengths, and $\beta$-N-acetylglucosaminidases (GlcNAcases, EC 3.2.1.52), which sequentially remove GlcNAc monomers from the reducing ends of chitin oligomers. Most bacteria secrete both endochitinases and GlcNAcases, which synergistically convert insoluble chitin into various chitoooligosaccharide fragments, yielding GlcNAc and GlcNAc$_2$ as end products.1–9 As classified by the Carbohydrate Active Enzyme (CAZy) database (www.cazy.org), family-18 glycosyl hydrolases (GH-18) act on carbohydrates, contain the catalytic ($\beta/\alpha$) TIM barrel domain, and catalyze the hydrolytic reaction through a conformation-retaining mechanism.10,11 To date, chitinase A and chitinase B are the two major enzymes in the GH-18 class, with well characterized structures and functions.1,12–16 The two enzymes bind chitin chains in opposite orientation, due to differences in their domain organization.

We had screened 14 species of marine bacteria in the Vibrio family for chitinase activity, and we found that V. harveyi (formerly V. carchariae) secreted a high level of chitinase A (named VhChiA), which degraded chitin polymer, generating GlcNAc$_2$ as the main product.6,17 The gene encoding VhChiA was subsequently cloned and expressed at a high level in E. coli M15 cells,18 which allowed its enzymic properties, substrate binding, and 3D-structure to be studied in detail.15,18,19 In common with all known family-18 chitinases, VhChiA catalyzes the hydrolytic reaction through the substrate-assisted mechanism16,17 and the catalytic role of the invariant acidic residue Glu315 was identified. Structural analysis showed the substrate-binding cleft of VhChiA to be a long, deep groove containing six GlcNAc-binding subsites, denoted (−4)(−3)(−2)(−1)(+1)(+2).15 Subsites (−4) to (−2) are designated the glycone binding sites, whereas subsites (+1) and (+2) are designated the aglycone binding sites.22 Chitin chain cleavage occurs between subsites (−1) and (+1). The critical subsite, (−1), is located at the bottom of the substrate-binding cleft, where Asp311-Asp313-Glu315 are arranged linearly as part of the DXDXE sequence motif. These residues have been reported to be essential in all known family-18 chitinases since they collaborate in the catalytic cycle of chitin degradation.14,21–23 In this study, we assigned Asp313 in the catalytic cycle by comparing the active site structures of VhChiA and Serratia marcescens ChiB (SmoChiB) and by investigating the pH-activity profiles of two variants of VhChiA by Asp313 substitution.

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Abbreviations: ChBD, chitin binding domain; DMAB, $p$-dimethylaminobenzaldehyde; GlcNAc, $\beta$-1–4 linked oligomers of $N$-acetyl-$\alpha$-glucosamine residues where $n = 1$–6; IPTG, isopropyl thi-$\beta$-D-galactoside; NRE, non-reducing end; PMSF, phenylmethylsulphonylfluoride; $p$NP, $p$-nitrophenol; RE, reducing end; WT, wild-type VhChiA
Materials and Methods

Bacterial strains and chemicals. Escherichia coli type strain DH5α was used for routine cloning, subcloning, and plasmid preparation. Supercompetent E. coli XL1Blue (Stratagene, La Jolla, CA) was the host strain for the production of mutagenized DNA. E. coli type strain M15 (Qiagen, Valencia, CA) and the pQE 60 expression vector harboring Chi A gene fragments were used for high-level expression of recombinant chitinases. Chitin from crab shells, chitooligosaccharides, and PNP-glycosides were purchased from Seikagaku (Bioactive, Bangkok, Thailand) or Sigma-Aldrich (Singapore). A QuickChange Site-Directed Mutagenesis Kit including pfu Turbo DNA polymerase was from Stratagene. Restriction enzymes and DNA modifying enzymes were from New England Biolabs, Inc. (Beverly, MA, USA).

Mutation design and site-directed mutagenesis. Point mutations were introduced into the wild-type chitinase A DNA by the PCR technique using a QuickChange Site-Directed Mutagenesis kit (Stratagene). VfChiA variants D313A and D313N were generated by introducing Ala and Asn codons into the wild-type DNA using mutagenic oligonucleotides designed and synthesized by the Bio Service Unit (BSU, Bangkok, Thailand). The oligonucleotide sequences used in site-directed mutagenesis are listed in Table 1. Successful mutagenesis was confirmed by automated DNA sequencing (BSU).

Protein expression and purification. The DNA fragment that encodes wild-type chitinase A (amino acid residues 22–597, without the 598–850 C-terminal fragment) was cloned into pQE60 expression vector. Recombinant WT and mutants was carried out following a protocol described previously. Briefly, cells were grown at 37°C in LB medium containing 100 μg/ml antibiotic until OD600 reached 0.6, and then chitinase expression was induced by the addition of isopropyl-thio-β-D-galactoside (IPTG) (0.5 mM) at 25°C for 18 h.

The cell pellet obtained after centrifugation was re-suspended in lysis buffer (20 mM Tris–HCl buffer pH 8.0, containing 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, and 1 mM 1,4-lactozyme), and then lysed on ice using a Sonopuls Ultrasonic homogenizer with a 6-mm diameter probe (50% duty cycle; amplitude setting, 20%; total time, 30 s, 6–8 times). Unbroken cells were removed by centrifugation at 12,000 × g for 1 h. The supernatant was immediately applied gravitationally to a Ni-NTA agarose affinity column (1 cm × 1 cm) (Qiagen, Hilden, Germany). The column was washed thoroughly with 5 mM imidazole, followed by 10 mM imidazole, and then eluted with 250 mM imidazole. The chitinase-containing fractions were analyzed by SDS-PAGE on a 12% acrylamide gel before several rounds of membrane centrifugation using Vivaspin-20 membrane concentrators (vivaspin, Hanover, Germany) for complete removal of imidazole.

The final concentration of the protein was determined by the Bradford method.

pH stability assay. Determination of the effect of pH on the stability of VfChiA was carried out in a 96-well microtiter plate. A reaction mixture containing 0.8 mmol enzyme was preincubated in the universal buffer (100 mM sodium citrate/citric acid, 200 mM sodium phosphate, and 100 mM NaCl) at various pH from 3–9, at 37°C. After 15 min, 1 mm PNP-GlcNAc2 was added, and the reaction mixture was incubated for a further 10 min. The reaction was terminated by the addition of 100 μl 3 M Na2CO3. The amount of p-nitrophenol (pNP) released by the hydrolytic action of the enzyme was determined spectrophotometrically at 405 nm in an Anthos Multilab 4000 Microplate Reader (Biochrom, Cambridge, UK). The molar quantity of the liberated pNP was estimated from a calibration curve constructed with a pNP standard varying from 0–15 nmol. The molecular activity of the enzyme was defined as nmol pNP produced per s per nmol enzyme.

Determination of pH activity profiles. Universal buffer was used to determine the pH activity profiles of VfChiA and the Asp313 mutants. The buffer contained 100 mM sodium citrate/citric acid, 200 mM sodium phosphate, and 100 mM NaCl. pH 3–9. Chitinase activity against pNP-GlcNAc2 was determined at various pH values. Reaction mixtures of 200 μl (prepared in a 96-well microtiter plate) contained various concentrations of the substrate (0–500 μM), 0.4 mM enzyme, and the assay buffer at desired pH. The pH activity was incubated at 37°C for 10 min with constant agitating at 200 rpm, and the reaction was terminated by the addition of 100 μl of 3 M Na2CO3. The initial rate of enzyme activity (v0) was estimated from the liberated pNP, as described above. The plots of the Kcat, kcat/Km values as a function of pH were constructed using a non-linear regression function available in GraphPad Prism version 5.0.

Chitin binding assay. Binding assays of three polysaccharides (colloidal chitin, crystalline α-chitin, and crystalline chitosan) to chitinase were performed at 0°C to minimize hydrolysis. Each reaction mixture (500 μL) contained 0.04 mmol enzyme and 1 mg polysaccharide in 20 mM Tris–HCl buffer, pH 8.0. The mixtures were incubated for 30 min, and the binding reaction was terminated by centrifugation at 12,000 × g, 4°C for 5 min. The concentration of the enzyme remaining in the supernatant (free enzyme) was determined by the Bradford method. From this, the concentration of the bound enzyme (Ebound) was calculated from the difference between the initial protein concentration (Etotal) and the free protein concentration (Efree) after binding. To determine adsorption isotherm, reaction mixtures (prepared as described above) containing various concentrations of chitinase (0–7 μM) were incubated with 1.0 mg of the tested polysaccharide for 30 min. After centrifugation, the concentrations of free enzyme in the supernatant were determined. The plots of [Etotal] vs [Efree] were constructed and the equilibrium dissociation binding constants (Kd) estimated, assuming one specific binding site, by a non-linear regression program available in GraphPad Prism v5.0.

Steady-state kinetics. The kinetic parameters of the chitinase variants were determined using pNP-GlcNAc2, chitooligosaccharides, or colloidal chitin as substrate. For pNP-GlcNAc2, the reaction was set up as described above with substrate concentrations varying from 0–500 μM. For chitooligosaccharides, reaction mixtures (200 μL) containing 0–500 μM GlcNAc2 and 0.8 nmol WT or 2.4 mmol D313A or D313N in 100 mM sodium acetate buffer, pH 5.5, were incubated at 37°C for 15 min. After boiling to 100°C for 10 min, the entire reaction mixture was subjected to DNS assay following the protocol described by Miller. The amounts of the product reactions generated from the chitin substrates were determined from a standard curve of GlcNAc2 (0–5 nmol). Kinetic values were evaluated from three independent sets of data using the non-linear regression function obtained from GraphPad Prism v5.0.

Results and Discussion

VfChiA is a family-18 chitinase that degrades chitin, releasing chitooligosaccharides, yielding GlcNAc2 as the
Table 2. Effects of Asp313 Mutations on the Kinetic Parameters of VhChiA

Kinetic studies with pNP-GlcNAc2 as substrate were carried out by a colorimetric assay, with pNP as standard. A kinetic study with chitooligosaccharides was carried out by a reducing sugar assay with a standard curve constructed from GlcNAc2 (for details of the experiments, see text). The values shown were obtained in three independent sets of experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>WT</th>
<th>D313A</th>
<th>D313N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>pNP-GlcNAc2</td>
<td>67 ± 4.5</td>
<td>82 ± 1.5</td>
<td>1,224</td>
</tr>
<tr>
<td>Chitotetraose</td>
<td>117 ± 25</td>
<td>38 ± 2.9</td>
<td>324</td>
</tr>
<tr>
<td>Chitopentose</td>
<td>85 ± 15</td>
<td>45 ± 2.4</td>
<td>529</td>
</tr>
<tr>
<td>Chitohexaose</td>
<td>45 ± 2.3</td>
<td>57 ± 0.7</td>
<td>1,267</td>
</tr>
</tbody>
</table>

*The values in brackets represent fold decrease of $k_{cat}/K_m$ in relation to the values of WT.

![Fig. 1. SDS-PAGE analysis of VhChiA.](image)

SDS-PAGE analysis of the chitinase variants, which were expressed in E. coli M15 and purified by Ni-NTA agarose affinity chromatography. The three forms of the enzyme were found to be relatively stable over a wide range of pH from 3 to 9 (data not shown). However, the molecular activities of D313A and D313N towards the single protein bands corresponding to the purified WT, and the D313A and D313N forms, each with the same apparent molecular weight of 63 kDa (Fig. 1).

The three forms of the enzyme were found to be relatively stable over a wide range of pH from 3 to 9 (data not shown). However, the molecular activities of D313A and D313N towards the pNP glycoside were greatly reduced relative to the activity of WT, by 30-fold and 20-fold respectively. As discussed in more detail below, the activity loss due to side-chain substitution of Aps313 was even more noticeable when the enzymes were tested with natural glycosides, especially with chitohexaose (Table 2).

The activity-pH profiles of the wildtype, D313A, and D313N were determined with pNP-GlcNAc2 as substrate (Fig. 2A–C). Among the three chitinase variants, the shapes of the plots of $k_{cat}$ (Fig. 2A), $K_m$ (Fig. 2B), and $k_{cat}/K_m$ (Fig. 2C) versus pH appeared similar, but with inflections at different pH values. The $k_{cat}$-pH and $k_{cat}/K_m$-pH profiles of WT were approximately bell-shaped, with maximum activity at around pH 5. This can be interpreted simply as a result of the ionization of the residue on the acidic side of the curve with a $pK_a$ of 4.2 ± 0.1. But $pK_a$, which represents the ionization of the second residue on the basic side of the curve, could not be determined accurately, since the shoulder seen between pH 6 and 9 indicates tight association of the second and third ionizing groups. Indeed, these ionizable groups help to maintain the hydrolytic activity of the enzyme at high pH values. This is not uncommon, as at least two GH-18 chitinases, one from Serratia sp. TKU020(29) and the other from Manduca sexta,30 have been reported to possess broad pH-activity profiles, with considerable retaining activity at alkali pH.
On the other hand, the $K_m$-pH profile of the WT has an inverse bell-shape, with $K_m$ increasing to about pH 5, while $k_{cat}$ and $k_{cat}/K_m$ decreased (Fig. 2B, as compared to Fig. 2A and C). The lowest $K_m$ was observed at exactly the pH optimum of the enzyme. As expected, mutants D313A and D313N had much lower $k_{cat}/K_m$ relative to the values for WT. Similar results have been reported for mutant DI42N for SmChBi23) and other family-18 chitinases.14,31,32) Significantly, the pH optimum for the D313 variants was shifted upward by 0.8 pH units to 6.0, indicating that the ionization state of Asp313 influences the microenvironment around the cleavage site. This accords completely with a previous study with SmChBi, in which Asp142 (equivalent to Asp313 in VhChiA) helped to lower $pK_a$ of catalytic residue Glu144 (equivalent to Glu315) by 0.8 units.23)

Substitution of the Asp313 to Ala or Asn severely impaired the catalytic activity of the enzyme bound best to colloidal chitin, followed by deacetylated chitin (crystalline chitosan). All forms of the enzyme bound best to colloidal chitin, followed by chitosan and crystalline chitin (Fig. 3).

The specific function of Asp313 in chitin binding was examined with different types of chitin derivative, including colloidal chitin, crystalline $\alpha$-chitin, and deacetylated chitin (crystalline chitosan). All forms of the enzyme bound best to colloidal chitin, followed by chitosan and crystalline $\alpha$-chitin (Fig. 3).

The binding capacity of each polysaccharide was found to be affected by how it is accessible to the enzyme’s binding cleft. Colloidal chitin (the best interacting molecule) is an acid-treated form of chitin, which appears more homogenous than the other polymers. Chitosan consists of fine particles that are moderately uniform, while crystalline chitin is flake-like and not homogenous in the buffered solution. This probably explains why chitosan reacted with the target enzymes better than crystalline chitin. As Fig. 3 also shows the Ala/Asn mutations of D313 caused a significant reduction in the binding ability of the enzyme towards all tested polysaccharides, the Ala mutant displaying greater effect than the Asn mutant.

Adsorption isotherms were found to obtain the equilibrium binding constant ($K_D$) of the individual enzymes. Non-linear (single-specific site binding) plots of bound versus free enzyme concentrations during titration of the three enzyme forms confirmed weakened binding by the mutated enzymes (Table 3). For colloidal chitin, the values of $K_D$ estimated for D313A (1.8 ± 0.3 $\mu$m) and D313N (1.6 ± 0.3 $\mu$m) were 2.6- and 2.3-fold respectively higher than the $K_D$ (0.7 ± 0.2 $\mu$m) for WT. These estimated $K_D$ values indicate relative substrate-binding affinities in the following order: WT > D313N > D313A. The $K_D$ values determined for crystalline chitin and chitosan (Table 3, rows 2 and 3) were greater than colloidal chitin, suggesting that colloidal chitin interacts with highest affinity to each chininase form. In accord with the results in the chitin binding assay (Fig. 3), crystalline chitin was the poorest binding partner, due to its highly compact structure, which does not allow it to be easily accessible by the enzyme molecule.

The increased $K_D$ in the D313A/N mutants was completely consistent with their increased $K_m$ over the $K_m$ of the WT over the entire range of pH (see Fig. 2B).

Figure 3. Chitin Binding Assays.

The binding assay set, described in the text, was incubated with colloidal chitin, crystalline $\alpha$-chitin, or crystalline chitosan for 30 min at 0°C. % Binding $= \left(\frac{E_f - E_i}{E_i}\right) \times 100$, where $E_i$ is the initial enzyme concentration and $E_f$ is the free enzyme concentration after binding. Black, gray, and hollow bars represent % binding of WT, D313A, and D313N respectively. Data are mean values obtained from three independent sets of experiment.

Table 3. Binding of Three Different Polysaccharides by V. harveyi Chitinase Variants

<table>
<thead>
<tr>
<th>Chitin derivatives</th>
<th>WT</th>
<th>D313A</th>
<th>D313N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{max}$ ($\mu$m/l)</td>
<td>$K_D$ ($\mu$m)</td>
<td>$B_{max}$ ($\mu$m/l)</td>
</tr>
<tr>
<td>Colloidal chitin</td>
<td>5.1 ± 0.2</td>
<td>0.7 ± 0.2 (1)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Crystalline chitin</td>
<td>6.0 ± 0.5</td>
<td>2.2 ± 0.5 (1)</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Chitosan</td>
<td>5.6 ± 0.3</td>
<td>1.9 ± 0.3 (1)</td>
<td>3.1 ± 0.4</td>
</tr>
</tbody>
</table>

*K_D values presented in brackets are to be compared with the values for the WT enzyme.
and with all the substrates tested (see Table 2). These results clearly indicate that Asp313 not only affects the catalytic step in chitin degradation, but also contributes significantly to the substrate-binding affinity of the enzyme. The estimated $K_D$ values shown in Table 3 are somewhat smaller than the corresponding $K_m$ values, by one order of magnitude for the WT and two orders of magnitude for the D313A and D313N mutants. This is not surprising since the $K_m$ derived from the steady-state reaction is not a simple function of the off-rate and on-rate constants (like $K_D$), but also depends on the catalytic rate constant. It is noticeable that mutations of Asp313 to Ala and Asn also reduced the maximum catalytic rate constant. It is reasonable that mutations of Asp313 to Ala and Asn also reduced the maximum catalytic rate constant.

Further analysis of the active site of the two enzymes complexed with their designated substrates revealed that the side-chain movement of Asp313 might initiate the catalytic cycle of chitin degradation, as has been proposed by Tews et al. and further refined by van Aalten et al. The active sites of VhChiA inactive mutant E315M (pdb code 3b9a), WT (pdb code 3b8s), and SmChiB mutant E144Q (pdb code 1e6n) were compared in the presence of GlcNAc$_6$ (for VhChiA) and GlcNAc$_5$ (for SmChiB). For better visualization of the correlation of Asp311-Asp313-Glu315, only the active sites of WT and SmChiB are displayed (Fig. 4). The catalytic domain of VhChiA and the ChBD at the C-terminus. The active site of VhChiA has a long, deep groove with openings at both ends, while that of SmChiB has a tunnel-like morphology.

Fig. 4. Comparison of the Active Sites of VhChiA and SmChiB.

A stick model of VhChiA superimposed on that of SmChiB. The catalytic domain of the VhChiA wildtype (pdb code 3b9a) is superimposed on that of SmChiB inactive mutant E144Q from S. marcescens (pdb code 1e6n) with RMSD of 2.041 Å for 241 C$_a$ atoms. The active-site structures of the two chitinases were displayed and presented by means of the Pymol program (http://www.pymol.org/). The important binding residues in VhChiA are colored dark gray, and the equivalent residues in SmChiB are colored light gray. The coordinates of GlcNAc$_6$ in VhChiA and GlcNAc$_5$ in SmChiA are shown as a line model, with GlcNAc$_6$ presented in dark gray and GlcNAc$_5$ in light gray.
to (+2) in VhChiA, whereas GlcNAc\(_5\) (light gray) occupied subsites (−2) to (+3) in SmChiB. The three invariant residues Asp311-Asp313-Glu315 of VhChiA had positions equivalent to Asp140-Asp142-Glu144 of SmChiB. Overall dissimilarities between the two structures are: i) The 2-acetamido group of GlcNAc in the catalytic cleft of VhChiA is oriented in a direction opposite to that in SmChiB, rendering its –NH group unable to interact with Asp313 unless it rotates to the same position as in SmChiB; and ii) The carboxylic side chain of Asp313 of VhChiA was rotated 109° downward relative to the position of Asp142 in SmChiB. This downward conformation was not seen in SmChiB. Asp313 cannot interact either with the –NH in the 2-acetamido group of −1GlcNAc, or with the \(\gamma\)-carboxylate in the side chain of Glu315, as they are simply too distant (5.0 Å for the NH-group and 6.0 Å for Glu315). On the other hand, such a conformation can potentially H-bond with –C=O in the 2-acetamido group of −1GlcNAc, which is only 3.5 Å distant.

The positional differences of −1GlcNAc and Asp313 observed between VhChiA and SmChiB (Fig. 4) provide an insight into how the side-chain of Asp313 and the 2-acetamido group can rotate and make initial contact before the distortion of −1GlcNAc subsequent to binding. These structures also suggest that the 2-acetamido side chain of Asp313 can assume initial orientations as seen in VhChiA upon binding to a chitin chain, both side chains subsequently rotating to adopt the orientation seen in SmChiB\(^{4}\) and also in SmChiA.\(^{5}\) In the light of this structural analysis, we propose a refined catalytic cycle for family-18 chitinases that probably involves four steps (instead of three as previously suggested), as illustrated in Fig. 5.

Fig. 5. The Refined Catalytic Cycle of Chitin Degradation.

The mechanism is further refined from that described by Tews et al.\(^{21}\) and van Aalten et al.\(^{13}\) Step 1: Pre-priming. An acidic pair is formed between Asp311 and Asp313. Step 2: Substrate binding. The Asp313 side chain detaches from Asp311 and rotates to form an H-bond with the \(\mathrm{C} =\mathrm{O}\) of the 2-acetamido group of −1GlcNAc. Step 3: Bond cleavage. The side chain of Asp313 and the 2-acetamido group rotate simultaneously. The Asp313 side chain forms two hydrogen bonds, with the –NH of 2-acetamido group and with the Glu315 side chain. The glycosidic bond is then cleaved by nucleophilic attack on C-1 by the carbonyl of the acetamido side chain, with protonation of the glycosidic oxygen by Glu315. Step 4: Formation of the reaction intermediate. Nucleophilic attack on the anomeric carbon (C-1 of −1GlcNAc) generates an oxazolinium ion intermediate, which is stabilized by Asp313. The cycle is completed after the second nucleophilic attack on the anomeric carbon by the –OH group of water, with retention of configuration. The Asp313 side chain then rotates back to its original conformation.
geometry of the carbonyl of the 2-acetamido group is maintained through an H-bond with the adjacent residue Tyr319. Step 4: Stabilization of the reaction intermediate. The oxazolinium ion intermediate is generated by nucleophilic attack on the anomeric carbon (C1) by –C=O of the 2-acetamido group of –1GlcNAc itself. The reaction intermediate is stabilized by Asp313 and is ready for a second displacement by the –OH group of a neighboring water molecule, with retention of the β conformation. The side chain of Asp313 then rotates back to its original position. In summary, structural information and kinetic data on the D313 variants suggest three essential roles of Asp313 in the various stages of the refined catalytic cycle. Firstly, it orients the 2-acetamido group of –1GlcNAc with a geometry favorable to its action as a nucleophile (Fig. 5, steps 2 and 3). Secondly, it helps to lower the pKₐ of Glu315, while orienting γ-COOH so as to facilitate proton abstraction by the O-glycosidic bond between the GlcNAc units at subsites (−1) and (+1) (Fig. 5, step 3). Thirdly, it stabilizes the reaction intermediate through a hydrogen bond formed between the –NH acetamido group and the β-COO⁻ group of Asp313 (Fig. 5, step 4).

Conclusions

The highly conserved DXDXE sequence motif is known to play a role in the catalytic mechanism of family-18 chitinases. In this study, Asp313 was mutated to Ala and Asn, generating two mutants, D313A and D313N. The pH-activity profiles of these mutants showed not only a profound reduction in the chitinolytic activity of the chitinase, but also a decrease in substrate binding affinity. The chitin binding assay confirmed that Asp313 contributes to the ability of VhChiA to bind chitin substrates. Replacement or removal of the Asp313 side chain causes a shift of the pH optimum to a more basic value, suggesting that Asp313 participates in the catalytic process by lowering the pKₐ of catalytic residue Glu315, promoting bond cleavage. A detailed inspection of the active site structures of VhChiA and SncChiB with bound chitin oligosaccharides provided additional information that coordinated rotations of the Asp313 side chain and the 2-acetamido group of the distorted sugar trigger Glu315 to act as a highly effective proton-donating residue. It is apparent that Asp313 plays multiple roles at various stages of the catalytic cycle of chitin degradation by VhChiA.

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