Azide anions inhibit GH-18 endochitinase and GH-20 Exo β -N-acetylglucosaminidase from the marine bacterium *Vibrio harveyi*

Received May 25, 2015; accepted August 2, 2015; published online September 1, 2015

Paknisa Sirimontree¹, Tamo Fukamizo² and Wipa Suginta^{1,*}

¹Biochemistry-Electrochemistry Research Unit, School of Biochemistry, Suranaree University of Technology, Nakhon Ratchasima, Thailand and ²Department of Advanced Biosciences, Kinki University, Nara, Japan

*Wipa Suginta, Biochemistry-Electrochemistry Research Unit, School of Biochemistry, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Tel: +66 44 226187, email: wipa@sut.ac.th

Vibrio harveyi is a bioluminescent marine bacterium that utilizes chitin as its sole source of energy. In the course of chitin degradation, the bacterium primarily secretes an endochitinase A (*VhChiA*) to hydrolyze chitin, generating chitooligosaccharide fragments that are readily transported into the cell and broken down to GlcNAc monomers by an exo β -N-acetylglucosaminidase (*VhGlcNAcase*). Here we report that sodium salts, especially sodium azide, inhibit two classes of these chitin-degrading enzymes (*VhChiA* and *VhGlcNAcase*) with distinct modes of action. Kinetic analysis of the enzymatic hydrolysis of *p*NP-glycoside substrates reveals that sodium azide inhibition of *VhChiA* has a mixed-type mode, but that it inhibits *VhGlcNAcase* competitively. We propose that azide anions inhibit chitinase activity by acting as strong nucleophiles that attack C_γ of the catalytic Glu or C_β of the neighbouring Asp residues. Azide anions may bind not only to the catalytic centre, but also to the other subsites in the substrate-binding cleft of *VhChiA*. In contrast, azide anions may merely occupy the small-binding pocket of *VhGlcNAcase*, thereby blocking the accessibility of its active site by short-chain substrates.

Keywords: chitin turnover/family 20 N-acetylglucosaminidase/family 18 chitinase/reversible inhibition/sodium azide.

Abbreviations: *p*NP, *p*-nitrophenol; *p*NP-GlcNAc, *p*-nitrophenyl-*N*-acetylglucosaminide; *p*NP-GlcNAc₂, *p*-nitrophenyl-di-*N*-acetylchitobioside; IPTG, isopropyl thio- β -D-galactoside; PMSF, phenylmethyl sulfonyl fluoride; *VhChiA*, *Vibrio harveyi* chitinase A; *VhGlcNAcase*, *Vibrio harveyi* β -N-acetylglucosaminidase.

Chitin is a homopolymer of *N*-acetylglucosamine units joined by β -1-4 glycosidic linkages. Chitin is highly abundant in marine environments, occurring in the structural skeletons of crustaceans (e.g. crabs, lobsters and shrimps), as well as in the internal shells of cephalopods, including squid and octopus. A complete degradation of chitin by marine bacteria of the *Vibrio* class involves the successive action of two classes of chitinolytic enzymes: 1) GH-18 endochitinases (EC 3.2.1.14) and GH-20 exo β -N-acetylglucosaminidases or GlcNAcases (EC 3.2.1.52) (1). Endochitinases catalyze the hydrolysis of insoluble chitin by randomly cleaving the internal glycosidic bonds within the chitin chain, yielding chitooligosaccharide fragments (2, 3) that are further hydrolyzed from the non-reducing end to produce GlcNAc monomers by β -N-acetylglucosaminidase (2, 4–6).

Vibrio harveyi is a Gram-negative marine bacterium that causes luminous vibriosis, a serious disease that affects commercially farmed fish and shellfish species world-wide (7). *V. harveyi* is responsible for a rapid turnover of chitin biomaterials, since it can grow rapidly using chitin as its sole source of energy (8). The chitin degradation pathway of *Vibrios* (here *V. harveyi*) has been proposed to involve a series of chitin-protein interactions. After chitin chemotaxis, the colonizing bacterium secretes a GH-18 endochitinase A (*VhChiA*) (1, 8) to degrade chitin biomaterials, yielding chitooligosaccharide fragments that are readily taken up through the outer membrane of the cell through a specific channel (chitoporin, *VhChiP*) (9, 10). In the periplasm, a GH-20 GlcNAcase (*VhGlcNAcase*) sequentially degrades the chitooligosaccharides to GlcNAc monomers, which are then transported further through the inner membrane, and finally metabolized in the cytoplasm to produce intermediates that are used for ATP production. Although *VhChiA* and *VhGlcNAcase* belong to different classes of glycoside hydrolases, both catalyse their hydrolytic reactions through the substrate-assisted retaining mechanism (3, 11, 12). Chitin degradation by *VhChiA* is achieved through the cleavage of the β -1,4 glycosidic bond between –1GlcNAc and +1GlcNAc by the catalytic residue Glu315, which donates a proton to the oxygen O4 of +1GlcNAc. The subsequent formation of an oxazolinium ion intermediate is then stabilized by the C2-acetamido group of the –1GlcNAc ring. In the subsequent step, a proton is withdrawn from a water molecule by the γ -COO[–] group of Glu315, and the remaining OH[–] is taken up

by the C1 of α -GlcNAc, yielding the shortened chitin chain, which retains the initial anomeric configuration (13, 14).

Sodium azide has been used in 'chemical rescue' experiments to probe the catalytic acid/base and the catalytic nucleophile residues of the retaining glycoside hydrolases, such as *Cellulomonas fimi* GH-85 exoglucanase/xylanase (15), *Bacillus licheniformis* GH-16 1,3-1,4- β -glucanase (16), *Streptomyces* sp. GH-1 β -glucosidase (17), *Geobacillus stearothermophilus* T-6 GH-51 α -L-arabinofuranosidase (18), *Streptomyces plicatus* GH-20 hexoxaminidase (*SpHex*) (19), *Sulfolobus solfataricus* GH-29 α -L-fucosidase (20), *Paenibacillus* sp. TS12 GH-3 glucosylceraminidase (21) and *Arthrobacter protophormiae* GH-85 endo- β -N-acetylglucosaminidase (Endo A) (22). Such studies demonstrated that sodium azide produced a significant recovery of the glycoside hydrolase activity of inactive mutants in which one of the catalytic pair of acidic amino acids was mutated. For example, the role of the key catalytic residues Glu134 and Glu138 of *Bacillus licheniformis* 1,3-1,4- β -glucanase was probed by activation of inactive mutants E134A and E138A by sodium azide. Addition of sodium azide reactivated the hydrolytic activity of mutant E138A, yielding a β -glycosyl azide product, arising from nucleophilic attack of azide anion on the glycosyl-enzyme intermediate in the deglycosylation step. Such results suggested that Glu138 was acting as a general acid-base residue. In contrast, azide anions reactivated the E134A mutant through a single inverting displacement, forming the α -glycosyl azide product in the glycosylation step, which suggested that Glu134 was the catalytic nucleophile (16).

Although the rescue activities of glycoside hydrolases by azide anions are well established, there is only one report of azide anions inhibiting the enzymatic activity of *Bacillus licheniformis* 1,3-1,4- β -glucanase (16). In this report, we demonstrate for the first time that high salt concentrations reduced the enzymatic activity of GH-18 *VhChiA* and GH-20 *VhGlcNAcase*. Because the active sites of the two enzyme species are dissimilar in shape and size, we provide a mechanistic theory of how azide anions could inhibit these different classes of chitin-degrading enzymes by different modes of action.

Materials and Methods

Bacterial strains, expression plasmids and chemicals

The genes encoding chitinase A (*VhChiA*) and β -N-acetylglucosaminidase (*VhGlcNAcase*) were isolated from the genome of the marine bacterium *Vibrio harveyi*, cloned into pQE60 expression vector and expressed in *E. coli* M15 (pREP) type strain as described elsewhere (23, 24). In this study, two DNA constructs, namely pQE-60/*ChiA* and pQE60/*GlcNAcase* were used for high-level expression of *VhChiA* and *VhGlcNAcase*. Chemicals and reagents used for protein expression, purification and characterization were of analytical grade. *p*-Nitrophenol (*pNP*) and *p*-nitrophenyl-N-acetyl-glucosaminide (*pNP-GlcNAc*) were products of Sigma-Aldrich (MO, USA). *p*-Nitrophenyl-di-N-acetyl-chitobioside (*pNP-GlcNAc₂*) was obtained from Toronto Research Chemicals (Ontario, Canada). Sodium salts were purchased from Carlo Erba (Milano, Italy).

Recombinant expression and protein purification

The recombinant plasmid containing the gene of interest was transformed into *E. coli* M15 competent cells, then a single ampicillin (Amp)/kanamycin (Kan) resistant colony was picked and grown overnight at 37° in LB broth, containing 100 μ g/ml Amp and 25 μ g/ml Kan, with shaking at 200 rpm. The freshly inoculated culture was diluted by a ratio of 1:100 with LB/Amp/Kan broth and further grown at 37° until the OD₆₀₀ reached 0.4–0.6. To induce protein expression, IPTG was added to a final concentration of 0.5 mM, and then the bacterial culture was further incubated overnight, at 25° for *VhChiA* or 20° for *VhGlcNAcase*, for an additional 18 h with shaking at 200 rpm. The IPTG-induced cells were harvested by centrifugation at 4,500 rpm at 4° for 30 min, then the cell pellet was resuspended in lysis buffer (20 mM Tris-HCl buffer, pH 8.0, containing 1 mg/ml lysozyme, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1% (v/v) Triton X-100, 2 mM MgCl₂, DNase I, 5 mM imidazole and 150 mM NaCl), and further incubated at room temperature for 30 min. Cell debris was removed by centrifugation at 12,000 rpm at 4° for 1 h, while supernatant, containing the recombinant protein, was collected for purification.

The first step of protein purification (both *VhChiA* and *VhGlcNAcase*) was carried out using Ni-NTA agarose resin (Qiagen, CA) at 4°. The supernatant containing soluble protein was gravitationally applied to a home-made column (1.0 $\times$ 1.0 cm²) packed with Ni-NTA agarose resin purchased from Qiagen GmbH (Hilden, Germany). After sample loading, the column was washed thoroughly with the equilibration buffer, followed by the equilibration buffer containing 5 and 20 mM imidazole, and then eluted with 250 mM imidazole. The eluted fractions were concentrated to 5 ml using a Vivaspin-20 membrane concentrator (M_r 10,000 cut-off, Vivascience AG, Hannover, Germany). The concentrated protein was further purified by gel filtration chromatography on a HiPrep 16/60 Sephacryl S-200 HR column connected to an ÄKTA purifier system (Amersham Bioscience, NJ). Fractions of 2 ml were collected and analysed by SDS-PAGE on a 12% acrylamide gel for verification of purity. The protein-containing fractions were pooled and concentrated to a small volume with the same type of Vivaspin membrane concentrator. The final protein concentration was determined by BCA protein assay kit (EMD Chemicals, CA).

Effects of sodium salts on specific enzymatic activity

Sodium salts of azide, formate, chloride, acetate and nitrate were used to investigate the effects of various anions on the activities of *VhChiA* and *VhGlcNAcase* toward their substrates *pNP-GlcNAc₂* and *pNP-GlcNAc*, respectively. A 100- μ l assay mixture contained 500 μ M substrate, 1 μ g *VhChiA* or 3 μ g *VhGlcNAcase* and 2 M sodium salts in 100 mM potassium acetate, pH 5.5 or 100 mM potassium phosphate, pH 7.5. The reaction mixture was incubated at 37° for 10 min with constant agitating, and then the enzymatic reaction was terminated by the addition of 100 μ l of 3 M Na₂CO₃. The amount of *p*-nitrophenol (*pNP*) released was determined spectrophotometrically at 405 nm in a Biochrom Anthos Multiread 400 Microplate Reader (Biochrom, Cambridge, UK). The molar concentrations of *pNP* were calculated from a calibration curve obtained with *pNP* standard varying from 0 to 20 nmol. One unit of enzyme is defined as releasing 1 nmol of *pNP* in 1 min at 37°.

Effects of sodium and potassium azides on enzymatic activity

The effect of cations on the enzymatic activities of *VhChiA* and *VhGlcNAcase* was investigated using *pNP*-glycosides as substrates. A 100- μ l assay mixture contained 500 μ M *pNP-GlcNAc₂* or *pNP-GlcNAc*, 1 μ g *VhChiA* or 3 μ g *VhGlcNAcase* and 2.0 M sodium azide or potassium azide in 100 mM potassium phosphate, pH 7.5. The reaction mixture was incubated at 37° for various times of 0, 2.5, 5, 10, 30 and 60 min with constant agitating, and the enzymatic reaction was then terminated and the amount of *pNP* released was calculated, as described in the previous section.

Effects of buffer concentrations on the enzyme activity

The concentrations of sodium and potassium phosphate buffers, pH 7.5 were varied to investigate the effects of buffer concentration on the activities of *VhChiA* and *VhGlcNAcase* toward *pNP-GlcNAc₂* and *pNP-GlcNAc*, respectively. A 100- μ l assay mixture contained 500 μ M *pNP-GlcNAc₂* or *pNP-GlcNAc*, 1 μ g *VhChiA* or 3 μ g *VhGlcNAcase* in various concentrations (0.1, 0.5, 1.0 and 2.0 M) of sodium or potassium phosphate buffer, pH 7.5. The assays were

performed and the amount of pNP released was calculated as described earlier.

Determination of reversible inhibition

A reaction mixture (500 µl) containing 250 µg *VhChiA* and 2 M sodium azide in 20 mM Tris-HCl, pH 8.0, was incubated at 37° with shaking. After 10 min, the enzyme was dialyzed against 20 mM Tris-HCl, pH 8.0 to remove sodium azide, and then the enzymatic activity was assayed as described earlier, with 500 µM pNP-GlcNAc₂ as substrate. To determine whether the inhibition by azide ion was reversible or irreversible, the enzyme activities before and after dialysis were compared.

Determination of structural integrity by fluorescence spectrophotometry

Fluorescence emission spectra of purified *VhChiA* and *VhGlcNAc*ase were obtained in different concentrations of sodium azide and sodium nitrate to examine the effects of sodium salts on the structural integrity of both enzymes. Reaction mixtures (500 µl) containing 2 µg of *VhChiA* or 4 µg of *VhGlcNAc*ase and 0, 0.2, 0.5, 1.0, 1.5 or 3.0 M sodium azide or sodium nitrate in 20 mM Tris-HCl, pH 8.0, were pre-incubated at 25° for 1 min with constant agitating. As a control, the enzyme was heated at 100° for 10 min or dissolved in 8 M urea in 20 mM Tris-HCl, pH 8.0 to denature the protein. Changes in intrinsic tryptophan fluorescence were directly monitored in a LS-55 fluorescence spectrometer (Perkin-Elmer, Bangkok, Thailand). The measurements were conducted at 25° with excitation wavelength 295 nm and emission intensities were collected over 300–500 nm, with the excitation and emission slit widths being kept at 5 nm. Each protein spectrum was corrected with the buffer spectrum, for the appropriate sodium azide or sodium nitrate concentration.

Determination of IC₅₀

Dose-response curves showing sodium azide and sodium nitrate inhibition of the enzymatic activity of *VhChiA* and *VhGlcNAc*ase were obtained using pNP-glycosides as substrates. Reaction mixtures (100 µl) contained 500 µM pNP-GlcNAc₂ or pNP-GlcNAc, 1 µg *VhChiA* or 3 µg *VhGlcNAc*ase, respectively, and varied concentrations of sodium azide or sodium nitrate with a 2-fold dilution series to obtain a concentration range of 0–4 M in 100 mM sodium phosphate buffer, pH 7.5, and enzymatic activity was measured as described earlier. The IC₅₀ value of each salt was obtained from a plot of the fractional activity against the logarithm of inhibitor concentrations using the 4-parameter curve fit function in GraphPad Prism v5.0. The *K_i* for *VhChiA* was obtained from the dose-response curve using equation 1 as described by Cheng and Prusoff (25),

$$IC_{50} = \frac{[S] + K_m}{\frac{K_m}{K_i} + \frac{[S]}{\alpha K_i}} \quad (1)$$

For *VhGlcNAc*ase, the *K_i* was estimated using equation 2 (25),

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad (2)$$

Kinetic analysis of enzyme inhibition

We investigated the mode of enzyme inhibition by sodium azide by treating the data according to the kinetics of reversible inhibition. The initial rate of pNP formation from pNP-glycoside hydrolysis by both enzymes was observed within the first 10 min of the reaction. Therefore, each assay reaction was carried out for only 10 min to ensure that the initial rate was obtained. Inhibitory effects on kinetic properties of *VhChiA* and *VhGlcNAc*ase were investigated using pNP-glycosides as substrates. Reaction mixtures (100 µl) contained 0–800 µM of pNP-GlcNAc or pNP-GlcNAc₂, 1 µg of *VhChiA* or 3 µg of *VhGlcNAc*ase, and different concentrations of sodium azide or sodium nitrate (0, 0.5, 1.0, 1.5 and 2.0 M for *VhChiA* or 0, 0.3, 0.4, 0.5, and 0.6 M for *VhGlcNAc*ase) in 100 mM sodium phosphate buffer, pH 7.5. Enzymatic release of pNP was estimated as described earlier.

The kinetic parameters (*V_{max}*, *K_m* and *k_{cat}*) were evaluated from the experiments carried out in triplicate using the nonlinear

regression function available in GraphPad Prism v5.0. The type of inhibition was assessed from the Lineweaver-Burk plots (also available in GraphPad Prism v5.0). Mixed-type inhibition is described by equation 3:

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \left(\frac{1 + \frac{[I]}{\alpha K_i}}{V_{max}} \right) \quad (3)$$

where *I* is the inhibitor, *S* is the substrate, *v₀* is the initial velocity, *V_{max}* is the maximal velocity, *K_m* is the Michaelis-Menten constant and *K_i* is the inhibitory constant. For simple competitive-type inhibition, the kinetic parameters were obtained by data-fitting based on the Michaelis-Menten equation and the substrate inhibition equation (equation 4).

$$v_0 = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [S]} \quad (4)$$

K_i and *αK_i* values were determined from Dixon plots of the slopes of Lineweaver-Burk plots or of the inverse apparent *V_{max}* from Michaelis-Menten plots, respectively, against sodium azide concentration. *K_i* represents the binding of inhibitor to the free enzyme, whereas the *αK_i* represents the binding of inhibitor to the enzyme-substrate complex. To further confirm the inhibition type, a linear transformation of the non-linear regression function was evaluated using equation 5.

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{max}} \quad (5)$$

Results

Sodium salts inhibit GH-18 endochitinase and GH-20 exoGlcNAcase

Sodium salts, including sodium azide, sodium formate, sodium acetate, sodium nitrate and sodium chloride were tested for their inhibitory effects on pNP-glycoside hydrolysis by GH-18 *VhChiA* and GH-20 *VhGlcNAc*ase. The results are shown in Figure 1. The two enzymes were most active at different pH values, the optimal pH for *VhChiA* being 5.5 and for *VhGlcNAc*ase 7.5. However, we carried out the initial assays at both pH conditions. The results showed that all sodium salts inhibited *VhChiA* activity at pH 7.5 more effectively than at pH 5.5 (Fig. 1A), with sodium azide showing the greatest inhibition. Chitinase activity remained ~30% in the presence of 2 M sodium azide, while other sodium salts showed moderate inhibition with the retention of activity >50%. Sodium salts also greatly inhibited *VhGlcNAc*ase activity at pH 7.5, while the activity at pH 5.5 in the presence or absence of sodium salts was insignificant (Fig. 1B). This result indicated that low pH was not a suitable condition for carrying out the *VhGlcNAc*ase assay. Figure 1B additionally shows that sodium nitrate acted much more effectively on *VhGlcNAc*ase than on *VhChiA*. The GlcNAcase activity in the presence of 2 M sodium azide and sodium nitrate was only 4 and 8% of the original activity, respectively.

Azide anions inhibit *Vibrio* chitinase and GlcNAcase

We subsequently investigated whether the inhibitory effect was caused by sodium cations or azide anions, by studying time courses of substrate hydrolysis in the presence of sodium azide and potassium azide. Supplementary Figure S1 shows plots of pNP

Fig. 1 Effects of sodium salts on the specific activity of *VhChiA* (A) and *VhGlcNAcase* (B). Reaction mixtures contained 1 μ g of *VhChiA* or 3 μ g of *VhGlcNAcase*, 500 μ M of *pNP*-GlcNAc₂ or *pNP*-GlcNAc, and 2 M sodium azide, sodium formate, sodium acetate, sodium nitrate or sodium chloride in 100 mM potassium acetate, pH 5.5 or 100 mM potassium phosphate, pH 7.5. Release of *pNP* was determined as described under Materials and Methods section. The error bars represent the standard errors from triplicate experiments.

Fig. 2 Effects of 0-3 M sodium azide on the structural integrity of *VhChiA* (A) and *VhGlcNAcase* (B) and of sodium nitrate on the structural integrity of *VhGlcNAcase* (C), investigated by fluorescence spectroscopy. The emission spectra were collected from 300 to 500 nm upon excitation at 295 nm. Shifts in the maximum emission wavelength and increases in fluorescence intensity occurred as a result of enzyme denaturation.

formation during hydrolysis of *pNP*-GlcNAc₂ by *VhChiA* (Supplementary Fig. S1A) and *pNP*-GlcNAc *VhGlcNAcase* (Supplementary Fig. S1B) over a period of 0–60 min. When compared with controls without

sodium salt, decreases in amount of the *pNP* product were detectable as early as 2.5 min in reaction mixes containing 2 M sodium azide or potassium azide. As already seen in Figure 1, the levels of inhibition by

Fig. 3 Dose-response plots of *VhChiA* fractional activity as a function of sodium azide concentrations (A) and *VhGlcNAcase* fractional activity as a function of sodium azide (circles) or sodium nitrate (squares) concentrations (B). The values of IC_{50} for sodium azide and sodium nitrate on the two enzymes were determined from this graph. Reaction mixtures (100 μ l), contained 500 μ M of *p*NP-GlcNAc₂ or *p*NP-GlcNAc, 1 μ g of *VhChiA* or 3 μ g of *VhGlcNAcase*, and concentrations of sodium azide or sodium nitrate varied from 0 to 4 M in 100 mM sodium phosphate buffer, pH 7.5. Release of *p*NP was determined as described under Materials and Methods. The error bars represent the standard errors from triplicate experiments.

sodium and potassium azides were much greater for *VhGlcNAcase* than for *VhChiA*. Comparing the two compounds (Supplementary Fig. S2), potassium azide inhibited both enzymes slightly more than sodium azide, but, given that there were only slight differences in activity in sodium azide (Supplementary Fig. S2A) and potassium azide (Supplementary Fig. S2B), the primary effect on both *VhChiA* and *VhGlcNAcase* was by azide anions.

Sodium azide inhibits the chitinase activity in a reversible manner

We evaluated whether inhibition by sodium azide was reversible or irreversible. Reaction mixtures containing *VhChiA* and 2 M sodium azide were incubated for various times, and after each time point of incubation was subjected to immediate dialysis. We observed that the activity before dialysis was 45% of the total activity, while after dialysis the activity was recovered to 75%, slightly less than before dialysis, indicating some loss of activity during dialysis (data not shown). The significant recovery of enzyme activity suggested that sodium azide acted on *VhChiA* in a reversible manner.

Sodium azide did not affect the structural integrity of *VhChiA* and *VhGlcNAcase*

Fluorescence spectroscopy was used to monitor changes in the molecular structure when sodium azide was added to the chitinase solution. For GlcNAcase, sodium nitrate was also tested, since it also significantly inhibited the enzyme. Figure 2 shows progressive decreases in the intensity of fluorescence emission at 300–500 nm upon excitation at 295 nm, in response to increasing concentrations of the inhibitor from 0 to 3 M; this is typical quenching of intrinsic fluorescence by large anions. However, no significant shift in the maximum emission wavelength was observed (Fig. 2A for *VhChiA* and 2B for *VhGlcNAcase*). In contrast, an increase in the fluorescence intensity, as well as a shift in the maximum

emission wavelength, was observed when both enzymes were subjected to complete denaturation by adding 8 M urea or heating at 100° for 10 min. When sodium nitrate was tested with *VhGlcNAcase*, similar results were obtained (Fig. 2C), with the fluorescence signal quenched more than when the enzyme was treated with sodium azide (Fig. 2B). A completely different pattern in the fluorescence intensity changes between salt addition and urea/heat denaturation suggested that sodium azide did not drastically destroy the structural integrity of both enzymes, whereas sodium nitrate may partially unfold the secondary structure of *VhGlcNAcase*.

Sodium azide inhibits *VhGlcNAcase* and *VhChiA* at high molar concentrations

The strength of inhibition by sodium azide/nitrate was further confirmed by dose-response experiments. Figure 3 shows a plot of fractional activity (v_i/v_0) of *VhChiA* (Fig. 3A) or *VhGlcNAcase* (Fig. 3B, circles) versus the logarithm of sodium concentration. A similar curve was plotted for the effect of sodium nitrate on *VhGlcNAcase* (Fig. 3B, squares). The IC_{50} value (concentration of the inhibitor producing half maximal inhibition) for sodium azide inhibition for *VhChiA* was estimated to be 0.4 ± 0.02 M, while the K_i value was estimated to be 1.40 ± 0.07 M. For *VhGlcNAcase*, the IC_{50} estimated for sodium azide inhibition was 0.3 ± 0.03 M and for sodium nitrate was 0.2 ± 0.02 M. K_i values calculated from the IC_{50} values obtained for *VhGlcNAcase* were 0.1 ± 0.02 and 0.1 ± 0.01 M, respectively. As will be shown later, the K_i values estimated from the dose-response curves are consistent with the K_i values obtained from Dixon plots, presented in Figure 4.

Sodium azide inhibits *VhChiA* by the mix-type mode, but *VhGlcNAcase* by the competitive mode

Figure 6A shows Michaelis-Menten plots of the initial velocity (v_0) of the *VhChiA* reaction versus the *p*NP-GlcNAc₂ concentration at various concentrations of

Fig. 4 Kinetic properties of *VhChiA* with *pNP-GlcNAc*₂ as substrate. Reaction mixtures (100 μ l), contained 1 μ g *VhChiA* and varied concentrations of *pNP-GlcNAc*₂ (0–800 μ M) with sodium azide 0 M (circles), 0.5 M (squares), 1 M (inverted triangles) or 2.0 M (diamonds) in 100 mM sodium phosphate buffer, pH 7.5. Release of *pNP* was determined as described under Materials and Methods section. Kinetic parameters were obtained from Michaelis-Menten plots (A) and the type of inhibition was assessed from Lineweaver-Burk plots (B). K_i and αK_i values were derived from Dixon plots (C and D). The error bars represent the standard errors from triplicate experiments.

Table I. Kinetic parameters of *VhChiA* in the presence of different NaN_3 concentrations

Inhibitor concentration (M)	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{mM}^{-1}$)
0	196 $\pm$ 25 (100)	2.40 $\pm$ 0.08 (100)	12.50 $\pm$ 0.30 (100)
0.5	164 $\pm$ 19 (84)	1.90 $\pm$ 0.08 (79)	11.70 $\pm$ 0.10 (94)
1.0	112 $\pm$ 15 (57)	1.20 $\pm$ 0.05 (50)	11.20 $\pm$ 0.70 (90)
1.5	92 $\pm$ 13 (47)	0.80 $\pm$ 0.02 (33)	9.20 $\pm$ 0.03 (74)
2.0	83 $\pm$ 12 (42)	0.60 $\pm$ 0.02 (25)	6.80 $\pm$ 0.20 (54)

The hydrolysis of *pNP-GlcNAc*₂ (0–800 μ M) was carried out with 1 μ g *VhChiA* and 0–2 M sodium azide in 100 mM phosphate buffer, pH 7.5 for 10 min at 37°C, and then the reaction was terminated with 100 μ l of 3 M Na_2CO_3 . Release of *pNP*, monitored at A_{405} , was converted to molar quantities using a calibration curve of *pNP* (0–20 nmol). The kinetic values (K_m and k_{cat}) were determined by nonlinear regression using GraphPad Prism version 5.0 (GraphPad Software, CA, USA). Numbers in brackets reveal the % relative K_m , k_{cat} and k_{cat}/K_m values of the enzyme with each sodium azide concentration, in comparison with the value without sodium azide (set to 100).

sodium azide. Apparent values of k_{cat} , K_m and k_{cat}/K_m were obtained by non-linear curve fitting. It appears that the catalytic rate constant (k_{cat}) obtained from the curve presented in Figure 4A decreased from 2.4 to 0.6 s^{-1} with increasing concentrations of sodium

azide from 0 to 2.0 M. Likewise, the apparent K_m also decreased from 196 to 83 μ M (Table I).

Figure 4B shows Lineweaver-Burk plots ($1/v_0$ versus $1/[S]$) of the same data. The double-reciprocal lines obtained at different concentrations of sodium azide intersected at a point close to -0.01 , -0.6 (x,y). This pattern indicates mixed-type inhibition, whereby sodium azide (I) may bind both to the free enzyme or to the enzyme-substrate complex. To obtain the values of K_i (the dissociation constant of inhibitor from EI complex) and αK_i (the dissociation constant from ESI complex), Dixon plots were constructed (Fig. 4C and D). Figure 4C is the Dixon plot of the slope of the primary plot (from the Lineweaver-Burk plot shown in Fig. 4B) against the inhibitor concentration, while Figure 4D is the plot of $1/V_{\text{max}}^{\text{app}}$ against the inhibitor concentration. The data suggest that sodium azide inhibits *VhChiA* with K_i for the EI complex of 1.5 ± 0.1 M (Fig. 4C) and αK_i for the ESI complex of 0.4 ± 0.02 M (Fig. 4D).

Patterns of inhibition were also assessed for *VhGlcNAcase*. Figure 5A and B presents the non-linear (Michaelis-Menten) plots of v_0 against the *pNP-GlcNAc* concentration at different concentrations of inhibitor. Curve fitting was conducted for individual concentrations of sodium azide (Fig. 5A) and sodium nitrate (Fig. 5B), yielding the apparent values of k_{cat} and K_m as shown in Table II. For both

Fig. 5 Kinetic properties of *VhGlcNAcase* with *pNP-GlcNAc* as substrate. Reaction mixtures (100 μ l), contained 3 μ g *VhGlcNAcase* and *pNP-GlcNAc* (0–800 μ M) with sodium azide or sodium nitrate 0 M (circles), 0.3 M (squares), 0.4 M (triangles), 0.5 M (inverted triangles) or 0.6 M (diamonds) in 100 mM sodium phosphate buffer, pH 7.5. Release of *pNP* was determined as described under Materials and Methods. Kinetic parameters of *VhGlcNAcase* with sodium azide and sodium nitrate were obtained from Michaelis-Menten plots (A) and (B), respectively. Types of inhibition of sodium azide (C) and sodium nitrate (D) were assessed from Lineweaver-Burk plots. K_i values of sodium azide and sodium nitrate derived from Dixon plots (E and F, respectively). The error bars represent the standard errors from triplicate experiments.

Table II. Kinetic parameters of *VhGlcNAcase* in the presence of different NaN_3 and NaNO_3 concentrations

Inhibitor concentration (M)	NaN_3			NaNO_3		
	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \text{ mM}^{-1}$)	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \text{ mM}^{-1}$)
0	238 $\pm$ 19 (100)	0.70 $\pm$ 0.02 (100)	3.10 $\pm$ 0.10 (100)	238 $\pm$ 19 (100)	0.70 $\pm$ 0.02 (100)	3.10 $\pm$ 0.10 (100)
0.3	438 $\pm$ 51 (184)	0.70 $\pm$ 0.02 (100)	1.60 $\pm$ 0.04 (52)	362 $\pm$ 44 (152)	0.70 $\pm$ 0.03 (100)	2.00 $\pm$ 0.10 (65)
0.4	554 $\pm$ 67 (233)	0.60 $\pm$ 0.08 (86)	1.20 $\pm$ 0.10 (39)	411 $\pm$ 44 (173)	0.60 $\pm$ 0.02 (86)	1.60 $\pm$ 0.20 (52)
0.5	608 $\pm$ 76 (255)	0.60 $\pm$ 0.01 (86)	1.00 $\pm$ 0.02 (32)	528 $\pm$ 58 (222)	0.60 $\pm$ 0.04 (86)	1.20 $\pm$ 0.02 (39)
0.6	679 $\pm$ 92 (285)	0.60 $\pm$ 0.02 (86)	0.80 $\pm$ 0.02 (26)	667 $\pm$ 77 (280)	0.60 $\pm$ 0.01 (86)	0.80 $\pm$ 0.03 (26)

The hydrolysis of *pNP-GlcNAc* (0–800 μ M) was carried out with 3 μ g *VhGlcNAcase* and 0–0.6 M sodium azide or sodium nitrate in 100 mM phosphate buffer, pH 7.5 for 10 min at 37°C, and the reaction was terminated with 100 μ l of 3 M Na_2CO_3 . Release of *pNP*, monitored at A_{405} , was converted to molar quantities using a calibration curve of *pNP* (0–20 nmol). The kinetic values (K_m and k_{cat}) were determined by non-linear regression using GraphPad Prism version 5.0 (GraphPad Software, CA, USA). Numbers in brackets reveal the % relative K_m , k_{cat} and k_{cat}/K_m values of the enzyme with each sodium azide or sodium nitrate concentration, in comparison with the value without sodium azide or sodium nitrate (set to 100).

Fig. 6 Proposed mechanism of inhibition of the hydrolytic activity of wild-type *VhChiA* by azide anion.

inhibitors, the catalytic rate constant (k_{cat}) of the enzyme decreases only slightly from the value without the inhibitor (from 0.7 to 0.6 s⁻¹). On the other hand, the apparent K_m was increased from 238 to 679 μM (sodium azide) and 238 to 667 μM (sodium nitrate) with increasing inhibitor concentrations (Table II).

The patterns of GlcNAcase inhibition were evaluated using Lineweaver-Burk plots. As seen from the figures, linear fits at different concentrations of sodium azide (Fig. 5C) or sodium nitrate (Fig. 5D) were found to meet the y-intercept at a value close to 0.3 (nmol⁻¹.min), indicating a competitive mode of inhibition. To obtain the apparent K_i , Dixon plots were constructed as shown in Figure 5E and F, in which the slopes of the primary plots (Lineweaver-Burk plots) were plotted against inhibitor concentrations, respectively. The data showed that sodium azide and sodium nitrate inhibited WT *VhGlcNAcase* with similar apparent K_i values (0.20 ± 0.03 M for sodium azide and 0.20 ± 0.05 M for sodium nitrate), respectively, suggesting that sodium azide and sodium nitrate inhibited the GlcNAcase activity with the same mode of action and similar affinity.

Discussion

Vibrio harveyi chitinase (*VhChiA*) is a GH-18 endochitinase that catalyzes chitin degradation into chitooligosaccharide fragments in a random manner, whereas *VhGlcNAcase* is a GH-20 exo β -*N*-acetylglucosaminidases and cleaves the chitooligosaccharide fragments in a sequential manner into GlcNAc monomers. As *VhChiA* and *VhGlcNAcase* primarily act on chitin and chitin degradation products, they are considered the major enzymes involved in the chitin degradation pathway of *V. harveyi*. Since the natural habitats of *V. harveyi* are open oceans, high salt concentrations may affect the physiological function of the chitin-degrading enzymes produced by this marine bacterium. In this study, we set out a series of experiments to investigate the effects of sodium salts on the hydrolytic activity of *VhChiA* and *VhGlcNAcase*. We found that all salts could inhibit *VhChiA*, as well as the

GlcNAcase activity to various extents, and all showed pronounced effects at high pH.

Sodium azide has been used in chemical 'rescue' to probe the mechanism of several glycoside hydrolases by serving as an alternative nucleophile, when the intrinsic nucleophilic sidechain essential for enzymic catalysis is deleted (16–23). However, in the presence of the natural catalytic nucleophile (i.e. in the native enzyme), the strongly nucleophilic azide anion was found instead to lower the enzyme activity, as clearly demonstrated here with GH-18 chitinase and GH-20 GlcNAcase.

Our kinetic analysis of reversible inhibition shows that sodium azide acts as a reversible inhibitor of *VhChiA*, and Lineweaver Burk plots in the presence of various concentrations of sodium azide indicated mixed-type inhibition. Scheme I represents the mechanism describing mixed-type inhibition, in which the inhibitor binds to either free enzyme (E) or to the enzyme-substrate complex (ES). In our data analysis using Dixon's plot, we obtained αK_i for ESI complex of 0.4 ± 0.2 M, which is about 4-fold less than K_i for EI complex (1.5 ± 0.1 M). These values indicate that sodium azide binds more strongly to the ligand-bound enzyme than to the free enzyme.

Scheme I The mechanism of mixed-type inhibition.

where K_m , K_i , αK_m and αK_i represent the various equilibrium dissociation constants for enzyme-substrate (ES), enzyme-inhibitor (EI) and enzyme-substrate-inhibitor (ESI) complexes.

It is important to note that K_m obtained for the substrate *p*NP-GlcNAc₂ binding to *VhChiA* is 0.2 mM. This value is 2,000-fold lower than K_i of sodium azide for the free enzyme. This suggests that sodium

azide is not a specific inhibitor for *VhChiA*, but it rather acts as a non-specifically in a partially reversible manner. This is not surprising, since sodium azide is commonly used as a chemical 'rescue' for various carbohydrate-active enzymes as already mentioned. In addition, we did not observe any inhibitory effect at a sodium azide concentration of <100 mM.

The kinetics of inhibition of *VhGlcNAcase*-catalyzed hydrolysis showed that sodium azide and sodium nitrate inhibited the enzyme competitively with *p*NP-GlcNAc. The mechanism of this type of inhibition is shown in scheme II:

Scheme II The mechanism of competitive inhibition.

where K_m and K_i represent the equilibrium dissociation constants of the ES and EI complexes.

We postulate that azide ion interacts with the carbon atom in the carbonyl group of the protonated side chain of the catalytic residues Asp313 and Glu315, thereby preventing the formation of hydrogen bonds between the two catalytic residues and also access of the incoming substrate to Glu315. Figure 6 shows how sodium azide may interrupt the catalytic cycle of *VhChiA* at the cleavage step. This simplified mechanism demonstrates that the azide ion, acting as a nucleophile, competes with the chitin substrate by interaction with the carbon atom in the carbonyl groups of Asp313 and Glu315, described as a 'nucleophilic substitution reaction'. The products of this reaction are hydroxyl ions (OH^-) and the enzyme in a form that is unable to attack the glycosidic bond at the cleavage site. Likewise, during inhibition of *VhGlcNAcase* by sodium azide or sodium nitrate, these ions may interact with the carbonyl group of the side chain of the catalytic residues in the catalytic mechanism, preventing access to the incoming substrate by these residues.

In this study, the inhibitory effects of sodium azide and sodium nitrate on *VhGlcNAcase* activity were greater than on *VhChiA*. Azide produced the most effective inhibition of both enzymes, perhaps because the size and shape of this compound best fits the structures of the active sites of *VhChiA* and *VhGlcNAcase*. The binding cleft of *VhChiA* has a long and deep groove, including six GlcNAc residue-binding subsites (−4)(−3)(−2)(−1)(+1)(+2) (26)). The kinetic studies showed that sodium azide inhibits the enzyme activity by binding more strongly to the enzyme-substrate complex than to the free enzyme. This may be because the azide ion is able to react not only with the carbonyl group of the catalytic residues but also with the substrate-binding residues located around the active

site of the enzyme. In contrast, the binding pocket of *VhGlcNAcase* contains four substrate binding subsites, designated (−1)(+1)(+2)(+3) (24), so it may allow azide or nitrate ion to react with the carbonyl group of the catalytic residues to inhibit the free enzyme activity easily.

In conclusion, the kinetics of inhibition suggest that sodium azide inhibits *VhChiA* by a mixed-type mode, whereas sodium azide and sodium nitrate were found to inhibit *VhGlcNAcase* activity in a simple competitive mode. The inhibitory effects of a on *VhChiA* and *VhGlcNAcase* suggested that both enzymes may adopt the substrate-assisted retaining mechanism in chitin degradation, but the mechanistic details of the catalysis (as accessed by the inhibition study) are not the same, owing to the dissimilar features in shape and molecular arrangement of the binding/catalytic residues that form the binding clefts of the two enzyme species.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We acknowledge Biochemistry-Electrochemistry Research Unit and Biochemistry Laboratory, the Center for Scientific and Technological Equipment, Suranaree University of Technology for providing all the facilities for this research. We greatly appreciate a critical proofreading of this manuscript by Dr David Apps, Centre for Integrative Physiology, School of Biomedical Sciences, University of Edinburgh, United Kingdom.

Funding

This work was financially supported by The Thailand Research Fund and Suranaree University of Technology through the Royal Golden Jubilee PhD. Scholarship to P.S. (PHD/0021/2552) and the TRF Basic Research Grant to W.S. (BRG578001).

Conflict of Interest

None declared.

References

1. Bassler, B.L., Yu, C., Lee, Y.C., and Roseman, S. (1991) Chitin utilization by marine bacteria. *J. Biol. Chem.* **266**, 24276–24286
2. Cohen-Kupiec, R., and Chet, I. (1998) The molecular biology of chitin digestion. *Curr. Opin. Biotechnol.* **9**, 270–277
3. Suginta, W., Vongsuwan, A., Songsiririttigul, C., Svasti, J., and Prinz, H. (2005) Enzymatic properties of wild-type and active site mutants of chitinase A from *Vibrio carchariae*, as revealed by HPLC-MS. *FEBS J.* **272**, 3376–3386
4. Matsuo, Y., Kurita, M., Park, J.K., Tanaka, k., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1999) Purification, characterization and gene analysis of *N*-acetylglucosaminidase from *Enterobacter* sp. G-1. *Biosci. Biotechnol. Biochem.* **63**, 1261–1268
5. Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H., and Morikawa Y. (1998) Purification and characterization of exo- β -D-glucosaminidase from a cellulolytic fungus, *Trichoderma reesei* PC-3-7. *Appl. Environ. Microbiol.* **64**, 890–895

6. Ueda, M., Fujita, Y., Kawaguchi, T., and Araai, M. (2000) Cloning, nucleotide sequence and expression of the β -N-acetyl-glucosaminidase gene from *Aeromonas* sp. No. 10S-24. *J. Biosci. Bioeng.* **89**, 164–169
7. Austin, B. and Zhang, X-H. (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett. Appl. Microbiol.* **43**, 119–124
8. Suginta, W., Robertson, P.A., Austin, B., Fry, S.C., and Fothergill-Gilmore, L.A. (2000) Chitinases from *Vibrio*: activity screening and purification of chiA from *Vibrio carchariae*. *J. Appl. Microbiol.* **89**, 76–84
9. Suginta, W., Chumjan, W., Mahendran, K.R., Janning, P., Schulte, A., and Winterhalter, M. (2013) Molecular uptake of chitooligosaccharides through chitoporin from the marine bacterium *Vibrio harveyi*. *PLoS One*. **8**, e55126
10. Suginta, W., Chumjan, W., Mahendran, K.R., Schulte, A., and Winterhalter, M. (2013) Chitoporin from *Vibrio harveyi*: a channel with exceptional sugar specificity. *J. Biol. Chem.* **288**, 11038–11046
11. Bortone, K., Monzingo, A.F., Ernst, S., and Robertus, J.D. (2002) The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. *J. Mol. Biol.* **320**, 293–302
12. Papanikolaou, Y., Prag, G., Tavlas, G., Vorgias, C.E., Oppenheim, A.B., and Petratos, K. (2001) High resolution structural analyses of mutant chitinase A complexes with substrates provide new insight into the mechanism of catalysis. *Biochemistry* **40**, 11338–11343
13. Suginta, W. and Sritho, N. (2012) Multiple roles of Asp313 in the refined catalytic cycle of chitin degradation by *Vibrio harveyi* chitinase A. *Biosci. Biotechnol. Biochem.* **76**, 2275–2281
14. Suginta, W., Pantoom, S., Prinz, H. (2009) Substrate binding modes and anomer selectivity of chitinase A from *Vibrio harveyi*. *J. Chem. Bio.* **2**, 191–202
15. MacLeod, A.M., Lindhorst, T., Withers, S.G., and Warren, R.A. (1994) The acid/base catalyst in the exoglucanase/xylanase from *Cellulomonas fimi* is glutamic acid 127: evidence from detailed kinetic studies of mutants. *Biochemistry* **33**, 6371–6376
16. Viladot, J.L., de Ramon, E., Durany, O., and Planas, A. (1998) Probing the mechanism of *Bacillus* 1,3-1,4- β -D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues. *Biochemistry* **37**, 11332–11342
17. Vallmitjana, M., Ferrer-Navarro, M., Planell, R., Abel, M., Ausín, C., Querol, E., Planas, A., and Pérez-Pons, J. A. (2001) Mechanism of the family 1 β -glucosidase from *Streptomyces* sp: catalytic residues and kinetic studies. *Biochemistry* **40**, 5975–5982
18. Shallom, D., Belakhov, V., Solomon, D., Shoham, G., Baasov, T., and Shoham, Y. (2002) Detailed kinetic analysis and identification of the nucleophile in α -L-arabinofuranosidase from *Geobacillus stearothermophilus* T-6, a Family 51 glycoside hydrolase. *J. Biol. Chem.* **277**, 43667–43673
19. Williams, S.J., Mark, B.L., Vocadlo, D.J., James, M.N.G., and Withers, S.G. (2002) Aspartate 313 in the *Streptomyces plicatus* hexosaminidase plays a critical role in substrate-assisted catalysis by orienting the 2-acetamido group and stabilizing the transition state. *J. Biol. Chem.* **277**, 40055–40065
20. Cobucci-Ponzano, B., Trincone, A., Giordano, A., Rossi, M., and Moracci, M. (2003) Identification of the catalytic nucleophile of the family 29 α -L-fucosidase from *Sulfolobus solfataricus* via chemical rescue of an inactive mutant. *Biochemistry* **42**, 9525–9531
21. Paal, K., Ito, M., and Withers, S.G. (2004) *Paenibacillus* sp. TS12 glucosylceramidase: kinetic studies of a novel sub-family of family 3 glycosidases and identification of the catalytic residues. *Biochem. J.* **378**, 141–149
22. Fujita, K., Sato, R., Toma, K., Kitahara, K., Suganuma, T., Yamamoto, K., and Takegawa, K. (2007) Identification of the catalytic acid base residue of arthrobacter endo- β -N-acetylglucosaminidase by chemical rescue of an inactive mutant. *J. Biochem.* **142**, 301–306
23. Suginta, W., Vongsuwan, A., Songsiriritthigul, C., Prinz, H., Estibeiro, P., Duncan, R.R., Svasti, J., and Fothergill-Gilmore, L.A. (2004) An endochitinase A from *Vibrio carchariae*: cloning, expression, mass and sequence analyses, and chitin hydrolysis. *Arch. Biochem. Biophys.* **424**, 171–180
24. Suginta, W., Chuenark, D., Mizuhara, M., and Fukamizo, T. (2010) Novel β -N-acetylglucosaminidases from *Vibrio harveyi* 650: cloning, expression, enzymatic properties, and subsite identification. *BMC Biochem.* **1**, 1–12
25. Cheng, Y.C and Prusoff, W.H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108
26. Songsiriritthigul, C., Pantoom, S., Aguda, A.H., Robinson, R.C., and Suginta, W. (2008) Crystal structures of *Vibrio harveyi* chitinase A complexed with chitooligosaccharides: Implications for the catalytic mechanism. *J. Struct. Biol.* **162**, 491–499