Structural Determinants of an Insect $\beta$-N-Acetyl-$\alpha$-hexosaminidase Specialized as a Chitinolytic Enzyme

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$\beta$-N-Acetyl-$\alpha$-hexosaminidase (EC 3.2.1.52), a member of the family 20 glycosyl hydrolases (GH20),5 is an enzyme that participates in the breakdown of glycosidic bonds of glycans, glycoproteins, and glycolipids. It has been postulated to have specialized physiological functions, including post-translational modification of $N$-glycans, degradation of glycoconjugates, and egg-sperm recognition. The structural basis for these specialized functions is still unclear.

It is interesting to note that insects have evolved to have more than one $\beta$-N-acetyl-$\alpha$-hexosaminidase, as revealed by genomic analysis of various insects, including Coleoptera, Diptera, Hymenoptera, Lepidoptera, Phthiraptera, and Hemiptera. The activities of insect $\beta$-N-acetyl-$\alpha$-hexosaminidases are not restricted to chitin degradation but are also associated with post-translational modification of $N$-glycans, degradation of glycoconjugates, and egg-sperm recognition, suggesting that these enzymes have rather versatile physiological functions in the growth and development of insects. Some of these physiological functions may overlap with those of the same enzymes found in higher organisms. Mammalian lysosomal $\beta$-N-acetyl-$\alpha$-hexosaminidases are mainly responsible for glycoconjugate degradation in lysosome (3). Likewise, $\beta$-N-acetyl-$\alpha$-hexosaminidases from the insects Bombyx mori (4) and Spodoptera frugiperda (5) have broad substrate specificity ranging from $N$-glycans to chitooligosaccharides, suggesting that they have the same function as their mammalian counterparts. Mammal $\beta$-N-acetyl-$\alpha$-hexosaminidases have been shown to be important for egg-sperm recognition (6), and the enzymes from Drosophila melanogaster sperm membrane also participate in the same process (7, 8). Plant $\beta$-N-acetyl-$\alpha$-hexosaminidases carry out post-translational modification of $N$-glycans (9, 10). Similarly, the enzymes from D. melanogaster (11) and S. frugiperda (12) (termed Fdls) also carry out post-translational modification of $N$-glycans, but they have a rather narrow substrate specificity. Comparative analysis of $\beta$-N-acetyl-$\alpha$-hexosaminidase genes in the co-
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lepteron, Tribolium castaneum, has provided molecular and biological evidence to support the hypothesis that each of the four TcNAGs among a total of seven β-N-acetyl-β-hexosaminidases has an essential and specific function in chitin degradation and/or N-glycan modification during development (13). Thus, it is interesting to know how these enzymes could carry out their specialized functions in terms of their structural features.

Seven crystal structures of GH20 β-N-acetyl-β-hexosaminidases have been obtained, including two human and five bacterial enzymes. Both the human HexA and HexB are the mammalian β-N-acetyl-β-hexosaminidases that degrade glyco-conjugate in the lysosome (14–16). The bacterial enzymes include SpHex (17, 18) and SmCHB (19, 20), which are found to efficiently degrade various glycosphingolipids (22). In addition, Paenibacillus sp. TS12, PsHex, that can efficiently degrade various glycosphingolipids (22). In addition, a bacterial enzyme found in the endocarditis pathogen, Streptococcus gordonii, is involved in the release of dietary carbohydrates (23). To our knowledge, no crystal structure of insect β-N-acetyl-β-hexosaminidase has yet been reported.

We have previously identified a β-N-acetyl-β-hexosaminidase (OfHex1) from the lepidopteran insect Ostrinia furnacalis (Asian corn borer), which we think may be involved in chitin degradation during insect metamorphosis (2). In the present study, we used RNA interference to demonstrate the vital role of OfHex1 during the pupation of O. furnacalis. We also resolved the crystal structures of free OfHex1 and OfHex1 in complex with the recently isolated inhibitor TMG- GlcNAc (21); and the enzyme from Paenibacillus sp. TS12, PsHex, that can efficiently degrade various glycosphingolipids (22). In addition, the enzyme found in the endocarditis pathogen, Streptococcus gordonii, is involved in the release of dietary carbohydrates (23). To our knowledge, no crystal structure of insect β-N-acetyl-β-hexosaminidase has yet been reported.

Gene Expression and RNAi—O. furnacalis was reared on an artificial diet. To analyze the temporal transcriptional pattern of OfHEX1 (encoding OfHex1), DP887769, total RNAs were extracted from the entire insect at the fourth instar larval, fifth instar larval, prepupal, pupal, and adult stages using RNAiso reagent (TaKaRa, Dalian, China). To analyze the temporal transcription pattern of OfHEX1 in different tissues, total RNAs were extracted from the integument and alimentary tract of the larva and subjected to RT-PCR using the Prime-Script RT-PCR kit (TaKaRa). The resulting cDNA obtained was subjected to real-time PCR using the OfHEX1-specific primers, 5′-TATGGGCCATCCAGCAGG-3′ (forward) and 5′-AGGAGAGCCCCCGTGTGTGT-3′ (reverse), and the Offp53 (encoding ribosomal protein S3, EU275206, as an internal reference)-specific primers, 5′-AGCGTTTTCAACAT-

CCCTGAAC-3′ (forward) and 5′-CACACCAGAGGAGCAG-3′ (reverse). Real-time PCR was performed using the SYBR PrimeScript RT-PCR Kit (TaKaRa) and Rotor-Gene 3000 (Corbett Research, Sydney, Australia).

For double-stranded RNA synthesis, a 246-bp fragment between nucleotides 151 and 356 in OfHEX1 was selected as template. Two primers, 5′-TAATAGCAGCTCATATAGGGCGTCAAGGCTAAGAGAACG-3′ (forward) and 5′-TAATACGACTTATAAGGGCGGTCAGGAGAACG-3′ (reverse), each with a T7 RNA polymerase sequence (underlined bases) flanking the 5′-end were used in the in vitro transcription performed with MEGAscript RNAi Kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Approximately 3 μg of OfHEX1 dsRNA (dsOfHEX1) was injected into the penultimate abdominal segment of day 2–5 instar larvae using a 10-μl syringe. Larvae injected with 3 μg of dsRNA of green fluorescent protein (dsGFP) were used as control. Injection was carried out for 100 individual larvae in each group. Two days after injection, three larvae were randomly selected for total RNA extraction. The remaining larvae were allowed to develop into the pupal stage. Three of these from the group injected with dsOfHEX1 that showed abnormal phenotypes and three from the control group were selected for transcriptional analysis of OfHEX1 by real-time PCR as described above.

Protein Expression and Purification—Expression and purification of OfHex1 were carried out as described previously (24). Briefly, the gene encoding OfHex1 was amplified from the vector CTB557-2-1 (kept in our laboratory) by PCR using forward primer 5′-GCTTACGTAGATTCGAGGAGTATGGGCGCTGGTG-3′ and reverse primer 5′-TTAATTCGGGCCGCTTAAATGATGATGATGATGGCGAATGCTACCCCTC-3′. The expressed OfHex1 contained a His6 tag at the C terminus. Single amino acid mutations were made by PCR using the following primer pairs: 5′-GCTTGTCTCTCTTACATCGGATGCGCAGC-3′ (forward) and 5′-GATTGAAGAAGCAGGTGTTATACGTCG-3′ (reverse) for E328Q. The expressed OfHex1 contained a His6 tag at the C terminus. Single amino acid mutations were made by PCR using the following primer pairs: 5′-GCTTGTCTCTCTTACATCGGATGCGCAGC-3′ (forward) and 5′-GATTGAAGAAGCAGGTGTTATACGTCG-3′ (reverse) for E328Q. The expressed OfHex1 contained a His6 tag at the C terminus. Single amino acid mutations were made by PCR using the following primer pairs: 5′-GCTTGTCTCTCTTACATCGGATGCGCAGC-3′ (forward) and 5′-GATTGAAGAAGCAGGTGTTATACGTCG-3′ (reverse) for E328Q. The PCR products were cloned into pPIC9 (Invitrogen) and transformed into Pichia pastoris strain GS115 by electroporation. Cells were cultured in BMMY broth (2% peptone, 1% yeast extract, 1% methanol, 1.34% yeast nitrogen base, and 0.1 M potassium phosphate (pH 6.0)) for 144 h, and methanol (1% of the total volume) was added every 24 h. Wild-type and mutant OfHex1 were purified from the culture supernatant by ammonium sulfate precipitation (65% saturation), affinity chromatography on an IMAC Sepharose High Performance column (GE Healthcare) followed by anion exchange chromatography on a Q Sepharose high performance column (GE Healthcare) (24).
Enzymology—Steady-state kinetics of wild-type and mutant OfHex1 were assayed using 4-methylumbelliferone-\(N\)-acyethyl-\(\beta\)-d-glucosaminide (4MU-\(\beta\)-GlcNAc) (Sigma) as substrate. The reaction mixture (100 \(\mu\)l) contained 1–16 \(\mu\)M 4MU-\(\beta\)-GlcNAc and an appropriate amount of enzyme in Britton-Robinson’s wide range buffer. After incubating at 25 °C for 5 min, 100 \(\mu\)l of 0.5 m glycine-NaOH (pH 10.3) was added to the sample to stop the reaction, and the fluorescence produced by the released 4-methylumbelliferone was quantified by a spectrofluorometer (Thermo Scientific Varioskan Flash, Thermo) using excitation and emission wavelengths of 360 and 405 nm, respectively.

Steady-state kinetics of wild type and of V327G and W490A mutants were also assayed using chitobiase ((GlcNAc)_2 Sigma) as substrate. The reaction mixture (50 \(\mu\)l) contained 33–100 \(\mu\)M (GlcNAc)_2 and 5 m\(\mu\)l sodium phosphate buffer (pH 7.0). After incubating at 25 °C for 10 min, the hydrolytic products were analyzed by HPLC using TSKgel 250 mm, Tosoh) on the Agilent 1200 HPLC system (Agilent) (2). The \(K_m\) and \(k_{cat}\) values of each enzyme were calculated by linear regression via Lineweaver-Burk plots.

The inhibition constants (\(K_i\)) were determined by steady-state kinetics using the same conditions, but the samples contained different concentrations of inhibitors. The \(K_i\) values were calculated by linear regression of data in Dixon plots.

Crystallization and Data Collection—Both native and inhibitor-bound OfHex1 were crystallized in the space group P3\(_2\)\(_1\) within 2 weeks by vapor diffusion at 4 °C. The mother liquor of the native protein consisted of 100 mM HEPES (pH 7.0), 200 mM MgCl\(_2\), and 30% PEG 400. The crystal of OfHex1 in complex with TMG-chitotriomycin was obtained by co-crystallization of the native protein with a 5-fold excess of TMG-chitotriomycin in the same mother liquor as that of native OfHex1, except at pH 7.5. Hanging drops were set up by mixing an aliquot of enzyme (7 mg/ml) with an equal amount of the mother liquor. Diffraction data were collected at the Shanghai Synchrotron Radiation Facility, BL-17U. All data collection and structure refinement are summarized in Table 1. The coordinates of the native OfHex1 and of the OfHex1-TMG-chitotriomycin complex were deposited in the Protein Data Bank with accession numbers of 3NSM and 3NSN, respectively. All structural figures were prepared by PyMOL (DeLano Scientific LLC, San Carlos, CA).

### RESULTS

Temporal and Spatial Transcriptional Patterns of OfHEX1—
The expression profile of OfHEX1 was analyzed by real-time RT-PCR. Expression of OfHEX1 was up-regulated before each molting stage during development of the late fourth instar larva (4L), late fifth instar larva (5LS), prepupa (PP), and late pupa (P3) (Fig. 1A). The expression level of OfHex1 reached its peak at the fifth instar day 5 and prepupa stages, which is about 10 times higher than that of fifth instar day 3 larva (5L3 in Fig. 1). This demonstrated that transcription of OfHEX1 was greatly up-regulated during pupation of O. furnacalis.

To determine whether transcription of OfHEX1 is tissue-specific, larvae of two representative developmental stages (fifth instar days 3 and 5) were selected, and the levels of OfHEX1 transcript in the integument and alimentary tracts were measured. Larvae at the fifth instar day 3 stage showed a considerable increase in body weight at a rate of 0.03 g/day, whereas those at the fifth instar day 5 stage had already stopped feeding and began to spin and prepare to pupate. As shown in Fig. 1B, the expression levels of OfHEX1 in the integument and alimentary tracts were similar at the fifth instar day 3 stage. However, at the fifth instar day 5 stage, the expression level of OfHex1 in the integument was up-regulated more than 3-fold but remained unchanged in the alimentary tract. This demonstrated that the up-regulation of OfHEX1 transcription was specific to the integument during the pupation of O. furnacalis.

RNAi of OfHEX1—Based on the data of temporal and spatial transcriptional patterns of OfHEX1, we proposed that this gene is involved in the pupation of O. furnacalis. To validate this proposal, gene-specific dsRNA was injected into fifth instar day 2 larvae of O. furnacalis to suppress the transcription of OfHEX1. Before pupation, most of the larvae injected with OfHex1 survived without any visible changes in phenotype and appeared similar to those injected with dsGFP. At the pupation stage, however, 20% of the dsOfHEX1-injected larvae showed abnormal phenotypes that were absent in dsGFP-injected larvae and died 1 or 2 days after pupation. The pupation process was obviously affected at different times (Fig. 1C).
Some abnormal phenotypes manifested only as a small opening at the back without further molting while the whole body still remained in the larval stage, but the white new cuticle was already visible at the opening (Fig. 1C, first abnormal sample). Some abnormal larvae shed half of their old cuticles, with the head capsules already moved to the middle of the body. The head and thorax became tanned, but the abdomen remained in the larval form (Fig. 1C, second abnormal sample). Other larvae with abnormal phenotypes shed most of the old cuticles and appeared as nascent pupae, but part of the old black cuticle with the head capsule still tightly adhered to the body and could not molt completely (Fig. 1C, third abnormal sample). Thus, it appeared that all of the O. furnacalis larvae with abnormal phenotypes failed to shed their old cuticles completely before new ones started to form underneath. Down-regulation of OfHEX1 in dsOfHEX1-injected O. furnacalis was observed not only in the larvae after 2 days of injection but also in the larvae at the emergence of the abnormal phenotype (Fig. 1D). The result demonstrated that OfHex1 plays a vital role in the degradation of old cuticle during the pupation stage of O. furnacalis. TcNAG1, a homolog of OfHEX1 from T. castaneum, has also been validated by RNAi to play a role in cuticular chitin turnover during insect metamorphosis (13). dsTcNAG1-injected insects failed to completely shed their old cuticles and finally died.

Overall Structure of an Insect β-N-Acetyl-α-hexosaminidase—OfHex1 was expressed as a secretory enzyme in P. pastoris and purified as described previously (24).

The crystal structure of the free OfHex1 reveals a homodimeric enzyme with the two monomers in the adjacent asymmetry units reconstructed by a crystallographic 2-fold symmetry axis adhering to each other in a side-by-side fashion. Each monomer is N-glycosylated at Asn164 and Asn375. All of the 12 cysteine residues of each monomer contribute to the intradisulfide bonds (Cys31–Cys59, Cys36–Cys55, Cys316–Cys373, Cys326–Cys331, Cys478–Cys491, and Cys585–Cys592).

The interface between two monomers is formed mainly by loop regions of the (β/α)8-barrel with a buried area of ~2,853 Å²/monomer, 2 times bigger than that of human dimeric HexB (1,612 Å²) or HexA (1,587 Å²) (Fig. 2A). Numerous hydrophobic bonds and five salt bridges at the dimer interface were revealed by PISA (available on the European Bioinformatics Institute Web site).

Each monomer of OfHex1 consists of a typical two-domain fold similar to those of the human (14–16) and bacterial (S. plicatus and Paenibacillus sp.) (17, 18, 22) enzymes. However, some significant differences can be seen, especially in the N and C termini as well as in the (β/α)8-barrel, where the active center is located.
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barrel segments. Like all known GH20 $\beta$-N-acetyl-$\delta$-hexosaminidasises, OfHex1 also does not have helices $\alpha$5 and $\alpha$7. Unlike the other enzymes, OfHex1 contains an extended loop ($L_{361-366}$, residues 361–366) instead of the $\beta$ strand. Because two catalytic residues, Asp$^{367}$ and Glu$^{368}$, are located at the end of $L_{361-366}$, this change may bring the catalytic residues into closer proximity with the substrate. Two other loops, residues 314–335 ($L_{314-335}$ in pink) and residues 478–496 ($L_{478-496}$ in green), are rigid but appear functional (Fig. 2, A and B). $L_{314-335}$ is located between $\beta$3 and $\alpha$3 and stabilized by two pairs of disulfide bonds, Cys$^{326}$–Cys$^{331}$ and Cys$^{316}$–Cys$^{373}$. $L_{478-496}$ appears as a twisted “8” and is positioned parallel to its counterpart in the adjacent monomer by numerous interactions, suggesting its role in dimerization.

Structural comparison showed that these two loops are present in bacterial GH20 $\beta$-N-acetyl-$\delta$-hexosaminidasises, SpHex (17, 18) and SmCHB (19, 20), although much variation in amino acid composition exists in these two loops of the enzymes. Because SpHex and SmCHB belong to chitinolytic bacteria, one of these two loops may play a role in substrate specificity. The C terminus of OfHex1 consists of an additional $\alpha$-helix followed by a loop-like structure (residues 575–594, in red) (Fig. 2A). These extra features may also be important for dimerization through interaction with domain II of the adjacent monomer.

Architecture of the Active Site—To gain insight into the active site of OfHex1, the crystal structure of the enzyme in complex with TMG-chitotriomycin was resolved to 2.1 Å (Fig. 3B). TMG-chitotriomycin is a linear pseudotetrasaccharide consisting of three sequentially arranged $\beta$-1,4-linked GlcNAs and one $N,N,N$-triMe-D-GlcNH$_2$ at the non-reducing end (Fig. 3A) (32, 33). Compared with other known inhibitors that have one sugar moiety, it is the best mimic of natural substrates (chitooligosaccharides) for investigating the substrate-binding mode.

Overall, TMG-chitotriomycin binds OfHex1 at a deeper and larger pocket compared with those of human HexA and HexB (Fig. 3C and supplemental Fig. S1). Unlike dimeric HexA (16) or HexB (14, 15), the active pocket of each OfHex1 monomer is isolated and self-stabilized. The active pocket can be divided into two parts: the −1 subsite for catalysis and the +1 subsite for binding the +1 sugar unit of substrates.

It is clear that numerous intermolecular interactions are involved in locking TMG-chitotriomycin into the active site and positioning it for intramolecular nucleophilic attack (Fig. 3C). Like all known GH20 $\beta$-N-acetyl-$\delta$-hexosaminidasises, the active site is highly conserved. Three tryptophan residues (Trp$^{424}$, Trp$^{448}$, and Trp$^{524}$) form a tight hydrophobic pocket (Fig. 3C). Together with Asp$^{367}$ and Tyr$^{375}$, these tryptophan residues form the wall and the bottom of the active site. A conserved catalytic triad is formed by residues Asp$^{249}$, His$^{303}$, and Glu$^{368}$ and stabilized by hydrogen bonds among these residues. The catalytic H$_2$O(II) (Fig. 3C), localized at the end of the catalytic triad, is stabilized by Glu$^{368}$ through a hydrogen bond. Another water molecule, H$_2$O(I), functions to stabilize Glu$^{328}$ and Glu$^{368}$ (Fig. 3C).

B-factors of each sugar unit of TMG-chitotriomycin were obtained: 20.94 (N,N,N-triMe-$\delta$-GlcNH$_2$), 23.72 (GlcNAcI), 41.18 (GlcNAcII), and 69.37 (GlcNAcIII). N,N,N-triMe-$\delta$-
GlcNH$_2$ binds tightly to the active pocket at the $-1$ subsite in $1^{\alpha}B$ conformation by stacking force provided by the indolyl group of Trp$^{490}$. Like (GlcNAc)$_2$, in the complex of SmCBH (19), the plane of the $-1$ sugar is rotated around the glycosidic linkage by about 90°. The positively charged $\text{N}_2\text{N}_4\text{N}_2\text{N}_4$-triMe group interacts with negatively charged catalytic Asp$^{367}$ and Glu$^{368}$ and forms hydrogen bonds with Asp$^{367}$, Glu$^{368}$, and Tyr$^{475}$.

In addition, other hydrogen bonds are formed between the sugar unit and active sites residues, including those between O-1 and Glu$^{368}$, O-3 and Arg$^{220}$, and O-4 and both Arg$^{220}$ and Glu$^{526}$ as well as between O-6 and both Asp$^{477}$ and Trp$^{490}$ (Fig. 3C). As revealed by structural alignment, the binding mode of N$_2$N$_2$N$_4$-triMe-D-GlcNH$_2$ is very similar to that of other known ligands of GH20 (2.48 Å) formed between O-3 and Glu$^{328}$ and between the nitro- gen of the acetamido group and Val$^{327}$. Both GlcNAcII and Glu$^{368}$ and forms hydrogen bonds with Asp$^{367}$, Glu$^{368}$, and Tyr$^{475}$.

The GlcNAcI component adjacent to N$_2$N$_2$N$_4$-triMe-D-GlcNH$_2$ is localized at the $+1$ subsite in the active pocket. It is sandwiched by the isopropyl group of Val$^{327}$ and the indolyl group of Trp$^{490}$ (Fig. 3C). The indolyl group of Trp$^{490}$ is stacked against the sugar ring in $1^C_4$ conformation, with a short hydrogen bond (2.48 Å) formed between O-3 and Glu$^{328}$ and between the nitrogen of the acetamido group and Val$^{327}$. Both GlcNAcII and GlcNAcIII are pointed away from the active pocket and are exposed to solvent. The GlcNAcII component in $1^C_4$ conformation is stabilized by two pairs of hydrogen bonds, one with Val$^{327}$ and the other with Trp$^{490}$ via a water molecule, thus blocking the entrance of the active pocket. The GlcNAcIII component in $5^S_2$ conformation remains pendulous.

The kinetics of V327G and W490A mutants, determined using 4MU-$\beta$-GlcNAc and (GlcNAc)$_2$ as substrates, are shown in Table 2. 4MU-$\beta$-GlcNAc contains the $-1$ sugar, whereas (GlcNAc)$_2$ contains both $-1$ and $+1$ sugars; therefore, 4MU-$\beta$-GlcNAc was used for investigating the catalytic activity of OfHex1 and (GlcNAc)$_2$ for sugar binding preference. The replacement of Val$^{327}$ with glycine resulted in slight decrease in $K_m$ and a 20–60% loss in catalytic activity as seen with a drop in $k_{cat}$ (Table 2), indicating that mutation at Val$^{327}$ affected both substrate binding and catalysis while having no effect on the enzyme’s preference toward $+1$ or $-1$ sugar binding. In contrast, replacement of Trp$^{490}$ with alanine led to a 13-fold increase in $K_m$ with (GlcNAc)$_2$ as a substrate and a 62% loss in catalytic activity when 4MU-$\beta$-GlcNAc was used as substrate, suggesting that Trp$^{490}$ is essential for binding the $+1$ sugar (Table 2). Further evidence supporting the important role of Trp$^{490}$ in binding the $+1$ sugar came from an inhibition study whereby the $K_i$ of W490A for TMG-chitotriomycin decreased by 2,277-fold relative to that of wild type, whereas the $K_i$ of V327G showed no change in $K_i$ (Table 3).

Inhibitor Binding Induced Significant Conformational Changes in the Active Site—Family 20 glycosyl hydrolases catalyze the breakdown of $\beta$-glycosidic bonds of various glycoconjugates from the non-reducing end by a mechanism known as the substrate assistant-retaining mechanism, which was first elucidated for bacterial and human $\beta$-N-acetyl-D-hexosaminidases (17–20). In this mechanism, the catalytic glutamate first acts as an acid that attacks the glycosidic oxygen and then as a base that abstracts a proton from a water molecule. The catalytic aspartate is required to stabilize the positively charged nitrogen of the 2-acetamido group, which is involved in the formation of a transient oxazolinium intermediate (17–20). As revealed by the crystal structure of OfHex1, the two catalytic residues (Glu$^{368}$ and Asp$^{367}$) the catalytic H$_2$O (H$_2$O(II)), and the catalytic triad (Asp$^{124}$ – His$^{303}$ – Glu$^{368}$) are highly conserved.
It is worthy of note that binding of TMG-chitotriomycin has induced significant conformational changes in OfHex1 compared with inhibitor-free enzyme (Fig. 5A). Because such an inhibitor-triggered conformational change has not been observed for any of the known GH20 β-N-acetyl-d-hexosaminidases, including the human enzymes HexA and HexB and the bacterial enzymes SpHex and SmCHB, (14–20), OfHex1 is therefore very different from other β-N-acetyl-d-hexosaminidases in this regard.

At the −1 subsite of the active pocket, Glu368 and Asp367 are rotated about 180° and 90°, respectively, after binding TMG-chitotriomycin. Two other residues, His303 and Trp448, are rotated about 30° and 45°, respectively (Fig. 5A). These movements have resulted in the establishment of two new hydrogen bonds, both of which are linked to Glu368: one connecting Glu368 with His303 via Asp249 and the other connecting Glu368 with Glu328 via the catalytic H2O (Fig. 5A). Mutation of Glu328 to alanine or glutamine resulted in a 19% decrease in catalytic activity in terms of $k_{cat}$ value (Table 2). We assume that the hydrogen bond networks function to stabilize Glu368.

It is very interesting to note that the movements of Glu368 together with Trp448 would lead either to an open- or close-active pocket, and the side chain of Trp448 would act as a lid for this pocket (Fig. 5B). After binding with TMG-chitotriomycin, the side chains of Glu368 and Trp448 are rotated to face the active site to form a closed hydrophobic pocket. In the absence of the inhibitor, the active site remains open and is accessible to solvent. The unique open-close mechanism of the active site would enable OfHex1 not only to carry out catalysis but also to facilitate substrate binding or release of product. Although no such conformational change has been found in other known GH20 enzymes (14–23), conformational change has been observed in GH84 β-N-acetyl-d-glucosaminidase (CpGH84) after binding with inhibitor due to a 180° rotation by the catalytic aspartate (Asp298) (34, 35). Before the binding of substrate, the side chain of the catalytic Glu368 of OfHex1 is connected to Thr427 via two hydrogen bonds, whereas Asp298 of CpGH84 is stabilized by an intramolecular hydrogen bond (34, 35).
The replacement of Trp448 by alanine resulted in a 2-fold increase in $K_m$ but a 927-fold decrease in $k_{cat}$ and a 1900-fold decrease in $k_{cat}/K_m$ (Table 2), meaning that the hydrophobic and large stereo indolyl group of Trp448 is essentially important for the “open-close” mechanism. Furthermore, when Trp448 was replaced by phenylalanine, which contains a plane-aromatic ring, little change in $K_m$ occurred, but $k_{cat}$ decreased more than 1,000-fold, resulting in an overall decrease in $k_{cat}/K_m$, indicating that the phenyl group cannot substitute for the indolyl group (Table 2). Trp448 is stabilized by a hydrogen bond with $L_{425–433}$ (residues 425–433) through its indolyl nitrogen.

At the $+1$ subsite, the GlcNAc1 component of TMG-chitotriomycin is sandwiched and stabilized by Val327 and Trp490. Compared with free OfHex1, the Val327 in the inhibitor-bound enzyme is rotated about 90° and moved slightly, and this has the effect of increasing the distance between Val327 and Trp490 from about 7 Å to 8.5 Å (Fig. 5A). Thus, the side chain of Val327 is hydrogen-bonded to GlcNAcII and GlcNAcII of TMG-chitotriomycin (Fig. 3C), and the isopro pyl group of Val327 is positioned parallel to the indolyl group of Trp490. In this state, the $+1$ sugar in TMG-chitotriomycin can be sandwiched firmly. Although OfHex1 and bacterial chitinolytic $β$-N-acetyl-$β$-hexosaminidases (SmCHB and SpHex) share a conserved $+1$ subsite architecture that is characterized by Val327 (Val493 in SmCHB and Val276 in SpHex) (17–20) (Fig. 2B), it is the only $β$-N-acetyl-$β$-hexosaminidase to show obvious conformational changes after ligand binding.

**DISCUSSION**

Insects contain several $β$-N-acetyl-$β$-hexosaminidases that have different physiological roles. However, until now, what determines the specialized physiological roles of these enzymes remains unknown.

We have shown here via RNAi that OfHex1 plays a key role during the pupation of *O. furnacalis*. OfHex1 shares 31–33% similarity in amino acid sequences with human lysosomal $β$-N-acetyl-$β$-hexosaminidases (human HexB as a representative) and 21–26% with bacterial chitinolytic $β$-N-acetyl-$β$-hexosaminidase.
hexosaminidases (SmCHB as a representative), and the structural comparison presented here has given some insights into how the insect chitinolytic $\beta$-N-acetyl-$\delta$-hexosaminidases may carry out the degradation of chitin.

**Insect OfHex1 Versus Human HexA and HexB—Human $\beta$-N-acetyl-$\delta$-hexosaminidases are known to be lysosomal enzymes having optimal pH at 3–4, whereas OfHex1 is an extracellular enzyme with optimal pH at 7. The physiological substrates for mammal lysosomal $\beta$-N-acetyl-$\delta$-hexosaminidases and their plant counterparts are mainly branching sugar chains on glycolipids and glycoproteins (3, 9, 10). In contrast, the physiological substrates of chitinolytic $\beta$-N-acetyl-$\delta$-hexosaminidases are linear chitooligosaccharides. The differences in substrate specificity between the chitinolytic and human $\beta$-N-acetyl-$\delta$-hexosaminidases can be explained by the differences in the structures of their active sites.

Chitinolytic $\beta$-N-acetyl-$\delta$-hexosaminidases, such as OfHex1 and SmCHB, have deeper substrate binding pocket that includes both $-1$ and $+1$ subsites, whereas lysosomal $\beta$-N-acetyl-$\delta$-hexosaminidases, such as the human enzymes, have only the $-1$ subsite (14–16). The two conserved residues, Trp$^{390}$ and Val$^{327}$, at the $+1$ subsite of OfHex1 are responsible for binding the $+1$ sugar by hydrogen bonds and $\pi-\pi$ stacking interactions as well as forming the walls of the $+1$ sugar-binding cleft in OfHex1. These two residues are structurally conserved in the bacterial enzymes (Trp$^{685}$ and Val$^{493}$ in SmCHB and Trp$^{108}$ and Val$^{276}$ in SpHex) (Fig. 2B) but are not present in human HexA and HexB (14, 15) (Fig. 2B). Thus, compared with OfHex1 as well as SmCHB and SpHex, the binding pockets of the human enzymes are exposed to physiological substrates (e.g. glycolipid $G_{\alpha 3}$), but only the $-1$ sugar can be accommodated. The rest of the molecule cannot be positioned in the binding pocket and instead remains exposed to solvent (14–16).

Furthermore, OfHex1 contains two conserved loops, $L_{314-335}$ and $L_{490-496}$ which are absent in human $\beta$-N-acetyl-$\delta$-hexosaminidases (Fig. 2B). It is worth noting that Val$^{327}$ and Trp$^{490}$ of OfHex1 are located within these two loops. Because these loops are localized at the entrance of the active pocket, they may serve as a scaffold for Val$^{327}$ and Trp$^{490}$ to stabilize the $+1$ sugar of the substrate. We think that all chitinolytic $\beta$-N-acetyl-$\delta$-hexosaminidases probably have a $+1$ subsite, which comprises the conserved tryptophan and valine, to stabilize the $+1$ sugar of the substrate. SmCHB and SpHex also contain these loops, although the amino acid sequences that constitute these loops are quite different except for the conserved valine and tryptophan (17–20). Thus, we believe these loop structures enable chitinolytic $\beta$-N-acetyl-$\delta$-hexosaminidases to bind long chained and linear chained substrates like chitoooligosaccharides, whereas HexA/B with a shallow active pocket is capable of binding branching N-glycans (e.g. $GlcNAC\beta1,2Man\alpha1,3$-$GlcNAC\beta1,2Man\alpha1,3$-$Man\beta1,4GlcNAC\beta1,4GlcNAC-PA) or branching substrates with bulky substituents, such as $G_{\alpha 3}$ and $G_{\alpha 2}$ gangliosides. OfHex1 cannot use GnGn-PA as a substrate (2).

**OfHex1 Versus Bacterial SmCHB—**Like OfHex1, SmCHB is a chitinolytic $\beta$-N-acetyl-$\delta$-hexosaminidase that could degrade chitoooligosaccharides and shares the conserved structural elements for binding the $+1$ sugar of the substrate. However, there are some differences between them. First, their overall structures are very different. OfHex1 is a homodimeric protein with two identical catalytic monomers, whereas SmCHB is a monomeric protein consisting of four domains, including one catalytic domain (19, 20). Therefore, although the two loops ($L_{314-335}$ and $L_{478-496}$ in OfHex1) at the $+1$ subsite can be found in the SmCHB (Fig. 2B), the loop length and amino acid composition as well as the roles in stabilizing the dimeric form are different.

OfHex1 is also distinguished from SmCHB by the big conformational changes between the free and inhibitor-bound enzymes. The structures of SmCHB as well as other known $\beta$-N-acetyl-$\delta$-hexosaminidases remain the same as their free forms after binding with inhibitors except for some minor conformational changes at the active pockets (19, 20). The conformational changes of OfHex1 initiated by binding with inhibitor has given rise to a unique mechanism that we called the “open-close” mechanism (Fig. 5C). In the “open” state (free enzyme), the side chain of Glu$^{368}$ interacts with Thr$^{427}$ via its side chain and nitrogen atom through hydrogen bonds of 2.71 and 2.94 Å, respectively. Thr$^{427}$ is further stabilized by His$^{433}$ through a hydrogen bond of 2.58 Å. In the “close” state (inhibitor-bound), the side chain of Glu$^{368}$ is rotated 180° to form hydrogen bonds with His$^{303}$, which is also rotated by about 30°. Together with Asp$^{494}$, a catalytic triad is formed, and this has the effect of extending the hydrogen bond between Thr$^{427}$ and His$^{433}$ to 2.69 Å. The loss of activity (~1389-fold) revealed by H433A mutant suggests that His$^{433}$ is very important with respect to stabilizing the hydrogen bond network necessary for catalytic efficiency.

**Selective Inhibition Mechanism of TMG-chitotriomycin—**TMG-chitotriomycin, which occurs naturally in Actinomycetes Streptomyces anulatus (32), has recently been synthesized by Yu et al. (33). It is the first reported inhibitor that shows specific inhibition against $\beta$-N-acetyl-$\delta$-hexosaminidases from chitin-containing organisms (32). The mechanism of selective inhibition has not yet been proven.

So far, based on the observations of intermolecular interactions between OfHex1 and TMG-chitotriomycin (Fig. 3C), the $N,N,N$-triMe-$\delta$-GlcNH$_2$ component appears to contribute most of the inhibitory activity of TMG-chitotriomycin, which functions as a substrate analog for $\beta$-N-acetyl-$\delta$-hexosaminidases. Therefore, one may deduce that $N,N,N$-triMe-$\delta$-GlcNH$_2$ alone would be a strong inhibitor or at least exhibits inhibitory activity. Usuki et al. (32) reported that $N,N,N$-triMe-$\delta$-GlcNH$_2$ is inactive against the insect GlcNAcase from Spodoptera littura. Similarly, we did not observe any inhibition exerted by $N,N,N$-triMe-$\delta$-GlcNH$_2$ against OfHex1 or against bacterial, plant, and mammalian $\beta$-N-acetyl-$\delta$-hexosaminidases, suggesting that other components of TMG-chitotriomycin are required for inhibition. Based on the crystal structure of OfHex1 complexed with TMG-chitotriomycin, two parts of this inhibitor are presumed to be responsible for the selective inhibition of OfHex1. First, the $+1$ subsite, which comprises Val$^{327}$, Trp$^{490}$, and Glu$^{368}$, can interact with the GlcNAcI component of TMG-chitotriomycin by both hydrophobic stacking and hydrogen bonding (Fig. 3C). These forces would stabilize GlcNAcI, which in turn sta-
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bilizes the N,N,N-triMe-β-GlcNH₂ component that directly binds to the −1 subsite in the active pocket. It is interesting to note that Val₁₃² and Trp₄⁹⁰ are conserved in the bacterial SpHex (Val²⁷⁶ and Trp²⁹⁰) and SmCHB (Val⁴⁹₃ and Trp⁶₈₅), both of which are capable of degrading chitooligosaccharides (Fig. 2B). Second, these chitinolytic β-N-acetyl-β-d-hexosaminidases, including OfHex1 and bacterial SpHex and SmCHB, have a deeper active pocket that can tightly bind both the −1 and +1 sugar units of GlcNAc, whereas human HexA and HexB have a shallower active pocket that merely binds the −1 sugar unit (supplemental Fig. S1). Without the presence of GlcNAcI at the open-close mechanism, as well as the unique architecture of OfHex1 with other enzymes revealed that OfHex1 is functionally specialized, and such a property is conferred by an oligosaccharide (Table 3). These results have confirmed that the −1 and +1 sugars of the inhibitor together with Trp₄⁹⁰ are essential for substrate binding. Because human HexA and HexB do not have the +1 subsite, it is not surprising that TMG-chitotriomycin is not an inhibitor for the human lysosomal β-N-acetyl-β-d-hexosaminidases. Thus, the mechanism of selective inhibition of TMG-chitotriomycin against OfHex1 is now elucidated.

In summary, OfHex1 is an insect chitinolytic β-N-acetyl-β-d-hexosaminidase that has been shown to play a vital role during the pupation of O. furnacalis. The structural alignment of OfHex1 with other enzymes revealed that OfHex1 is functionally specialized, and such a property is conferred by an open-close mechanism, as well as the unique architecture of the substrate binding site of the enzyme. Two residues, Trp₄⁴₈ and Trp₄⁹⁰, have been proven to be highly essential for catalysis and inhibition by TMG-chitotriomycin, as seen with a loss of more than 2,000-fold activity when these two residues were mutated. This work is the first to identify an important insect β-N-acetyl-β-d-hexosaminidase by functional and structural methods and may provide important clues for the design of species-specific pesticides.

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